Structure of Rhodotorucine *A*, a Peptidyl Factor, Inducing Mating Tube Formation in *Rhodospiridium toruloides*

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Rhodotorucine *A*, which induces mating tube formation in *Rhodospiridium toruloides*, was found to be a novel peptide composed of eleven amino acid residues with a lipophilic group at the C-terminus. The amino acid sequence of the peptide was determined by Edman degradation and enzymatic hydrolyses. The C-terminus of rhodotorucine *A* was revealed to be S-farnesylcysteine by proton magnetic resonance, mass spectrometry and chemical synthesis. We proposed the following structure for rhodotorucine *A*.

H-Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg-Asn-Gly-Cys(S-farnesyl)-OH.

In the sexual process of *Rhodospiridium toruloides*, two mating type cells designated as type *A* and *a* conjugate to form filamentous dikaryon mycelium.¹⁾ At the initial stage of this process, *A* type cells secrete a peptidyl factor, which induces the mating tube formation of *a* type cells.²⁾ Recently, we have succeeded in the isolation^{3,4)} of the factor named as rhodotorucine *A*, and reported its structure preliminarily.^{5,6)} In this paper, we wish to describe the structural determination of rhodotorucine *A* in detail.

Based on the amino acid analysis of the acid hydrolyzate (Table I), the composition of ten amino acid residues in rhodotorucine *A* was established as Asp 1, Thr 1, Ser 1, Glu 1, Pro 1, Gly 1, Ile 1, Tyr 1, Trp 1 and Arg 1. The UV spectrum (Fig. 1) also indicated that the peptide contained one residue each of tryptophan and tyrosine. Besides these amino acids, about 0.05 molar ratio of cystine and trace amount of cysteic acid were observed in the amino acid analysis. Treatment of rhodotorucine *A* with performic acid followed by acid hydrolysis did not recover one molar ratio of cysteic acid. This suggested the presence of an unknown amino acid which was decomposed on acid hydrolysis to give a trace of cystine and cysteic acid.

N-Terminal amino acid was determined by dansyl (DNS) method with some modifications.⁷⁾ After hydrolysis of dansylated peptide with 5.7 N HCl, DNS-amino acid was detected by a thin-layer chromatography (TLC) on polyamide sheets. As O, N-di-DNS-Tyr, N-DNS-Tyr and O-DNS-Tyr were identified on TLC, N-terminus of rhodotorucine *A* was determined to be tyrosine.

Then, we applied Edman dansyl procedure for the sequence analysis. Edman degradation was performed according to the procedure described by Hartley⁸⁾ by use of 40 μ g of the peptide with the scaling down method.⁷⁾ At the fifth step of degradation, no DNS-amino acid was detected. This suggested the occurrence of tryptophan in this position, which was further confirmed by enzymatic hydrolyses as described later. Thus the DNS-amino acids up to the eighth step were identified as shown in Table II.

In order to differentiate glutamic acid and glutamine or aspartic acid and asparagine, direct Edman degradation was carried out by use of 200 μ g of rhodotorucine *A*. The phenylthiohydantoin (PTH) amino acid was identified on polyamide TLC with a fluorescence indicator.⁹⁾ At the third step of degradation, only PTH-Glu was detected and there was no

FIG. 2. Chromatography of Peptide Fragments on Sephadex G-25 Column (1.0 × 68 cm).

Fraction size, 1.85 ml; flow rate, 8.1 ml/hr; , peptide fragments. A) Hydrolyzate of Tr-1 with α -chymotrypsin. B) Hydrolyzate of CT-1 with thermolysin.

Tr-2 gave colorless crystals when recrystallized from aqueous methanol. Hydrolysis of Tr-2 with aminopeptidase M liberated asparagine, glycine and an unknown amino acid. On acid hydrolysis with 5.7 N HCl, Tr-2 gave aspartic acid, glycine and small amounts of cystine and cysteic acid. As Tr-2 was insoluble in water and easily extracted with *n*-butanol, the unknown amino acid would contain a lipophilic moiety and a cysteine residue.

The structure of the lipophilic amino acid was disclosed by Fourier transfer proton magnetic resonance (FT-PMR) and mass spectrometric analyses of Tr-2. As shown

in Fig. 3, the FT-PMR spectrum of Tr-2 revealed the signals characteristic of a farnesyl moiety; four vinyl methyl protons at δ 1.60 (6H) and 1.66 (6H), allyl methylene protons at δ 2.0 (8H) and vinyl methine protons at δ 5.0~5.3 (3H). Moreover, signals at δ 2.6~3.5 attributable to methylene protons of asparagine and cysteine and to S-allyl methylene protons (S-CH₂-CH=CH-) were observed together with solvent signals. This suggested that cysteine and farnesyl moieties constitute the unknown amino acid.

Tr-2 was acetylated with a mixture of acetic anhydride and acetic anhydride d₆ (1:1) in methanol. In this condition carboxyl group was esterified with methanol. The chemical ionization (CI) mass spectrum of the product is shown in Fig. 4. Singlet fragment ion peaks are easily distinguishable from doublet ion peaks containing acetylated N-terminus.¹⁰⁾ Among the former, the peaks at *m/e* 69, 137, 205 and 239 are considered to have originated from the S-farnesyl moiety. Strong doublet ion peaks *m/e* 157 (160) and 214 (217) are unambiguously identified as [Ac-Asn]⁺ and [Ac-Asn-Gly]⁺. Other doublet ion peaks larger than *m/e* 214 (217) must contain the fragments of the unknown amino acid. Significant doublet ion peaks *m/e* 332 (335) and 536 (539) are explained as [Ac-Asn-Gly-Cys S-)-OMe]⁺H-NH₃ and [Ac-Asn-Gly-Cys (S-farnesyl)-OMe]⁺H-NH₃ respectively. The high resolution electron impact ionization (EI) mass spectrum of acetylated Tr-2 methyl ester did not give the parent ion, but it revealed the peaks with the following element compositions; 69 (C₆H₉), 137 (C₁₀H₁₇), 204 (C₁₅H₂₄), 237 (C₁₅H₂₆S₁), 157 (C₆H₉N₂O₃), 214 (C₈H₁₂N₂O₃) and 332 (C₁₂H₁₈N₃O₆S₁). These data strongly support the assumption of the ion structures in the CI mass spectrum. Thus, the structure of H-Asn-Gly-Cys(S-farnesyl)-OH was proposed for Tr-2.

TABLE III. ENZYMATIC HYDROLYSES OF RHODOTORUCINE A

Peptide fragment	Th-1	Th-3	CT-2	Tr-2
N-Terminal amino acid	Tyr	Ile	Thr	Asn
Amino acid sequence	Tyr-Pro-Glu	Ile-Ser-Trp	Thr-Arg	Asn-Gly-X

FIG. 3. FT-PMR Spectrum of Tr-2 in CDCl_3 : CD_3OD : D_2O (1: 1: 1), 100 MHz, Pulse, 42 μsec ; Interval, 5 sec; Repetition, 46 Times.

FIG. 4. CI-mass Spectrum of Acetylated Tr-2 Methyl ester.
Sample temp., 150°C; reactant gas, isobutane (99.99%); ion accelerating voltage, 100 eV.

FIG. 5. PMR Spectrum of *S-trans-trans*-Farnesylcysteine in CDCl_3 : CD_3OD : D_2O (1: 1: 1).

For further confirmation, synthesis of Tr-2 using L-amino acids was performed as follows.* Asn-Gly-Cys (SH)-OH was treated with farnesyl bromide in a mixture of methanol-*n*-butanol-water (1:1:1) containing an excess amount of sodium bicarbonate. The reaction mixture was purified on Sephadex LH-20 column with methanol to give H-Asn-Gly-Cys(S-farnesyl)-OH, though the yield was poor. *R_f* values of the synthetic peptide on silica gel TLC in several solvent systems were identical with those of natural Tr-2. The mass spectrum of Ac-Asn-Gly-Cys (S-farnesyl)-OMe was also identical with that of natural acetylated Tr-2 methyl ester. Therefore, the structure of Tr-2 was confirmed as H-Asn-Gly-Cys (S-farnesyl)-OH.

The stereochemistry of the farnesyl moiety was predicted to be all *trans* based on the chemical shifts of methyl protons of Tr-2 in CDCl₃: CD₃OD: D₂O (1:1:1) as compared with *trans-trans*-farnesol.¹¹⁾ To confirm this, *S-trans-trans*-farnesylcysteine was synthesized from cysteine and *trans-trans*-farnesylbromide. As shown in Fig. 5, the chemical shifts of methyl protons of *S-trans-trans*-farnesylcysteine were identical with those of natural Tr-2. Thus, the stereochemistry of farnesyl moiety was decided to be all *trans*.

Consequently, we proposed the structure (I) for rhodotorucine A

Recently, we have succeeded in the synthesis of rhodotorucine A with solution method using L-amino acids, and the synthetic peptide showed the same biological activity as natural rhodotorucine A. This confirmed the correctness of the structure of rhodotorucine A. Details of the synthesis will be reported elsewhere.¹²⁾

As for the sex hormone of yeasts, an oligo-

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peptide named α substance-I_A or α factor has been isolated as the regulator of the mating reaction in *Saccharomyces cerevisiae*.^{13, 15)} The peptide is composed of amino acids commonly found in proteins. On the other hand, rhodotorucine A is a lipophilic peptide with a novel amino acid, S-farnesylcysteine, at the C-terminus. As far as we know, this type of lipophilic peptide is the first isolated from nature as a regulatory substance. The synthetic peptide which had no alkyl side chain at the C-terminus did not show the biological activity even at a high concentration.¹²⁾ This indicated that the farnesyl moiety in rhodotorucine A is indispensable for inducing the mating tube formation of *a* type cells.

Recently, Sakagami *et al.* isolated a conjugation tube inducing hormone named tremmerogen A-10 in *Tremella mesenterica* Fr. They also predicted the presence of S-alkylated cysteine at the C-terminus, though the whole structure is not yet clarified.¹⁶⁾

Rhodotorucine A did not induce the mating tube formation of *T. mesenterica* Fr., and tremmerogen A-10 also did not induce the mating tube formation of *R. toruloides*. This suggested the peptide sequence was also important to show biological activity.¹⁷⁾

Biological study of the mating reaction in *R. toruloides*^{18~20)} and the syntheses of the analo-

gues of rhodotorucine A are in progress.

EXPERIMENTAL

Amino acid analyses were run on a Hitachi KLA-3B or a JEOL JLC-6AH amino acid analyser. The PMR spectra were measured with a JEOL FX-100 or a PMX-60 spectrometer with a tetramethylsilane as an internal standard. The CI and EI mass spectra were recorded on a Hitachi RMU-6MG mass spectrometer with a direct inlet system. The high resolution EI mass spectrum was recorded with a Hitachi RMU-7M mass spectrometer. The UV spectrum was obtained

with a Hitachi 124 spectrophotometer and UV absorptions were monitored with a ISCO UV-5 monitor. The specific rotation was measured with a JASCO DIP-180 automatic polarimeter using 10 cm cell. Melting points were measured on a microscope hot plate and were uncorrected.

Amino acid analyses

Rhodotorucine *A* or each peptide fragment (50~200 μ g) was hydrolyzed with freshly distilled 0.2 ml of 5.7 *N* HCl (constant boiling) in a small evacuated tube at 105°C for 36 hr. When the peptide contained tryptophan, 2% thioglycolic acid was added to HCl and hydrolyzed in the same way. After reaction, HCl was evaporated over KOH in a vacuum desiccator and the resulting residue was subjected to the amino acid analysis.

Performic acid oxidation

A performic acid solution was prepared from 9 ml of formic acid and 1 ml of 30% H_2O_2 at 0°C for 1 hr. Rhodotorucine *A* (200 μ g) was dissolved in 200 μ l of the performic acid solution and kept at 0°C for 4 hr. Then 30 μ l of 48% HBr was added to the solution and evaporated *in vacuo* at 40°C. The residue was dissolved in 100 μ l of water, lyophilized and hydrolyzed with 5.7 *N* HCl in the same conditions as described in amino acid analyses.

Dansyl Edman degradations

N-Terminal amino acid was determined by dansyl (DNS) method with some modifications.⁷⁾ The peptide (2 μ g) was treated with 2 μ l of 0.2 *M* NaHCO_3 and 2 μ l of 0.3% DNS-chloride in acetone at 45°C for 30 min in a micro test tube. The product was hydrolyzed with 5.7 *N* HCl at 100°C for 16 hr.

Edman degradation was carried out by use of 40 μ g of the peptide. A sample in a small stoppered tube was dissolved in 20 μ l of pyridine-water (1:1). One μ l of phenylisothiocyanate was added to the solution and kept at 45°C for 90 min under N_2 gas with occasional shaking. After lyophilization of the reactant, 20 μ l of trifluoroacetic acid (TFA) was added to the residue and the solution was kept at 45°C for 30 min under N_2 gas. The TFA was evaporated under N_2 stream, and 200 μ l of water was added to the residue. The water solution was shaken twice with 100 μ l of *n*-butyl acetate. A small portion (10~20 μ l) was taken from the aqueous residue for the N-terminus determination and the remainder was lyophilized for further degradation.

The DNS-amino acid obtained at acid hydrolysis was identified by TLC on polyamide sheets (5 $\times$ 5 cm, Cheng Ching Trading Co.) with two-dimensional development by the following solvent systems; A) H_2O -28% $\text{NH}_3(\text{H}_2\text{O})$ (100:1) for the first direction and *n*-

heptane-*n*-BuOH-HCOOH (10:10:1) for the second direction; B) H_2O -HCOOH (200:3) for the first direction, benzene-AcOH (9:1) for the second direction and AcOEt-MeOH-AcOH (20:1:1) for the third run in the same direction as the second. The solvent system B was found suitable for the mutual separation of DNS-Glu and DNS-Asp.⁷⁾

Direct Edman degradations

Direct Edman degradation was performed by use of 200 μ g of rhodotorucine *A* as described by Sauer *et al.*²⁰⁾ Phenylthiohydantoin amino acids were detected by TLC on polyamide sheets (5 $\times$ 5 cm) with two-dimensional development by use of toluene-*n*-hexane-AcOH (60:30:16) containing butyl PBD [2-(4-*t*-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazol] as a fluorescence indicator and H_2O -AcOH (3:1).⁹⁾

Enzymatic hydrolyses

Rhodotorucine *A* (6 mg) was dispersed in 20 ml of 0.1 *M* ammonium acetate buffer pH 7.0 and digested with 120 μ g of TPCK trypsin (EC 3.4.21.4, Worthington Biochemical Co.) for 20 hr at 30°C. The reaction mixture was extracted with 10 ml of *n*-BuOH three times and combined BuOH layers were washed twice with 10 ml of water. The aqueous layer gave octapeptide Tr-1 and the butanol layer gave crude Tr-2.

Tr-1 (3.0 mg) was dissolved in 3.0 ml of 0.1 *M* ammonium acetate buffer pH 7.0 and digested with 30 μ g of α -chymotrypsin (EC 3.4.21.1, Miles Research Products Co.) for 18 hr at 30°C. The reaction mixture was concentrated and subjected to a Sephadex G-25 (Super fine, Pharmacia) column chromatography (1.0 $\times$ 68 cm) eluted with 0.1 *M* AcOH. Fractions 24~28 gave CT-2 (0.4 mg) and fractions 36~38 gave CT-1 (2.3 mg).

CT-1 (1.5 mg) was dissolved in 1.5 ml of 0.1 *M* ammonium acetate buffer pH 7.0 containing 5 mM of CaCl_2 and digested with 30 μ g of thermolysin (EC 3.4.24.4, Daiwakasei Co. Tokyo) for 12 hr at 40°C. The reaction mixture was again purified with the same Sephadex G-25 column eluted with 0.1 *M* AcOH. Fractions 23~27 gave Th-1 (0.5 mg) and fractions 31~35 gave Th-3 (0.5 mg).

Each peptide fragment (10~20 μ g) was digested with aminopeptidase M (EC 3.4.11.2, Protein Research Foundation, Osaka) at 30°C in ammonium acetate buffer pH 7.0, enzyme: substrate=1:20 (wt/wt). Amino acids were identified with DNS method described before.

Tr-2

Crude Tr-2 was recrystallized from aqueous methanol (fine needles, 1.29 mg). *Rf* values on silica gel TLC; solvent system A) *n*-BuOH-AcOH- H_2O (4:1:5, upper layer) 0.47, B) methylethylketone-

pyridine-AcOH-H₂O (70:15:2:15) 0.53. Tr-2 was insoluble in water and soluble in pyridine and a mixture of CHCl₃:MeOH:H₂O (1:1:1). The PMR spectrum is shown in Fig. 3.

Tr-2 (10~20 µg) was acetylated with a mixture of acetic anhydride and acetic anhydride d₆ (1:1) in 10% methanol solution (30 µl) for 2 hr at room temp. In this condition the carboxyl group was easily methylated. After evaporation of excess reagent, the residue was subjected to EI and CI mass spectrometric analyses, sample temp.; 120~180°C, ion accelerating voltage; 20 eV and 70 eV (EI), 100 eV (CI), reactant gas; isobutane, 99.99%. Figure 3 shows a CI mass spectrum of acetylated Tr-2 methyl ester. High resolution EI mass spectrum (70 eV, 140°C) of this sample revealed peaks [*m/e* observed and error (mMU)] 69.0687 (−1.6) C₅H₉, 137.1326 (−0.3) C₁₀H₁₇, 157.0586 (−2.5) C₈H₉ N₂O₈, 204.1874 (−0.2) C₁₈H₂₄, 214.0803 (−2.3) C₈H₁₂ N₃O₄, 237.1695 (2.0) C₁₅H₂₅S₁, 332.0967 (5.2) C₁₂H₁₅ N₃O₈S₁.

Synthesis of *S-trans-trans*-farnesylcysteine

trans-trans-Farnesol was prepared from *trans*-geranyl acetone²²⁾ and purified by Lobar silica gel column (3.7×44 cm, Merck) with solvent system *n*-hexane-AcOEt (4:1) to remove slight contamination of *trans-cis* isomer. This was converted to a bromide by treatment with *N*-bromosuccinimide and methylsulfide according to the procedure described by Corey.²⁸⁾

Cysteine (150 mg) and the bromide (200 mg) were dissolved in 20 ml of *n*-BuOH-MeOH-H₂O (1:1:1). Guanidine carbonate (150 mg) was added to the solution which was stirred at room temp. for 18 hr. After addition of 20 ml of water to this solution, the reaction mixture was extracted three times with each 10 ml of *n*-BuOH. Evaporation of the solvent gave crude *S*-farnesylcysteine which was recrystallized from aqueous methanol (100 mg), [α]_D²⁰ −30.2 (*c*=0.01) mp 148~150°C (gradually decomp.). The PMR spectrum is shown in Fig. 5. *R_f* values on silica gel TLC; solvent system A) 0.68, C) BuOH-AcOH-pyridine-H₂O (15:3:10:12) 0.83, CI-MS (N-Ac, OMe) *m/e*: 382 (M⁺H), 350, 205, 178, 137 and 69.

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