CYCLOPEPTIDE ALKALOIDS. PHENCYCLOPEPTINES FROM *CEANOTHUS SANGUINEUS*

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ABSTRACT.—Field desorption mass spectroscopy has identified five phencyclopeptines in the crude alkaloidal extracts of *Ceanothus sanguineus*. A new paired-ion hplc system for the separation of these alkaloids in discussed. Amino acid analysis, electron impact mass spectroscopy, and ^{1}H nmr spectroscopy have established the the structures of six phencyclopeptines including two isomeric compounds **5** and **6**. The structure of **2** has not been previously reported.

Ceanothus sanguineus Pursh., a species of the family Rhamnaceae commonly known as Redstem Ceanothus for its red-purple branches, inhabits wooded slopes, open hills, flats, and ledges from Northern California northward into British Columbia, and eastward into Idaho and Montana (1). In the present report, as part of a systematic study of the alkaloids of Californian *Ceanothus* species, we describe the identification of six cyclopeptide alkaloids from crude extracts of the root bark of this shrub. Before chromatographic separation, however, the number and nominal masses of the constituents of the crude acidic extract were ascertained by field desorption (fd) mass spectrometry (2). This composite fd mass spectrum of the alkaloidal mixture revealed the presence of five major components with molecular ions *m/e* 504, 520, 534, 559, and 573 (figure 1). After high performance liquid chromatography (hplc) (figure 2), the structures of phencyclopeptines **1–6**, including those of the two isomers with molecular weight of 534 (Table 1), were established by electron impact (ei) mass spectrometry, ^{1}H nmr spectroscopy, and amino acid analysis. Of the six components, five have been previously reported (3–7) while **2** is a new compound.

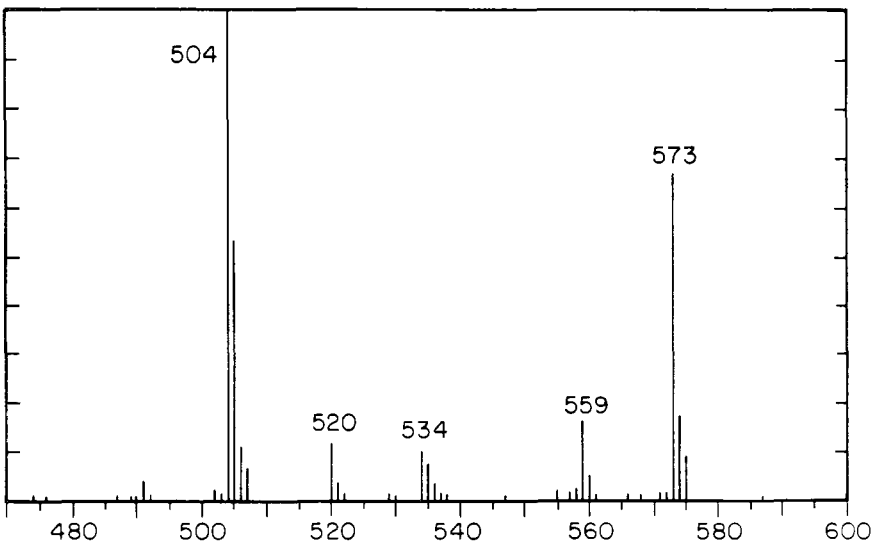


FIG. 1. Field desorption mass spectrum of the crude acidic, alkaloid extract of *Ceanothus sanguineus* (emitter current 16mA).

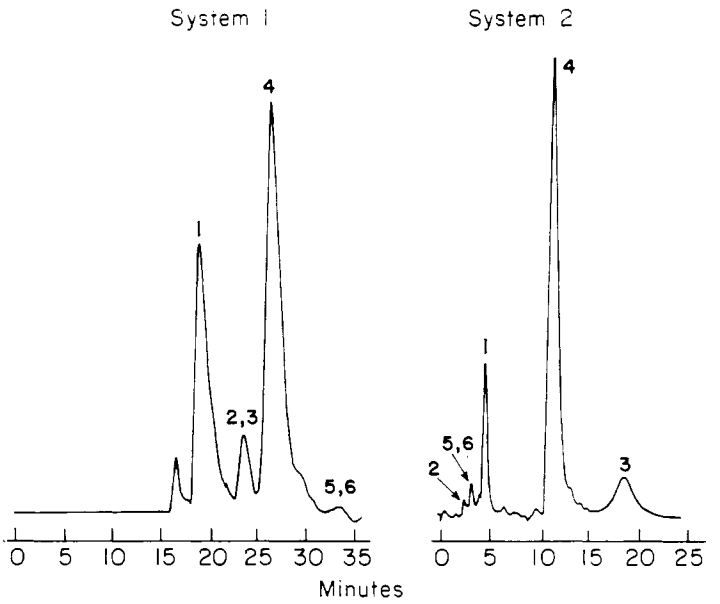


FIG. 2. Hplc of crude alkaloidal mixtures from *C. sanguineus*. Hplc systems employed: 1) LiChrosorb RP-18 (10μ, 10 x 250 mm); mobile phase 0.015M perfluorobutyric acid/CH_3CN (6/4 v/v); flow rate 1.6 ml/min/25-C; A 254 nm, injection volume 10 μl; C=3.8 mg/ml. 2) Waters Porasil (10μ, 3.2 x 280 mm); mobile phase $CHCl_3$/hexane/triethylamine (95/5/0.1 v/v); flow rate 1.6 ml/min; 41-C; A 280 nm; injection volume 10μl, C=3.5 mg/ml.

EXPERIMENTAL[1]

PLANT MATERIAL.[2]—Root bark of *C. sanguineus* was collected from plants in Klamath National Wilderness on Hawkinsville Road, 5.5 miles west of Hawkinsville on Humbug Mountain.

EXTRACTION PROCEDURE. ISOLATION OF CRUDE ALKALOIDAL CONSTITUENTS.—The extraction procedure described in an earlier report (4) was used. The total crude alkaloid yield was 0.22% of the root bark.

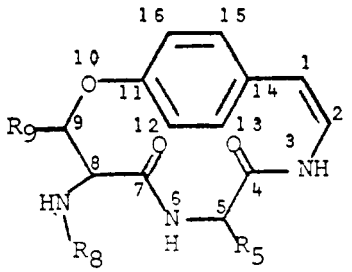
FIELD DESORPTION MASS SPECTROSCOPY.—As shown in figure 1, the fd mass spectrum of the crude alkaloidal mixture reveals five major components with nominal masses as follows: *m/e* 504, 520, 534, 559 and 573.

[1]Hplc was performed with a Spectra Physics Model SP 3500B chromatograph and a model 748 oven, Santa Clara, CA. Uv absorbance was monitored with an Altex Model 151 Dual Wavelength Detector, Altex Scientific Inc., Berkeley, CA. Hplc grade solvents obtained from Burdick and Jackson Laboratories, Muskegon, MI; perfluorobutyric acid from ICN Pharmaceuticals, Inc., Plainview, N.Y.; and water purified with a Milli-Q system, Millipore Corp., Bedford, MA, were used for hplc. Uncorrected micromelting points (μmp) were determined on a Kofler Micro Hot Stage. A model AEI-MS12 mass spectrometer, AEI Scientific Apparatus Ltd., Manchester, England with INCOS Data System and a Hitachi M-52 mass spectrometer were used for low resolution mass spectrometry. High resolution EI mass spectrometry was obtained at the Space Sciences Laboratory, Biomedical Mass Spectrometry Resource, University of California, Berkeley, on a Kratos/AEI-MS-902 mass spectrometer with a Logos-2 Data System interfaced with a Xerox Sigma 7 Computer. Fd mass spectra were acquired on the same instrument equipped with a combined Fd-Ei ion source.

Amino acid analyses were performed on a Beckman 120C Chromatograph, Fullerton, CA. ^1H nmr were taken in $CDCl_3$ solution ($CHCl_3$ at 7.25 ppm) at 22° on a homemade spectrometer based on a Bruker 63-KG magnet operating at 270 mHz with a Nicolet 1180 data system. Evaporations were done in vacuo with a Buchi rotary evaporator.

[2]The plant material was collected and extracted by Frederick K. Klein.

TABLE 1. Phencyclopeptines of *Ceanothus sanguineus*.



	R_5	R_8	R_9	MW
1, 5-Benzyl-8-N-(N'-methylprolyl)-9-isopropyl phencyclopeptine (Ceanothine-B)[a]	$CH_2C_6H_5$	NMePro	$CH(CH_3)_2$	504
2, 5-sec-Butyl-8-N-(N'-methyl-phenylalanyl)-9-isopropylphency-clopeptine	$CH(CH_3)CH_2CH_3$	NMePhe	$CH(CH_3)_2$	520
3, 5-β-Indolylmethyl-8-N-(N',N'-dimethylvalyl)-9-isopropyl-phencyclopeptine[b]	-β-indolyl-CH_2	NMe_2Val	$CH(CH_3)_2$	559
4, 5-β-Indolylmethyl-8-N-(N',N'-dimethylisoleucyl)-9-isopropyl-phencyclopeptine (Discarine-B)[c]	-β-indolyl-CH_2	NMe_2Ileu	$CH(CH_3)_2$	573
5, 5-Isobutyl-8-N-(N',N'-dimethyl-phenylalanyl)-9-isopropylphency-clopeptine (Frangufoline)[d]	$CH_2CH(CH_3)_2$	NMe_2Phe	$CH(CH_3)_2$	534
6, 5-sec Butyl-8-N-(N',N'-dimethyl-phenylalanyl)-9--isopropylphency-clopeptine (Adouetine Y[f] or Myrianthine B)[e]	$CH(CH_3)CH_2CH_3$	NMe_2Phe	$CH(CH_3)_2$	534

[a]First identified in *Ceanothus americanus* L. (2).
[b]First identified in *Ceanothus integerrimus* H. and A. (4).
[c]First identified in *Discaria longespina* H. and A. (5).
[d]First identified in *Rhamnus frangula* L. (6).
[e]First identified in *Waltheria americana* L. (7).

HPLC ISOLATION OF PHENCYCLOPEPTINES.—Preparative hplc was performed with two systems (figure 2).

System one used a LiChrosorb RP-18 column (10μ, 10 x 250 mm, E. M. Merck). The crude alkaloidal mixture was dissolved in methanol and filtered through a 5 micron teflon filter. Injection volumes ranged from 100 to 200 μl at a concentration of 3–4 mg/ml. The mobile phase was a 65/35 (v/v) mixture of 0.015*M* aqueous perfluorobutyric acid and acetonitrile. The chromatography was done at room temperature with a flow rate of 1–2.5 ml/min. Alkaloidal components were detected at 254 nm. Figure 2 shows a typical hplc tracing. The collected fractions were evaporated *in vacuo* and the residues were dissolved in 1.5*M* ammonium hydroxide (4 ml) and extracted with methylene chloride (3 x 1.5 ml). The combined organic layers were extracted with water (1 x 1.5 ml) and then dried under a stream of nitrogen.

The second system used a Waters Porasil column (10μ, 3.2 x 250 mm). The crude alkaloidal mixtures were dissolved in chloroform, and chromatography was performed at 37–41° with a mobile phase of chloroform-hexane-triethylamine, 95/5/0.1. Alkaloids were detected at 280 nm as shown in fig. 2. Fractions collected were immediately evaporated *in vacuo* and dried under high vacuum. Relative weight percents of *C. sanguineus* phencyclopeptines (hplc system 2) were **1** (37%), **2** (3%), **3** (12%), **4** (43%), 5 and **6** (6%).

STRUCTURES OF PHENCYCLOPEPTINES FROM *C. sanguineus*.[3]

5-BENZYL-8-N-(N'-METHYLPROLYL)-9-ISOPROPYLPHENCYCLOPEPTINE (CEANOTHINE B).—**1**. $C_{29}H_{36}N_4O_4$; μmp 225°, lit. (8) mp 238.5–240.5°; ms: M^+ $C_{29}H_{36}N_4O_4$ requires 504.2736, found

[3]For complete mass spectra, see Table 2. For amino acid analyses, each fraction was hydrolyzed with 1 ml 6N HCl with 50 μl 5% phenol (aq) for 16 h at 11° in an evacuated ampule.

504.2795, bp $C_5H_{10}N$ requires 84.0813, found 84.0809; amino acid analysis 110°C/16 h: phenylalanine (1.0); hplc-system, retention time in min: 1, 19.5; 2, 4.5 (figure 2); ^{1}H nmr ($CDCl_3$)—0.91 (d, 3H, J=6.7Hz, R9–CH_3), 1.24 (d, 3H, 6.7Hz, R9–CH_3), 1.63 (m, 1H, R9–CH), 1.7–1.9 (m, 2H, R8–γ1 CH_2 and β1 CH_2), 1.98 (s, 3H, N–CH_3), 2.1–2.2 (m, 1H, R8–γ2 CH_2), 2.2–2.3 (m, 1H, R8–β2 CH_2), 2.68 (dd, 1H, 4.3Hz, 10.6Hz, R8–δ1 CH_2), 2.85 (dd, 1H, 8.2Hz, -14.7Hz, R5–CH_2), 3.01 (m, 1H, R8–δ2 CH_2), 3.08 (dd, 1H, 4.2Hz, -14.7Hz, R5–CH_2), 4.3–4.4 (m, C5–H), 4.34 (dd, 1H, 7.0Hz, 10.0Hz C8–H), 4.93 (dd, 1H, 2.0Hz, 7.0Hz C9–H), 5.99 (d, 1H, 6.9Hz C1–H), 6.39 (d, 1H, 7.4Hz R8–αCH), 6.4–6.5 (m, N3–H), 6.66 (m, 1H, C2–H), 7.0–7.3 (m, 9H, aromatic, R5–ϕ, C12,13,15,16–H's), 7.75 (d, 1H, 10.0Hz, C8–NH). This nmr spectrum is identical with that obtained from an authentic sample of Ceanothine B from *Ceanothus americanus* L. (3).

5-SEC BUTYL-8-N-(N'-METHYLPHENYLALANYL)-9-ISOPROPYLPHENCYCLOPEPTINE **2**.—$C_{30}H_{40}N_4O_4$; μmp 229°; ms: M^+ *m/e* 520, M–91 $C_{23}H_{33}N_4O_4$requires 429.2502, found 429.2506, bp $C_9H_{12}N$ requires 134.0970, found 134.0966; amino acid analysis 110°/16 h: isoleucine (1.0); hplc-1, 24.0; 2, 2.5 (figure 2).

5-β-INDOLYLMETHYL-8-N-(N',N'-DIMETHYLVALYL)-9-ISOPROPYLPHENCYCLOPEPTINE **3**.—$C_{32}H_{41}N_5O_4$; μmp 229°, lit. (4) μmp 233°; ms: M^+ *m/e* 559, M–2H $C_{32}H_{39}N_5O_4$ requires 557.3002, found 557.2957, M–43 *m/e* 516, bp $C_6H_{14}N$ requires 100.1126, found 100.1125; amino acid analysis 110°/16h: no amino acids observed; hplc-1, 24.0; 2, 18.7 (figure 2).

5-β-INDOLYLMETHYL-8-N-(N',N'-DIMETHYLISOLEUCYL)-9-ISOPROPYLPHENCYCLOPEPTINE (DISCARINE B) **4**.—$C_{33}H_{43}N_5O_4$; μmp 233°, lit (4) μmp 233°; ms: M^+ $C_{33}H_{43}N_5O_4$ requires 573.3315, found 573.3264, M–57 $C_{29}H_{34}N_6O_4$ requires 516.2611, found 516.2644, bp $C_7H_{16}N$ requires 114.1283, found 114.1281; amino acid analysis 110°C/16h: tryptophan (low recovery); hplc-1, 26.7; 2, 11.1 (figure 2); ^{1}H nmr identical with previously reported spectra (4, 5).

5-ISOBUTYL-8-N-(N',N'-DIMETHYLPHENYLALANYL)-9-ISOPROPYLPHENCYCLOPEPTINE (FRANGUFOLINE) **5** and 5-SEC BUTYL-8-N-(N',N'-DIMETHYLPHENYLALANYL)-9-ISOPROPYLPHENCYCLOPEPTINE (ADOUETINE-Y[1]) **6**.[4]—$C_{31}H_{42}N_4O_4$; ms: M^+ *m/e* 534, M–91 $C_{24}H_{35}N_4O_4$ requires 443.2658, found 443.2649, bp $C_{10}H_{14}N$ requires 148.1126, found 148.1118; μmp 261° (lit. (6, 9) mp 289–290° for **5**, and lit. (10), 244° for **6**; amino acid analysis 110°C/16h: ile-leu, 2/1; ^{1}H nmr, high field region: δ 0.38 (d, 2H, J=6.7Hz, ileu-γ-CH_3), 0.60 (d, 1H, 6.7Hz leu δCH_3), 0.64 (d, 1H, 6.7Hz leu δCH_3), 0.68 (m, 2H, ile δCH_3), 1.01 (d, 3H, J=6.4Hz, R9–Me), 1.27 (d, 3H, J=6.7Hz, R9–Me); tlc Analtech Silica Gel G (250μ)-eluant, chloroform-ether-methanol, 45/15/1, R_f 0.40 (**5**) and 0.33 (**6**), and 0.33 (**6**), lit. (9) R_f 0.66 (**5**); 0.58 (**6**); lit. (11) R_f 0.44 (**5**); 0.35 (**6**); hplc-1, 33.5; 2, 3.2 (figure 2).

DISCUSSION

In contrast to mass spectrometry methods used in previous phytochemical investigations of *Ceanothus*, we have employed field desorption (fd) mass spectrometry as a means to rapidly determine the alkaloid composition in crude extracts (2). The fd analysis of crude alkaloidal mixtures from *Ceanothus sanguineus* revealed parent ions of five phencyclopeptines (figure 1). Given this result, the development of a chromatographic system to resolve all five components became our initial goal.

When the reversed phase hplc system using 0.001% NH_4OH (aq)/acetonitrile mixtures (4) proved unsuccessful, a paired-ion, reverse phase hplc system using 0.01*N* perfluorobutyric acid/acetonitrile mixtures was devised (fig. 2, system 1). The use of perfluorobutyric acid decreased column degradation and tailing of peaks observed with the alkaline eluants. In addition, this paired-ion proved to be superior to the sulfonic acids due to its high volatility and ease of removal. A silica hplc system was employed to separate phencyclopeptines **2** and **3** which co-chromatographed in system 1 (figure 2, system 2).

The structural assignments of the hplc-purified phencyclopeptine components of *Ceanothus sanguineus* are based primarily on their characteristic electron impact mass spectra (table 2). With the exception of the tryptophan-containing phencyclopeptines **2** and **3**, the assignment of the ring amino acid residue (R_5) is based on acidic hydrolysis of the purified phencyclopeptine followed by amino

[4]In reference 10 the authors give the name Myrianthine B to a compound of the same structure named Adouetine Y' in an earlier report (7).

TABLE 2. Low resolution mass spectra of hplc purified components of *Ceanothus sanguineus*.[a]

Fragment	Compound				
	1	**2**	**3**	**4**	**5,6**
M^+	504	520	559	573	534
BP a	84	134	100	114	148
b	461	429	516	516	443
c	195	181	195	195	195
d	167	153	167	167	167
e	337	303	376	376	303
f	190	190	190	190	190
g	421	387	460	460	387
h	308	274	347	347	274
i	135	135	135	135	135
j	378	344	417	417	344
k	244	210	283	283	210
l	216	182	255	255	182
m	97	97	97	97	97
n			170	170	
o	120	86	159	159	86
p	91		130	130	
other					519[b]
	489[b]	463[d]			370[f]
	475[c]	133[e]			398[g]
					133[h]

[a]Fragment ions according to fragmentation scheme in ref. (4).
[b]M^+–15.
[c]M^+–29.
[d]M^+–C_4H_9.
[e]a–H.
[f]g–OH.
[g]b–$NH(CH_3)_2$.

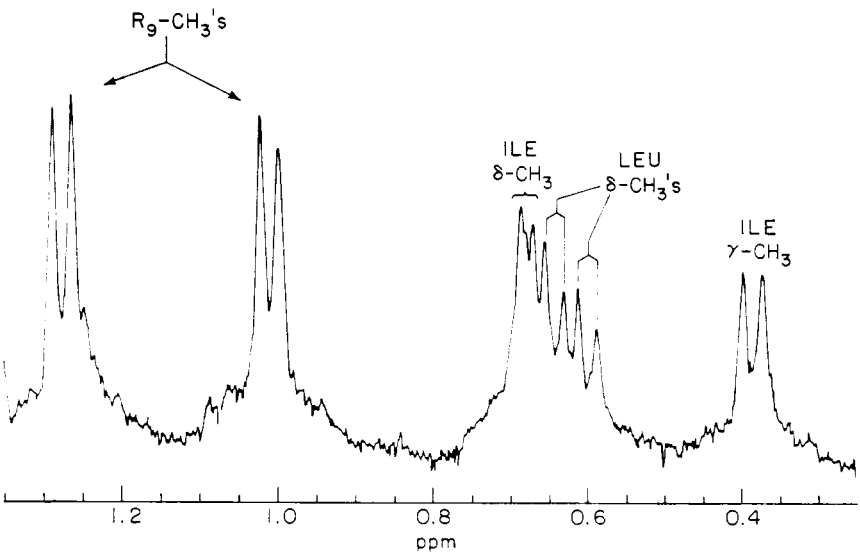


FIG. 3. Expanded view of high field region of ^1H nmr of 2:1 Myrianthine-B **6** and Frangulanine **5** mixture.

acid analysis. Amino acid analysis reveals that the two isomeric phencyclopeptines, frangufoline **5** and adouetine Y' **6**, which are unseparable by reversed phase hplc (figure 2, system 1), are present in a ratio of 1 to 2, respectively. Further evidence for the mixture of these two isomers is provided by ^{1}H nmr spectroscopy and thin layer chromatography. As shown in figure 3 the two doublets at 0.59 and 0.64 ppm have been assigned to the leucine δ-methyl groups of frangufoline **5**, in agreement with the reported values of 0.60 and 0.65 ppm (12). The assignments of the doublet at 0.38 ppm and the multiplet at 0.69 ppm to the isoleucine γ- and δ-methyl groups of adouetine-Y' **6** are consistent with the literature (10). Integration of these signals confirms the 1 to 2 ratio of leucine to isoleucine indicated by amino acid analysis. The two large doublets at 1.01 and 1.27 ppm (figure 3), assigned to the R_9 methyls of both **5** and **6**, are consistent with the literature values of 0.99 and 1.25 ppm for **5** (12) and 0.99 and 1.23 ppm for **6** (10). Furthermore, silica tlc ($CHCl_3$) of this mixture of isomers reveals two spots with R_f 0.40 and 0.33, which agrees well with the reported chromatographic data for these two phencyclopeptines (10).

In an earlier report describing the isolation and characterization of alkaloids from *Ceanothus integerrimus*, the chemotaxonomic utility of the phencyclopeptines was discussed. In the future, the use of field desorption/collison induced dissociation (fd/cid) mass spectrometry employing the linked B/E scan (2) may provide a useful and rapid approach to the analysis of individual phencyclopeptines in crude plant extracts without the need for chromatographic separation.

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