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MACROCYCLIC PYRROLIZIDINE ALKALOIDS FROM *SENECIO ANONYMUS*. SEPARATION OF A COMPLEX ALKALOID EXTRACT USING DROPLET COUNTER-CURRENT CHROMATOGRAPHY[1]

LEON H. ZALKOW,* CLARITA F. ASIBAL, JAN A. GLINSKI, SANDRA J. BONETTI, LESLIE T. GELBAUM,[2] DONALD VANDERVEER, and GARTH POWIS[3]

School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332

ABSTRACT.—Ten 12-membered macrocyclic pyrrolizidine alkaloids, all of them esters of the necines, retronecine or otonecine, have been isolated from *Senecio anonymus*. The separation, carried out by droplet counter-current chromatography, afforded senecionine [**1**], integerrimine [**2**], retrorsine [**3**], senkirkine [**5**], neosenkirkine [**6**], otosenine [**10**], hydroxysenkirkine [**7**], and a new alkaloid given the trivial name anonamine [**9**]. Traces of usaramine [**4**] and another new alkaloid, hydroxyneosenkirkine [**8**], were detected by ^{1}H nmr. In addition, the previously unreported 3aβ-hydroxy-4-ethoxy-2,6-perhydroindoledione [**11**] was isolated. X-ray structures were obtained for neosenkirkine [**6**], hydroxysenkirkine [**7**], anonamine [**9**], and [**11**]. ^{1}H-^{13}C heteronuclear shift correlated nmr (HETCOR) provided unambiguous chemical shift assignments for ^{13}C-nmr data. Antitumor activity was assayed using the A204-rhabdomyosarcoma cell line in soft agarose.

Of the pyrrolizidine alkaloid (PA) containing plants, the genus *Senecio* (Compositae), having the largest number of species (ca. 1450), has generated numerous studies. The most current lists (1–3) of investigated species, however, indicate that only about 10% of the genus has thus far been studied. Most of these contain hepatotoxic PAs (esters of 1,2- unsaturated necines), and a number of these hepatotoxic alkaloids have been shown to be mutagenic. Pyrrolizidine alkaloids and their pyrrolic metabolites have been implicated in megalocytosis and mitotic inhibition (1), and recently semisynthetic pyrrolizidine alkaloid *N*-oxides have been investigated as antitumor agents (4,5). The work described in this paper, which continues our earlier work (6) on isolation and structure elucidation of pyrrolizidine alkaloids, involves the separation of pyrrolizidine alkaloids from a locally abundant species, *Senecio anonymus* Wood (formerly called *Senecio smallii*) from which a cytotoxic compound, jacaranone ethyl ester, was previously isolated in our laboratory (7).

RESULTS AND DISCUSSION

Examination of the alkaloidal fraction from *S. anonymus* led to the identification of ten 12-membered macrocyclic pyrrolizidine alkaloids. Four of these, senecionine [**1**] (8,9), integerrimine [**2**] (9,10), retrorsine [**3**] (10–12), and usaramine [**4**], known also as mucronatinine (10,13), are esters of retronecine while the six remaining, senkirkine [**5**] (14–16), neosenkirkine [**6**] (17,18), hydroxysenkirkine [**7**] (15,19), hydroxyneosenkirkine [**8**], anonamine [**9**], and otosenine [**10**] (20,21), are esters of otonecine.

The macrocyclic ester rings are formed by six different but closely related necic dicarboxylic acids. Thus, **1** and **5** are esters of senecic acid, **2** and **6** are esters of integerrinecic acid, **3** and **7** are esters of isatinic acid, and **4** and **8** are esters of *trans*-isatinic acid, while **10** is an ester of jacobinic acid and **9** is an ester of 7-hydroxyintegerrinecic acid. Since senecic and integerrinecic acids as well as isatinic and *trans*-isatinic acids are

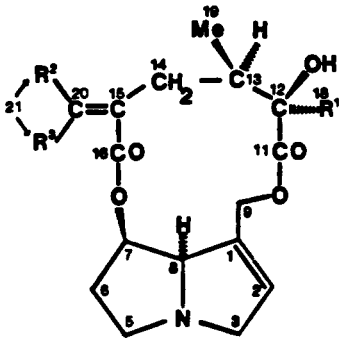
[1]Taken from the Ph.D. dissertation of Clarita Florendo Asibal, School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332, June, 1987.

[2]Research Center for Biotechnology, Georgia Institute of Technology, Atlanta, Georgia 30332.

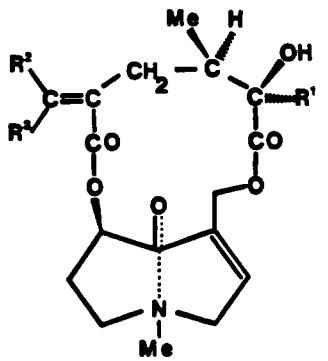
[3]Department of Oncology, Mayo Clinic and Foundation, Rochester, Minnesota 55905.

geometric isomers, the eight esters **1–8** constitute four pairs of alkaloids differing only in the configuration of the C-15–C-20 double bond. Therefore, the physical properties of these geometric isomers are very similar, posing a serious problem in separation.

The initial EtOH plant extract was partitioned to give the *N*-oxides in an H_2O layer and the free bases in a $CHCl_3$ layer. The ratio of *N*-oxides to free bases varied from 6:1 to 10:1 depending on the plant parts (leaves, flowers, stems). Separate analyses of the extracts from the flowers, leaves, and stems combined with roots revealed a specific distribution of the alkaloids. Thus, the major PAs from the flowers were **10**, **1**, and **3**, from the leaves **5**, **6**, and **7**, and from the stems and roots **5**, **6**, **3**, and **1**. The total estimated alkaloid content of the whole plant was 0.02%, and the relative percentages of the various alkaloids were **5** (41.0), **6** (20.5), **1** (13.3), **7** (7.8), **10** (6.7), **3** (4.1), **9** (3.8), **2** (2.0), **8** (0.5), and **4** (0.3%).



1 $R^1=R^3=Me$, $R^2=H$ (senecionine)
2 $R^1=R^2=Me$, $R^3=H$ (integerrimine)
3 $R^1=CH_2OH$, $R^2=H$, $R^3=Me$ (retrorsine)
4 $R^1=CH_2OH$, $R^2=Me$, $R^3=H$ (usaramine)



5 $R^1=R^3=Me$, $R^2=H$ (senkirkine)
6 $R^1=R^2=Me$, $R^3=H$ (neosenkirkine)
7 $R^1=CH_2OH$, $R^2=H$, $R^3=Me$ (hydroxysenkirkine)
8 $R^1=CH_2OH$, $R^2=Me$, $R^3=H$ (hydroxyneosenkirkine)
9 $R^1=Me$, $R^2=CH_2OH$, $R^3=H$ (anonamine)
10 $R^1=R^3=Me$, $R^2=H$, epoxide (15*S*, 20*S*) in place of Δ^{15} (otosenine)

Several chromatographic methods, including gravity column, tlc, centrifugal tlc, hplc, and droplet counter-current chromatography (dccc) were evaluated for their efficiency in the separation of the complex alkaloid mixture. Finally, dccc was chosen as the most suitable method for preparative separation. As we have observed many times, PAs tend to adsorb irreversibly to solid phases such as alumina, silica, or reversed-phase in

hplc, resulting in large losses. Dccc, a technique based on partition between two immiscible liquid phases, is free of this drawback. Moreover, with a properly selected solvent system, it gives good separation with low solvent consumption (separation of 3 g of a crude mixture consumed only about 4 liters of solvent mixture).

In an attempt to find a solvent system most suitable for our needs, we measured the partition coefficients of the model compounds 9-benzoylretronecine, indicine, monocrotaline, and retronecine in the three solvent systems containing $CHCl_3$-MeOH-H_2O in ratios of 13:7:4, 7:13:8, and 5:6:4 and in $CHCl_3$-C_6H_6-MeOH-H_2O, 5:5:7:2 (22). This group of model compounds provides a representation of low, medium, and high polarity PAs. The first three solvent systems gave fairly similar results and were suitable for separation of more polar PAs, while the C_6H_6-containing system was found to be better suited for moderately polar PAs. Because most of the alkaloids present in *S. anonymus* are moderately polar, this system was selected for the initial run.

The collected fractions were monitored by tlc and by 300-MHz ^{1}H nmr. The ^{1}H-nmr spectra were especially useful in determining compositions of the fractions containing mixtures of *cis-trans* isomeric alkaloids; these could be distinguished by their H-20 absorptions which occur at δ 6.5–6.8 for the *cis* isomers **2**, **4**, **6**, and **8** and about δ 5.7–5.9 for the *trans* isomers **1**, **3**, **5**, and **7**. Overall results of the dccc separation exceeded our expectation, affording pure samples of 8 out of a total of 10 alkaloids present in the plant plus the non-alkaloidal compound **11**. Only two very minor components, **4** and **8**, have not been fully separated from their stereoisomers **3** and **7**, respectively; their ^{1}H-nmr data were acquired from the most enriched fractions. Attempts to separate the pairs of *cis-trans* diastereomers using other chromatographic methods (gravity columns, traditional and centrifugal tlc, and reversed-phase hplc) resulted only in slight enrichments, low recoveries, and/or broad peaks.

The stereoisomers **1-2**, **3-4**, **5-6**, and **7-8** differ only in the configuration about the C-15–C-20 double bond. ^{1}H-nmr spectroscopy readily distinguishes between stereoisomeric pairs by the chemical shift positions of the H-20 quartet which appears at δ 5.70–5.86 for the *trans* isomers **1**, **3**, **5**, and **7** and at δ 6.49–6.77 for the *cis* isomers **2**, **4**, **6**, and **8** (see Table 1). In the new alkaloid, anonamine [**9**], instead of a quartet, an AB pattern is seen at δ 6.63 for H-20. This signal was shown by both decoupling experiment and ^{1}H-^{1}H shift correlated spectroscopy (COSY; Figure 1) to be coupled to the two sets of doublet-of-doublets at δ 4.19 and 4.40. Anonamine [**9**] differs from neosenkirkine [**6**] only by the replacement of the C-21 methyl with a hydroxymethylene group. The postulated structure of another new alkaloid which we have named hydroxyneosenkirkine [**8**] is based on a comparison of its ^{1}H-nmr spectrum with those of hydroxysenkirkine [**7**] and neosenkirkine [**6**]. The ^{1}H-nmr spectra of **7** and **8** are very similar, displaying characteristic and essentially identical AB patterns for the H_2-18 at δ 3.66 and 3.74 in **7** and at δ 3.69 and 3.76 in **8**. The two spectra differ significantly only in the areas of olefinic absorption. Isomer **8** also resembles **6**, where the H-20 absorption is found at δ 6.71. The structures of **6**, **7**, and **9** were confirmed by single crystal X-ray crystallographic analyses (23). Structures of the other alkaloids were established by melting points, optical rotations, high resolution ms and nmr, and direct comparisons with authentic samples (**1**, **3**, **10**) and/or with literature data (**2**, **4**, **5**).

^{13}C-nmr spectra were obtained for all the alkaloids except **4** and **8** (see Table 2), and unambiguous assignments for all H-containing carbons were based on ^{1}H-^{13}C heteronuclear shift correlated nmr (HETCOR; Figure 2). For example, the clear distinction between the chemical shifts of C-2 and C-20 in retrorsine [**3**], which were previously interchanged (24) is illustrated in Figure 3. Table 2 indicates chemical shifts that were misassigned or interchanged in earlier reports.

As seen in Table 2, *cis* isomers **2**, **6**, and **9** show more shielded C-14's with values of

TABLE 1. 400 MHz ^{1}H nmr of Secopyrrolizidine Alkaloids from *Senecio anonymus*.

Proton	Compound				
	5	**6**	**7**	**8**	**9**
2	6.11 t (J = 2 Hz)	6.14 br s	6.15 br s	6.20 br s	6.12 br s
3β	3.21 dt (J = 19,2 Hz)	3.18 dt (J = 19,3 Hz)	3.24 ABq (J = 18 Hz)	3.24 ABq (J = 18 Hz)	3.20 dt (J = 18,2 Hz)
3α	3.40 d (J = 19 Hz)	3.35 d (J = 19 Hz)	3.41 ABq (J = 18 Hz)	3.37 ABq (J = 18 Hz)	3.39 d (J = 18 Hz)
5β	2.71 m	2.68 m	2.73 dt (J = 12,4 Hz)	2.80 m	2.74 m
5α	2.86 dt (J = 19,2 Hz)	2.79 m	2.86 m	2.87 m	2.84 m
6β	2.33 m	2.34 m	2.29 m	2.34 m	2.30 m
6α	2.53 m	2.36 m	2.53 m	2.42 m	2.48 m
7	4.96 t (J = 3 Hz)	4.93 t (J = 5 Hz)	4.97 t (J = 3 Hz)	4.99 t (J = 3 Hz)	5.00 t (J = 5 Hz)
9u	4.33 d (J = 11 Hz	4.39 d (J = 11 Hz)	4.41 d (J = 11 Hz)	4.50 d (J = 11 Hz)	4.33 d (J = 11 Hz)
9d	5.39 d (J = 11 Hz)	5.35 d (J = 11 Hz)	5.39 d (J = 11 Hz)	5.37 d (J = 11 Hz)	5.28 d (J = 11 Hz)
13	1.67 m	1.87 d (J = 7 Hz)	1.75 m		1.92 m
14β	1.77 d (J = 13 Hz)	1.99 d (J = 12 Hz)	1.82 d (J = 13 Hz)		2.18 d (J = 14 Hz)
14α	2.28 d (J = 13 Hz)	2.20 d (J = 12 Hz)	2.33 d (J = 13 Hz)		2.19 d (J = 14 Hz)
18	1.31 s	1.31 s	3.66 AB (J = 11 Hz)	3.69 ABq (J = 11 Hz)	1.28 s
			3.74 AB (J = 11 Hz)	3.76 ABq (J = 11 Hz)	
19	0.89 d (J = 6 Hz)	0.87 d (J = 7 Hz)	0.84 d (J = 6 Hz)	0.84 d (J = 7 Hz)	0.87 d (J = 7 Hz)
20	5.85 dq (J = 7,1 Hz)	6.71 q (J = 8 Hz)	5.86 dq (J = 7,1 Hz)	6.77 q (J = 7 Hz)	6.63 AB (J = 5 Hz)
21	1.88 dd (J = 7,2 Hz)	1.76 d (J = 7 Hz)	1.89 dd (J = 7,2 Hz)	1.78 d (J = 7 Hz)	4.19 dd (J = 14,5 Hz)
					4.40 dd (J = 14,5 Hz)
NMe	2.07 s	2.09 s	2.10 s	2.11 s	2.06 s

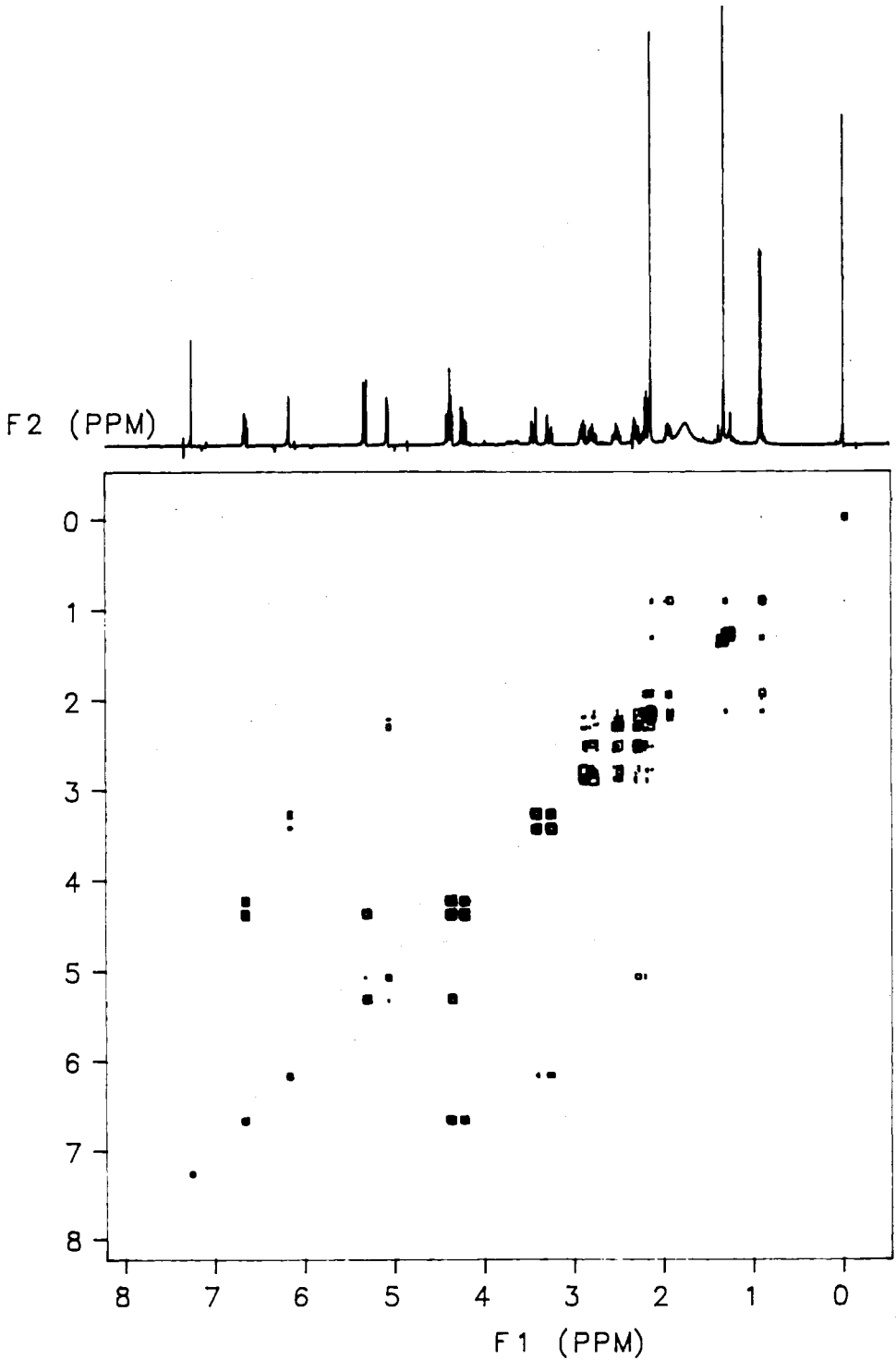


FIGURE 1. ^{1}H-^{1}H shift correlated spectroscopy (COSY) of anonamine [9].

TABLE 2. ^{13}C nmr of 12-Membered Macrocyclic Pyrrolizidine Alkaloids.

Carbon	Compound							
	1	**2**	**3**	**5**	**6**	**7**	**9**	**10**
1	(131.41)[a]	(131.63)[d]	(132.38)[b]	134.25	134.30	133.82	134.08	134.08
2	136.60	136.59	(136.85)[d]	137.28	137.45	136.71	137.40	137.00
3	(62.87)[f]	62.62	(62.89)[b]*	(58.53)[a]	58.16	56.72	58.88	(58.80)[f]
5	53.03	53.15	53.05	53.13	53.09	53.31	53.69	52.94
6	(34.79)[d]	33.88	(37.97)[a]	(36.28)[d]	34.93	35.83	35.36	(36.96)[d,f]*
7	(74.93)[f]*	75.51	(75.12)[c]	78.00	79.06	77.87	78.48	78.29
8	(77.62)[f]*	77.15	(77.56)[c]	191.77	192.15	185.30	191.71	190.69
9	(60.64)[f]	60.99	(61.26)[b]*	(64.27)[a]	64.70	64.04	64.74	(64.00)[f]
11	178.19	177.63	175.50	177.97	178.07	175.09	178.30	(177.55)[f]**
12	76.75	76.59	81.32	77.31	76.57	81.02	76.37	76.81
13	(38.35)[e]	39.56	35.77	38.51	39.53	35.56	38.30	38.35
14	(38.23)[e,d]	29.68	(34.87)[a]	(37.60)[d]	28.87	37.17	30.34	(35.37)[d,f]*
15	(133.05)[a]	(133.81)[d]	(131.23)[b]	131.76	132.48	130.79	132.34	63.59
16	167.55	168.92	167.37	166.39	167.87	160.85	166.72	(167.87)[f]**
18	24.92	25.29	66.88	24.47	24.74	66.30	25.52	23.75
19	11.09	11.97	11.84	10.87	11.46	11.29	12.46	12.35
20	134.13	135.22	(134.58)[d]	136.99	138.00	137.22	140.61	55.86
21	14.99	14.32	15.21	15.22	14.46	15.10	59.28	13.44
22	—	—	—	40.35	41.05	40.47	41.10	39.86

[a–f]Values in parentheses are interchangeable in the reference indicated by the superscript: [a]Molyneux *et al.* (32), [b]Drewes *et al.* (24), [c]Mody *et al.* (33), [d]Jones *et al.* (34), [e]Liang and Roeder (8), [f]Roder *et al.* (20). Asterisks indicate couplings within a particular reference.

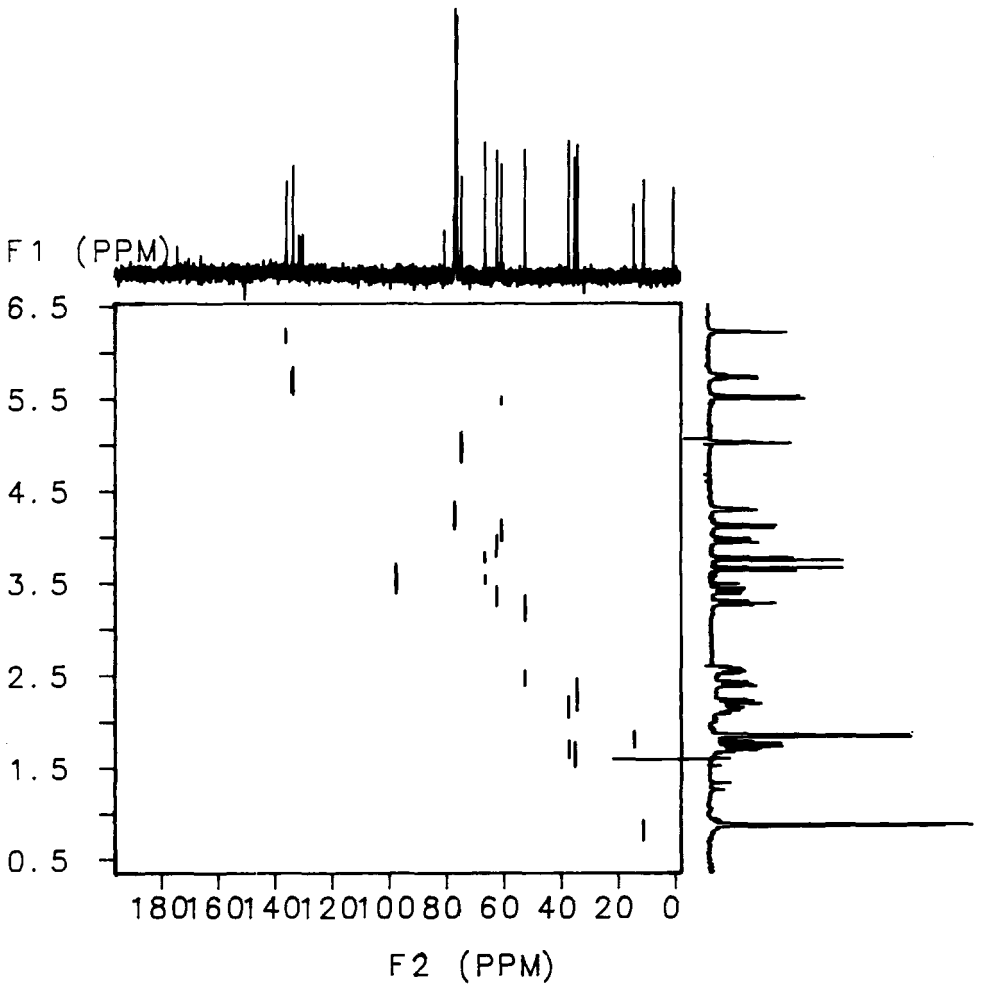


FIGURE 2. ^{1}H-^{13}C heteronuclear shift correlated nmr (HETCOR) of retrorsine [**3**].

29.68, 28.87, and 30.34 ppm, respectively, because of the *cis* interaction with the C-21 groups, while corresponding values for the *trans* isomers **1**, **3**, **5**, **7**, and **10** are 38.23, 34.87, 37.60, 37.17, and 35.37, respectively. The change of the C-18 methyl group in **5** to a hydroxymethylene group in **7** is evident from the downfield shift of this carbon from 24.47 to 66.30 ppm. In a similar manner, the change from a C-21 methyl group in **6** to hydroxymethylene group in **9** results in a chemical shift change from 14.46 to 59.28 ppm.

There are a number of reports on the co-occurrence of the diasteromers **1-2** (11,16,25), **3-4** (9,25), and **5-6** (17,18). For the pair **7-8**, this is the first report of isomer **8** and only the third time that **7** has been reported (15,19). On the basis of the literature cited above, it seemed to be an unusual coincidence to find 4 pairs of geometric isomers in one plant. Therefore, we attempted to examine the possibility of *cis-trans* interconversion by subjecting the *N*-oxide of **1** to the same work-up as that used for the crude extract, including zinc-acid reduction. The test revealed no formation of the isomeric **2**. *Cis-trans* isomerization has been induced by uv irradiation (25) to convert **4** into **3** and by bromination/debromination (26) to convert **3** into **4**.

The identity of a new, non-alkaloidal compound, isolated from the crude alkaloidal extract, was established as 3a-hydroxy-4-ethoxy-2,6-perhydroindoledione [**11**] by X-

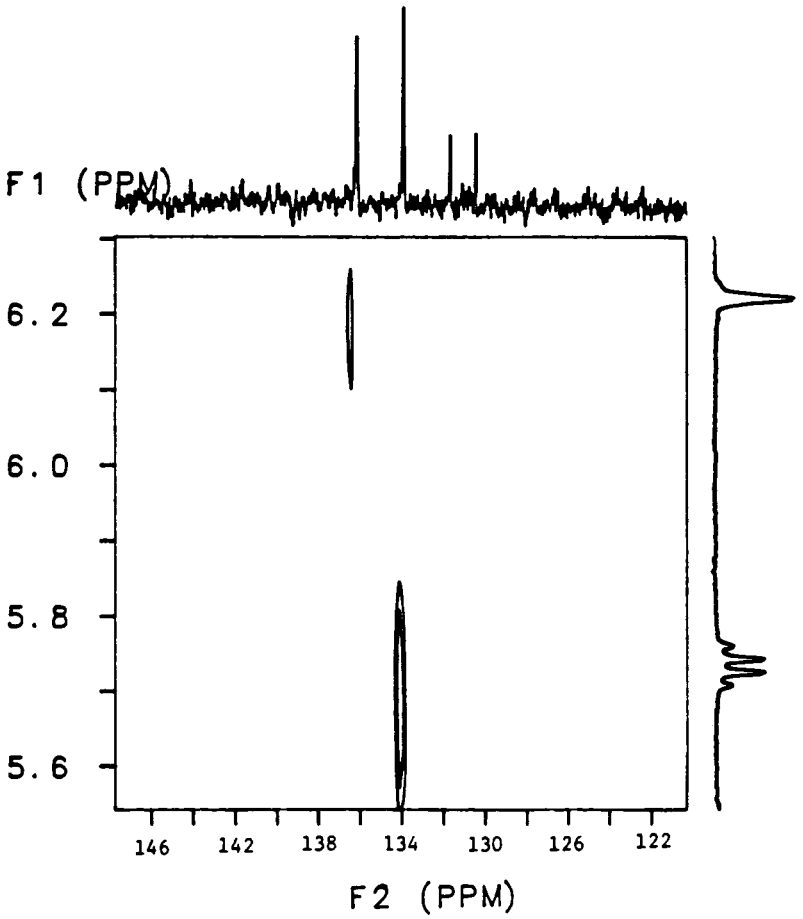


FIGURE 3. Expanded portion of ^{1}H-^{13}C HETCOR nmr of retrorsine [**3**].

ray analysis.[4] A quadrant of data (±h, +k, +l) was collected on a Syntex $P2_1$ diffractometer using omega scans. A total of 1884 unique data were measured out to $2\theta = 50°$, and 1197 reflections were used in a full-matrix least-squares refinement on F. The refinement converged at $R = 0.072$, and $Rw = 0.68$ for 151 parameters varied. Most of the hydrogens were located from a difference Fourier map and were included in the refinement at fixed positions. The hydrogens on C-10 and C-11 were calculated as members of fixed groups. Figure 4 shows a computer-drawn picture of **11**. The compound bears close resemblance to a number of natural products isolated recently from algae (27,28), but contrary to these, it contains a *trans*-fused 2,6-perhydroindoledione skeleton. The monoclinic crystal belonging to the space group $P2_1/n$ showed unit cell parameters $a = 5.6772(8)$, $b = 26.831(4)$, $c = 7.174(2)$ Å, $\beta = 101.00(2)°$, $Z = 4$, $D_c = 1.317$ g cm^{-3}, $\lambda = 0.71969$ Å and has a center of inversion, indicating that the crystal was racemic. Severe overlap of the nmr signals even at 400 MHz made it necessary to determine the chemical shifts of the individual protons using a COSY experiment. Analysis of the COSY data indicated that H-3α, H-5α, and H-7α overlapped at

[4]Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, UK.

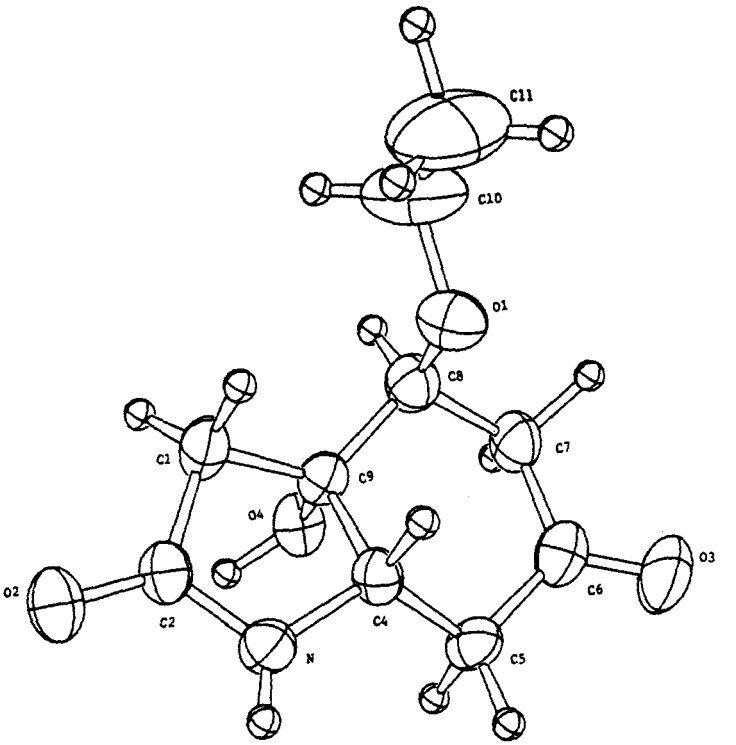
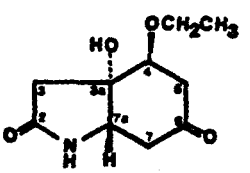


FIGURE 4. Computer-drawn picture of compound **11**.

δ 2.83 and in addition, H-5β and H-7β overlapped at δ 2.53. All chemical shift and coupling constant data are consistent with the structure determined from the X-ray analysis. The origin of **11** remains unclear; most likely it is not a natural product but rather an artifact arising from jacaranone [**12**], a known constituent of *S. anonymus* (7), during the work-up, which included the use of EtOH and NH_3. Reexamination of the crude plant extract, avoiding the use of NH_3 and EtOH, did not lead to the detection of **11**. Alkaloidal fractions frequently are found to contain neutral compounds carried along in the routine work-ups of plant material.

The relative in vitro cytotoxicities of some of the isolated alkaloids and related compounds were measured against the A204 human rhabdomyosarcoma cell line using the soft agar colony forming assay (Table 3). Indicine *N*-oxide was used as an internal standard for each group of compounds in Table 3, because it is a pyrrolizidine alkaloid that was selected for clinical development as an antitumor agent by the National Cancer Institute (29). Compounds **1**, **2**, **3**, **9**, and **10** show similar cytotoxicity, while **5**, **6**, and **7** are less cytotoxic, but all of the compounds tested were more cytotoxic than indicine *N*-oxide in this system. Senecionine *N*-oxide exhibited the same cytotoxicity as senecionine. In the bottom of Table 3 (last 3 entries) monocrotaline [**14**], the semisynthetic compound **15**, and their *N*-oxides are compared with indicine [**13**] and



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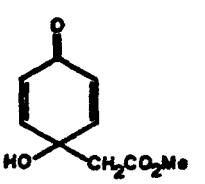

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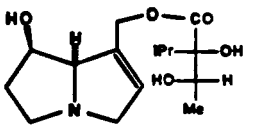

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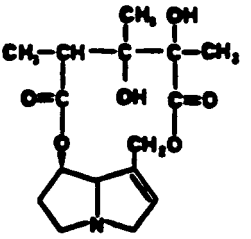

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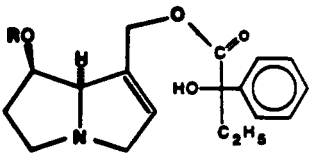

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its *N*-oxide. Semisynthetic **15** *N*-oxide shows excellent cytotoxicity in this assay and excellent in vivo activity (5). We have found an excellent correlation between cytotoxicity against the A204 rhabdomyosarcoma cell line and in vivo activity for a large number of semisynthetic compounds such as **15**, and in every case the *N*-oxides were more cytotoxic than the free bases.[5] The semisynthetic bases were not screened in vivo because of their expected hepatotoxicity. The natural alkaloids indicine [**13**] and monocrotaline [**14**] are more cytotoxic than their corresponding *N*-oxides, but neither monocrotaline nor its *N*-oxide shows much activity in this test.

None of the naturally occurring macrocyclic alkaloids reported in this study would be expected to be useful antitumor agents because their hepatotoxicity would severely limit their usefulness (1).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All separations were carried out using a Buchi 670 dcc Chromatograph equipped with 300 tubes of 2.7 mm i.d. plus 200 tubes of 3.0 mm i.d. The flow rates varied between 15 and 24 ml/h, and eluates were collected by an automatic fraction collector. The separation process was monitored by a combination of tlc analysis on EM Al_2O_3 150 F-254 plates developed in a mixture of toluene with 5 to 15% MeOH or in $CHCl_3$ with 5 to 10% MeOH and ^{1}H-nmr analysis. All ^{1}H- and ^{13}C-nmr spectra were obtained using a Varian XL-400 spectrometer operating at 399.934 MHz and 100.575 MHz, respectively. Chemical shifts are reported relative to residual $CHCl_3$ (7.24 ppm) for ^{1}H and to $CDCl_3$ (77.0 ppm) for ^{13}C. ^{1}H-^{13}C heteronuclear shift correlated nmr spectra were collected as a 128 × 4096 data matrix using the pulse sequence HETCOR supplied with the Varian 6.1 c software (30,31). This was processed using pseudo echo weighting to a 512 × 2048 data matrix for plotting. The hplc experiments were performed on a LDC Constametric III pump equipped with a Rheodyne 7120 sample injector and either a Universil C_{18}, 25 cm × 4.6 mm or an ALLTECH C_{18}, 10μ, 25 cm × 10 mm column and a Holochrome Gilson uv detector (at 220 nm). Two solvent systems were employed: 20–35% EtOH in 0.01 M $(NH_4)_2CO_3$ and 10–50% MeCN in 0.01 M $(NH_4)_2CO_3$ (pH 7.6). Optical rotations were taken with a Perkin-Elmer 141 polarimeter. Mass spectra were obtained on a Varian MAT 112S spectrometer interfaced with an SS200 data system. Melting points were taken on a Kofler hot stage and are corrected.

[5]L.H. Zalkow, unpublished results.

TABLE 3. Cytotoxicity against A204 Rhabdomyosarcoma Cell Line In Vitro.[a]

Compound	Base IC_{50}[b] (μg/ml)	*N*-Oxide IC_{50}[b] (μg/ml)
1	150±6	150±6
2	120±5	
3	120±5	
5	260±20	
6	221+13	
7	360+50	
9	153+4	
10	130+9	
13		440±20
14	316±95	721±38
15	>100	11±0
13	34±11	125±22

[a]See Experimental section for details of preparation of soft agarose cultures. Cultures were conducted in quadruplicate to allow reliable estimates of the variance of the IC_{50} to be obtained. Control cultures with vehicle alone were always run at the same time. Dose-response curves were constructed using at least four drug concentrations to produce between 10 and 99.9% inhibition of cell growth. Dose-response curves were constructed on at least three different preparations.

[b]To obtain the IC_{50}, the drug concentration producing 50% inhibition of cell growth, and its variance, the dose-response data was fitted to a monoexponential curve using a NONLIN nonlinear least squares regression analysis program. Variance of IC_{50} was obtained from the variance of the intercept and slope using Taylor series expansion. Values are mean ± SE.

IN VITRO CYTOTOXICITY.—Soft agarose cultures of A204 human rhabdomyosarcoma cells were performed as follows: Each 35-mm culture dish contained a base layer consisting of 0.5 ml Dulbecco's modified Eagle's medium containing 10% fetal calf serum with 0.5% agarose (growth medium). On day 0 cells in bulk culture were dissociated with trypsin and EDTA, washed once in growth medium, and subcultured by layering 1×10^4 viable cells in 0.5 ml growth medium with 0.3% agarose over each base layer. Cultures were examined with the aid of an inverted stage microscope, and only cultures containing uniformly distributed single cell suspensions (< 10 30-μ cell cultures and no 60-μ clusters) were accepted for subsequent evaluation. Cultures were maintained in cell culture incubators at 37°, 5% CO_2, 95% air, and 100% humidity. On day 1 (24 h later) an upper layer of 1 ml growth medium with and without the compound under investigation was added to the dishes. After 24 h, the upper layer of medium was removed by aspiration, agarose culture surfaces washed once with 0.5 ml prewarmed growth medium, and then overlaid with 1 ml of fresh growth medium. Colony formation was examined at daily intervals by conventional light microscopy. Cell lines form a sufficient number of detectable colonies (> 60-μ diameter) for analysis following 7 to 9 days incubation. Viable colonies were stained using a metabolizable tetrazolium salt (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride), and colonies were counted using a Bausch & Lomb FAS-II image analysis system. Cultures were conducted in quadruplicate. Control cultures without drug were run at the same time.

ISOLATION OF THE ALKALOIDAL FRACTION.—Flowering *S. anonymus* was collected 10 miles south of Atlanta, Georgia, in the third week of May 1984, and identified by Dr. Caywood Chapman, Department of Science, Gordon Junior College; a voucher specimen is on deposit at the Georgia Institute of Technology. Different parts of the plant—flowers, leaves, and stems + roots—were dried separately. The air-

dried flowers (3.3 kg) were macerated in a blender with 95% EtOH and allowed to soak in about 5 gal of solvent for at least 2 days at room temperature. The solvent was replaced with fresh solvent three times, and the combined extract was evaporated on a rotary evaporator to leave 0.7 kg of a dark residue. Out of this, 333 g was partitioned between H_2O and $CHCl_3$ (2.0 liters each). The organic layer gave 60.6 g of material which was partitioned between 0.25 liter each of hexane and 90% aqueous MeOH. From the aqueous MeOH layer 15.0 g of a residue was obtained. This was dissolved in 200 ml of 5% NaOH and extracted five times with 300 ml of Et_2O. The Et_2O extract was then washed three times with 150 ml of 10% HCl. Basification of the combined aqueous layer with excess of NH_4OH, followed by extraction with $CHCl_3$, drying over $MgSO_4$, and evaporation in vacuo, yielded 106 mg of a crude alkaloidal fraction (0.007% dry wt). The aqueous layer, 2.0 liters, from the initial $CHCl_3$-H_2O partition was treated with 120 ml of concentrated H_2SO_4 and 24 g of Zn powder. The reaction mixture was stirred overnight and the decanted solution extracted with 4 × 400 ml of $CHCl_3$. The aqueous phase was then made alkaline with excess of aqueous NH_3 and extracted four times with 400 ml of $CHCl_3$. The combined $CHCl_3$ extract was dried over $MgSO_4$, filtered, and evaporated in vacuo leaving 1.28 g of a crude alkaloid fraction (0.04% dry w).

The air-dried leaves, 3.37 kg, were processed in the same manner as the flowers, giving 930 g of a concentrated EtOH extract. The workup afforded 187 mg of the free alkaloid fraction plus 1.98 g after Zn reduction of the *N*-oxides (0.006% and 0.06% dry wt, respectively).

Due to a very low content of PA in the stalks and roots, the EtOH extract (311 g) obtained from 3.0 kg of the dry material was subjected to Zn-acid reduction without prior separation of the free alkaloids. The work-up gave 372 mg of the alkaloid mixture (0.01% dry wt).

Combined leaves and flowers (4.3 kg), collected in the third week of May 1985 in the same vicinity as previously (voucher specimen at Georgia Institute of Technology), were extracted, as before, to give 879 g of concentrated EtOH extract which was directly reduced to give 4.67 g of crude alkaloid fraction (0.01% dry wt).

The dcc chromatograph was filled with the stationary phase consisting of the lower layer of a solvent mixture prepared from $CHCl_3$-C_6H_6-MeOH-H_2O (5:5:7:2). The alkaloidal fraction of *S. anonymus* (1985 collection), 3.0 g, dissolved in 15 ml of a 1:1 mixture of the upper and lower phases was aspirated into the instrument followed by the mobile phase (upper layer). The fractions, of 20-ml vol, afforded the following alkaloids: 23–26, **9** (37 mg); 27–29, **11** (30 mg); 30, **7** (17 mg); 31–34, **7** + **8** (64 mg); 37–43, **10** (65 mg); 58–60, **3** + **4** (13 mg); 61–64, **3** (30 mg); 68–69, **5** (29 mg); 70–77, **5** + **6** (556 mg); 78–84, **6** (15 mg). From fraction 87 on, the stationary phase started to be pumped out and collected, giving: 114–115, **1** (20.5 mg); 116–119, **1** + **2** (121.5 mg), 120, **2** (8 mg).

SEPARATION OF SENKIRKINE [**5**] AND NEOSENKIRKINE [**6**].—A mixture containing **5** and **6** (ca. 1:1) (158.7 mg) was subjected to dccc in the solvent system $CHCl_3$-C_6H_6-MeOH-H_2O (5:5:7:2) in ascending mode. At the flow rate of 15 ml/h, fractions of 10 ml were collected. Pure **5** (24.8 mg) was eluted in fractions 133–146 while pure **6** (5.7 mg) was obtained from fractions 176–182. Fractions 147–175 contained mixtures of varying ratios of **5** and **6**.

SEPARATION OF HYDROXYSENKIRKINE [**7**] AND HYDROXYNEOSENKIRKINE [**8**].—A mixture of **7** and **8** (ca. 9:1) (94 mg) was subjected to chromatography in the solvent system $CHCl_3$-MeOH-H_2O (13:7:4) in descending mode. At flow rate of 24 ml/h, 20-ml fractions were collected. Fractions 36–37 afforded a mixture of **7** and **8** (9.5 mg) in a 1:2 ratio. Pure **7** (44.1 mg) was obtained from fractions 43–49. Intervening fractions contained varying amounts of both isomers.

ATTEMPTED SEPARATION OF RETRORSINE [**3**] AND USARAMINE [**4**].—A mixture of **3** and **4** (93:7) (40 mg) was subjected to dccc using the solvent system $CHCl_3$-MeOH-H_2O (13:7:4) in descending mode. At flow rate of 24 ml/h, 20-ml fractions were collected. Fraction 14 (20.5 mg) afforded **3** with traces of **4**, and fractions 15–17 (16 mg) afforded pure retrorsine. Fraction 14 was rerun under the same conditions, but no pure usaramine was obtained. Pure **3** (16 mg) was obtained in fractions 17–22.

SEPARATION OF SENECIONINE [**1**] AND INTEGERRIMINE [**2**].—A mixture of **1** and **2** (1:1) was subjected to fractional crystallization from Me_2CO utilizing the fact that **1** was less soluble than **2** in the solvent.

ANONAMINE [**9**].—Mp 202°, $[\alpha]^{27}D$ +33.5° (c = 1.0, $CHCl_3$); eims *m/z* (%) 100 (15), 110 (46), 122 (29), 123 (49), 124 (19), 150 (16), 151 (63), 168 (50), 169 (24), 248 (21), 266 (13), 282 (23), $[M]^+$ 381 (2); cims $[MH]^+$ 382 (100); exact mass calcd for $C_{19}H_{27}NO_7$, 381.1788, found 381.1742.

3aβ-HYDROXY-4-ETHOXY-2,6-PERHYDROINDOLEDIONE [**11**].—Mp 170°; eims *m/z* (%) 43 (100), 44 (42), 55 (24), 70 (28), 71 (23), 73 (24), 97 (16), 99 (39), 125 (37), 141 (40), 150 (36), 167 (25), 184 (2), 195 (5), 213 (0.8), 214 (0.6); cims $[MH]^+$ 214 (100); exact mass calcd for $C_{10}H_{14}NO_4$ $[MH]^+$ 214.1185, found by cims 214.1048; ^1H nmr ($CDCl_3$) δ 1.1 (t, J = 7 Hz, CH_2Me), 3.60 and 3.35 (each dq, $J \approx 9.5$, 7.0 Hz, CH_2Me), 4.04 (dd, J = 13.7, 4.9 Hz, H-4), 3.72 (dd, J = 4.7, 1.7 Hz, H-7a), 2.83

and 2.21 (each d, J = 15.0 Hz, H_2-3), 2.81 (m, H-5), 2.52 (d, J = 15.5 Hz, H-5), 2.85 (m, H-7), 2.53 (d, J = 13.8 Hz, H-7).

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