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Polymer-bound reagents for the introduction of spacer-modified biotin labels

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Abstract—We have developed a method for the chemoselective introduction of spacer modified biotin labels into unprotected multifunctional amines. A range of novel biotin spacer conjugates attached to a polymer-bound sulfonamide anchor was prepared using established amide bond forming procedures. After chemical transformation of the attachment site by alkylation, the resulting reactive species were utilized as N-selective polymer-supported biotinylation reagents. The labeled compounds, obtained in good to excellent yield and purity, are free of residual biotin and possess a custom tailored distance from the immobilization site being especially suited for the immobilization on streptavidin-functionalized dextran layers of surface plasmon resonance detector chips. In addition, derivatives displaying a phenyl group were synthesized in order to demonstrate the versatility of the procedure for the simultaneous introduction of spacer-modified biotin and a UV-light absorbing moiety. The formation of biotin sulfoxides in the presence of in situ generated peroxides was investigated and is discussed. Our results suggest that this derivatization technique is a useful addition to the existing biotin labeling protocols.

1. Introduction

The avidin–biotin and streptavidin–biotin complex has become a very useful and extremely versatile, general mediator in a wide range of bioanalytical applications.[1] Biotin coupled to low- or high-molecular-weight molecules, can still be recognized by the glycoprotein avidin and the protein streptavidin, respectively, with dissociation constants of the high affinity complex formed in the pico- to femtomolar range. The bicyclic hexahydro-2-oxo-1*H*-thieno[4,3-*d*]imidazol ring system and the valeric acid side chain of the biotin molecule are essential for the specific recognition by these proteins whereas modification of the carboxyl group of the valeric acid moiety can be used for the design of biotinyl derivatives with retained molecular recognition capability.

Recently, the use of the avidin–biotin and streptavidin–biotin complex has been extended to a novel application for drug development using biosensors based on the principles of surface plasmon resonance (SPR).[2] For this type of intermolecular interaction studies, streptavidin modified dextran covered gold surface chips are commercially available. Thus, the streptavidin–biotin interaction in which biotin is used to anchor biological ligands represents a useful tool for the study of molecular interactions with biological target molecules by SPR ligand-fishing experiments.

For all applications employing biotinylated compounds in SPR assays, residual biotin originating from use of excess reagent during covalent attachment to the substrate must be removed efficiently after the biotinylation process to avoid blocking of streptavidin sites on the sensor chip surface. Furthermore, derivatives with long spacer arms (a minimum of six carbon atoms) are recommended to minimize sterical hindrance and facilitate streptavidin binding.[3] To control the exact biotinylation site in multi-functional substrates, regio-, and chemoselective labeling is crucial. Although a wide range of biotinylation reagents is available commercially, none of them accomplishes these requirements needed for a biosensor assay.[4]

Herein, we describe a new, simple, and efficient polymer-assisted method to chemoselectively label multi-functional amines with a custom tailored biotin label, to be utilized for SPR experiments using a biacore system in particular or for any other bioanalytical application needed.

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2. Results

Our group established a polymer-assisted solution-phase (PASP) protocol for the synthesis of biological active adenosine analogs employing PASP synthesis for the N-selective acylation of the amino group of various amino deoxyadenosines.[5,6] This protocol seemed to be ideally suited for the synthesis of various polymer-bound biotinylation reagents to covalently label multi-functional amines with biotin.

Initially, we prepared a polymer-bound, chemoselective biotinylation reagent applying the safety-catch linker introduced by Kenner et al.[7] Biotin was coupled to the sulfamoyl group of the Kenner safety-catch linker attached to aminomethylated polystyrene, activated with bromoacetonitrile and in the following chemoselectively transferred to the amino function of the target compound **1a–d** yielding **3a–d** in excellent yield and purity (Fig. 1 and Table 1).[8]

Besides the advantage of chemoselective acylation properties, the Kenner safety-catch linker is also suitable for on-bead modifications of attached building blocks permitting the incorporation of spacer atoms between the biotin moiety and the compound to be labeled. Thus, we introduced various amino acid residues between the biotin and amine moiety of compounds **3a–c**. Thereby, appropriate Fmoc protected amino acids were immobilized via the Kenner safety-catch linker on amino-

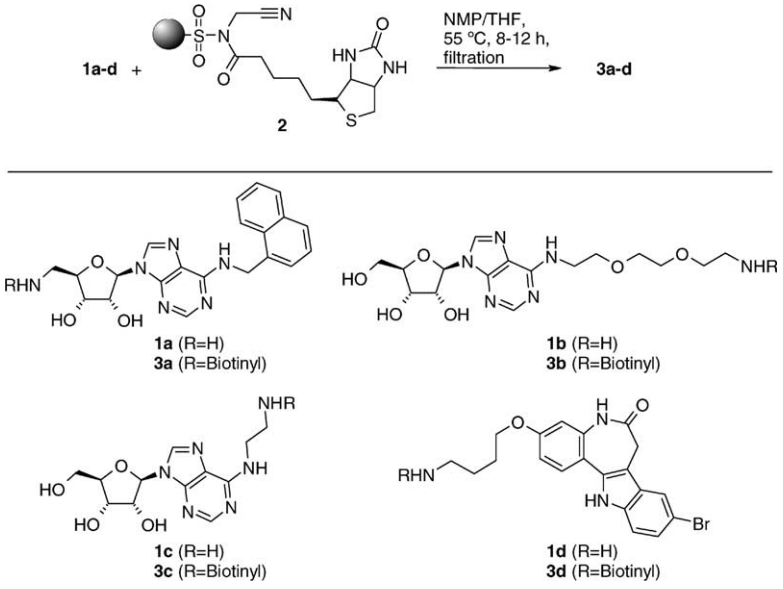


Figure 1. Biotinylated compounds **3a–d** obtained via PASP synthesis.

Table 1. Yield and purity of biotinylated compounds **3a–d**, of spacer-modified biotinylated compounds **6a–c** and **12**, of appropriate (spacer)-modified biotinylated derivatives **13a–f** resulting from peroxide mediated sulfoxidation

Product	From R–NH_2 (Compounds **1a–d**)	Spacer in construct *substrate*-spacer-*biotinyl-sulfoxides*	Sufficient purity[a] (>80%)	Yield[b] (%)
Biotinylated derivatives				
3a	**1a**	—	Prior to MPLC	95
3b	**1b**	—	Prior to MPLC	97
3c	**1c**	—	Prior to MPLC	95
3d	**1d**	—	Prior to MPLC	80
6a	**1a**	–$CO(CH_2)_5NH$–	After MPLC	79
6b	**1b**	–$CO(CH_2)_{10}NH$–	After MPLC	79
6c	**1c**	–L-Phenylalanyl–	Prior to MPLC	81
12	**1a**	–$[CO(CH_2)_5NH]_4$–	Prior to MPLC	88
Derivatives from peroxide mediated sulfoxide formation				
13a	**1a**	—	Prior to MPLC	97
13b	**1a**	–$CO(CH_2)_3NH$–	Prior to MPLC	97
13c	**1a**	–$CO(CH_2)_5NH$–	Prior to MPLC	95
13d	**1b**	–$CO(CH_2)_{10}NH$–	Prior to MPLC	80
13e	**1a**	–$[CO(CH_2)_5NH]_2$–	Prior to MPLC	91
13f	**1c**	–$CO(CH_2)_5NH$–L-Phenylalanyl–	after MPLC	71

[a] All compounds were purified by a single semi-preparative mid-pressure LC (MPLC) run under standard conditions that were not optimized for each individual compound. Purity of reaction product prior to purification was estimated using the 100% method, UV-detection at 254 nm.
[b] Product containing fractions were collected and evaporated, the yield is reported as yield of purified material.

methylated polystyrene. After cleavage of the Fmoc protecting group with 20% piperidine in DMF, biotin was attached via in situ anhydride formation to the amino function of the polymer-supported building block yielding the appropriate biotin labels. Finally, the spacer-biotin-loaded sulfonamide linker was alkylated with bromoacetonitrile leading to activated polymer-bound *N*-cyanomethyl analogs **4** and **5** ready for the chemoselective transfer to the primary amino function of the target compound yielding compounds **6a**–**c** in good yield and purity (Scheme 1 and Table 1).

To establish customized polymer-bound biotinylating reagents, flexible spacers of various lengths were generated via on-bead construction of amide chains, demonstrated in the eight step synthesis shown below (Scheme 2).

Initially, 6-(Fmoc-amino) caproic acid was immobilized on aminomethylated polystyrene via the Kenner safety-catch linker **7** yielding the polymer-bound *N*-acylsulfonamide **8** being stable under basic and strongly nucleophilic reaction conditions.[9,10] After cleavage of the Fmoc protecting group, three additional 6-(Fmoc-amino) caproic acid equivalents were attached to the polymer-supported building block and deprotected, stepwise, yielding derivative **10**. In the following, biotin was attached to the amino function of **10** via in situ anhydride formation.

The spacer modified polymer-bound biotin label **11** was again activated with bromoacetonitrile yielding the activated analog to be transferred to the primary amino group of the unprotected multi-functional adenosine scaffold **1a** by agitation in *N*-methylpyrrolidone (NMP) at 55 °C chemoselectively (not shown), leading to **12** in good yield and purity (Fig. 2).

According to conditions described above, biotinylated compounds **3a**–**d**, **6a**–**c**, and **12** were synthesized in excellent to good yield and purities (Table 1).

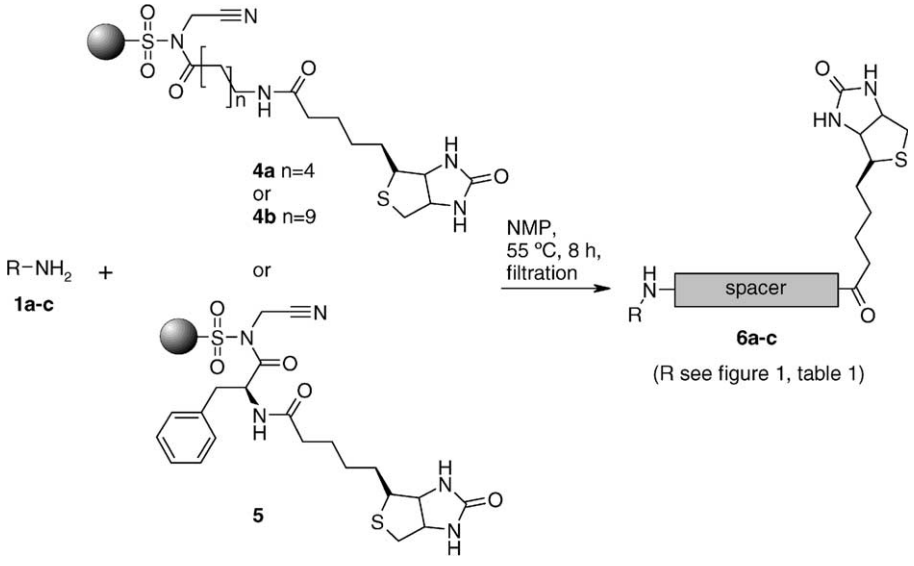
When transferring the activated polymer-supported construct to the aminofunction of the adenosine scaffold in THF of commercial quality at 55 °C, unintended sulfoxide formation of the biotin moiety occurred, in most of the cases quantitatively (Fig. 3; Table 1; R groups see Fig. 1).

In order to widen the scope of the reaction reported, phenylalanine, was introduced (see entry **6c**, **13f**), to obtain a biotinylation reagent concurrently including a UV-quenching moiety, that is of interest for labeling compounds such as amino sugars.

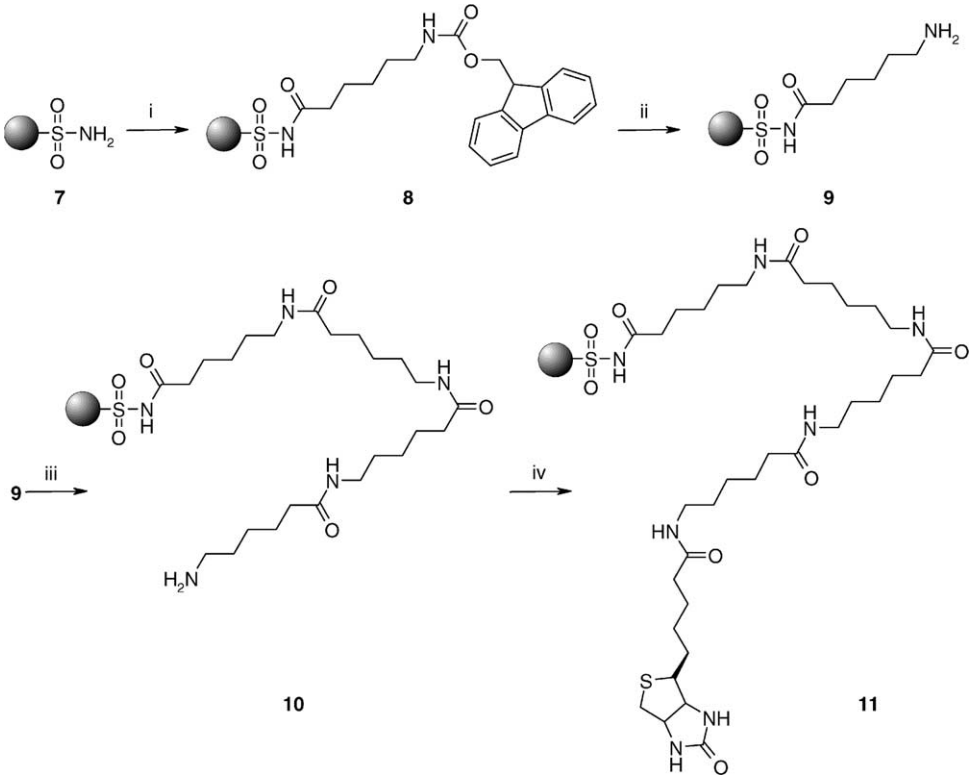
3. Discussion

The above demonstrated on-bead construction protocol permits the simple preparation of custom tailored biotin labels using established amide bond forming procedures. PASP synthesis leads to simple product isolation because the excess of polymer-bound reagent that is applied to drive reactions to completion can be removed by filtration. This allows for the fast and convenient access of biotinylated compounds in high yield and purity.

When using biotinylated compounds in an SPR assay, chemoselective labeling is crucial to know the exact binding site of the molecule. Control of the labeling site is required due to the fact, that a biotin label might inactivate the compounds binding ability when being incorporated into the wrong region of the molecule. As shown above, the N-selective acylation observed with alkylated acyl sulfonamides offers the opportunity to incorporate the biotin label into the desired region of the molecule.[11] Additionally, due to the N-selective acylation property of our approach, no protecting group operations are needed.



Scheme 1. PASP synthesis of spacer-modified biotinylated compounds **6a**–**c**.



Scheme 2. On-bead construction of spacer-modified biotin conjugate **11**. Reagents and conditions: (i) 6-(Fmoc-amino) caproic acid, DMF/DCM, DIC, DMAP, ambient temperature, 6 h; (ii) 20% piperidine in DMF, ambient temperature, 30 min; (iii) (a) 6-(Fmoc-amino) caproic acid, DMF/DCM, DIC, DMAP, ambient temperature, 6 h; (b) 20% piperidine in DMF, ambient temperature, 30 min; step iii repeated three times; (iv) Biotin, DMF, DIC, HOBt, DIPEA, ambient temperature, 24 h.

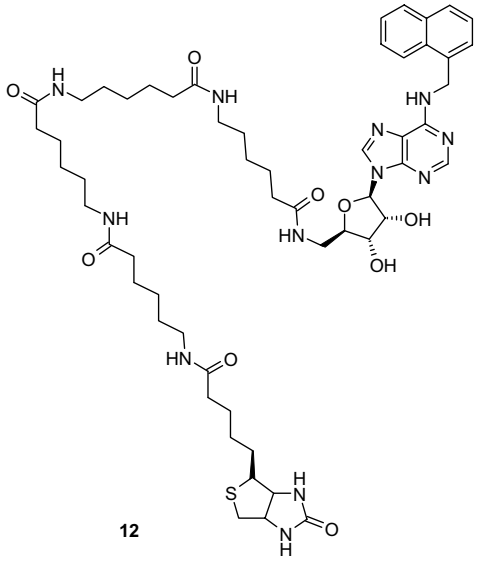


Figure 2. Custom tailored biotin label introduced into a multi-functional amine.

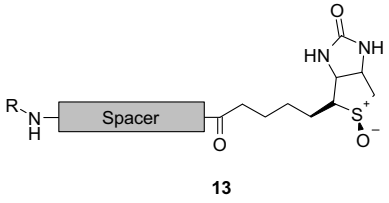


Figure 3. Product from peroxide mediated sulfoxide formation.

Derived from our findings, Gelb and co-workers could show additional aspects of the N-selective acylation with polymer-bound *N*-acylsulfonamides, recently.[12] They confirmed that the reaction of activated *N*-acylsulfonamides with amino groups displaying different chemical properties could be fine tuned by variation of the reaction temperature. At room temperature, only amino functions connected to a primary carbon atom react with the immobilized and activated polymer-bound *N*-acylsulfonamide. When increasing the temperature up to 55 °C, primary amino groups bound to a secondary carbon atom undergo complete reaction, concurrently. This adjustable chemoselectivity is impressive because it is superior to the results obtained using enzymatic N-selective acylation of amino deoxynucleosides, to date. The first report on enzymatic N-selective acylation of 3′,5′-diamino-3′,5′-dideoxy pyrimidine nucleosides using N-selective acylation mediated by *Candida antarctica* lipase B, *Pseudomonas cepacia* lipase (PSL) or immobilized PSL was published by Lavandera et al. in 2001.[13] The reaction produced in most of the cases reported mixtures of the possible monoamides and a diamide.

Besides the necessity of chemoselectivity, additional requirements for the biotinylation process have to be

fulfilled. For the reason that excess biotin blocks streptavidin on the sensor chip surface, the absence of biotin is of utmost importance for reproducible binding to streptavidin on the sensor chip surface. During PASP synthesis, excess biotin remains coupled to the polymer support and can thus be easily removed by simple filtration whereas the biotinylated compound stays in solution. The degree of biotinylation should ideally be kept low; only one biotin label per molecule is sufficient and optimal. Uncontrolled higher biotinylation degrees per molecule affect the optimal display of the ligand bound to the sensor chip surface and again influence the reproducibility of binding. Derivatives with long spacer arms (a minimum of six carbon atoms) are recommended to minimize sterical hindrance and to facilitate streptavidin binding.

Although a wide range of biotinylation reagents is available commercially (Fig. 4), none of them accomplishes all of the requirements needed for performing SPR experiments. The use of commercially available biotinylation reagents **14a–e** will undoubtedly in many cases yield quantitative reactions. However, the need for protecting group operations as well as the separation of the product from excess reagent in the cases of **14a** and **14b** and **14d** and **14e**, respectively, by for example, HPLC, dialysis, or affinity chromatography are time consuming; often excess biotin remains in the sample. Reagents **14d** and **14e** display the advantage of an incorporated flexible spacer. Whereas **14d** is well established, **14e** represents a novel labeling agent, available from Quantabiodesign. This compound shows improved water-soluble characteristics, which is an interesting feature for lipophilic compounds to be studied. This is especially important when employing the biacore system for SPR experiments because solubility of the sample in aqueous buffers is required.

Polymer-bound **14c** allows for easy removal, and thus enables enforcement of reaction completeness by using a large excess of reagent. However, due to the high reactivity of the linkage, it is not useful for the on-bead construction of biotin-spacer conjugates.

Via on-bead construction of amide chains, customized spacers can be obtained by varying the building blocks as shown in Table 1. Since polymer-bound acyl sulfonamides like the Kenner linker also possess a high orthogonal stability in basic and strong nucleophilic reaction media, they again play a key role in the reaction pattern. This property enables to work under drastic conditions, so that a wide range of on-bead spacer construction is possible. Alkylation of the safety-catch linker with electron withdrawing cyanomethyl groups leads to a highly reactive analog that can easily be cleaved with a broad range of nucleophiles. Therefore, the polymer-assisted solution-phase protocol allows for the incorporation of spacers of variable length and characteristics (such as water solubility, UV-absorbing properties). Thus, labels can be designed according to the demands of each compound to be labeled; no excess biotin remains in the sample.

The known, almost exclusive formation of α-sulfoxides that we observed when the reaction was performed in THF, can easily be avoided resorting to solvents that are not prone to peroxide formation.[14] Solvents like NMP or DMF lead to excellent swelling of the resin particles,

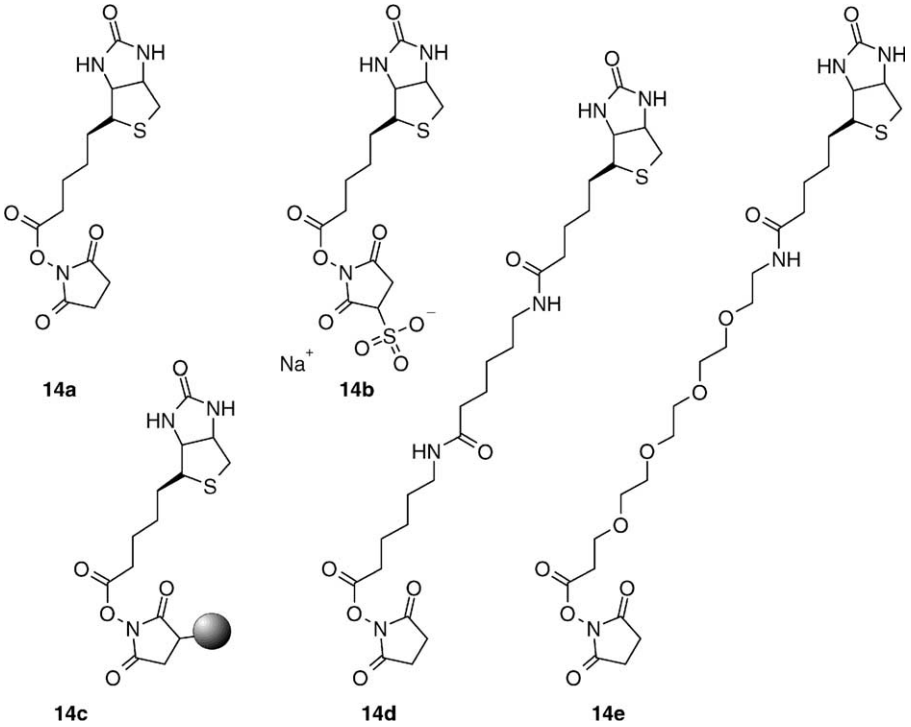


Figure 4. Representative commercially available biotinylation reagents.

as well. In our hands, this proved to be a straightforward way to circumvent the observed side reaction. However, peroxides preformed in THF that completely oxidize polymer-bound biotin might be useful for the development of biotin sulfoxide labels. Biotin sulfoxide is suited for rendering attached molecules even more water soluble. Preliminary SPR experiments comparing the immobilization properties of biotin and biotin sulfoxide labels showed, that biotin sulfoxide labeled compounds also tightly bind to the streptavidin surface of the sensor chip, but to a somewhat lesser extend. These findings will be further investigated by our group.

4. Conclusion

A rapid and efficient polymer-assisted approach to biotinylated nucleoside derivatives bearing an amine functionality has been developed. The protocol reported enables the incorporation of spacers of various lengths and chemical characteristics via on-bead construction. Custom tailored spacers are obtained using established amide bond forming procedures. Our results suggest that this derivatization technique is a useful addition to the existing biotin labeling protocols, in particular for introducing custom tailored spacer-biotin anchors as prerequisite in the analysis of drug candidates using streptavidin chips via SPR methodology.

5. Experimental

5.1. General experimental

Identity of all compounds was assigned by NMR spectroscopy. ^{1}H NMR spectra were recorded on a Bruker AMX 400 spectrometer. *J* values are given in Hz. Sample purity was calculated from chromatographic purification profiles. Yields are reported as isolated material. MPLC simultaneous purity analyses/purifications were performed using a Büchi 681 pump (flow rate 10 mL/min, MeOH/H_2O 30:70) and an UV-detector (254 nm) with Merck 310-25 Lobar-LiChroprep^{TM}-RP-18 columns. High resolution MS data of pure compounds were obtained on a Finnigan MAT 95 XL (ESI, methanol/water (1:1, v/v) infusion at 2 μL/min with polypropylene glycol as reference).

Introduction of spacer atoms (General procedure A) and biotinylation (General procedure B) was carried out according to procedures reported for other carboxylic acids with the deviations described in the following paragraphs.[6,15]

5.1.1. General procedure A for the on-bead construction of spacer atoms. DMF (10 mL) was added to dry 4-sulfamoylbenzoylaminomethyl polystyrene (2.0 g) with an initial loading level of 1.23 mmol/g.[6,10] The resin was allowed to swell at room temperature for 30 min. Separately, an appropriate Fmoc-aminocarboxylic acid equivalent (9.8 mmol, 4 equiv) was dissolved in DMF (5–7.5 mL) and preactivated for 30 min via in situ anhydride formation with *N*,*N*-diisopropylcarbodiimide (DIC) (1,5 mL, 9.8 mmol, 4 equiv). CAUTION: *N*,*N*-diisopropylcarbodiimide may lead to severe allergic reactions, strictly avoid skin contact. After adding DMAP (7 mg, 0.05 mmol) and dichloromethane (2 mL) the coupling mixture was subsequently poured onto to the swollen resin and the resulting reaction mixture was agitated at room temperature for 6 h. The resin beads were filtered off and washed exhaustively with DMF (three times 5 mL) and methanol (five times 5 mL). To obtain a quantitative acylation, the coupling reaction was performed in duplicates for all Fmoc-aminoacids. After cleavage of the Fmoc protecting group with 20% piperidine in DMF, further building blocks (appropriate Fmoc-aminoacids or biotin) were attached to the polymer support.

5.1.2. General procedure B for the biotinylation. Biotin (2.4 g, 10 mmol) was suspended in DMF (30 mL) at 50 °C and preactivated via in situ anhydride formation by adding 780 μL (5 mmol) *N*,*N*-diisopropylcarbodiimide. After 3 h, first *N*-ethyl-*N*,*N*-diisopropylamine (DIPEA) (580 μL, 3.4 mmol) and 1-hydroxybenzotrizole (HOBt) (1.4 g, 10 mmol) and in the following the coupling mixture were added to the swollen resin. The resulting reaction mixture was shaken at room temperature for 24 h. The resin beads were filtered off and washed exhaustively with DMF, dichloromethane, and methanol, three times 5 mL, each. Again, to reach a quantitative reaction the biotinylation was carried out in duplicates.

5.1.3. General procedure C for activating the sulfonamide linker and transferring the activated biotin label to the target amine. The sulfonamide linker was activated for cleavage by alkylation with 640 μL of bromoacetonitrile (9 mmol) and 340 μL of DIPEA (2 mmol) in 4 mL 1-methylpyrrolidone (NMP) overnight. The resin beads were washed with dimethylsulfoxide (five times 5 mL) and NMP (once 5 mL). The polymer-bound, activated building block was transferred to the amino group of 10 μmol of **1a**–**d**, by adding a solution of **1a**–**d** in 1 mL NMP to a slurry of 400 mg of the appropriate resin in 4 mL NMP and shaking at 55 °C for 10 h yielding compounds **3a**–**d**, **6a**–**c**, and **12**.

5.2. 5′-(6-Biotinylamidohexanoylamino)-5′-deoxy-N^6-(1-naphthylmethyl)adenosine 6a

NMR δ_H (ppm) (400 MHz, DMSO-d_6, Me_4Si): 8.48 (b s, 1H), 8.37 (b s, 1H), 8.25–8.23 (m, 2H), 8.15 (m, 1H), 7.95 (m, 1H), 7.82 (m, 1H), 7.71 (t, 1H, $J = 5.5$ Hz), 7.59–7.52 (m, 2H), 7.47–7.41 (m, 2H), 6.41 (s, 1H), 6.34 (s, 1H), 5.88 (d, 1H, $J = 6.4$ Hz), 5.46 (m, 1H), 5.24 (m, 1H), 5.20 (b s, 2H), 4.71 (m, 1H), 4.30 (m, 1H), 4.11 (m, 1H), 4.05 (b s, 1H), 3.97 (b s, 1H), 3.07 (m, 1H), 3.02–2.97 (m, 2H), 2.80 (dd, 1H, $J = 5.1$ Hz, 12.5 Hz), 2.18–

2.10 (m, 2H), 2.02 (t, 2H, $J = 7.4$ Hz), 1.53–1.16 (m, 12H). HRESI-MS found 768.3269. $C_{37}H_{47}N_9O_6S$. $[M+H]^+$ requires 768.3268.

5.3. N^6-(2-{2-[2-(11-Biotinylamido-undecanoylamino)ethoxy]ethoxy}ethyl)adenosine 6b

NMR δ_H (ppm) (400 MHz, DMSO-d_6, Me_4Si): 8.36 (s,1H), 8.22 (s, 1H), 7.82–7.71 (m, 3H), 6.41 (s, 1H), 6.35 (s, 1H), 5.89 (d, 1H, $J = 6.1$ Hz), 5.44 (d, 1H, $J = 6.4$ Hz), 5.39 (m, 1H), 5.19 (d, 1H, $J = 4.6$ Hz), 4.60 (m, 1H), 4.30 (m, 1H), 4.16–4.13 (m, 2H), 3.96 (m, 1H), 3.70–3.48 (m, 10H), 3.39–3.36 (m, 2H), 3.09 (m, 1H), 3.02–2.97 (m, 2H), 2.83–2.79 (dd, 1H, $J = 5.1$ Hz; $J = 12.5$ Hz), 2.03 (t, 2H, $J = 7.4$ Hz), 1.65–1.22 (m, 22H). HRESI-MS found 830.4210. $C_{37}H_{61}N_9O_9S$. $[M+Na]^+$ requires 830.4210.

5.4. N^6-[2-((2*S*)-Biotinylamido-3-phenylpropionylamino)ethyl]adenosine 6c

NMR δ_H (ppm) (400 MHz, DMSO-d_6, Me_4Si): 8.36 (s, 1H), 8.22 (b s, 1H), 8.12 (t, 1H, $J = 5.3$ Hz), 8.01 (d, 1H, $J = 8.4$ Hz), 7.85 (m, 1H), 7.24–7.10 (m, 5H), 6.38 (s, 1H), 6.34 (s, 1H), 5.90 (d, 1H, $J = 6.26$ Hz), 5.40 (b s, 3H), 4.60 (t, 1H, $J = 5.4$ Hz), 4.45 (m, 1H), 4.30 (m, 1H), 4.15 (m, 1H), 4.09 (m, 1H), 3.97 (m, 1H), 3.68 (m, 1H), 3.57–3.51 (m, 3H), 3.05–2.94 (m, 3H), 2.83–2.76 (m, 2H), 2.59 (d, 1H, $J = 12.5$ Hz), 2.03 (t, 2H, $J = 7.4$ Hz), 1.60–1.15 (m, 6H). HRESI-MS found 706.2753. $C_{31}H_{41}N_9O_7S$. $[M+Na]^+$ requires 706.2747.

5.5. 5′-(6-{6-[6-(6-Biotinylamido-hexanoylamino)hexanoylamino]hexanoylamino}hexanoylamino)-5′-deoxy-N^6-(1-naphthylmethyl)adenosine 12

NMR δ_H (ppm) (400 MHz, DMSO-d_6, Me_4Si): 8.50 (b s, 1H), 8.38 (b s, 1H), 8.24–8.23 (m, 2H), 8.17 (t, 1H, $J = 5.4$ Hz), 7.95 (m, 1H), 7.82 (m, 1H), 7.74–7.69 (m, 4H), 7.59–7.52 (m, 2H), 7.46–7.41 (m, 2H), 6.41 (s, 1H), 6.35 (s, 1H), 5.87 (d, 1H, $J = 6.4$ Hz), 5.48 (d, 1H, $J = 6.1$ Hz), 5.26 (b s, 1H, $J = 4.6$ Hz), 5.19 (b s, 2H), 4.70 (m, 1H), 4.30 (m, 1H), 4.12 (m, 1H), 4.03 (m, 1H), 3.95 (m, 1H), 3.08 (m, 1H), 3.00–2.96 (m, 8H), 2.83–2.79 (dd, 1H, $J = 12.5$ Hz; $J = 5.1$ Hz), 2.11 (t, 2H, $J = 7.4$ Hz), 2.05–1.98 (m, 8H), 1.65–1.15 (m, 30H). HRESI-MS found 1107.5774. $C_{55}H_{80}N_{12}O_9S$. $[M+Na]^+$ requires 1107.5789.

Target compounds (**13a**–**f**) were obtained as described in general procedures A–C with the deviation of using THF as solvent instead of NMP. After activating the sulfonamide linker the resin beads were washed with DMSO and THF, five times 5 mL and three times 5 mL, respectively. The polymer-bound, activated building block was transferred to the amino group of 10 μmol of **1a**–**c**, by adding a solution of **1a**–**c** in 0.5 mL NMP to a slurry of 400 mg of the appropriate resin in 4 mL THF and shaking at 55 °C for 10 h resulting.

5.6. 5′-Deoxy-N^6-(1-naphthylmethyl)-5′-[5-(3a*S*,4*S*,5-*S*,6a*R*)(2,5-dioxo-octahydro-5λ^4-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]adenosine 13a

NMR δ_H (ppm) (400 MHz, DMSO-d_6, Me_4Si): 8.49 (b s, 1H), 8.37 (b s, 1H), 8.25–8.23 (m, 2H), 8.16 (t, 1H, $J = 5.6$ Hz), 7.95 (m, 1H), 7.81 (m, 1H), 7.59–7.52 (m, 2H), 7.46–7.41 (m, 2H), 6.78 (s, 1H), 6.67 (s, 1H), 5.88 (d, 1H, $J = 6.4$ Hz), 5.46 (d, 1H, $J = 6.1$ Hz), 5.24 (d, 1H, $J = 4.8$ Hz), 5.19 (b s, 2H), 4.70 (m, 1H), 4.43 (m, 1H), 4.31 (m, 1H), 4.05 (m, 1H), 3.97 (m, 1H), 2.93–2.84 (m, 2H), 2.15 (t, 2H, $J = 7.4$ Hz), 1.73–1.33 (m, 6H). HRESI-MS found 649.2561. $C_{31}H_{36}N_8O_6S$. $[M+H]^+$ requires 649.2558.

5.7. 5′-Deoxy-N^6-(1-naphthylmethyl)-5′-{4-[5-(3a*S*,4*S*,5*S*,6a*R*)(2,5-dioxo-octahydro-5λ^4-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]butanoylamino}adenosine 13b

NMR δ_H (ppm) (400 MHz, DMSO-d_6, Me_4Si): 8.51 (b s, 1H), 8.39 (b s, 1H), 8.25–8.20 (m, 3H), 7.95 (m, 1H), 7.83–7.78 (m, 2H), 7.60–7.52 (m, 2H), 7.46–7.41 (m, 2H), 6.79 (s, 1H), 6.69 (s, 1H), 5.87 (d, 1H, $J = 6.6$ Hz), 5.45 (d, 1H, $J = 6.1$ Hz), 5.27 (d, 1H, $J = 4.6$ Hz), 5.19 (b s, 2H), 4.70 (m, 1H), 4.43 (m, 1H), 4.30 (m, 1H), 4.04 (m, 1H), 3.95 (m, 1H), 3.04–2.99 (m, 2H), 2.92–2.87 (m, 2H), 2.13 (t, 2H, $J = 7.4$ Hz), 2.05 (t, 2H, $J = 7.3$ Hz), 1.64–1.32 (m, 8H). HRESI-MS found 734.3093. $C_{35}H_{43}N_9O_7S$. $[M+H]^+$ requires 734.3085.

5.8. 5′-Deoxy-N^6-(1-naphthylmethyl)-5′-{6-[5-(3a*S*,4*S*,5*S*,6a*R*)(2,5-dioxo-octahydro-5λ^4-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]hexanoylamino}adenosine 13c

NMR δ_H (ppm) (400 MHz, DMSO-d_6, Me_4Si): 8.51 (b s, 1H), 8.39 (b s, 1H), 8.25–8.23 (m, 2H), 8.18 (t, 1H, $J = 5.4$ Hz), 7.95 (m, 1H), 7.82 (m, 1H), 7.74 (t, 1H, $J = 5.6$ Hz), 7.60–7.52 (m, 2H), 7.47–7.41 (m, 2H), 6.80 (s, 1H), 6.69 (s, 1H), 5.88 (d, 1H, $J = 6.4$ Hz), 5.20 (b s, 2H), 4.71 (m, 1H), 4.45 (m, 1H), 4.32 (m, 1H), 4.02 (m, 1H), 3.97 (m, 1H), 3.02–2.97 (m, 2H), 2.98–2.86 (m, 2H), 2.12 (t, 2H, $J = 7.4$ Hz), 2.05 (t, 2H, $J = 7.4$ Hz), 1.55–1.18 (m, 12H). HRESI-MS found 762.3397. $C_{37}H_{47}N_9O_7S$. $[M+H]^+$ requires 762.3398.

5.9. 5′-Deoxy-N^6-(1-naphthylmethyl)-5′-{11-[5-(3a*S*,4*S*,5*S*,6a*R*)(2,5-dioxo-octahydro-5λ^4-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]undecanoylamino}adenosine 13d

NMR δ_H (ppm) (400 MHz, DMSO-d_6, Me_4Si): 8.36 (s, 1H), 8.22 (s, 1H), 7.81 (t, 1H, $J = 5.6$ Hz), 7.78 (b s, 1H), 7.73 (t, 1H, $J = 5.3$ Hz), 6.79 (s, 1H), 6.69 (s, 1H), 5.89 (d, 1H, $J = 6.4$ Hz), 5.45 (d, 1H, $J = 6.4$ Hz), 5.40 (m, 1H), 5.20 (d, 1H, $J = 4.6$ Hz), 4.60 (m, 1H), 4.45 (m, 1H), 4.32 (m, 1H), 4.15 (m, 1H), 3.96 (m, 1H), 3.70–3.48 (m, 8H), 3.39–3.35 (m, 2H), 3.19–3.14 (m, 2H), 3.02–2.97 (m, 2H), 2.94–2.86 (m, 2H), 2.08–2.01 (m, 4H), 1.78–1.70 (m, 2H), 1.57–1.34 (m, 6H), 1.22 (s, 16H).

HRESI-MS found 897.4069. $C_{37}H_{61}N_9O_{10}S$. $[M+Na]^+$ requires 897.4057.

5.10. 5′-Deoxy-*N*⁶-(1-naphthylmethyl)-5′-(6-{6-[5-(3a*S*,4*S*,5*S*,6a*R*)(2,5-dioxo-octahydro-5λ⁴-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]hexanoylamino}hexanoylamino)adenosine 13e

NMR δ_H (ppm) (400 MHz, DMSO-d_6, Me_4Si): 8.51 (b s, 1H), 8.38 (b s, 1H), 8.24–8.17 (m, 3H), 7.95 (m, 1H), 7.82 (m, 1H), 7.75–7.69 (m, 2H), 7.60–7.53 (m, 2H), 7.46–7.41 (m, 2H), 6.79 (s, 1H), 6.69 (s, 1H), 5.87 (d, 1H, $J = 6.4$ Hz), 5.49 (d, 1H, $J = 5.6$ Hz), 5.26 (b s, 1H), 5.18 (b s, 2H), 4.70 (m, 1H), 4.45 (m, 1H), 4.32 (m, 1H), 4.04–4.03 (m, 1H), 3.96 (m, 1H), 3.00–2.86 (m, 6H), 2.11 (t, 2H, $J = 7.4$ Hz), 2.08–1.98 (m, 4H), 1.55–1.15 (m, 18H). HRESI-MS found 897.4069. $C_{43}H_{58}N_{10}O_8S$. $[M+Na]^+$ requires 897.4057.

5.11. 5′-Deoxy-*N*⁶-(1-naphthylmethyl)-5′-(6-{2-(2*S*)-[5-(3a*S*,4*S*,5*S*,6a*R*)(2,5-dioxo-octahydro-5λ⁴-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-3-phenylpropionylamino}hexanoylamino)adenosine 13f

NMR δ_H (ppm) (400 MHz, DMSO-d_6, Me_4Si): 8.36 (s, 1H), 8.22 (s, 1H), 8.03 (d, 1H, $J = 8.7$ Hz), 7.94–7.86 (m, 3H), 7.27–7.15 (m, 5H), 6.79 (s, 1H), 6.70 (s, 1H), 5.89 (d, 1H, $J = 6.1$ Hz), 5.45–5.40 (m, 2H), 5.38–5.36 (dd, 2H, $J = 2.1$ Hz, 6.0 Hz), 5.20 (d, 1H, $J = 4.6$ Hz), 4.61 (m, 1H), 4.46 (m, 1H), 4.31 (m, 1H), 4.15 m, 1H), 3.96 (m, 1H), 3.68 (m, 1H), 3.58–3.52 (m, 3H), 3.06–2.85 (m, 5H), 2.08–2.01 (m, 4H), 1.48–1.11 (m, 12H). HRESI-MS found 813.3726. $C_{37}H_{52}N_{10}O_9S$. $[M+H]^+$ requires 813.3718.

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