Phytochemistry, Vol 28, No 4, pp 1073 1075, 1989
Printed in Great Britain

0031 9422/89 $3 00+0 00

ECDYSTERONE FROM STEM OF *DIPLOCLISIA GLAUCESCENS*

B. M RATNAYAKE BANDARA,* LALITH JAYASINGHE,* VERANJA KARUNARATNE,* G PERCY WANNIGAMA,* MICHAEL BOKEL,† WOLFGANG KRAUS† and SUBRAMANIAM SOTHEESWARAN‡

*Department of Chemistry, University of Peradeniya, Peradeniya, Sri Lanka; †Institut für Chemie, Universität Hohenheim, 7000 Stuttgart, F R G, ‡School of Pure and Applied Sciences, University of the South Pacific, P O Box 1168, Suva, Fiji

(*Received* 11 *August* 1988)

Key Word Index—*Diploclisia glaucescens*; Menispermaceae, stigmasterol, ecdysterone, saponins, ^{13}C NMR, NOE, bioactivity

Abstract—The stem of *Diploclisia glaucescens* afforded ecdysterone in a high yield of over 3%. ^{13}C NMR of the tetraacetate and NOE studies on the triacetate provided further data on the structure and conformation of this phytoecdysteroid Hemolytic, insecticidal and spermicidal activity are reported for the compound.

INTRODUCTION

Diploclisia glaucescens (Bl.) Diels (= *Cocculus macrocarpus* W. & A.) (Menispermaceae) is a creeper growing in the mid-country regions of South India and Sri Lanka. The leaves have been used in the treatment of biliousness and venereal diseases [1]

The presence of alkaloids in the leaves and twigs of the plant has been indicated in a preliminary survey [2] Chemical investigation of the seeds of the plant gave five phytoecdysteroids showing activity against larvae of the European corn borer, *Ostrinia nubialis* [3]. The principal phytoecdysteroid, isolated in a yield of 0.46% was ecdysterone (20-hydroxyecdysone) (**1**).

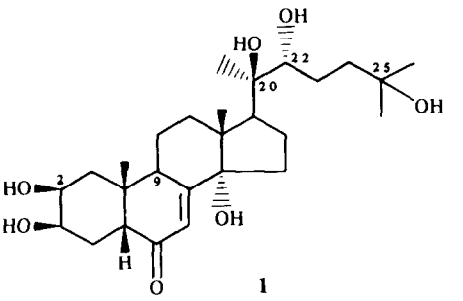
RESULTS AND DISCUSSION

The present investigation began with a search for saponins in the plant The dry mature stem of *D. glaucescens* was defatted with petrol and the residue extracted with methanol. A *n*-butanol extract of the methanol extract gave a positive response for saponins in both the froth and hemolysis tests The methanol extract also showed reasonable molluscicidal activity. At a concentration of 200 ppm, the minimum for activity, the methanol extract caused 100% mortality of *Biomphalaria glabrata* snails, one of the intermediate hosts of the *Schistosoma* parasite

Separation of the methanol extract by adsorption chromatography over silica gel gave stigmasterol (**2**) and ecdysterone (20-hydroxyecdysone) (**1**), in yields of 0 03 and 3.2% respectively. The residual unresolved mixture gave strongly positive froth and hemolysis tests for saponins.

Stigmasterol (**2**) and ecdysterone (20-hydroxyecdysone) (**1**) were identified by spectroscopic means as well as by comparison with authentic samples. The 2,3,22-triacetate (**3**) and 2,3,22,25-tetraacetate (**4**) of ecdysterone were prepared and their physical data [4, 5] also used to confirm the identity of ecdysterone. The ^{13}C NMR spectrum recorded for the tetraacetate constitutes additional physical data.

The *cis*-A/B ring system in ecdysterone (**1**) can exist in either of two conformations, of which the higher energy



1

conformation has the hydroxyl group at C-2 and the methyl group at C-10 in 1,3-diaxial positions. In the preferred conformation, where these two groups are diequatorial, H-2 and H-9 are much close to each other than in the conformation of higher energy. X-Ray crystallographic analysis shows that ecdysterone adopts the preferred conformation in the solid state [6]. Our studies show that this conformation is retained even in solution for the triacetate since a positive NOE is observed between H-2 (δ 5.05) and H-9 (δ 3.10) in its ^{1}H NMR ($CDCl_3$).

Ecdysterone (**1**) and related steroids, termed ecdysteroids, are the moulting hormones of arthropods. Early workers isolated such ecdysteroids in minute yields (< 0.000005%) from the arthropods, themselves [7]. Following the isolation of 25-deoxyecdysterone [8] in 0.05% yield from *Podocarpus nakaii*, a Taiwanese plant, several workers reported the isolation of ecdysteroids in higher yields from other plant sources [9]. Larger quantities of these phytoecdysteroids were thus available for biological testing. In the present investigation, the yield (3 2%) of ecdysterone from the mature stem of *D. glaucescens* surpasses the yields so far recorded in the isolation of any ecdysteroid from a natural source.

Saponins are reported in the literature as showing interesting bioactivities such as hemolytic, insecticidal, antibacterial [10], molluscicidal [11], antifungal [12], anti-inflammatory [13] and spermicidal [14]. Since ecdysterone resembles a saponin in containing both hydrophilic and hydrophobic parts in the molecule, it was also

subjected to tests for the same bioactivities reported for saponins

Ecdysterone (**1**) showed strongly positive froth and hemolysis tests and showed moderate insecticidal activity (LD_{50} 1 8 mg/kg against the groundnut aphid, *Aphis craccivora*) Ecdysterone also showed significant spermicidal activity (a concentration of 20 mg/ml giving 100% immotility of spermatozoa of fresh human semen within 20 sec) Ecdysterone (**1**) showed no activity as a molluscicide (against *B glabrata* snails), no antifungal activity (against *Cladosporium cladosporioides*), no antibacterial activity (against *Mycobacterium fortuitum*, *Escherichia coli* and *Staphylococcus aureus*) and no anti-inflammatory activity (leucotriene, LTB_4, assay)

EXPERIMENTAL

Mps uncorr Chemical shifts are given in δ(ppm) with TMS as int standard Assignment of chemical shifts in ^{13}C NMR were made with the aid of DEPT analysis

Plant material D glaucescens was kindly identified and collected in May from the Central Province of Sri Lanka by Professor S Balasubramaniam, Department of Botany, University of Peradeniya, Sri Lanka

Extraction and isolation The dry, ground, mature stem of *D glaucescens* (500 g) was sequentially extracted with hot petrol (40–60°) and hot MeOH Evaporation of the MeOH gave a dark brown solid (60 g) A portion (15 g) was separated on a column of 200 g of silica gel (Merck, Art 9385) by medium pressure liquid chromatography with petrol, EtOAc and MeOH as eluants Stigmasterol (**2**) (30 mg) and ecdysterone (**1**) (4 g) were eluted in order of increasing polarity and further purified by prep TLC on silica gel followed by recrystallization

Stigmasterol (**2**) was obtained as colourless needles from MeOH–CH_2Cl_2, mp 156–158 , $[\alpha]_D^{22} - 52\,5^\circ$ ($CHCl_3$, *c* 0 40), identical with an authentic sample (mmp, co TLC, IR and ^1H NMR at 60 MHz) Reaction with pyridine and Ac_2O gave stigmasteryl acetate as colourless needles from MeOH–EtOAc, mp 128–130°, $[\alpha]_D^{22} - 51\,4^\circ$ ($CHCl_3$, *c* 0 70)

Ecdysterone (**1**) was obtained as colourless needles from MeOH–EtOAc, mp 242–244°, $[\alpha]_D^{22} + 52\,2^\circ$ (MeOH, *c* 0 23), identical with an authentic sample (mmp, co TLC and IR), IR ν_{max}^{KBr} cm^{-1} 3450 (OH), 2950, 1660 (C=O), 1450, 1390, 1060, 880, UV λ_{max}^{EtOH} nm (log ε) 243 (4 06) unchanged on addition of N NaOH; ^1H NMR (C_5D_5N), δ 1 05, 1 20, 1 60 (each 3H, *s*, H-19, H-18, H-21), 1 35 (6H, *s*, H-26, H-27), 3 00 (1H, *m*, H-9), 3 60 (1H, *m*,H-2), 3 85 (1H, *m*, H-3), 4 20 (1H, *m*, H-22), ^{13}C NMR see Table 1, EIMS *m/z* (rel int) 481 $[M+1]^+$ (< 1), 442 (1), 426 (2), 345 (4), 327 (5), 149 (100), FDMS *m/z* 481 $[M+1]^+$

Triacetate (**3**) *and tetraacetate* (**4**) Compound **1** (250 mg) was allowed to react overnight with Ac_2O (2 ml) and pyridine (2 ml) and a mixture of acetates (300 mg) obtained Separation by prep TLC on silica gel gave ecdysterone 2,3,22-triacetate (**3**) (210 mg) and ecdysterone 2,3,22,25-tetraacetate (**4**) (70 mg) The triacetate (**3**) was obtained as colourless needles from MeOH–EtOAc, mp 145–147°, $[\alpha]_D^{22} + 68$ (MeOH, *c* 0 25), ^1H NMR ($CDCl_3$) δ 0 85, 1 03, 1 26 (each 3H, *s*, H-18, H-19, H-21), 1 21 and 1 23 (each 3H, *s*, H-26/H-27), 2 46 (1H, *m*, H-5), 3 10 (1H, *m*, H-9), 4 84 (1H, *m*, H-22), 5 05 (1H, *dt*, *J* = 12 5, 5 and 2 5 Hz, H-2), 5 35 (1H, *m*, H-3), 5 87 (1H, *m*, H-7), ^{13}C NMR see Table 1, EIMS *m/z* (rel int) 279 (10), 167 (15), 149 (100), FDMS *m/z* (rel int) 607 $[M+1]^+$ (100), 58 (58) ($[M+1]^+$ by accurate mass measurement 607 375, $C_{33}H_{51}O_{10}$ requires 607 348) The tetraacetate (**4**) was obtained as colourless needles from MeOH–EtOAc, mp 197–199°, $[\alpha]_D^{22}$ + 76.9° (MeOH, *c* 0 13), ^1H NMR ($CDCl_3$) δ 0 85, 1 03, 1 26 (each 3H, *s*, H-18, H-19, H-21), 1 41 and 1 44 (each 3H, *s*, H-26/H-27), 2 31 (1H, *m*, H-5), 3 13 (1H, *m*, H-9), 4 80 (1H, *m*, H-22), 5 05 (1H, *dt*, *J* = 12 5, 5 and 2 5 Hz, H-2), 5 33 (1H, *m*, H-3), 5 86 (1H, *m*, H-7), ^{13}C NMR see Table 1, EIMS *m/z* (rel int) 510 (2), 492 (3), 459 (1), 447 (5), 429 (7), 327 (8), 43 (100), FDMS *m/z* (rel int) 649 $[M+1]^+$ (100), 631 (2), 58 (20)

Table 1 ^{13}C NMR spectral data of **1** (C_5D_5N), **3** and **4** ($CDCl_3$)

C	1	3	4
1	37 96	38 34	38 29
2	68 03	67 04	66 98
3	68 12	68 62	68 57
4	32 41	34 00	33 98
5	51 37	50 92	50 89
6	203 43	202 09	201 94
7	121 64	121 58	121 54
8	166 08	164 52	164 45
9	34 43	33 57	33 55
10	38 64	38 34	38 29
11	21 11	20 46	20 53
12	31 96	31 07	31 06
13	48 09	47 48	47 42
14	84 16	84 48	84 41
15	31 98	31 52	31 59
16	21 47	28 60	29 17
17	50 08	49 51	49 54
18	17 87	17 42	17 38
19	24 46	23 78	23 76
20	76 82	77 74	77 00
21	21 68	21 11	21 06
22	77 52	79 74	79 33
23	27 45	24 75	24 55
24	42 62	40 24	37 49
25	69 52	70 58	81 73
26	29 99	29 19	25 91
27	30 09	30 18	26 25

Hemolysis test This test was carried out using the standard cup method on sheep blood agar gel

Acknowledgements– The authors thank the Natural Resources, Energy and Science Authority of Sri Lanka for a research grant They thank the Stiftung Volkswagenwerk, F R G for generous provision of spectral data They also thank Dr R W Miller (USDA, Peoria) for an authentic sample of ecdysterone, Dr K Hostettmann (Lausanne, Switzerland) for molluscicidal testing, Dr Rosalinda Solevilla (Manila, Philippines) for anti-inflammatory testing, Prof W D Ratnasooriya (University of Colombo) for spermicidal testing, Mr K A N P Bandara (Peradeniya) for insecticidal testing; Prof M R M Pinto and Dr N K B Adikaram (Peradeniya) for antibacterial and antifungal testing, respectively)

REFERENCES

1 Chopra, R N, Nayar, S L and Chopra, I C (1956) *Glossary of Indian Medicinal Plants*, pp 72 and 99 Council of Scientific and Industrial Research, New Delhi

2 Sultanbawa, M U S, Wanniigama, G P, Bandaranayake, W M, Kumar, V, Gunatilaka, A A L, Marikar, F A, Balasubramaniam, S and Arsecularatne, S N (1978) *Lloydia* **41**, 597

3 Miller, R W, Clardy, J, Kozlowski, J, Mikolajczak, K L,

Plattner, R D., Powell, R G., Smith, C. R., Weisleder, D and Zheng Qi-Tai (1985) *Planta Med.* **51**, 40.
4 Galbraith, M N. and Horn, D H. S (1969) *Aust. J Chem.* **22**, 1045.
5. Nakano, K., Nohara, T., Tomimatsu, T. and Nishikawa, M (1982) *Phytochemistry* **21**, 2749
6. Dammeier, B. and Hoppe, W (1971) *Chem Ber* **104**, 1660
7 Karlson, P (1956) in *Vitamins and Hormones*, Vol. 14, p 227. Academic Press, New York
8 Nakanishi, K., Koreeda, M, Sasaki, S, Chang, M L and Hsu, H. Y (1966) *Chem. Commun.* 915
9 Hikino, H and Hikino, Y (1970) in *Progress in the Chemistry of Organic Natural Products* (Herz, W, Grisebach, H and Scott, A I, eds) Vol. 28, p 256 Springer, Vienna
10. Tschesche, R and Wulff, G (1977) in *Progress in the Chemistry of Organic Natural Products* (Herz, W., Grisebach, H and Kirby, G W eds) Vol 30, p 461 Springer, Vienna
11 Hostettmann, K (1984) *Naturwissenschaften* **71**, 247
12. Moreno, M and Rodriguez, V. M (1981) *Phytochemistry* **20**, 1446
13 Woo, W S., Shin, K H and Kang, S. S (1976) *Kor J Pharmacogn.* **7**, 47
14 Waller, D P, Zaneveld, L. J D and Fong, H H S (1980) *Contraception* **22**, 183