Journal of Natural Products
Vol. 47, No. 5, pp. 796-801, Sep-Oct. 1984

ANTINEOPLASTIC AGENTS, 99. *AMARYLLIS BELLADONNA*[1]

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ABSTRACT.—*Amaryllis belladonna* bulbs were examined for constiuents inhibitory against the murine P-388 lymphocytic leukemia (PS system). Two in vitro active alkaloids, acetylcaranine (**2**; 9PS ED_{50} 0.23 μg/ml) and ambelline (**3**; 9PS ED_{50} 1.6 μg/ml), were isolated accompanied by undulatine. However, the non-chiral anhydrolycorinium chloride (**5**) was found to be the principal antineoplastic (3 PS, 64-69% life extension at dose levels 10 to 20 mg/kg in vivo, ED_{50} 1.4 μg/ml in vitro) component. Quaternary chloride **5** has not been located previously among plant or animal biosynthetic products.

Plants of the Amaryllidaceae family are well known for a variety of reasons. Many are attractive ornamentals (daffodils) and valuable reservoirs of alkaloids. Some have a long history of use in the primitive treatment of cancer. The "belladonna lily" or "naked-lady," *Amaryllis belladonna* L., is known for each purpose.[2] For example, *A. belladonna* has found use in Java for "swelling", a presumed synonym for cancer (2). Two other members of this genus, *Amaryllis formossisima* L. and *Amaryllis zeylanica* L., have also been employed in folk medicine as a cancer treatment (3). In a collaborative study conducted by Drs. Charlson (Macquarie University) and Hartwell (U.S. National Cancer Institute),[3] an Australian collection of *A. belladonna* bulbs were shown (5) to yield fractions with a confirmed level (up to T/C 203% at 50 mg/kg) of activity against the *in vivo* murine P-388 lymphocytic leukemia (3 PS system). One of the antineoplastic components was found to be lycorine (**1**, 3 PS, T/C 135% at 75 mg/kg), a ubiquitous Amaryllidaceae alkaloid,[4] but a more potent antineoplastic agent was not isolated.

In our laboratory, a commercial collection of *A. belladonna* (88.9 kg of bulbs) was extracted using the CH_2Cl_2-MeOH (followed by dilution with H_2O) technique (14). The CH_2Cl_2 phase was further separated (Scheme 1) employing a solvent partitioning sequence (14): 9:1↦3:2 MeOH-H_2O with hexane↦CH_2Cl_2. Separation was guided by the PS in vitro (9 PS) and in vivo (3 PS) bioassays. By this means, the most encouraging activity was located in the aqueous MeOH fraction. After concentration, solution in MeOH and addition of Me_2CO, solvent was removed from the solution phase, and the residue was dissolved in H_2O. When the resulting aqueous solution was extracted with *n*-BuOH, activity was distributed in both solvents.

Before we were certain that the most promising component resided primarily in the aqueous MeOH fraction, we chromatographed CH_2Cl_2 partition fraction on neutral alumina, followed by silica gel, which afforded in vitro active acetylcaranine (**2**, 9 PS ED_{50}, 0.23 μg/ml) and ambelline (**3**, 9 PS ED_{50}, 1.6 μg/ml)[4]; however, 3 PS in vivo activity was not realized at the dose levels (5↦200 and 1.25↦10 mg/kg, respectively)

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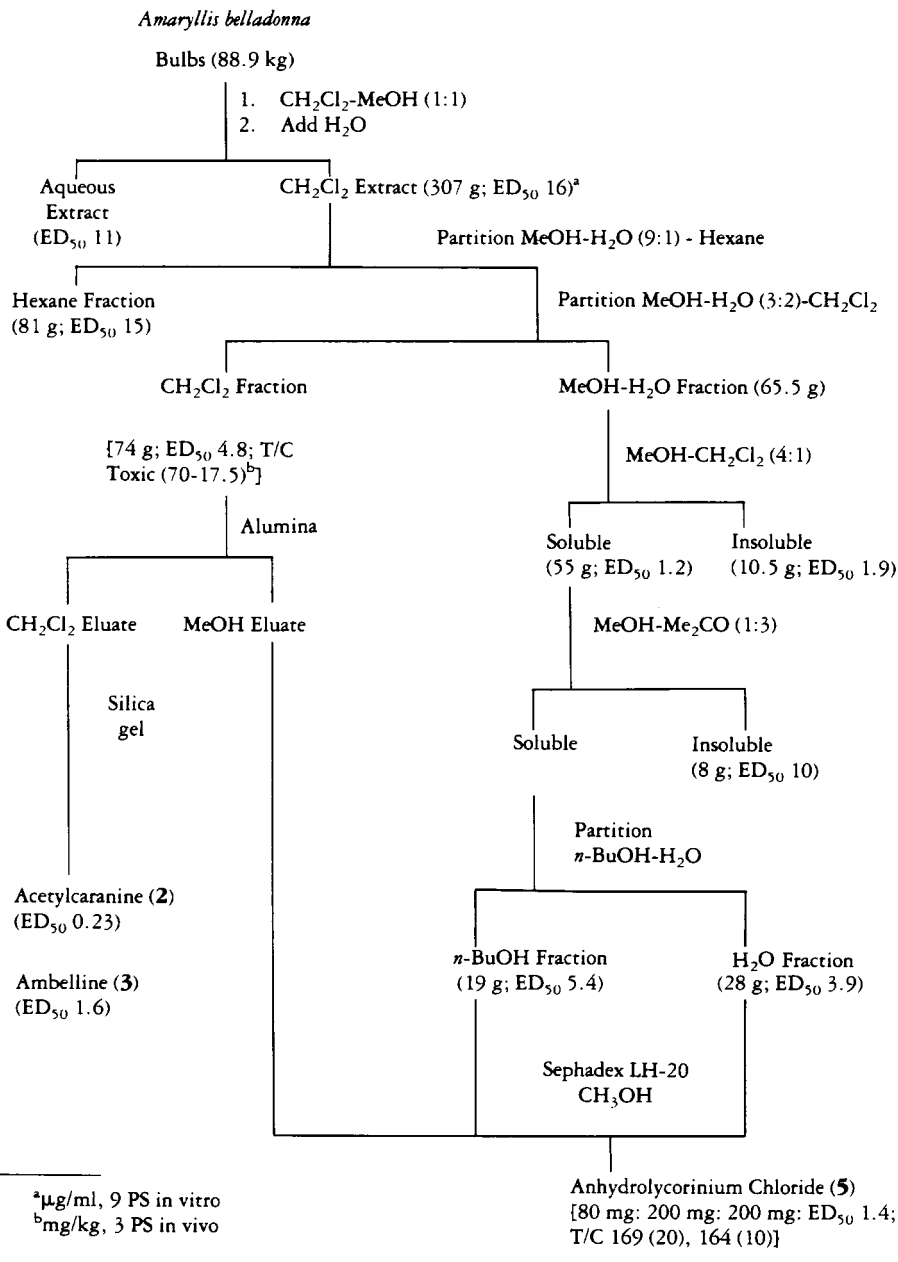
[1]For part 98, see Pettit *et al.* (1).

[2]The bulbs of *A. belladonna* are known in Southern Africa to produce a cardiac-type poison; and the flower, an antispasmodic action (4).

[3]We are pleased to thank Drs. A.J. Charlson, J.L. Hartwell and M.I. Suffness for informing us of these useful results prior to publicaion (5).

[4]In a very important study (6) of the hybrid ¾ *A. belladonna* L. × ¼ *Brunsvigea gigantea* Heister, the bulbs were found to contain acetylcaranine (0.14%, **2**), lycorine (0.048%, **1**), ambelline (0.044%, **3**), undulatine (0.04%, **4**), belladine (0.015%), and buphonidrine (0.0079%). For related studies and isolation of other *A. belladonna* (and hybrid) bulb alkaloids, refer to references 7-13.

SCHEME 1. Extraction and Isolation Sequence

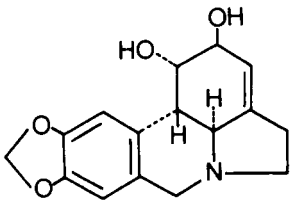


tested. Gel permeation chromatography (Sephadex LH-20) of a 92:8 CH_2Cl_2-MeOH fraction from the silica gel step led to undulatine (**4**, 9 and 3 PS inactive).[4,5]

When a MeOH fraction from the alumina column was separated on a column of Sephadex LH-20, the most prominent PS active (T/C 164-169% at dose levels of 10-20 mg/kg, ED_{50}, 1.4 μg/ml) component was isolated as a pale yellow solid (**5**, 0.08 g). At this point, it became clear that the markedly 3 PS in vivo active component was quite H_2O soluble and most probably was concentrated in the above-mentioned *n*-BuOH

[5]Due to Prof. Wildman's (6) death it was not possible to obtain authentic specimens of alkaloids **3** and **4** for direct comparison. However, the spectral data we analyzed for ambelline and undulatine were in complete accord with those respective structures.

and/or H_2O partition fractions. Each of these fractions, on chromatography (gel filtration) using Sephadex LH-20 and recrystallization (MeOH), afforded 0.2 g of the 3 PS active constituent as pale yellow needles (**5**).



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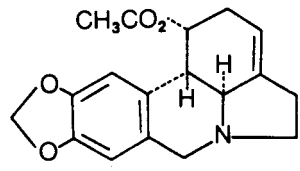

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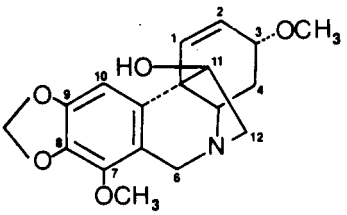

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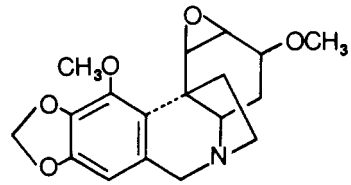

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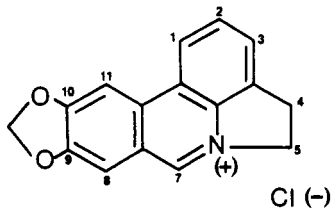

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Because of solubility behavior, high decomposition point (264-270°), and color, a quaternary ammonium halide type system was anticipated for the yellow anticancer component. Support for this assumption was obtained when an aqueous solution of the substance gave a strong test for chloride ion with $AgNO_3$ and yielded a crystalline perchlorate salt with perchloric acid. Spectral data, particularly uv and pmr, obtained from the chloride salt was consistent with a phenanthridinium structure such as **5**. The pmr spectrum showed triplets corresponding to two protons each at δ 3.65 and 5.07 ppm that individually collapsed upon irradiation of the adjoining set and were assigned, respectively, to ring positions 4 and 5. The signals at 6.38 (methylenedioxy methylene), 7.62, and 7.68 (protons at C-8 and C-11), 7.8 (protons at C-1, 2 and 3) and 9.26 ppm (H at C-7) were interpreted as indicated and led us to assign structure **5**. Interestingly, chloride **5** was described nearly 30 years ago as a degradation product of lycorine (**1**), known as anhydrolycorinium chloride or isolycorine hydrochloride (15,16). Meanwhile, Cheng and colleagues (17) completed a total synthesis[6] of chloride **5**, and our specimen was found to be identical with the synthetic product.[7] Mutual identity of the *A. belladonna* and synthetic specimens of chloride **5** adds to those relatively rare occasions[8] where a total synthesis has preceded discovery of the natural product.

The fact that quaternary chloride **5** represents one of the most PS-inhibitory biosynthetic products of *A. belladonna* may explain, along with the relatively low yield,

[6]Performed as part of the National Cancer Institute's drug synthesis program. The synthetic specimen exhibited 3 PS T/C 127-153% over the dose range 6.25-25 mg/kg.

[7]Kindly provided by Drs. C.C. Cheng, V.L. Narayanan, and Mr. L.H. Kedda.

[8]By coincidence, belladine from an *A. belladonna* hybrid provides another such example (18,19)

previous experience with loss of activity during isolation. Perhaps lycorine (**1**) and/or derivatives such as chloride **5** may account for the 3 PS in vivo activity of certain related species such as *Brunsvigia radulosa* Herb. (5). Other quaternary isoquinoline chlorides exhibiting 3 PS in vivo activity are fagaronine and nitidine chloride (20).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All solvents were redistilled. Sephadex LH-20 (particle size 25-100 μ) was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. Other column chromatographic separations were performed with silica gel (70-230 mesh) and neutral aluminum oxide (activity I, both from E. Merck, Darmstadt). The fraction collection was partially automated using a Gilson microfractionator. Tlc was performed with silica gel GHLF and Alumina GF precoated (250 μ) plates supplied by Analtech, Inc. The plates were visualized by uv and/or Dragendorff's reagent.

The mutual identity of authentic and isolated specimens was confirmed by ir spectral (on KBr) comparisons. All melting points are uncorrected and were observed utilizing a Kofler-type melting point apparatus. Optical rotations were determined in $CHCl_3$ solution with a Perkin-Elmer model 241 polarimeter. Infrared spectra were recorded with Perkin-Elmer model 299 and Nicolet MX-1 FT-IR spectrophotometers. The uv spectra were measured in MeOH solution with a Hewlett-Packard model 8450A spectrophotometer. Pmr spectra were measured using Varian XL-100 and Bruker WH-90 spectrometers and cmr spectra were measured at 22.63 MHz with a Bruker WH-90 spectrometer. The nmr spectra were recorded in $CDCl_3$ solution using TMS as internal reference unless otherwise stated, and δ values are reported in ppm. Mass spectra were obtained using Varian MAT 731 and MAT 312 spectrometers. Fractions and pure compounds were tested for PS activities according to established protocols (21) under auspices of the National Cancer Institute.

PLANT MATERIAL.—A commercial collection of the bulbs (88.9 kg, PR 54095; B663685) of *A. belladonna* was provided by the Economic Botany Laboratory, Agricultural Research Center–East, USDA, Beltsville, MD, under the joint NCI-USDA program directed by Drs. M.I. Suffness and J.A. Duke.

PLANT EXTRACTION.—Dried, finely ground bulbs (88.9 kg) of *A. belladonna* were extracted with a mixture (600 liters) of CH_2Cl_2 and MeOH (1:1) at ambient temperature. The extract was separated into CH_2Cl_2 and aqueous phases on addition of 25% H_2O. The CH_2Cl_2 fraction (307 g, 9 PS, ED_{50} 16 μg/ml) was found to be toxic (70 to 17.5 mg/kg) in the 3 PS *in vivo* system. The aqueous fraction was nontoxic and inactive (70 to 8.75 mg/kg).

SOLVENT PARTITION SEQUENCE.—The CH_2Cl_2 fraction (307 g) was successively partitioned between MeOH-H_2O (9:1) and hexane and MeOH-H_2O (3:2) and CH_2Cl_2. Removal of solvents from the hexane, CH_2Cl_2, and MeOH-H_2O solutions gave, respectively, 81 g (9 PS, ED_{50} 15 μg/ml), 74 g (9 PS, ED_{50} 4.8 μg/ml, 3 PS toxic 150 to 18.5 mg/kg), and 65.5 g fractions. The MeOH-H_2O fraction (65.5 g) was treated with a mixture of MeOH-CH_2Cl_2 (4:1), and the solution was filtered whereby the insoluble material (10.5 g, 9 PS, ED_{50} 1.9 μg/ml; 3 PS non-toxic and inactive 150 to 18.75 mg/kg) was separated from the soluble portion (55 g, 9 PS, ED_{50} 1.2 μg/ml; 3 PS toxic 150 to 18.5 mg/kg).

ISOLATION OF ACETYLCARANINE (**2**), AMBELLINE (**3**), AND UNDULATINE (**4**).—A 32-g aliquot of the CH_2Cl_2 partition fractions was adsorbed on neutral alumina (150 g). Rapid elution, first with CH_2Cl_2 (500 ml), gave a fraction (28 g, 9 PS, ED_{50} 3.6 μg/ml; 3 PS toxic 150 to 18.5 mg/kg), and then with MeOH (500 ml), another fraction (3.5 g, 9 PS, ED_{50} <1.0 μg/ml, 3 PS toxic 150 to 18.5 mg/kg). A 5-g aliquot of the CH_2Cl_2 eluate was adsorbed on a column of silica gel (150 g) and eluted with mixtures of CH_2Cl_2 containing increasing amounts of MeOH. Elution with CH_2Cl_2-MeOH (98:2) followed by crystallization from Me_2CO-hexane afforded acetylcaranine as needles (**2**, 400 mg, 0.0058%[9] 9 PS, ED_{50} 0.23 μg/ml). Continued elution with CH_2Cl_2-MeOH (92:8) led to a mixture (0.4 g) enriched in undulatine. Final elution with CH_2Cl_2-MeOH (85:15) followed by crystallization from Me_2CO yielded ambelline (**3**, 50 mg, 0.0007%, 9 PS, ED_{50} 1.6 μg/ml, 3 PS toxic at 9.6 and 4.8 and inactive at 2.4 and 1.2 mg/kg). The mixture (0.4 g) enriched in undulatine was subjected to gel permeation chromatography on Sephadex LH-20 using MeOH as eluent. Pure undulatine (**4**, 230 mg, 0.0033%, 9 PS, ED_{50} 21 μg/ml, 3 PS toxic at 32 and 16 and inactive at 8 and 4 mg/kg) was obtained upon crystallization from MeOH.

CHARACTERIZATION OF ACETYLCARANINE (**2**).—Acetylcaranine (**2**) exhibited the following physical properties: mp 195° [lit. (7), mp 184-185°], $[\alpha]^{32}D$ −175.61° [c, 1.64, $CHCl_3$; lit. (7), $[\alpha]^{22.5}D$ −177.5°]. Eims *m/z* 313 (M^+, $C_{18}H_{19}NO_4$); uv λ max (log ε), 236 (3.52) and 294 (3.55) nm; ir ν max (KBr) 1727, 1505, 1487, 1377, 1264, 1249, 1239, 1219, 1034, and 902 cm^{-1}; pmr[10] δ 1.92

[9]Percent yields are given on the basis of dry weight of plant material. The separation methods were not optimized, and samples at each step were consumed in biological testing.

(3H, s, $OCOCH_3$), 2.48 (8H, m), 3.32 (1H, m), 3.54 (1H, d, J=15 Hz, H-7α), 4.16 (1H, d, J=15 Hz, H-7β), 5.4 (1H, bs, H-1), 5.90 (1H, bs, H-3), 5.92 (2H, s, OCH_2O), 6.58 (1H, s), 6.74 (1H, s) (H-8 and 11); cmr[10] 170.79 (s, $OCOCH_3$), 146.42 (s), 146.13 (s) (C-9 and 10), 139.53 (s, C-3a), 129.55 (s, C-7a), 127.73 (s, C-11a), 114.19 (d, C-3), 107.29 (d, C-11), 105.02 (d, C-8), 100.89 (t, OCH_2O), 66.52 (d, C-1), 61.25 (C-11c), 56.99 (C-7), 53.71 (C-5), 43.57 (C-11b), 33.34 (t), 28.62 (t) (C-2 and 4), 21.22 (q, $OCOCH_3$). The identity was confirmed by direct comparison with an authentic sample [(7) ir and mmp).

CHARACTERIZATION OF AMBELLINE (**3**).—Ambelline (**3**) showed mp 254-255° [lit. (7), mp 260-261°], $[\alpha]^{32}D$ +43.05° [c, 1.51, $CHCl_3$; [lit. (7), $[\alpha]^{26}D$ +32.3°]; Eims m/z 331 (M^+, $C_{18}H_{21}NO_5$); uv λ max (log ε) 287 (3.17) nm, ir ν max (KBr) 3440, 3080, 1480, 1040, 940 cm^{-1}; pmr[10] δ 3.35 (3H, s, 3-OCH_3), 3.89 (1H, d, J=17 Hz, H-6α), 3.92 (3H, s, 7-OCH_3), 4.30 (1H, d, J=17 Hz, H-6β), 5.93 (2H, s, OCH_2O), 6.02 (1H, dd, $J_{1,2}$=10 Hz, $J_{2,3}$=5 Hz, H-2), 6.54 (1H, d, J=10 Hz, H-1), 6.59 (1H, s, H-10); cmr[10] 148.27 (s, C-9), 141.13 (s, C-7), 134.47 (s, C-8), 132.12 (s, C-10a), 131.83 (d, C-2), 126.21 (d, C-1), 118.35 (s, C-6a), 100.80 (d, C-10), 100.34 (t, OCH_2O), 86.53 (d, C-3), 72.37 (d, C-11), 62.91, 62.59 (C-4a and 12), 59.27, 59.17 (C-6 and OCH_3), 56.51 (q, OCH_3), 48.20 (s, C-10b), 28.76 (t, C-4). The physical data was consistent with the ambelline structure (7).

CHARACTERIZATION OF UNDULATINE (**4**).—Undulatine (**4**) exhibited mp 156-157° [lit. (6), mp 149-151°] $[\alpha]^{32}D$ −31.97° [c, 1.47 $CHCl_3$, lit. (6) $[\alpha]^{25}D$ −31.8°]. Eims m/z 331 (M^+, $C_{18}H_{21}NO_5$); uv λ max (log ε) 287 (3.25) nm; ir ν max (KBr) 2940, 2925, 2882, 1616, 1478, 1279, 1116, 1100, 1088, 1050 cm^{-1}; pmr [10] 3.42 (3H, s, 3-OCH_3), 3.69 (1H, d, J=17 Hz, H-6α), 3.96 (3H, s, 10-OCH_3), 4.21 (1H, d, J=17 Hz, H-6β), 5.86 (2H, s, OCH_2O), 6.62 (1H, s, H-7); cmr[10] 148.08 (s, C-8), 141.09 (s, C-10), 139.07 (s, C-9), 133.39 (s, C-6a), 117.95 (s, C-10a), 100.6 (t, OCH_2O), 96.44 (d, C-7), 74.99 (d, C-3), 61.28 (d, C-4a), 59.04 (q, OCH_3), 58.71, 57.48, 55.11, 53.87, 52.60 (C-1,2,6,12 and OCH_3), 41.52 (s, C-10b), 39.31 (t, C-11), 25.31 (t, C-4).

To a solution of undulatine (**4**, 25 mg) in Et_2O (1 ml) containing a few drops of EtOH was added (dropwise) an EtOH solution of perchloric acid. The crystalline perchlorate (25 mg) separated within a few minutes, mp 229°.

The physical data recorded for undulatine (**4**) and its perchlorate are in close agreement with reported (6) values.

ISOLATION AND CHARACTERIZATION OF ANHYDROLYCORINIUM CHLORIDE.—The MeOH eluate (12 g) from alumina chromatography of the CH_2Cl_2 partition fraction (see below) was chromatographed on Sephadex LH-20 (600 g) using MeOH as eluent to give anhydrolycorinium chloride (**5**, 80 mg), which crystallized as pale yellow needles from MeOH. Alternatively, the MeOH-H_2O partition fraction (55 g, 9 PS ED_{50} 1.2 μg/ml, 3 PS toxic 150 to 18.5 mg/kg) was dissolved in MeOH (200 ml). On addition of Me_2CO (600 ml), a solid (8 g, 9 PS, ED_{50} 10 μg/ml) separated and was collected by filtration. The MeOH-Me_2CO soluble material was partitioned between *n*-BuOH and H_2O to afford, respectively, 19 g (9 PS, ED_{50} 5.4 μg/ml, 3 PS toxic at 75 to 18.75 mg/kg) and 28 g (9 PS, ED_{50} 3.9, 3 PS toxic at 75 to 18.75 mg/kg) fractions.

The *n*-BuOH and H_2O soluble fractions upon chromatography on Sephadex LH-20 (600 g), using MeOH as eluent followed by crystallization from MeOH, each provided pale yellow needles of anhydrolycorinium chloride [**5**, 200 mg in each case, total yield 0.0004%, 9 PS, ED_{50} 1.4 μg/ml, 3 PS T/C 169 (20), 164 (10 mg/kg)].

Anhydrolycorinium chloride (**5**) displayed the following physical properties: decomp. 264-270° [lit. (17), mp 282-284°; lit. (15), decomp, 280-285°] uv λ max (log ε) 258 (4.51), 267 (4.47), 279 (4.46) and 341 (4.08) nm; ir ν max (KBr) 3437, 3392 (due to H_2O of crystallization) 3016, 1502, 1476, 1424, 1276, 1265, 1030, 925 cm^{-1}; pmr (D_2O solution using DSS as internal reference) 3.65 (2H, t, J=6 Hz, H-4), 5.07 (2H, t, J=6 Hz, H-5), 6.38 (2H, s, OCH_2O), 7.62 (1H, s), 7.68 (1H, s) (H-8 and 11), 7.8 (3H, m, H-1, 2 and 3), 9.26 (1H, s, H-7); cmr (D_2O solution using TMS as external reference) 157.24 (s), 151.04 (s) (C-9 and 10), 144.67 (d, C-7), 137.26 (s), 136.41 (s), 133.13 (s), 132.45 (d), 127.25 (d), 123.48 (s), 122.80 (s), 120.46 (d), 108.11 (d), 105.54 (t, OCH_2O), 101.45 (d), 56.57 (t, C-5), 28.24 (t, C-4). The identity was confirmed by direct comparison with an authentic synthetic specimen (17).

To a solution of anhydrolycorinium chloride (**5**, 1 mg) in EtOH (1 ml) was added an EtOH solution (0.1 ml) of perchloric acid [prepared from 0.2 ml of perchloric acid (70-72%) and EtOH (10 ml)]. The precipitate (separated immediately) was crystallized from hot H_2O (containing a trace of EtOH) to afford yellow needle shaped crystals of anhydrolycorinium perchlorate (1 mg), mp >380°; ir ν max (KBr) 3435 (due to H_2O of crystallization) 1611, 1475, 1260, 1121, 1108 cm^{-1}.

[10]Tentative assignments of pmr and cmr data have been made on the basis of comparison with that published for known compounds (22,23).

Anal. calcd for $C_{16}H_{12}ClNO_6 \cdot 3/2\ H_2O$: C, 51.00; H, 3.98; Cl, 9.43; N, 3.72. Found: C, 51.14; H, 3.62; Cl, 8.85; N, 3.65.

ACKNOWLEDGMENTS

The necessary financial assistance for this investigation was provided by Contract NO1-CM-97297 with the Division of Cancer Treatment, NCI, National Institute of Health, DHHS, Grants CA-16049-06 and 08 awarded by the National Cancer Institutes, Mrs. Mary Dell Pritzlaff, the Olin Foundation (Spencer T. and Ann W.), the Fannie E. Rippel Foundation, Mrs. Eleanor W. Libby, the Donald Ware Waddell Foundation, Mrs. Pearl Spear, and Mr. Robert B. Dalton. For other very necessary assistance, we are pleased to thank Drs. M.I. Suffness, J.D. Douros, J.A. Duke, D.L. Herald, C.L. Herald, and J.E. Leet.

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Received 14 October 1983