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CYTOTOXIC STEROIDS OF *GELSEMIUM SEMPERVIRENS*

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ABSTRACT.—A new pregnane derivative, 12β-hydroxy-5α-pregn-16-ene-3,20-dione [**1**], along with the known derivative 12β-hydroxy-pregna-4,16-diene-3,20-dione [**2**] have been isolated from a MeOH extract of the stem of *Gelsemium sempervirens* and found to be the principal cytotoxic entities. The ^{13}C-nmr spectra of both compounds were assigned by comparison with other pregnane analogs thereby allowing confirmation of the stereochemistry at C-5 in compound **1**. Heteronuclear 2D correlation and selective INEPT experiments indicated the need to revise a number of ^{13}C-nmr assignments of pregn-4,16-dien-3,20-dione. Nine indole alkaloids, gelsemine, gelsevirine, 21-oxogelsemine, gelsedine, 14β-hydroxygelsedine, gelsenicine, humantenidine, humantenirine, and koumidine were found to be inactive in the KB and P-388 cytotoxicity test systems.

Our previous chemical studies of the stems of *Gelsemium sempervirens* (L.) Jaume St.-Hilaire (Loganiaceae) resulted in the isolation and identification of 14β-hydroxygelsedine (1), gelsemine (2), gelsevirine, and 21-oxogelsemine (3). Besides gelsedine (4), four other indole alkaloids, gelsenicine (=humantenimine) (5,6), humantenidine (7), humantenirine (8), and koumidine (9), which were reported recently from Chinese *Gelsemium (G. elegans)*, were isolated for the first time from the stem, roots, and leaves of *G. sempervirens* (10).

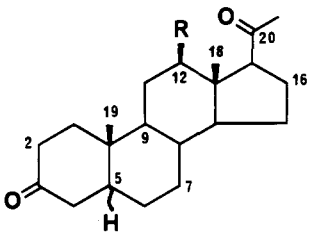
As part of our program on the isolation of plant anticancer agents, we found that extracts of *G. sempervirens* displayed activity in the PS and KB test system in vitro (11,12). Bioassay-directed fractionation of the extract through column chromatography and preparative tlc afforded two active principles identified as compounds **1** and **2**, while the nine indole alkaloids were found to be inactive. In this report, we wish to present the structure elucidation of the two active principles.

Compounds **1** and **2** showed many similar chromogenic and spectroscopic properties justifying the presence of an α,β-unsaturated CO group, a 12β-hydroxyl group (δ 3.69, J=5 and 11 Hz) and similar environments for CH_3-21 (δ 2.38 ppm) and CH_3-18 (δ 0.89 ppm) in both isolates.

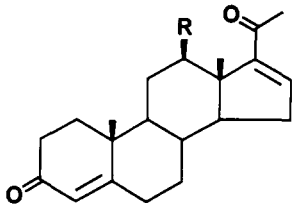
The mass spectrum of **2**, however, exhibited a molecular ion at *m/z* 328, 2 amu less than that of **1** (M^+,330) revealed as an additional double bond by the presence of an olefinic proton at δ 5.74 for H-4. Deshielding by the $\Delta^{4,5}$-bond caused the CH_3-19 to shift downfield to δ 1.22 ppm from δ 1.05 in compound **1**. The mass spectral fragmentations of **1** and **2** were in agreement with the pattern reported for pregnene derivatives (13,14), with prominent ions at *m/z* 124, 123, and 43. Compound **2** was, therefore, identified as 12β-hydroxy-pregna-4,16-diene-3,20-dione. This pregnane derivative, previously isolated only from *Anodendron affine* (Apocynaceae), was identified by direct comparison with an authentic sample (15).

Compound **1** showed a molecular ion at *m/z* 330, consistent with a molecular formula of $C_{21}H_{30}O_3$. No signal was observed at δ 5.74 for H-4 in the ^{1}H-nmr spectrum, and the isolate was, therefore, assigned the structure 12 β-hydroxy-pregn-16-ene-3,20-dione.

It is well established that the chemical shift of C-19 differs by 11-12 ppm between 5α-H and 5β-H steroids due to the shielding of the C-19 methyl carbon (16). The chemical shift of C-19 in compound **1** indicated the presence of a 5α-pregnane-3,20-dione [**3**] rather than a 5 β-pregnane-3,20-dione [**4**] (Table 1). The close chemical shifts of the methyl group carbons, C-18 and C-19, in **1** were defined by a 1D



	R	5-H	
1	OH	α	$\Delta^{16,17}$
3	H	α	
4	H	β	



	R
2	OH
5	H

heteronuclear correlation experiment (17). This represents the first reported isolation of 12 β-hydroxy-5α-pregn-16-ene-3,20-dione [**1**].

In order to assign the ^{13}C-nmr spectrum of compound **2**, commercially available pregna-4,16-diene-3,20-dione [**5**] was chosen as the model compound. The ^{13}C-nmr spectrum of **5** was first determined by Reich *et al.* in 1969 (18) and refined by Blunt *et al.* in 1977 (16), based on comparison with a series of steroids. Probably due to solvent effects, the spectrum measured previously in $CDCl_3$ and dioxane differed from the spectrum obtained in $CDCl_3$ in this study (Table 1). The assignments for C-16 and C-17 need to be revised, based on the APT and hetero 2D experiments, in as much as correspondence was observed between the most downfield proton (H-16) at δ 6.75 and the

TABLE 1. Carbon-13 Spectral Data of 12 β-Hydroxy-5α-pregn-16-ene-3,20-dione [**1**], 12 β-Hydroxy-pregna-4,16-diene-3,20-dione [**2**], 5 α-Pregnane-3,20-dione [**3**], 5 β-Pregnane-3,20-dione [**4**], and Pregna-4,16-diene-3,20-dione [**5**]

Carbon	Compounds					
	1[a]	**2**[a]	**3**[a,b]	**4**[a,b]	**5**[b,c]	**5**[a,d]
1	37.96	35.30	38.5	37.1	36.0	34.90
2	37.96	33.83	38.0	37.0	34.1	33.32
3	211.54	198.86	212.3	212.3	197.6	198.45
4	44.32	124.13	44.5	42.3	124.4	123.23
5	46.43	169.87	46.6	44.2	169.7	170.22
6	30.77	32.45	28.8	25.8	32.8	32.06
7	29.26	30.97	31.6	26.6	32.5[e]	31.18
8	32.31	32.52	35.4	35.6	34.4	33.14
9	52.38	52.39	53.7	40.8	54.8	53.44
10	35.62	38.45	35.8	34.9	39.0	38.08
11	28.52	28.75	21.2	21.2	21.2	20.12
12	73.28	73.11	38.9	39.2	35.2	33.84
13	51.34	51.76	44.1	44.2	46.6	45.38
14	53.58	53.14	56.4	56.6	56.3	54.99
15	32.00	31.99	24.3	24.4	32.3[e]	31.49
16	149.65	149.37	22.9	23.0	155.9[f]	143.68
17	155.11	154.95	63.6	63.9	143.5[f]	154.63
18	11.58	11.56	13.4	13.5	16.0	15.22
19	11.16	16.97	11.4	22.6	17.1	16.57
20	198.93	199.26	210.0	208.8	195.7	195.79
21	26.58	26.65	31.4	31.4	26.7	26.52

[a]Measured in $CDCl_3$.
[b]Data from Blunt and Stothers (16).
[c]Measured in $CDCl_3$+dioxane.
[d]Data from this study with purchased compound.
[e,f]Assignments may need to be revised.

carbon resonance at 143.68 ppm. Selective INEPT spectra (19) obtained through the irradiation of H_3-18, H_3-19, H-4, or H-16, with coupling constants of 8 or 5 Hz, permitted observation of the corresponding carbons 2 or 3 bonds away, i.e. C-12, C-14, and C-17; C-1, C-5, and C-9; C-2, C-10, and C-6; C-14, C-15, and C-13, C-20, respectively. In this way, the unambiguous carbon-13 assignments of compound **5** in $CDCl_3$ were established. The close chemical shifts of C-6, C-7, and C-15 were differentiated as discussed above, and as a result, the previous assignments of C-6 and C-15 should be revised.

The ^{13}C-nmr assignments for **2** were made by comparison with those established for **5**. Substitution by the 12-OH induced downfield shifts for C-12 (+39), C-13 (+6), and C-11 (+9) and upfield shifts for C-14 (−2) and C-9 (−1) as anticipated (20).

Compound **1** displayed the same C,D ring structure as **2**, and its ^{13}C-nmr spectral data for the A,B rings were closer to those of 5α-pregnane-3,20-dione [**3**]. In order to differentiate the close chemical shifts of C-18 and C-19, irradiation of the downfield satellite of H-19 at δ 1.05, besides enhancing the corresponding carbon at δ 11.6, affected the resonance at δ1.01 (H-6) demonstrating the corresponding carbon shift to be δ 30.77. In the same way, irradiation of H-18 (effect of H-9) or H-21 (effect of H-7) afforded the corresponding carbon assignments, permitting the close chemical shifts of C-6 and C-7 to be unambiguously assigned. The ^{1}H nmr of compound **1** could be assigned by a homonuclear COSY experiment, although some of the contours overlapped substantially.

Compounds **1** and **2** are the first pregnane derivatives to be isolated from the Loganiaceae. A closely related cytotoxic pregnane derivative was recently reported from *Stizophylum ruparium* (Bignoniaceae) (21).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined by means of a Kofler hotplate and are uncorrected. The uv spectrum was obtained with a Beckman model DU-7 spectrophotometer. The ir spectrum was determined on a Nicolet MX-1 interferometer. The ^{1}H-nmr spectra were recorded on a Nicolet NMC 360 (360MHz) with a Nicolet Fourier-Transform attachment using $CDCl_3$ as solvent and TMS as an internal standard. The ^{13}C-nmr spectra were obtained on a Nicolet NMC 360 instrument operating at 90.8 MHz. The mass spectra were obtained with a Finnigan/MAT 112S double focussing spectrometer operating at 70 eV. Optical rotations were measured with a Perkin-Elmer, Model 241 polarimeter. Silica gel for chromatography was purchased from E. Merck, Darmstadt, W. Germany, and preparative tlc plates were purchased from Analtech, Newark, Deleware. 16-Dehydroprogesterone (=pregn-4,16-dien-3,20-dione) was purchased from Sigma (St. Louis, Missouri) and after tlc analysis was used without further purification.

PLANT MATERIAL.—Dried stem material of *G. sempervirens* was collected during the spring of 1983 from Texas and identified by Dr. C.W. Morden. Voucher specimens are deposited in the S.M. Tracy Herbarium, Texas A&M University, College Station, Texas, and the Field Museum of Natural History, Chicago, Illinois.

EXTRACTION AND PURIFICATION.—Compounds **1** and **2** were isolated from the stem material of *G. sempervirens* through MeOH extraction, acid-base work-up, and column chromatography as described in a previous publication (1).

ISOLATION OF 12β-HYDROXY-5α-PREGN-16-ENE-3,20-DIONE [**1**].—The petroleum ether-EtOAc (8:2) eluent (0.42 g) from the column chromatography was further subjected to preparative tlc on silica gel initially developed in $CHCl_3$-MeOH-$(C_2H_5)_2NH$ (200:1:1) to afford a band at Rf 0.26, which was further triple developed in the system $CHCl_3$-MeOH-$(C_2H_5)_2NH$ (99:1:1). The Me_2CO eluent of the band at Rf 0.8 was washed with hexane and recrystallized from Me_2CO to afford needles of **1** (23 mg), mp 231-233°; $[\alpha]_D$+27° (*c* 0.03, MeOH); uv λ max (MeOH) 246 nm (log ε 4.06); ir ν max (KBr) 3390, 2947, 1714, 1637, 1580, 1429, 1367, 1248, 1022 cm^{-1}; ^{1}H nmr (360 MHz, $CDCl_3$) δ 6.98 (1H, dd, *J*=1.9, 3.2 Hz, H-16), 5.83 (1H, s, D_2O exchangeable, OH-12), 3.69 (1H, dd, *J*=5.0, 10.6 Hz, H-12), 2.43 (1H, q, *J*=3.3 Hz, H-15α), 2.40 (1H, m, H-4β), 2.38 (3H, s, H_3-21), 2.34 (2H, m, H_2-2), 2.27 (1H, d, *J*=14.1 Hz, H-4α), 2.18 (1H, ddd, *J*=1.6, 5.8, 12.6 Hz, H-7β), 2.04 (2H, ddd, *J*=2.2, 6.4, 13.2 Hz,

H-1α, H-15β), 1.92 (1H, dt, J=4.7, 13.3 Hz, H-11), 1.45 (1H, dd, J=2.4, 13.2 Hz, H-11), 1.30 (7H, m, H-5, H-7α, H-8, H-6β, H-11β, H-14, and H-1β), 1.05 (3H, s, CH_3-19), 1.01 (1H, dd, J=5.4, 12.0 Hz, H-6α), 0.95 (1H, dd, J=5.0, 9.8 Hz, H-9), 0.89 (3H, s, CH_3-18); ^{13}C nmr see Table 1; ms *m/z* (rel. int.) 330 (M^+, 25), 315 (10), 297 (7), 123 (56), 43 (100).

ISOLATION OF 12β-HYDROXY-PREGNA-4,16-DIENE-3,20-DIONE [**2**].—The petroleum ether-EtOAc (8:3) eluent from the column chromatography of the total alkaloid fraction was subjected to repeated preparative tlc using petroleum ether-C_6H_6-EtOAc-$(C_2H_5)_2NH$ (5:2:2:0.8) as the eluent and further purified on a silica gel plate eluting with $CHCl_3$-MeOH-$(C_2H_5)_2NH$ (99:0.5:0.5). The band at Rf 0.21 was removed and processed to afford colorless needles of **2** (8.5 mg): mp 232-233°; $[\alpha]_D$+115° (*c* 0.17, MeOH); uv λ max 244 nm (log ε 4.55); ir ν max (KBr) 3370, 1673, 1638, 1440, 1371, 1244, 1018, 862 cm^{-1}; ^{1}H nmr (360 MHz, $CDCl_3$) δ 6.98 (1H, dd, J=2.0, 3.3 Hz, H-16), 5.86 (1H, s, D_2O exchanged, OH-12), 2.39 (3H, s, CH_3-21), 1.22 (3H, s, CH_3-19), 0.92 (3H, s, CH_3-18); ^{13}C nmr see Table 1; ms *m/z* (rel. int.) 328 (M^+, 8), 313, (2), 285 (1), 124 (13), 123 (15), 43 (100).

BIOLOGICAL ACTIVITY OF **1** AND **2**.—The isolates were evaluated for cytotoxicity according to established protocols (11,12). It was found that compounds **1** and **2** were cytotoxic in the PS and KB test systems in vitro (ED_{50} 0.9, 0.7 in PS and 2.8, 2.0 μg/ml in KB, respectively).

CHROMATOGRAPHY OF BASIC FRACTION.—The total alkaloid extract obtained previously (1) was subjected to column chromatography on silica eluting with petroleum ether and increasingly polar mixtures of petroleum ether and EtOAc to successively afford ursolic acid (35 mg, 0.008%), scopoletin (884 mg, 0.29%), gelsemine (2.4 g, 0.08%), 21-oxogelsemine (4 mg, 0.0001%) (3), gelsevirine (25 mg, 0.06%), gelsedine (7 mg, 0.0002%), 14-hydroxygelsedine (15 mg, 0.0005%), gelsenicine (6 mg, 0.0002%), humantenidine (1.5 mg, 0.0003% of leaves), humantenirine (15 mg, 0.0005%), and koumidine (15 mg, 0.0005%). None of the isolated alkaloids displayed activity in the KB or P-388 test systems in vitro (11).

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