Reductive Alkylation of 9-Amino-xanthen-3-yloxymethylpoly(styrene): a Novel Procedure for the Synthesis of Peptidyl *N*-Alkyl Amides by Fmoc/Bu^t Chemistry

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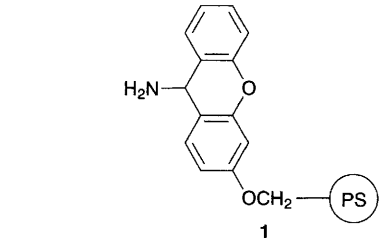
Reductive *N*-alkylation of the 'amide' anchor resin, 9-amino-xanthen-3-yloxymethyl poly(styrene) **1**, by sequential treatment with alkyl aldehydes under mild acidic conditions followed by $NaBH_4$ in MeOH–DMF, and then followed by optimised Fmoc/Bu^t solid-phase peptide synthetic procedures afforded an unique class of peptidyl *N*-alkyl amides in excellent yields.

Peptidyl amides are an important group of naturally occurring molecules which includes the family of mammalian hormones, *e.g.* cholecystokinins, gonadotropin-releasing hormone and oxytocin. In addition, several peptidyl amides (indolicidin, protegrins) with potent antimicrobial activities have recently been characterised and implicated to play an essential role in the host innate defence mechanisms.[1] The recent development in Fmoc/Bu^t solid-phase peptide chemistry employing acid-labile alkoxybenzhydrylamine-, trialkoxybenzylamine- or trityl-amine-based 'amide' linker/anchor resins has enabled the chemical synthesis of these peptidyl amides to be achieved using mild and efficient procedures.[2]

However, similar efficient Fmoc/Bu^t strategies for the synthesis of peptidyl *N*-alkyl amides have not been reported. To date, such peptidyl *N*-alkyl amides may be obtained by laborious and inefficient methods, such as alkylaminolysis of the resin-bound peptidyl 4-formamido-benzyl ester[3] or amidation of the side-chain protected N^{α}-Fmoc/Boc peptide acids by carbodiimide activation in the presence of HOBt–alkylamine salts,[4] as expected, both procedures commonly suffer from epimerization of the *C*-terminus amino acid residue. We now describe an efficient and facile route to peptidyl *N*-alkyl amides, by firstly reductive *N*-alkylation of either 9-amino-xanthen-3-yloxymethyl polystyrene† (PS)[5] **1** or 4-(amino-(2′,4′-dimethoxyphenyl)-methyl)-phenoxyacetamido-PEG PS,†[6] **2** resin and then followed by peptide assembly using solid-phase Fmoc/Bu^t chemistry. Here, mild acidolysis with 90% *v/v* TFA, in the presence of carbocation scavengers, yielded the desired peptides in excellent yields and purities. This conceptually novel strategy has been applied for the synthesis of several *N*-alkyl hexapeptide amides that are based on an antibacterial peptide selected from a combinatorial library.[7]



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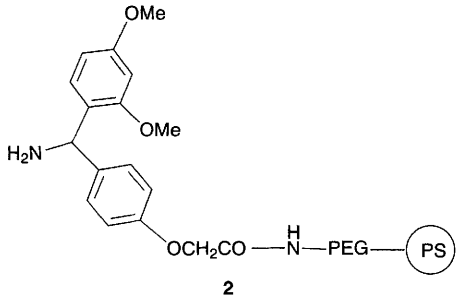

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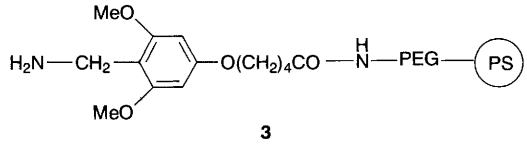

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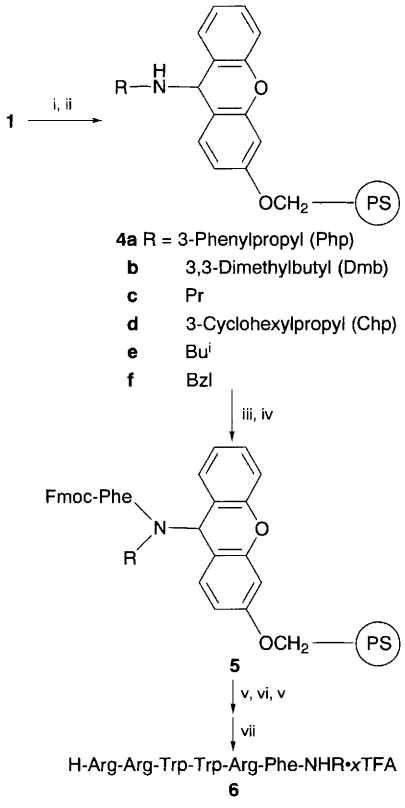
Much of the successful application of *N*-reductive alkylation in solid-phase peptide synthesis has previously been carried out for the synthesis of the ψ[CH_2NH] pseudopeptides. This is achieved by the *in situ* treatment of the amine moiety on a resin-bound amino acid residue with excess N^{α}-BoC-amino aldehyde and $NaBH_3CN$ in acidified DMF.[8] However, our initial attempts to utilise this methodology for reductive *N*-alkylation of **1** using either equivalent or fourfold excess of 3-phenyl-propionaldehyde–$NaBH_3CN$ gave disappointing yields of the desired anchor resin **4a**. With equivalent amount of reagents, the typically observed low yield (*ca.* 20%) was found to be a result of inefficient reductive *N*-alkylation, whilst with excess reagents, we anticipated that the poor yield was due to significant formation of the dialkylated[9] product.

We have now established that the 'amide' anchor resin **1** can be efficiently mono-alkylated by a simple two-step procedure, involving firstly its treatment with excess alkyl aldehyde in 1% *v/v* AcOH–DMF followed by reduction of the imine thus formed using excess $NaBH_4$ in MeOH–DMF (Scheme 1) to afford the key derivatives **4** in *ca.* 95% yields.‡ The initial condensation reaction, with a range of alkyl aldehydes, proceeded quickly and was found to be complete within 1 h (negative to 2,4,6-trinitrobenzenesulphonic acid amine test). These imine **1a–e** intermediates were then reduced using $NaBH_4$ in MeOH–DMF. In all cases, the solvent mixture was found to be crucial since negligible reduction (0–1%) was observed in DMF. This observation suggests participation of MeOH in the reduction transition state, and is consistent with the proposed mechanism[10] for the $NaBH_4$ reduction of ketones in the presence of hydroxylic groups or solvents. Curiously, rather poor reduction (< 10%) was observed in the propan-2-ol–DMF mixture, probably as a consequence of steric hindrance (with the alcoholic solvent). In our studies, the efficiency for the reduction of the resin-bound imine **1** is estimated indirectly by the RP-HPLC analysis ratio of Fmoc-amino acid-NH**R** : Fmoc-amino acid-NH_2 (Fig. 1, Table 1), obtained by reacting DMF-washed crude **4** with carboxyl-activated Fmoc-amino acid to yield initially **5**, which is followed by acidolytic treatment.‡ Under the acylation conditions used, any imine-**1** still present undergoes hydrolytic cleavage to regenerate **1** which in turn is *N*-acylated.

In a similar approach, efficient reductive alkylation has also been achieved on the alkoxybenzhydrylamine linker resin **2** (see Table 1). In contrast, we were unable to obtain stable imines using 5-(4-aminomethyl-3,5-dimethoxyphenoxy)-valeramido-PEG PS **3**[11] and hence is not applicable for the synthesis of peptidyl *N*-alkyl amides using our strategy.

In view of the possible problems associated with the acylation of sterically hindered secondary amines, we next investigated coupling reagents for the attachment of Fmoc-amino acids to **4**. In initial studies, Fmoc-Phe-OH was selected for coupling to **4a–e** for two reasons: the amino acid residue displays intermediate steric hindrance, and occurs at the *C*-terminus in the model antibacterial peptide.[7] Thus, using the recently reported azabenzotriazole-based reagents,[12] we have

found that Fmoc-Phe-OH, carboxyl-activated using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)–1-hydroxy-7-azabenzotriazole (HOAt)–DIPEA was efficiently coupled (18 h),‡ to give **5a–e** in >94% yields (Table 1); here, the extent of reaction is estimated by the Fmoc loading.† However, extensive studies on the acylation of **4e** (Table 1) indicate that this reaction is somewhat perturbed with Fmoc-amino acids containing highly sterically hindered side-chains, *e.g.* Pro and Val. In these cases, the level of acylation is in fact optimised since re-coupling does not improve the Fmoc loading; any potentially reactive resin-bound secondary amines were then capped with Ac_2O, and hence will not affect the quality of the subsequently assembled peptides. Interestingly, the acylation of both *N*-Php- and *N*-Bu^i-**2** gave modest yields (Table 1), probably reflecting the increased steric demands imposed on the secondary amines by the adjacent 2,4-dimethoxyphenyl group. In addition, as predicted by the increased steric effect, the acylation of **4f**, obtained by reductive alkylation using benzaldehyde, with Fmoc-Phe-OH afforded a comparatively lower yield.



Scheme 1 Solid-phase strategy for the synthesis of the antibacterial peptidyl *N*-alkyl amides. *Reagents and conditions*: i, alkyl aldehyde (5 equiv.) in 1% *v/v* AcOH–DMF, 1.5 h; ii, $NaBH_4$ (8 equiv.) in MeOH–DMF, 2 h; iii, Fmoc-Phe-OH–HATU–HOAt–DIPEA (1 : 1 : 1 : 2, 4–8 equiv.), 18 h; iv, Ac_2O, 15 min; v, 20% piperidine–DMF; vi, standard Fmoc/Bu^t solid-phase procedures (1.5 h acylation);§ vii, TFA–Pr$^i_3$SiH–1,2-ethanedithiol–H_2O (90 : 1 : 4.5 : 4.5), 3 h, 30 °C.

The utility of **5a–f** is illustrated by the synthesis of our model hexapeptides **6a–f**.§,¶ The assembled peptidyl-**5a–f** were obtained in *ca.* 90% yields and **6a–f** in excellent purities and yields (80–95%, see Fig. 2), thus establishing the stability of the secondary amido anchor linkage to repeated standard Fmoc/Bu^t synthetic conditions and the lability of the peptidyl alkyl amino xanthene bond to mild acidolysis, respectively

In summary, the above described strategy, particularly *N*-reductive alkylation of **1** followed by optimised Fmoc/Bu^t procedures, offers a simple and convenient method for the solid-

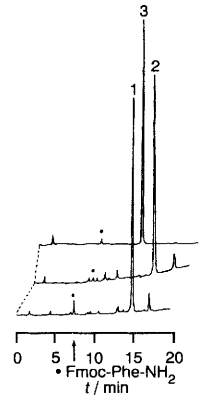
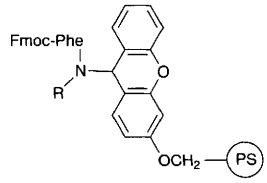
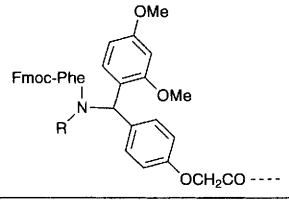


Fig. 1 Analysis of crude Fmoc-Phe alkyl amides by HPLC on Hypersil Pep C_{18} column¶ eluted with a linear gradient from 50 to 100% **B** in 20 min and monitoring the eluate at 254 nm. 1 = Fmoc-Phe-NHPhp, electrospray-MS MH^+ calc. 505.59, found 505.65; 2 = Fmoc-Phe-NHDmb, calc. 471.57, found 471.40; 3 = Fmoc-Phe-NHBu^i, calc. 443.53, found 443.35.

Table 1 Efficiencies of the synthetic procedures

Derivatised 'amide' linker/anchor resin	*N*-alkyl group, **R**	Estimated % purity[a]	Fmoc-amino acid loading† (mmol g^{-1})
	Php	92	0.33 (94%)
	Dmb	98	0.37 (99%)
	Pr	98[b]	0.35 (99%)
	Chp	94[b]	0.34 (96%)
	Bu^i	98	0.36 (99%)
	Bzl	98	0.19 (55%)
Fmoc-Asp(OBu^t)-N(**R**)----	Bu^i	92	0.36 (100%)
Fmoc-Ser(Bu^t)-N(**R**)----	Bu^i	97	0.31 (88%)
Fmoc-Pro-N(**R**)----	Bu^i	93	0.24 (67%)
Fmoc-Val-N(**R**)----	Bu^i	94	0.18 (50%)
	Php	96	0.12 (62%)
	Bu^i	93	0.12 (62%)

[a] Based on Fmoc-amino acid-NH**R** : Fmoc-amino acid-NH_2 ratio determined by RP-HPLC. [b] Inferred from the peptide ratio **6c**: Arg-Arg-Trp- Trp-Arg-Phe-NH_2 and **6d**: Arg-Arg-Trp-Trp-Arg-Phe-NH_2, respectively.

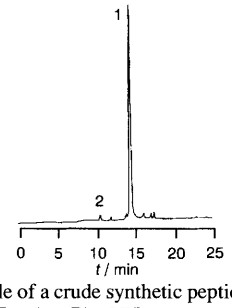


Fig. 2 RP-HPLC¶ profile of a crude synthetic peptide assembled on **4a**. 1 = **6a**; 2 = Arg-Arg-Trp-Trp-Arg-Phe-NH_2.

phase synthesis of peptidyl *N*-alkyl amides and should find a broad application in peptide chemistry including the ability to increase the diversity of peptidyl amide combinatorial libraries.

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Footnotes

† The commercially available *N*-Fmoc-**1** (0.38 mmol g^{-1}) and *N*-Fmoc-**3** (Fmoc-PAL-PEG-PS, 0.17 mmol g^{-1}) were Fmoc-deprotected with 20% *v*/*v* piperidine in DMF immediately prior to reductive alkylation. The linker resin **2** was found to have a loading of 0.21 mmol g^{-1}.

The resin loading is based on spectrophotometric determination of the Fmoc-derived chromophore liberated upon treatment with 20% piperidine–DMF using ε_{290} = 5 261 dm^3 mol^{-1} cm^{-1}.

‡ Typically, **1** (0.05 mmol) suspended in DMF (*ca.* 1 ml) was treated with the aldehyde (0.25 mmol) and acetic acid (10 μl) with agitation over 45 min. The resin was washed with DMF, and the chemical reaction was repeated to ensure complete formation of the imine. The imine-**1** anchor resin is stable to DMF wash; this was confirmed by the negative amine test after washing with DMF at 2.8 ml min^{-1} for 5 min. To a stirred suspension of the DMF-washed resin in DMF (1 ml) was then added MeOH (0.4 ml) and followed immediately by portion-wise addition of $NaBH_4$ (0.40 mmol) over 1 h. The reaction mixture was stirred for a further 1 h, and the resin was then extensively DMF-washed to yield **4**. *N*-Acylation of **4** was carried out using HATU-activated Fmoc-amino acids (4–8 equiv.) in DMF (*ca.* 0.3 mol dm^{-3}) overnight.

Fmoc-amino acid-NH**R** was obtained by acidolysis of **5** using TFA–Pr^i_3SiH–H_2O (90:2:8) for 30 min at ambient temperature.

§ Solid-phase assembly of synthetic peptides were accomplished by Fmoc continuous-flow procedures using a Millipore PepSynthesizer 9050. Unless otherwise stated, carboxyl activation was achieved by the mixture *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)–HOBt–*N*,*N*-diisopropylethylamine (DIPEA) (1:1:2 molar ratios).

¶ Crude peptides were analysed by RP-HPLC on Hypersil Pep C_{18} column (4.6 × 150 mm). The elution gradient was 20 to 60% **B** in 25 min at 1.20 ml min^{-1} (**A** = 0.06% aq. TFA, **B** = 0.06% TFA in 90% aq. MeCN) and the eluate was monitored at 220 nm.

All purified synthetic peptides gave expected plasma desorption-MS data: **6a** calc. MH^+ 1124.3, found 1124.2; **6b** calc. 1090.4, found 1090.9; **6c** calc. 1048.3, found 1048.5; **6d** calc. 1130.4, found 1130.8; **6e** calc. 1062.3, found 1062.7; **6f** calc. 1096.2, found 1096.9.

References

1 M. E. Selsted, M. J. Novotny, W. L. Morris, Y. Q. Tang, W. Smith and J. S. Cullor, *J. Biol. Chem.*, 1992, **267**, 4292; C. Zhao, L. Liu and R. I. Lehrer, *FEBS Lett.*, 1994, **346**, 285.
2 E. Atherton and R. C. Sheppard, *Solid Phase Peptide Synthesis: A Practical Approach*, IRL, Oxford, 1989; G. B. Fields and R. L. Noble, *Int. J. Peptide Protein Res.*, 1990, **35**, 161.
3 R. C. Sheppard and B. J. Williams, *Int. J. Peptide Protein Res.*, 1982, **20**, 451.
4 M. Bodánszky, M. A. Bednarek and A. Bodánszky, *Int. J. Peptide Protein Res.*, 1982, **20**, 387.
5 P. Sieber, *Tetrahedron Lett.*, 1987, **28**, 2107; W. C. Chan, P. D. White, J. Beythien and R. Steinauer, *J. Chem. Soc., Chem. Commun.*, 1995, 589.
6 H. Rink, *Tetrahedron Lett.*, 1987, **28**, 3787; M. S. Bernatowicz, S. B. Daniels and H. Köster, *Tetrahedron Lett.*, 1989, **30**, 4645.
7 R. A. Houghten, S. E. Blondelle and J. H. Cuervo, in *Innovation and Perspectives in Solid-Phase Synthesis*, ed. R. Epton, Intercept, Andover, 1992, p. 237.
8 D. H. Coy, S. J. Hocart and Y. Sasaki, *Tetrahedron*, 1988, **44**, 835; P. T. Ho, D. Chang, J. W. X. Zhong and G. F. Musso, *Peptide Res.*, 1993, **6**, 10.
9 J.-P. Salvi, N. Walchshofer and J. Paris, *Tetrahedron Lett.*, 1994, **35**, 1181.
10 D. C. Wigfield and F. W. Gowland, *Tetrahedron Lett.*, 1976, 3373; D. C. Wigfield and F. W. Gowland, *J. Org. Chem.*, 1977, **42**, 1108; S. H. J. Thompson, M. F. Mahon, K. C. Molloy, M. S. Hadley and T. Gallagher, *J. Chem. Soc., Perkin Trans. 1*, 1995, 379.
11 F. Albericio, N. Kneib-Cordonier, S. Biancalana, L. Gera, R. I. Masada, D. Hudson and G. Barany, *J. Org. Chem.*, 1990, **55**, 3730.
12 L. A. Carpino, *J. Am. Chem. Soc.*, 1993, **115**, 4397; L. A. Carpino, A. El-Faham, C. A. Minor and F. Albericio, *J. Am. Chem. Soc., Chem. Commun.*, 1994, 201.