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Synthesis of tools for raising antibodies against moenomycin epitopes and initial immunological studies

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Abstract—The moenomycins A and C_1 as well as penta-, di- and monosaccharide analogues have been conjugated to BSA and biotin, respectively. The moenomycin A–BSA conjugates have been used to raise polyclonal antibodies. It has been demonstrated that the antisera recognize moenomycin A.

1. Introduction

In view of the problem of antibiotic resistance[1] anti-infectives with novel modes of action are desperately needed. The transglycosylation reaction,[2] the second last step in the biosynthesis of peptidoglycan (the main structural polymer of the bacterial cell wall) occurs at the outside of the cytoplasmic membrane and is catalyzed by membrane proteins designated as bifunctional (class A) high molecular mass penicillin-binding proteins (PBPs).[3] The reaction came recently into focus as a promising new target for antiinfectives for a number of reasons: (i) the methods for isolating the enzyme(s) that are involved have improved considerably,[4,5] (ii) one of the substrates of the transglycosylation step, the so-called lipid II, can now be made in sufficient amounts,[6] and (iii) new and efficient in vitro test systems have been developed which conveniently allow to monitor the inhibition of the incorporation of lipid II into uncross-linked peptidoglycan[6,7] and binding of inhibitors to the enzyme, respectively.[8]

The assembly of the peptidoglycan polysaccharide strands from lipid II is blocked by certain glycopeptides,[9] ramoplanin,[10] lantibiotics such as nisin.[11] and the moenomycin-type antibiotics.[12] Ramoplanin and nisin interfere with lipid II. The mechanism of the glycopeptides that inhibit the transglycosylation reaction is not clear.[13] The moenomycins (see Scheme 1) are the only compounds known to inhibit the enzyme itself[2] (i.e. the transglycosylase domain of the bifunctional high-molecular PBPs). Based on structure–activity relationships,[14] it is assumed that they are anchored to the cytoplasmic membrane via the lipid part[15] and that the C–E–F trisaccharide part then blocks highly selectively the binding site of the growing polysaccharide strand at the enzyme.[4,16,17]

The moenomycins are, thus, unique tools for investigating both the transglycosylation step and the corresponding enzyme(s) as well as for the development of new transglycosylase inhibitors. For some time we have been involved in exploiting these tools based on the chemistry which permits to prepare compounds **2a**,[18] **2b**,[19] and **9b**.[20] Both the amino groups in **2b** and **9b**, and the thiol group in **2a** are orthogonal in their reactivities to all other functional groups present in **2b**, **9b**, and **2c** and have been used for immobilization reactions at chromatographic supports[5] and surface plasmon resonance (SPR) sensor chips,[8] respectively, and for the introduction of reporter groups allowing (i) to isolate PBP 1b by affinity chromatography,[5] (ii) to study binding of moenomycin analogues to PBP 1b by SPR,[8] (iii) to investigate the interaction of moenomycin with artificial membranes making use of fluorescence methods,[15] (iv) to perform initial studies aimed at identifying the moenomycin binding site at the enzyme by affinity labeling,[21] (v) to isolate aptamers that bind to moenomycin in the nanomolar range,[22] and (vi) to execute fluorescence correlation spectroscopy measurements.[23]

The incentive of selecting moenomycin aptamers arose from the idea that it would be useful to be able to detect moenomycin and moenomycin analogues (for example biosynthetic precursors[24] or metabolites) with high selectivity and sensitivity. Parallel to the selection of aptamers

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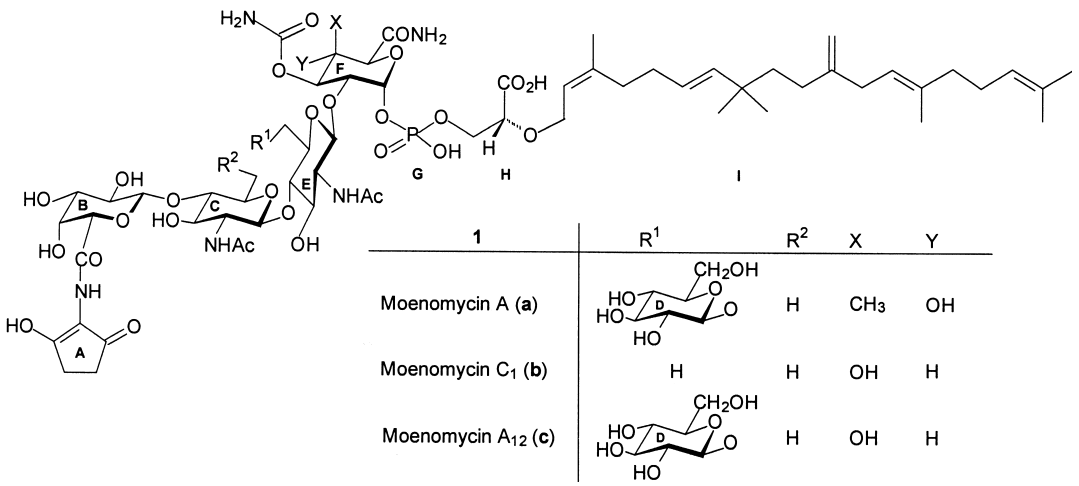
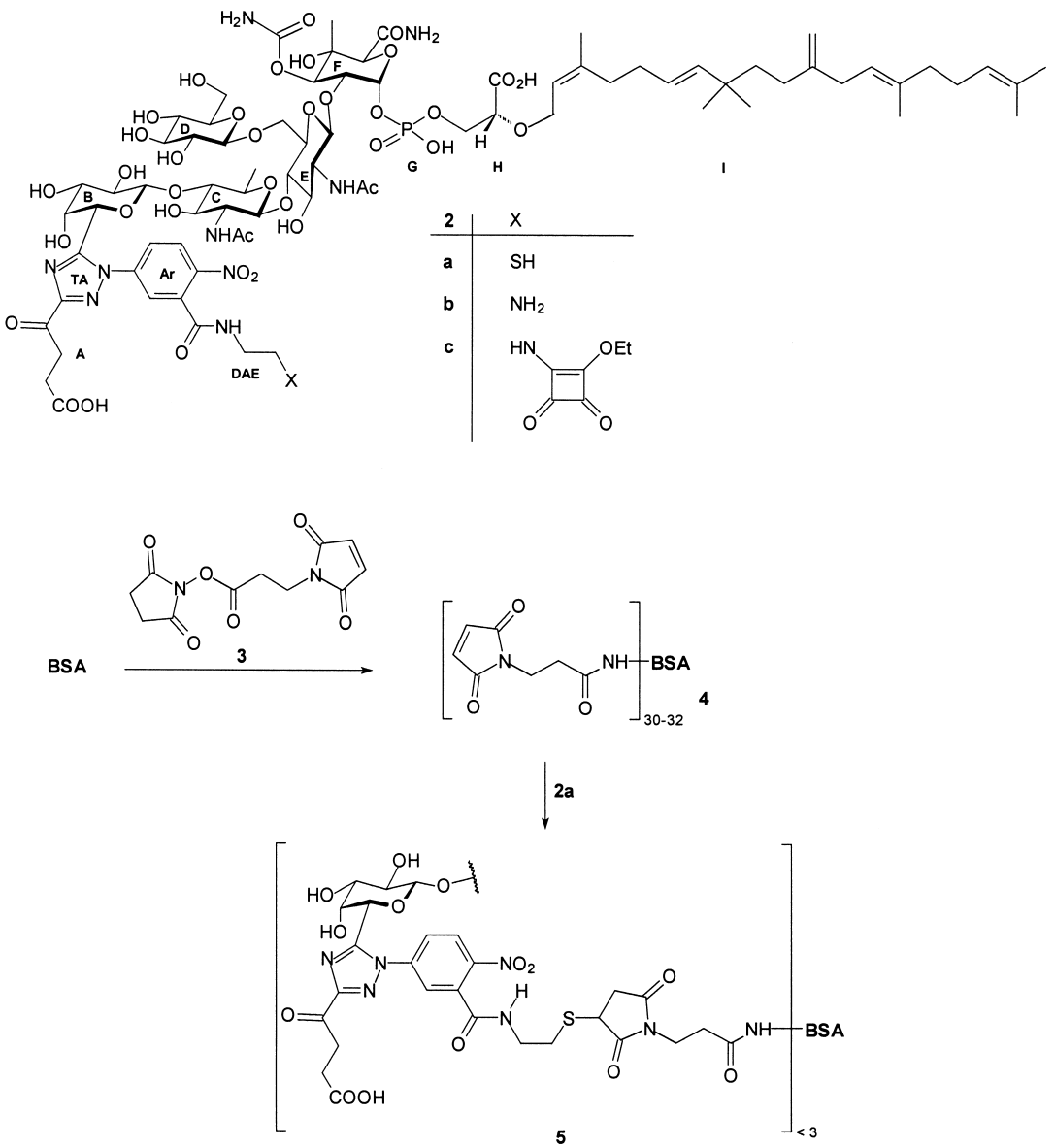
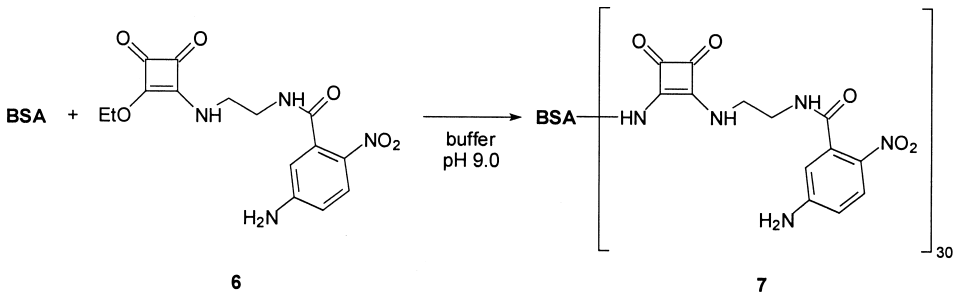


Scheme 1.



Scheme 2.



Scheme 3.

and with the same intention we have prepared moenomycin–bovine serum albumin (BSA) conjugates and have used them to raise anti-moenomycin antibodies. This is the subject of the present publication.

2. Results and discussion

2.1. Coupling of moenomycin to BSA

In order to exploit the SH-labeled moenomycin derivative **2a**, BSA was activated by reaction of the lysine amino groups with the heterobifunctional reagent **3**. SDS-PAGE showed a new band and MALDI TOF indicated that about 30–32 lysine residues had been converted to 3-maleinimidopropionamides. BSA derivative **4** was subsequently treated with the thiol **2a** (in fact a thiol mixture obtained from the moenomycin complex in which moenomycin A (**1a**) is the major component[25]) for 1 d at 5°C.

Purification was achieved by ultrafiltration. An aliquot was analyzed by MALDI TOF MS. The molecular ion signal was centered at m/z=79,000 indicating that on an average three moenomycin units were coupled to BSA. 2-Mercaptoethanol was then added to cap all unreacted maleimide groups (1 d at 20°C) (Scheme 2).

It seemed desirable to develop a conjugation procedure not demanding a capping step, which means changing the sequence of the two conjugation steps. Squaric acid diethyl ester was used for this purpose as a bifunctional linker. It is known that under neutral conditions only one of the ethoxy groups can be replaced by primary or secondary amines whereas the second one reacts only under basic conditions to form the corresponding diamide.[26,27] In a model experiment BSA was treated with 133 equiv. of squaric acid amide ester **6** (prepared from 5-amino-*N*-[2-aminoethyl)-2-nitrobenzamide and diethyl squarate) at pH 9.0 for 64 h at 20°C. After removing inorganic salts and the excess of **6** by ultrafiltration the residue was analyzed by MALDI TOF MS. From the molecular mass difference (center of the conjugate molecular mass around m/z=78,369 and the BSA molecular mass (internal standard) at m/z=68,932) it was concluded that an average of 30 residues of **6** were attached to BSA, a value well in accord with the fact that out of the 59 lysine groups present in BSA 30–35 are available for conjugation reactions (Scheme 3).[28]

Compound **2b** (obtained from pure moenomycin A) was first converted to squaric acid amide ester **2c** at pH 7.3 and this in turn coupled to BSA at pH 9.0 using an excess of 5 equiv. (a), 10 equiv. (b), and 20 equiv. (c) of **2c**. After removing inorganic salts and the excess of **2c** by ultrafiltration at 4°C the conjugates **8** were analyzed by MALDI TOF

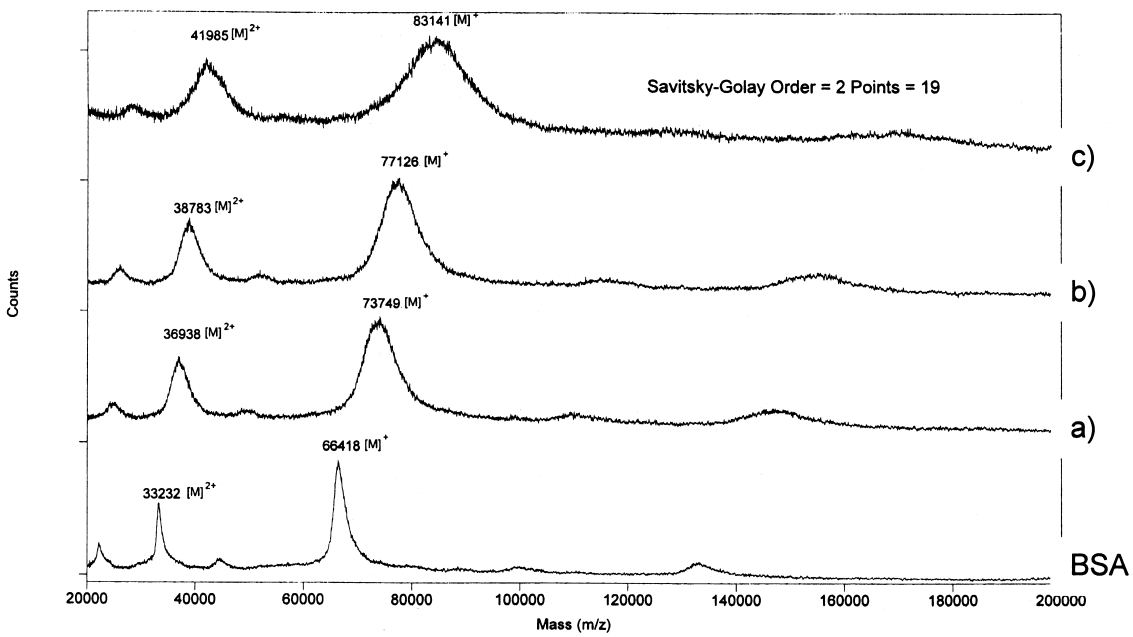
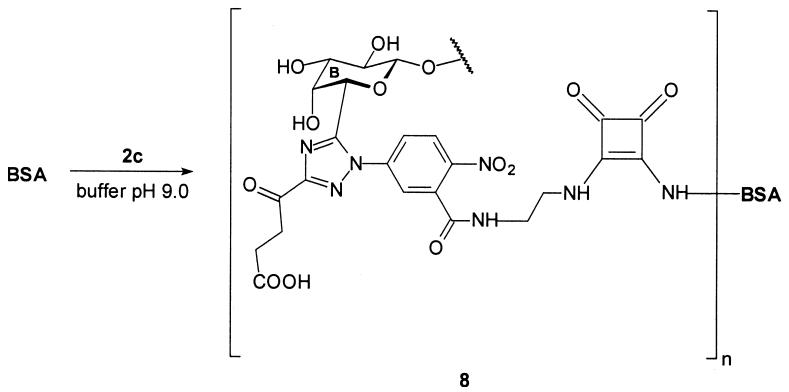


Figure 1. MALDI-TOF MS spectra of BSA and moenomycin–BSA conjugates **8** (experiments a–c, see text).



Scheme 4.

MS with BSA as external standard (m/z=66,418). The molecular ion signal of the conjugates **8** were centered at m/z=73,749 (a), m/z=77,126 (b) and m/z=83,141 (c) corresponding to average loading values of 3.9, 5.6 and 8.8, respectively (see Fig. 1). The ESI ICR MS with the deconvoluted molecular ion peak of **8** (from experiment a) centered at m/z=74,000 (BSA at m/z=66,400) confirmed the MALDI results (Scheme 4).

2.2. Coupling of pentasaccharide 9c to BSA

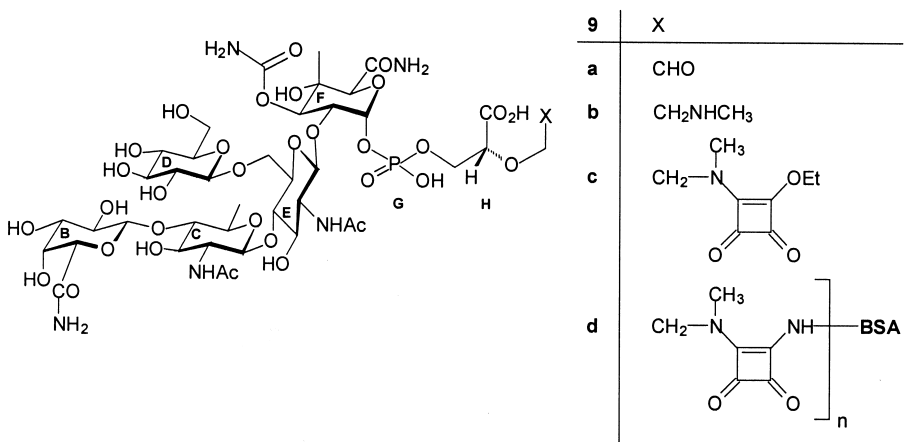
It is known from structure–activity studies[14] as well as from surface plasmon resonance measurements[8] that the antibiotic activity of the moenomycins is critically dependent on the sugar units and that there is a specific binding interaction of the sugar components with PBP 1b. It seemed, thus, useful to prepare conjugates of BSA and haptens consisting of moenomycin carbohydrate units that would allow to raise antibodies against selected areas of the moenomycin sugar chain (Scheme 5).

The known moenomycin pentasaccharide degradation product **9b**[20] was coupled to diethyl squarate at pH 7.0 to give ester amide **9c** in 61% yield. High resolution mass spectra were in accord with structure **9c**. As usual a mixture of obviously two conformers was observed by NMR giving rise to two *N*-CH_3-3 signals at δ=37.72 and 37.91 ppm.[26] Subsequently, **9c** was coupled to BSA at pH 9.0. Three experiments were performed with different **9c**–BSA ratios (1:9 (a), 1:18 (b), 1:43 (c)) to give **9d**. According to the MALDI TOF MS analysis on an average from 2.2 to 4.7 hapten units were attached to BSA (see Fig. 2).

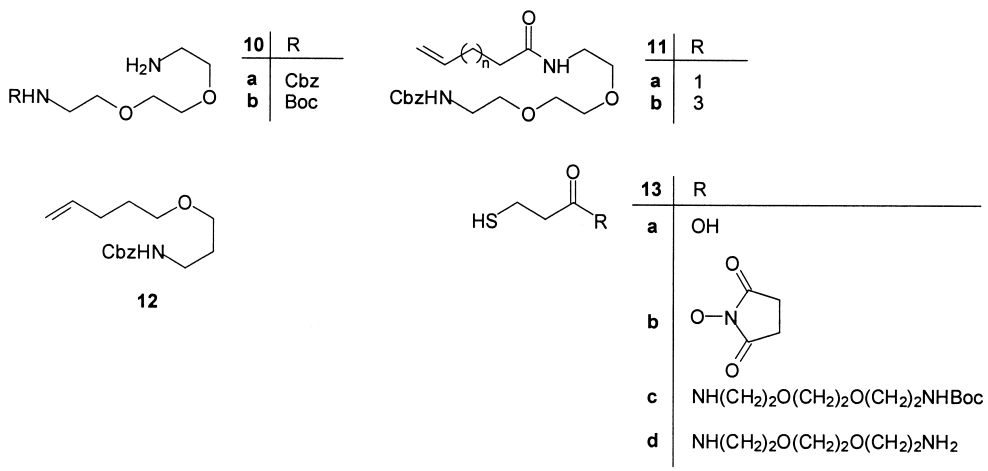
2.3. Coupling of the moenomycin C_1 and A_{12} type disaccharide 18b to BSA

It has been speculated that the branching methyl group present in unit F of moenomycin A (**1a**) and related antibiotics is introduced at a late stage of the biosynthesis and that compounds of the moenomycin C_1 and A_{12} type[25,29] (see **1b** and **1c**) with a D-galacturonamide part F are the biogenetic precursors.[24] With this in mind we have prepared a number of D-galacturonic acid-derived conjugates. In the course of this study some linker chemistry was tested. The olefinic linkers **11a** and **11b** were obtained from **10a**[30] and 4-pentenoic acid and 6-heptenoic acid, respectively, after activating the carboxylic acids with carbonyldiimidazole. Compound **12** was obtained from the Boc derivative of 3-bromopropylammonium bromide and 4-penten-1-ol by Williamson etherification. Finally, 3-mercaptopropionic acid (**13a**) was converted into hydroxysuccinimide derivative **13b**.[31] The latter compound on reaction with **10b**[32] furnished **13c** which was deprotected to give **13d** (Scheme 6).

Disaccharide **16a** on ozonolysis in methanol provided aldehyde **14** in 94% yield. **14** gave **15b** after reductive amination with **10a** (→**15a**, 76%) and hydrogenolytic



Scheme 5.



Scheme 6.

deprotection (61%). Allyl derivative **16a** on attempted metathesis reaction in $CHCl_3$ solution using the first generation Grubbs catalyst[33] failed to react with **11a**. The olefin **11b** with a longer distance between the double bond and the amide group provided **17a** in 21% yield as a mixture of stereoisomers. The cross metathesis of **16a** with **12** gave the best results in this series (→**17b**, 37%). **17a** and **17b** on hydrogenation of the double bond and concomitant removal of the protecting groups led to **19a** (84%) and **19b** (93%), respectively. Addition of **13d** to the allyl group of the disaccharide **16b** by irradiation at 254 nm[34] provided **18a** in 71% yield. **18a** on reaction with diethyl squarate at pH 7.2 furnished the ester amide **18b** in 66% yield. The structure of **18b** was in agreement with the NMR and high resolution mass spectra. Again, the ^{13}C NMR spectra showed two sets of signals for some of the carbons indicating the existence of two conformers of **18b** in solution. **18b** (15 equiv.) was coupled to BSA at pH 9.0 to furnish **18c**. After purification by ultrafiltration the MALDI TOF MS analysis revealed that an avarage of 12–13 units of **18b** were loaded onto BSA (Scheme 7).

2.4. Coupling of 21e, a monosaccharide analogue of moenomycins A_{12} and C_1, to BSA

Reaction of the known galacturonic acid derived compound **20a**[35] with the acid chloride of allyl hydrogen succinate yielded **20b** (77% yield). Subsequently, the acetal protecting group was removed quantitatively by treatment with 20% acetic acid at 50°C. The NMR spectra of **21a** had to be taken in polar solvents such as methanol-d_4 or pyridine-d_5, since in $CDCl_3$ solution only broad and unresolved signals resulted. The carbamoyl group was then introduced using the well-established two-step procedure:[35] (i) reaction with trichloroacetyl isocyanate (1.2 equiv., −6°C) to give the trichloroacetyl urethane, and (ii) reductive removal of the trichloroacetyl group with Zn dust in methanol. **21b** was obtained in 69% yield alongside with the 4-isomer **21c** (2%) and the dicarbamoyl derivative **21d** (10%). The structures followed from the characteristic ^1H and ^{13}C NMR chemical shifts (see Section 3). Selective cleavage of the allyl ester of **21b** was achieved according to Kunz and co-workers[36] by Pd(0)-mediated allyl transfer onto a soft nucleophile. Thus,

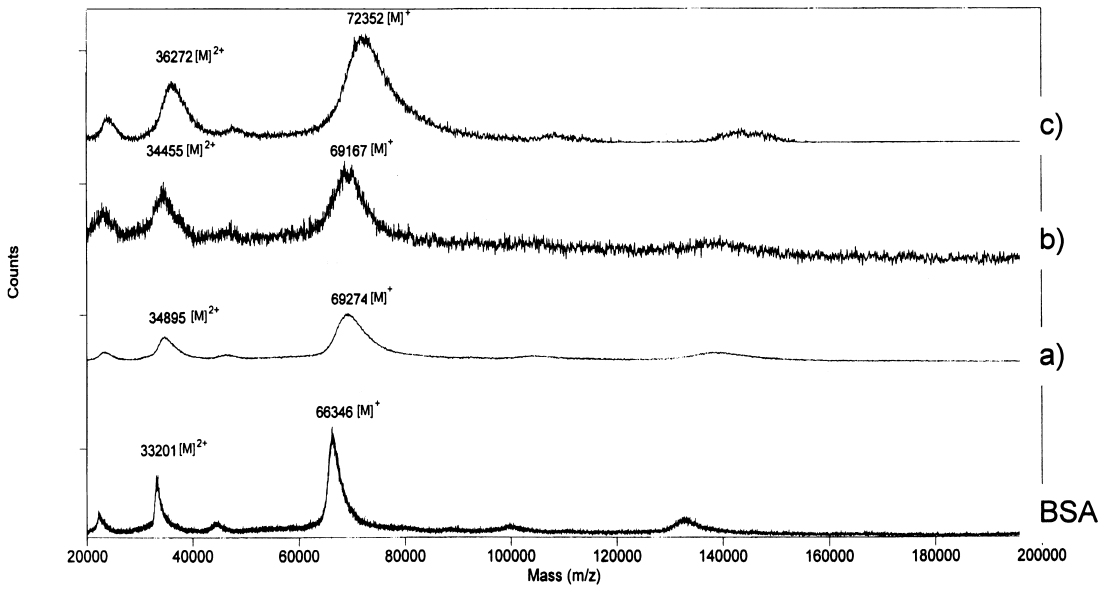
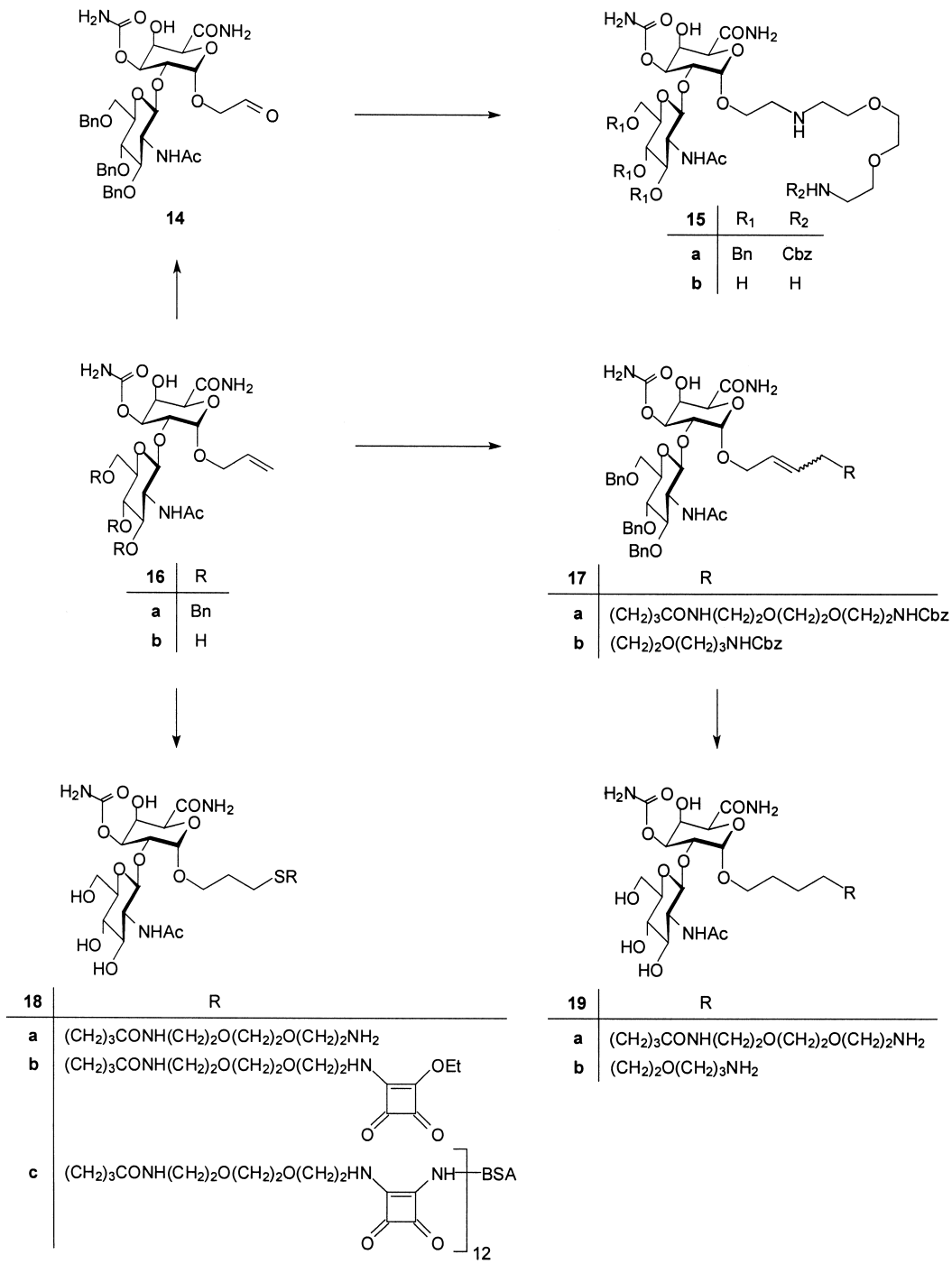


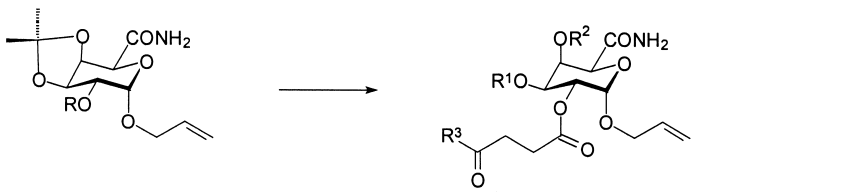
Figure 2. MALDI-TOF MS spectra of BSA and pentasaccaride–BSA conjugates **9d** (experiments a–c, see text).



Scheme 7.

21b furnished **21e** on treatment with 10 mol% of freshly prepared $Pd[PPh_3]_4$ and morpholine in THF. After removal of *N*-allyl morpholine and excess of morpholine with Dowex 50 (H^+) **21e** was isolated in 89% yield (Scheme 8).

Compound **21e** was activated through reaction with *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-diaminopropyl)carbodiimide (EDC) in DMF and coupled in a model experiment to glycine benzyl ester to give **21f** in moderate yield (not optimized). For the conjugation to BSA **21e** was activated in DMF solution as described above. Then BSA (3×10^{-3} equiv., dissolved in 50 mM phosphate buffer, pH 7.2) was added. The reaction mixture was left at 20°C for several days. The adduct **21g** was purified either by careful dialysis against water or by ultrafiltration. SDS-PAGE (see Fig. 3) clearly showed the conversion of BSA into a new product. A more specific characterization was achieved by MALDI TOF mass spectrometry. Fig. 4 shows the co-mass spectra of the BSA conjugate **21g** and BSA which was added as an internal standard. Setting the BSA molecular mass to 66,497 the (somewhat broader) adduct peak had its intensity maximum at m/z=72,541 demonstrating that an average of 17 molecules of **21e** had been coupled to one BSA molecule (see Fig. 4).



20	R
a	H
b	$CO(CH_2)_2C(O)OAll$

21	R^1	R^2	R^3
a	H	H	OAll
b	$CONH_2$	H	OAll
c	H	$CONH_2$	OAll
d	$CONH_2$	$CONH_2$	OAll
e	$CONH_2$	H	OH
f	$CONH_2$	H	$NH{-}CH(CH_2OCH_2Ph){-}C(=O)$
g	$CONH_2$	H	$NH{-}CH(CH_2{-}BSA){-}C(=O)$

Scheme 8.

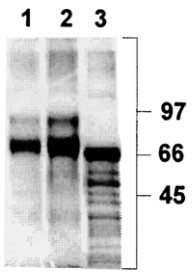


Figure 3. SDS PAGE of the conjugation experiment. Lanes 1, 2: reaction product, lane 3: BSA.

2.5. Some biotin conjugates

A number of moenomycin-derived supports for affinity chromatography have already been reported.[5,22] We wish to add three more compounds which could be useful for the affinity purification of anti-moenomycin antibodies. Moenomycin A derivatives **2a** and **2b** have been coupled to biotin previously.[18,19] Following the reported protocol[18] moenomycin C_1 (**1b**) was converted into thiol derivative **22b** via **22a**. The thiol grouping in **22b** was used to prepare the moenomycin C_1–biotin adduct **22c** by 1,4-additions to a known biocetin-derived maleimide (cf. formula **22c**). **22c**

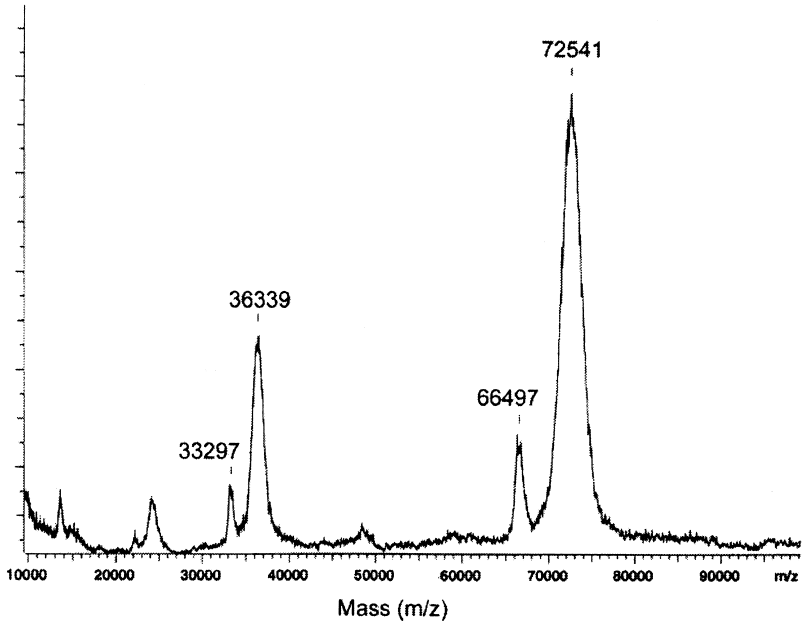
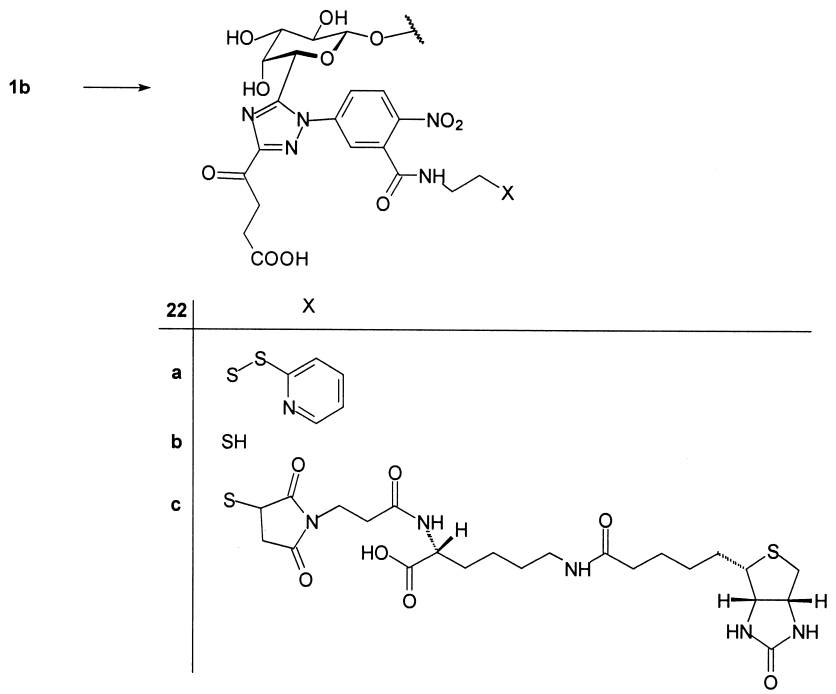


Figure 4. MALDI TOF MS spectrum of conjugate **21g**. BSA was added as an internal reference.



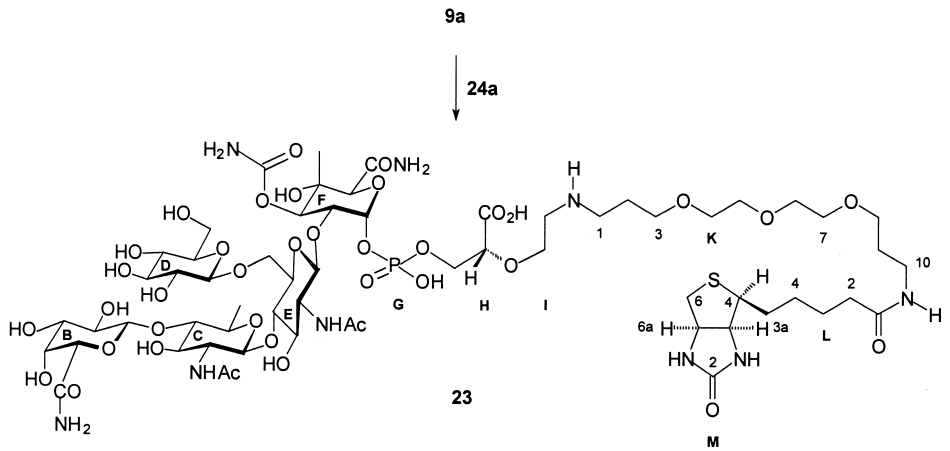
Scheme 9.

exhibited somewhat reduced antibiotic properties when compared with moenomycin A (Scheme 9).

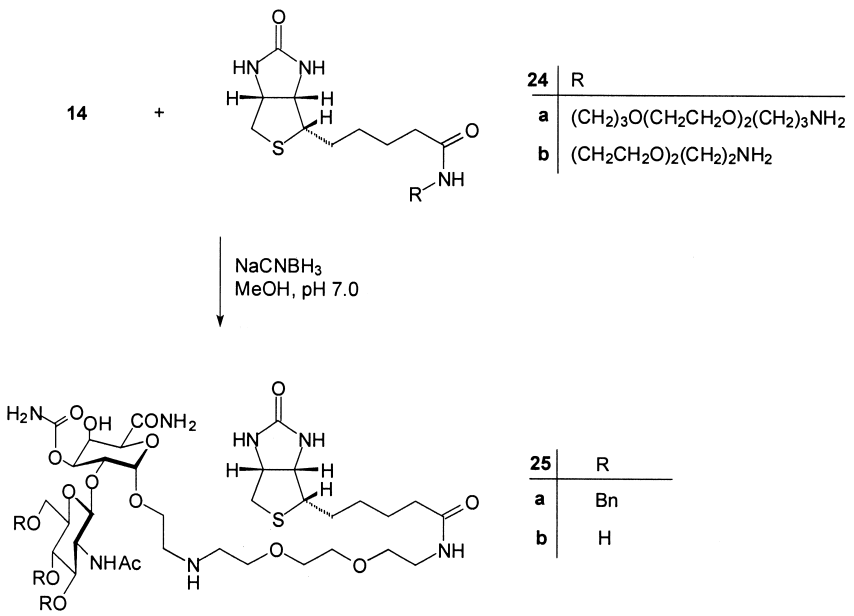
The pentasaccharide-derived biotin derivative **23** was obtained from **9a** and **24a** by reductive amination as reported above. Analogously the disaccharide-derived biotin adduct **25b** was prepared from **16a** by a sequence of (i) ozonolysis, (ii) reductive amination with **24b**, and (iii) hydrogenolytic removal of the benzyl protecting groups (Schemes 10 and 11).

2.6. Biochemical results

Rabbits were immunized by injection of moenomycin–BSA conjugates **8** (from experiments a–c) to raise polyclonal antisera against moenomycin. First screening of the blood sera for anti-moenomycin antibodies was performed in enzyme-linked immunosorbent assays (ELISA). Microtiter plates were coated with moenomycin–KLH (obtained from **2a** and maleimide-activated KLH), moenomycin–BSA **8**, and the complex of streptavidin and biotinylated moenomycin derivative **16c**, respectively. Antiserum was added in serial dilutions. For the ELISAs anti-rabbit antibodies conjugated with peroxidase were employed. Each of these different ELISA strategies showed increasing antibody concentrations recognizing moenomycin during the immunization procedure (Fig. 5(A)). After 20 injections, the immunization procedure was stopped (Fig. 5(B)). Alternatively, a Western blot analysis was performed with **8** and anti-moenomycin antiserum as primary antibody. After incubation with alkaline phosphatase-conjugated anti-rabbit antibodies the Western blot showed specific coloring but not in control experiments with BSA confirming the presence of antibodies recognizing moenomycin (Fig. 6). Furthermore, **2b** was coupled to a SPR sensor chip as described previously.[5] SPR experiments with antiserum as soluble analyte confirmed the specificity of the antiserum for moenomycin



Scheme 10.



Scheme 11.

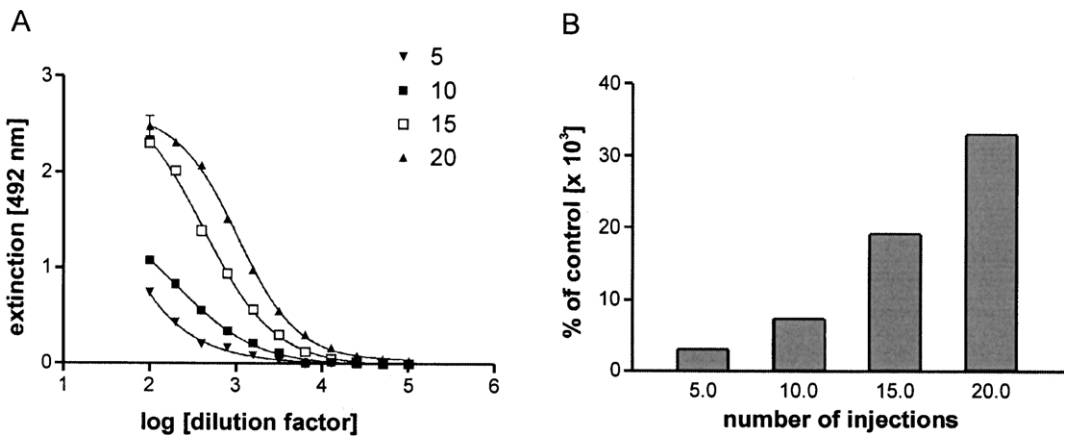


Figure 5. ELISA. (A) Determination of the titer after 5, 10, 15 and 20 injections of **8**. Microtiter plates were coated with **8**. (B) Percentual increasing of the immune response (dilution 1:1600) during the immunization procedure according to the ELISA.

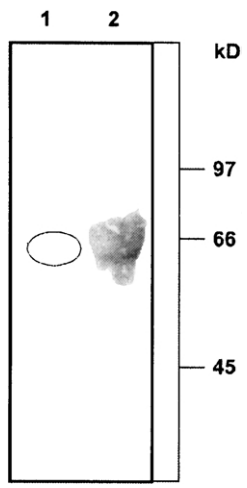


Figure 6. Western blotting for the determination of anti-moenomycin antibodies. Incubation of antiserum was performed in the presence of 1% BSA for neutralization of anti-BSA antibodies. (1) 15 μg BSA. (2) 15 μg moenomycin–BSA.

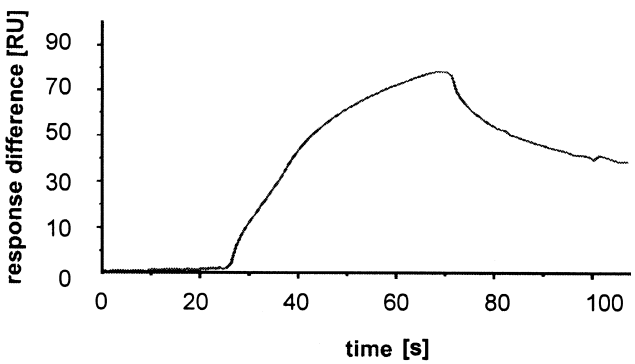


Figure 7. SPR sensorgram of the injection of rabbit anti-moenomycin antibodies. The graph displays the chance of the response during and after the injection of antiserum. Control experiments were done with preserum. Running and binding buffer: HBS-EP, pH 7.4. Protein concentrations: 2.5 μg/mL, flow 20 μL/min, contact time 45 s, 25°C.

not coupled to BSA (Fig. 7). However, affinities have not yet been determined. In all experiments anti-BSA antibodies which were also produced during the immunization procedure were neutralized with BSA.

3. Experimental

3.1. Methods and materials

For flash chromatography (FC), see Ref. 37. The matrix for the FAB mass spectra was 3-nitrobenzyl alcohol. For HPLC the following instrumentation was used: Analytical HPLC: Jasco PU-980 pump with Uniflows Degasys DG-1310 system, Sepsil column (C18, 5 μm, 250 mm×2.1 mm), Sepsil precolumn (C18, 5 μm, 20 mm×2.1 mm), flow rate 0.5 mL/min, sample volume 20 μL, eluent: a 63:37 mixture of buffer (KH_2PO_4 (0.6 g), $K_2HPO_4×3H_2O$ (26.2 g), 1-heptanesulfonic acid, sodium salt monohydrate (3.0 g), water, final volume 1 L) and acetonitrile (adjusted to pH 8 with phosphoric acid),[38] detection with the Jasco MD-910 diode array detector, data processing with the DP-L910-V software. Preparative HPLC: Jasco PU-987 pump, Jasco 875-UV UV–Vis detector, Sepsil column (C18, 10 μm, 250 mm×20 mm), 0.5 mL sample volume. Either an Amicon gas-pressurized cell (model 8050) with an Amicon membrane YM 3 (3000 Da cut-off) or reversed phase chromatography (HP-20 resin, swollen in methanol for 12 h, washed with acetone, water, 0.1 M NaOH, water, 0.1 M HCl, water, methanol, water) or gel filtration (PD-10 prepacked columns, Sephadex G-25M, 9.1 mL) were used for the removal of low molecular weight impurities and inorganic salts. Microsep centrifugal concentrators (PALL FILTRON, 30 kDa cut-off, Beckman Avanti 30 centrifuge, fixed-angle rotor at 25°, 7500***g***, 4°C) and Amicon gas-pressurized cell (model 8050) with an Amicon membrane YM 30 (30 kDa cut-off) were used for the purification of the BSA preparations. For ion exchange chromatography Dowex W 50 and Lewatit S 100, respectively, were used. NMR equipment: NMR: UNITY 400 (Varian), DRX 400 (Bruker), DRX 600 (Bruker), GEMINI 200 (Varian), GEMINI 2000 (Varian). All spectra have been analyzed as described in the individual procedures. For brevity, signals of protecting groups and spacers are described only once. Mass spectrometry: EI MS: MAT-731 (Varian), FAB MS: VG Autospec (Fisons, matrix: 3-nitrobenzylalcohol), ESI MS: FT-ICR-MS Apex II (Bruker Daltonics, water–methanol). The MALDI TOF mass spectra were acquired with the Reflex™ (Bruker) and the Voyager™ (PerSeptive Biosystems, nitrogen laser at 337 nm, Delayed Extraction™), matrices: sinapic acid, solvent: 30% acetonitrile in ultrapure water (18 MΩ cm)+trifluoroacetic acid (3%); 2,4,6-trihydroxyacetophenone (THAP) (10 mg/1 mL) dissolved in water–acetonitrile 1:1, the solutions of analytes were applied to dried matrix (fast-evaporation method); 2,5-dihydroxybenzoic acid (DHB) dissolved in ultrapure water (ca 10 mg/1 mL), the sample of BSA–moenomycin conjugates in ultrapure water (1 mg/1 mL) was premixed with the matrix solution in a 1:1 v/v ratio and applied onto the plate (1 μL) (dried-droplet method). Following the molecular formula two masses are always communicated, the first was calculated using the International Atomic Masses, the second is the mono-isotopic mass. IR: Genesis FTIR (ATI Mattson).

3.2. Reaction of BSA with 3

To a stirred solution of BSA (SERVA, 10 mg) in phosphate buffer (9 mL, pH 7.0, 50 mmol) a solution of 3-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl) propionic acid 2,5-dioxo-pyrrolidin-1-yl ester (**3**) (10 mg, 0.038 mmol) in dimethylformamide (1 mL) was added. The reaction mixture was stirred at 5°C for 3 d. Removal of low molecular weight reaction products was accomplished by dialysis against phosphate buffer (Pierce Slide-A-Lyzer™ 10 K Dialysis Cassette). The MALDI TOF MS (matrix: sinapic acid, internal calibration with BSA, m/z=66,892) displayed an average m/z of 71,441, corresponding to an average loading value of 32.

3.3. Flavomycin–BSA conjugate 5

To a solution of the above activated BSA (**4**) in phosphate buffer (5 mL, pH 7.0, 50 mM) a solution of **2a**[18] (prepared from Flavomycin®, vide supra, 10 mg, 78 equiv.) in phosphate buffer was added. The reaction mixture was stirred for 1 d at 5°C. An aliquot was dialyzed against water (Pierce Slide-A-Lyzer™ 10K Dialysis Cassette). The MALDI TOF MS (DHB matrix, external calibration with BSA, m/z=66,418) displayed a mean m/z of 77,015, corresponding to an average protein loading of 3.0. Then mercaptoethanol (0.1 mL) was added and stirring was continued for 1 d. Removal of low molecular weight reaction products was accomplished by dialysis against phosphate buffer (Pierce Slide-A-Lyzer™ 10K Dialysis Cassette).

3.3.1. 5-Amino-*N*-[2-(2-ethoxy-3,4-dioxo-1-cyclobuten-1-ylamino)ethyl]-2-nitrobenzamide (6). A solution of 5-amino-*N*-(2-aminoethyl)-2-nitro-benzamide (15 mg, 0.067 mmol) and 3,4-diethoxy-3-cyclobutene-1,2-dione (10 mg, 0.058 mmol) in ethanol (2.2 mL) was stirred at ambient temperature. Progress of the reaction was controlled by TLC ($CHCl_3$–methanol 10:2, R_f=0.15). After 16 h all diethyl squarate was consumed. Solvent evaporation and FC ($CHCl_3$–methanol 5:1) provided 20 mg (99%) of pure **6**. ^{1}H NMR (200 MHz, CD_3OD, homo decoupling): δ=1.45 (t, J=7.0 Hz, 3H, OCH_2CH_3), 3.50–3.85 (m, 4H, CH_2-1^{Ar}, CH_2-2^{Ar}), 4.72 (m, 2H, OCH_2CH_3), 6.52 (d, J_{6-4}=2.4 Hz, 1H, 6^{Ar}-H), 6.65 (dd, J_{4-3}=9.2 Hz, J_{4-6}=2.4 Hz, 1H, 4^{Ar}-H), 7.96 (d, J_{3-4}=9.2 Hz, 1H, 3^{Ar}-H). ^{13}C NMR (50 MHz, CD_3OD): δ=16.0 (OCH_2CH_3), 41.1, 41.4 (C-1^{DAE}), 44.8 (C-2^{DAE}), 70.9 (OCH_2CH_3), 113.2, 114.2 (C-6^{Ar}, C-4^{Ar}), 128.9 (C-3^{Ar}), 135.1 (C-1^{Ar}), 137.6 (C-2^{Ar}), 156.5 (C-5^{Ar}), 171.7 ($CONH^{Ar}$), the squaric acid signals could not be identified. $C_{15}H_{16}N_4O_6$ (348.31, 348.10698), FAB MS: m/z=371.1 $[M+Na]^+$, 349.1 $[M+H]^+$.

3.4. Coupling of 6 to BSA

To a solution of BSA (5 mg, 0.075 μmol) in borax buffer (0.8 mL, 0.01 M, pH 9.0) a suspension of **6** (3.5 mg, 0.01 mmol) in borax buffer (0.6 mL, 0.01 M, pH 9.0) with a few drops of methanol and EtOH (to make the solution homogeneous) was added and the mixture was stirred at 20°C under argon. After 64 h the solution was filtered and

freed from inorganic salts and starting **6** by ultrafiltration. Freeze drying gave 4 mg of **7**. MALDI TOF MS (THAP matrix, internal standard BSA, *m/z*=68,932): *m/z*=78,369 $[M]^+$, 39,688 $[M]^{2+}$ indicating an average loading of BSA with 30 residues of **6**.

3.5. Preparation of BSA–moenomycin conjugates 8

Compound **2c** (1.7 mg, 0.87 μmol (experiment a), 6.1 mg, 3.0 μmol (experiment b), 23.6 mg, 12 μmol (experiment c)), and BSA (Serva, 10 mg, 0.15 μmol (experiment a), 20 mg, 0.3 μmol (experiment b), 40 mg, 0.6 μmol (experiment c)) in borax buffer, pH 9.03 (prepared from 100 mL 0.025 M borax and 80 mL 0.1 M $KHCO_3$, 1 mL (experiment a), 2 mL (experiment b), 3 mL (experiment c)) were stirred under argon at 20°C for 7 d. Inorganic salts were removed by ultrafiltration. After lyophilization products **8**, 9.3 mg (experiment a), 23.3 mg (experiment b) and 60.0 mg (experiment c) were obtained. MALDI TOF MS (DHB matrix, BSA as external standard with *m/z*=66,418) showed for experiment a: *m/z*=73,749 $[M]^+$, 36,938 $[M]^{2+}$; experiment b: *m/z*=77,126 $[M]^+$, 38,783 $[M]^{2+}$ and experiment c: *m/z*=83,141 $[M]^+$, 41,985 $[M]^{2+}$ that correspond to an average protein loading of 3.9 (experiment a), 5.6 (experiment b) and 8.8 (experiment c). ESI FT ICR MS (BSA as external standard with deconvoluted peak at *m/z*=66,400): **8a** (from experiment a) deconvoluted peak at *m/z*=74,000 that correspond to an average protein loading of 4.0.

3.5.1. (*R*)-2-{2-[(2-Ethoxy-3,4-dioxo-1-cyclobuten-1-yl)methylamino]ethoxy}-3-({β-D-galactopyranuronamidosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyl-oxy}-hydroxy-phosphoryloxy)-propionic acid (9c). To a solution of amine **9b** (100 mg, 83 mmol) in phosphate buffer (5 mL, pH 7.0, 1 M) 3,4-diethoxy-3-cyclobuten-1,2-dion (141 mg, 830 mmol) was added dropwise and the mixture was stirred at 20°C for 24 h (during that time 3,4-diethoxy-3-cyclobuten-1,2-dions was dissolved slowly). In regular time intervals the pH was adjusted to 7.0 by adding saturated $NaHCO_3$ solution. The reaction mixture was then directly passed through Sephadex® LH-20 (elution with 1:4 water–CH_3OH). All fractions containing product were combined and the solvent was evaporated. The residue was adsorbed onto Kieselguhr (500 mg) and placed on top of a FC column. Elution with ethyl acetate–isopropanol–water 4:5:4, solvent evaporation, filtration of the product-containing fractions through a Sephadex® LH-20 column (elution with 1:4 water–methanol), solvent evaporation, and lyophilization provided **9c** (67 mg, 61%). ^{1}H NMR (400 MHz, H,H COSY, D_2O): δ=1.18 (s, CH_3-4^F), 1.34 (bs, a doublet in the 200 MHz spectrum, CH_3-6^C), 1.36–1.40 (CH_3-6^K), 1.97, 2.04 (s, $NHCOCH_3^E$, s, $NHCOCH_3^C$), 3.24 (m, 2^D-H), 4.16 (s, 4^B-H, 5^B-H), 4.45 (d, J_{1D-2D}=7.3 Hz, 1^D-H), 4.96 (m, J_{2F-3F}=9.8 Hz, 3^F-H), 5.73 (m, 1^F-H). ^{13}C NMR (50 MHz, D_2O): δ=14.9 (CH_3-4^F), 15.3 (CH_3-6^K), 16.9 (CH_3-6^C), 22.6 ($NHCOCH_3^E$, $NHCOCH_3^C$, C-5^K), 37.7–37.9 (CH_3-3^I, two steroisomers), 55.4–55.7 (C-2^E, C-2^C), 61.0 (C-6^D), 67.3 (C-1^I), 69.1, 69.9, 70.8, 71.3, 72.4, 72.7, 73.0, 73.3, 73.7, 74.5, 75.1, 76.0, 76.2, 76.6, 76.7 (C-5^C, C-4^D, C-4^B, C-2^B, C-3^B, C-3^C, C-5^F, C-6^E, C-5^B, C-3^E, C-2^F, C-4^F, C-5^E, C-2^D, C-3^F, C-5^D, C-3^D), 80.1 (C-4^E), 83.3 (C-4^C), 94.6 (d, C-1^F), 101.4, 102.3, 102.8, 103.4 (C-1^C, C-1^E, C-1^B, C-1^D), 158.4 ($OCONH_2^F$), 172.9, 173.4, 174.4, 174.8 ($CONH_2^B$, $CONH_2^F$, $NHCOCH_3^E$, $NHCOCH_3^C$), 176.6 (C-3^H). The signals of the quaternary carbons of the squaric acid part were not found. ^{31}P NMR (81 MHz, D_2O): δ=−2.54. $C_{48}H_{75}N_6O_{35}P$ (1327.12, 1326.40), ESI MS: *m/z*=1347.3748 (1347.3758) $[M+Na-2H]^-$, 1325.3941 (1325.3938) $[M-H]^-$, 662.1917 (662.1933) $[M-2H]^{2-}$.

3.6. Coupling of 9c to BSA

To a solution of BSA (20 mg, 0.3 μmol) in borate buffer (from 0.05 M sodium tetraborate (50 mL) and 0.2 M boric acid (50 mL), pH 9.0, 2 mL) a solution of **9c** (a) 3.6 mg (2.7 μmol), (b) 7.2 mg (5.4 μmol), (c) 18 mg (13 μmol) in borate buffer (pH 9.0, 2 mL) was added and the mixtures were stirred at 20°C for 48 h. The reaction mixture was then directly passed through a Sephadex® G-15 column (elution with water). After solvent evaporation and lyophilization the conjugates **9d** (a) (21 mg), (b) (19.8 mg) and (c) (22 mg) were obtained and analysed by MALDI TOF MS (DHB matrix, BSA as external standard with *m/z*=66,346): **9d** (a) *m/z*=69,274 $[M]^+$, 34,895 $[M]^{2+}$, corresponding to an average protein loading of 2.3; **9d** (b) *m/z*=69,167 $[M]^+$, 34,455 $[M]^{2+}$, corresponding to an average protein loading of 2.2 and **9d** (c) *m/z*=72,352 $[M]^+$, 36,272 $[M]^{2+}$, corresponding to an average protein loading of 4.7.

3.6.1. Benzyl {2-[2-(2-pent-4-enoylamino-ethoxy)-ethoxy]-ethyl}-carbamate (11a). A solution of 4-pentenoic acid (306 mg, 3.06 mmol) and *N*,*N*′-carbonyldiimidazole (545 mg, 3.36 mmol) in dry pyridine (20 mL) was stirred for 1 h at 20°C. **10a** (949 mg, 3.36 mmol) was added and the mixture was stirred at 20°C for 2 h. Solvent evaporation and FC ($CHCl_3$–methanol 19:1) furnished **11a** (902 mg, 81%, based on 4-pentenoic acid) as a pale yellow oil. R_f=0.27 ($CHCl_3$–methanol 19:1). IR (film): 3315, 2927, 2870, 1714, 1651, 1543, 1257, 1134, 1115 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.15–2.44 (m, 4H, C*H*$_2$CONH, CH_2=CHC*H*$_2$), 3.31–3.63 (m, 12H, 2×C*H*$_2$NH, 4×CH_2O), 4.92–5.01 (m, 1H, C*H*H=CHCH$_2$), 5.05–5.12 (m, 3H, CH_2^{Cbz}, CH*H*=CHCH$_2$), 5.40 (bs, 1H, NH), 5.67–5.89 (m, 1H, CH_2=C*H*CH$_2$), 6.10 (bs, 1H, NH), 7.26–7.36 (m, 5H, Ar–Hs^{Cbz}). ^{13}C NMR (75 MHz, $CDCl_3$, APT): δ=29.6 (+, CH_2=CH*C*H$_2$), 35.7 (+, *C*H$_2$CONH), 39.1, 40.8 (+, 2×CH_2NH), 66.7 (+, CH_2^{Cbz}), 69.9–70.2 (+, CH_2O-signals), 115.4 (+, *C*H$_2$=CHCH$_2$), 128.2–128.5 (−, Ar–CH^{Cbz}), 136.5 (+, Ar–Cq^{Cbz}), 137.1 (−, CH_2=*C*HCH$_2$), 156.5 (+, C=O^{Cbz}), 172.4 (+, CONH). $C_{19}H_{28}N_2O_5$ (364.44, 364.20), FAB MS: *m/z*=365.2 $[M+H]^+$.

3.6.2. Benzyl {2-[2-(2-hept-6-enoylamino-ethoxy)-ethoxy]-ethyl}-carbamamate (11b). 11b was prepared as described for **11a**. R_f=0.25 (petroleum ether–ethyl acetate–methanol 10:10:3). ^{1}H NMR (300 MHz, $CDCl_3$, H,H COSY): δ=1.37–1.51 (m, 2H, CH_2=CHCH$_2$C*H*$_2$), 1.60–1.74 (m, 2H, C*H*$_2$CH$_2$CONH), 2.04–2.14 (m, 2H, CH_2=CHC*H*$_2$), 2.14–2.25 (m, 2H, C*H*$_2$CONH), 3.39–3.52 (m, 4H, 2×C*H*$_2$NH), 3.53–3.71 (m, 8H, 4×C*H*$_2$O), 4.94–5.08 (m, 2H, C*H*$_2$=CHCH$_2$), 5.14 (bs, 2H, CH_2^{Cbz}), 5.34 (bs,

1H, NH), 5.75–5.90 (m, 1H, CH_2=C*H*CH_2), 5.99 (bs, 1H, NH), 7.35–7.43 (m, 5H, Ar–Hs^{Cbz}). ^{13}C NMR (75 MHz, $CDCl_3$, H,C COSY, APT): δ=25.4 (+, *C*H_2CH_2CONH), 28.7 (+, CH_2=CHCH_2*C*H_2), 33.7 (+, CH_2=CH*C*H_2), 36.7 (+, *C*H_2CONH), 39.4, 41.1 (+, 2×CH_2NH), 67.0 (+, CH_2^{Cbz}), 70.3–70.5 (+, CH_2O-signals), 114.9 (+, *C*H_2=$CHCH_2$), 128.4–128.8 (−, Ar–CH^{Cbz}), 136.7 (+, Ar–Cq^{Cbz}), 138.8 (−, CH_2=*C*HCH_2), 156.9 (C=O^{Cbz}), 173.5 (CONH). $C_{21}H_{32}N_2O_5$ (392.50, 392.23), FAB MS: *m*/*z*=393.2 $[M+H]^+$, 415.2 $[M+Na]^+$.

3.6.3. Benzyl (3-pent-4-enyloxy-propyl)-carbamamate (12). The mixture of sodium hydride (60% in oil, 697 mg, 17.4 mmol) and 4-penten-1-ol (1.0 g, 11.6 mmol) in dry DMF (5 mL) was stirred at 20°C for 30 min. At 0°C a solution of *tert*-butyl 3-bromopropylcarbamate[39] (4.14 g, 17.4 mmol) in dry DMF (10 mL) was added dropwise. The mixture was stirred at 0°C for 15 min and at 20°C for 4 h. Work-up and FC (cyclohexane–CH_2Cl_2–ethyl acetate 5:5:1) gave *tert*-butyl 3-pent-4-enyloxy-propylcarbamate (17 g, 41%, based on 4-penten-1-ol) as a colorless oil. The latter compound was deprotected by treatment with 20% TFA in CH_2Cl_2, to give, after work-up 3-pent-4-enyloxy-propylamine (309 mg, 95%). To a solution of this amine (250 mg, 1.74 mmol) and triethylamine (266 μL, 1.92 mmol) in CH_2Cl_2 (30 mL) at −50°C a solution of benzyl chloroformate (271 μL, 1.92 mmol) in dichloromethane (50 mL) was added dropwise within 30 min. The mixture was then stirred at −50°C for 30 min and at 20°C for 2 h. Work-up and FC (cyclohexane–CH_2Cl_2–ethyl acetate 5:5:1) furnished **12** (412 mg, 85%) as a colorless oil. R_f=0.28 (cyclohexane–CH_2Cl_2–ethyl acetate 5:5:1). IR (film): 3332, 2937, 2864, 1718, 1703, 1529, 1255, 1113 cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$, H,H COSY): δ=1.59–1.85 (m, 4H, 2×C*H*$_2CH_2O$), 2.05–2.19 (m, 2H, CH_2=CHC*H*$_2$), 3.26–3.53 (m, 6H, C*H*$_2$NHCbz, 2×CH_2O), 4.92–5.08 (m, 2H, C*H*$_2$=$CHCH_2$), 5.10 (bs, 2H, CH_2^{Cbz}), 5.27 (bs, 1H, NHCbz), 5.70–5.91 (m, 1H, CH_2=C*H*CH_2), 7.27–7.47 (m, 5H, Ar–Hs^{Cbz}). ^{13}C NMR (50 MHz, $CDCl_3$): δ=29.0, 29.8, 30.4 (2×*C*H_2CH_2O, CH_2=CH*C*H_2), 39.6 (CH_2NHCbz), 66.6 (CH_2^{Cbz}), 69.4, 70.5 (2×CH_2O), 114.8 (*C*H_2=$CHCH_2$), 128.1–128.6 (Ar–CH^{Cbz}), 136.9 (Ar–Cq^{Cbz}), 138.3 (CH_2=*C*HCH_2), 156.5 (C=O^{Cbz}). $C_{16}H_{23}NO_3$ (277.36, 277.17), FAB MS: *m*/*z*=278.2 $[M+H]^+$, ESI ICR MS: *m*/*z*=278.1753 (calcd 278.17507) $[M+H]^+$, 300.15724 (calcd 300.15701) $[M+Na]^+$.

3.6.4. *N*-{2-[2-(2-{2-Mercaptoacetamido}ethoxy)ethoxy]-ethyl} *tert*-butyl carbamate (13c). To a stirred solution of **13b** (600 mg, 2.42 mmol) and triethylamine (489 mg, 4.83 mmol) in dichloromethane (10 mL) at 20°C a solution of *N*-{2-[2-(2-aminoethoxy)ethoxy]ethyl} *tert*-butyl carbamate (**10b**)[32] (491 mg, 2.42 mmol) in dichloromethane (50 mL) was added slowly and the mixture was stirred at 20°C for 2 h. Usual work-up and FC (cyclohexane–ethyl acetate–methanol 10:10:1) provided **13c** (763 mg, 94%) as pale yellow oil. R_f=0.13 (cyclohexane–ethyl acetate–methanol 10:10:1). IR (KBr): 3338, 2972, 2931, 2870, 1707, 1655, 1535, 1452, 1363, 1275, 1252, 1171, 1111 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$, H,H COSY): δ=1.44 (s, 9H, $C(CH_3)_3$), 1.60 (t, $J_{SH,CH2}$=8.2 Hz, 1H, SH), 2.49 (t, *J*=6.7 Hz, 2H, CH_2CO), 2.78 (dt, 2H, C*H*$_2$SH), 3.25–3.33 (m, 2H, C*H*$_2$NHBoc), 3.41–3.49 (m, 2H, C*H*$_2$-NHCO), 3.50–3.64 (2×CH_2O, OC*H*$_2CH_2O$), 5.05 (bs, 1H, N*H*Boc), 6.25 (bs, 1H, N*H*CO). ^{13}C NMR (75 MHz, $CDCl_3$): δ=20.6 (CH_2SH), 28.5 ($C(CH_3)_3$), 39.4, 40.3, 40.5 (*C*H_2CO, *C*H_2NHCO, CH_2NHboc), 69.9, 70.2, 70.3, 70.4 (2×CH_2O, OC*H*$_2CH_2O$), 79.5 ($C(CH_3)_3$), 156.1 (*C*O$C(CH_3)_3$), 170.9 (CONH). $C_{14}H_{28}N_2O_5S$ (336.45, 336.17), ESI ICR MS: *m*/*z*=337.1793 (calcd 337.1792) $[M+H]^+$, 359.1919 (calcd 359.1611) $[M+Na]^+$, 671.3354 (calcd 671.3354) $[disulfide+H]^+$.

3.6.5. *N*-{2-[2-(2-Aminoethoxy)ethoxy]ethyl}mercapto-acetamide (13d). Compound **13c** (500 mg, 1.49 mmol) was dissolved in a 20:80 mixture of trifluoroacetic acid and dichloromethane (5 mL) and the mixture was stirred at 20°C for 4 h. Solvent evaporation and FC ($CHCl_3$–methanol–water 10:10:2) furnished 490 mg of **13d** (as a salt) as a yellow oil. R_f=0.33 ($CHCl_3$–methanol–water 10:10:2). IR (film): 3373, 1676, 1641, 1441, 1188, 1138 cm^{-1}. ^{1}H NMR (300 MHz, D_2O, H,H COSY): δ=2.55 (t, *J*=6.6 Hz, 2H, C*H*$_2$CONH), 2.76 (t, 2H, C*H*$_2$SH), 3.19 (m, 2H, C*H*$_2NH_2$), 3.41 (t, *J*=5.5 Hz, 2H, C*H*$_2$NHCO), 3.64 (t, 2H, CH_2O), 3.70 (s, 4H, OC*H*$_2$C*H*$_2$O), 3.74 (t, *J*=5.1 Hz, 2H, CH_2O). ^{13}C NMR (75 MHz, D_2O): δ=20.1 (CH_2SH), 39.0, 39.3, 39.5 (*C*H_2CONH, CH_2NH, CH_2NH_2), 66.6, 69.0, 69.6, 69.8 (4×CH_2O), 174.7 (CONH). $C_9H_{20}N_2O_3S$ (236.33, 236.12), FAB MS: *m*/*z*=237.1 $[M+H]^+$, 259.1 $[M+Na]^+$.

3.6.6. Allyl-2-*O*-(2-acetamido-3,4,6-*O*-tribenzyl-2-deoxy-β-D-glucopyranosyl)-3-*O*-carbamoyl-α-D-galactopyranosiduronamide (16a). The compound was prepared as described for allyl-2-*O*-(2-acetamido-3,4,6-*O*-triacetyl-2-deoxy-β-D-glucopyranosyl)-3-*O*-carbamoyl-α-D-galacto-pyranosiduronamide.[35] R_f=0.17 ($CHCl_3$–methanol 6:1). ^{1}H NMR (400 MHz, pyridine-d_5, H,H COSY): δ=2.19 (s, 3H, NHCOC*H*$_3$), 3.59–3.62 (m, 1H, 5^E-H), 3.76–3.96 (m, 4H, 2^E-H, 4^E-H, CH_2-6^E), 4.11 (dd, *J*=13.0, 5.1 Hz, 1H, CH_2=CHCH*H*), 4.23 (dd, 1H, CH_2=CHC*H*H), 4.56–5.08 (m, 10H, CH_2–Hs^{benzyl}, 2^F-H, 5^F-H, 3^E-H, CH*H*=$CHCH_2$), 5.33 (d, *J*=17.1 Hz, 1H, C*H*H=$CHCH_2$), 5.49 (bs, 1H, 4^F-H), 5.56–5.66 (m, 2H, 1^E-H, 1^F-H), 5.79–5.97 (m, 2H, 3^F-H, CH_2=C*H*CH_2), 7.24–7.56 (Ar–Hs^{benzyl}), 7.77 (s, 1H, CONH), 8.46 (s, 1H, CONH), 9.10 (d, $J_{2E,NH}$=7.3 Hz, 1H, N*H*COCH$_3$). ^{13}C NMR (100 MHz, pyridine-d_5, H,C COSY, APT): δ=23.6 (−, NHCO*C*H_3), 58.4 (−, C-2^E), 68.9 (−, C-4^F), 69.0 (+, CH_2=CH*C*H_2), 69.6 (+, C-6^E), 73.0, 73.0 (−, C-5^F, C-3^F), 73.5 (+, CH_2^{benzyl}), 74.7, 74.8 (+, 2×CH_2^{benzyl}), 75.0 (−, C-2^F, C-5^E), 79.2 (−, C-4^E), 81.6 (−, C-3^E), 99.3 (−, C-1^F), 102.0 (−, C-1^E), 116.9 (+, *C*H_2=$CHCH_2$), 127.7–128.7 (−, Ar–CH^{benzyl}), 134.7 (−, CH_2=*C*HCH_2), 139.0, 139.2, 139.6 (+, Ar–Cq^{benzyl}), 157.8 (+, $OCONH_2$), 171.2, 172.1 (+, NH*C*OCH_3, C-6^F). $C_{39}H_{47}N_3O_{12}$ (749.82, 749.32), FAB MS: *m*/*z*=772.3 $[M+Na]^+$.

3.6.7. Allyl-2-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3-*O*-carbamoyl-α-D-galactopyranosiduronamide (16b). A mixture (degassed by sonication while purging with argon) of allyl-2-*O*-(2-acetamido-3,4,6-*O*-triacetyl-2-deoxy-β-D-glucopyranosyl)-3-*O*-carbamoyl-α-D-galacto-pyranosiduronamide[35] (200 mg, 330 μmol) and 2:1 methanol–water (15 mL) was cooled to 0°C, 0.3 M LiOH (4.4 mL) was added and the reaction mixture was stirred

at 0°C for 30 min followed by 15 min at 20°C. After addition of Dowex W X2 (H^+, 2.0 g) the mixture was stirred at 20°C for 30 min. The resin was filtered off and washed with 2:1 methanol–water and water. After solvent evaporation the residue was adsorbed onto kieselguhr and purified by FC ($CHCl_3$–methanol–water 9:6:1.4) to give after lyophilization 135 mg (86%) **16b** as a white solid. R_f=0.10 ($CHCl_3$–methanol–water 9:6:1.4). ^{1}H NMR (300 MHz, D_2O, H,H COSY): δ=1.94 (s, 3H, NHCOCH_3), 3.32–3.48 (m, 3H, 3^E-H, 4^E-H, 5^E-H), 3.58–3.70 (m, 2H, 2^E-H, 6^E-H), 3.84 (d, 1H, 6^E-H′), 3.95 (dd, $J_{2F,3F}$=10.4 Hz, $J_{1F,2F}$=3.8 Hz, 1H, 2^F-H), 4.02–4.19 (m, 2H, CH_2=CHCH_2), 4.31 (dd, $J_{3F,4F}$=3.3 Hz, $J_{4F,5F}$=1.3 Hz, 1H, 4^F-H), 4.38 (d, 1H, 5^F-H), 4.46 (d, $J_{1E,2E}$=8.5 Hz, 1H, 1^E-H), 4.89 (dd, 1H, 3^F-H), 5.15–5.21 (d, 1H, CH*H*=CHCH$_2$), 5.23–5.33 (m, 2H, 1^F-H, C*H*H=CHCH$_2$), 5.82–5.96 (m, 1H, CH_2=C*H*CH$_2$). ^{13}C NMR (75 MHz, D_2O, H,C COSY): δ=22.4 (NHCO*C*H$_3$), 55.6 (C-2^E), 61.0 (C-6^E), 68.1 (C-4^F), 69.2 (CH_2=CH*C*H$_2$), 70.0 (C-4^E), 70.3 (C-5^F), 71.3 (C-3^F), 73.8 (C-3^E), 75.1, (C-2^F), 75.9 (C-5^E), 97.6 (C-1^F), 102.9 (C-1^E), 119.1 (*C*H$_2$=CHCH$_2$), 133.3 (CH_2=*C*HCH$_2$), 157.9 (OCONH$_2$), 173.5, 174.9 (NH*C*OCH$_3$, C-6^F). $C_{18}H_{29}N_3O_{12}$ (479.44, 479.18), FAB MS: *m*/*z*=480.2 [M+H]$^+$, 486.2 [M+Li]$^+$, 502.1 [M+Na]$^+$.

3.6.8. 2-Oxoethyl 2-*O*-(2-acetamido-3,4,6-*O*-tribenzyl-2-deoxy-β-D-glucopyranosyl)-3-*O*-carbamoyl-α-D-galactopyranosiduronamide (14). Through a solution of **16a** (160.0 mg, 213 μmol) in methanol (50 mL) O_3/O_2 (Fischer OZON 502, flow rate 50 L/h=2 g/h O_3) was passed at −78°C for 5 min. Then oxygen was bubbled through the solution for 15 min and Ar for another 15 min. The mixture was allowed to warm to ambient temperature. Dimethylsulfide (200 μL) was added and the mixture was stirred at 20°C for 2 h. TLC ($CHCl_3$–MeOH 3:1) showed the formation of a single product which was obtained by solvent evaporation and lyophilization. An aliquot was submitted to FC ($CHCl_3$–MeOH 4:1) in order to remove small amounts of DMSO to give a white solid (94%). R_f=0.23 ($CHCl_3$–methanol 4:1). ^{13}C NMR (75 MHz, pyridine-d$_5$, APT): characteristic signals at δ=23.6 (NHCO*C*H$_3$), 54.3 (CH(O*C*H$_3$)OH)), 58.1, 58.2 (C-2^E), 69.7 (C-6^E), 79.2, 79.3 (C-4^E), 81.9, 82.0 (C-3^E), 90.0 (CH(OH)$_2$), 97.0, 97.1 (*C*H(OCH$_3$)OH)), 100.4, 100.7, 101.0 (C-1^F), 102.4, 102.6 (C-1^E), 127.8–128.9 (Ar–CH^{benzyl}), 139.0–139.8 (Ar–Cq^{benzyl}), 157.9–158.0 (OCONH$_2$), 171.3–172.4 (NH*C*OCH$_3$, C-6^F). $C_{38}H_{45}N_3O_{13}$ (751.79, 751.30, aldehyde), $C_{38}H_{47}N_3O_{14}$ (769.80, 769.30, aldehyde hydrate), $C_{39}H_{49}N_3O_{14}$ (783.83, 783.32, hemiacetal), FAB MS: *m*/*z*=752.3 [M+H]$^+$ (aldehyde), 774.3 [M+Na]$^+$ (aldehyde), 784.3 [M+H]$^+$ (hemiacetal), 792.3 [M+Na]$^+$ (aldehyde hydrate), 806.3 [M+Na]$^+$ (hemiacetal).

3.6.9. 2-*O*-(2-Acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-1-*O*-[2-(2-{2-[2-(benzyloxycarbonylamino)ethoxy]ethoxy}ethylamino)ethyl]-3-*O*-carbamoyl-α-D-galactopyranuronamide (15a). A solution of **10a** (82.6 mg, 293 μmol) in methanol (10 mL) was adjusted to pH 7 (with acetic acid in methanol). Solutions of **14** (110 mg, 146 μmol) in methanol (30 mL) and sodium cyanoborohydride (18.4 mg, 293 μmol) in methanol (1 mL) were added successively and the mixture was stirred at 20°C for 18 h. Solvent evaporation, Sephadex LH 20 LC (MeOH–H_2O 3:1), FC ($CHCl_3$–MeOH–H_2O 12:4.5:1), another Sephadex LH 20 LC (MeOH–H_2O 3:1), and lyophilization provided **15a** (149 mg, 76%) as a white solid. R_f=0.29 ($CHCl_3$–methanol–water 12:4.5:1). ^{1}H NMR (400 MHz, pyridine-d$_5$, H,H COSY): δ=2.32 (s, 3H, NHCOCH_3), 3.42–3.66 (m, 15H, including 5^E-H), 3.80–3.91 (m, 2H, 6^E-H, 4^E-H), 3.95–4.05 (3H, including 6^E-H′), 4.28–4.36 (m, 1H, 2^E-H), 4.65–4.72 (m, 2H, including 3^E-H), 4.74–4.79 (m, 2H, including, 5^F-H), 4.91–5.00 (m, 2H, including 2^F-H), 5.40–5.45 (m, 2H, 1^E-H, 4^F-H), 5.53 (d, $J_{1F,2F}$=3.6 Hz, 1H, 1^F-H), 5.60 (dd, $J_{2F,3F}$=10.6 Hz, $J_{3F,4F}$=3.0 Hz, 1H, 3^F-H), 7.72 (bs, 1H, CONH), 8.14 (bs, 1H, NHCbz), 8.43 (s, 1H, CONH), 9.63 (d, $J_{2E,NH}$=8.9 Hz, 1H, N*H*COCH$_3$). ^{13}C NMR (100 MHz, pyridine-d$_5$, HMQC): δ=23.6 (NHCO*C*H$_3$), 56.4 (C-2^E), 68.8 (C-4^F), 69.5 (C-6^E), 73.0 (C-3^F), 73.4, 73.5 (CH$_2^{benzyl}$, C-5^F), 74.8, 74.8, 75.3 (CH$_2^{benzyl}$, C-5^E, C-2^F), 78.93 (C-4^E), 82.72 (C-3^E), 100.2 (C-1^F), 103.7 (C-1^E), 157.4, 158.0 (C=O^{Cbz}, OCONH$_2$), 171.5, 171.9 (NH*C*OCH$_3$, C-6^F). $C_{52}H_{67}N_5O_{16}$ (1018.13, 1017.46), ESI ICR MS: *m*/*z*=1018.4632 (calcd 1018.4656) [M+H]$^+$.

3.6.10. 2-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-1-*O*-(2-{2-[2-(2-aminoethoxy)ethoxy]ethylamino}ethyl)-3-*O*-carbamoyl-α-D-galactopyranuronamide (15b). A mixture of **15a** (50 mg, 49.1 μmol) and 10% Pd/C (25 mg) in 2:1:0.5 methanol–acetic acid–water (5 mL) was stirred under hydrogen for 48 h at 20°C.[40] The catalyst was removed by filtration through Celite® (elution with 2:1 methanol–water (5 mL)). Solvent evaporation, FC ($CHCl_3$–methanol–water 10:10:2:0.1), Sephadex LH-20 LC (methanol–water 3:1), and lyophilization gave **15b** (30 mg, 61%) as a white solid. R_f=0.05 ($CHCl_3$–methanol–water–HCO_2H 10:10:2:0.1). ^{1}H NMR (400 MHz, D_2O, H,H COSY): δ=2.00 (s, 3H, NHCOCH_3), 3.40–3.45 (m, 2H, 4^E-H, 5^E-H), 3.45–3.53 (m, 1H, 3^E-H), 3.66–3.84 (m, 13H, including 2^E-H, 6^E-H), 3.84 (1H, d, *J*=12.2 Hz, 6^E-H′), 4.00–4.08 (m, 2H, including 2^F-H), 4.35–4.38 (m, 1H, 4^F-H), 4.44 (bs, 1H, 5^F-H), 4.52 (d, $J_{1E,2E}$=8.3 Hz, 1H, 1^E-H), 4.96 (dd, $J_{2F,3F}$=10.5 Hz, $J_{3F,4F}$=3.2 Hz, 1H, 3^F-H), 5.28 (d, $J_{1F,2F}$=3.4 Hz, 1H, 1^F-H). ^{13}C NMR (100 MHz, D_2O, HMQC): δ=22.9 (NHCO*C*H$_3^E$), 56.0 (C-2^E), 60.8 (C-6^E), 68.6 (C-4^F), 70.2, 70.2, 70.3 (C-7^{SPA}, C-8^{SPA}, C-4^E), 71.1, 71.2 (C-5^F, C-3^F), 74.5 (C-3^E), 76.2, 76.2 (C-2^F, C-5^E), 99.2 (C-1^F), 103.6 (C-1^E), 158.4 (OCONH$_2$), 173.8, 175.1 (NH*C*OCH$_3$, C-6^F). $C_{23}H_{43}N_5O_{14}$ (613.62, 613.28), ESI ICR MS: *m*/*z*=614.2885 (calcd 614.2879) [M+H]$^+$, 636.2705 (calcd 636.2699) [M+Na]$^+$.

3.6.11. 2-*O*-(2-Acetamido-3,4,6-*O*-tribenzyl-2-deoxy-β-D-glucopyranosyl)-1-*O*-[(Ξ)-8-(2-{2-[2-(benzyloxycarbonylamino)ethoxy]ethoxy}ethylamino)-8-oxooct-2-enyl]-3-*O*-carbamoyl-α-D-galactopyranuronamide (17a). A suspension of **16a** (84 mg, 112 μmol) and **11b** (88 mg, 224 μmol) in ethanol-free chloroform (40 mL) was degassed flushing with argon and sonication. This solution was transferred via a canula into a degassed solution containing the Grubbs catalyst (catalytic amount) in chloroform (5 mL). The mixture was left at 35°C for 18 h under Argon. Solvent evaporation and FC (petroleum ether–ethyl acetate–methanol 2:2:1) furnished 43 mg (49%) of not consumed **11b**, 25 mg (29%) of the homo metathesis product of **11b**, 45 mg (54%) of **16a** and a

fraction containing **17a**. The **17a** fraction was adsorbed onto kieselguhr and further purified by FC ($CHCl_3$–methanol 3:1) to give 26 mg (21%) of **17a** as a white solid. R_f=0.08 (petroleum ether–ethyl acetate–methanol 2:2:1). ^{1}H NMR (600 MHz, pyridine-d_5, H,H COSY): δ=2.17, 2.18 (2×s, 3H, NHCOCH_3), 3.53–3.70 (m, 13H, including 5^E-H), 3.78–3.96 (m, 4H, 2^E-H, 4^E-H, CH_2-6^E), 4.59–4.79 (Ar–Hs^{benzyl}, 3^E-H), 4.85–5.01 (Ar–Hs^{benzyl}, 2^F-H, 5^F-H), 5.43–5.69 (m, 5H, 1^F-H, 1^E-H, 4^F-H, 2^{SPA}-H, 3^{SPA}-H), 5.79–5.85 (m, 1H, 3^F-H), 8.15 (s, 1H, NHCbz), 8.43 (s, 2H, NH, CONH), 9.03–9.12 (m, 1H, N*H*COCH$_3$). ^{13}C NMR (150 MHz, pyridine-d_5, HMQC): δ=23.6 (NHCO*C*H$_3$), 58.4 (C-2^E), 68.9 (C-4^F), 69.7, 69.8 (C-6^E, two isomers), 73.0, 73.1, 73.1, 73.2 (C-5^F, C-3^F, two isomers, respectively), 75.0, 75.1 (C-5^E, C-2^F), 79.3 (C-4^E), 81.6 (C-3^E), 99.3, 99.6 (C-1^F, two isomers), 102.0, 102.1 (C-1^E, two isomers), 126.2, 126.6 (C-2^{SPA}, two isomers), 133.6, 134.1 (C-3^{SPA}, two isomers), 157.4, 157.8 (C=O^{Cbz}, $OCONH_2$), 171.2, 172.1 and 172.2, 173.1 and 173.15 (NH*C*OCH$_3$, C-6^F, C-8^{SPA}). $C_{58}H_{75}N_5O_{17}$ (1114.26, 1113.52), ESI ICR MS: *m*/*z*=1114.5259 (calcd 1114.5231) $[M+H]^+$, 1136.5080 (calcd 1136.5050) $[M+Na]^+$.

3.6.12. 2-*O*-(2-Acetamido-3,4,6-*O*-tribenzyl-2-deoxy-β-D-glucopyranosyl)-1-*O*-{6-[3-(benzyloxy-carbonylamino)propoxy]-hexyl}-3-*O*-carbamoyl-α-D-galactopyranuronamide (17b). The CMR of **16a** (95 mg, 127 μmol) and **12** (70 mg, 253 μmol) was performed as described for **17a**. FC ($CHCl_3$–methanol 6:1) gave 13 mg (18%) of **12**, 37 mg (52%) of the homo metathesis product of **12**, and 48 mg (38%) of **17b** as a white solid. 53 mg (56%) of **16a** were recovered. R_f=0.19 ($CHCl_3$–methanol 6:1). ^{1}H NMR (400 MHz, pyridine-d_5, H,H COSY): δ=2.17, 2.18 (2s, 3H, NHCOCH_3), 3.60–3.67 (m, 1H, 5^E-H), 3.80–3.96 (m, 4H, 2^E-H, 4^E-H, CH_2-6^E), 4.59–4.79 (CH_2^{benzyl}, 3^E-H), 4.90 (bs, 1H, 5^F-H), 4.93–5.07 (CH_2^{benzyl}, 2^F-H), 5.43–5.73 (m, 5H, 1^F-H, 1^E-H, 4^F-H, 2^{SPA}-H, 3^{SPA}-H), 5.81–5.87 (m, 1H, 3^F-H), 7.76 (s, 1H, CONH), 7.90–8.00 (NHCbz), 8.47 (s, 1H, CONH), 9.07 (d, $J_{2E,NH}$=7.3 Hz, 1H, N*H*COCH$_3$). ^{13}C NMR (100 MHz, pyridine-d_5, HMQC): δ=23.6 (NHCO*C*H$_3$), 58.3 (C-2^E), 68.9 (C-4^F), 69.6, 70.1 (C-6^E, two isomers), 73.0–73.1 (C-5^F, C-3^F), 75.0–75.1 (C-5^E, C-2^F), 79.2 (C-4^E), 81.6 (C-3^E), 99.3, 99.5 (C-1^F, two isomers), 102.0, 102.1 (C-1^E, two isomers), 126.6, 126.7 (C-2^{SPA}, two isomers), 133.0, 133.8 (C-3^{SPA}, two isomers), 157.2, 157.7 (C=O^{Cbz}, $OCONH_2$), 171.2, 172.1 (NH*C*OCH$_3$, C-6^F). $C_{53}H_{66}N_4O_{15}$ (999.12, 998.45), ESI ICR MS: *m*/*z*=999.4605 (calcd 999.4597) $[M+H]^+$, 1021.44249 (calcd 1021.4417) $[M+Na]^+$.

3.6.13. 2-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-1-*O*-(8-{2-[2-(2-aminoethoxy)ethoxy]-ethylamino}-8-oxooctyl)-3-*O*-carbamoyl-α-D-galactopyranuronamide (19a). A mixture of **17a** (17 mg, 15.3 μmol) and 10% Pd/C (15 mg) in 4:1:0.5 methanol–acetic acid–water (5 mL) was stirred under hydrogen for 18 h at 20°C. The catalyst was removed by filtration through Celite® (elution with 2:1 methanol–water (5 mL). FC ($CHCl_3$–methanol–water–HCO_2H 5:5:1:0.1), Sephadex LH-20 LC (methanol–water 3:1) and lyophilzation provided 9.1 mg (84%) **19a** as a white solid. R_f=0.14 ($CHCl_3$–methanol–water–HCO_2H 5:5:2:0.1). ^{1}H NMR (400 MHz, D_2O, H,H COSY): δ=1.87 (s, 3H, NHCOCH_3), 3.19–3.32 (m, 4H, CH_2-10^{SPA}, 4^E-H, 5^E-H), 3.37 (t, *J*=9.0 Hz, 1H, 3^E-H), 3.43–3.63 (m, 12H, including 2^E-H, 6^E-H), 3.77 (d, *J*=12.0 Hz, 1H, 6^E-H′), 3.85 (dd, $J_{2F,3F}$=10.4 Hz, $J_{1F,2F}$=3.4 Hz, 1H, 2^F-H), 4.21–4.27 (m, 2H, 4^F-H, 5^F-H), 4.37 (d, $J_{1E,2E}$=8.5 Hz, 1H, 1^E-H), 4.79 (dd, $J_{3F,4F}$=1.8 Hz, 1H, 3^F-H), 5.14 (d, 1H, 1^F-H). ^{13}C NMR (100 MHz, D_2O, HMQC): characteristic signals at δ=22.9 (NHCO*C*H$_3$), 36.3 (C-7^{SPA}), 39.6, 39.8 (C-10^{SPA}, C-17^{SPA}), 54.8 (C-2^E), 71.8 (C-3^F), 74.3 (C-3^E), 76.0 (C-2^F), 98.5 (C-1^F), 103.2 (C-1^E). $C_{24}H_{44}N_4O_{13}$ (711.76, 711.35), ESI ICR MS: *m*/*z*=712.3634 (calcd 712.3611) $[M+H]^+$, 734.3467 (calcd 734.3430) $[M+Na]^+$.

3.6.14. 2-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-1-*O*-[6-(3-aminopropoxy)-hexyl]-3-*O*-carbamoyl-α-D-galactopyranuronamide (19b). Compound **17b** (39 mg, 39 μmol) was hydrogenated as described for **17a**. FC ($CHCl_3$–methanol–water–HCO_2H 9:6:1.4:0.1), Sephadex LH-20 LC (methanol–water 3:1) and lyophilization furnished 21.8 mg (93%) **19b** as a white solid. R_f=0.08 ($CHCl_3$–methanol–water–HCO_2H 9:6:1.4:0.1). ^{1}H NMR (400 MHz, D_2O, H,H COSY): δ=2.01 (s, 3H, NHCOCH_3), 3.36–3.75 (m, 11H, including 2^E-H, 3^E-H, 4^E-H, 5^E-H, 6^E-H), 3.84 (dd, *J*=12.2 Hz, $J_{5E,6E}$=1.9 Hz, 1H, 6^E-H′), 4.00 (dd, $J_{2F,3F}$=10.6 Hz, $J_{1F,2F}$=3.9 Hz, 1H, 2^F-H), 4.36–3.39 (m, 2H, 4^F-H, 5^F-H), 4.52 (d, $J_{1E,2E}$=8.5 Hz, 1H, 1^E-H), 4.89 (dd, $J_{3F,4F}$=3.2 Hz, 1H, 3^F-H), 5.27 (d, 1H, 1^F-H). ^{13}C NMR (100 MHz, D_2O, HMQC): δ=22.8 (NHCO*C*H$_3$), 56.0 (C-2^E), 61.5 (C-6^E), 68.5 (C-4^F), 70.6, 70.7 (C-4^E, C-5^F), 71.8 (C-3^F), 74.3 (C-3^E), 75.6, (C-2^F), 76.3 (C-5^E), 98.7 (C-1^F), 103.4 (C-1^E), 158.4 ($OCONH_2$), 173.9, 175.4 (NH*C*OCH$_3$, C-6^F). $C_{24}H_{44}N_4O_{13}$ (596.63, 596.29), ESI ICR MS: *m*/*z*=597.2973 (calcd 597.29776) $[M+H]^+$.

3.6.15. 2-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-1-*O*-[3-(3-{2-[2-(2-aminoethoxy)ethoxy]ethylamino}-3-oxopropylthio)propyl]-3-*O*-carbamoyl-α-D-galactopyranuronamide (18a). A solution of **16b** (70 mg, 146 μmol) and **13d** (207 mg, 876 μmol) in ultrapure water (1 mL) was degassed (sonication while purging with argon) and irradiated at 10°C in a quartz cuvette for 2 h at 254 nm (Rayonet reactor, RPR-100). Solvent evaporation and FC ($CHCl_3$–methanol–water–HCO_2H 9:6:1.4:0.1) followed by Sephadex LH-20 chromatography (elution with methanol–water 3:1) gave after lyophilization **18a** (74 mg, 71%) as a white solid. R_f=0.10 ($CHCl_3$–methanol–water–HCO_2H=9:6:1.4:0.1). ^{1}H NMR (400 MHz, D_2O, H,H COSY): δ=1.95 (s, 3H, NHCOCH_3), 3.30–3.39 (m, 4H, 4^E-H, 5^E-H, CH_2-9^{SPA}), 3.41–3.48 (m, 1H, 3^E-H), 3.59–3.70 (m, 9H, including 2^E-H, 6^E-H), 3.84 (d, *J*=12.4 Hz, 1H, 6^E-H′), 3.94 (dd, $J_{1,2}$=10.6 Hz, $J_{2,3}$=3.9 Hz, 1H, 2^F-H), 4.29–4.33 (m, 1H, 4^F-H), 4.36 (bs, 1H, 5^F-H), 4.46 (d, $J_{1,2}$=8.5 Hz, 1H, 1^E-H), 4.88 (dd, $J_{3,4}$=3.2 Hz, 1H, 3^F-H), 5.21 (d, 1H, 1^F-H). ^{13}C NMR (100 MHz, D_2O, HMQC): δ=22.9 (NHCO*C*H_3^E), 56.1 (C-2^E), 61.5 (C-6^E), 68.6 (C-4^F), 70.6 (C-4^E), 70.8 (C-5^F), 71.7 (C-3^F), 74.3 (C-3^E), 75.6 (C-2^F), 76.4 (C-5^E), 98.6 (C-1^F), 103.4 (C-1^E), 158.4 ($OCONH_2$), 173.9, 175.3, 175.3 (NH*C*OCH$_3$, C-6^F, C-7^{SPA}). $C_{27}H_{49}N_5O_{15}S$ (715.77, 715.29), ESI ICR MS: *m*/*z*=716.3015 (calcd 716.3019) $[M+H]^+$, 738.2838 (calcd 738.2838) $[M+Na]^+$.

3.6.16. 2-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-3-*O*-carbamoyl-1-*O*-{3-[3-(2-{2-[2-(2-ethoxy-3,4-dioxo-

cyclobut-1-en-1-ylamino)ethoxy]ethoxy}ethylamino)-3-oxopropylthio]propyl}-α-D-galactopyranuronamide (18b). To a solution of **18a** (50 mg, 69.8 μmol) in 0.5 M phosphate buffer (pH 7.2, 3 mL) a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (35.6 mg, 210 μmol) in ethanol (0.5 mL) was added and the mixture was stirred at 20°C for 18 h. After solvent evaporation buffer salts and the excess of 3,4-diethoxy-3-cyclobuten-1,2-dione were removed by Sephadex LH-20 chromatography (elution with methanol–water 3:1). The remaining material was adsorbed to kieselguhr and placed on top of a FC column. Elution with ethyl acetate–methanol–water 6:3:1) followed by Sephadex LH-20 chromatography (elution with methanol–water 3:1) and lyophilization furnished **18b** (39 mg, 66%) as a white solid. R_f=0.16 (ethyl acetate–methanol–water 6:3:1). ^{1}H NMR (400 MHz, D_2O, H,H COSY): δ=1.37–1.44 (m, 3H, $OCH_2CH_3^{SA}$), 2.00 (s, 3H, $NHCOCH_3^E$), 3.38–3.45 (m, 2H, 4^E-H, 5^E-H), 3.47–3.53 (m, 1H, 3^E-H), 3.61–3.85 (m, 12H, including 2^E-H, 6^E-H), 3.90 (bd, J=12.2 Hz, 1H, 6^E-H′), 4.00 (dd, $J_{1,2}$=10.8 Hz, $J_{2,3}$=3.9 Hz, 1H, 2^F-H), 4.35–4.38 (m, 1H, 4^F-H), 4.42 (d, 1H, 5^F-H), 4.52 (d, $J_{1,2}$=8.3 Hz, 1H, 1^E-H), 4.64–4.75 (m, 2H, $OCH_2CH_3^{SA}$ partially hidden by the water signal), 4.94 (dd, $J_{3,4}$=3.4 Hz, 1H, 3^F-H), 5.27 (d, 1H, 1^F-H). ^{13}C NMR (100 MHz, D_2O, HMQC): δ=15.7 ($OCH_2CH_3^{SA}$), 22.9 ($NHCOCH_3^E$), 56.1 (C-2^E), 61.6 (C-6^E), 68.6 (C-4^F), 70.6 (C-4^E), 70.8 (C-5^F), 71.3 ($OCH_2CH_3^{SA}$), 71.8 (C-3^F), 74.4 (C-3^E), 75.6 (C-2^F), 76.4 (C-5^E), 98.7 (C-1^F), 103.4 (C-1^E), 158.4 ($OCONH_2$), 173.9, 174.4, 175.2, 175.3 ($NHCOCH_3$, C=C^{SA}, C-6^F, C-7^{SPA}), 177.8, 178.1 (C=C^{SA}), 184.0, 189.6 (2×C=O^{SA}). $C_{33}H_{53}N_5O_{18}S$ (839.87, 839.31), FAB MS: m/z=840.3 [M+H]$^+$, 862.3 [M+Na]$^+$. ESI ICR MS: m/z=840.3179 (calcd 840.3179) [M+H]$^+$, 862.3003 (calcd 862.2998) [M+Na]$^+$.

3.7. Conjugate 18c

A solution of **18b** (5.7 mg, 6.7 μmol) in 0.1 M carbonate buffer (pH 9.0, 0.5 mL) was added to a solution of BSA (30 mg, 0.45 μmol) in 0.1 M carbonate buffer (pH 9.0, 1.2 mL) and the mixture was stirred at 20°C for 40 h. Then TLC (ethyl acetate–methanol–water 6:3:1, R_f of **18b**: 0.16) indicated the complete consumption of **18b**. The conjugate was purified by ultrafiltration (30,000 cut off) to provide after lyophilization 31 mg of the conjugate **18c**. MALDI TOF MS (BSA as internal standard, m/z around 66,590) displayed for the conjugate a molecular peak maximum around m/z=76,560 corresponding to an average haptene–BSA ratio of 12:13.[41]

3.7.1. Allyl 2-*O*-[3-(allyloxycarbonyl)-propionyl]-3,4-*O*-isopropylidene-α-D-galactopyranosiduronamide (20b). To an ice-cold solution of allyl hydrogen succinate (62.8 mg, 0.39 mmol) in 300:1 CH_2Cl_2–DMF (300 μL) oxalyl chloride (38 μL, 54.6 mg, 0.43 mmol) was added dropwise. The mixture was stirred at 0°C for 1 h and 2 h at 20°C. The solution of the acid chloride was then added to an ice-cold solution of **20a** (100.0 mg, 0.37 mmol) in CH_2Cl_2 (400 μL) and pyridine (700 μL). The mixture was stirred at 0°C for 4 h and at 20°C for 15 h. Excess acid chloride was destroyed by addition of 2-propanol (500 μL). After 30 min at 20°C solvents were evaporated. LC (petrol–ethyl acetate 1.2:1→1:2) provided **20b** (115.9 mg, 77%) and recovered **20a** (11.4 mg). IR ($CHCl_3$): 1740 (C=O), 1700 (C=C), 1380, 1220, 1160, 1080, 1030, 990 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$, homo decoupling): δ=1.36, 1.51 (2s, 6H, $C(CH_3)_2$), 2.66–2.75 (m, 4H, CH_2-2^{succ}, CH_2-3^{succ}), 4.02 (dddd, 1H, 1^{allyl1}-H), 4.18 (dddd, 1H, 1′^{allyl1}-H), 4.41 (dd, $J_{3,4}$=5.4 Hz, 1H, 3^F-H), 4.54 (d, 1H, 5^F-H), 4.60 (ddd, $J_{1,2}^{allyl-2}$=3.8 Hz, 2H, CH_2-1$^{allyl-2}$), 4.63 (dd, $J_{4,5}$=2.7 Hz, 1H, 4^F-H), 4.96 (dd, $J_{2,3}$=7.8 Hz, 1H, 2^F-H), 5.09 (d, 1H, $J_{1,2}$=3.6 Hz, 1^F-H), 5.23, 5.24 (2dddd, J_{cis}=10.4 Hz, 2H, $3_{trans}^{allyl-1}$-H, $3_{trans}^{allyl-2}$-H), 5.30, 5.32 (2dddd, J_{trans}=17.3 Hz, 2H, $3_{cis}^{allyl-1}$-H, $3_{cis}^{allyl-2}$-H), 5.72, 6.50 (concentration dependent, 2s, b, 2H, $CONH_2^F$), 5.80–5.89 (m, 2H, 2$^{allyl-1}$-H, 2$^{allyl-2}$-H). ^{13}C NMR (50 MHz, $CDCl_3$, C,H COSY, APT): δ=26.7, 28.3 ($C(CH_3)_2^F$), 29.5, 29.5 (C-2^{succ}, C-3^{succ}), 65.9 (C-1$^{allyl-2}$), 69.1 (C-5^F), 69.8 (C-1$^{allyl-1}$), 71.9 (C-2^F), 73.5 (C-3^F), 73.9 (C-4^F), 95.8 (C-1^F), 110.6 ($C(CH_3)_2$), 118.7, 118.8 (C-3$^{allyl-1}$, C-3$^{allyl-2}$), 132.5, 133.5 (C-2$^{allyl-1}$, C-2$^{allyl-2}$), 170.6, 172.1, 172.4 ($CONH_2$, 2C=O^{succ}). calcd C 55.20, H 6.58, N 3.39, found C 55.65, H 6.20, N 3.57. $C_{19}H_{27}NO_9$ (413.42, 413.17), FAB MS: m/z=436.1 [M+Na]$^+$, 414.1 [M+H]$^+$.

3.7.2. Allyl 2-*O*-[3-(allyloxycarbonyl)-propionyl]-α-D-galactopyranosiduronamide (21a). A mixture of **20b** (2.22 g, 5.4 mmol) and 20% acetic acid (97 mL) was stirred at 50°C for 8 h. Solvent evaporation (codestillation with toluene), dissolving the residue in water and lyophilization, followed by LC (petrol–$CHCl_3$–methanol 1:1:0.2) furnished **21a** (1.9695 g, 98%). IR ($CHCl_3$): 3550–3200, 3020, 2920, 1740, 1690, 1220, 1150, 1100, 1050, 1030 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ=4.03 (dd, $J_{2,3}$=10.4 Hz, $J_{3,4}$=3.0 Hz, 1H, 3^F-H), 4.265 (s, 1H, 5^F-H), 4.269 (d, 1H, 4^F-H), 5.02 (dd, 1H, 2^F-H), 5.16 (d, $J_{1,2}$=3.6 Hz, 1H, 1^F-H), 7.31, 7.34 (2s, b, $CONH_2^F$). ^{13}C NMR (75 MHz, CD_3OD, C,H COSY): δ=68.6 (C-3^F), 71.3 (C-5^F), 72.4 (C-2^F), 72.8 (C-4^F), 97.0 (C-1^F), 173.7, 173.9, 174.1 ($CONH_2^F$, 2C=O^{succ}). $C_{16}H_{23}NO_9$ (373.36, 373.14), calcd C 51.47, H 6.21, N 3.75, found C 51.60, H 5.90, N 3.70, FAB MS: m/z=769.2 [2M+Na]$^+$, 747.2 [2M+H]$^+$, 396.0 [M+Na]$^+$, 374.0 M+H]$^+$.

3.8. Conversion of 21a to 21b

To a solution of **21a** (1.45 g, 3.9 mmol) in CH_2Cl_2 (300 mL) trichloroacetyl isocyanate (560 μL, 4.7 mmol) was added at −6°C. The mixture was stirred at −6°C for 3.5 h. Excess reagent was destroyed by addition of CH_3OH (16 mL) and stirred at 20°C for 1.5 h. After solvent evaporation the residue was redissolved in CH_3OH (190 mL). Zn dust (2.53 g, 38.8 mmol) was added and the mixture was vigorously stirred at 20°C for 12 h. Solids were removed by filtration and washed carefully with CH_3OH. The combined filtrates were evaporated. FC ($CHCl_3$–CH_3OH 9:1) provided **21b** (1.1097 g, 69%), alongside with the 4-isomer (**21c**, 30.7 mg, 2%) and the 3,4-dicarbamoyl derivative of **21d** (179.9 mg, 10%).

3.8.1. Allyl 2-*O*-[3-(allyloxycarbonyl)-propionyl]-3-*O*-carbamoyl-α-D-galactopyranosiduronamide (21b). IR (KBr): 3650–3200, 2930, 1740, 1690, 1390, 1330, 1150, 1090, 1050, 1030 cm^{-1}. ^{1}H NMR (300 MHz, pyridine-d_5, homo decoupling): δ=4.88 (d, $J_{4,5}$=1.1 Hz, 1H, 5^F-H), 5.48 (broad s, w_{1-2}≈7 Hz, 1H, 4^F-H), 5.52 (d, $J_{1,2}$=3.7 Hz, 1H,

1^F-H), 5.94 (dd, $J_{2,3}$=10.8 Hz, $J_{3,4}$=3.2 Hz, 1H, 3^F-H), 6.13 (dd, 1H, 2^F-H), 7.60, 7.88, 7.96, 8.48 (4s, $CONH_2^F$, $OCONH_2^F$). ^{1}H NMR (400 MHz, DMSO-d_6, H,H COSY): δ=4.08 (s, 1H, 5^F-H), 4.26 (dd, $J_{3,4}$=2.9 Hz, $J_{4,OH}$=6.2 Hz, 1H, 4^F-H), 4.91 (dd, $J_{2,3}$=10.9 Hz, 1H, 3^F-H), 5.04 (d, $J_{1,2}$=3.7 Hz, 1H, 1^F-H), 5.10 (dd, 1H, 2^F-H), 5.46 (d, 1H, 4^F-OH), 6.57 (s, b, 2H, $OCONH_2^F$), 7.08, 7.31 (2s, 2H, $CONH_2^F$). ^{13}C NMR (50 MHz, pyridine-d_5, C,H COSY, APT): δ=69.1 (C-4^F), 69.9 (C-2^F), 71.4 (C-3^F), 73.4 (C-5^F), 96.7 (C-1^F), 157.8 ($OCONH_2^F$), 172.1, 172.1, 172.8 ($CONH_2^F$, 2C=O^{succ}). $C_{17}H_{24}N_2O_{10}$, (416.38, 416.14), FAB MS: *m*/*z*=439.0 $[M+Na]^+$, 417.0 $[M+H]^+$.

3.8.2. Allyl 2-*O*-[3-(allyloxycarbonyl)-propionyl]-4-*O*-carbamoyl-α-D-galactopyranosiduronamide (21c). IR (KBr): 3610–3180, 1720, 1710, 1640, 1380, 1320, 1210, 1150, 1040, 1020 cm^{-1}. ^{1}H NMR (200 MHz, pyridine-d_5):[42] δ=4.85 (dd, $J_{3,4}$=3.4 Hz, 1H, 3^F-H), 4.94 (d, $J_{4,5}$=1.2 Hz, 1H, 5^F-H), 5.52 (d, $J_{1,2}$=3.7 Hz, 1H, 1^F-H), 5.68 (dd, $J_{2,3}$=10.5 Hz, 1H, 2^F-H), 6.50 (dd, 1H, 4^F-H), 7.52 (broad s, $OCONH_2^F$), 7.77, 8.49 (2s, $CONH_2^F$). ^{13}C NMR (50 MHz, pyridine-d_5): δ=67.1 (C-3^F), 69.4 (C-$1^{allyl\text{-}1}$), 71.9 (C-2^F), 72.9 (C-4^F), 73.1 (C-5^F), 96.7 (C-1^F), 158.13 ($OCONH_2^F$), 170.9, 172.2, 172.9 ($CONH_2^F$, 2C=O^{succ}). $C_{17}H_{24}N_2O_{10}$, (416.38, 416.14), FAB MS: *m*/*z*=855.3 $[2M+Na]^+$, 833.3 $[2M+H]^+$, 439.1 $[M+Na]^+$, 417.1 $[M+H]^+$.

3.8.3. Allyl 2-*O*-[3-(allyloxycarbonyl)-propionyl]-3,4-di-*O*-carbamoyl-α-D-galactopyranosiduronamide (21d). IR (KBr): 3600–3140, 1730, 1670, 1380, 1320, 1140, 1060, 1030, 990 cm^{-1}. ^{1}H NMR (300 MHz, pyridine-d_5, homo decoupling): δ=4.94 (broad s, 1H, 5^F-H), 5.51 (d, $J_{1,2}$=3.5 Hz, 1H, 1^F-H), 5.73 (dd, $J_{2,3}$=10.9 Hz, 1H, 2^F-H), 6.07 (dd, $J_{3,4}$=3.3 Hz, 1H, 3^F-H), 6.72 (s, 1H, 4^F-H), 7.40–7.80 (s, $OCONH_2^F$), 7.58, 7.65, 8.50 (3s, $CONH_2^F$, $OCONH_2^F$). ^{13}C NMR (75 MHz, pyridine-d_5, C,H COSY, APT): δ=68.7 (C-3^F), 69.3 (C-2^F), 70.1 (C-4^F), 70.8 (C-5^F), 95.9 (C-1^F), 156.9, 157.1 ($2OCONH_2^F$), 170.0, 171.7, 172.2 ($CONH_2^F$, 2C=O^{succ}). $C_{18}H_{25}N_3O_{11}$ (459.41, 459.15), FAB MS: *m*/*z*=941.2 $[2M+Na]^+$, 482.1 $[M+Na]^+$, 460.1 $[M+H]^+$.

3.8.4. Allyl 2-*O*-succinyl-3-*O*-carbamoyl-α-D-galacto-pyranosiduronamide (21e). To a solution of **21b** (219.0 mg, 0.53 mmol) in THF (5.3 mL) freshly prepared $Pd(PPh_3)_4$ (67.7 mg, 0.06 mmol), dissolved in THF (5.7 mL), and morpholine (67 μL, 0.78 mmol) were added. The yellow solution was stirred at 20°C for 1 h. During this time a white solid precipitated. After solvent evaporation the residue was redissolved in CH_3OH and Dowex 50 (H^+) was added. The mixture was stirred. After filtration the resin was several times rinsed with CH_3OH. The combined filtrates were evaporated. The residue was partitioned between CH_2Cl_2 and water. The aqueous phase was several times extracted with CH_2Cl_2 and then freeze-dried to give pure **21e** (176.6 mg, 89%). IR (KBr): 3660–3090, 1730, 1690, 1680, 1590, 1560, 1400, 1380, 1150, 1090, 1060, 1020, 990 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6, H,H COSY): δ=4.08 (s, 1H, 5^F-H), 4.26 (m, 1H, 4^F-H), 4.90 (dd, $J_{2,3}$=10.9 Hz, $J_{3,4}$=3.1 Hz, 1H, 3^F-H), 5.04 (d, $J_{1,2}$=3.7 Hz, 1H, 1^F-H), 5.10 (dd, 1H, 2^F-H), 5.45 (d, $J_{4,OH}$=6.3 Hz, 1H, 4^F-OH), 6.56 (s, 2H, $OCONH_2^F$), 7.08, 7.30 (2s, 2H, $CONH_2^F$), 11.7–12.7 (s, broad, 1H, $COOH^{succ}$). ^{13}C NMR (100 MHz, DMSO-d_6, C,H COSY, APT): δ=67.5 (C-4^F), 68.2 (C-2^F), 69.4 (C-3^F), 71.5 (C-5^F), 95.1 (C-1^F), 156.3 ($OCONH_2^F$), 170.2, 172.1 ($CONH_2^F$, C-1^{succ}), 173.4 (C-4^{succ}). $C_{14}H_{20}N_2O_{10}$, (376.32, 376.11), FAB MS: *m*/*z*=421.1 $[M+2Na-H]^+$, 399.1 $[M+Na]^+$, 377.1 $[M+H]^+$.

3.8.5. Allyl 2-*O*-[3-(benzyloxycarbonylmethyl-carbamoyl)-propionyl]-3-*O*-carbamoyl-α-D-galactopyranosiduronamide (21f). To a solution of **21e** (25.3 mg, 0.067 mmol) in DMF (>99.5%, 150 μL) a solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (19.5 mg, 0.102 mmol) in bistilled water (22 μL) and after 15 min a solution of *N*-hydroxysuccinimide (NHS, 11.9 mg, 0.100 mmol) in DMF (>99.5%, 13.5 μL) were added. The reaction mixture was stirred at 20°C for 20 h. Then solutions of EDC·HCl (19.5 mg, 0.102 mmol) in bidistilled water (22 μL) and NHS (11.9 mg, 0.100 mmol) in DMF (>99.5%, 13.5 μL) were added. Stirring was continued for another 3 h. The mixture was then cooled to 4°C and a solution of benzyl glycinate hydrochloride (20.4 mg, 0.101 mmol) in bidistilled water (110 μL, and neutralized with 5% aq $NaHCO_3$) was added. The reaction mixture was stirred at 4°C for 24 h and then freeze-dried. FC ($CHCl_3$–CH_3OH–AcOH 90:7:1) provided pure **21f** (4.7 mg, 13%), a fraction of slightly impure **21f** (14.2 mg, ≈40%), and **21e** contaminated with benzyl glycinate (16.7 mg). ^{1}H NMR (200 MHz, DMSO-d_6, homo decoupling; H,H COSY): δ=3.91 (d, $J_{NH,CH}$=5.9 Hz, 2H, CO–NH–CH_2–COO–), 4.09 (s, 1H, 5^F-H), 4.28 (s, broad, w_{1-2}≈6.8 Hz, 1H, 4^F-H), 4.91 (dd, $J_{2,3}$=10.7 Hz, $J_{3,4}$=2.6 Hz, 1H, 3^F-H), 5.03–5.12 (m, 2H, 1^F-H, 2^F-H), 5.40–5.60 (s, b, 1H, 4^F-OH), 6.61 (s, 2H, $OCONH_2^F$), 7.12 (s, 1H, $CONH_2^F$), the second $CONH_2^F$ signal was hidden by aromatic proton signals, 7.38 (m, 5H, Ar–H), 8.44 (t, 1H, CO–N*H*–CH_2–COO). $C_{23}H_{29}N_3O_{11}$ (523.50, 523.18), FAB MS: *m*/*z*=562.1 $[M+K]^+$, 546.1 $[M+Na]^+$, 524.1 $[M+H]^+$.

3.9. Coupling of 21e to BSA

To a solution of **21e** (9.6 mg, 0.026 mmol) in analytical grade DMF (60 μL) a solution of 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC·HCl, 8.8 mg, 0.046 mmol) in water (15 μL) was added and after 45 min a solution of *N*-hydroxysuccinimide (NHS, 4.5 mg, 0.038 mmol) in analytical grade DMF (5 μL). The reaction mixture was stirred at 20°C for 23 h. After this time again solutions of EDC·HCl (8.8 mg, 0.046 mmol) in water (15 μL) and after 20 min of NHS (4.5 mg, 0.038 mmol) in DMF (>5 μL) were added. The mixture was stirred at 20°C for 2 h. Then, at 4°C a cooled solution of BSA (5.6 mg/mL 50 mM PBS, pH 7.2, 2.8 mL) was added. The reaction mixture was stirred at 4°C for 12 d. Low-molecular weight impurities were removed by ultrafiltration at 2°C. In a second experiment purification was performed by dialysis. After lyophilization 18.8 mg of the conjugate **21g** were obtained. SDS-PAGE indicated the conversion of BSA. The MALDI TOF MS (external calibration, mean of 8 spectra, each obtained from 100 scans) displayed *m*/*z*=72,569 $[M+H]^+$±141 corresponding to hapten–carrier ratio 17.

3.9.1. 2-*O*-{2-Acetamido-4-*O*-[2-acetamido-4-*O*-((5*R*)-5-{5-(3-carboxy-propionyl)-2-[4-nitro-3-(2-pyridin-2-yldithio-ethylcarbamoyl)-phenyl]-2*H*-[1,2,4]triazol-3-yl}-α-L-arabinopyranosyl)-2,6-dideoxy-β-D-glucopyranosyl]-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-methylene-nonodeca-2,6,13,17-tetraenyloxy)-ethoxy]-hydroxy-phosphoryl}-α-D-galactopyranuronamide (22a). Compound **22a** was prepared as described for **2a**.[18] Lyophilization, followed by ultrafiltration and FC ($CHCl_3$–MeOH–H_2O 18:11:2.7) provided **22a** in 65% yield. R_t: 20.1 min (RP-HPLC: ^{1}H NMR (400 MHz, D_2O): (characteristic signals): δ=0.94 (s, CH_3-23^I, CH_3-24^I), 1.22, 1.30 (2×d, J=5.1, 5.9 Hz, CH_3-6^C, CH_3-6^E), 1.59, 1.60 (2×s, CH_3-20^I, CH_3-21^I), 1.66 (s, CH_3-19^I), 1.73 (s, CH_3-25^I), 1.85–2.15 (m, CH_2-10^I, CH_2-15^I, CH_2-16^I, CH_2-5^I, CH_2-4^I), 1.98 (s, NHCOCH_3^E), 2.00 (s, NHCOCH_3^C), 2.73 (d, J=7.3 Hz, CH_2-12^I), 3.10 (t, CH_2-2^{Cys}), 4.45–4.50 (m, H-5^F), 5.00 (bd, J=8.8 Hz, 3^F-H), 5.10–5.20 (m, 13^I-H, 17^I-H), 5.30–5.50 (m, 2^I-H, 7^I-H, 6^I-H), 5.70–5.80 (m, 1^F-H), 7.22–7.32 (m, 1H, 4^{py}-H), 7.75–7.90 (m, 2H, 3^{py}-H, 2^{py}-H), 7.94 (d, 1H, 2^{Ar}-H), 8.04 (bd, J=8.8 Hz, 1H, 4^{Ar}-H), 8.35 (d, J=4.4 Hz, 1H, 5^{py}-H), 8.51 (d, J=8.8 Hz, 1H, 5^{Ar}-H). $C_{76}H_{107}N_{10}O_{31}S_2P$ (1751.82, 1750.63), FAB MS: m/z=1752.1 $[M+H]^+$, 1774.3 $[M+Na]^+$, 1795.6 $[M+2Na-H]^+$.

3.9.2. 2-*O*-{2-Acetamido-4-*O*-[2-acetamido-4-*O*-((5*R*)-5-{5-(3-carboxy-propionyl)-2-[3-(2-mercapto-ethylcarbamoyl)-4-nitro-phenyl]-2*H*-[1,2,4]triazol-3-yl}-α-L-arabinopyranosyl)-2,6-dideoxy -β-D-glucopyranosyl]-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-methylene-nonodeca-2,6,13,17-tetraenyloxy)-ethoxy]-hydroxy-phosphoryl}-α-D-galactopyranuronamide (22b). To a solution of dithiothreitol (24.6 mg, 0.16 mmol) in methanol (10 mL) a solution of **22a** (28.0 mg, 16 μmol) in methanol (5 mL) was added slowly. The mixture was stirred at 20°C for 5 h. Progress of the reaction was controlled by RP-HPLC. Lyophilization followed by removal of low molecular weight impurities by ultrafiltration furnished **22b** (21.3 mg, 81%). R_t: 12.2 min (RP-HPLC). $C_{71}H_{104}N_9O_{31}SP$ (1642.68, 1641.63), FAB MS: m/z=1665.6 $[M+Na]^+$, 1687.7 $[M+2Na-H]^+$.

3.9.3. 2-*O*-[2-Acetamido-4-*O*-(2-acetamido-4-*O*-{(5*R*)-5-[2-(3-{2-[(*RS*)-1-(2-{(*R*)-1-carboxy-5-[5-((3a*S*)-2-oxo-(3a*r*,6a*c*)-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4*t*-yl)-pentanoylamino]-pentylcarbamoyl}-ethyl)-2,5-dioxo-pyrrolidin-3-ylthio]-ethylcarbamoyl}-4-nitro-phenyl)-5-(3-carboxy-propionyl)-2*H*-[1,2,4]triazol-3-yl]-α-L-arabinopyranosyl}-2,6-dideoxy-β-D-glucopyranosyl)-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-methylene-nonodeca-2,6,13,17-tetraenyloxy)-ethoxy]-hydroxy-phosphoryl}-α-D-galactopyranuronamide (22c). A solution of **22b** (15.0 mg, 9 μmol) and *N*-(3-maleimidopropionyl)-biocytin (80% purity, Fluka, 4.6 mg, 7 μmol) in water (10 mL) was stirred 5 h at 20°C. The reaction was followed by RP-HPLC (diode array detector). Lyophilization, followed by FC ($CHCl_3$–MeOH–H_2O 18:11:2.7) provided 11.1 mg (56%) of **22c**. R_t: 10.3 min (RP-HPLC). ^{1}H NMR (400 MHz, D_2O, H,H COSY): (characteristic signals): δ=0.88 (s, CH_3-23^I, CH_3-24^I), 1.22, 1.30 (2×d, J=6.5, 6.3 Hz, CH_3-6^C, CH_3-6^E), 1.27–1.33 (m, 9^I-H), 1.53, 1.55 (2×s, CH_3-20^I, CH_3-21^I), 1.60 (s, CH_3-19^I), 1.67 (s, CH_3-25^I), 1.82–1.87 (m, CH_2-10^I), 1.93 (s, NHCOCH_3^E), 1.96 (s, NHCOCH_3^C), 2.51–2.57 (m, CH_2-3$^{\beta a}$), 2.65–2.70 (m, CH_2-6^{BTR}, CH_2-12^I), 2.89 (dd, J=12.8, 5.6 Hz, CH_2'-6^{BTR}), 3.08 (t, 2^{Lys}-H), 4.31 (dd, J=8.2, 4.8 Hz, 3a^{BTR}-H), 4.50 (dd, J=8.2, 4.9 Hz, 6a^{BTR}-H), 4.92 (dd, J=10.4, 3.6 Hz, 3^F-H), 5.07–5.12 (m, 13^I-H, 17^I-H), 5.23–5.38 (m, 2^I-H, 7^I-H, 6^I-H), 5.68–5.72 (m, 1^F-H), 7.94 (bs, 1H, 2^{Ar}-H), 7.97 (bd, J=9.4 Hz, 1H, 4^{Ar}-H), 8.44 (d, J=8.6 Hz, 1H, 5^{Ar}-H). $C_{94}H_{137}N_{14}O_{38}S_2P$ (2166.28, 2164.84), FAB MS: m/z=2189.7 $[M+Na]^+$, 2202.7 $[M+K]^+$, 2210.8 $[M+2Na-H]^+$.

3.9.4. (*R*)-2-(16-Biotinamido-7,10,13-trioxa-3-azahexadecyloxy)-3-({β-D-galactopyranuronamidosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy}hydroxy-phosphoryloxy)-propionic acid (23). To a solution **9a** (80 mg, 67 μmol) in phosphate buffer (pH 7.0, 623 μL, 50 mM) and methanol (1.250 mL) a solution of **24a** (Pierce, 116.5 mg, 268 μmol) in methanol (625 μL) was added. The pH was readjusted to 7.0 and a solution of $NaBH_3CN$ (8.6 mg, 134 μmol) in methanol (156 μL) was added. The mixture was stirred at 20°C for 48 h and then directly filtered through a Sephadex® LH-20 column (elution with H_2O–CH_3OH 1:4). All fractions containing **23** were combined, the solvents were evaporated and the residue was adsorbed on kieselguhr (300 mg). This material was transferred to the top of a FC column. Elution with FSC (ethyl acetate–*i*-propanol–H_2O 6:4:2) and subsequent removal of inorganic material via Sephadex® LH-20 chromatography (elution with H_2O–CH_3OH 1:4) provided after lyophilization 26 mg (26%) of **23**. ^{1}H NMR (400 MHz, D_2O, H,H COSY): δ=1.18 (s, CH_3-4^F), 1.33 (d, J_{5-6}=5.9 Hz, CH_3-6^C), 1.30–1.40 (m, CH_2-4^L), 1.50–1.70 (m, CH_2-3^L, CH_2-5^L), 1.71–178 (m, CH_2-9^K), 1.95–2.00 (m, CH_2-2^K), 1.99, 2.05 (s, NHCOCH_3^E, s, NHCOCH_3^C), 2.21 (dd, J_{2-3}=7.0 Hz, CH_2-2^L), 2.72–2.75, 2.93–2.97 (m, CH_2-6^M), 3.13 (dd, J_{1-2}=7.5 Hz, CH_2-1^K), 3.19–3.23 (m, CH_2-10^K), 3.23–3.27 (m, 2^D-H), 3.52–3.54 (m, CH_2-8^K), 3.58–3.62 (m, CH_2-3^K), 3.60–3.70 (m, CH_2-4^K-CH_2-7^K), 4.18 (s, H-4^B, H-5^B), 4.36–4.38 (m, 3a^M-H), 4.40 (s, 5^F-H), 4.47 (d, J_{1-2}=7.8 Hz, 1^D-H), 4.57–4.58 (m, 6a^M-H), 4.96 (d, J_{2-3}=10.8 Hz, 3^F-H), 5.77 (q, J_{1F-2F}=3.4 Hz, J_{1F-P}=5.9 Hz, 1^F-H). ^{13}C NMR (50 MHz, D_2O, APT): δ=14.9 (CH_3-4^F), 16.8 (CH_3-6^C), 22.5, 22.6 (NHCO*C*H$_3^E$, NHCO*C*H$_3^C$), 25.3, 25.7, 27.9, 28.1, 28.4 (C-2^K, C-9^K, C-3^L, C-4^L, C-5^L), 35.7, 36.5 (C-10^K, C-2^L), 39.9 (C-6^M), 45.4 (C-1^K), 47.5 (C-2^I), 55.3, 55.5, 55.7 (C-2^E, C-2^C, C-4^M), 60.4 (C-6a^M), 61.0 (+) (C-6^D), 62.3 (C-3a^M), 65.1 (+) (C-1^I), 67.1 (C-1^H), 68.1–76.2 (C-5^C, C-4^D, C-4^B, C-2^B, C-3^B, C-6^E, C-5^B, C-3^E, C-3^C, C-5^F, C-2^F, C-4^F, C-5^E, C-2^D, C-3^F, C-5^D, C-3^D, C-3^K-C-8^K), 80.0 (C-4^E), 80.9 (C-2^H), 83.2 (C-4^C), 94.6 (C-1^F), 101.4, 102.3, 102.8, 103.4 (C-1^C, C-1^E, C-1^B, C-1^D), 158.3 (O*C*ONH$_2^E$), 165.5 (C-2^M), 172.9, 173.4, 174.3, 174.7 (CONH$_2^B$, CONH$_2^F$, NH*C*OCH$_3^E$, NH*C*OCH$_3^C$), 176.6, 176.9 (C-3^H, C-1^L). The signals in the region 65–80 ppm are broad. ^{31}P NMR (81 MHz, D_2O): δ=2.19. $C_{61}H_{104}N_9O_{37}PS$ (1618.57, 1617.59), ESI MS:

m/z=1616.5989 (calcd 1616.5919) $[M-H]^-$, 807.7931 (calcd 807.7923) $[M-2H]^{2-}$.

3.9.5. 2-*O*-(2-Acetamido-3,4,6-*O*-tribenzyl-2-deoxy-β-D-glucopyranosyl)-1-*O*-(2-{2-[2-(2-biotinamidoethoxy)-ethoxy]ethylamino}ethyl)-3-*O*-carbamoyl-α-D-galacto-pyranuronamide (25a). A solution of **14** (49.8 mg, 133 μmol) in methanol (10 mL) was adjusted to pH 7 with methanolic solution of acetic acid. Solutions of **24b** (Pierce, 50 mg, 66 μmol) in methanol (20 mL) and of sodium cyanoborohydride (8.35 mg, 133 μmol) in methanol (0.5 mL) were added successively and the mixture was stirred at 20°C for 18 h (Ar atmosphere). The reaction mixture was adsorbed to kieselguhr and the material placed on top of a FC column. Elution with $CHCl_3$–methanol–HCO_2H 10:10:0.1, followed by Sephadex LH-20 chromatography (elution with methanol–water 3:1) and lyophilization provided 43 mg (58%) of **25a** as a white powder. R_f=0.12 ($CHCl_3$–methanol–water 12:6:1). ^1H NMR (400 MHz, D_2O, H,H COSY): δ=1.14–1.28 (m, 2H, CH_2-4^{bio}), 1.31–1.61 (m, 4H, CH_2-3^{bio}, CH_2-5^{bio}), 1.89 (s, 3H, NHCOCH_3), 2.07 (t, $J_{2,3}$=7.1 Hz, 2H, CH_2-2^{bio}), 2.56 (d, *J*=13.2 Hz, 1H, CH_2-6^{bio}), 2.74 (dd, $J_{6,6a}$=4.6 Hz, 1H, CH_2-6^{bio}), 3.38–3.84 (m, 15H, 2^E-H, 3^E-H, 4^E-H, 5^E-H, CH_2-5^{SPA}, CH_2-7^{SPA}, CH_2-8^{SPA}, CH_2-10^{SPA}, CH*H*-1^{SPA}, CH_2-6^E), 3.93–4.03 (m, 2H, 2^F-H, CH*H*-1^{SPA}), 4.17 (m, 1H, $3a^{bio}$-H), 4.30–3.66 (m, 10H, 1^E-H, 4^E-H, 5^E-H, $6a^{bio}$-H, CH_2^{benzyl}), 5.98 (dd, $J_{2,3}$=10.5 Hz, $J_{3,4}$=3.2 Hz, 1H, 3^F-H), 5.26 (d, $J_{1,2}$=3.4 Hz, 1H, 1^F-H), 6.76–7.44 (m, 15H, Ar–Hs^{benzyl}). ^{13}C NMR (100 MHz, D_2O, APT, HMQC): δ=22.8 (NHCO*C*H_3), 25.7, 28.2, 28.5 (C-3^{bio}, C-4^{bio}, C-5^{bio}), 35.9 (C-2^{bio}), 39.3 (C-11^{SPA}), 40.2 (C-6^{bio}), 47.0 (C-2^{SPA}, C-4^{SPA}), 55.4 (C-2^E), 55.8 (C-4^{bio}), 60.6 (C-$6a^{bio}$), 62.4 (C-$3a^{bio}$), 63.9 (C-1^{SPA}), 66.3 (C-5^{SPA}), 68.4 (C-4^F, C-6^E), 69.4 (C-10^{SPA}), 70.0, 70.2 (C-7^{SPA}, C-8^{SPA}), 71.0, 71.1 (C-5^F, C-3^F), 73.6 (CH_2^{benzyl}), 73.9 (C-4^E), 75.3, 75.7 (2×CH_2^{benzyl}), 76.3 (C-2^F), 78.0, 82.6 (C-3^E, C-5^E), 99.0 (C-1^F), 103.3 (C-1^E), 128.7–129.4 (Ar–CH^{benzyl}), 137.5, 137.6, 138.2 (Ar–Cq^{benzyl}), 158.2 ($OCONH_2$), 165.6 (C-2^{bio}), 173.6, 174.2 (NH*C*OCH$_3$, C-6^F), 176.7 (C-1^{bio}). $C_{54}H_{75}N_7O_{16}S$ (1110.29, 1109.50), FAB MS: *m/z*=1110.5 $[M+H]^+$, 1124.5 $[M+Na]^+$, ESI ICR MS: *m/z*=1110.5065 (calcd 1110.5064) $[M+H]^+$, 1132.4889 (calcd 1132.4883) $[M+Na]^+$.

3.9.6. 2-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-1-*O*-(2-{2-[2-(2-biotinamidoethoxy)ethoxy]ethylamino}ethyl)-3-*O*-carbamoyl-α-D-galactopyranuronamide (25b). To a solution of **25a** (19 mg, 17.1 μmol) in methanol–acetic acid–water (2:1:0.5, 5 mL) 10% Pd/C (20 mg) was added and the mixture was stirred under hydrogen at 35°C for 24 h. Filtration through celite and washing with 2:1 methanol–water, solvent evaporation, FC ($CHCl_3$–methanol–water–HCO_2H 9:6:1.4:0.1), Sephadex LH-20 chromatography (methanol–water 3:1), and lyophilization furnished 9.3 mg (64%) of the biotinylated disaccharide **25b** as white solid. R_f=0.09 ($CHCl_3$–methanol–water–HCO_2H 9:6:1.4:0.1). ^1H NMR (400 MHz, D_2O, H,H COSY): δ=1.43–1.55 (m, 2H, CH_2-4^{bio}), 1.59–1.86 (m, 4H, CH_2-3^{bio}, CH_2-5^{bio}), 2.11 (s, 3H, NHCOCH_3), 2.34 (t, $J_{2,3}$=7.2 Hz, 2H, CH_2-2^{bio}), 2.85 (d, *J*=12.7 Hz, 1H, CH_2-6^{bio}), 3.06 (dd, $J_{6,6a}$=5.3 Hz, 1H, CH_2-6^{bio}), 3.37–3.50 (m, 7H, 4^{bio}-H, CH_2-2^{SPA}, CH_2-4^{SPA}, CH_2-11^{SPA}), 3.51–3.55 (m, 2H, 4^E-H, 5^E-H), 3.58–3.64 (m, 1H, 3^E-H), 3.71 (t, $J_{10,11}$=5.5 Hz, 2H, CH_2-10^{SPA}), 3.76–3.83 (m, 5H, CH_2-7^{SPA}, CH_2-8^{SPA}, 2^E-H), 3.85–3.94 (m, 4H, CH_2-5^{SPA}, C*H*H-1^{SPA}, 6^E-H), 4.00 (d, *J*=12.0 Hz, 1H, 6^E-H′), 4.11–4.19 (m, 2H, 2^F-H, C*H*H-1^{SPA}), 4.47–4.51 (m, 2H, 4^F-H, $3a^{bio}$-H), 4.55 (d, $J_{4,5}$=1.1 Hz, 1H, 5^F-H), 4.65–4.70 (m, 2H, 1^E-H, $6a^{bio}$-H), 5.12 (dd, $J_{2,3}$=10.6 Hz, $J_{3,4}$=3.2 Hz, 1H, 3^F-H), 5.43 (d, $J_{1,2}$=3.9 Hz, 1H, 1^F-H). ^{13}C NMR (100 MHz, D_2O, APT, HMQC): δ=22.9 (NHCO*C*H_3^E), 25.8, 28.3, 28.5 (C-3^{bio}, C-4^{bio}, C-5^{bio}), 36.1 (C-2^{bio}), 39.5 (C-11^{SPA}), 40.3 (C-6^{bio}), 47.3, 47.6 (C-2^{SPA}, C-4^{SPA}), 56.0 (C-2^E, C-6^{bio}), 60.9 (C-6^E, C-$6a^{bio}$), 62.7 (C-$3a^{bio}$), 64.5 (C-1^{SPA}), 66.3 (C-5^{SPA}), 68.6 (C-4^F), 69.6 (C-10^{SPA}), 70.1, 70.3, 70.3 (C-7^{SPA}, C-8^{SPA}, C-4^E), 71.1 (C-5^F), 71.3 (C-3^F), 74.5 (C-3^E), 76.1 (C-2^F), 76.2 (C-5^E), 99.2 (C-1^F), 103.6 (C-1^E), 158.4 ($OCONH_2$), 166.0 (C-2^{bio}), 173.8, 175.0 (NH*C*OCH$_3$, $CONH_2$), 177.6 (C-1^{bio}). $C_{33}H_{57}N_7O_{16}S$ (839.91, 839.36), ESI ICR MS: *m/z*=840.3658 (calcd 840.3655) $[M+H]^+$, 862.3486 (calcd 862.3475) $[M+Na]^+$.

3.10. Immunization procedure

Rabbits were immunized by injection of moenomycin–BSA conjugates to raise polyclonal antisera against moenomycin. Approximately 500 μg of purified protein (moenomycin–BSA **8**) in phosphate buffered saline (PBS) was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into the rabbit. Subsequent injections (500 μg of **8** in PBS) were given in 14–28 d intervals. Last injections contained additionally incomplete Freund's adjuvant. Three days later, antiserum was obtained and screened for anti-moenomycin antibodies. For further experiments blood sera were incubated for 2 h at room temperature, centrifuged (3000***g***, 10 min, 4°C), incubated again (56°C, 45 min), and stored at −70°C.

3.11. Preparation of moenomycin–KLH

2 mg of **2a** and 2 mg of maleimide–activated KLH (keyhole limpet hemocyanin, Pierce) were mixed with 400 μL of 0.05 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl, 0.1 M EDTA for 2 h. Non reacted maleimide groups were blocked with cysteine for 1 h at room temperature. The preparation was dialysed by ultrafiltration. Quantitation of coupling efficiency was performed by determination of thiol groups with Ellmann's reagent[43] or, alternatively, by HPLC analysis as described previously.[22]

3.12. Screening of anti-moenomycin antibodies

Screening of the blood sera was performed in an enzyme-linked immunosorbent assay (ELISA) and by Western blotting. Polystyrene microtiter plates (Maxisorp or Polysorp, NUNC) with high binding capacity were coated with 500 ng moenomycin–KLH/mL or 150 nM **8**, or 10 μg streptavidin/mL in 0.05 M sodium carbonat buffer, pH 9.6 and incubated overnight at 4°C. Streptavidin-coated microtiter plates were further incubated with 10 μg of a the moenomycin A-biotin derivative reported in Ref. 18 in the same buffer for 1 h. Control ELISA plates were coated with KLH, BSA and streptavidin, respectively. The plates were washed with 50 mM Tris–HCl, pH 10.2, 150 mM NaCl, 0.05% (w/v) Tween 20 (washing buffer A), blocked

with 1–2% BSA or Tween 20 in washing buffer A, and washed again. Antisera were added in serial dilutions in 100 μL/well in washing buffer A for 2 h. For screening on **8** coated plates the antisera were preincubed with 2 mg/mL BSA for neutralization of anti-BSA antibodies. After washing (3×, washing buffer A) the plates were incubated with 100 μL/well of pork anti-rabbit antibodies conjugated with peroxidase (1:2000) for 1 h in washing buffer A. Colorimetric analysis was performed by addition of *o*-phenylene diamine (0.7 mg/mL) and 1% H_2O_2 in 50 mM Na_2HPO_4, pH 5.0, 20 mM citrate. The absorbance was monitored at a wavelength of 492 nm. Antibodies that reacted with control wells were considered to be of undesired specifity.

3.13. Separation, Western blot analysis and detection of anti-moenomycin antibodies

8 and BSA (15 μg each) were denatured at 95°C for 5 min in sodium dodecyl sulfate-mercaptoethanol buffer and submitted to analysis by electrophoresis on a 12.5% sodium dodecyl sulfate-homogeneous polyacrylamide gel as described by Laemmli.[31] The 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 blocked with 50 mM Tris, 150 mM NaCl, 2% Tween 20, washed with washing buffer A, incubated with rabbit moenomycin-antiserum as primary antibody (1:800) in washing buffer A, 1% BSA, washed again, and incubated with pork-anti-rabbit antibodies conjugated with alkaline phosphatase as secondary antibodies. Binding was visualized with 0.25 mg/mL nitro-blue-tetrazoliumchloride (NBT)/0.125 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in 25 mM Tris, 100 mM NaCl, 5 mM $MgCl_2{\cdot}6H_2O$. Control experiments were performed with preserum. Protein concentrations were determined by the bicinchoninic acid (BCA) method,[33] using bovine serum albumin as a standard.

3.14. Surface plasmon resonance measurements

SPR measurements were performed with a Biacore® 3000 (BIACORE) with research grade sensor chips (Biacore 3000 Control Software 3.1.1, BIACORE). (a) Coupling of **2b** to a sensor chip (CM5, Biacore) was performed as previously described.[5] (b) SPR measurements of moenomycin-antiserum to immobilized **2b**: Following the immobilization of **2b**, antiserum (2.5 μg/mL) was injected into both flow cells at a flow rate of 20 μL min^{-1} and contact times 60–600 s in HBS buffer. Control experiments were performed with preserum.

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