European Commission

Draft Assessment Report prepared according to the Commission Regulation (EU) N° 1107/2009

24-Epibrassinolide

Volume 3 – B.7 (AS)

Rapporteur Member State : Austria

Version History

When	What
2018/05	Initial DAR

Table of contents

B.7. RESIDUE DATA 4

B.7.1. STORAGE STABILITY OF RESIDUES 9

B.7.2. METABOLISM, DISTRIBUTION AND EXPRESSION OF RESIDUES 10

B.7.2.1. Plants 10
B.7.2.2. Poultry 64
B.7.2.3. Lactating ruminants 64
B.7.2.4. Pigs 64
B.7.2.5. Fish 64

B.7.3. MAGNITUDE OF RESIDUE TRIALS IN PLANTS 65

B.7.4. FEEDING STUDIES 68

B.7.4.1. Poultry 68
B.7.4.2. Ruminants 68
B.7.4.3. Pigs 68
B.7.4.4. Fish 68

B.7.5. EFFECTS OF PROCESSING 69

B.7.5.1. Nature of the residue 69
B.7.5.2. Distribution of the residue in peel and pulp 69
B.7.5.3. Magnitude of residues in processed commodities 69

B.7.6. RESIDUES IN SUCCEEDING OR ROTATIONAL CROPS 70

B.7.6.1. Metabolism in rotational crops 70
B.7.6.2. Magnitude of residues in rotational crops 70

B.7.7. OTHER STUDIES 71

B.7.7.1. Effect on the residue level in pollen and bee products 71

B.7.8. REFERENCES RELIED ON 72

B.7. RESIDUE DATA

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 7.2.1-1). 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 7.2.1-1). 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 polyhydroxysteroids, 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 7-1).

Table 7-1 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

No. of carbon	Type of B-ring	Substituent in A-ring	Representative(s)
		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 7-1).

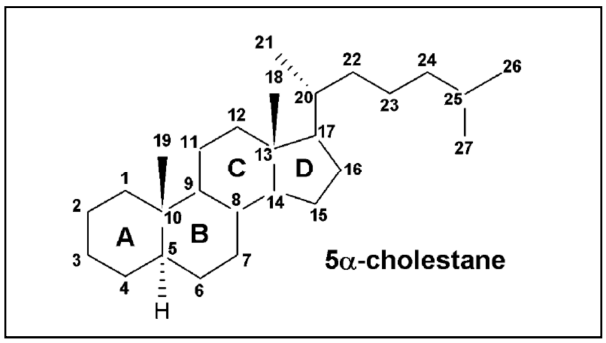
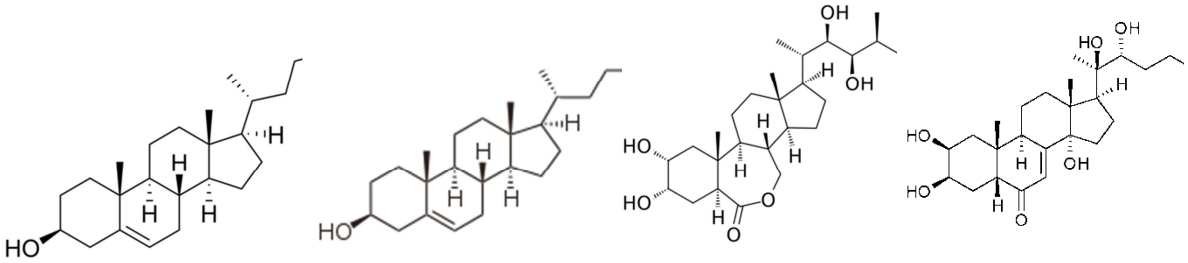


Figure 7-1: 5α-cholestane as the skeleton of Brassinosteroid synthesis. Structural differences occur in rings A and B, and in substituents in the side chain $C_{20} – C_{27}$.

Campesterol (Figure 7-2) derives from 5α-cholestane and, with its attached alcohol group, chemically represents a sterol, like e.g. the animal sterol cholesterol or the insect derived molting hormone ecdysterone. 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).



Campesterol Cholesterol Brassinolide Ecdysteron

Figure 7-2: 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).

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 leafs (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 7.2.1-1) and thus 24-Epibrassinolide has no relevant toxicity hazard towards humans.

The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) has concluded that plant sterols (which includes 24-Epibrassinolide) are not only of low risk for the human consumer but necessary for a healthy diet as they are contributing to lowering the LDL-cholesterol levels, which is pivotal for the prevention of coronary heart diseases. Therefore, a daily intake of up to 3 g of plant sterols per day is highly recommended by EFSA (2012) (see chapter B.6.9.4, Epidemiological studies, Section Toxicology).

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*., 2014) 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. Vorbrodt *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 COMMISSION REGULATION (EU) 2017/1432 of 7 August 2017 amending Regulation (EC) No 1107/2009 of the European Parliament and the Council concerning the placing of plant protection products on the market as regards the criteria for the approval of low-risk active substances.

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.

Annex IV of Regulation (EC) 396/2005 of the European Parliament and of the Council on Maximum Residue Levels for pesticides contains a list of active substances for which maximum residue levels (MRLs) are not required. The criteria are listed in SANCO/11188/2013 Rev. 2, 14 September 2015: Guidance document on criteria for the inclusion of active substances into Annex IV of Regulation (EC) N° 396/2005.

Active substances of no toxicological concerns Criterion 3: This group consists of the active substances that fulfill all of the following criteria: ADI and ARfD are not needed, they are low risk substances [in the meaning of point 5 of Annex II of Regulation (EC) No 1107/2009 i.e., do not show any of the following properties: carcinogenic, mutagenic, toxic to reproduction, sensitising chemicals, very toxic or toxic, corrosive, endocrine disruptor, neurotoxic, immunotoxic] and they do not produce any adverse effect up to test guideline limit doses. In that case, no more information on residues in registered uses in EU is necessary.

Natural exposure is higher than the one linked to the use as PPP (criterion 4): substances which exhibit a higher natural background level in food than is expected from PPP use(s) (please see Table 7.2.1-1). Active substances for which exposure via usual diet is higher than the one through the use as PPP might also be eligible for the inclusion in Annex IV. This group also includes well-defined compounds, even if their toxicity is not negligible, if consumer exposure to the compound or its degradates via usual diet is higher than the one through use as PPP. In that case, no more information on residues in registered uses in EU is necessary.

24-Epibrassinolide has a very low toxicity profile and is ubiquitous distributed in the plant kingdom (please see Table 7.2.1-1) and therefore fulfils criterion 3 of SANCO/11188/2013 Rev. 2 of 14 September 2015: *The compound has no identified hazardous properties*. In addition, criterion 4 of SANCO/11188/2013 Rev. 2 of 14 September 2015: *Natural exposure is higher than the one linked to the use as PPP,* is met. Therefore, inclusion into Annex IV of Regulation (EC) N° 396/2005 is requested, as no maximum residue levels are required.

For the inclusion of the active substance 24-Epibrassinolide and the representative formulation Sunergist (0.01 % 24-Epibrassinolide) in Annex I, data to support the application for inclusion is provided in the following section. Studies, where available, are summarised under the respective data points. In some cases, public literature is used to address data points. In the case where published literature is used to address a data point, an extended summary of the published literature is provided and cited.

In the case where published literature is used to scientifically justify why a study was not deemed necessary to be conducted or as supporting information, only authors and year is given in the text, while full bibliographical information can be found in "Annex I: Publications to support evaluation" at the end of each section. Relevant

literature from the EFSA- compliant literature search, which has to be evaluated on full-text level, is discussed under the respective data point.

B.7.1. STORAGE STABILITY OF RESIDUES

Brassinosteroids, including 24-Epibrassinolide are naturally occurring plant constituents, present in higher plants, lower plants, including algae, mosses, the "living fossil" *Equisetum* (Table 7.2.1-1) as well as certain fungi. 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). It is therefore impossible to distinguish between a possible residue resulting from the use of the natural-identical active substance 24-Epibrassinolide and the similar natural substance ubiquitously present in the environment.

The concentration of Brassinosteroids in plants is regulated by a complex system of feedback pathways 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. Different homeostatic mechanisms, including feedback inhibition of Brassinosteroids and catabolic inactivation, play a role in the maintenance of the equilibrium of bioactive Brassinosteroid in plants. Brassinosteroids exogenously applied to shoots and leafs are metabolised within 24-96 h after application (Yokota *et al*. 1992; Winter *et al*., 1997 and Husar *et al*., 2011).

Furthermore, 24-Epibrassinolide is an implicit candidate for inclusion in Annex IV of Regulation (EC) 396/2005 as it fulfils criteria 3 and 4 of SANCO/11188/2013 Rev. 2, 14 September 2015. Therefore, no more information on residues in EU is necessary.

Studies on the stability of residues were therefore not conducted.

B.7.2. METABOLISM, DISTRIBUTION AND EXPRESSION OF RESIDUES

B.7.2.1. Plants

24-Epibrassinolide is an implicit candidate for inclusion in Annex IV of Regulation (EC) 396/2005 as it fulfils criteria 3 and 4 of SANCO/11188/2013 Rev. 2, 14 September 2015. Therefore, no more information on residues in EU is necessary.

Nevertheless, public literature data are described to address the data points.

Distribution and transport of Brassinosteroids in plants

Brassinosteroids, including 24-Epibrassinolide are naturally occurring plant constituents, found throughout the plant kingdom as well as some fungi (Table 7.2.1-1). 24-Epibrassinolide elicits and activates the plant's self-defence mechanisms meditating the plant's resistance to unfavourable environmental factors, stress and diseases.

Brassinosteroids are present in all plant organs such as pollens, 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 with 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 (Table 7.2.1-1). The contents of Brassinosteroids, which play a major role in fruit ripening in grapes, vary during the ripening process between 0.75 and 2.6 µg/kg fresh weight (Symons *et al*. 2006). Based on the results of their investigations in grapes, the authors conclude that Brassinosteroids may play a key role in fruit ripening of non-climacteric plants such as grapes, citrus and strawberries.

The concentration of Brassinosteroids in plants is regulated by a complex system of feedback pathways 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.

In addition, different homeostatic mechanisms, including feedback inhibition of Brassinosteroids and catabolic inactivation, play a role in the maintenance of the equilibrium of bioactive Brassinosteroid in plants. Brassinosteroid synthesis is also triggered for example by microorganisms. Asari *et al*. (2017) measured the Brassinosteroid levels in plants after inoculation with the rhizobacterium *Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5113. Brassinolide (approx.0.01 fg/mg FW*), castasterone (approx. 0.13 fg/mg FW*) and teasterone (approx. 3.5 fg/mg FW*) levels were significantly increased in the roots.

Table 7.2.1-1 is a summary table based on open literature and without any claim to completeness. It is to be expected that Brassinosteroids are also ubiquitous in other organisms not included in this table.

Table 7.2.1-1 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 (*Lolium perenne* L.)	pollen	BR (1)	0.001	Taylor *et al*., 1993[3]
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

Family/Species	Examined part	Brassino-steroid[1]	content µg/kg fr. wt.[2]	References
				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				
Hemp (*Cannabis sativa* L.)	seeds	CS	600	Takatsuto *et al*., 1996b

Family/Species	Examined part	Brassino-steroid[1]	content µg/kg fr. wt.[2]	References
	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, 2000a[3]; Nomura *et al*., 2001
	20-days-old	BR (5)	0.1-0.79	Bancos *et al.*, 2002

Family/Species	**Examined part**	**Brassino-steroid[1]**	**content µg/kg fr. wt.[2]**	**References**
	shoots			
	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
	breaking wall	24-epiBL	628	Pan *et al*., 2012

Family/Species	Examined part	Brassino-steroid[1]	content µg/kg fr. wt.[2]	References
	pollen			
	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, 1987c ; 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

Family/Species	Examined part	Brassino-steroid[1]	content µg/kg fr. wt.[2]	References
	shoot	BR (6)	0.047-5.2	Nomura *et al*., 1997, 1999, 2001
	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]

Family/Species	Examined part	Brassino-steroid[1]	content µg/kg fr. wt.[2]	References
Orange (*Citrus sinensis* Osbeck)	pollen	BL	36.2	Motegi *et al*., 1994
	pollen	CS	29.4	Motegi *et al*., 1994
Theaceae				
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]

Family/Species	Examined part	Brassino-steroid[1]	content µg/kg fr. wt.[2]	References
Convolvulaceae				
Pharbitis purpurea Voigt	seeds	CS	1.1	Suzuki *et al*., 1985
	seeds	BR (1)	0.2	Suzuki *et al*., 1985
Cucurbitaceae				
Cucurbita moschata Duch.	seeds	BL, CS	unspecified	Jang *et al*., 2000
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]

Family/Species	**Examined part**	**Brassino-steroid[1]**	**content µg/kg fr. wt.[2]**	**References**
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				
Cryptomeria japonica D. Don.	pollen, anther	28-homoBL, BR (8)	unspecified	Yokota *et al*., 1998, Watanabe *et al*., 2000
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

Family/Species	Examined part	Brassino-steroid[1]	content µg/kg fr. wt.[2]	References
	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
	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

Family/Species	Examined part	Brassino-steroid[1]	content µg/kg fr. wt.[2]	References
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
	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	cultured cells	BL	125.1 µg/kg dr. wt	Stirk *et al*., 2013

Family/Species	Examined part	Brassino-steroid[1]	content µg/kg fr. wt.[2]	References
(Acutodesmus acuminatus)				
	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
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

Family/Species	Examined part	Brassino-steroid[1]	content µg/kg fr. wt.[2]	References
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).

Some studies have been performed to test the long distance transport between plant organs of applied and endogenous Brassinosteroids.

According to the review paper on Brassinosteriod transport in plants by Symons *et al*. (2008), long distance transport of Brassinosteriods between different plant organs is unlikely to occur. This is supported by several studies on long distance transport of Brassinosteroids. Symons and Reid (2004) evaluated the transport of Brassinosteroids by grafting Brassinosteroid-deficient mutants to wild-type plants; no change to the phenotype was observed. Furthermore, Brassinosteroid levels in the remaining internodes did not decrease after the removal of the apical bud or mature leaves as in the case of, for example, auxin and gibberellin (Symons and Reid, 2004). The authors further observed that Brassinosteriods produced in planta are only transported over short distances to the exterior surface of the producing cell or to the surfaces of neighboring cells.

This is consistent with the results of studies showing that Brassinosteroid biosynthesis genes are widely expressed in plant tissues, and transcript levels are generally higher in tissues with high Brassinosteroid levels (reviewed by Nomura and Bishop, 2006). Thus, it is likely that endogenous Brassinosteroid levels in plant tissues are primarily regulated through the tissue-specific control of Brassinosteroid synthesis, catabolism, and inactivation rather than through long-distance transport (Symons and Reid, 2004).

However, some transport studies have been performed with exogenously applied Brassinosteroids on roots that show root to shoot movement. After the application of ^{3}H-labeled Brassinolide or ^{3}H-labeled Castosterone to the roots of rice plants, Yokota *et al*. (1992) observed that approximately 10 % of the radioactivity taken up by the plant was found in the shoot after only 6 h. The major part was in the form of unmetabolized Brassinosteroids, suggesting that applied Brassinosteroids taken up through the roots were translocated, unchanged, to the shoots (Yokota *et al*., 1992; see below for executive study summary).

Similarly, in both cucumber and wheat seedlings a rapid transfer of radioactivity to the shoot after ^{14}C-labeled 24-Epibrassinolide was applied to the roots (Nishikawa *et al*., 1994), was observed. However, in this study it was not determined whether the radioactivity found in the shoot was in the form of unmetabolized Brassinosteroids or more polar Brassinosteroid metabolites. Therefore, it is unclear if the movement of radioactivity observed in this study accurately represents the movement of a Brassinosteroids in its bioactive form (see below for executive study summary).

Consistent with the results of Yokota *et al*. (1992) and Nishikawa *et al*. (1994), Brassinosteroid-deficient *Arabidopsis* mutants having an abnormal shoot phenotype can be restored to normal growth when these plants are grown on media containing bioactive Brassinosteroids (Choe *et al*., 1998) suggesting that root-to shoot transfer is possible.

However, when applied directly to shoots and leaves, exogenous Brassinosteroids appear to be relatively immobile. For instance, when ^{3}H-labeled Brassinolide or ^{3}H-labeled Castosterone was applied to pea leaves, it entered the leaves, but was not transported to other plant organs (Symons and Reid, 2004; see below for executive study summary).

Similarly, the majority of exogenous, radiolabeled Brassinolide and Castosterone incorporated into the leaves of rice remained in the treated leaves 24 h after it was applied (Yokota *et al*., 1992). In this last study, some radioactivity was detected in the roots after 72 h, mostly in the form of water-soluble Brassinosteroid metabolites (Yokota *et al*., 1992). In wheat, radioactivity did not move from the treated leaf, even 7 days after exogenous ^{14}C-labeled 24-Epibrassinolide was applied, while in cucumber, some radioactivity moved out of the leaf but not until after 3 days (Nishikawa *et al*., 1994).

Data point addressed:	CA 6.2.1/01
Author(s) (year):	Yokota, T., Higuchi, K., Kosaka, Y., Takahashi, N. (1992)
Title:	TRANSPORT AND METABOLISM OF BRASSINOSTEROIDS IN RICE
Laboratory report / project Number (Doc. No.):	Not applicable (092-098)
Testing facility:	Not applicable
Published:	Yes (Progress in Plant Growth Regulation, 1992, 13, 298-305)
Test guideline used:	Not indicated
Deviations:	None
GLP:	No

Executive Summary
In this work, the transport and metabolism of Brassinosteroids was investigated by applying either ^{3}H labeled Brassinolide, ^{3}H labeled 24-Epibrassinolide or ^{3}H labeled Castasterone to roots or leaves of rice. Furthermore, the metabolism of ^{3}H labeled Castasterone was investigated by using the rice lamina inclination bioassay.

Long distance transport of unmetabolized Brassinosteroids is possible after the application to roots. After the application of ^{3}H-labeled Brassinolide or ^{3}H-labeled Castosterone to the roots of rice plants, it was observed that approximately 10 % of the radioactivity taken up by the plant was found in the shoot after only 6 h.

No or very slow long distance transport of Brassinosteroids after the application of ^{3}H labeled 24-Epibrassinolide to leaves of rice is possible. After 24 h most of the radioactivity incorporated was localized in the treated leaves. However, after 72 h some radioactivity was slowly transported to the roots. Most of the radioactivity found in the roots after 72 h was water-soluble. It can therefore be concluded that no movement from unmetabolized Brassinosteroids did occur but from metabolized water-soluble substances.

I. MATERIALS AND METHODS

A. Materials

^{3}H labeled Brassinolide, ^{3}H labeled 24-Epibrassinolide and ^{3}H labeled Castasterone have been synthesized according to Yokota *et al*. (1990). The specific activities of these ^{3}H labeled Brassinosteroids were 8 x 10^{13} Bq/mol.

B. Study design and methods

1. Application of ^{3}H radiolabeled Brassinolide, 24-Epibrassinolide and Castasterone to roots of rice

1.1. Feeding
Rice *(Oryza sativa* L. cv. Koshihikari) seedlings were grown under natural light in a biotron (day/night, 25 °C/20 °C) in Kimura's solution ($(NH_4)_2SO_4$ 48.2 mg, K_2SO_4 15.9 mg, $MgSO_4$ 65.9 mg, KNO_3 18.5 mg, $Ca(NO_3)_2$ 59.9 mg, KH_2P0_4 24.8 mg, Fe-citrate 5 mg in 1 liter of water) adjusted to pH 5.2.

Roots of the 10 seedlings at 3.5th to 4th leaf stages were dipped in Kimura's solution (100 ml) containing a radioactive Brassinosteroids (10 µCi) in a 100 ml conical flask shaded with aluminum foil. A set of seedlings was incubated for 6 h in the biotron. The seedlings were washed with water and dissected into roots, shoots and seeds prior to extraction with methanol. Another set of seedlings was further grown for 7 d in fresh nutrient solution without radiolabeled Brassinosteroids.

1.2. Extraction
Tissues were extracted with methanol by grinding with quartz sand in a mortar. The extracts were subjected to partitioning between chloroform and water at pH 3 prior to evaporation to dryness. The chloroform-soluble fractions were dissolved in chloroform and were passed through Sep-Pak silica columns (Waters). The columns were eluted with 1 % methanol in chloroform (10 ml) and then with pure methanol (10 ml). Most of the radioactivity was recovered in the latter fractions (82-94 % in the shoot extracts and 90-99 % in the root extracts). Only the methanol eluates were further analyzed by HPLC using a Senshu Pak C_{18} column (6 x 200 mm) with 45 % acetonitrile at a flow rate of 1.5 ml/min at room temperature. The water-soluble fraction,

dissolved in water (15 ml), was passed through a Sep-Pak C_{18} silica column and was eluted with methanol (10 ml).

1.3. Solvolysis

Samples were reacted with ethyl acetate saturated with 2 N sulphuric acid overnight at 37 °C. The mixture was washed twice with water and evaporated to dryness.

2. Application ^{3}H labeled Brassinolide and ^{3}H labeled Castasterone to of rice leaf surfaces

Five rice seedlings at the 4th leaf stage were placed in a Kimura's solution in a 50 ml vial shaded with aluminium foil. Radiolabeled Brassinosteroids (0.04 µCi) dissolved in methanol (5 µl) was applied to the upper surface of the 3rd leaf blade. These experiments were performed in duplicate. The seedlings were grown in a biotron and then dissected into treated leaf blades, other parts of shoots, seeds and roots. The leaf blades were rinsed with methanol (10 ml) for 30 s and then with chloroform (10 ml) for 30 s. The plant tissues were extracted as stated above.

3. Application of ^{3}H labeled Castasterone to etiolated rice-lamina explants

3.1. Feeding

Second lamina were cut 0.7 cm above and below the joints from etiolated rice seedlings, which were grown after Arima *et al*. (1984). In each feeding, 150 explants were floated on 0.01 ppm solution (10 ml) of ^{3}H labeled Castasterone (5 µCi/µg) at 30 °C in the dark.

3.2. Extraction

The tissues were washed with water three times, and extracted with methanol (30 ml) using a polytron homogenizer (Kinematica). The extract was dissolved in 70 % acetonitrile (20 ml) and passed through a C_{18} silica column (2 g, 100-200 mesh, Senshu Kagaku). The column was washed with 70 % acetonitrile (10 ml). The combined eluates, in which more than 95 % of the radioactivity was recovered, were analyzed by HPLC as described above (column temperature, 40 °C).

3.3. Hydrolysis

Alkaline hydrolysis

Fractions were hydrolyzed with IN NaOH (5 ml) for 1 h with or without refluxing, acidified to pH 3 and passed through a column of Sep-Pak C_{18}. Extraction from the column was done using methanol.

Acid hydrolysis

Fractions were refluxed in 1N HCl for 1 h and treated as stated above.

Enzymatic hydrolysis

Metabolites were incubated with 50 µg of alkaline phosphatase (Boehringer-Mannheim Grade 1, spec. act. >2500 U/mg) dissolved in 100 µl of TBS buffer (Tris - HCl 6.05 g, NaCl 8.80 g and $MgCl_2$ 0.203 g in 1 liter of water) at 37 °C for 2 days. The reaction mixture was treated as stated above.

II. RESULTS AND DISCUSSION

1. Transport and metabolism ^{3}H radiolabeled Brassinolide, 24-Epibrassinolide and Castasterone to applied to roots in green rice seedlings

After 6-h feeding to the roots, radiolabeled Brassinosteroids were incorporated in both, roots and shoots (Table 7.2.1-2). For each Brassinosteroid applied, the shoots contained about one-tenth of the radioactivity found in the roots. This indicates that Brassinosteroids taken up through roots are translocated to shoots. Interestingly, ^{3}H labeled Castasterone was incorporated at a higher rate than ^{3}H radiolabeled Brassinolide and 24-Epibrassinolide (Table 7.2.1-2). The incorporation rates of the latter two were nearly equal. In each case the major part of the radioactivity recovered from the roots and shoots was chloroform-soluble and the minor part was water-soluble (Table 7.2.1-2). A major component in the chloroform-soluble fractions was an unmetabolized Brassinosteroid.

When seedlings fed with radiolabeled Brassinosteroids were further grown without Brassinosteroids for 7 days, the radioactivity in the chloroform-soluble fractions from both the roots and shoots decreased to a large extent (Table 7.2.1-2). In contrast, radioactivity in the water-soluble fractions from the roots decreased to a lesser extent, and in those from the shoots it even increased. The HPLC analysis of the chloroform-soluble fractions indicated that polar metabolites were the major components, while unchanged Brassinosteroids were minor

components. This is quite different from the data obtained from the 6-h feeding experiment. Thus, during 7 days, Brassinosteroids were metabolized to water-soluble substances as well as to chloroform-soluble polar ones.

The water-soluble metabolites were retained little on reversed phase HPLC using 30 % acetonitrile as the mobile phase (data not shown), suggesting that they are not glycosides. When the mobile phase was acidified with acetic acid (1 %, v/v), the retention times of the metabolites increased, suggesting the presence of an acidic moiety (data not shown). Sulphate esters of animal steroid hormones are cleavable by solvolysis. Upon solvolysis, the metabolites of ^{3}H radiolabeled Brassinolide and Castasterone released chloroform-soluble substances in 46 % and 59 % yields, respectively. The products contained compounds with polarities similar to those of the free Brassinosteroids as examined by TLC and reversed phase HPLC (data not shown). A similar result was obtained by alkaline hydrolysis of the ^{3}H radiolabeled Castasterone metabolites. Therefore, the water-soluble metabolites might include sulfate ester-like compounds.

Table 7.2.1-2 Distribution of radioactivity after application of ^{3}H radiolabeled Brassinolide, 24-Epibrassinolide and Castasterone to roots in green rice seedlings

Tissue	Time after 6 h-feeding	Incorporation (%)					
		Brassinolide feeding		24-Epibrassinolide feeding		Castasterone feeding	
		$CHC1_3$ phase	H_20 phase	$CHC1_3$ phase	H_20 phase	$CHC1_3$ phase	H_20 phase
Root	0 h	1.47 (1.17)	0.52	1.63 (1.30)	0.12	4.62 (3.18)	1.35
	7 days	0.15 (0.032)	0.32	0.05 (0.023)	0.10	0.13 (0.007)	0.64
Shoot	0h	0.20 (0.18)	0.04	0.17 (0.12)	0.03	0.44 (0.38)	0.04
	7 days	0.05 (0.009)	0.06	0.06 (0.011)	0.06	0.11 (0.003)	0.18

Incorporation rates were expressed as % of the radioactivity applied. Figures between brackets refer to unmetabolized Brassinosteroids

2. Transport and metabolism of ^{3}H labeled Brassinolide and ^{3}H labeled Castasterone applied to leaf surface of rice

^{3}H labeled Brassinolide and ^{3}H labeled Castasterone applied to leaf surface of rice behaved similarly. During tire incubation, they were absorbed only in part by the plant tissues and most of the radioactivity applied was recovered in the washings (Table 7.2.1-3). No radioactivity was found in the seeds. After 24 h most of the radioactivity incorporated was localized in the treated leaves. However, after 72 h some radioactivity also accumulated in the roots (Table 7.2.1-3). Thus, it seems that a slow transport of Brassinosteroids or its metabolites from leaves to roots can occur. Most of the radioactivity found in the roots after 72 h was water-soluble (Table 7.2.1-3).

Table 7.2.1-3 Distribution of radioactivity after application of ^{3}H radiolabeled Brassinolide and Castasterone to leaf surface of rice.

Compound applied	Time after application (h)	Incorporation (%)				
		Treated leaf	Root	Other tissue[1]	Leaf washing	Total recovery
Brassinolide	6	0.55	0.09	0.08	96.56	97.28
	24	0.94	0.06	0.04	95.80	96.84
	72	0.61(0.12)	0.21(0.18)	0.09	95.01	95.92
Castasterone	6	0.67	0.08	0.05	98.14	98.94
	24	0.55	0.07	0.03	97.98	98.63
	72	0.59(0.10)	0.17(0.15)	0.09	97.73	98.58

[1]The data are tentative because of quenching. Figures between brackets denote radioactivity in water phase after partitioning between chloroform and water.

3. Metabolism of ^{3}H labeled Castasterone in etiolated rice lamina explants

Rice lamina inclination bioassay using etiolated rice lamina explants is the most sensitive method to detect Brassinosteroids. Etiolated rice lamina explants were incubated with 0.01 ppm solution of ^{3}H labeled Castasterone for 72 h. The ^{3}H labeled Castasterone was rapidly incorporated by the tissues, up to 41 % of the total amount applied, after 72 h (Table 7.2.1-4). The whole extracts were analyzed by reversed-phase HPLC.

The radioactivity corresponding to intact ^{3}H labeled Castasterone did not clearly increase with the incubation time. It is likely that ^{3}H labeled Castasterone loosely adsorbed to the leaf surface was also extracted. Major metabolites of ^{3}H labeled Castasterone were polar compounds which were eluted with little retention in HPLC. They continuously increased during 72 h (Table 7.2.1-4). At the end of the feeding, the polar metabolites accounted for up to 23 % of the radioactivity initially present in the medium.

The polar metabolites in 72 h-incubation were analyzed by reversed-phase HPLC using 33 % acetonitrile. The metabolites were not retained under this condition, indicating that they are not glycosidic. The metabolites were separable into two zones by reversed-phase HPLC with a gradient from 10 to 45 % acetonitrile (1 % acetic acid) (data not shown). After hydrolysis using sodium hydroxide, hydrochloric acid or alkaline phosphatase, followed by methylation with diazomethane, the metabolites obtained from the two zones seemed not to be changed as examined by reversed-phase HPLC (data not shown). It seems that etiolated rice leaf explants metabolize ^{3}H labeled Castasterone differently from intact rice roots.

Table 7.2.1-4 Distribution of radioactivity and bending angle of laminae after application of ^{3}H labeled Castasterone to etiolated rice lamina explants.

Time after the start of n incubation (h)	Bending angle[3]	Incorporation (%)		
		Total radioactivity	After HPLC CS zone (ft. 17-19)	Polar metabolite zone (fr. 2-6)
1	0.8	7.70	6.98	0.03
3	1.0	12.76	12.10	0.44
7	7.6	8.96	7.12	1.60
12	14.6	26.84	23.29	2.84
24	68.4	26.28	19.97	6.28
48	130.1	21.21	8.06	12.00
72	125.7	41.01	16.32	23.66

[a]Mean of ten explants.

III. CONCLUSIONS

After the application of ^{3}H-labeled Brassinolide or ^{3}H-labeled Castosterone to the roots of rice plants it was observed that approximately 10 % of the radioactivity taken up by the plant was found in the shoot after only 6 h. The major part was in the form of unmetabolized Brassinosteroids, suggesting that applied Brassinosteroids taken up through the roots were translocated, unchanged, to the shoots. After 7 day incubation, the fractions changed and Brassinosteroids were metabolized to water-soluble substances as well as to chloroform-soluble polar ones.

After 24 h most of the radioactivity incorporated was localized in the treated leaves. However, after 72 h some radioactivity was slowly transported to the roots. Most of the radioactivity found in the roots after 72 h was water-soluble. It can therefore be concluded that no movement from unmetabolized Brassinosteroids did occur but from metabolized water-soluble substances.

Data point addressed:	CA 6.2.1/02
Author(s) (year):	Nishikawa, N., Toyama, S., Shida, A., Futatsuya, F. (1994)
Title:	THE UPTAKE AND THE TRANSPORT OF 14C-LABELED EPIBRASSINOLIDE IN INTACT SEEDLINGS OF CUCUMBER AND WHEAT
Laboratory report / project Number (Doc. No.):	Not applicable (092-088)
Testing facility:	Not applicable
Published:	Yes (Journal of Plant Research, 1994, 107, 125-130)
Test guideline used:	Not indicated
Deviations:	None
GLP:	No

Executive Summary
The uptake and the transport of exogenously applied 24-Epibrassinolide in seedlings of cucumber and wheat were examined by autoradiography using ^{14}C-24-Epibrassinolide. ^{14}C-24-Epibrassinolide was applied to roots, young and mature leaves, and the shoot apex. When applied to roots, ^{14}C-24-Epibrassinolide was readily taken up and was swiftly transported throughout both plant species. When ^{14}C-24-Epibrassinolide was applied to the adaxial surface of a young cucumber leaf, it was readily taken up, but was very slowly transported. In cucumber leaves, ^{14}C-24-Epibrassinolide was transported throughout the treated leaf after 3 days of treatment, and then it was transported to upper leaves from the treated leaf after 7 days. Some 6.3 % of applied ^{14}C-24-Epibrassinolide was transported to the newly expanded leaves. In wheat leaves, ^{14}C-24-Epibrassinolide was transported only in the apical direction from the treated spot after 3 days of treatment, but it was not transported from the treated leaf to the other leaves or organs even after 7 days. Some 1.3 % of applied ^{14}C-24-Epibrassinolide was transported to the tip area of the treated leaf. These results indicate that exogenous 24-Epibrassinolide applied to intact plants is acropetally transported.

I. MATERIALS AND METHODS

Seeds of wheat (*Triticum aestivum* L. cv. Norin 61) and cucumber (*Cucumis sativus* L. cv. Tokiwajibai) were sown in vermiculite under natural light at 20 to 32 °C. After 7 to 10 days, young seedlings were transferred to solution culture. Seedlings of wheat were cultured in Kimurashi-A medium (Kimura 1931) containing 100 mg/l of SiO_2. Seedlings of cucumber were cultured in Otsuka No. 1 and No. 2 (Otsuka Kagaku Co., Ltd.) medium that are nutrient solutions prepared for solution culture.

After 2 weeks, when the 3rd leaves emerged from the 2nd leaf sheaths, seedlings of wheat were used for the experiments. After 3 weeks, when the 3rd leaves began to expand, seedlings of cucumber were used. ^{14}C-24-Epibrassinolide (specific activity; 56.78 Ci/mol) was obtained from Nippon Kayaku Co., Ltd.. ^{14}C-24-Epibrassinolide was stored in 99 % ethanol and was diluted as required for individual experiments.

Roots of each seedling were immersed in 10 ml of solution culture medium containing 5.6 µCi of ^{14}C-24-Epibrassinolide for 3 h, were washed with distilled water once to remove the excess ^{14}C-24-Epibrassinolide, and then were cultured by solution culture in a growth chamber under 16.5 W/m^2 of light intensity, day and night 14h/10h, and at 25 °C. The stock solution was diluted in distilled water containing 0.1 % of detergent (Polyoxyethylene sorbitan trioleate). Twenty-microliter droplets containing 0.24 µCi of ^{14}C-24-Epibrassinolide were applied to each plant with a micropipette.

After 0.5 to 1 h, the applied droplets were dry and the treated seedlings were cultured by solution culture as described above for the required period. Each sample was dried immediately with an electric iron without further removal of ^{14}C-24-Epibrassinolide from the surface and was placed in contact with an X-ray film for 14 to 35 days at 4 °C in dark. To estimate the radioactivity of a plant, the dried leaf or petiole was cut into small pieces and each of them was added to 10 ml of a scintillation fluid (Toluene-PPO). Radioactivity was counted with a liquid scintillation counter (Packard TRI-CARB liquid scintillation spectrometer model 3255).

II. Results

1. Root application

In cucumber, ^{14}C radioactivity had been transported throughout the leaves within 1 h of pulse labelling. After 7 days of culture, ^{14}C was transported to the newly expanded 4th leaf. In wheat, ^{14}C remained at the root just after

pulse labelling, and then it arrived at the tip of the 2nd leaf after 6 h (data not shown). After 7 days of culture, ^{14}C was transported to the newly expanded 3rd leaf.

2. Shoot application

When ^{14}C-24-Epibrassinolide was applied to the shoot apex of cucumber in which the 3rd leaf was expanding, it was taken up easily in the 3rd leaf and the newly expanded 4th leaf after 7 days of culture. ^{14}C was transported along the vein, and radioactivity was especially concentrated in the margin of the 3rd and 4th leaves. However, ^{14}C was not transported to the 1st and the 2nd leaves from the shoot apex. When ^{14}C-24-Epibrassinolide was applied on the adaxial surface of the fully expanded 2nd leaf of cucumber, it was transported throughout the 2nd leaf after 7 days of culture. Some 3.0 % of applied ^{14}C-24-Epibrassinolide was transported from the treated spot.

In contrast, when ^{14}C-24-Epibrassinolide was applied on the abaxial surface of the fully expanded 2nd leaf of cucumber, it was transported only in the apical direction from the treated spot after 7 days of culture. ^{14}C-24-Epibrassinolide was taken up more easily from the adaxial surface of the leaf than from the abaxial surface. When ^{14}C-24-Epibrassinolide was applied on the adaxial surface of the 3rd leaf of cucumber, it was transported throughout the 3rd leaf and to the newly expanded 4th and 5th leaves through the petiole of the 3rd leaf after 7 days of culture. Some 6.3 % of applied ^{14}C-24-Epibrassinolide was transported from the treated leaf to the newly expanding leaves.

When applied on the abaxial surface of the young 3rd leaf, ^{14}C was transported only in the apical direction from the treated spot after 7 days of culture. When ^{14}C-24-Epibrassinolide was applied on the adaxial or abaxial surfaces of the 2nd leaf of wheat, it was slightly transported only in the apical direction from the treated spot after 7 days of culture. Some 1.1 % of applied ^{14}C-24-Epibrassinolide was transported to the tip area of the treated leaf.

As in cucumber, ^{14}C-24-Epibrassinolide applied to the young 3rd leaf of wheat was taken in more easily from the adaxial than the abaxial leaf surface. A little ^{14}C was transported downward from the treated spot, but almost all ^{14}C was transported in the apical direction. Some 1.3 % of applied ^{14}C-24-Epibrassinolide was transported to the tip area of the treated leaf.

III. DISCUSSION AND CONCLUSIONS

When ^{14}C-24-Epibrassinolide was applied to roots, it was readily taken up and was swiftly transported throughout both plants, wheat and cucumber. ^{14}C-24-Epibrassinolide was probably transported through the xylem. In wheat, it took about 6 h for ^{14}C-24-Epibrassinolide transportation from root to shoot. Under this condition, the uptake of ^{14}C-24-Epibrassinolide was so small that radioactivity in the shoot was detected only with difficulty after 6 h from the beginning of the treatment.

In contrast, when ^{14}C-24-Epibrassinolide was applied to the leaf, the uptake of ^{14}C-24-Epibrassinolide was remarkably different for various foliar applications of droplets. Namely, in cucumber, ^{14}C-24-Epibrassinolide was taken up most easily from the adaxial surface of the young leaf. When ^{14}C-24-Epibrassinolide was applied on the adaxial surface, and the treated side was then turned downward, ^{14}C was not transported from the treated spot (data not shown). ^{14}C-24-Epibrassinolide was not transported, even if the applied ^{14}C-24-Epibrassinolide abaxial surface was placed to turn upward or downward (data not shown). This suggests that the difficulty for uptake and transport of ^{14}C-24-Epibrassinolide from the leaf surface may be dependent on its structural and physiological characteristics, e.g. hairs, cuticle and other cell wall components.

^{14}C-24-Epibrassinolide was absorbed most easily from the younger leaves of wheat, but the remarkable difference between the adaxial surface and the abaxial surface was not observed. The transport of foliar applied ^{14}C to other leaves in cucumber is very striking, but not in wheat even after 7 days of culture.

In this study, the transport of Brassinosteroids was generally very slow when it was applied to leaves. When ^{14}C-24-Epibrassinolide was applied to the shoot apex of cucumber, it was not transported to the old leaves. When ^{14}C-24-Epibrassinolide was applied to the adaxial surface of young leaves of cucumber, ^{14}C-24-Epibrassinolide was basipetally transported in the petiole and then was acropetally transported to the upper leaves. ^{14}C-24-Epibrassinolide was probably transported through the phloem.

Data point addressed:	CA 6.2.1/03
Author(s) (year):	Symons, G.M., Reid, J.B. (2004)
Title:	BRASSINOSTEROIDS DO NOT UNDERGO LONG-DISTANCE TRANSPORT IN PEA. IMPLICATIONS FOR THE REGULATION OF ENDOGENOUS BRASSINOSTEROID LEVELS
Laboratory report / project Number (Doc. No.):	Not applicable (092-095)
Testing facility:	Not applicable
Published:	Yes (Plant Physiology, 2004, 135, 2196-2206; doi: 10.1104/pp.104.043034)
Test guideline used:	Not indicated
Deviations:	None
GLP:	No

Executive Summary
It is widely accepted that Brassinosteroids are important regulators of plant growth and development. However, in comparison to the other classical plant hormones, such as auxin, relatively little is known about Brassinosteroid transport and its potential role in the regulation of endogenous Brassinosteroid levels in plants. Here, the authors show that end-pathway Brassinosteroids in pea (*Pisum sativum*) occur in a wide range of plant tissues, with the greatest accumulation of these substances generally occurring in the young, actively growing tissues, such as the apical bud and young internodes. However, despite the widespread distribution of Brassinosteroids throughout the plant, the authors found no evidence of long-distance transport of these substances between different plant tissues. For instance, the authors show that the maintenance of steady-state Brassinosteroid levels in the stem does not depend on their transport from the apical bud or mature leaves. Similarly, reciprocal grafting between the wild type and the Brassinosteroid-deficient lkb mutants demonstrates that the maintenance of steady-state Brassinosteroid levels in whole shoots and roots does not depend on either basipetal or acropetal transport of Brassinosteroids between these tissues. Together, with results from ^{3}H-Brassinosteroid plant feeding studies, these results demonstrate that Brassinosteroids do not undergo long-distance transport in pea. The widespread distribution of end-pathway Brassinosteroids and the absence of long-distance Brassinosteroid transport between different plant tissues provide significant insight into the mechanisms that regulate Brassinosteroid homeostasis in plants.

I. MATERIALS AND METHODS

1. Plant Materials and Growth Conditions

The pure lines of garden pea (*Pisum sativum*) used in this study were Hobart line 107 (cv Torsdag, wild type) and the single-gene Brassinosteroid mutant lines NGB5862 (lkb, semi-erectoides) and NGB5865 (lka, semi-erectoides). NGB5862 and NGB5865 were both derived from Torsdag by mutagenesis with ethyl methanesulfonate. Mutant lkb has been shown to be a lesion in the gene homologous to DIM/distilled water F1 in Arabidopsis, while lka has been shown to be a lesion in the pea homolog of the Arabidopsis BRI1 gene.

Seeds were sown 2 to 3 cm deep in 14 cm slim-line pots containing a 1:1 (v/v) mixture of vermiculite and 10 mm dolerite chips topped with 4 cm of pasteurized peat/sand potting mixture. All plants were grown under an 18 h photoperiod in a heated greenhouse, with the natural day-length extended at its beginning and end with light from 40 W cool-white fluorescent tubes and 100 W incandescent bulbs, providing about 25 µM m^{-2} s^{-1} at pot top. Nutrient was applied weekly in the form of Aquasol (Hortico, Melbourne, Australia). Node counts commenced from the first scale leaf as node 1; internode 1 was the internode between nodes 1 and 2.

2. Harvest Procedure

All plants utilized for hormone quantification were harvested after the leaf at node 8 was fully expanded (approximately 30 d old). In these experiments, plant shoots and roots were separated at node 0. Shoots were either left whole or separated into individual tissue types, while the roots were left whole and washed free of excess soil. All plant tissues used for hormone analysis were weighed and then immediately immersed in cold (-20 °C) 80 % (v/v) methanol.

3. Extraction, Purification, and Gas Chromatography-Mass Spectrometry Quantification of Brassinosteroids

Procedures for the extraction, purification, and gas chromatography-mass spectrometry quantification of endogenous Brassinosteroids, indole-3-acetic acid (IAA), Gibberellin (GA_1), and abscisic acid (ABA) have been previously outlined in Symons and Reid (2003a) (cited in Symons and Reid, 2004).

4. Metabolism and Transport of ^{3}H-Brassinolide and ^{3}H- Castasterone

A total of 1 x 10^6 dpm of radiolabeled Brassinosteroids (^{3}H-Brassinolide and ^{3}H- Castasterone; 0.78 Ci mM^{-1}) was applied in 5 ml of ethanol to either the youngest expanded leaf (at node 9) or directly to the apical bud of intact, 33-d-old wild-type plants. Forty-eight hours later, the site of Brassinosteroids application and all adjacent shoot tissues (including individual leaves, internodes, and the apical bud) were harvested separately, frozen in liquid nitrogen, and stored at -20 °C. Tissue samples were homogenized and Brassinosteroids were extracted in a mixture of 80 % methanol and 20 % distilled water. The level of radioactivity in the individual tissue samples was determined by radiocounting (using a Beckman LS 6500 scintillation counter; Beckman Instruments, Fullerton, CA). Metabolism of the radiolabeled Brassinosteroid content was analyzed by subjecting extracts from the treated tissues to HPLC radiocounting. Details of the HPLC system and the solvent program were as outlined in Symons and Reid (2003a) (cited in Symons and Reid, 2004).

II. RESULTS AND DISCUSSION

1. Spatial Distribution of Brassinosteroids in Wild-Type Plants

Brassinosteroids were detected in all tissue types tested, including the apical bud, mature leaves, stem, and roots, although Brassinosteroids levels varied greatly between different tissue types (Table 7.2.1-5). For instance, in the shoot, the levels of Castasterone, 6-Deoxocastasterone, and Typhasterol were higher in young, actively growing tissues, such as the apical bud, and lowest in the mature leaves (Table 7.2.1-5). This spatial distribution of end-pathway Brassinosteroids in the shoot follows a similar pattern to the observed distribution of indole-3-acetic acid (IAA), gibberellin (GA_1), and abscisic acid (ABA) which was also generally higher in young, actively growing tissues of the apical bud than in mature leaves (Table 7.2.1-6). In the roots, Brassinosteroids levels were significantly lower than in shoot tissues, as Castasterone and Typhasterol levels were below detection limits and 6-Deoxocastasterone levels were between 5- and 18-fold lower than in the shoot tissues (Table 7.2.1-5). Brassinolide levels were below detection limits in all of the tissue types studied (Table 7.2.1-5 to Table 7.2.1-7).

Table 7.2.1-5 Distribution of endogenous brassinosteroids in tissues of wild-type pea plants

Tissue Type	Brassinosteroid			
	Brassinolide	**Castasterone**	**6-Deoxocastasterone**	**Typhasterol**
Apical bud	nd	0.26 ± 0.05	5.29 ± 0.46	0.09
Stem	nd	0.21 ± 0.01	2.97 ± 0.20	0.04
Leaves	nd	0.17 ± 0.01	2.06 ± 0.04	nd
Roots	nd	nd	0.30	nd

All plants had eight fully expanded leaves at the time of harvest. Values represent the mean endogenous hormone levels ± the se, obtained from three separate replicate experiments. nd, not detected. Typhasterol levels in the apical bud and stem and 6-Deoxocastasterone levels in the roots were detected in one replicate only.

Table 7.2.1-6 Effect of removing the apical bud (decapitation) on the endogenous IAA, GA_1, and ABA levels in apical, stem, and leaf issues of wild-type pea plants

Tissue	Treatment	Hormone level ng/g fresh weight		
		IAA	**GA_1**	**ABA**
Apical bud	Intact	82.6 ± 71	6.8 ± 0.3	4.2 ± 0.1
	Decapitated	-	-	-
Stem	Intact	100.4 ± 4.8	1.2 ± 0.04	1.7 ± 0.1
	Decapitated	9.8 ± 2.5	0.08 ± 0.003	0.8 ± 0.02
Leaves	Intact	16.9 ± 3.2	0.7 ± 0.1	2.2 ± 0.2
	Decapitated	8.2 ± 0.1	0.5 ± 0.2	2.5 ± 0.2

All plants had eight fully expanded leaves at the time of harvest. Values represent the mean endogenous hormone levels ± the se, derived from the same two replicate experiments as the results outlined in Table 7.2.1-7.- Not applicable.

2. Comparative Distribution of Brassinosteroids in Wild-Type, lka, and lkb Plants

In a previous study, Brassinosteroid levels were elevated in whole shoots of lka mutant plants and reduced in whole shoots of lkb mutant plants, in comparison with the wild type. This is consistent with the finding that the lkb mutation blocks the biosynthesis of Brassinosteroids, while the lka mutation causes impaired perception of these substances. In this study, the authors show that these changes in endogenous Brassinosteroids in lka and lkb mutant plants (compared to the wild type) are consistent across a wide range of different shoot tissues and are not restricted to any specific location (Table 7.2.1-7). For instance, Brassinosteroid levels were consistently reduced (2.5- to 3.5-fold for Castasterone, 20- to 37-fold for 6-Deoxocastasterone) in the apical buds, shoots, and leaves of lkb plants, in comparison to the wild type. Similarly, Brassinosteroid levels were consistently increased (6- to 7-fold for Castasterone, 1.8- to 2.2-fold for 6-Deoxocastasterone, and 1.4- to 2.6-fold for Typhasterol in the apical buds, shoots, and leaves of lka plants, in comparison to the wild type (Table 7.2.1-7)). Interestingly, even in lka plants, where Castasterone levels were increased up to 7-fold in comparison with the wild type, Brassinolide was still below detection limits, estimated to be approximately 0.005 ng/g of fresh weight (FW). A comparison of the Castasterone and 6-Deoxocastasterone levels in wild-type, lka, and lkb mutant plants from this study also provides an insight into the possible regulation of the Brassinosteroid pathway. For instance, in wild-type pea plants, the ratio of 6-Deoxocastasterone to Castasterone is consistently around 6:1 in all tissue types tested, while in the lka and lkb mutants this ratio is consistently around 2:1 or less (Table 7.2.1-7). This may suggest that the conversion of 6-Deoxocastasterone to Castasterone is increased in the lkb and lka mutants, most likely in response to the perceived or real deficiency of active Brassinosteroids in these plants, respectively.

Table 7.2.1-7 Comparative distribution of endogenous brassinosteroids in apical, stem, and leaf tissues of wild-type and Brassinosteroid mutant plants

Tissue	Genotype	Brassinosteroid Level ng/g fresh weight				
		Brassinolide	Castasterone	6-Deoxo-castasterone	Typhasterol	Ratio 6-Deoxo-castasterone to Castasterone
Apical bud	Wild type	nd	0.62	4.10	0.20	6.6
	lka	nd	4.40	9.12	0.83	2.1
	lkb	nd	0.17	0.20	nd	1.2
Stem	Wild type	nd	0.41	2.62	0.11	6.4
	lka	nd	2.52	5.52	0.15	2.2
	lkb	nd	0.16	0.07	nd	0.4
Leaf	Wild type	nd	0.21	1.20	0.03	5.7
	lka	nd	1.49	2.26	0.08	1.5
	lkb	nd	0.06	0.05	nd	0.8

All plants had eight fully expanded leaves at the time of harvest. Values were obtained from a single replicate consisting of between 50 and 80 plants of each genotype. nd, Not detected.

3. Influence of the Apical Bud on Brassinosteroid Levels in the Shoots of Wild-Type Plants

Removal of the apical bud (decapitation) did not cause a reduction in Brassinosteroid levels in either stem or leaf tissues of wild-type plants 48 h after the apical bud was removed (Table 7.2.1-8).
Indeed, Castasterone and 6-Deoxocastasterone levels were actually slightly increased in the internodes and leaves of decapitated plants. However, with the exception of the slight increase in Castasterone levels (P, 0.05) in the stem tissues of decapitated plants, the changes were not statistically significant. In contrast, the levels of the other classical plant hormones, IAA, GA_1, and ABA, were all dramatically reduced in stem tissues after decapitation (in comparison with levels in intact plants; see Table 7.2.1-6). For instance, IAA, GA_1, and ABA levels were reduced (10-, 15-, and 2-fold, respectively, compared to intact plants) in stem tissues 48 h after decapitation (Table 7.2.1-6). Decapitation also resulted in a reduction in IAA levels (2-fold compared to intact plants) in the mature leaves, but did not cause any significant change in GA_1 or ABA levels in these tissues (Table 7.2.1-6).

Table 7.2.1-8 Effect of removing the apical bud (decapitation) on the levels of endogenous brassinosteroids in stem and leaf tissues of wild-type pea plants

Tissue	Treatment	Brassinosteroid ng/g fresh weight			
		Brassinolide	**Castasterone**	**6-Deoxocastasterone**	**Typhasterol**
Stem	Intact	nd	0.21 ± 0.02	2.78 ± 0.05	nd
	Decapitated	nd	0.31 ± 0.01	2.92 ± 0.19	nd
Leaves	Intact	nd	0.13 ± 0.02	2.10 ± 0	nd
	Decapitated	nd	0.14 ± 0.01	2.14 ± 0.18	nd

All plants had 8 fully expanded leaves at the time of harvest. Plants were decapitated by removing the apical bud via a cut made directly above node 8. Values represent the mean endogenous hormone levels ± the se, 48 h after decapitation. Values were derived from two separate replicate experiments. nd, not detected.

4. Influence of Mature Leaves on Brassinosteroid Levels in the Shoots of Wild-Type Plants

Removal of the three youngest expanded leaves (defoliation) also did not result in a dramatic change in Brassinosteroid levels in either internode or apical tissues of wildtype plants 48 h after the leaves were removed (Table 7.2.1-9). Castasterone, 6-Deoxocastasterone, and Typhasterol levels in the apical bud were slightly decreased in response to defoliation (Table 7.2.1-9). In contrast, Castasterone and 6-Deoxocastasterone levels in the stem tissue remained unchanged, while Typhasterol levels were again decreased slightly after defoliation (Table 7.2.1-9).

Table 7.2.1-9 Effect of removing mature leaves (defoliation) on the distribution of endogenous brassinosteroids in the apical and stem tissues of wild-type pea plants

Tissue	Treatment	Brassinosteroid ng/g fresh weight			
		Brassinolide	**Castasterone**	**6-Deoxocastasterone**	**Typhasterol**
Apical Bud	Intact	nd	0.55 ± 0.06	3.25 ± 0.02	0.039 ± 0.008
	Defoliated	nd	0.43 ± 0	3.01 ± 0.01	0.023 ± 0.003
Stem	Intact	nd	0.43 ± 0.01	2.50 ± 0.28	0.037
	Defoliated	nd	0.43 ± 0.03	2.49 ± 0.05	0.022 ± 0.001

All plants had eight fully expanded leaves at the time of harvest. Plants were defoliated by separating the petiole from the stem at nodes 6, 7, and 8. Stipules were left intact. Values represent the mean endogenous Brassinosteroid levels 6 the se, 48 h after defoliation. Values were derived from two separate replicate experiments. nd, not detected.

5. Influence of the Roots on Brassinosteroid Levels in the Shoots

Grafting an lkb shoot onto a wild-type rootstock did not restore either the endogenous Brassinosteroid levels in, or the phenotype of, the lkb shoot to that of the wild type. In this case, the endogenous Brassinosteroid levels and the shoot phenotype were both comparable to the shoots of selfgrafted lkb plants. Similarly, when a wild-type shoot was grafted onto an lkb rootstock, the presence of the lkb rootstock did not alter Brassinosteroid levels in the wildtype shoot. In this case, Brassinosteroid levels in the shoot were comparable to those in the shoots of self-grafted wild-type plants. This suggests that the maintenance of steady-state Brassinosteroid levels in the shoot is not dependent on Brassinosteroid synthesis in, or acropetal (root to shoot) Brassinosteroid transport from the roots.

6. Influence of the Shoots on Brassinosteroid Levels in the Roots

Grafting a wild-type shoot onto an lkb rootstock did not restore Brassinosteroid levels in the lkb root to that of the wild type. Indeed, Brassinosteroid levels in the root were below detection limits, which is similar to the situation in the roots of self-grafted lkb plants. This suggests that the maintenance of steady-state Brassinosteroid levels in the roots is not dependent on Brassinosteroid synthesis in, or basipetal (shoot to root) transport from the shoots.

7. Transport of Exogenously Applied, Radiolabeled Brassinosteroids

Radiolabeled Brassinosteroids (1 x 10^6 dpm in 5 ml ethanol of ^{3}H-Brassinolide or ^{3}H-Castasterone; 0.78 Ci/mM), applied either to the youngest expanded leaf or directly to the apical bud of wild-type plants, were not detected in adjacent stem or leaf tissues 48 h later (data not shown). Recovery of the radioactivity at the site of application was consistent with the amount of substrate applied to the plants (data not shown), and HPLC radiocounting confirmed that the majority of radioactivity recovered remained in its original form (^{3}H-Brassinolide or ^{3}H-Castasterone) and was not broken down or metabolized during the experiment (Fig. 7-3).

Application of unlabeled Brassinolide (200 ng in 5 ml of ethanol) to the youngest expanded leaf of Brassinosteroid-deficient lkb plants caused a localized increase in leaflet elongation and lightening of the leaf color, both of which were confined to the site of application. The localized nature of this growth response resulted in an abnormal leaf shape and curling of the leaflet. However, application of Brassinolide to the youngest expanded leaf did not alter the growth of adjacent expanding stem tissue, suggesting that there was little or no transport of the exogenous Brassinolide from these tissues into the stem (data not shown).

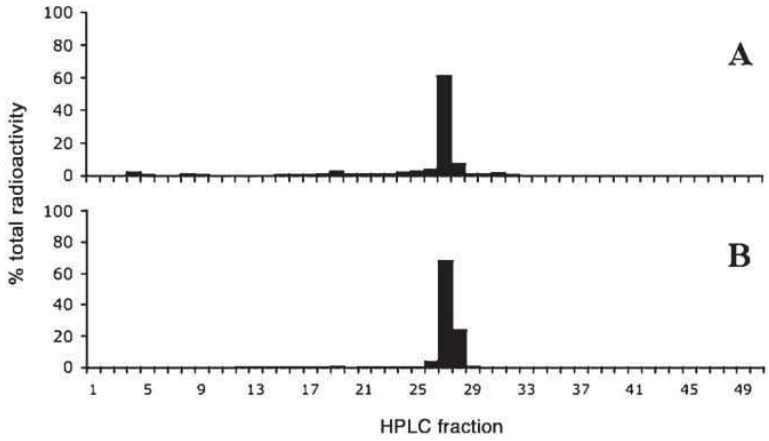


Figure 7-3 HPLC chromatogram

A) HPLC chromatogram showing the retention time for authentic ^{3}H-Castasterone. Peak height was determined by HPLC radiocounting.
B) HPLC chromatogram showing the metabolism of ^{3}H-Castasterone 48 h after application to mature leaf tissues of wild-type plants. A total of 1 X 10^{6} dpm of ^{3}H-Castasterone was applied to the leaflets at node 9 of intact wild-type plants. After 48 h, all leaf tissue (including the stipules, tendrils, and petioles) at node 9 was harvested, homogenized, and extracted in 80 % methanol. Metabolism of ^{3}H-Castasterone was analyzed by HPLC radio-counting. Similar results were obtained when ^{3}H-Castasterone was applied to the apical bud and when ^{3}H-Brassinolide was applied to either leaves or the apical bud (data not shown).

III. CONCLUSIONS

The study shows that many of the classical plant hormones undergo some form of long-distance transport around the plant. In contrast, the results from this study provide no evidence for the long-distance transport of endogenous Brassinosteroids.

The available evidence, including the occurrence of late-pathway Brassinosteroids in a wide range of plant tissues, the apparent lack of Brassinosteroid transport between these different tissue types and the expression of genes involved in Brassinosteroid biosynthesis, suggest that Brassinosteroids may be synthesized and act at least in the same tissues, or perhaps even in the same cells.

Based on the above-described studies, it could therefore be concluded that long distance transport of aerial applied Brassinosteroids is unlikely to occur, and if, only very slow movement is expected.

Metabolic Pathway of 24-Epibrassinolide and Brassinosteroids in plants

The metabolic/catabolic pathway of 24-Epibrassinolide and Brassinosteroids in plants has not yet been completely elucidated. Nevertheless, some studies have been performed on the metabolic pathway in different plants and fungi. There is no uniform degradation pathway but different plant and fungal species or groups have their own pathways. As Brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

The plant metabolism process of 24-Epibrassinolide includes hydrogenation, hydroxylation, esterification and glycosylation (Table 7.2.1-10; Bajguz *et al*., 2007; Fujioka and Yokota, 2003). The metabolic pathway is not completely known and strongly depends on the plant species, the plant organ and the developmental stage. In tomato cells, the major metabolites of 24-epibrasinolide are 25-Hydroxy-24-Epibrassinolide and 6-Hydroxy-24-Epibrassinolide that is further metabolised to 25-β-D-glucosyloxy-24-epiepibrasinolide and 25-hydroxy-24-Epibrassinolide (Hai *et al*., 1995; Schneider *et al*., 1994). In seradella cells, 24-Epibrassinolide is converted to

3,24-diepibrasinolide which is further metabolised to a mixture of 3-laurate, 3-myristate and 3-palmitate (Kolbe *et al*., 1995). In cucumber, 24-Epibrassinolide is metabolized to 2,24-diepibrassinolide in the second leaves and petioles, but not in hypocotyls and roots (Nishikawa *et al*., 1995).

Different homeostatic mechanisms exist to maintain the Brassinosteroid equilibrium, including the feedback inhibition of Brassinosteroid production. The spatial and temporal regulation of its homeostasis in the different plant tissues or on cellular level is crucial for normal growth and development (Symons *et al*., 2008). Most of the Brassinosteroid-spesific biosynthesis genes (DET2, DWF4, CDP, BR6ox1 and ROT3) are feedback regulated (Saini *et al*., 2015). In addition, catabolic inactivation is also considered to play a role in the regulation of bioactive Brassinosteroid levels (Fujioka and Yokota, 2003).

Brassinosteroids are catabolically altered or conjugated, with some modifications yielding inactive products. Various processes like acylation, sulphonation, glycosylation hydroxylation etc. play a role in maintaining the optimum levels of bioactive Brassinosteroids in the cells. Several genes and enzymes have been identified to be involved in the Brassinosteroid inactivation processes for example the *Arabidopsis thaliana* cytochrome P450 monooxygenase, which causes catabolic inactivation through hydroxylation (Winter *et al*., 1997). Another class of Brassinosteroid conjugates, which are inactive, are glucosides. Whilst enzymes mediating C-2 glucosylation of Brassinosteroids are still unknown, it was shown by Husar *et al*. (2011) that 23-Oglucosylation of Brassinosteroids in *A. thaliana* is catalyzed by UGT73C5, a UDP-glycosyltransferase (UGT).

It is also known that Brassinosteroids are metabolized by certain microorganisms. 24-Epicastasterone and 24-Epibrassinolide for example are transformed by the fungus *Cunninghamella echinulata* to give the corresponding 12β-hydroxylated compounds (eg. 12β-hydroxy-24-Epibrassinolide) (Voigt *et al*., 1993).

The following table lists metabolic reactions of 24-Epibrassinolide as described in literature.

Table 7.2.1-10: Metabolic reactions of 24-Epibrassinolide (Fujioka and Yokota, 2003; Bajguz *et al*., 2007)

Reaction	Site	Substrate	Product	Plant	Reference
Hydrogenation	2α-OH	24-Epibrassinolide	2,24-diepibrassinolide	Cucumber *(C. sativus)*	Nishikawa *et al*., 1995
	3αOH	24-Epibrassinolide	3,24-diepibrassinolide	Serradella (*O. sativus)*	Kolbe *et al*., 1996
Hydroxylation	C-12	24-Epibrassinolide	12β-hydroxy-24-Epibrassinolide	Fungi *(C. echinulata)*	Voigt *et al*., 1993
	C-20	3,24-diepbrassinolide	20-hydroxy-3,24-diepibrassinolide	Serradella (*O. sativus)*	Kolbe *et al*., 1996
	C-25	24-Epibrassinolide	25-hydroxy-24-Epibrassinolide	*Tomato (L. esculentum)*	Winter *et al*., 1997 Hai *et al*., 1995 Schneider *et al*., 1994
	C-25	3,24-diepibrassinolide	25-hydroxy-3,24-diepibrassinolide	Serradella (*O. sativus)*	Kolbe *et al*., 1996
	C-26	24-Epibrassinolide	26-hydroxy-24-Epibrassinolide	Tomato *(L. esculentum)*	Winter *et al*., 1997 Hai *et al*., 1995 Schneider *et al*., 1994
Side chain cleavage	C-20/22	3,24-diepibrassinolide	3,24-diepibrassinolide C_{21}-catabolite	Serradella (*O. sativus)*	Kolbe *et al*., 1996
Esterification	3β-OH	3,24-diepibrassinolide	3,24-diepibrassinolide-3-palmitate ($R = C_{15}H_{31}$) 3,24-diepibrassinolide-3-myristate ($R = C_{13}H_{27}$) 3,24-diepibrassinolide-3-	Serradella (*O. sativus)*	Kolbe *et al*., 1995

Reaction	Site	Substrate	Product	Plant	Reference
			laurate ($R = C_{11}H_{23}$)		
Glycosylation	25-OH	25-hydroxy-24-Epibrassinolide	25-hydroxy-24-Epibrassinolide-25-*O*-β-glucoside	Tomato *(L. esculentum)*	Winter *et al*., 1997 Hai *et al*., 1995 Schneider *et al*., 1994
	26-OH	26-hydroxy-24-epibrassinolide	26-hydroxy-24-Epibrassinolide-26-*O*-b-glucoside	Tomato *(L. esculentum)*	Winter *et al*., 1997 Hai *et al*., 1995 Schneider *et al*., 1994

Data point addressed:	CA 6.2.1/04
Author(s) (year):	Nishikawa, N., Shida, A., Toyama, S. (1995)
Title:	METABOLISM OF 14C-LABELED EPIBRASSINOLIDE IN INTACT SEEDLINGS OF CUCUMBER AND WHEAT
Laboratory report / project Number (Doc. No.):	Not applicable (092-087)
Testing facility:	Not applicable
Published:	Yes (Journal of Plant Research, 1995, 108, 65-69)
Test guideline used:	Not indicated
Deviations:	None
GLP:	No

Executive Summary
The metabolism of exogenously applied ^{14}C-24-Epibrassinolide in seedlings of cucumber and wheat was examined. Total lipids were extracted with isopropanol and chloroform, and then partitioned with water. More than 80 % of radioactivity was distributed in the chloroform-phase. The concentrated chloroform-phase was applied to a silica gel plate and was developed with chloroform-ethanol (5:1 v/v), R_f (retardation factor) value of original, ^{14}C-24-Epibrassinolide was approximately 0.6. In harvested 2 day-culture cucumber leaves, three peaks were detected at R_f 0.11, 0.47 and 0.84. In harvested 2 day-culture cucumber petioles, however, a major peak was detected at R_f 0.90. However, ^{14}C-24-Epibrassinolide was hardly metabolized in hypocotyls and roots after 2 days.

In wheat leaves, harvested just after pulse labelling, a peak was detected at R_f 0.63. By further analysis of this peak using ODS-HPLC, however, an original peak of ^{14}C-24-Epibrassinolide and two metabolites having higher polarity were detected. In wheat leaves harvested after 2 day-culture, the profile of TLC scanning was similar to that just after pulse labelling, although, an original peak of ^{14}C-24-Epibrassinolide was no longer detected by ODS-HPLC. In wheat roots, ^{14}C-24-Epibrassinolide was hardly metabolized. These results indicate that ^{14}C-24-Epibrassinolide occurring in leaves and petioles is metabolized to produce several kinds of metabolites.

I. MATERIALS AND METHODS

A. Materials

1. Plant materials
Seeds of wheat (*Triticum aestivum* L, cv. Norin 61) and cucumber (*Cucumis sativus* L, cv, Tokiwajibai) were sown in vermiculite under natural light at 20 to 32 °C. After 7 to 10 days, young seedlings were transferred to hydroponic culture. Wheat seedlings were cultured in Kimura-si-A medium (Kimura 1931) containing 100 mg/l of SiO_2. Cucumber seedlings were cultured in Otsuka No. 1 and No. 2 (Otsuka Kagaku Co., Ltd.) medium that are nutrient solutions prepared for hydroponic cultures. Approximately 20-day-old, plants were used as experimental materials.

2. Brassinosterold
^{14}C-24-Epibrassinolide (58.78 Ci/mol) was supplied from Nippon Kayaku Co., Ltd. ^{14}C-24-Epibrassinolide was stored in 99 % ethanol and diluted as required for individual experiments. 24-Epibrassinolide was labelled on the 4th carbon on the ring A.

B. Study design and methods

1. Treatment with brassinosteroid
Roots of two cucumber seedlings were immersed in 10 ml of distilled water containing 5.6 µCi of ^{14}C-24-Epibrassinolide for 3 h, and roots of 4 wheat seedlings in 10 ml of distilled water containing 11.2 µCi of, ^{14}C-24-Epibrassinolide for 6 h. After pulse labelling, the roots were washed with distilled water three times to remove the excess ^{14}C-24-Epibrassinolide, and were then cultured in a culture solution in a growth chamber under 16.5 W/m^3 of light intensity, day and night 14 h/10 h, and at 25 °C. The 2nd leaves, petioles of the 2nd leaves, hypocotyls, and the roots of the cucumber, as well as the 2nd leaves and roots of wheat were harvested and stored at -20 °C.

2. Estimation of radioactivity in a plant

After measuring the fresh weight, the leaves, petioles, hypocotyls, and roots were dried at 60 °C for 24 h. To estimate the radioactivity, the dried tissue was cut into small pieces and added to 10 ml of a scintillation fluid (Toluene-PPO). Radioactivity was counted by a liquid scintillation counter (Packard TRI-CARBIR> liquid scintillation spectrometer model 3255).

3. Extraction of metabolites

Leaves (2 to 4 g), petioles (2 to 3 g), hypocotyls (3 to 4 g), and roots (2 to 3 g) were extracted twice with boiled iso-propanol, isopropanol-chloroform (1:1, v/v) and then chloroform. The mixed extracts were partitioned with distilled water twice. Radioactivity in the chloroform phase and the aqueous phase was counted with a liquid scintillation spectrometer. The chloroform phase was concentrated below 40 °C *in vacuo* to give the total lipids.

4. Thin-layer chromatography

The total lipids were applied to a silica gel plate (Silicagel 70FM-Plate Wako, Wako Pure Chemical Ind. Ltd.), and were developed with chloroform-ethanol (5; 1 v/ v). Radioactive spots on each lane of the TLC plates were detected by a radio scanner (Aloka JTC-201). Radioactive spots, in which R_f values were about 0.6, were recovered from the TLC plates, concentrated *in vacuo*, and then prepared for HPLC.

5. High-performance liquid chromatography

A Shimadzu Model LC-4A chromatograph equipped with a radio analyser (Aloka RLC-551) was employed. A reversed-phase column of STR ODS-II (150 mm X 4.0 mm I.D., Shimadzu Techno Research, INC.) was used at 45 °C. The mobile phase was acetonitrile-water (45:55 v/v) at a flow-rate of 1.0 ml/min.

II. RESULTS

The uptake of ^{14}C-24-Epibrassinolide in each tissue was examined. The radioactivity in each tissue was almost unchanged during the 7 days of culture (data not shown). In every tissue of the cucumber, more than 80 % of radioactivity was distributed in the chloroform phase (Table 7.2.1-11). The metabolites were present in the chloroform phase.

Table 7.2.1-11: Percentage of radioactivity in chloroform phase and aqueous phase

Plant material	Culture days	Percentage of radioactivity (%)	
		CHCh-soluble	H20-soluble
Cucumber			
Leaf	0	90.6	9.4
	2	86.5	13.5
	7	83.2	16.8
Petiole	0	88,3	11.7
	2	95.5	4.5
	7	96.6	3.4
Root	0	97.1	2.9
	2	94.4	5.6
	7	83.9	16.1
Wheat			
Leaf	0	92.2	7.8
	2	82.2	17.8
	7	78.0	22.0
Root	0	98.5	1,5
	2	70,7	29.3
	7	67.0	33.0

Profiles of TLC-scannings of the total lipids extracted from different tissues of cucumber cultured for 2 days were strikingly different among the tissues. In the 2nd leaves, three peaks were detected at R_f 0.11, 0.47 and 0.84. Besides ^{14}C-24-Epibrassinolide, a metabolite having higher polarity was also detected by further HPLC analysis of the peak 2.

From a calculation of the peak area, 88.6 % of ^{14}C-24-Epibrassinolide in the 2nd leaves was metabolized. In the 2nd leaves of cucumber cultured for 7 days, the profile of TLC-scanning was similar to that of the two day

cultured. ^{14}C-24-Epibrassinolide, however, was not detected any longer by HPLC (data not shown). In roots, a peak was detected at R_f 0.55. In petioles, two peaks were detected at R_f 0.64 and 0.90, and the rates of peak area were 28.5 % and 71.5 %, respectively.

In hypocotyls, a peak was detected at R_f 0.59. From a time-course experiment, ^{14}C-24-Epibrassinolide was not metabolized in leaves after 3 h of pulse labelling. The metabolites in leaves, however, were detected after 1 day (data not shown).

In TLC-scanning of total lipids extracted from the 2nd leaves of wheat a peak was detected at R_f 0.63. Besides ^{14}C-24-Epibrassinolide, two metabolites having higher polarity, were detected by HPLC. However, ^{14}C-24-Epibrassinolide was completely metabolized to a compound having higher polarity. Although a peak having less polarity, which was detected in cucumber leaves, was not detected, a peak having higher polarity (R_f=0.11) was also detected by TLC after 7 days. In roots of wheat, the radioactivity in the aqueous-phase increased up to 33 % after 7 days, however, the compound in the chloroform phase was ^{14}C-24-Epibrassinolide (data not shown).

III. DISCUSSION AND CONCLUSION

In cucumber, ^{14}C-24-Epibrassinolide was metabolized in the 2nd leaves and petioles after 2 day-culture, but not in hypocotyls and roots. In wheat, ^{14}C-24-Epibrassinolide was completely metabolized in leaves after 2 day-culture, but some 64.5 % of ^{14}C-24-Epibrassinolide was not metabolized in roots even after 7 day-culture. These results indicate that ^{14}C-24-Epibrassinolide metabolism probably takes place in leaves and petioles.

In this study, the radioactivity was mainly distributed to the chloroform-phase, however, the chemical structure of these metabolites have not been identified yet. The metabolism of Brassinosteroids was occurring primarily in the shoots. Both polar and less polar metabolites were detected in cucumber, but the former were only detected in wheat. It is uncertain why the metabolism in shoots and roots differs.

Although the physiological activity of these metabolites in intact plants has not been verified yet, it is well known that Brassinosteroids promote the elongation of cucumber hypocotyl. In this study, a less polar metabolite was detected after elongation. Therefore, it seems that a triggering role of Brassinosteroid for hypocotyl elongation is plausible in the case of cucumber. The metabolite in the cucumber petioles seems to be hardly transported, because of less polarity. Therefore, it was suggested that this metabolite was not transported from leaves, but metabolized in petioles. In wheat the original ^{14}C-24-Epibrassinolide was gradually metabolized to compounds having higher polarity during culture.

Data point addressed:	CA 6.2.1/05
Author(s) (year):	Kolbe, A., Schneider, B., Porzel, A., Adam, G. (1996)
Title:	METABOLISM OF 24-EPI-CASTASTERONE AND 24-EPI-BRASSINOLIDE IN CELL SUSPENSION CULTURES OF ORNITHOPUS SATIVUS
Laboratory report / project Number (Doc. No.):	Not applicable (092-086)
Testing facility:	Not applicable
Published:	Yes (Phytochemistry, 1996, 41 (1), 163-167)
Test guideline used:	Not indicated
Deviations:	None
GLP:	No

Executive Summary
Exogenously applied 24-Epibrassinolide and 24-Epicastasterone are transformed by cell suspension cultures of *Ornithopus sativus* in a multistep pathway to give 20-keto-pregnane derivatives. As intermediates of these degradations 20*R*-hydroxy-3,24-bisepibrassinolide and 20*R*-hydroxy-3,24-bisepicastasterone, respectively, as well as 20/i-dihydroxy-5a-pregnane-6,20-dione were isolated from the culture medium and their structures were elucidated by means of 1HNMR and mass spectrometry. In addition to side-chain cleavage, 25-hydroxylation to give 25-hydroxy-3,24-bisepibrassinolide was also observed.

I. MATERIALS AND METHODS

1. Radiochemicals and measurement of radioactivity
[5,7,7-^{3}H]24-Epibrassinolide and [5,7,1-^{3}H]24-Epicastasterone with sp. act. of 10.3 and 4.8 Mβq/mmol respectively, were used in the experiments described in this paper. Radioactivity was measured by liquid scintillation counting (LSC). Before LSC the solid samples were incinerated. The radioactive zones of the TLC plates were analyzed with an automatic TLC linear analyzer. For quantification of metabolites the ratio of peak areas from TLC radioscans were used.

2. Cell cultures and application.
The suspended cells of *Ornithopus sativus* cultures were grown in a Linsmaier-Skoog medium at 26 °C on a gyratory shaker (125 r.p.m) in the dark in 300 ml Erlenmeyer flasks containing 150 ml cell suspension. Subculturing was performed every 7 days using an inoculum of approximately 40 ml.

The ethanolic solution (< 1 ml) of [5,7,7-^{3}H]24-Epicastasterone and [5,7,7-^{3}H]24-Epibrassinolide, respectively, (final concentration: 3.3 µM) were added to the cell suspension cultures at day 4 of the growth cycle. The cell suspensions were held under identical conditions for another 24 h, 36 h or 4 days, respectively.

3. Isolation and purification of compounds 4,5,11 and 16
The cells were harvested by suction filtration through a nylon mesh. The filtrate (culture medium) was extracted with $CHCl_3$. The $CHCI_3$ extract was purified after concentrating *in vacuo* by TLC and reversed-phase HPLC.

4. *Isolation and purification of compound 3*
The cells were homogenized with an Ultra-turrax grinder at room temperature in 80 % aqueous, MeOH, filtered and washed with MeOH. The aqueous solution remaining after concentration of the combined filtrates *in vacuo* at less than 40 °C was extracted with $CHCI_3$. The $CHCI_3$ was removed *in vacuo* and the residue was extracted with Me_2CO. The Me_2CO fraction contained fatty acyl esters and compound 3, which was separated by TLC and reversed-phase HPLC.

5. Spectrometry methods.
EI-MS (70 eV) was performed with an AMD 402 mass spectrometer. NMR experiments were carried out on a VARIAN UNITY 500 spectrometer it 499.84 MHz (^{1}H) using a NALORAC 3 mm micro-sample inverse-detection probe. $CDCl_3$ was used as solvent. Two-dimensional COSY, HMQC and HMBC spectra were recorded according to standard VARIAN pulse programs.

II. RESULTS AND DISCUSSION

Tritium-labelled 24-Epicastasterone and 24-Epibrassinolide (3.3 µM), respectively, were applied to sterile cell suspension cultures of *0. sativus* (Brot.) at day 4 of the growth cycle. After an incubation period of 24 h, 36 h and 4 days, respectively, the cells were separated from the medium and both fractions were individually studied. As shown in Fig. 7.2.1-2, there was a clear compartmentalization of the different types of metabolites between the cells (about 75 % of the radioactivity after application of **1** and 60 % for **2**) and the medium (25 % and 40 %, respectively). While in the medium mainly non-conjugated metabolites were found, the cells contained a major fraction of lipophilic and a minor fraction of hydrophilic conjugates. The lipophilic conjugates recently were identified as fatty acyl esters of 3,24-bisepicastasterone (**7-9,** please see 7.2.1-2) and 3,24-bisepibrassinolide, respectively (**13-15**).
The hydrophilic compounds, probably glycosides were not further investigated. The culture medium, both after application of **1** and **2**, respectively, contained several non-conjugated metabolites, which belong to metabolic sequences leading to 2α,3β-dihydroxy-B-homo-6a-oxa-5α-pregnane-6,20-dione (**12**) and 2α,3β,6β trihydroxy-5α-pregnane-20-one (**6**), respectively (Figure 7.2.1-2).

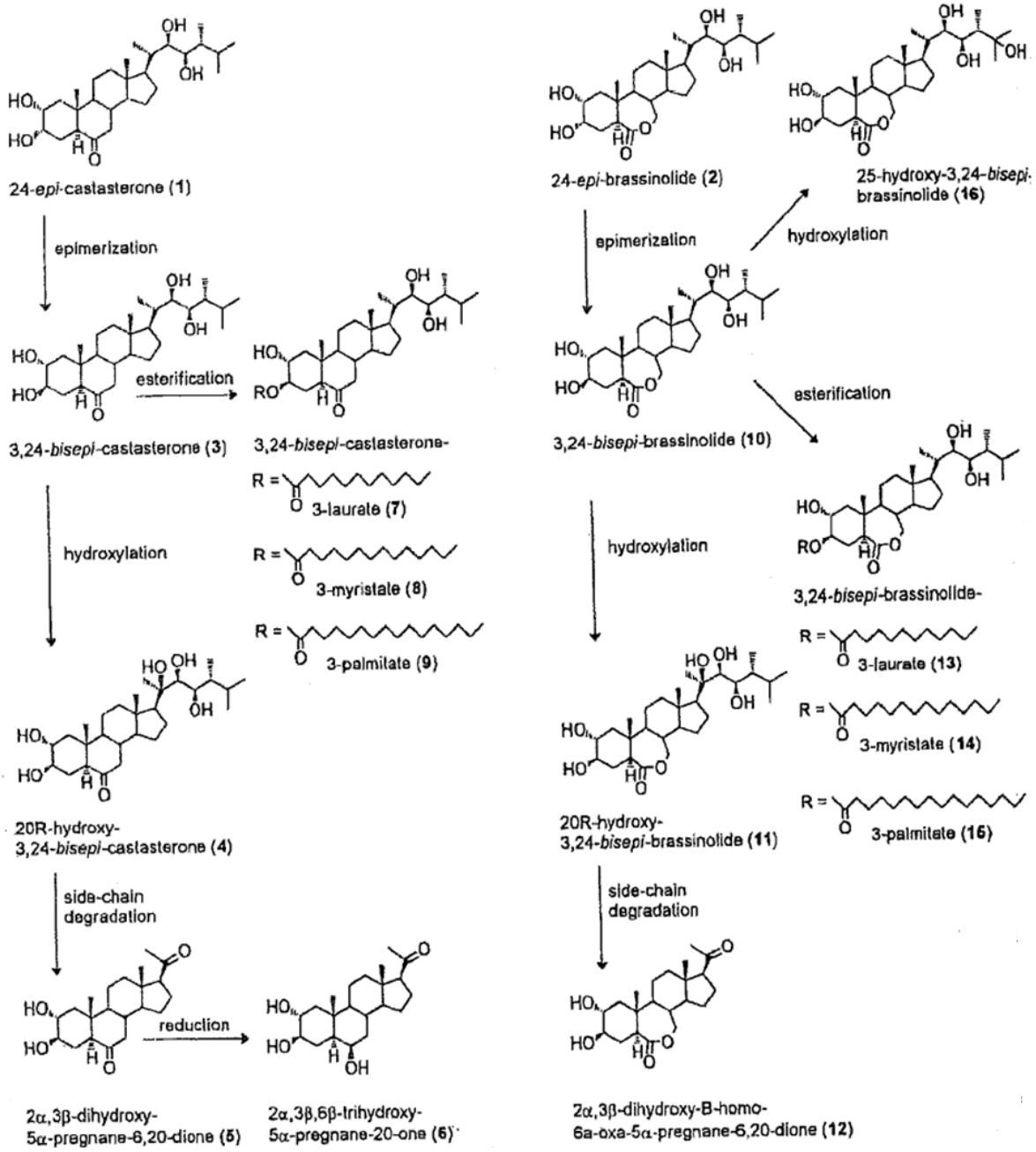


Figure 7.2.1-4 The metabolism of 24-Epicastasterone and 24-Epibrassinolide in cell suspension cultures of *Ornithopus sativus*

Each degradation product was assigned a number which are referred to in the text (**bold**).

In experiment 1 the cells were harvested 36 h after application of 24-Epicastasterone (**1**). The chloroform extract of the filtrate contained 75 % of the radioactivity of the culture medium. TLC indicated one radioactive peak with R_f*0.24*. Further purification and separation were achieved using TLC followed by reversed-phase HPLC (gradient 2, R_t 17.9 min), The compound with R_t 17.9 min was found to be 20*R*-hydroxy-3,24-bisepicastasterone (**4**). The EI-mass spectrum of 20*R*-hydroxy-3,24-bisepicastasterone showed the parent peak, an intensive peak at m/z 349, indicating predominant fission between C-20/C-22 and peaks owing to a stepwise loss of three H_2O from this fragment.

The ^{1}HNMR spectrum of 20*R*-hydroxy-3,24-bisepicastasterone showed only three methyl doublets (δ 0.95, 0.93 and 0.87) but three methyl singlets (δ1.34, 0.84, 0.83), indicating hydroxylation at C-20 or C-24. In the multiple-bond ^{1}H-^{13}C shift correlation experiment (HMBC) the carbon signal at δ 54.9 exhibits correlation peaks, to both methyl singlets at δ 1.34 and δ 0.84. Therefore, these methyl signals had to be assigned to methyl-21 and methyl-

18, respectively, because only these two methyl signals are expected to give a mutual correlation to one carbon signal (C-17).

Methyl-21 gives two further correlations with the carbon signals at δ 77.8 and δ 75.4. One of these signals (δ 75.4) shows a correlation with a proton signal (δ 3.35) in the direct ^{1}H-^{13}C shift correlation experiment (HMQC), whereas no correlation for the ^{13}C signal at 5 δ 77.8 were found. Thus, the latter signal was assigned to the quaternary carbon C-20. Further detailed analysis of the ^{1}H-^{1}H (COSY), direct ^{1}H-^{13}C (HMQC) and long-range ^{1}H-^{13}C (HMBC) shift-correlation two-dimensional NMR spectra yielded the assignments of almost all proton and carbon signals. The results of NOE difference suggests, as most probable, the 20*R*-configuration. The halfwidth of the ^{1}H multiplet of H-3 (Δ½ = 24Hz) and the upfield shift of H-5α in comparison with 24-Epicastasterone (δ 2.32 and 2.69, respectively) prove the epimerization at C-3.

The cells obtained in experiment 1 (cell harvest 36 h after application of 24-Epicastasterone) were extracted with 80 % aqueous methanol. After purification of the chloroform fraction of this extract by means of TLC (R_f 0.25) and reversed-phase HPLC (gradient 3), the fraction with R_t 25.4 min was found to be 3,24-bisepicastasterone (**3**). The HNMR signals of 3,24-bisepicastasterone were assigned by comparison with the ^{1}H chemical shifts of 24-Epicastasterone. While the side-chain signals of 3,24-bisepicastasterone are identical to those of 24-Epicastasterone, the A-ring signals are different. In particular the upfield shift of H-3 (δ 3.39, *ddd)* and the ^{1}H-^{1}H coupling constants (11.7/9.0/4.9 Hz) indicate an axial position of this proton and hence epimerization at C-3.

The chloroform extract of the cell culture medium obtained 24 h after administration of 24-Epibrassinolide (experiment 2) was subjected to TLC. The radioscan indicated a major labeled zone with *Rf* 0.49 and a minor one with *Rf* 0.38. The latter radioactive fraction was purified twice by reversed-phase HPLC (gradient 1, R_t 10-13 min; gradient 2, R_t 15 min; see methods). Structure elucidation of the obtained compound by ^{1}H-^{1}H-COSY and EIMS revealed 20*R*-hydroxy-3,24-bis-Epibrassinolide **(11).** The EI mass spectrum showed the parent peak and the peak due to the fission between C-22/C-23 both of very low intensity. As was found for 20*R*-hydroxy-3,24-bisepicastasterone (**4**)**,** the fission of 20*R*-hydroxy-3,24-bis-epibrassinolide **(11)** takes place predominantly between C-20/C-22 followed by stepwise loss of H_2O. Considering chemical shifts and coupling patterns, the ^{1}HNMR spectrum of 20*R*-hydroxy-3,24-bis-epibrassinolide **(11)** shows side-chain methyl signals nearly identical to those of 20*R*-hydroxy-3,24-bisepicastasterone (**4**)**,** indicating a 20β-hydroxylation for this metabolite also. Again, H-3 exhibits a large linewidth and H-5α *(δ* 2.90) a high-field shift of about 0.2 ppm in comparison with 24-bisepibrassinolide **(2).** Thus, H-3 has to be axial and therefore in the β-position.

As previously described, 3,24-bisepi-brassinolide **(10)** *(*R_f 0.49; gradient 1, R_t 16.6 min) has been isolated from the chloroform extract of the medium. From the same TLC zone another metabolite was purified by reversed-phase HPLC (gradient 1, R_t 12.4 min). Structure elucidation by El-mass spectrometry and ^{1}HNMR revealed 25-hydroxy-3,24-bisepibrassinolide **(16).** The El-mass spectrum showed the parent peak and peaks resulting from the fission between C-22/C-23 and stepwise loss of three H_2O. In contrast to the 20-hydroxy compounds **4** and **11,** preferred fragmentation of **16** occurred between C-22 and C-23. Thus, the fragments m/z 379 and m/z 365, respectively, represent diagnostic ions of pen-tahydroxylated Brassinosteroids, derived from 24-bisepibrassinolide **(2),** for 25-hydroxylation in the first case and for 20-hydroxylationin the second. The 'HNMR methyl signals of **16** show the typical chemical shifts and coupling patterns for an 24-Epibrassinolide side-chain with 25-hydroxylation (two doublets at *δ* 1.00 and 0.82; four singlets at *δ*1.30,1.28, 0.98 and 0.71). The chemical shift of H-5α and the large halfwidth of the H-3 signal indicate epimerization at C-3.

In experiment 3 the cells were harvested 4 days after application of 24-epi-castasterone **(1).** In this experiment the chloroform extract of the medium contained 70 % of the radioactivity of the culture medium. TLC indicated two radioactive peaks with *Rf* 0.22 and *Rf* 0.38 in the ratio 2:1. Purification was achieved using TLC followed by reversed-phase HPLC (R_t 10.2 and R_t 11.4 min, isocratic mode). As previously reported, the compound with R_t 10.2 min was 2α,3β,6β-trihydroxy-5α-pregnane-20-one **(6)**. On the basis of El-mass spectrometry and ^{1}HNMR analysis, the structure of the compound with R_t 11.4 min was proved to be 2α,3β-dihydroxy-5α-pregnane-6,20-dione **(5).** The El-mass spectrum showed m/z 348 (38) $[M]^+$ and *m/z* 330 (24) $[M\text{-}H_2O]^+$. The $_{1}$HNMR spectrum of 2α,3β-dihydroxy-5α-pregnane-6,20-dione **(5)** exhibited only two methyl singlets in the high-field methyl region (δ 0.81 and 0.62), indicating degradation of the side chain. The methyl singlet at *δ*2.13 is assigned to methyl-21 in a 20-keto-pregnane side-chain moiety. The halfwidth of the H-3 proton signal (25 Hz) as well as the ^{1}H chemical shift of H-5α (*δ* 2.34. as derived from the ^{1}H-^{1}HCOSY spectrum) prove the epimerization at C-3, Only two proton signals appear in the low-field region (*δ* 3.61 H-2β; *δ* 3.40 H-3α). In addition, no significant shift of the proton signals of H-5α and methyl-19 were observed compared with **4,** indicating that the 6-keto group in **5** was unchanged.

III CONCLUSION

In this report four new metabolites of exogenously applied Brassinosteroids in cell suspension cultures of *Ornithopus sativus* were described. 20-Hydroxy-3,24-bisepicastasterone (4) and 2α,3β-dihydroxy-5α-pregnane-6.20-dione **(5),** isolated after application of 24-epicastasterone, are intermediates of the degradation pathway to 2α,3β,6β-trihydroxy-5a-pregnane-20-one **(6).** These metabolites complete the metabolic scheme of 24-epicasterone in *Ornithopus sativus* cell suspension cultures. First, 24-epi-castasterone **(1)** is epimerized at C-3 to 3,24-bisepicastasterone **(3).** This epimerization probably follows a redox mechanism via the corresponding 3-ketone. In the following step hydroxylation at C-20 takes place. This 20-hydroxy compound **(4)** is split between C-20/C-22 to give 2α,3β-dihydroxy-5α-pregnane-6,20-dione **(5).** Reduction of the latter compound at the 6-keto-group in the final step revealed 2α,3β,6β-dihydroxy-5a-pregnane-20-one **(6).**

The metabolism of 24-Epibrassinolide **(2)** follows an analogous pathway except the reduction of the 6-keto group in the last step, which is not operating with the lactone structure. Assuming an oxidative mechanism of this side-chain cleavage, 2-hydroxy-3,4-dimethylpentanoic acid should be expected as a side-chain fragment. An analogous compound, 4-hydroxy-4-methylpentanoic acid was found as a metabolite of 20-hydroxyecdysone in insects and in crustaceous organisms. However, in these experiments no pentanoic acid fragment could be detected because of the lack of a label in the side chain. The hydroxylation at C-20 is especially remarkable, yielding a hitherto unknown type of pentahydroxylated Brassinosteroid.

Both 3,24-bisepicastasterone **(3)** and 3,24-bisepibrassinolide **(10)** are branching points in the metabolism of **1** and **2.** In addition to the pregnane pathway, conjugation with fatty acids to acyl conjugates **7-9** and **13-15,** respectively, was observed. Furthermore, in the experiments with 24-Epibrassinolide **(2),** hydroxylation at C-25 occurred. This reaction is well known for other steroid compounds, e.g. for ecdysones, and was recently observed by us also for **1** and **2** in tomato cell cultures. In contrast to tomato, 25-hydroxylation 25-hydroxylated compound **16** was found in the nonglucosylated state.

These results demonstrate close similarity to the metabolism of ecdysones in insects. Like *O. sativus* cell suspension cultures, insects are able to split the bond between C-20 and C-22. Insects are also able to epimerize the hydroxyl-group of ecdysone at position C-3 and to hydroxylate the molecule at position C-20. In contrast to the cell suspension cultures of *O. sativus,* insects do not form conjugates with fatty acids at position C-3, but at C-22.

Data point addressed:	CA 6.2.1/06
Author(s) (year):	Winter, J., Schneider, B., Strack, D., Adam, G. (1997)
Title:	ROLE OF A CYTOCHROME P450-DEPENDENT MONOOXYGENASE IN THE HYDROXYLATION OF 24-EPI-BRASSINOLIDE
Laboratory report / project Number (Doc. No.):	Not applicable (092-097)
Testing facility:	Not applicable
Published:	Yes (Phytochemistry, 1997, 45 (2), 233-237)
Test guideline used:	Not indicated
Deviations:	None
GLP:	No

Executive Summary
24-Epibrassinolide, exogenously applied to cell suspension cultures of *Lycopersicon esculentum* is hydroxylated at C-25 and C-26, respectively, followed by glucosylation of the newly formed hydroxyl group. Treatment of the cell cultures with the specific cytochrome P450 inhibitors, clotrimazole and ketoconazole, resulted in a strong decrease of only the C-25 hydroxylation, whereas hydroxylation at C-26 was not affected. The common cytochrome P450 inducers, ethanol, $MnCl_2$, phenobarbital, pregnenolone 16a-carbonitrile or clofibrate, did not induce hydroxylation activity at C-25 or at C-26. In addition, substrate analogues (22S,23S-homobrassinolide, 24-Epicastasterone, ecdysone, and 20-OH-ecdysone) were not accepted. Only application of 24-Epibrassinolide and Brassinolide resulted in an increased activity of both the C-25- and C-26-hydroxylases. For further examination of the molecular level of this inducing effect, the influence of the protein biosynthesis inhibitor cycloheximide has been studied. Thus, increase of both hydroxylase activities is obviously based on gene expression by action of the substrates, 24-Epibrassinolide and Brassinolide.

I. MATERIALS AND METHODS

1. Radiochemicals and measurement of radioactivity.
[5,7,7-^{3}H]24-Epibrassinolide sp. act. of 80 MBq/mmol was obtained from Dr A. Kolbe. [l-^{14}C]. Lauric acid with a sp.act. of 2.15 GBq/mmol was obtained from Amersham. Radioactivity was measured by liquid scintillation counting (LSC.). TLC plates were analysed for radioactive compounds with an automatic TLC linear analyser. For quantitation of metabolites, the ratios of peak areas from TLC radioscans were used. All measurements were performed at least twice. The data shown came from one representative experiment.

2. Biochemicals.
Clotrimazole, pregnenolone 16a-car-bonitrile, cycloheximide, and 12-hydroxydodecanoic acid were obtained from Sigma; lauric acid, phenobarbital, clofibrate, ecdysone, 20-hydroxyecdysone and $MnCl_2$ from Fluka; ketoconazole from ICN, and Brassinolide from Beak Technologies Inc. 24-Epibrassinolide, 24-Epicastasterone and 22S,23S-Homobrassinolide were received from Dr B. Voigt (Halle).

3. Cell culture
Plant cell cultures of *Lycopersicon esculentum* Mill, were obtained from the cell culture laboratory of Prof. Zenk (Munich, Germany). The suspended cells were grown in Linsmaier-Skoog medium at 23 °C on a gyratory shaker under constant diffuse light (4.4 µmol m^{-2} sec^{-1}) in 140 ml conical flasks containing 40 ml cell suspension.

4. Administration of labelled 24-Epibrassinolide
EtOH solutions of [5,7,7-^{3}H]24-Epibrassinolide were administered to the cell cultures 72 h after subculturing under sterile conditions.

5. Administration of [1-^{14}C] lauric acid
Cell suspension cultures were incubated for 2 h with 2 mM of an n-hexane solution of [1-^{14}C]lauric acid (132 kBq $mmol^{-1}$).

6. Isolation of metabolites
The Brassinosteroids were purified, Lauric acid and 12-hyd-roxydodecanoic acid were isolated by TLC (Et_20-n-hexane-HCOOH 60:30:1).

7. Time-dependency

Cell suspension cultures were incubated with [5,7,7-^{3}H]24-Epibrassinolide for increasing time periods up to 72 h. The incubation was stopped by filtration of the cells that were subsequently extracted with MeOH.

8. Inhibition and induction

Cell suspension cultures were incubated with 50 µM of clotrimazole and ketoconazole, respectively, 8 h prior to the application of [5,7,7-^{3}H]24-Epibrassinolide. The incubation time was 12 h, which seemed to be optimal for detecting the substrate and the products. Thus, inhibitory effects could be well investigated. For inhibiting protein biosynthesis cycloheximide (7 mg in EtOH) was added to the cells 1 h prior to the application of the inducing compounds. Induction experiments were carried out by treating the cells with the inducing substance (dissolved in DMSO or EtOH) 24 h prior to the application of [5,7,7-^{3}H]24-Epibrassinolide. The incubation period was 8.5 h, which seemed to be optimal for investigating inducing effects, since not too much of the substrate was metabolized.

II. RESULTS AND DISCUSSION

The time course of metabolism of exogenously applied 24-Epibrassinolide in cell suspension cultures of *Lycopersicon esculentum* has been studied over 72 h. [5,7,7-^{3}H]24-Epibrassonolide was used in final concentrations of 1.5 µM. The absorption of radioactivity by cultured cells proceeds very rapidly. After 2 h 60 % of the supplied radioactivity was taken up. The conversion to the 25- and 26-β-D-glucopyranosyloxy derivatives, respectively, occurs between 8 and 24 h after administration of 24-Epibrassinolide (Table 7.2.1-12). After 24 h the substrate was totally converted in equal ratio of either product that did not change significantly up to 72 h. To investigate whether the metabolism of 24-Epibrassinolide is dose-dependent; experiments with different amounts of the substrate were carried out. It could be shown that raising the concentration of the substrate from 2 to 7 µM and 20 µM was followed by a dramatic decrease of the conversion to the 25- and 26-metabolites. The conversion rate with 7 µM was only 50 % compared with the value obtained with 2 µM. With a substrate concentration of 20 µM no products were detectable.

Table 7.2.1-12: Time dependent metabolism of exogenously applied [5,7,7-3H]24-Epibrassinolide to cell suspension cultures of *Lycopersicon esculentum*. The C-25- and C-26-metabolites were detected as their glucosides.

Time (h)	25-OH- %	26-OH- %	24-Epibrassinolide %
8	n.d.	n.d.	100
12	34	42	24
16	40	51	9
24	37	63	n.d.

The biosynthesis of Brassinosteroids is characterized by the introduction of various hydroxyl groups to the phytosterol precursor. At least some of these hydroxylations are assumed to be cytochrome P450 dependent, strongly suggesting a similar biosynthetic pathway as described for the ecdysteroid metabolism. To demonstrate the cytochrome P450 characteristics of both hydroxylases, cell suspension cultures were treated with the specific imidazole-based P450 inhibitors clotrimazole and ketoconazole. Both compounds only inhibited the conversion of 24-Epibrassinolide to the 25-hydroxy metabolite, whereas hydroxylation at C-26 was not negatively affected.

Cytochrome P450 enzymes are involved in biosynthesis and in detoxification processes of numerous substrates. Therefore, studies of the metabolism of both enzyme activities involved in the hydroxylation at C-25 and C-26 of 24-Epibrassinolide have been investigated for their deducibility by substances with diverse inducing spectra. Ethanol, $MnCl_2$ and phenobarbital are known for their ability to unspecifically induce numerous cytochromes P450. Thus, these compounds exhibit a broad spectrum of induction, in contrast to pregnenolone 16a-carbonitrile and clofibrate, which only have inducing effects on distinct cytochromes P450 and, therefore, show a narrow spectrum.

Table 7.2.1-13: Effect of cytochrome P450-inducing compounds on the C-25- and C-26-hydroxylation of exogenously administered [5, 7, 7-^{3}H]24-Epibrassinolide in cell suspension cultures of *Lycopersicon esculentum*. The C-25- and C-26-metabolites were detected as their glucosides. The control values were arbitrarily set 100 % for each metabolite

Compound	Concentration	C-25 Hydroxylase activity (%)	C-26 Hydroxylase activity (%)

Compound	**Concentration**	**C-25 Hydroxylase activity (%)**	**C-26 Hydroxylase activity (%)**
Ethanol	300 mM	110	91
Phenobarbitol	5 mM	70	84
$MnCl_2$	5 mM	60	63
Clofibrate	4 ρM	71	110
Pregnenolone 16a-carbonitrile	4 ρM	108	113
Ecdysone	4 ρM	88	94
20-Hydroxyecdysone	4 ρM	90	101

As shown in Table 7.2.1-13, both enzyme activities could not be increased by these commonly used P450-inducers. Furthermore, no substrate analogues, such as ecdysteroids (ecdysone, 20-hydroxyecdysone), nor even the Brassinosteroids 22S, 23S-Homobrassinolide and 24-Epicastasterone showed any positive effect on hydroxylase activities. Only the substrates 24-Epibrassinolide and Brassinolide increased the activity of the C-25- and C-26-hydroxylase, respectively. Thus, the induction of both hydroxylases seems to be highly specific and requires essentially the combination of the lactone and the (22R, 23R)-diol side-chain moiety. On the other hand, the configuration of the 24-methyl group seems not to be important, because both 24-epimers, Brassinolide and 24-Epibrassinolide, induced both hydroxylase activities. These structural requirements for an increase of C-25- and C-26-hydroxylase activities indicate a strict regulation. Such a conclusion is also verified by the observation that no hydroxylase activity could be detected after inhibiting protein biosynthesis by cycloheximide.

Furthermore, the possible hydroxylation by an unspecific cytochrome P450-dependent lauric acid-ω-hydroxylase could be excluded since no activity of this enzyme was detectable, employing [1-^{14}C]lauric acid. This also indicates that both hydroxylation reactions are involved in the natural pathway of these compounds. In addition, the inhibitory effect of substrate concentrations in a low micro-molar range (7 µM; 20 µM) supports this assumption.

The studies showed that hydroxylation at C-25 and C-26 is catalysed by two different enzymes. To confirm this observation, inhibitory studies were done, which demonstrated clearly that the specific cytochrome P450 inhibitors only affect hydroxylation at C-25. The increase in hydroxylase activity at C-26 was based on the availability of more substrate, since the competing enzyme, C-25-hydroxylase, is inhibited. These data indicate that the C-25-hydroxylase is a typical cytochrome P450-protein.

Data point addressed:	CA 6.2.1/07
Author(s) (year):	Hai, T., Schneider, B., Adam, G. (1995)
Title:	METABOLIC CONVERSION OF 24-EPI-BRASSINOLIDE INTO PENTAHYDROXYLATED BRASSINOSTEROID GLUCOSIDES IN TOMATO CELL CULTURES
Laboratory report / project Number (Doc. No.):	Not applicable (092-083)
Testing facility:	Not applicable
Published:	Yes (Phytochemistry, 1995, 40 (2), 443-448)
Test guideline used:	Not indicated
Deviations:	None
GLP:	No

Executive Summary
Two isomeric metabolites, 25-β-D-glucopyranosyloxy-24-Epibrassinolide and 26-β-D-glucopyranosyloxy-24-Epibrassinolide, have been formed in cell suspension cultures of *Lycopersicon esculentum* from exogenously applied 24-Epibrassinolide. The two-step metabolic process involved hydroxylation of the side-chain at C-25 and C-26, respectively, followed by glucosidation of the newly formed hydroxyl groups. The ratio between both metabolites was significantly altered by *in vivo* treatment of the cell cultures with various cytochrome P-450-specific inhibitors, indicating the involvement of two different enzyme systems. Biosynthetically prepared 25-hydroxy-24-Epibrassinolide, reapplied to cell cultures, was exclusively glucosylated at the 25-hydroxyl group, strongly suggesting regiospecificity of the corresponding glucosyltransferase.

I. MATERIALS AND METHODS

1. Radiochemicals and measurement of radioactivity
The synthesis of [5,7,7-^{3}H]24-Epibrassinolide **(1)** with spicific activity of 22.2 MBq mmol^{-1} was used. Radioactivity of all fractions was measured by liquid scintillation counting (LSC). TLC plates were analysed for radioactive zones with an automatic TLC linear analyser. For quantification of metabolites the ratios of peak areas from TLC radioscans were used.

2. Cell culture
Plant cell cultures of *Lycopersicon esculentum* were obtained from the cell culture laboratory Munich. The suspended cells were grown in Linsmaier-Skoog medium at 20 °C on a gyratory shaker (100 rpm) under constant diffuse light (600 lux) in 300-ml Erlenmeyer flasks containing 150 ml cell suspension. Subculturing was performed every 7 days using an inoculum of *ca*. 70 ml.

3. Administration of labeled precursors and inhibitors
The filter-sterilized ethanolic solutions (< 1 ml) of [5,7,7-^{3}H]24-Epibrassinolide **(1)** (final concentration of cell Suspension 10^{-5} M), [5,7,7-^{3}H]25-hydroxy-24-Epibrassinolide **(2)** (10^{-5} M), and [5,7,7,-^{3}H]26-hydroxy-24-Epibrassinolide **(4)** (10^{-5} M) were administered to the cell cultures at day 3 of growth. The cell suspensions were maintained under identical conditions for another 7 (following administration of **1**) and 4 (**2, 4**) days. Inhibitors were applied simultaneously with the Brassinosteroids as filter-sterilized ethanolic or aqueous solutions (100 µl). The air and gas stream composed of CO and O_2, or N_2, respectively, were sterilized by filtration and bubbled through the cell suspensions with a flow rate of 4 ml min^{-1}.

4. Isolation and purification of metabolites
Compound **3** (TLC, $CHCl_3$-MeOH, 9:1 and 4:1) has been isolated by published standard procedure. Compound **5** was isolated and purified as described for **3** with the following changes: reverse phase HPLC (Nucleosil C18; 10 µm, 250x10 mm; flow rate 4 ml min^{-1}; detection UV 204 nm and LSC of aliquots: gradient MeCN-H_2O from 1:3 to 9:11 in 15 min). Following enzymatic hydrolysis, Compounds **2** and **4**, respectively, were subjected to preparation. TLC (CH_2Cl_2 -MeOH, 9:1) and reverse phase HPLC (Nucleosil C8; 10 µm, 250x10 mm; flow rate 4 ml min^{-1}; detection UV 204 nm and LSC of aliquots: MeCN-$H_2$0 2:3 for 2 (*R*, 8.9 min); MeCN for 3 (*R*, 7 min).

6. Spectrometric methods
FAB-MS was performed with an AMD 402 mass spectrometer; negative ionization 4 kV; positive ionization 9 kV. NMR spectra were recorded on a Varian Unity 500 spectrometer at 499.84 MHz (^{1}H) and 125.7 MHz (^{13}C) in CD_3OD, with TMS as intitial standard.

II. RESULTS

In the following, description of the respective results covers only the conversion of 24-Epibrassinolide, as further information is not needed for description of the catabolic pathway, covered in this dossier part.

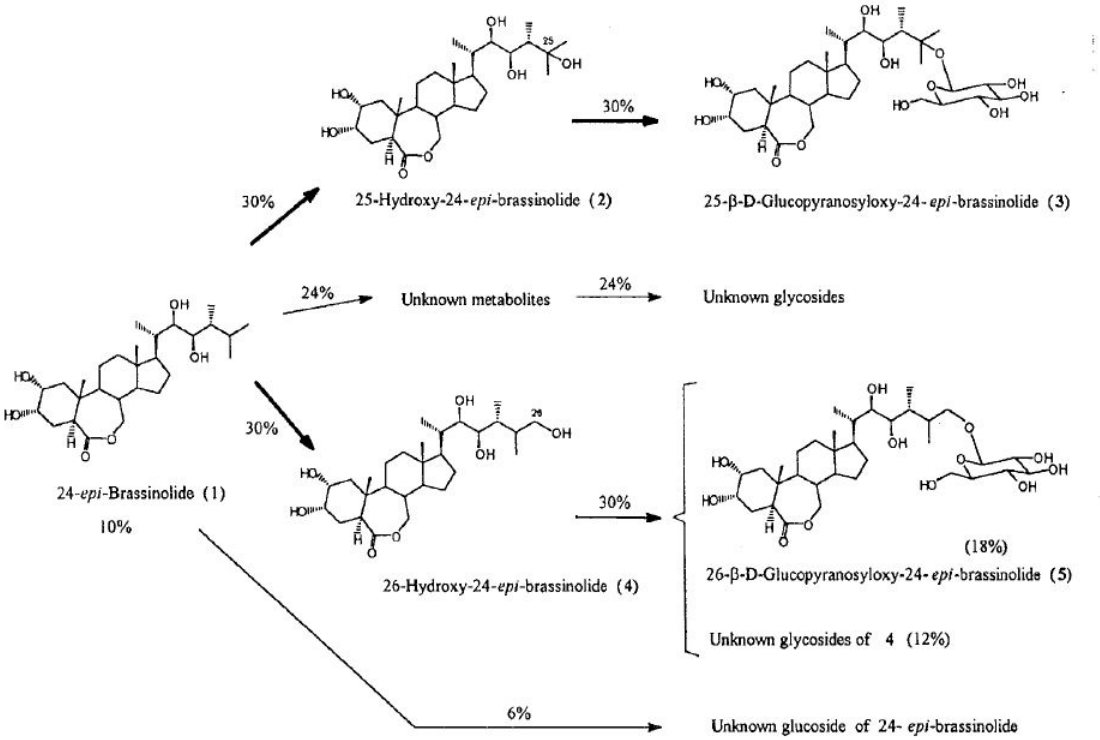


Figure 6.2.1-3: Metabolism of 24-Epibrassinolide (1) in cultured cells of *Lycopersicon esculentum*

Each degradation product was assigned a number which are referred to in the text (**bold**).

As shown by TLC of the cell extracts, 24-Epibrassinolide (**1**), after administration to cell suspension cultures of *Lycopersicon esculentum,* was converted into several hydrophilic metabolites. The TLC radioscan of an 80 % aqueous methanol extract obtained at day 4 of a characteristic experiment exhibited two major peaks of compounds **3** (30 %, R_f 0.46) and **5** (30 %, R_f 0.32). The structure of Compound **3** has recently been reported as 25-β-D-glucopyranosyloxy-24-Epibrassinolide. Compound **5** is accompanied by several other glycosides. The glycosidic character of this product was shown by enzymatic hydrolysis using cellulase as well as ß-glucosidase to give the aglycone **4** (R_f 0.32). The major compound of this fraction (60 %) was purified by chromatographic methods including reverse-phase HPLC (R_t 8.5 min) as the final step. The FAB-mass spectrum of **5** resembled that of compound **3**. Both spectra exhibited identical fragmentation patterns and relative intensities with negligible differences between the corresponding peaks, suggesting that both compounds were isomers bearing an additional hydroxyl group in comparison with the parent compound **1**: (negative ionization) m/z 657 $[M - H]^-$ and 495 [aglycone - H]$^-$; (positive ionization) m/z 659 $[M + H]^+$ and 681 $[M + Na]^+$, aglycone fragments m/z 497 $[aglycone + H]^+$, 479 $[aglycone + H - H_2O]^+$, 461 $[aglycone + H - 2H_20]^+$, 443 $[aglycone + H - 3H_20]^+$ and 409 (bond fission between C-23 and C-24). These data indicated an additional fifth hydroxyl group within the aglycone, located in the terminal part of the side-chain.

The NMR analysis of **5** (^1H-^1H COSY, HMBC, HMQC) confirmed an unchanged sterol ring system and the position of the new hydroxyl function at one of the terminal methyl groups. As was assumed for similar side-chain hydroxylations in the ecdysteroid series, this newly functionalized methyl group may be C-26. The NMR data also indicated a β-D-glucosidic bond between 26-OH and the sugar moiety. Thus, the structure of **5** was shown to be 26-β-D-glucopyranosyloxy-24-Epibrassinolide. Both glucosides **3** and **5** bear the glucose moiety at the new hydroxyl group. Thus, the metabolic conversion of **1** to **3** and **5**, respectively, is a two-step process including hydroxylation and subsequent glucosidation (Figure 6.2.1-3). Different from this finding, the known 23-*O*-glucosides of Brassinosteroids are formed without preceding hydroxylation. Obviously, the pentahydroxylated Brassinosteroids **2** and **4** are much better substrates for glucosylation than **1**. This was

supported by the observation that compounds **2** and **4**, after an incubation period of four days, were not detectable in free form in the cultured cells but were completely glucosylated, while 10 % of the parent compound **1** remained non-metabolized. 24-Epibrassinolide (**1**) was directly glycosylated in small amounts to an unknown glucoside which was characterized by enzymatic hydrolysis. Furthermore, several highly hydrophilic minor glycosides of unknown structure were detected.

III. CONCLUSION

The superior bioactivity of 25-hydroxy-24-Epibrassinolide (**2**), and the regiospecificity of both the C-25 hydroxylase and 25-*O*-glucosyltransferase, suggested that metabolite **2** and its 25-*O*-glucoside **3** are not detoxification products of exogenously applied 24-Epibrassinolide (**1**) but could be regarded as final members of the biosynthetic chain of Brassinosteroids.

Data point addressed:	CA 6.2.1/08
Author(s) (year):	Schneider, B., Kolbe, A., Porzel, A., Adam, G. (1994)
Title:	A METABOLITE OF 24-EPI-BRASSINOLIDE IN CELL SUSPENSION CULTURES OF LYCOPERSICON ESCULENTUM
Laboratory report / project Number (Doc. No.):	Not applicable (092-092)
Testing facility:	Not applicable
Published:	Yes (Phytochemistry, 1994, 36 (2), 319-321)
Test guideline used:	Not indicated
Deviations:	None
GLP:	No

Executive Summary
Exogenously applied 24-Epibrassinolide is converted into 25-β-D-glucosyloxy-24-Epibrassinolide by cell suspension cultures of *Lycopersicon esculentun*. The structure was fully elucidated by FAB-MS and NMR analysis.

I. MATERIALS AND METHODS

Radiochemicals and measurement of radioactivity
Compound 1 with a sp. act. of 22.2 M Bq $mmol^{-1}$ was used. Radioactivity of all fractionss was measured by liquid scintillation counting (LSG). Before TSC, the solid samples were incinerated. TLC plates were analysed for radioactive zones by means of an automatic TLC linear analyser. For quantification of metabolites the ratios of peak areas from TLC radioscans were used.

Cell culture and administration of 1
Plant cell cultures of *L. esculentum* were obtained from the cell culture laboratory in Munich. The suspended cells were grown in Linsmaier-Skoog medium at 20 °C on a gyratory shaker (100 rpm) under constant diffuse light (600 lux) in 300-ml Erlenmeyer flasks containing 150 ml cell suspension. Subculturing was performed every 7 days using an inoculum of ca 70 ml, filter-sterilized ethanolic solutions (< 1 ml) of [5,7,7-^{3}H]24-Epibrassinolide (final concentration of cell suspension 10^{-5} M) were administered to the cell cultures on day 3 of growth. The cell suspensions were maintained under identical conditions for another 7 days.

Isolation and purification of 3
The cells were harvested by suction filtration through a nylon mesh, homogenized with an ultra-turrax homogenizer at room temp, in 80 % aqueous MeOH, filtered and washed with MeOH, The aqueous solution, remaining after concentration of the combined filtrates *in vacuo* at less than 40 °C, was purified by absorption chromatography (XAD-2). The aqueous efflux, the 40 % aqueous MeOH and the MeOH eluates from the column contained no significant radioactivity and were discarded. The 60 % aqueous eluate was subjected to ion exchange chromatography (DEAE A-25, elution with MeOH). The combined radioactive fractions were purified by TLC (Merck silica gel 60; 0.5 mm layer for preperation mode; silica gel sheets for analytical mode; twice developed in MeOH-$CHCl_3$ 9:1 and 4:1) and reversed phase HPLC (Nucleosil C18; 10 pi; 250 x 4 mm; flow rate 4 ml min detection UV 204 nm and LSC of aliquots; gradient MeCN-H_2O from 3:7 to 1:1 in 15 min).

Enzymatic hydrolysis
A sample of **3** (50 kBq) was dissolved in 1 ml Mcllvain buffer, pH 4.0 and incubated with 1 mg cellulase for 20 hr at 37 °C. The EtOAc extract of this solution contained 41 kBq. Radio-TLC indicated the presence of radioactive **2**.

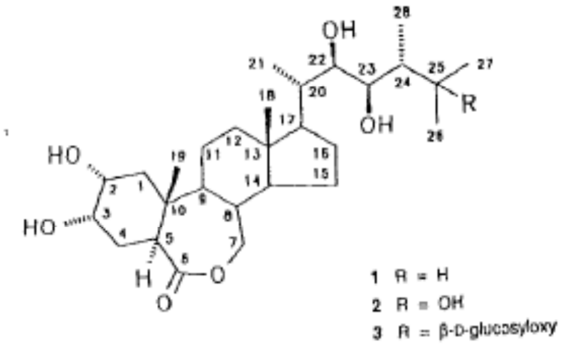


Figure 7-5 Structure of 24-Epibrassinolide (1) 25-hydroxy-24-Epibrassinolide (2) and 25β-D-glucosyloxy-24-Epibrassinolide (3)

Each degradation product was assigned a number which are referred to in the text (**bold**).

Spectrometric methods

FAB-MS: AMD 402 mass spectrometer; neg, ionization 4kV; pos. ionization 9kV. NMR: 499.84 MHz (^{1}H) and 125,7 MHz (^{13}C in CD_3OD, TMS as international standard.

II. RESULTS

24-Epibrassinolide (**1**), was applied to sterile cell suspension cultures at day 3 of the growth cycle. Compared with untreated controls, **1** did not influence normal cell growth when applied in 10^{-5} M concentration. As indicated by radioactivity measurements after application of [5,7,7-^{3}H]24-Epibrassinolide, **1** was rapidly taken up by the cells. In a characteristic experiment, after four days only 7.5 % of the recovered radioactivity was still found in the medium. The remainder was absorbed by the cells (methanol extract 88.3 %; insoluble material 4.2 %). Up to day 7 of the experiment, this ratio showed little change, i.e. 5.8 % of radioactivity in the medium and 94.2 % in the cells (88.4 %; 5.8 %) on day 7. At this time, the cells were harvested and extracted with 80 % aqueous methanol. Radio-TLC indicated nearly complete conversion of the substrate to several hydrophilic compounds. On day 7 of the experiment, **1** (R_f 0.86) was detectable only in traces. The major component (29 % of the extracted radioactivity, R_f 0.38), when subjected to enzymatic hydrolysis using cellulase, yielded a less polar compound, **2** (R_f 0.7). This behaviour indicated a glycoside. After removal of matrix compounds by means of absorption chromatography, ion exchange chromatography, TLC and reversed phase HPLC (R_t 6.5 min) spectroscopic data indicated that the new metabolite was 25-β-D-glucosyloxy-24-Epibrassinolide (**3**).

The FAB mass spectra (negative ionization) confirmed both the additional hydroxyl group and the hexose moiety of 3: *m/z* 657 $[M-H]^-$ (rel. int. 100) and 495 [aglycone - H]$^-$ (17). The positive FAB mass spectrum showed the molecular ion peaks m/z 659 $[M+H]^+$ (rel. int. 11) and 681 $[M+Na]^+$ (38), and aglycone fragments *m/z* 497 [aglycone + H]$^+$ (11), 479 [aglycone + H – H_2O]$^+$ (23), 461 [aglycone + H - $2H_2O$]$^+$ (9) and 443 [aglycone + H - $3H_2O$]$^+$ (6). Furthermore, a typical fragment at *m/z* 409 (100), due to bond fission between C-23 and C-24, indicated an unchanged Brassinosteroid ring system with a vicinal 22,23-diol function. Therefore, the fifth hydroxyl must be situated in the terminal part of the side chain beyond C-23.

The ^{1}HNMR spectrum of **3** showed two methyl doublets and four methyl singlets, two of them at remarkably low field (δ 1.36 and 1.30). In the low field region 14 proton signals were found, five of them identical in chemical shifts and coupling patterns with the ^{1}H NMR signals of H-2ß, H-3β, H-5α, H-7α and H-7β of 1. Thus, ring A and B of **3** have to be unchanged in comparison with the parent compound **1**. As indicated in the ^{1}H-^{1}H COSY spectrum, seven low-field proton signals belong to a sugar unit, the coupling constants of the sugar protons indicated the presence of β-D-glucose. The remaining low-field signals at 53.67 and 3.54 gave a mutual cross-peak in the homonuclear COSY experiment. The signals could be assigned to H-22 and H-23, respectively, by a one-bond ^{1}H-^{13}C shift correlation experiment, since the assignments of the corresponding carbon signals were known from the multiple-bond ^{1}H-^{13}C shift correlation spectrum (HMBC). The assignments of the carbon signals were based on the HMBC experiment and known ^{1}HNMR assignments via ^{1}H-^{13}C shift correlation. The HMBC spectrum allowed the unambiguous assignment of all side chain carbon signals, as well as the position of the sugar moiety. The quaternary carbon signal at δ84.1 could be assigned to C-25, indicating this position to be

hydroxylated. Furthermore, the HMBC correlation between the anomeric proton H-1' of glucose and C-25 proved the position of the sugar moiety at 25-OH. This was supported by ROESY cross-peaks between H-1' and H_3-26/H_3-27 indicating close spatial proximity of the sugar and these methyl groups. Thus, from this data, the structure of metabolite **3** is 25-β-D-glucosyloxy-24-Epibrassinolide.

III. CONCLUSION

25-β-D-glucosyloxy-24-Epibrassinolide represents the first Brassinosteroid of plant origin with a hydroxyl group in the C-25 position. Hitherto, only two synthetic Brassinosteroid-like compounds with 25-OH were mentioned which, however, carry no hydroxyl group at C-23, Furthermore, **3** is the first Brassinosteroid glucoside to not have the glucose moiety at 23-OH.

Data point addressed:	CA 6.2.1/09
Author(s) (year):	Voigt, B., Porzel, A., Naumann, H., Hoerhold-Schubert, C., Adam, G. (1993)
Title:	HYDROXYLATION OF THE NATIVE BRASSINOSTEROIDS 24-EPICASTASTERONE AND 24-EPIBRASSINOLIDE BY THE FUNGUS CUNNINGHAMELLA ECHINULATA
Laboratory report / project Number (Doc. No.):	Not applicable (092-096)
Testing facility:	Not applicable
Published:	Yes (Steroids, 1993, 58 (7), 320-323)
Test guideline used:	Not Indicated
Deviations:	None
GLP:	No

Executive Summary
24-Epicastasterone and 24-Epibrassinolide, two naturally occurring phytohormones of the Brassinosteroid type, were transformed by the fungus *Cunninghamella echinulata* to give the corresponding 12ß-hydroxylated compounds. The structures of these compounds were determined by spectroscopic methods, especially heterocorrelated two-dimensional nuclear magnetic resonance investigations. In the rice lamina inclination test the 12ß-hydroxylation lowered the bioactivity at 0.1 ppm to 10 % in comparison with that of the corresponding parent hormones. The described hydroxylations represent the first biotransformations of native Brassinosteroids.

I. MATERIAL AND METHODS

24-Epicastasterone (**1**) and 24-Epibrassinolide (**4**) were synthesized from ergosterol. Melting points were determined on a Boetius hot stage microscope and are corrected. Specific rotations were determined in methanol on a Zeiss Instrument Polamat A. Infrared spectra were recorded on a Zeiss spectrometer IR 75, and ultraviolet (UV) spectra were recorded on a Zeiss Specord UV-VIS instrument. ORD measurements were made on a Jasco spectropolarimeter ORD-UV 5. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker A M 500 and AC 300 instruments. The mass spectra were obtained with an AMD 402 instrument. Silica gel 60 (grain size 0.040 to 0.063 mm, Merck) was used for column chromatography. Preparative high-performance liquid chromatography (HPLC) was performed on a Knauer instrument on LiChrospher 100 RP 18.5 /im with MeOH/H_2O 7:3 v/v and UV detection at 210 nm. A culture of fungus strain *C. echinulata* IMEX 43918 taken from the collection of the Institute of Microbiology and Experimental Therapy, Jena, was maintained on agar slants: malt extract 4 %, yeast extract 0.3 %, agar 1.5 %. Cultures inoculated onto this medium were incubated at 25 °C for 5 to 7 days. For the precultivation, the cells were washed off into 500-ml shake flasks containing 100 ml of medium as described above with the exception of agar. The flasks were incubated on a rotary shaker (180 rpm) at 28 °C for 2 days. Ten milliliters of the preculture was transferred into shake flasks with 100 ml of the same medium. After 48 hours, 25 mg of Brassinosteroids **1** and **4**, respectively, dissolved in 1 ml of methanol were added. The flasks were reincubated for 5 days. After this incubation period, the entire medium was extracted for the isolation of the transformation products with n-butanol. The purification of the raw material was performed by column chromatography (SiOa) and by HPLC.

12ß-Hydroxy-24-epicastasterone (2)
Elution of the silica gel column with ethyl acetate/methanol 9:1 v/v first afforded 22 mg starting compound **1**. Further elution gave crude *12ß-Hydroxy-24-epicastasterone*, which was purified by preparative HPLC, leading to 14 mg (14 %, calculated for reacted **1**) of the more polar compound **2** with melting point (mp) 242-245 C (needles from methanol); $[\alpha]^{24}{}_D$7.3° (c=0.241, MeOH); IR (KBr), ν_{max}: 3,400 (OH), 1,705 cm^{-1} (ketone); UV (MeOH), λ_{max} : 282 nm (ε 190); ORD: $[\Phi]_{280}$ + 343°, $[\Phi]_{307}$ - 229°, a = - 6 ; EI-MS (m/z): 480 (M^+, $C_{28}H_{48}O_6$, 20%), 361 (480-C_6H_{13}O-H2O, 10 %), 343 (36I-H_2O, 100 %), 325 (343-H_2O, 62 %), 303 (480-C_9H_{19}O2-H_2O, 50 %); FABMS (m/z): 481 (M^+ + 1, 100%), 463 (481-18, 17 %), 445 (481-2 H_2O, 50 %); ^{1}H NMR (500 MHz, $CDCI_3$), (δ): 0.748 (s, 18-H_3), 0.754 (s, 19-H_3), 0.827 (d, J = 7.1 Hz, 28-H_3), 0.844 and 0.909 (d, J = 6.9 Hz and d, 6.9 Hz, 26-H_3 and 27-H_3), 1.072 (d, J = 7.2 Hz, 21-H_3), 2.68 (dd, J = 12.5/2.7 Hz, 5a-H), 3.35 (dd, J = 6.4/4.9 Hz, 23-H), 3.56 (dd, J = 10.9/4.6 Hz, 12a-H), 3.71 (ddd, J = 11.8/4.9/3.1 Hz, 2β-H), 3.75 (dd, J = 4,9/1.2 Hz, 22-H), 4.01 (ddd, J = 2.5/2.5/2.5 Hz. 3β-H). ^{13}C NMR.

Pentaacetyl derivative (3)
Compound **2** (10 mg) was acetylated with Ac_2O-pyridine (each 0 5 ml) for 20 hours at room temperature to give 9 mg (76%) **3** with mp 158 -161 C (from $CHCI_3$); $[\alpha]^{24}{}_D$-13.6°(c=0.214, MeOH); IR ($CHCI_3$), ν_{max}:1,720 cm^{-1}

(CO); UV (MeOH), λ_{max}: 279 nm, (ε 200); ORD: $[\Phi]_{272}$ + 1,210°, $[\Phi]_{302}$ - 962°, a = -22; EI-MS (m/z): 690 (M^+, 1 %), 630 (M^+ - AcOH, 100%); 588 (8%); 570 (M^+ - 2AcOH, 27 %); 555 (14 %); 528 (588 - AcOH, 18 %); 511 (555 – CO_2, 35 %); 487 (35 %); 445 (32 %); 427 (50 %); 415 (13 %); 385 (32 %); 367 (12 %); 343 (45 %); 325 (38 %). ^{1}H NMR (300 MHz, $CDCI_3$) (δ): 0.815 and 0.826 (2 x s, 18-H_3 and 19-H_3), 0.831, 0.848, 0.934, and 0.980 (4 x d, J = 6.9 Hz, 6.8 Hz, 6.9 Hz; 6.9 Hz, 21-H_3, 26-H_3, 27-H_3; and 28-H_3), 1.987, 2.037, 2.043, 2.070, and 2.087, (5 x s, 5 x OAc) 2.339 (dd, J=13.4/4.4 Hz, 7α-H), 2.568 (dd, J=11 8/3.9Hz, 5α-H), 4.664 (dd, J=10.8/4.6Hz, 12α-H). 4.915 (ddd, J=10.2/6.8/3.0 Hz, 2β-H), 5.029 (dd, J=6.4/5.8 Hz, 23-H), 5.283 (dd, J=6.5/1.2 Hz, 22-H), 5.373 broad (s, 3β-H).

12β-Hydroxy-24-epibrassinolide (5)
Elution of the silica gel column with ethyl acetate/methanot 9:1 v/v gave 19 mg starting material **4** and crude **5**, which was purified by preparative HPLC leading to 13 mg (12 %) of pure **5** with mp 218 to 221 C (needles from MeOH); $[a]^{26}_D$ + 25.7° (c=0.219, MeOH); IR (KBr), v_{max}: 3,400 (OH), 1,725 cm^{-1} (lactone); EIMS (mlz): 496 (M^+, $C_{28}H_{48}0_7$, 1%), 395 (M^+-C6H,,0, 32%), 377 (395-H,0, 62%), 341 (377-H,0, 64%); FAB-MS (m/z): 497 (M^+ + 1,48 %), 479 (497-H_2O, 24 %), 461 (479-H_2O, 75 %), 443 (461-H_2O, 33%), 377 (M^+ - $C_6H_{13}O$-H_20, 17 %).

Pentaacetyl derivative (6)
Compound **5** (10 mg) was acetylated with Ac_2O/pyridine (each 0.5 ml) for 20 hours to give 6 mg (65 %) **6** with mp 139 to 142 C (needles from $CHCL_3$/n-hexane); $[\alpha]^{25}_D$ + 29.6° (c = 0.227, MeOH) ; IR($CHCl_3$), v_{max}: 1,730, 1,720cm^{-1} (acetyland lactone); EI-MS (m/z): 706 (M^+, 1 %), 647 (M^+ - CH_3C0O^-, 2 %), 586 (M^+ - 2AcOH, 4 %), 564 (6 %), 544 (10 %), 527 (12 %), 503 (25 %), 461 (100 %), 443 (21 %), ^{1}H NMR (300 MHz, $CDCl_3$) (δ): 0.833, 0.848, 0.938, and 0.973 (4 x d, J=6.8 Hz, 6.7 Hz, 6.9 Hz, and 6.8 Hz, 21-H_3, 26-H_3, 27-H_3, and 28-H_3), 0.845 (s, 18-H_3), 0.976 (s, 19-H_3), 2.000. 2.030, 2.041, 2.077, and 2.133 (5 x s, 5 x OAc), 2.970 (dd, J=12.3/4.5 Hz, 5α-H), 4.018 (dd, J=12.6/9.2 Hz, 7α-H), 4.146 (br d, J=12.6 Hz, 7β-H), 4.606 (dd, J=10.4/4.6 Hz, 12α-H), 4.844 (ddd, J=12.4/4.7/2.6 Hz, 2β-H), 5.019 (dd, J=6.3/5.8 Hz, 23-H), 5.268 (dd, J=6.4/1.3 Hz, 22-H), 5.359, broad (s, 3/3-H).

II. RESULTS

The authors have studied the microbial transformation of the two naturally occurring Brassinosteroids 24-Epicastasterone (**1**) and 24-Epibrassinolide (**4**) by the fungus *C. echinulata* IMET 43918. This fungus is usually known for hydroxylations in 6ß-, 11α-, 11β-, 15β-, and 17α positions. Upon exposure of 24-Epicastasterone to the growing culture of this fungus after silica gel chromatography and HPLC purification, the hitherto not described 12β-hydroxylated compound **2** was isolated in 14% yield. The UV absorption maximum at 282 nm and the negative cotton effect with a = -6 shows the remained 6-carbonyl function in **2**. The EI and FAB mass spectra indicate the elemental composition of $C_{28}H_{48}O_6$. The ^{1}H NMR spectrum of **2** shows in comparison with that of **1** an additional double doublet in the low-field region (at δ 3.56). According to the found coupling constants (10.9 Hz, 4.6 Hz), this methine proton has to be axial; thus, the corresponding OH group must be equatorial. The assignment of the ^{1}H NMR signals of H_3-I8 and H_3-I9 was done by the direct heteronuclear ^{1}H, ^{13}C shift correlation two-dimensional (2D) NMR spectrum (XHCORRD) using the known ^{13}C chemical shifts of C-18 and C-19.

Furthermore, in the XHCORRD spectrum the proton signal at 3.56 ppm correlates with the carbon signal at 77.8 ppm. Assignment of this ^{13}C signal was done using a proton-detected multiple bond ^{1}H, ^{13}C shift correlation 2D NMR experiment (HMBC), which gives correlation peaks for protons and carbons in the case of ^{1}H-^{13}C couplings via two or three bonds. The found correlation of the ^{13}C Signal at 77.8 ppm with the proton signal of the 18 methyl group in the HMBC spectrum indicates the assignment C-12 for this carbon signal. In an HMBC spectrum, the ^{1}H 18-methyl signal is expected to give correlations with C-12, C-13, C-14, and C-17. The additional OH group is bound to one of these four carbons, but only C-12 gives a methine signal in this case. Thus, the position of the additional hydroxyl group is 12β. This is supported by the upfield shift of C-18 (δ 8.5; Δδ-3,2) in **2** compared with **1** (δ11.7), which is a result of the y-gauche effect of 12β-OH on methyl group 18. Assignment of ^{13}C NMR signals of **1** and **2** was done according to the strategy used for Brassinosteroids of the lactone series. The introduction of the additional OH function is also confirmed by formation of the pentaacetyl derivative **3** with a molecular ion at m/z 690 for the elemental composition of $C_{38}H_{58}O_{11}$ and the appearance of five acetyl singlets in its ^{1}H NMR spectrum. The double doublet at δ 4.66 (J = 10.8/4.6 Hz) for the axial 12α-H confirms the 12β configuration of the newly introduced oxygen function.

In the same manner as described for 24-Epicastasterone (**1**), 24-Epibrassinolide (**4**) was also microbially transformed to give after purification 12β-hydroxy-24-Epibrassinolide (**5**) with a molecular ion at m/z 496 in the

EI-MS spectrum for $C_{28}H_{48}O_7$. Compound **5** was characterized as its pentaacetyl derivative **6** (m/z 706), with NMR data corresponding with the given structure. The double doublet (J=10.4/4.6 Hz) at 4.61 ppm in the ^1H NMR spectrum of **6** (H-12α) especially verifies the presence of the 12β-function. For studies on structure-activity relationships, the highly sensitive rice lamina inclination test using the cultivar Koshirikari was applied as the assay for the new Brassinosteroids **2** and **5**. Preliminary experiments showed that, similar to the 12β-hydroxylated (22S,23S)-homobrassinolide, the activity of 12β-hydroxy-24-Epicastasterone (**2**) and 12β-hydroxy-24-Epibrassinolide (**5**) is lowered to 10 % at 0.1 ppm in comparison with that of the corresponding parent hormones **1** and **4**.

Data point addressed:	CA 6.2.1/10
Author(s) (year):	Husar, S., Berthiller, F., Fujioka, S., Rozhon, W., Khan, M., Kalaivanan, F., Elias, L., Higgins, G.S., Li, Y., Schuhmacher, R., Krska, R., Seto, H., Vaistij, F.E., Bowles, D., Poppenberger, B. (2011)
Title:	OVEREXPRESSION OF THE UGT73C6 ALTERS BRASSINOSTEROID GLUCOSIDE FORMATION IN ARABIDOPSIS THALIANA
Laboratory report / project Number (Doc. No.):	Not applicable (092-148)
Testing facility:	Not applicable
Published:	Yes (BMC Plant Biology 2011)
Test guideline used:	Not indicated
Deviations:	None
GLP:	No

Executive Summary
Brassinosteroids (BRs) are signaling molecules that play essential roles in the spatial regulation of plant growth and development. In contrast to other plant hormones, Brassinosteroids act locally, close to the sites of their synthesis, and thus homeostatic mechanisms must operate at the cellular level to equilibrate Brassinosteroid concentrations.

Whilst it is recognized that levels of bioactive Brassinosteroids are likely adjusted by controlling the relative rates of biosynthesis and by catabolism, few factors, which participate in these regulatory events, have as yet been identified. Previously the authors have shown that the UDP-glycosyltransferase *UGT73C5* of *Arabidopsis thaliana* catalyzes 23-*O*-glucosylation of Brassinosteroids and that glucosylation renders Brassinosteroids inactive. This study identifies the closest homologue of *UGT73C5*, *UGT73C6*, as an enzyme that is also able to glucosylate Brassinosteroids *in planta*.

This approach identified novel Brassinosteroid catabolites, which are considered to be Brassinosteroid-malonylglucosides, and provided first evidence indicating that glucosylation protects Brassinosteroids from cellular removal. The physiological significance of Brassinosteroid glucosylation, and the possible role of *UGT73C6* as a regulatory factor in this process are discussed in light of the results presented.

I. MATERIALS AND METHODS

Plant material and growth conditions
A. thaliana ecotype Columbia-0 (Col-0) was used as the wild type for all experiments carried out in this study. For phenotypic analysis, if not indicated differently, plants were cultivated in a growth room with long-day growth conditions (16 h white light, 80-100 $\mu mol \cdot m^{-2} \cdot s^{-1}$/8 h dark) at 21 ± 2 °C. ATS media was used for plant growth under sterile conditions.

Chemicals
Brassinolide and Castasterone were purchased from Synthchem Inc. (Waterloo, Ontario, Canada). 24-Epibrassinolide was obtained from Sigma-Aldrich (St. Louis, USA). Stock solutions of 100 µg/mL in ethanol were made and stored in amber screw vials at -20 °C. The BR-Glcs were stored in amber screw vials at -20 °C as 50 µg/mL stocks in ethanol. Water for LC was purified using a MilliQ system (Millipore, Molsheim, France). LC gradient grade methanol, acetonitrile and sodium chloride (p.a.) were purchased from Merck (Merck KGaA, Darmstadt, Germany). LCMS grade formic acid was obtained from Sigma-Aldrich (St. Louis, USA). Ethyl acetate was supplied by Carl Roth (Karlsruhe, Germany). Strata Si-1 silica gel SPE cartridges (500 mg, 6 mL) and security guard C18 precolumns were acquired from Phenomenex (Aschaffenburg, Germany).

Sample preparation for the analysis of metabolism of Brassinolide and Castasterone in plants
Fifty eleven-day-old seedlings of Col-0, *UGT73C6oe*, *UGT73C6ko* and *UGT73C5oe*, grown on ATS plates, were transferred to sterile Erlenmeyer flasks containing 30 ml liquid ATS media and incubated on a shaker (60 rpm) in continuous light (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$) conditions at 21 °C ± 2 °C. 24 h after transfer of the plants, Brassinolide or Castasterone were added to an end concentration of 1 µg/ml (2.1 and 2.2 µM, respectively) and the seedlings were incubated for the indicated periods of time. The plant material (on average 0.8 to 1.0 g) was

then harvested, ground in liquid nitrogen and extracted twice with 5 ml aqueous methanol (50+50, v+v). The methanolic plant extracts (10 ml) were dried down under a gentle stream of nitrogen at 40 °C and re-dissolved in 2.5 ml saturated NaCl solution and 2.5 ml water. Liquid/liquid extraction was performed 3 times with 5 ml ethyl acetate each. The ethyl acetate phases were combined, dried down under nitrogen and re-dissolved in 1 ml ethyl acetate. Strata Si-1 silica gel SPE cartridges were conditioned with 5 ml acetonitrile and equilibrated with 10 ml ethyl acetate before the sample was applied. The cartridges were washed with 5 mL ethyl acetate, removing most of the chlorophyll. The Brassinosteroids and their glucosides were eluted with 5 ml of ethyl acetate/methanol 20/80 (v/v), dried under nitrogen and reconstituted in 1 ml 70 % methanol for analysis by LC-HRMS. For the time course experiment samples were taken before and 6, 12, 24, 48 and 96 h after addition of Brassinolide (1 µg/ml).

Liquid chromatography high-resolution mass spectrometry (LC-HRMS) for the analysis of BRs and their glucosides

A LC-HRMS method was developed for the quantification of Brassinosteroids and BR-Glcs as well as their metabolites produced in plant tissues. An Accela HPLC pump (Thermo Fisher Scientific, Waltham, MA, USA) together with a Mistral column thermostat (Maylab, Thermo Fisher Scientific) and a PAL HTC autosampler (CTC Analytics, Zwingen, Switzerland) were coupled to a LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific). Separation was performed on a Hypersil Gold column (150 × 2.1 mm, 3 µm particle size; Thermo Fisher Scientific) at 25 °C. Gradient separation used water with 0.1 % formic acid as solvent A and methanol with 0.1 % formic acid as solvent B. 50 % B were kept for 1 min, then a linear gradient reached 100 % B at 12 min. After a 5 min washing step with 100 % B, the solvent composition was changed back to 50 % B within one min and the column was re-equilibrated till the end of the run at 32 min. A divert valve redirected the eluent into the ion source between 8 and 13 min to minimize unnecessary contamination of the MS. A flow rate of 250 µl/min was chosen, the injection volume was 5 µl.

Ionization was performed in the electrospray positive mode at 300 °C with the following settings: sheath gas flow 45, aux. gas flow 5, source voltage 4 kV, capillary voltage 5 V, tube lens 200 V. Centroid FTMS data were acquired from m/z 200-1000 with a resolution of 60.000. The sodium adduct of n-butylbenzenesulfonamide (nBBS; *m/z* 236.071570) was found to be ubiquitous in our system and was used as lock mass. Instrument control and data evaluation was performed with Xcalibur 2.0.7. For the latter, a mass tolerance of 5 ppm was allowed for the following masses: $[BL+Na]^+$ *m/z* 503.3343; $[CS + Na]^+$ m/z 487.3394; $[BL\text{-glucosides} + Na]^+$ *m/z* 665.3871; $[CS\text{-glucosides} + Na]^+$ *m/z* 649.3922. Retention times were: BL23G: 9.54 min; BL2G: 9.74 min; CS23G: 10.25 min; CS2G: 10.32 min; BL22G: 10.72 min; BL3G: 10.79 min; BL: 10.97 min; CS22G: 11.34 min; CS3G 11.50 min; CS 11.60 min. External standard calibration was performed with 1/x weighted models. While the parent substances Brassinolide and Castasterone showed highly linear correlations from 1-1000 ng/ml, quadratic models were used for all eight glucosides over the same concentration range. Recovery and repeatability was tested by spiking 100 ng/ml of all analytes in liquid media or methanolic extracts of untreated plants in quadruplicates before clean-up and measurement.

II. RESULTS

In the following, description of the respective results covers only the conversion of 24-Epibrassinolide, as further information is not needed for description of the catabolic pathway, covered in this dossier part.

UGT73C6 catalyzes 23-O-glucosylation of Castasterone and Brassinolide in Planta

UGT73C6 has previously been characterized as a UDP glucose:flavonol-3-O-glycoside-7-*O*-glucosyltransferase, based on a decrease in quercetin-3-O-rhamnoside-7-*O*-glucoside accumulation in flowers of a *UGT73C6* knockout (*UGT73C6ko*) line and a respective catalytic activity *in vitro*. To investigate the possibility that *UGT73C6*, in addition to its role in quercetin-3-O-rhamnoside glucosylation, can also catalyze Brassinosteroid glucosylation in planta, it was anticipated to analyze Brassinosteroid glucoside formation in plants altered in *UGT73C6* expression. For this purpose a LC-HRMS method was developed, which is outlined in the experimental procedures section. As reference standards Castasterone-2-*O*-glucoside (CS-2Glc), Castasterone-3-*O*-glucoside (CS-3Glc), Castasterone-22-*O*-glucoside (CS-22Glc), Castasterone-23-*O*-glucoside (CS-23Glc), Brassinolide-2-*O*-lucoside (BL-2Glc), Brassinolide-3-*O*-glucoside (BL-3Glc), Brassinolide-22-*O*-glucoside (BL-22Glc) and Brassinolide-23-*O*-glucoside (BL-23Glc) were used. The identification was based on retention times and mass spectra, by direct comparison of standards and metabolites. Recovery rates for all measured analytes were between 83 % and 93 % (except for Castasterone with 63 % recovery), with a repeatability ranging from 1.8 % to 3.9 %. Preliminary experiments showed that, endogenous Brassinosteroid glucosides were below the limit of detection in untreated plants, also with the newly developed LC-HRMS method. Thus, Brassinosteroid

glucoside formation was investigated in plants treated with Castasterone or Brassinolide. Ten-day-old light-grown seedlings of wild type and *UGT73C5oe* plants, as well as *UGT73-C6oe* and *UGT73C6ko* plants, were incubated in media containing either Castasterone or Brassinolide for 48 h and metabolites formed were measured by LC-HRMS. The results of the plant feeding studies showed that in plants over-expressing the *UGT73C6*, in correspondence with plants overexpressing *UGT73C5*, *CS-23Glc* and BL-23Glc formation was strongly increased (Table 7.2.1-14) whereas CS-2Glc and BL-2Glc levels appeared unaltered. In seedlings of *UGT73C6ko* plants no statistically significant differences in Brassinosteroid glucosylation activities to wild type were found. Interestingly *CS-23Glc* and *BL-23Glc* were not only present in plant extracts, but were also detected in the media, in which the plants had been incubated for the plant feeding studies (Table 7.2.1-14).

Table 7.2.1-14: Glucosides of Brassinosteroids measured in seedlings of *UGT73C5oe*, *UGT73C6oe* and wild type used in Brassinosteroid plant feeding studies

Brassinosteroids	**Col-0** ng/g fw	***UGT73C6oe*** ng/g fw
6-DeoxoCT	1.29/1.08	1.33/0.78
CT	n.d./ n.d.	n.d./ n.d.
6-DeoxoTE	0.06/0.03	0.07/0.02
6-Deoxo3DT	0.36/0.17	0.46/0.21
TE	n.d./ n.d.	n.d./ n.d.
6-DeoxoTY	0.78/0.84	0.62/0.82
TY	0.24/0.23	0.14/0.14
6-DeoxoCS	1.44/1.83	0.94/1.28
CS	0.60/0.50	0.31/0.25
BL	n.d./ n.d.	n.d./ n.d.

Kinetics of Brassinolide-23-Oglucoside formation

To investigate the conversion of Brassinolide into BL-23Glc in *UGT73C5oe* and *UGT73C6oe* plants over time, a timecourse feeding study was initiated. Eleven-day-old seedlings of wild type Col-0, *UGT73C5oe* and *UGT73C6oe* were incubated with 1 µg/ml (2.1 µM) of Brassinolide. Samples were harvested in a time-course manner and *BL-23Glc* formation was determined in tissue extracts by LC-HRMS. As shown in Figure 6.2.1-5a, Brassinolide was rapidly incorporated, as evidenced by a strong increase in endogenous Brassinolide amounts following Brassinolide application. In wild type seedlings Brassinolide levels increased rapidly for 12 h following Brassinolide application, before they started to decline. Brassinolide levels in *UGT73C5oe* and *UGT73C6oe* also increased for approximately 12 h post application of Brassinolide, however Brassinolide amounts only reached about 50 % of the levels, which were accumulated in wild type (Figure 6.2.1-5a). 96 h post application, Brassinolide levels in both wild type and *UGT73C5oe* and *UGT73C6oe* lines had dropped to levels below the limit of detection.

BL-23Glc formation in wild type seedlings slowly increased for 24 h, before BL-23Glc amounts started to decrease. In *UGT73C5oe* and *UGT73C6oe* plants the concentration of BL-23Glc strongly increased for approximately 12 h, reaching amounts which were approximately 10-fold higher, than those measured in wild type (Figure 7.2.1-4). In summary exogenously applied Brassinolide was rapidly incorporated by both wild type and *UGT73C5oe* and *UGT73C6oe* plants and was thereafter efficiently removed. In contrast, following its formation, BL-23Glc was maintained at elevated levels in plant tissues.

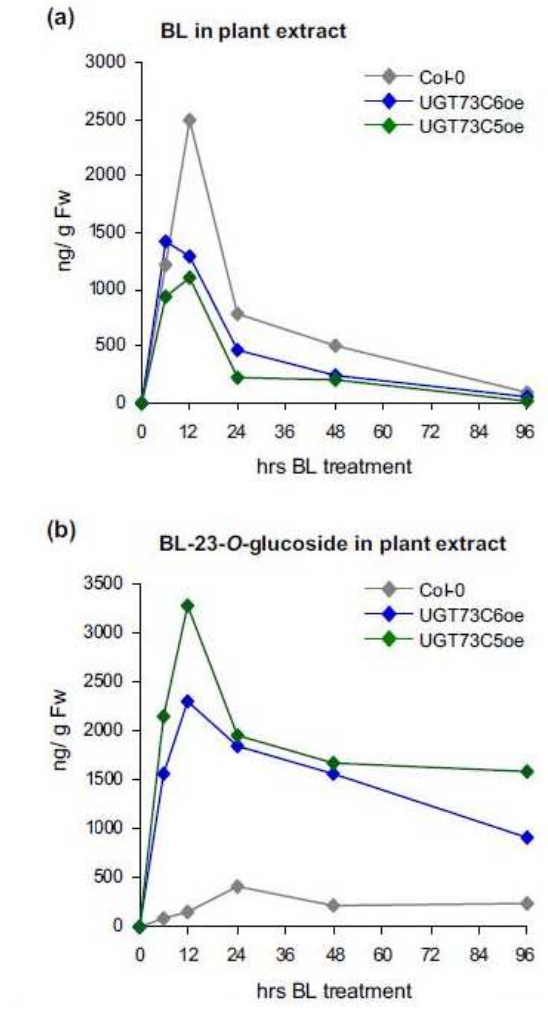


Figure 7.2.1-6: Brassinolide and BL-23Glc levels formed in seedlings of A. thaliana used in Brassinolide feeding studies over time, analyzed by LC-HRMS.

Eleven-day-old seedlings were incubated for the indicated periods of time in ATS media supplemented with 30 µg Brassinolide, and Brassinolide contents were quantified from plant extracts by LCHRMS analysis. (a) Brassinolide and (b) BL-23Glc levels are shown in ng/g Fw.

Catabolic Fate of Brassinosteroid-23-O-glycosides

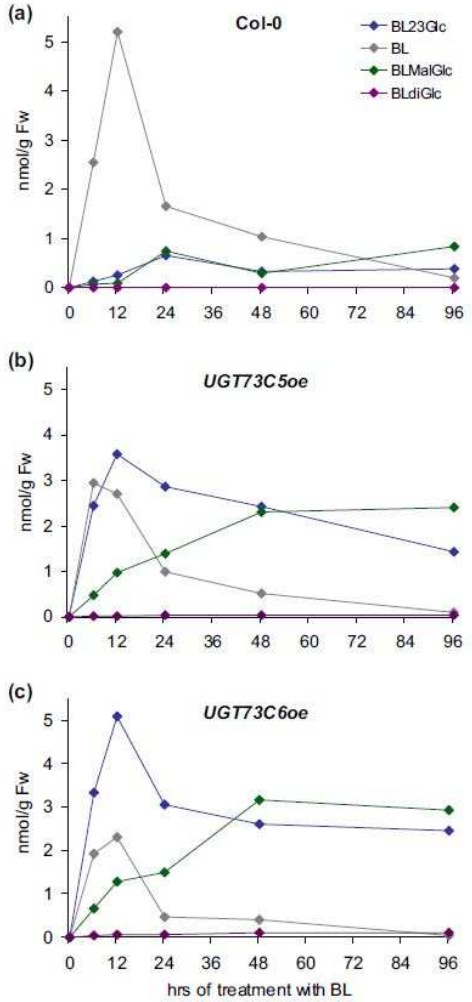
The decrease of BL-23Glc levels in plant tissues, starting at 12 h post application of Brassinolide in *UGT73C5oe* and *UGT73C6oe* seedlings and at 24 h in wild type, indicated that the *BL-23Glc* formed was either immobilized, degraded or was further modified to yield products, which escaped detection. Also, previously it had been shown that in Brassinolide-feeding studies of wild type *A. thaliana*, an initial increase in BL-23Glc formation was followed by a decrease, indicating a further metabolization. Thus it was of interest to investigate the catabolic fate of externally applied Castasterone and Brassinolide. LC-HRMS was used to analyze Brassinosteroid conjugates in seedlings of wild type, *UGT73C5oe* and *UGT73C6oe* plants, following 48 h of incubation with either Castasterone or Brassinolide. In addition to significant amounts of Brassinosteroid-23Glc, minor peaks corresponding to Brassinosteroid-2Glc, Brassinosteroid-sulfate and Brassinosteroid-hydroxide were found. Moreover, very interestingly, a previously unknown substance with a mass of *m/z* 751.3877 eluted at 9.61 min (compared to 9.54 min of BL-23Glc), in seemingly high abundance, from the column. According to accurate mass measurements the compound was tentatively identified as Brassinolide-malonylglucoside (BL-MalGlc). The theoretical mass of the sodium adduct of this substance is 751.3875 (0.2 ppm deviation), the only possible sum formula is $C_{37}H_{60}O_{14}$ (subtracting the sodium adduct; nitrogen rule applied; max. 1 ppm mass deviation; max. 10 nitrogen, 30 oxygen, 100 carbon and 200 hydrogen atoms). As malonylglucosides are formed from glucosides it is highly likely that the compound is Brassinolide-23-O-malonylglucoside (BL-23MalGlc). Similarly, when plants fed with Castasterone were analyzed for Castasterone-catabolites a peak at 10.29 min

(compared to 10.25 min of CS-23G), showing a *m/z* of 735.3929, appeared and was tentatively identified as Castasterone-malonylglucoside (CS-MalGlc). Only one sum formula is conceivable when applying the criteria outlined above, namely $C_{37}H_{60}O_{13}$ (mass deviation 0.4 ppm). Again, it seems highly likely that the substance is Castasterone-23-O-malonylglucoside (CS-23MalGlc). In addition to the putative Brassinosteroid-MalGlcs Brassinosteroid-diglucosides (BR-diGlc) were also identified. Interestingly, both the formation of the putative BR-MalGlcs and the Brassinosteroid-diglucoside was increased in UGT73C6oe and UGT73C5oe seedlings as compared to those of wild type indicating that they were formed from BR-23Glcs (Figure 6.2.1-6). In analogy to BR-23Glc both BR-diGlc and the putative BR-MalGlc were not only detected in plant extracts, but were also present in the media in which plants had been incubated for the feeding studies; thus BR-Glcs formed in planta were released to the media. In summary, these results suggest that 23-O-glucosides of Brassinolide and Castasterone are further modified by malonylation in planta.

Kinetics of Brassinolide-glucoside catabolism in UGT73C5oe and UGT73C6oe plants

To determine the kinetics of formation of the putative BR-MalGlc and BR-diGlc, the samples of the time-course Brassinolide plant feeding studies were analyzed for an occurrence of BL-23Glc catabolites. At present no analytical standard is available for BL-MalGlc to accurately quantify its amounts. However, as a rough estimate the same response factor as for BL-23Glc was assumed, allowing for a semi-quantitative estimation of BL-MalGlc concentrations.

Similarly the concentration of BL-diGlc was estimated by assuming the same response factor as for BR-23Glc. The results are illustrated in Figure 6.2.1-6 and show levels of BL-MalGlc and BL-diGlc in nmol/g Fw, as compared to Brassinolide and BL-23Glc amounts in seedlings of Col-0, *UGT73C5oe* and *UGT73C6oe*. All BL-Glcs detected were present in all analyzed lines however, in wild type BL-diGlc was close to the limit of detection with the applied LC-HRMS method. Amounts of the putative BL-MalGlc increased in wild type for 12 h and were then sustained (Figure 6.2.1-6a). Similarly, the kinetics of putative BL-MalGlc formation in *UGT73C5oe* and *UGT73C6oe* lines were characterized by an increase to a plateau concentration within 48 h of feeding, which was then sustained for the rest of the experiment (Figure 6.2.1-6b,c). This is in contrast to Brassinolide and BL-23Glc levels, which decreased in both wild type and *UGT73C5oe* and *UGT73C6oe* after having reached a peak. Interestingly, a drop in BL-23Glc amounts correlated with a corresponding increase in putative BL-MalGlc in *UGT73C5oe* and *UGT73C6oe* plants, supporting the notion that BL-23Glc was converted to BL-23MalGlc. In summary, the results show that in Brassinolide feeding studies of wild type, and *UGT73C5oe* and *UGT73C6oe* plants a decrease in BL-23Glc levels correlated with an increase in putative BL-MalGlc, showing that BL-23Glc was further conjugated. As opposed to Brassinolide and BL-23Glc the putative BL-MalGlc did not decrease after an initial increase, suggesting that malonylation may protect 23-*O*-glucosylated Brassinolide from removal, in the soluble fractions analyzed.



The values shown are nmol/g Fw.

Figure 7.2.1-7: Analysis of wild type and *UGT73C5oe* and *UGT73C6oe* seedlings, used in Brassinolide plant feeding studies, for the occurrence of BL-MalGlc over time.

III. CONCLUSION

In summary, this study provides evidence that in addition to *UGT73C5*, also its closest homologue *UGT73C6*, is able to catalyze 23-*O*-glucosylation of the bioactive Brassinosteroids Castasterone and Brassinolide in planta. Future studies will address the question, if Brassinosteroid glucosylation is a physiological role of both UGTs, and if this potential multiplicity may provide a highly flexible system for homeostatic adaptation at a cellular level.

OVERALL CONCLUSION

Brassinosteroids, including 24-Epibrassinolide, are naturally occurring plant constituents, found throughout the plant kingdom as well as some fungi. 24-Epibrassinolide elicits and activates the plant's self-defence mechanisms meditating the plant's resistance to unfavourable environmental factors, stress, and diseases.

They are present in higher plants, lower plants, including algae, mosses, the "living fossil" *Equisetum* and in certain fungi (Table 7.2.1-1). 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 the highest contents of Brassinosteroids with 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 (Table 7.2.1-1).

The concentration of Brassinosteroids in plants is regulated by a complex system of feedback pathways and Brassinosteroids are constantly synthesised, metabolised, activated and inactivated depending on the plant's needs as well as environmental cues.

Thus, endogenous Brassinosteroid levels in plant tissues are primarily regulated through the tissue-specific control of Brassinosteroid synthesis, catabolism, and inactivation rather than through long-distance transport. Brassinosteriods produced *in planta* are only transported over short distances to the exterior surface of the producing cell or to the surfaces of neighboring cells.

Although Brassinosteroids are taken up through the roots and are translocated unchanged from roots to shoots through the plant's phloem, exogenously applied Brassinosteroids to shoots and leafs are not transported throughout the plant or only at a very low level.

The metabolic/catabolic pathway of 24-Epibrassinolide and Brassinosteroids in plants has not yet been completely elucidated. Nevertheless, some studies have been performed on the metabolic pathway in different plants and fungi. There is no uniform degradation pathway, but different plant and fungal species or group have their own pathways. As Brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

These processes strongly depend on the plant species, the plant organ and the developmental stage. Different homeostatic mechanisms are thought to operate to maintain the Brassinosteroid equilibrium, including the feedback inhibition of Brassinosteroid production. The spatial and temporal regulation of its homeostasis at the tissue or at cellular level is crusial for normal growth and development of plants. In addition, catabolic inactivation is also considered to play a role in the regulation of bioactive Brassinosteroid levels.

Brassinosteroids are catabolically altered or conjugated, with some modifications yielding inactive products. Various processes like acylation, sulphonation, glycosylation hydroxylation etc. play a role in maintaining the optimum levels of the bioactive brassinosteroids in the cells.

Brassinosteroids exogenously applied to shoots and leafs are metabolised within 24-96 h after application (Yokota *et al*. 1992; Winter *et al*., 1997 and Husar *et al*., 2011).

In addition to that, for agricultural purposes, only very low amounts of the natural-identical synthesized molecule, 24-Epibrassinolide, are used and thus the artificial release will influence natural background levels only to a limited extend. This is for example highlighted by Khripach *et al.* (2000). For typical quantities of Brassinosteroids of 5-50 mg per hectare used in agriculture the authors calculate for the highest dosage an average Brassinosteroids concentration of 2.1 x 10^{-3} nmol/g plant biomass assuming a total weight of 50 tons per hectare and full absorption by plants. The authors conclude that this is close to the natural Brassinosteroids concentration in plants. This is also compliant with the proposed application rates for the representative formulation (please refer to Document D3).

B.7.2.2. Poultry

As Brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones. Animals such as poultry are constantly exposed to 24-Epibrassinolide e.g. through consumption of plants as well as other natural foods (Table 7.2.1-1). Furthermore, no different metabolic pathways of the natural-identical synthesized molecule, 24-Epibrassinolide, to the natural occurring 24-Epibrassinolide, are expected.

B.7.2.3. Lactating ruminants

As Brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones. Lactating ruminants are constantly exposed to 24-Epibrassinolide e.g. through consumption of plants (Table 7.2.1-1). Furthermore, no different metabolic pathway of the natural-identical synthesized molecule, 24-Epibrassinolide, to the natural occurring 24-Epibrassinolide, are expected.

B.7.2.4. Pigs

As Brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones. Pigs are constantly exposed to 24-Epibrassinolide e.g. through consumption of plants as well as other natural foods (Table 7.2.1-1). Furthermore, no different metabolic pathway of the natural-identical synthesized molecule, 24-Epibrassinolide, to the natural occurring 24-Epibrassinolide, are expected.

B.7.2.5. Fish

As Brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones. Animals are constantly exposed to 24-Epibrassinolide e.g. through consumption of plants, in case of fish especially algae and water plants (Table 7.2.1-1). Furthermore, no different metabolic pathway of the natural-identical synthesized molecule, 24-Epibrassinolide, to the natural occurring 24-Epibrassinolide, are expected.

B.7.3. MAGNITUDE OF RESIDUE TRIALS IN PLANTS

Brassinosteroids, including 24-Epibrassinolide are naturally occurring plant constituents found throughout the plant kingdom. They are present in higher plants, lower plants, including algae, mosses, the "living fossil" *Equisetum* (Table 7.2.1-1) as well as certain fungi. It is therefore impossible to distinguish between a possible residue resulting from the use of the natural-identical active substance 24-Epibrassinolide and the similar natural substance ubiquitously present in the environment.

In addition, different homeostatic mechanisms, including feedback inhibition of Brassinosteroids and catabolic inactivation, play a role in the maintenance of the natural equilibrium of bioactive Brassinosteroids in plants. Brassinosteroids exogenously applied to shoots and leafs are metabolised within 24-96 h after application (Yokota *et al.* 1992; Winter *et al*., 1997 and Husar *et al*., 2011). The concentration of Brassinosteroids in plants is regulated by a complex system of feedback pathways 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.

Furthermore, very low concentrations of 24-Epibrassinolide (0.01 %) are intended to be applied as a spray application and residues only below the default MRL of 0.01 mg/kg or below the natural background level are expected.

Table 7.3-1 represents the worst-case residues in mg/kg harvested crop for the representative uses assuming no drift, 100 % crop interception by harvested crop and all applications conducted on the day of harvest. Crop yield statistics from DESTAT and FAOSTAT for the year 2015 was used as representative yields. Furthermore, residue predictions were calculated for crops where available natural background levels are available using the same assumptions as described below (Table 7.3-2).

Based upon the possible worst-case residue calculations for the representative uses, all crop residues are below the default MRL of 0.01 mg/kg except for wine and table grapes (0.01320 mg/kg). As assumption of no drift, 100 % crop interception by harvested crop, all applications conducted simultaneously at the day of harvest are a more than conservative approach, the residue levels are much lower for wine and table grapes in practice. Furthermore, based upon the theoretical application of 24-Epibrassinolide on rice, buckwheat, carrot, apple, tea and potatoes and reference values for natural concentrations available (please refer to Table 7.3-2), all residue levels are below the default MRL of 0.01 mg/kg or below the measured natural background level (Table 7.3-2). In addition, no degradation or catabolism was assumed.

Table 7.3-1: Worst-case residues in mg/kg harvested crop based upon the representative crops

Family /Species	Maximal water volume per application (L/ha)	dilution rate	g a.s./ha	Maximal number of applications	Maximal total rate (mg a.s./ha)	Yield kg/ha	BBCH last application	Residue-calculation (mg/kg)	Rating
Wine grapes and table grapes	1000	1:2000	0.05	3	0,15	11.282[2]	85	0.01320	>default MRL of 0.01 mg/kg
Lettuce (F)	400	1:1000	0.04	2	0,08	33.960[1]	41	0.00234	< default MRL of 0.01 mg/kg
Lettuce (G)	400	1:1000	0.04	2	0,08	39.830[1]	41	0.00199	< default MRL of 0.01 mg/kg
Sugarbeet	800	1:2000	0.04	3	0,12	72.170[1]*	39	0.00165	< default MRL of 0.01 mg/kg
Cucumber (G)	1000	1:2000	0.05	3	0,15	221.120[1]	69	0.00067	< default MRL of 0.01 mg/kg

1Yield per hectare from DESTATIS (Statistisches Bundesampt Deutchland) for the year 2015
2Wine-must yield per hectare from DESTATIS (Statistisches Bundesampt Deutchland) for the year 2015, converted to fruit (factor 0.78).

Table 7.3-2: Worst-case residues in mg/kg harvested crop based upon background levels measured

Family/Species	Examined part	Content 24-EpiBL mg/kg fr. wt. [1]	Maximal water volume per application (L/ha)	Dose per application dilution rate 1:2000) g a.s./ha	Maximal number of applications	Maximal total rate (mg a.s./ha)	Yield kg/ha	Residue-calculation (mg/kg)	Rating
Rice (*Oryza sativa* L.)	grains	0.216	1000	0.05	3	0.15	6.315[3]	0.0236	< below the measured background level
Common buckwheat (*Fagopyrum esculentum* Moench)	grains	0.378	1000	0.05	3	0.15	1.332[3]	0.1118	< below the measured background level
Wild carrot (*Daucus carota* ssp. Sativus L.)	root	0.00043	600	0.03	3	0.09	54.600[2]	0.0016	< default MRL of 0.01 mg/kg
Apple (*Malus domestica*)	fruit	0.027	1500	0.07	3	0.22	30.994[2]	0.0072	< default MRL of 0.01 mg/kg < below the measured background level

Family/Species	**Examined part**	**Content 24-EpiBL mg/kg fr. wt.** [1]	**Maximal water volume per application (L/ha)**	**Dose per application dilution rate 1:2000) g a.s./ha**	**Maximal number of applications**	**Maximal total rate (mg a.s./ha)**	**Yield kg/ha**	**Residue-calculation (mg/kg)**	**Rating**
Green tea	leaves	0.1	1000	0.05	3	0.15	4.133[3]	0.0360	< below the measured background level
Potato (*Solanum tuberosum*)	tuber	0.0375	600	0.03	3	0.09	43.810[2]	0.0020	< default MRL of 0.01 mg/kg < below the measured background level

[1] Khripach *et al*., 2013
[2] Yield per hectare from DESTATIS (Statistisches Bundesampt Deutschland) for the year 2015
[3] Yield per hectare from FAOSTAT 2014

B.7.4. FEEDING STUDIES

24-Epibrassinolide has no relevant toxicity hazard towards humans, mammals or animals, including aquatic organisms. Humans, mammals and fish are constantly exposed to 24-Epibrassinolide either directly through the consumption of primary material such as plants, plant organs (e.g. natural contents in seeds, roots, and leafs 0.22 - 378 µg/kg), natural foods such as honey (7.4 µg/kg) or algae (e.g. Bajguz A., 2009; please also refer to Table 7.2.1-1) or indirectly.

The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) has concluded that plant sterols (which includes 24-Epibrassinolide) are not only of low risk for the human consumer but necessary for a healthy diet as they are contributing to lowering the LDL-cholesterol levels, which is pivotal for the prevention of coronary heart diseases. Therefore, a daily intake of up to 3 g of plant sterols per day is highly recommended by EFSA (please refer to chapter B.6.9.4, Epidemiological studies, Section toxicology).

This was also confirmed in humans and rats, where the effect of Brassinolide on cholesterol levels was evaluated. In both, humans and rats, a decrease in total serum cholesterol from initial elevated values was observed (Statsenko *et al*., 2008; Zhabinskii *et al*., 2015).

Furthermore, as described in a review by Zhabinskii *et al*, (2015), 24-Epibrassinolide treatment of fingerlings of diverse fish species (e.g. black sea salmon, carp, Russian sturgeon and silver carp) lead to significantly less negative effects through toxicants ($CuSO_4$, phenol) contained in the water and treatment of sturgeon eggs with 24-Epibrassinolide was found to increase fecundation, hatching and larvae/fingerling survival (higher resistance to stress). This effect was also found for phytophaguous fishes (grass carp and silver carp).

Structurally, the Brassinolide receptor system is related to the TGF-β transmembrane receptor kinase signal transduction pathway in animals (Ross, 2010). However, the effect of Brassinolide has been validated as limited to higher protein metabolism, not linked to any sex hormone receptor (Oklestkova *et al.,* 2015). Brassinosteroids act independently of the genome via cell-surface signalling, involving indirect inactivation of the plant glycogen synthetase kinase 3 (GSK3)-like kinase BIN2 (Esposito, 2011). In animals, lipophilic steroids bind to steroid receptors, located either in the cytosol or in the nucleus by diffusing through the plasma membrane. This ligand binding leads to conformal change and dimerization, targeting direct DNA binding. In experimental binding assays, Brassinolide has been shown not to be capable of binding to these mammalian-specific receptors (Oklestkova *et al.,* 2015). This has been confirmed by genome alignment, not revealing *BRI1*-like gene sequences in mammals and, vice versa, no animal-receptor similarity in plants (Ross, 2010).

Due to the daily natural exposure of poultry, ruminants, pigs and fish to Brassinosteroids, feeding studies are not considered necessary.

B.7.4.1. Poultry

Please refer to B.7.4. above.

B.7.4.2. Ruminants

Please refer to B.7.4. above.

B.7.4.3. Pigs

Please refer to B.7.4. above.

B.7.4.4. Fish

Please refer to B.7.4. above.

B.7.5. EFFECTS OF PROCESSING

Brassinosteroids, including 24-Epibrassinolide are naturally occurring plant constituents found throughout the plant kingdom. 24-Epibrassinolide elicits and activates the plant's self-defence mechanisms meditating the plant's resistance to unfavourable environmental factors, stress, and diseases.

Brassinosteroids are present in higher plants, lower plants, including algae and mosses, the "living fossil" *Equisetum* and in some fungi (Table 7.2.1-1). 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 the highest contents of Brassinosteroids with 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 (Table 7.2.1-1). Furthermore, Brassinosteroids are also naturally present in 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 7.2.1-1).

However, it is impossible to distinguish between a possible residue resulting from the use of the natural-identical active substance 24-Epibrassinolide and the similar natural substance, ubiquitously present in the environment.

Due to the natural concentrations of 24-Epibrassinolide in natural and processed food commodities, studies on the effect and magnitude of 24-Epibrassinolide on processing is not considered necessary.

B.7.5.1. Nature of the residue

Please refer to B.7.5. above.

B.7.5.2. Distribution of the residue in peel and pulp

Please refer to B.7.5. above.

B.7.5.3. Magnitude of residues in processed commodities

Please refer to B.7.5. above.

B.7.6. RESIDUES IN SUCCEEDING OR ROTATIONAL CROPS

Brassinosteroids, including 24-Epibrassinolide are naturally occurring plant constituents found throughout the plant kingdom. 24-Epibrassinolide elicits and activates the plant's self-defence mechanisms meditating the plant's resistance to unfavourable environmental factors, stress, and diseases.

They are present in higher plants, lower plants, including algae and mosses, the "living fossil" *Equisetum* and in some fungi (Table 7.2.1-1). Brassinosteroids are present in all plant organs such as pollens, 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.

Furthermore, 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, as for other phytosterols, Brassinosteroids and their breakdown products 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. No information on exact concentrations of Brassinosteroids in different soil types is available. For vermicompost, Aremu *et al*., 2015 measured Brassionsteroid concentrations between 3.084 ng and 3.809 ng per liter of vermicompost leachate (fg/mL).

Because Brassinosteroids are ubiquitous, phylogenetically ancient phytohormones that are naturally occurring throughout the plant kingdom, studies on the residues in rotational crops is not considered necessary.

Furthermore, 24-Epibrassinolide is a moderately hydrophobic organic compound with a log Pow of 2.0 (please refer to Vol. 4, B.2) and will be spontaneously transferred from soil to the plant root by a diffusion-driven process. 24-Epibrassinolide is considered a "natural-identical synthesized molecule", no differences in behaviour compared to the naturally available 24-Epibrassinolide are expected.

B.7.6.1. Metabolism in rotational crops

Please refer to B.7.6. above.

B.7.6.2. Magnitude of residues in rotational crops

Please refer to B.7.6. above.

B.7.7. OTHER STUDIES

No further studies were conducted.

B.7.7.1. Effect on the residue level in pollen and bee products

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 the highest contents of Brassinosteroids with a range of 0.001 – 6400 µg/kg fresh weight (Table 7.2.1-1). Furthermore, natural concentrations of up to 7.4 µg/kg have been observed in honey.

Additionally, it is impossible to distinguish between a possible residue resulting from the use of the natural-identical active substance 24-Epibrassinolide and the similar natural substance ubiquitously present in the environment and especially in pollen.

Based on the above justification, the determination of residues in pollen and bee products are not considered necessary.

B.7.8. REFERENCES RELIED ON

B.7.8.1. Scientific peer-reviewed open literature

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".

Data point addressed:	CA 9.1/01
Author(s) (year):	Reisinger, T., Huber, L. (2017)
Title:	LITERATURE REVIEW REPOT - ACTIVE SUBSTANCE: 24-Epibrassinolide
Laboratory report / project Number (Doc. No.):	PP309-00002
Testing facility:	Scientific Consulting Company, Bad Kreuznach, Germany
Published:	No
Test guideline used:	
Deviations:	None
GLP:	No

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.

In total, 854 records were retrieved from bibliographic databases and were screened by expert reviewers for relevance. Based on the evaluation of the summary records (titles/abstracts) 845 publications were assessed as obviously not relevant for the EU-data requirements related to side-effects on human health, non-target species and the environment for the active substance 24-Epibrassinolide.

Nine full-text documents were assessed in detail. One of these publications did not provide relevant information for the dossier preparation or risk assessment purposes and was as well assessed as obviously not relevant for the EU-data requirements.

Eight publications were selected to provide relevant information and will be cited in the supplementary dossiers under point CA 5.2, CA 5.8.4, CA 8.2.6, CA 8.3.1, CA 8.4.2 and CA 8.7.

B.7.8.2. Studies submitted by the applicant

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 6/01	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 6/02	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 6/03	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 6/04	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 6/05	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 6/06	Thompson, M.J. Mandava, N.	1979	SYNTHESIS OF BRASSINO STEROIDS: NEW PLANT-GROWTH-PROMOTING	N	N		nr	N

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
	Flippen-Anderson, J.L. Worley, J.F. Dutky, S.R. Robbins, W.E. Lusby, W.		STEROIDS Report No.: na (092-063) The Journal of Organic Chemistry, 1979, 44 (26), 5002-5004 Not GLP, published					
KCA 6/07	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 6/08	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 6/09	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 6/10	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	N	N		nr	N

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
			Not GLP, published					
KCA 6/11	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 6/12	Eremina, M. Unterholzner, S.J. Rathnayake, A. Castellano, M. Khan, M. Kugler, K.G. May, S.T. Mayer, K.F. 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 6/13	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 6/14	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,	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

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
	J.							
KCA 6/15	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 6/16	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 6/17	Mudge, S.M. Joao A.F. Bebianno, M. East, J.A. Barreira, L.A.	1999	STEROLS IN THE RIA FORMOSA LAGOON, PORTUGAL Report No.: na (092-169) Water Research, 1999, 33 (4), 1038-1048 Not GLP, published	N	N		nr	N
KCA 6/18	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 6/19	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 6/20	Voigt, B. Porzel, A. Naumann, H. Hoerhold-	1993	HYDROXYLATION OF THE NATIVE BRASSINOSTEROIDS 24-EPICASTASTERONE AND 24-	N	N		nr	N

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
	Schubert, C. Adam, G.		EPIBRASSINOLIDE BY THE FUNGUS CUNNINGHAMELLA ECHINULATA Report No.: na (092-096) Steroids, 1993, 58, 320-323 Not GLP, published					
KCA 6/21	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 6/22	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 6/23	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 6.1/01	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

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 6.1/02	Yokota, T. Higuchi, K. Kosaka, Y. Takahashi, N.	1992	TRANSPORT AND METABOLISM OF BRASSINOSTEROIDS IN RICE Report No.: na (092-098) Progress in Plant Growth Regulation, 1992, 13, 298-305 Not GLP, published	N	N		nr	N
KCA 6.1/03	Winter, J. Schneider, B. Strack, D. Adam, G.	1997	ROLE OF A CYTOCHROME P450-DEPENDENT MONOOXYGENASE IN THE HYDROXYLATION OF 24-EPI-BRASSINOLIDE Report No.: na (092-097) Phytochemistry, 1997, 45 (2), 233-237 Not GLP, published	N	N		nr	N
KCA 6.1/04	Husar, S. Berthiller, F. Fujioka, S. Rozhon, W. Khan, M. Kalaivanan, F. Elias, L. Higgins, G.S. Li, Y. Schuhmacher, R. Krska, R. Seto, H. Vaistij, F.E. Bowles, D. Poppenberger, B.	2011	OVEREXPRESSION OF THE UGT73C6 ALTERS BRASSINOSTEROID GLUCOSIDE FORMATION IN ARABIDOPSIS THALIANA Report No.: na (092-148) BMC Plant Biology, 2011, 11(51), 1-14 Not GLP, published	N	N		nr	N
KCA 6.2.1/01	Yokota, T. Higuchi, K. Kosaka, Y. Takahashi, N.	1992	TRANSPORT AND METABOLISM OF BRASSINOSTEROIDS IN RICE Report No.: na (092-098) Progress in Plant Growth Regulation, 1992, 13, 298-305 Not GLP, published	N	N		nr	N
KCA 6.2.1/02	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)	N	N		nr	N

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
			Journal of Plant Research, 1994, 107, 125-130 Not GLP, published					
KCA 6.2.1/03	Symons, G.M. Reid, J.B.	2004	BRASSINOSTEROIDS DO NOT UNDERGO LONG-DISTANCE TRANSPORT IN PEA. IMPLICATIONS FOR THE REGULATION OF ENDOGENOUS BRASSINOSTEROID LEVELS Report No.: na (092-095) Plant Physiology, 2004, 135, 2196-2206; doi: 10.1104/pp.104.043034 Not GLP, published	N	N		nr	N
KCA 6.2.1/04	Nishikawa, N. Shida, A. Toyama, S.	1995	METABOLISM OF 14C-LABELED EPIBRASSINOLIDE IN INTACT SEEDLINGS OF CUCUMBER AND WHEAT Report No.: na (092-087) Journal of Plant Research, 1995, 108, 65-69 Not GLP, published	N	N		nr	N
KCA 6.2.1/05	Kolbe, A. Schneider, B. Porzel, A. Adam, G.	1996	METABOLISM OF 24-EPI-CASTASTERONE AND 24-EPI-BRASSINOLIDE IN CELL SUSPENSION CULTURES OF ORNITHOPUS SATIVUS Report No.: na (092-086) Phytochemistry, 1996, 41 (1), 163-167 Not GLP, published	N	N		nr	N
KCA 6.2.1/06	Winter, J. Schneider, B. Strack, D. Adam, G.	1997	ROLE OF A CYTOCHROME P450-DEPENDENT MONOOXYGENASE IN THE HYDROXYLATION OF 24-EPI-BRASSINOLIDE Report No.: na (092-097) Phytochemistry, 1997, 45 (2), 233-237 Not GLP, published	N	N		nr	N
KCA 6.2.1/07	Hai, T. Schneider, B. Adam, G.	1995	METABOLIC CONVERSION OF 24-EPI-BRASSINOLIDE INTO PENTAHYDROXYLATE	N	N		nr	N

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
			D BRASSINOSTEROID GLUCOSIDES IN TOMATO CELL CULTURES Report No.: na (092-083) Phytochemistry, 1995, 40 (2), 443-448 Not GLP, published					
KCA 6.2.1/08	Schneider, B. Kolbe, A. Porzel, A. Adam, G.	1994	A METABOLITE OF 24-EPI-BRASSINOLIDE IN CELL SUSPENSION CULTURES OF LYCOPERSICON ESCULENTUM Report No.: na (092-092) Phytochemistry, 1994, 36 (2), 319-321 Not GLP, published	N	N		nr	N
KCA 6.2.1/09	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 6.2.1/10	Husar, S. Berthiller, F. Fujioka, S. Rozhon, W. Khan, M. Kalaivanan, F. Elias, L. Higgins, G.S. Li, Y. Schuhmacher, R. Krska, R. Seto, H. Vaistij, F.E. Bowles, D. Poppenberger, B.	2011	OVEREXPRESSION OF THE UGT73C6 ALTERS BRASSINOSTEROID GLUCOSIDE FORMATION IN ARABIDOPSIS THALIANA Report No.: na (092-148) BMC Plant Biology, 2011, 11(51), 1-14 Not GLP, published	N	N		nr	N
KCA 6.2.1/11	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	N	N		nr	N

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
			Not GLP, published					
KCA 6.2.1/12	Symons, G.M. Davies, C. Shavrukov, Y. Dry, I.B. Reid, j.B. Thomas, M.R.	2006	GRAPES ON STEROIDS. BRASSINOSTEROIDS ARE INVOLVED IN GRAPE BERRY RIPENING Report No.: na (092-183) Plant Physiology, 2006, 140, 150-158; doi: 10.1104/pp.105.070706 Not GLP, published	N	N		nr	N
KCA 6.2.1/13	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 6.2.1/14	Bajguz, A. Tretyn, A.	2003	THE CHEMICAL STRUCTURES AND OCCURRENCE OF BRASSINOSTEROIDS IN PLANTS Report No.: na (092-145) Brassinosteroids. Chapter 1, 2003, 1-44 Not GLP, published	N	N		nr	N
KCA 6.2.1/15	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 6.2.1/16	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

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 6.2.1/17	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 6.2.1/18	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 6.2.1/19	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 6.2.1/20	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 6.2.1/21	Suzuki, Y. Yamaguchi, I. Yokota, T.	1986	IDENTIFICATION OF CASTASTERONE, TYPHASTEROL AND TEASTERONE FROM	N	N		nr	N

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
	Takahasi, N.		THE POLLEN OF ZEA MAYS Report No.: na (092-053) Agricultural and Biological Chemistry, 1986, 50 (12), 3133-3138 Not GLP, published					
KCA 6.2.1/22	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 6.2.1/23	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 6.2.1/24	Suzuki, H. Fujioka, S. Yokota, T. Murofushi, N. Sakurai, A.	1994	IDENTIFICATION OF BRASSINOLIDE, 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	N	N		nr	N
KCA 6.2.1/25	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,	N	N		nr	N

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
			58 (5), 986-989 Not GLP, published					
KCA 6.2.1/26	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 6.2.1/27	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 6.2.1/28	Soene, 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 6.2.1/29	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

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 6.2.1/30	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 6.2.1/31	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 6.2.1/32	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 6.2.1/33	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 6.2.1/34	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 6.2.1/35	Arima, M. Yokota, T. Takahashi, N.	1984	IDENTIFICATION AND QUANTIFICATION OF BRASSINOLIDE-RELATED STEROIDS IN THE INSECT GALL AND HEALTHY TISSUES OF THE	N	N		nr	N

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
			CHESTNUT PLANT Report No.: na (092-008) Phytochemistry, 1984, 23 (8), 1587-1591 Not GLP, published					
KCA 6.2.1/36	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 6.2.1/37	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 6.2.1/38	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 6.2.1/39	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)	N	N		nr	N

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
			Plant & Cell Physiology, 1996, 37 (8), 1201-1203 Not GLP, published					
KCA 6.2.1/40	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 6.2.1/41	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 6.2.1/42	Bancos, S. Nomura, T. Sato, T. Molnar, G. Bishop, G.J. Koncz, C. Yokota, T. Nagy, F. Szekeres, M.	2002	REGULATION OF TRANSCRIPT LEVELS OF THE ARABIDOPSIS CYTOCHROME P450 GENES INVOLVED IN BRASSINOSTEROID BIOSYNTHESIS Report No.: na (092-161) Plant Physiology, 2002, 130, 504-513; DOI: 10.1104/pp.005439 Not GLP, published	N	N		nr	N
KCA 6.2.1/43	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 6.2.1/44	Choe, S. Fujioka, S. Noguchi, T.	2001	OVEREXPRESSION OF DWARF4 IN THE BRASSINOSTEROID	N	N		nr	N

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
	Takatsuto, S. Yoshida, S. Feldmann, K.A.		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					
KCA 6.2.1/45	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 6.2.1/46	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 6.2.1/47	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 6.2.1/48	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

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 6.2.1/49	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 6.2.1/50	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 6.2.1/51	Pan, J. Hu, Y. Liang, T. Li, G.	2012	PREPARATION OF SOLID-PHASE MICROEXTRACTION FIBERS BY IN-MOLD COATING STRATEGY FOR DERIVATIZATION ANALYSIS OF 24-EPIBRASSINOLIDE IN POLLEN SAMPLES Report No.: na (092-041) Journal oh Chromatography A, 2012, 1262, 49-55; doi: 10.1016/j.chroma.2012.09.008 Not GLP, published	N	N		nr	N
KCA 6.2.1/52	Baba, J. Yokota, T. Takahashi, N.	1983	BRASSINOLIDE-RELATED NEW BIOACTIVE STEROIDS FROM DOLICHOS LABLAB SEED	N	N		nr	N

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
			Report No.: na (092-011) Agricultural and Biological Chemistry, 1983, 47 (3), 659-661 Not GLP, published					
KCA 6.2.1/53	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 6.2.1/54	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 6.2.1/55	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 6.2.1/56	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

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 6.2.1/57	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
KCA 6.2.1/58	Yokota, T. Koba, S. Kim, S.K. Takatsuto, S. Ikekawa, N. Sakakibara, M. Okada, K. Mori, K. Takahashi, N.	1987	DIVERSE STRUCTURAL VARIATIONS OF THE BRASSINOSTEROIDS IN PHASEOLUS VULGARIS SEED Report No.: na (092-076) Agricultural and Biological Chemistry, 1987, 51 (6), 1625-1631 Not GLP, published	N	N		nr	N
KCA 6.2.1/59	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 6.2.1/60	Kim, T.-W. Park, S.-H. Han, K.-S. Choo, J. Lee, J.S. Hwang, S. Kim, S.-K.	2000	OCCURRENCE OF TEASTERONE AND TYPHASTEROL, AND THEIR ENZYMATIC CONVERSION IN PHASEOLUS VULGARIS Report No.: na (092-035) Bulletin-Korean Chemical Society, 2000, 21 (4), 373-374 Not GLP, published	N	N		nr	N
KCA 6.2.1/6	Kim, S.-K.	1991	NATURAL OCCURRENCES OF	N	N		nr	N

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
1			BRASSINOSTEROIDS Report No.: na (092-033) ACS Symposium series, 1991, 474, Chapter 3, 26-35 Not GLP, published					
KCA 6.2.1/62	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 6.2.1/63	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 6.2.1/64	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 6.2.1/65	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	Motegi, C.	1994	IDENTIFICATION OF	N	N		nr	N

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
6.2.1/66	Takatsuto, S.		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					
KCA 6.2.1/67	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 6.2.1/68	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 6.2.1/69	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 6.2.1/70	Fujioka, S. Inoue, T. Takatsuto, S. Yanagisawa, T. Yokota, T. Sakurai, A.	1995	IDENTIFICATION OF A NEW BRASSINOSTEROID, CATHASTERONE, IN CULTURED CELLS OF CATHARANTHUS ROSEUS AS A BIOSYNTHETIC PRECURSOR OF TEASTERONE	N	N		nr	N

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
			Report No.: na (092-017) Bioscience, Biotechnology and Biochemistry, 1995, 59 (8), 1543-1547 Not GLP, published					
KCA 6.2.1/71	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 6.2.1/72	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 6.2.1/73	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 6.2.1/74	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 6.2.1/7	Yamamoto, R.	2001	BRASSINOSTEROID LEVELS INCREASE	N	N		nr	N

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
5	Fujioka, S. Demura, T. Takatsuto, S. Yoshida, S. Fukuda, H.		DRASTICALLY PRIOR TO MORPHOGENESIS OF TRACHEARY ELEMENTS Report No.: na (092-066) Plant Physiology, 2001, 125, 556-563 Not GLP, published					
KCA 6.2.1/76	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 6.2.1/77	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 6.2.1/78	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 6.2.1/79	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

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 6.2.1/80	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 6.2.1/81	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 6.2.1/82	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 6.2.1/83	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	Yokota, T.	1983	2-	N	N		nr	N

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
6.2.1/84	Arima, M. Takahashi, N. Takatsuto, S. Ikekawa, N. Takematsu, T.		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					
KCA 6.2.1/85	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 6.2.1/86	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 6.2.1/87	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 6.2.1/88	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,	N	N		nr	N

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
			xxx, 1-10; doi: 10.1016/j.phytochem.2016.12.020 Not GLP, published					
KCA 6.2.1/89	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 6.2.1/90	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 6.2.1/91	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 6.2.1/92	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

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 6.2.1/93	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 6.2.1/94	Nomura, T. Bishop, G.J.	2006	CYTOCHROME P450S IN PLANT STEROID HORMONE SYNTHESIS AND METABOLISM Report No.: na (092-089) Phytochemistry Reviews, 2006, 5, 421-432; DOI 10.1007/s11101-006-9024-2 Not GLP, published	N	N		nr	N
KCA 6.2.1/95	Choe, S. Dilkes, B.P. Fujioka, S. Takatsuto, S. Sakurai, A. Feldmann, K.A.	1998	THE DWF4 GENE OF ARABIDOPSIS ENCODES A CYTOCHROME P450 THAT MEDIATES MULTIPLE 22ALPHA-HYDROXYLATION STEPS IN BRASSINOSTEROID BIOSYNTHESIS Report No.: na (092-080) The Plant Cell, 1998, 10, 231-243 Not GLP, published	N	N		nr	N
KCA 6.2.1/96	Bajguz, A.	2007	METABOLISM OF BRASSINOSTEROIDS IN PLANTS Report No.: na (092-079) Plant Physiology and Biochemistry, 2007, 45, 95-107; doi:10.1016/j.plaphy.2007.01.002 Not GLP, published	N	N		nr	N
KCA 6.2.1/97	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 6.2.1/98	Kolbe, A. Schneider, B.	1995	ACYL-CONJUGATED METABOLITES OF BRASSINOSTEROIDS	N	N		nr	N

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
	Porzel, A. Schmidt, J. Adam, G.		IN CELL SUSPENSION CULTURES OF ORNITHOPUS SATIVUS Report No.: na (092-085) Phytochemistry, 1995, 38 (3), 633-636 Not GLP, published					
KCA 6.2.1/99	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 6.2.1/100	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 6.3/01	Yokota, T. Higuchi, K. Kosaka, Y. Takahashi, N.	1992	TRANSPORT AND METABOLISM OF BRASSINOSTEROIDS IN RICE Report No.: na (092-098) Progress in Plant Growth Regulation, 1992, 13, 298-305 Not GLP, published	N	N		nr	N
KCA 6.3/02	Winter, J. Schneider, B. Strack, D. Adam, G.	1997	ROLE OF A CYTOCHROME P450-DEPENDENT MONOOXYGENASE IN THE HYDROXYLATION OF 24-EPI-BRASSINOLIDE Report No.: na (092-097) Phytochemistry, 1997, 45 (2), 233-237 Not GLP, published	N	N		nr	N
KCA 6.3/03	Husar, S. Berthiller, F.	2011	OVEREXPRESSION OF THE UGT73C6 ALTERS	N	N		nr	N

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
	Fujioka, S. Rozhon, W. Khan, M. Kalaivanan, F. Elias, L. Higgins, G.S. Li, Y. Schuhmacher, R. Krska, R. Seto, H. Vaistij, F.E. Bowles, D. Poppenberger, B.		BRASSINOSTEROID GLUCOSIDE FORMATION IN ARABIDOPSIS THALIANA Report No.: na (092-148) BMC Plant Biology, 2011, 11(51), 1-14 Not GLP, published					
KCA 6.3/04	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 Doc. No.: 092-030 (Not applicable) National Academy of Sciences of Belarus, 2013, 57 (2), 63-69 Not GLP, published	N	N		nr	N
KCA 6.4/01	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 6.4/02	Statensko, E.A. Korolevich, K. Zhabinski, V. Samusevich, M.	2008	METHODS OF CORRECTION OF LIPID METABOLISM IN ATHLETES Report No.: na (092-093) Voennye medicina (Military Medicine), 2008, 9, 102-104 Not GLP, published	N	N		nr	N

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 6.4/03	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 6.4/04	Ross, J.J. Reid, J.B.	2010	EVOLUTION OF GROWTH-PROMOTING PLANT HORMONES Report No.: na (092-091) Functional Plant Biology, 2010, 37, 795-805 Not GLP, published	N	N		nr	N
KCA 6.4/05	Oklestkova, J. Rarova, L. Kvasnica, M. Strnad, M.	2015	BRASSINOSTEROIDS: SYNTHESIS AND BIOLOGICAL ACTIVITIES Report No.: na (092-090) Phytochemistry Reviews, 2015, 14, 1053-1072; DOI 10.1007/s11101-015-9446-9 Not GLP, published	N	N		nr	N
KCA 6.4/06	Esposito, D. Komarnytsky, S. Shapses, S. Raskin, I.	2011	ANABOLIC EFFECT OF PLANT BRASSINOSTEROID Report No.: na (092-081) FASEB Journal, PMC3177571, 2011, 25 (10), 3708-3719; doi: 10.1096/fj.11-181271 Not GLP, published	N	N		nr	N
KCA 6.5/01	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 6.6/01	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 6.6/02	Aremu, A.O.	2015	EVIDENCE OF PHYTOHORMONES	N	N		nr	N

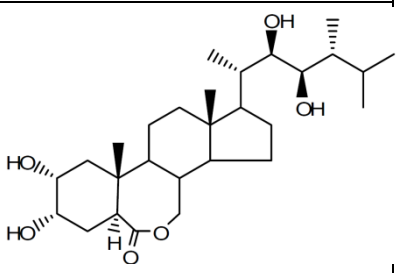
Data Point	Author(s)	Year	Title Compagny Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
	Stirk, W.A. Kulkarni, M.G. Tarkowska, D. Tureckova, V. Gruz, J. Subrtova, M. Pencik, A. Novak, O. Dolezal, K. Strnad, M. Van Staden, J.		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					
KCA 6.6/03	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 6.6/04	Mudge, S.M. Joao A.F. Bebianno, M. East, J.A. Barreira, L.A.	1999	STEROLS IN THE RIA FORMOSA LAGOON, PORTUGAL Report No.: na (092-169) Water Research, 1999, 33 (4), 1038-1048 Not GLP, published	N	N		nr	N
KCA 6.7.1/01	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 6.7.1/02	Statensko, E.A. Korolevich, K. Zhabinski, V. Samusevich, M.	2008	METHODS OF CORRECTION OF LIPID METABOLISM IN ATHLETES Report No.: na (092-093) Voennye medicina (Military Medicine), 2008, 9, 102-104	N	N		nr	N

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
			Not GLP, published					
KCA 6.10.1/ 01	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

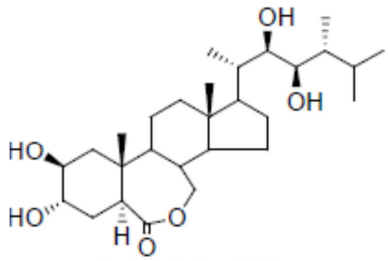
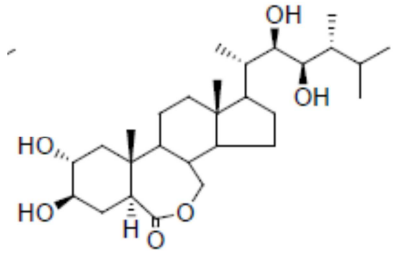
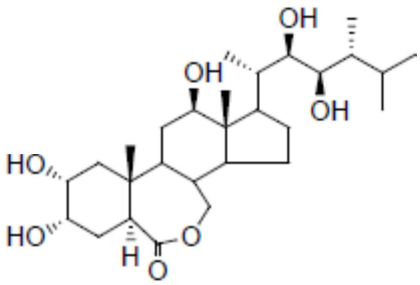
B.7.9. APPENDICES

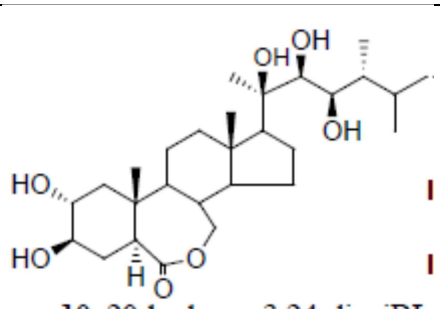
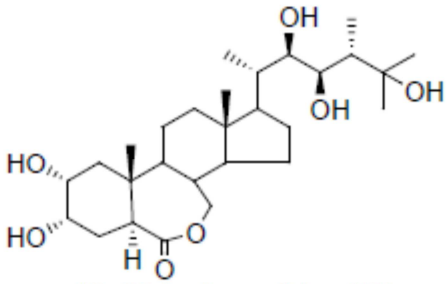
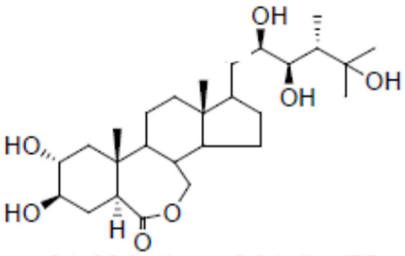
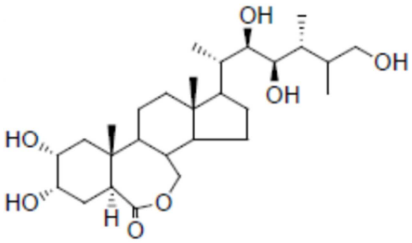
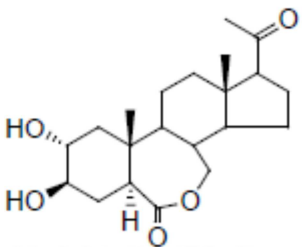
B.7.9.1. List of metabolites

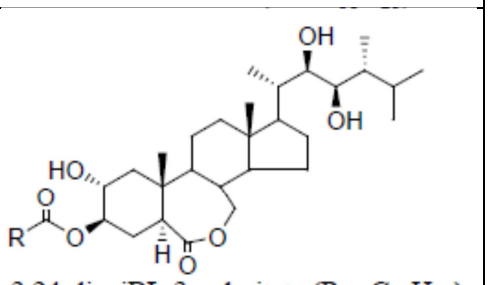
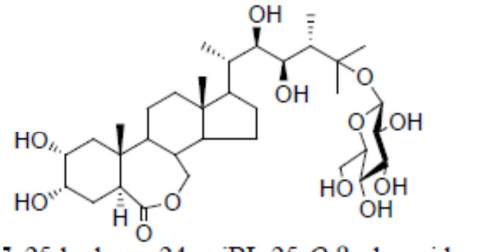
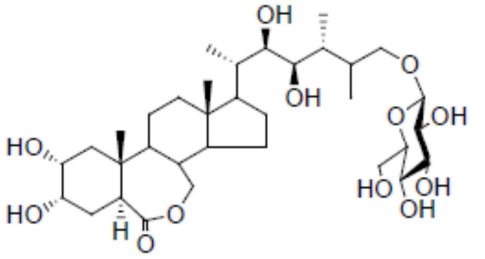
Substances; structures, codes, synonyms

Code Number (Synonyms)	Description	Compound found in:	Structure
Name (iso): 24-Epibrassinolide Synonym: Brassinolide, 24-epi-Brassinolide CAS No: 78821-43-9	(22R,23R,24R)-2α,3α,22,23-tetrahydroxy-24-methyl-β-homo-7-oxa-5-cholestan-6-one	***Natural occurrence in:*** Soil Water sediment systems higher plants, lower plants, including algae, mosses and in certain fungi	

Metabolites and breakdown products; structures, codes, synonyms

Degradation product	Substrate	Reaction	Site	Compound found in:	Structure
2,24-diepibrassinolide	24-Epibrassinolide	Hydrogenation	2α-OH	Cucumber *(C. sativus)*	 **2** 2,24-diepiBL
3,24-diepibrassinolide	24-Epibrassinolide		3αOH	Serradella (*O. sativus)*	 **5** 3,24-diepiBL
12β-hydroxy-24-Epibrassinolide	24-Epibrassinolide	Hydroxylation	C-12	Fungi *(C. echinulata)*	 **8** 12β-hydroxy-24-epiBL

Degradation product	**Substrate**	**Reaction**	**Site**	**Compound found in:**	**Structure**
20-hydroxy-3,24-diepibrassinolide	3,24-diepbrassinolide		C-20	Serradella (*O. sativus)*	 **10** 20-hydroxy-3,24-diepiBL
25-hydroxy-24-Epibrassinolide	24-Epibrassinolide		C-25	Tomato *(L. esculentum)*	 **12** 25-hydroxy-24-epiBL
25-hydroxy-3,24-diepibrassinolide	3,24-diepibrassinolide		C-25	Serradella (*O. sativus)*	 **14** 25-hydroxy-3,24-diepiBL
26-hydroxy-24-Epibrassinolide	24-Epibrassinolide		C-26	Tomato *(L. esculentum)*	 **17** 26-hydroxy-24-epiBL
3,24-diepibrassinolide C_{21}-catabolite	3,24-diepibrassinolide	Side chain cleavage	C-20/22	Serradella (*O. sativus)*	 **23** 3,24-diepiBL C_{21}-catabolite

Degradation product	**Substrate**	**Reaction**	**Site**	**Compound found in:**	**Structure**
3,24-diepibrassinolide-3-palmitate (R = $C_{15}H_{31}$) 3,24-diepibrassinolide-3-myristate (R = $C_{13}H_{27}$) 3,24-diepibrassinolide-3-laurate (R = $C_{11}H_{23}$)	3,24-diepibrassinolide	Esterification	3β-OH	Serradella (*O. sativus)*	 3,24-diepiBL-3-palmitate (R = $C_{15}H_{31}$) 3,24-diepiBL-3-myristate (R = $C_{13}H_{27}$) 3,24-diepiBL-3-laurate (R = $C_{11}H_{23}$)
25-hydroxy-24-Epibrassinolide-25-*O*-β-glucoside	25-hydroxy-24-Epibrassinolide	Glycosylation	25-OH	*Tomato (L. esculentum)*	 7 25-hydroxy-24-epiBL-25-*O*-β-glucoside
26-hydroxy-24-Epibrassinolide-26-*O*-b-glucoside	26-hydroxy-24-epibrassinolide		26-OH	*Tomato (L. esculentum)*	 9 26-hydroxy-24-epiBL-26-*O*-β-glucoside