The Characterization and Structure−Activity Evaluation of Toxic Norditerpenoid Alkaloids from Two *Delphinium* Species

Gary D. Manners,*,† Kip E. Panter,‡ James A. Pfister,‡ Michael H. Ralphs,‡ and Lynn F. James‡

Western Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 800 Buchanan Street, Albany, California, and Poisonous Plant Research Laboratory, Agricultural Research Service, United States Department of Agriculture, 1150 E. 1400 N, Logan, Utah

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A new *N*-(methylsuccinimido)anthranoyllycoctonine norditerpenoid alkaloid, geyerline, has been isolated and characterized from extracts of the poisonous larkspur *Delphinium glaucum*. A previously described norditerpenoid alkaloid, grandiflorine, has also been isolated from *Delphinium geyeri*. Both alkaloids are closely related structurally to the potent neurotoxin methyllycaconitine, established as the primary toxin in many larkspurs poisonous to cattle. Mouse bioassay tests showed grandiflorine to possess toxicity comparable to methyllycaconitine, while its synthetically derived monoacetate, grandiflorine acetate, and geyerline are significantly less toxic.

Larkspur species (*Delphinium* sp.) constitute the single largest poisonous-plant threat to grazing cattle on open rangelands of the western United States and are annually responsible for significant economic losses for western ranchers.[1] Two larkspurs, *D. glaucum* and *D. geyeri*, are responsible for recurring cattle-poisoning episodes throughout their ranges. *D. glaucum* is a tall larkspur indigenous to higher elevations of the mountainous west, ranging from California to Alaska and eastward to Colorado, whereas *D. geyeri* is an intermediate larkspur found in the high plains areas of Wyoming, Utah, and Colorado east to Nebraska.

The *N*-(methylsuccinimido)anthranoyllycoctonine (MSAL) norditerpenoid alkaloids, commonly found in the larkspurs have been established as potent mammalian toxins[2] and are considered to be the causative agents in cattle poisoning deaths.[3] The MSAL norditerpenoid alkaloid methyllycaconitine (MLA) (**4**) is recognized as a highly efficient postsynaptic antagonist of nicotinic acetylcholine receptors (nAChR) in insects and mammals.[4−7] Cattle suffering larkspur toxicosis display clinical signs consistent with paralytic effects associated with the antagonistic competitive binding of nAChR sites at the neuromuscular boundary.

The α-bungerotoxin binding specificity of MLA has led to its utilization as a pharmacological probe to characterize nAChR sites,[8,9] and efforts have been directed to the preparation of semisynthetic model probe compounds based upon MLA.[10−12] Utilizing a mouse bioassay to examine structure−activity relationships among the MSAL norditerpenoid alkaloids, we have recently reported[13] three structural prerequisites (a tertiary alkaloid nitrogen, an *ortho*-imide-substituted C-18 benzoate ester, and specific functionality at C-14) necessary for the toxicity of these compounds in mammals.

We now report the isolation, characterization, and toxicological evaluation of grandiflorine (**1**) from *D. glaucum*, its synthetic monoacetate (**2**), and a new norditerpenoid alkaloid, geyerline (**3**), from *D. geyeri*. The observed toxicological properties of these three alkaloids will be discussed in the context of previously presented structure−activity data for other MSAL norditerpenoid alkaloids obtained from poisonous larkspur species.[13]

Results and Discussion

Grandiflorine (**1**) was obtained from an ethyl alcohol extract of *D. glaucum* leaf and stem plant material collected in the Sierra Nevada mountains of California. This alkaloid has been previously described from the Chinese larkspur *D. grandiflorum*.[14] Because experimental details for the characterization of **1** from *D. grandiflorum* are not readily accessible, we here provide experimental data for the isolation and characterization of **1** from *D. glaucum*.

The ^{1}H NMR spectrum of **1** displayed resonances distinctive for MSAL norditerpenoid alkaloids (Table 1), including methyl protons of the alkaloid *N*-ethyl group (δ_H 1.12), the methyl group of the *N*-(methylsuccinimido)anthranoyl ester group (δ_H 1.47), three methoxyl groups (δ_H 3.27, 3.37, 3.41), downfield resonances for the methine proton at C-14 (δ_H 3.63), the C-18 methylene protons (δ_H 4.07; δ 4.19), and lowfield aromatic protons (δ_H 7.48−8.08). These proton resonances are closely comparable to those reported for MLA[15] (**4**) and isodelectine[16] (**8**). Grandiflorine (**1**) primarily differs from MLA (**4**) with the presence of resonances for three rather than four methoxyl groups and a carbinol methine proton resonance at δ_H 3.75 in the ^{1}H NMR spectrum. Conversely, the chemical shift of the carbinol methine proton resonance in **1** is closely comparable to the resonance assigned to the C-1 carbinol methine proton in isodelectine (**8**) (δ_H 3.73), and the chemical shift of the three methoxyl groups of **8** are similar to those in **1** (δ_H 3.29, 3.36, 3.41). A ^{1}H COSY experiment applied to **1** established a carbinol methine proton vicinal to methylene protons in a $-CHOH-CH_2-CH_2-$ fragment, confirming the presence of a hydroxyl at C-1 in grandiflorine (**1**).

Assignment of ^{13}C resonances for **1** (Table 1), as established by DEPT and one-bond HETCOR experi-

* To whom correspondence should be addressed: Tel.: (510) 559-5813. Fax: (510) 559-5828. E-mail: gmann@pw.usda.gov.
† Western Regional Research Centre.
‡ Poisonous Plant Research Laboratory.

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Table 1. ^{13}C and ^{1}H NMR Chemical Shift Assignments for Grandiflorine (**1**), Grandiflorine Acetate (**2**), Geyerline (**3**)[a]

^{13}C	(**1**)	(**2**)	(**3**)	^{1}H	(**1**)	(**2**)	(**3**)
C-1	72.4	77.3	84.1	H-1β	3.75 br t	4.79 dd (J = 7.2, 10.2)	3.43 m
C-2	26.9	27.2	26.2	H-2a, 2b	1.58, 1.68 m	1.60, 1.68 m	1.65, 1.79 m
C-3	29.1	29.0	28.3	H-3	1.80 m	1.70 m	2.17 m
C-4	36.7	37.5	37.8				
C-5	45.2	50.2	50.3	H-5	1.90 br s	1.83 br s	1.72 br s
C-6	90.7	90.5	90.9	H-6α	3.98 br s	3.92 br s	3.57 br s
C-7	87.8	88.6	88.7				
C-8	78.4	77.4	77.4				
C-9	43.3	43.0	43.7	H-9	2.97 m	3.14 m	3.10 m
C-10	43.9	45.3	45.9	H-10	2.04 m	2.23 m	1.92 m
C-11	49.5	48.1	49.1				
C-12	30.4	29.7	29.9	H-12a, 12b	1.78, 2.08 m	1.71, 2.00 m	1.90, 2.50 m
C-13	37.7	38.0	38.1	H-13	2.43 dd (J = 4.2, 7.0)	2.34 dd (J = 4.2, 7.8)	2.33 dd (J = 4.8, 6.8)
C-14	84.5	83.3	83.5	H-14β	3.63 t (J = 4.6)	3.56 t (J = 4.8)	3.61 t (J = 4.4)
C-15	33.5	33.5	33.3	H-15a, 15b	1.80, 2.61 m	1.76, 2.78 m	1.63 dd (J = 5.6, 16.0), 2.71 dd (J = 9.6, 16.0)
C-16	82.9	83.0	74.9	H-16α	3.30 m	3.32 m	4.78 dd (J = 5.6, 9.6)
C-17	65.7	63.6	64.7	H-17	2.87 s	3.00 s	3.02 s
C-18	69.2	69.2	69.7	H-18a, 18b	4.08, 4.19 d (J = 11.2)	4.03, 4.14 d (J = 11.6)	4.05, 4.13 d (J = 11.2)
C-19	56.9	52.9	52.6	H-19a, 19b	2.53, 2.59 d (J = 11.0)	2.53, 2.70 d (J = 11.2)	2.47, 2.70 d (J = 11.2)
NCH_2CH_3	50.2	50.4	51.2	CH_3CH_2N	2.85, 3.00 m	3.07 m	2.78, 2.96 m
NCH_2CH_3	13.4	13.9	14.3	CH_3CH_2N	1.12 t (J = 7.0)	1.11 t (J = 7.2)	1.06 t (J = 7.2)
CH_3O (1)			55.9				3.24s
CH_3O (6)	57.7	56.3	57.9	CH_3O (6)	3.41 s	3.39 s	3.42 s
CH_3O (14)	57.9	58.2	58.4	CH_3O (14)	3.37 s	3.34 s	3.36 s
CH_3O (16)	56.2	56.3		CH_3O (16)	3.27 s	3.35 s	
C=O(ester)	164.2	164.1	164.4				
Ar C-1	126.9	126.9	127.2				
Ar C-2	133.1	133.0	133.3				
Ar C-3	129.4	129.4	129.6	Ar H-3	7.48 dd (J = 1.0, 8.0)	7.28 dd (J = 1.0, 8.0)	7.28 dd (J = 1.0, 8.0)
Ar C-4	133.7	133.7	133.9	Ar H-4	7.62 dt (J = 2.0,9.2)	7.56 dt (J = 2.0, 9.2)	7.54 dt (J = 2.0, 9.2)
Ar C-5	130.8	131.0	131.0	Ar H-5	7.76 dt (J = 2.0,9.2)	7.69 dt (J = 2.0, 9.2)	7.68 dt (J = 2.0, 9.2)
Ar C-6	130.0	130.0	130.3	Ar H-6	8.02 dd (J = 1.0,8.0)	8.05 dd (J = 1.0, 8.0)	8.04 dd (J = 1.0, 8.0)
Suc $C^{1'}$=O	175.8	175.8	175.9				
Suc $C^{2'}$	35.3	35.2	35.1	Suc H-2	3.08 br	3.08 br	3.08 br
Suc $C^{3'}$	36.9	37.0	37.1	Suc H-3a, 3b	2.50 m	2.50 m	2.50 m
Suc $C^{4'}$=O	179.7	179.7	180.0				
Suc $C^{2'}$-CH_3	16.3	16.4	16.5	Suc CH_3	1.47 m	1.47 m	1.45 m
CH_3C=O		21.9	21.7	$OCOCH_3$		2.06 s	2.06 s
CH_3C=O		170.3	170.9				

[a] Solvent $CDCl_3$. Chemical shifts in ppm downfield from TMS. Coupling constants in parentheses are in Hz.

ments, was also comparable to that of MLA (**4**) and of isodelectine (**8**). Grandiflorine (**1**) displayed a resonance for C-1 at lower field than the C-1 of **4** and has one less methoxyl carbon resonance. In comparison to **8**, with the exception of differences in the observed resonances associated with the ester moiety, the chemical shifts of carbons 1−19 are very similar. The comparability of the ^{1}H and ^{13}C NMR spectra of **1** with **8** and with other norditerpenoid alkaloids[15,17] also implies a C-1α, C-6β, C-14α, and C-16β substituent configuration in **1**.

In the presence of Ac_2O−pyridine at room temperature, grandiflorine (**1**) formed the monoacetate **2** (amorphous solid, $C_{38}H_{50}N_2O_{11}$). The ^{1}H NMR spectrum of **2** showed a resonance for a single alkyl acetate (δ_H 2.06), and the resonance attributed to the carbinol methine at C-1 in **1** (δ_H 3.75) is shifted to lower field (δ_H 4.79) in **2**. Specific carbon and proton resonances of **2** were established by DEPT, one-bond HETCOR spectra, and ^{1}H COSY experiments (Table 1). Confirmation evidence for a C-1 monoacetate in **2** is provided by the observed downfield shift of the resonance assigned to C-1 in **1** from δ_C 72.4 to δ_C 77.3 in the ^{13}C NMR spectrum (Table 1) of **2**. The combined spectral evidence obtained for **1** and **2** and the comparison of spectral data with those of known norditerpenoid alkaloids[15,17] defined grandiflorine monoacetate (**2**) as 1α-acetoxy-7,8-diol-20-ethyl-6β,14α,16β-trimethoxy-4-([{2-[3-methyl-2,5-dioxo-1-pyrrolidinyl]benzoyl}oxy]methyl)aconitane.

Geyerline (**3**) ($C_{38}H_{50}N_2O_{11}$) was obtained as an amorphous solid from an alkaloidal fraction of an ethanolic extract of *D. geyeri* stems and leaves. The ^{1}H NMR spectrum of **3** (Table 1) also displayed methyl protons of an *N*-ethyl group (δ_H 1.06), a methyl resonance of the *N*-(methylsuccinimido)anthranoyl ester group (δ_H 1.45), C-18 methylene protons (δ_H 4.05, 4.13), and lowfield aromatic protons (δ_H 7.26−8.08) distinctive of an MSAL norditerpenoid alkaloid. Also prominent in the ^{1}H NMR spectrum were singlet resonances for an acetate group (δ_H 2.06) and three methoxyl groups (δ_H 3.24, 3.36, 3.42). The spectrum also featured resonances for a methoxyl-associated methine (δ_H 3.61) and a carbinol acetate methine (δ_H 4.78), which compare closely with those observed for resonances assigned for protons at C-14 and C-1 in a C-16 acetate-substituted norditerpenoid alkaloid (**10**) synthesized from ajacine[18] (**9**).

The ^{13}C spectral assignments for geyerline (**3**) by DEPT and one-bond HETCOR experiments were similar to those of grandiflorine acetate (**2**), except for the transposition of the resonances assigned to C-1 and C-16 (**3** vs **2**) (Table 1). Conversely, the chemical shifts of these resonances in **3** agree closely with those reported for C-1 and C-16 in **10** (δ_C 83.9, 74.9).[18] The one-bond HETCOR spectrum of **3** assigned two lowfield multiple resonances (δ_H 3.61, 4.78) to methine protons at the C-14 and C-16 carbons. A ^{1}H COSY experiment clearly established the acetate carbinol methine proton interaction with the methylene protons assigned to H-15 in **3** and a weak interaction of that proton with the methine proton assigned to H-13 in **3**. In addition, the ^{1}H COSY experiment clearly established interactions of the me-

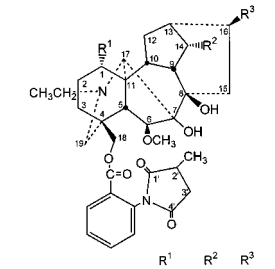
Table 2. Toxicity of Norditerpenoid Alkaloids in Mice[a]

alkaloid	mice (no.)	LD_{50} (mg/kg) (calcd)
grandiflorine (**1**)	27	8.3
grandiflorine acetate (**2**)	2	100[b]
geyerine (**3**)	2	60[b]
methyllycaconitine (**4**)[13]	15	7.5
14-deacetylnudicauline (**5**)[13]	18	4.0
nudicauline (**6**)[13]	23	2.7
barbinine (**7**)[13]	6	57[b]

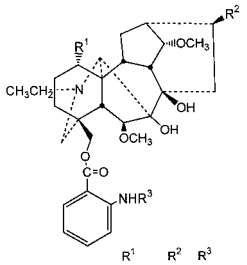
[a] Intravenous injection. [b] Estimated LD_{50}, insufficient number of mice to calculate LD_{50}.

thine proton at C-14 with H-13 and H-9. These NMR data place the acetate at C-16 in **3**.

The configuration at C-1, C-6, C-14, and C-16 can be established through the similarity of ^{13}C and ^{1}H NMR data to those reported for the synthetic acetate analogue (**10**) of ajacine[18] (**9**). Supporting evidence of the β substitution of the acetate at C-16 in **3** is described by the observed coupling between the methine proton at C-16 and the methylene protons at C-15 (J_{16}, $J_{15a} = 9.6$ Hz; J_{16}, $J_{15e} = 5.6$). The NMR spectral data established that **3** is 16β-acetoxy-7,8-diol-20-ethyl-1α,6β,14α-trimethoxy-4-([{2-[3-methyl-2,5-dioxo-1-pyrrolidinyl]-benzoyl}oxy]methyl)aconitane. Geyerline (**3**) is the first naturally occurring MSAL norditerpenoid alkaloid to have an acetate at C-16.



	R^1	R^2	R^3
Grandiflorine (**1**)	OH	OCH_3	OCH_3
Grandiflorine Acetate (**2**)	OAc	OCH_3	OCH_3
Geyerline (**3**)	OCH_3	OCH_3	OAc
Methyllycaconitine (**4**)	OCH_3	OCH_3	OCH_3
14-Deacetylnudicauline (**5**)	OCH_3	OH	OCH_3
Nudicauline (**6**)	OCH_3	OAc	OCH_3
Barbinine (**7**)	OCH_3	=O	OCH_3



	R^1	R^2	R^3
Isodelectine (**8**)	OH	OCH_3	H
Ajacine (**9**)	OCH_3	OCH_3	Ac
(**10**)	OCH_3	OAc	Ac

The toxicity and structure−activity relationship of MSAL norditerpenoid alkaloids were obtained as follows. In the mouse bioassay of compounds **1**−**3** (Table 2), grandiflorine (**1**) was found to be the most toxic, with an observed LD_{50} of 8.3 mg/kg. Acetylation of the C-1 hydroxyl group of **1** significantly reduced the observed toxicity, with grandiflorine acetate (**2**) having an estimated LD_{50} of 100 mg/kg. Geyerline (**3**) was also found to have a significantly lower toxicity than **1**, with an estimated LD_{50} of 60 mg/kg.

Grandiflorine (**1**) occurs with MLA (**4**) in *D. glaucum*, but at much lower concentrations (about 10−20% of MLA levels). Because its toxicity is high, the concentration of **1** must be taken into account in assessing the total toxic threat that *D. glaucum* poses to cattle exposed to this tall larkspur. Geyerline (**3**) also occurs in much lower amounts than MLA (<2%) in *D. geyeri*. Because **3** is much lower in toxicity than MLA, its contribution to the toxicity of *D. geyeri* is very low and can probably be ignored in evaluating the toxic threat of this larkspur species to cattle. HPLC analysis of the low larkspur *Delphinium andersonii* has established the presence of this alkaloid at low levels.

The significantly lower toxicity of grandiflorine acetate (**2**) and geyerline (**3**) and the limited availability of these compounds restricted the ability to obtain a calculated LD_{50} in the mouse bioassay (Table 2). However, the magnitude of the difference in estimated toxicity of grandiflorine acetate (**2**) and geyerline (3) vs the calculated toxicity of grandiflorine (**1**) and MLA (**4**) supplies evidence that specific functionality at C-1, C-14, and C-16 in the MSAL norditerpenoid alkaloids affects their toxic action in mammals. Correlating the nature of the functional group at C-1 and C-16 to the differences in the toxicity of **1**−**4** implies that a specific relationship may exist between functionality at these positions and nAChR binding affinity (methoxyl>hydroxyl>>acetate). On the basis of our previous study,[13] the functionality at C-14 of these alkaloids appears to have an exactly opposite relationship to their toxicity (acetate>hydroxyl>methoxyl>carbonyl) as displayed by MLA (**4**), 14-deacetylnudicauline (**5**), nudicauline (**6**), and barbinine (**7**) (Table 2).

Our earlier study of structure−activity relationships for MSAL norditerpenoid alkaloids established succinimidization of the anthranilic acid amino group and the nature of the functionality at C-1, C-14, and C-16 as important secondary factors affecting the ability of these alkaloids to bind to nAChR.[13] This investigation expands information about the impact of changes in functionality at C-1, C-14, and C-16 on the toxicity of this group of alkaloids. The apparent contrary nature of the influence of functional groups at C-1 and C-16 compared to those at C-14 on the toxicity of these alkaloids suggests that opposing mechanisms may be responsible for the effects of functional groups at these positions on their toxicity. Further validation of this structure−activity relationship could be important in establishing characteristics of these alkaloids, which could be exploited to reduce their threat to livestock.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker 400 MHz spectrometer in $CDCl_3$ with TMS as internal standard. Mass spectra were obtained on a VG 7070 mass spectrometer.

Plant Materials. *D. glaucum* plant material was collected at the flowering stage from Bell Meadow near Pinecrest Lake, Stanislaus County, CA. *D. geyeri* was collected at the vegetative stage 10 miles southeast of Cheyenne, WY. Voucher specimens for both plant species were placed in the herbarium at Utah State University, Logan, Utah.

Plant Extraction. Plant material was air-dried and ground in a hammermill to pass a 1/8″ screen. Dried plant material from both *D. glaucum* (1.7 kg) and *D. geyeri* (3.6 kg) were subjected to the same extraction and preliminary crude alkaloid isolation protocol. The plant

material was Soxhlet extracted (5 days) with 95% EtOH. The EtOH extract was concentrated to near dryness, and 1.5% H_2SO_4 (800 mL) was added. The acid−extract mixture was stirred until acid-soluble materials were dissolved, then extracted with $CHCl_3$ (3 × 800 mL), and the $CHCl_3$ extract was discarded. The aqueous acidic solution was cooled (5 °C), adjusted to pH 9.5 with 20% NaOH, and extracted with $CHCl_3$ (3 × 800 mL). The $CHCl_3$ extract was dried ($MgSO_4$) and concentrated to dryness to afford an alkaloid extract from *D. glaucum* (22.7 g) and from *D. geyeri* (57.8 g).

Grandiflorine (1) and Grandiflorine Acetate (2). The alkaloid extract obtained from *D. glaucum* (22.7 g) was dissolved in $CHCl_3$ (20 mL) and an aliquot (10 mL) applied to an open preparative column (10 cm × 40 cm) packed with deactivated alumina. The column was eluted with hexane−95% isopropyl alcohol (IPA) (86:14), and four 1-L fractions were collected. The MSAL alkaloid content of each fraction was determined by analytical HPLC;[19] fraction 4 was determined to contain the unknown alkaloid (**1**) and was concentrated to dryness (387 mg), redissolved in a minimal amount of $CHCl_3$, and subjected to repetitive preparative HPLC chromatography (Spherisorb alumina, 5 μ, 20 mm × 250 mm, hexane−95% IPA (80:20). The norditerpenoid alkaloid (UVdetection at 280 nm) was eluted at a high retention time (18.6 min). Fractions containing **1** from multiple HPLC runs were combined and concentrated to dryness (25 mg). The combined fraction was dissolved in $CHCl_3$ and subjected to semipreparative HPLC chromatography [Spherisorb alumina, 5 μ, 10 mm × 250 mm, hexane−95% IPA (84:16)]. The purified grandiflorine (**1**) (19 mg) (amorphous solid) was collected: $[\alpha]^{23}_D$ +49.2 (*c* 0.61, $CHCl_3$); ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 668 $[M]^+$ (5), 653 (50), 651 (54), 637 (68), 635 (100), 380 (17), 278 (36), 216 (30), 149 (26); HREIMS *m/z* 668.32866 (calcd for $C_{36}H_{48}N_2O_{10}$, 668.33089).

Grandiflorine (5 mg) was dissolved in Ac_2O (500 μL), and pyridine (10 μL) was added. The solution was allowed to stand overnight at room temperature before workup in the usual manner to yield grandiflorine monoacetate (**2**) (amorphous solid, 5 mg): $[\alpha]^{23}_D$ −3.1 (*c* 0.92, $CHCl_3$); ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 710 $[M]^+$ (4), 695 (6), 679 (11), 677 (17), 651 (100); HREIMS *m/z* 710.34085 (calcd for $C_{38}H_{50}N_2O_{11}$ 710.34146).

Geyerline (3). An aliquot of the $CHCl_3$ alkaloid extract (25 gm) was redissolved in a minimal amount of $CHCl_3$ and applied to a deactivated alumina open chromatography column (75 mm × 500 mm). The column was eluted with hexane−95% IPA (88:12) and ten 500-mL fractions were collected. The MSAL norditerpenoid alkaloid content of the fractions was determined by HPLC analysis.[19] Fractions 4 and 5 were found to contain MLA and related alkaloids and were combined, concentrated to dryness (combined wt 880 mg), and subjected to repeated preparative HPLC (Spherisorb alumina, [5 μ, 20 mm × 250 mm, hexane−95% IPA (85:15)]. A 280-nm UV-absorbing norditerpenoid alkaloid, eluting immediately after MLA, was collected. Fractions containing the unknown norditerpenoid alkaloid from repetitive chromatography were combined (12 mg) and subjected to semipreparative HPLC [Spherisorb alumina, [5 μ, 10 mm × 250 mm, hexane−95% IPA (88:12)]. Purified geyerline (9 mg, amorphous solid) was collected: $[\alpha]^{23}_D$ +32.3 (*c* 0.28, $CHCl_3$); ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 710 $[M]^+$ (6), 692 (46), 679 (100), 661 (24), 632 (34), 353 (12), 325 (48), 306 (19), 280 (27); HREIMS *m/z* 710.33780 (calcd for $C_{38}H_{50}N_2O_{11}$ 710.34146).

Mouse Bioassay. Individual alkaloids were suspended in physiological buffered saline solution, and the pH was lowered to 4.0−4.5 with 40% HCl to achieve solubility. The solutions were stored in injection vials for toxicity testing.

White Swiss−Webster male mice (25−35 g) were weighed after a 24-h fast and injected intravenously. Time of injection, clinical effects, and time of death were noted and recorded. The relative toxicity and amount of alkaloid available dictated the use of a modified up-and-down method[20] to provide sufficient information for the calculation of LD_{50} values. This method results in variations in the number of mice per test group. LD_{50} values were calculated using a log-probit method.[21]

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