Novabiochem®
Innovations: 3/11

Use and applications of Dbz resins in native chemical ligation

Dawson's Dbz linker [1, 2] is an important new tool for making the peptide thioester component for native chemical ligation (NCL) reactions by Fmoc SPPS. The linker consists of 3,4-diaminobenzoic acid which is attached *via* the carboxyl group to a TFA-cleavable amino functionalized resin. Peptide chain extension is performed on one of the anilino groups, followed by formation of an 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 (Figure 1).

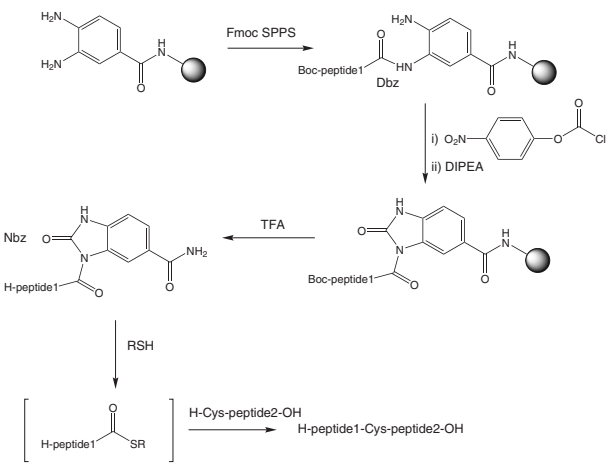

Fig. 1: Use of Dbz resins in NCL.

This innovation describes the special considerations involved with the use of this resin, and presents methods and strategies of how to use it successfully in synthesis.

Synthesis design & general considerations

Branching & truncations

The success of the Dbz approach rests on being able to fully acylate only one of the two linker amines with the *C*-terminal amino acid residue and to avoid acylation of the unprotected amine during chain extension. Incomplete acylation leads to formation of *C*-terminally truncated peptides as new chains are propagated by acylation of any unreacted amines during subsequent coupling cycles. On the otherhand, overacylation results to formation of branched peptides with chains growing off both linker amines (Figure 2). Therefore, the selection of acylation method of attachment of the *C*-terminal residue and subsequent couplings is critical if good results are to be obtained (Method 1).

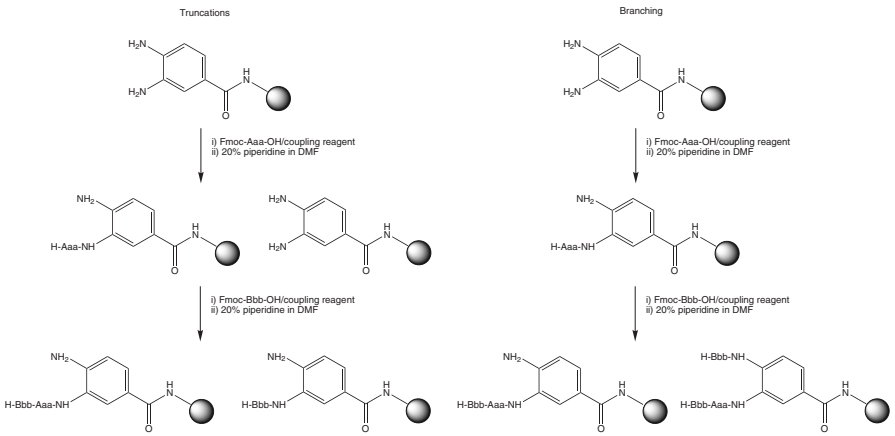

Fig. 2: Possible side-products involving Dbz linker.

Particularly problematic is the coupling of glycine residues, especially if they occur close to the C-terminus of the peptide. This reactive and unhindered amino acid can couple to the free Dbz-amino group if uronium or phosphonium activation is used. For example, Figure 3 shows the crude product obtained in the synthesis of H-Ala-Gly-Tyr-Glu-Phe-Lys-Gly-Lys-Leu-Dbz when HCTU was used for all couplings. Extensive branching results from additional acylation of the second linker amino group by glycine, and to lesser extent by other amino acids, under these conditions. In our hands best results are obtained if glycine residues are introduced using Fmoc-Gly-OPfp/HOBt. This precaution may be unnecessary once the peptide is extended beyond 20 residues, as hindrance should reduce the reactivity of unprotected Dbz amine. Blocking of the *N*-terminal amino group, prior to

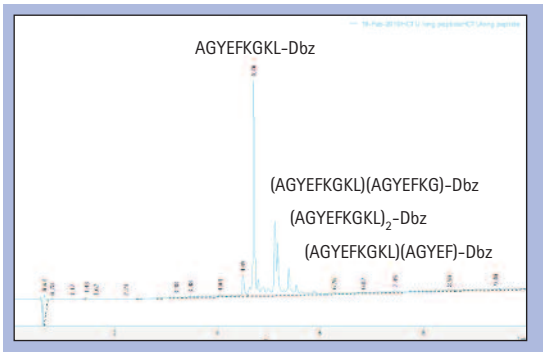

Fig. 3: HPLC profile of crude H-Ala-Gly-Tyr-Glu-Phe-Lys-Gly-Lys-Leu-Dbz prepared with HCTU.

activation of the linker with p-nitrophenyl chloroformate, is essential. The easiest and simplest way is to use a Boc-amino acid to introduce the final residue. If N-terminal capping is necessary, it must be done using very mild reagents if blocking of the second amine is to be avoided. Recently, Dawson has found *N*-terminal acylation could be performed successfully using N,N-diacetylaminoquinazolinone [2c]. Boc-N_3 should be suitable for introduction of an *N*-terminal Boc group.

Recently, the use of Alloc protection for blocking the second amino group has been advocated as a way to avoid all issues with branching and truncation [3] (Figure 4). Dbz resins are supplied as a mixture of 74% 3-Fmoc-Dbz, 17% 4-Fmoc-Dbz and 6% *bis*-Fmoc-Dbz. Capping the resin with Alloc-Cl prior to removal of the Fmoc group will thus reduce the maximum potential for branching or truncation to 6%. For hindered amino acids, it has been found necessary to load the resin prior to capping with Alloc. The Alloc group must be cleaved off with Pd(0) before conversion of the Nbz form.

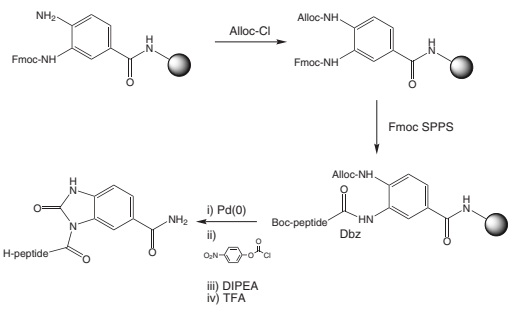

Fig. 4: Alloc protection strategy [3].

General synthetic procedures

Loading of the C-terminal residue

Loading of the C-terminal residue is best achieved using Method 1. With synthesizers that use dry reagents, such as the ABI 443A, loading of the first residue can be automated by using a mixture of Fmoc-amino acid and the appropriate coupling reagent packed in a cartridge or vial, with activation effected by addition of DIPEA in DMF. For subsequent amino acids, the standard pre-dissolved activator can be used. Some optimization of the loading reaction may be required to maximize loading and minimize branching. Treatment of a small sample of loaded resin with TFA and analysis by HPLC of the cleaved product can be helpful in monitoring this process.

Method 1: Loading Dawson Dbz resins

1. Pre-swell the resin (0.1 mmole) in DCM for 60 mins and wash with DMF. Remove Fmoc group with 20% piperidine in DMF and wash with DMF.
2. **Ile, Val, Thr, Pro, Arg:** Add Fmoc-Aaa-OH (0.6 mmole), HATU (0.6 mmole) and DIPEA (0.9 mmole). Agitate gently for 1 h. Wash resin with DMF and repeat coupling.
   Gly: Add Fmoc-Gly-OPfp (0.6 mmole) and HOBt (0.6 mmole). Agitate gently for 1 h.
   Other amino acids: Add Fmoc-Aaa-OH (0.6 mmole), HCTU (0.6 mmole) and DIPEA (0.9 mmole). Agitate gently for 1 h. .
3. Check loading using Method 3-6, p. 3.6 of the 2010/2011 Novabiochem catalog. Alternatively, wash a sample of resin with DCM and treat with 95% TFA aq. for 30 min. Anaylze cleaved product by HPLC.

Chain extension

In general, strong activators like HATU or HCTU should be avoided as their use can lead to branching. In our hands, HBTU/HOBt appears to work well for coupling of all residues except Gly, where the use of the pre-formed OPfp in conjunction with HOBt gives minimal branching.

Conversion to Nbz

Activation of the linker by conversion to the Nbz is achieved according to Method 2. The reaction is usually quantitative. With high loaded resins like Dawson Dbz AM resins, some cross-linking of Dbz moieties can occur. In our experience, much cleaner results are obtained with low-loaded resins like Dawson Dbz NovaSyn® TGR resin. Treatment of the Nbz resin with TFA releases the fully deprotected peptide-Nbz, which can be used directly in the NCL reaction. The Nbz peptide is obtained as a mixture of regioisomers.

Method 2: Synthesis & Activation

1. Extend peptide chain using HBTU/HOBt/DIPEA activation, except for Gly which should be introduced using Fmoc-Gly-OPfp/HOBt. The N-terminal residue must be introduced using a Boc-amino acid. Wash the resin with DMF and DCM.
2. Add *p*-nitrophenyl chloroformate (0.5 mmole) in DCM and leave to gently agitate under N_2 for 1 h. Wash resin with DCM and add 0.5 M DIPEA in DMF (10 ml) and leave for 30 min. Wash resin with DMF and DCM.
3. Cleave peptide with TFA/water/TIS 95:2.5:2.5 for 3 h.

NCL

Ligation using peptide-Nbz derivatives as latent thioesters can be carried out according to Method 3.

Method 3: Ligation of unprotected peptide fragments

1. Dissolve purified peptide-Nbz (1 eq.) and N-terminal-Cys peptide (1.5 eq.) in a screw-cap tube containing degassed ligation buffer (0.2 M phosphate buffer, 6 M guanidine hydrochloride, 0.2 M 4-mercaptophenylacetic acid, 0.02M TCEP, pH 7.0). The final concentration of the peptides should be approximately 2 mM.
2. Monitor the progress of the reaction by HPLC.
3. Acidify the reaction with TFA (0.1% by volume of solution), lyophilize and purify by standard procedures.

Exemplar synthesis

Application 1:

Boc-Ala-Leu-Tyr(tBu)-Glu(OtBu)-Phe-Lys(Boc)-Leu-Lys(Boc)-Leu-Dbz NovaSyn® TGR resin was prepared automatically on an ABI 433A. The C-terminal residue, Leu, was coupled using Fmoc-Leu-OH/HCTU/DIPEA (5:5:10) for 1 h. All subsequent acylation reactions were carried out using 5-fold excesses of Fmoc-amino acids activated with 1 eq. of HBTU in the presence of 1 eq. HOBt and 2 eq. of DIPEA. The N-terminal residue was introduced using Boc-Ala-OH. A coupling time of 60 min was used throughout. Fmoc removal was effected with 20% piperidine in NMP (2 x 3 min). A small sample of the intermediate Dbz peptide was cleaved from the resin for analysis by treatment with TFA/ TIS /water 95:2.5:2.5 for 3 h (Fig. 5). The remaining resin was converted to the Nbz according to Method 2, cleaved with TFA as previously described and analyzed. The crude product and H-Cys-Phe-Ala-Pro-Leu-Val-OH were ligated as described in Method 3. The reaction was allowed to stand for 3 h. Analysis of the total reaction mixture (Fig. 6) indicated complete reaction.

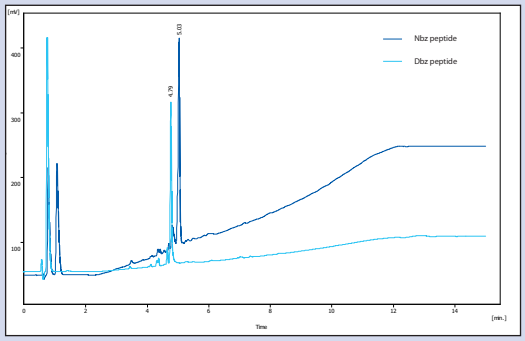

Fig. 5: HPLC profiles of crude H-Ala-Leu-Tyr-Glu-Phe-Lys-Leu-Lys-Leu-Dbz (light blue) and H-Ala-Leu-Tyr-Glu-Phe-Lys-Leu-Lys-Leu-Nbz (dark blue).

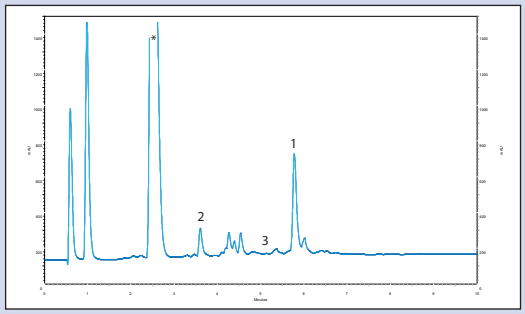

Fig. 6: HPLC profiles of ligation reaction between H-Ala-Leu-Tyr-Glu-Phe-Lys-Leu-Lys-Leu-Nbz and H-Cys-Phe-Ala-Pro-Leu-Val-OH. [1]Ligation product; [2]unreacted H-Cys-Phe-Ala-Pro-Leu-Val-OH; [3]H-Ala-Leu-Tyr-Glu-Phe-Lys-Leu-Lys-Leu-Nbz; *4-mercaptophenylacetic acid.

Ordering information

855142	Dawson Dbz NovaSyn ®TGR resin	1 g
NEW		5 g
855131	Dawson Dbz AM resin	1 g
		5 g
		25 g

References

1. J. B. Blanco-Canosa & P. E. Dawson (2008) *Angew. Chem. Int. Ed.*, **47**, 6851.
2. a) Z. Harpaz, et al. (2010) *ChemBioChem*, **11**, 1232; b) B. L. Pentelute, et al. (2010) *ACS Chem. Biol.*, **5**, 359; c) T. K. Tiefenbrunn, et al. (2010) *Biopolymers*, **94**, 405.
3. S. K. Mahto, et al (2011) *Biopolymers*, **96,** 429; poster abstr. P008.