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STRUCTURE-ACTIVITY RELATIONSHIPS OF NORDITERPENOID ALKALOIDS OCCURRING IN TOXIC LARKSPUR (*DELPHINIUM*) SPECIES

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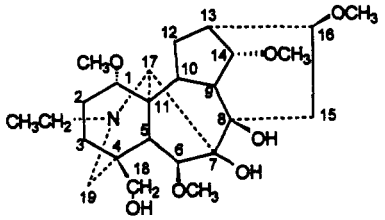
ABSTRACT.—Fourteen norditerpenoid alkaloids present in larkspur (*Delphinium*) species associated with cattle poisoning on grazing land in the western United States have been toxicologically assessed in a mouse bioassay. Toxicity data for these alkaloids have established the tertiary nitrogen atom and anthranilic acid esterification as important structural features necessary to impart toxicity to lycoctonine-type norditerpenoid alkaloids. Variation in C-14 functionality of the toxic alkaloids is also a factor that influences toxicity in these compounds. The relationship of the structure-activity information of this study to previous in vitro neuromuscular studies is discussed.

Larkspur (*Delphinium*) species continue to be a significant cause of cattle poisoning on rangelands of the western United States (1). Presently, managing cattle to avoid access to larkspur remains the only economically effective means to reduce cattle losses on rangelands with larkspur infestations. Western larkspur species are categorized as low larkspurs, plains larkspurs, or tall larkspurs according to growth and habitat characteristics. Cattle poisonings are associated with representatives of each category of larkspur; however, the tall larkspurs commonly found in higher elevation grazing areas of the mountainous west are considered the major cause of cattle losses due to larkspur poisoning (2).

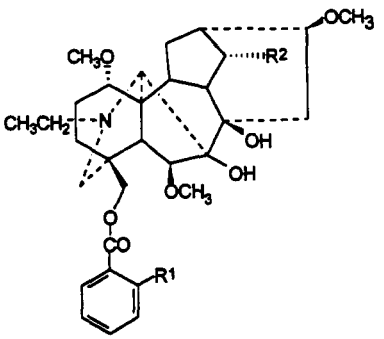
Norditerpenoid alkaloids occur as prominent constituents of poisonous larkspurs in either of two structural types: (1) the lycoctonine-type (e.g., lycoctonine, **1**), and (2) the 7,8-methylenedioxylycoctonine (MDL)-type (e.g., deltaline, **2**). More than 40 norditerpenoid alkaloids of these two types have been identified in various western larkspur species, and toxicity data has been accumulated for 23 of these alkaloids (3,4).

Among the lycoctonine-type norditerpenoid alkaloids, those which are esterified with *N*-(methylsuccinyl)anthranilic acid at the C-18 alcohol [e.g., *N*-(methylsuccinimido)anthranoyllycoctonine (MSAL) norditerpenoid alkaloids] are the most toxic (3–5). When the MSAL norditerpenoid alkaloid methyllycaconitine [**3**] was administered to cattle by intravenous injection, observed clinical signs of toxicosis were found to be consistent with field and feeding trial observations of larkspur-poisoned cattle (6). These observations, and the presence of methyllycaconitine (MLA) [**3**] in all species of western larkspur which have been chemically examined, establish MLA [**3**] as a primary toxin in the poisoning of rangeland stock consuming larkspur.

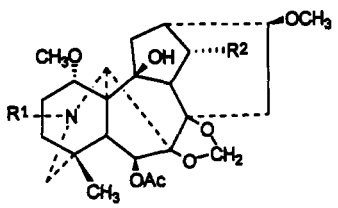
Clinical observations of cattle exposed to MLA [**3**] suggest a neuromuscular site of action. In a neuromuscular in vitro test system, MLA [**3**] was confirmed to be highly toxic, with a curare-like action associated with the postsynaptic blockage of nicotinic cholinergic receptors (7,8). MLA [**3**] was also found to be a potent competitor of α-



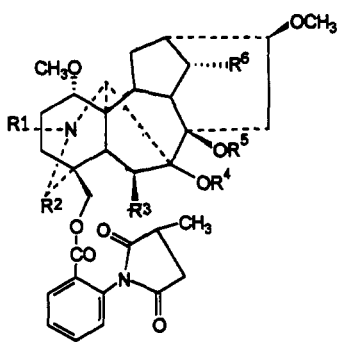
Lycoctonine [1]



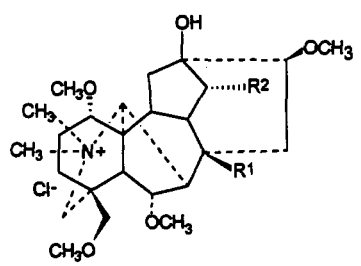
	R¹	R²
Anthranoyllycoctonine [10]	NH_2	OCH_3
Delavaine A+B [12]	$NHCOCH(CH_3)CH_2COOCH_3$ or $NHCOCH_2CH(CH_3)COOCH_3$	OCH_3
Delsemine [17]	$NHCOCH(CH_3)CH_2CONH_2$ or $NHCOCH_2CH(CH_3)CONH_2$	OCH_3
18-*O*-Benzoyllycoctonine [18]	H	OCH_3
Andersonine [21]	$NHCOCH(CH_3)CH_2COOCH_3$ or $NHCOCH_2CH(CH_3)COOCH_3$	OAc
Andersonidine [22]	NH_2	OAc



	R¹	R²
Deltaline [2]	Et	OCH_3
Dictyocarpine [11]	Et	OH
N-Desethyldeltaline [16]	H	OCH_3



	R¹	R²	R³	R⁴	R⁵	R⁶
Methyllycaconitine [3]	Et	H_2	OCH_3	H	H	OCH_3
Nudicauline [4]	Et	H_2	OCH_3	H	H	OAc
14-Deacetylnudicauline [5]	Et	H_2	OCH_3	H	H	OH
Barbinine [6]	Et	H_2	OCH_3	H	H	=O
Elatine [7]	Et	H_2	OCH_3	- CH_2 -		OCH_3
Glaudelsine [8]	Et	H_2	OH	H	H	OCH_3
Elanine [9]	H	=O	OCH_3	H	H	$OCOCH(CH_3)C_2H_5$
N-Desethylmethyllycaconitine [13]	H	H_2	OCH_3	H	H	OCH_3
Anhweidelphinine [14]	-	H	OCH_3	H	H	OCH_3
Zaliline [15]	H	=O	OCH_3	H	H	OCH_3



	R¹	R²
Delphinine methochloride [19]	OAc	OBz
Delphonine methochloride [20]	OH	OH

bungarotoxin (αBGT) binding to nicotinic acetylcholine receptors (nAChR) in the nanomolar range in insects (9). Examination of the ability of MLA [3] to inhibit the binding of αBGT to rat brain membranes, frog and human muscle extracts, and a human muscle cell line revealed a higher affinity of the alkaloid for the brain sites in the rat than the muscle sites (10). Experiments with cultured fetal rat hippocampal tissues showed MLA [3] to be a specific, concentration-dependent, reversible, voltage-independent antagonist to acetylcholine and anatoxin-induced neuronal whole-cell currents at picomolar concentrations (11). MLA [3] was also found to inhibit αBGT binding to the rat hippocampal membrane nAChR, suggesting a competitive mode of action with the ability to reversibly protect the receptor from an irreversible blockage by αBGT. The affinity and potency of MLA [3] in binding assays and functional studies showed it to

bind better than the snake α-toxins, but to be reversible like the curarimimetic agents. Two recent examinations of MSAL and MDL norditerpenoid alkaloids relate structural characteristics of the alkaloids to their binding affinity to vertebrate and invertebrate neural membranes (12,13). Examination of the binding affinity of nudicauline [**4**], 14-deacetylnudicauline [**5**], methyllycaconitine [**3**], barbinine [**6**], and deltaline [**2**] to lizard muscle nAChR revealed distinct differences in the binding affinities among the MSAL norditerpenoid alkaloids, and between the MSAL norditerpenoid alkaloids and the MDL norditerpenoid alkaloid deltaline [**2**] (nudicauline [**4**] >14-deacetylnudicauline [**5**] >methyllycaconitine [**3**] >>barbinine [**6**] >deltaline [**2**]) (12). In a study of norditerpenoid inhibition to α-bungarotoxin in rat and insect neural membranes, the MSAL norditerpenoid alkaloids methyllycaconitine [**3**], 14-deacetylnudicauline [**2**], elatine [**5**], glaudelsine [**21**], and elanine [**22**] were found to be the most potent binding inhibitors in the rat neuronal tissue (methyllycaconitine [**3**] >elatine [**7**] >14-deacetylnudicauline [**5**] >elanine [**9**] >glaudelsine [**8**]), while three MDL norditerpenoid alkaloids and nine other C_{19} norditerpenoid alkaloids showed much lower binding affinity (13).

The variation in antagonism of the nAChR binding by the MSAL alkaloids indicates that specific structural requirements are necessary to impart and/or enhance the mammalian toxicity of norditerpenoid alkaloids. In this study we have examined the toxicity of several synthetic and naturally occurring norditerpenoid alkaloids in an effort to establish specific information about the structural features and functionality necessary for their toxicity in mammalian systems.

RESULTS AND DISCUSSION

The toxicological data (Table 1) obtained in this investigation define the potential mammalian toxicity of a wide range of norditerpenoid alkaloids. In agreement with previous studies (5,6), the MSAL norditerpenoid alkaloids nudicauline [**4**], 14-deacetylnudicauline [**5**], methyllycaconitine (MLA) [**3**], and elatine [**7**] were found to be highly toxic, with LD_{50} values of between 2.7 and 10 mg/kg. Delavaine A+B [**12**], a naturally occurring norditerpenoid alkaloid obtained from MLA [**3**] through transesterification of the *n*-methylsuccinyl ring of MLA [**3**], displays toxicity similar to

TABLE 1. Toxicity of Norditerpenoid Alkaloids in Mice.[a]

Alkaloid	Number of Mice	LD_{50}[b] (calculated)	LD_{50}[c] (estimated)
Nudicauline [**4**]	23	2.7	
14-Deacetylnudicauline [**5**]	18	4.0	
Methyllycaconitine [**3**]	15	7.5	
Delavaine A+B [**12**]	23	3.3	
Elatine [**7**]	15	9.2	
Anthranoyllycoctonine [**10**]	19	20.8	
Barbinine [**6**]	6		57
N-Desethylmethyllycaconitine [**13**]	6		100
Deltaline [**2**]	23	200.5	
Anhweidelphinine [**14**]	1		>177
N-Desethyldeltaline [**16**]	12	210	
Zaliline [**15**]	1		>230
Dictyocarpine [**11**]	20	282.9	
Lycoctonine [**1**]	17	443.5	

[a]Intravenous injection.
[b]*Mg/kg*.
[c]Insufficient mice tested for a calculated LD_{50} value to be determined.

14-deacetylnudicauline [**5**]. In the case of anthranoyllycoctonine [**10**], complete removal of the linear or cyclic methylsuccinyl group reduces toxicity by a factor of more than two times in comparison with MLA [**3**]. Barbinine [**6**], in comparison with other MSAL norditerpenoid alkaloids, is significantly less toxic when administered intravenously in the mouse bioassay. This comparative reduction is consistent with earlier observations obtained when barbinine [**6**] was administered subcutaneously to mice (5).

The MDL norditerpenoid alkaloids deltaline [**2**] and dictyocarpine [**11**] are much lower in toxicity than the MSAL norditerpenoid alkaloids, with LD_{50} values of 133 mg/kg and 350 mg/kg, respectively. The naturally occurring *N*-desethyl MSAL norditerpenoid alkaloids anhweidelphinine [**14**] (estimated LD_{50} >177 mg/kg) and zaliline [**15**] (estimated LD_{50} >230 mg/kg) also are much less toxic than the MSAL norditerpenoid alkaloids. Removal of the *N*-ethyl group from MLA [**3**] and deltaline [**2**] lowered their toxicity to LD_{50} value of 100 mg/kg (*N*-desethylmethyllycaconitine [**13**]) and 210 mg/kg (*N*-desethyldeltaline [**16**]), respectively. Lycoctonine [**1**], the unesterified parent alkaloid of the MSAL norditerpenoids alkaloids, showed the lowest toxicity of any alkaloid in the test system (443.5 mg/kg).

Evaluation of the toxicity data obtained in this investigation establishes two structural features (an *N*-ethyl bicyclo substituted tertiary alkaloid nitrogen atom and a C-18 anthranilic acid ester) to be necessary to impart toxicity to the norditerpenoid alkaloids. Additionally, the toxicity data establish that two other structural features (functionality at the anthranilic acid amine nitrogen and at C-14) can enhance that toxicity. The 10- to 400-fold higher toxicity of the MSAL norditerpenoid alkaloids **3–5**, **7**, and **12** compared with the toxicity values of the non-anthranilic acid esterified parent alkaloid lycoctonine [**1**], and the *N*-desethyl alkaloids (**13–16**), and the reduced toxicity of *N*-desethyldeltaline [**16**] compared with deltaline [**2**], validate the requirement for both the presence of an *N*-ethyl bicyclo tertiary nitrogen atom and a C-18 anthranilic acid ester for toxicity among the norditerpenoid alkaloids.

The observed toxicity of the MSAL norditerpenoid alkaloids [**3–5**] and delavaine A+B [**12**] in comparison with anthranoyllycoctonine [**10**] delineates the importance of an amide or succinimide functionality at the anthranilic acid amine group to the enhancement of the toxicity among the toxic larkspur alkaloids. Additional evidence for the importance of a functionality at the anthranilic acid amine group to toxicity enhancement among the norditerpenoid alkaloids is found in a discussion of structure-activity relationships for some MLA [**3**] related alkaloids (3). In vitro measurements of miniature end-plate potentials in a frog muscle preparation revealed delsemine [**17**] to be only slightly less potent than MLA [**3**] and 18-*O*-benzoyllycoctonine [**18**] to be about twice as active as lycoctonine [**1**].

The lower toxicity of barbinine [**6**] compared with other larkspur MSAL norditerpenoid alkaloids and the variation in toxicity among the MSAL alkaloids [**3–5**] in the mouse bioassay defines the oxidative state of the C-14 carbon and the substitution of the C-14 secondary alcoholic function as important structural factors in the ultimate toxicity of the MSAL norditerpenoid alkaloids. The direct comparability of the toxicity results for nudicauline [**4**], 14-deacetylnudicauline [**5**], methyllycaconitine (MLA) [**3**], and barbinine [**6**] in the mouse, with in vitro test results for the binding affinity of these alkaloids to lizard muscle nAChR (12), further corroborates the importance of the oxidative state and functionality of the C-14 carbon atom to the toxicity of the MSAL norditerpenoid alkaloids. The observed differences in the binding affinity of 14-deacetylnudicauline [**5**], methyllycaconitine (MLA) [**3**] and elanine [**9**] to rat brain nAChR (13) is fully consistent with the influence of C-14 functionality on MSAL norditerpenoid alkaloid toxicity in mammals. The minimal change in toxicity observed

for elatine [**7**] compared with MLA [**3**] in this study and in the examination of competitive binding to rat nAChR (13) also suggests that the unsubstituted 7,8 dihydroxy functionality common to many norditerpenoid alkaloids is not essential to toxicity. Based upon the toxicity data of this study, anthranoyllycoctonine [**10**] appears to be a pivotal toxic alkaloid precursor in the transition of the norditerpenoid alkaloids from low/moderate toxicity to high toxicity.

The structure-activity data established in this study serve to confirm a previously suggested molecular basis for the pharmacological specificity of MLA [**3**] modeling techniques, MLA [**3**] and acetylcholine were determined to be conformationally comparable and to have a suitable template fit with the rigid nicotinic antagonist cytisine (10). Based upon the projected fit of MLA [**3**] to the nicotinic pharmacophore, the tertiary nitrogen atom of MLA [**3**] and the quaternary nitrogen of acetylcholine were considered capable of undergoing equivalent electrostatic interaction with a receptor binding site. The observed necessity for a toxic norditerpenoid alkaloid to possess a tertiary nitrogen and an anthranilic acid ester in this study is fully consistent with the previous proposed rationale. Further support for the importance of the electronic state of the alkaloid nitrogen to the toxicity of the norditerpenoid alkaloids is seen in the high potency of the semi-synthetic drugs delphinine methochloride [**19**] and delphonine methochloride [**20**] (14). These compounds (with a quaternary alkaloid nitrogen) display higher potencies than observed for MLA [**3**] as curariform neuromuscular blockers, thereby offering further evidence for the alkaloid nitrogen as the primary binding site for electrostatic interaction with the nicotinic receptor site. Examination of the pharmacophore models used to develop the structural rationale for binding (10) offers no evidence for the role of the toxicity-enhancing constituents on the anthranilic acid moiety or at C-14 of the lycoctonine moiety of the MSAL norditerpenoid alkaloid. We can speculate that they provide spatial and/or secondary bonding enhancement at the nicotinic binding site.

Twenty-two norditerpenoid alkaloids have been characterized in *Delphinium* species which possess the structural features established in this study to be necessary for moderate to high mammalian toxicity. Seven of these alkaloids [**3–6**, **10**, **21**, **22**] have been identified in eight larkspur species (*D. brownii, D. bicolor, D. nuttallianum, D. nudicaule, D. andersonii, D. glaucescens, D. occidentale, D. barbeyi*) indigenous to the western United States and Canada which are commonly associated with cattle poisoning (5, 8, 15–21). The widespread occurrence of these alkaloids among these toxic western larkspurs is consistent with the continuous poisoning losses of cattle on larkspur-infested rangelands. The information obtained in this study relating the toxicity of these compounds to particular structural features will enable the development of methodology to neutralize the toxicity of these compounds and thereby alleviate the poisoning threat to cattle posed by larkspur on the open range.

EXPERIMENTAL

GENERAL EXPERIMENTAL METHODS.—^{1}H- and ^{13}C-nmr spectra were recorded on a Bruker 400 MHz spectrophotometer. Mass spectra were obtained on a VG 7070 mass spectrometer.

ALKALOIDS FROM NATURAL SOURCES.—Eight of the norditerpenoid alkaloids included in this study were obtained from EtOH extracts of ground, dried whole plants of two *Delphinium* species. Nudicauline [**4**] was obtained from *Delphinium andersonii* Gray (15), and 14-deacetylnudicauline [**5**], methyllycaconitine (MLA) [**3**], barbinine [**6**], anthranoyllycoctonine [**10**], deltaline [**2**], dictyocarpine [**11**], and lycoctonine [**1**] were obtained from *Delphinium barbeyi* Huth. (5,16). Four of the alkaloids [**1**, **2**, **10**, **11**] were obtained as crystalline products. The amorphous alkaloids [**3–5**, **12**] were rechromatographed for purity utilizing centrifugal tlc (22) and hplc (23) methods. The identity and purity of both naturally occurring and synthetic norditerpenoid alkaloids included in this study were confirmed by comparison of physical properties (mp,

mmp) with those of authentic alkaloid samples, or through a comparison of spectral data (ms, nmr) with the spectral properties of authentic alkaloids or reported spectra for authentic alkaloids (24,25).

SYNTHESIZED ALKALOIDS.—Five of the alkaloids included in this study (**7**, **12–15**) were obtained synthetically from MLA using previously described synthetic methods. Delavaine A+B [**12**] was obtained (yield 27%) from methyllycaconitine (MLA) [**3**] upon overnight refluxing with MeOH and subsequent chromatographic separation by centrifugal tlc according to the method of Pelletier *et al.* (26). Elatine [**7**] was obtained (yield 17%) from methyllycaconitine (MLA) [**3**] by reaction with formaldehyde according to the procedure of Pelletier *et al.* (27). *N*-Desethylmethyllycaconitine [**13**] was obtained (yield 37%) from MLA [**3**], rather than from methyllycaconitine hydroiodide, according to the method of Sun *et al.* (28). Anhweidelphinine [**14**] (yield 16%) and zaliline [**15**] (yield 10%) were synthesized from the synthetic *N*-desethylmethyllycaconitine [**13**] according to the procedure of Sun and Benn (29).

N-Desethyldeltaline [**16**] was synthesized from deltaline [**2**] by adaptation of the method of Sun *et al.* (28). Deltaline [**2**] (2.14 g, 0.33 mmol) and mercuric acetate (5.25 g, 1.65 mmol) were dissolved in 2.5% HOAc (100 ml), refluxed (1 h), and stirred (12 h). The reaction mixture was filtered, basified (pH 10, NH_4OH) and extracted with $CHCl_3$. The $CHCl_3$ extract was dried (Mg_2SO_4) and evaporated to dryness. The product was chromatographed [Si gel, $CHCl_3$-MeOH-NH_4OH (15:1:0.1)], collected, and concentrated to yield **16** (amorphous, 0.97 g, 60%); $[\alpha]^{21}D$ −15.8°, ($CHCl_3$); hreims *m/z* 479.24815 observed $C_{25}H_{37}NO_8$, requires 479.25192; ^1H nmr ($CDCl_3$, 400 MHz) δ 0.91 (3H, s, CH_3-18), 2.10 (3H, s, $OCOCH_3$), 3.15 (1H, t, *J*=8 Hz, H-16α), 3.36 (1H, d, *J*=5 Hz, H-9), 3.30 (3H, s, OMe), 3.31 (3H, s, OMe), 3.45 (3H, s, OMe), 3.62 (1H, t, *J*=6 Hz, H-1α), 4.12 (1H, dd, *J*=4 Hz, H-14α), 4.90 (1H, s, OCH_2O), 5.06 (1H, s, OCH_2O), 5.59 (1H, s, H-6α); ^{13}C nmr ($CDCl_3$) δ 21.7 (q, $COCH_3$), 25.7 (t, C-2), 26.4 (q, C-18), 33.1 (t, C-3), 33.5 (s, C-4), 34.7 (t, C-15), 38.6 (d, C-13), 39.7 (t, C-12), 48.4 (d, C-5), 50.5 (d, C-9), 52.6 (t, C-19), 55.5 (q, OMe-1′), 56.3 (t, OMe-14′), 56.4 (s, C-11), 57.7 (t, OMe-16′), 59.2 (d, C-17), 78.0 (d, C-1), 79.3 (d, C-6), 81.3 (d, C-16), 81.8 (d, C-14), 82.2 (s, C-10), 84.2 (s, C-8), 88.7 (s, C-7), 94.4 (t, O-CH_2-O), 169.8 (s, C=O).

MOUSE BIOASSAY.—Individual alkaloids were suspended in physiological buffered saline, and the pH was lowered with 40% HCl to achieve solubility. Alkaloids **1–10** were solubilized over a pH range of 4.0–4.5. Alkaloids **11–14** were solubilized over the pH range 5.4–6.6. 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 (iv). 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 (30) 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 (31).

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