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Antineoplastic Agents. 587. Isolation and Structure of 3-Epipancratistatin from *Narcissus* cv. Ice Follies[1]

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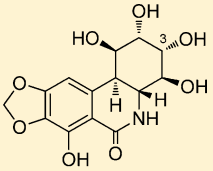
Supporting Information

ABSTRACT: Bioassay-guided (cancer cell line) separation of an extract prepared from *Narcissus* cv. Ice Follies (from The Netherlands) led to the isolation of a new Amaryllidaceae isocarbostiryl, 3-epipancratistatin (**1b**), as well as narciclasine (**2**). This *Narcissus* cultivar was found to be a good source of narciclasine. The structure of **1b** was established by high-resolution mass and high-field 2D NMR spectroscopic analyses. Against a panel of murine and human cancer cell lines, 3-epipancratistatin (**1b**) led to cell growth inhibition (GI_{50} 2.2−0.69 μg/mL) some 100× less than that found for pancratistatin (**1a**) and narciclasine (**2**), thereby revealing an important configurational requirement in **1a** for strong cancer cell growth inhibition.



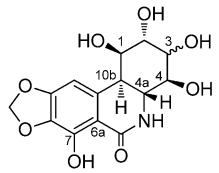
1b

The Amaryllidaceae plant family genera and particularly the *Narcissus* genus have a long and quite notable place in the history of traditional and Western medicine.[2] Research concerning the discrete constituents had a formal beginning in 1877 with a description of lycorine from *Narcissus pseudonarcissus*.[3a] Presently, well over 100 Amaryllidaceae nitrogen-containing compounds are known, and some of the most promising members with antineoplastic properties have been nicely reviewed.[3] Current progress in preclinical development of the isocarbostyril pancratistatin (**1a**) has been summarized in other important reviews,[4] and efforts toward efficient total syntheses continue.[5]

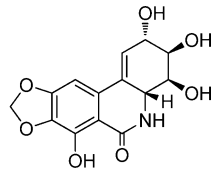
Importantly, the biological foundation for advancement of pancratistatin (**1a**), in the form of its very soluble phosphate prodrug,[6] has continued to increase rapidly. Illustrative are the recent experimental results confirming that **1a** very selectively sends a range of cancer cell types, including metastatic prostate cancer, breast cancer, and colon carcinoma, as well as ex vivo models of leukemia, into apoptosis without toxicity to non-cancerous counterpart cell lines; the mode of action appears to involve selective targeting of cancer cell mitochondria.[7]

While investigating *Narcissus* varieties from horticultural sources in order to increase the availability of narciclasine (**2**)[8] for use in our semisyntheses of **1a**,[9] we found *Narcissus* cv. Ice Follies[10] to be a new and useful source of **2**. During a scale-up isolation of **2**, we detected the presence of a new isocarbostyril clearly related to pancratistatin that offered the prospect of finding useful anticancer activity and new SAR data. The isolation and structure elucidation proceeded as follows.

The bulbs (4000 kg) of *Narcissus* cv. Ice Follies were extracted with CH_3OH. The extract was partitioned between 9:1 CH_3OH−H_2O and hexane, and the hexane-soluble lipid fraction was removed. The aqueous phase was then partitioned between 3:2 CH_3OH−H_2O and CH_2Cl_2, and the CH_2Cl_2 fraction was separated. After removal of CH_3OH from the aqueous layer, the remaining H_2O phase was extracted with EtOAc followed by *n*-butanol. The CH_2Cl_2, EtOAc, and *n*-butanol fractions significantly inhibited the murine lymphocytic leukemia cell line (P388: ED_{50} 0.24, 0.034, and 0.92 μg/mL, respectively). Bioassay-guided (P388) separation of these three fractions was conducted using a series of gel permeation and partition chromatographic separations on Sephadex LH-20 columns, followed by final purification by crystallization, to afford 3-epipancratistatin (**1b**, 50 mg, 1.25×10^{-6} %, P388 ED_{50} 0.69 μg/mL) and narciclasine (**2**, 26 g, 6.5×10^{-4} %, P388 ED_{50} 0.018 μg/mL).



1a, 3-β-OH, (+)-pancratistatin
1b, 3-α-OH



2, narciclasine

3-Epipancratistatin (**1b**) crystallized from MeOH as a colorless solid. The molecular formula of **1b** was established by high-resolution mass spectroscopy as $C_{14}H_{15}NO_8$, which is also the molecular formula of pancratistatin (**1a**). The ^1H NMR, ^{13}C NMR, and HMQC spectra of **1b** indicated the presence of six aromatic carbons, including one methine carbon (δ_H 6.49, δ_C 97.7) and five quaternary carbons (δ_C 152.1, 145.4, 135.8, 131.7, 107.4), as well as one OCH_2O group (δ_H 6.02, 6.04, δ_C 103.4), one amide unit (δ_{NH} 7.49, δ_{CO} 169.4), and six sp^3-methine groups, of which four were oxygenated (δ_C/δ_H 67.3/4.27, 72.6/3.82, 70.4/3.55, 71.9/3.54, 54.0/3.37, 38.3/2.94). The six exchangeable protons in the ^1H NMR spectrum were

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Table 1. Murine P388 Lymphocytic Leukemia (ED_{50}) and Human Cancer Cell Line Values (GI_{50} μg/mL)

	cancer cell line[a]						
	P388	BXPC-3	MCF-7	SF-268	NCI-H460	KM20L2	DU-145
1a[11]	0.017	0.02	0.023	0.014	0.032	0.025	0.015
1b	0.69	1.7	2.2	0.74	0.85	2.3	1.3

[a]In DMSO. Cancer type: P388 (murine lymphocytic leukemia); BXPC-3 (pancreas adenocarcinoma); MCF-7 (breast adenocarcinoma; SF 268 (CNS glioblastoma); NCI-H460 (lung large cell); KM 20L2 (colon adenocarcinoma); DU-145 (prostate carcinoma).

removed by addition of D_2O, revealing the presence of a phenolic OH group (δ_H 13.02), an amide NH group (δ_H 7.49), and four additional OH groups (δ_H 5.11, 4.94, 4.62, 5.03). Thus all the atoms of **1b** by NMR spectroscopy matched the molecular formula $C_{14}H_{15}NO_8$ from its high-resolution mass spectrum.

In an analysis of the H−H COSY spectrum, the six sp^3 protons were found to interact in a sequence that indicated a six-membered ring. The four OH signals at δ_H 5.11, 4.94, 4.62, and 5.03 were assigned to positions 1 to 4 according to the H−H COSY correlations between the OH groups and the adjacent protons. The aromatic ring, six-membered ring, and amide segments were further connected by analysis of cross signals in the HMBC spectrum, which led to the conclusion that compound **1b** had the same planar structure as pancratistatin (**1a**).

The relative configuration of **1b** was elucidated by interpretation of the ROESY spectrum and coupling constant values. The large coupling constant between H-10b and H-4a ($J_{H\text{-}10b/H\text{-}4a}$ = 12.6 Hz) suggested a *trans*-fused B/C ring system, with each proton having an axial orientation as in pancratistatin (**1a**). In the ROESY spectrum of **1b**, cross-peaks for axial-oriented H-10b with the 2-OH group and H-4 were observed (δ_H/δ_H = 2.94/4.94 and 2.94/3.54, respectively). An unambiguous relationship was observed between the axial-oriented 1-OH group and H-3 (δ_H/δ_H 5.11/3.55). Although the water peak interfered, ROESY cross-peaks were noted for H-4a (δ_H 3.37) with 1-OH and H-3. For comparison, a NOESY experiment using an authentic pancratistatin (**1a**) sample was performed, which showed the NOE effects between H-4a and 1-OH (δ_H/δ_H 3.70/4.82), H-4a and 3-OH (3.70/5.07), and H-4 and 2-OH (3.72/5.36). All these results described 1−3−5 space effects in a chair conformation of the six-membered ring and implied an α (equatorial) assignment for the 3-OH group of **1b**, the reverse of the β (axial) 3-OH in pancratistatin (**1a**). Furthermore, a closer comparison of the ^{1}H NMR spectra of **1b** and **1a** clearly showed that the difference in configuration of the 3-OH group in **1b** appeared in chemical shift changes for H-2, H-3, H-4, and H-5, as well as the 1-OH and 3-OH groups, relative to those of **1a**. Also, a 1−3 effect resulted in an upfield shift of 4 ppm for the C-4a signal in the ^{13}C spectrum (from δ_C 50 for **1a** to δ_C 54 for **1b**).

The growth inhibitory properties of *Narcissus* cv. Ice Follies constituent **1b** were evaluated using the murine P388 lymphocytic leukemia cell line and a selection of human cancer cell lines (Table 1) and compared to those of its C-3 epimer, compound **1a**. As we have consistently found, pancratistatin (**1a**) exhibited strong inhibition against growth of the murine P388 lymphocytic leukemia and a minipanel of human tumor cell lines. The new isocarbostyril 3-epipancratistatin (**1b**) also exhibited significant growth inhibitory activity against the same cancer cell lines but was over 100× less inhibitory than **1a**, thereby revealing a very important configurational requirement for stronger cancer cell growth inhibitory activity.

EXPERIMENTAL SECTION

General Experimental Procedures. All solvents used in the extraction and isolation processes were redistilled prior to use. Both gel and partition column chromatography was performed with Sephadex LH-20 from Pharmacia. The uncorrected melting points were observed using a Fisher-Johns apparatus. The IR spectra were obtained with a Thermo Nicolet Avatar 360 ESP spectrometer. ^{1}H and ^{13}C NMR spectra were recorded using a Varian Inova 500 MHz instrument. High-resolution APCI mass spectra were obtained employing a JEOL-LC Mate LCMS system.

Plant Material. The bulbs of *Narcissus* cv. Ice Follies were grown commercially (K. Van Bourgondien and Sons, Virginia Beach, VA, USA) in 1997. A voucher specimen (B849350) was deposited in the herbarium of the Cancer Research Institute at Arizona State University.

Extraction and Isolation. All the separations were guided by bioassay results using the murine P388 lymphocytic leukemia (P388) and a minipanel of human cancer cell lines. The dry *Narcissus* bulbs (4000 kg) were placed in barrels and soaked in CH_3OH for at least a week, and an extract was prepared[8b] and processed in 1 kg batches as follows. The bulb extract was partitioned between 9:1 CH_3OH−H_2O (8 L) and hexane (8 L), and the CH_3OH−H_2O layer was further extracted with hexane (4 × 8 L). The hexane layers were combined and dried, and water (4 L) was added to the methanolic phase to give a 3:2 CH_3OH−H_2O layer, which was extracted with CH_2Cl_2 (3 × 8 L). The CH_2Cl_2 fractions were combined and dried. The CH_3OH was removed from the aqueous layer in vacuo, and the remaining H_2O phase was made up to 4.5 L and extracted with EtOAc (5 × 4.5 L), followed by *n*-BuOH (4 × 4.5 L), to give EtOAc and 1-butanol fractions. This process was repeated and the final extracts were combined to give CH_2Cl_2 (3.12 kg, P388 ED_{50} 0.24 μg/mL), EtOAc (935 g, P388 ED_{50} 0.034 μg/mL), and *n*-butanol (3.45 kg, P388 ED_{50} 0.92 μg/mL) extracts. These fractions contained narciclasine (**2**, detected by TLC), and each was dissolved in CH_3OH and subjected to gel permeation column chromatography on Sephadex LH-20 (100 × 15 cm). Continued elution with CH_3OH provided 45 g of a crude narciclasine-containing fraction, which was subjected to repeated partition column chromatography on Sephadex LH-20 (5 × 110 cm) employing hexane−CH_2Cl_2−CH_3OH (3:1:1) as eluent to afford 50 mg of 3-epipancratistatin (**1b**) and 26 g of pure narciclasine (**2**).

*3-Epipancratistatin (**1b**):* colorless solid that crystallized from CH_3OH; mp 293−295 °C; IR (film) ν_{max} 3421, 3227, 2924, 1670, 1463, 1342, 1088, 1032 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 13.02 (1H, s, 7-OH), 7.49 (1H, s, 5-NH), 6.49 (1H, s, H-10), 6.04, 6.02 (1H each, d, J = 1.5 Hz, H-8a), 5.11 (1H, d, J = 4.8 Hz, 1-OH), 5.03 (1H, d, J = 3.6 Hz, 4-OH), 4.94 (1H, d, J = 3.6 Hz, 2-OH), 4.62 (1H, d, J = 4.5 Hz, 3-OH), 4.27 (1H, br s, H-1), 3.82 (1H, br s, H-2), 3.55 (1H, br s, H-3), 3.54 (1H, br s, H-4), 3.37 (1H, dd, J = 13.5, 9.6 Hz, H-4a), 2.94 (1H, d, J = 12.6 Hz, H-10b); ^{13}C NMR (DMSO-d_6) δ 169.4 (C-6), 152.1 (C-9), 145.4 (C-7), 135.8 (C-10a), 131.7 (C-8), 107.4 (C-6a), 101.8 (C-8a), 97.7 (C-10), 72.6 (C-2), 71.9 (C-4), 70.4 (C-3), 67.3 (C-1), 54.0 (C-4a), 38.3 (C-10b); HRAPCI m/z 326.0987 $[M + H]^+$ (calcd for $C_{14}H_{16}NO_8$, 326.0876). Compound **1b** recrystallized from DMSO−H_2O: mp 297−299 °C; anal. C 46.91%, H 4.78%, N 3.82%, calcd for $C_{14}H_{15}NO_8 \cdot 2H_2O$, C 46.50%, H 5.26%, N 3.87%.

Cancer Cell Line Procedures. Inhibition of human cancer cell line growth was determined using the NCI's sulforhodamine B assay as previously described.[12] In summary, cells in a 5% fetal bovine serum/RPMI1640 medium solution were inoculated in 96-well plates and

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incubated for 24 h. The compounds were then added as serial dilutions. After 48 h, the plates were fixed with trichloroacetic acid, stained with sulforhodamine B, and then read with an automated microplate reader. Growth inhibition of 50% (GI_{50}, or the drug concentration causing a 50% reduction in the net protein increase) was calculated from optical density data with Immunosoft software.

The murine lymphocytic leukemia P388 cells[13] were incubated for 24 h in a 10% horse serum/Fisher medium solution followed by a 48 h incubation with serial dilutions of each compound. Cell growth inhibition (ED_{50}) was then calculated using a Z1 Beckman/Coulter particle counter.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{13}C NMR spectra of compound **1b** and a table of the data (Table 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

In memory of Professor Norman Farnsworth (1930−2011), a world-class pharmaceutical and natural products scientist who will be deeply missed.

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