New Cytotoxic Indole Alkaloids from *Tabernaemontana calcarea* from the Madagascar Rainforest[1]

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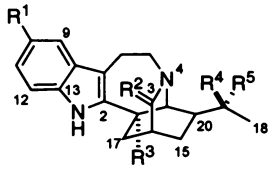
Bioassay-directed fractionation of the alkaloid portion of a CH_2Cl_2−MeOH extract of *Tabernaemontana calcarea* resulted in the isolation of the three new cytotoxic indole alkaloids, **1**−**3**, and the 12 known alkaloids voacangine (**4**), isovoacangine (**5**), coronaridine (**6**), 11-hydroxycoronaridine (**7**), voacristine (**8**), 19-*epi*-voacristine (**9**), isovoacristine (**10**), ibogamine (**11**), 10-methoxyibogamine (**12**), 11-methoxyibogamine (**13**), heyneanine (**14**), and 19-*epi*-heyneanine (**15**). The structures of the new compounds **1**−**3** were elucidated on the basis of extensive 1D and 2D NMR spectroscopic interpretation. All the compounds exhibited cytotoxic activity against the A2780 ovarian cancer cell line.

In our continuing research on the isolation of bioactive compounds from the Suriname and Madagascar rainforests as a part of the mission of the International Cooperative Biodiversity Group (ICBG),[2] we obtained a sample of a CH_2Cl_2−MeOH extract of *Tabernaemontana calcarea* Pichon (Apocynaceae) from the National Cancer Institute. *T. calcarea* is a forest understory shrub widespread in western and northern Madagascar.

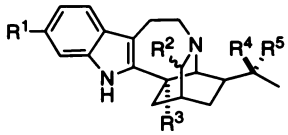
Tabernaemontana is a large genus that is both chemically complex and interesting. There are about 100 species of *Tabernaemontana*,[3] widely distributed in the tropics, with about 18 in Africa, 15 in Madagascar (one of which also occurs in the Comores and Seychelles), one in the Mascarenes, 21 in tropical Asia, and about 50 in the neotropics. Oddly, no species are shared between these five geographic regions. Thus Africa, Madagascar, the Mascarenes, Asia, and the New World tropics all have completely distinct groups of species.

The genus is a very rich source of a number of indole alkaloids with intriguing carbon skeletons and novel biological activities.[4−7] The extract of *T. calcarea* was selected for bioassay-guided fractionation on the basis of its cytotoxicity, with an IC_{50} of 9.3 μg/mL against the A2780 ovarian cancer cell line. Previous phytochemical studies of this species, also known as *Pandaca calcarea*, resulted in the isolation of several alkaloids.[8] The alkaloid portion of the crude extract after extensive chromatography followed by reversed-phase HPLC furnished the three new indole alkaloids **1**−**3** and 12 known derivatives, voacangine (**4**),[7] isovoacangine (**5**),[9] coronaridine (**6**),[10] 11-hydroxycoronaridine (**7**),[11] voacristine (**8**),[7] 19-*epi*-voacristine (**9**),[12] isovoacristine (**10**),[7] ibogamine (**11**),[13] 10-methoxyibogamine (**12**),[13] 11-methoxyibogamine (**13**),[13] heyneanine (**14**),[12] and 19-*epi*-heyneanine (**15**).[12]

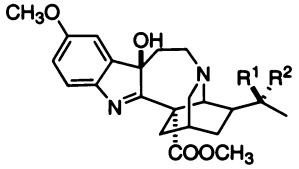
Compound **1** was isolated as a pale yellow optically active oil whose molecular formula was established as $C_{22}H_{26}N_2O_5$ from its HRFABMS, APT (Attached Proton Test), and ^{13}C NMR spectral data. The UV spectrum of **1** showed absorptions at 224, 280, and 294 nm, similar to those of ibogamine alkaloids,[7] suggesting its indole alkaloid nature. The IR absorption bands at 3250 and 1732 cm^{-1} suggested the presence of NH/OH and carbonyl functional groups, respectively, in **1**. It gave a yellow color spot on normal-phase TLC ($CHCl_3$−MeOH, 95:5) with $FeCl_3$−$HClO_4$ spray reagent (1 mL of 0.5 M aqueous $FeCl_3$ in 50 mL of 35% $HClO_4$). The fragment ions observed at *m/z* 380, 367, and 329 in the EIMS indicated the successive loss of water, OCH_3, and $COOCH_3$ molecules from the molecular ion. The



1 $R^1 = OCH_3$; $R^2 = O$; $R^3 = COOCH_3$; $R^4 = H$; $R^5 = OH$
4 $R^1 = OCH_3$; $R^2 = H_2$; $R^4 = R^5 = H$; $R^3 = COOCH_3$
6 $R^1 = R^4 = R^5 = H$; $R^2 = H_2$; $R^3 = COOCH_3$
8 $R^1 = OCH_3$; $R^2 = H_2$; $R^3 = COOCH_3$; $R^4 = OH$; $R^5 = H$
9 $R^1 = OCH_3$; $R^2 = H_2$; $R^4 = H$; $R^3 = COOCH_3$; $R^5 = OH$
11 $R^1 = R^3 = R^4 = R^5 = H$; $R^2 = H_2$
12 $R^1 = OCH_3$; $R^2 = H_2$; $R^3 = R^4 = R^5 = H$
14 $R^1 = R^3 = R^5 = H$; $R^2 = H_2$; $R^4 = OH$
15 $R^1 = R^3 = R^4 = H$; $R^2 = H_2$; $R^5 = OH$
16 $R^1 = R^3 = R^4 = H$; $R^2 = O$; $R^5 = OH$



3 $R^1 = OCH_3$; $R^2 = OH$; $R^3 = R^4 = R^5 = H$
5 $R^1 = OCH_3$; $R^2 = R^4 = R^5 = H$; $R^3 = COOCH_3$
7 $R^1 = OH$; $R^2 = R^4 = R^5 = H$; $R^3 = COOCH_3$
10 $R^1 = OCH_3$; $R^2 = R^5 = H$; $R^3 = COOCH_3$; $R^4 = OH$
13 $R^1 = OCH_3$; $R^2 = R^3 = R^4 = R^5 = H$



2 $R^1 = H$; $R^2 = OH$
17 $R^1 = OH$; $R^2 = H$

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Table 1. ^{1}H NMR Data for Compounds **1**−**3**[a] ($CDCl_3$, 500 MHz)

position	**1**	**2**	**3*R***	**3*S***
3		2.75 dt (9.1, 2.1) 2.92 m	4.39 d (1.4)	4.77 dd (7.3, 1.6)
5	3.16 m, 4.43 m	2.84 m, 3.58 m	1.91 m, 2.04 m	1.91 m, 2.04 m
6	3.21 m	2.82 m, 2.92 m	3.02 m, 3.18 m	3.02 m, 3.18 m
9	6.89 d (2.2)	6.91 d (2.2)	7.30 d (8.6)	7.30 d (8.6)
10			6.73 dd (8.6, 2.2)	6.73 dd (8.6, 2.2)
11	6.80 dd (8.6, 2.2)	6.82 dd (8.6, 2.1)		
12	7.12 d (8.8)	7.35 d (8.4)	6.75 d (8.5)	6.75 d (8.5)
14	2.63 br d (8.2)	2.40 m	1.98 m	1.98 m
15	1.38 m 1.95 ddd (13.6, 9.8, 3.0)	1.80 m, 1.88 m	1.16 m, 1.94 m	1.16 m, 1.94 m
16			2.76 m	2.76 m
17	2.34 ddt (13.6, 5.2, 2.8) 2.63 dd (13.5, 1.5)	1.86 m 2.52 dt (13.8, 2.1)	2.15 m	2.15 m
18	1.30 d (6.2)	1.08 d (6.4)	0.89 t (7.2)[b]	0.87 t (7.1)[b]
19	3.82 qd 6.6, 1.8	3.80 qd, 6.2, 1.8	1.48 m	1.48 m
20	1.78 m	1.50 m	1.46 m	1.46 m
21	4.62 br s	4.09 br s	3.49 d (2.2)	3.49 d (2.2)
OCH_3	3.84 s	3.81 s	3.82 s	3.82 s
$COOCH_3$	3.72 s	3.73 s		
NH	7.84 br s		7.54 br s[c]	7.52 br s[c]

[a] Assignments made on the basis of COSY and HMQC spectral data and comparison with the literature values.[4,9−16] [b,c] Values having the same superscripts between an *R*/*S* pair are interchangeable.

Table 2. ^{13}C NMR Data for Compounds **1**−**3**[a] ($CDCl_3$, 125 MHz)

carbon	**1**	**2**	**3*R***	**3*S***
2	134.2	186.2	140.6[b]	140.2[b]
3	172.8	47.2	91.6	82.8
5	42.4	47.4	54.0[c]	53.5[c]
6	21.2	32.6	21.7[d]	21.4[d]
7	109.3	87.9	108.7	108.7
8	128.2	143.2	124.2	124.2
9	100.6	107.4	118.6	118.5
10	154.2	158.6	108.8	108.8
11	112.7	113.4	156.0	155.9
12	111.5	121.6	94.4	94.4
13	130.9	143.5	135.2	135.1
14	38.1	26.5	24.7	28.3
15	27.5	28.3	33.0[e]	32.9[e]
16	55.8	53.6	41.3[f]	41.1[f]
17	36.0	36.3	33.2[g]	33.1[g]
18	21.6	22.0	12.1[h]	12.0[h]
19	70.1	70.4	27.7[i]	27.0[i]
20	41.4	40.3	40.2[j]	40.1[j]
21	54.6	54.4	59.1[k]	58.8[k]
OCH_3	56.0	55.8	56.0[l]	55.1[l]
$COOCH_3$	175.9	173.3		
$COOCH_3$	53.2	53.6		

[a] Assignments made on the basis of HMQC and HMBC and comparison with the literature data.[4,9−16] [b−l] Values having the same superscripts between an *R*/*S* pair are interchangeable.

^{1}H NMR spectrum showed the presence of a doublet of doublets at δ 6.80 (1H, J = 8.6, 2.2 Hz), three doublets at δ 7.12 (1H, J = 8.8 Hz), 6.89 (1H, J = 2.2 Hz), and 1.30 (3H, J = 6.2 Hz), an oxymethine proton as a quartet of doublets at δ 3.82 (1H, J = 6.6, 1.8 Hz), two methoxyl groups at δ 3.84 (s, 3H) and 3.72 (s, 3H), four methylenes between δ 1.38 and 4.43, and three methines at δ 1.78, 2.63, and 4.62. The ^{13}C NMR values for all 22 carbons were assigned on the basis of HMQC and HMBC spectral data and are given in Table 2. The ^{13}C NMR spectrum of **1** indicated the presence of five sp^2 quaternary carbons, three sp^2 methines, one sp^3 quaternary carbon, three sp^3 methyls, four sp^3 methylenes, four sp^3 methines, and two carbonyl groups in its structure. A search in the literature established that the ^{1}H and ^{13}C NMR spectral data of **1** were similar to those of voacristine (**8**)[7] in the indole part and of 3-oxo-19-*epi*-heyneanine (**16**)[14] in the terpenoid part of the molecule. The structure was further supported by COSY (H-5/H-6; H-11/H-12; H-14/H-15, H-17; H-19/H-18, H-20; H-20/H-15, H-19, H-21) and HMBC (H-6/C-2, C-5, C-7; H-9/C-7, C-8, C-10; H-11/C-9, C-10, C-12; H-12/C-10, C-11, C-13; H-14/C-3, C-15, C-16, C-17; H-17/C-3, C-14, C-16; H-19/C-15, C-18, C-20, C-21; H-21/C-2, C-16, C-17, C-20) correlations. The stereochemistry of the hydroxy group at the C-19 position in **1** was assigned by analogy with the chemical shifts of related C-19 hydroxyindole alkaloids. Thus, 19*S* compounds have a chemical shift for the C-15 carbon at about 23 ppm, which is shifted downfield by about 6.7 ppm from its position in 19*R* compounds. Similarly, the values for C-21 in the 19*R* epimers are shifted upfield by about 5 ppm to about 54.7 ppm compared to the 19*S* epimers, which have the chemical shift of the C-21 carbon at about 60 ppm.[7] The chemical shifts of C-15 (27.5 ppm) and C-21 (54.6 ppm) in **1** are very close to those of **9** [C-15 (28.5 ppm) and C-21 (54.1 ppm)], but not to that of the epimeric compound **8** [C-15 (23.7 ppm) and C-21 (60.6 ppm)],[12] suggesting the *R* configuration for the hydroxy group at C-19. On the basis of the above spectral data, **1** was assigned as 3-oxo-19-*epi*-voacristine.

Compound **2** was also obtained as an optically active oil, and its molecular formula was deduced as $C_{22}H_{28}N_2O_5$ by HRFABMS and ^{13}C NMR spectral data. Its UV and IR spectra showed the presence of an indolenine[10] chromophore with NH/OH and carbonyl functional groups. On normal-phase TLC ($CHCl_3$−MeOH, 95:5) it gave a dark brown colored spot after spraying with $FeCl_3$−$HClO_4$ spray reagent; this contrasted with the yellow color observed for **1**. The ^{1}H NMR spectrum of **2** showed the presence of a doublet of doublets at δ 6.82 (1H, J = 8.6, 2.1 Hz), three doublets at δ 7.35 (1H, J = 8.4 Hz), 6.91 (1H, J = 2.2 Hz), and 1.08 (3H, J = 6.4 Hz), an oxymethine as a quartet of doublets at δ 3.80 (1H, J = 6.2, 1.8 Hz), and two methoxyl groups at δ 3.81 (s, 3H) and 3.73 (s, 3H), very similar to **1**. In the aliphatic region, it showed the presence of five methylenes and three methines, as compared with four methylenes and three methines in **1**. The ^{13}C NMR values for all the carbons were assigned on the basis of HMQC and HMBC spectra and are given in Table 2. The ^{13}C NMR spectrum of **2** did not have a signal corresponding to that of the carbonyl group at the C-3 position of **1**, indicating that a methylene group has replaced the C-3 amide carbonyl group. Although the ^{1}H NMR spectra of **1** and **2**

Table 3. Cytotoxicities of Compounds **1**−**15**[a]

compound	IC_{50} (μg/mL)
1	6.8
2	10.8
3	7.9
4	10.4
5	9.4
6	9.9
7	4.8
8	11.0
9	4.0
10	9.6
11	3.5
12	10.2
13	4.9
14	10.7
15	8.9

[a] Concentration of each compound that inhibited 50% (IC_{50}) of the growth of the A2780 mammalian cell line according to the procedure described,[2,17] with actinomycin D (IC_{50} 1−3 ng/mL) as the positive control. Each value is the average of two independent determinations.

were quite similar, the ^{13}C NMR spectral data differed significantly. The ^{13}C NMR spectrum of **2** showed the presence of four sp^2 quaternary carbons, three sp^2 methines, and a quaternary sp^3 carbon, as compared with five sp^2 quaternary carbons and three sp^2 methines in **1**, suggesting that the indole moiety of **1** was present as an indolenine moiety in **2**. A close comparison of the ^1H and ^{13}C NMR data of **2** with those of voacristine hydroxyindolenine (**17**)[10] indicated that the compounds were almost identical, differing primarily in the carbon chemical shift values of C-15 and C-20. This suggested that **2** has a hydroxyindolenine structure, and this general skeleton was supported by the key HMBC correlations: H-5/C-6, C-7; H-6/C-2, C-5, C-7, C-8; H-17/C-2, C-14, C-15, C-16; and H-21/C-2, C-16, C-17, C-19, C-20. The carbon values for C-15 and C-21 were observed at δ 28.3 and 54.4, which are very close to those of **1**, suggesting the *R* configuration for the C-19 hydroxy group. The same compound was reported earlier as a synthetic product from the oxidation of 19-*epi*-voacristine,[15] but the NMR data have not been published. On the basis of the above spectral data, **2** was assigned as 19-*epi*-voacristine hydroxyindolenine.

Compound **3** was isolated as a colorless oil and determined to have the molecular formula $C_{20}H_{26}N_2O_2$ by HRFABMS. It gave a yellow-colored spot on normal-phase TLC ($CHCl_3$−MeOH, 90:10) with $FeCl_3$−$HClO_4$ spray reagent, similar to that observed with **1**. It was obtained as an inseparable mixture of two compounds. The NMR spectrum of **3** (Tables 1 and 2) indicated the presence of an 11-methoxyibogamine (**13**) skeleton having an additional hydroxy group at the C-3 position. This additional hydroxyl group existed as an approximately 1:1 mixture of 3*R* and 3*S* epimers, interconvertible through the open-chain form. The ^1H NMR chemical shifts for most protons in the two epimers of **3** had the same values, except for the C-3 hydroxymethine protons, the C-18 terminal methyl protons, and the NH protons. There are many reports in the literature in support of the presence of mixtures of 3*R* and 3*S* epimers in the genus *Tabernaemontana*.[7,9,16] These data show that the ^1H NMR chemical shift for the oxymethine proton in the 3*S* epimer appears at lower field than that of the 3*R* proton, and vice versa for the ^{13}C NMR values. Thus, on the basis of the NMR chemical shift analogy reported for these epimers, the oxymethine proton observed as a doublet of doublets at δ 4.77 (J = 7.3, 1.6 Hz) correlating to the carbon value δ 82.8 was assigned to the 3*S* epimer and the other oxymethine observed at δ 4.39 (d, J = 1.4 Hz), with the carbon value δ 91.6, was assigned to the 3*R* epimer. The ^1H and ^{13}C NMR spectral data for the two epimers of **3** were assigned in comparison with **13** and similar compounds representing 3*R*/3*S* epimers in the literature. On the basis of the above spectral data, **3** was assigned as 3*R*/3*S*-hydroxytabernanthine.

All isolated compounds were tested for cytotoxicity against the A2780 ovarian cancer cell line. As shown in Table 3, all the isolated compounds (**1**−**15**) were found to be marginally active, with IC_{50} values ranging from 3.5 to 11.0 μg/mL.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR and UV spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. NMR spectra were obtained on a JEOL Eclipse 500 spectrometer. Mass spectra were obtained on a JEOL HX-110 instrument. The chemical shifts are given in δ (ppm) with TMS as internal reference and coupling constants in Hz. Si gel (Merck 230−400 mesh) and reversed-phase Si gel (LRP-2, 200 μm) were used for column chromatography. Reversed-phase HPLC was performed on a Shimadzu LC-10AT instrument with an ODS C_{18} column.

Cytotoxicity Bioassays. The A2780 ovarian cancer cell line assay was performed at Virginia Polytechnic Institute and State University as previously reported.[2,17]

Plant Material. The sample of *Tabernaemontana calcarea* (Apocynaceae) was collected at Antsiranana in the Ankarana Special Reserve, ca. 5 km NW of Park Village near the Besaboba stream. Collection was from a dry river bed in primary forest, and the specimen collected consisted of leaves and immature fruits. Collection was made on April 27, 1993, by D. K. Harder, M. C. Merello, S. G. Razafimandimbison, and T. G. Razafindrabaeza from the Missouri Botanical Garden and was given the number Harder et al. 1760. The plant was identified according to Leeuwenberg,[3] and duplicate voucher specimens are preserved at Centre National d'Application et des Recherches Pharmaceutiques in Antananarivo, Madagascar; Missouri Botanical Garden, St. Louis, Missouri; Museum National d'Histoire Naturelle Herbarium in Paris, France; Parc de Tsimbazaza Herbarium in Antananarivo, Madagascar; and the Nationaal Herbarium Nederland, Wageningen University Branch.

Extract Preparation. Field-dried plant was ground in a hammermill, then extracted by overnight percolation at room temperature with a 1:1 mixture of reagent-grade CH_2Cl_2−MeOH. The solvent was withdrawn quickly by suction, and the specimen was covered with 100% MeOH. After 30 min the MeOH wash was drained into the same flask as the CH_2Cl_2−MeOH extract, and the combined organic solvent extracts were evaporated on a rotary evaporator below 40 °C to give a thick concentrate, which was transferred into a glass bottle for storage. The bottle was dried overnight under high vacuum to give the dried CH_2Cl_2−MeOH extract N037509.

Extraction and Isolation. The crude extract (3.50 g) was suspended in diethyl ether (200 mL) and extracted with 5% AcOH (3 × 200 mL). The pH of the acid layer was then adjusted to 10 with NH_4OH and extracted with $CHCl_3$−IPA (9:1, 3 × 250 mL). The organic layer was concentrated to yield a dark brown residue (0.85 g) having an IC_{50} of 6.6 μg/mL. The residue (0.80 g) was fractionated over a Si gel column using $CHCl_3$−MeOH (100:0 to 2:1) to furnish 13 fractions (A−M), of which fractions A−C and E−G were found to be the most active. Fraction A on reversed-phase preparative TLC (MeOH−H_2O, 70:30) followed by reversed-phase HPLC with the mobile phase CH_3CN−H_2O (80:20) yielded the two known alkaloids **7** (1.4 mg) and **8** (1.4 mg). Fraction B on reversed-phase preparative TLC (MeOH−H_2O, 70:30) followed by reversed-phase HPLC with the mobile phase CH_3CN−H_2O (70:30) yielded the new alkaloid **3** (0.8 mg) and the three known alkaloids **4** (0.9 mg), **9** (1.2 mg), and **11** (1.6 mg). Fraction C on column chromatography over RP C_{18} using MeOH−H_2O (80:

20) followed by reversed-phase HPLC with the mobile phase CH_3CN-H_2O (75:25) furnished the new alkaloid **1** (1.2 mg) and the known alkaloid **15** (1.5 mg). Fraction E on reversed-phase preparative TLC (MeOH−H_2O, 85:15) followed by reversed-phase HPLC with the mobile phase CH_3CN-H_2O (90:10) yielded the four known compounds **5** (0.8 mg), **6** (0.7 mg), **10** (4.2 mg), and **12** (2.3 mg). Fraction F on reversed-phase preparative TLC (MeOH−H_2O, 90:10) followed by reversed-phase HPLC with the mobile phase CH_3CN-H_2O (90:10) yielded the new alkaloid **2** (1.2 mg). Fraction G on reversed-phase preparative TLC (MeOH−H_2O, 90:10) yielded the two known compounds **13** (2.2 mg) and **14** (1.5 mg). The 12 known compounds **4**−**15** were identified by comparison of their spectral data with literature values.[7,9−13]

19-*epi*-3-Oxovoacristine (1): pale yellow oil; $[\alpha]_D$ −10° (*c* 0.42, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 224 (4.14), 280 (3.82), 294 (3.45); IR ($CHCl_3$) ν_{max} 3250, 3050, 1732, 1650, 1450, 1320 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m*/*z* 398 $[M]^+$ (100), 380 (18), 367 (23), 329 (14), 328 (16), 244 (12), 182 (16), 154 (8), 136 (16), 122 (18); HRFABMS *m*/*z* 399.1926 $[M + H]^+$ (calcd for $C_{22}H_{27}N_2O_5$ 399.1920).

19-*epi*-Voacristine hydroxyindolenine (2): colorless oil; $[\alpha]_D$ +6° (*c* 0.36, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 220 (3.92), 283 (4.12), 292 (3.76), 302 (3.62), 316 (3.51); IR ($CHCl_3$) ν_{max} 3320, 2965, 1727, 1540, 1435, 1280, 1125 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m*/*z* 400 $[M]^+$ (100), 368 (12), 341 (23), 308 (16), 281 (23), 280 (12), 243 (15), 182 (24), 154 (12), 142 (18), 136 (14), 122 (21); HRFABMS *m*/*z* 341.1862 $[M - CH_3COO]^+$ (calcd for $C_{20}H_{25}N_2O_3$ 341.1865).

3*R*/*S*-Hydroxytabernanthine (3): colorless oil; $[\alpha]_D$ −17° (*c* 0.24, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 222 (4.02), 286 (3.68), 294 (3.26); IR ($CHCl_3$) ν_{max} 3345, 2965, 1650, 1450, 1325 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m*/*z* 326 $[M]^+$ (100), 308 (12), 295 (15), 291 (23), 280 (12), 243 (15), 182 (24), 154 (12), 142 (18), 136 (14), 122 (24); HRFABMS *m*/*z* 325.1917 $[M - H]^+$ (calcd for $C_{20}H_{25}N_2O_2$ 325.1916).

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Supporting Information Available: ^1H NMR spectra for compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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