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209. Shuji Takahashi, Mamoru Arai, and Eiji Ohki*1 : Chemical Studies on Azalomycins. I. Preliminary Study on Azalomycin-B.

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A macrolide antibiotic produced by *Streptomyces hygroscopicus* var. *azalomyceticus*, azalomycin-B (I), which is activity against gram-positive bacteria and *Trichomonas vaginalis*, was investigated as a preliminary study for the structural elucidation. A molecular formula of I was tentatively established as $C_{56}H_{92}O_{19}$. It was also found that an $\alpha,\beta,\gamma,\delta$-unsaturated ester function (X) existed as the chromophor part of the molecule.

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In the course of a systematic search for new antibiotics, one of the present authors (M.A)[1~3] isolated and characterized several kinds of new antibiotics from the culture broth of *Streptomyces hygroscopicus* var. *azalomyceticus*. Azalomycin B,*2,3 one of these antibiotics, was investigated for its structural elucidation; and it was found to be one of macrolide antibiotics with high molecular weight which forms the subject of this paper.

Azalomycin-B (I), which was purified by several recrystallization from ethyl acetate, decomposed at 210~212° (originally given as 185~187°[3]). It formed colorless needles in the solvent, but drying up of standing in air rapidly liberated the solvent of crystallization to give an amorphous powder. The analytical data showed C, 62.85% and H, 8.57%, without nitrogen, sulfur, halogen, or O-methyl group.*4 The purified sample was insoluble in acetone, ethanol, benzene, or ether, slightly soluble in methanol and ethyl acetate, but rather soluble in a mixed solvent of methanol-ethyl acetate or methanol-benzene than in either pure solvent.

The strong hydroxyl absorption at 3450 cm^{-1} in its infrared spectrum showed the presence of abundant hydroxyl functions and the double-bond region absorptions at 1700, 1645, and 1620 cm^{-1}, with a broad absorption of ultraviolet spectrum at 252 mμ, suggested the presence of an aliphatic conjugated ketone or ester system in the molecule.

Acetylation of I afforded the acetate (II), m.p. 210~215°, and benzoylation with *p*-bromobenzoyl chloride gave the *p*-bromobenzoate (III), m.p. 197°. The latter was further acetylated to give *p*-bromobenzoate acetate (IV), m.p. 166~167°. Hydrogenation of I in dimethylformamide*5 over palladium charcoal or platinum oxide afforded perhydro compound (V), m.p. 190~192°. Perhydro-acetate (VI), m.p. 155°, obtained by acetylation of V, was identical with the sample obtained by hydrogenation of II, and VI

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*2 Arcamone, *et al.* reported elaiophylin, an antibiotic produced by *Streptomyces melanosporus*, which is considered to be identical with azalomycin-B from its physical constants and biological properties (F. M. Arcamone, C. Bertazzolim, M. Ghione, T. Scotti : Giornale di Microbiologia, **7**, 2037 (1959)).

*3 Although gram-positive bacteria were previously reported[2] as the sole group of microorganisms sensitive to azalomycin-B, further biological studies revealed that *Trichomonas vaginalis* is also inhibited by the antibiotic at a minimal inhibitory concentration of 12.5 μg./ml.

*4 The presence of methoxyl group, which was shown by elementary analysis in the previous paper,[3] may be ascribed to adhesive contamination of methanol of crystallization.

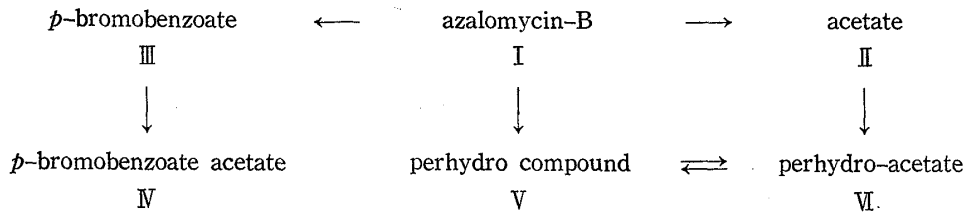
*5 Hydrogenation of I in other solvents was unfavorable because of insolubility of V in them.

1) M. Arai : Japan. Pat. 16584/1959 (May 24, 1959). It was also presented at the 115th Meeting of Japan Antibiotics Research Association, May 25, 1959.

2) *Idem* : J. Antibiotics, Ser. A, **13**, 46 (1960).

3) *Idem* : *Ibid.*, **13**, 51 (1960).

was also saponified with mild alkali to give V in a small yield; these facts indicated that there was no substantial decomposition in the molecule by these reactions.



Each of these derivatives did not form any stable crystal suitable for X-ray analysis because of adhesive contamination of the solvent of recrystallization as I itself. Attempted preparation of other crystalline derivatives was also not successful; thus the structural determination by X-ray diffraction was unfavorable. Mass spectrometry could also make no direct contribution since the antibiotics had many hydroxyl groups and was unstable to heat.

Either azalomycin-B or its derivatives is so sensitive to acid or alkali that even the short-time treatment decomposed the compound completely to give a complex mixture which was shown by many spots revealed on thin-layer chromatogram.

As for the molecular weight of the antibiotic (I), 272 for $C_{14}H_{24}O_5$, which was described in a previous paper,[3] should be corrected to a higher value. Rast method (camphor) did not give any reproducible constant depression. Vapor pressure determination (osmometer) of I did not give any reliable data because of its insolubility in common solvents, except in purified methanol, in which it fell to 713.

X-Ray measurement of I was carried out on a crystal coated with polyvinyl acetate. The crystal was monoclinic, the space group being $P2_1$ or $P2_1/m$. Molecular weights, 1032 ($Z=2$) and 516 ($Z=4$), thus obtained were only approximate, because there was a considerable inaccuracy in the determination of density and solvent content. These data at least served to eliminate the early C_{14} formula,[3] although the interpretation of results obtained in these ways was not obvious. Mass spectrometry*[6] of I carried out by direct inlet method, showed that the highest peak fell in the range of 668~672; but there was no proof for the mass number to exhibit a molecular ion peak. Mass spectrometry of the acetate (II) showed rather a deep fragmentation. Moreover, the possibility of determining a molecular ion by sililated derivatives on mass spectrometry was also supposed to be remote due to the difficulty of blocking all hydroxyl groups by sililation.

Since the presence of an ester or a lactone function was suggested from the infrared band at 1700 cm^{-1} in I and 1720 cm^{-1} in the perhydro compound (V), potentiometric titrations were attempted on these compounds. However, both direct titrations with alkali at 60° in aqueous ethylene glycol and back titration in an excess of alkali gave very obscure and variable results.

Nuclear magnetic resonance (NMR) spectrum of the acetate (II) exhibited two signals of the same intensity at 2.00 and 2.19 p.p.m., which suggested the presence of two or more even-numbered acetyl groups of different nature. Molecular weight of II deduced by comparing integration of the acetyl peaks with that obtained by adding a certain amount of acetanilide, fell in the range of 550~600 for diacetate and 1100~1200 for

*[6] Mass spectrometry was carried out on "Hitachi" RMU-6D mass spectrometer. Authors gratefully acknowledge the kind help of Professor T. Goto, Nagoya University, in the mass spectrometric measurement.

tetraacetate. In order to test the accuracy of these results,*7 ^{14}C-labeled acetylation[4] was carried out; I and aniline were acetylated with the same labeled acetic anhydride and comparison of the radioactivities of II and acetanilide thus obtained gave 614 for the molecular weight of II as the diacetate, and 1224 as the tetraacetate. As the former was ruled out from consideration of mass spectroscopic data, a molecular formula of $C_{56}H_{88}O_{15}(OCOCH_3)_4$ (calcd. mol. wt., 1236) was deduced for II. This was also approximately consistent with 1224 for II obtained by vapor pressure determination. Thus, a molecular formula for azalomycin-B (I) was derived from the C_{64} formula of II as $C_{56}H_{92}O_{19}$ (calcd. mol. wt., 1068), which was also consistent with 1032 obtained for I by X-ray measurement. As shown in Table I, values calculated from the C_{56} formula of I were approximately satisfactory for analytical data for all of the derivatives, assigning III as di-*p*-bromobenzoate and IV as di-*p*-bromobenzoate diacetate. The

Table I. Values (%) from Elemental Analyses of Azalomycin-B Derivatives

Derivatives	Formula	Analysis (%) Calcd. C	H	mol. wt.	Found C	H	mol. wt.
Azalomycin-B (I)	$C_{56}H_{92}O_{19}$	62.92	8.61	1068	62.85	8.57	713
Acetate (II)	$C_{64}H_{100}O_{23}$	62.14	8.09	1236	62.14	8.16	1224
Perhydro-acetate (VI)	$C_{64}H_{108}O_{23}$	61.74	8.68	1242	61.21	8.53	1236
Perhydro compound (V)	$C_{56}H_{100}O_{19}$	62.45	9.29	1076	60.29	9.65	—
p-Bromobenzoate (III)	$C_{70}H_{98}O_{21}Br_2$	58.58	6.83	1434	58.53	6.67	—
		Br : 11.16			Br : 11.64		
p-Bromobenzoate acetate (IV)	$C_{74}H_{102}O_{23}Br_2$	58.50	6.72	1514	58.23	6.68	1600
		Br : 10.54			Br : 11.49		

hydrogen uptake of I and II corresponded to about 1 mole/258 g. and 1 mole/300 g., respectively. Recalculation based on the C_{56} formula of I suggested octahydro compounds for V and VI.

However, considering the known difficulty[5] of molecular formula determination in the chemistry of these macrolide antibiotics, it should be remembered that this C_{56} formula for I is only a tentative one, which may serve to further investigation on the structure, and would be reexamined and corrected after complete chemical structure was elucidated.

Chromophor Group——As mentioned before, the antibiotic (I) or its acetate (II) has a strong absorption at 252 mμ in the ultraviolet region, which disappeared in the perhydro derivatives (V or VI). This absorption, together with the infrared data in the double-bond region, was similar to those of sorbic acid and its homolog as shown in Table II; and it suggested the presence of $\alpha,\beta,\gamma,\delta$-unsaturated ester function in the molecule. Further, NMR analysis of I and its decoupling studies,*8 which were shown in Fig. 1, in a low field (7~5 p.p.m.) also indicated the presence of an unsaturated ester system like VII shown in Table II.

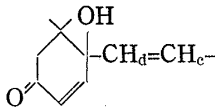
*7 Acetoxyl determination for II was hopeless because II easily decomposed and gave some volatile organic acids by mild treatment with alkali.

*8 We are grateful to Dr. T. Hino, National Institute of Radiological Sciences, and Professor S. Ito, Tohoku University, for their kind help on NMR spectrometry.

4) E. Harri, W. Loeffler, H. P. Sigg, H. Stahelin, Ch. Stoll, Ch. Tamm, D. Wiesinger : Helv. Chim. Acta, **45**, 839 (1962).

5) R. B. Woodward : Angew. Chem., **69**, 50 (1957).

Table II. Physical Data of $\alpha,\beta,\gamma,\delta$-Unsaturated Acid Derivatives

	UV ($m\mu$)	IR (cm^{-1})	NMR (p.p.m.)(J=c.p.s.)
Sorbic acid CH_3-CH_d=CH_c-CH_b=CH_a-COOH	254	1695, 1639, 1618	H_a 5.79(J_{ab}=16); H_b 7.36(J_{ab}=16); H_c, H_d 6.2(multiplet)
Methylsorbic acid CH_3-CH_d=CH_c-$C(CH_3)$=CH_a-COOH	255	1686, 1637, 1603	H_a 5.6; H_c 6.16(multiplet); H_d 7.57(J_{cd}=16)
Abscisin II 	246	1678, 1623, 1603	H_a 5.79; H_c 7.81(J_{cd}=16); H_d 7.57(J_{cd}=16)
Azalomycin-B (I) [-CH_e-CH_d=CH_c-CH_b=CH_a-CO-O-] (VII)	252	1700, 1645, 1620	H_a 5.6(J_{ab}=15); H_b 6.79(J_{ab}=15, J_{bc}=11); H_c 6.07(J_{bc}=11, J_{cd}=15); H_d 5.62(J_{bc}=15, J_{de}=10)
Azalomycin-B acetate (II)	252	1745(acetyl) 1700, 1648, 1621	H_a 5.82(J_{ab}=15); H_b 6.74(J_{ab}=15, J_{bc}=11); H_c 6.00(J_{bc}=11, J_{cd}=15); H_d 5.80(J_{cd}=15)

In addition, oxidation of I with concentrated nitric acid did not give any fatty acid except acetic acid and propionic acid, while oxidation of the perhydro compound (V), under the same condition, afforded α-methylpimelic acid (VIII) which was identified by gas chromatographic study of acidic oxidation products. This longer-chain acid VIII may be assumed to originate in the chromophor moiety of the unsaturated ester system and, therefore, its production suggests the presence of a sequence like IX in the perhydro compound (V), and X in I.

Furthermore, the intensity (log ε 4.46) of the ultraviolet absorption of I, calculated on the basis of the C_{56} formula, suggests the independent presence of two of the unsaturated ester system (X) in the antibiotic molecule.

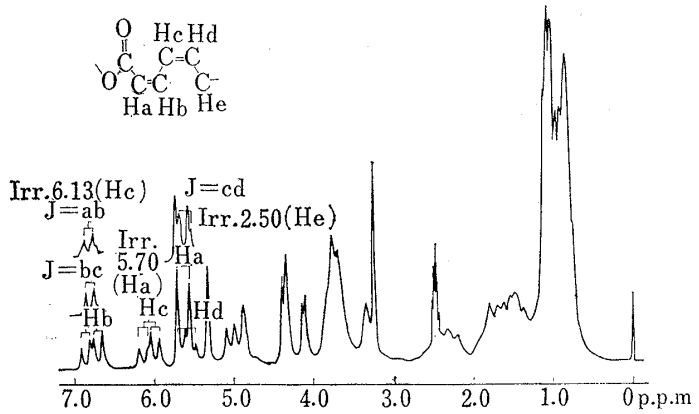


Fig. 1. Azalomycin-B (100 Mc. DMS[d_6], int. ref. TMS)

HOOC-$CH(CH_3)$-CH_2-CH_2-CH_2-CH_2-COOH

VIII

-O-C(-)-$CH(CH_3)$-CH_2-CH_2-CH_2-CH_2-COO-

IX

-O-C(-)-$CH(CH_3)$-CH=CH-CH=CH-COO-

X

Experimental

Melting points are not corrected. Ultraviolet spectra were determined in 95% EtOH on a Beckman Model DK-2 and infrared spectra on Perkin-Elmer Model 21. Proton magnetic resonance spectra were taken on a Varian A-60 or HA-10*8 spectrometer with Me_4Si as an internal standard. Molecular weight determinations were carried out in benzene on a Vapor Pressure Osmometer Model 301A "Mechrolab." All specimen analysed were dried at 25°/0.5 mm. for 8 hr., and the data were given as a mean value of a few runs. Analyses of gas-liquid chromatography were conducted with a Shimadzu Model GC-IB programmed vapor-phase chromatograph.

Azalomycin-B (I)——For production of azalomycin-B, *Streptomyces hygroscopicus* var. *azalomyceticus* was grown in a medium containing 2% dried yeast, 0.5% soybean meal, 2% glycerol, 0.3% NaCl, and 0.2% K_2HPO_4, pH 7.0, at 27° for 50 hr. in a submerged culture. After filtration of the culture broth, the mycelial cake was extracted with aqueous acetone. The precipitate, obtained from the remaining

aqueous solution by concentration of the solvent *in vacuo* from the combined extracts, was collected, and which was extracted with MeOH, and further purified by passing through the columns of Duolite A-2(CH_3COO^-) and activated carbon. Azalomycin-B and -M[6] were separated from azalomycin-F[2,3] by addition of BuOAc to the concentrated eluate. The BuOAc-rich layer involving the former two antibiotics was concentrated *in vacuo* to precipitate both antibiotics. Azalomycin-B was then crystallized from MeOH-EtOAc (1:1) mixture in which azalomycin-M and other impurities were easily soluble. From 6000 L. of the culture broth, 165 g. of the crude crystal of azalomycin-B were obtained.

One gram of the crude azalomycin-B was recrystallized by prolonged heating under reflux with 200 ml. of EtOAc until it dissolved, the solution was evaporated to half its volume, and allowed to cool. When stored for a long-time, the antibiotic is partly decomposed and it becomes difficult to purify only by repeated recrsystallization. In such a case, refluxing of the material in H_2O shortly before recrystallization for taking off some water-soluble impurities is advisable. The product, 300 mg. of the crystals obtained in this way, came as fine needles which melted at 210~212° under decomposition. The melting point was dependent on the rate of heating and was less reliable than its rotation, $[\alpha]_D^{18}$ −48°(c=1, MeOH). Azalomycin-B (I) is insoluble in ether, EtOH, PrOH, or BuOH, almost insoluble in H_2O, slightly soluble in MeOH, EtOAc, or $CHCl_3$ and more soluble in aqueous MeOH, MeOH-EtOAc, or MeOH-benzene than in any of the pure solvent. I also dissolves freely in pyridine or dimethylformamide. UV λ_{max} 252 mμ ($E^{1\%}_{1cm}$ 620). IR ν^{Nujol}_{max} cm^{-1} : 3450 (OH), 1700, 1645, and 1620 (double bond region).

Attempted Molecular Weight Determination of Azalomycin-B (I) by X-Ray Diffraction——Crystals of I obtained by recrystallization from EtOAc, without drying, were allowed to stand for a few minutes in 5~10% AcOEt solution of polyvinyl acetate and collected. X-Ray measurement was carried out on a monoclinic crystal thus obtained. Cell constants (Å) : a=22.44±0.02; b=10.05±0.07; c=17.11±0.10; β=113°40′ ±20′. Cell volume : V=3534±41 Å^3. Space group : $P2_1$ (Z=2) or $P2_1/m$ (Z=4). Density was determined by the application of a flotation method at 26° in toluene-CCl_4 in which any damage of crystals or desolvation of the solvent was not observed. Mean value for six runs was ρ=1.155±0.001.

The content of the solvent was determined by measurement of the loss in weight of crystals under drying *in vacuo* (0.01 mm., 40°, 10 hr.). Its mean value for three runs was 16.1±1%. Based on these data, molecular weight was calculated as 1032 (Z=2) or 516 (Z=4). The experimental error was around 4%.

Azalomycin-B Acetate (II)——To a solution of 10 g. of I in 50 ml. of anhyd. pyridine 15 ml. of Ac_2O was added dropwise. The mixture was allowed to stand overnight at room temperature and poured into 100 ml. of ice-water. Crystals thus obtained were recrystallized twice from AcOEt or H_2O-acetone to 7.1 g. of the acetate (II) as needles, m.p. 210~215°. IR ν^{Nujol}_{max} cm^{-1} : 3450 (OH), 1758 (acetoxyl), 1690, 1645, 1620 (double bond region). UV λ_{max} 252 ($E^{1\%}_{1cm}$ 530).

Molecular Weight Determination of the Acetate (II) by ^{14}C-Labeled Acetylation——To a solution of 1 g. of I in 8 ml. of anhyd. pyridine 2 ml. of ^{14}C-labeled Ac_2O (2 mc. in 5 ml.) was added and treatment of the mixture as described above afforded labeled II, m.p. 210~215°. Standard acetanilide, m.p. 112~115°, was also prepared by adding 1 ml. of the same ^{14}C-labeled Ac_2O to a solution of 1 ml. of aniline in 4 ml. of anhyd. pyridine. Each specimen was dissolved in 10 ml. of dioxane and 1 ml. of the solution was diluted with 15 ml. of the isotope solution (PPO 8 g., POPOP 0.25 g., dioxane 1000 g., toluene 15 ml.). Radioactivities of these specimens were determined on Packard Model 3002 as follows :

Sample (mg.)	10^4 c.p.m.	10^4 d.p.m/mg.	Mean value
Acetanilide			
1.014	2.2465	2.214	
1.025	2.2329	2.178	
1.027	2.2446	2.185	2.192×10^4 d.p.m./mg.
Acetate (III)			
2.000	1.9381	0.9695	
2.009	1.9470	0.9691	
3.314	3.1810	0.9598	0.966×10^4 d.p.m./mg.

Calculated molecular weight : 306 for the monoacetate, 614 for diacetate, and 1224 for the tetracetate.

***p*-Bromobenzoate (III) and *p*-Bromobenzoate Acetate (IV)**——To a solution of 1 g. of I in 30 ml. of anhyd. pyridine 1.4 g. of *p*-bromobenzoylchloride was added in small portions under cooling. The mixture was allowed to stand overnight at room temperature, poured into ice-water, and extracted with ether. The extract washed successively with dil. K_2CO_3 solution, dil. HCl, and H_2O. After drying over anhyd. Na_2SO_4, the extract was evaporated. The residue was recrystallized from EtOAc to obtain *p*-bromobenzoate (III) as needles, m.p. 197°(decomp.). IR ν^{Nujol}_{max} cm^{-1} : 3460, 1730, 1700, 1645, 1620, 1595, 755.

Further acetylation of III with Ac_2O-pyridine gave *p*-bromobenzoate acetate (IV) as needles, m.p. 166~167°. IR ν^{Nujol}_{max} cm^{-1} : 3450, 1752, 1735, 1700, 1645, 1620, 1595, 755.

Perhydro-acetate (VI)——A solution of 2 g. of II in 100 ml. of MeOH was hydrogenated over 1 g. of 5% Pd-C. During 2 hr., uptake of 150 ml. of H_2 was observed at 25° and it almost ceased. The solution

6) H. Okazaki, M. Arai : Japan. Pat. 58456/1964 (Oct. 14, 1964).

was filtered, evaporated in reduced pressure, and the residue was recrystallized from AcOEt or acetone-H_2O to yield 1.8 g. of perhydro-acetate (Ⅵ), as prisms, m.p. 155°. IR ν_{max}^{Nujol} cm^{-1}: 3450 (OH), 1745 (acetoxyl), 1711.

Perhydro Compound (Ⅴ)——A solution of 15 g. of I in 800 ml. of dimethylformamide was hydrogenated over 7 g. of 5% Pd-C. During 2 hr., 1300 ml. of H_2 was absorbed at 24°. After removal of the catalyst, the solution was concentrated to 100 ml. under reduced presssure, followed by addition of 200 ml. of acetone, and the mixture was allowed to stand overnight in a refrigerator. Thirteen grams of crystals of Ⅴ thus obtained was recrystallized from $CHCl_3$-MeOH (1:1) mixture to give 11 g. of Ⅴ as a powder, m.p. 190~192°. IR ν_{max}^{Nujol} cm^{-1}: 3571 (OH), 1712 (CO). *Anal.* Found: C, 60.29, 60.00; H, 9.65, 9.59. The analytical values were not reliable because of adhesive contamination of MeOH, which was checked by NMR spectrometry.

Acetylation of Ⅴ with Ac_2O-pyridine afforded the perhydro-acetate (Ⅵ), almost in a quantitative yield. The sample was identical in m.p., mixed m.p. and infrared spectrum comparison with the acetate (see above).

Oxidation of Azalomicin-B (I) and Perhydro Compound (Ⅴ)——A solution of 3 g. of Ⅴ in 50 ml. of conc. HNO_3 (sp. gr. 1.42) was heated at 100° for 2 days under stirring. The reaction mixture was diluted with H_2O and concentrated to a half volume under reduced pressure. This procedure was repeated three times for repelling volatile acids.

The distillate was made alkaline with dil. NaOH and evaporated to dryness. The residue was again diluted with H_2O, followed by acidification with dil. H_2SO_4, and extracted with ether. After drying over anhyd. Na_2SO_4, the extract was concentrated to a few ml. This concentrate was found to contain acetic acid and propionic acid, as testified by gas chromatography (DC-550-stearic acid, 2.25 m., 120°, 60 ml./min.).

The previously obtained concentrate made alkaline with dil. NaOH, evaporated to dryness, and extracted with MeOH. The MeOH extract was acidified with conc. HCl and excess of CH_2N_2 was added under cooling. After standing for 1 hr., the mixture was diluted with H_2O and extracted with ether. The concentrate of the ether extract was found to contain methyl ester of α-methylpimelic acid, accompanied with a small amount of esters of glutaric acid, adipic acid, and some unidentified substances by gas chromatography (polyester succinate, 2.25 m., 196°, 60 ml./min.).

Oxidation of I with HNO_3 was also carried out under the same condition used for Ⅴ. The presence of acetic acid and propionic acid in the volatile part of the oxidation product was clarified, but other higher homologs, including α-methylpimelic acid, was not detected from the non-volatile part of the oxidation product.

We are grateful to Dr. G. Sunagawa, Manager, and Drs. I. Iwai and H. Okazaki, Assistant Managers of the Laboratories, for their guidance.