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24-Epibrassinolide

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Rapporteur Member State : Austria

Version History

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B.6. TOXICOLOGY AND METABOLISM DATA

General Introduction

Brassinosteroids, including 24-Epibrassinolide are naturally occurring, plant growth promoting molecules, present in higher plants, lower plants, including algae, mosses, the "living fossil" *Equisetum* as well as some fungi (Takatsuto *et al*., 1990a, Table 6-2). Brassinosteroids are present in all plant organs such as pollen, anthers, seeds, leaves, stems, roots, flowers, grains and fruits with the highest concentrations found in pollen, seeds and fruits (Zhu *et al*., 2013) and considered an obligatory plant constituent. Pollen and immature seeds show contents of Brassinosteroids in a range of 0.001 – 6400 µg/kg fresh weight, while shoots and leaves usually show lower concentrations of 0.001 – 100 µg/kg fresh weight. Fruits, e.g. apples contain 10-35 µg/kg fresh weight (Table 6-2). The concentration of Brassinosteroids in plants is regulated by a complex system of feedback pathways (e.g. Saini *et al*., 2015) and Brassinosteroids are constantly synthesised, metabolised, activated and inactivated depending on the plant's needs as well as environmental cues. The concentrations of Brassinosteroids are continuously fluctuating - spatially and temporally: in a single plant, different concentrations can be measured simultaneously in different plant organs, cell structures and cells as well as in the same location at different times (e.g. Symons *et al*., 2008).

Brassinosteroids represent ubiquitous, phylogenetically ancient phytohormones that promote growth in land plants as well as in green freshwater algae. According to Kutschera and Wang (2012), Brassinosteroids may have evolved in the Pre-Cambrian, at a time during the evolution of life on earth, when the split between uni- and multicellular green algae (which later gave rise to the embryophytes) had not yet occurred.

24-Epibrassinolide was first synthesized in 1979 (Thompson *et al*., 1979). Ten years later the natural occurrence of 24-Epibrassinolide in the plant kingdom was demonstrated by isolation and detection of 24-Epibrassinolide in *Vicia faba* pollen (Ikekawa *et al*., 1988) for the first time. Isolation of 24-Epibrassinolide and other Brassinosteroids, respectively, from natural materials is a complicated and expensive process. Therefore, 24-Epibrassinolide is chemically synthesized, identical to the naturally occurring 24-Epibrassinolide and is considered a "natural-identical synthesized molecule".

Brassinosteroids, which belong to the class of polyhdroxysteroids, can be divided into free as well as conjugated signal molecules. They are classified by their alkyl-substitutions in the side chain, as C_{27}, C_{28} or C_{29} Brassinosteroids (Table 6-1).

Table 6-1: **Division of free brassinosteroids according to number of carbon in structure and different types of B-ring and substituents in the A-ring**

Brassinolide (BL), Castasterone (CS), Cathasterone (CT), Dolicholide (DL), Dolichosterone (DS), Dehydroteasterone (DT), Methyl (Me), Secasterone (SE), Teasterone (TE), Typhasterol (TY)

No. of carbon	Type of B-ring	Substituent in A-ring	Representative(s)
C27	7-Oxalactone	C(2α,3α)-OH	28-norBL
	6-Oxo	C(2α,3α)-OH	28-norCS
		C3α-OH	28-norTY
	6-Deoxo	C(2α,3α)-OH	6-deoxo-28-norCS
		C3α-OH	6-deoxo-28-norTY, 3-epi-6-deoxo-28-norCT
		C3β-OH	6-deoxo-28-norTE, 6-deoxo-28-norCT
		C3-oxo group	3-dehydro-6-deoxo-28-norTE, 3-keto-22-epi-28-norCT
C28	7-Oxalactone	C(2α,3α)-OH	BL, **24-epiBL**, 23-dehydroBL, DL
		C(2α,3β)-OH	3-epi-23-dehydroBL, 3-epiBL
		C(2β,3α)-OH	2-epi-23-dehydroBL
		C(2β,3β)-OH	2,3-diepi-23-dehydroBL
		C3α-OH	2-deoxyBL, 7-oxTY
		C3β-OH	7-oxTE
	6-Oxo	C(2α,3α)-OH	CS, 24-epiCS, DS
		C(2α,3β)-OH	3-epiCS, 3,24-diepiCS
		C(2β,3α)-OH	2-epiCS
		C(2β,3β)-OH	2,3-diepiCS
		C(1β,2α,3α)-OH	1β-OH-CS
		C(1α,2α,3β)-OH	3-epi-1α-OH-CS
		C3α-OH	TY
		C3β-OH	TE, CT
		C3-oxo group	3-DT (3-dehydroTE)
		C(2β,3β)-epoxide	SE, 24-epiSE
		C(2α,3α)-epoxide	2,3-diepiSE
		$\Delta^{2,3}$	Secasterol
	6-Deoxo	C(2α,3α)-OH	6-deoxoCS, 6-deoxo-24-epiCS, 6-deoxoDS
		C(2α,3β)-OH	3-epi-6-deoxoCS
		C3α-OH	6-deoxoTY, 3-epi-6-deoxoCT
		C3β-OH	6-deoxoTE, 6-deoxoCT
		C3-oxo group	6-deoxo-3DT (3-dehydro-6-deoxoTE)
	6-Hydroxy	C(2α,3α)-OH	6α-OH-CS
C29	7-Oxalactone	C(2α,3α)-OH	28-homoBL, 28-homoDL
	6-Oxo	C(2α,3α)-OH	28-homoCS, 28-homoDS, 25-MeDS, 25-MeCS
		C(2β,3α)-OH	2-epi-25-MeDS, 2-epi-25-MeCS
		C(2β,3β)-OH	2,3-diepi-25-MeDS, 2,3-diepi-25-MeCS
		C3α-OH	28-homoTY, 2-deoxy-25-MeDS
		C3β-OH	28-homoTE, 3-epi-2-deoxy-25-MeDS
	6-Deoxo	C(2α,3α)-OH	6-deoxo-28-homoDS, 6-deoxo-25-MeDS

Most Brassinosteroids, including the physiologically most important C_{28} Brassinolides, are synthesized by the precursor campesterol via a common 5α-cholestane skeleton. Structural variations are synthesized by differences in orientation of the oxygenated functions in rings A and B, and by different substituents in the side chain (Bajguz, 2011, Figure 6-1).

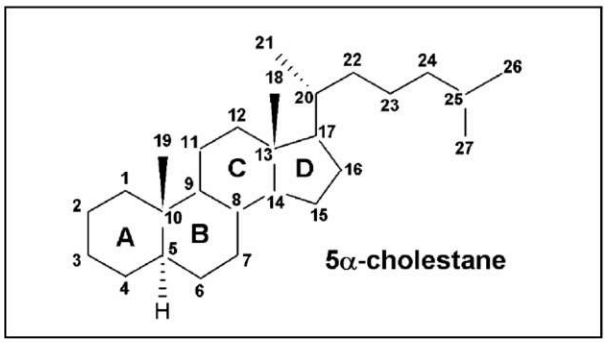

Figure 6-1: 5α-cholestane as the skeleton of Brassinosteroid synthesis. Structural differences occur in rings A and B, and in substituents in the side chain C_{20} – C_{27}.

Campesterol (Figure 6-2) derives from 5α-cholestane and, with its attached alcohol group, chemically represents a sterol, like e.g. the animal sterol cholesterol (Figure 6-2) or the insect derived molting hormone ecdysterone (Figure 6-2). Campesterol exhibits a double bond from carbon 5 to carbon 6 and therefore can be defined as a Δ^5 sterol. During synthesis of 24-Epibrassinolide, campesterol becomes fully saturated (Δ^0) by creation of an additional carbonyl bond and attachment of hydroxyl groups to the side chains. Therefore, 24-Epibrassinolide represents no longer a sterol but a stanol as, per definition, stanols are saturated or reduced sterols that share structural similarities with the campesterol/cholesterol skeleton. Due to the fact that alkenes (double bonds between carbons), as found in other plant steroids, display a chemical bond of higher reactivity, 24-Epibrassinolide has to be differentiated concerning its structural chemistry for the absence of an alkene group. This difference for example minimizes stanol absorption in the mammalian intestines (Bajguz, 2011).

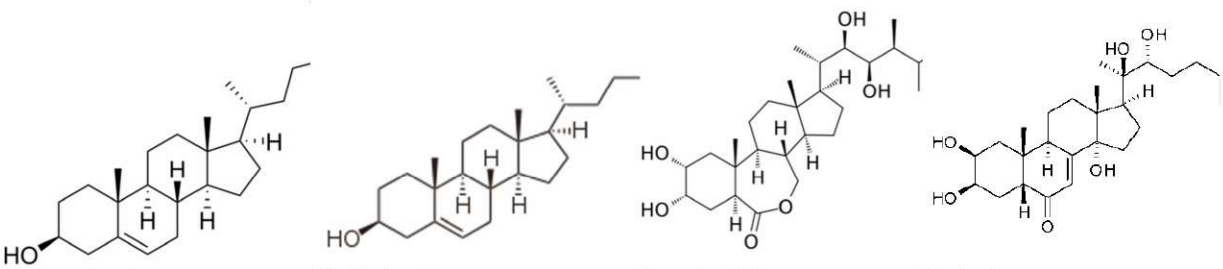

Figure 6-2: Structure of the (a) precursor Campesterol, (b) the animal derived molecule Cholesterol, (c) the active substance 24-Epibrassinolide, and (d) the insect hormone Ecdysterone.

24-Epibrassinolide belongs, besides 28-Homobrassinolide and Brassinolide, to the most biologically active Brassinosteroids, all three of them having similar chemical structures. 28-homobrassinolide and 24-Epibrassinolide differ from Brassinolide by the substituent in the side chain at C24 or by its configuration at C24, respectively (Khripach *et al.*, 2000). All three act in low concentrations between 0.1 – 0.001 ppm (Ikekawa and Zhao, 1991).

Table 6-2 is a summary table based on open literature and without any claim to completeness. It is to be expected that Brassinosteroids are also ubiquitous distributed in organisms not included in this table.

Table 6-2: Natural occurrence and concentrations of Brassinosteroids in higher and lower plants, fungi, processed and unprocessed foodstuffs

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
Monocotyledons				
Arecaceae				
Date palm (*Phoenix dactylifera*)	pollen	24-epiCS	unspecified	Zaki *et al.*, 1993[3]
Gramineae				
Perennial ryegrass	pollen	BR (1)	0.001	Taylor *et al.*, 1993[3]

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
(*Lolium perenne* L.)				
Rice (*Oryza sativa* L.)	shoot	BL	unspecified	Abe *et al*., 1984b; Abe 1991[3]
	shoot	CS	0.014	Abe *et al*., 1984b; Abe 1991[3]
	shoot	BR (1)	0.008	Abe *et al*., 1984b; Abe 1991[3]
	bran	BR (3)	unspecified	Abe *et al*., 1995a
	seeds	CS, BR (2)	unspecified	Park *et al*., 1994b
	grains	24-epiBL	216	Khripach *et al*., 2013
	grains	BL	29	Khripach *et al*., 2013
	grains	28-homoBL	4.4	Khripach *et al*., 2013
Canary grass (*Phalaris canariensis)*	seeds	CS	5	Shimada *et al*.,1996[3]
	seeds	BR (1)	0.7	Shimada *et al*.,1996[3]
Common wheat (*Triticum aestivum* L.)	grain	CS, BR (4)	unspecified	Yokota *et al*, 1994
Rye (*Secale cereal*)	seeds	CS, BR (4)	unspecified	Schmidt *et al*., 1995b[3]
	leaves	BR (3)	0.02-0.052	Antonchick *et al*., 2003[3]
	roots	BR (2)	0.032-0.107	Antonchick *et al*., 2003[3]
Maize (*Zea mays* L.) - dent corn	pollen	CS	120	Suzuki *et al*., 1986
	pollen	BR (2)	4.1-6.6	Suzuki *et al*., 1986
	roots	CS	0.3	Kim *et al*., 2000a
Maize (*Zea mays* L.) - sweet corn	pollen	CS	27.2	Gamoh *et al*., 1990[3]
	pollen	BR (2)	16.9-18.3	Gamoh *et al*., 1990[3]
Liliaceae				
Asian fawnlily (*Erythronium japonicum* Decne)	pollen, anther	BR (1)	5	Yasuta *et al*., 1995
Lilium elegans Thunb.	pollen	CS	10-50	Suzuki *et al*., 1994b ; Yasuta *et al*., 1995
Liliaceae				
	pollen	BL	1-5	Suzuki *et al*., 1994b ; Yasuta *et al*., 1995
	pollen	BR (2)	1-50	Suzuki *et al*., 1994b ; Yasuta *et al*., 1995
Lilium longiflorum Thunb.	pollen	BR (1)	3180	Abe, 1991[3] ; Abe *et al*., 1994 ; Asakawa *et al*.,1994, 1996 ; Soeno *et al*., 2000
Lilium longiflorum Thunb.	anther	BL, CS	unspecified	Abe, 1991[3] ; Abe *et al*., 1994 ; Asakawa *et al*.,1994, 1996 ; Soeno *et al*., 2000
	anther	BR (5)	20-2440	Abe, 1991[3] ; Abe *et al*., 1994 ; Asakawa *et al*.,1994, 1996 ; Soeno *et al*., 2000
Garden tulip (*Tulipa gesneriana* L.)	pollen	BR (1)	unspecified	Abe, 1991[3]
Typhaceae				
Broadleaf cattail (*Typha latifolia*)	pollen	BR (2)	68	Schneider *et al*., 1983[3]; Abe, 1991[3]
Dicotyledons – Apetalae				
Betulaceae				
Common alder (*Alnus glutinosa* (L.))	pollen	BL, CS	unspecified	Plattner *et al*., 1986
Cannabaceae				

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
Hemp (*Cannabis sativa* L.)	seeds	CS	600	Takatsuto *et al*., 1996b
	seeds	BR (1)	1800	Takatsuto *et al*., 1996b
Caryophyllaceae				
Gypsophilla perfoliata L.	seeds	24-epiBL	unspecified	Schmidt *et al*., 1996
Sticky catchfly (*Lychnis viscaria* L.)	seeds	24-epiCS, BR (1)	unspecified	Friebe *et al*., 1999[3]
Chenophyllaceae				
Beet (*Beta vulgaris* L.)	seeds	CS, 24-epiCS	unspecified	Schmidt *et al*., 1994[3]
Fagaceae				
Japanese chestnut (*Castanea crenata* Sieb. Et Zucc.)	galls	BL	0.001-12	Yokota *et al*., 1982a, Ikeda *et al*., 1983, Ikekawa & Takatsuto, 1984
	galls	CS	0.011-11.43	Yokota *et al*., 1982a, Ikeda *et al*., 1983, Ikekawa & Takatsuto, 1984
	galls	BR (2)	0.011-26	Yokota *et al*., 1982a, Ikeda *et al*., 1983, Ikekawa & Takatsuto, 1984
	shoot	BR (1)	15-30	Arima *et al*., 1984
	leaves	CS	2-6	Arima *et al*., 1984
Polygonaceae				
Common buckwheat (*Fagopyrum esculentum* Moench)	pollen	BL	5	Takatsuto *et al*., 1990b
	pollen	CS	7.1	Takatsuto *et al*., 1990b
	grains	24-epiBL	378	Khripach *et al*, 2013
	grains	BL	40	Khripach *et al*, 2013
	grains	28-homoBL	8.1	Khripach *et al*, 2013
Pieplant (*Rheum rhabarbarum* L.)	panicles	BL, CS, 24-epiCS	unspecified	Schmidt *et al*., 1995a[3]
Dicotyledons – Chloripetalae				
Apiaceae				
Asian pennywort (*Centella asiatica*)	leaves	CS	unspecified	Sondhi *et al*., 2010
Celery (*Apium graveolens* L.)	seeds	BR (1)	unspecified	Schmidt *et al*., 1995c[3]
Wild carrot (*Daucus carota* ssp. Sativus L.)	seeds	BL, CS, 24-epiCS	unspecified	Schmidt *et al*., 1998[3]
	root	24-epiBL	0.43	Khripach *et al*., 2013
	root	BL	1.5	Khripach *et al*., 2013
	root	28-homoBR	0.83	Khripach *et al*., 2013
	root	24-epiCS	0.23	Khripach *et al*., 2013
	whole plant	24-epiBL	0.745	Swaczynová *et al*., 2007
	whole plant	BL	0.644	Swaczynová *et al*., 2007
	whole plant	CS	0.316	Swaczynová *et al*., 2007
	whole plant	24-epiCS	0.642-1.19	Swaczynová *et al*., 2007
Brassicaceae				
Arabidopsis thaliana (L.) Heynh.	shoot	BL	0.04	Fujioka *et al*., 1996, 1997, 2000a[3]; Nomura *et al*., 2001
	shoot	CS	0.75	Fujioka *et al*., 1996, 1997, 2000a[3]; Nomura *et al*., 2001
	shoot	BR (9)	0.025-1.96	Fujioka *et al*., 1996, 1997,

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
				2000a[3]; Nomura *et al*., 2001
	20-days-old shoots	BR (5)	0.1-0.79	Bancos *et al.*, 2002
	20-days-old shoots	CS	0.15	Bancos *et al.*, 2002
	seeds	24-epiBL	0.22	Fujioka *et al*., 1998[3]
	seeds	BL	0.5-1.9	Fujioka *et al*., 1998[3]
	seeds	CS	0.4-5	Fujioka *et al*., 1998[3]
	seeds	BR (4)	0.5-5.4	Fujioka *et al*., 1998[3]
	seeds	24-epiBL	0.22	Schmidt *et al*., 1997
	seeds	CS	0.36	Schmidt *et al*., 1997
	root callus	BL, BR (1)	unspecified	Konstantinova *et al*., 2001[3]
	20-days-old roots	BR (5)	0.09-1.8	Bancos *et al.*, 2002
	20-days-old roots	CS	0.035	Bancos *et al.*, 2002
	seedlings	BR (10)	unspecified	Choe *et al*., 2001 ; Fujioka *et al*. 2002
	whole plant	24-epiBL	3.634-4.566	Swaczynová *et al*., 2007
	whole plant	BL	1.245	Swaczynová *et al*., 2007
	whole plant	CS	0.562	Swaczynová *et al*., 2007
	apical shoot	BR (6)	0.03-7.93	Shimada *et al*., 2003
	apical shoot	CS	2.02	Shimada *et al*., 2003
	stem	BR (5)	0.14-2.64	Shimada *et al*., 2003
	stem	CS	0.40	Shimada *et al*., 2003
	cauline leaves	BR (5)	0.11-4.33	Shimada *et al*., 2003
	cauline leaves	CS	0.31	Shimada *et al*., 2003
	rosette leaves	BR (5)	0.06-2.85	Shimada *et al*., 2003
	rosette leaves	CS	0.13	Shimada *et al*., 2003
	silique	BR (5)	0.36-8.89	Shimada *et al*., 2003
	silique	CS	0.94	Shimada *et al*., 2003
Chinese Cabbage (*Brassica campestris var. pekinensis* L.)	seeds	BL	940	Abe *et al*., 1982, 1983[3]; Ikekawa *et al*., 1984[3]
	seeds	CS	1600	Abe *et al*., 1982, 1983[3]; Ikekawa *et al*., 1984[3]
	seeds	28-homoCS	130	Abe *et al*., 1982, 1983[3]; Ikekawa *et al*., 1984[3]
	seeds	BR (2)	780-1300	Abe *et al*., 1982, 1983[3]; Ikekawa *et al*., 1984[3]
	immature seeds and sheaths	BL	0.0094	Ikekawa & Takatsuto, 1984
	immature seeds and sheaths	CS	0.0016	Ikekawa & Takatsuto, 1984
	immature seeds and sheaths	BR (3)	0.0013-0.00078	Ikekawa & Takatsuto, 1984
Indian mustard (*Brassica juncea* L.)	fresh leaves	24-epiBL	unspecified	Kanwar *et al*., 2013
Oilseed rape (*Brassica napus* L.)	pollen	BL	100	Grove *et al*., 1979

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
	breaking wall pollen	24-epiBL	628	Pan *et al.*, 2012
	pollen	BL	101.664	Swaczynová *et al.*, 2007
	pollen	CS	12.166	Swaczynová *et al.*, 2007
Radish (*Raphanus sativus* L.)	seeds	BL	0.3	Schmidt *et al.*, 1991[3], 1993b[3]
	seeds	CS	0.8	Schmidt *et al.*, 1991[3], 1993b[3]
	seeds	BR (2)	unspecified	Schmidt *et al.*, 1991[3], 1993b[3]
	germinated seeds	BL	0.45	Schmidt *et al.*, 1991[3]
	germinated seeds	CS	0.4	Schmidt *et al.*, 1991[3]
Fabaceae				
Lablab bean (*Dolichos lablab* L.)	seeds	BR (4)	12-160	Baba *et al.*, 1983; Yokota *et al.*, 1982b[3], 1983b, 1984
	seeds	BL, CS, BR (2)	unspecified	Baba *et al.*, 1983; Yokota *et al.*, 1982b[3], 1983b, 1984
Dolichos lablab	immature seeds	Homodolicholide	0.353	Yokota *et al.*, 1983b
False acacia (*Robinia pseudo-acacia*)	pollen	CS, BR (2)	unspecified	Abe *et al.*, 1995b
Broad bean (*Vicia faba* L.)	pollen	24-epiBL	5	Park *et al.*, 1987; Ikekawa *et al.*, 1988
	pollen	BL	190	Park *et al.*, 1987; Ikekawa *et al.*, 1988
	pollen	CS, BR (1)	unspecified	Park *et al.*, 1987; Ikekawa *et al.*, 1988
	pollen	BL	181	Gamoh *et al.*, 1989[3]
	pollen	CS	134	Gamoh *et al.*, 1989[3]
	pollen	BR (2)	537-628	Gamoh *et al.*, 1989[3]
Serradella (*Ornithopus sativus* Brot.)	seeds	CS	5	Schmidt *et al.*, 1993a[3]
	seeds	24-epiCS	25	Schmidt *et al.*, 1993a[3]
	shoot	CS, 24-epiCS, BR (3)	unspecified	Spengler *et al.*, 1995[3]
Common bean (*Phaseolus vulgaris* L.)	seeds	24-epiCS, BL, CS, BR (22)	unspecified	Yokota *et al.*, 1983c, 1987 ; Kim *et al.*, 1987, 1988[3], 2000b ; Kim, 1991 ; Park *et al.*, 2000
	10-day-old seedlings	24-epiBL	<0.346	Swaczynová *et al.*, 2007
	10-day-old seedlings	BL	0.471	Swaczynová *et al.*, 2007
	10-day-old seedlings	CS	0.967	Swaczynová *et al.*, 2007
Goa bean (*Psophocarpus tetragonolobus* (Stickm.) DC.)	seeds	BL, CS, BR (2)	unspecified	Takatsuto, 1994[3]
Pea (*Pisum sativum* L.)	seeds	BL, CS, BR (3)	unspecified	Yokota *et al.*, 1996[3]
	shoot	BL	0.2-0.8	Nomura *et al.*, 1997, 1999, 2001
	shoot	CS	0.4-2.4	Nomura *et al.*, 1997, 1999, 2001
	shoot	BR (6)	0.047-5.2	Nomura *et al.*, 1997, 1999, 2001

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
	15- days-old shoots	BR (5)	0.073-11.7	Bancos *et al.*, 2002
	15- days-old shoots	CS	0.69	Bancos *et al.*, 2002
	shoots (36 d old)	BL	0.164	Nomura *et al.*, 1997
	shoots (36 d old)	CS	0.355	Nomura *et al.*, 1997
	shoots (36 d old)	BR (1)	3.133	Nomura *et al.*, 1997
	6 months old plants	BL	0.84	Nomura *et al.*, 1997
	6 months old plants	CS	2.36	Nomura *et al.*, 1997
	6 months old plants	BR (1)	0.995	Nomura *et al.*, 1997
	49-d-old shoots	CS	0.491	Nomura *et al.*, 1999
	49-d-old shoots	BR (7)	0.02-2.937	Nomura *et al.*, 1999
	15- days-old roots	BR (6)	0.002-5.1	Bancos *et al.*, 2002
	15- days-old roots	BL	0.024	Bancos *et al.*, 2002
	15- days-old roots	CS	0.038	Bancos *et al.*, 2002
Hamamelidaceae				
Distylium racemosum Sieb. Et Zucc.	galls	CS	2500	Ikekawa *et al.*, 1984[3]
	galls	BR (1)	5	Ikekawa *et al.*, 1984[3]
	leaves	BL	0.023	Ikekawa *et al.*, 1984[3], Abe *et al.*, 1994
	leaves	CS	0.13	Ikekawa *et al.*, 1984[3], Abe *et al.*, 1994
	leaves	BR(4)	0.016-0.16	Ikekawa *et al.*, 1984[3], Abe *et al.*, 1994
Myrtaceae				
Eucalyptus calophylla R. Br.	pollen	BL	unspecified	Takatsuto, 1994[3]
Eucalyptus marginata Sn.	pollen	BR (1)	unspecified	Takatsuto, 1994[3]
Rosaceae				
Loquat (*Eriobotyra japonica* (Thunb.) Lindl.)	flower, buds	CS	unspecified	Takatsuto, 1994[3]
Apple (*Malus domestica*)	fruit	24-epiBL	27	Khripach *et al*, 2013
	fruit	BL	35	Khripach *et al*, 2013
	fruit	28-homoBL	10	Khripach *et al*, 2013
Rutaceae				
Bael tree (*Aegle marmelos* Corr.)	leaves	24-epiBL	unspecidied	Sondhi *et al.*, 2008
Satsuma orange (*Citrus unshiu* Marcov.)	pollen	BL, CS, BR (2)	unspecified	Abe, 1991[3]
Orange (*Citrus sinensis* Osbeck)	pollen	BL	36.2	Motegi *et al.*, 1994
	pollen	CS	29.4	Motegi *et al.*, 1994
Theaceae				

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
Chinese Tea (*Thea sinensis* L.)	leaves	BL	0.006	Abe *et al.* 1983[3], 1984a; Morishita *et al.*, 1983[3]; Ikekawa *et al.*, 1984[3]
	leaves	CS	0.1	Abe *et al.* 1983[3], 1984a; Morishita *et al.*, 1983[3]; Ikekawa *et al.*, 1984[3]
	leaves	BR (4)	<0.001-0.06	Abe *et al.* 1983[3], 1984a; Morishita *et al.*, 1983[3]; Ikekawa *et al.*, 1984[3]
	seeds	BR (6)	unspecified	Kaur *et al.*, 2002[3]
Green tea	leaves	24-epiBL	100	Khripach *et al*, 2013, Gupta *et al.*, 2004
	leaves	BL	0.0046	Ikekawa & Takatsuto, 1984
	leaves	CS	0.11	Ikekawa & Takatsuto, 1984
	leaves	BR (6)	0.002	Ikekawa & Takatsuto, 1984, Gupta *et al.*, 2004
Dicotyledons – Sympetalae				
Apocynaceae				
Catharanthus roseus G. Don.	culture cells	BL	0.4-8.7	Choi *et al.*, 1993, 1996[3], 1997[3] ; Fujioka *et al.*, 1995, 2000b[3] ; Park *et al.*, 1989 ; Suzuki *et al.*, 1993[3], 1994a[3], c, 1995 ; Yokota *et al.*, 1990 ; Choe *et al.*, 2001 ; Fujioka *et al.*, 2002
	culture cells	CS	0.6-4.5	Choi *et al.*, 1993, 1996[3], 1997[3] ; Fujioka *et al.*, 1995, 2000b[3] ; Park *et al.*, 1989 ; Suzuki *et al.*, 1993[3], 1994a[3], c, 1995 ; Yokota *et al.*, 1990 ; Choe *et al.*, 2001 ; Fujioka *et al.*, 2002
	culture cells	BR (17)	0.047-30	Choi *et al.*, 1993, 1996[3], 1997[3] ; Fujioka *et al.*, 1995, 2000b[3] ; Park *et al.*, 1989 ; Suzuki *et al.*, 1993[3], 1994a[3], c, 1995 ; Yokota *et al.*, 1990 ; Choe *et al.*, 2001 ; Fujioka *et al.*, 2002
Asteraceae				
Common sunflower (*Helianthus anuus* L.)	pollen	BL	106	Takatsuto *et al.*, 1989
	pollen	CS	21	Takatsuto *et al.*, 1989
	pollen	BR (1)	65	Takatsuto *et al.*, 1989
	breaking wall pollen	24-epiBL	1930	Pan *et al.*, 2012
Solidago altissima L.	shoot	BL	unspecified	Takatsuto, 1994[3]
Zinnia elegans L.	culture cells	CS, BR (4)	unspecified	Yamamoto *et al.*, 2001
Boraginaceae				
Echium plantagineum L.	pollen	BL	unspecified	Takatsuto, 1994[3]
Convolvulaceae				
Pharbitis purpurea Voigt	seeds	CS	1.1	Suzuki *et al.*, 1985
	seeds	BR (1)	0.2	Suzuki *et al.*, 1985
Cucurbitaceae				
Cucurbita moschata	seeds	BL, CS	unspecified	Jang *et al.*, 2000

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
Duch.				
Lamiaceae				
Perilla frutescens (L.) Britt.	seeds	CS	unspecified	Park *et al*., 1994b
Plantaginaceae				
Coastal water hyssop (*Bacopa monnieri* L.)	Fresh leaves	24-epiBL	unspecified	Tripathi & Sharma, 2015
Rubiaceae				
Coffee (Coffea arabica)	bean	24-epiBL	30	Khripach *et al*., 2013
	bean	BL	250	Khripach *et al*., 2013
	bean	28-homoBL	23	Khripach *et al*., 2013
Solanaceae				
Tobacco (*Nicotiana tabacum* L.)	culture cells	CS	unspecified	Park *et al*., 1994b
Tomato (*Lycopersicum esculentum* Mill.)	shoot	CS	0.2	Yokota *et al*., 1997d
	shoot	BR (2)	0.03-1.7	Yokota *et al*., 1997d
	shoot (dwarf mutant)	BL	<0.001	Bishop *et al*., 1999
	shoot (dwarf mutant)	CS	0.2	Bishop *et al*., 1999
	shoot (dwarf mutant)	BR (10)	<0.001-52	Bishop *et al*., 1999
	36- days-old shoots	BR (5)	0.016-0.64	Bancos *et al.*, 2002
	36- days-old shoots	CS	0.14	Bancos *et al.*, 2002
	36- days-old roots	BR (5)	0.062-2.8	Bancos *et al.*, 2002
	36- days-old roots	CS	0.011	Bancos *et al.*, 2002
Potato (*Solanum tuberosum*)	tuber	24-epiBL	37.5	Khripach *et al*., 2013
	tuber	BL	10	Khripach *et al*., 2013
	tuber	28-homoBL	1.5	Khripach *et al*., 2013
	tuber	Epi-CS	1.7	Khripach *et al*., 2013
Gymnosperms				
Cupressaceae				
Cupressus arizonica Greene	pollen	BL	<1	Griffiths *et al*., 1995
	pollen	CS	1000	Griffiths *et al*., 1995
	pollen	BR (7)	2-6400	Griffiths *et al*., 1995
Ginkgoaceae				
Ginkgo biloba L.	seeds	BR (1)	15	Takatsuto *et al*., 1996a
Pinaceae				
Picea sitchensis Trantv. ex Mey	shoot	CS	5	Yokota *et al*., 1985[3]
	shoot	BR (1)	7	Yokota *et al*., 1985[3]
Pinus silvestris L.	cambial region	BL, CS	unspecified	Kim *et al*., 1990
Pinus thunbergii Parl.	pollen	BR (1)	89.5	Yokota *et al*., 1983a
Taxodiaceae				
Crypromeria japonica D. Don.	pollen, anther	28-homoBL, BR (8)	unspecified	Yokota *et al*., 1998, Watanabe *et al*., 2000

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
Lower plants				
Athyriaceae				
Black lady fern (*Deparia japonica*)	fertile frond	CS	0.008	Yokota *et al*., 2017
	fertile frond	BR (7)	0.013-4.867	Yokota *et al*., 2017
Asian common ladyfern (*Athyrium yokoscense*)	reproductive frond	CS	0.002	Yokota *et al*., 2017
	reproductive frond	BR (6)	0.073-4.807	Yokota *et al*., 2017
Dennstaedtiaceae				
Eagle fern (*Pteridium aquilinum*)	vegetative frond	CS	0.003	Yokota *et al*., 2017
	vegetative frond	BR (7)	0.021-1.873	Yokota *et al*., 2017
Dryopteridaceae				
Wood fern (*Dryopteris crassirhizoma*)	fertile frond	CS	0.024	Yokota *et al*., 2017
	fertile frond	BR (3)	0.019-0.802	Yokota *et al*., 2017
Autumn fern (*Dryopteris erythrosora*)	reproductive shoot	CS	0.005	Yokota *et al*., 2017
	reproductive shoot	BR (6)	0.008-20.87	Yokota *et al*., 2017
Cyrtomium laetevirens	reproductive shoot	CS	0.002	Yokota *et al*., 2017
	reproductive shoot	BR (5)	0.006-3.172	Yokota *et al*., 2017
Equisetaceae				
Field Horsetail (*Equisetum arvense* L.)	whole plant	CS	0.17	Takatsuto *et al*., 1990a
	whole plant	BR (3)	0.15-0.75	Takatsuto *et al*., 1990a
	shoot	CS	0.003-0.008	Yokota *et al*., 2017
	shoot	BR (8)	0.02-2	Yokota *et al*., 2017
Funariaceae				
Spreading earth-moss (*Physcomitrella patens*)	protonema	CS	0.004	Yokota *et al*., 2017
	protonema	BR (8)	0.008-1.122	Yokota *et al*., 2017
Lygodiaceae				
Vine-like fern (*Lygodium japonicum*)	vegetative frond	CS	0.016	Yokota *et al*., 2017
	vegetative frond	BR (7)	0.005-25.41	Yokota *et al*., 2017
Marchantiaceae				
Common liverwort (*Marchantia polymorpha* L.)	culture cells	BR (3)	unspecified	Park *et al*., 1999
	thallus	CS	0.006-0.038	Yokota *et al*., 2017
	thallus	BR (6)	0.001-0.139	Yokota *et al*., 2017
	on agar medium	CS	0.007	Yokota *et al*., 2017
	on agar medium	BR (5)	0.002-0.119	Yokota *et al*., 2017
Onocleaceae				
Bead fern (*Onoclea sensibilis*)	vegetative frond	CS	0.003	Yokota *et al*., 2017

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
	vegetative frond	BR (3)	0.063-0.19	Yokota *et al.*, 2017
Fiddlehead fern (*Matteuccia struthiopteris*)	vegetative frond	CS	0.016	Yokota *et al.*, 2017
	vegetative frond	BR (3)	0.15-1.175	Yokota *et al.*, 2017
Osmundaceae				
Asian royal fern (*Osmunda japonica*)	vegetative frond	CS	0.004-0.005	Yokota *et al.*, 2017
	vegetative frond	BR (11)	0.007-202.9	Yokota *et al.*, 2017
Selaginellaceae				
Spikemoss (*Selaginella moellendorffii*)	frond	CS	0.02	Yokota *et al.*, 2017
	frond	BR (2)	<0.042-0.084	Yokota *et al.*, 2017
Blue Spikemoss (*Selaginella uncinata*)	frond	CS	0.006	Yokota *et al.*, 2017
	frond	BR (6)	0.007-0.275	Yokota *et al.*, 2017
Thelypteridaceae				
Japanese Beech Fern (*Thelypteris decursive-pinnata*)	fertile frond	CS	0.015	Yokota *et al.*, 2017
	fertile frond	BR (7)	0.025-5.119	Yokota *et al.*, 2017
Marsh fern (*Thelypteris palustris*)	vegetative frond	BR (6)	0.002-1.122	Yokota *et al.*, 2017
Algae				
Chaetophoraceae				
Green algae *(Stigeoclonium nanum*)	cultured cells	BL	168.7 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	144.9 µg/kg dr. wt	Stirk *et al.*, 2013
Chlamydomonadaceae				
Chlamydomonas reinhardtii	cultured cells	BL	162.9 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	153.8 µg/kg dr. wt	Stirk *et al.*, 2013
Protococcus viridis	cultured cells	BL	211.6 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	134.8 µg/kg dr. wt	Stirk *et al.*, 2013
Chlamydomonadaceae				
	cultured cells	BL		Stirk *et al.*, 2013
Chlorellaceae				
Green algae (*Clorella vulgaris*)	cultured cells	BL	0.07	Bajguz, 2009
Green algae (*Clorella vulgaris*)	cultured cells	CS	0.47	Bajguz, 2009
Green algae (*Clorella vulgaris*)	cultured cells	BR (5)	0.18-0.39	Bajguz, 2009
Green algae (*Clorella pyrenoidosa*)	cultured cells	BL	253 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	158 µg/kg dr. wt	Stirk *et al.*, 2013
Green algae (*Clorella vulgaris*)	cultured cells	BL	193.3 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	215.3 µg/kg dr. wt	Stirk *et al.*, 2013
Green algae (*Clorella minutissima*)	cultured cells	BL	306.5 µg/kg dr. wt	Stirk *et al.*, 2013

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
	cultured cells	CS	215.3 µg/kg dr. wt	Stirk *et al.*, 2013
Chlorococcaceae				
Green algae *Chlorococcum ellipsoideum*	cultured cells	BL	168.7 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	105.7 µg/kg dr. wt	Stirk *et al.*, 2013
Green algae *Nautococcus mamillatus*	cultured cells	BL	115.8 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	99.9 µg/kg dr. wt	Stirk *et al.*, 2013
Green algae *Spongiochloris excentrica*	cultured cells	BL	131.2 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	108.5 µg/kg dr. wt	Stirk *et al.*, 2013
Coccomyxaceae				
Green algae Coccomyxa sp.	cultured cells	BL	205.8 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	177.1 µg/kg dr. wt	Stirk *et al.*, 2013
Hydrodictyaceae				
Green algae *(Hydrodictyon reticulatum* (L.) Lager	cultured cells	24-epiCS	0.3	Yokota *et al.*, 1987b[3]
	cultured cells	28-homoCS	4	Yokota *et al.*, 1987b[3]
Klebsormidiaceae				
Green algae *(Klebsormidium flaccidum)*	cultured cells	BL	548.7 µg/kg dr. wt	Stirk *et al.*, 2013
		CS	429.1 µg/kg dr. wt	Stirk *et al.*, 2013
Neochloridaceae				
Green algae (*Poloidion didymos*)	cultured cells	BL	167.3 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	172.8 µg/kg dr. wt	Stirk *et al.*, 2013
Palmellaceae				
Green algae (*Gyoerffyana humicola)*	cultured cells	BL	270.9 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	201.1 µg/kg dr. wt	Stirk *et al.*, 2013
Prasiolaceae				
Green algae *(Stichococcus bacillaris)*	cultured cells	BL	291.8 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	242.7 µg/kg dr. wt	Stirk *et al.*, 2013
Protosiphonaceae				
Green algae *(Protosiphon botryoides)*	cultured cells	BL	100.6 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	74 µg/kg dr. wt	Stirk *et al.*, 2013
Scenedesmaceae				
Green algae *(Acutodesmus acuminatus)*	cultured cells	BL	125.1 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	105.5 µg/kg dr. wt	Stirk *et al.*, 2013
Green algae *(Acutodesmus incrassatulus)*	cultured cells	BL	124.8 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	92.6 µg/kg dr. wt	Stirk *et al.*, 2013
Green algae *(Desmodesmus armatus)*	cultured cells	BL	125.1 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	109.3 µg/kg dr. wt	Stirk *et al.*, 2013

Family/Species	Examined part	Brassino-steroid[1]	Content µg/kg fr. wt.[2]	References
Green algae *(Scotiellopsis terrestris)*	cultured cells	BL	336.9 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	235.9 µg/kg dr. wt	Stirk *et al.*, 2013
Green algae *(Coelastrum microporum)*	cultured cells	BL	199.2 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	158.3 µg/kg dr. wt	Stirk *et al.*, 2013
Selenastraceae				
Green algae *(Monoraphidium contortum)*	cultured cells	BL	284.9 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	195 µg/kg dr. wt	Stirk *et al.*, 2013
Green algae *(Raphidocelis subcapitata)*	cultured cells	BL	58.6 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	58.7 µg/kg dr. wt	Stirk *et al.*, 2013
Trebouxiaceae				
Green algae *(Myrmecia bisecta)*	cultured cells	BL	202.4 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	164.3 µg/kg dr. wt	Stirk *et al.*, 2013
Ulotrichaceae				
Green algae (Ulothrix sp.)	cultured cells	BL	84.9 µg/kg dr. wt	Stirk *et al.*, 2013
	cultured cells	CS	74.2 µg/kg dr. wt	Stirk *et al.*, 2013
Crystoseiraceae				
Brown algae (*Cystoseira myrica* (Gmelin) Agardh)	whole plant	BR	unspecified	Hamdy *et al.*, 2009
Fungi				
Cercospora arachidicola	unspecified	unspecified	unspecified	Zakharychev, 1999[3] in Tsavkelova *et al.*, 2006
Processed foods				
Juice and Wines				
Apple juice	juice	24-epiBL	12	Khripach *et al.*, 2013
Apple juice	juice	BL	1.7	Khripach *et al.*, 2013
Apple juice	juice	28-homoBL	3	Khripach *et al.*, 2013
Grape juice	juice	24-epiBL	1.7	Khripach *et al.*, 2013
Grape juice	juice	BL	1.8	Khripach *et al.*, 2013
Grape juice	juice	28-homoBL	0.4	Khripach *et al.*, 2013
Pineapple juice	juice	24-epiBL	3	Khripach *et al.*, 2013
Pineapple juice	juice	BL	1.6	Khripach *et al.*, 2013
Pineapple juice	juice	28-homoBL	0.5	Khripach *et al.*, 2013
Birch juice	juice	24-epiBL	0.5	Khripach *et al.*, 2013
Birch juice	juice	BL	1.2	Khripach *et al.*, 2013
Birch juice	juice	28-homoBL	0.1	Khripach *et al.*, 2013
Dry red wine (Merlot)	wine	24-epiBL	3	Khripach *et al.*, 2013
Dry red wine (Merlot)	wine	BL	10	Khripach *et al.*, 2013
Dry red wine (Merlot)	wine	28-homoBL	4.2	Khripach *et al.*, 2013
Honey				
Honey		24-epiBL	7.4	Khripach *et al.*, 2013
Honey		BL	1	Khripach *et al.*, 2013

[1] 24-epiBL = 24-Epibrassinolide; 24-epiCS=24-Epicastasterone (precursor of 24-Epibrassinolide); BL = Brassinolide; CS=Castasterone (precursor of Brassinolide); 28-homoBL = 28-Homobrassinolide; 28-homoCS = 28-Homocastasterone (precursor of 28-Homobrassinolide); BR (Nr.)= Other Brassinosteroids (Number)

[2] Amount of Brassinosteroid is expressed in µg/kg fresh weight, if not specified otherwise

[3] Cited in the review publications Bajguz and Tretyn (2003) and Hayat and Ahmad (2011).

24-Epibrassinolide elicits and activates the plant's self-defence mechanisms mediating the plant's resistance to unfavourable environmental conditions, (e.g. salinity, drought, cold and heat stress) and fungal diseases.

Application of brassinosteroids leads to a complex sequence of biochemical reactions such as activation or suppression of key enzymatic reactions, induction of protein synthesis and the production of various chemical defence compounds (Bajguz and Hayat, 2009). Brassinosteroid treated plants are not only more tolerant to biotic but also to abiotic stresses, providing a solution for problems that could arise in agriculture in the course of the climate change (Eremina *et al*., 2016).

Humans are constantly exposed to 24-Epibrassinolide through consumption of plants and plant organs, e.g. seeds, roots, and leaves (0.22 - 378 µg/kg), as well as other natural and processed foods such as honey (7.4 µg/kg), fruit juices (0.5 - 12 µg/kg) and wine (3 µg/kg) (Table 6-2).

Plant sterols and stanols are used as dietary food supplements, and their safety and efficacy have been reviewed by EFSA in several publications (see examples below). Generally, up-take of up to 3 g of plant sterols/stanols per day is considered safe and beneficial:

> EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2014. Scientific Opinion on the modification of the authorisation of a health claim related to plant sterol esters and lowering blood LDL-cholesterol; high blood LDL-cholesterol is a risk factor in the development of (coronary) heart disease pursuant to Article 14 of Regulation (EC) No 1924/2006, following a request in accordance with Article 19 of Regulation (EC) No 1924/2006. EFSA Journal 2014;12(2):3577, 14 pp. doi:10.2903/j.efsa.2014.3577
>
> EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA); Scientific Opinion on the substantiation of a health claim related to 3 g/day plant stanols as plant stanol esters and lowering blood LDL-cholesterol and reduced risk of (coronary) heart disease pursuant to Article 14 of Regulation (EC) No 1924/2006. EFSA Journal 2012;10(5):2692. [18 pp.]. doi:10.2903/j.efsa.2012.2692

Composition of these food supplements, in agreement with Decision 2004/336/CE of 31 March 2004, is:

> β-sitosterol < 80%
> β-sitostanol < 15%
> Campesterol < 40%
> Campestanol < 5%
> Stigmasterol < 30%
> Brassicasterol < 3%
> and other sterols/stanols < 3%

Brassinosteroids are also non-toxic to non-target organisms. Mammals, aquatic organisms, insects, and soil organisms are constantly exposed to Brassinosteroids through the consumption of Brassinosteroids contained in higher and lower plants (present in soil, fresh- and seawater). Furthermore, no effects on soil microorganisms are expected. Not only are certain soil microorganisms able to metabolize Brassinosteroids, but some microorganisms are also able to synthesize Brassinosteriods themselves (Tsavkelova *et al*., 2006).

Non-target soil organisms are constantly exposed to Brassinosteroids, not only from the constant release of Brassinosteroid from decaying plant material (e.g. Aremu *et al*., 2015) but also from the Brassinosteroid precursors, campesterol, sitosterol, and stigmasterol. These precursors are known root exudates and are involved in the mediation of interactions in the rhizosphere, which includes the symbiotic associations with beneficial microbes, such as mycorrhizae, rhizobia, and plant growth-promoting rhizobacteria (PGPR) (Badri and Vivanco, 2009).

Due to the constant formation and decomposition of plant root systems, the presence of seeds, pollen, and decomposing plant material and the release of Brassinosteroids from decomposing organic matter (e.g. Aremu *et al*., 2015) as well as the vast number of other Brassinosteroid producing organisms such as algae in the environment, Brassinosteroids – and other phytosterols – are naturally present in all environmental compartments including soil e.g. Aremu *et al*., 2015) and water-bodies including sediment (Hassett & Lee, 1977; Mudge *et al*., 1999).

In addition to that, bioaccumulation is not expected as Brassinosteroids are readily absorbed and metabolised by higher and lower plants (e.g. Nishikawa *et al.*, 1994), diatoms (e.g. Mekhalfi *et al.,* 2012), green algae (e.g. Bajguz, 2011), fungi (e.g. Voigt *et al*., 1993), mycobacteria (e.g. Vorbrot *et al.,* 1991), and cyanobacteria (e.g. Saygideger

and Deniz, 2008). As Brassinosteroids are phylogenetically ancient phytohormones, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones. It was further found that Brassinosteroid synthesis in plants is naturally triggered for example by microorganisms (Asari *et al.*, 2017).

24-Epibrassinolide can be considered as low risk active substance in accordance with Regulation (EC) 1107/2009, Annex II point 5, as it is not classified as carcinogenic, mutagenic, toxic to reproduction, sensitising, very toxic or toxic, explosive or corrosive and it is not considered persistent, bio-accumulating, endocrine disrupting or neuro- or immunotoxic. Further, it fulfils all low risk criteria indicated in Regulation (EU) 2017/1432 of 7 August 2017. In addition, it is a natural, ubiquitous occurring plant molecule, which is expected to have no negative effects on the environment, non-target organism or humans.

B.6.1. ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION IN MAMMALS

No guideline-compliant studies on ADME behaviour of 24-epibrassinolide have been conducted.
Several articles have been cited by the applicant illustrating that plant sterols are ubiquitously present in plants and thus, humans are continually exposed to them. Dedicated studies are not considered necessary since natural background exposure via food is considered higher than exposure through the use as a plant protection product.

Brassinosteroids are found throughout the plant kingdom and are widely distributed in lower and higher plants. Until today, 69 natural brassinosteroids have been identified and have been detected in all plant organs, including pollen, anthers, seeds, leaves, stems, roots, flowers, fruits and grains. Through the dietary intake of plants and plant products, humans and animals are constantly exposed to Brassinosteroids and their metabolites. Highest concentrations of brassinosteoids in food are found in vegetable oils and nuts (Ogbe RJ, 2015).
The highest concentrations of Brassinosteroids in plants are found in pollen, immature seeds and fruits at a range of 1–100 µg/kg fresh weight, while shoots and leaves usually contain lower amounts of 0.01–0.1 µg/kg fresh weight, for details see general introduction.

A review article published in 2015 summarizes much of the current knowledge on phytosterol occurrence in plants, metabolism in mammals, and their current use as food additives (**Ogbe RJ, 2015**), the section on absorption and metabolism from this review is copied here in this section:
[…]
Though various diets contain similar amounts of phytosterols and cholesterol, serum phytosterol concentrations are usually several hundred times lower than serum cholesterol levels in humans. It was reported that less than 10% of dietary phytosterols are systematically absorbed, in contrast to about 50 – 60% of dietary cholesterol. Like cholesterol, phytosterols are incorporated into mixed micelles before they are taken up by enterocytes. Once inside the enterocytes their systemic absorption is inhibited by the activity of efflux transporters, consisting of a pair of ATP-binding cassette (ABC) proteins known as ABCG5 and ABCG8. ABCG5 and ABCG8 each forms one half of a transporter that secretes phytosterols and unesterified cholesterol from the enterocyte into the interestinal lumen. Phytosterols are secreted back into the intestine by ABCG5/G8 transporters at a much greater rate than cholesterol, resulting in much lower intestinal absorption of dietary phytosterol than cholesterol. Within the enterocytes, phytosterols are not as readily esterified as cholesterol, so they are incorporated into chylomicrons at much lower concentrations. Those phytosterols that are incorporated into chylomicrons enter blood circulation and are taken up by the liver. Once inside the liver, phytosterols are metabolized into cholesterol and other metabolites, by the action of several enzymes and a key enzyme called cholesterol 7α-hydroxylase into bile acids, and rapidly secreted into bile by hepatic ABC G5/G8 transporters. This enzyme is a regulatory enzyme in bile acids biosynthesis. Even though cholesterol could also be secreted into bile, the rate of phytosterol secretion into bile is greater than cholesterol secretion. Therefore, the low serum concentrations of phytosterols compared to cholesterol can be explained by decreased intestinal absorption and increased excretion of phytosterols into bile.
[…]

Through the dietary intake of plants and plant products, humans and animals are constantly exposed to brassinosteroids and their metabolites. Harmful effects are not likely from the use of 24-epibrassinolide in agriculture. In the light of these considerations, unnecessary animal testing should be avoided. The available literature indicates that plant sterols are metabolized alongside with cholesterol to bile acids and rapidly excreted.

The table below summarizes scientific literature submitted by the applicant on ADME behavior of brassinosteroids and RMS conclusion on relevance / reliability in the last column. No adverse data or data raising concern was identified in any of the publications submitted.

Table 6.1-1: Summary of Other Scientifically Relevant Information submitted by the applicant on ADME

Study Title	Reference Report number	Article type	Study Content	Relevance / Reliability
A REVIEW ON DIETARY PHYTOSTEROLS: THEIR OCCURRENCE, METABOLISM AND HEALTH BENEFITS Report No.: na (092-147) Asian Journal of Plant Science and Research, 2015, 5(4) 10-21 Not GLP, published	Ogbe RJ, 2015 092-147	Review	Survey of scientific literature about the occurrence of phytosterols, their bioavailability and metabolism, biological activities and health benefits. Contains also information on the safety and any notable adverse effect associated with the consumption of these phytochemicals.	Highly relevant, concise summary of up-to-date knowlegde on plant sterol use, metabolism and health effects
SUPPRESSION OF CHLORELLA VULGARIS GROWTH BY CADMIUM, LEAD, AND COPPER STRESS AND ITS RESTORATION BY ENDOGENOUS BRASSINOLIDE	Bajguz A et al, 2011 092-103	Research article	In order to elaborate their roles in plants subjected to heavy metals stress, *Chlorella vulgaris* cultures were treated with 10^{-8} M brassinolide (BL) were exposed to 10^{-6} to 10^{-4} M heavy metals (cadmium, lead and copper). Application of BL to *C. vulgaris* cultures reduced heavy metals stress and effects on growth, prevented chlorophyll, monosaccharides, and protein loss, and increased phytochelatins content. The arrested growth of *C. vulgaris* cells treated with heavy metals was restored by the coapplication of BL.	Not relevant, no information on ADME behavior of brassinolides in mammalian organisms
BRASSINOSTEROID SIGNALLING	Zhu JY et al, 2013 092-165	Review	The authors provide an overview of the brassinosteroid signalling network in plants and explain how this steroid hormone functions as a master regulator of plant growth, development and metabolism.	Not relevant, no information on ADME behavior of brassinolides in mammalian organisms
TWENTY YEARS OF BRASSINOSTEROIDS: STEROIDAL PLANT HORMONES WARRANT BETTER CROPS FOR THE XXI CENTURY	Khripach VA et al, 2000 092-029	Review	Reviews many aspects of brassinolide biology like occurrence and biological effects in plants.	Not relevant, no information on ADME behavior of brassinolides in mammalian organisms
ENZYME IMMUNOASSAY OF (24R)-BRASSINOSTEROIDS	Khripach VA et al, 2007 092-119	Research article	Describes the generation of several antibodies that bind to various brassinosteroids.	Not relevant, no information on ADME behavior of brassinolides in mammalian organisms
STEROID PLANT HORMONES: EFFECTS OUTSIDE PLANT KINGDOM	Zhabinskii VN et al, 2015 092-099	Review	Reviews available knowledge on effects of steroid plant hormones on insects, funghi, fish, protozoa, and warm blooded animals. Results of pharmacokinetic studies with radiolabelled 24-epibrassinolide in rats are described: ADME of EBl was	Contains relevant information on tissue distribution of 24-epibrassinolide, but low reliability as no experimental

Study Title	Reference Report number	Article type	Study Content	Relevance / Reliability
			studied in rats by intragastric administration of its ^{3}H-Iabelled form. It was well absorbed from the gastrointestinal tract following the administration and quickly distributed to blood, liver, intestine, lungs and kidneys. The serum highest radioactivity was reached in 30 min after administration. The serum half-life was about 3 h after administration. Similarly, the highest activity in liver also took place after 30 min and then it gradually decreased. The accumulation of ^{3}H-24-epibrassinolide (and/or its metabolites) went slower in kidneys, where its highest level could be seen after 6 h. The quickest 24-epibrassinolide-accumulating organ was found to be the small intestine, where only 15 min were needed to reach its highest concentration. Since significant amounts of ^{3}H-24-epibrassinolide and (or) products of its biotransformation were found in kidneys, urine and faeces of experimental animals, it was concluded that these are the major ways for its elimination from the body.	details are reported.
24-EPIBRASSINOLIDE PHARMACOKINETIC STUDIES	Sauchuk AL et al, 2016 092-124	Oral communication	Plasma concentration profiles and pharmacokinetic parameters have been obtained following single dose intragastric or intraperitoneal administration of 24-epibrassinolide (150 µg per 200 g) in Wistar rats. 24-epibrassinolide was detected using a developed ELISA method. The blood plasma highest concentration was reached in 5-10 min after administration. The plasma elimination half-life of 24-epibrassinolide was about 50 min after administration.	Contains relevant information on tissue distribution and elimination of 24-epibrassinolide, but low reliability as no experimental details are reported.
INTERACTION OF HUMAN STEROID 7ALPHA-HYDROXYLASES WITH BRASSINOSTEROIDS	Dzichenka YV et al, 2016 092-116	Abstract	CYP7A1, but not CPY7B1 or CYP39A1, binds to epibrassinolide.	Low relevance and low reliability as no experimental details are reported
ISOLATION AND CHARACTERIZATION OF BRASSINOSTEROIDS FROM ALGAL CULTURES OF CHLORELLA	Bajguz A, 2009 092-013	Short communication	Seven brassinosteroids, including teasterone, typhasterol, 6-deoxoteasterone, 6-deoxotyphasterol, 6-deoxocastasterone, castasterone and brassinolide, were identified by	Not relevant, no information on ADME behavior of brassinolides in mammalian

Study Title	Reference Report number	Article type	Study Content	Relevance / Reliability
VULGARIS BEIJERINCK (TREBOUXIOPHYCEAE)			GC-MS in *Chlorella vulgaris.*	organisms
HYPOCHOLESTEROL-EMIC MECHANISM OF CHLORELLA: CHLORELLA AND ITS INDIGESTIBLE FRACTION ENHANCE HEPATIC CHOLESTEROL CATABOLISM THROUGH UP-REGULATION OF CHOLESTEROL 7α-HYDROXYLASE IN RATS	Shibata, S et al, 2007 092-151	Research article	The study authors conclude that *Chlorella* powder enhances the hepatic degradation of cholesterol by up-regulating the expression of CYP7A1 in rats with or without diet-induced hypercholesterolemia. Based on data on hepatic enzyme activities and gene expression profiles, induction of CYP7A1 expression is considered to be the most important mechanism in the liver contributing to the hypocholesterolemic effect of *Chlorella* powder.	Not relevant, no information on ADME behavior of brassinolides

B.6.1.1. Absorption, distribution, metabolism and excretion by oral route

No data available, not required.

B.6.1.2. Absorption, distribution, metabolism and excretion by other routes

No data available, not required.

B.6.2. ACUTE TOXICITY

Results of the available guideline-compliant studies on 24-epibrassinolide are considered conclusive and sufficient for classification.

For acute oral toxicity, the LD_{50} value observed in the available guideline-compliant study is higher than 5000 mg/kg bw.
Subsances whose LD_{50} is ≥ 2000 mg/kg bw shall not be classified for acute toxicity via the oral route according to the criteria in CLP Annex I, 3.1.2 to 3.1.3.4.

For acute dermal toxicity, the LD_{50} value observed in the avialable guideline-compliant study is higher than 2000 mg/kg bw.
Subsances whose LD_{50} is ≥ 2000 mg/kg bw shall not be classified for acute toxicity via the dermal route according to the criteria in CLP Annex I, 3.1.2 to 3.1.3.4.

For acute inhalation toxicity, the LC_{50} value observed in the available guideline-compliant study is higher than 1.08 mg/L (highest attainable concentration, dust).
Subsances whose LD_{50} is ≥ 5 mg/L shall not be classified for acute toxicity via the inhalation route according to the criteria in CLP Annex I, 3.1.2 to 3.1.3.4.

For skin irritation, no oedema or erythema were observed in the available guideline-compliant study. According to the criteria in CLP, this substance shall not be classified for skin irritation.

For eye irritation, no ocular or iridal effects were observed in the available guideline-compliant study. According to the criteria in CLP, this substance shall not be classified for eye irritation.

For skin sensitisation, no effects were observed in the available guideline-compliant study, according to the criteria in CLP, this substance shall not be classified for eye irritation.

Table 6.2-1: Summary of Available Guideline-Compliant Studies on Acute Toxicity

Parameter	Species	Result	Classification/ remarks	Reference
Acute oral	Wistar Rat	LD_{50} > 5000 mg/kg bw (female)	None	[redacted] (2017)
Acute dermal	Wistar Rat	LD_{50} > 2000 mg/kg bw (male/female)	None	[redacted] (2017)
Acute inhalation (4 hours)	Wistar Rat	LC_{50} > 1.08 mg/L (male/female)	None	[redacted] (2017)
Skin irritation	Young Adult New Zealand White Rabbits	Non Irritant	None	[redacted] (2017)
Eye irritation	Young Adult New Zealand White Rabbits	Non Irritant	None	[redacted] (2017)
Skin sensitisation (Magnusson and Kligman)	Albino Dunkin Hartley Guinea Pig	Not sensitising	None	[redacted] (2017)

Table 6.2-2: Summary of Other Scientifically Relevant Information on Acute Toxicity from Open Literature

Parameter	Species	Result	Classification/ remarks	Reference
Acute oral and dermal toxicity, skin and eye irritation	Rat, mouse, and rabbit	Studies performed at the Sanitary-Hygienic Institute of Belarus for 24-epibrassinolide are cited: Acute toxicity (oral route): LD_{50} > 1000 mg/kg (female mouse) LD_{50} > 2000 mg/kg (male/female rat)	No classification required, no experimental details are reported, no specific references are	Khripach, V., et al. (2000)

Parameter	Species	Result	Classification/ remarks	Reference
		Acute toxicity (dermal route) LD_{50} > 2000 mg/kg (male/female rat) Skin and Eye irritation: Negative (rabbit)	cited in the article	
Acute oral and dermal toxicity, and eye irritation	Rat, mouse, and rabbit	The toxicity of epibrassinolide was investigated by researchers at Nippon Kayaku Co. Acute toxicity (oral route): LD_{50} > 1000 mg/kg (mouse) LD_{50} > 2000 mg/kg (rat) Acute toxicity (dermal route): LD_{50} > 2000 mg/kg (rat) Eye irritation: Rabbit, 0.01% solution: negative	No classification required, no experimental details are reported, no specific references are cited in the article	Ikekawa, N., and Zhao, Y. J. (1991)
Acute oral toxicity, and skin sensitisation	Mouse	Acute toxicity (oral route): LD_{50} > 5000 mg/kg (mouse) Skin sensitisation: Mouse, intracutaneously: negative	No classification required, no experimental details are reported	Zhabinskii, V. N., et al. (2014)

B.6.2.1. Oral

B.6.2.1.1. Acute Oral Toxicity Study in Rats

Annex point	CA 5.2.1/01
Reference:	ACUTE ORAL TOXICITY STUDY IN RATS WITH 24-EPIBRASSINOLIDE (TGAI)
Author(s), year:	[redacted] (2017)
Report/Doc. number:	6113 (521-001)
Guideline(s):	OECD No. 423 (2002), OPPTS 870.1100, OPPTS 870.1000
GLP:	Yes
Deviations from OECD 423 (2002):	None
Acceptability:	Yes

Executive summary
The study was performed to assess the oral toxicological potential of the test item 24-Epibrassinolide (TGAI) in an *in vivo* test using Wistar rats. The test item was administered by oral gavage at a single dose of 5000 mg/kg body weight to three female animals. The test item was formulated in a vehicle (refined groundnut oil) at a concentration of 500 mg/mL. The dose volume was 10 mL/kg body weight. No clinical signs were observed and no substance related effects could be reported. No abnormalities were observed in any of the treated animals during necropsy at terminal sacrifice.
Based on the results, the median lethal dose of 24-Epibrassinolide (TGAI) after single oral administration to female rats, observed over a period of 14 days is:

LD_{50} Females > 5000 mg/kg body weight

I. MATERIALS AND METHODS

A. Test material and vehicle

1 Test materials

Test substance	24-Epibrassinolide (TGAI)
CAS no.	78821-43-9
Lot/batch	002-20150112
Purity	91.2% w/w
Expiry date	January 11, 2017
Storage conditions:	Room Temperature (20°C to 30°C)
Vehicle	Refined Groundnut Oil

2 Test animals

Species	Rat
Strain	Wistar Rats (*Rattus norvegicus)*
Age	9-10 weeks
Bodyweight at dosing	180.3 - 183.4 g
Source	[redacted]
Acclimation period	6 – 9 days
Diet	*Ad libitum* Teklad Certified Global 14% Protein Rodent Maintenance Diet (Batch No.2014C-111215MA) from ENVIGO
Water	*Ad libitum* tap water
Housing	Individually
Temperature	20.8 - 23.0°C
Humidity	55 – 65%
Ventilation	≥ 10 times/hour
Photoperiod	12 hours light / 12 hours dark

B. Study design and method

Test substance preparation and administration

The 24-Epibrassinolide (TGAI) content obtained in the dose concentration analysis was ranged from 98.33% to 98.87%. The dose formulations were prepared shortly before each dosing. The starting dose was 300 mg/kg body weight based on Annex 2c, OECD Guideline 423, adopted 17th December 2001.

The test item 24-Epibrassinolide (TGAI) was administered by oral gavage at a single dose of 5000 mg/kg body weight in three animals after being fasted for approximately 17 hours. The test item was formulated in a vehicle (refined groundnut oil) at a concentration of 500 mg/mL. The administration volume was 10 mL/kg body weight. Individual doses were calculated based on the fasted bodyweight of each animal which was recorded on the day of dosing (Day 0). Homogeneity of the test item in the vehicle was maintained during administration using a magnetic stirrer.

Main test

The animals received a single dose of the test item by oral gavage administration at 5000 mg/kg body weight after being fasted for approximately 17 hours, but with free access to drinking water. Initially animal no. 01 was dosed. As no mortality was observed, the remaining two animals were dosed with same amount of test item. Food was provided again at approximately 3 hours after dosing for all animals.

The administration volume was 10 mL/kg body weight. The animals were dosed using 15 G oral plastic feeding tubes.

All animals survived throughout the experimental period, no further testing was carried out.

Mortality and clinical observations

The animals were observed twice daily during the acclimatization period and mortality/viability and clinical signs were recorded. All animals were observed for clinical signs during first 30 minutes and at approximately 1, 2, 3 and 4 hours post test item administration on test day 0 and once daily during test days 1-14. Mortality/viability was recorded during first 30 minutes and at approximately 1, 2, 3 and 4 hours post administration on test day 0 (in common with the clinical signs) and twice daily during days 1-14 (once on day of sacrifice).

Body weight

Body weights were recorded on test day 0 (prior to administration), as well as test days 7 and 14.

Necropsy

All treated animals were euthanised at the end of the observation period by carbon dioxide asphyxiation in euthanasia chamber and discarded after the gross/macroscopic pathological changes were observed and recorded. No organs or tissues were retained.

C Statistics

Data were not analysed statistically.

II. RESULTS AND DISCUSSION

Mortality and clinical signs

No mortality was observed in any of the animals treated at 5000 mg/kg body weight

All the animals treated at the dose of 5000 mg/kg body weight did not display any clinical signs at approximately 30 minutes, at 1, 2, 3 and 4 hours on day 0 post test item administration until the last day of observation period (day 14).

Table 6.2.1.1-1: Acute oral toxicity: clinical signs

Dose	Animal Number	Sex	Test days																		
			0*					1	2	3	4	5	6	7	8	9	10	11	12	13	14
			0.5	1	2	3	4														
5000 mg/kg body weight	01	F	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	02	F	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	03	F	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Key: mg/kg = milligram/kilogram, F = Female, 1 = Normal,

No clinical signs were evident in any animal during the acclimatization period.

* Examinations were performed within the first 0.5 hours and at approximately 1, 2, 3 and 4 hours post treatment on test day 0.

Body weights
All the animals had gained body weight by day 7 and 14 as compared to day 0

Table 6.2.1.1-2: Acute oral toxicity: body weights

Dose	Animal Number	Sex	Test day 0 (treatment) (g)	Test day 7 (g)	Test day 14 (g)
5000 mg/kg body weight	01	Female	183.4	199.2	214.4
	02	Female	182.4	197.9	212.8
	03	Female	180.3	195.8	209.7

Key: mg/kg = milligram/kilogram, g = gram

Necropsy
No abnormalities were observed in any of the treated animals at necropsy.

Table 6.2.1.1-3: Acute oral toxicity: Macroscopic findings

Dose	Animal Number	Sex	Mode of death	Macroscopic findings
5000 mg/kg body weight	01	Female	Terminal Sacrifice	No Abnormality Detected
	02	Female	Terminal Sacrifice	No Abnormality Detected
	03	Female	Terminal Sacrifice	No Abnormality Detected

Key: mg/kg = milligram/kilogram, g = gram

III CONCLUSION
Based on the above results, the median lethal dose of 24-Epibrassinolide (TGAI) after single oral administration to female rats, observed over a period of 14 days, is:

LD_{50} > 5000 mg/kg body weight

B.6.2.2. Dermal

B.6.2.2.1. Acute Dermal Toxicity Study in Rats

Annex point	CA 5.2.2/01
Reference:	ACUTE DERMAL TOXICITY STUDY IN RATS WITH 24-EPIBRASSINOLIDE (TGAI)
Author(s), year:	[redacted] (2017)
Report/Doc. number:	6114 (522-001)
Guideline(s):	OECD No. 402 (1987), OPPTS 870.1200, OPPTS 870.1000
GLP:	Yes
Deviations from OECD 402 (1987):	None
Acceptability:	Yes

Executive summary
The study was performed to assess the dermal toxicological potential of the test item 24-Epibrassinolide (TGAI) in an *in vivo* test using Wistar rats.
Five male and five female Wistar Rats were treated by a single dermal application at the dose of 2000 mg/kg body weight. One day before treatment, the back of the animals was clipped with electric clipper exposing an area of approximately 10% of the total body surface. The skin reactions were assessed. All the treated animals appeared normal and no systemic or local signs of toxicity were observed from day 0 after treatment until the end of the observation period (day 14). All the animals survived until the end of the experimental period.
No abnormalities were observed in any of the treated animals during necropsy.
Based on these results, the median lethal dose of 24-Epibrassinolide (TGAI) in male and female rats after a single dermal administration, observed over a period of 14 days, was estimated to exceed 2000 mg/kg bw.

- Dermal LD_{50} Males > 2000 mg/kg bw
  Females > 2000 mg/kg bw
  Combined > 2000 mg/kg bw

I. MATERIALS AND METHODS

A. Test material and vehicle

1 Test materials

Test substance	24-Epibrassinolide (TGAI)
CAS no.	78821-43-9
Lot/batch	002-20150112
Purity	91.2% w/w
Vehicle	Distilled Water
Expiry date	January 11, 2017
Storage conditions:	Room Temperature (20°C to 30°C)

2 Test animals

Species	Rat
Strain	Wistar Rats (*Rattus norvegicus)*
Age	11-12 weeks
Bodyweight at dosing	Males: 252.3 to 260.1 g Females: 223.4 to 235.2 g
Source	[redacted]
Acclimation period	6 days
Diet	*Ad libitum* Teklad Certified Global 14% Protein Rodent Maintenance Diet (Batch No. 2014C-111215MA) from ENVIGO
Water	*Ad libitum* tap water
Housing	Individually
Temperature	20.6 - 22.9°C
Humidity	53 - 65%
Ventilation	≥ 10 times/hour
Photoperiod	12 hours light / 12 hours dark

B. Study design and method

Test substance preparation and administration
Five male and five female Wistar rats were treated with 24-Epibrassinolide (TGAI) by a single dermal application at the dose of 2000 mg/kg body weight. The test item was applied as delivered by the sponsor moistened with distilled water.
Since test item related mortality was not observed in any of the treated animals, the experiment was completed as limit test only. One day before treatment, the back of the animals were clipped (with electric clipper), exposing an area of approximately 10% of the total body surface.
On test day 0, the test item was transferred to a surgical gauze patch and moistened with a minimum amount of distilled water and applied to the intact skin. This gauze patch was covered with a semi-occlusive dressing. The dressing was wrapped around the abdomen and anchored with non-irritating adhesive tape.
After the 24-hour application period, the dressings were removed and the skin was gently wiped with cotton soaked in distilled water. The skin reactions were assessed.

Mortality and clinical observations
The animals were observed daily during the acclimatization period and mortality/viability and clinical signs were recorded. All the animals were observed for clinical signs during the first 30 minutes and at approximately 1, 2, 3 and 4 hours post treatment on day 0 and once daily during test days 1-14. Mortality/viability was recorded during the first 30 minutes and at approximately 1, 2, 3 and 4 hours post application on test day 0 (in common with the clinical signs) and twice daily during days 1-14 (once on the day of sacrifice). Local signs/skin reactions were observed daily from test days 1-14 (in common with clinical signs).

Body weight
Body weights were recorded on days 0 (prior to application), as well as 7 and 14.

Necropsy
All animals were sacrificed at the end of the observation period by carbon dioxide asphyxiation in euthanasia chamber and discarded after the gross/macroscopic pathological changes were observed and recorded. No organs or tissues were retained.

C. Statistics
Data were not analysed statistically.

II. RESULTS AND DISCUSSION

Mortality and clinical signs
All the treated animals appeared normal and no systemic or local signs of toxicity were observed from day 0 of observation period till the last day of observation period (day 14). All the animals survived till the end of the experimental period. No mortality was observed in any of the treated animals.

Table 6.2.2.1-1: Mortality / Clinical Signs/ Local Signs / Skin Reactions

Dose	Sex	Animal Number	Test day																		
			0*					1	2	3	4	5	6	7	8	9	10	11	12	13	14
			0.5	1	2	3	4														
2000 mg/kg body weight	Male	01	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		02	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		03	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		04	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		05	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Female	06	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		07	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		08	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		09	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Key: mg/kg = milligram/kilogram, 1 = Normal.

* Examinations/observations were performed during the first 0.5 hour and at approximately 1, 2, 3 and 4 hours after test item application on test day 0.

Body weights
The body weight of the animals was within the normal range of variability commonly recorded for this species, strain and age. There was body weight gain in all the animals by days 7 and 14 when compared to day 0.

Table 6.2.2.1-2: Body Weights

Dose	Animal Number	Sex	Test day 0 (treatment) (g)	Test day 7 (g)	Test day 14 (g)
2000 mg/kg body weight	01	Male	257.8	280.7	304.2
	02	Male	252.3	275.7	298.5
	03	Male	260.1	282.6	305.0
	04	Male	253.9	277.5	300.2
	05	Male	258.4	281.8	303.4
	06	Female	223.4	239.2	254.6
	07	Female	234.2	249.0	263.5
	08	Female	227.0	239.8	255.2
	09	Female	235.2	250.5	265.2
	10	Female	224.6	241.1	256.5

Key: mg/kg = milligram/kilogram, g = gram

Necropsy
No abnormalities were detected in any of the treated animals during necropsy at terminal sacrifice.

Table 6.2.2.1-3: Macroscopic Findings

Dose	Sex	Animal Number	Mode of Death	Macroscopic Findings
2000 mg/kg body weight	Male	01	Terminal Sacrifice	No Abnormality Detected
		02	Terminal Sacrifice	No Abnormality Detected
		03	Terminal Sacrifice	No Abnormality Detected
		04	Terminal Sacrifice	No Abnormality Detected
		05	Terminal Sacrifice	No Abnormality Detected
	Female	06	Terminal Sacrifice	No Abnormality Detected
		07	Terminal Sacrifice	No Abnormality Detected
		08	Terminal Sacrifice	No Abnormality Detected
		09	Terminal Sacrifice	No Abnormality Detected
		10	Terminal Sacrifice	No Abnormality Detected

Key: mg/kg = milligram/kilogram.

III CONCLUSION

The median lethal dose of 24-Epibrassinolide (TGAI), following single dermal application in Wistar rats of either sexes, observed over a period of 14 days are:

LD_{50} (rat) > 2000 mg/kg body weight

B.6.2.3. Inhalation

B.6.2.3.1. Acute Inhalation Toxicity Study in Rats

Annex point	CA 5.2.3/01
Reference:	ACUTE INHALATION TOXICITY STUDY IN RATS WITH 24-EPIBRASSINOLIDE (TGAI)
Author(s), year:	[redacted] (2017)
Report/Doc. number:	6118 (520-001)
Guideline(s):	OECD No. 423 (2002), OPPTS 870.1100, OPPTS 870.1000
GLP:	Yes
Deviations from OECD 403 (2009):	None
Acceptability:	Yes

Executive summary
The purpose of this study was to assess the acute inhalation toxicity of 24-Epibrassinolide in rats.
Three male and three female rats were exposed to 24-Epibrassinolide (TGAI) for a period of 4 hours/single exposure, at a concentration of 1.08 mg/L air (maximum attainable concentration).
After the treatment, the rats were observed for a total of 14 days. Mortality, clinical signs, bodyweights and feed and water consumption were recorded during the observation period and all animals were examined macroscopically at scheduled necropsy.
During exposure, no mortality was observed and all animals appeared normal throughout the experimental period.
No abnormalities were detected in any of the animals on necropsy at the end of observation period.

- **LC_{50} Males > 1.08 mg/L (maximum attainable concentration)**
  Females > 1.08 mg/L (maximum attainable concentration)
  Combined > 1.08 mg/L (maximum attainable concentration)

I. MATERIALS AND METHODS

A Test material and vehicle

1 Test materials

Test substance	24-Epibrassinolide (TGAI)
CAS no.	78821-43-9
Lot/batch	002-20150112
Purity	91.2% w/w
Expiry date	January 11, 2017
Storage conditions:	Room Temperature (20°C to 30°C)

2 Test animals

Species	Rat
Strain	Wistar Rats (*Rattus norvegicus*)
Age	9 - 10 weeks
Bodyweight at dosing	Males: 209.2 - 210.6 g Females: 197.3 - 198.4 g
Source	[redacted]
Acclimation period	6 days
Diet	*Ad libitum* Teklad Certified Global 14% Protein Rodent Maintenance Diet (Batch No.2014C-111215MA) from ENVIGO
Water	*Ad libitum* tap water
Housing	Individually
Temperature	20.6 – 22.6°C
Humidity	54 - 65%
Ventilation	≥ 10 times/hour
Photoperiod	12 hours light / 12 hours dark

B. Study design and method

Test substance preparation and administration
Test item was used as supplied and aerosolized without any additional preparation. Two groups of rats were exposed continuously for 4 hours (nose-only) to test atmospheres containing the dust of 24-Epibrassinolide (TGAI).

Inhalation Exposure System
The dust generators produced and maintained a uniform test item distribution in the atmosphere, providing a constant stream of "fresh" test item to each animal, and precluding re-breathing of the exhaled air. The animals were confined separately in restraint tubes which were positioned radially around the flow-past, nose-only exposure chamber
A dust aerosol was generated from the test item using a rotating brush aerosol generator (CR3020) connected to a micronising jet mill. The aerosol generated was then discharged into the exposure chamber.

Chamber atmosphere analysis
The concentration of the test item in the chamber was analyzed by collecting samples at 1, 2, 3 and 4 hours after chamber equilibrium time during the exposure period. Samples in triplicate were taken for each time point, for dose concentration analysis. Gravimetric determinations of aerosol concentration were performed using Whatman filter (GF/C), loaded in a 47 mm in-line stainless steel filter sampling device. These determinations were performed 5 times during the whole exposure period.
The particle size distribution was measured gravimetrically two times during each exposure. Mass Median Aerodynamic Diameters (MMAD) and Geometric Standard Deviations (GSD) were calculated.

Range Finding Study
A dust aerosol was generated from the test item using a rotating brush aerosol generator and piston speed set at a rate 100 mL/min. The actual concentration achieved at this speed was 1.05 mg/L and the nominal concentration was 5.86 mg/L. One male and one female rat were exposed to this concentration for 4 hours and observed for 7 days (Group – I). All animals survived at the above concentration. Both animals showed dullness at 30 min post exposure. From day 1 of observation period, both the animals appeared normal throughout the experimental period. Subsequently these animals were euthanized without necropsy.

Limit test
Based on the range finding study, rats (Group – II; 3 males and 3 females) were exposed to 1.08 mg/L (actual concentration) and 6.25 mg/L (nominal concentration) for 4 hours. To attain this nominal concentration, dust aerosol was generated from the test item using a rotating brush aerosol generator and piston speed was set at a rate 100 mL/min.
Attempt was made to achieve a higher concentration by increasing the speed set rate greater than 100 mL/min, but the generation of aerosol was poor at this speed. Hence, the concentration of 1.08 mg/L achieved at the speed rate of 100 mL/min was considered as the maximum attainable concentration.

Mortality and clinical observations
The rats were observed twice daily during the acclimatization period, at 1, 2, 3 and 4 hours during exposure and 30 minutes after exposure of day 0. During the 14-day observation period rats were examined at least twice daily.
Observations included, but were not limited to: changes in the skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behaviour pattern.

Bodyweight
All rats were observed on the test days 0 (prior to exposure), 1, 3, 7 and 14.

Necropsy
At the end of the 14-day observation period, all surviving rats were sacrificed. The gross/macroscopic pathological changes were observed with particular reference to any changes in the respiratory tract and recorded. Since no treatment related lesions were observed, organs or tissues were not retained for histopathology.

C. Statistics
No statistical analysis was performed since the study was terminated using single exposure group only.

II. RESULTS AND DISCUSSION

Based on the range finding study, Group of 3 male and 3 female rats was exposed to 1.08 mg/L (actual concentration) and 6.25 mg/L (nominal concentration).

Table 6.2.3.1-1: Gravimetric Determination of Aerosol Concentration - Range finding study (1.05 mg/L air)

Sampling time	Sample Volume (L)	Amount of test Item on Filter (Gravimetric)	Gravimetric aerosol Concentration (mg/L air)
12:05 pm - 12:06 pm	1	0.95	0.95
01:03 pm - 01:04 pm	1	1.05	1.05
02:01 pm - 02:02 pm	1	1.10	1.10
03:00 pm - 03:01 pm	1	1.08	1.08
03:58 pm - 03:59 pm	1	1.06	1.06
Mean			**1.05**
S.D.			**0.06**
N			**5**

Table 6.2.3.1-2: Gravimetric Determination of Aerosol Concentration - Main study (1.08 mg/L air)

Sampling time	Sample Volume (L)	Amount of test Item on Filter (Gravimetric)	Gravimetric aerosol Concentration (mg/L air)
11:35 am - 11:36 am	1	0.98	0.98
12:20 pm - 12:21 pm	1	1.09	1.09
01:15 pm - 01:16 pm	1	1.16	1.16
02:10 pm - 02:11 pm	1	1.11	1.11
03:04 pm - 03:05 pm	1	1.08	1.08
Mean			**1.08**
S.D.			**0.07**
N			**5**

Mortality
No mortalities were observed during the experimental period.

Clinical signs
All animals appeared normal throughout the experimental period.

Body weights
All animals had gained body weight by days 1, 3, 7 and 14 as compared to day 0.

Table 6.2.3.1-3: Summary of bodyweights (mean)

Animal Numbers	Sex	Test day 0 (Exposure) (g)	Test day 1 (g)	Test day 3 (g)	Test day 7 (g)	Test day 14 (g)
03	Male	210.6	211.3	214.8	221.5	255.8
04	Male	209.6	210.2	214.3	220.6	253.9
05	Male	209.2	209.9	214.0	220.3	252.4
Mean		**209.80**	**210.47**	**214.37**	**220.80**	**254.03**
S.D.		**0.72**	**0.74**	**0.40**	**0.62**	**1.70**
N		**3**	**3**	**3**	**3**	**3**
06	Female	198.4	198.9	200.4	210.4	225.3
07	Female	197.3	197.8	199.6	208.3	224.6
08	Female	198.2	198.5	200.4	206.9	220.8
Mean		**197.97**	**198.40**	**200.13**	**208.53**	**223.57**
S.D.		**0.59**	**0.56**	**0.46**	**1.76**	**2.42**
N		**3**	**3**	**3**	**3**	**3**

Key: mg/L = milligram/Litre, g = gram, S.D. = Standard deviation, N = No. of animals

Necropsy
No abnormalities were detected in any of the animals on necropsy at the end of observation period, except for animal No. 06 which showed a slightly distended uterus with water content. This finding was considered as being incidental.

Table 6.2.3.1-4: Main study - Group II (1.08 mg/L air)

Animal Number	Sex	Mode of Death	Macroscopic Findings
03	Male	Terminal sacrifice	NAD
04	Male	Terminal sacrifice	NAD
05	Male	Terminal sacrifice	NAD
06	Female	Terminal sacrifice	*Uterus-distended with water content, slightly.
07	Female	Terminal sacrifice	NAD
08	Female	Terminal sacrifice	NAD

Key: mg/L = milligram/Litre; NAD – No Abnormality Detected
* Incidental finding

III. CONCLUSION

No mortalities or abnormalities were observed during the experimental period.

The LC_{50} (4-hours exposure) of 24-Epibrassinolide (TGAI) obtained in this study, based on combined gender mortality data, was estimated to be greater than 1.08 mg/L air (maximum attainable concentration).

LC_{50} > 1.08 mg/L air (maximum attainable concentration)

B.6.2.4. Skin irritation

B.6.2.4.1. Acute Dermal Irritaion/ Corrosion Study in Rabbits

Annex point	CA 5.2.4/01
Reference:	ACUTE DERMAL IRRITATION/CORROSION STUDY IN RABBITS WITH 24-EPIBRASSINOLIDE (TGAI)
Author(s), year:	[redacted] (2017)
Report/Doc. number:	6115 (565-001)
Guideline(s):	OECD No. 404 (2015), OPPTS 870.2500, OPPTS 870.1000
GLP:	Yes
Deviations from OECD 404 (2015):	None
Acceptability:	Yes

Executive summary

The study was performed to assess the dermal toxicological potential of the test item 24-Epibrassinolide (TGAI) in an *in vivo* test using rabbits.
The test item was applied by topical (semi-occlusive) application of 0.5 g to the intact skin of the left flank of male rabbits for an exposure period of 4 hours.
All the treated animals appeared normal and no systemic or local signs of toxicity were observed from day 0 of observation period after treatment until the end of observation period (day 14). The test item did not induce any degree of erythema or oedema. All the animals survived until the end of the experimental period.
No abnormalities were observed in any of the treated animals during necropsy at terminal sacrifice.
Based on these results, 24-Epibrassinolide (TGAI) is classified as "Non Irritant" to the rabbit's skin.

- **Non Irritant**

I. MATERIALS AND METHODS

A. Test material and vehicle

1 Test materials

Test substance	24-Epibrassinolide (TGAI)

CAS no.	78821-43-9
Lot/batch	002-20150112
Chemical Purity	91.2% w/w
Expiry date	January 11, 2017
Storage conditions:	Room Temperature (20 °C to 30 °C)
Vehicle	Distilled Water

2 Test animals

Species	Rabbit
Strain	Young Adult New Zealand White Rabbits (*Oryctolagus cuniculus)*
Age	12-14 weeks
Bodyweight at dosing	2.2162 - 2.4627 kg
Source	[redacted]
Acclimation period	6 - 9 days
Diet	*Ad libitum* Teklad Certified Global 14% Protein Rodent Maintenance Diet (Batch No.2014C-111215MA) from ENVIGO
Water	*Ad libitum* tap water
Housing	Individually
Temperature	20.6 - 22.9°C
Humidity	55 – 67%
Ventilation	≥ 10 times/hour
Photoperiod	12 hours light / 12 hours dark

B. Study design and method

Test substance preparation and administration
Approximately 24 (± 2) hours before application of the test item, both flanks were clipped with a clipper, exposing an area of approximately 100 cm^2 (10 cm x 10 cm). Animals without any skin injury or overt signs of irritation were used in the test.
The test item, 0.5 g (per animal) was weighed, moistened with distilled water and applied to the intact skin.
A single male rabbit was treated first. As no severe skin reactions were observed until 72 hours (approximately) post exposure, the test was completed by exposing the remaining two male rabbits.
On test day 0, the test item was transferred to a surgical gauze patch and moistened with 0.5 mL of distilled water and applied to the intact skin. This gauze patch was covered with a semi-occlusive dressing. The dressing was wrapped around the abdomen and anchored with non-irritating adhesive tape. The duration of exposure was 4 hours (approximately), after which the dressing was removed and the skin was gently wiped clean with cotton soaked in distilled water.
The skin reaction was assessed according to the numerical scoring system listed in the OECD guideline No. 404, "Grading of Skin Reactions" (28th July 2015), at approximately 1, 24, 48 and 72 hours post removal of the dressing, gauze patch and unabsorbed test item.

Mortality and clinical observations
The animals were observed daily during the acclimatization period and mortality/viability and clinical signs were recorded. All the animals were observed for clinical signs during first 30 minutes and at approximately 1, 2, 3 and 4 hours post treatment on day 0 and once daily during test days 1-14. Mortality/viability was recorded during the first 30 minutes and at approximately 1, 2, 3 and 4 hours post application on test day 0 (in common with the clinical signs) and twice daily during days 1-14 (once on the day of sacrifice). Local signs/skin reactions were observed daily from test days 1-14 (in common with clinical signs).

Bodyweight
Body weights were recorded on days 0 (prior to application), 7 and 14.

Necropsy
All animals were sacrificed at the end of the observation period by carbon dioxide asphyxiation in euthanasia chamber and discarded after the gross/macroscopic pathological changes were observed and recorded. No organs or tissues were retained.

C. Statistics

Data were not analysed statistically.

II. RESULTS AND DISCUSSION

Mortality and clinical signs

All the treated animals appeared normal and no systemic or local signs of toxicity were observed from day 0 of observation period till the last day of observation period (day 14). All the animals survived till the end of the experimental period. No mortality was observed in any of the treated animals

The scoring of skin reactions were undertaken at approximately 1, 24, 48, and 72 hours post exposure for Animal No. 01, 02 and 03 (post removal of the dressing, gauze patch and unabsorbed test item). All individual mean scores for erythema/eschar formation for Animal No. 01, 02 and 03 at 24, 48 and 72 hours were 0.00. All individual mean scores of oedema for Animal No. 01, 02 and 03 at 24, 48 and 72 hours were also 0.00.
The test item did not induce any degree of erythema or oedema in Animal No. 01, 02, and 03 at approximately 1, 24, 48 and 72 hours observation post exposure (post removal of the dressing, gauze patch and unabsorbed test item).
The test item did not result in any coloration on the treated skin of any rabbit.
No corrosive effects were observed on the skin of treated animals.

Table 6.2.4.1-1: Mortality / Clinical Signs/ Local Signs / Skin Reactions

Animal No.	Sex	Test day*							
		0		1		2		3	
		M	E	M	E	M	E	M	E
01	Male	1	1	1	1	1	1	1#	-
02		1	1	1	1	1	1	1#	-
03		1	1	1	1	1	1	1#	-

Key: M = Morning check, E = Evening check, 1 = Normal.
*Examinations were performed twice daily.
No clinical signs were evident in any animal during the acclimatization.
Animal sacrificed after 72 hours.

Table 6.2.4.1-2: Skin Irritation Scores - Individual mean values after 24, 48 and 72 hours (treated site)

Animal Number	01		02		03	
Observation	**Erythema**	**Oedema**	**Erythema**	**Oedema**	**Erythema**	**Oedema**
Mean Score	0.00	0.00	0.00	0.00	0.00	0.00

Body weights

The body weights of all the animals were considered to be within the normal range of variability commonly observed for this species, strain and age (Table 6.2.4.1-3).

Table 6.2.4.1-3: Body Weights

Body weight in kg				
Animal Number	**Sex**	**First Day of Acclimatization**	**Day of Treatment**	**Last Day of Observation**
01	Male	2.3527	2.4627	2.5316
02		2.0823	2.2519	2.3242
03		2.0315	2.2162	2.2819

Key: kg = kilogram

Necropsy

No abnormalities were detected in any of the treated animals during necropsy at terminal sacrifice.

Table 6.2.4.1-4: Macroscopic findings

Animal Number	Sex	Mode of death	Macroscopic Findings
01	Male	Terminal Sacrifice	No Abnormality Detected
02		Terminal Sacrifice	No Abnormality Detected

03		Terminal Sacrifice	No Abnormality Detected

III CONCLUSION

Observed over a period of 72 hours following single dermal application, 24-Epibrassinolide (TGAI) is classified in male rabbits as:

"Not Irritant"

B.6.2.5. Eye irritation

B.6.2.5.1. Acute Eye Irritaion/ Corrosion Study in Rabbits

Annex point	CA 5.2.5/01
Reference:	ACUTE EYE IRRITATION/CORROSION STUDY IN RABBITS WITH 24-EPIBRASSINOLIDE (TGAI)
Author(s), year:	[redacted] (2017)
Report/Doc. number:	6116 (566-001)
Guideline(s):	OECD No. 405 (2012), OPPTS 870.2400, OPPTS 870.1000
GLP:	Yes
Deviations from OECD 405 (2012):	None (latest version of TG published 09, 2017; experimental period was in 2016)
Acceptability:	Yes

Executive summary

The acute eye irritation/corrosion study of 24-epibrassinolide (TGAI) in rabbits was investigated according to OECD test guideline 405. The test item, 0.1 g was placed in the conjunctival sac of the left eye of a single male rabbit. As no severe eye reactions were observed upto 72 hours approximately post treatment, the treatment was completed using the remaining two male rabbits.

The scoring of eye reactions was performed at 24, 48, 72 hours post test item instillation, the mean score was calculated across 3 scoring intervals (24, 48 and 72 hour post instillation).

The individual mean score for corneal opacity, iris effects, conjunctivae and chemosis for Animal No. 01, 02 and 03 was 0.00, 0.00, 0.00 and 0.00.

In Animal No. 01, 02 and 03, test item instillation in left eye of the rabbit caused slight redness of conjunctivae at 1 hour post test item instillation on day 0. The eye lesion was reversed at 24 hour observation and the treated eye appeared normal for all animals. The test item did not induce any corneal opacity, lesion in iris and chemosis throughout the experimental period. After 72 hour observation, the animals were sent for terminal sacrifice.

No clinical signs suggestive of systemic toxicity were observed in any of the animals throughout the acclimatization and post treatment periods.

The body weights of all the animals were considered to be within the normal range of variability commonly observed for this species, strain and age.

No gross pathological abnormalities were detected in any of the treated animals during necropsy at terminal sacrifice.

- **Not eye irritant**

I. MATERIALS AND METHODS

A. Test material and vehicle

1 Test materials

Test substance	24-Epibrassinolide (TGAI)
CAS no.	78821-43-9
Lot/batch	002-20150112
Chemical purity	91.2% w/w
Expiry date	January 11, 2017

Storage conditions:	Room Temperature (20°C to 30°C)
Vehicle	Distilled Water

2 Test animals

Species	Rabbit
Strain	Young Adult Male New Zealand White Rabbits (Oryctolagus cuniculus)
Age	12 - 14 weeks
Bodyweight at dosing	1.9319 -2.2158 kg
Source	[redacted]
Acclimation period	6 – 9 days
Diet	*Ad libitum* Teklad Certified Global 14% Protein Rodent Maintenance Diet (Batch No. 2014C-111215MA) from ENVIGO
Water	*Ad libitum* tap water
Housing	Individually
Temperature	20.9 - 22.4°C
Humidity	54 - 64%
Ventilation	≥ 10 times/hour
Photoperiod	12 hours light / 12 hours dark

B. Study design and method

Test substance preparation
A formulation of 1% (w/v) aqueous suspension was prepared. The average pH was found to be 6.55 (from solubility and pH Test).

Main test
Pre treatment
Approximately 60 minutes prior to test item instillation, Buprenorphine 0.01 mg/kg was administered by subcutaneous injection and 5 minutes prior to test item instillation, two drops of topical ocular anesthetic (0.5% Proparacaine hydrochloride) was instilled in each eye. The eye (right eye) not treated with test item, but treated with topical anaesthetics served as control.

Post treatment
Buprenorphine 0.01 mg/kg and Meloxicam 0.5 mg/kg was administered by subcutaneous injection approximately eight hours after test item instillation. After the initial 8-hour post treatment, Buprenorphine 0.01 mg/kg was administered by subcutaneous injection to the animals every 12 hours, in conjunction with Meloxicam 0.5 mg/kg every 24 hours up to 72 hour for all the treated animals.

Mortality and clinical observations
Mortality and viability was observed at least twice daily from commencement of acclimatization of the animals to the termination of the test.
Clinical signs were observed once daily from commencement of acclimatization of the animals and twice daily with a minimum of 6 hours between observations from the test item instillation to the termination of the test.

Ocular observations
The eye reactions were assessed according to the numerical scoring system listed in the OECD guidelines No. 405, "Grading of Ocular Lesions" (2nd of October, 2012). The eyes were examined at approximately 1, 24, 48 and 72 hours post instillation of test item. Fluorescein sodium ophthalmic strip and Neitz ophthalmoscope (Model; BX α-13) were used for scoring of eye lesions of all the rabbits.
The scores of each animal at the recording interval (24, 48, 72 hours) were used in calculating the respective mean values for each type of lesion.

Body weight
Body weight was assessed on the days of commencement of acclimatization, test item instillation and at termination of observation.

Necropsy

All animals were sacrificed at the end of the observation period and discarded after the gross/macroscopic pathological changes were observed and recorded.
No organs or tissues were retained.

C. Statistics
No statistical analysis was performed.

III. RESULTS AND DISCUSSION

Mortality and clinical signs
No mortalities were observed during the experimental period.
No clinical signs were observed in any of the animals throughout the acclimatization and post treatment periods.

Table 6.2.5.1-1: Mortality and Clinical Signs

Animal No.	Sex	Test day*							
		0		1		2		3	
		M	E	M	E	M	E	M	E
01	Male	1	1	1	1	1	1	1#	-
02		1	1	1	1	1	1	1#	-
03		1	1	1	1	1	1	1#	-

Key: M = Morning check, E = Evening check, 1 = Normal.
*Examinations were performed twice daily with the minimum interval of 6 hours.
No clinical signs were evident in any animal during the acclimatization.
Animal sacrificed after 72 hour observation.

Ocular observations
The scoring of eye reactions was performed at 1, 24, 48, 72 hours, for Animal No. 01, 02 and 03 post test item instillation. The mean score was calculated across 3 scoring intervals (24, 48 and 72 hour post treatment) for each animal for corneal opacity, iris, conjunctival redness and chemosis. The individual mean score of opacity, iris, conjunctivae and chemosis for Animal No. 01, 02 and 03 was 0.00.

Table 6.2.5.1-2: Eye Irritation Scores - Mean values after 24, 48 and 72 hours (treated - left eye)

Animal Number	Sex	Eye reaction			
		Corneal Opacity	Iris	Conjunctivae	Chemosis
01	Male	0.00	0.00	0.00	0.00
02		0.00	0.00	0.00	0.00
03		0.00	0.00	0.00	0.00

Table 6.2.5.1-3: Eye Irritation Scores - Individual values (treated - left eye)

Sex	Male														
Animal Number	01					02					03				
Eye Reactions	At hour					At hour					At hour				
	PE	1	24	48	72	PE	1	24	48	72	PE	1	24	48	72
Corneal Opacity	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Area of Opacity	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Iris	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Conjunctivae	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0
Chemosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Key: PE = Pre Exposure, Note: For scoring

In Animal No. 01, 02 and 03, test item instillation in left eye of the rabbit caused slight redness of conjunctivae at 1 hour post test item instillation on day 0. The eye lesions were reversible at 24 hour observation and the treated eyes appeared normal for all animals. The test item did not induce any corneal opacity, lesion in iris and chemosis throughout the experimental period. After 72 hour observation, the animals were necropsied.
The test item did not result in any coloration of the treated eyes of the rabbits.

No corrosive effects were observed on the cornea of the treated rabbits.

Body weights
The body weights of all the animals were considered to be within the normal range of variability commonly observed for this species, strain and age.

Table 6.2.5.1-4: Individual Animal Body weights

Animal Number	Sex	First Day of Acclimatization (kg)	Pre Treatment (kg)	Pre Termination (kg)
01	Male	2.0735	2.2158	2.2829
02		1.7573	2.0001	2.0657
03		1.6355	1.9319	1.9938

Key: kg = kilogram

Necropsy
No gross pathological abnormalities were observed in any of the treated animals during necropsy at terminal sacrifice.

Table 6.2.5.1-5: Gross / Macroscopic findings

Animal Number	Sex	Mode of death	Gross/Macroscopic Observations
01	Male	Terminal Sacrifice	NAD
02	Male	Terminal Sacrifice	NAD
03	Male	Terminal Sacrifice	NAD

Key: NAD – No Abnormality Detected

III CONCLUSION
24-Epibrassinolide (TGAI) is classified as **"Not Irritating"** to rabbit eyes.

B.6.2.6. Skin sensitization

Annex point	CA 5.2.6/01
Reference:	CONTACT HYPERSENSITIVITY IN ALBINO GUINEA PIGS, MAXIMIZATION TEST (MAGNUSSON AND KLIGMAN METHOD) WITH 24-EPIBRASSINOLIDE (TGAI)
Author(s), year:	[redacted] (2017)
Report/Doc. number:	6117 (567-001)
Guideline(s):	OECD No. 406 (1992), OPPTS 870.2600, OPPTS 870.1000
GLP:	Yes
Deviations from OECD 406 (1992):	None
Acceptability:	Yes

Executive summary
In order to assess the allergenic potential of 24-Epibrassinolide (TGAI), the Maximization Test was performed in 10 treated (Male) and 5 control (Male) Albino Dunkin Hartley Guinea Pigs, in accordance with OECD Guideline No. 406.
The intradermal induction of sensitisation in the test group was performed in the dorsal skin from the scapular region with a 0.1 % dilution of the test item in propylene glycol and in an emulsion of Freund's Complete Adjuvant (FCA)/Physiological saline.
One day before the epidermal induction, the scapular region was painted with 0.5 mL of 10% sodium lauryl sulphate in Vaseline, in order to create local irritation. The epidermal induction of sensitization was conducted for 48 hours under occlusion with the test item at 100% one week after the intradermal induction. The animals of the control

group were intradermally induced with propylene glycol and FCA/Physiological saline and epidermally induced with distilled water under occlusion.
Two weeks after epidermal induction the test and the control animals were challenged by epidermal application of the test item at 100% and distilled water under occlusion. Cutaneous reactions were evaluated at 24 hour and 48 hours after removal of the dressing.
Based on the findings in the adjuvant sensitisation test (M&K-test) in guinea pigs, 24-Epibrassinolide (TGAI) is considered to be not skin sensitising.

I. MATERIALS AND METHODS

A. Test material and vehicle

1 Test materials

Test substance	24-Epibrassinolide (TGAI)
CAS no.	78821-43-9
Lot/batch	002-20150112
Purity	91.2% w/w
Expiry date	January 11, 2017
Storage conditions:	Room Temperature (20°C to 30°C)
Vehicle	Propylene glycol (intradermal induction) Distilled water (epidermal induction)

2 Test animals

Species	Guinea Pig
Strain	Male Albino Dunkin Hartley Guinea Pig (*Cavia porcellus)*
Age	8 - 10 weeks
Bodyweight at dosing	306.9 – 343.5 g
Source	[redacted]
Acclimation period	13 days
Diet	*Ad libitum* Teklad Certified Global 14% Protein Rodent Maintenance Diet (Batch No. 2014C-111215MA) from ENVIGO
Water	*Ad libitum* tap water
Housing	Individually
Temperature	20 ± 3°C
Humidity	30 - 70 %
Ventilation	≥ 10 times/hour
Photoperiod	12 hours light / 12 hours dark

B. Study design and method

Test substance preparation and administration
For the intradermal injection, an amount of 1000.04 mg of test item was mixed with Propylene Glycol and the final volume was made up to 10 mL. Thus, the concentration was 1%.
A ten-fold dilution was made in propylene glycol resulting in a concentration of 0.1% to be used as test solution for the main study.

For the epidermal application, 200 mg of 100% test item was moistened with 0.2 mL of distilled water and applied.
For the challenge epidermal application, 200 mg of 100% test item was moistened with 0.2 mL of distilled water and applied.

Intradermal Induction (Day 0)
An area of dorsal skin from the scapular region (approximately 6 x 8 cm) was clipped with a clipper or depilated with an approved depilatory cream. Three pairs of intradermal injections (0.1 mL/site) were given just within the boundaries of a 4 x 6 cm area in the cleared region as follows:

Test Group

Injection 1	1:1 (v/v) mixture of Freund's Complete Adjuvant and physiological saline.
Injection 2	The test item in its required concentration (0.1 %) is selected in Propylene glycol (w/v).
Injection 3	The test item at the selected concentration (0.1 %) in Propylene glycol formulated in a 1:1 (v/v)

	mixture of Freund's Complete Adjuvant and physiological saline. (1:1 (v/v) mixture of Injection 1 and injection 2).

Control Group

Injection 1	1:1 (v/v) mixture of Freund's Complete Adjuvant and physiological saline.
Injection 2	Propylene glycol.
Injection 3	A 50% v/v formulation of the vehicle in a 1:1 (v/v) mixture of Freund's Complete Adjuvant and physiological saline (1:1 (v/v) mixture of Injection 1 and injection 2).

Injections 1 and 2 were given close to each other and nearest the head, while injection 3 was given towards the caudal part of the test area.
The skin reaction of this intradermal induction was observed at 24 (± 2) hours after intradermal injection according to the Magnusson and Kligman grading scale.

Epidermal Applications of Sodium Lauryl Sulphate (Day 6)
Approximately 24 hours before the application of 10% sodium lauryl sulphate in vaseline, the scapular region (approximately 6 x 8 cm) of all the guinea pigs, were clipped with a clipper or depilated with an approved depilatory cream. On day 6, the scapular region was painted with 0.5 mL of 10% sodium lauryl sulphate in vaseline, in order to create a local irritation.

Epidermal Application (Day 7)
On test day 7, one week after the intradermal injections, approximately, 2 x 4 cm patch of filter paper was saturated with 0.2 mL of 100% test item as such was placed over the right scapular area of the test animals.
The patch was covered by aluminium foil and secured with an adhesive tape. The dressings were removed after an exposure period of approximately 48 hours.
The guinea pigs of the control group were treated with distilled water only on the scapular region.
The reaction sites were assessed 24 hours after removal of the bandage for erythema and oedema according to the method of Magnusson and Kligman.

First Challenge (Day 21)
The test and control guinea pigs were challenged two weeks after the epidermal induction application. Hair was removed with the help of a clipper from an area of approximately 5 x 5 cm on the left and right flank of each guinea pig 24 hours prior to the application. Two patches (approximately 3 x 3 cm) of filter papers, saturated with 200 mg of 100% non-irritating concentration of test item moistened with 0.2 mL of distilled water was applied to the left flank, while distilled water was applied on the right flank for all the animals using the same method as for the epidermal application. The dressings were left in place for approximately 24 hours.
Approximately, 48 hours from the start of the challenge application, the skin reaction was observed and recorded according to the numerical grading system.
Approximately 24 hours after this observation a second observation (72 hours from the start of the challenge application) was made and once again recorded.

Mortality and clinical observations
The guinea pigs were observed twice daily from delivery of the animals to the termination of the test. Clinical signs have been observed daily.

Body weight
Body weight was measured on the day of acclimatization start, during randomization, before treatment and at the end of the experiment.

Necropsy
All surviving animals were sacrificed at the end of the observation period by carbon dioxide asphyxiation in euthanasia chamber and discarded after the gross/macroscopic pathological changes were observed and recorded. No organs or tissues were retained.

C. Statistics
Descriptive statistics (mean and standard deviation) were calculated for body weights. No inferential statistics were used.

II. RESULTS AND DISCUSSION

After intradermal induction
The findings at 24 hour observation after intradermal injection were, 6 out of 10 animals showed discrete erythema (grade 1) in the treated group. There were no findings in the control animals

Table 6.2.6-1: Skin reaction after the intradermal injection of 24-Epibrassinolide (TGAI) (0.1%) during induction period

Animal No.	Sex	REACTION READINGS AFTER INJECTION	
		24 hours at left scapular region	24 hours at right scapular region
09	Male	1	1
10		1	1
11		1	1
12		0	0
13		0	0
14		1	1
15		0	0
16		1	1
17		1	1
18		0	0

After epidermal induction
The findings at 24 hour observation after patch removal were, 5 out of 10 animals showed positive skin reactions (grade 1) in the treated group. There were no findings in the control group.

Table 6.2.6-2: Skin response after the epidermal application of 24-Epibrassinolide (TGAI) (100%) during induction period (test group)

Animal No.	Sex	REACTION READINGS AFTER REMOVAL OF BANDAGE
		24 hours at Scapular
09	Male	1
10		1
11		1
12		0
13		0
14		1
15		0
16		1
17		0
18		0

After challenge
No positive skin reactions were observed in the animals when treated with the test item at 100% concentration on left flank at 24 hour and 48 hour observation after patch removal.

Table 6.2.6-3: Skin reactions after the Challenge Procedure

	After 24 hours	After 48 hours
	positive / total (% positive of total)	positive / total (% positive of total)
CONTROL GROUP		
24-Epibrassinolide (TGAI), (100%) (left flank)	0 / 5 (0%)	0 / 5 (0%)
Distilled water only (right flank)	0 / 5 (0%)	0 / 5 (0%)
TREATMENT GROUP		

24-Epibrassinolide (TGAI), (100%) (left flank)	0 / 10 (0%)	0 / 10 (0%)
Distilled water only (right flank)	0 / 10 (0%)	0 /10 (0%)

The results obtained from test animals following the challenge application was compared with the results seen in control animals.
No allergic reaction was found at the challenge site.
The test animals showed no evidence of contact hypersensitivity as there was no dermal reaction resulting from the challenge application in the treated group.
For evaluation of the incidence index both test and control animals were used. The incidence index is an expression of the number of animals showing a response of grade 1 or greater at the 24 and 48 hour reading out of the total animals in the group (this is expressed in percent).

The sensitivity and reliability of the experimental technique employed was confirmed by the use of 2-mercaptobenzothiazole as a positive control.

Mortality and clinical signs
No mortality was observed in any of the treated animals during the experimental period.
No signs of systemic toxicity were observed in the animals either from the control or treated group.

Body weight
Normal body weight gain was observed in all the animals by the end of the experimental period.

Table 6.2.6-4: Summary of Body Weights

Sex	Group	Mean ± Standard Deviation	
		Pre Treatment	**Pre Sacrifice**
Male	Control Group	327.44 ± 11.497 (n = 5)	375.74 ± 11.645 (n = 5)
	Test Group	328.60 ± 11.609 (n = 10)	374.51±12.21 (n = 10)

Key: n = Number of Animals

Necropsy
No abnormalities were detected in any of the treated animals during necropsy at terminal sacrifice.

III. CONCLUSION
Based on the above findings in an adjuvant sensitization test (M&K-test) in guinea pigs 24-Epibrassinolide (TGAI) is considered to be **non-sensitising**.

B.6.2.7. Phototoxicity

24-Epibrassinolide does not exceed the trigger value of the extinction coefficient ε = 10 L x mol^{-1} x cm^{-1} within the range of 290 – 700 nm. Thus, a phototoxicity study is not required.

B.6.3. SHORT-TERM TOXICITY

A 90-day study in rats was submitted by the applicant. At the highest dose level tested, body weights were statistically significantly reduced in male animals from day 71 in male animals. Food consumtion was reduced in male and female animals at the highest dose level, body weights were not statistically significanly reduced in female animals. A NOAEL of 300 mg/kg bw/day is proposed based on an overal reduction in food consumption over the entire study period in the highest dose group (not statistically significant), and statistically significantly reduced body weights in the highest dose group in male animals. No concern was raised by public literature. Brassinosteroids are naturally found throughout the plant kingdom and are widely distributed in lower and higher plants. Through the dietary intake of plant and plant products, humans and animals are constantly exposed to Brassinosteroids and their metabolites. Harmful effects are not likely from the repeated oral exposure to 24-Epibrassinolide. Thus, unnecessary animal studies should be avoided, and no further data was considered necessary.

Table 6.3-1: Summary of Available Guideline-Compliant Studies on Short Term Toxicity

Study	Dose levels tested	NOAEL / remarks	Reference
REPEATED DOSE 90 DAYS ORAL TOXICITY STUDY IN RAT WITH 24-EPIBRASSINOLIDE (TGAI)	Range finding study: 0, 500, 1000, 1500 mg/kg bw/day Main study: 0, 100, 300, 1000 mg/kg bw/day	Range finding study: Decreased feed consuption at 1500 mg/kg bw/day in the first 1-2 weeks Main study: NOAEL = 300mg/kg bw/day (overall reduced food consumption and reduced body weight in male animals at 1000 mg/kg bw/day)	[redacted] 2017

Table 6.3-2: Summary of Other Scientifically Relevant Information on Short Term Toxicity

Study title	Reference Report number	Article type	Study content	Relevance/ Relieability
BIOSYNTHESIS AND METABOLISM OF BRASSINOSTEROIDS	Fujioka, S. et al., 2003 092-082	Review	Reviews the biosynthesis and metabolism of brassinosteroids in plants.	Not relevant, no information on short term toxicity in mammalian organisms
USING PEST TO STUDY THE INTERACTIONS OF BRASSINOLIDE AND OTHER PLANT GROWTH REGULATORS	Sasse, J.M. 092-163	Research report	Analyses effects of brassinolides on dwarf peas.	Not relevant, no information on short term toxicity in mammalian organisms
BRASSINOSTEROIDS FROM SEEDS OF ARABIDOPSIS THALIANA	Schmidt, J. et al., 1997 092-048	Research article	Extracts of seeds of *Arabidopsis thaliana* (ecotype 24) were analysed for the presence of free and conjugated brassinosteroids. 24-epibrassinolide (ca 220 ng/kg) and castasterone (ca 360 ng/kg) were isolated and identified.	Not relevant, no information on short term toxicity in mammalian organisms
SUPPRESSION OF CHLORELLA VULGARIS GROWTH BY CADMIUM, LEAD, AND COPPER STRESS AND ITS RESTORATION BY ENDOGENOUS BRASSINOLIDE	Bajguz, A. et al., 2011 092-103	Research article	In order to elaborate their roles in plants subjected to heavy metals stress, *Chlorella vulgaris* cultures were treated with 10^{-8} M brassinolide (BL) were exposed to 10^{-6} to 10^{-4} M heavy metals (cadmium, lead and copper). Application of BL to *C. vulgaris* cultures reduced the accumulation of heavy metals stress on growth, prevented chlorophyll, monosaccharides, and protein loss, and	Not relevant, no information on short term toxicity in mammalian organisms

			increased phytochelatins content. The arrested growth of *C. vulgaris* cells treated with heavy metals was restored by the coapplication of BL.	
STEROID PLANT HORMONES: EFFECTS OUTSIDE PLANT KINGDOM	Zhabinskii, V.N. et al., 2015 092-099	Review	Reviews available knowledge on effects of steroid plant hormones on insects, funghi, fish, protozoa, and warm blooded animals. A four week study in rats is mentionend with dose levels of 2 or 20 µg/kg of 24-epibrassinolide in feed. In rats fed with high-cholesterol diet, the intake of a daily dose of 2 µg/kg of 24-epibrassinolide for 4 weeks reduced the plasma concentration of total cholesterol for 34% and triglycerides for 58%. In comparison with control animals fed 24-epibrassinolide, plasma concentration of vitamin A and vitamin E increased for 16% and 53%, correspondingly. In rats fed with high-cholesterol diet, the intake of a daily dose of 20 µg/kg of 24-epibrassinolide for 4 weeks reduced the plasma concentration of total cholesterol for 44%, triglycerides for 68% and low-density lipoprotein for 11% in comparison with control animals that received the high-cholesterol diet only.	Low relieablilty, no experimental details are reported

B.6.3.1. Oral 28-day study

No guideline-compliant studies available, not required.

B.6.3.2. Oral 90- day study

Annex point	CA 5.3.2/01
Reference:	REPEATED DOSE 90 DAYS ORAL TOXICITY STUDY IN RAT WITH 24-EPIBRASSINOLIDE (TGAI)
Author(s), year:	[redacted] (2017)
Report/Doc. number:	6120 (533-001)
Guideline(s):	OECD No. 408 (1998)
GLP:	Yes
Deviations from OECD 408 (1998):	Minor deviations
Acceptability:	Yes

Executive summary

The purpose of this study was to evaluate the subchronic toxicity and to determine the no-observed-adverse-effect level (NOAEL) after repeated dosing of 24-Epibrassinolide to rats over 90 days (13 weeks), as well as the recovery period (17 weeks respectively).

24-Epibrassinolide (TGAI) was administered by gavage once daily to a group of Wistar rats of both sexes for a period of 90 days at doses of 100, 300 and 1000 mg/kg body weight/day. No test item related changes in the body weights, food consumption, ophthalmological, haematological, urine and clinical biochemistry parameters were noted in the groups of the main test. Macroscopic examination revealed no abnormality attributable to the treatment

at the highest dose (1000 mg/kg bw/d) and there were no treatment related microscopic findings across different groups of both sexes.

- **NOAEL = 1000 mg/kg bw/day.**

I. MATERIALS AND METHODS

A. Test material and vehicle

1 Test materials

Test substance	24-Epibrassinolide (TGAI)
CAS no.	78821-43-9
Lot/batch	002-20150112
Chemical purity	91.2% w/w
Expiry date	January 11, 2017
Storage conditions:	Room Temperature (20°C to 30°C)
Vehicle	0.1% Sodium Carboxymethyl Cellulose

2 Test animals

Species	Rat
Strain	Wistar Rats (*Rattus norvegicus)*
Age	6 - 8 weeks
Bodyweight at dosing	Male: 141.4 – 183.5 g Female: 118.3 – 154.8 g
Source	[redacted]
Acclimation period	7-9 days
Diet	*Ad libitum* Teklad Certified Global 14% Protein Rodent Maintenance Diet (Batch No. 2014C-111215MA) from ENVIGO
Water	*Ad libitum* tap water
Housing	Individually
Temperature	22 ± 3°C
Humidity	30 - 70%
Ventilation	≥ 10 times/hour
Photoperiod	12 hours light / 12 hours dark

B. Study design and method

Test substance preparation and administration
The test substance was formulated in 0.1% Sodium Carboxymethyl Cellulose as the vehicle and prepared shortly before dosing on every day.
The test item was administered to the rats, for the period of 90 consecutive days by oral route using oral gavage needle.

Sampling
Samples in triplicate for high and low dose formulations were taken for stability analysis. Samples in triplicate for each dose formulation were taken from lower, middle and top layers for homogeneity test. A sample in triplicate for each dose formulation was taken for dose concentration.

Dose Range Finding
Dose range finding study was carried out to confirm the high dose level for the main study. A total of three groups (low, intermediate and high), consisting of 5 male and 5 female rats were treated for a period of two weeks at dose levels of 500, 1000 and 1500 mg/kg bw/day. All animals were observed for mortality/viability, clinical signs of toxicity, body weight and food consumption. At the end of treatment all animals were necropsied. Descriptions of all macroscopic abnormalities were recorded. Organs as specified in the study plan were weighed. Samples of the organs/tissues (as listed in the study plan) were preserved until start of experiment. Collected organ(s)/tissue(s), which showed significance, were subjected for histopathology examination. Organs/tissues, which did not show significant changes compared to controls were discarded at the start of the main study.

Table 6.3.2-1: Animal Groups of Dose Range Finding Tests

Number of Groups	4
Number of animals per group	Group 1 – control (Vehicle): 5 males, 5 females
	Group 2 – 500 mg/kg: 5 males, 5 females
	Group 3 – 1000 mg/kg: 5 males, 5 females
	Group 4 – 1500 mg/kg: 5 males, 5 females
Total number of animals	40 (20 males and 20 females)

No mortality occurred during the study period. No clinical signs of illness were observed in any of the animals during treatment. No statistically significant changes in food consumption, body weight or body weight gain were observed. However, there was a dose-related decrease in food consumption (exceeding 10% at the high dose level) compared with controls in males during Weeks 1 and 2 and in females during Week 1 of treatment.

Main test

Groups of ten animals each of both sexes were treated for 13 weeks with concentrations of 0 (control), 100 (low dose), 300 (intermediate dose) and 1000 mg/kg bw/day (high dose). In addition, two recovery groups of each 5 males and 5 females, one for the control and one for the hogh dose group, were included in the main test (13 weeks treatment, 4 weeks recovery).

Table 6.3.2-2: Animal Groups of Main Test

Number of Groups	06
Number of animals per group	Group1 – Control (Vehicle): 10 males, 10 females
	Group 2 – 100 mg/kg bw/day: 10 males, 10 females
	Group 3 – 300 mg/kg bw/day: 10 males, 10 females
	Group 4 – 1000 mg/kg bw/day: 10 males, 10 females
	Group 1R – Control recovery: 5 males, 5 females
	Group 4R – 1000 mg/kg recovery: 5 males, 5 females
Total number of animals	100 (50 males and 50 females)

Clinical Laboratory Investigations

Blood and urine samples for hematology and clinical biochemistry were collected from all animals, in week 13 for Groups 1-4 and in week 17 for Groups 1R and 4R. Urine samples were collected in the morning before the blood collection. Blood samples were drawn from the retro-orbital plexus using a micro-hematocrit heparinised glass capillary tube, under the influence of isoflurane inhalation anaesthesia. Blood samples were centrifuged, and plasma was separated for clinical biochemistry. Hematological and Biochemical analysis was done using Hematology analyser and Biochemistry analyser, respectively.

Mortality and clinical observations

Cage-side observation for mortality and moribundity was performed at least twice per day in all animals.
Clinical examination, including palpation of masses, was conducted at least once per week, twice daily on first three days of treatment.
Clinical observations include changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (lacrimation, piloerection, pupil size, and unusual respiratory pattern).

All animals were observed for the Functional Observational Battery (FOB) during week 12 (G1, G2, G3, G4, G1R and G4R) and during week 17 (G1R and G4R) of the experiment as per score and grade.

Table 6.3.2-3: Mortality and clinical observations time schedule

Mortality / Viability		Twice daily
General Clinical Observations		
	Acclimatization Period	Once daily
	Treatment Period	Twice daily on first 3 days of treatment; once daily thereafter
	Recovery Period	Once daily
Detailed Clinical Observations		Once prior to the first exposure and weekly thereafter
Food Consumption		
	Treatment Period	Once weekly
	Recovery Period	Once weekly

Body Weights		
	Treatment Period	Once weekly
	Recovery Period	Once weekly
Ophthalmoscopy		Prior to the administration of the test item (in 20% of total population) and during week 13 in all animals of group 1 and 4. Tropicamide was used as a mydriatic agent.
Functional Observation Battery		1. Sensory reactivity to stimuli was assessed as per the methods employed by Modified Irwin. 2. Motor activity was assessed with automated IR activity system and rota rod for each animal. 3. Forelimb grip strength was measured using a force gauge.

Organ Weights
The following organs were weighed and recorded on the scheduled dates of necropsy.

Brain	Spleen	Thymus	Epididymides	Liver	Uterus
Heart	Kidneys	Testes	Ovaries	Adrenals	

Wet weight was taken as soon as possible after dissection to avoid drying. Paired organs were weighed together. Relative organ weights were calculated by relating the absolute organ weights to the last determined (terminal) individual body weight and to the brain weight.

Necropsy
The animals were sacrificed at the end of the treatment for gross pathology examination.
All surviving animals of allocation A of the main study (Group 1 to Group 4) were sacrificed at the end of week 13 and animals of allocation B (Group 1R and Group 4R) were sacrificed at the end of week 17 of the experimental period. All animals were weighed and necropsied. Descriptions of all macroscopic abnormalities were recorded.
Full histopathology was performed on the preserved organs and tissues of all animals in the control and high dose groups and examined microscopically.

C. Statistics
The following statistical methods were used to analyze the body weight, functional observation battery, food consumption, organ weights as well as clinical pathology data.

- Data was summarized in tabular form. Statistical analysis was performed using statplus program.
- All the data was checked for normality with Shapiro-Wilk W test
- Data for each group of animals was subjected to analysis of variance (ANOVA). Values were given as mean ± standard deviation (SD).
- t-test was used to compare the difference between treated and control groups. Statistical significances of differences were calculated with one-way analyses of variance.
- $P \leq 0.05$ (5% level of significance) was considered to represent the significance in the respective parameters.

II. RESULTS AND DISCUSSION

Analytical results
The concentration of test item (24-Epibrassinolide (TGAI)) in the dose formulation was determined after derivatisation of the active substance 24-epibrassinolide (TGAI) with phenylboronic acid by HPLC analysis.
The analytical method for determination of the test item concentration in the dose formulation was validated and the detector response was found to be linear ($r^2 = 1000$) in the range of 200 to 600 mg/L concentration.
Accuracy and precision of the method were assessed by five recovery determinations each on two concentration levels.

Main study

Mortality and clinical signs
Mortality was not observed in any of the animals of the different treatment groups during the study. No clinical signs of illness were observed in any of the animals during treatment and recovery period.
Ophthalmological examiniation did not reveal any abnormalities in the control and high dose treated animals at the end of treatment period.

Body weights
In males, the body weights were significantly decreased in the high dose group (1000 mg/kg bw/d, G4) when compared with the control group (G1) animals on days 71, 78, 85 and 90. The male animals of the treated recovery group (1000 mg/kg bw/d, G4R) revealed a statistically significant decrease in body weight from day 50 to termination on day 118 when compared to the vehicle control group (G1R). Since the decreases in body weight of male animals were below 10% when compared to control values and were accompanied by decreased food consumption, they were considered to be without toxicological relevance.

Table 6.3.2-4 Summary of body weights [g] in male rats

Day		**Dose [mg/kg bw/d]**					
		0	**100**	**300**	**1000**	**0 Recovery**	**1000 Recovery**
		G1	**G2**	**G3**	**G4**	**G1R**	**G4R**
1	**Mean**	160	159.2	159.2	158.7	161.8	162
	SD	13.34	12.38	12.51	12.1	11.74	11.73
8	**Mean**	179	178.9	178.6	178.6	181.2	181.8
	SD	12.75	12.05	12.43	11.15	12.5	12.79
15	**Mean**	199	196.2	198.2	195.9	196.3	196.4
	SD	11.17	9.14	13.29	11.32	12.42	10.32
22	**Mean**	235	232.6	234.2	229.2	229.9	229.5
	SD	6.81	9.92	9.91	11.59	10.96	7.31
29	**Mean**	273.5	270.5	279.5	270.9	270.1	273.8
	SD	10.39	17.32	11.91	14.4	7.67	10.61
36	**Mean**	309.3	305.1	316.6	315.2	316.6	307.6
	SD	15.51	20.02	13.86	14.7	9.54	7.54
43	**Mean**	347.4	341.4	350.3	345.8	354.6	341.4
	SD	15.2	22.55	13.99	16.15	14.91	7.8
50	**Mean**	376	368	374.5	374.1	386.2	**364.6***
	SD	15.46	20.96	13.35	19.29	12.95	8.64
57	**Mean**	406.4	393.5	399.1	395.5	413.7	**387.2***
	SD	15.27	19.58	14.82	19.88	13.16	11.85
64	**Mean**	430.7	414.3	417.9	415.6	441.8	**407.8***
	SD	14.59	18.82	18.44	19.28	13.07	7.67
71	**Mean**	451.6	429.4	430.6	**429.0***	460.3	**420.3***
	SD	14.7	20.71	18.46	19.88	12.56	7.84
78	**Mean**	463.6	**441.0***	442.9	**439.2***	474.9	**434.1***
	SD	13.83	20.63	18.34	20.07	11.53	9.99
85	**Mean**	475.1	**452.4***	454.2	**450.0***	489.4	**445.2***
	SD	14.67	21.96	17.51	19.33	11.81	9.99
91	**Mean**	483.9	463.4	462.3	**459.7***	502.6	**457.2***
	SD	14.89	20.8	18.03	19.26	11.54	11.6
98	**Mean**	-	-	-	-	511.7	**465.4***
	SD	-	-	-	-	11.85	11.82
105	**Mean**	-	-	-	-	521.5	**473.2***
	SD	-	-	-	-	10.97	10.81
112	**Mean**	-	-	-	-	529.8	**484.6***
	SD	-	-	-	-	11.11	11.61
118	**Mean**	-	-	-	-	539.5	**496.6***
	SD	-	-	-	-	10.95	14.29

SD: standard deviation
Number of animals: Group 1-4: 10 animals/group, Recovery groups: 5 animals/group
* Significant at $p \leq 0.05$ level (for groups G2, G3 and G4 compared to control group G1; for recovery group G4R compared to recovery control group G1R)

In females, there were only minor, statistically not significant differences in body weight in the treated groups (G1 to G4).
Statistically significant decreases in body weight were observed in female animals of the high dose recovery group (1000 mg/kg bw/d, G4R) when compared to the control recovery group (G1R) from day 43 to termination on day 118. The decrease in body weight was slightly above 10% when compared to control values on days 71, 78 and 85. This effect is likely attributable to the slight decrease in food consumption observed in G4R females. Interestingly, the control female animals of the G1R group showed consistently higher body weights than those of the G1 group of the same treatment week.
As the effects on body weight were only observed in the female animals of the high dose recovery group (1000 mg/kg bw/d, G4R) but not in those of the high dose group (1000 mg/kg bw/d, G4), they were considered of no toxicological relevance by the study authors.

Table 6.3.2-5: Summary of body weights [g] in female rats

Week		Dose [mg/kg bw/d]					
		0	100	300	1000	0 Recovery	1000 Recovery
		G1	G2	G3	G4	G1R	G4R
1	Mean	138.2	137.5	137.3	137.6	141.9	141.7
	SD	12.66	12.47	12.18	12.84	11.31	11.06
8	Mean	147.3	147.2	146.8	146.7	151.7	151.4
	SD	12.64	12.16	12.29	13.34	12.22	11.23
15	Mean	159.3	158.5	159.9	159.5	163.0	162.4
	SD	12.62	12.11	13.57	14.35	11.26	11.59
22	Mean	177.8	178.3	180.9	178.9	182.8	180.6
	SD	11.63	12.88	14.27	12.36	9.83	12.22
29	Mean	195.8	197.7	201.8	197.3	203.9	197.4
	SD	13.59	14.22	14.22	13.06	8.84	13.80
36	Mean	211.6	216.8	219.2	212.6	223.8	211.1
	SD	14.19	16.29	14.69	10.98	11.56	11.03
43	Mean	223.6	228.9	229.7	221.9	236.7	**220.0***
	SD	13.44	15.82	15.50	10.86	11.17	11.23
50	Mean	235.3	240.2	240.9	231.2	248.4	**230.1***
	SD	12.64	14.12	15.92	10.20	12.15	9.50
57	Mean	248.2	253.1	250.3	240.9	261.9	**240.0***
	SD	12.75	15.54	15.59	8.78	12.27	9.13
64	Mean	260.7	265.0	260.4	251.6	275.7	**250.3***
	SD	12.97	15.69	16.00	7.97	12.91	9.52
71	Mean	272.9	273.5	268.8	260.5	286.4	**256.9***
	SD	12.33	15.29	15.73	8.24	15.04	8.74
78	Mean	280.8	283.0	277.1	268.6	294.2	**263.3***
	SD	12.09	19.12	15.85	9.12	15.20	9.62
85	Mean	288.0	290.4	284.7	275.0	303.1	**271.5***
	SD	12.04	19.23	14.99	9.79	16.61	9.35
91	Mean	294.6	297.1	292.0	282.0	310.9	**280.3***
	SD	11.82	20.42	15.23	10.89	17.37	10.82
98	Mean	-	-	-	-	315.9	**286.2***
	SD	-	-	-	-	16.35	11.63
105	Mean	-	-	-	-	323.1	**292.6***
	SD	-	-	-	-	17.05	10.81
112	Mean	-	-	-	-	328.3	**298.1***
	SD	-	-	-	-	16.33	11.71
118	Mean	-	-	-	-	335.2	**304.0***
	SD	-	-	-	-	16.51	11.68

SD: standard deviation
Number of animals: Group 1-4: 10 animals/group, Recovery groups: 5 animals/group
* Significant at $p \leq 0.05$ level (compared to recovery control group G1R)

The body weight gain of male animals of all treatment groups was comparable with the control group animals during the entire dosing period up to day 57 and was consistently decreased thereafter in all treatment groups until termination on day 90. The treated animals of the high dose recovery group (1000 mg/kg bw/d, G4R) showed a decrease in body weight gain from day 50 to day 118, however, only one single statistically significant decrease was observed on day 78 when compared to the vehicle controls (G1R).

In females, the body weight gain of the treated groups was comparable to the vehicle control group throughout the study period. The body weight gain of the female animals in the recovery group (1000 mg/kg bw/d, G4R) was consistently lower throughout the study period. Only one single significant decrease was observed on day 78 when compared to the vehicle controls (G1R).

The variations in the body weight gain were considered non relevant as they were related to slightly lowered food consumption.

Food Consumption

Male animals:
In the first week, a statistically significant decrease in food consumption was observed in male animals of all treated groups when compared to the vehicle control group. From week 2 to week 7 statistical significant decreases were observed at doses of 300 (G3) and 1000 mg/kg bw/d (G4) when compared with the vehicle control group. From week 8 to experimental termination in week 13 there were only minor differences in food consumption of the treated male animals in comparison to the vehicle treated male animals. The food consumption of the treated male animals in the recovery group (1000 mg/kg bw/d, G4R) was consistently lower than that of the vehicle control animals of the recovery group (G1R) throughout the study course, however, the difference revealed no statistical significance.

Female animals:
In female animals only minor differences were observed between the treated groups and the vehicle control group from week 1 to study termination in week 13. The food consumption in female animals was consistently lower than that of the vehicle control animals from week 8 to termination in week 17, however, it was not significantly decreased during this treatment period.

Table 6.3.2-6: Summary of food consumption [g] per animal per day in male rats

Dose [ppm]		Week													Average [g/rat/d]
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	
0 (G1)	**Mean**	13.80	14.30	15.14	16.57	16.69	17.07	17.40	21.59	21.64	20.37	21.76	21.75	30.39	19.11
	SD	0.334	0.193	0.492	0.657	0.413	0.715	0.491	0.701	0.810	3.252	0.447	0.268	0.754	
100 (G2)	**Mean**	**13.10***	13.52	14.17	15.15	15.76	16.27	17.15	21.29	21.58	21.82	21.84	21.67	29.89	18.71
	SD	0.576	0.549	0.676	0.854	1.061	0.749	1.101	0.482	0.524	0.403	0.649	0.554	0.702	
300 (G3)	**Mean**	**11.65***	**12.11***	**12.81***	**13.94***	**14.19***	**14.74***	**14.90***	21.45	21.30	22.06	21.71	21.72	30.07	17.9
	SD	0.149	0.682	0.783	0.742	0.478	0.442	0.546	0.130	0.423	0.421	0.433	0.371	1.109	
1000 (G4)	**Mean**	**11.59***	**12.25***	**12.74***	**13.48***	**13.88***	**14.30***	**14.90***	20.80	21.40	21.32	21.26	21.57	29.38	17.61
	SD	0.277	0.579	0.703	0.973	0.940	0.815	0.652	0.465	0.555	0.643	0.379	0.439	0.678	

SD: standard deviation
Number of cages: 5 cages/group
* Significant at $p \leq 0.05$ level

Table 6.3.2-7: Summary of food consumption [g] per animal per day in male rats recovery group

Dose [ppm]		Week																	Average [g/rat/d]
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	
Group 1R (G1R)	**Mean**	13.90	14.25	15.43	16.56	16.67	17.21	18.14	24.75	24.83	25.29	25.94	25.90	29.74	26.38	25.83	25.81	29.87	22.15
	SD	1.30	1.23	2.09	2.84	2.78	3.12	3.05	4.61	5.11	5.56	6.48	6.87	8.03	8.07	9.26	8.90	8.68	
Group 4R (G4R)	**Mean**	13.10	12.99	13.54	14.12	14.52	15.27	15.64	23.79	23.27	23.05	23.60	23.92	27.16	23.71	23.97	24.05	28.54	20.25
	SD	2.07	1.80	1.97	2.22	2.05	2.05	2.30	5.48	6.07	5.87	6.84	6.86	7.71	7.15	7.51	8.01	7.94	

Table 6.3.2-8: Summary of food consumption [g] per animal per day in female rats

Dose [ppm]		Week													Average [g/rat/d]
		1	2	3	4	5	6	7	8	9	10	11	12	13	
0 (G1)	Mean	10.70	11.36	12.01	12.47	12.68	13.47	13.94	17.53	18.55	18.53	18.63	18.90	26.33	15.78
	SD	0.818	1.063	0.972	1.081	0.834	0.564	0.452	0.564	0.205	0.704	0.852	0.501	1.423	
100 (G2)	Mean	10.72	11.24	11.61	11.87	12.48	12.80	13.24	16.72	**16.89***	17.74	18.05	18.47	25.70	15.19
	SD	1.294	1.237	1.204	1.138	1.208	1.243	1.243	0.515	0.966	0.299	0.284	0.348	0.996	
300 (G3)	Mean	10.15	10.95	11.55	11.86	12.29	13.04	13.39	16.97	17.79	18.07	18.22	18.36	26.30	15.30
	SD	1.038	0.676	0.908	0.863	0.986	0.767	0.817	0.671	0.251	0.807	1.002	0.722	0.456	
1000 (G4)	Mean	9.70	10.15	10.85	11.27	11.81	12.41	12.65	**18.61***	18.17	**16.98***	17.93	18.23	26.10	14.99
	SD	0.938	0.683	0.970	0.702	0.755	0.521	0.803	0.430	1.031	0.819	0.086	0.480	1.017	

SD: standard deviation
Number of cages: 5 cages/group
* Significant at $p \leq 0.05$ level

Table 6.3.2-9: Summary of food consumption [g] per animal per day in female rats recovery group

Dose [ppm]		Week																	Average [g/rat/d]
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
Group 1R (G1R)	Mean	11.20	11.70	12.17	13.29	12.95	13.15	13.64	19.39	21.03	20.75	21.11	22.44	26.04	22.30	22.24	21.27	24.33	18.18
	SD	1.761	2.281	2.386	2.853	1.566	1.670	1.615	0.735	2.809	3.317	4.246	5.300	6.255	4.866	5.982	5.211	5.235	
Group 4R (G4R)	Mean	10.52	11.72	12.03	12.68	12.51	13.11	13.69	16.62	17.35	17.85	18.44	18.70	21.12	18.96	19.30	19.28	23.14	16.30
	SD	2.110	2.344	2.484	3.214	1.992	1.677	1.464	2.158	2.733	3.229	2.593	2.806	3.181	2.575	2.952	2.161	5.069	

Clincial Pathology

Hematology

No toxicologically relevant findings were noted in hematology parameters at the treatment and recovery period.

Hematology parameters such as erythrocyte count (RBC), hemoglobin (Hb), reticulocytes (RET), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), platelet (thrombocyte) count (PLT), total leukocyte count (WBC), differential leukocyte count (DC) and activated partial thromboplastin time (APTT) did not show any toxicologically relevant finding in both sexes.

However, in the 1000 mg/kg bw/d group (G4) there was a single significant decrease in prothrombin time (PT) in males when compared with the control group (G1). The difference was minor and no effect was observed in the recovery group. Thus, it was considered as being without toxicological relevance.

Table 6.3.2-14 Hematology Summary (Males)

Group	**PT**
	Sec
G1 (0 mg/kg bw/d)	15.45
G2 (100 mg/kg bw/d)	15.02
G3 (300 mg/kg bw/d)	14.70
G4 (1000 mg/kg bw/d	**14.44***
G1R (0 mg/kg bw/d)	14.82
G4R (1000 mg/kg bw/d)	15.06

*Significant at $p < 0.05$ level with group 1

Clinical Biochemistry

No toxicologically relevant findings were noted in clinical biochemistry parameters at the treatment and recovery period.

Clinical biochemical parameters like glucose (GLU), creatinine (CREA), cholesterol (CHOL), triglycerides (TRIGL), alanine aminotransferase (ALT), alkaline phosphatase (ALP), sodium (Na), potassium (K), chloride (Cl), total protein (TPO), albumin (ALB), globulin (GLB) and A G ratio did not show any toxicologically relevant findings in both sexes in the treatment and recovery groups.

A single significant increase in AST was observed in male animals in the high dose group of 1000 mg/kg bw/d (G4) when compared to the vehicle control. There were no significant changes observed in the male recovery group animals.

Table 6.3.2-15 Clinical Biochemistry Summary (Males)

Group	**AST**
	U/L
G1 (0 mg/kg bw/d)	58.88
G2 (100 mg/kg bw/d)	59.04
G3 (300 mg/kg bw/d)	58.57
G4 (1000 mg/kg bw/d	**74.19***
G1R (0 mg/kg bw/d)	58.80
G4R (1000 mg/kg bw/d)	70.02

* Significant at $p < 0.05$ level

In female animals of the recovery group (G4R), a significant decrease in bilirubin (BIL) was observed when compared to the vehicle control group (G1R). However, these changes observed were not considered to be treatment related as these values were within the historical control range and histopathology revealed no abnormalities. Furthermore, there was no change in BIL in the treatment groups without recovery (G2, G3 and G4).

Table 6.3.2-16 Clinical Biochemistry Summary (Females)

Group	BIL
	µmol/L
G1 (0 mg/kg bw/d)	1.43
G2 (100 mg/kg bw/d)	1.68
G3 (300 mg/kg bw/d)	1.53
G4 (1000 mg/kg bw/d	1.30
G1R (0 mg/kg bw/d)	1.64
G4R (1000 mg/kg bw/d)	**0.64***

* Significant at $p < 0.05$

Urine Analysis

Urine analysis parameters (specific gravity, colour, clarity, pH, erythrocytes, leukocytes, urobilinogen, bilirubin, ketone bodies, proteins and glucose and microscopic examination) did not reveal any test item related changes in both sexes during treatment and recovery period.

In male animals of the recovery group (1000 mg/kg bw/d, G4R), a significant increase in epithelial cells and pus was noticed when compared to the vehicle control (G1R) group, whereas no changes were observed in the treatment groups without recovery (G2, G3 and G4) compared to controls (G1).

Table 6.3.2-17 Urine analysis Recovery Summary (Males)

RECOVERY		ERY	LEU	EPI	RBC	PUS	CAST	CRY
		/nL	/uL	/filed	/field	/filed	/field	/field
Group 1R	Mean	0.00	100.00	0.40	0.00	1.00	0.00	2.20
	SD	0.000	0.000	0.548	0.000	0.000	0.000	0.447
Group 4R	Mean	0.00	165.00	**1.20***	0.00	**1.60***	0.00	3.00
	SD	0.000	190.066	0.447	0.000	0.548	0.000	0.707

*Significant at $p < 0,05$ level with group (G1R)

In female animals of the low dose group of 100 mg/kg bw/d (G2) a significant increase in urine volume was observed when compared to the control group (G1). The variations were considered incidental in the absence of a dose dependent trend and in the absence of relevant histopathological changes.

Functional Observation Battery

The home cage, hand-held, open field, stimulus response, IR activity, neuromuscular measurements (grip strength) and rota rod parameter of all treatment groups and the recovery group were comparable to the vehicle control animals. Neurological evaluation for individual animals, as applicable, was recorded in the form of scores. In male animals of the high dose recovery group (1000 mg/kg bw/d, G4R), a statistically significant increase in slow movement of IR activity was noticed when compared to the vehicle control group (G1R), which however was not observed in the treatment groups without recovery (G2, G3 and G4).

Necropsy

Organ Weights

No test item related changes were observed in absolute and relative organ weights of male (Table 5.3.2-19) and female animals (Table 5.3.2-20) during treatment and recovery period.

In male animals, a statistically significant increase in absolute and relative spleen weight and relative kidney weight was observed at 1000 mg/kg bw/d (G4). The increase was dose-related; however, histopathological examination revealed no abnormalities in spleen and kidney and no significant organ weight effects were observed in the recovery group animals (1000 mg/kg bw/d, G4R). The males animals of the recovery group (1000 mg/kg bw/d, G4R) showed significant increases in relative liver, testes, epididymides and brain weight. No significant effects on these organ weight were observed in the 90 day treatment groups without recovery (G2, G3 and G4) and there were no test item related abnormalities in histopathological examination.

A statistically significant decrease in absolute thymus weight and an increased relative heart weight was observed in female animals of the recovery group (1000 mg/kg bw/d, G4R). The relative brain weight was significantly increased in females in the treatment groups 300 (G3) and 1000 mg/kg bw/d (G4). In addition, the female recovery

treatment group of 1000 mg/kg bw/d (G4R) showed a significant increase in relative brain weight. However, there were no relevant findings attributable to test item treatment in histopathological examination in the respective organs.

A statistically significant decrease in spleen weight relative to brain was observed in male animals of 1000 mg/kg bw/d (G4). Females of 1000 mg/kg bw/d recovery group (G4R) showed a significant decrease in thymus weight relative to brain. However, there were no relevant microscopic findings observed attributable to test item in the respective organs.

The changes observed in organ weight of male and females rats were not considered to be test item related, but to be a results of changes in body weight or considered as biological variation.

Table 6.3.2-18 Organ Weights (gram) – Summary males

		Group 1	Group 2	Group 3	Group 4	Group 1R	Group 4R
Body Weight	**Mean**	472.44	452.02	452.08	447.86	524.90	475.72
	SD	15.32	20.91	17.48	20.10	8.04	18.67
	N	10	10	10	10	05	05
Adrenals	**Mean**	0.0662	0.0788	0.0849	0.0759	0.0654	0.0628
	SD	0.0157	0.0139	0.0207	0.0151	0.0091	0.0067
	N	10	10	10	10	05	05
Kidneys	**Mean**	2.3336	2.1936	2.3886	2.5138	2.3338	2.3978
	SD	0.2223	0.4636	0.2356	0.2069	0.3935	0.1755
	N	10	10	10	10	05	05
Liver	**Mean**	12.6218	12.0192	12.8301	13.6152	11.4254	12.3658
	SD	1.1652	1.0885	1.5486	2.0690	1.3760	1.4254
	N	10	10	10	10	05	05
Heart	**Mean**	1.3284	1.2493	1.2779	1.3625	1.3809	1.3427
	SD	0.1413	0.1599	0.0818	0.1137	0.1518	0.1871
	N	10	10	10	10	05	05
Thymus	**Mean**	0.5065	0.5082	0.4796	0.4598	0.5243	0.5122
	SD	0.1335	0.1027	0.0892	0.0721	0.1294	0.1219
	N	10	10	10	10	05	05
Spleen	**Mean**	0.7279	0.7382	0.7870	**0.8422***	0.7270	0.7413
	SD	0.0607	0.0790	0.0853	0.0942	0.0871	0.0660
	N	10	10	10	10	05	05
Testes	**Mean**	4.0625	4.0428	3.9203	4.2332	3.9811	4.2751
	SD	0.4974	0.3337	0.5152	0.3802	0.4191	0.3176
	N	10	10	10	10	05	05
Epididymides	**Mean**	1.7313	1.7946	1.7668	1.7899	1.6226	1.6966
	SD	0.1549	0.2727	0.3092	0.1081	0.1346	0.1256
	N	10	10	10	10	05	05
Brain	**Mean**	2.2360	2.1579	2.2230	2.2858	2.1939	2.2695
	SD	0.0775	0.0620	0.1227	0.1295	0.0787	0.0811
	N	10	10	10	10	05	05

* Significant at $p \leq 0.05$ level

Table 6.3.2-19 Organ Weights (gram) – Summary females

		Group 1	Group 2	Group 3	Group 4	Group 1R	Group 4R
Body Weight	**Mean**	297.12	287.45	283.50	272.21	322.10	292.84
	SD	16.91	22.05	15.15	11.45	16.54	11.02
	N	10	10	10	10	05	05
Adrenals	**Mean**	0.0896	0.0918	0.0945	0.0860	0.0842	0.0883
	SD	0.0178	0.0138	0.0090	0.0125	0.0156	0.0154
	N	10	10	10	10	05	05
Kidneys	**Mean**	1.5110	1.5654	1.5477	1.5113	1.6716	1.6221
	SD	0.2198	0.1097	0.0914	0.1272	0.1392	0.1002
	N	10	10	10	10	05	05
Liver	**Mean**	7.1515	7.3196	7.4839	7.1292	7.6005	7.8740

		Group 1	Group 2	Group 3	Group 4	Group 1R	Group 4R
	SD	1.4944	0.7080	0.7317	0.6729	1.0094	0.6735
	N	10	10	10	10	05	05
Heart	**Mean**	0.9575	0.9069	0.9728	0.8754	0.9115	0.9270
	SD	0.1479	0.1201	0.0991	0.1137	0.0590	0.0451
	N	10	10	10	10	05	05
Thymus	**Mean**	0.3794	0.3863	0.4234	0.4147	0.4897	**0.4090***
	SD	0.0397	0.0738	0.0947	0.0835	0.0415	0.0509
	N	10	10	10	10	05	05
Spleen	**Mean**	0.5271	0.5132	0.5397	0.5454	0.4963	0.4987
	SD	0.0629	0.0685	0.0558	0.1073	0.0325	0.0477
	N	10	10	10	10	05	05
Ovaries	**Mean**	0.1489	0.1482	0.1548	0.1997	0.1617	0.1557
	SD	0.0237	0.0293	0.0212	0.1332	0.0258	0.0340
	N	10	10	10	10	05	05
Uterus	**Mean**	0.7466	0.7474	0.8075	0.7508	0.7685	0.7038
	SD	0.2467	0.1882	0.2764	0.1453	0.3788	0.0827
	N	10	10	10	10	05	05
Brain	**Mean**	1.9891	2.0744	2.1251	2.0151	2.0465	2.0612
	SD	0.1410	0.0943	0.1120	0.1126	0.1197	0.0549
	N	10	10	10	10	05	05

* Significant at $p \leq 0.05$ level

Macroscopic Findings
Necropsy was performed at the end of the treatment and recovery period and no test item related findings were observed macroscopically in all treated male and female animals. No gross abnormality was observed in control group animals. Macroscopically, a distended uterus with watery content was observed in three female animals of the control group (G1) and two female animals of the 300 mg/kg bw/d dose (G3) group. These findings were considered as incidental.

Microscopic Findings
Microscopic examination revealed no abnormality attributable to the test item 24-Epibrassinolide (TGAI) in the high dose group (1000 mg/kg bw/d, G4) group. Most of the findings in tissues evaluated were within normal histological background data for this rat strain.

The histopathological findings observed in various tissues during evaluation of the test item group animals were comparable with the control group and were considered incidental. These changes observed can usually be considered to be species, age, gender, physiological or mode of death related and are covered in background historical data of pathology.

III. CONCLUSION

Based upon the results results obtained, the following can be concluded:

The test item 24-Epibrassinolide (TGAI) was administrated daily by oral route to Wistar rats for a period of 90 consecutive days. A recovery period of 28 days without test item treatment was included in this study.

Three groups consisting of 10 male and 10 female rats each were treated at dose levels of 100, 300 and 1000 mg/kg bw/d respectively. Concurrently, vehicle control and test item high dose recovery groups (1000 mg/kg bw/d) were used in the present study.

All animals were observed for mortality/viability, detailed clinical signs and clinical signs of toxicity. Food consumption and body weight were recorded weekly during the treatment period. All animals were subject to Functional Observation Battery (FOB) examination during treatment period (week 13) and recovery (week 17). At the end of treatment and recovery periods all the animals were necropsied for gross and histopathology examinations.
All the animals survived up to the scheduled sacrifice.
No test item related changes in clinical signs, detailed clinical observation, ophthalmic examination findings, food consumption and body weight changes were noted.

Minor changes in hematological, clinical biochemistry, body weight, functional observation battery, body weight gain, organ weight and urine analysis were recorded at the end of treatment and recovery periods, but the variations were considered incidental as there was no dose dependent trend in the observed variations and the respective tissues revealed no abnormalities in macroscopic and histopathological examination.

Decreases in overall food consumption over the entire study period in both genders (not statistically significant), statistically significantly reduced body weights in male animals, and non-statistically significant body weight reductions in female animals were abserved at the highest dose level.

In conclusion, under the conditions of this experiment the **No Observed adverse Effect Level (NOAEL) was determined to be 300 mg/kg body weight.**

B.6.3.3. Other routes

No guideline-compliant studies available, not necessary.

B.6.4. GENOTOXICITY

The genotoxic potential of 24-Epibrassinolide was investigated in a number of different genotoxicity tests.
24-Epibrassinolide (TGAI) was negative in the bacterial reverse mutation test (Ames) and the mammalian cell gene mutation test (HPRT). In the chromosome aberration test, 24-Epibrassinolide (TGAI) showed no potential for clastogenicity with and without metabolic activation. Open literature did not raise any concerns regarding possible genotoxic or mutagnic effects of 24-epibrassinolide. No data on genotoxicity/ mutagenicity in humans is available.According to these findings, and due to ubiquitous lifetime exposure to 24-epibrassinloide, no *in vivo* studies are considered necessary.

Table 6.4-1: Summary of Available Guideline-Compliant Studies on Genotoxicity

Study	Result	Classification/remarks	Reference
Bacterial Reverse Mutation Test (AMES)	No increase in revertants, either with or without metabolic activation	None	Srilatha S. (2017)
In vitro Mammalian Cell Gene Mutation Test (HPRT)	Non-mutagenic, either with or without metabolic activation	None	Wollny H.-E. (2017)
In vitro Mammalian Chromosome Aberration Test	Non-clastogenic either with or without metabolic activation	None	Kandula S.R. (2017)

Table 6.4-2: Summary of Other Scientifically Relevant Information on Genotoxicity

Study title	Reference/ Report number	Article type	Study content	Relevance/ Relieablilty
STEROID PLANT HORMONES: EFFECTS OUTSIDE PLANT KINGDOM	Zhabinskii VN et al, 2015 092-099	Review	Reviews available knowledge on effects of steroid plant hormones on insects, funghi, fish, protozoa, and warm blooded animals. Results of an AMES test (*S. typhimurium*, TA100) and an assay with phage lambda are mentioned, both negative for mutagenic / genotoxic activity	Low relieablilty, no experimental details are reported
ENZYME IMMUNOASSAY OF THE CONTENT OF ENDOGENOUS BRASSINOSTEROIDS IN PHYTOGENIC FOOD PRODUCTS	Khripach, V.A. et al, 2013 092-030	Research article	Describes the development of a quantitative ELISA assay for 24-epibrassinolide and 24-epicasterone	Not relevant, no information on genotoxicity/ mutagenicity
TWENTY YEARS OF BRASSINOSTEROIDS: STEROIDAL PLANT HORMONES WARRANT BETTER CROPS FOR THE XXI CENTURY	Khripach VA et al, 2000 092-029	Review	Reviews many aspects of research on brassinosteroids. An Ames test for mutagenic activity carried out at the Scientiic Research Center of Toxicologic and Hygienic Regulation of Biopreparations of Russia, with or without metabolic activation, is mentioned and was negative (Salmonella typhimurium TA 1534, TA 1537, TA 1950, TA 98, TA 100). Also, in micronuclear or chromosome abberation tests (mice CBAB1/6) neither 24-epibrassinolide nor Epin (a plant protection product) caused spontaneous mutations.	Low relieability, no experimental details are reported

B.6.4.1. In vitro studies

B.6.4.1.1. Bacterial Reverse Mutation Test

Annex point	CA 5.4/01
Reference:	BACTERIAL REVERSE MUTATION ASSAY WITH 24-EPIBRASSINOLIDE (TGAI)
Author(s), year:	Srilatha, S. (2017)
Report/Doc. number:	6119 (557-001)
Guideline(s):	OECD No. 471 (1997)
GLP:	Yes
Deviations from OECD 471 (1997):	none
Acceptability:	Yes

Executive Summary

This study was performed to assess the mutagenic potential of 24-Epibrassinolide (TGAI) to induce gene mutations in comparison to vehicle (solvent) control according to the plate incorporation method (Trial I) and the pre-incubation method (Trial II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100 and TA 102.

The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the negative, vehicle and positive controls was tested in triplicates. The test item was tested at concentrations 0.0125, 0.0396, 0.1252, 0.3956 and 1.25 mg/plate, both in the presence (+S9) and absence (-S9) of metabolic activation.

No substantial increase in revertant colony numbers in any of the tester strains was observed following treatment with 24-Epibrassinolide (TGAI) at any dose level in both the confirmatory trials, neither in the presence nor in the absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

The solvent and positive controls induced the appropriate responses in the corresponding strains.

It was concluded that 24-Epibrassinolide (TGAI) did not show a mutagenic potential in bacteria.

- **Non-mutagenic in the bacterial reverse mutation assay**

I MATERIALS AND METHODS

A Materials

1 Test materials

Test substance	24-Epibrassinolide (TGAI)
Lot/batch	002-20150112
Chemical purity	91.2% w/w

2 Test system

Strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535, and TA1537) were used in this study.

Regular checking (once in three months) of the properties of the *Salmonella typhimurium* strains regarding the membrane permeability and ampicillin and tetracycline resistance as well as histidine dependence is performed.

B Study design

Storage of tester strains and pre-culture

The strain stock cultures are stored in ampoules with nutrient broth + 9% DMSO (Sigma D-5879) in deep freezer (-60°C to -80°C).

From the thawed ampoules of the strains, loop full of bacterial culture were transferred into Erlenmeyer flasks containing nutrient medium.

Preparation of S9 mix

Aroclor 1254 induced S9 was stored at -60°C to -80°C inside the deep freezer. The protein concentration in the S9 fraction was 37.4 mg/mL. Each batch of S9 mix was tested with 2-aminoanthracene as well as benzo [a] pyrene for its efficiency.

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution, to result in a final concentration of approximately 10% v/v in the S9 mix.
Cofactor solution contained following quantity of chemicals in 500 mL of RO Water.

$MgCl_2$	1.0 g in 500mL
KCl	1.35 g in 500mL
D-Glucose-6-phosphate	0.8 g in 500mL
β-NADP	1.75 g in 500mL
Na_2HPO_4	6.4 g in 500mL
$NaH_2PO_4.H_2O$	1.4 g in 500mL
S9	10% (v/v)

Solvent and positive controls
To select an appropriate solvent and dose concentration range of the test item to be tested in pre-experiment, solubility and precipitation test were performed. In these tests 1.25 mg/plate dissolved in DMSO at 50 mg/mL was selected as highest concentration for the pre-experiment.
Concurrent negative (RO water) and solvent (DMSO) controls were performed.
Positive control substances were chosen as follows:

Table 6.4.1.1-1: List of positive control substances

Without metabolic activation				**With metabolic activation**
Strain	TA 1535, TA 100	TA 1537, TA 98	TA 102	TA 1535, TA 1537, TA 98, TA 100 and TA 102
Substance	Sodium azide, NaN_3	4-Nitro-o-phenylenediamine, 4-NOPD	Methyl methane sulfonate, MMS	2-Aminoanthracene, 2-AA
CAS No	26628-22-8	99-56-9	66-27-3	613-13-8
Purity	99.8%	>98%	99.9%	97.5%
Dissolved in	RO water	DMSO	RO water	DMSO
Concentration	10 µg/plate	10 µg/plate in strain TA 98, 50 µg/plate in strain TA 1537	4.0 µL/plate	2.5 µg/plate [10.0 µg/plate in TA 102]

Pre-experiment
To evaluate the toxicity of the test item a pre-experiment was performed with strains TA 98 and TA 100. Eight concentrations were tested for toxicity and mutation induction in triplicates:
0.0004, 0.0013, 0.0040, 0.0125, 0.0396, 0.1252, 0.3956 and 1.25 mg/plate
The experimental conditions in this pre-experiment were the same as described for the trial I (Plate incorporation test). Toxicity of the test item results in a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn.
The pre-experiment was reported as a part of the main experiment (trial I), because the following criterion was met: Evaluable plates at five concentrations or more.

Reverse mutation tests (Experiment I and II)
Two reverse mutation tests were performed independently, Trial I and II, in the presence and absence of a metabolic activation system. For each strain and dose level, including the controls, three plates (triplicate) were used. From the results of the dose range-finding test**,** the following dose levels were chosen for each test.

Trial I
With and without S9 mix
All strains: 0.0125, 0.0396, 0.1252, 0.3956 and 1.25 mg/plate

Trial II
With and without S9 mix
All strains: 0.0125, 0.0396, 0.1252, 0.3956 and 1.25 mg/plate

Treatment procedures
In the pre-incubation assay 100 µL test solution, 500 µL S9 mix/S9 mix substitution buffer and 100 µL bacterial suspensions were mixed in a test tube and incubated at 37°C for 60 minutes. After pre-incubation 2.0 mL overlay agar (47°C) was added to each tube. The mixture was poured on minimal agar plates.
After solidification the plates were incubated in inverted position for 48 hours at 37°C.

Colony count
The colonies were counted manually. The mean values of the plates for each concentration together with standard deviation were compared to the spontaneous reversion rates. Microsoft Office Excel based calculation were used for descriptive statistical analysis.

Validity criteria
A test item is considered as a mutagen, if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100 and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed.
A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration.
An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.
A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of solvent control such an increase is not considered biologically relevant.

C Statistics
No statistical analysis was conducted.

II RESULTS AND DISCUSSION

Cytotoxicity assay
The pre-experiment was performed with TA 100 and TA 98 strain of *Salmonella typhimurium* and with eight different concentrations of the test item prepared with half log intervals. The top concentration 1.25 mg/plate was selected based on the occurrence of precipitation. The following concentrations were selected for the pre-experiment: 0.0004, 0.0013, 0.0040, 0.0125, 0.0396, 0.1252, 0.3956 and 1.25 mg/plate.
Cytotoxicity was evaluated based on the reduction of revertant colony count and bacterial background lawn.
At concentration 1.25 mg/plate, there was no reduction in colony count but diminution or clearing of background lawn was observed which indicates cytotoxicity of test item at this concentration. Slight precipitation was observed at 1.25 mg/plate which did not interfere with colony scoring. There was no reduction in colony count as well as background lawn at concentrations 0.0004, 0.0013, 0.0040, 0.0125, 0.0396, 0.1252 and 0.3956 mg/plate, both in the absence and presence of metabolic activation, when compared to that of the vehicle control group.
Based on the results of the pre-test, 1.25 mg/plate was selected as the highest dose for the main study both, in the absence (-S9) and in the presence (+S9) of metabolic activation.

Table 6.4.1.1-2: Revertant Count for Pre-experiment

Dose (mg/plate)	R	Without metabolic activation (-S9)		With metabolic activation (+S9)	
		TA100	TA 98	TA100	TA 98
NC (0.00)	R1	88	24	80	19
	R2	72	27	76	16
	R3	101	29	106	15
VC (0.00)	R1	94	18	120	19
	R2	86	22	110	19
	R3	108	25	119	19
T1	R1	103	26	120	26

(0.0004)	R2	110	20	90	20
	R3	116	15	64	15
T2 (0.0013)	R1	98	17	80	25
	R2	106	25	93	20
	R3	89	28	126	14
T3 (0.0040)	R1	111	15	82	20
	R2	100	17	72	18
	R3	94	19	93	19
T4 (0.0125)	R1	83	25	81	17
	R2	74	26	109	15
	R3	69	28	105	15
T5 (0.0396)	R1	78	26	94	24
	R2	96	28	120	18
	R3	108	30	116	20
T6 (0.1252)	R1	115	27	80	19
	R2	106	29	98	15
	R3	91	32	82	19
T7 (0.3956)	R1	68	29	70	19
	R2	74	26	96	15
	R3	85	27	86	14
T8 (1.25)	R1	69(Sppt)	28(Sppt)	68(Sppt)	16(Sppt)
	R2	70(Sppt)	26(Sppt)	66(Sppt)	19(Sppt)
	R3	68(Sppt)	22(Sppt)	81(Sppt)	14(Sppt)
PC	R1	754	476	1772	1152
	R2	822	582	2012	756
	R3	986	634	1872	924

R = Replicate
NC = Negative control
VC = Vehicle control
T = Test concentration (T8: Highest, T1: Lowest)
Sppt = Slight precipitation
PC = Positive control
4-NOPD [10 µg/plate]: TA 98 without metabolic activation
Sodium azide [10 µg/plate]: TA 100 without metabolic activation
2-Aminoanthracene [2.5 µg/plate]: TA98, TA100 with metabolic activation

Experiment I and II

Trial I was performed with five concentrations of test item along with the negative, vehicle and positive controls with the remaining three strains i.e. TA 1537, TA1535 and TA 102 by the plate incorporation method.

For the TA 98 and TA l00 revertant colony counts were directly incorporated in the trial I from the pre-experiment up to the required five concentrations 0.0125 mg/plate (T4) to 1.25 mg/plate (T8).

For trial I the following concentrations of test item were applied with half log intervals:

0.0125, 0.0396, 0.1252, 0.3956 and 1.25 mg/plate both, in the absence (-S9) as well as in the presence of metabolic activation (+S9).

The plates were treated and incubated at 37°C for 48 hours. No relevant increases in the revertant colony count in any of the five strains were reported at any of the test concentrations. The slight dose-related increase observed in trial I for strain TA 1537 with and without metabolic activation is regarded as being biologically irrelevant since the threshold was not exceeded at any of the concentrations applied and the values were in the range of the historical control data. Cytotoxicity was observed at the highest concentration 1.25 mg/plate evident as clearing of the background lawn. Slight precipitation was observed at 1.25 mg/plate which did not interfere with colony scoring.

Positive controls resulted in an unequivocal response in all the five tester strains when compared to the concurrent negative/solvent controls.

Table 6.4.1.1-3: Mean Revertant Count in Plate Incorporation Method (TRIAL I)

Dose (mg/plate)	In the presence of Metabolic Activation (+S9)									
	TA 1537		TA 1535		TA 98		TA 100		TA 102	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
NC	4.33	1.53	10.33	1.53	16.67	2.08	87.33	16.29	276.00	15.10

(0.00)										
VC (0.00)	3.67	1.15	8.00	1.00	19.00	0.00	116.33	5.51	277.33	14.74
T1 (0.0125)	3.33	0.58	10.33	2.08	15.67	1.15	98.33	7.21	266.67	10.26
T2 (0.0396)	4.33	1.15	11.33	2.52	20.67	3.06	110.00	15.14	306.00	8.00
T3 (0.1252)	5.67	1.15	14.33	1.53	17.67	2.31	86.67	14.00	285.33	9.45
T4 (0.3956)	7.33	0.58	13.67	1.53	16.00	2.65	84.00	9.87	294.00	15.10
T5 (1.25)	8.33	0.58	16.33	1.15	16.33	2.52	71.67	13.11	274.00	8.00
PC	160.00	34.77	426.00	103.94	944.00	198.76	1885.33	120.55	1618.67	180.14

Dose (mg/plate)	**In the Absence of Metabolic Activation (-S9)**									
	TA 1537		**TA 1535**		**TA 98**		**TA 100**		**TA 102**	
	MEAN	**SD**	**MEAN**	**SD**	**MEAN**	**SD**	**MEAN**	**SD**	**MEAN**	**SD**
NC (0.00)	3.67	0.58	13.33	4.04	26.67	2.52	87.00	14.53	242.67	1.15
VC (0.00)	4.00	1.73	9.67	1.15	21.67	3.51	96.00	11.14	269.33	15.53
T1 (0.0125)	3.67	1.15	11.00	1.00	26.33	1.53	75.33	7.09	268.67	14.19
T2 (0.0396)	5.00	0.00	12.67	0.58	28.00	2.00	94.00	15.10	297.33	10.07
T3 (0.1252)	5.67	1.15	14.33	1.53	29.33	2.52	104.00	12.12	277.33	15.28
T4 (0.3956)	7.33	0.58	13.67	1.53	27.33	1.53	75.67	8.62	284.67	10.07
T5 (1.25)	8.33	0.58	16.00	1.00	25.33	3.06	69.00	1.00	290.67	12.06
PC	135.67	23.86	949.33	108.25	564.00	80.52	854.00	119.26	1826.67	138.58

NC = Negative Control, **VC =** Vehicle control**, T =** Test concentration (T5: Highest, T1: Lowest), **SD** = Standard Deviation
PC = Positive control
2-Aminoanthracene [2.5 µg/plate]: TA 1537, TA 1535, TA 98, TA 100
2- Aminoanthracene [10 µg/plate]: TA 102
Sodium azide [10 µg/plate]: TA 1535, TA 100
4-NOPD: TA 1537 [50 µg/plate] TA 98 [10 µg/plate]
Methyl methanesulfonate [4 µL/plate]: TA 102

Trial II was performed independently with all the five tester strains along with the negative, vehicle and positive controls by pre-incubation method for the confirmation of the trial I results.
The following concentrations of the test item with half log intervals were applied 0.0125, 0.0396, 0.1252, 0.3956 and 1.25 mg/plate both in the presence (+S9) as well as in the absence (-S9) of metabolic activation.
The concentrations of the positive controls were identical to those used in trial I. The test item, negative and positive controls were pre-incubated along with 500 µL of metabolic activation mix (+S9)/Buffer (-S9) and 100 µL of bacterial culture for 60 minutes at 37°C inside the incubator.
After pre-incubation, 2 mL of top agar was mixed with the pre-incubation mixture and poured on minimal glucose agar plates. The treated plates were incubated for 48 hours inside the incubator.
No relevant increase in the revertant counts was observed in any of the five tester strains pre-incubated with the test item. Cytotoxicity was observed at the highest concentration 1.25 mg/plate evident as clearing of the background lawn. Slight precipitation was observed at 1.25 mg/plate which did not interfere with colony scoring.
The positive controls showed an unequivocal increase in revertant counts with all the five tester strains and compared to the respective controls used, thus confirming the non-mutagenic activity of the test item.

Table 6.4.1.1-4: Mean Revertant Count in Pre-incubation method (TRIAL II)

Dose (mg/plate)	In the presence of Metabolic Activation (+S9)									
	TA 1537		TA 1535		TA 98		TA 100		TA 102	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
NC (0.00)	4.67	2.89	7.67	1.15	15.00	1.00	95.67	7.64	266.00	12.00
VC (0.00)	3.67	0.58	9.33	0.58	16.33	1.53	93.33	7.57	294.67	12.06
T1 (0.0125)	4.67	0.58	10.67	1.53	21.33	4.16	98.00	5.29	300.67	11.72
T2 (0.0396)	4.67	1.53	12.33	1.53	22.00	4.00	96.00	8.00	285.33	10.26
T3 (0.1252)	6.33	0.58	13.33	1.53	24.67	3.06	109.33	2.31	279.33	14.74
T4 (0.3956)	7.00	1.00	15.33	0.58	25.00	2.65	103.67	6.66	295.33	7.02
T5 (1.25)	8.67	1.15	11.33	2.52	21.00	2.65	99.67	7.51	294.67	15.53
PC	172.00	23.58	242.67	64.17	970.67	91.66	1074.00	251.72	1276.00	175.45

Dose (mg/plate)	In the Absence of Metabolic Activation (-S9)									
	TA 1537		TA 1535		TA 98		TA 100		TA 102	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
NC (0.00)	3.33	0.58	8.67	1.53	15.67	0.58	91.67	8.50	254.67	11.37
VC (0.00)	3.67	0.58	9.33	1.53	18.00	2.00	93.67	8.50	276.00	9.17
T1 (0.0125)	4.67	0.58	13.00	1.00	19.33	3.06	103.33	7.23	296.67	3.06
T2 (0.0396)	5.33	0.58	12.67	0.58	21.00	3.61	92.67	12.22	279.33	6.11
T3 (0.1252)	6.67	0.58	16.00	1.00	21.00	3.00	105.67	9.50	288.67	9.02
T4 (0.3956)	6.00	2.00	13.33	3.06	22.33	3.21	105.00	9.54	292.67	9.02
T5 (1.25)	6.67	1.53	10.33	1.53	29.33	1.15	107.33	6.11	283.33	8.33
PC	133.00	12.49	960.00	109.67	636.67	169.47	1062.67	90.43	1505.33	311.59

NC = Negative Control, **VC** = Vehicle control**, T =** Test concentration (T5: Highest, T1: Lowest), **SD** = Standard Deviation
PC = Positive control
2-Aminoanthracene [2.5 µg/plate]: TA 1537, TA 1535, TA 98, TA 100
2- Aminoanthracene [10 µg/plate]: TA 102
Sodium azide [10 µg/plate]: TA 1535, TA 100
4-NOPD: TA 1537[50 µg/plate] TA 98[10 µg/plate]
Methyl methanesulfonate: [4 µL/plate]: TA 102

III CONCLUSIONS

The test item 24-Epibrassinolide (TGAI) was assessed for its potential to induce gene mutations according to the plate incorporation method (Trial I) and the pre-incubation method (Trial II) using *Salmonella typhimurium* tester strains TA 1535, TA 1537, TA 98, TA 100 and TA 102 along with different positive controls for different strains.
The assay was performed in two independent experiments both with and without liver microsomal activation based on the results of the pre-experiment. Each concentration and the controls were tested in triplicates. The test item was tested at concentrations 0.0125, 0.0396, 0.1252, 0.3956 and 1.25 mg/plate, both in the absence (-S9) as well as in the presence of metabolic activation (+S9).

At concentration 1.25 mg/plate, clearing of background lawn was observed which indicates cytotoxicity of the test item at this concentration. Slight precipitation was observed at 1.25 mg/plate which did not interfere with colony scoring (trial I and trial II). No substantial increase in revertant colony numbers in any of the tester strains were observed following treatment with 24-Epibrassinolide (TGAI) at any dose level in both trials, neither in the presence nor in the absence of metabolic activation (S9 mix). Tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance was observed for strain TA 1537 with and without metabolic activation. This finding is regarded as being biologically irrelevant since the threshold was not exceeded at any of the concentrations applied.
The numbers of revertants in the negative, vehicle and positive controls are within the 95% confidence interval of the historical data and therefore, the study is considered acceptable.
The reference mutagens showed a distinct increase in induced revertant colonies in all the tester strains both in the presence (+S9) as well as in the absence (-S9) of metabolic activation without showing any signs of cytotoxicity.
It can be concluded that the test item 24-Epibrassinolide (TGAI) did not induce gene mutations either by base pair substitution or by frame shifts in the genome of the strains used under the conditions of the assay with and without metabolic activation.

B.6.4.1.2. In vitro Mammalian Cell Gene Mutation Test

Annex point	CA 5.4/02
Reference:	24-EPIBRASSINOLIDE (TGAI): GENE MUTATION ASSAY IN CHINESE HAMSTER V79 CELLS IN VITRO (V79/HPRT)
Author(s), year:	Wollny, H.-E. (2017)
Report/Doc. number:	6119 (557-001)
Guideline(s):	OECD No. 476 (2015)
GLP:	Yes
Deviations from OECD 476 (2015):	None
Acceptability:	Yes

Executive summary
The study was performed to investigate the potential of 24-Epibrassinolide (TGAI) to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster. The experiment was performed with a treatment time of 4 hours with and without metabolic activation

The maximum test item concentration of the pre-experiment (2000 µg/mL) was chosen with respect to the current OECD guideline 476. The concentration range of the main experiment was limited by cytotoxicity and precipitation of the test item.

No substantial and reproducible dose dependent increase of the mutation frequency was observed in the main experiment.

Appropriate reference mutagens, used as positive controls, induced a distinct increase in mutant colonies and thus, showed the sensitivity of the test system and the activity of the metabolic activation system.

In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells. Therefore, 24-Epibrassinolide is considered to be not mutagenic.

- **Non-mutagenic in the mammalian cell gene mutation test (HPRT assay)**

I MATERIALS AND METHODS

A Materials

1 Test materials

Test substance	24-Epibrassinolide (TGAI)
Lot/batch	002-20150112
Chemical purity	91.2%*
Expiry Date	11 January 2017
Storage Conditioins	Room Temperature

*Dose calculation was not adjusted to purity.

2 Positive Control Substances

Without metabolic activation	
Test substance	EMS; ethylmethane sulfonate
Dissolved in	Nutrient medium
Chemical purity	99%
Final Concentration	0.3 mg/mL = 2.4 mM

With metabolic activation	
Test substance	DMBA; 7,12-dimethylbenz(a)anthracene
Dissolved in	DMSO; dimethyl sulfoxide
Chemical purity	≥ 95 %
Final Concentration	2.3 µg/mL = 8.9 µM

3 Test system

The test was performed in cells of the V79 cell line in the cell bank of Envigo CRS GmbH. Thawed stock cultures were propagated at 37°C in 75 cm^2 plastic flasks. About 2-3 x 10^6 cells were seeded into each flask with 15 mL of MEM (minimal essential medium) containing Hank's salts supplemented with 10% foetal bovine serum (FBS), neomycin (5 µg/mL) and amphotericin B (1%). All incubations were done at 37°C with 1.5% carbon dioxide (CO_2) in humidified air.

B Study design

Preparation of the test solution

Immediately before treatment, the test item was suspended (pre-experiment) or dissolved (main experiment) in DMSO. The final concentration of DMSO in the culture medium was 0.5% (v/v). The solvent was chosen based on its solubility properties and its compatibility with the cell cultures.
All formulations were prepared freshly before treatment and used within two hours of preparation. The formulation was assumed to be stable for this period unless specified otherwise by the Sponsor. The osmolarity and the pH-value were determined in culture medium of the solvent control and at the maximum concentration in the pre-experiment without metabolic activation.

Metabolic activation system (S9-mix)

Phenobarbital/-naphthoflavone induced rat liver S9 was used as metabolic activation system.
An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).
The protein concentration of the S9 preparation was 29.8 mg/mL in the pre-experiment and in the main experiment.

Pre-test

A pre-test was performed in order to determine the concentration range for the mutagenicity experiment. The general culture conditions and experimental conditions in this pre-test were the same as described for the mutagenicity experiment below. In this pre-test the colony forming ability of approximately 500 single cells (duplicate cultures per concentration level) after treatment with the test item was observed and compared to the controls. Toxicity of the test item is indicated by a reduction of the cloning efficiency (CE).

Dose selection

The pre-experiment was performed in the presence and absence (4 h treatment) of metabolic activation. Test item concentrations between 3.1 µg/mL and 2000 µg/mL were tested with respect to the current OECD guideline.
In the pre-experiment the test medium was checked for precipitation or phase separation at the beginning and at the end of treatment (4 hours) prior to removal to the test item.
The dose range of the main experiment was set according to data generated in the pre-experiment. The individual concentrations were spaced by a factor of 2.0.
To overcome problems with possible deviations in toxicity the main experiment was started with more than four concentrations.

Main mutation test

Two to four days after sub-cultivation stock cultures were trypsinized at 37°C for approximately 5 to 10 minutes. The enzymatic digestion was stopped by adding complete culture medium with 10% FBS and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.2% in saline.
Afterwards the cells were treated according to table 6.4.1.2-5 and subsequently subcultivated, immediately after end of treatment.
Following the expression time of 7 days five 75 cm² cell culture flasks were seeded with about 4 to 5 x 10^5 cells each in medium containing 6-TG. Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability (cloning efficiency II).
The cultures were incubated at 37°C in a humidified atmosphere with 1.5% CO_2 for about 8 days. The colonies were stained with 10% methylene blue in 0.01% KOH solution.
The stained colonies with more than 50 cells were counted. In doubt the colony size was checked with a preparation microscope.

Table 6.4.1.2-1: Doses applied in the gene mutation assay with 24-Epibrassinolide (TGAI)

exposure period	S9 mix	concentrations in µg/mL					
		Main experiment					
4 hours	-	**1.6**	**3.1**	**6.3**	**12.5**	25.0 P	50.0 P
4 hours	+	**3.1**	**6.3**	**12.5**	**25.0 P**	50.0 P	100.0 P

P = Precipitation visible at the end of treatment
Concentrations in **bold** letters were chosen for the mutation rate analysis

The cultures at the two highest concentrations with and without metabolic activation were not continued due to exceedingly severe cytotoxic effects.

C Statistics
A linear regression (least squares, calculated using a validated excel spread sheet) was performed to assess a possible dose dependent increase of mutant frequencies. The numbers of mutant colonies generated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.
A t-Test was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to evaluate an isolated increase of the mutation frequency at a test point exceeding the 95% confidence interval. Again a t-test is judged as significant if the p-value (probability value) is below 0.05.

However, both, biological and statistical significance were considered together.

Interpretation of results
A test item is classified as clearly mutagenic if, in any of the experimental conditions examined, all of the following criteria are met:

a) At least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
b) The increase is dose-related when evaluated with an appropriate trend test,
c) Any of the results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits).

II RESULTS AND DISCUSSION

Pre-test
In the pre-experiment a relevant cytotoxic effect, indicated by a relative cloning efficiency of 50% or below was observed at 24.7 and 74.1 µg/mL without metabolic activation. In the presence of metabolic activation no relevant cytotoxic effect was determined up to the highest concentration.
Precipitation occurred at 24.7 µg/mL and above after 4 hours treatment without metabolic activation and 74.1 µg/mL and above with metabolic activation.
There was no relevant shift of pH and osmolarity of the medium even at the maximum concentration of the test item.

Table 6.4.1.2-2: Toxicity Data, pre-experiment (TGAI), dose selection for main experiment

Test group	conc.	S9 mix	Duration of treatment	Cells seeded	Number of colonies per flask found			CE absolute	CE relative	precipitation
	µg/mL		(h)	I / II	I	II	mean	%	%	
Solvent control with DMSO		-	4	506	234	251	242.5	47.9	100.0	
Test item	3.1	-	4	506	237	228	232.5	45.9	95.9	
Test item	6.2	-	4	506	260	278	269.0	53.2	110.9	
Test item	12.3	-	4	506	203	194	198.5	39.2	81.9	
Test item	24.7	-	4	506	92	73	82.5	16.3	34.0	precipitation
Test item	74.1	-	4	506	117	96	106.5	21.0	43.9	precipitation
Test item	222.2	-	4	506	166	153	159.5	31.5	65.8	precipitation
Test item	666.7	-	4	506	159	182	170.5	33.7	70.3	precipitation
Test item	2000.0		4	506	192	205	198.5	39.2	81.9	precipitation
Solvent control with DMSO		+	4	506	237	264	250.5	49.5	100.0	
Test item	3.1	+	4	506	251	281	266.0	52.6	106.2	
Test item	6.2	+	4	506	250	279	264.5	52.3	105.6	
Test item	12.3	+	4	506	263	271	267.0	52.8	106.6	
Test item	24.7	+	4	506	261	248	254.5	50.3	101.6	
Test item	74.1	+	4	506	251	244	247.5	48.9	98.8	precipitation
Test item	222.2	+	4	506	266	252	259.0	51.2	103.4	precipitation
Test item	666.7	+	4	506	271	244	257.5	50.9	102.8	precipitation
Test item	2000.0	+	4	506	228	226	227.0	44.9	90.6	precipitation

Main mutation test

Dose selection was made based on the results of the pre-test, 4 concentrations were chosen for mutation rate analysis (see table 6.4.1.2-1). The cultures at the two highest concentrations with and without metabolic activation were not continued due to precipitation of the test substance in the culture medium and exceedingly severe cytotoxic effects.

In the main experiment with and without S9 mix the range of the solvent controls was from 9.1 up to 30.4 mutant colonies per 10^6 cells; the range of the groups treated with the test item was from 10.9 up to 46.7 mutant colonies per 10^6 cells. The highest solvent control value of 30.4 colonies per 10^6 cells without metabolic activation slightly exceeded the 95% confidence interval but the mean value of both parallel cultures (14.3 and 30.4, equal to a mean of 22.4 colonies per 10^6 cells) remained well within this interval.
EMS (300 µg/mL) and DMBA (2.3 µg/mL) were used as positive controls and showed a distinct increase in induced mutant colonies.
The results are displayed in Table 6.4.1.2-3.

No relevant and reproducible increase in mutant colony numbers/10^6 cells was observed in the main experiment up to the maximum concentration. The 95% confidence interval was exceeded without metabolic activation at 1.6 and 3.1 µg/mL in the first culture and at 3.1, 6.3, and 12.5 µg/mL in culture II. In the presence of metabolic activation the 95% confidence interval was exceeded at 3.1, 12.5, and 25.0 µg/mL in culture I and at 6.3 µg/mL in culture II. However, there was no dose dependent increase as indicated by a lacking statistical significance with the linear regression analysis. Therefore, these increases in the number of mutant colonies/10^6 cells were considered as biologically not relevant.
Appropriate reference mutagens, used as positive controls, induced a distinct increase in mutant colonies and thus, showed the sensitivity of the test system and the activity of the metabolic activation system.

Table 6.4.1.2-3: Main Experiment: Cloning efficiency and Mutation Rates (Culture I and II) evaluated in the gene mutation assay with 24-Epibrassinolide (TGAI)

	conc.	P	S9 mix	Relative cloning efficiency I	Relative cell densitiy	rel. adjusted cloning efficacy I	Mutant colonies / 10^6 cells	95% confidence interval of HCD range	relative cloning efficiency I	Relative cell densitiy	rel. adjusted cloning efficacy I	Mutant colonies / 10^6 cells	95% confidence interval of HCD range
	µg/mL			%	%	%			%	%	%		
Experiment I / 4 h treatment				Culture I					Culture II				
Solvent control with DMSO			-	100.0	100.0	100.0	14.3	0.2 - 29.7	100.0	100.0	100.0	**30.4**	0.2 - 29.7
Positive control (EMS)	300.0		-	83.9	61.7	51.7	**123.2**	0.2 - 29.7	100.1	102.2	102.4	**127.4**	0.2 - 29.7
Test item	1.6		-	67.8	66.2	44.9	**36.5**	0.2 - 29.7	87.7	85.8	75.3	23.0	0.2 - 29.7
Test item	3.1		-	49.2	76.6	37.7	**41.7**	0.2 - 29.7	68.0	92.6	63.0	**46.7**	0.2 - 29.7
Test item	6.3		-	69.3	71.0	49.2	18.8	0.2 - 29.7	73.8	84.3	62.2	**40.4**	0.2 - 29.7
Test item	12.5		-	23.1	73.1	16.9	28.2	0.2 - 29.7	46.6	74.1	34.6	**33.5**	0.2 - 29.7
Test item	25.0	P	-	15.2	11.3	1.7	#		#	6.8	#		
Test item	50.0	P	-	10.3	21.6	2.2	#		#	7.9	#		
Solvent control with DMSO			+	100.0	100.0	100.0	27.6	0.6 – 28.7	100.0	100.0	100.0	9.1	0.6 – 28.7
Positive control (DMBA)	2.3		+	106.1	69.4	73.6	**134.3**	0.6 – 28.7	86.2	76.3	65.8	**76.1**	0.6 – 28.7
Test item	3.1		+	102.5	84.5	86.6	**40.2**	0.6 – 28.7	89.7	107.0	96.1	17.1	0.6 – 28.7
Test item	6.3		+	97.0	84.0	81.5	15.3	0.6 – 28.7	91.8	102.3	94.0	**35.4**	0.6 – 28.7
Test item	12.5		+	102.0	82.3	83.9	**35.8**	0.6 – 28.7	87.3	135.2	118.1	10.9	0.6 – 28.7
Test item	25.0	P	+	103.9	79.0	82.1	**38.0**	0.6 – 28.7	21.0	81.2	17.1	20.3	0.6 – 28.7
Test item	50.0	P	+	7.2	26.3	1.9	#		4.4	50.9	2.2	#	
Test item	100.0	P	+	2.5	42.0	1.0	#		0.7	69.5	0.5	#	

P = Precipitation
# = culture not continued due to cytotoxicity
Formatting in **bold** indicates results outside of the 95% confidence interval of the historical negative control data range

Statistical analysis
A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. No significant dose dependent trend of the mutation frequency indicated by a probability value of < 0.05 was determined in any of the experimental groups. Additionally, a t-test was performed to evaluate a significant increase of the mutation frequency at individual concentrations compared to the corresponding solvent controls. The only significant t-test result was noted at an intermediate concentration of 3.1 µg/mL without metabolic activation. As there clearly was no dose dependent increase, the isolated significant t-test was considered as irrelevant.

Table 6.4.1.2-4: Linear Regression analysis of experimental groups against solvent control cultures

Linear Regression	
experimental group	**p-value**
culture I without S9 mix	0.985
culture II without S9 mix	0.739
culture I with S9 mix	0.546
culture II with S9 mix	0.847
t-Test	
cultures I and II at 1.6 µg/mL without S9	0.226
cultures I and II at 3.1 µg/mL without S9	**0.001**
cultures I and II at 6.3 µg/mL without S9	0.295
cultures I and II at 12.5 µg/mL without S9	0.079
cultures I and II at 3.1 µg/mL with S9	0.087
cultures I and II at 6.3 µg/mL with S9	0.195
cultures I and II at 12.5 µg/mL with S9	0.465
cultures I and II at 25.0 µg/mL with S9	0.106

III CONCLUSIONS
In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.
Therefore, 24-Epibrassinolide (TGAI) is considered to be non-mutagenic in this HPRT assay.

B.6.4.1.3. In vitro Mammalian Chromosome Aberration Test

Annex point	CA 5.4/03
Reference:	IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST USING HUMAN PERIPHERAL BLOOD LYMPHOCYTES WITH 24-EPIBRASSINOLIDE (TGAI)
Author(s), year:	Kandula, S. R. (2017)
Report/Doc. number:	6121 (557-003)
Guideline(s):	OECD No. 473 (replaced 2014, adopted 2016)
GLP:	Yes
Deviations from OECD 473 (2016):	None
Acceptability:	Yes

Executive summary

The *in vitro* chromosomal aberration assay was performed to assess the potential of 24-Epibrassinolide (TGAI) to induce structural/numerical chromosomal aberrations in human peripheral blood lymphocyte cultures.
The experiment was performed both in the presence and in the absence of metabolic activation (1 and 2% S9 mix) after 48 h mitogenic stimulation at concentrations of 0.063, 0.125, and 0.25 mg/mL. The cells arrested in metaphase were analysed microscopically and number of metaphases were recorded in different fields to determine the mitotic index. The number of cells with aberrations was recorded to calculate percent aberrant cells.
A moderate cytotoxic effect was observed after treatment with 0.25 mg/mL in both experimental parts. The reduction in the mitotic index was around 35% in the absence of S9 mix and 40% in the presence of S9 mix.
No relevant increase in cells carrying chromosomal aberrations was observed when compared to the values of the negative and solvent control and no evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.

I MATERIALS AND METHODS

A Materials

1 Test materials

Test substance	24-Epibrassinolide (TGAI)
Lot/batch	002-20150112
Chemical purity	91.2% w/w
Expiry Date	January 11, 2017

2 Test system

Blood samples have been obtained by vein puncture of three healthy male donors (non-smoker, non-alcoholic), not receiving medication for at least 3 months and being 32, 29 and 33 years of age, for the cytotoxicity experiment, experiment I and experiment II, respectively. The lymphocytes of the respective donors have been shown to respond well to stimulation of proliferation with PHA and to positive control substances. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.

B Study design

Preparation of test substance solution

Dose selection was performed according to the OECD Guideline No. 473 for *in vitro* Mammalian Chromosome Aberration Test.

Based on the solubility, precipitation and pH test, dimethyl sulfoxide (DMSO) was selected as solvent for the study. The concentration of 50 mg/mL was used to give a final treatment concentration of 0.5 mg/mL in the culture medium. Slight precipitation was observed at concentrations of 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL. Hence, 0.25 mg/mL was selected as the highest concentration for treatment in the cytotoxicity experiment (Table 6.4.1.3-1) both in the presence and in the absence of metabolic activation.

Table 6.4.1.3-1: Precipitation Test for Cytotoxicity Experiment

Precipitation Record				
Sample ID	**Volume of Test item preparation**	**Volume of RPMI Media**	**Concentration mg/ml**	**Result**
C	80 µL of A	7.92 mL	1.0 mg/mL	Precipitation
D	80 µL of B	7.92 mL	0.5 mg/mL	Slight Precipitation
E	80 µL (40 µL of B + 40 µL of DMSO)	7.92 mL	0.25 mg/mL	Slight Precipitation
F	80 µL (20 µL of B + 60 µL of DMSO)	7.92 mL	0.125 mg/mL	Slight Precipitation

A: 100 mg/mL solution of test substance in DMSO; B: 50 mg/mL solution of test substance in DMSO

The test substance was not corrected for purity and suspended in DMSO. The highest concentration was prepared immediately before each treatment and lower concentrations by serial dilutions. The final concentration of DMSO in the medium was 1% or lower after the treatment with the test substance solutions.

Concurrent negative controls (Phosphate Buffer Saline) and solvent controls (DMSO) were performed. Positive controls were conducted as following:

Without metabolic activation

Name	EMS; ethyl methanesulfonate
CAS Number	62-50-0
Dissolved in	Culture medium (RPMI 1640)
Final Concentration	600 µg/mL

With metabolic activation

Name	CPA; cyclophosphamide monohydrate
CAS Number	6055-19-2
Dissolved in	Culture medium (RPMI 1640)
Final Concentration	30 µg/mL

Solutions were prepared on the day of experiment. The stability of the positive control substance in solution is proven by the mutagenic response in the expected range.

Cytotoxicity Experiment

To evaluate the toxicity of the test item, a cytotoxicity assay was performed both in the presence and absence of metabolic activation. Three test item concentrations (0.125, 0.25 and 0.5 mg/mL) were selected based on the solubility, precipitation and pH test of the test item. Cytotoxicity was determined by reduction in the mitotic index in comparison with solvent control.

The procedure for conducting the cytotoxicity experiment was the same as in the main experiments up to the scoring of the mitotic index, except slide coding.

Slight precipitation in the culture media was observed at 0.25 mg/mL and above at the beginning of treatment.

Dose selection

Based on observations of precipitation and the reduction in mitotic index observed in the cytotoxicity experiment, the following doses were selected for both main experiments:

0.063, 0.125 and 0.25 mg/mL in the absence (-S9) as well as in the presence of metabolic activation (+S9).

Preparation of S9 mix

An appropriate quantity of S9 supernatant is thawed and mixed with S9 cofactor solution, to result in a final concentration in the S9 mix of approximately 1% v/v for Phase I of experiment

$MgCl_2$	1.00 g
KCl	1.35 g
D-Glucose-6-phosphate	0.08 g
Na_2HPO_4	6.40 g
$NaH_2PO_4.H_2O$	1.40 g
β-NADP	1.75 g
S9	1% (v/v)

Main experiment

The treatment of cultures with the test item was made in two independent experiments. Minimum three different concentrations were selected for the main test, both in the presence and absence of metabolic activation. Negative control, solvent control and positive control were maintained, both in the presence and absence of metabolic activation system. Duplicate cultures were maintained for each test concentration and controls.

Table 6.4.1.3-2: Timeframe for the Main Experiment

	Without S9 mix		With S9 mix	
	Experiment I	**Experiment II**	**Experiment I**	**Experiment II**
Exposure period	4 h	24 h	4 h	4 h
Recovery	20 h	-	20 h	20 h
Harvesting	24 h	24 h	24 h	24 h

Key: h = hour

Experiment I
In experiment I, the cultures were exposed to 24-Epibrassinolide (TGAI) for 4 hours, both, in the absence and in the presence of metabolic activation (1%). In Experiment I, test item concentrations applied were 0.063, 0.125 and 0.25 mg/mL. The duration of exposure to the test item in the presence of metabolic activation was 4 hours and in the absence of metabolic activation the duration of exposure was 24 hours (Table 6.4.1.3-2).

Experiment II
Experiment II was performed to confirm the negative results obtained in the first experiment. In Experiment II, test item concentrations applied were 0.063, 0.125 and 0.25 mg/mL both in absence and in presence of metabolic activation (2%). The duration of exposure to the test item in the presence of metabolic activation was 4 hours and in the absence of metabolic activation the duration of exposure was 24 hours (Table 6.4.1.3-2).

Analysis of Metaphase Cells
The slides were prepared by dropping the cell suspension onto a clean ice-chilled microscope slide.
A minimum of 1000 cells were counted in different fields of slide per culture and the number of metaphases were recorded for mitotic index (MI) calculation. At least 150 well spread metaphase plates per culture were scored for cytogenetic damage on coded slides. Chromosomal and chromatid breaks, acentric fragments, deletions, exchanges, pulverization, polyploidy (including endoreduplication) and disintegrations were recorded as structural chromosomal aberrations. Gaps were recorded as well, but they were not included in the calculation of the aberration rates. Only metaphases with 46 ± 2 centromere regions were included in the analysis. To describe a cytotoxic effect the mitotic index (% cells in mitosis) was determined.

Validity of study
Acceptance of this test is based on the following criteria

- The concurrent negative control is considered acceptable for addition to the laboratory historical negative control database.
- Concurrent positive controls should induce responses that are compatible with those generated in the historical positive control data base.
- Adequate number of cells and concentrations are analysable

Evaluation of results
Providing that all acceptability criteria are fulfilled, a test item is considered to be clearly positive if in any of the experimental conditions examined:

- At least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
- If the increase is dose-related when evaluated with an appropriate trend test
- Any of the results are outside the distribution of the historical negative control data

Providing that all acceptability criteria are fulfilled, a test item is considered clearly negative if in all experimental conditions examined:

- None of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
- If there is no dose-related increase when evaluated with an appropriate trend test
- All results are inside the distribution of the historical negative control data

C Statistics

Statistical significance was confirmed by means of the non-parametric Mann Whitney Test. The rationale for selecting statistical test is due to the fact that the results are in % for the count (Percentage Aberrant Cells) and only two replicates per concentration were taken. Hence a non parametric Mann Whitney Test was used. However, both biological and statistical significance should be considered together.
If the above mentioned criteria for the test item are not clearly met, the classification with regard to the historical data and the biological relevance is discussed and/or a confirmatory experiment is performed.
An increase in the number of polyploid cells may indicate that the test item has the potential to inhibit mitotic processes and to induce numerical chromosomal aberrations. An increase in the number of cells with endoreduplicated chromosomes may indicate that the test item has the potential to inhibit cell cycle progress.

II RESULTS AND DISCUSSION

Cytotoxicity Experiment

The cytotoxicity of 24-Epibrassinolide (TGAI) was evaluated in the absence and presence of metabolic activation (1% S9 mix) at concentrations of 0.125, 0.25 and 0.5 mg/mL.
The highest applied test item concentration of 0.5 mg/mL of culture media showed a reduction in the mitotic index of approximately 72% in the absence of metabolic activation and approximately 77% in the presence of metabolic activation, when compared to the respective solvent control. The test item concentration 0.25 mg/mL showed a reduction in the mitotic index of approximately 32% in the absence of metabolic activation and approximately 25% in the presence of metabolic activation, when compared to the respective solvent control.
Slight precipitation in the culture medium was observed at 0.25 mg/mL and above at the beginning of treatment.
Hence, 0.25 mg/mL was selected as the highest concentration for the main study both in the presence and in the absence of metabolic activation.

Table 6.4.1.3-3: Mitotic Index of the Cytotoxicity Test

Treatment	R	Mitotic Index (%)					
		In the Absence of Metabolic Activation (-S9)			In the Presence of Metabolic Activation (1% S9)		
		Mitotic Index	Mean	Percent Reduction	Mitotic Index	Mean	Percent Reduction
NC	R1	9.07	9.02	0	9.47	9.33	0
	R2	8.96			9.19		
VC (DMSO)	R1	9.17	9.12	0	9.36	9.42	0
	R2	9.07			9.48		
T1 (0.125 mg/mL)^{P}	R1	8.59	8.53	6.51	8.93	8.84	6.15
	R2	8.47			8.76		
T2 (0.25 mg/mL)^{P}	R1	6.59	6.23	31.69	7.40	7.08	24.84
	R2	5.87			6.77		
T3 (0.5 mg/mL)^{P}	R1	2.78	2.58	71.71	2.78	2.19	76.76
	R2	2.38			1.60		
PC	R1	8.47	8.57	6.01	9.46	9.31	1.21
	R2	8.68			9.15		

Key: R = Replicate, NC = Negative control, VC = Solent (Vehicle) control, T = Test item concentration , ^{P} = Slight precipitation, PC = Positive control

Main experiment

Experiment I

In experiment I, the cultures were exposed to 24-Epibrassinolide (TGAI) for 4 hours, both, in the absence and in the presence of metabolic activation (1% S9 mix).
A moderate cytotoxic effect was observed after treatment with 0.25 mg/mL in both experimental parts. The reduction in the mitotic index was 34.8% in the absence of S9 mix and 39.96% in the presence of S9 mix.
No relevant increase in cells carrying chromosomal aberrations was observed at concentrations 0.063, 0.125 and 0.25 mg/mL (0.667% aberrant cells in the absence of S9 mix; 0.333, 0.667 and 0.667%, respectively, in the presence of S9 mix) when compared to the values of the negative and solvent control (0.333 and 0.667 %, respectively, both in the absence and presence of S9 mix).
No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.
Treatment with ethyl methane sulfonate (600 µg/mL) in the absence of metabolic activation and with cyclophosphamide monohydrate (30 µg/mL) in the presence of metabolic activation (1% S9 mix) showed distinct increases in cells with structural aberrations. Even though the analysis did not reveal any statistical significance, the increase was biologically relevant.

Table 6.4.1.3-4: Mitotic Index of Experiment I

Treatment	Mitotic Index (%)			
	Experiment I			
	In the Absence of Metabolic Activation (-S9)		In the Presence of Metabolic Activation (1% S9)	
	Mean	Percent Reduction	Mean	Percent Reduction
NC	9.54	0	9.56	0
VC (DMSO)	9.49	0	9.31	0
T1 (0.063 mg/mL)	8.92	6.03	8.72	6.37
T2 (0.125 mg/mL)	8.82	7.03	8.58	7.84
T3 (0.25 mg/mL)	6.19	34.80	5.59	39.96
PC	9.03	4.88	8.82	5.25

Key: NC = Negative control, VC = Solent (Vehicle) control, T = Test item concentration, PC = Positive control

Table 6.4.1.3-5: Percent Aberrant Cells of Experiment I

Treatment	Percent Aberrant Cells	
	Experiment I	
	In the Absence of Metabolic Activation (-S9)	In the Presence of Metabolic Activation (1% S9)
	Mean	Mean
NC	0.333	0.333
VC	0.667	0.667
T1 (0.063 mg/mL)	0.667	0.333
T2 (0.125 mg/mL)	0.667	0.667
T3 (0.25 mg/mL)	0.667	0.667
PC	8.667	9.667

Key: NC = Negative control, VC = Solent (Vehicle) control, T = Test item concentration, PC = Positive control

Experiment II

Experiment II was performed to confirm the negative results obtained in the first experiment. In Experiment II, test item concentrations applied were 0.063, 0.125 and 0.25 mg/mL in culture, both in absence and in presence of metabolic activation (2%). The duration of exposure to the test item in the presence of metabolic activation was 4 hours and in the absence of metabolic activation 24 hours.
A moderate cytotoxic effect was observed after treatment with 0.25 mg/mL in both experimental parts. The reduction in the mitotic index was 35.11% in the absence of S9 mix and 40.19% in the presence of S9 mix.
No relevant increase in cells carrying chromosomal aberrations was observed at concentrations 0.063, 0.125 and 0.25 mg/mL (0.667% aberrant cells in the absence of S9 mix; 0.333, 0.667 and 0.667%, respectively, in the presence of S9 mix) when compared to the values of the negative and solvent control (0.667% in the absence of S9 mix; 0.333 and 0.667%, respectively, in the presence of S9 mix).
No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.
Treatment with ethyl methanesulfonate (600 µg/mL) in the absence of metabolic activation and cyclophosphamide monohydrate at the concentration (30 µg/mL) in the presence of metabolic activation (2%) showed distinct increases with structural aberrations. Though the analysis did not reveal any statistical significance, the increase was biologically significant.
The increased frequency of aberrations observed in the concurrent positive control groups demonstrated the sensitivity of the test system, suitability of the methods and conditions employed in the experiments.

Table 6.4.1.3-6: Mitotic Index of Experiment II

Treatment	Mitotic Index (%)			
	Experiment II			
	In the Absence of Metabolic Activation (-S9)		In the Presence of Metabolic Activation (2% S9)	
	Mean	Percent Reduction	Mean	Percent Reduction
NC	9.46	0	9.46	0
VC	9.29	0	8.93	0
T1 (0.063 mg/mL)	8.62	7.17	8.43	5.59
T2 (0.125 mg/mL)	8.53	8.20	7.98	10.57
T3 (0.25 mg/mL)	6.03	35.11	5.34	40.19
PC	8.94	3.81	8.73	2.24

Key: NC = Negative control, VC = Solent (Vehicle) control, T = Test item concentration, PC = Positive control

Table 6.4.1.3-7: Percent Aberrant Cells Experiment II

Treatment	Percent Aberrant Cells	
	Experiment II	
	In the Absence of Metabolic Activation (-S9)	In the Presence of Metabolic Activation (2% S9)
	Mean	Mean
NC	0.667	0.333
VC	0.667	0.667
T1 (0.063 mg/mL)	0.667	0.333
T2 (0.125 mg/mL)	0.667	0.667
T3 (0.25 mg/mL)	0.667	0.667
PC	9.667	9.333

Key: NC = Negative control, VC = Solent (Vehicle) control, T = Test item concentration, PC = Positive control

Validity of the study and Statistics
Statistical significance at the $p < 0.05$ was evaluated by means of the non-parametric Mann-Whitney test.

The result of treatment groups and positive controls were compared with negative control. For the positive controls, though the analysis did not reveal any statistical significance, the increase was biologically significant.
The increased frequency of aberrations observed in the concurrent positive control groups demonstrated the sensitivity of the test system, and the suitability of the methods and conditions employed in the experiment. From the results of this study, it is concluded that 24-Epibrassinolide (TGAI) is non-clastogenic both in the presence (1 and 2%) and in the absence of metabolic activation under the specified conditions.

Table 6.4.1.3-8: Biometry and Statistical Significance

Solvent control Versus test group	Experiment I			
	-S9		+S9	
	p value	Significance	p value	Significance
0.063 mg of Test item/mL Culture media (T1)	0.4386	-	0.2453	-
0.125 mg of Test item/mL Culture media (T2)	0.4386	-	0.4386	-
0.25 mg of Test item/mL Culture media (T3)	0.4386	-	0.4386	-
Positive Control	0.1213	-	0.1213	-

Solvent control Versus test group	Experiment II			
	-S9		+S9	
	p value	Significance	p value	Significance
0.063 mg of Test item/mL Culture media (T1)	0.4386	-	0.2453	-
0.125 mg of Test item/mL Culture media (T2)	0.4386	-	0.4386	-
0.25 mg of Test item/mL Culture media (T3)	0.4386	-	0.4386	-
Positive Control	0.1213	-	0.1213	-

Key: - = not significant

III CONCLUSIONS

In conclusion, under the experimental conditions and results of this study, 24-Epibrassinolide (TGAI) is considered "**non-clastogenic**" both in the presence (1% and 2%) and in the absence of metabolic activation.

B.6.4.2. In vivo studies in somatic cells

No studies submitted, not considered necessary

B.6.4.3. In vivo studies in germ cells

No studies submitted, not considered necessary

B.6.5. LONG-TERM TOXICITY AND CARCINOGENESIS

No guideline-compliant studies were submitted, due to the ubiquitous presence of brassinosteroids in plant material and therefore continuous lifetime exposure, conduct of long term toxicity/ carcinogenicity studies is not considered required. The literature review and the publications submitted by the applicant did not raise any concerns regarding potential carcinogenic or other toxic effects after long term exposure.

Table 6.5-1: Summary of Other Scientifically Relevant Information on Long-Term Toxicity and Carcinogenesis

Study title	Reference Report number	Article type	Study content	Relevance/ Relieability
STEROID PLANT HORMONES: EFFECTS OUTSIDE PLANT KINGDOM	Zhabinskii V.N. et al, 2015 092-099	Review	Reviews available knowledge on effects of steroid plant hormones on insects, funghi, fish, protozoa, and warm blooded animals. Discusses possible effects of brassinosteroids on blood cholesterol in rats. Application of 24-epibrassinolide to rats with normal blood cholesterol level in daily doses of 0.2, 2, 20, or-200 µg/kg for 36 weeks gave a dose dependent 9-25% reduction in blood cholesterol levels.	Low reliablilty, no experimental details are reported
ENZYME IMMUNOASSAY OF THE CONTENT OF ENDOGENOUS BRASSINOSTEROIDS IN PHYTOGENIC FOOD PRODUCTS	Khripach, V.A. et al, 2013 092-030	Research article	Describes the development of a quantitative ELISA assay for 24-epibrassinolide and 24-epicasterone	Not relevant, no information on long-term toxicity/ carcinogenicity
ISOLATION AND CHARACTERIZATION OF BRASSINOSTEROIDS FROM ALGAL CULTURES OF CHLORELLA VULGARIS BEIJERINCK	Bajguz, A., 2009 092-013	Research article	Describes isolation and identification of seven brassinosteoids from Chlorella vulgaris	Not relevant, no information on long-term toxicity/ carcinogenicity

(TREBOUXIOPHYCEAE)				
HYPOCHOLESTEROLEMIC MECHANISM OF CHLORELLA: CHLORELLA AND ITS INDIGESTIBLE FRACTION ENHANCE HEPATIC CHOLESTEROL CATABOLISM THROUGH UP-REGULATION OF CHOLESTEROL 7α-HYDROXYLASE IN RATS	Shibata, S. et al, 2007 092-151	Research article	The study authors conclude that *Chlorella* powder enhances the hepatic degradation of cholesterol by up-regulating the expression of CYP7A1 in rats with or without diet-induced hypercholesterolemia. Based on data on hepatic enzyme activities and gene expression profiles, induction of CYP7A1 expression is considered to be the most important mechanism in the liver contributing to the hypocholesterolemic effect of *Chlorella* powder.	Not relevant, no information on long-term toxicity/ carcinogenicity
ISOLATION AND CHARACTERIZATION OF 24-EPIBRASSINOLIDE FROM BRASSICA JUNCEA L. AND ITS EFFECTS ON GROWTH, NI ION UPTAKE, ANTIOXIDANT DEFENSE OF BRASSICA PLANTS AND IN VITRO CYTOTOXICTY	Kanwar M.K. et al, 2013 092-118	Research article	Analysis of effects of 24-epibrassinolide on Ni-toxicity in *Brassica juncea*	Not relevant, no information on long-term toxicity/ carcinogenicity
BRASSINOSTEROIDS CAUSE CELL CYCLE ARREST AND APOPTOSIS OF HUMAN BREAST CANCER CELLS	Steigerova, J. et al, 2010 092-125	Research article	Investigates the effects of 24-Epibrassinolide and 28-homocastaster-one on human breast cancer cell lines (MCF-7 and MDA-MB-468). MCF-7 cells were treated with 40 µM 28-homoCS or 60 µM 24-Epibrassinolide, and MDA-MB-468 cells were treated with 65 µM 28-homoCS or 68 µM 24-epibrassinolide for different timepoints. Effects on cell cycle, cell cycle proteins, apoptosis induction and estrogen receptor localization were monitored. The figures are not readable due to poor quality resolution and black and white images.	Not relevant, no information on long-term toxicity/ carcinogenicity
MECHANISMS OF NATURAL BRASSINOSTEROID-INDUCED APOPTOSIS OF PROSTATE CANCER CELLS	Steigerova, J. et al, 2012 092-126	Research article	The study examines the mechanism of the antiproliferative activity of 28-homocastasterone and 24-epibrassinolide in hormone-sensitive and -insensitive (LNCaP and DU-145) human prostate	Not relevant, no information on long-term toxicity/ carcinogenicity

			cancer cell lines. LNCaP cells were treated with 45 µM 28-homoCS and 60 µM 24-epibrassinolide, and DU-145 cells were treated with 45 µM 28-homoCS and 65 µM 24-epibrassinolide. Effects on cell cycle and apoptosis induction were monitored.	

B.6.6. REPRODUCTIVE TOXICITY

The oral administration of 24-epibrassinolide (TGAI) to pregnant Wistar rats by oral gavage from gestation day 5 to day 19 at dose levels of 100, 300 and 1000 mg/kg bw/day did not result in treatment related maternal and embryofoetal toxicity or mortalities. Hence, the No Observed Adverse Effect Level (NOAEL) of test item 24-epibrassinolide (TGAI) for maternal and embryofoetal toxicity in Wistar rats via the oral route was found to be the highest dose level employed, i.e. 1000 mg/kg bw/day.

In a study performed with homobrassinolide, no effects were observed up to 1000 mg/kg bw/day, which was the highest dose level tested in the main study. In a range finding experiment, significant toxicity was observed at 2000 and 3000 mg/kg bw/day.

Due to ubiquitous occurrence of 24-epibrassinolide and lifetime exposure via food, no further studies on reproductive toxicity are considered necessary.

Table 6.6-1 Summary of Available Guideline-Compliant Studies on Developmental Toxiciy

Species	Dose levels	Classification/remarks	Reference
Rat (Wistar)	Range finding study: 0, 100, 300, 1000 mg/kg bw/day(gavage) Main Study: 0, 100, 300, 1000 mg/kg bw/day (gavage)	Range finding study: No effects at 1000 mg/kg bw/day Main study: No effects up to the highest dose tested; NOAEL (maternal) 1000 mg/kg bw/d NOAEL (embryofœtal) 1000 mg/kg bw/d	[redacted] (2017)

Table 6.6-2 Summary of Other Scientifically Relevant Information on Developmental Toxiciy

Species	Dose levels	Classification/remarks	Reference
Rat (Wistar), Study performed with homobrassinolide	Range finding study: 1000, 2000, 3000 mg/kg bw/day (gavage) Main study: 0, 100, 1000 mg/kg bw/day (gavage)	Range finding study: 1000, 2000, 3000 mg/kg bw/day (gavage) Main study: 0, 100, 1000 mg/kg bw/day (gavage)	Murkunde and Murthy, (2010)

B.6.6.1. Generational studies

No studies submitted, not considered necessary.

B.6.6.2. Developmental toxicity studies

B.6.6.2.1. Developmental Toxicity Studies in Rats

Annex point	KCA 5.6.2/01
Reference:	PRENATAL DEVELOPMENTAL TOXICITY STUDY IN WISTAR RATS WITH 24-EPIBRASSINOLIDE (TGAI)
Author(s), year:	[redacted] (2017)
Report/Doc. number:	6642 (551-001)
Guideline(s):	OECD No. 414 (2001)
GLP:	Yes
Deviations from OECD 414 (2001):	None
Acceptability:	Yes

Executive summary

The purpose of this study was to investigate the effects of 24-Epibrassinolide (TGAI) on the pregnant female rat, as well as the embryonic and foetal development.

The test item, 24-epibrassinolide (TGAI), formulated in 0.1% sodium carboxymethyl cellulose was administered once daily by oral gavage to three treatment groups of twenty four pregnant female Wistar rats per group from gestation day 5 to day 19 at dose levels of 100, 300 and 1000 mg/kg bw/d. A control group of twenty four females was administered with vehicle (0.1% Sodium Carboxymethyl Cellulose) alone.

No clinical signs of illness were observed in any of the animals during treatment period. No test item related effects were observed in the body weight, body weight changes, corrected body weight gain and feed consumption.

The evaluation of the reproductive organs of the dam, the gravid uterine weight, ovarian weight, corpora lutea count, total implants, placental weight, pre and post implantation loss, litter size, viability of the foetus, foetus weight, foetal sex ratio, foetal anogenital distance and the external, visceral and skeletal examination of the foetuses, revealed that 24-epibrassinolide (TGAI) did not produce any maternal or embryofoetal toxicity at 100, 300 and 1000 mg/kg bw/day.

Hence, under the present experimental conditions, the No Observed Adverse Effect Level (NOAEL) of test item 24-epibrassinolide (TGAI) for maternal and embryofoetal toxicity in Wistar rats via oral route was found to be the highest dose level employed, i.e. 1000 mg/kg bw/day.

- **NOAEL (maternal and developmental toxicity): 1000 mg/kg bw/day**

I MATERIALS AND METHODS

A Materials

1 Test materials

Test substance	24-epibrassinolide(TGAI)
Lot/batch	002-20150112
Chemical purity	91.2% w/w

2 Test animals

Species	Rat
Strain	Wistar (*Rattus norvegicus*)
Age	14 – 16 weeks
Total number of animals used	68 males and 116 females
Bodyweight at dosing	Male: 307.2 – 383.5 g Female: 221.1 – 285.6 g
Source	[redacted]
Acclimation period	Five days
Diet	*Ad libitum* Teklad Certified Global 14% Protein Rodent Maintenance Diet (Batch No.2014C-111215MA) from ENVIGO
Water	*Ad libitum* fresh tap water
Housing	Polycarbonate cages
Temperature	22 ± 3°C
Humidity	30 - 70%

Ventilation	10 air changes / hour
Photoperiod	12 hours light / 12 hours dark

B Study design

Preparation of the test solution
An appropriate amount of test substance was mixed with the vehicle 0.1% sodium carboxymethyl cellulose (Na CMC) solution. The dose formulation was prepared shortly before each dosing.

Dose selection and administration
The doses were selected based on a GLP Dose Range finding (DRF) study in which 5 mated female rats for each dose level were treated from day 5 to day 19 of gestation at dose levels of 0, 100, 300 and 1000 mg/kg body weight per day. Control group animals were treated with vehicle (0.1% Sodium carboxymethyl cellulose).

The test solutions were administered by gavage from gestation days (GD) 5-19. The dosing volume was set at 10 mL/kg bw and individual dosing volumes were calculated based on the body weights on the day of dosing.

Table 6.6.2.1-1: Study outline of Dose range finding study

Study group	Dose [mg/kg bw]	Concentration [mg/mL]	Number of copulated females
Control (vehicle)	0	0	5
Low dose	100	10	5
Middle dose	300	30	5
High dose	1000	100	5

Main Study
The test solutions were administered by oral gavage commenced on day 5 of gestation and continued until day 19 of gestation.

Table 6.6.2.1-2: Main study doses

Study group	Dose [mg/kg bw]	Concentration [mg/mL]	Number of copulated females
Control (vehicle)	0	0	24
Low dose	100	10	24
Middle dose	300	30	24
High dose	1000	100	24

Mating
Animals from the dose range finding study and the main study were paired on a one male to one female basis. Female animals were transferred to male cage for mating and examined to vaginal smear or the presence of a copulation plug in the vagina after separation on the following morning.

The stage of estrus cycles was determined.

The presence of sperm in the vaginal smear was considered as positive evidence of mating (Day 0 of gestation). The precoital interval was found to be one to eight days.

Mortality and clinical observations
The condition of the animals was recorded including mortality, any behavioural changes, and all signs of toxicity.
All animals were observed for mortality and general appearance once on day of receipt and twice daily thereafter.
Placental signs were observed for vaginal bleeding between day 13 to 17 of gestation.

Table 6.6.2.1-3: Observations of the dams

Mortality / Viability	Once on day of receipt and twice daily thereafter. Once on day of necropsy
Clinical Signs	Cage-side clinical observations
- Acclimatization Period	Once daily

- Treatment Period	Twice daily on initial 3 days after treatment and once daily thereafter
Body Weights	
- Acclimatization Period	Once weekly
- Premating	On first day of pairing
- Gestation	On gestation day 0, 3, 5, 8, 11, 14, 17 and 20
Feed Consumption	
- Gestation	On gestation day 3, 5, 8, 11, 14, 17 and 20
Examination of placental signs	Observed for vaginal bleeding between day 13 to 17 of gestation

Body weights
Body weight of all animals was measured on GD 0 (day of successful copulation) and on GD 3, 5, 8, 11, 14, 17 and 20 (day of autopsy).

Feed consumption
The amounts of feed supplied or remaining in each cage were measured on GD 3, 5, 8, 11, 14, 17 and 20.

Necropsy
All surviving female animals were weighed and sent for necropsy on 20th day of gestation. All surviving animals were sacrificed under carbon dioxide asphyxiation. Descriptions of all macroscopic abnormalities were recorded. No test item related macroscopic abnormality was observed in any of the dam; hence the tissues were not preserved for histological examination. Ovaries of all the female animals were weighed.

Male animals were sacrificed by carbon dioxide asphyxiation after confirmation of last mating.

Uterine contents
At termination, the uteri were removed and the pregnancy status of the animals was ascertained. Gravid uteri including the cervix and placenta were weighed. The uteri of females were examined for the presence and number of implantation sites and the number of corpora lutea in the ovaries were determined for pregnant animals. The weight of the ovaries was determined. The uterine content was examined for number of resorption, live and/or dead foetuses. The degree of resorption was described in order to estimate the relative time of death of the conceptus. Uteri that appeared non-gravid were further examined by 5% ammonium sulphide staining to reveal any early resorption or post implantation loss.

Foetal examinations
External examination
The foetuses were removed, identified, weighed, and sexed and anogenital distance was measured and evaluated for external malformation/variation. Viability assessments of the foetuses were done and live pups were sacrificed. One-half of the foetuses independent of sex were processed for skeletal alterations and remaining half was examined for visceral alterations.
On completion of external examination the foetuses were eviscerated and placed in 70% isopropyl alcohol/ethanol.

Visceral examination
The foetuses were observed by Staple's dissection technique for visceral malformations/alterations. The heads of foetuses were cut between the jaws and below the ears and stored in modified Davidson's fixative for at least 7 days after necropsy and sectioned and examined by Wilsons's Cross-Sectioning technique.

Skeletal examination
All foetuses were dehydrated in 70% isopropyl alcohol/ethanol, macerated and stained with Alizarin red S in 1% KOH solution, cleared with 70% IPA, Glycerol and benzyl alcohol (2:2:1) solution, preserved in 70% IPA and Glycerol (1:1) solution.
The stained specimens were placed in 75% glycerol for at least 24 hours and finally stored in pure glycerol with a pinch of thymol crystals. The skeletal specimens were examined for malformations/alterations by using stereomicroscope.

C Evaluations
The following data was evaluated:
Pregnant females:

Mortality, clinical signs
Body weight (BW), body weight change and feed consumption (mean ± SD)

$$\text{BW change} = \frac{\text{BW on particular interval – BW previous interval}}{\text{Initial BW}} \times 100$$

Corrected body weight (CBW) gain (mean ± SD)

$$\text{CBW gain} = (\text{BW on } 20^{th} \text{ day of gestation} - \text{BW on } 5^{th} \text{ day of gestation}) - \text{Gravid uterine weight}$$

The feed consumption was calculated per animal and per feed consumption interval using following formula.

$$FC = \frac{C}{AD}$$

Where
FC is feed consumption in grams per animal per day,
C is measured feed consumption in grams per cage over the consumption interval, and
AD is total consumption days over for animal in the cage during the consumption interval.

Necropsy and caesarian section data:
Gravid uterine weight, necropsy findings, ovarian weight, placental weight; number of corpora lutea (CL), number of implantations, number and percent of live and dead foetuses and resorptions, number and percent of pre- and post-implantation losses (early and late embryonic death) (mean ± SD)

$$\text{Pre implantation loss (\%)} = \frac{\text{No. of CL – No .of implants}}{\text{No. of CL}} \times 100$$

$$\text{Post implantation loss (\%)} = \frac{\text{Total number of resorption}}{\text{No. of implants}} \times 100$$

Foetuses:
Number and percent of live offspring, sex ratio, and foetal body weight by sex and with sexes combined, external, visceral (soft tissue), and skeletal malformations and other relevant alterations

$$\text{Live foetus (\%)} = \frac{\text{No. of Live Foetus}}{\text{No. of implants}} \times 100$$

$$\text{Dead foetus (\%)} = \frac{\text{No. of Dead Foetus}}{\text{No. of implants}} \times 100$$

$$\text{Sex Ratio (\%)} = \frac{\text{No. of Male Foetuses}}{\text{Total No. of Foetuses}} \times 100$$

$$\text{Variation/Malformation (\%)} = \frac{\text{No. of foetuses with abnormality/group}}{\text{Total No. of foetuses/group}} \times 100$$

D Statistics
The following statistical methods were used to analyze the body weight, body weight change, corrected body weight (CBW) gain, feed consumption, reproduction and external, visceral and skeletal alterations/variations:

Data were summarized in tabular form. Statistical analysis was performed using the statplus program (version 4.9). All the data were checked for Normality with Shapiro-Wilk W test and for Homogeneity with Bartlett Chi-Square test. Each group of animals was subjected to Analysis of Variance (ANOVA). Values were given as mean ± standard deviation (SD).

$P \leq 0.05$ (5% level of significance) was considered to represent significance in the respective parameters, $P > 0.05$ was considered not significant.

II RESULTS AND DISCUSSION

Analytical analysis of the test solution

The concentration analyzed before dosing (on date 16/12/2016) of the formulation of the low (Group 2), intermediate (Group 3) and high dose (Group 4) on 5th day of gestation was in agreement with the target concentration (i.e., ranged between 98.24 to 99.61 %). The formulations of Group 2, Group 3 and Group 4 collected before dosing on 5th day of gestation were homogenous (i.e. % coefficient of variation ranged in between 0.17 to 1.04).

Table 6.6.2.1-4: Dose Concentration analysis of 24-epibrassinolide

Replication	Dose Concentration analysis of 24-epibrassinolide (TGAI) (Date of sampling: 16-12-2016)		
	10.0 (mg/mL)	**30.0 (mg/mL)**	**100.0 (mg/mL)**
1	9.884	29.722	98.571
2	9.896	30.242	97.932
3	9.863	29.685	98.227
Mean ± SD	9.881 ± 0.02	29.883 ± 0.31	98.243 ± 0.32
Mean Recovery (%)	98.81	99.61	98.24
RSD (%)	0.17	1.04	0.33

Stability of dose formulations
The stability of the dose formulation was performed after study initiation date and before experimental start date. The stability of the dose formulation at 10 mg/mL and 100 mg/mL was checked at 0, 6 and 24 hours period. The dose formulations were found to be stable up to 24 hours.

A. MATERNAL TOXICITY

Mortality and Clinical Signs
No mortality was observed in any of the animal from day of treatment till the end of experiment.
No clinical signs were observed in any of the animals throughout the acclimatization and experimental periods.

Body weight
No significant difference in the body weight and body weight change (%) was observed in pregnant female animals of the low (100 mg/kg bw/d), intermediate (300 mg/kg bw/d) and high dose (1000 mg/kg bw/d) groups when compared to the control group.

Feed Consumption
No test-item related difference was observed in feed consumption between control and treated groups. A significant increase in feed consumption was observed on gestation day 17 in the high dose group (1000 mg/kg bw/d) when compared to the control (0 mg/kg bw/d) group and on gestation day 20 in the high dose group (1000 mg/kg bw/d) when compared to the control group. The significant increase in feed consumption is considered as biological variation and of no toxicological significance.

Table 6.6.2.1-5: Summary of feed consumption

Group		FEED CONSUMPTION (G/ANIMAL/DAY)						
		Gestation Day						
		0-3	**3-5**	**5-8**	**8-11**	**11 - 14**	**14-17**	**17-20**
G1 (0 mg/kg bw/d)	**Mean**	16.37	17.43	17.36	18.83	20.57	21.89	23.35
	SD	1.94	1.93	2.01	1.78	1.98	1.99	2.61
G2 (100 mg/kg bw/d)	**Mean**	16.64	17.20	18.11	18.93	20.57	21.79	2441
	SD	1.78	2.28	2.13	1.94	2.48	2.19	2.00
G3 (300 mg/kg bw/d)	**Mean**	16.42	17.19	17.99	20.17	21.99	23.26	24.84
	SD	1.43	2.18	1.93	1.95	2.04	2.04	2.08
G4 (1000 mg/kg bw/d)	**Mean**	16.14	16.73	17.67	19.61	21.55	**23.49***	**25.69***
	SD	1.74	2.04	1.86	2.11	1.77	1.62	1.46

*Significant at $p < 0.05$ level with Group 1

Reproductive data
All female animals showed positive evidence of mating. All the female animals showed normal pattern of estrus cyclicity i.e. not more than 5 days before conception.

The number of pregnant female animals was found to be 21 females each in the control group and intermediate dose group (300 mg/kg bw/d), 20 females in the low dose group (100 mg/kg bw/d) and 22 females in the high dose group (1000 mg/kg bw/d).

Gross Macroscopic Examination
No abnormalities were detected in any of the treatment and control group animal during necropsy at terminal sacrifice.

Cesarean Section Data

Maternal data
No test-item related significant difference was observed in the gravid uterine weight, ovarian weight, placental weight, corpora lutea count, number and live foetuses, early and late resorption, total resorption and implants, number and percent of pre and post implantation losses in pregnant female animals between control and treated groups. No dead foetus was observed in any of the dams of control and all treatment groups. However, a significant decrease in pre implantation loss was observed in the high dose group (1000 mg/kg bw/d) when compared to the control (0 mg/kg bw/d), low dose group (100 mg/kg bw/d) and intermediate dose group (300 mg/kg bw/d). Since the animals were not dosed during gestation day 0 to 5, the decrease in pre implantation loss was concluded to be of no test item related effect.

Table 6.6.2.1-6: Summary of Cesarean Section Observations

Parameter	**Group**	**G1**	**G2**	**G3**	**G4**
	Dose (mg/kg bw/d)	**0**	**100**	**300**	**1000**
	No. of Dams	24	24	24	24
	No. of Litters	21	20	21	22
Gravid Uterine Weight	**Mead**	44.7520	46.6225	48.8010	50.6866
	SD	18.0318	16.7835	15.1220	17.5685
Ovarian weight (G)	**Mean**	0.1424	0.1410	0.1619	0.1478
	SD	0.0402	0.0224	0.0492	0.0339
Placental wei«ht (G)	**Mem**	0.4957	0.4864	0.4836	0.4887
	SD	0.1466	0.1123	0.1330	0.1328
Total Live Foetuses	**Number**	178	181	202	214
	Mtad	8.48	9.05	9.62	9.73
	SD	4.01	3.59	3.02	3.74
No. uf CL	**Mean**	12.14	12.40	13 24	12.12
	SD	3.10	3.00	2.21	2.06
No. of Implantation	**Mean**	9.14	9.75	10.33	10.41
	SD	4.00	3.73	2.96	3.38
Early Resorption	**Mean**	0.67	0.55	0.71	0.64
	SD	0.86	1.19	0.96	0.90
Late Resorption	**Mean**	0	0.15	0	0.05
	SD	0.00	0.67	0.00	0.21
Pre implantation loss	**Mean**	3.00	2.65	2.90	**1.91***
	SD	2.61	2.50	2.57	3.15
	%	26.91	22.49	22.33	14.91
Post implantation loss.	**Mean**	0.67	0.70	0.71	0.68
	SD	0.86	1.81	0.96	0.99
	%	11.86	5.88	6.97	11.28
Dam with resorption/s	**Number**	10	6	9	9

*Significant at $p < 0.05$ level

Table 6.6.2.1-7: Summary of litter data

Parameter	**Group**	**G1**	**G2**	**G3**	**G4**

	Dose (mg/kg bw)	0	100	300	1000
	No. of Dams	24	24	24	24
	No. of Litters	21	20	21	22
	Total No. of Foetuses	178	181	202	214
	Mean Litter Size	8.48	9.05	9.62	9.73
Live Foetuses	**Number**	178	181	202	214
	Mean	8.48	9.05	9.62	9.73
	SD	4.01	3.59	3.02	3.74
Dead Foetuses	**Number**	0	0	0	0
	Mean	0	0	0	0
	SD	0.00	0.00	0.00	0.00
Live Male Foetuses	**Number**	99	86	100	104
	Mean	4.71	4.30	4.86	4.73
	SD	2.37	1.87	2.13	2.45
Live Female Foetuses	**Number**	79	95	100	110
	Mean	3.76	4.75	4.76	5.00
	SD	2.05	2.63	2.49	2.29

Foetus Weight
No significant difference was observed in foetus weight (combined and sex wise) between control and treated groups.

Anogenital distance and sex ratio
No significant difference was observed in anogenital distance (sex wise) and the sex ratio between control and treated groups.

Table 6.6.2.1-8: Summary of the foetal weight, anogenital distance and placental weight

Group		Foetus Weight / Litter (G)	Foetus Weight (G)		Anogenital Distance (mm)		Placental weight (G)
			Male	Female	Male	Female	
G1 (0 mg/kg bw)	**Mean**	3.1963	3.2524	3.1346	4.42	2.37	0.4957
	SD	0.6703	0.6824	0.6562	0.54	0.50	0.1466
	N	178	99	79	99	79	192
G2 (100 mg/kg bw)	**Mean**	3.2650	3.3660	3.1736	4.24	2.15	0.4864
	SD	0.6003	0.6263	0.5636	0.46	0.36	0.1123
	N	181	86	95	86	95	195
G3 (300 mg/kg bw)	**Mean**	3.3026	3.3478	3.2565	4.16	2.21	0.4836
	SD	0.6891	0.6471	0.7299	0.48	0.47	0.1330
	N	202	102	100	102	100	217
G4 (1000 mg/kg bw)	**Mean**	3.3398	3.4252	3.2591	4.37	2.27	0.4887
	SD	0.5390	0.5204	0.5462	0,48	0.46	0.1328
	N	214	104	110	104	110	229

B. DEVELOPMENTAL TOXICITY
The number of foetuses showing external, visceral and skeletal variation in treatment groups was comparable with control group.

External examination

No test-item related variations/malformations were observed in foetuses subjected for external examination.

External examination of foetuses revealed nine small sized foetuses each in the control (0 mg/kg bw/d) and high dose (1000 mg/kg bw/d) groups, two small sized foetuses in the low dose (100 mg/kg bw/d) and eleven small sized foetuses in the intermediate dose (300 mg/kg bw/d) group. Pale body was observed in two foetuses in control (0 mg/kg bw/d) group and three foetuses each in intermediate dose (300 mg/kg bw/d) and high dose (1000 mg/kg bw/d) groups.

No significant difference was observed in external findings in all treatment groups. The variations observed were randomly distributed across the groups. Hence, they were considered to be incidental and of no toxicological significance.

Table 6.6.2.1-9: Summary of External Findings

Group		**G1**	**G2**	**G3**	**G4**
Dose (mg/kg bw)		**0**	**100**	**300**	**1000**
No. of Litter Examined		21	20	21	22
No. of litter Affected		7	2	9	6
No. of litter Affected (%)		33.3	10.0	42.86	27.27
No. of Foetuses Examined		178	181	202	214
Variation Incidence – Number (%)					
No. of Foetus with Variations	**Total Variations**	9 (5.06)	2 (1.10)	11 (5.45)	9 (4.21)
	a) Body: Small in size	9 (5.06)	2 (1.10)	11 (5.45)	9 (4.21)
	b) Body: Pale	2 (1.12)	0 (0.00)	3 (1.49)	3 (1.40)

Number in parenthesis indicates variation incidence in %

Visceral Findings

Visceral examination of foetuses revealed small sized spleen in six foetuses of the control group, two foetuses in the low dose group (100 mg/kg bw/d), four foetuses in the intermediate dose group (300 mg/kg bw/d) and seven foetuses in the high dose group (1000 mg/kg bw/d). Pale spleen was observed in nine foetuses of the control group, five foetuses each in the low (100 mg/kg bw/d) and intermediate (300 mg/kg bw/d) dose group and seven foetuses in the high dose group (1000 mg/kg bw/d). Convoluted ureters were observed in ten foetuses each of the control group and the intermediate dose group (300 mg/kg bw/d), six foetuses in the low dose group (100 mg/kg bw/d) and twelve foetuses in the high dose group (1000 mg/kg bw/d).

No statistical significance was observed when comparing the variations expressed on a foetus/litter basis to the control group. Hence, they were considered to be incidental and of no toxicological significance.

Table 6.6.2.1-10: Summary of Visceral Findings

Group		**G1**	**G2**	**G3**	**G4**
Dose (mg/kg bw)		**0**	**100**	**300**	**1000**
No. of Litter Examined		21	20	21	22
No. of Litter Affected		16	7	12	14
Litter Affected (%)		76.19	35.00	57.14	63.64
No. of Foetuses Examined		84	85	98	101
Variation Incidence – Number (%)					
No. of Foetus with Variations	**Total Variations**	23 (27.38)	10 (11.76)	16 (16.33)	20 (19.80)
	Spleen: Small in size	6 (7.14)	2 (2.35)	4 (4.08)	7 (6.93)
	Spleen: Pale	9 (10.71)	5 (5.88)	5 (5.10)	7 (6.93)

	Ureters: Convoluted	10 (11.90)	6 (7.06)	10 (10.20)	12 (11.88)

Number in parenthesis indicates variation incidence in %

Skeletal Findings

Skeletal examination of foetuses revealed dumbbell shaped thoracic vertebrae in 12 foetuses of control (0 mg/kg bw/d), 7 foetuses of the low dose group (100 mg/kg bw/d), 12 foetuses in the intermediate dose group (300 mg/kg bw/d) and 17 foetuses in the high dose group (1000 mg/kg bw/d).

Bipartite thoracic vertebrae was observed in 2 foetuses of the control (0 mg/kg bw/d), 3 foetuses of the low dose group (100 mg/kg bw/d), 3 foetuses in the intermediate dose group (300 mg/kg bw/d) and 2 foetuses in the high dose group (1000 mg/kg bw/d).

Not ossified second sternal centers was observed in 6 foetuses of the control (0 mg/kg bw/d), 6 foetuses of the low dose group (100 mg/kg bw/d), 4 foetuses in the intermediate dose group (300 mg/kg bw/d) and 5 foetuses in the high dose group (1000 mg/kg bw/d). Whereas not ossified first sternal center was observed only in one foetus of the high dose group (1000 mg/kg bw/d).

Not ossified manubrium was recorded in 1 foetus of the control (0 mg/kg bw/d) and 1 foetus of the high dose group (1000 mg/kg bw/d).

Xyphiod was not ossified in 15 foetuses of the control (0 mg/kg bw/d), 26 foetuses of the low dose group (100 mg/kg bw/d), 25 foetuses in the intermediate dose group (300 mg/kg bw/d) and 10 foetuses in the high dose group (1000 mg/kg bw/d).

Waviness of right and left ribs was observed in 2 foetuses of the control (0 mg/kg bw/d), 1 foetuses of the low dose group (100 mg/kg bw/d), 1 foetuses in the intermediate dose group (300 mg/kg bw/d) and there was no foetus in the high dose group (1000 mg/kg bw/d) having waviness of ribs. While only 1 foetus of the low dose group (100 mg/kg bw/d) was having supernumerary ribs (left and right).

These variations were isolated and when expressed on a foetus/litter basis, they were found to be not statistically significant.

Type and distribution of variations noted during skeletal examination at the dose levels of 100, 300 and 1000 mg/kg bw/d did not indicate any test item-related effects, they were considered to be incidental and of no toxicological significance.

Table 6.6.2.1-11: Summary of Skeletal Findings

Group	**G1**	**G2**	**G3**	**G4**
Dose (mg/kg bw)	**0**	**100**	**300**	**1000**
No. of Litter Examined	21	20	21	22
No. of Litter affected	12	14	15	16
Litter Affected (%)	57.14	70.0	71.43	72.73
No. of Foetuses Examined	94	96	104	113
Variation Incidence – Number (%)				
Total Variation	25 (26.60)	35 (36.46)	33 (31.73)	26 (23.01)
Thoracic Vertebra				
Dumbbell shaped	12 (12.77)	7 (7.29)	12 (11.54)	17 (15.04)
Bipartite	2 (2.13)	3 (3.13)	3 (2.88)	2 (1.77)
Sternal centers (1)				
Not ossified	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.88)
Sternal centers (2)				
Not ossified	6 (6.38)	6 (6.25)	4 (3.85)	5 (4.42)
Manubrium				
Not ossified	1 (1.06)	0 (0.00)	0 (0.00)	1 (0.88)
Xyphiod				
Not ossified	15 (15.96)	26 (27.08)	25 (24.04)	10 (8.85)
Ribs - Left				
Waviness	2 (2.13)	1 (1.04)	1 (0.96)	0 (0.00)

Supernumerary	0 (0.00)	1 (1.04)	0 (0.00)	0 (0.00)
Ribs - Right				
Waviness	2 (2.13)	1 (1.04)	1 (0.96)	0 (0.00)
Supernumerary	0 (0.00)	1 (1.04)	0 (0.00)	0 (0.00)

Number in parenthesis indicates variation incidence in %

III CONCLUSIONS

The oral administration of 24-epibrassinolide (TGAI) to pregnant Wistar rats by oral gavage from gestation day 5 to day 19 at dose levels of 100, 300 and 1000 mg/kg bw/d did not result in treatment related maternal and embryofoetal toxicity or mortalities.

Hence, the No Observed Adverse Effect Level (NOAEL) of test item 24-epibrassinolide (TGAI) for maternal and embryofoetal toxicity in Wistar rats via the oral route was found to be the highest dose level employed, i.e. 1000 mg/kg bw/day.

Reference:	DEVELOPMENTAL TOXICITY OF HOMOBRASSINOLIDE IN WISTAR RATS
Author(s), year:	Murkunde, Y. V., Murthy, P. B., 2010
Report/Doc. number:	KCA 5.6/0001 (092-121)
Guideline(s):	Gaitonde Committee Guideline for Registration of Crop Protection/ Growth Promoter Product. Report of the sub-committee on pesticide toxicology, 6.3.0 C. iii (2) Effect of pesticides on reproductive processes-teratology study. Study design generally similar to OECD No. 414 (2001)
GLP:	No
Deviations from Guideline 414 (2001)	Not applicable
Acceptability:	Yes

Executive summary

Brassinosteroids (BRs) are close analogues of animal cholesterol. Brassinosteroids have shown their great value as yield promoters of a variety of plants. In view of its steroidal moiety and recent use in agriculture in many countries, the teratogenic potential of homobrassinolide (HBR) was evaluated in Wistar rats. Homobrassinolide was administered by oral gavage at doses 0, 100, and 1000 mg/kg body weight per day in water during gestation days (GD) 6 to 15 in groups of 20 mated females. Maternal and embryo-fetal toxicity was analyzed by studying effects such as clinical signs, mortality/morbidity, abortions, body weight, feed consumption, and pregnancy data, gravid uterine weights, implantation losses, litter size, external, visceral, and skeletal malformations. No treatment-related effect was observed on any of the maternal/fetal endpoints in any dose group. From the results, it can be concluded that HBR is non-teratogenic at doses as high as up to 1000 mg/kg body weight/day in Wistar rats.

- **NOAEL (maternal and embryofoetal toxicity) = 1000 mg/kg bw/day**

I MATERIALS AND METHODS

Materials

1 Test materials

Test substance	Homobrassinolide (Trade name: Combine)
Lot/batch	Not reported
Supplier	Messer's Godrej Agrovet Ltd, Mumbai, India
Chemical purity	86.3%

2 Test animals

Species	Rat
Strain	Wistar (*Rattus norvegicus*)
Age	Not reported
Total number of animals used	Preliminary study: 9 female rats divided into three groups Male rats: not reported Main study: 60 female rats divided into 3 groups;

	male rats: not reported
Body weight at dosing	Male: not reported Female: 200-225 g
Source	Fredrick Institute of Plant Protection and Toxicology (IIBAT), Padappai, India
Acclimation period	Seven days
Diet	*Ad libitum* standard rat pellet feed (Messer's Amrut Laboratory Animals Feed, Maharashtra, India
Water	*Ad libitum* filtered water
Housing	Standard polypropylene cages, individual housing
Temperature	22 ± 3°C
Humidity	30 - 70%
Ventilation	Not reported
Photoperiod	12 hours light / 12 hours dark

Study design

Preparation of the test solution
Distilled water was used as vehicle, the administered dose volume (10 mL/ kg body weight) was adjusted on weekly body weight measurements during the treatment period.

Dose selection and administration
The doses were selected based on a preliminary dose range finding study.
3 groups of pregnant rats containing 3 animals each were treated with 1000, 2000, and 3000 mg/ kg bw/day of test substance per gavage. The test solutions were administered from gestation days (GD) 6-15.
Signs of toxicity, body weight, feed consumption, gross pathology, and external examination of fetuses were monitored.
Animals treated with 3000 mg/kg body weight/day of Combine (HBR) were sacrificed humanely on GD 14 and 15 because of moribund condition (2/3). Rats treated with 2000 mg/kg body weight/day showed a very high decrease in body weight and feed consumption, and exhibited signs of toxicity like abortion (1/3), dullness, lethargy, and anorexia during GD 8 to GD 17. Animals treated with 1000 mg/kg body weight exhibited dullness and lethargy during GD 8 to GD 10, which resolved subsequently.

Main Study
Based on preliminary study results, 3 groups were maintained in the main study. Mated females were allocated to one of these 3 groups (20/group) by stratified randomization; mean body weight was restricted not to exceed by 20% between the groups. Group 1 served as the control receiving distilled water alone, groups 2 and 3 received 100 mg/kg body weight/day and 1000 mg/kg body weight/day of HBR, respectively. Dosing was done from GD 6 to GD 15.

Mating
Animals were tested for their phase in the estrous cycle and those animals in the pro-estrous phase were selected for mating. Mating was done in a ratio of 1:1. Vaginal plug was used to confirm mating and was designated as gestation day 0 (GD 0).

Maternal Endpoints
Body weights of all dams were recorded on GD 0, 7, 14, and 20.
Feed consumption was recorded daily and reported as an average on GD 0, 7, 14, and 20.
Animals were observed for mortality, morbidity, and any overt clinical signs of toxicity during dosing, postdosing, and at sacrifice.
On GD 20, all animals were sacrificed by cervical dislocation. The abdominal cavity was opened to expose the reproductive system and the pregnancy status was determined. Uterine components were examined for the number of implantations, implantation defects, resorptions (early and late), dead/live fetuses, and number of corpora lutea. Uteri/unilateral uterine horns with no visible implantation sites were stained with 10% ammonium sulfide.
Dams were exhaustively examined at necropsy; cervical, thoracic, and abdominal viscera were inspected for abnormalities.

Foetal examinations
Fetuses and placentas were examined in situ and carefully separated from the dams. All fetuses were weighed, sexed, and observed for morphological abnormalities.
Fetuses were sacrificed by intraperitoneal administration of thiopental sodium and preserved in appropriate fixatives.

About two thirds of the fetuses were preserved in 70% propanol and analyzed for skeletal defects. After 15 to 20 days of preservation, the foetuses were skinned and eviscerated, macerated in 1% KOH followed by staining with Alizarin Red S and carefully examined for any skeletal abnormalities. The remaining one third of the fetuses was preserved in Bouin fixative and sectioned serially for detection of visceral abnormality, if any.

Statistics

The statistical significance of differences between the means of body weight, feed consumption, resorptions, fetal weights, total number of live fetuses, and number of male and female fetuses was assessed by 1-way analysis of variance (ANOVA). Values were expressed as mean + SD. One-way ANOVA was used to compare the values between different groups and P values less than 0.05 were considered statistically significant.

II RESULTS AND DISCUSSION

A. MATERNAL TOXICITY

There was no morbidity or mortality at any dose tested. Clinical signs of toxicity were unremarkable. No significant changes to body weights and feed consumption were recorded in females from the treated group when compared with the control.

Table 6.6.2.1-12: Summary of body weights and feed consumption (dams)

Table 1. Summary of Body Weight (grams) of Dams During Gestation Period[a,b]

Days	Control	100 mg/kg bw	1000 mg/kg bw
0	210.15 ± 2.51	214.30 ± 5.46	212.10 ± 4.53
7	233.45 ± 3.32	237.40 ± 6.52	235.65 ± 4.59
14	257.30 ± 6.55	260.70 ± 6.05	258.95 ± 5.23
20	276.10 ± 3.48	281.05 ± 7.63	278.25 ± 6.95

Abbreviation: bw, body weight.
[a] Values are expressed as mean ± SD (n = 20).
[b] None of the treated values differed significantly from the control ($P > .05$).

Table 2. Summary of Feed Consumption (grams) of Dams During Gestation Period[a,b]

Days	Control	100 mg/kg bw	1000 mg/kg bw
0	17.71 ± 1.31	19.03 ± 1.05	18.70 ± 1.31
7	21.74 ± 1.48	23.58 ± 1.09	22.20 ± 1.73
14	25.58 ± 1.39	27.75 ± 1.34	26.15 ± 2.01
20	30.11 ± 1.77	32.20 ± 1.23	31.13 ± 1.97

Abbreviation: bw, body weight.
[a] Values are expressed as mean ± S.D (n = 20).
[b] None of the treated values differed significantly from the control ($P > .05$).

There was no incidence of spontaneous abortion or premature delivery in any of the groups. No remarkable gross pathological changes were observed in dams of any group including the control (data not shown). No changes in the weights of gravid uterus in any of the treated groups were recorded when compared with the control.

No statistically significant changes were observed regarding the mean number of corpora lutea and the implantation sites among the various treatment groups. Early and late resorptions, mean litter size, and viable fetuses were comparable in all the groups.

Table 6.6.2.11-13: Uterine and foetal data

Parameters	Control	100 mg/kg bw	1000 mg/kg bw
Maternal pregnancy status			
No. of treated females	20	20	20
No. of dead/euthanized/removed	0	0	0
No. of pregnant at sacrifice	20	20	20
Resorptions			
Early resorptions (mean ± SD)[a]	0.3 ± 0.4	0.2 ± 0.5	0.3 ± 0.6
Late resorptions (mean ± SD)[a]	0.2 ± 0.4	0.2 ± 0.5	0.2 ± 0.4
Litters with resorptions	7	5	5
Fetal observations			
No. of live fetuses (mean ± SD)[a]	9.2 ± 1.4	8.7 ± 0.9	9.5 ± 1.2
No. of male fetuses (mean ± SD)[a]	4.6 ± 1.1	4.3 ± 0.8	4.8 ± 0.9
No. of female fetuses (mean ± SD)[a]	4.6 ± 1.1	4.4 ± 0.9	4.8 ± 1.0
Fetal weight (mean ± SD)[a]	3.9 ± 0.4	3.9 ± 0.3	3.7 ± 0.6
Fetuses with external observation (total fetuses)	0 (184)	0 (173)	0 (190)
Fetuses with visceral observation (total fetuses)	6 (63)	6 (59)	3 (66)
Fetuses with skeletal variants (total fetuses)	5 (121)	7 (114)	6 (124)

Abbreviation: bw, body weight.
[a] None of the treated values differed significantly from the control ($P > .05$).

B. DEVELOPMENTAL TOXICITY

Mean fetal weights and sex ratio were similar among the groups. No fetal external malformations were observed. Treatment-related visceral malformations were not observed in any treated group. External hydrocephalus and hemoperitoneum (each 1 of 66 fetuses) was observed at the 1000 mg/kg body weight dose, and dilated renal pelvis was observed (2 of 59) in 100 mg/kg body weight group along with other commonly occurring spontaneous findings which were scattered among all the groups. No skeletal malformations were observed in any of the group that could be attributed to test substance. However, routinely recorded skeletal variations such as supernumerary, rudimentary ribs, incomplete ossification of ribs, and bi-lobed/doubled centrum were found in all the groups.

Table 6.6.2.1-1413: Foetal visceral and skeletal observations

Observations	Control	100 mg/kg bw	1000 mg/kg bw
Visceral observation			
External hydrocephalus	1/63 (1)	0/59	1/66 (1)
Hemoperitoneum	0/63	0/59	1/66 (1)
Hematoma, subcutis, neck region	2/63 (1)	0/59	0/66
Internal hydrocephalus	1/63 (1)	2/59 (1)	0/66
Dilated renal pelvis	0/63	2/59 (2)	0/66
Hydronephrosis unilateral	2/63 (2)	0/59	0/66
Hemorrhage in nasal sinuses	0/63	1/59 (1)	0/66
Subcutaneous edema	2/63 (1)	2/59 (1)	0/66
Skeletal observation			
Supernumerary rib (unilateral)	2/121 (2)	2/114 (2)	3/124 (2)
Supernumerary rib (bilateral)	1/121 (1)	2/114 (2)	1/124 (1)
Rudimentary rib	0/121	1/114 (1)	0/124
Incomplete ossification of rib	2/121 (1)	0/114	2/124 (2)
Bilobed/bifurcated/double centrum	0/121	2/114 (1)	0/124

Abbreviation: bw, body weight.
[a] Numerals in parenthesis indicate litter incidence.

III CONCLUSIONS

Murkunde and Murthy (2010) published a study on the developmental toxicity of the brassinosteroid 28-Homobrassinolide, in Wistar rats. 28-Homobrassinolide was administered by oral gavage at doses of 0, 100, and 1000 mg/kg body weight in water during gestation days (GD) 6 to 15 to groups of 20 mated females. Maternal and embryo-fetal toxicity was analysed by studying effects such as clinical signs, mortality/morbidity, abortions, body weight, feed consumption, pregnancy data, gravid uterine weights, implantation losses, litter size, external, visceral, and skeletal malformations. No treatment-related effect was observed on any of the maternal/fetal endpoints in any dose group. From the results, it can be concluded that 28-Homobrassinolide is non-teratogenic at doses up to 1000 mg/kg body weight/day in Wistar rats.

B.6.6.2.2. Developmental Toxicity Studies in Rabbits

No studies submitted, not considered necessary.

B.6.7. NEUROTOXICTY

Through the dietary intake of plants and plant products, humans and animals are constantly exposed to brassinosteroids and their metabolites. Due to absence of neurotoxic effects from available guideline toxicity studies, and no indications from published literature for neurotoxic potential, no neurotoxicity studies are considered necessary.

Table 6.7-1: Summary of Other Scientifically Relevant Information on Neurotoxicity

Study title	Reference Report number	Article type	Study content	Relevance/ Relieability
24-EPIBRASSINOLIDE, A PHYTOSTEROL FROM THE BRASSINOSTEROID FAMILY, PROTECTS DOPAMINERGIC CELLS	Carange, J. et al, 2011 092-104	Research article	The study analyzes the ability of 24-epibrassinolide to protect mammalian neuronal PC12 cells from l-methyl-4-	Low relevance, cell line study, no indications for (neuro)toxic effects on cells

AGAINST MPP^+-INDUCED OXIDATIVE STRESS AND APOPTOSIS			phenylpyridinium (MPP^+) - induced oxidative stress and consequent apoptosis in dopaminergic neurons. 24-epibrassinolide reduces oxidative stress and inhibits induction of apoptosis at nM concentrations.	
BRASSINOSTEROIDS AND ANALOGS AS NEUROPROTECTORS: SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIPS	Ismaili, J. et al, 2012 092-117	Research article	Potential neuroprotective effects of 9 brassinosteroids, among them 24-epibrassinolide, were tested in neuronal PC12 cells. Six of the tested brassinolides exhibited protective effects at nM concentrations against l-methyl-4-phenylpyridinium (MPP^+) - induced toxicity.	Low relevance, cell line study, no indications for (neuro)toxic effects on cells
INHIBITION OF H_2O_2-INDUCED DNA DAMAGE IN SINGLE CELL GEL ELECTROPHORESIS ASSAY (COMET ASSAY) BY CASTASTERONE ISOLATED FROM LEAVES OF CENTELLA ASIATICA	Sondhi, N. et al, 2010 092-155	Research article	The paper examines the effects of a plant extract containing catasterone on H_2O_2 induced oxidative stress in human blood lymphocytes.	Not relevant, no information on neurotoxicity

B.6.7.1. Neurotoxicity studies in rodents

Not required.

B.6.7.2. Delayed polyneuropathy studies

Not required.

B.6.8. OTHER TOXICOLOGICAL STUDIES

B.6.8.1. Toxicity studies on metabolites and relevant impurities

Two QSAR studies performed with the OECD-toolbox were submitted. The first one is comparing 24-epibrassinolide to homobrassinolide, and the second one is comparing the properties of 24-epibrassinolide to its impurities[redacted]

The results of these analyses indicate that bridging between 24-epibrassinolide and homobrassinolide is justified, since both molecules contain the same functional groups.
Impurities[redacted]do not have any additional alerting structures when compared to 24-epibrassinolide except for a positive call in the BfR model for skin irritation/corrosion for impurity[redacted]Given the specified level of impurity[redacted](see confidential section), this alert for potential irritating properties is not considered to be of concern. No QSAR analysis was run [redacted] 24-Epibrassinolide, [redacted]

Table 6.8.1-1: Summary of QSAR studies submitted on 24-epibrassinolide and its impurities

Parameter investigated	Model	Result	Classification/ remarks	Reference

Parameter investigated	Model	Result	Classification/ remarks	Reference
Similarity between 24-epibrassinolide and homobrassinolide	OECD-QSAR Toolbox (Version 3.4)	Read across between the two molecules is supported by this analysis, as both substances have the same profiles in the QSAR analysis	Ambigous results for the endpoint 'Estrogen receptor binding', as contradicting results are obtained with two different predicitve models	Wildemann T., 2015
Similarity between 24-epibrassinolide and impurities [redacted]	OECD-QSAR Toolbox (Version 3.4)	Comparing the impurities [redacted] to 24-Epibrassinolide no additional alert was found regarding the following endpoints: systemic toxicity, genotoxicity, carcinogenicity, endocrine disruption, reproductive and developmental toxicity and skin sensitisation. Impurity [redacted] met the inclusion criteria for skin irritation/corrosion, while 24-Epibrassinolide and the impurities [redacted] did not meet the criteria for inclusion.	No additional toxcological alerts compared to 24-epibrassinolide were predicted for impurities [redacted] except for a positive prediction on skin irritation/corrosion for Impurity [redacted]	Wildemann, T., Roth, T., 2015

Reference:	QSAR Analysis on the toxicological similarities between 24-Epibrassinolide and Homobrassinolide
Author(s), year:	Wildemann, T., 2015
Report/Doc. number::	PP309-00002, 092-129
Guideline(s):	Not applicable
GLP:	No
Deviations from guideline	Not applicable
Acceptability:	Yes

24-epibrassinolide and homobrassinolide were compared. The molecules are identical except a different sidechain on C24 (-CH3 for 24-epibrassinolide and –C2H5 for homobrassinolide).
Since both substances have the same functional groups, the predictions on the different endpoints are the same. In conclusion, bridging from 24-epibrassinolide to homobrassinolide is considered justified.

Table 6.8-2: Results of the QSAR analysis

Property / endpoint profiler	Active substance	
	Homobrassinolide	**24-Epibrassinolide**
Predefined		
Substance type	Discrete chemical	Discrete chemical
Chemical category	Not categorised	Not categorised
Empiric		

Lipinski Rule Oasis	Bioavailable	Bioavailable
Organic functional groups	Alcohol Alkane, branched with tertiary carbon Cycloalkane Dihydroxyl group Fused saturated carbocycles Fused saturated heterocycles Isopropyl Lactone Saturated heterocyclic fragment	Alcohol Alkane, branched with tertiary carbon Cycloalkane Dihydroxyl group Fused saturated carbocycles Fused saturated heterocycles Isopropyl Lactone Saturated heterocyclic fragment
Organic functional groups (nested)	Alkane, branched with tertiary carbon Cycloalkane Dihydroxyl group Fused saturated heterocycles Isopropyl Lactone Overlapping groups Saturated heterocyclic fragment	Alkane, branched with tertiary carbon Cycloalkane Dihydroxyl group Fused saturated heterocycles Isopropyl Lactone Overlapping groups Saturated heterocyclic fragment
Organic functional groups (US EPA)	Aliphatic Carbon [CH] Aliphatic Carbon [-CH2-] Aliphatic Carbon [-CH3] Carbonyl, aliphatic attach [-C(=O)-] Cyclic ester Ester, aliphatic attach [-C(=O)O] Fused Aliphatic ring unit Hydroxy, aliphatic attach [-OH] Miscellaneous sulfide (=S) or oxide (=O) Olefinic carbon [=CH- or =C<] Tertiary Carbon	Aliphatic Carbon [CH] Aliphatic Carbon [-CH2-] Aliphatic Carbon [-CH3] Carbonyl, aliphatic attach [-C(=O)-] Cyclic ester Ester, aliphatic attach [-C(=O)O] Fused Aliphatic ring unit Hydroxy, aliphatic attach [-OH] Miscellaneous sulfide (=S) or oxide (=O) Olefinic carbon [=CH- or =C<] Tertiary Carbon
Organic functional groups, Norbert Haider (checkmol)	1,2-diol Alcohol Carbonic acid derivative Carboxylic acid derivative Carboxylic acid ester Heterocyclic compound Hydroxy compound Lactone Secondary alcohol	1,2-diol Alcohol Carbonic acid derivative Carboxylic acid derivative Carboxylic acid ester Heterocyclic compound Hydroxy compound Lactone Secondary alcohol
Tautomers unstable	Stable form	Stable form

Table 6.8-3: Mechanistic results of the QSAR analysis

Property / endpoint profiler	Active substance	
	Homobrassinolide	**24-Epibrassinolide**
DNA binding by OASIS v.1.3	No alert found	No alert found
DNA binding by OECD	No alert found	No alert found
DPRA Cysteine peptide depletion	Not possible to classify according to these rules	Not possible to classify according to these rules
DPRA Lysine peptide depletion	Not possible to classify according to these rules	Not possible to classify according to these rules
Estrogen receptor binding	Strong binder, OH group	Strong binder, OH group

Protein binding by OASIS v.1.3	No alert found	No alert found
Protein binding by OECD	Acylation >> Direct Acylation Involving a Leaving group >> Acetates	Acylation >> Direct Acylation Involving a Leaving group >> Acetates
Protein binding potency	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)
Superfragments	No superfragment	No superfragment
Toxic hazard classification Cramer (extension)	High (Class III)	High (Class III)
Toxic hazard classification Cramer (original)	High (Class III)	High (Class III)

Table 6.8-4: Endpoint specific results of the QSAR alanysis

Property / endpoint profiler	**Active substance**	
	Homobrassinolide	**24-epibrassinolide**
Carcinogenicity (gentox/nongenox) alerts by ISS	Structural alert for nongenotoxic carcinogenicity Substituted n-alkylcarboxylic acids (Nongenotox)	Structural alert for nongenotoxic carcinogenicity Substituted n-alkylcarboxylic acids (Nongenotox)
DART scheme v.1.0	Not known precedent reproductive and developmental toxic potential	Not known precedent reproductive and developmental toxic potential
DNA alerts for AMES, MN and CA by OASIS v.1.3	No alert found	No alert found
Eye irritation/corrosion exclusion rules by BfR	(!Undefined)Group All Lipid Solubility < 0.01 g/kg Group All Melting Point > 200 C Group C Melting Point > 55 C Group C Molecular Weight > 380 g/mol	(!Undefined)Group All Lipid Solubility < 0.01 g/kg Group All Melting Point > 200 C Group C Melting Point > 55 C Group C Molecular Weight > 380 g/mol
Eye irritation/corrosion inclusion rules by BfR	Inclusion rules not met	Inclusion rules not met
in vitro mutagenicity (Ames test) alerts by ISS	No alert found	No alert found
in vivo mutagenicity (Micronucleus) alerts by ISS	H-acceptor-path3-H-acceptor	H-acceptor-path3-H-acceptor
Keratinocyte gene expression	Not possible to classify according to these rules	Not possible to classify according to these rules
Oncologic primary classification	Not classified	Not classified
Protein binding alerts for Chromosomal aberration by OASIS v1.1	No alert found	No alert found
Protein binding alerts for skin sensitization by OASIS v1.3	No alert found	No alert found

Respiratory sensitisation	No alert found	No alert found
Retinoic acid receptor binding	Not possible to classify according to these rules	Not possible to classify according to these rules
rtER expert system ver. 1- USEPA	No alert found	No alert found
skin irritation/corrosion exclusion by BfR	(!Undefined)Group All Lipid Solubility < 0.01 g/kg (!Undefined)Group C Surface Tension > 62 mN/m Group All Melting Point > 200 C Group C Melting Point > 55 C Group C Molecular Weight > 350 g/mol Group C Vapour Pressure < 0.0001 Pa	(!Undefined)Group All Lipid Solubility < 0.01 g/kg (!Undefined)Group C Surface Tension > 62 mN/m Group All Melting Point > 200 C Group C Melting Point > 55 C Group C Molecular Weight > 350 g/mol Group C Vapour Pressure < 0.0001 Pa

Reference:	QSAR Analysis on the toxicological similarities between 24-Epibrassinolide and its impurities [redacted]
Author(s), year:	Wildemann, T., Roth, T., 2015
Report/Doc. number:	PP309-00002, 092-128
Guideline(s):	Not applicable
GLP:	No
Deviations from guideline	Not applicable
Acceptability:	Yes

During 5-Batch analysis of 24-Epibrassinolide, [redacted] impurities have been found. For 24-Epibrassinolide and the impurities [redacted], the QSAR Toolbox (version 3.4) was used to identify relevant structural alerts for different toxicological endpoints, potential mechanism or mode of action and to evaluate their potential toxicity based on their chemical structure. Impurity [redacted] 24-Epibrassinolide because of [redacted]. As the QSAR Toolbox [redacted], [redacted] and 24-Epibrassinolide. Thus, no separate QSAR analysis was conducted for impurity [redacted]

Within this report, it has been found that the active substance 24-Epibrassinolide and the impurities [redacted] are bioavailable, while impurity [redacted] is not bioavailable.

None of the substances has a positive alert for the profilers or the profilers related to genotoxicity with the exception of the profiler 'in vivo mutagenicity (micronucleus) alerts by ISS'. For this profiler, 24-Epibrassinolide and [redacted] impurities have a positive alert.

For the active substance 24-Epibrassinolide negative results are available for the Ames, HPRT and chromosome aberration tests. Based on the in vitro tests, it is not expected that 24-Epibrassinolide has any potential for genotoxicity, including chromosomal damage. Thus, the positive alert for in vivo mutagenicity (micronucleus) can be considered to be false-positive for 24-Epibrassinolide and [redacted] impurities.

24-Epibrassinolide and the impurities [redacted] have positive alerts for the mechanistic profiler 'estrogen receptor binding'. However, this alert is not confirmed by the profiler rtER expert system.

In addition to that, all substances were negative for developmental and reproductive toxicity. With regard to skin irritation/corrosion, the impurity [redacted] met the rules for inclusion for skin irritation/corrosion, while 24-Epibrassinolide and the impurities [redacted] were not classifiable.

B.6.8.2. Supplementary studies on the active substance

The studies summarized below were identified in the systematic literature review. No studies raising concerns regarding potential adverse toxicological effects or effects relevant for classification were identified in in the literature search.

Annex point	KCA 5.8.2/01
Reference:	HAEMATOPOIESIS AND STEROID METABOLISM PARAMETERS IN ANIMALS EXPOSED TO EPIBRASSINOLIDE (English translation)
Author(s), year:	Nadzharyan, L. A., et al, 2006
Report/Doc. number:	092-122
Guideline(s):	Not applicable, public literature
GLP:	No
Deviations from guideline	Not applicable
Acceptability:	Limited reliability due to poor reporting of experimental details, purity of starting material is not described, low resolution graphics, and poor legibility of figures

In the acute experiment to evaluate genotoxic properties, adult male CBA mice weighing 20±2 g were used. The substance was dissolved in physiological solution with added Tween (Sigma-Aldrich, USA) and injected intraperitoneally at a dose of 500 mg/kg.
A significant increase in the number of cells with aberrations was observed. Parameters for haematopoiesis, hormonal status, and the activity of certain enzymes associated with steroid metabolism were studied in subchronic and chronic experiments on outbred white rats under conditions of daily intragastric administration of doses of 0.00002-0.2 mg/kg for 60, 90, or 240 days.
The findings of this study are judged to be of low reliability due to to deficiencies in experimental design and reporting.

Annex point	KCA 5.8.2/02
Reference:	THE EFFECT OF EPIBRASSINOLIDE ON IMMUNE SYSTEM PARAMETERS
Author(s), year:	Voytovich, A. M., et al, 2006 (English translation)
Report/Doc. number:	092-127
Guideline(s):	Not applicable, public literature
GLP:	No
Deviations from guideline	Not applicable
Acceptability:	Limited relieability due to poor reporting of experimental details, purity of starting material is not described, low resolution graphics, and poor legibility of figures

Solution of Tween and 24-Epibrassinolide did not statistically significantly alter the relative mass coefficients of the thymus in mice during acute injection over time, compared to injection of physiological saline.
In the chronic experiment no increased thymocyte cell death was detected during that period. The thymus changed over the time period but did not differ from control group. The results of the contact studies were not statistically significant compared to the control and therefore, 24-Epibrassinolide has been declared as allergy-safe. Thus, no adverse effect related to the substance 24-Epibrassinolide has been observed.
The findings of this study are judged to be of low reliablitiy due to to deficiencies in experimental design and reporting.

Annex point	KCA 5.8.2/03
Reference:	BRASSINOSTEROIDS INHIBIT IN VITRO ANGIOGENESIS IN HUMAN ENDOTHELIAL CELLS
Author(s), year:	Rarova, L., et al, 2012
Report/Doc. number:	092-123
Guideline(s):	Not applicable, public literature
GLP:	No
Deviations from guideline	Not applicable
Acceptability:	Yes

Antiangiogenic activity of various brassinosteroid plant hormones and their derivative cholestanon was investigated in human umbilical vein endothelial cells (HUVEC) and in human microvascular endothelial cells (HMEC-1). 24-Epibrassinolide did inhibit growth of HUVEC (IC_{50} > 50 µM) and HMEC cells (IC_{50} =42.7 µM), inhibited migration at a concentration of 30 µM, but had no detectable influence on tube formation. The authors also observed a slight increase in the SubG1 fraction in the cell cycle analysis, which indicates induction of apopotosis.
In transgene reporter assays, 24-epibrassinolide did not show agonistic activity on Estrogen Receptor (ER) α, Estrogen Receptor β, Androgen Receptor, Glucocorticoid Receptor, Mineralcorticoid receptor, or the Progesterone Receptor (EC_{50} > 100 µM, for comparison: EC_{50} of estradiol to estrogen receptor α in the same assay was 22 pM).
24-Epibrassinolide showed weak antagonistic activity on ERα (IC_{50} was 14.8 µM) and no activity on the other receptors (IC_{50} > 100 µM). The IC_{50} of ICI 182 780, a high affinity ERα antagonist, was 2 nM in the same assay.
In an Estrogen Receptor competitor assay, 24-epibrassinolide showed no binding activity (IC_{50} > 100 µM, estradiol had IC_{50} values of 8.6 nM (ERα) and 8.1 nM (ERβ) in the same assay.

The results of this study indicate that 24-epibrassinolide inhibits growth and migration of HUVEC and HMEC cells *in vitro*.
In a reporter assay in stably transfected cell lines, 24-epibrassinolide showed antagonistic effects on ERα at concentrations of about 14.8 µM (for comparison: IC_{50} of ICI 182 780 in the same assay was 2 nM, which is 7400-fold lower).
In a receptor binding assay, no activity was observed (IC_{50} > 100 µM).
Based on these results it is considered unlikely that 24-epibrassinolide will act as a modulator of steroid signalling *in vivo*.

B.6.8.3. Studies on endocrine disruption

From the data available on 24-epibrassinolide, there are no indications that the active substance could act as endocrine disruptor.

Brassinosteroids (BRs) are natural phytohormones, found in many plant species, with highest contents in seed, pollen and fruits (Carange et al., 2011, Zhu et al., 2013). As plant sterols they regulate hormonal balance, activation of protein and nucleic acid synthesis, enzyme activity, and growth promotion. In addition, they mediate augmented resistance to unfavourable environmental factors, stress, and disease. Exogenous exposure has been connected to specific antioxidative effects, via improvement of synthesis of photosynthetic pigments and antioxidant enzymes activity (Niu et al., 2016).

According to the adopted proposal for endocrine disrupting properties (SANTE/11992/2017 Rev.0), an endocrine disruptor shows an adverse effect in an intact organism or its progeny, has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system, and the adverse effect is a consequence of the endocrine mode of action.
There are no indications for an endocrine disruption potential of 24-Epibrassinolide from the available data. No adverse effects were observed in any of the available guideline studies. In addition, the literature search did not reveal any relevant publication demonstrating endocrine disrupting properties in mammals.

There is at present no indication that 24-Epibrassinolide acts as an endocrine disruptor.

Furthermore, 24-Epibrassinolide is not mentioned in any list of chemicals for which endocrine disrupting properties are proved or suspected (e.g. the lists of chemicals for Tier 1 screening in the EDSP of the US-EPA (2009) or the list in the PIP report from 2009 (Position paper on the potential impact of proposed changes to EU pesticide regulations on ACP countries)).

A study performed with homobrassinolide including a Hershberger assay, was submitted with the dossier. As both substances are highly similar and thus, read across is considered justified, the results of this study are reported below:

Annex point	KCA 5.8.3/04
Reference:	ANABOLIC EFFECT OF PLANT BRASSINOSTEROID
Author(s), year:	Esposito, D., et al, 2011
Report/Doc. number:	092-081
Guideline(s):	None, public literature
GLP:	No
Deviations from guideline	Not applicable
Acceptability:	Yes

In this study, the effects of homobrassinolide (HB) on rat skeletal muscle cells and in Wistar rats were examined.
In vitro, homobrassinolide stimulated protein synthesis and inhibited protein degradation in L6 rat skeletal muscle cells (EC_{50} 4 µM) mediated in part by PI3K/Akt signaling pathway.
In an *in vitro* competitive binding assay to the rat nuclear androgen receptor, homobrassinolide showed no significant binding from concentrations of 0.01 µM up to 10 µM. In the same assay, methandrostenolone as positive control produced specific binding to the androgen receptor with an IC_{50} of 24 nM.

Oral administration of HB (20 or 60 mg/kg/d for 24 days) to healthy rats fed normal diet (protein content 23.9%) increased food intake, body weight gain, lean body mass, and gastrocnemius muscle mass as compared with vehicle-treated controls. The effect of homobrassinolide administration increased slightly in animals fed a high-protein diet (protein content 39.4%).

Both oral (up to 60 mg/kg) and subcutaneous (up to 4 mg/kg) administration of homobrassinolide showed low androgenic activity when tested in a 10 day Hershberger assay with orchiectomized (ORX) Wistar rats (6 animals per group). The positive control group received 0.4 mg/kg per day of testosterone propionate (TP) subcutaneously.

Weights of androgen-sensitive tissues from sham-treated and ORX rats treated with HB

Admini-stration and treatment group	Tissue				
	Ventral prostate (mg)	Seminal vesicles (mg)	Bulbocavernosus/levator ani (mg)	Glans penis (mg)	Cowper's gland (mg)
Oral					
Sham	222.5 ± 15.2***	515.0 ± 12.9***	517.0 ± 10.5***	83.7 ± 0.8***	26.7 ± 1.8***
ORX	19.2 ± 2.5	33.3 ± 3.0	123.7 ± 6.8	45.7 ± 1.5	10.8 ± 1.3
ORX + HB20	26.0 ± 2.5	37.3 ± 3.5	109.2 ± 8.6	55.3 ± 1.7*	11.2 ± 0.7
ORX + HB60	23.0 ± 2.4	34.7 ± 3.3	137.7 ± 9.9	59.6 ± 1.6**	12.5 ± 1.1
ORX + TP0.4[a]	110.50 ± 9.5***	262.5 ± 12.5***	382.0 ± 22.0***	93.8 ± 4.2***	34.5 ± 4.7***
Subcutaneous					
ORX	22.2 ± 3.1	27.8 ± 1.3	109.2 ± 6.4	41.3 ± 2.6	11.3 ± 0.7
ORX + HB0.4	18.3 ± 1.2	29.0 ± 3.1	131.3 ± 7.1	50.7 ± 1.9	11.3 ± 0.5
RX + HB4	23.2 ± 1.2	30.2 ± 1.2	147.5 ± 6.9*	56.3 ± 3.3*	10.5 ± 0.3
ORX + TP0.4	92.8 ± 6.3***	228.7 ± 35.3	293.8 ± 19.0***	72.3 ± 4.5***	34.3 ± 1.9***

Rats were fed normal diet (23.9% protein content) and gavaged daily with 20 or 60 mg/kg body weight of HB or subcutaneously injected with 0.4 and 4 mg/kg body weight of HB for 10 d. Results are expressed as means ± SE.

*$P < 0.05$,
**$P < 0.01$,
***$P < 0.001$ *vs.* ORX; 1-way ANOVA and Dunnett's posttest.
[a]Teststerone propionate (TP) was given as subcutaneous injection at 0.4 mg/kg and served as positive control.

The authors conclude that oral application of homobrassinolide triggers a selective anabolic response with minimal or no androgenic side-effects.

B.6.9. MEDICAL DATA AND INFORMATION

B.6.9.1. Medical surveillance on manufacturing plant personnel and monitoring studies

Manufacturing of the substance is closely observed by the "Production Safety Law of the [redacted]" (2002). According to this law every employee has to pass the education and training programs regarding production safety before being allowed to start to work in the company. Under this law regularly health checks, appraisals, supervisions and controls have to be conducted to secure health and safety of every employee. According to the notifier, as well as government audits, there are no reported employee related health effects of workplace safety accident issues (Suntton statement, Wang, S., 2016). The production in premises with exposure is hereby arranged by one shift manager, a foreman and six factory workers. No respective report is available.

B.6.9.2. Data collected on humans

New active substance, no data available.

B.6.9.3. Direct observation

New active substance, no data available.

B.6.9.4. Epidemiological studies

No studies addressing the effects of 24-epibrassinlode are available in English.

In a review article by Zhabinskii et al. (2015), two studies are cited examining the effects of 24-epibrassinolide on cholesterol levels in humans. Both articles are not available in English, and no further experimental details are reported in the review article:

> Statsenko EA. Prophylactic and correction of functional state among athletes of high qualifying categories under training process [Doctoral thesis]. Moscow: Federal Scientific Center of physical Culture and Sport: 2013,
>
> Statsenko EA Korolevich MP, Seregkina TV, Paramonova NA, Ostapenko VA, Ryibkina IL, et al. Methods of correction of lipid metabolism in athletes. Voennaya Medicina [Military Medicine] 2008;9:102-4).

In Statsenko et al 2013, volunteers (10 people) with hypercholesterolemia were assigned to consume daily 15 µg of 24-epibrassinolide. Participants experienced a decrease in total serum cholesterol from initially elevated values of 5.70 to 4.73 mmol/L. Analysis of the lipid profile showed that the observed changes were to a greater extent due to the reducing the content of LDL fraction from 4.03 to 2.97 mmol/L.
Statsenko et al 2009 describes a study undertaken with subjects with a normal level of cholesterol. Both the control and experimental group consisted of 30 healthy volunteers. Each person from the experimental group daily received 15 µg of 24-epibrassinolide during 1 month. Before and immediately after finishing the experiment all volunteers were subjected to complex investigations including basic laboratory tests. There were no significant differences in hematological and biochemical parameters between the two groups except for the level of cholesterol. Statistically significant decreases of cholesterol and triglycerides levels were noted (38% for cholesterol and 41% for triglycerides).

As plant sterols and stanols in general are used as food supplements, there are several evaluations by different regulatory agencies available addressing their safety and beneficial effects on blood cholesterol levels: While the exact content of 24-epibrassinolide in these supplements is not known and presumably rather low (see Decision 2004/336/CE of 31 March 2004), these evaluations clearly show that consumption of plant sterols and stanols at up to 3 g/day can be considered safe and beneficial.

Effects of plant sterols and plant stanols on human cholesterol levels have been evaluated by the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) (Scientific Opinion on the substantiation of a health claim related to 3 g/day plant sterols/stanols and lowering blood LDL-cholesterol and reduced risk of (coronary) heart disease pursuant to Article 19 of Regulation (EC) No 1924/2006, EFSA Journal 2012;10(5):2693, Scientific Opinion of the Panel on Dietetic Products Nutrition and Allergies on a request from the European Commission and a similar request from France in relation to the authorization procedure for health claims on plant stanols and plant sterols and lowering/reducing blood LDL-cholesterol pursuant to Article 14 of Regulation (EC) No 1924/2006, EFSA Journal (2009) 1175, 1-9).
The Panel concluded that for an intake of 1.5 - 2.4 g/d an average reduction of between 7 and 10.5% of LDL-cholesterol can be expected (EFSA, 2009). In its opinion adopted on 26 April 2012, the EFSA Panel extended the conclusion "that plant sterols and stanol esters at a daily intake of 3 g (range 2.6 - 3.4 g) sterols/stanols in matrices approved by the Regulation (EC) No 376/2010 (yellow fat spreads, dairy products, mayonnaise and salad dressings) lower LDL-cholesterol by 11.3% (95% CI: 10.0 – 12.5). The minimum duration required to achieve the maximum effect of plant sterols and stanols on LDL-cholesterol lowering is two to three weeks." (EFSA, 2012).

In 2010 the Canadian Bureau of Nutritional Sciences "Health Canada" evaluated the effect of plant sterols on blood cholesterol (Health Canada 2010). They evaluated study results of 84 randomized controlled trials (comprising 141 pertinent trial arms) published from 1994 to 2007 and found an overall 8.8% reduction in LDL-cholesterol with an average intake of 2 g/day of plant sterols. A dose-response relationship was observed up to about 3 g/day in these studies which included doses ranging from about 0.5 g/day to 9.0 g/day. Health Canada concluded that sufficient scientific evidence exists to support a relationship between phytosterol consumption and blood cholesterol lowering, while having no detrimental effect on HDL-cholesterol levels, leading to an overall improvement in blood lipid profile.

In the United States, plant sterols and stanols added to a variety of food products are generally recognized as safe (GRAS) by the FDA. The authors conclude that there is scientific evidence to support the fact that phytosterols and their derivatives have several biological activities which promote the health of animals, humans and micro-organisms with only few adverse effects, such as occur in phytosterolemia, a rare genetic disorder. These health benefits include reduction of plasma total and LDL cholesterol levels, which decrease the risk of cardiovascular diseases; anti-inflammatory activities; prevention of colon, breast and prostate cancers, and treatment of benign prostatic hyperplasia. Therefore, regular consumption of plant sterols and stanols in natural foods not exceeding 3 g/day is considered healthy to man and animals.

In a review article (Komosinska-Vassev, K., et al, 2015) the authors claim that bee pollen contains 1.1% of phytosterols and detail positive effects of bee pollen on rats and rabbits, as well as on human beings.

Furthermore, in an epidemiological estimation of the average daily intake of plant sterols in Finland it is reported that the mean intake for a randomized population study of 334 subjects with a 3 day record food data, was 305 mg/d for men and 237 mg/d for women (Valsta, et al, 2004). Cereals, margarine, vegetables and vegetable oils were the main sources of plant sterols.

B.6.9.5. Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical test

No clinical signs or symptoms of poisonings have been observed in any of the acute exposure studies performed with 24-Epibrassinolide, and no clinical signs or symptoms of poisoning are anticipated to result from accidental human exposure.

B.6.9.6. Proposed treatment: first aid measures, antidotes, medical treatment

No antidote treatment is foreseen.
As a general hygienic measure in case of direct contact with the active ingredient, the following first aid instructions should be followed:

Inhalation: Move person to fresh air.
Skin: Wash off immediately with soap and water.
Eyes: Hold open eye and rinse slowly and gently with water, also under the eyelids, for at least 15 minutes. If skin irritation occurs, seek medical advice/attention.
Ingestion: Rinse out mouth thoroughly with water.

Most important symptoms and effects, both acute and delayed:
No symptoms and effects have been reported.

Indication of any immediate medical attention and special treatment needed:
Medical treatment is to be carried out symptomatically.

B.6.10. REFERENCES RELIED ON

A literature search for the active substance 24-Epibrassinolide was performed in accordance to the provisions of the EFSA Guidance “Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) 1107/2009”.

Reference:	LITERATURE REVIEW REPORT - ACTIVE SUBSTANCE: 24-Epibrassinolide
Author(s), year:	Reisinger, T., Huber, L. (2017)
Report/Doc. number:	PP309-00002
Guideline(s):	EFSA Guidance "Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009; EFSA Journal 2011, 9(2):2092"
GLP:	Not applicable
Deviations from Guideline	Not applicable, literature search is performed according to the cited guidance document
Acceptability:	Yes

The objective of the literature search was the assessment of scientific peer-reviewed open literature published within the last 10 years and dealing with side-effects on health, the environment and non-target species for the active substance 24-Epibrassinolide.

Literature was searched accessing the databases: AGRICOLA, BIOSIS, CABA, EMBASE, ESBIOBASE, HCAPLUS, MEDLINE, PASCAL, PQSCITECH, TOXCENTER via the service provider STN-International. The search strategy was based on a single concept search (CAS number and chemical names).

The search has been carried out on 09.11.2016 (calendar week 45).

In a first step the CAS REGISTRY database was accessed and the CAS number was searched to retain information on identity and substance names/synonyms.
The following list of substance specific search terms generated was used as query to search the STN databases:

CAS Number: 78821-43-9

Chemical names:

- (1R,3AS,3BS,6AS,8S,9R,10AR,10BS,12AS)-1-((1S,2R,3R,4R)-2,3-DIHYDROXY-1,4,5-TRIMETHYLHEXYL)HEXADECAHYDRO-8,9-DIHYDROXY-10 A,12A-DIMETHYL-6H-BENZ(C)INDENO(5,4-E)OXEPIN-6-ONE
- (22R,23R,24R)-2.ALPHA.,3.ALPHA.,22,23-TETRAHYDROXY-B-HOMO-7-OXA-5.ALPHA.-ERGOSTAN-6-ONE
- EPIBRASSINOLIDE R
- EPIBRASSINOLIDE
- 126721-49-1
- 24(R)-EPIBRASSINOLIDE
- 24-EPI-BRASSINOLIDE
- 24-EPIBRASSINOLIDE
- 6H-BENZ(C)INDENO(5,4-E)OXEPIN-6-ONE, 1-(2,3-DIHYDROXY-1,4,5 TRIMETHYLHEXYL)HEXADECAHYDRO-8,9-DIHYDROXY-10A,12ADIMETHYL-,(1R-(1.ALPHA.(1S*,2R*,3R*,4R*),3A.BETA.,3B.ALPHA.,6A.BETA.,8.BETA.,9.BETA.,10A/BI
- 72075-02-6
- 78821-43-9

Trade name:

- EPIN

In total, 3861 hits were retrieved, which were further filtered by publication type (patents were excluded) and date (publication year > 2005).
After removal of duplicates, 854 records were retrieved form bibliographic databases and screened for relevance by expert reviewers based on their titles.

At this step, 802 references were excluded.

For the remaining 52 records, complete reference information and abstracts were retrieved from the bibliographic databases and screened by the reviewers for relevance with respect to the relevant EU data

requirements related to side-effects on human health, non-target species and the environment for the active substance 24-Epibrassinolide.

At this step, 43 references were excluded.

Table 6.10-1: Overview on records retireved and study selection process

Results of the study selection process	**n**
Total number of summary records retrieved after all searches of peer-reviewed	854
Number of summary records excluded from the search results after assessment screening of the summary records (title / abstract) for relevance	845
Number of summary records retrieved from bibliographic databases	54
Total number of full-text documents assessed in detail	9
Number of full text documents included in the assessment	8

Nine full-text documents were assessed in detail based on the Klimisch score (J. Klimisch, M. Andreae and U. Tillmann (1997) A Systematic Approach for Evaluating the Quality of Experimental Toxicological and Ecotoxicological Data Regulatory Toxicology and Pharmacology Vol 25 pp 1-5).

The list of relevance criteria (modified Klimisch score) is displayed in the following table.

Table 6.10-2: Modified Klimsch criteria for relevance for each data requirement

Data requirements (indicated by the corresponding EU data point)	**Criteria for relevance**	
General criteria for relevance considered for all data requirements indicated by the corresponding EU data points	1	Publication scientifically sound
	2	Does provide relevant information for dossier preparation or risk assessment purpose
	3	Method validated
	4	Documentation sufficient for assessment
	5	Does meet important criteria of today standard methods
	6	No relevant methodological deficiencies
	7	Suitable test system
	8	Document does not contain already identified results

As the relevance of the publications was evaluated by the (modified) Klimisch score, the score for the excluded publications uses the negation of the above listed criteria (e.g. Criterium 3“Method validated” would turn to “Method NOT validated” if the method is not validated and the publication is excluded based on this criterium.

After assessment of full texts, eight publications were considered relevant and relieable and included in the assessment report under the respective subheadings (see following table), references *in italics* were included in the human health section.

Table 6.10-3: References included in the assessment report after full text relevance assessment

Author(s)	**Data requirement (indicated by the corresponding EU Annex point)**	**Year**	**Title**	**Source**
Chuda-Mickiewicz, B., et al.	CA 8.3.1	2009	The Role of Phytohormones in Instrumental Insemination of Queen Bees	Journal of Apicultural Science, 53(2): 91-96
Mekhalfi, M., et al.	CA 8.2.6	2012	Consequences of the presence of 24 - epibrassinolide , on cultures of a diatom, Asterionella formosa	Biochimie 94(5): 1213-1220

Nadzharyan, L. A., et al.	*CA 5.8.2*	*2006*	*Hemopoiesis- and steroid metabolism indices in animals under epibrassinolide effect*	*Sovremennye Problemy Toksikologii 2: 43-48*
Ohri, P., et al.	CA 8.4.2	2008	Studies on Meloidogyne incognita under influence of 24 - epibrassinolide	Annals of Plant Protection Sciences 16(1): 198-202
Rarova, L., et al.	*CA 5.8.2*	*2012*	*Brassinosteroids inhibit in vitro angiogenesis in human endothelial cells*	*Steroids, 77(13): 1502-1509*
Rupinder, K., et al.	CA 8.4.2	2013	Alterations in antioxidative enzymes in Meloidogyne incognita females treated with 24 - epibrassinolide	Indian Journal of Nematology 43(2): 219-221
Voitovich, A. M., et al.	*CA 5.8.2 CA 5.8.4*	*2006*	*Epibrassinolide effect on immune system parameters*	*Sovremennye Problemy Toksikologii 3: 33-37*
Waisi, H., et al.	CA 8.7	2015	Bacteriostatic effect of 24 - epibrassinolide against erwinia amylovora isolates	Acta Microbiologica Hellenica, 60(3): 2015-2024

Table 6.10-4: References excluded from the assessment report after full text relevance assessment

Author(s)	Year	Title	Source	Klimsch score	Reason(s) for not including the study
Sysa, A. G., et al.,	2010	Effect of the structure of the brassinosteroid side chain on monooxygenase activity of liver microsomes	Prikladnaia biokhimiia i mikrobiologiia, Vol. 1, pp 29-34	2; Does not provide relevant information for dossier preparation or risk assessment purpose	Study shows the dependence of the side chain of Brassinosteroids for its effect on the microsomal enzymatic system. 24-Epibrassinolde did not reveal an adverse effect up to a concentration of 250 uM. No other effect on the monooxygenase system could be determined. Thus, for 24-BR no adverse effect is shown.

Detailed lists of all studies screened can be retrieved from the literature review report.

Data Point	Author(s)	Year	Title Compagny Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation

KCA 5/01	Takatsuto, S. Abe, H. Gamoah, K.	1990	EVIDENCE FOR BRASSINOSTEROIDS IN STROBILUS OF EQUISETUM ARVENSE L. Report No.: na (092-059) Agricultural and Biological Chemistry, 1990, 54 (4), 1057-1059 Not GLP, published	N	N		nr	N
KCA 5/02	Zhu, J.-Y. Sae-Seaw, J. Wang, Z.-Y.	2013	BRASSINOSTEROID SIGNALLING Report No.: na (092-165) Development, 2013, 140(8), 1615-1620; doi: 10.1242/dev.060590 Not GLP, published	N	N		nr	N
KCA 5/03	Saini, S. Sharma, I. Pati, P.K.	2015	VERSATILE ROLES OF BRASSINOSTEROID IN PLANTS IN THE CONTEXT OF ITS HOMOEOSTASIS, SIGNALING AND CROSSTALKS Report No.: na (092-182) Frontiers in Plant Science, 2015, 6, 950; doi: 10.3389/fpls.2015.00950 Not GLP, published	N	N		nr	N
KCA 5/04	Symons, G.M. Ross, J.J. Jager, C.E. Reid, J.B.	2008	BRASSINOSTEROID TRANSPORT Report No.: na (092-094) Journal of Experimental Botany, 2008, 59 (1), 17-24; doi:10.1093/jxb/erm098 Not GLP, published	N	N		nr	N
KCA 5/05	Kutschera, U. Wang, Z.-Y.	2012	BRASSINOSTEROID ACTION IN FLOWERING PLANTS: A DARWINIAN PERSPECTIVE Report No.: na (092-036) Journal of Experimental Botany, 2012, 63 (10), 3511-3522; doi:10.1093/jxb/ers065 Not GLP, published	N	N		nr	N
KCA 5/06	Thompson, M.J. Mandava, N. Flippen-Anderson, J.L. Worley, J.F. Dutky, S.R. Robbins, W.E. Lusby, W.	1979	SYNTHESIS OF BRASSINO STEROIDS: NEW PLANT-GROWTH-PROMOTING STEROIDS Report No.: na (092-063) The Journal of Organic Chemistry, 1979, 44 (26), 5002-5004 Not GLP, published	N	N		nr	N
KCA 5/07	Ikekawa, N. Nishiyama, F. Fujimoto, Y.	1988	IDENTIFICATION OF 24-EPIBRASSINOLIDE IN BEE POLLEN OF THE BROAD BEAN, VICIA FABA L. Report No.: na (092-027) Chemical and Pharmaceutical Bulletin, 1988, 36 (1), 405-407 Not GLP, published	N	N		nr	N
KCA 5/08	Bajguz, A.	2011	SUPPRESSION OF CHLORELLA VULGARIS GROWTH BY CADMIUM, LEAD, AND COPPER STRESS AND ITS RESTORATION BY ENDOGENOUS BRASSINOLIDE Report No.: na (092-103) Archives of Environmental Contamination and Toxicology, 2011, 60, 406-416; DOI 10.1007/s00244-010-9551-0 Not GLP, published	N	N		nr	N
KCA 5/09	Khripach, V. Zhabinskii, V. De Groot, A.	2000	TWENTY YEARS OF BRASSINOSTEROIDS: STEROIDAL PLANT HORMONES WARRANT BETTER CROPS FOR THE XXI CENTURY Report No.: na (092-029) Annals of Botany, 2000, 86, 441-447; doi:10.1006/anbo.2000.1227 Not GLP, published	N	N		nr	N
KCA 5/10	Ikekawa, N. Zhao, Y.-J.	1991	APPLICATION OF 24-EPIBRASSINOLIDE IN AGRICULTURE Report No.: na (092-026) ACS Symposium series, 1991, 474, Chapter 24, 280-291 Not GLP, published	N	N		nr	N
KCA 5/11	Bajguz, A. Tretyn, A.	2003	THE CHEMICAL STRUCTURES AND OCCURRENCE OF BRASSINOSTEROIDS IN PLANTS Report No.: na (092-145)	N	N		nr	N

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KCA 5/12	Hayat, s. Ahmad, A.	2011	BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE Report No.: na (092-146) Springer Verlag, 2011, 1-477, DOI 10.1007/978-94-007-0189-2; ISBN: 978-94-007-0188-5 Not GLP, published	N	N		nr	N
KCA 5/13	Abe, H. Nakamura, K. Morishita, T. Uchiyama, M. Takatsuto, S. Ikekawa, N.	1984	ENDOGENOUS BRASSINOSTEROIDS OF THE RICE PLANT: CASTASTERONE AND DOLICHOSTERONE Report No.: na (092-004) Agricultural and Biological Chemistry, 1984, 48 (4), 1103-1104 Not GLP, published	N	N		nr	N
KCA 5/14	Abe, H. Takatsuto, S. Nakayama, M. Yokota, T.	1995	28-HOMOTYPHASTEROL, A NEW NATURAL BRASSINOSTEROID FROM RICE (ORYZA SATIVA L.) BRAN Report No.: na (092-006) Bioscience, Biotechnology and Biochemistry, 1995, 59 (2), 176-178 Not GLP, published	N	N		nr	N
KCA 5/15	Park, K.-H. Park, J.-D. Hyun, K.-H. Nakayama, M. Yokota, T.	1994	BRASSINOSTEROIDS AND MONOGLYCERIDES IN IMMATURE SEEDS OF CASSIA TORA AS THE ACTIVE PRINCIPLES IN THE RICE LAMINA INCLINATION BIOASSAY Report No.: na (092-046) Bioscience, Biotechnology and Biochemistry, 1994, 58 (7), 1343-1344 Not GLP, published	N	N		nr	N
KCA 5/16	Khripach, V.A. Litvinovskaya, R.P. Kurtikova, A.L. Drach, S.V. Pryadko, A.G. Mirantsova, T.V. Baranovskiy, A.V.	2013	ENZYME IMMUNOASSAY OF THE CONTENT OF ENDOGENOUS BRASSINOSTEROIDS IN PHYTOGENIC FOOD PRODUCTS Report No.: na (092-030) National Academy of Sciences of Belarus, 2013, 57 (2), 63-69 Not GLP, published	N	N		nr	N
KCA 5/17	Yokota, T. Nakayama, M. Wakisaka, T. Schmidt, J. Adam, G.	1994	3-DEHYDROTEASTERONE, A 3,6-DIKETOBRASSINOSTEROID AS A POSSIBLE BIOSYNTHETIC INTERMEDIATE OF BRASSINOLIDE FROM WHEAT GRAIN Report No.: na (092-078) Bioscience, Biotechnology and Biochemistry, 1994, 58 (6), 1183-1185 Not GLP, published	N	N		nr	N
KCA 5/18	Suzuki, Y. Yamaguchi, I. Yokota, T. Takahasi, N.	1986	IDENTIFICATION OF CASTASTERONE, TYPHASTEROL AND TEASTERONE FROM THE POLLEN OF ZEA MAYS Report No.: na (092-053) Agricultural and Biological Chemistry, 1986, 50 (12), 3133-3138 Not GLP, published	N	N		nr	N
KCA 5/19	Kim, S.-K. Chang, S.C. Lee, E.J. Chung, W.-S. Kim, Y.-S. Hwang, S. Lee, J.S.	2000	INVOLVEMENT OF BRASSINOSTEROIDS IN THE GRAVITROPIC RESPONSE OF PRIMARY ROOT OF MAIZE Report No.: na (092-034) Plant Physiology, 2000, 123, 997-1004 Not GLP, published	N	N		nr	N
KCA 5/20	Yasuta, E. Terahata, T. Nakayama, M. Abe, H. Takatsuto, S. Yokota, T.	1995	FREE AND CONJUGATED BRASSINOSTEROIDS IN THE POLLEN AND ANTHERS OF ERYTHRONIUM JAPONICUM DECNE Report No.: na (092-067) Bioscience, Biotechnology and Biochemistry, 1995, 59 (11), 2156-2158 Not GLP, published	N	N		nr	N
KCA	Suzuki, H.	1994	IDENTIFICATION OF BRASSINOLIDE,	N	N		nr	N

5/21	Fujioka, S. Yokota, T. Murofushi, N. Sakurai, A.		CASTASTERONE, TYPHASTEROL AND TEASTERONE FROM THE POLLEN OF LILIUM ELEGANS Report No.: na (092-054) Bioscience, Biotechnology and Biochemistry, 1994, 58 (11), 2075-2076 Not GLP, published					
KCA 5/22	Abe, H. Honjo, C. Kyokawa, Y. Asakawa, S. Natsume, M. Narushima, M.	1994	3-OXOTEASTERONE AND THE EPIMERIZATION OF TEASTERONE: IDENTIFICATION IN LILY ANTHERS AND DISTYLIUM RACEMOSUM LEAVES AND ITS BIOTRANSFORMATION INTO TYPHASTEROL Report No.: na (092-005) Bioscience, Biotechnology and Biochemistry, 1994, 58 (5), 986-989 Not GLP, published	N	N		nr	N
KCA 5/23	Asakawa, S. Abe. H. Kyokawa, Y. Nakamura, S. Natsume, M.	1994	TEASTERONE 3-MYRISTATE: A NEW TYPE OF BRASSINOSTEROID DERIVATIVE IN LILIUM LONGIFLORUM ANTHERS Report No.: na (092-009) Bioscience, Biotechnology and Biochemistry, 1994, 58 (1), 219-220 Not GLP, published	N	N		nr	N
KCA 5/24	Asakawa, S. Abe, H. Nishikawa, N. Natsume, M. Koshioka, M.	1996	PURIFICATION AND IDENTIFICATION OF NEW ACYL-CONJUGATED TEASTERONES IN LILY POLLEN Report No.: na (092-010) Bioscience, Biotechnology and Biochemistry, 1996, 60 (9), 1416-1420 Not GLP, published	N	N		nr	N
KCA 5/25	Soeno, K. Kyokawa, Y. Natsume, M. Abe, H.	2000	TEASTERONE-3-O-ß-D-GLUCOPYRANOSIDE, A NEW CONJUGATED BRASSINOSTEROID METABOLITE FROM LILY CELL SUSPENSION CULTURES AND ITS IDENTIFICATION IN LILY ANTHERS Report No.: na (092-050) Bioscience, Biotechnology and Biochemistry, 2000, 64 (4), 702-709 Not GLP, published	N	N		nr	N
KCA 5/26	Plattner, R.D. Taylor, S.L. Grove, M.D.	1986	DETECTION OF BRASSINOLIDE AND CASTASTERONE IN ALNUS GLUTINOSA (EUROPEAN ALDER) POLLEN BY MASS SPECTROMETRY/MASS SPECTROMETRY Report No.: na (092-047) Journal of Natural Products, 1986, 49 (3), 540-545 Not GLP, published	N	N		nr	N
KCA 5/27	Takatsuto, S. Abe, H. Yokota, T. Shimada, K. Gamoh, K.	1996	IDENTIFICATION OF CASTASTERONE AND TEASTERONE IN SEEDS OF CANNABIS SATIVA L. Report No.: na (092-062) Japan Oil Chemists' Society, 1996, 45 (9), 871-873 Not GLP, published	N	N		nr	N
KCA 5/28	Schmidt, J. Boehme, F. Adam, G.	1996	24-EPIBRASSINOLIDE FROM GYPSOPHILA PERFOLIATA Report No.: na (092-049) Zeitschrift für Naturforschung, 1996, 51 c, 897-899 Not GLP, published	N	N		nr	N
KCA 5/29	Yokota, T. Arima, M. Takahashi, N.	1982	CASTASTERONE, A NEW PHYTOSTEROL WITH PLANT-HORMONE POTENCY, FROM CHESTNUT INSECT GALL Report No.: na (092-072) Tetrahedron letters, 1982, 23 (12), 1275-1278 Not GLP, published	N	N		nr	N
KCA 5/30	Ikeda, M. Takatsuto, S. Sassa, T. Ikekawa, N. Nukina, M.	1983	IDENTIFICATION OF BRASSINOLIDE AND ITS ANALOGUES IN CHESTNUT GALL TISSUE Report No.: na (092-024) Agricultural and Biological Chemistry, 1983, 47 (3), 655-657 Not GLP, published	N	N		nr	N
KCA	Ikekawa, N.	1984	MICROANALYSIS OF BRASSIOSTEROIDS IN PLANTS	N	N		nr	N

5/31	Takatsuto, S.		BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY Report No.: na (092-025) Mass Spectroscopy, 1984, 32 (1), 55-70 Not GLP, published					
KCA 5/32	Asakawa, S. Abe. H. Kyokawa, Y. Nakamura, S. Natsume, M.	1994	TEASTERONE 3-MYRISTATE: A NEW TYPE OF BRASSINOSTEROID DERIVATIVE IN LILIUM LONGIFLORUM ANTHERS Report No.: na (092-009) Bioscience, Biotechnology and Biochemistry, 1994, 58 (1), 219-220 Not GLP, published	N	N		nr	N
KCA 5/33	Arima, M. Yokota, T. Takahashi, N.	1984	IDENTIFICATION AND QUANTIFICATION OF BRASSINOLIDE-RELATED STEROIDS IN THE INSECT GALL AND HEALTHY TISSUES OF THE CHESTNUT PLANT Report No.: na (092-008) Phytochemistry, 1984, 23 (8), 1587-1591 Not GLP, published	N	N		nr	N
KCA 5/34	Takatsuto, S. Omote, K. Gamoh, K. Ishibashi, M.	1990	IDENTIFICATION OF BRASSINOLIDE AND CASTASTERONE IN BUCKWHEAT (FAGOPYRUM ESCULENTUM MOENCH) POLLEN Report No.: na (092-060) Agricultural and Biological Chemistry, 1990, 54 (3), 757-762 Not GLP, published	N	N		nr	N
KCA 5/35	Sondhi, N. Bhardwaj, R. Kaur, S. Chandel, M. Kumar, N. Singh, B.	2010	INHIBITION OF H2O2-INDUCED DNA DAMAGE IN SINGLE CELL GEL ELECTROPHORESIS ASSAY (COMET ASSAY) BY CASTASTERONE ISOLATED FROM LEAVES OF CENTELLA ASIATICA Report No.: na (092-155) Health, 2010, 2 (6), 595-602; doi:10.4236/health.2010.26088 Not GLP, published	N	N		nr	N
KCA 5/36	Swaczynova, J. Novak, O. Hauserova, E. Fuksova, K. Sisa, M. Kohout, L. Strnad, M.	2007	NEW TECHNIQUES FOR THE ESTIMATION OF NATURALLY OCCURRING BRASSINOSTEROIDS Report No.: na (092-057) Journal of Plant Growth Regulation, 2007, 26, 1-14; DOI: 10.1007/s00344-006-0045-2 Not GLP, published	N	N		nr	N
KCA 5/37	Fujioka, S. Choi, Y.-H. Takatsuto, S. Yokota, T. Li, J. Chory, J. Sakurai, A.	1996	IDENTIFICATION OF CASTASTERONE, 6-DEOXOCASTASTERONE, TYPHASTEROL AND 6-DEOXOTYPHASTEROL FROM THE SHOOTS OF ARABIDOPSIS THALIANA Report No.: na (092-018) Plant & Cell Physiology, 1996, 37 (8), 1201-1203 Not GLP, published	N	N		nr	N
KCA 5/38	Fujioka, S. Li, J. Choi, Y.-H. Seto, H. Takatsuto, S. Noguchi, T. Watanabe, T. Kuriyama, H. Yokota, T. Chory, J. Sakurai, A.	1997	THE ARABIDOPSIS DEETIOLATED2 MUTATNT IS BLOCKED EARLY IN BRASSINOSTEROID BIOSYNTHESIS Report No.: na (092-019) The Plant Cell, 1997, 9, 1951-1962 Not GLP, published	N	N		nr	N
KCA 5/39	Nomura, T. Sato, T. Bishop, G.J. Kamiya, Y. Takatsuto, S. Yokota, T.	2001	ACCUMULATION OF 6-DEOXOCATHASTERONE AND 6-DEOXOCASTASTERONE IN ARABIDOPSIS, PEA AND TOMATO IS SUGGESTIVE OF COMMON RATE-LIMITING STEPS IN BRASSINOSTEROID BIOSYNTHESIS Report No.: na (092-040) Phytochemistry, 2001, 57, 171-178 Not GLP, published	N	N		nr	N
KCA 5/40	Bancos, S. Nomura, T.	2002	REGULATION OF TRANSCRIPT LEVELS OF THE ARABIDOPSIS CYTOCHROME P450 GENES INVOLVED	N	N		nr	N

	Sato, T. Molnar, G. Bishop, G.J. Koncz, C. Yokota, T. Nagy, F. Szekeres, M.		IN BRASSINOSTEROID BIOSYNTHESIS Report No.: na (092-161) Plant Physiology, 2002, 130, 504-513; DOI: 10.1104/pp.005439 Not GLP, published					
KCA 5/41	Schmidt, J. Altmann, T. Adam, G.	1997	BRASSINOSTEROIDS FROM SEEDS OF ARABIDOPSIS THALIANA Report No.: na (092-048) Phytochemistry, 1997, 45 (7), 1325-1327 Not GLP, published	N	N		nr	N
KCA 5/42	Choe, S. Fujioka, S. Noguchi, T. Takatsuto, S. Yoshida, S. Feldmann, K.A.	2001	OVEREXPRESSION OF DWARF4 IN THE BRASSINOSTEROID BIOSYNTHETIC PATHWAY RESULTS IN INCREASED VEGETATIVE GROWTH AND SEE YIELD IN ARABIDOPSIS Report No.: na (092-015) The Plant Journal, 2001, 26 (6), 573-582 Not GLP, published	N	N		nr	N
KCA 5/43	Fujioka, S. Takatsuto, S. Yoshida, S.	2002	AN EARLY C-22 OXIDATION BRANCH IN THE BRASSINOSTEROID BIOSYNTHETIC PATHWAY Report No.: na (092-020) Plant Physiology, 2002, 130 (2), 930-939; doi/10.1104/pp.008722 Not GLP, published	N	N		nr	N
KCA 5/44	Shimada, Y. Goda, H. Nakamura, A. Takatsuto, S. Fujioka, S. Yoshida, S.	2003	ORGAN-SPECIFIC EXPRESSION OF BRASSINOSTEROID-BIOSYNTHETIC GENES AND DISTRIBUTION OF ENDOGENOUS BRASSINOSTEROIDS IN ARABIDOPSIS Report No.: na (092-162) Plant Physiology, 2003, 131, 287-297; DOI: 10.1104/pp.013029 Not GLP, published	N	N		nr	N
KCA 5/45	Abe, H. Morishita, T. Uchiyama, M. Marumo, S. Munakata, K. Takatsuto, S. Ikekawa, N.	1982	IDENTIFICATION OF BRASSINOLIDE-LIKE SUBSTANCES IN CHINESE CABBAGE Report No.: na (092-002) Agricultural and Biological Chemistry, 1982, 46 (10), 2609-2611 Not GLP, published	N	N		nr	N
KCA 5/46	Ikekawa, N. Takatsuto, S.	1984	MICROANALYSIS OF BRASSIOSTEROIDS IN PLANTS BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY Report No.: na (092-025) Mass Spectroscopy, 1984, 32 (1), 55-70 Not GLP, published	N	N		nr	N
KCA 5/47	Kanwar, M.K. Bhardwaj, R. Chowdhary, S.P. Arora, P. Sharma, P. Kumar, S.	2013	ISOLATION AND CHARACTERIZATION OF 24-EPIBRASSINOLIDE FROM BRASSICA JUNCEA L. AND ITS EFFECTS ON GROWTH, NI ION UPTAKE, ANTIOXIDANT DEFENSE OF BRASSICA PLANTS AND IN VITRO CYTOTOXICTY Report No.: na (092-118) Acta Physiologiae Plantarum, 2013, 35, 1351-1362; DOI 10.1007/s11738-012-1175-8 Not GLP, published	N	N		nr	N
KCA 5/48	Grove, M.D. Spencer, G.F. Rohwedder, W.K. Mandava, N. Worley, J.F. Warthen, J.D. Steffens, G.L. Flippen-Anderson, J.L. Cook, J.C.	1979	BRASSINOLIDE, A PLANT GROWTH-PROMOTING STEROID ISOLATED FROM BRASSICA NAPUS POLLEN Report No.: na (092-022) Nature, 1979, 281, 216-217 Not GLP, published	N	N		nr	N
KCA 5/49	Pan, J. Hu, Y. Liang, T.	2012	PREPARATION OF SOLID-PHASE MICROEXTRACTION FIBERS BY IN-MOLD COATING STRATEGY FOR DERIVATIZATION ANALYSIS OF 24-	N	N		nr	N

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KCA 5/50	Baba, J. Yokota, T. Takahashi, N.	1983	BRASSINOLIDE-RELATED NEW BIOACTIVE STEROIDS FROM DOLICHOS LABLAB SEED Report No.: na (092-011) Agricultural and Biological Chemistry, 1983, 47 (3), 659-661 Not GLP, published	N	N		nr	N
KCA 5/51	Yokota, T. Baba, J. Takahashi, N.	1983	BRASSINOLIDE-RELATED BIOACTIVE STEROLS IN DOLICHOS LABLAB: BRASSINOLIDE, CASTASTERONE AND A NEW ANALOG HOMODOLICHOLIDE Report No.: na (092-073) Agricultural and Biological Chemistry, 1983, 47 (6), 1409-1411 Not GLP, published	N	N		nr	N
KCA 5/52	Yokota, T. Baba, J. Koba, S. Takahashi, N.	1984	PURIFICATION AND SEPARATION OF EIGHT STEROIDAL PLANT-GROWTH REGULATORS FROM DOLICHOS LABLAB SEED Report No.: na (092-075) Agricultural and Biological Chemistry, 1984, 48 (10), 2529-2534 Not GLP, published	N	N		nr	N
KCA 5/53	Abe, H. Takatsuto, S. Okuda, R. Yokota, T.	1995	IDENTIFICATION OF CASTASTERONE, 6-DEOXOCASTASTERONE, AND TYPHASTEROL IN THE POLLEN OF ROBINIA PSEUDO-ACACIA L. Report No.: na (092-007) Bioscience, Biotechnology and Biochemistry, 1995, 59 (2), 309-310 Not GLP, published	N	N		nr	N
KCA 5/54	Park, K.-H. Yokota, T. Sakurai, A. Takahashi, N.	1987	OCCURRENCE OF CASTASTERONE, BRASSINOLIDE AND METHYL 4-CHLOROINDOLE-3-ACETATE IN IMMATURE VICIA FABA SEEDS Report No.: na (092-044) Agricultural and Biological Chemistry, 1987, 51 (11), 3081-3086 Not GLP, published	N	N		nr	N
KCA 5/55	Yokota, T. Morita, M. Takahashi, N.	1983	6-DEOXOCASTASTERONE AND 6-DEOXODOLICHOSTERONE: PUTATIVE PRECURSORS FOR BRASSINOLIDE-RELATED STEROIDS FROM PHASEOLUS VULGARIS Report No.: na (092-074) Agricultural and Biological Chemistry, 1983, 47 (9), 2149-2151 Not GLP, published	N	N		nr	N
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KCA 5/57	Kim, S.-K. Yokota, T. Takahashi, N.	1987	25-METHYLDOLICHOSTERONE, A NEW BRASSINOSTEROID WITH A TERTIARY BUTYL GROUP FROM IMMATURE SEED OF PHASEOLUS VULGARIS Report No.: na (092-032) Agricultural and Biological Chemistry, 1987, 51 (8), 2303-2305 Not GLP, published	N	N		nr	N
KCA	Kim, T.-W.	2000	OCCURRENCE OF TEASTERONE AND TYPHASTEROL,	N	N		nr	N

5/58	Park, S.-H. Han, K.-S. Choo, J. Lee, J.S. Hwang, S. Kim, S.-K.		AND THEIR ENZYMATIC CONVERSION IN PHASEOLUS VULGARIS Report No.: na (092-035) Bulletin-Korean Chemical Society, 2000, 21 (4), 373-374 Not GLP, published					
KCA 5/59	Kim, S.-K.	1991	NATURAL OCCURRENCES OF BRASSINOSTEROIDS Report No.: na (092-033) ACS Symposium series, 1991, 474, Chapter 3, 26-35 Not GLP, published	N	N		nr	N
KCA 5/60	Park, S.C. Kim, T.-W. Kim, S.-K.	2000	IDENTIFICATION OF BRASSINOSTEROIDS WITH 24R-METHYL IN IMMATURE SEEDS OF PHASEOLUS VULGARIS Report No.: na (092-043) Bulletin-Korean Chemical Society, 2000, 21 (12), 1274-1276 Not GLP, published	N	N		nr	N
KCA 5/61	Nomura, T. Nakayama, M. Reid, J.B. Takeuchi, Y. Yokota, T.	1997	BLOCKAGE OF BRASSINOSTEROID BIOSYNTHESIS AND SENSITIVITY CAUSES DWARFISM IN GARDEN PEA Report No.: na (092-038) Plant Physiology, 1997, 113, 31-37 Not GLP, published	N	N		nr	N
KCA 5/62	Nomura, T. Kitasaka, Y. Takatsuto, S. Reid, J.B. Fukami, M. Yokota, T.	1999	BRASSINOSTEROID/STEROL SYNTHESIS AND PLANT GROWTH AS AFFECTED BY IKA AND IKB MUTATIONS OF PEA Report No.: na (092-039) Plant Physiology, 1999, 119, 1517-1527 Not GLP, published	N	N		nr	N
KCA 5/63	Sondhi, N. Bhardwaj, R. Kaur, S. Kumar, N. Singh, B.	2008	ISOLATION OF 24-EPIBRASSINOLIDE FROM LEAVES OF AEGLE MARMELOS AND EVALUATION OF ITS ANTIGENOTOXITIY EMPLOYING ALLIUM CEPA CHROMOSOMAL ABERRATION ASSAY Report No.: na (092-154) Plant Growth Regulation, 2008, 54, 217-224; DOI: 10.1007/s10725-007-9242-7 Not GLP, published	N	N		nr	N
KCA 5/64	Motegi, C. Takatsuto, S.	1994	IDENTIFICATION OF BRASSINOLIDE AND CASTASTERONE IN THE POLLEN OF ORANGE (CITRUS SINENSIS OSBECK) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY Report No.: na (092-037) Journal of Chromatography A, 1994, 658, 27-30 Not GLP, published	N	N		nr	N
KCA 5/65	Abe, H. Morishita, T. Uchiyama, M. Takatsuto, S. Ikekawa, N.	1984	A NEW BRASSINOLIDE-RELATED STEROID IN THE LEAVES OF THEA SINENSIS Report No.: na (092-003) Agricultural and Biological Chemistry, 1984, 48 (8), 2171-2172 Not GLP, published	N	N		nr	N
KCA 5/66	Gupta, D. Bhardwaj, R. Nagar, P.K. Kaur, S.	2004	ISOLATION AND CHARACTERIZATION OF BRASSINOSTEROIDS FROM LEAVES OF CAMELLIA SINENSIS (L.) O. KUNTZE Report No.: na (092-153) Plant Growth Regulation, 2004, 43, 97-100 Not GLP, published	N	N		nr	N
KCA 5/67	Choi, Y.-H. Inoue, T. Fujioka, S. Saimoto, H. Sakurai, A.	1993	IDENTIFICATION OF BRASSINOSTEROID-LIKE ACTIVE STUBSTANCES IN PLANT-CELL CULTURES Report No.: na (092-016) Bioscience, Biotechnology and Biochemistry, 1993, 57 (5), 860-861 Not GLP, published	N	N		nr	N
KCA 5/68	Fujioka, S. Inoue, T. Takatsuto, S.	1995	IDENTIFICATION OF A NEW BRASSINOSTEROID, CATHASTERONE, IN CULTURED CELLS OF CATHARANTHUS ROSEUS AS A BIOSYNTHETIC	N	N		nr	N

	Yanagisawa, T. Yokota, T. Sakurai, A.		PRECURSOR OF TEASTERONE Report No.: na (092-017) Bioscience, Biotechnology and Biochemistry, 1995, 59 (8), 1543-1547 Not GLP, published					
KCA 5/69	Park, K.-H. Saimoto, H. Nakagawa, S. Sakurai, A. Yokota, T. Takahashi, N. Syono, K.	1989	OCCURRENCE OF BRASSINOLIDE AND CASTASTERONE IN CROWN GALL CELLS OF CATHARANTHUS ROSEUS Report No.: na (092-045) Agricultural and Biological Chemistry, 1989, 53 (3), 805-811 Not GLP, published	N	N		nr	N
KCA 5/70	Suzuki, H. Fujioka, S. Takatsuto, S. Yokota, T. Murofushi, N. Sakurai, A.	1995	BIOSYNTHESIS OF BRASSINOSTEROIDS IN SEEDLINGS OF CATHARANTHUS ROSEUS, NICOTIANA TABACUM, AND ORYZA SATIVA Report No.: na (092-056) Bioscience, Biotechnology and Biochemistry, 1995, 59 (2), 168-172 Not GLP, published	N	N		nr	N
KCA 5/71	Yokota, T. Ogino, Y. Takahashi, N. Saimoto, H. Fujioka, S. Sakurai, A.	1990	BRASSINOLIDE IS BIOSYNTHESIZED FROM CASTASTERONE IN CATHARANTHUS ROSEUS CROWN GALL CELLS Report No.: na (092-077) Agricultural and Biological Chemistry, 1990, 54 (4), 1107-1108 Not GLP, published	N	N		nr	N
KCA 5/72	Takatsuto, S. Yokota, T. Omote, K. Gamor, K. Takahashi, N.	1989	IDENTIFICATION OF BRASSINOLIDE, CASTASTERONE AND NORCASTASTERONE (BRASSINONE) IN SUNFLOWER (HELIANTHUS ANNUUS L.) POLLEN Report No.: na (092-058) Agricultural and Biological Chemistry, 1989, 53 (8), 2177-2180 Not GLP, published	N	N		nr	N
KCA 5/73	Yamamoto, R. Fujioka, S. Demura, T. Takatsuto, S. Yoshida, S. Fukuda, H.	2001	BRASSINOSTEROID LEVELS INCREASE DRASTICALLY PRIOR TO MORPHOGENESIS OF TRACHEARY ELEMENTS Report No.: na (092-066) Plant Physiology, 2001, 125, 556-563 Not GLP, published	N	N		nr	N
KCA 5/74	Suzuki, Y. Yamaguchi, I. Takahasi, N.	1985	IDENTIFICATION OF CASTASTERONE AND BRASSINONE FROM IMMATURE SEEDS OF PHARBITIS PURPUREA Report No.: na (092-052) Agricultural and Biological Chemistry, 1985, 49 (1), 49-54 Not GLP, published	N	N		nr	N
KCA 5/75	Jang, M.-S. Han, K.-S. Kim, S.-K.	2000	IDENTIFICATION OF BRASSINOSTEROIDS AND THEIR BIOSYNTHETIC PRECURSORS FROM SEEDS OF PUMPKIN Report No.: na (092-028) Bulletin-Korean Chemical Society, 2000, 21 (2), 161-164 Not GLP, published	N	N		nr	N
KCA 5/76	Tripathi, S. Sharma, P.	2015	CHARACTERIZATION OF BRASSINOSTEROID ISOLATED FROM BACOPA MONNIERI L. AND THEIR FREE RADICAL SCAVENGING ACTIVITY Report No.: na (092-156) International Journal of Science and Research (IJSR), 2015, 4 (4), 2738-2742 Not GLP, published	N	N		nr	N
KCA 5/77	Yokota, T. Nomura, T. Nakayama, M.	1997	IDENTIFICATION OF BRASSINOSTEROIDS THAT APPEAR TO BE DERIVED FROM CAMPESTEROL AND CHOLESTEROL IN TOMATO SHOOTS Report No.: na (092-070) Plant & Cell Physiology, 1997, 38 (11), 1291-1294 Not GLP, published	N	N		nr	N

KCA 5/78	Bishop, G.J. Nomura, T. Yokota, T. Harrison, k. Noguchi, T. Fujioka, S. Takatsuto, S. Jones, J.D.G. Kamiya, Y.	1999	THE TOMATO DWARF ENZYME CATALYSES C-6 OXIDATION IN BRASSINOSTEROID BIOSYNTHESIS Report No.: na (092-014) Proceedings of the National Academy of Sciences, 1999, 96, 1761-1766 Not GLP, published	N	N		nr	N
KCA 5/79	Griffiths, P.G. Sasse, J.M. Yokota, T. Cameron, D.W.	1995	6-DEOXOTYPHASTEROL AND 3-DEHYDRO-6-DEOXOTEASTERONE, POSSIBLE PRECURSORS TO BRASSINOSTEROIDS IN THE POLLEN OF CUPRESSUS ARIZONICA Report No.: na (092-021) Bioscience, Biotechnology and Biochemistry, 1995, 59 (5), 956-959 Not GLP, published	N	N		nr	N
KCA 5/80	Takatsuto, S. Abe, H. Shimada, K. Nakayama, M. Yokota, T.	1996	IDENTIFICATION OF TEASTERONE AND 4-DESMETHYLSTEROLS IN THE SEEDS OF GINKGO BILOBA L. Report No.: na (092-061) Japan Oil Chemists' Society, 1996, 45 (12), 1349-1351 Not GLP, published	N	N		nr	N
KCA 5/81	Kim, S.-K. Abe, H. Anthony Little, C.H. Pharis, R.P.	1990	IDENTIFICATION OF TWO BRASSINOSTEROIDS FROM THE CAMBIAL REGION OF SCOTS PINE (PINUS SILVERSTRIS) BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY, AFTER DETECTION USING A DWARF RICE LAMINA INCLINATION BIOASSAY Report No.: na (092-031) Plant Physiology, 1990, 94, 1709-1713 Not GLP, published	N	N		nr	N
KCA 5/82	Yokota, T. Arima, M. Takahashi, N. Takatsuto, S. Ikekawa, N. Takematsu, T.	1983	2-DEOXYCASTASTERONE, A NEW BRASSINOLIDE-RELATED BIOACTIVE STEROID FROM PINUS POLLEN Report No.: na (092-071) Agricultural and Biological Chemistry, 1983, 47 (10), 2419-2420 Not GLP, published	N	N		nr	N
KCA 5/83	Yokota, T. Higuchi, K. Takahashi, N. Kamuro, Y. Watanabe, T. Takatsuto, S.	1998	IDENTIFICATION OF BRASSINOSTEROIDS WITH EPIMERIZED SUBSTITUENTS AND / OR THE 23-OXO GROUP IN POLLEN AND ANTHERS OF JAPANESE CEDAR Report No.: na (092-068) Bioscience, Biotechnology and Biochemistry, 1998, 62 (3), 526-531 Not GLP, published	N	N		nr	N
KCA 5/84	Watanabe, T. Yokota, T. Shibata, K. Nomura, T. Seto, H. Takatsuto, S.	2000	CRYPTOLIDE, A NEW BRASSINOLIDE CATABOLITE WITH A 23-OXO GROUP FROM JAPANESE CEDAR POLLEN/ANTHER AND ITS SYNTHESIS Report No.: na (092-065) Journal of Chemical Research (S), 2000, 18-19 Not GLP, published	N	N		nr	N
KCA 5/85	Park, S.-H. Han, K.-S. Kim, T.-W. Shim, J.-K. Takatsuto, S. Yokota, T. Kim, S.-K.	1999	IN VIVO AND IN VITRO CONVERSION OF TEASTERONE TO TYPHASTEROL IN CULTURED CELLS OF MARCHANTIA POLYMORPHA Report No.: na (092-042) Plant & Cell Physiology, 1999, 40 (9), 955-960 Not GLP, published	N	N		nr	N
KCA 5/86	Yokota, T. Ohnishi, T. Shibata, K. Asahina, M. Nomura, T. Fujita, T. Ishizaki, K. Kohchi, T.	2017	OCCURRENCE OF BRASSINOSTEROIDS IN NON-FLOWERING LAND PLANTS, LIVERWORT, MOSS, LYCOPHYTE AND FERN Report No.: na (092-069) Phytochemistry, 2017, xxx, 1-10; doi: 10.1016/j.phytochem.2016.12.020 Not GLP, published	N	N		nr	N

KCA 5/87	Stirk, W.A. Balint, P. Tarkowska, D. Novak, O. Strnad, M. Oerdoeg, V. van Staden, J.	2013	HORMONE PROFILES IN MICROALGAE: GIBBERELLINS AND BRASSINOSTEROIDS Report No.: na (092-051) Plant Physiology and Biochemistry, 2013, 70, 348-353; doi: 10.1016/j.plaphy.2013.05.037 Not GLP, published	N	N		nr	N
KCA 5/88	Bajguz, A.	2009	ISOLATION AND CHARACTERIZATION OF BRASSINOSTEROIDS FROM ALGAL CULTURES OF CHLORELLA VULGARIS BEIJERINCK (TREBOUXIOPHYCEAE) Report No.: na (092-013) Journal of Plant Physiology, 2009, 166, 1946-1949; doi:10.1016/j.jplph.2009.05.003 Not GLP, published	N	N		nr	N
KCA 5/89	Hamdy, A.-H. A. Aboutabl, E.A. Sameer, S. Hussein, A.A. Diaz-Marrero, A.R. Darias, J. Cueto, M.	2009	3-KETO-22-EPI-28-NOR-CATHASTERONE, A BRASSINOSTEROID-RELATED METABOLITE FROM CYSTOSEIRA MYRICA Report No.: na (092-023) Steroids, 2009, 74, 927-930; doi: 10.1016/j.steroids.2009.06.008 Not GLP, published	N	N		nr	N
KCA 5/90	Tsavkelova, E.A. Klimova, S.Y. Cherdyntseva, T.A. Netrusov, A.I.	2006	HORMONES AND HORMONE-LIKE SUBSTANCES OF MICROORGANISMS: A REVIEW Report No.: na (092-064) Applied Biochemistry and Microbiology, 2006, 42 (3), 229-235 Not GLP, published	N	N		nr	N
KCA 5/91	Bajguz, A. Hayat, S.	2009	EFFECTS OF BRASSINOSTEROIDS ON THE PLANT RESPONSES TO ENVIRONMENTAL STRESSES Report No.: na (092-133) Plant Physiology and Biochemistry, 2009, 47, 1-8; doi:10.1016/j.plaphy.2008.10.002 Not GLP, published	N	N		nr	N
KCA 5/92	Eremina, M. Unterholzner, S.J. Rathnayake, A.I. Castellanos, M. Khan, M. Kugler, K.G. May, S.T. Mayer, K.F.X. Rozhon, W. Poppenberger, B.	2016	BRASSINOSTEROIDS PARTICIPATE IN THE CONTROL OF BASAL AND ACQUIRED FREEZING TOLERANCE OF PLANTS Report No.: na (092-136) Proceedings of the National Academy of Sciences, 2016, 113 (40), E5982-E5991 Not GLP, published	N	N		nr	N
KCA 5/93	Aremu, A.O. Stirk, W.A. Kulkarni, M.G. Tarkowska, D. Tureckova, V. Gruz, J. Subrtova, M. Pencik, A. Novak, O. Dolezal, K. Strnad, M. Van Staden, J.	2015	EVIDENCE OF PHYTOHORMONES AND PHENOLIC ACIDS VARIABILITY IN GARDEN-WASTE-DERIVED VERMICOMPOST LEACHATE, A WELL-KNOWN PLANT GROWTH STIMULANT Report No.: na (092-158) Plant Growth Regulation, 2015, 75 (2), 483-492; DOI: 10.1007/s10725-014-0011-0 Not GLP, published	N	N		nr	N
KCA 5/94	Badri, D.V. Vivanco, J.M.	2009	REGULATION AND FUNCTION OF ROOT EXUDATES Report No.: na (092-012) Plant, Cell and Environment, 2009, 32, 666-681; doi: 10.1111/j.1365-3040.2009.01926.x Not GLP, published	N	N		nr	N
KCA 5/95	Hassett, J.P. Fred Lee, G. Lee, F.G.	1977	STEROLS IN NATURAL WATER AND SEDIMENT Report No.: na (092-168) Water Research, 1977, 11, 983-989 Not GLP, published	N	N		nr	N
KCA 5/96	Mudge, S.M. Joao A.F.	1999	STEROLS IN THE RIA FORMOSA LAGOON, PORTUGAL Report No.: na (092-169)	N	N		nr	N

	Bebianno, M. East, J.A. Barreira, L.A.		Water Research, 1999, 33 (4), 1038-1048 Not GLP, published					
KCA 5/97	Nishikawa, N. Toyama, S. Shida, A. Futatsuya, F.	1994	THE UPTAKE AND THE TRANSPORT OF 14C-LABELED EPIBRASSINOLIDE IN INTACT SEEDLINGS OF CUCUMBER AND WHEAT Report No.: na (092-088) Journal of Plant Research, 1994, 107, 125-130 Not GLP, published	N	N		nr	N
KCA 5/98	Mekhalfi, M. Avilan, L. Lebrun, R. Botebol, H. Gontero, B.	2012	CONSEQUENCES OF THE PRESENCE OF 24-EPIBRASSINOLIDE, ON CULTURES OF A DIATOM, ASTERIONELLA FORMOSA Report No.: na (092-109) Biochimie, 2012, 94, 1213-1220; doi: 10.1016/j.biochi.2012.02.011 Not GLP, published	N	N		nr	N
KCA 5/99	Vorbrodt, H.-M. Adam, G. Porzel, A. Hoerhold, C. Daenhardt, S. Boehme, K.-H.	1991	MICROBIAL DEGRADATION OF 2 ALPHA, 3 ALPHA-DIHYDROXY-5 ALPHA-CHOLESTAN-6-ONE BY MYCOBACTERIUM VACCAE Report No.: na (092-157) Steroids, 1991, 56, 586-588 Not GLP, published	N	N		nr	N
KCA 5/100	Voigt, B. Porzel, A. Naumann, H. Hoerhold-Schubert, C. Adam, G.	1993	HYDROXYLATION OF THE NATIVE BRASSINOSTEROIDS 24-EPICASTASTERONE AND 24-EPIBRASSINOLIDE BY THE FUNGUS CUNNINGHAMELLA ECHINULATA Report No.: na (092-096) Steroids, 1993, 58, 320-323 Not GLP, published	N	N		nr	N
KCA 5/101	Saygideger, S. Deniz, F.	2008	EFFECT OF 24-EPIBRASSINOLIDE ON BIOMASS, GROWTH AND FREE PROLINE CONCENTRATION IN SPIRULINA PLATENSIS (CYNOPHYTA) UNDER NaCl STRESS Report No.: na (092-176) Plant Growth Regulation, 2008, 56, 219-223; DOI: 10.1007/s10725-008-9310-7 Not GLP, published	N	N		nr	N
KCA 5/102	Asari, S. Tarkowska, D. Rolcik, J. Novak, O. Palmero, D.V. Bejai, S. Meijer, J.	2017	ANALYSIS OF PLANT GROWTH-PROMOTING PROPERTIES OF BACILLUS AMYLOLIQUEFACIENS UCMB5113 USING ARABIDOPSIS THALIANA AS HOST PLANT Report No.: na (092-181) Planta, 2017, 245, 15-30; DOI: 10.1007/s00425-016-2580-9 Not GLP, published	N	N		nr	N
KCA 5/114	Wildemann, T	2015	QSAR ANALYSIS ON THE TOXICOLOGICAL SIMILARITIES BETWEEN 24-EPIBRASSINOLIDE AND HOMOBRASSINOLIDE Report No.: na (092-129) Not GLP, not published	N	Y	nr	Suntton GmbH	N
KCA 5/115	Wildemann, T Roth T	2015	QSAR ANALYSIS ON THE TOXICOLOGICAL SIMILARITIES BETWEEN 24-EPIBRASSINOLIDE AND ITS IMPURITIES [redacted] Report No.: na (092-128) Not GLP, not published	N	Y	nr	Suntton GmbH	N
KCA 5.1/01	Bajguz, A.	2011	SUPPRESSION OF CHLORELLA VULGARIS GROWTH BY CADMIUM, LEAD, AND COPPER STRESS AND ITS RESTORATION BY ENDOGENOUS BRASSINOLIDE Report No.: na (092-103) Archives of Environmental Contamination and Toxicology, 2011, 60, 406-416; DOI 10.1007/s00244-010-9551-0 Not GLP, published	N	N		nr	N
KCA 5.1/02	Zhu, J.-Y. Sae-Seaw, J. Wang, Z.-Y.	2013	BRASSINOSTEROID SIGNALLING Report No.: na (092-165) Development, 2013, 140(8), 1615-1620; doi: 10.1242/dev.060590 Not GLP, published	N	N		nr	N

KCA 5.1/03	Khripach, V. Zhabinskii, V. De Groot, A.	2000	TWENTY YEARS OF BRASSINOSTEROIDS: STEROIDAL PLANT HORMONES WARRANT BETTER CROPS FOR THE XXI CENTURY Report No.: na (092-029) Annals of Botany, 2000, 86, 441-447; doi:10.1006/anbo.2000.1227 Not GLP, published	N	N		nr	N
KCA 5.1/04	Khripach, V.A. Sviridov, O.V. Pryadko, A.G. Litvinovskaya, R.P. Drach, S.V. Matveentsev, V.D. Novik, T.V. Mikhailopulo, K.I. Zhabinskii, V.N. Zavadskaya, M.I. Aver'kova, M.A. Drachenova, O.A. Chashchina, N.M.	2007	ENZYME IMMUNOASSAY OF (24R)-BRASSINOSTEROIDS Report No.: na (092-119) Russian Journal of Bioorganic Chemistry, 2007, 33 (3), 347-353; DOI: 10.1134/S1068162007030120 Not GLP, published	N	N		nr	N
KCA 5.1/05	Zhabinskii, V.N. Khripach, N.B. Khripach, V.A.	2015	STEROID PLANT HORMONES: EFFECTS OUTSIDE PLANT KINGDOM Report No.: na (092-099) Steroids, 2015, 97, 87-97; doi: 10.1016/j.steroids.2014.08.025 Not GLP, published	N	N		nr	N
KCA 5.1/06	Sauchuk, A.L. Litvinovskaya, R.P. Nasek, V. Sanko-Schislenok, E.	2016	24-EPIBRASSINOLIDE PHARMACOKINETIC STUDIES Report No.: na (092-124) 23rd Conference on Isoprenoids and National Academy of Sciences of Belarus, Chemical Series 2016, N 3 (Oral Communications P51-52) Not GLP, published	N	N		nr	N
KCA 5.1/07	Dzichenka, Y.V. Usanov, S.A.	2016	INTERACTION OF HUMAN STEROID 7ALPHA-HYDROXYLASES WITH BRASSINOSTEROIDS Report No.: na (092-116) 23rd Conference on Isoprenoids and National Academy of Sciences of Belarus, Chemical Series 2016, N 3, 1-128 (Oral Communications P43-44) Not GLP, published	N	N		nr	N
KCA 5.1/08	Bajguz, A.	2009	ISOLATION AND CHARACTERIZATION OF BRASSINOSTEROIDS FROM ALGAL CULTURES OF CHLORELLA VULGARIS BEIJERINCK (TREBOUXIOPHYCEAE) Report No.: na (092-013) Journal of Plant Physiology, 2009, 166, 1946-1949; doi:10.1016/j.jplph.2009.05.003 Not GLP, published	N	N		nr	N
KCA 5.1/09	Shibata, S. Hayakawa, K. Egashira, Y. Sanada, H.	2007	HYPOCHOLESTEROLEMIC MECHANISM OF CHLORELLA: CHLORELLA AND ITS INDIGESTIBLE FRACTION ENHANCE HEPATIC CHOLESTEROL CATABOLISM THROUGH UP-REGULATION OF CHOLESTEROL 7?-HYDROXYLASE IN RATS Report No.: na (092-151) Bioscience, Biotechnology Biochemistry, 2007, 71 (4), 916–925 Not GLP, published	N	N		nr	N
KCA 5.2/01	[redacted]	2017	ACUTE ORAL TOXICITY STUDY IN RATS WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6113 (521-001) [redacted] GLP, unpublished	Y	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH	N
KCA 5.2/02	[redacted]	2017	ACUTE DERMAL TOXICITY STUDY IN RATS WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6114 (522-001)	Y	Y	New study necessary for the approval	Suntton Gmb	N

			[redacted] GLP, unpublished			of 24-Epibrassinolide	H	
KCA 5.2/03	[redacted]	2016	ACUTE INHALATION TOXICITY STUDY IN RATS WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6118 (520-001) [redacted] GLP, unpublished	Y	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH	N
KCA 5.2/04	[redacted]	2017	ACUTE DERMAL IRRITATION/CORROSION STUDY IN RABBITS WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6115 (565-001) [redacted] GLP, unpublished	Y	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH	N
KCA 5.2/05	[redacted]	2017	ACUTE EYE IRRITATION/CORROSION STUDY IN RABBITS WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6116 (566-001) [redacted] GLP, unpublished	Y	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH	N
KCA 5.2/06	[redacted]	2017	CONTACT HYPERSENSITIVITY IN ALBINO GUINEA PIGS, MAXIMIZATION TEST (MAGNUSSON AND KLIGMAN METHOD) WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6117 (567-001) [redacted] GLP, unpublished	Y	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH	N
KCA 5.2/07	Khripach, V. Zhabinskii, V. De Groot, A.	2000	TWENTY YEARS OF BRASSINOSTEROIDS: STEROIDAL PLANT HORMONES WARRANT BETTER CROPS FOR THE XXI CENTURY Report No.: na (092-029) Annals of Botany, 2000, 86, 441-447; doi:10.1006/anbo.2000.1227 Not GLP, published	N	N		nr	N
KCA 5.2/08	Ikekawa, N. Zhao, Y.-J.	1991	APPLICATION OF 24-EPIBRASSINOLIDE IN AGRICULTURE Report No.: na (092-026) ACS Symposium series, 1991, 474, Chapter 24, 280-291 Not GLP, published	N	N		nr	N
KCA 5.2.1/01	[redacted]	2017	ACUTE ORAL TOXICITY STUDY IN RATS WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6113 (521-001) [redacted] GLP, unpublished	Y	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH	N
KCA 5.3/01	Fujioka, S. Yokota, T.	2003	BIOSYNTHESIS AND METABOLISM OF BRASSINOSTEROIDS Report No.: na (092-082) Annual Review of Plant Biology, 2003, 54, 137-164 Not GLP, published	N	N		nr	N
KCA 5.3/02	Sasse, J.M.	1989	USING PEST TO STUDY THE INTERACTIONS OF BRASSINOLIDE AND OTHER PLANT GROWTH REGULATORS Report No.: na (092-163) Proceedings of the Plant Growth Regulator Society of America, 16th Annual Meeting, 1989, 82-87 Not GLP, published	N	N		nr	N
KCA 5.3/03	Schmidt, J. Altmann, T. Adam, G.	1997	BRASSINOSTEROIDS FROM SEEDS OF ARABIDOPSIS THALIANA Report No.: na (092-048) Phytochemistry, 1997, 45 (7), 1325-1327 Not GLP, published	N	N		nr	N
KCA 5.3.2/01	[redacted]	2016	REPEATED DOSE 90 DAYS ORAL TOXICITY STUDY IN RAT WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6120 (530-001) [redacted]	Y	Y	New study necessary for the approval of 24-	Suntton GmbH	N

			GLP, unpublished			Epibrassinolide		
KCA 5.3.2/02	Bajguz, A.	2011	SUPPRESSION OF CHLORELLA VULGARIS GROWTH BY CADMIUM, LEAD, AND COPPER STRESS AND ITS RESTORATION BY ENDOGENOUS BRASSINOLIDE Report No.: na (092-103) Archives of Environmental Contamination and Toxicology, 2011, 60, 406-416; DOI 10.1007/s00244-010-9551-0 Not GLP, published	N	N		nr	N
KCA 5.4/01	Srilatha, S.	2017	BACTERIAL REVERSE MUTATION ASSAY WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6119 (557-001) RCC Laboratories India Private Limited, Hyderabad, India GLP, unpublished	N	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH	N
KCA 5.4/02	Wollny, H.-E.	2017	24-EPIBRASSINOLIDE (TGAI): GENE MUTATION ASSAY IN CHINESE HAMSTER V79 CELLS IN VITRO (V79/HPRT) Report No.: 1775001 (557-002) Envigo CRS GmbH, Rossdorf, Germany GLP, unpublished	N	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH	N
KCA 5.4/03	Kandula, S.R.	2017	IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST USING HUMAN PERIPHERAL BLOOD LYMPHOCYTES WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6121 (557-003) RCC Laboratories India Private Limited, Hyderabad, India GLP, unpublished	N	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH Sunergist Co., Ltd.	N
KCA 5.4.1/04	Zhabinskii, V.N. Khripach, N.B. Khripach, V.A.	2015	STEROID PLANT HORMONES: EFFECTS OUTSIDE PLANT KINGDOM Report No.: na (092-099) Steroids, 2015, 97, 87-97; doi: 10.1016/j.steroids.2014.08.025 Not GLP, published	N	N		nr	N
KCA 5.4.2/01	Khripach, V.A. Sviridov, O.V. Pryadko, A.G. Litvinovskaya, R.P. Drach, S.V. Matveentsev, V.D. Novik, T.V. Mikhailopulo, K.I. Zhabinskii, V.N. Zavadskaya, M.I. Aver'kova, M.A. Drachenova, O.A. Chashchina, N.M.	2007	ENZYME IMMUNOASSAY OF (24R)-BRASSINOSTEROIDS Report No.: na (092-119) Russian Journal of Bioorganic Chemistry, 2007, 33 (3), 347-353; DOI: 10.1134/S1068162007030120 Not GLP, published	N	N		nr	N
KCA 5.4.2/02	Khripach, V. Zhabinskii, V. De Groot, A.	2000	TWENTY YEARS OF BRASSINOSTEROIDS: STEROIDAL PLANT HORMONES WARRANT BETTER CROPS FOR THE XXI CENTURY Report No.: na (092-029) Annals of Botany, 2000, 86, 441-447; doi:10.1006/anbo.2000.1227 Not GLP, published	N	N		nr	N
KCA 5.5/01	Zhabinskii, V.N. Khripach, N.B. Khripach, V.A.	2015	STEROID PLANT HORMONES: EFFECTS OUTSIDE PLANT KINGDOM Report No.: na (092-099) Steroids, 2015, 97, 87-97; doi: 10.1016/j.steroids.2014.08.025 Not GLP, published	N	N		nr	N
KCA 5.5/02	Khripach, V.A. Sviridov, O.V. Pryadko, A.G. Litvinovskaya, R.P. Drach, S.V. Matveentsev,	2007	ENZYME IMMUNOASSAY OF (24R)-BRASSINOSTEROIDS Report No.: na (092-119) Russian Journal of Bioorganic Chemistry, 2007, 33 (3), 347-353; DOI: 10.1134/S1068162007030120 Not GLP, published	N	N		nr	N

	V.D. Novik, T.V. Mikhailopulo, K.I. Zhabinskii, V.N. Zavadskaya, M.I. Aver'kova, M.A. Drachenova, O.A. Chashchina, N.M.							
KCA 5.5/03	Bajguz, A.	2009	ISOLATION AND CHARACTERIZATION OF BRASSINOSTEROIDS FROM ALGAL CULTURES OF CHLORELLA VULGARIS BEIJERINCK (TREBOUXIOPHYCEAE) Report No.: na (092-013) Journal of Plant Physiology, 2009, 166, 1946-1949; doi:10.1016/j.jplph.2009.05.003 Not GLP, published	N	N		nr	N
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KCA 5.8.3/ 01	Carange, J. Longpre, F. Daoust, B. Martinoli, M.-G.	2011	24-EPIBRASSINOLIDE, A PHYTOSTEROL FROM THE BRASSINOSTEROID FAMILY, PROTECTS DOPAMINERGIC CELLS AGAINST MPP+-INDUCED OXIDATIVE STRESS AND APOPTOSIS Report No.: na (092-104) Journal of Toxicology, 2011, Article ID 392859, 1-13; doi:10.1155/2011/392859 Not GLP, published	N	N		nr	N
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KCA 5.9.2/ 07	Komosinska-Vassev, K. Olczyk, P. Kazmierczak, J. Mencner, L. Olczyk, K.	2015	BEE POLLEN: CHEMICAL COMPOSITION AND THERAPEUTIC APPLICATION Report No.: na (092-120) Evidence-Based Complementary and Alternative Medicine, 2015, Article ID 297425, 1-6; DOI: 10.1155/2015/297425 Not GLP, published	N	N		nr	N
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na = not applicable / ni = not indicated / nr = not relevant