ORGANIC LETTERS

2002
Vol. 4, No. 17
2893−2895

Synthesis of Tyrocidine A and Its Analogues by Spontaneous Cyclization in Aqueous Solution

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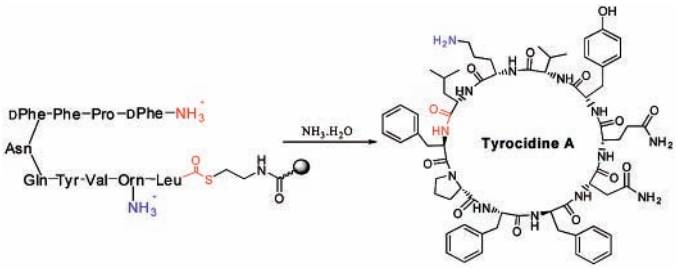
Received June 7, 2002

ABSTRACT



Head-to-tail cyclization of peptides is a multistep process involving tedious C-terminal activation and side chain protection. Here we report a facile, quantitative cyclization method in aqueous ammonia solution for the total syntheses of the cyclic decapeptide antibiotic Tyrocidine A and its analogues from their fully deprotected linear thioester precursors on a solid support. This novel aqueous method is conformation-dependent and may be applicable to syntheses of other natural cyclic peptides.

Tyrocidine A, similar to its well-studied relative gramicidin S, is a cyclic decapeptide antibiotic adopting a preformed β-pleated sheet conformation under physiological conditions.[1] Although the mode of action of the β-sheet antimicrobial peptides has not been elucidated, it has been suggested that the bacterial membrane is their primary target, where their accumulation results in disruption of its normal barrier function.[2] Development of resistance to these peptide antibiotics is unlikely since it requires significant alternation of the lipid composition for the cell wall. So far, no resistance has been found for these peptide antibiotics.[3] Therefore, both natural cyclic peptides represent an attractive class of antibacterial agents for new drug discovery, in light of the fact that widespread antibiotic resistance has become a serious threat to public health.[4]

The major disadvantage of tyrocidine A and gramicidin S is their low specificity toward microorganisms. Although both compounds have high antimicrobial activities, they also disrupt higher mammalian cell membranes, as indicated by their high hemolytic activity. Generation of analogues of these peptides for structure−activity relationship studies aiming to find drug candidates with high antimicrobial and low hemolytic activities has thus become an area of active research.[5] However, synthesis of these cyclic peptides is a challenge. The difficulty lies in the C-terminus activation and cyclization of the linear precursor from solid-phase peptide synthesis. Traditionally, *N*-hydroxysuccinimide ester (ONSu)[6] and azide methods[7] are most often used for the peptide cyclization. Both of these protocols are multistep processes suffering from low yield and racemization.[8]

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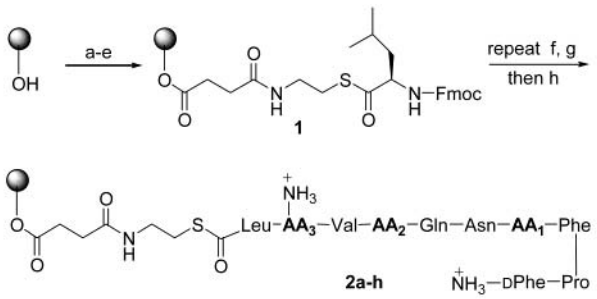
10.1021/ol0263191 CCC: $22.00 © 2002 American Chemical Society
Published on Web 07/30/2002

Inspired by the high-yield formation of a cyclic product in our recent treatment of an on-resin thioester linear precursor of tyrocidine A with aqueous ammonia solution,[9] we explored the possibility of developing this cyclization phenomenon into a general synthetic method for tyrocidine A and its analogues.

Using Fmoc solid-phase peptide synthesis (SPPS) and an optimized Fmoc deprotection method,[9] a fully deprotected linear peptide thioester **2a** mimicking the biosynthetic precursor of tyrocidine A[10] was synthesized on TentaGel-OH (Scheme 1). The on-resin linear peptide thioester was

Scheme 1



a. Succimic anhydride, DMAP, 48h;
b. $(HCl.NH_2CH_2CH_2S)_2$, DMAP, *i*-Pr_2EtN, DMF;
c. $(CH_3CO)_2O$;
d, $HOCH_2CH_2SH$;
e. Fmoc-L-Leu-OH, HOBt, DIC, DMF;
f. 80 mM DBU, 74 mM HOBt, DMF, 3 x 1 min;
g. Fmoc-amino acid (Boc-D-Phe-OH for the last residue), DIC, HOBt, 2h;
h. CF_3COOH:phenol:*i*-Pr_3SiH:H_2O = 88:5:5:2, 1 h.

	AA_1	AA_2	AA_3
2a	DPhe	Tyr	Orn
2b	DPhe	Tyr	Lys
2c	DPhe	Phe	Orn
2d	DPhe	Phe	Lys
2e	Tyr	Tyr	Orn
2f	Tyr	Tyr	Lys
2g	Tyr	Phe	Orn
2h	Tyr	Phe	Lys

subsequently treated with 7 M aqueous ammonia solution, and the product was collected for structural characterization. As illustrated in Figure 1A, LC-ESIMS analysis showed that a cyclic product was formed with a measured molecular ion of 1270.59 ($[M + 1]^+$), consistent with the calculated molecular weight of 1269.65. Furthermore, the linear hydrolytic or aminolytic products (**4a** or **5a**, respectively, Scheme 2) were not detected,[11] indicating quantitative conversion of the linear precursor to the cyclic product.

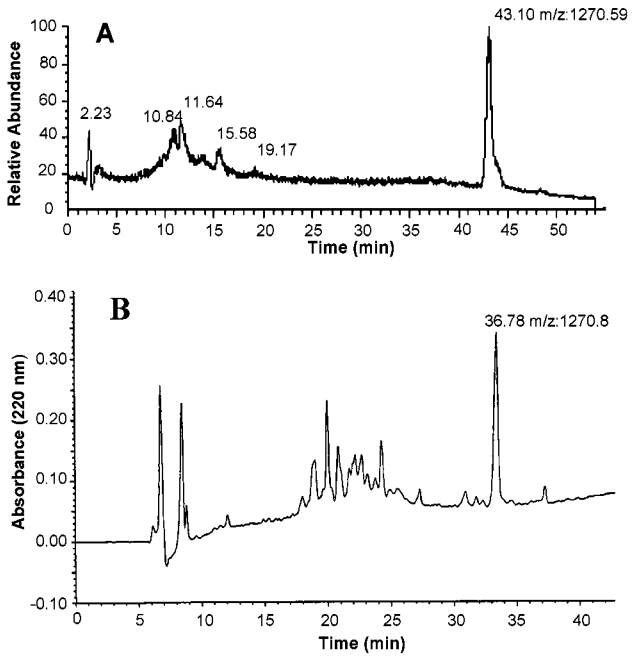


Figure 1. LC-ESI-MS analysis (**A**) and semipreparative HPLC chromatogram (**B**) of the cleavage product of the linear precursor **2a** by 7 M $NH_3 \cdot H_2O$.

Since there are two active amine groups on the fully deblocked linear precursor **2a**, the cyclic product identified in the LC-MS analysis (Figure 1, retention time 43.10 min) could be either the expected tyrocidine A or the aminolysis product of the C-terminal thioester by δ-NH_2 of the ornithine in the sequence. To distinguish these two possibilities, semipreparative reverse-phase HPLC was used to purify the cyclic product, which (Figure 1B, retention time 36.78 min) was verified by molecular weight determination with FAB-MS. The purified product was analyzed by ^1H NMR spectroscopy and found to be identical to the natural tyrocidine A reported earlier.[1b,c] In addition, the minimum inhibition concentration (MIC) of the purified product was determined with a standard method for a *Bacillus substilis* strain to be 12 μg/mL, consistent with that of 20 μg/mL for the wild-type tyrocidine A.[12] On the basis of these results, the cyclic product from the linear precursor **2a** is indeed the wild-type tyrocidine A (**3a**). Thus, the deblocked peptide thioester precursor cyclized quantitatively to form the specific head-to-tail product under treatment of 7 M aqueous ammonia, regardless of the δ-NH_2 on the ornithine residue.

The high specificity and quantitative yield of the cyclization reaction is likely due to the formation of a certain conformation that favors the ring closure. Resembling the cyclic product shown to adopt a rigid antiparallel β-pleated sheet structure by forming four interstrand hydrogen bonds,[1b,c] the linear precursor **2a** could fold into a similar conformation

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(11) The conclusion was drawn from the absence of elution peaks with the molecular ions $[M + 1]^+$ or $[M + 2]^{2+}$ of 1288.67 or 644.8, respectively, for **4a** and 1287.67 or 644.4, respectively, for **5a** in the LC-ESI-MS analysis.

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Table 1. Product Distribution of the Cleavage Reaction of the Linear Thioester Precursor **2a**−**h** in 7 M $NH_3 \cdot H_2O$ Solution[a]

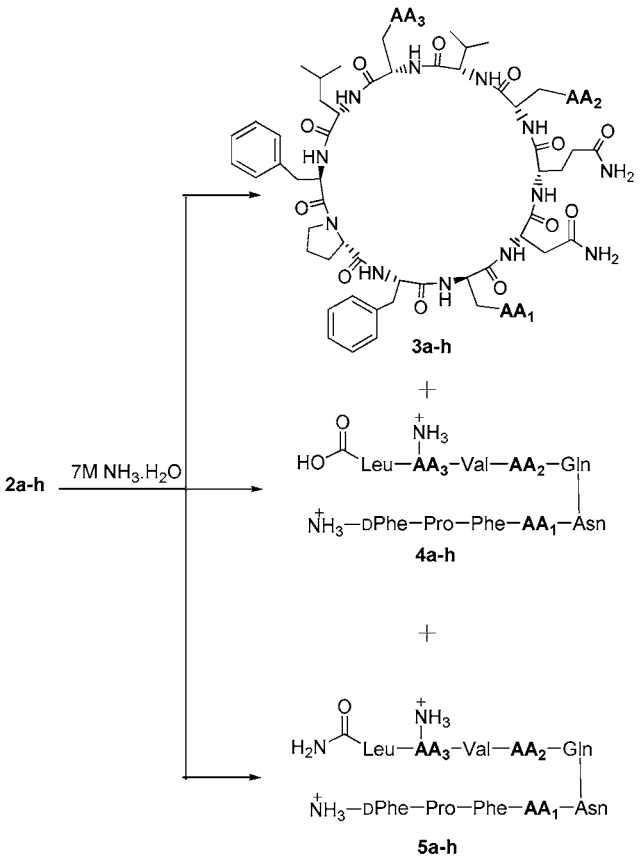
		linear products[b]				cyclic product			
precursor	AA_1	calcd mass	$[M+2]^{2+}$	$[M+1]^+$	rt (min)	calcd mass	$[M+1]^+$	rt (min)	ratio[c] (cyclic:linear)
2a (wt)	DPhe	1287.67		not detected		1269.66	1270.59	43.10	>55:1
2b	DPhe	1301.68		not detected		1283.67	1284.59	42.78	>46:1
2c	DPhe	1271.67		not detected		1253.66	1254.50	44.14	>40:1
2d	DPhe	1285.69		not detected		1267.68	1268.59	44.04	>45:1
2e	Tyr	1303.66	653.06	1305.49	9.0	1285.65	1286.51	37.05	<1:35
2f	Tyr	1317.68	660.14	1318.49	9.6	1299.67	1300.55	37.02	<1:20
2g	Tyr	1287.67	644.61	1288.51	10.5	1269.66	1270.51	37.97	<1:19
2h	Tyr	1301.68	652.14	1302.52	9.0	1283.67	1284.57	37.64	<1:30

[a] Results are derived from the LC-ESIMS analysis. Samples were run on a ThermoQuest Hypersil Elite C18 column (2.1 × 100 mm, 5 μm) coupled to a Finnigan LCQ Classic mass spectrometer. HPLC conditions: flow rate at 0.2 mL/min; linear gradient from 20 to 30% acetonitrile in water within 5 min, 30% acetonitrile for the next 20 min, and finally a linear gradient from 30 to 80% acetonitrile within 25 min. [b] Hydrolytic and aminolytic products **4** and **5** are inseparable under the HPLC conditions. They are different by approximately 0.5 and 1.0 in mass for the molecular ions of $[M+2]^{2+}$ and $[M+1]^+$, respectively. Data for the hydrolytic product **4** are given in the table above. [c] Ratio was calculated by assuming that the detection limit of the LC-MS analysis is 1% of the total ion intensity over the entire elution process.

that brings the aminoxy and carboxy termini into close proximity to facilitate the cyclization reaction.

On the basis of this assumption, the preference of the ammonia cleavage reaction for the cyclic product over the aminolytic or hydrolytic product will disappear if this conformation is disrupted. To test this, we chose to change the D-Phe[4] to L-Tyr[4] at the position of AA_1 (Scheme 1), a β-turn residue in tyrocidine A[1b,c] believed to be crucial to the formation of the β-sheet structures of the cyclic product and the precursor. In addition, Phe and Lys were simultaneously introduced into the positions of Tyr[7] (AA_2) and Orn[9] (AA_3), respectively, and all eight possible sequences were synthesized as shown in Scheme 1. The LC-ESIMS results of the cleavage products from 7 M $NH_3 \cdot H_2O$ treatment are summarized in Table 1. As expected, only cyclic products are formed when the D-configuration at position-4 (AA_1) is maintained, whereas hydrolytic and aminolytic products dominate the reaction when the amino acid at position-4 (AA_1) is changed to L-Tyr. These results show that the ring closure reaction is indeed dependent on formation of a Tyrocidine A-resembling conformation of the linear precursor.

Scheme 2



In summary, we have found a quantitative cyclization method for the total syntheses of the cyclic decapeptide antibiotic Tyrocidine A and its analogues from their fully deprotected linear thioester precursors on a solid support. This novel aqueous method is conformation-dependent and may be applicable to syntheses of other natural cyclic peptides.

Acknowledgment. This work was supported by the Innovation and Technology Fund (ITS/119/00) from the Innovation and Technology Commission of the Government of the Hong Kong Special Administrative Region and the HKUST (DAG01/02.SC09). We also thank the Department of Chemistry at the HKUST for providing a postdoctoral fellowship to Dr. G. Xie and a studentship to Mr. X. Wu.

Supporting Information Available: Experimental procedures for the solid-phase peptide synthesis of **2a**−**h** and MIC determination of **3a**, ^{1}H NMR and FAB-MS of tyrocidine A (**3a**), and LC-ESIMS results for cleavage products of **2a**−**h**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL0263191