Moenomycin-Mediated Affinity Purification of 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 at enzymes such as the penicillin binding protein 1b (PBP 1b). This binding has been employed to isolate PBP 1b by affinity chromatography. Suitable ligands have been prepared from moenomycin A and coupled both to affinity supports and to surface plasmon resonance sensor surfaces. The reactions that take place upon immobilization of the ligands to the affinity support and the sensor surface, respectively, have been studied in detail. With the help of surface plasmon resonance the optimal conditions for binding of PBP 1b to moenomycin-derivated ligands have been established. For the first time the selective binding of the moenomycin sugar moiety to the enzyme has been demonstrated.

KEYWORDS:

affinity ligands · antibiotics · glycolipids · peptidoglycan · transglycosylases

Introduction

Antibiotic resistance in bacterial pathogens has become a serious problem for human health, and countering this problem requires both the development of antiinfectives with novel modes of action and a deeper mechanistic understanding of already existing drugs.[1]

The shape of bacteria relies on a net-like multilayer polymer surrounding the cell. The polymer, peptidoglycan, consists of repeating β-1,4-linked *N*-acetylglucosaminyl-*N*-acetylmuramyl units cross-linked through short peptide side chains.[2] The biosynthesis of peptidoglycan is an essential pathway for bacteria and has no direct counterpart in eukaryotic cells. Defects or disruption of peptidoglycan or inhibition of its biosynthesis lead to cell lysis caused by osmotic pressure. The distinct stages of peptidoglycan biosynthesis offer attractive targets for the development of selective antibacterial agents.

The *Escherichia coli* peptidoglycan biosynthesis, which starts from a membrane-bound undecaprenyl-linked disaccharide precursor (lipid II), is completed by two successive reactions, a transglycosylation reaction leading to unbranched glycan strands[3] and a transpeptidation reaction cross-linking the peptide units of different strands.[2] Both reactions are catalyzed by the major high molecular weight penicillin-binding proteins (PBPs).[4] PBPs such as PBP 1a and 1b are bifunctional enzymes with two separate active sites, one for transglycosylation and one for transpeptidation. Each of these domains can specifically be 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 a number of antibiotics, including the moenomycins.[5] Of these, the moenomycins are the only compounds known to inhibit the enzyme (through the transglycosylase domain).[3] The structure – activity relationship of the moenomycins has been studied extensively,[6] and a mechanism for their mode of action has been proposed.[7, 8] It is assumed that they are anchored to the cytoplasmic membrane through the moenocinol lipid part, followed by highly selective recognition of the oligosaccharide moiety at a substrate binding site of the enzyme, most probably the binding site of the growing glycan chain (the glycosyl donor). Whereas the mechanism of the transpeptidation reaction is reasonably understood, the active site of the transglycosylase is still unknown and the mechanism of the transglycosylation step is largely unexplored. The moenomycins are a unique tool for elucidating the structure of the enzyme and the detailed mechanism of the transglycosylation reaction. Here we describe their use for the purification of the enzyme.

Traditional affinity chromatography has previously been employed for the purification of the membrane-bound PBP 1b

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*Supporting information for this article is available on the WWW under http://www.chembiochem.com or from the author. It includes the full IUPAC names of compounds **3b**, **5**, **11**, **1c**.*

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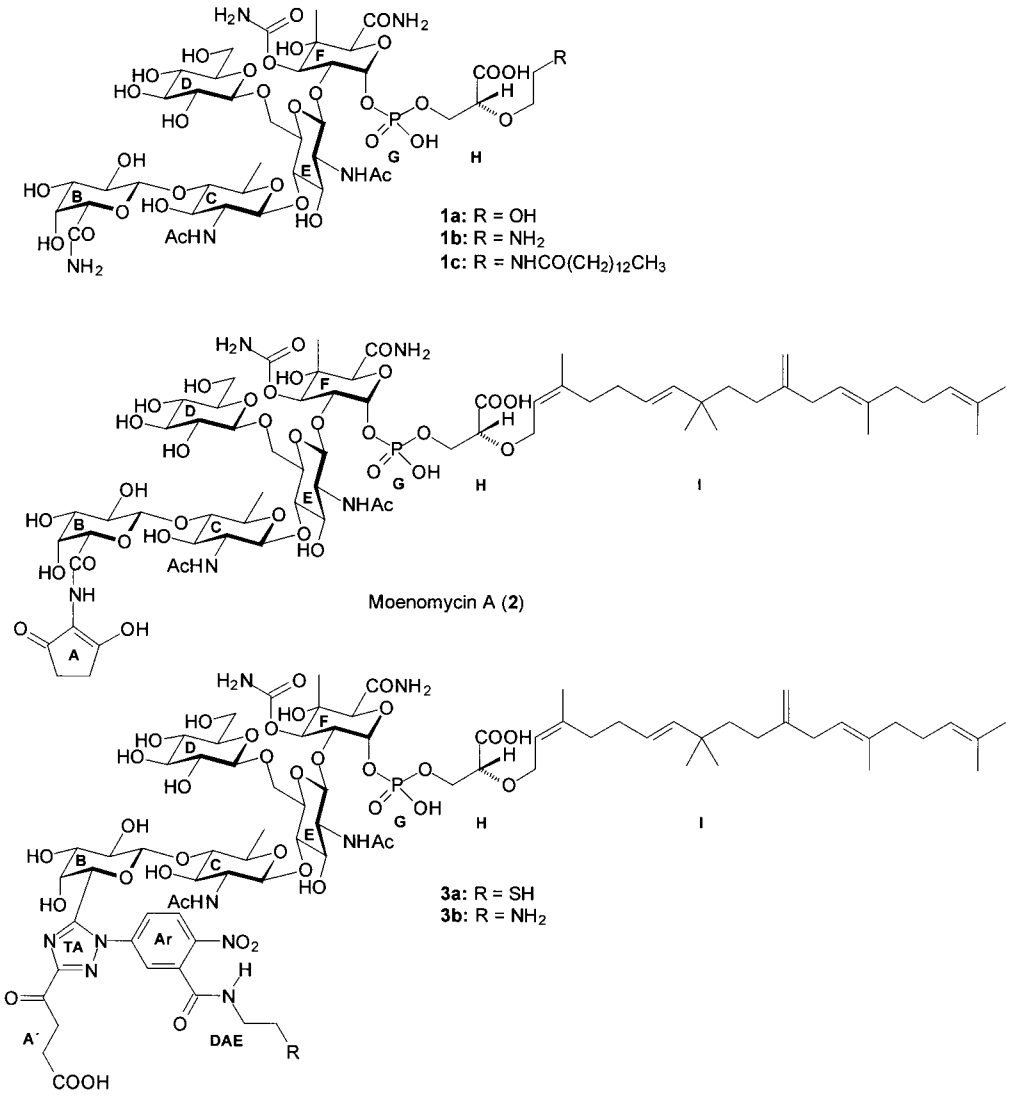
by using the interaction of β-lactam antibiotics with the transpeptidase domain of PBP 1b.[9–12] However, this method has a serious disadvantage: cleavage of the covalent bond between the immobilized antibiotic and PBP 1b for releasing the enriched protein from the affinity matrix requires drastic elution conditions (1 M hydroxylamine) that cause protein inactivation. Consequently, the isolated enzyme looses an appreciable part of its activity under these conditions.[13] A second procedure for the affinity isolation of PBP 1b requires the genetic introduction of an amino-terminal hexahistidine tag[14] and purification on a Ni^{2+}–nitrilotriacetic acid (Ni-NTA) agarose column.[8, 15] The reversible and noncovalent binding of the moenomycins to the active site of the transglycosylase domain offers the opportunity to perform an efficient affinity purification of PBP 1b without genetic manipulation. Here we describe the use of well-characterized moenomycin sepharoses for separation and enrichment of PBP 1b, a process combining the advantages of a very selective method with mild conditions to obtain highly purified protein from crude membrane extracts in a single chromatography step.

Results and Discussion

Immobilization of moenomycin A on sepharose

We have previously shown that reaction of diazonium salts with the enolized β-diketone unit A allows the introduction of functional groups into moenomycin (**2**) with reactivities orthogonal to those of all other functional groups.[16] In addition, this reaction occurs at a site that is of minor importance for the antibiotic activity.[6]

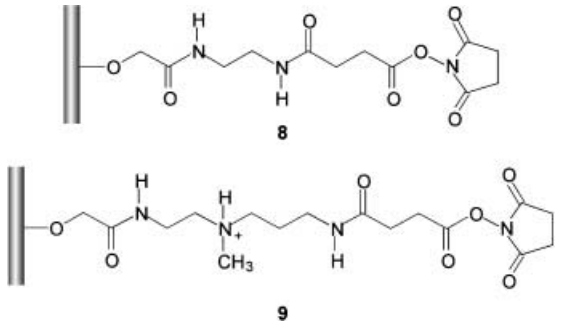
The primary coupling product undergoes a Japp-Klingemann rearrangement and the formed amidrazone cyclizes to give a triazol, the final product of the reaction (Scheme 1). By this sequence the thiol-substituted compound **3a** was prepared and used for the synthesis of a number of bioconjugates by 1,4-addition to maleimides[16] and by disulfide formation.[17] We report here that the amino compound **3b** is also available by this route.[18] The heterobifunctional reagent **6** was prepared from 5-amino-2-nitrobenzoic acid by using Staab's procedure.[19, 20] After complete formation (according to thin-layer chromatog-



Scheme 1. *Moenomycin A and derivatives with amino and thiol groups, respectively.*

raphy (TLC)) of the imidazolide (structure not shown), a large excess of ethylene diamine was added. Due to the different nucleophilicities of aromatic and aliphatic amino groups and the large excess of ethylene diamine used only the latter reacted with the imidazolide to form amide **6** in good yield. The selective formation of the aromatic diazonium salt from **6** is the consequence of the different pK values of the amino groups. Under the conditions of the diazonium salt formation the aliphatic amino group is protected by protonation. Amino compound **3b** appears to be a much more useful ligand than thiol **3a** since more coupling reactions are available for the preparation of bioconjugates including addition to isothiocyanates to give thioureas, reaction with activated carboxylic acids to provide amides, and use of the bifunctional linker squaric acid diethyl ester for the attachment to other amines.

For the immobilization at a chromatography support, reaction of **3b** with an *N*-hydroxysuccinimide (NHS) activated acid was used. Thus, **3b** was coupled with the two sepharose derivatives **8** and **9** (Affi-Gels 10 and 15, respectively; Scheme 2). The

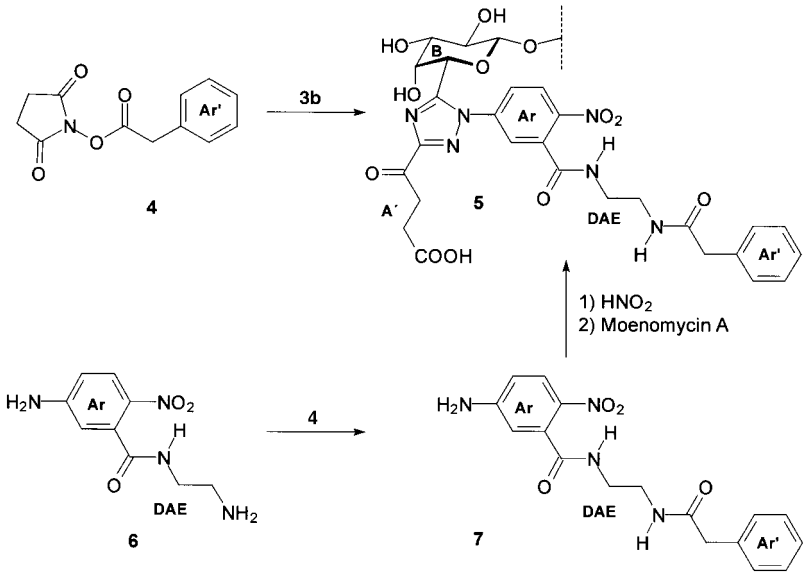


Scheme 2. *Affi-Gels 10 (**8**) and 15 (**9**).*

reaction was shown to proceed rapidly in 0.1 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (pH 7.4) or in methanol/water solution and was complete after 1 – 2 h. The amount of immobilized **3b** was determined by UV spectroscopic quantitation (280 nm) before and after immobilization, with simultaneous suppression of the NHS absorption. The data revealed a coupling efficiency of 50%. Nonfunctionalized moenomycin A (**2**) was coupled to an extent of less then 5% even after prolonged reaction times. The affinity sepharoses were stable for about 2 – 3 weeks when stored at 4 °C in binding buffer or 20% methanol, as determined by binding of protein PBP 1b to the immobilized moenomycin derivative.

The coupling of a compound like **3b** with its many functional groups to **8** and **9** poses, of course, the problem of selectivity. Thus, a model experiment was performed with NHS-activated phenylacetic acid **4**. This compound seemed to be a good model for the NHS-activated acid spacer on the matrix because it was anticipated that the structure elucidation of the coupling product of **3b** with **4** would be rather straightforward and that the reactivity of **4** would match that of the NHS-activated spacer acid.

Compound **4** was obtained from phenylacetyl chloride and *N*-hydroxysuccinimide. The reaction of **3b** with an excess of **4** was performed in methanolic solution at 20 °C under argon. After flash chromatography (FC) and gel filtration (to remove the excess of **4**) a pure acylated product was isolated in good yield. The reaction of **3b** with **4** was also performed in HEPES buffer (pH 7.4). Progress of the reaction was monitored by reversed-phase high-pressure liquid chromatography (HPLC). It was observed that under these conditions the consumption of **3b** (retention time (R_t) = 10.8 min) was complete after 1 h. Under both conditions the same product was formed (HPLC analysis). It was anticipated to have structure **5** (Scheme 3). The ^{1}H and ^{13}C NMR spectroscopic analysis with the help of two-dimensional (2D) experiments showed the presence of all expected structural units. All ^{1}H and ^{13}C signals (with the exception of some sugars signals) could be assigned. However, a definite proof of structure **5** by NMR spectroscopic methods failed. Thus, in the HMBC spectrum, long-range coupling of the protons at the C-2 position of the phenylacetic acid part ($\delta = 3.50$) and of protons with the signal at $\delta = 3.40$ to the phenylacetic acid C-1 atom ($\delta = 174.27$) was observed. The $\delta = 3.40$ signal could, however, not be assigned with certainty to the CH_2-2 group of the 1,2-diaminoethane part since it overlapped with signals of the sugar units. The ^{1}H,^{15}N COSY spectra in H_2O/D_2O (9:1) or in [D_6]DMSO were also inconclusive. The structure of **5** was therefore proven by chemical means, that is, by an unequivocal synthesis, because of these difficulties. Treatment of **6** with 0.9 of an equivalent of **4** in methanol/DMF solution gave **7** in 72% yield after FC. The HMBC spectrum of **7** in [D_6]DMSO showed long-range couplings of the CH_2-2 group (1,2-diaminoethane unit), the CH_2-2 group of phenylacetic acid part, and the amide proton CON*H* (phenylacetamide) to the C-1 atom of the phenylacetic acid unit. The protons CH_2-1 (DAE unit), the aromatic protons 6^{Ar}-H and 3^{Ar}-H,

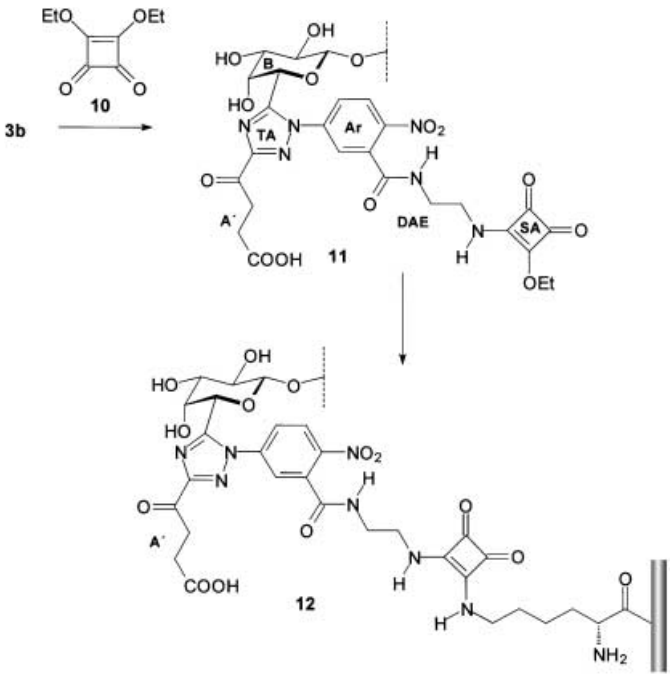


Scheme 3. *Selective acylation of the amino group of **3b**.*

and the benzamide CONH^{Ar} had long-range couplings to the benzamide CO carbon atom. These results fully confirm structure **7** and prove that the aliphatic amino group of **6** had been acylated. Diazotation of **7** and reaction of the diazonium salt with moenomycin A (**2**) were performed under the conditions used for the preparation of **3 b**. The product was obtained in 51 % yield after purification. From its mode of formation it must have structure **5**. The ^{1}H and ^{13}C NMR spectra of **5** obtained this way were identical to those of the compound prepared from **3 b** and **4**. Also the high-resolution electrospray ionization (ESI) ion cyclotron resonance (ICR) mass spectrometry (MS) of both compounds were practically identical. These results proved that the compound obtained from **3 b** and **4** did indeed have structure **5**. In agreement with this, there was no product formation when moenomycin A (**2**) was treated with **4** under the conditions of the reaction between **3 b** and **4**, even after 10 days (TLC control).

Recently, Rechenberg and Höltje published the preparation of a moenomycin sepharose and reported that the coupling of the ligand was achieved by treatment of NHS-activated sepharose (Affi-Gel 10, BIO-RAD) directly with moenomycin A (**2**).[21] Rechenberg and Höltje claimed that they immobilized 30 mg of moenomycin A per mL of the gel. According to the manufacturer's manual the coupling capacity is 15 μmol mL^{-1} (which corresponds to 23 mg of moenomycin A). The Rechenberg and Höltje result would thus indicate a 130 % coupling efficiency. Even with **3 b** we were unable to reach a substitution degree of higher than 50 %. In addition, as described above, in our hands there was no reaction between moenomycin A and an NHS-activated carboxylic acid in the methanolic and buffer systems, respectively. In conclusion, Rechenberg and Höltje have most likely used a column with nonspecifically bound (without covalent bonds) moenomycin A in their experiments.

A second immobilization procedure made use of the bifunctional linker squaric acid diethyl ester (**10**).[22] Thus, **3 b** on treatment with **10** in either phosphate buffer (pH 7.3) or methanolic solution provided **11** in yields of 56 % and 67 %, respectively. The reaction of **11** and lysine sepharose 4B was then performed in buffer solution at pH 9.1 to give **12** (Scheme 4).



Scheme 4. *Immobilization of **3 b** on Lysine Sepharose 4B.*

Purification of PBP 1b by using immobilized 3 b

Isolation of PBP 1 b[23] from membranes of *E. coli* strain JM109/pJP13, which overexpresses PBP 1b,[24, 25] was achieved by extraction of the membrane fraction with 1 % (w/v) detergent and precipitation with ammonium sulfate (after extraction with sarcosyl), followed by ampicillin affinity chromatography as described previously.[12] The eluate was dialyzed against the buffer used in the experiments described below. This sample was used to optimize the conditions for the moenomycin affinity chromatography by surface plasmon resonance (SPR) experiments.[26] For this purpose, **3 b** was immobilized on a sensor chip surface carrying NHS-activated carboxylic acid groups. The ampicillin-purified PBP 1b was injected as soluble analyte under a series of different parameters and the interaction between PBP 1b and the immobilized **3 b** was detected on line. For regeneration of the sensor surface, bound PBP 1b was competitively eluted with moenomycin A (**2**). This analytical system allowed important quantitative information to be obtained about the binding profile of PBP 1b to conjugated **3 b** under different conditions (see the Experimental Section for buffer systems, pH values, salt concentrations, detergents, additives), and also allowed the development of a conceptionally new method for the isolation of PBP 1b with moenomycin derivatives conjugated to sepharose 4B. The interpretation of the SPR sensorgrams revealed that 10 – 25 mM HEPES or tris(hydroxymethyl)aminomethane (Tris)/maleate buffer (pH 5.5 – 6.5), 100 – 300 mM NaCl, and 0.5 – 1 % Triton X-100 or β-octylglucoside are optimal for binding of PBP 1b to immobilized **3 b**. These conditions differ strongly from those used for the affinity separation of PBP 1b through the ampicillin method. The optimal binding conditions could be easily transferred to the preparative affinity column with **3 b** coupled to Affi-Gel 10 (**8**) and 15 (**9**) and permitted the isolation and enrichment of PBP 1b by competitive elution with moenomycin A (**2**). This chromatography procedure offers an alternative for the enrichment of PBP 1b from crude membrane extracts under much milder conditions than those used in the chromatography with ampicillin. Proteins were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining and Western blotting with anti-PBP 1b antibodies (Figure 1).

Purification of PBP 1 b by using immobilized 1 b

As discussed above it is the current opinion that moenomycin A, after membrane anchoring, interacts highly selectively with PBP 1b through its sugar moiety. It seemed rewarding to make use of these interactions for a more rational affinity isolation of

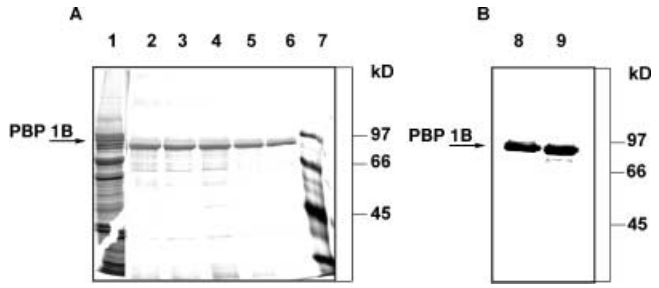


*Figure 1. Purification of PBP 1b by using immobilized **3 b**. A) Proteins were separated by SDS-PAGE and visualized by silver staining. Lane 1: membrane extract; lanes 2 – 6: fractions 2 – 6 after affinity chromatography; lane 7: molecular weight standard. B) Western blotting. Lane 8: fraction 2; lane 9: fraction 3.*

PBP 1b. Moenomycin fragment **1 a** which is inactive in vivo lacks the chromophore unit A and the lipid moiety but contains the complete oligosaccharide part. It is obtained from moenomycin A by ozonolysis followed by $NaBH_4$ reduction.[27] In preliminary SPR competition experiments constant amounts of PBP 1b were preincubated with fragment **1 a** at different ratios and the response of PBP 1b binding to the immobilized moenomycin **3 b** in the presence of fragment **1 a** was detected. Pleasingly, the antibiotically inactive fragment **1 a** displayed a measurable affinity towards PBP 1b. These results allowed a second affinity chromatography system to be established for PBP 1b. Fragment **1 b** (obtained from **2** by ozonolysis and subsequent reductive amination[28]) was immobilized on NHS-activated sepharoses **8** or **9**. The conjugated amount was calculated either from the UV absorption of the released NHS or by determining the mass of unbound **1 b** after gel filtration and lyophilization. In a model experiment, reaction of **1 b** with activated myristic acid (Staab procedure) occurred exclusively at the amino group to provide amide **1 c** in 42 % yield after purification. The spectroscopic data are in accord with structure **1 c**. The new amide ^{13}C signal appeared at $\delta = 177.3$. Under the conditions optimized by SPR a single purification step making use of **1 b** sepharose was sufficient to obtain pure PBP 1b (see Figure 2).

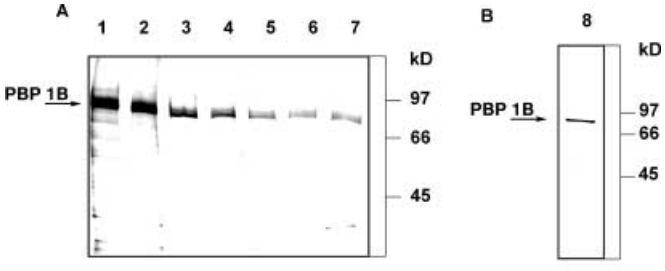


*Figure 2. Purification of PBP 1b by using immobilized **1 b**. A) Proteins were separated by SDS-PAGE and visualized by silver staining. Lanes 1 – 7: fractions 2 – 8 after affinity chromatography. B) Western blotting. Lane 8: pooled fractions 2 – 4.*

Conclusion

The enzyme PBP 1b represents an important target for antibiotics. The moenomycins have been shown to interact reversibly with the PBP 1b transglycosylase domain. These interactions were used to isolate PBP 1b in a single affinity-chromatographic step employing two well-characterized new moenomycin affinity supports. A second very important result is the first direct experimental proof of the interaction of PBP 1b with the pentasaccharide part of moenomycin A.

Experimental Section

Moenomycin A was a gift of BC Biochemie GmbH (Frankfurt)and was purified prior to use as described previously.[29]

5-Amino-N-(2-amino-ethyl)-2-nitrobenzamide (6): *N*,*N*′-Carbonyl diimidazole (CDI; 840 mg, 5.2 mmol) was added to a solution of 5-amino-2-nitrobenzoic acid (653 mg, 3.6 mmol) in pyridine (20 mL) and the mixture was stirred at 20 °C for 1 h. Progress of the reaction was monitored by TLC (MeOH/$CHCl_3$ (1:1), $R_f = 0.41$). Then, a solution of ethylene diamine (1120 mg, 14 mmol) in pyridine (20 mL) was added and the mixture was stirred at 0 °C for 1 h and at 80 °C for 3 h (TLC in MeOH, $R_f = 0.24$)). The solvent was removed under reduced pressure and the residue was dissolved in methanol. Kieselguhr was added and after solvent evaporation the residue was transferred onto the top of a chromatography column. Flash column chromatography (FC) with MeOH as the eluent provided 0.62 g (77 %) of pure **6**. mp: 157 – 158 °C (petroleum ether); IR (KBr): $\tilde{\nu} = 3405, 3212, 1646, 1587, 1488, 1446, 1319, 1257\ cm^{-1}$; UV ($H_2O$): λ_{max} (ε) = 384 nm (12 160); ^1H NMR (200 MHz, [D_6]DMSO): $\delta = 2.62$ (t, 2 H, $J_{2\text{-}1} = 6.4$ Hz, CH_2-2^{DAE}), 3.16 (dt, 2 H, $J_{1\text{–}2} = 6.0$, $J_{1\text{–NH}} = 5.9$ Hz, CH_2-1^{DAE}), 6.46 (d, 1 H, $J_{6\text{–}4} = 2.6$ Hz, 6^{Ar}-H), 6.59 (dd, 1 H, $J_{4\text{–}3} = 9.1$, $J_{4\text{–}6} = 2.5$ Hz, 4^{Ar}-H), 6.74 (s, 2 H, NH_2^{Ar}), 7.89 (d, 1 H, $J_{3\text{–}4} = 9.1$ Hz, 3^{Ar}-H), 8.29 (t, 1 H, $J_{\text{NH–}1} = 5.6$ Hz, CON*H*^{Ar}); ^1H NMR (200 MHz, CD_3OD): $\delta = 2.84$ (t, 2 H, $J_{2\text{–}1} = 6.1$ Hz, CH_2-2^{DAE}), 3.41 (t, 2 H, $J_{1\text{–}2} = 6.1$ Hz, CH_2-1^{DAE}), 6.60 (d, 1 H, $J_{6\text{–}4} = 2.5$ Hz, 6^{Ar}-H), 6.69 (dd, 1 H, $J_{4\text{–}6} = 2.5$ Hz, $J_{4\text{-}3} = 9.1$ Hz, 4^{Ar}-H), 8.01 (d, 1 H, $J_{3\text{–}4} = 9.1$ Hz, 3^{Ar}-H); ^{13}C NMR (50 MHz, [D_6]DMSO, APT): $\delta = 41.21$ (C-2^{DAE}), 42.92 (C-1^{DAE}), 111.98, 112.33 (C-6^{Ar}, C-4^{Ar}), 127.43 (C-3^{Ar}), 132.94 (C-1^{Ar}), 137.31 (C-2^{Ar}), 154.75 (C-5^{Ar}), 167.22 (*C*ONH^{Ar}); ^{13}C NMR (50 MHz, CD_3OD): $\delta = 41.79$ (C-2^{DAE}), 43.62 (C-1^{DAE}), 113.21, 114.11 (C-6^{Ar}, C-4^{Ar}), 128.73 (C-3^{Ar}), 134.92 (C-1^{Ar}), 137.60 (C-2^{Ar}), 156.26 (C-5^{Ar}), 171.51 (*C*ONH^{Ar}); calcd for $C_9H_{12}N_4O_3$: 224.21, 224.09094; fast atom bombardment (FAB) MS (matrix: 3-nitrobenzyl alcohol): *m*/*z* found: 225.1 $[M+H]^+$.

Moenomycin A amino derivative 3 b: 5-Amino-*N*-(2-amino-ethyl)-2-nitrobenzamide (**6**; 51 mg, 0.23 mmol) was dissolved in 6 % aqueous HCl (2 mL) and the solution was cooled in an ice bath. A solution of $NaNO_2$ (19 mg, 0.27 mmol) in water (0.8 mL) was added slowly to this solution and the resulting mixture was stirred for 15 min in an ice bath. The solution of the diazonium salt was slowly (in 0.3 mL portions) added to a stirred solution of moenomycin A (**2**; 300 mg, 0.19 mmol) and NaOAc (3 g) in water (150 mL). The progress of the reaction was monitored by HPLC (RP_{18} column; phosphate buffer (pH 8.0)/acetonitrile (6:4); flow rate: 0.5 mL min^{-1}) and TLC (*n*PrOH/H_2O (7:3); $R_f = 0.31$, $R_{f\,\text{moenomycin A}} = 0.45$). It was observed that after 1 h a product was formed (amidrazone, $R_t = 13.2$ min, $\lambda_{max} = 380$ nm). After 27 h this product had been 90 % converted into the final product (triazole, $R_t = 11.2$ min, $\lambda_{max} = 279$ nm). The reaction mixture was stirred at 20 °C under an argon atmosphere for an additional 24 h. Then the solution was filtered and inorganic salts were removed by FC (RP_{18} column; H_2O; 300 mL; the compound was eluted with MeCN/H_2O (1:2)) or by ultrafiltration (YM3 membrane, Amicon). The organic solvent was removed under reduced pressure and the water was removed by lyophilization. The product was purified by FC (SiO_2, *n*PrOH/H_2O (7:3)) to give 269 mg (78 %) of pure compound **3 b**. UV (H_2O): λ_{max} (ε) = 274 nm (6700); ^1H NMR (600 MHz,

CD_3OD/D_2O (5:1), $^1H,^1H$ COSY): $\delta = 0.95$ (s, 6H, CH_3-23,24^I), 0.97 (t, impurities from RP_{18} column), 1.25 (s, 3H, CH_3^F), 1.31 (d, 3H, $J =$ 5.8 Hz, CH_3-6^C), 1.35 (m, 2H, CH_2 – 9^I), 1.43 (m, impurities from the RP_{18} column), 1.60 (s, 3H, CH_3-20^I), 1.61 (s, 3H, CH_3-21^I), 1.67 (s, 3H, CH_3-19^I), 1.75 (s, 3H, CH_3-25^I), 1.89 (m, 2H, CH_2-10^I), 1.98 – 2.19 (m, 14H, CH_2-4, CH_2-5, CH_2-15, CH_2-16^I, and 2.04, 2.05 ($CH_3CONH^{C,E}$)), 2.62 (br s, 2H, CH_2-3$^{A'}$), 2.69 (d, 2H, $J_{12-13} = 7.3$ Hz, CH_2-12^I), 2.87 (t, impurities from the RP_{18} column), 2.97 (t, impurities from the RP_{18} column), 3.22 – 4.55 (m, many overlapping signals of the sugar protons and 3.33 (CH_2-2$^{A'}$), 3.76 (2^F-H), 4.05/4.18 (CH_2-1^I)), 5.09 (m; 3H, 13^I-H, 17^I-H, 3^F-H), 5.22 (s, 1H), 5.28 (dt, 1H, $J_{6-7} = 15.2$, $J_{6-5} = 6.9$ Hz, 6^I-H), 5.35 (d, 1H, $J_{7-6} = 15.7$ Hz, 7^I-H,), 5.43 (t, 1H, $J_{2-1} = 6.3$ Hz, 2^I-H), 5.87 (m, 1H, 1^F-H), 8.11 (d, 1H, $J_{2-6} = 2.1$ Hz, 2^{Ar}-H), 8.21 (dd, 1H, $J_{6-2} = 2.1$, $J_{6-5} =$ 8.9 Hz, 6^{Ar}-H), 8.43 (d, 1H, $J_{5-6} = 8.4$ Hz, 5^{Ar}-H); ^{13}C NMR (75 MHz, CD_3OD/D_2O (5:1), APT, HMQC): $\delta = 13.85$, 16.12 (CH_3^F), 16.25 (C-21^I), 17.81 (C-6^C), 17.94 (C-20^I), 23.44, 23.49 ($CH_3CONH^{C,E}$), 23.94 (C-25^I), 25.93 (C-19^I), 27.36 (C-16^I), 27.77, 27.80 (C-23, C-24^I), 30.28, 31.74 (C-3$^{A'}$), 31.96 (C-10^I), 32.42 (C-5^I), 33.21 (C-4^I), 35.89 (C-12^I), 36.28 (C-8^I), 38.89 (C-2^{DAE}), 39.96 (C-1^{DAE}), 40.42 (C-2$^{A'}$), 40.58 (C-15^I), 42.66 (C-9^I), 56.46, 56.96 (C-2^{C,E}), 62.20 (C-6^D), 67.05 (C-1^I), 68.67 (C-3^H), 69.78, 70.90, 71.11, 71.31, 71.98, 72.92, 73.22, 73.39, 73.66, 74.27, 74.65, 74.80, 75.96, 77.44, 77.58, 78.25 (unassigned signals of the sugar carbons), 81.30 (br, C-2^H), 81.80 (C-4^E), 83.66 (C-4^C), 95.75 (C-1^F), 102.45 (C-1^C), 103.05 (C-1^E), 103.78 (C-1^D), 104.51 (C-1^B), 109.34 (C-22^I), 122.86 (C-2^I), 123.40 (C-13^I), 125.15 (C-17^I), 126.35 (C-2^{Ar}), 126.83 (C-6^I), 127.42 (C-6^{Ar}), 128.60 (C-5^{Ar}), 132.53 (C-18^I), 134.33 (C-3^{Ar}), 137.35 (C-14^I), 141.35 (C-3^I,7^I), 142.78 (C-4^{Ar}), 147.18 (C-1^{Ar}), 151.27 (C-11^I), 155.16 (C-5TA), 159.15 ($OCONH_2^F$), 159.99 (C-3TA), 168.74 ($CONH^{Ar}$), 174.32 (C-6^F), 174.45 ($CH_3CONH^{C,E}$), 177.33 (C-1^H), 180.25 ($COOH^{A'}$), 195.27 (C-1$^{A'}$); ^{13}C NMR (100 MHz, D_2O, HMQC): $\delta = 15.18$ (CH_3^F), 15.95 (C-21^I), 17.06 (C-6^C), 17.72 (C-20^I), 22.73 ($CH_3CONH^{C,E}$), 23.63 (C-25^I), 25.78 (C-19^I), 26.73 (C-16^I), 27.37 (C-23, 24^I), 29.95 (C-3$^{A'}$), 31.02 (C-10^I), 31.53 (br, C-5^I, C4^I), 32.25 (C-2$^{A'}$), 34.98 (C-12^I), 35.49 (C-8^I), 37.90 (C-2^{DAE}), 38.87 (C-1^{DAE}), 39.85 (C-15^I), 41.74 (C-9^I), 55.09 (br, C-2^E, C-2^C), 60.90 (C-6^D), 65.00 – 78.00 (br, sugar carbons), 79.37, 80.09 (C-2^H, C-4^E), 81.98 (C-4^C), 94.63 (C-1^F), 101.11 (C-1^C), 102.63, 103.34 (br, C-1^{E,D,B}), 108.93 (C-22^I), 121.31 (C-2^I), 122.29 (C-13^I), 124.51 (C-17^I), 125.64 (C-2^{Ar}), 125.94 (C-6^I), 126.94, 127.81 (C-5, 6^{Ar}), 131.09 (C-18^I), 133.23 (C-3^{Ar}), 136.18 (C-14^I), 140.44 (C-3^I), 141.46 (C-7^I, C-4^{Ar}), 146.19 (C-1^{Ar}), 149.51 (C-11^I), 153.77 (C-5TA), 158.21 ($OCONH_2^F$), 158.82 (C-3TA), 167.58 ($CONH^{Ar}$), 172.72 (C-6^F), 173.91, 174.22 ($CH_3CONH^{C,E}$), 176.92 (C-1^H), 181.20 ($COOH^{A'}$), 195.24 (C-1$^{A'}$); calcd for $C_{78}H_{117}N_{10}O_{37}P$: 1817.80, 1816.73187; FAB MS: *m/z* found: 1817.6 $[M+H]^+$; ESI ICR MS (neg. mode): *m/z* found: 1815.71756 (calcd: 1815.72397) $[M-H]^-$, 907.35634 (calcd: 907.35804) $[M-2H]^{2-}$.

2,5-Dioxo pyrrolidine-1-yl phenylacetate (4): Commercial phenylacetyl chloride (0.4 mL, 2.2 mmol, $\rho = 1.167$) was slowly added to a solution of *N*-hydroxysuccinimide (0.25 g, 2.1 mmol) in dry CH_2Cl_2 (4 mL) and Et_3N (0.75 mL), and the mixture was stirred at 20 °C for 3 h. Then, Kieselguhr was added to the reaction mixture, and the solvents and the remaining reactant were removed by distillation at 10^{-2} mbar. The residue was purified twice by FC ($CHCl_3$/EtOAc (4:1), $R_f = 0.55$) gave 200 mg (41%) of pure **4**. mp: 118.5 °C (cyclohexane); IR (KBr): $\tilde{\nu} = 3438$, 1814, 1779, 1733, 1363, 1207, 1068, 736, 709, 646 cm^{-1}; UV (CH_3OH): λ_{max} (ε) = 299.5 nm (3239); ^1H NMR (200 MHz, $CDCl_3$): $\delta = 2.76$ (s, 4H, CH_2 – 3^{pyr}, CH_2 – 4 ^{pyr}), 3.92 (s, 2H, CH_2-2), 7.35 (m, 5H, 2-, 4-, 5-, 6-H$^{Ar'}$); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 25.59$ (C-3^{pyr}, C-4^{pyr}), 37.61 (C-2), 127.82 (C-4$^{Ar'}$), 128.86 (C-3$^{Ar'}$, C-5$^{Ar'}$), 129.33 (C-2$^{Ar'}$, C-6$^{Ar'}$), 131.48 (C-1$^{Ar'}$), 166.83 (C-1), 169.13 (C-2^{pyr}, C-5^{pyr}); calcd for $C_{12}H_{11}NO_4$: 233.22, 233.06881; FAB MS: *m/z* found: 234.1 $[M+H]^+$.

***N*-[2-(5-Amino-2-nitrobenzamido)ethyl]benzeneacetamide (7)**: Compound **4** (34 mg, 0.14 mmol) in MeOH (0.5 mL) and DMF (0.2 mL) was added dropwise to a stirred solution of amine **6** (36 mg, 0.16 mmol) dissolved in MeOH (0.5 mL). The reaction mixture became heterogeneous. More DMF (1.3 mL) was added until a homogeneous mixture resulted. This solution was stirred at 20 °C for 3 h (progress of the reaction was monitored by TLC ($MeOH/CHCl_3$ (1:5), $R_f = 0.54$)). The solvents were removed under reduced pressure (10^{-2} mbar). The residue was purified three times by FC ($MeOH/CHCl_3$ (1:5)) and then a further two times by FC ($MeOH/CHCl_3$ (3:10)) to give 38 mg (72%) of pure **7**. ^1H NMR (600 MHz, $[D_6]$DMSO, HMBC): $\delta = 3.21$ (m, 2H, CH_2-2^{DAE}),[30] 3.24 (m, 2H, CH_2-1^{DAE}),[30] 3.42 (s, 2H, CH_2-2), 6.48 (d, 1H, $J_{6-4} = 2.2$ Hz, 6^{Ar}-H), 6.61 (dd, 1H, $J_{4-6} = 2.3$, $J_{4-3} = 9.4$ Hz, 4^{Ar}-H), 6.74 (s, 2H, NH_2-5^{Ar}), 7.20 – 7.33 (m, 5H, 2-, 3-, 4-, 5-, 6-H$^{Ar'}$), 7.91 (d, 1H, $J_{3-4} = 9.4$ Hz, 3^{Ar}-H,), 8.04 (br, 1H, N*H*CO-CH_2-Ar'), 8.34 (br, 1H, N*H*CO-Ar); ^{13}C NMR (50 MHz, $[D_6]$DMSO, HMBC): $\delta = 38.79$ (C-2^{DAE}),[30] 39.40 (C-1^{DAE} under solvent signals, HMBC),[30] 43.07 (C-2), 112.52 (C-6^{Ar}), 113.05 (C-4^{Ar}), 126.96 (C-4$^{Ar'}$), 128.07 (C-3^{Ar}), 128.83 (C-3$^{Ar'}$, C-5$^{Ar'}$), 129.65 (C-2$^{Ar'}$, C-6$^{Ar'}$), 133.59 (C-2^{Ar}), 136.94 (C-1$^{Ar'}$), 137.61 (C-1^{Ar}), 155.32 (C-5^{Ar}), 167.91 ($CONH^{Ar}$), 170.95 (C-1); calcd for $C_{17}H_{18}N_4O_4$: 342.45, 342.13280; FAB MS: *m/z* found: 343.13 $[M+H]^+$, 365.10 $[M+Na]^+$.

Moenomycin A model conjugate 5:

a) Synthesis from **3b** and **4**: A solution of **4** (26 mg, 0.113 mmol) in MeOH (0.5 mL) was added to a solution of **3b** (119 mg, 0.075 mmol) in MeOH (10 mL). The not completely clear mixture (which became clear after a few hours) was stirred at 20 °C under argon for 43 h. Then, Kieselguhr was added to the reaction mixture, and the solvents were evaporated under reduced pressure. The residue was transferred to a FC column. Elution with *n*PrOH/H_2O (7:2) ($R_f = 0.34$) led to a fraction containing **5** and **4**. Gel filtration chromatography (LH-20 column, MeOH/H_2O (3:1)) was used to remove the remaining starting material **4**. Solvent evaporation and lyophilization gave 65 mg (45%) of pure product **5**.

b) HPLC investigation of the reaction of **3b** with **4**: **3b** (5.7 mg, 0.0031 mmol) and **4** (0.14 mg, 0.0006 mmol) in Hepes buffer (10 mL, pH 7.4) were stirred under argon at 20 °C. Progress of the reaction was monitored by HPLC (C-18 column, phosphate buffer (pH 8.1)/acetonitrile (6:4); flow rate: 0.5 mL min^{-1}) with the samples injected directly onto the HPLC column. After 15 min the ratio of **5**:**3b** was 1:10 (**5**: $R_t = 13.8$ min, $\lambda_{max} = 279$ nm; NHS, $R_t = 5.1$ min; **3b**, $R_t =$ 10.8 min, $\lambda_{max} = 279$ nm). HPLC analysis after 1 h, 1.5 h, 4.5 h, and 23 h indicated the following ratios of **5**:**3b** (at 279 nm): 1:3.2, 1:2.9, 1:2.8, and 1:2.8, respectively. This means that the reaction was practically finished after 1 h.

c) **5** from **2** and the diazonoum salt derived from **7:** A solution of the amine **7** (40 mg, 0.117 mmol) in 6% aqueous HCl (1.5 mL) was cooled in an ice bath. A solution of $NaNO_2$ (21 mg, 0.30 mmol) in water (1 mL) was added dropwise and the mixture was stirred in an ice bath for 30 min. This diazonium salt solution was added dropwise to a precooled solution of moenomycin A (**2**; 178 mg, 0.11 mmol) and NaOAc (1.4 g) in water (90 mL). The cooling bath was removed and the mixture was stirred at 20 °C under argon for 42 h. Then inorganic salts were removed by FC (RP_{18} column; water; 200 mL). Further elution with H_2O/MeCN (2:1) gave a crude product which was purified by FC (SiO_2, *n*PrOH/H_2O (7:2), $R_f = 0.34$). Removal of acetonitrile under reduced pressure and water by lyophilization gave 115 mg (51%) of pure **5**. UV (CH_3OH): λ_{max} (ε) = 267.0 nm (14761); the ^1H and ^{13}C NMR spectra of the samples of **5** prepared by the methods described in (a) and (c) were completely identical; ^1H NMR (600 MHz, $CD_3OD/[D_6]$DMSO (17:1), $^1H,^1H$ COSY, HMQC, HMBC): $\delta = 0.94$ (s, 6H, CH_3-23^I, CH_3-24^I), 1.20 (s, 3H, CH_3^F), 1.33 (br, 5H, CH_2-9^I, CH_3-6^C), 1.56 (s, 3H, CH_3-20^I), 1.57 (s, 3H, CH_3-21^I), 1.63 (s, 3H, CH_3-19^I), 1.72 (s, 3H, CH_3-25^I), 1.86 (m, 2H, CH_2-10^I), 1.97 (br, 8H, $CH_3CONH^{C,E}$, 16^I-H_a, 15^I-H_a), 2.05 – 2.15 (br m, 15^I-H, 16^I-H_b, CH_2-4^I, 5^I-

H), 2.55 (CH_2-3$^{A'}$, under solvent signals), 2.65 (d, 2H, $J_{12-13}=9.5$ Hz, CH_2-12^{I}), 3.14 – 3.56 (broad overlapping signals of the sugar protons and 3.35 – 3.55 (CH_2-1, 2^{DAE}, HMQC), 3.50 (s, CH_2-2)), 5.00 – 5.15 (m, 3H, 13^{I}-H, 17^{I}-H, 3^{F}-H), 5.22 – 5.43 (m, 3H, 6^{I}-H, 7^{I}-H, 2^{I}-H), 5.83 (br, 1H, 1^{F}-H), 7.10 – 7.30 (m, 5H, 2-, 3-, 4-, 5-, 6-H $^{Ar'}$), 7.92 (br, 1H, 2^{Ar}-H), 8.06 (br, 1H, 6^{Ar}-H), 8.30 (br, 1H, 5^{Ar}-H); ^{13}C NMR (150 MHz, CD_3OD/[D_6]DMSO (17:1), HMQC, HMBC): $\delta=16.26$ (C-21^{I}), 16.63 (CH_3^{F}), 17.94 (C-20^{I}, C-6^{C}), 23,59 ($NHCOCH_3^{C,E}$), 24.23 (C-25^{I}), 26.08 (C-19^{I}), 27.71 (C-16^{I}), 27.96 (C-23, C-24^{I}), 32.02 (C-3$^{A'}$), 32.36 (C-10^{I}), 32.75 (C-5^{I}), 33.44 (C-4^{I}), 35.92 (C-12^{I}), 36.51 (C-8^{I}), 39.76 (C-2^{DAE}, HMQC), 40.95 (C-15^{I}), 41.08 (C-1^{DAE}, HMBC), 42.85 (C-9^{I}), 43.96 (C-2), 55.78 (C-2^{E}), 56.63 (C-2^{C}), 62.78 (C-6^{D}), 65 – 80 (br, unassigned signals of the sugar carbons), 82.39 (C-4^{E}), 85.33 (C-4^{C}), 95.83 (C-1^{F}), 102.93 (C-1^{C}), 104.11, 104.59 (C-1^{B}, C-1^{E}), 105.19 (C-1^{D}), 109.39 (C-22^{I}), 123.46 (C-13^{I}), 125.40 (C-17^{I}), 126.31 (C-2^{Ar}), 126.90 (C-6^{I}), 127.28 (C-5^{Ar}), 127.88 (C-4$^{Ar'}$), 128.79 (C-6^{Ar}), 129.62 (C-2$^{Ar'}$, C-6$^{Ar'}$), 130.31 (C-3$^{Ar'}$, C-5$^{Ar'}$), 132.22 (C-18^{I}), 135.09 (C-3^{Ar}), 137.06 (C-1$^{Ar'}$), 137.35 (C-14^{I}), 141.59 (C-7^{I}), 142.42 (C-1^{Ar}), 147.71 (C-4^{Ar}), 151.03 (C-11^{I}), 155.48 (C-5TA), 158.95 ($OCONH_2^{F}$), 160.26 (C-3TA), 167.60 ($CONH^{Ar}$), 173.44, 173.78, 174.15 ($CH_3CONH^{C,E}$, $CONH_2^{F}$), 174.27 (C-1), 176.70 (br, $COOH^{H}$), 179.82 (br, $COOH^{A'}$), 194.63 (C-1$^{A'}$); calcd for $C_{86}H_{123}N_{10}O_{38}P$ (1935.94, 1934.77373); ESI ICR MS (neg. mode) (BA 4-25): *m/z* found: 977.37325 (calcd: 977.37001) $[M+Na-3H]^{2-}$, 966.37863 (calcd: 966.37897) $[M-2H]^{2-}$, 643.91955 (calcd: 643.91667) $[M-3H]^{3-}$.

Control experiment: Treatment of moenomycin A with **4**: **2** (20 mg, 0.012 mmol) and **4** (4.4 mg, 1.5 equiv) in methanol (5.4 mL) were stirred under argon at ambient temperature for 10 days and then at 50 °C overnight. No products were observed by TLC (*n*PrOH/H_2O (7:2)).

Moenomycin A squaric ester amide conjugate 11:

a) Preparation in buffer solution: A solution of **3b** (90 mg, 0.049 mmol) and 3,4-diethoxy-3-cyclobutene-1,2-dione (**10**) (28 mg, 0.162 mmol) in 0.5 M phosphate buffer (10 mL, pH 7.3) was stirred at ambient temperature under argon for 48 h. Progress of the reaction was monitored by TLC (*n*PrOH/H_2O (7:2), $R_f=0.35$, $R_{f\,3b}=0.15$) and HPLC (RP_{18} column, phosphate buffer/acetonitrile (6:4); flow rate: 0.3 mL min^{-1}; $R_t=27.3$ min, $R_{t\,3b}=25.7$ min). The inorganic salts were removed by ultrafiltration (YM3 membrane, Amicon). Any water was lyophilized and the crude product was purified by FC (SiO_2, *n*PrOH/H_2O (7:2)). The organic solvent was removed under reduced pressure and the water was lyophilized to leave 53 mg (56%) of pure **11**.

b) Preparation in methanolic solution: **3b** (40 mg, 0.022 mmol) and **10** (5.2 mg, 1.4 equiv) in methanol (10 mL) were stirred at 20 °C under argon for 29 h. Progress of the reaction was monitored by TLC (*n*PrOH/H_2O (7:2), $R_f=0.35$). Then Kieselguhr was added and the solvent was evaporated under reduced pressure. The Kieselguhr containing the adsorbed compound was added to a FC column (SiO_2) and the compound was eluted with *n*PrOH/H_2O (7:2). Solvent evaporation and water lyophilization gave 29 mg (67%) of pure product **11**.

UV (H_2O): λ_{max} (ε) = 267.5 nm (19400); (MeOH): λ_{max} (ε) = 267.0 nm (35570); ^{1}H NMR (600 MHz, CD_3OD/D_2O (13:8), ^{1}H,^{1}H COSY): $\delta=0.95$ (s, 6H, CH_3-23, CH_3-24^{I}), 1.25 (s, 3H, CH_3^{F}), 1.35 (m, 5H, CH_3-6^{C}, CH_2-9^{I}), 1.44 (t, 3H, $J=6.9$ Hz, OCH_2CH_3), 1.58 (s, 3H, CH_3-20^{I}), 1.59 (s, 3H, CH_3-21^{I}), 1.65 (s, 3H, CH_3-19^{I}), 1.75 (s, 3H, CH_3-25^{I}), 1.88 (m, 2H, CH_2-10^{I}), 2.00 – 2.20 (13H, CH_2-4^{I}, CH_2-5^{I}, CH_2-15^{I}, CH_2-16^{I} and 2.06 (CH_3CONH-$^{C\,or\,E}$), 2.07 ($CH_3CONH^{E\,or\,C}$)), 2.66 (br, 4H, CH_2-12^{I}, CH_2-3$^{A'}$), 3.10 – 4.90 (broad overlapping signals of the sugar protons and 3.36 (CH_2-2$^{A'}$, ^{1}H,^{1}H COSY), 4.65 – 4.73 (OCH_2CH_3, ^{1}H,^{1}H COSY)), 5.05 – 5.12 (m, 3H, 13^{I}-H, 17^{I}-H, 3^{F}-H), 5.28 (br, 1H, 6^{I}-H), 5.34 (d, 1H, $J_{7-6}=15.7$ Hz, 7^{I}-H), 5.43 (br s, 1H, 2^{I}-H), 5.85 (br s, 1H, 1^{F}-H), 7.94/7.97 (br s/s, 1H, 2^{Ar}-H), 8.08 (br m, 1H, 5^{Ar}-H), 8.48 (m, 1H, 6^{Ar}-H); ^{13}C NMR (150 MHz, CD_3OD/D_2O (13:8), HMQC): $\delta=16.10$ (CH_3^{F}, OCH_2CH_3), 16.29 (C-21^{I}), 17.66 (C-6^{C}), 18.03 (C-20^{I}), 23.45 ($CH_3CONH^{C,E}$), 24.06 (C-25^{I}), 26.12 (C-19^{I}), 27.38 (C-16^{I}), 27.85 (C-23^{I}, C-24^{I}), 31.08 (C-3$^{A'}$), 32.02 (C-10^{I}), 32.39 (C-5^{I}), 33.09 (C-4^{I}), 35.86 (C-12^{I}), 36.24 (C-8^{I}), 40.55 (C-15^{I}), 41.03, 41.57 (C-1^{DAE}, probably due to two conformations), 42.62 (C-9^{I}), 44.57, 44.70 (C-2^{DAE}), 55.98 (C-2^{C}), 56.18 (C-2^{E}), 62.16 (C-6^{D}), 66.95, 68.34, 69.81, 70.02, 70.41 (OCH_2CH_3), 71.13, 71.36, 71.49, 71.91, 71.98, 72.91, 73.17, 73.51, 73.81, 74.18, 74.52, 75.97, 77.28, 77.43, 77.98 (unassigned signals mostly of the sugar carbons), 80.56 (br, C-2^{H}), 81.74 (C-4^{E}), 84.03 (C-4^{C}), 95.61 (C-1^{F}), 102.37 (C-1^{C}), 103.54 (C-1^{E}), 104.28, 104.36 (C-1^{B,D}), 109.49 (C-22^{I}), 122.38 (C-2^{I}), 123.31 (C-13^{I}), 125.16 (C-17^{I}), 125.97 (C-2^{Ar}), 126.71 (C-6^{I}), 127.77, 129.01(C-6^{Ar}, C-5^{Ar}), 132.36 (C-18^{I}), 134.47 (C-3^{Ar}), 137.19 (C-14^{I}), 141.35 (C-7^{I}), 141.86 (C-3^{I}), 142.15 (C-4^{Ar}), 147.30 (C-1^{Ar}), 150.92 (C-11^{I}), 154.98 (C-5TA), 159.09 ($OCONH_2^{F}$), 159.86 (C-3TA), 168.09 ($CONH^{Ar}$), 173.94, 174.54, 174.77 ($CONH_2^{F}$, $CH_3CONH^{C,E}$), 177.15 (br, C-1^{H}), 178.24, 178.79, 179.95 184.37 (C-1, 2^{SA}, $COOH^{A'}$), 190.06, 193.11 (C-3, 4^{SA}), 195.31 (C-1$^{A'}$); calcd for $C_{84}H_{121}N_{10}O_{40}P$: 1941.90, 1940.74792; FAB MS: *m/z* found: 1985.6 $[M-H+2Na]^+$, 1963.7 $[M+Na]^+$; ESI ICR MS: *m/z* found: 980.35465 (calcd: 980.35711) $[M-3H+Na]^{2-}$, 969.36430 (calcd: 969.36606) $[M-2H]^{2-}$, 645.90906 (calcd: 645.90807) $[M-3H]^{3-}$.

Control experiment with moenomycin A: Moenomycin A (**2**; 10 mg, 0.0063 mmol) and **10** (1 mg, 0.006 mmol) in methanol (10 mL) and Et_3N (2 drops) were stirred at 20 °C under argon. Progress of the reaction was monitored by TLC (*n*PrOH/H_2O (10:3)). Even after 21 h, no new products were observed.

Moenomycin A derivative 1c: A solution of myristic acid (43.2 mg, 189 μmol) und CDI (30.7 mg, 189 μmol) in THF (1 mL) was stirred at 20 °C for 1 h. The solvent was evaporated under reduced pressure. A suspension of **1b** (45 mg, 38 μmol) in DMSO (2 mL) was added and the mixture was sonicated with stirring at 20 °C for 12 h. The reaction mixture was then filtered through a Sephadex LH-20 column (elution with H_2O/CH_3OH (1:4)). After solvent evaporation the residue was adsorbed onto Kieselguhr (300 mg) and this material was transferred to a FC column. Elution with ethyl acetate/*i*PrOH/H_2O (6:4:3) provided a specimen of **1c** that was further purified by chromatography on a Sephadex PD-10 column (elution with H_2O) to give, after lyophilization of the water, 22 mg (42%) of pure **1c**. ^{1}H NMR (400 MHz, D_2O, H,H COSY): $\delta=0.76$ (br s, CH_3-14^{K}), 1.13 (s, CH_3-4^{F}), 1.10 – 1.25 (m, CH_2-4^{K} – CH_2-13^{K}), 1.28 (d, $J_{5C-6C}=5.9$ Hz, CH_3-6^{C}), 1.44 – 1.56 (br m, $J_{2K-3K}=7.1$ Hz, CH_2-3^{K}), 1.93, 2.00 (2 × s, $NHCOCH_3^{E}$, $NHCOCH_3^{C}$), 2.17 (dd, $J_{2K-3K}=7.1$ Hz, CH_2-2^{K}), 3.20 (dd, $J_{1D-2D}=8.0$ Hz, $J_{2D-3D}=8.4$ Hz, H-2^{D}), 4.12 (s, H-4^{B}, H-5^{B}), 4.36 (s, H-5^{F}), 4.42 (d, $J_{1D-2D}=8.0$ Hz, H-1^{D}), 4.93 (d, $J_{2F-3F}=10.3$ Hz H-3^{F}), 5.69 (m, H-1^{F}); ^{13}C NMR (100 MHz, D_2O, APT): $\delta=14.37$ (CH_3-14^{K}), 16.15 (CH_3-4^{F}), 17.78 (CH_3-6^{C}), 23.40 ($NHCOCH_3^{E}$, $NHCOCH_3^{C}$), 25.70, 26.78 (C-13^{K}, C-3^{K}), 30.03 – 30.33 (C-4^{K} – C-11^{K}), 32.66 (C-12^{K}), 34.75 (C-2^{K}), 40.49 (C-2^{I}), 56.43 – 56.56 (C-2^{E}, C-2^{C}), 62.38 (C-6^{D}), 68.34 (C-1^{H}), 69.62 – 78.27 (C-1^{I}, C-5^{C}, C-4^{D}, C-4^{B}, C-2^{B}, C-3^{B}, C-6^{E}, C-5^{B}, C-3^{C}, C-5^{F}, C-3^{E}, C-5^{F}, C-2^{F}, C-4^{F}, C-5^{E}, C-2^{D}, C-3^{F}, C-5^{D}, C-3^{D}), 81.89 (C-4^{E}), 84.44 (C-4^{C}), 95.66 (C-1^{F}), 102.71, 103.36, 104.11, 104.41 (C-1^{C}, C-1^{E}, C-1^{B}, C-1^{D}), 159.11 ($OCONH_2^{F}$), 173.93, 174.21, 174.47, 174.69 ($CONH_2^{B}$, $CONH_2^{F}$, $NHCOCH_3^{E}$, $NHCOCH_3^{C}$), 176.54 – 177.26 (C-3^{H}, C-1^{K}), some of the signals were rather broad; ^{31}P NMR (81 MHz, D_2O): $\delta=-1.32$; calcd for $C_{55}H_{95}N_6O_{33}P$: 1399.35, 1398.56; ESI ICR MS: *m/z* found: 1397.59111 (calcd: 1397.59049) $[M-H]^-$, 698.28128 (calcd: 698.27661) $[M-2H]^{2-}$.

Expression and preparation of PBP 1b: The protein was purified from the membrane fraction of *E. coli* strain JM109 [K-12 *recA*1 Δ(*lac-pro*AB) *endA*1 *gyrA*96 *thi*1 *hsdR*17 *supE*44 *relA*1 F′ (*tra-D*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] carrying the plasmid pJP13.[24, 25] 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_{550nm}) of 0.25, and the temperature was increased from 30 to 42 °C to induce transcription and overexpression of PBP 1b. After 4 h cells were harvested by centrifugation (5000 × *g*) and washed, and the cell pellet was frozen at −20 °C overnight. The cells were resuspended in 0.05 M 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) detergent (sarcosyl or Triton-X 100) and phenylmethanesulfonyl 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 and the pellet was extracted again with 1 M NaCl. Following the extraction with sarcosyl in buffer A or 0.05 M sodium phosphate buffer (pH 6.8), the membrane proteins were fractionated with ammonium sulfate on ice for 2 h or overnight at 4 °C. The fraction that precipitated at 0–41% saturation was collected by centrifugation. Extracts or ammonium sulfate pellets at protein concentrations of 25–30 mg mL^{-1} as quantified by the bicinchoninic acid (BCA) method[33] were stored at −70 °C. For further purification steps, the ammonium sulfate pellet was dissolved in buffer A or 0.05 M sodium phosphate buffer (pH 6.8) and dialyzed against the binding buffer required for affinity chromatography with ampicillin.

Coupling of 3b, 2, and 1b to the affinity support (Affi-Gel 10 or 15): The immobilization of moenomycin derivates to Affi-Gel 10 (**8**) or 15 (**9**) (BIO-RAD) was performed both in organic solvents and aqueous buffer solutions. The gel/**3b** suspension (4.5 mM **3b** per mL of gel in 0.1 M Hepes (pH 7.4) or methanol/water (1:2)) was gently agitated on a shaker for 1–2 hours at 20 °C or overnight at 4 °C. The immobilization of fragment **1b** (4.1 and 9.6 mM **1b** per mL of gel) was performed under the same conditions in methanol/water (1:2). The immobilization of moenomycin A (**2**, 9.5 mM moenomycin A per mL of gel) was performed over 48–72 hours at 4 °C, as described by von Rechenberg and Höltje.[21] Blocking of residual active esters was accomplished by adding 1 M ethanolamine (pH 8.5, 0.1 mL) per mL of gel after reaction for 1 h. Blocking can also performed in 0.1 M Tris-HCl (pH 8.0) for 3–4 hours at 20 °C or overnight at 4 °C. The gel was extensively washed with different buffers (variation of pH values and ionic strengths) to remove reactants and to elute adsorbed substances. Unbound sample was recovered and reused after purification. The amount of uncoupled **3b** or **2** was determined by dilution of an aliquot of the unbound effluent into 0.01 M HCl and measurement of the absorbance at 280 nm before and after coupling. Uncoupled **1b** was indirectly determined by measurement of the absorbance of released *N*-hydroxysuccinimide at 280 nm, or after gel filtration chromatography (30 g of Sephadex LH-20 or Sephadex PD-10 (Pharmacia); eluent: water/methanol (1:3) or water, respectively) and lyophilization, or by HPLC (5 μm LiChrospher 100 RP-18e; eluent: acetonitrile/buffer (40:60) where buffer = sodium heptanesulfonate (3 g), $K_2HPO_4 \cdot 3H_2O$ (26.2 g), KH_2PO_4 (0.6 g) in water to a final volume of 1 L (pH 8.2); flow rate: 0.5 mL min^{-1}; detection: diode array at 278 nm). When not in use, the column was stored at 4 °C in aqueous solution containing 0.2% sodium azide or 20% methanol.

Coupling of 11 to Lysine Sepharose 4B: The commercial Lysine Sepharose 4B (Pharmacia, 2 g, 0.032–0.056 mmol of active lysine amino groups) was washed with distilled water (500 mL). The mixture of **11** (149.6 mg, 0.077 mmol) and Lysine Sepharose (approximately 8 mL of gel) in buffer (10 mL; pH 9.1) was shaken for 19 h. The mixture was poured into a small column and washed with solution 1 (70 mL; 1 M NaCl (50 mL) and 0.05 M phosphate buffer (pH 7.5, 50 mL)) and then with solution 2 (50 mL; phosphate buffer (pH 7.5)). The collected eluents were desalted by UF (YM3) and, after lyophilization, 45 mg of pure **11** were recovered. The column was again washed three times repeating the above cycle of solution 1 (50 mL) then solution 2 (50 mL). Neither TLC nor HPLC indicated the presence of **11** in these solutions. It was calculated that nearly 96% of the lysine groups had reacted with **11** (54 μmol were immobilized) with this method.

Purification of PBP 1b: After membrane extraction with sarcosyl and precipitation with ammonium sulfate, the fraction containing PBP 1b was dialyzed against buffer A containing 1% sarcosyl and 1 M NaCl. Preparation of ampicillin/CH-Sepharose 4B and purification of PBP 1b by ampicillin affinity chromatography was performed as described previously.[12] In order to remove hydroxylamine the eluate was dialyzed against buffers as described below. The suspension was concentrated with polyethylene glycol 20 000 or Sephadex G-100 or G-200 at 4 °C, stored at −70 °C, and used as protein for SPR measurements. Extraction of membrane proteins for purification of PBP 1b with moenomycin affinity chromatography must be performed with buffer A or 0.05 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100. Following the extraction, the buffer of the mixture was exchanged by dialysis against binding buffer (25 mM sodium phosphate buffer, 10 mM Tris-maleate, 10 mM Hepes, or 25 mM Tris-HCl; pH 6.6–7.2; 150–300 mM NaCl; 1% Triton X-100). The suspension was applied to a moenomycin–sepharose column (2–3 mL bed volume) equilibrated in the same buffer. After 10 min incubation the column was intensely washed once with binding buffer (200 mL). From the moenomycin column, PBP 1b was mostly eluted in the first ten fractions (1.5 mL per fraction) with 100 or 200 μM moenomycin A (**2**) in binding buffer (flow rate: 0.2 mL min^{-1}). The fractions were collected, characterized by SDS-PAGE and Western blotting, and pooled.

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.[31] 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.[32] 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 porcine antirabbit 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,[33] 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 **3b** to a sensor chip (CM5, BIACORE): An *N*-hydroxysuccinimide ester was formed on the sensor chip surface by treating the carboxymethyl groups with a mixture of 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 **3b**. The matrix of the analytical flow cell and also the reference cell were activated during 7 min with 0.05 M NHS and 0.2 M EDC at a flow rate of 5 μL min^{-1}. Up to 40% loading of the carboxymethyl groups can be achieved.[34] **3b** (0.1–0.5 mg mL^{-1}) was dissolved in buffer (0.01 M Hepes (pH 7.4), 0.15

NaCl, 3 mM EDTA, 0.005 (v/v) surfactant P20) and injected into the analytical flow cell over 14 min at a flow rate of 5 μL min^{-1}. The activation time could be modified in order to tune the degree of immobilization. Residual active ester groups in both cells that remained unsaturated after immobilization of **3b** were subsequently deactivated over 7 min with 1 M ethanolamine at pH 8.5.

b) SPR measurements of PBP 1b and immobilized **3b**: Following the immobilization of **3b**, 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. A series of different protein concentrations (10 nM – 10 μM) was injected into both flow cells. The interaction was investigated with variety of different parameters (50 – 200 μL protein solution, flow rates of 5 – 35 μL min^{-1}, and contact times from 60 – 600 s). Stock solutions of all further additives (Triton X-100, β-octylglucosid, sarcosyl, sodium deoxycholate, SDS, NaCl, $MgCl_2$) were diluted in different binding buffer systems (sodium phosphate buffer, Tris-maleate, Hepes, or Tris-HCl with variation of molarity (10 – 250 mM) and pH values (4 – 8)) to different concentrations (0.1 – 1% detergent, 0 – 1000 mM salt). Binding buffers and running buffers were identical or well-balanced to eliminate bulk effects. The regeneration of the sensor chip surface was performed with 50 – 1000 μM moenomycin A (**2**) solution or 1% SDS in running buffer until no binding could be detected anymore and the response baseline was reached. For the competition experiments in solution, constant amounts of PBP 1b (400 nM) were mixed with **1a** in different concentrations (100 – 1000 μM) and preincubated at 20 °C for 5 min. An aliquot of this test reaction was injected at flow rates of 20 – 35 μL min^{-1} and the response of protein binding to immobilized **3b** in the presence of **1a** was detected.

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