ORGANIC LETTERS

2003
Vol. 5, No. 10
1749−1752

Biomimetic Synthesis of Gramicidin S and Analogues by Enzymatic Cyclization of Linear Precursors on Solid Support

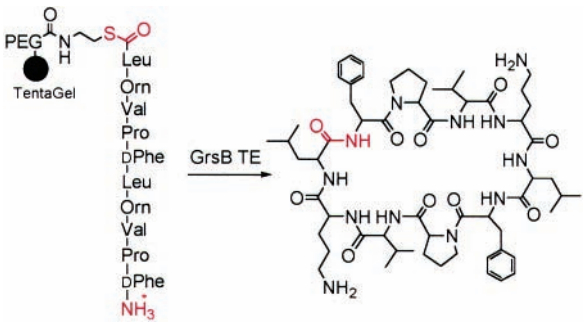
Xiaoming Wu, Xianzhang Bu, Ka Man Wong, Weili Yan, and Zhihong Guo*

Department of Chemistry and Biotechnology Research Institute, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China

chguo@ust.hk

Received March 12, 2003

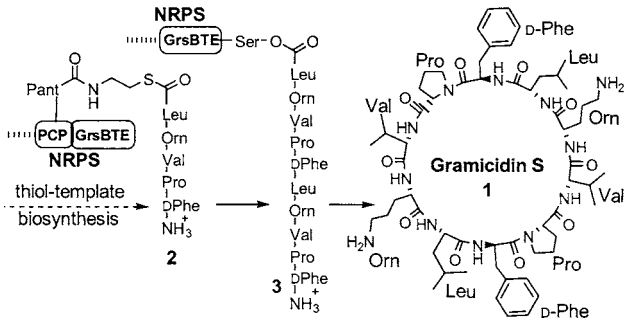
ABSTRACT



Gramicidin S is a potent decapeptide antibiotic with high hemolytic activity but is unlikely to provoke microbial resistance. Here we demonstrate that gramicidin thioesterase (GrsB TE) correctly cyclizes immobilized linear decapeptide precursors into head-to-tail products, indicating its suitability for parallel solid-phase synthesis of gramicidin analogues from linear precursors on solid support. This chemoenzymatic method will enable the optimization of the therapeutic index of the natural product to fight microbial resistance.

Widespread resistance of microbial pathogens to currently available antibiotics has become a serious public health threat.[1] To contain the resistance, considerable interest has been focused on the amphiphilic peptide antibiotics, including gramicidin S (**1**, Scheme 1),[2] which has hydrophobic side chains on one side of its rigid antiparallel β-pleated sheet structure and hydrophilic side chains on the other side.[3] This natural product is an attractive target for new drug discovery because it acts on cell membranes where its accumulation results in disruption of the barrier functions[4] and no resistance to this antibiotic has been found so far.[5] In addition, development of resistance to such a compound is unlikely

Scheme 1. Dimerization and Cyclization of Pentapeptide Precursor in Gramicidin S Biosynthesis



(1) (a) Neu, H. C. *Science* **1992**, *257*, 1064. (b) Cohen, M. L. *Science* **1992**, *257*, 1050.

(2) For examples, see: (a) Dathe, M.; Wieprecht, T.; Nikolenko, H.; Handel, L.; Maloy, W. L.; MacDonald, K.; Beyermann, M.; Bienert, M. *FEBS Lett.* **1997**, *403*, 208. (b) Oren, Z.; Hong, J.; Shai, Y. *J. Biol. Chem.* **1997**, *272*, 14643. (c) Shai, Y.; Oren, Z. *J. Biol. Chem.* **1996**, *271*, 7305. (d) Oren, Z.; Shai, Y. *Biochemistry* **1997**, *36*, 1826. (e) Dathe, M.; Shumann, M.; Wieprecht, T.; Winkler, A.; Beyermann, M.; Krause, E.; Matsuzaki, K.; Murase, O.; Bienert, M. *Biochemistry* **1996**, *35*, 12612. (f) Kondejewski, L. H.; Jelokhani-Niaraki, M.; Farmer, S. W.; Bruce, L. Kay, C. M.; Sykes, B. D.; Hancock, R. E. W.; Hodges, R. S. *J. Biol. Chem.* **1999**, *274*, 13181.

10.1021/ol034437y CCC: $25.00
Published on Web 04/16/2003

because it requires significant alteration of the lipid composition. However, the effort to increase its therapeutic index, namely, minimizing its high hemolytic activity while maintaining its high antibiotic activity, has been hampered by the limited number of accessible analogues of the natural product through traditional synthetic methods.[6]

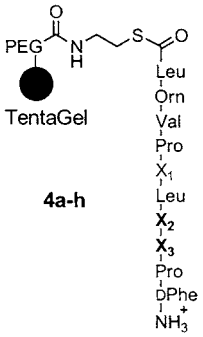
One way to circumvent this problem is to take advantage of enzymes in gramicidin S biosynthesis for chemoenzymatic analogue synthesis. The natural product is a cyclic decapeptide antibiotic composed of two repetitive pentapeptide units that is produced by *Bacillus brevis*.[7] It is synthesized by a thiol-template mechanism catalyzed by modular nonribosomal peptide synthases (NRPS) GrsA and GrsB.[8] Of particular interest is the last functional domain of GrsB, which is predicted to be a thioesterase (GrsB TE) responsible for dimerizing the linear pentapeptide precursor generated by the NRPS and then cyclizing the dimer head-to-tail to release the product gramincidin S (Scheme 1).

In its putative form, GrsB TE was found to retain the dimerization and cyclization power toward the pentapeptide substrate (**2**) coupled to a *N*-acetylcysteaminyl (NAC) group at carboxy terminus, a mimic of the native phosphopantetheinyl spacer linking the linear peptide and the peptidyl carrier protein (PCP). However, the yields of the dimer and the cyclic product were very low, whereas the hydrolysis of the thioester dominated the enzyme-catalyzed reaction.[9] The abortive thioester hydrolysis was avoided when the linear dimerized decapeptide precursor (**3**) was used directly as the substrate for GrsB TE. In homogeneous solution, the enzyme was further demonstrated to tolerate change in the linear peptide sequence and be capable of cyclizing peptide precursors with variable sizes. These investigations show the potential of the thioester in parallel generation of the cyclic peptide analogues for the natural product.

The most efficient way to generate analogues of the natural product is to use the thioesterase to cyclize an array of linear decapeptide precursors through the combinatorial solid-phase peptide synthesis (SPPS). Feasibility of this strategy has been proved by the successful synthesis of tyrocidine A analogues by TycC TE,[10] an NRPS thioesterase homologous to GrsB TE. Here we explore the activity of the gramicidin S thioesterase toward linear precursor substrates on solid support and test its suitability for solid-phase parallel synthesis of the natural product analogues.

The gramicidin S thioesterase gene was amplified from *B. brevis* (ATCC 9999) genomic DNA and expressed in the vector pFAB5c.His.[11] The 34-kD thioesterase with a histidine tag at the carboxy terminus was purified from an *E. coli* host, using metal-chelating affinity chromatography and anion-exchange column chromatography, in good yield (~1.0 mg L^{-1}) and high purity (>95% pure by SDS−PAGE). The expressed protein was tested active toward NAC-pentapeptide and pentapeptide dimer with a comparable k_{cat} and K_M as reported.[9]

The fully deprotected biosynthetic decapeptide precursor of gramicidin S (**4a**, Figure 1) was synthesized on TentaGel-OH resin, using a modified Fmoc deprotection method for solid-phase peptide synthesis.[12] This precursor, similar to that for tyrocidine A,[13] was found to cyclize spontaneously into head-to-tail product (**1**) in ammonia solution, providing a convenient way to quantify the total amount of correct linear decapeptide precursor.



PEG = polyethyleneglycol

	X_1	X_2	X_3
4a	DPhe	Orn	Val
4b	DPhe	Lys	Val
4c	DAla	Orn	Val
4d	DAla	Lys	Val
4e	DPhe	Orn	Phe
4f	DPhe	Lys	Phe
4g	DAla	Orn	Phe
4h	DAla	Lys	Phe

Figure 1. Structures of the linear precursors on TentaGel resin for cyclization by GrsB TE.

To test the activity of the enzyme toward the solid-supported substrate, freshly deprotected linear peptide on the resin (50 mg, loading 0.20 mmol g^{-1}) was extensively washed with methanol and double-deionized water and immersed in 100 μL of the GrsB TE (100 μg mL^{-1}) in 25 mM 3-(*N*-morpholino)propanesulfonate (MOPS) buffer (pH 7.0) in a 1.5-mL microfuge tube. The suspension was constantly swirled and incubated at 37 °C for 3 h. After the incubation, the reaction was quenched by addition of 100 μL of 1.7% trifluoroacetic acid in H_2O. The resin was separated from the supernatant and washed thrice with 1 mL

(3) (a) Hodgkin, D. C.; Oughton, B. M. *Biochem. J.* **1957**, *65*, 752. (b) Stern, A.; Gibbons, W. A.; Craig, L. C. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *61*, 734. (c) Ohnishi, M.; Urry, D. W. *Biochim. Biophys. Res. Commun.* **1969**, *36*, 194.

(4) Prenner, E. J.; Lewis, R. N. A. H.; McElhaney, R. N. *Biochim. Biophys. Acta* **1999**, *1462*, 201.

(5) Hancock, R. E. W. *Lancet* **1997**, *349*, 418.

(6) Waki, M.; Izumiya, N. In *Biochemistry of Peptide Antibiotics: Recent Advances in the Biotechnology of β-Lactams and Microbial Bioactive Peptides*; Kleinkaug, H., von Döhren, H., Eds.; Walter de Gruyter: Berlin, 1990, pp 205−244.

(7) (a) Gause, G. G.; Brazhnikova, M. G. *Nature* **1944**, *154*, 703. (b) Izumiya, N.; Kato, T.; Aoyagi, H.; Waki, M.; Kondo M. In *Synthetic Aspects of Biologically Active Cyclic Peptides-Gramicidin and Tyrocidines*; Kodansha Ltd. and Wiley: Tokyo, 1979.

(8) (a) Krätzschmar, J.; Krause, M.; Marahiel, M. A. *J. Bacteriol.* **1989**, *171*, 5422. (b) Kleinkauf, H.; von Döhren, H. *Eur. J. Biochem.* **1990**, *192*, 1. (c) Turgay, K.; Krause, M.; Marahiel, M. A. *Mol. Micriobiol.* **1992**, *6*, 529. (d) Kleinkauf, H.; von Döhren, H. *Eur. J. Biochem.* **1996**, *236*, 335. (e) von Döhren, H.; Keller, U.; Vater, J.; Zocher, R. *Chem. Rev.* **1997**, *97*, 2675. (f) Marahiel, M. A.; Stachelhaus, T.; Mootz, H. D. *Chem. Rev.* **1997**, *97*, 2661.

(9) (a) Trauger, J. W.; Kohli, R. M.; Mootz, H. D.; Marahiel, M. A. Walsh, C. T. *Nature* **2000**, *407*, 215. (b) Trauger, J. W.; Kohli, R. M.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7092. (c) Kohli, R. M.; Trauger, J. W.; Schwarzer, D.; Marahiel, M. A.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7099.

(10) Kohli, R. M.; Walsh, C. T.; Burkart, M. D. *Nature* **2002**, *418*, 658.

(11) Orum, H.; Andersen, P. S.; Oster, A.; Johansen, L. K.; Riise, E.; Bjørnvad, M.; Svendsen, I.; Engberg, J. *Nucleic Acids Res.* **1993**, *21*, 4491.

(12) Bu, X.; Xie, G.; Law, C. W.; Guo, Z. *Tetrahedron Lett.* **2002**, *43*, 2419.

(13) Bu, X.; Wu, X.; Xie, G.; Guo, Z. *Org. Lett.* **2002**, *4*, 2893.

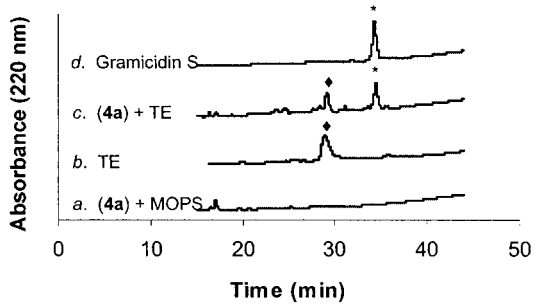


Figure 2. HPLC analysis of the turnover product of gramicidin thioesterase-catalyzed cyclization of the linear decapeptide precursor (**4a**) on a solid support. (♦) TE = gramicidin thioesterase, GrsB TE; (*) gramicidine S.

of methanol. The solution phase was combined and dried in vacuo, and the turnover product was dissolved in 500 μL of methanol. As a negative control, the same amount of substrate-bearing resin was processed in parallel using pure buffer instead of the enzyme solution to obtain a control product.

As shown in Figure 2, HPLC analysis of the turnover product showed there were two major peaks at retention time (t_R) 29.1 and 32.4 min (trace c). Both peaks are associated with the enzyme treatment of the immobilized linear precursor (**4a**, Figure 1) because they are absent in the chromatogram (trace a, Figure 2) resulting from the MOPS buffer treatment. The peak at 29.1 min was found to be from the thioesterase GrsB TE in comparison to the chromatogram for the pure enzyme (trace b). The other peak at 32.4 min was determined to be the expected product, gramicidin S (**1**), because it showed elution time identical with that of the authenticated natural product (trace d). This product identification is further supported by the FAB-MS analysis that showed only one molecular ion peak at 1141.9 ($[M + 1]^+$), consistent with the calculated mass of 1140.71 for the expected cyclic product, gramicidin S, for the enzyme catalyzed reaction.

Finally, the enzyme treatment of the resin-bound precursor (**4a**) was scaled up, and the product at 32.4 min was isolated by semipreparative HPLC and dried in vacuo. Its ^{1}H NMR spectrum was found to be completely consistent with that of the wild-type gramicidin S.[3b] On the basis of these results, the thioesterase GrsB TE indeed correctly cyclizes the heterogeneous decapeptide precursor (**4a**) to form the cyclic gramicidin S (**1**).

From the HPLC and mass spectroscopic analyses, apparently gramicidin S formation dominated the enzyme-catalyzed cyclization reaction, with negligible amount of hydrolytic product that should appear at about 22 min in the HPLC elution chromatogram. This conclusion was further confirmed by LC-MS analysis of the turnover product.

However, it was also found that only a fraction of the linear decapeptide precursor on the resin was converted into gramicidin S product since treatment of the same amount of resin in 7 M aqueous ammonia solution produced substantially greater amount of the same product. By comparison of their HPLC chromatograms and co-injection, it was found that only 28% of the linear precursor was cyclized by GrsB TE. Increasing the enzyme concentration (from 100 to 500 μg mL^{-1}) or the incubation time at 37 °C did not increase the product yield. These results indicate that the majority of the linear precursor is hidden in the resin, and only a portion of it is exposed on the particle surface and accessible for the enzyme GrsB TE. Although this result contradicts that from a recent investigation in which TentaGel-supported substrates were found to be inaccessible to enzymes,[14] it is otherwise consistent with the results of other investigations of enzyme activity toward substrates on the same solid support.[15]

Seven other linear decapeptides (**4b**−**4h**, Figure 1) with variations in the wide-type gramicidin S precursor were synthesized to test GrsB TE activity toward immobilized unnatural substrates. The enzyme conversion and the turnover

Table 1. Product Characterization of the Cyclization of Linear Decapeptide Precursors **4a**−**4h** catalyzed by GrsB TE[a]

precursors	X_1	X_2	X_3	t_R (min)	calcd mass	$[M + H]^+$	overall yield[b]	ratio (cyclic:linear)[c]	% precursor accessible by GrsB TE[d]
4a (wt)	DPhe	Orn	Val	32.4	1140.7	1141.7	5.5	8:1	28
4b	DPhe	Lys	Val	30.0	1154.7	1155.6	9.2	10:1	28
4c	DAla	Orn	Val	27.1	1064.7	1065.6	13.4	9:1	18
4d	DAla	Lys	Val	26.8	1078.7	1079.4	8.8	5:1	9
4e	DPhe	Orn	Phe	32.4	1188.7	1189.6	7.1	4:1	29
4f	DPhe	Lys	Phe	32.2	1202.7	1203.5	16.8	9:1	35
4g	DAla	Orn	Phe	29.3	1112.7	1113.5	18.9	8:1	39
4h	DAla	Lys	Phe	29.0	1126.7	1127.6	9.2	10:1	37

[a] Molecular ion is from mass spectroscopic analysis under FAB mode. Other results are derived from the HPLC analyses and purification performed with Waters 600E system with a reverse-phase semipreparative XTerra RP_{18} column, 7 μm, 7.8 mm × 300 mm. Separation conditions were 3.0 mL min^{-1} flow rate, a linear gradient of 80% to 20% A in 25 min, 20% to 0% A in another 10 min, washed with 100% B for 10 min, and then calibrated at 80% A for 15 min. Solution A was 0.1% TFA in double-deionized H_2O; solution B was 0.1% TFA in acetonitrile. [b] Overall HPLC-isolated yield of the head-to-tail cyclic products based on the original loading value of the resin after 7 M NH_3·H_2O treatment. [c] Ratio of the cyclic product peak area to that over 22−25 min on the HPLC chromatograms where the linear hydrolytic products appear. [d] Percentage of the linear precursor cyclizable by the enzyme is calculated as the ratio of cyclic product peak area on the HPLC chromatogram from GrsB TE (500 μg mL^{-1}), after subtraction of that from blank MOPS incubation, to that from the 7 M NH_3·H_2O treatment of identical amount of the precursor-bound resin.

products were characterized as summarized in Table 1. The thioesterase was found to convert the analogous precursors into cyclic products with high selectivity, similar to the formation of gramicidin S from its biosynthetic precursor. HPLC purification and subsequent ^{1}H NMR spectroscopic analyses showed that the resulting cyclic products were formed through the head-to-tail cyclization of the corresponding precursors.

Interestingly, these analogous precursors, like the wide-type one, underwent spontaneous head-to-tail cyclization to form corresponding gramicidin S analogues in ammonia solution, enabling quantification of the total amount of the linear precursors on the resin. Again, it was found that only a small portion of the linear precursors were accessible to the enzyme, regardless of the enzyme concentration and reaction time, leading to lower product yields. Despite this shortfall, these results nevertheless prove that the thioesterase GrsB TE is active toward heterogeneous native substrate and its analogues. In addition, the low availability of linear precursors to the enzyme can be ameliorated by using the more accessible solid supports.[14] Furthermore, the observation that the linear precursors can selectively cyclize spontaneously is an interesting finding that may lead to an even simpler synthetic method for gramicidin analogues.

In summary, we have demonstrated that the putative gramicidin thioesterase (GrsB TE) is active toward heterogeneous substrate analogues immobilized on solid support through a poly(ethylene glycol) linker. Considering the high substrate flexibility of the thioesterase,[9] this result indicates that the enzyme is feasible for generation of diverse cyclic peptides by cyclizing the linear precursors from combinatorial solid-phase peptide synthesis. In turn, this chemoenzymatic method will provide convenient access to large amount of gramicidin analogues for lead discovery against microbial resistance.

Acknowledgment. This work was supported in part by the Innovation and Technology Fund (ITS/119/00) and the RGC (DAG01/02.SC09) from the Government of the Hong Kong Special Administrative Region. We also thank the Department of Chemistry at the HKUST for providing postgraduate studentships to X.W. and K.M.W.

Supporting Information Available: Experimental procedures, LC-ESIMS spectrum of **4a** enzyme cyclization product, and ^{1}H NMR spectra of cyclization products of **4a**−**4h**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL034437Y

(14) Kress, J.; Zanaletti, R.; Amour, A.; Ladlow, M.; Frey, J. G.; Bradley, M. *Chem. Eur. J.* **2002**, *8*, 3769.

(15) (a) Vagner, J.; Barany, G.; Lam, K. S.; Krchnak, V.; Sepetov, N. F.; Ostrem, J. A.; Strop, P.; Lebl, M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8194. (b) Reents, R.; Jeyaraj, D. A.; Waldmann H. *Adv. Synth. Catal.* **2001**, *343*, 501.