Shamsul Hayat · Mohammad Yusuf
Renu Bhardwaj · Andrzej Bajguz *Editors*

Brassinosteroids: Plant Growth and Development

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Preface

Brassinosteroids are endogenous plant growth-promoting hormones found throughout the plant kingdom that influence cellular expansion and proliferation, and the phenotype of mutant affected in brassinosteroid biosynthesis and signaling clearly shows that these plant steroids are essential regulators of physiological processes including organ elongation, vascular differentiation, male fertility, timing of senescence, and leaf development. Several books covering various aspects of brassinosteroid biology and chemistry appeared in 1991, 1999, 2003, and 2011. However, in the past 7 years, a great deal of progress has been made in understanding specific components of brassinosteroid signal transduction and in clarifying mechanism by which brassinosteroid perception ultimately results in changes in the expression of specific genes associated with different developmental programs. The number of physiological processes known to involve brassinosteroid action has also expanded, and significant experiments quantifying the utility of brassinosteroid application in practical agriculture have been documented. Therefore, it is a need of the hour to gather the information in a book form.

The book is comprised of 16 chapters. Chapter 1 of this book gives a survey of diversity of brassinosteroids in plants. Chapter 2 deals with the currently available data of brassinosteroids in microalgae, which has not been covered in any earlier volume of brassinosteroids. The recent progress in brassinosteroids in cereals is covered in Chap. 3. Chapter 4 summarizes the importance of fluoroxyl and hydroxyl substitutions in brassinosteroids for shooting control and the use of in vitro-grown shoots as test systems. Chapter 5 deals with the role of brassinosteroids in plant response to stress. Physiological action of brassinosteroids which depends on their concentration discussed in a Chap. 6 is solely for the role of brassinosteroids during senescence. Regulation of photosynthesis is discussed in Chap. 7. Chapter 8 deals with the genetic and molecular bases of brassinosteroid metabolism and interactions with other phytohormones. In Chap. 9, transformation of matter and energy in crops under the influence of brassinosteroids is briefly described. Chapter 10 covers the use of transcriptomics and proteomics techniques to study the regulation of brassinosteroids in plants. In Chap. 11, the interplay between antioxidant enzymes and brassinosteroids in the control of plant development and stress tolerance is discussed.

Chapter 12 possesses the information of brassinosteroids in relation to horticultural crops. A current scenario on the role of brassinosteroids in plant defense triggered in response to biotic challenges has been discussed in Chap. 13. Anticancer potential of brassinosteroids is described in Chap. 14. Chapter 15 covers the potential of brassinosteroids in abiotic stress tolerance. Finally, a cross talk of brassinosteroid with other phytohormones is summarized in Chap. 16.

This book is not an encyclopedia of review but includes a selected collection of newly written, integrated, and illustrated chapters describing our knowledge of brassinosteroids. The aim of this book is to tell all about brassinosteroids by the present time. The various chapters incorporate both theoretical and practical aspects and may serve as a baseline information for future researches through which significant developments are possible. It is intended that this book will be useful to the students, teachers, and researchers, both in universities and research institutes especially in relation to biological and agricultural sciences.

With great pleasure, we extend our sincere thanks to all the contributors for their timely response, their excellent and up-to-date contributions, and their consistent support and cooperation. We are thankful to all who has helped us in any way during the preparation of this volume. We are extremely thankful to Springer Nature for the expeditious acceptance of our proposal and completion of the review process. Subsequent cooperation and understanding of their staff are also gratefully acknowledged. We express our sincere thanks to the members of our family for all the support they provided and the neglect and loss they suffered during the preparation of this book.

Finally, we are thankful to the Almighty who provided and guided all the channels to work in cohesion of the idea to the development of the final version of this treatise *Brassinosteroids: Plant Growth and Development* until the successful completion of the job.

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Al Ain, UAE   Mohammad Yusuf
Amritsar, India   Renu Bhardwaj
Bialystok, Poland   Andrzej Bajguz

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About the Editors and Contributors

Editors

Dr. Shamsul Hayat is Professor in the Department of Botany, Aligarh Muslim University, Aligarh, India. He received his Ph.D. degree in Botany from Aligarh Muslim University, Aligarh, India. Before joining the department as faculty, he has worked as Research Associate and Young Scientist in the same department. He has also worked as Associate Professor in King Saud University, Riyadh, Saudi Arabia, and as a BOYSCAST Fellow at the National Institute of Agrobiological Sciences, Tsukuba, Japan. His major areas of research are plant hormone, nanoscience, and abiotic stress in plants. It has been reported from his group that phytohormone such as brassinosteroids and salicylic acid play an important role in increasing the photosynthetic efficiency of the plant and regulate the antioxidant system even under abiotic stress. He is also studying the protein profiling in hormone-treated plants under abiotic stress. Dr. Hayat has been awarded Prof. Hira Lal Chakravarty Award by the Indian Science Congress Association, Kolkata, India; Associate of the National Academy of Agricultural Sciences, New Delhi, India; BOYSCAST Fellow by the Department of Science and Technology, Government of India, New Delhi; and Young Scientist by the Association of the Advancement of Science, Aligarh, India. He has been the Principal Investigator of the various projects sanctioned by different agencies and guided five students for the award of Ph.D. degree and two students for the award of M.Phil. degree. Dr. Hayat has published more than 160 research papers and chapters in leading journals of the world such as *Environmental and Experimental Botany*, *Plant Physiology and Biochemistry*, *Environmental Pollution*, *Nitric Oxide*, *Protoplasma*, *Plant Signaling & Behavior*, *Photosynthetica*, and *Acta Physiologiae Plantarum* with good citation records (Citation: 7900 and h index 36) and also published 10 books by Kluwer Academic, Springer, Wiley-VCH, Science Publisher, and Narosa Publishing House. Dr. Hayat has presented his work at several national and international conferences in Japan, Brazil, Spain, China, and Saudi Arabia. He is a regular reviewer and is on the panel of the editorial boards of

national and international journals and is also member of important national and international scientific societies.

Dr. Mohammad Yusuf is an Instructor (Lecturer) in the Department of Biology, United Arab Emirates University, Al Ain, UAE. Mohammad received his Ph.D. in Plant Physiology and M.Sc. in Botany (Plant Physiology) from Aligarh Muslim University, India. He has worked as the SERB-Young Scientist and also an awardee of the prestigious Dr. D.S. Kothari Postdoctoral Fellowship from the Government of India. He has also been invited to present his work at Huazhong Agricultural University (HAU), Wuhan, China. Mohammad's research contribution includes more than 40 research articles in the international journal of repute and also more than 1500 google scholar citations with 19 h-index. He is exploring phytohormone-mediated abiotic stress tolerance mechanism in crop plants through proline metabolism.

Prof. Renu Bhardwaj is serving Guru Nanak Dev University for more than 27 years in the research area of Plant Growth Regulations and Stress Physiology and has published more than 250 research papers in the journals of national and international repute. She has been on various administrative positions like Director IQAC and Professor-in-Charge Examinations. She has been awarded INSA Visiting Fellowship, CSIR Visiting Associateship, FNESA Award, and FLS, London. She completed 15 major research projects and guided more than 30 Ph.D. students. She has also organized more than 20 national/international conferences. She has collaborated research both at national and international levels.

Dr. Andrzej Bajguz is Professor in the University of Bialystok, Poland. In 1994, he obtained his M.Sc. degree in Biology at Warsaw University, Branch in Bialystok, his Ph.D. in 1998 at the Nicholas Copernicus University in Torun, and Habilitation in Biological Sciences in 2012. He works at the University of Bialystok. He is an author or a coauthor of 85 original and review papers, chapters, and 6 books. He is a member of the Polish Biochemical Society, the Polish Botanical Society, the Polish Society of Experimental Plant Biology, and the Federation of European Societies of Plant Biology. He is also a member of the editorial board of the *Journal of Plant Growth Regulation* and *Frontiers in Plant Science* and an expert of the National Science Centre in Poland. Fields of his scientific interest are phytohormones, especially brassinosteroids—their physiology and biochemistry in lower plants. Within the last 20 years, the dominant area of his scientific experiments was hormonal growth and development regulation of the algae, mainly through brassinosteroids.

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Chapter 1
The Brassinosteroids Family – Structural Diversity of Natural Compounds and Their Precursors

Marco Antonio Teixeira Zullo and **Andrzej Bajguz**

Abstract The members of the brassinosteroids family, defined as the 3-oxygenated (20β)-5α-cholestane-22α,23α-diols or their derived compounds isolated from plants, bearing additional alkyl or oxy substituents, are presented. Further, brassinosteroids are grouped into C_{27}, C_{28}, and C_{29} depending upon the number of carbons in their skeletons. Their structural variations occur due to the substitution in A and B-rings as well in the side chain. They occur in both free and conjugated forms to sugars, fatty and inorganic acids. Their presence in Algae, Bryophyta, Pteridophyta and Angiosperms indicates a ubiquitous distribution in the plant kingdom. The related brassinosteroids precursors, as well as their occurrence, are also presented. Brassinosteroids are considered as the 6th class of plant hormones which have been established after the discovery of brassinolide and other related compounds.

Keywords Natural brassinosteroids · Brassinosteroids precursors · Brassinosteroids occurrence

1 Introduction

Intrigued with previous reports of growth regulating properties of pollen extracts, Mitchell and Whitehead (1941) examined the growth responses and histological changes that resulted from the application of ethereal extracts of corn pollen on intact bean plants or on the cut surfaces of decapitated stems. They observed that the first internode of the plants where these extracts were applied grew significantly more and faster than the untreated ones or treated with some known auxins, as well as gained more fresh and dry weights than the controls. They demonstrated that

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these were light dependent phenomena, and due to cell elongation rather than cell division. When applied to tap roots, these extracts inhibited root elongation and provoked the appearance of small tumors distal to the application point. When these pollen extracts where applied to the cut surfaces of decapitated stems they caused pronounced radial elongation of epidermal, cortical parenchyma, and endothelial cells. Later Mitchell et al*.,* reported that immature bean seeds also contained plant growth-stimulating hormones (Mitchell et al. 1951) and that *Brassica napus* pollen contained new, yet unknown, hormones they called *brassins* (Mitchell et al. 1970), all of them with properties similar to those reported earlier (Mitchell and Whitehead 1941).

About 60 kinds of pollen were then screened for plant growth activity in the bean second internode assay, and "a few samples, notably the pollen from rape plant (*Brassica napus* L.) and alder tree (*Alnus glutinosa* L.), produced an unusual response that combined elongation (the typical gibberellin response) with swelling and curvature" (Mandava 1988). At the same time, some experiments showed that application of brassins to young bean and Siberian elm tree plants promoted overall plant growth (Mitchell and Gregory 1972), what led United States Department of Agriculture (USDA) to initiate an effort aimed to explore the agricultural perspectives of brassins and to isolate their component(s). After processing 500 libers of rape pollen, finally, the USDA team announced the isolation and structure elucidation of the active principle, brassinolide (**1**) (Grove et al. 1979), the first plant hormone of steroidal nature, presenting, unlike animal steroidal hormones, (*i*) a 22α,23α-dihydroxylated campestane side chain, (*ii*) a B-ring lactone, and, (*iii*) a 2α,3α-dihydroxylated ring A. Bean second internodes exhibited elongation, curvature, swelling and even splitting when treated with increasing amounts of brassinolide (**1**) (Grove et al. 1979) (Fig. 1.1), a very distinct effect never observed with any other known plant hormone. Its isolation was followed by its partial synthesis (Fung and Siddall 1980; Ishiguro et al. 1980) and of its analogues (Thompson et al. 1979, 1981, 1982; Mori 1980; Takatsuto et al. 1981; Sakakibara and Mori 1982; Sakakibara et al. 1982; Mori et al. 1982), some later recognized as plant hormones themselves.

The early synthetic work furnished many compounds with similar or weaker brassin activity, what prompted natural products chemists to search for brassinolide related compounds in plant species other than rape. To the first of them, the 6-ketosteroid castasterone (**2**) (Yokota et al. 1982a), the putative biosynthetic precursor of brassinolide (**1**), followed that of dolicholide (**3**) (Yokota et al. 1982b), dolichosterone (**4**) (Baba et al. 1983), both with a 24-methylene-5α-cholestane structure, and 28-homodolichosterone (**11**) (Baba et al. 1983), with a 24(*E*)-ethylidene-5α-cholestane skeleton instead of a 5α-campestane basis as in brassinolide (**1**) and castasterone (**2**), and then a multitude of brassinosteroids (BRs) of different side chain structures and oxygenation patterns were isolated, giving rise to the class of brassinosteroids phytohormones, the components of which will be described ahead.

Fig. 1.1 Natural brassinosteroids

BR₂₂ (22) BR₂₃ (23) BR₂₄ (24)
BR₂₅ (25) BR₂₆ (26) BR₂₇ (27)
BR₂₈ (28) BR₂₉ (29) BR₃₀ (30)
BR₃₁ (31) BR₃₂ (32) BR₃₃ (33)
BR₃₄ (34) BR₃₅ (35) BR₃₆ (36)
BR₃₇ (37) BR₃₈ (38) BR₃₉ (39)
BR₄₀ (40) BR₄₁ (41) BR₄₂ (42)

Fig. 1.1 (continued)

BR₄₃ (43)

BR₄₄ (44)

BR₄₅ (45)

BR₄₆ (46)

BR₄₇ (47)

BR₄₈ (48)

BR₄₉ (49)

BR₅₀ (50)

BR₅₁ (51)

BR₅₂ (52)

BR₅₃ (53)

BR₅₄ (54)

BR₅₅ (55)

BR₅₆ (56)

BR₅₇ (57)

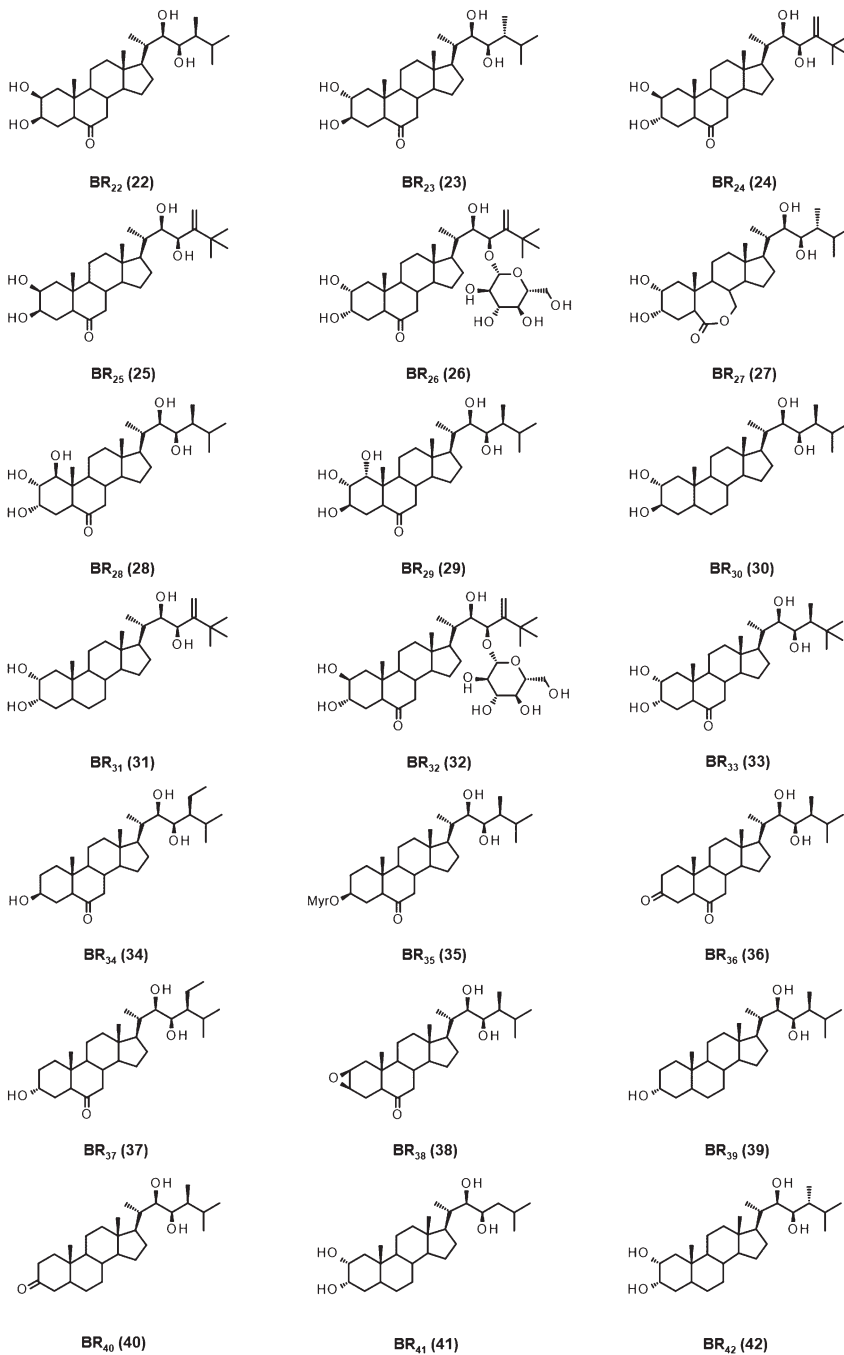**BR₅₈ (58)**

BR₅₉ (59)

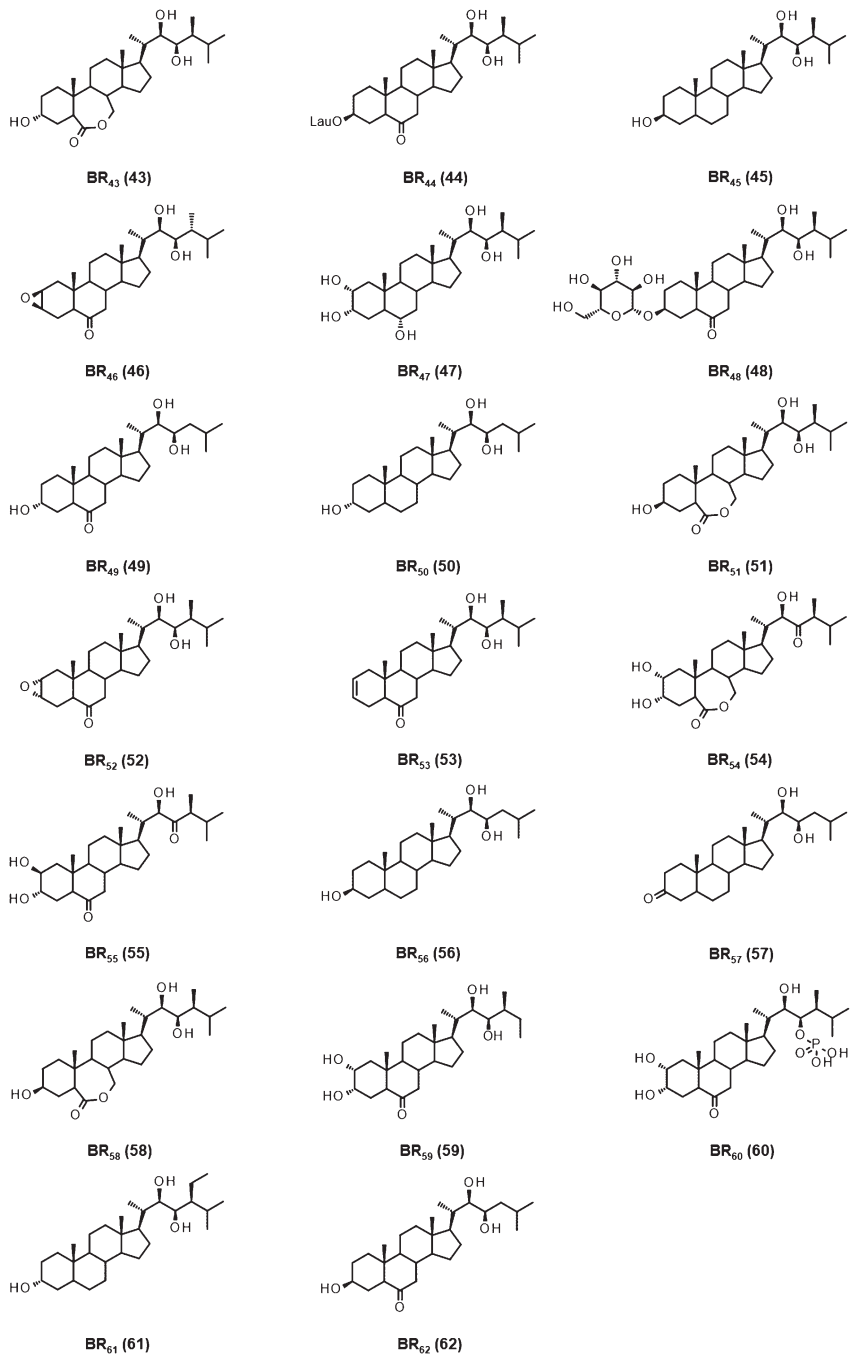**BR₆₀ (60)**

BR₆₁ (61)

BR₆₂ (62)
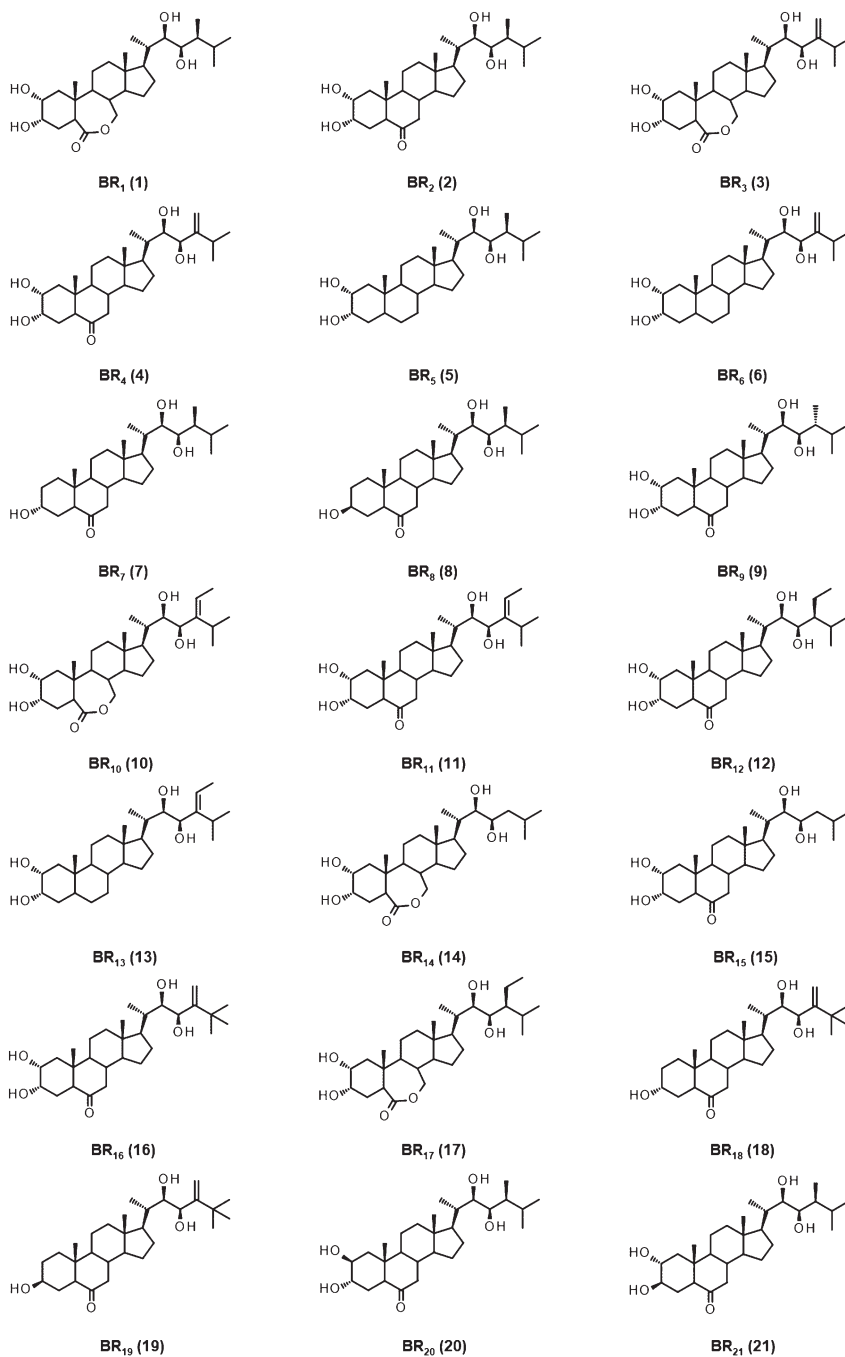
Fig. 1.1 (continued)

2 Natural Brassinosteroids

About sixty compounds with structures related to that of brassinolide (**1**) were isolated from or detected in plant materials in the last forty years (see Table 1.1 and Fig. 1.1). They were found in 26 species of 6 families of Algae, in 2 species of 2 families of Bryophyte, in 15 species of 8 families of Pteridophyte, in 6 species of 4 families of Gymnospermae, in 74 species of 35 families of Angiospermae (in 18 species of 6 families of Monocotyledoneae and 56 species of Dicotyledoneae), and in some plant derived products. About 15 biosynthetic precursors of brassinosteroids, some presenting brassinosteroid activity themselves, were found in many plant species.

Table 1.1 First report of a natural brassinosteroid

BR_n	Trivial name	References
1	Brassinolide	Grove et al. (1979)
2	Castasterone	Yokota et al. (1982a)
3	Dolicholide	Yokota et al. (1982b)
4	Dolichosterone	Baba et al. (1983)
5	6-Deoxocastasterone	Yokota et al. (1983c)
6	6-Deoxodolichosterone	Yokota et al. (1983c)
7	Typhasterol	Schneider et al. (1983)
8	Teasterone	Abe et al. (1984a)
9	24-Epicastasterone	Yokota et al. (1987b)
10	28-Homodolicholide	Yokota et al. (1983b)
11	28-Homodolichosterone	Baba et al. (1983)
12	28-Homocastasterone	Abe et al. (1983)
13	6-Deoxo-28-homodolichosterone	Yokota et al. (1987c)
14	28-Norbrassinolide	Abe et al. (1983)
15	28-Norcastasterone	Abe et al. (1983)
16	25-Methyldolichosterone	Kim et al. (1987)
17	28-Homobrassinolide	Ikekawa et al. (1984)
18	2-Deoxy-25-methyldolichosterone	Takahashi et al. (1988)
19	3-Epi-2-deoxy-25-methyldolichosterone	Yokota and Takahashi (1988)
20	2-Epicastasterone	Takahashi et al. (1988)
21	3-Epicastasterone	Takahashi et al. (1988)
22	2,3-Diepicastasterone	Takahashi et al. (1988)
23	3,24-Diepicastasterone	Takahashi et al. (1988)
24	2-Epi-25-methyldolichosterone	Takahashi et al. (1988)
25	2,3-Diepi-25-methyldolichosterone	Takahashi et al. (1988)
26	23-*O*-β-*D*-Glucopyranosyl-25-methyldolichosterone	Yokota et al. (1987a)
27	24-Epibrassinolide	Ikekawa et al. (1988)
28	1β-Hydroxycastasterone	Takahashi et al. (1988)

Table 1.1 (continued)

BR$_n$	Trivial name	References
29	1α-Hydroxy-3-epicastasterone	Kim (1991)
30	3-Epi-6-deoxocastasterone	Kim (1991)
31	6-Deoxo-25-methyldolichosterone	Kim (1991)
32	23-*O*-β-*D*-Glucopyranosyl-2-epi-25-methyldolichosterone	Kim (1991)
33	25-Methylcastasterone	Taylor et al. (1993)
34	28-Homoteasterone	Schmidt et al. (1993b)
35	Teasterone-3-myristate	Asakawa et al. (1994)
36	3-Dehydroteasterone	Abe et al. (1994)
37	28-Homotyphasterol	Abe et al. (1995a)
38	Secasterone	Schmidt et al. (1995b)
39	6-Deoxotyphasterol	Griffiths et al. (1995)
40	3-Dehydro-6-deoxoteasterone	Griffiths et al. (1995)
41	6-Deoxo-28-norcastasterone	Spengler et al. (1995)
42	6-Deoxo-24-epicastasterone	Spengler et al. (1995)
43	2-Deoxybrassinolide	Schmidt et al. (1995c)
44	Teasterone-3-laurate	Asakawa et al. (1996)
45	6-Deoxoteasterone	Fujioka et al. (1998b)
46	24-Episecasterone	Friebe et al. (1999)
47	6α-Hydroxycastasterone	Fujioka et al. (2000b)
48	3-*O*-β-*D*-Glucopyranosylteasterone	Soeno et al. (2000b)
49	28-Nortyphasterol	Fujioka et al. (2000a)
50	6-Deoxo-28-nortyphasterol	Yokota et al. (2001)
51	3-Epibrassinolide	Konstantinova et al. (2001)
52	2,3-Diepisecasterone	Antonchick et al. (2003)
53	Secasterol	Antonchick et al. (2003)
54	Cryptolide	Watanabe et al. (2000)
55	23-Dehydro-2-epicastasterone	Hwang et al. (2006)
56	6-Deoxo-28-norteasterone	Bhardwaj et al. (2007)
57	3-Dehydro-6-deoxo-28-norteasterone	Bhardwaj et al. (2007)
58	3-Epi-2-deoxybrassinolide	Katsumata et al. (2008)
59	26-Norcastasterone	Son et al. (2013)
60	Castasterone 23-phosphate	Kim et al. (2015)
61	6-Deoxo-28-homotyphasterol	Xin et al. (2016)
62	28-Norteasterone	Oklestkova et al. (2017)

The finding that the rice lamina inclination assay, developed by Maeda (1965), to test for auxin activity could be used to detect the activity of brassinosteroids at even nanomolar or subnanomolar concentrations (Wada et al. 1981) and the development of a microanalytical method for the quantification of 22α, 23α-dihydroxybrassinosteroids (Takatsuto et al. 1982), allowed a rapid expansion of the number of known brassinosteroids. The first brassinosteroids isolated presented, as common features, (*i*) a 5α-cholestane or a 6,7-seco-5α-cholestane

derived skeleton, (*ii*) ring A with one to three oxygen functions (one always at carbon 3), (*iii*) ring B fully saturated or with varying degree of oxidation at carbon 6, (*iv*) all-*trans* ring junctions and (*v*) 22α,23α-dihydroxylation. In this sense, 3-oxygenated (20β)-5α-cholestane-22α,23α-diols of plant origin, bearing additional alkyl or oxy substituents, were considered as natural brassinosteroids (Zullo and Adam 2002). A more restricted definition states that, in the biosynthetic route to a brassinosteroid lactone, "one would consider as brassinosteroids only those compounds originated after the 22α,23α-dihydroxylation (i.e., those between teasterone or 6-deoxoteasterone and brassinolide), and hence as *brassinosteroid precursors* those before dihydroxylation occurs (i.e., those compounds up to cathasterone and 6-deoxocathasterone)" (Zullo et al. 2003; Zullo and Kohout 2004). After then some other brassinosteroids presenting 2,3-epoxy, 23-dehydro, 23-glycosidic, 23-ester functions, or 26-nor side chain or even 2,3-unsaturation were isolated, "allowing to consider as *natural brassinosteroids* the 3-oxygenated (20β)-5α-cholestane-22α,23α-diols or their derived compounds isolated from plants, bearing additional alkyl or oxy substituents" (Zullo 2018).

The unconjugated brassinosteroids so far isolated present 27 (C_{27}), 28 (C_{28}) or 29 (C_{29}) carbons, with 5α-cholestane or 26-nor-5α-campestanes (= 26-nor-24α-methyl-5α-cholestane) structures for the C_{27} series, 5α-campestane (= 24α-methyl-5α-cholestane), 5α-ergostane (= 24β-methyl-5α-cholestane) or 24-methylene-5α-cholestane skeletons for the C_{28} series, and 5α-sitostane (= 24α-ethyl-5α-cholestane), 24(*Z*)-ethylidene-5α-cholestane, 25-methyl-5α-campestane and 24-methylene-25-methyl-5α-cholestane structures for the C_{29} series (Fig. 1.2). Only one of the side chains of isolated brassinosteroids is of a 26-nor sterol, although C_{26}-demethylation of brassinosteroids have been demonstrated in metabolic studies with some species (Joo et al. 2012, 2015; Kim et al. 2000a, b). From the 12 different side chains of natural brassinosteroids, 9 of them present 22α,23α-dihydroxylation, while one presents a 22α-hydroxy-23-oxo group, another one presents conjugation of one glucose unit at the 23α-hydroxyl, and a last different side chain shows phosphorylation at the 23α-hydroxyl. Feeding studies shows that side chain glucosylation can occur at either C-23 (Poppenberger et al. 2005) or C-22 (Soeno et al. 2006), and also at C-25 or C-26 after hydroxylation at these carbons (Hai et al. 1996). Phosphorylation (Kim et al. 2015) and sulfonation (Rouleau et al. 1999) have been demonstrated to occur at the side chain of brassinosteroids, but while the first occurs at C-23, the second occurs at C-22, at least with the actual experimental data available.

It is known that the bioactivity of brassinosteroids is dependent on the structure of the side chain and of the A/B rings (Takatsuto et al. 1983b; Takatsuto et al. 1983a; Brosa et al. 1996; Takatsuto et al. 1987; Mandava 1988; Liu et al. 2017; Zullo and Adam 2002). Regarding to the side chain, as general rules, employing the rice lamina inclination assay on any of its versions (Maeda 1965; Wada et al. 1981; Fujioka et al. 1998a), for the same A/B ring structures, 22α,23α-dihidroxybrassinosteroids of the brassinolide series are so active as of the 28-homobrassinosteroids series (Takatsuto et al. 1983a), and more active than those of 24-epi- or 28-norbrassinosteroids (Takatsuto et al. 1983a; Wada et al. 1983),
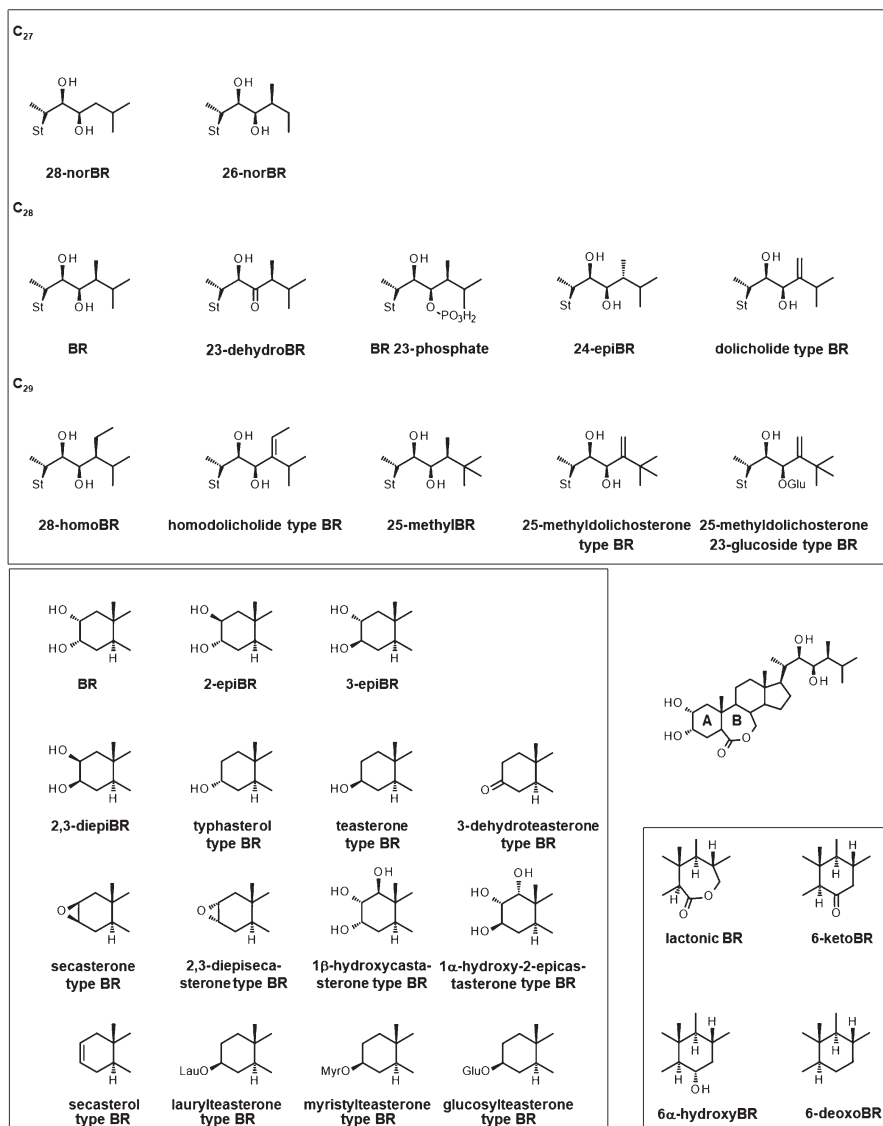
Fig. 1.2 Brassinolide and structural variations of brassinosteroids

which are more active than 26-norbrasssinosteroids (Kim et al. 2000a; Watanabe et al. 2001). 23-Dehydrogenation (Watanabe et al. 2001), or conjugation at one of the side chain hydroxyls (Suzuki et al. 1993b; Kim et al. 2015; Rouleau et al. 1999), diminishes (Yokota et al. 1998; Suzuki et al. 1993b) or abolishes the biological activity (Kim et al. 2015; Rouleau et al. 1999), an effect contrary to that observed with 25-methylation (Mori and Takeuchi 1988). It is to note that the relative

biological activity of brassinosteroids vary according to the biological assay performed for their evaluation, not only in relation to the side chain but also to the other active sites of their molecules (Takatsuto et al. 1983b; Watanabe et al. 2001; Zullo and Adam 2002; Liu et al. 2017).

A greater structural variation is observed in ring A, with 15 different structures reported, ranging from $\Delta^{2,3}$-unsaturated to trioxygenated and conjugated brassinosteroids: even so, this variation still does not reflect all the possible substructures at this ring, presumed either by efforts of large scale isolation of brassinosteroids (Kim 1991; Fujioka 1999), or by the study of the metabolism of brassinosteroids (Zullo 2018). The biological activity for brassinosteroids with A/B *trans* ring junctions increases as substitution in ring A changes in the order 3β-hydroxy ≤ 3-oxo ≤ 3α-hydroxy < 2α,3α-dihydroxy, and diminishes as deviates from these patterns (Mandava 1988; Zullo and Adam 2002; Liu et al. 2017; Takatsuto et al. 1987; Fujioka et al. 1995a).

The structural variations in ring B reflect the main steps in the biosynthesis of brassinosteroids (Vriet et al. 2013), being more active as its oxidation state increases (Mandava 1988) sequentially from the 6-deoxo to the 6α-hydroxy to the 6-oxo and to the 7-oxalactone types. Therefore, brassinosteroids can be classified, according to the B ring structure, as: (a) **6-oxo-7-oxalactonic brassinosteroids:** (*i*) 2α,3α-dihydroxylated: brassinolide (**1**), dolicholide (**3**), 28-homodolicholide (**10**), 28-nor-brassinolide (**14**), 28-homobrassinolide (**17**), 24-epibrassinolide (**27**), cryptolide (**54**); (*ii*) 2α, 3β-dihydroxylated: 3-epibrassinolide (**51**); (*iii*) 3α-hydroxylated: 2-deoxybrassinolide (7-oxatyphasterol, **43**); (*iv*) 3β-hydroxylated: 3-epi-2-deoxy-brassinolide (7-oxateasterone, **58**); (b) **6-oxo (or 6-keto) brassinosteroids:** (*i*) 2α,3α-dihydroxylated: castasterone (**2**), dolichosterone (**4**), 24-epicastasterone (**9**), 28-homodolichosterone (**11**), 28-homocastasterone (**12**), 28-norcastasterone (**15**), 25-methyldolichosterone (**16**), 25-methylcastasterone (**33**), 26-norcastasterone (**59**); (*ii*) 2β,3α-dihydroxylated: 2-epicastasterone (**20**), 2-epi-25-methyldolichosterone (**24**), 23-dehydro-2-epicastasterone (**55**); (*iii*) 2α,3β-dihydroxylated: 3-epi-castasterone (**21**), 3,24-diepicastasterone (**23**); (*iv*) 2β,3β-dihydroxylated: 2,3-diepi-castasterone (**22**), 2,3-diepi-25-methyldolichosterone (**25**); (*v*) 3α-monohydroxylated: typhasterol (**7**), 2-deoxy-25-methyldolichosterone (**18**), 28-homotyphasterol (**37**), 28-nortyphasterol (**49**); (*vi*) 3β-monohydroxylated: teasterone (**8**), 3-epi-2-deoxy-25-methyldolichosterone (**19**), 28-homoteasterone (**34**), 28-norteasterone (**62**); (*vii*) 1β,2α,3α-trihydroxylated: 1β-hydroxycastasterone (**28**); (*viii*) 1α,2α,3β-dihydroxylated: 1α-hydroxy-3-epicastasterone (**29**); (*ix*) 2α,3α-epoxide: 2,3-diepise-casterone (**52**); (*x*) 2β,3β-epoxide: secasterone (**38**), 24-episecasterone (**46**); (*xi*) Δ^2-olefin: secasterol (**53**); (*xii*) 3β-conjugates: teasterone-3-myristate (**35**), teast-erone-3-laurate (**44**), 3-O-β-D-glucopyranosylteasterone (**48**); (*xiii*) 23α-conjugates: 23-*O*-β-*D*-glucopyranosyl-25-methyldolichosterone (**26**), 23-*O*-β-*D*-glucopyranosyl-2-epi-25-methyldolichosterone (**32**), castasterone 23-phosphate (**60**); (*xiv*) 3-dehydro: 3-dehydroteasterone (**36**); (c) **6α-hydroxybrassinosteroids:** 6α-hydroxycastasterone (**47**); (d) **6-deoxobrassinosteroids:** (*i*) 2α,3α-dihydroxylated: 6-deoxocastasterone (**5**), 6-deoxodolichosterone (**6**), 6-deoxo-

28-homodolichosterone (**13**), 6-deoxo-25-methyldolichosterone (**31**), 6-deoxo-28-norcastasterone (**41**), 6-deoxo-24-epicastasterone (**42**); (*ii*) 2α,3β-dihydroxylated: 3-epi-6-deoxocastasterone (**30**); (*iii*) 3α-monohydroxylated: 6-deoxo-typhasterol (**39**), 6-deoxo-28-nortyphasterol (**50**), 6-deoxo-28-homotyphasterol (**61**); (*iv*): 3β-monohydroxylated: 6-deoxoteasterone (**45**), 6-deoxo-28-norteasterone (**56**); (*v*) 3-dehydro: 3-dehydro-6-deoxoteasterone (**40**), 3-dehydro-6-deoxo-28-norteasterone (**57**).

3 Brassinosteroids Precursors

A series of papers revealed the main steps of brassinosteroids biosynthesis, from the plant sterols to the brassinosteroid lactones, especially that from campesterol (**CR**) or campestanol (**CN**) to brassinolide (**1**). From these studies it became clear that, if the natural brassinosteroids can be easily recognized from their chemical structures, similar observation does not happen with their precursors (see Fig. 1.3 and Table 1.2). The first experiments established the biosynthesis of brassinolide (**1**) from teasterone (**8**) via, sequentially, 3-dehydroteasterone (**36**), typhasterol (**7**), and castasterone (**2**) (Suzuki et al. 1993a, 1994a, c) (follow by Fig. 1.4). Soon after it was found that campesterol (**CR**) was converted to campestanol (**CN**) and to 6α-hydroxycampestanol (**63**), 6-oxocampestanol (**64**), 22α-hydroxy-6-oxocampestanol (**65**), named cathasterone, and this one to teasterone (**8**) (Fujioka et al. 1995b). The complete biosynthetic sequence of brassinolide starting from campesterol (**CR**) via cathasterone (**65**) is known as the early C-6 oxidation pathway (a route in which C-6 oxidation occurs earlier than 22α,23α-dihydroxylation).

The frequent isolation or detection of 6-deoxobrassinosteroids brought the suspicion that another biosynthetic route to brassinosteroid lactones could exist. Feeding experiments with labeled precursors established the sequence 6-deoxoteasterone (**45**), 3-dehydro-6-deoxoteasterone (**40**), 6-deoxotyphasterol (**39**), 6-deoxocastasterone (**5**), castasterone (**2**), brassinolide (**1**), which was called the late C-6 oxidation pathway (a route in which C-6 oxidation occurs later than 22α, 23α-dihydroxylation) (Choi et al. 1997). It was further demonstrated the conversion of campestanol (**CN**) to 6-deoxoteasterone (**45**) through 6-deoxocathasterone (**66**) (Bishop et al. 1999), and the presence of 3-epi-6-deoxocathasterone (**67**), a putative brassinosteroid precursor, in cultured cells of *Catharanthus roseus* (Fujioka et al. 2000b).

A thorough examination of the sterols present in cultured cells of *C. roseus* and in *Arabidopsis* seedlings, conjugated with metabolic studies with deuterated substrates, revealed that the conversion of campesterol (**CR**) to campestanol (**CN**) occurs through campest-4-en-3-one (**4en3one**) and campestan-3-one (**3one**) (Fujioka et al. 2002). Moreover, it revealed the operation of intermediates in the conversion of campesterol (**CR**) to 6-deoxocathasterone (**66**), originating
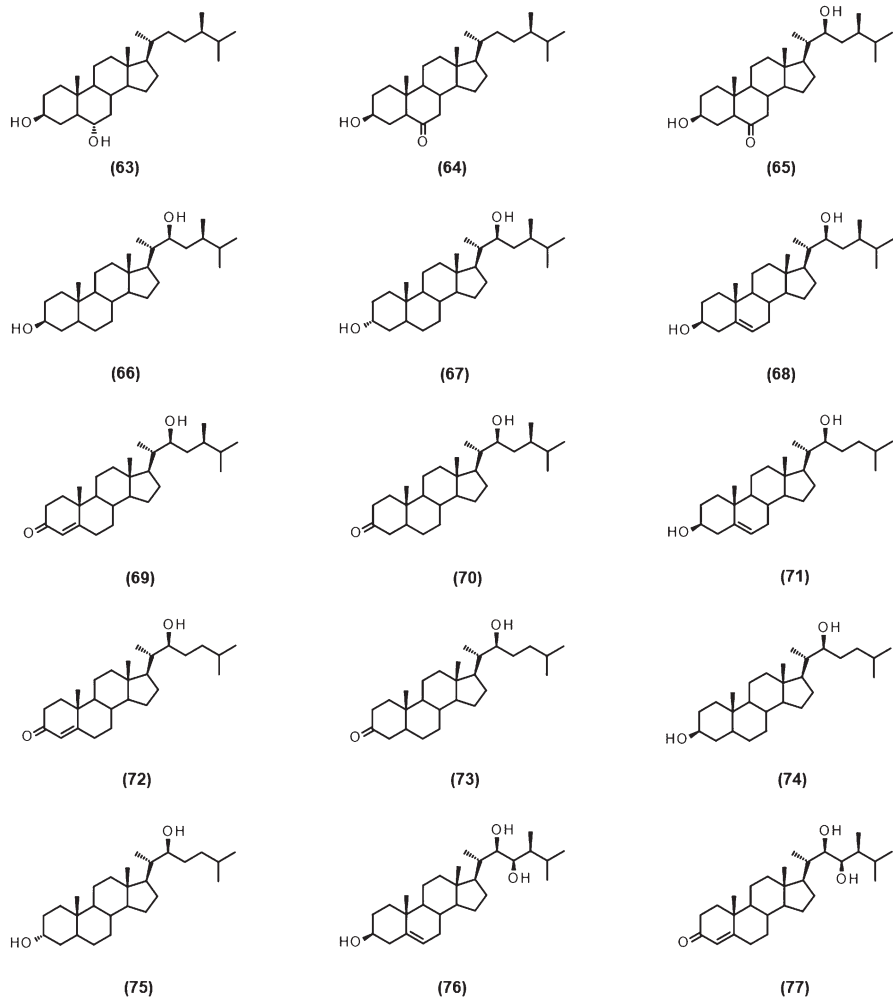
Fig. 1.3 Brassinosteroids precursors

22α-hydroxycampesterol (**68**), 22α-hydroxycampest-4-en-3-one (**69**), and 22α-hydroxy-5α-campestan-3-one (**70**) from, respectively, campesterol (CR), campest-4-en-3-one (**4en3one**) and campestan-3-one (**3one**). In the same extracts were found also the 28-norhomologues 22α-hydroxycholesterol (**71**), 22α-hydroxycholest-4-en-3-one (**72**), 22α-hydroxy-5α-cholestan-3-one (**73**), 6-deoxo-28-norcathasterone (**74**) and 3-epi-6-deoxo-28-norcathasterone (**75**). Later, studying the action of *Arabidopsis* CYP90C1 and CYP90D1, it was found that these enzymes act on 3-epi-6-deoxocathasterone (**67**), 22α-hydroxycampesterol (**68**), 22α-hydroxy-5α-campestan-3-one (**70**), and 22α-hydroxycampest-4-en-3-

Table 1.2 Brassinosteroid precursors

Compound	Trivial name	References
63	6α-Hydroxycampestanol	Fujioka et al. (1995b)
64	6-Oxocampestanol	Fujioka et al. (1995b)
65	Cathasterone	Fujioka et al. (1995b)
66	6-Deoxocathasterone	Bishop et al. (1999)
67	3-Epi-6-deoxocathasterone	Fujioka et al. (2000b)
68	22α-Hydroxycampesterol [(22*S*)-22-Hydroxycampesterol]	Fujioka et al. (2002)
69	22α-Hydroxycampest-4-en-3-one [(22*S*,24*R*)-22-Hydroxyergost-4-en-3-one]	Fujioka et al. (2002)
70	22α-Hydroxycampestan-3-one [(22*S*,24*R*)-22-Hydroxy-5α-ergostan-3-one]	Fujioka et al. (2002)
71	22α-Hydroxycholesterol [(22*S*)-28-Nor-22-hydroxycampesterol]	Fujioka et al. (2002)
72	22α-Hydroxycholest-4-en-3-one [(22*S*)-28-Nor-22-hydroxyergost-4-en-3-one]	Fujioka et al. (2002)
73	22α-Hydroxycholestan-3-one [(22*S*)-28-Nor-22-hydroxy-5α-ergostan-3-one]	Fujioka et al. (2002)
74	6-Deoxo-28-norcathasterone	Fujioka et al. (2002)
75	3-Epi-6-deoxo-28-norcathasterone	Fujioka et al. (2002)
76	22α,23α-Dihydroxycampesterol [(22*R*,23*R*)-22,23-Dihydroxycampesterol]	Ohnishi et al. (2006b)
77	22α,23α-Dihydroxycampest-4-en-3-one [(22*R*,23*R*)-22,23-Dihydroxycampest-4-en-3-one]	Ohnishi et al. (2006b)

one (**69**) to yield, respectively, 6-deoxotyphasterol (**39**), 22α, 23α-dihydroxycampesterol (**76**), 3-dehydro-6-deoxoteasterone (**40**), and 22α, 23α-dihydroxycampest-4-en-3-one (**77**), revealing a new shortcut in the biosynthesis of brassinosteroids. Compounds **63-77**, isolated from plant material, present side chains with no oxygen function or 22α-monohydroxylated or 22α,23α-dihydroxylated and rings A/B typical of common plant sterols (as 3β-hydroxy-Δ^5-sterols or 3β-hydroxy-5α-stanols) or less usual ones [like Δ^4-sten-3-ones (Franke et al. 2004; Georges et al. 2006; Pinto et al. 2002) or 5α-stan-3-ones (Guillen and Manzanos 2001)] or reflecting the steps for the construction of typical A/B rings of brassinosteroids (5α-stan-3β,6α-diols, 5α-stan-3β-ol-6-one, 5α-stan-3α-ol) (Fig. 1.5). None of these fragments, *per se*, can be attributed exclusively to brassinosteroids (Zullo 2018).

It is to note that only brassinosteroids precursors of campestane and cholestane skeletons had been isolated to date, what does not exclude the possibility of similar biosynthetic reactions can occur at the remaining skeletons (ergostane, sitostane, 24-methylenecholestane, 24-ethylidenecholestane, 25-methylcampestane and 24-methylene-25-methylcholestane), for all the possible sequences in the grid (as shown in Fig. 1.4) or through conversions of skeletons while functional-
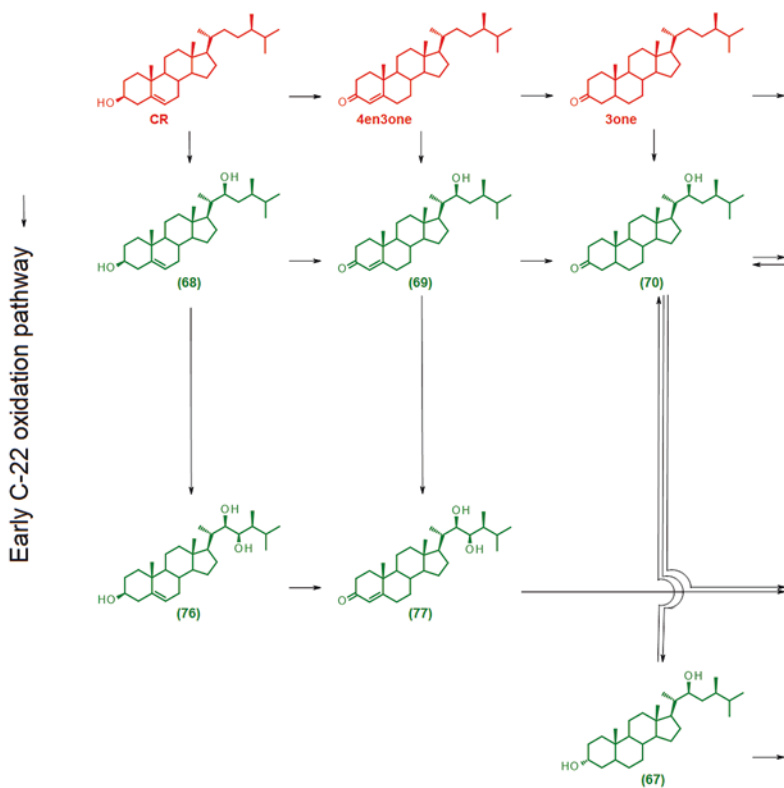
Fig. 1.4 Biosynthesis of brassinolide (1) from campesterol (red: sterols; green: brassinosteroids precursors; blue: brassinosteroids). Adapted from Zullo 2018

izing them towards the synthesis of castasterone-like or brassinolide-like brassinosteroids, what could explain the isolation or detection of brassinosteroids of different skeletons in the same plant materials. The fact that total sterols usually comprise $2–3 \times 10^{-3}$ g/g of plant dry weight (Benveniste 2004) and that brassinosteroids are present usually in $10^{-12}–10^{-9}$ g/g fresh weight in plant material (Bajguz and Tretyn 2003; Takatsuto 1994), immersed in a matrix of tens of compounds of similar structure (and, hence, of similar polarity and similar chromatographic behavior), turns a very difficult task to determine the brassinosteroids profile of a given plant material, including the compounds of transient existence,
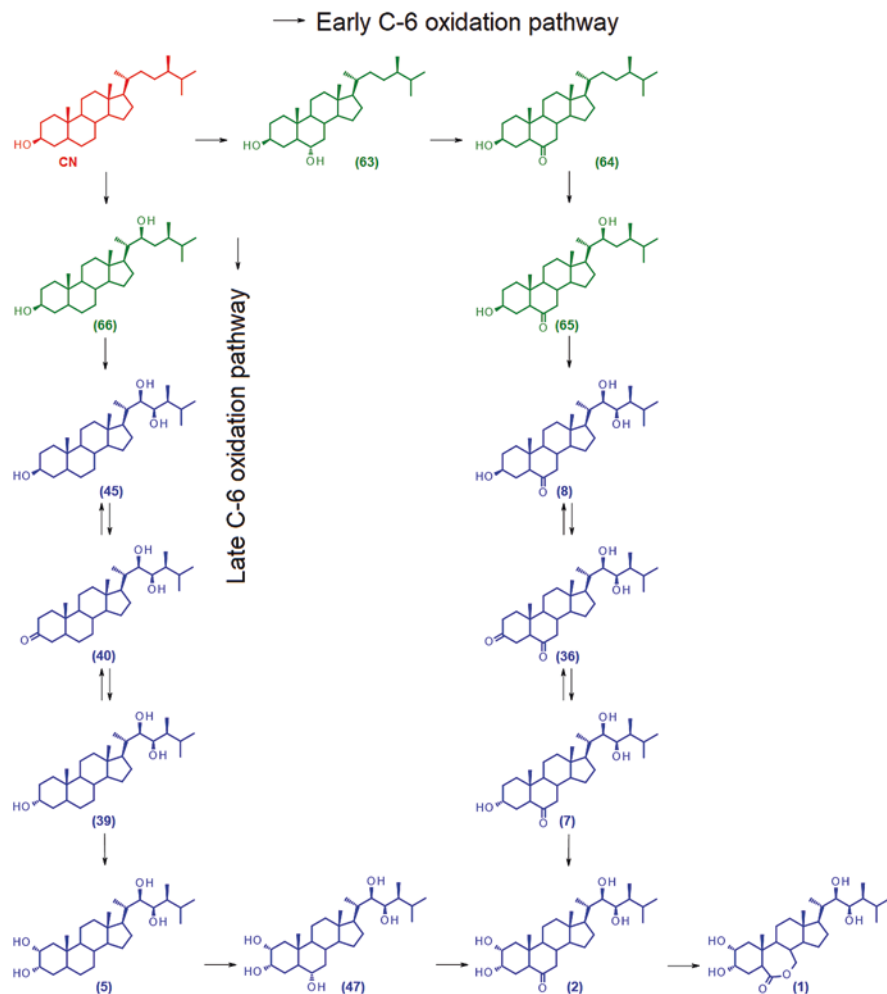
Fig. 1.4 (continued)

like their precursors, can explain why precursors of different skeletons have not been isolated yet.

4   Brassinosteroids with Partially Elucidated Structure

A few natural brassinosteroids were isolated in pure state in enough amount to iden-tify them by the usual spectroscopic methods, but usually they are detected by com-parison with authentic compounds prepared by synthesis. Sometimes, due to small

Fig. 1.5 Fragments found in brassinosteroids precursors
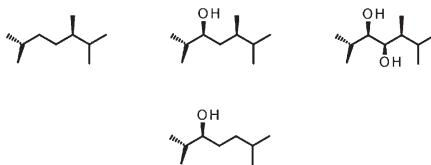
Fragments found in the side chain
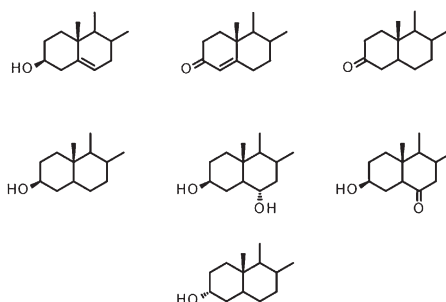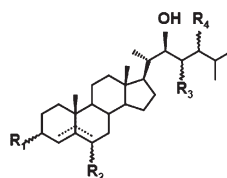
Fragments found in rings A and B

amounts of samples, to similar spectroscopic characteristics but different chromato-graphic behavior, it is not possible to determine the structure of all compounds pres-ent in a given brassinosteroids extract. Eventually the complete structure of one of these compounds is correctly elucidated.

One of the richest sources of brassinosteroids, the seeds of kidney beans, pres-ents about 60 compounds of partially known structure (Hwang et al. 2006), for which some of them were described (Yokota et al. 1987c) (see Fig. 1.6). Among them is cited 1 isomer of 6-deoxo-28-homodolichosterone (**78**), 4 isomers of castasterone (**79**), 1 isomer of a hydroxylated castasterone (**80**), 2 isomers of 28-homocastasterone (**81**), 3 isomers of a homologue of dolichosterone (**82**), 1 iso-mer of a brassinolide derivative with 14 atomic units higher (**83**), 1 isomer of a brassinolide derivative with 44 atomic units higher (**84**), 1 isomer of dolicholide (**85**), 1 isomer of dolicholide with an extra oxygen (**86**), another one with an extra hydroxyl (**87**), a dolicholide derivative 28 atomic units higher (**88**), and another one
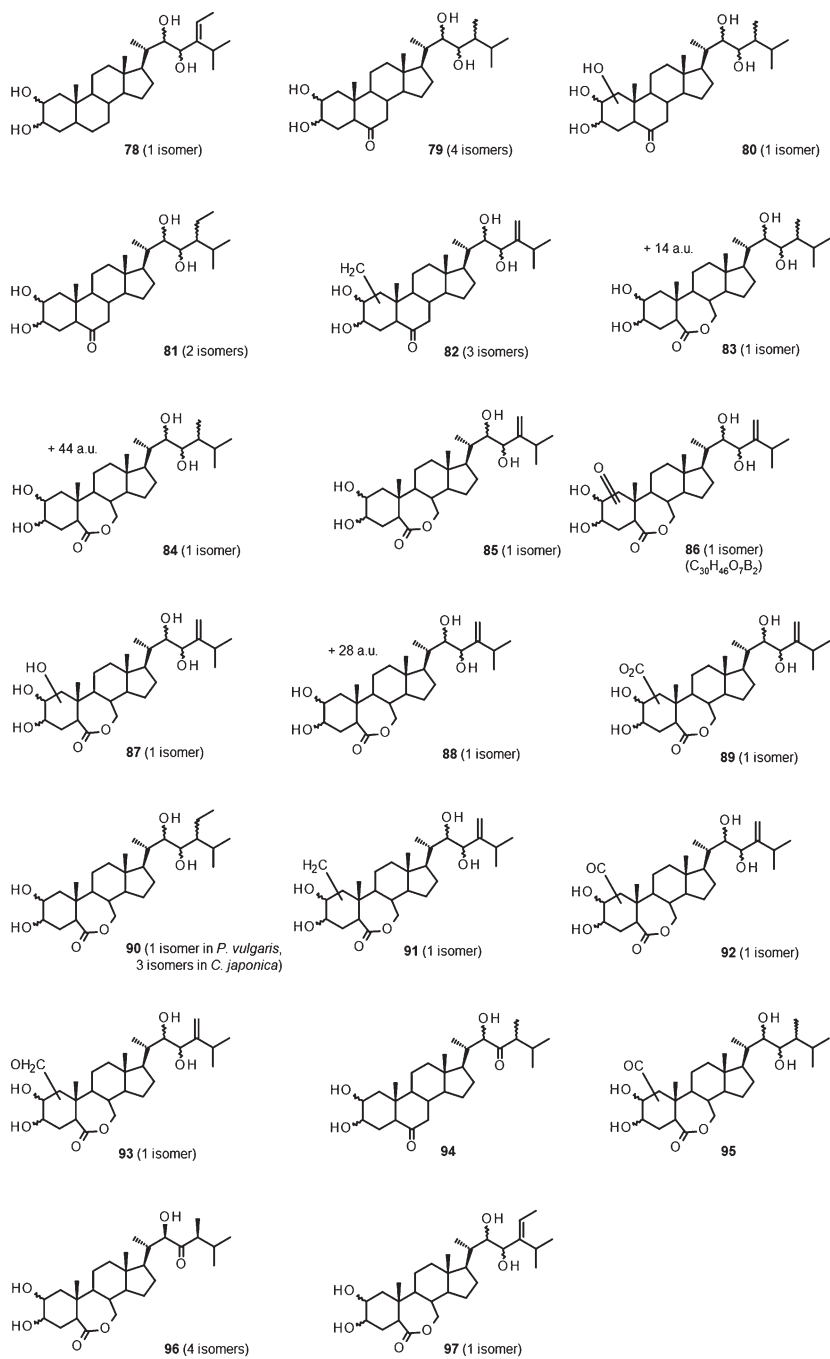
Fig. 1.6 Brassinosteroids with partially elucidated structure