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Chemical synthesis of a homoserine-mutant of the antibacterial, head-to-tail cyclized protein AS-48 by α -ketoacid–hydroxylamine (KAHA) ligation†

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An antibacterial cyclic AS-48 protein was chemically synthesized by α -ketoacid–hydroxylamine (KAHA) ligation. Initial challenges associated with the exceptionally hydrophobic segments arising from the amphiphilic nature of the protein were resolved by the development of bespoke reaction conditions for hydrophobic segments, using hexafluoroisopropanol (HFIP) as a co-solvent. The synthetic protein displays similar biological activity and properties to those of the native protein. To support the current understanding of its antibacterial mode of action, we demonstrate the ability of AS-48 to be incorporated into synthetic multilamellar vesicles (MLVs).

Introduction

AS-48 is a cyclic antibacterial protein produced by *Enterococcus faecalis*.^{1,2} At 70 residues it is, together with uberolysin, the largest member of the class of circular bacteriocins and its structure and bactericidal mechanism have been extensively studied.^{3–7} It consists of five amphipathic α -helices and adopts a saposin fold. Owing to its cyclic nature it is exceptionally thermodynamically stable and shows an increased resistance to proteolytic degradation.^{8,9} It is active against a wide range of pathogenic bacteria – including *L. monocytogenes* and *B. cereus* – and has been proposed as a biopreservative and as a treatment for *acne vulgaris*.^{2,10} These unique properties and the cyclic nature of AS-48 make it a formidable target for chemical synthesis.^{11–20}

During the preparation of this manuscript, Tam published a chemoenzymatic synthesis of cyclic bacteriocins²¹ – including AS-48 – by butelase-mediated cyclization^{22–24} of linear unprotected peptide precursors. Although an elegant approach, it required both a folded cyclization precursor and an enzyme that is not broadly available.²⁵ Tam also reported that preparation of this protein by chemical synthesis using native chemical ligation was unsuccessful due to the low solubility of the extremely hydrophobic peptide segments, prompting us to disclose our own work on the chemical synthesis of this cyclic protein.

In this report, we document the chemical synthesis of AS-48 by α -ketoacid–hydroxylamine (KAHA) ligation, an amide-forming ligation reaction that is particularly well-suited for assembling hydrophobic peptides and proteins. KAHA ligation relies on the chemoselective reaction of α -ketoacids with hydroxylamines and tolerates both organic and aqueous solvents.^{26,27} We have developed cyclic hydroxylamines that form serine or homoserine residues upon ligation.^{28–30} The requisite peptide α -ketoacids and hydroxylamines can be conveniently prepared by Fmoc–SPPS and we have applied this reaction to the synthesis of numerous linear proteins as well as small cyclic peptides.^{31,32} The homoserine-forming variant, which uses (*S*)-5-oxaproline as the ligation partner, has the unique property of forming depsipeptides as the primary ligation products, a feature that can greatly aid in the preparation of hydrophobic sequences.

Results and discussion

Preliminary studies

In preliminary studies on the synthesis of AS-48 we identified the hydrophobicity of the peptide segments as the main obstacle. In the first attempt to synthesize AS-48 1–44 by Fmoc–SPPS, after 19 residues it became impossible to analyze the peptide by RP-HPLC. Only by employing Liu's removable poly-arginine solubilizing tag³³ at a glycine residue were we able to isolate the segment.

Design

In order to reduce the synthetic overhead, we desired to develop a synthesis strategy unconstrained by the need for additional steps and non-standard manipulations of the peptide sequences. This required careful selection of the ligation sites to

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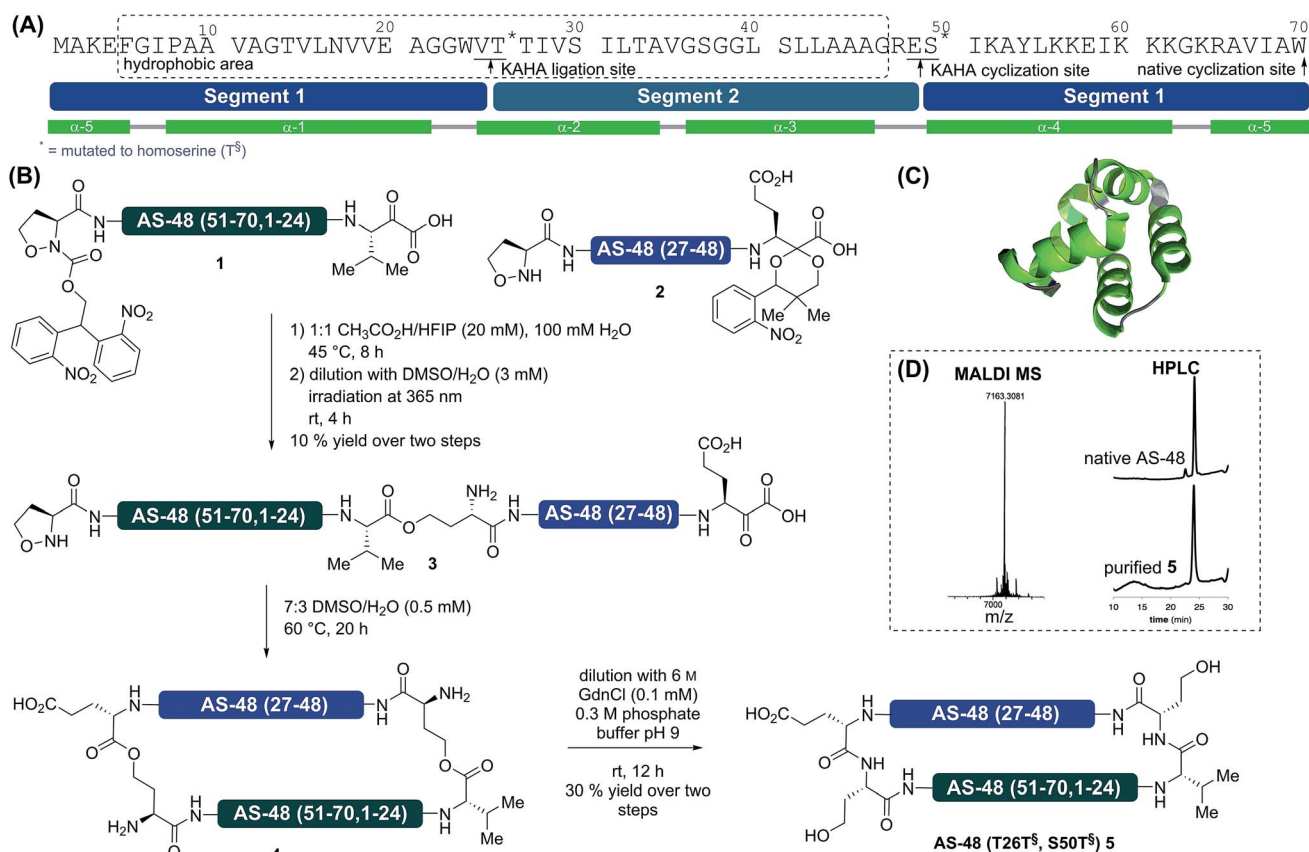
adjust the hydrophobicity of each segment while avoiding mutations possibly detrimental to biological activity. The synthesis of a cyclic peptide facilitates the selection of non-conventional strategies, as any amino acid pair can serve as a potential cyclization site.

Based on several considerations, including the predicted hydrophobicity of the segments, the secondary structure of the cyclization site, and the desire to place the two non-canonical homoserine residues at positions that would not disturb the biological activity, we selected V25T26 as the first ligation site and E49S50 as the cyclization site (Scheme 1). In order to minimize handling and purification of the hydrophobic segment and linear peptide, we sought to utilize photolabile protecting groups for the 5-oxaproline in segment 2 and for the α -ketoacid in segment 1. This would allow the release of the linear cyclization precursor 3 immediately after ligation. For the α -ketoacid we used our recently reported protecting group, which is introduced as a linker attached to a solid support.³⁴ To avoid the formation of diastereomers on the (*S*)-5-oxaproline residue, we used the achiral photolabile protecting group 2,2-bis(2-nitrophenyl)ethoxycarbonyl (di-NPEOC).³⁵

Protein synthesis

We prepared both segments 1 and 2 by Fmoc-SPPS and subjected them to our standard ligation conditions (15 mM, 9 : 1 DMSO/H₂O, 0.1 M oxalic acid, 60 °C). Possibly due to the extremely hydrophobic nature of the segments, we could not observe any conversion. Variation of the solvent ratio and switching the organic solvent component to *N*-methylpyrrolidinone (NMP) did not improve the outcome; increasing the temperature to 95 °C led to decomposition.

We speculated that the steric bulk of the valine α -ketoacid might hamper the reactivity, and resynthesized segment 1, replacing valine with leucine α -ketoacid at the C-terminus. As this ligation also failed we excluded steric repulsion as the likely cause for the lack of reactivity and considered aggregation or the formation of other perturbing structures. As both hexafluoroisopropanol (HFIP) and acetic acid have a pronounced effect on protein secondary and tertiary structure, we attempted the ligation in 1 : 1 HFIP/CH₃CO₂H.^{36,37} Gratifyingly, we observed almost complete conversion after 8 h. The reaction proceeded sufficiently fast at lower temperatures than usually employed for KAHA ligation (45 °C compared to 60 °C).



Scheme 1 Synthesis of AS-48 by KAHA ligation of two segments. (A) Sequence of AS-48 with division into two peptide segments. The dashed box indicates an area of mainly hydrophobic residues. Alpha helices are depicted in green, loop and coil regions in gray. The asterisks (*) indicate residues mutated to Hse in the synthesis by KAHA ligation. (B) Synthesis by a two-segment strategy with photoprotected 5-oxaproline segment 2 and photoprotected α -ketoacid segment 1. (C) Three dimensional structure of native AS-48. (D) MALDI mass spectrum and analytical HPLC traces at 220 nm of purified cyclic AS-48 5 and comparison with HPLC trace of native AS-48.



presumably due to their cyclic structure. This experiment indicates that AS-48 is able to be incorporated into membranes without the need for specific receptors or other active mechanisms and supports the currently assumed bactericidal mechanism of action.

Conclusions

In summary, we have reported the chemical synthesis of a biologically active variant of AS-48. This work further establishes important features of the KAHA ligation: its utility for the synthesis of highly hydrophobic proteins by forming deipeptide ligation products and operating under acidic conditions in the presence of organic solvents – in this case HFIP/CH₃CO₂H – that excel at dissolving even difficult sequences. It is notable that the cyclization proceeded well on the denatured, linear protein, in contrast to enzymatic cyclizations that require a prefolded structure. The ease of preparing long linear peptides by KAHA ligation of segments bearing photoprotected α -ketoacids and hydroxylamines by Fmoc-SPPS will make this approach ideal for preparing other types of cyclic proteins.

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Notes and references

- M. Sánchez-Hidalgo, M. Montalbán-López, R. Cebrián, E. Valdivia, M. Martínez-Bueno and M. Maqueda, *Cell. Mol. Life Sci.*, 2011, **68**, 2845–2857.
- M. J. Grande Burgos, R. P. Pulido, M. del Carmen López Aguayo, A. Gálvez and R. Lucas, *Int. J. Mol. Sci.*, 2014, **15**, 22706–22727.
- R. Cebrián, M. Martínez-Bueno, E. Valdivia, A. Albert, M. Maqueda and M. J. Sánchez-Barrena, *J. Struct. Biol.*, 2015, **190**, 162–172.
- B. Samyn, M. Martínez-Bueno, B. Devreese, M. Maqueda, A. Gálvez, E. Valdivia, J. Coyette and J. Van Beeumen, *FEBS Lett.*, 1994, **352**, 87–90.
- M. J. Sánchez-Barrena, M. Martínez-Ripoll, A. Gálvez, E. Valdivia, M. Maqueda, V. Cruz and A. Albert, *J. Mol. Biol.*, 2003, **334**, 541–549.
- C. González, G. M. Langdon, M. Bruix, A. Gálvez, E. Valdivia, M. Maqueda and M. Rico, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 11221–11226.
- D. J. Craik, *Science*, 2006, **311**, 1563–1564.
- M. Sánchez-Hidalgo, A. M. Fernández-Escamilla, M. Martínez-Bueno, E. Valdivia, L. Serrano and M. Maqueda, *Protein Pept. Lett.*, 2010, **17**, 708–714.
- E. S. Cobos, V. V. Filimonov, A. Gálvez, M. Maqueda, E. Valdivia, J. C. Martínez and P. L. Mateo, *FEBS Lett.*, 2001, **505**, 379–382.
- R. Cebrián, S. Arévalo, S. Ananou, S. Arias-Santiago, C. Riazzo, M. Dolores Rojo, M. P. Bermudez-Ruiz, E. Valdivia, M. Martínez-Bueno and M. Maqueda, *PeerJ Prepr.*, 2016, DOI: 10.7287/peerj.preprints.2107v1.
- S. Bondalapati, M. Jbara and A. Brik, *Nat. Chem.*, 2016, **8**, 407–418.
- T. J. Harmand, C. E. Murar and J. W. Bode, *Curr. Opin. Chem. Biol.*, 2014, **22**, 115–121.
- R. Kleineweischede and C. P. R. Hackenberger, *Angew. Chem., Int. Ed.*, 2008, **47**, 5984–5988.
- E. Saxon, J. I. Armstrong and C. R. Bertozzi, *Org. Lett.*, 2000, **2**, 2141–2143.
- B. L. Nilsson, L. L. Kiessling and R. T. Raines, *Org. Lett.*, 2000, **2**, 1939–1941.
- Y. Zhang, C. Xu, H. Y. Lam, C. L. Lee and X. Li, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, 201221012.
- J.-X. Wang, G.-M. Fang, Y. He, D.-L. Qu, M. Yu, Z.-Y. Hong and L. Liu, *Angew. Chem., Int. Ed.*, 2015, **54**, 2194–2198.
- E. Boll, J.-P. Ebran, H. Drobecq, O. El-Mahdi, L. Raibaut, N. Ollivier and O. Melnyk, *Org. Lett.*, 2015, **17**, 130–133.
- T. Moyal, S. N. Bavikar, S. V. Karthikeyan, H. P. Hemantha and A. Brik, *J. Am. Chem. Soc.*, 2012, **134**, 16085–16092.
- J. A. Camarero, J. Pavel and T. W. Muir, *Angew. Chem., Int. Ed.*, 1998, **37**, 347–349.
- X. Hemu, Y. Qiu, G. K. T. Nguyen and J. P. Tam, *J. Am. Chem. Soc.*, 2016, **138**, 6968–6971.
- G. K. T. Nguyen, A. Kam, S. Loo, A. E. Jansson, L. X. Pan and J. P. Tam, *J. Am. Chem. Soc.*, 2015, **137**, 15398–15401.
- G. K. T. Nguyen, X. Hemu, J.-P. Quek and J. P. Tam, *Angew. Chem., Int. Ed.*, 2016, **55**, 12802–12806.
- G. K. T. Nguyen, Y. Qiu, Y. Cao, X. Hemu, C.-F. Liu and J. P. Tam, *Nat. Protoc.*, 2016, **11**, 1977–1988.
- At the present time, active butelase cannot be produced by recombinant expression. Approximately 1 mg is obtained by extraction of 1 kg of pods of *Clitoria ternatea*. See: G. K. T. Nguyen, S. Wang, Y. Qiu, X. Hemu, Y. Lian and J. P. Tam, *Nat. Chem. Biol.*, 2014, **10**, 732–738.
- J. W. Bode, R. M. Fox and K. D. Baucom, *Angew. Chem., Int. Ed.*, 2006, **45**, 1248–1252.
- F. Rohrbacher, T. G. Wucherpfennig and J. W. Bode, in *Protein Ligation and Total Synthesis II*, ed. L. Liu, Springer International Publishing, 2014, pp. 1–31.
- V. R. Pattabiraman, A. O. Ogunkoya and J. W. Bode, *Angew. Chem., Int. Ed.*, 2012, **51**, 5114–5118.
- T. G. Wucherpfennig, F. Rohrbacher, V. R. Pattabiraman and J. W. Bode, *Angew. Chem., Int. Ed.*, 2014, **53**, 12244–12247.
- I. Pusterla and J. W. Bode, *Nat. Chem.*, 2015, **7**, 668–672.
- T. J. Harmand, C. E. Murar and J. W. Bode, *Nat. Protoc.*, 2016, **11**, 1130–1147.
- F. Thuaud, F. Rohrbacher, A. Zwicky and J. W. Bode, *Org. Lett.*, 2016, **18**, 3670–3673.
- J.-S. Zheng, M. Yu, Y.-K. Qi, S. Tang, F. Shen, Z.-P. Wang, L. Xiao, L. Zhang, C.-L. Tian and L. Liu, *J. Am. Chem. Soc.*, 2014, **136**, 3695–3704.



- 34 F. Thuaud, F. Rohrbacher, A. Zwicky and J. W. Bode, *Helv. Chim. Acta*, 2016, **99**, 868–894.
- 35 A. Hasan, K.-P. Stengele, H. Giegrich, P. Cornwell, K. R. Isham, R. A. Sachleben, W. Pfeleiderer and R. S. Foote, *Tetrahedron*, 1997, **53**, 4247–4264.
- 36 N. Hirota, Y. Goto and K. Mizuno, *Protein Sci.*, 1997, **6**, 416–421.
- 37 F. Shen, S. Tang and L. Liu, *Sci. China: Chem.*, 2011, **54**, 110–116.
- 38 During the cyclization, a small amount of amide product (S7; see the ESI†) at the cyclization site is formed. 4 rearranges quickly to S7† and more slowly to 5. Based on these observations, we assume that the rearrangement at E49T⁸50 proceeds quickly, whereas the rearrangement at V25T⁸26 is slower.
- 39 Two days after the first antibacterial assay, the experiment was repeated with unchanged activity.
- 40 M. Sánchez-Hidalgo, M. Martínez-Bueno, A. M. Fernández-Escamilla, E. Valdivia, L. Serrano and M. Maqueda, *J. Antimicrob. Chemother.*, 2008, **61**, 1256–1265.
- 41 R. Cebrián, M. Maqueda, J. L. Neira, E. Valdivia, M. Martínez-Bueno and M. Montalbán-López, *Appl. Environ. Microbiol.*, 2010, **76**, 7268–7276.
- 42 V. L. Cruz, J. Ramos, M. N. Melo and J. Martínez-Salazar, *Biochim. Biophys. Acta, Biomembr.*, 2013, **1828**, 2524–2531.
- 43 A. Gálvez, M. Maqueda, M. Martínez-Bueno and E. Valdivia, *J. Bacteriol.*, 1991, **173**, 886–892.
- 44 S. D. Tilley and M. B. Francis, *J. Am. Chem. Soc.*, 2006, **128**, 1080–1081.
- 45 In preliminary experiments using 80 μM of wildtype AS-48 we had observed precipitation after incubation of MLVs with 80 μM AS-48 at pH 8 without SDS. In a control experiment, MLVs were incubated with pH 8 buffer without AS-48. No precipitation was observed. This indicated that – similar to the assumed biological mode of action – AS-48 was able to lyse MLVs. A low concentration of SDS (7 mM) proved to be effective to prevent lysis of the MLVs.

