CYCLOTHIAZOMYCIN, A NOVEL POLYTHIAZOLE-CONTAINING PEPTIDE WITH RENIN INHIBITORY ACTIVITY

TAXONOMY, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL CHARACTERIZATION

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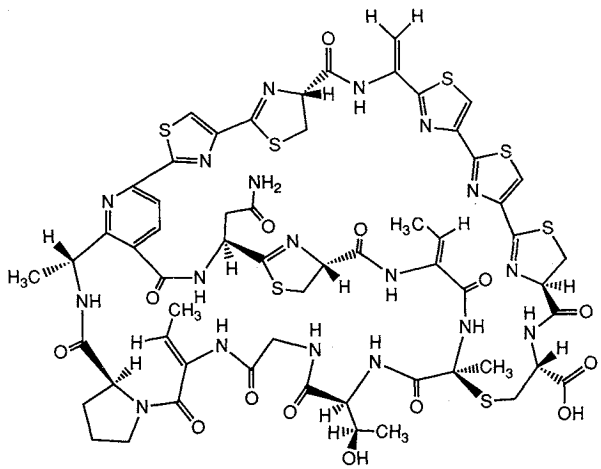
Cyclothiazomycin is a novel renin inhibitor produced by *Streptomyces* sp. NR0516. It was isolated from fermentation broth by extraction with butyl alcohol, QAE-Toyopearl column chromatography and preparative HPLC. Cyclothiazomycin, which was determined to be a unique polythiazole-containing bicyclic peptide, exhibited inhibitory activity against human plasma renin with IC_{50} being 1.7 μM.

The renin angiotensin system is implicated in several forms of hypertension. As a highly specific aspartic protease, renin cleaves angiotensinogen to form angiotensin I. This metabolic intermediate is further cleaved by angiotensin converting enzyme (ACE) to yield angiotensin II, which causes vasoconstriction and stimulates secretion of aldosterone and catecholamine leading to elevation of blood pressure. In this cascade reaction, renin is a rate-limiting enzyme[1,2]. Therefore, renin inhibitors are expected to the most effective in preventing these disorders.

Fig. 1. Structure of cyclothiazomycin.



In recent years, many renin inhibitors have been designed by the peptide mimetic approach[3~5]. In order to obtain further detailed information for the medicinal chemistry of the renin inhibitors, however, it is worth while to seek new types of inhibitors from microorganisms. In the course of a renin inhibitor screening program, cyclothiazomycin (Fig. 1) was isolated from the fermentation broth of *Streptomyces* sp. NR0516. The structure of cyclothiazomycin was determined to be a unique polythiazole-containing bicyclic peptide by spectral analysis[6,7]. In this paper, taxonomy, fermentation, isolation and physico-chemical properties of cyclothiazomycin are reported.

Taxonomy of the Producing Strain

The producing organism, strain NR0516 was isolated from a soil sample collected at Kamakura, Kanagawa, Japan. The spore chains were of the spirals type and each had more than 30 spores per chain. The spores were oval ($0.8 \sim 1.1 \times 1.0 \sim 1.2\ \mu m$) and their surface was smooth. Sclerotic granules, sporangia or flagellated spores were not observed. The cultural and physiological characteristics and the utilization of carbohydrate, which were observed after incubation at 27°C for 2 weeks, are shown in Tables 1, 2 and 3, respectively. The substrate mycelia grew well on all the media tested and were either colorless, yellow or yellowish olive. The aerial mycelia were formed abundantly on yeast extract-malt extract agar, oatmeal

Table 1. Cultural characteristics of *Streptomyces* sp. NR0516.

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Sucrose-nitrate agar	Good, yellow	Thin, pale yellow	Yellow	Pale yellow
Yeast extract-malt extract agar (ISP No.2)	Good, pale yellow	Abundant, light olive gray	Pale yellow	Pale yellow
Oatmeal agar (ISP No. 3)	Good, pale yellowish brown	Abundant, brownish gray	Light olive	Dark yellow
Inorganic salts-starch agar (ISP No. 4)	Good, grayish olive	Abundant, olive gray	Yellowish brown	Light olive
Glycerol-asparagine agar (ISP No. 5)	Good, yellow	Moderate, olive gray	Yellow	Pale yellow
Glucose-asparagine agar	Good, yellow	Thin, light gray	Yellow	Pale yellow
Tyrosine agar (ISP No. 7)	Good, colorless	Moderate, light gray	Light brownish gray	None
Nutrient agar	Good, colorless	Thin, white	Colorless	None

The color names used in this table were based on the Color Standerd (Nihon Shikisai Co., Ltd.).

Table 2. Physiological characteristics of *Streptomyces* sp. NR0516.

Melanin formation	−
Starch hydrolysis	+
Gelatin liquefaction	+
Milk coagulation	−
Milk peptonization	+
Nitrate reduction	−

+: Positive, −: negative.

Table 3. Carbohydrate utilization of *Streptomyces* sp. NR0516.

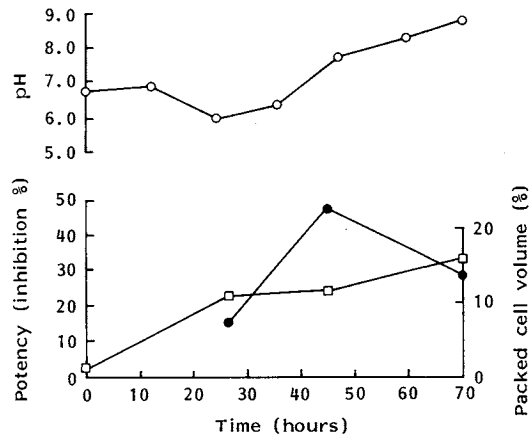
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	+
i-Inositol	+
L-Rhamnose	+
Raffinose	+
D-Mannitol	+

+: Utilized.

agar and inorganic salts-starch agar, and showed grayish color after sporulation. A soluble pigment was yellow on most media. Whole cell hydrolysates of strain NR0516 contained L,L-diaminopimelic acid. Glycine was detected in the cell wall. The strain was thereby placed in the type I cell wall group of LECHEVALIER *et al.*[8]. Based on the taxonomic properties described above, the producing strain NR0516 was assigned to the genus *Streptomyces*. The definite nomenclature of the producing organism will be reported elsewhere.

Fig. 2. Time course of fermentation in a 50-liter jar fermenter.

○ pH, □ packed cell volume, ● potency (10% conc broth).



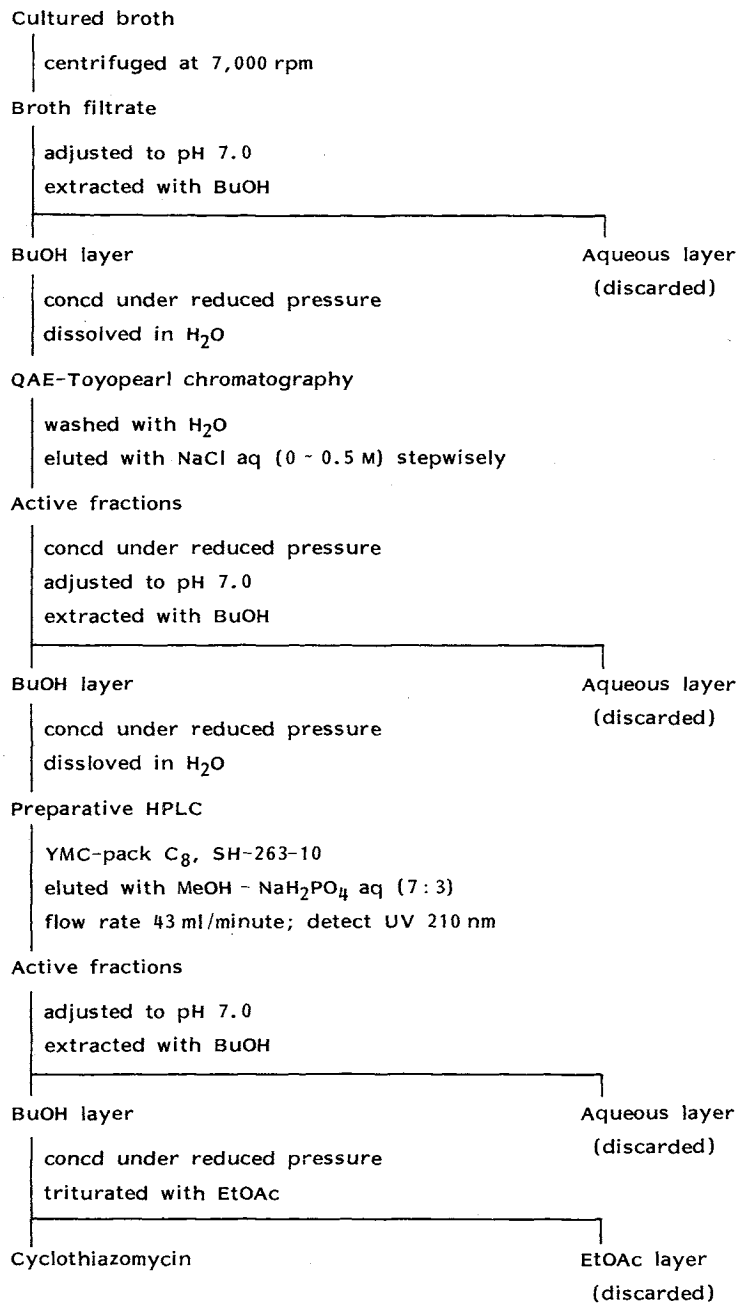
Fermentation

The frozen mycelial suspension of *Streptomyces* sp. NR0516 (15 ml) was inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of a medium consisting of glucose 1.5%, soluble starch 1.0%, Polypeptone 0.5%, meat extract 0.75%, yeast extract 0.1%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.2% and Nissan Disfoam CA-115 0.05%. The medium was adjusted to pH 7.0 before addition of $CaCO_3$. The inoculated flask was incubated on a rotary shaker at 27°C for 3 days at 190 rpm to make a seed culture. The resultant seed culture (12 ml) was transferred into a 3-liter baffled Erlenmeyer flask containing 600 ml of the same medium as above followed by incubation on a rotary shaker at 27°C for 3 days at 100 rpm. This seed culture (600 ml) was transferred into a 50-liter jar fermenter containing 30 liters of the same medium as described above except that 0.3% of Nissan Disfoam CA-115 was added to the medium. The fermentation was carried out at 27°C with aeration of 30 liters per minute and agitation at 350 rpm for 69 hours (Fig. 2).

Isolation

Cyclothiazomycin was isolated from the broth filtrate as outlined in Fig. 3 and the activity was monitored by testing renin inhibitory activity. Human plasma renin activity was measured by radio-immuno assay (MEDIPRO) for angiotensin I which was generated during incubation of plasma at 37°C for 3 hours. The broth filtrate (3.2 liters) was adjusted to pH 7.0 with aqueous NaOH and extracted with butyl alcohol. The organic layer was concentrated under reduced pressure. The residue was dissolved in 100 ml of H_2O and then applied to a QAE-Toyopearl (coarse grade) column and eluted stepwisely with aqueous NaCl solutions (0, 0.3, 0.4 and 0.5 M). The active fractions collected were then adjusted to pH 7.0 with aqueous NaOH and extracted with butyl alcohol. The organic layer was evaporated under reduced pressure to give crude cyclothiazomycin (351 mg). This crude powder was purified by HPLC using a Toso HLC-837 system with a YMC-pack C_8 (30 mm × 250 mm) and eluted with a mixture of methanol and 0.05 M aqueous NaH_2PO_4 solution (7:3) at a flow rate of 43 ml/minute. Cyclothiazomycin detected by UV absorption at 210 nm produced a peak with a retention time of 8.5 minutes. The eluate containing cyclothiazomycin was concentrated to a small volume and then extracted with butyl alcohol at pH 7.0 for desalting. The organic layer was evaporated under reduced pressure and the residue was triturated with ethyl acetate to give a light brown powder (215 mg) of pure cyclothiazomycin.

Fig. 3. Isolation procedure of cyclothiazomycin.



Physico-chemical Properties

Selected physico-chemical properties of cyclothiazomycin are presented in Table 4. Cyclothiazomycin obtained as a sodium salt is soluble in water, chloroform-methanol mixture and mixed aqueous-organic solvents such as MeOH-H_2O, dimethyl sulfoxide (DMSO)-H_2O and acetone-H_2O. It is slightly soluble in methanol, DMSO and *N*,*N*-dimethylformamide. Cyclothiazomycin is detected on TLC plates using short-

wavelength UV light and ninhydrin reagent for visualization. Its UV spectrum (Fig. 4) is similar to that of the peptide antibiotics containing a polythiazole residue including saramycetin[9,10], cystamycin[11], jingsimycin[12] and 5102-2[13], the structures of which are still unknown. The IR spectrum (Fig. 5) shows absorbance at 3350 and 1670 cm^{-1} consistent with a peptide structure. The molecular formula ($C_{59}H_{64}N_{18}O_{14}S_7$) was determined based on HRFAB mass using a dual target probe (Obsd 1495.2733, Calcd 1495.2790 $(M+Na)^+$), elemental analysis, and ^{13}C and ^1H NMR spectroscopic analyses (Figs. 6 and 7).

Fig. 4. UV spectrum of cyclothiazomycin.

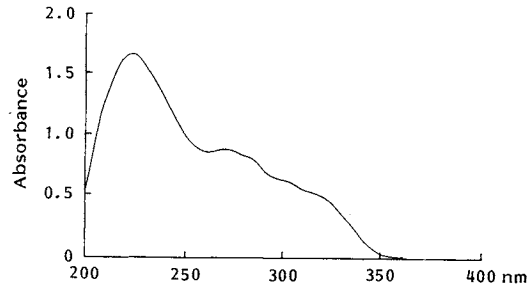


Table 4. Physico-chemical properties of cyclothiazomycin.

Nature	Light brown amorphous powder
Formula	$C_{59}H_{64}N_{18}O_{14}S_7$
MW	1,472
MP	>210°C (dec)
$[\alpha]_D^{24}$ (c 0.69, H_2O)	+46°
HRFAB-MS	Calcd for $C_{59}H_{64}N_{18}O_{14}S_7 \cdot Na$ $(M+Na)^+$: 1,495.2790
	Obsd: 1,495.2733
Elemental analysis	Calcd for $C_{59}H_{63}N_{18}O_{14}S_7Na \cdot 7H_2O$:
	C 43.70, H 4.75, N 15.56, S 13.81, Na 1.41
	Obsd: C 43.66, H 5.25, N 15.18, S 13.82, Na 1.50
UV λ_{max}^{MeOH} (ε) nm[a]	222 (87,000), 270 (41,000), 284 (sh), 300 (sh), 320 (sh)
IR ν_{max} (KBr) cm^{-1}	3350, 1670, 1625, 1615, 1515, 1440, 1385, 1290, 1240, 1170, 1120, 1075, 1040
Amino acid analysis	Gly ×1, L-Thr ×1, L-Asp ×1, L-Pro ×1, L-Cys>3

[a] No change of UV spectrum was observed in acidic and basic solution.

Fig. 5. IR spectrum of cyclothiazomycin.

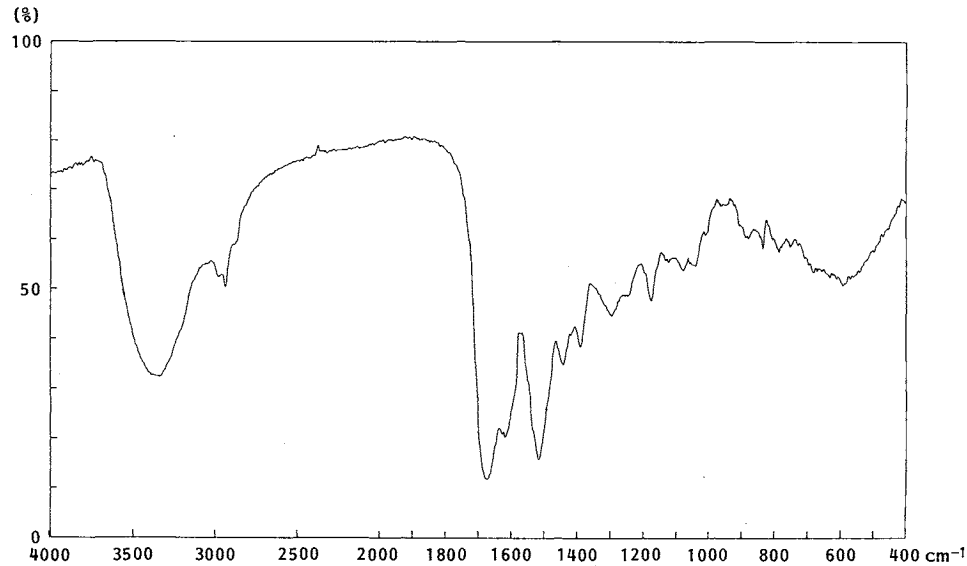


Fig. 6. 500 MHz ^{1}H NMR spectrum of cyclothiazomycin in CD_3OH-H_2O.
Solvent peaks were suppressed by homogate decoupling.

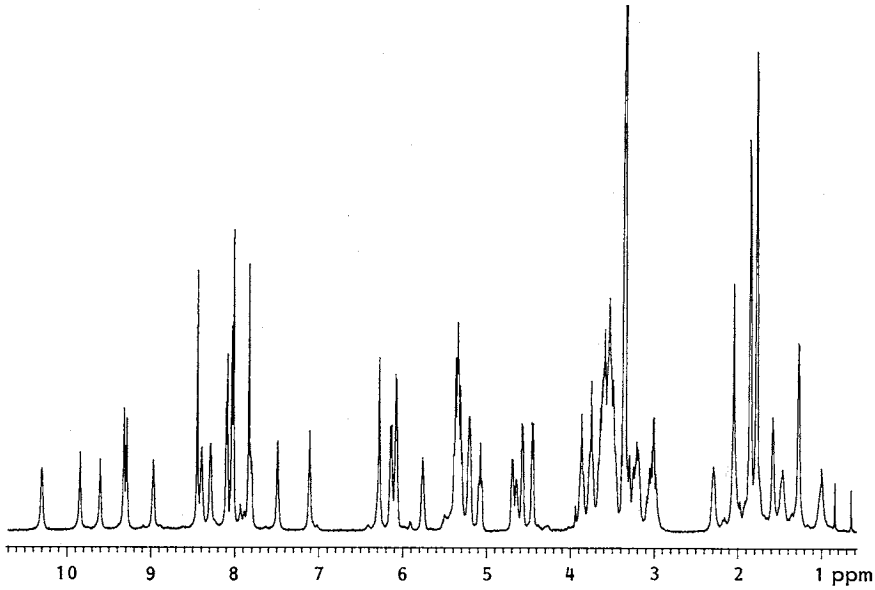
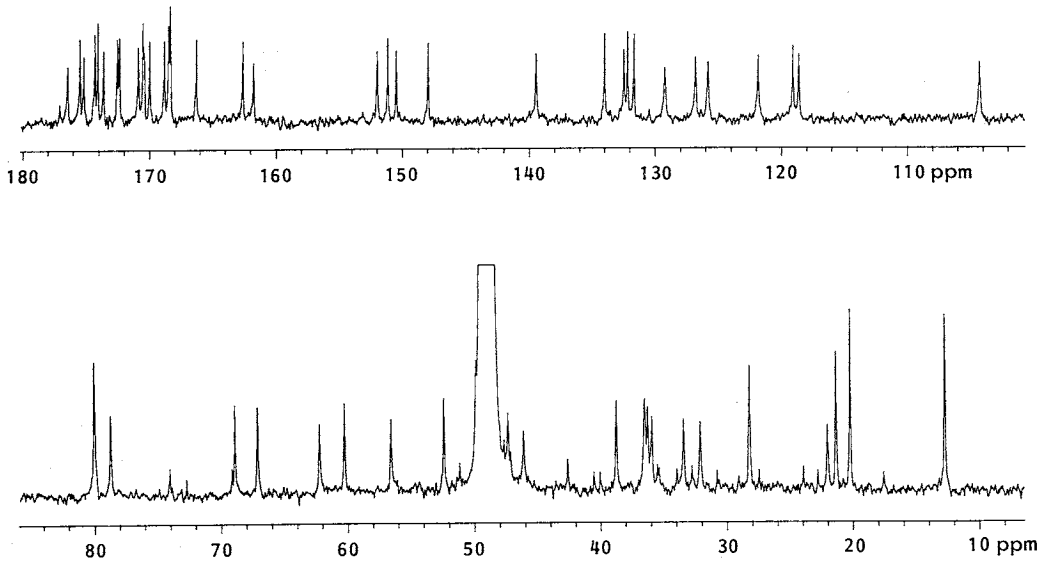


Fig. 7. 125 MHz ^{13}C NMR spectrum of cyclothiazomycin in CD_3OH-H_2O.



Biological Characteristics

Cyclothiazomycin exhibited inhibitory activity against human plasma renin with an IC_{50} of 1.7 μM. This activity was much greater than that against rat plasma renin (IC_{50} 81 μM) and dog plasma renin (IC_{50} 177 μM). Cyclothiazomycin showed selective activity against renin; no inhibitory activity was obtained up to 272 μM against porcine pepsin, another aspartic protease, and several other enzymes

belonging to serine-, thiol- or metallo-proteases. Cyclothiazomycin also showed weak anti-fungal activity. The detailed biological study will be reported in due course.

Discussion

Cyclothiazomycin was isolated from the cultured broth of *Streptomyces* sp. NR0516 by solvent extraction, QAE-column chromatography and preparative HPLC. Since cyclothiazomycin exhibited inhibitory activity against human plasma renin, it may be interesting to investigate the renin inhibitory activity of other polythiazole-containing peptides. Since cyclothiazomycin showed no blood pressure decrease in sodium-depleted squirrel monkeys after po (3 mg/kg) and iv (1 mg/kg) administration (data not shown), it is not suitable for therapeutic use. We believe, however, that clarification of the active site of cyclothiazomycin will provide structure-activity information for medicinal chemistry to prepare useful analogues and that screening of renin inhibitors from nature will provide non-peptide lead compounds for this therapeutic area.

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