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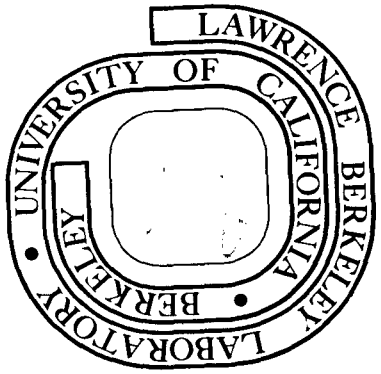

DISCLAIMER

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Cyclopeptide Alkaloids. Phencyclopeptines[1] from the Polymorphic Species Ceanothus integerrimus.

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ABSTRACT.--Seven cyclopeptide alkaloids, phencyclopeptines **1-7**, have been found distributed among three forms of the shrub *Ceanothus integerrimus*. Chemical degradation, mass spectroscopy, and ^{1}H NMR spectroscopy have established structures for these seven compounds, three of which have been previously reported. The utility of cyclopeptide alkaloid structure and distribution for chemotaxonomic assignments is discussed.

Ceanothus integerrimus ("Deer Brush") is a polymorphic species of the family Rhamnaceae occurring from southern Washington through California into western New Mexico. Although as many as eight varieties of this semi-deciduous shrub have been characterized, only two of the seven found in California are present in significant population. C. integerrimus H. and A. var integerrimus inhabits the inner South Coast Range and C. integerrimus var californicus (Kell.) G. T. Benson is found in the Sierra Nevada northward through the Cascade and Klamath Ranges (1).

LC 1

As part of more comprehensive alkaloid structure studies of Pacific North American Rhamnaceae, we have begun a phytochemical investigation of Ceanothus integerrimus. Our investigation of three specimens of this shrub, one of C. integerrimus var californicus, and two from different populations of C. integerrimus var integerrimus has led to the identification of four new cyclopeptide alkaloids, phencyclopeptines 1-4, in addition to three previously reported alkaloids 5-7 (Table 1). Employing reversed phase high performance liquid chromatography (HPLC) and mass and ^{1}H NMR spectroscopy, the distribution of phencyclopeptines among the three plants was determined (Figures 1, 2 and 3; Tables 1 and 2).

EXPERIMENTAL[2]

PLANT MATERIAL.[3]-- Root bark of C. integerrimus var integerrimus was obtained from its type locality in the Santa Cruz Mountains of California and from a population in the North Coast Ranges of Mendocino Co., California, while root bark of C. integerrimus var. californicus came from its type locality in the Sierra Nevada Mountains of Calaveras Co. California. Counting annuli revealed the plant from Santa Cruz Co. was 11 years old, the one from Mendocino Co. was 16 years old, and the var. californicus was considerably older. Herbarium voucher specimens were submitted to the University Herbarium, University of California, Berkeley, California.

EXTRACTION PROCEDURE.-- Plant material (500 g), frozen in liquid nitrogen, was ground to a fine powder in a Waring blendor and extracted with 0.1N HCl (2x2 liters) over a period of 8-12 hours at room temperature. After filtration, the extracts were combined, adjusted to pH 10 with sat. NaOH, and extracted with CH_2Cl_2 (2x1 liter). The combined CH_2Cl_2 layers were concentrated to 100 ml, and extracted with 0.1N HCl (5x20 ml) or until further acid extracts were alkaloid free. The combined acid extracts were made alkaline with sat. Na_2CO_3 to pH 10, extracted with CH_2Cl_2 (5x50 ml), and evaporated, affording the following alkaloidal yields: C. integerrimus var integerrimus (Santa Cruz Co.), 0.09%; C. integerrimus var californicus, 0.33%; C. integerrimus var integerrimus (Mendocino Co), 0.14% (root bark).

HPLC ISOLATION OF PHENCYCLOPEPTINES. -- Semi-preparative HPLC was performed on a LiChrosorb C2 column (10 μ, 10x150 mm or 10x250

mm, E. M. Merck). The crude alkaloidal mixtures were dissolved in 1/1 methanol/acetonitrile at a concentration of 3 mg/ml, and injection volumes ranged from 10-250 µl. The mobile phase was a mixture of acetonitrile and 0.0015% (v/v) aq ammonia with the aqueous ammonia comprising 10-30%, the flow rate was usually 2 ml/min, and the temperature was maintained at 40°C. Alkaloid components were detected at 254 nm. Figure 1 shows a typical HPLC tracing for the alkaloid mixtures from each plant variety; 10-20 injections provided sufficient material of each component for structural analysis. Fractions were evaporated in vacuo and dried under high vacuum immediately after collection.

YIELDS OF PHENCYCLOPEPTINE COMPONENTS FROM C. integerrimus.--

C. integerrimus var integerrimus (Santa Cruz Co.). Of the eight components separated by HPLC shown in Figure 1, five showed mass spectral patterns characteristic of the phencyclopeptine nucleus. These components were obtained in the following relative yield: 7 (70%), 2 (16%), 4 (10%), 3 (4%), 5 trace.

C. integerrimus var integerrimus (Mendocino Co). Three of seven components contained the phencyclopeptine nucleus, 6 (70%), 5 (15%), and 1 (15%).

C. integerrimus var californicus. Four phencyclopeptines were identified by mass spectroscopy in relative amounts as follows: 7 (45%), 5 (45%), 4 (5%) and 3 (5%).

fn 4 STRUCTURES[4] OF PHENCYCLOPEPTINE COMPONENTS FROM C. integerrimus.

5-β-Indolylmethyl-8-N-methylvalyl-9-phenylphencyclopeptine 1. $C_{34}H_{37}N_5O_4$; µmp >350°; MS: M^+ $C_{34}H_{37}N_5O_4$ requires 579.2845, found 579.2788, M-43 $C_{31}H_{30}N_5O_4$ requires 536.2298, found 536.2302, BP

$C_5H_{12}N$ requires 86.0970, found 86.0970 (see Figure 2 for complete mass spectra); amino acid analysis after acid hydrolysis: no amino acids observed; ^1H NMR, high field region:[5] δ 0.27 (d, 3H, J=6.9 Hz val-γ-CH_3), 0.54 (d, 3H, J=6.9 Hz val-γ-CH_3).

5-β-Indolylmethyl-8-N,N-dimethylvalyl-9-isopropylphencyclopeptine 2. $C_{32}H_{41}N_5O_4$; μmp 233°; MS: M^+ $C_{32}H_{41}N_5O_4$ requires 559.3158, found 559.3146, M-43 m/e 516, BP $C_6H_{14}N$ requires 100.1126, found 100.1130 (see Figure 2); ^1H NMR, high field region: δ 0.84 (d, 3H, J=6.8 Hz, $(CH_3)_2CH$, 0.93 (d, 3H, J=6.8 Hz, $(CH_3)_2CH$, 0.96 (d, 3H, J=6.9 Hz, val-γ-CH_3), 1.18 (d, 3H, J=6.9 Hz val-γ-CH_3).

5-Benzyl-8-N,N-dimethylisoleucyl-9-phenylphencyclopeptine 3. $C_{34}H_{40}N_4O_4$; μmp >350°; MS: M^+ m/e 568, M-57, $C_{30}H_{31}N_4O_4$ requires 511.2345, found 511.2332, BP $C_7H_{16}N$ requires 114.1282, found 114.1279 (see Figure 2); ^1H NMR, high field region: δ 0.18 (d, 3H, J=6.9 Hz ileu-γ-CH_3), 0.80 (t, 3H, J=6.9 Hz, ileu-δ-CH_3).

5-Isobutyl-8-N-methylisoleucyl-9-phenylphencyclopeptine 4. $C_{30}H_{40}N_4O_4$; μmp 213°; MS: M^+ $C_{30}H_{40}N_4O_4$ requires 520.3049, found 520.3053, M-57 $C_{26}H_{31}N_4O_4$ requires 463.2345, found 463.2356, BP $C_6H_{14}N$ requires 100.1126 found 100.1131 (see Figure 2); amino acid analysis after acid hydrolysis: 1.0 leucine; ^1H NMR, high field region: δ 0.57 (d, 3H, J=6.9 Hz, ileu-γ-CH_3), δ 0.66 (m, 6H, ileu-δ-CH_3 and leu(C5)-δ-CH_3), 0.76 (d, 3H, J=6.5 Hz, leu-(C5)-δ-CH_3).

5-β-Indolylmethyl-8-N,N-dimethylisoleucyl-9-isopropylphencyclopeptine (Discarine B) 5. $C_{33}H_{43}N_5O_4$; μmp 233°, lit (2) mp 235-236°; MS: M^+ $C_{33}H_{43}N_5O_4$ requires 573.3315, found 573.3297, M-57 m/e 516, BP $C_7H_{16}N$ requires 114.1282 found 114.1284 (see Figure 2), ^1H NMR (identical to literature (2,3)), high field region: δ 0.82 (d, 3H,

J=6.7Hz, ileu-γ-CH_3), 0.90 (t, 3H, J=7.5Hz, ileu-δ-CH_3), 0.91 (d, 3H, J=6.8Hz, $(CH_3)_2CH$), 1.18 (d, 3H, J=6.8Hz, $(CH_3)_2CH$).

5-β-Indolylmethyl-8-N,N-dimethylvalyl-9-phenylphencyclopeptine (Integerrine) 6. $C_{35}H_{39}N_5O_4$; µmp 246°, lit (4) mp 258°; MS: M^+ $C_{35}H_{39}N_5O_4$ requires 593.3002, found 593.2924, M-43 m/e 550, BP $C_6H_{14}N$ requires 100.1126, found 100.1127 (see Figure 2); ^1H NMR, high field region: δ 0.16 (d, 3H, J=6.8 Hz, val-γ-CH_3), 0.70 (d, 3H, J=6.8 Hz, val-γ-CH_3).

5-Isobutyl-8-N,N-dimethylisoleucyl-9-phenylphencyclopeptine (Integerrenine) 7. $C_{31}H_{42}N_4O_4$; µmp 259°, lit (5) mp 278°; MS: M^+ $C_{31}H_{42}N_4O_4$ requires 534.3205, found 534.3200, M-57 $C_{27}H_{33}N_4O_4$ requires 477.2502, found 477.2515, BP $C_7H_{16}N$ requires 114.1282, found 114.1283 (see figure 2); ^1H NMR (identical to literature (5)), high field region: δ 0.36 (d, 3H, J=6.7Hz, ileu-γ-CH_3), 0.78 (d, 3H, J=6.5Hz, leu-δ-CH_3), 0.85 (d, 3H, J=6.5Hz, leu-δ-CH_3), 0.86 (t, 3H, J=7.3Hz, ileu-δ-CH_3).

DISCUSSION

The identification of the HPLC-purified constituents of C. integerrimus is based mainly on their characteristic electron impact mass spectra. According to the fragmentation schemes previously proposed (6,7) (Figure 2), the mass spectra of the seven alkaloids from the three plants (Table 2) confirm the structural assignments made in Table 1. In addition, the total alkaloid acid hydrolytic products from each plant (Table 3) are consistent with the distribution of phencyclopeptines 1-7 among the three plants shown in Figure 3. Since tryptophan is destroyed by acid hydrolysis and N-alkylated amino acids are not detected by the usual automatic amino acid analysis due to their low color yield, the failure to detect any other amino acids in the acidic hydrolysate of C. integerrimus var. integerrimus (Mendocino Co.) corroborates the observation of only indolic phencyclopeptine components in this plant.

Leucine, isoleucine, and valine and their methylated derivatives were distinguished from one another by mass spectroscopy and ^1H NMR spectroscopy, and amino acid analysis in some cases. Amino acid analysis of the acid hydrolysates of each HPLC-purified phencyclopeptine confirmed the identity of the ring amino acid (R_5) suggested by mass spectroscopy. Fragments produced from the rearrangement of the base peak (BP, a) in the mass spectrum of the phencyclopeptine provided diagnostic evidence for the structure of the N-alkylated amino acid residue, R_8 (Table 2).

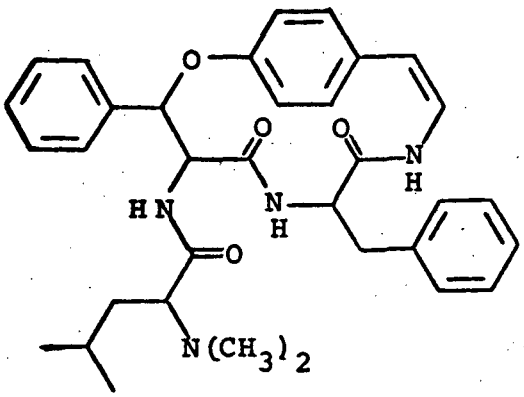
^1H NMR spectroscopy furnished the most definitive information regarding the nature of the N-terminal amino acid moiety (R_8),

since the two methyl groups of isoleucine manifest different multiplicity in their NMR signals, the γ-methyl being a doublet and the δ-methyl a triplet. Both the δ-methyls of leucine and the γ-methyls of valine are two sets of doublets. Furthermore, the chemical shifts of the methyl groups on the N-terminal amino acid (R_8) are also diagnostic. In the cases of phencyclopeptines where R_9 is phenyl, a pronounced upfield shift (as much as 0.6 ppm) has been observed in the N-methyl and γ-methyl resonances of the N-terminal amino acids (5). Such high field resonances do not occur in the spectra of alkaloids which have N-terminal leucine residues since there are no γ-methyl groups. Thus the chemical shift of the leucine δ-methyls in crenatine A 9 occur within the expected range (8), two doublets at δ 0.78 and 0.83 ppm in $CDCl_3$, whereas the doublet occurring at 0.24 ppm in the spectrum of integerrine 6 is attributable to the γ-methyl of the N-terminal isoleucine residue.

LC 8

Our observation of unusually high field doublets in the spectra of phencyclopeptines 1, 3, 4 and 7 as well, establishes that the N-terminal amino acids are either derivatives of valine or isoleucine. Such high field resonances were not observed in the ^1H NMR spectrum of discarine B 5 and phencyclopeptine 2 in agreement with the literature (2,3).

It is unusual that only one of the phencyclopeptines, discarine B 5, found in C. integerrimus var integerrimus from Santa Cruz Co. was observed in the extract of the plant of the same species from Mendocino Co. (Figure 3). In contrast, the total alkaloidal mixture from var. integerrimus of Santa Cruz Co. and that from var. californius contained four common phencyclopeptines 3, 4, 5 and 7.



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Integerressine 8 has been reported as the major alkaloid of C. integerrimus var integerrimus roots, integerrenine 7 as a minor alkaloid, and integerrine 6 as a trace component (4,5). Our results are different from this reported estimation. In the extract of C. integerrimus var integerrimus from Santa Cruz Co., integerrenine 7 was the major alkaloid whereas integerrine 6 and integerressine 8 were absent. On the other hand, integerrine 6 was the major constituent of C. integerrimus var integerrimus obtained from Mendocino Co.

Conservative botanical opinion has been that the polymorphic forms of C. integerrimus may represent responses to varying amount of moisture and therefore should be included in a single species C. integerrimus H. and A. (1). It is possible that qualitative differences in alkaloid composition between plants from different populations of C. integerrimus may similarly reflect the response of the plants to local environmental conditions.

The phytochemical investigation of C. integerrimus also poses a difficult challenge to both the botanist and the chemist because interspecific hybridization within the genus Ceanothus is widespread. Thus the variation in the alkaloidal characters could be representa-

tive of the degree of interspecific hybridization in Ceanothus. This concept might explain the disparities among the alkaloid contents of the three examples of C. integerrimus var integerrimus examined in this investigation and those observed by others (4,5). Furthermore, the reported association of nitrogen-fixing actinomycetes LC 9 with the roots of Ceanothus (9) as well as with other plants which produce phencyclopeptines may implicate the symbionts in the production of cyclopeptide alkaloids. These intriguing possibilities further complicate the phytochemical investigation of C. integerrimus and will be addressed in future studies of Ceanothus.

The chemotaxonomic utility of the phencyclopeptines must rely upon the examination of many plants from each different population of C. integerrimus. The procedure outlined here, involving standardized isolation, HPLC purification, and mass spectral identification, provides a quick and objective means upon which to base plant taxonomic and evolutionary relationships.

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FOOTNOTES

[1]We propose the name phencyclopeptine to represent the fundamental para-bridged 14-membered ring nucleus most common in this large class of widely occurring alkaloids. This basic nucleus and the numbering system shown in Table 1 allow individual alkaloids to be simply and unambiguously designated. Thus we can avoid the multitude of trivial names based on botanical anagrams that have no structural significance.

[2]HPLC was performed with a Spectra Physics Model SP3500B 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 from Burdick and Jackson Laboratories, Muskegon, MI, and water purified with a Milli-Q system, Millipore Corp. Bedford, MA, were used for HPLC. Uncorrected melting points were determined on a Kofler Micro Hot Stage (μmp). A model AEI-MS12 mass spectrometer, AEI Scientific Apparatus Ltd, Manchester, England, with INCOS data system was used for determining low resolution mass spectra. High resolution mass spectra were obtained with a Consolidated Electrodynamics CEC-110B instrument. Amino acid analyses were performed on a Beckman 120C Chromatograph, Fullerton, CA, unless otherwise indicated ^{1}H NMR spectra were taken in $CDCl_3$ solution ($CHCl_3$ at 7.21 ppm) at 22°C on a home-made spectrometer based on a Bruker 63kG magnet operating at 270 MHz with a Nicolet 1180 data system. Evaporations were done in vacuo with a Buchi rotary evaporator.

[3]Identification of plant materials was performed by Dr. L. R. Heckard, University of California, Berkeley, CA, and Dr. M. A. Nobs, Carnegie Institution of Washington, Stanford, CA. All three plants were collected in the months of May and June.

[4]Compounds of the same structure isolated from different plants had the same mp's and ^{1}H NMR spectra.

[5]A complete analysis of the ^{1}H NMR spectra of the phencyclopeptine system will be dealt with in a future report.

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Fig. 1. HPLC of crude alkaloidal extracts of the polymorphic species C. integerrimus.

HPLC system employed: LiChrosorb C2 (10 µ, 10x150 mm); mobile phase CH_3CN/10% aq. NH_3 (9/1, v/v); flow rate, 2 ml/min; 35°C; A 254 nm; injection volume, 100 µl; c ~3 mg/ml.

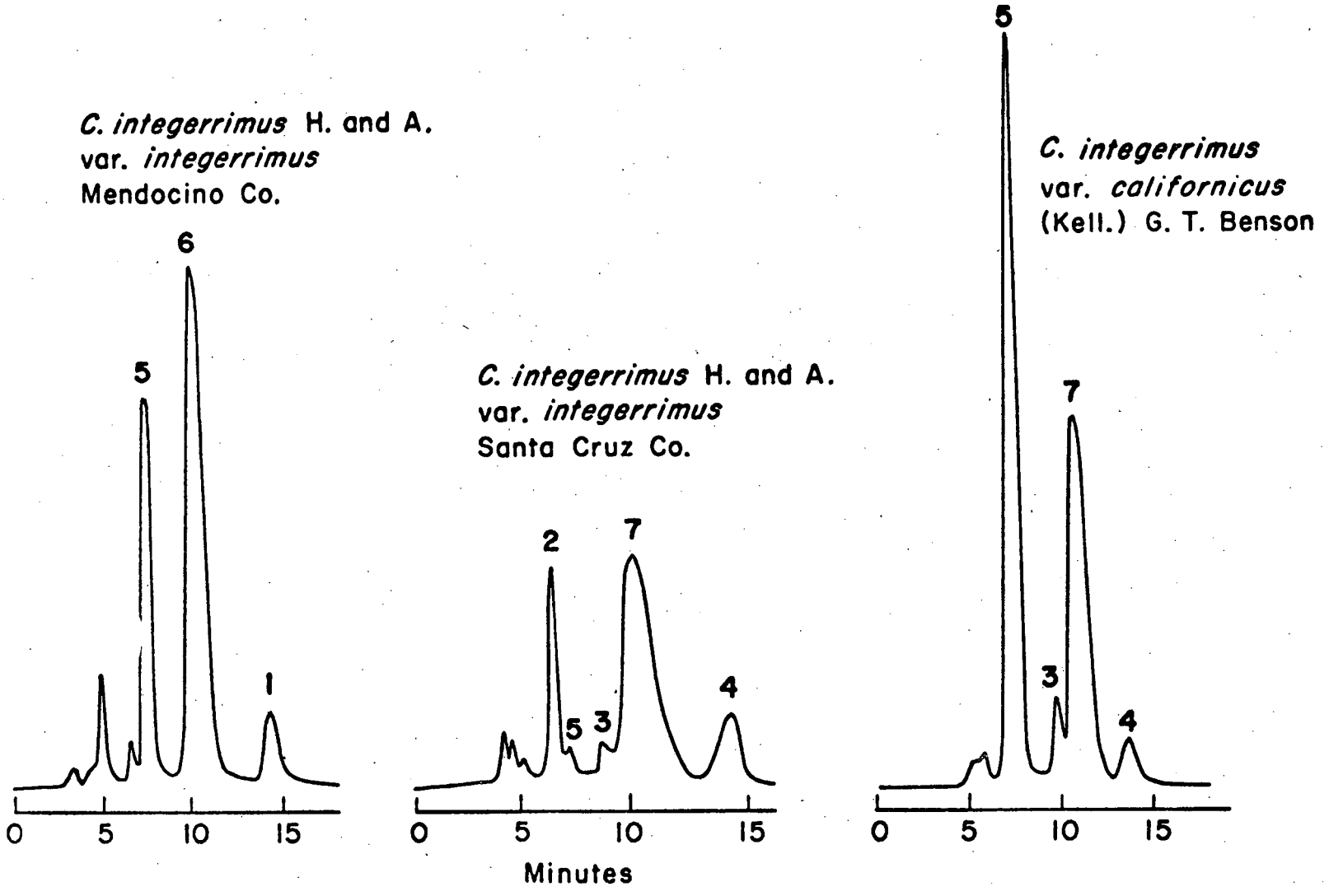

C. integerrimus H. and A.
var. *integerrimus*
Mendocino Co.
6
5
1
0
5
10
15
C. integerrimus H. and A.
var. *integerrimus*
Santa Cruz Co.
2
7
5
3
4
0
5
10
15
Minutes
5
C. integerrimus
var. *californicus*
(Kell.) G. T. Benson
7
3
4
0
5
10
15

Fig. 2. Electron impact mass spectral fragmentation of phencyclopeptines.

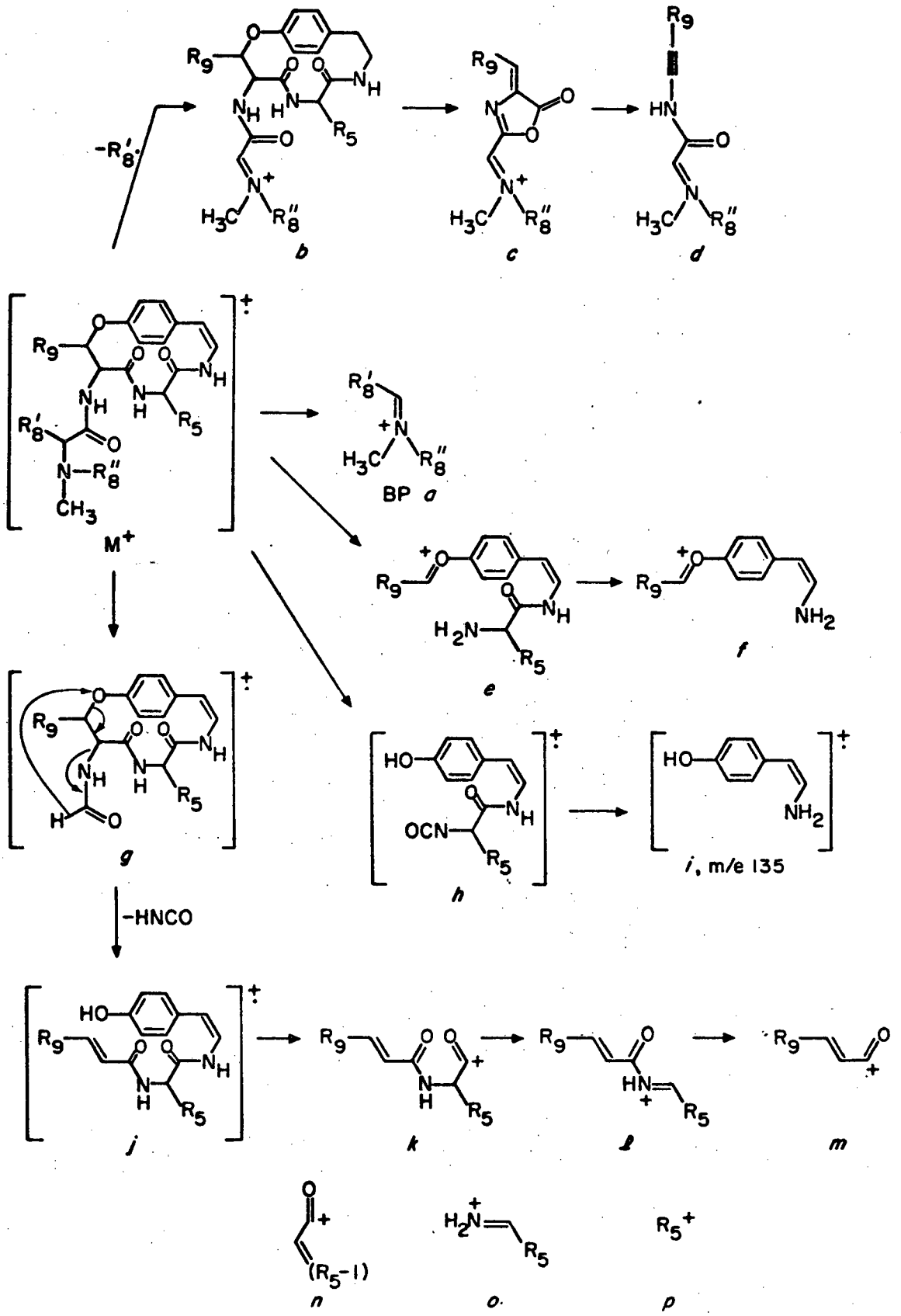

-R'8·
b
c
d
M+
BP a
e
f
g
h
i, m/e 135
-HNCO
j
k
l
m
n
o
p

Fig. 3. Phencyclopeptine distribution in C. integerrimus var integerrimus H. and A. from Santa Cruz Co., C. integerrimus var integerrimus H. and A. from Mendocino Co., and C. integerrimus var californicus (Kell) and G. T. Benson.

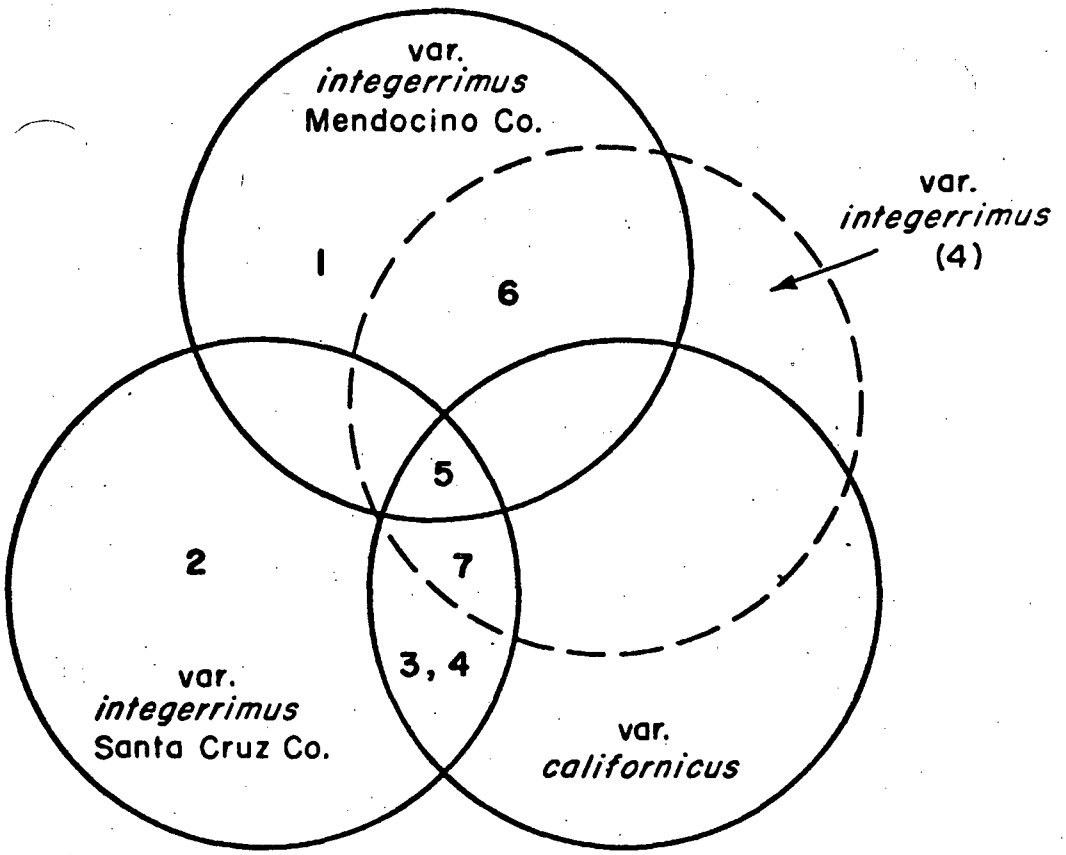
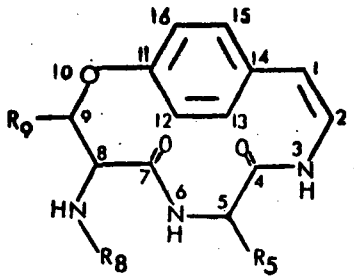

var.
integerrimus
Mendocino Co.
var.
integerrimus
(4)
1
6
5
2
7
3, 4
var.
integerrimus
Santa Cruz Co.
var.
californicus

Table 1. Phencyclopeptine constituents of Ceanothus integerrimus.



	R_9	R_5	R_8	MW
1, 5-β-Indolylmethyl-8-N-methylvalyl-9-phenylphencyclopeptine	C_6H_5	β-indolyl-CH_2	NMeVal	579
2, 5-β-Indolylmethyl-8-N,N-dimethylvalyl-9-isopropylphencyclopeptine	$(CH_3)_2CH$	β-indolyl-CH_2	NMe_2Val	559
3, 5-Benzyl-8-N,N-dimethylisoleucyl-9-phenylphencyclopeptine	C_6H_5	$C_6H_5CH_2$	NMe_2Ile	568
4, 5-Isobutyl-8-N-methylisoleucyl-9-phenylphencyclopeptine	C_6H_5	$(CH_3)_2CHCH_2$	NMeIle	520
5, 5-β-Indolylmethyl-8-N,N-dimethylisoleucyl-9-isopropylphencyclopeptine (Discarine B)[a]	$(CH_3)_2CH$	β-indolyl-CH_2	NMe_2Ile	573
6, 5-β-Indolylmethyl-8-N,N-dimethylvalyl-9-phenylphencyclopeptine (Integerrine)[b]	C_6H_5	β-indolyl-CH_2	NMe_2Val	593
7, 5-Isobutyl-8-N,N-dimethylisoleucyl-9-phenylphencyclopeptine (Integerrenine)[c]	C_6H_5	$(CH_3)_2CHCH_2$	NMe_2Ile	534
8, 5-Benzyl-8-N-methylvalyl-9-phenylphencyclopeptine (Integerressine)[d]	C_6H_5	$C_6H_5CH_2$	NMe_2Val	554

[a]First identified in Discaria longespina H. and A. (2). [b]First identified in C. integerrimus H. and A. (4). [c]First identified in C. integerrimus H. and A. (5). [d]Identified constituent of C. integerrimus H. and A. (3), not observed in any of the three plants in the present investigation.

Table 2. Mass spectra of the HPLC purified phencyclopeptine components of C. integerrimus.

Fragment[a]	Compound						
	1	2	3	4	5	6	7
M+	579[b]	559[b]	568	520[b]	573[b]	593[b]	534[b]
BP a	86[b]	100[b]	114[b]	100[b]	114[b]	100[b]	114[b]
b	536[b]	516	511[b]	463[b]	516	550	477[b]
c	215	195	229	215	195	229	229
d	187	167	201	187	167	201	201
e	410[c]	376[c]	371	337	376		337
f	224[c]	190	224	224	190	224	224
g	494			421			
h	347[c]	347[c]	308	274	347	347[c]	
i	135	135	135	135	135	135	135
j	451	417[c]	412[c]	378			
k	317[c]	283	278[c]	244	283	317	244
l	289	255[c]	250[c]	216	255		216
m	131	97[c]	131	131	97	131	131
n	170[c]	170	131	97	170	170	97
o	159	159	120	86	159	159	86
p	130	130	91	57	130	130	57
other	117	117	98	505[d]	117	117	519[d]
				491[e]	85[g]	85[g]	505[e]
				477[f]			491[f]

Footnotes to Table 2:

[a]Fragment ions refer to structures in Figure 2.

[b]High resolution mass spectral data obtained.

[c]Weak ion intensity in some spectra.

[d]M^+-15 [e]M^+-29 [f]M^+-43

[g]Fragments from rearrangement of BP, diagnostic of N-alkylated amino acid N-terminal moiety: m/e 58, MeLeu; 72, Me_2Leu; 85, Me_2Val and Me_2Ileu. Taken from ref (5).

Table 3. Amino acid identification and yields from acid hydrolysis of *C. integerrimus* root bark total alkaloid mixtures.[a]

Product	*C. integerrimus* var. *integerrimus* Santa Cruz Co.	*C. integerrimus* var. *integerrimus* Mendocino Co.	*C. integerrimus* var. *californicus*
NH_3	769	516	590
Ile	-	-	6
Leu	352	-	190
Phe	29	-	26

[a]Crude alkaloid mixtures (250 mg) were hydrolyzed with 1-2 ml 6N HCl containing 1 drop glacial acetic acid for solubilization in sealed ampules for 24 h at 135°C. Yields are reported in nanomoles/250 mg mixture hydrolyzed.

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