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Moenomycin A: Reactions at the Lipid Part. New Structure-Activity Relations

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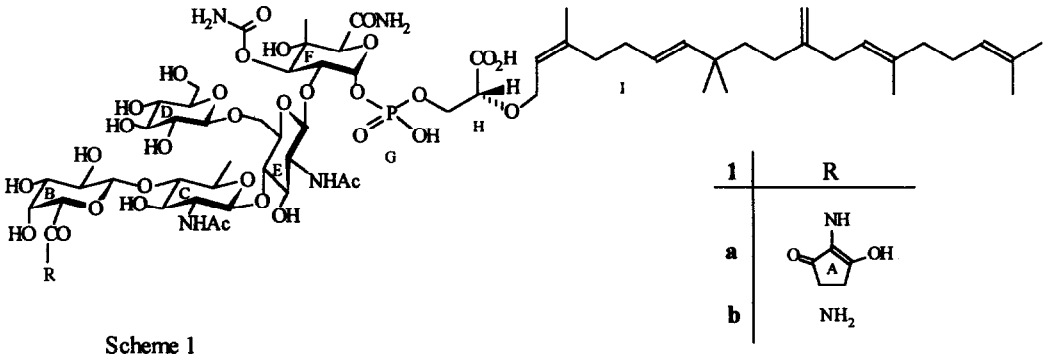
Abstract - Methods for the removal and the oxidation of the lipid moiety of the antibiotic moenomycin A have been studied. Combined with biochemical studies, the results demonstrate that antibiotic activity is closely related with the integrity of the lipid unit.

Introduction

Transglycosylase inhibition may be the basic mechanism of future therapeutics. The first compound which was shown to inhibit specifically the transglycosylase, one of the enzymes that catalyzse the formation of peptidoglycan from a membrane intermediate, was moenomycin A.[1] Recently, new moenomycin-type antibiotics have been fully characterized.[2,3]

A wealth of structure-activity relations has accumulated in recent years by analyzing the transglycosylase inhibiting properties of both moenomycin (A,[4] C_3,[2] C_1[3]) degradation products as well as of synthetic compounds.[5]



Scheme 1

What has never been probed specifically is the role of the lipid moiety of the moenomycins. Until now, it is only known, that hydrogenation of the moenocinol part of moenomycin A does not lead to a decrease in antibiotic activity.

In the early phase of the moenomycin investigations, moenomycin A has been degraded by mild acid treatment. A so-called delipido moenomycin A was isolated but never structurally characterized. This compound was inactive and this was taken as evidence, that the lipid part is essential for antibiotic activity.[6]

In this publication we wish to report on results which shed some light both on the chemistry of the moenomycin-bound lipid part and its importance as far as transglycosylase inhibiting and antibiotic activity is concerned.

Selective removal of the moenocinol unit from moenomycin A (1a→1b)

It was expected that it would be difficult to find reactions affecting the lipid moiety selectively without involving the very sensitive chromophore unit A. From previous work, it was known that hydrogenation of moenomycin A to give the decahydro derivative has to be carefully controlled, otherwise the chromophore part is also reduced.[7] In order to avoid these complications, the chromophore unit which is known not to be essential for antibiotic activity was oxidatively removed by treatment with $K_3[Fe(CN)_6]$[8] in alkaline solution to give **1b**. Progress of this degradation reaction could be followed only with great difficulties by ordinary means (HPLC, TLC). UV was of no help, since the UV bands of the chromophore unit[9] of **1a** were covered by bands of the reagent. Finally, circular dichroism (CD) turned out to be a very useful tool. Figure 1 shows the CD spectrum of moenomycin A (**1a**) at different pH values. The band at longer wavelength (275 nm) could be used as a measure of the concentration of **1a** in the oxidation solution. The rotational strength at different times have been determined and plotted against time. The results are shown in Figure 2. The oxidation was performed at different pH values. At pH 7 and pH 9 the half-life of **1a** was almost equal whereas at pH 5 it was twice as long.

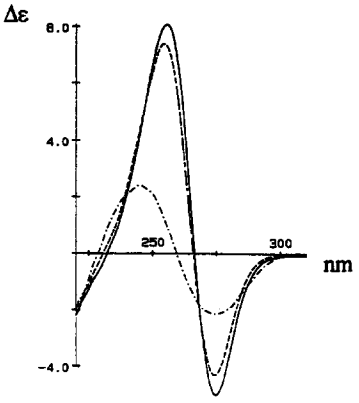


Figure 1. CD spectrum of **1a** in basic (--), neutral (- -) and acidic (-·-) solution.

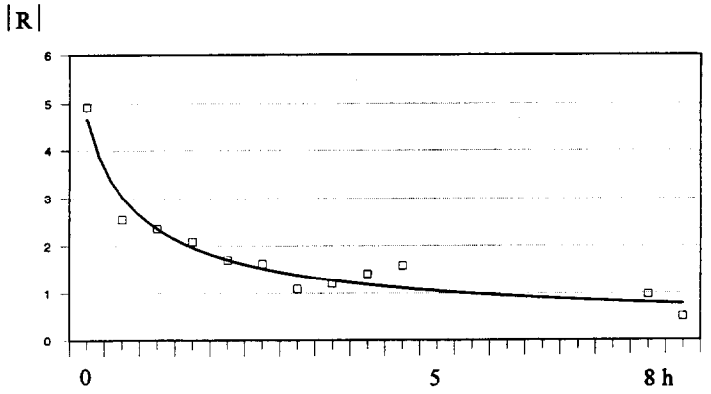
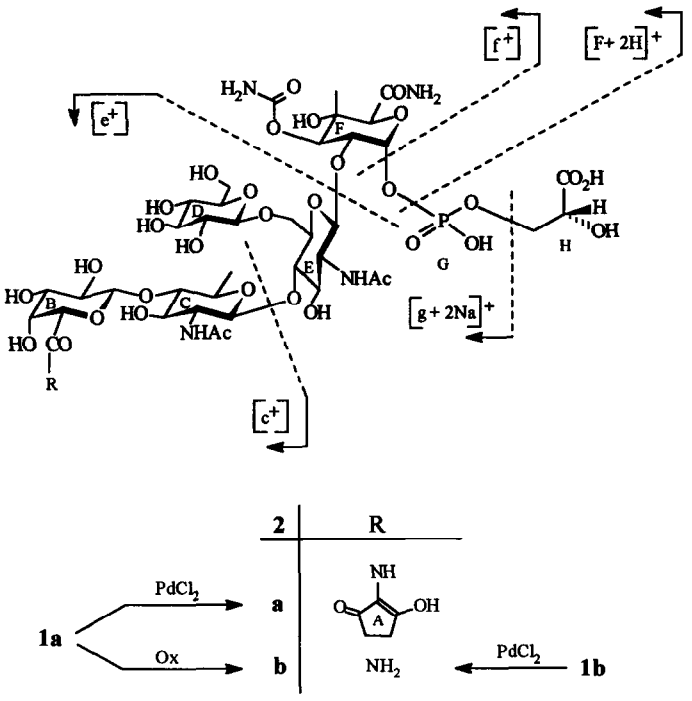


Figure 2. Reaction of **1a** (2.5 mg in 1 ml H_2O) with $K_3[Fe(CN)_6]$ (20 equiv.) at pH 7 |R| = rotational strength (absolute value) of the longer wavelength band.



Scheme 2

In order to remove the lipid moiety from **1b** recourse was made to one of the methods which are known to (oxidatively) cleave allyl ethers.[10] Thus, treatment of **1b** with palladium chloride in an acetate buffer[11] furnished **2b** which after purification was isolated in 49% yield. Both chromatographic purification and spectroscopic characterization of the very polar delipido compounds turned out to be extremely difficult. For the isolation of **2b** the reaction mixture was freed from inorganic salts by gel permeation chromatography (Bio-Gel P-2), the organic compounds were separated by silica gel chromatography (elution with ethyl acetate - 2-propanol - water 6:4:3), and, finally, from the fraction containing **2b** inorganic materials (from silica gel) were removed by Sephadex G-15 gel filtration.

A beautiful ^{13}C-NMR spectrum of **2b** was obtained, fully in accord with the proposed structure (see Experimental). On the contrary, mass spectra were only obtained after much experimentation. FAB mass spectra were usually of low quality, but in the case of **2b** ions at m/z 1168.3 ($[M+Na]^+$) and m/z 1190.3 ($[M+2Na-H]^+$) did support the structural assignment.

On the other hand, electro spray (ES) mass spectrometry under carefully selected conditions (cone voltage) provided nice results. In the positive ion mode the spectra were obtained from acetonitrile - water - formic acid 50:50:0.5 solution, in the negative ion mode from acetonitrile - water 50:50 solution. In the negative ion mode the spectrum of **2b** exhibited signals at 1166.8 ($[M+Na-2H]^-$) and 1144.7 ($[M-H]^-$). In the positive ion

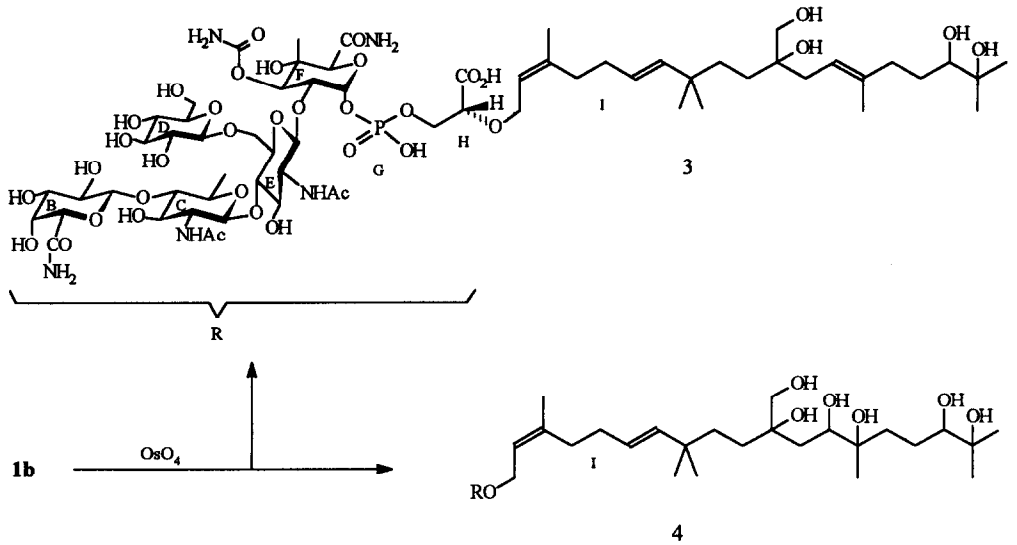
mode very informative peaks were observed both in the molecular ion region and corresponding to fragments formed by cleavage of the glycosidic and phosphate ester bonds, respectively, as indicated in formula **2**.

With low intensity a cluster of signals at m/z = 1607.2/1627.3/1645.6 was observed as well as doubly charged counter parts. The origin of these peaks is at present not understood. The ^{13}C NMR spectrum of **2b** did not contain any indication of an impurity.

When moenomycin A itself was submitted to the allyl ether cleavage conditions, after separation similar to those described above compound **2a** was isolated in 31% yield. ^{13}C NMR and ^{31}P NMR, as well as FAB and ES (positive mode) mass spectra were fully in accord with structure **2a**.

It is an old observation, that *pure* moenomycin on standing slowly decomposes to antibiotically inactive compounds which from RP 18 columns are eluted with short retention times. Details of the degradation process were unknown. We have now been able to isolate from many years old moenomycin samples the main decomposition product which turned out to be identical with **2a** (on the basis of chromatographic behaviour, ^{13}C NMR and mass spectra). The structural assignment was confirmed by ozonolytic removal[12] of the chromophore unit whereupon **2b** was obtained. The mechanism of the decomposion reaction - either hydrolytic or oxidative - remains to be established.

Interestingly, in the course of the present investigations, we observed another oxidative removal of the lipid moiety. When moenomycin A was treated in methanolic solution with ^{t}butyl hydroperoxide in the presence of a catalytic amount of iron (III) tetrakis (pentafluorophenyl) porphyrin chloride ($(F_{20}TPP)FeCl$) both the lipid and the chromophore part were lost to give **2b**. The mechanism of this cleavage reaction is at present unknown. Under the same conditions **1b** was converted into **2b**.



Scheme 3

The last-mentioned reactions were originally performed with the intention to introduce an oxygen functionality into the moenocinol unit.[13] A successful outcome of such a reaction was then realized when **1b**was treated with catalytic osmium tetroxide and $K_3[Fe(CN)_6]$ as cooxidant.[14] In agreement with published work,[15,16] the allylic double bond was not attacked. The separation of the reaction products was very difficult, but we were able to isolate a tetrahydroxy and a hexahydroxy compound (**3** and **4**). Structural assignments rest on correct FAB mass spectra and on an analysis of the double bond region of the ^{13}C NMR spectra. Comparison of the spectra of **1b** and **3** clearly indicated lack of olefinic carbon signals for C-11, C-22, and C-18. Applying the same procedure to the analysis of the spectrum of **4** showed the absence of C-11, C-22, C-18, and C-14 olefinic carbon signals.

Antibiotic properties of the moenomycin A derivatives.

Minimum inhibitory concentrations (MIC) against various microorganisms have been determined by a serial two-fold agar dilution method (Müller Hinton Agar). The results are collected in Table 1.

Table 1. Minimum inhibitory concentrations of some moenomycin A derivatives against various test organisms (moenomycin A is included for comparison).

	MIC values (µg/ml)					
test organism	**1a**	**1b**	**2a**	**2b**	**3**	**4**
Strept. pyog. A77	<0.002	0.013	12.5	6.25	6.25	>100
Strept. faecium MD8b	>100	>100	>50	>100	>100	>100
S. aureus SG 511	0.025	0.025	>50	>100	>100	>100
S. aureus 285	0.025	0.025	>50	>100	100	>100
S. aureus 503	0.049	0.013	>50	>100	>100	>100
E. coli 078	25	25	>50	>100	>100	>100
E.coli DC0	25	12.5	>50	>100	>100	>100
E. coli DC2	12.5	12.5	>50	>100	>100	>100
KL. aerog 1522E	100	50	>50	>100	>100	>100
Ent. cloacae 1321E	1.563	6.25	>50	>100	>100	>100
Ps. aerug. 1771	50	25	>50	>100	>100	>100
Ps. aerug. 1771M	3.125	3.125	>50	>100	>100	>100

The inhibitory effect of **1b** and a number of degradation products directly on the transglycosylation reaction was studied with the *in vitro* assay developed earlier in one of our laboratories[17] using a crude extract from an over producer *E.coli JA200 plc19-19* and as substrate the lipid intermediate which is the immediate precursor of uncross-linked peptidoglycan. Table 2 summarizes the results.

Table 2. Effect of moenomycin A derivatives **3** and **4** on the in-vitro formation of uncross-linked peptidoglycan by transglycosylation

	% Inhibition		
compound		3	4
concentration (μg/ml)	10 1 0.1	100 100 20	63 20 -

Finally, inhibition of the UDP-N-acetylmuramyl pentapeptide-dependent incorporation of [^{14}C]UDP-N-acetylglucosamine into cross-linked high-molecular weight peptidoglycan was studied with a slightly modified[18] version of the assay described by Izaki, Matsuhashi, and Strominger.[19] In Table 3 the results are found.

Table 3. Effect of some moenomycin A derivatives on the in-vitro UDP-N-acetylmuramyl pentapeptide-dependent incorporation of [^{14}C]UDP-N-acetylglucosamine into cross-linked high-molecular weight peptidoglycan.

	% Inhibition						
compound		1a	1b	2a	2b	3	4
concentration (μg/ml)	10 1 0.1	98 81 72	85 89 83	65 7 0	88 46 0	84 61 2	64 10 0

The results depicted in Tables 1 and 3 confirm the previous observation, that the chromophore part of moenomycin (A) has no importance as far as structure activity relations are concerned: **1b** is as active as moenomycin A (**1a**) itself. The data in Table 1 also show that the presence of the moenocinol unit is a prerequisite of the antibiotic activity of moenomycine-type compounds: Compounds **2a** and **2b** are completely inactive. Comparison of the data summarized in Tables 1, 2, and 3 reveals one further very interesting point. For the first time dramatic differences of *in-vivo* activity against Staph.aureus and *in-vitro* inhibition of the transglycosylase can be noticed. Whereas hexahydroxy compound **4** is inactive both *in-vivo* and *in-vitro*, the tetrahydroxy moenomycin A derivative **3** is inactive *in-vivo* but shows high activity in the *in-vitro* test systems. This observation seems to indicate that an unpolar lipid part is necessary to attach the antibiotic to the cytoplasmic membrane of the test organisms whereas in the *in-vitro* systems the enzyme active site may be reached more directly. In one test system (cf. Table 3) even **2b** exhibits distinct activity whereas it is inactive *in-vivo*.

EXPERIMENTAL

The following equipment was used. NMR: AM-400 (Bruker); FAB MS: MAT 731 (Varian) with a modified Saddle Field Source (Ion Tech Ltd.) or VG AUTOSPEC, matrix: lactic acid; ES MS: VG BIOTECH BIO-Q in the positive (solvent acetonitrile - water - formic acid 50:50:0.5) and negative ion mode (solvent: acetonitrile - water 50:50), error -0.3 - -0.5 Da; HPLC: (i) Model 6000 (Waters-Associates Inc.), UV detector LC-3 (Pye Unicam at 254 nm), (ii) system Constametric III (LDC), UV detector Spectromonitor III (LDC at 254 nm); ozonolysis (ozone generator Fischer Labortechnik). Solvents were degassed by sonification (15 min), the flow rate was in all cases 0.8 ml/min. For gel permeation chromatography Bio-Gel® P-2 (BIO RAD, particle size: <45 μm, flow rate: 8 ml/h) and Sephadex G-10 and G-15 (Pharmacia, particle size 40-120 μm, flow rate: 1.5 ml/min) were used. For elution a Duramat pump (CfG) was used. Two molecular masses are always communicated, the first was calculated using the International Atomic Masses, the second refers to ^{12}C, ^{1}H, ^{16}O, ^{14}N, ^{31}P (mono-isotopic masses). For all other experimental techniques, see ref..[5]

2-0-{2-Acetylamino-4-O-[2-acetylamino-4-O-((5S)-5-carbamoyl-ß-L-arabinopyranosyl)-2,6-dideoxy-ß-D-glucopyranosyl]-2-deoxy-6-O-ß-D-glucopyranosyl-ß-D-glucopyranosyl}-3-O-carbamoyl-1-O-{[(S)-2-carboxy-2-((2Z, 6E, 13E)-3,8,8,14,18-pentamethyl-11-methylene-nonadeca-2,6,13,17-tetraen-1-yloxy)-ethoxy]-hydroxyphosphoryl}-4-C-methyl-α-D-glucopyranuronamide (1b)

To **1a** (1.0001 g, 0.6323 mol), dissolved in water (70 ml) solutions of K_2CO_3 (5.1135 g, 0.037 mol) in water (15 ml) and $K_3[Fe(CN)_6]$ in water (15 ml) were added at 0°C. The mixture was stirred for 30 min at 0°C, then (2 h) at 40°C. Progress of the reaction was monitored as described above and by TLC ($CHCl_3$ - methanol - water 18:11:2.7). The inorganic salts were removed by reversed phase chromatography (HP 20, 60 g, elution with H_2O (200 ml) and methanol (800 ml)). After solvent evaporation and lyophilization the mixture was separated by LC (SiO_2, 2-propanol - 25 per cent NH_3 - water 10:3:0.5). After evaporation and lyophilization pure **1b** was obtained (0.5484 g, 58%).- $C_{64}H_{104}N_5O_{32}P$ (1486.52, 1485.64), FAB MS: m/z 1547.1 ($[M+Na+K-H]^+$), 1531.1 ($[M+2Na-H]^+$), 1509.0 ($[M+Na]^+$), 982.2 ($[f+Na-H]^+$), 750.2 ($[e+Na-H]^+$), 549.2 ($[M-f+H+Na]^+$).- ^{13}C NMR ($CDCl_3/CD_3OD/D_2O$ 18:11:2.7): δ = 173.5, 173.3, 172.9, 172.5 ($NH\underline{C}OCH_3^{C, E}$, C-$6^{F, B}$), 157.9 ($O\underline{C}ONH_2^F$), 149.9 (C-$11^I$), 142.0 (C-$3^I$), 140.6 (C-$7^I$), 136.3 (C-$14^I$), 131.3 (C-$18^I$), 125.3, 124.2, 122.0 (C-2, C-6, C-13, C-17 of unit I), 108.3 (C-22^I), 102.9, 102.6, 101.5 (C-$1^{B, C, D, E}$), 83.1 (C-4^C), 80.1 - 68.0 (unassigned signals), 61.0 (C-6^D), 55.4, 54.7 (C-$2^{C, E}$), 41.5, 39.7 (C-9^I, C-15^I), 35.4, 34.8 (C-8^I, C-12^I), 32.3, 31.6, 31.3 (C-4, C-5, C-10 of unit I), 27.0. 26.6, 25.4, 23.4, 22.6, 22.5 (C-16, C-19, C-23, C-24, C-25 of unit I), 17.4, 16.9, 15.7, 15.3 ($NHCO\underline{C}H_3^{C, E}$, C-$20^I$, C-$21^I$).

Cleavage of 1b with $PdCl_2$ in NaOAc - AcOH solution

To a solution of **1b** (0.0504 g, 0.0339 mmol) in 0.1 mol/l NaOAc in 20:1 acetic acid-water (3 ml) $PdCl_2$ (0.0386 g, 0.22 mmol) was added slowly (within 28 h). First (23 h) the mixture was stirred at 20°C, then (8 h) at 40°C. Progress of the reaction was monitored by TLC (ethyl acetate - 2-propanol - water 4:5:5 and $CHCl_3$ - methanol - water 18:11:2.7). When **1b** could not be detected anymore (after 31 h) solids were removed by centrifugation. After solvent evaporation and lyophilization inorganic salts were removed by gel filtration (Bio-Gel P-2, bed volume 74 ml, sample volume 2.5 ml, eluant H_2O). After lyophilization the mixture was separated by LC (SiO_2, ethyl acetate - 2-propanol - water 6:4:3) to provide fractions containing **2b**, free of other degradation products. Finally, inorganic impurities (from SiO_2) were removed by gel filtration (Sephadex G-15, bed volume 108 ml, sample volume 3 ml, eluent H_2O). After lyophilization pure **2b** was obtained (0.0189 g, 49%). The purity was controlled in the following TLC systems: ethyl acetate - 2-propanol - water 4:5:5, 2-propanol - 2 mol/l NH_3 7:3, 1-propanol - 2 mol/l NH_3 7:3, 2-propanol - 25 per cent NH_3 -water 6:3:1, $CHCl_3$ - methanol - water 14:15:5, $CHCl_3$ - methanol - water 16:12.5:4.

2-0-{2-Acetylamino-4-O-[2-acetylamino-4-O-((5S)-5-carbamoyl-ß-L-arabinopyranosyl)-2,6-dideoxy-ß-D-glucopyranosyl]-2-deoxy-6-O-ß-D-glucopyranosyl-ß-D-glucopyranosyl}-3-O-carbamoyl-1-O-{[(S)-2-carboxy-2-hydroxy)-ethoxy]-hydroxyphosphoryl}-4-C-methyl-α-D-glucopyranuronamide (2b)

$C_{39}H_{64}N_5O_{32}P$ (1145.93, 1145.33).- FAB MS: m/z 1190.3 ($[M+2Na-H]^+$), 1168.3 ($[M+Na]^+$).- ES MS (negative ion mode): m/z 1166.8 ($[M-2H+Na]^-$), 1144.7 ($[M-H]^-$), 571.9 ($[M-2H]^{2-}$).- ES MS (positiv ion mode): m/z 1628.5 ($[M'+Na]^+$), low intensity, 1212.5 ($[M+3Na-2H]^+$), 1190.6 ($[M+2Na-H]^+$), 1168.6 ($[M+Na]^+$), 1102.7 ([g+2Na]$^+$), 1000.6 ([F+2H+Na-H]$^+$), 982.6 ([f+Na-H]$^+$), 836.2 ($[M'+3Na-2H]^{2+}$), 826.1 ($[M'+2Na-H]^{2+}$), 814.9 ($[M'+Na]^{2+}$), 750.5 ([e+Na-H]$^+$).- ^{13}C NMR (D_2O - CD_3OD = 1:1): δ = 179,6 (C-1^H), 176.3, 176.0. 175.3 broad signal ($NH\underline{C}OCH_3^{C,E}$, C-$6^{F,B}$), 160.3 ($O\underline{C}ONH_2^F$), 105.6, 105.1, 104.4, 103.8 (C-$1^{B,C,D,E}$), 96.7 (d, $J_{C,P}$ = 7,7 Hz, C-1^F), 85.5 (C-4^C), 82.7 (C-4^E or C-2^H), 79.1 (d, C-2^H or C-2^F), 78.5-71.1 (unassigned signals), 63.3 (C-6^D), 57.7 (C-2^E), 57.5 (C-2^C), 24.63, 24.56 ($NHCO\underline{C}H_3^{C,E}$), 18.9 (C-$6^C$), 17.2 (C-$4^F$).

Removal of the lipid part from 1a with $PdCl_2$

To solution of **1a** (0.1006 g, 0.0636 mmol) in 0.1 mol/l NaOAc in 20:1 acetic acid-water (5.5 ml) $PdCl_2$ (0.0781 g, 0.4405 mmol) was added slowly (within 23 h). First (21 h) the mixture was stirred at 20°C, then (6 h) at 40°C. Progress of the reaction was monitored by TLC (ethyl acetate - 2-propanol - water 4:5:5). When **1a** could not be detected anymore (after 27 h) solids were removed by centrifugation. After solvent evaporation and lyophilization inorganic salts were removed by gel filtration (Sephadex G-10, bed volume 83 ml, sample volume 3ml, eluent H_2O). After lyophilization the mixture was separated by LC (SiO_2, 2-propanol - 25 per cent NH_3 - water 6:3:0.1 (TLC analysis: 2-propanol - 25 per cent NH_3 - water 6:3:1). The most polar compound, **2a**, was obtained in 31% yield after evaporation and lyophilization.

2-0-{2-Acetylamino-4-O-[2-acetylamino-4-O-((5S)-5-N-(1-hydroxy-3-oxo-1-cyclopenten-2-yl)-carbamoyl-ß-L-arabinopyranosyl)-2,6-dideoxy-ß-D-glucopyranosyl]-2-deoxy-6-O-ß-D-glucopyranosyl-ß-D-glucopyranosyl}-3-O-carbamoyl-1-O-{[(S)-2-carboxy-2-(hydroxy)-ethoxy]-hydroxyphosphoryl}-4-C-methyl-α-D-glucopyranuronamide (2a)

$C_{44}H_{68}N_5O_{34}P$ (1242.01, 1241.35).- FAB MS: m/z 1264.1 ($[M+Na]^+$), 1242.1 ($[M+H]^+$), 824.2 ([e]$^+$).- ES MS (positiv ion mode): m/z 1264.7 ($[M+Na]^+$), 1242.7 ($[M+H]^+$), 824.8 ([e]$^+$), 459.3 ([c]$^+$).- ^{13}C NMR (D_2O - CD_3OD = 1:1): δ = 200.4 (C-1^A, C-3^A), 175.2, 174.3, ($NH\underline{C}OCH_3^{C,E}$, C-$6^F$), 170.7 (C-$6^B$), 159.3 ($O\underline{C}ONH_2^F$), 104.4 broad signal, 103.6, 102.8 (C-$1^{B,C,D,E}$), 95.8 (d, C-1^F), 84.7 (C-4^C), 81.5 (C-4^E or C-2^H), 78.2 broad signal (C-2^H or C-2^F), 77.5-69.6 (unassigned signals), 62.3 (C-6^D), 57.0 (C-2^E), 56.2 (C-2^C), 31.4 (C-4^A, C-5^A), 23.6 broad signal ($NHCO\underline{C}H_3^{C,E}$), 18.1 (C-$6^C$), 16.2 (C-$4^C$).- ^{31}P NMR (D_2O - CD_3OD = 1:1, 85 per cent H_3PO_4 as external standard): δ = -1.1.

Isolation of 2a from old moenomycin samples

a) From an old sample of a mixture of moenomycins (kept at 37°C for 3 years, 2 g) in 2 runs the moenomycins were removed by gel filtration (Pharmacia/LKB Superdex 75, 26 mm x 600 mm, mobile phase: 0.05 mol/l phosphate buffer at pH 7, flow rate: 6 ml/min). Fractions with low retention times were collected and freeze-dried. Inorganic salts were removed by reversed-phase chromatography (RP 18, an aqueous solution was applied on the column, elution with acetonitrile). After solvent evaporation and lyophilization 1 g of moenomycin degradation products were obtained. 0.2101 g of this sample were separated by LC (100 g SiO_2, elution with ethyl acetate - 2-propanol - water 5:4:3, TLC monitoring with ethyl acetate - 2-propanol - water 4:5:5). Fractions containing **2a** were collected, evaporated and freeze-dried. The residue was taken up in water (2 ml) and freed from inorganic material by gel filtration (Sephadex G-15, bed volume 108 ml, elution with water). After solvent evaporation and lyophilization **2a** (0.1013 g) was obtained. The R_f- values of this sample in several TLC systems (ethyl acetate - 2-propanol - water 4:5:5, 2-propanol - 25 per cent NH_3 - water 6:3:1), the HPLC retention time (3.2 min, RP 18, elution with a 80:20 mixture of solution A and acetonitrile. Solution A: Sodium heptanesulfonate (3 g), K_2HPO_4 (15.5 g), KH_2PO_4 (1.0 g) and water, final volume: 1 l), the ^{13}C NMR and ES MS (positive mode) spectra of this sample were identical with those of the compound described above.

b) A 16 years old specimen of moenomycin A (3.5477 g) was separated by MPLC (415 g of SiO_2, elution with 2-propanol - 25 per cent NH_3 - water 6.5:3:0.1 (4 l), then 2-propanol - 25 per cent NH_3 - water 6:3:0.2 (2 l), and finally methanol (2 l), monitoring the separation by TLC (ethyl acetate - 2-propanol - water 4:5:5) yielded (after solvent evaporation and lyophilization) a sample of **2a** (0.4142 g), which according to ^{13}C NMR contained about 10% of an impurity. The ES MS (positive ion mode) indicated that the sample of **2a** contained **2b**.

Conversion of 2a into 2b

A sample of pure **2a** (0.3080 g, 0.2480 mmol) was dissolved in methanol (300 ml). At -78°C ozonized oxygen was passed through the solution for 10 min (20 l/h = 1.19 mmol O_3/min.). The reaction flask was flushed with O_2 for 15 min. An excess of dimethyl sulfide was added and the mixture was allowed to warm to 20°C and was stirred at this temperature 17 h. Solvents were evaporated and the residue was freeze-dried after addition of water. MPLC (2-propanol - 25 per cent NH_3 - water 6.5:3:0.2) allowed only partial separation. Two pure compounds were obtained. One of them (17.4 mg, 6%) was identical in all respects (^{13}C NMR, ^{31}P NMR, FAB-MS, ES-MS (negative mode) with **2b**. The structure of the other, less polar reaction product (28.6 mg) remains to be established.

Cleavage of 1a with (F_{20}TPP)FeCl and ^{t}butyl hydroperoxide

To a solution of **1a** (0.2515 g, 0.1589 mmol) in methanol (5 ml) (F_{20}TPP)FeCl (0.0001 g, 0.0009 mmol) and ^{t}butyl hydroperoxide (445 µl, 1.5887 mmol) were added. The mixture was stirred (5 h) at 20°C. The progress of the reaction was monitored by TLC (2-propanol - 25 per cent NH_3 - water 6:3:1). After evaporation and several times repeated LC (SiO_2, 2-propanol - 25 per cent NH_3 - water 6:3:0.2) pure **2b** was obtained (0.0262 g, 14%).

Cleavage of 1b with (F_{20}TPP)FeCl and ^{t}butyl hydroperoxide

To a solution of **1b** (0.2313 g, 0.1557 mmol) in methanol (5 ml) (F_{20}TPP)FeCl (0.8 mg, 0.0008 mmol) and ^{t}butyl hydroperoxide (436 µl, 1.5565 mmol) were added. The mixture was stirred (5 h) at 20°C. The progress was monitored by TLC (2-propanol - 2 mol/l NH_3 7:3). After evaporation and several times repeated LC (SiO_2, 2-propanol - 25 per cent NH_3 - water 6:3:0.2) pure **2b** was obtained (0.0306 g, 17%).

Dihydroxylation of 1b with OsO_4

To a solution of **1b** (0.5400 g, 0.3635 mmol) in ^{t}butanol (15 ml) and water (15 ml) $K_3[Fe(CN)_6]$ (1.1998 g, 3.6441 mmol) and K_2CO_3 (0.5099 g, 3.6894 mmol) were added. After addition of OsO_4 (0.0039 g, 0.0153 mmol in 195 µl H_2O) the solution was stirred (3 h) at 40°C. Progress of the reaction was monitored by TLC (2-propanol - 2 mol/l NH_3 7:3). The excess of OsO_4 was destroyed by addition of Na_2SO_3 (0.4600 g, 3.6567 mmol). and stirring was continued for 14 h at 20°C. After evaporation the mixture was diluted with water. The inorganic salts were removed by reversed phase chromatography (HP 20, 30 g, elution with H_2O (150 ml) and methanol (500 ml)). Evaporation and lyophilization followed by reversed phase MPLC (RP 18, methanol - water - CH_3CN 1:6:1) provided pure **3** (0.0228 g, 4%) and **4** (0.0539 g, 9%).

2-0-{2-Acetylamino-4-O-[2-acetylamino-4-O-((5S)-5-carbamoyl-ß-L-arabinopyranosyl)-2,6-dideoxy-ß-D-glucopyranosyl]-2-deoxy-6-O-ß-D-glucopyranosyl-ß-D-glucopyranosyl}-3-O-carbamoyl-1-O-{[(S)-2-carboxy-2-((2Z, 6E, 13E)-11, 17, 18-trihydroxy-11-hydroxymethyl-3,8,8,14,18-pentamethyl--nonadeca-2,6,13-trien-1-yloxy)-ethoxy]-hydroxyphosphoryl}-4-C-methyl-α-D-glucopyranuronamide (3)

$C_{64}H_{108}O_{36}N_5P$ (1554.55, 1553.65).- FAB MS: m/z 1630.6 ($[M+2K-H]^+$); 1614.6 ($[M+Na+K-H]^+$); 1592.6 ($[M+K]^+$); 1576.6 ($[M+Na]^+$). ^{13}C NMR (D_2O - CD_3OD 1:1): δ = 177.4 (C-1^H), 175.0. 174.8, 174.0. 173.8 ($NH\underline{C}OCH_3$,$^{C, E}$, C-$6^{B, F}$), 159.1 ($O\underline{C}ONH_2^F$), 142.4, 141.6 (C-3^I, C-7^I), 138.7 (C-14^I), 126.5, 122.1, 120.2 (C-2, C-6, C-13 of unit I), 104.2, 103.9, 103.4, 102.5 (C-$1^{B, C, D, E}$), 95.7 (C-1^F), 84.2 (C-4^C), 81.5-66.7 (unassigned signals), 62.0 (C-6^D), 56.5, 56.1 (C-$2^{C, E}$), 37.7-15.9 (unassigned signals).

2-0-{2-Acetylamino-4-O-[2-acetylamino-4-O-((5S)-5-carbamoyl-ß-L-arabinopyranosyl)-2,6-dideoxy-ß-D-glucopyranosyl]-2-deoxy-6-O-ß-D-glucopyranosyl-ß-D-glucopyranosyl}-3-O-carbamoyl-1-O-{[(S)-2-carboxy-2-((2Z, 6E)-11, 13, 14, 17, 18-pentahydroxy-11-hydroxymethyl-3,8,8,14,18-pentamethyl-nonadeca-2,6-dien-1-yloxy)-ethoxy]-hydroxyphosphoryl}-4-C-methyl-α-D-glucopyranuronamide (4)
$C_{64}H_{110}O_{38}N_5P$ (1588.56, 1587.66).- FAB MS: m/z 1664.5 ([M+2K-H]$^+$), 1648.6 ([M+Na+K-H]$^+$), 1626.6 ([M+K]$^+$), 1610.6 ([M+Na]$^+$), 1588.6 ([M+H]$^+$), 960.3 ([f]$^+$), 728.2 ([e]$^+$).- ^{13}C NMR (D_2O - CD_3OD 1:1): δ = 175.0. 174.8, 174.0. 173.9 ($NH\underline{C}OCH_3$[C, E], C-6[B, F]), 159.1 ($O\underline{C}ONH_2$[F]), 141.2, 138.8 (C-3[I], C-7[I]), 127.4, 120.2 (C-2[I], C-6[I]), 104.3, 10.9, 103.4, 102.6 (C-1[B, C, D, E]), 95.7 (C-1[F]), 84.2 (C-4[C]), 81.4-68.1 (unassigned signals), 62.1 (C-6[D]), 56.5, 56.2 (C-2[C, E]), 39.7-16.0 (unassigned signals).

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