Phytochemistry, Vol. 26, No. 2, pp. 503–506, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00
Pergamon Journals Ltd.

BRASSINOSTEROIDS AND STEROLS FROM A GREEN ALGA, *HYDRODICTYON RETICULATUM*: CONFIGURATION AT C-24

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(*Received* 6 *May* 1986)

Key Word Index—*Hydrodictyon reticulatum*; Chlorophyceae; green alga; brassinosteroids; (24*S*)-24-ethylbrassinone; 24-epicastasterone; phytosterols; cholesterol; 24-methylcholesterol; 24-ethylcholesterol.

Abstract—Two brassinosteroids, (24*S*)-24-ethylbrassinone [(22*R*,23*R*,24*S*)-2α,3α,22,23-tetrahydroxy-24-ethyl-5α-cholestan-6-one] and 24-epicastasterone [(22*R*,23*R*,24*R*)-2α,3α,22,23-tetrahydroxy-24-methyl-5α-cholestan-6-one] have been identified from *Hydrodictyon reticulatum*. Examination of the sterols of this alga has established that 24-ethylcholesterol is predominantly the 24α-epimer, but 24-methylcholesterol is a mixture of the 24α- and 24β-epimers. Thus, similarity with respect to the C-24 configuration was observed between the brassinosteroids and 4-demethylsterols.

INTRODUCTION

The possible occurrence of plant hormones in algae has been examined by many investigators. Physicochemical evidence has been obtained for the presence of IAA [1, 2] and cytokinins [3] in some algae. No solid evidence for the occurrence of GAs in algae has been obtained, but biological activities due to GAs have been found in the extracts of various algae [4, 5]. Although ABA has not been found in algae, lunularic acid has been considered as the substitute [6]. Therefore, it is most likely that plant hormones will be important in growth and physiological phenomena of algae.

Brassinosteroids, a new class of steroidal plant hormones, have been demonstrated to have strong growth-promoting activity and have been characterized from various higher plants ranging from angiosperms (monocots and dicots) to a gymnosperm [7]. However, no indication for the presence of brassinosteroids in algae has been reported. In this work, we investigated a freshwater green alga, *Hydrodictyon reticulatum*, for the presence of brassinosteroids.

RESULTS AND DISCUSSION

A chloroform-soluble fraction obtained from *H. reticulatum* was partitioned between hexane and 80% methanol. Brassinosteroid activity was found in the 80% methanol fraction based on a rice-lamina inclination assay. This was separated into neutral and acidic fractions. Major biological activity was found in the neutral fraction, being estimated to be equivalent to 5 μg of brassinolide, while the biological activity of the acidic fraction was about one-tenth that of the neutral fraction. The neutral fraction was purified on a silica gel column eluted step-wise with increasing amounts of methanol in chloroform. Eluates with 4% and 5% methanol in chloroform were biologically active and, therefore, were combined, then subjected to Sephadex LH-20 chromatography using a methanol–chloroform mobile phase. The activity was found in the elution volume corresponding to that of typical brassinosteroids. Upon reversed-phase high-performance liquid chromatography (HPLC) [8], two fractions (A and B) of biological activity were separated as shown in Fig. 1.

Fraction A was analysed by GC/MS after methane boronation [9]. The peak observed in the reconstructed ion current trace had the same R_t as the bismethaneboronate of (22*R*,23*R*,24*S*)-24-ethylbrassinone (**1**) (Table 1) and gave a mass spectrum identical to the bismethaneboronate of **1**. Major fragment ions were observed at *m/z* (rel. int.): 526 $[M]^+$ (24), 441 (12), 358 (21), 287 (12), 169 (100). Compound **1** and its configurational isomers (**2**–**4**) with respect to C-22, C-23 and C-24 [10] were examined for their behaviour in TLC, GC (Table 1) and reversed-phase HPLC (Fig. 1), confirming that the brassinosteroid in fraction A should be compound **1**. Fraction B, with only weak activity, was also analysed by GC/MS after methaneboronation. The peak with R_t of 8.52 min exhibited a mass spectrum identical to that of the bismethaneboronate of castasterone (**5**), *m/z* (rel. int.): 512 $[M]^+$ (43), 497 (3), 441 (13), 358 (27), 287 (22), 155 (100), but the R_t was larger than that of the bismethaneboronate of **5** (Table 1). This fact indicates that the brassinosteroid in this fraction must be an epimer of castasterone. Precise comparison of its chromatographic (GC and HPLC) behaviour, with those of authentic epimers (**5**, **6** and **7**) led to the identification of compound B as (22*R*,23*R*,24*R*)-castasterone (**6**) (Fig. 1 and Table 1). The amounts of **1** and **6** in *H. reticulatum* were estimated to be 4.0 and 0.3 μg/kg fr. wt, respectively, based on GC-selected ion monitoring (SIM) of the active fraction obtained after Sephadex LH-20 chromatography. These values are obviously underestimates because of possible losses during the sample purification.

The present work demonstrates for the first time that

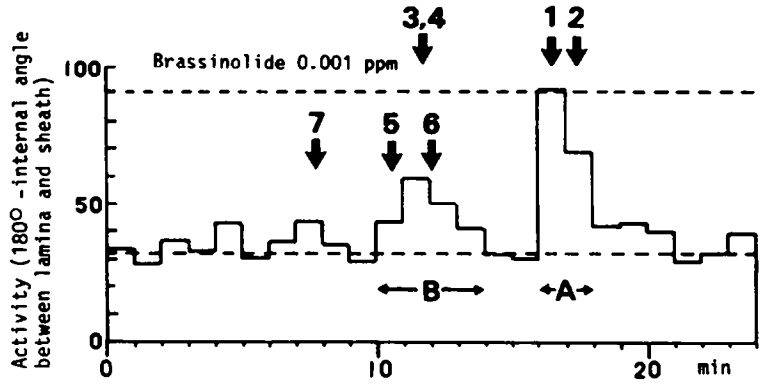


Fig. 1. Distribution of biological activity determined by the rice-lamina inclination test after reversed-phase HPLC of the partially purified extract from *H. reticulatum*. The arrows denote the elution positions of authentic specimens.

Table 1. Chromatographic data of fractions A and B, and synthetic epimers of ethylbrassinone and castasterone

Compound	TLC R_f*	GC/MS R_t (min)†
Fraction A	0.42	9.20
(22*R*,23*R*,24*S*)-Ethylbrassinone (1)	0.42	9.20
(22*R*,23*R*,24*R*)-Ethylbrassinone (2)	0.42	9.63
(22*S*,23*S*,24*S*)-Ethylbrassinone (3)	0.52	9.12
(22*S*,23*S*,24*R*)-Ethylbrassinone (4)	0.52	8.79
Fraction B	0.43	8.52
(22*R*,23*R*,24*S*)-Castasterone (5)	0.39	7.90
(22*R*,23*R*,24*R*)-Castasterone (6)	0.43	8.50
(22*S*,23*S*,24*R*)-Castasterone (7)	0.49	7.58

*Silica gel HPTLC (Merck) was used with a 6:1 mixture of chloroform–ethanol as solvent. TLC plates were sprayed with 70% sulphuric acid and then heated for visualization as bluish-purple spots under UV (365 nm). The detection limit was less than 0.01 μg.

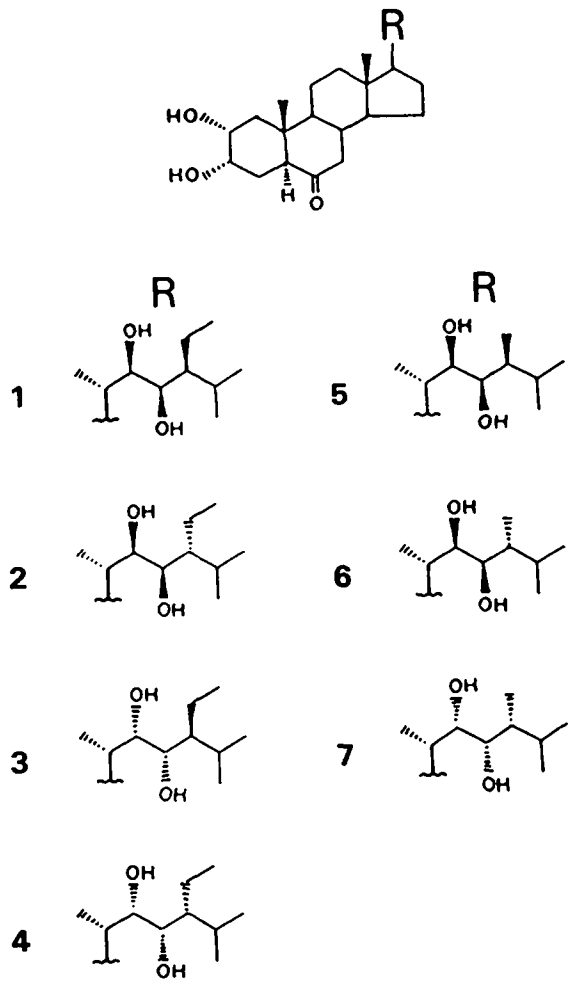
†GC conditions are described in the Experimental.

brassinosteroids occur in algae and also that a 24*R*-type brassinosteroid is present in plants. It, however, seemed unexpected that (22*R*,23*R*,24*S*)-24-ethylbrassinone (1), the major brassinosteroid in *H. reticulatum*, has a 24α-alkyl substituent (α is taken to mean the substituent pointing towards the observer [11]), because algal sterols have generally been believed to have a 24β-alkyl substituent [12, 13]. This question prompted us to analyse the sterols contained in *H. reticulatum*.

The 4-demethylsterol fraction obtained from the hexane fraction was acetylated and then purified on a column of silica gel impregnated with silver nitrate, affording a mixture of cholesteryl acetate and its 24-alkyl homologues. This was subjected to reversed-phase HPLC with a methanol–hexane mobile phase, affording cholesteryl acetate, 24-methylcholesteryl acetate and 24-ethylcholesteryl acetate in a 31:1:68 ratio. The ^{1}H NMR spectrum (400 MHz) of the 24-ethylcholesteryl acetate was identical to that of authentic sitosteryl acetate (24α-isomer) and also was in good agreement with the literature data [11, 14–16]. Thus the 24-ethylcholesterol in *H. reticulatum* consisted predominantly of the 24α-isomer. The *Hydrodictyon* 24-methylcholesteryl acetate was found to be a mixture of the 24α- and 24β-isomers from comparison of the ^{1}H NMR data with those reported in the literature [11, 14–17]. By comparing the intensities of the doublets for the C-26 and C-27 protons with those of the authentic 24α-methylcholesteryl acetate, the mixture was estimated to be composed of *ca* 80% of the α-isomer and *ca* 20% of the β-isomer.

Huneck [18] once reported that spinasterol is a major sterol component in *H. reticulatum*, but his conclusion should be reconsidered in the light of our data. It is striking from phylogenetic and taxonomical aspects that the configuration at C-24 of the 24-alkylsterols in *H. reticulatum* are quite analogous to those reported for tracheophytes [11, 14, 15, 17, 19] rather than green algae [12, 13, 20]. As concerns other green algae, their 24-alkylsterols are known to have the 24β-configuration although, in some cases, this assignment was tentative [21, 22]. It is interesting that the 24β-methylsterol isolated from *Nitella flexilis* (Chalophyta) contains a small amount (10–15%) of 24α-isomer [23].

Since it was found that *H. reticulatum* contains sterols



which have the same C-24 configuration as the endogenous brassinosteroids, it seems rational that both the brassinosteroids and sterols in *H. reticulatum* might be synthesized in a way analogous to the biosynthesis of tracheopyte sterols, especially with respect to the formation of the alkyl group at C-24 [14, 17, 24, 25].

EXPERIMENTAL

General methods. GC/MS, GC and SIM were carried out as described by Yokota *et al.* [7] except that, in GC/MS, a 2% OV-1 column (1 m × 2.6 mm i.d.) was used at 255° with a 40 ml flow rate of He. In ^{1}H NMR (400 MHz), TMS was used as internal standard and the resolution was 0.25 Hz.

Plant materials. Hydrodictyon reticulatum (L.) Lagerheim was collected from concrete-made water channels leading to paddy fields in Utsunomiya University during May 1984. The water temp. was *ca* 15°. The alga was centrifuged to remove excess H_2O using a washing machine.

Authentic materials. Compounds 1–7 were gifts from Prof. N. Ikekawa, Tokyo Institute of Technology, and Dr. S. Takatsuto, Joetsu University of Education. Brassinolide was a gift from Dr. F. Fujita, Zen-Noh. Authentic sitosteryl acetate and campesteryl acetate were prepared from β-sitosterin (50–60% purity), purchased from Fluka, using the procedure used for the purification of *Hydrodictyon* sterols (see below). The campesteryl acetate obtained contained a trace amount of the 24β-epimer (NMR).

Bioassay. The rice-lamina inclination test was carried out using the cultivar Koshihikari as described by Arima *et al.* [26].

Extraction. H. reticulatum (3.85 kg fr. wt) was extracted with MeOH. The MeOH extract was reduced to the aq. phase *in vacuo*, and extracted 3 × with $CHCl_3$. The $CHCl_3$ extracts were combined and reduced to dryness *in vacuo* (66 g). The resulting oily material was dissolved in hexane (1.5 l.) and partitioned 3 × against 80% MeOH (1.5 l., 2 × 1 l.), giving a hexane fraction (43.6 g) and an 80% MeOH fraction. The latter, dissolved in EtOAc (1 l.), was washed 3 × with 0.2 M K_2HPO_4 (0.3 l.) to give a neutral EtOAc fraction (21 g). The aq. phase was acidified and extracted with EtOAc to give an acidic fraction (2.4 g).

Purification of brassinosteroids. The neutral EtOAc fraction was purified on a column packed with 200 g silica gel. Elution was carried out step-wise with $CHCl_3$ containing 0, 2.5, 3, 4, 5, 7, 9, 11, 15 and 20% MeOH (1.5 l. each). The 4% and 5% MeOH eluates (0.29 g) which contained biological activity were combined and chromatographed on a Sephadex LH-20 column (900 × 26.6 mm i.d.) using a mixture of $CHCl_3$–MeOH (1:4) at a flow rate of 25 ml/hr. Successive 10 ml fractions were collected and biological activity was detected in fractions 33–35. A portion of fraction 34, which was the most active, was subjected to HPLC on a 150 × 10 mm i.d. column packed with 5 μm Develosil ODS and eluted at a flow rate of 3 ml/min with a 1:1 mixture of MeCN–H_2O. Fractions were collected at 1 min intervals (Fig. 1). The active fractions (A and B) were subjected to GC/MS analysis.

Purification of sterols. A portion (11.3 g) of the hexane fraction was saponified by reflux with 5% KOH in 80% EtOH. The recovered non-saponifiable lipid (0.93 g) was chromatographed on silica gel (30 g), eluted with a mixture of hexane and CH_2Cl_2 (1:1). A fraction containing the 4-demethylsterols (230 mg) was acetylated (Ac_2O–pyridine) and fractionated on a column packed with a 4:1 mixture of silica gel and $AgNO_3$ (22 g) eluted with 20% C_6H_6 in hexane (250 ml) and 30% C_6H_6 in hexane (400 ml) [19]. Fractions were collected every 10 ml and examined by silica gel–$AgNO_3$ TLC using EtOH-free $CHCl_3$ as solvent [16]. The 20% C_6H_6 eluates (209 mg) and the first 7 fractions eluted with 30% C_6H_6 (10 mg) contained compound(s) with R_f (0.68) identical to authentic sitosteryl acetate, but the latter fractions contained a trace amount of compound(s) with R_f (0.61) identical to authentic stigmasteryl acetate. A portion (30 mg) of the former was purified by HPLC on a Senshu Pak ODS-4241-H (250 × 20 mm i.d.) column eluted with 3% hexane in MeOH at a flow rate of 9.9 ml/min: *ca* 6 mg steryl acetate mixture in 2.5 ml of the elution solvent was introduced per injection. Thus peaks (UV 212 nm) with R_ts of 55.7, 60.9 and 67.2 min afforded cholesteryl acetate (8.6 mg), 24-methylcholesteryl acetate (0.3 mg) and 24-ethylcholesteryl acetate (18.5 mg), respectively. These were subjected to MS and 400 MHz ^{1}H NMR examination.

Cholesteryl acetate. MS *m/z*: 428 $[M]^+$ (trace), 368 $[M-60]^+$ (base); ^{1}H NMR ($CDCl_3$): δ0.677 (3H, *s*, H-18), 0.863 (3H, *d*, *J* = 6.6 Hz, H-26 or H-27), 0.866 (3H, *d*, *J* = 6.6 Hz, H-27 or H-26), 0.914 (3H, *d*, *J* = 6.4 Hz, H-21), 1.018 (3H, *s*, H-19) [11, 16].

24-Methylcholesteryl acetate. MS *m/z*: 442 $[M]^+$ (trace), 382 $[M-60]^+$ (base); ^{1}H NMR ($CDCl_3$): δ0.678 (3H, *s*, H-18), 0.773 (3H, *d*, *J* = 6.4 Hz, H-28), 0.784, 0.802 (3H, *d*'s, *J* = 7.6, 6.8 Hz, respectively, H-27), 0.851 (3H, *d*, *J* = 6.8 Hz, H-26), 0.911, 0.919 (3H, *d*'s, *J* = 6.4 Hz, H-21), 1.018 (3H, *s*, H-19).

24-Ethylcholesteryl acetate. MS *m/z*: 456 $[M]^+$ (trace), 396 $[M-60]^+$ (base); ^{1}H NMR ($CDCl_3$): δ0.678 (3H, *s*, H-18), 0.814 (3H, *d*, *J* = 7.3 Hz, H-27), 0.835 (3H, *d*, *J* = 7.1 Hz, H-26), 0.844 (3H, *t*, *J* = 8.2 Hz, H-29), 0.920 (3H, *d*, *J* = 6.4 Hz, H-21), 1.018 (3H, *s*, H-19).

Acknowledgements—We would like to thank Prof. N. Ikekawa, Dr. S. Takatsuto and Dr. F. Fujita for gifts of synthetic brassinosteroids; Mr. K. Furihata for the ^{1}H NMR measurement; and Lion Co. Ltd. for extraction of the plant material.

REFERENCES

1. Abe, H., Uchiyama, M. and Sato, R. (1972) *Agric. Biol. Chem.* **36**, 2259.
2. Jacobs, W. P., Falkenstein, K. and Hamilton, R. H. (1985) *Plant Physiol.* **78**, 844.
3. Stephen, A. B. T., MacLeod, J. K., Palni, L. M. S. and Letham, D. S. (1985) *Phytochemistry* **24**, 2611.
4. Graebe, J. E. and Ropers, H. J. (1978) in *Phytohormones and Related Compounds—A Comprehensive Treatise* (Letham, D. S., Goodwin, P. B. and Higgins, T. J. V., eds) p. 107. Elsevier/North-Holland, Amsterdam.
5. Furuya, M. and Takeno, K. (1983) in *The Biochemistry and Physiology of Gibberellins* (Crozier, A., ed.) Vol. 2, p. 189. Prager, New York.
6. Milborrow, B. V. (1978) in *Phytohormones and Related Compounds—A Comprehensive Treatise* (Letham, D. S., Goodwin, P. B. and Higgins, T. J. V., eds) p. 295. Elsevier/North-Holland, Amsterdam.
7. Yokota, T., Arima, M., Takahashi, N. and Crozier, A. (1985) *Phytochemistry* **24**, 1333.
8. Yokota, T., Baba, J., Koba, S. and Takahashi, N. (1984) *Agric. Biol. Chem.* **48**, 2529.
9. Takatsuto, S., Ying, B., Morisaki, M. and Ikekawa, N. (1982) *J. Chromatogr.* **239**, 233.
10. Takatsuto, S., Yazawa, N., Ikekawa, N., Morishita, T. and Abe, H. (1983) *Phytochemistry* **22**, 1393.
11. Nes, W. R., Krevitz, K. and Behzadan, S. (1976) *Lipids* **11**, 118.
12. Nes, W. R. (1977) *Adv. Lipid Res.* **15**, 233.
13. Patterson, G. W. (1971) *Lipids* **6**, 120.
14. Zakelj, M. and Goad, L. J. (1983) *Phytochemistry* **22**, 1931.
15. Sica, D., Piccialli, V. and Masullo, A. (1984) *Phytochemistry* **23**, 2609.
16. Rubinstein, I., Goad, L. J., Clague, A. D. H. and Mulheirn, L. J. (1976) *Phytochemistry* **15**, 195.
17. Scheid, F., Rohmer, M. and Benveniste, P. (1982) *Phytochemistry* **21**, 1959.
18. Huneck, S. (1969) *Phytochemistry* **8**, 1313.
19. Nes, W. R., Krevitz, K., Joseph, J. and Nes, W. D. (1977) *Lipids* **12**, 511.
20. Patterson, G. W. (1974) *Comp. Biochem. Physiol.* **47B**, 453.
21. Fattorusso, E., Magno, S. and Mayol, L. (1980) *Experientia* **36**, 1137.
22. Napoli, L. D., Magno, S., Mayol, L. and Novellino, E. (1982) *Phytochemistry* **21**, 1993.
23. Wright, J. L. C., McInnes, A. G., Shimizu, S., Smith, D. G., Walter, J. A., Idler, D. and Khalil, W. (1978) *Can. J. Chem.* **56**, 1898.
24. Seo, S., Uomori, A., Yoshimura, Y. and Takeda, K. (1984) *J. Chem. Soc. Chem. Commun.* 1174.
25. Bladocha, M. and Benveniste, P. (1985) *Plant Physiol.* **79**, 1098.
26. Arima, M., Yokota, T. and Takahashi, N. (1984) *Phytochemistry* **23**, 1587.