Peptidal Sex Hormones Inducing Conjugation Tube Formation in Compatible Mating-Type Cells of *Tremella mesenterica*

Abstract. *The pair of peptidal sex hormones (tremerogen A-10 and tremerogen a-13) that induce conjugation tube formation in compatible type cells (A and a types) of* Tremella mesenterica *were isolated. Tremerogen A-10 is a dodecapeptide and tremerogen a-13, a tridecapeptide. In both peptides, the sulfhydryl group of the cysteines at the carboxyl terminus was blocked by farnesyl moieties.*

Tremella mesenterica, a basidiomycetous jelly fungus, is dimorphic, and produces a haploid yeastlike cell as well as dikaryotic mycelium. In the homokaryotic phase, each of the compatible mating-type cells (A and a types) propagates by budding. When the A- and a-type cells are mixed, conjugation tube formation occurs and dikaryotic mycelium is formed by their mating (*1*). In 1965, Bandoni reported that the A- and a-type cells produced diffusible hormones that induce conjugation tube formation in cells of the opposite type (*2*). To clarify the mating process of *T. mesenterica* from a chemical standpoint, we attempted to determine the structures of this pair of hormones. We first isolated the hormone tremerogen A-10 (**1**) (Fig. 1), which is produced by the A-type cell (IFO 9310) and induces conjugation tube formation in the a-type cell (IFO 9313) (*3–5*). We now describe the isolation and structure of tremerogen a-13 (**2**) (Fig. 1), a hormone, produced by the a-type cell, that induces conjugation tube formation in the A-type cell.

The a-type cell was cultured for 48 hours in a jar fermenter, with aeration and agitation at 23°C; the medium consisted of 1 percent glucose, 0.05 percent yeast extract, and 0.1 percent Soytone (*p*H 7.0). The grown cells were removed by centrifugation, and the supernatant (25 liters per batch) was used for isolation of the hormone (Table 1). Biological activity was assayed by a method similar to that reported in (*6*).

In the final step of purification, the active fractions were pure enough to give only one positive spot with iodine or fluorescamine (Fluram, Roche) on silica gel (Merck) thin-layer chromatography under each of two solvent systems: (i) *n*-butyl alcohol, acetic acid, and water (12:3:5) at R_F = 0.29, and (ii) *sec*-butyl alcohol and 3 percent NH_4OH (25:11) at R_F = 0.26. The isolated hormone, tremerogen a-13, had a specific activity of 1.0 ng per unit. This hormone is a peptide, because its biological activity was lost by treatment with Pronase (Kakenkagaku) and trypsin (Sigma). When tremerogen a-13 was hydrolyzed with 5.7*N* hydrochloric acid at 110°C for 20 hours, the amino acid ratio of the hydrolyzate was Asp, 2.00 (*2*); Ser, 0.99 (*1*); Glu, 1.07 (*1*); Pro, 0.95 (*1*); Gly, 4.88 (*5*); Val, 1.07 (*1*); Arg, 0.97 (*1*); Cys, 0.36 (*1*). The tryptophanyl residue was not present in this peptide, as judged from the weak absorbance at 280 nm.

The amino acid sequence of tremerogen a-13 was determined by the microscale dansyl–Edman procedure combined with the Edman method (Table 2) (*3*). In the proton magnetic resonance spectrum of tremerogen a-13, a signal at δ 0.97 (6H) was assigned to the methyl groups of a valyl residue, while three singlets at δ 1.62 (6H), 1.68 (3H), and 1.69 (3H) were ascribable to four olefinic methyls, indicating the presence of the *trans,trans*-farnesyl moiety (Fig. 2).

The structure of the carboxyl terminus and the presence of the farnesyl group were confirmed as follows. Tremerogen a-13 was treated with thermolysin (Daiwa). The digested solution was extracted with *n*-butyl alcohol, and then the organic phase was washed with water. Amino terminal analysis of the extract revealed only dansyl-Val, and the extract also gave one spot detectable with iodine or fluorescamine on silica gel thin-layer chromatography under two solvent systems: (i) *n*-butyl alcohol, acetic acid, and water (12:3:5) at R_F = 0.68, and (ii) *sec*-butyl alcohol and 3 percent NH_4OH (25:11) at R_F = 0.36.

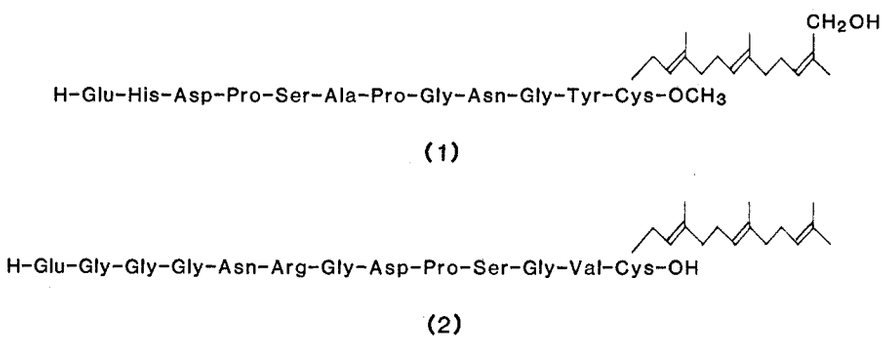

Fig. 1. The structures of tremerogen A-10 (**1**) and tremerogen a-13 (**2**). Glu, glutamic acid; Gly, glycine; Asn, asparagine; Arg, arginine; Asp, aspartic acid; Pro, proline; Ser, serine; Val, valine; and Cys, cysteine.

Table 1. Isolation of tremerogen a-13. *Bioassay*: A-type cells (IFO 9310) were cultured for 72 hours in medium containing 0.4 percent glucose and 0.4 percent Soytone at 26.5°C. The culture medium was diluted with eight volumes of sterilized water, and 1.0 ml of diluted medium containing A-type cells was added to the freeze-dried samples from each of the purification steps. After incubation at 26.5°C for 18 hours, one drop of assay medium was put on a glass slide and observed microscopically for conjugation tube formation. We define one unit of tremerogen a-13 as the minimum amount that can induce conjugation tube formation in 30 percent of the A-type cells in this bioassay. *Purification*: charcoal (Wakojunyaku) (25 g), successively treated with 3 percent EDTA, acetic acid, and pyridine was added to the supernatant of the a-type cell culture (25 liters). After being shaken for 3 hours, the charcoal was collected by filtration and packed into a column which was then eluted with 50 percent pyridine. The eluent was concentrated under vacuum and lyophilized. The active material was dissolved in 0.05*M* ammonium acetate buffer (*p*H 8.5) and applied to a QAE-Sephadex A-25 column equilibrated with the same buffer. The column was then washed with 0.05*M* pyridine acetate buffer (*p*H 7.0), and the active fraction was recovered with 0.05*M* pyridine acetate buffer (*p*H 5.0). Ammonium acetate was added to the active fraction to the final concentration of 2*M*. This solution was charged on Octyl-Sepharose CL-4B (Pharmacia), the column was washed with 10 percent ethyl alcohol in 0.05*M* ammonium acetate, and eluted with a gradient of 10 to 80 percent ethyl alcohol in 0.05*M* ammonium acetate. The active fractions were combined, concentrated, and lyophilized, then subjected to Sephadex LH-20 column chromatography with 80 percent methyl alcohol in 0.05*M* ammonium acetate.

Purification step	Total dry weight (mg)	Total activity (units)	Specific activity (μg/unit)
Culture medium (25 liters)	250,000	400,000	620
Charcoal	1,800	270,000	6.7
QAE-Sephadex A-25		140,000	
Octyl-Sepharose CL-4B	0.2	100,000	0.002
Sephadex LH-20	0.1	100,000	0.001

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Table 2. Amino acid sequence analysis of tremerogen a-13. Abbreviations: dansyl, 1-dimethylaminonaphthalen-5-sulfonyl; PTH, phenylthiohydantoin.

Analysis	Degradation step												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Dansyl-amino acid	Glu	Gly	Gly	Gly	Asp	Arg	Gly	Asp	Pro	Ser	Gly	Val	Cys (O_3H)
PTH-amino acid		Glu				Asn			Asp				
Possible sequence	H-Glu	- Gly	- Gly	- Gly	- Asn	- Arg	- Gly	- Asp	- Pro	- Ser	- Gly	- Val	- Cys-X

This indicated that the glycyl-valyl bond was hydrolyzed. The lipophilic fragment (**3**) was extracted with *n*-butyl alcohol, and the extract was treated with acetic anhydride and methanol to yield the acetylated derivative (**4**). The structure of **4** was determined from its mass spectrum (Fig. 3) to be *N*-acetylvalyl-(*S*-farnesyl)-cysteine methyl ester. However, a carbomethoxyl signal was not observed in the proton magnetic resonance spectrum of the original peptide. Thus, the ester group in **4** must be formed during the acetylation of **3** (*7*). Moreover, thermolysin has no amidase and esterase activity (*8*). In conclusion, the carboxyl terminal amino acid of tremerogen a-13 is *S-trans,trans*-farnesylcysteine. The whole structure of this hormone (**2**) is shown in Fig. 1. Tremerogen a-13 is a tridecapeptide and is unusual in containing five glycyl residues, three of which exist in the repeated sequence -Gly-Gly-Gly-.

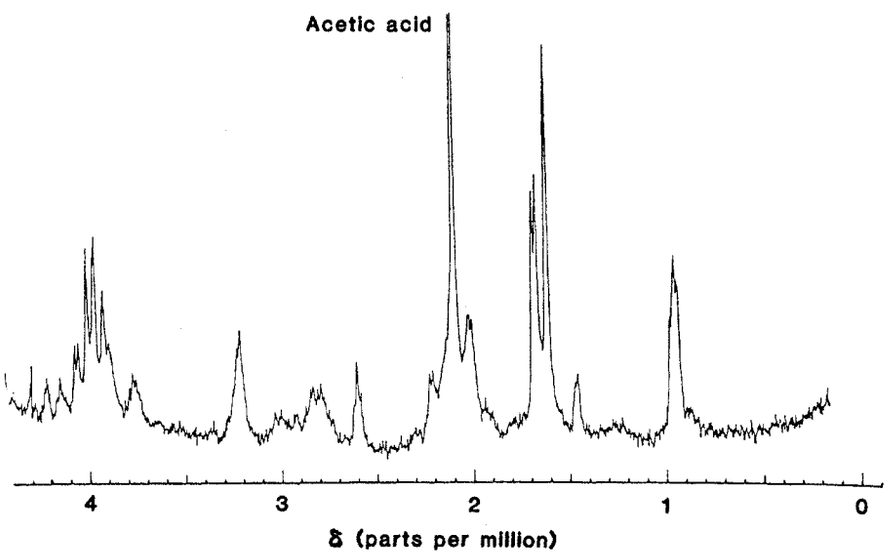

Fig. 2. Proton magnetic resonance spectrum of tremerogen a-13 (instrument, JEOL FX-400 FT-NMR). The sample (0.1 mg) was dissolved in 0.3 ml of D_2O containing 0.5 percent formic acid.

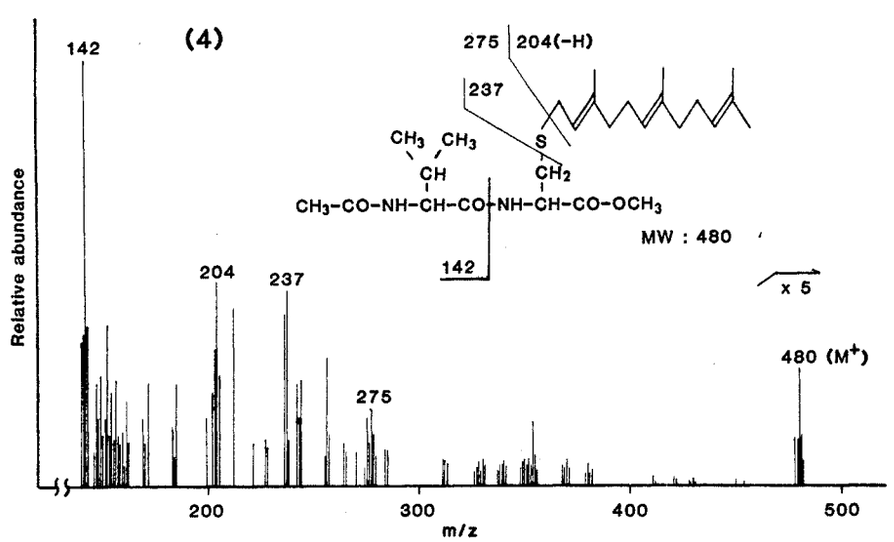

Fig. 3. The mass spectrum of the acetylated derivative (**4**) of the lipophilic fragment; *m/z* is mass-to-charge ratio.

Both tremerogen A-10 and tremerogen a-13 have amino-terminal glutamic acid and the amino acid sequence -Asp-Pro-Ser- in their molecules. The carboxyl terminal amino acid sequences in these hormones are similar, the most striking feature being the farnesyl skeletons blocking the sulfhydryl groups of cysteines. A study of synthetic analogs of tremerogen A-10 suggests that the lipophilic side chain has an important role in biological activity (*9*). The carboxyl terminus of tremerogen a-13 is free, whereas that of tremerogen A-10 is blocked. The study of synthetic analogs (*9*) has revealed a requirement for a blocked carboxyl terminus in tremerogen A-10 agonists.

In addition to the tremerogens, the structures of two peptidal hormones have been determined: (i) α factor, a dodecapeptide from *Saccharomyces cerevisiae* belonging to the Ascomycetes (*10, 11*) and (ii) rhodotorucine A, an undecapeptide from *Rhodosporidium torutoides* belonging to the Basidiomycetes (*12*). It is interesting that rhodotorucine A also has *S*-farnesylcysteine as its carboxyl-terminal amino acid.

In *T. mesenterica*, the structures of yeast sex hormones from two compatible mating-type cells have been confirmed. Although the isolation of *a* factor of *S. cerevisiae* has been reported, the structure has not yet been determined (*13*). The single pair of hormones, tremerogen A-10 and tremerogen a-13, should make a contribution to studies on sexual reproduction in fungi and the cell differentiation process in eukaryotes.

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14. We thank M. Fujino and C. Kitada for useful advice, N. Ootake and H. Seto for cultivation of *T. mesenterica* and the measuring of the 400-MHz FT-PMR spectrum, and T. Noguchi for the amino acid analysis. This work was partly supported by a grant from the Ministry of Education, Science, and Culture, Japan.

28 January 1981

Physical Dependence on Morphine Fails to Develop During the Hibernating State

Abstract. *Physical dependence on morphine occurs in a typical fashion during the active state of the mammalian hibernator* Citellus lateralis, *but does not occur when morphine exposure is confined to the hibernating state. Morphine exposure during hibernation can produce stereotyped behavior, thus demonstrating partial responsiveness of the central nervous system to opioids during this natural state.*

In the mammalian central nervous system (CNS), the development of physical dependence is a direct consequence of prolonged opioid action. This phenomenon, commonly characterized by a constellation of autonomic and behavioral signs that appear after abrupt or antagonist-precipitated withdrawal of the opioid agent (*1*), has been observed in response to various opioid doses, durations of exposure, routes of administration, and environmental conditions (*2*).

We now report that, rather than being an inevitable consequence of prolonged exposure of the CNS to opioids, physical dependence may be a state-dependent phenomenon. It develops in a typical fashion in the mammalian hibernator when morphine is administered during the euthermic (nonhibernating) state—that is, while brain activity is comparable to that of mammals that do not hibernate. However, no physical dependence is apparent when morphine exposure is confined to deep hibernation, a state that is initiated and maintained by the CNS (*3*) and that is characterized by striking changes in brain electrophysiological and neurochemical parameters (*4*).

California golden-mantled ground squirrels (*Citellus lateralis*) of both sexes, with no previous exposure to drugs, were studied. During the euthermic state, two 75-mg morphine sulfate pellets (*5*) were implanted in the interscapular region while the animals were lightly anesthetized with ether. After a 72-hour exposure to morphine in their home cages, the animals were transferred to a plexiglass test chamber (17.5 by 21.5 by 24.5 cm; floor covered with wood shavings and cotton nesting material) for determination of the abstinence syndrome. A 20-minute period was allowed for behavioral observation and acclimation. The abstinence syndrome was precipitated with naloxone HCl (1 mg/kg, subcutaneously), and the signs displayed after the naloxone injection were recorded for 30 minutes.

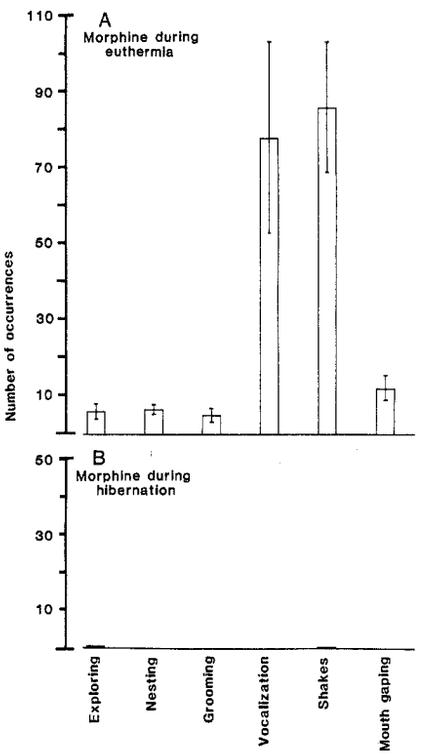

Fig. 1. Quantified signs of abstinence precipitated by naloxone during the nonhibernating (euthermic) state in ground squirrels implanted with morphine pellets. (A) Strong abstinence syndrome (N = 10). (B) No abstinence syndrome (N = 6). Exploring and body shakes were present in negligible amounts. Brackets denote standard error of mean.

Hibernating animals received the same drug treatment as the euthermic animals. The morphine pellets were implanted during the early phase of the animals' hibernation bout (individual period of hibernation); 72 hours later, they were transferred to the plexiglass test chamber and handled briefly to stimulate full arousal from hibernation (*6*). Immediately after reaching the euthermic state, the animals were observed for 20 minutes before naloxone injection (1 mg/kg, subcutaneously) and for the 30-minute period after precipitation of the abstinence syndrome, as described above.

Control animals (euthermic and hibernating) received implants of two placebo pellets (*5*); they were tested 72 hours later with naloxone (1 mg/kg, subcutaneously). All experiments were conducted during the winter season, with the animals maintained throughout at an ambient temperature of 5°C in dim illumination. The onset and offset of illumination paralleled the natural light cycle. All animals in this study had been cycling in and out of hibernation bouts in a normal fashion. Experiments on the effects of morphine exposure during euthermia were conducted in animals that were naturally in this phase of the hibernation cycle at the time of testing.

Euthermic animals (N = 10) displayed heightened locomotor activity during the first 12 hours of morphine exposure that resulted in destruction (flattening) of their normally well-maintained nest. This was followed for the rest of the 72-hour period by a depressed phase that was typified by quiet, sedentary behavior. No animal entered hibernation during this period. Injection of naloxone after the 72-hour administration of morphine precipitated a vigorous abstinence syndrome incorporating a constellation of 14 signs. Six of these signs (exploratory behavior, nesting behavior, grooming, vocalization, body shakes, and a yawn-like gaping of the mouth) were counted each time they occurred (Fig. 1A). The other eight signs, tabulated simply as present or absent during the 30-minute postnaloxone period, included chewing, milky dacryorrhea, digging, dyspnea, eye twitch, flattened posture, ptosis, and a forward thrust of the tail. Vocalization (consisting of a mixture of trilling and chirping sounds) and body shakes (head or trunk) were the most prominently displayed of the counted

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