Chem. Pharm. Bull. 30(1) 223—229 (1982)

Bioconversion and Biosynthesis of 16-Membered Macrolide Antibiotics. XXII.[1] Biosynthesis of Tylosin after Protylonolide Formation

SATOSHI ŌMURA,* NORIAKI SADAKANE and HAJIME MATSUBARA

School of Pharmaceutical Sciences, Kitasato University and The Kitasato Institute, Minato-ku, Tokyo 108, Japan

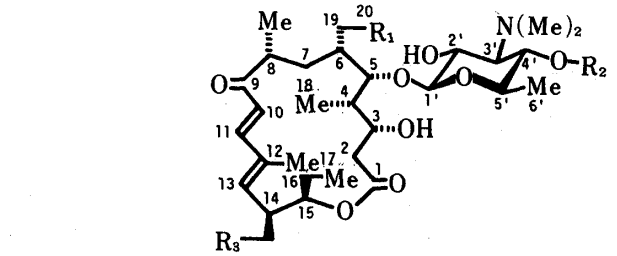
(Received May 20, 1981)

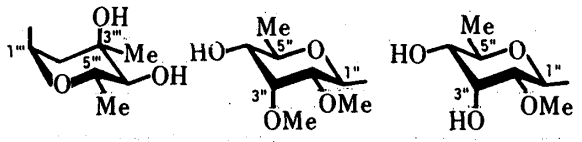
In order to clarify the later stages of tylosin biosynthesis, the biotransformation of tylosin-related compounds to tylosin was examined in a tylosin-producing strain, *Streptomyces fradiae* KA-427, with the aid of cerulenin, which is an inhibitor of fatty acid and polyketide biosynthesis.

Protylonolide (**9**), 5-*O*-mycaminosylprotylonolide (**11**), deepoxycirramycin A_1 (**12**), 20-deoxy-5-*O*-mycaminosylrelonolide (**13**), 5-*O*-mycaminosyltylonolide (**3**) and demycarosyltylosin (**2**) were bioconverted to tylosin. However, 23-hydroxyprotylonolide (**10**), 20-deoxydemycarosylrelomycin (**14**) and 20-deoxy-23-*O*-mycinosylrelonolide (**16**) were bioconverted not to tylosin, but to 20-deoxyrelomycin (**15**). Thus, the biosynthetic pathway *via* compounds **11** and **3** is proposed.

Keywords——biosynthesis; tylosin; 16-membered macrolide; cerulenin; protylonolide; bioconversion

Tylosin (**1**), a 16-membered macrolide antibiotic produced by *Streptomyces fradiae* KA-427 (C-373), was first reported by McGuire *et al.*[2] in 1961. Macrocin (**7**), a related antibiotic, was

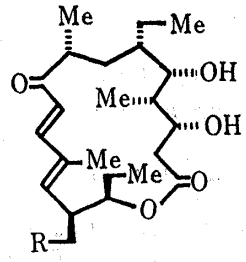




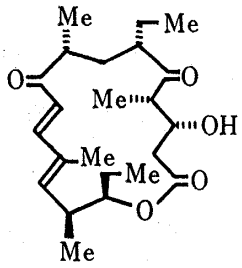
mycarosyl mycinosyl 3-demethoxy-3-hydroxymycinosyl

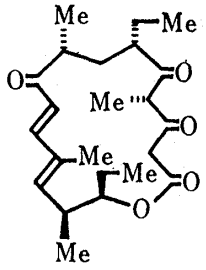
	R_1	R_2	R_3
1: tylosin	CHO	mycarosyl	-*O*-mycinosyl
2: demycarosyltylosin (=desmycosin)	CHO	H	-*O*-mycinosyl
3: 5-*O*-mycaminosyltylonolide	CHO	H	OH
4: relomycin	CH_2OH	mycarosyl	-*O*-mycinosyl
5: demycarosylrelomycin	CH_2OH	H	-*O*-mycinosyl
6: 5-*O*-mycaminosylrelonolide	CH_2OH	H	OH
7: macrocin	CHO	mycarosyl	-*O*-3-demethoxy-3-hydroxymycinosyl
8: lactenocin	CHO	H	-*O*-3-demethoxy-3-hydroxymycinosyl
11: 5-*O*-mycaminosylprotylonolide	Me	H	H
12: deepoxycirramycin A_1	CHO	H	H
13: 20-deoxy-5-*O*-mycaminosylrelonolide	Me	H	OH
14: 20-deoxydemycarosylrelomycin	Me	H	-*O*-mycinosyl
15: 20-deoxyrelomycin	Me	mycarosyl	-*O*-mycinosyl

Fig. 1. Continued



	R
9: protylonolide	H
10: 23-hydroxyprotylonolide	OH
16: 20-deoxy-23-*O*-mycinosylrelonolide	-*O*-mycinosyl





17: 5-dehydroprotylonolide **18**: 3,5-didehydroprotylonolide

Fig. 1. Structures of Tylosin and Related Compounds

isolated from the cultures of the same microorganism.[3] Relomycin (**4**) was described as a metabolite from *S. hygroscopicus*.[4] Desmycosin (**2**) and lactenocin (**8**) were reported by Hamill and co-workers[3,5] as hydrolysis products of tylosin and macrocin, respectively. Later, these two antibiotics were detected in the fermentation broth of the tylosin producer.[6] The structures of tylosin[7–9] and related compounds are presented in Fig. 1.

Along with these findings, biosynthetic relations of these antibiotics were reported. Seno and his co-workers[6] proposed a scheme for the terminal steps in tylosin biosynthesis, in which tylosin (**1**) is synthesized *via* lactenocin (**8**), a diglycosylated intermediate, as shown in Fig. 2. We showed that the aglycone of tylosin (**1**) is derived from two acetates, five propionates and one butyrate, using carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy.[10,11] However, the steps after aglycone formation, such as oxidations of the aglycone and subsequent glycosidations, have not been studied in detail.

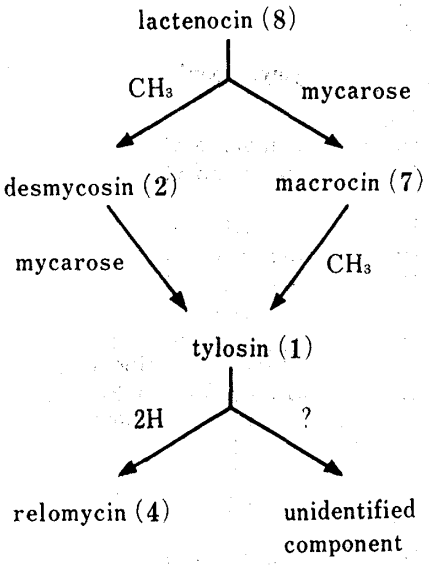


Fig. 2. Proposed Scheme for the Terminal Steps in Tylosin Biosynthesis[6]

Recently, we isolated protylonolide from a mycaminose idiotroph of *S. fradiae* KA-427.[12,13] Protylonolide (**9**) is a 16-membered lactone with the fundamental carbon skeleton of tylosin aglycone, and is a precursor of tylosin, since it is efficiently converted to tylosin by the parent strain of *S. fradiae* KA-427.[12] In order to clarify further tylosin biosynthesis, many derivatives (Fig. 1) of protylonolide, tylosin and related compounds were synthesized chemically,[14] and biotransformation of these derivatives was carried out using *S. fradiae* KA-427 grown in the presence of cerulenin, an inhibitor of *de novo* synthesis of macrolide antibiotics.[15–18]

In the present work, we investigated tylosin biosynthesis after protylonolide formation with special regard to the order of the reactions occurring in the conversion of protylonolide to tylosin, namely, oxidations at the C-20 and C-23 positions and glycosidations by three sugars. The results are discussed in connection with Seno's proposal. The synthesis, biological activity and structure-activity relationship of the derivatives have been described.[14]

Materials and Methods

Preparation of Tylosin-related Compounds——Demycarosyltylosin (**2**),[7] 5-*O*-mycaminosyltylonolide (**3**),[7] protylonolide (**9**),[12] 23-hydroxyprotylonolide (**10**),[14] 5-*O*-mycaminosylprotylonolide (**11**),[14] deepoxycirramycin A_1 (**12**),[14] 20-deoxy-5-*O*-mycaminosylrelonolide (**13**),[14] 20-deoxydemycarosylrelomycin (**14**)[14] and 20-deoxy-23-*O*-mycinosylrelonolide (**16**)[14] were prepared by the methods described in the cited references.

5-Dehydroprotylonolide (**17**) and 3,5-didehydroprotylonolide (**18**) were obtained as described below.

Two hundred mg of protylonolide (**9**) were stirred in 10 ml of acetone in an ice bath. To the above solution, 400 mg of CrO_3 and 4 ml of pyridine were added and the reaction mixture was stirred for 30 h. The mixture was poured into 100 ml of 0.5 N HCl, and filtered. The reaction products were extracted with three portions of 50 ml of $CHCl_3$. The solvent layer was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The residue was applied to a silica gel column, which was developed with benzene–acetone (50: 1) to give 150 mg of **17** and 20 mg of **18**.

5-Dehydroprotylonolide (**17**): UV λ_{max}^{MeOH} nm (ε): 280 (21630). MS *m/z*: 392 (M^+, 392.260; Calcd for $C_{23}H_{36}O_5$: 392.256). *Rf*=0.44 (silica gel thin layer chromatography (TLC), benzene–acetone (10: 1)).

3,5-Didehydroprotylonolide (**18**): UV λ_{max}^{MeOH} nm (ε): 277 (17680), $\lambda_{max}^{0.1N\ NaOH\text{-}MeOH}$ nm (ε): 273 (20460). MS *m/z*: 390 (M^+, 390.237; Calcd for $C_{23}H_{34}O_5$: 390.241). *Rf*=0.67 (silica gel TLC, benzene–acetone (10: 1). Coloration: $FeCl_3$ (+).

Method of Biotransformation——The tylosin-producing strain, *S. fradiae* KA-427, was cultured in a 500 ml Sakaguchi flask containing 100 ml of a medium composed of 1.0% glucose, 2.0% starch, 0.5% peptone, 0.5% yeast extract, 0.3% L-asparagine and 0.4% $CaCO_3$ (adjusted to pH 7.4 prior to autoclaving). In order to inhibit the *de novo* synthesis of tylosin, 40 μg/ml of cerulenin was added to the medium initially and at intervals of 24 h. After 48 h cultivation, 50 μg/ml of a test compound was added to the culture, and the cultivation was continued for a further 48 h. The culture broth was centrifuged and the supernatant was extracted with an equal volume of benzene. The benzene layer was concentrated to dryness and subjected to silica gel TLC, developed with $CHCl_3$–MeOH–conc. NH_4OH (10: 1: 0.01). The microbial transformation of each compound was monitored by means of a chromatogram scanner (Model CS-920, Shimadzu Seisakusho Co., Ltd.) at 282 nm. For the structural confirmation of the compounds formed, each compound isolated on TLC was extracted with $CHCl_3$–MeOH, and subjected to mass and NMR spectroscopies.

Results and Discussion

Eleven compounds were prepared chemically and subjected to bioconversion by the tylosin-producing strain *S. fradiae* KA-427 in the presence of cerulenin. As representatives of these experiments, TLC profiles of the products formed from 20-deoxy-5-*O*-mycaminosylrelonolide (**13**) and from 20-deoxydemycarosylrelomycin (**14**) are shown in Fig. 3. Table I

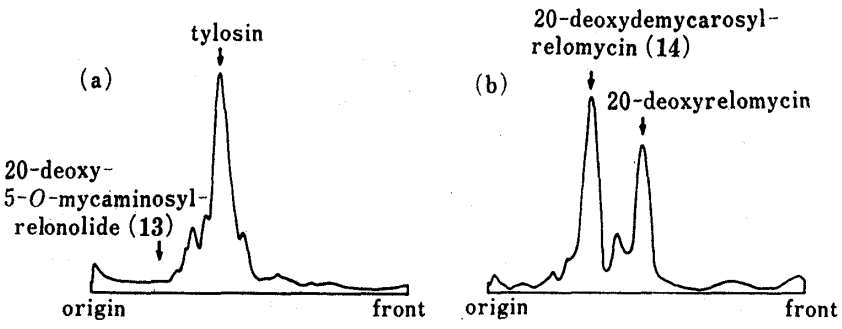


Fig. 3. Bioconversion of 20-Deoxy-5-*O*-mycaminosylrelonolide (**13**) (a) and 20-Deoxydemycarosylrelomycin (**14**) (b) by *S. fradiae* KA-427 in Cerulenin-supplemented Culture

The bioconversion was analyzed by silica gel TLC (solvent $CHCl_3$: MeOH: conc. NH_4OH=10: 1: 0.01) scanned at 282 nm.

Table I. Bioconversion of Tylosin-related Compounds by *S. fradiae* KA-427 in the Presence of Cerulenin, which inhibits Lactone Synthesis

Compound added	Compound formed (%)[a]				
	Tylosin (1)	Relomycin (4)	20-Deoxy-relomycin (15)	Unidentified compounds	Starting material (%)[a]
Protylonolide (9)	73	1	0	21	5
23-Hydroxyprotylonolide (10)	0	0	16	65[b]	19
5-*O*-Mycaminosylprotylonolide (11)	29	4	18	35	14
Deepoxycirramycin A_1 (12)	45	2	0	37	16
20-Deoxy-5-*O*-mycaminosylrelonolide (13)	86	1	0	11	0
20-Deoxydemycarosylrelomycin (14)	0	0	35	14	51
5-*O*-Mycaminosyltylonolide (3)	60	6	0	21	13
Demycarosyltylosin (2)	62	4	0	6	28
20-Deoxy-23-*O*-mycinosylrelonolide (16)	0	0	1	1	98
5-Dehydroprotylonolide (17)	5	0	0	0	95
3,5-Didehydroprotylonolide (18)	5	0	0	5	90

a) The conversion (%) of each compound was calculated from the peak area detected with a Shimadzu chromatoscanner (Model CS-920) at 282 nm.
b) A characteristic peak (55% of the total peak area) identical with that of 20-deoxy-23-*O*-mycinosylrelonolide (16) was observed.

summarizes the percent peak areas of tylosin and related products on the TLC as detected by a chromatogram scanner at 282 nm.

The biotransformation products, tylosin (**1**), relomycin (**4**), 20-deoxyrelomycin (**15**) and 20-deoxy-23-*O*-mycinosylrelonolide (**16**), were identified by high resolution mass spectrometry in comparison with corresponding authentic samples. According to our working model of the biosynthetic pathway, the formation of 20-deoxyrelomycin (**15**) was expected as a major product from 20-deoxydemycarosylrelomycin (**14**), and it was identified from mass fragmentation and NMR data as shown in Fig. 4. The sum of the peak areas of the minor compounds which appeared in the TLC of the bioconvertants is indicated as "unidentified compounds" except for 20-deoxy-23-*O*-mycinosylrelonolide (**16**), which was formed from compound **10** and identified by comparison with an authentic sample.

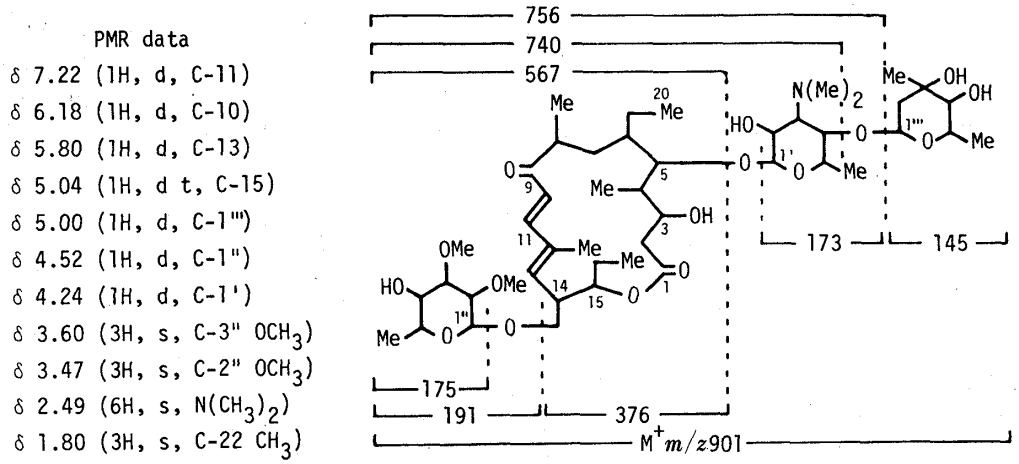


Fig. 4. PMR and Mass Fragmentation of 20-Deoxyrelomycin (**15**)

As shown in Table I, compounds **2**, **3**, **9**, **11**, **12** and **13** were efficiently converted to tylosin. On the other hand, compounds **10**, **14**, and **16** were converted not to tylosin, but to 20-deoxy-relomycin (**15**) by a process involving the glycosidations, but not oxidation of the C-20 methyl group to aldehyde.

Several reactions such as condensation of acetates (or malonates), propionates (or methylmalonates) and butyrate (or 2-ethylmalonate), ring closure for the lactone formation and reduction of carbonyl groups to give a hydroxyl derivative are involved in the biosynthesis of protylonolide (**9**). It is of interest to know the order of these reactions. In order to get information on this problem, bioconversion was carried out with two compounds, 5-dehydroprotylonolide (**17**) and 3,5-didehydroprotylonolide (**18**), both of which have carbonyl groups related to a hypothetical intermediate polyketide, and might be the immediate precursor of protylonolide. Only 5% tylosin formation was observed from each compound (Table I). In view of this small value of conversion to tylosin, it is difficult to conclude that these compounds are precursors of protylonolide. The synthesis of 3-dehydroprotylonolide, which is also one of the possible precursors of protylonolide, and transformation studies are in progress.

From these results, we conclude that tylosin is biosynthesized in *S. fradiae* KA-427 through the pathways shown in Fig. 5, in which the routes confirmed by the present experiments are indicated by thick arrows.

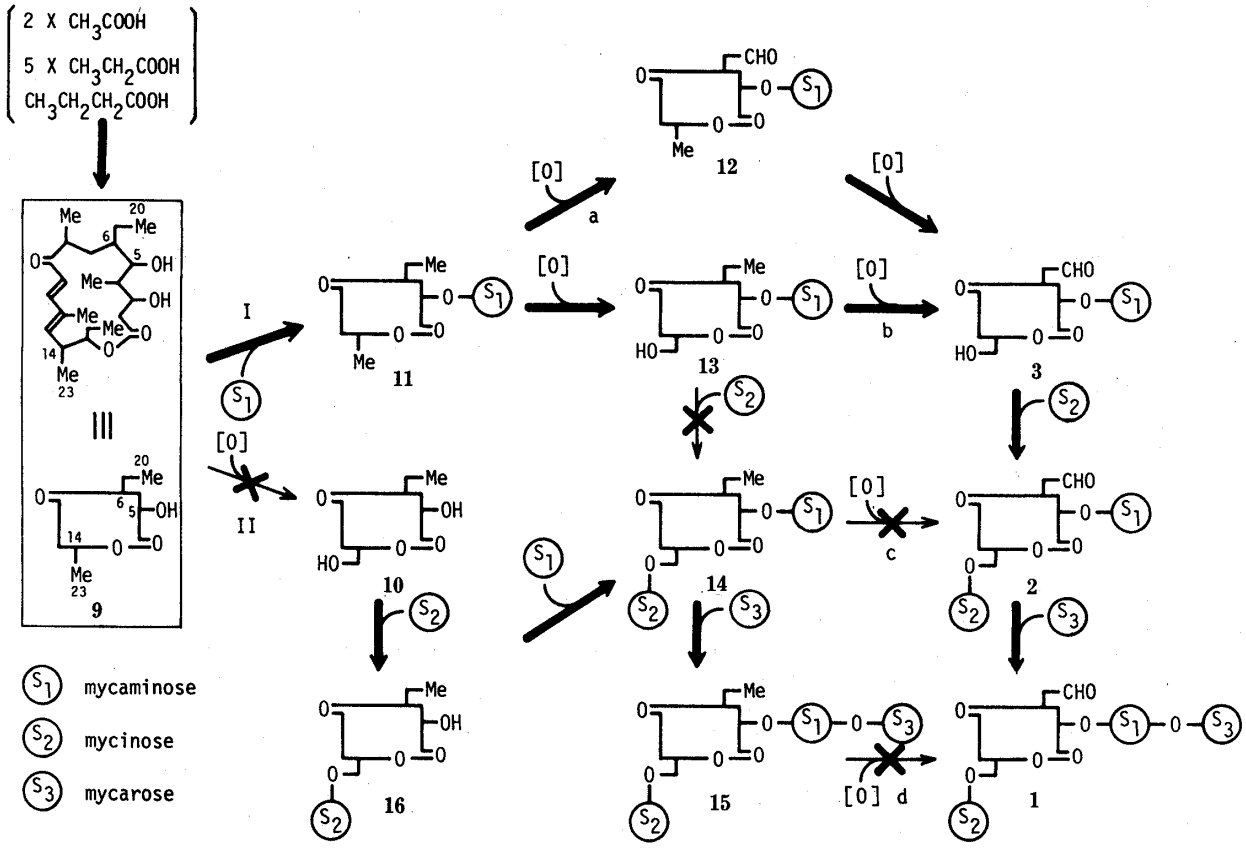


Fig. 5. Bioconversion of Tylosin-related Compounds and Postulated Pathway of Tylosin Biosynthesis in *S. fradiae* KA-427

The fact that the C-20 methyl group was oxidized in compounds **11** and **13**, but not in compounds **14** and **15**, suggests that the enzyme involved in the C-20 methyl oxidation is highly specific. On the other hand, glycosidation by mycarose occurred in compounds **2** and **14** in the present experiments, and in protylonolide and demycarosylrelomycin as already reported[13,18] showing that the specificity of the glycosylase is not high. Such a difference in substrate specificity of the enzymes involved in the biosynthesis of the antibiotic may account for the phenomenon that antibiotics are generally produced as many components in which some partial structures are different.

Seno *et al.* reported two pathways from lactenosin (**8**), which possesses a 3-demethoxy-3-

hydroxymycinosyl moiety, to tylosin *via* desmycosin (**2**) or macrosin (**7**), as shown in Fig. 2. These pathways could be inserted between 5-*O*-mycaminosyltylonolide (**3**) and tylosin (**1**) in Fig. 5, so that one of the two (*i.e.*, the one through desmycosin (**2**)) is superimposed on our pathway. However, our present experiments could not detect the formation of these intermediates (indicated in Fig. 2). Since mycinose is a structural constituent of many macrolide antibiotics, such as chalcomycin,[19] neutramycin,[20] angolamycin[21] and mycinamicin,[22] as well as compounds **15** and **16** reported here, an enzyme involved in the methylation of the 3-demethoxy-3-hydroxymycinosyl moiety may have low substrate specificity, or different enzymes may serve for the methylation in different producers of these antibiotics. Erythromycin C was transformed to erythromycin A by *S*-adenosyl methionine: erythromycin C *O*-methyltransferase, which was reported to have strict substrate specificity.[23] In view of these variations of methyl transfer to sugar hydroxyl, further experiments on tylosin biosynthesis in cell-free systems are required to determine the substrate specificity of the enzyme.

As shown in Fig. 2, Seno *et al.* reported that tylosin (**1**) is a precursor of relomycin (**4**), while we have reported the interconversion of the aldehyde and the hydroxymethyl group in the stage before mycarose is introduced at the 4-*O*-position of mycaminose.[18]

In the present study, cerulenin was used as an efficient tool to set up conditions such that bioconversion could be easily monitored without using radioactive substrates, in the same way as reported in biosynthetic studies of spiramycin,[16] leucomycin,[17] tylosin[18] and nanaomycin.[24] From this experience with cerulenin, it is considered that the formation of the polyketide is more sensitive to cerulenin than that of β-ketoacyl-ACP in fatty acid biosynthesis. This is presumably the basis of the successful use of cerulenin in strain improvement in the fermentation of the polyketide antibiotic, daunomycin.[25] Further applications of cerulenin seem likely not only in studies of lipid biochemistry, but also for the improvement of industrial production of antibiotics.

Acknowledgement The authors thank Dr. L.E. Day of Eli Lilly and Company for kindly supplying the tylosin-producing strain *S. fradiae*. Thanks are also due to Dr. Y. Tanaka, Kitasato University, for stimulating discussions.

References and Notes

1) Part XXI of this series: Y. Tanaka, Y. Takahashi, R. Masuma, Y. Iwai, H. Tanaka, and S. Omura, *Agric. Biol. Chem.*, **45**, 2475 (1981).
2) J.M. McGuire, W.S. Boniece, C.E. Higgens, M.M. Hoehn, W.M. Stark, J. Westhead, and R.N. Wolfe, *Antibiot. Chemother.*, **11**, 320 (1961).
3) R.L. Hamill and W.M. Stark, *J. Antibiot.*, **17**, 133 (1964).
4) H.A. Whaley, E.L. Patterson, A.C. Dornbush, E.J. Backus, and N. Bohonos, *Antimicrob. Agents and Chemother.*, **1963**, 45, (1964).
5) R.L. Hamill, M.E. Haney, M. Stamper, and P.F. Wiley, *Antibiot. Chemother.*, **11**, 328 (1961).
6) E.T. Seno, R.L. Pieper, and F.M. Huber, *Antimicrob. Agents and Chemother.*, **11**, 455 (1977).
7) R.B. Morin, M. Gorman, R.L. Hamill, and P.V. Demarco, *Tetrahedron Lett.*, **1970**, 4737.
8) S. Ōmura, A. Nakagawa, A. Neszmélyi, Stephan D. Gero, A.-M. Sequlchre, F. Piriou, and G. Lukacs, *J. Am. Chem. Soc.*, **97**, 4001 (1975).
9) S. Ōmura, H. Matsubara, A. Nakagawa, A. Furusaki, and T. Matsumoto, *J. Antibiot.*, **33**, 915 (1980).
10) S. Ōmura, A. Nakagawa, H. Takeshima, J. Miyazawa, C. Kitao, F. Piriou, and G. Lukacs, *Tetrahedron Lett.*, **1975**, 4503.
11) S. Ōmura, H. Takeshima, A. Nakagawa, J. Miyazawa, F. Piriou, and G. Lukacs, *Biochem.*, **16**, 2860 (1977).
12) S. Ōmura, C. Kitao, and H. Matsubara, *Chem. Pharm. Bull.*, **28**, 1963 (1980).
13) S. Ōmura, N. Sadakane, C. Kitao, H. Matsubara, and A. Nakagawa, *J. Antibiot.*, **33**, 913 (1980).
14) H. Matsubara, K. Miyano, A. Nakagawa, and S. Ōmura, *Chem. Pharm. Bull.*, **30**, 97 (1982).
15) S. Ōmura, *Bact. Rev.*, **40**, 681 (1976).
16) S. Ōmura, C. Kitao, H. Hamada, and H. Ikeda, *Chem. Pharm. Bull.*, **27**, 176 (1979).
17) C. Kitao, J. Miyazawa, and S. Ōmura, *Agric. Biol. Chem.*, **43**, 833 (1979).
18) S. Ōmura, C. Kitao, J. Miyazawa, H. Imai, and H. Takeshima, *J. Antibiot.*, **31**, 254 (1978).

19) P.W.K. Woo, H.W. Dion, and Q.R. Bartz, *J. Am. Chem. Soc.*, **86**, 2724 (1964).
20) L.A. Mitscher and M.P. Kunstamann, *Experientia*, **25**, 12 (1969).
21) M. Brufani and W. Keller-Schierlein, *Helv. Chim. Acta*, **49**, 1962 (1966).
22) M. Hayashi, M. Ohno, and S. Satoi, *J. Chem. Soc. Chem. Comm.*, **1980**, 119.
23) J.W. Corcoran, "Methods in Enzymology," Vol. 43, ed. by J.H. Hash, Academic Press, New York, 1975, p. 487.
24) C. Kitao, H. Tanaka, S. Minami, and S. Ōmura, *J. Antibiot.*, **33**, 711 (1980).
25) J.C. McGuire, G. Glotfelty, and R.J. White, *FEMS Microb. Lett.*, **9**, 141 (1980).