ISOLATION AND STRUCTURAL ELUCIDATION OF NEW CYCLOTETRAPEPTIDES, TRAPOXINS A AND B, HAVING DETRANSFORMATION ACTIVITIES AS ANTITUMOR AGENTS

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(Received for publication April 26, 1990)

New cyclotetrapeptides, trapoxins A and B were isolated from the culture broth of *Helicoma ambiens* RF-1023. These compounds exhibit detransformation activities against *v-sis* oncogene-transformed NIH3T3 cells (*sis*/NIH3T3) as antitumor agents. The structures were found to be new cyclotetrapeptides, *cyclo*[(*S*)-phenylalanyl-(*S*)-phenylalanyl-(*R*)-pipecolinyl-(2*S*,9*S*)-2-amino-8-oxo-9,10-epoxydecanoyl-] for trapoxin A and *cyclo*[(*S*)-phenylalanyl-(*S*)-phenylalanyl-(*R*)-prolyl-2-amino-8-oxo-9,10-epoxydecanoyl-] for trapoxin B, by X-ray analysis, mass spectrometric, NMR and chemical studies.

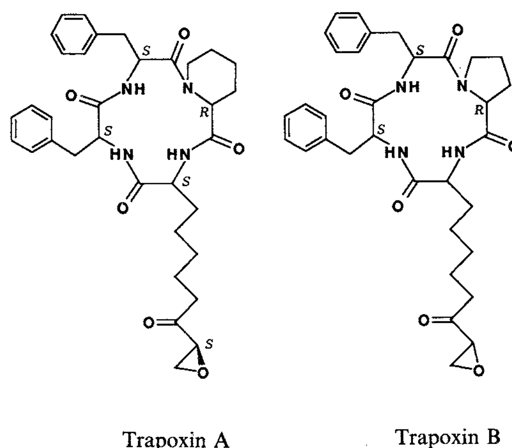
In the course of screening compounds for detransformation activity[1)] as antitumor antibiotics, the new cyclotetrapeptides designated trapoxins A (**1a**) and B (**1b**), were isolated from a fermentation broth of *Helicoma ambiens* RF-1023. The taxonomy, fermentation, isolation, physico-chemical properties, biological properties and structures of **1a** and **1b**, which are related to the antibiotics WF-3161,[2~4)] HC-toxin,[5~8)] chlamydocin,[9)] Cyl-1[10)] and Cyl-2,[11,12)] are described in this paper.

Materials and Methods

Taxonomy

Taxonomic studies of strain RF-1023 are summarized as follows. Colonies grow slowly on V8-juice agar. Conidiophores are simple or have a few branches. The lower sterile part is straight and rigid, the upper fertile part is frequently geniculate and bent, brown in the middle part and very pale brown toward the tip, 65~270 μm in length and 3.0~4.5 μm in width. Conidia are 14~22 μm in diameter across the coil; the conidial filament is 5.5~9.0 μm at the widest part, 7~9-septate. The basal cell is *U*-shaped with an indistinct scar, has a highly guttulate content, is pale to light olive colored. Based on the taxonomic properties described above, strain RF-1023 has been identified as *H. ambiens* Morgan (1892).[13)] Strain RF-1023 has been deposited with the Fermentation Research Institute,

Fig. 1. Structure of trapoxins A and B.



Agency of Industrial Science and Technology, Japan, under the accession No. FERM BP-2751.

Fermentation

A slant culture of strain RF-1023 was used to inoculate a seed medium (100 ml) containing Polypeptone 1.0%, glucose 2.0%, beef extract 0.3%, yeast extract 0.2%, NaCl 0.1%, tap water (pH 7.0) in a 500-ml Sakaguchi flask, and cultured at 28°C on a rotary shaker at 120 rpm for 72 hours. The seed culture was then used at the rate of 4% to inoculate 100 ml of the production medium in each of twenty four 500-ml Erlenmeyer flasks and cultivation was carried out for 10~14 days at 28°C under agitation at 180 rpm. The production medium contained 1 liter of 20% potato decoction and sucrose 20 g (pH 7.0).

Isolation

Since the activity was observed in broth filtrate and mycelia, the active compounds were extracted from both fractions after separation by centrifugation. The filtrate was extracted twice with 600 ml of ethyl acetate. The extracts were washed with NaCl solution, dried over anhydrous sodium sulfate and evaporated *in vacuo* to dryness, giving 317 mg of crude extract I. On the other hand, the mycelia were extracted with 500 ml of methanol. The methanol extract was concentrated *in vacuo* to about 50 ml and extracted twice with 100 ml of ethyl acetate. After the usual workup, a crude extract II of 240 mg was obtained. The combined extracts (557 mg) were subjected to preparative TLC, giving A and A+B fractions (Merck KGF: solvent; toluene - EtOAc (1 : 1); Rf: A fraction = 0.20, A+B fraction = *ca.* 0.15; detection, UV 254 nm). The latter fraction was further purified by preparative TLC to afford the A and B fractions, 55 mg and 72 mg, respectively (solvent: CH_2Cl_2 - MeOH (9 : 1), Fraction A: Rf = 0.60, B: Rf = 0.50). The A fraction (245 mg) was recrystallized from 2-PrOH - H_2O to yield 184 mg of trapoxin A (**1a**) as colorless needles of mp 173~174°C, which was used for X-ray crystallographic analysis. Recrystallization of the B fraction (72 mg) from MeOH afforded 70 mg of trapoxin B (**1b**) as colorless needles of mp 188~190°C.

Amino Acid Analysis

A mixture of sample (0.5 mg) and 6 N HCl (0.5 ml) was heated in a sealed glass tube at 110°C for 20 hours. After cooling, the reaction mixture was evaporated *in vacuo* to dryness, and one portion of the residue was dissolved with a solution of sodium citrate buffer of pH 2 to make a mixture of 20 nmol/ml. The mixture was analyzed with the amino acid autoanalyzer.

Determination of Absolute Configurations of Amino Acids

The acid hydrolysates of **1a** and **1b** were analyzed by HPLC on chiral columns. Rt's were described.

(a) Phenylanine (Phe): D-Phe; Rt = 6.4 minutes, L-Phe; Rt = 7.9 minutes, Phe of trapoxin A (L-Phe); Rt = 8.1 minutes, Phe of trapoxin B (L-Phe); Rt = 8.2 minutes. Condition of HPLC: Column, Crownpak CR 4 × 150 mm (Daicel Chemical Industries, Ltd.); solvent, 0.1% $HClO_4$; flow rate, 0.8 ml/minute; detection, UV 210 nm.

(b) Proline (Pro): L-Pro; Rt = 4.7 minutes, D-Pro; Rt = 7.5 minutes, Pro of trapoxin B (D-Pro); Rt = 7.7 minutes. Condition of HPLC: Column, TSK gel Enantio L1 5 μm 4.6 × 150 mm (Tosoh MFG Co., Ltd.); solvent, 0.25 mM $CuSO_4$, flow rate; 1.0 ml/minute, detection; UV 254 nm.

(c) Pipecolic Acid (Pip): L-Pip; Rt = 6.2 minutes, D-Pip; Rt = 5.2 minutes, Pip of trapoxin A (D-Pip); Rt = 5.2 minutes. Condition of HPLC: column, TSK gel Enantio L1 5 μm 4.6 × 150 mm (Tosoh MFG Co., Ltd.); solvent, 0.25 mM $CuSO_4$; flow rate, 1.0 ml/minute; detection, UV 254 nm.

Biological Assays

a) Detransformation: The ability of trapoxins A and B to flatten the *sis* oncogene-transformed NIH3T3 cells (*sis*/NIH3T3) was examined. Five thousand cells of *sis*/NIH3T3 were inoculated into each well of a 96 well plate in 100 μl of DULBECCO's modified minimum essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS, Flow Laboratories). After overnight incubation at 37°C, the cells were given 100 μl D-MEM (10% FBS) containing various concentrations of trapoxins A and B. With further incubation at 37°C, the morphological change of the cells was observed under a microscope, and the minimum concentrations of trapoxins A and B needed for the flattening were determined.

b) Growth Inhibition: Colorimetric MTT assay[14] was used to determine the inhibitory activity of

trapoxins A and B against the growth of *sis*/NIH3T3. Five thousand cells of *sis*/NIH3T3 were put into each well of a 96 well plate in 100 μl of D-MEM (10% FBS), and incubated overnight at 37°C in a 5% CO_2 incubator. Different concentrations of trapoxins A and B were added to the cells, which were incubated for 48 hours at 37°C. MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide} at 150 μg was added to the cell culture, and the plate was incubated at 37°C for 4 hours. The acid-SDS solution (100 μl of 0.01 N HCl in 10% SDS solution) was inoculated into blue crystals. The plate was read at 570 nm using an autoreader (Dynatech).

Results and Discussion

Structural Elucidation of Trapoxins A (1a) and B (1b)

The physico-chemical properties of **1a** and **1b** are shown in Table 1. These compounds give positive color reactions for Dragendorff and phosphomolybdic acid, but not for ninhydrin. The ^1H and ^{13}C NMR spectra are shown in Figs. 2 and 3. The molecular formulas of **1a** and **1b** were determined as $C_{34}H_{42}N_4O_6$ and $C_{33}H_{40}N_4O_6$, respectively, by HR liquid SI-MS (HRLSI-MS) and elemental analysis.

Table 1. Physico-chemical properties of trapoxins A and B.

	Trapoxin A	Trapoxin B
Appearance	Colorless needles	Colorless needles
MP (°C)	173 ~ 174 (2-PrOH - H_2O)	188 ~ 190 (MeOH)
Molecular formula	$C_{34}H_{42}N_4O_6$ (602)	$C_{33}H_{40}N_4O_6$ (588)
HRLSI-MS $(M+H)^+$ (*m/z*)	603.3178	589.3020
Elemental analysis	$C_{34}H_{42}N_4O_6 \cdot \frac{1}{5}H_2O$	$C_{33}H_{40}N_4O_6 \cdot \frac{1}{2}H_2O$
Calcd:	C 67.35, H 7.05, N 9.24	C 66.31, H 6.91, N 9.37
Found:	C 67.40, H 7.13, N 9.42	C 66.00, H 6.78, N 9.40
$[\alpha]_D^{25}$ MeOH	−63.7° (*c* 0.515)	−75.3° (*c* 0.517)
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	End absorption, 235 (sh, 75)	End absorption, 235 (sh, 90)
IR λ_{max} ($CHCl_3$) cm^{-1}	3410, 1710, 1680, 1522	3402, 1706, 1678, 1521
Amino acid analysis	L-Phe (2), D-Pip (1)	L-Phe (2), D-Pro (1)

Fig. 2. ^1H NMR spectra of trapoxins A and B (200 MHz, $CDCl_3$).
(A) Trapoxin A, (B) trapoxin B.

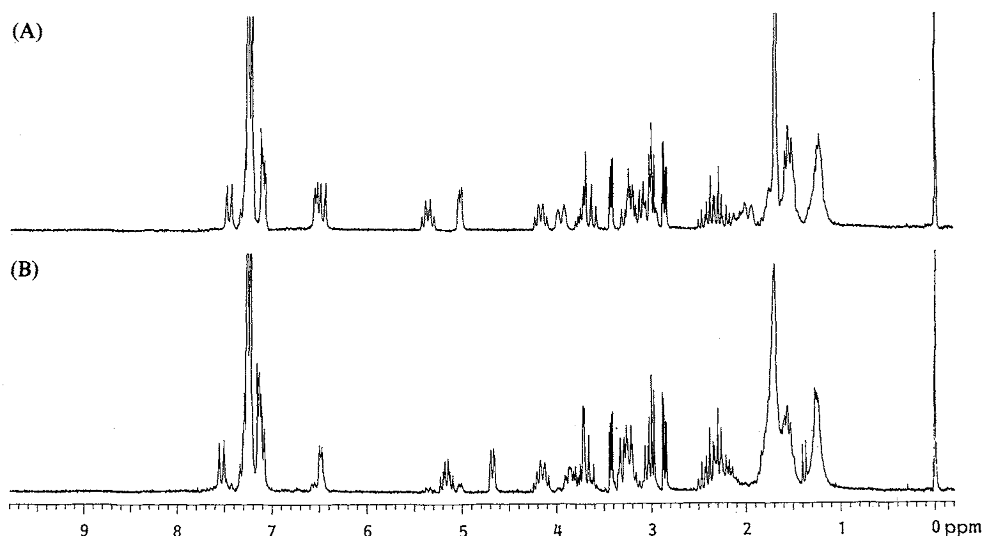
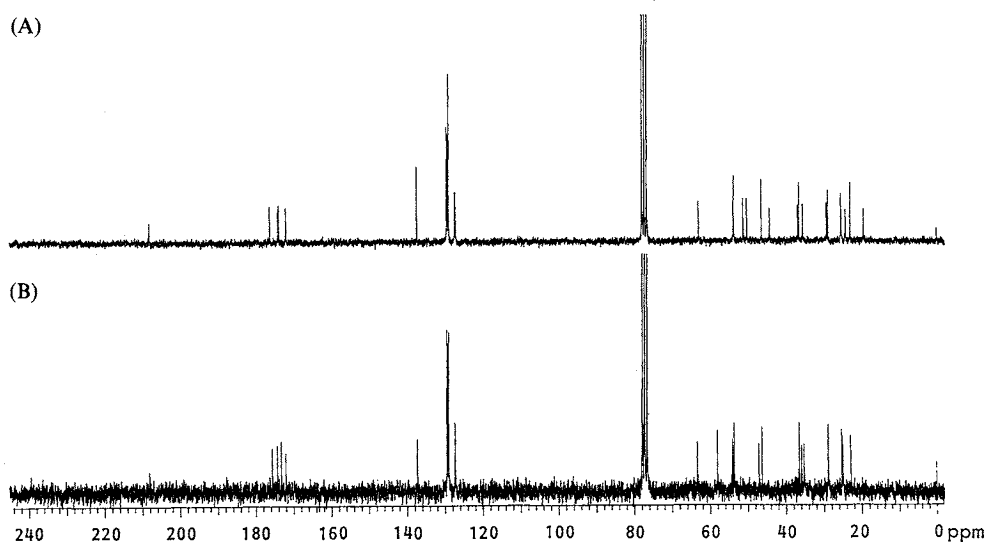


Fig. 3. ^{13}C NMR spectra of trapoxins A and B (50 MHz, $CDCl_3$).
(A) Trapoxin A, (B) trapoxin B.



The UV spectra of these compounds had the end absorption and shoulder band at 235 nm. The IR spectra of **1a** and **1b** showed absorption bands at 3410, 1710, 1680 and 1522 cm^{-1} for the former, and at 3402, 1706, 1678 and 1521 cm^{-1} for the latter, suggesting the presence of peptides in the molecules. Acid hydrolysis of **1a** (6 N HCl, 110°C, 20 hours) afforded Phe and Pip at a molar ratio 2:1, and that of **1b** gave Phe and Pro at a molar ratio 2:1. The absolute configuration of these amino acids were determined as L-Phe, D-Pip and D-Pro by HPLC using chiral columns. The ^1H and ^{13}C NMR spectra of **1a** and **1b** closely resembled each other, although the number of methylene groups of **1b** was one less than that of **1a**. The ^1H NMR spectra of **1a** and **1b** indicated the presence of a terminal epoxy group at δ 2.86 (1H, dd, J=2.4 and 5.8 Hz), 2.98 (1H, dd, J=4.6 and 5.8 Hz) and 3.43 (1H, dd, J=2.4 and 4.6 Hz) for **1a**, and at δ 2.86 (1H, dd, J=2.4 and 5.8 Hz), 3.00 (1H, dd, J=4.6 and 5.8 Hz) and 3.42 (1H, dd, J=2.4 and 4.6 Hz) for **1b**. In the ^{13}C NMR spectra, these compounds displayed the signals due to a terminal epoxy group attached to a ketone at δ 46.38 (t), 53.82 (d) and 208.24 (s) for **1a**, and at δ 46.37 (t), 53.81 (d) and 208.25 (s) for **1b**, and the signals corresponding to four amide carbonyl carbons, δ 172.05 (s), 173.95 (s), 174.22 (s), and 176.35 (s) for **1a**, and δ 172.15 (s), 173.32 (s), 174.34 (s) and 175.74 (s) for **1b**. Reduction of the epoxy ketone group of **1a** with diphenylselenide and sodium borohydride[15] gave β-hydroxyketone (**2**) (LSI-MS: m/z 605 $(M+H)^+$). These results suggest that **1a** and **1b** have 2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe) residue, which contains five methylene groups and an epoxy ketone moiety, as reported previously.[2~12] In the LSI-MS, the characteristic peaks, expressed by the immonium ions ($^+NH_2$=CHR), of amino acid residues constituting **1a** and **1b**, were observed at m/z 84 ($C_5H_{10}N$, Pip), 120 ($C_8H_{10}N$, Phe) and 170 ($C_9H_{16}NO_2$, Aoe) for **1a**, and at m/z 70 (C_4H_8N, Pro), 120 (Phe) and 170 (Aoe) for **1b** (Fig. 4).[10] These elemental compositions of the peaks were confirmed by HRLSI-MS.

Based on NMR and LSI-MS analysis, it was concluded that **1a** and **1b** were the cyclotetrapeptides composed of two Phe, a Pip and an Aoe for **1a**, and of two Phe, a Pro and an Aoe for **1b**, and there were three possible amino acid sequences, *cyclo*[-Phe-Phe-Pip-Aoe-] (**I**), *cyclo*[-Pip-Phe-Phe-Aoe-] (**II**) and *cyclo*[-Phe-Pip-Phe-Aoe-] (**III**) for **1a** as shown in Fig. 5.

Fig. 4. LSI-MS of trapoxins A and B.

(A) Trapoxin A, (B) trapoxin B.

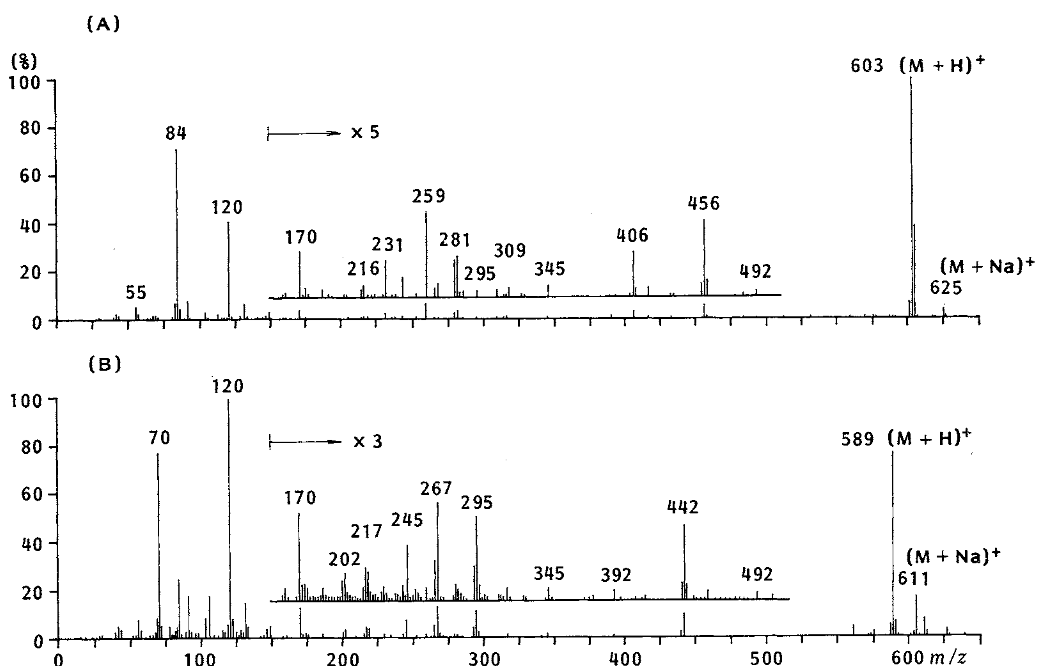
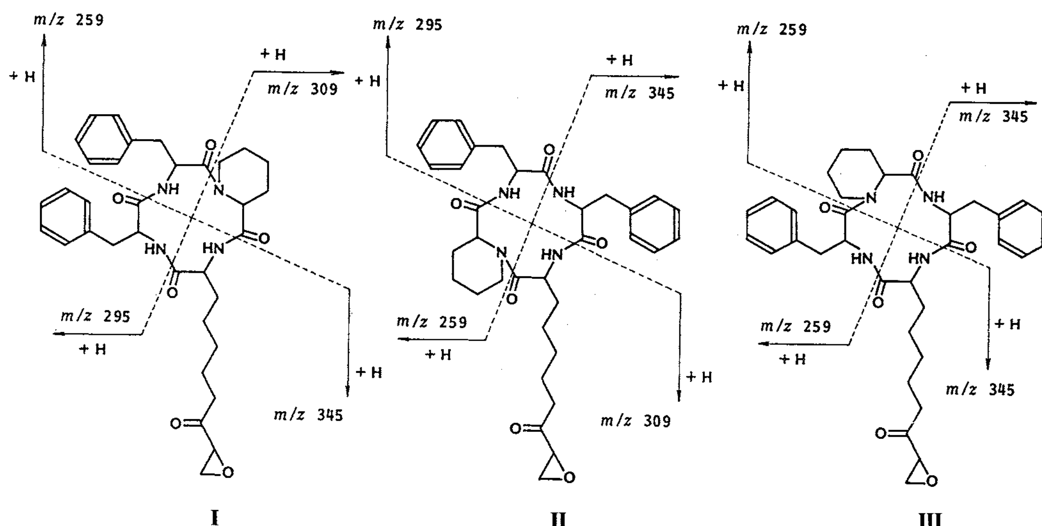
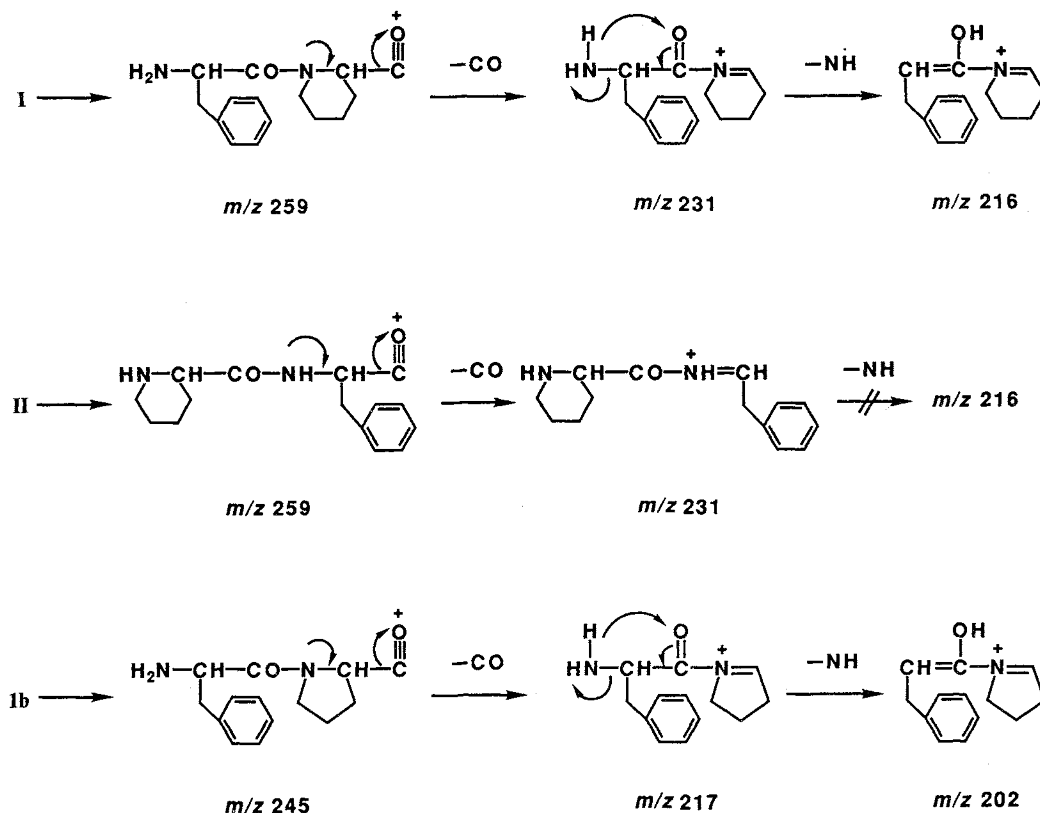


Fig. 5. Three possible structures of trapoxin A.



Further structural elucidation was carried out by LSI-MS. In the HRLSI-MS of **1a**, the protonated molecule $(M+H)^+$ showed at m/z 603.3178 ($C_{34}H_{43}N_4O_6$). The peak at m/z 456.2505 ($C_{25}H_{34}N_3O_5$) was assigned to the fragment ion generated by the repulsion of Phe from $(M+H)^+$. The peak at m/z 309.1803 ($C_{16}H_{25}N_2O_4$) was attributed to the fragment ion $(Pip\text{-}Aoe)^+$ or $(Aoe\text{-}Pip)^+$, which could not be produced from *cyclo*[-Phe-Pip-Phe-Aoe-] (**III**). The peak at m/z 259.1447 ($C_{15}H_{19}N_2O_2$) ascribable to the

Fig. 6. Fragmentation paths of trapoxins A and B.

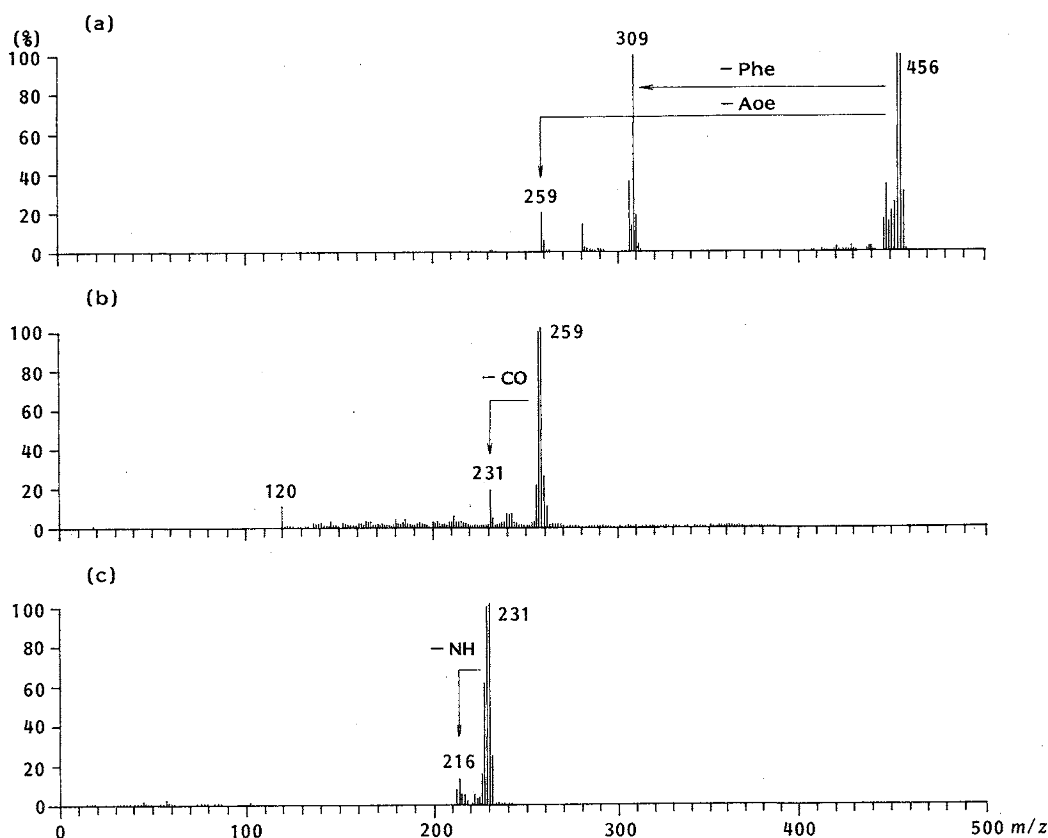


fragment ion (Phe-Pip)$^+$ successively decomposed to the peak at *m/z* 231.1485 ($C_{14}H_{19}N_2O$) by a loss of CO, which was followed by a loss of NH to give the peak at *m/z* 216.1395 ($C_{14}H_{18}NO$). This fragmentation path, which was unable to arise from (Pip-Phe)$^+$ as shown in Fig. 6, was supported by the metastable ion (MI) spectra obtained by B/E linked scan technique as shown in Fig. 7.

Therefore, *cyclo*[-Pip-Phe-Phe-Aoe-] (II) was excluded by the fragmentation path described above. Thus, the amino acid sequence of **1a** was determined as *cyclo*[-Phe-Phe-Pip-Aoe-] (I). The relative configuration of **1a** was confirmed by X-ray crystallographic analysis (R value is 0.04)[16] (Fig. 8), and its absolute configuration was unambiguously determined as *cyclo*[(*S*)-Phe-(*S*)-Phe-(*R*)-Pip-(2*S*,9*S*)-Aoe-] on the base of previously mentioned amino acid chirality analysis. In a similar manner, the HRLSI-MS of **1b** showed fragment ion peaks at *m/z* 589.3020 ($C_{33}H_{41}N_4O_6$, $(M+H)^+$), 442.2341 ($C_{24}H_{32}N_3O_5$, $(M+H-Phe)^+$), 295.1657 ($C_{15}H_{23}N_2O_4$, (Pro-Aoe)$^+$), 245.1289 ($C_{14}H_{17}N_2O_2$, (Phe-Pro)$^+$), 217.1342 ($C_{13}H_{17}N_2O$) and 202.1235 ($C_{13}H_{16}NO$), and the fragmentation path, *m/z* 245→*m/z* 217→*m/z* 202 was supported by the MI spectra of **1b** (Fig. 6). These facts also established the amino acid sequence of **1b** as *cyclo*[-Phe-Phe-Pro-Aoe-]. Since **1a** and **1b** are produced from the same strain, we assume that the configuration Aoe of **1b** is identical with that of **1a**. However, the stereochemistry of Aoe in **1b** remains ambiguous.

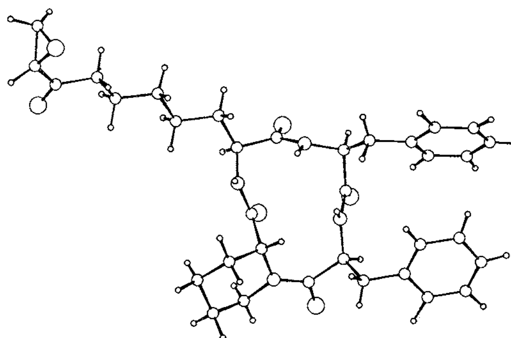
In conclusion, trapoxins A and B are new cyclotetrapeptides similar to the antibiotics WF-3161, HC-toxin, chlamydocin, Cyl-1 and Cyl-2, which are composed of a unique amino acid of Aoe and three

Fig. 7. MI spectra of *m/z* 456, 259 and 231 from trapoxin A.
(a) *m/z* 456, (b) *m/z* 259, (c) *m/z* 231.



amino acids.

Fig. 8. X-ray crystal structure of trapoxin A.



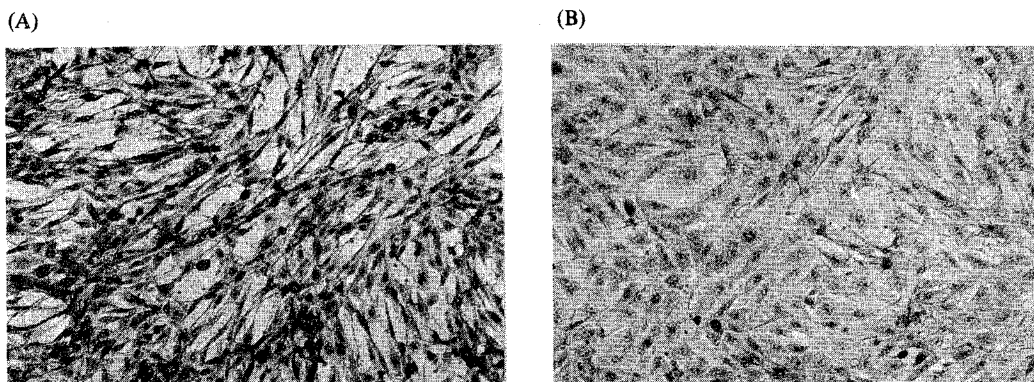
Biological Activities of Trapoxin A

The detransformation activities of trapoxins A (**1a**) and B (**1b**) against *sis* oncogene-transformed NIH3T3 cells were almost the same. A lower concentration of **1a** (1 ng/ml) was enough to change the transformed phenotype of *sis*/NIH3T3 into the flat one, compared to the concentration (~200 ng/ml) which showed 50% inhibition of the growth of the same cells. The detransformation activity was visible within 6 hours after drug administration, which showed that this observation was not related to selection of the flat and drug-insensitive cells, but to the direct action of the drug on the cells. **1a** had no effect even at the concentration of 250 μg/ml against human red blood cells on incubation at 37°C for 2 days.

Cyclo[(*S*)-Phe-(*S*)-Phe-(*R*)-Pip-(*S*)-2-amino-8-oxo-10-hydroxydecanoyl-] (**2**) did not show detransformation activity.

Fig. 9. Morphology of *sis* oncogene-transformed NIH3T3 cells.

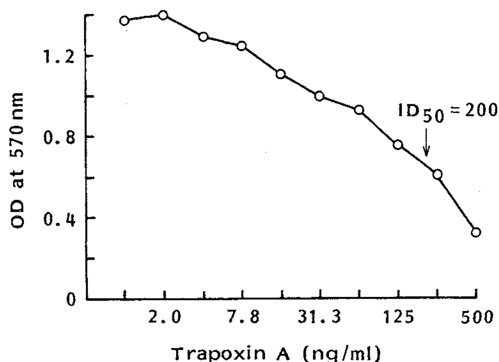
(A) Treated with trapoxin A, (B) not treated.



Experimental

NMR spectra were measured with a Varian VXR-200 spectrometer in $CDCl_3$ solution with TMS as the internal standard. Mass spectra were obtained with a Hitachi M-90 mass spectrometer and IR spectra with a Jasco DS-403G spectrometer. Amino acid analysis was carried out with a Hitachi amino acid autoanalyzer 835.

Fig. 10. Inhibition of trapoxin A on the growth of *sis* oncogene-treated NIH3T3 cells.



Conversion of **1a** into *cyclo*[(*S*)-Phe-(*S*)-Phe-(*R*)-Pip-(*S*)-2-amino-8-oxo-10-hydroxydecanoyl-] (**2**)

To a dissolved solution of diphenyl diselenide (8 mg, 0.025 mmol) in dry EtOH (0.2 ml) was added $NaBH_4$ (1.9 mg) and AcOH (0.5 μl) under argon with stirring. To the mixture was added a solution of **1a** (10 mg) in EtOH - THF (1 : 1) (100 μl) and the resulting mixture was stirred at room temperature for 30 minutes. The reaction mixture was extracted with EtOAc (2.5 ml). The extract was washed with sat NaCl, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to dryness, leaving a residue (9 mg). This was purified by preparative TLC (Merck KGF, CH_2Cl_2 - MeOH (1 : 1), Rf = 0.5), affording **2** as an oil (8 mg, yield 80%). **2**: LSI-MS: m/z 605 $(M+H)^+$ (Calcd for $C_{34}H_{44}N_4O_6+H$). ^1H NMR δ 3.85 (2H, m), 2.62 (2H, t, J=5 Hz), 2.39 (2H, t, J=7 Hz). ^{13}C NMR δ 19.45 (t), 23.39 (t), 24.17 (t), 25.14 (t), 25.30 (t), 28.47 (t), 28.99 (t), 35.47 (t), 36.83 (t), 43.21 (t), 44.21 (t), 44.58 (t), 50.20 (d), 51.16 (d), 53.71 (d), 58.11 (t), 62.90 (d), 127.19 (d), 127.41 (d), 129.05 (d) (×4), 129.37 (d) (×2), 129.57 (d) (×2), 137.44 (s), 137.52 (s), 172.15 (s), 173.99 (s), 174.21 (s), 176.37 (s), 212.28 (s).

NMR Data of Trapoxin A (**1a**)

^1H NMR δ 7.46 (1H, d, J=10 Hz), 7.27 (10H, s), *ca.* 7.11 (1H, m), *ca.* 6.46 (2H, m), 5.36 (1H, q, J=9 Hz), 5.02 (1H, d, J=5 Hz), 4.17 (1H, q, J=9 Hz), 3.96 (1H, d, J=9 Hz), 3.43 (1H, dd, J=2.4 and 4.6 Hz), 2.98 (1H, dd, J=4.6 and 5.8 Hz), 2.86 (1H, dd, J=2.4 and 5.8 Hz), 4.20~2.80 (m), 2.50~1.70 (m), 1.56 (2H, m), 1.46 (2H, br). ^{13}C NMR δ 19.43 (t), 22.94 (t), 24.17 (t), 25.39 (t), 25.40 (t), 28.82 (t), 29.18 (t), 35.45 (t), 36.49 (t), 36.80 (t), 44.20 (t), 46.38 (t), 50.23 (d), 51.20 (d), 53.69 (d), 53.82 (d), 63.07 (d), 127.18 (d), 127.43 (d), 129.04 (d) (×4), 129.35 (d) (×2), 129.56 (d) (×2), 137.44 (s) (×2), 172.05 (s), 173.95 (s), 174.22 (s), 176.35 (s), 208.24 (s).

NMR Data of Trapoxin B (**1b**)

^{1}H NMR δ 7.65 (1H, d, J=10 Hz), 7.13 (2H, m), *ca.* 6.40 (1H, br), 7.29~7.23 (10H, m), 5.18 (1H, m), 4.68 (1H, d, J=5 Hz), 4.15 (1H, m), 3.85 (1H, m), *ca.* 3.72 (1H, m), 3.42 (1H, dd, J=2.4 and 4.6 Hz), 3.00 (1H, dd, J=4.6 and 5.8 Hz), 2.86 (1H, dd, J=2.4 and 5.8 Hz), 2.50~1.20 (m). ^{13}C NMR δ 22.99 (t), 25.02 (t), 25.14 (t), 25.41 (t), 28.88 (t), 29.00 (t), 35.29 (t), 35.99 (t), 36.51 (t), 46.37 (t), 47.20 (t), 53.70 (d), 53.81 (d), 54.22 (d), 58.15 (d), 63.49 (d), 127.27 (d), 127.42 (d), 129.03 (d) ($\times$2), 129.15 (d) ($\times$2), 129.36 (d) ($\times$2), 129.55 (d) ($\times$2), 137.44 (s), 137.47 (s), 172.15 (s), 173.32 (s), 174.34 (s), 175.74 (s) and 208.25 (s).

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