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Synthesis of 'difficult' peptide sequences: application of a depsipeptide technique to the Jung–Redemann 10- and 26-mers and the amyloid peptide Aβ(1–42)

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Abstract—Recently a 26-mer peptide **1** incorporating Ser and Thr was described as a 'difficult' sequence that could not be synthesized by standard methods. If the first Ser residue was used to incorporate a depsipeptide unit, the resulting hybrid was readily assembled. The 26-mer ester was then converted to the native peptide by an O→N acyl shift. The technique may be general for other systems containing appropriate Ser and Thr units and was demonstrated here for the case of the amyloid peptide Aβ(1–42).
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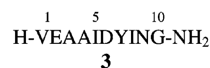
In 1996 Jung and Redemann (JR)[1] reported that 26-mer **1** represented a 'difficult' peptide sequence in that an attempted automated assembly by standard (N-TBTU)[2] Fmoc-based methodology gave none of the desired peptide. On the other hand substitution of the acid fluoride-forming reagent TFFH[3] for N-TBTU gave the desired peptide with a purity of approximately 40%. The published ESI-MS trace of the crude product obtained via N-TBTU showed that the major deletion peptides come within the first nine C-terminal amino acids suggesting that even the corresponding 10-mer, **2** would probably also represent a difficult sequence and therefore an excellent short model for the testing of peptide methodology.

Previously,[4] we have made extensive use of ACP decapeptide **3** for this purpose but this sequence is truly difficult only under demanding conditions, for example, when using a limited excess (0.5 molar equiv) of amino acid and a short coupling time (1.5 min).



As expected when assembly of **2** was examined under standard conditions (N-HBTU; 7 min preactivation; 30 min coupling; 15 min deblocking; 4 equiv excess Fmoc amino acid; 8 equiv DIEA) major deletion peptides were

Abbreviations: ACP = Acyl carrier protein decapeptide (64–75); Boc = *t*-butyloxycarbonyl; $(Boc)_2O$ = di-*t*-butyldicarbonate; Bsmoc = 1,1-dioxobenzo[*b*]thiophene-2-ylmethyloxycarbonyl; DCM = dichloromethane; DIEA = diisopropylethylamine; DMAP = 4-(dimethylamino)pyridine; Fmoc = 9-fluorenemethyloxycarbonyl; N-HATU = 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide; N-HBTU = 1-[bis(dimethylamino)-methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide; N-TBTU = 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium tetrafluoroborate 3-oxide; TFA = trifluoroacetic acid; TFFH = tetramethylfluoroformamidinium hexafluorophosphate; Trt = trityl = triphenylmethyl.

Keywords: Depsipeptides; O/N shift; Difficult sequences; Protecting groups; Bsmoc group; Amyloid peptide.

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obtained in significant amounts [Fig. 1; des-Thr (7.2%), des-Trp (15.9%), des-Phe (4.5%), des-(Trp, Thr) (8.6%), des-(Trp, Phe) (7.5%), and des-(Trp, Phe, Thr) (2.3%)].

Using an ABI 433A peptide synthesizer and standard instrument files for a 20-μmol scale synthesis with a 5-equivalent excess of Fmoc amino acid, it was established that the deblocking difficulties commenced at amino acid number 5 (Leu) as shown in Figure 2A. Since deblocking of the Bsmoc[5] residue is considerably faster than for the Fmoc analog, an initial attempt using **2** as a test sequence involved a comparison of assembly via Fmoc and Bsmoc chemistry. As expected the latter led to a more effective synthesis, for example, with 5 equiv of protected amino acid, 10 equiv of DIEA as base, and 5 equiv of N-HATU as coupling reagent with a 30 s preactivation period and 2 × 4 min for deblocking,[6] Fmoc and Bsmoc assembly led to decapeptide **2** in yields of 40.7% and 48.8%, respectively.

Other interesting results emerged upon a careful examination of the LC–MS traces for the Fmoc synthesis (Fig. 1A–C, Supplementary data). A significant peak was observed at 33.9 min, which exhibited the same molecular weight as the desired 10-mer **2** and separated from it by only 2.4 min, thus suggesting that it represented an epimer arising from loss of configuration at one of the constituent amino acids during activation. Of the

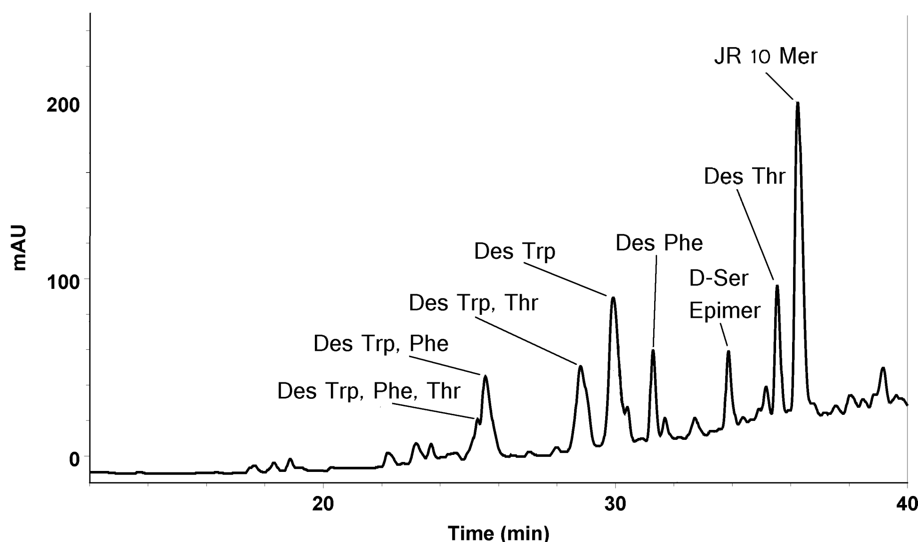


Figure 1. UV chromatogram (220 nm) of crude peptide **2** obtained via standard Fmoc/N-HBTU chemistry. For LC details see Supplementary data.

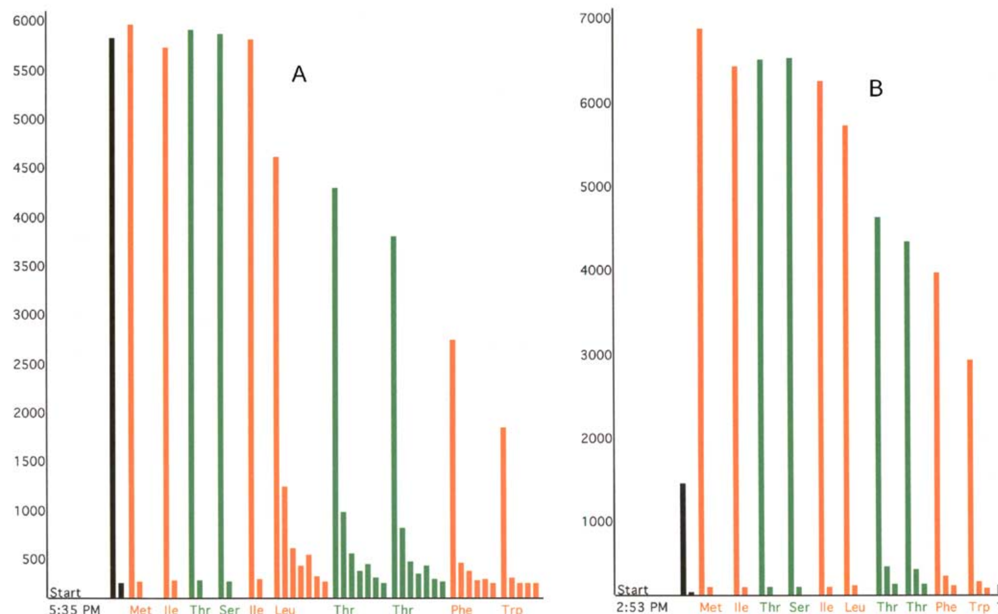


Figure 2. UV monitoring traces for the assembly of peptide **2** on an ABI 433A peptide synthesizer. (A) Fmoc chemistry monitored at 301 nm; (B): Bsmoc chemistry, monitored at 310 nm. For details see Supplementary data.

various amino acids present the culprit was suspected to be serine based on previous experience with this amino acid.[7] This supposition was confirmed by authentic syntheses of the seven nonthreonine epimers theoretically possible by loss of configuration at a single amino acid (Fig. 7a–g, Supplementary data). Comparison of HPLC retention times and co-injections showed that only the authentic D-Ser epimer co-eluted with the side product (Figs. 8B, 9A–C, Supplementary data). Quantitative chiral amino acid analysis established the absence of the three D-allo threonine epimers.

Significant formation of the side product can be avoided simply by reducing the preactivation time from 7 min to 30 s. The general effect of preactivation time on loss of configuration during the coupling of sensitive systems has been noted previously.[8]

Further examination of LC–MS traces of the 10-mer revealed a second group of peaks, which exhibited the same molecular weight as the desired peptide (Fig. 5, Supplementary data). Three such peaks were located and since they were found at significantly lower retention times relative to the authentic 10-mer they could not reasonably be assigned to other epimeric species. Instead these were suspected to be the various N→O shift isomers arising from the presence of serine and threonine in the original sequence. As reported previously[9] for other sequences such rearrangements can occur during the final acidic treatment, which serves to deblock all side chain protecting groups and release the peptide from the resin. The fact that these three peaks disappear from the HPLC traces upon treatment with dilute ammonium hydroxide (reversal of the N→O shift) is consistent with this suggestion.

Further studies were carried out to identify these isomers, which were found at 20.2, 20.9, and 24.8 min relative to the main peak at 32.6 min. The intensities of these three peaks totalled 12% relative to the main peak set to 100%. All four of the theoretically possible depsipeptides having a single ester bond at either serine or each of the three threonine units were synthesized manually (Fig. 6a–d, Supplementary data).

The synthetic method is illustrated for depsipeptide **9** in Scheme 1 and involves the coupling of Fmoc-Ser(Trt)-OH to the threonine unit, removal of the Fmoc residue and its substitution by the Boc group. Selective deblocking of the trityl residue in the presence of the newly introduced Boc function via 1% TFA in DCM in the presence of triethylsilane leads to the unprotected hydroxyl residue, which is then acylated via Bsmoc-Ile-F[10] in the presence of DMAP to give depsipeptide **7**.

The remainder of the synthesis proceeds in the normal manner via Fmoc-protected amino acids following removal of the Bsmoc residue. After completion of the sequence final cleavage gives the unprotected depsipeptide **9** as the TFA salt. From Fmoc-Thr(Trt)-OH analogous methods were used to obtain the three remaining depsipeptides **10–12**.

The structures of all four depsipeptides were confirmed by LC–MS analysis along with co-injections of each of the samples **9–12** with the crude sample of the all-L peptide (Fig. 7A–E, Supplementary data). Only depsipeptide **9** was absent in the sample of all-L 10-mer **2** showing that all three threonine units, but not the serine unit, were subject to the N→O shift during the assembly of **2**. Coincidence of each of the unknown peaks via co-injection studies with each of the authentic depsipeptide samples confirms their identity. The four depsipeptides differed from the all-L amide **2** in that in the ESI-MS traces the intensity of the doubly charged molecular ion (*m*/*z* 606.5) exceeds that for the singly charged species (*m*/*z* 1211.5). This is in line with the fact that in the depsipeptides a second free amino group is present, which favors the formation of doubly charged ions. The same effect was seen for the three impurity peaks observed during the synthesis of **2**.

Another curiosity regarding the authentic syntheses of the four depsipeptides **9–12** was that syntheses of **9** and **10**, sequences in which the ester unit appears within the first four amino acids, were far more efficient (56–73%) than the syntheses of **11** and **12** (11–19%) in which cases the ester unit comes toward the end of the sequence. Clearly, whatever effect, perhaps aggregation,

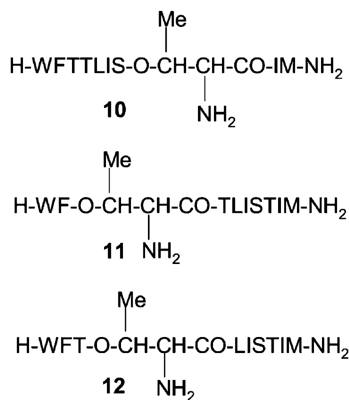
Fmoc-Thr(t-Bu)-Ile-Met-NH—(P) **4** —i., ii.→ Fmoc-Ser(Trt)-Thr(t-Bu)-Ile-Met-NH—(P) **5** —i., iii.→

Boc-Ser(Trt)-Thr(t-Bu)-Ile-Met-NH—(P) **6** —iv., v.→ Bsmoc-Ile-O-CH$_2$-CH(NH-Boc)-CO-Thr(t-Bu)-Ile-Met-NH—(P) **7**

—i., vi.→ Fmoc-Trp-Phe-Thr(t-Bu)-Thr(t-Bu)-Leu-Ile-O-CH$_2$-CH(NH-Boc)-COThr(t-Bu)-Ile-Met-NH—(P) **8**

—i., vii.→ H-Trp-Phe-Thr-Thr-Leu-Ile-O-CH$_2$-CH(NH$_2$)-COThr-Ile-Met-NH$_2$ **9**

Scheme 1. Reagents and conditions : (i) 20% v/v piperidine/DMF, 7 min; (ii) Fmoc-Ser(Trt)-OH, N-HATU, DIEA, 30 min; (iii) Boc$_2$O/DMF/DIEA/2 × 30 min; (iv) DCM/TFA/triethylsilane/94/1/5 2 min × 3 times; (v) Bsmoc-Ile-F/DMAP/DCM/2h × 2 times; (vi) Fmoc-Leu-OH N-HATU, DIEA, DMF, etc., for all other amino acids; (vii) TFA/thioanisole/1,2-ethanedithiol/anisole 90/5/3/2 2 h.



which accounts for the difficulty of synthesizing the JR sequence, which is known to begin at amino acid number 5, is overcome by the presence of the ester linkage built into positions 6–7 or 7–8. The ammonium hydroxide-induced O→N shift for the authentic depsipeptide was examined in the case of **9** and **10**, with reversal occurring more readily for the latter.

In view of the tendency for serine to undergo loss of configuration, as noted above, it was not unusual that coupling of the serine residue to the threonine hydroxyl unit in the synthesis of **10** via the DCC/DMAP technique was accompanied by extensive epimerization (up to 22.5%). Considering that synthetic difficulties in the synthesis of **2** appear to have been avoided in the syntheses of **9** and **10** it was expected that one could merely continue the assembly to the end of the 26-mer sequence and then effect the O→N shift and thereby obtain the native amide sequence in good yield. In order to avoid loss of configuration at serine this was done for sequence **9** and in fact an excellent synthesis of the 26-mer resulted. The progress of the final step (O→N shift) is shown in Figure 3.

This new method may well prove to be applicable to any difficult sequence for which serine or threonine units are found at appropriate positions.[11] It may be noted that these are precisely the systems for which the 'pseudoproline' method of Mutter et al.[12] for the synthesis of difficult sequences was designed. It was shown as part of the present work that the Mutter technique, involving the simultaneous introduction of Ile^{22} and Ser^{23} via the dipeptide Fmoc-Ile-Ser($\psi^{Me,Me}$pro)-OH also provided an excellent route to the full 26-mer **1**.

With both depsipeptide and amide forms of 26-mer **1** available, pure samples were obtained and subjected to IR studies in order to characterize the structural effects presumably responsible for the facile synthesis of the ester form. In the IR spectrum the amide form, upon solution in D_2O, shows the characteristic band at $1621\,cm^{-1}$, which indicates the presence of significant amounts of intermolecular β-sheet structures whereas the depsipeptide analog exhibits the spectrum of an unordered peptide, with a broad band at $1647\,cm^{-1}$.

We also applied the method to amyloid peptide Aβ(1–42) **13**, which has been reported to be a difficult sequence for SPPS, because of incomplete couplings that also can be avoided by using Fmoc-amino acid fluorides.[13]

Using an ABI 433A peptide synthesizer and a standard Fmoc-chemistry protocol (N-TBTU, double couplings), Aβ(27–42) was assembled automatically. The next coupling involving Boc-Ser and the subsequent O-acylation via Fmoc-Gly were carried out manually, and the remainder of the synthesis was completed automatically. Direct use of unprotected Boc-Ser-OH in place of the O-trityl Fmoc analog used in the synthesis of **9** reduces the number of steps required in the generation of the depsipeptide unit. The resulting crude product (depsi isomer) was of excellent quality although it contained a significant by-product Aβ(26–42), as a result of incomplete O-acylation. Compared with the pseudoproline methodology, a very important advantage of the depsipeptide method results from the fact that the conformation-

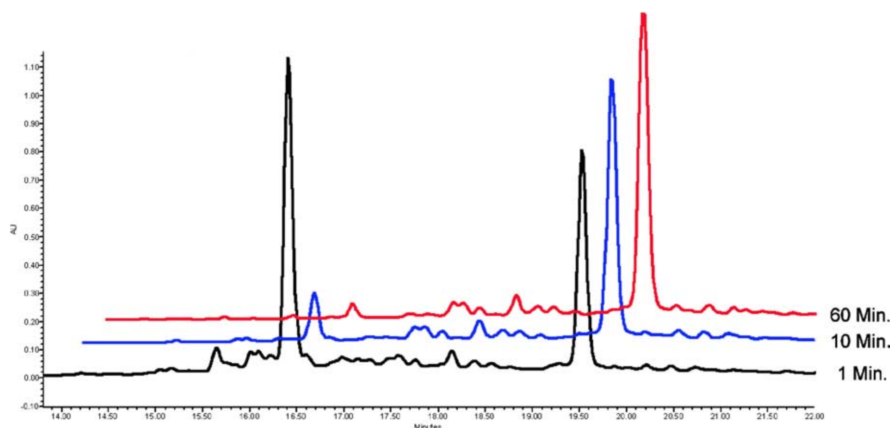


Figure 3. Progress of the O→N shift of depsi(22–23) JR 26-mer (rt 15.5 min) to the native JR 26-mer (rt 19.0 min) upon treatment with ammonia over a period of 1 h. See Supplementary data for details.

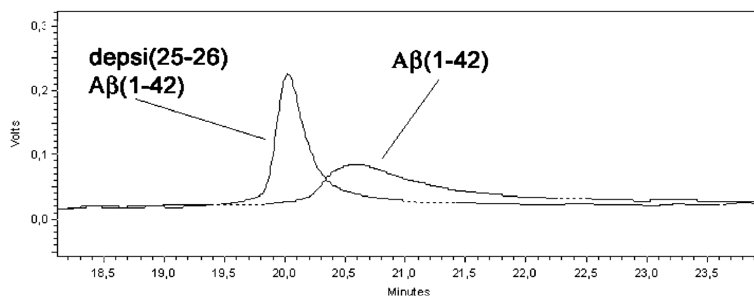


Figure 4. Comparison of purified depsiAβ(1–42) before and after treatment at pH 8.5 (application of the same quantity of both peptides to the HPLC column).

disrupting modification introduced by the depsipeptide unit is still present during chromatographic purification of the deblocked peptide. The suppression of conformation-induced association leads to an increased solubility and narrower peaks for the depsi isomer compared to the amide isomer, thus allowing a much more efficient purification. The HPLC behavior of the two forms is shown in Figure 4.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2004.07.162. Experimental details for solid phase syntheses as well as authentic syntheses of various appropriate epimers and all four N→O rearrangement products and details of how some were established to be present in test peptide sequences by LC–MS techniques.

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