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Review

Brassinosteroids: distribution in plants, bioassays and microanalysis by gas chromatography–mass spectrometry

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ABSTRACT

During the past decade, studies on brassinosteroids (BRs) have greatly widened the knowledge of new steroidal plant hormones. This review summarizes studies on BRs from the viewpoints of distribution in plants, bioassays and a microanalytical method using gas chromatography–mass spectrometry with selected ion monitoring (GC–MS-SIM). A highly sensitive and specific bioassay employed to isolate brassinolide from rape pollen is bean-second internode assay. The rice-lamina inclination test and wheat leaf unrolling test are now widely and routinely used mainly in Japan as highly sensitive and specific bioassays during the purification steps of BRs from the plant sources. When a highly purified fraction containing a very small amount of BRs is obtained, the fraction is derivatized with methaneboronic acid to form a bismethaneboronate of BRs and then analysed by GC–MS-SIM. The rice-lamina inclination test and the GC–MS-SIM microanalytical method have contributed greatly to studies on the identification of many natural BRs and also to their screening and distribution in the plant kingdom. So far about 30 natural BRs have been characterized in a number of higher plants (phanerogams) and also in some lower plants (cryptogams). These data strongly suggest that BRs occur widely in the plant kingdom, as in the case of other known plant hormones, and that BRs play some physiological functions in plant growth and development.

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1. INTRODUCTION

Brassinolide (BL), present at 0.1 mg/kg in rape (*Brassica napus* L.) pollen, was the first steroidal plant growth promoter to be isolated, in 1979 [1]. In 1982, another new BL-related steroid termed castasterone (CS) was isolated from the insect galls of chestnut (*Castanea crenata*) [2]. CS differs from BL only in B-ring functionality. BL, CS and their structurally related steroidal compounds are known collectively as brassinosteroids (BRs). After the discovery of BL, the chemical synthesis of BL and its related compounds, the physiological study of them, the study of structure–activity relationships and screening of natural BRs in the plant kingdom were intensively and extensively carried out by many scientists. Following the basic investigations, some BRs (mainly BL, 24-epiBL and 28-homoBL) have been tested for many years for their practical applications in agriculture. These BRs have been shown to possess the following characteristics: promotion of germination and plant growth, raising of ripening, thickening promotion, recovery from stresses under various conditions unfavourable for plants and effects on flowering or its differentiation. Several excellent reviews on these subjects are available [3–14].

Microanalytical methods for BRs have also been developed: (i) gas chromatographic–mass spectrometric (GC–MS) analysis of BRs as bismethaneboronate derivatives [15] or methaneboronate–trimethylsilyl derivatives [16], (ii) high performance liquid chromatographic analysis of BRs as bisboronate derivatives having a fluorophore or an electrophore [17] and (iii) radioimmunoassay for BRs [18]. Among these microanalytical methods, GC–MS analysis has contributed greatly to the study of the identification and characterization of a number of natural BRs. At present about 30 BRs (Fig. 1) have been chemically identified from plants sources. Some reviews on microanalytical methods of BRs are also available [17,19–21].

This review summarizes research on BRs with respect to the distribution of BRs in the plant kingdom, bioassays for BRs and microanalytical methods for BRs using GC–MS with selected ion monitoring (SIM).

2. DISTRIBUTION OF BRASSINOSTEROIDS IN PLANT KINGDOM

Since the discovery of BL and CS, intensive and extensive studies on the isolation of new BRs from plant sources and on screening of BRs in the plant kingdom have been made mainly by Japanese scientists. In these studies, a sensitive and specific bioassay, the rice-lamina inclination test [22], and a GC–MS analysis [15,16] have been very effective and useful. So far about 30 BRs have been isolated and their structures have been chemically characterized [5,14]. It is now believed that BRs are ubiquitously distributed in the plant kingdom.

2.1. *Brassinosteroids in higher plants (dicots)*

The occurrence of BRs in a number of dicots plants has been reported: BL, CS, 28-norBL and 28-homoCS from the sheaths and immature seeds of Chinese cabbage (*Brassica campestris* L. var. *pekinensis*) [23,24], CS from the seeds of persimmon (*Diospyros kaki* Thunb.) [25], BL and CS from the pollen of European alder, *Alnus glutinosa* (L.) Gaertn [26], BL, CS and 28-norCS from the pollen of sunflower (*Helianthus annuus* L.) [27], BL from the stems of *Solidago altissima* L. [28], BL and CS from the pollen of *Cistus hirusutum* [29], BL and CS from the crown gall cells of *Catharanthus roseus* Don [30], BL and CS from the pollen of buckwheat (*Fagopyrum esculentum* Moench) [31], BL, CS, 28-homoCS, 28-norCS, typhasterol (2-deoxyCS, TyS) and teasterone (TeS, 3-epimer of TyS) from the leaves of green tea (*Thea sinensis* L.) [24,32,33], CS from the pollen and anther of green tea [34], CS from the flower buds of loquat (*Eriobotrya japonica* Lindl.) [35], CS and 28-norCS from the immature seeds of *Pharbitis purpurea* Voigt [36], dolichosterone (DS) from the pollen of *Eucalyptus marinata* [29], BL from the pollen of *Eucalyptus calophylla* [29], BL, CS and 6-deoxo-CS from the insect galls [2,37,38] and CS and 6-deoxoCS from the shoots, leaves and flower buds [38] of chestnut (*Castanea crenata* Sieb. et Zucc.), dolicholide (DL), DS, 28-homoDL, 28-homoDS, 6-deoxoCS and 6-deoxoDS from the immature seeds of *Dolichos*

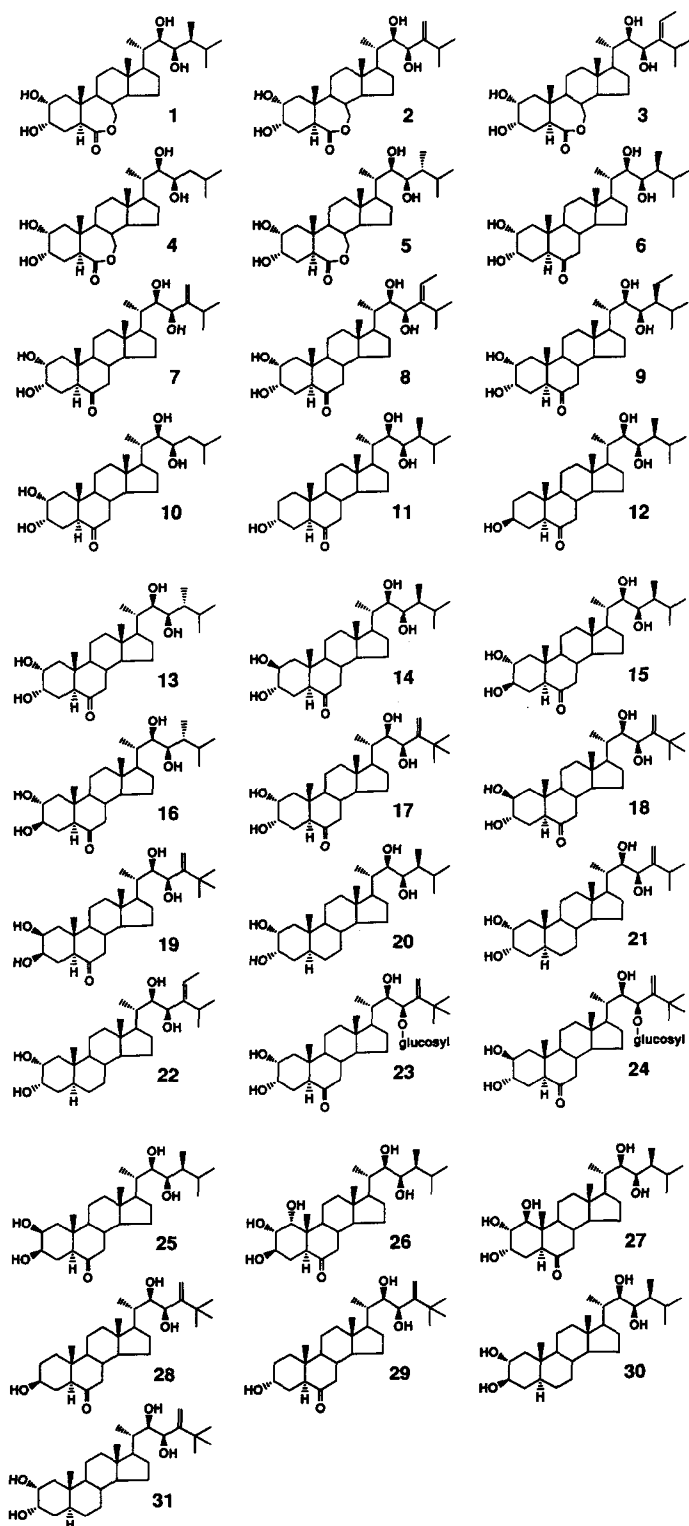

Fig. 1. Structures of natural brassinosteroids. **1** = Brassinolide; **2** = dolicholide; **3** = 28-homodolicholide; **4** = 28-norbrassinolide; **5** = 24-epibrassinolide; **6** = castasterone; **7** = dolichosterone; **8** = 28-homodolichosterone; **9** = 28-homocastasterone; **10** = 28-norcastasterone; **11** = typhasterol; **12** = teasterone; **13** = 24-epicastasterone; **14** = 2-epicastasterone; **15** = 3-epicastasterone; **16** = 3,24-diepicastasterone; **17** = 25-methyldolichosterone; **18** = 2-epi-25-methyldolichosterone; **19** = 2,3-diepi-25-methyldolichosterone; **20** = 6-deoxocastasterone; **21** = 6-deoxodolichosterone; **22** = 6-deoxo-28-homodolichosterone; **23** = 23-O-β-D-glucopyranosyl-25-methyldolichosterone; **24** = 23-O-β-D-glucopyranosyl-2-epi-25-methyldolichosterone; **25** = 2,3-diepicastasterone; **26** = 3-epi-1α-hydroxycastasterone; **27** = 1β-hydroxycastasterone; **28** = 3-epi-2-deoxy-25-methyldolichosterone; **29** = 2-deoxy-25-methyldolichosterone; **30** = 3-epi-6-deoxocastasterone; **31** = 6-deoxo-25-methyldolichosterone.

lablab L. [39–42], BL and CS from the immature seeds [43] and BL, 24-epiBL, CS and 28-norCS from the pollen [44] of broad bean (*Vicia faba* L.), BL, CS, 28-homoCS, 6-deoxo-28-homoCS and 6-deoxoCS from the immature seeds of *Piophocarpus tetragonolobus* [45], CS from the shoots of *Pisum sativum* L. cv. Holland [46], CS and 28-norCS from the insect galls and BL, CS, 28-norBL and 28-norCS from the leaves of *Distylium racemosum* Sieb. et Zucc. [47], BL, CS, TyS and TeS from the pollen of *Citrus unshiu* Marcov. [34], BL and CS from the pollen of orange (*Citrus sinensis* Osbeck) [48], BL and CS from the pollen of *Echium piantagineum* [29], BL and CS from the pollen of *Banksia grandis* [29] and BL and CS from the seeds of *Raphanus sativus* var. Remo [49]. The occurrence of more than 30 BRs in the immature seeds of *Phaseolus vulgaris* L. has been reported [50–56] and BRs whose structures have been established are BL, CS, DL, DS, 6-deoxoCS, 6-deoxoDS, 6-deoxo-28-homoDS, 2-epiCS, 3-epiCS, 2,3-diepiCS, 3,24-diepiCS, 1β-hydroxyCS, 3-epi-1α-hydroxy-CS, 3-epi-6-deoxoCS, 25-methylDS, 2-epi-25-methylDS, 2,3-diepi-25-methylDS, 2-deoxy-25-methylDS, 3-epi-2-deoxy-25-methylDS and 6-deoxo-25-methylDS. Among them, 25-methylDS is an interesting compound because it is the first 25-methylated BR and its biological activity is about ten times higher than that of its non-25-methylated counterpart, DS. Two conjugated BRs, 23-O-β-D-glucopyranosyl-25-methylDS and 23-O-β-D-glucopyranosyl-2-epi-25-methylDS have also been isolated from the *Phaseolus vulgaris* L. seeds.

Biological activity characteristic to BRs has also been detected by the bean second-internode assay and the rice-lamina inclination test, which are sensitive and specific bioassays for BRs, in the following dicot plant extracts: the pollens of *Sinapis arvensis* L. (=*Brassica kaber* L.) [3], *Sisymbrium irio L.* [3], *Rhus* sp. [3], *Leucopogon conostephioides* [29], thistle (*Carduus nutans* L.) [3], horse chestnut (*Aesculus hippocastanum* L.) [3], elm (*Ulmus* spp.) [3], pear (*Pyrus communis* L.) [3], hawthorn (*Crataegus sp.*) [3], *Robinia pseudo-acacia* L. [57], *Echium vulgare* L. [3] and rye (*Secale cereal* L.) [3].

2.2. *Brassinosteroids in higher plants (monocots)*

The presence of BRs in monocots has been demonstrated; TyS and TeS from the pollen of cat-tail (*Typha latifolia* L.) [34,58], CS and DS from rice shoots (*Oryza sativa*) [59], CS, TyS and TeS from the pollen (dent corn) [60] and CS from the immature seeds (sweet corn) [61] of corn (*Zea mays* L.), BL, CS and 28-homoCS from the immature seeds of wheat (*Triticam aestivum* L.) [62], BL, CS and TyS from the pollen of lily (*Lillium longiflorum* cv. Georgia) [34], BL, CS, TyS and TeS from the pollen of lily (*Lilium elegans* Thumb.) [63] and TyS from the pollen of tulip (*Tulipa gesneriana* L.) [34].

2.3. *Brassinosteroids in higher plants (gymnosperms)*

The occurrence of BRs in gymnosperms has been reported: TyS and CS from the pollen of Japanese black pine (*Pinus thunbergii* Parl.) [64], CS and TyS from the shoots of sitka spruce (*Picea sitchensis* Bong Carr) [65], and BL and CS from cambial scrapings of Scots pine (*Pinus silverstris*) [66] and DL and several unknown BRs from the pollen and anthers of a Japanese cedar (*Cryptomeria japonica* D. Don) [67].

2.4. *Brassinosteroids in lower plants*

Some lower plants (cryptogams) have been investigated for the presence of BRs. Green alga, *Hydrodictyon reticulatum* (L.) Lagerheim, has been reported to contain 24-epiCS and 28-homoCS [68], and in fern, *Equisetum arvense* L., specific biological activity for BRs has been obtained by the rice-lamina inclination test and some BRs have been tentatively identified by high-performance liquid chromatographic analysis [69]. The presence of BR-like bioactive substances in a highly purified fraction from *Chrollera pyrenoidosa*, which was obtained by the rice assay, has recently been reported [70]. Although these three studies strongly suggest that BRs also occur in lower plants, the wide occurrence of BRs in lower plants remains to be clarified.

2.5. *Distribution in plants*

From the above-described work on the identification of BRs in higher plants (phanerogams), it has been proved that BRs are contained in as many as 22 families and 39 genuses (27 of dicots, 8 of monocots and 4 of gymnosperms). In addition, the occurrence of BR-like bioactive substances has been suggested in 13 families and 17 genuses from the specific bioassays and chromatographic behaviour. Some studies on the occurrence of BRs in lower plants have also reported. Therefore, these screening data on BRs strongly suggest that BRs occur widely in the plant kingdom, as in the case of other known phytohormones, and that BRs play some physiological functions in plant growth and development.

Among the plants so far investigated, CS occurs most frequently, followed by BL. Therefore, these two BRs are believed to be important. In most plants, several kinds of BRs are found. In this respect, it is interesting that more than 30 BRs including unknown compounds (partial structures being determined by GC–MS analysis) occur in immature seeds of *Phaseolus vulgaris* [51]. Based on the data, it is likely that the number of natural BRs will increase in the future.

2.6. *Content of brassinosteroids in plants*

As far as the amount of BRs contained in plant tissues is concerned, pollens are the richest sources of BRs (*ca.* 10–100 μg/kg), immature seeds have also high contents of BRs (*ca.* 1–100 μg/kg) while shoots and leaves have lower levels (*ca.* 10–100 ng/kg) [3,4,7]. Although roots have not yet been examined, it has recently been suggested by the rice-lamina inclination test that a tuber of potato (*Solanum tuberosum*), a tap root of carrot (*Daucus carota* var. *sativus*) and a tuberous root of sweet potato (*Ipomoea batatas*) contain 6-keto-type (CS-type) BR-like bioactive substances [71]. Another interesting tissue is insect gall. The galls of *Castanea crenata* and *Distylium racemosum* have higher levels of BRs (several μg/kg) than the normal tissues [38,47]. Another tissue with a BR content is the crown gall (nopaline type) cells of *Catharanthus roseus* [30]. The crown gall cells have higher contents of BL and CS (*ca.* 30–40 μg/kg) than the normal cells.

It is known that in the same plant tissues, the young growing tissues are likely to have higher contents of BRs than old tissues. In *Dolichos lablab* immature seeds, the BR content is higher at a younger stage of the seed [4]. In the pollens of green tea (*Thea sinensis*) and lily (*Lillium longiflorum*), the bioactivity by rice-lamina inclination test increased as pollens grew mature and reached a maximum value just before anthesis; after the anthesis, the activity decreased [34]. These results suggest the possibility that BRs play an important role in regenerative growth regulation. Another interesting study was the comparative quantification of BRs with seeds of *Raphanus sativus* var. Remo [49]. It has been found by a GC–MS-SIM analysis that the ratio of BL to CS is significantly different in germinated seeds and in resting seeds, indicating an increase in BL formation during germination. The result suggests that BL could play an important role in germination.

2.7. *Biosynthesis of brassinosteroids*

A structural relationship between phytosterols and BRs could be suggested in which all naturally occurring BRs possess carbon skeletons identical with those of common phytosterols (*e.g.*, campesterol, 24-methylenecholesterol, isofucosterol, sitosterol and cholesterol). Therefore, BRs may be speculatively regarded as the enzymatic oxidation products of phytosterols with the corresponding carbon skeletons. Studies of the biosynthesis of BRs has just started. Yokota *et al.* [72] have proved that BL is biosynthesized from CS in crown gall cells of *Catharanthus roseus*. By employing the feeding experiment using deuterium-labelled BRs, it has recently been proved that the biosynthetic pathway TeS → TyS → CS → BL operates in both the crown gall cells and the normal cells of *Catharanthus roseus* [73,74]. However, a major part of the biosynthesis from phytosterols remains to be investigated.

3. BIOASSAYS USED TO GUIDE FRACTIONATION OF BRASSINOSTEROIDS

Since the isolation of BL, BL and its related compounds have been tested by a number of bioassays originally designed for known plant hormones. BRs have been shown to have a broad spectrum of biological activities [3,4,8]. Structure–activity relationships of BRs have also been clarified by bean second-internode bioassay, bean first-internode bioassay, raphanus test, tomato test and rice-lamina inclination test [75–78].

The development of bioassays for the isolation of bioactive compounds from natural sources has played an important role in recent natural product chemistry. For the isolation and purification of BRs from plant sources, highly sensitive and specific bioassays are essential, because of the very low concentration of BRs in plants. The bean-second internode assay was used to isolate BL from rape pollen [1], and the rice-lamina inclination test was used to isolate CS from chestnut insect galls [2]. After these studies, the latter bioassay has been widely employed in Japan to isolate successfully many BRs from a number of plant sources, because of its simplicity, high sensitivity and specificity for BRs.

The following three bioassays have been employed for the BR purification procedure to guide the fractionation: (i) bean second-internode bioassay, (ii) rice-lamina inclination test and (iii) wheat leaf unrolling test. Although the bean second-internode bioassay has historical significance (by employing the assay, BL was isolated from the rape pollen in 1979 [1]), the most frequently employed rice-lamina inclination test and a convenient wheat leaf unrolling test are described in this section.

3.1. Rice-lamina inclination test

It has been found by Wada *et al.* [22] that the rice-lamina inclination test is a highly sensitive and specific bioassay for BRs. The bioassay has been used to guide the fractionation during the purification procedure of the plant extracts, successfully resulting in the isolation and identification of a number of BRs with both lactone and ketone groups in the B-ring. 2-DeoxyBRs (TyS and TeS) have also been isolated by this bioassay [33,58,64]. The rice test is routinely employed in the purification steps mainly by Japanese scientists. In the rice test with cultivars Arborio J-1 and Nihonbare, a linear correlation was obtained between $5 \cdot 10^{-3}$ and $5 \cdot 10^{-5}$ μg/ml for BL and CS [79]. The induced angles leveled off at higher concentrations. BRs including 2-deoxy compounds showed very strong and specific activity at very low concentrations. Indole-3-acetic acid (IAA) was tested and was found to produce only a weak effect, five orders of magnitude less than BL. Cytokinins were inactive and actually counteracted the effect of BL. Abscisic acid (ABA) also counteracted the effect of BL. This assay is therefore highly specific for BRs and is also the most sensitive, concentrations as low as 0.05 ng/ml of BL being readily detected.

3.2. Wheat leaf unrolling test

The wheat leaf unrolling test has been found to be a convenient bioassay [80], in which BRs show strong activity. BL and CS dramatically stimulated wheat leaf unrolling, their activity being dose dependent. At 0.5 ng/ml both compounds markedly stimulated unrolling and, at 0.01 μg/ml or higher, BL produced complete unrolling of the leaf segments to about 3.6 mm. This assay is about one tenth as sensitive as the rice-lamina inclination test, but it is much simpler to carry out. GA_3 produced only slight unrolling at 0.1–10 μg/ml, as did the cytokinin 6-(3-methyl-2-butenyl)aminopurine. However, zeatin, 6-(4-hydroxy-3-methyl-2-butenyl)aminopurine, caused complete unrolling at 1 μg/ml and had a measurable effect at 0.001 μg/ml. ABA, IAA and indoleacetonitrile inhibited unrolling of leaf segments.

There have been some reports of the isolation of BRs from plant sources, employing the wheat leaf unrolling test as a bioassay: BL was isolated in pure form from the stems of *Solidago altissima* L. [28] and CS was identified by GC–MS analysis of the highly purified fraction of the immature seeds of corn [61]. Similarly, BL, CS and 28-homoCS were identified in the immature seeds of wheat (*Triticam aestivum* L.) [62]. These

reports suggest that isolation and purification of BRs from plants are successfully guided by the wheat leaf unrolling test. Because of its simpler manipulation than the rice-lamina inclination test, the wheat leaf unrolling test will replace the rice assay.

Highly sensitive and specific bioassays are essential for studies of the isolation of BRs from plant sources, because the contents of BRs in plants are extremely low. The above-described bioassays, in particular the rice-lamina inclination test, have greatly contributed to the study of BRs and will continue to do so in the future.

4. GC–MS-SIM OF BRASSINOSTEROIDS

It is well known that the content of BRs in plants is very low. In most instances the isolation of BRs in pure form is time consuming and tedious work. BRs are highly polar and involatile compounds. Therefore, in gas-phase analysis, conversion of BRs into volatile derivatives makes it easy to characterize BRs in a partially purified bioactive fraction by GC–MS or GC–MS-SIM. The GC–MS-SIM microanalytical method for BRs was developed by Takatsuto and co-workers [15,16,47].

4.1. GC–MS-SIM

BRs are converted into volatile derivatives based on the presence of two sets of vicinal diol groups in BRs. Considering its application to the analysis of natural BRs, the desired derivatives of BRs are bismethaneboronates (BMBs), because methaneboronic acid is a specific reagent for a vicinal diol function, allowing easy separation from other contaminants originating from plant sources. The derivative is suitable for gas-phase analysis and also for the analysis of fragment ions in electron impact (EI) mass spectra. The BMBs of BL, 28-homoBL, 28-norBL and their corresponding 6-keto analogues were well separated by GC using packed and capillary columns (capillary columns showed better resolution) and gave sharp peaks. A pair of 24-epimers of BL and that of CS were completely separated by GC using a capillary column.

MS fragmentation patterns of BMBs of typical BRs are summarized in Fig. 2. For BMBs of saturated BRs such as BL and CS, the fragment ions resulting from C_{23}–C_{24} fission, C_{20}–C_{22} fission and C_{17}–C_{20} fission are characteristic ions. The 6-ketone derivatives generally showed stronger molecular ions than the 7-oxalacetone derivatives. The ions at *m/z* 374 (lactones) and *m/z* 358 (ketones) were accompanied by hydrogen transfer. Thus, the fragment ions at *m/z* 457, 374, 345 and 177 (the assignment is shown in Fig. 2) are common for lactone-type BRs, whereas ions at *m/z* 441, 358 and 329 are common for ketone-type BRs. The ketone derivatives also gave another common fragment ion at *m/z* 287 resulting from C_{14}–C_{15} and C_{13}–C_{17} fissions. The ions corresponding to the cyclic boronate moiety of the side-chain part (C_{20}–C_{22} fission) are base peaks in both lactone- and ketone-type BRs. However, in the case of BMBs of unsaturated BRs such as DL and DS, which possess a C-24(28) double bond, different fragments ions were observed and most of them resulted from the cleavages of the cyclic boronate moiety of the side-chain part. These ions are *m/z* 427, 403, 385, 124 and 82 for the DL derivative and *m/z* 411, 387, 369, 124 and 82 for the DS derivative, as shown in Fig. 2. Another remarkable difference is that hydrogen transfer observed from C_{20}–C_{22} fission in the saturated series is not recorded in the case of the unsaturated series, but two-hydrogen transfer from the C_{17}–C_{20} fission is observed for the unsaturated series. The BMBs of 28-homoDL and 28-homoDS showed similar fragmentation patterns to those of DL and DS, respectively. However, salient differences in the relative intensities of the ions resulting from C_{24}–C_{25} fission are observed; *m/z* 497 (relative intensity 100%) for 28-homoDL BMB *vs.* *m/z* 483 (2.6%) for DL BMB and *m/z* 481 (100%) for 28-homoDS BMB *vs.* *m/z* 467 (5.3%) for DS BMB. Similar fragment patterns in EI-MS were reported for the BMBs of 6-deoxoCS and 6-deoxoDS [50]. In addition to the characteristic fragment ions derived from the side-chain cleavages as described above, the ions at *m/z* 288, 273 and 205 resulting from ring C and D fissions were observed. More detailed data on the fragmentation of BMBs of

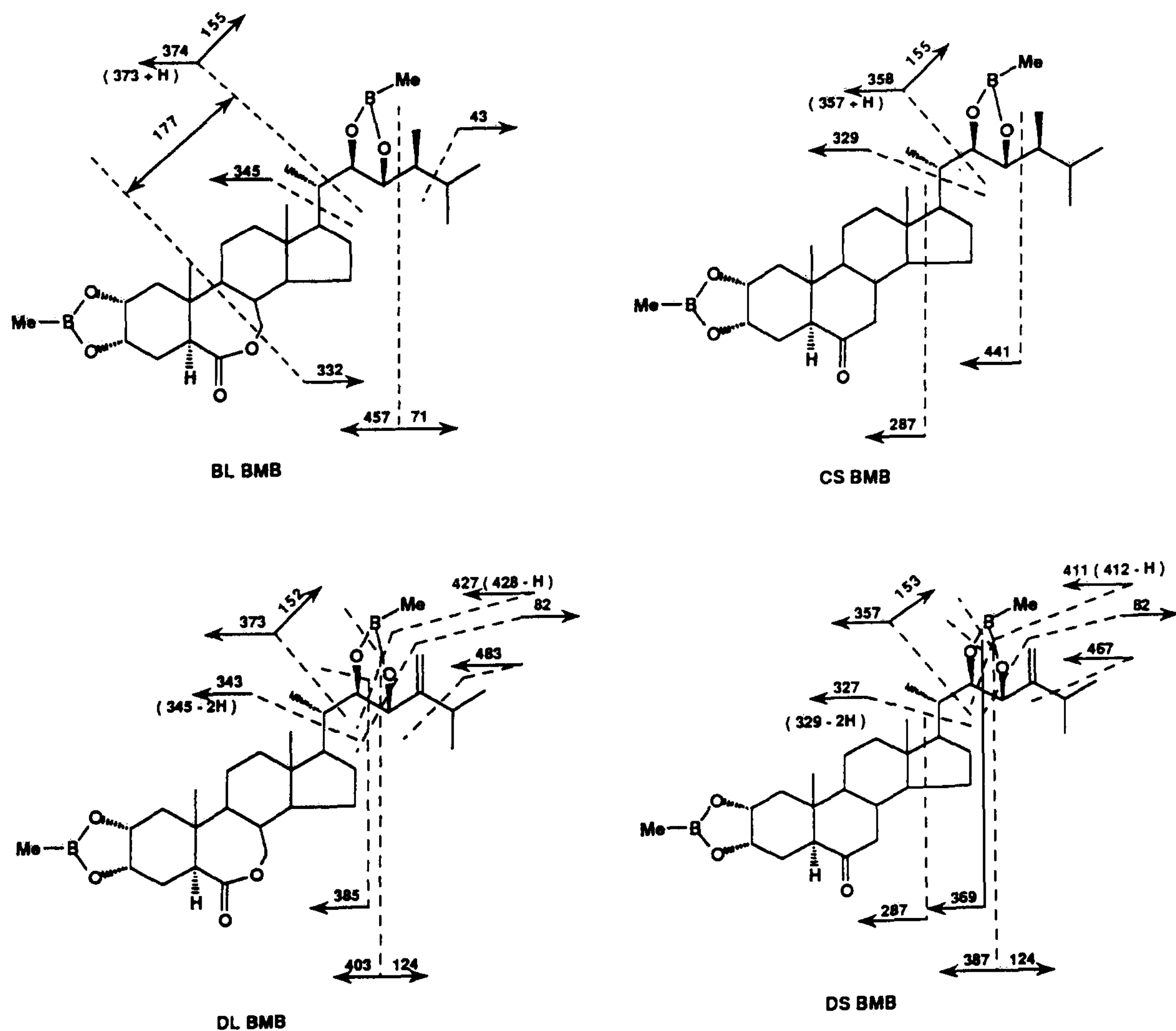

Fig. 2. Mass fragmentation of typical BR BMBs.

BRs in EI/MS were described in a previous review [19].

GC–MS analysis of 2-deoxyBRs (*e.g.*, TeS, TyS) has also been developed [16]. Since the 2-deoxyBRs have a vicinal diol function in the side-chain and an isolated hydroxyl group in the A-ring, the diol was first methaneboronated and then the remaining 3-hydroxyl group was trimethylsilylated. The resulting methaneboronate–trimethylsilyl (MB–TMS) derivative is also suitable for gas-phase analysis and also for analysis of fragment ions in EI-MS. The MB–TMS derivatives of TeS, TyS and their 28-homo and 28-nor analogues were well separated by GC using packed and capillary columns and gave sharp peaks. The fragmentation patterns in the EI mass spectra of MB–TMS derivatives of several representative 2-deoxyBRs have been summarized in a previous paper [16].

In the chemical ionization (CI) mass spectra of BMBs of BRs, the derivatives afforded ions of $M + 1$ as a base peak, $M + 1 - 60$, and also weak

ions resulting from C_{17}–C_{20} and C_{20}–C_{22} fissions. There is not much difference between the saturated and unsaturated BR derivatives.

4.2. Applications

In order to determine very small amounts of BRs in a bioactive fraction from plant sources, computerized SIM using a GC–CI-MS system is suitable and effective in detecting BRs, because the molecular ions of the BMBs of BRs are base peaks, making it easy to monitor these ions in GC–MS-SIM for screening of natural BRs. The presence of these molecular ions could be used to detect BRs. The GC–MS-SIM method was capable of detecting BRs at the picogram level. GC–CI-MS-SIM has been successfully applied to the identification of BRs with two sets of vicinal diols. Using this technique, BL (detected as its BMB, *m/z* 529) and CS (BMB, *m/z* 513) were found in the crude bioactive fractions obtained from the immature seeds and sheaths of Chinese cabbage, *Brassica campestris* var. *pekinensis* [23], the leaves of green tea, *Thea sinensis* [32], the insect galls of chestnut tree, *Castanea* spp. [37] and the leaves and insect galls of *Distylium racemosum* Sieb. et Zucc [47]. In the bioactive fraction from the aerial part of rice plant, *Oryza sativa*, CS and DS were detected as their BMBs [59]. From the historical points of view, it is of interest to note that BRs are contained in *Distylium racemosum* Sieb. et Zucc. GC–MS analysis coupled with the rice-lamina inclination test has made it clear that the *Distylium* factors obtained from the leaves of *Distylium racemosum* Sieb. et Zucc. in 1968 by Marumo and co-workers [81] are unequivocally BRs (BL, 28-norBL, CS and 28-norCS) [47]. A detailed story of this is described in a review [82]. Our microanalytical method was very effective in the structure determination of BRs in the immature seeds of *Phaseolus vulgaris* cv. Kentucky Wonder [50]. As the amounts of isolated BRs were very small and they were mixed with structurally closely related compounds, their structures were determined, only by GC–MS as their BMB derivatives, to be 6-deoxoCS, 6-deoxoDS, CS and DS. In screening for new BRs, TeS and TyS have been identified for the first time as MB–TMS derivatives in the leaves of green tea (*Thea sinensis*) [33].

GC–MS has been applied successfully to identify traces of natural BRs from plant sources, as described above. When a sufficient amount of purified BRs was obtained from plants, the full mass spectrum is taken by EI-MS. A recent example is the identification of three BRs in the pollen of sunflower (*Helianthus annuus* L.) [27]. The methanol extract of the pollen was subjected to solvent partitioning to obtain a chloroform-soluble neutral fraction. This fraction was successively purified by silica gel column chromatography, normal-phase preparative thin-layer chromatography (p-TLC) and Sephadex LH-20 column chromatography to give a highly purified bioactive fraction. The fraction was derivatized with methaneboronic acid and the resulting BMBs were analysed by GC–EI-MS, using a capillary column. A total ion chromatogram is presented in Fig. 3. The retention times of 12.13, 13.20 and 15.06 min and the full mass spectra of the peaks were identical with those of the BMBs of authentic 28-norCS, CS and BL, respectively. The amounts of these BRs are 65, 21 and 106 ng/g, respectively. Hence the bioactive BRs contained in the sunflower pollen were rigorously identified.

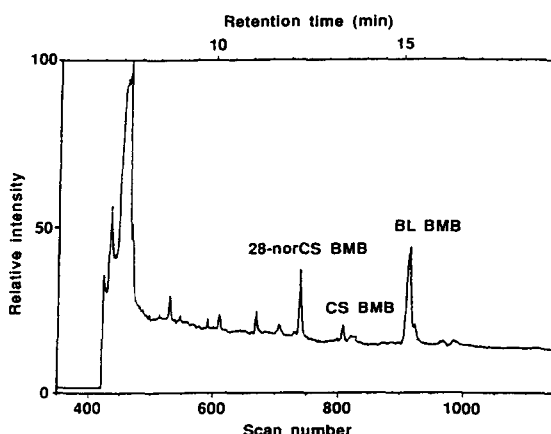

Fig. 3. Total ion chromatogram obtained in GC–MS of the methaneboronated highly purified fraction obtained from sunflower pollen.

Rigorous identification was also achieved by GC–high-resolution (HR)-MS-SIM. This technique, capable of detecting BRs at the picogram level, was applied to the identification of BRs in the pollens of broad bean (*Vicia faba* L.) [44] and buckwheat (*Fagopyrum esculentum* Moench) [31]. A recent example is the identification of BRs in the buckwheat pollen [31]. As the content of BRs in the buckwheat pollen was found to be very small by the bioassay and the BRs were extremely contaminated with unknown natural products, several chromatographic methods, including silica gel adsorption chromatography, normal-phase p-TLC, activated charcoal chromatography and reversed-phase p-TLC, were employed. The bioactive fraction thus obtained was derivatized and analysed by the GC–HR-MS-SIM method using a capillary column in the EI-MS mode. Under our GC–MS conditions, authentic BMBs of CS and BL were eluted at 13.56 and 15.16 min, respectively. The SIM results for the derivatized samples obtained from the active fraction are presented in Fig. 4. Monitoring of the molecular ion of CS BMB at *m/z* 512.3842 and that of BL BMB at *m/z* 528.3791 exhibited sharp peaks with the same retention times as those of authentic BMBs, thereby establishing rigorously the presence of BL (5.0 ng/g) and CS (7.1 ng/g) in the buckwheat pollen.

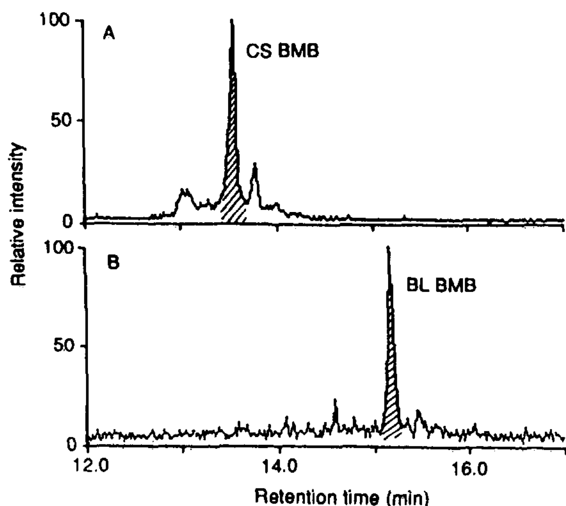

Fig. 4. GC–HR-MS-SIM of the BMBs of CS and BL obtained from buckwheat pollen. (A) Detection of CS BMB by monitoring the molecular ion at *m/z* 512.3842; (B) detection of BL BMB by monitoring the molecular ion at *m/z* 528.3791.

Deuterium-labelled BRs, [26,28-$^2H_6$]BL, [26,28-$^2H_6$]CS, [26,28-$^2H_6$]TyS and [26,28-$^2H_6$]TeS, have been synthesized for use as internal standards [83]. Quantitative analysis of natural BRs by GC–MS employing the deuterated BRs has been carried out [46]. In order to understand the growth retardation mechanism of (*S*)-uniconazole, the shoots of *Pisum sativum* L. treated with (*S*)- and (*R*)-uniconazoles were analysed in terms of the levels of the endogenous GAs, BRs and phytosterols. Only referring to BRs, it is of interest to examine whether uniconazoles modify the biosynthesis of BRs. BRs contained in the shoots of *P. sativum* L. were extracted, purified and analysed by GC–MS. GC–MS analysis of the active fraction led to the identification of CS: *m/z* (relative intensity) 512 (M^+, 54%), 155 (100%). GC–MS-SIM determination using an internal standard (d_6-CS) revealed that the content of CS in the control plants was 0.9 ng/g fresh mass and, after treatment with (*S*)- and (*R*)-uniconazoles, reduced to 54% and 34% of the controls, respectively. The result suggests that the altered metabolism of BRs is probably involved in the action mechanism of (*S*)-uniconazole.

Deuterium-labelled BRs have also been used in the biosynthetic study of natural BRs [73,74,84]. When [26,28-$^2H_6$]TyS, which is a hypothetical precursor of CS, was fed to cultured crown gall cells of *Catharanthus roseus*, conversion of [26,28-$^2H_6$]TyS into [26,28-$^2H_6$]CS and [26,28-$^2H_6$]BL was shown by GC–MS analysis of the metabolite [73,84]. In a similar feeding experiment employing [26,28-$^2H_6$]TeS and [26,28-$^2H_6$]TyS, it has recently been proved that the biosynthetic pathway TeS→TyS→CS→BL operates in both the crown gall cells and the normal cells of *Catharanthus roseus* [74]. Thus, our GC–MS method is useful and effective for the biosynthetic study of natural BRs.

An alternative highly sensitive GC–MS technique involves tandem MS (MS–MS). In this technique, the first mass filter is used to select the ion of interest from all the other ions

produced from the matrix. This ion is usually the molecular ion in the EI mode or the protonated molecule (MH^+) in the CI mode. These selected ions then undergo collisionally activated dissociation to produce daughter ions, which are then separated by a second mass filter and analysed. GC–MS–MS has been applied to the identification of BRs in the pollen of European alder, *Alnus glutinosa* (L.) Gaertn [26]. The crude bioactive fraction obtained from the pollen was derivatized with methaneboronic acid and the resulting derivatives were analysed by GC–MS–MS in the CI mode, because the protonated molecular ions of BR BMBs are produced as base peaks in the CI mode. BL and CS have been identified in the pollen as their BMBs.

The GC–MS-SIM microanalytical method for BRs has most frequently been employed in analytical studies of trace levels of BRs in plants and it has greatly contributed to studies of the identification of many natural BRs and also their distribution in the plant kingdom.

5. CONCLUSIONS

Studies on BRs have greatly widened our knowledge of the chemistry and plant physiology of BRs in the past 10 years and more. With respect to the microanalysis of BRs, highly sensitive and specific bioassays and microanalytical methods including GC–MS-SIM, HPLC and radioimmunoassay have been developed. Combination of the bioassays and the GC–MS-SIM microanalysis has led successfully to the identification of more than 30 naturally occurring BRs. So far, in as many as 22 families and 39 genuses BRs have been identified and, in addition, in 13 families and 17 genuses, the occurrence of BR-like bioactive substances has been suggested. These screening data strongly indicate a ubiquitous distribution of BRs in the plant kingdom. In these studies the rice-lamina inclination test and our GC–MS method have been effectively employed. The microanalytical methods in combination with the bioassays will contribute to more detailed studies of the physiological mechanism of BRs, because they are essential for the identification and determination of endogenous BRs which are involved in plant growth and development.

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