Three New Toxic Norditerpenoid Alkaloids from the Low Larkspur *Delphinium nuttallianum*

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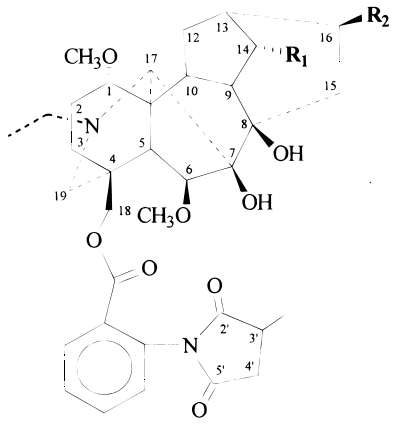
Three new *N*-(methylsuccinimido)anthranoyllycoctonine norditerpenoids, given the names bearline (**1**), 14-acetylbearline (**2**), and 16-deacetylgeyerline (**3**), were isolated from the extract of the low larkspur *Delphinium nuttallianum*. The structures of the individual alkaloids were determined by ^{1}H and ^{13}C NMR spectroscopy and HRMS. These alkaloids are structurally related to the neurotoxic alkaloid methyllycaconitine (**4**) and may be important in establishing the toxicity of low larkspurs to cattle. A mouse bioassay was used to measure the LD_{50} values for two of the new alkaloids (**1** and **2**), as sufficient quantities of **3** were not available for toxicity testing. A structurally related alkaloid, geyerline (**7**), was isolated from *D. geyeri* in sufficient quantities for toxicity testing. The toxicities of **1**, **2**, and **7** were found to be comparable to that of **4**, with calculated LD_{50} values in mice of 5.7, 3.3, and 6.2 mg/kg, respectively.

The larkspurs (*Delphinium* species) are considered to be one of the most serious poisonous plant problems for livestock on the rangelands of the western United States.[1] The low larkspur, *Delphinium nuttallianum* Pritz., is one species from which a large number of diterpenoid and norditerpenoid alkaloids have been isolated and characterized, particularly from plants found in British Columbia.[2−7] Less is known of the alkaloid content of plants found in areas of Utah, Arizona, Wyoming, and Colorado. We have analyzed a number of low larkspur samples from these areas specifically for the more toxic *N*-(methylsuccinimido)-anthranoyllycoctonine (MSAL) norditerpenoid alkaloids using electrospray mass spectrometry.[8]

Typically, the low larkspur samples analyzed have been found to contain the alkaloids methyllycaconitine (**4**), nudicauline (**5**), and 14-deacetylnudicauline (**6**) as the major toxic alkaloids, with minor amounts of geyerline (**7**), grandiflorine,[9] and several unknown alkaloids in trace amounts. From one particular collection near Bear Lake, Utah, we detected several of these unknown alkaloids in quantities larger than in the other collections and postulated structures of three new alkaloids based on the use of electrospray mass spectrometry and sequential tandem mass spectrometry (MS^n).[8] We have since collected more plant material from the Bear Lake location to supply sufficient material for isolation of low milligram quantities of the three new alkaloids. We now report the isolation and structural characterization, by HRMS and ^{1}H and ^{13}C NMR, of these three compounds as well as the mammalian toxicity for two of the alkaloids as measured in a mouse bioassay procedure.

Results and Discussion

A crude alkaloid fraction was obtained from the aerial parts of *D. nuttallianum* after extraction with methanol and subsequent partitioning of the extract using standard acid/base partition procedures. The alkaloid fraction was further separated using repetitive preparative liquid chromatography. The HPLC eluent was monitored by UV (280 nm), and fractions were collected based on detected peaks. Alkaloid content of individual fractions was then identified using direct injection electrospray mass spectrometry (ESMS) off-line. In addition to **4**, **5**, **6**, and **7**, three compounds with molecular weights of 696, 738, and 668 were detected, corresponding to the previously postulated structures bearline (**1**), 14-acetylbearline (**2**), and 16-deacetylgeyerline (**3**), respectively.[8]



	R_1	R_2
Bearline (**1**)	OH	OAc
14-Acetylbearline (**2**)	OAc	OAc
16-Deacetylgeyerline (**3**)	OCH_3	OH
Methyllycaconitine (**4**)	OCH_3	OCH_3
Nudicauline (**5**)	OAc	OCH_3
14-Deacetlynudicauline (**6**)	OH	OCH_3
Geyerline (**7**)	OCH_3	OAc

Compound **1** was isolated in sufficient purity and amount for further structural characterization by 1D and 2D NMR techniques. Compound **2** was isolated in lesser amounts and as a mixture with **7**, but was shown to be the acetyl derivative of **1** after acetylation of **1** with acetic anhydride yielded a compound with HPLC retention time and a MS/

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Table 1. ^{13}C and ^{1}H NMR Chemical Shift Assignments for Compounds **1**, **2**, and **3**[a]

^{13}C	**1**	**2**	**3**	^{1}H	**1**	**2**	**3**
C-1	84.2	83.8	84.7	H-1β	2.95 m	2.98 m	2.97 m
C-2	25.6	25.9	25.5	H-2	2.02, 2.19 m	2.07, 2.15 m	2.11, 2.19 m
C-3	28.1	27.5	27.5	H-3	1.57, 1.70 m	1.57, 1.73 m	1.55, 1.75 m
C-4	37.8	37.6	37.5				
C-5	50.0	49.9	51.1	H-5	1.78 br s	1.76 br s	1.78 br s
C-6	90.4	90.6	90.8	H-6α	3.89 br s	3.87 br s	3.86 br s
C-7	88.2	88.3	88.7				
C-8	77.2	77.0	77.4				
C-9	44.9	42.5	43.7	H-9	3.08 m	3.24 m	3.23 m
C-10	45.6	45.4	45.9	H-10	1.96 m	1.98 m	1.80 m
C-11	48.6	48.9	49.1				
C-12	29.7	32.1	29.9	H-12	1.92, 2.48 m	1.91, 1.97 m	1.78, 1.86 m
C-13	39.5	38.6	38.1	H-13	2.30 t (5.2)	2.37 t (5.6)	2.37 br s
C-14	73.8	75.2	83.5	H-14β	4.10 t (4.2)	4.76 t (4.8)	3.67 m
C-15	33.6	33.1	33.3	H-15	1.63 dd (5.4, 16.8), 2.74 dd (9.2, 16.8)	1.56 dd (5.6, 16.8), 2.71 dd (9.2, 16.8)	1.72, 2.71 m
C-16	74.7	74.4	72.2	H-16α	4.89 dd (5.4, 9.2)	4.86 dd (5.6, 9.2)	3.67 m
C-17	64.9	64.4	64.7	H-17	3.02 m	3.02 d (2.0)	3.06 m
C-18	69.4	69.3	69.6	H-18	4.09, 4.12 d (11.2)	4.08 br s	4.07, 4.14 d (11.2)
C-19	52.3	52.3	52.4	H-19	2.47, 2.74 d (11.4)	2.43, 2.70 d (12.0)	2.43, 2.72 d (11.6)
NCH_2CH_3	51.2	51.0	51.1	CH_3CH_2N	2.82, 2.96 m	2.79, 2.94 m	2.82, 2.97 m
NCH_2CH_3	14.2	14.1	14.2	CH_3CH_2N	1.07 t (7.1)	1.06 t (7.2)	1.06 t (7.2)
CH_3O (1)	55.9	55.7	56.0	CH_3O (1)	3.25 s	3.24 s	3.25 s
CH_3O (6)	58.3	58.2	58.2	CH_3O (6)	3.37 s	3.36 s	3.39 s
CH_3O (14)			58.4	CH_3O (14)			
CH_3O (16)				CH_3O (16)			3.45 s
C=O(ester)	164.2	164.1	164.4				
Ar C-1	127.2	127.6	127.2				
Ar C-2	133.1	133.1	133.3				
Ar C-3	129.4	129.5	129.6	Ar H-3	7.25 dd (1.0, 8.0)	7.27 dd (1.0, 8.0)	7.28 dd (1.0, 8.0)
Ar C-4	133.7	133.7	133.9	Ar H-4	7.55 dt (2.0, 9.2)	7.55 dt (2.0, 9.2)	7.53 dt (2.0, 9.2)
Ar C-5	131.0	131.0	131.0	Ar H-5	7.69 dt (2.0, 9.2)	7.69 dt (2.0, 9.2)	7.69 dt (2.0, 9.2)
Ar C-6	130.1	130.1	130.3	Ar H-6	8.04 dd (1.0, 8.0)	8.04 dd (1.0, 8.0)	8.05 dd (1.0, 8.0)
Suc 2 C=O	175.8	175.9	175.9				
Suc C-3	35.3	35.3	35.1	Suc H-3′	3.08 br s	3.04 br s	3.06 br s
Suc C-4	37.0	37.6	37.1	Suc H-4′	2.50 m	2.51 m	2.54 m
Suc 5 C=O	179.8	179.8	180.0				
Suc 3-CH_3	16.4	16.4	16.5	Suc CH_3	1.46 br s	1.47 br s	1.47 br s
CH_3C=O (14)		21.4		CH_3C=O (14)		2.02 s	
CH_3C=O (14)		171.4					
CH_3C=O (16)	21.5	21.4		CH_3C=O (16)	2.06 s	2.08 s	
CH_3C=O (16)	170.5	170.4					

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

MS product ion spectrum that were identical to **2**. Compound **3** was isolated in only a small quantity but with sufficient purity that its structural designation was confirmed by ^{1}H and ^{13}C NMR spectroscopy and by acetylation to yield **7**.

The ^{1}H and ^{13}C NMR spectra of **1**, **2**, and **3** (Table 1) are closely comparable to the MSAL norditerpenoid alkaloids **4**, **5**, **6**, and **7** previously described in *Delphinium* species.[10] The ^{1}H NMR spectra displayed distinctive resonances for the methyl and methylene groups of the ethyl substituted tertiary alkaloid nitrogen (δ_H ca. 1.07; δ_H ca. 2.80, ca. 3.00), the methyl group on the succinimido ring of the *N*-(methylsuccinimido)anthranoyl ester moiety (δ_H ca. 1.47), methoxyl groups (δ_H 3.2−3.4), and aromatic protons of the anthranoyl ester (δ_H 7.2−8.0). These resonances are common to all of the MSAL norditerpenoid alkaloids.[11] Methine protons associated with the oxygen functionality at C-1, -6, -14, -16, and -18 are also common and appeared in the δ_H 2.9−4.9 ppm range. Carbons with oxygen functionality (C-1, -6, -7, -8, -14, -16, and -18) are also distinctive in the ^{13}C NMR spectra of these alkaloids and appear in the δ_C 70−90 ppm range (Table 1). The ^{13}C NMR spectra of the MSAL norditerpenoid alkaloids also feature prominent resonances for the six benzene ring carbons of the anthranoyl ester (δ_C 125−135) and the carbonyls of the ester and succinimido moiety (δ_C 163−177).

The ^{1}H NMR spectrum of **1** displayed singlet resonances for an alkyl acetate (δ_H 2.06) and two methoxyl groups (δ_H 3.25, 3.37) in addition to the characteristic norditerpenoid alkaloid NMR resonances described above (Table 1). Resonances for four methine and two methylene protons were located in the δ_H 2.9−5.0 ppm range. A single proton resonance at δ_H 4.89 ppm was assigned as an acetoxy-associated methine on the basis of chemical shift comparison to similar protons in **5** (δ_H 4.76) and **7** (δ_H 4.78). A second methine signal was found at δ_H 4.10. A comparison of the chemical shift of this proton with the C-14 carbinol proton of **6** and one-bond HETCOR correlation of the proton with an oxygen-substituted carbon (δ_C 73.8) was consistent with hydroxyl substitution at C-14 in **1**. The proton−proton COSY spectrum established correlation of the low-field methine proton with the methylene protons at C-15 (δ_H 1.63, 2.74) and correlation of the carbinol proton with the C-13 methine proton and the C-9 methine (δ_H 3.08). COSY data and ^{1}H and ^{13}C NMR comparison of **1** to **5** and **7** established acetoxy substitution at C-16 and hydroxyl substitution at C-14 in **1**. The remaining proton resonances in the region were assigned to the C-18 methylene (δ_H 4.09, 4.12) and the C-6 methine (δ_H 3.89). The lack of coupling between the H-16 proton and the proton at C-13 and the coupling constants between the H-16 proton and the methylene protons at C-15 (J = 9.2, 5.4) were consistent with a β configuration of the acetate at C-16. Compound **1**

Table 2. Toxicity of Compounds **1**, **2**, and **7** in Mice[a]

alkaloid	mice (na)	LD_{50} (mg/kg)	95% CI
bearline (**1**)	17	5.7	5.5−5.9
14-acetylbearline (**2**)	14	3.3	2.8−3.9
geyerline (**7**)	19	6.2	5.7−6.6

[a] Intravenous injection.

was therefore established to be 16β-acetoxy-7,8,14α-triol-20-ethyl-1α,6β-dimethoxy-4-([{2-[3-methyl-2,5-dioxo-1-pyrrolidinyl]-benzoyl}oxy]methyl)aconitane.

The ^{1}H NMR spectrum of **2**, the monoacetyl product of **1**, displayed resonances for two alkyl acetates (δ_H 2.02, 2.08), two methoxyls (δ_H 3.24, 3.36), and four methine, and two methylene proton resonances between δ_H 2.90 and 5.0 ppm. The chemical shifts of two methine proton multiplets at δ_H 4.76 and 4.86 were consistent with the C-14 and C-16 acetoxy methine protons observed for **5** (δ_H 4.75) and **7** (δ_H 4.78). A comparison of the ^{13}C NMR spectra of **1** and **2** showed a significant shift of C-14 to lower field (δ_C 73.8 to δ_C 75.2). The chemical shift of C-14 is closely comparable to that of the acetoxy-substituted C-14 of **5** (δ_C 75.9). From the COSY experiment, correlations were observed between the lowest field methine proton resonance (δ_H 4.86) and resonances for the C-15 (δ_H 1.56, 2.71) protons. The other low-field methine signal (δ_H 4.76) was observed to be correlated to proton signals for the C-13 (δ_H 2.37) proton and the C-9 (δ_H 3.24) proton. These data placed acetoxy functionality at C-14 and C-16 and described **2** as 14α,-16β-diacetoxy-7,8-diol-20-ethyl-1α,6β-dimethoxy-4-([{2-[3-methyl-2,5-dioxo-1-pyrrolidinyl]-benzoyl}oxy]methyl)-aconitane.

Compound **3** displayed resonances for three methoxyls (δ_H 3.25, 3.39, 3.45) and overlapping multiplet resonances for two methines at δ_H 3.67 ppm in the ^{1}H NMR. Comparison of the ^{13}C NMR to that of **7** revealed a shift to higher field (δ_C 74.9 to 72.2) for the C-16 resonance and close comparability of methoxyl resonances in the ^{1}H and ^{13}C NMR spectra, and the locations of the two oxygenated methines were consistent with the assigned designation for **3**. A portion of **3** was acetylated with acetic anhydride to yield **7** and identified by comparison of HPLC retention time, co-injection, and elution with a standard sample of **7** and analysis by ES/MS/MS, supporting the identification of **3** as 7,8,16β-trihydroxy-20-ethyl-1α,6β,14α-trimethoxy-4-([{2-[3-methyl-2,5-dioxo-1-pyrrolidinyl]-benzoyl}oxy]-methyl)aconitane.

The toxicity of **1** and **2** was tested in a mouse bioassay. Compounds **1** and **2** were highly toxic, with measured LD_{50} values of 5.7 mg/kg and 3.3 mg/kg, respectively (Table 2). The observed toxicity of **1** and **2** is comparable to that reported for the MSAL alkaloids **5** and **6** (4.0 mg/kg and 2.7 mg/kg), and they differ structurally only in the C-16 acetoxy substitution.[12] Compounds **1** and **2** appeared to be considerably more toxic than the C-16 acetoxyl-substituted alkaloid **7** as previously reported.[9] However, insufficient quantities of **7** were available for toxicity testing and only an estimated LD_{50} value was reported. Therefore, **7** was isolated from *D. geyeri* in sufficient quantities (12 mg) for further toxicological testing and yielded a calculated LD_{50} value of 6.2 mg/kg (n = 19), which is comparable to those of **1** and **2**. Geyerline was the first reported naturally occurring MSAL norditerpenoid alkaloid to have an acetoxy at the C-16 position. It is important to note that the toxicity of this group of alkaloids (C-16 acetoxy substituted MSAL norditerpenoid alkaloids) is sufficient to warrant a thorough accounting of these compounds when considering the overall toxicity of certain species of low and plains larkspur plants (*D. geyeri*, *D. nuttallianum*, and *D. andersonii*).

The mouse bioassay results from this study further clarify the structure−neurotoxicity relationships of the larkspur norditerpenoid alkaloids. It was previously established that a methyl succinimide-substituted anthranylic acid ester of the norditerpenoid alkaloid lycoctonine is necessary for neurotoxicity in mice, and comparison of the toxicity of **4**, **5**, and **6** and barbinine (C=O, C-14; OMe, C-16) suggested that the toxicological efficacy of these alkaloids was related to the type of substituents at C-14 and C-16.[12] A mouse bioassay evaluation of grandiflorine (OH, C-1) and of grandiflorine acetate and **7** led to speculation that a specific relationship may exist between the functionality at C-1, -16 (methyl>hydroxyl≫acetate) and at C-14 (acetate>hydroxyl> methoxyl>carbonyl) of these alkaloids.[9] In the current study, the improved accuracy for the toxicity testing of **7** coupled with the observed toxicity of **1** and **2** established that an increase in the toxicity of these compounds can be related to a substituent change (acetate>hydroxyl>methoxyl) at both C-14 and C-16. This structure−activity relationship for functionality at C-16 is contrary to the earlier speculation,[9] which was based upon an estimated toxicity evaluation of **7**. Toxicological evaluation of the as yet unknown C-1 (methoxyl), C-14, -16 (hydroxyl)-substituted norditerpenoid alkaloid and accurate toxicological evaluation of grandiflorine acetate would provide the conclusive evidence to associate the nature of substitution at C-1, -14, and -16 to the neurotoxicity of these alkaloids.

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. HRMS were obtained on a VG 7070 mass spectrometer, at 70 eV for electron impact; methane was the reagent gas for chemical ionization. Electrospray mass spectra (ESMS) were obtained on a Finnigan LCQ using conditions previously described.[8]

Plant Material. The aerial plant parts of *D. nuttallianum* (Ranunculaceae) were collected east of Bear Lake, UT, during May 1997 and 1998 (Intermountain Herbarium, Utah State University, voucher #226055). *D. geyeri* (Ranunculaceae) was collected northwest of Fort Collins, CO.

Plant Extraction and Crude Alkaloid Isolation. Plant material was air-dried and ground in a Wiley mill to pass a 1-mm screen. The dried plant material (*D. nuttallianum*, 60 g) was placed in a large Erlenmeyer flask and extracted with 750 mL of methanol by mechanical stirring overnight. The solvent was removed under reduced pressure, and the recovered methanol was used to extract the plant material a second time for 15 min; it was then filtered, and the solvent was again removed. The combined plant extract was then partitioned between 1% H_2SO_4 and $CHCl_3$ (200 mL each). The acid solution was separated and extracted with fresh $CHCl_3$ (100 mL), then made basic with the addition of concentrated NH_4OH (pH = 9) and extracted twice with $CHCl_3$ (200 mL). The combined $CHCl_3$ extract was dried over anhydrous Na_2SO_4, filtered, and the solvent removed under reduced pressure to yield the crude alkaloid fraction (120 mg). Four other 60-g aliquots of plant material were treated in the same manner, yielding a total crude alkaloid fraction of 590 mg.

Bearline (1). Initially, a portion of the crude alkaloid mixture (20 mg) was separated using semipreparative HPLC: Spherisorb alumina, 5 μm, 130 Å, 250 × 10 mm (Keystone Scientific); isocratic elution with hexane/95% 2-propanol (85:15); UV (280 nm) detection. Fractions were collected based on the UV chromatogram and then each fraction was monitored by ESMS. Fraction 4 was determined to contain the unknown alkaloid with MH^+ = 697 by ESMS (2.6 mg). Further HPLC runs of the crude alkaloid fraction afforded bearline (**1**) (28 mg) (amorphous solid): ^{1}H and ^{13}C NMR, see Table 1; HRCIMS [MH^+] m/z 697.33640 (calcd for $C_{37}H_{49}N_2O_{11}$, 697.33364).

14-Acetylbearline (2). Compound **1** (12 mg) was dissolved in acetic anhydride (500 μL) and pyridine (20 μL) and allowed to react for 18 h at ambient temperature (ca. 20 °C). Water (4 mL) was added and the solution mechanically rotated for 5 min. Concentrated ammonium hydroxide was added to adjust the pH to 9, and the sample was extracted twice with $CHCl_3$ (3 mL). The $CHCl_3$ extracts were dried by filtering through anhydrous Na_2SO_4 and the solvent evaporated under a stream of nitrogen at 70 °C. The product was separated by HPLC using the conditions above to afford purified **2** (6.1 mg) (amorphous solid): ^{1}H and ^{13}C NMR, see Table 1; HRCIMS [MH^+] *m*/*z* 739.35013 (calcd for $C_{39}H_{51}N_2O_{12}$, 739.34420). This product was found to have the identical HPLC retention time and ESMS data as that of the naturally occurring compound detected in the original crude alkaloid mixture.

16-Deacetylgeyerline (3). Semipreparative HPLC chromatography of the crude alkaloid mixture also afforded a fraction eluting as a shoulder just before the peak corresponding to **1**. This fraction was rechromatographed several times where only the first half of the peak was collected, eliminating contamination from **1**, to yield **3** (3.3 mg) (amorphous solid): ^{1}H and ^{13}C NMR (Table 1); HREIMS [M^+] *m*/*z* 668.3224 (calcd for $C_{36}H_{48}N_2O_{10}$, 668.3309).

A portion of compound **3** (0.1 mg) was dissolved in acetic anhydride (100 μL) and pyridine (10 μL) and then allowed to react for 18 h. The sample was worked up as above to yield the acetyl product, which was found to be identical to **7** by HPLC/MS retention time (coelution) and tandem mass spectrometry.

Geyerline (7). Compound **7** was isolated from *D. geyeri* plant material using procedures previously described.[9] Extraction of 185 g of dried plant material followed by acid/base workup, open column chromatography (neutral alumina), and repeated isolation by HPLC yielded geyerline (12 mg).

Mouse Bioassay. Known amounts of individual alkaloids were suspended in physiological buffered saline solution, and the pH was lowered to 4.0−5.5 with 2 M HCl to achieve solubility. The solutions were stored in sterile injection vials for toxicity testing.

White Swiss-Webster male mice (12−25 g) were weighed after a 24-h fasting 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[13] method 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.[14]

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