A New Type of Safety-catch Linker cleaved by Intramolecular Activation of an Amide Anchorage and allowing Aqueous Processing in Solid-phase Peptide Synthesis†

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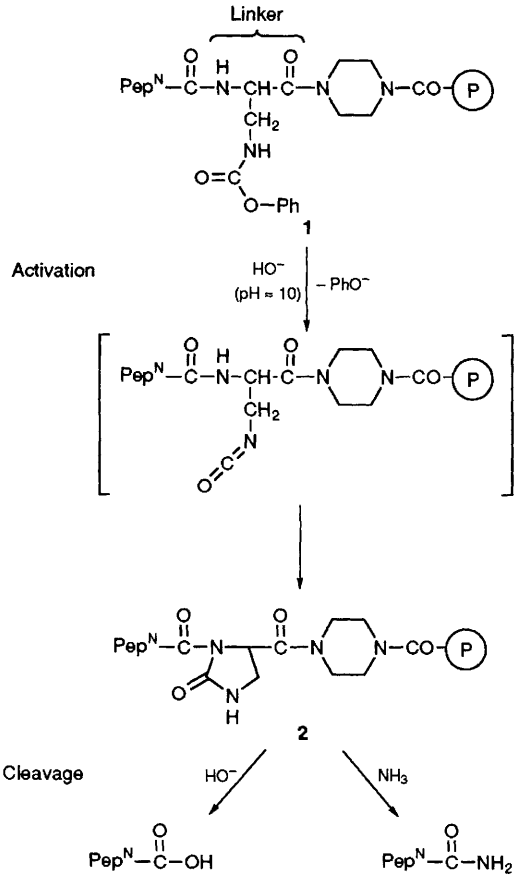
The N^3-phenyloxycarbonyl-L-2,3-diaminopropionic acid residue behaves as a versatile linker [Dpr(Phoc)linker] displaying high stability under neutral and acidic conditions but undergoes activation under mild alkaline conditions for the release of peptide acids or amides by nucleophilic cleavage.

Solid-phase peptide synthesis is usually achieved in non-aqueous solvents with the help of various linkers.[1] Most of them are not suitable for the use of water, even though aqueous processing is attractive, notably in large-scale synthesis, to avoid expensive and hazardous chemicals. In the Boc-solid-phase‡ method for example, aqueous processing can be easily introduced[2] since aqueous mineral acids remove Boc-protecting groups,[3] but the available ester linkers that are cleavable under acceptable conditions, such as slightly activated esters, are not stable enough in neutral and acidic aqueous media, as shown for example by a study on the glycolamide ester linker.[2,4]

This led us to devise a new type of safety-catch linker based on an amide anchorage, as stable as any peptide bond during peptide chain elongation, but one that could be cleaved by nucleophiles after activation by an intramolecular reaction under mild alkaline conditions (Scheme 1). The Dpr(Phoc) residue was selected as a first example because of (*i*) the resistance of phenyl carbamates to acidolysis,[5] (*ii*) their ability to generate an isocyanate at alkaline pH with suitable rates,[6,7] (*iii*) the expected fast and selective intramolecular reaction of this electrophile with the nearest amide group[7] leading to a five-membered ring in the intermediate **2**, (*iv*) the regioselective cleavage of cyclic acylureas of this type,[8] (*v*) the ready access to the linkage agent and its straightforward attachment to a support. Moreover, selectivity in ring closure and in cleavage had already been observed in related procedures[9] for the modification of peptides and proteins at asparagine or aspartic acid residues *via* an isocyanate generated by Hofmann or Lossen rearrangements.

The linkage agent protected for Boc-chemistry was synthesized in two steps (Scheme 2) from Boc-asparagine which was submitted to a Hofmann rearrangement under mild acidic conditions.[10]§ Boc-Dpr was then treated with phenyl chloroformate giving Boc-Dpr(Phoc) **3** [overall yield 60%, FAB-MS *m/z* 325 (M + H$^+$) 347 (M + Na$^+$), satisfactory elemental analyses ($C_{15}H_{20}N_2O_6$), HPLC and 250 MHz ^{1}H NMR data].

The applicability of the new linker to Boc-chemistry and its suitability for aqueous processing were demonstrated by the synthesis of various peptides on the hydrophilic polyacrylamide support Expansin (methyl ester content 0.86 mmol g^{-1}) which was first derivatized with *N*-Boc-piperazine (0.75 ± 0.02 mmol g^{-1})¶ and then submitted to the following synthetic cycle: (*i*) Boc group removal by aq. 6 mol dm^{-3} HCl for 35 min,[2] (*ii*) washes with water, DMF, (*iii*) coupling of Boc-amino acids, the linkage agent **3** included, in DMF as preformed HOBt esters using *in situ* neutralization,[11]‖ (*iv*) washes with DMF, water.



Scheme 1 Activation and cleavage of the Dpr(Phoc) linker (Pep^N = amino terminal part of peptide)

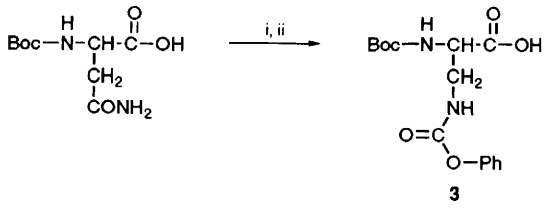
† Presented in part at the 22nd European Peptide Symposium, Interlaken, Switzerland, Sept. 13–19, 1992.

‡ Abbreviations used: Boc = *tert*-butyloxycarbonyl, DIEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, Dpr = L-2,3-diaminopropionic acid, Fmoc = fluoren-9-ylmethoxycarbonyl, HOBt = 1-hydroxybenzotriazole, Phoc = phenyloxycarbonyl, TBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid.

§ The published procedure was satisfactorily modified by using *I*,*I*-diacetoxyiodobenzene instead of *I*,*I*-[bis(trifluoroacetoxy)]iodobenzene and by avoiding the use of pyridine.

¶ The starting resin (a generous gift from Expansia, Aramon, France) was modified by (*i*) hydrolysis of methyl esters (0.1 mol dm^{-3} NaOH, 2 h), (*ii*) washes with 1 mol dm^{-3} HCl, H_2O, DMF, (*iii*) reaction with *N*-Boc-piperazine, TBTU, DIEA in DMF. The nearly quantitative functionalization was estimated by Cl$^-$ titration after removal of the Boc group by aq. HCl.

‖ 0.5 mol dm^{-3} TBTU-HOBt (3 equiv. each) in DMF was added to Boc-amino acid (3.3 equiv.) and DIEA (3 equiv.) in DMF, the mixture was allowed to stand for 7 min and added to the resin with DIEA (1 equiv.). Completion, mostly reached within 30 min, was monitored by the ninhydrin test.



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Scheme 2 Synthesis of Boc-Dpr(Phoc) **3**; i, $PhI(OAc)_2$ in dimethylformamide–water; ii, PhOCOCl, $NaHCO_3$ in water

Boc-Tyr-Gly-Gly-Phe-Leu (protected Leu-enkephalin) was thus assembled on 0.504 g of Boc-piperazine resin (overall weight increase 0.278 g, theoretically 0.281 g). The cleavage was carried out without isolation of the acylurea **2**, by two successive treatments with 0.04 mol dm^{-3} NaOH (2 equiv., 1 h) in Pr^iOH–water 7:3. After neutralization, UV monitoring of the hydrolysate indicated the presence of phenol and a tyrosine-containing peptide in more than 90% yield. After Pr^iOH evaporation and acidification with $KHSO_4$, the crude peptide was extracted by AcOEt, isolated (0.244 g, 98%, Fig. 1) and purified over silica gel column with CH_2Cl_2–MeOH–AcOH 85:15:1 as eluent (0.226 g, 91%, Fig. 1).**

Four acetylated short peptide models, Ac-Arg(NO_2)-Xaa (Xaa = Gly, Leu, Val) and Ac-Trp-Pro-Gly, and a decapeptide (LH–RH)†† were also assembled on the resin in high yields as shown by the resin weight increase and the UV absorption of hydrolysates which were obtained after alkaline treatment (0.03 mol dm^{-3} NaOH) and characterized by a single HPLC peak except in the case of LH–RH (*ca.* 60% purity). All the acetylated dipeptide models were cleaved from the resin in excellent yields determined by the UV absorption of the nitroguanidine group after completion of the alkaline hydrolysis (over 95% of the quantitative value for Xaa = Gly, Leu; 83% for Xaa = Val‡‡), although the rates decreased when the sterical hindrance of the C-terminal residue was increased.[4]

These results are consistent with a high selectivity in the formation and the cleavage of the acylurea **2**. This is also supported by the negative ninhydrin test obtained in each case after completion of the alkaline cleavage, showing that competitive isocyanate hydrolysis or ring opening, which would lead to detectable amino groups on the resin, does not occur.

Acid and amide C-terminal forms of LH–RH were released satisfactorily.[4] A comparison with the model Ac-Trp-Pro-Gly, which bears the same Pro-Gly C-terminal end, indicated no significant effect of peptide length on the rates of activation and cleavage of the linker. Peptide–amide yields depend on the difference in rate between formation and cleavage of the acylureas **2** under the conditions chosen for activation. By using an aqueous buffer at pH 10 before ammonolysis (Pr^iOH saturated with NH_3, 24 h), 60–80% yields were obtained. They might be improved by the use of non-aqueous media for activation. The purity of the crude peptide-amides was very similar to that of the acids obtained by hydrolysis.§§

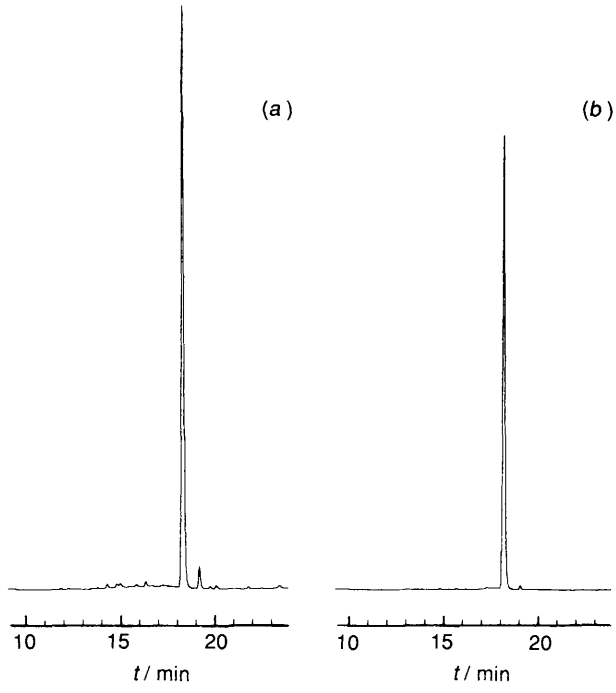

Fig. 1 HPLC profiles of crude (*a*) and purified (*b*) Boc-Leu-enkephalin. HPLC conditions: Brownlee Spheri-5 RP-18 column; buffer A, 0.1% aq. TFA; B, MeCN (0.06% TFA); linear gradient 15–90% B over 25 min; detection 220 nm. The impurity peak eluting at 19.2 min is the des-glycine Boc-tetrapeptide.

The high yields in peptides show the high resistance of the linker to acidic aqueous media. A similar stability was observed under the moderately to strongly acidic organic conditions involved in conventional Boc-chemistry. For example, the purity of Ac-Arg(NO_2)-Gly and its quantitative recovery upon hydrolysis were unchanged after a 15 h treatment of the starting peptide-resin by 50% TFA in CH_2Cl_2. A similar result was obtained with Ac-Trp-Pro-Gly using the stronger conditions of side-chain deprotection (1 mol dm^{-3} TFMSA-scavengers in TFA, 0 °C, 1.5 h). Lastly, protected Leu-enkephalin and LH–RH were successfully synthesized using 50% TFA in CH_2Cl_2 for Boc removal.¶¶

In summary, the Dpr(Phoc) linker is compatible with Boc-chemistry in aqueous or non-aqueous media. Easily synthesized, its insertion on the support is straightforward, as is the anchoring of the C-terminal residue by amide and not ester bond formation, ensuring high substitution, no racemization and no diketopiperazine formation at the dipeptide stage. Additionally, numerous applications are expected from the orthogonal scheme for side-chain deprotection and peptide cleavage, as for example fragment synthesis and release of peptides cleared of deprotection contaminants. Lastly, this type of linker, both highly stable but easily cleavable in aqueous media, could be helpful for certain biological uses of synthetic peptides. However, application to

** FAB-MS, *m/z* 656 (M + H^+), 678 (M + Na^+), 694 (M + K^+); amino acid ratios (6 mol dm^{-3} HCl hydrolysate): Gly 2.01, Leu 0.98, Tyr 0.99, Phe 0.94.

†† Side-chain protections used: mesitylene-2-sulfonyl (Arg), 2,6-dichlorobenzyl (Tyr), benzyl (Ser), and 2,4-dinitrophenyl (His); removal before the cleavage of the linker: (*i*) thiophenol (20 equiv.) in DMF, 1 h, (*ii*) 1 mol dm^{-3} TFMSA in TFA-*m*-cresol-thioanisole-ethanedithiol 10:1:1:1, 0 °C, 1 h.

‡‡ A higher yield of 90% was estimated by considering the alkaline decomposition of the chromophore, which could not be neglected in this case of slower hydrolysis.

§§ LH–RH was identified as the major component of the crude peptide–amide by comparison with an authentic sample by HPLC and HPCE. HPLC monitoring at various stages of the peptide elongation showed that most side-products resulted from the non-optimized synthesis protocol.

¶¶ However, quantitative couplings using the *in situ* neutralization procedure described above often required further addition of DIEA, probably because of an incomplete removal of TFA from the polyacrylamide resin by CH_2Cl_2 and DMF washes.

base-sensitive peptides may be difficult, as with any base-labile linkers. We are currently investigating related linkers (*i*) to carry out the cleavage under less basic conditions and (*ii*) to examine how this new kind of linker could be adapted to Fmoc-chemistry.

Received, 28th July 1993; Com. 3/04520J

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