Peptide Alkaloids from *Ceanothus americanus* L. (Rhamnaceae)[1]

Robert E. Servis,[2] Alvin I. Kosak,[2] R. Tschesche,[3] E. Frohberg,[3] and H.-W. Fehlhaber[3]

Contribution from the Department of Chemistry, New York University, New York, New York 10003, and the Institute of Organic Chemistry, University of Bonn, 53 Bonn, Germany. *Received March 1, 1969*

Abstract: Five more peptide alkaloids have been isolated from the root bark of *Ceanothus americanus* L. Their structures were elucidated using the informations obtained from hydrolysis of the dihydro derivatives and from their mass spectrometric fragmentation reactions. Three of the alkaloids have been found to be identical with alkaloids known from other plants, *i.e.*, frangulanine (IV), adouétine-X (V), and adouétine-Y (VI). Two alkaloids represent compounds not described up to now, they have been named ceanothine-D (VII) and ceanothine-E (VIII).

In the course of the last 5 years numerous peptide alkaloids have been isolated in six different laboratories from several Rhamnaceae[1b,1d,4-15] as well as other species of the subclass Dicotyledoneae.[16-21] With the possible exception of zizyphine[4] and lasiodine-A,[15] all these alkaloids posses an analogous skeleton: a 14-membered cyclic system, incorporating a *p*-hydroxystyrylamine (in exceptional cases[18-20] replaced by *p*-hydroxy-2-phenylethylamine with an additional oxygen function at the benzylic carbon atom), an amino acid, and a β-hydroxyamino acid, attached to a terminal N-methylated amino acid (or dipeptide) residue.

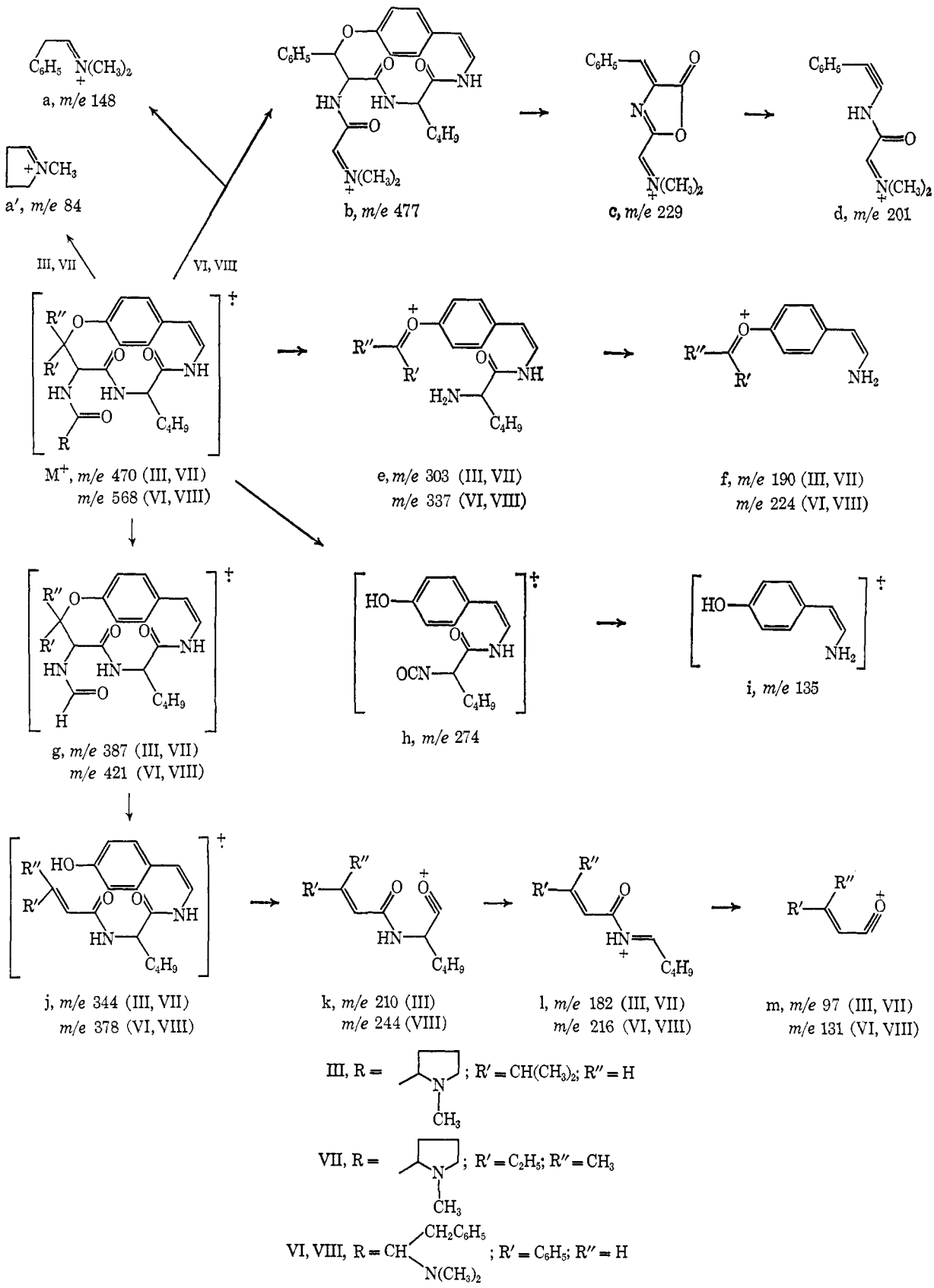
Ceanothus americanus, Jersey Tea, has been known to contain alkaloids since 1884,[22] but the first reasonably pure alkaloid, ceanothine, was not isolated until 1933.[23] In 1965, two different groups[5,6] succeeded in isolating several pure components from the complex mixture of alkaloids that is initially extracted from the root bark of the shrub. That same year, a proposal appeared[7] for the structure of ceanothine, renamed ceanothine-B.[6] More recent work[1b,15] has indicated that the correct structure is represented by formula I. The isolation and structure elucidation of another alkaloid, americine (II), was recently reported by Klein and Rapoport.[13]

For another alkaloid previously isolated by Warnhoff and coworkers,[6] ceanothine-C, structure III was proposed by one of us[24] on the basis of the mass spectrum which had been obtained from a small sample kindly provided by Professor Warnhoff. Since the details of this structure elucidation have not been published, they will be given here. The mass spectrum of III is reproduced in Figure 1. To confirm the assignment of the fragment ions, extensive high-resolution mass measurements were made (see Table II in the Experimental Section). The mass spectrometric fragmentation of ceanothine-C closely follows the previously stated[9] fragmentation scheme that proved to be highly characteristic of alkaloids of this type,[24] and which has been successfully used in numerous cases[1d,9-12,15,17] to elucidate the structure of peptide alkaloids.

The typically large base peak at *m/e* 84, originating from the amine fragment of the terminal amino acid, is characteristic of N-methylproline (ion a′, see Scheme I) and the ion *m/e* 135 (i) indicates the presence of a *p*-hydroxystyrylamine group. The peak at *m/e* 97 corresponds to the fragment ion m, R′ plus R″ being equal to C_3H_8, which has been shown[6,9,24] to arise from the etherified β-hydroxyamino acid moiety. Since the mass spectrum displays a prominent peak at M − 43, 70% of which is due to the elimination of a C_3H_7 radical arising from the common α-cleavage[25] of the ether function to form the oxonium ion $>CHCH{=}O^+C_6H_4-$, the R′ and R″ groups must be equal to C_3H_7 and H, respectively. The hydroxyamino acid is, therefore, β-hydroxyleucine. At *m/e* 86, the amine fragment of the amino acid incorporated in the ring system, $H_2N^+{=}CHC_4H_9$, can be detected. As discussed previously,[24] no distinction between leucine and isoleucine is possible.

(1) (a) Paper II on Ceanothus alkaloids by the New York University group. (b) I, R. E. Servis and A. I. Kosak, *J. Amer. Chem. Soc.*, **90**, 4179 (1968). (c) Paper VII in the series deals with alkaloids from Rhamnaceae by the Bonn University group. (d) Paper VI: R. Tschesche, L. Behrendt, and H.-W. Fehlhaber, *Chem. Ber.*, **102**, 50 (1969).
(2) New York University.
(3) University of Bonn.
(4) (a) E. L. Ménard, J. M. Muller, A. F. Thomas, S. S. Bhatnagar, and N. J. Destoor, *Helv. Chim. Acta*, **46**, 1801 (1963); (b) E. Zbiral, E. L. Ménard, and J. M. Muller, *ibid.*, **48**, 404 (1965).
(5) H. Saltzman, Ph.D. Thesis, New York University, 1965.
(6) E. W. Warnhoff, S. K. Pradhan, and J. C. N. Ma, *Can. J. Chem.*, **43**, 2594 (1965).
(7) E. W. Warnhoff, J. C. N. Ma, and P. Reynolds-Warnhoff, *J. Amer. Chem. Soc.*, **87**, 4198 (1965).
(8) R. Tschesche, R. Welters, and H.-W. Fehlhaber, *Chem. Ber.*, **100**, 323 (1967).
(9) R. Tschesche, J. Rheingans, H.-W. Fehlhaber, and G. Legler, *ibid.*, **100**, 3924 (1967).
(10) R. Tschesche, H. Last, and H.-W. Fehlhaber, *ibid.*, **100**, 3937 (1967).
(11) R. Tschesche, E. Frohberg, and H.-W. Fehlhaber, *Tetrahedron Lett.*, 1311 (1968).
(12) R. Tschesche and H. Last, *ibid.*, 2993 (1968).
(13) F. K. Klein and H. Rapoport, *J. Amer. Chem. Soc.*, **90**, 2388 (1968).
(14) F. K. Klein and H. Rapoport, *ibid.*, **90**, 3576 (1968).
(15) J. Marchand, M. Pais, X. Monseur, and F.-X. Jarreau, *Bull. Soc. Chim. Fr.*, in press. The remittance of the manuscript prior to publication by Professor Goutarel is gratefully acknowledged.
(16) M. Pais, J. Mainil, and R. Goutarel, *Ann. Pharm. Fr.*, **21**, 139 (1963).
(17) M. Pais, J. Marchand, F.-X. Jarreau, and R. Goutarel, *Bull. Soc. Chim. Fr.*, 1145 (1968).
(18) M. Pais, X. Monseur, X. Lusinchi, and R. Goutarel, *ibid.*, 817 (1964).
(19) M. Pais, F.-X. Jarreau, X. Lusinchi, and R. Goutarel, *Ann. Chim.*, 83 (1966).
(20) (a) M. Pais, J. Marchand, X. Monseur, F.-X. Jarreau, and R. Goutarel, *C. R. Acad. Sci., Paris*, **264**, 1409 (1967); (b) M. Pais, J. Marchand, G. Ratle, and F.-X. Jarreau, *Bull. Soc. Chim. Fr.*, 2979 (1968).
(21) R. Tschesche and I. Reutel, *Tetrahedron Lett.*, 3817 (1968).
(22) J. H. M. Clinch, *Amer. J. Pharm.*, **56**, 131 (1884).
(23) A. Bertho and W. S. Liang, *Arch. Pharm.*, **271**, 273 (1933).
(24) H.-W. Fehlhaber, *Z. Anal. Chem.*, **235**, 91 (1968).
(25) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Mass Spectrometry of Organic Compounds," Holden-Day, Inc., San Francisco, Calif., 1967, p 227.

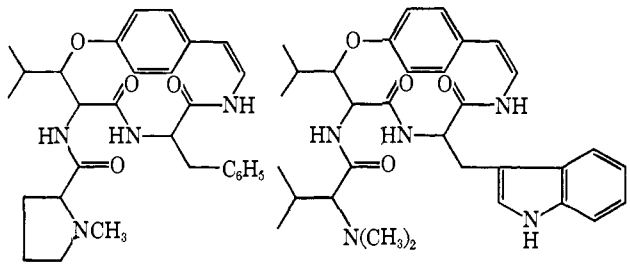
Scheme I



The mutual combination of the amino acids can be deduced from the following fragments: the ions f (*m/e* 190), h (*m/e* 274), and e (*m/e* 303) indicate that the *p*-hydroxystyrylamine moiety is bonded both with the β-hydroxyleucine and the C_6 amino acid (leucine or isoleucine); in addition, the ions k (*m/e* 210) and l (*m/e* 182) prove these latter two amino acids to be attached to each other, and establish the presence of the 14-membered cyclic system in III. Since we did not succeed in isolating ceanothine-C, we were unable to support structure III by chemical investigations.

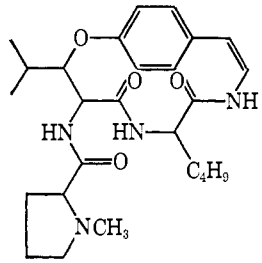
We have obtained, however, five other alkaloids from

Chart I

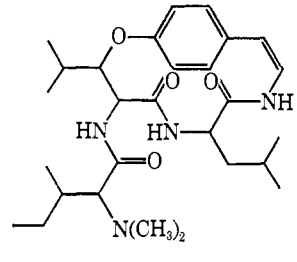


I, ceanothine–B

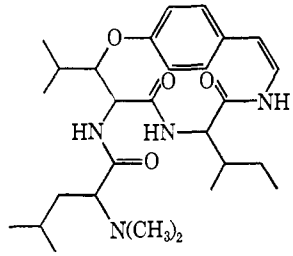
II, americine



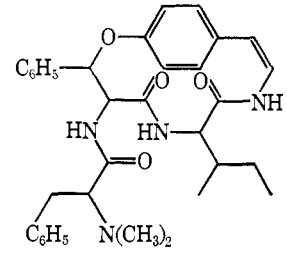
III, ceanothine–C



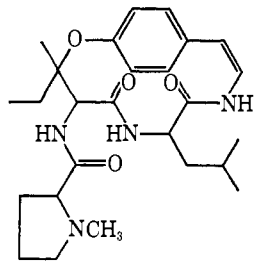
IV, ceanthamine–A (frangulanine)



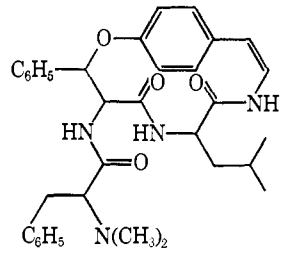
V, adouetine–X (ceanothamine–B)



VI, adouetine–Y



VII, ceanothine–D



VIII, ceanothine–E

the root bark of *Ceanothus americanus*. They were isolated using tlc, either by means of multiple developments on silica gel plates or by *seriatim* combination of silica gel plates and neutral alumina plates of activity 2.5.[26] The structure of each alkaloid was then elucidated on the basis of its mass spectrometric fragmentation and the information obtained from acid hydrolysis of its dihydro derivative.[27] The hydrolysis products were analyzed by paper chromatography, by tlc, by vpc after conversion to their trimethylsilyl derivatives, and by a combination of spectroscopic evidences. In each case, the

(26) The activity of the alumina tlc plates was of primary importance, since plates of lower activity were not effective. The activity was determined according to K. Randerath, "Thin-Layer Chromatography," translated by D. D. Libman, 2nd ed, Academic Press, New York, N. Y., 1966, p 13.

(27) Hydrolysis was performed on the dihydro derivatives in order to identify the *p*-tyramine moiety formed by the hydrogenation of the double bond. The *p*-hydroxystyrylamine moiety of the parent alkaloids cannot be detected because of its decomposition during acidic hydrolysis.

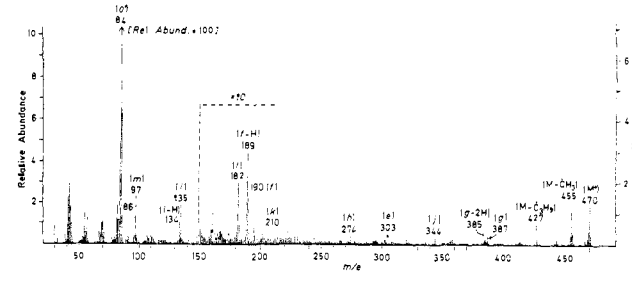


Figure 1. Mass spectrum of ceanothene-C (III).

compounds were compared with known reference standards.

The structures of the five alkaloids are represented by formulas IV–VIII (Chart I). Two of them, IV and V, correspond to alkaloids isolated by Warnhoff and coworkers.[6]

Ceanothamine-A. The first alkaloid gave a mass spectrum (parent peak at *m/e* 500) which, when recorded under the same conditions, was fully identical with the mass spectrum of frangulanine previously isolated from *Rhamnus frangula*.[10] As illustrated above, the mass spectrum completely reflects the structural properties of a peptide alkaloid, the remaining uncertainties[24] being only the position of the ethereal oxygen in the styrylamine moiety and the differentiation between leucine and isoleucine, when one of these amino acids is incorporated in the ring system. Acid hydrolysis of the dihydro derivative of IV yielded N,N-dimethylisoleucine, leucine, *p*-tyramine, and small amounts of β-hydroxyleucine, glycine, and 2-keto-4-methylpentanoic acid, the latter two compounds obviously originating from the known[28] decomposition reactions of β-hydroxyleucine. These findings confirmed, in agreement with the physical properties, that this alkaloid is indeed identical with frangulanine (IV).

Adouétine-X. The mass spectrum of the second alkaloid also displayed a molecular ion peak at *m/e* 500 as does frangulanine. The fragment ion peaks are also analogous except for one important difference: the amine fragment of the terminal amino acid (base peak at *m/e* 114) underwent further fragmentation not by loss of an ethyl radical as in the case of frangulanine (→ *m/e* 85), but by elimination of propene generating an *m/e* 72 ion, a reaction characteristic of N,N-dimethylleucine.[9,10,24] A detailed comparison of the mass spectrum showed it to be completely identical with the mass spectra of adouétine-X[10,16,17,29] and franganine.[12] The only difference between these two peptide alkaloids is the α-amino acid incorporated in the ring system, the former alkaloid containing isoleucine, the latter leucine.

By acidic hydrolysis of the dihydro derivative of V isoleucine, tyramine, N,N-dimethylleucine, and small amounts of β-hydroxyleucine and 2-keto-4-methylpentanoic acid were identified, thus indicating that the alkaloid is identical with adouétine-X (V). This conclusion was confirmed by comparing the nmr spectra of both samples[29] which were recorded in $CDCl_3$ with the aid of a time-averaging computer since the compounds were but sparingly soluble in this solvent.

(28) Th. Wieland, H. Cords, and E. Keck, *Chem. Ber.*, 87, 1312 (1954).

(29) We are indebted to Professor Goutarel for a sample of adouétine-X.

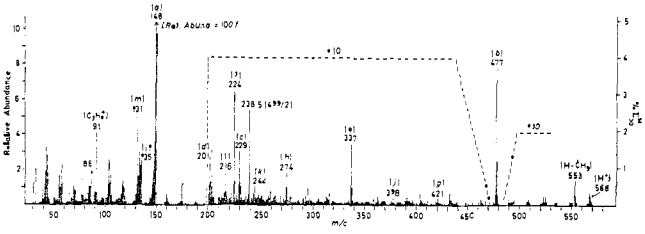


Figure 2. Mass spectrum of ceanothene-E (VIII).

The physical properties, the molecular weight, and the nature of the amino acids suggested that the alkaloids IV and V correspond to ceanothamine-A and ceanothamine-B isolated by Warnhoff and coworkers[6] from *C. americanus* and believed by them to be stereoisomers. Since the physical properties of both alkaloids are very similar, positive identification cannot be made in this way. The actual names of the two ceanothamines were assigned by Warnhoff[6] on the basis of their R_f values (with ceanothamine-A being the faster moving component). The difference in the R_f values of frangulanine (IV) and adouétine-X (V) is very small in a variety of solvent systems on silica gel plates. When, however, ethyl acetate is the solvent a remarkable divergence in R_f values is observed and frangulanine is unequivocally the faster moving compound. It should, therefore, correspond to ceanothamine-A, and adouétine-X should be identical with ceanothamine-B.

Since both ceanothamines[6] are identical to well-known alkaloids, their names should be removed from the literature. This appears to be even more appropriate because Warnhoff[6] had chosen these names only in order to indicate the relative R_f values displayed during the initial tlc separation of the alkaloids regardless of the total structure of the alkaloid. Since such a differentiation seems to us to give rise to confusion, we propose designating all alkaloids isolated from *C. americanus*, irrespective of the structure of their terminal amino acids, as *ceanothines*.

Adouétine-Y. The mass spectrum of the third alkaloid (parent ion peak at *m/e* 568) is dominated by a large base peak at *m/e* 148 (ion a, see Scheme I) which, together with a prominent peak at M − 91 (b),[30] strongly suggests the presence of a terminal N,N-dimethylphenylalanine moiety. Further peaks in the lower mass region at *m/e* 135, 131, and 86, corresponding to the fragment ions i, m (R′ = C_6H_5, R″ = H), and $H_2N^+{=}CHC_4H_9$, indicate the presence of *p*-hydroxystyrylamine, β-phenylserine,[9,24] and a leucine isomer, respectively. The peaks at *m/e* 229 and 201 (c, d) indicate the N,N-dimethylamino acid to be attached to the β-phenylserine moiety which, in turn as can be seen from a peak at *m/e* 224 (f), is joined by an ether linkage of the *p*-hydroxystyrylamine residue. Peaks at *m/e* 274 and 337 (h, e) demonstrate that the styrylamine is attached to the leucine isomer which must also be combined with the β-phenylserine fragment owing to the occurence of a fragment peak at *m/e* 216 (l). Hence, it follows that this alkaloid possesses a ring system derived from a leucine isomer, *p*-hydroxystyrylamine, β-phenylserine, and N,N-dimethylphenylalanine residue.

(30) Ion b in this case produces relatively abundant secondary fragments of mass number 432 (b − $HN(CH_3)_2$) and 404 (b − $HN(CH_3)_2$ − CO), a behavior which has been observed previously with two other peptide alkaloids, see ref 1d and 12.

Acidic hydrolysis of the dihydro derivative yielded isoleucine, tyramine, N,N-dimethylphenylalanine, phenylpyruvic acid (the desamination product of β-phenylserine), and a small amount of β-phenylserine. The structure of this alkaloid is, therefore, represented by formula VI, which is, indeed, identical with the known structure of adouétine-Y previously isolated from *Waltheria americana* by Goutarel and coworkers.[16,17] The physical properties are in good agreement with this conclusion.

Ceanothine-D. From the fourth alkaloid a mass spectrum was obtained that is very similar to the mass spectrum of ceanothine-C (III) discussed above. In addition to the molecular ion peak at *m/e* 470, peaks corresponding to the fragment ions a′, e, f, f − H, g, g − 2H, h, i, j, l, and m occur at the same mass numbers as in the mass spectrum of III, showing that the alkaloid must be an isomer of ceanothine-C.

A significant difference between the two spectra exists, however, in the upper mass range. In contrast to III, this alkaloid had at M − 43 a peak of only negligible size, but a very intense peak at M − 29 (by far the largest peak above mass number 200). According to high-resolution mass measurements, this fragment ion arose from the loss of an ethyl radical. In analogy to the formation of the M − 43 ion from III, the formation of this ion must be due to the α-cleavage[25] of the ether function to yield the oxonium ion $>CHC(CH_3){=}O^+$-C_6H_4−; this indicates that the R′ and R″ residues of the β-hydroxyamino acid (*cf.* Scheme I) must be C_2H_5 and CH_3, *i.e.*, a β-hydroxyisoleucine moiety is present.

This suggestion was confirmed by acidic hydrolysis of the dihydro derivative, after which traces of β-hydroxyisoleucine as well as 2-keto-3-methylpentanoic acid (the desamination product of β-hydroxyisoleucine) were detected. Besides these, tyramine, N-methylproline, and leucine were identified. According to these findings the alkaloid must have the structure given in formula VII and has been named ceanothine-D.

The nmr spectrum (in $CDCl_3$) of ceanothine-D supports the proposed structure. The five methyl absorptions show the anticipated chemical shifts and splitting patterns: the singlet of the N-methyl group is situated at δ 2.27 ppm, the two methyl groups of the leucine moiety give rise to a pair of doublets (J = 6.5 Hz) centered at 1.29 and 0.98 ppm,[31] the ethyl residue of the β-hydroxyisoleucine moiety produces a triplet at 0.87 ppm (J = 6.0 Hz), and the methyl group at the carbon atom adjacent to the ether oxygen gives a singlet at 1.27 ppm.

Ceanothine-E. The mass spectrum of the fifth alkaloid which we isolated from this shrub is reproduced in Figure 2. Apart from differences in peak intensities, it is largely identical with the mass spectrum of adouétine-Y (VI). The fragment ions, the assignments of which were confirmed by high-resolution mass measurements (see Table II), coincide with those discussed in the case of adouétine-Y, *i.e.*, the fragmentation reactions completely follow the scheme presented above with R′ being C_6H_5 and R″ being equal to H. Since the physical properties of the two compounds differ considerably from one another, this alkaloid must be an isomer of VI, the point of difference being the α-amino acid incorporated in the ring system.

(31) The nonequivalence of the methyl groups of the leucyl moiety is obvious; *cf.* R. H. Bible, "Interpretation of NMR Spectra... an Empirical Approach," Plenum Press, New York, N. Y., 1965, p 75.

This hypothesis was tested by acidic hydrolysis of the dihydro derivative. In the reaction mixture N,N-dimethylphenylalanine, tyramine, β-phenylnaphthalene (which is known to be generated by treatment of β-phenylserine with acid[9,32]), and *leucine* were identified. This alkaloid has therefore, the structure VIII, and has been named ceanothine-E.

Experimental Section

Melting points were determined on a Nalge-Axelrod hot stage. Optical rotations were obtained with a Rudolph photoelectric polarimeter, Model 200, or a Perkin-Elmer polarimeter, Model 141. Nuclear magnetic resonance spectra were recorded on a Varian Associates Model A-60 spectrometer. The mass spectra of compounds, V, VI, and VII were obtained with a Varian Associates M-66 cycloidal mass spectrometer with a probe temperature of approximately 200° (direct glass inlet) at 70 eV. The mass spectra of compounds, III, IV, and VIII were obtained with a double-focusing A.E.I. MS-9 mass spectrometer at an ion source temperature of approximately 200° (direct insertion technique) at 70 eV. An F & M Model 500 gas chromatograph fitted with a 12 ft × 0.25 in. column packed with 20% SE-52 silicone gum on 60/80 Gas-Chrom Z was used. Hydrogenations were conducted in a microhydrogenator manufactured by Hans Hösli, Bischofszell, Switzerland. High-resolution data of all appropriate fragements were in accordance with expectations.

Isolation of the Alkaloids. A. The mixture of alkaloids isolated from the dried, ground root bark of the shrub was chromatographed on preparative silica gel tlc plates (0.5 mm) using chloroform–methanol (97:3) as the solvent. A typical plate showed six major bands in addition to the origin and several smaller bands: the six major bands showed R_f values of 0.68, 0.63, 0.48, 0.44, 0.38, and 0.31. The respective fractions were removed from the adsorbent by extraction overnight in a Soxhlet extractor with methanol. The methanol was then evaporated to dryness, the residue extracted with a mixture of chloroform–methanol (3:1), the extract filtered, and the filtrate was evaporated to dryness. Each of the fractions was composed of more than one component but could not be well separated on silica gel plates. They were further purified by chromatography on neutral alumina plates which had been activated by heating under vacuum (1.0 mm) at 120°.[26] The development solvent was chloroform–acetone (85:15). Each band was scraped off the plate, the alkaloid was eluted with chloroform–methanol (3:1) and filtered, and the solvent was removed under vacuum. The resultant white-to-yellow solid was recrystallized from an appropriate solvent to yield in all cases white needles. In this way frangulanine (IV), adouétine-X (V), adouétine-Y (VI), and ceanothine-D (VII) were isolated; their physical properties are listed in Table I.

Table I. Physical Properties of the Peptide Alkaloids

Alkaloid	Solvent used for recrystallization	Mp, °C	$[\alpha]_D$ ($-CHCl_3$)	Mol wt (mass spectrum
Ceanothamine-A[a] (IV)	Chloroform	277–278	−293	500
Adouétine-X[b] (V)	Chloroform	278–280	−338	500
Adouétine-Y[c] (VI)	Chloroform–ether	287–289	−213	568
Ceanothine-D (VII)	Chloroform–ether	227–229	−347	470
Ceanothine-E (VIII)	CH_2Cl_2–ether	238–239	−285	568

[a] Data given[10] for "Frangulanine": mp 275–276°, $[\alpha]_D$ −288. Reference 6 gave mp 276–279°. [b] Lit. mp 277–279°,[16,17] 279–280°;[6] $[\alpha]_D$ −316,[16,17] −370[6] (ref 6 gave data for "ceanothamine-B"). [c] Lit.[16,17] mp 292°; $[\alpha]_D$ −230.

B. The mixture of alkaloids was repeatedly chromatographed on preparative silica gel plates using benzene–ethyl acetate–acetone (8:4:1) and benzene–acetonitrile (2:1) as solvents. In each case the fastest and the slowest moving band were eluted with methanol. In this way frangulanine (IV) and ceanothine-E (VIII) could be isolated. Both compounds were recrystallized from methylene chloride–ether. Their physical properties are given in Table I.

Hydrogenation. The conditions were the same for the hydrogenation of all peptide alkaloids. Approximately 30 mg (in the case of VIII 5 mg) of the alkaloid was added to 20 mg of 10% palladium–carbon in 2 ml of ethanol in a hydrogen atmosphere. After flushing with a stream of nitrogen, the catalyst was filtered and the filtrate evaporated to yield the dihydro derivative of the alkaloid. This was subjected to acidic hydrolysis without additional purification.

Hydrolysis. Total acidic hydrolysis of the dihydro alkaloids was conducted by heating them at 110° in a sealed tube with 6 *N* hydrochloric acid. After vacuum desiccation of the reaction mixture, it was analyzed by the following techniques.

Analysis of the Hydrolysis Mixture by Paper Chromatography. One portion of the reaction mixture was spotted on Whatman no. 1 chromatographic paper and developed with the upper phase of the mixture *n*-butyl alcohol–acetic acid–water (4:1:5)[33] in accordance with the classical procedure of Levy and Chung.[34] The component amino acids were detected with ninhydrin, the N-methylated amino amino acids with iodine or morin,[35] and tyramine with *p*-diazobenzenesulfonic acid.[36] In all cases, the identity of a component of the hydrolysis mixture was confirmed by comparison with a reference compound of known structure. The data obtained from hydrolysis of the dihydro alkaloids are contained in Table II.

Analysis of the Hydrolysis Mixture by Thin Layer Chromatography. In order to differentiate unambiguously between leucine and isoleucine and the corresponding N-methylated amino acids, the hydrolysis mixture was chromatographed on cellulose tlc plates using the solvent butanone–pyridine–acetic acid–water (70:15:15:2).[37] The observed R_f values were leucine 0.59, isoleucine 0.52, N,N-dimethylleucine 0.71, and N,N-dimethylisoleucine 0.63. The spots were detected with ninhydrin and iodine, respectively. If the hydrolysis mixture was chromatographed on silical gel plates with *n*-hexane as the solvent, the β-phenylnaphthalene (from decomposition of β-phenylserine[32]) was identified.

Analysis of the Hydrolysis Mixture by Gas Chromatography. Another part of the hydrolysis mixture was treated with a mixture of hexymethyldisilazane, trimethylchlorosilane, and pyridine (3:1:9 v/v),[38] bis(trimethylsilyl)acetamide,[39] or bis(trimethylsilyl)trifluoroacetamide ("Regisil").[40] After addition of the silylating reagent(s), the mixture was left to react overnight. Since TMS derivatives are very sensitive to moisture, they were prepared in rubber ("Viton") stoppered serum vials and the contents were removed by syringe. A sample of it was injected into the vpc apparatus and the column temperature was raised from 75 to 225° at the rate of 5.6°/min. Confirmation of the identity of a component was made at constant column temperature by comparison with a reference compound of known structure. The retention times of the various TMS derivatives can be found in Table II.

Analysis of the Hydrolysis Mixture with Mass Spectrometry. The hydrolysis mixture was introduced into the sample chamber and the temperature of the probe was slowly raised from about 75 to 200°; the mass spectrum slowly changed. At any one temperature, the spectrum recorded was that of a mixture.[8] By consideration of which ions enlarged as others decreased, it was possible to match the spectra with those of the reference compounds of known structure, since each sample had two, and usually three or four large, characteristic ions: for instance, *m/e* 86 for leucine and isoleucine in addition to *m/e* 44 for leucine and *m/e* 75 and 57 for isoleucine; *m/e* 74, 91 and 120 for phenylalanine; *m/e* 102 and 148 for N,N-dimethylphenylalamine; *m/e* 30, 77, 107, 108 for tyramine. Usually the hydrolysis was not complete, and the dihydroalkaloid minus the terminal could be detected by its large molecular ion.[1b,8] An example of this type of ion is *m/e* 395 for adouétine-Y and *m/e* 361 for frangolanine adouétine-X, and ceanothine-D. This technique

(32) F. Bettzieche, *Z. Physiol. Chem.*, **150**, 177 (1925).

(33) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(34) A. L. Levy and D. Chung, *Anal. Chem.*, **25**, 396 (1953).

(35) Reference 26, p 54.

(36) E. Stahl, "Dunnschichtchromatographie," Springer-Verlag, Berlin, 1962, p 501; prepared from L. Fieser, "Organic Experiments," 3rd ed, D. C. Heath, Boston, 1964, p 192.

(37) (a) P. Wollenweber, *J. Chromatogr.*, **9**, 369 (1962); (b) M. Justisz and P. de la Llosa, *Bull. Soc. Chim. Fr.*, 2913 (1963).

(38) *E.g.*, see C. C. Sweely, R. Bently, M. Makita, and W. W. Wells, *J. Amer. Chem. Soc.*, **85**, 2497 (1963).

(39) J. F. Klebe, H. Finkbeiner, and D. M. White, *ibid.*, **88**, 3390 (1966).

(40) "Regisil" is sold by Regis Chemical Co., Chicago, Ill.

Table II. Identification of Products from Acid Hydrolysis of the Dihydro Derivatives by Paper Chromatography and by Vapor Phase Chromatography (VPC) of the Trimethylsilyl (TMS) Derivatives

Compound	Paper chromatography R_f[a]	Method of detection[b]	Vpc of TMS derivatives, retention time, min[c] 125°	175°	Compound identified in the reaction obtained from the alkaloid IV	V	VI	VII	VIII
N-Methylproline	0.77	M, J	2.25					×	
N,N-Dimethylleucine	0.81	M, J	2.75			×			
N,N-Dimethylisoleucine	0.79	M, J	2.80		×				
N,N-Dimethylphenylalanine	0.81	M, J		3.12			×		×
Leucine	0.74	N	3.88		×			×	×
Isoleucine	0.70	N	4.38	1.00		×	×		
β-Hydroxyleucine	0.44	N	11.70	2.10	×	×			
β-Hydroxyisoleucine	0.41	N		2.42				×	
2-Keto-4-methylpentanoic acid	0.27	D	3.38		×	×			
2-Keto-3-methylpentanoic acid	0.25	D	3.42					×	
Glycine	0.17	N			×				
β-Phenylserine	0.51	N		5.88			×		×
Phenylpyruvic acid	0.34	D		4.90			×		
Tyramine	0.68	S		3.88	×	×	×	×	×

[a] Whatman no. 1 paper; solvent *n*-butyl alcohol–acetic acid–water (4:1:5). [b] The methods of detection are symbolized in the following way: N = ninhydrin, M = morin, D = 2,4-dinitrophenylhydrazine, S = *p*-diazobenzenesulfonic acid, J = iodine. [c] Flow rate: 91 ml/min; the temperatures are isothermal.

was only used to confirm the results of the previous two procedures, since it does not produce completely unambiguous results.

Reference Compounds. Leucine, isoleucine, β-phenylserine, 2-keto-4-methylpentanoicacid, and phenylpyruvic acid were commercial samples. N-Methylproline, N,N-dimethylleucine, N,N-dimethylisoleucine, and N,N-dimethylphenylalanine were prepared by reductive methylation using procedures outlined in the literature.[41,42] β-Hydroxyleucine was prepared by the procedure of Buston and Bishop.[43] β-Hydroxyisoleucine was prepared by the following reaction sequence. *sec*-Butyl methyl ketone was brominated to the α,α′-dibromo ketone.[44] By treatment with sodium hydroxide this dibromo ketone yielded 3-methylpent-2-enoic acid[45] which by action of aqueous bromine solution gave 2-bromo-3-hydroxy-3-methylpentanoic acid.[46] The bromine was exchanged for an amino group with aqueous ammonia to yield β-hydroxyisoleucine.[47]

2-Keto-3-methylpentanoic acid was prepared according to the procedure of Holden, *et al.*[47]

Acknowledgment. Dr. Harold Saltzman, Miss Danute Zukas, Mr. Michael Lukacovic, and Mr. Horst Rindermann have made valuable contributions to this work. The Bonn University group is indebted to the Stiftung Volkswagenwerk for having made possible the purchase of the MS-9 mass spectrometer and to the Deutsche Forschungsgemeinschaft for financial support.

(41) V. M. Ingram, *J. Biol. Chem.*, **202**, 193 (1953).
(42) R. E. Bowman and H. H. Stroud, *J. Chem. Soc.*, 1342 (1950).
(43) H. W. Buston and J. Bishop, *J. Biol. Chem.*, **215**, 217 (1955).
(44) R. B. Wagner and J. A. Moore, *J. Amer. Chem. Soc.*, 72, 974 (1950).
(45) C. Rappe and R. Adestrom, *Acta Chem. Scand.*, **19**, 273 (1965).
(46) H. E. Carter and C. L. Zirkle, *J. Biol. Chem.*, **178**, 710 (1949).
(47) J. T. Holden, R. B. Wildman, and E. E. Snell, *ibid.*, **191**, 560 (1951).