Brassinosteroid phytohormones - structure, bioactivity and applications

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Brassinosteroids are a new class of plant hormones with a polyoxygenated steroid structure showing pronounced plant growth regulatory activity. This review covers their natural occurrence, biological and chromatographic methods for their detection, biosynthesis and metabolism, biological activity, structure-activity relationships and prospective agricultural uses.

Key words: brassinolide, brassinosteroids.

Hormônios vegetais brassinosteroídicos – estrutura, bioatividade e aplicações: Os brassinosteróides são uma nova classe de hormônios vegetais com estrutura esteroídica polioxigenada, dotados de pronunciada atividade reguladora do crescimento vegetal. Esta revisão cobre sua ocorrência natural, os métodos biológicos e cromatográficos para sua detecção, sua biossíntese e metabolismo, a atividade biológica, as relações estrutura-atividade e suas perspectivas de uso agrícola.

Palavras-chave: brassinolídeo, brassinosteróides.

INTRODUCTION

Since the 1930's, USDA researchers have found that pollen extracts promote plant growth (Mandava, 1988) and a first paper was published in 1941 reporting that application of hexanic extracts of maize pollen to the first internode of young bean seedlings produced marked elongation of the treated internode (Mitchell and Whitehead, 1941), a response also obtained using extracts of immature bean seeds (Mitchell et al., 1951).

In the 1960's the USDA started a research program aimed to find new plant hormones, leaded by J. W. Mitchell (Maugh II, 1981). The hypothesis to be tested was that pollens would have an elevated concentration of plant hormones and there was a great probability of finding new physiologically active substances in them.

Employing the bean second internode bioassay, pollen extracts of around 60 different plant species were tested (Mandava and Mitchell, 1971): rape (*Brassica napus* L.) and alder tree (*Alnus glutinosa* L.) pollens produced an unexpected response, combining elongation (typical of gibberellins) with swelling and curvature of the treated internode. These researchers proposed that the rape pollen contained a new group of lipidic plant hormones, which they called *brassins* (Mitchell et al., 1970). Mandava, Mitchell and co-workers reported the occurrence of an active fraction of the brassins containing, mainly, glucosyl esters of fatty acids (Mandava and Mitchell, 1972; Mandava et al., 1973). Further work revealed that although these esters promoted elongation, they were not able to reproduce all of the observed response (Grove et al., 1978). Brassins, however, were able to increase plant growth, crop yield and seed viability (Mitchell and Gregory, 1972; Gregory, 1981; Meudt et al., 1984).

In 1975 a research project to identify and synthesize the active compounds in brassins, evaluate their effect on the yield of selected crops (such as wheat, maize, soybean and potato), and evaluate their growth regulating properties in green-houses and in the field was begun (Mandava, 1988; Steffens, 1991).

For isolating the brassins active compounds, about 250 kg of bee collected rape pollen were extracted with isopropanol in batches of 25 kg (Mandava et al., 1978). The extracts were partitioned between carbon tetrachloride, methanol and water. The methanolic fractions were chromatographed in a series of silica columns, a process that reduced the biologically active material to 100 g. Final purification, followed by the bean second internode assay, was accomplished by column chromatography and high performance liquid chromatography, affording 10 mg of a crystalline substance, called brassinolide (1, figure 1). Its structure was elucidated by spectroscopic methods including X-ray analysis and can be systematically designated as (22R,23R,24S)-2α,3α,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one (Grove et al., 1979; see the Appendix for notation of steroids).

At the time brassinolide (1) was pointed out to be the first phytosteroid with plant growth-promoting activity, even in very minute amounts and concentrations, presenting a 6-oxo-7-oxalactone function and two groups of *cis*-vicinal hydroxyls, one at carbons 2 and 3 and the other at carbons 22 and 23, resembling in some way the ecdysteroids.

A few years later Japanese scientists isolated castasterone (9; Yokota et al., 1982a; figure 1) from chestnut gall tissue, the ketone that was then thought to be the putative precursor of brassinolide. Soon afterwards they were able to identify, as a mixture of brassinolide-like substances (Ikekawa et al., 1984), the *Dystilium* factors A1 and B, isolated from the leaves of *D. racemosum* (Marumo et al., 1968), that were active in the rice lamina inclination bioassay.

After the first chemical syntheses of brassinolide (Fung and Siddall, 1980; Ishiguro et al., 1980), a great effort is being made to synthesize it and similar compounds (the *brassinosteroids*), to isolate new brassinosteroids, elucidate their biosynthetic routes, verify their biological activities and their agricultural applications.

NATURAL BRASSINOSTEROIDS

Since the isolation of brassinolide (1), a series of brassinosteroids [such as dolicholide (3), 28-homodolicholide (4), castasterone (9), dolichosterone (11), 28-homodolichosterone (12) and typhasterol (25); see figure 1] have been isolated from plant sources and fully characterized by the usual spectroscopic methods. The vast majority of the more than 50 hitherto known natural brassinosteroids were detected in different organs of plants in several families by gas or liquid chromatography combined with mass spectrometry and comparison with authentic samples (Adam and Marquardt, 1986; Singh and Bhardwaj, 1986; Mandava, 1988; Abreu, 1991; Takatsuto, 1994; Fujioka and Sakurai, 1997a; Adam et al., 1999; Fujioka, 1999).

Brassinosteroids can be derived from the 5α-cholestane carbon skeleton bearing the following structural characteristics:

i) ring A mono- to trioxygenated, always oxygenated at carbon 3;

ii) ring B presenting a 6-oxo-7-oxalactone or a 6-oxo function or full saturation;

iii) *all-trans* junctions of rings A - D;

iv) 22α,23α-dihydroxylated, mostly alkylated at carbon 24, sometimes methylated at carbon 25 and sometimes unsaturated between carbons 24 and 28.

These characteristics lead to considering as *natural brassinosteroids* the 3-oxygenated (20β)-5α-cholestane-22α,23α-diols of plant origin, bearing additional alkyl or oxy substituents (see general structures 52 and 52a, figure 2). They can also occur conjugated especially with sugars or fatty acids. *Brassinosteroid analogues* are compounds that show any structural similarity with natural brassinosteroids and/or brassinolide activity (Zullo et al., 2002).

The occurrence of the natural brassinosteroids is described in table 1, according to the plant source. They were isolated or detected in algae, pteridophytae, gimnosperms and angiosperms (mono- and dicotyledons), indicating a probable ubiquitous distribution in the plant kingdom.

Some of the biosynthetic precursors of the brassinosteroids, such as cathasterone (53; Fujioka et al., 1995; figure 3), 6-deoxocathasterone (54), 3-epi-6-deoxocathasterone (55; Fujioka et al., 2000b) and 6-deoxo-28-norcathasterone (54a, Yokota et al., 2001), as well as catabolites, such as cryptolide (56; Watanabe et al., 2000), are in some instances considered as brassinosteroids themselves, but they do not fulfill all the structural requirements.

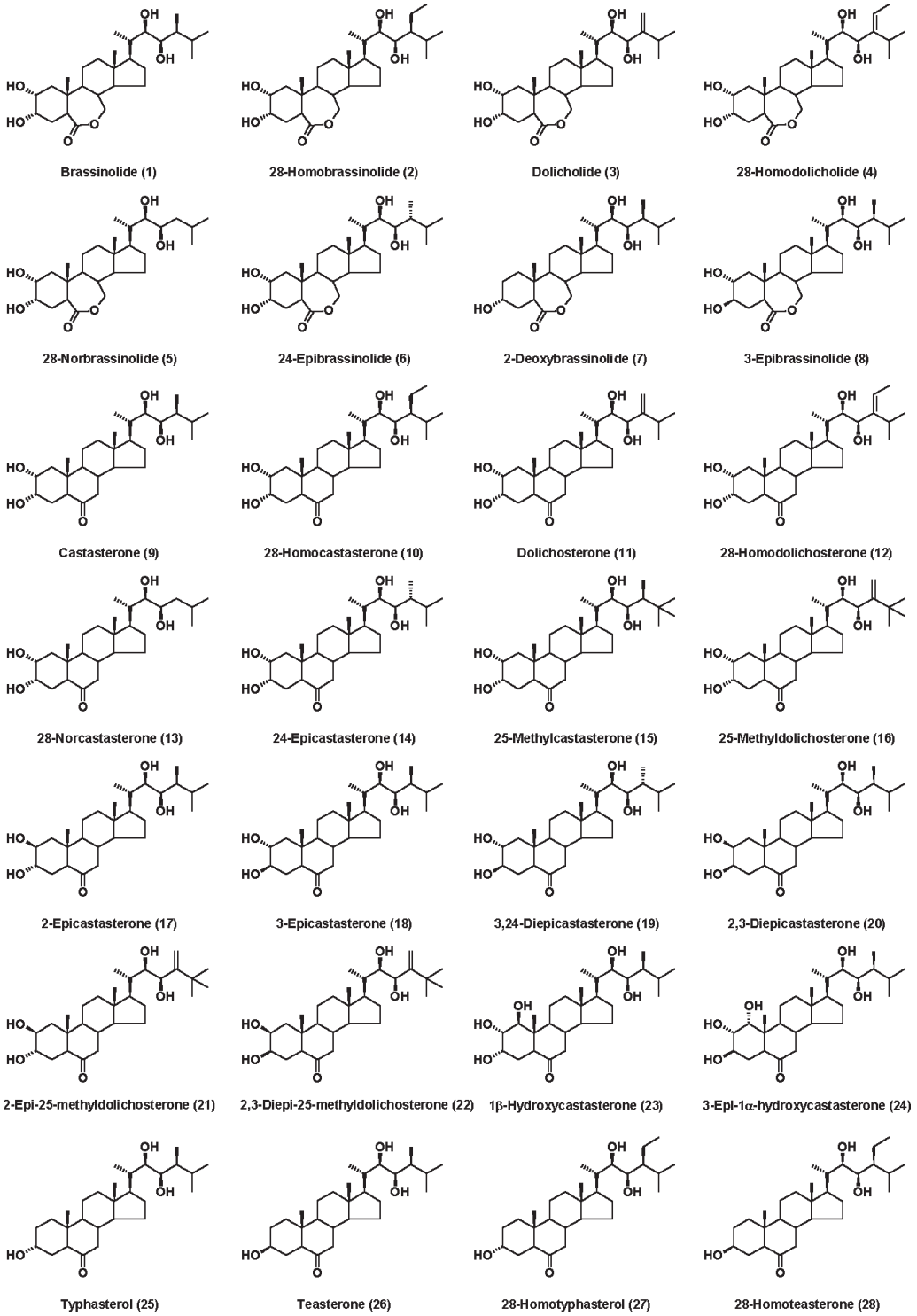

Figure 1. Natural brassinosteroids.

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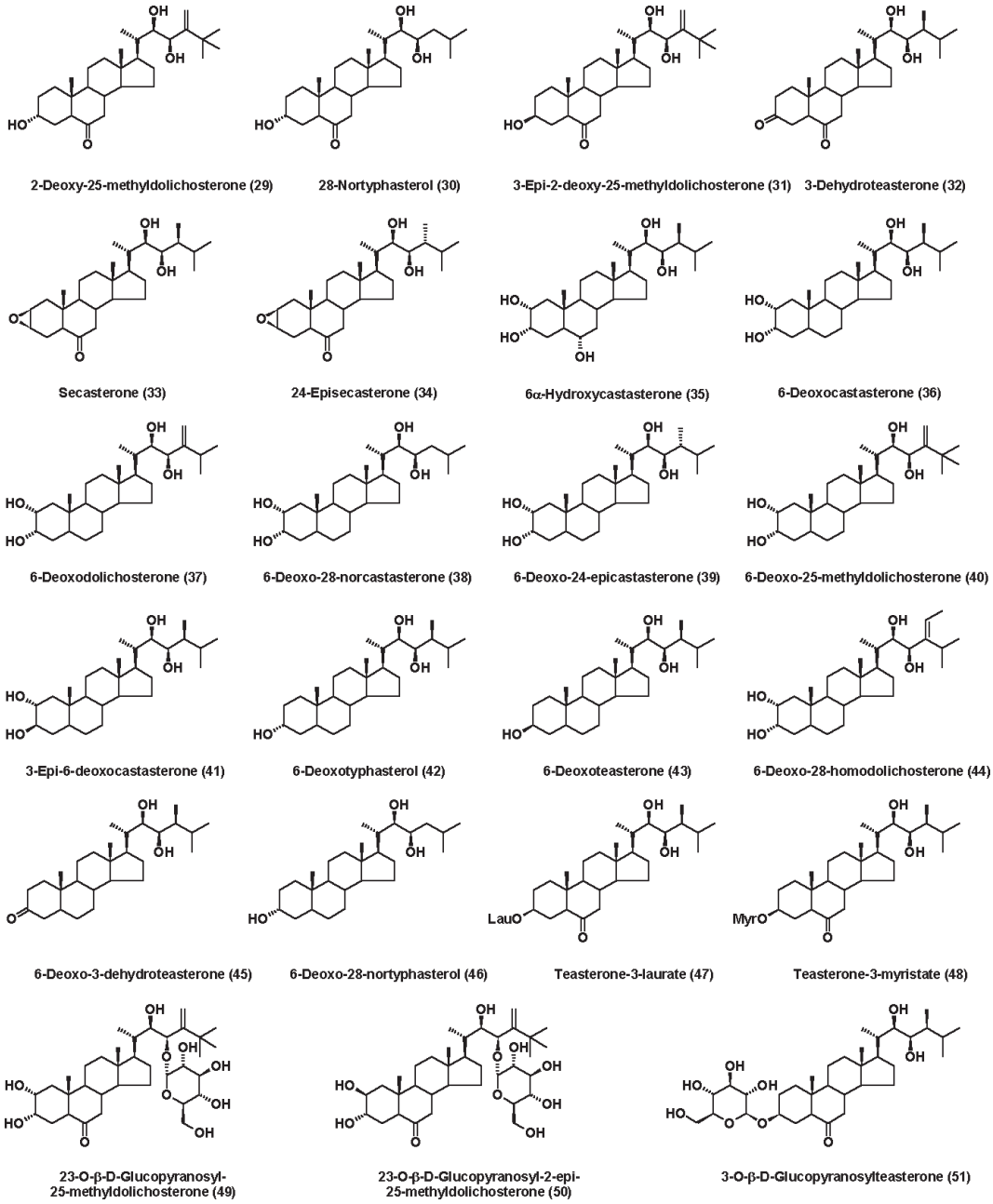

Figure 1. Natural brassinosteroids (continued).

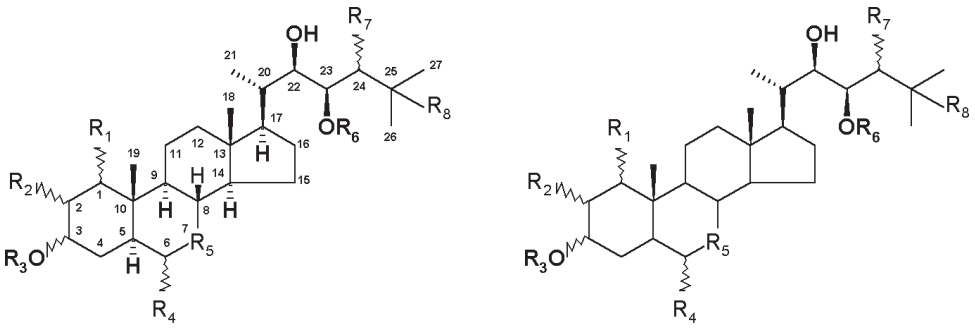

Figure 2. General formulae of natural brassinosteroids.

Table 1. Occurrence of natural brassinosteroids

Species	Family	Plant part	Brassinosteroids	Reference
Alnus glutinosa (L.) Gaertn.	Betulaceae	pollen	1, 9	Plattner et al., 1986
Apium graveolens L.	Umbelliferae	seeds	7	Schmidt et al., 1995c
Arabidopsis thaliana (L.) Heynh.	Brassicaceae	*bri1-5* seedling	1, 9, 13, 30	Fujioka et al., 2000a
		root-callus suspension cultures	1, 8	Konstantinova et al., 2001
		seeds	6, 9	Schmidt et al., 1997
		seeds, siliques	1, 9, 25, 36, 42, 43	Fujioka et al., 1998
		shoots	9, 25, 36, 42	Fujioka et al., 1996
Banksia grandis Willd.	Proteaceae	pollen	1, 9	cf. Takatsuto, 1994
Beta vulgaris L.	Chenopodiaceae	seeds	9, 14	Schmidt et al., 1994
Brassica campestris var. pekinensis Lour.	Brassicaceae	seeds, sheat	1, 2, 5, 9, 10, 13	Abe et al., 1982, 1983; Arima et al., 1984; Ikekawa and Takatsuto, 1984
Brassica napus L.	Brassicaceae	pollen	1, 9	Grove et al., 1979; Ikekawa et al., 1984
Cannabis sativa L.	Cannabaceae	seeds	9, 26	Takatsuto et al., 1996b; Gamoh et al., 1996
Cassia tora L.	Fabaceae	seeds	1, 9, 13, 25, 26	Park et al., 1993a, 1994b
Castanea crenata Sieb. Et Zucc	Fagaceae	galls	1, 9, 13, 36	Abe et al., 1983; Ikeda et al., 1983; Ikekawa et al., 1984; Yokota et al., 1982a
		shoots	9, 36	Arima et al., 1984
Catharanthus roseus G. Don.	Apocynaceae	crown gall cells	1, 9	Park et al., 1989
		culture cells	1, 9, 25, 26, 35, 36, 39, 43	Choi et al., 1993, 1997; Fujioka et al., 2000b
Cistus hirsutum Theill.	Cistaceae	pollen	1, 9	cf. Takatsuto, 1994
Citrus sinensis Osbeck	Rutaceae	pollen	1, 9	Motegi et al., 1994
Citrus unshiu Marcov.	Rutaceae	pollen	1, 9, 25, 26	Abe, 1991; cf. Takatsuto, 1994
Cryptomeria japonica D. Don.	Taxodiaceae	pollen, anthers	2, 3, 4, 25, 32	cf. Takatsuto, 1994; Yokota et al., 1998; Watanabe et al., 2000
Cucurbita moschata Duchesne	Cucurbitaceae	seeds	1, 9	Jang et al., 2000
Cupressus arizonica E. Greene	Cupressaceae	pollen	1, 9, 10, 11, 25, 26, 32, 36, 42, 45	Griffiths et al., 1995
Daucus carota ssp. sativus L.	Apiaceae	seeds	1, 9, 14	Schmidt et al., 1998
Diospyros kaki Thunb.	Ebenaceae	seeds	9	cf. Takatsuto, 1994
Distylium racemosum Sieb et Zucc.	Hammamelidaceae	galls	9, 13	Ikekawa et al., 1984
		leaves	1, 5, 9, 13, 32	Abe et al., 1994
Dolichos lablab Adans.	Leguminosae	seeds	1, 3, 4, 5, 9, 11, 12, 36, 37	Baba et al., 1983; Yokota et al., 1982b, 1983b, 1984
Echium plantagineum L.	Boraginaceae	pollen	1	cf. Takatsuto, 1994
Equisetum arvense L.	Equisetaceae	strobilus	5, 9, 11, 13	Takatsuto et al., 1990a
Eriobottrya japonica Lindl.	Rosaceae	flower buds	9	cf. Takatsuto, 1994
Erythronium japonicum Decne	Liliaceae	pollen, anthers	25	Yasuta et al., 1995
Eucalyptus calophylla R. Br.	Myrtaceae	pollen	1	cf. Takatsuto, 1994
Eucalyptus marginata Sn.	Myrtaceae	pollen	11	cf. Takatsuto, 1994
Fagopyrum esculentum Moench	Polygonaceae	pollen	1, 9	Takatsuto et al., 1990b
Gingko biloba L.	Gingkoaceae	seeds	26	Takatsuto et al., 1996a
Gypsophyla perfoliata L.	Caryophyllaceae	seeds	6	Schmidt et al., 1996
Helianthus annuus L.	Asteraceae	pollen	1, 9, 13	Takatsuto et al., 1989
Hydrodiction reticulatum (L.) Lagerheim	Hydrodictyaceae	green alga	10, 14	Yokota et al., 1987a
Lilium elegans Thunb.	Araceae	pollen	1, 9, 25, 26	Susuki et al., 1994b
Lilium longiflorum Thunb.	Araceae	anthers	1, 9, 25, 32, 47, 48, 51	Abe, 1991; Abe et al., 1994; Asakawa et al., 1994;

Continue

Table 1. Occurrence of natural brassinosteroids (continued).

Species	Family	Plant part	Brassinosteroids	Reference
				Soeno et al., 2000b
		culture cells	26, 47, 48	Soeno et al., 2000a
		pollen	47, 48	Asakawa et al., 1996
Lolium perene L.	Poaceae	pollen	15	Taylor et al., 1993
Lychnis viscaria L.	Caryophyllaceae	seeds	14, 34	Friebe et al., 1999
Lycopersicon esculentum Mill.	Solanaceae	2 month old plants	9, 36, 42, 43, 45	Bishop et al., 1999
		roots and shoots	38, 46	Yokota et al., 2001
		shoots	9, 13, 36	Yokota et al., 1997
Marchantia polymorpha L.	Marchantiaceae	whole bodies	9	Kim et al., 2002
Ornithopus sativus Brot.	Fabaceae	seeds	9, 14	Schmidt et al., 1993a
		shoots	9, 14, 36, 38, 39	Spengler et al., 1995
Oryza sativa L.	Poaceae	bran	27, 28, 36	Abe et al., 1995a
		seeds	9, 26, 36	Ikekawa and Takatsuto, 1984; Park et al., 1993c, 1994a
		shoots	1, 9, 11	Abe et al., 1984b; Shim et al., 1998
Perilla frutescens Britton.	Labiatae	seeds	2, 9	Park et al., 1993b, 1994a
Phalaris canariensis L.	Poaceae	seeds	9, 26	Shimada et al., 1996
Pharbitis purpurea Voigt	Convolvulaceae	seeds	9, 13	Suzuki et al., 1985
Phaseolus vulgaris L.	Fabaceae	seeds	1, 3, 9, 16, 17, 18, 19, 20, 21, 22, 23,24, 25, 26, 29, 31, 36, 37, 40, 41, 44, 49, 50	Kim, 1988; Kim et al., 1988; Yokota et al., 1983c, 1987b; Kim et al., 2000
Phoenix dactilifera L.	Arecaceae	pollen	14	Zaki et al., 1993
Picea sitchensis (Bong.) Carr	Pinaceae	shoots	9, 25	Yokota et al., 1985
Pinus silvestris Lour.	Pinaceae	cambial region	1, 9	Kim et al., 1990
Pinus thubergii Parl.	Pinaceae	pollen	9, 25	Yokota et al., 1983a
Pisum sativum L.	Fabaceae	seeds	1, 7, 9, 25, 36	Yokota et al., 1996
		shoots	1, 9, 36	Nomura et al., 1997
Psophocarpus tetragonolobus DC	Fabaceae	seeds	1, 9, 10, 36	cf. Takatsuto, 1994
Raphanus sativus L.	Brassicaceae	seeds	1, 9, 26, 28	Schmidt et al., 1991, 1993b
Rheum barbarum L.	Polygonaceae	panicles	1, 9, 14	Schmidt et al., 1995a
Robinia pseudo-acacia Oswald	Fabaceae	pollen	9, 25, 36	Abe et al., 1995b
Secale cereale L.	Poaceae	seeds	9, 10, 13, 25, 26, 33, 36	Schmidt et al., 1995b
Solidago altissima L.	Asteraceae	stem	1	cf. Takatsuto, 1994
Sporobolus stapfianum Gand.	Graminae		1, 3, 9	Sasse et al., 1998
Thea sinensis L.	Theaceae	leaves	1, 9, 10, 13, 25, 26	Abe et al., 1983, 1984a; Ikekawa and Takatsuto, 1984; Morishita et al., 1983
Triticum aestivum L.	Poaceae	grain	9, 25, 26, 32, 36	Yokota et al., 1994
Tulipa gesneriana L.	Liliaceae	pollen	25	Abe, 1991; cf. Takatsuto, 1994
Typha latifolia G. F. W. Mey.	Typhaceae	pollen	25	Schneider et al., 1983
Vicia faba L.	Fabaceae	pollen	1, 6, 9, 11, 13	Ikekawa et al., 1988; Park et al., 1988
		seeds	1, 9	Park et al., 1988; Park and Hyun, 1987
Zea mays L.	Poaceae	pollen	9, 10, 13, 25, 26	Suzuki et al., 1986; Gamoh et al., 1990a
		seeds	9, 13, 26, 36	Park et al., 1995

Some other natural products (57-75; figure 4) related to brassinosteroids, many of them occurring in the immature *Phaseolus vulgaris* L. seeds (Kim, 1991; Kim et al., 1994) have been reported, but their structures are incompletely elucidated (Fujioka, 1999). They include configurational isomers of known brassinosteroids (57-59, 61-69), of a brassinosteroid catabolite (60) and brassinosteroids bearing extra oxygen or carbonyl bearing carbon atom in ring A (70-75).

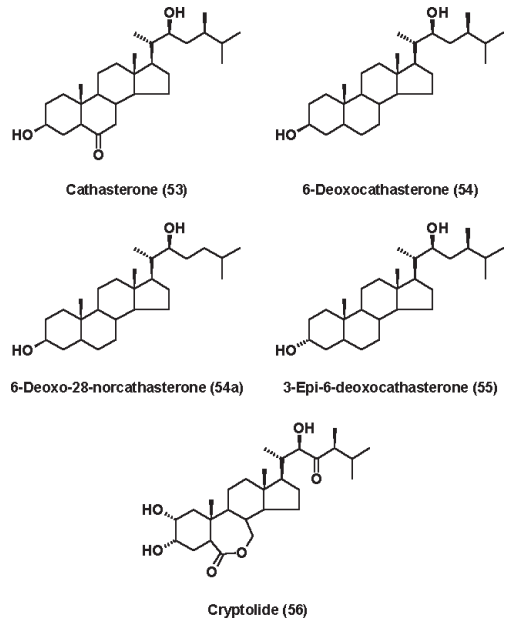

Figure 3. Biosynthetic precursors and a catabolite of brassinosteroids.

DETECTION AND CHROMATOGRAPHIC ANALYSIS OF BRASSINOSTEROIDS

Due to the very small amount in which brassinosteroids are found in plants (ca. 10-100 μg.kg^{-1} in pollen, 1-100 μg.kg^{-1} in immature seeds and 10-100 ng.kg^{-1} in shoots and leaves; Adam and Marquardt, 1986; Mandava, 1988; Takatsuto, 1994), special methods were developed for their detection and identification, as their isolation in pure state would demand a great amount of plant material and be very tedious and expensive. The extraction of brassinosteroids from the plant material can be achieved with partition and chromatographic processes in which extraction with methanol or methanol/ethyl acetate followed by partition between water/chloroform and 80% methanol/*n*-hexane is used as a standard procedure. A sensitive bioassay is necessary to monitor the brassinosteroid containing fractions during the chromatographic steps.

The biological detection of brassinosteroids was initially performed by the bean second internode bioassay, a test gradually substituted by the rice lamina inclination bioassay (Wada et al., 1981, 1984; Takeno and Pharis, 1982; Kim et al., 1990) and the wheat leaf unrolling bioassay (Wada et al., 1985; Takatsuto, 1994), while the immunological methods are less used (Horgen et al., 1984; Yokota et al., 1990; Schlagenhaufer et al., 1991; Taylor et al., 1993).

Due to the need to detect brassinosteroids in plant sources, a micromethod was very quickly developed for screening and quantification of these kinds of compounds (Takatsuto et al., 1982), that consists in reacting the *cis*-dihydroxy function with methaneboronic acid, affording its methane- or bismethaneboronate, e.g. brassinolide bismethaneboronate (76). Eventually isolated hydroxyl is trimethylsilylated after boronation to afford, e.g. in case of 2-deoxybrassinolide (7), the mixed derivative (77; figure 5). The derivatives are analysed by gas chromatography, with retention times sensitive to small variations in the structure of the brassinosteroids. The boronates are analysed by mass spectrometry, either by electron impact or chemical ionization. Selective scan ion monitoring can also be used, due to the regularity of the fragmentation pattern of the different types of brassinosteroids (Adam et al., 1996, 1999). The method is being routinely used in the detection of brassinosteroids because its detection limit is less than 10 pg (Ikekawa and Takatsuto, 1984; Ikekawa et al., 1984; Takatsuto, 1994).

High performance liquid chromatography is the method of choice for final purification in the isolation of natural brassinosteroids, but it is unusual for their detection in plant sources (Konstantinova et al., 2001). Reversed phase high performance liquid chromatography is less sensitive than gas chromatography for the detection and quantification of brassinosteroids, with a detection limit in the range of 25-100 pg, but with response linearity in the range of 25 pg-40 ng, in the best instances, and precision around 3 %. Using the vicinal hydroxyls as derivatization sites the α-naphthylboronic (78; Gamoh et al., 1988), 9-phenanthrylboronic (79; Takatsuto et al., 1989, 1990b), 1-cyanoisoindolyl-2-*m*-phenylboronic (80; Gamoh and Takatsuto, 1989), dansylaminophenylboronic (81, Gamoh et al., 1990a), *m*-aminophenylboronic (82; Gamoh et al., 1992) and ferrocenylboronic (83; Gamoh et al., 1990b) acids reacted with brassinosteroids to obtain, quantita-

tively, the derivatives 84 (figure 6). The naphthylboronates 84 are ultraviolet detected, with absorption maxima at 280 nm and detection limit of 100 pg, the ferrocenylboronates 84 are electrochemically detected with detection limit of 50 pg, while the boronates derived from 79-81 are indicated for fluorimetric detection with detection limits of, respectively, 50 pg, 20 pg and 25 pg. These methods have also been used to identify and quantify brassinosteroids in plant sources (Takatsuto et al., 1989, 1990a, b; Gamoh and Takatsuto, 1994; Motegi et al., 1994).

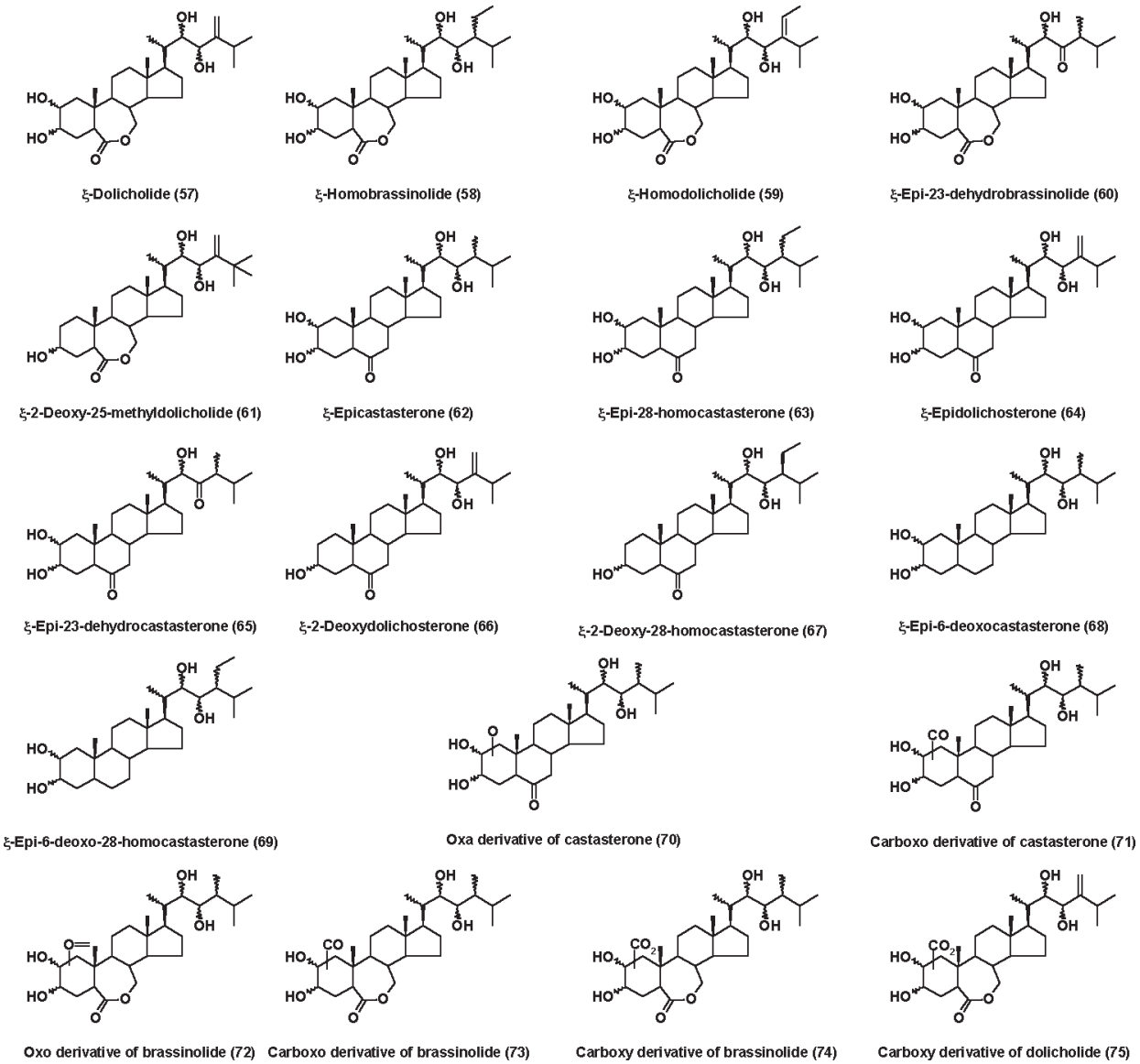

Figure 4. Natural products related to brassinosteroids with incompletely elucidated structure.

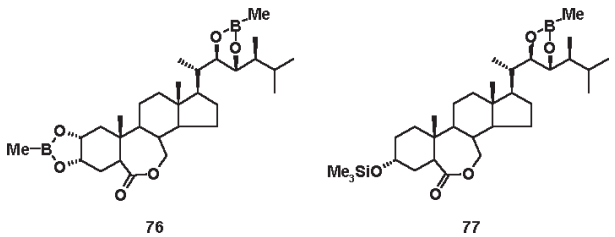

Figure 5. Brassinosteroid derivatives for gas chromatography/mass spectrometry.

A quite different strategy for the detection of brassinosteroids employs dansylhydrazine (85; figure 6) to prepare the fluorescent dansylhydrazones of 6-oxobrassinosteroids followed by the dansylaminophenylboronation of vicinal hydroxyls. The sensitivity of the method is only 1.5 ng for the hydrazone of 24-epicastasterone (86) and it has the advantage that even precursors lacking the 22α,23α-diol side chain can be detected. The subsequent dansylaminophenylboronation of the hydrazone brings the sensitivity of the method to 100 pg (Winter et al., 1999).

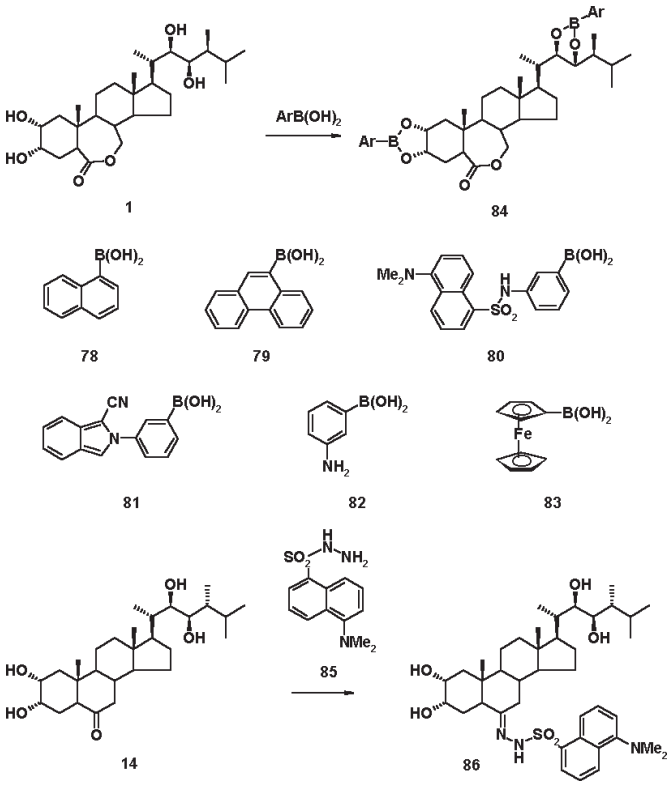

Figure 6. Brassinosteroid derivatives for reversed phase high performance liquid chromatography.

BIOSYNTHESIS AND METABOLISM OF BRASSINOSTEROIDS

The elucidation of brassinosteroid biosynthesis (Sakurai, 1999) and metabolism (Adam and Schneider, 1999; Schneider, 2002) is important for determining what are their biologically active forms and for understanding how their endogenous levels are regulated to promote adequate plant growth and development.

The first system used for studying brassinosteroid biosynthesis was feeding culture cells of *Catharantus roseus* G. Don. (Madagascar periwinkle) with deuterium labeled precursors of brassinolide (1), where campesterol (87) was the main component of the sterol fraction, and trace the deuterium labeling in the brassinosteroid fraction. The discoveries of brassinosteroid biosynthesis deficient mutants in *Arabidopsis thaliana*, *Pisum sativum* and *Lycopersicon esculentum* allowed the clarification of some steps in brassinolide biosynthetic pathways. In this case, partial recovery of their growth or development is rescued by exogenous application of brassinolide and its precursors or putative precursors. The blocked step is recognized as the one in which administration of a compound does not change the mutant phenotype. It was soon recognized that campesterol biosynthesis deficient mutants presented brassinosteroid deficiency, and in this case analysis of sterol composition aids to locate the specific biosynthetic step blocked. It should be mentioned that brassinosteroid-insensitive mutants, i.e., mutants that can respond to all other plant hormones but not to brassinosteroids, were also recognized in *Arabidopsis*, pea and tomato (Clouse and Sasse, 1998; Clouse and Feldmann, 1999).

It was verified that brassinolide biosynthesis begins by the reduction of campesterol (87) to campestanol (88), which is oxidized to 6α-hydroxycampestanol (89) and this to 6-oxocampestanol (90; Suzuki et al., 1995a). Parallel experiments showed that cathasterone (53) is the biosynthetic precursor of typhasterol (25) and teasterone (26; Fujioka et al., 1995), but the conversion of 6-oxocampestanol (90) to cathasterone (53) or to teasterone (26) could not be demonstrated (Fujioka et al., 1995; Suzuki et al., 1995b; Fujioka and Sakurai, 1997b). It was observed that teasterone (26) and typhasterol (25) are interconvertible in periwinkle, tobacco and tomato and that typhasterol (25) is oxidized to castasterone (9) and then to brassinolide (1; Suzuki et al., 1994a, 1995a). In periwinkle, tobacco and rice castasterone (9) is also isomerized to 3-epicastasterone (18; Suzuki et al., 1995a). As a result, a probable biosynthetic route to brassinolide (1) is shown in figure 7. Due to the initial conversion of campestanol (88) to 6α-hydroxycampestanol (89), this route is called the "early C-6 oxidation pathway" (Fujioka and Sakurai, 1997a, b).

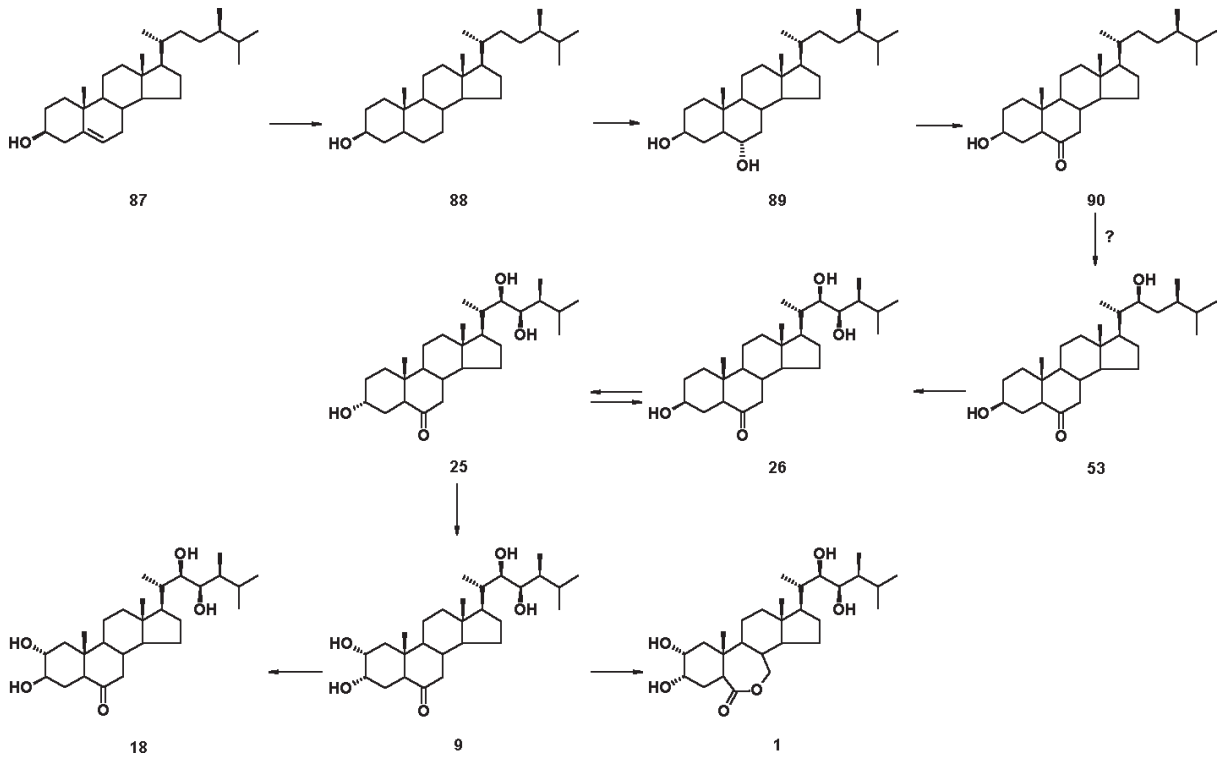

Figure 7. Biosynthesis of brassinolide via the early C-6 oxidation pathway.

The 6-deoxobrassinosteroids, presenting weak brassinolide activity, were initially considered as inactivation products of the 6-oxobrassinosteroids. As they were detected in an increasing number of plant species and, in many cases, were observed the presence of the pair 6-deoxocastasterone (36)/castasterone (9), the hypothesis arose that the 6-deoxobrassinosteroids could also be biosynthetic precursors of 6-oxobrassinosteroids. In cultured periwinkle, tobacco and rice cells the conversion of 6-deoxocastasterone (36) to castasterone was observed (9; Choi et al., 1996). In cultured cells of periwinkle the presence of 6-deoxotyphasterol (42) and 6-deoxoteasterone (43) was also observed and, using labeled compounds, the conversions of 6-deoxoteasterone (43) to 3-dehydro-6-deoxoteasterone (45) and then to 6-deoxotyphasterol (42), to 6-deoxocastasterone (36) and to castasterone (9), as shown in figure 8, could be demonstrated (Choi et al., 1997). This route is called the "late C-6 oxidation pathway". It was verified that both early and late C-6 oxidation pathways operate simultaneously in periwinkle, but there is no conversion of 6-deoxoteasterone (43), 3-dehydro-6-deoxoteasterone (45) or 6-deoxotyphasterol (42) to their 6-oxo counterparts. These studies could not say how campestanol (88) is converted to 6-deoxoteasterone (43). Recent studies, however, have revealed that 6-deoxotyphasterol (42) is converted to typhasterol (25) in *Arabidopsis*, to a marginal extent (Noguchi et al., 2000).

Although there are important differences in the biosynthesis of plant sterols, a commonly accepted route for the biosynthesis of campesterol (87) and campestanol (88) is depicted in figure 9 (Asami and Yoshida, 1999). Plant sterols are biosynthesized from mevalonic acid, which originates squalene-2,3-oxide (91) that cyclizes to cycloartenol (92). This compound is homologated to 24-methylenecycloartenol (93) and demethylated to cycloeucalenol (94), which is isomerized to obtusifoliol (95). Subsequent demethylation gives rise to 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol (96), that is reduced to 4α-methylfecosterol (97). Isomerization of the $\Delta^{8(9)}$ double

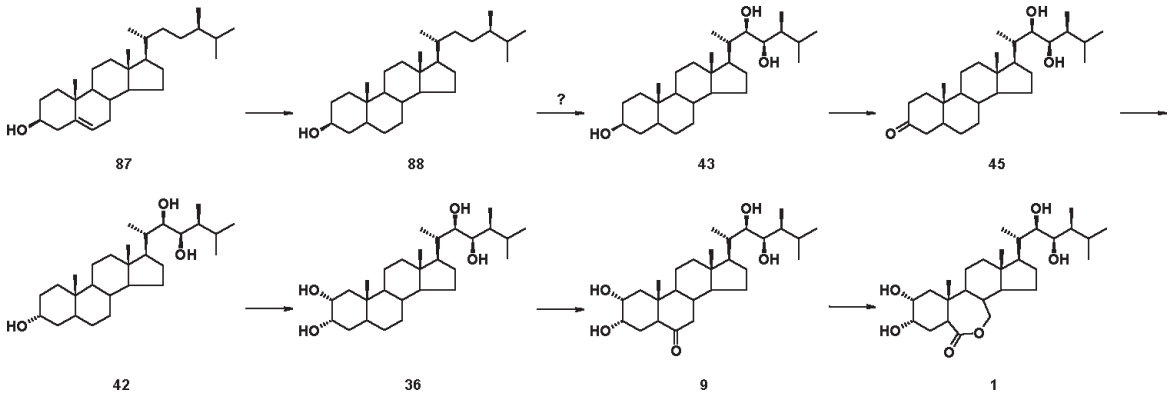

Figure 8. Biosynthesis of brassinolide via the late C-6 oxidation pathway.

bond to $\Delta^{7(8)}$ originates 24-methylenelophenol (98), that is demethylated to episterol (99). This compound suffers dehydrogenation to 5-dehydroepisterol (100) and hydrogenation to 24-methylenecholesterol (101). Isomerization of the $\Delta^{24(28)}$ double bond to $\Delta^{24(25)}$ produces 24-methyldesmosterol (102), that is reduced to campesterol (87). Oxidation of this sterol to (24R)-24-methyl-4-cholesten-3-one (103) is followed by saturation of the olefinic double bond to (24R)-methyl-5α-cholestan-3-one (104) and reduction of the carbonylic function for campestanol (88) production.

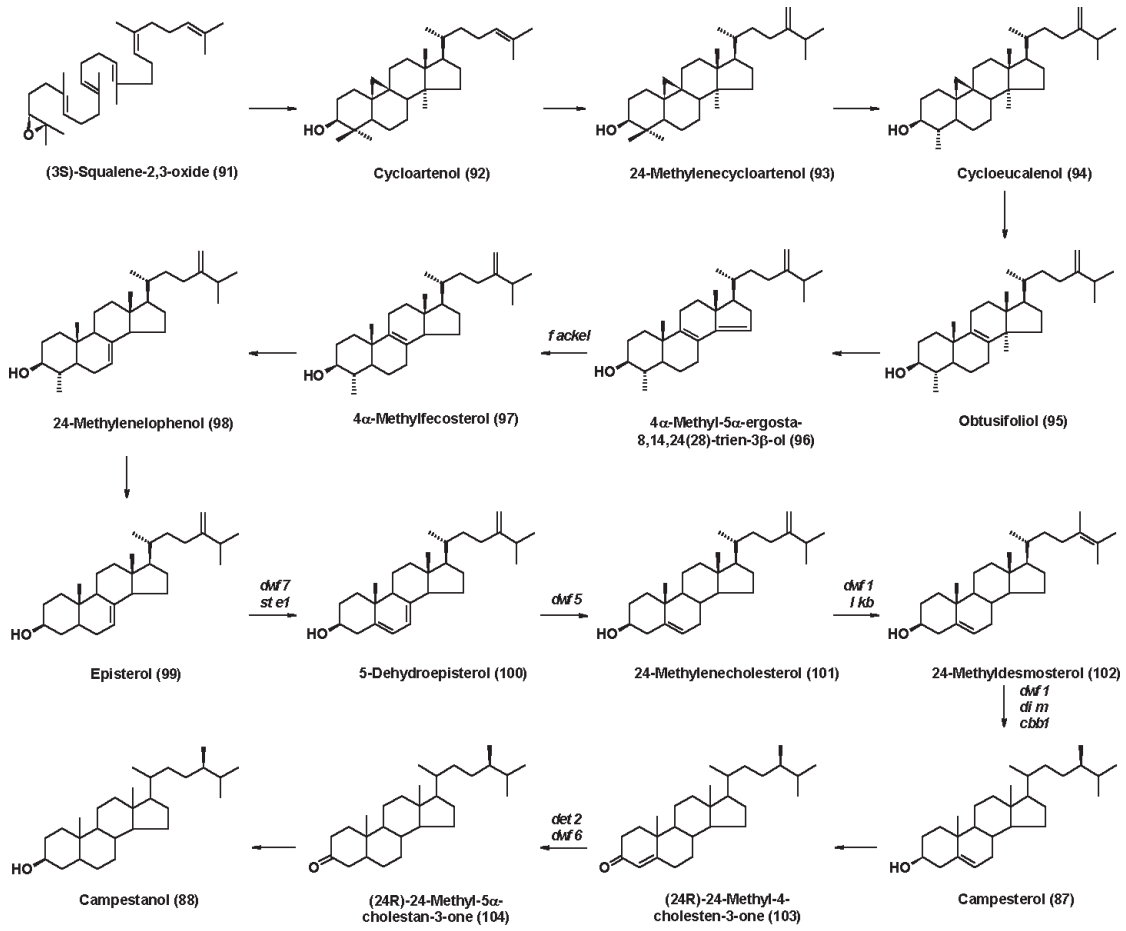

Figure 9. Biosynthesis of campestanol (88) from (3S)-squalene-2,3-oxide (91).

The 14α-demethylation of obtusifoliol (95) to 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol (96) is encoded by a CYP51 enzyme (steroid 14α-demethylase) and *Arabidopsis* antisense *AtCYP51* transgenic plants showed dwarfism during early development, slow growth during maturation, a high obtusifoliol (95) content but did not show phytosterol deficiency (Kushiro et al., 2001). In the *Arabidopsis fackel* mutant seedlings a high content of 96 and $\Delta^{8,14}$-unsaturated sterols was observed, and the wild phenotype was not rescued by brassinosteroid application, indicating the blockage of the conversion of 96 to 4α-methylfecosterol (97; Jang et al., 2000; Schrick et al., 2000). Two *Arabidopsis* mutants, *dwf7* and *ste1*, were recognized to be unable to perform the conversion of episterol (99) to 5-dehydroepisterol (100), and a third one, *dwf5*, is blocked in the conversion of 5-dehydroepisterol (100) to 24-methylenecholesterol (101). The *Arabidopsis dwf1* mutant is defective in the conversion of the last compound to campesterol (87). The mutants *dim* and *cbb1* are defective in the conversion of 24-methyldesmosterol (102) to campesterol (87). The *Arabidopsis* mutants *det2* and *dwf6* are defective in the reduction of (24R)-24-methyl-4-cholesten-3-one (103) to (24R)-methyl-5α-cholestan-3-one (104). The blocked biosynthetic step in the garden pea *lkb* mutant is the conversion of 24-methylenecholesterol (101) to campesterol (87; Nomura et al., 1999), more probably the isomerization of 101 to 24-methyldesmosterol (102).

A small number of brassinosteroid biosynthesis mutants were recognized in the steps between campestanol (88) and brassinolide (1; figure 10). In the *Arabidopsis dwf4* mutant the conversions of campestanol (88) to 6-deoxocathasterone (54) and of 6-oxocampestanol (90) to cathasterone (53) are blocked, indicating that both substrates (88 and 90) are recognized by the same 22α-hydroxylase (Choe et al., 1998). In the *Arabidopsis* mutants *cpd* (Szekeres et al., 1996), *dwf3* and *cbb3,* the blocked brassinolide biosynthesis steps are the 23-hydroxylations of 6-deoxocathasterone (54) to 6-deoxoteasterone (43) and of cathasterone (53) to teasterone (26). The tomato *dpy* mutant, an intermediate dwarf with severely altered morphology, is rescued by spraying with 6-deoxoteasterone (43) and subsequent precursors of brassinolide (1) in the late C-6 oxidation pathway, but not by 6-deoxocathasterone (54), cathasterone (53) or their precursors (Clouse and Feldmann, 1999). In tomato the late C-6 oxidation pathway seems to be the major route in brassinolide biosynthesis. Analysis of the brassinosteroid fraction in the extreme dwarf (d^x) tomato mutant showed that brassinolide biosynthesis is blocked in the conversion of 6-deoxocastasterone (36) to castasterone (9; Bishop et al., 1999), as evidenced by the low castasterone (9) and high 6-deoxocastasterone (36) contents in the mutant compared to the wild type.

The conversion of teasterone (26) to typhasterol (25) and then to castasterone (9) was also observed in cultured cells of *Marchantia polymorpha* (Park et al., 1999; Kim et al., 2001), while in *Phaseolus vulgaris* the *in vitro* enzymatic conversion of teasterone (26) to typhasterol (25) was confirmed to be a two-step reaction with the intermediacy of 3-dehydroteasterone (32; Kim et al., 2000). Although this is evidence that the pathways depicted in figure 10 are common for brassinolide (1) biosynthesis in plant species other than *Arabidopsis*, pea and tomato (Nomura et al., 2001), they may not be simply extended to the synthesis of other lactones. In the case of 28-norbrassinosteroids one would expect that they could be derived from cholesterol (105), in a series of reactions similar to those occurring from campesterol (87). Metabolic experiments with deuterium labeled castasterone (9) in *Arabidopsis*, rice, tomato and periwinkle detected 28-norcastasterone (13) as a catabolite of castasterone (9; Fujioka et al., 2000a). The detection of 28-nortyphasterol (30) in *Arabidopsis* (Fujioka et al., 2000a) and of 6-deoxo-28-norcathasterone (54a), 6-deoxo-28-nortyphasterol (46) and 6-deoxo-28-norcastasterone (38) in tomato (Yokota et al., 2001) are indications that both early and late oxidation pathways are operative for the synthesis of 28-norbrassinosteroids from a suitable precursor such as cholestanol (88a). Feeding experiments with labeled campestanol (88), cholestanol (88a) and cholesterol (105) in *Arabidopsis*, tobacco and periwinkle revealed that cholesterol (105) is converted to 4-cholesten-3-one (103a), cholestanol (88a) and 6-oxocholestanol (90a), but the conversion ratios of cholesterol (105) to cholestanol (88a) are much smaller than those of campestanol (88) to cholestanol (88a, Nakajima et al., 2002), so that it is unlikely that 28-norbrassinosteroids are preferably biosynthesized from cholesterol (105) but more probably from campesterol (87; see figure 11).

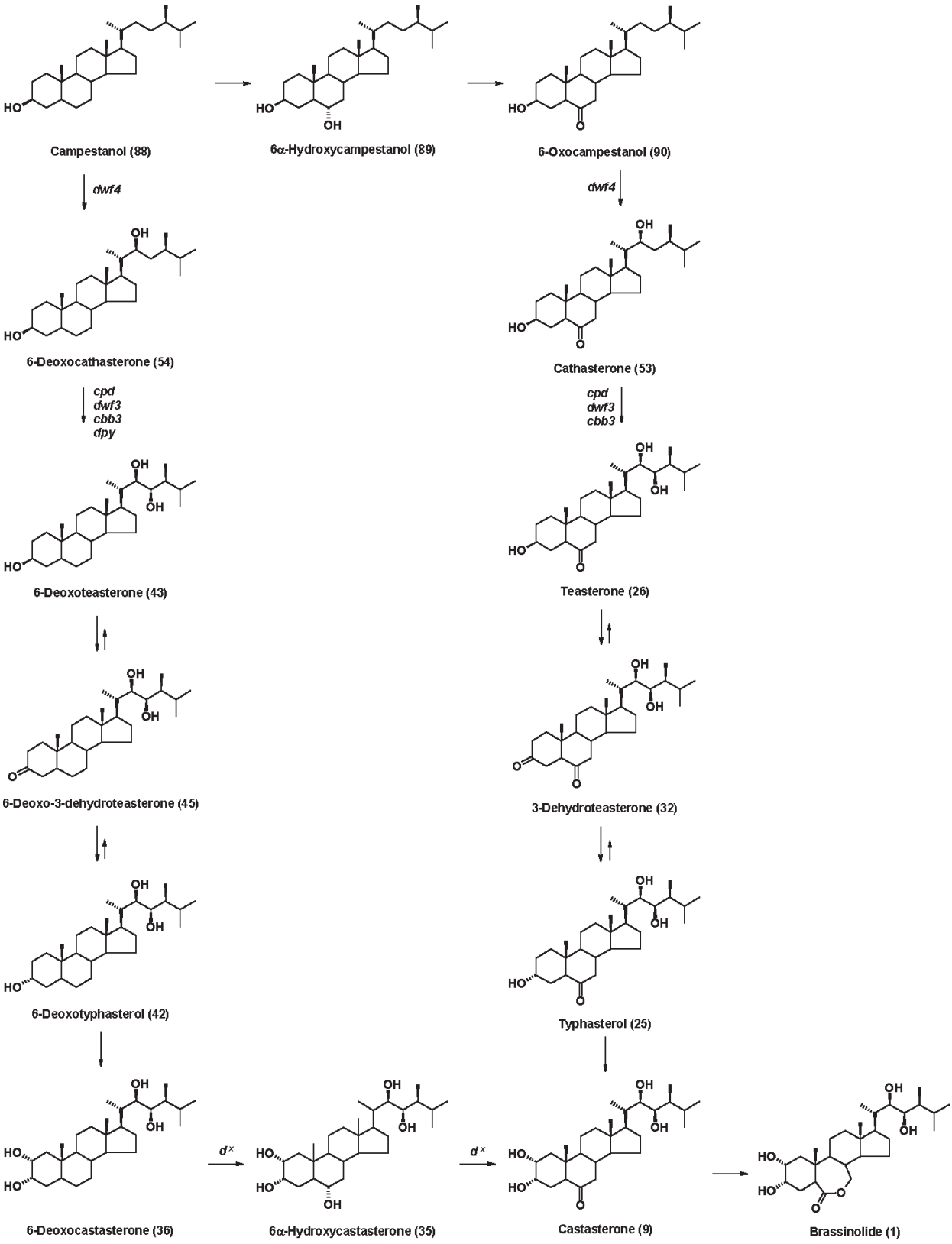

Figure 10. Biosynthesis of brassinolide (1) from campestanol (88).

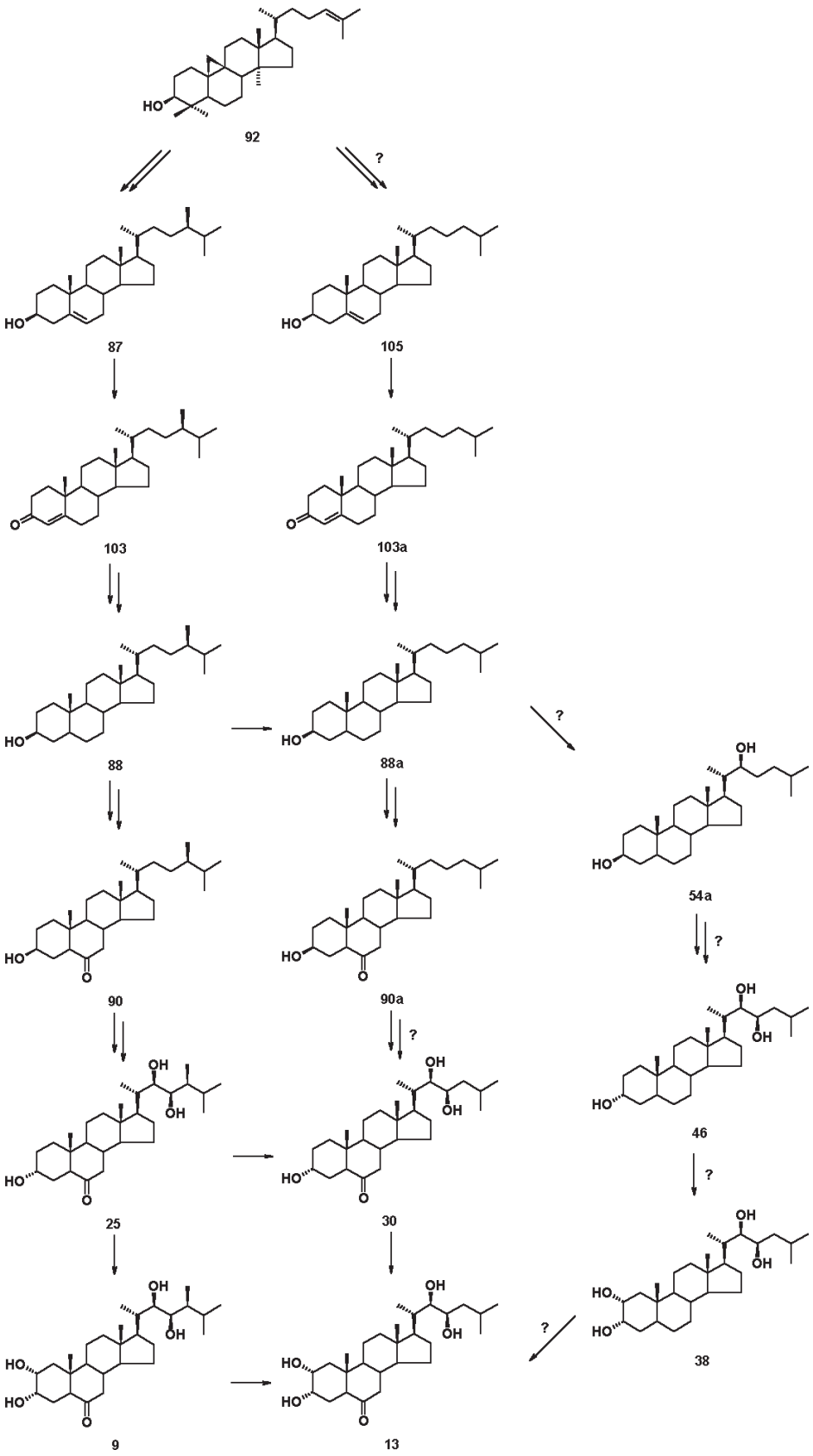

Figure 11. Biosynthesis of 28-norcastasterone (13).

The brassinosteroid metabolism was mainly studied in cultured cells of tomato and serradella using the corresponding 5,7,7-*tris*-tritiated brassinosteroids (Kolbe et al., 1992) as monitors in the feeding experiments. Cell suspension cultures of tomato convert 3-dehydro-24-epiteasterone (106), a putative precursor of 24-epibrassinolide (6), to 24-epiteasterone (107) and 24-epityphasterol (108; Kolbe et al., 1998) and also the conjugated brassinosteroids 3-*O*-β-D-glucopyranosyl-24-epiteasterone (109; Kolbe et al., 1998), 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-24-epiteasterone (110) and 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl-24-epiteasterone (111; Kolbe et al., 1997; figure 12). The enzymatic conversion of 24-epiteasterone (107) to 3-dehydro-24-epiteasterone (106) was monitored in cytosolic tomato and *Arabidopsis thaliana* fractions using fluorescent tagging and HPLC analysis. Inhibition experiments with cathasterone (53), 6-deoxocathasterone (54) and 6-deoxoteasterone (43) indicated that the corresponding 3β-dehydrogenase is rather substrate specific for β-dehydrogenation of 24-epiteasterone (107; Stündl and Schneider, 2001).

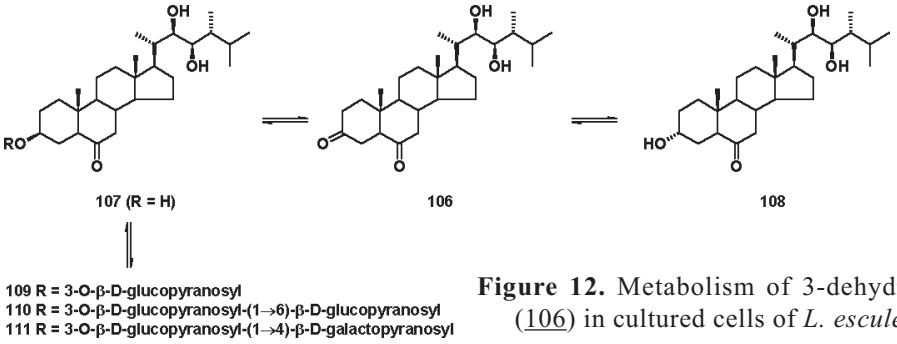

Figure 12. Metabolism of 3-dehydro-24-epiteasterone (106) in cultured cells of *L. esculentum*.

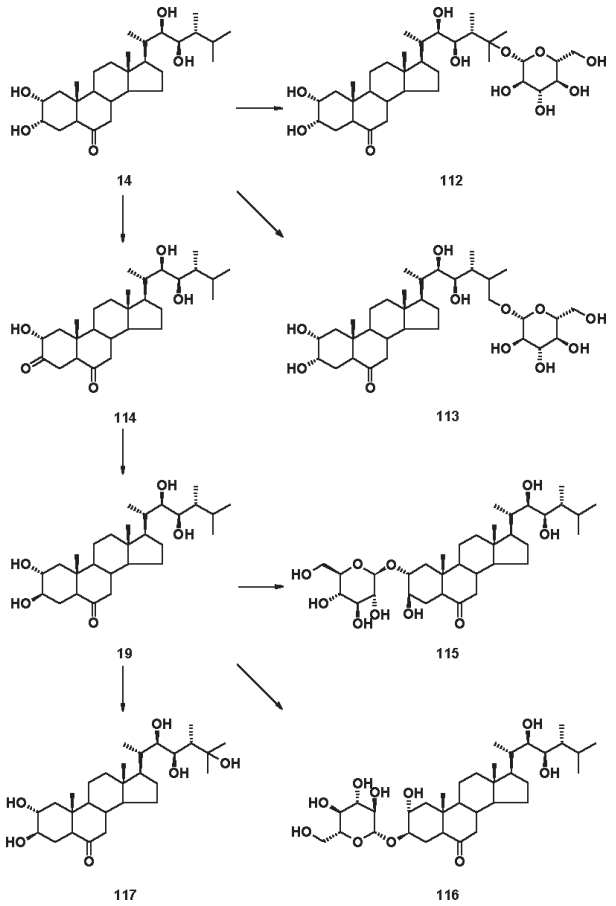

Figure 13. Metabolism of 24-epicastasterone (14) in cultured cells of *L. esculentum*.

In tomato, 24-epicastasterone (14) is hydroxylated and glucosylated at C-25 or C-26, yielding 112 and 113, or is dehydrogenated to 3-dehydro-24-epicastasterone (114), that is reduced to 3,24-diepicastasterone (19). This compound can be glucopyranosylated at C-2 or C-3 yielding 115 and 116 or hydroxylated at C-25 resulting in 25-hydroxy-3,24-diepicastasterone (117; Hai et al., 1996; figure 13).

24-Epibrassinolide (6; figure 14) was transformed to the glucopyranosides 118 and 119, while 25-hydroxy-24-epibrassinolide (120), obtained by enzymatic hydrolysis of 118, was transformed exclusively to the 25-glucoside 118 in cultured cells of tomato (Schneider et al., 1994; Hai et al., 1995). These hydroxylations are performed by two distinct enzymes, and 25-hydroxylase proved to be a cytochrome P450 protein, while the 26-hydroxylase seems to be a flavin-containing monooxygenase (Winter et al., 1997).

It was verified that serradella (*Ornithopus sativus* Brot.) cell cultures degrade 24-epicastasterone (14) up to 2α,3β,6β-trihydroxy-5α-pregnane-20-one (121; Kolbe et al., 1994; figure 15), and fatty esters 122-124 of 3,24-diepicastasterone (19) were also produced (Kolbe et al., 1995). Trihydroxyketone 121 is formed via transformation of 24-epicastasterone (14) to 3,24-diepicastasterone (19), which is oxidized to 20*R*-hydroxy-3,24-diepicastasterone (125) and further to the pregnanedione 126 followed by reduction (figure 15).

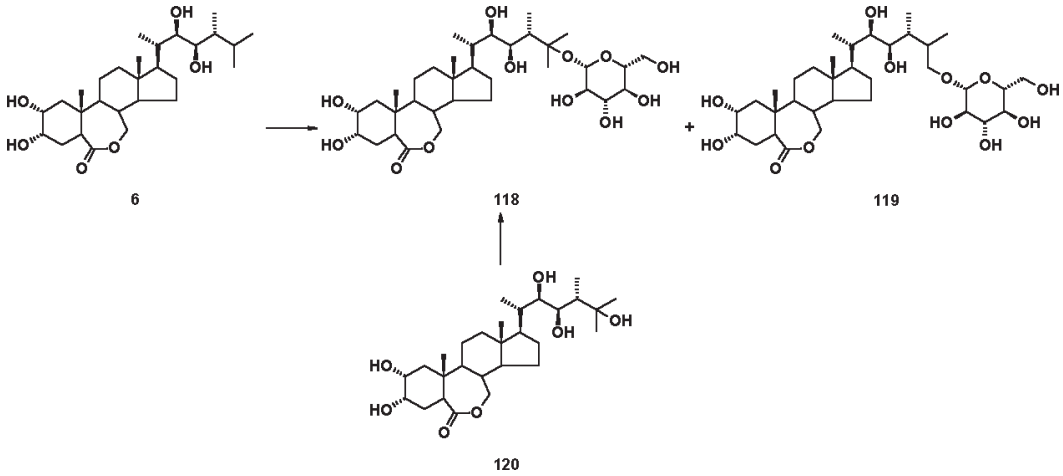

Figure 14. Metabolism of 24-epibrassinolide (6) by cultured cells of *L. esculentum*.

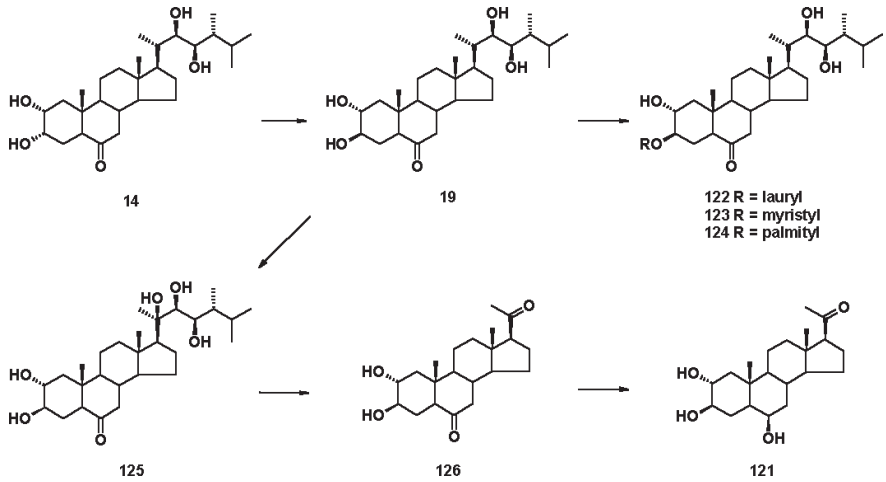

Figure 15. Metabolism of 24-epicastasterone (14) by cell culture of *O. sativus*.

In the same system, 24-epibrassinolide (6; figure 16) was transformed into 2α,3β-dihydroxy-B-homo-7-oxa-5α-pregnane-6,20-dione (127; Kolbe et al., 1994), the esters 128-130 (Kolbe et al., 1996) and into 25-hydroxy-3,24-diepibrassinolide (131) and 20*R*-hydroxy-3,24-diepibrassinolide (132), through the initial conversion of 24-epibrassinolide (6) to 3,24-diepibrassinolide (133; Kolbe et al., 1996).

A purified recombinant *Brassica napus* steroid sulfotransferase expressed by *Escherichia coli* catalyses the enzymatic sulfonation of brassinosteroids and precursors specifically at position 22, as exemplified in figure 17 (Rouleau et al., 1999). It exhibited highest affinity for 24-epicathasterone (134), followed by 24-epiteasterone (107).

In first experiments on microbial transformations of brassinosteroid, incubation of 24-epibrassinolide (6) with the fungus *Cunninghamella echinulata* yielded 12β-hydroxy-24-epibrassinolide (137) and the same 12β-hydroxylation was also observed with 24-epicastasterone (14; Voigt et al., 1993a). On the other hand, the fungus *Cochliobolus lunatus* transformed 24-epicastasterone (14) to the corresponding 15β-hydroxylated compound 138 (Voigt et al., 1993b; figure 18).

The analogue 2α,3α-dihydroxy-6-oxocholestane (139), when incubated with the fungus *Mycobacterium vaccae*, yielded 2α,3α,6α-trihydroxy-5α-androstane-17-one (140) and 2α-hydroxy-4-androstene-3,17-dione (141; Vorbrodt et al., 1991; figure 18).

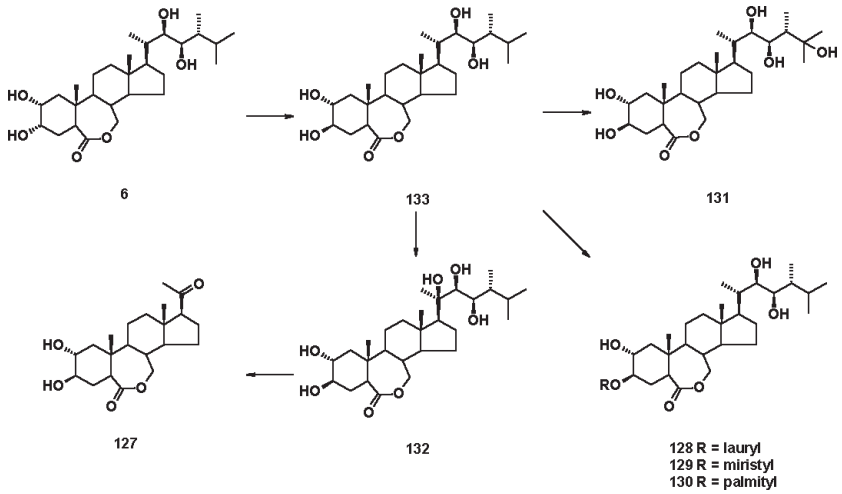

Figure 16. Metabolism of 24-epibrassinolide (6) by culture cells of *O. sativus*.

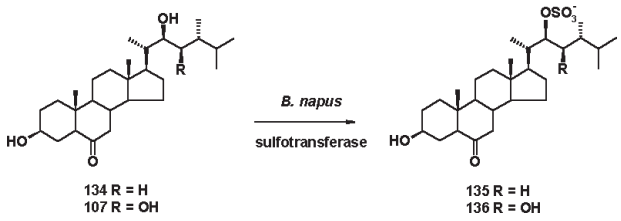

Figure 17. Sulfonation of brassinosteroids and precursors by a *Brassica napus* steroid sulfotransferase.

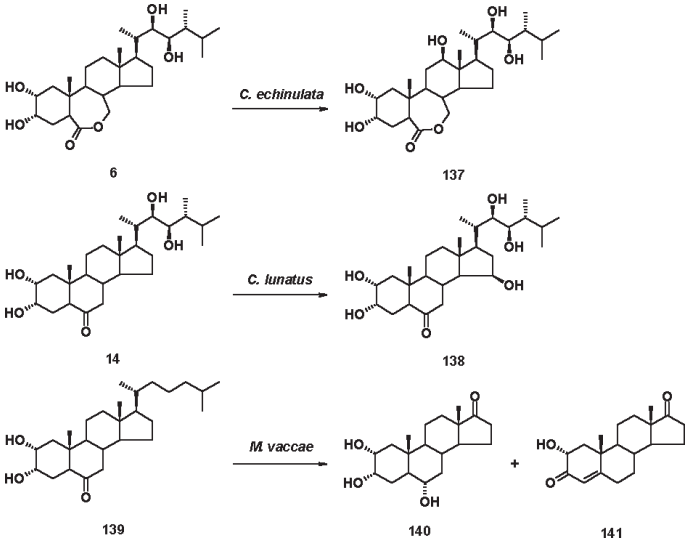

Figure 18. Brassinosteroid transformations by fungi.

BIOLOGICAL ACTIVITY ON INSECTS

Brassinosteroids show striking structural similarities with arthropod hormones of the ecdysteroid type such as 20-hydroxyecdysone (142; Adler and Grebenok, 1995; Lafont 1997), which led to several studies on the bioactivity of brassinosteroids and analogues on insects. Inhibiting and antiecdysone effects have been observed in the course of such investigations (Richter and Koolman, 1991).

Thus, from a series of tested compounds, castasterone (9) and 22,23-diepi-28-homobrassinolide (143), a synthetic 22β,23β-stereoisomer of 28-homobrassinolide (2), inhibited the evagination of imaginal disks of the *Phormia terranovae* fly (Hetru et al., 1986). The 22,23-diepi-28-homocastasterone analogue 144 and lactone 143, to a lesser extent, bound competitively to ecdysteroid receptors from larvae of the *Calliphora vicina* blowfly, representing first antiecdysones (Lehmann et al., 1988). Compounds 143 and 144 were shown to act as weak inhibitors of binding of the ecdysteroid ponasterone A to the intracellular ecdysteroid receptor from the epithelial cell line from *Chironimus tentans* and gave morphological effects and inhibition of chitin synthesis similar to the moulting hormones (Spindler et al., 1992). Compound 144 also exhibited a binding affinity to an ecdysteroid receptor in last instar larvae of the *Galleria mellonella* wax moth similar to ecdysone (Sobek et al., 1993) but did not act as ecdysone antagonist in the salivary gland degeneration in *Amblyomma hebraeum* (Charrois et al., 1996). In the *Drosophila melanogaster*

II cell bioassay natural brassinosteroids showed no agonistic or antagonist activity (Dinan et al., 2001). When a series of synthetic brassinosteroid/ecdysone hybrid molecules was checked in the same test system, only compound 145, exhibiting the 14α-hydroxy-7-en-6-one function characteristic of ecdysteroids like 142, showed a weak agonistic activity (Voigt et al., 2001). Using cultured imaginal wing discs from last-instar larvae of the *Spodoptera littoralis* cotton leafworm, both native brassinosteroids 24-epibrassinolide (6) and 24-epicastasterone (14) caused 50% competition for binding with the tritiated ecdysteroid ponasterone A but no induction of evagination (Smagghe et al., 2002).

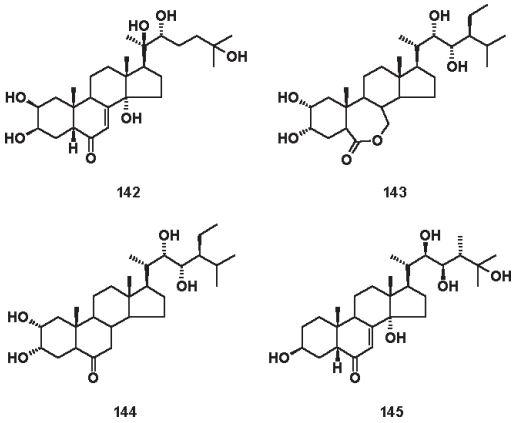

Figure 19. Structures of compounds 142-145.

In other studies the cockroach *Periplaneta americana* has been used as preferred model. In feeding experiments 22,23-diepi-28-homobrassinolide (143) provoked moulting retardation by about 11 days with the highest applied doses (Richter et al., 1987). Similarly to the effects of the hormone 20-hydroxyecdysone (142) dose-dependent neurodepressing effects were observed with compounds 144 and, to a lesser extent, compound 143 on *Periplaneta americana* indicating an ecdysteroid agonistic activity (Richter and Adam, 1991). Also the first evidence for a metabolic transformation of a brassinosteroid in insects has been shown recently with this species (Schmidt et al., 2000). Thus, an organspecific epimerization of the brassinosteroid to 2,24-diepicastasterone (146; figure 20) could be detected in female insects when 24-epicastasterone (14) was fed to the cockroach. The metabolite was observed only in the ovaries but not in the testes of the insect and was identified by GC-MS comparison with a synthesized sample (Voigt et al., 2002).

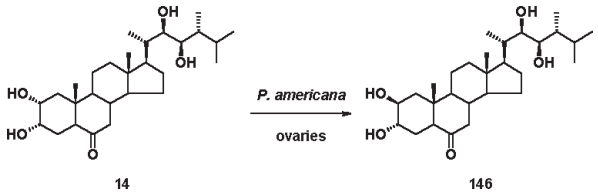

Figure 20. Brassinosteroid biotransformation by *P. americana*.

The above-mentioned results indicate a series of biological effects of brassinosteroids on insects including *in vitro* cell culture and *in vivo* whole larvae. More detailed biological and biochemical studies using the structural multitude of brassinosteroids are necessary and could lead to new strategies to influence ecdysteroid-dependent steps of insect development and new pathways for insect pest control.

BIOLOGICAL ACTIVITY AND STRUCTURE-ACTIVITY RELATIONSHIPS

The biological activity of brassinosteroids was initially evaluated by the bean second internode assay (Grove et al., 1979; Thompson et al., 1981, 1982; Mandava, 1988). In this test auxins and cytokinins are not detected and gibberellins elongate the treated and upper internodes. Brassinosteroids promote cell division and elongation, swelling, curvature and splitting of the treated internode: these morphological alterations are concentration dependent.

The bean first internode assay, used for evaluating the auxin-induced growth, was also employed for testing the structure-activity relationships of brassinosteroids (Thompson et al., 1982; Meudt and Thompson, 1983; Mandava, 1988; Fuendjiep et al., 1989).

The rice lamina inclination assay, based on a test originally developed for auxins (Maeda, 1965), was modified for brassinosteroid detection (Wada et al., 1981, 1984). While this assay has a limit of detection of 50 ppm for indolacetic acid, the limit is 0.5 ppb for brassinolide (1) and 5 ppb for 28-homobrassinolide (2). A modification, employing rice lamina of the whole seedlings pre-treated with IAA, diminished the limit of brassinolide (1) detection to 0.1 ppb (Takeno and Pharis, 1982). This test is considered as specific for brassinosteroids and is employed to detect and follow the purification of these natural products (Takatsuto, 1994; Adam et al., 1996).

The wheat leaf unrolling bioassay, introduced in 1985, responds to brassinolide (1) and castasterone (9) at a limit of detection of 0.5 ng.mL^{-1} (0.5ppb), with complete unrolling at brassinolide concentrations equal or higher than 10 ng.mL^{-1}. In this assay gibberellic acid and cytokinins produce a small effect in the concentration range of 0.1-10 µg.mL^{-1} and zeatin causes slight to complete unrolling at 1ng-1 µg.mL^{-1}, while abscisic acid, indolacetic acid and indolacetonitrile inhibit unrolling (Wada et al., 1985).

Other assays are less frequently employed to evaluate brassinosteroids structure-activity relationships, such as the mung bean epicotyl elongation assay (Gregory and Mandava, 1982), the radish (Takatsuto et al., 1983b, 1984) and tomato (Takatsuto et al., 1983b) hypocotyl elongation assays, and auxin-induced ethylene production by etiolated mung bean segments (Arteca et al., 1985).

Although the above biological assays are not equivalent, they allowed the establishment of relatively safe structural activity relationships (Adam and Marquardt, 1986; Singh and Bhardwaj, 1986; Mandava, 1988; Abreu, 1991), with the aid of a series of brassinosteroid analogues.

As a general rule, the most bioactive brassinosteroids are of the 6-oxo-7-oxalactone type, followed by the 6-oxo brassinosteroids and the 6-deoxo brassinosteroids, that are almost inactive (Mandava, 1988).

Transforming 6-oxo-7-oxalactone to ether, thialactone, lactam, 6-oxa-7-oxolactone, 6-aza-7-oxalactone and 6-aza-7-thiolactone (Okada and Mori, 1983a; Kishi et al., 1986; Takatsuto et al., 1987) dramatically reduce the brassinolide activity [e.g. brassinolide (1, 10,000) ≈ 28-homobrassinolide (2, ≈ 10,000) > 6-deoxo-28-homobrassinolide (147, 100) ≈ 7-aza-28-homobrassinolide (148, 100) > 7-thia-28-homobrassinolide (149, 10), while 6-oxa-7-oxo-28-homobrassinolide (150) presents about 1% of the bioactivity of 28-homobrassinolide (2; Takatsuto et al., 1987) and 6-aza-7-oxo-28-homobrassinolide (151; Anastasia et al., 1984) and 6-aza-7-thia-28-homobrassinolide (152) are inactive (Okada and Mori, 1983b)]. Moving from the lactone to the 6-ketone it is observed that the brassinolide activity decreases from 100% to 50% in the pair brassinolide (1)/castasterone (9; Takatsuto et al., 1983a) in the rice lamina inclination assay, while 24-epicastasterone (14) is about 3 times more active than castasterone (9) in the bean second internode assay (Thompson et al., 1982). The introduction of a C-7/C-8 double bond in 24-epicastasterone (14) reduces the bioactivity of 7-dehydro-24-epicastasterone to one tenth (153; Takatsuto et al., 1987) while in the pair 22,23,24-triepicastasterone (155)/7-dehydro-22,23,24-triepicastasterone (154) the biological activity decreases about one hundred times. The introduction of a hydroxyl at 5α decreases the brassinolide activity ca. 1,000 times when moving from 7-dehydro-24-epicastasterone (153) to 7-dehydro-5α-hydroxy-24-epicastasterone (156) and about 100 times in the pair 7-dehydro-22,23,24-triepicastasterone (155)/7-dehydro-5α-hydroxy-22,23,24-triepicastasterone (157; Takatsuto et al., 1987). A less dramatic decrease in bioactivity on the rice lamina inclination assay has also been reported when a 5α-hydroxyl function is introduced on 28-homocastasterone (10; Brosa et al., 1998; Brosa, 1999; Ramírez et al., 2000a). While the introduction of a 5α-fluoro group in 28-homocastasterone (10) decreases its bioactivity by one order of magnitude, the same introduction in 28-homoteasterone (28) or in 28-homotyphasterol (27) slightly increases their bioactivity (Ramírez et al., 2000b). The absence of an oxygen function at ring B decreases the brassinolide activity significantly, as in the case of 6-deoxocastasterone (36) that shows only 1 % of the castasterone bioactivity of castasterone (9; Yokota et al., 1983c; see structures of compounds 147-157 in figure 21).

The effect of ring A substituents on brassinolide activity was studied in some detail in the 28-homobrassinolide (2) series (Takatsuto et al., 1987): changing the hydroxyls from 2α,3α to 3α,4α either in 28-homobrassinolide (2) or in 6-oxa-7-oxo-28-homobrassinolide (150) reduces the bioactivity of 158 and 159 in one order of magnitude. 2-Deoxy-28-homobrassinolide (160) is about 100 times less active than 28-homobrassinolide (2), while 28-homotyphasterol (27) is about ten times less active than 28-homocastasterone (10). Their 3β-isomers 3-epi-2-deoxy-28-homobrassinolide (161) and 28-homoteasterone (28) are also ten times less active than 28-homobrassinolide (2) and 28-homocastasterone (10), respectively. While 3-dehydro-2-deoxy-28-homobrassinolide (162) and 2,3-dideoxy-28-homobrassinolide (163) are about ten times less active than 28-homobrassinolide (2), 3-dehydro-28-homoteasterone (164) and 2,3-dideoxy-28-homoteasterone (165) are, respectively, ten and one hundredfold less active than 28-homocastasterone (10). 3-Dehydroteasterone (32), secasterone (33) and 2,3-diepisecasterone (166) show, respectively, 74%, 59% and 89% of the bioactivity of 24-

epicastasterone (14) in the rice lamina inclination assay (Voigt et al., 1995; see structures of compounds 158-166 in figure 22). The replacement of 3-hydroxy function by a 3-fluoro group in either 28-homotyphasterol (27) or 28-homoteasterone (28) yields compounds active at the rice lamina inclination assay at dosages equal or higher than 50 ng per plant, but not as active as their parent compounds (Galagovsky, 2001).

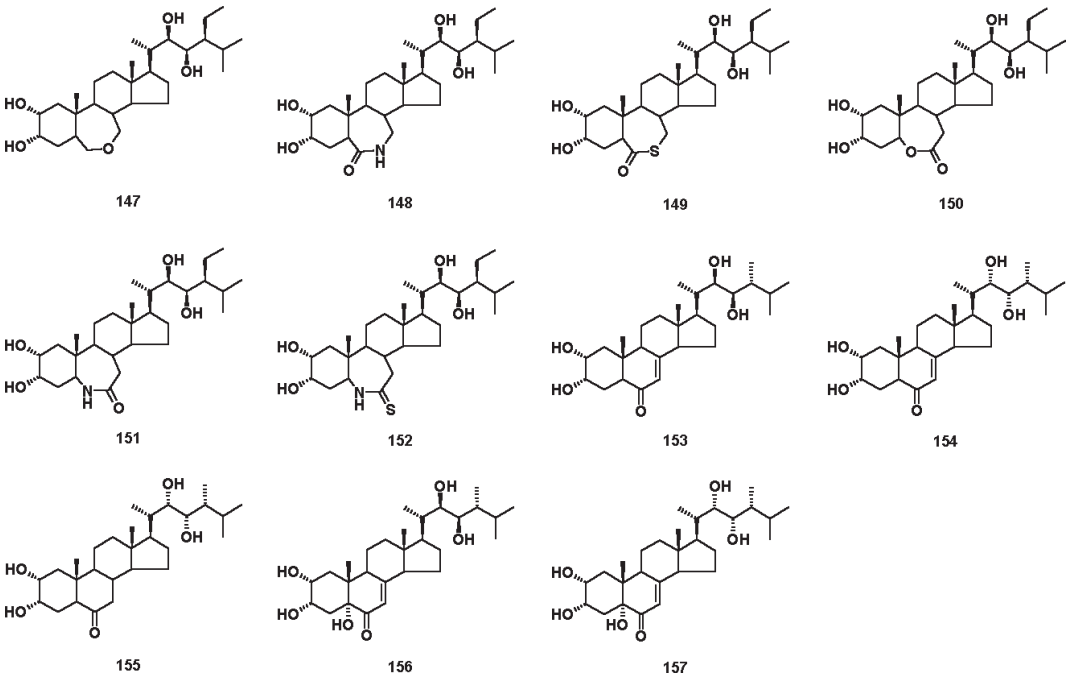

Figure 21. Structures of brassinosteroid analogues 147-157.

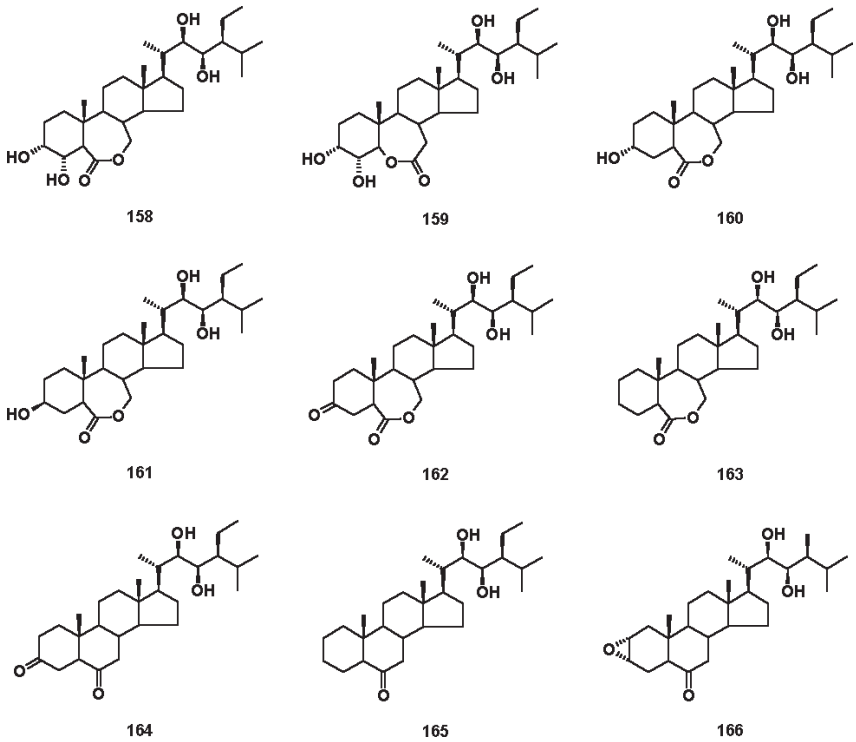

Figure 22. Structures of brassinosteroid analogues 158-166.

Although brassinosteroids with *cis* A/B ring fusion (5β configuration) have not yet been isolated from natural sources, they were initially synthesized to explore their potential antiecdysteroid activity (Brosa et al., 1994). Evaluation of the brassinolide activity of these compounds by the rice lamina inclination assay, employing the Bahia cultivar and a single brassinosteroid concentration, 1µg per segment, showed that either 28-homobrassinolide (2) or 2,3,5-triepi-28-homobrassinolide (167) showed 87% of the brassinolide (1) bioactivity. When the configuration of the side chain changes to 22β,23β the bioactivity of 22,23-diepi-28-homobrassinolide (143) becomes two times higher than for 2,3,5,22,23-pentaepi-28-homobrassinolide (168; 14 % and 6 % respectively). In the 6-oxo series, 28-homocastasterone (10) presents 97 % of the brassinolide activity, while 2,3,5-triepi-28-homocastasterone (169) only 51 % (Brosa et al., 1996). However, evaluation of synthesized 5-epibrassinolide (170a, Seto et al. 1998) and 2,3,5-triepibrassinolide (170b) showed a nearly complete loss of bioactivity in the rice lamina inclination assay indicating that *trans*-fusion of rings A/B play an essential role (Seto et al., 1999; see structures of compounds 167-170 in figure 23).

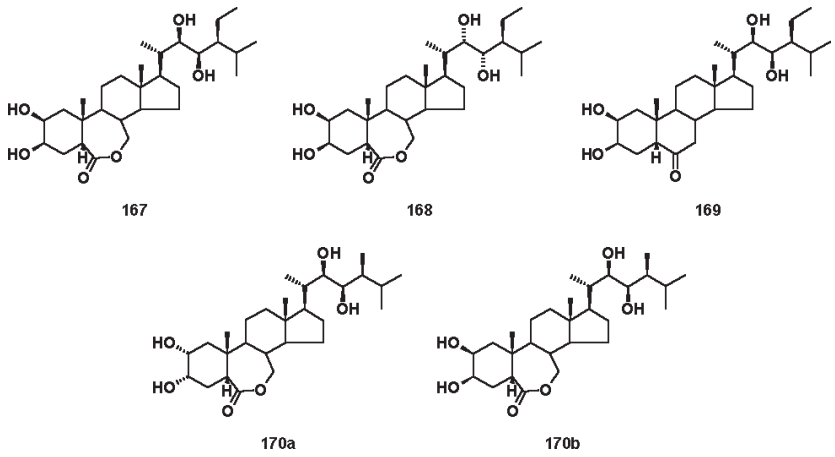

Figure 23. Structures of brassinosteroid analogues 167-170.

Many papers have dealt with the relationship between the side chain structure and brassinolide activity. When the 24-substituents of the 22α,23α-brassinosteroids were examined, the order of brassinolide activity is brassinolide (1) > 24-epibrassinolide (6) > 28-homobrassinolide (2) > 24-epi-28-homobrassinolide (171) > dolicholide (3) > 28-homodolicolide (4) > 28-norbrassinolide (5), a decreasing order that is also observed in the 6-oxo series in the bean second internode assay (Mandava, 1988) and in the rice lamina inclination assay (Takatsuto et al., 1983a). Introduction of a methyl group at C-25 increases the brassinolide activity ten times, at least in the pairs brassinolide (1)/25-metilbrassinolide (172) and dolichosterone (11)/25-methyldolichosterone (16; Mori and Takeuchi, 1988), while the removal of the methyl groups at C-25, resulting in 26,27-dinorbrassinolide (173) or in 26,27-dinorcastasterone (174) does not affect the brassinolide activity at the rice lamina inclination assay, compared to their parent compounds (Takatsuto et al., 1984). In contrast to these findings, 26,28-dinorbrassinolide (175) and 26,28-dinorcastasterone (176) only show bioactivity only at 10 mg per plantlet against activity at 0.01 mg per plantlet for brassinolide (1) and 0,1 mg per plantlet for castasterone (9; Thompson et al., 1982). As the side chain is reduced so is also the biological activity: in this way the bisnorcholanelactone 177 and the androstanelactone 178 show only 2 % and 0.001 % of the brassinolide (1) activity, respectively (Kondo and Mori, 1983; see structures of compounds 171-178 in figure 24). 21-Carboxipregnanelactones (Cerny et al., 1987) and 22-alkoxybisnorcholanelactones (Kerb et al., 1983) also show brassinolide activity.

When the configuration of the side chain hydroxyls was analyzed, those presenting 22α,23α stereochemistry are more active than those presenting 22β,23β configuration, whether for 6-oxobrassinosteroids or for lactones, no matter what bioassay is employed (Thompson et al., 1979, 1981, 1982; Takatsuto et al., 1983a, 1987). When the ef-

fect of the alkyl substituent at C-24 and the stereochemistry of the hydroxyls at C-22 and C-23 were analyzed jointly, the relative order of bioactivity changes according to the alkyl substituent, the ring B structure and the bioassay employed: even so the 22α,23α,24α isomers are always the most actives (Thompson et al., 1981, 1982; Takatsuto et al., 1983a). The 22α,23β- or the 22β,23α-brassinosteroids present little bioactivity (Takatsuto et al., 1983a, b; Fuendjiep et al., 1989). Elimination of one hydroxyl, as in 23-deoxy-28-norbrassinolide (179) decreases the bioactivity (Kondo and Mori, 1983; Takatsuto et al., 1983b) [as occurs with cathasterone (53; Fujioka et al., 1995) and the cholestanelactone 180 (Takematsu, 1982)], that is suppressed with the elimination of both side chain hydroxyls (Thompson et al., 1982; Kondo and Mori, 1983; Takatsuto et al., 1983b). Side chain brassinosteroid glycosides are less bioactive than their aglycones, as happens to the 23-*O*-β-D-glucopyranosylbrassinolide (181; Yokota et al., 1991; Suzuki et al., 1993). This kind of conjugation is considered to be a mechanism for brassinosteroids deactivation. Luo et al. (1998) prepared a series of methyl ethers of brassinolide (1) to prevent such conjugation and, employing the rice lamina inclination assay, verified that, while brassinolide 23-methyl ether (182) showed weak or low activity even at high dosage (1,000 ng per plant), the 22-methyl ether 183 showed an activity comparable to 24-epibrassinolide (6) at dosages up to 100 ng per plant and the 22,23-dimethyl ether 184 even at dosages up to 1,000 ng per plant (see structures of compounds 179-184 in figure 25). Another way of deactivation was the recently shown enzymatic sulfonation of several brassinosteroids, including 24-epibrassinolide (6), with a steroid sulfotransferase from *Brassica napus*. This sulfonation abolished the biological activity in the bean second internode bioassay and was demonstrated to be specific for the hydroxyl at position 22 of brassinosteroids (Rouleau et al., 1999).

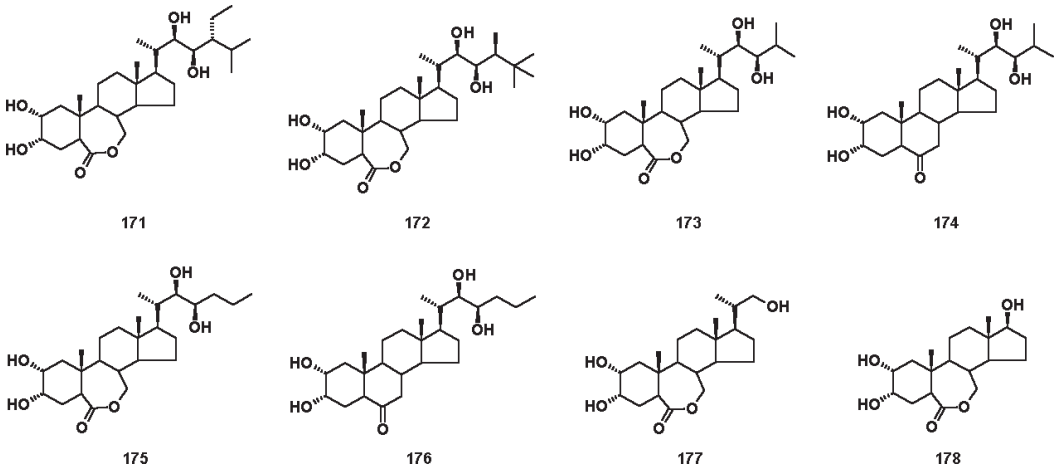

Figure 24. Structures of brassinosteroid analogues 171-178.

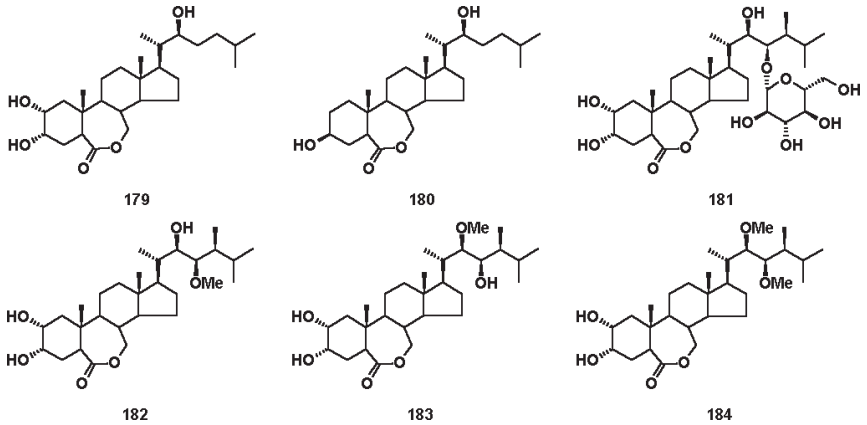

Figure 25. Structures of brassinosteroid analogues 179-184.

A series of brassinosteroid analogues, such as 17-esters of androstanelactones (Kohout, 1989), 2α,3α,17β-trihydroxy-5α-androstane-6-one (Gaudinova et al., 1995), hemiesters, orthoesters and ketals of 2α,3α-cholestanediol (Kerb et al., 1982a), 22-ethers of lactone 177 (Kerb et al., 1983), esters of 28-homobrassinolide (Kerb et al., 1982b) and 2-deoxybrassinosteroids (Abe and Yuya, 1993), also show brassinolide activity. The spirostanes 185-188 (Marquardt et al., 1989; Arteaga et al., 1997), spirosolanes 189 and 190 (Quyen et al., 1994a), epiminocholestanes 191 and 192 (Quyen et al., 1994a) and solanidanes 193 and 194 (Quyen et al., 1994b) analogues also show bioactivity. A series of the first ten nonsteroidal brassinosteroid analogues was synthesized recently (Andersen et al., 2001), and the compounds 195 and 196, when co-applied with indolacetic acid, promoted rice lamina inclination at dosages as low as 0.01 ng and 0.001 ng per plant, respectively. Very recently the heterodimer hybrid 197 of 24-epicastasterone and dexamethasone was synthesized to study the regulation of protein-protein interactions, to trigger signal transduction pathways and to detect ligand-protein receptor interactions (Kolbe et al., 2002). A series of inclusion complexes of brassinosteroid (Durán Caballero et al., 1999) or of the spirostanic brassinosteroid analogues (De Azevedo et al., 2001a) in cyclodextrins were prepared aiming to improve the brassinolide activity what was achieved with the 24-epibrassinolide/β-cyclodextrin inclusion complex 198 (De Azevedo et al., 2001b, 2002; see structures of compounds 185-198 in figure 26).

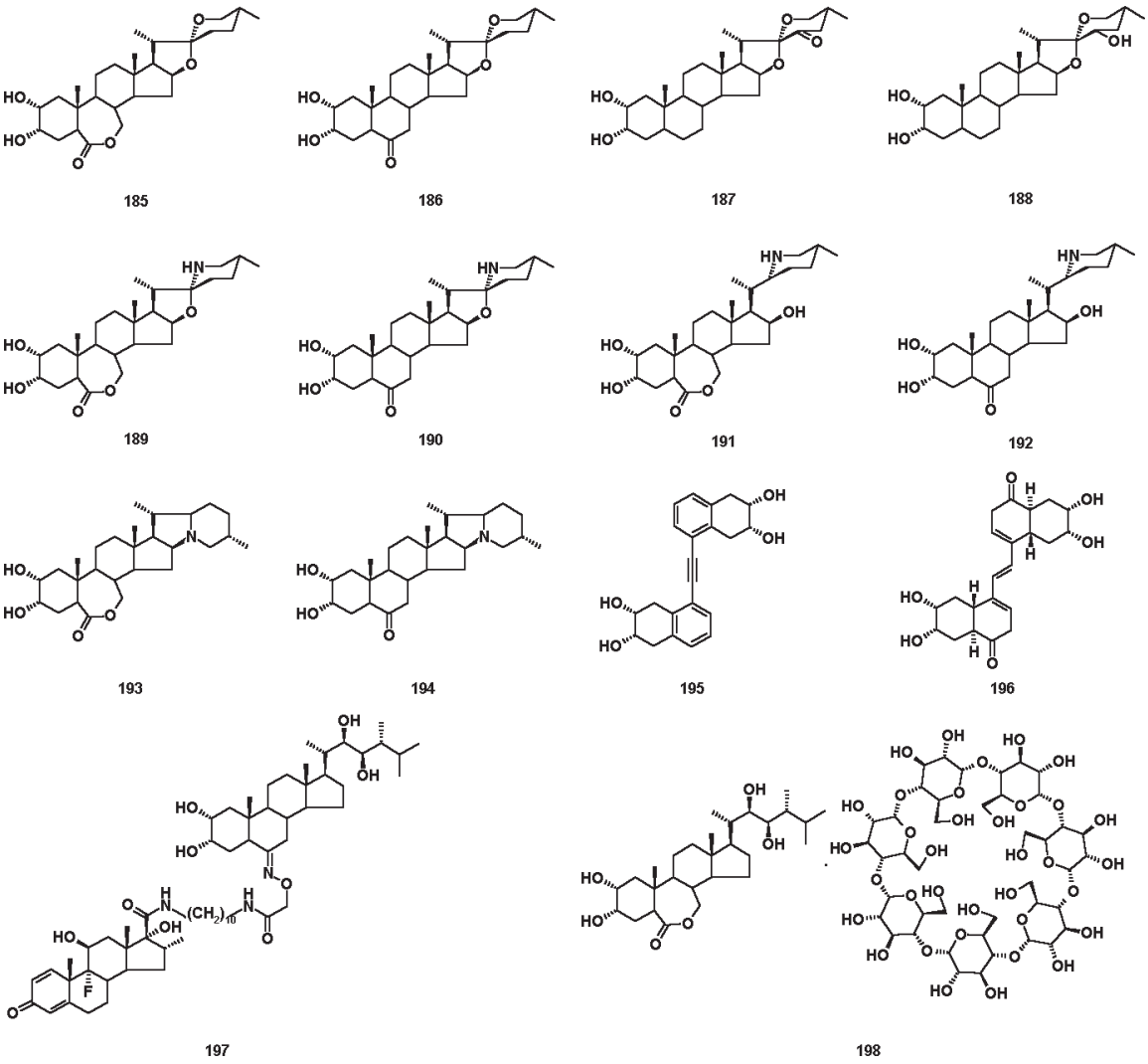

Figure 26. Structures of brassinosteroid analogues 185-198.

It is usually assumed that a brassinosteroid is linked to its receptor site through three points: the 2α,3α-hydroxyls (Wada and Marumo, 1981), the B ring lactone and the 22α,23α-hydroxyls (Kishi et al., 1986). It was formerly considered that the receptor affinity to the 2α,3α-hydroxyls would be greater than to the 22α,23α-hydroxyls, as variations in side chain structure are less influential in brassinolide activity than variations in the ring A structure (Takatsuto et al., 1983b). A study on quantitative structure-activity relationships indicates, however, that the contributions of the ring A and the side chain hydroxyls configurations are 25 % and 35 % of the total of brassinolide activity, but also that the activity of a brassinosteroid or analogue would be greater as greatest would be the similarity between the compound and brassinolide (1) itself (Brosa et al., 1996). Further improvement of the methodology for predicting the activity of a brassinosteroid or analogue takes into account the putative H-bonding interactions in the brassinosteroid-receptor complex (Brosa, 1999).

A look at the reactions involved in the metabolism of a brassinosteroid (see the general formula in figure 27 summarizing the reactions observed in 24-epibrassinosteroids) suggests that, as different enzymes catalyze different transformations, and as these enzymes can be located at different organelles inside the cell, there is not a single receptor site for a brassinosteroid but there are different receptor sites in different enzymes in which different brassinosteroid molecules are able to exhibit one of the many brassinosteroid physiological activities. Each receptor site must need different structural requirements for exhibiting the maximal activity, and this may be the reason why there are different structure-activity relationships according to the bioassay employed.

The almost rigid structure of the steroidal nucleus of the brassinosteroids is confirmed by molecular orbital calculations, nuclear magnetic resonance experiments and X-ray diffraction studies, revealing that, in the 5α-series, the A and C rings assume a chair conformation, observed also in the ring B of 6-oxobrassinosteroids, while in the 6-oxo-7-oxalactones the 7-membered B ring tends to lie in the same plane as rings C and D. In the 5β-series brassinosteroids, the ring A also adopts a chair conformation, but it sets almost perpendicularly to the plane formed by the rings B, C and D (see partial structures 199-202 in figure 28).

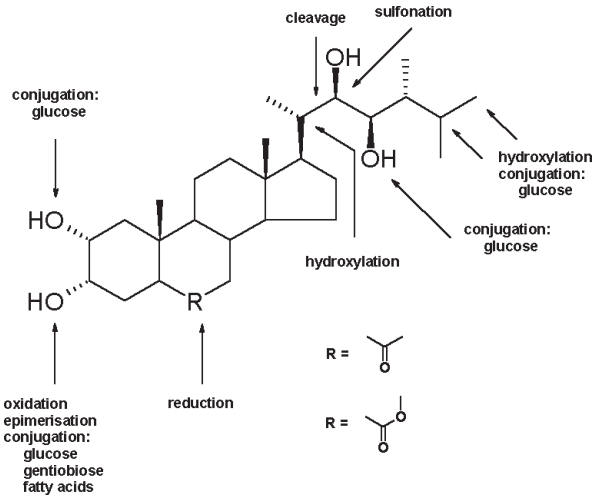

Figure 27. Summary of metabolic reactions observed in 24-epibrassinosteroids.

In regard to the more flexible side-chain conformation, a series of 10 brassinosteroids were investigated by means of detailed NMR investigations, molecular modeling studies, and compared with data from X-ray analysis. For the most bioactive compound brassinolide (1) the majority of conformations in solution showed a side-chain bent towards the β-face of the steroid skeleton, whereas for the less active members like 24-epibrassinolide conformations with straight side-chains or side-chains bent towards α-face are preferred (see partial structures 203 and 204 in figure 28; Stoldt et al., 1997; Drohsin et al., 2001). While these models are valuable approaches for the design of new brassinosteroid analogues, it must be remembered that they may not furnish the actual active conformation of a brassinosteroid inside the receptor site of an enzyme.

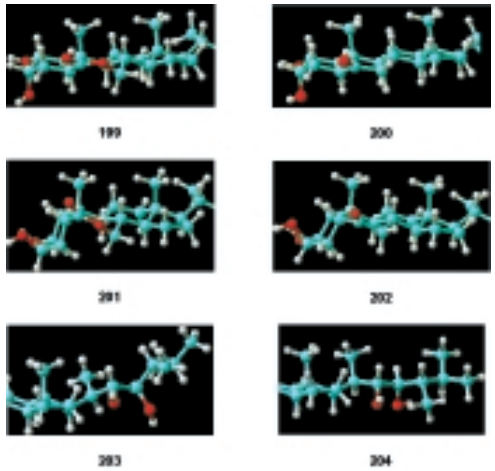

Figure 28. Hypothetical active conformations of the steroidal nuclei and side chains of brassinosteroids.

In summary, the following structural features are important for exhibiting a high brassinolide activity: i) a 6-oxo-7-oxalactone function; ii) 2α,3α hydroxyls; iii) 22α,23α hydroxyls; iv) 24-alkyl substitution; v) A/B-*trans* ring conjunction.

PROSPECTIVE AGRICULTURAL USES

Since the beginning of the research on the isolation of brassinosteroids from plant sources, brassins proved to be able to promote plant growth (Mitchell and Gregory, 1972), as well as its acceleration (Gregory, 1981; Braun and Wild, 1984; Meudt et al., 1984).

With the isolation of brassinolide (1) and the synthesis of similar compounds, brassinosteroids were shown to be useful to increase crop yield: by using brassinolide (1) bean crop yield increased, shown by the increase of 41 % to 51 % in the weight of seeds per plant and the leaf weight of two different lettuce varieties increased by about 25 % (Meudt et al., 1983).

Treatment of rice plantlets with a 5 ppm solution of brassinolide (1) caused an increase of 22 % in fresh weight and 31.5 % in dry weight of seeds per plant in the Taebaik cultivar (Lim, 1987). It was also reported to increase plant growth speed, root size, and root and stem dry weight (Kim and Sa, 1989), to reduce the toxicity of 2,4-D and butachlor to the plantlets (Choi et al., 1990) and to increase the percentage of ripe grains when cultivated at low temperature (Irai et al., 1991).

In barley (cv. Nosovsky 9), brassinolide (1), 28-homobrassinolide (2) and 24-epibrassinolide (6) increased the activity of endospermic α-amylase, the weight of seeds per ear, the weight of 1,000 seeds and the crop yield, besides increasing the stem diameter, causing an increased resistance to lodging (Prusakova et al., 1995).

In corn (cv. Kwangok) the ear fresh weight increased by about 7 % and seed dry weight increased by 11 % to 14 % by using brassinosteroids, while in the cv. Danok 1 the effect of these treatments was depressive (Lim and Han, 1988).

The application of 24-epibrassinolide (6) or 22,23,24-triepibrassinolide (205; figure 25) on wheat increased panicle weight by 25-33 % and seed weight by 4-37 % and decreased the sterile portion of the ear by 25-62 % (Takematsu et al., 1988).

Experiments performed at the Instituto Agronomico (M. A. T. Zullo, unpublished results obtained between 1986 and 1988 with samples given by Professor Nobuo Ikekawa) with 24-epibrassinolide (6), 24-epicastasterone (14), 22,23,24-triepibrassinolide (205) and 22,23,24-triepicastasterone (155) allowed the observation of increases in crop yields in wheat (up to 18 % in seed weight per ear), soybean (up to 22 % in seed weight per plant) and bean (up to 83 % in seed weight per plant in the Carioca-80 cultivar).

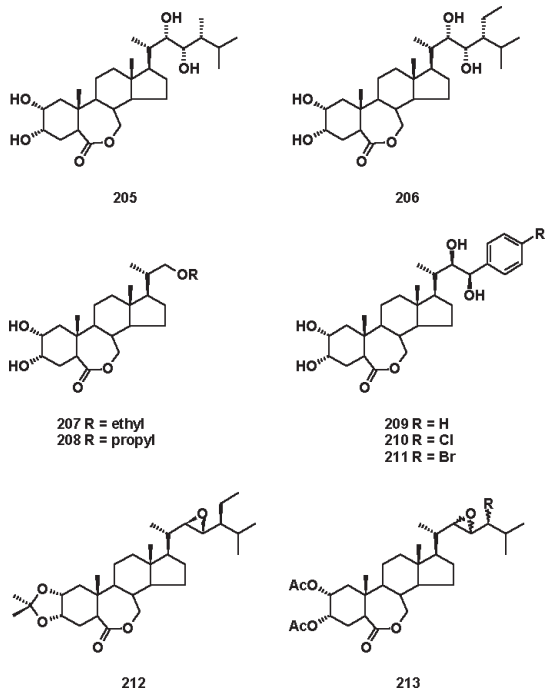

Figure 29. Structures of brassinosteroid analogues 205-213.

Application of 24-epibrassinolide (6) at 1 ppb increased the root growth of chick-pea, cv. Pusa 256, by 25 % (Singh et al., 1993). The application of the same compound to three different chick-pea cultivars, at the flowering stage, caused increases in seed yield, crop index, 100 seeds dry weight and in protein and soluble sugars of the seeds (Ramos, 1995; Ramos et al., 1995, 1997). In this case the crop yield (in $kg.ha^{-1}$) increased by 86 %, 76 % and 61 % for the cultivars IAC India-4, IAC Mexico and IAC Marrocos, respectively.

The application of 24-epibrassinolide (6) or 24-epicastasterone (14) on coffee caused no significant effect on seed setting, seed size or yield (Mazzafera and Zullo, 1990). *Coffea stenophylla* calli grew up to 237 % between 60 and 130 days of culturing in the presence of 24-epibrassinolide (6), compared with growth of up to 49 % in the absence of this compound (Ramos et al., 1987).

It was noted that brassinolide (1) promoted potato tuber development, inhibited its germination during storage and increased resistance to infections by *Phytophthora infestans* and *Fusarium sulfureum* (Kazakova et al., 1991).

28-Homobrassinolide (2) and its 22,23-diepimer 143 caused an increase of 5-24 % in the size and of 23-59 % in the fresh weight of azuki bean plants, as well as increases of 3-30 % in the size and of 8-28 % in the fresh weight of rape plants. The application of 22,23,24-triepi-28-homobrassinolide (206) increased tomato fruit setting by 43-111 %, while with 28-homobrassinolide (2) this increase was 118-129 % (Mori et al., 1986).

The application of brassinolide (1) on orange trees during flowering increased fruit setting, while when applied during fruit growth it decreased the physiological drop of fruits, causing an increased number of fruits per plant, accompanied by an increase in the average fruit weight and in the brix/acidity ratio. The increase in fruit setting due to decreasing physiological fruit dropping was also observed in lemon, peach, pear, persimmon and apple. In *Citrus unshiu* increased juice production and a higher brix/acidity ratio was observed (Kuraishi et al., 1991). In *Citrus madurensis* Lour. brassinolide (1) retarded fruit abscission was observed (Iwaori et al., 1990).

The application of the ethers 207 and 208 (figure 29), active in the bean second internode assay, increased leaf width in sugar beet and the lateral diameter of the root (Kerb et al., 1986).

Some brassinosteroid analogues, synthesized for long lasting activity in the field, at first showed some usefulness in agricultural practice. So the phenylbrassinosteroids 209-211 (figure 29), applied to corn plants, increased their sizes by 14-15 % and their weight by 23-36 % (Hayashi et al., 1989). The lactone 212 (figure 29), an intermediate in the synthesis of 28-homobrassinolide (2), increased radish fresh weight by 13-22 %, wheat seed weight by 11-22 %, grapevine cluster weight by 9-18 %, onion fresh bulb weight by 11-18 % and rice plant weight by 21-22 % (Kamuro et al., 1990). The mixture of epoxides 213 (figure 29) increased the size of soybean and corn plants and the dry weight of corn seeds (Takatsuto et al., 1990c).

It has been shown in studies with arborescent plant species that pretreatment with 22,23-diepi-28-homobrassinolide (143) induced increase in rooting and rooting quality in cuttings taken from mature Norway spruce donor plants and improved their viability (Rönsch et al., 1993).

Micropropagation processes of tropical plants, such as cassava (*Manihot esculenta* Crantz), yam (*Dioscorea alata* L.) and pineapple (*Ananas comosus* L. Merril), can be improved by the use of 28-homocastasterone (10) or 3β-acetyl-28-homoteasterone, as suggested in a preliminary study (Bieberach et al., 2000). Treatment of shoot apices of the marubakaido apple rootstock [*Mallus prunifolia* (Willd.) Borkh] with 5α-fluoro-28-homocastasterone increased the apple rootstock multiplication rate up to 112 % (Schaefer et al., 2002).

Growth stimulating effects were also found in studies on higher fungi when the cultivation of *Psilocybe cubensis* as well as of *Gymnopilus purpuratus* in the presence of 10^{-2} ppm brassinosteroid 143 resulted in a two to three-fold growth acceleration with an increasing number of fruiting bodies from 1-2 to 4-7 in the first flush (Gartz et al., 1990; Adam et al., 1991).

Many other examples of brassinosteroid use for increasing crop yield can be found in the literature (Kamuro and Takatsuto, 1999; Khripach et al., 1999; Núñez Vázquez and Robaina Rodríguez, 2000).

The brassinosteroids can be mixed with solid excipients (such as talc, mica, diatomaceous earth, clay), pastes (such as lanolin) or liquids (usually water or hydroalcoholic mixtures) for use as powders, pellets, tablets, pastes, suspensions, solutions, in the presence or not of emulsifiers that help homogenize the preparation. The application can be made by spraying, spreading, coating or dipping the plants or their organs or the soil. The amount of brassinosteroid to be applied varies with the brassinosteroid structure, the formulation employed, the kind of plant to be treated and the effect desired. Usually the concentration of the brassinosteroid in the preparation ranges from 0.01 to 100 ppm, and it can be applied with other agrochemicals, such as other plant hormones or growth regulators, fertilizers, herbicides, insecticides and other adjuvants (Mori, 1984).

Although many brassinosteroids, such as 24-epibrassinolide (6), are commercially available and employed in some countries, more accurate studies on dosage, method and time of application, fit brassinosteroid suitability for the plant or cultivar, and association with other phytohormones are needed, since many of the results were obtained by experiments performed in greenhouses or small fields. The preliminary results regarding the increases of crop yield and antistress effects on sev-

eral plants at very low doses, and the fact of being easily metabolized, as seen for tomato and serradella (Adam and Schneider, 1999; Schneider, 2002), recommend brassinosteroids as ecologically safe plant growth promoters (Kamuro and Takatsuko, 1999; Khripach et al., 2000) with promising properties for practical use in agriculture and horticulture.

CONCLUSION

Even after more than 20 years of the isolation of brassinolide (1) and other natural brassinosteroids, there is a continued effort to isolate or detect these natural products from or in many plant species, to improve the biological or physical chemical methods for their detection, to elucidate their biosynthesis and metabolism and to prospect their bioactivity and agricultural uses.

The exploitation of brassinosteroids physiological activity (Sasse, 1999), the comprehension of the molecular mechanisms of their activity (Clouse, 1997; Altmann 1999) and the synthesis of natural and artificial brassinosteroids (Back et al., 1997; Khripach et al., 1999) are other areas of intense activity that soon will allow the general employment of these substances in agricultural practice, due to their peculiar characteristics in promoting plant growth, increasing crop yield and resistance to biotic and abiotic stress, and on being ecologically safe plant growth promoters.

APPENDIX

Steroids are compounds containing the gonane skeleton, usually methylated at carbons 10 (C-19) and 13 (C-18), and a side chain extending from carbon 17 (i). For substituents above the steroidal nucleus, i.e., those pointing in the same direction as carbons 18 and 19 the β designation is given, and for those below the steroidal nucleus the α notation is assigned (ii). There are two different conventions for the designation of the configuration of the substitutents in the side chain. The first is the Fieser-Plattner convention, according to which the side chain is placed so the longest chain extends upward from the ring D and under the plane of the drawing (Fieser and Fieser, 1948; Plattner, 1951a, 1951b). The side chain substituents project above this plane: those appearing at the right side of the chain are designed as α, and those appearing at the left as β (iii and iv, for the cholestane side chain). The second convention uses the sequence rules of Cahn, Ingold and Prelog (Cahn et al., 1966), according to which, briefly, when substituents of a saturated carbon atom, arranged in decreasing order of atomic number, are viewed so that the substituent of least precedence is on the remote side of the carbon, are arranged clockwise, this carbon is designated as *R*, and if they are arranged counterclockwise, this carbon is designated as *S*. The application of these rules to the side chain of brassinolide (1) and 22,23-diepibrassinolide are presented in formulae v and vi, respectively. The second convention is adopted for the official nomenclature of steroids (Joint Commission on Biochemical Nomenclature, 1989). The symbol ξ is used for designating a stereocenter of unknown configuration.

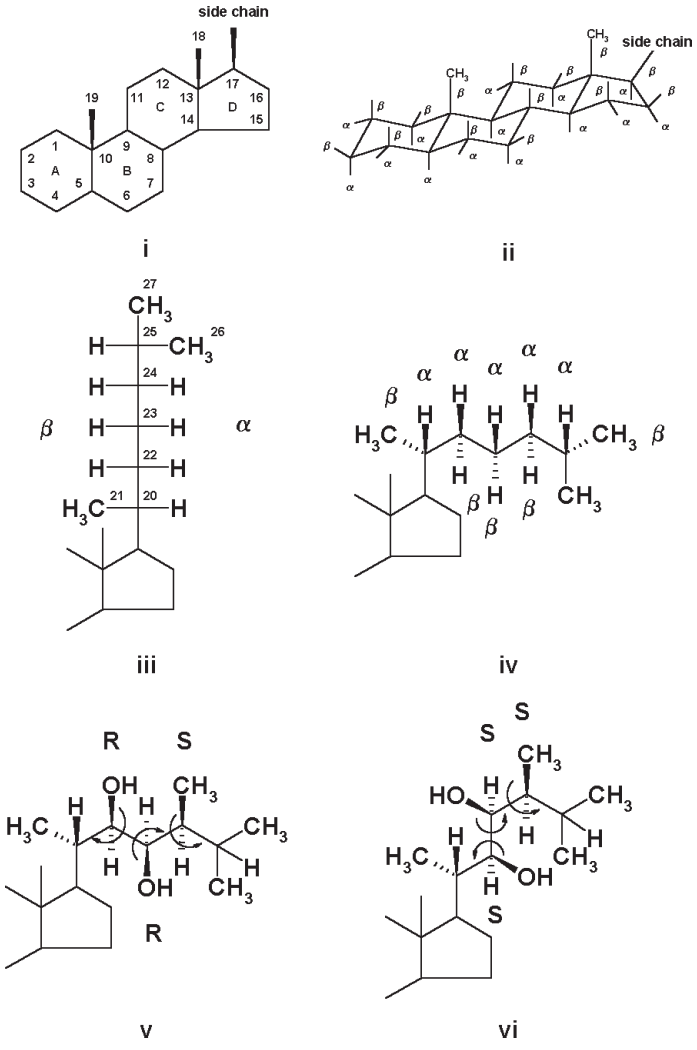

Figure 30. Notations for the steroid system.

NOTE ADDED IN PROOF

A recent publication describes the identification of the seven new brassinosteroid precursors 214-220 of the 22α-hydroxy type (figure 31) in cultured cells of *C. roseus* and in *A. thaliana* seedlings of wild phenotype and *det2-1* mutant [Fujioka S, Takatsuto S, Yoshida S (2002) An early C-22 oxidation branch in the brassinosteroid biosynthetic pathway. Plant Physiology 130:930-939].

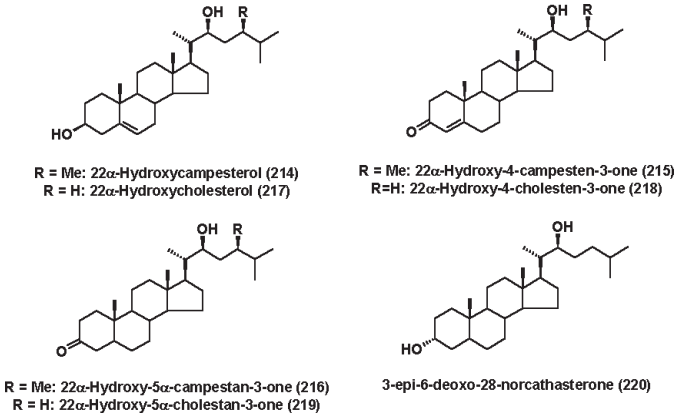

Figure 31. New brassinosteroid precursors detected in *C. roseus* and *A. thaliana*.

Feeding *C. roseus* cultured cells or 7-day old *Arabidopsis* seedlings of the wild phenotype with hexadeuterated 22α-hydroxycampesterol (214) resulted in the detection of labeled 22α-hydroxy-4-campesten-3-one (215), 22α-hydroxy-5α-campestan-3-one (216), 6-deoxocathasterone (54) and 3-epi-6-deoxocathasterone (55), while administration of 214 to *Arabidopsis* seedlings of the *det2-1* mutant resulted in 16 % conversion to 22α-hydroxy-4-campesten-3-one (215) and 0.3 % conversion to 6-deoxocathasterone (54), accompanied by the accumulation of 22α-hydroxy-4-campesten-3-one (215). Feeding *C. roseus* cultured cells or 7-day old *Arabidopsis* seedlings of the wild phenotype with hexadeuterated 6-deoxocathasterone (54) resulted in the detection of 3-epi-6-deoxocathasterone (55) and 22α-hydroxy-5α-campestan-3-one (216). Either labeled 6-deoxotyphasterol (42) or 6-deoxoteasterone (43) were detected in these experiments.

It was also found that administration of 22α-hydroxycampesterol (214), 22α-hydroxy-4-campesten-3-one (215), 22α-hydroxycholesterol (217) or 22α-hydroxy-4-cholesten-3-one (218) failed to rescue the wild phenotype when applied to dark or light grown *Arabidopsis det2-1* mutants. On the other hand, 22α-hydroxy-5α-campestan-3-one (216), 6-deoxocathasterone (54), 22α-hydroxy-5α-cholestan-3-one (219) and 6-deoxo-28-norcathasterone (54a) rescued the wild phenotype when applied to dark or light grown *Arabidopsis det2-1* mutants, the last two less effectively.

These findings led the authors to propose the operation of an early C-22 oxidation subpathway in the biosynthesis of brassinosteroids (figure 32), which could probably be linked to the late C-6 oxidation pathway via the 23α-hydroxylation of either 3-epi-6-deoxocathasterone (55) or 6-deoxocathasterone (54) to, respectively, 6-deoxotyphasterol (42) or 6-deoxoteasterone (43). These results suggest that brassinosteroids are biosynthesized through a metabolic grid instead of two distinct or linked main pathways.

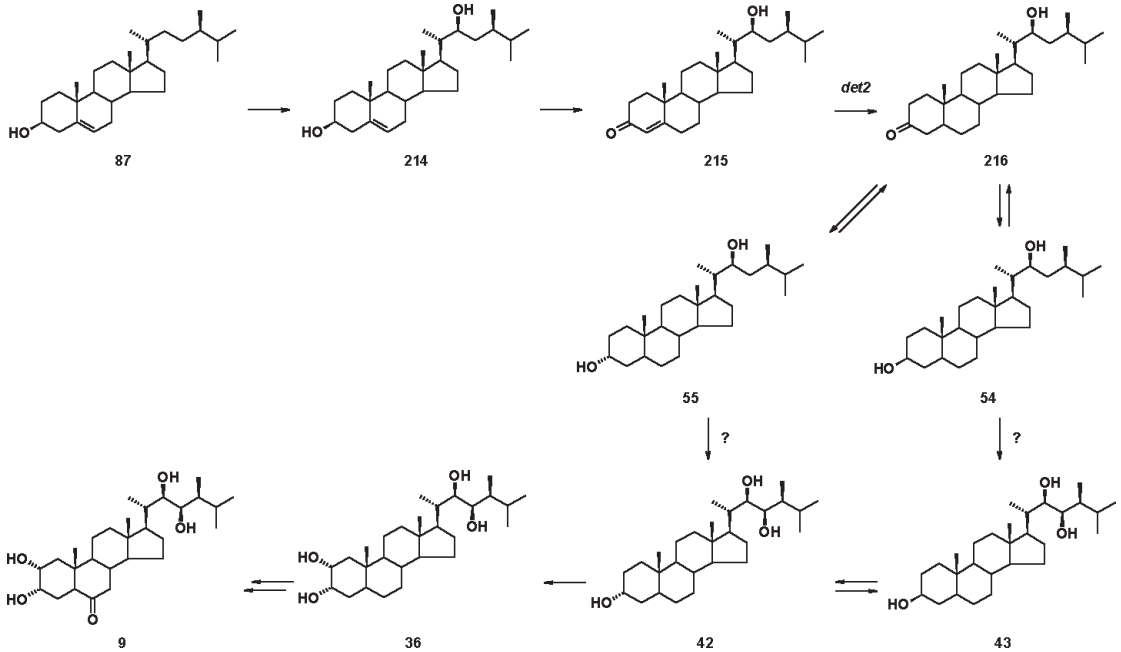

Figure 32. Early C-22 oxidation subpathway in brassinosteroid biosynthesis.

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