Synthesis and Cytotoxic Activity of 11-Nitro and 11-Amino Derivatives of Acronycine and 6-Demethoxyacronycine

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Condensation of 2-chloro-3-nitrobenzoic acid with either 5-amino-7-methoxy-2,2-dimethyl-2*H*-chromene or 5-amino-2,2-dimethyl-2*H*-chromene afforded diphenylamines 14 and 15. Trifluoroacetic anhydride mediated cyclization gave the corresponding acridones 16 and 17, which were subsequently *N*-methylated and reduced to 11-aminoacronycine and 11-amino-6-demethoxyacronycine.

These two amino compounds, which gave stable water soluble salts, were 2- to 3-fold more potent than acronycine or 6-demethoxyacronycine in inhibiting L1210 cell proliferation.

Key words acronycine; 11-aminoacronycine; 11-amino-6-demethoxyacronycine; cytotoxicity

The acridone alkaloid acronycine (**1**), which was first isolated from *Acronychia baueri* SCHOTT (Rutaceae) in 1948 was later found to be a potent anticancer agent.[1—5] It is of interest because of its broad spectrum of activity, including numerous solid tumors.[2—6] Nevertheless, clinical trials have been severely hampered by its very low water-solubility and have therefore given only poor results.[7]

In this context, the development of derivatives bearing amino groups and able to give water-soluble salts appeared to us highly desirable. The only previous efforts towards this aim led to the synthesis of acronycine analogues with a dialkylaminoalkoxy substituent instead of a methoxy at 6-position.[8] Some of these derivatives, exemplified by **2**, exhibited cytotoxic and antitumor activities but spontaneously decomposed into the inactive noracronycine (**3**) in an acidic medium. Consequently, such 6-dialkylaminoalkoxy-3,12-dihydro-3,3,12-trimethyl-7*H*-pyrano[2,3-*c*]acridin-7-ones, which did not give stable water-soluble salts, were not further developed. More recently, 11-methoxyacronycine (**4**), first obtained by total synthesis[9] and later isolated from various *Citrus* species[10,11] was shown to be significantly active when tested against HL-60 promyelocytic leukemic cells.[12,13] Consequently, we decided to introduce an aromatic amino group at 11-position, considering it should most probably not affect the activity.

This paper deals with the synthesis and cytotoxic properties of 11-nitroacronycine (**5**) and 11-aminoacronycine (**6**) and of their 6-demethoxy counterparts, 11-nitro-6-demethoxyacronycine (**7**) and 11-amino-6-demethoxyacronycine (**8**), since 6-demethoxyacronycine (**9**) was recently shown to exhibit cytotoxic activity within the same range of magnitude as acronycine itself.[14] It should be noted that 11-nitronoracronycine (**10**) had been previously synthetized and did not show any significant cytotoxicity, as most "nor" derivatives in the series.[15]

Chemistry

The key-step of our approach was an Ullmann condensation[16] of 2-chloro-3-nitrobenzoic acid (**11**) with either 5-amino-7-methoxy-2,2-dimethyl-2*H*-chromene (**12**)[17] or 5-amino-2,2-dimethyl-2*H*-chromene (**13**)[14,18,19] to afford the corresponding carboxylic diphenylamines **14** and **15**, respectively. Cyclization to 3,12-dihydro-3,3-dimethyl-6-methoxy-11-nitro-7*H*-pyrano[2,3-*c*]acridin-7-one (**16**) and 3,12-dihydro-3,3-dimethyl-11-nitro-7*H*-pyrano[2,3-*c*]acridin-7-one

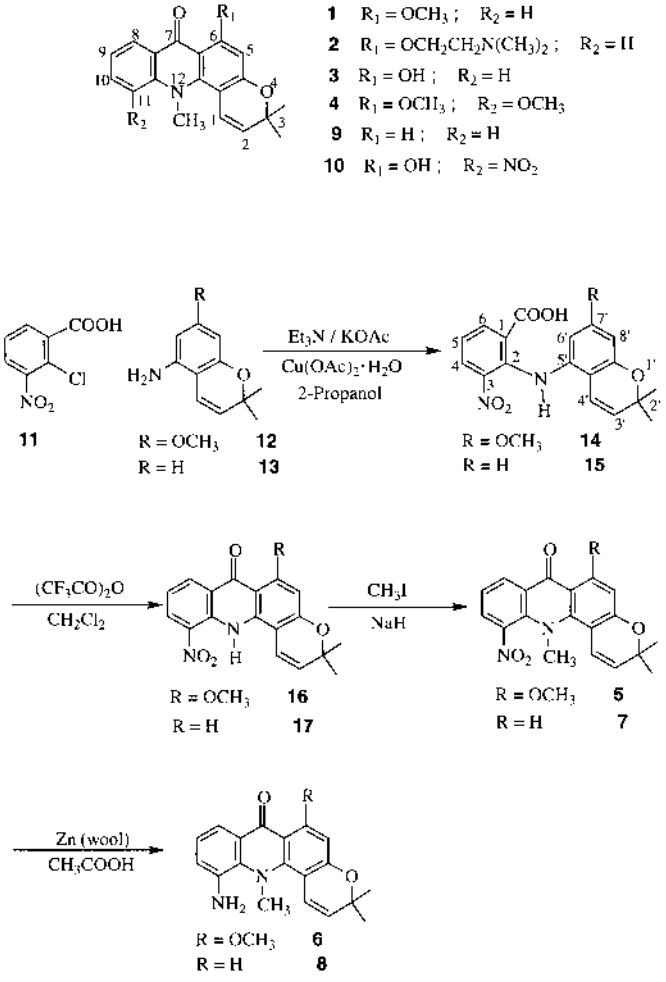

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Table 1. Inhibition of L1210 Cell Proliferation by Compounds **16**, **17**, **5**, **7**, **6** and **8** in Comparison with **1** and **9**

Compound	**1**	**9**	**16**	**17**	**5**	**7**	**6**	**8**
IC_{50} (μM)	27	29.9	39.3	70.4	32.7	70.7	18.8	9.4

(**17**) was obtained by the use of trifluoroacetic anhydride in dichloromethane, which had previously given excellent results in the course of syntheses of acronycine[20] and 6-demethoxyacronycine.[14] Presence of a methoxy group on the chromenyl moiety of the starting material had a dramatic influence on the evolution and yield of the reaction, since **16** was obtained in almost quantitative yield from **14** within 5 min, whereas **17** was only obtained in 78% from **15** within 24 h under optimized conditions. Methylation at N-12 was ensured by the use of methyl iodide in the presence of sodium hydride in dimethylformamide, to give **5** and **7**, respectively. Finally, reduction of the aromatic nitro group by zinc wool in acetic acid afforded the desired **6** and **8**.

Pharmacology

The study of the biological properties of the new pyranoacridone derivatives was carried out *in vitro* on the L1210 murine leukemia cell line. The results (IC_{50}) are reported in Table 1. Nitro derivatives **5**, **7**, **16**, and **17** were devoid of significant antiproliferative activity. In contrast, amino compounds **6** and **8** were, as expected, 2- to 3-fold more potent than acronycine and 6-demethoxyacronycine in inhibiting the proliferation of L1210 cells.

Results and Discussion

Considering the structure–activity relationships in the acronycine series, it appears that compounds bearing an amino substituent at the 11-position exhibit cytotoxic activities within the same range of magnitude as the parent compounds. This conclusion is in full agreement with previous observations stating that acronycine derivatives with hydroxy or alkoxy substituents at 11-position or at 10- and 11-positions on A ring are cytotoxic.[12,13] Interestingly, both **6** and **8** gave stable water soluble salts, which should further allow parenteral formulation of these drugs.

Experimental

Chemistry Mass spectra were recorded with a Nermag R-10-10C spectrometer using electron impact (MS) and/or desorption chemical ionization (DCI-MS; reagent gas: NH_3) techniques. UV spectra (λ_{max} in nm) were determined in spectroscopic grade MeOH on a Beckman Model 34 spectrophotometer. IR spectra (ν_{max} in cm^{-1}) were obtained in potassium bromide pellets on a Perkin-Elmer 257 instrument. ^{1}H-NMR (δ[ppm], J[Hz]) and ^{13}C-NMR spectra were recorded at 300 MHz and 75 MHz, respectively, using a Bruker AC-300 spectrometer. Column chromatography was conducted using flash silica gel 60 Merck (40—63 μm) with an overpressure of 300 mbars.

2-[(2,2-Dimethyl-7-methoxy-2*H*-chromen-5-yl)amino]-3-nitrobenzoic Acid (14) A mixture of 5-amino-7-methoxy-2,2-dimethyl-2*H*-chromene (**12**) (0.205 g, 1 mmol), 2-chloro-3-nitrobenzoic acid (**11**) (0.250 g, 1.24 mmol), potassium acetate (240 mg), cupric acetate monohydrate (9 mg), and triethylamine (0.23 ml) in 2-propanol was heated under reflux for 48 h. The reaction mixture was evaporated under reduced pressure and the residue was partitioned between CH_2Cl_2 (15 ml) and 1 N aqueous HCl (10 ml). The aqueous phase was extracted with CH_2Cl_2 (2×5 ml). The combined organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. Column chromatography (eluent: CH_2Cl_2) afforded **14** as an amorphous solid (0.295 g, 79%). IR (KBr) cm^{-1}: 3340, 2825, 1680, 1570, 1345, 1120, 910. UV λ_{max} (MeOH) nm (log ε): 268 (4.28), 315 (3.95). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.45 (s, 6H, ($C\underline{H}_3$)$_2$), 3.65 (s, 3H, O-$C\underline{H}_3$), 5.56 (d, 1H, J=10 Hz, C3′-H), 5.97 (d, 1H, J=2 Hz, C8′-H), 6.16 (d, 1H, J=2 Hz, C6′-H), 6.51 (d, 1H, J=10 Hz, C4′-H), 6.94 (t, 1H, J=8 Hz, C5-H), 8.06 (dd, 1H, J=8, 2 Hz, C6-H), 8.20 (dd, 1H, J=8, 2 Hz, C4-H), 9.50 (br s, 1H, D_2O exch., N-H), 12,00 (br s, 1H D_2O exch., COO$\underline{H}$). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 27.7 (q, ($\underline{C}H_3$)$_2$), 55.2 (q, O-$\underline{C}H_3$), 76.2 (s, C-2′), 97.7 (d, C-8′), 99.4 (d, C-6′), 108.2 (s, C-4′a), 117.1 (d, C-5), 117.4 (d, C-4′), 117.9 (s, C-1), 128.2 (d, C-3′), 131.8 (d, C-4), 137.2 (d, C-6), 137.7 (s, C-5′), 139.1 (s, C-3), 141.6 (s, C-2), 155.3 (s, C-8′a), 160.5 (s, C-7′), 170.7 (s, $\underline{C}$OOH). MS *m/z*: 370 (M^+), 355, 337, 323, 308. *Anal*. Calcd for $C_{19}H_{18}N_2O_6$: C, 61.62; H, 4.90; N, 7.56. Found: C, 61.80; H, 4.87; N, 7.63.

2-[(2,2-Dimethyl-2*H*-chromen-5-yl)amino]-3-nitrobenzoic Acid (15) Condensation of 5-amino-2,2-dimethyl-2*H*-chromene (**13**) (0.088 g, 0.50 mmol) with **11** (0.120 g, 0.52 mmol) under conditions similar to those described for the preparation of **14** afforded **15** (0.125 g, 73%) as an amorphous solid. IR (KBr) cm^{-1}: 3320, 3000, 1670, 1445, 1265, 770. UV λ_{max} (MeOH) nm (log ε): 208 (4.50), 262 (4.29), 284 (4.18). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.50 (s, 6H, ($C\underline{H}_3$)$_2$), 5.72 (d, 1H, J=10 Hz, C3′-H), 6.44 (dd, 1H, J=8, 1 Hz, C8′-H), 6.58 (dd, 1H, J=8, 1 Hz, C6′-H), 6.62 (d, 1H, J=10 Hz, C4′-H), 6.94 (t, 1H, J=8 Hz, C5-H), 6.96 (t, 1H, J=8 Hz, C7′-H), 8.08 (dd, 1H, J=8, 2 Hz, C6-H), 8.21 (dd, 1H, J=8, 2 Hz, C4-H), 9.55 (br s, 1H, D_2O exch., N-H), 11.50 (br s, 1H D_2O exch., COO$\underline{H}$). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 27.7 (q, ($\underline{C}H_3$)$_2$), 75.8 (s, C-2′), 111.9 (d, C-8′), 113.8 (d, C-5), 115.3 (s, C-4′a), 117.3 (2d, C-6′, C-4′), 117.8 (s, C-1), 128.9 (d, C-3′), 131.3 (d, C-4), 132.0 (d, C-7′), 136.9 (s, C-5′), 137.4 (d, C-6), 138.9 (s, C-3), 142.3 (s, C-2), 154.1 (s, C-8′a), 171.6 (s, $\underline{C}$OOH). DCI-MS *m/z*: 341 $(M+H)^+$, 323, 307, 240. *Anal*. Calcd for $C_{18}H_{16}N_2O_5$: C, 63.52; H, 4.74; N, 8.23. Found: C, 63.41; H, 4.69; N, 8.31.

3,12-Dihydro-3,3-dimethyl-6-methoxy-11-nitro-7*H*-pyrano[2,3-*c*]acridin-7-one (16) To a solution of **14** (0.074 g, 0.20 mmol) in CH_2Cl_2 (3 ml) was added trifluoroacetic anhydride (0.3 ml). The mixture was stirred at 20 °C for 5 min, evaporated under reduced pressure, and taken up by CH_2Cl_2 (10 ml) and saturated aqueous $NaHCO_3$. The aqueous phase was extracted with CH_2Cl_2 (2×5 ml). The combined organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure, to afford **16** as a reddish amorphous solid (0.068 g, 97%). IR (KBr) cm^{-1}: 3380, 2860, 1610, 1520, 1310, 1200, 750. UV λ_{max} (MeOH) nm (log ε): 237 (4.27), 278 (4.32). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.50 (s, 6H, ($C\underline{H}_3$)$_2$), 4.00 (s, 3H, O-CH_3), 5.75 (d, 1H, J =10 Hz, C2-H), 6.30 (s, 1H, C5-H), 6.60 (d, 1H, J=10 Hz, C1-H), 7.28 (t, 1H, J=8 Hz, C9-H), 8.59 (dd, 1H, J=8, 2 Hz, C10-H), 8.75 (dd, 1H, J=8, 2 Hz, , C8-H), 11.30 (br s, 1H, D_2O exch., N-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 27.8 (q, ($\underline{C}H_3$)$_2$), 56.2 (q, O-$\underline{C}H_3$), 77.7 (s, C-3), 95.4 (d, C-5), 100.3 (s, C-12b), 106.7 (s, C-6a), 114.2 (d, C-1), 119.8 (d, C-9), 125.5 (s, C-7a), 128.5 (d, C-2), 130.2 (d, C-10), 133.4 (s, C-12a), 135.0 (s, C-11a), 136.2 (d, C-8), 137.8 (s, C-11), 159.0 (s, C-4a), 162.7 (s, C-6), 174.8 (s, C-7). MS *m/z*: 352 (M^+), 337. *Anal*. Calcd for $C_{19}H_{16}N_2O_5$: C, 64.77; H, 4.58; N, 7.95. Found: C, 64.92; H, 4.49; N, 8.03.

3,12-Dihydro-3,3-dimethyl-11-nitro-7*H*-pyrano[2,3-*c*]acridin-7-one (17) Cyclization of **15** (0.077 g, 0.23 mmol) under conditions similar to those described for the preparation of **16**, but with a reaction time of 24 h, afforded **17** (0.057 g, 78%) as an amorphous solid. IR (KBr) cm^{-1}: 3330, 2990, 1600, 1290, 745. UV λ_{max} (MeOH) nm (log ε): 246 (4.61), 269 (4.50). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.55 (s, 6H, ($C\underline{H}_3$)$_2$), 5.87 (d, 1H, J=10 Hz, C2-H), 6.68 (d, 1H, J=10 Hz, C1-H), 6.84 (d, 1H, J=9 Hz, C5-H), 7.30 (t, 1H, J=8 Hz, C9-H), 8.23 (d, 1H, J=9 Hz, C6-H), 8.67 (dd, 1H, J=8, 2 Hz, C10-H), 8.82 (dd, 1H, J=8, 2 Hz, C8-H), 9.90 (br s, 1H, D_2O exch., N-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 27.8 (q, (CH_3)$_2$), 78.4 (s, C-3), 107.1 (s, C-12b), 114.4 (2d, C-5, C-1), 116.0 (s, C-6a), 119.9 (d, C-9), 124.0 (s, C-7a), 128.7 (d, C-2), 131.0 (d, C-10), 131.2 (d, C-6), 134.2 (s, C-12a), 135.8 (s, C-11a), 136.0 (s, C-11), 136.3 (d, C-8), 158.3 (s, C-4a), 175.8 (s, C-7). MS *m/z*: 322 (M^+), 307, 261. *Anal*. Calcd for $C_{18}H_{14}N_2O_4$: C, 67.08; H, 4.38; N, 8.69. Found: C, 67.21; H, 4.41; N, 8.77.

11-Nitroacronycine (5) To a cooled solution of **16** (0.080 g, 0.23 mmol) in dimethylformamide (7 ml) was added methyl iodide (0.4 ml) and sodium hydride (0.30 g of 50% oil dispersion). The mixture was refluxed under Ar for 48 h. Ice water (15 ml) was added and the mixture was extracted with CH_2Cl_2 (20 ml). The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure, to give **5** as an amorphous solid (0.048 g, 58%). IR (KBr) cm^{-1}: 2960, 1595, 1565, 1350, 765. UV λ_{max} (MeOH) nm (log ε): 278 (4.53), 412 (3.73). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.53 (s, 6H, ($C\underline{H}_3$)$_2$), 3.47 (s, 3H, N-$C\underline{H}_3$), 3.97 (s, 3H, O-$C\underline{H}_3$), 5.64 (d, 1H, J=10 Hz, C2-H), 6.38 (s, 1H, C5-H), 6.61 (d, 1H, J=10 Hz, C1-H), 7.32 (t,

1H, J=8 Hz, C9-H), 8.12 (dd, 1H, J=8, 2 Hz, C10-H), 8.56 (dd, 1H, J=8, 2 Hz, C8-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 27.2 (q, ($\underline{C}H_3)_2$), 47.0 (q, N-$\underline{C}H_3$), 56.4 (q, O-$\underline{C}H_3$), 77.8 (s, C-3), 96.0 (d, C-5), 105.2 (s, C-12b), 111.0 (s, C-6a), 120.5 (d, C-1), 121.7 (d, C-2), 125.4 (d, C-9), 129.6 (d, C-8), 130.0 (s, C-7a), 132.4 (d, C-10), 140.3 (s, C-12a), 140.9 (s, C-11a), 148.2 (s, C-11), 160.1 (s, C-4a), 162.3 (s, C-6), 176.0 (s, C-7). MS m/z: 366 (M^+), 351. *Anal*. Calcd for $C_{20}H_{18}N_2O_5$: C, 65.57; H, 4.95; N, 7.65. Found: C, 65.47; H, 5.02; N, 7.56.

11-Nitro-6-demethoxyacronycine (7) Methylation of **17** (0.040 g, 0.12 mmol) with methyl iodide (0.2 ml) and sodium hydride (0.24 g of 50% oil dispersion) in dimethylformamide (4 ml) under conditions similar to those described for the preparation of **5** afforded **7** (0.37 g, 92%) as an amorphous solid. IR (KBr) cm^{-1}: 2990, 1600, 1280, 765. UV λ_{max} (MeOH) nm (log ε): 254 (4.43), 275 (4.38), 284 (4.33), 404 (3.65). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.55 (s, 6H, ($C\underline{H}_3)_2$), 3.56 (s, 3H, N-$C\underline{H}_3$), 5.73 (d, 1H, J=10 Hz, C2-H), 6.68 (d, 1H, J=10 Hz, C1-H), 6.88 (d, 1H, J=9 Hz, C5-H), 7.36 (t, 1H, J=8 Hz, C9-H), 8.17 (dd, 1H, J=8, 2 Hz, C10-H), 8.21 (d, 1H, J=9 Hz, C6-H), 8.65 (dd, 1H, J=8, 2 Hz, C8-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 27.1 (q, ($\underline{C}H_3)_2$), 46.6 (q, N-$\underline{C}H_3$), 78.4 (s, C-3), 111.4 (s, C-12b), 114.3 (d, C-1), 119.4 (s, C-6a), 120.5 (d, C-5), 121.5 (d, C-6), 127.5 (d, C-9), 128.6 (d, C-2), 128.8 (s, C-7a), 130.3 (d, C-8), 132.4 (d, C-10), 141.2 (s, C-11a), 141.4 (s, C-11), 146.0 (s, C-12a), 159.7 (s, C-4a), 176.4 (s, C-7). MS m/z: 336 (M^+), 321, 306, 274. *Anal*. Calcd for $C_{19}H_{16}N_2O_4$: C, 67.85; H, 4.79; N, 8.33. Found: C, 67.83; H, 4.71; N, 8.39.

11-Aminoacronycine (6) Zinc wool (0.112 g) was added portionwise to a solution of **5** (0.030 g, 0.08 mmol) in acetic acid (1 ml) and water (0.15 ml). The mixture was stirred at room temperature for 30 min, filtered, alkalinized with 10% aqueous NH_4OH, and extracted with EtOAc (2× 10 ml). The combined organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. Column chromatography (eluent: CH_2Cl_2) afforded **6** as an amorphous solid (0.018 g, 65%). IR (KBr) cm^{-1}: 2980, 2920, 1580, 1200, 760. UV λ_{max} (MeOH) nm (log ε): 267 (4.02), 380 (3.37). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.54 (s, 6H, ($C\underline{H}_3)_2$), 3.36 (s, 3H, N-$C\underline{H}_3$), 3.69 (br s, 2H, D_2O exch., NH_2), 3.96 (s, 3H, O-$C\underline{H}_3$), 5.63 (d, 1H, J=10 Hz, C2-H), 6.34 (s, 1H, C5-H), 6.66 (d, 1H, J=10 Hz, C1-H), 6.96 (dd, 1H, J=8, 1.5 Hz, C10-H), 7.14 (t, 1H, J=8 Hz, C9-H), 7.69 (dd, 1H, J=8, 1.5 Hz, C8-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 27.4 (q, ($\underline{C}H_3)_2$), 45.7 (q, N-$\underline{C}H_3$), 56.2 (q, O-$\underline{C}H_3$), 76.8 (s, C-3), 95.9 (d, C-5), 105.0 (s, C-12b), 113.4 (s, C-6a), 116.9 (d, C-9), 119.0 (d, C-1), 119.2 (s, C-7a), 119.4 (s, C-11a), 119.8 (d, C-2), 124.4 (d, C-10), 126.0 (d, C-8), 138.4 (s, C-11), 150.7 (s, C-12a), 158.9 (s, C-4a), 162.0 (s, C-6), 179.8 (s, C-7). MS m/z: 336 (M^+), 321, 306, 277. *Anal*. Calcd for $C_{20}H_{20}N_2O_3$: C, 71.41; H, 5.99; N, 8.33. Found: C, 71.27; H, 6.05; N, 8.37.

11-Amino-6-demethoxyacronycine (8) Reduction of **7** (0.090 g, 0.27 mmol) under conditions similar to those described for the preparation of **6** afforded **8** (0.35 g, 42%) as an amorphous solid. IR (KBr) cm^{-1}: 2980, 1590, 1275, 750. UV λ_{max} (MeOH) nm (log ε): 265 (4.34), 402 (3.47). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.54 (s, 6H, ($C\underline{H}_3)_2$), 3.42 (s, 3H, N-$C\underline{H}_3$), 3.97 (br s, 2H, D_2O exch., NH_2), 5.72 (d, 1H, J=10 Hz, C2-H), 6.73 (d, 1H, J=10 Hz, C1-H), 6.81 (d, 1H, J=9 Hz, C5-H), 7.02 (dd, 1H, J=8, 2 Hz, C10-H), 7.17 (t, 1H, J=8 Hz, C9-H), 7.78 (dd, 1H, J=8, 2 Hz, C8-H), 8.15 (d, 1H, J=9 Hz, C6-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 27.3 (q, ($\underline{C}H_3)_2$), 45.6 (q, N-$\underline{C}H_3$), 78.3 (s, C-3), 112.0 (s, C-12b), 113.7 (d, C-1), 117.0 (d, C-9), 119.8 (2d, C-5, C-6), 120.6 (s, C-6a), 124.2 (d, C-10), 127.6 (s, C-7a), 128.1 (d, C-8), 128.5 (d, C-2), 138.1 (s, C-11a), 138.8 (s, C-11), 148.7 (s, C-12a), 158.4 (s, C-4a), 179.7 (s, C-7). MS m/z: 306 (M^+), 291, 276. *Anal*. Calcd for $C_{19}H_{18}N_2O_2$: C, 74.49; H, 5.92; N, 9.14. Found: C, 74.57; H, 6.01; N, 9.07.

Biological Pharmacology Cytotoxicity: Murine leukemia L1210 cells from the American Type Culture Collection (Rockville Pike, MD) were grown in RPMI medium 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES buffer (pH 7.4). The cytotoxicity was measured by microculture tetrazolium assay essentially as described.[21] Cells were exposed for 48 h to nine graded concentrations in triplicate of the test drug. Results are expressed as IC_{50} (mean, n=3), which is defined as the drug concentration inhibiting the absorbance by 50% with respect to that of untreated cells.

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