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Chemical Studies on the Mistletoe. II.[1] The Structure of Viscumamide, a New Cyclic Peptide Isolated from *Viscum album* Linn. *var. coloratum* Ohwi

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A new cyclic peptide, viscumamide ($C_{30}H_{55}N_5O_5$) was isolated from *Viscum album* Linn. *var. coloratum* Ohwi, and the structure Ia ($\overline{\text{→L-Leu→L-Ileu→L-Leu→L-Ileu→L-Leu}}$) was assigned to this substance from gas-chromatographic studies on the products of partial hydrolysis.

During our studies on the constituents of a mistletoe, *Viscum album* Linn. *var. coloratum* Ohwi, we isolated a new cyclic peptide and named it viscumamide.

The mistletoe which occurred on a hackberry (*Celtis sinensis* Pers. *var. japonica* Nakai) was collected and extracted with methanol in the usual way. The extract was fractionated into the neutral, basic, and acidic fractions. The neutral fraction was chromatographed on a column of activated alumina and gave a crystalline substance of mp 349.5—351 °C. This substance, viscumamide is optically active, $[\alpha]_D^{25}$ −49.1°. Elemental analyses and the mass spectrum (Fig. 1) show that viscumamide has a composition of $C_{30}H_{55}N_5O_5$ and a molecular weight of 565.8. The infrared spectrum of this substance (Fig. 2) shows absorption bands at

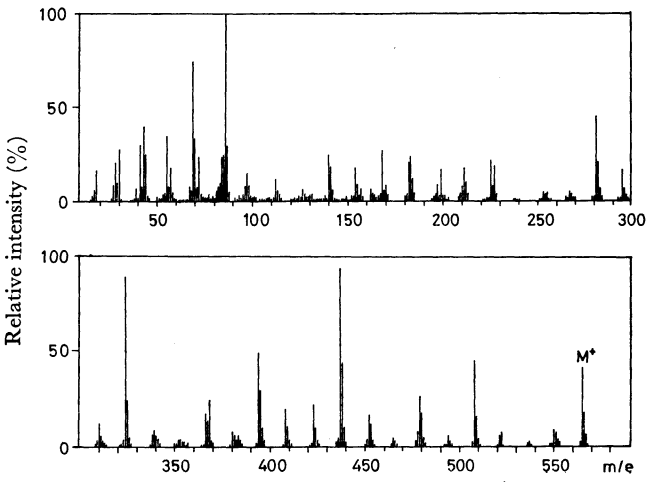


Fig. 1. Mass spectrum of viscumamide.

1) Part I: A. Sakurai and Y. Okumura, Reports of Faculty of Science, Shizuoka University, **6**, 63 (1971).

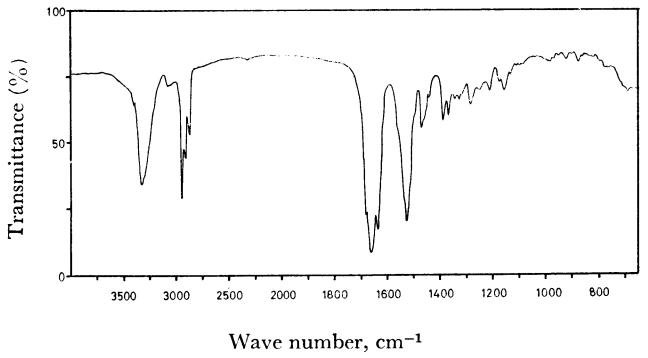
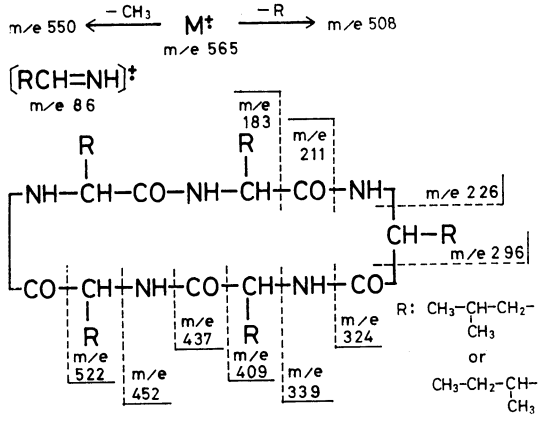


Fig. 2. Infrared spectrum of viscumamide.

3315, 3050, 1658, and 1530 cm^{-1} ascribed to secondary amide groups, and shows no absorption band indicative of the existence of a free amino group or a carboxyl group. The potentiometric titration of viscumamide in a dilute hydrochloric acid solution with potassium hydroxide shows that the substance has no dissociating group. And this substance shows a negative reaction with ninhydrin and is sparingly soluble in numerous solvents. These results suggest a cyclic structure linked by five peptide bonds for viscumamide.

Hydrolysis of viscumamide with 6M hydrochloric acid according to the method described by Harfenist[2] gave a mixture of leucine (60.6%) and isoleucine (39.4%). Gas-chromatographic analysis of the hydrolysate after esterification and treatment with trifluoroacetic anhydride[3] also showed to be a mixture of leucine and isoleucine, and showed the absence of alloisoleucine.



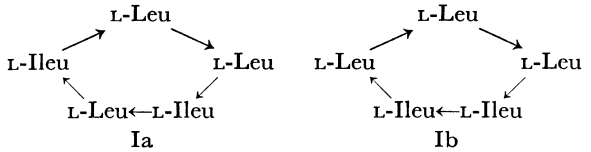
Scheme.

The mass spectrum of the homocyclic hexapeptide[4] has been investigated and shows that its fragmentation obeys the simple rules established with cyclodipeptides[5] as follows: the lower mass range is governed by the amine fragments ($RCH{=}\overset{+}{N}H_2$), while in the higher mass range expulsion of HNCO followed by several alternating losses of RCH=NH and carbon monoxide can be discerned. The mass spectrum of viscumamide shows fragmentations according to the fragmentation rules of these cyclopeptides. A primary process in the fragmentation of viscumamide is the loss of a HNCO fragment from $M^{\ddagger}$ (*m/e* 565) which gives a fragment ion peak at *m/e* 522. Then, the successive eliminations of RCH=NH (R=*i*-butyl or *s*-butyl) followed by loss of carbon monoxide occur, and intensive fragment ion peaks are formed at *m/e* 437, 409, 324, 296, 211, and 183, respectively. The amine fragments ($RCH{=}\overset{+}{N}H_2$) in the lower mass range appear at the same mass number, *m/e* 86, as the base peak. These results lead to a fragmentation sequence and a possible partial structure for viscumamide, which is shown in the scheme.

Table. Specific optical rotation of amino acids

Amino acid		$[\alpha]_D^{27}$	*c* in 20% HCl
Amino acids isolated from the hydrolysate of viscumamide	Leucine	+12.1°	0.40
	Isoleucine	+34.8°	0.39
Amino acids treated under the same conditions as the hydrolysis of viscuammide	L-Leucine	+13.2°	0.40
	L-Isoleucine	+33.8°	0.41

Specific optical rotation of leucine and isoleucine, which were isolated from the hydrolysate of viscumamide by chromatography on a column of Dowex 50×4, are summarized in the table and are in good agreement with the data of L-leucine and L-isoleucine, respectively, which were treated under the same conditions as the hydrolysis of viscumamide. These results indicate that this substance is constructed of three molecules of L-leucine and two molecules of L-isoleucine. And this leads to two possible structures, Ia and Ib for viscumamide.



Partial hydrolysis of viscumamide with alcoholic hydrochloric acid gave a mixture of five substances, which were analyzed by gas-chromatography after esterification and treatment with trifluoroacetic anhydride. The gas-chromatogram of this mixture showed five peaks.

The substances of the first and the second peaks in order of the retention time in the gas-chromatogram were identified as *n*-butyl *N*-trifluoroacetylisoleucinate and *n*-butyl *N*-trifluoroacetylleucinate, respectively. After being fractionated by gas-chromatography, the substances of the third, the fourth and the fifth peaks were hydrolyzed respectively with 6M hydrochloric acid. And each hydrolysate was analyzed by gas-chromatography in the form of *N*-trifluoroacetylated *n*-butyl ester. The gas-chromatograms of the hydrolysates showed that upon hydrolysis, both the substances of the third and the fourth peaks gave isoleucine and leucine in equal amounts, and that the substance of the fifth peak gave only leucine. By comparison with

2) E. J. Harfenist, *J. Amer. Chem. Soc.*, **75**, 5528 (1953).
3) G. W. Gehrke and W. M. Lamkin, *Chem. Eng. News*, **42**, 62 (1964).
4) K. Heyns and H.F. Grützmacher, *Ann. Chem.*, **669**, 189 (1963).
5) H. J. Svec and G. A. Junk, *J. Amer. Chem. Soc.*, **86**, 2278 (1964).

synthetic samples of dipeptide, the substances of the third, the fourth and the fifth peaks were identified as *n*-butyl *N*-trifluoroacetylisoleucylleucinate, *n*-butyl *N*-trifluoroacetylleucylisoleucinate and *n*-butyl *N*-trifluoroacetylleucylleucinate, respectively.

Thus, partial hydrolysis of viscumamide gave leucine, isoleucine, isoleucylleucine (II), leucylisoleucine (III), and leucylleucine (IV). The absence of isoleucylisoleucine (V) in the partial hydrolysate eliminates the validity of the structure Ib for viscumamide, and all experimental results described above support the structure of Ia for viscumamide.

Experimental

All melting points are uncorrected. Infrared spectrum was measured in KBr disk with a model EPI-G3 Hitachi spectrophotometer and gas-chromatogram was measured with a model K-53 Hitachi gas-chromatograph.

Extraction and Isolation of Viscumamide. The mistletoe (10 kg)[6] was extracted with methanol in the usual way, and the extract was concentrated under reduced pressure. The concentrate was dissolved in ethyl acetate and fractionated into the basic, acidic, and neutral fractions by shaking with dilute hydrochloric acid and then with sodium carbonate solution. The neutral fraction was concentrated to a syrup *in vacuo*. This syrup was digested with boiling *n*-hexane and the insoluble residue was collected by filtration. The faintly brown residue (18.2 g) was dissolved in hot methanol (100 ml), and activated alumina (50 g) was added to this solution and stirred. The suspension was evaporated to dryness *in vacuo*. The alumina which adsorbed the residue was placed on the top of a column of activated alumina (450 g), and the column was eluted with solvents. Elution with a mixture of ethyl acetate and ethanol and recrystallization from ethanol gave colorless needles, mp 349.5—351 °C (158 mg, 0.0158% from wet plant). $[\alpha]_D^{25}$ −49.1° (*c* 0.199, ethanol).

Found: C, 63.52; H, 10.07; N, 12.55%. Calcd for $C_{30}H_{55}N_5O_5$: C, 63.68; H, 9.80; N, 12.38%.

Potentiometric Titration of Viscumamide. Viscumamide (5.6 mg) in 0.01M hydrochloric acid (20 ml) was titrated with 0.1M sodium hydroxide using a Hiranuma Rat-1(P) autotitrator. The titration curve was identical with that of blank titration. This results showed that the substance has no dissociating group.

Hydrolysis of Viscumamide. Viscumamide (1.51 mg) in 6M hydrochloric acid (2 ml) was heated in a sealed tube at 110 °C for 120 hr according to the method described by Harfenist.[2] The reaction mixture was evaporated to dryness *in vacuo* and was dissolved in water. This solution was analyzed with a model KLA-3B Hitachi amino acid analyzer and showed to be a mixture of 0.91 mg of leucine (60.6%) and 0.59 mg of isoleucine (39.4%) at the recovery rate of 86.3%.

Separation of Leucine and Isoleucine from the Hydrolysate of Viscumamide. Viscumamide (100 mg) in 6M hydrochloric acid (20 ml) was hydrolyzed in the same manner as described above. The reaction mixture evaporated to dryness *in vacuo*, was dissolved in water (5 ml) and was submitted to chromatography on a column of Dowex 50 X 4 (150×2 cm, H^+ form, 200—400 mesh; developer: hydrochloric acid, pH value being changed continuously from 1M hydrochloric acid (1 l) to 12M hydrochloric acid (1 l). The fractions containing a mixture of leucine and isoleucine were evaporated to dryness *in vacuo*, and the residue of the mixture was chromatographed again in the same manner. The respective fractions containing pure leucine and isoleucine were evaporated to dryness *in vacuo*, and dissolved in ethanol (2 ml) and treated with ethyl acetate. The solutions were allowed to stand at 0 °C. Leucine hydrochloride (15 mg) and isoleucine hydrochloride (13 mg) were obtained as colorless powder.

Specific rotations of these amino acid hydrochlorides were measured in 20% hydrochloric acid, and the data were summarized in the table.

Preparation of Amino Acids for the Measurement of Specific Rotation. L-Leucine and L-isoleucine were respectively treated with 6M hydrochloric acid in the same manner as described above. The solutions were evaporated to dryness *in vacuo*. The residue was dissolved in ethanol and treated with ethyl acetate. The specific rotations of the resulted L-leucine hydrochloride and L-isoleucine hydrochloride were measured and the data were summarized in the table.

Preparation of L-Isoleucyl-L-Leucine (II). To a solution containing carbobenzoxy-L-isoleucine[7] (1.6 g) in dry chloroform (20 ml), dry triethylamine (0.6 g) was added at 0 °C and ethyl chloroformate (0.65 g) was added to this solution. After 30 min methyl L-leucinate dissolved in chloroform (10 ml) was added in about 20% excess of the calculated amount. The solution was allowed to stand at room temperature for 1 hr and then washed successively with 1M hydrochloric acid, sodium bicarbonate, and water. The solution was dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was dissolved in ether (10 ml) and treated with petroleum ether (15 ml, bp 50—80 °C). After being chilled, methyl carbobenzoxy-L-isoleucyl-L-leucinate (1.9 g) was obtained as colorless needles; 83% of the theoretical yield and mp 121.5—122 °C.

The above-described ester (1.5 g) in acetone (15 ml) was saponified by treating with 1M sodium hydroxide (4.2 ml) at room temperature for 45 min. The solution was acidified until acid to Congo red with concentrated hydrochloric acid and was concentrated *in vacuo*. Then the product was extracted with ethyl acetate and the extract was dried over sodium sulfate. By adding petroleum ether, carbobenzoxy-L-isoleucyl-L-leucine (1.2 g) was obtained as colorless crystals; 85% of the theoretical yield and mp 130—131 °C (lit,[8] mp 130—131 °C).

The carbobenzoxydipeptide (500 mg) was hydrogenated in methanol (6 ml) containing water (0.30 ml) and glacial acetic acid (0.30 ml) in the presence of palladium black. The catalyst was removed by filtration and the filtrate was concentrated *in vacuo* with repeated additional methanol. Ether was added to complete the crystallization. After recrystallization from methanol–water, L-isoleucyl-L-leucine (243 mg)was obtained as colorless needles; 71% of the theoretical yield and $[\alpha]_D^{18}$ −11° (*c* 1, 1M sodium hydroxide).

Found: C, 58.71; H, 10.02; N, 11.36%. Calcd for $C_{12}H_{24}N_2O_3$: C, 58.99; H, 9.90; N, 11.47%.

Preparation of L-Leucyl-L-isoleucine (III). L-Leucyl-L-isoleucine was prepared in 58% yield in the same manner as described above *via* methyl carbobenzoxy-L-leucyl-L-isoleucinate, colorless needles, mp 64.5—65.5 °C (lit,[9] mp 64—65 °C) and carbobenzoxyl-L-leucyl-L-isoleucine, colorless crystals, mp 99—101 °C (lit,[10] mp 101—101.5 °C) from carbo-

6) The mistletoe was collected at Futamata of Tenryu City in the winter of 1969.

7) H. E. Carter, R. L. Frank, and H. W. Johnston, "Organic Synthesis," Coll. Vol., 3, p. 167 (1955).

8) D. Theodoropoulos and L. C. Craig, *J. Org. Chem.*, **20**, 1169 (1955).

9) K. Isono and R. W. Curtis, *Phytochemistry*, **3**, 276 (1964).

10) E. Smith, D. H. Spackman, and W. J. Polglase, *J. Biol. Chem.*, **199**, 803 (1952).

benzoxy-L-leucine[6] (1.6 g) and methyl L-isoleucinate (1.0 g); colorless needles, $[\alpha]_D^{20}$ +19° (*c* 1, water) (lit,[10] $[\alpha]_D^{23}$ +20.9° (*c* 1, water)).

Found: C, 54.49; H, 9.78; N, 10.48%. Calcd for $C_{12}H_{24}N_2O_3 \cdot H_2O$: C, 54.94; H, 9.99; N, 10.68%.

Preparation of L-*Leucyl*-L-*leucine (IV).* L-Leucyl-L-leucine was prepared in 65% yield in the same manner as described above *via* methyl carbobenzoxy-L-leucyl-L-leucinate, colorless needles, mp 98.5—99 °C (lit,[10] mp 97.5—98.5 °C) and carbobenzoxy-L-leucyl-L-leucine, colorless crystals, mp 98.5—100 °C (lit,[10] mp 98—101 °C) from carbobenzoxy-L-leucine[7] (1.6 g) and methyl L-leucinate (1.0 g); colroless needles, $[\alpha]_D^{20}$ −13.6° (*c* 5, 1M sodium hydroxide)).

Found: C, 55.11; H, 9.49; N, 10.69%. Calcd for $C_{12}H_{24}N_2O_3 \cdot H_2O$: C, 54.94; H, 9.99; N, 10.68%.

Preparation of L-*Isoleucyl*-L-*isoleucine (V).* L-Isoleucyl-L-isoleucine was prepared in 63% yield in the same manner as described above *via* methyl carbobenzoxy-L-isoleucyl-L-isoleucinate, colorless needles, mp 132—132.5 °C and carbobenzoxy-L-isoleucyl-L-isoleucine, colorless crystals, mp 120.5—122°C from carbobenzoxy-L-isoleucine[7] (1.6 g) and methyl L-isoleucinate (1.0 g); colorless needles, $[\alpha]_D^{20}$ +7° (*c* 0.1, water).

Found: C, 55.05; H, 9.79; N, 10.79%. Calcd for $C_{12}H_{24}N_2O_3 \cdot H_2O$: C, 54.94; H, 9.99; N, 10.68%.

Preparation of N-*Trifluoroacetylated Amino Acid* n-*Butyl Esters and* N-*Trifluoroacetylated Dipeptide* n-*Butyl Esters.* These substances were prepared by a modification of the procedure described by Gehrke and Lamkin.[3] One mg of amino acid or dipeptide was treated with methanol (1 ml) saturated with hydrogen chloride at room temperature for 4 hr. The reaction mixture was evaporated to dryness *in vacuo*, and the residue was dissolved in *n*-butanol (1 ml) saturated with hydrogen chloride. And then the mixture was heated in a sealed tube at 120 °C for 2 hr. After the solvent was removed *in vacuo*, the resulted *n*-butyl ester was treated with trifluoroacetic anhydride (1 ml) in dichloromethane (1 ml) at room temperature for 15 min. After the solvent was removed *in vacuo*, the residue was dissolved in dichloromethane and was submitted to gas-chromatography.

Gas-Chromatogram of the Hydrolysate of Viscumamide. The hydrolysate of viscumamide was esterified and was treated with trifluoroacetic anhydride in the same manner as described above. The resulted *N*-trifluoroacetylated amino acid *n*-butyl esters showed the identity with authentic *n*-butyl *N*-trifluoroacetylleucinate and *n*-butyl *N*-trifluoroacetylisoleucinate, but not with authentic *n*-butyl *N*-trifluoroacetylalloisoleucinate in gas-chromatography (column: 3 mm×4 m stainless steel column, stationary phase; 20% diethylene glycol adipate on Celite 545, column bath temperature; 175 °C, carrier gas; He, 20 ml/min). The retention times of *n*-butyl *N*-trifluoroacetylleucinate, *n*-butyl *N*-trifluoroacetylisoleucinate and *n*-butyl *N*-trifluoroacetyl-alloisoleucinate were 31.6, 25.6, and 24.2 min, respectively.

Partial Hydrolysis of Viscumamide. Viscumamide (10 mg) in a mixture of 6M hydrochloric acid (10 ml) and ethanol (10 ml) was refluxed for 4 hr. After being evaporated to dryness *in vacuo*, the hydrolysate was esterified and treated with trifluoroacetic anhydride in the same manner as described above. The resulted *N*-trifluoroacetylated amino acid *n*-butyl esters and *N*-trifluoroacetylated dipeptide *n*-butyl esters were submitted to gas-chromatography (column; 3 mm×2 m stainless steel column, stationary phase; 20% diethylene glycol adipate on Celite 545, column bath temperature; 200 °C, carrier gas; He, 40 ml/min) and fractionated. The chromatogram of this mixture showed five peaks and the retention times of these peaks were 1.0, 1.2, 20.4, 22.1, 23.5 min. The fractions of the first and the second peaks in order of the retention time showed the identity with authentic *n*-butyl *N*-trifluoroacetylisoleucinate and *n*-butyl *N*-trifluoroacetylleucinate, respectively. The fraction of the third peak was hydrolyzed with 6M hydrochloric acid, and then was esterified and treated with trifluoroacetic anhydride in the same manner as described above. The reaction mixture showed the identity with a mixture (1 : 0.99) of authentic *n*-butyl *N*-trifluoroacetylisoleucinate and *n*-butyl *N*-trifluoroacetylleucinate in gas-chromatography. The fraction of the fourth peak was treated in the same manner as the fraction of the third peak and showed the identity with a mixture (0.96 : 1) of authentic *n*-butyl *N*-trifluoroacetylisoleucinate and *n*-butyl *N*-trifluoroacetylleucinate. The fraction of the fifth peak was treated in the same manner and showed the identity with only authentic *n*-butyl *N*-trifluoroacetylleucinate. And the fractions of the third, the fourth and the fifth peaks showed to be *n*-butyl *N*-trifluoroacetylisoleucylleucinate, *n*-butyl *N*-trifluoroacetylleucylisoleucinate and *n*-butyl *N*-trifluoroacetylleucylleucinate, respectively, by gas-chromatographic comparison with the synthetic samples.

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