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STRUCTURE OF RHODOTORUCINE A, A NOVEL LIPOPEPTIDE, INDUCING MATING TUBE FORMATION IN RHODOSPORIDIUM TORULOIDES

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SUMMARY Rhodotorucine A is a peptidyl factor which induces mating tube formation in Rhodosporidium toruloides. The amino acid sequence of the factor was determined by Edman degradation and enzymatic hydrolysis. Rhodotorucine A was shown to contain a lipophilic amino acid, S-farnesyl cysteine, at C-terminus 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 yeast, Rhodosporidium toruloides, the mating reaction between two haploid cells designated A and a type (1) is controlled by pheromone-like substances secreted from each mating type cell (2). At the initial step of the mating reaction, A type cells secrete a factor which induces mating tube formation in the opposite a type cells (3). Recently, we succeeded in the isolation of the factor from the culture filtrate of A type cells (4-5) and proposed the partial structure of the principle (6). The factor, named rhodotorucine A, showed biological activity at a concentration of 8 ng/ml. In this paper, we wish to report the complete structure of rhodotorucine A, which contains a novel amino acid, S-farnesyl cysteine, at the C-terminus.

MATERIALS AND METHODS

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Rhodotorucine A Pure rhodotorucine A was obtained from the culture filtrate of yeast strain IFO M919 (A type cells) as described before (4). TLC* of the sample on silica gel plates (Merck GF 254 0.25 mm in thickness) using four solvent systems revealed a single spot, which was positive to ninhydrin, chlorine-o-tolidine-KI, Ehrlich's and Sakaguchi's reagents. Solvent systems; (A) n-BuOH-AcOH-water (4:1:5, upper phase), Rf 0.29, (B) n-BuOH-n-PrOH-0.2 M AcOH (2:1:3, upper phase), Rf 0.37, (C) n-BuOH-pyridine-water-AcOH (15:10:12:3), Rf 0.75 and (D) sec-BuOH saturated with water, Rf 0.45.

Enzymatic cleavage of rhodotorucine A Cleavage of rhodotorucine A into peptide fragments was performed in the following way. Trypsin (EC 3.4.21.4, Worthington Biochemical Co.) [1.5-20 hrs, substrate:enzyme=50:1 (wt/wt), 0.1M ammonium acetate pH 7.0, 30°C], α-chymotrypsin (EC 3.4.21.1, Miles Research Products) [4-18 hrs, 20:1, 0.1M ammonium acetate pH 7.0, 25°C], thermolysin (EC 3.4.24.4, Daiwakasei, Tokyo) [4-20 hrs, 20:1, 0.1M ammonium acetate pH 7.0 40°C] and aminopeptidase M (EC 3.4.11.2, Protein Research Foundation, Osaka) [2-20 hrs, 20:1, 0.1M ammonium acetate pH 7.0, 25-40°C].

Peptide fragments were purified on Sephadex G-25 (Supper Fine, Pharmacia) columns (1.0x70 cm) with 0.1M AcOH

Proton magnetic resonance and mass spectra of peptide frgments PMR* spectra of peptide fragments were measured by a JEOL FX-100 spectrometer. Peptides were methylated with 5% dry HCl in methanol for one hr at room temp. and successively acetylated with a mixture of acetic anhydride and acetic anhydride d6 (Merck) (1:1) in 50% aqueous methanol for 2 hrs at room temp. EI* and CI* mass spectra were measured by a Hitachi RMU-6MG mass spectrometer. High resolution mass spectrum was measured by a Hitachi RMU-7M mass spectrometer. Sample temp.; 120°-180°C, ion accelerating voltage; 70 eV (EI) 100 eV (CI), reactant gas; isobutane 99.99%.

RESULTS AND DISCUSSION

The amino acid composition of rhodotorucine A was estimated to be Asp 1, Thr 1, Ser 1, Glu 1, Pro 1, Gly 1, Ile 1, Tyr 1, Trp 1, Arg 1 based on the amino acid analysis and measurement of UV absorption (4). Besides these amino acids, about 0.05-0.16 molar ratio of cystine was always observed in amino acid analyses. This suggested the presence of an additional amino acid component which was decomposed on acid hydrolysis to give a trace of cystine and cysteic acid.

We proposed the partial structure (I) for rhodotorucine A by dansyl*-Edman degradation and direct Edman degradation (6).

H-Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg-Asx-Gly-X

(I)

*Abbreviations: TLC, thin layer chromatography; PMR, proton magnetic resonance; EI, electron impact; CI, chemical ionization; dansyl-, 1-dimethylamino-naphthalene-5-sulfonyl-.

Table I. Enzymatic hydrolyses of rhodotorucine A

Peptide fragments	Th-1	Th-3***	CT-2	Tr-2
N-terminal amino acid*	Tyr	Ile	Thr	Asn
Amino acid composition**	Glu, Pro, Tyr	Ser. Ile, Trp	Thr, Arg	Asn, Gly, X
Amino acid sequence	Tyr-Pro-Glu	Ile-Ser-Trp	Thr-Arg	Asn-Gly-X

* N-terminal amino acids were identified by dansylation method.
** Amino acid compositions were identified by 6N HCl hydrolyses containing 2% thioglycolic acid or enzymatic hydrolyses with aminopeptidase M.
*** Th-2 was obtained from direct hydrolysis of Tr-1 with thermolysin (6).

Digestion of rhodotorucine A was performed with several kinds of peptidases. Treatment of lyophilized powder of rhodotorucine A with trypsin in ammonium acetate buffer gave two peptide fragments Tr-1 and Tr-2: Tr-2 was extracted with n-butanol, while Tr-1 remained in the aqueous phase. Tr-1 was digested with α-chymotrypsin to give two fragments CT-1 and CT-2. CT-1 gave two further fragments Th-1 and Th-3 on digestion with thermolysin. Determination of amino acid composition and N-terminal amino acid of each fragment confirmed the proposed structure of rhodotorucine A (Table I).

Tr-2 containing the C-terminus gave colorless crystals when recrystallized from aqueous methanol. Treatment of Tr-2 with aminopeptidase M liberated successively asparagine, glycine and an unknown lipophilic amino acid. On acid hydrolysis with 6N HCl, Tr-2 gave aspartic acid, glycine and small amounts of cystine and cysteic acid. Thus the unknown amino acid, which gave cystine and cysteic acid on acid hydrolysis, should be attached to Asn-Gly-residue of Tr-2 as the C-terminus of rhodotorucine A.

The structure of the lipophilic amino acid was disclosed by PMR and mass spectrometric analyses of Tr-2. As shown in Fig. 1, the Fourier transfer PMR

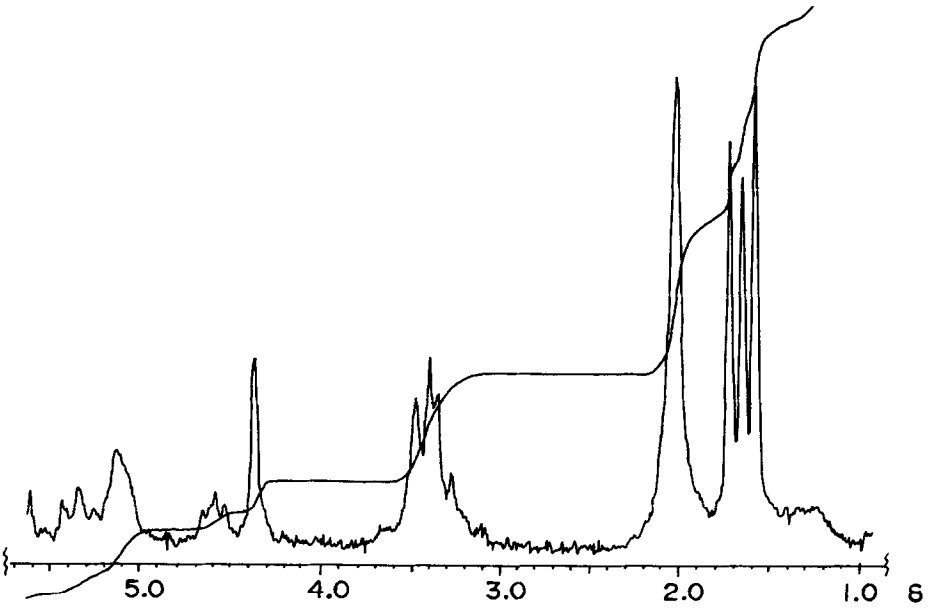

Fig. 1 Fourier transfer proton magnetic resonace spectrum of Tr-2 in $C_5D_5N^+D_2O$

of Tr-2 revealed the signals characteristic of farnesyl moiety: four vinyl methyl protons at δ 1.60 (6H), 1.66 (3H) and 1.72 (3H), allyl methylene protons at δ 2.0 (8H) and vinyl methine protons at δ 5.0-5.3 (3H). Moreover, signals at δ 3.1-3.7 (6H) attributable to methylene protons of asparagine and cysteine and to S-allyl methylene protons (S-$\underline{CH_2}$-CH=CH-) were observed. This suggested that cysteine and farnesyl moiety constitute the C-terminal amino acid.

Tr-2 was methylated with 5% dry HCl in methanol and then acetylated with a mixture of acetic anhydride and acetic anhydride d_6 (1:1) in aqueous methanol. CI mass spectrum of the product is shown in Fig. 2. Singlet fragment ion peaks are distinguishable from doublet ion peaks containing acetylated N-terminus (7). 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 $[Ac\text{-}Asn\text{-}]^+$

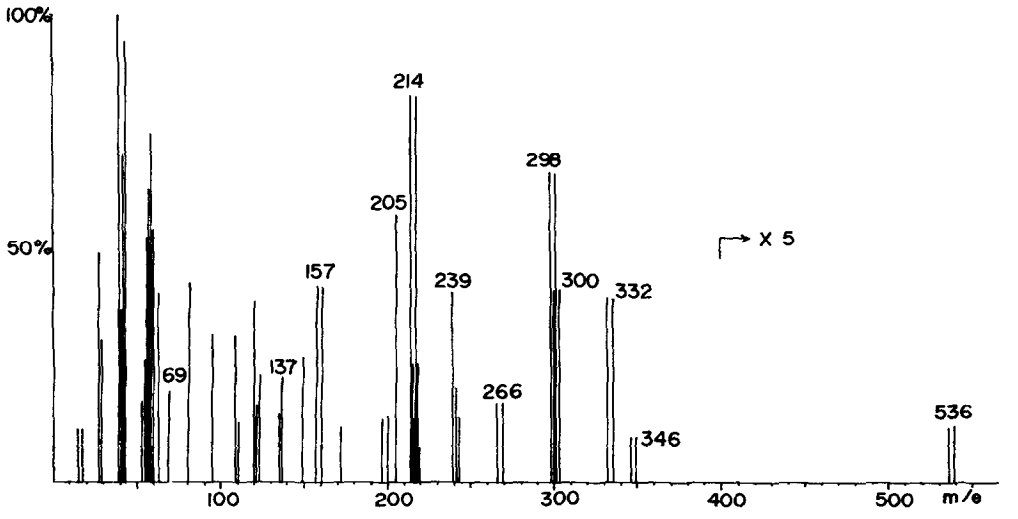

Fig. 2 Chemical ionization mass spectrum of acetylated Tr-2 methyl ester Sample temp.; 150°C, reactant gas; isobutane (99.99%), ion accelerating voltage; 100eV.

and $[Ac\text{-}Asn\text{-}Gly\text{-}]^+$. 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 $[Ac\text{-}Asn\text{-}Gly\text{-}(S\overset{\}}{\{})\text{-}OMe]^+H\text{—}NH_3$ and $[Ac\text{-}Asn\text{-}Gly\text{-}Cys(S\text{-}farnesyl)\text{-}OMe]^+H\text{—}NH_3$ respectively. The high resolution 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(C_5H_9), 137($C_{10}H_{17}$), 204($C_{15}H_{24}$), 237($C_{15}H_{25}S_1$), 157($C_6H_9N_2O_3$), 214($C_8H_{12}N_3O_4$) and 332($C_{12}H_{18}N_3O_6S_1$). 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.

For further confirmation, synthesis of Tr-2 was performed as follows*. H-Asn-Gly-Cys(SH)-OH was treated with all trans 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 with methanol to give H-Asn-Gly-Cys(S-farnesyl)-OH, though the yield was not excellent. Rf

*Details of the synthesis will be reported elsewhere.

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 completely identical with that of natural acetylated Tr-2 methyl ester.

Therfore we proposed the structure (II) for rhodotorucine A

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

(II)

The stereochemistry of farnesyl moiety was predicted to be all trans based on the chemical shifts of methyl protons of Tr-2 in $CDCl_3$:CD_3OD:D_2O (1:1:1), [δ 1.60 (6H) and 1.66 (6H)] (8).

Recently, an oligopeptide named α-substance I_A (α factor) has been isolated as the regulator of the mating reaction in Saccharomyces cerevisiae (9-12). 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-farnesyl cysteine, at the C-terminus. As far as we know, this type of lipopeptide is the first isolated from nature as a regulatory substance. The lipophilic nature of the side chain in rhodotorucine A might play an important role when the peptide is adsorbed at the surface of a type cells in the early stage of the mating process (13-14).

Syntheses of rhodotorucine A and its analogues are in progress.

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REFERENCES

1. Banno, I. (1967) J. Gen. Appl. Microbiol. 13, 167-196
2. Abe, K., Kusaka, I. and Fukui, S. (1975) J. Bacteriol., 122, 710-718
3. Kamiya, Y., Yamamoto, K., Sakurai, A., Tamura, S., Abe, K. and Fukui, S. (1975) Proc. Japan Acad., 51, 571-576
4. Kamiya, Y., Sakurai, A., Tamura, S., Abe, K., Tsuchiya, E. and Fukui, S. (1977) Agric. Biol. Chem., 41, 1099-1010
5. Kamiya, Y., Sakurai, A., Tamura, S., Takahashi, N., Abe, K., Tsuchiya, E. and Fukui, S. (1978) Agric. Biol. Chem., 42 in press.

6. Kamiya, Y., Sakurai, A., Tamura, S., Takahashi, N., Abe, K., Tsuchiya, E. and Fukui, S. (1978) Agric. Biol. Chem., 42, 209-211
7. Gray, W. R., Wojcik, L. H. and Futrell, J. H. (1970) Biochem. Biophys. Res. Comm., 41, 1111-1119
8. Bates, R. B. and Gale, D. M. (1960) J. Amer. Chem. Soc., 82, 5749-5751
9. Sakurai, A., Tamura, S., Yanagishima, N. and Shimoda, C. (1976) Agric. Biol. Chem., 40, 1057-1058
10. Sakurai, A., Tamura, S., Yanagishima, N. and Shimoda, C. (1977) Agric. Biol. Chem., 41, 395-398
11. Stötzler, D., Kiltz, H. H. and Duntze, W. (1976) Eur. J. Biochem., 69 397-400
12. Ciejek, E., Thorner, J. and Geier, M. (1977) Biochem. Biophys. Res. Comm. , 78, 952-961
13. Tsuchiya, E., Abe, K. and Fukui, S. Submitted to J. Gen. Appl. Micobiol. (1978)
14. Abe, K, Tsuchiya, E., Kusaka, I., Fukui, S. and Kusanagi, A. (1977) J. Gen. Appl. Microbiol., 23, 157-181