TMC-66, a New Endothelin Converting Enzyme Inhibitor Produced by *Streptomyces* sp. A5008

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A new endothelin converting enzyme (ECE) inhibitor, TMC-66 was isolated from the fermentation broth of *Streptomyces* sp. A5008. The structure of TMC-66 was elucidated by spectroscopic analyses to be a new member of benzo[*a*]naphthacenequinone class of antibiotics. TMC-66 had a highly selective inhibitory activity for ECE with an IC_{50} value of 2.9 μM. Taxonomy of the producing strain is also described.

Endothelin (ET) is a 21-residue potent vasoconstrictive peptide produced by vascular endothelial cells from the 38-residue inactive precursor, big endothelin (big ET), *via* specific cleavage at Trp-21-Val-22[1,2]. Endothelin converting enzyme (ECE) catalyzes the conversion and constitutes a potential regulatory step for the production of ET[3]. ECEs have been cloned from rat[4], bovine[5], and human[6], respectively, and reported to be a membrane-bound neutral metalloprotease, which is related to neutral endopeptidase 24.11 (NEP)[7]. ECE inhibitors would be therapeutically useful for treatment of the diseases involving ECE such as hypertension.

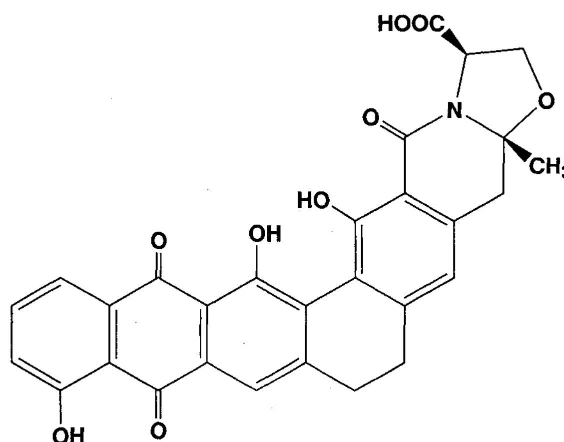
In the course of our screening for selective ECE inhibitors, a new inhibitor, designated as TMC-66 (Fig. 1), was isolated from the culture broth of *Streptomyces* sp. A5008. In this paper, we report the taxonomy and fermentation of the producing strain, isolation, physico-chemical properties, structure determination and biological activities of TMC-66.

Result

Taxonomy

The cultural characteristics of the producing strain A5008 are summarized in Table 1. The substrate mycelium developed well and branched irregularly. Each spore chain, which was of spiral type, had more than 30 spores per chain. The spores were cylindrical, 0.4~0.6×0.7~1.1 μm, and their surface was smooth (Fig. 2). Fragmentation of substrate mycelium, sporangia, or motile spores was not observed. LL-Diaminopimelic acid was detected in the whole-cell hydrolysate of the strain, indicating that the cell wall belongs to type I. On the basis of these morphological and chemical characteristics, the strain A5008 was assignable to the genus *Streptomyces*.

Fig. 1. Structure of TMC-66.
(Relative Stereochemistry only)

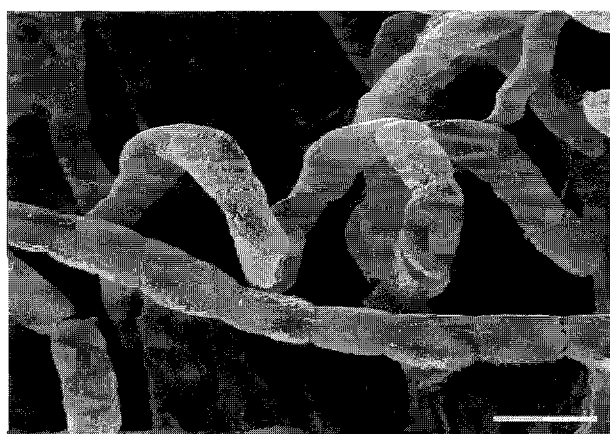


Isolation

The fermentation broth (30 liters) was extracted with 1-butanol (15 liters) and the extract was concentrated to dryness. The butanol extract was suspended in water (0.4 liter) and extracted with ethyl acetate (0.4 liter). The extract was concentrated *in vacuo*, and the residual solid was dissolved in 90% aqueous methanol, and partitioned against *n*-hexane. The aqueous methanol phase was evaporated to yield a crude solid of TMC-66 (7.3 g). The solid was chromatographed on a column of reversed phase silica gel (YMC GEL ODS A60) developed with a mixture of acetonitrile and 10 mM phosphate buffer, pH 3.5, (4 : 6). The active factions were pooled and concentrated *in vacuo* to give an aqueous solution, which was desalted by extraction with ethyl acetate. The extract was concentrated and further purified by Sephadex LH-20 column chromatography with a mixture of dichloromethane - methanol (1 : 1) as an eluant. Evaporation of the active eluate afforded pure TMC-66 (53.2 mg).

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. A5008 grown on yeast extract - malt extract agar.

Bar represents 1 μm.



Physico-chemical Properties

The physico-chemical properties of TMC-66 are summarized in Table 2. It was soluble in methanol, chloroform, DMSO, alkaline water and acetone but practically insoluble in *n*-hexane, neutral and acidic water. The molecular formula of TMC-66 was determined to be $C_{29}H_{21}NO_9$ on the basis of its HR-FAB-MS, ^{1}H and ^{13}C NMR spectral data. The characteristic UV and visible absorption (215, 245, 310 (sh), 365 and 465 nm in methanol) suggested the presence of fused aromatic rings in TMC-66. The IR

Table 1. Cultural characteristics of strain A5008.

Medium		
Yeast extract-malt extract agar (ISP No.2)	G:	Good
	AM:	Abundant, Dull reddish orange (3-15-5)[a]
	R:	Brownish black (6-11-1)
	S:	Reddish brown (2-13-5)
Oatmeal agar (ISP No.3)	G:	Moderate
	AM:	None
	R:	Reddish brown (3-14-5)
	S:	None
Inorganic salts-starch agar (ISP No.4)	G:	None
	AM:	None
	R:	
	S:	None
Glycerol-asparagine agar (ISP No.5)	G:	Good
	AM:	Abundant, Pale reddish orange (3-17-4)
	R:	Reddish black (2-11-1)
	S:	Light brown (5-16-3)
Peptone-yeast extract iron agar (ISP No.6)	G:	None
	AM:	None
	R:	
	S:	None
Tyrosine agar (ISP No.7)	G:	Good
	AM:	Abundant, pale reddish orange (3-17-4)
	R:	Brownish black (3-11-1)
	S:	None

[a] Color codes from the Guide to Color Standard.

Abbreviation: G, Growth; AM, Aerial mycelium; R, Reverse side color; S, Soluble pigment.

Table 2. Physico-chemical properties of TMC-66.

Appearance	Reddish brown powder
MP	215-220°C (dec)
$[\alpha]_D^{24}$	-327 ° (*c* 0.01, $CHCl_3$)
Mass spectrum	
APCI-MS (*m/z*)	$528(M+H)^+$
HR-FAB-MS (*m/z*)	
Found :	$528.1307(M+H)^+$
Calcd :	$528.1295(M+H)^+$
Molecular formula	$C_{29}H_{21}NO_9$
UV λ_{max} (MeOH) nm (ε)	215(27,400), 245(28,600) 310(sh, 11,700), 365(4,400), 465(10,500)
IR ν_{max} (KBr) cm^{-1}	3450, 1725, 1620, 1275

spectrum also inferred the presence of carboxylic acid (a carbonyl band at $1725\,cm^{-1}$ in its free form and $1610\,cm^{-1}$ in its sodium salt), quinone ($1610\,cm^{-1}$), and hydroxyl ($3450\,cm^{-1}$) groups.

Structure of TMC-66

The ^1H and ^{13}C NMR spectral data of TMC-66 (obtained from ^1H, ^{13}C, DEPT and HMQC spectra) are summarized in Table 3. The ^{13}C NMR spectrum displayed 29 signals composed of $-CH_3\times1$, $-CH_2-\times4$, $>CH-\times1$, $=CH-\times5$, $>C<\times14$ and $>C=O\times4$.

The extensive NMR studies including ^1H-^1H COSY, HMBC and selected INEPT experiments revealed the planar structure of TMC-66 as shown in Fig. 3. The ^1H-^1H COSY spectrum connected a sequence of 5-H_2 to 6-H_2, 10-H to 11-H and 16-H to 17-H_2. In the HMBC and selected INEPT experiments, the methylene protons (δ 3.19, 19-H) were coupled to C-2, C-3, C-4 and C-18; 4-H to C-1, C-2, C-5, C-14b and C-19; 5-H to C-4a and C-14b; 6-H to C-6a, C-7 and C-14a; 7-H to C-6, C-7a, C-8, C-13a and C-14a. The carbonyl carbons C-8 (δ 187.3) and C-13 (δ 187.6), which were coupled to 7-H and 12-H, respectively, were observed at low-field due to the hydrogen bondings. The low-field hydrogen-bonding proton (δ 13.26) was assigned to 9-OH by the long range couplings to C-8a, C-9 and C-10. Similarly, the proton (δ 12.41), coupled to C-13a, C-14 and C14a, was assigned to 14-OH. The above NMR data, together with its UV and visible absorption spectral data, led us to interpret the presence of benzo[*a*]naphthacenequinone in TMC-66 molecule (Fig. 3).

The long range couplings from 16-H to C-15, C-18 and C-20; 17-H_2 to C-18 and C-20; 21-H_3 to C-18 and C-19, and the geminal coupling constant $J_{17Ha-17Hb}=8.8\,Hz$ indicated the existence of a five-membered ring system (Fig. 3). The observed NOE's between 16-H and 17-Ha; 17-Ha and 19-Ha; 17-Hb and 21-H_3; 19-Hb and 21-H_3 confirmed the partial structure from C-16 to C-19, and revealed the relative stereochemistry of these rings (Fig. 4). Based on above results, the structure of TMC-66 was determined as shown in Fig. 1.

Biological Activity

TMC-66 inhibited the activity of ECE from rat lung in a dose-dependent manner with the IC_{50} value of 2.9 μM. We performed a detailed kinetic analysis to elucidate the mechanism of inhibition of TMC-66 (Fig. 5). The *Km* value of ECE from rat lung was 0.42 μM in our system. A reciprocal plot analysis revealed that TMC-66 behaved as a competitive inhibitor. The *Ki* value of TMC-66 was calculated to be 3.1 μM.

To investigate its enzyme selectivity, we tested the inhibitory activity of TMC-66 towards NEP and trypsin. TMC-66 inhibited neither of these protease activities up to 100 μM.

Table 3. ^{1}H and ^{13}C NMR data of TMC-66.

Position	δ_C[a]		δ_H[b]
1	158.5	s[c]	
2	109.1	s	
3	137.1	s	
4	117.8	d	6.84 (1H, s)[d]
4a	147.4	s	
5	29.4	t	2.92 (1H, m), 2.75 (1H, m)
6	29.9	t	2.78 (1H, m), 2.65 (1H, m)
6a	149.6	s	
7	117.7	d	7.72 (1H, s)
7a	131.1	s	
8	187.3	s	
8a	116.0	s	
9	161.8	s	
10	124.4	d	7.36 (1H, dd, 2.6, 7.0)
11	137.1	d	7.80 (1H, m)
12	119.0	d	7.79 (1H, m)
12a	133.3	s	
13	187.6	s	
13a	114.7	s	
14	159.6	s	
14a	127.9	s	
14b	118.0	s	
15	164.6	s	
16	56.5	d	4.75 (1H, dd, 6.2, 8.2)
17	67.4	t	4.53 (1H, dd, 8.2, 8.8)
			4.17 (1H, dd, 6.2, 8.8)
18	93.7	s	
19	39.9	t	3.19 (2H, s)
20	170.6	s	
21	22.8	q	1.45 (3H, s)
1-OH			12.57 (1H, s)
9-OH			13.26 (1H, s)
14-OH			12.41 (1H, s)

[a] 100 MHz in DMSO-d_6 at 90°C.
[b] 400 MHz in DMSO-d_6 at 90°C.
[c] Multiplicity.
[d] Proton number, Multiplicity and coupling constants in Hz.

Fig. 3. Structure of TMC-66 was elucidated by NMR data.

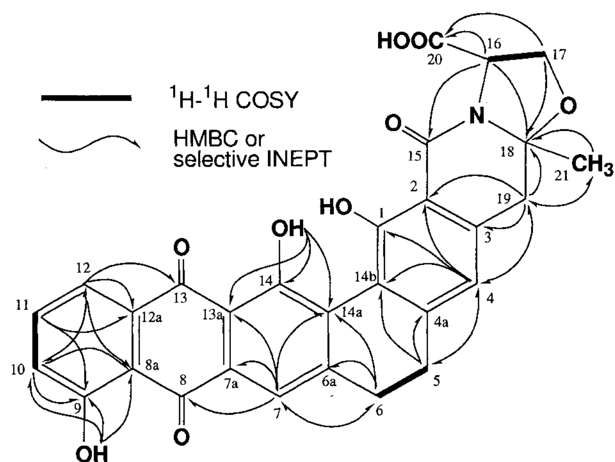


Fig. 4. Relative stereochemistry of TMC-66.

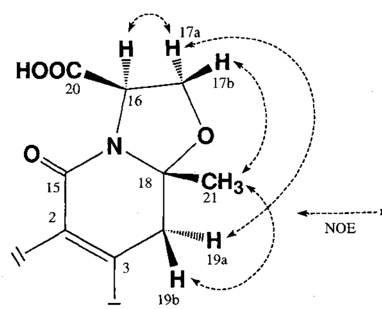
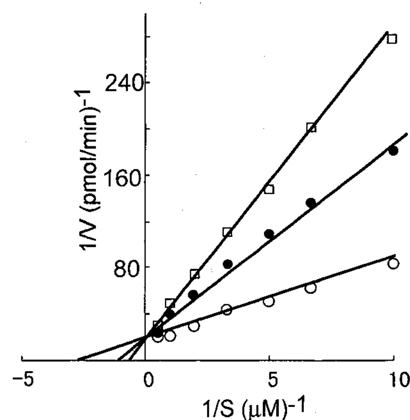


Fig. 5. Lineweaver-Burk plot for inhibition of ECE by TMC-66.

○ None, ● 3 μM TMC-66, □ 6 μM TMC-66.



Discussion

We found the highly selective ECE inhibitor designated as TMC-66 from the culture broth of *Streptomyces* sp. A5008. This compound consists of a benzo[*a*]-naphthacenequinone and an oxazolidine ring. While many compounds having a benzo[*a*]naphthacenequinone structure, *e.g.* WS79089A, B, C[8], benaphthamycin[9] and

pradimicins[10] had been already reported, a polycyclic structure with an oxazolidine is rare, the only precedent in nature is cervinomycin, a xhanthon anti-mycoplasmal antibiotic[11].

It had been reported that WS79089B inhibited ECE potently ($IC_{50}=0.14\,\mu M$), and pradimicin did weakly ($IC_{50}=60\sim120\,\mu M$)[8]. Pradimicin has a sugar and an amino sugar attached to the benzo[*a*]naphthacenequinone moiety, whereas TMC-66 and WS79089B have small or rigid substituents. These results suggest that the benzo[*a*]-naphthacenequinone structure might be responsible for exhibiting ECE inhibitory activity, and that a freely rotatable bulky moiety on the benzo[*a*]naphthacenequinone seems to interfere with the potency.

Experimental

General

^1H and ^{13}C NMR spectra were obtained with a JEOL GSX-400 NMR spectrometer and chemical shifts are given in ppm (δ) relative to TMS as an internal standard. Mass spectra, UV spectra and IR spectra were obtained by a JEOL JMS HX-100 spectrometer, a Shimadzu UV-2200A spectrophotometer and a JASCO model 100 infrared spectrophotometer, respectively. Melting point was determined with a Yanaco MP-2S micro melting point apparatus and was uncorrected. Optical rotation was determined with a Horiba SEPA-200 high sensitive polarimeter.

Taxonomic Studies

Cultural characteristics were determined by the methods of SHIRLING and GOTTLIEB[12]. The substrate and aerial mass colors were assigned by Guide to Color Standard, 1954 (Japan Color Research Institute). Morphological characteristics were observed with a scanning electron microscope (Hitachi S-4200). 2,6-Diaminopimelic acid in the whole cell was analyzed by the method of BECKER *et al.*[13] and by the method of HASEGAWA *et al.*[14].

Fermentation

A loopful of spores of *Streptomyces* sp. A5008 was inoculated into 500-ml Erlenmeyer flasks containing 70 ml of a seed medium composed of glucose 0.5%, glycerol 2.0%, soybean meal 2.0%, yeast extract 0.2%, $CaCO_3$ 0.4% and NaCl 0.25%. The inoculated flasks were incubated on a rotary shaker at 220 rpm at 27°C for 3 days. The seed culture thus obtained (300 ml) was transferred into a 50-liter jar fermentor containing 30 liters of a production medium composed of glucose 0.5%, glycerol 10%, fish meal 2.0%, yeast extract 0.2%, $CaCO_3$ 0.4 % and NaCl 0.25%. Fermentation was carried out at 27°C for 5 days with agitation of 200~600 rpm and aeration at 15 liters per minute to maintain the dissolved oxygen at 10% of the saturated concentration. The production of the inhibitor was monitored by the biological assay of ECE inhibition.

Preparation of ECE and NEP

ECE and NEP were partially purified from lung and kidney, respectively, of Wister rats as described by SHIMA *et al.*[15]. Briefly, a solubilized fraction of the membrane-bound activity was subjected to WGA-agarose column chromatography, and the active fractions obtained were used as an enzyme source of ECE. For preparation of NEP, WGA agarose was substituted with ConA-agarose.

Enzyme Assays

ECE activity was measured by the sandwich enzyme immunoassay developed by SHIMA *et al.*[16]. Big-ET was used as a substrate. Assays of NEP and trypsin activities were carried out by the fluorometric and colorimetric methods, respectively, as described previously[15,17].

Acknowledgments

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