A Surface Plasmon Resonance Analysis of the Interaction between the Antibiotic Moenomycin A and Penicillin-Binding Protein 1b

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The antibiotic moenomycin A inhibits the biosynthesis of peptidoglycan, the main structural polymer of the bacterial cell wall. The inhibition is based on a reversible binding of the antibiotic to one of the substrate binding sites in enzymes such as penicillin-binding protein (PBP) 1b. A novel assay based on surface plasmon resonance (SPR) has been established that can be used to investigate selective binding of the moenomycin sugar moiety and other transglycosylase inhibitors to this enzyme. Suitable ligands were prepared from moenomycin A and coupled to SPR sensor surfaces. Moenomycin analogues with structural variations were used to perform competitive SPR experiments with PBP 1b. The SPR results confirm for the first time that the trisaccharide fragment of moenomycin A (C-E-F-G-H-I) is the minimal structure that possesses all moieties sufficient for biological activity and for affinity towards PBP 1b. The method seems to be appropriate for use in screens for transglycosylase inhibitors that bind to the moenomycin-binding site of the enzyme.

KEYWORDS:

antibiotics · glycolipids · surface plasmon resonance · transglycosylase

Introduction

The shape of bacteria relies on a netlike multilayer polymer that surrounds the cell. The polymer, peptidoglycan, consists of repeating β-1,4-linked *N*-acetylglucosaminyl-*N*-acetylmuramyl units crosslinked by short peptide side chains.[1] Disruption of peptidoglycan biosynthesis leads to cell lysis caused by osmotic pressure. In view of the problem of antibiotic resistance,[2] this biosynthetic pathway is intensively studied with the aim of developing anti-infectives with novel modes of action as well as gaining a deeper mechanistic understanding of already existing drugs.[3]

Peptidoglycan biosynthesis is completed by two consecutive polymerization reactions that occurr at the outer face of the cytoplasmic membrane. The first reaction is a glycosyltransfer reaction (transglycosylation)[4] that is believed to proceed in such a way that the growing peptidoglycan chain, linked to a C_{55} lipid by a pyrophosphate bridge, acts as the glycosyl donor, whilst a disaccharide intermediate, the so-called lipid II, is the glycosyl acceptor. This mode of glycan chain elongation has been demonstrated for a poorly lytic mutant of *Bacillus licheniformis*.[4] Subsequently, a transpeptidation reaction takes place, which crosslinks the peptide units of different strands and releases a D-Ala residue.

The two polymerization reactions in *Escherichia coli* have been demonstrated to be catalyzed by the high molecular weight penicillin-binding proteins (PBPs).[5] Of these, PBP 1b has been studied in great detail. This protein is a bifunctional enzyme with two separate active sites, one for transglycosylation and the other one transpeptidation. Each of these domains can be specifically inhibited by antibiotics. While β-lactam antibiotics exert their action by covalent binding to an essential serine residue in the transpeptidase domain, the transglycosylation step of cell wall assembly can be blocked by certain glycopeptides,[6] ramoplanin,[7] the lantibiotics,[8] and the moenomycin-type antibiotics.[9] Of these molecules, the moenomycins are the only compounds known to inhibit the transglycosylase function of the enzyme.[4]

The moenomycins (for example, moenomycin A (**1a**; Scheme 1) exert high antibiotic activity solely against Gram-positive bacteria.[10] In order to better understand this selectivity, the minimum inhibitory concentrations (MICs) of moenomycin A against a number of test organisms have been studied in the presence of polymyxin B nonapeptide (PMBNP), a compound well-known for its ability to increase the permeability of the outer membrane of Gram-negative bacteria.[11] It was found that PMBNP increases the sensitivity of *E. coli* (which is Gram-negative) to moenomycin A dramatically in a dose-dependent manner. As expected, no effect was found for *Staphylococcus aureus* (Gram-positive). These results were taken as evidence

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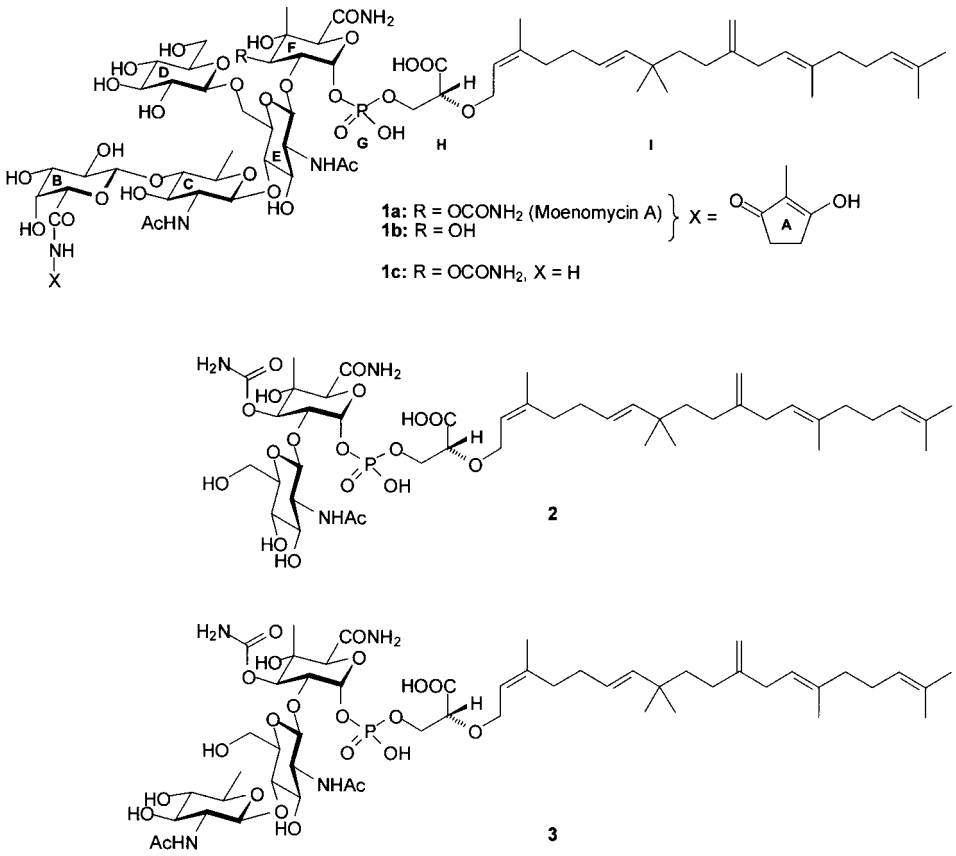


Scheme 1. *Meonomycin A (**1a**) and two analogues that differ only in the oligosaccharide structure.*

that the low antibiotic activity of the moenomycin-type antibiotics against Gram-negative bacteria results from the low permeability of the outer membrane to these compounds.[9] In keeping with this result, *E. coli* BAS849, which is characterized by outer membrane defects, is as sensitive towards moenomycin A as *Staph. aureus*.[12] In addition, in a number of different assays with membrane fractions of *E. coli*[6, 9, 13–15] as well as ether-[6, 16, 17] or toluene-permeabilized[18] *E. coli* cells, the inhibition of peptidoglycan biosynthesis and, more specifically, the inhibition of the transglycosylation step[16] in Gram-negative bacteria by the moenomycins, has been demonstrated. Finally, in assays with purified *E. coli* enzymes and lipid II as the substrate, the moenomycins have been found to inhibit the formation of non-cross-linked peptidoglycan.[19–21]

In vitro and in vivo activities (in *E. coli* and *Staph. aureus* systems, respectively) give similar results for the moenomycins and many moenomycin analogues but there are a number of notable exceptions (Table 1). Thus, disaccharide analogue **2** is active in *E. coli* based assays, whereas it is inactive against *Staph. aureus*. A similar behavior has been observed for **4a**/**4b**[22] and the semisynthetic lipid-modified analogue **5** (Scheme 2).[18] The reasons for these discrepancies are at present elusive. It has been speculated that access to the enzyme is different in the in vivo and the in vitro test systems.[9]

Herein, we have now develop a novel enzyme-based assay for transglycosylase inhibitors of the moenomycin type, the results of which correlate well with the *Staph. aureus* MIC values. The assay is based on competition experiments that use surface plasmon resonance (SPR).

Results and Discussion

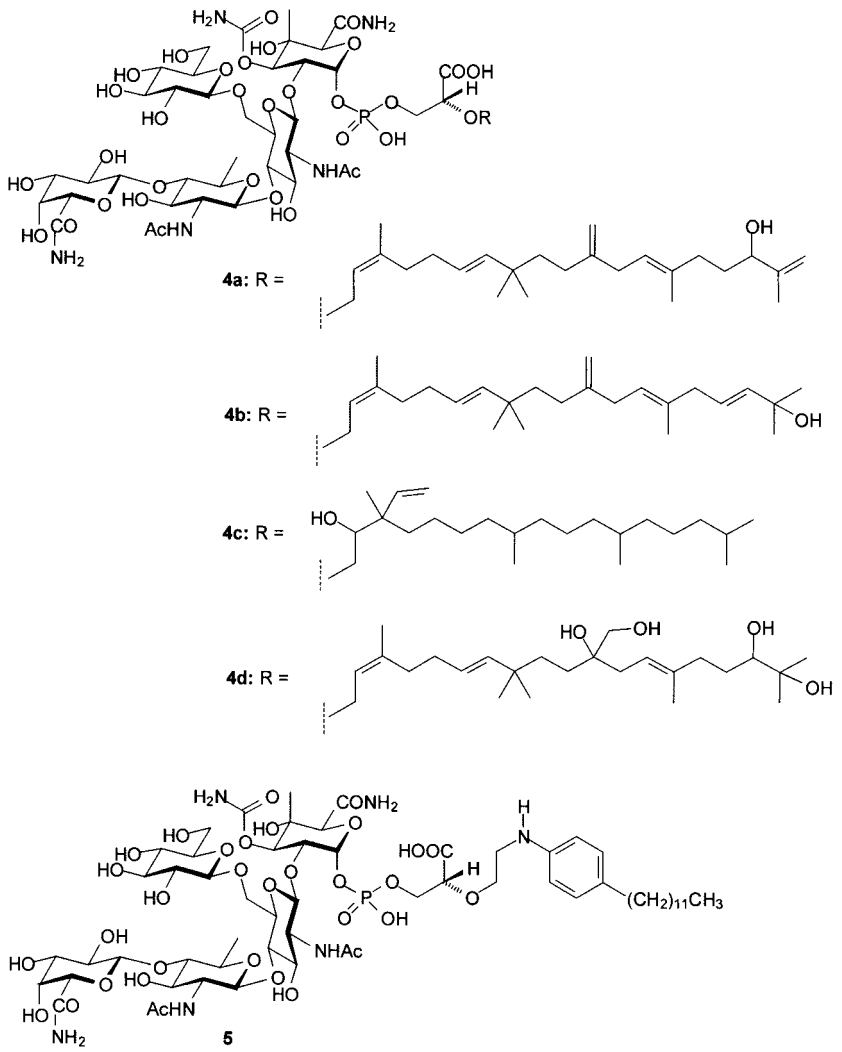
SPR competition experiments

Isolation and purification of PBP 1b[23, 24] was performed as described previously by using ampicillin affinity chromatogra-

Table 1. *Comparison of in vivo and in vitro activities for different moenomycin derivatives.*

Compound	MIC [M] (*Staph. aureus* test system)[a]	Inhibition concentrations [M] (*E. coli* test system)	B_{50} [M]
1a	6.9×10^{-9}	6.3×10^{-7}[39] (100%[b])	1.3×10^{-4}
		IC_{50}: 1.5×10^{-8}[12]	
1b	2.7×10^{-6}	n.d.	5×10^{-4}
1c	1.1×10^{-8}	6.7×10^{-7}[33] (89%[b])	1.3×10^{-4}
2	$> 1.6 \times 10^{-5}$	1.0×10^{-6}[9,c] (100%[b])	1.9×10^{-3}
3	1.1×10^{-6}	8.6×10^{-7}[35] (100%[b])	2.6×10^{-4}
4a/**4b**	2.06×10^{-6}	6.7×10^{-7}[22] (100%[b])	n.d.
4c	1.4×10^{-6}	n.d.	n.d.
4d	$> 6.4 \times 10^{-5}$	6.4×10^{-7}[33] (61%[b])	n.d.
5	$> 2.2 \times 10^{-5}$	5×10^{-9}[18]	n.d.
7	$> 2.6 \times 10^{-5}$	n.d.	3.3×10^{-3}
8	$> 3.6 \times 10^{-5}$	n.d.	9.4×10^{-3}
9	$> 4.8 \times 10^{-5}$	n.d.	9.4×10^{-3}
10	9.5×10^{-5}	n.d.	> 1

[a] Various strains. [b] Inhibition of the transglycosylase. [c] Determined for the disaccharide analogue with the saturated lipid moiety. n.d. = not determined.

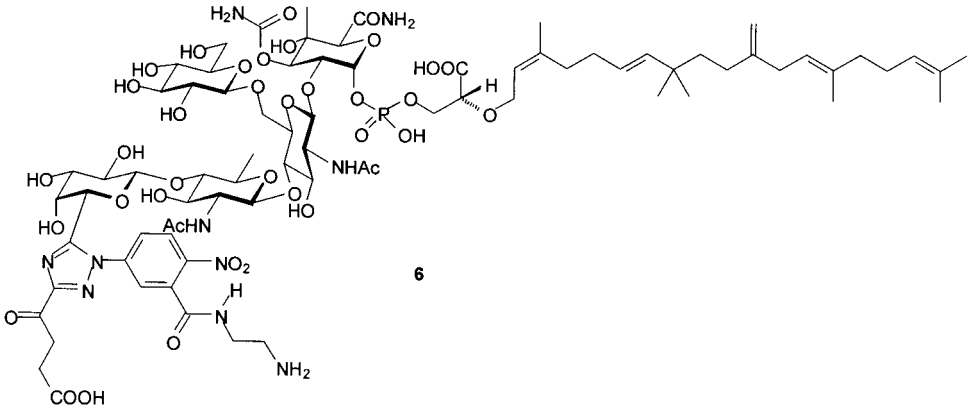


Scheme 2. *Meonomycin A analogues that inhibit biosynthesis of peptidoglycan in* E. coli *but not in* Staph. aureus.

phy.[19, 25] The affinity eluate was dialyzed against the buffer used in the experiments described below. In the purification process proteins were analyzed by SDS-PAGE and visualized by silver staining and Western blotting with anti-PBP 1b antibodies. For the competition SPR experiments,[26] moenomycin derivative **6** (Scheme 3)[27] was immobilized on a sensor chip surface, which carried *N*-hydroxysuccinimide-activated carboxylic acid groups. We have shown previously that reaction with PBP 1b occurs exclusively at the amino group of **6**.[28] PBP 1b was injected as soluble analyte at different concentrations and the SPR response that resulted from the interaction between PBP 1b and the immobilized **6** was found to be concentration dependent (Figure 1). The PBP 1b that was bound to the immobilized **6** was then competitively eluted with moenomycin A. Competition experiments in solution were also performed. Thus, equimolar solutions of PBP 1b were incubated with moenomycin A at various concentrations to allow complex formation before injection. As expected, the relative binding response decreased with increasing moenomycin concentrations, that is, binding was proportional to the concentration of protein with free binding sites (Figure 2).

It was evident from the experiment displayed in Figure 2A that SPR experiments could be used to compare the affinities of moenomycin and its structural analogues. Moenomycin A and analogue **7**[29] (Scheme 4) were each preincubated with PBP 1b at exactly equal concentrations and were then injected onto the same sensor chip. The much higher response of the **7**/PBP 1b sample agrees nicely with the low antibiotic activity of this compound. For an extended series of binding experiments we chose, however, to perform competitive elution of PBP 1b bound to immobilized **6** as described above. To minimize the known problem of rebinding, we used a low surface loading and a high eluent solution flow.

Moenomycin analogues with systematic structural variations were injected at different concentrations for competitive elution of PBP 1b. The extent of elution was extracted from the difference betweeen the binding response (in relative units; RU) at the onset and that at the end of the dissociation phase (the elution volumes were constant for all compounds), for example as shown in Figure 2B. The concentration (B_{50}) of each moenomycin analogue that resulted in 50% elution was calculated by nonlinear regression. The B_{50} values provided valuable information in the form of a relative affinity ranking set, even if absolute interaction constants were not estimated.[30]



Scheme 3. *The moenomycin derivative that was immobilized on a sensor chip surface for SPR experiments.*

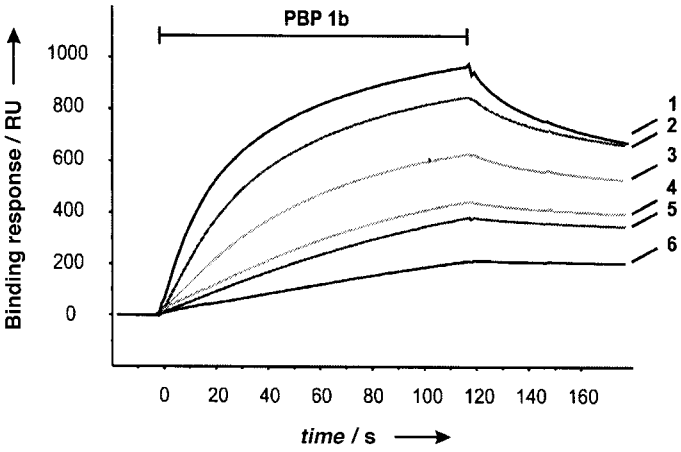


Figure 1. Sensorgrams for different concentrations of PBP 1b. The protein, at concentrations of (1) 1.6 μM, (2) 0.8 μM, (3) 0.4 μM, (4) 200 nM, (5) 160 nM, and (6) 80 nM, was injected over a sensor surface, which held immobilized ***6*** *(flow rate: 25 μL min⁻, contact time: 120 s, 25 °C). The sensorgrams were corrected by subtraction of the signal from a reference cell.*

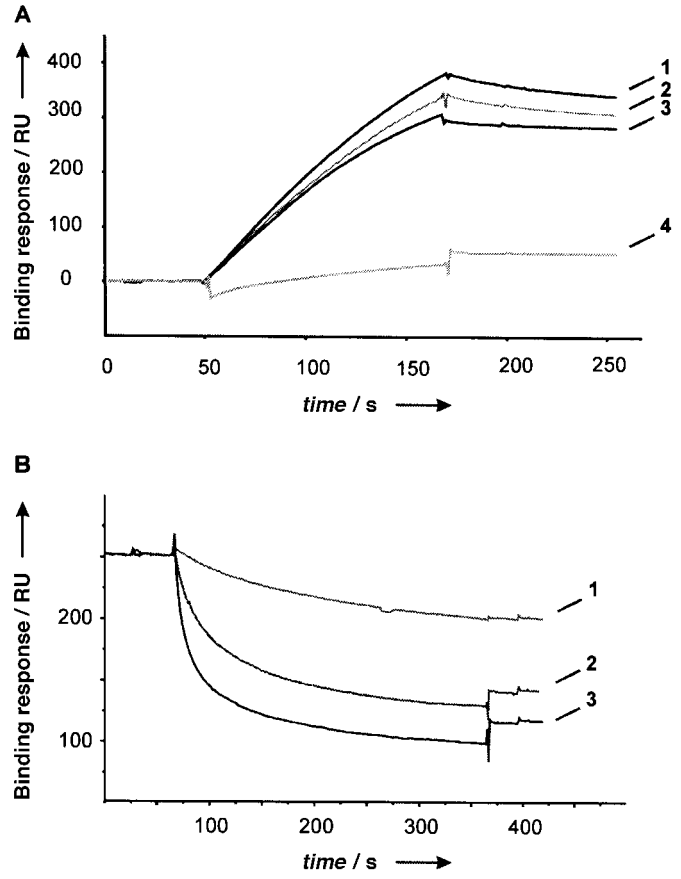


*Figure 2. A) Competition experiment in solution. Solutions of PBP 1b (160 nM) were incubated (5 min) with an equimolar amount of moenomycin A (****1 a****) or pentasaccharide* ***7*** *at different concentrations to allow complex formation prior to injection (flow rate: 25 μL/min, contact time: 120 s, 25 °C). Primary association data for PBP 1b (1) alone and in the presence of (3) 100 μM or (4) 900 μM moenomycin (****1 a****) or (2) with 900 μM pentasaccharide* ***7*** *are shown. B) Competitive elution (flow rate: 25 μL min⁻, contact time: 300 s, 25 °C): Immediate injection of moenomycin (****1 a****) at concentrations of (1) 0.1 mM, (2) 0.5 mM, and (3) 1 mM following PBP 1b (80 nM) injection.*

In the first series moenomycin A (**1 a**), disaccharide **2**, and trisaccharide **3**[29], which differ only in the oligosaccharide structure, were injected and analyzed. The trisaccharide **3** gave a relative extent of dissociation of PBP 1b from the surface of 88% at 1 mM **3**, 66% at 0.5 mM, and 26% at 0.1 mM, and showed a slightly reduced affinity for the protein ($B_{50} = 2.6 \times 10^{-4}$ M) compared to moenomycin **1 a** (1 mM: 100%, 0.5 mM: 82%, 100 μM: 42% dissociation; $B_{50} = 1.3 \times 10^{-4}$ M) under the experimental conditions used. However, the disaccharide **2** displayed a highly reduced affinity (27% dissociation at 1 mM; $B_{50} = 1.9 \times 10^{-3}$ M). These results reveal that the affinities of **1 a**, **2**, and **3** for PBP 1b correlate nicely with the MIC values: trisaccharide **3**, which is still active as an antibiotic (see Table 1), displays a high affinity for PBP 1b, whereas the antibiotically inactive **2** binds to the enzyme to a much lesser extent. In order to probe the influence of the lipid part of the molecules, an elution experiment was performed with compound **10**,[31] which contains only units G,H, and I of moenomycin A (see Scheme 1 for structures of these units). **10** was unable to dissociate PBP 1b from its complex with immobilized **6**.

Decarbamoyl moenomycin (**1 b**)[32] and derivative **1 c**[33] provided a nice confirmation of the reliability of the method. We have shown previously that removal of the carbamoyl group from moenomycin to give **1 b** is accompanied by the loss of antibiotic activity.[32] In keeping with this, **1 b** showed a markedly reduced affinity for PBP 1b ($B_{50} = 5 \times 10^{-4}$ M) in the SPR elution experiments when compared to moenomycin A (**1 a**). On the other hand removal of the chromophore part from **1 a** to give **1 c** is neither of consequence for the antibiotic activity nor for binding to the enzyme preparation (see Table 1).

Until now, selective recognition of the carbohydrate part of moenomycin by the enzyme has been postulated on the basis of structure–activity relationships. This binding was, however, virtually inaccessible by direct experimental means. The new assay seemed to offer the opportunity to demonstrate the binding unequivocally for the first time. Thus, the delipido derivatives **7**, **8**, and **9** were tested under the above experimental conditions in a second series of SPR elutions. The antibiotically inactive pentasaccharide **7** still displayed a definite, although much reduced, affinity for PBP 1b ($B_{50} = 3.3 \times 10^{-3}$ M) and even the tri- and disaccharides **8** and **9** showed very weak competition with **6** for PBP 1b binding ($B_{50} = 9.4 \times 10^{-3}$ M).

Conclusions

A new assay has been developed the results of which are in agreement with the MIC values (measured in *Staph. aureus*) of moenomycin and its structural analogues. The SPR results confirm for the first time that the trisaccharide fragment C-E-F-G-I-H (**3**) can be considered as the minimal structure that possesses all moieties sufficient for biological activity and for affinity towards the biological target PBP 1b. In contrast to the in vivo results, the lipid moiety is less important for in vitro binding of PBP 1b to moenomycin analogues. However, units G,H, and I alone are incapable of binding to PBP 1b, which demonstrates again the importance of the sugar moiety.

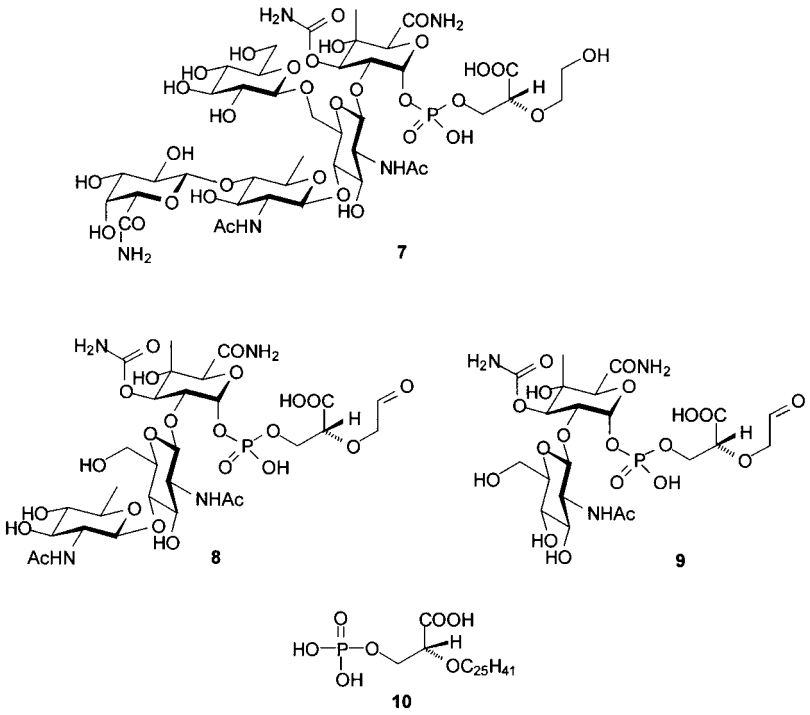
The information acquired from this assay may help to find novel transglycosylase inhibitors. Other applications are supported by the new assay, such as affinity purification of PBP 1b[28] and affinity labeling of the moenomycin binding site at the enzyme.[34]

Experimental Section

Moenomycin A was a gift from BC Biochemie GmbH (Frankfurt) and was purified prior to use as described previously.[35]

2-*O*-{2-Acetamido-4-*O*-[2-acetamido-2,6-dideoxy-β-D-glucopyranosyl]-3-*O*-carbamoyl-1-*O*-[(*R*)-2-carboxy-2-((2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylennonadeca-2,6,13,17-tetraen-1-yloxy)-ethoxy]-hydroxyphosphoryl}-4-*C*-methyl-α-D-glycopyranuronamide (2): A 40 °C solution (300 μL) of sodium periodate ($NaIO_4$; 1.05 g, 5 mmol) and sodium acetate trihydrate (1.38 g, 10 mmol) in 50% aqueous acetic acid (12 mL), which had been heated to 80 °C until a clear solution resulted, was added to a solution of **3** (30 mg, 26 μmol) in water (50 μL). The mixture was stirred at 40 °C for 5 h. Excess $NaIO_4$ was destroyed by addition of ethanediol (15 μL) and stirring at 20 °C for 1 h. At 0 °C a solution of ammonia (25%, 1 mL) was added and the mixture was stirred at 0 °C for 12 h. After neutralization with acetic acid, the reaction mixture was directly filtered through an LH-20 column (elution with water/methanol (1:4)). The solvent was evaporated and lyophilization was carried out before the product was purified by ultrafiltration (water (8 mL), Amicon YM1, 1000 Da molecular weight cut-off). After lyophilization, pure **2** (16 mg, 64%) was obtained. ^{1}H NMR (partly broad signals by micelle formation,[27] H,H COSY, 400 MHz, CD_3OD): $\delta = 0.95$ (br s, CH_3-23, CH_3-24^I), 1.23 (s with long range coupling, CH_3-4^F), 1.15 – 1.40 (m, CH_2-9^I), 1.59 (s), 1.60 (s), 1.66 (s), 1.73 (s, CH_3-19^I, CH_3-20^I, CH_3-21^I, CH_3-25^I), 1.86 – 1.91 (m, CH_2-10^I), 2.01 (s, NHCO-CH_3^E), 1.95 – 2.20 (m, CH_2-16^I, CH_2 – 15^I, CH_2-5^I, CH_2-4^I), 2.68 (d, CH_2-12^I), 5.08 – 5.15 (H-3^F, H-13^I, H-17^I), 5.25 – 5.45 (H-2^I, H-6^I, H-7^I), 5.94 (m, H-1^F) ppm, $J_{12I,13I} = 7.3$ Hz; ^{13}C NMR (50 MHz, CD_3OD, the sugar moiety carbons gave broad signals): $\delta = 16.25$ (CH_3-4^F, C-21^I), 17.87 (C-20^I), 23.46 (NHCOCH_3^E), 23.97 (C-25^I), 27.83 (C-19^I, C-16^I, C-23^I, C-24^I), 32.09 – 33.29 (C-5^I, C-10^I, C-4^I), 35.93 (C-8^I), 36.36 (C-12^I), 40.69 (C-15^I), 42.71 (C-9^I), 57.13 (C-2^E), 62.20 (C-6^E), 67.08, 68.41 (C-1^I, C-1^H), 71.49 – 79.15 (C-5^F, C-3^E, C-2^F, C-4^F, C-5^E, C-3^F, C-4^E), 95.87 (C-1^F), 103.88 (C-1^E), 109.36 (C-22^I), 122.84 (C-2^I), 123.40 (C-13^I), 125.19 (C-17^I), 126.83 (C-6^I), 141.42 (C-3^I, C-7^I), 159.33 (O$CONH_2^F$), 174.29 – 174.49 (NH$COCH_3^E$, $CONH_2^F$) ppm; ^{31}P NMR (150 MHz, D_2O): $\delta = -1.66$ ppm; calcd for $C_{44}H_{72}N_3O_{18}P$: 962.04, 961.48; FAB MS: *m/z* found: 1000.2 $[M+K]^+$, 984.3 $[M+Na]^+$.

Expression and preparation of PBP 1b: The protein was purified from the membrane fraction of *E. coli* strain JM 109 (K-12 *rec*A1 Δ(*lac-pro*AB) *end*A1 *gyr*A96 *thi*1 *hsd*R17 *sup*E44 *rel*A1 F′ [*tra-D*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]) carrying the plasmid pJP13. This strain expresses the structural gene of PBP 1b of *E. coli*. Cells were grown in modified Lennox broth (Bacto-Trypton (10 g L^{-1}), yeast extract (5 g L^{-1}), NaCl (5 g L^{-1}), ampicillin (100 μg mL^{-1})) to an optical density (OD_{550}) of 0.25, and the temperature was increased from 30 °C to 42 °C to induce transcription and overexpression of PBP 1b. After 4 h, cells were harvested by centrifugation (5000 × *g*), washed, and the cell pellet was frozen at −20 °C overnight. The cells were resuspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 7.6) containing 0.1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol (buffer A) or 0.05 M sodium phosphate buffer (pH 6.8) and sonicated (18 μm, 3 × 30 s). The lysed cell suspension was centrifuged at 100 000 × *g* for 90 min to obtain the membrane fraction. The supernatant was discarded. The pellet was resuspended in the same buffer and mixed with an equal volume of buffer containing 2% (w/v) Triton-X 100 and phenyl-



Scheme 4. Moenomycin analogues used in SPR competition experiments.

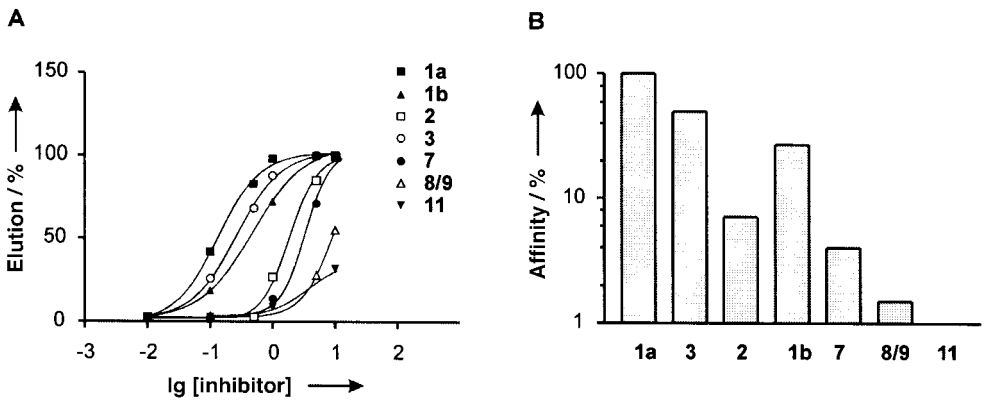


Figure 3. A) Determination of B_{50} values: elution response difference versus concentration for seven moenomycin derivatives. The elution response was normalized to the dissociation phase of PBP 1b in the absence of any test compound. The data were obtained by nonlinear regression analysis. B) B_{50} values were used to calculate the affinity of PBP 1b for different moenomycin derivatives and plotted on a logarithmic scale to display proportional changes.

methanesulfonyl fluoride (final concentration 2 mM) at 4 °C overnight or at 30 °C for 30 min to extract PBP 1b. Insoluble residues were removed by centrifugation. The extract was stored at −70 °C at a final protein concentration of 25 – 30 mg mL^{-1}, as quantified by the bicinchoninic acid (BCA) method.[36]

Purification of PBP 1b: Following membrane extraction, PBP 1b was purified by ampicillin affinity chromatography, as described previously.[19, 25] In order to remove hydroxylamine, the eluate was dialyzed against binding buffer (10 mM Tris-maleate (pH 6.6), 150 mM NaCl, 1 % Triton-X 100) after the chromatography step. The suspension was stored at −70 °C and used as protein for SPR measurements.

Separation, detection of PBP 1b, and protein determination: Samples prepared as described above were denatured at 95 °C for 5 min in SDS/mercaptoethanol buffer and submitted to analysis by electrophoresis on an 8.5 % SDS homogeneous polyacrylamide gel as described by Laemmli.[37] Proteins were detected by silver staining or Western blotting after separation of the mixture. For Western blotting, gels were transferred to nitrocellulose membranes (0.45 μm) in a semidry apparatus for 15 – 45 min.[38] Transfer of proteins onto the membrane was confirmed by staining with Ponceau S. Membranes were incubated with a rabbit anti-PBP 1b antibody as primary antibody and with a pork anti-rabbit alkaline phosphatase conjugated secondary antibody. PBP 1b was visualized with nitro-blue-tetrazoliumchloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Protein concentrations were determined by the BCA method,[36] with bovine serum albumin as a standard.

Surface plasmon resonance measurements: All SPR measurements were performed with a Biacore 3000 apparatus (BIACORE) with research grade sensor chips (Biacore 3000 Control Software 3.1.1, BIACORE).

a) Coupling of **6** to a sensor chip (CM5, BIACORE): An *N*-hydroxysuccinimide ester was formed on the sensor chip surface by treating the carboxy groups with a mixture of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) according to a procedure recommended by BIACORE. This active ester was used for the immobilization of **6**. The matrix of the analytic flow cell and also the reference cell were activated over 7 min with 0.05 M NHS and 0.2 M EDC at a flow rate of 5 μL min^{-1}. **6** (0.1 – 0.5 mg mL^{-1}) was dissolved in buffer (0.01 M 2-[4-(hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (pH 7.4), 0.15 M NaCl, 3 mM ethylenediaminetetraacetate, 0.005 (*v*/*v*) surfactant P20) and injected into the analytic flow cell over 14 min at a flow rate of 5 μL min^{-1}. Up to 300 RU loading of the carboxy groups was achieved. Residual active ester groups in both cells that had not reacted were subsequently deactivated over 7 min with 1 M ethanolamine at pH 8.5.

b) SPR competition experiments: Following the immobilization of **6**, the whole system was rinsed thoroughly with previously filtered running buffer at high flow rates. A purified and characterized solution of PBP 1b was carefully defrosted in ice and centrifuged to remove undissolved particles. The protein was injected in binding buffer (10 mM Tris-maleate, pH 6.6, 150 mM NaCl, 1 % Triton-X 100) at constant concentrations (50 nM – 500 nM) and bound to the immobilized **6** (the binding buffer and running buffer were identical). Moenomycin analogues were dissolved in binding buffer and injected at different concentrations to elute the bound protein. The interaction was investigated under constant conditions (125 μL substance solution, 25 μL min^{-1} flow rate). The difference in response indicated the percentage extent of elution of the analogue in comparison to the reference compound moenomycin. The regeneration of the sensor surface was performed by using 50 – 1000 μM moenomycin (**1 a**) in running buffer in order to remove any protein that was still bound to the surface.

c) Data analysis: Binding response (RU) was recorded continuously and presented as a sensorgram. The extent of elution was extracted from the difference between the RU values at the onset and at the end of the dissociation phase and these differences were used as report points to monitor the elution level. A plot of elution response difference versus substance concentration was used to estimate the concentration corresponding to 50 % elution. This B_{50} value was used as a measure of affinty under the experimental conditions. B_{50} values were determined by nonlinear regression analysis with the GraphPad Prism version 3.02 program.

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