Structures of Tremerogens *A*-9291-I and *A*-9291-VIII: Peptidyl Sex Hormones of *Tremella brasiliensis*†

Yoshihiro Ishibashi, Youji Sakagami, Akira Isogai, and Akinori Suzuki*

ABSTRACT: Tremerogens *A*-9291-I through *A*-9291-VIII are peptidyl sex hormones (*A* factors) of *Tremella brasiliensis* that are secreted by the *A* cells into the culture medium and able to induce conjugation tube formation in the *a* cells. The structures of tremerogens *A*-9291-I (Ia, *A* factor proper) and *A*-9291-VIII (Ib, most abundant component) have been determined by dansyl–Edman procedure, fast atom bombardment mass spectra, and nuclear magnetic resonance spectra, together with characterization of thermolytic and tryptic peptides. Tremerogen *A*-9291-VIII differs from *A*-9291-I by the absence of a methyl ester at the C-terminus. Both hormones exhibit microheterogeneity of glycine and serine at the fourth residues, which was confirmed by characterization of the tryptic peptides, and have a unique isoprenoidal moiety blocking the sulfhydryl group of the C-terminal cysteine residues.

The species of *Tremella*, heterothallic basidiomycetous fungi, are dimorphic: yeast cells in the haplophase and mycelia with clamps in the diplophase. The stages of the sexual conjugation are (1) formation of long filamentous conjugation tubes in the yeast cells, (2) tube fusion at the apex, and (3) initiation of the dikaryotic mycelium.

In *Tremella mesenterica*, Bandoni (1963) showed that the stages from the conjugation tube formation to fusion at the apex are controlled by a single pair of alleles (*A* and *a* factors) and that the initiation of the dikaryotic mycelium is controlled by multiple alleles (*B* factors). Furthermore, Bandoni (1965) demonstrated the existence of conjugation tube inducing substances (=hormones) that are produced constitutively under the genetical control of a pair of alleles (*A* and *a*). The *A* factor, produced by the *A* cells and effective to the *a* cells, and the *a* factor, vice versa, were isolated and chemically identified by our group (*A* factor, named tremerogen *A*-10, and *a* factor, tremerogen *a*-13; Sakagami et al., 1981).

Flegel (1981) has described the advantage of sequencing peptidyl sex hormones of yeasts belonging to a single genus for phylogeny. From this standpoint, it is interesting that *Tremella brasiliensis*, a species very closely related to *T. mesenterica*, has a mating system similar to that of *T. mesenterica* (R. J. Bandoni, unpublished data) and that the cells of *T. brasiliensis* do not respond to the hormones from the cells of *T. mesenterica* (Ishibashi et al., 1983). To elucidate the species specificity of the sex hormones of the Basidiomycetes on the molecular basis, we attempted to characterize the hormones of *T. brasiliensis*.

Previously, we reported the isolation of the *A* factors, tremerogens *A*-9291-I and *A*-9291-II, from the culture filtrate of the *A* cells of *T. brasiliensis* (Ishibashi et al., 1983). Further study of the *A* factors revealed the presence of six other active substances, and this diversity of *A* factors was interesting in their biological function and particularly biosyntheses. In this paper, we describe the isolation of tremerogens *A*-9291-III through *A*-9291-VIII and the structure determination of tremerogens *A*-9291-I and *A*-9291-VIII in detail.

Experimental Procedures

Microbe Strains and Culture Conditions. Tremella brasiliensis IFO 9291 strain (mating type *AB*) was used for the production of *A* factors, and IFO 9290 strain (*ab*) was used for the bioassay of *A* factors. Culture conditions for these strains and method of bioassay were described previously (Ishibashi et al., 1983).

Chemicals. Sequanal-grade pyridine (Tokyo Kasei Kogyo Co., Ltd.), phenyl isothiocyanate, and TFA[1] (Wako Pure Chemical Industries, Ltd.) were used for sequence analyses. Thermolysin was purchased from Seikagaku Kogyo Co., Ltd. *N,O*-Bis(trimethylsilyl)acetamide was from Nakarai Chemical Co. Hydrogen chloride–methanol reagent 5 [5% (w/w) hydrogen chloride] from Tokyo Kasei Kogyo Co., Ltd., was used for the esterification of A-VIII. Deuterized solvents were from Merck. Develosil ODS was obtained from Nomura Chemical Co., Ltd. The Nucleosil 5 CN packed column was obtained from Senshu Scientific Co., Ltd. All other reagents were of the highest quality commercially available.

Instruments. Amino acid analyses were carried out with a Hitachi 385 amino acid analyzer. Proton NMR spectra were measured with a JEOL FX-400 spectrometer. FAB-MS and GC–MS spectra were run with a JEOL JMS DX-300 mass spectrometer. SIMS spectra were measured with a Hitachi M-80A mass spectrometer. Preparative and analytical HPLC were performed with a SP 8700 solvent delivery system. PTH-amino acids were chromatographically identified with a JASCO Tri-roter III liquid chromatograph.

Isolation of Tremerogen A-9291. Isolation procedures were as described previously (Ishibashi et al., 1983). In the final purification step (step 4), the fractions corresponding to peaks I–VIII in Figure 1 were collected. The yields of each compound were calculated from absorbance at 220 nm (=peak area), with bovine serum albumin as standard, and were not corrected.

Amino Acid Sequence Analysis. Dansyl–Edman analysis was performed according to the scaling-down method reported by Kimura (1974). To differentiate Asp and Asn at the *n*th residue, *n*-butyl acetate extract in the *n*th degradation step of the Edman procedure was treated with 1.0 M HCl at 80 °C for 10 min to yield the corresponding PTH-amino acid. The

† From the Department of Agricultural Chemistry, the University of Tokyo, Bunkyo-ku, Tokyo 113, Japan. *Received May 24, 1983.* This work was partly supported by a grant from the Ministry of Education, Science and Culture of Japan.

[1] Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; SIMS, secondary ion mass spectrometry; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; GC–MS, gas chromatography–mass spectrometry; DNS, dansyl (5-dimethylaminonaphthalene-1-sulfonyl); PTH, phenylthiohydantoin; A-I, A-II, etc., tremerogens *A*-9291-I, *A*-9291-II, etc.; t_R, retention time; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance.

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PTH-amino acid was subjected to HPLC analysis on a Develosil ODS-5 column (4.6 × 250 mm). The conditions were as follows: solvent, a linear gradient of 12.5–25% acetonitrile (15 min), followed by 25% isocratic elution in 0.01 M ammonium acetate; flow rate, 1.0 mL/min; detection, absorbance at 269 nm; oven temperature, 60 °C. PTH-Asp had a t_R of 5.8 min; PTH-Asn had a t_R of 13.3 min.

Thermolysin Digestion of Tremerogen A-9291-I. A-I (ca. 100 μg) was incubated with 2 μg of thermolysin in 100 μL of 0.1 M ammonium acetate (pH 7.2) at 40 °C for 100 min. After incubation, the lipophilic fragment was extracted with 1-butanol (100 μL, 3 times), concentrated by evaporation at 50 °C, and lyophilized.

Interconversion of Tremerogen A-9291-I and A-9291-VIII. (i) A-I (ca. 10 μg) was dissolved in 150 μL of 0.1 M sodium hydroxide and kept at 45 °C for 2.5 h. The reaction was stopped by addition of 300 μL of 1% formic acid. After lyophilization, the product was subjected to HPLC analysis. (ii) A-VIII (ca. 30 μg) was dissolved in 30 μL of HCl–methanol reagent and let stand at room temperature. After 6 h, the reaction was terminated by addition of an excess volume (ca. 500 μL) of water. After lyophilization, the product was subjected to bioassay and HPLC analysis. Chromatographic conditions were as follows: column, Develosil ODS-3 (4.6 × 250 mm); solvent, a linear gradient of 20–50% acetonitrile in 0.01 M ammonium acetate (30 min); flow rate, 0.8 mL/min; detection, absorbance at 220 nm. A-VIII had a t_R of 14.8 min; A-I had a t_R of 25.9 min.

Identification of Lipophilic Moiety of Tremerogen A-9291-VIII. The lipophilic side chain of A-VIII was identified by the same manner as that of tremerogen *A*-10 (Sakagami et al., 1979). A-VIII (2 mg) was dissolved in 400 μL of 3% formic acid and 50 μL of freshly distilled methyl iodide. The solution was stirred for 18 h at room temperature in the dark. The solution was then extracted 4 times with 200 μL of chloroform–methanol (9:1 v/v), and the chloroform–methanol extract containing the isoprenoidal moiety was subjected to GC–MS analysis (see paragraph at end of paper regarding supplementary material).

After removal of the solvents, the extract was dissolved in 20 μL of pyridine, and 2 μL of *N,O*-bis(trimethylsilyl)acetamide was added to afford the Me_3Si diether of the isoprenoidal moiety. GC–MS conditions were as follows: column, OV-1 (0.3 × 100 cm); oven temperature, 170 °C; injection temperature, 230 °C; ion accelerating voltage, 70 eV.

The aqueous phase was lyophilized and redissolved in 600 μL of 0.2 M ammonium acetate (pH 7.5). The solution was reduced with 60 μL of 2-mercaptoethanol for 18 h at room temperature. After a washing with ethyl acetate, the reaction mixture was purified by HPLC on a Develosil ODS-3 column (6 × 200 mm). The conditions used were as follows: solvent, a linear gradient of 0–40% acetonitrile in 0.01 M ammonium acetate (45 min); flow rate, 1.0 mL/min; detection, absorbance at 220 nm. *S*-Methyltremerogen *A*-9291-VIII had a t_R of 14.2 min (186 μg).

Trypsin Digestion of Tremerogen A-9291-VIII. A-VIII (2 mg) was incubated with 20 μg of trypsin in 500 μL of 0.1 M ammonium acetate (pH 7.0) at 30 °C for 3 h. The reaction mixture was then boiled (to inactivate the enzyme) and lyophilized. The resultant fragments were purified by HPLC on a Nucleosil 5 CN column (4.6 × 250 mm). The conditions used were as follows: solvent, a linear gradient of 0–30% acetonitrile in 0.01 M ammonium acetate (30 min); flow rate, 1.0 mL/min; detection, absorbance at 220 nm. The N-terminal fragment had a t_R of 3.0 min (173 μg); the C-terminal fragment had a t_R of 17.0 min (666 μg).

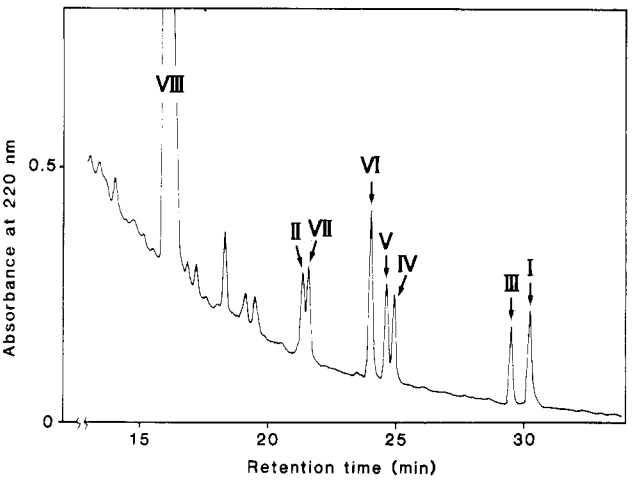


FIGURE 1: Isolation of tremerogens *A*-9291-I through *A*-9291-VIII. Develosil ODS-3 column (6 × 200 mm) was eluted with a linear gradient of 20–40% acetonitrile in 0.01 M ammonium acetate (33.3 min). Flow rate was 1.0 mL/min. I, II, etc., tremerogens *A*-9291-I, *A*-9291-II, etc.

Table I: Specific Activity and Yields of Tremerogens *A*-9291-I through *A*-9291-VIII

	sp act. (ng/unit)	yield (μg/L)
I	4.8	4–40
II	4.8	1–10
III	125	3–30
IV	125	1–20
V	125	1–20
VI	125	1–20
VII	125	1–10
VIII	1000	40–160

The N-terminal fragment was separated into two components by HPLC on a Develosil ODS-3 column (4.6 × 250 mm) with isocratic elution of 0.1% aqueous TFA: N1, t_R 11.2 min (36 μg); N2, t_R 12.5 min (35 μg). For characterization of N1 and N2, 5 μg was used for amino acid analysis, 25 μg for sequence analysis, and the remainder for measurement of SIMS.

Results

Isolation of Six Other Compounds with A Factor Activity. In the previous paper, we described the isolation and the characterization of tremerogens *A*-9291-I and *A*-9291-II, sex hormones of *Tremella brasiliensis*, that can induce conjugation tube formation in the *a* cells (Ishibashi et al., 1983). The typical profile of the final purification step, HPLC on Develosil ODS-3, is shown in Figure 1. High biological activity was found at two peaks corresponding to tremerogens *A*-9291-I and *A*-9291-II. Since several peaks other than A-I and A-II also proved to be active in the extensive bioassay, the active fractions were isolated and named tremerogens *A*-9291-III through *A*-9291-VIII.

A-I through A-VIII were always isolated, but the yields of them varied somewhat in different preparations (Table I). Their yields were always in the order A-VIII > A-I > A-III > A-II and A-IV through A-VII. A-I and A-II were the most active components, and effective at a concentration as low as 5 ng/mL. The activity of A-III through A-VII is only 3.1% of that of A-I and A-II. A-VIII is the least active but the most abundant component.

Structural Analysis of Tremerogen A-9291-I. A-I has been characterized to be a peptide composed of Asx (2), Ser (4–5),

Table II: Characterization of Tremerogen *A*-9291-VIII and Its Tryptic Fragments

	tremerogen *A*-9291-VIII	C-terminal fragment	N-terminal fragment[a]	
			N1	N2
MS	1529 $(M + H)^+$,[b] 1559 $(M + H)^+$[b]	970 $(M + H)^+$[b]	578 $(M + H)^+$[c]	608 $(M + H)^+$[c]
Asp	1.79 (2)[d]	1.21 (1)	1.00 (1)	1.00 (1)
Ser	3.73 (4–5)	1.97 (2)	1.76 (2)	2.67 (3)
Gly	4.44 (4–5)	3.24 (3)	2.02 (2)	1.15 (1)
Ala	1.00 (1)	1.00 (1)		
Arg	1.00 (1)		0.96 (1)	0.98 (1)
Pro	1.02 (1)	0.94 (1)		
Cys[e]	0.57 (1)	0.42 (1)		
sequence		Asp-Pro-Gly-Ala-Ser-Ser-Gly-Gly-Cys	Asp-Ser-Gly-*Gly*-Ser-Arg	Asp-Ser-Gly-*Ser*-Ser-Arg

[a] The N-terminal fragment was separated into N1 and N2 by HPLC in the ratio of 1:1. [b] Measured by FAB-MS. [c] Measured by SIMS. [d] Amino acid ratios of 5.7 M HCl hydrolysate are presented; given in parentheses are amino acid composition as determined by sequence analysis. [e] Cysteine was estimated from cystine.

Gly (4–5), Ala (1), Arg (1), Pro (1), and Cys (1), with Asx at the N-terminus (Ishibashi et al., 1983). The amino acid sequence was determined by the dansyl–Edman procedure as H-Asp-Ser-Gly-(Ser or Gly)-Ser-Arg-Asp-Pro-Gly-Ala-Ser-(Ser or Gly)-Gly-Gly-Cys-X. Acid and amide groups were differentiated by identifying the extracted anilinothiazolinone after conversion to the phenylthiohydantoin. In the 14th degradation step, a weak spot of DNS-cysteic acid was newly detected on a polyamide sheet, and the 15th residue was estimated to be cysteine. In this sequence, the 4th and the 12th residues could not be definitely determined. The ambiguity may be caused by (1) contamination of a peptide that was not degraded in the preceding step and (2) possible microheterogeneity of glycine and serine. Contamination of free amino acids is inevitable in the manual dansylation, so it might have caused the ambiguity also. The FAB mass spectrum of A-I gave two ion peaks at m/z 1543 and 1573, both of which seemed to be quasi molecular ions (Ishibashi et al., 1983). This 30 mass units difference was explainable by the difference of molecular weights between glycine and serine; therefore, it was hypothesized that A-I had the microheterogeneity of glycine and serine at either 4th or 12th residues.

However, the peptide composed of 15 amino acid residues did not account for the molecular weights of 1542 and 1572, so it was expected that A-I may contain a lipophilic moiety like tremerogens *A*-10 and *a*-13. To manifest the lipophilic moiety, an NMR spectrum of A-I was measured. In the 400-MHz ^1H NMR spectrum (Figure 2), three olefinic methyls (δ 1.6–1.8), three olefinic protons (δ 5.2–5.4), and olefinic hydroxymethyl (δ 4.1) were observed, and they were ascribed to (2*E*,6*E*,10*Z*)-3,7,11-trimethyl-2,6,10-dodecatrien-12-ol group (structure III, Chart I), which had been already found to block the thiol group of the cysteine residue in tremerogen *A*-10 (Sakagami et al., 1979).

The NMR spectrum also showed a carbomethoxyl signal at δ 3.7 ppm. The location of the methyl ester was confirmed as follows. A-I (100 μg) was digested with thermolysin, and the lipophilic fragment was extracted with 1-butanol. The fragment was shown to be heterogeneous by N-terminal analysis with the dansyl method (DNS-Ala, DNS-Ser, and DNS-Gly were detected), and however, little aspartic acid was detected in the amino acid analysis of the acid hydrolysate of the fragment (Ser, 1.49; Gly, 2.35; Ala, 1.00; Cys_2, 0.35; Asp, 0.14). Since the amount obtained was so scarce (20 μg), the extract was characterized without further purification. In the ^1H NMR spectrum of the extract, the signals of both the isoprenoidal moiety and the carbomethoxy group appeared in the same ratio as those in the ^1H NMR spectrum of the intact peptide; thus the possibility that the methyl ester may block

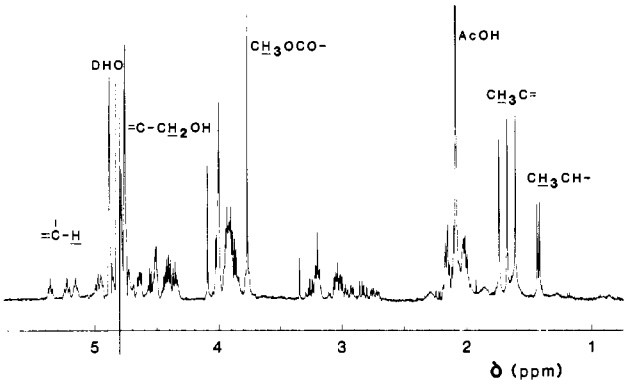
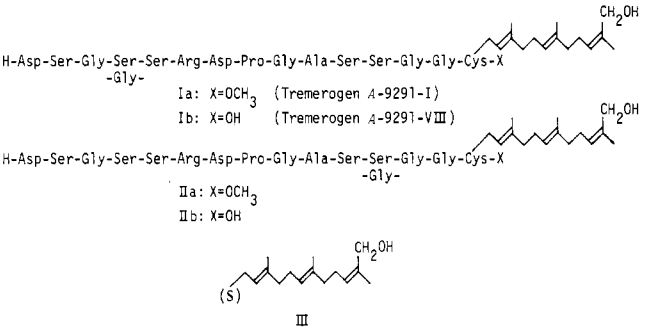


FIGURE 2: Proton NMR spectrum of tremerogen *A*-9291-I. Conditions were as follows: solvent, D_2O containing 0.5% formic acid; accumulation, 1160 times; gated-decoupled mode (irradiated at δ 4.8 ppm).

Chart I



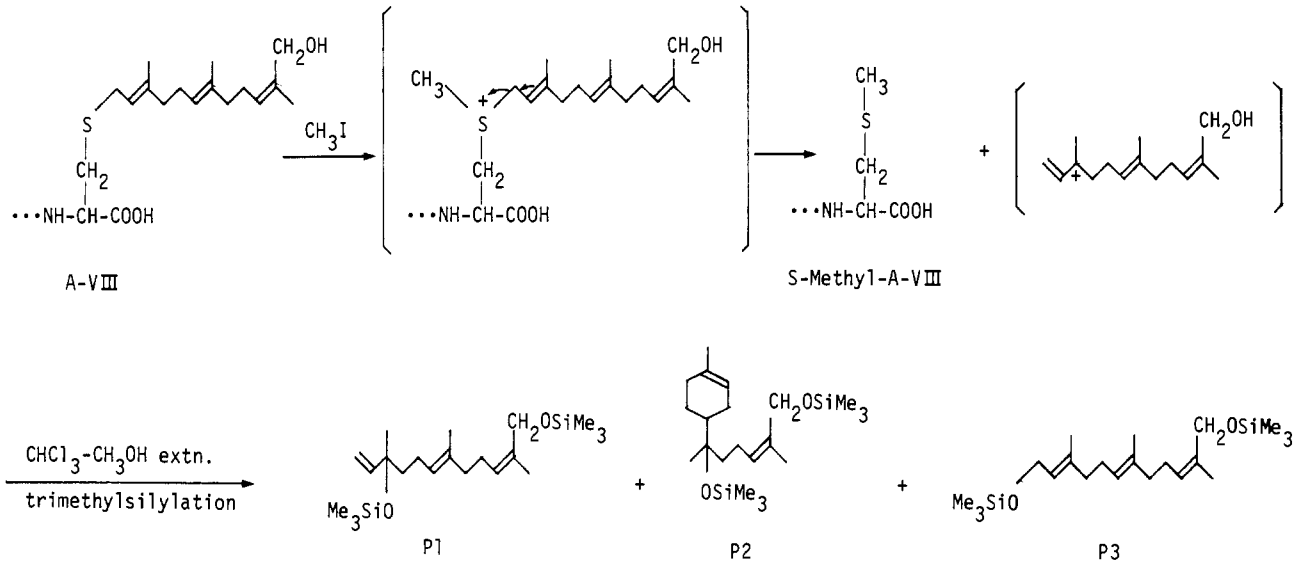
the β-carboxyl group of an aspartic acid residue (1st or 7th) was excluded. Necessarily, the methyl ester blocks the C-terminal carboxyl group.

The above-mentioned results lead to the conclusion that the structure of A-I is either Ia or IIa (Chart I). The attachment of the isoprenoidal moiety to the sulfur atom of the cysteine, which was speculative to this point, was confirmed as described later.

Structural Analysis of Tremerogen A-9291-VIII. A-VIII was the most abundant component of tremerogen *A*-9291, and occurred 4–50 times as much as A-I (Table I). Judging from the behavior during purification procedures, A-VIII appeared to be a peptide related to A-I, though the activity of A-VIII is only 0.4% of that of A-I.

The NMR spectrum of A-VIII was quite similar to that of A-I, except that a carbomethoxy signal was absent. The amino acid analysis of the acid hydrolysate of A-VIII gave the same molar ratios as A-I (Table II). The result of sequence analysis was also similar, and the same sequence as A-I was disclosed.

Scheme I



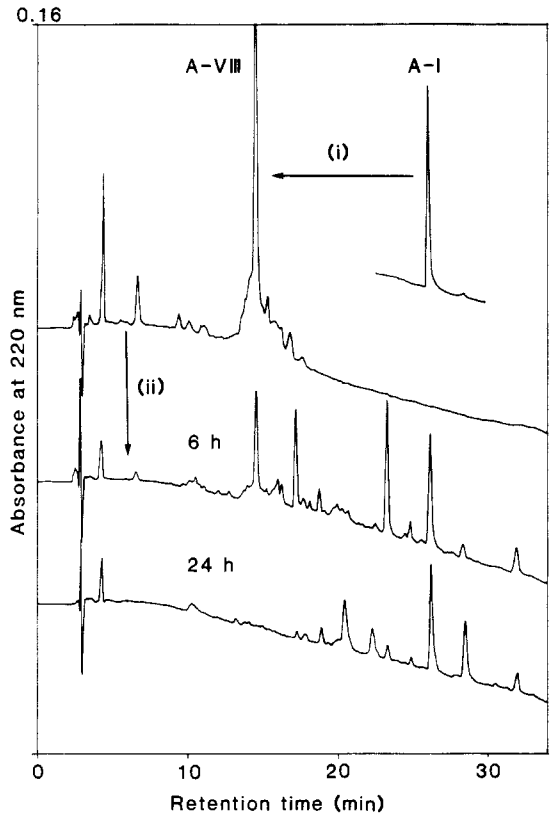


FIGURE 3: HPLC analysis of the interconversion of tremerogens *A*-9291-I and *A*-9291-VIII: (i) 0.1 M NaOH (45 °C, 2.5 h); (ii) HCl–methanol reagent.

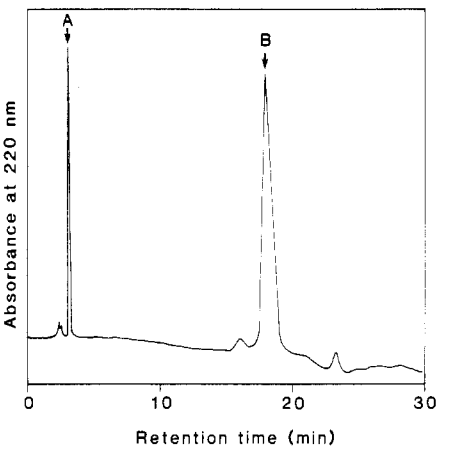


FIGURE 4: Purification of tryptic digest of tremerogen *A*-9291-VIII. Conditions were as follows: column, Nucleosil 5 CN (4.6 × 250 mm); solvent, a linear gradient of 0–30% acetonitrile in 0.01 M ammonium acetate (30 min); flow rate, 1.0 mL/min. (A) N-Terminal fragment; (B) C-terminal fragment.

In the FAB mass spectrum of A-VIII, two ion peaks were observed at m/z 1529 and 1559. It was natural to assume that the two ions represent the microheterogeneity of A-VIII like the case of A-I.

Consequently, it was estimated that A-VIII differs from A-I by the absence of a methyl ester at the C-terminus (Ib or IIb). This estimation was confirmed by the interconversion of A-I and A-VIII. The product of treatment of A-I with 0.1 M sodium hydroxide coeluted with A-VIII on an analytical HPLC. Conversely, A-VIII treated with HCl–methanol exhibited biological activity at the same concentration as A-I, and its HPLC analysis (Figure 3) revealed A-I as one of the esterified products. In Figure 3, the other products which were not further characterized may contain a methyl ester(s) in the aspartic acid residue(s).

Confirmation of the Lipophilic Side Chain of Tremerogen A-9291-VIII. To locate the isoprenoidal moiety and to confirm its structure, direct characterization of the isolated isoprenoid was necessary. Jones et al. (1975) reported that the *S*-methyl group of the methionine residue in sperm whale myoglobin can be changed to *S*-[^{13}C]methyl by treating the protein with [^{13}C]methyl iodide, followed by reduction with dithioerythritol. Kamiya et al. (1979) has demonstrated that the reaction is also applicable to the liberation of isoprenoidal side chains from S-alkylated cysteines, and described the formation of nerolidol, α-bisabolol, and farnesol from *S*-farnesylcysteine by a method similar to ours.

In our experiments, A-VIII was treated with methyl iodide in 3% formic acid, and the modifying group, released as alcohol, was extracted with chloroform–methanol, converted to its Me_3Si diether, and subjected to GC–MS analysis. The results paralleled those of tremerogen *A*-10 (Sakagami et al., 1979), and three peaks were detected in gas chromatography (P1, P2, and P3, eluted in this order). In the light of the works of Kamiya et al. (1979), the structures of P1, P2, and P3 were presumed as illustrated in Scheme I, and the structure of the isoprenoidal moiety was presumed as (2*E*,6*E*,10*Z*)-3,7,11-

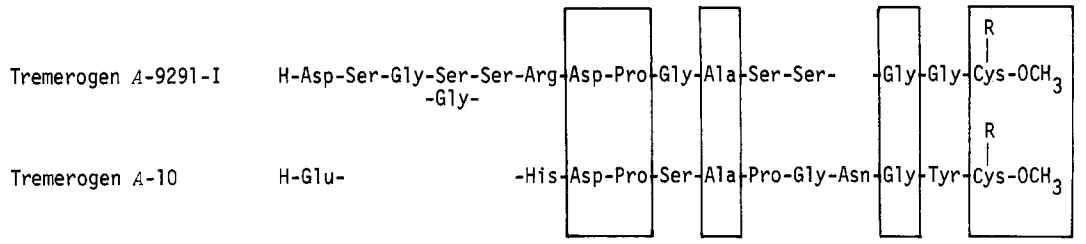


FIGURE 5: Sequence homology of tremerogens *A*-9291-I and *A*-10. R, oxidized farnesyl group (structure III).

trimethyl-2,6,10-dodecatrien-12-ol. To confirm the validity of the structure including the geometry of the double bonds, *E,E,Z* and *E,E,E* isomers were synthesized (Y. Morishita et al., unpublished results). In the NMR spectrum of the synthetic *E,E,Z* isomer, the signals of the olefinic methyls and the olefinic hydroxymethyl were almost identical with those in the NMR spectrum of A-VIII (olefinic protons were not well resolved in the 100-MHz NMR), and its Me_3Si diether showed the same retention time and identical mass spectrum with P3 in the GC–MS analysis.

The aqueous phase was reduced with mercaptoethanol to eliminate any *S*-dimethylsulfonium salt. The peptide moiety was purified by HPLC on ODS. In its NMR spectrum, signals due to olefinic methyls, olefinic protons, and olefinic hydroxymethyl had disappeared, and a new signal ascribable to *S*-methyl appeared at δ 2.2. This result supported the attachment of the isoprenoidal moiety to the sulfur atom of the cysteine residue. The isoprenoidal moiety was liberated by the treatment with methyl iodide alone, and it may be ascribed to the property of this allyl-type substituent and the relative stability of the resultant tertiary carbonium ion.[2]

The S-alkylated cysteine had been detected as cystine in the amino acid analysis of A-I and A-VIII. The fact is rationalized that the bond connecting the sulfur and the isoprenoid may be cleaved under the conditions of the conventional acid hydrolysis with 5.7 M HCl. This may also be specific for the allyl-type substituents.[3]

Confirmation of Microheterogeneity of Tremerogens A-9291-VIII and A-9291-I. To determine the 4th and the 12th residues and to confirm the possible microheterogeneity, we decided to characterize the tryptic fragments of A-VIII. A-VIII was digested with trypsin, and the digested mixtures were purified by HPLC on a Nucleosil 5 CN column (Figure 4).

The results of the characterization of the tryptic fragments are summarized in Table II. The FAB mass spectrum of the C-terminal fragment gave quasi molecular ions at *m/z* 970 $(M + H)^+$ and 992 $(M + Na)^+$, indicating that the C-terminal fragment is homogeneous. Together with the result of amino acid analysis of the C-terminal fragment, the 12th residue of A-VIII must be serine. The conclusion was supported by the sequence analysis of the C-terminal fragment, although it remains to be elucidated why DNS-Gly was detected in the 12th degradation step of the dansyl–Edman analysis of the original peptide.

On the other hand, the FAB mass spectrum of the N-terminal fragment gave two quasi molecular ions at *m/z* 578 and 608, suggesting that the microheterogeneity was located at the fourth residue. Rechromatography of the N-terminal fragment on Develosil ODS-3, with 0.1% TFA as the mobile phase, afforded two peaks (N1 and N2, eluted in this order). The structures of N1 and N2 were determined by SIMS, amino acid analyses, and sequence analyses as shown in Table II. Furthermore, A-I also afforded N1 and N2 in the ratio of 1:1 by the treatment with trypsin.

The isolation and the characterization of N1 and N2 verified the presence and the location of microheterogeneity. Thus, the amino acid sequences of A-I and A-VIII were confirmed, and the structures of A-I and A-VIII were established as Ia and Ib, respectively.

Discussion

In the course of the investigation of the purification procedures, only A-I and A-II were isolated as the *A* factors of *Tremella brasiliensis*. A-I and A-II represented about 85% of the activity recovered, and their specific activity was in the same order as other basidiomycetous sex hormones such as tremerogen *A*-10 (1 ng/mL; Sakagami et al., 1978), tremerogen *a*-13 (1 ng/mL; Yoshida et al., 1981), and rhodotorucine *A* (8 ng/mL; Kamiya et al., 1978). The fractions corresponding to the other peaks that appeared in the final purification step (Figure 1) were bioassayed at unusually high concentrations to clarify whether they had activity to some extent or not, and all were revealed weakly active.

A-I and A-II are the proper *A* factors on the basis of specific activity, and A-I may be the most significant component of A-I through A-VIII, since the yield of A-I is always higher than that of A-II. The low but real activity of A-III through A-VIII would imply structural relatedness to A-I and A-II. Actually, A-VIII turned out to be A-I with a free C-terminus. The structure–activity relation of A-I and A-VIII would indicate that the C-terminal methyl ester function plays an important part in the biological activity.

A-VIII cannot be an artifact of the purification, since significant loss of activity was never observed in the course of purification. From the yields and specific activity of A-I and A-VIII, one can easily understand that if A-VIII existed as A-I in the culture filtrate, the activity of the culture filtrate would have been higher (4–10 times) than it was. It is conceivable, however, that A-VIII is an inactivated product of A-I by the *A* cells during cultivation. In this connection, it should be noted that other workers have isolated the degradation products of α-mating factor, another peptidyl sex hormone of the yeast *Saccharomyces cerevisiae* from the culture filtrate of the α-cells (Tanaka & Kita, 1977). Interestingly, the inactivation mechanism in *T. brasiliensis* seems of the esterase type, while that in *S. cerevisiae* is proteolytic. Yet the pos-

[2] *S*-Allylcysteine [TLC (1-butanol–AcOH–H_2O, 12:3:5) R_f 0.50; ^1H NMR (D_2O) δ 3.3 (d, 2 H, J = 9 Hz), 5.3 (m, 2 H), 5.9 (m, 1 H)] and *S*-propylcysteine [TLC R_f 0.52; ^1H NMR (TFA) δ 1.0 (t, 3 H, J = 6.8 Hz), 1.7 (m, 2 H, J = 6.8 Hz), 2.7 (t, 2 H, J = 6.8 Hz)] were prepared by the method of Kamiya et al. (1979) in which L-cysteine reacted with allyl bromide and propyl iodide, respectively, and treated in the same manner. In this model experiments, *S*-methylcysteine (7.6%) was yielded from *S*-allylcysteine, while *S*-propylcysteine did not change. Since we could not detect sulfonium salt formation, the conditions may not be optimum for sulfonium salt formation. However, *S*-methyl-A-VIII was produced quantitatively from A-VIII (assessed from HPLC analysis). The reason for the low yield observed with *S*-allylcysteine is that the resultant carbonium ion is a primary one, which is less stable than the alkyl cation released from A-VIII.

[3] Hydrolysis of *S*-allylcysteine yielded cystine (10%), while hydrolysis of *S*-propylcysteine did not.

sibility that A-VIII is a precursor of A-I has not been completely excluded.

A-I and A-VIII are novel pentadecapeptides with an isoprenoidal moiety and have microheterogeneity at the fourth residues. The relative proportions of [Gly^4]*A* factor and [Ser^4]*A* factor were almost in the ratio of 1:1, being estimated from the yields of N1 and N2. The cause of the microheterogeneity is not explained at present. Recently, the structure of the gene for a putative precursor of α-mating factor of *S. cerevisiae* was reported, and the precursor was shown to contain four tandem copies of α-factor (Kurjan & Herskowitz, 1982). It is probable that in *T. brasiliensis* half of the copies code for [Ser^4]*A* factor and half for [Gly^4]*A* factor, though ribosomal synthesis of tremerogens has not been confirmed yet. Work on the biogenesis of tremerogens will be exciting, since they are unique lipopeptides having an isoprenoidal moiety in the molecule.

From the phylogenetic point of view, A-I may be compared with tremerogen *A*-10 (Figure 5). The two have five common residues, including the S-alkylated cysteine methyl ester. Furthermore, five residues can be attributable to single base substitutions. Thus, the close relatedness of *T. brasiliensis* and *T. mesenterica* is illustrated from the structures of sex hormones as well as from a morphological basis. The lack of cross activity would indicate that the species specificity is determined by the amino acid sequences of the hormones. Anyway, we may draw common structural features of the sex hormones in the genus *Tremella*: the N-terminus is an acidic amino acid residue, while the C-terminus is an S-alkylated cysteine residue. We are proceeding with the structural analysis of tremerogens *A*-9291-II through *A*-9291-VII.

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Supplementary Material Available

Results of GC–MS analysis of the isoprenoidal moiety (2 pages). Ordering information is given on any current masthead page.

Registry No. III, 88801-91-6; A-I, 86753-86-8; A-II, 86753-87-9; A-III, 88887-24-5; A-IV, 88887-25-6; A-V, 88887-26-7; A-VI, 88887-27-8; A-VII, 88887-29-0; A-VIII, 88887-30-3.

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