Novabiochem®

Practical aspects of the use of the Dbz linker for making thioesters by Fmoc SPPS

R. Behrendt[1] & P. White[2]

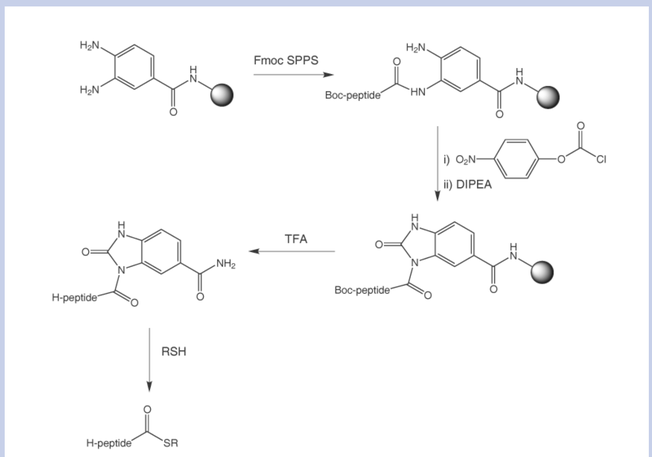
[1]Novabiochem, Merck & Cie, Im Laternenacker 5, CH-8200 Schaffhausen, Switzerland

[2]Novabiochem, Merck Chemicals Ltd., Beeston, Notts, NG9 2JR, U.K.

Introduction

Dawson's Dbz linker [1] is an important new tool for making peptide thioesters by Fmoc SPPS. The linker consists of 3,4-diaminobenzoic acid which is attached *via* the carboxyl group to an amino functionalized resin. Peptide chain extension is performed on one of the anilino groups, before formation of a imidazolidinone (Nbz) with *p*-nitrophenyl chloroformate, and cleavage form the resin with TFA. The peptide Nbz is used directly in chemical ligation reactions to generate *in situ* the desired peptide thioester.

Fig. 1: Synthesis of thioesters using Dawson resin.



During our attempts to incorporate this new linker into our routine synthesis of peptide thioesters, we observed formation of branched peptides resulting from acylation of the second anilino group, particularly with glycine. In this poster we present the results from our experiments to overcome this problem.

Results and discussion

The success of Dawson's approach using the Dbz linker rests on being able to fully acylate only one of the two linker amines with the C-terminal amino acid residue. Incomplete acylation will lead to formation of C-terminally truncated peptides as new chains are propagated by acylation of any unreacted amines during subsequent coupling cycles. Overacylation will lead to formation of branched peptides with chains growing off both linker amines (Figure 2).

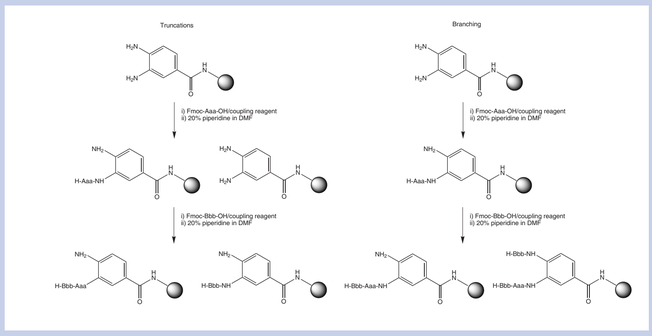


Fig. 2: Possible side-reactions involving Dbz linker.

.

Table 1 shows the procedures for loading of the C-terminal residue given in the supplementary information of their paper together with additional methods kindly provided in a subsequent personal communication. The requirement for special loading conditions for different residues emphasises the care that must be taken to achieve selective and complete acylation a single linker amine.

Table 1: Reported conditions for use of Dbz linker. Concentrations given are reaction end-point concentrations.

Amino acid	Conditions
C-terminal Gly	0.15 M HBTU/HOBt/DIPEA 1:1:1.5[1] 0.15 M HCTU/HOBt/DIPEA 1:1:1.5[2]
C-terminal Ile, Val, Thr, Pro, Arg	0.15 M HATU/DIPEA 1:1:1.5[1]
C-terminal others	0.25 M HBTU/DIPEA 1:1:1.5[1] 0.25 M HCTU/DIPEA 1:1:1.5[2]
general chain extension	0.15 M HBTU/HOBt/DIPEA 1:1:1.5[1] 0.45 M HCTU/HOBt/DIPEA 1:1:2[2]

For our routine synthesis of thioesters, we require a robust and generic approach that is adaptable to the automated synthesizers available to us. Clearly, whilst an approach requiring the use of multiple coupling reagents of varying and dilute concentrations in a single synthesis is amenable for manual synthesis, it is not easily adaptable to our instruments, particulary the ABI 433A. Therefore, we were interested to see how well Dawson's Ddz approach would work on our ABI synthesizer using a single generic protocol.

Initially, we undertook the synthesis of peptide 1 (Table 2) as shown in Table 3 using HCTU, as the use of this reagent appears from the information in Table 1 to be appropriate for both initial loading of the C-terminal Leu residue and the addition of subsequent amino acids. The results of this synthesis were satisfactory (Figure 3).

Table 2: Sequences prepared in this study.

	Peptide sequence
1	H-Ala-Leu-Tyr-Glu-Phe-Lys-Leu-Lys-Leu-Dbz
2	H-Ala-Gly-Tyr-Glu-Phe-Lys-Gly-Lys-Leu-Dbz
3	H-Ala-Gly-Tyr-Glu-Phe-Lys-Gly-Lys-Gly-Dbz

Table 3: Synthesis conditions.

	Conditions
Resin	Dawson AM resin
Instrument	ABI 433A
Coupling	Fmoc-Aaa-OH (0.15 M) : Coupling reagent : DIPEA (1:1:2)
Deblock	20% Piperidine (3 or 4 times 3 min)
Cleavage	TFA/water/TIS (95:2.5:2.5) for 3 h

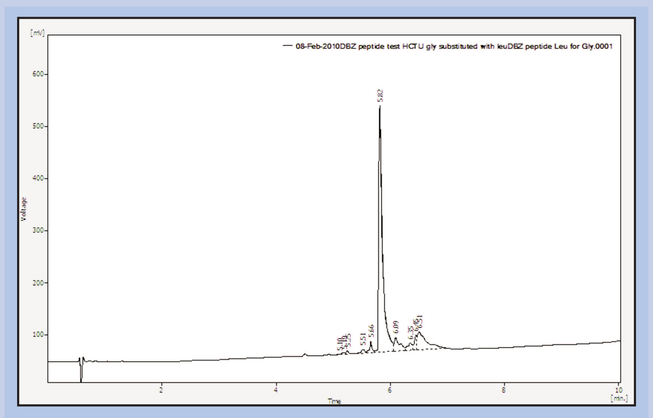


*Fig. 3: HPLC profile of peptide **1** prepared using HCTU.*

Encouraged we then attempted the synthesis of an analogous sequence, peptide **2**. HPLC (Figure 4) and MALDI-TOF (Figure 5a) revealed a very different result: in addition to branching at the C-terminal Leu residue, branching also occurred whenever a Gly residue was introduced into the sequence. Clearly, HCTU activation is not appropriate for introduction of Gly residues with this linker.

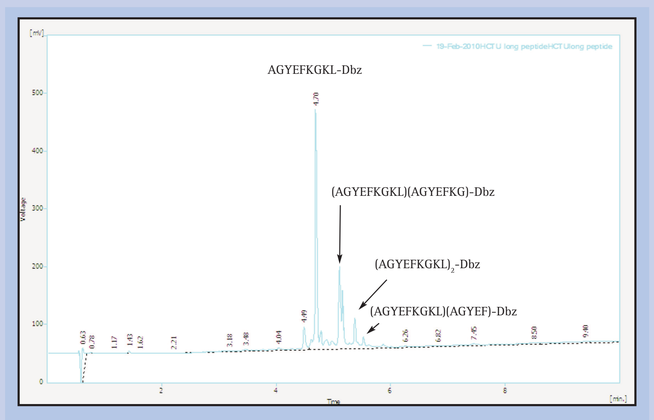


*Fig. 4: HPLC profile of peptide **2** using HCTU.*

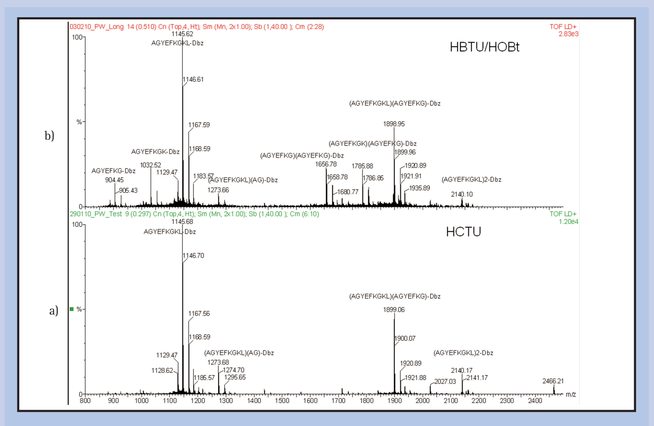


Fig. 5: MALDI-TOF spectra of peptide 2 prepared a) with HCTU or b) HBTU/HOBt.

The synthesis was then repeated using HBTU/HOBt activation, to determine whether milder activation would decrease the branching at Gly. HPLC (Figure 6) and MALDI (Figure 5b) analysis of the crude product revealed the formation of an even more complex mixture of peptides. In addition to a small amount of C-terminal Leu and Gly-branched peptides, C-terminal truncation peptides and branched peptides derived from these were also present. This result is of concern since this would indicate that HBTU/HOBt activation is not sufficiently powerful to completely load Fmoc-Leu onto the linker in 1 h but still capable of initiating the formation of branched peptides. In practical terms, formation of truncated sequences is far more problematic than that of branched ones since they can be the origin of further branching, leading to very complex mixtures of products.

We, therefore, decided to examine the use of even milder carboxyl activation of the introduction of Gly and decided to use an OPfp ester with HOBt catalysis with HCTU for the coupling of all other residues. HPLC analysis (Figure 7) of the product obtained from this synthesis indicated that the levels of Gly-branching had been reduced from 7 to 2% compared to the synthesis (Table 4). However, reducing these by-products enabled the other branching by-products arising from acylation of the second linker amine by each of the C-terminal 5 residues to be identified, indicating HCTU is causing overacylation even with residues other than Gly. Re-examination of the data from this and previous syntheses identified branching originating from Phe to be particularly problematic.

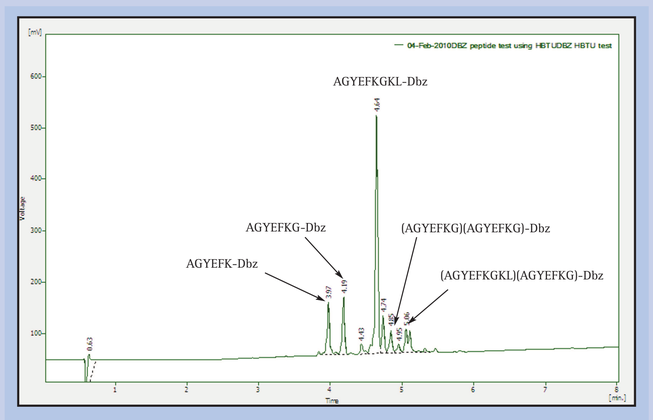


*Fig. 6: HPLC profile of peptide **2** prepared with HBTU/HOBt.*

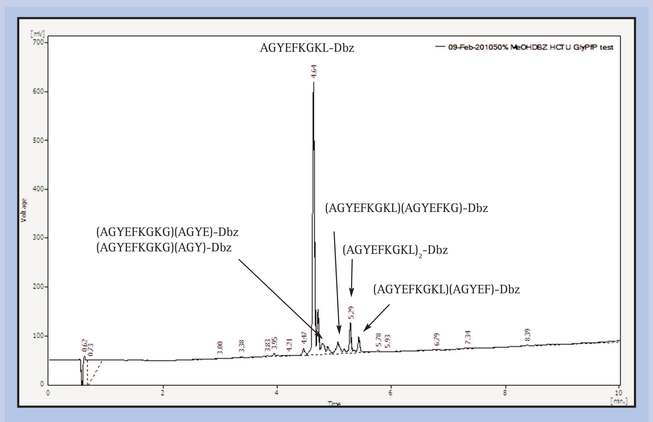


*Fig. 7: HPLC profile of peptide **2** prepared with HCTU and GlyOPfp.*

Table 4: Principle by-products produced in synthesis of peptide 2.

Peptide	HCTU	HBTU/HOBt	HCTU/Gly-OPfp
(AGYEFKGKL)(AGYEFKG)Dbz	17	7	2
(AGYEFKGKL)(AGYEFKGKL)Dbz	7	0	8
(AGYEFKGKL)(AGYEF)Dbz	2	0	5
(AGYEFKGK)Dbz	0	13	
(AGYEFKG)Dbz	0	13	

Given the moderate success of using OPfp/HOBt activation in reducing branching, the synthesis of peptide **3** was also attempted using DIPCDI/Oxyma to determine whether avoiding base mediated coupling reagents would reduce the extent of branching. Unfortunately, significant branching at Gly and Phe was again observed (Figure 8).

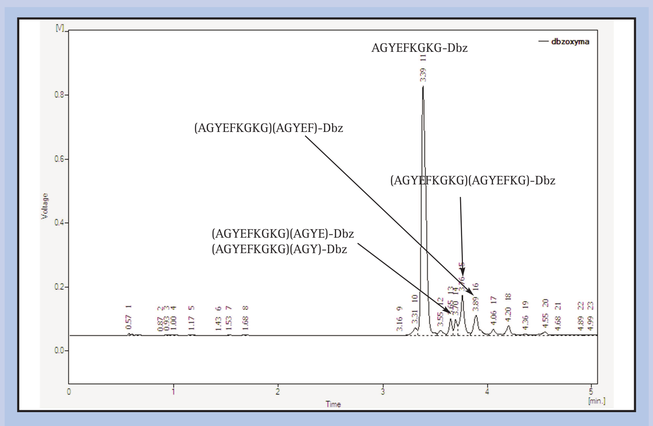


*Fig. 8: HPLC profile of peptide **3** prepared using DIPCDI/Oxyma Pure.*

Conclusions

- Unfortunately, a generic coupling strategy for loading of the C-terminal residue to the Dbz linker and chain extension could not be found. Complete loading of the C-terminal residue is critical if truncated and truncated/branches by-products are to be avoided. Preparation of resin from Dbz linker preloaded with the C-terminal amino acid is best approach to avoid these problems.
- Formation of branched peptides occurs whenever Gly (and to a lesser extent Phe) is coupled to the linker. This problem can be minimised by using only mildly activated Gly derivatives such as OPfps, or reducing reagent concentration and time.
- Small amounts of branched peptides also form at other residues. Therefore, mild activation is again recommended. Extended or double couplings, or strong activation is likely to introduction extensive branching. Therefore, the Dbz approach is unlikely to be useful for making thioesters of difficult peptides.
- Appropriate protection of one of the anilino groups will eliminate all the above issues and is the subject of ongoing research.

References

1. J. B. Blanco-Canosa & P. E. Dawson (2008) *Angew. Chem. Int. Ed.* **47**, 6851.

2. P. E. Dawson, personal Communication.

MERCK