RAMOPLANIN (A-16686), A NEW GLYCOLIPODEPSIPEPTIDE ANTIBIOTIC

III. STRUCTURE ELUCIDATION†

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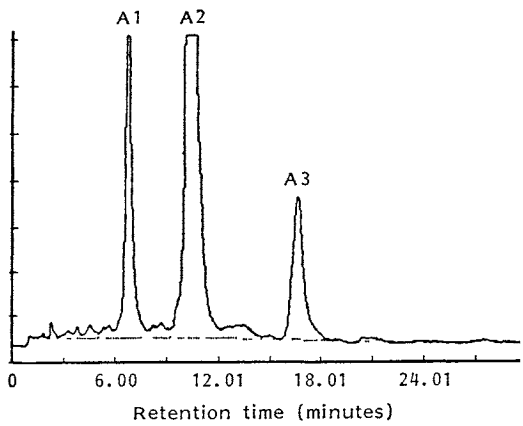
By combination of chemical, ^{1}H and ^{13}C NMR, and mass spectrometric studies, the structures of the three components of the antibiotic ramoplanin (A-16686), produced by *Actinoplanes* sp. ATCC 33076, have been elucidated. All the components have structures formed by a common depsipeptide skeleton carrying a dimannosyl group and are differentiated by the presence of various acylamide moieties, derived from C_8, C_9 and C_{10} fatty acids.

A-16686,†† an antibiotic produced by *Actinoplanes* sp. ATCC 33076,[1] is active against aerobic and anaerobic Gram-positive bacteria, including methicillin-resistant Staphylococci and bacteria resistant to ampicillin and/or erythromycin.[2~5] In particular, it is very active against a series of clinical isolates of strains of *Propionibacterium acnes*.[6] Preliminary physico-chemical characterization[1] indicated that A-16686 is formed by a peptidic core carrying two D-mannose units.

Reversed-phase high pressure liquid chromatography (HPLC) showed at an early stage that it consists of three related factors, designated A1, A2 and A3, as shown in Fig. 1. Single factors A1, A2 and A3 were separated by reversed-phase preparative HPLC (Silica gel-C_{18}) and isolated as water soluble dihydrochlorides. Most of the studies have been done on factor A2, which is present in 80% ratio in the complex.

Fig. 1. HPLC profile[a] of A-16686 (retention times: A1, 6.79; A2, 11.31; A3, 16.61).



[a] Liquid chromatograph: Hewlett-Packard 1084 B with UV detector (254 nm), column: Erbasil C-18, 5 μm, 4.6×150 mm, mobile phase: 0.05 M NaH_2PO_4 - CH_3CN (65 : 35), flow rate: 1.5 ml/minute, injection: 20 μl (0.5 mg/ml in water).

Fast Atom Bombardment (FAB) Mass Spectrometry

The FAB spectra of the single components of the antibiotic gave isotopic clusters of the quasi molecular ions in agreement with the molecular formulas of the free bases (Table 1). Fragment ion clusters due to loss of 324 units, attributed to a dimannose unit, were observed. Also the exact mass of the lowest isotope of the

† Some of these data were presented at the 2nd International Symposium on "New Bioactive Metabolites from Microorganisms", Gera (GDR), May 2, 1988.

†† Ramoplanin is the International Nonproprietary Name.

Table 1. Molecular formulas, weights and ions.

Factor	Formula	MW	$[M+H]^+$ cluster peaks, *m/z* (relative abundance)
A1	$C_{118}H_{152}ClN_{21}O_{40}$	2,540.068	2,539, 2,540, 2,541, 2,542 (65, 100, 85, 80)
A2	$C_{119}H_{154}ClN_{21}O_{40}$	2,554.095	2,553, 2,554, 2,555, 2,556 (77, 92, 100, 79)
A3	$C_{120}H_{156}ClN_{21}O_{40}$	2,568.122	2,567, 2,568, 2,569, 2,570 (50, 91, 100, 89)

Table 2. Amino acids and chiralities.

Amino acids	Number of units	Chirality
Threo-β-hydroxyaspartic acid (OH-Asp)	1	L
Aspartic acid	1	L
Allo-threonine (*allo*-Thr)	3	2D+1L
Glycine	1	
Alanine	1	D
4-Hydroxyphenylglycine (Hpg)	5	2D+3L
Leucine	1	L
Phenylalanine	1	L
3-Chloro-4-hydroxyphenylglycine (Chp)	1	L
Ornithine (Orn)	2	2D

$[M+H]^+$ clusters agreed within 30 ppm with the calculated masses, as determined by external reference peak matching.

Amino Acid Composition

Preliminary structural studies[1] had shown that by hydrolysis of each factor aspartic acid, glycine, alanine, 4-hydroxyphenylglycine (Hpg), leucine, phenylalanine, chlorohydroxyphenylglycine, and ornithine (Orn), plus two unknown amino acids, were isolated.

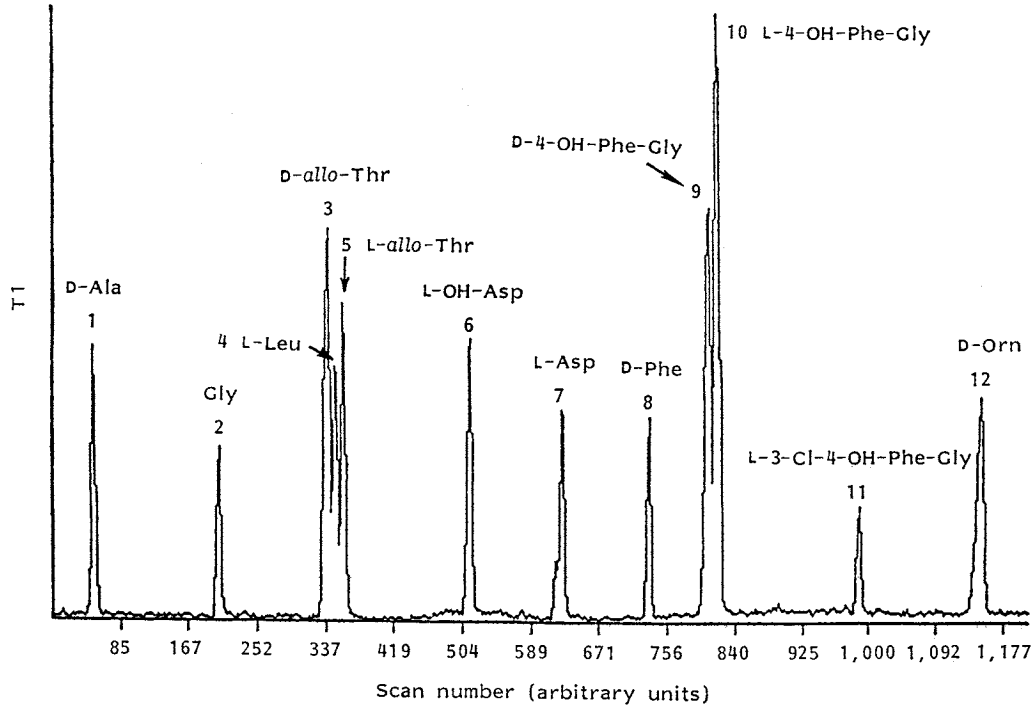
To determine the structure of the two undefined amino acids and the substituent position in the chlorohydroxyphenylglycine A-16686 was hydrolyzed with 6 N HCl at 105°C for 20 hours and the amino acids were separated by ion exchange column chromatography. The unknown amino acids were unequivocally identified by comparison (^1H NMR, GC-MS) with authentic samples as β-hydroxyaspartic acid and *allo*-threonine (*allo*-Thr). The structure of chlorohydroxyphenylglycine was established as 3-chloro-4-hydroxy by comparison with a sample prepared by synthesis following a general procedure described in the literature.[7]

Quantitative HPLC determination of the amino acids together with ^1H[8] and ^{13}C NMR investigations suggested that each factor likely has the same peptide core (Table 2).

Configuration of the Amino Acids

Factors A1, A2 and A3 were separately hydrolyzed (6 N HCl, 105°C, 20 hours) and the mixtures of amino acids were derivatized and examined by GC-MS on a chiral column (Fig. 2). Chiralities were assigned (Table 2) by comparison with optically active amino acids or with their racemic mixtures (in the case of *threo*-β-hydroxyaspartic acid (OH-Asp) and 3-chloro-4-hydroxyphenylglycine (Chp)) since on the chiral column used D-enantiomers show lower retention times than the related L-enantiomers.[9,10] For *allo*-Thr and Chp the ratio of the respective enantiomers was determined basing on the ratio of the areas of the peaks in the GC chromatogram.

Fig. 2. GC-MS separation[a] of the *N*-pentafluoropropionyl propyl esters of the peptidic hydrolysate of A-16686 factor A2.



[a] Gas chromatograph Hewlett-Packard model 5840A equipped with a capillary column OS 6411 25 m × 0.2 mm (CGC Analytic Labor, Massingen (FRG)). Column temp 80°C for 4 minutes, then 170°C (4°C/minute); flow He 1.05 kg/cm²; source temp 200°C. Mass spectrometer Hewlett-Packard model 5985B, EI 70 eV, EM 2200 V.

Acid-base Ionization

By acid-base titration in water of A-16686 factor A2 the presence of 2 mol of HCl per mol of antibiotic was confirmed. Furthermore two functions were clearly detectable at an average *pKa* value of about 8.0 and three at a value of about 10.0, which were in total attributed to five phenolic functions. When the titration was repeated in CH_3OH - H_2O (4 : 1) the titration slopes practically disappeared because their *pKa* values increased, thus confirming that five ionizable groups have to be attributed to acidic functions, *i.e.*, five phenols. The titration slope around *pKa* 11 remained unchanged, and this was indicative of basic functions, *i.e.*, amino groups. In water the pH value 9.25 corresponds to the isoelectric point and to the minimum of solubility. At this pH the compound is in the form of zwitterion formed between the two stronger phenolic functions and the two amino groups of the two Orn moieties. Titration with 0.1 N $HClO_4$ in glacial AcOH confirmed the presence of two basic functions.

Characterization of the Lactone Bond

Each of the A-16686 factors show the main IR bands listed in Table 3. The bands at 1760 and 1225 cm^{-1} suggested the presence of a lactone bond. The action of dilute bases at ambient temperature resulted in a loss of the 1760 cm^{-1} band and in a shift of the two absorption bands at 232 and 270 nm in the UV spectrum to a high intensity band at 250 nm (Table 4), indicating a transformation

into an "open" compound, designated A-16686-acid. To establish which amino acid contributed to the lactone bond with its carboxylic group, A-16686 was reduced with $Ca(BH_4)_2$, then hydrolyzed and analyzed for its amino acid composition (Scheme 1).

Bidimensional TLC of the hydrolysate showed the lack of Chp, while a new compound appeared. This latter was isolated and recognized (^{1}H NMR, MS) to be 2-amino-2-(3-chloro-4-hydroxyphenyl)-ethanol, whose structure was confirmed by comparison with an authentic sample.

The identification of the hydroxylamino acid contributing to the lactone bond with its hydroxyl group was accomplished by reacting both A-16686 and A-16686-acid derivative with phenylisocyanate,[11] followed by acid hydrolysis (Scheme 1) and HPLC quantitative amino acid determination (Table 5). Only the amount of hy-

Table 3. Assignments of the main bands of A-16686 IR spectrum.[a]

Bands ν (cm^{-1})	Attribution
3500~3100	νNH and νOH
1760	νC=O, lactone
1630	νC=O, amide I
1510	δNH, amide II
1225	νC–O, lactone
1065~980	νC–O, sugars
840 and 815	γCH, aromatic

[a] Recorded with a Perkin-Elmer 580 spectrophotometer in Nujol mull.

Table 4. UV bands and absorptivities (a) of A-16686 in water solutions at different pH values.[a]

	λ_{max} (nm)	a
0.1 N HCl	232	21.1
	270	10.5
pH 5.5	232	21.8
	270	10.5
pH 7.5	232	22.1
	268	11.0
pH 8.5	232	21.2
	258	12.1
pH 9.2	233	20.5
	256	15.9
0.1 N KOH	250	30.0

[a] Recorded with a double beam spectrophotometer Perkin-Elmer model 320.

Scheme 1.

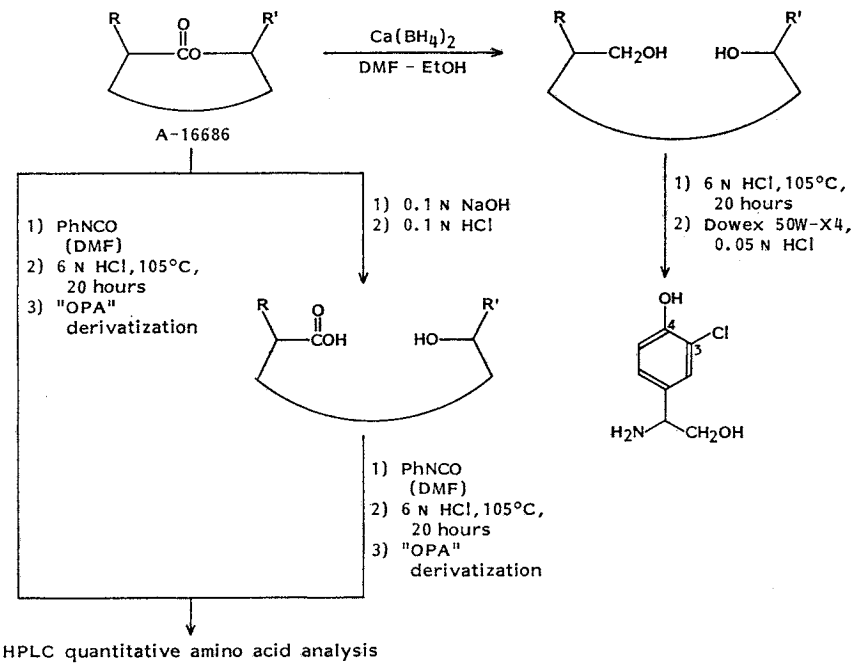
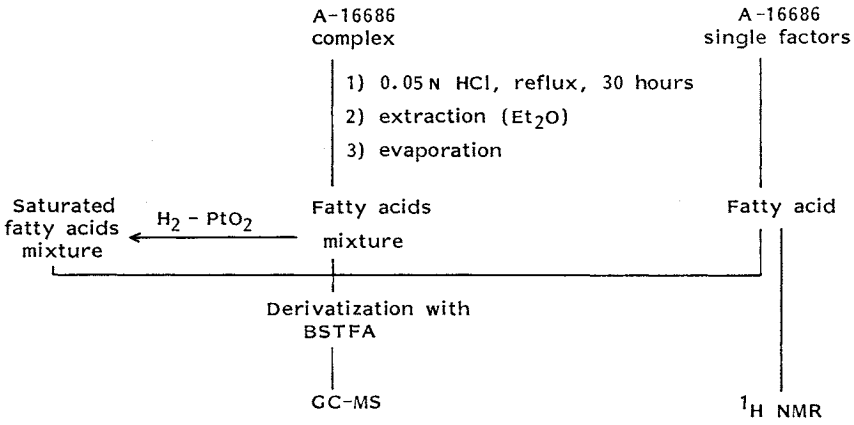


Table 5. Molar ratio of amino acids (Ala=1) in the hydrolysates of A-16686 and A-16686-acid before and after treatment with phenylisocyanate (PhNCO).

	OH-Asp	Asp	Gly	Ala	*allo*-Thr	Hpg	Chp	Phe	Leu	Orn
A-16686	1	1	1	1	3	5	1	1	1	2
A-16686+PhNCO	1	1.1	1.2	1	0.6	2.4	0.9	0.4	1	0.7
A-16686-acid	1	1	1.3	1	2.9	5.1	1	0.9	1	2.3
A-16686-acid+ PhNCO	0.5↓	1.1	1.1	1	0.2	1	0.7	0.4	1	0.5

Scheme 2.



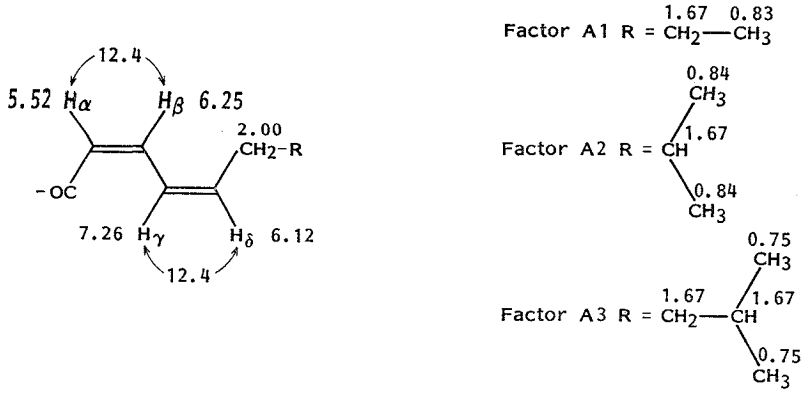
droxyaspartic acid decreased for A-16686-acid, thus indicating that this amino acid was involved in the lactone linkage with its hydroxyl group.

Nature of Fatty Acids

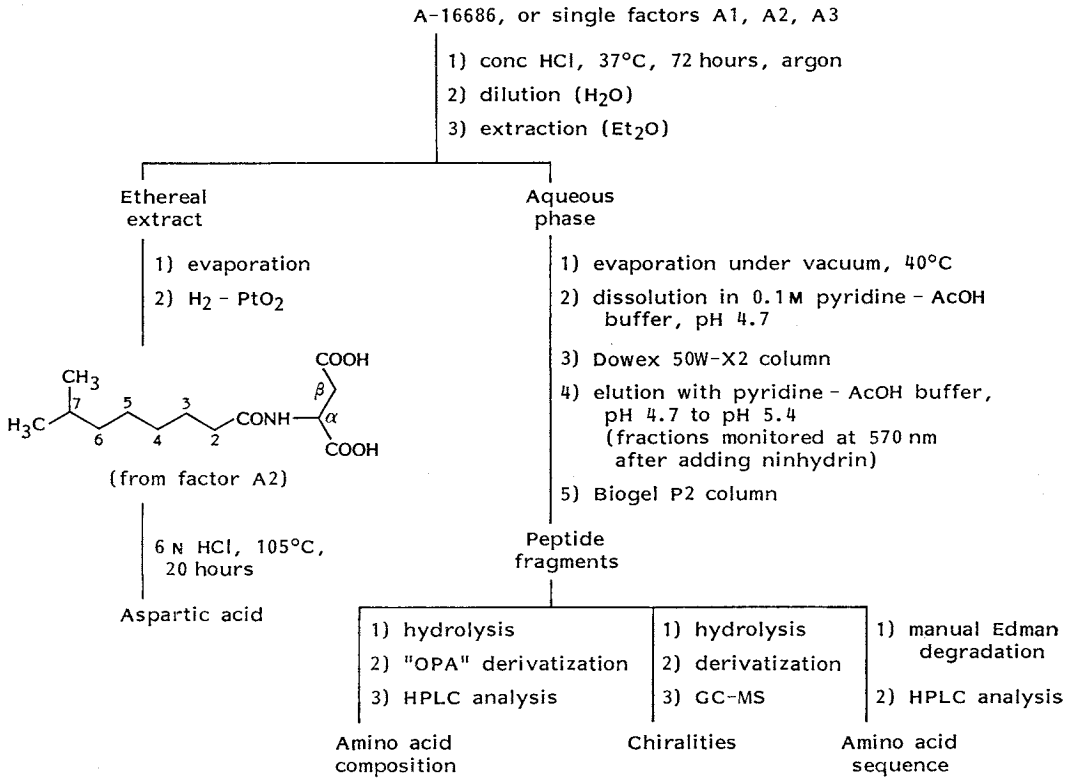
The analysis of the ethereal extract of the hydrolysate gave indication of the presence of fatty acids. Therefore, their nature was investigated as shown in Scheme 2 by hydrolyzing A-16686 and single factors under mild conditions, and derivatizing the residue of the ethereal extract with bis-(trimethylsilyl)trifluoroacetamide (BSTFA). Three peaks were detected by GC-MS analysis in the ratio approximately corresponding to that of the three factors of the complex. Their mass spectra gave molecular ions (M^+) at *m/z* 212, 226 and 240, and typical common fragments at *m/z* 169 and 155, thus indicating the presence of propyl, butyl and pentyl radicals and the existence of the unsaturated moiety $C_4H_4COOSi(CH_3)_3$. The GC-MS analysis of the same fatty acids mixture after hydrogenation over PtO_2 gave three peaks in the same ratio as before, showing M^+ at *m/z* 216, 230 and 244, whose fragmentation pattern on the basis of library data corresponded to that of trimethylsilyl esters of octanoic, 7-methyloctanoic and 8-methylnonanoic acids. These results indicate that the unsaturated acids obtained are octa-2,4-dienoic acid and the homologues 7-methylocta- and 8-methylnona-2,4-dienoic acids.

These structures were confirmed by the data of the ^1H NMR spectra of the factors (Fig. 3). The resonances of the diene part of the fatty acid was common to all three components, and the coupling constants and nuclear Overhauser enhancement (NOE)-measurement indicate the *cis*, transoid, *cis* configuration system for the 2,4-conjugated double bonds. Thus the acyl moieties characterize the three factors of A-16686 and account for the differences of 14 daltons shown by the molecular weights.

Fig. 3. ^1H NMR (500 MHz, H_2O - DMSO (4 : 1), pH 4.6) chemical shifts (δ) of the acyl moieties of A-16686 factors.



Scheme 3.



Amino Acid Sequence Determination

A-16686 factor A2 was hydrolyzed under mild conditions (conc HCl, 37°C, 72 hours) to establish the amino acid sequence, to assign the individual chiralities to the three *allo*-Thr and to the five Hpg and to identify the amino acid linked to the fatty acid moiety.

The operating procedure is shown in Scheme 3. From the ethereal extract a residue was obtained which was hydrogenated with PtO_2 to give (^1H NMR, GC-MS) the amide of 7-methyloctanoic acid

Table 6. Amino acid sequence of peptide fragments.

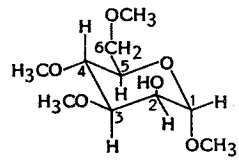
NMR sequence	Fragments									Ethereal extract	Chiralities
	I	II	III	IV	V	VI	VII	VIII	IX		
MOA										⌉	
1 Asp										⌋	L
2 OH-Asp		⌈									L
3 Hpg		⌋									D
4 Orn		⌊									D
5 *allo*-Thr	⌈						⌈				D
6 Hpg	⌋						⌋				L or D
7 Hpg							⌊				D or L
8 *allo*-Thr			⌈					⌈	⌈		L
9 Phe			⌋					⌋	⌋		L
10 Orn								⌊	⌊		D
11 Hpg									⌊		L
12 *allo*-Thr	⌈			⌈							D
13 Hpg	⌋			⌋							L
14 Gly				⌊							
15 Leu						⌈					L
16 Ala					⌈	⌋					D
17 Chp					⌊	⌊					L

MOA: 7-Methylocta-2,4-dienoic acid (factor A2).

with aspartic acid, which, by further hydrolytic treatment, gave aspartic acid. From the aqueous layer nine diagnostic fragments (I~IX) were obtained. Amino acid analysis and successive manual Edman degradation established the sequences shown in Table 6. Fragments containing *allo*-Thr and Hpg were examined for their chiralities as previously described (GC-MS). The results did not allow the assignment of the chiralities of the Hpg units in positions 6 and 7.

Corresponding results were obtained from factors A1 and A3. ^{1}H NMR studies[8] were in agreement with these data and confirmed that the core peptide was identical for the three factors.

Table 7. ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125.76 MHz, $CDCl_3$) NMR data of methyl 3,4,6-trimethylmannopyranoside (δ; J in Hz).

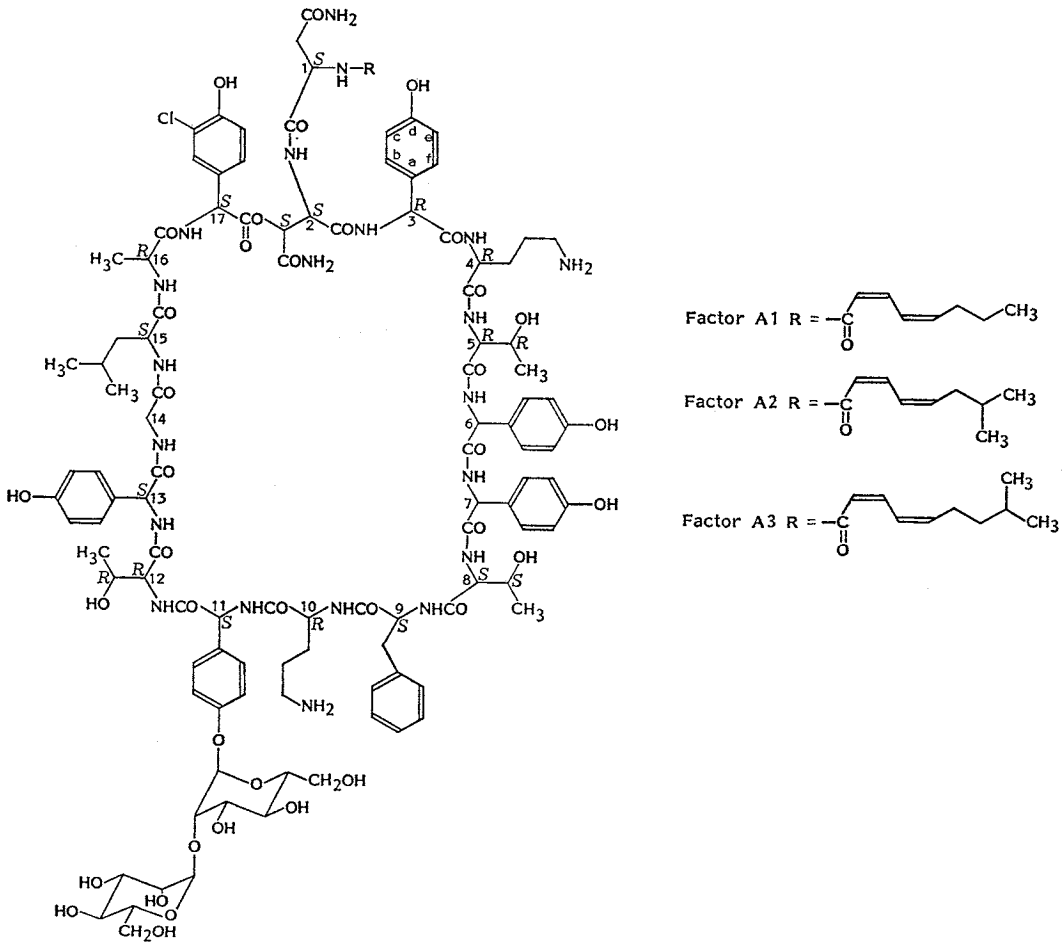


Position	^{1}H	^{13}C
1	4.78 d $^3J_{1,2}$=1.7	100.4
2	4.01 dd $^3J_{2,3}$=3.3	67.6
3	3.46 dd $^3J_{3,4}$=9	81.5
4	3.42 d $^3J_{4,5}$=9	75.9
5	3.58 m	70.6
6	3.61 br s	71.5
1-OCH_3	3.37 s	54.9
3-OCH_3	3.42 s	57.6
4-OCH_3	3.48 s	59.2
6-OCH_3	3.52 s	60.6

Asparagine and β-Hydroxyasparagine

Both Asp and OH-Asp were initially assumed to have the carboxylic groups free, but later it was demonstrated that they are present as primary amides. In fact, while the acid-base ionization data previously discussed do not show free carboxylic groups, two equivalent of ammonia per mol of each factor are titrated in the hydrolysates. In addition, ^{15}N NMR confirmed the total number of N atoms in the molecule as 21 and showed that the two terminal NH_2 of the Orn units appear at −346.8 ppm and the remaining 19 N atoms in the interval from −261.3 to −285.5 ppm. These latter N atoms exceed by two the number calculated

Fig. 4. Structures of A-16686.



from the amino acids.

Structure and Position of the Sugar Moiety

A-16686 was permethylated by the HAKOMORI[12] method and then methanolized. The crude reactants was submitted to silica gel flash chromatography obtaining two sugars, *i.e.*, permethylated D-mannose and tetramethyl-D-mannose. The position of the free OH of this latter was established by ^{1}H and ^{13}C NMR spectroscopy (Table 7). This is consistent with the presence of the dimannosyl moiety suggested by FAB-MS data.

Except for the ^{13}C-resonance of C-2 the carbons C-1, C-3, C-4, and C-6 showed a $^{3}J_{CH}$ long range coupling from four attached methoxy groups, as demonstrated by a COLOC-experiment (CH-correlation by long range coupling).[18] Therefore the structure of methyl 3,4,6-trimethylmannopyranoside was attributed to the first sugar of the dimannosyl unit.

^{1}H NMR spectra of each factor show the two anomeric protons of the dimannose at 5.02 and 5.70 ppm (*J ca.* 2 Hz), thus indicating an α configuration. The two anomeric carbons of the dimannosyl unit are clearly distinguished in the ^{13}C NMR spectrum of single factors (δ 98.6 and 103.7). The position of the attachement to the peptidic part of the molecule was established by NOE spec-

troscopy correlation experiments on single factors.[8,14] Through-space interactions were shown between the anomeric proton of the dimannose unit at δ 5.70 and the aromatic *ortho* and *para* protons (δ 7.34 and 6.95) of the Hpg unit at 11 (Fig. 4).

Conclusions

All the experiments presented above and the ^{1}H NMR studies described in the accompanying paper[8] reveal that antibiotic A-16686 is formed by three factors which have a common cyclic depsipeptide skeleton composed by seventeen amino acids and a dimannosyl unit bound to the hydroxyl of Hpg unit at 11. Three different diunsaturated fatty acid residues acylating the free amino group of the extracyclic asparagine differentiate the three components of the complex (Fig. 4).

Up to now very few glycolipodepsipeptide antibiotics have been isolated: Pantomycin[15] where an undefined carbohydrate moiety seems to be incorporated into a stendomycin-like lipodepsipeptidic molecule, and herbicolin A.[16]

Experimental

^{1}H NMR spectra were recorded on Bruker AM 250 or AM 500 spectrometer equipped with an Aspect 3000 computer at 40°C unless otherwise stated (internal standard TMS, δ=0.00). ^{13}C NMR spectra were recorded on the Bruker AM 500 spectrometer at 125.76 MHz at 40°C and at room temperature. ^{15}N NMR spectra were recorded on the Bruker AM 500 spectrometer at 50.69 MHz in H_2O - DMSO (1 : 4) solution at 60°C.

FAB-MS were obtained in positive ion mode, using glycerol added with a trace of HCl as a matrix, with a Kratos MS 50 instrument and a Xenon beam energy of 8 KeV.

GC-MS was run with a Hewlett-Packard model 5985B instrument under electron impact (EI) at 70 eV, ion source 200°C. Mass spectra were automatically taken at 1 second intervals.

Separation of A-16686 Factors A1, A2 and A3

The A-16686 complex was obtained as previously described.[1] The separation of the single factors was carried out on a Lichrosorb RP 18, 10 μm (Merck, Art 9334), packed in a 250×50-mm stainless-steel column connected to a Waters pump model 590 and an UV detector Waters LC Lambda-Max model 481. Before the injection the column was equilibrated with 1 liter of buffer (0.05 M $HCOONH_4$ - CH_3CN, 64 : 36) at the flow rate of 30 ml/minute. The sample (5 ml, 100 mg/ml in H_2O) was introduced by using a Model 7010 injection valve (Rheodyne) with laboratory-made 5 ml sample loop. The column was eluted with the same mobile phase and flow rate as above and the fractions were monitored at λ=285 nm. Homogeneous fractions were collected, desolventized and concentrated to dryness under vacuum by adding BuOH to avoid foaming. The solid residue was suspended several times in abs EtOH to remove $HCOONH_4$. When all the $HCOONH_4$ was removed (negative Nessler test) the solid residue was dissolved in 0.001 N HCl and freeze-dried. Starting from 500 mg of A-16686 complex 50 mg of component A1, 300 mg of component A2 and 27 mg of component A3 were obtained.

Determination of Amino Acid Composition

A-16686 (1.5 g) was dissolved in 6 N HCl (150 ml) and treated at 105°C for 20 hours in a sealed tube. The acidic solution was extracted with Et_2O and concentrated under vacuum.

The residue was chromatographed on a column of Dowex 50W-X12 (H^+) (54×2 cm) eluting with 0.5 N HCl (10 liters), 0.75 N HCl (1.6 liters), 1 N HCl (1 liter), and 2 N HCl (1.5 liters) with an elution speed of 50 ml/hour. Fractions of 12 ml each were collected and checked for homogeneity and purity on cellulose HPTLC by using as solvents S_1: BuOH - AcOH - H_2O (4 : 1 : 5, upper phase) and S_2: pyridine - H_2O (4 : 1). From the column were obtained in the order: OH-Asp (fractions 25~31), Asp (59~68), Asp and *allo*-Thr (69~72), *allo*-Thr (73~83), Gly (123~127), Gly and Ala (128~139), Ala (140~150), Hpg (325~334), Hpg and Leu (335~485), Phe and Chp (786~1,109), and Orn

(1,110~1,145). The structure of the already identified amino acids Asp, Gly, Ala, Hpg, Leu, Phe and Orn was confirmed by bidimensional cellulose HPTLC (elution first with S_1 and S_2 after) and ^1H NMR in comparison with authentic samples.

From fractions 786~1,109 Chp was isolated after another ion exchange chromatography on Dowex 50W-X4 (53×1.2 cm) eluting with a linear gradient developed from 0.5 N HCl (1 liter) to 1 N HCl (1 liter) and elution speed of 25 ml/hour. Fractions containing Chp were concentrated under vacuum. The residue was dissolved in water, pH adjusted at 4 with NH_4OH and the solution concentrated again. The residue was crystallized from H_2O to give white crystals of pure Chp: MP 228°C (230°C, see later), literature 221~222°C,[17] 217°C,[18] 217~218°C.[19] ^1H NMR and mass spectrum were identical with those of an authentic sample of Chp prepared by ourselves for this purpose.

The amino acids OH-Asp and *allo*-Thr were obtained as white solids from fractions 25~31 and 73~83, respectively. Their structures were identified by NMR and mass spectra which were identical with those of commercially available authentic samples of OH-Asp and D- and L-*allo*-Thr

Synthesis of 3-Chloro-4-hydroxyphenylglycine (Chp)

To a cooled (0°C) suspension of 2-benzoylamino-2-hydroxyacetic acid (3.9 g, 0.02 mol) in a mixture of 97% H_2SO_4 (2 ml) and AcOH (18 ml) 2-chlorophenol (6.04 g, 5 ml, 0.05 mol) was added with magnetic stirring. Stirring was continued for 48 hours and the mixture was poured into ice (100 g) and solid $NaHCO_3$ (40 g) and extracted with EtOAc. The aqueous phase was acidified with conc HCl and extracted again with EtOAc. The EtOAc solution was washed with brine, dried over $MgSO_4$, filtered and evaporated under vacuum, The residue was crystallized from EtOAc - petroleum ether to give white crystals (3.9 g, 64%) of pure *N*-benzoyl-3-chloro-4-hydroxyphenylglycine: MP 191°C.

Anal Calcd for $C_{15}H_{12}ClNO_4$: C 58.93, H 3.96, N 4.58, Cl 11.59.
Found: C 58.59, H 3.94, N 4.59, Cl 11.85.

N-Benzoyl-3-chloro-4-hydroxyphenylglycine (3.8 g, 0.012 mol) was refluxed for 6 hours with conc HCl (250 ml) with mechanical stirring. The acidic phase was extracted with Et_2O, concentrated under vacuum (20 ml) and the pH was adjusted at 4 with conc NH_4OH. A white solid precipitated that was collected and dried (2.18 g, 87.2%), mp 221~222°C. 0.2 g of this solid was crystallized from H_2O (70 ml), filtered and dried at 70°C for 5 hours under vacuum to give white crystals of pure Chp: MP 230°C; ^1H NMR (250 MHz, DMSO - CF_3COOD) δ 7.60 (Hb, J_{meta}=2.2 Hz), 7.33 (Hf), 7.11 (He, J_{ortho}=7.6 Hz), 5.07 (s, CH).

Anal Calcd for $C_8H_8ClNO_3$: C 47.66, H 4.00, N 6.95, Cl 17.58.
Found: C 47.52, H 4.01, N 6.93, Cl 17.62.

Determination of the Chiralities of the Amino Acids

A-16686 factor A2 (20 mg) was hydrolyzed with 3 ml of 6 N HCl at 105°C for 20 hours. The reaction mixture was washed with EtOAc and evaporated to dryness under vacuum. The residue was dissolved in a few milliliters of water then evaporated again for three times, and finally lyophilized. The solid residue was treated in sealed ampoules with PrOH - dry HCl at 90°C for 1 hour. After cooling the solvents were evaporated with an helium stream at 50°C. Pentafluoropropionic anhydride was added to the residue dissolved in CH_2Cl_2, and the reaction mixture was left at room temperature for 1 hour. The excess of the reactant and solvents were eliminated with helium as above, and the residue was dissolved in a few milliliters of CH_2Cl_2 and injected in the GC-MS apparatus using a capillary column CGC Analytic Labor, OS 6411 (25 m×0.2 mm) at 80°C for 4 minutes, then to 140°C at 4°C/minute. Carrier gas: Helium 1.05 kg/cm². Factors A1 and A3 were treated in the same manner.

Characterization of Lactone Bond

Reduction of A-16686 with $Ca(BH_4)_2$: To a cooled (0~5°C) solution of $NaBH_4$ (1.5 g) in abs EtOH (150 ml) finely ground $CaCl_2$ (2.5 g) was added with magnetic stirring. After 1.5 hours A-16686 (1 g) dissolved in dry DMF (60 ml) was added dropwise. The cooled reaction mixture was stirred for 24 hours then cautiously poured into water (600 ml). The pH was adjusted at 5 with dil HCl and the solution was desalted on a XAD-2 column by eluting first with H_2O to remove salts and then with the mixture 0.01 N HCl - CH_3CN (1:1) to recover the reduced peptide. Fractions containing the

peptide were combined and concentrated to dryness under vacuum by adding BuOH to avoid foaming.

The residue was hydrolyzed with 6 N HCl at 105°C for 20 hours. The acidic solution after extraction (EtOAc) was concentrated under vacuum. Bidimensional cellulose HPTLC on the residue [first run BuOH - AcOH - H_2O (4 : 1 : 5, upper layer), second run pyridine - H_2O (4 : 1); spots of amino acids located by spraying with ninhydrin and heating at 120°C for 5 minutes] showed the lack of the spot corresponding to Chp and the appearance of a spot with the highest Rf not present in the acidic hydrolysate obtained from parent A-16686. The residue was chromatographed on a Dowex 50W-X4 column by eluting with 0.05 N HCl. The fractions containing the wanted compound were combined and concentrated. The oily residue was purified again by preparative layer chromatography [SiO_2, 5 mm thick, BuOH - AcOH - H_2O (4 : 1 : 5, upper layer)] to obtain 2-amino-2-(3-chloro-4-hydroxyphenyl)ethyl alcohol. ^1H NMR (250 MHz, DMSO-d_6) δ 3.45 (m, CH, partly covered by the water signal), 3.83 (m, CH_2), 6.92 (d, CH-5), 7.11 (dd, ^3J=8.8 Hz, CH-6), 7.32 (d, ^4J=2.5 Hz, CH-2). To prepare a sample for comparison Chp (30 mg) was converted to the related methyl ester (MeOH, HCl) and reduced with $Ca(BH_4)_2$ following the procedure reported above. The amino alcohol so obtained displayed TLC, NMR and MS spectra identical to those of the product obtained from A-16686.

Preparation of A-16686-acid

A solution of A-16686 (1 g) in 0.1 N NaOH (10 ml) was left at room temperature for 0.5 hour, then 0.1 N HCl (10 ml) was added with stirring. The precipitate was filtered and dried at 40°C under vacuum to give 0.84 g of the title compound.

Quantitative Amino Acid Determination of A-16686 and A-16686-acid

Phenylisocyanate Derivatives after Hydrolysis: A-16686 (100 mg) and A-16686-acid (100 mg) were separately dissolved in DMF (2 ml). To each solution phenylisocyanate (300 μl) was added. Reaction mixtures were left at room temperature for 48 hours, then quenched by adding H_2O (20 ml) and filtered. The solid precipitates were collected and hydrolyzed to the amino acid components in the PICO-TAG Work Station (Waters) by heating peptides (0.5~1 mg) with 6 N HCl at 105°C for 20 hours. Acidic hydrolysates were concentrated to dryness under vacuum in the same work station. The dry peptide hydrolysates were dissolved in 200 μl of a mixture CH_3CN - H_2O (1 : 1) and derivatized with *o*-phthalaldehyde (OPA)[20~22] solution† (25 μl) and after 1 minute the mixtures were injected in the HPLC instrument.

HPLC Conditions: Column: Ultrasphere ODS (Beckman) 5 μm (250×4.6 mm), detector: Fluorescence Spectrophotometer 650-10S (Perkin-Elmer), excitation at 360 nm and emission measured at 455 nm, eluent: solvent A was 0.05 M AcONa - MeOH - THF (80 : 19 : 1) (pH 6.8), solvent B was 0.05 M AcONa - MeOH (20 : 80) (pH 6.8), gradient program (minutes) %B: (0) 0, (5) 0, (11) 40, (21) 40, (34) 100, (38) 100, (40) 0, flow rate: 1 ml/minute, loop 10 μl.

Fatty Acids Determination

A solution of A-16686 (2.5 g) in 0.05 N HCl (150 ml) was heated at reflux for 30 hours, then the reaction mixture was extracted with Et_2O. The ethereal extract was evaporated and the residue (100 mg) was divided in two portions. One was treated with BSTFA and analyzed by GC-MS under the conditions described later on. The second one was dissolved in EtOAc and hydrogenated over PtO_2 at room temperature and atmospheric pressure. The mixture was filtered to remove the catalyst and the filtrate was concentrated to dryness. The residue was derivatized with BSTFA and examined by GC-MS in comparison with octanoic acid and decanoid acid.

About 0.5 mg of each fatty acids mixture was added to 0.2 ml of anhydrous pyridine and 0.2 ml of BSTFA in a sealed vial heated at 80°C for 15 minutes. After cooling 1 μl of the reaction mixture was injected into a Hewlett-Packard model 5985B, using a capillary column H.P. (25 m×0.2 mm) made of fused silica wall-coated with phenyl-methyl silicon fluid, and a programmed temperature rise 60~250°C at 10°C/minute. Flow rate: helium, 1.5 ml/minute, varied according to the needs

† OPA (500 mg) was dissolved in a mixture of MeOH (10 ml) and 2-mercaptoethanol (0.4 ml). This solution was diluted to 100 ml with a buffer solution of 0.2 M H_3BO_3 at pH 9.5 (pH was adjusted with 4 N NaOH).

of detection sensitivity. Injection port temperature 250°C. Retention times of the three main peaks before and after hydrogenation: 4.9, 5.6, 6.7 minutes and 4.4, 5.2, 6.2 minutes.

Amino Acid Sequence Determination

a) *N*-7-Methyloctanoyl Aspartic Acid: A degased solution of A-16686 factor A2 (0.82 g) in conc HCl (350 ml) was maintained at 37°C for 72 hours under argon atmosphere, diluted with water while cooling and extracted (Et_2O). The ethereal extract was dried (Na_2SO_4) and evaporated to yield an oily residue (40 mg). This was dissolved in EtOAc (10 ml), PtO_2 (20 mg) was added and the mixture was submitted to hydrogenation at room temperature and atmospheric pressure. At the end the catalyst was removed by filtration and the filtrate was concentrated to dryness under vacuum to give a residue (20 mg) of *N*-isononanoyl aspartic acid: ^1H NMR (250 MHz, $CDCl_3$) δ 7.97 (1H, d, NH), 4.51 (1H, m, CHα), 2.63 and 2.51 (2H, dd, $CH_2\beta$), 2.08 (2H, t, CH_2-2), 1.47 (3H, m, CH_2-3 and CH-7), 1.07~1.04 (6H, m, CH_2-4,5,6), 0.84 (6H, d, two CH_3).

A portion of the compound was submitted to further hydrolysis with 6 N HCl (1 ml) at 105°C for 20 hours. After dilution with water (5 ml) the reaction mixture was extracted with pentane. The aqueous layer was evaporated to dryness, taken up in water and freeze-dried. A parallel run on TLC cellulose (BuOH - pyridine - AcOH - H_2O, 15 : 10 : 3 : 12) with an authentic sample of Asp gave identical Rf 0.22. The identification with Asp was also confirmed by HPLC after derivatization with OPA.

b) Separation of Peptide Fragments: The aqueous phase was evaporated under vacuum at 40°C, dissolved in 0.1 M pyridine - AcOH buffer (pH 4.7) and chromatographed on a Dowex 50W-X2 column (50×1.5 cm) at 37°C with a flow rate of 60 ml/hour. About 500 ml of the following buffers were successively passed through the column: 0.1 M, 0.02 M, and 0.4 M pyridine - AcOH (pH 4.7) then 1.07 M, and 2.23 M pyridine - AcOH (pH 5.4). Fractions of 5.5 ml were collected and monitored (every fourth test tube) spectrophotometrically (570 nm) after adding ninhydrin and heating at 110°C for 10 minutes. Fractions containing the same peptides (TLC-cellulose, BuOH - pyridine - AcOH - H_2O, 15 : 10 : 3 : 12) were pooled, concentrated under vacuum and freeze-dried. Further purification was done on a Biogel P2 (Bio-Rad) column (45×1.8 cm) eluted with 10% AcOH if necessary. The composition of each peptide fragment was determined after acid hydrolysis by OPA amino acid analysis as described previously.

c) Edman Degradation of Peptide Fragments: The amino acid sequence of each peptide was established by manual Edman degradation.[23~25] A typical run is reported: The pure peptide (1~3 μmol) was put into a 50×5-mm test tube and submitted to coupling with freshly prepared mixture (25 μl) of EtOH - H_2O - triethylamine - phenylisothiocyanate (7 : 1 : 1 : 1). The test tube was put in the reactor of a PICO-TAG work station, the air was substituted with nitrogen and the sample was heated at 50°C for 0.5 hour. The solvent was evaporated under vacuum (0.05~0.1 mmHg) and H_2O (25 μl) was added. The resulting suspension was washed 3 times with heptane - EtOAc (10 : 1) and 4 times with heptane - EtOAc (1 : 1). The organic phases, containing excess reagent, were eliminated with the aid of a syringe and the aqueous phase was taken to dryness. The resulting phenylthiocarbamylpeptide was cleaved with conc HCl (about 15 μl) at room temperature for 5 minutes and again taken to dryness. The so formed thiazolinone of the first amino acid was suspended in 0.01 N HCl (25 μl) and extracted 4 times with Et_2O. The aqueous phase was submitted to the second degradation step. The ethereal extracts were taken to dryness. The residue was dissolved in CH_3CN and injected into the HPLC apparatus. The assignments of PTH amino acids was done in comparison with PTH standard of the same amino acid.

Eluent: Solvent A: 0.01 N CH_3COONa (pH 4.5), solvent B: CH_3CN, gradient program (minutes) %B: (0)24, (6)37, (30)37, (31)24, flow rate: 1 ml/minute, temperature 62°C, detector; UV 254 nm.

d) Configuration of the Amino Acids: Peptide fragments containing *allo*-Thr and Hpg (Table 6) were examined as previously described.

Permethylation and Methanolysis of A-16686

A stirred mixture of 55% NaH in oil dispersion (21.8 g) and dry DMSO (150 ml) under N_2 atmosphere was heated at 60°C until evolution of H_2 ceased (about 2 hours). The solution was cooled at

room temperature and a solution of A-16686 (dried for 3 days in a dessicator over P_2O_5) (5 g) in dry DMSO (125 ml) was added along with CH_3I (200 ml) during 40 minutes. After 4 hours at room temperature H_2O (300 ml) and $CHCl_3$ (300 ml) were added to give a two-phase system. The $CHCl_3$ layer was separated and washed with an equal volume of 5% $Na_2S_2O_3$. The washed $CHCl_3$ layer was dried over $MgSO_4$, filtered and concentrated under vacuum. The oily residue was dissolved in MeOH (150 ml) containing 10% HCl and refluxed for 20 hours. The acidic solution was neutralized by adding solid $NaHCO_3$, NaCl was filtered off and the clear solution was concentrated under vacuum. The oily residue showed to contain two compounds (TLC, SiO_2, hexane - Me_2CO (3 : 7)) having Rf 0.65 and 0.33. The two compounds were separated by flash chromatography on SiO_2 by eluting with a mixture hexane - Me_2CO (95 : 5). The compound having Rf 0.65 resulted to be methyl 2,3,4,6-tetramethylmannopyranoside: ^1H NMR (250 MHz, $CDCl_3$) δ 4.79 anomeric proton, 3.52, 3.49, 3.47, 3.41 and 3.38 (5 CH_3); MS M^+ absent, fragmentation in agreement with literature data.[26] The compound having Rf 0.33 resulted to be methyl 3,4,6-trimethylmannopyranoside. ^1H NMR see Table 7; MS M^+ absent, fragmentation in agreement with literature data.[27]

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