nature chemistry

SUPPLEMENTARY INFORMATION

https://doi.org/10.1038/s41557-019-0256-3

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Identification of autoinducing thiodepsipeptides from staphylococci enabled by native chemical ligation

Bengt H. Gless[1], Martin S. Bojer[2], Pai Peng[2], Mara Baldry[2], Hanne Ingmer[2] and Christian A. Olsen[1]*

[1]Center for Biopharmaceuticals, Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark. [2]Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark. *e-mail: cao@sund.ku.dk

Supplementary Information

Identification of autoinducing thiodepsipeptides from staphylococci enabled by native chemical ligation

Bengt H. Gless[1], Martin S. Bojer[2,3], Pai Peng[2], Mara Baldry[2], Hanne Ingmer[2,3], Christian A. Olsen[1]*

[1]Center for Biopharmaceuticals and Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100, Copenhagen, Denmark.

[2]Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, DK-1870 Frederiksberg C, Denmark.

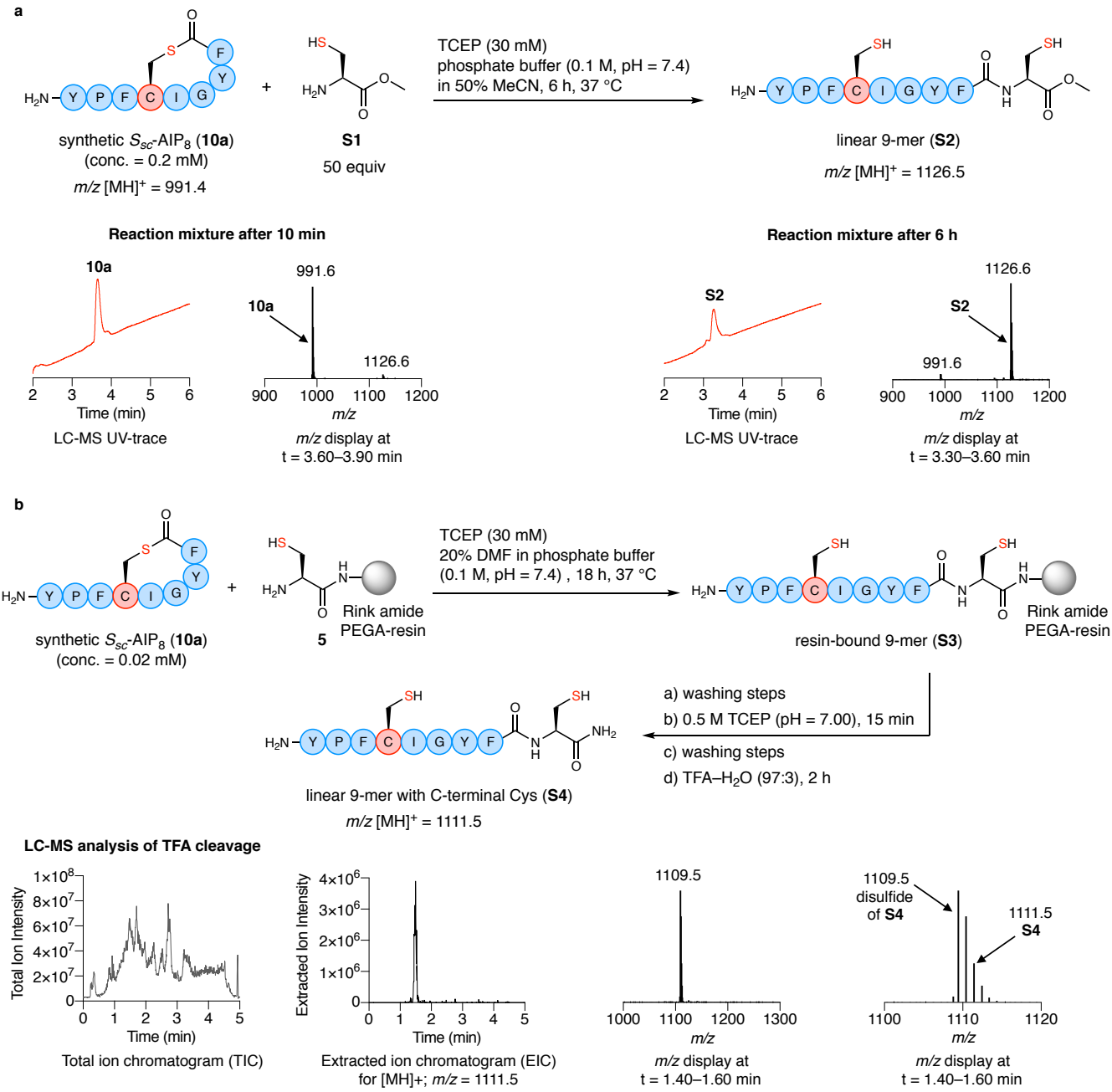
[3]Centre for Bacterial Stress Response and Persistence, University of Copenhagen, DK-2200, Copenhagen, Denmark.

*Correspondence to: cao@sund.ku.dk

Table of Contents

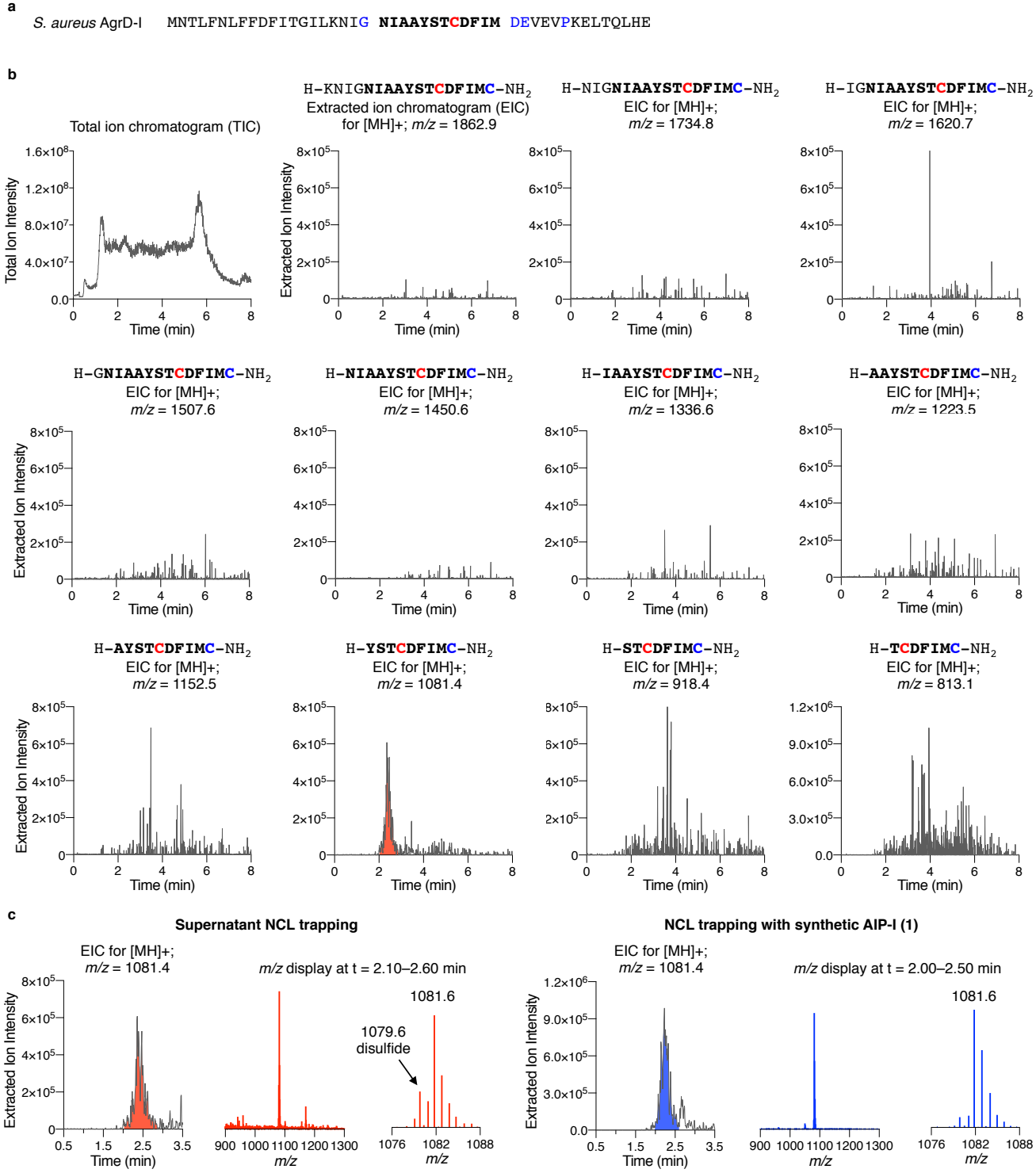
Supplementary figures

Development of native chemical ligation trapping with synthetic AIPs

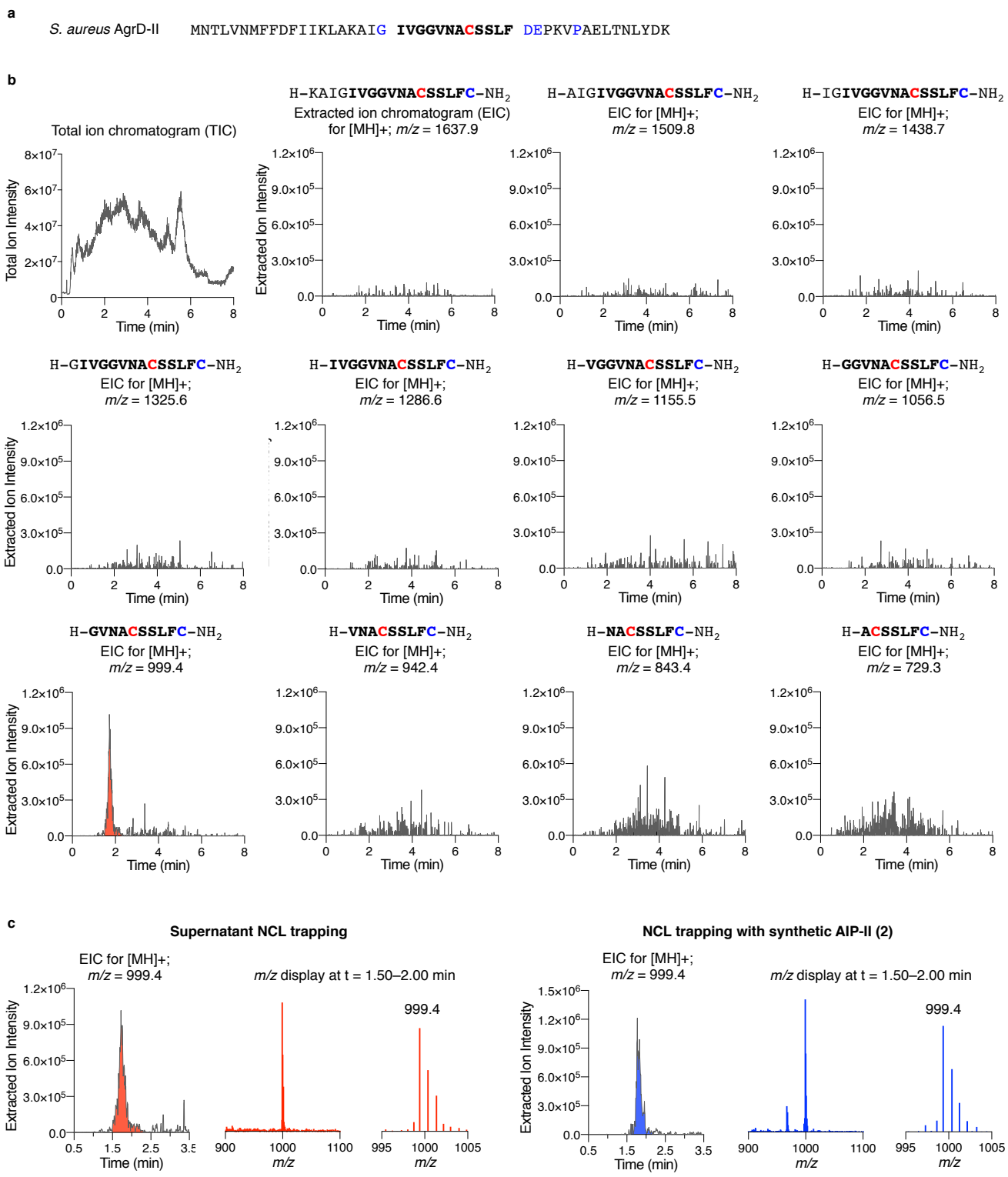


Supplementary Figure 1. Development of native chemical ligation (NCL) trapping with synthetic AIPs. a, NCL of S_{sc}-AIP$_8$ (**10a**) and H-Cys-OMe (**S1**) (50 equiv) in the presence of TCEP (30 mM) at pH = 7.4 went to completion after 6 h. **b**, NCL trapping of S_{sc}-AIP$_8$ (**10a**). The AIP **10a** (conc. = 0.02 mM) was trapped using Cys-Rink amide PEGA resin (**5**) (50 equiv) in 20% DMF in phosphate buffer in the presence of TCEP (30 mM) overnight at 37 °C to give resin-bound linear 9mer peptide (**S3**). The peptidyl-resin **S3** was washed a) 3 × DMF, 3 × MeOH, 3 × H_2O, b) treated with a TCEP solution (0.5 M) for 15 min, washed again c) 3 × H_2O, 3 × MeOH, 3 × CH_2Cl_2 and dried under suction. The dried resin **S3** was treated with a cleavage cocktail (TFA–H_2O, 97:3) for 2 h to release the linear 9-mer peptide with a C-terminal cysteine residue (**S4**). The TFA was evaporated and the remaining residue taken up in 50% MeCN in H_2O (100 µL) and analysed by LC-MS. The extracted ion chromatogram (EIC) for *m/z* [MH]$^+$ = 1111.5 of **S4** showed a strong signal at t_R = 1.50 min and the corresponding *m/z* of **S4** as well as the *m/z* of the internal disulfide of **S4** were the only signals present at t_R = 1.40–1.60 min.

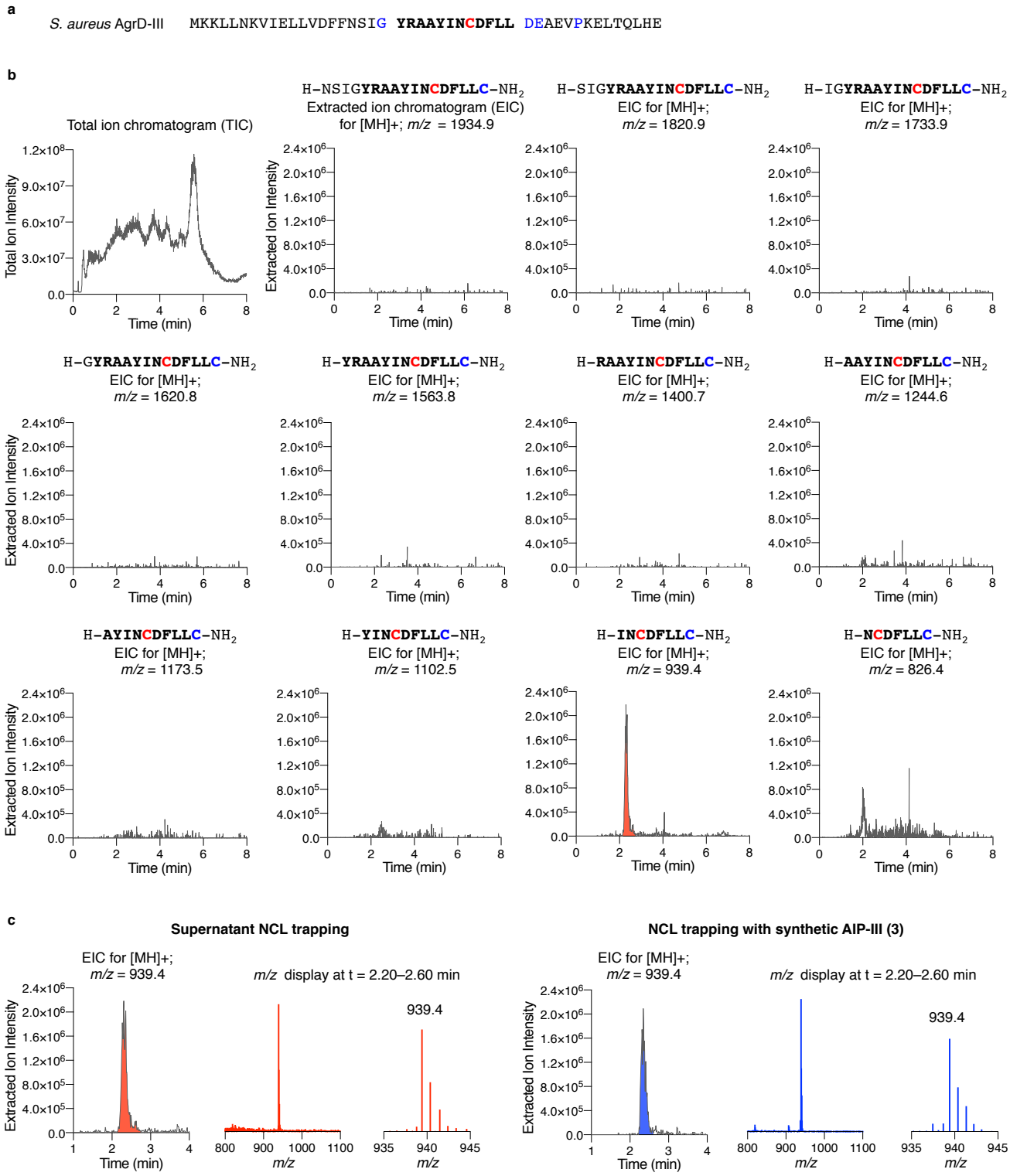
LC-MS traces of sequence-guided identification of AIPs



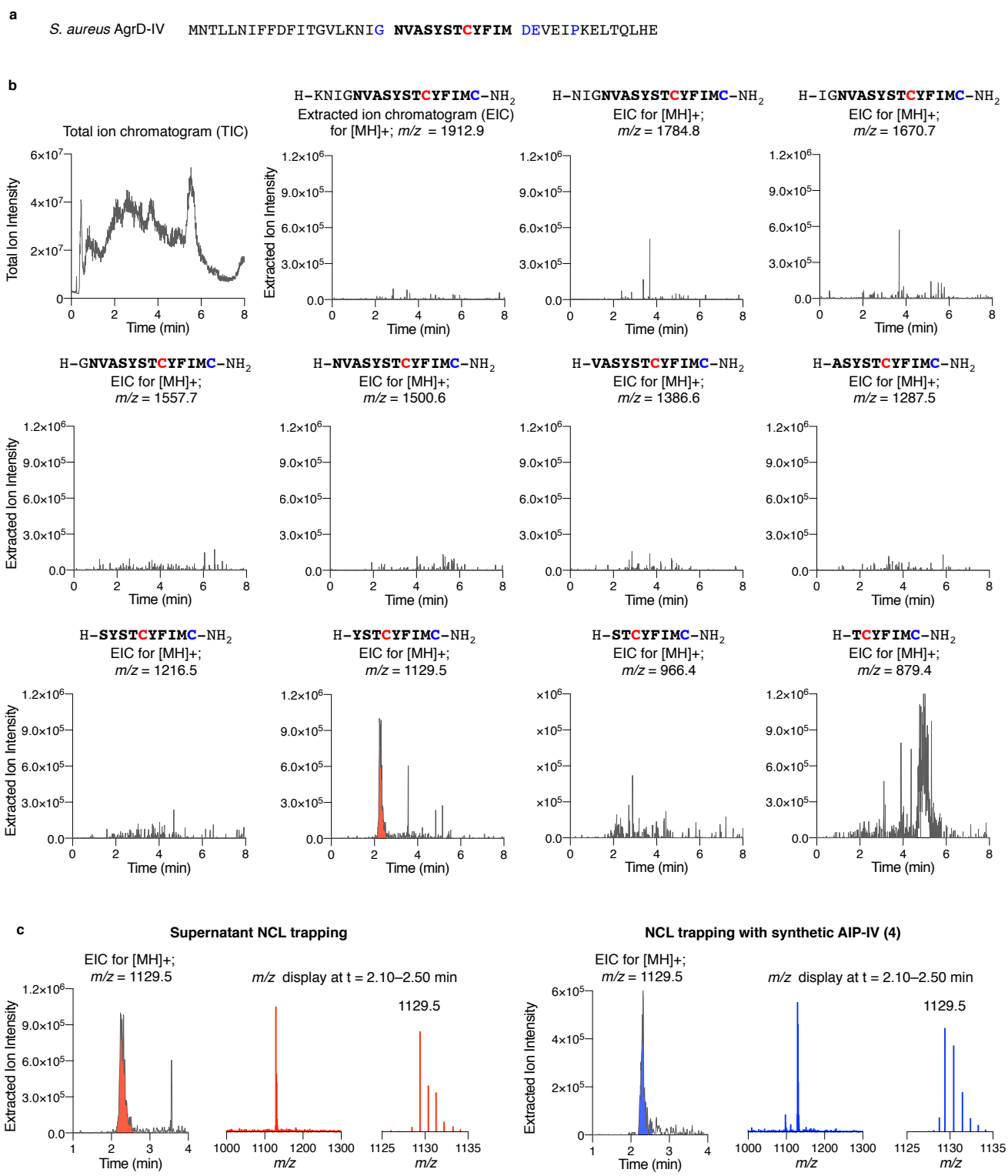
Supplementary Figure 2. Sequence-guided identification of *S. aureus* AIP-I (1) from bacterial supernatant. **a**, AgrD sequence of *S. aureus agr*-I (UniProtKB: Q53643). **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped AIP-I (**1**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear AIP-I sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic AIP-I (**1**) confirms the identity of trapped AIP-I (**1**) from bacterial supernatant.



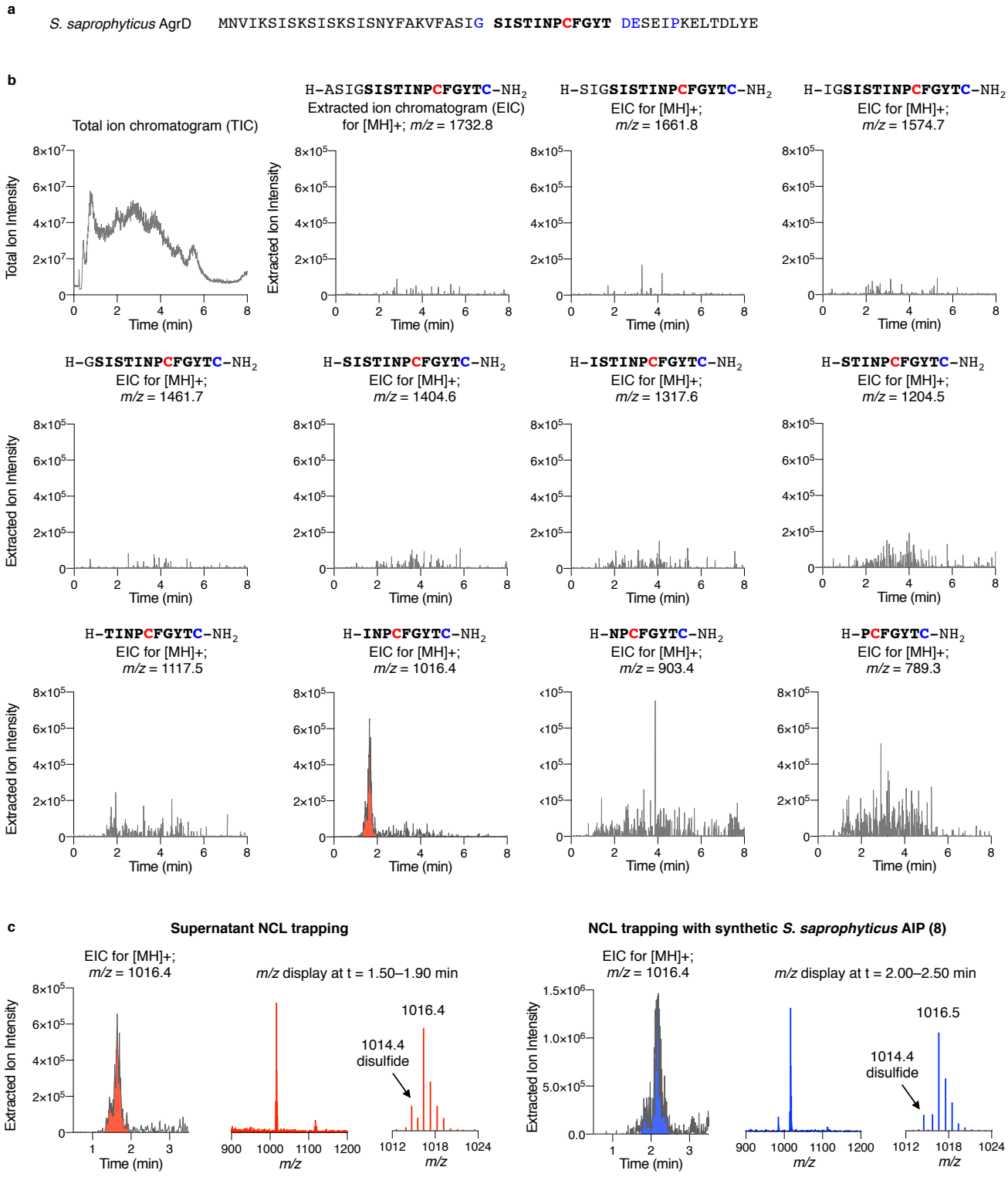
Supplementary Figure 3. Sequence-guided identification of *S. aureus* AIP-II (2) from bacterial supernatant. a, AgrD sequence of *S. aureus agr*-II (UniProtKB: O33586). **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped AIP-II (**2**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear AIP-II sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic AIP-II (**2**) confirms the identity of trapped AIP-II (**2**) from bacterial supernatant.



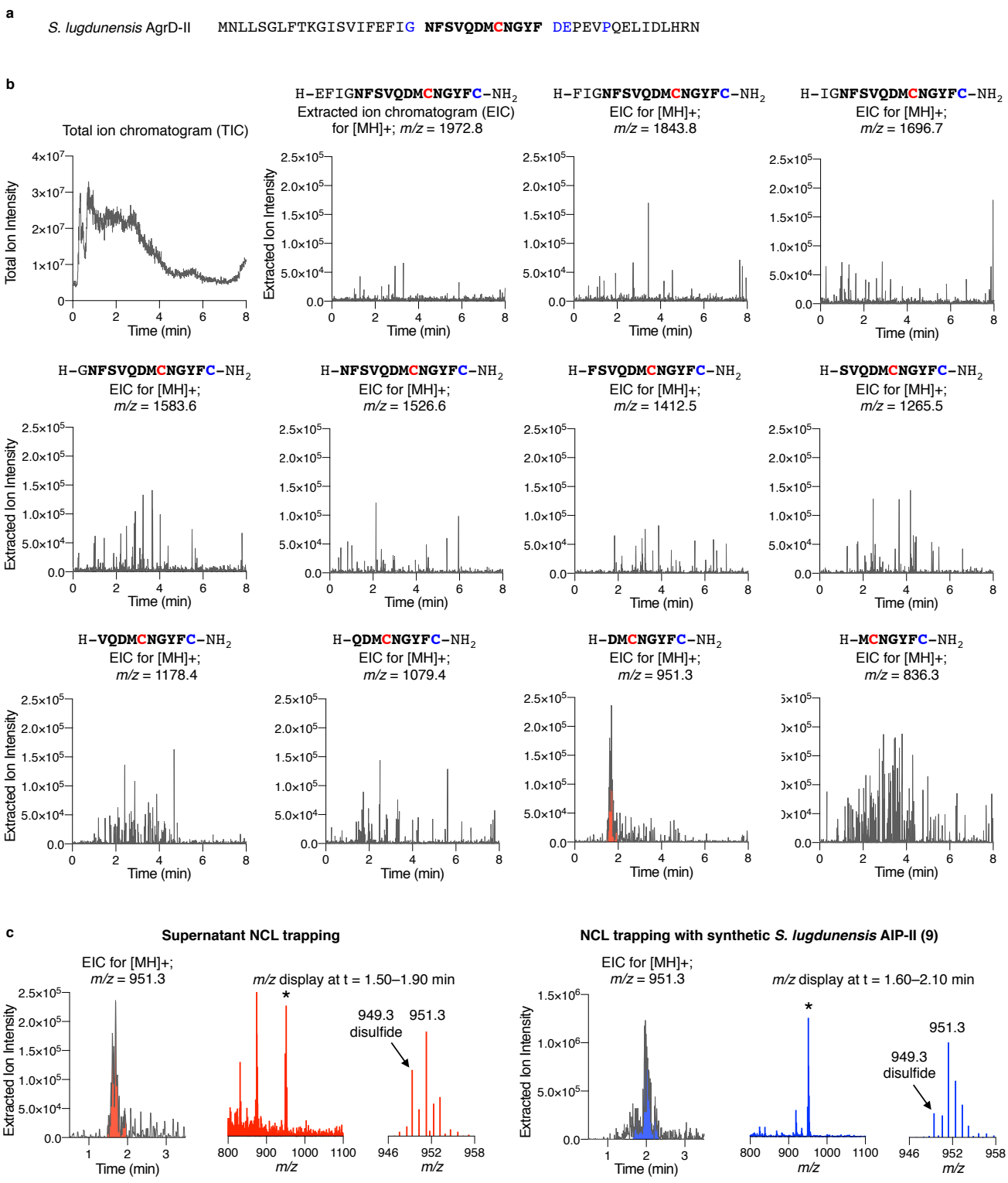
Supplementary Figure 4. Sequence-guided identification of *S. aureus* AIP-III (3) from bacterial supernatant. a, AgrD sequence of *S. aureus agr*-III (UniProtKB: O33589). **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped AIP-III (**3**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear AIP-III sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic AIP-III (**3**) confirms the identity of trapped AIP-III (**3**) from bacterial supernatant.



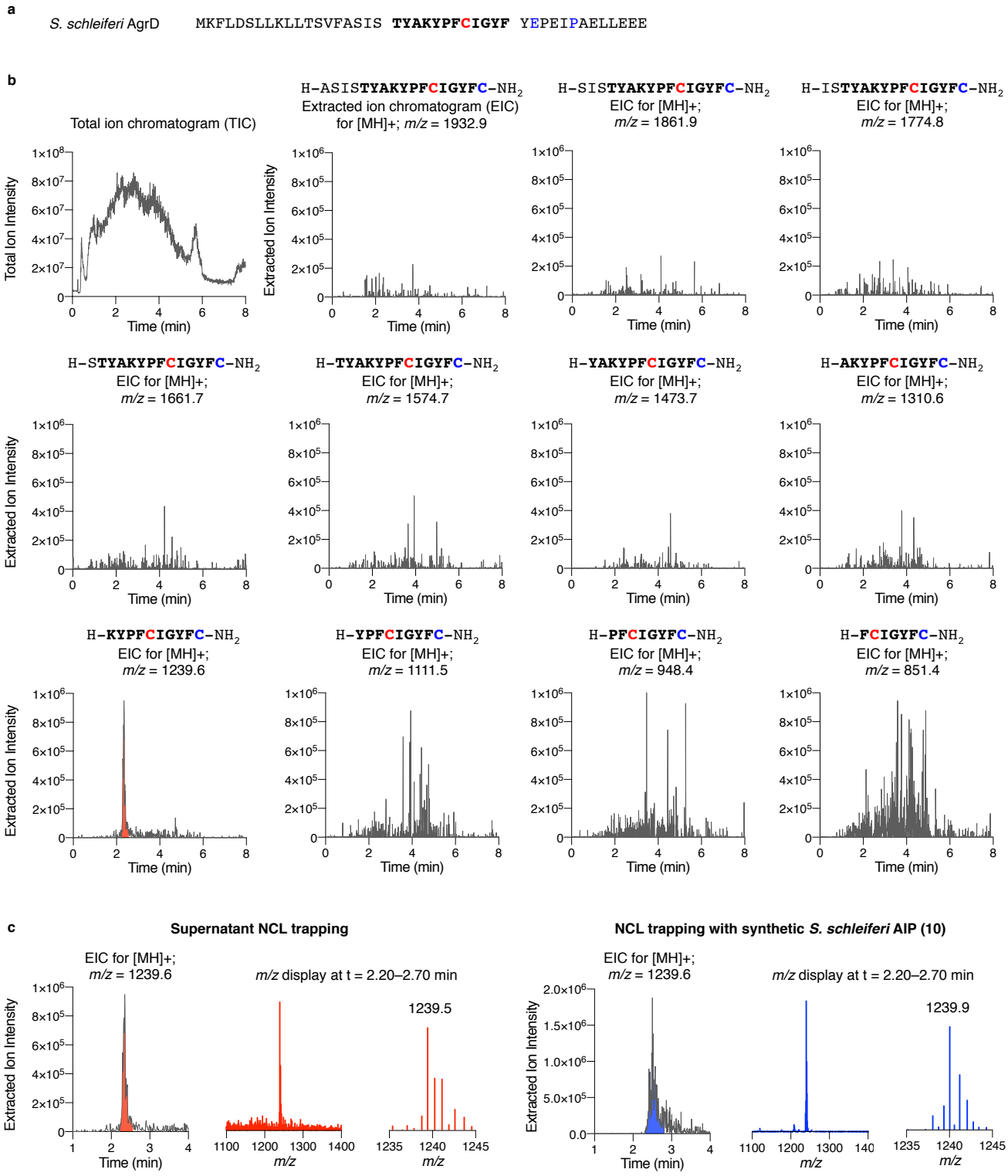
Supplementary Figure 5. Sequence-guided identification of *S. aureus* AIP-IV (4) from bacterial supernatant. a, AgrD sequence of *S. aureus agr*-IV (UniProtKB: Q9L561). **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped AIP-IV (**4**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear AIP-IV sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic AIP-IV (**4**) confirms the identity of trapped AIP-IV (**4**) from bacterial supernatant.



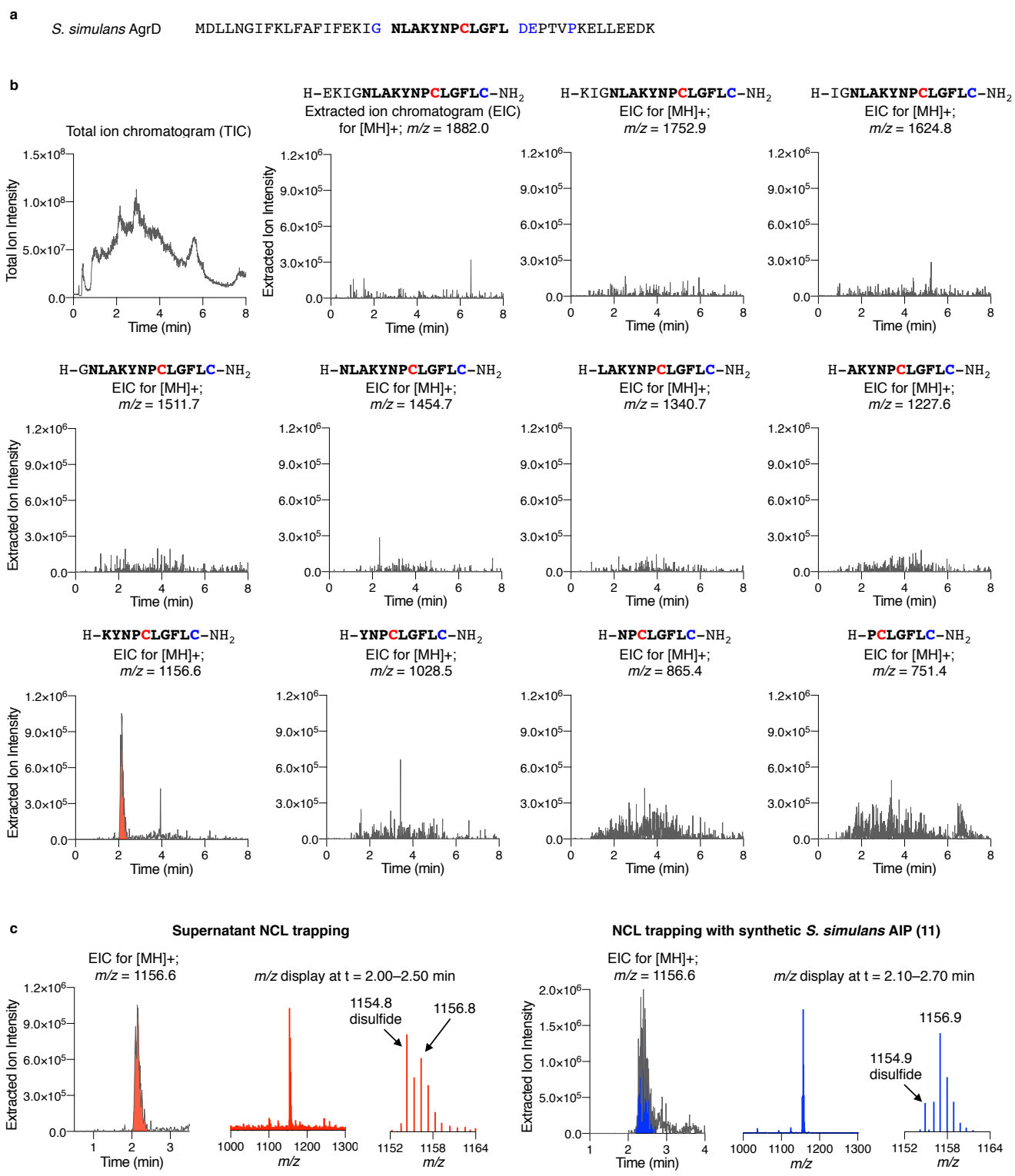
Supplementary Figure 6. Sequence-guided identification of *S. saprophyticus* AIP (S_{sa}-AIP, 8) from bacterial supernatant. **a**, AgrD sequence of *S. saprophyticus* sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped S_{sa}-AIP (**8**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. saprophyticus* AIP sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic S_{sa}-AIP (**8**) confirms the identity of trapped S_{sa}-AIP (**8**) from bacterial supernatant.



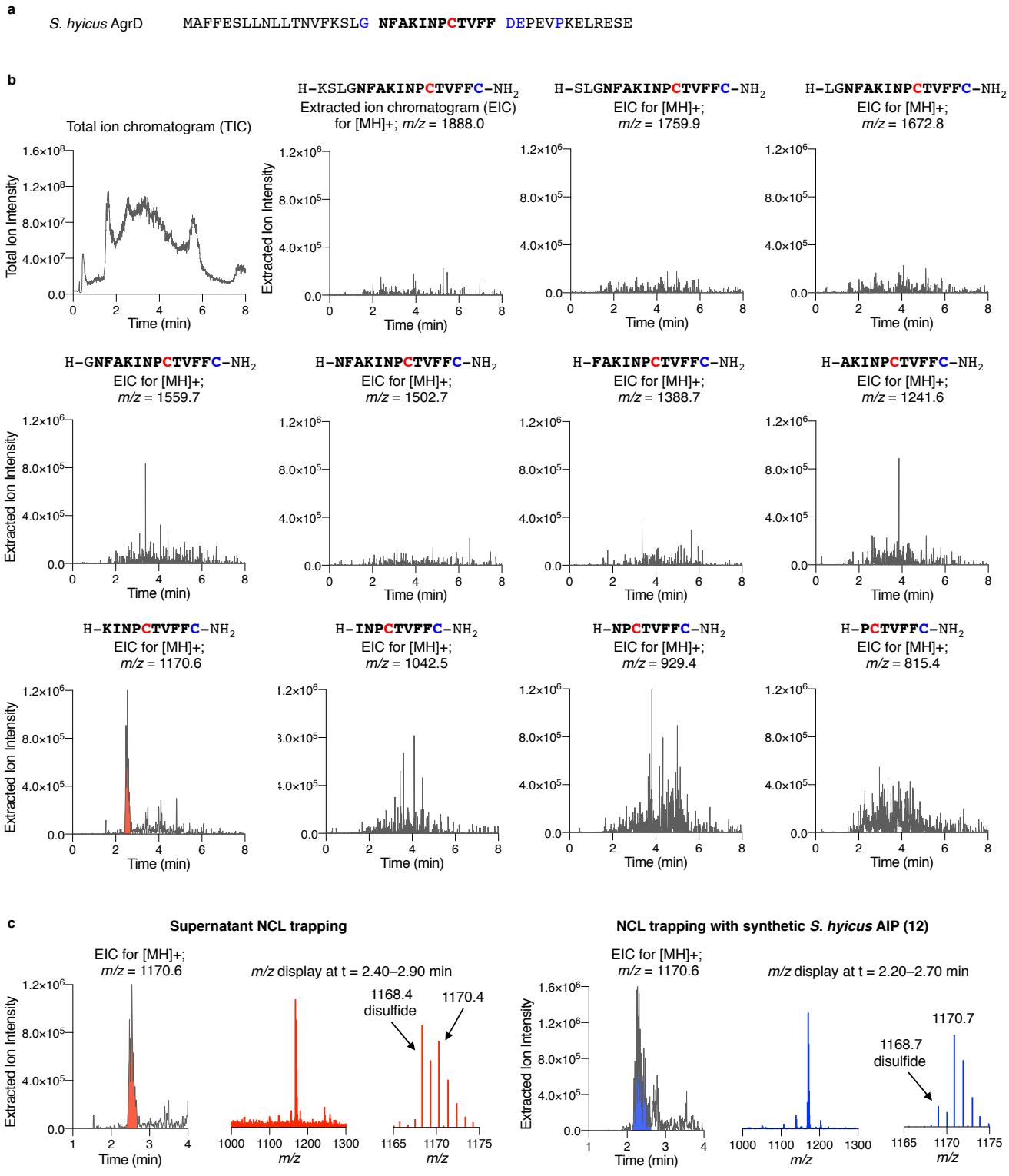
Supplementary Figure 7. Sequence-guided identification of *S. lugdunensis* AIP-II (S_{lu}-AIP-II, 9) from bacterial supernatant. a, AgrD sequence of *S. lugdunensis agr*-II sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped S_{lu}-AIP-II (**9**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. lugdunensis* AIP-II sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic S_{lu}-AIP-II (**9**) confirms the identity of trapped S_{lu}-AIP-II (**9**) from bacterial supernatant.



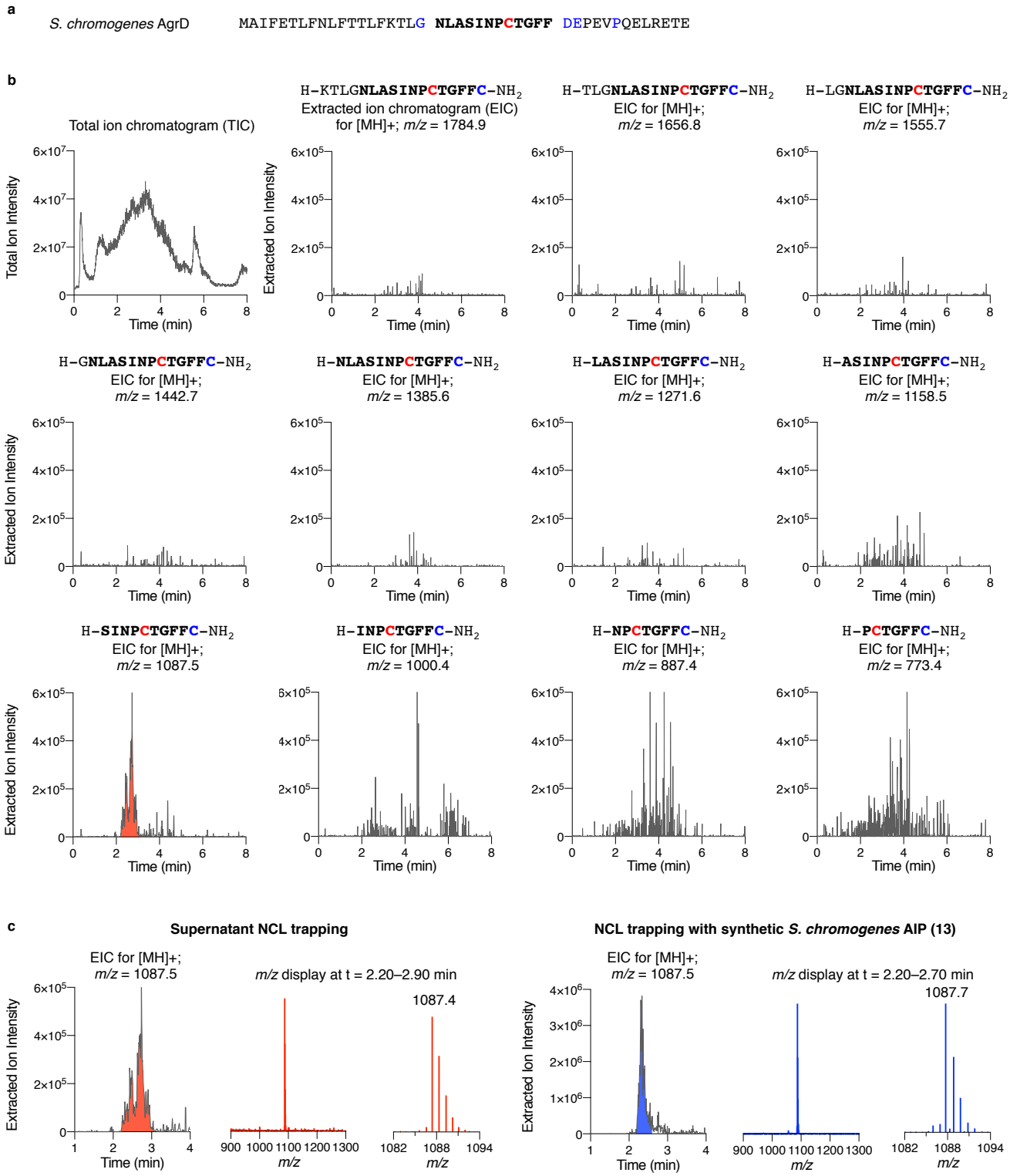
Supplementary Figure 8. Sequence-guided identification of *S. schleiferi* AIP (S_{sc}-AIP, 10) from bacterial supernatant. a, AgrD sequence of *S. schleiferi* sequenced in previous study (NCBI accession: PRJEB1574).[1] **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped S_{sc}-AIP (**10**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. schleiferi* AIP sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic S_{sc}-AIP (**10**) confirms the identity of trapped S_{sc}-AIP (**10**) from bacterial supernatant.



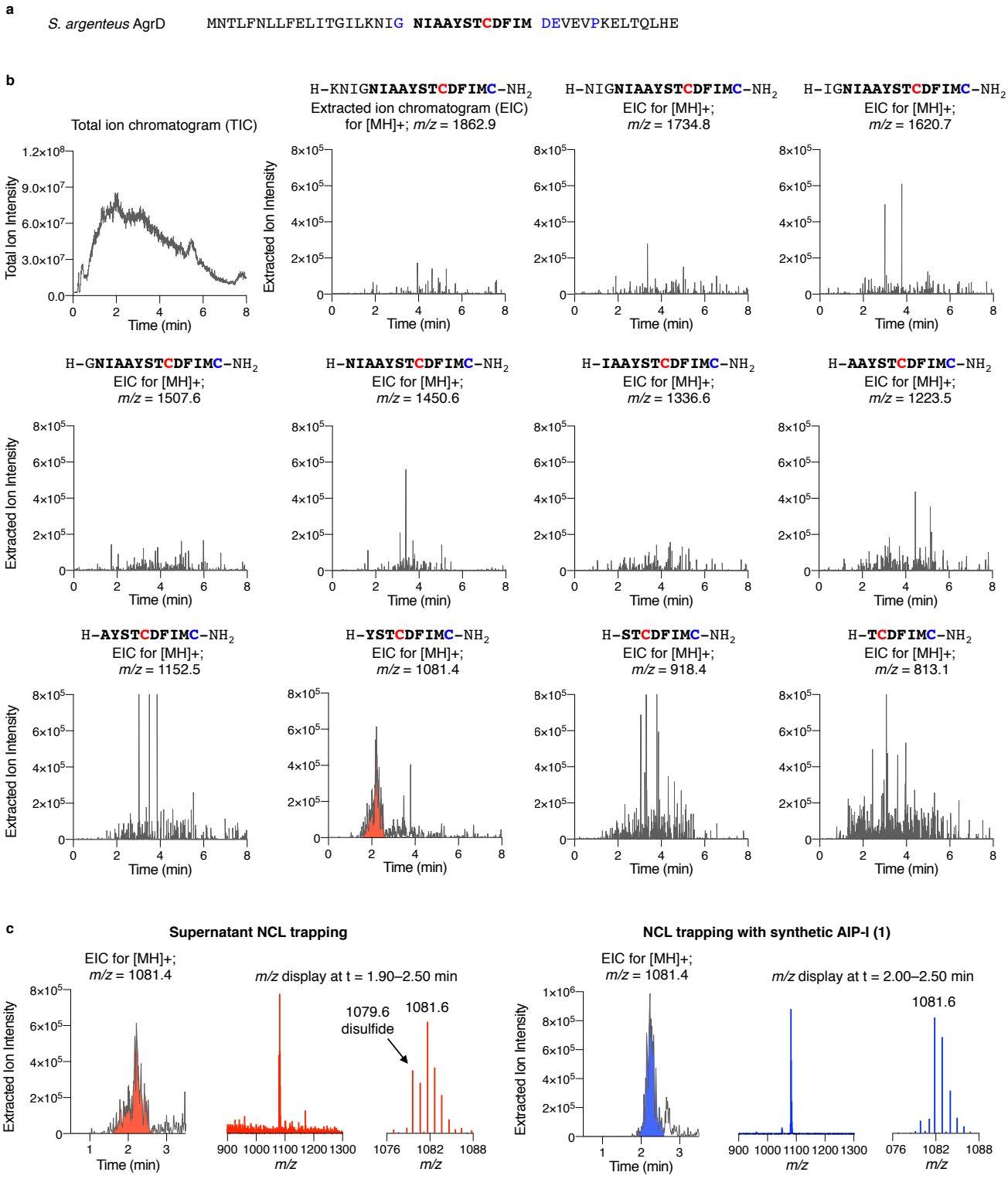
Supplementary Figure 9. Sequence-guided identification of *S. simulans* AIP (S_{si}-AIP, 11) from bacterial supernatant. a, AgrD sequence of *S. simulans* sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped S_{si}-AIP (**11**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. simulans* AIP sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic S_{si}-AIP (**11**) confirms the identity of trapped S_{si}-AIP (**11**) from bacterial supernatant.



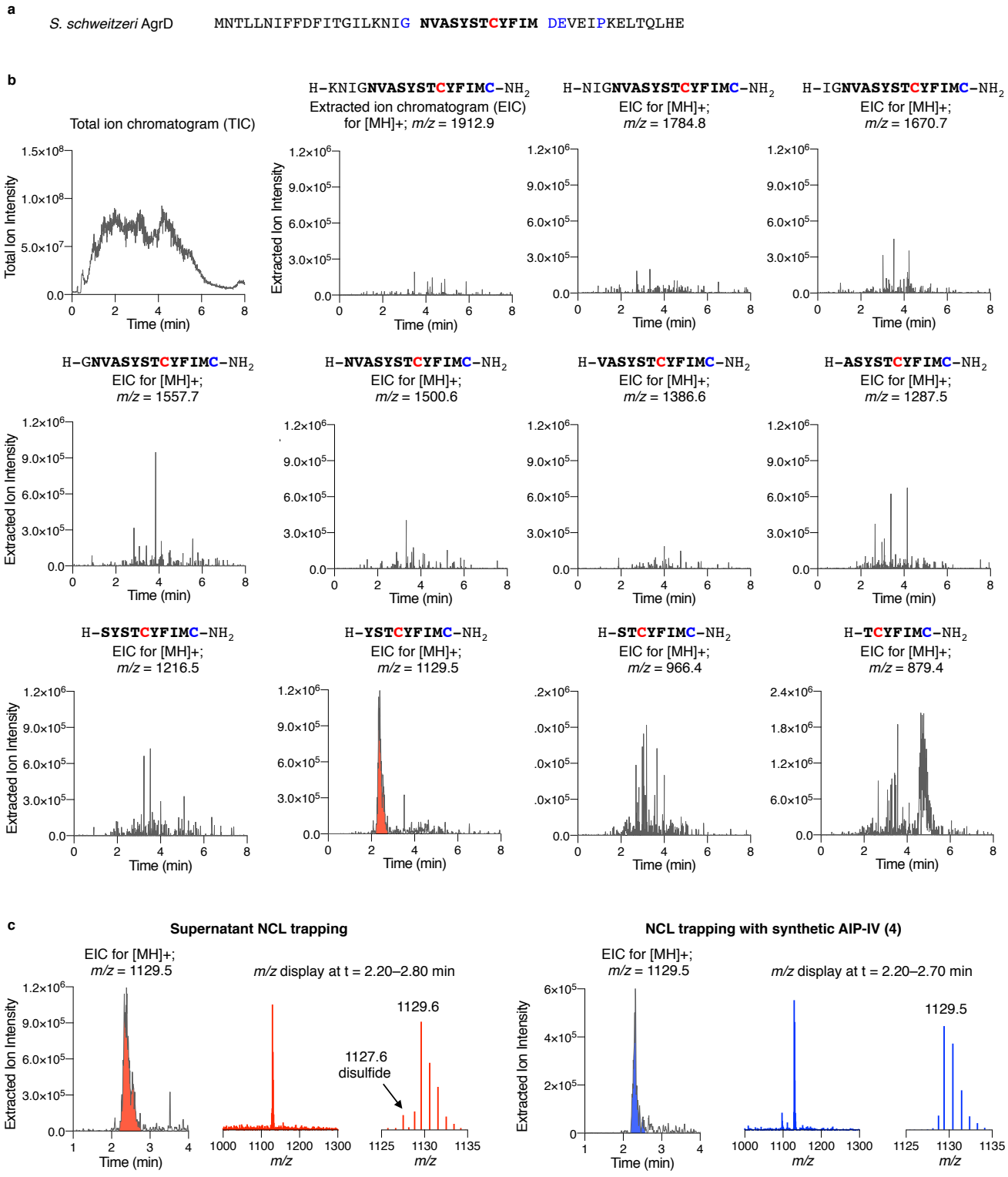
Supplementary Figure 10. Sequence-guided identification of *S. hyicus* AIP (S_{hy}-AIP, 12) from bacterial supernatant. a, AgrD sequence of *S. hyicus* sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped S_{hy}-AIP (**12**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. hyicus* AIP sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic S_{hy}-AIP (**12**) confirms the identity of trapped S_{hy}-AIP (**12**) from bacterial supernatant.



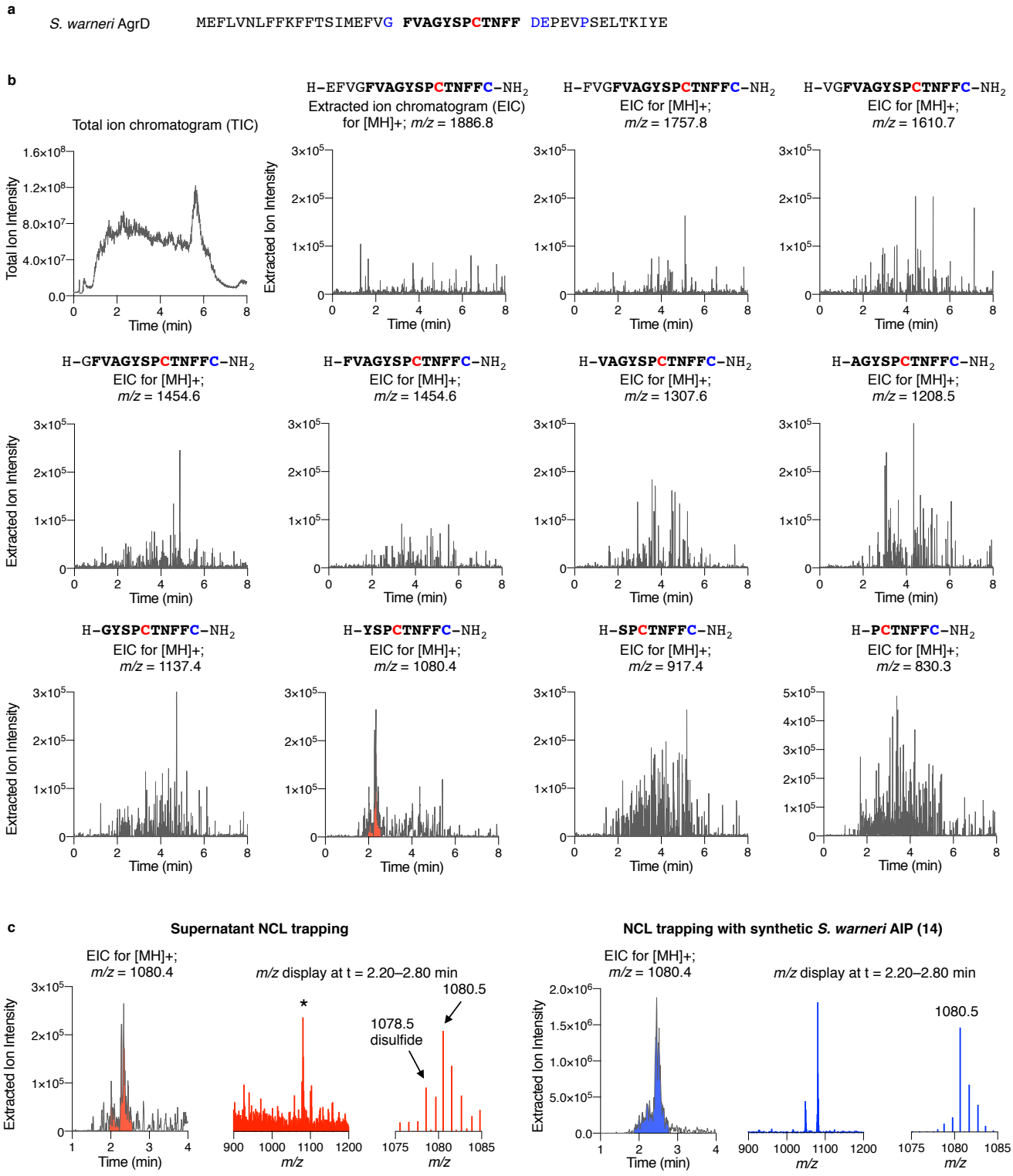
Supplementary Figure 11. Sequence-guided identification of *S. chromogenes* AIP (S_{ch}-AIP, 13) from bacterial supernatant. a, AgrD sequence of *S. chromogenes* sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped S_{ch}-AIP (**13**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. chromogenes* AIP sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic S_{ch}-AIP (**13**) confirms the identity of trapped S_{ch}-AIP (**13**) from bacterial supernatant.



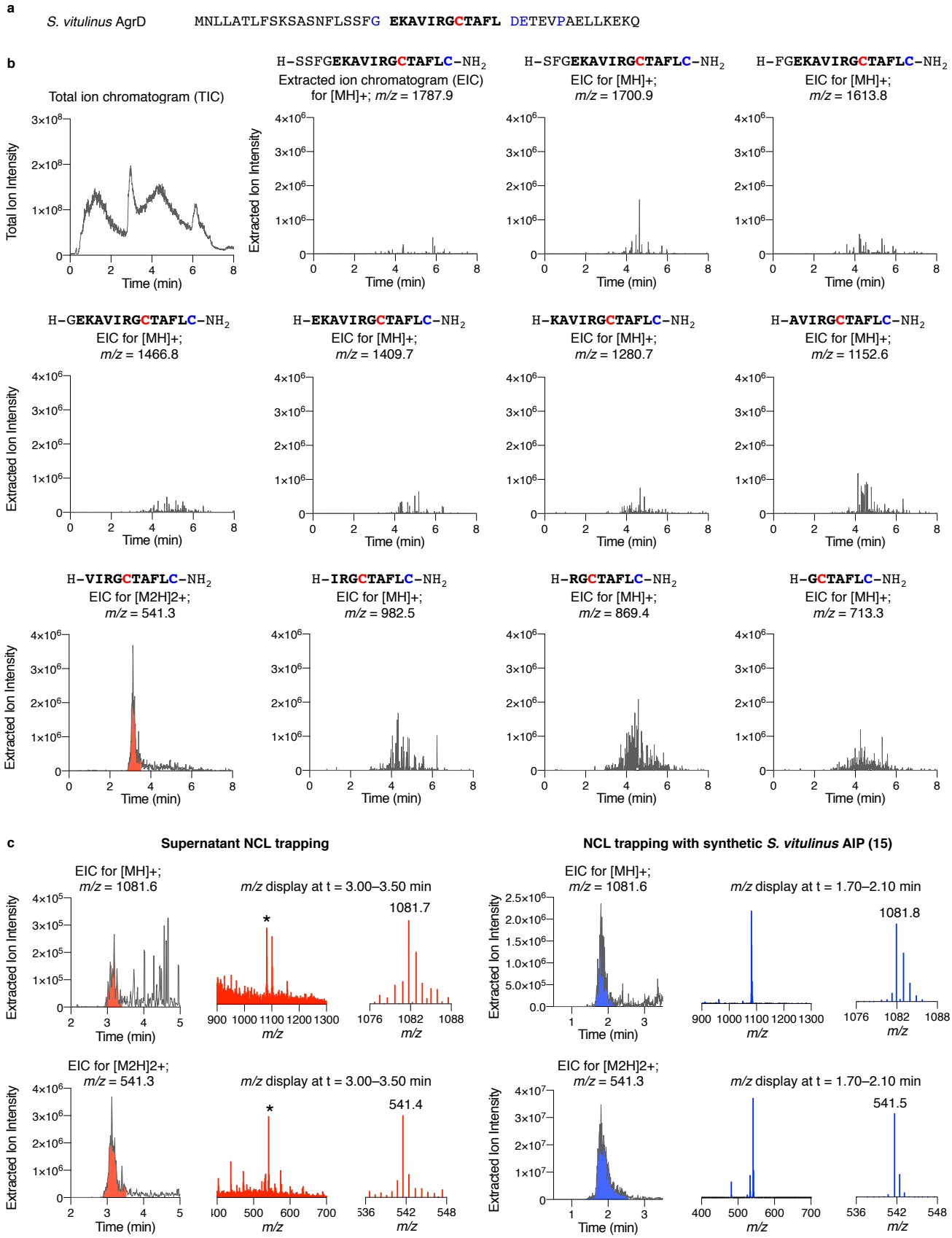
Supplementary Figure 12. Sequence-guided identification of *S. argenteus* AIP, which is identical to *S. aureus* AIP-I (1), from bacterial supernatant. a, AgrD sequence of *S. argenteus* sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped *S. argenteus* AIP and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. argenteus* AIP sequences including a C-terminal cysteine. (**C**) NCL trapping of synthetic *S. aureus* AIP-I (**1**) confirms the identity of trapped *S. argenteus* AIP from bacterial supernatant.



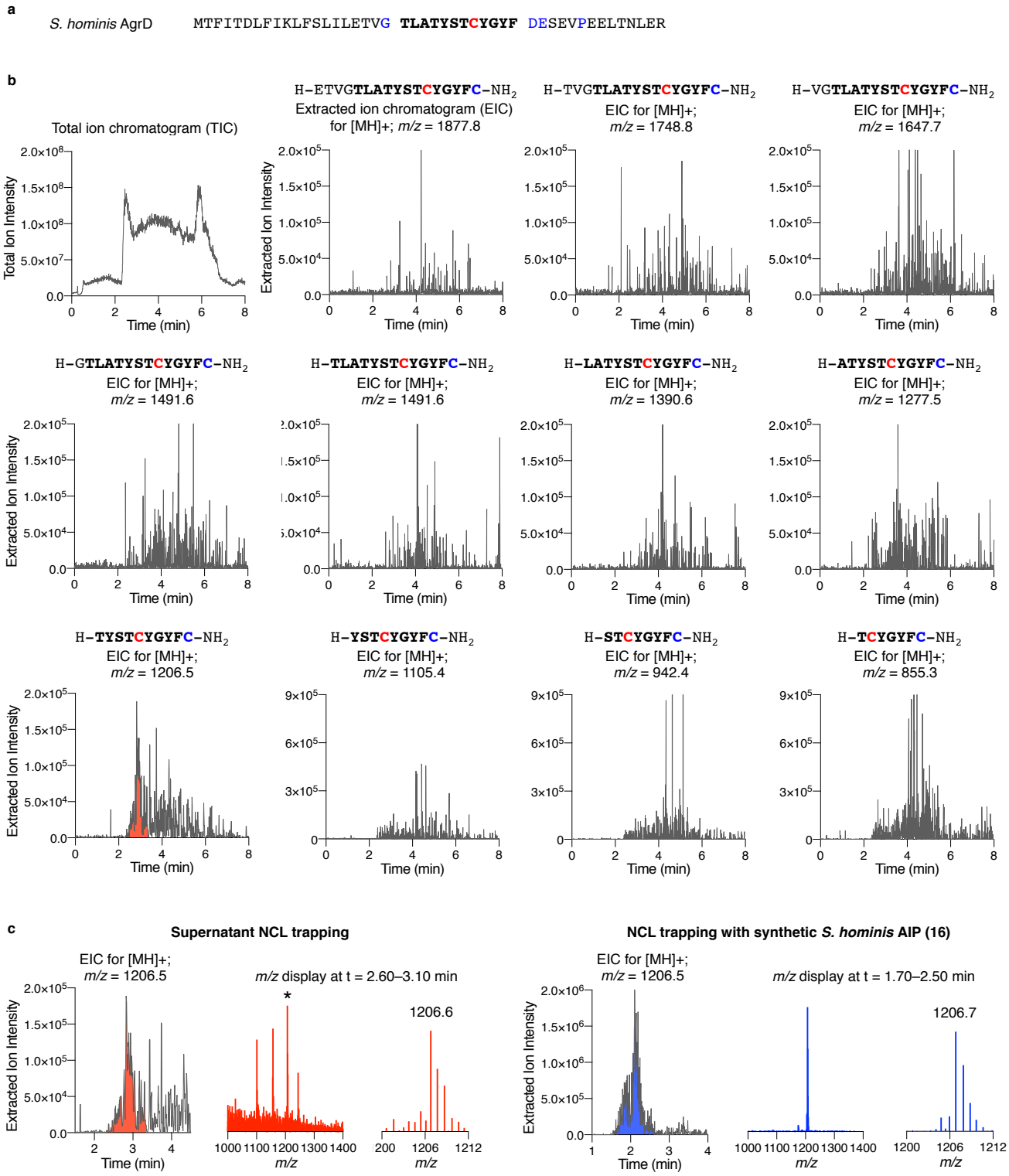
Supplementary Figure 13. Sequence-guided identification of *S. schweitzeri* AIP, which is identical to *S. aureus* AIP-IV (4), from bacterial supernatant. a, AgrD sequence of *S. schweitzeri* sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped *S. schweitzeri* AIP and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. schweitzeri* AIP sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic *S. aureus* AIP-IV (**4**) confirms the identity of trapped *S. schweitzeri* AIP from bacterial supernatant.



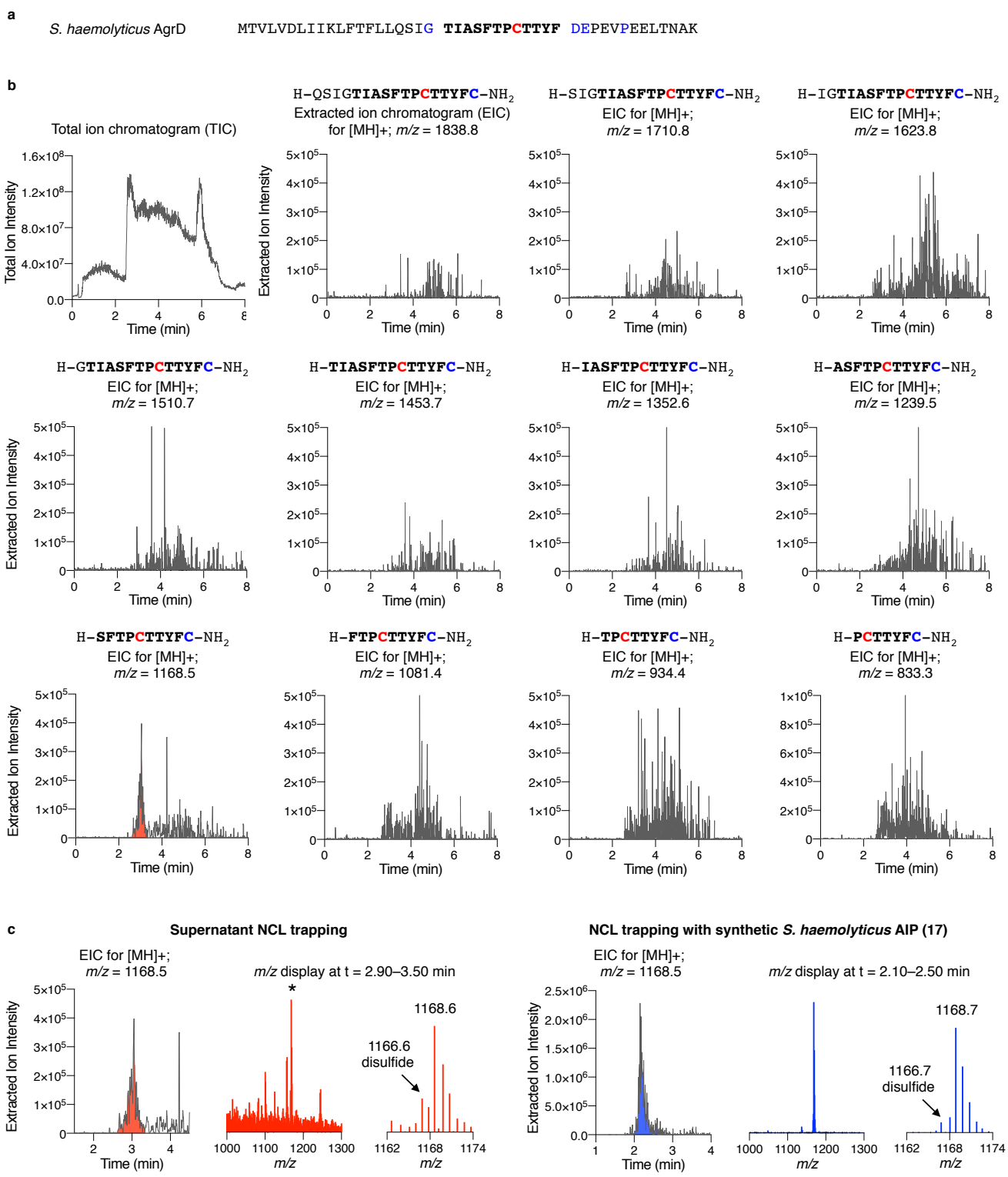
Supplementary Figure 14. Sequence-guided identification of *S. warneri* AIP (S_{wa}-AIP, 14) from bacterial supernatant. a, AgrD sequence of *S. warneri* sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped S_{wa}-AIP (**14**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. warneri* AIP sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic S_{wa}-AIP (**14**) confirms the identity of trapped S_{wa}-AIP (**14**) from bacterial supernatant.



Supplementary Figure 15. Sequence-guided identification of *S. vitulinus* AIP (S_{vi}-AIP, 15) from bacterial supernatant. a, AgrD sequence of *S. vitulinus* sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped S_{vi}-AIP (**15**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. vitulinus* AIP sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic S_{vi}-AIP (**15**) confirms the identity of trapped S_{vi}-AIP (**15**) from bacterial supernatant.

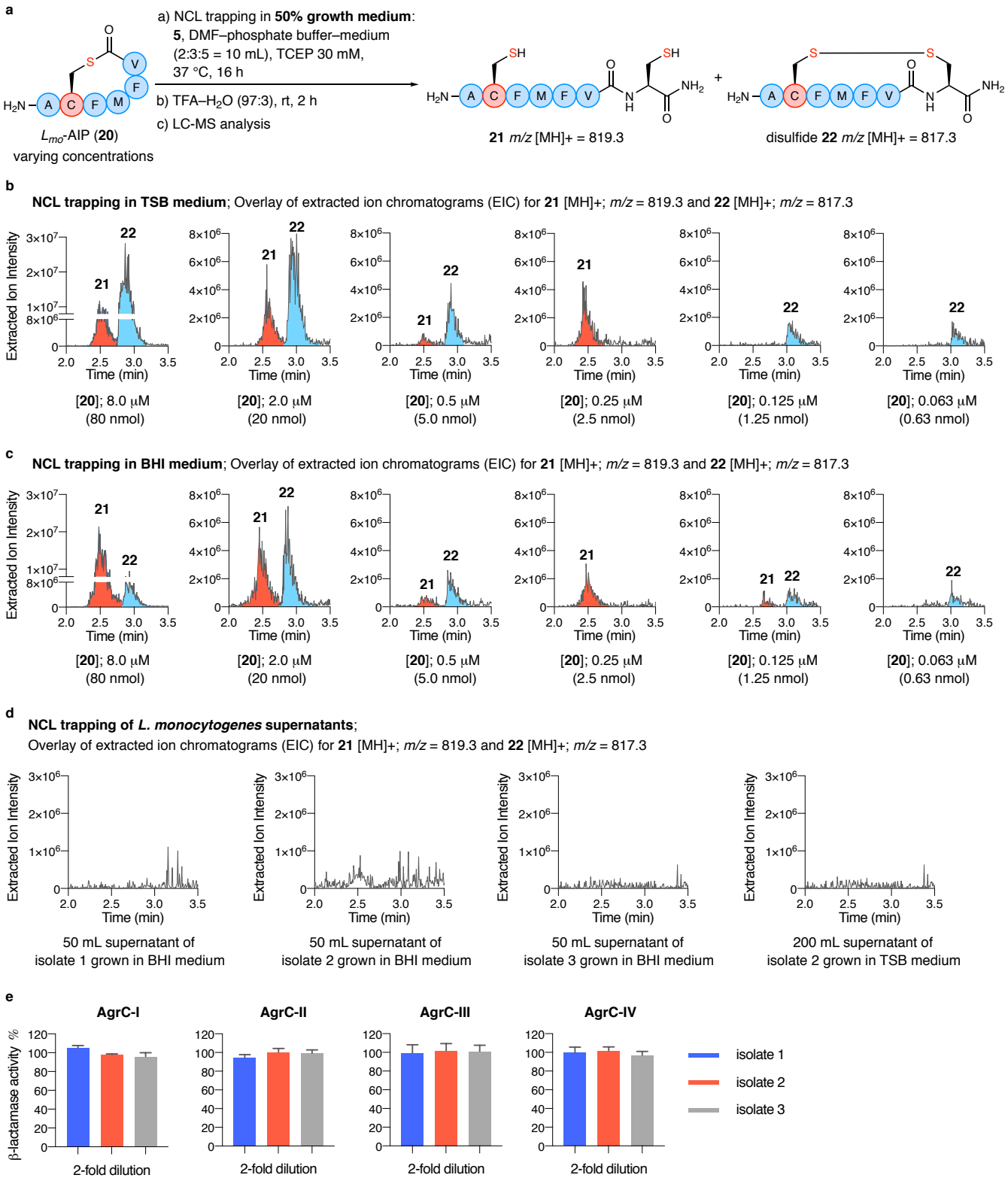


Supplementary Figure 16. Sequence-guided identification of *S. hominis* AIP (S_{ho}-AIP, 16) from bacterial supernatant. a, AgrD sequence of *S. hominis* sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped S_{ho}-AIP (**16**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. hominis* AIP sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic S_{ho}-AIP (**16**) confirms the identity of trapped S_{ho}-AIP (**16**) from bacterial supernatant.



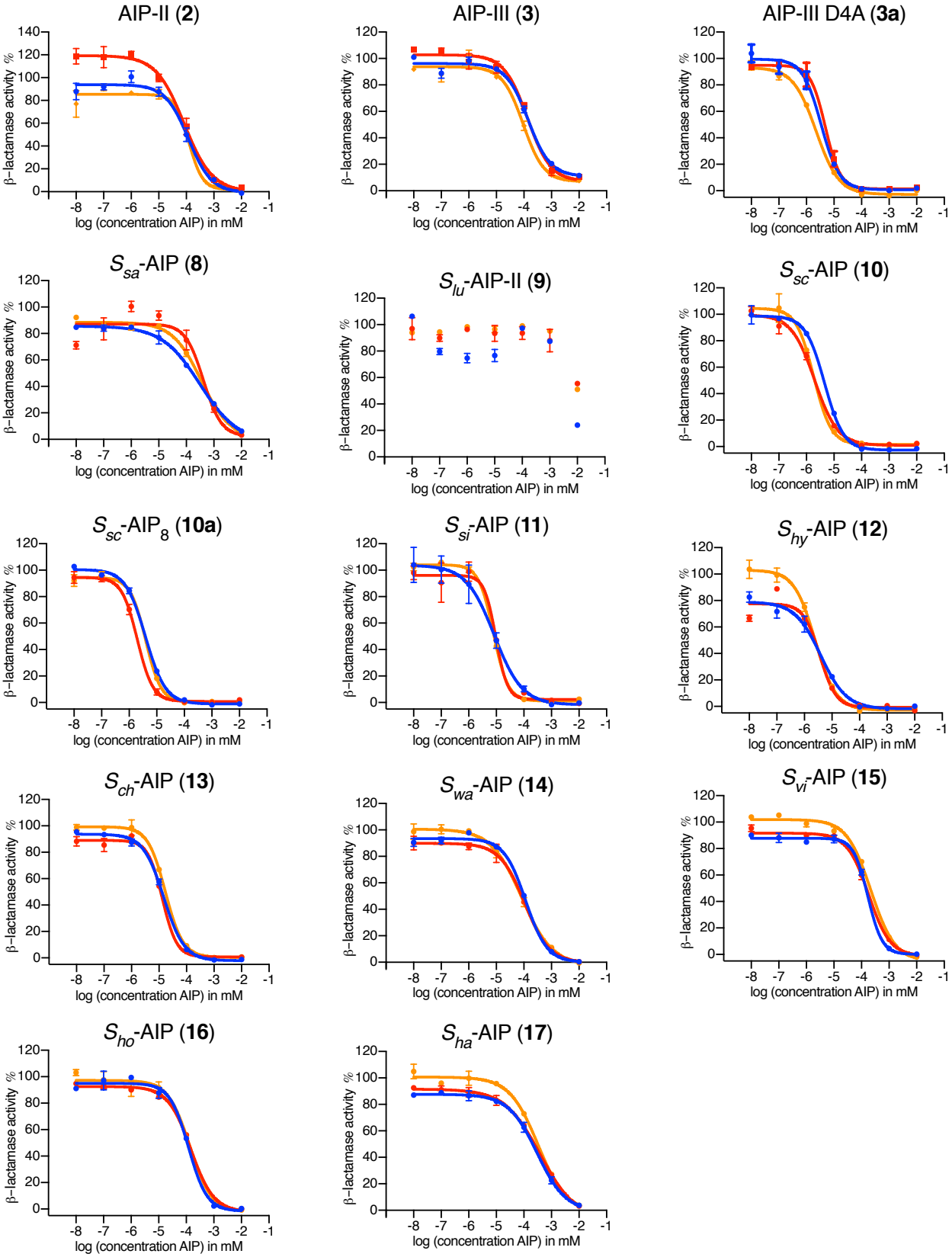
Supplementary Figure 17. Sequence-guided identification of *S. haemolyticus* AIP (S_{ha}-AIP, 17) from bacterial supernatant. a, AgrD sequence of *S. haemolyticus* sequenced in this study. **b**, Total ion chromatogram (TIC) of the TFA cleavage solution of trapped S_{ha}-AIP (**17**) and extracted ion chromatograms (EIC) for *m/z* = [MH]+ of possible linear *S. haemolyticus* AIP sequences including a C-terminal cysteine. **c**, NCL trapping of synthetic S_{ha}-AIP (**17**) confirms the identity of trapped S_{ha}-AIP (**17**) from bacterial supernatant.

Investigation of NCL trapping of the *L. monocytogenes* AIP

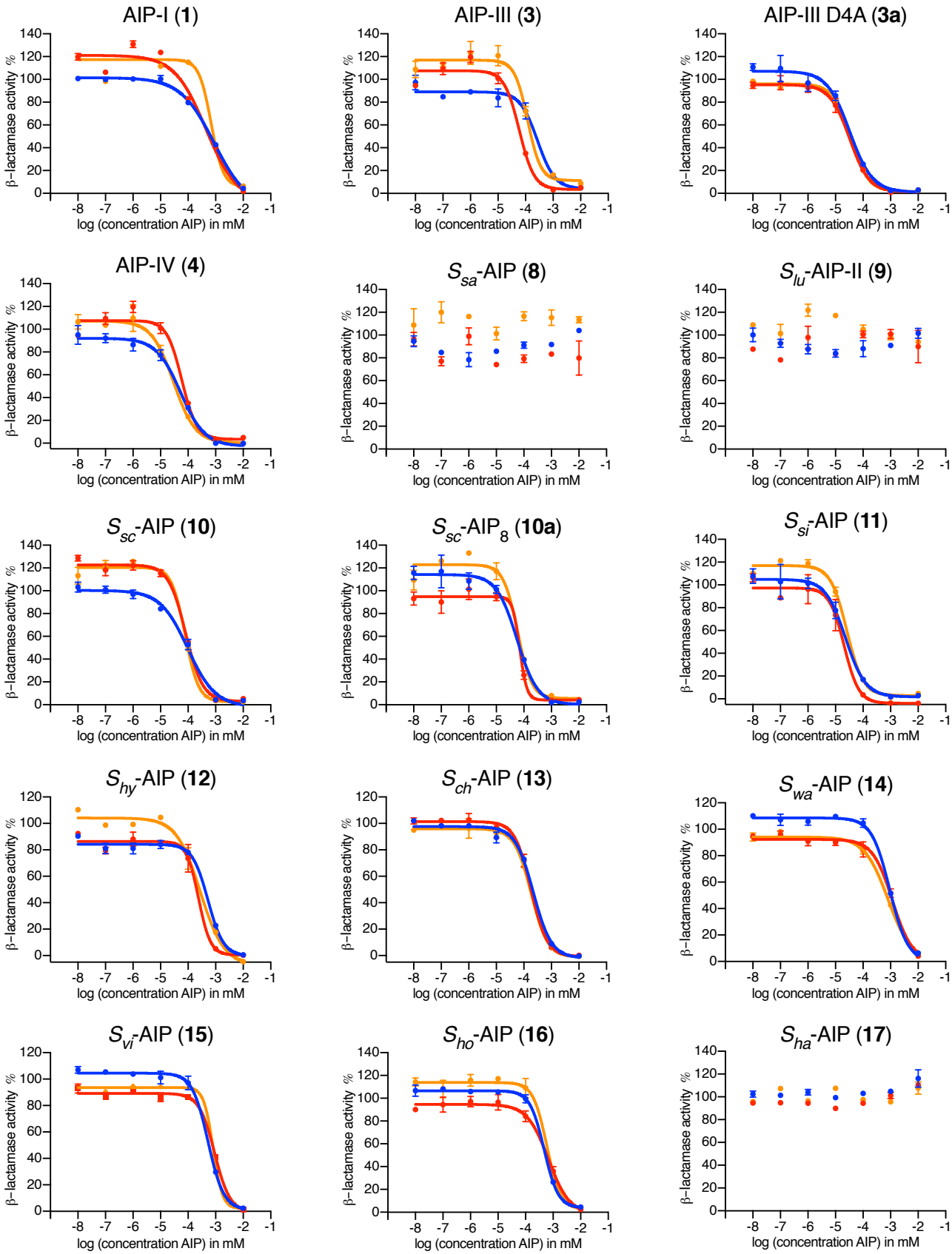


Supplementary Figure 18. Investigation of NCL trapping of *L*$_{mo}$-AIP (20). a, NCL trapping of synthetic *L*$_{mo}$-AIP (**20**) in 50% TSB or BHI medium with varying concentrations (8.0–0.063 µM) in a total volume of 10 mL. **b**, Overlaid extracted ion chromatograms (EICs) $[M+H]^+$ for **21** and **22** of NCL trapping performed in TSB medium. **c**, Overlaid EICs $[M+H]^+$ for **21** and **22** of NCL trapping performed in BHI medium. **d**, Overlaid EICs [MH]+ for **21** and **22** of NCL trapping performed on three *L. monocytogenes* isolates in different media. **e**, *β*-Lactamase inhibition assays performed with supernatants of three *L. monocytogenes* isolates. The shown error bars are the standard error of the mean (SEM) of two individual assays.

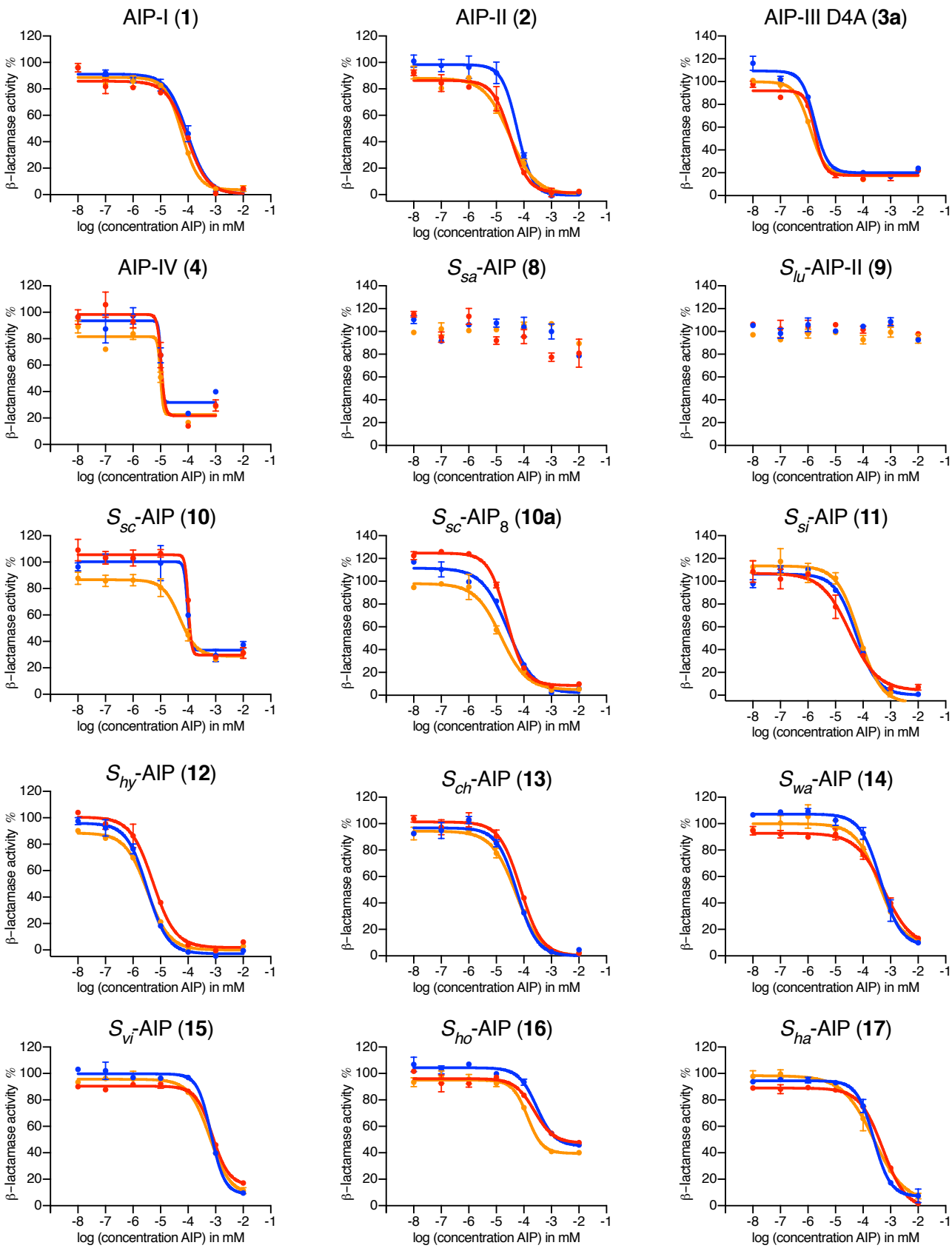
IC_{50}/EC_{50} Curves from β-lactamase assays



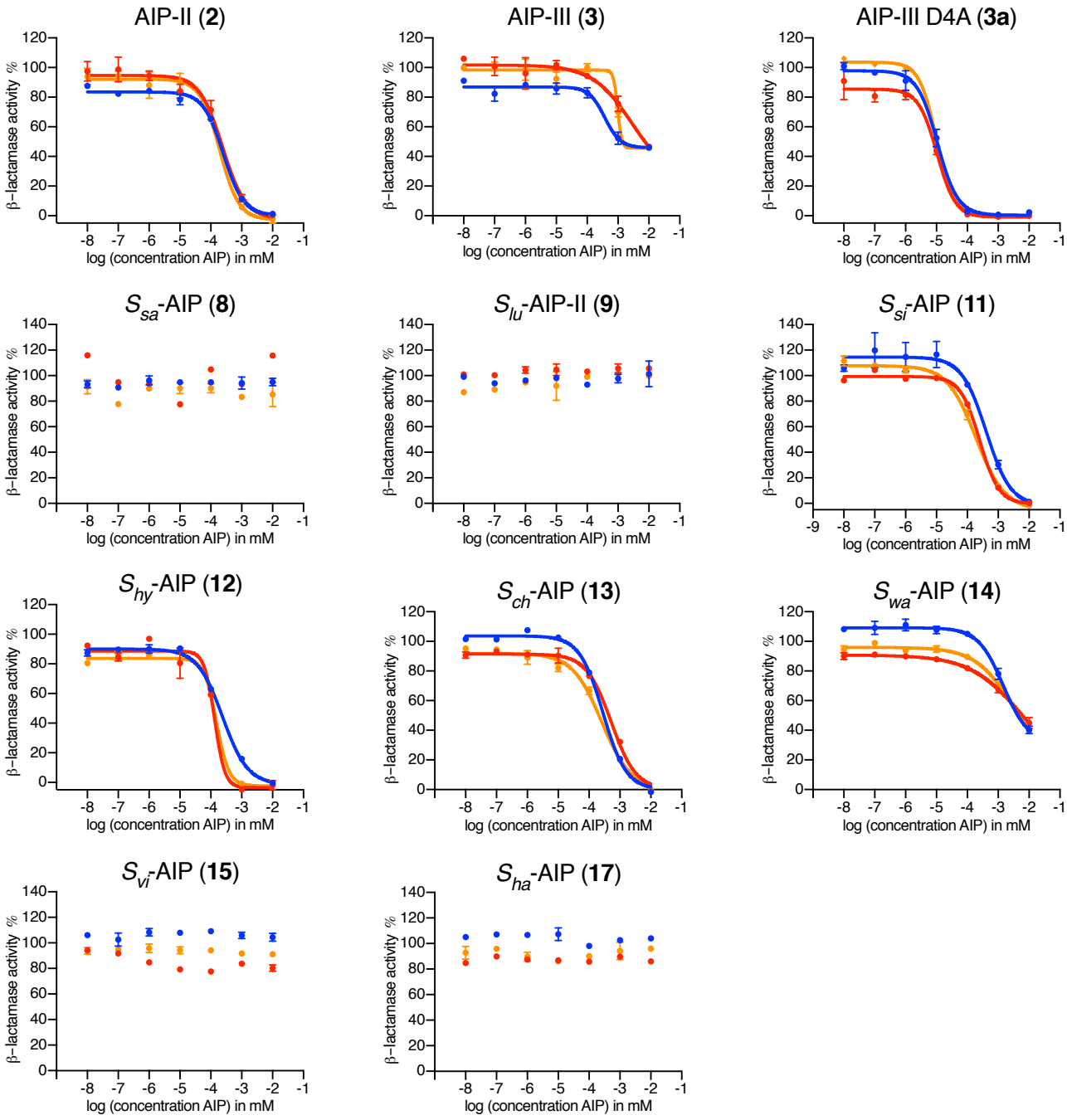
Supplementary Figure 19. IC_{50} curves for inhibition of *S. aureus* AgrC-I. Inhibition properties of synthetic AIPs (10 mM to 10 pM) were determined through β-lactamase activity using the *S. aureus* AgrC-I reporter strain in the presence of 100 nM AIP-I (**1**). The shown error bars are the standard error of the mean (SEM) of two individual assays performed in biological triplicate..



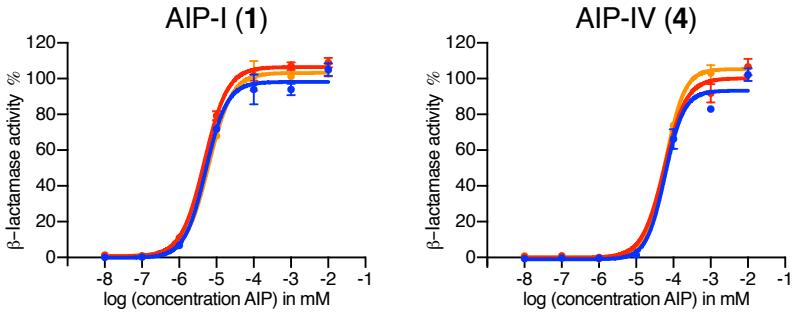
Supplementary Figure 20. IC_{50} curves for inhibition of *S. aureus* AgrC-II. Inhibition properties of synthetic AIPs (10 mM to 10 pM) were determined through *β*-lactamase activity using the *S. aureus* AgrC-II reporter strain in the presence of 100 nM AIP-II (**2**). The shown error bars are the standard error of the mean (SEM) of two individual assays performed in biological triplicate.



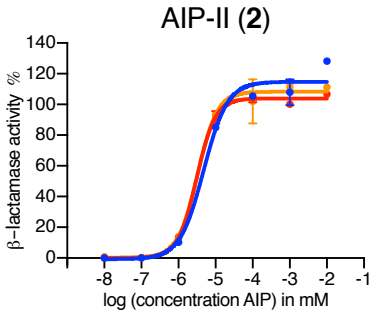
Supplementary Figure 21. IC_{50} curves for inhibition of *S. aureus* AgrC-III. Inhibition properties of synthetic AIPs (10 mM to 10 pM) were determined through *β*-lactamase activity using the *S. aureus* AgrC-III reporter strain in the presence of 100 nM AIP-III (**3**). The shown error bars are the standard error of the mean (SEM) of two individual assays performed in biological triplicate.



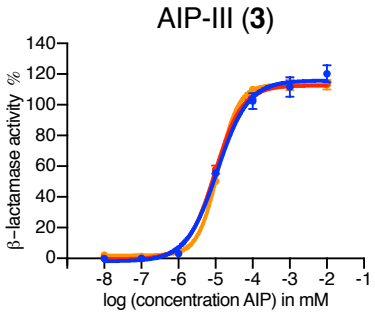
Supplementary Figure 22. IC_{50} curves for inhibition of *S. aureus* AgrC-IV. Inhibition properties of synthetic AIPs (10 mM to 10 pM) were determined through *β*-lactamase activity using the *S. aureus* AgrC-IV reporter strain in the presence of 100 nM AIP-IV (**4**). The shown error bars are the standard error of the mean (SEM) of two individual assays performed in biological triplicate.



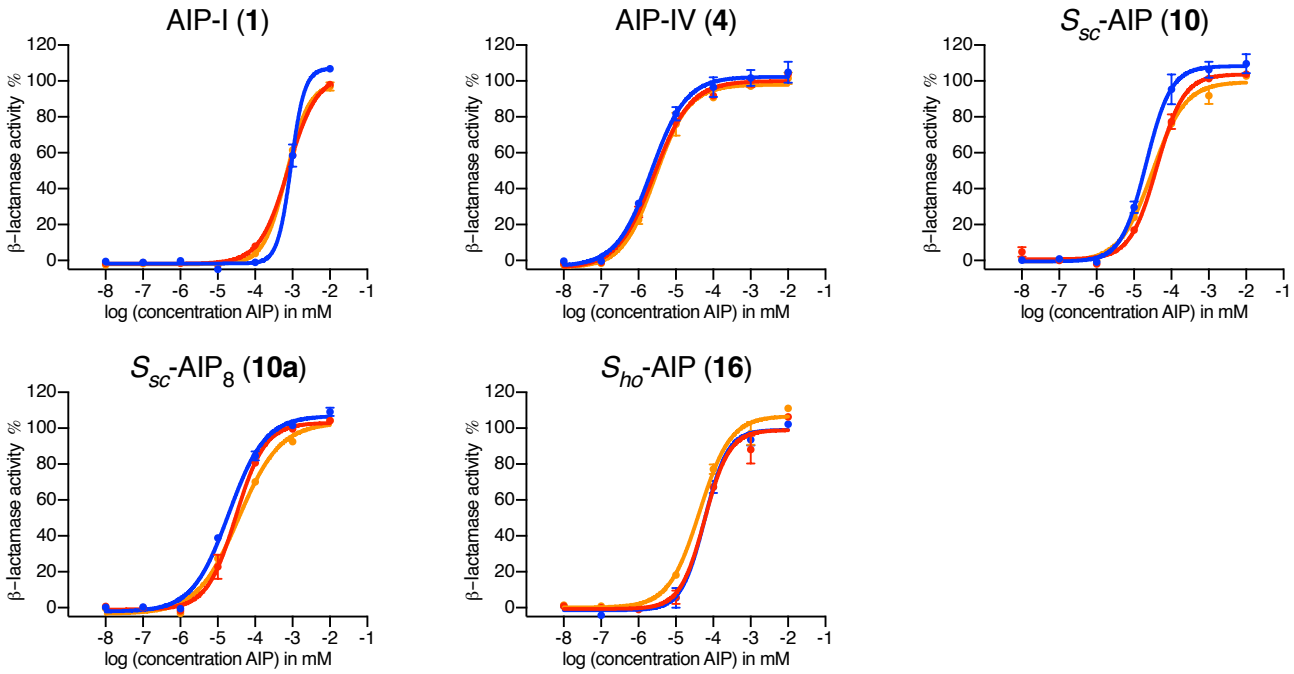
Supplementary Figure 23. EC_{50} curves for activation of *S. aureus* AgrC-I. Activation properties of synthetic AIPs (10 mM to 10 pM) were determined through *β*-lactamase activity using the *S. aureus* AgrC-I reporter strain. The shown error bars are the standard error of the mean (SEM) of two individual assays performed in biological triplicate.



Supplementary Figure 24. EC_{50} curves for activation of *S. aureus* AgrC-II. Activation properties of synthetic AIPs (10 mM to 10 pM) were determined through *β*-lactamase activity using the *S. aureus* AgrC-II reporter strain. The shown error bars are the standard error of the mean (SEM) of two individual assays performed in biological triplicate.



Supplementary Figure 25. EC_{50} curves for activation of *S. aureus* AgrC-III. Activation properties of synthetic AIPs (10 mM to 10 pM) were determined through *β*-lactamase activity using the *S. aureus* AgrC-III reporter strain. The shown error bars are the standard error of the mean (SEM) of two individual assays performed in biological triplicate.



Supplementary Figure 26. EC_{50} curves for activation of *S. aureus* AgrC-IV. Activation properties of synthetic AIPs (10 mM to 10 pM) were determined through *β*-lactamase activity using the *S. aureus* AgrC-IV reporter strain. The shown error bars are the standard error of the mean (SEM) of two individual assays performed in biological triplicate.

Supplementary tables

Supplementary Table 1. Bacterial strains used in this study.

Name	Characteristics	Reference
E. coli IM08B	Host strain used for cloning	(2)
S. aureus 8325-4	*agr*-I type strain	Lab stock
S. aureus RN6607	*agr*-II type strain	Lab stock
S. aureus MW2	*agr*-III type strain	Lab stock
S. aureus 4850	*agr*-IV type strain	Lab stock
P3-*blaZ*/p*argC*-I	*S. aureus* RN10829 reporter expressing AgrC-I	(3)
P3-*blaZ*/p*argC*-II	*S. aureus* RN10829 reporter expressing AgrC-II	This study
P3-*blaZ*/p*argC*-III	*S. aureus* RN10829 reporter expressing AgrC-III	This study
P3-*blaZ*/p*argC*-IV	*S. aureus* RN10829 reporter expressing AgrC-IV	This study
S. lugdunensis	Strain ID A1, human nose isolate	This study
S. saprophyticus	Human nose isolate	This study
S. schleiferi	Strain ID 30743, dog isolate	(1)
S. hiycus	Pig nose isolate	This study
S. simulans	Pig nose isolate	This study
S. chromogenes	Pig nose isolate	This study
S. schweitzeri	Strain ID 59	This study
S. argenteus	Strain ID 60	This study
S. warneri	Strain ID 5811483, human nose isolate	This study
S. hominis	Strain ID 9525	(1)
S. vitulinus	Strain ID 62, horse isolate	(1)
S. haemolyticus	Strain ID1312, dog isolate	(1)
L. monocytogenes isolate 1	Strain ID 10403S	Lab stock
L. monocytogenes isolate 2	Strain ID N53-I	Lab stock
L. monocytogenes isolate 3	Strain ID EGD	Lab stock

Supplementary Table 2. Primers used for AgrD sequencing.

Species	Forward oligonucleotide (5'-3')	Reverse oligonucleotide (5'-3')
S. lugdunensis	GTCATCATTGTTGTGCCACATTC	GCAGCGTGTACTCGTAGTATG
S. saprophyticus	TGTTTTACTACCTTGGATAGTCGG	GTGGTGATAATGACAACTGCATAC
S. hyicus	TCTCTCGAGAGGTCGTAATGG	GCTATTCACATGGCGCTCATG
S. simulans	ATGTAAACAAGGAAATGAAGATACTAGC	ATGGACTTGCTCTTCTATTCCATG
S. chromogenes	CTACGATAATTAATTTCTCGCGACG	GTTTTTCACATGGGGCTCATG
S. schweitzeri	CAAACCAGGCATGTCATCTTCG	TTAGATATGCTCCTGCAGCAAC
S. argenteus	CCAGTTCGCCACGTATCTTC	GAAATTTTGCCAATATAGGCATACATAAC
S. warneri	TGACATAGTCATGTCGAAACTTACG	GCAACCTATACCTAAACGCTTAGTG
S. hominis	GTTGGTTTACCATGGCTCATTG	GTGTATTCATAATACGTTTCAATTTCTTCC
S. vitulinus	CTATTCATCAATAGTCTTATCAAAACAGC	CAAAGTCCACATTTTTATGCACC
S. haemolyticus	GTGACATATGGTGTTGCACTTATC	CAAGACAAGCGAAATAATTGTAAGATACG

Supplementary Table 3. Previously published and sequenced AgrD sequences used for AIP identification.

Bacterial strain	AgrD amino acid sequence	Uniprot/NCBI
S. aureus agr-I	MNTLFNLFFDFITGILKNIGNIAAYSTCDFIMDEVEVPKELTQLHE	Uniprot: Q53643
S. aureus agr-II	MNTLVNMFFDFIIKLAKAIGIVGGVNACSSLFDEPKVPAELTNLYDK	Uniprot: O33586
S. aureus agr-III	MKKLLNKVIELLVDFFNSIGYRAAYINCDFLLDEAEVPKELTQLHE	Uniprot: O33589
S. aureus agr-IV	MNTLLNIFFDFITGVLKNIGNVASYSTCYFIMDEVEIPKELTQLHE	Uniprot: Q9L561
S. saprophyticus	MNVIKSISKSISKSISNYFAKVFASIGSISTINPCFGYTDESEIPKELTDLYE	NCBI: QBG64256
S. lugdunensis agr-II	MNLLSGLFTKGISVIFEFIGNFSVQDMCNGYFDEPEVPQELIDLHRN	NCBI: QBG64255
S. schleiferi	MKFLDSLLKLLTSVFASISTYAKYPFCIGYFYEPEIPAELLEEE	NCBI: PRJEB15874
S. simulans	MDLLNGIFKLFAFIFEKIGNLAKYNPCLGFLDEPTVPKELLEEDK	NCBI: QBG64258
S. hiycus	MAFFESLLNLLTNVFKSLGNFAKINPCTVFFDEPEVPKELRESE	NCBI: QBG64257
S. chromogenes	MAIFETLFNLFTTLFKTLGNLASINPCTGFFDEPEVPQELRETE	NCBI: QBG64259
S. argenteus	MNTLFNLLFELITGILKNIGNIAAYSTCDFIMDEVEVPKELTQLHE	NCBI: QBG64261
S. schweitzeri	MNTLLNIFFDFITGILKNIGNVASYSTCYFIMDEVEIPKELTQLHE	NCBI: QBG64260
S. warneri	MEFLVNLFFKFFTSIMEFVGFVAGYSPCTNFFDEPEVPSELTKIYE	NCBI: QBG64262
S. vitulinus	MNLLATLFSKSASNFLSSFGEKAVIRGCTAFLDETEVPAELLKEKQ	NCBI: QBG64264
S. hominis	MTFITDLFIKLFSLILETVGTLATYSTCYGYFDESEVPEELTNLER	NCBI: QBG64263
S. haemolyticus	MTVLVDLIIKLFTFLLQSIGTIASFTPCTTYFDEPEVPEELTNAK	NCBI: QBG64265
L. monocytogenes	MKNMNKSVGKFLSRKLEEQSMKVADSSMSKACFMFVYEPKSPFVKMQEKNENK	Uniprot: A8E0G2_LISMN

Materials and methods

General information

Reagents and materials. Bacteria were cultured in tryptic soya broth (TSB) medium and on tryptic soya agar (TSA) plates (both Oxoid) supplemented with 10 μg/ml chloramphenicol (Sigma-Aldrich) when appropriate. *N*α-Fmoc protected and *N*α-Boc protected amino acids, 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorophosphate (HBTU) and 2-(1*H*-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorphosphate (HATU) were obtained from ChemImpex and PepChem. Aminomethyl ChemMatrix resin was obtained from PCAS BioMatrix. Aminomethyl PEGA resin, *N*,*N*-diisopropylethylamine, 4-nitrophenylchloroformate and maleic acid (qNMR grade) were obtained from obtained from Sigma-Aldrich. Nitrocefin was obtained from Oxoid. All other chemicals used were obtained in the highest available purity from CombiBlocks or IrisBiotech. All solvents used were of analytical grade and purchased from Fisher Scientific. Manual solid-phase peptide synthesis was performed in polypropylene syringes equipped with fitted disks, purchased from Torviq.

Analysis and purification. Analytical high-performance liquid chromatography (HPLC) was performed on a C18 Phenomenex Luna column (2.6 μm, 100 Å, 150 × 4.60 mm) using an Agilent 1100 series system equipped with a diode array ultraviolet detector. A gradient with eluent A (water–MeCN–TFA, 95:5:0.1, v/v/v) and eluent B (0.1% TFA in MeCN) rising linearly from 0 to 95% of B over 30.0 min was applied at a flow rate of 1 mL min^{-1} to determine the purity of peptides (λ = 210 nm). Ultra-high-performance liquid chromatography (UPLC) mass spectrometry (MS) analyses were performed on a Phenomenex Kinetex column (1.7 μm, 100 Å, 50 × 2.10 mm) using a Waters Acquity ultra-HPLC system. A gradient with eluent C (0.1% HCOOH in water) and eluent D (0.1% HCOOH in MeCN) rising linearly from 0 to 95% of D over 5.20 min at a flow rate of 0.6 mL min^{-1} was applied to analyse reaction mixtures. Preparative HPLC purification was performed on a C18 Phenomenex Luna column (5 μm, 100 Å, 250 × 20 mm) using an Agilent 1260 LC system equipped with a diode array ultraviolet detector. Various gradients with eluent A and eluent B at a flow rate of 20 mL min^{-1} were applied for the purification. Fractions containing the purified target peptide were identified using UPLC-MS or matrix assisted laser desorption ionisation–time of flight mass spectrometry (MALDI-TOF MS). Selected fractions were pooled and lyophilized. MALDI-TOF mass spectra were recorded with a Bruker microflex bench-top MALDI using a matrix of alpha-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid in water/MeCN (1:1, v/v) containing 0.1% TFA. The observed *m/z* corresponded to the monoisotopic ions, unless otherwise stated. High-resolution mass spectra (HRMS) were recorded using a maXis G3 quadrupole time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) source or on an Agilent 1290 UHPLC equipped with a diode array detector and coupled to Agilent 6550 QTOF mass spectrometer operated in positive electrospray or on a Bruker Solarix WR by either MALDI, or electrospray ionization (ESI). Nuclear magnetic resonance (NMR) spectra were recorded at 298 K using a Bruker Avance III HD (^{1}H NMR and ^{13}C NMR recorded at 600 MHz and 150 MHz, respectively). Chemical shifts are reported in parts per million (ppm) relative to the deuterated solvent peak of DMSO-d_6 (δ_H = 2.50 ppm; δ_C = 39.52 ppm) as internal standard.

Sequence-guided identification of autoinducting peptides (AIPs)

Sequencing of bacterial isolates. The AgrD-encoding chromosomal region was sequenced for strains in which their genome sequences were unknown. Bacterial DNA was extracted from a single colony by resuspension in water containing 100 µg/ml lysostaphin and incubation at 37 °C for 30 minutes followed by boiling (99 °C, 10 min) and removal of cell debris by centrifugation. The DNA was used as template in a PCR reaction (Phusion Hot Start II DNA Polymerase, Thermo Fisher Scientific) using primers designed to amplify approximately 1000 bp encompassing the *agrD* sequence from respective species (Supplementary Table 2) deduced from publicly available genomes. PCR fragments were purified (GeneJET PCR Purification Kit, Thermo Fisher Scientific) and sequenced bi-directionally at Macrogen using the same oligonucleotides.

Preparation of bacterial supernatants. Bacterial isolates were streaked on agar plates and grown over night at 37 °C. Single colonies were then inoculated in 50 mL TSB media overnight at 37 °C in an incubator with 200 rpm shaking. Overnight cultures were centrifuged with 8000 rpm at 4 °C and supernatants filtered through a sterile filter (0.22 µm) and lyophilised. The lyophilised supernatants were stored at -20 °C until they were used.

Preparation of Cys-Rink resin (5). Amino PEGA resin (1.0 g, 0.37 mmol/g) was placed in a polypropylene syringe equipped with a fritted disk, swelled in DMF for 30 min and washed with DMF (5 × 1 min). Fmoc-Rink-amide linker (998 mg, 1.85 mmol, 5.0 equiv), HATU (688 mg, 1.81 mmol, 4.9 equiv), and *i*-Pr_2NEt (644 µL, 3.70 mmol, 10.0 equiv) were pre-incubated in DMF (10.0 mL) for 2 min and then added to the resin. After 2 h, the resin was washed with DMF (3 × 1 min), MeOH (3 × 1 min), and CH_2Cl_2 (3 × 1 min) and treated with a capping solution (Ac_2O–*i*-Pr_2NEt–CH_2Cl_2, 2:2:6, v/v/v, 10.0 mL). After 2 h, the resin was washed with DMF (3 × 1 min), MeOH (3 × 1 min), and CH_2Cl_2 (3 × 1 min). The resin was then treated with piperidine in DMF (1:4, v/v, 10.0 mL) (1 × 2 min, 1 × 20 min) and washed with DMF (3 × 1 min), MeOH (3 × 1 min), and CH_2Cl_2 (3 × 1 min). Fmoc-Cys(S*t*-Bu)-OH (479 mg, 1.11 mmol, 3.0 equiv), HATU (408 mg, 1.07 mmol, 2.9 equiv), and *i*-Pr_2NEt (386 µL, 2.22 mmol, 6.0 equiv) were pre-incubated in DMF (10.0 mL) for 2 min and then added to the resin. After 2 h, the resin was washed with DMF (3 × 1 min), MeOH (3 × 1 min), and CH_2Cl_2 (3 × 1 min) and dried under high vacuum for 16 h.

Native chemical ligation (NCL) trapping of AIPs from bacterial supernatants. Fmoc-Cys(StBu)-Rink-PEGA resin (**5**) (50 mg) was placed in a 10 mL polypropylene syringe equipped with a fritted disk, swelled in DMF for 30 min and washed with DMF (5 × 1 min). The resin was treated with piperidine in DMF (1:4, v/v, 2.0 mL) (1 × 2 min, 1 × 20 min) and washed with DMF (3 × 1 min), MeOH (3 × 1 min), and H_2O (3 × 1 min). The resin was treated with a TCEP solution (0.5 M, pH = 7.00, 0.5 mL) for 15 min and subsequently washed with H_2O (3 × 1 min), MeOH (3 × 1 min) and DMF (3 × 1 min). Lyophilised supernatant (original volume = 50 mL) was dissolved in 10 mL trapping buffer (phosphate buffer [0.1 M, pH = 7.4]–DMF–TCEP solution [0.5 M, pH = 7.0] = 7.4:2:0.6, v/v/v) and the solution was added to the resin. The syringe containing the trapping mixture was

agitated at 37 °C overnight. The trapping solution was removed from resin and the resin subsequently washed with DMF (3 × 1 min), MeOH (3 × 1 min), and H_2O (3 × 1 min). The resin was then treated with a TCEP solution (0.5 M, pH = 7.00, 0.5 mL) for 10 min and subsequently washed with H_2O (3 × 2 mL). A solution of DMF in H_2O (10 mL, 1:1, v/v) was added to the resin and the resin was agitated at 37 °C. After 30 min, was the resin washed with DMF (3 × 1 min), MeOH (3 × 1 min), and DCM (3 × 1 min) and dried under suction for 15 min. The dried resin was treated with a cleavage cocktail (1.5 mL, TFA–MilliQ water, 97:3, v/v) for 2 h at room temperature. The peptide containing cleavage solution was removed from the resin, collected and the resin rinsed with neat TFA (1.0 mL). The combined TFA fractions were evaporated under N_2 stream to near dryness, redissolved in a solution of MeCN in H_2O (100 µL, 1:1, v/v) and filtered (0.22 µm). The solution was analysed by LC-MS as descripted.

Sequence-guided LC-MS analysis. The filtered TFA cleavage solution was analysed using an Waters Acquity ultra-HPLC system equipped with a Phenomenex Kinetex column (1.7 µm, 100 Å, 50 × 2.10 mm) applying a gradient with eluent C (0.1% HCOOH in water) and eluent D (0.1% HCOOH in MeCN) rising linearly from 0 to 95% of D over 10.0 min at a flow rate of 0.6 mL min^{-1} and an injection volume of 40 µL. The total ion chromatograms (TIC) were analysed by displaying extracted ion chromatograms (EIC) of *m/z* $[M+H]^+$ values of the possible linear peptides with an additional *C*-terminal cysteine and amide functionality based on the obtained AgrD sequence.

Synthesis of autoinducing peptides (AIP)

General protocol for automated peptide synthesis. Automated peptide synthesis was carried out on a Biotage SyroWave™ synthesiser using standard Fmoc SPPS chemistry. The following Fmoc-protected amino acids with side chain protecting groups were used: Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*-Bu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*-Bu)-OH, Fmoc-Thr(*t*-Bu)-OH, Fmoc-Tyr(*t*-Bu)-OH, and Fmoc-Val-OH. The following Boc-protected amino acids with side chain protecting groups were used as *N*-terminal amino acids: Boc-Ala-OH, Boc-Asp(O*t*-Bu)-OH, Boc-Lys(Boc)-OH and Boc-Tyr(*t*-Bu)-OH.

SPPS was performed on 0.02 mmol scale using MeDbz-Gly-ChemMatrix (**15**)[4] resin containing additional 5% MeDbz-Rink-ChemMatrix. Fmoc deprotection was performed in two stages: 1) piperidine in DMF (2:3, v/v) for 3 min and 2) piperidine in DMF (1:4, v/v) for 12 min. The deprotection was followed by washing with DMF (2 × 45 s), CH_2Cl_2 (1 × 45 s), and DMF (2 × 45 s). The first coupling reaction was performed as double coupling using Fmoc-Xaa-OH (5.0 equiv to the resin loading), HATU (4.9 equiv) and *i*-Pr_2NEt in NMP (10 equiv, 2.0 M) in DMF (final concentration = 0.2 M) for 90 min. The remaining coupling reactions were performed as double couplings with Fmoc-Xaa-OH (5.0 equiv to the resin loading), HBTU (4.9 equiv) and *i*-Pr_2NEt in NMP (10 equiv, 2.0 M) in DMF (final concentration = 0.2 M) for 40 min for each coupling. The last amino acid was incorporated as Boc-Xaa-OH.

General procedure for N-acyl-benzimidazolinone (Nbz) formation. After automated peptide elongation, the resin (0.02 mmol, 1.0 equiv) was transferred into a polypropylene syringe equipped with a fritted disk using CH_2Cl_2 and the resin was then washed with CH_2Cl_2 (5 × 1 min). A solution of 4-nitrophenyl-chloroformate (20.1 mg, 0.10 mmol, 5.0 equiv) in CH_2Cl_2 (1.0 mL) was added to the resin and the suspension was agitated for 30 min. The resin was then washed with CH_2Cl_2 (2 × 1 min) and the procedure was repeated. The resin was then washed with CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min) and a solution of *i*-Pr_2NEt (87 μL, 0.50 mmol, 25.0 equiv) in DMF (1.0 mL) was added to the resin. After 15 min, the resin was washed with DMF (3 × 1 min) and the procedure was repeated. The resin was then washed with DMF (3 × 1 min), *i*-Pr_2NEt in DMF (5%, v/v) (3 × 1 min), DMF (3 × 1 min), MeOH (3 × 1 min), and CH_2Cl_2 (3 × 1 min) and dried under high vacuum.

General procedure for on-resin cyclisation/cleavage. Dried peptidyl MeNbz-Gly-ChemMatrix resin (0.02 mmol, 1.0 equiv) containing additional 5% MeNbz-Rink-ChemMatrix was placed in a polypropylene syringe equipped with a fritted disk and treated with a deprotection/cleavage cocktail (2.0 mL, TFA–*i*-Pr_3SiH–water, 94:3:3, v/v/v) for 1 h. The TFA solution was collected for assessment of the peptide synthesis and the resin was washed with CH_2Cl_2 (3 × 1 min), DMF (3 × 1 min), and CH_2Cl_2 (3 × 1 min) and dried under suction for several minutes. The cyclisation buffer (5.0 mL, phosphate buffer (0.2 M, pH = 6.8)–MeCN, 1:1, v/v) was added to the resin (final concentration = 4.0 mM) and the suspension was agitated at 50 °C for 2 h. The solution was removed from the resin, collected and the resin was rinsed with fresh cyclisation buffer. The combined peptide-containing washings were pooled and purified by preparative RP-HPLC. Fractions containing pure peptide were lyophilised to afford the desired cyclised peptide.

β-Lactamase assay for IC_{50}/EC_{50} determination

Sample concentration determination. Concentrations of DMSO stock solutions for compounds containing tyrosine were determined on a NanoDrop^{TM} One^{C} microvolume UV-spectrophotometer from ThermoFisher Scientific by UV absorbance at 280 nm according to the following equation:

$$c\left(\frac{mg}{mL}\right) = \frac{A280 \times MW \times \text{dilution factor}}{e}$$

A280 = Absorbance at 280 nm [AU]; e = molar extinction coefficient [number of tyrosine residues × 1200 AU/mmol/mL]

Concentrations of DMSO stock solutions for compounds without the tyrosine chromophore were determined by quantitative NMR relative to the signal (δ_H = 6.24 ppm, 2H) of the internal standard maleic acid (qNMR grade) by using the following equation:

$$c\,(\text{peptide})\left(\frac{mmol}{L}\right) = \frac{I(\text{peptide}) \times N(\text{maleic acid}) \times c(\text{maleic acid})}{I(\text{maleic acid}) \times N(\text{peptide})}$$

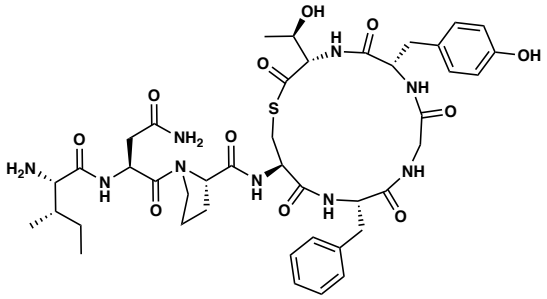
I = Integral of chosen proton signal; N = Number of protons; c = concentration of diluted DMSO stock

Construction of reporter strains and plasmids. *β*-Lactamase reporter strains for AIP-II, AIP-III, and AIP-IV were constructed by replacement cloning of *agrC*-II, *agrC*-III, and *agrC*-IV into p*agrC*-I, respectively. Fragments encoding the respective receptor variants were amplified by PCR (Phusion Hot Start II DNA Polymerase, Thermo Fisher Scientific) using chromosomal DNA templates obtained from strains RN6607 (type II), MW2 (type III), and RN4850 (type IV). Forward primers 5'-GATACACTGCAGGGCGTAAACGCTTGCAGC-3'(type II), 5'-GATACACTGCAGCTATAAGAGAAAGTGTGATAGTAAGTGGAAGC -3' (type III), and 5'-GATACACTGCAGGTACATGTTATTTCATAATGGACGAAGTTG -3' (type IV) were combined with a common reverse primer, 5'- GATACAGGTACCCATACATTCACATCCTTATGGCTAGTTG -3'. Purified nucleotide fragments were subsequently cloned into p*agrC*-I by directional cloning using PstI and KpnI restriction enzymes and T4 DNA ligase combined with dephosphorylation of the vector with FastAP Thermosensitive Alkaline Phosphatase (all enzymes from Thermo Fisher Scientific), generating p*agrC*-II, p*agrC*-III, and p*agrC*-IV, respectively. Plasmid constructs were generated in *E. coli* IM08B from where they were transformed into *S. aureus* RN10829.[5]

Assay protocol for IC_{50}/EC_{50} determination. The *β*-lactamase reporter strains RN10829 [(P2-*agrA*:P3-*blaZ*)],[5] with p*agrC*-I[3] or p*agrC*-II, p*agrC*-III or p*agrC*-IV substituting the native *agr* locus with a chromosomal integration of P2-*agrA* and P3-*blaZ* and a plasmid from which a wild-type variant of the corresponding AgrC is expressed, were used to asses inhibition and activation of the AgrC receptor via *β*-lactamase activity in response to varying concentrations of the QS modifying peptides. Overnight cultures of the reporter strains in TSB medium were diluted 1:250 in fresh TSB medium and grown to OD_{600} = 0.35–0.45 (early exponential phase) at 37 °C. For inhibition assays, peptide stock solutions (10 μL) in 1:10 serial dilutions (final concentrations = 10 μM–10 pM) were added to each well of a 96-well plate as well as stock solutions (10 μL) of cognate AIP (final concentration = 100 nM) followed by 80 μL of bacterial cells. For activation assays, peptide stock solutions (10 μL) in 1:10 serial dilutions (final concentrations = 10 μM–10 pM) were added to each well of a 96-well plate as well as TSB medium (10 μL) followed by 80 μL of bacterial cells. Positive controls were wells replacing peptide stock solution with TSB medium (10 μL) and negative controls were wells replacing both peptide and cognate AIP stock solution with TSB medium (20 μL). The 96-well plates were incubated at 37 °C shaking at 200 rpm for 1 h and immediately frozen down at -80 °C to minimize growth during nitrocefin treatment. Next, the 96-well plates were thawed and OD_{600} values were determined using a plate-reader for normalization followed by addition of 50 μL of nitrocefin solution to the wells (final concentration = 33.3 μg/mL). *β*-Lactamase activity was monitored at OD_{486} every 20 s for 10 min at 37 °C using a plate-reader. Linear nitrocefin conversion rates were plotted to obtain IC_{50}/EC_{50} values by non-linear regression with variable slope using GraphPad Prism 7.0 software. Assays were performed at least as duplicate determinations in biological triplicate.

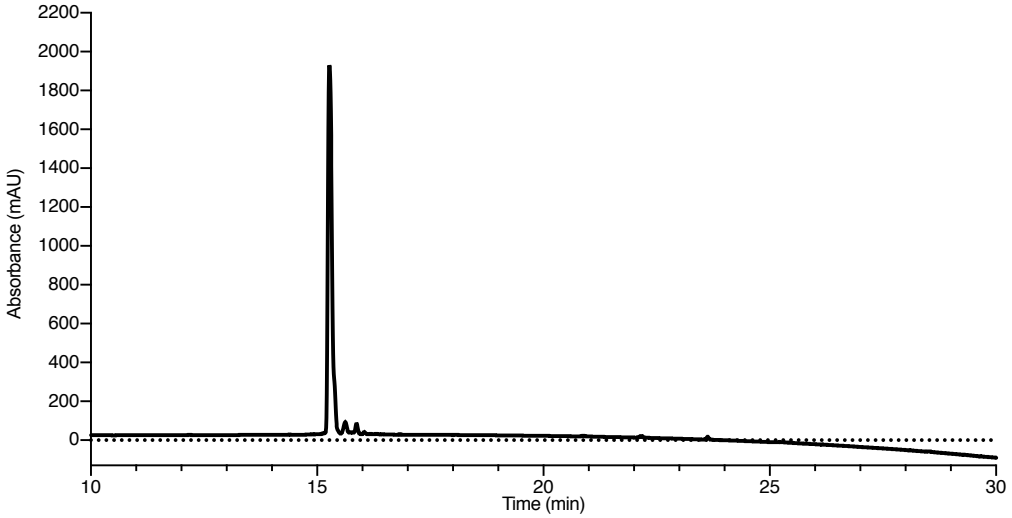
Compound characterisation

***S. saprophyticus* AIP (S_{sa}-AIP, 8)**



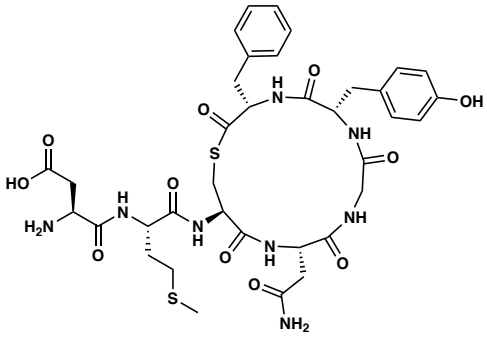
$C_{42}H_{57}N_9O_{11}S$
MW: 896.03

The peptide was synthesised using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *S. saprophyticus* AIP (**8**) as a fluffy white solid after lyophilisation (2.6 mg, 15%). Analytical HPLC t_R = 15.3 min, purity 95% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{42}H_{58}N_9O_{11}S^+$ 896.3971; found 896.3962. Major conformer (conformers were observed in a ration of 1:0.1): ^{1}H NMR (600 MHz, DMSO-d_6) δ = 0.84 (t, *J* = 7.3 Hz, 3H), 0.88 (d, *J* = 6.9 Hz, 3H), 1.05 (d, *J* = 6.4 Hz, 3H), 1.08–1.15 (m, 1H), 1.41–1.49 (m, 1H), 1.63–1.69 (m, 1H), 1.74–1.80 (m, 1H), 1.81–1.93 (m, 3H), 2.42 (dd, *J* = 15.9, 7.5 Hz, 1H), 2.57–2.64 (m, 2H), 2.69–2.80 (m, 2H), 2.86 (dd, *J* = 13.9, 5.9 Hz, 1H), 3.05 (dd, *J* = 14.1, 3.3 Hz, 1H), 3.09 (dd, *J* = 13.0, 4.9 Hz, 1H), 3.18–3.23 (m, 1H), 3.53–3.68 (signals partially observed), 3.82 (dd, *J* = 14.0, 5.1 Hz, 1H), 4.25–4.34 (m, 2H), 4.35 (ddd, *J* = 11.1, 8.2, 5.0 Hz, 1H), 4.44 (dd, *J* = 9.7, 1.9 Hz, 1H), 4.49 (qd, *J* = 6.4, 1.9 Hz, 1H), 4.61 (td, *J* = 9.4, 5.9 Hz, 1H), 4.88 (q, *J* = 6.9 Hz, 1H), 4.93 (br s, 1H), 6.63–6.68 (m, 2H), 6.99 (s, 1H), 7.05–7.09 (m, 2H), 7.13–7.24 (m, 5H), 7.46 (s, 1H), 7.94 (d, *J* = 9.7 Hz, 1H), 8.03–8.09 (m, 4H), 8.12 (d, *J* = 9.4 Hz, 1H), 8.54 (t, *J* = 5.5 Hz, 1H), 8.67 (d, *J* = 7.1 Hz, 1H), 8.86 (d, *J* = 8.2 Hz, 1H), 9.21 (br s, 1H).



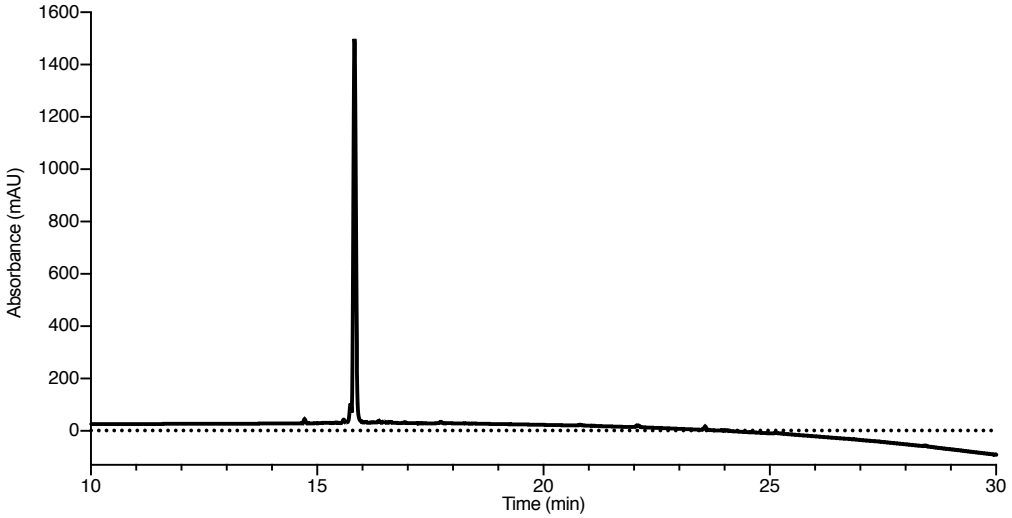
Analytical HPLC of purified *S. saprophyticus* AIP (**8**): t_R = 15.3 min (λ = 210 nm).

***S. lugdunensis* AIP-II (*S*$_{lu}$-AIP-II, 9)**



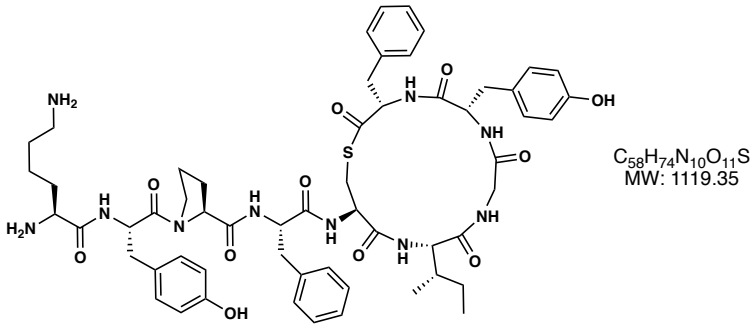
$C_{36}H_{46}N_8O_{11}S_2$
MW: 830.93

The peptide was synthesised on 10 μmol scale using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *S. lugdunensis* AIP-II (**9**) as a fluffy white solid after lyophilisation (1.1 mg, 13%). Analytical HPLC t_R = 15.7 min, purity 95% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{36}H_{47}N_8O_{11}S_2^+$ 831.2800; found 831.2791. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 1.75–1.83 (m, 1H), 1.86–1.96 (m, 1H), 2.04 (s, 3H), 2.36 (dd, *J* = 14.3, 10.4 Hz, 1H), 2.39–2.49 (m, 4H), 2.59 (dd, *J* = 14.3, 4.3 Hz, 1H), 2.67 (dd, *J* = 17.6, 8.7 Hz, 1H), 2.78 (dd, *J* = 13.0, 10.1 Hz, 1H), 2.84 (dd, *J* = 17.6, 3.9 Hz, 1H), 2.92 (dd, *J* = 14.1, 11.1 Hz, 1H), 3.17 (dd, *J* = 13.0, 4.7 Hz, 1H), 3.38–3.44 (m, 1H), 3.85 (dd, *J* = 14.5, 4.6 Hz, 1H), 4.07–4.16 (m, 2H), 4.38–4.45 (m, 2H), 4.59 (ddd, *J* = 11.1, 9.2, 3.9 Hz, 1H), 4.69 (dt, *J* = 8.8, 6.8 Hz, 1H), 6.61 (d, *J* = 8.5 Hz, 2H), 6.84–6.89 (m, 3H), 7.19–7.26 (m, 3H), 7.26–7.32 (m, 2H), 7.34 (s, 1H), 8.18 (t, *J* = 5.3 Hz, 1H), 8.22 (d, *J* = 9.3 Hz, 1H), 8.30 (d, *J* = 7.8 Hz, 1H), 8.38 (d, *J* = 8.7 Hz, 1H), 8.42 (d, *J* = 7.8 Hz, 1H), 8.63 (d, *J* = 7.8 Hz, 1H), 9.19 (s, 1H).

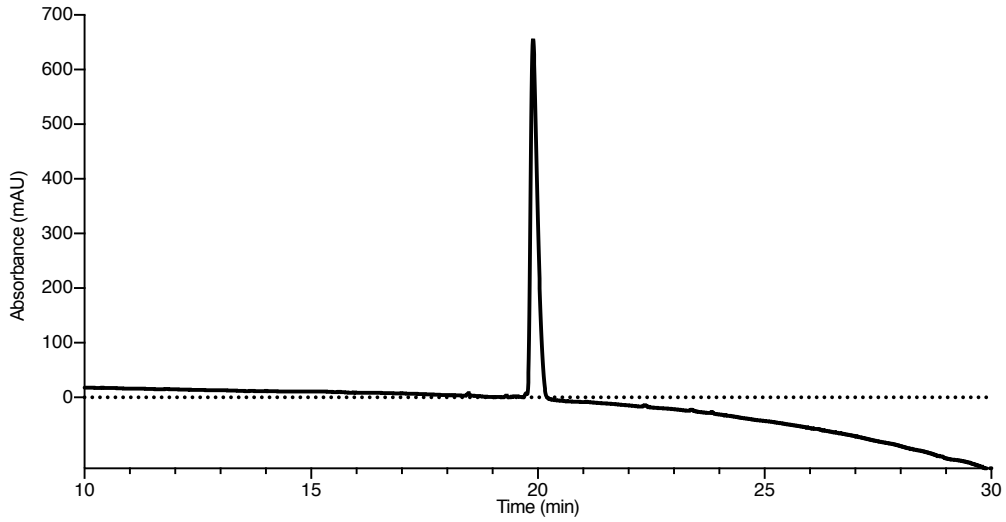


Analytical HPLC of purified *S. lugdunensis* AIP-II (**9**): t_R = 15.7 min (λ = 210 nm).

***S. schleiferi* AIP (S_{sc}-AIP, 10)**

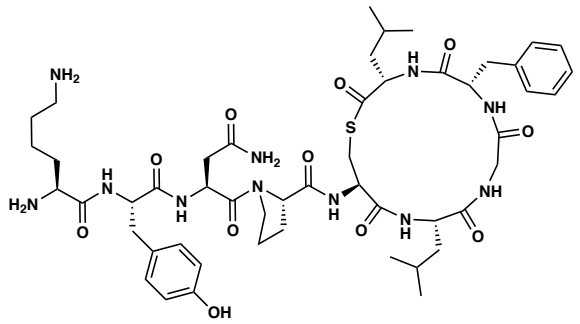


The peptide was synthesised using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *S. schleiferi* AIP (**10**) as a fluffy white solid after lyophilisation (9.1 mg, 41%). Analytical HPLC t_R = 19.9 min, purity 96% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{58}H_{75}N_{10}O_{11}S^+$ 1119.5332; found 1119.5307. Major conformer (conformers were observed in a ration of 1:0.25): ^{1}H NMR (600 MHz, DMSO-d_6): δ = 0.79–0.85 (m, 6H), 1.01–1.12 (m, 1H), 1.24–1.39 (m, 2H), 1.45–1.58 (m, 4H), 1.65–1.76 (m, 4H), 1.76–1.86 (m, 2H), 1.90–1.97 (m, 1H), 2.29 (dd, *J* = 14.2, 11.2 Hz, 1H), 2.63 (dd, *J* = 13.0, 11.1 Hz, 1H), 2.65–2.82 (m, 4H), 2.84–2.96 (m, 2H), 3.04–3.12 (m, 2H), 3.21 (dd, *J* = 13.0, 5.2 Hz, 1H), 3.26–3.33 (m, 1H), 3.35 (dd, *J* = 14.6, 3.4 Hz, 1H), 3.43–3.50 (m, 1H), 3.60–3.66 (m, 1H), 3.70–3.76 (m, 1H), 3.86–3.94 (m, 1H), 4.10–4.20 (m, 2H), 4.27 (dd, *J* = 8.4, 4.1 Hz, 1H), 4.41–4.48 (m, 1H), 4.51–4.66 (m, 3H), 6.62 (d, *J* = 8.4 Hz, 2H), 6.66–6.72 (m, 2H), 6.90–6.98 (m, 2H), 7.12 (d, *J* = 8.5 Hz, 2H), 7.15–7.25 (m, 6H), 7.26–7.35 (m, 4H), 7.65 (d, *J* = 8.0 Hz, 1H), 7.70–7.77 (m, 3H), 8.04–8.11 (m, 5H), 8.18 (d, *J* = 9.4 Hz, 1H), 8.66 (d, *J* = 7.9 Hz, 1H), 8.73 (d, *J* = 7.3 Hz, 1H), 8.94 (t, *J* = 5.6 Hz, 1H), 9.10–9.42 (m, 2H).

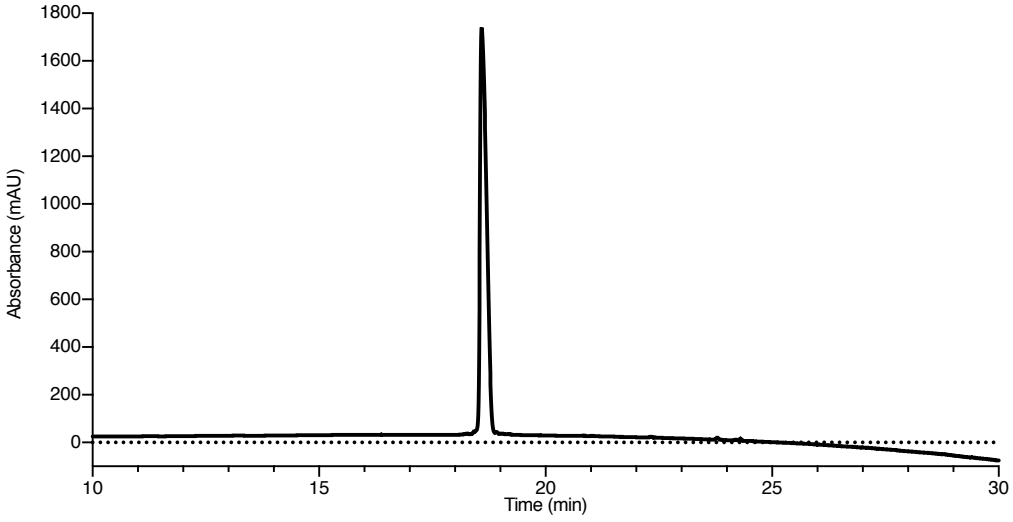


Analytical HPLC of purified *S. schleiferi* AIP (**10**): t_R = 19.9 min (λ = 210 nm).

***S. simulans* AIP (S_{si}-AIP, 11)**

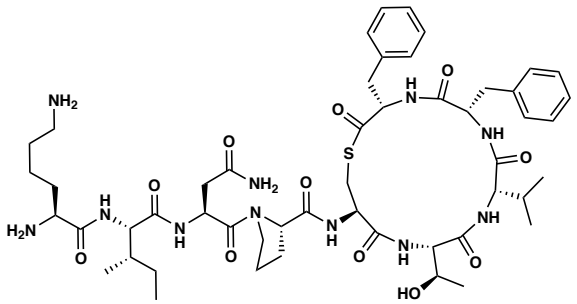


The peptide was synthesised using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *S. simulans* AIP (**11**) as a fluffy white solid after lyophilisation (8.4 mg, 41%). Analytical HPLC t_R = 18.7 min, purity 99% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{50}H_{74}N_{11}O_{11}S^+$ 1036.5284; found 1036.5257. Major conformer (conformers were observed in a ration of 1:0.1): ^{1}H NMR (600 MHz, DMSO-d_6) δ = 0.81 (d, J = 6.5 Hz, 3H), 0.83 (d, J = 6.4 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H), 0.92 (d, J = 6.4 Hz, 3H), 1.27–1.37 (m, 3H), 1.42–1.48 (m, 1H), 1.48–1.56 (m, 3H), 1.58–1.66 (m, 1H), 1.65–1.74 (m, 3H), 1.78–1.87 (m, 4H), 1.89–1.96 (m, 1H), 2.37 (dd, J = 15.6, 6.8 Hz, 1H), 2.56–2.64 (m, 2H), 2.68 (dd, J = 14.0, 8.7 Hz, 1H), 2.72–2.82 (m, 3H), 2.88 (dd, J = 14.2, 4.8 Hz, 1H), 3.11 (dd, J = 13.1, 5.0 Hz, 1H), 3.18 (dd, J = 14.2, 3.7 Hz, 1H), 3.27–3.31 (m, 1H), 3.50–3.60 (signals partially observed), 3.73–3.78 (m, 2H), 4.25 (ddd, J = 11.1, 7.9, 5.2 Hz, 1H), 4.35–4.48 (m, 4H), 4.52 (td, J = 8.2, 5.0 Hz, 1H), 4.81 (q, J = 7.1 Hz, 1H), 6.64 (d, J = 8.5 Hz, 2H), 6.94 (s, 1H), 7.01 (d, J = 8.5 Hz, 2H), 7.18–7.22 (m, 1H), 7.23–7.30 (m, 4H), 7.46 (s, 1H), 7.70–7.79 (m, 3H), 8.02–8.11 (m, 6H), 8.49–8.55 (m, 2H), 8.70 (d, J = 8.5 Hz, 1H), 8.74 (t, J = 5.5 Hz, 1H), 9.24 (br s, 1H).



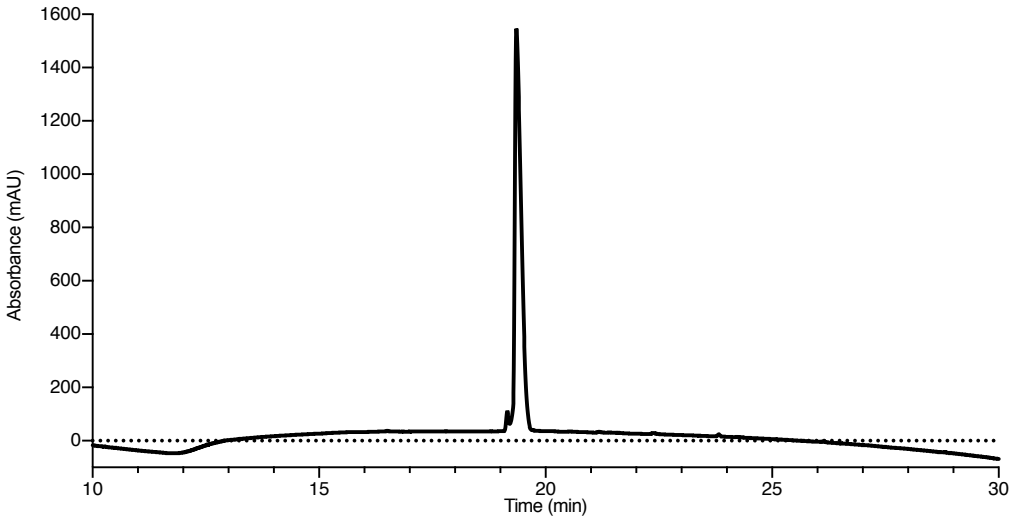
Analytical HPLC of purified *S. simulans* AIP (**11**): t_R = 18.7 min (λ = 210 nm).

***S. hyicus* AIP (S_{hy}-AIP, 12)**



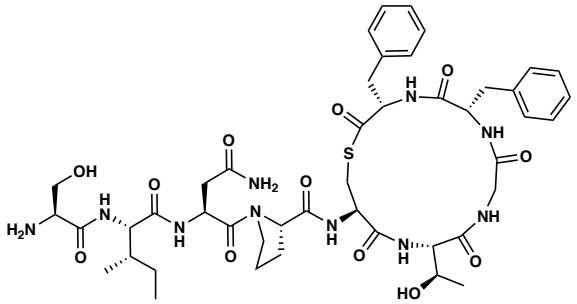
$C_{51}H_{75}N_{11}O_{11}S$
MW: 1050.29

The peptide was synthesised using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *S. hyicus* AIP (**12**) as a fluffy white solid after lyophilisation (5.4 mg, 26%). Analytical HPLC t_R = 19.5 min, purity 95% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{51}H_{76}N_{11}O_{11}S^+$ 1050.5441; found 1050.5411. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 0.72 (d, *J* = 6.7 Hz, 3H), 0.79–0.86 (m, 9H), 1.02 (d, *J* = 6.0 Hz, 3H), 1.04–1.12 (m, 1H), 1.27–1.36 (m, 2H), 1.39–1.47 (m, 1H), 1.49–1.57 (m, 2H), 1.64–1.72 (m, 3H), 1.78–1.84 (m, 1H), 1.84–1.89 (m, 1H), 1.90–1.97 (m, 2H), 1.97–2.05 (m, 1H), 2.42 (dd, *J* = 15.7, 7.1 Hz, 1H), 2.59 (dd, *J* = 15.7, 6.6 Hz, 1H), 2.74–2.81 (m, 3H), 2.81–2.84 (m, 2H), 3.00 (t, *J* = 12.5 Hz, 1H), 3.16 (dd, *J* = 12.5, 3.7 Hz, 1H), 3.24 (dd, *J* = 14.0, 3.8 Hz, 1H), 3.62–3.69 (m, 1H), 3.70–3.78 (m, 1H), 3.83–3.89 (m, 1H), 3.95–4.03 (m, 3H), 4.08 (dd, *J* = 10.1, 8.1 Hz, 1H), 4.25 (dd, *J* = 8.7, 7.3 Hz, 1H), 4.32 (dd, *J* = 8.4, 3.8 Hz, 1H), 4.42 (q, *J* = 7.5 Hz, 1H), 4.48 (ddd, *J* = 12.0, 8.0, 3.9 Hz, 1H), 4.82 (q, *J* = 7.1 Hz, 1H), 5.08 (br s, 1H), 6.82–6.87 (m, 2H), 6.93 (s, 1H), 7.10–7.16 (m, 5H), 7.20–7.28 (m, 3H), 7.52 (s, 1H), 7.72–7.79 (m, 4H), 7.82 (d, *J* = 10.1 Hz, 1H), 8.13 (d, *J* = 5.4 Hz, 3H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.39 (d, *J* = 8.7 Hz, 1H), 8.42–8.51 (m, 2H), 9.02 (d, *J* = 7.6 Hz, 1H).



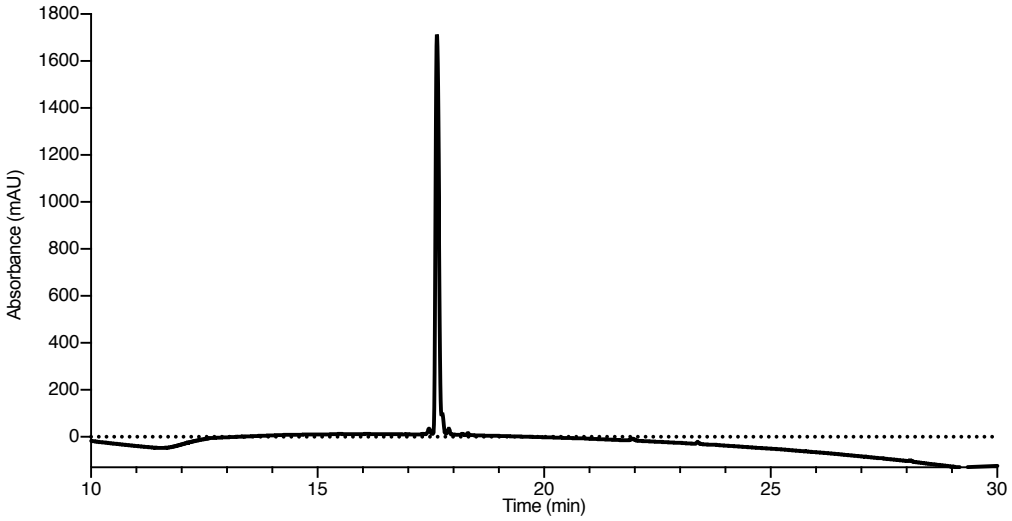
Analytical HPLC of purified *S. hyicus* AIP (**12**): t_R = 19.5 min (λ = 210 nm).

***S. chromogenes* AIP (S_{ch}-AIP, 13)**



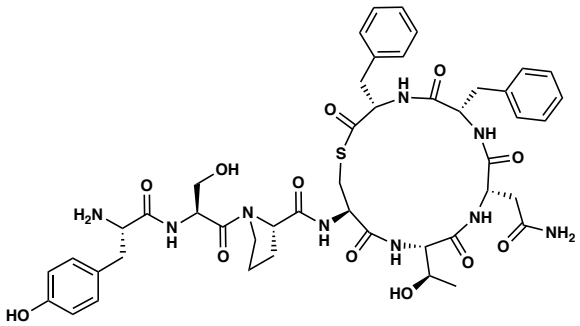
$C_{45}H_{62}N_{10}O_{12}S$
MW: 967.11

The peptide was synthesised using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *S. chromogenes* AIP (**13**) as a fluffy white solid after lyophilisation (5.5 mg, 28%). Analytical HPLC t_R = 17.8 min, purity 95% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{45}H_{63}N_{10}O_{12}S^+$ 967.4342; found 967.4337. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 0.79–0.85 (m, 6H), 1.03 (d, *J* = 6.3 Hz, 3H), 1.04–1.11 (m, 1H), 1.36–1.45 (m, 1H), 1.66–1.74 (m, 1H), 1.81–1.90 (m, 3H), 1.92–2.01 (m, 1H), 2.37–2.46 (m, 2H), 2.60 (dd, *J* = 15.6, 7.0 Hz, 1H), 2.74 (dd, *J* = 14.4, 3.9 Hz, 1H), 2.76–2.87 (m, 2H), 3.14 (dd, *J* = 13.0, 4.3 Hz, 1H), 3.38 (dd, *J* = 14.1, 4.0 Hz, 1H), 3.60–3.68 (m, 4H), 3.69–3.77 (m, 2H), 3.89–3.95 (m, 1H), 3.96–4.02 (m, 1H), 4.17 (dd, *J* = 8.9, 3.9 Hz, 1H), 4.22–4.29 (m, 2H), 4.32–4.40 (m, 2H), 4.70 (ddd, *J* = 11.1, 9.5, 4.0 Hz, 1H), 4.77 (q, *J* = 7.0 Hz, 1H), 4.89 (br s, 1H), 5.50 (br s, 1H), 6.97 (s, 1H), 7.09–7.13 (m, 2H), 7.16–7.28 (m, 6H), 7.28–7.34 (m, 2H), 7.47 (s, 1H), 7.53 (d, *J* = 8.9 Hz, 1H), 8.08 (d, *J* = 5.0 Hz, 3H), 8.20 (d, *J* = 9.5 Hz, 1H), 8.24 (d, *J* = 7.9 Hz, 1H), 8.27–8.34 (m, 2H), 8.39 (d, *J* = 8.8 Hz, 1H), 8.59 (d, *J* = 7.9 Hz, 1H).



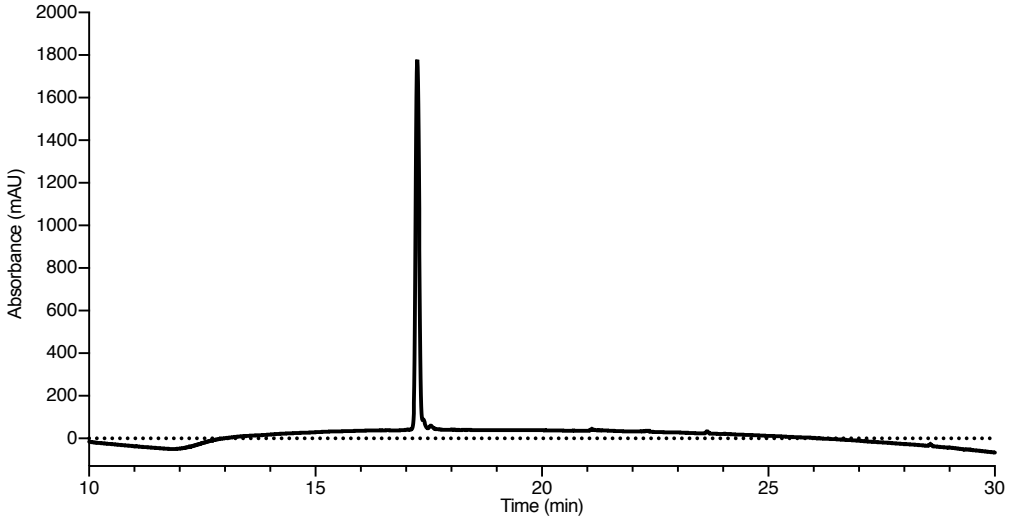
Analytical HPLC of purified *S. chromogenes* AIP (**13**): t_R = 17.8 min (λ = 210 nm).

***S. warneri* AIP (*S*$_{wa}$-AIP, 14)**



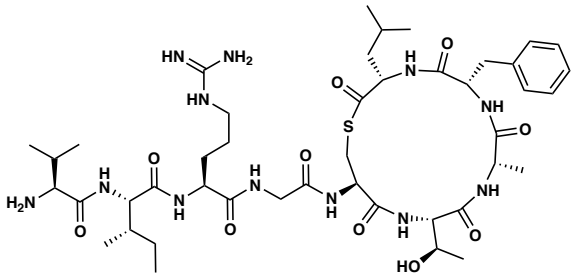
$C_{46}H_{57}N_9O_{12}S$
MW: 960.07

The peptide was synthesised using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *S. warneri* AIP (**14**) as a fluffy white solid after lyophilisation (7.9 mg, 41%). Analytical HPLC t_R = 17.4 min, purity 97% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{46}H_{58}N_9O_{12}S^+$ 960.3920; found 960.3934. Major conformer (conformers were observed in a ration of 1:0.22): ^{1}H NMR (600 MHz, DMSO-d_6) δ = 0.99 (d, *J* = 6.1 Hz, 3H), 1.72–1.80 (m, 1H), 1.81–1.96 (m, 2H), 2.01–2.11 (m, 1H), 2.49–2.53 (signal partially observed), 2.59 (dd, *J* = 15.1, 6.1 Hz, 1H), 2.76–2.85 (m, 3H), 2.86–2.92 (m, 2H), 2.96 (dd, *J* = 14.3, 5.3 Hz, 1H), 3.18 (dd, *J* = 12.7, 3.8 Hz, 1H), 3.30 (dd, *J* = 14.0, 3.8 Hz, 1H), 3.50–3.60 (m, 2H), 3.62–3.71 (m, 2H), 3.97–4.03 (m, 2H), 4.03–4.12 (m, 2H), 4.27–4.37 (m, 1H), 4.36–4.47 (m, 2H), 4.52 (q, *J* = 7.1 Hz, 1H), 4.65 (q, *J* = 7.0 Hz, 1H), 4.93–5.10 (m, 2H), 6.69 (d, *J* = 8.1 Hz, 2H), 6.99–7.10 (m, 7H), 7.16–7.26 (m, 6H), 7.57 (s, 1H), 7.81 (d, *J* = 8.9 Hz, 1H), 7.99 (d, *J* = 9.3 Hz, 1H), 8.06 (d, *J* = 4.5 Hz, 3H), 8.37 (d, *J* = 7.5 Hz, 1H), 8.46 (d, *J* = 7.9 Hz, 1H), 8.71 (d, *J* = 8.4 Hz, 1H), 8.78 (d, *J* = 7.7 Hz, 1H), 9.37 (br s, 1H).



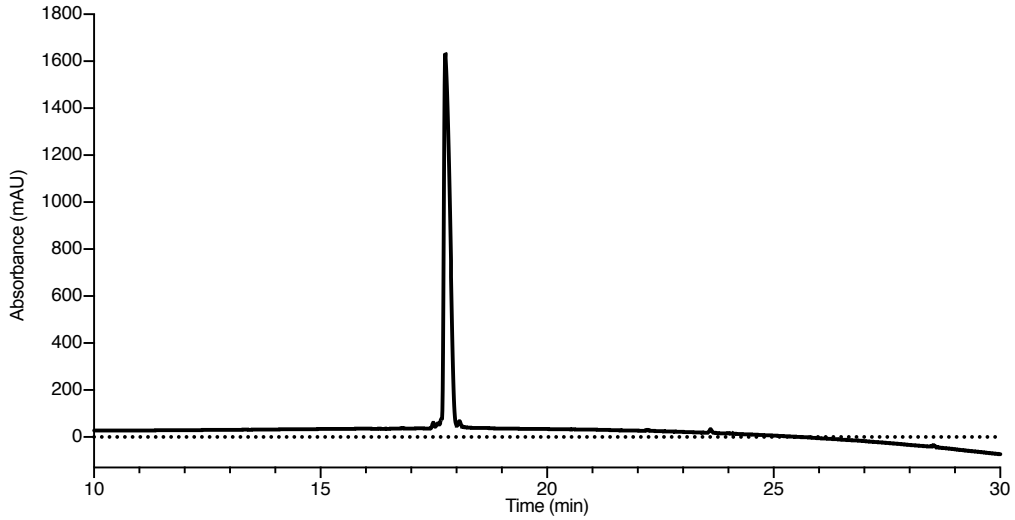
Analytical HPLC of purified *S. warneri* AIP (**14**): t_R = 17.4 min (λ = 210 nm).

***S. vitulinus* AIP (S_{vi}-AIP, 15)**



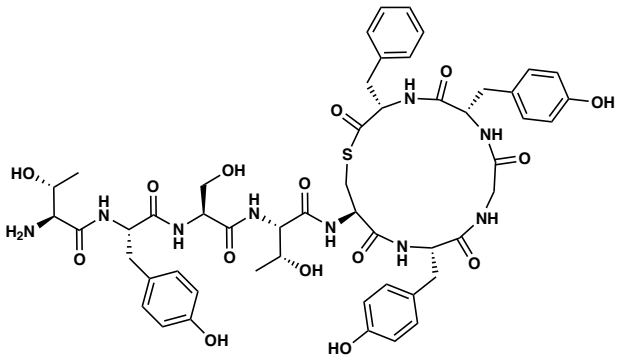
$C_{44}H_{72}N_{12}O_{10}S$
MW: 961.19

The peptide was synthesised using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *S. vitulinus* AIP (**15**) as a fluffy white solid after lyophilisation (9.4 mg, 49%). Analytical HPLC t_R = 17.8 min, purity 98% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{44}H_{73}N_{12}O_{10}S^+$ 961.5288; found 961.5283. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 0.77 (d, *J* = 6.5 Hz, 3H), 0.81 (t, *J* = 7.4 Hz, 3H), 0.82–0.87 (m, 6H), 0.87–0.91 (m, 6H), 0.99 (d, *J* = 6.2 Hz, 3H), 1.01–1.10 (m, 1H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.34–1.60 (m, 5H), 1.60–1.70 (m, 4H), 2.00 (dq, *J* = 13.5, 6.8 Hz, 1H), 2.89 (t, *J* = 12.3 Hz, 1H), 3.00 (dd, *J* = 13.4, 6.4 Hz, 1H), 3.05–3.12 (m, 4H), 3.63–3.70 (m, 2H), 3.78 (dd, *J* = 17.0, 6.3 Hz, 1H), 3.96–4.03 (m, 2H), 4.11–4.24 (m, 4H), 4.30 (td, *J* = 8.3, 6.6 Hz, 1H), 4.37 (ddd, *J* = 11.9, 7.9, 4.1 Hz, 1H), 5.01 (br s, 1H), 7.16–7.22 (m, 3H), 7.26–7.30 (m, 2H), 7.69 (t, *J* = 5.8 Hz, 1H), 7.77 (d, *J* = 8.4 Hz, 1H), 7.88 (d, *J* = 8.6 Hz, 1H), 8.07 (d, *J* = 5.4 Hz, 3H), 8.20 (d, *J* = 7.9 Hz, 1H), 8.26 (d, *J* = 7.9 Hz, 1H), 8.29–8.38 (m, 3H), 8.59 (d, *J* = 8.6 Hz, 1H).

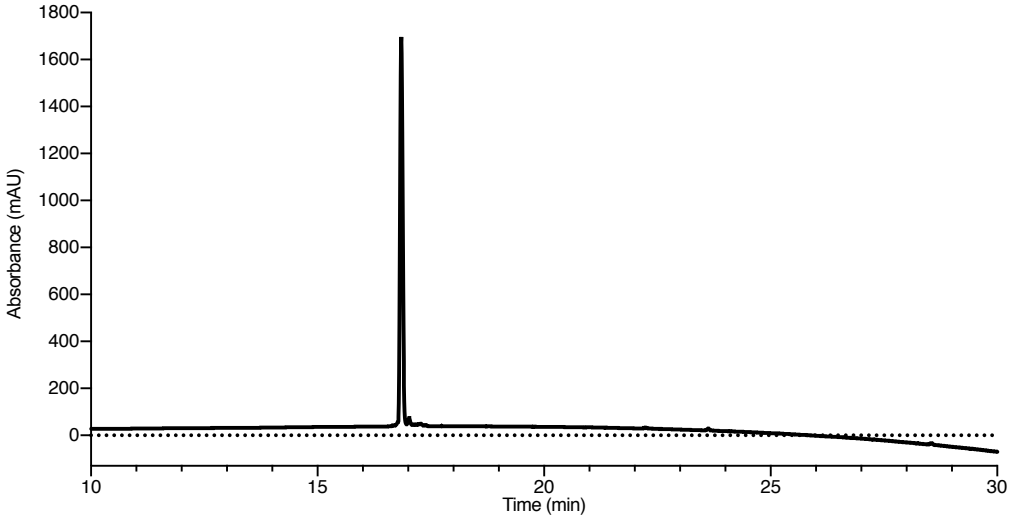


Analytical HPLC of purified *S. vitulinus* AIP (**15**): t_R = 17.8 min (λ = 210 nm).

***S. hominis* AIP (S_{ho}-AIP, 16)**

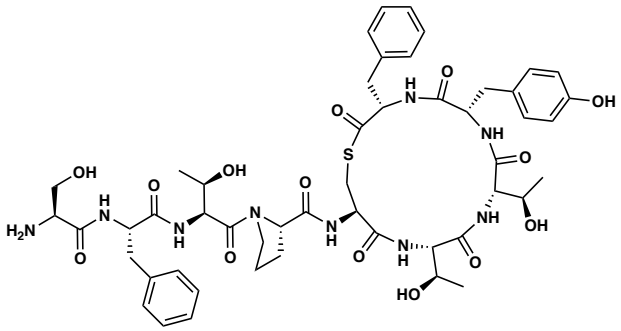


The peptide was synthesised using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *S. hominis* AIP (**16**) as a fluffy white solid after lyophilisation (8.2 mg, 38%). Analytical HPLC t_R = 16.9 min, purity 96% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{52}H_{64}N_9O_{15}S^+$ 1086.4237; found 1086.4221. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 0.99 (d, *J* = 6.3 Hz, 3H), 1.17 (d, *J* = 6.3 Hz, 3H), 2.31 (dd, *J* = 14.3, 10.9 Hz, 1H), 2.62 (dd, *J* = 13.0, 10.4 Hz, 1H), 2.66–2.80 (m, 4H), 2.99 (dd, *J* = 14.3, 3.9 Hz, 1H), 3.04 (dd, *J* = 14.3, 11.3 Hz, 1H), 3.16–3.27 (m, 2H), 3.36 (signal partially observed), 3.54–3.62 (m, 2H), 3.60–3.67 (m, 1H), 3.80 (dd, *J* = 14.1, 4.6 Hz, 1H), 3.82–3.90 (m, 1H), 4.00–4.06 (m, 1H), 4.13 (ddd, *J* = 11.4, 8.0, 3.8 Hz, 1H), 4.23 (dd, *J* = 8.4, 3.5 Hz, 1H), 4.41 (q, *J* = 6.3 Hz, 1H), 4.44–4.54 (m, 2H), 4.57–4.64 (m, 2H), 4.89 (d, *J* = 5.0 Hz, 1H), 5.14 (t, *J* = 5.3 Hz, 1H), 5.51 (d, *J* = 4.9 Hz, 1H), 6.59–6.64 (m, 4H), 6.64 (d, *J* = 8.5 Hz, 2H), 6.92 (d, *J* = 8.5 Hz, 2H), 7.00 (d, *J* = 8.5 Hz, 2H), 7.08 (d, *J* = 8.5 Hz, 2H), 7.21–7.25 (m, 1H), 7.27–7.34 (m, 4H), 7.74 (d, *J* = 8.4 Hz, 1H), 7.96 (d, *J* = 8.0 Hz, 1H), 7.98–8.04 (m, 3H), 8.18 (d, *J* = 9.2 Hz, 1H), 8.31 (d, *J* = 9.0 Hz, 1H), 8.36 (d, *J* = 7.5 Hz, 1H), 8.48–8.54 (m, 2H), 8.63 (t, *J* = 5.6 Hz, 1H), 9.18 (s, 1H), 9.19–9.24 (m, 2H).

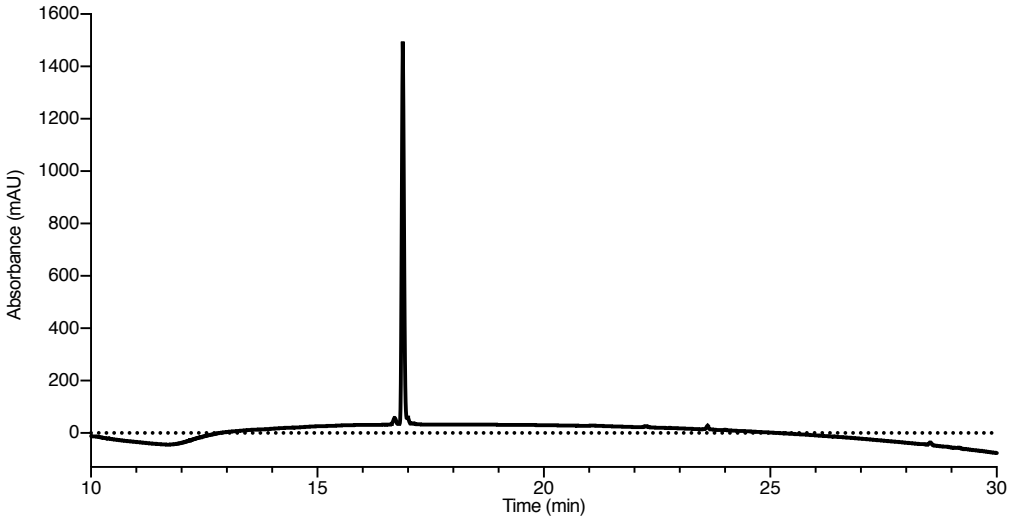


Analytical HPLC of purified *S. hominis* AIP (**16**): t_R = 16.9 min (λ = 210 nm).

***S. haemolyticus* AIP (S_{ha}-AIP, 17)**

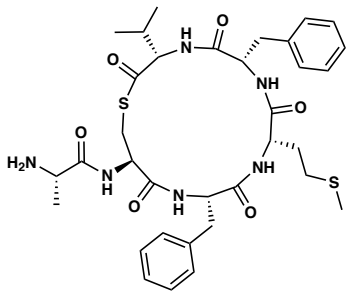


The peptide was synthesised using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *S. haemolyticus* AIP (**17**) as a fluffy white solid after lyophilisation (2.5 mg, 12%). Analytical HPLC t_R = 17.0 min, purity 96% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{50}H_{66}N_9O_{14}S^+$ 1048.4444; found 1048.4411. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 1.00–1.03 (m, 6H), 1.19 (d, J = 6.3 Hz, 3H), 1.70–1.77 (m, 1H), 1.76–1.84 (m, 1H), 1.89–1.96 (m, 1H), 1.99–2.09 (m, 1H), 2.67–2.79 (m, 3H), 2.83 (dd, J = 13.9, 8.6 Hz, 1H), 2.96 (t, J = 12.3 Hz, 1H), 3.05 (dd, J = 14.0, 4.2 Hz, 1H), 3.17–3.21 (m, 2H), 3.63–3.70 (m, 2H), 3.72–3.82 (m, 2H), 3.81–3.85 (m, 1H), 3.86–3.91 (m, 1H), 3.92–3.96 (m, 1H), 3.97–4.02 (m, 2H), 4.07 (dd, J = 9.3, 2.9 Hz, 1H), 4.23 (dd, J = 10.1, 4.0 Hz, 1H), 4.34 (td, J = 8.4, 5.9 Hz, 1H), 4.38 (dd, J = 8.1, 5.1 Hz, 1H), 4.42 (t, J = 7.5 Hz, 1H), 4.51 (ddd, J = 12.0, 8.0, 3.9 Hz, 1H), 4.71 (td, J = 8.3, 4.3 Hz, 1H), 4.78 (br s, 1H), 4.99–5.07 (m, 2H), 5.48 (br s, 1H), 6.67 (d, J = 8.5 Hz, 2H), 6.76–6.81 (m, 2H), 6.95 (d, J = 8.5 Hz, 2H), 7.10–7.16 (m, 3H), 7.17–7.28 (m, 5H), 7.92 (d, J = 10.1 Hz, 1H), 7.96 (d, J = 9.3 Hz, 1H), 8.04–8.10 (m, 4H), 8.42 (d, J = 7.4 Hz, 1H), 8.55 (d, J = 8.1 Hz, 1H), 8.62 (d, J = 7.9 Hz, 1H), 8.86 (d, J = 7.5 Hz, 1H), 9.25 (s, 1H).

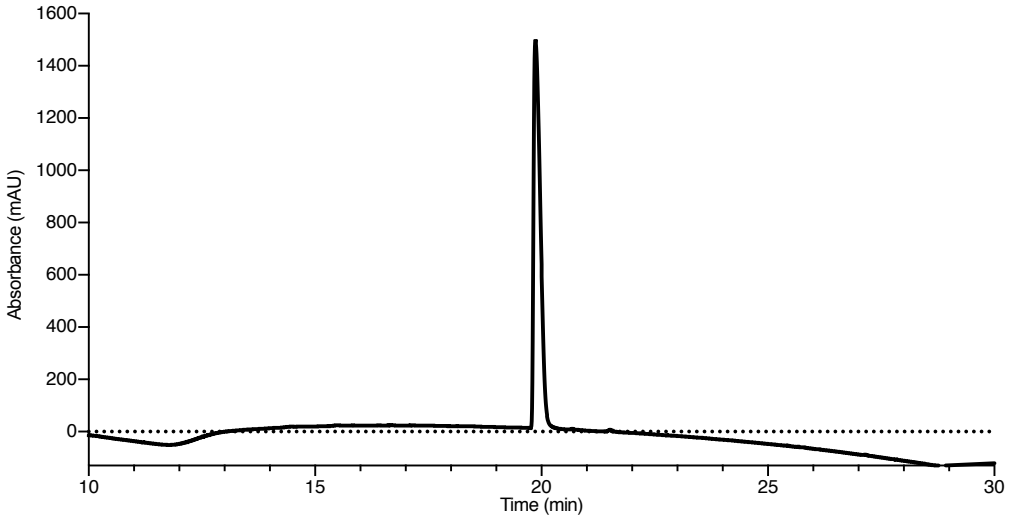


Analytical HPLC of purified *S. haemolyticus* AIP (**17**): t_R = 17.0 min (λ = 210 nm).

***L. monocytogenes* AIP (L_{mo}-AIP, 20)**



The peptide was synthesised using the general procedure for on-resin cyclisation/cleavage. Purification by preparative RP-HPLC afforded *L. monocytogenes* AIP (**20**) as a fluffy white solid after lyophilisation (3.0 mg, 21%). Analytical HPLC t_R = 20.0 min, purity 99% (λ = 210 nm). UPLC-MS (ESI) *m/z*: $[M+H]^+$ Calcd for $C_{34}H_{47}N_6O_6S_2^+$ 699.30; found 699.29. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 0.90–0.95 (m, 6H), 1.15 (d, J = 6.9 Hz, 3H), 1.75–1.81 (m, 2H), 1.96 (s, 3H), 2.04–2.15 (m, 2H), 2.38–2.45 (m, 1H), 2.69–2.74 (m, 1H), 2.82 (dd, J = 13.9, 10.0 Hz, 1H), 2.94 (dd, J = 13.9, 5.2 Hz, 1H), 3.15–3.24 (m, 2H), 3.29–3.35 (m, 1H), 3.74–3.81 (m, 1H), 3.94 (q, J = 7.3 Hz, 1H), 4.20 (ddd, J = 12.0, 7.6, 4.7 Hz, 1H), 4.37 (ddd, J = 11.2, 8.4, 4.4 Hz, 1H), 4.40–4.46 (m, 1H), 4.47 (dd, J = 9.8, 4.3 Hz, 1H), 7.14–7.21 (m, 3H), 7.20–7.26 (m, 5H), 7.26–7.32 (m, 2H), 8.04–8.10 (m, 4H), 8.38 (d, J = 8.8 Hz, 1H), 8.63 (d, J = 9.8 Hz, 1H), 8.66 (d, J = 7.8 Hz, 1H), 8.82 (d, J = 8.4 Hz, 1H).



Analytical HPLC of purified *L. monocytogenes* AIP (**20**): t_R = 20.0 min (λ = 210 nm).

Supplemental references

1. Canovas, J., Baldry, M., Bojer, M. S., Andersen, P. S., Gless, B. H., Grzeskowiak, P. K., Stegger, M., Damborg, P., Olsen, C. A., Ingmer, H. Cross-talk between *Staphylococcus aureus* and other staphylococcal species via the *agr* quorum sensing system. *Front. Microbiol.* **7**, 1733 (2016).
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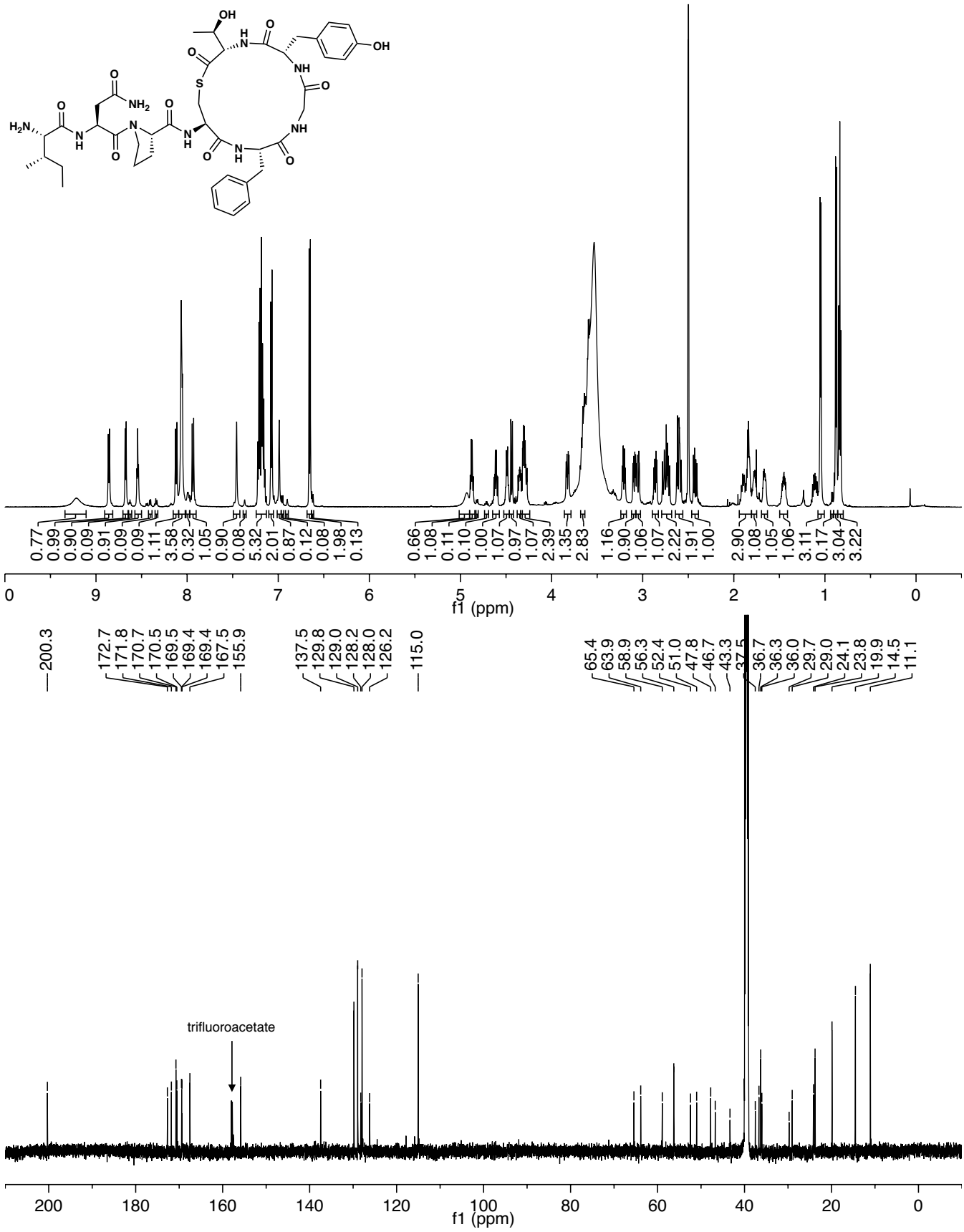
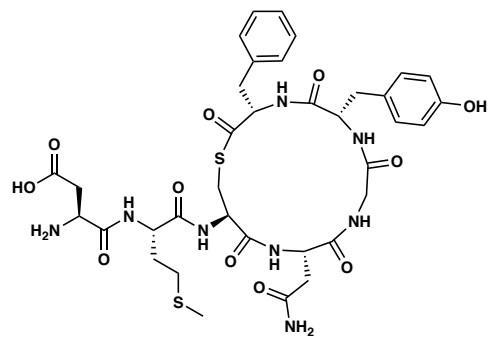
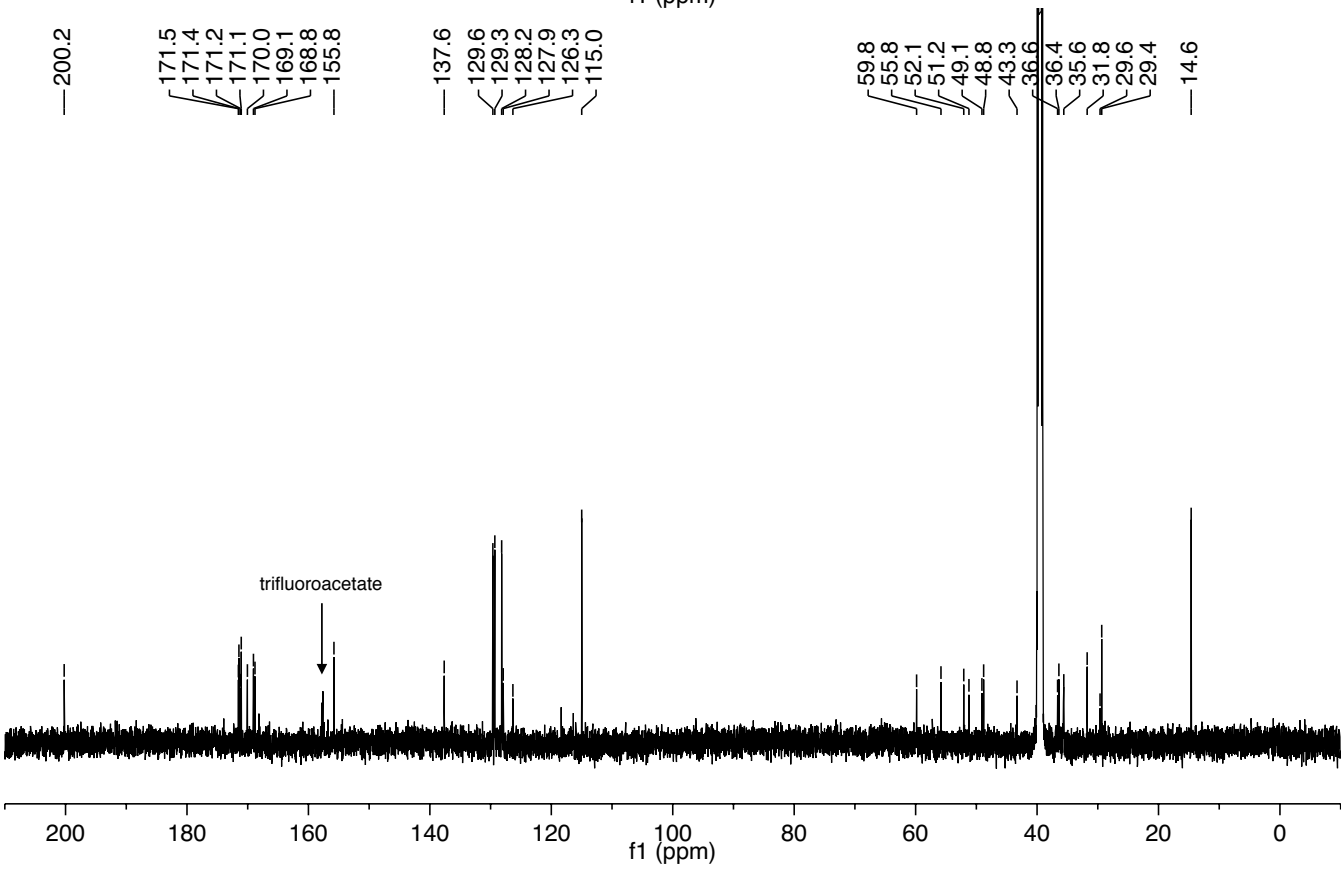
Copies of NMR spectra

***S. saprophyticus* AIP (8)**

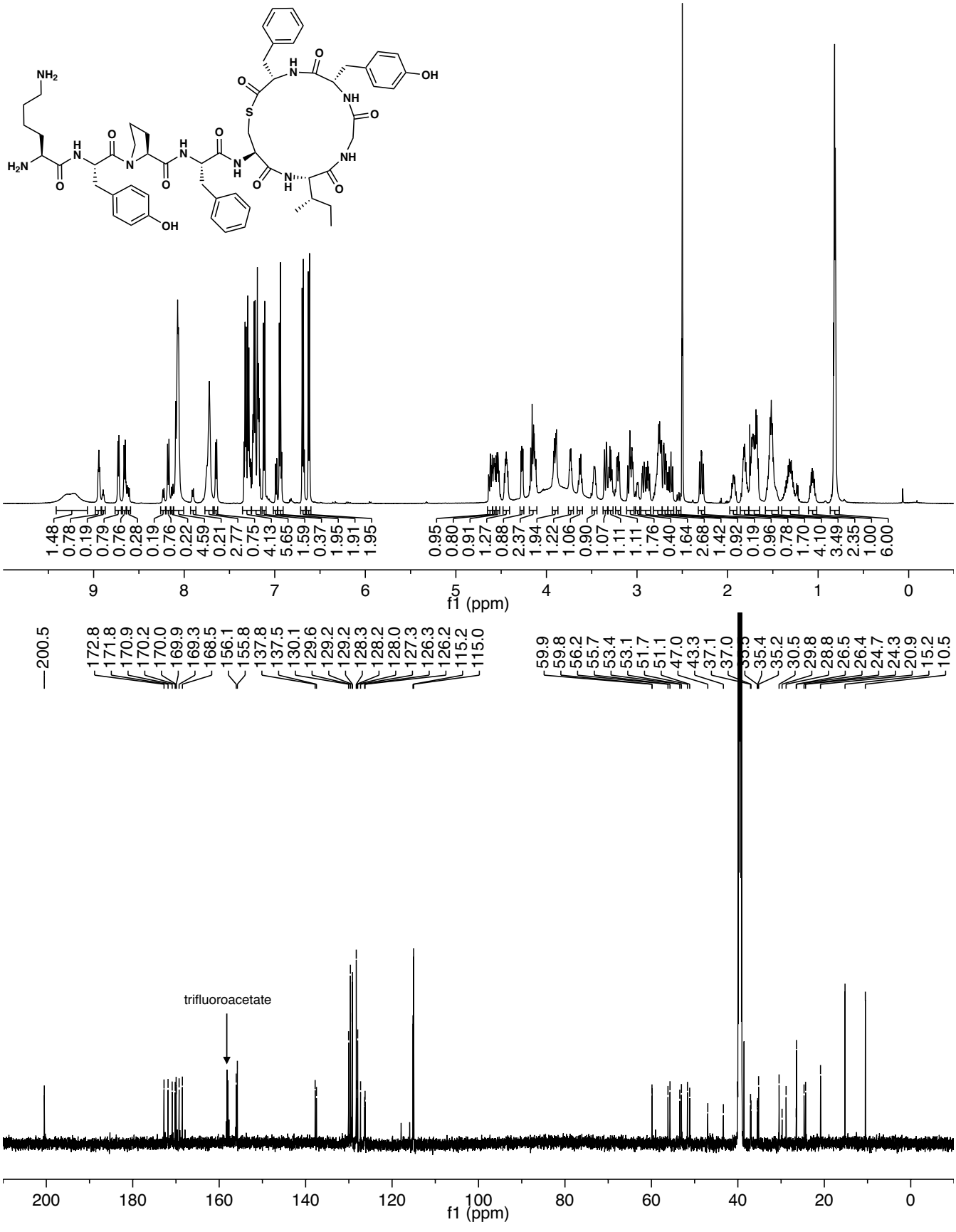


***S. lugdunensis* AIP-II (9)**

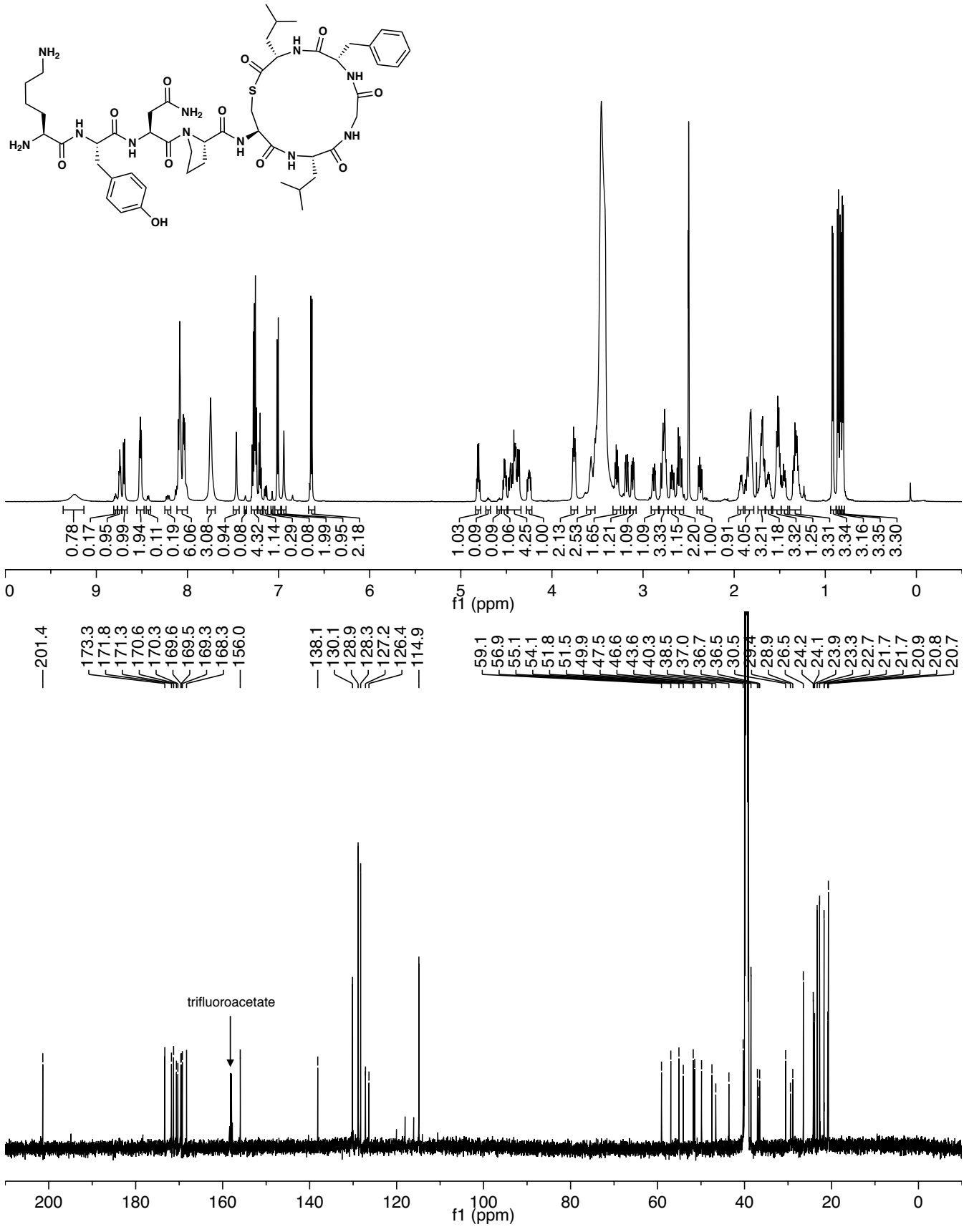




***S. schleiferi* AIP (10)**



***S. simulans* AIP (11)**



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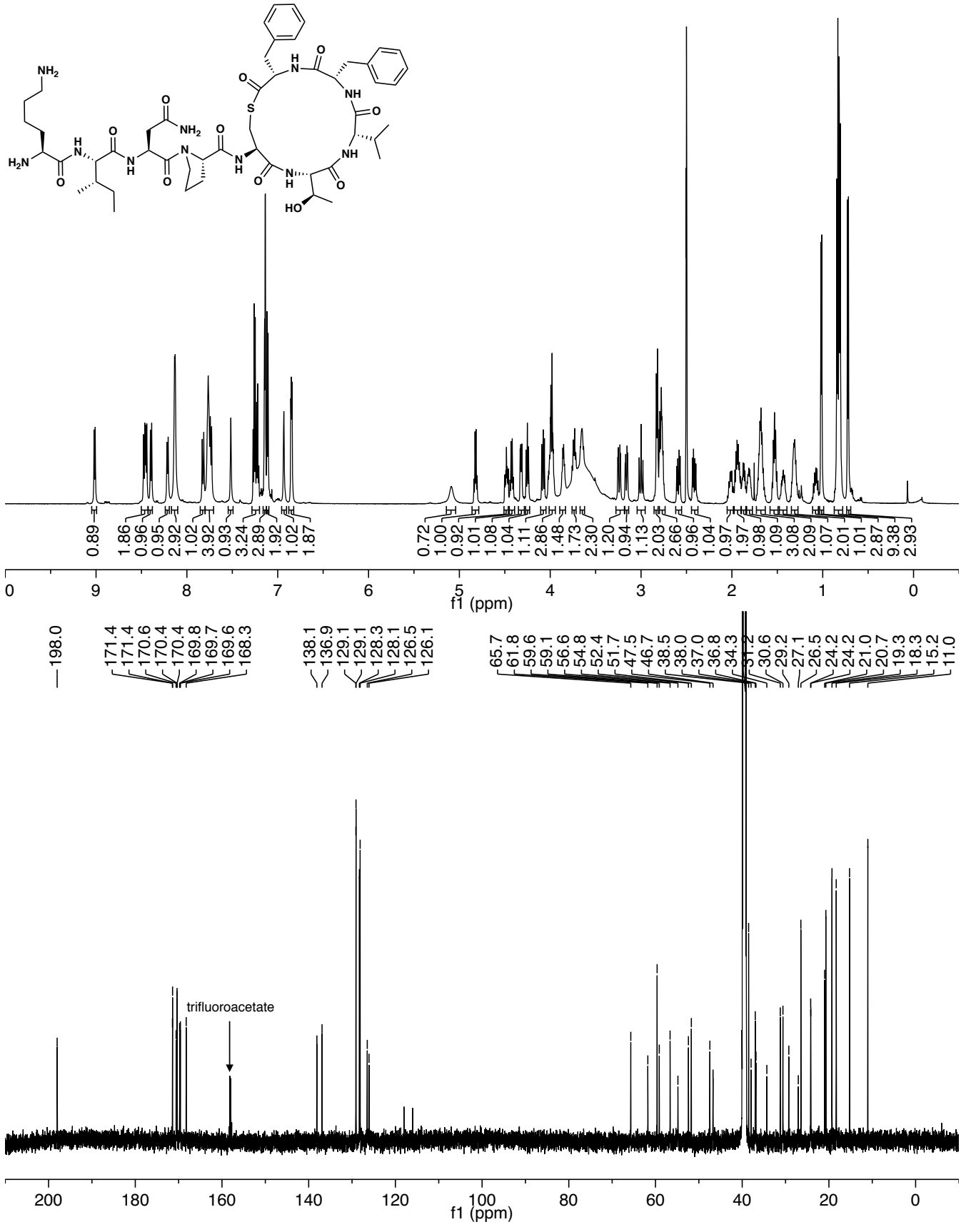
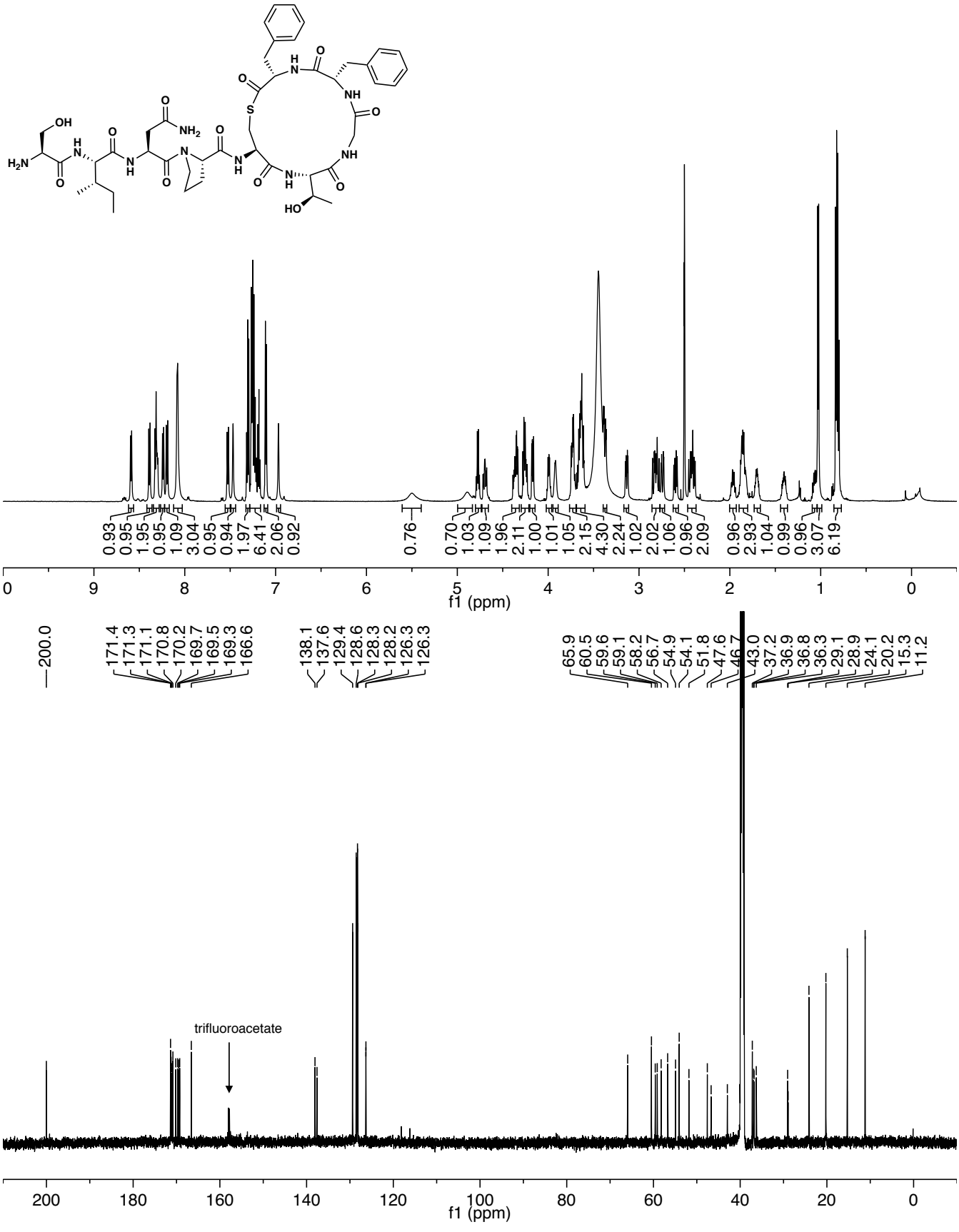
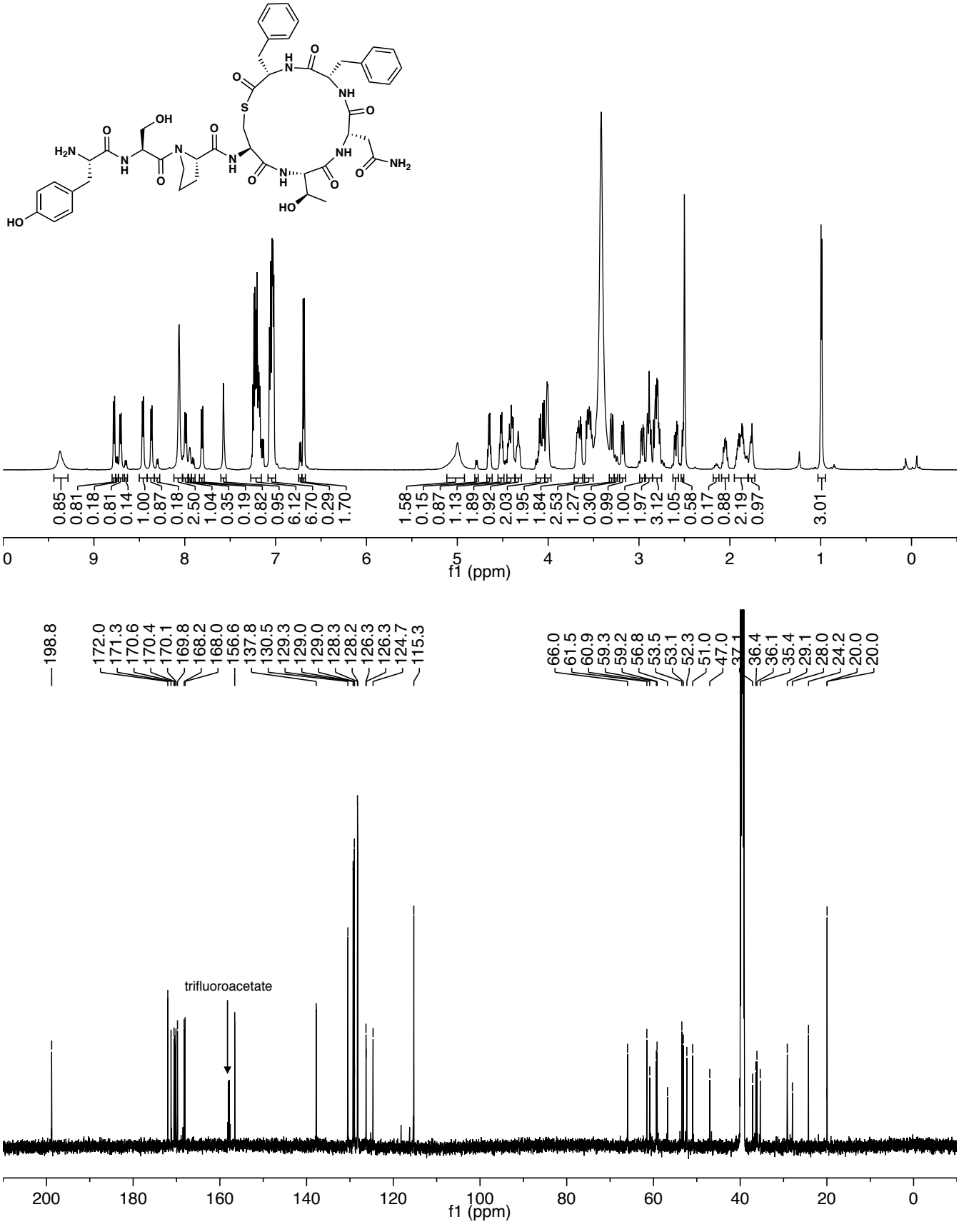


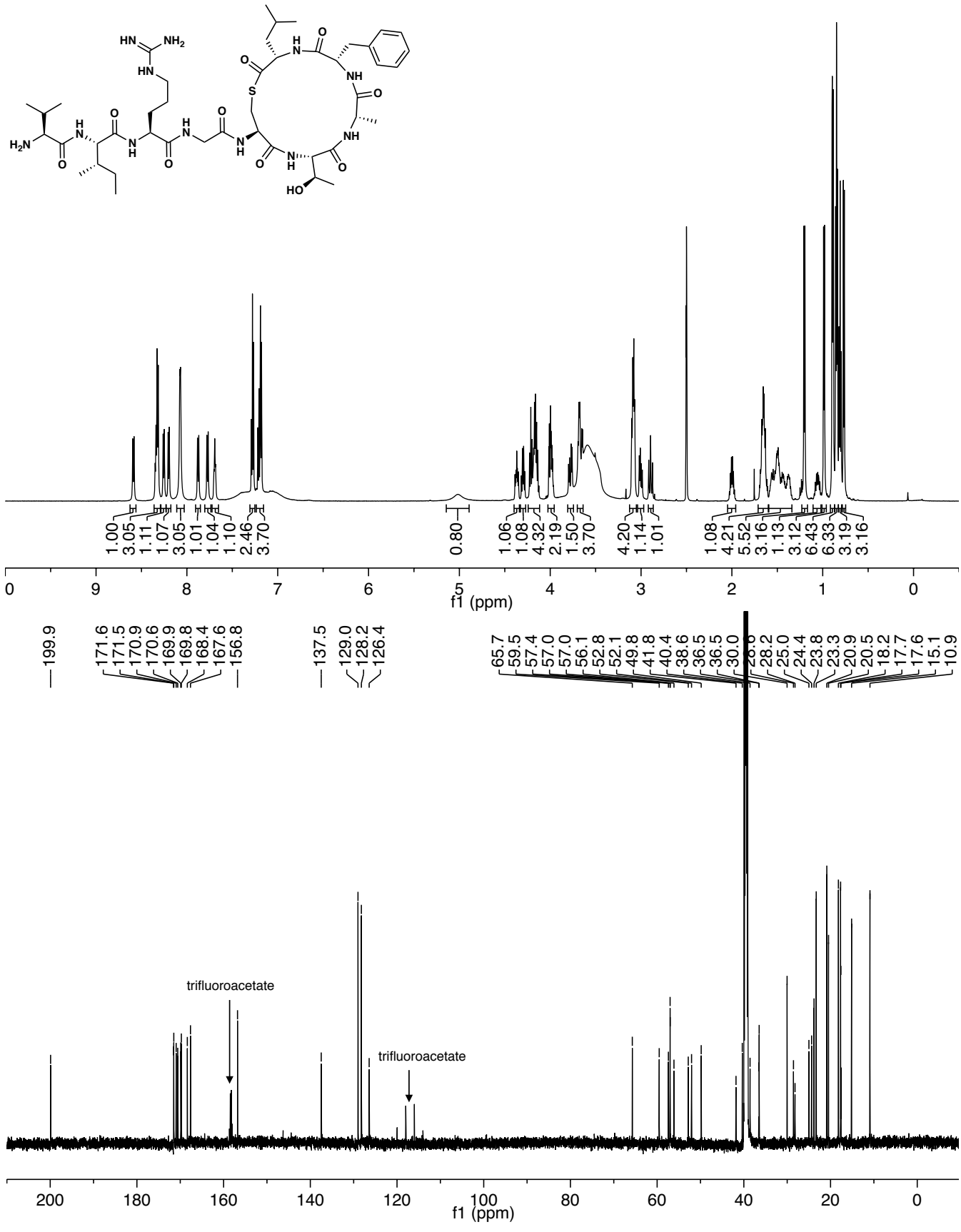
***S. chromogenes* AIP (13)**



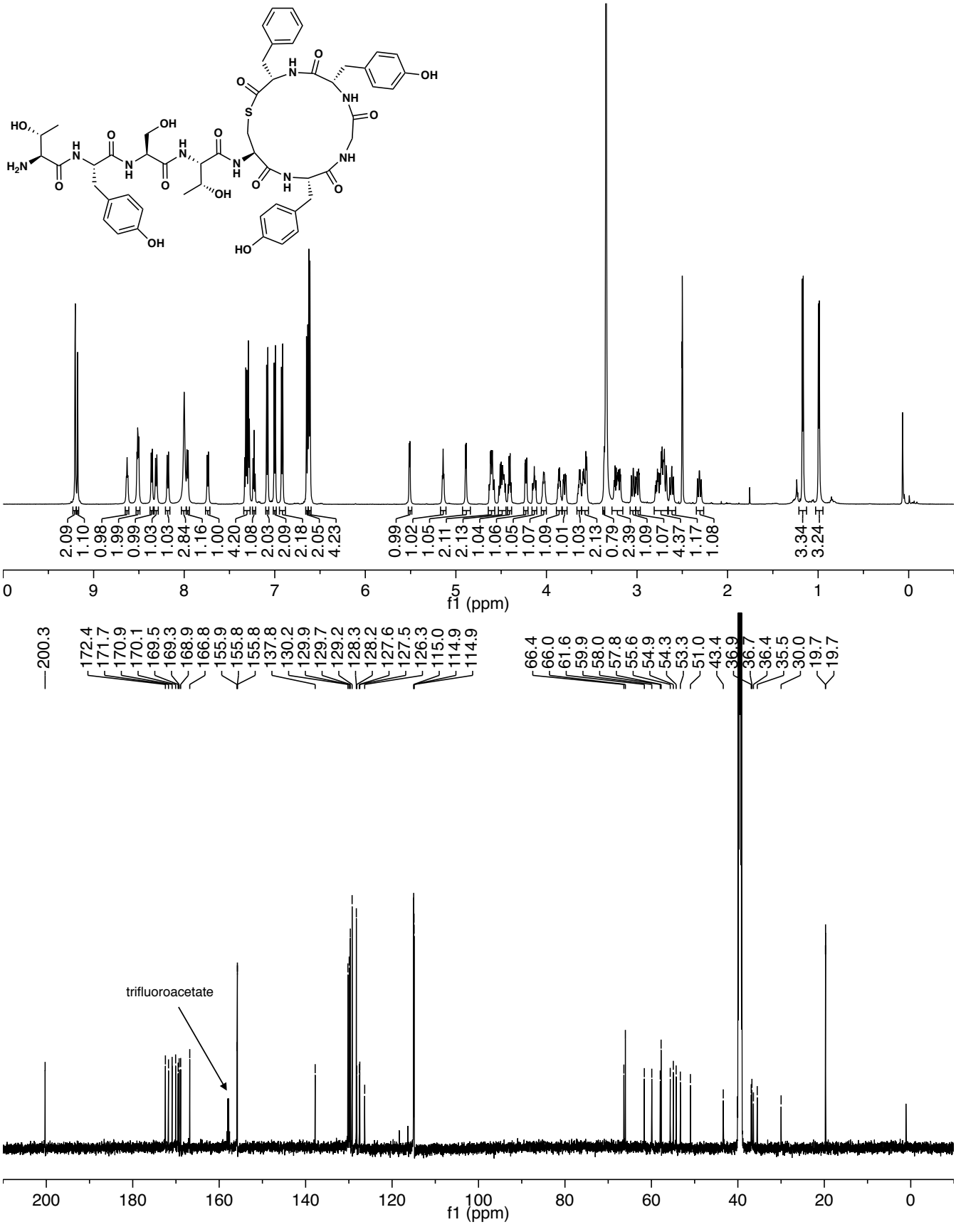
***S. warneri* AIP (14)**



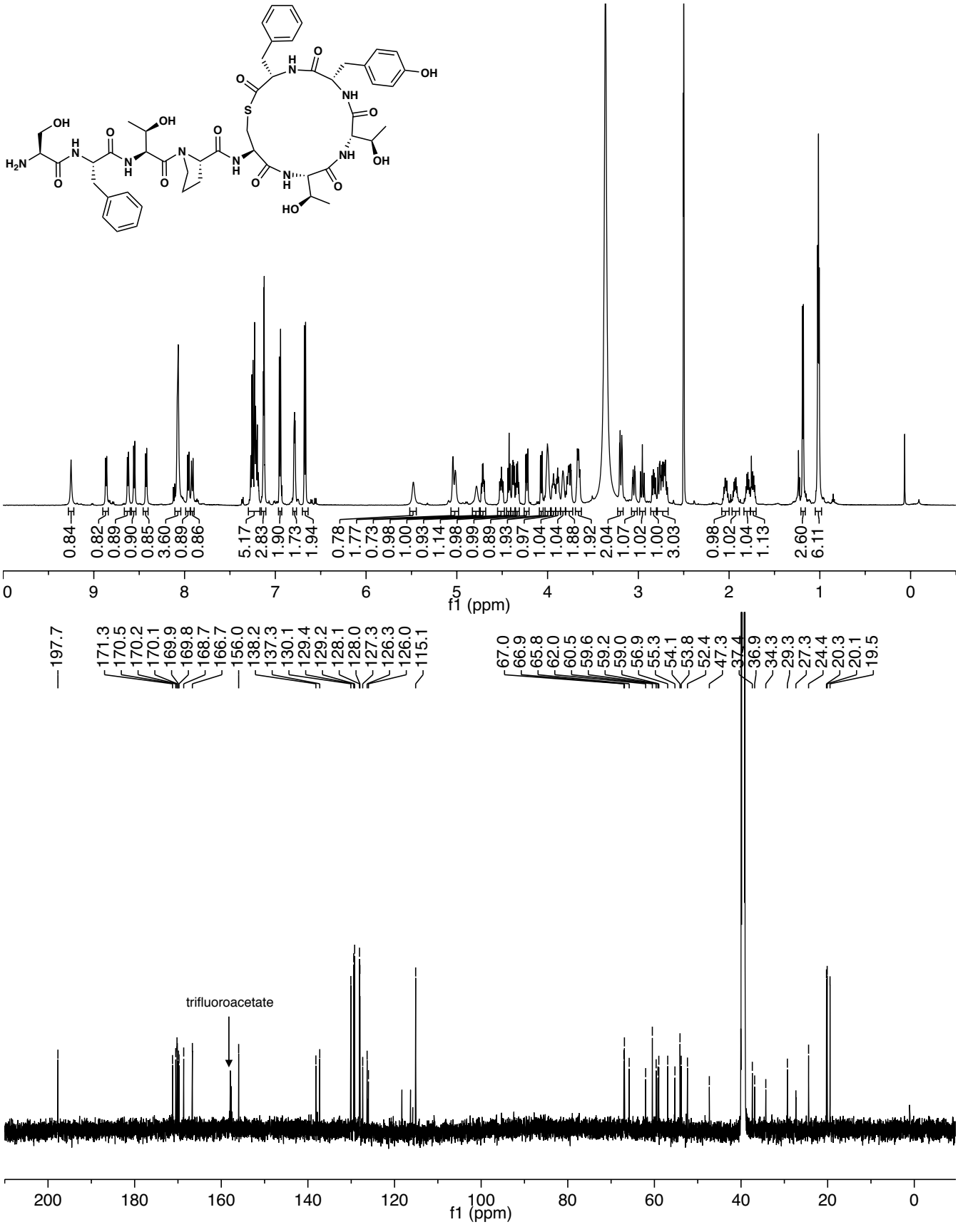
***S. vitulinus* AIP (15)**



***S. hominis* AIP (16)**



***S. haemolyticus* AIP (17)**



***L. monocytogenes* AIP (20)**