JOC Notes

An Automatic Solid-Phase Synthesis of Peptaibols

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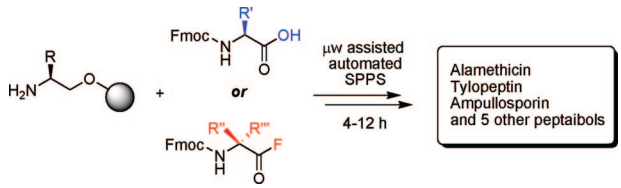

An automated approach to peptaibols using microwave-assisted solid-phase peptide synthesis is demonstrated with a combination of HBTU and acid fluoride mediated couplings for normal and α,α-dialkylated amino acids, respectively. The method is utilized for the automated synthesis of several full-length peptaibols, including alamethicin, tylopeptin, ampullosporin, bergofungin, cervinin, trikoningin, trichogin, and peptaibolin, reducing both synthesis time and costs significantly as compared to other approaches. Furthermore, the use of noncommercially available reagents is minimized.

Solid-phase peptide synthesis (SPPS) has become the preferred method for the synthesis of small peptides since its implementation in the 1960s by Merrifield.[1] The solid-phase approach is especially attractive due to the possibility of full automation of the synthesis process using robotic peptide synthesizers. Two automatic SPPS techniques are dominant, differing in the *N*-α-protecting group on the peptidyl-resin, namely the Boc- and the Fmoc-approach,[2,3] the latter becoming increasingly popular as the use of hazardous hydrofluoric acid is avoided.

Notwithstanding the success of SPPS, the synthesis of certain peptides containing highly hindered nonproteinogenic amino acids is still not considered straightforward. One such class of peptides is the peptaibols, which have received considerable interest due to their membrane-disrupting abilities leading to antibiotic activity.[4,5] This holds, for example, for the intensely studied peptaibol, alamethicin, which is considered an excellent model for ion-channel behavior and *trans*-lipid pore formation.[6] Peptaibols contain a high proportion of the α,α-dialkylated amino acids, α-aminoisobutyric acid (Aib) and/or L-isovaline (Iva), which, due to their increased steric bulk, couple to the peptidyl resin in poor yields using standard SPPS coupling reagents such as HBTU, HATU, or PyBOP.[7,8] Because of these difficulties, peptaibols or peptaibol fragments have been synthesized using a variety of other methods such as solution-phase segment condensations,[9] expression,[10] and SPPS approaches including the solid-phase azirine/oxazolone method[11] and the amino acid fluoride approach.[7,12] All these methods, however, suffer from an intense consumption of man-hours due to their use of noncommercially available reagents and in most cases a lack of automation. Exactly this problem stimulated the development of an automated procedure for peptaibol synthesis presented here.

To facilitate our labeling studies of alamethicin and other peptaibols,[13] we sought an effective, cheap, and automated solid-phase synthesis of these peptides, using commercially available reagents whenever possible and applying a minimum of reprogramming of the peptide synthesizer. Our initial attempts using standard HBTU-mediated Fmoc SPPS under microwave irradiation failed. Likewise, in situ TFFH activation, which we utilized previously for the semiautomatic synthesis of alamethicin,[13] was not directly transferable to any of our fully automatic synthesizers, although an automated synthesis of

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TABLE 1. Coupling Cycles Used during SPPS of Peptaibols[a]

Std. cycle	Acid fluoride cycle	Std. double cycle
1) Fmoc deprotection	1) Fmoc deprotection	1) Fmoc deprotection
2) Drain and wash	2) Drain and wash	2) Drain and wash
3) Add amino acid	3) Add amino acid fluoride	3) Add amino acid
4) Add HBTU	4) Add DIPEA	4) Add HBTU
5) Add DIPEA	5) μW heating 5 min	5) Add DIPEA
6) μW heating 5 min	6) Drain and wash	6) μW heating 5 min
7) Drain and wash		7) Drain
		Repeat 3) to 7)
		8) Wash

[a] For a detailed description of the coupling conditions see the Experimental Section and the Supporting Information.

TABLE 2. Methods Used for SPPS of Peptaibols (Exemplified by Alamethicin F30)

Method A
Ac-UPUAUAQUVUGLUPVUUEQ-Phol
Method B
Ac-UPUAUAQUVUGLUPVUUEQ-Phol

Black: Std. cycle, red: Acid fluoride cycle, blue: Std. double cycle.

alamethicin derivatives using TFFH activation with custom programmed cycles has been reported once.[14]

Instead, we considered the acid fluoride method, utilizing isolated and purified amino acid fluorides for each coupling. However, to avoid the tedious synthesis of a range of these acid fluorides we decided to attempt using them for the α,α-dialkylated amino acids Aib and Iva only, while maintaining standard HBTU conditions for the remaining couplings. The acid fluorides of Aib or Iva are easily synthesized using the method of Carpino and co-workers[15] and are stable for months in the freezer. They are also stable in a solution of DMF for several hours.[16]

Modern peptide synthesizers, such as the one used in this work coupled to a microwave, allow for easy customization of the couplings for each individual amino acid. As depicted in Table 1, we used three different coupling cycles for the automated peptaibol synthesis, namely the factory-installed standard cycle (black) and acid fluoride cycle (red), in which an amino acid fluoride is added and the HBTU addition is removed, and finally the factory installed double cycle (blue) where the HBTU-mediated coupling process is repeated twice prior to deprotection (Table 1). The use of HOBt as a additive is not necessary when using microwave-assisted SPPS, as it has no effect on racemization levels.

The cycles are applied to peptaibol synthesis in two ways, method A and B, depending on the difficulty of the peptide sequence (Table 2). Method A simply uses the standard cycle for proteinogenic amino acids (including hydroxyproline) and the acid fluoride cycle for the α,α-dialkylated amino acids (Aib [U] and Iva [J]). For longer peptaibols, the yields of the coupling of certain proteinogenic amino acids onto the α,α-dialkylated amino acids are no longer high enough to achieve an acceptable crude purity. Therefore, method B expands on method A by using the standard double cycle on all proteinogenic amino acids that are coupled onto an α,α-dialkylated amino acid. The methods are exemplified for the synthesis of alamethicin F30 in Table 2 using the color-coding of the three different cycles shown in Table 1.

Initially, we tested method A for the synthesis of short peptaibols, namely peptaibolin (5 residues) and trichogin A IV (11 residues), and we were pleased to see that this straightforward method gave the desired peptides in very good crude purities of 94% and 96%, respectively, which enabled us to isolate them in good yields using HPLC (Table 3, entries 1 and 2). Encouraged by these results, we proceeded to test the method for the synthesis of the 20 residue peptaibol alamethicin. However, in this case, method A proved inadequate as the crude mixture contained a large proportion of deletion products (Table 3 entry 10). Especially apparent were products involving valine (V) and glutamine (Q) deletions. The nature of the deletion products led us to conclude that the limitations of method A when synthesizing longer peptaibols were due to low yields of peptide coupling of some of the proteinogenic amino acids onto Aib. As we wanted to avoid the synthesis of further amino acid fluorides, we tested method B (Table 2) on the alamethicin synthesis, where couplings onto α,α-dialkylated amino acids are performed using a double HBTU cycle. Gratifyingly, method B gave a sufficiently pure crude product providing alamethicin in a 24% yield after HPLC purification (Table 3, entry 11). Furthermore, the result showed that when coupling two consecutive Aib residues onto the peptidyl resin using acid fluorides the use of a double acid fluoride cycle is not required for the second Aib coupling.

To further explore the scope of the methods, we gradually increased the length of the peptaibol sequences from 5 to 20 while also synthesizing peptaibols containing isovaline and hydroxyproline residues. The 11 residue peptaibol trikoningin KB I was successfully synthesized in an isolated 21% yield using method A, albeit with a relatively low crude purity of 35% (Table 3, entry 4). Employing method B to the synthesis of trikoningin only increased the crude and isolated yields slightly (Table 3, entry 5), indicating that some peptaibols contain "difficult" sequences not related to low coupling yields, which can normally be increased by employing method B. Cervinin I containing 12 residues was synthesized in a 20% yield employing method A (Table 3, entry 6), whereas the 14 peptaibol bergofungin D was isolated in a 11% yield using method B (Table 3, entry 7). The synthesis of bergofungin demonstrates that peptaibols containing hydroxyproline (O) residues are also available applying the present approach. The decreased yield of bergofungin D prompted us to use method B for the longer peptaibols ampullosporin I and tylopeptin A. Ampullosporin was isolated in a good yield of 22% *even though the sequence contains three consecutive Aib residues* (Table 3, entry 8). The successful synthesis of tylopeptin in an isolated yield of 12% illustrates the ability of method B to also incorporate the highly hindered isovaline residues (J), which are common in peptaibols (Table 3, entry 9).[17] Thus a variety of peptaibols have been successfully synthesized using an automated approach, incorporating Aib, Iva and hydroxyproline residues, as well as other proteinogenic amino acids.

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(17) The lower yield obtained in the synthesis of tylopeptin A is due to the fact that considerable amounts of the Iva deletion product were obtained. Two peaks were observed for the purified tylopeptin A due to the presence of two epimers of this peptide as racemic Iva was employed.

TABLE 3. Automated SPPS of Peptaibols[a]

entry	peptaibol	sequence[b]	method	synthesis time[c] (h)	crude purity (%)	isolated yield[e] (%)
1	peptaibolin	Ac-LULU-Phol	A	4	94	71
2	trichogin A IV	Oc-UGLUGGLUGI-Lol	A	6	96	20
3			A[d]	16	89	24
4	trikoningin KB I	Oc-UGVUGGVUGI-Lol	A	6	35	21
5			B	7	46	24
6	cervinin 1	Ac-LUPULUPAUPV-Lol	A	6	29	20
7	bergofungin D	Ac-VUUVGLUUOQUOU-Phol	B	8	24	11
8	ampullosporin I	Ac-WAUULUQUUUQLUQ-Lol	B	9	27	22
9	tylopeptin A	Ac-WVUJAQAUSUALUQ-Lol	B	9	28	12
10	alamethicin F30	Ac-UPUAUAQUVUGLUPVUUEQ-Phol	A	11	<15	
11			B	12	53	24

[a] All syntheses were performed on a CEM Liberty microwave-assisted peptide synthesizer on a 0.1 mmol scale using preloaded chlorotrityl resin. [b] U = Aib, *J* = (*Rac*)-Iva, *O* = hydroxyproline, Phol = L-phenylalaninol, Lol = leucinol, Oc = $HO(CH_2)_7(CO)-$. [c] Excluding acetylation and cleavage. [d] The 5 min microwave coupling step was replaced by a 1 h room-temperature coupling step. [e] Purities of isolated peptides were all >90%.

It is evident from the results of Table 3 that the crude purity of the peptides does not always correlate well with the isolated yields (compare, for example, entries 1 and 2). We believe that this discrepancy is mainly due to differences in the degree of precipitation for both the peptaibols themselves and perhaps more importantly the impurities of the crude peptide during isolation.

Although microwave heating has been shown to improve HBTU-mediated coupling of Aib residues in solution-phase chemistry,[18] we performed the synthesis of trichogin A IV without the use of microwaves and obtained a similar result (Table 3, entries 2 and 3). This implies that the method is easily transferable to other peptide synthesizers without microwave capabilities. However, it should be pointed out that the synthesis time is much longer as coupling times required 1 h rather than 5 min under microwave heating.

Figure 1 illustrates the effect of employing method B when method A proved insufficient in the synthesis of alamethicin F30. When method A is employed the crude HPLC trace in Figure 1a clearly shows a complex mixture with three main products, none of which corresponds to alamethicin. On the other hand, when method B was used the HPLC trace of the crude mixture (Figure 1b) shows a main product corresponding to the purified alamethicin illustrated in Figure 1c.

In summary, we have demonstrated a procedure for the fast automated synthesis of peptaibols that utilizes a combination of standard HBTU mediated solid phase peptide synthesis when coupling proteinogenic amino acids, and acid fluoride peptide synthesis when coupling α,α-dialkylated amino acids. The method requires a minimum of synthetic effort prior to the SPPS as only the syntheses of the Fmoc-amino acid fluorides of Aib and/or Iva are required. The solid phase synthesis time was 12 h or less, enabling overnight production of even the longest peptaibols in good yields. Thus, this method is sufficiently efficient and robust to allow for the expedient synthesis of eight different full-length peptaibols as presented in this paper.

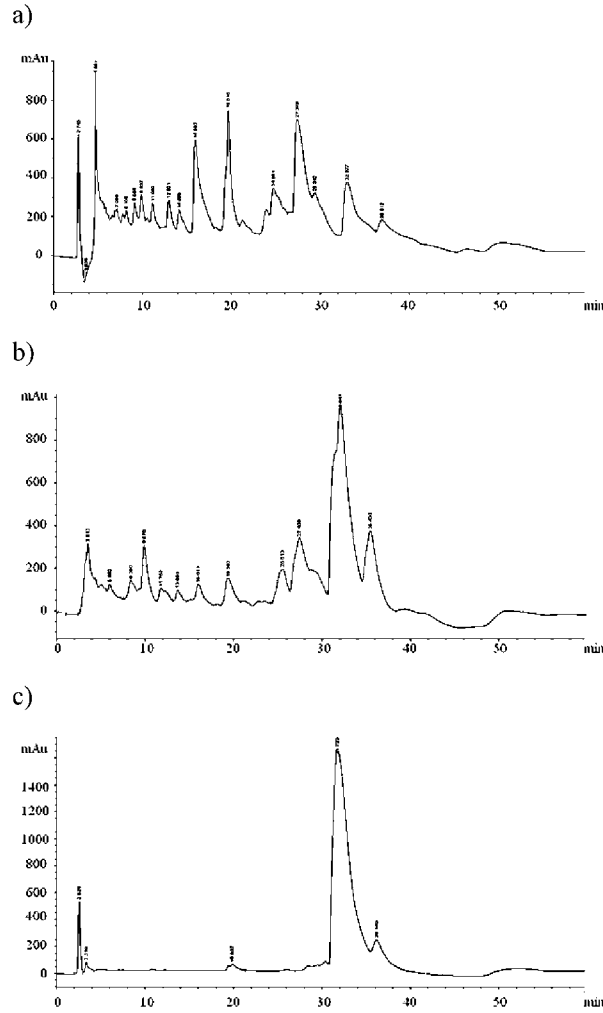

FIGURE 1. Analytical HPLC trace of (a) crude alamethicin using method A, (b) crude alamethicin using method B, and (c) purified alamethicin.

Experimental Section

Alamethicin F30 (Ac-UPUAUAQUVUGLUPVUUEQ-Phol). The peptide was synthesized by applying method B on a microwave-assisted peptide synthesizer using a 2-chlorotrityl chloride resin preloaded with phenylalaninol (0.10 mmol). After transfer to the reaction vessel, the resin was swelled using the standard protocol of the SPPS machine involving 15 min immersion in 10 mL of 1:1 DMF/CH_2Cl_2. The cycle used for each individual amino acid of alamethicin is depicted in Tables 1 and 2. The experimental conditions (see also the Supporting Infomation) of the individual operations of the coupling cycles were as follows:

Fmoc Deprotection. 20% piperidine in DMF (7.0 mL) was added, and microwave heating was employed for 30 s giving an end temperature of 33 °C. After draining, 20% piperidine in DMF (7 mL) was added again and the mixture microwave heated for 3 min reaching an end temperature of 75 °C. Nitrogen gas agitation was used during heating.

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Drain and Wash. Washings (4×) were performed using 7.0 mL of DMF with nitrogen gas agitation.

Addition of Amino Acid. Amino acids were added as 0.20 M solutions in DMF (2.5 mL, 0.5 mmol).

Addition of Amino Acid Fluoride. Amino acid fluorides were added as 0.20 M solutions in DMF (2.5 mL, 0.5 mmol).

Addition of HBTU. 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was added as a 0.50 M solution in DMF (1.0 mL, 0.5 mmol).

Addition of DIPEA. Diisopropylamine was added as a 2.00 M solution in *N*-methylpyrrolidone (0.5 mL, 1.0 mmol).

Microwave Heating. Microwave heating was employed for 5 min giving an end temperature of 80 °C. Nitrogen gas agitation was used during heating.

After completion of the automatic synthesis following the final Fmoc-deprotection cycle, the peptidyl-resin was washed twice with 10 mL of CH_2Cl_2 before being removed from the synthesizer. *N*-Terminal acetylation was performed by treating the resin with Ac_2O (0.70 mmol) and DIPEA (1.40 mmol) in DMF (2 mL) for 45 min. Cleavage and deprotection of the peptide was performed by treating the resin with 5 mL of TFA/CH_2Cl_2/H_2O/TIPS 47:47:4:2 (v/v) for 60 min. The cleavage mixture was concentrated to approximately 1 mL under reduced pressure, followed by precipitation in cold *tert*-butyl methyl ether. Repeated centrifugation, decantation, and trituration (3 times) followed by lyophilization gave the crude peptide (173 mg, 53% purity). The crude peptide was dissolved in 1:1 MeCN/H_2O and purified by semipreparative HPLC-purification using a linear gradient from 40 to 60% of a solution of 0.1% TFA in MeCN in a solution of 0.1% TFA in H_2O over 20 min with a flow rate of 5 mL/min, giving alamethicin F30 (47.8 mg, 24% yield, 95% purity). MALDI-TOF MS: $C_{92}H_{150}N_{22}O_{25}$ [M + Na^+] calcd 1986.1, found 1986.6.

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Supporting Information Available: Experimental procedures for all compounds, spectroscopic data for compounds **1**−**5**, and HPLC chromatograms for the synthesized peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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