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

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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 difficult⁴ 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-princi-

ple 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–streptavidin 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).

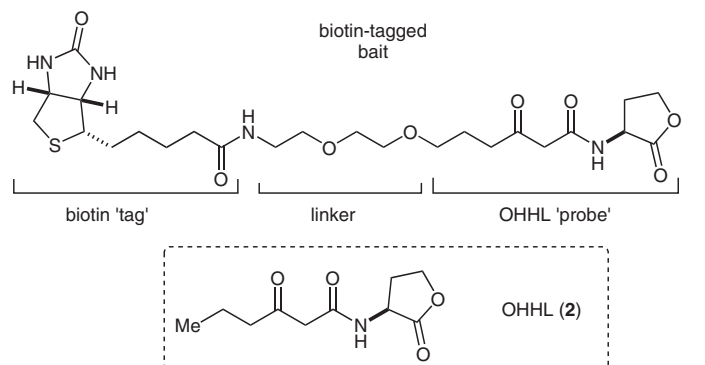


Figure 1 The quorum-sensing molecule OHHL (**2**) is incorporated into the bait **1** and attached to the D-biotin tag via a linker. *N*-(3-Oxohexanoyl)-L-homoserine lactone (**2**) and the bait compound **1** both contain the L-homoserine lactone head group.

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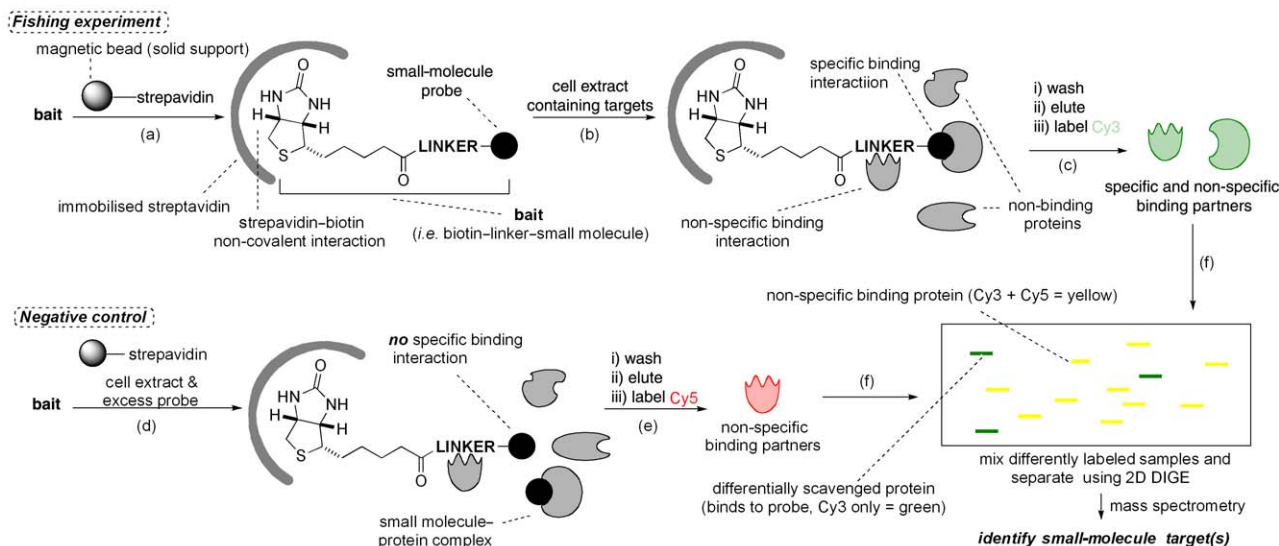


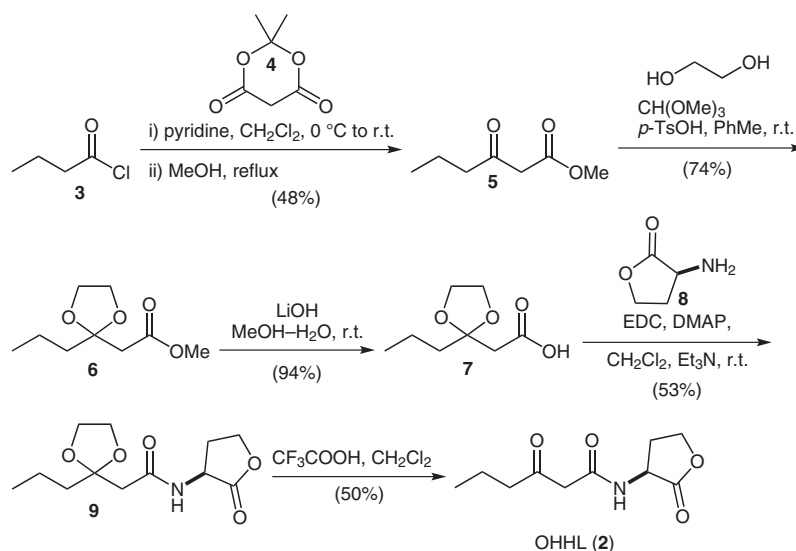
Figure 2 A schematic overview of target identification using biotin-mediated affinity-capture combined with 2D DIGE. (a) The bait (in which the small-molecule probe is attached to biotin via a linker) is immobilized on streptavidin-containing insoluble magnetic beads. (b) This complex is incubated with 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, streptavidin 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).

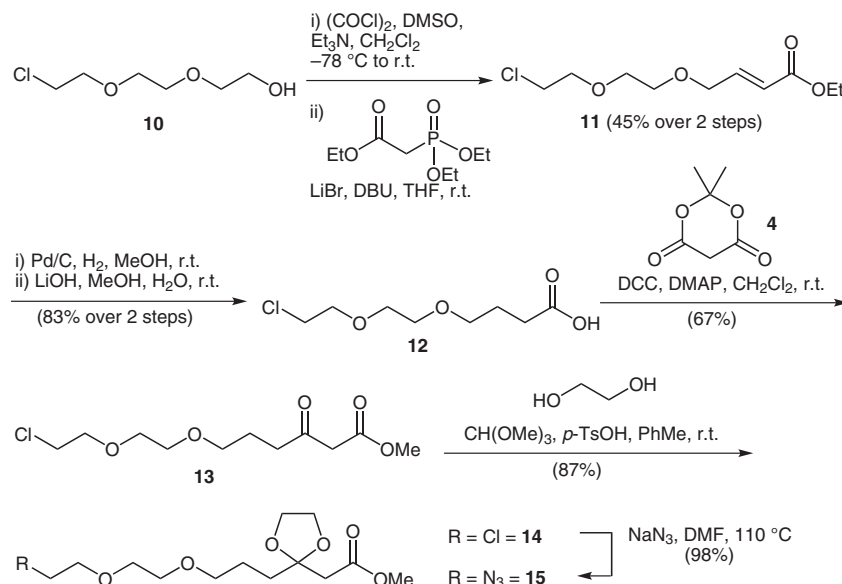
As a proof-of-principle, we chose to explore the feasibility of our approach using a known small-molecule–protein

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**)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**

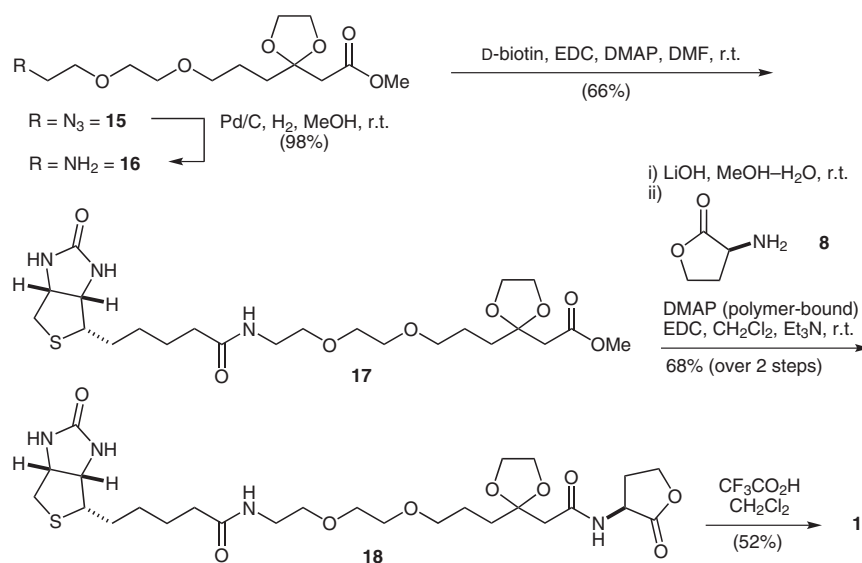
and ester cleavage gave access to the acid **7**. An EDC coupling of **7** with the amine **8** then furnished the protected target compound **9** which was treated with TFA to give OHHL (**2**). The spectral data obtained¹¹ was consistent with that previously reported¹² and the optical rotation was consistent with authentic OHHL.¹³ Furthermore, binding studies with CarR using fluorescence quenching gave a K_d value ($K_d = 1.5 \mu\text{M}$) 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

- (1) For reviews on chemical genetics, see: (a) MacBeath, G. *Genome Biol.* **2001**, 2, 2005.1. (b) Spring, D. R. *Chem. Soc. Rev.* **2005**, 34, 472. (c) Walsh, D. P.; Chang, Y.-T. *Chem. Rev.* **2006**, 106, 2476.
- (2) The complementary approach, reverse chemical genetics, involves modulating a known protein and analyzing the resulting phenotype.^{1b}
- (3) For reviews on DOS, see: (a) Spandl, R. J.; Thomas, G. L.; Diaz-Gavilan, M.; O'Connell, K. M. G.; Spring, D. R. *Chem. Rec.* **2008**, 129. (b) Nielsen, T. E.; Schreiber, S. L. *Angew. Chem. Int. Ed.* **2008**, 47, 48. (c) Spandl, R. J.; Spring, D. R.; Bender, A. *Org. Biomol. Chem.* **2008**, 6, 1149. (d) Tan, D. S. *Nat. Chem. Biol.* **2005**, 1, 74. (e) Thomas, G. L.; Wyatt, E. E.; Spring, D. R. *Curr. Opin. Drug Discovery Dev.* **2006**, 9, 700.
- (4) For reviews and approaches to solving the target identification problem, see: (a) Ahn, Y. H.; Chang, Y. T. *Acc. Chem. Res.* **2007**, 40, 1025. (b) Wong, C. C.; Cheng, K. W.; He, Q. Y.; Chen, F. *Proteomics: Clin. Appl.* **2008**, 2, 338. (c) Zheng, X. F. S.; Chan, T. F.; Zhou, H. H. *Chem. Biol.* **2004**, 11, 609.
- (5) Burdine, L.; Kodadek, T. *Chem. Biol.* **2004**, 11, 593.
- (6) For reviews of quorum sensing involving N-acylated homoserine lactones, see: (a) Hodgkinson, J. T.; Welch, M.; Spring, D. R. *ACS Chem. Biol.* **2007**, 2, 715. (b) Geske, G. D.; O'Neill, J. C.; Miller, D. M.; Wezeman, R. J.; Mattmann, M. E.; Lin, Q.; Blackwell, H. E. *ChemBioChem* **2008**, 9, 389.
- (7) For selected recent examples, see: (a) Thomas, G. L.; Bohner, C. M.; Williams, H. E.; Walsh, C. M.; Ladlow, M.; Welch, M.; Bryant, C. E.; Spring, D. R. *Mol. Biosyst.* **2006**, 2, 132. (b) Welch, M.; Mikkelsen, H.; Swatton, J. E.; Smith, D.; Thomas, G. L.; Glansdorp, F. G.; Spring, D. R. *Mol. Biosyst.* **2005**, 1, 196. (c) Welch, M.; Dutton, J. M.; Glansdorp, F. G.; Thomas, G. L.; Smith, D. S.; Coulthurst, S. J.; Barnard, A. M. L.; Salmond, G. P. C.; Spring, D. R. *Bioorg. Med. Chem. Lett.* **2005**, 15, 4235. (d) Glansdorp, F. G.; Thomas, G. L.; Lee, J. J. K.; Dutton, J. M.; Salmond, G. P. C.; Welch, M.; Spring, D. R. *Org. Biomol. Chem.* **2004**, 2, 3329.
- (8) A solution-phase route to OHHL(**2**): Dekhane, M.; Douglas, K. T.; Gilbert, P. *Tetrahedron Lett.* **1996**, 37, 1883.
- (9) The synthesis of native N-acylated homoserine lactones [including OHHL(**2**)] and non-natural analogues on solid support: (a) Geske, G. D.; O'Neill, J. C.; Blackwell, H. E. *ACS Chem. Biol.* **2007**, 2, 426. (b) Geske, G. D.; O'Neill, J. C.; Miller, D. M.; Mattmann, M. E.; Blackwell, H. E. *J. Am. Chem. Soc.* **2007**, 129, 13613. (c) Geske, G. D.; Wezeman, R. J.; Siegel, A. P.; Blackwell, H. E. *J. Am. Chem. Soc.* **2005**, 127, 12762.
- (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-Oxohehexanoyl)-L-homoserine lactone(**2**): $R_f = 0.23$ (SiO₂; EtOAc-PE, 8:2). IR (neat): $\nu_{\max} = 3301$ (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, CDCl₃): $\delta = 7.73$ (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, COCH₂CO), 2.68–2.58 [1 H, br m, C(3)H_aH_b], 2.47 (2 H, t, $J = 7.3$ Hz, CH₃CH₂CH₂), 2.30–2.16 [1 H, br m, C(3)H_aH_b], 1.54 (2 H, sext, $J = 7.3$ Hz, CH₃CH₂CH₂), 0.86 (2 H, t, $J = 7.5$, CH₃CH₂CH₂). ¹³C NMR (100 MHz, CDCl₃): $\delta = 206.1$ (C), 175.2 (C), 166.9 (C), 65.9 (CH₂), 48.9 (CH), 48.7 (CH₂), 45.4 (CH₂), 29.2 (CH₂), 16.8 (CH₂), 13.4 (CH₃). HRMS: m/z calcd for C₁₀H₁₅NO₄Na⁺: 236.0899; found [ESI – Na⁺]: 236.0892; Δ ppm = –1.5; mp 80–81 °C (EtOAc-PE). $[\alpha]_D^{25} +7.36$ (c 0.95, CHCl₃).
- (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, CHCl₃). Sigma OHHL $[\alpha]_D^{25} +8.5$ (c 0.12, CHCl₃). 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, CHCl₃). 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·H₂O (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). $R_f = 0.36$ (SiO₂; CH₂Cl₂-MeOH, 85:15). IR (neat): $\nu_{\max} = 3391$ (s, br), 2932 (w, br), 1766 (m), 1645 (s) br, 1549 (s), 1474 (m), 1355 (m), 1063 (s) cm⁻¹. ¹H NMR (400 MHz, CD₃OD): $\delta = 4.69$ –4.52 [2 H, m, NHCHCH₂S 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, NHCHCH₂S and C(4)H_aH_b], 4.13–3.94 [4 H, m, C(OCH₂CH₂O)CH₂], 3.67–3.54 (6 H, m, OCH₂CH₂O and NHCH₂CH₂O), 3.54 (2 H, t, $J = 6.1$ Hz, OCH₂CH₂CH₂C), 3.35–3.47 (2 H, br m, NHCH₂CH₂O), 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(OCH₂CH₂O)CH₂CO 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, CH₂CH₂CH₂CH₂CONH], 1.88–1.55 (8 H, CH₂CH₂CH₂CH₂CONH and OCH₂CH₂CH₂C), 1.53–1.41 (2

H, m, CH₂CH₂CH₂CH₂CONH). ¹³C NMR (100 MHz, CD₃OD): δ = 174.5 (C), 173.3 (C), 168.9 (C), 163.1 (C), 107.7 (C), 69.3 (CH₂), 68.4 (CH₂), 68.2 (CH₂), 67.7 (CH₂), 64.5 (CH₂), 63.4 (CH₂), 60.5 (CH), 58.8 (CH), 54.1 (CH), 47.0 (CH), 42.2 (CH₂), 38.3 (CH₂), 37.5 (CH₂), 33.9 (CH₂), 32.5 (CH₂), 26.9 (CH₂), 26.7 (CH₂), 26.5 (CH₂), 24.0 (CH₂), 22.0 (CH₂). LCMS (MeCN): 587 [MH]. HRMS: *m/z* calcd for C₂₆H₄₃N₄O₉S₁⁺: 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), CH₂Cl₂ (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 (SiO₂; CH₂Cl₂–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, CD₃OD): δ = 4.58 [1 H, dd, *J* = 10.8 9.3, C(2)H], 4.53–4.40 [2 H, m, NHCHCH₂S and C(4)H_aH_b], 4.38–4.23 [2 H, m, NHCHCH₂S and C(4)H_aH_b], 3.62–3.50 (6 H, m, OCH₂CH₂O and NHCH₂CH₂O), 3.46 (2 H, t, *J* = 6.3 Hz, OCH₂CH₂CH₂), 3.39–3.32 [4 H, m, NHCH₂CH₂O and C(O)CH₂C(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 OCH₂CH₂CH₂), 2.62–2.52 [1 H, m, C(3)H_aH_b], 2.37–2.17 [3 H, m, C(3)H_aH_b, CH₂CH₂CONH], 1.84 [2 H, quin, *J* = 6.9 Hz, OCH₂CH₂CH₂C(O)], 1.77–1.53 (4 H, m, CH₂CH₂CH₂CH₂), 1.44 (2 H, quin, *J* = 3.9 Hz, CH₂CH₂CH₂CH₂). ¹³C NMR (100 MHz, CD₃OD): δ = 197.5 (C), 175.7 (C), 174.8 (C), 167.9 (C), 164.7 (C), 69.8 (CH₂), 69.7 (CH₂), 69.2 (CH₂), 65.9 (CH₂), 61.9 (CH), 60.2 (CH), 55.6 (CH), 48.7 (CH), 48.5 (CH₂), 39.6 (CH₂), 39.0 (CH₂), 38.9 (CH₂), 35.4 (CH₂), 28.3 (CH₂), 28.2 (CH₂), 28.1 (CH₂), 25.4 (CH₂), 23.3 (CH₂). HRMS: *m/z* calcd for C₂₄H₃₉N₄O₈S⁺: 543.2489; found [ESI – H⁺]: 543.2497; Δppm = +0.7. [α]_D²⁵ +11.7 (c 0.54, CHCl₃).