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Efficient Microwave-Assisted One Shot Synthesis of Peptaibols Using Inexpensive Coupling Reagents

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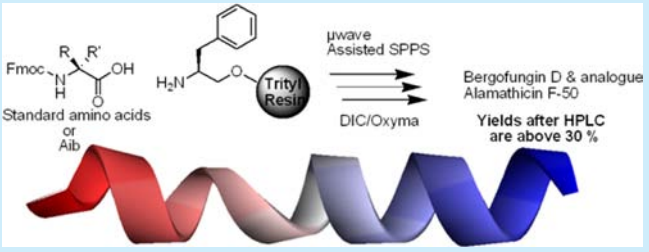
Supporting Information

ABSTRACT: A diisopropylcarbodiimide/Oxyma (ethyl 2-cyano-2-(hydroxyimino)acetate) coupling cocktail was successfully incorporated into the automated microwave-assisted synthesis of two peptaibols and one analog, whose previously reported syntheses were complicated by steric hindrance. This method utilizes commercially available reagents and affords alamethicin F50/5 and bergofungin D in high yields and purities along with an appreciable reduction of synthesis time and cost when compared to previously reported methods.



α-Helices account for 30% of a protein's secondary structure while β-strands account for 20%; therefore, considerable interest in the design and application of small helical *de novo* peptides can be observed in the literature.[1,2] For example, peptide helices have been exploited as protein/protein interaction disruptors,[3,4] and studying their self-association provides clues toward understanding their pathogenicity.[5] These exquisite properties allow helices to find numerous applications in medicine as well as in the design of nanomaterials.[6,7]

Studies concerning the development of peptides designed to self-associate or to adopt a stable helical confirmation led us to consider peptaibols as a template. Peptaibols are a class of nonribosomally synthesized peptides from a diverse set of fungi that include *Trichoderma*, *Gliocladium*, and *Stibella*.[8] Usually linear, these peptides contain 4 to 20 residues and are characterized by a high content of α,α-dialkyl α-amino acids such as α-aminoisobutyric acid (Aib, U) and D- or L-isovaline (Iva). They possess an acetylated N-terminus along with a β-amino alcohol, generally phenylalaninol (Fol) or leucinol (Lol), at their C-terminus. These peptides show antibiotic activity through formation of voltage-dependent pores in biological membranes.[9] Pore formation of this type requires that peptides have a high propensity to form 3_{10}- or α-helical structures, a conformation sterically induced by the constrained C_α carbon of the Aib residue.[10,11] Because of their intriguing channel forming properties and small size, peptaibols have attracted considerable interest as model peptides and numerous syntheses have been reported.[12] However, the synthesis of peptaibols is significantly hampered by steric hindrance in residues such as Aib, where reactivity during the coupling reaction is diminished in comparison to other amino acid residues. Overcoming the poor reactivity of such residues requires the use of very efficient but expensive coupling reagents that sufficiently activate the Aib carboxylic functional group. These include HATU, HCTU, TFFH, and more recently COMU.[13] Most of the reported syntheses of peptaibols rely on in-solution strategies based on segment condensation that lead to tedious flash chromatography separations to purify each fragment and the final compound.[14,15] It is noteworthy that this arduous solution-based approach is still a popular method for synthesizing peptaibols.[16,17] Consequently, solid-phase peptide synthesis (SPPS) is a convenient alternative that avoids lengthy intermediate purification steps.[19] However, few examples of peptaibols synthesized on solid-phase resin have been reported thus far.[20,21] The present paper focuses on the synthesis of two peptaibols: alamethicin F50/5, which is considered the archetype of peptaibols, and bergofungin D, which has been synthesized previously with only an 11% yield.[21−25] Using the method herein both compounds were obtained cost effectively by SPPS in high yield and purity.

To compare *de novo* peptaibols on the basis of architectural design it is necessary to optimize the synthetic strategy for preparing peptaibols of varying lengths. A cost-effective and automated synthetic strategy would avoid the following characteristics: (1) The use of expensive coupling reagents such as HATU or TFFH (TFFH was shown to be efficient for the synthesis of ampullosporin A, but was not sufficiently automated[21,22]); (2) the preparation of ready-to-couple amino acids such as α-amino acid fluoride;[20,21] (3) reprograming the peptide synthesizer or changing coupling reagents during the synthesis;[21,22] (4) the use of double-coupling steps for Aib residues or subsequent residues also sterically hindered by Aib.[21]

An Fmoc-based strategy in conjunction with a DIPC/ethyl 2-cyano-2-(hydroxyimino) acetate (Oxyma) coupling cocktail proved ideal for accomplishing these goals. Encouraging results

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have been observed previously by Albericio and co-workers for the coupling of Aib residues in model peptides such as the enkephalin analog YUUFLNH$_2$ and by our group for the cyclization of marine peptide; however, this approach has yet to be applied to peptaibols.[28,29] Among the numerous advantages of incorporating these reagents are reduced racemization and a lower cost, making them 15 and 30 times cheaper than HATU and TFFH, respectively. As reported for the enkephalin analog, it was necessary to use a 4 h double-coupling step at the Aib residue to drive the synthesis.[28] More recently, microwave irradiation has been effective in shortening the reaction time and increasing product purity, while being compatible with both oxyma-derived COMU and DIC/Oxyma activation methods.[30,31]

To test the DIC/Oxyma and microwave combination approach, two challenging peptaibols were selected. Alamethicin F50/5 (**1**) (Figure 1) was chosen for its 20-residue length

AcUPUAUAQUVUGLUPVUUQQFol (**1**)
AcVUUVGLUUOQUOUFol (**2**)
AcVUUVGLUUPQUPUFol (**3**)

Figure 1. Structures of alamethicin F50/5 (**1**), bergofungin D (**2**), and its analog (**3**). U: Aib, O: Hyp.

which contains eight Aib (U) residues, two of which are located at adjacent positions and two of which are coupled to sterically hindered proline. Bergofungin D (**2**) (Figure 1) is challenging because it contains two UU motifs, one of which is followed by hydroxyproline (O) as well as another UO motif. Previous methods offered only satisfactory crude purity (<24%) and low purified yield (11%) despite synthesis optimization.[21] Compound **3** is an analog of compound **2** in which the (2*S*,4*R*)-4-hydroxyproline residue is replaced with a proline residue for initial synthesis optimization at lower cost.

Solid-phase synthesis was carried out using a CEM Liberty One peptide synthesizer. Compounds **1**, **2**, and **3** were assembled on 0.28 g, 0.1 mmol of a preloaded Fmoc-phenylalaninol *o*-chlorotrityl resin (0.36 mmol/g). The optimized protocol for the synthesis of the target peptides was accomplished on a 0.1 mmol scale using Fmoc-protected amino acids, DIC, and Oxyma each in 5-fold excess with regard to the resin capacity. The most efficient reaction conditions affording complete coupling of Aib were found to be microwave irradiation at 70 °C for 20 min. A 20% solution of piperidine in DMF was used for Fmoc deprotection after each coupling step, and the fulvene−piperidine adduct was quantified by UV absorption (301 nm) to estimate coupling efficacy (Supporting Information (SI)). Acetylation was performed on a solid support by treatment with a solution of acetic anhydride in DMF for 10 min. After completion peptides were cleaved from the resin using dilute TFA in dichloromethane to ensure complete peptide cleavage without degradation of the acid labile Aib-Pro bond.

Compounds **1**, **2**, and **3** were obtained in high crude purity (Table 1), as determined by HPLC for which an evaporative light scattering detector was used to detect even small amounts of impurities (Figure 2).[33,34]

The three compounds were purified by semipreparative HPLC, and single products were obtained. LC/MS electrospray analysis provided the singly, doubly, and triply charged pseudomolecular ions $[M + H]^+$, $[M + 2H]^{2+}$, and $[M + 3H]^{3+}$ for each of the compounds (Table 2 and SI). During

Table 1. Analysis and Purity of Synthesized Peptides

compd	t (h)[a]	crude purity (%)	reported crude purity (%)	purified yields (% - *mg*)	reported purified yields (%)
1	15	91	53[b]	35 - *68*	24[b]
2	10	97	24[b]	50 - *72*	11[b]
3	10	95	–	49 - *68*	

[a]Total duration of the synthesis (h: hour). [b]Reference 21.

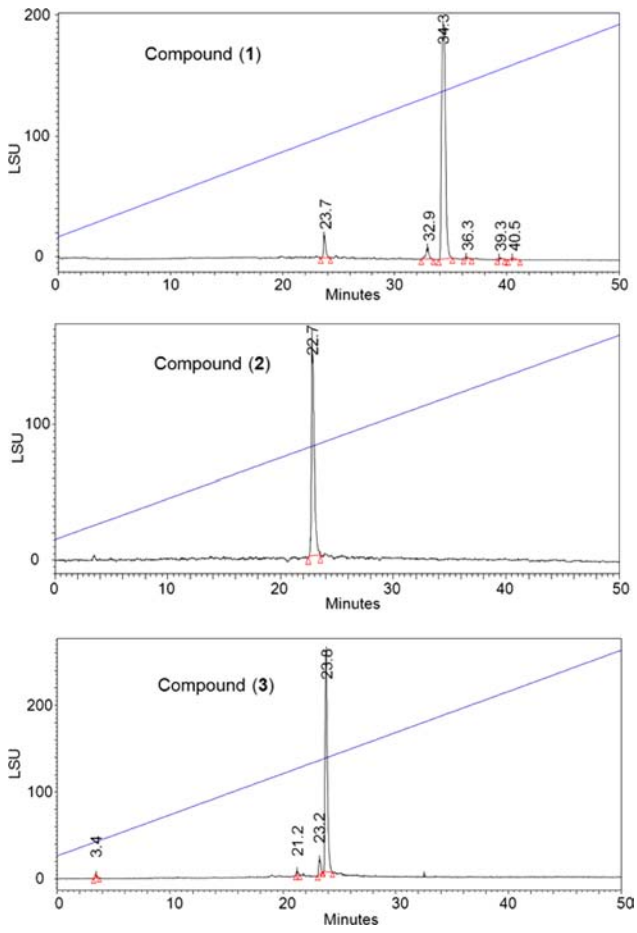

Figure 2. HPLC profiles of the crude alamethicin F50/5 (**1**), bergofungin D (**2**), and its analog (**3**) with ELSD detection. The HPLC analysis was conducted on a Waters 2695 HPLC system with a Grace Vydac 218MS 5 μM C-18 column (250 mm × 4.6 mm) using a gradient mixture of water with 0.1% FA (Buffer A) and ACN with 0.1% FA (Buffer B). All compounds were eluted with a 0.9 mL/min flow rate from 10% B to 100% B over 50 min.

Table 2. Observed Ions in Full ESI Mass Spectra of Compounds 1, 2, and 3

compd	$[M + H]^+$	$[M + 2H]^{2+}$	$[M + 3H]^{3+}$	b ions	y ions
1	1963.52	981.95	654.92	b_{13}: 1189.09	y_7: 774.22
2	1425.97	714.26		b_8: 751.05 b_{11}: 1076.85	y_6: 676.15 y_3: 350.07
3	1393.92	697.61		b_8: 751.06 b_{11}: 1060.83	y_6: 644.11 y_3: 334.07

analysis the labile Aib-Pro peptide bond breaks to yield the expected fragments b_{13} and y_7 of **1** even in full scan mode.[35]

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The same phenomenon is observed at the two Aib-Hyp peptide bonds of **2** and two Aib-Pro peptide bonds of **3** yielding fragments b_8 and b_{11} with their corresponding fragments y_6 and y_3 (Table 2 and SI).

An obvious concern when dealing with peptide couplings and deprotection at elevated temperature is the risk of amino acid racemization.[36] However, the peptides we have synthesized do not contain any amino acids that are known to be prone to racemization such as Cys, His, or Ser. Furthermore even for these amino acids specific coupling conditions under microwave irradiation are reported to suppress the racemization by lowering the temperature to 50 °C.[37] Therefore based on these previous reports a study of the racemization was deemed unnecessary, and this was confirmed by HPLC which has only shown sharp peaks and ^{1}H NMR studies for which the CHα and NH regions are well-defined (SI).

These results demonstrate a rapid and efficient method for the synthesis of peptaibols offering products of higher purities and yields than the previously reported methods (Table 1). This method allows the use of inexpensive resins and of a low cost DIC/Oxyma-based activation scheme with standard reagent excesses. These results highlight the effectiveness of Oxyma in SPPS by demonstrating a significant improvement in speed and efficiency, particularly for the synthesis of difficult sterically hindered peptides. Future synthesis of these peptides should take advantage of the recent evolution of peptide synthesizers.[38]

■ ASSOCIATED CONTENT

Supporting Information

UV quantification of the fulvene−piperidine adduct obtained after Fmoc deprotection with piperidine. HPLC trace of purified peptides. LCMS analysis of pure peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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