A83586C, A NEW DEPSIPEPTIDE ANTIBIOTIC

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A culture identified as *Streptomyces karnatakensis* was found to produce a novel cyclic hexadepsipeptide antibiotic designated A83586C. The structure was elucidated by X-ray crystallography, and full ^{1}H and ^{13}C NMR assignments are reported. The absolute configuration was confirmed by the detection of D-threonine in the acid hydrolysate of A83586C. A83586C had potent Gram-positive activity *in vitro* but lacked *in vivo* efficacy in mice.

During the course of screening for biologically active metabolites, culture A83586, subsequently identified as a strain of *Streptomyces karnatakensis*, was isolated. This culture, which produced a complex of piericidins[1], was also found to produce an antibiotic possessing potent Gram-positive activity. Isolation of this antibiotic, designated A83586C, yielded a crystalline material which was shown by X-ray analysis to be a cyclic hexadepsipeptide closely related to azinothricin[2]. The absolute stereochemistry was determined to be the same as in azinothricin through the detection of D-threonine in an acid hydrolysate of A83586C. In fact, A83586C differs from azinothricin only in the presence of *N*-hydroxy-L-alanine instead of *N*-hydroxy-*O*-methyl-L-serine, and in having a methyl instead of an ethyl group at C-37. Other antibiotics containing the piperazic acid moiety include the monamycins[3], and PD 124,966[4] (C. D. RITHNER; personal communication).

Taxonomy

Culture A83586 was isolated from a soil collected in Guam. The culture was characterized following methods outlined by the International Streptomyces Project (ISP)[5]. ISCC-NBS Centroid Color Charts, standard sample No. 2106 and the Color Harmony Manual (4th Ed., 1958, Container Corporation of America, Chicago, Illinois) were used to identify the colors of the reverse side and aerial spore mass respectively.

The culture grew well on both complex and defined media. Aerial spore mass color was predominantly gray, although some white and pale yellow were observed. The reverse color was yellowish brown to grayish yellow. Soluble pigments were not produced. The cultural characteristics of A83586 on various ISP media are presented in Table 1.

The culture A83586 produces an extensive substrate mycelium. Aerial hyphae produce long, compact chains of spores arranged in spirals consisting of 5 or more coils. There are >10 spores per chain. The spore surface is covered with long profuse hairs (Fig. 1). Thus the morphology is spiral (S) and spore surface hairy (HA).

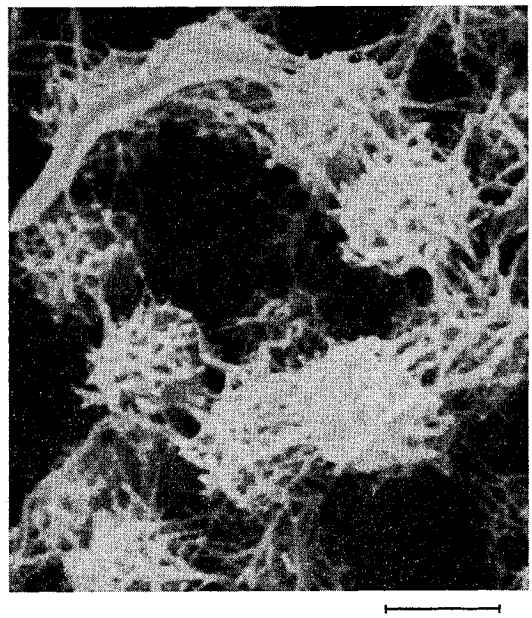
Culture A83586 is characterized by a very narrow carbohydrate utilization pattern. It utilized the following carbohydrates: D-Galactose, glucose, glycerol, glycogen, maltose, mannose, D-trehalose, and sodium butyrate. It was unable to utilize adonitol, D- and L-arabinose, cellobiose, cellulose, dextrin, dulcitol, ethanol, *i*-erythritol, D-fructose, *i*-inositol, inulin, D-lactose, D-mannitol, D-melezitose,

Table 1. Cultural characteristics of A83586.

ISP 2	G:	Abundant
	R:	72.d.OY
	Am:	Abundant; white
	Sp:	None
ISP 3	G:	Good
	R:	90.gy.Y
	Am:	Fair; gray
	Sp:	None
ISP 4	G:	Abundant
	R:	70.d.OY
	Am:	Fair; gray
	Sp:	None
ISP 5	G:	Abundant
	R:	72.d.OY
	Am:	Good; pale yellow
	Sp:	None
ISP 7	G:	Abundant
	R:	78.d.yBr
	Am:	Good; pale yGN to gray
	Sp:	None
No. 172	G:	Good
	R:	90.gy.Y
	Am:	Fair; white
	Sp:	None
CZAPEK'S solution agar	G:	Fair
	R:	92.y white
	Am:	Fair; white
	Sp:	None

G: Growth, R: reverse, Am: aerial mycelium, Sp: soluble pigment.

Fig. 1. Electronmicrograph showing spores of *Streptomyces karnatakensis*, grown on ISP medium 3 at 30°C for 14 days.
Bar equals 1.0 μm.



D-melibiose, α-methyl-D-glucoside, D-raffinose, L-rhamnose, D-ribose, salicin, sorbitol, L-sorbose, sucrose, xylitol and xylose.

Culture A83586 hydrolyzed starch, produced catalase, and reduced nitrates, but did not produce H_2S. Melanoid pigments were not produced on ISP No. 1, ISP No. 6, or ISP No. 7. It was resistant to bacitracin 10 U, cephalothin 30 μg, gentamicin 10 μg, lincomycin 2 μg, neomycin 30 μg, oleandomycin 15 μg, benzylpenicillin 10 U, streptomycin 10 μg and tobramycin 10 μg. It was sensitive to rifampin 5 μg, tetracycline 30 μg and vancomycin 30 μg.

Hydrolyzed whole cells contain LL-diaminopimelic acid. No diagnostic sugars were observed. Therefore A83586 has a type I cell wall and an N.C. sugar pattern[6].

The chemotaxonomic properties and the general cultural and morphological characteristics of A83586 support the assignment of this strain to the genus *Streptomyces*. Study of the characteristics of similar strains in the literature indicates that A83586 is most similar to, and therefore classified as, a strain of *Streptomyces karnatakensis*[7].

Fermentation and Isolation

Stock cultures of A83586 were suspended in glycerol (10%) - lactose (5%) solution and preserved by storage in liquid nitrogen. These suspensions served as inoculum for a medium containing glucose 1%, soluble starch 2%, yeast extract 0.5%, NZ-Amine A (Sheffield Products) 0.5%, and $CaCO_3$ 0.1% in deionized water. The medium was adjusted to pH 7.2 with aqueous NaOH and 50 ml portions

were dispensed into 250-ml Erlenmeyer flasks prior to autoclaving. Inoculated flasks were incubated for 72 hours at 30°C on a rotatory shaker orbiting in a 5.07-cm circle at 250 rpm. The resulting culture was transferred into 400-ml volumes of the same medium in 1-liter Erlenmeyer flasks. After an additional incubation period of 48 hours, the mycelial culture was used as inoculum (2%) for a 14-liter bioreactor of conventional design with an operating volume of 10 liters. The bioreactor medium contained SAG 471 (Union Carbide) 0.02%, polypropylene glycol (MW 2,000) 0.01%, glucose 1%, blackstrap molasses 2%, Bacto-peptone (Difco) 0.5%, and $CaCO_3$ 0.2% in deionized water (pH 6.8 prior to autoclaving). During the fermentation, dissolved oxygen was maintained at 50~80% of air saturation after the initial 24 hours. The pH rose steadily to a final value of 7.8~8.0 when the fermented broth was harvested after an incubation period or 66 hours at 30°C.

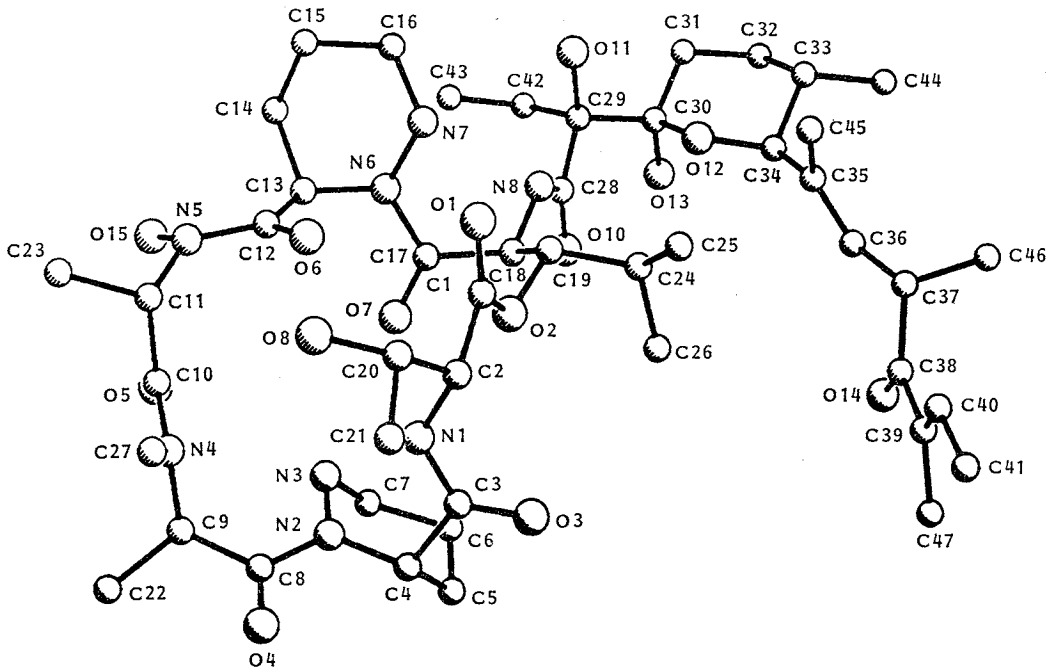
A83586 whole broth (10 liters) was stirred with 10 liters of ethyl acetate, filtered through diatomaceous earth, and the layers separated. The aqueous layer was re-extracted with 4 liters of fresh ethyl acetate and the organic layers were combined and evaporated *in vacuo*. The residue (8 g) was dissolved in 20 ml of chloroform, and chromatographed on 500 ml of Silica gel 60 using a linear gradient of *n*-hexane - chloroform (1 : 3) to chloroform - methanol (94 : 6). The fractions containing antimicrobial activity vs. *Micrococcus luteus* were combined and chromatographed on reverse phase (ODS) silica gel using a methanol - H_2O gradient (1 : 1 to 100 : 0). A83586C crystallized directly from the C_{18} column fractions upon standing to yield 10 mg colorless needles: MP 189~191°C; fast atom bombardment mass spectra (FAB-MS) *m/z* 983.5623 $(M+Li)^+$ (calcd for $C_{47}H_{76}N_8O_{14}Li$: 983.5641); field desorption mass spectra (FD-MS) *m/z* 976; IR (KBr) cm^{-1} 3400, 2980, 1725, 1663, 1641, 1631, 1505, 1493, 1457, 1443, 1393, 1314, 1280, 1238, 1000; UV λ_{max}^{MeOH} nm 235 (sh); $[\alpha]_D^{25}$ +116.1° (*c* 0.2, $CHCl_3$).

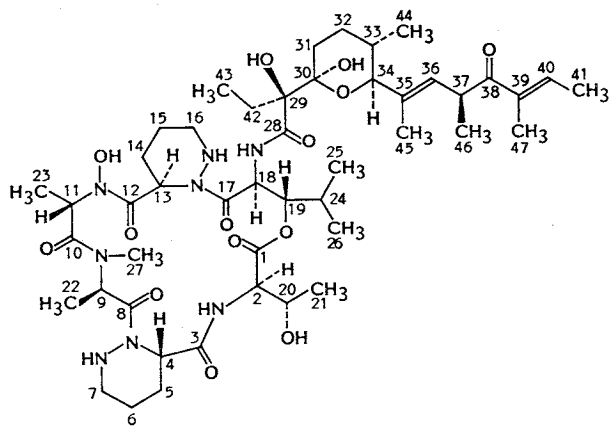
Structure

A83586C was recrystallized from toluene to give colorless needles in the space group $P2_12_12_1$, $Z=4$, with unit cell dimensions of $a=9.818(2)$ Å, $b=19.620(4)$ Å, $c=30.357(8)$ Å. The calculated density was 1.214 gcm^{-3}. A total of 4413 unique reflections was measured on an automated four-circle diffractometer using monochromatic copper radiation. The structure was solved using the Random Tangent method of the SHEXTL program library (G. M. SHELDRICK, 1981) and was refined by the least squares method with anisotropic temperature factors for all atoms except hydrogen. Hydrogen atoms were included with isotropic temperature factors at calculated positions. The final R-factor was 0.0570 for 3369 observed reflections. Tables of the atomic coordinates, bond lengths, bond angles, and anisotropic temperature factors have been deposited at the Crystallographic Data Center. Fig. 2 shows the X-ray structure of A83586C.

The absolute stereochemistry of A83586C was determined by detection of D-threonine in the acid hydrolysate. Ten mg of A83586C was hydrolyzed in refluxing 6 N HCl for 21 hours, then dried *in vacuo*. The residue was triturated with 1.5 ml of 0.2 M Tris-HCl buffer, which was further diluted 1 : 5 with buffer and the pH adjusted to 2.5 with HCl. This sample was then derivatized by the method of ASWAD[8] which yields chiral *o*-phthaldialdehyde derivatives of primary amines. Analysis of these derivatives by HPLC using a Beckman Ultrasphere ODS column (4.6 mm i.d. ×250 mm) with a gradient of sodium acetate buffer (0.05 M, pH 5.9) - 80% MeOH in buffer, 90 : 10 to 70 : 30 in 20 minutes with the gradient started at 1 minute, yielded base line resolution of the D- and L-enantiomers of threonine (elution times of 17.4 and 18.6 minutes, respectively). Quantization by this method gave a value

Fig. 2. X-Ray structure of A83586C.





A83586C

of 784 nmol/ml D-threonine vs. 739 nmol/ml (D-, L-) threonine obtained on a Beckman model 6300 amino acid analyzer.

Both the ^{1}H and ^{13}C NMR spectra of antibiotic A83586C were fully assigned, using a variety of two-dimensional (2D) NMR techniques and the known structure of the compound. Spectra were recorded in $CDCl_3$ solution at 27°C on a Bruker AM500 spectrometer; the sample concentration was 37 mg in 0.4 ml. ^{1}H and ^{13}C spectra for A83586C are shown in Figs. 3 and 4. The assignments were developed as follows: First, the 47 carbons and their associated proton resonances were sorted into 12 methyls, 9 methylenes, 14 methines, and 12 quaternary carbons, using the carbon-proton correlation experiment XHCORRD. Next, all of the protonated carbons were assigned to substructures

Fig. 3. ^{1}H NMR spectra of A83586C (500 MHz, $CDCl_3$).

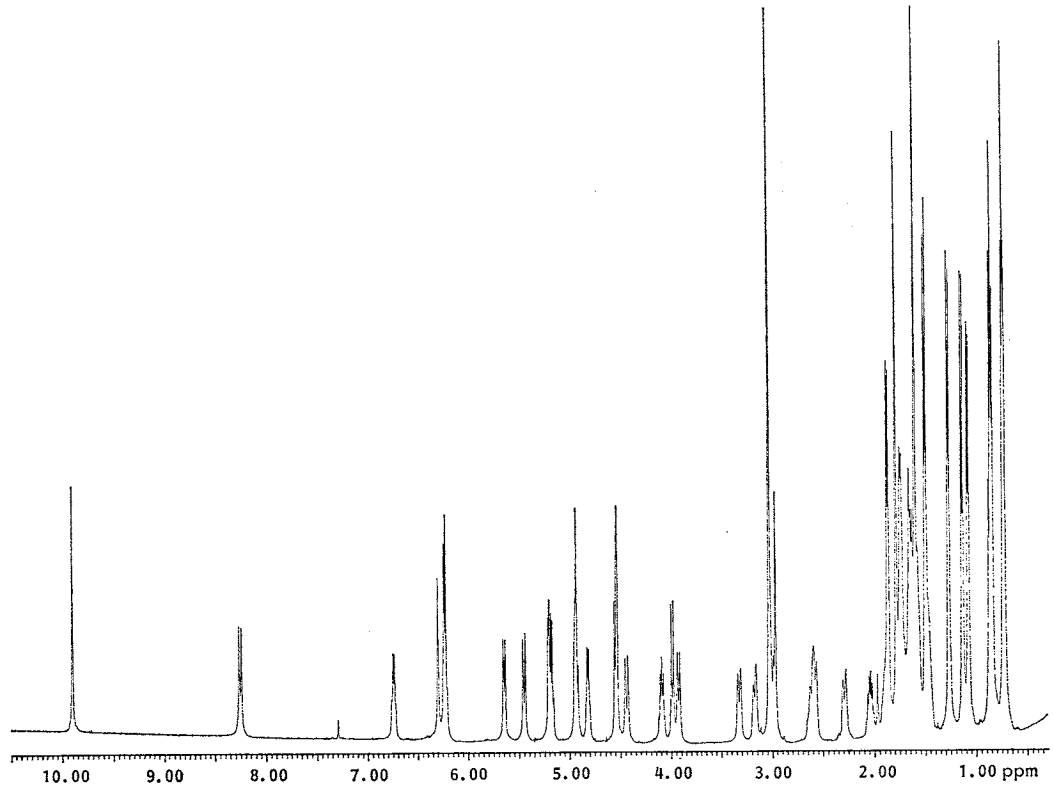
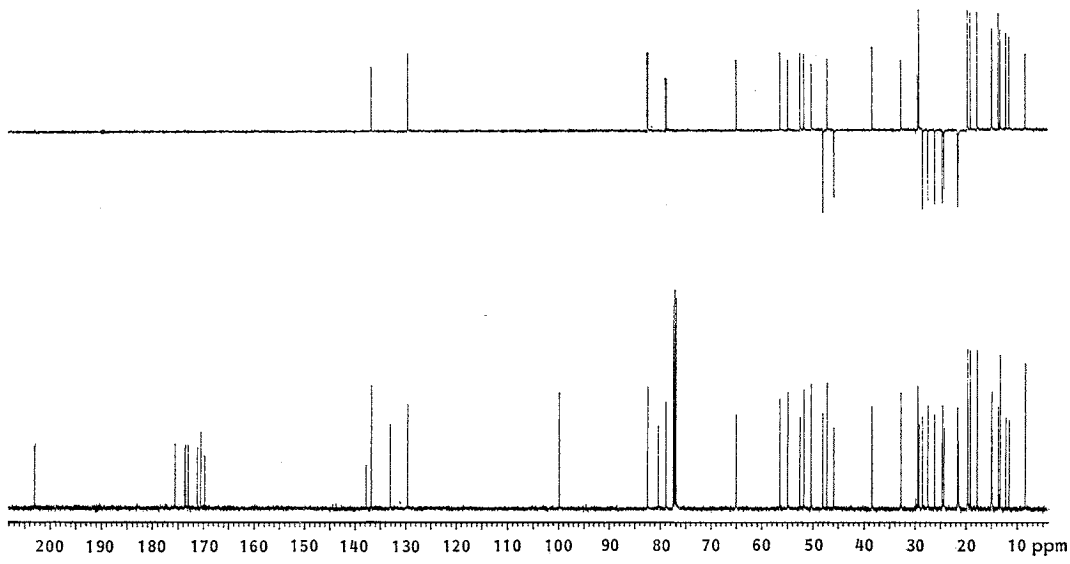
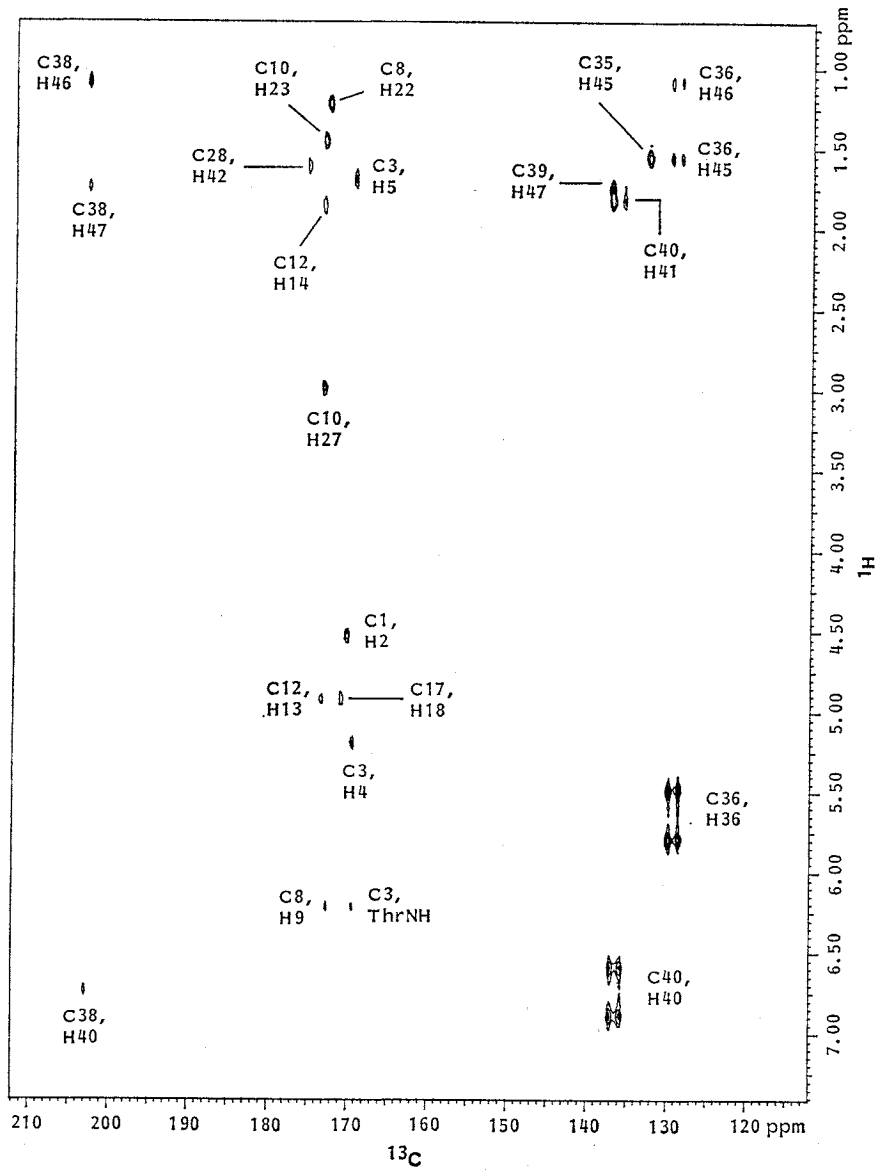


Fig. 4. ^{13}C NMR spectra of A83586C (125 MHz, $CDCl_3$).



based on correlations derived from proton-proton correlation 2D NMR experiments (COSY and COSYRCT). All protonated carbons were assigned in this manner for the sidechain, the β-hydroxyleucine and the threonine residue. In addition there were the two *N*-substituted alanine residues

Fig. 5. Long-range ^{1}H-^{13}C NMR spectra of A83586C.



and the two piperazic acid (Pip) residues; their proton assignments in ppm are shown below:

	α-H	CH_3			
Ala No. 1	5.16	1.48			
Ala No. 2	6.20	1.26			
	α	β	γ	δ	NH
Pip No. 1	5.20	2.56, 1.72	1.56	3.31, 2.62	3.90
Pip No. 2	4.93	2.27, 1.88	1.62, 1.46	3.17, 2.98	4.43

The final steps required to complete the total assignment were to distinguish between these pairs of

Table 2. ^{13}C and ^{1}H NMR assignments for A83586C ($CDCl_3$; TMS 0 ppm).

	Position	^{13}C δ (ppm)	^{1}H δ (ppm)		Position	^{13}C δ (ppm)	^{1}H δ (ppm)
1	Thr CO	170.38	—	22	*N*-CH_3-Ala β	13.21	1.26
2	Thr α	56.36	4.53	23	*N*-OH-Ala β	13.52	1.48
	Thr NH	—	6.26	24	β-OH-Leu γ	29.38	1.73
3	Pip CO	169.63	—	25	β-OH-Leu δ	19.50	0.72
4	Pip α	52.43	5.20	26	β-OH-Leu δ	14.77	0.83
5	Pip β	24.52	2.56, 1.72	27	*N*-CH_3	29.13	3.02
6	Pip γ	21.52	1.56	28		175.44	—
7	Pip δ	47.94	3.31, 2.62	29		80.25	—
	Pip NH	—	3.90	30		99.73	—
8	*N*-CH_3-Ala CO	172.87	—	31		28.44	1.72
9	*N*-CH_3-Ala α	47.13	6.20	32		27.40	1.64
10	*N*-OH-Ala CO	173.40	—	33		32.70	1.46
11	*N*-OH-Ala α	50.25	5.16	34		82.34	3.97
	N-OH	—	9.82	35		132.96	—
12	Pip CO	173.55	—	36		129.53	5.62
13	Pip α	51.67	4.93	37		38.33	4.09
14	Pip β	24.22	2.27, 1.88	38		202.93	—
15	Pip γ	21.36	1.62, 1.46	39		137.68	—
16	Pip δ	45.79	3.17, 2.98	40		136.67	6.75
	Pip NH	—	4.43	41		14.88	1.86
17	β-OH-Leu CO	171.04	—	42		26.05	2.03, 1.65
18	β-OH-Leu α	54.82	4.92	43		8.32	0.84
19	β-OH-Leu β	78.41	5.45	44		17.69	0.71
	β-OH-Leu NH	—	8.24	45		12.10	1.59
20	Thr β	64.92	4.80	46		19.55	1.12
21	Thr γ	19.01	1.07	47		11.45	1.78

residues and to identify the 12 carbon singlets. These steps were accomplished using a carbon-proton correlation experiment which retains the long-range correlations[9,10]; the microprogram name BAX52.AU reflects the origin of the experiment in reference 9. Several of the useful correlations are shown in Fig. 5 and the complete assignments are given in Table 2.

Biological Activity

A83586C showed very potent activity *in vitro* vs. Gram-positive bacteria (Table 3), with MICs of 0.008~0.06 μg/ml vs. *Staphylococcus* and *Streptococcus* sp., and no activity vs. Gram-negative bacteria. In a protection test vs. *Staphylococcus aureus* in mice, A83586C was inactive at doses of 4.2 mg/kg, and lethal at doses of 9.3 mg/kg. A83586C also exhibited an IC_{50} of 0.0135 μg/ml vs. CCRF-CEM cells, a human T-cell leukemia line[11].

Table 3. Antimicrobial activity of A83586C.

Organism	MIC (μg/ml)
Staphylococcus aureus	0.008
S. epidermidis	0.008
Streptococcus pyogenes	0.008
S. pneumoniae	0.008
S. faecalis	0.06
Haemophilus influenzae	64
Escherichia coli	>128
Klebsiella pneumoniae	>128
Enterobacter cloacae	>128
Salmonella typhi	>128
Pseudomonas aeruginosa	>128
Serratia marcescens	64
Shigella sonnei	>128
Morganella morganii	>128
Citrobacter freundii	>128

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