Separation and Detection of Macrolide Antibiotics by HPLC Using Macrolide-imprinted Synthetic Polymers as Stationary Phases

MARTIN SIEMANN†, LARS I. ANDERSSON†† and KLAUS MOSBACH*

Pure and Applied Biochemistry, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden

(Received for publication June 17, 1996)

Erythromycin and oleandomycin are macrolide antibiotics belonging to the erythromycin type and tylosin is one of the compounds of the structurally very related carbomycin family[1] (Fig. 1). In particular erythromycin has, since its discovery in the early 1950s, been one of the very widely used antibiotics, useful in the treatment of a variety of infectious deseases[2~4]. Several methods for the determination of these antiobitics in plasma and tissue, including microbial test systems[5], radioimmunoassay[6] and liquid chromatography[7,8], have been described.

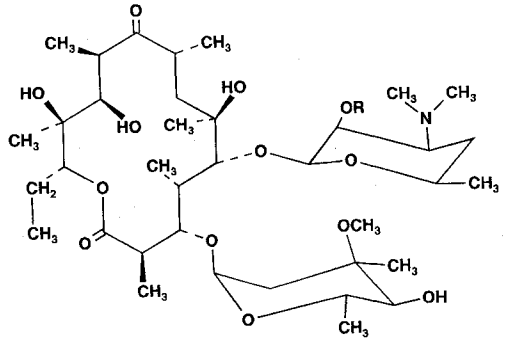
Molecular imprinting[9] of organic compounds is increasingly recognised as a facile technique for the preparation of polymeric materials containing recognition sites of predetermined specificity. The resulting polymers retain a memory for the print molecule and can be used in the HPLC mode for separation of structurally similar substances, and (in case of single enantiomer imprinting) resolution of the enantiomers of the print species[10]. Polymerisation of monomers, which are selected for their ability to engage in non-covalent interactions with specific functionality of the print molecule, constitutes the key step. Subsequent removal of the print molecule exposes sites within the polymer matrix whose chemical and steric topography is defined by the print molecule present during its preparation. These memory sites enable the polymer to later rebind the print molecule, in many instances with binding affinities and specificities approaching those demonstrated by antigen-antibody systems[11,14]. This paper describes the molecular imprinting of some macrolide antibiotics and the use of the resultant polymers as stationary phases with predetermined selectivity in HPLC.

Materials and Methods

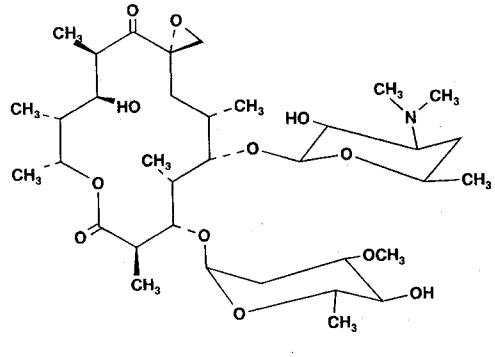
Polymer Preparation

Polymers were prepared according to a method described previously[10]. Imprint molecule; 149 mg (0.20 mmol) of erythromycin A, 151 mg (0.22 mmol) of oleandomycin, or 183 mg (0.17 mmol) of tylosin, 3.96 g

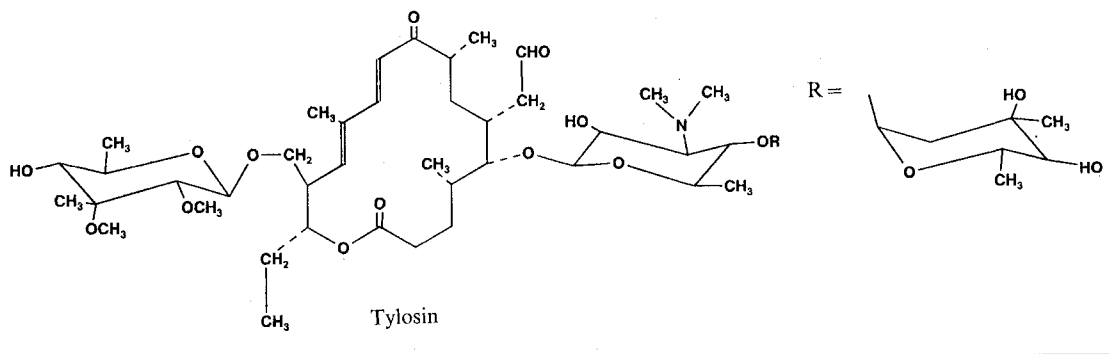
Fig. 1. Structures according to MERCK INDEX[13] of the macrolides antibiotics.



Erythromycin A
R=H



Oleandomycin



Tylosin

Present address: † Institute of Biochemical Engineering, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany.
†† Astra Pain Control, Pharmaceutical and Analytical R & D, Bioanalytical Chemistry, S-151 85 Södertälje, Sweden.

of ethylene glycol dimethacrylate and 344 mg of methacrylic acid were weighed into 20 ml borosilicate test tubes and dissolved in 6 ml of dried methylene chloride. A blank polymer was produced by omitting the print molecule. The mixtures were cooled on ice, sonicated and sparged with nitrogen for 5 minutes. Fifty mg of azobis-(isobutyronitrile) (initiator) was added and the ampoules were placed under a UV-source (366 nm) for 16 hours at 4°C. The bulk polymers were ground in a mechanical mortar (Retsch, Haan, Germany) and wet-sieved through a 25 μm sieve (Retsch, Haan, Germany). Fines were removed by repeated sedimentation from acetonitrile and the polymer particles were finally dried under vacuum.

HPLC Analysis

Polymer particles were packed into stainless steel columns (200 × 4.6 mm) as described in[10]. In order to extract the imprint species, the columns were washed on line with methanol - acetic acid (9 : 1; v/v; 1 liter) and then with the eluent. Twenty μl of 0.1 mM samples (dissolved in the eluent) were analysed isocratically at a flow rate of 1 ml/minute. The eluents were: 0.5% acetic acid in chloroform (erythromycin polymer); 1% acetic acid in chloroform (oleandomycin polymer); 1% acetic acid in methylene chloride (tylosin polymer). Acetone was used as a void marker. Retention factors were calculated using $k' = (V_R - V_0)/V_0$, where V_R is the retention volume of the compound and V_0 is the void volume of the system. Separation factor is $\alpha = k'_1/k'_2$, where k'_1 is the retention factor recorded for the print species and k'_2 is that for the second compound. Plate numbers (N), which were around 5000 m^{-1} for all columns, was calculated using standard chromatographic theory[12].

Results and Discussion

Molecular imprinting of macrolide antibiotic structures were done in methacrylic acid-ethylene glycol dimethacrylate copolymers. The resultant molecularly imprinted polymers (MIPs) were packed into HPLC-columns and their specific separation abilities were analysed, as has been described in previous studies of imprinted polymers[10]. For each compound the retention factor, k', is related to the relative binding affinity of the compound to the imprinted polymer. The extent of retention of a given compound on a column is, however, composed of two factors: the specific binding to the imprinted sites and non-specific retention due to interaction with the surface of the polymer. Non-specific interactions could be reduced to a minimum by addition of small amounts of acetic acid to the eluent[10].

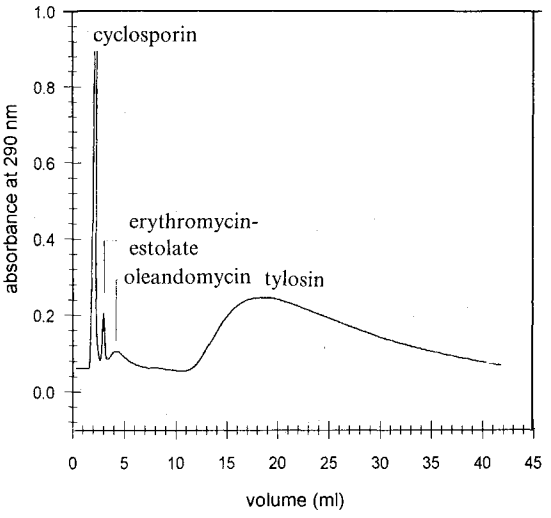
All polymers showed strongest affinity for the compound that was present during its preparation (Table 1). In all instances, other macrolides were less well recognised, *i.e.* other macrolides eluted earlier than the print species. On the column packed with oleandomycin MIP, the print molecule was completely separated from mixtures with erythromycin and tylosin, with separation factors (α) of 6.8 and 5.7, respectively. The same is true for erythromycin and tylosin MIPs. On the column packed with erythromycin MIP, erythromycin is more strongly retained than oleandomycin and tylosin ($\alpha = 5.0$ and 2.4, respectively). On the tylosin column, a much higher retention factor was recorded for tylosin than for oleandomycin and erythromycin; in this instance $\alpha =$ 8.2 and 2.9 were obtained. A non-imprinted reference polymer, made under otherwise identical conditions but by omitting the print molecule, was not able to retain any of the compounds tested. With the same mobile phase as used for the imprinted columns, all compounds eluted with the void ($k' = 0.0$). In all instances, the increased retention of the imprint species relative to other compounds is high, indicating a high specificity of each MIP. In this context, unrelated compounds, such as cyclosporin and rifampicin, eluted with the void ($k' = 0.0$) or was slightly retained ($k' = 1.0$) on the oleandomycin polymer (Table 1), which further demonstrate the specificity of the imprints. The slight retention of rifampicin may be explained by some structural similarities to the ansa chain of rifampicin to the macrolide ring of the imprint species. The very high separations obtained make these polymers useful in

Table 1. Chromatographic data obtained by HPLC analysis of imprinted polymers.

Test compound	Imprint molecule					
	Oleandomycin		Erythromycin		Tylosin	
	k'	α	k'	α	k'	α
Oleandomycin	**6.8**	1.0	1.4	5.0	1.3	8.0
Erythromycin	1.0	6.8	**7.0**	1.0	3.7	2.7
Tylosin	1.2	5.7	2.9	2.4	**10.7**	1.0
Erythromycin estolate	1.3	5.2	0.7	10.0	0.3	35.7
Erythr. ethyl-succinate	0.9	7.6	1.7	4.1	0.1	107.0
Erythromycin stearate	2.0	3.4	0.7	10.0	1.0	10.7
Spiramycin	1.2	5.7	2.7	2.6	9.6	1.1
Rifampicin	1.0	6.8	n.d.	n.d.	n.d.	n.d.
Cyclosporin	0.0	∞	0.0	∞	0.0	∞

Retention factor, k', and separation factor α, were calculated as described in Materials and Methods. n.d.: not determined.

Fig. 2. Separation of a mixture of each 1 mg/ml of cyclosporin, erythromycin and oleandomycin, and 0.1 mg/ml of tylosin on the polymer with the imprints against tylosin.



The eluent was methylene chloride containing 1% acetic acid at a flow rate of 1 ml/minute. Detection was at 290 nm. Note the strong adsorption of tylosin at this wavelength.

chromatographic separations of the imprint species from a mixture of antibiotics and related compounds (Fig. 2). The data presented here demonstrate the ability of molecular imprinting to provide an easy access to stationary phases with high specificity of the desired macrolide structure.

At least in one instance, the polymer was able to detect subtle structural differences. On the erythromycin column, 2'-esters of erythromycin eluted much earlier than erythromycin itself did. The parent compound was separated from its ethyl succinate, stearate and estolate ester derivatives with separation factors of 7.6, 5.2 and 3.4, respectively (Table 1). In contrast, the bulky molecules tylosin and spiramycin gave similar retention values on the tylosin column, with spiramycin eluting only slightly prior to tylosin (Table 1). A possible explanation is their identical disaccharide unit combined with the many structural similarities in the macrocyclic ring.

In conclusion, we have demonstrated the molecular imprinting of macrolide antibiotics, a structurally complex class of compounds having a large ring structure containing many chemical functionalities. The resultant polymer demonstrated efficient recognition of their respective imprinted macrolides and may eventually be used in a chromatographic mode for analytic purposes or isolation of these antibiotics from fermentation broths.

References

1) KELLER-SCHIERLEIN, W.: Chemie der Makrolith-Antibiotika. Fortschr. Chem. Org. Naturst. 30: 313~460, 1973
2) WASHINGTON, J. A. & W. R. WILSON: Erythromycin: A microbial and clinical perspective after 30 years of clinical use (part 1). Mayo Clin. Proc. 60: 189~203, 1985
3) WASHINGTON, J. A. & W. R. WILSON: Erythromycin: A microbial and clinical perspective after 30 years of clinical use (part 2). Mayo Clin. Proc. 60: 271~278, 1985
4) WESTPHAL, J. F.; D. VETTER & J. M. BROGARD: Hepathic side-effects of antibiotics. J. Antimicrob. Chemother. 33: 387~401, 1994
5) HUCK, T. A.; N. PORTER & M. E. BUSHEL: Positive selection of antibiotic-producing soil isolates. J. Gen. Microbiol. 137: 2321~2329, 1991
6) TANAKA, Y.; K. KIMURA, Y. KOMAGATA, K. TSUZUKI, H. TOMADA & S. ŌMURA: Radioimmunoassay for erythromycin derivatives. J. Antibiotics 41: 258~260, 1988
7) KHAN, K.; J. PAESEN, E. ROETS & J. HOOGMARTENS: Analysis of erythromycin A and its metabolites in biological samples by liquid chromatography with post-column ion-pair extraction. L. Liquid Chrom. 17: 4195~4212, 1994
8) JANECEK, M.; M. A. QUILLIAM, M. R. BAILEY & D. H. NORTH: Determination of erythromycin A by liquid chromatography and electrochemical detection, with application to salmon tissue. J. Chromat. Biomed. Appl. 619: 63~69, 1993
9) MOSBACH, K.: Molecular imprinting. Trends Biochem. Sci. 19: 9~14, 1994
10) ANDERSSON, L. I. & K. MOSBACH: Enantiomeric resolution on molecularly imprinted polymers prepared with only non-covalent and non-ionic interactions. J. Chromatogr. 516: 313~322, 1990
11) VLATAKIS, G.; L. I. ANDERSSON, R. MÜLLER & K. MOSBACH: Drug assay using antibody mimics made by molecular imprinting. Nature 361: 645~647, 1993
12) UNGER, K. K.: Packings and stationary phases in chromatographic techniques. *In* Chromatographic Science Series, 47: Marcel Dekker, New York, Basel, 1990
13) MERCK & CO., INC: The Merck Index. An encyclopedia of chemicals, drugs, and biologicals. *Ed.*, S. BUDAVARI *et al.*, Merck & Co., Inc., Rahway, N.J., U.S.A., 1989
14) SIEMANN, M.; L. I. ANDERSSON & K. MOSBACH: Selective recognition of the herbicide atrazine by noncovalent molecularly imprinted polymers. J. Agric. Food Chem. 44: 141~145, 1996

Cladinose Analogues of Sixteen-membered Macrolide Antibiotics

II. Preparation of Pharmacokinetically Improved Analogues *via* Biotransformation

KEIICHI AJITO*, KEN-ICHI KURIHARA, SEIJI SHIBAHARA, OSAMU HARA, AKIRA SHIMIZU, MINAKO ARAAKE and SHOJI OMOTO

Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., Morooka-cho, Kohoku-ku, Yokohama 222, Japan

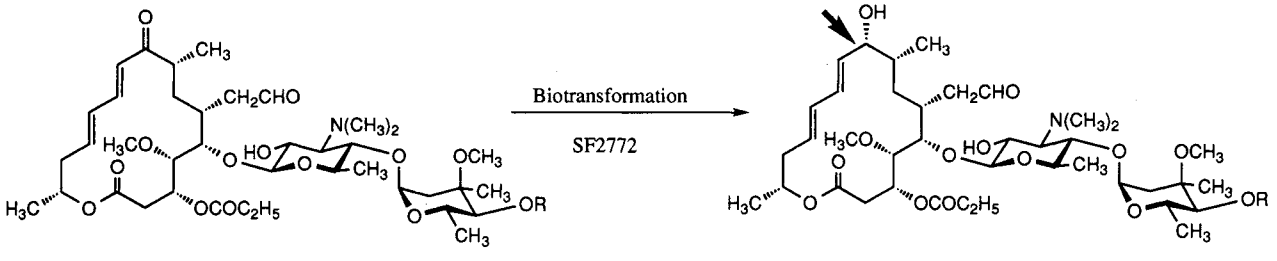
(Received for publication June 20, 1996)

Sixteen-membered macrolide antibiotics[1] have been used clinically for many years because of their excellent efficacy and safety. Since, they do not always exhibit satisfactory pharmacokinetics[2] compared with fourteen-membered macrolides, the design and synthesis of analogues showing improved pharmacokinetics would be an important achievement from the clinical point of view. As part of our program in this area, we have recently designed, synthesized and studied 4-*O*-alkyl-L-cladinose analogues, compounds (**1**~**5**), including 9-dehydro-3″-*O*-methyl-4″-*O*-(3-methylbutyl)-3-*O*-propionylleucomycin V[3], compound **3** (Scheme 1). In this communication, we wish to report the preparation of the potentially more useful C-9 hydroxyl analogues, compounds **6**~**10**. The reported compound **8** exhibited dramatically improved pharmacokinetics in mice.

As we reported in our previous paper[3], one of the 4-*O*-alkyl-L-cladinose analogues, **3**, showed better pharmacokinetics in mice compared to midecamycin A_3 (Fig. 1) having 4-*O*-acylated sugar moiety. Pharmacokinetic comparison between midecamycin A_1 and A_3 in mice *in vivo*[4] (Fig. 1) led us to reduce a carbonyl group at the C-9 position to generate an α-hydroxyl group stereoselectively. As a result of this modification, improvement in both serum concentration and urinary excretion *in vivo* was achieved. Some methods for reducing the C-9 carbonyl of sixteen-membered macrolides to the alcohol through synthetic approaches[5~7] and biochemical approaches[8~11] have been reported. After careful consideration of these methodologies, we chose the biotransformation[9,10] of these compounds because of the exclusive stereoselectivity and ease of procedure.

Bioconversion of a C-9 carbonyl to a corresponding α-hydroxyl group was successfully completed in spite of the presence of an unnatural sugar (4-*O*-alkyl-L-cladinose). In general, the medium† was pipetted in 80 ml portions into three 500 ml-Erlenmeyer's flasks and sterilized at 120°C for 30 minutes. The medium in each flask was inoculated with 1.6 ml of a frozen seed of a non-producing mutant of *Streptomyces mycarofaciens* SF-837, for example *Streptomyces mycarofaciens* SF2772[4] strain, having a cell density of 10~15% which was then incubated at 28°C for 24 hours while shaking. *Streptomyces mycarofaciens* SF2772 has been deposited with Fermentation Research Institute of agency of Industrial Science and Technology, of 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki 305, Japan, under the accession number FERM BP-4465. SF2772 strain can be constructed by artificial mutagenesis of the SF-837 strain by known methods. *Streptomyces mycarofaciens* SF-837 has been deposited with Fermentation Research Institute of Agency of Industrial Science and Technology under the accession number FERM P-262 and with American Type Culture Collection under the accession number of ATCC21454 and now available to the public.

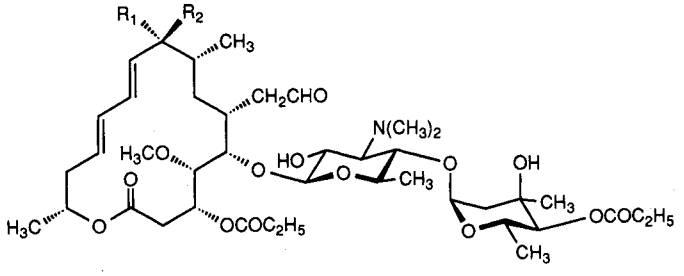
Scheme 1.



	R	
1	$CH_2CH{=}CH_2$	**6**
2	$CH_2CH_2CH_2CH_3$	**7**
3	$CH_2CH_2CH(CH_3)_2$	**8**
4	$CH_2CH_2CH_2CH_2CH_2CH_3$	**9**
5	$CH_2C_6H_5$	**10**

† A medium comprising 2.0% of glucose, 1.0% of polypeptone, 0.05% of dipotassium hydrogenphosphate, 0.05% of magnesium sulfate heptahydrate and 0.3% of sodium chloride was adjusted to pH 7.0 and sterilized prior to the use.

Fig. 1. Structure of midecamycins.



Midecamycin A_1 R_1 = OH, R_2 = H

Midecamycin A_3 R_1, R_2 = O

Table 1. Antibacterial activities of **1**~**10** and midecamycin A_1 (MIC, μg/ml).

Test organisms	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	Midecamycin A_1
Staphylococcus aureus 209P JC-1	3.13	0.20	0.20	0.78	0.20	3.13	0.39	0.20	0.39	3.13	0.39
S. aureus M133	12.5	0.78	0.78	1.56	1.56	6.25	0.78	0.78	1.56	12.5	0.78
S. aureus M126	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
S. aureus MS15026	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
S. aureus MS15027	12.5	0.78	0.78	1.56	1.56	6.25	0.78	0.78	1.56	6.25	0.78
S. epidermidis ATCC 14990	25	1.56	0.78	1.56	3.13	12.5	1.56	1.56	1.56	25	1.56
Micrococcus luteus ATCC 9341	0.39	0.05	0.05	0.10	0.10	0.39	0.05	0.05	0.10	0.78	0.05
Enterococcus faecalis W-73	6.25	3.13	1.56	6.25	1.56	6.25	3.13	3.13	3.13	6.25	3.13
Streptococcus pneumoniae IP692	1.56	0.10	0.10	0.20	0.20	0.78	0.05	0.10	0.20	0.78	0.39
S. pneumoniae Type I	1.56	0.20	0.20	0.20	0.20	0.78	0.20	0.20	0.20	1.56	0.39
S. pyogenes Cook	1.56	0.10	0.05	0.20	0.20	0.78	0.20	0.10	0.20	0.78	0.20
Escherichia coli NIHJ JC-2	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Klebsiella pneumoniae PCI602	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Branhamella catarrhalis W-0500	12.5	0.78	0.78	1.56	1.56	6.25	1.56	0.78	1.56	6.25	3.13
B. catarrhalis W-0506	6.25	0.78	0.78	1.56	1.56	6.25	1.56	1.56	1.56	6.25	1.56
Haemophilus influenzae 9334	NT	NT	NT	NT	NT	50	6.25	6.25	6.25	50	3.13
Mycoplasma pneumoniae FH	NT	NT	NT	NT	NT	NT	1.56	1.56	1.56	NT	1.56
M. pneumoniae Mac	NT	NT	NT	NT	NT	NT	0.10	0.05	0.20	NT	0.10
M. pneumoniae FH-P24	NT	NT	NT	NT	NT	NT	0.10	0.025	0.20	NT	0.10
M. pneumoniae Numata	NT	NT	NT	NT	NT	NT	0.05	0.025	0.05	NT	0.05

NT; Not tested.

Next, 1.2 ml of a methanol solution containing 20 mg of the carbonyl compound was added to each flask in 0.4 ml portions and the incubation was continued at 28°C for 17 hours while shaking. After the completion of the incubation, the culture was centrifuged at 3000 rpm for 10 minutes. Thus 180 ml of a transparent culture supernatant was obtained while the solid matters including the cells were removed. To the solid matters was added 120 ml of water and the mixture was stirred followed by centrifugation. The washing liquor thus obtained was combined with the above-mentioned transparent culture supernatant. After adjusting the mixture to pH 9 with a 1 N aqueous solution of sodium hydroxide, the conversion product was extracted with 300 ml portions of ethyl acetate twice. The ethyl acetate layer was dried over anhydrous sodium sulfate and then filtered. The filtrate was concentrated under reduced pressure and the residue thus obtained was purified by preparative TLC [developing system: chloroform/methanol (10:1)]. Thus, 9~12 mg (45~60%) of the desired α-hydroxyl compounds were obtained with trace of a starting material. For example, evidence of the desired α-hydroxyl stereochemistry for compound **8** was demonstrated by ^{1}H NMR experiments (δ 4.07 (1H, dd, $J_{8,9}=4.0$ Hz, $J_{9,10}=10.0$ Hz, 9-H))[6].

The antibacterial activities *in vitro* of novel 4-*O*-alkyl-L-cladinosyl derivatives (**6**~**10**), compared with corresponding midecamycin A_1, are shown in Table 1††. Although compounds **7** and **8** displayed similar *in vitro* activities to midecamycin A_1, their activities against *Streptococcus pneumoniae* were clearly enhanced compared with midecamycin A_1. The activity of **8** against *Mycoplasma pneumoniae* was also quite high. As expected, stereoselective reduction at the C-9 position of 9-keto derivatives did not reduce their original antibacterial activities.

†† All biological studies were done as described in our previous paper. See ref. 3.

Table 2. Physico-chemical properties of **6**~**10**.

Compound (**6**): EI-MS *m/z* 811 $(M)^+$; $[\alpha]_D$ −55° (*c* 0.6, CH_3OH); MP 101~106°C; ^1H NMR (400 MHz, $CDCl_3$) δ 0.99 (3H, d, 19-H), 1.16 (3H, d, 6′-H), 1.22 (3H, t, 3-$OCOCH_2CH_3$), 1.23 (3H, d, 6″-H), 1.24 (3H, s, 3″-CH_3), 1.26 (3H, d, 16-H), 1.57 (1H, dd, 2″-Hax), 1.89 (1H, m, 8-H), 2.24 (1H, brd, 2-H), 2.24 (1H, d, 2″-Heq), 2.51 and 2.64 (each 2H, dq, 3-$OCOCH_2CH_3$), 2.65 (6H, s, 3′-$N(CH_3)_2$), 2.76 (1H, dd, 2-H), 2.84 (1H, brdd, 17-H), 2.87 (1H, d, 4″-H), 3.25 (3H, s, 3″-OCH_3), 3.26 (1H, brd, 4-H), 3.28 (1H, dq, 5′-H), 3.50 (1H, t, 4′-H), 3.57 (3H, s, 4-OCH_3), 3.87 (1H, brd, 5-H), 4.07 (1H, dd, 9-H), 4.11 and 4.19 (each 2H, brdd, 4″-$OCH_2CH=CH_2$), 4.40 (1H, dq, 5″-H), 4.54 (1H, d, 1′-H), 4.91 (1H, d, 1″-H), 5.03 (1H, ddq, 15-H), 5.13 (1H, brd, 3-H), 5.17 and 5.23 (each 2H, brd, 4″-$OCH_2CH=CH_2$), 5.62 (1H, dd, 10-H), 5.79 (1H, ddd, 13-H), 5.95 (1H, ddt, 4″-$OCH_2CH=CH_2$), 6.08 (1H, brdd, 12-H), 6.68 (1H, dd, 11-H), 9.63 (1H, s, 18-H).

Compound (**7**): EI-MS *m/z* 827 $(M)^+$; $[\alpha]_D$ −50° (*c* 0.9, CH_3OH); MP 99~101°C; ^1H NMR (400 MHz, $CDCl_3$) δ 0.91 (3H, t, 4″-$OCH_2CH_2CH_2CH_3$), 0.98 (3H, d, 19-H), 1.15 (3H, d, 6′-H), 1.21 (3H, t, 3-$OCOCH_2CH_3$), 1.22 (3H, d, 6″-H), 1.24 (3H, s, 3″-CH_3), 1.26 (3H, d, 16-H), 1.37 (2H, m, 4″-$OCH_2CH_2CH_2CH_3$), 1.57 (1H, dd, 2″-Hax), 1.60 (2H, m, 4″-$OCH_2CH_2CH_2CH_3$), 1.89 (1H, m, 8-H), 2.23 (1H, d, 2″-Heq), 2.24 (1H, brd, 2-H), 2.51 and 2.64 (each 2H, dq, 3-$OCOCH_2CH_3$), 2.62 (6H, s, 3′-$N(CH_3)_2$), 2.76 (1H, dd, 2-H), 2.78 (1H, d, 4″-H), 2.85 (1H, brdd, 17-H), 3.22 (1H, brdd, 2′-H), 3.25 (1H, brd, 4-H), 3.25 (3H, s, 3″-OCH_3), 3.28 (1H, dq, 5′-H), 3.48 (1H, t, 4′-H), 3.57 (3H, s, 4-OCH_3), 3.57 and, 3.62 (each 2H, dt, 4″-$OCH_2CH_2CH_2CH_3$), 3.87 (1H, brd, 5-H), 4.07 (1H, dd, 9-H), 4.39 (1H, dq, 5″-H), 4.53 (1H, d, 1′-H), 4.89 (1H, d, 1″-H), 5.03 (1H, ddq, 15-H), 5.13 (1H, brd, 3-H), 5.62 (1H, dd, 10-H), 5.79 (1H, ddd, 13-H), 6.08 (1H, brdd, 12-H), 6.67 (1H, dd, 11-H), 9.63 (1H, s, 18-H).

Compound (**8**): EI-MS *m/z* 841 $(M)^+$; $[\alpha]_D$ −49° (*c* 0.7, CH_3OH); MP 98~100°C; ^1H NMR (400 MHz, $CDCl_3$) δ 0.89 (6H, d, 4″-$OCH_2CH_2CH(CH_3)_2$), 0.99 (3H, d, 19-H), 1.15 (3H, d, 6′-H), 1.22 (3H, t, 3-$OCOCH_2CH_3$), 1.23 (3H, d, 6″-H), 1.24 (3H, s, 3″-CH_3), 1.26 (3H, d, 16-H), 1.57 (1H, dd, 2″-Hax), 1.69 (1H, m, 4″-$OCH_2CH_2CH(CH_3)_2$), 1.89 (1H, m, 8-H), 2.22 (1H, d, 2″-Heq), 2.24 (1H, brd, 2-H), 2.51 and 2.64 (each 2H, dq, 3-$OCOCH_2CH_3$), 2.62 (6H, s, 3′-$N(CH_3)_2$), 2.76 (1H, dd, 2-H), 2.78 (1H, d, 4″-H), 2.85 (1H, brdd, 17-H), 3.22 (1H, brdd, 2′-H), 3.25 (3H, s, 3″-OCH_3), 3.26 (1H, brd, 4-H), 3.28 (1H, dq, 5′-H), 3.48 (1H, t, 4′-H), 3.57 (3H, s, 4-OCH_3), 3.60 and 3.64 (each 2H, dt, 4″-$OCH_2CH_2CH(CH_3)_2$), 3.87 (1H, brd, 5-H), 4.07 (1H, dd, 9-H), 4.39 (1H, dq, 5″-H), 4.53 (1H, d, 1′-H), 4.89 (1H, d, 1″-H), 5.03 (1H, ddq, 15-H), 5.13 (1H, brd, 3-H), 5.62 (1H, dd, 10-H), 5.79 (1H, ddd, 13-H), 6.08 (1H, brdd, 12-H), 6.68 (1H, dd, 11-H), 9.63 (1H, s, 18-H).

Compound (**9**): EI-MS *m/z* 855 $(M)^+$; $[\alpha]_D$ −50° (*c* 0.8, CH_3OH); MP 96~102°C; ^1H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, t, 4″-$OCH_2CH_2(CH_2)_3CH_3$), 0.99 (3H, d, 19-H), 1.15 (3H, d, 6′-H), 1.22 (3H, t, 3-$OCOCH_2CH_3$), 1.23 (3H, d, 6″-H), 1.24 (3H, s, 3″-CH_3), 1.26 (3H, d, 16-H), 1.57 (1H, dd, 2″-Hax), 1.61 (2H, m, 4″-$OCH_2CH_2(CH_2)_3CH_3$), 1.89 (1H, m, 8-H), 2.23 (1H, d, 2″-Heq), 2.24 (1H, brd, 2-H), 2.51 and 2.64 (each 2H, dq, 3-$OCOCH_2CH_3$), 2.63 (6H, s, 3′-$N(CH_3)_2$), 2.76 (1H, dd, 2-H), 2.78 (1H, d, 4″-H), 2.85 (1H, brdd, 17-H), 3.22 (1H, brdd, 2′-H), 3.25 (1H, brd, 4-H), 3.25 (3H, s, 3″-OCH_3), 3.28 (1H, dq, 5′-H), 3.48 (1H, t, 4′-H), 3.55 and 3.61 (each 2H, dt, 4″-$OCH_2CH_2(CH_2)_3CH_3$), 3.57 (3H, s, 4-OCH_3), 3.87 (1H, brd, 5-H), 4.07 (1H, dd, 9-H), 4.39 (1H, dq, 5″-H), 4.53 (1H, d, 1′-H), 4.89 (1H, d, 1″-H), 5.03 (1H, ddq, 15-H), 5.13 (1H, brd, 3-H), 5.62 (1H, dd, 10-H), 5.79 (1H, ddd, 13-H), 6.08 (1H, brdd, 12-H), 6.68 (1H, dd, 11-H), 9.63 (1H, s, 18-H).

Compound (**10**): SI-MS *m/z* 862 $(M+H)^+$; $[\alpha]_D$ −52° (*c* 0.8, CH_3OH); MP 112~116°C; ^1H NMR (400 MHz, $CDCl_3$) δ 0.98 (3H, d, 19-H), 1.15 (3H, d, 6′-H), 1.15 (3H, s, 3″-CH_3), 1.21 (3H, t, 3-$OCOCH_2CH_3$), 1.23 (3H, d, 6″-H), 1.26 (3H, d, 16-H), 1.57 (1H, dd, 2″-Hax), 1.89 (1H, m, 8-H), 2.22 (1H, d, 2″-Heq), 2.24 (1H, brd, 2-H), 2.31 (1H, brdd, 17-H), 2.51 and 2.64 (each 2H, dq, 3-$OCOCH_2CH_3$), 2.62 (6H, s, 3′-$N(CH_3)_2$), 2.76 (1H, dd, 2-H), 2.84 (1H, brdd, 17-H), 3.00 (1H, d, 4″-H), 3.25 (1H, brd, 4-H), 3.25 (3H, s, 3″-OCH_3), 3.28 (1H, dq, 5′-H), 3.49 (1H, t, 4′-H), 3.57 (3H, s, 4-OCH_3), 3.87 (1H, brd, 5-H), 4.07 (1H, dd, 9-H), 4.45 (1H, brdq, 5″-H), 4.54 (1H, d, 1′-H), 4.62 and 4.70 (each 2H, d, 4″-$OCH_2C_6H_5$), 4.90 (1H, d, 1″-H), 5.03 (1H, ddq, 15-H), 5.13 (1H, brd, 3-H), 5.62 (1H, dd, 10-H), 5.79 (1H, ddd, 13-H), 6.08 (1H, brdd, 12-H), 6.68 (1H, dd, 11-H), 7.3~7.4 (5H, m, 4″-$OCH_2C_6H_5$), 9.63 (1H, s, 18-H).

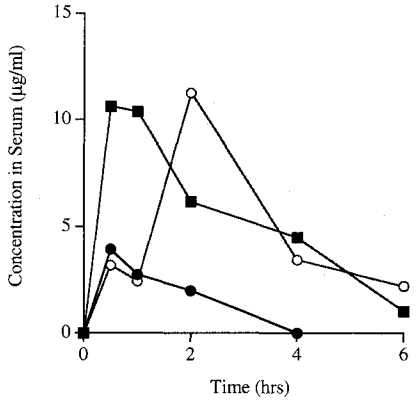
These compounds (**7**~**9**) also exhibited good metabolic stability in rat plasma *in vitro*. After 24 hours of incubation[††], 71, 85, and 79% of initial activity (t=0) was observed against *Micrococcus luteus* for compound **7**, **8**, and **9**, respectively, while only 2% activity remained for midecamycin A_1. Thus, these 4-*O*-alkyl-L-cladinosyl analogues proved to be more stable than midecamycin A_1 *in vitro*. Improved stability is best explained by inability of neutral sugars to be metabolized by esterase[12].

Compound (**8**), which was the best compound in terms of potency and stability, was finally examined *in vivo*. Serum concentrations of antibiotics in mice after 200 mg/kg oral administration[†††] are shown in Fig. 2. Serum concentration of **8** was dramatically higher and longer lasting than midecamycin A_1 and its AUC was also greater than that of midecamycin A_1. The maximum concentration of **8** in serum was comparable to that of

[†††] A test compound was mixed with a 0.2% aqueous solution of CMC to give a concentration of 4.0 mg/ml and a 1.0 ml portion of the resulting emulsion was orally administered to 4 weeks old male Jcl: ICR mice. Blood was collected from the armpits of the mice 0.5, 1, 2 ,4 and 6 hours after the administration of the test compound (n=2). The collected blood was allowed to stand at 0°C for 2 hours and centrifuged at 3000 rpm for 20 minutes to obtain serum. To the serum was added an equivalent volume of 50% CH_3CN-0.05 M phosphate buffer (pH 7.0). The resulting mixture served as a serum sample. The concentration of the test compound in the serum sample was measured by a bioassay method using *M. luteus* ATCC 9341.

Fig. 2. Concentration in serum.

200 mg/kg, mouse, n=2, p.o., ○ **8**, ● midecamycin A_1, ■ clarithromycin.



clarithromycin. Moreover urinary recovery of **8** within 24 hours in mice (200 mg/kg, p.o.)†††† was 20%, while less than 1% for midecamycin A_1, but 24% for clarithromycin. These excellent pharmacokinetics of **8** could be mainly explained by metabolic stability of the dialkyl neutral sugar.

These results demonstrate the possibility to design and synthesize sixteen-membered macrolide derivatives exhibiting similar efficacy to the second generation fourteen-membered macrolides, so-called *new* macrolides such as clarithromycin. They also point the way for discovery of highly potent and pharmacokinetically excellent derivatives in the sixteen-membered macrolide fields.

Acknowledgments

We wish to thank Drs. S. INOUYE and S. GOMI for valuable discussions and encouragement during this study. We are also grateful to Dr. T. ISHIZUKA for metabolic stability tests in rat plasma and Miss. N. KIKUCHI for her technical assistance.

References

1) ŌMURA, S. (*Ed.*): Macrolide Antibiotics. Chemistry, Biology, and Practice. Academic Press Inc., 1984
2) OKAMOTO, R.; T. FUKUMOTO, K. IMAFUKU, T. OKUBO, K. KIYOSHIMA, A. TAKAMATSU & T. TAKEUCHI: Screening for 16-membered macrolide-transforming microorganisms. J. Ferment. Technol. 57: 519~528, 1979
3) KURIHARA, K.; K. AJITO, S. SHIBAHARA, T. ISHIZUKA, O. HARA, M. ARAAKE & S. OMOTO: Cladinose analogues of sixteen-membered macrolide antibiotics. I. Synthesis of 4-*O*-alkyl-L-cladinose analogues *via* glycosylation. J. Antibiotics 49: 582~592, 1996
4) AJITO, K.; K. KURIHARA, A. SHIMIZU, S. GOMI, N. KIKUCHI, M. ARAAKE, T. ISHIZUKA, A. MIYATA, O. HARA & S. SHIBAHARA (Meiji Seika Kaisha, LTD.): 16-Membered macrolide derivatives and process for producing the same. United States Patent 5,407,918, Apr. 18, 1995
5) FREIBERG, L. A.; R. S. EGAN & W. H. WASHBURN: The synthesis of 9-*epi*-leucomycin A_3. The revised configurational assignment of C-9 in natural leucomycin A_3. J. Org. Chem. 39: 2474~2475, 1974
6) ŌMURA, S.; H. SAKAKIBARA, T. FUJIWARA & M. AIZAWA: 9-*epi*-Leucomycin A_5 synthesis and antimicrobial activity. J. Antibiotics 34: 1577~1580, 1981
7) TSUZUKI, K.; H. MATSUBARA, A. NAKAGAWA & S. ŌMURA: Synthesis and antimicrobial activities of 9-*O*-acyl derivatives of tylosin and demycarosyltylosin. J. Antibiotics 39: 1784~1787, 1986
8) SUZUKI, M.; N. NAGAHAMA, Y. SEKI & T. YAMAGUCHI (Tanabe Seiyaku Co., Ltd.), Jpn. Kokai 126880 ('75), Oct. 6, 1975
9) NAGAOKA, K.; H. OGAWA, Y. MATSUHASHI, K. WATANABE, T. MATSUNOBU, T. SHOMURA & H. GOI (Meiji Seika Kaisha., Ltd.), Jpn. Kokai 8793 ('79), Jan. 23, 1979
10) MATSUHASHI, Y.; H. OGAWA & K. NAGAOKA: The enzymatic interconversion between midecamycin A_1 and A_3. J. Antibiotics 37: 777~779, 1979
11) ŌMURA, S.; C. KITAO & N. SADAKANE: The microbial transformation of tylosin by the spiramycun-producing strain, *Streptomyces ambofaciens* KA-1028. J. Antibiotics 33: 911~912, 1980
12) SHOMURA, T.; S. SOMEYA, K. UMEMURA, M. NISHIO & S. MURATA: Metabolism of 9,3″-diacetylmidecamycin. I. The metabolic fate of 9,3″-diacetylmidecamycin. Yakugaku Zasshi (Japanese) 102: 781~795, 1982

†††† Subsequently, 200 mg/kg of a test compound was orally administered to three mice in the same manner as described above. The mice were put in a metabolic cage MM type (Sugiyamagen Co., Tokyo, Japan) and urine was collected 2, 4 and 24 hours after the administration. The collected urine was filtered through a filter having a pore size of 0.45 mm (Millipore) and was mixed with an equivalent volume of 50% CH_3CN-0.05 M phosphate buffer (pH 6.5) to serve as an urine sample. The bioassay was carried out by *M. luteus* to determine the concentration of the test compound in the urine sample and the recovery in the urine was calculated.