Synthesis and Biological Activity of Brassinolide and Its 22β,23β-Isomer: Novel Plant Growth-Promoting Steroids

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Received 8-25-81

ABSTRACT

Brassinolide (2α,3α,22α,23α-tetrahydroxy-24α-methyl-B-homo-7-oxa-5α-cholestan-6-one), a novel plant growth-promoting steroid isolated from rape pollen, and its hitherto unknown 22β,23β-isomer were synthesized from a C-24 epimeric 60:40 mixture of 22-dehydrocampesterol (24α-methyl) and brassicasterol (24β-methyl) from oysters. The method of synthesis favored the formation of the 22β,23β-isomer by better than 4:1. Comparative plant growth-promoting capabilities of brassinolide, both natural and synthetic, and its three side chain cis-glycolic isomers in the bean second internode bioassay showed that the natural and synthetic brassinolides were equally active and caused splitting of the internode at the 0.1 μg level. The least active was the 22β,23β-isomer of brassinolide. The isomers with the 22α, 23α and 24β, and the 22β, 23β and 24β configurations were highly active and were required at about 10 times the concentration of brassinolide to cause the same physiological response. In the bean first internode bioassay, an auxin-induced growth test system which employs isolated bean plant segments, the isomer with 22β, 23β and 24β configuration caused a greater response than brassinolide. Two of the four tetrahydroxy ketones obtained in the synthesis of the isomers were also active in both assays.

INTRODUCTION

Brassinolide (I, 2α,3α,22α,23α-tetrahydroxy-24α-methyl-B-homo-7-oxa-5α-cholestan-6-one), a novel plant growth promoting steroidal lactone isolated from the pollen [1-4] of rape (Brassica napus L.), is perhaps the most important discovery to the plant physiologists and biochemists since the discovery of gibberellic acid. Subsequent to the identification of brassinolide [5], two side chain 22,23-cis-glycolic C-24 methyl isomers of I were synthesized [6]. These brassinosteroids, 2α,3α,22α,23α-tetrahydroxy-24β-methyl-B-homo-7-oxa-5α-cholestan-6-one (II) and 2α,3α,22β,23β-tetrahydroxy-24β-methyl-B-homo-7-oxa-5α-

cholestan-6-one(III), like brassinolide (I), showed brassin activity in a bean bioassay at hormonal levels (10^{-6} - 10^{-9} M). Brassinolide, though not readily attainable by synthesis, has recently been synthesized via C_{22} steroids at two different laboratories [7,8]. We now report the synthesis of brassinolide and its hitherto unknown 22β,23β-isomer from 22-dehydrocampesterol, and the comparative plant growth-promoting effects of I and its three side chain 22,23-cis-glycolic C-24 isomers in two different bioassay systems.

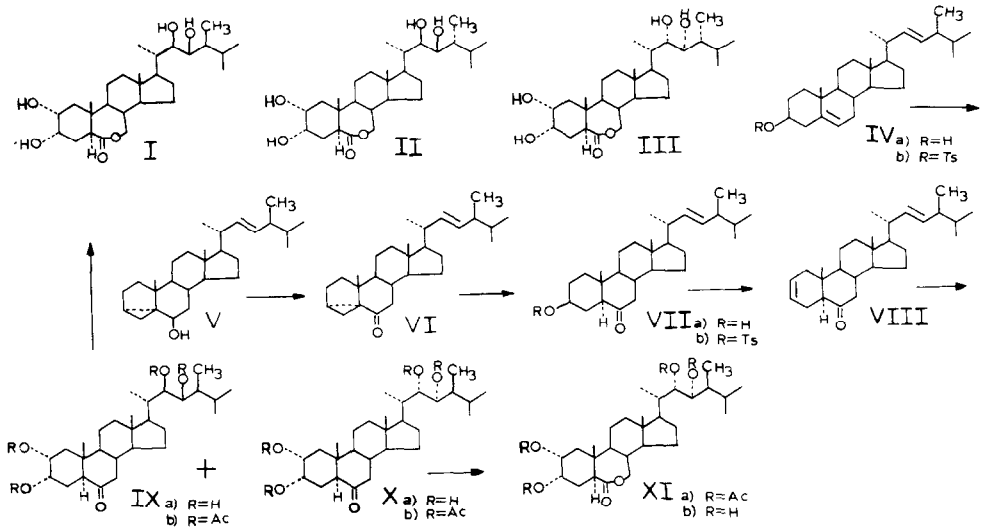

Figure 1. Synthetic scheme for brassinolide (I) and its 22β,23β-isomer (XIb)

EXPERIMENTAL

Instrumentation: Melting points were determined on the Kofler block [9] and are uncorrected. Rotations were determined at 25° of about 1% solutions of compounds in chloroform. Infrared spectra were obtained with a Perkin-Elmer Model 221 prism grating spectrophotometer. EI-MS were recorded on an LKB Model 9000 GC-MS (LKB-Produkter AB, Stockholm) equipped with a Varian Spectro System 100 MS data system. The samples were introduced directly into the ion chamber and the ionization energy was 70 ev. CI-MS were obtained with a Finnigan 4000 mass spectrometer. Ammonia chemical ionization was used to produce ions, and samples were introduced by way of the solid probe. The source temperature was 160°.

Isolation of Sterols: The oysters, C. viriginica (150 liters), were obtaned from a commercial source, and lipids were isolated and separated to yield sterol fractions as previously reported [10].

Bean Second Internode Bioassay: This test system [11] was used to evaluate the growth-promoting activity of natural and synthetic brassinolide (I) and its three isomers (II, III and XIa). Plants were grown in growth rooms (temperature 25-27°, light 7.5 Klux for 12 hr.), and groups of three 6-day-old bean (Phaseolus vulgaris L., Pinto var.) seedlings with second internodes 2 mm long were treated with the different concentrations of the test compounds in 250 µg lanolin. The control plants were treated with lanolin alone. Measurements were taken after 4 days, and those of the treated and control plants were compared, and the percentage of increase in internode elongation due to treatment was used as a measure of the activity of these compounds. Since the test plants showed not only elongation but also curvature, swelling and splitting of the internodes, depending on dosage of brassinosteroid, these growth responses were rated on a 0 to 5 scale.

Bean First Internode Curvature Bioassay: The growing of etiolated bean (P. vulgaris L. cv. Bush Burpee Stringless) seedlings and collecting of the excised first internodes (4.0-5.0 cm long) from the 6-day-old bean seedlings were conducted according to a published procedure [13]. One hundred nanograms of test compounds, solubilized in ethanol, was applied to the base of excised bean internode sections 1 hr prior to the application of 0.1 nM of auxin (indole-3-acetic acid, IAA). The control plant was treated with auxin alone. The growth of the sections was measured as previously reported [12], 1, 2, and 3 hr after the application of auxin.

Materials: Silicic acid (Unisil 100 to 200 mesh) was obtained from Clarkson Chemical Co., (Williamsport, PA). Neutral alumina was obtained from ICN Nutritional Biochemicals (Cleveland, OH), and the required amount of water was added to make activity Grade II or III alumina. High performance thin-layer chromatography (HP-TLC plates) 10 x 10 cm Silica Gel 60F-254 (E. Merck, Darmstadt, Germany) were used for Nano-TLC and Analab Anasil H scored plates for regular TLC analyses.

Isolation and/or Synthesis of Steroids IVa-VI: The 4,4-desmethyl sterols from oyster, Crassostrea virginica, were chromatographed as acetates on 20% w/w silver nitrate-silicic acid, and the C-24 epimeric mixture of (22E)-24-methylcholesta-5,22-dien-3β-yl acetate [m.p. 149-150°; IR(CS_2) ν_{max} 1735 (CH_3CO), 968 cm^{-1} (E Δ^{22}-bond)] was isolated as previously reported [10]. Conventional GLC analyses on 1% OV-17 or 0.75% SE-30 showed only one peak and it corresponded to a retention time identical to that of the GLC peak for brassicasterol acetate (24β- methylcholesta-5,22-dien-3β-yl acetate). The acetate (1.25 g) was saponified under reflux conditions for 3 hr with a mixture of 4% potassium carbonate in 100 ml of 70% aqueous methanol that contained 25 ml of benzene. The reaction mixture was distilled until no benzene appeared in the distillate, and then was diluted with water and ice. The precipitated sterol (IVa) was collected and dried: weight 1.1 g; m.p. 151-154°; MS $C_{28}H_{46}O$ (m/z, rel. intensity, %), 398 (M^+, 40) 383

(M - CH_3, 6), 380(M - H_2O, 21) 327(M - C_5H_{11}), 300 (M - C_7H_{14}, 29), 271(27), 255(56), 213(19), 133(35), 81(58), 69(100). Both TLC and conventional GLC analyses of the sterol indicated a single component. Analysis of a sample of the trimethylsilyl ether of the sterol by GLC on a glass capillary column coated with SP-2340 [13], however, indicated as expected, a 60:40 mixture of 22-dehydrocampesterol (IVa, 24α-methyl-cholesta-5,22-dien-3β-ol) and brassicasterol (24β- methylcholesta-5,22-dien-3β-ol), respectively. Since there are presently no known physical methods of separating C-24 epimeric sterols other than by capillary GLC on SP-2340 [13], the synthesis of brassinolide was conducted with this mixture, and the four side chain cis-glycolic isomers obtained from the oxidation of VIII were separated at this stage. (The structures in Figure 1 show only the 24α-methyl orientation). Tosylation of IVa (Figure 1) gave 1.3 g of IVb which upon solvolysis in 325 ml of acetone, 125 ml of water and 1.5 g of potassium bicarbonate according to reported method [14] gave 989 mg of the crude i-sterol (V), m.p. 85-87°. Oxidation of V with chromic acid in pyridine, followed by work-up in the usual manner [15,16] gave 923 mg of crude VI. A 60-mg sample was transferred to a chromatographic column of 5.0 g hexane-washed neutral activity Grade II alumina, and was eluted with 25-ml volumes of hexane and 2, 4, 6, and 8% ether in hexane. The eluate was collected and monitored by TLC analysis. The fractions eluted with hexane containing 4, 6, and 8% ether yielded a product that, when recrystallized from dilute acetone, gave 35 mg of VI as rosettes, m.p. 103-106°, $[\alpha]^{25}D$ + 25°; IR(CS_2) ν_{max}, 1685 cm^{-1} (6-ketone); MS $C_{28}H_{44}O$ (m/z, rel. intensity, %), 396(M^+, 73), 381(M - CH_3, 12), 353(M - CH_3 - CO, 24), 298(M - C_7H_{14}, 44), 271(65), 257(13), 245(23), 243(19), 161(28), 135(51), 55(100).

3β-Hydroxy-24-Methyl-5α-cholest-22-en-6-one (VIIa): To 900 mg of crude VI in 20 ml of glacial acetic acid was added 5 ml of 5 N sulfuric acid solution and the mixture was refluxed for 2 hr [16]. The solution was cooled and diluted with crushed ice and water, and the precipitate was collected and dried. The precipitate with 4 g of potassium carbonate in 100 ml of methanol and 20 ml of water was heated under reflux conditions for 3 hr. Methanol was distilled off until the compound began to precipitate from solution. The solution was cooled, and diluted with water and ice, and the precipitate was collected and dried under vacuum at 65°. The precipitate was chromatographed on a column (2.5 x 8 cm) containing 30 g of benzene-hexane (1:1)-washed activity Grade II neutral alumina. The column was eluted with 100-ml portions (50-ml fraction collected once the compound started to emerge from the column) of benzene/hexane (1:1); benzene; ether/benzene (1:1); ether; and ether containing 1, 2, and 3% methanol. The fractions were monitored by TLC, and the fractions eluted with 1 to 3% methanol in ether were combined to give 520 mg of VIIa. A sample recrystallized from hexane-acetone gave needles: m.p. 161-163°; $[\alpha]^{25}D$ - 21°; IR(CCl_4) ν_{max} 3605 (hydroxyl), 1710(C=O), and 968 cm^{-1} (E Δ^{22}-bond); MS $C_{28}H_{46}O_2$ (m/z, rel. intensity, %), 414(M^+, 25), 396(M - H_2O, 16) 353(M - CH_3 - H_2O - CO, 9), 316(M - C_7H_{14}, 17), 301(10), 287(27), 217(26), 177(13), 123(38), 109(46), 69(97), 55(100).

24-Methyl-5α-cholesta-2,22-dien-6-one(VIII): A mixture of 500 mg of

VIIa, 500 mg of p-toluenesulfonyl chloride, and 5 ml of pyridine was allowed to react for 18 hr at room temperature. The solution was poured into water and crushed ice, and the precipitate was collected and dried to give 670 mg of the crude tosylate(VIIb), m.p. 167-169°. The 670 mg of VIIb was heated under reflux conditions with 670 mg of lithium bromide and 7 ml of dimethylformamide for 45 min. The reaction mixture was cooled and diluted with water and crushed ice, and the precipitate was collected and dried under vacuum. The precipitate was placed on a column (2.5 x 6.2 cm) containing Unisil and was eluted with 50-ml volumes of hexane, and hexane containing 2, 4 and 6% ether. The fraction eluted with 4% ether gave 413 mg of chromatographically pure VIII. A 70-mg sample, recrystallized from methanol, gave 60 mg of needles m.p. 90-91°; $[\alpha]_D^{25}$ + 20°.

2α,3α,22α,23α-Tetrahydroxy-24α-methyl-5α-cholestan-6-one (IXa) and 2α,3α,22β,23β-Tetrahydroxy-24α-methyl-5α-cholestan-6-one (Xa): To 650 mg (513 mg required) of osmium tetroxide in 30 ml of dry benzene was added 400 mg of VIII in 2 ml of benzene followed by 2 drops of pyridine. The mixture was allowed to stand at room temperature in the dark for 90 hr during which time the black osmate ester had precipitated. Some of the solvent was removed under vacuum at 30°; and the residue, in 110 ml of 80% aqueous ethanol and 3 g of sodium bisulfite, was refluxed for 4 hr. The mixture was then distilled until no more benzene appeared in the distillate. The solution was filtered while hot and the black inorganic precipitate was washed with ethanol, and most of the solvent of the filtrate was then removed under vacuum. The filtrate was chilled and diluted with water, and the precipitate was collected and dried under vacuum to give 409 mg of crystalline material. Analysis by TLC on an HP-TLC plate, developed 3 times in chloroform/ethanol (15:1) with wick gave three major spots with Rf's of 0.51, 0.48, and 0.33 and a minor component with an Rf of 0.28 (The steroids responsible for these spots are henceforth referred to as S-0.51, S-0.48, S-0.33, and S-0.28). The Rf values of 0.48 and 0.33 were identical to the Rf's of previously synthesized [6] 2α,3α,22β,23β-tetrahydroxy-24β-methyl-5α-cholestan-6-one and 2α,3α,22α,23α-tetrahydroxy-24β-methyl-5α-cholestan-6-one, respectively (these compounds come from the 40% brassicasterol present in starting material). The mixture of compounds was place on a 50-g column (2.2 x 13cm) of chloroform/benzene (9:1)-washed alumina (activity Grade III) and was eluted with 100-ml volumes of the following (50-ml fractions collected once material started to emerge from column): chloroform/benzene (9:1), chloroform/benzene (95:5), chloroform, and chloroform containing 1, 2, 3, 4, 5, 6, 10, 15, 20, 25, 40, 50% methanol, and 100% methanol. The fractions, monitored by HP-TLC showed that those eluted with 1% and 2% methanol in chloroform contained S-0.51 and some apolar material. The fraction eluted with the first 50 ml of chloroform/3% methanol gave 93 mg of the chromatographically pure S-0.51; m.p. 209-210°. The fractions eluted with the second 50 ml of chloroform/3% methanol and with the subsequent solvents ending with chloroform/5% methanol all showed some of the material with an Rf of S-0.51 and were therefore combined (mixture A) and was set aside for rechromatography. The fractions eluted with solvents beginning with chloroform/10% methanol and ending with 100% methanol all contained mostly the steroids with S-0.33 and S-0.28 hence, they were also

combined (mixture B) and set aside for rechromatography.

Mixture A was rechromatographed on 28 g of activity Grade III neutral alumina in a tapered column (2.2 id x 1.2 cm then 1 x 28 cm) with the same solvents as above and yielded an additional 40 mg of pure S-0.51 in the fractions eluted with chloroform/2% methanol and chloroform/3% methanol. The subsequent fractions that still contained a small amount of S-0.51 though now showing the compound with an Rf value S-0.48 as the major component, were combined and rechromatographed on 30 g of alumina (1.9 x 10.4 cm) with the above solvents. An additional 5 mg of the of S-0.51 was obtained in the chloroform/1% and 2% methanol fractions. The fractions eluted with chloroform/4% to 6% methanol yielded 55 mg of chromatographically pure S-0.48, m.p. 182-183°. Its m.p., Rf and IR spectrum were identical with those of authentic 2α,3α,22β,23β-tetrahydroxy-24β-methyl-5α-cholestan-6-one [6].

The different batches of chromatographically purified S-0.51 were combined and recrystallized from ethyl acetate to give 130 mg of 2α,3α,22β,23β-tetrahydroxy-24α-methyl-5α-cholestan-6-one (Xa): m.p. 210-211°; $[\alpha]^{25}D$ + 0; MS $C_{28}H_{48}O_5$ (m/z, rel. intensity, %), 464 (M^+, <1) 446 (M - H_2O, 1), 393(6), 364 (M - $C_6H_{12}O$, 100), results from cleavage of 22,23-carbon bond, 345(93), 327(61), 287(39), 263(23), 245(32), 175(27), 173(29), 147(26), 137(24), 107(59), 94(97).

Since the steroidal ketones were so strongly held to the alumina column, mixture B (containing S-0.33 and S-0.28) was chromatographed on a tapered column of 15 g of chloroform-washed Unisil (2 id x 3.4 and 1 id x 28 cm). The column was eluted with 50-ml portions (25-ml fractions collected once material started to emerge from column) of chloroform and of chloroform containing 1, 2, 3, 4, 5, 6, 7, and 34% methanol. The fraction eluted with chloroform/2 to 4% methanol gave 50 mg of S-0.33, m.p. 240-242°. The m.p., Rf, and IR spectrum of this compound were identical to those of authentic 2α,3α,22α,23α-tetrahydroxy-24β-methyl-5α-cholestan-6-one[6].

The fraction eluted with the first 25 ml of chloroform/5% methanol consisted of approximately a 55:45 mixture of S-0.33 and S-0.28, respectively. The second 25-ml eluate of chloroform/5% methanol and the first 25-ml eluate of chloroform/6% methanol gave the chromatographically pure S-0.28. Rechromatography of the nearly 1:1 fraction on the same size column and eluting with the same solvents as before gave an additional 10 mg of 2α,3α,22α,23α-tetrahydroxy-24β-methyl-5α-cholestan-6-one and an additional 8 mg of S-0.28. Recrystallization of the combined S-0.28 from ethyl acetate yielded 24.4 mg of 2α,3α,22α,23α-tetrahydroxy-24α-methyl-5α-cholestan-6-one (IXa) as rectangular plates m.p. 250-251°; $[\alpha]^{25}D$ - 7°; MS $C_{28}H_{48}O_5$ (m/z, rel. intensity, %) showed no M^+, 446(M - H_2O, <1), 393(2), 364(M - $C_6H_{12}O$, 100) results from cleavage of 22,23-carbon bond, 345(90), 327(62), 287(32), 263(21), 245(24), 175(23), 173(25), 147(20), 137(20), 107(46), 95(65).

2α,3α,22β,23β-Tetrahydroxy-24α-methyl-B-homo-7-oxa-5α-cholestan-6-one (XIa): About 177 mg of the amorphous tetraacetate Xb, m.p. 108-110° [prepared from the acetylation of 130 mg of Xa with acetic anhydride-pyridine (1:3) for 18 hr at 65°], in 2 ml of dichloromethane was added to a cold solution of 5 ml of dichloromethane that contained 490 mg of trifluoroacetic anhydride, and 0.06 ml of 50% hydrogen peroxide. The reaction mixture was then allowed to stand at room temperature for 1.5

hr at the end of which TLC analysis indicated that the oxidation had gone to completion and that the 7-oxa ketone contained about 12% of the more polar 6-oxa ketone. The reaction mixture was partitioned against cold aqueous, 2% potassium carbonate, and the organic phase was washed with water, dried over anhydrous sodium sulfate and evaporated under vacuum. The residue was chromatographed on 25 g of benzene/30% chloroform-washed Unisil (1 x 22 cm). The column was eluted with 50-ml portions (25-ml fractions collected once the compound started to emerge from the column) of benzene/chloroform mixtures. The chloroform concentration was 30% initially and was raised in 1% increments. The fractions were monitored by TLC [solvent system, benzene/ethyl acetate (6:1), developed 3 times], and those eluted with 40 to 42% chloroform in benzene showed one spot. These fractions were combined to give 135 mg of XIa. Attempted recrystallization from hexane-acetone did not yield any crystalline product. The residue was heated with 500 mg of potassium carbonate in 25 ml of 70% aqueous methanol under reflux conditions for 3 hr, and the hot solution was acidified with 6 N hydrochloric acid solution. Removal of methanol and subsequent recrystallization of the precipitate from ethyl acetate gave 80 mg of XIb, m.p. 229-230°; $[\alpha]^{25}D$ + 40°; EI-MS $C_{28}H_{48}O_6$, showed no M^+ at m/z 480, the first observable peak at m/z 462($M - H_2O$, <1), and other observable ions at m/z 447($M - H_2O - CH_3$, 1), 409(5), 380($M - C_6H_{12}O$, 25) results from cleavage of 22,23-carbon bond, 350(24), 343(21), 333(12), 319(10), 303(16), 301(13), 285(15), 208(20), 189(25), 177(38), 107(78), 81(99), 71(97); CI-MS (ammonia) 498($M + NH_4$,39), 481(M + 1,34), 478(21), 463($M + 1 - H_2O$, 13) 396(55), 378(33), 368(100), 350(69), 348(33), 334(15), 190(14), 177(14), 148(54).

2α,3α,22α,23α-Tetrahydroxy-24α-methyl-B-homo-7-oxa-5α-cholestan-6-one (I): To a 5° solution of 2 ml of dichloromethane that contained 149 mg of trifluoroacetic anhydride and 0.02 ml of 50% hydrogen peroxide was added 29.6 mg of the crude tetraacetate IXb, m.p. 215-217° (Lit. [8] m.p. 215-217°), in 2 ml of dichloromethane. (The tetraacetate was prepared by the acetylation of 21.4 mg of IXa as in the preparation of Xb). The mixture was allowed to react at room temperature for 3.5 hr, worked-up and chromatographed on a tapered column of 15 g of Unisil (2.5 id x 3.34 and 1 x 28 cm) as in the purification of XIa. Recrystallization of the combined chromatographically pure fractions gave 18.2 mg of the tetracetate of I: m.p. 212-214°; $[\alpha]^{25}D$ + 40. Saponification in 3 ml of 2% potassium carbonate - 70% aqueous methanol as in the preparation of XIb gave 13.5 mg of brassinolide (I), m.p. 278-281° (decomp). Compound I, recrystallized from ethyl acetate, melted at 279-281°(decomp.) [Lit. (5) m.p. 274-275°]; EI-MS $C_{28}H_{48}O_6$ (m/z, rel. intensity, %), 480(M^+, <1) 462($M - H_2O$, 1), 447($M - H_2O - CH_3$), 409(3), 380($M - C_6H_{12}O$, 33), 379(37), 361(42), 350(19), 343(22), 333(9), 319(10), 303(14), 285(12), 208(15), 189(23), 177(22), 107(52), 81(76), 71(55); CI-MS(ammonia) 498 ($M + NH_4$, 50), 481(M + 1, 14) 480(14), 478(15), 463($M+1-H_2O$, 13), 462(14), 396(43), 378(69), 368(82), 366(64), 350(100), 348(54), 336(20), 334(12), 177(14), 148(96). The EI-MS, CI-MS, and TLC analyses (Table 1) agreed well with those of natural brassinolide.

RESULTS AND DISCUSSION

Chemistry

Brassinolide (I) and its 22β,23β-cis-glycol isomer (XIb, 2α,3α,22β,23β-tetrahydroxy-24α-methyl-B-homo-7-oxa-5α-cholestan-6-one) [17] were synthesized according to reported [6] general sequence of reactions, as indicated in the experimental section and in Figure 1. Since a C-24 epimeric sterol mixture of 22-dehydrocampesterol and brassicasterol (60:40) was used as starting material, compounds V-VIII were also mixtures of 24α- and 24β-methylsteroids. The structures in Figure 1, however, show only the 24α-methyl orientation.

The oxidation of VIII (24α-methyl) with osmium tetroxide gave the expected cis 2α,3α-cis-diol orientation [18] but the point of attack on the side chain occurred mainly from the back side, because better than 80% of the 22β,23β-isomer (Xa) and less than 20% of the desired 22α,23α-isomer (IXa) were formed. On the other hand the oxidation of the 24β-methyl compound of VIII in the present work and also previously [6], gave approximately a 1:1 mixture of the 22β,23β- and 22α,23α-tetrahydroxy ketones. It is obvious from an inspection of a molecular model of VIII that the 24α-methyl and ring D of the steroid nucleus greatly hinder a frontal attack of the bulky osmium tetroxide molecule on the side chain. Similarly, we have observed that the osmium tetroxide oxidation of a compound like VIII, but with a 24α-ethyl substituent, gave better than 85% of the 22β,23β-cis-glycolic isomer [19].

Our method of synthesis of brassinolide (I) and its 22β,23β-isomer permitted the synthesis of these compounds without having to be concerned about the final orientation of the C-20 and C-24 methyl groups. Although the yield of I was quite low, the overall yields of

both I and its 22β,23β-isomer, were better than 12%. Clearly, the large differences in the Rf's of the ketones IXa and Xa make for an easy separation of these compounds. Thus, the yields of I and XIb should increase if they are synthesized via pure 22-dehydrocampesterol. Presently, 22-dehydrocampesterol offers the best and most rapid route to XIb. It may also be the most rapid route for obtaining I, even though the yield should be low, as indicated by its synthesis from a mixture of 22-dehydrocampesterol and brassicasterol.

Previous reports of the synthesis of brassinolide by others [7,8], and the present synthesis of brassinolide and its 22β,23β-isomer (XIb), and our earlier synthesis of the steroidal lactones II and III [6] now complete the series and make available I and all of its 22,23-cis-glycolic isomers for physiological studies. We have also presented their TLC data in Table 1.

Table 1. TLC analysis of natural and synthetic brassinolide and its three isomers and their corresponding tetraacetates.

Brassinosteroids	Orientation of substituents at C-22	C-23	C-24	Rf Values* Tetrahydroxy**	Tetraacetate***
Natural I	α	α	α	0.42	0.22
Synthetic I	α	α	α	0.42	0.22
II	α	α	β	0.48	0.26
III	β	β	β	0.60	0.39
XIb	β	β	α	0.64	0.42

* From HP-TLC plates
** TLC plate developed 2 times (in chamber with wick) in chloroform/ethanol (7:1)
*** TLC plate developed 3 times (in chamber with wick) in benzene/ethyl acetate (6:1)

Biology

Brassinolide, both natural and synthetic, and its three 22,23-cis glycolic C-24 methyl isomers, were evaluated in two bioassay systems: 1) specific for gibberellic acid type cell elongating compounds, and 2) the other for measuring auxin type growth response. For evaluation in the bean second internode bioassay, we have used two scoring methods 1) percentage elongation over controls, and 2) a growth response code on a 0-5 scale (Fig. 2 and Table 2 - see numbers in parenthesis). The latter method of scoring accounts for the overall brassin activity, which is the induction of a unique biological response of cell division and cell elongation that result in elongation, curvature, swelling, and finally splitting of the treated bean internodes [5].

Table 2. Effects of brassinosteroids and their concentrations on the elongation and growth response of internodes in the bean second internode bioassay.

Brassinosteroid	% Elongation over controls and growth responses*			
	10µg	1µg	0.1µg	0.01µg
Natural I	271 (5)	187 (5)	200 (5)	163 (4)
Synthetic I	136 (5)	136 (5)	156 (5)	183 (4)
II	237 (5)	178 (5)	228 (4)	224 (3)
III	163 (5)	136 (5)	152 (3)	140 (2)
XIb	257 (5)	129 (4)	116 (2)	80 (1)

*Growth response of the treated internodes was rated arbitrarily on a 0 to 5 scale (see numbers in parenthesis): 0. no elongation; 1. elongation only; 2. elongation with slight curvature and swelling; 3. elongation with good curvature and swelling; 4. elongation with excellent curvature and swelling; and 5. elongation with split internodes.

Using the response code, we concluded that the natural and synthetic brassinolides (I) were equally active and induced the same type of growth responses (Table 2). The least active compound was XIb

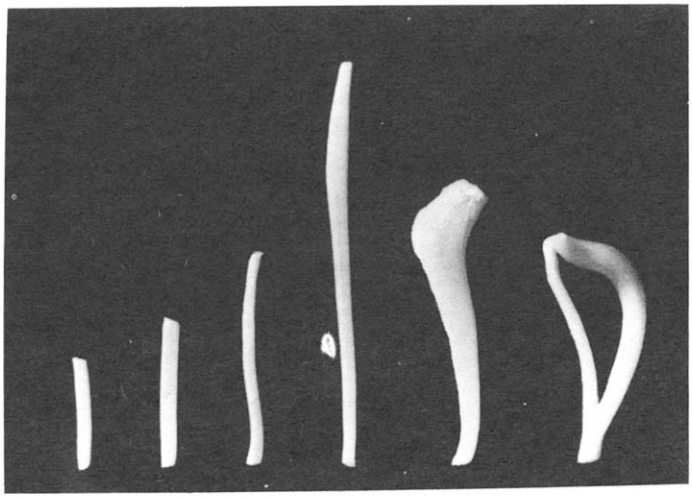

Figure 2. Plant growth responses in bean second internode bioassay. Excised internodes (rated on 0 to 5 scale) are shown in the picture.

with 22β-OH, 23β-OH, and 24α-Me orientation. As in a previous study [6], very good biological activity was shown by II (22α-OH, 23α-OH and 24β-Me) and by III (22β-OH, 23β-OH and 24β-Me) and they elicited the same types of responses under the concentrations examined (Table 2). The results suggest that compound II was slightly more active than compound III and that they are about one-tenth as active as brassinolide [6].

Brassinolide and its isomers evaluated at the 100 ng level in a bean first internode bioassay [12] for their effectiveness in stimulating auxin-induced growth showed that three of the four compounds (I-III) caused a rapid growth response within 1 hr in the presence of exogenous auxin, and that the internodes continued to grow at a high rate during the second and third hours of the experiment (Table 3). Compound III (22β-OH, 23β-OH and 24β-Me substitution) caused the highest rate of growth and brassinolide the next highest rate. Compound XIb caused the

least amount of growth during the first 2 hours, but was the second most active of the four during the third hour of the experiment. Internodes treated with a brassinosteroid and auxin elongated more than those

Table 3. Effect of brassinolide and its isomers on the elongation of internodes in the bean first internode bioassay.

Brassinosteroid	Growth rate (mm/hr)* Hours after treatment			Total Growth
	1	2	3	(mm)
Control (IAA)	1.69 ± 0.26a	1.13 ± 0.16a	0.57 ± 0.16a	3.40
I	2.48 ± 0.23b	1.76 ± 0.28bc	1.04 ± 0.13b	5.28
II	2.19 ± 0.31ab	1.68 ± 0.17b	1.17 ± 0.15bc	5.04
III	3.22 ± 0.31c	2.24 ± 0.32c	1.36 ± 0.15c	6.82
XIb	1.96 ± 0.22a	1.60 ± 0.19b	1.24 ± 0.20bc	4.80

* Means followed by the same letter are not significantly different at the 5% level by Duncan's multiple range test.

treated with only auxin (control).

Interestingly, we found that the synthetic precursor ketone to II and the ketone IXa also showed biological activity in both tests. Not only was the ketone IXa more active than the precursor ketone to II, but it was also as active as compounds II and III in both assays.

Previously we demonstrated that compound II and III could be synthesized in gram quantities [6], from the readily available plant sterol, ergosterol. We have also found that II and III were comparable to brassinolide (I) in types of physiological responses they induced but that they were less potent. It has been suggested that our marine environment is perhaps the best source of such sterols as 22-dehydrocampesterol, which is rather uncommon in higher plants [20]. It is likely that a rich marine source of 22-dehydrocampesterol can be found in the near future, at which time brassinolide synthesis reported herein

would be commercially feasible. Until then, our scheme for synthesizing II and III [6] affords a feasible commercial approach, should there be a continued demand for brassinosteroids in practical agriculture to improve crop efficiency and crop yields.

ACKNOWLEDGEMENT

Thanks are extended to G. W. Patterson for his help in making available 24-methylcholesta-5,22-dienol fraction from oysters under the U.S.D.A. broad form cooperative agreement (12-14-1001-238). We thank R. H. Thompson, Jr., for the capillary GLC analysis of the 24-methylcholesta-5,22-dienol fraction from the oyster, and C. Fulton for technical assistance.

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