Short Communications

Occurrence of three new brassinosteroids: brassinone, (24S)-24-ethylbrassinone and 28-norbrassinolide, in higher plants

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Summary. Three new brassinolide-like steroids, a new class of plant growth promoter, have been isolated from higher plants. These bioactive steroids were identified as (22R,23R)-2α,3α,22,23-tetrahydroxy-B-homo-7-oxa-5α-cholestan-6-one (**III**, norbrassinolide), its 6-ketone analogue named brassinone (**IV**) and the 24-ethyl analogue of (**IV**) named (24S)-24-ethylbrassinone (**VI**).

Recently, new plant growth-promoting steroids, brassinolide (**I**) and its 6-ketone analogue, castasterone (**II**), have been isolated from the pollen of rape (*Brassica nupus* L.)[1] and from chestnut insect galls (*Castanea* spp.)[2], respectively. These bioactive steroids, termed brassinosteroids[3], constitute a new class of plant growth regulator. However, the possibility of a wide distribution of brassinosteroids in the plant kingdom, and their physiological functions in plants, have not yet been investigated in detail. A probable biosynthetic precursor of brassinolide (**I**) is 24-methylenecholesterol or campesterol. Since sitosterol and cholesterol are widely distributed in the plant kingdom, the presence of 28-norbrassinolide (**III**) and 28-homobrassinolide (**V**) or their corresponding 6-ketone analogues (**IV**) and (**VI**) might be expected. Therefore, for direct comparison with natural steroids, (**III**), (**V**), (**IV**) and (**VI**) were synthesized[4,5]. We focused our attention on the isolation and identification of these new brassinosteroids in higher plants. Using computerized mass fragmentography, 3 new brassinosteroids,

brassinone, (22R,23R)-2α,3α,22,23-tetrahydroxy-5α-cholestan-6-one (**IV**), (24S)-24-ethyl-brassinone, (22R,23R,24S)-2α,3α,22,23-tetrahydroxy-24-ethyl-5α-cholestan-6-one (**VI**) and 28-norbrassinolide, (22R,23R)-2α,3α,22,23-tetrahydroxy-B-homo-7-oxa-5α-cholestan-6-one (**III**) were identified in Chinese cabbage, in green tea and in chestnut insect galls. We now report the identification and biological activity of these new brassinosteroids.

More than 10 years ago we reported the isolation of novel plant growth regulators[6] which possess a specific and marked activity against the lamina inclination in the rice-lamina joint test; these were tentatively named the *Distylium* factors A₁, A₂ and B. *Distylium* factors were found to be distributed widely in a variety of higher plants, but the very limited quantity in a plant precluded their structure elucidation at that time. Recently, brassinolide and its synthetic analogue, homobrassinolide, were demonstrated to promote dramatically lamina inclination at very low concentrations[7]. These facts indicate the wide distribution
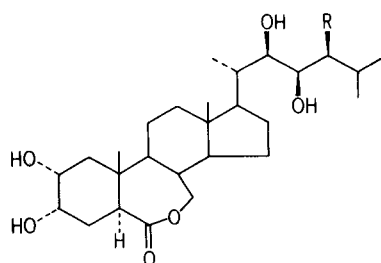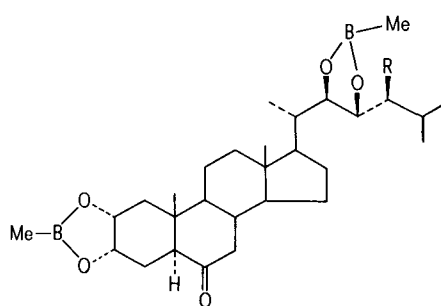

I R = CH₃
III R = H
V R = CH₂CH₃
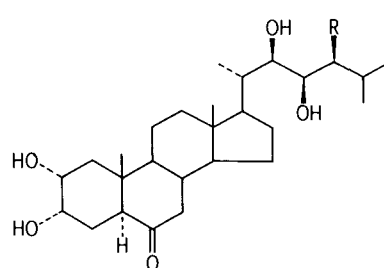
II R = CH₃
IV R = H
VI R = CH₂CH₃
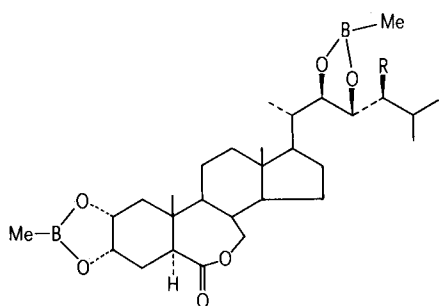
VII R = H
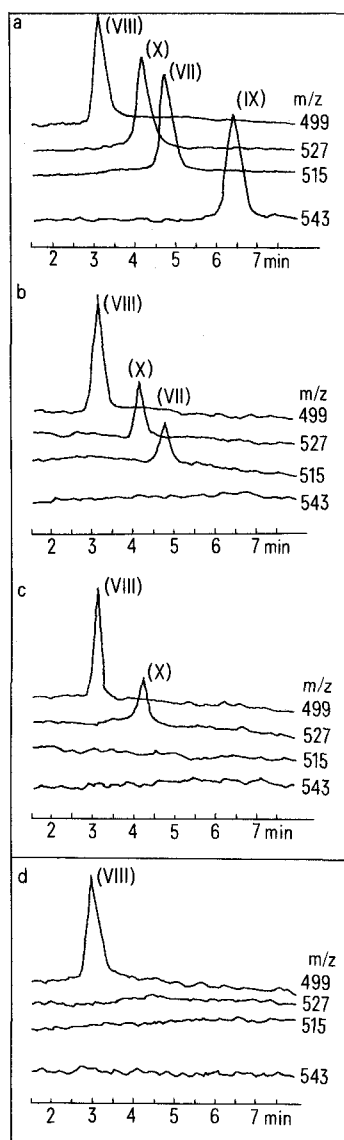IX R = CH₂CH₃

VIII R = H
X R = CH₂CH₃

of brassinolide-like substances in the plant kingdom. The lamina joint test, a highly specific and sensitive assay for brassinosteroids, is useful for predicting the presence of brassinosteroid-like substances in plants and effective for the extremely small amount of brassinosteroids in plants during purification. Furthermore, the microanalytical technique for brassinolide and its synthetic analogues as their bismethaneboronate derivatives using computerized mass fragmentography is most useful for detection of brassinosteroids[8]. Using the bioassay test for monitoring every step of the isolation procedures and the microanalytical method, brassinolide (I) and its 6-ketone analogue (II) have been recently identified in several kinds of plants. In our pre-

Identification of brassinone (IV), (24S)-24-ethylbrassinone (VI) and 28-norbrassinolide (III) in higher plants by computerized mass fragmentography. a Standard mixture; b in Chinese cabbage; c in green tea; d in chestnut insect gall. A Shimadzu GC-MS 6020 gas chromatograph-mass spectrometer with chemical ionization sources and a SCAP-1123 were used. A column packed with 2% OV-17 on Chromosorb W (80–100 mesh) (0.5 m×2 mm ID) was used at 290 °C; the reagent gas was isobutane; the carrier gas (He) flow rate was 30 ml/min; electron energy, 150 eV; box current, 150 µA; acceleration high voltage, 3.5 kV; ion source temperature, 250 °C.

vious papers, the detailed isolation procedure and the identification of (I) and (II) were reported for 3 kinds of plant, the immature seeds and sheaths (320 kg) of the Chinese cabbage (Brassica campestris var. pekinensis)[9], leaves (65 kg) of green tea (Thea sinensis)[10] and insect galls (3.5 kg) of the chestnut tree (Castanea spp.)[11].

Computerized mass fragmentography was applied to the active fractions obtained in our previous papers[9-11] in order to detect new brassinosteroids. For detection of these new brassinosteroids, the strong molecular ions (M+1), m/z 499, 527, 515 and 543, corresponding to the bismethaneboronates of brassinone (IV), (24S)-24-ethylbrassinone (VI), 28-norbrassinolide (III) and 28-homobrassinolide (V), respectively, were used for selected ion monitoring in GC-CIMS[12] with 2% OV-17 column packing. Selected ion monitoring (m/z 499, 527, 515 and 543, respectively) of the boronate of each active fraction clearly indicates the presence of (VII), (VIII) and (X) in the Chinese cabbage (fig. b), (VIII) and (X) in green tea (fig. c), and (VIII) in the chestnut insect galls (fig. d). Similar results were obtained with OV-101 column packing.

The amount of brassinone (IV), (24S)-24-ethylbrassinone (VI) and 28-norbrassinolide (III) in Chinese cabbage (320 kg) was 250 ng, 40 ng and 400 ng, respectively, compared with 3 µg of brassinolide (I). On the other hand, the amount of (IV) and (VI) in green tea (65 kg) was 130 ng and 30 ng, respectively. In this plant castasterone (II) (7.2 µg) is a major brassinosteroid. In the chestnut insect galls (3.5 kg) the amount of (IV) (40 ng) was approximately equal to that of (II). Thus, in the present investigation, brassinone (IV), a possible biosynthetic precursor to (III), (24S)-24-ethylbrassinone (VI) and 28-norbrassinolide (III) were identified for the first time in these plants as naturally-occurring brassinosteroids, suggesting the wide distribution of brassinosteroids in the plant kingdom.

The promoting effect of new brassinosteroids on rice-lamina inclination was measured. The lamina joint sections, excised from the 2nd leaves of etiolated seedlings (Oryza sativa L. cv. Arborio J-1) and cultivated at 28 °C in darkness for 6 days, were used in all the experiments. After having been floated in distilled water for 24 h at 28 °C in the dark, 10 were transferred to 1 ml of 2.5 mM potassium maleate solution containing a finite amount of a test sample and the magnitude of the induced angle between lamina and sheath was measured after incubation for 48 h under the same conditions of temperature and lighting. In the presence of 0.001 µg/ml of brassinolide or its 6-ketone analogue[13], laminae were bent sharply at the lamina joint to the abaxial side at an angle of 140° or more, whereas indolyl-3-acetic acid showed only weak activity, about 105° at 50 µg/ml. The new brassinosteroids (III, IV and VI), although synthetic compounds, were also used for bioassay, and also promoted lamina inclination remarkably at the very low concentration of 0.001 µg/ml. Examination of the comparative activity of these new brassinosteroids at the concentration which gives rise to an angle between lamina and sheath of about 140°, showed that (VI) had the strongest activity, nearly equal to that of (II) but several times lower than that of (I). The activity of (IV) was equivalent to (III) or somewhat weak, being about one tenth that of (VI). The structure-activity relationship of these naturally-occurring brassinosteroids and related synthetic analogues will be reported in a forthcoming paper[14].

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Synthesis and biological activities of [β-Malyl¹]- and [β-Malyl¹, Leu⁸]-angiotensin II analogues[1]

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Summary. The synthesis of [β-Malyl¹]- and [β-Malyl¹, Leu⁸]-angiotensin II using a solid phase procedure is reported. The replacement of the N-terminal amino group of aspartic acid by a hydroxyl group gives analogues with lower affinity than [Asn¹]- and [Asn¹, Leu⁸]-AII. However, the isoster [β-Malyl¹]-AII shows higher potency than [Asn¹]-AII and this may be due to metabolic or enzymatic resistance.

We wish to describe the syntheses of the agonist and the [Leu⁸]-antagonist isosters of angiotensin II, in which the N-terminal primary amino group was replaced by a hydroxyl group. These isosters, [β-Malyl¹]- and [β-Malyl¹, Leu⁸]-AII contain the α-hydroxy-analogue of L-aspartic acid at position 1. The L-malic acid was bound via its β-carboxyl group instead of its α because [β-Asp¹]-AII is well known to exhibit higher potency than the natural hormone[4,5]. The pharmacological activities of these analogues were investigated to provide further information about the importance of the N-terminus amino group and the basicity of its nitrogen for maximum agonistic and antagonistic propterties.

Methods. Firstly, the synthesis of α-benzyl-L-malate was achieved by 2 independent routes, and its structure was confirmed by ¹H NMR and other physical techniques. L-Malic acid, when treated with benzyl bromide in the presence of triethylamine, gave dibenzyl-L-malate (I). The homogeneous oily product was tritylated in the presence of pyridine and the dibenzyl-O-trityl-L-malate (II) obtained, owing to the well known steric hindrance of the trityl group[6], was selectively saponified with 2N LiOH (2:1 molar ratio). The resulting α-benzyl-O-trityl-L-malate (III) was isolated as the dicyclohexylammonium salt (IV) in crystalline form. On the other hand, the disilver salt of L-malic acid was treated with thionyl chloride to give the malic anhydride[7], which, without purification, was further treated with benzyl alcohol. The α-benzyl-L-malate (V) thus obtained was isolated as the dicyclohexylammonium salt (VI) and its structure was confirmed as follows. Compound VI, after being treated with 5% citric acid, washing with water and drying, was tritylated in the presence of pyridine. The obtained α-benzyl-O-trityl-L-malate and the corresponding dicyclohexylammonium salt, were proved to be identical with those obtained by the former route (table 1). The ¹H NMR-data of α-benzyl-O-trityl-L-malate (obtained by both routes) showed an A₂ singlet at δ=4.75 ppm due to the α CH₂ benzylic protons and no singlet at

lower field. In contrast dibenzyl-O-trityl-L-malate indicates an A₂ singlet at δ=5.0 ppm for the β CH₂ benzylic protons. *[β-Malyl¹]-angiotensin II.* The protected octapeptide was prepared by the solid phase method of Merrifield[8] on a 1% crosslinked chloromethyl polystyrene containing 0.75 meqCl/g (Bio-Beads S-X1; 200–400 mesh). A substitution of 0.67 mmoles of Boc-Phe per g of resin was obtained[9]. The remainder of the synthesis was performed with 1.49 g of substituted resin. The Boc group was used for the protection of the α-amino functions with the side-chain protecting groups indicated in parentheses. Pro, His(Tos), Ile, Tyr(O-2-Br-Z), Val, Arg(NO₂). Couplings were performed with 2.5 equiv. of t-Boc-protected amino acids and DCC (0.25 M in CH₂Cl₂) for 120 min. The completion of the coupling reaction was confirmed by the Kaiser ninhydrin test[10]. For the incorporation of α-benzyl-malate, the corresponding dicyclohexylammonium salt was neutralized, and the oily product was coupled to the heptapeptide polymer using a 3-fold molar excess of DCC and HOBt until a negative ninhydrin test was obtained (5 h) Boc-protecting groups were removed at each stage by treatment with 50% CF₃COOH in CH₂Cl₂ containing 2% anisole (20 ml, 20 min). Neutralization was effected with 10% triethylamine in CH₂Cl₂ (20 ml, 5 min).

Cleavage of the peptide from the resin and removal of the protecting groups was accomplished with HF containing 10% by volume of anisole for 1 h at 0 °C[11]. After removal of HF and drying under vacuum the resin was washed several times with ether and then was extracted with acetic acid (2 M). Lyophilization of the latter extract yielded the crude peptide in a solid form. A portion of this product (~ 300 mg) was purified by gel filtration on sephadex G-15 column (2.6 × 100 cm) using 1 M acetic acid as the eluent, followed by partition chromatography on sephadex G-25 M on 2 successive columns using the solvent systems n-butanol-acetic acid-pyridine-water (30:6:20:24) and n-butanol-acetic acid-water (4:1:5). Overall yield was 30% based on the quantity of Boc-Phe originally attached to the