STRUCTURE AND BIOLOGICAL ACTIVITY OF LIPIARMYCIN B

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Actinoplanes deccanensis ATCC 21983, the producer of antibiotics lipiarmycin A3 and A4, furnished also a related antibiotic designated lipiarmycin B, active against Gram-positive bacteria, including anaerobes, and against *Neisseria*. The structures of the two major components, B3 and B4, were elucidated from their physico-chemical properties, ^{1}H and ^{13}C NMR spectra and fast atom bombardment mass spectra data in comparison with lipiarmycins A3 and A4.

Fermentation of *Actinoplanes* strain ATCC 21983 produced the antibiotic lipiarmycin[1,2], later called lipiarmycin A, which is a complex of two minor factors and two major factors A3 and A4. The structures of lipiarmycins A3 and A4 have been elucidated by chemical degradation and NMR studies[3~5] (Fig. 1) and presented at the 14th IUPAC Symposium on the Chemistry of Natural Products (Poznan, 1984)[4].

Pilot plant fermentations furnished a crude material which by chromatographic techniques was shown to contain an additional factor designated lipiarmycin B. This report describes the isolation, physico-chemical properties and biological activity of lipiarmycin B. The structures of the two major components of lipiarmycins B, B3 and B4, were elucidated by chemical and NMR studies in comparison with lipiarmycins A3 and A4.

Isolation of Lipiarmycin B

The fermentation of *Actinoplanes deccanensis* ATCC 21983 and the recovery were carried out as previously described[1]. Two main spots attributable to biologically active substances were revealed in the crude materials by TLC under the conditions described in Table 1. The spot with the higher Rf (which under these conditions appears homogeneous) corresponds to lipiarmycin A and the other to lipiarmycin B.

Pure lipiarmycin B was obtained by silica gel chromatography under pressure followed by a purification step at atmospheric pressure.

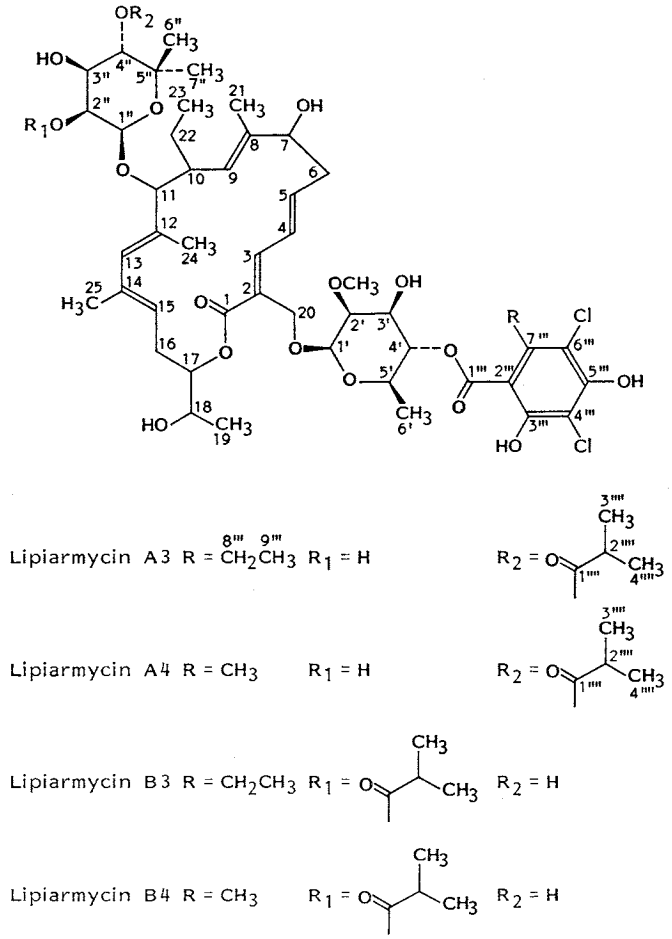
Lipiarmycins B3 and B4

Silanized silica gel TLC and reversed phase HPLC revealed that lipiarmycin B is composed of two minor and two major factors. The latter have been designated lipiarmycins B3 and B4. Suitable quantities were prepared by preparative HPLC or by flash chromatography.

Physico-chemical Properties of Lipiarmycin B

Some physico-chemical properties are shown in Table 1 in comparison with those of lipiarmycin

Fig. 1.



In accordance with the journal's instructions for standardizing the representation of 18-membered ring macrolides the structure and numbering reported in ref 9 has been used instead of those previously published by us[4,5] which are shown in Fig. 4.

A. Lipiarmycin B is a white powder, less soluble in water and in non-aqueous solvents and less lipophilic than lipiarmycin A. The UV spectra suggested that their chromophoric skeletons are very similar. The elemental analysis and osmometric data are in agreement with the formula $C_{51\sim52}H_{72\sim74}Cl_2O_{18}$, which is the same as that of lipiarmycin A. Fast atom bombardment mass spectra (FAB-MS) in the positive ion spectrum in thioglycerol matrix exhibited $(M+Na+thioglycerol)^+$ ions at *m/z* 1,187 and *m/z* 1,173. Thus, the molecular formulas $C_{52}H_{74}Cl_2O_{18}$ and $C_{51}H_{72}Cl_2O_{18}$ were definitely assigned to B3 and B4, as previously to A3 and A4.

All of the above data supported the hypothesis that B3 and B4 differ by a CH_2 group and are isomers of A3 and A4.

Structure Assignment

The ^1H and ^{13}C NMR resonances have been completely assigned (Table 2). Comparison of the ^1H NMR spectra of lipiarmycins B3 and A3[5] indicates that the two molecules differ only in the substitution pattern of the 5-methylrhamnose moiety since the other resonances are within 0.08 ppm.

Table 1. Physico-chemical properties[a].

	Lipiarmycin B	Lipiarmycin A
Appearance	White powder	White crystal powder
MP (°C)[b]	134~140	167~173
Anal[c] C	58.78	58.60
H	6.95	6.94
Cl	6.87	6.59
$[\alpha]_D^{20}$	−8.8° (*c* 1.97, MeOH)	−5.5° (*c* 1.98, MeOH)
HPLC retention time (minutes)	2.25 (B3); 1.95 (B4)	2.33 (A3); 2.03 (A4)
TLC, Rf		
CH_2Cl_2 - MeOH (9 : 1)	0.30	0.42
EtOAc	0.27	0.40
2-PrOH - CH_2Cl_2 (3 : 7)	0.66	0.73
UV λ_{max} nm ($E_{1cm}^{1\%}$)		
MeOH	230 (360), 270 (sh), 315 (117)	232 (354), 268 (sh), 315 (108)
0.1 N HCl	227 (376), 268 (202)	232 (338), 272 (207)
Buffer pH 7.5	237 (341), 277 (200), 315 (sh)	238 (331), 275 (194)
0.1 N NaOH	240, 270, 320 (sh)	235 (370), 268 (sh)
MW (osmometric)	1,072	1,060
pK_{MCS}	6.95	6.8

[a] See the Experimental section.

[b] DSC-TG shows an endothermic peak at 60~120°C, due to the weight loss of the imbibition solvents, and an endothermic peak between 130~140°C, followed by decomposition.

[c] On a sample dried at 140°C in an inert atmosphere.

Scheme 1.

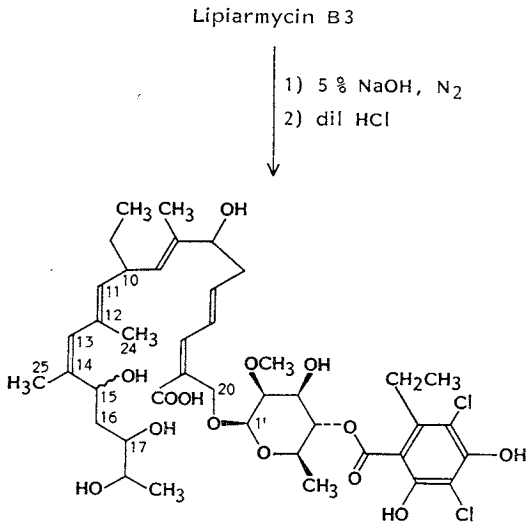


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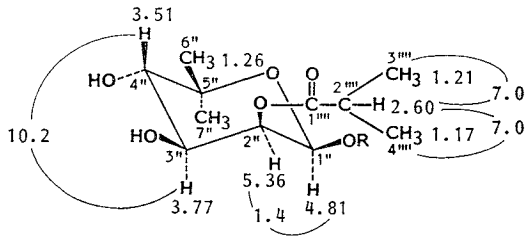
Fig. 2.



Chemical shift (ppm) and coupling constants (Hz) in acetone-d_6 exhibited by the protons of the 2-*O*-isobutyryl-5-methyl-β-rhamnoside (shown D-($^4C_1$)).

This was confirmed chemically; both lipiarmycins B3 and A3 gave, upon alkaline hydrolysis followed by strong acidification, the same mixture (**5**) of the two diastereoisomeric compounds at C-15, whose structures were assigned earlier[5] (Scheme 1). As the formation of compounds **5** proceeds *via* the expulsion of the above mentioned 5-methylrhamnose unit, it follows that lipiarmycins B3 and A3 differ only in the nature of this sugar. The structure of this sugar in lipiarmycin B3 was determined to be 2-*O*-isobutyryl-5-methylrhamnose by analysis of the chemical shifts and coupling constants, the results of which are presented in Fig. 2. In particular, the downfield shift (1.39 ppm) and the upfield shift

Table 2. ^{1}H and ^{13}C NMR chemical shifts (ppm) for lipiarmycins B3 and B4 in acetone-d_6.

Atom	δ_H[a]		δ_C[b]	Atom	δ_H		δ_C
	B3	B4	B3		B3	B4	B3
1			167.61 s	1′	4.69	4.68	101.82 d
2			125.44 s	2′	3.61	3.61	81.57 d
3	7.22	7.23	145.19 d	3′	3.81	3.82	72.34 d
4	6.63	6.62	128.22 d	4′	5.11	5.10	77.63 d
5	5.95	5.95	143.04 d	5′	3.60	3.62	70.62 d
6a	2.69	2.71	37.25[c] t	6′	1.32	1.30	18.16 q
6b	2.50	2.50		2′-OCH_3	3.53	3.52	61.65 q
7	4.26	4.26	72.87 d	1‴			169.52 s
8			136.58[d] s	2‴			110.40[e] s
9	5.19	5.19	124.26 d	3‴			156.07 s
10	2.58	2.58	42.26 d	4‴			108.27 s
11	3.65	3.67	92.38 d	5‴			153.82 s
12			136.06[d] s	6‴			114.60[e] s
13	5.83	5.83	133.98 d	7‴			142.70 s
14			135.86 s	8‴a	3.00	2.55	26.14 t
15	5.64	5.64	126.52 d	8‴b	3.00		
16a	2.77	2.78	28.49[c] t	9‴	1.23		14.31 q
16b	2.43	2.44		1″	4.81	4.81	94.77 d
17	4.74	4.74	78.35 d	2″	5.36	5.35	72.92 d
18	4.04	4.03	68.03 d	3″	3.77	3.77	70.34 d
19	1.19	1.19	20.43 q	4″	3.51	3.49	74.98 d
20a	4.60	4.60	63.46 t	5″			75.09 s
20b	4.42	4.42		6″	1.26	1.26	29.02 q
21	1.63	1.63	15.16 q	7″	1.11	1.11	17.56 q
22a	1.85	1.85	26.14 t	1‴′			176.61 s
22b	1.25	1.25		2‴′	2.60	2.60	34.82 d
23	0.78	0.79	11.19 q	3‴′	1.21[f]	1.21[g]	19.59[h] q
24	1.80	1.80	13.66 q	4‴′	1.17[f]	1.17[g]	19.37[h] q
25	1.75	1.75	17.44 q				

[a] Lipiarmycins B3 and B4 exhibit OH resonances at δ_H 10.63 (3‴-OH), 9.24 (5‴-OH) and 3.3~4.3 (5 aliphatic OH), and δ_H 10.84 (3‴-OH), 9.24 (5‴-OH) and 3.3~4.3 (5 aliphatic OH), respectively.

[b] Small letters refer to multiplicities arising from coupling with directly bonded protons; s: singlet, d: doublet, t: triplet, q: quartet.

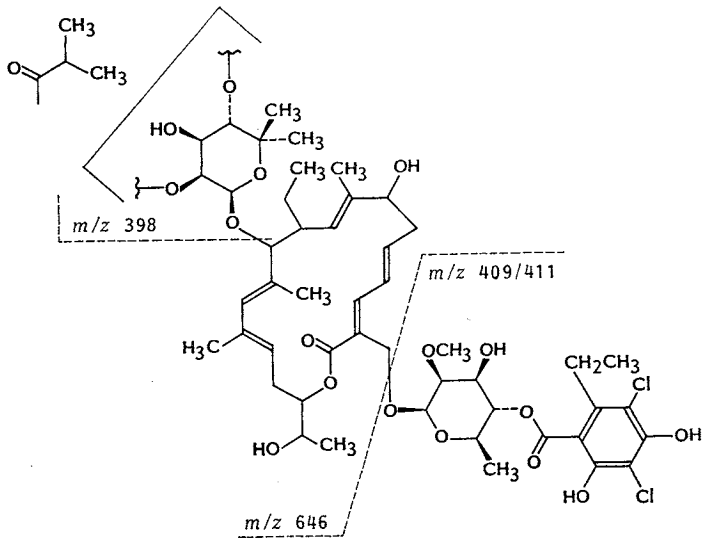
[c~h] Assignments may be interchanged.

(−1.49 ppm) experienced by 2″-H and 4″-H in lipiarmycin B3 when compared with their chemical-shift values in lipiarmycin A3[5] indicate that the isobutyryl residue (which in lipiarmycin A3 is attached to the oxygen in position 4″) in lipiarmycin B3 esterifies the 2″-OH. Moreover, the close similarity between the vicinal coupling values exhibited by the methine protons in lipiarmycins A3 and B3 ($\Delta \leq$ 0.3 Hz) implies that in both the compounds the 5-methylrhamnose sugar is in the chair conformation with the anomeric proton axially disposed and has the same relative configuration. We have no proof of its absolute configuration which is arbitrarily assumed to be β-D-($^4C_1$) in Fig. 2.

Finally, the value of 155 Hz exhibited by the anomeric C-1″ in the fully ^{1}H-coupled ^{13}C NMR spectrum confirms that this sugar is equatorially linked to the rest of the molecule, as in lipiarmycin A3[5,6]. Therefore, the structure of lipiarmycin B3 is as reported in Fig. 1.

The structure of lipiarmycin B4 was determined to be that shown in Fig. 1, because its ^{1}H NMR spectrum in acetone-d_6 (see Table 2) is similar to within 0.02 ppm to that of lipiarmycin B3, the only difference being the presence of an aromatic methyl group (8‴-H_3 2.55 ppm) in B4 instead of an

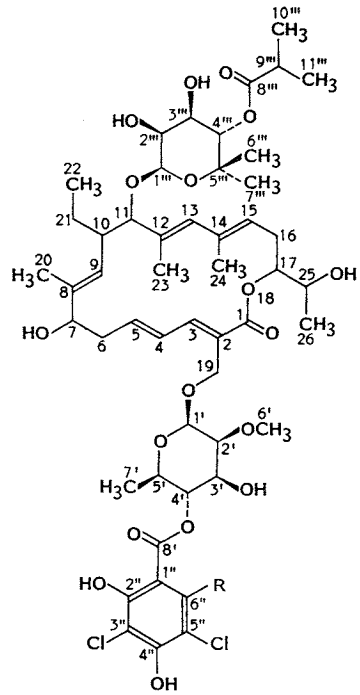
Fig. 3.



ethyl group.

The mass spectra obtained by liquid chromatography (LC)/MS in negative chemical ionization confirmed the MWs. Some significant fragments are present (Fig. 3). Compounds B3 and A3 both gave fragment ions at *m/z* 409/411 (B4 and A4 at *m/z* 395/397), confirming that lipiarmycins B3 and A3 differ from lipiarmycins B4 and A4 in the alkyl substituents on the aromatic ring. From both lipiarmycins B3 and A3 a fragment at *m/z* 646 ($M-C_{16}H_{20}Cl_2O_8$) is formed, confirming that the difference between them is in the position of the substituent isobutyryl group. In addition, the mass spectra of lipiarmycins B3 and A3 showed large differences in fragment ion abundances at *m/z* 932/934, 878/880, 824/826 which can be used for distinguishing the two compounds.

Fig. 4.



Lipiarmycin A3 R = CH_2CH_3

Lipiarmycin A4 R = CH_3

Recently, a strain of *Micromonospora echinospora* was found to produce a complex of lipiarmycin-like antibiotics named clostomicins A, B_1, B_2, C and D[7]. On the basis of the published data clostomicins B_1 and B_2 appear to be identical to lipiarmycins A3 and B3. After sending this paper to the journal, another complex of related antibiotics designated tiacumicins has been described[8,9]. The structure of tiacumicin B seems to correspond to that of lipiarmycin A3, and that of tiacumicin C to lipiarmycin B3.

Table 3. *In vitro* antimicrobial activity[a].

Organism	MIC (μg/ml)	
	Lipiarmycin B	Lipiarmycin A
Staphylococcus aureus Tour	32	2
S. aureus Tour[b]	128	32
S. epidermidis ATCC 12228	16	0.5
S. haemolyticus L602[c]	32	4
Streptococcus pyogenes C203	64	8
S. pneumoniae UC41	64	32
S. faecalis ATCC 7080	16	2
Neisseria gonorrhoeae L997[c]	32	2
Escherichia coli SKF 12140	>128	>128
Clostridium perfringens ISS 30543	0.03	0.03
C. difficile ATCC 9689	0.13	0.03
Propionibacterium acnes ATCC 6919	32	1
P. acnes L1560[c]	16	1
P. acnes L1565[c]	16	1
Bacteroides fragilis ATCC 23745	>128	>128

[a] MIC for aerobes were determined by microtiter dilution (media: Oxoid Iso-Sensitest broth for Staphylococci, *S. faecalis*, *E. coli*; Difco Todd-Hewitt broth for other Streptococci; Difco GC base broth +1% BBL IsoVitaleX for *N. gonorrhoeae*). MIC for anaerobes were determined on Oxoid Wilkins-Chalgren agar. Inocula were 10^4 cfu per ml or per spot. Incubation time, at 37°C, was 48 hours for *N. gonorrhoeae* (in 5% CO_2) and for anaerobes (in anaerobic gas); overnight for other species.

[b] 30% Bovine serum added.

[c] Clinical isolate.

Biological Activity

The MIC of lipiarmycin B were compared with those of lipiarmycin A. In addition to the activities previously reported[2] the lipiarmycins are active against *Neisseria gonorrhoeae* (Table 3). Lipiarmycin B is less active than lipiarmycin A against all bacterial species (Table 3). Thus, the position of the isobutyryl residue on methylrhamnose affects *in vitro* activity. The mechanism of action of lipiarmycin B is the same as that of lipiarmycin A[2], *i.e.*, inhibition of RNA synthesis. In mice with septicemia arising from ip infection with *Streptococcus pyogenes* C203, lipiarmycin B was inactive at doses of up to 250 mg/kg, both orally and sc.

Experimental

MP's are uncorrected.

TLC were run on silica gel plates (Merck F254) and on silanized silica gel plates (Merck HF 254) to a distance of 150 mm. Detection: UV light at 254 nm; bioautography with *Staphylococcus aureus* ATCC 6538 (at least 100 μg of lipiarmycin B and 20 μg of lipiarmycin A must be spotted).

HPLC was run with a Constametric III pump (LDC Milton Roy) equipped with a Reodyne 7125 injector and a Perkin-Elmer LC-15 detector at 254 nm. Column: Spheri 5 Brownlee Labs RP-8 (250×4.6 mm). Eluent: CH_3CN - H_2O (3:1). Injection: 20 μl. Flow rate: 1 ml/minute.

The *pK* values were determined in Methyl Cellosolve - water (4:1) by titration with 0.1 N NaOH.

IR spectra were recorded with a Perkin-Elmer 177 instrument.

UV spectra were run on a Beckman DK-2 spectrophotometer.

^1H (300.13 MHz) and ^{13}C (75.47 MHz) NMR spectra were recorded on a Bruker CXP-300 spectrometer; chemical shifts are from TMS (internal standard, 0.00 ppm).

LC/MS was performed by a direct inlet system on an HP 5985 B instrument in negative ionization. The instrument was equipped with the HPLC apparatus described above, eluent mixture CH_3CN - H_2O (1 : 1). The eluents were used as reactant gases for ionization. E=200 eV; source temp 250°C; EM 2200 V.

Osmometric determinations were made with a Knauer apparatus in $CHCl_3$ at 37°C.

Separation of Lipiarmycin B

The antibiotic activity present in filtered broth, in powders, and in chromatographic fractions was assayed by the disk diffusion method on Penassay agar medium, pH 6.6. *Bacillus cereus* var *mycoides* ATCC 9634 (sensitivity range 30 to 250 μg/ml) was used for more concentrated preparations and *Micrococcus luteus* ATCC 9341 (0.3 to 10 μg/ml) for less concentrated preparations. The harvested broth (2,500 liters) was extracted with BuOH at neutral pH. The extracts were washed with BuOH - saturated water, concentrated under vacuum and poured into a large amount of petroleum ether. The precipitate was collected and dried (spectrophotometric titer 60%, microbiological titer 48%).

The separation was carried out under pressure with the Chromatospac Prep 100 (Jobin-Yvon) apparatus. The column was packed at a pressure of 10 bar with 1.5 kg of Silica gel 60 H (Merck) slurried with a mixture of CH_2Cl_2 - MeOH (95 : 5). A solution of 19 g of the crude in 200 ml of CH_2Cl_2 - MeOH (9 : 1) was applied to the column, which was eluted at a pressure of 8 bar with CH_2Cl_2 - MeOH (95 : 5) up to fraction 22, then with CH_2Cl_2 - MeOH (9 : 1).

The following fractions (700 ml) were collected and checked by TLC:

1~2 undefined substances;
3~6 lipiarmycin A;
7~15 lipiarmycin A+undefined substances;
16~20 lipiarmycin A+lipiarmycin B;
21~35 lipiarmycin B+impurities (trace amount).

Fractions 21~35 were combined and concentrated at 40°C under vacuum to a small volume. Upon addition of Et_2O and petroleum ether, a solid separated which was filtered off, dissolved in 40 ml of EtOAc and chromatographed on 60 g of Silica gel 60 (0.06~0.2 mm) (Merck) slurred in EtOAc and eluted with EtOAc. Fractions of 25 ml were collected. Fractions 6, 7 and 8 were combined and evaporated to dryness. The residue was dissolved in a small volume of MeOH by gentle heating to 40°C; Et_2O was added to cloudiness, then petroleum ether. The separated solid was filtered off and dried under vacuum at 50°C, yielding 1.2 g of lipiarmycin B.

Separation of Lipiarmycins B3 and B4

Lipiarmycin B (100 mg) was adsorbed onto the top of a chromatographic column filled with flash RP-C18 silica gel and eluted under N_2 pressure with acetone - H_2O (2 : 1); the elution was monitored using silanized silica gel plates. The fractions containing homogeneous compounds were collected, evaporated and chromatographed on PLC Silica gel with CH_2Cl_2 - MeOH (15 : 1) as eluant to give pure compounds B3 (40 mg) and B4 (10 mg).

Lipiarmycin B3 is a glassy solid from EtOAc - hexane; mp 148~153°C; $[\alpha]_D^{20}$ −11.7° (*c* 0.1, MeOH); TLC (acetone - 0.1% aq Na_2SO_4, 1 : 1) Rf 0.39; IR ν_{max} cm^{-1} 1730 (aliphatic ester), 1690 (unsaturated lactone), 1650 (aromatic ester).

Anal Calcd for $C_{52}H_{74}Cl_2O_{18}$: C 59.03, H 7.05, Cl 6.7.
Found: C 58.8, H 7.0, Cl 6.6.

Lipiarmycin B4 is a white powder from Et_2O - hexane; mp 115~117°C; $[\alpha]_D^{20}$ −5.9° (*c* 0.1, MeOH); TLC Rf 0.46 (with the same eluent mixture reported above).

Hydrolysis of Lipiarmycin A3 to Isomeric Compounds **5**

Lipiarmycin B3 (100 mg) was treated with 5% NaOH for 20 hours at room temp. The solution was then acidified with dilute HCl and extracted with EtOAc. After evaporation of the solvent, the solid residue was treated with 5 ml of 0.5 N HCl - MeOH (1 : 2) for 10 minutes, at 60°C under N_2; the solution was extracted with EtOAc. PLC of the extract using hexane - EtOAc (1 : 1) as solvent gave compounds **5** identical in mp, TLC and NMR with the compound isolated from lipiarmycin A3[5].

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