New methoxy-substituted 9-phenylxanthen-9-ylamine linkers for the solid phase synthesis of protected peptide amides

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New methoxy-substituted 9-phenylxanthen-9-ylamine linkers are described for the solid phase synthesis of peptide amides *via* the Fmoc strategy; depending on the cleavage conditions, the peptide amides can be cleaved rapidly and in high purity in their protected or deprotected forms.

Efficient stepwise solid-phase syntheses of C-terminal peptide amides yielding products of high purity are of interest for several reasons: (i) a considerable number of hormones, including secretin, calcitonin, oxytocin, thyroliberin and luliberin, bear a C-terminal acid amide function, (ii) several peptidyl amides, such as indolicin or protegrins, have potent antimicrobial activities, (iii) changing the C-terminal carboxy function of a biologically active peptide into a peptidyl or peptidyl N-alkyl amide often leads to analogues with more favourable or prolonged activity, and (iv) fully protected, but N-terminally free, peptide amides are needed for the assembly of longer chains.[1]

Highly acid-labile linkers are desirable, especially for the synthesis of peptide fragments in the protected form *via* the efficient Fmoc–Bu^t SPPS strategy, as they allow the cleavage of peptide amides under very mild conditions. The xanthenylamine linker (XAL) system has been described as a useful handle for the synthesis of peptide amides,[2,3] but the conditions (30 min with 1% TFA–CH_2Cl_2 v/v)[1] used for their cleavage from the resin also cause, for example, loss of the trityl protection group from His, and it is therefore not suitable for the preparation of side chain-protected peptide segments with *e.g.* His(Trt)-containing peptide amides.[4] The rapid acidolysis (50% TFA, < 5 min) of the 9-phenylxanthen-9-yl (Pixyl) protection group for the carboxy amide function of asparagine and glutamine recently developed in our laboratory[5] prompted us to increase the acid lability of XAL by introducing methoxy-substituted phenyl residues in position 9.

The starting material, 3-hydroxyxanthen-9-one **1**, was synthesized in good yield *via* a three-step procedure from *o*-chlorobenzoic acid and *m*-methoxyphenol.[6,7] Treatment of **1** with sodium methoxide and chloromethylated polystyrene in *N*,*N*-dimethylacetamide (DMA) yielded resin **2**, free of chloride. The resulting xanthenol resins **3a** and **3b** were prepared from **2** *via* a Grignard reaction using the corresponding bromo reagents in THF. The disappearance of the CO band in the IR spectrum at 1645 cm^{-1} indicated that the reaction had gone to completion. Both resins, **3a** and **3b**, were converted with AcCl in toluene into the chloro forms, and then by treatment with ammonia in CH_2Cl_2 into the amino compounds **4a** and **4b** (Scheme 1).‡

As a test case, Fmoc-Gly-OH was coupled to the resin using DCC (3 equiv., 2 h) and the remaining amino groups were capped with Ac_2O–Pr$^i_2$EtN (1 : 1) in CH_2Cl_2 for 20 min. To determine the extent of functionalization of the resin, the Fmoc-group was cleaved with 25% piperidine–DMF and the solution of the 9-fluorenylmethyl piperidine adduct was measured by UV spectroscopy at λ = 290 nm. The loading determined was 0.52 and 0.58 mmol Fmoc-Gly g^{-1} resin for **5a** and **5b**, respectively. Other Fmoc amino acids were attached to the resins and the degree of resin loading was found to be satisfactory, even with highly sterically hindered amino acids such as Fmoc-Val-OH (> 0.43 mmol g^{-1}) and Fmoc-Asn(Trt)-OH (> 0.35 mmol g^{-1}).

Kinetic studies were performed to compare the cleavage times of Fmoc amino acids from the resins **5a** and **5b** with those

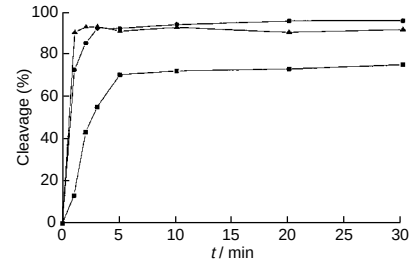
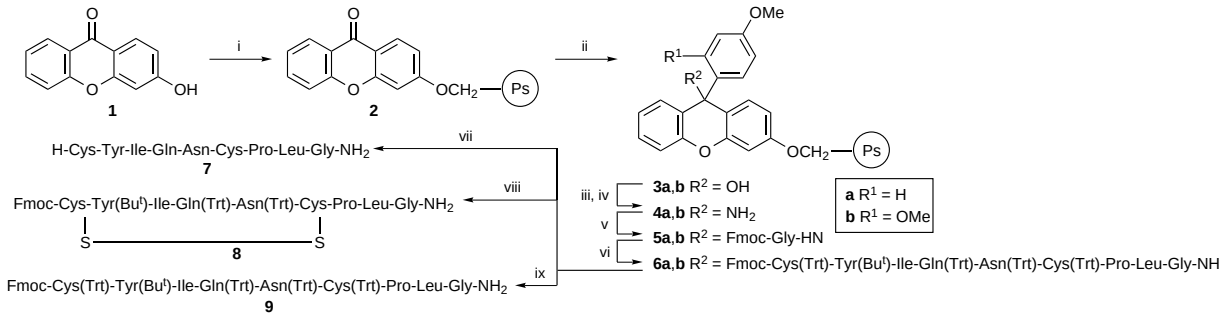


Fig. 1 Cleavage kinetics of Fmoc-Gly-NH_2 from (▲) **5b**, (●) **5a** and (■) 9-aminoxanthen-3-yloxymethyl polystyrene resin (XAL)



Scheme 1 *Reagents and conditions*: i, chloromethylated polystyrene (1.7 mmol g^{-1}, 2.5 equiv.) DMA, 65 °C, 15 h; ii, Mg (10 equiv.) and 4-bromoanisole (10 equiv.) (for **a**) or 1-bromo-2,4-dimethoxybenzene (10 equiv.) (for **b**), THF, 12 h, reflux; iii, AcCl (20 equiv.) PhMe, 40 °C, 1 h; iv, 50% NH_3 in CH_2Cl_2, −45 °C to room temp., 1 h; v, Fmoc-Gly-OH, DCC, CH_2Cl_2; vi, standard Fmoc–Bu^t solid phase procedures (see footnote ‖); vii, 10% TFA–CH_2Cl_2, 15 min, TFA–thioanisole–ethanedithiol–H_2O–PhOH (10 : 0.5 : 0.5 : 0.5 : 0.5, v/v/v/v/w), TFA, 2 h, room temp.; viii, I_2 (10 equiv.) in 0.5% TFA–CH_2Cl_2 (v/v), 3–5 min; ix, 1% TFA–CH_2Cl_2 (v/v), 3–10 min

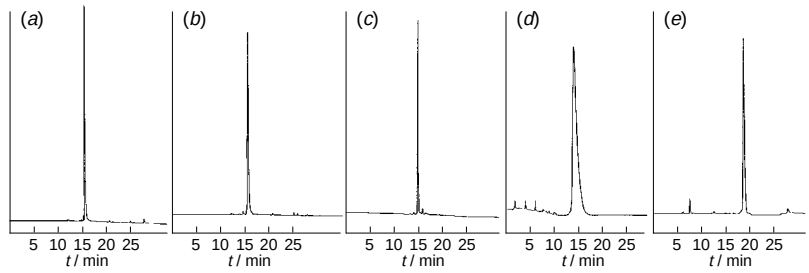


Fig. 2 Reversed-phase high performance liquid chromatograms of synthetic oxytocin: (*a*) deprotected crude product **7** cleaved from **6a**; (*b*) deprotected crude product **7** cleaved from **6b**; (*c*) oxidized unprotected crude product cleaved from **6b**; (*d*) protected crude product **9** cleaved from **6b**; (*e*) protected oxidized crude product **8** cleaved from **6b**. Column: Nucleosil 5 μm C_{18} 4.6 × 250 mm. Eluents: [for (*a*)–(*c*)] A = H_2O–TFA (1000 : 0.25), B = MeCN–H_2O–TFA (600 : 400 : 0.2); [for (*d*) and (*e*)] A = H_2O–TFA (1000 : 0.25), B = MeCN–TFA (1000 : 0.2). Gradients (A : B): [for (*a*)–(*c*)] 0–1 min (95 : 5), 1–31 min (95 : 5 to 5 : 95); [for (*d*)] 0–1 min (20 : 80), 1–11 min (20 : 80 to 0 : 100), 11–30 min (0 : 100); [for (*e*)] 0–1 min (40 : 60), 1–16 min (40 : 60 to 0 : 100), 16–30 min (0 : 100). Flow rate: 1 ml min^{-1}. Detection: λ = 214 nm. Concentration: 1 mg ml^{-1}.

from the 9-aminoxanthen-3-yloxymethyl polystyrene support (Novabiochem). The Fmoc-Gly-resins were treated with 1% TFA in CH_2Cl_2 and aliquots (*ca*. 5 mg) were periodically withdrawn from the reaction vessel to determine the degree of cleavage *via* UV spectroscopy,§ demonstrating the expected much higher acid lability of the new linkers compared to the commercially available XAL (Fig. 1).

The practicability of the new linker system is illustrated by the efficient synthesis of our model peptide oxytocin [Fmoc-Cys(Trt)-Tyr(Bu^t)-Ile-Gln(Trt)-Asn(Trt)-Cys(Trt)-Pro-Leu-Gly-NH_2], chosen as a test sequence for the synthesis of a disulfide-bridged protected peptide amide. The linear sequence **7** was smoothly synthesized batchwise using the Fmoc–Bu^t strategy.¶ After incorporation of the last amino acid and removal of the N-terminal Fmoc group, the peptide was cleaved with 10% TFA–CH_2Cl_2 over 15 min. To remove the side chain protecting groups, the product was treated with TFA–ethanedithiol(EDT)–thioanisole(TA)–H_2O–PhOH (10 : 0.5 : 0.5 : 0.5 : 0.5, v/v/v/v/w), followed by diethyl ether precipitation and lyophilisation. The linear product **7** was obtained in 83 and 90% yield from **6a** and **6b**, respectively, demonstrating the stability of the peptidyl linker bond under the standard basic Fmoc–Bu^t synthetic conditions. The crude reduced oxytocin showed excellent purity (Fig. 2) and was oxidized by air in NH_4Ac buffer.‖

To yield the protected linear oxytocin **9** the cleavage was performed with 1% TFA–CH_2Cl_2 for 10 min from **6a** (80% yield) and 5 min from **6b** (93% yield).** The versatility of the linker is further illustrated by the simple and efficient method of oxidation with iodine during the cleavage. To obtain the cyclic protected oxytocin **8**, the product was cleaved with 0.5% TFA–CH_2Cl_2 (v/v) and the intramolecular disulfide bridge was simultaneous formed by oxidation with I_2 (10 equiv.) over 5 min for **6a** and 3 min for **6b** in 81 and 89% yield, respectively.††

Our results show that the introduction of the methoxy-substituted phenyl residue in position 9 of the xanthenyl system increased the acid lability as expected. The high yields and the excellent purity of the synthetic peptides produced on the resins show that the peptidyl linker bond is stable under the basic Fmoc–Bu^t synthetic conditions and that these new linkers allow a far faster cleavage of peptide amides compared to the commercially available XAL. The linker is also suitable for the convenient preparation of cystine-containing protected peptide amides by simultaneous iodine oxidation and cleavage of the products. Therefore, our new linker can be recommended for the synthesis of peptide amides in both unprotected and protected form, and is almost free of side products.

Footnotes

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‡ The chloromethylated polystyrene (1.7 mmol g^{-1}) was treated with 2.5 equiv. of 3–hydroxyxanthen-9-one **1** and 2.5 equiv. of MeONa in DMA at 65 °C for 15 h. The Beilstein test and microanalysis for chloride were negative. The Grignard reactions of the 9-oxoxanthen-3-yloxymethyl polystyrene resin **2** were performed in THF refluxing for 12 h with the Mg adducts 4-bromoanisole and 1-bromo-2,4-dimethoxybenzene, respectively. The resins **3a** and **3b** were transformed into the dark red halide forms over 1 h with AcCl in toluene at 70 °C. To yield the orange 9-amino-9-(4-methoxyphenyl)xanthen-3-yloxymethyl and 9-amino-9-(2,4-dimethoxyphenyl)xanthen-3-yloxymethyl polystyrene resins **4a** and **4b**, respectively, the resins were treated with a cold (−45 °C) solution of ammonia in CH_2Cl_2 (50%) and allowed to warm to room temperature (1 h).

§ A 100 mg batch of each Fmoc-Gly-resin (**5a**, **5b** and XAL) was placed in a reaction vessel and allowed to swell in CH_2Cl_2 for 10 min, then treated with 20 ml of 1% TFA–CH_2Cl_2 (v/v); periodically, *ca*. 5 mg samples were taken out and washed immediately (3 × CH_2Cl_2, 3 × DMF 3 × MeOH, 2 × CH_2Cl_2 and Et_2O). To control the release of the amino acid, the remaining Fmoc groups were cleaved with 25% piperidine–DMF for determination by UV spectroscopy at λ = 290 nm, using the rule of Lambert–Beer.

¶ The solid phase syntheses of oxytocin were accomplished by Fmoc batchwise procedures using an ECOSYN P peptide synthesizer (Eppendorf-Biotronik). Carboxy activation was achieved by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and Pr^i_2EtN in DMF except for the Fmoc-Cys(Trt)OH residues, which were activated with diisopropylcarbodiimide and hydroxybenzotriazole (HOBt) in CH_2Cl_2 to prevent epimerization during the activation.[8] The Fmoc group was cleaved with 25% piperidine in DMF.

‖ The air oxidation of the synthetic oxitocin was performed in a 10^{-4} m solution in 0.1 m NH_4Ac buffer at pH 7.5 in 2 h.

** The peptidyl resins were treated repeatedly with 1% TFA in CH_2Cl_2 and the solutions were filtered off after a short period of time (*ca*. 1 min) into a vessel containing 3% pyridine in CH_2Cl_2 to neutralize the TFA. The resulting mixture was extracted three times with H_2O, evaporated to remove the CH_2Cl_2 and Bu^{t}OH–H_2O (4 : 1) was added before the product **9** was lyophilised.

†† For the cleavage and simultaneous oxidation, the peptidyl resins (**6a** and **6b**) were treated with a solution of 0.5% TFA and 10 equiv. of I_2 in CH_2Cl_2. The same procedure was then used as described in footnote ††, except that the excess of iodine was removed by an additional washing step with a 0.05 m $Na_2S_2O_3$ solution. The synthetic peptides gave the expected FAB, IS or MALDI-MS data.

References

1 B. Riniker, A. Flörsheimer, H. Fretz, P. Sieber and B. Kamber, *Tetrahedron*, 1993, **49**, 9307.
2 Y. Han, S. L. Bontems, P. Hegyes, M. C. Munson, C. A. Minor, S. A. Kates, F. Albericio and G. Barany, *J. Org. Chem.*, 1996, **61**, 6326.
3 P. Sieber, *Tetrahedron Lett.*, 1987, **28**, 2107.
4 M. Meisenbach, G. Grübler, G. Paulus and W. Voelter, in *Peptides*, ed. H. L. S. Maia, 1994, ESCOM, 1995, p. 265.
5 H. Echner and W. Voelter, in *Peptides*, ed. C. H. Schneider and A. N. Eberle, 1992, ESCOM, 1993, p. 285.
6 C. Somlai, L.Nyereges, B. Penke, P. Hegyes and W. Voelter, *J. Prakt. Chem.*, 1994, **336**, 429.
7 H. Echner and W. Voelter, in *Peptide Chemistry*, ed. N. Yanaihara, 1992, ESCOM, 1993, p. 113.
8 T. Kaiser, G. J. Nicholson, H. J. Kohlbau and W. Voelter, *Tetrahedron Lett.*, 1996, **37**, 1187.

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