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2 CYCLOPEPTIDE ALKALOIDS. PHENCYCLOPEPTINES FROM
3 CEANOETHUS SANGUINEUS.
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1 ABSTRACT. -- Field desorption mass spectroscopy has
2 identified five phencyclopeptides in the crude alkaloidal
3 extracts of Ceanothus sanguineus. A new paired-ion HPLC
4 system for the separation of these alkaloids is discussed.
5 Amino acid analysis, electron impact mass spectroscopy, and
6 ¹H NMR spectroscopy have established the structures of six
7 phencyclopeptides including two isomeric compounds 5 and 6.
8 The structure of 2 has not been previously reported.

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1 Ceanothus sanguineus Pursh., a species of the family Rhamnaceae
2 commonly known as Redstem Ceanothus for its red-purple branches,
3 inhabits wooded slopes, open hills, flats, and ledges from Northern
4 California northward into British Columbia, and eastward into Idaho
LC 1 5 and Montana (1). In the present report, as part of a systematic
6 study of the alkaloids of Californian Ceanothus species, we describe
7 the identification of six cyclopeptide alkaloids from crude extracts
8 of the root bark of this shrub. Before chromatographic separa-
9 tion however, the number and nominal masses of the constituents of
10 the crude acidic extract were ascertained by field desorption
LC 2 11 (FD) mass spectrometry (2). This composite FD mass spectrum of
12 the alkaloidal mixture revealed the presence of five major
13 components with molecular ions m/e 504, 520, 534, 559, and 573
Fig. 1 14 (Figure 1). After high performance liquid chromatography (HPLC)
Fig. 2 15 (Figure 2), the structures of phencyclopeptides 1-6, including
Table 1 16 those of the two isomers with molecular weight of 534 (Table 1),
17 were established by electron impact (EI) mass spectrometry,
18 ¹H NMR spectroscopy, and amino acid analysis. Of the six com-
LC 3-7 19 ponents, five have been previously reported (3-7) while 2 is a
20 new compound.

EXPERIMENTAL¹

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.² ISOLATION OF CRUDE ALKALOIDAL
CONSTITUENTS.-- The extraction procedure, as 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.

HPLC ISOLATION OF PHENCYCLOPEPTINES. -- Preparative HPLC
was performed with two systems (Figure 2).

System one used a LiChrosorb RP-18 column (10 μ , 10x250 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 μ l at a concentration of 3-4 mg/ml. The
mobile phase was a 65/35 (v/v) mixture of 0.015M aqueous perfluoro-
butyric 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 1.5M NH₄OH (4 ml) and extracted with CH₂Cl₂
(3x1.5 ml). The combined organic layers were extracted with H₂O
(1x1.5 ml) and then dried under a stream of nitrogen.

The second system used a Waters Porasil column (10 μ , 3.2x250 mm).

1 The crude alkaloidal mixtures were dissolved in CHCl_3 , and
 2 chromatography was performed at 37-41°C with a mobile phase of
 3 CHCl_3 /hexane/triethylamine, 95/5/0.1. Alkaloids were detected at
 4 280 nm as shown in Fig. 2. Fractions collected were immediately
 5 evaporated in vacuo and dried under high vacuum. Relative weight
 6 percents of C. sanguineus phencyclopeptides (HPLC System 2) were
 7 1 (37%), 2 (3%), 3 (12%), 4 (43%), 5 and 6 (6%).

l.c.

fn 3

8 STRUCTURES OF PHENCYCLOPEPTINES FROM C. sanguineus.³

9 5-Benzyl-8-N-(N'-methylpropyl)-9-isopropylphencyclopeptide

LC 8

10 (Ceanothine B). 1. $\text{C}_{29}\text{H}_{36}\text{N}_4\text{O}_4$; mp 225°, lit. (8) mp 238.5-240.5°;
 11 MS: M^+ $\text{C}_{29}\text{H}_{36}\text{N}_4\text{O}_4$ requires 504.2736, found 504.2795, BP $\text{C}_5\text{H}_{10}\text{N}$
 12 requires 84.0813, found 84.0809; amino acid analysis 110°C/16 h:
 13 phenylalanine (1.0); HPLC-system, retention time in min: 1, 19.5;
 14 2, 4.5 (Figure 2); ^1H NMR (CDCl_3) - 0.91 (d, 3H, $J=6.7\text{Hz}$, R9- CH_3),
 15 1.24 (d, 3H, 6.7Hz , R9- CH_3), 1.63 (m, 1H, R9-CH), 1.7-1.9 (m, 2H,
 16 R8- γ 1 CH_2 and β 1 CH_2), 1.98 (s, 3H, N- CH_3), 2.1-2.2 (m, 1H, R8- γ 2
 17 CH_2), 2.2-2.3 (m, 1H, R8- β 2 CH_2), 2.68 (dd, 1H, 4.3Hz , 10.6Hz ,
 18 R8- δ 1 CH_2), 2.85 (dd, 1H, 8.2Hz , -14.7Hz , R5- CH_2), 3.01 (m, 1H, R8-
 19 δ 2 CH_2), 3.08 (dd, 1H, 4.2Hz , -14.7Hz , R5- CH_2), 4.3-4.4 (m, C5-H),
 20 4.34 (dd, 1H, 7.0Hz , 10.0Hz C8-H), 4.93 (dd, 1H, 2.0Hz , 7.0Hz
 21 C9-H), 5.99 (d, 1H, 6.9Hz C1-H), 6.39 (d, 1H, 7.4Hz R8- α CH), 6.4-6.5
 22 (m, N3-H), 6.66 (m, 1H, C2-H), 7.0-7.3 (m, 9H, aromatic, R5- ϕ , C12,13,
 23 15,16-H's), 7.75 (d, 1H, 10.0Hz , C8-NH). This nmr spectrum is
 24 identical with that obtained from an authentic sample of Ceanothine B
 25 from Ceanothus americanus Linn. (3).

26 5-sec Butyl-8-N-(N'-methylphenylalanyl)-9-isopropylphencyclo-
 27 peptide 2. $\text{C}_{30}\text{H}_{40}\text{N}_4\text{O}_4$; mp 229°; MS: M^+ m/e 520, M-91 $\text{C}_{23}\text{H}_{33}\text{N}_4\text{O}_4$

1 requires 429.2502, found 429.2506, BP $C_9H_{12}N$ requires 134.0970, found
 2 134.0966; amino acid analysis 110°C/16 h: isoleucine (1.0); HPLC-1,
 3 24.0; 2, 2.5 (Figure 2).

4 5- β -Indolylmethyl-8-N-(N',N'-dimethylvalyl)-9-isopropylphencyclo-
 5 peptide 3. $C_{32}H_{41}N_5O_4$; mp 229°, lit. (4) mp 233°; MS: M^+ m/e 559,
 6 M-2H $C_{32}H_{39}N_5O_4$ requires 557.3002, found 557.2957, M-43 m/e 516, BP
 7 $C_6H_{14}N$ requires 100.1126, found 100.1125; amino acid analysis 110°C/
 8 16h: no amino acids observed; HPLC-1, 24.0; 2, 18.7 (Figure 2).

9 5- β -Indolylmethyl-8-N-(N',N'-dimethylisoleucyl)-9-isopropyl-
 10 phencyclopeptide (Discarine B) 4. $C_{33}H_{43}N_5O_4$; mp 233°, lit (4)
 11 mp 233°; MS: M^+ $C_{33}H_{43}N_5O_4$ requires 573.3315, found 573.3264,
 12 M-57 $C_{29}H_{34}N_5O_4$ requires 516.2611, found 516.2644, BP $C_7H_{16}N$ requires
 13 114.1283, found 114.1281; amino acid analysis analysis 110°C/16h:
 14 tryptophan (low recovery); HPLC-1, 26.7; 2, 11.1 (Figure 2); 1H NMR
 LC 9 identical with previously reported spectra (4,9).

15 5-Isobutyl-8-N-(N',N'-dimethylphenylalanyl)-9-isopropylphen-
 16 cyclopeptide (Frangufoline) 5 and 5-sec Butyl-8-N-(N',N'-dimethyl-
 17 phenylalanyl)-9-isopropylphencyclopeptide (Adouetine-Y') 6.⁴
 18 $C_{31}H_{42}N_4O_4$; MS: M^+ m/e 534, M-91 $C_{24}H_{35}N_4O_4$ requires 443.2658,
 19 found 443.2649, BP $C_{10}H_{14}N$ requires 148.1126, found 148.1118; mp
 20 261° (lit. (6) mp 289-290° for 5, and lit. (10), 244° for 6; amino
 21 acid analysis 110°C/16h: ile/leu, 2/1; 1H NMR, high field region:
 22 δ 0.38 (d, 2H, J=6.7 Hz, ileu- γ -CH₃), 0.60 (d, 1H, 6.7 Hz leu δ CH₃),
 23 0.64 (d, 1H, 6.7Hz leu δ CH₃), 0.68 (m, 2H, ile δ CH₃), 1.01 (d, 3H,
 24 J=6.4Hz, R9-Me), 1.27 (d, 3H, J=6.7Hz, R9-Me); TLC Analtech Silica
 25 Gel G (250 μ)-eluant, $CHCl_3/Et_2O/MeOH$, 45/15/1, R_f 0.40 (5) and 0.33
 26 (6), lit. (9) R_f 0.66 (5); 0.58 (6); lit. (11) R_f 0.44 (5); 0.35 (6);
 LC 11 HPLC-1, 33.5; 2, 3.2 (Figure 2).

DISCUSSION

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

10 When the reversed phase HPLC system using 0.001% NH_4OH (aq)/
11 acetonitrile mixtures (4) proved unsuccessful, a paired-ion, reverse
12 phase HPLC system using 0.01N perfluorobutyric acid/acetonitrile
13 mixtures was devised (Fig. 2, system 1). The use of perfluoro-
14 butyric acid decreased column degradation and tailing of peaks
15 observed with the alkaline eluants. In addition, this paired-ion
16 proved to be superior to the sulfonic acids due to its high
17 volatility and ease of removal. A silica HPLC system was employed
18 to separate phencyclopeptides 2 and 3 which co-chromatographed in
19 system 1 (Figure 2, system 2).

20 The structural assignments of the HPLC-purified phencyclopeptide
21 components of *Ceanothus sanguineus* are based primarily on their
Table 2 22 characteristic electron impact mass spectra (Table 2). With the
23 exception of the tryptophan-containing phencyclopeptides 2 and 3,
24 the assignment of the ring amino acid residue (R_5) is based on
25 acidic hydrolysis of the purified phencyclopeptide followed by
26 amino acid analysis. Amino acid analysis reveals that the two
27 isomeric phencyclopeptides, frangufoline 5 and adouetine Y' 6,

1 which are unseparable by reversed phase HPLC (Figure 2, system 1),
2 are present in a ratio of 1 to 2, respectively. Further evidence for
3 the mixture of these two isomers is provided by ^1H NMR spectroscopy
4 and thin layer chromatography. As shown in Figure 3 the two
5 doublets at 0.59 and 0.64 ppm have been assigned to the leucine
6 δ -methyl groups of franguloline 5, in agreement with the reported
7 values of 0.60 and 0.65 ppm (12). The assignments of the doublet
8 at 0.38 ppm and the multiplet at 0.69 ppm to the isoleucine γ - and
9 δ -methyl groups of adouetine-Y' 6 are consistent with the literature
10 (10). Integration of these signals confirms the 1 to 2 ratio of
11 leucine to isoleucine indicated by amino acid analysis. The two large
12 doublets at 1.01 and 1.27 ppm (Figure 3), assigned to the R_g methyls
13 of both 5 and 6, are consistent with the literature values of 0.99
14 and 1.25 ppm for 5 (12) and 0.99 and 1.23 ppm for 6 (10). Further-
15 more, silica tlc (CHCl_3) of this mixture of isomers reveals two spots
16 with R_f 0.40 and 0.33, which agrees well with the reported chromato-
17 graphic data for these two phencyclopeptides (10).

18 In an earlier report describing the isolation and characteri-
19 zation of alkaloids from Ceanothus integerrimus, the chemotaxonomic
20 utility of the phencyclopeptides was discussed. In the future, the
21 use of Field Desorption/Collision Induced Dissociation (FD/CID) Mass
22 Spectrometry employing the linked B/E scan (2) may provide a useful
23 and rapid approach to the analysis of individual phencyclopeptides in
24 crude plant extracts without the need for chromatographic separation.

LC 12
Fig. 3

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26

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1

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4 We thank Dr. Ken Straube for acquiring FD spectra. Willy C. Shih
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6 nmr spectra. We are especially indebted to Dr. Frederick K. Klein
7 for the collection of plant material and the isolation of total
8 crude alkaloids.

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Footnotes

¹HPLC 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 Lackson 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 (μ mp) 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 CDCl₃ solution (CHCl₃ at 7.25 ppm) at 22°C 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.

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

1 ³For complete mass spectra see Table 2. For amino acid
2 analyses, each fraction was hydrolyzed with 1 ml 6N HCl with 50
3 µl 5% phenol (aq) for 16 h at 110°C in an evacuated ampule.

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

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Table 1. Phencycloptines of Ceanothus sanguineus

	<u>R₅</u>	<u>R₈</u>	<u>R₉</u>	<u>MW</u>
1, 5-Benzyl-8-N-(N'-methylpropyl)-9-isopropyl phencycloptine (Ceanothine-B) ^a	CH ₂ C ₆ H ₅	NMePro	CH(CH ₃) ₂	504
2, 5-sec Butyl-8-N(N'-methylphenylalanyl)-9-isopropylphencycloptine	CH(CH ₃)CH ₂ CH ₃	NMePhe	CH(CH ₃) ₂	520
3, 5-β-Indolylmethyl-8-N-(N',N'-dimethylvalyl)-9-isopropylphencycloptine ^b	-β-indolyl-CH ₂	NMe ₂ Val	CH(CH ₃) ₂	559
4, 5-β-Indolylmethyl-8-N-(N',N'-dimethylisoleucyl)-9-isopropylphencycloptine (Discarine-B) ^c	-β-indolyl-CH ₂	NMe ₂ Ileu	CH(CH ₃) ₂	573
5, 5-Isobutyl-8-N-(N',N'-dimethylphenylalanyl)-9-isopropylphencycloptine (Franguline) ^d	CH ₂ CH(CH ₃) ₂	NMe ₂ Phe	CH(CH ₃) ₂	534
6, 5-sec Butyl-8-N-(N',N'-dimethylphenylalanyl)-9-isopropylphencycloptine (Adouetine Y' or Myrianthine B) ^e	CH(CH ₃)CH ₂ CH ₃	NMe ₂ Phe	CH(CH ₃) ₂	534

- ^a First identified in Ceanothus americanus L (2). ^b First identified in Ceanothus integriramus 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).

TABLE 2. Low Resolution Mass Spectra of HPLC Purified Components of Ceanothus sanguineus^a

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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⁺-C₄H₉ ^e a-H ^f g-OH ^g b-NH(CH₃)₂

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 (10 μ , 10 x 250 mm); mobile phase 0.015M perfluorobutyric acid/CH₃CN (6/4 v/v); flow rate 1.6 ml/min/25°C; A 254 nm, injection volume 10 μ l; C=3.8 mg/ml.

2) Waters Porasil (10 μ , 3.2 x 280 mm); mobile phase CHCl₃/hexane/triethylamine (95/5/0.1 v/v); flow rate 1.6 ml/min; 41°C; A 280 nm; injection volume 10 μ l, C=3.5 mg/ml.

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

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