Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 2449–2453

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Side-Chain-to-Tail Thiolactone Peptide Inhibitors of the Staphylococcal Quorum-Sensing System

R. John Scott,[a] Lu-Yun Lian,[c] S. Hanna Muharram,[b] Alan Cockayne,[b] Stewart J. Wood,[a] Barrie W. Bycroft,[a] Paul Williams[a,b] and Weng C. Chan[a,*]

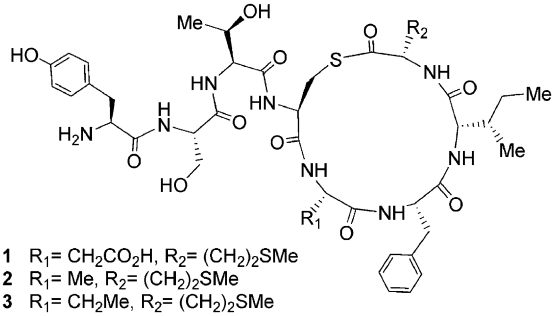
[a]*School of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK*
[b]*Institute of Infection, Immunity & Inflammation, University of Nottingham, University Park, Nottingham NG7 2RD, UK*
[c]*Department of Biomolecular Sciences, University of Manchester Institute of Science & Technology, The Mill, Sackville Street, Manchester M60 1QD, UK*

Received 7 April 2003; revised 14 May 2003; accepted 15 May 2003

Abstract—The expression of many staphylococcal virulence factors are regulated by the *agr* locus via a two-component signal transduction system (TCSTS), which is activated in response to a secreted autoinducer peptide (AIP). By exploiting the unique chemical architecture of the naturally occurring AIP-1, several potent inhibitors of staphylococcal TCSTS were designed and synthesized using either a linear or branched solid-phase approach. These inhibitors are competitive binders and contain the crucial 16-membered side-chain-to-tail thiolactone peptide pharmacophore.

Staphylococcus aureus is a versatile pathogen capable of causing life-threatening infections that are mediated primarily through the phenotypic expression of virulence factors.[1,2] The *agr* locus in *S. aureus*, which consists of two divergent operons expressed from the promoters P2 and P3, is critically involved in the expression of virulence determinants.[1,3] The P2 operon encodes for elements of a quorum-sensing system, these being proteins necessary for the biosynthesis of an autoinducer molecule, AIP, and the two-component sensor (AgrC): response regulator (AgrA) proteins. Activation of the TCSTS is mediated by interaction of the autoinducer with its cognate sensor protein resulting in upregulation of transcription both from promoter P2, amplifying the response, and from promoter P3, initiating the production of RNA III. Ultimately, the RNA III upregulates the production of several exotoxins and enzymes, and represses the expression of a range of bacterial cell-surface proteins.[3,4]

The staphylococcal autoinducer molecule, for example, AIP-1 **1**, is peptidic, comprising of a 16-membered macrocyclic thiolactone attached to a tri- or tetra-peptide 'tail'. The unusual thiolactone structure is derived from the C-terminal carboxyl group being bonded to the sulfhydryl group of an N-terminally located cysteine residue. Although four *S. aureus* AIPs have been reported to-date, they differ in their primary sequence but retained the unique thiolactone structure. Interestingly, these AIPs are capable of cross-inhibiting the activities of non-self staphylococcal TCSTS.[3,5,6]



1 R_1= CH_2CO_2H, R_2= $(CH_2)_2SMe$
2 R_1= Me, R_2= $(CH_2)_2SMe$
3 R_1= CH_2Me, R_2= $(CH_2)_2SMe$

The inhibition of staphylococcal quorum-sensing systems, specifically the TCSTS, have been shown to abolish the production of enterotoxin C3, lipase and toxic shock syndrome toxin-1.[3,6] Moreover, following internalization by epithelial cells, the ability of the *S. aureus* to effect endosomal escape and intracellular replication

*Corresponding author. Tel.: +44-115-9515080; fax: +44-115-9515078; e-mail: weng.chan@nottingham.ac.uk

0960-894X/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0960-894X(03)00497-9

appeared to be dependent on the *agr*-regulated production of exoproteins.[7] Thus, the blockade of staphylococcal TCSTS constitutes a unique therapeutic approach,[8] which is based on the attenuation of bacterial virulence and hence disrupting the capacity of the bacteria to cause infection.

During the course of our structure–activity relationship (SAR) studies of AIP-1, we discovered a potent TCSTS inhibitor, (Ala[5])AIP-1 **2**.[6] The dose–response (*agr-blaZ* reporter[1]) curve, which was acquired in the presence of 0.25 μM AIP-1, was reminiscent of a competitive acceptor antagonist.[6] Thus, functionally similar compounds will now be referred to as AgrC antagonists. The sensor-kinase AgrC is comprised of two large domains, that is, an N-terminal sensor domain displaying transmembrane helices and extracellular loops, and a cytoplasmic C-terminal histidine kinase domain.[1,3] We herein report our endeavors in the development of a practical and efficient methodology for the solid-phase synthesis of these macrocyclic peptides. The overall synthetic strategy is based on solid-phase assembly of either a linear or branched protected peptide (see Fig. 1), release from the solid support, and followed by polymer-supported carbodiimide mediated cyclization in solution. We have applied these synthetic methods for the syntheses of three other potent AgrC antagonists **3**–**5**. A noteworthy feature of the antagonists **4** and **5** is the presence of the (4-substituted phenoxy)butyryl group which functions as a Tyr-surrogate at the N-terminus of the exocyclic 'tail'.

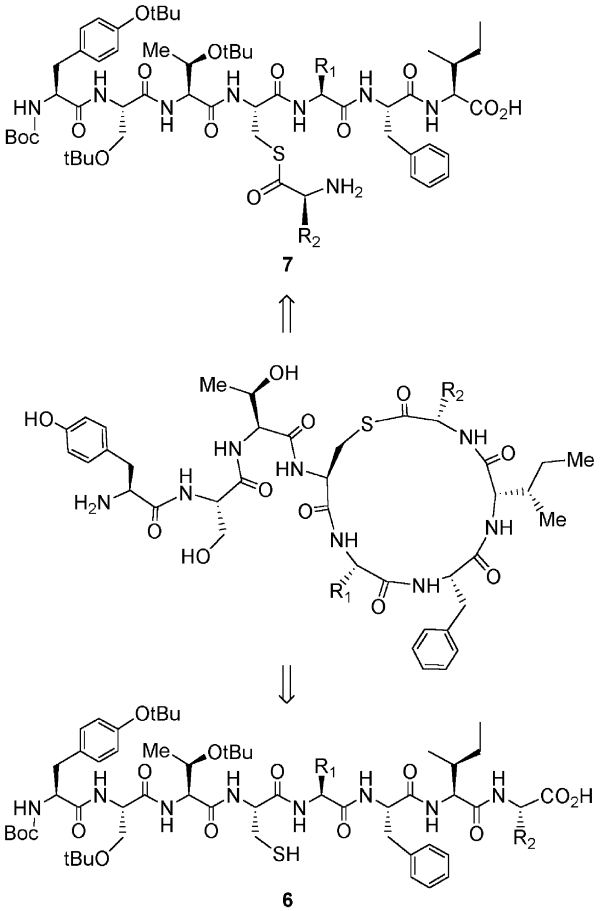
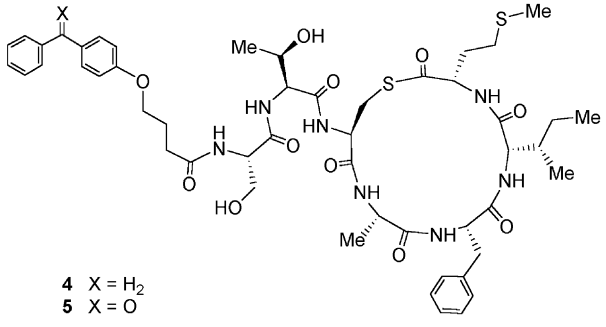


Figure 1. The linear **6** and branched **7** retro-synthetic analyses to the side-chain-to-tail thiolactone peptides.

Two contrasting synthetic strategies were initially investigated for the total synthesis of (Ala[5])AIP-1 **2**. Since the required peptide chains were assembled using standard Fmoc/*t*Bu solid-phase synthesis conditions[9] and followed by regioselective unmasking of the reactive groups at the macrocyclization sites, trityl-based protecting groups were utilized to mask these crucial reactive functionalities.

For the linear approach, in which the macrocyclization was carried out through the formation of a thioester bond between the Cys sulfhydryl and C-terminus carboxyl groups, the required protected peptide **6** (R_1 = Me, R_2 = $(CH_2)_2$SMe) was obtained via standard Fmoc/tBu solid-phase peptide assembly[9] on *N*-Fmoc-Met-O-2-chlorotrityl polystyrene.[10] Notably, the Cys residue was installed using four-equivalent excess of the *S*-4-methoxytrityl protected *N*-Fmoc-cysteine, which was activated using *N*,*N*′-diisopropylcarbodiimide (DIPCDI) in the presence of 1-hydroxybenzotriazole (HOBt).[11] Subsequent treatment of the assembled peptide-resin with TFA:Et_3SiH:CH_2Cl_2 (1:1:98 v/v) for 30–40 min resulted in the chemoselective acidolysis of the 2-chlorotrityl ester resin linkage and *S*-4-methoxytrityl to afford the partially protected octapeptide **6** (R_1 = Me, R_2 = $(CH_2)_2$SMe) in typically quantitative yields. Partial purification of **6** was accomplished by filtration of the acidolytic resin suspension into pyridine:MeOH (1.5:75 v/v), evaporation of the filtrate to dryness in vacuo, trituration of the residual material with ice-cold water, and finally drying the precipitated peptide in vacuo. A small fraction of the partially protected peptide **6** was exposed to TFA:H_2O:Et_3SiH:EtSMe (90:5:3:2) for 1 h, followed by RP-HPLC analysis,[12] which revealed a peptide purity >95%.



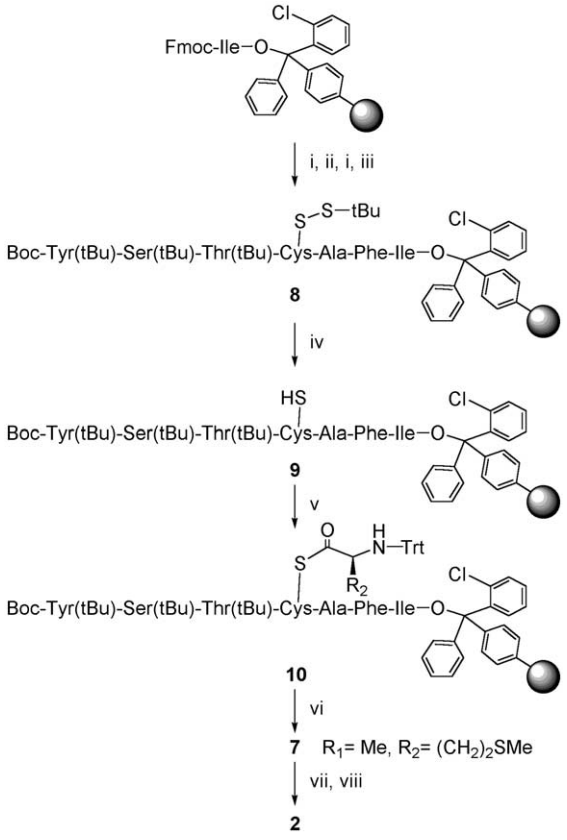
4 X = H_2
5 X = O

Macrocyclization was then achieved by exposing a dilute solution of **6** in $CHCl_3$ (1–2 mM) to *N*-polystyrene methyl-*N*′-cyclohexylcarbodiimide (5 equiv), in the presence of 7-aza-1-hydroxybenzotriazole[13] and 4-dimethylaminopyridine (0.1 equiv) for 3–4 days at ambient temperature. The polymer-supported carbodiimide (2% DVB, 200–400 mesh) is a commercially available analogue of *N*-polystyrene methyl-*N*′-isopropylcarbodiimide.[14] Following a standard workup, 90% TFA-mediated acidolytic treatment of the crude protected macrocyclic peptide afforded the desired

peptide analogue **2** as a white amorphous solid in good yield and purity >40%. Significantly, RP-HPLC analysis[12] showed virtually absence of the linear peptide and only a small amount (<5%) of the D-Met[8] diastereoisomer. Following purification by RP-HPLC, the thiolactone octapeptide **2** was obtained as a lyophilized powder (15–20% yield).[15]

We then focused our attention on the branched approach for the synthesis of the cyclic peptide **2**. In this strategy, the thioester bond was formed on the solid-phase and the final macrocyclization of the partially protected peptide **7** was through an amide bond between the Ile[7]-carboxyl and Met[8]-amine groups. We envisaged that this branched strategy has a number of merits, including (i) formation of the 'difficult' thioester bond can be forced to completion by exploiting solid-phase methods, and (ii) has a readily accessible site (on a pre-assembled peptide sequence) for the installation of unique *C*-terminal amino acids, since this position has been shown to have profound effect on Agr-binding.[6]

Scheme 1 outlines the overall synthetic method for the branched strategy. The key features are: (i) the Cys residue was installed as the *S*-*t*butylsulphenyl (S*t*Bu)[16] protected amino acid, in which the thio-group was chemoselectively and efficiently unmasked[17] on the solid-phase under the mild reducing conditions, 10 equiv Bu_3P in isopropanol-DMF (1:10) within 5–6 h at room temperature—we found that the Bu_3P-mediated deprotection was incomplete (~30% in 5 h) when THF was used as the solvent, (ii) *S*-acylation was achieved by repeated treatment with activated Trt-Met-OH[18] to afford the polymer-bound branched peptide **10**, and (iii) concomitant release from resin and *N*-deprotection by mild acidolytic treatment of **10** to afford the partially protected peptide **7** in good yield and purity (>95%). Macrocyclization was then undertaken using the previously established conditions, that is, a $CHCl_3$ solution of **7** (2 mM) was added polymer-bound carbodiimide and 7-aza-1-hydroxybenzotriazole (HOAt). Following workup and 90% TFA-mediated acidolytic treatment of the crude protected macrocyclic peptide, the peptide **2** was obtained in acceptable yield and purity. However, following purification by RP-HPLC, **2** was obtained in yield (10–15%) that is comparable to the linear synthetic strategy. Nonetheless, we are confident that the branched strategy will facilitate the combinatorial synthesis of AIPs.



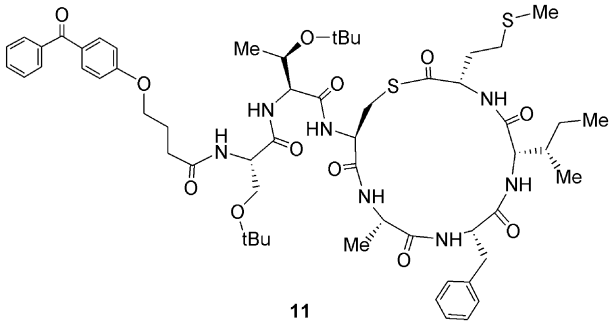
Scheme 1. Reagents and conditions: (i) 20% piperidine-DMF; (ii) Fmoc-amino acid:TBTU:HOBt:DIEA (1:1:1:2, 4–8 equiv); (iii) Boc_2O (4–8 equiv), 18 h; (iv) Bu_3P (10 equiv), *i*PrOH-DMF (1:10), 6 h; (v) (a) Trt-Met-OH (10 equiv)-DIPCDI (5 equiv), DMAP (0.01 equiv), CH_2Cl_2-DMF (1:1), 3 h, (b) Trt-Met-OH (5 equiv)-DIPCDI (2.5 equiv), DMAP (0.01 equiv), 6 h, (c) repeat (b); (vi) TFA-Et_3SiH-CH_2Cl_2 (1:1:98), 40 min; (vii) polymer-bound carbodiimide:HOAt (5:1 equiv), $CHCl_3$, 72 h; (viii) (a) TFA:H_2O:Et_3SiH:EtSMe (90:5:3:2), 1 h; (b) purification by RP-HPLC using Kromasil C8 preparative column (10×150 mm).

Compared to our lead compound **2**, we previously observed that the Gly-analogue, (Gly[5])AIP-1 displayed ~40-fold decreased in activity as an antagonist at the AgrC-1 acceptor.[6] We hypothesized that increasing the side-chain hydrophobicity of the crucial amino acid-5 would enhance potency. Thus, the methyl homologue, (Abu[5])AIP-1 **3** was synthesized using the solid-phase strategies outlined above and purified by RP-HPLC to afford **3** as a lyophilized powder (10–19% yield).[19]

The effect of introducing Tyr[1]-surrogates on the activity of our lead compound **2** was investigated. Our approach here was to replace the Tyr residue with aromatic-bearing acyl groups with increased hydrophobicity. Thus, we have selected 4-benzylphenoxyalkanoyl as the generic *des*-amino-Tyr-surrogate, which was installed at the N-terminus using 4-benzoylphenoxyalkanoic acid building blocks. The reasons for utilizing this synthon approach are two-fold: (i) structural variants of benzoylphenol are readily accessed by the Friedel–Crafts acylation of substituted benzene using *p*-methoxybenzoyl chloride,[20] and (ii) biaryl ketones are photoactivatable[21] and hence the 4-benzoylphenoxyalkanoyl-derivatized AIP-1 could be used for photolabelling studies of AgrC. The ketone functionality in the benzoylphenoxy moiety was readily reduced by a TFA:Et_3SiH mixture, which is also used for the global deprotection of synthesized peptide.

The synthesis of *N*-4-(4-benzylphenoxy)butyryl-(*des*-Tyr[1]; Ala[5])AIP-1 **4** was accomplished using the linear approach. Thus, following solid-phase assembly of the linear peptide, the N-terminus was acylated using DIPCDI-activated 4-benzoylphenoxybutyric acid (mp 94–95 °C), which was readily prepared by *O*-alkylation of 4-hydroxybenzophenone with ethyl 4-bromobutyrate followed by saponification using methanolic aqueous

NaOH. The partially protected peptide intermediate was obtained by 1% TFA-mediated acidolysis, which was then subjected to polymer-bound carbodiimide-mediated macrocyclization. The cyclization reaction was carried out in $CHCl_3$:DMF (~40:1) due to solubility issues. Treatment of the crude protected cyclic peptide **11** with TFA:H_2O:Et_3SiH:EtSMe (90:5:3:2) resulted in global deprotection and reduction of the ketone functionality to afford **4**, which was purified by RP-HPLC (yield 5–10%).[22] The observed low HPLC-purified yield was partly due the unexpected poor solubility of the peptide **4** in either water, MeCN or aqueous MeCN.



11

In addition, the peptide analogue **5**[23] was obtained by treatment of the protected cyclic peptide **11** with TFA:H_2O:*i*Pr_3SiH:EtSMe (90:5:3:2) for 30 min. The sterically hindered *i*Pr_3SiH and a shorter reaction time were used in order to minimize undesired reduction of the benzophenone moiety.

The synthetic AIP-1 peptide derivatives were tested for their ability to inhibit staphylococcal TCSTS, specifically their capacity to antagonize AgrC-1 and AgrC-2 acceptors. The bacterial cell assay is based on the ability of these compounds to competitively block the activity of AIP-1 and AIP-2 within their specific *S. aureus* strains. The biological activity was monitored by the robust *agr-blaZ* reporter[1,3,6] that entailed the P3-driven transcription of a β-lactamase and the spectrophotometric analysis of the hydrolysis of its substrate, nitrocefin. The results are summarized in Table 1.

Unexpectedly, the (Abu[5])-analogue **3** showed lower inhibitory potency compared to the lead peptide **2** as an AgrC-1 antagonist. The hydrophobicity and/or steric bulk of the amino acid-5 side-chain are likely to contribute to the decreased binding capacity of the antagonist **3**. Our recent ^{1}H NMR studies (temperature coefficient and NOESY) established that the 16-membered macrocyclic domain in both the agonist **1** and the antagonist **2** display similar and well-defined conformation that is stabilized by two intramolecular H-bonds — the H-bond donors being the backbone amide NHs of the Asp/Ala[5] and Ile[7] residues (data will be reported elsewhere). We suspect that the side-chain-to-tail thiolactone pharmacophore is the 'address' region of the peptide ligands, which is in-part responsible for molecular recognition.

Table 1. Inhibition (IC_{50} nM)[a] of staphylococcal AgrC determined using competitive assays and *S. aureus* Group I and II engineered with the *agr-blaZ* reporter,[1,3,6] RN6390B:pRN6683 and SA564:pRN6683, respectively

AIP-1 derivatives	IC_{50} (nM)	
	AgrC-1	AgrC-2
(Ala[5])AIP-1, **2**	21±9	4±4
3	137±65	2.8±0.4
4	295±22	19±14
5	303±164	18±5

[a]Assays were carried out at least in triplicate, and sigmoidal dose-response curves were generated using PRISM3 program.

In addition, it was gratifying to observe that both analogues **4** and **5**, comprising the installed *des*-amino-Tyr-surrogates, exhibited good antagonistic potency though somewhat lower compared to the octapeptide **2**. The good binding capacity of the analogue **5**, which contained a photophore tag, will facilitate further work in obtaining a detailed map of the AgrC ligand-binding site.

Of particular significance is the exceptionally high inhibitory potency of the AIP-1 analogues **2**–**5** towards the AgrC-2 acceptor, to which the natural agonist is AIP-2[1]. We believe that these new antagonists will provide an invaluable platform for the design and synthesis of the next generation of global AgrC antagonists, especially those with structural variants of 4-benzylphenoxyalkanoyl installed at the N-terminus.

Acknowledgements

We are grateful for funding from BBSRC, UK (a studentship to S.J.W.) and MRC, UK.

References and Notes

1. Ji, G.; Beavis, R. C.; Novick, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12055.
2. (a) For example: Wesson, C. A.; Liou, L. E.; Tood, K. M.; Bohach, G. A.; Trumble, W. R.; Bayles, K. W. *Infect. Immun.* **1998**, *66*, 5238. (b) Lammers, A.; Nuijten, P. J. M.; Smith, H. E. *FEMS Microbiol. Lett.* **1999**, *180*, 103. (c) Nilsson, I. M.; Hartford, O.; Foster, T.; Tarkowski, A. *Infect. Immun.* **1999**, *67*, 1045. (d) Mullarky, I. K.; Su, C.; Frieze, N.; Park, Y. H.; Sordillo, L. M. *Infect. Immun.* **2001**, *69*, 45. (e) Kielian, T.; Cheung, A.; Hickey, W. F. *Infect. Immun.* **2001**, *69*, 6902.
3. (a) Ji, G.; Beavis, R. C.; Novick, R. P. *Science* **1997**, *276*, 2027. (b) Lina, G.; Jarraud, S.; Ji, G.; Greenland, T.; Pedraza, A.; Etienne, J.; Novick, R. P.; Vandenesch, F. *Mol. Microbiol.* **1998**, *28*, 655. (c) Dufour, P.; Jarraud, S.; Vandenesch, F.; Greenland, T.; Novick, R. P.; Bes, M.; Etienne, J.; Lina, G. *J. Bacteriol.* **2002**, *184*, 1180.
4. (a) Morfeldt, E.; Tegmark, K.; Arvidson, S. *Mol. Microbiol.* **1996**, *21*, 1227. (b) Benito, Y.; Kolb, F. A.; Romby, P.; Lina, G.; Etienne, J.; Vandenesch, F. *RNA* **2000**, *6*, 668.
5. (a) Mayville, P.; Ji, G.; Beavis, R.; Yang, H.; Goger, M.; Novick, R. P.; Muir, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1218. (b) Lyon, G. J.; Mayville, P.; Muir, T. W.; Novick, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13330. (c) Jarraud, S.; Lyon, G. J.; Figueiredo, A. M. S.; Gérard, L.; Vandenesch, F.; Etienne, J.; Muir, T. W.; Novick, R. P. *J. Bacteriol.* **2000**, *182*, 6517.

6. McDowell, P.; Affas, Z.; Reynolds, C.; Holden, M. T. G.; Wood, S. J.; Saint, S.; Cockayne, A.; Hill, P. J.; Dodd, C. E. R.; Bycroft, B. W.; Chan, W. C.; Williams, P. *Mol. Microbiol.* **2001**, *41*, 503.
7. Qazi, S. N. A.; Counil, E.; Morrissey, J.; Rees, C. E. D.; Cockayne, A.; Winzer, K.; Chan, W. C.; Williams, P.; Hill, P. J. *Infection Immun.* **2001**, *69*, 7074.
8. (a) Williams, P. *Expert Opin. Ther. Targets* **2002**, *6*, 1. (b) Stephenson, K.; Hoch, J. A. *Pharmacol. Ther.* **2002**, *93*, 293.
9. *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*; Chan, W. C.; White, P. D., Eds.; Oxford University Press: Oxford, 2000.
10. Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos, G. *Int. J. Peptide Protein Res.* **1991**, *37*, 513.
11. Han, Y.; Albericio, F.; Barany, G. *J. Org. Chem.* **1997**, *62*, 4307.
12. Kromasil 100-C8, linear elution gradient 20–60% **B** in 25 min at 1.1 mL min^{-1}. Column dimension: 4.6×150 mm; effluent was monitored at 220 nm; **A**: 0.06% aq TFA; **B**: 0.06% TFA in MeCN-H_2O (9:1).
13. Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397.
14. (a) Weinshenker, N. M.; Shen, C. M. *Tetrahedron Lett.* **1972**, *32*, 3281. (b) Weinshenker, N. M.; Shen, C. M.; Wong, J. Y. *Org. Synth. Coll. Vol.* **1988**, *6*, 951.
15. ^{1}H NMR (600 MHz, DMSO-d_6, 303 K) δ_H 9.08 (Met^8 NH), 8.70 (Ser^2 NH), 8.26 (Ile^7 NH), 8.25 (Cys^4 NH), 8.00 (Ala^5 NH), 7.95 (Thr^3 NH), 7.80 (Phe^6 NH), 7.28, 7.20 (2×m, Phe^6 ArHs), 7.08, 6.70 (Tyr^1 ArHs), 5.24 (Ser^2 C_βOH), 4.95 (Thr^3 C_βOH), 4.54 (Ser^2 C_αH), 4.50 (Phe^6 C_αH), 4.28 (Cys^4 C_αH), 4.27 (Thr^3 C_αH), 4.25 (Met^8 C_αH), 4.10 (Thr^3 C_βH), 4.03 (Ala^5 C_αH), 4.00 (Tyr^1 C_αH), 3.77 (Ile^7 C_αH), 3.69, 3.56 (2×m, Ser^2 $C_\beta H_2$), 3.20 (Cys^4 C_βH), 3.03 (Tyr^1 C_βH), 2.96 (Phe^6 $C_\beta H_2$), 2.90 (Cys^4 C_βH), 2.78 (Tyr^1 C_βH), 2.54 (Met^8 C_γH), 2.51 (s, Met^8 CH_3), 2.45 (Met^8 C_γH), 2.19 (Met^8 C_βH), 1.94 (Met^8 C_βH and Ile^7 C_βH), 1.18 (Ile^7 $C_\gamma H_2$), 1.06 (Thr^3 CH_3 and Ala^5 CH_3), 0.82 (Ile^7 $C_{\gamma'}H_3$), 0.78 (Ile^7 $C_\delta H_3$); ES–MS^+ *m*/*z* 917.27 (MH^+), calcd 917.39; RP-HPLC Kromasil C8, 20-60% B in 25 min, t_R 19.6 min.
16. Weber, U.; Hartter, P.; Hoppe-Seyler's, Z. *Physiol. Chem.* **1970**, *351*, 1384.
17. (a) Eritja, R.; Ziehler-Martin, J. P.; Walker, P. A.; Lee, T. D.; Legesse, K.; Albericio, F.; Kaplan, B. E. *Tetrahedron* **1987**, *43*, 2675. (b) Beekman, N. J. C. M.; Schaaper, W. M. M.; Tesser, G. I.; Dalsgaard, K.; Kamstrup, S.; Langeveld, J. P. M.; Boshuizen, R. S.; Meloen, R. H. *J. Peptide Res.* **1997**, *50*, 357.
18. Barlos, K.; Papaioannou, D.; Theodoropoulos, D. *J. Org. Chem.* **1982**, *47*, 1324.
19. ^{1}H NMR (600 MHz, DMSO-d_6, 303 K) δ_H 9.10 (Met^8 NH), 8.63 (br s, Ser^2 NH), 8.46 (Abu^5 NH), 8.31 (Phe^6 NH), 8.25 (Cys^4 NH), 8.20 (br s, Ile^7 NH), 7.92 (Thr^3 NH), 7.86 (Tyr^1 NH_2), 7.28, 7.20 (2×m, Phe^6 ArHs), 7.08, 6.70 (Tyr^1 ArHs), 5.22 (Ser^2 C_βOH), 4.90 (Thr^3 C_βOH), 4.54 (Ser^2 C_αH), 4.50 (Phe^6 C_αH), 4.30 (Abu^5 C_αH), 4.28 (Thr^3 C_αH), 4.25 (Cys^4 C_αH), 4.22 (Met^8 C_αH), 4.08 (Thr^3 C_βH), 3.92 (Tyr^1 C_αH), 3.80 (Ile^7 C_αH), 3.69, 3.54 (2×m, Ser^2 $C_\beta H_2$), 3.20 (Cys^4 C_βH), 3.02 (Tyr^1 C_βH), 2.90 (Cys^4 C_βH), 2.78 (Tyr^1 C_βH), 2.65 (Phe^6 C_βH), 2.55 (Met^8 C_γH), 2.50 (Phe^6 C_βH), 2.50 (s, Met^8 CH_3), 2.45 (Met^8 C_γH), 2.20, 1.95 (Met^8 $C_\beta H_2$), 1.94 (Ile^7 C_βH), 1.69, 1.52 (Abu^5 $C_\beta H_2$), 1.32 (Abu^5 CH_3), (1.18 (Ile^7 $C_\gamma H_2$), 1.06 (Thr^3 CH_3), 0.84 (Ile^7 $C_{\gamma'}H_3$), 0.76 (Ile^7 $C_\delta H_3$); ES–MS^+ *m*/*z* 931.35 (MH^+), calcd 931.41; RP-HPLC Kromasil C8, 20–60% B in 25 min, t_R 20.7 min.
20. Atkinson, G. E.; Fischer, P. M.; Chan, W. C. *J. Org. Chem.* **2000**, *65*, 5048.
21. Dorman, G. D.; Prestwich, G. D. *Biochemistry* **1994**, *33*, 5661.
22. ^{1}H NMR (600 MHz, DMSO-d_6, 303 K) δ_H 9.10 (Met^8 NH), 8.26 (Ile^7 NH), 8.20 (Cys^4 NH), 8.04 (Ser^2 NH), 7.95 (Ala^5 NH), 7.90 (BnPh ArH), 7.82 (Phe^6 NH), 7.75 (BnPh ArH), 7.70 (Thr^3 NH), 7.45, 7.30 (BnPh ArHs), 7.28, 7.20 (2×m, Phe^6 ArHs), 7.14, 7.08, 6.85 (BnPh ArHs), 5.05 (Ser^2 C_βOH), 4.90 (Thr^3 C_βOH), 4.50 (Phe^6 C_αH), 4.40 (Ser^2 C_αH), 4.28 (Cys^4 C_αH), 4.24 (Met^8 C_αH), 4.21 (Thr^3 C_αH), 4.05 (Thr^3 C_βH), 4.02 (Ala^5 C_αH), 3.95 (PhOC$H_2$$CH_2$), 3.87 (PhC$H_2$Ph), 3.78 ($Ile^7$ C_αH), 3.62, 3.55 (2×m, Ser^2 $C_\beta H_2$), 3.20 (Cys^4 C_βH), 2.98 (Phe^6 $C_\beta H_2$), 2.91 (Cys^4 C_βH), 2.55 (Met^8 C_γH), 2.52 (s, Met^8 CH_3), 2.45 (Met^8 C_γH), 2.34 (CH_2CH_2CO), 2.20 (Met^8 C_βH), 1.95 (Met^8 C_βH and Ile^7 C_βH), 1.94 (PhOCH_2C$H_2$$CH_2$CO), 1.19 ($Ile^7$ $C_\gamma H_2$), 1.05 (Ala^5 CH_3), 1.02 (Thr^3 CH_3), 0.80 (Ile^7 $C_{\gamma'}H_3$), 0.76 (Ile^7 $C_\delta H_3$); ES–MS^+ *m*/*z* 1006.37 (MH^+), calcd 1006.44; RP-HPLC Kromasil C8, 50–100% B in 20 min, t_R 10.8 min.
23. ES–MS^+ *m*/*z* 1020.76 (MH^+), calcd 1020.42; RP-HPLC Kromasil C8, 50–100% B in 20 min, t_R 8.9 min.