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Synthesis of biotinylated muramyl tripeptides with NOD2-stimulating activity

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ABSTRACT

Muramyl di- and tri-peptides are putative activators of the innate immune system through stimulation of the NOD2 receptor. To provide tools for the clarification of the mechanism of this activation we isolated different UDP-muramyl tripeptides (Lys- and DAP-type) from bacteria and used them to synthesize biotinylated derivatives. All biotinylated compounds retained their ability to activate NOD2 in a cell-based test system and are therefore suitable for binding studies aimed at identifying the appropriate pattern recognition receptor(s).

The cytosolic nucleotide binding oligomerization domain-proteins (NOD-proteins) NOD1 and NOD2 play an important role in innate immunity as sensors for components derived from bacterial peptidoglycan (PGN). These proteins are assigned to the NLR-family whose members share a tripartite domain structure of which the LRR domains of NOD1 and NOD2 are thought to interact with bacterial PGN part structures as an initial step in the induction of pro-inflammatory processes.[1,2] Although the pathogen-associated molecular patterns (PAMPs) of NOD1 and NOD2 have been identified,[3] the molecular mechanism of the induction remains unknown.[4] Both proteins are associated with human inflammatory diseases. For example, three common mutations in or near the LRR-domain of NOD2 (R702W, G908R, L1007insC) have been found in Crohn's disease patients.[5,6] That these mutations occur in the supposed ligand binding region of NOD2 clearly demonstrates the need for specific tools for analyzing interactions between (the LRR domains of) NOD-proteins and their respective peptidoglycan PAMPs.

The required minimal motif for triggering NOD2 muramyl dipeptide **1** (MDP; MurNAc-L-Ala-D-Glx, Fig. 1) has been identified. If the second amino acid is a D-*iso*-glutamine (*i*Q, **1a**) or a D-glutamate (E, **1b**) there is no significant effect on the potency of stimulating proinflammatory processes as measured by the triggering of TNF-α production in the whole blood test.[7] The Lys-type muramyl tripeptide **2a** (MTP (Lys); MurNAc-L-Ala-D-Glu-L-Lys, Fig. 1) as well as the bacterial cell wall precursor UDP-MTP (Lys-type (**2b**) and DAP-type (**3b**), Fig. 1) have been found to possess approximately equal NOD2-activity as MDP (E, **1b**).[3] Thus, biotinylated derivatives of such muropeptides should be very attractive tools for binding studies.

Presently only one biotinylated MDP is commercially available (**4**, Fig. 1).[8] Here, the biotin-label is bound via a 6-(6-(6-amino-hexanamido)hexanamido)hexanoic acid-linker to the *O*-6-position of the *N*-acetyl-muraminic acid. A similar derivatization strategy has been used by Grimes et al.[9]; they synthesized C6-amino derivatives of biotinylated MDP. Alternatively, we considered the carbohydrate moiety of MDP to be the key element for specific interaction with NOD2. Hence, we used the distal ε-amino function of (UDP-)MTPs (**2**, **3**, Fig. 1) for biotinylation. In this way the minimal motif for recognition by NOD2 — the MDP — is chemically unmodified, which may be ideal for the envisaged binding studies.

We isolated the bacterial cell wall precursor UDP-MTP (Lys-type) (**2b**) and UDP-MTP (DAP-type) (**3b**) from *Staphylococcus aureus* SA113 and from *Bacillus cereus* T, using a modification of the protocol of Kohlrausch and Höltje.[10] As a first synthesis strategy, these precursors were reacted with Sulfo-NHS-Biotin (**5a**) or Sulfo-NHS-LC-LC-Biotin (**5b**) in phosphate buffer (pH 8.0) for 1 h at 27 °C to give the respective biotinylated derivatives **6a**,[11] **6b** and **7** after HPLC purification. We found that the use of at least 1.5 equiv of biotinylation reagent (**5a** or **5b**) was necessary to achieve sufficient yields (Table 1). In case of the biotinylation of **3b** with **5a**, only the reaction at higher temperature (50 °C) led to a full conversion, but resulted in a decreased yield (of compound **7**). It is noteworthy that only in the reaction of **2b** with **5b** the complete conversion of starting material resulted in the highest yield. Subsequent acidic hydrolysis of **6a**, **6b** and **7** followed by another HPLC purification step led then to the biotinylated muropeptides

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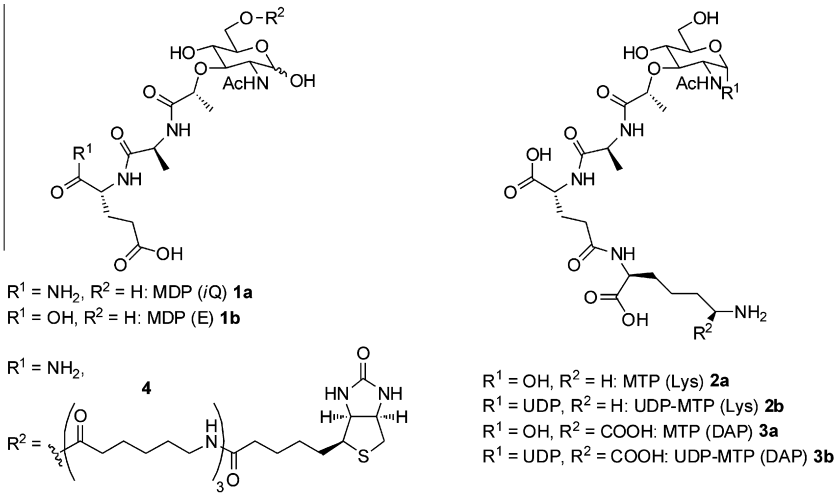


Figure 1. Chemical structures of muramyl dipeptides **1**, (UDP-)muramyl tripeptides **2** and **3** as well as commercially available biotin-MDP **4**.

Table 1
Yields of biotinylation of UDP-muropeptides **2b** and **3b** using different conditions

UDP-muropeptide	Equiv of biotinylation reagent	Temperature (°C)	Yield
2b	2.0 equiv of **5a**	27	59% (of **6a**)*[a]
2b	1.5 equiv of **5a**	27	71% (of **6a**)
2b	1.25 equiv of **5a**	27	32% (of **6a**)
2b	1.0 equiv of **5a**	27	23% (of **6a**)
2b	2.0 equiv of **5b**	27	66% (of **6b**)*
2b	1.5 equiv of **5b**	27	42% (of **6b**)
3b	2.0 equiv of **5a**	27	48% (of **7**)
3b	2.0 equiv of **5a**	50	34% (of **7**)*
3b	1.5 equiv of **5a**	27	45% (of **7**)

[a] The marked reactions (*) showed a full conversion of starting material (**2b** or **3b**).

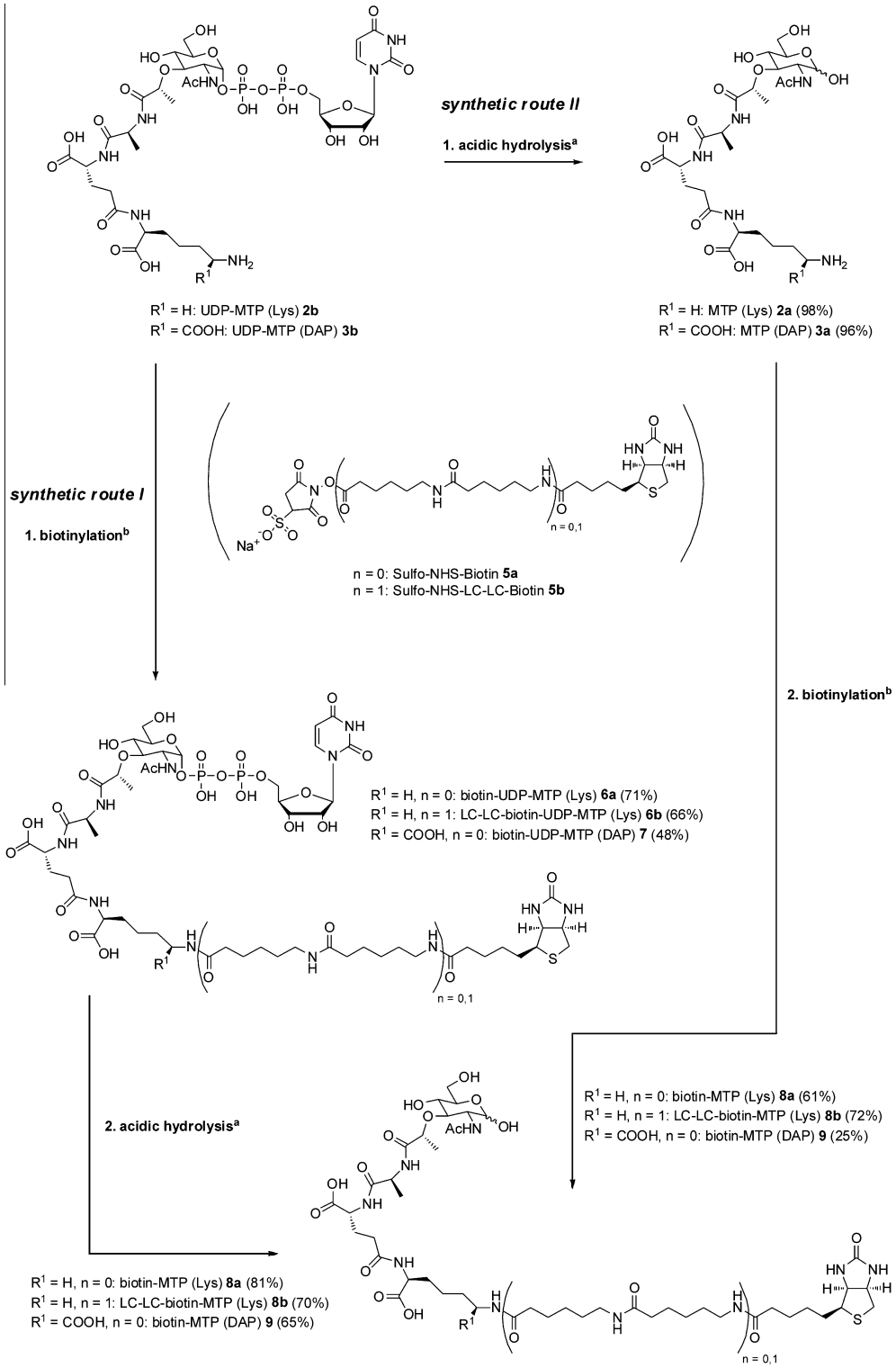
8a[12] (81%), **8b** (70%) and **9** (65%). The reaction steps for this synthetic route I, including the yields for the respective best conditions (see Table 1), are depicted in Scheme 1.

A second possible synthesis strategy was to hydrolyse the UDP-muramyl tripeptides **2b** and **3b** first to prepare the respective MTPs **2a** (98%) and **3a** (96%). Biotinylation of these compounds led to compounds **8a**, **8b** and **9**. These biotinylations were performed with a reaction time of 1 h at 27 °C with 1.5 or 2.0 equiv of biotinylation reagents **5a** or **5b** (Table 2). In the biotinylation reaction of **2a** the use of 2.0 equiv **5a** led to a twofold yield compared to the reaction with 1.5 equiv. In contrast, in the LC-LC-biotinylation of **2a**, the use of 1.5 or 2.0 equiv **5b** led to **8b** in comparable yields. As it was the case with UDP-MTP (DAP) (**3b**) the biotinylation of MTP (DAP) (**3a**) resulted in a much lower yield compared to the reaction of the respective Lys-type counterpart. The steps for synthetic route II including the yields for the respective best conditions (see Table 2) are summarized in Scheme 1, too. Comparing the reaction strategies for the synthesis of **8a** both routes resulted in comparable overall yields (58% (I) vs 60% (II) over two steps). For the synthesis of **8b** strategy II is more advantageous (46% (I) vs 71% (II) over two steps). In case of **9** the synthesis via strategy I is slightly improved (32% (I) vs 24% (II) over two steps).

In summary, we synthesized biotinylated derivatives of both cell wall precursors (UDP-MTPs) and possible cell wall degradation products (MTPs) from Gram-positive (Lys-type) and Gram-negative (DAP-type) cell walls via two different two-step synthetic routes in acceptable to good yields.

To determine if the modified (UDP-)muramyl tripeptides were able to induce an inflammatory response via the activation of NOD2, all muramyl peptide derivatives were tested in NOD2-transfected HEK293 cells and compared with their unmodified counterparts.[13] As depicted in Figure 2 all modified compounds retained their ability to activate NOD2. Only biotin-MTP (DAP) (**9**) as well as its unmodified counterpart (MTP (DAP), **3a**) were unable to stimulate NOD2 (as it is known for the latter compound).[3] Comparing the values for biotinylated MTPs (Lys) **8a** and **8b** to **2a** it can be stated that at low concentration (0.1 μM) direct biotinylation of the ε-amino function of lysine without a LC-LC-linker seems to be favorable. Comparing biotinylated UDP-MTPs (Lys) **6a** and **6b** to **2b** as well as biotinylated (**7**) and unmodified (**3b**) UDP-MTP (DAP) the chemical modification had no significant influence on the stimulatory potency in NOD2-transfected HEK293 cells. In conclusion, biotin-MTP (Lys) (**8a**) and biotinylated UDP-MTPs (**6a**, **6b**, **7**) are not only as active as their unmodified counterparts but have also a similar NOD2 activity compared with MDP (*i*Q) (**1a**). Furthermore, **8a** has a significantly higher potency to stimulate NOD2 than commercially available biotin-MDP (*i*Q) (**4**) especially at low and therefore physiological concentrations. This is an additional proof for our observation that for the biotinylation of muropeptides the use of a long chain-linker is not advantageous.

The biotinylated (UDP-)MTPs we have synthesized, especially compound **8a**, are very promising tools for clarifying the molecular mechanism(s) whereby the human immune system recognizes bacterial peptidoglycan part structures and in identifying the



Scheme 1. Synthetic routes I and II. [a]0.1 M HCl, 100 °C, 10 min. [b]**5a** or **5b**, phosphate buffer (pH 8.0), 27 °C, 1 h.

Table 2
Yields of biotinylation of muropeptides **2a** or **3a** using different equivalents of biotinylation reagent **5a** or **5b**

Muropeptide	Equiv of biotinylation reagent	Yield
2a	2.0 equiv of **5a**	61% (of **8a**)
2a	1.5 equiv of **5a**	32% (of **8a**)
2a	2.0 equiv of **5b**	69% (of **8b**)
2a	1.5 equiv of **5b**	72% (of **8b**)
3a	2.0 equiv of **5a**	25% (of **9**)

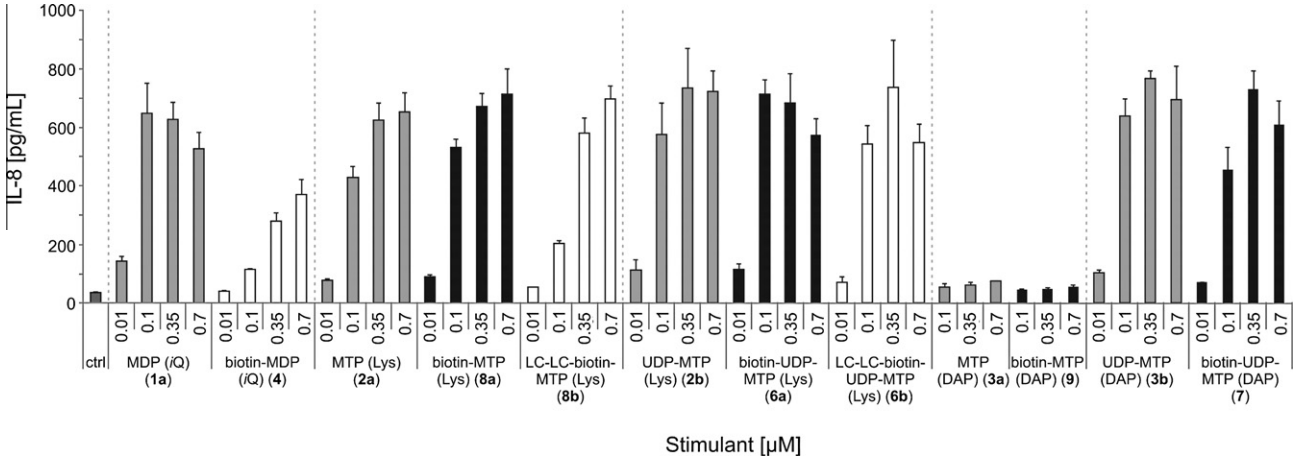


Figure 2. Test for the ability of unlabeled and biotinylated (UDP-)muropeptides to activate NOD2 in transiently NOD2-transfected HEK293 cells. Cells were incubated with different concentrations (0.01, 0.1, 0.35, 0.7 μM) of the indicated stimulant and the secretion of IL-8 was measured by ELISA as a marker of cell activation; dark grey bar: negative control, light grey bars: unmodified (UDP-)muropeptides, black bars: biotinylated (UDP-)muropeptides, white bars: (LC-)LC-LC-biotinylated (UDP-)muropeptides. Data shown as means ± SEM and represent one of three independent experiments; assays were performed in triplicates.

receptor(s) for these potent immunostimulatory bacterial compounds.

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Supplementary data

Supplementary data (isolation procedures for **2b** and **3b**, experimental procedures, NMR and HR-MS data for all isolated and synthesized compounds, cell-based assay) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.04.005.

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11. *Biotin-UDP-MTP (Lys)* (**6a**). ^{1}H NMR (700 MHz, D_2O): δ = 7.96 (d, J = 8.1 Hz, 1H, H-6$_U$), 5.99 (d, J = 4.8 Hz, 1H, H-1$_{Rib}$), 5.97 (d, J = 8.3 Hz, 1H, H-5$_U$), 5.50–5.46 (m, 1H, H-1$_{MurNAc}$), 4.63–4.59 (m, 1H, H-8$_{Biotin}$), 4.42 (dd, J = 7.9, 4.5 Hz, 1H, H-7$_{Biotin}$), 4.39–4.36 (m, 1H, H-2$_{Rib}$), 4.38–4.35 (m, 1H, H-3$_{Rib}$), 4.36–4.32 (m, 1H, H-2$_{iGlu}$), 4.30 (q, J = 7.2 Hz, 1H, CH$_{Ala}$), 4.31–4.27 (m, 1H, H-4$_{Rib}$), 4.30–4.27 (m, 1H, H-2$_{Lys}$), 4.26–4.22 (m, 1H, C*H*CH$_{3MurNAc}$), 4.26–4.22 (m, 1H, H-5a$_{Rib}$), 4.21–4.17 (m, 1H, H-5b$_{Rib}$), 4.15–4.11 (m, 1H, H-2$_{MurNAc}$), 3.98–3.93 (m, 1H, H-5$_{MurNAc}$), 3.90–3.86 (m, 1H, H-6a$_{MurNAc}$), 3.83 (dd, J = 12.3, 4.1 Hz, 1H, H-6b$_{MurNAc}$), 3.79 (dd, J = 9.7, 9.5 Hz, 1H, H-3$_{MurNAc}$), 3.64 (dd, J = 9.7, 9.4 Hz, 1H, H-4$_{MurNAc}$), 3.35–3.31 (m, 1H, H-6$_{Biotin}$), 3.19 (t, J = 6.6 Hz, 2H, H-6$_{Lys}$), 3.00 (dd, J = 13.0, 5.0 Hz, 1H, H-9a$_{Biotin}$), 2.78 (d, J = 13.0 Hz, 1H, H-9b$_{Biotin}$), 2.37 (t, J = 7.4 Hz, 2H, H-4$_{iGlu}$), 2.25 (t, J = 7.0 Hz, 2H, H-2$_{Biotin}$), 2.22–2.17 (m, 1H, H-3a$_{iGlu}$), 2.02 (s, 3H, COC*H*$_{3MurNAc}$), 2.02–1.97 (m, 1H, H-3b$_{iGlu}$), 1.91–1.83 (m, 1H, H-3a$_{Lys}$), 1.77–1.69 (m, 1H, H-3b$_{Lys}$), 1.75–1.68 (m, 1H, H-5a$_{Biotin}$), 1.70–1.59 (m, 2H, H-3$_{Biotin}$), 1.61–1.55 (m, 1H, H-5b$_{Biotin}$), 1.56–1.51 (m, 2H, H-5$_{Lys}$), 1.43 (d, J = 7.2 Hz, 3H, CH$_{3Ala}$), 1.45–1.40 (m, 2H, H-4$_{Lys}$), 1.46–1.39 (m, 2H, H-4$_{Biotin}$), 1.39 (d, J = 6.7 Hz, 3H, CHC*H*$_{3MurNAc}$) ppm. ^{13}C NMR (176 MHz, D_2O): δ = 177.5 (*C*OCH$_{2Biotin}$), 177.4 (COOH$_{Lys}$), 176.5 (COCH$_{MurNAc}$), 176.2 (COOH$_{iGlu}$), 175.8 (CO$_{iGlu}$), 175.4 (CO$_{Ala}$), 175.1 (*C*OCH$_{3MurNAc}$), 167.1 (C-4$_U$), 166.2 (NH*C*ONH$_{Biotin}$), 152.7 (C-2$_U$), 142.5 (C-6$_U$), 103.5 (C-5$_U$), 95.5 (d, J = 5.2 Hz, C-1$_{MurNAc}$), 89.3 (C-1$_{Rib}$), 84.1 (d, J = 8.6 Hz, C-4$_{Rib}$), 80.7 (C-3$_{MurNAc}$), 78.8 (*C*HCH$_{3MurNAc}$), 74.6 (C-2$_{Rib}$), 73.8 (C-5$_{MurNAc}$), 70.5 (C-3$_{Rib}$), 69.0 (C-4$_{MurNAc}$), 65.9 (d, J = 4.3 Hz, C-5$_{Rib}$), 63.0 (C-7$_{Biotin}$), 61.2 (C-6$_{MurNAc}$), 61.1 (C-8$_{Biotin}$), 56.2 (C-6$_{Biotin}$), 54.3 (d, J = 7.9 Hz, C-2$_{MurNAc}$), 54.2 (C-2$_{Lys}$), 53.6 (C-2$_{iGlu}$), 50.4 (CH$_{Ala}$), 40.5 (C-9$_{Biotin}$), 39.8 (C-6$_{Lys}$), 36.4 (C-2$_{Biotin}$), 32.5 (C-4$_{iGlu}$), 31.2 (C-3$_{Lys}$), 28.7 (C-5$_{Lys}$), 28.7 (C-4$_{Biotin}$), 28.5 (C-5$_{Biotin}$), 27.8 (C-3$_{iGlu}$), 26.0 (C-3$_{Biotin}$), 23.4 (C-4$_{Lys}$), 23.0 (COC*H*$_{3MurNAc}$), 19.5 (CHC*H*$_{3MurNAc}$), 17.7 (CH$_{3Ala}$) ppm. ^{31}P NMR (284 MHz, D_2O): δ = −10.6 (d, J = 18.2 Hz, P$_{Rib}$), −12.4 (d, J = 18.2 Hz, P$_{MurNAc}$) ppm. HR-MS: Calcd for $C_{44}H_{69}N_9O_{26}P_2S$: m/z = 1233.3550. Found: m/z = 1233.3569.
12. *Biotin-MTP (Lys)* (**8a**). ^{1}H NMR (700 MHz, D_2O): δ = 5.16 (d, J = 3.5 Hz, 1H, H-1α_{MurNAc}), 4.68 (d, J = 8.5 Hz, 1H, H-1β_{MurNAc}), 4.63–4.59 (m, 2 × 1H, H-8α_{Biotin}, H-8β_{Biotin}), 4.42 (dd, J = 7.9, 4.5 Hz, 2 × 1H, H-7α_{Biotin}, H-7β_{Biotin}), 4.36–4.31 (m, 2 × 1H, H-2α_{iGlu}, H-2β_{iGlu}), 4.35–4.32 (m, 2 × 1H, CHα_{Ala}, CHβ_{Ala}), 4.33–4.28 (m, 1H, *CH*CH$_3\alpha_{MurNAc}$), 4.28 (dd, J = 9.0, 5.0 Hz, 2 × 1H, H-2α_{Lys}, H-2β_{Lys}), 4.24 (q, J = 6.7 Hz, 1H, *CH*CH$_3\beta_{MurNAc}$), 3.96 (dd, J = 10.5, 3.5 Hz, 1H, H-2α_{MurNAc}), 3.91 (dd, J = 12.2, 1.7 Hz, 1H, H-6aβ_{MurNAc}), 3.90–3.86 (m, 1H, H-5α_{MurNAc}), 3.85 (dd, J = 12.1, 2.0 Hz, 1H, H-6aα_{MurNAc}), 3.81 (dd, J = 12.2, 5.0 Hz, 1H, H-6bα_{MurNAc}), 3.82–3.76 (m, 1H, H-2β_{MurNAc}), 3.76 (dd, J = 12.3, 5.6 Hz, 1H, H-6bβ_{MurNAc}), 3.71 (dd, J = 10.1, 9.2 Hz, 1H, H-3α_{MurNAc}), 3.58 (dd, J = 9.7, 9.4 Hz, 1H, H-4α_{MurNAc}), 3.55 (dd, J = 9.5, 9.3 Hz, 1H, H-4β_{MurNAc}), 3.51 (dd, J = 9.8, 9.0 Hz, 1H, H-3β_{MurNAc}), 3.50–3.46 (m, 1H, H-5β_{MurNAc}), 3.36–3.31 (m, 2 × 1H, H-6α_{Biotin}, H-6β_{Biotin}), 3.23–3.15 (m, 2 × 2H, H-6α_{Lys}, H-6β_{Lys}), 3.00 (dd, J = 13.0, 5.0 Hz, 2 × 1H, H-9aα_{Biotin}, H-9aβ_{Biotin}), 2.78 (d, J = 13.0 Hz, 2 × 1H, H-9bα_{Biotin}, H-9bβ_{Biotin}), 2.36 (d, J = 7.7 Hz, 2 × 2H, H-4α_{iGlu}, H-4β_{iGlu}), 2.25 (t, J = 7.2 Hz, 2 × 2H, H-2α_{Biotin}, H-2β_{Biotin}), 2.22–2.15 (m, 2 × 1H, H-3aα_{iGlu}, H-3aβ_{iGlu}), 1.98 (s, 3H, COCH$_3\alpha_{MurNAc}$), 1.97 (s, 3H, COCH$_3\beta_{MurNAc}$), 2.03–1.96 (m, 2 × 1H, H-3bα_{iGlu}, H-3bβ_{iGlu}), 1.90–1.83 (m, 2 × 1H, H-3aα_{Lys}, H-3aβ_{Lys}), 1.76–1.71 (m, 4 × 1H, H-5aα_{Biotin}, H-5aβ_{Biotin}, H-3bα_{Lys}, H-3bβ_{Lys}), 1.71–1.60 (m, 2 × 2H, H-3α_{Biotin}, H-3β_{Biotin}), 1.63–1.55 (m, 2 × 1H, H-5bα_{Biotin}, H-5bβ_{Biotin}), 1.56–1.49 (m, 2 × 2H, H-5α_{Lys}, H-5β_{Lys}), 1.44 (d, J = 6.9 Hz, 3H, CH$_3\beta_{Ala}$), 1.43 (d, J = 7.1 Hz, 3H, CH$_3\alpha_{Ala}$), 1.40 (d, J = 6.5 Hz, 3H, CHC*H*$_3\alpha_{MurNAc}$), 1.39 (d, J = 6.0 Hz, 3H, CHC*H*$_3\beta_{MurNAc}$), 1.46–1.38 (m, 2 × 2H, H-4α_{Biotin}, H-4β_{Biotin}), 1.44–1.36 (m, 2 × 2H, H-4α_{Lys}, H-4β_{Lys}) ppm. ^{13}C NMR (176 MHz, D_2O): δ = 177.7 (COOHα_{Lys}, COOHβ_{Lys}), 177.5 (COCH$_2\alpha_{Biotin}$, COCH$_2\beta_{Biotin}$), 176.7 (COOHα_{iGlu}, COOHβ_{iGlu}), 176.5 (COCHα_{MurNAc}), 176.3 (COCHβ_{MurNAc}), 175.8 (COα_{iGlu}, COβ_{iGlu}), 175.3 (COβ_{Ala}), 175.3 (COα_{Ala}), 175.1 (*C*OCH$_3\beta\beta_{MurNAc}$), 174.8 (COCH$_3\alpha_{MurNAc}$), 166.2 (NH*C*ONHα_{Biotin}, NH*C*ONHβ_{Biotin}), 95.8 (C-1β_{MurNAc}), 91.8 (C-1α_{MurNAc}), 83.3 (C-3β_{MurNAc}), 80.4 (C-3α_{MurNAc}), 78.9 (*C*HCH$_3\beta_{MurNAc}$), 78.6 (CHCH$_3\alpha_{MurNAc}$), 76.6 (C-5β_{MurNAc}), 72.4 (C-5α_{MurNAc}), 69.9 (C-4α_{MurNAc}), 69.7 (C-4β_{MurNAc}), 63.0 (C-7α_{Biotin}, C-7β_{Biotin}), 61.6 (C-6β_{MurNAc}), 61.4 (C-6α_{MurNAc}), 61.1 (C-8α_{Biotin}, C-8β_{Biotin}), 57.0 (C-2β_{MurNAc}), 56.2 (C-6α_{Biotin}, C-6β_{Biotin}), 54.5 (C-2α_{MurNAc}), 54.3 (C-2α_{Lys}, C-2β_{Lys}), 53.8 (C-2α_{iGlu}, C-2β_{iGlu}), 50.4 (CHα_{Ala}), 50.4 (CHβ_{Ala}), 40.5 (C-9α_{Biotin}, C-9β_{Biotin}), 39.8 (C-6α_{Lys}, C-6β_{Lys}), 36.4 (C-2α_{Biotin}, C-2β_{Biotin}), 32.5 (C-4α_{iGlu}, C-4β_{iGlu}), 31.3 (C-3α_{Lys}, C-3β_{Lys}), 28.7 (C-5α_{Lys}, C-5β_{Lys}, C-4α_{Biotin}, C-4β_{Biotin}), 28.5 (C-5α_{Biotin}, C-5β_{Biotin}), 28.0 (C-3α_{iGlu}, C-3β_{iGlu}), 26.0 (C-3α_{Biotin}, C-

$3\beta_{Biotin}$), 23.4 (C-4α_{Lys}, C-4β_{Lys}), 23.1 ($COCH_3\beta_{MurNAc}$), 22.9 ($COCH_3\alpha_{MurNAc}$), 19.5 ($CHCH_3\alpha_{MurNAc}$, $CHCH_3\beta_{MurNAc}$), 17.7 ($CH_3\alpha_{Ala}$, $CH_3\beta_{Ala}$) ppm. Anomeric ratio: α/β = 0.60:0.40. HR-MS: Calcd for $C_{35}H_{57}N_7O_{15}S$: m/z = 847.3633. Found: m/z = 847.3632.

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