A pH cleavable linker for zone diffusion assays and single bead solution screens in combinatorial chemistry

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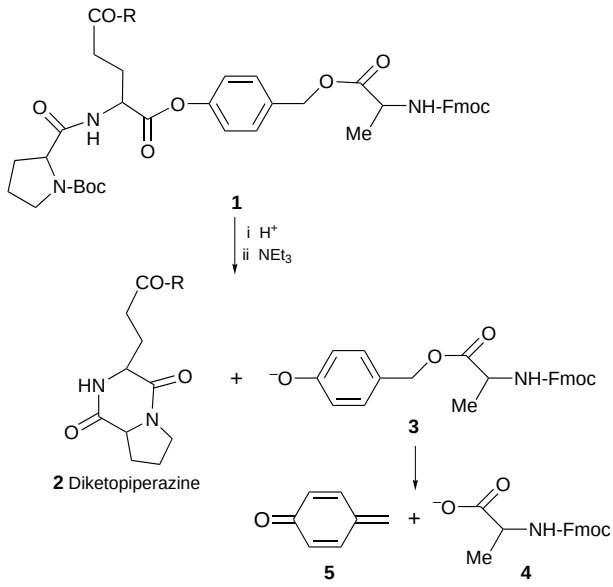
A safety catch linker has been developed that allows efficient compound release into buffered aqueous solutions and agarose gels.

Linkers, acting as the selectively cleavable bridge between the solid support and the compound of interest, play a pivotal role in solid phase, combinatorial and peptide chemistry. This role is fundamental, not only in dictating the range of chemistry which can be carried out, but also in determining the particular screening approach to the library in question.[1] At the present time there are a limited number of linkers available that are 'biocompatible' in terms of linkers being directly cleavable within the assay. The best known examples are light cleavable linkers;[2] the internal imidazole catalysed, ester cleavable linkers of Frank[3] and the iminodiacetic acid based linkers of Lebl.[4] All of these have a number of attractive features but also limitations for screening.

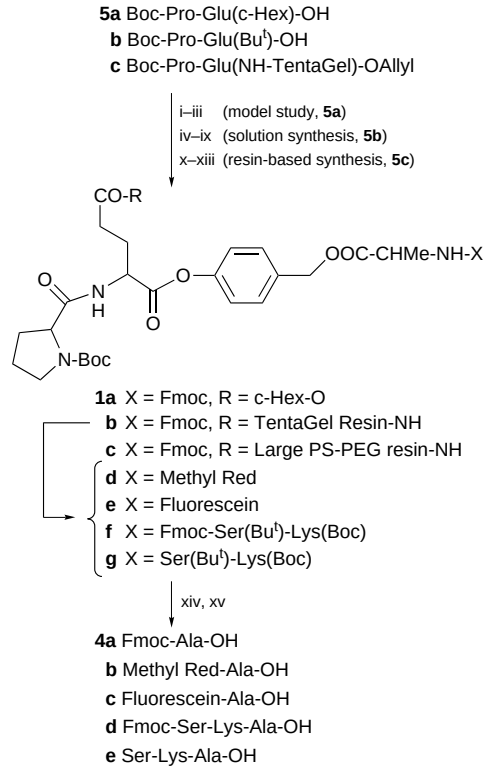
We now report the development of a highly efficient linker which cleaves at pH 8, following acidic (safety catch) activation. The activated linker rapidly cleaves in buffered solution and within agarose gel within a time frame that allows the gel to set before compound release but fast enough to allow complete cleavage within a few hours. The key mechanistic features of the linker **1** are diketopiperazine **2** formation, taking place at pH 7–8, which releases a derivatised hydroxymethylphenol(ate) **3**. This then undergoes a facile 1,6-elimination process to give the product **4** and the quinone methide by-product **5** (see Scheme 1). The process was initially investigated in solution using **1a** (R = cyclohexylO) which was synthesised as shown in Scheme 2. Cleavage was monitored by TLC following removal of the Boc group and treatment with NEt_3, and resulted in rapid cleavage (< 1 h) of the linker and quantitative release of Fmoc-Ala-OH.



Scheme 1

Having ascertained the practicalities of cleavage the first resin-based experiments were initiated by the synthesis of **1b** and by its simple derivatisation to give **1d**–**g**† to look at the ensuing release of **4a**–**e**. The linkers were initially synthesised in solution and attached to the resin prior to derivatisation since in our view quantification of linker efficiency/kinetics is most reliable with homogeneous material coupled to the solid support. Subsequently the solution synthesis was replaced by an efficient resin based synthesis as shown in Scheme 2.

Cleavage from the resin was followed by UV analysis in real time, RP-HPLC [with samples being removed and quenched (0.1% TFA) before injection] and quantification using internal standards. Although TentaGel is compatible with most solvents and hence a wide range of compounds, we were interested in the variation of cleavage rate with a number of different compounds. Compounds released in this study included Fmoc-Ala-OH, Methyl Red-Ala-OH, Fluorescein-Ala-OH and two tripeptides Fmoc-Ser-Lys-Ala-OH and H-Ser-Lys-Ala-OH. The



Scheme 2 *Reagents and conditions*: i, 4-HOC_6H_4CHO, DCC, 98%; ii, $NaBH_3CN$, 65%; iii, Fmoc-Ala, DCC, DMAP, 68%; iv, 4-HOC_6H_4CHO, DCC, 90%; v, $NaBH_3CN$, 80%; vi, Fmoc-Ala, DCC, DMAP, 79%; vii, CF_3CO_2H–CH_2Cl_2 (1 : 1), 100%; viii, Boc_2O, 60%; ix, TentaGel Resin-S NH_2 or 'big' PS-PEG-NH_2 beads, DIC, HOBt; x, $Pd(PPh_3)_4$, dimedone; xi, 4-HOC_6H_4CHO, DCC; xii, $NaBH_3CN$; xiii, Fmoc-Ala, DCC, DMAP; xiv, CF_3CO_2H–CH_2Cl_2 (1 : 1); xv, K_2HPO_4 (50 mM, pH 8)

resins used were TentaGel-S-NH_2 and aminomethyl polystyrene beads (150–300 μm, 2 mmol g^{-1}) made water-compatible by the method of Hudson.[5] Cleavage results are shown in Fig. 1.

The half-lives of cleavage from TentaGel-S-NH_2 in 50 mM phosphate buffer (pH 8.0) ranged from 10–75 min depending on the compound being cleaved with yields greater than 70% in all cases.‡ As with photocleavable linkers[6] we observed that hydrophobic compounds were released much more slowly into the aqueous assay solution than hydrophilic, a factor which must have implications for single bead library screening. The intermediate **3** was identified by HPLC and ES-MS in the cleavage of **1b**. The large water compatible beads **1c** were studied for the release of Fmoc-Ala-OH. The half-life for cleavage in this case was 800 min, which compares with 75 min for Fmoc-Ala-OH being released from TentaGel resin. Clearly the difference illustrates the effect of bead size on the diffusion of materials into and out of the large beads (these beads swell to about 600–700 μm in water), but also we believe it is associated with the smaller PEG chains on our larger beads compared to those found on TentaGel.

Beads **1b**,**e** were used in a zone diffusion type assay. The Boc safety catch was removed and the beads added to molten agarose (45 °C) and poured into petri dishes. Fluorescent zones were clearly visible around beads **1e** after 2–3 h in the agarose gel. Beads **1b** were left in the gel for 12 h, following which 5% TFA–H_2O was added, the gel melted and the beads removed and thoroughly washed. Fmoc analysis indicated that 70% of the material had been cleaved from the resin. Since the half-life of cleavage for **1b** in solution is 75 min this experiment demonstrates that materials are cleaved into the agarose gel with relatively good efficiency, although as expected more slowly than into free solution.

In summary, we have developed what we believe is a practical example of a "pH" labile linker for solid phase and combinatorial chemistry. Importantly we have shown that the linker is stable to the base treatments required during Fmoc peptide chemistry and that it is efficiently cleaved in buffered aqueous solutions following synthesis. The one negative side is the quinone methine that may need quenching in the assay. We believe that linkers of this type have huge potential in solid phase chemistry for the screening of compound libraries using single bead solution screens and zone diffusion assays.

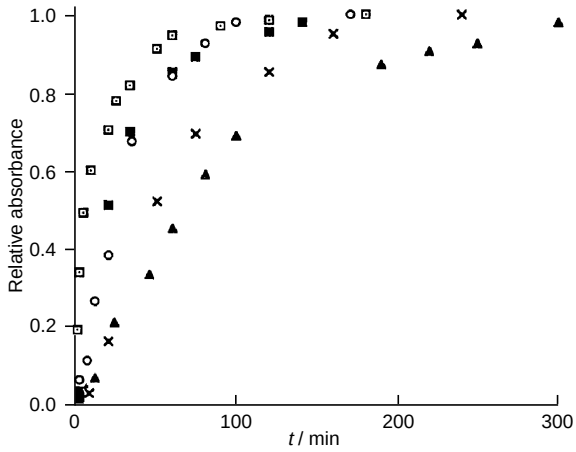


Fig. 1 Kinetics of compound release from **1b**,**d**–**g**: (x) Methyl Red-Ala, (▲) Fmoc-Ala, (■) Fluor-Ala, (○) Fmoc-Ser-Lys-Ala, (⊡) Ser-Lys-Ala

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Footnotes

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† *Selected data* for Boc-Pro-Glu(OH)OPhCH_2OAla-Fmoc (*E*/*Z* forms exist about the Boc-Pro urethane): δ_H(300 MHz, CD_3OD) 1.38–1.51 (12 H, m, *Boc*-Pro and C*H*$_3$CH), 1.65–2.06 and 2.08–2.45 [6 H, 4 br m, Pro-CH_2 (β and γ) and Glu-CH_2 (β)], 2.48–2.63 [2 H, m, Glu-CH_2 (γ)], 3.37–3.48 and 3.49–3.64 [2 H, 2 br m, Pro-CH_2 (δ)], 4.15–4.20 (1 H, t, *J* 7, FmocCH), 4.26–4.41 [2 H, 2 m, Ala and Pro CH (α)], 4.30 (2 H, d, *J* 7, FmocCH_2), 4.68 [1 H, m, Glu-CH (α)], 5.16 (2 H, ABq, *J* 7, ArCH_2), 7.07 (2 H, d, *J* 8, Ar), 7.30 (2 H, t, *J* 8, Fmoc), 7.39 (2 H, d, *J* 8, Ar), 7.40 (2 H, t, *J* 7, Fmoc), 7.66 (2 H, d, *J* 7, Fmoc), 7.79 (2 H, d, *J* 7, Fmoc); δ_C(75 MHz, CD_3OD) 17.45 (*C*H_3-Ala), 24.46 [Pro-*C*H_2 (γ)], 27.24 [Glu-*C*H_2 (β)], 28.63 (Me_3), 31.07 [Glu-*C*H_2 (γ)], 32.47 [Pro-*C*H_2 (β)], 47.86 [Pro-*C*H_2 (δ)], 48.23 [Ala-*C*H (α)], 51.10 (Fmoc-*C*H), 53.38 [Glu-*C*H (α)], 61.52 [Pro-*C*H (α)], 67.05 (*C*H_2Ar), 67.95 (Fmoc-*C*H_2), 81.53 (*C*Me_3), 120.90, 122.63, 126.16, 128.12, 128.74, 130.30 (ArCH), 135.16, 142.48, 145.14, 151.84, (ArC), 156.0, 158.33 (urethanes), 171.46, 171.62, 174.34 (esters and amide), 176.05 (acid); TLC R_f 0.3 (49 : 1 EtOAc–AcOH); mp 102–104 °C; ES-MS (+ve): *m*/*z* 766.3 $(M + Na)^+$, 781.4 $(M + K)^+$; $\nu_{max}(CHCl_3)/cm^{-1}$ 3424, 3351, 2979, 1725, 1681, 1508; HRMS: expected for $C_{40}H_{46}O_{11}N_3$, 744.3132. Found, 744.3166. Compounds **1d**,**f**,**g** were synthesised from **1b** in a conventional manner using DIC–HOBt couplings and 20% piperidine–DMF deprotections. Compound **1e** was prepared using Fluorescein isocyanate.

‡ Based on the initial dry weight substitution of TentaGel. We have found it very hard to return TentaGel to its free flowing form after solvation, thus yields probably represent a minimum value. A control cleavage in which the safety catch had not been removed showed no compound release after 4 h.

References

1 M. A. Gallop, R. W. Barrett, W. J. Dower, S. P. A. Fodor and E. M. Gordon, *J. Med. Chem.*, 1994, **37**, 1233; N. K. Terrett, M. Gardner, D. W. Gordon, R. J. Kobylecki and J. Steele, *Tetrahedron*, 1995, **51**, 8135; M. Rinnova and M. Lebl, *Collect. Czech. Chem. Commun.*, 1996, **61**, 171.

2 J. J. Burbaum, M. H. Ohlmeyer, J. C. Reader, I. Henderson, L. W. Dillard, G. Li Troy, T. L. Randle, N. H. Sigal, D. Chelsky and J. J. Baldwin, *Proc. Natl. Acad. Sci. USA*, 1995, **91**, 6027; J. J. Baldwin, J. J. Burbaum, I. Henderson and M. H. Ohlmeyer, *J. Am. Chem. Soc.*, 1995, **117**, 5588; C. P. Holmes and D. G. Jones, *J. Org. Chem.*, 1995, **60**, 2318.

3 S. Hoffmann and R. Frank, *Tetrahedron Lett.*, 1994, **35**, 7763.

4 M. Lebl, M. Patek, P. Kocis, V. Krchnak, V. J. Hruby, S. E. Salmon and K. S. Lam, *Int. J. Pept. Protein Res.*, 1993, **41**, 201; S. E. Salmon, K. S. Lam, M. Lebl, A. Kandola, P. S. Khattri, S. Wade, M. Patek, P. Kocis, V. Krchnak, D. Thorpe and S. Felder, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 11 708; P. Kocis, V. Krchnak and M. Lebl, *Tetrahedron Lett.*, 1993, **34**, 7251.

5 R. M. Cook, J. H. Adams and D. Hudson, *Tetrahedron Lett.*, 1994, **35**, 6777.

6 N. Bouloc, M. Phil Thesis, *Diffusion Zone Assays for Bio-Active libraries—Light Mediated Compound Release*, University of Southampton, 1996.

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