Notes

A NEW ISOCOUMARIN ANTIBIOTIC, Y-05460M-A

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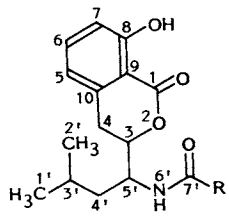
A new antibiotics, named Y-05460M-A, was isolated from the culture broth of *Bacillus* sp. Y-05460M. The strain was isolated from a soil sample collected at Iriomote island, Okinawa prefecture, Japan. Y-05460M-A is a basic, substituted isocoumarin antibiotic, active against Gram-positive and Gram-negative bacteria. It also showed a cytotoxic activity against P388 lymphatic leukemia. Moreover it exhibited an antiulcer activity (data not shown).

The culture medium was composed of 4.0% white dextrine, 1.0% glucose, 4.0% peanut powder, 1.0% gluten meal, 0.05% $MgSO_4$, 0.2% $CaCO_3$ and 0.0004% $CoCl_2$ (pH 7.0). The fermentation was done with three 30-liter jar fermenters at 37°C for 4 days.

The following isolation procedures were performed by monitoring the activity against *Staphylococcus aureus* FDA 209P. The culture broth (50 liters), adjusted to pH 3, was centrifuged with centrifugal separator. Y-05460M-A was extracted twice from the supernatant with EtOAc (80 liters) at pH 9, and it was extracted into the aqueous layer (50 liters) at pH 3. Then it was reextracted from the aqueous layer with EtOAc (50 liters) at pH 9. The extract was dried with anhydrous Na_2SO_4 and concentrated *in vacuo* to syrup (19.02 g). The syrup was dissolved with 15 ml of $CHCl_3$-MeOH (2:1). It was chromatographed on silica gel column (5 i.d. × 150 cm) with the solvent $CHCl_3$-MeOH (4:1). The column chromatography on silica gel was repeated with the solvent EtOAc-MeOH (4:1).

Fig. 1. Structures of Y-05460M-A (**I**) and related isocoumarine compounds.



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Y-05460M-A (**I**): OH OH NH_2 $C(12')H_3$ / $C(8')H-C(9')H-C(10')H-C(11')H-C(13')H_3$

Baciphelacin: CH_3 OH OH NH_2 / $CH-CH-CH-CH-CH_2-CH_3$

Amicoumacin-A: OH OH NH_2 / $CH-CH-CH-CH_2-CONH_2$

AI-77-B: OH OH NH_2 / $CH-CH-CH-CH_2-COOH$

Table 1. NMR spectral data for Y-05460M-A (**I**) ($CDCl_3$).

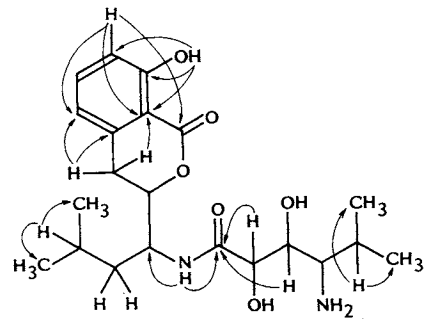
^{13}C shift	^{1}H shift	Assignment	^{13}C shift	^{1}H shift	Assignment
173.4	—	7′	39.8	1.40	4′
169.8	—	1	30.1	3.07	4
162.0	—	8		2.84	
139.5	—	10	27.5	2.24	11′
136.5	7.36	6	24.6	1.64	3′
118.4	6.68	5	23.2	0.91	1′, 2′
116.2	6.82	7	21.7	0.87	
108.1	—	9	20.1	1.00	12′, 13′
81.2	4.59	3	18.9	1.05	
72.1	4.53	8′		10.65	OH (C-8)
69.7	4.26	9′	—	7.82	NH_2 (C-10′)
61.6	3.21	10′	—	7.56	NHCO
49.3	4.29	5′	—	5.85	OH (C-8′)
39.8	1.78	4′	—	5.15	OH (C-9′)

δ ppm.

Fractions which were pure on TLC with $CHCl_3$-MeOH (4:1) as the solvent were combined and evaporated. Y-05460M-A was obtained 1.95 g as a white powder.

Y-05460M-A gave positive color reactions with ninhydrin and $FeCl_3$ reagents. The mp is 104~106°C. The ultraviolet spectrum, λ_{max}^{MeOH} nm (ε): 208 (27,300), 246 (6,400), 314 (4,380), and the infrared spectrum, ν_{max}^{KBr} cm^{-1}: 3300, 2900, 1680, 1540, 1230, indicated the presence of a benzoic acid moiety with a hydroxyl group on the benzene ring, and an amide group. The molecular formula $C_{21}H_{32}N_2O_6$ was determined by HR-MS, *m/z* 408.22608, and by the carbon number in the ^{13}C NMR spectrum. With the consideration of chemical shifts, the ^{1}H-^{1}H and ^{13}C-^{1}H COSY spectra revealed two partial structures, i) $-C(8')H(OH)-C(9')H(O-)-CH(NH_2)-CH(CH_3)_2$, and ii) $-CH_2-C(3)H(O-)-C(5')H-(NH-)-CH_2-CH(CH_3)_2$. In partial structure i), the coupling between hydroxyl proton and 8′-H was observed; amine protons were observed at δ 7.82 ppm. In partial structure ii), the coupling between amine proton and 5′-H was observed. The assignments of ^{1}H and ^{13}C NMR signals are shown in Table 1. The HMBC spectrum clarified the posision of the hydroxyl group as *ortho* from the carbonyl group and allowed to combine the benzoic acid moiety with the partial structures i) and ii). The result of the HMBC spectrum is shown in Fig. 2. By comparing the ultraviolet spectrum of Y-05460M-A with those of isocoumarin compounds, such as baciphelacin[1] λ_{max}^{MeOH} nm (ε): 210 (23,600), 246 (6,950), 314 (4,530), amicoumacin-A[2] λ_{max}^{MeOH} nm (ε): 208 (27,300), 247 (6,400), 315 (4,380), and AI-77-B[3] λ_{max}^{MeOH} nm (ε): 246 (6,250), 314 (4,450),

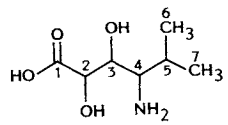
Fig. 2. Long range couplings observed by HMBC experiment.



we came to the conclusion that the chromophore of Y-05460M-A is the same as that of those compounds. Therefore the structure of Y-05460M-A is **I**. The structure was confirmed by the acid product (**II**), $COOH-CH(OH)-C(3)H(OH)-CH(NH_2)-CH(CH_3)_2$, MW 177. **II** was obtained as follows: After hydrolysis of **I** (38 mg) with 6 N HCl, 110°C for 21 hours, the reaction mixture was dried up *in vacuo*. The residue was solved with 10 ml water and adjusted to pH 7. The same amount of EtOAc was added and the mixture was shaked. The aqueous layer was removed and dried. It was solved with a small amount of *n*-PrOH-H_2O (4:1) and chromatographed on silica gel (1 i.d. × 50 cm) with *n*-PrOH-H_2O (9:1) and then with *n*-PrOH-H_2O (4:1). By monitoring the TLC with *n*-PrOH-H_2O (4:1) as the solvent and H_2SO_4 as the color reagent, **II** was collected and dried. 6.9 mg of **II** was obtained. In the ^{1}H NMR spectrum of **II**, the chemical shift of 3-H, δ 4.18 ppm, was almost the same as that of

Table 2. NMR spectral data for II (D_2O).

^{13}C shift	^{1}H shift	Assignment
180.5	—	1
76.1	4.22	2
73.1	4.18	3
63.2	3.16	4
30.0	2.17	5
21.8	1.05	6
20.6	1.05	7



II

δ ppm.

Table 3. Antimicrobial activity of Y-05460M-A (I).

Test organisms	MIC (μg/ml)	Test organisms	MIC (μg/ml)
Staphylococcus aureus FDA 209P JC-1	6.25	*Mycobacterium smegmatis* ATCC 607	>100
S. epidermidis IID 866	12.5	*Escherichia coli* O-1	100
Streptococcus pyogenes Cook	12.5	*Klebsiella pneumoniae* ATCC 10031	12.5
Enterococcus faecalis IID 682	50.0	*Pseudomonas aeruginosa* NCTC 10490	>100
E. faecium CAY 09-1	12.5	*Flavobacterium* 633	12.5

Table 4. Antitumor activity of Y-05460M-A (I) against P388 leukemia.

Drug	Dose (mg/kg/day) × days	Median life span (days)	T/C (%)	Survival ratio (23 days)
Saline	—	11.0	100	0/12
Y-05460M-A	1.56 × 5 ip	11.0	100	0/8
	3.13 × 5 ip	12.5	114	0/8
	6.25 × 5 ip	13.0	118	0/8
Mitomycin C	0.5 × 5 ip	21.5	195	2/8

Y-05460M-A, δ 4.26 ppm, and did not show a characteristic shift to higher field. So the position of the ester bond in Y-05460M-A is confirmed not to be C-9′ but C-3. The assignments of ^{1}H and ^{13}C NMR signals of II is shown in Table 2.

Structurally related compounds, such as baciphelacin (an antibacterial and antiviral), amicoumacin-A (an antibacterial, antiinflammatory and antiulcer) and AI-77-B (a gastroprotective) were also isolated from the *Bacillus* group, their structures are shown in Fig. 1. But xenocoumarins[4] (a gastroprotective) was isolated from *Xenorhabdus*.

Y-05460M-A is active against Gram-positive and less active against Gram-negative bacteria. The MIC values of Y-05460M-A are listed in Table 3. Y-05460M-A exhibited cytotoxic activity against the lymphoid leukemia cell lines L1210 and P388 with IC_{50} values of 0.13 and 0.045 μg/ml, respectively. The antitumor activity of Y-05460M-A was determined in mice against P388 cells. Y-05460M-A was given intraperitoneally once a day for five consecutive days, administration started from the next day after intraperitoneal tumor inplantation. Antitumor activity was evaluated by the median life span. Although Y-05460M-A showed a strong cytotoxicity *in vitro*, it exhibited only weak antitumor activity *in vivo*. The result is shown in Table 4.

Acknowledgments

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References

1) OKAZAKI, H.; T. KISHI, T. BEPPU & K. ARIMA: A new antibiotic, baciphelacin. J. Antibiotics 28: 717~719, 1975
2) ITOH, J.; S. OMOTO, T. SHOMURA, N. NISHIZAWA, S. MIYADO, Y. YUDA, U. SHIBATA & S. INOUYE: Amicoumacin-A, a new antibiotic with strong

antiinflammatory and antiulcer activity. J. Antibiotics 34: 611~613, 1981

3) SHIMOJIMA, Y.; H. HAYASHI, T. OOKA & M. SHIBUKAWA: Production, isolation and pharmacological studies of AI-77s. Agric. Biol. Chem. 46: 1823~1829, 1982

4) MCINERNEY, B. V.; W. C. TAYLOR, M. J. LACEY, R. J. AKHURST & R. P. GREGSON: Biological active metabolites from *Xenorhabdus* spp. Part 2. Benzopyran-1-one derivatives with gastroprotective activity. J. Nat. Prod. 54: 785~795, 1991