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Calcium-Promoted Hydrolysis of *N*-Acylureas Allows Mild Release of Peptides Anchored with the Dpr(Phoc) Linker to Hydrophilic Resins

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Abstract: Calcium chloride is an efficient additive for promoting the release of short peptide models anchored with Dpr(Phoc) linker to hydrophilic solid-phase synthesis supports. It was shown to induce a moderate to marked (especially for *C*-terminal proline peptides) increase in the rate of alkaline hydrolytic cleavage, without epimerization at the *C*-terminal residue, while substantially reducing the hydroxide ion concentration. Base-promoted side-reactions are therefore expected to be slowed down.
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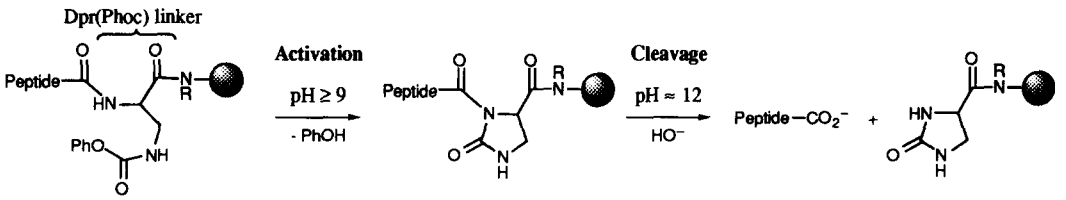
The safety-catch Dpr(Phoc) linker (Dpr = L-2,3-diaminopropionic acid, Phoc = phenyloxycarbonyl) suitable for Boc[1] and Fmoc solid-phase peptide synthesis,[2] is cleaved under aqueous alkaline conditions ([HO^-] = 0.01-0.05 M) which could make its application to base-sensitive peptides difficult. This limitation prompted us to undertake investigations aimed at finding a catalytic way that would allow a reduction in the hydroxide ion concentration. In the usual two-stage procedure, the second stage (*i.e.* cleavage) requires more alkaline conditions than the activation step and thus had to be the target of catalysis.



Described herein is the efficiency of calcium chloride to promote alkaline cleavage of the Dpr(Phoc) linker. Our approach was founded on mechanistic grounds. As for other *N*-acylureas,[3] alkaline hydrolysis of the *N*-acylimidazolidin-2-ones involved in Dpr(Phoc) linker cleavage must predominantly take place through a transition state bearing two negative charges. This led us to the hypothesis of electrostatic stabilization of the transition state by complexation with multivalent cations. To our knowledge, such catalysis has never been reported for *N*-acylurea hydrolysis, though derivatives of several metals, including calcium and magnesium, have been used as additives for related reactions[4] and for transesterification of peptide esters.[5]

We synthesized several models **1-4** (Table 1) consisting of peptidic material bound to polyacrylamide (Expansin) or polyethyleneglycol grafted on polystyrene (TentaGel) resins, which were selected as solid supports owing to their hydrophilic character.[6] Attachment of the Dpr(Phoc) linker to resins bearing a secondary (instead of primary) amino group precludes 6-membered ring formation which might compete with the desired cyclization during activation. Expansin was thus derivatized with piperazine[1] and TentaGel with

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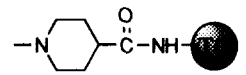
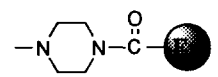
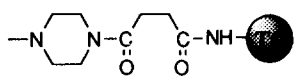
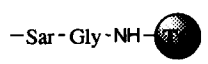
Table 1. Reactivity of Dpr(Phoc) Linker in Peptide-resins **1-4** at 20.0 °C.[a]

Entry	Peptide-resin :	Peptide model — Dpr(Phoc) — Derivatization linkage	Salt	$t_{1/2}$ Activation	$t_{1/2}$ Peptide release
				Solvent: H_2O	
1	**1a**	Bz-Gly-  TentaGel	no salt [b]	8 min	5 h
2			0.25 M $CaCl_2$ [c]	10 min	85 min
3			0.75 M KCl [d]	3 min	3.5 h
4			0.75 M KCl [e]	12 min	12.5 h
				Solvent: *i*PrOH-H_2O 7:3	
5	"	"	"	no salt	< 2 min	17 min
6				0.25 M $CaCl_2$	11 min	25 min
7	**1b**	Bz-Leu-Phe-	"	no salt	1 min	1.1 h
8				0.25 M $CaCl_2$	20 min	35 min
9	**1c**	Bz-Val-	"	no salt	2 min	23 h
10				0.25 M $CaCl_2$	18 min	8 h
11	**1d**	Bz-Pro-	"	no salt	1.5 min	24 h
12				0.25 M $CaCl_2$	15 min	3 h
13				0.75 M LiCl	5 min	10 h
14	**2**	"  Expansin	no salt	1 min	> 24 h
15			0.1 M $CaCl_2$	n.d.	2.5 h
16			0.25 M $CaCl_2$	8.5 min	85 min
17			0.5 M $CaCl_2$	n.d.	50 min
18	**3**	" 	0.25 M $CaCl_2$	9 min	110 min
19	**4**	" 	0.25 M $CaCl_2$	9 min	85 min

[a] unless otherwise specified, 4 x 10^{-3} M total hydroxide was introduced in the medium, that includes precipitated and incompletely dissociated $Ca(OH)_2$. General procedure, unless otherwise specified: the resin (10-25 mg in order to release *ca* 5 x 10^{-6} mol of peptide) was allowed to swell in solvent or in salt solution (4.8 cm^3) for 30 min and the reaction was initiated by the addition of 0.1 N NaOH (0.2 cm^3). At 0.25 M $CaCl_2$, homogeneous solutions are obtained in water, but precipitation occurs in *i*PrOH-H_2O 7:3 (filtrate titration showed that the concentration of soluble hydroxides is 2.5 x 10^{-3} M); [b] pH = 11.58; [c] pH = 11.25; [d] pH = 11.65; [e] pH = 11.25, obtained by adding 0.2 cm^3 0.1 N NaOH to 12.3 cm^3 KCl solution.

isonipecotic acid, succinylpiperazine or the dipeptide Sar-Gly. This derivatization, as well as the introduction of Dpr(Phoc) and peptide elongation, were carried out using Boc-chemistry.[7]

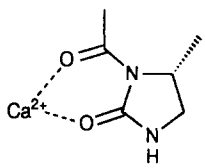
Peptide-resins were treated with diluted NaOH, in water or *i*PrOH-H_2O 7:3, and the effect of different salts ($CaCl_2$, $MgCl_2$, KCl, LiCl) on the consecutive activation and cleavage reactions was studied (Table 1). These reactions were monitored by sampling, then HPLC analysis of phenol and the peptide model, respectively. On completion, no yield decrease was observed in the presence of salts.[8] In water, the effect of $CaCl_2$ was determined on the Bz-Gly model **1a**. A 3.5-fold increase in the cleavage rate was observed at 0.25 M $CaCl_2$ (entries 1 and 2). However, $CaCl_2$ alters the pH (because of incomplete dissociation of $Ca(OH)_2$) and the ionic strength. A better estimate of the effect of Ca^{2+} ions is therefore obtained by comparison of entry 2 with entry 4, which describes an assay performed under comparable experimental conditions (same ionic strength and initial pH), leading to a *ca.* 9-fold rate increase.[9]

Since higher rates were obtained in *i*PrOH-H_2O 7:3 (entries 5 and 6), the study was carried on with this solvent on peptide resins **1a-d** which bear various *C*-terminal residues covering a wide reactivity range. The effect of $CaCl_2$ addition on activation and cleavage steps resembles that observed in water. However, the *ca.* 10-fold reduction in the activation rates at 0.25 M $CaCl_2$ indicates a marked decrease in [HO^-] due to precipitation of $Ca(OH)_2$ and probably to its weaker dissociation than in water. As a result, the activation step can significantly limit the rate of release for peptides that have a rapid Ca^{2+}-mediated cleavage step, as illustrated by Bz-Gly and Bz-Leu-Phe models. The most marked calcium effect on peptide release is displayed by the Bz-Pro model. In addition, the absence of epimerization at the *C*-terminal residue, previously noticed with the Dpr(Phoc) linker alone, and its preservation with the additive are clearly demonstrated with resin **1b** since the diastereomer Bz-Leu-D-Phe was not detected in the hydrolysates (< 0.3% by HPLC).[10]

The influence of the support on the rates was studied using Bz-Pro models. The two first peptide-resins prepared from TentaGel (**1d** and **3**) do not exhibit as good a reactivity as obtained with the model derived from Expansin (**2**), even when the derivatization linkage contains the piperazine moiety of resin **2** (entry 18). This is probably due to the greater hydrophily of the polyacrylamide matrix, as supported by the recovery of the best reactivity with Tentagel derivatized with the most hydrophilic moiety (entry 19).

The results of the experiments carried out with K^+ (entries 3 and 4) and Li^+ (entry 13) salts show the specificity of the reaction with Ca^{2+} ions. The extent of the rate increases obtained with the latter rules out a mere ionic strength effect and strongly supports catalysis. A very slow reaction (< 2% cleavage in 24 h) was observed with 0.25 M $MgCl_2$ due to nearly complete precipitation of $Mg(OH)_2$.

Amide carbonyl groups can bind calcium in favourable structures, as observed in proteins.[11] Here, the interactions might involve two carbonyl groups or more in the sequence of amide or peptide bonds present in peptide-resins near the cleavage site.[12] One possibility could be chelation by the imide moiety (scheme). This requires the *cis*-conformation which was found to be the less stable in a closely related *N*-acylimidazolidin-2-one structure because of dipole-dipole repulsion of the two carbonyl groups.[13] But the *cis-trans* equilibrium would probably be shifted upon chelation, as supported by the similar chelating properties of chiral *N*-acylimidazolidin-2-ones, which have been proposed to explain the diastereoselectivity of reactions involving these compounds.[14] Anyway, association at an early stage is not a prerequesite for catalysis, and thus Ca^{2+} catalysis may be simply explained, as proposed in our initial hypothesis, by Ca^{2+} coordination at the transition state with atoms bearing the negative charges and perhaps with neighbouring carbonyl groups.



This Ca^{2+} catalysis must be applicable to longer peptides since it accelerated release of the decapeptide Tyr-Asp-Pro-Ala-(Pro)$_6$ from Expansin.[2] About 90% cleavage within 3.5 h was obtained under the same experimental conditions as those of entry 16. This is consistent with the reactivity of Bz-Pro resin **2**, showing, in this case, no significant influence of peptide length on the Ca^{2+} effect. Moreover, under preparative conditions[2] which require more hydroxide for more peptide to be released, we noted (as expected) higher $Ca(OH)_2$ precipitation upon preparation of the cleavage medium (0.51 cm^3 1 N NaOH added to 5 cm^3 0.6 M $CaCl_2$ in iPrOH-H_2O 7:3). The fact that the cleavage rate was very close to that given in entry 17 shows that

[HO^-] was probably limited to the same low level by precipitation and incomplete dissociation of $Ca(OH)_2$.[15] Such limitation is of interest because of its expected slowing down effect on base-promoted side-reactions.

In conclusion, this work should lead to new procedures for the mild release of peptides and other carboxylic compounds synthesized on a solid support with the Dpr(Phoc) linker or its cyclic urea derivative. At this time it can be predicted that, compared to the uncatalyzed method, the Ca^{2+}-mediated release of peptides will enable a shortening of reaction times for usually difficult cleavages (e.g. hindered *C*-terminus) and, in all cases, an advantageous reduction in the hydroxide ion concentration ([HO^-] < 4 x 10^{-3} M). Such process may also find useful applications in other fields of chemistry, such as the removal of imidazolidin-2-one[13,14] and closely related chiral auxiliaries[16] from *N*-acyl derivatives in stereoselective synthesis.

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6. Expansin resin was a generous gift from Expansia, F-30390 Aramon, France; TentaGel S NH_2 was purchased from Rapp Polymere, D-7400 Tubingen, Germany.
7. *N*-Boc-isonipecotic acid is commercially available from Bachem. *N*-Boc-*N'*-(3-carboxypropionyl)piperazine was synthesized by reaction of succinic anhydride with piperazine (1.5 equiv) in dioxane-water in the presence of Na_2CO_3 (3 equiv), separation of the monosubstituted derivative on cation exchange resin, and protection with $(Boc)_2O$ in the presence of aq. NaOH; overall yield 56%; m.p. 125-126°C; FAB-MS *m/z* 287 ($M+H^+$); ^1H NMR [250 MHz, $CDCl_3$)] δ 1.47 (s, 9H), 2.69 (m, 4H), 3.4-3.65 (m, 8H), 9.6 (s, 1H).
   Peptide elongation: (i) Boc removal: trifluoroacetic acid (TFA)-CH_2Cl_2 3:7 (30 min) followed by CH_2Cl_2, MeOH, and DMF washes for TentaGel resin, or 6 N HCl (60 min) followed by H_2O and DMF washes for Expansin resin; (ii) 1 h coupling using hydroxybenzotriazole (HOBt) esters (7 min preactivation) preformed from Boc-AA (3.3 equiv), 2-(2H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (3 equiv), HOBt (3 equiv), and diisopropylethylamine (4.5 equiv); DMF washes followed by CH_2Cl_2 or H_2O washes.
8. Except for the incomplete reaction with $MgCl_2$, no decrease in peptide yield was observed compared to the cleavage without salt which is nearly quantitative as previously established (ref. 1). For example, in the case of Bz-Leu-Phe synthesis, Boc-Dpr(Phoc) Tentagel resin (0.207 mmol/g determined by alkaline treatment followed by HPLC analysis of PhOH) gave resin **1b** (0.181 mmol Dpr(Phoc)/g determined as above, theoretically 0.196 mmol/g). Calcium-promoted cleavage of **1b** under the conditions of Table 1 (entry 8) was nearly quantitative as shown by HPLC analysis of the hydrolysate indicating a 0.174 mmol dipeptide release per g of resin **1b**. HPLC analysis: Brownlee Spheri-5 RP-18 column; buffer A, 0.1% aq. TFA; B, MeCN (0,06% TFA); linear gradient 30-80% B over 25 min; detection 220 nm; t_R 5.7 min PhOH; t_R 14.1 min Bz-Leu-Phe; PhOH and Bz-Leu-Phe determination was performed after calibration with standard solutions of pure materials.
9. The small experimental effect on the activation step (entries 1 and 2) is mostly accounted for by the two opposite effects of ionic strength and pH, as shown by comparison of entries 1 with 3, and 3 with 4, respectively.
10. See note 8 for HPLC conditions; no peak detected at t_R 14.5 min for Bz-L-Leu-D-Phe [assumed to have the same retention time as the enantiomer Bz- D-Leu-L-Phe observed in the diastereomeric mixture that we intentionally prepared by coupling Bz-L-Leu-OSu with L-Phe (suspension in DMF) in the presence of excess DIEA].
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12. Binding affinity would be rather weak since no levelling off of the rate is observed up to 0.5 M $CaCl_2$ (entry 17).
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15. Under preparative conditions, rates of peptide release are therefore expected to be close to that given in Table 1. In addition, solid and undissociated calcium hydroxides supply the stoichiometric amount of HO^- ions required for complete cleavage as fast as they are used by peptide release.
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