CHEMICAL MODIFICATION OF STREPTOVARICIN C

I. 19-O-SUBSTITUTED DAMAVARICIN C*[1]

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(Received for publication February 12, 1975)

Damavaricin C*, a degradative derivative of streptovaricin C, has reduced antibiotic activity relative to streptovaricin C. It has, however, a new phenolic hydroxyl group at the C-19 position of the naphthoquinone ring on which various groups can be substituted through an ether linkage. A series of 19-O-substituted derivatives of damavaricin C has been synthesized. The preparation of these derivatives, their *in vitro* antibacterial activities, *in vitro* inhibition of *E. coli* RNA polymerase, and lethal activity on the membrane mutants of *E. coli* are reported. It is believed that the original biological activity of damavaricin C is retained and that introduction of the functional groups at the C-19 position has increased the membrane diffusibility of the molecule.

Streptovaricins were discovered by SIMINOFF *et al.*[2] and have been studied for their potential antitubercular activity. A chemical study of the streptovaricins has already been made by RINEHART and his collaborators.[3] In addition to notable antibacterial activity, streptovaricins have an intriguing property which has attracted many chemists' and biologists' interest. They act on reverse transcriptase of some RNA oncogenic viruses which may be involved in the process of viral transformation.[4]

Relatively little has been reported on the chemical modification of the streptovaricins compared with rifamycins, which also belong to the ansa ring antibiotics.

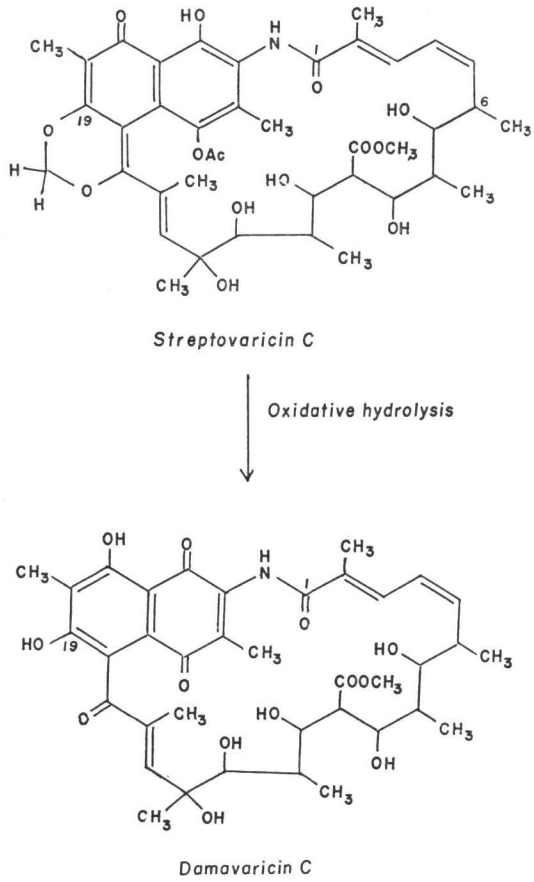
We wish to describe here the preparation of a series of 19-O-substituted damavaricin C derivatives and their comparative biological activities *in vitro*.

Crude streptovaricin complex was supplied by the Upjohn Co., Kalamazoo, Michigan, U.S.A. Streptovaricin C, the most abundant component of the streptovaricin complex, was isolated in pure crystalline form by a combined technique using silica gel chromatography and gel filtration on Sephadex LH-20 with acetone.

Upon bubbling air or oxygen through a solution of streptovaricin C (**1**), $C_{40}H_{51}NO_{14}$,[3] it was converted to an oxidative degradation product, damavaricin C (**2**), $C_{37}H_{47}NO_{13}$, m.p. 284~285°C. The NMR spectrum of **2** lacked both the methylenedioxy protons at δ 5.18 (d) and δ 5.83 (d) as well as the O-acetyl protons at δ 1.96 present in **1**. Moreover, the IR

* Damavaricin C and other damavaricins were first prepared by P.K. MARTIN in Dr. K.L. RINEHART's laboratory (Ph. D. Thesis, University of Illinois (1967). That work is briefly described in the report in this issue (K.L. RINEHART, Jr., F.J. ANTOSZ, P.V. DESHMUKH, K. KAKINUMA, P.K. MARTIN, B.I. MILAVETZ, K. SASAKI, T.R. WITTY, L.H. LI and F. REUSSER: J. Antibiotics 29: 201~203, 1976).

Scheme 1. Conversion of streptovaricin C into damavaricin C.

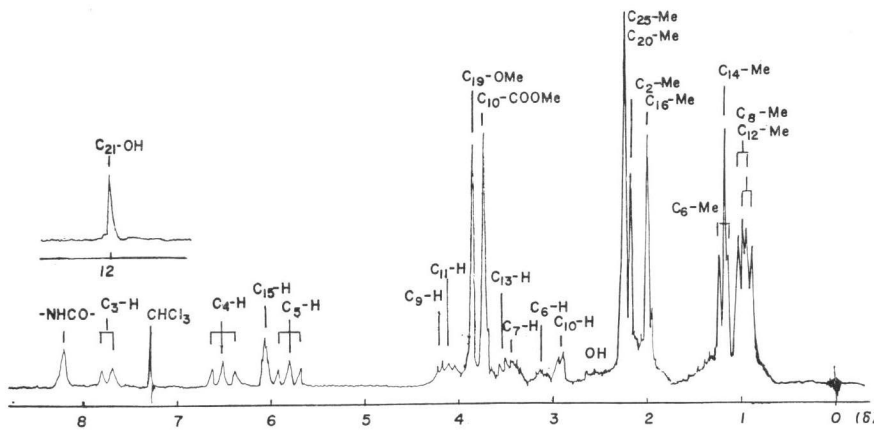


spectrum of **2** lacked the absorption corresponding to an O-acetyl group on an aromatic ring observed at 1765 cm^{-1} in **1**.

Therefore, the loss of C_3H_2O during the reaction is ascribed to the hydrolysis of the methylenedioxy and the O-acetyl groups while the loss of H_2 results, from the autoxidation of a 1, 4-dihydronaphthoquinone to a 1,4-naphthoquinone (Scheme 1).

Damavaricin C (**2**) has a newly formed phenolic hydroxyl group at the C-19 position of the naphthoquinone ring, thus enabling the preparation of a series of 19-O-substituted derivatives, as listed in Table 1. The etherification of **2** was carried out in good yield by treating it with silver oxide and a bromo- or iodo-compound. For example, 19-O-methyldamavaricin C (**3**) ($C_{38}H_{49}NO_{13}H_2O$, m.p. 165 167°C; M^+ 727) was prepared by the reaction of **2** with silver oxide and methyl iodide in methanol. The NMR spectrum of **3** showed the newly formed 19-O-methyl protons at δ 3.83 while absorption for a strongly chelated phenolic hydroxyl proton at C-21 still remained, at δ 11.98 (Fig. 1). A series of 19-O-substituted derivatives was prepared (Table 1) essentially by the same procedure. These derivatives are generally yellow substances sparingly soluble in water. Their structures were confirmed on the basis of chemical behavior

Fig. 1. The N.M.R. spectrum of 19-O-methyldamavaricin C (**3**) (100 MHz; in $CDCl_3$ and TMS as internal reference).

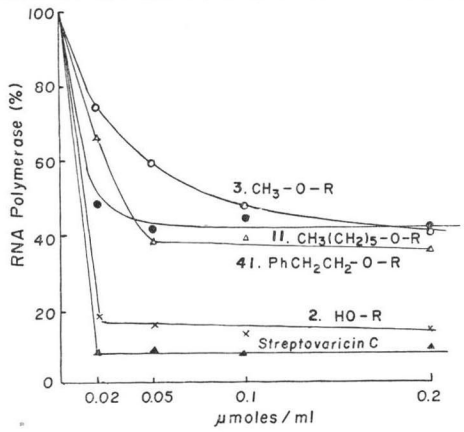


and physical data, such as NMR, IR, UV and mass spectra. The UV spectra in methanol are very similar to that of the methyl ether (**3**), with maxima at 257~258, 310~315 (including inflection), and 420~425 nm. It is interesting that the chromophore of these derivatives is very similar to that of rifamycin S.

Three parameters for assessing the biological activity of damavaricin C derivatives were employed: (1) antibacterial activity *in vitro*, (2) inhibitory activity against *Escherichia coli* RNA polymerase *in vitro*, and (3) antibacterial activity against membrane mutants of *E. coli* *in vitro*.

Table 1 shows that **2** has reduced antibacterial activity relative to streptovaricin C, while many of the derivatives with an ether linkage at the C-19 position of the naphthoquinone ring have equal or better antibacterial activity. In this series of *n*-alkyl ethers the biological activity against *Staphylococcus aureus* and *Bacillus subtilis* (Table 1) seems to be related to the number of the methylene units in the alkyl chain. The antibacterial activity against *Mycobacterium smegmatis* does not closely parallel the activities against the other bacteria. It is noteworthy that none of the derivatives examined thus far showed activity against *E. coli* or *Pseudomonas aeruginosa* at 100 μg/ml.

Fig. 2. Inhibition of RNA polymerase *in vitro* by various derivatives.
Assay mixture contained in a total volume of 0.1 ml: Tris-HCl buffer (pH 8.0), 2 μmol: $MgCl_2$, 1 μmol; mercaptoethanol, 0.2 μmol; EDTA, GTP, ATP, CTP, 0.025 μmol each; KCl, 10 μmol; calf thymus DNA, 15 μg; C^{14}-UTP (48 mCi/m mol), 0.025 μCi. The reaction mixture was incubated at 37°C for 20 minutes.



It is well known that streptovaricins as well as rifamycins inhibit DNA-dependent RNA polymerase *in vitro*[4b,5,6] Therefore, the inhibitory effect of the representative derivatives **2**, **3**, **10**, and **41** on *E. coli* RNA polymerase was compared with that of streptovaricin C (Fig. 2). It is interesting to note that damavaricin C (**2**), which has low antibacterial activity, inhibited RNA polymerase almost to the same degree as streptovaricin C (**1**) while some derivatives with greater antibacterial activity were less inhibitory in this assay (Fig. 2, Table 1). A question arises: Why are damavaricin C derivatives which block RNA polymerase unable to inhibit *E. coli* growth?

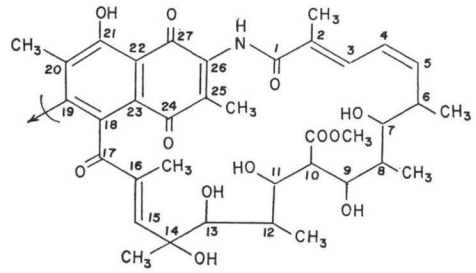
One plausible explanation is that the permeability barriers of *E. coli* and *P. aeruginosa* toward the derivatives are quite different from those of other bacteria. Hence, if mutations leading to an alteration of surface structure occur in *E. coli*, it would be expected that some derivatives could be found with activity against such membrane mutants.

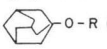
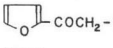
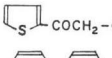
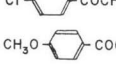
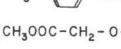
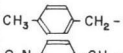
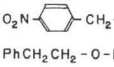
Three kinds of mutants were examined. Tol A, B and C are membrane mutants which absorb colicins, protein antibiotics, on the cell surface but are not killed by them. These mutants were reported to show pleiotropic alterations of permeability properties to various antibiotics[7,8] and they are sensitive to various derivatives (Table 2). However, another membrane mutant, *E. coli* deficient in phospholipase A, which hydrolyzes diacylglycerophos-

pholipid to monoacylglycerophospholipid, is not sensitive to the derivatives.[9] The cell membranes of colicin-tolerant mutants showed increased permeability toward derivatives which had no effect on the wild type of *E. coli* or the phospholipase deficient mutant. Since most phospholipid acyl groups are composed of oleic acid when a mutant requiring oleic acid is cultured in a medium supplemented with this acid, the membrane shows different permeability properties.[10,11] As opposed to the colicin-tolerant mutants with altered membrane protein,[7,8] a mutant with altered lipid components showed a selective permeability toward the drugs.

Table 1. Antibacterial activity (μg/ml) of damavaricin C derivatives *in vitro*.

R =



Compound *	*S. aureus* FDA 209P	*B. subtilis*	*Mycobact.* ATCC 607	*E. coli*	Compound *	*S. aureus* FDA209P	*B. subtilis*	*Mycobact.* ATCC 607	*E. coli*
Streptovaricin C (1)	2	10	≧100	>100	Ph-O-CH_2CH_2-O-R (25)	2	>100	0.5	>100
H-O-R (2)	50	>100	>100	>100	NC-$(CH_2)_3$-O-R (26)	10	20	5	>100
CH_3-O-R (3)	1	10	20	>100	C_6H_{11}-O-R (27)	50	50	50	>100
CH_3CH_2-O-R (4)	1	0.5	20	>100	 O-R (28)	50	N.D.	1	>100
$CH_3(CH_2)_2$-O-R (5)	10	5	5	>100	CH_3COCH_2-O-R (29)	1	2	100	>100
$(CH_3)_2$-CH-O-R (6)	50	50	50	>100	Ph-$COCH_2$-O-R (30)	5	20	20	>100
$CH_3(CH_2)_3$-O-R (7)	10	5	20	>100	 $COCH_2$-O-R (31)	0.5	N.D.	20	>100
$CH_3(CH_2)_4$-O-R (8)	10	5	20	>100	 $COCH_2$-O-R (32)	5	N.D.	10	>100
$(CH_3)_2CHCH_2CH_2$-O-R (9)	10	5	20	>100	 $COCH_2$-O-R (33)	20	N.D.	10	>100
$CH_3(CH_2)_5$-O-R (10)	5	2	2	>100	Cl-  $COCH_2$-O-R (34)	10	10	50	>100
$CH_3(CH_2)_6$-O-R (11)	2	N.D.	0.5	>100	CH_3O-  $COCH_2$-O-R (35)	5	5	5	>100
$CH_3(CH_2)_7$-O-R (12)	>100	50	10	>100	$CH_3OOC-CH_2$-O-R (36)	10	10	50	>100
$CH_3(CH_2)_8$-O-R (13)	>100	N.D.	5	>100	H_2NCOCH_2-O-R (37)	100	N.D.	20	>100
$CH_3(CH_2)_{10}$-O-R (14)	>100	50	N.D.	>100	$PhCH_2$-O-R (38)	2	N.D.	20	>100
$CH_3(CH_2)_{11}$-O-R (15)	>100	>100	20	>100	CH_3-  CH_2-O-R (39)	5	N.D.	0.5	>100
$CH_3(CH_2)_{17}$-O-R (16)	>100	>100	N.D.	>100	O_2N-  CH_2-O-R (40)	5	N.D.	1	>100
$HOCH_2CH_2$-O-R (17)	50	50	>100	>100	$PhCH_2CH_2$-O-R (41)	10	5	10	>100
Cl-CH_2CH_2-O-R (18)	1	1	N.D.	>100	Ph-COO-CH_2CH_2-O-R (42)	5	N.D.	1	>100
 -CH-CH_2-O-R (19)	10	N.D.	2	>100	19-O-Methyldamavaricin A (43)**	5	10	20	>100
Cl-$CH_2CH_2CH_2$-O-R (20)	5	2	20	>100	19-O-Methyldamavaricin B (44)**	0.5	N.D.	2	>100
CH_2=$CHCH_2$-O-R (21)	2	1	20	>100					
$HOCH_2(CH_2)_3$-O-R (22)	5	5	0.5	>100					
$(C_2H_5O)_2CHCH_2$-O-R (23)	20	50	N.D.	>100					
CH_2=CH-O-CH_2CH_2-O-R (24)	10	20	N.D.	>100					

N.D.: not determined

* Microanalysis and low resolution mass spectral data agree with the molecular formula assigned.

** The compounds **43** and **44** were prepared from streptovaricins A and B, respectively, by the same reaction procedure used for the preparation of **3**.

Table 2. Antibacterial activity (μg/ml) of damavaricin C derivatives against membrane mutants of *E. coli*.

Compound	Tol A B mutants	Tol C mutant	Phospholipase A deficient mutant	Oleic acid auxotroph
Streptovaricin C (**1**)	1	5	>100	50
H−O−R (**2**)	20	50	>100	N.D.
CH_3−O−R (**3**)	5	5	>100	100
CH_3CH_2−O−R (**4**)	2	5	>100	100
$CH_3CH_2CH_2$−O−R (**5**)	5	5	>100	>100
$CH_3(CH_2)_5$−O−R (**10**)	10	100	>000	>100
CH_2=$CHCH_2$−O−R (**21**)	5	5	>100	>100
CH_3COCH_2−O−R (**29**)	5	10	>100	>100
$PhCH_2$−O−R (**38**)	5	5	>100	>100
$PhCH_2CH_2$−O−R (**41**)	10	50	>100	N.D.

Only the methyl ether (**3**) and ethyl ether (**4**) were effective against this mutant (Table 2).

The results described above indicate that damavaricin C retains its original biological activity at the target enzyme and that the groups introduced to the C-19 position of the molecule confer increased diffusibility to the derivatives, thereby facilitating cell penetration.

As described in the introduction, the streptovaricin complex has been reported to possess, in addition to its antibacterial activity, an inhibitory effect on reverse transcriptase of RNA tumor virus or the inhibition of focus formation of animal cells by RNA tumor virus *in vitro*.[4a,4b,12] None of the streptovaricin derivatives thus far reported, however, had an inhibitory effect on the growth of tumor cells *in vitro*. Since the derivatives of streptovaricin C reporpted here have different antibacterial spectra due to the differing permeability properties of the molecules, it is tempting to ask whether or not some of them may acquire biological activity on animal cells *in vitro*.

Preliminary experiment showed that some of them were active on animal cells *in vitro* including normal and viral transformed cells derived from rat, mouse and man.[1] Details will be published in a subsequent paper.[13]

Experimental

General

Melting points are uncorrected and were determined on a Yanaco micro hot-stage apparatus. Infrared spectra were obtained on a Hitachi spectrophotometer, Model EPI-G2, and ultraviolet spectra on a Hitachi spectrophotometer, Model 124. Nuclear magnetic resonance spectra were determined in deutrochloroform on a Varian Associates spectrometer, Model HA-100; chemical shift values (δ) are reported in p.p.m. from internal TMS. Mass spectra were obtained on a JEOL mass spectrometer, Model JMS 01SG-2, with heated direct inlet system. Molecular weights were established by low resolution mass spectrometry. Microanalyses were obtained at the Institute of Physical and Chemical Research, Wako-shi, Saitama, Japan.

Separation of streptovaricin C.

Column chromatographic separation of the streptovaricin components was accomplished on silica gel (0.063~0.200 mm, E. Merck), according to the procedure of RINEHART, *et al.*,[4b] followed by gel filtration over Sephadex LH-20, employing acetone as solvent. The order of elution of the streptovaricin components by the gel filtration was streptovaricin E,A,B,D,G,C, and J.

Streptovaricin C (**1**) was recrystallized from ethanol-*n*-hexane as orange-red prisms, m.p. 195~196°C, and identified with standard streptovaricin C (kindly supplied by Dr. K.L. RINEHART, Jr., University of Illinois, U.S.A.) by thin-layer chromatography (GF254, E. Merck), UV, NMR, and mass spectrometric analyses. The yield of streptovaricin C, the most abundant component of the streptovaricin complex (supplied by The Upjohn Co., Kalamazoo, Michigan, U.S.A.), was about 20 %.

Preparation of damavaricin C

Procedure A. Oxygen was bubbled for 1 hour through a solution of 4 g of streptovaricin C and 500 mg of potassium hydroxide in 100 ml of methanol at room temperature. The mixture was evaporated to a small volume under reduced pressure at 45°C, diluted with water, neutralized with 6 N hydrochloric acid, and extracted with ethyl acetate. The extract was washed with water followed by saline solution, dried, and evaporated to dryness under reduced pressure to an orange-red oil. The oil was recrystallized from acetone to give yellow crystals (1.28 g). The mother liquor was evaporated to dryness *in vacuo* and the resulting oil was chromatographed on silica gel with 10 % methanol in chloroform. The red oil from appropriate fractions gave an additional 300 mg of product, melting 284~285°C; λ_{max}^{MeOH} 243, 310, 430 nm (e 44,500, 16,800 inflection, 6,940); $\lambda_{max}^{0.01N\ NaOH\text{-}MeOH}$ 237, 291, 344, 424, 550 nm (e 48,900, 33,300, 12,600 inflection, 10,800, 6,140); pKa′ 5.77 and 10.9; M^+ (molecular ion peak) 713.

Anal. Calcd. for $C_{87}H_{47}NO_{13}$: C, 62.26; H, 6.64; N, 1.96.
Found: C, 62.19; H, 6.70; N, 1.78.

Procedure B. Air was bubbled for 1 hour through a solution of 6 g of streptovaricin C in 400 ml of methanol at room temperature, 200 ml of conc. ammonium hydroxide was added, and the bubbling was continued for an additional 2 hours. The reaction mixture was evaporated at 40°C to the one-third of of its volume under reduced pressure, and the dark solution was acidified to pH 3 with 6 N hydrochloric acid to give a yellow brown precipitate. The collected precipitate was washed with acetone, giving **2** as a yellow crystalline powder (2.8 g) (a single purple spot on t.l.c.). An additional 570 mg of **2** was obtained from the mother liquor by the same chromatographic procedure described in Procedure A.

Etherification of damavaricin C

The 19-O-substituted derivatives of damavaricin C were synthesized following two main procedures. Procedure A consisted of treating damavaricin C (n moles) with silver oxide (0.75 n mole) in methanol at room temperature for 1~2 hours to form a silver salt of **2** which was treated with the appropriate iodo-compound (5~10 n moles) at room temperature for 0.5~1 hour. The desired product was separated by column chromatography on silica gel and/or gel filtration on Sephadex LH-20 with acetone. Procedure B differed from A in that a longer reaction time (15~20 hours) and an excess of silver oxide (5 n moles) plus 5~10 n moles of a bromo compound were used. The derivatives obtained were checked by t.l.c. on silica gel plates using 10 % methanol in chloroform as eluant. Examples follow.

19-O-Methyldamavaricin C (**3**)

Procedure A. To a suspension of 1.42 g of damavaricin C in 70 ml of methanol was added 360 mg of silver oxide, and the mixture was stirred at room temperature for 1 hour. To this was added 1.5 g of methyl iodide, the reaction mixture was stirred at room temperature for 45 minutes and filtered through Celite, and the filtrate was evaporated under reduced pressure, to give a red oil. The oil was recrystallized from acetone-*n*-hexane, giving yellow needles, m.p. 165~167°C (single spot on t.l.c.); λ_{max}^{MeOH} 257, 310, and 423 nm (e 47,400, 17,800, and 8,600); mol. wt. 727 (mass spectrum).

Anal. Calcd. for $C_{35}H_{49}NO_{13}\cdot H_2O$: C, 61.17; H, 6.89; N, 1.88.
Found: C, 61.18; H, 6.68; N, 1.71.

19-O-Phenacyldamavaricin C (**30**)

Procedure B. To a suspension of 713 mg of damavaricin C in 50 ml of methanol was

added 1.16 g of silver oxide. This was stirred at room temperature for 1.5 hours. Two g of α-bromoacetophenone were added, and stirring was continued for 18 hours. The reaction mixture was filtered through Celite, and the filtrate was evaporated under reduced pressure to give a red oil. The oil was dissolved in a small amount of chloroform and precipitated by adding *n*-hexane. This crude product was chromatographed on Sephadex LH-20 with acetone. The appropriate fractions gave an orange-red oil (single spot on t.l.c.) which crystallized from acetone - *n*-hexane as yellow needles (340 mg), m.p. 155~156°; λ_{max}^{MeOH} 246, 308, and 422 nm (e 67,200, 20,500, and 9,500); M^+ 831 (mass spectrum).

Anal. Calcd. for $C_{45}H_{53}NO_{14}\cdot H_2O$: C, 63.59; H, 6.52; N, 1.65.
Found: C, 63.86; H, 6.37; N, 1.63.

19-O-Cyclohexyldamavaricin C (**27**)

Procedure B. A suspension of 750 mg of damavaricin C in 80 ml of methanol and 928 mg of silver oxide was stirred at 25°C for 2 hours after which 3.26 g of cyclohexyl bromide was added. The reaction was maintained at room temperature for 20 hours and filtered, and the filtrate was evaporated under reduced pressure to give a red oil. The oil was chromatographed on silica gel with 3 % methanol in chloroform. Combination of appropriate fractions yielded an orange oil which was recrystallized from acetone - *n*-hexane, giving yellow crystals (185 mg), m.p. 164~166°; λ_{max}^{MeOH} 261, 316, and 425 nm (e 46,600, 15,100, and 7,400); mol. wt. 795 (mass spectrum).

Anal. Calcd. for $C_{48}H_{57}NO_{13}\cdot 1/2H_2O$: C, 64.16; H, 7.26; N, 1.74.
Found: C, 64.20; H, 7.27; N, 1.65.

Biological tests

In vitro activity. The antimicrobial activity of these new derivatives was assayed by determining the minumum inhibitory concentrations (MIC) against *Staphylococcus aureus* FDA 209P (ATCC 6538-P), *Bacillus subtilis* ATCC 6633, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, and *Pseudomonas aeruginosa* IFO using the serial dilution technique in nutrient broth. The MIC was the lowest concentration of antibiotic which prevented visible growth after an 18~48 hour incubation at 37°C.

Colicin-tolerant mutants of *E. coli*, tol A, B and C mutants, were isolated by BERNSTEIN *et al.*[7,8] A phospholipase A deficient mutant and one requiring oleic acid were supplied by Dr. S. NOJIMA, The University of Tokyo, Tokyo.[9,8]

RNA polymerase assay. DNA-dependent RNA polymerase of *E. coli* was measured by the method of BURGESS.[14] RNA polymerase was provided by Dr. H. IKEDA, Institute of Medical Science, The University of Tokyo, Tokyo.

Acknowledgment

The authors would like to express their thanks to Mr. K. MOMOKI of the Kaken Chemical Co., Ltd. for his excellent technical assistance.

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