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A New Safety-Catch Peptide-Resin Linkage for the Direct Release of Peptides into Aqueous Buffers

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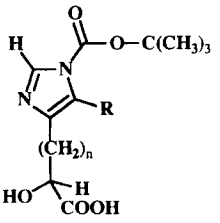
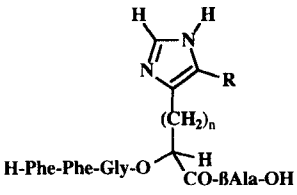
Abstract : The preparation and application of a new safety-catch peptide-resin linkage orthogonal to Fmoc/tBu SPPS[1] is reported. This linkage allows the fast cleavage of non-modified peptide acids under neutral conditions directly into aqueous buffers exploiting the hydrolysis of an ester bond by intramolecular catalysis.

The concept of an orthogonal peptide-resin linkage allows the final deprotection and removal of contaminating chemicals while the peptide remains attached to the support which then can be followed by the direct release into an aqueous buffer of choice. Thus, it has some very attractive features compared to conventional one-step deprotection strategies. It greatly facilitates the small scale simultaneous preparation and biological-activity screening of a plurality of individual peptides. Furthermore, it can improve the preparation of multicomponent peptide mixtures (libraries) by avoiding the risk of losing individual components during work-up and purification, because of their very different physicochemical properties. Following this concept, Bray et al.[2,3] reported linkages for the direct release of C-terminally modified peptides and peptide amides using the well known diketopiperazine formation for the former and the gas phase cleavage of an ester bond with ammonia vapour in the latter. These elegant methods stimulated us to design a corresponding linkage for the solid phase synthesis of non-modified peptide acids.

The linker we have developed is based on the well known glycolic acid anchor[4] from which the peptide acid has to be cleaved by treatment with 10 mM NaOH (pH 12). Our idea was the incorporation of a basic group which will enhance the hydrolysis of the ester bond by intramolecular catalysis.



(1/2)



(3/4)

H-Phe-Phe-Gly-O-CH_2-CO-ßAla-OH

(5)

Figure 1 *: Structures of the linkage reagents and the model compounds described in the text. (1) and (3) : R = CH_3 , n = 0; (2) and (4) : R = H, n = 1.*

This was realized by substitution with a series of imidazole side chain groups (Fig. 1). During peptide assembly the catalytic effect of the base must be 'turned off' by a linker protecting group, which reduces the basicity of the imidazole ring. To fit this additional group into the overall N^{α}-Fmoc/tBu-protection scheme, we have selected here the Boc-group for histidine protection. The final acidic deprotection of the peptide simultaneously removes Boc from the linker and 'turns on' the catalytic effect, which is still blocked however under acidic work-up by the protonation of the imidazole ring.

Linker **1** was synthesized starting from the corresponding imidazolecarbaldehyde via a cyanhydrine reaction[5] followed by the introduction of the Boc-group with tert-butoxycarbonylazide or pyrocarbonicacid-tert-butylester[6]. Linker **2** was obtained directly from the commercially available 3-imidazol-4-yl-2-hydroxy-propanoic acid[6]. To study the cleavage of peptides from the linkage in solution, linker compounds **1** and **2**, as well as glycolic acid for reference, were coupled to ß-alanine loaded p-benzyloxybenzyl-polystyrene ('Wang-Resin'). The resulting resins were loaded with Fmoc-glycine by MSNT/MeIm activation[7] and the tripeptide sequence H-Phe-Phe-Gly was assembled by standard procedures using DIC/HOBt in DMF for coupling and 20% piperidine in DMF for Fmoc cleavage[8]. Quantitative monitoring of the dibenzofulvene-piperidine adduct during peptide assembly showed that the assembly of the peptide sequence proceeded almost quantitatively. Thus, any premature hydrolysis of the ester bond to the linker during peptide synthesis is also prevented by BOC-protection of the imidazole ring. Deprotection with TFA/DCM (1:1) containing 2.5% triisobutylsilane and 2% water led to compounds **3**, **4** and **5** (Fig. 1). These compounds were stable in acidic media such as 50% TFA in DCM for several months. This proves that the ester bond to the deprotected linkage is sufficiently stable under work-up conditions.

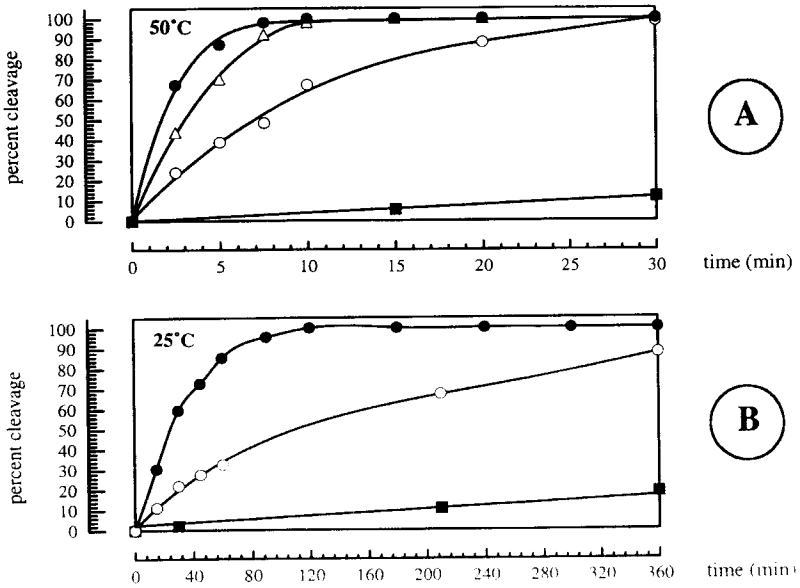


Figure 2 : *Kinetics of peptide release from various linkers in solution. A : (•) compound (3) in 0.01 M KH_2PO_4/Na_2HPO_4 (pH 7.5), (△) compound (3) in 0.01 M KH_2PO_4/Na_2HPO_4 (pH 7.0), (○) compound (4) in 0.01 M TEAAc (pH 7.3), (■) compound (5) in 0.01 M KH_2PO_4/Na_2HPO_4 (pH 7.5) at 50°C; B : (•) compound (3), (○) compound (4) and (■) compound (5) in 0.01 M KH_2PO_4/Na_2HPO_4 (pH 7.5) at 25°C.*

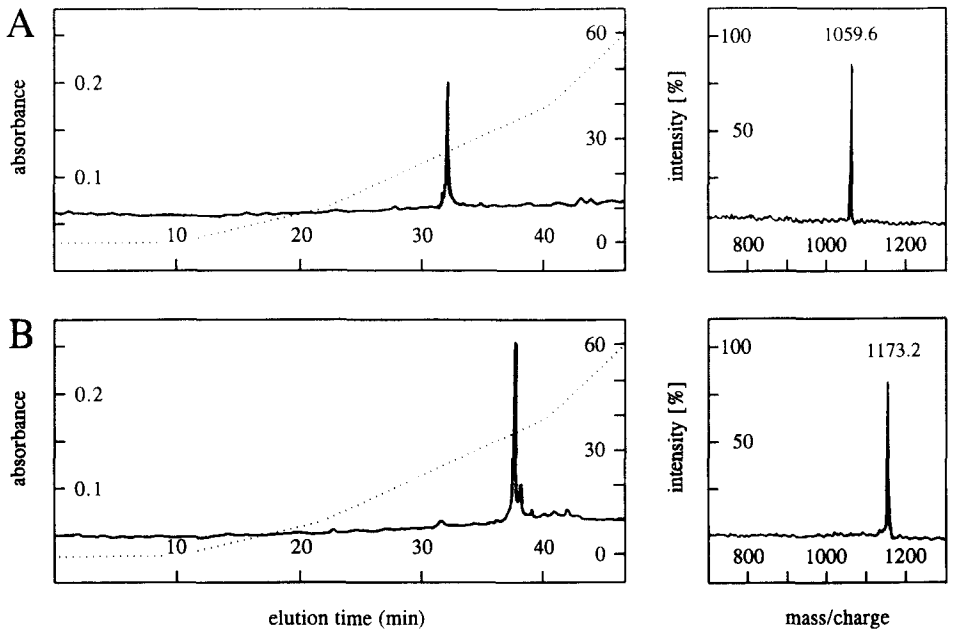


Figure 3 : *RP-C_{18}-HPLC-profiles and MALDI-TOF mass spectra of crude peptides H-GYKDGNEYI-OH (A) and H-DRWIIGLNKG-OH (B) [see also Tab.1] synthesized on TentaGel S Amine® resin derivatized with **1**; HPLC: eluent (H_2O/CH_3CN (0.1 % TFA)); MALDI-TOF (matrix : sinapinic acid).*

On changing the pH to neutral or near neutral, the desired tripeptide H-Phe-Phe-Gly-OH was liberated at a much higher hydrolysis rate than that of the unsubstituted glycolic acid linker (Fig. 2). The reaction rate depends on the pH as well as on the concentration and type of buffer used. In the best case, the reaction is complete after 5-7 min at 50°C in 0.01 M phosphate-buffer at pH 7.5 for the anchor **1**. This linkage also allows the cleavage of peptides into the volatile 0.01 M triethylammonium acetate buffer (pH 7.3). The reaction rate is much smaller (complete after 12 h at 25°C for compound **3**) than with other buffers, but the peptide acids can be obtained free of salt by lyophilisation.

Table 1 : *Characterization of crude peptides synthesized on TentaGel S Amine derivatisized with linkage **1**.*

Peptide No.[a)]	Sequence[b)]	Molecular Mass		Quantity[c)]	Yield
		(calc. [M])	(obt.,$[M+H]^{\oplus}$)	[nmol]	[%]
A	H-GYKDGNEYI-OH	1058.4	1060	750	63
B	H-DRWIIGLNKG-OH	1171.8	1173	390	33
C	H-TEGPGVRYPL-OH	1088.6	1089	900	75
D	H-VYAKECTGLK-OH	1111.6	1113	960	80

a) RP-C_{18}-HPLC and MALDI-TOF-spectra of peptide A and B are shown in Fig. 3; b) synthesized with Fmoc-Cys(Trt)-OH and Fmoc-Trp(Boc)-OH using TBTU/N-methylmorpholine activation (scale 1.2 µmol based on first amino acid loading); c) determind by total hydrolysis and quantitative amino acid analysis.

Thus, the designed intramolecular catalysis of the ester cleavage indeed efficiently takes place, presumably *via* correct coordination of a water molecule by the π-nitrogen of the imidazole moiety or the intermediate formation of a peptidyl-imidazolide, which is immediatly hydrolysed in the aquaeous media to the free peptide acid. These assumed mechanisms might explain the remarkable difference between the hydrolytic stability of **3** and **4**.

In order to exemplify the application of the new linkage reagent in multiple peptide synthesis, several peptides were synthesized on TentaGel S Amine® resin using the multiple peptide synthesizer AMS 422 (ABIMED-Analysentechnik/Langenfeld-Germany). Linker **1** was directly coupled to the amino function of the support and the C-terminal amino acid was esterified to the linkage with MSNT/MeIm in DCM[7]. After the final acidic deprotection and successive extractions (each 3 x 10 min) with MeOH/H_2O (1:1) containing 0.1% HCl and 1 M acetic acid, cleavage in 0.01M phosphate buffer (pH 7.5) at 50°C for 25 min gave the desired peptide acids shown in Tab. 1 and Fig. 3.

References and Notes

1. Special abbreviation used : SPPS : Solid Phase Peptide Synthesis; Fmoc : 9-fluorenylmethoxycarbonyl-; Boc : tert-butoxycarbonyl-; TFA : trifluoroacetic acid; DCM : dichloromethane, MSNT : mesitylenesulfonyl-3-nitro-1.2.4-triazole; MeIm : N-methylimidazole.

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5. Synthetic procedure : 18.25 g (0.17 mol) 4-methyl-5-1H-imidazolylcarbaldehyde was dissolved in 80 ml water and 23.65 g (0.36 mol) potassium cyanide, 30 ml conc. hydrochloric acid and 66 ml acetic acid anhydride were added at 0°C. The mixture was stirred for 1 h at 0°C. After additional 24 h at RT 15 ml conc. hydrochloric acid and 66 ml acetic acid anhydride were added and the mixture were stirred for 24 h at RT. After addition of 250 ml water and 250 ml conc. hydrochloric acid the mixture was stirred under reflux for 2 h. 250 ml hydrochloric acid was added and the mixture was stirred for additional 12 h at 80°C. Crystalisation from water/actone yields 2-[1H-5-methyl-imidazol-4-yl]-2-hydroxyacetic acid in 42%.- m.p. 175°C.- ^{1}H-NMR (400 MHz, D_2O) : δ = 8.57 (s, 1H, 2-H), 5.59 (s, 1H, C<u>H</u>OH), 2.33 (s, 3H, CH_3).- ^{13}C-NMR (75 MHz, D_2O) : δ = 174.0 (s, COOH), 135.2 (d, C-2), 132.3 (s, C-5), 119.9 (d, C-4), 65.5 (d, <u>C</u>HOH), 9.5 (q, CH_3).- MS (FAB) : m/z (3-NBA) = 157 ($[M+H]^{\oplus}$).

6. Synthetic procedure for **1** and **2** : 0.025 mmol 2-[5-methyl-imidazol-4-yl]-2-hydroxyacetic acid or 3-imidazol-4-yl-2-hydroxy-propanoic acid (obtained from SIGMA Chemical Co.) were suspended in DMF at 4°C and 1.1 eq. tert-butoxycarbonyl azide (CAUTION : explosive) or pyrocarbonic acid tert-butyl-ester and 3 eq. triethylamine were added under stirring. After additional 2d at 4°C the mixture was evaporated to dryness, suspended in dioxane, filtered off and lyophilized from dioxane. Both linkage reagents were obtained as triethylammonium salts.- **1** : yield 67 % (white solid).- ^{1}H-NMR (400 MHz, $CDCl_3$) : δ = 8.15 (s, 1H, 2-H), 5.14 (s, 1H, C<u>H</u>OH), 2.46 (s, 3H, CH_3), 1.58 (s, 9H, CH_3).-^{13}C-NMR (75 MHz, $CDCl_3$) : δ = 174.6 (s, COOH), 147.1 (s, N-COO-), 137.0 (d, C-2), 136.2 (s, C-5), 127.2 (s, C-4), 86.4 (s, <u>C</u>$(CH_3)_3$), 66.5 (d, CHOH), 27.8 (q,CH_3).- MS (FAB) : m/z (3-NBA) = 279 ($[M+Na]^{\oplus}$), 257 ($[M+H]^{\oplus}$), 201 ($[M-57+H]^{\oplus}$, tBu).- **2** : yield 87 % (white solid).- ^{1}H-NMR (300 MHz, $CDCl_3$) : δ = 8.15 (d, 1H, 2-H, $^4J_{H,H}$= 1.25 Hz), 7.27 (s, 1H, 4-H), 4.39 (t, 1H, C<u>H</u>OH, $^3J_{H,H}$=4.5 Hz), 3.14 (AB-dd, 1H, -CH_2-, $^2J_{H,H}$=14.8 Hz, $^3J_{H,H}$=4.0 Hz), 3.02 (AB-dd, 1H, -CH_2-, $^2J_{H,H}$=14.8 Hz, $^3J_{H,H}$=5.0 Hz), 1.57 (s, 9H, CH_3).- ^{13}C-NMR (75 MHz, $CDCl_3$) : δ = 176.1 (s,COOH), 146.3 (s, N-COO), 136.8 (d, C-2), 135.9 (s, C-5), 116.1 (d, C-4), 86.4 (s, <u>C</u>$(CH_3)_3$), 68.5 (d, CHOH), 67.0 (t, -CH_2-), 27.8 (q, CH_3).- MS (FAB, DCHA-derivative) : m/z (3-NBA) = 295 ($[M+K]^{\oplus}$), 279 ($[M+Na]^{\oplus}$), 257 ($[M+H]^{\oplus}$), 201 ($[M-57]^{\oplus}$).

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