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George R. Pettit, George R. Pettit III, Ralph A. Backhaus, Michael R. Boyd, and Alan W. Meerow

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ANTINEOPLASTIC AGENTS, 256.[1] CELL GROWTH INHIBITORY ISOCARBOSTYRILS FROM *HYMENOCALLIS*

GEORGE R. PETTIT,* GEORGE R. PETTIT III, RALPH A. BACKHAUS,

Cancer Research Institute, Department of Chemistry and Department of Botany, Arizona State University, Tempe, Arizona 85287-1604

MICHAEL R. BOYD,

Laboratory of Drug Discovery Research and Development, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

and ALAN W. MEEROW

University of Florida, IFAS, Fort Lauderdale Research and Education Center, 3205 College Avenue, Fort Lauderdale, Florida 33314

ABSTRACT.—The bulbs of *Hymenocallis littoralis*, collected in Hawaii and horticulturally grown in Arizona, and bulbs of *Hymenocallis caribaea* and *Hymenocallis latifolia*, collected in Singapore, were found to contain a cytotoxic, isocarbostyril-type biosynthetic product, 7-deoxy-*trans*-dihydronarciclasine [**2**]. This new compound inhibited the cytopathicity and/or replication of various viruses. Companion cytotoxic constituents of *H. littoralis* and *Hymenocallis* sp. were found to be pancratistatin [**1**], narciclasine [**5**], and 7-deoxynarciclasine [**4**]. These four compounds, along with four other closely related compounds, were comparatively evaluated in the National Cancer Institute's in vitro cytotoxicity panel. Although there were striking differences in overall potency, some of the compounds shared a highly characteristic differential cytotoxicity profile against the 60 diverse human tumor cell lines comprising the NCI panel. As a group, the melanoma subpanel lines were most sensitive; certain individual lines within other subpanels (eg., NSC lung, colon, brain, renal) were as much as a thousand-fold or more sensitive than the less sensitive lines.

The 4th century B.C. physician Hippocrates of Cos recommended a pessary prepared from narcissus oil (probably *Narcissus poeticus* L., Amaryllidaceae) for the management of uterine tumors (2). In the 1st century A.D., Pliny the Elder recorded the topical use of this extract (3,4) and another from *Narcissus pseudonarcissus* (5–7) for tumors (8). Arabian, North African, Central American, and Chinese medical practitioners of the middle ages continued applications of the oil of narcissus in cancer treatment. A number of species in other genera of the Amaryllidaceae were also utilized in primitive treatment of cancer (9). One of these was *Hymenocallis caribaea* (L. emend Gawler) Herbert, employed in early European medicine for inflammatory tumors (10).

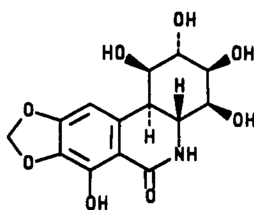
In 1984, *Pancratium littorale* Jacq. (Amaryllidaceae), reidentified in the present study as *Hymenocallis littoralis* (Jacq.) Salisb., was found (11) to contain a new antineoplastic isocarbostyril, named pancratistatin (1). That discovery directed our attention to *H. caribaea* and related *Hymenocallis* species. Meanwhile, pancratistatin proved to be very effective against the murine P-388 lymphocytic leukemia in vitro (ED_{50} 0.01 μg/ml) and in vivo (38–106% life extension at 0.75–12.5 mg/kg). Pancratistatin also inhibited growth of the in vivo murine M-5076 ovary sarcoma (53–84% life extension at 0.38–3.0 mg/kg). This compound has been the subject of preclinical drug development both in the United States and in Europe, and clinical investigation of the agent is anticipated. Recently, pancratistatin has been found to exhibit strong anti-RNA virus activity (12). When injected into mice infected with Japanese encephalitis, pancratistatin increased

[1]For Part 255, see Ludueña *et al.* (1).

survival by 100%, and it showed activity against two other RNA flaviviruses (yellow fever and dengue) and against the bunyaviruses Punta Tora and Rift Valley fever (12).

Since pancratistatin [**1**] may become medicinally important and its synthesis (to date) is a time-consuming and expensive multi-step process (13), a biotechnological approach to increasing supplies was undertaken with *H. caribaea,* one of the first plants selected for evaluation. This species and two other related *Hymenocallis* species produce, instead of pancratistatin, a new biosynthetic product determined to be 7-deoxy-*trans*-dihydronarciclasine [**2**].

Collections of *H. caribaea, Hymenocallis latifolia* (Mill.) Roemer, and *Hymenocallis* sp. bulbs were each extracted employing a CH_2Cl_2/MeOH procedure (14) followed by addition of H_2O. Bioassay-guided isolation of cytotoxic constituents employed the P388 murine leukemia cell line. The aqueous phase was extracted with *n*-BuOH, and the alcohol fraction was further separated by gel permeation chromatography on Sephadex LH-20 using MeOH as eluent. Fraction collection was monitored by Si gel tlc [CH_2Cl_2-MeOH (3:1)]. Crystallization of a component with tlc R_f value 0.37 from *H. caribaea* and *H. latifolia* gave pure 7-deoxy-*trans*-dihydronarciclasine [**2**] (P388 ED_{50} 0.02 μg/ml). Investigation of *Hymenocallis* sp. revealed the component with tlc R_f 0.37 to be a mixture of 7-deoxy-*trans*-dihydronarciclasine [**2**] and 7-deoxynarciclasine [**4**]. The band at tlc R_f 0.46 proved to be a mixture of pancratistatin [**1**] and narciclasine [**5**]. A new collection of *H. littoralis* gave isocarbostyril [**2**], which was not detected in our earlier (10) investigation of this species.



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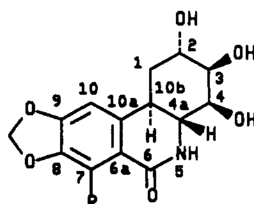

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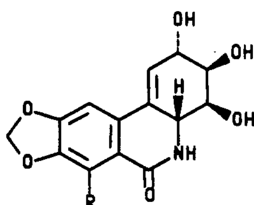

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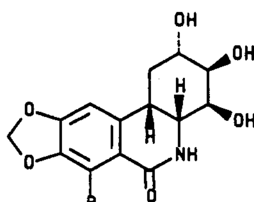

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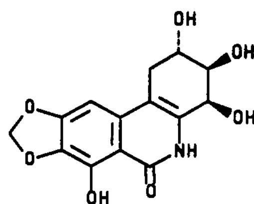

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Racemic 7-deoxy-*trans*-dihydronarciclasine was synthesized as a mixture of B/C ring juncture *cis* and *trans* isomers in 1968 (15,16). Ten years later racemic **2** was characterized as part of another synthetic study concerned with plant growth regulators (17). The present research represents the first time chiral 7-deoxy-*trans*-dihydronarciclasine has been discovered and characterized as a natural product. In addition to its cytotoxicity, this compound has also shown activity against a series of RNA viruses (12). The cytotoxicity of the 7-deoxy-*trans* isomer **2** led us to examine the corresponding *trans*-dihydronarciclasine [**3**] prepared (12) by hydrogenation of narciclasine [**5**], and it displayed substantial activity (ED_{50} 0.02 μg/ml) against the P388 leukemia. However, 7-deoxynarciclasine [**4**] appeared to be rather modestly cytotoxic, and the corresponding *cis* isomers **6** and **7** (14) were found to be weak or essentially inactive, as was isonarciclasine [**8**] (14).

Because of the continuing interest in pancratistatin and/or related compounds for drug development, structure-activity comparison of all eight of the above-named compounds was made using the United States Natonal Cancer Institute's (NCI) "disease-oriented" in vitro primary antitumor screen (18,19). To enhance the precision of comparisons, each compound was tested eight separate times, over three different concentration ranges (10^{-4}, 10^{-5}, and 10^{-6} M upper limits). In each concentration range, five dilutions (10-fold each) of drug were tested against each of the 60 human tumor lines comprising the NCI panel. The concentration-response curves thus generated were used to calculate and construct the corresponding individual as well as "averaged" GI_{50}, TGI, and LC_{50} mean graphs (18–20). From these, the mean response values for each cell line as well as the overall panel values for each response parameter were determined. Likewise, the characteristic "fingerprints" or patterns of differential cellular sensitivity to each compound were compared by a computerized pattern-recognition algorithm (20). The results of these analyses are summarized in Table 1.

Three of the compounds (6–8) were only very weakly active or inactive in the NCI screen. However, the other five (1–5) showed a characteristic and reproducible pattern of differential cytotoxicity against the panel of 60 cell lines (Table 1). Although there was

TABLE 1. Results of Comparative Antitumor Evaluations of Isocarbostyrils in the NCI In Vitro Primary Screen.[a]

Compound	Mean Panel GI_{50} ($\times 10^{-8}$ M)[b]	Compare Correlation Coefficient
1	9.12	1.00
2	6.76	0.89
3	1.26	0.92
4	14.5	0.90
5	1.55	0.90
6	9550	<0.6
7	380	<0.6
8	1180	<0.6

[a]All compounds were tested eight times, at three different concentration ranges (10^{-4}, 10^{-5} and 10^{-6} M upper limits, $\log_{10}$ dilutions×5) against the entire panel of 60 human tumor cell lines comprising the NCI screen.

[b]Standard errors averaged less than 10% of the respective means.

[c]Correlation coefficients from the Compare pattern-recognition algorithm were calculated by computer using the LC_{50}-centered mean graph profiles of differential cellular sensitivities to each of the eight compounds. The LC_{50} mean graph profile of **1** was used as the benchmark or "seed" for all of the comparisons.

as much as a tenfold difference in potency among the active compounds, the differential cellular sensitivity profiles were remarkably similar, as confirmed by computer analysis (Table 1). As a group, the melanoma subpanel lines were most sensitive, and certain individual lines within other subpanels (e.g., NSC lung, colon, brain, renal) were as much as 1000 times more sensitive, with respect to the LC_{50} response parameter, than the least sensitive lines comprising the panel. The characteristic LC_{50} mean graph profile shared by this group of compounds did not show a comparably high correlation with any known conventional class of antitumor compound. A more specific example of individual cell line sensitivities to one of the compounds [**2**] is provided in the Experimental section.

From evidence now in hand, 7-deoxy-*trans*-dihydronarciclasine [**2**] appears to be the principal substance responsible for the antitumor activity attributed to *H. caribaea*. Indeed, it seems very likely that pancratistatin and related *trans*-dihydrocarbostyrils, as well as narciclasine, constitute the most important antineoplastic components of Amaryllidaceae species used in the folk medical treatment of cancer. Given the screening results described above, a comparative in vivo evaluation of compounds **1–5** in xenograft models incorporating selected sensitive cell lines identified by the in vitro screen may be warranted. Moreover, an enhanced focus of future clinical testing of pancratistatin or related compounds upon particularly sensitive tumor types (e.g., melanoma) might be justified by the present results from the NCI evaluation.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All solvents used for chromotography were redistilled. Tlc was performed on Si gel GHLF uniplates (0.25 mm layer thickness) supplied by Analtech Inc., using CH_2Cl_2-MeOH (3:1) as mobile phase and ceric sulfate spray reagent for development. Sephadex LH-20 (particle size 25–100 μ) was supplied by Pharmacia Co. A Gilson Microfractionator was used for fractionations. Mp's were determined using an Electrothermal 9100 instrument, and optical rotations were measured with a Perkin-Elmer Model 241 Automatic Polarimeter. Ir spectra were obtained using a Nicolett MX-1 FT spectrophotometer. The ^{1}H- and ^{13}C-nmr spectra were recorded with a Varian-Gemini 300 MHz spectrometer using DMSO as internal standard. Eims were determined employing a Finnigan-MAT mass spectrometer (Model 312).

PLANT COLLECTION.—Specimens of *H. latifolia* (2.5 kg fresh wt, bulbs), *H. caribaea* (8.6 kg fresh wt, bulbs), and *Hymenocallis* sp. (1.04 kg fresh wt, bulbs) were obtained in Singapore from commercial suppliers and taxonomically identified by one of us (A.M.). Voucher specimens are maintained in our Institute and at the University of Florida. *H. littoralis* was grown at our Institute by tissue culture methods (21) and by separating and replanting asexually produced offsets. The original bulb shipment (1.5 kg fresh wt) of *H. littoralis* came from the University of Hawaii, and a voucher specimen is deposited at the Harold L. Lyon Arboretum, University of Hawaii. Originally, this plant was identified as *P. littorale* (11) but was recently taxonomically corrected (by A.M.). Flowers from our greenhouse plants were utilized for the definitive taxonomic studies. Unfortunately, *Hymenocallis* sp. could not be conclusively identified as the flowers were unavailable.

GENERAL EXTRACTION AND SOLVENT PARTITION PROCEDURE.—In a typical experiment, chopped bulbs (1.0 kg) of *H. littoralis* were extracted with MeOH-CH_2Cl_2 (1:1) (3 liters) at room temperature for 15 days. The extract was filtered through muslin, and the CH_2Cl_2 phase was separated by the addition of 20% by volume of H_2O. The aqueous phase was concentrated to 300 ml, and the pale yellow solution was extracted with *n*-BuOH (3×30 ml). The alcohol extract was concentrated to a 1.9 g fraction. By the same general procedure, *H. caribaea* (8.6 kg, dried bulbs) gave a 1.5 g *n*-BuOH fraction, *H. latifolia* (1.0 kg, dried bulbs) produced a 0.7 g *n*-BuOH fraction, and *Hymenocallis* sp. (1.04 kg, dried bulbs) afforded a 2.0 g *n*-BuOH fraction.

Isolation of 1,3,4,4a,5,11b-hexahydro-2,3,4-trihydroxy[1,3]dioxolo[4,5-j]phenanthridin-6(2H)-one [**2**] *(7-deoxy-*trans*-dihydronarciclasine).*—The following isolation of 7-deoxy-*trans*-dihydronarciclasine from *H. caribaea* illustrates the general procedure employed with each of the four *Hymenocallis* species. The *n*-BuOH fraction (1.5 g) from the extraction and solvent partitioning separation of *H. caribaea* was treated as follows: dilution of the *n*-BuOH residue (1.5 g) with MeOH (10.0 ml) followed by Me_2CO (50 ml) caused a milky white solid to form. The insoluble material was collected and the filtrate evaporated to dryness. The residue

was again redissolved in 4.0 ml of MeOH, and the resulting reddish-orange solution was chromatographed (fractions were monitored by tlc) on a column of Sephadex LH-20 (80 g, 106×3 cm) using MeOH as eluent. Fractions containing the single component with tlc R_f 0.37 were allowed to evaporate slowly (and undisturbed) for several days, whereupon colorless sharp needles appeared (33.0 mg yield, 3.3×10^{-3}%): melting at 303–304°; $[\alpha]^{25}$D +138° (c=0.96, DMSO); eims m/z $[M]^+$ 293 ($C_{14}H_{15}O_6N$); uv λ max (EtOH) (log ε) 217 (3.70), 260 (3.45), 304 (3.54) nm; ir (KBr) ν max 3553, 3490, 3427, 3209, 3059, 2920, 1667, 1612, 1495, 1462, 1400, 1380, 1365, 1349, 1297, 1268, 1237, 1186, 1169, 1130, 1110, 1100, 1073, 1045, 1035, 982 cm^{-1}; ^{1}H nmr (300 MHz, DMSO-d_6) δ 1.59 (1H, dt, J=2.3 Hz, 13.4 Hz) 2.12 (1H, brd, J=13.3 Hz) 2.84 (1H, dt, J=3.45 Hz, 12.78 Hz), 3.23 (1H, m), 3.68 (2H, brs), 3.84 (1H, brs), 4.78 (1H, brs, OH), 4.91 (2H, brs, OH), 6.03 (2H, s), 6.89 (2H, s, N-H, 10-H), 7.30 (1H, s). Isocarbostyril **2** was found to be identical (tlc, mp, ir, ^{1}H-nmr) with an authentic sample (14) prepared by hydrogenation of narciclasine.

BIOLOGICAL EVALUATION.—The rationale and the technical details of how the NCI in vitro primary screening assay is performed and data calculations applied are described elsewhere (18–20,22). As a specific example, the negative $\log_{10}$ of GI_{50}, TGI, and LC_{50} values, respectively, obtained for compound **2** are listed below, in sequence, following the individual cell line names: CCRF-CEM (7.46, 6.28, >4.00) HL-60TB (7.49, 6.72, 4.52), K-562 (7.33, 5.96 >4.00), MOLT-4 (7.35, 5.72, >4.00), RPMI-8226 (7.02, 6.11, >4.00), SR (7.28, 5.49, 4.25); A549/ATCC (7.00, 5.89, >4.00), EKVX (6.48, 5.43, 4.09), HOP-18 (6.80, 5.77, 4.10), HOP-62 (7.19, 6.30, 5.00), HOP-92 (7.12, 6.13, >4.00), NCI-H226 (6.82, 6.12, 5.06), NCI-H23 (7.27, 6.44, 5.19), NCI-H322M (7.15, 6.15, 4.24), NCI-H460 (7.41, 6.09, 4.28), NCI-H522 (7.43, 6.62, 5.42), LXFL-529 (7.33, 6.15, 4.57); DMS-114 (7.30, 6.29, 4.85), DMS-273 (7.21, 6.22, 4.92); COLO 205 (7.27, 6.70, 6.34), DLD-1 (7.36, 6.08, >4.00), HCC-2998 (7.11, 6.09, 4.80), HCT-116 (7.48, 6.40, 4.62), HCT-15 (7.07, 5.92, 4.07), HT 29 (7.21, 6.09, >4.00), KM12 (7.33, 6.04, 4.62), KM20L2 (7.29, 6.04, 4.18), SW-620 (7.19; 5.62, >4.00); SF-268 (7.15, 5.82, 4.20), SF-295 (6.85, 6.04, 4.38), SF-539 (7.22, 6.55, 5.85), SNB-19 (7.38, 6.00, 4.03), SNB-75 (7.22, 6.36, 4.62), SNB-78 (7.06, 6.04, 4.08), U251 (7.32, 5.89, 4.43), XF-498 (7.09, 6.14, 4.68); LOX IMVI (7.48, 6.80, 6.15), MALME-3M (7.12, 6.38, 5.07), M14 (7.19, 6.46, 5.77), M19-MEL (7.40, 6.29, 5.28), SK-MEL-2 (7.42, 6.48, 5.26), SK-MEL-28 (7.09, 6.19, 4.35), SK-MEL-5 (7.34, 6.68, 5.92), UACC-257 (6.85, 5.74, 4.34), UACC-62 (7.00, 6.25, 4.96); IGROV1 (6.85, 5.96, 4.17), OVCAR-3 (7.08, 6.30, 4.34), OVCAR-4 (7.44, 6.37, 4.77), OVCAR-5 (6.49, 5.62, >4.00), OVCAR-8 (7.15, 5.64, 4.14), SK-OV-3 (7.04, 5.96, >4.00); 786-0 (6.96, 5.68, 4.24), A498 (6.64, 5.96, 5.03), ACHN (7.09, 6.10, 4.96), CAKI-1 (6.72, 5.85, >4.00), RXF-393 (7.14, 6.38, 5.60), SN12C (6.85, 5.74, 4.74), TK-10 (6.60, 5.72, >4.00), UO-31 (7.00, 6.02, 4.35).

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