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Identification and isolation of lantibiotics from culture: a bioorthogonal chemistry approach†

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A distinguishing feature of the lantibiotic family of cyclic peptides is the presence of thioethers. Treatment of a lantibiotic with an alkaline solution at high pH gives rise to a β -elimination reaction yielding the corresponding ring opened precursor, containing a dehydro-amino acid residue. We here reveal in a proof-of-concept study that a ring opened lantibiotic (mersacidin) can be captured for pull-down from a culture broth, subsequently released and identified by mass spectrometry.

Introduction

Due to the rapid spread of drug-resistant infectious diseases a point of no return has been reached where novel antibiotics are an absolute necessity.¹ However, it has become increasingly difficult to find novel classes of antibiotics with efficacy against multi-drug resistant pathogens such as MDR-TB (multi-drug resistant *Mycobacterium tuberculosis*), MRSA (methicillin-resistant *Staphylococcus aureus*) and the rapidly emerging MDR Gram-negative pathogens. Actinomycetes are prolific antibiotic producers, and full genome sequencing efforts have established that even the widely studied species are still relatively untapped sources of natural products. Around 20 new gene clusters encoding natural products were found on the genome of the actinomycete *Streptomyces coelicolor* alone.² This suggests the presence of silent antibiotics, and new approaches are required to identify these.³ One promising class of antibiotics is that of the lantibiotics, peptide antibiotics that are characterized by the presence of one or more cyclic thioether linkages.⁴ Nisin, the first discovered lantibiotic and produced by the firmicute *Lactococcus lactis*, is used as a food preservative and no significant bacterial resistance has thus far been encountered.⁵ At present, more than 50 different lantibiotics produced by Gram-positive bacteria are known with diverse structure and function, and due to the next-generation sequencing technology many new lantibiotic gene clusters are being discovered. Lantibiotics show substantial specificity for bacterial cell membrane components, including lipid II and phosphoethanolamine, and act on pathogenic

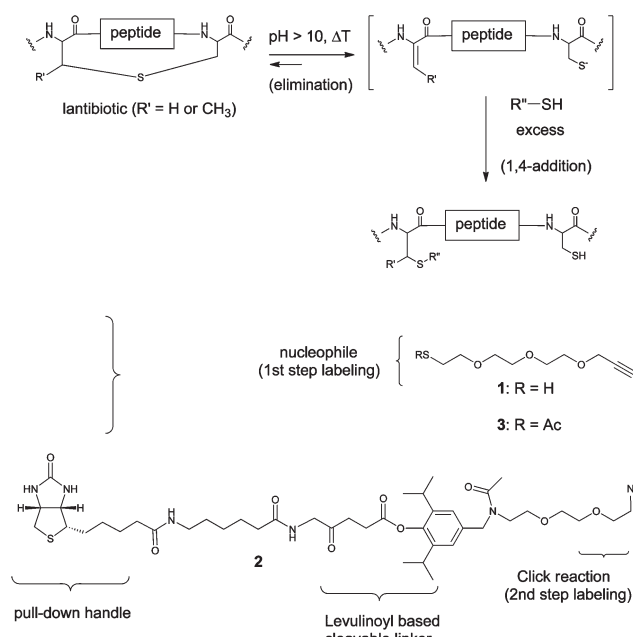
bacteria by pore formation or inhibition of peptidoglycan biosynthesis.⁶ Some lantibiotics combine more than one mode of action, allowing them to escape resistance mechanisms.⁷ These properties classify the lantibiotics as an interesting class of molecules and a viable target for the discovery and development of novel antimicrobial therapeutics.⁸

Lantibiotics are ribosomally synthesized as linear pro-peptides and post-translationally modified to their biologically active thioether containing forms. Following secretion, the leader peptide is removed, creating the mature lantibiotic (typically around 2 kDa). Studies of the biosynthesis of lantibiotics suggest that the post-translational modifications generally involve a two-step procedure in which threonine and serine residues of the precursor oligopeptides are enzymatically dehydrated to form dehydrobutyrine and dehydroalanine residues, respectively.⁹ These unsaturated amino acid residues serve as electrophiles on which the thiolates of cysteine residues can attack *via* intra-molecular 1,4-addition followed by stereoselective protonation of the resulting enolate to create a β -methylanthionine or lanthionine moiety, respectively. Lantibiotics containing several thioether rings may result from programmed post-translational modification steps. Recently, it was found that even more complex ring structures can be obtained when the resulting enolate is not protonated but serves as a nucleophile in an additional intramolecular 1,4-addition reaction.¹⁰ The thioether rings of certain lantibiotics can be chemically reversibly opened following an elimination reaction at high pH and elevated temperature (Scheme 1). The resulting ring opened intermediates can be trapped by the addition of an excess of a thiol reagent, such as mercaptoethanol. Several ring opened derivatives can thus be prepared and this strategy has been successfully applied for the sequence analysis of the lantibiotics galidermin, Pep5 and actagardine using NMR spectroscopy.¹¹ It occurred to us that this chemical feature of lantibiotics may be generally applied in a two-step labeling strategy¹² for the discovery of novel

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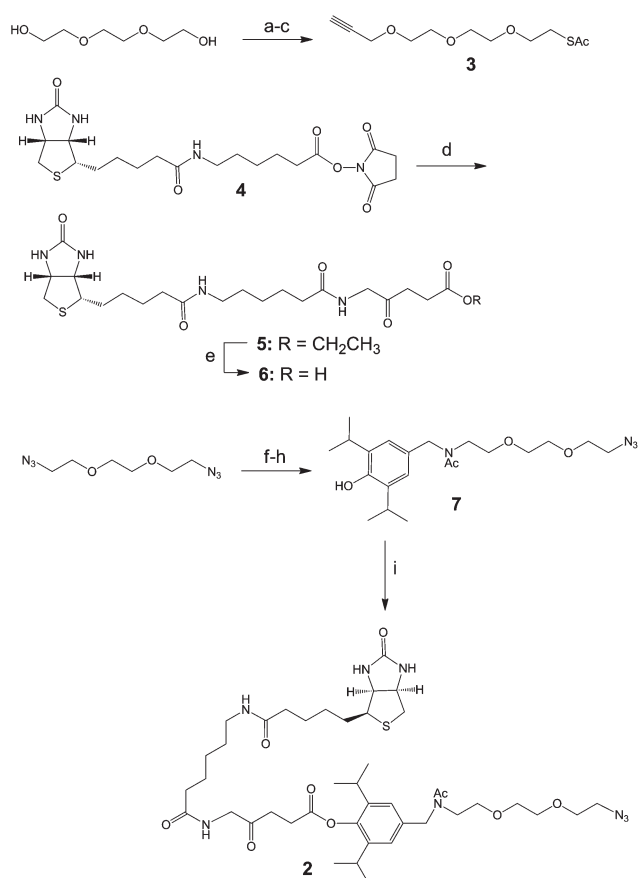
Scheme 1 The opening of a thioether linkage of a lantibiotic antimicrobial peptide and the trapping of the ring opened intermediate using excess thiol reagent, and the structures of the designed probes **2** and **1** (generated *in situ* from **3**) used in the two-step labeling strategy.

lantibiotics produced by bacillus and streptomyces bacterial strains. For this purpose we designed the probes **2** and **3** (Scheme 1). The free thiol **1**, generated *in situ* from **3** under the strong basic reaction conditions of the elimination reaction, is equipped with an acetylene moiety for a projected click reaction¹³ with the azide moiety of probe **2**. Probe **2** contains a biotin handle for recognition using streptavidin beads and a hydrazine sensitive cleavable linker we previously described for chemical proteomics purposes.¹⁴ We here reveal that probes **2** and **3** can be used in a two-step labeling protocol to identify the known lantibiotic mersacidin¹⁵ from *Bacillus subtilis* fermentation broth.

Results and discussion

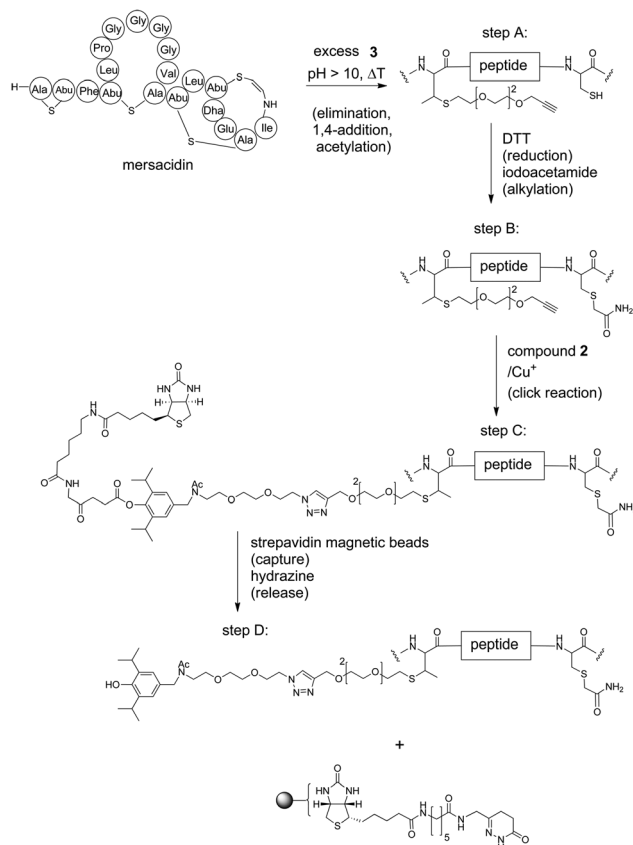
The synthesis of compounds **2** and **3** is described in Scheme 2. Triethylene glycol was alkylated using sodium hydride and propargyl bromide, subsequently converted into its tosylate and treated with potassium thioacetate to obtain compound **3** in 25% yield over 3-steps. Compound **4**¹⁷ was treated with 5-azido-4-oxopentanoate in the presence of 10% Pd/C to give **5** in 73% yield. Ester **5** was readily saponified to provide compound **6** in 87% yield. Carboxylic acid **6** was reacted with the phenol **7**, obtained in a three step one pot procedure from 1,2-bis(2-azidoethoxy)ethane, in the presence of EDC/DMAP to give product **2** in only 15% yield. The low yield of the former reaction is likely due to severe steric hindrance of the aromatic hydroxyl.

Once synthesized, we proceeded to evaluate their reactivity using the known lantibiotic mersacidin ($C_{80}H_{120}N_{20}O_{21}S_4$, MW 1824.78 Da), containing three methyllanthionine residues, one dehydroalanine residue and one *S*-aminovinyl-2-methylcysteine moiety.¹⁶ We first investigated the efficiency of our probes using



Scheme 2 (a) NaH, propargyl bromide in THF. (b) TsCl, NEt₃ in CH₂Cl₂. (c) KSAC in DMF. (d) Ethyl 5-azido-4-oxopentanoate, Pd/C, H₂ in methanol. (e) 2 M NaOH in methanol. (f) PPh₃, THF/H₂O 4/1 v/v. (g) 4-Hydroxy-3,5-diisopropylbenzaldehyde, NaOH, NaBH₄ in methanol. (h) Ac₂O. (i) Compound **6**, EDC, DMAP in DMF.

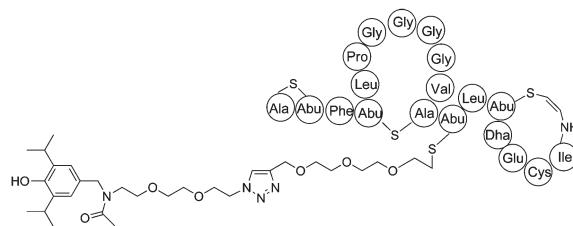
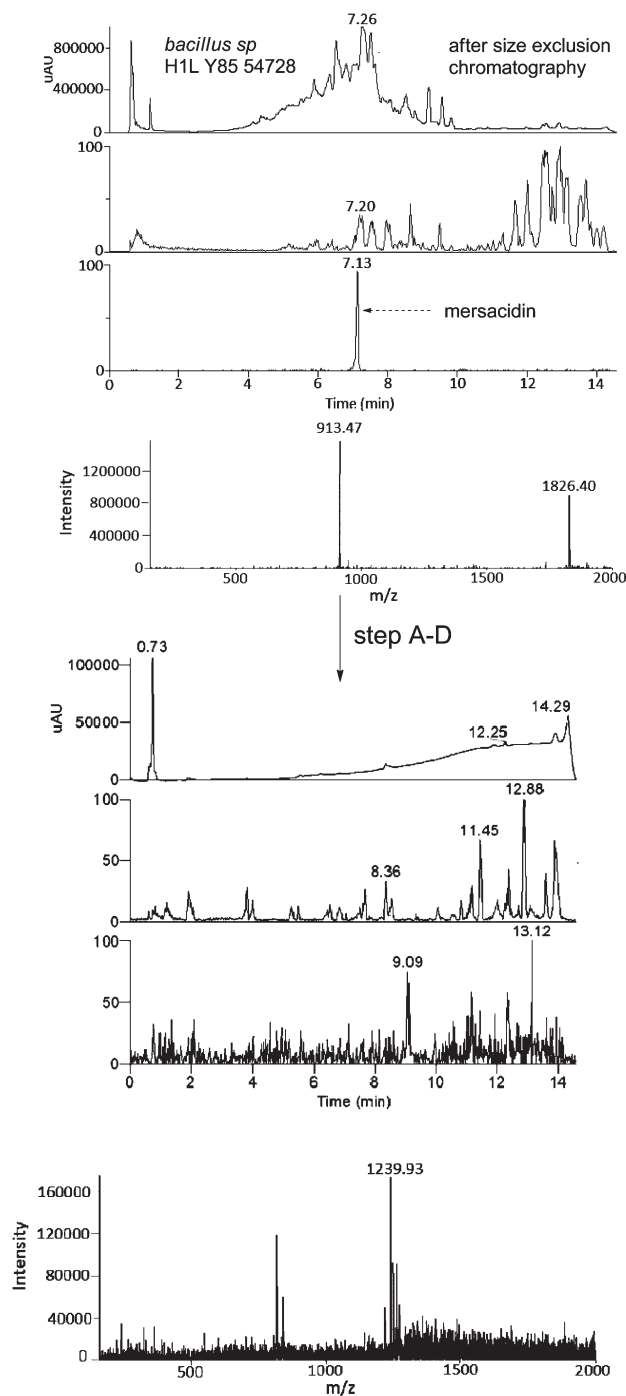
pure mersacidin (Scheme 3) and used the optimized conditions to identify this lantibiotic from *B. subtilis* (Scheme 4). Complete conversion of mersacidin was accomplished in aqueous methanol at pH > 10 at 50 °C for 1 h using 30 equiv. of compound **3** (Scheme 2, step A). LC/MS analysis showed that mersacidin was completely converted into its anticipated products with the addition of one, two and three sulfide-alkyne moieties (HSA) (Fig. S1, ESI†). This is indicated by the ions m/z 1037.00 Da [$M + HSA + Ac + 2H^+$]²⁺, 1139.00 Da [$M + 2HSA + Ac + 2H^+$]²⁺ and 1240.67 Da [$M + 3HSA + Ac + 2H^+$]²⁺. By performing MS/MS sequencing it was found that the single mersacidin modified product is labeled at position 13 in the sequence (Fig. S2, ESI†). The additional acetyl group in the products at the N-terminus is probably obtained by a reaction involving the deacetylation of **3**. In addition, the *in situ* generated reactive intermediate **1** was found to dimerize during the reaction. To prevent labeling *via* S–S bond formation, a reduction step (using DTT) and an alkylation step (using iodoacetamide) were introduced (Scheme 3 step B, LC/MS analysis is shown in Fig. S3, ESI†). Copper(I)-catalyzed azide–alkyne cycloaddition between the alkyne modified mersacidin and biotin–azide **2** (Scheme 3, step C) was complete within 17 h at room temperature according to LC/MS analysis (Fig. S4, ESI†). The corresponding labeled



Scheme 3 Protocol for the two-step labeling of the lantibiotic mersacidin.

products are indicated by ions m/z 1466.53 Da $[M + \text{HSA} + \text{Ac} + \text{Biotin} + 2\text{H}^+]^{2+}$ and 1494.60 Da $[M + \text{HSA} + \text{Ac} + \text{AA} + \text{Biotin} + 2\text{H}^+]^{2+}$. With these products in hand the optimal conditions for the cleavage of the levulinolate-linkage were examined (Fig. S5, ESI†) and subsequently applied by pull down of the labeled products using streptavidin coated magnetic beads followed by treatment of the beads with 0.1 M hydrazine at 37 °C for 4 h (Scheme 3, step D). The resulting solution was characterized with LC/MS. The corresponding products were found as a major (m/z 1240.00 Da $[M + \text{HSA} + \text{Ac} + \text{Biotin-C} + 2\text{H}^+]^{2+}$) and a minor signal (m/z 1268.47 Da $[M + \text{HSA} + \text{Ac} + \text{IAA} + \text{biotin-C} + 2\text{H}^+]^{2+}$, Fig. S6, ESI† HRMS, Fig. S7†).

In the key experiment, *Bacillus* sp. H1L Y 85 54728 was incubated, the supernatant collected and the crude products applied on a size-exclusion chromatography column. As indicated by LC/MS mersacidin is present in the fermented sample along with numerous other components, and the size-exclusion chromatography step thus mainly serves to concentrate the fermentation sample (Scheme 4). The obtained sample was treated with the above described protocol steps A–D involving reaction with *in situ* generated probe **1** at high pH and elevated temperature, followed by S–S bond reduction, alkylation, dialysis, click reaction using probe **2**, pull down with streptavidin-coated magnetic beads and hydrazine cleavage. The resulting products were analyzed with LC/MS. Following this protocol mersacidin was isolated from culture and identified by the mass of the resulting derivative with mass m/z 1239.93 Da $[M + \text{HSA} + \text{Ac} + \text{biotin-}$



Scheme 4 Two-step labeling strategy for the identification of mersacidin from cultured *Bacillus* sp. H1L Y85 54728.

$C + 2H^+]$ (Scheme 4, the *S*-acetamide alkylated product could not be clearly detected).

Conclusion

Two probes were designed and synthesized for a two-step labeling strategy to identify lantibiotic peptides following opening of one (or more) of the characteristic thio-ether linkages. Using this strategy, involving a first step labeling *via* an elimination/1,4-addition reaction, a second step labeling with a click reaction, and pull-down of the modified peptide products using streptavidin beads and hydrazine-induced release, we positively identified the lantibiotic mersacidin in a fermentation sample of *Bacillus* sp. H1L Y85 54728. The concentration of mersacidin in a fermented sample is typically 1–10 mg L⁻¹. Our procedure involves the use of a size-exclusion chromatography step to concentrate the sample. We are currently evaluating the here-described approach to identify unknown lantibiotics from cultures produced by actinomycetes.

Experiment section

General methods

All reagents used were purchased from commercial sources and were used without further purification. Solvents that were used in reactions were stored over 4 Å molecular sieves, except methanol and acetonitrile, which were stored over 3 Å molecular sieves. Molecular sieves were flame dried before use. Tetrahydrofuran was distilled over LiAlH₄ prior to use. The eluents ethyl acetate (EtOAc) and petroleum ether (40–60 °C boiling range) were distilled prior to use. Mersacidin was provided by Novacta (UK). *Bacillus* was cultured in an Innova 4000 incubator shaker. Column chromatography was performed on Screening Devices b.v. Silica Gel, with a particle size of 40–63 µm and a pore diameter of 60 Å. Unless stated otherwise, all reactions were monitored by TLC on Merck aluminum sheets (Silica gel 60 F254). Spots were detected under UV light (254 nm), and/or by spraying with a solution of (NH₄)₆Mo₇O₂₄·4H₂O (25 g L⁻¹) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g L⁻¹) in 10% sulfuric acid, or a solution of KMnO₄ (20 g L⁻¹) and K₂CO₃ (10 g L⁻¹) in water, followed by charring at *ca.* 150 °C. ¹H NMR spectra were recorded at 298 K on a Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are referenced to CDCl₃ (7.26 ppm, CDCl₃) or CD₃OD (3.31 ppm, CD₃OD) or D₂O (4.78 ppm, D₂O). ¹³C NMR APT spectra were recorded at 100 MHz, and chemical shifts are referenced to CDCl₃ (77.23 ppm, CDCl₃) or CD₃OD (48.9 ppm, CD₃OD). ¹H NMR data are reported as though they are first order, and the peak assignments were made by 2D-NMR spectroscopy (¹H–¹H COSY and HMQC). IR spectra were recorded on a Perkin Elmer Paragon 1000 FT-IR Spectrometer. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile 50/50 (v/v) and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution *R* = 60 000 at *m/z* 400 (mass range *m/z* = 150–2000) and dioctylphthalate (*m/z* = 391.28428) as a “lock mass”. The high-resolution mass

spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3 µm C18 50 × 4.60 mm column (detection simultaneously at 214 and 254 nm), coupled to a PE Sciex API 165 mass spectrometer with ESI (System A) or a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200–600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI (System B). The applied buffers were H₂O, ACN and 1.0% aq. TFA. The gradient used was 10% → 90% ACN/0.1% aq. TFA.

S-2-(2-(2-(Prop-2-ynyloxy)ethoxy)ethoxy)ethyl ethanethioate (3). To a stirred solution of triethylene glycol (5.30 g, 35 mmol) and NaH (60% in mineral oil, 1.4 g, 35 mmol) in THF (100 mL) at 0 °C after 30 min was added propargyl bromide (3.80 mL, 80% in toluene, 35 mmol) and stirring was continued for 30 min. The reaction mixture was diluted with ether (200 mL) and washed with water (50 mL), brine (50 mL) and dried over MgSO₄. The organic solution was concentrated and the residue was purified by silica gel column chromatography (EtOAc : petroleum ether, elution with 1 : 2 → 3 : 1 v/v) to give 2.43 g of a colorless oil. 1.75 g (9.3 mmol) of this intermediate was dissolved in CH₂Cl₂ (20 mL), treated with Et₃N (3 mL) and subsequently tosyl chloride (2.30 g, 12.1 mmol) was added slowly at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and at room temperature for 1 h, then poured onto water (10 mL), and extracted with ether (3 × 20 mL). The organic layer was washed with brine (10 mL), dried with MgSO₄ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc : petroleum ether, elution with 1 : 10 → 1 : 1 v/v) to give 2.53 g of a colorless oil. This intermediate was dissolved in DMF (20 mL) and treated with KSAC (1.69 g, 14.8 mmol). The solution was stirred for 4 h at 70 °C, and concentrated under diminished pressure. The remaining reddish oil was purified by silica gel column chromatography (EtOAc : petroleum ether, elution with 1 : 10 to 4 : 1 v/v) to give **3** (1.5 g, 25% yield in three steps) as a colorless oil; *R*_f 0.55 (EtOAc : petroleum ether 1 : 2 v/v); ¹H NMR (400 MHz, CDCl₃) δ 4.21 (d, *J* = 2.4 Hz, 2 H), 3.72–3.63 (m, 8 H), 3.60 (t, *J* = 6.4 Hz, 2 H), 3.10 (t, *J* = 6.4 Hz, 2 H), 2.44 (t, *J* = 2.4 Hz, 1 H), 2.34 (s, 3 H); ¹³C NMR (100 MHz, CDCl₃) δ 195.74, 79.83, 74.71, 70.73, 70.65, 70.48, 69.94, 69.28, 58.59, 30.76, 29.02; IR neat (cm⁻¹) 3251.9, 2867.7, 1686.1, 1353.5, 1096.7, 1032.7, 626.2; HRMS *m/z* calculated for (M + H⁺) C₁₁H₁₉O₄S 2479.0926. Found: 2479.0999.

Ethyl 5-azido-4-oxopentanoate. To a solution of ethyl 5-bromo-4-oxopentanoate¹⁶ (0.35 g, 1.57 mmol) in THF (5 mL) was added a solution of NaN₃ (0.20 g, 3.14 mmol) in H₂O (3 mL) at 0 °C. Stirring was continued for 1 h at room temperature and then the reaction mixture was diluted with H₂O (10 mL) and extracted with EtOAc (3 × 10 mL). The organic layer was washed with brine (10 mL), dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc : petroleum ether 1 : 20 v/v) to give ethyl 5-azido-4-oxopentanoate (0.27 g, 95%) as a slightly yellow oil; *R*_f 0.5 (EtOAc : petroleum ether 1 : 8 v/v); ¹H NMR (400 MHz, CDCl₃) δ 4.13 (q, *J* = 7.2 Hz, 2 H), 4.04 (s, 2 H),

2.72 (ddd, $J = 8.8, 6.8, 1.7$ Hz, 2 H), 2.66 (ddd, $J = 8.8, 6.8, 1.7$ Hz, 2 H), 1.26 (t, $J = 7.2, 3$ H); ^{13}C NMR (100 MHz, CDCl_3) δ 203.08, 172.31, 60.89, 57.49, 34.40, 27.85, 14.11; IR neat (cm^{-1}) 2984.1, 2103.4, 1727.5, 1203.0; HRMS m/z calculated for ($\text{M} + \text{H}^+$) $\text{C}_7\text{H}_{12}\text{N}_3\text{O}_3$ 186.0800. Found: 186.2195.

Ethyl 4-oxo-5-(6-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)hexanamido)pentanoate (5). Biotin-AHA-NHS **4**¹⁷ (0.1 g, 0.22 mmol) and ethyl 5-azido-4-oxopentanoate (49 mg, 0.26 mmol) were dissolved in methanol (20 mL). To this solution was added 10% Pd/C (5 mg) and stirring was continued for 4 h under a hydrogen atmosphere. The solution was concentrated and the residue was purified by silica gel column chromatography (CH_2Cl_2 : CH_3OH , elution with 30:1 $\rightarrow$ 7:1, v/v) to give compound **5** (80 mg, 73%) as a white amorphous solid; R_f 0.19 (CH_2Cl_2 : CH_3OH 10:1 v/v); ^1H NMR (400 MHz, CD_3OD) δ 4.52 (dd, $J = 7.8, 5.0$ Hz, 1 H), 4.33 (dd, $J = 7.8, 4.5$ Hz, 1 H), 4.13 (q, $J = 7.1$ Hz, 2 H), 4.09 (s, 2 H), 3.26–3.18 (m, 1 H), 2.96 (dd, $J = 12.7, 5.0$ Hz, 1 H), 2.79 (t, $J = 6.4$ Hz, 2 H), 2.73 (d, $J = 12.7$ Hz, 1 H), 2.61 (t, $J = 6.4$ Hz, 1 H), 2.30 (t, $J = 7.4$ Hz, 1 H), 2.22 (t, $J = 7.3$ Hz, 1 H), 1.78–1.39 (m, 14 H), 1.26 (t, $J = 7.1$ Hz, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ 205.03, 175.04, 174.58, 172.95, 164.70, 61.99, 60.34, 60.23, 55.62, 48.33, 39.67, 38.80, 35.42, 35.18, 33.83, 28.69, 28.38, 28.10, 27.37, 26.08, 25.52, 25.11, 13.10; IR neat (cm^{-1}) 3314.7, 2936.0, 1710.0, 1640.8, 1538.0, 1423.8, 1197.5. HRMS m/z calculated for ($\text{M} + \text{H}^+$) $\text{C}_{23}\text{H}_{39}\text{N}_4\text{O}_6\text{S}$ 499.2512. Found: 499.2582.

4-Oxo-5-(6-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)hexanamido)pentanoic acid (6). To a solution of ester **5** (80 mg, 0.16 mmol) in methanol (10 mL) was added NaOH (2 M, 1.5 mL). The solution was stirred at room temperature for 3 h, and then adjusted to pH 5 by adding HCl (2 M). The solution was concentrated under reduced pressure and after adding H_2O (5 mL) sonicated for 5 min at room temperature. The resulting white solid was filtered to obtain the product **6** (65 mg, 87%) without the need for further purification; ^1H NMR (400 MHz, DMSO) δ 12.15 (s, 1 H), 8.11 (s, 1 H), 7.74 (s, 1H), 6.44 (s, 1 H), 6.37 (s, 1 H), 4.31 (s, 1 H), 4.13 (s, 1 H), 3.92 (s, 2 H), 3.34 (s, 4 H), 3.10–2.04 (m, 13 H), 1.60–1.26 (m, 12 H); ^{13}C NMR (100 MHz, DMSO) δ 206.19, 174.11, 172.91, 172.23, 163.17, 61.49, 59.64, 55.89, 48.77, 40.37, 38.74, 35.67, 35.45, 34.43, 29.44, 28.68, 28.49, 27.87, 26.55, 25.79, 25.43; IR neat (cm^{-1}) 3315.7, 2939.9, 1699.8, 1638.9, 1537.8, 1264.0; HRMS m/z calculated for ($\text{M} + \text{H}^+$) $\text{C}_{21}\text{H}_{35}\text{N}_4\text{O}_6\text{S}$ 471.2199. Found: 471.2272.

***N*-(2-(2-(2-Azidoethoxy)ethoxy)ethyl)-*N*-(4-hydroxy-3,5-diisopropylbenzyl)acetamide (7).** 1,2-Bis(2-azidoethoxy)ethane (0.24 g, 1.29 mmol) was dissolved in a mixture of THF and H_2O (5 mL, 4:1 v/v). To this solution was added PPh_3 (0.34 g, 1.29 mmol) and stirring was continued for 17 h. The solution was diluted with H_2O (10 mL) and washed with ether (2 $\times$ 10 mL). The water layer was concentrated under reduced pressure and the crude product was dissolved in methanol (10 mL). The reaction mixture was cooled to 0 $^\circ\text{C}$ and 4-hydroxy-3,5-diisopropylbenzaldehyde (0.16 g, 0.77 mmol) was added. The solution was adjusted to pH 8 with the aid of a 5 M NaOH solution. After stirring for 2 h at room temperature,

the reaction mixture was cooled to -10 $^\circ\text{C}$ and NaBH_4 (30 mg, 0.77 mmol) was added. The reaction mixture was stirred for 30 min, then allowed to warm to room temperature and stirring was continued for another 30 min. The reaction mixture was then adjusted to pH 6 by adding acetic acid. To the solution was added acetic anhydride (5 mL) and stirring was continued for 2 h. The reaction mixture was diluted with ether (20 mL) and washed with 1 M NaOH (5 mL), water (5 mL), brine (5 mL) and dried over MgSO_4 . The organic layer was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (EtOAc :petroleum ether, elution with 1:2 $\rightarrow$ 3:1 v/v) to give compound **7** (0.27 g, 87%) as a colorless oil; R_f 0.29 (EtOAc :petroleum ether 1:1 v/v); ^1H NMR (400 MHz, CDCl_3) δ 6.93, 6.83 (2 $\times$ s, 2 H), 5.52, 5.38 (2 $\times$ s, 1 H), 4.58 (s, 2 H), 3.76–3.72 (m, 1 H), 3.68–3.52 (m, 9 H), 3.41–3.36 (m, 2 H), 3.18 (sept, $J = 7.0$ Hz, 2 H), 2.20, 2.15 (2 $\times$ s, 2 H), 1.23 (d, $J = 7.0$ Hz, 12 H); ^{13}C NMR (100 MHz, CDCl_3) δ 171.27 (b), 171.12 (a), 149.45 (b), 149.39 (a), 134.53 (2 C, b), 134.07 (2 C, a), 129.17(a), 128.26 (b), 123.45 (2 C, a), 121.32 (2 C, b), 70.98, 70.87, 70.75 (b), 70.70 (a), 70.42 (b), 70.40(a), 69.39 (a), 69.22 (b), 53.63 (a), 51.00 (b), 47.81 (a), 45.99 (b), 27.02 (2 C), 22.68 (4 C), 21.66 (a), 21.63 (b); R_f 0.29 (EtOAc :petroleum ether 1:1 v/v); IR neat (cm^{-1}) 3342.1, 2960.3, 2111.9, 1624.3, 1471.9, 1287.7, 1202.7, 1123.5, 784.1. HRMS m/z calculated for ($\text{M} + \text{H}^+$) $\text{C}_{21}\text{H}_{35}\text{N}_4\text{O}_4$ 407.2580. Found: 407.2649.

4-((*N*-(2-(2-(2-Azidoethoxy)ethoxy)ethyl)acetamido)methyl)-2,6-diisopropylphenyl 4-oxo-5-(6-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)hexanamido)pentanoate (2). Acid **6** (18 mg, 0.038 mmol), phenol **7** (31 mg, 0.078 mmol) and EDC (15 mg, 0.078 mmol) were taken up in DMF (3 mL). To the suspension was added DMAP (5 mg, 0.04 mmol) and stirring was continued for 16 h at 70 $^\circ\text{C}$. The reaction mixture was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (CH_2Cl_2 : CH_3OH , elution with 30:1 $\rightarrow$ 7:1 v/v) to give product **2** (4.8 mg, 15%) as a colorless oil; R_f 0.6 (CH_2Cl_2 : CH_3OH 8:1 v/v); ^1H NMR (400 MHz, CD_3OD) δ 7.07, 6.99 (2 $\times$ s, 2 H), 4.72, 4.63 (2 $\times$ s, 2 H), 4.48 (dd, $J = 7.9, 4.7$ Hz, 1 H), 4.29 (dd, $J = 7.9, 4.3$ Hz, 1 H), 4.08 (s, 2 H), 3.66–3.52 (m, 8 H), 3.37–3.34 (m, 2 H), 3.22–3.16 (m, 3 H), 2.99–2.88 (m, 5 H), 2.70 (d, $J = 12.7$ Hz, 1 H), 2.28 (t, $J = 7.4$ Hz, 2 H), 2.24, 2.12 (2 $\times$ s, 2 H), 2.19 (t, $J = 7.3$ Hz, 2 H), 1.75–1.36 (m, 14 H), 1.60 (d, $J = 6.5$ Hz, 12 H); ^{13}C NMR (100 MHz, CD_3OD) δ 206.04, 176.50, 176.01, 174.16, 173.40, 166.12, 146.17 (a), 146.13 (b), 134.54 (2 C, a), 142.07 (2 C, b), 137.23 (b), 126.53 (a), 124.66 (2 C, b), 122.98 (2 C, a), 71.79, 71.67, 71.53 (b), 71.51 (a), 71.23 (b), 71.20 (a), 70.32 (a), 70.12 (b), 63.42, 61.65, 57.06, 54.27 (a), 51.82, 49.80 (b), 49.71 (a), 47.16 (b), 41.10, 40.23, 36.85, 36.60, 35.16, 34.72, 30.12, 29.82, 29.53, 28.63 (4 C), 27.51, 26.96, 26.57, 21.85 (2 C), 21.75; IR neat (cm^{-1}) 3290.0, 2926.3, 2109.1, 1695.9, 1653.9, 1559.9, 1458.0, 1142.0, 668.0. HRMS m/z calculated for ($\text{M} + \text{H}^+$) $\text{C}_{42}\text{H}_{67}\text{N}_8\text{O}_9\text{S}$ 859.4673. Found: 859.4751.

Bacterial strain culture conditions

For the preparation of culture stocks, the *Bacillus* sp. H1L Y85 54728 was grown in a nutrient broth at 30 $^\circ\text{C}$ for 24 h. A colony

was transferred from the solid medium to a 3 mL tryptone soy broth and incubated for 24 h at 200 rpm and 30 °C. The culture was diluted 1 : 1 with sterile 20% glycerol and stored at –80 °C. The seed culture (0.1 mL) was inoculated into 50 mL mersacidin production medium 2 × BPM ((NH₄)₂SO₄ (50 mM), MgSO₄ (2 mM), CaCl₂ (1 mM), FeSO₄ (0.35 mM), MnSO₄ (1 mM), Tris-maleate buffer pH 7 (0.1 M), glucose (0.4 M), potassium phosphate buffer pH 7 (0.04 M)) in a 200 mL spring flask and was shaken at 30 °C and 200 rpm for 4–5 days. The culture (50 mL) was centrifuged at 5000 rpm for 3 × 10 min. The supernatant was collected and lyophilized. The foamy residue was treated with methanol (50 mL) and sonicated for 10 min. The suspension was centrifuged and the supernatant was collected and concentrated to a small volume and applied on a Sephadex LH 20 column using methanol as an eluent. The high molecular weight fractions were collected, concentrated under reduced pressure to give a sample of 8 mg. LC/MS analysis (Scheme 3) showed that mersacidin (C₈₀H₁₂₀N₂₀O₂₁S₄ calculated mass 1824.78) was present in the sample in a small amount, indicated by ions m/z 913.27 Da [M + 2H]²⁺ and 1826.47 Da [M + H]⁺, retention time 7.18 min.

Protocol for the two-step labeling, pull-down, release and identification of the lantibiotic mersacidin from culture

Step A: opening of the thioether linkage(s) and labeling. To the obtained cultured sample (3 mg) in a 0.5 mL Eppendorf vial were added thio-alkyne **3** (2 mg, 8.1 μmol), H₂O (10 μL) and EtOH (20 μL). The reaction mixture was carefully flushed with argon for 2 min and a 1 M NaOH solution (8.1 μL) was added. The reaction mixture was shaken vigorously (vortex) and stirred at 50 °C for 1 h and subsequently quenched with a 1 M acetic acid solution (8.1 μL). The reaction mixture was shaken vigorously (vortex) and centrifuged for 3 min at 5000 rpm using an Eppendorf centrifuge. The supernatant was discarded to obtain a white precipitate.

Step B: reduction of S–S linkages and reaction of the free thiols with iodoacetamide. The white precipitate was dissolved in EtOH and H₂O (40 μL, 1 : 1 v/v) and a solution of DTT (60 μL) as a 0.2 M solution in a 1 : 1 mixture of EtOH and 50 mM aq. (NH₄)₂CO₃ was added. The Eppendorf vial was incubated for 30 min at 37 °C using a water bath. Subsequently, iodoacetamide (60 μL) as a 0.6 M solution in a 1 : 1 mixture of EtOH and 50 mM aq. (NH₄)₂CO₃ was added and the reaction mixture was shaken for 30 min at room temperature in the dark. The reaction mixture was dialysed with MWCO 1200 cellulose tubing in a mixture of EtOH and H₂O (1 : 1, v/v) for 24 h. The resulting solution was collected and concentrated under reduced pressure in an Eppendorf centrifuge.

Step C: click reaction and pull-down using magnetic streptavidin beads. The residue was dissolved in a mixture of *t*-butanol and ethanol (10 μL, 2 : 1 v/v). The solution was flushed carefully with argon and a solution of the biotin probe **2** (0.5 mg, 0.58 μmol) in *t*-butanol and ethanol (5 μL, 2 : 1 v/v) was added. To the stirred reaction mixture were added CuSO₄ (0.09 mg, 0.58 μmol) and sodium ascorbate (0.12 mg, 0.58 μmol). Stirring was continued for 16 h followed by concentration of the reaction

mixture. The residue was dissolved in methanol (20 μL) and pull-down (PD) buffer (50 mM Tris·HCl pH 7.5, 150 mM NaCl) (1 mL) to afford a clear solution. The solution was incubated with 50 μL pre-washed MyOne T1 Streptavidin grafted magnetic beads (Invitrogen) at 4 °C with vigorous shaking for 1 h. The beads were stringently washed with 300 μL PD buffer with 0.1% SDS, 2 × 300 μL PD buffer, 2 × 300 μL wash buffer I, 2 × 300 μL wash buffer II and 2 × 300 μL water.

Step D: release from the beads and analysis. To the biotin captured magnetic beads was added hydrazine (300 μL, 100 μM) and the sample was shaken at 37 °C for 2 h (or at room temperature for 17 h). The supernatant was removed and lyophilized to give a white powder, which was dissolved in a mixture of *t*-butanol, acetonitrile and water (40 μL, 1 : 1 : 1, v/v) that was analyzed with LC/MS. From this sample the modified mersacidin product was identified having a retention time of 9.05 min and m/z 1239.93 Da [M + HSA + Ac + biotin-C + 2H]²⁺ (Scheme 3).

Analysis of the protocol

Opening of the thioether linkage(s) and labeling. A mixture of pure mersacidin (0.25 mg, 0.137 μmol) kindly provided by the company Novacta and thio-alkyne **3** (1.01 mg, 4.11 μmol, 30 equiv.), H₂O (10 μL), EtOH (10 μL) in a 0.5 mL Eppendorf vial was carefully flushed with argon for 2 min and 1 M NaOH (4.1 μL, 4.11 μmol, 30 equiv.) was added. The mixture was stirred at 50 °C for 1 h. A solution of 1 M acetic acid (4.1 μL) was added to the reaction mixture. The solution was vortexed and centrifuged. The supernatant was discarded and the white precipitate was dissolved in EtOH and H₂O (40 μL, 1 : 1, v/v). The reaction mixture was analyzed by LC/MS. From the spectrum shown in Fig. S1,† mersacidin was modified at one, two and three positions in the sequence. This is indicated by the ions m/z 1037.00 Da [M + HSA + Ac + 2H]²⁺, C₉₁H₁₄₀N₂₀O₂₅S₅²⁺, calculated mass 1036.44, 1139.00 Da [M + 2HSA + Ac + 2H]²⁺, C₁₀₀H₁₅₆N₂₀O₂₈S₆²⁺, calculated mass 1138.48 and 1240.67 Da [M + 3HSA + Ac + 2H]²⁺, C₁₀₉H₁₇₂N₂₀O₃₁S₇²⁺, calculated mass 1240.52, within 7.33–9.38 min retention times. The modified mersacidin product [M + HSA + Ac + 2H]²⁺ was sequenced to show that the modification site is at position 13 in the sequence (Fig. S2†). From the LC/MS data it was also observed that the reactive compound **1**, which is formed *in situ* via deacetylation of **3** in the strong basic medium, also formed a dimer during the reaction conditions (ion m/z 406.60 Da, C₁₈H₃₁O₆S₂⁺, calculated mass 407.15). To exclude labeling via the formation of S–S linkages we included a reduction and alkylation step in our protocol.

Reduction of S–S linkages and reaction of the free thiols with iodoacetamide. The above obtained reaction mixture was treated with a solution of 0.2 M DTT (40 μL) in a 1 : 1 mixture of EtOH and 50 mM aq. (NH₄)₂CO₃ for 30 min at 37 °C, and was alkylated with iodoacetamide (40 μL, 0.6 M in a 1 : 1 mixture of EtOH and 50 mM aq. (NH₄)₂CO₃) for 30 min at room temperature in the dark. The mixture was dialysed with MWCO 1200 cellulose tubing in a mixture of EtOH and H₂O (1 : 1, v/v) for 24 h. The resulting solution was collected and analyzed with

LC/MS (Fig. S3†). The spectrum showed that the thiol groups of the thioether opened and modified mersacidin reacted with the iodoacetamide as indicated by the ions m/z 1065.80 Da $[M + \text{HSA} + \text{Ac} + \text{IAA} + 2\text{H}^+]^{2+}$, $\text{C}_{93}\text{H}_{143}\text{N}_{21}\text{O}_{26}\text{S}_5^{2+}$, calculated mass 1064.95, rt 7.96 min; 1167.73 Da $[M + 2\text{HSA} + \text{Ac} + \text{IAA} + 2\text{H}^+]^{2+}$, $\text{C}_{102}\text{H}_{159}\text{N}_{21}\text{O}_{29}\text{S}_6^{2+}$, calculated mass 1166.99, rt 8.54 min. The product with ion 1037.40 Da $[M + \text{HSA} + \text{Ac} + 2\text{H}^+]^{2+}$, rt 8.25, 8.48 min, did not change.

Click reaction. Pure mersacidin (2.1 mg, 1.15 μmol) was labeled as described using compound **3** (8.5 mg, 34.5 μmol) under basic conditions 1 M NaOH (34.5 μmol), reduced with 0.2 M DTT (400 μL) in a 1 : 1 mixture of EtOH and 50 mM aq. $(\text{NH}_4)_2\text{CO}_3$, alkylated with iodoacetamide (400 μL) 0.6 M in a 1 : 1 mixture of EtOH and 50 mM aq. $(\text{NH}_4)_2\text{CO}_3$ and dialysed. The obtained reaction mixture was dissolved in a mixture of *t*-butanol and ethanol (30 μL , 2 : 1, v/v). The solution was flushed carefully with argon for 2 min and a solution of the biotin probe **2** (0.98 mg, 1.15 μmol) in *t*-butanol and ethanol (10 μL , 2 : 1 v/v), CuSO_4 (0.18 mg, 1.15 μmol) and sodium ascorbate (0.23 mg, 1.15 μmol) were added. The reaction mixture was stirred for 16 h at room temperature and analyzed by LC/MS. The corresponding 2-step labeled products are indicated by ions m/z 1466.53 Da $[M + \text{HSA} + \text{Ac} + \text{Biotin} + 2\text{H}^+]^{2+}$, $\text{C}_{133}\text{H}_{206}\text{N}_{28}\text{O}_{34}\text{S}_6^{2+}$, calculated mass 1465.67, with rt 8.31 min, 1494.60 Da $[M + \text{HSA} + \text{Ac} + \text{IAA} + \text{Biotin} + 2\text{H}^+]^{2+}$, $\text{C}_{135}\text{H}_{209}\text{N}_{29}\text{O}_{35}\text{S}_6^{2+}$, calculated mass 1494.18, with rt 7.94 min (Fig. S4†).

Reactivity of the cleavable linker. The biotin labeled mersacidin dissolved in methanol (21 μL) was divided over six 0.5 mL Eppendorf vials and two different concentrations of hydrazine (0.05 M, 0.1 M) were added and each of the two sets was stirred at room temperature, 37 °C and 50 °C, respectively. The reactions were monitored by LC/MS. It was found that the samples using 0.1 M hydrazine, at room temperature for 17 h or at 37 °C for 4 h, gave complete conversion, as shown in Fig. S5.† The cleaved products were found to be ions m/z 1239.93 Da $[M + \text{HSA} + \text{Ac} + \text{biotin-C} + 2\text{H}^+]^{2+}$ with rt 9.00 min, 1268.93 Da $[M + \text{HSA} + \text{Ac} + \text{IAA} + \text{biotin-C} + 2\text{H}^+]^{2+}$ with rt 8.53 min.

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