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Dramatically enhanced N→O acyl migration during the trifluoroacetic acid-based deprotection step in solid phase peptide synthesis

Louis A. Carpino,[a,*] Eberhard Krause,[b] Calin Dan Sferdean,[a] Michael Bienert[b] and Michael Beyermann[b,*]

[a]*Department of Chemistry, University of Massachusetts, Amherst, MA 01003, USA*
[b]*Institute of Molecular Pharmacology, Peptide Chemistry Group, 13125 Berlin, Germany*

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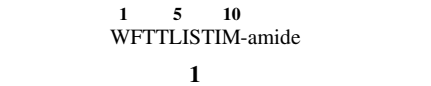
Abstract—N→O acyl migrations at serine or threonine residues in peptides or proteins have previously been observed upon treatment with strong acids. Here we show that the extent of such N→O shifts depends on the peptide sequence and even in the presence of moderately acidic trifluoroacetic acid, as during Fmoc or Bsmoc-based solid phase peptide synthesis, may give rise to large amounts of depsipeptide by-products.

The reversible pH dependent N,O-acyl migration of β-amino alcohols was first investigated by Bergmann et al.[1] in 1923. Such N→O acyl migrations have been the basis of several attempts to cleave peptide chains specifically next to Ser or Thr residues. For example, silk fibroin was treated with sulfuric acid to cause an N→O shift at various serine sites. This was followed by acetylation of the free amino groups generated and subsequent hydrolysis of the ester bonds formed during the shift process.[2] Other strong acids such as HF[3] or BF_3/HCO_2H[4] have also been used for this purpose.

In addition the use of strong acids in peptide chemistry has led to undesired side product formation due to such N→O shifts. For example, esterification of insulin with HCl/MeOH was accompanied by an N→O shift at threonine residue B27[5] and liquid HF and CF_3SO_3H have to be used with considerable care during peptide synthesis.[6] Thus for BOC-based solid phase synthesis, it has been recommended[7] that following the final deblocking step in liquid HF, a precautionary treatment with dilute aqueous bicarbonate may be advised in appropriate cases.

If only moderately strong acids, such as trifluoroacetic acid (TFA) are used, acid-catalyzed N→O shifts are expected to be avoided.[8,9] Indeed, while using Fmoc chemistry for peptide assembly with a final TFA treatment for the removal of all protecting groups, N→O acyl shifts have not been recognized as leading to serious side reactions and thus are not even mentioned in recent textbooks on Fmoc-based peptide synthesis.[10] In agreement with this perception, while inspecting very carefully for side products in the case of the synthesis of a 'difficult' peptide sequence, the C-terminal 10-mer **1** of the Jung–Redemann 26-mer **2**[11] using both Fmoc and Bsmoc-based solid phase syntheses we observed,[12] in addition to a number of other side products (not shown here), only small amounts of isomeric side products,



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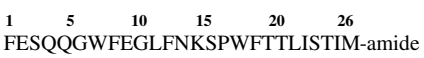

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Keywords: N→O Acyl migration; Depsipeptides; Trifluoroacetic acid deblocking; D-Amino acid-containing peptides.
* Corresponding authors. Tel.: +1 413 545 2027; fax: +1 413 545 2667 (L.A.C.); tel.: +49 309 479 3272; fax: +49 309 479 3159 (M.B.); e-mail addresses: carpino@chem.umass.edu; beyermann@fmp-berlin.de

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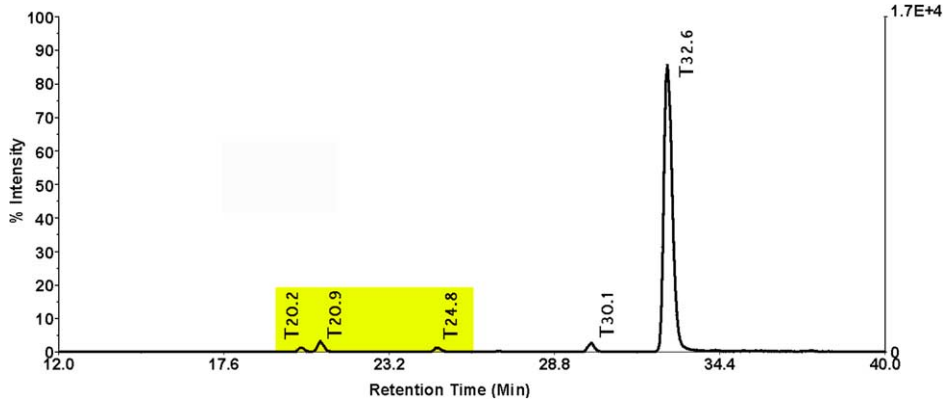

Figure 1. Extracted ion chromatogram (*m/z* 606.3; 1211.6) of peptide **1** obtained via Bsmoc chemistry.

which did not appear to be epimers in view of their very different HPLC retention behavior relative to the desired product (Δt_R = 8–12 min). See Figure 1.

This group of isomeric peptides (t_R = 20.2, 20.9, 24.8 min) has been identified[12,13] as the corresponding N→O shift isomers of **1** at the various threonine sites. In confirmation, these materials were converted to the desired peptide **1** by treatment with dilute ammonium hydroxide solution, which effects the corresponding O→N shifts.

Figure 1 shows a small amount of a D-epimer (t_R 30.1 min) that is also formed during synthesis and in the course of identifying this material as the D-Ser epimer we had occasion to synthesize a number of analogs of **1** bearing a single D-amino acid. We were surprised to observe unexpectedly large amounts of the corresponding depsipeptides[14] in some of these cases, namely those involving D-Thr[8], D-Thr[4], and D-Thr[3] (Fig. 2A–C).

The amount of depsipetides formed based on the UV integral data is shown in Table 1.

Relatively moderate depsipeptide formation occurred in the case of the D-Phe and D-Leu analogs and much smaller amounts in all other cases examined (see Supplementary data).

The results show clearly that the extent of the N→O shift is dependent on the position of the D-amino acid within the chain. D-Amino acid incorporation may result in an increased turn formation tendency around this position, which could also favor formation of a hydroxyoxazolidine, which has been suggested[1,2] as an intermediate in N,O-acyl migration. It may be pertinent that in the case of aspartimide formation, another side reaction which occurs during peptide synthesis via intramolecular cyclization, the incorporation of D-amino acids next to aspartic acid residues has also been shown to increase its extent.[15] Interestingly, the crystal struc-

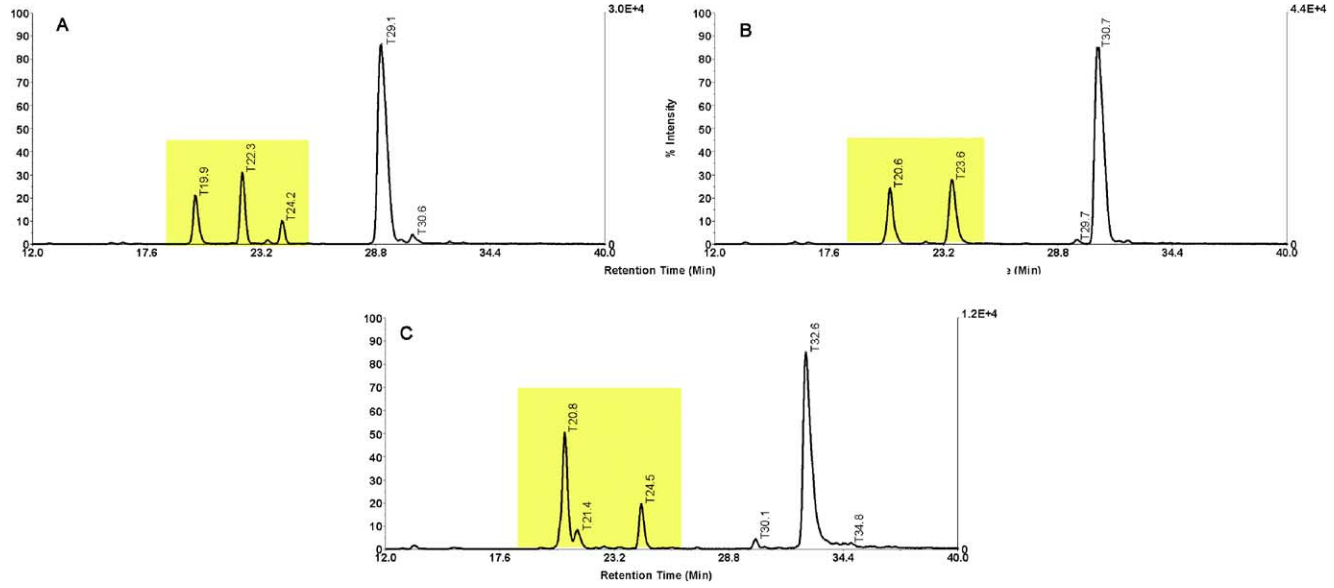

Figure 2. (A) Extracted ion chromatogram (*m/z* 606.3; 1211.6) of the D-Thr[8] epimer. (B) Extracted ion chromatogram (*m/z* 606.3; 1211.6) of the D-Thr[4] epimer. (C) Extracted ion chromatogram (*m/z* 606.3; 1211.6) of the D-Thr[3] epimer.

Table 1. Formation of isomeric side products of D-amino acid analogs of the Jung–Redemann 10-mer calculated from HPLC chromatograms (220 nm)

Peptide	RT (min) of the main peak	RT (min) of depsipeptides	Peak area (mAU min) UV 220 nm	Area % based on normalization of the main peak to 100%
All-L 10-mer (obtained via Bsmoc chemistry)	**32.6**		**77.1**	
		20.2	**1.0**	**1.3**
		20.9*	4.3	5.6
		24.8	**0.5**	**0.6**
D-Trp analog	**38.1**		**32.6**	
		23.9*	0.6	1.8
		24.8	**1.2**	**3.7**
		25.6*	1.1	3.4
D-Phe analog	**35.4**		**53.3**	
		24.1 + 24.5*	6.7	12.6
		27.9*	1.4	2.6
D-Thr^{3} analog	**32.6**		**127.7**	
		20.8*	28.9	22.6
		21.4*	16.8	13.2
		24.5	**3.1**	**2.4**
D-Thr^{4} analog	**30.7**		**287.5**	
		20.6*	39.9	13.9
		23.6	**29.8**	**10.4**
D-Leu analog	**30.8**		**101.9**	
		21.0	**3.6**	**3.5**
		24.7	**1.6**	**1.6**
		25.4	**0.6**	**0.6**
allo-D-Ile^{6} analog	**34.0**		**177.7**	
		25.8*	6.9	3.9
		26.6*	5.4	3.0
		27.9*	1.5	0.8
D-Ser analog	**30.0**		**337.2**	
		19.7*	8.4	2.5
		20.5	**13.3**	**3.9**
		24.2	**4.2**	**1.2**
D-Thr^{8} analog	**29.1**		**280.7**	
		19.9*	40.1	14.3
		22.3	**22.2**	**7.9**
		24.2	**31.6**	**11.3**
Allo-D-Ile^{9} analog	**32.6**		**315.4**	
		25.1*	n.d.	n.d.
		28.1*	5.2	1.6
D-Met analog	**29.3**		**45.2**	
		19.6	**0.8**	**1.8**
		21.6	**2.2**	**4.9**
		24.0	**2.3**	**5.1**

Extracted ion chromatograms (*m*/*z* 606.3, 1211.6) recorded by on-line ESI-MS were used to identify the depsipeptides (drastically increased intensity of the doubly charged peak).

* Overlapping peaks detected by on-line ESI-MS.

tures of proteins that autoproteolyze via an N→O shift, such as glycosylasparaginase precursors, show a tight turn around the scissile peptide bond.[16]

In conclusion, N→O acyl migration at serine or threonine sites in peptides does not occur only under strongly acidic conditions, but also can occur with moderately strong acids such as TFA, which is the standard reagent for the final deblocking step in Fmoc-based peptide synthesis. The extent of a particular N→O shift occurring under such conditions is strongly dependent on primary structure and can be clearly influenced by the introduction of a D-amino acid into a peptide chain. Details of the structural changes, which accompany such a substitution are currently unknown.

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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.2004.12.089. Experimental details for the synthesis of the various D-analogs of **1** and their LC/MS and HPLC/MS characteristics.

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9. For a remarkable example of the difference between TFA and a stronger acid (e.g., methanesulfonic acid, MSA) see: Fujino, M.; Wakimasu, M.; Shinagawa, S.; Kitada, C.; Yajima, H. *Chem. Pharm. Bull.* **1978**, *26*, 539, These workers prepared mammalian glucagon by a solution-based segment coupling process using Boc/Bn chemistry. Five of the six segments used in the process contained unprotected serine or threonine units. TFA was used to deblock the N-terminal Boc protectant from each segment in preparation for segment coupling. No contamination due to the formation of depsipetides occurred whereas when the same segments were treated with MSA impurities arising from N→O shifts were encountered. The final deblocking to give the unprotected 29-mer required MSA to remove benzyl-based side chain protection and in this case, it was only after treatment with 0.5 N aqueous ammonia (O→N reversal) that a pure peptide could be obtained.
10. (a) *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*; Chan, W. C., White, P. D., Eds.; Oxford University Press: Oxford, UK, 2003; (b) *Synthetic Peptides. A User's Guide*; Grant, G. A., Ed.; Oxford University Press: Oxford, UK, 2002.
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13. Authentic samples of the three threonine-linked depsipeptides were synthesized by standard methods and HPLC-based co-injection experiments confirmed their probable identity with the three unknown peaks. While not an absolute proof of structure since the small peaks in question were not isolated and sequenced, the corresponding serine-linked depsipeptide was synthesized in the same way and shown definitively by a similar experiment to be absent from the mixture of peptides obtained by assembly of all-L peptide **1**.
14. The term 'depsipeptide' refers here to the homomeric species in which the peptide is O-linked at serine or threonine in place of the normal amide linkage. For a recent discussion of the nomenclature and structural representation of these materials see: Filip, S. V.; Cavelier, F. *J. Pept. Sci.* **2004**, *10*, 115.
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