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MOENOMYCIN A:
A STRUCTURAL REVISION AND NEW STRUCTURE-ACTIVITY RELATIONS

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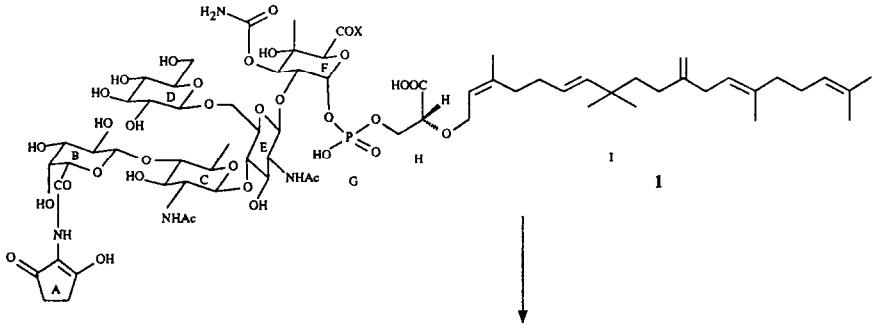
Abstract - A detailed FAB MS analysis combined with NMR and chemical results requires the structure of moenomycin A to be revised from **1a** to **1b**. New biochemical results seem to support the assumption that in the region F-G-H of **1b** the structural requirements for antibiotic activity are rather strict

Introduction

Moenomycin A and related antibiotics interfere with the biosynthesis of peptidoglycan, the main structural component of the bacterial cell wall With cell-free systems from Escherichia coli it was demonstrated that moenomycin A inhibits the formation of the linear glycan strands of peptidoglycan from the last membrane precursor by its inhibitory effect on the enzyme penicillin-binding protein 1b (PBP 1b) [1] On the basis of degradation and spectroscopic studies structure **1a** was proposed for moenomycin A [2] New spectroscopic and chemical results as detailed below require the structure of moenomycin A to be revised from **1a** to **1b**, i e unit F contains a carboxamide function rather than a free carboxylic acid group. The same structural change is necessary for compounds **2-8** and **12**, which have been prepared from **1b** in order to define the minimum structural requirements for the antibiotic activity of moenomycin A (Scheme 1) [3]

Mass Spectra of Compounds **1b**, **2b**, **8b**, **7b**, **12b**

Structure **1a** as originally derived from degradation experiments[2a] was later largely confirmed by ^{13}C NMR spectroscopy and FAB MS [2b] The latter spectra besides apparently proving the molecular mass provided structural informations by a number of fragments formed through cleavage of glycosidic bonds [2b] Recently we noted, however, the rather unexpected formation of "odd-electron molecular ions" from some moenomycin A degradation products, a



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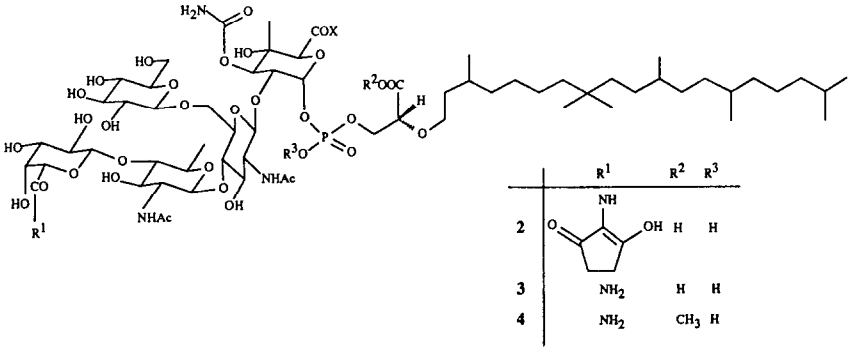
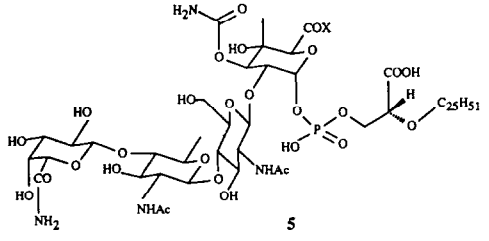


	R^1	R^2	R^3
2		H	H
3	NH_2	H	H
4	NH_2	CH_3	H



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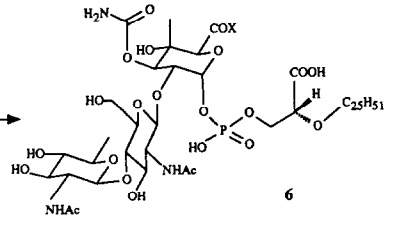

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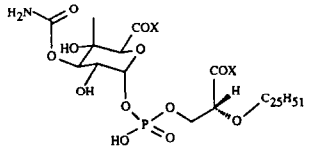

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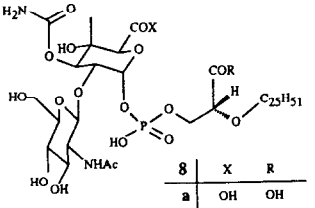


8	X	R
a	OH	OH
b	NH_2	OH
c	NH_2	OCH_3
d	OCH_3	OCH_3

1-7	X
a	OH
b	NH_2

Scheme 1

property that could not be explained [3] With more advanced FAB MS instrumentation available the mass spectral behaviour of moenomycin A and some of its derivatives was, therefore, reinvestigated

1a has the molecular formula $C_{69}H_{107}N_4O_{35}P$ and consequently a mono-isotopic molecular mass of 1582 6 Da The previously reported FAB mass spectra of moenomycin A exhibited parent ion peaks at m/z 1605 and 1621 which were assumed to correspond to the MNa^+ and MK^+ ions, respectively [2b] The instrumentation used at that time allowed only the determination of nominal masses, particularly when dealing with such relatively high molecular weight compounds

Using a modern mass spectrometer/data aquisition system which is capable to perform mass determinations with an accuracy of better than 0 2 Da even under low-resolution conditions, we obtained the FAB mass spectra presented in Figures 1a and 1b These spectra exhibit (slightly depending on the FAB matrix used) a parent ion peak at m/z 1582.7 and/or a series of peaks resulting from the exchange of one or two hydrogen atoms against sodium or potassium (m/z 1604 6 1582 7 +Na-H, 1620 6 1582 7 +K-H, 1626 7 1582 7 +2Na-2H) [4] The occurance of radical cations in FAB MS is extremly rare,[5] and, in our experience, is never observed, when alkali ions are attached [6]

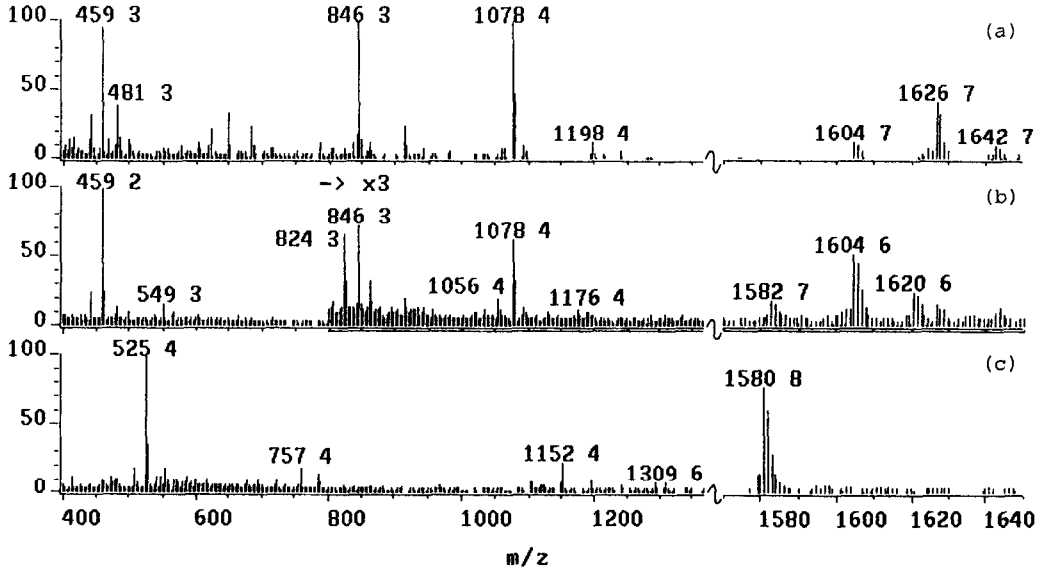
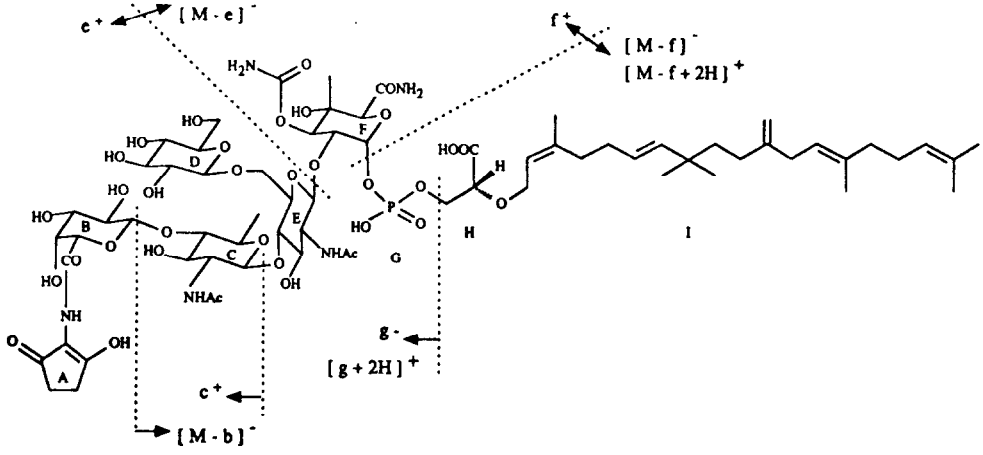


Fig 1 FAB mass spectra of moenomycin A (**1b**) a) matrix 3-nitro benzyl alcohol, positive ions, b) matrix glycerol, positive ions, c) matrix glycerol, negative ions The molecular ion regions are shown with an expanded mass scale



Scheme 2.

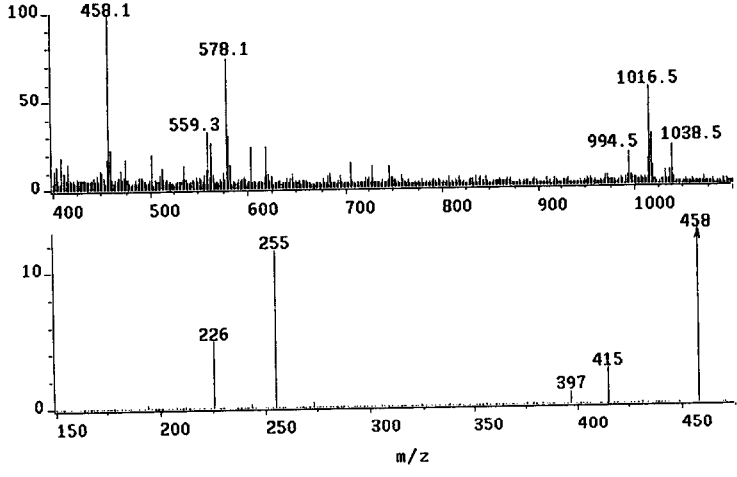
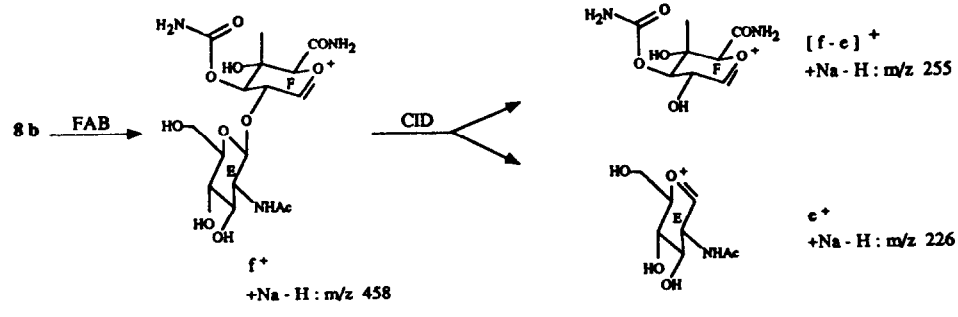


Fig. 2.

a) FAB mass spectrum of **8b**, matrix 3-nitro benzyl alcohol

b) CID spectrum of the fragment ion m/z 458 of **8b**.



Scheme 3.

This means, that the observed parent ion of moenomycin A should represent the protonized molecular ion and that the molecular mass of moenomycin A is in fact 1581 7 (± 0.1) Da, one mass unit less than structure **1a** requires This result is confirmed by the negative ion FAB MS which exhibits the even-electron anion $[M-H]^-$ at m/z 1580.8 (Fig 1c) High-resolution mass measurements then unequivocally demonstrated that the molecular formula of moenomycin A is $C_{69}H_{108}N_5O_{34}P$ (see Table 4), i.e one oxygen atom has to be replaced by an NH group in **1a**.

In order to identify the part of the molecule to be modified, the mass spectrometric fragmentation of moenomycin A (**1**), its decahydro derivative (**2**) and the degradation products **8**[3] and **12**[3] was examined carefully. It was found that all structurally relevant fragment ions can be assigned as summarized in Scheme 2: Cleavage of the glycosidic bonds of the pyranose units B, C, E, and F gives rise to the formation of cations c^+, e^+, f^+, stabilized by the pyranose oxygen and, as counterparts, of the alcoxide ions ($[M-b]^-$, $[M-e]^-$). Cleavage of either phosphoric acid diester bond yields the protonized phosphoric acid monoesters ($[M-f+2H]^+$, $[g+2H]^+$) and the corresponding phosphate anions ($[M-f]^-$, g^-), respectively All fragment ions unambiguously identified in the positive ion MS are summarized in Table 1, most of them appear also as sodium attachment ions This complication is, as expected, absent in the negative ion spectrum of moenomycin A (see Fig.1c. m/z 1309 6 = $[M-b]^-$, 757.4 = $[M-e]^-$, 525.4 = $[M-f]^-$, 1152.4 = g^-)

From the mass difference of fragment ions e^+ and f^+ as well $[M-e]^-$ and $[M-f]^-$, viz 232 Da, follows that subunit F (as neutral molecule) must have an even-numbered mass and, therefore, contains two nitrogen atoms. In keeping with this, monosaccharidic degradation product **7b** has an even-numbered molecular mass ($C_{36}H_{69}N_2O_{13}P$, calc M = 768 5, obs m/z 769 5 = $[M+H]^+$, 791 5 = $[M+Na]^+$). Further confirmation was achieved through "isolation" of unit F of **8b** by an MS/MS experiment (Fig 2a and 2b, Scheme 3) Through collison-induced decomposition (CID) the disaccharide fragment f^+ was cleaved into the two monosaccharide cations e^+ and $[f-e]^+$, the latter corresponding to unit F and again proving its elemental composition

In conclusion, the mass spectrometric data of moenomycin A and its degradation products clearly demonstrate that unit F contains a second nitrogen function, presumably a carboxamide function instead of the carboxylic acid group

Table 1. FAB MS data of moenomycin A, decahydro moenomycin A and degradation products **8b** and **12b** (positive ions, mono-isotopic masses).

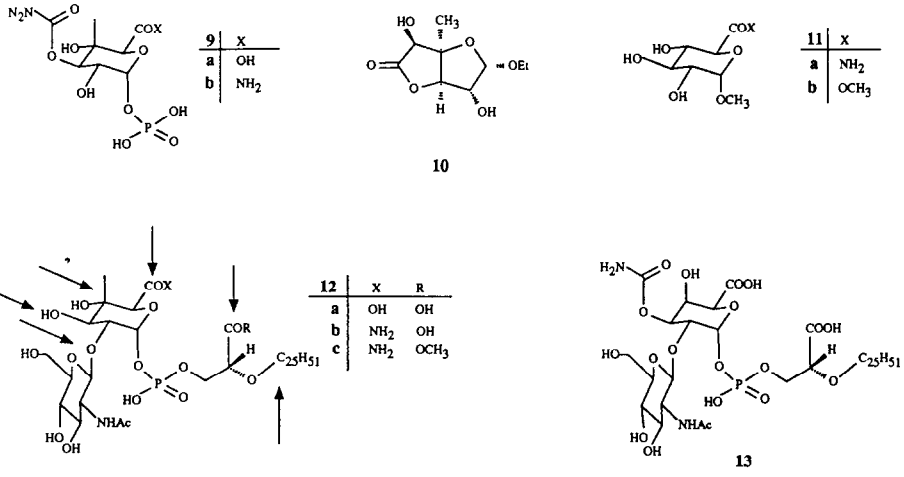
	1b		**2b**	**8b**	**12b**	
molecular formula	$C_{69}H_{108}N_5O_{34}P$		$C_{69}H_{118}N_5O_{34}P$	$C_{44}H_{82}N_3O_{18}P$	$C_{43}H_{81}N_2O_{17}P$	
M calculated	1581.7		1591.8	971.5	928.5	
matrix used	NBA	glycerol	NBA	NBA	NBA	glycerol
molecular ions:						
$[M+H]^+$		1582.7				929.5
$[M+Na]^+$	1604.7	1604.6	1614.8	994.5		951.5
$[M+K]^+$		1620.6				967.5
$[M+2Na-H]^+$	1626.7		1636.7	1016.5	973.7	
$[M+Na+K-H]^+$	1642.7		1652.7			
$[M+3Na-2H]^+$				1038.5		
fragment ions:						
c^+	459.3	459.2	459.3			
$[c+Na-H]^+$	481.3		481.3			
e^+		824.3				
$[e+Na-H]^+$	846.3	846.3	846.3			
f^+		1056.4				393.2
$[f+Na-H]^+$	1078.4	1078.4	1078.4	458.1	415.2	
$[M-f+Na+H]^+$		549.3	559.5	559.3		
$[g+Na+H]^+$		1176.4				
$[g+2Na]^+$	1198.4		1198.4	578.1		

The Structure of Unit F in Moenomycin A

Structural assignment of unit F rests mainly on degradation products **9** and **10** isolated after acid cleavage of moenomycin A.[7,8] Constitution as well as relative and absolute configuration of **10** have unambiguously been confirmed by synthesis.[9] In the ^{13}C and ^1H NMR spectra of **9** the positions of both the carbamoyl and the phosphate groups were clearly visible. The peak at m/z = 331 in the field desorption mass spectrum was taken as the molecular ion peak and, consequently, the compound was assigned structure **9a**.[5] Reexamining compound **9** by FAB MS showed in the molecular ion region peaks at m/z 331, 353 and 375 which correspond to $[M+H]^+$, $[M+Na]^+$, and $[M+2Na-H]^+$, respectively. From this result it follows that compound **9** has the elemental composition $C_8H_{15}N_2O_{10}P$ rather than $C_8H_{14}NO_{11}P$ as originally assumed, in agreement with structure **9b**. Loss of phosphoric acid accounts for the oxonium ion peak at m/z 233 which is accompanied by m/z 255 (233+Na-H).

Fig.3a shows the low field region of the ^1H NMR spectrum of **9b** in 4:1 $[D_6]$DMSO-H_2O solution comprising the 1-H signal at δ = 5.4,[10] a broad two-

proton multiplet around δ = 6 4, and two one-proton signals around 7 4 When measured in [D6]DMSO-D2O solution (Fig 3b) the same part of the spectrum exhibits only the 1-H signal, definitely confirming the presence of two NH_2 groups in **9b** and hence in compounds **1b-8b**, and **12b** By comparison with the spectrum of reference compound **11a** (see Experimental) the signals around δ = 7 4 in the spectrum of **9b** may be attributed to the uronamide function



Scheme 4

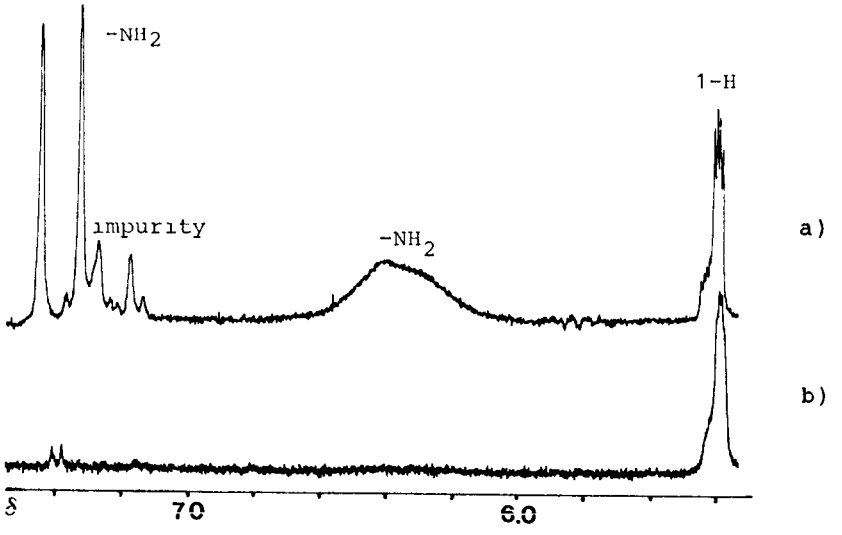


Fig. 3. ^1H NMR spectrum (low field region) of **9b**
a) in [D6]DMSO-H2O 4 1, b) in [D6]DMSO-D2O 4 1

In order to obtain further insight into the structural requirements for the antibiotic activity, especially with respect to unit F, we wished to convert **8b** (the smallest fully active degradation product of **1b**[3]) into **8a** There exists, of course, a great number of methods for this task We used an indirect approach based on the observation that treatment with Dowex 50 resin (H^+ form) in methanol solution (2d at 20°C) cleanly converted methyl α-D-glucopyranosiduronamide (**11a**) into the ester **11b** (99% yield) Subjecting **8b** to the same conditions led to the formation of monoester **8c** (quantitative yield), the structural assignment of which rests on FAB MS results (see Experimental). In the same way the decarbamoyl product **12b** was esterified to give **12c**. Further treatment of **8c** with Dowex 50 resin in methanol at 50°C under optimized conditions (in order to avoid undesired decomposition) provided diester **8d** in 85% yield Treatment of **8d** with LiOH in methanol then gave the desired dicarboxylic acid **8a** (80%) that has the structure originally assigned to the disaccharidic degradation product of moenomycin A [3]

In the FAB mass spectrum of **8a** (Fig 4) besides $[M+Na]^+$ = m/z 995 6 and $[M+2Na-H]^+$ 1017 6, the major fragment ions are $[M-f+Na+H]^+$ = m/z 559 5 and $[f+Na-H]^+$ = m/z 459 2 The latter ion undergoes collisional-induced decomposition to give the monosaccharide ions $[f-e+Na-H]^+$ = m/z 256 and $[e+Na-H]^+$ = m/z 226 (cf Scheme 3) If compared with **8b**, striking differences are obvious with respect to the relative abundances and the appearence of additional CID fragment ions (m/z 354 = 459 - H_2NCO_2H - CO_2, m/z 324 = 459 - H_2NCO_2H - $OCHCO_2H$) Quite similar mass spectra were, however, obtained from the dicarboxylic acid **13**, the synthesis of which was recently reported [11]

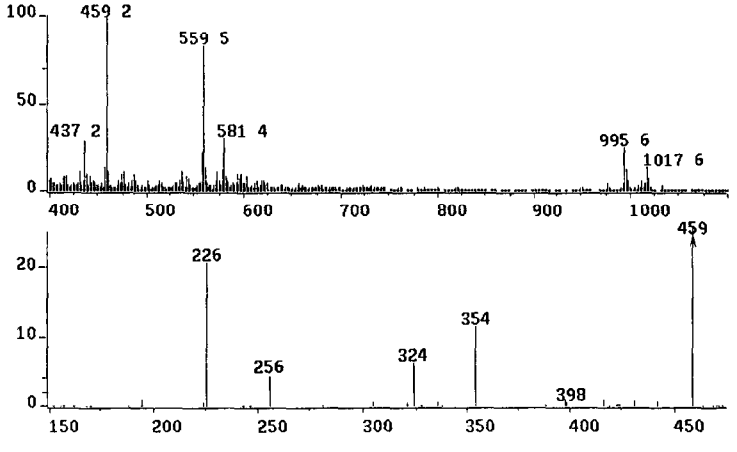


Fig 4

a) FAB mass spectrum of **8a**, matrix 3-nitro benzyl alcohol

b) CID spectrum of the fragment ion m/z 459 of **8a**

A final comment concerns compound **3b**. After treatment of **3b** with diazomethane two products were isolated.[2b] One was a monomethyl derivative with the glyceric acid unit esterified,[2b] which now must be assigned structure **4b**.[12] The second compound was believed to be a triester of **3a**. This assignemt is necessarily incorrect, and the product was probably a mixture of two stereoisomeric diesters (diastereomeric at P) of **3b**

Biological Activity of Compounds **8a**, **8b**, **8c**, **7b**, and **12b**

It is very interesting to compare the inhibitory effect of the new compounds in the *in-vitro* assay for the transglycosylation reaction with previous results (see Table 2) Degradation product **8b** may serve as the reference compound, it is as active as moenomycin A [3] Previously, we reported, that removal of unit E from **8b** (**8b**-->**7b**), as well as cleaving off the carbamoyl group (**8b**-->**12b**) leads to less efficient inhibitors [3] The same decrease in inhibitory efficiency is now found on replacement of the primary amide function by the free carboxylic acid group (**8b**-->**8a**) A free acid function in the glyceric acid unit seems to be of paramount importance for the inhibition of the transglycosylase reaction of these moenomycin A analogues, since methyl ester **8c** is devoid of any activity at least at 10 µg/ml, confirming the report that methyl **4b** is antibiotically inactive [2b]

Table 2 Effect of some moenomycin A degradation products on the in vitro formation of peptidoglycan by transglycosylation

Product	Final concentration (µg/ml)	% Inhibition
8b[3]	10	100
	1	100
	0 1	45
7b[3]	100	100
	10	100
	1	53
12b[3]	100	100
	10	56
	1	0
8c[a]	10	0
8a[a]	10	43
	1	26
	0 1	0

[a] Assays were carried out as described previously [13]

From these results one may conclude, that a large part of moenomycin A (units A, B, C, D) is unimportant for the inhibition of the transglycosyla-

ting enzyme whereas especially in the region F-G-H the structural requirements for antibiotic activity seem to be very strict (see arrows in formula **12**) Work is now in progress to determine the effect of structural variations at C-4 of the uronic acid unit F on the biological activity (cf ref [11])

For comparison, in Table 3 the MIC values of some moenomycin A degradation products are listed. It is more difficult to find a clear relation between structure and enzyme inhibition, since most probably penetration ability also plays a major rôle. But again it is obvious that esterification of the glyceric acid unit destroys the antibiotic activity (see **8c** in Table 3)

Table 3 Minimal inhibitory concentrations (in μg/ml) of some moenomycin A degradation products against various test organisms.

	Staph aureus 503	Strept. pyogenes A 308	Strept pyogenes A77	Pseud aeruginosa 1771m	E coli DC2
8b[3]	12.50	12.50	1 56	12.50	50
7b[3]	>50	6.25	3.13	>50	>50
12b[3]	>50	>50	12 50	>50	>50
8c	>100	12 5	12 5	>100	>100
8a[a]	>100	25	6 25	>100	>100

[a]Determined as described previously [3]

Experimental

Instrumentation and materials used ^{1}H and ^{13}C NMR AM 400 (Bruker), medium pressure liquid chromatography (MPLC). 20 0 cm X 1 5 cm glass tubes filled with 9 g SiO_2 (Grace, 35-70 μm) and a Duramat pump, normal column chromatography (SC) SiO_2 (ICN Silica 63-100) The ion exchange resin used was Dowex 50W X 10, 20/50 mesh (Fluka), H^+ form, carefully washed with methanol

Mass spectrometry
Instrument: VG Analytical ZAB2-SEQ (BEQQ confguration) with data system 11-250J, equipped with the standard VG FAB ion source and a caesium FAB gun operated at 35 kV - Low-resolution FAB MS 1 μl of a sample solution in methanol or dimethyl sulfoxide was added to 2 μl of glycerol or 3-nitro benzyl alcohol (NBA) on the FAB target, R P - 3,000, scan speed 30 s/dec, mass calibration with CsI - High-resolution FAB MS A 10% solution of polyethyleneglycol-1540 in NBA was added to the mixture of sample and matrix mixture as internal reference, the proper reference sample ratio was adjusted after the first measurement (if necessary), R P = 10,000, 30 sec linear voltage scan over a mass range of 70 Da, raw data accumulation of 7-9 scans using the VG MCA software, mass determination by software interpolation Mean values of three indepedent measurements are listed in Table 2 - CID MS collisional activation in the third field-free region of the mass spectrometer using argon gas (ca 30 eV), quadrupole scan over a mass range of 2000 Da in 10 sec, raw data accumulation (MCA) of 10-20 scans, mass calibration using CsI

Table 4 High-resolution MS data of **1b**, **8b**, and **8a**.

		[M+H]$^+$		[M+Na]$^+$		[M+2Na-H]$^+$	
	mol formula	calc	obs	calc	obs	calc	obs
1b	$C_{69}H_{108}N_5O_{34}P$	1582 669	1582 667	1604 651	1604 652	1626 633	1626 636
8b	$C_{44}H_{82}N_3O_{18}P$			994 523	994 525	1016 505	1016 505
8a	$C_{44}H_{81}N_2O_{19}P$			995 507	995 509	1017 489	1017 492

Degradation product **9b**
$C_8H_{15}N_2O_{10}P$ (330 2[14a], 330 1[14b]). For spectral data, see text and ref.[8]

11b from 11a
11a[15] (40 mg, 0 193 mmol), and Dowex/H$^+$ resin (3 g) were stirred in dry methanol (5 ml) at 20°C for 2d Filtration, solvent evaporation, and lyophilization provided pure **11b**[16] (40 mg, 99%) - ^{1}H NMR (400 MHz, d_6-DMSO-H_2O 4 1) δ = 3 24 (OCH_3), 4 57 (1-H), 7 16 and 7 61 (NH_2)

2-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-3-O-carbamoyl-1-O-{[(R)-2-methoxycarbonyl-2-(3,8,8,11,14,18-hexamethyl-nonadecyloxy)-ethoxyl-hydroxy-phosphoryl}-4-C-methyl-α-D-glucopyranuronamide (**8c**)
8b (75 5 mg, 0 078 mmol) and Dowex/H$^+$ resin (6 g) were stirred in methanol (10 ml) at 20°C for 2d Filtration, solvent evaporation, and MPLC ($CHCl_3$-CH_3OH-H_2O 18.11 2) provided **8c** (43 2 mg, 56%) - ^{13}C NMR (100 6 MHz, $CDCl_3$-CD_3OD-D_2O 18 11 2 7, DPT) δ = 52 8 (OCH_3), 56 2 (C-2^E), 61 2 (C-6^E), 66 0 (C-3^H), 70 4 (CH), 70 6 (C-1^I), 72 7 (CH), 73 5 (C-4^F), 74.5 (CH), 75 0 (CH), 76 7 (CH), 78 4 (C-2^F), 79 1 (C-2^H), 95 1 C-1^F), 103 2 (C-1^E), 158 6 ($OCONH_2$), 172 3 (CO), 173 8 (CO) - $C_{45}H_{84}N_3O_{18}P$ (986 2[14a], 985 6[14b]), FAB MS (NBA) m/z 1030 5 = [M+2Na-H]$^+$, 595 3 = [M-f+2Na]$^+$, 573 3 = [M-f+Na+H]$^+$, 458 1 = [f+Na-H]$^+$

2-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-1-O-{[(R)-2-methoxycarbonyl-2-(3,8,8,11,18-hexamethyl-nonadecyloxy)-ethoxyl-hydroxy-phosphoryl}-4-C-methyl-α-D-glucopyranuronamide (**12c**)
12c was prepared from **11b** (2 4 mg) as described for **8c** - $C_{44}H_{83}N_2O_{17}P$ (943 1[14a], 942 5[14b]), FAB MS (NBA) m/z 987 5 = [M+2Na-H]$^+$, 595 3 = [M-f+2NA]$^+$, 573 3 = [M-f+Na+H]$^+$, 415.1 = [f+Na-H]$^+$

Methyl 2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3-O-carbamoyl-1-O-{[(R)-2-methoxycarbonyl-2-(3,8,8,14,18-hexamethyl-nonadecyloxy)-ethoxyl-hydroxy-phosphoryl}-4-C-methyl-α-D-glucopyranuronate (**8d**)
8c (29 1 mg, 0 030 mmol) and Dowex/H$^+$ resin (5g) were stirred in methanol (10 ml) for 16h at 50°C (optimized conditions after monitoring test reactions by TLC, $CHCl_3$-CH_3OH-H_2O 18 11 2 7) After filtration, solvent evaporation, and MPLC ($CHCl_3$-CH_3OH-H_2O 18 11 1 5) gave **8c** (22 2 mg) and **8d** (6 3 mg, 85%, yield corrected for recovered **8c**) - ^{13}C NMR (100 6 MHz, $CDCl_3$-CD_3OD-D_2O 18 11 2.7) δ = 51 6 (OCH_3), 51 8 (OCH_3), 55 0 (C-2^E), 59 9 (C-6^E), 64 9 (C-3^H), 69 1, 69 3, 69 4, 72 2, 73 2, 73 4, 74 1, 75 5, 77 5 (C-2^F), 78 1 (C-2^H), 94 1 (C-1^F), 102 3 (C-1^E), 157 6 ($OCONH_2$), 169.1 (CO), 171 1 (CO) - $C_{46}H_{85}N_2O_{19}P$ (1001 2[14a], 1000 6[14b]), FAB MS (NBA) m/z 1045 6 = [M+2Na-H]$^+$, 595 4 = [M-f+2Na]$^+$, 573 4 = [M-f+Na+H]$^+$, 473 2 = [f+Na-H]$^+$

2-O-(2-Acetamido-2-deoxy-β-glucopyranosyl-)-3-O-carbamoyl-1-O-{[(R)-2-carboxy-2-(3,8,8,11,14,18-hexamethyl-nonadecyloxy)-ethoxyl-hydroxy-phosphoryl}-4-C-methyl-α-D-glucopyranuronic acid (**8a**)
A mixture of **8d** (10 mg, 0 01 mmol), methanol (0 85 ml), and aq LiOH (0 3 mol/l, 230 µl) was stirred for 7 5 h at 20°C Excess base was neutralized

with Dowex-50/H$^+$ resin Filtration, solvent evaporation, and SC (7g SiO_2, $CHCl_3$-CH_3OH-H_2O 18 11 1.5) provided **8a** (7 3 mg, 80%).- $C_{44}H_{81}N_2O_{19}P$ (973 1[14a], 972 5[14b]), FAB MS (NBA): c.f. Fig 4a, Table 4

Synthetic compound **13**.[11]
$C_{43}H_{79}N_2O_{19}$ (928 1[14a], 927 5[14b]), FAB MS (NBA). m/z 1003 5 = $[M+2Na-H]^+$, 981 5 = $[M+Na]^+$, 559 4 = $[M-f+Na+H]^+$, 445 1 = $[f+Na-H]^+$. CID MS of m/z 445 m/z 384 = $[445-H_2NCO_2H]^+$, 340 = $[445-H_2NCO_2H-CO_2]^+$, 310 = $[445-H_2NCO_2H-OCHCO_2H]^+$, 242 = $[f-e+Na-H]^+$, 226 = $[e+Na-H]^+$

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References and Notes

[1] For a summary, see van Heijenoort,J , van Heijenoort,Y ,Welzel,P in "Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function", eds Actor,P., Daneo Moore,L ,Higgins, M L ,Salton, M R J , Shockmann,G.D. American Society for Microbiology, Washington 1988, p 549-557

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[4] All compounds discussed here show a pronounced affinity towards alkali cations. Even after treatment with ion exchange resin intensive Na/K attachment ions were observed, especially when 3-nitro benzyl alcohol (NBA) was used as liquid matrix With glycerol this phenomenon is less marked but the overall sensitivity decreases For that reason we used NBA for most measurements

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[12] The captions to Figures 4 and 5 in ref [2b] have been changed by mistake To the monomethyl derivative (**4b** in the present publication) corresponds Fig 5 in ref [2b] and to the second methylation product Fig 4

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