Synthesis and Characterization of the 47-Residue Heterodimeric Antimicrobial Peptide Distinctin, Featuring Directed Disulfide Bridge Formation

Daniel G. Mullen,[1,2] Raffaello Verardi,[1] Fernando Porcelli,[3] Andrea Scaloni,[4] George Barany,[2] Gianluigi Veglia[1,2]

[1]*Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN 55455*

[2]*Department of Chemistry, University of Minnesota, Minneapolis, MN 55455*

[3]*Department for Innovation in Biological, Agro-Food and Forest Systems, DIBAF, University of Tuscia, Viterbo, Italy*

[4]*Proteomics & Mass Spectrometry Laboratory, ISPAAM, National Research Council, 80147 Naples, Italy*

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ABSTRACT:

Distinctin, a 47-residue heterodimeric peptide with potent antimicrobial activity, comprises two monomeric units linked covalently by a disulfide bond between Cys19 from the 22-residue A chain and Cys23 from the 25-residue B chain. Previous synthetic strategies involved assemblies of the two individual chains, followed by their co-oxidation to form the connecting disulfide bridge, and resulted in a mixture of three species: two homodimers and one heterodimer. Here, we report synthesis of exclusively heterodimeric distinctin, using recently developed tactics for directed disulfide bridge formation. Material prepared this way was characterized and found to be suitable for more detailed structural studies. © 2012 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 98: 479–484, 2012.

Keywords: *directed disulfide bridge formation; solid-phase synthesis; distinctin; antimicrobial peptides; solution NMR*

This article is dedicated to the late Michael and Kate Bárány, in tribute to their outstanding contributions to understanding the biochemistry and biophysics of peptides and proteins.

Additional Supporting Information may be found in the online version of this article.

Correspondence to: Gianluigi Veglia, 6-155 Jackson Hall, 321 Church St SE, Minneapolis, MN 55455; e-mail: vegli001@umn.edu

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INTRODUCTION

The increasing resistance of bacteria to antibiotics continues to be a serious global emergency for human health.[1–5] To counteract this threat, researchers are seeking and studying novel alternatives, including antimicrobial agents that are unaffected by acquired resistance. In particular, antimicrobial peptides (AMPs) represent a viable approach to solve this problem.[6–10] AMPs evolved as the first chemical defense mechanism of eukaryotic cells against bacteria, protozoa, fungi, and viruses; they show broad spectra of activity, and represent potential chemical templates for developing antibiotics.[11–16] Some AMPs are already in use to treat topical infections and have been commercialized by pharmaceutical companies.[17–19]

Therefore, there is an increasing need to elucidate structural information on AMPs alone and during interactions in membrane environments, to better understand their mechanisms of action and to improve their design for potential therapeutic applications.

NMR spectroscopy has played a major role in research on AMPs in model membrane systems, and has inspired the development of new AMPs with increased potencies and selectivities by comparison to those that occur naturally.[20–22] In addition, antimicrobial polymers have been designed and synthesized.[23,24] Both liquid- and solid-state NMR have been

applied.[25–31] Solid-state NMR can give structural and dynamic information about the large-size complexes formed by lipid bilayers with peptides.[26,32–35] Since the appropriate NMR techniques generally require milligram amounts of isotopically labeled peptides, there has been a significant effort to develop efficient procedures to obtain sufficient quantities of such peptides.

The present report focuses on distinctin, a 47-residue peptide originally extracted from *Phyllomedusa distincta*, a tree frog that lives in the forests of Brazil.[36] Distinctin preferentially interacts with negatively charged membranes and is active against Gram-positive and Gram-negative bacteria.[37–39] According to recent findings on murine models, this peptide may be successfully used to treat topical infections and for embedding of medical devices.[37,40] Structurally, distinctin consists of two linear chains of 22 (A chain) and 25 (B chain) residues, linked by a disulfide bridge that connects Cys19 of the A chain with Cys23 of the B chain (Figure 1). Previous synthetic schemes to create distinctin relied on air oxidation to form the crosslinking disulfide.[38,39] Such methods suffer from the drawback that, in addition to the desired A–B heterodimeric distinctin, the A–A and B–B homodimers are also produced, in a molar ratio corresponding to the relative thermodynamic stabilities. Since all three species have similar HPLC retention times, the required purification step poses difficulties. Furthermore, nonselective air oxidation results in substantial diversion of material into undesired homodimeric by-products.

In contrast, we report here the chemical synthesis of distinctin by using a directed disulfide synthetic strategy that creates *only* the wanted heterodimeric product. Our overall strategy, and the specific tactics to implement it, allows for the efficient and cost-effective production of material that incorporates stable isotopic labels at discrete positions along the polypeptide chains, as is needed for conformational studies and investigations on the interactions between distinctin and lipid membranes by solid-state NMR spectroscopy.[35]

MATERIALS AND METHODS

General

All solvents were of HPLC grade. DIEA and TFA were of Sequalog/peptide synthesis grade from Fisher. Fmoc amino acids, HBTU, and HOBt were from AnaSpec. Preloaded Fmoc-AA-PEG-PS resins were purchased from Applied Biosystems. Isotopically labeled amino acids with *N*-Fmoc protection were from Cambridge Isotopes. All other reagents were from Sigma-Aldrich. Analytical and preparative RP-HPLC was conducted with water and CH_3CN solvent mixtures, each containing 0.1% TFA on Vydac 218TP54 C18 columns, 4.6 mm × 250 mm, 5.0 μ particles.

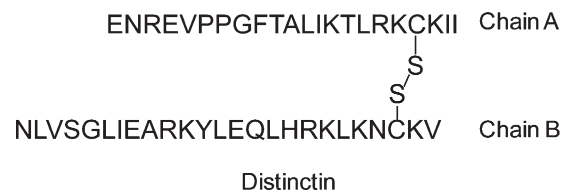


FIGURE 1 Primary structure and disulfide bridging of distinctin. Both *N*-termini are amino groups, and both *C*-termini are carboxyl groups.

Peptide Synthesis

SPPS of Distinctin Chains. Linear sequences were assembled on PEG-PS resins, with HBTU/DIEA as the coupling reagent, on a CEM Liberty peptide synthesizer with the default instrument Fmoc-based SPPS protocols. Isotopically labeled amino acids were double coupled using one equivalent of the Fmoc derivative for each cycle, mediated by the BOP reagent (1.0 eq.) and DIEA (2.0 eq.) as base. Subsequently, a single coupling with unlabeled Fmoc-amino acid 4.0 equiv) was run to react any remaining amine groups. For the chain that contained the isotopic labels, FmocCys(Trt) was used, while FmocCys(Acm) was incorporated into the unlabeled chain. The *N*-terminal Fmoc group was removed in the last instrument cycle.

Distinctin A Chain Containing Cys(Scm) (3). To convert Cys(Acm) to Cys(Scm) on-resin, the peptide-resin (220 mg, 25 μmol) was first swollen by extensive washing in CH_2Cl_2. After the peptide-resin was suspended in CH_2Cl_2 (4 mL), neat methoxycarbonylsulfenyl chloride (3.0 μL, 33 μmol) was added, and the mixture was shaken for 3 h. The peptide-resin was then washed with CH_2Cl_2 (3 × 5 mL) and dried. The peptide was cleaved from the resin with freshly prepared reagent K[41] [TFA—phenol—thioanisole—water—ethanedithiol (82.5:5:5:5:2.5 v/v)] (5 mL) under argon for 2 h, precipitated with ether (80 mL), and centrifuged to collect a pellet which was washed twice with ether. The crude peptide was dissolved in 0.1% aqueous TFA (20 mL), filtered, and purified by RP-HPLC. Yield: 12.1 mg (18%);T_r = 43 min; purity by RP-HPLC: 98%; deconvoluted ESI-MS: calculated mass 2615.4, observed mass 2615.4.

Distinctin B Chain Containing ^{15}N, ^{13}C-G5, ^{15}N-L6, ^{15}N-Y12, ^{15}N-L13 (4). The peptide was cleaved from the resin with freshly prepared reagent K (5 mL) under argon for 3 h, precipitated with ether (80 mL), and centrifuged to form a pellet which was washed twice with ether. The crude peptide was dissolved in 0.1% aqueous TFA, filtered, and purified by RP-HPLC. Yield: 8.0 mg (9%); T_r = 40 min; purity by RP-HPLC: 95%; deconvoluted ESI-MS: calculated mass 2957.7, observed mass 2956.5.

Distinctin (5) by Directed Disulfide Formation. The peptides were dissolved in 0.1% aqueous TFA at 1.0 mg/mL: [A chain] = 0.38 m*M*, [B chain] = 0.34 m*M*. $Zn(OAc)_2$ dihydrate (3.0 mg, 13.5 μmol, 5.0 equiv) was added to the B chain solution (8.0 mL, 2.7 μmol), followed by the A chain solution (7.4 mL, 2.8 μmol, 1.05 equiv); at this point, the homogeneous solution had a pH value of about 2. To start the reaction, 0.10*M* acetate buffer (pH = 5.0, 1.0 mL) was added, and the pH of the reaction was adjusted into the range of 5.0–5.5 by careful dropwise addition of 1.0*N* aqueous NaOH. The reaction was monitored by HPLC and judged to be

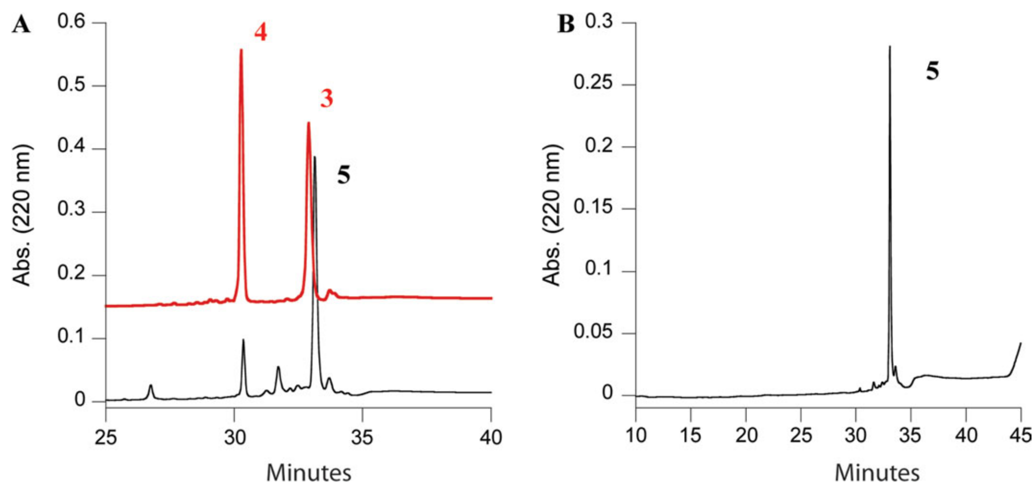
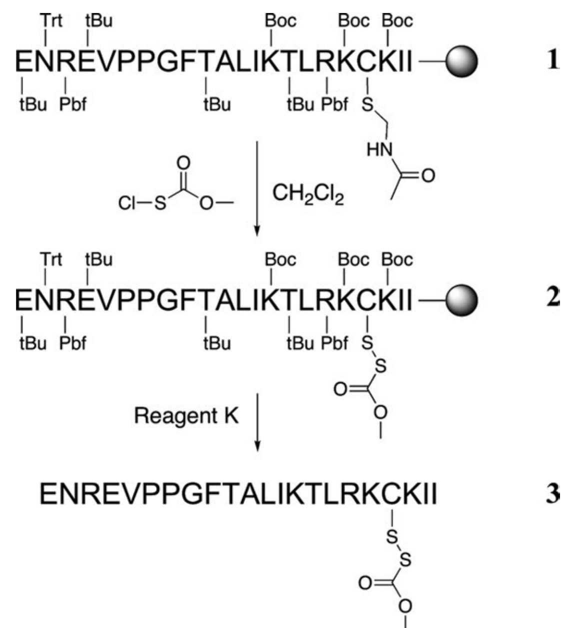


FIGURE 2 (A) RP-HPLC monitoring of directed disulfide formation reaction (Scheme 2). Red trace: initial mixture of the reacting peptides at pH ~ 2, before the pH was adjusted to initiate the reaction. Note starting **3** and **4**. Black trace: peptide mixture after 1 h of reaction following pH adjustment to a value of 5.3; chromatogram shows formation of **5** and complete consumption of limiting **3**. (B) RP-HPLC of distinctin (**5**), after purification. All chromatograms involve a gradient from 10 to 50% (B), over 40 min, flow 1.0 mL/min.

complete within 1 h (Figure 2). The pH was then lowered into the range of 2–3 by addition of neat TFA, the reaction was filtered, and purification by RP-HPLC followed. Yield: 6.7 mg (45%); T_r = 43 min; purity by RP-HPLC: 92%; deconvoluted ESI-MS: calculated mass 5484.6, observed mass 5483.4. For the syntheses of distinctin A chain containing free cysteine residue and distinctin B chain containing Cys(Scm), see Supporting Information.

RESULTS AND DISCUSSION

We report here a directed disulfide synthesis strategy for the unambiguous and cost-effective production of heterodimeric distinctin molecules needed for NMR structural investigations. The application of directed methods to the peptide field dates to independent pioneering work of Kamber and Hiskey[42,43] in the 1970s, and the overall principles and practices have been reviewed several times.[44–46] Our preferred method of directed disulfide formation is to react two chains, the first containing a free cysteine thiol, and the second containing an activated Cys(Scm) residue, with one another in solution.[47–50] In turn, we have found that the most robust and effective way to introduce the needed Cys(Scm) activation is to first carry out solid-phase peptide synthesis (SPPS) for assembly of a chain that incorporates a Cys(Acm) residue, and then treating the completed peptide-resin with methoxycarbonysulfenyl chloride to effect the Acm to Scm transformation (Scheme 1).[49,51] This procedure has several advantages, including (i) potential side reactions such as sulfenylation of Tyr or His residues[52–54] are avoided, because the peptide-resin is still globally protected at this stage; (ii) reaction stoichiometry is much easier to control during on-resin conversion, with respect to corresponding reactions in solution; and (iii) an intermediate purification step for isolation of the Cys(Acm)-containing peptide is no longer necessary, thus increasing the overall yield. The Scm group is stable throughout the standard acid/scavenger conditions used to release peptides from the support after SPPS, as well as during RP-HPLC purification. Moreover, peptides that contain a Cys(Scm) residue can be readily lyophilized and then stored

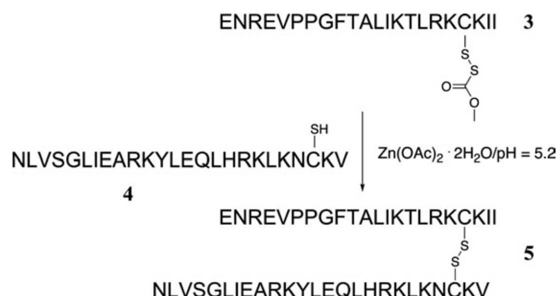


SCHEME 1 On-resin conversion of Cys(Acm) to Cys(Scm) in protected distinctin A-chain resin (*N*-terminus free from Fmoc removal step, conventional Wang-type handle), and subsequent cleavage from the resin with reagent K.

at 0°C for several weeks. For the synthetic work reported here that is directed towards distinctin, we have found that on-resin conversion of Cys(Acm) to Cys(Scm) worked equally well on either the A or B chains (data not shown); we choose to install the Cys(Scm) residue in whatever chain did not contain isotopic labels. Even though the Cys(Acm) residues that were to be converted in each case occur rather close to the respective *C*-termini in the sequences of greater than 20 residues, and one might be concerned a priori with hypothetical accessibility problems, this turned out to not be an issue in the actual practice.

In previous work, we found that further transformations of the Cys(Scm) group on full length peptides while still attached to the solid support, be they reduction with DTT or thiolytic displacement, gave varying results that seemed to depend on the location of the Cys(Scm) group in the sequence.[51] Thus, we elected to carry out the directed disulfide reaction in solution, where it can be monitored readily by RP-HPLC. In our experience, the solution directed disulfide formation step (Scheme 2) is best conducted under mildly acidic conditions with Zn^{2+} catalysis,[50] building on a precedent of Naider for alkylation of cysteine-containing peptides.[55] Under such acidic reaction conditions, unwanted side reactions such as air oxidation of chains (leading to homodimers) and disulfide exchange (scrambling of chains) are minimized or avoided entirely. The reaction of the Cys(Scm)-containing A chain (**3**) with the isotopically labeled B chain (**4**) was observed to proceed cleanly and with good kinetics in the pH range of 5.0–5.5, as established by an acetate buffer (Figure 2A). Distinctin (**5**) was the major product formed, and the reaction reached completion without further side product formation once the limiting reagent, the Cys(Scm)-containing A chain, was consumed. Because primarily **5** was produced in this reaction, with negligible levels of the two possible symmetrical disulfide-linked homodimers, RP-HPLC purification was straightforward (Figure 2B).

The synthetic strategy reported here is particularly conducive to the production of materials required for NMR stud-



SCHEME 2 Directed disulfide formation strategy for synthesis of distinctin, as catalyzed by $Zn(OAc)_2$ at pH 5.0–5.5.

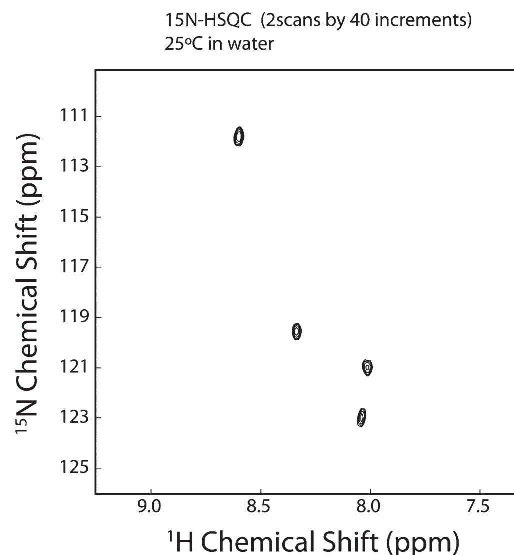


FIGURE 3 [^{1}H-^{15}N]-HSQC spectrum of purified distinctin in aqueous buffer. The sample contained the following isotopic labels in the B chain: ^{15}N, ^{13}C-G5, ^{15}N-L6, ^{15}N-Y12, and ^{15}N-L13. All of the expected resonances appeared in the peptide fingerprint.

ies. To conserve expensive ^{13}C and/or ^{15}N-labeled amino acids during chain assembly by stepwise SPPS, the appropriate Fmoc-amino acids were double coupled on a Liberty microwave synthesizer by using only one equivalent per cycle. A third coupling reaction followed with unlabeled Fmoc-amino acid was performed, so that acylation would go to completion, thereby preventing the formation of deletion peptides missing a residue at those positions that were to be labeled. When using this plan for the synthesis of the distinctin B chain, where four residues were labeled, we found upon electrospray mass spectrometry that a mass that was lower by about one atomic mass unit was reported for the product. This result means that incorporation of labels in each position was not quite quantitative. However, the ^{1}H-^{15}N HSQC NMR spectrum of the purified distinctin product showed four distinct alpha cross peaks of approximate equal intensity (Figure 3). This result validates our overall strategy, which will now allow us to move forward with planned solid-state NMR experiments.

CONCLUSIONS

We report here a new synthesis for the AMP distinctin that features directed disulfide bridge formation. Unlike the previous synthetic method that generated a statistical mixture of homodimers along with desired heterodimer, our scheme makes possible the ready isolation of milligram quantities of pure distinctin, including variants with site-specific incorporation of isotopic labeled residues. Synthetic distinctin con-

taining several ^{15}N-amino acids was characterized by NMR. A high-quality [^{1}H-^{15}N]-HSQC spectrum was obtained, indicative of a well-folded polypeptide. Our overall synthetic process is straightforward, economical, and likely generalizable to the synthesis of other unsymmetrical disulfide-containing molecules of both peptidic and nonpeptidic nature. Our laboratory is now poised to address further structural characterization of distinctin by solution and solid-state NMR, including studies of how the peptide interacts with membrane-mimetic systems.

REFERENCES

1. Rubinchik, E.; Schneider, T.; Elliott, M.; Scott, W. R.; Pan, J.; Anklin, C.; Yang, H.; Dugourd, D.; Muller, A.; Gries, K.; Straus, S. K.; Sahl, H. G.; Hancock, R. E. Antimicrob Agents Chemother, 2011, 55, 2743–2754.
2. Paterson, D. L. Curr Opin Pharmacol 2006, 6, 486–490.
3. Ho, J.; Tambyah, P. A.; Paterson, D. L. Curr Opin Infect Dis 2010, 23, 546–553.
4. Giamarellou, H.; Poulakou, G. Drugs 2009, 69, 1879–1901.
5. Giamarellou, H. Int J Antimicrob Agents 2010, 36(Suppl 2), S50–S54.
6. Wu, G.; Li, X.; Fan, X.; Wu, H.; Wang, S.; Shen, Z.; Xi, T. Peptides, 2011, 32, 1139–1145.
7. Venugopal, D.; Klapper, D.; Srouji, A. H.; Bhonsle, J. B.; Borschel, R.; Mueller, A.; Russell, A. L.; Williams, B. C.; Hicks, R. P. Bioorg Med Chem 2010, 18, 5137–5147.
8. Mechkarska, M.; Ahmed, E.; Coquet, L.; Leprince, J.; Jouenne, T.; Vaudry, H.; King, J. D.; Conlon, J. M. Comp Biochem Physiol C Toxicol Pharmacol 2010, 152, 467–472.
9. Bommarius, B.; Kalman, D. IDrugs 2009, 12, 376–380.
10. Abbanat, D.; Morrow, B.; Bush, K. Curr Opin Pharmacol 2008, 8, 582–592.
11. Hata, T. R.; Gallo, R. L. Semin Cutan Med Surg 2008, 27, 144–150.
12. Izadpanah, A.; Gallo, R. L. J Am Acad Dermatol 2005, 52, 381–390; quiz 391–382.
13. Lai, Y.; Gallo, R. L. Trends Immunol 2009, 30, 131–141.
14. Murakami, M.; Lopez-Garcia, B.; Braff, M.; Dorschner, R. A.; Gallo, R. L. J Immunol 2004, 172, 3070–3077.
15. Jenssen, H.; Hamill, P.; Hancock, R. E. Clin Microbiol Rev 2006, 19, 491–511.
16. Schittek, B.; Paulmann, M.; Senyurek, I.; Steffen, H. Infect Disord Drug Targets 2008, 8, 135–143.
17. Brogden, K. A. Nat Rev Microbiol 2005, 3, 238–250.
18. Gordon, Y. J.; Romanowski, E. G.; McDermott, A. M. Curr Eye Res 2005, 30, 505–515.
19. Marr, A. K.; Gooderham, W. J.; Hancock, R. E. Curr Opin Pharmacol 2006, 6, 468–472.
20. Avrahami, D.; Oren, Z.; Shai, Y. Biochemistry 2001, 40, 12591–12603.
21. Bechinger, B. Adv Exp Med Biol 2010, 677, 24–30.
22. Hong, J.; Oren, Z.; Shai, Y. Biochemistry 1999, 38, 16963–16973.
23. Mensa, B.; Kim, Y. H.; Choi, S.; Scott, R.; Caputo, G. A.; DeGrado, W. F. Antimicrob Agents Chemother 2011, 55, 5043–5053.
24. Tew, G. N.; Scott, R. W.; Klein, M. L.; Degrado, W. F. Acc Chem Res 2009, 43, 30–39.
25. Porcelli, F.; Verardi, R.; Shi, L.; Henzler-Wildman, K. A.; Ramamoorthy, A.; Veglia, G. Biochemistry 2008, 47, 5565–5572.
26. Bechinger, B.; Gierasch, L. M.; Montal, M.; Zasloff, M.; Opella, S. J. Solid State Nucl Magn Reson 1996, 7, 185–191.
27. Georgescu, J.; Munhoz, V. H.; Bechinger, B. Biophys J 2010, 99, 2507–2515.
28. Mascioni, A.; Porcelli, F.; Ilangovan, U.; Ramamoorthy, A.; Veglia, G. Biopolymers 2003, 69, 29–41.
29. Porcelli, F.; Buck, B.; Lee, D. K.; Hallock, K. J.; Ramamoorthy, A.; Veglia, G. J Biol Chem 2004, 279, 45815–45823.
30. Porcelli, F.; Buck-Koehntop, B. A.; Thennarasu, S.; Ramamoorthy, A.; Veglia, G. Biochemistry 2006, 45, 5793–5799.
31. Epand, R. F.; Maloy, L.; Ramamoorthy, A.; Epand, R. M. Biophys J 2010, 98, 2564–2573.
32. Aisenbrey, C.; Bertani, P.; Bechinger, B. Methods Mol Biol 2010, 618, 209–233.
33. Gehman, J. D.; Luc, F.; Hall, K.; Lee, T. H.; Boland, M. P.; Pukala, T. L.; Bowie, J. H.; Aguilar, M. I.; Separovic, F. Biochemistry 2008, 47, 8557–8565.
34. Lam, Y. H.; Hung, A.; Norton, R. S.; Separovic, F.; Watts, A. Proteins 2010, 78, 858–872.
35. Verardi, R.; Traaseth, N. J.; Shi, L.; Porcelli, F.; Monfregola, L.; de Luca, S.; Amodeo, P.; Veglia, G.; Scaloni, A. Biochim Biophys Acta 2011, 1808, 34–40.
36. Batista, C. V.; Scaloni, A.; Rigden, D. J.; Silva, L. R.; Rodrigues Romero, A.; Dukor, R.; Sebben, A.; Talamo, F.; Bloch, C. FEBS Lett 2001, 494, 85–89.
37. Cirioni, O.; Ghiselli, R.; Orlando, F.; Silvestri, C.; De Luca, S.; Salzano, A. M.; Mocchegiani, F.; Saba, V.; Scalise, G.; Scaloni, A.; Giacometti, A. Crit Care Med 2008, 36, 2629–2633.
38. Raimondo, D.; Andreotti, G.; Saint, N.; Amodeo, P.; Renzone, G.; Sanseverino, M.; Zocchi, I.; Molle, G.; Motta, A.; Scaloni, A. Proc Natl Acad Sci USA 2005, 102, 6309–6314.
39. Resende, J. M.; Moraes, C. M.; Munhoz, V. H.; Aisenbrey, C.; Verly, R. M.; Bertani, P.; Cesar, A.; Pilo-Veloso, D.; Bechinger, B. Proc Natl Acad Sci USA 2009, 106, 16639–16644.
40. Giacometti, A.; Cirioni, O.; Ghiselli, R.; Orlando, F.; Silvestri, C.; Renzone, G.; Testa, I.; Mocchegiani, F.; Della Vittoria, A.; Saba, V.; Scaloni, A.; Scalise, G. J Biomed Mater Res A 2007, 81, 233–239.
41. King, D. S.; Fields, C. G.; Fields, G. B. Int J Pept Protein Res 1990, 36, 255–266.
42. Kamber, B. Helv Chim Acta 1973, 56, 1370–1381.
43. Hiskey, R. G.; Muthukumaraswamy, N.; Vunnam, R. R. J Org Chem 1975, 40, 950–953.
44. Andreu, D.; Albericio, F.; Sole, N. A.; Munson, M. C.; Ferrer, M.; Barany, G. Methods Mol Biol 1994, 35, 91–169.
45. Annis, I.; Hargittai, B.; Barany, G. Methods Enzymol 1997, 289, 198–221.
46. Akaji, K.; Kiso, Y. In Synthesis of Peptides and Peptidomimetics; Goodman, M.; Felix, A.; Moroder, L.; Toniolo, C., Eds.; Georg ThiemeVerlag Stuttgart: New York, 2004.
47. Chen, L.; Bauerova, H.; Slaninova, J.; Barany, G. Pept Res 1996, 9, 114–121.
48. Chen, L.; Golser, R.; Machova, A.; Slaninova, J.; Barany, G. J Med Chem 1999, 42, 5002–5009.

49. Chen, L.; Zoulikova, I.; Slaninova, J.; Barany, G. J Med Chem 1997, 40, 864–876.
50. Wollack, J. W.; Zeliadt, N. A.; Mullen, D. G.; Amundson, G.; Geier, S.; Falkum, S.; Wattenberg, E. V.; Barany, G.; Distefano, M. D. J Am Chem Soc 2009, 131, 7293–7303.
51. Mullen Daniel, G.; Weigel, B.; Barany, G.; Distefano Mark, D. J Pept Sci 2010, 16, 219–222.
52. Kamber, B.; Hartmann, A.; Eisler, K.; Riniker, B.; Rink, H.; Sieber, P.; Rittel, W. Helv Chim Acta 1980, 63, 899–915.
53. Scoffone, E.; Fontana, A.; Rocchi, R. Biochemistry 1968, 7, 971–979.
54. Buku, A.; Altmann, R.; Wieland, T. Justus Liebigs Annalen der Chemie 1976, 417–431.
55. Naider, F. R.; Becker, J. M. Biopolymers 1997, 43, 3–14.