Synthesis of a Biotin-Labeled Quorum-Sensing Molecule: Towards a General Method for Target Identification

Richard J. Spandl,[a] Rebecca L. Nicholson,[a] David M. Marsden,[a] James T. Hodgkinson,[a] Xianbin Su,[a] Gemma L. Thomas,[a] George P. C. Salmond,[b] Martin Welch,[b] David R. Spring*[a]

[a] Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, UK
Fax +44(1223)336362; E-mail: drspring@ch.cam.ac.uk

[b] Department of Biochemistry, University of Cambridge, Downing Site, Cambridge, CB2 1QW, UK

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Dedicated to Professor Sir Jack Baldwin in celebration of his 70th birthday

Abstract: The synthesis of bacterial quorum-sensing regulator *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) and biotin-tagged OHHL is reported. The latter will be applied to developing a general method to address the 'target identification problem' in chemical genetics.

Key words: drugs, high-throughput screening, proteins, OHHL, quorum sensing

Chemical genetics describes the use of small molecules to explore biological systems by perturbing protein function.[1] Initially, in forward chemical genetics,[2] compound collections are screened to identify novel bioactive molecules (protein modulators) in suitably designed phenotypic assays. Whereas combinatorial methods such as diversity-oriented synthesis (DOS) can provide small-molecule collections,[3] subsequent target identification is notoriously difficult[4] and has previously been described as the 'missing link' in chemical genetics.[5] To this end, we report the synthetic studies associated with a potential solution to this problem: the use of biotin-mediated affinity-capture combined with 2D difference gel electrophoresis (DIGE). The key focus of this article is the synthesis of the biotin-tagged 'bait' **1** [the bait portion of **1** is the quorum-sensing compound *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL, **2**)],[6] which will be used in proof-of-principle experiments (Figure 1). A simple and expedient synthesis of OHHL (**2**) is also reported.

The most common method of target identification involves attaching the novel bioactive small molecule under investigation (the 'probe'), via a linker, to an insoluble polymer support.[5] The small molecule's macromolecular targets can then be extracted from the cell extract by virtue of specific binding. Subsequent elution and separation of the binding proteins using polyacrylamide gel electrophoresis, followed by mass spectrometry (e.g., MALDI-TOF and LC-MSMS) allows the small molecule's binding partners to be identified.

To overcome the drawbacks of this approach, that is, the lack of generality and lack of sensitivity, we hope initially to scavenge protein targets using a bait in which the small molecule is attached via a linker to biotin (Figure 1).

The resulting bait-protein construct could then be immobilized on (magnetic) streptavidin beads as a result of the strong noncovalent biotin–stepavidin interaction. Subsequent washing, binding-partner elution (using the native small molecule), and protein labeling (with the dyes Cy3 or Cy5) would then allow target identification using 2D DIGE and mass spectrometry. This method of electrophoresis would allow a greater sensitivity compared to alternative 1D separation techniques and should allow the identification of weak binding-protein partners (Figure 2).

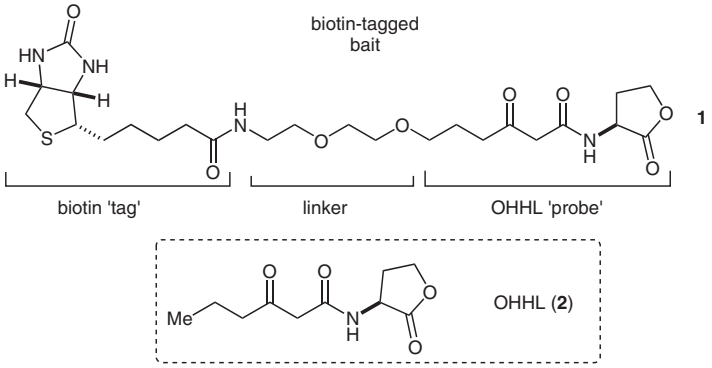

Figure 1 The quorum-sensing molecule OHHL (**2**) is incorporated into the bait **1** and attached to the D-biotin tag via a linker. *N*-(3-Oxohexanoyl)-L-homoserine lactone (**2**) and the bait compound **1** both contain the L-homoserine lactone head group.

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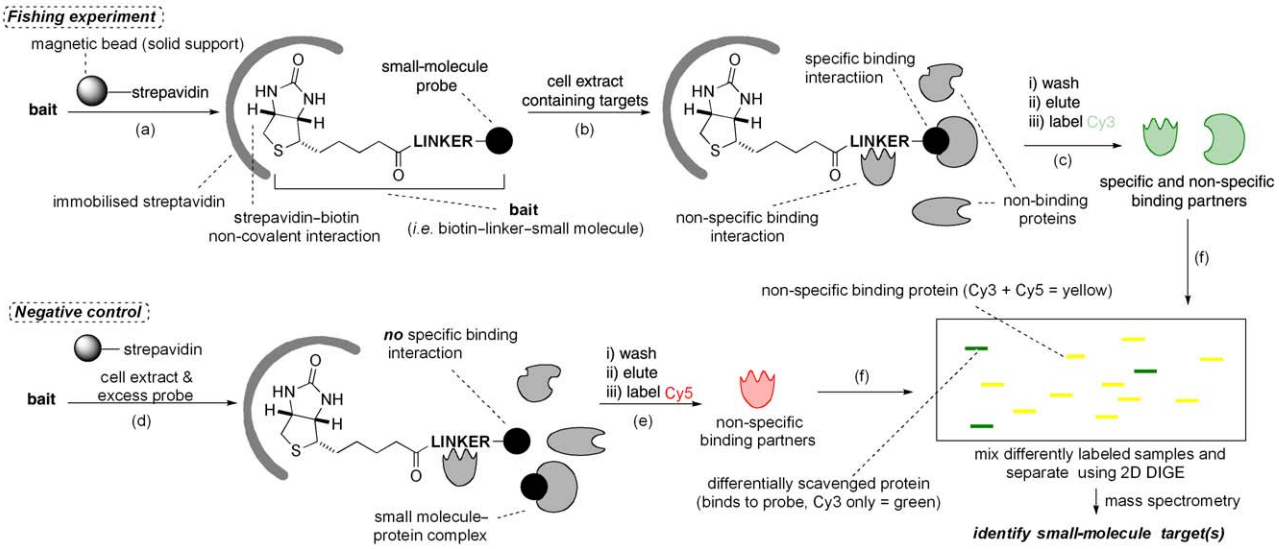
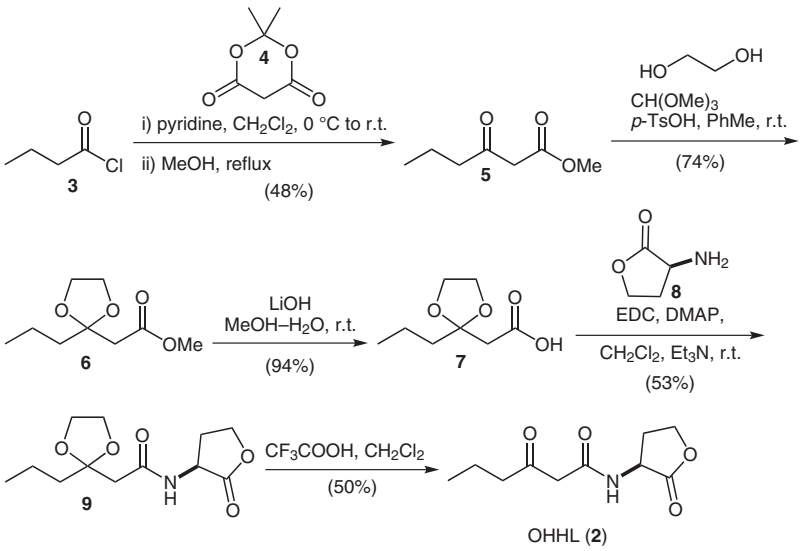


Figure 2 A schematic overview of target identification using biotin-mediated affinity-capture combined with 2D DIGE. (a) The bait (in which the small-molecule probe is attached to biotin via a linker) is immobilized on strepavidin-containing insoluble magnetic beads. (b) This complex is incubated with cell extract and, as a result of both specific and nonspecific binding interactions, proteins bind. (c) The bound proteins are eluted and labeled with Cy3 (green). (d) A negative control experiment is also performed where the bait, strepavidin beads, cell extract, and the small-molecule probe are incubated initially. As a result of competition, specific binding events are minimized with the bait. (e) After elution, the nonspecific binding proteins are labeled with Cy5 (red). (f) The differentially labeled samples from both experiments are mixed and 2D DIGE is performed to separate the binding proteins. The resulting gel is visualized and specific binders appear green (Cy3) where as nonspecific binders appear yellow (as both Cy3 and Cy5 tagged proteins are present). The specific binders, and hence small-molecule protein targets, can then be identified using mass spectrometry.

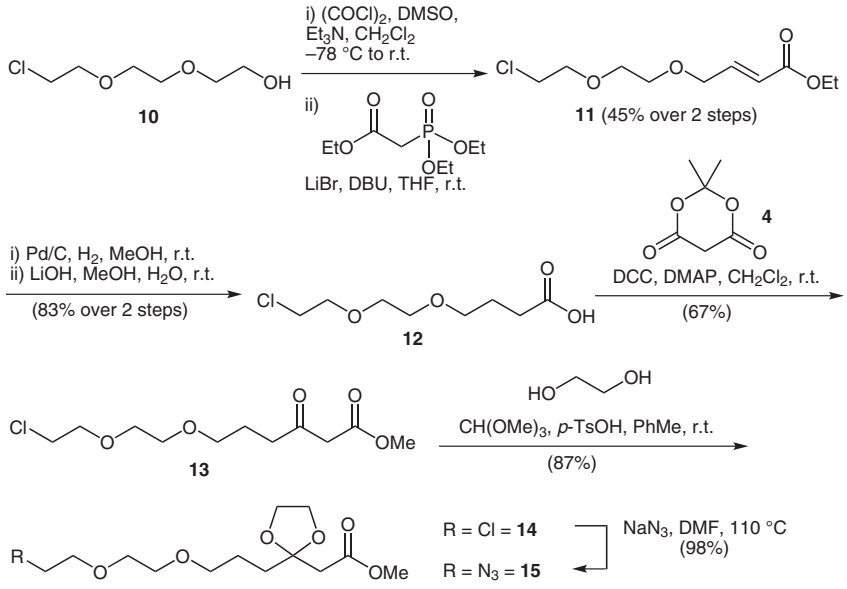
Furthermore, by the addition of the native small molecule in the scavenging step, specific binding interactions resulting from the biomolecule could be minimized (by competition) and hence control experiments could be performed. A comparative analysis, taking advantage of the different protein dyes used in either the 'fishing' or 'control' experiments, that is, in the presence and absence of small-molecule competition, would allow nontarget molecule specific interactions (i.e., that bind due to the tag and/or linker) to be identified (Figure 2).

As a proof-of-principle, we chose to explore the feasibility of our approach using a known small-molecule–protein interaction. Consistent with our group's continued interest in quorum sensing,[7] the interaction of the OHHL (**2**) with the protein CarR was investigated. In the Gram-negative bacteria *Erwinia carotovora* OHHL (**2**) binds to CarR and regulates virulence factor expression.[7c] Initially, the synthesis of OHHL (**2**) was explored. Although the synthesis of OHHL (**2**)[8] and its analogues have been published previously,[9] our approach provides an efficient alternative (Scheme 1).[10]

Butyryl chloride **3** was firstly reacted with Meldrum's acid **4** and refluxed in methanol thus yielding the 1,3-diketone architecture **5**. Subsequent ketal protection, to give **6**,



Scheme 1 The synthesis of OHHL (**2**)



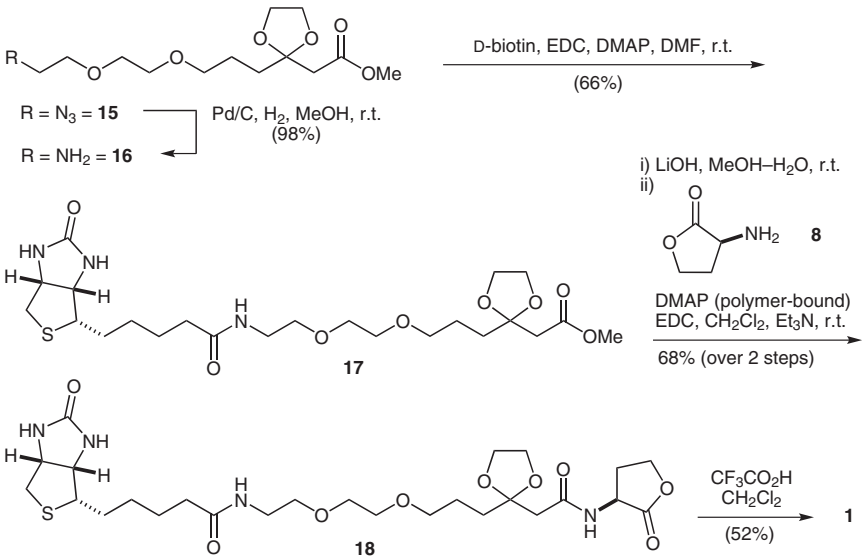
Scheme 2 The synthesis of azide **15**

and ester cleavage gave access to the acid **7**. An EDC coupling of **7** with the amine **8** then furnished the protected target compound **9** which was treated with TFA to give OHHL (**2**). The spectral data obtained[11] was consistent with that previously reported[12] and the optical rotation was consistent with authentic OHHL.[13] Furthermore, binding studies with CarR using fluorescence quenching gave a K_d value (K_d = 1.5 μM) identical to that reported previously.[7c]

With OHHL (**2**) in hand, the target molecule **1** was envisaged where the OHHL probe was attached to biotin through a linker. Initially, using the triethylene glycol monochlorohydrin **10** as the starting unit, the azide intermediate **15** was synthesized in good yield over seven steps (Scheme 2) via the compounds **11**–**14**.

With the ester moiety of **15** acting as a potential site of attachment for the homoserine lactone head group (via the acid) and the azide acting as a potential site of attachment of biotin (via the amine), two alternative synthetic strategies were possible. In the former approach, after initial attachment of the homoserine lactone head group, the biotinylation would occur last. Since the purification of biotinylated compounds can be problematic, this route was initially investigated. However, problems associated with selective removal of the protecting groups hindered our efforts. Consequently, the alternative route, with biotinylation occurring earlier in the sequence, was found to be more fruitful (Scheme 3).

After reduction of **15** to give **16** and an EDC-mediated coupling with D-biotin to give **17**, subsequent hydrolysis, and coupling to amine **8** furnished the protected precursor **18** in reasonable yield.[14] The synthesis of **1** could then be completed by ketal deprotection.



Scheme 3 Synthesis of the biotinylated bait **1**

In summary, we report the efficient synthesis of the quorum-sensing molecule OHHL (**2**) and the biotin-tagged analogue **1**. The binding properties of **1** with CarR are currently being investigated. Results from these investigations and the proof-of-principle target identification experiments will be published in due course.

Acknowledgment

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References and Notes

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(2) The complementary approach, reverse chemical genetics, involves modulating a known protein and analyzing the resulting phenotype.[1b]

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(10) Analogues of the related signaling molecule *N*-3-(oxododecanoyl)-L-homoserine lactone (OdDHL), used in *Pseudomonas aeruginosa*, have been synthesized by coupling using the acid, Meldrum's acid, and the amine in one pot: Chhabra, S. R.; Harty, C.; Hooi, D. S. W.; Daykin, M.; Williams, P.; Telford, G.; Pritchard, D. I.; Bycroft, B. W. *J. Med. Chem.* **2003**, *46*, 97; in our hands this method proved less fruitful than the stepwise method employed therein.

(11) *N*-(3-Oxohexanoyl)-L-homoserine lactone(**2**): R_f = 0.23 (SiO_2; EtOAc–PE, 8:2). IR (neat): ν_{max} = 3301 (w, br), 2965 (w), 2878 (w), 1774 (s), 1716 (m), 1649 (s), 1535 (m), 1379 (m), 1221 (m), 1169 (s), 1021 (m) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.73 (1 H, br s, CONH), 4.63–4.51 [1 H, br m, C(2)H], 4.43 [1 H, br t, *J* = 9.1 Hz, C(4)H_aH_b], 4.27–4.18 [1 H, br m, C(4)H_aH_b], 3.42 (2 H, s, $COCH_2CO$), 2.68–2.58 [1 H, br m, C(3)H_aH_b], 2.47 (2 H, t, *J* = 7.3 Hz, $CH_3CH_2CH_2$), 2.30–2.16 [1 H, br m, C(3)H_aH_b], 1.54 (2 H, sext, *J* = 7.3 Hz, $CH_3CH_2CH_2$), 0.86 (2 H, t, *J* = 7.5, $CH_3CH_2CH_2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 206.1 (C), 175.2 (C), 166.9 (C), 65.9 (CH_2), 48.9 (CH), 48.7 (CH_2), 45.4 (CH_2), 29.2 (CH_2), 16.8 (CH_2), 13.4 (CH_3). HRMS: *m/z* calcd for $C_{10}H_{15}NO_4Na^+$: 236.0899; found [ESI – Na^+]: 236.0892; Δppm = –1.5; mp 80–81 °C (EtOAc–PE). $[\alpha]_D^{25}$ +7.36 (*c* 0.95, $CHCl_3$).

(12) Blackwell, H. E.; Geske, G. D.; Wezeman, R. J. WO 2006/084056 A2, **2006**.

(13) Compound **2**: $[\alpha]_D^{25}$ +7.36 (*c* 0.95, $CHCl_3$). Sigma OHHL $[\alpha]_D^{25}$ +8.5 (*c* 0.12, $CHCl_3$). These specific rotation values are slightly lower than those reported by Blackwell and co-workers,[12a] that is, $[\alpha]_D^{25}$ +12.2 (*c* 2.7, $CHCl_3$). Although some racemization may have occurred during the synthesis reported here, this did not affect binding of CarR. In our hands coupling with HOBt was less successful.

(14) Polymer-bound DMAP was required in the final EDC-mediated coupling to aid purification. The reaction products and DMAP had very similar R_f values.

Synthesis of 18

A round-bottom flask, equipped with a magnetic stirrer, containing the ester **17** (529 mg, 1.02 mmol), $LiOH·H_2O$ (98 mg, 2.33 mmol) and 66% aq MeOH (25 mL) was stirred at r.t. for 16 h. The solvent was removed in vacuo to give the lithium salt of the corresponding acid (structure not shown) as a white solid (550 mg). The salt was used in subsequent reactions without further purification. A round-bottom flask, equipped with a magnetic stirrer, containing the lithium salt (0.55 g, 1.09 mmol), EDC (0.27 g, 1.42 mmol), polymer-bound DMAP (5 mmol/g, 1.1 g, 5.46 mmol), and DMF (40 mL) was stirred at r.t. for 15 min before being charged with L-homoserine lactone hydrobromide (1.02 g, 5.6 mmol) and stirred at r.t. for 16 h. The crude reaction mixture was filtered and solvent removed in vacuo. The crude product was purified by column chromatography to give **18** as a colorless oil (0.43 g, 68% over 2 steps).

R_f = 0.36 (SiO_2; CH_2Cl_2–MeOH, 85:15). IR (neat): ν_{max} = 3391 (s, br), 2932 (w, br), 1766 (m), 1645 (s) br, 1549 (s), 1474 (m), 1355 (m), 1063 (s) cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ = 4.69–4.52 [2 H, m, $NHCHCH_2S$ and C(2)H], 4.48 [1 H, t, *J* = 9.2 Hz, C(4)H_aH_b], 4.41–4.29 [2 H, m, $NHCHCH_2S$ and C(4)H_aH_b], 4.13–3.94 [4 H, m, $C(OCH_2CH_2O)CH_2$], 3.67–3.54 (6 H, m, OCH_2CH_2O and $NHCH_2CH_2O$), 3.54 (2 H, t, *J* = 6.1 Hz, $OCH_2CH_2CH_2C$), 3.35–3.47 (2 H, br m, $NHCH_2CH_2O$), 3.30–3.22 (1 H, m, SCH), 2.97 (1 H, dd, *J* = 12.7, 5.1 Hz, SCH_aH_b), 2.75 (1 H, d, *J* = 12.7 Hz, SCH_aH_b), 2.66–2.49 [3 H, m, $C(OCH_2CH_2O)CH_2CO$ and C(3)H_aH_b)], 2.43–2.30 [1 H, m, C(3)H_aH_b], 2.26 (2 H, t, *J* = 7.1 Hz, $CH_2CH_2CH_2CH_2CONH$], 1.88–1.55 (8 H, $CH_2CH_2CH_2CH_2CONH$ and $OCH_2CH_2CH_2C$), 1.53–1.41 (2

H, m, $CH_2CH_2CH_2CH_2CONH$). ^{13}C NMR (100 MHz, CD_3OD): δ = 174.5 (C), 173.3 (C), 168.9 (C), 163.1 (C), 107.7 (C), 69.3 (CH_2), 68.4 (CH_2), 68.2 (CH_2), 67.7 (CH_2), 64.5 (CH_2), 63.4 (CH_2), 60.5 (CH), 58.8 (CH), 54.1 (CH), 47.0 (CH), 42.2 (CH_2), 38.3 (CH_2), 37.5 (CH_2), 33.9 (CH_2), 32.5 (CH_2), 26.9 (CH_2), 26.7 (CH_2), 26.5 (CH_2), 24.0 (CH_2), 22.0 (CH_2). LCMS (MeCN): 587 [MH]. HRMS: *m/z* calcd for $C_{26}H_{43}N_4O_9S_1^+$: 587.2746; found [ESI – H^+]: 587.2746; Δppm = +0.1.

Synthesis of 1

A round-bottom flask, equipped with a magnetic stirrer and open to air, containing the acetal **18** (140 mg, 0.23 mmol), CH_2Cl_2 (2.5 ml), and TFA (0.25 mL, 33.6 mmol) at r.t. was stirred for 2.5 h and the solvent was removed in vacuo. The crude product was purified by column chromatography to give the title compound **1** (66 mg, 52%) as a colorless oil. R_f = 0.21 (SiO_2; CH_2Cl_2–MeOH, 9:1). IR (neat): ν_{max} = 3292 (w, br), 2926 (w, br), 1774 (m), 1671 (s, br), 1541 (m), 1469 (m), 1332 (m), 1200(s), 1175 (s), 1127 (s), 1020 (m) cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ = 4.58 [1 H, dd, *J* = 10.8 9.3, C(2)H], 4.53–4.40 [2 H, m, $NHCHCH_2S$ and $C(4)H_aH_b$], 4.38–4.23 [2 H, m, $NHCHCH_2S$ and $C(4)H_aH_b$], 3.62–3.50 (6 H, m, OCH_2CH_2O and $NHCH_2CH_2O$), 3.46 (2 H, t, *J* = 6.3 Hz, $OCH_2CH_2CH_2$), 3.39–3.32 [4 H, m, $NHCH_2CH_2O$ and $C(O)CH_2C(O)$], 3.24–3.16 (1 H, m, SCH), 2.93 (1 H, dd, *J* = 5.1, 12.7 Hz, SCH_aH_b), 2.74–2.63 (3 H, m, SCH_aH_b and $OCH_2CH_2CH_2$), 2.62–2.52 [1 H, m, $C(3)H_aH_b$], 2.37–2.17 [3 H, m, $C(3)H_aH_b$, CH_2CH_2CONH], 1.84 [2 H, quin, *J* = 6.9 Hz, $OCH_2CH_2CH_2C(O)$], 1.77–1.53 (4 H, m, $CH_2CH_2CH_2CH_2$), 1.44 (2 H, quin, *J* = 3.9 Hz, $CH_2CH_2CH_2CH_2$). ^{13}C NMR (100 MHz, CD_3OD): δ = 197.5 (C), 175.7 (C), 174.8 (C), 167.9 (C), 164.7 (C), 69.8 (CH_2), 69.8 (CH_2), 69.7 (CH_2), 69.2 (CH_2), 65.9 (CH_2), 61.9 (CH), 60.2 (CH), 55.6 (CH), 48.7 (CH), 48.5 (CH_2), 39.6 (CH_2), 39.0 (CH_2), 38.9 (CH_2), 35.4 (CH_2), 28.3 (CH_2), 28.2 (CH_2), 28.1 (CH_2), 25.4 (CH_2), 23.3 (CH_2). HRMS: *m/z* calcd for $C_{24}H_{39}N_4O_8S^+$: 543.2489; found [ESI – H^+]: 543.2497; Δppm = +0.7. $[\alpha]_D^{25}$ +11.7 (*c* 0.54, $CHCl_3$).