Liquid-phase Peptide Synthesis on Polyethylene Glycol (PEG) Supports using Strategies Based on the 9-fluorenylmethoxycarbonyl Amino Protecting Group: Application of PEGylated Peptides in Biochemical Assays

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Abstract: Stepwise synthetic assembly of polypeptide chains reversibly linked to polyethylene glycol represents a hybrid between traditional solution and solid-phase chemistries and combines the inherent advantages of both approaches. The technical simplicity and scalability of the liquid-phase peptide synthesis method renders it particularly attractive for multiple parallel syntheses, combinatorial approaches and the large-scale preparation of peptides. The versatile protection strategy based on the N^α-fluorenylmethoxycarbonyl group commonly used in solid-phase peptide synthesis was adapted to the liquid-phase approach. Fluoride ions were used rather than the conventional organic base piperidine for the repetitive amino-deprotection step. Using a range of acid- and base-labile linkers between the polymer and the peptide, it was shown that free and fully side-chain protected peptides can be obtained using our version of the liquid-phase peptide synthesis method. Protocols for simultaneous multiple syntheses requiring a minimum of equipment are presented and the use of polyethylene glycol-bound peptides in biochemical binding and functional assay systems is demonstrated. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: liquid-phase peptide synthesis (LPPS); Fmoc protecting group; polyethylene glycol (PEG); polymer support; screening

INTRODUCTION

Liquid-phase peptide synthesis (LPPS), i.e. a hybrid approach between solution- and solid phase methods in which protecting groups based on dissolving polymers are employed, was first described in 1972 [1]. Polyethylene glycols (PEGs)

are particularly suitable because of their solubility properties. LPPS protocols based on N^α-Boc protecting group peptide chemistry were elaborated in detail some time ago [2]. However, because they are laborious, LPPS methods were not used widely until the recent advent of combinatorial synthesis. Liquid-phase methods are inherently suitable for parallel and combinatorial synthesis approaches, where reactions at high concentration in homogeneous medium, as well as predictable and ready isolation/purification of intermediates and products are important. Thus combinatorial LPPS using essentially the original chemistry is now possible [3,4]. Furthermore, the attractiveness of liquid-phase strategies in the large scale- and combinatorial synthesis of macromolecules as well

Abbreviations: Amino acid and peptide nomenclature conforms to IUPAC-IUB rules (*J. Peptide Sci.* 1999, **5**: 465–471). Other abbreviations: ABHL, alkoxybenzhydryl linker; DBF, dibenzofulvene; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HMPA, 4-hydroxymethyl-phenoxyacetyl; HMBA, 4-hydroxymethylbenzoyl; LPPS, liquid-phase peptide synthesis; NMP, *N*-methylpyrrolidinone.

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as organic molecules in general are now starting to be appreciated [5–11].

As far as can be ascertained, there has been only one report [12] describing LPPS methods based on N^{α}-Fmoc protecting group peptide chemistry [13]. Because this chemistry is currently the most versatile in terms of available starting materials, as well as orthogonality between N^{α}-, side chain- and C-terminal protecting groups, we sought to develop Fmoc-based LPPS. Here we present our results, including replacement of the traditional Fmoc deprotection reagent piperidine with TBAF.H_2O/DMF, as well as procedures for peptide chain assembly in which the standard filtration steps for the isolation of solid intermediates are replaced by centrifugation/decantation techniques. Application of these procedures then permits efficient, rapid and cost-effective simultaneous multiple syntheses on practically any scale. If PEGylated peptides are suitable for the biochemical or physiological use intended, syntheses can be performed on HO-PEG-OMe **1a** or H_2N-PEG-OMe **1b** directly by anchoring of the C-terminal Fmoc-amino acid to the PEG hydroxy or amino group via an ester or amide bond. Although a number of linker-modified PEGs have

been reported [14,15], these are generally not suitable for Fmoc-based syntheses, with the exception of photo-labile linkers [16,17]. Thus if free peptide acids or amides are desired, the standard acid- and base-labile linkers used in Fmoc-based SPPS [18,19] are applicable and the derivatives HMPA-NH-PEG-OMe **2**, HMBA-NH-PEG-OMe **3** and Fmoc-[Rink linker]-NH-PEG-OMe **4** [20] are suitable starting materials. Additionally, a PEG derivative containing a linker [21] with enhanced acid lability (ABHL-NH-PEG-OMe, **5**) was investigated (Figure 1).

Synthesis of polypeptides beyond the chain-length conveniently achievable by step-wise SPPS has become an area of great interest. While native chemical ligation of terminally modified peptides shows promise, most convergent approaches are still based on condensation of fully protected peptide segments in solution or on solid supports. Traditionally, protected segments have been assembled in solution; more recently, solid-phase segment-condensation techniques have become important, mostly because excess of acyl components in the condensation reactions can readily be employed. For the latter method, protected segments are typically assembled on supports equipped with acid super-labile linkers
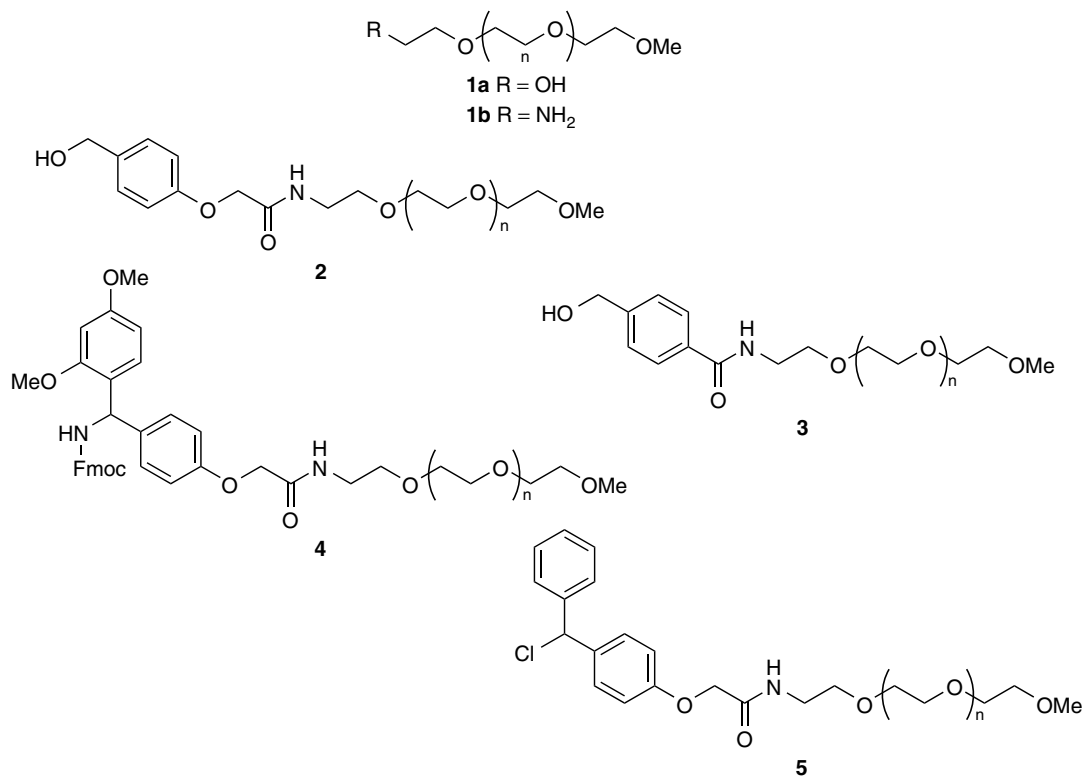

Figure 1

using Fmoc-based protection strategy. The protected peptides are then removed from the support using very mild acidolysis (e.g. with AcOH or HFIP). The limiting factors here are the lack of efficient purification techniques for protected peptide segments and the poor availability (in terms of preparative methods or cost) of suitable synthesis resins. For these reasons we sought to develop a PEG support bearing a linker permitting efficient detachment of protected peptides. We show that Fmoc-Xaa-O-[4-carboxytrityl]-NH-PEG-OMe **6** (Figure 2) fulfils this criterion. We demonstrate the utility of this novel support for the synthesis of protected peptide segments using the Fmoc LPPS method.

MATERIALS AND METHODS

Materials

HO-PEG-OMe 5000 was obtained from Fluka AG, Buchs, Switzerland. H_2N-PEG-OMe was purchased from Shearwater Polymers Europe, Enschede, Netherlands (product M-NH2-5000, M_r = 5254, 91%–92% amine substitution); larger batches were prepared as described [14]. Peptide synthesis grade DMF and TFA, as well as HOAt were from PerSeptive Biosystems, Hertford, UK. Amino acid derivatives, coupling reagents and linkers were from Calbiochem-Novabiochem AG, Läufelfingen, Switzerland, and Bachem AG, Bubendorf, Switzerland. $TBAF \cdot H_2O$ was from Fluka (Buchs, Switzerland). Other reagents and solvents were of the highest commercially available grade and were used without purification. DMF and CH_2Cl_2 were stored over molecular sieves 4A. The Fmoc-amino acid side-chain protecting groups used were as follows: t-butyl ethers and esters for Asp, Glu, Ser, Thr and Tyr; Boc for Lys; Trt for His, Gln and Asn; Pmc for Arg. The acidolysis reagent was as follows: $PhOH/H_2O/PhSMe/HS-(CH_2)_2$-SH/TFA (0.75 : 0.5 : 0.5 : 0.25 : 10, w/v/v/v/v) [22].
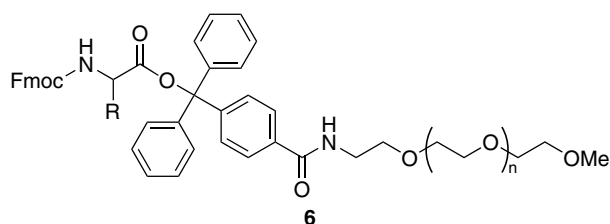

6

Figure 2

General

RP-HPLC was performed using Vydac 218TP54 (4.6 × 250 mm) and 218TP1022 (12 × 250 mm) columns for analytical and preparative work, respectively. Flow rates of 1 ml/min (analytical) and 9 ml/min (preparative) were used. Gradient elution was with increasing amounts of MeCN in H_2O, containing 0.1% TFA, over 20 min (analytical) or 40 min (preparative). Eluants were monitored using UV detectors (λ = 200–300 nm). NMR spectra were recorded on a Bruker DPX300 instrument. Chemical shifts (δ) are in ppm relative to $SiMe_4$. Mass spectra were obtained using a DE MALDI-TOF instrument (Dynamo; ThermoBioAnalysis); the matrix used was α-cyano-4-hydroxycinnamic acid [23]. Flash chromatography was performed as described [24] using Merck silica gel 60, 230–400 mesh. For TLC Merck silica gel 60 F_{254} pre-coated glass plates were used. Detection was by UV light and ninhydrin reagent. Automated SPPS was carried out on an ABI 433A (Applied Biosystems) synthesiser according to the manufacturer's protocols ('FastMoc' chemistry).

HMPA-NH-PEG-OMe (2)

4-Hydroxymethylphenoxyacetic acid (2.77 g, 15.2 mmol) was suspended in CH_2Cl_2 (20 ml) and DMF was added until dissolution was complete (ca. 15 ml). The mixture was stirred and DIC (1.19 ml, 7.6 mmol) was added and stirring continued for 15 min. H_2N-PEG-OMe (19.97 g, 3.8 mmol) in CH_2Cl_2 (50 ml) and HOBt (10.30 g, 7.6 mmol) in DMF (50 ml) were added. The reaction was allowed to proceed for 2 h. CH_2Cl_2 was then removed under reduced pressure and the residual solution was treated with Et_2O (1 l) and cooled. Precipitated product was filtered, washed with cold Et_2O and dried. Pure title compound (19.56 g, 95%) was obtained by recrystallisation from EtOH (200 ml) and drying *in vacuo*. Polymer loading (by ^{1}H-NMR): 88%.

HMBA–NH-PEG-OMe (3)

This compound (18.94 g, 93%) was obtained from 4-hydroxymethylbenzoic acid (2.31 g, 15.2 mmol) using the same procedure as for the preparation of **2**. Polymer loading (by ^{1}H-NMR): 86%.

Esterification of 1, 2 and 3 with Fmoc-amino acids

This was carried out by reaction of polymers **1**–**3** with 2 mol eq each of Fmoc-amino acid

and DIC in the presence of 0.1 mol eq of *N*,*N*-dimethylaminopyridine in CH_2Cl_2/DMF (4 : 1) for 3 h. The products were precipitated twice from Et_2O and recrystallised from EtOH. Polymer substitution levels of 0.13–0.16 mmol/g (40%–70%) were measured by titration of the amino groups after Fmoc-deprotection.

Fmoc-(Rink linker)-NH-PEG-OMe (4)

p-{(*R*, *S*)-α-[1-(9*H*-Fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl}phenoxyacetic acid (4.86 g, 9 mmol) and HOAt (1.22 g, 9 mmol) were dissolved in DMF (100 ml). To this was added a solution of H_2N-PEG-OMe (15.76 g, 3 mmol) in CH_2Cl_2 (100 ml), followed by DIC (1.64 ml, 10.5 mmol). The reaction was stirred for 2 h. CH_2Cl_2 was evaporated and Et_2O (0.7 l) was added. After cooling, the precipitated product was filtered, washed with cold Et_2O, dried and recrystallised from EtOH to afford the title compound (15.48 g, 89%). Polymer loading (by ^{1}H-NMR): 80%.

ABHL-NH-PEG-OMe (5)

4-Hydroxyacetophenone was alkylated with ethyl iodoacetate and saponified to afford the linker precursor 4-benzoylphenoxyacetic acid as described [21]. NH_2-PEG-OMe (15.76 g, 3 mmol) was dissolved in CH_2Cl_2 (100 ml) and added to a solution containing the precursor (2.31 g, 9 mmol) and HOAt (1.22 g, 9 mmol) in DMF (100 ml). DIC (1.64 ml, 10.5 mmol) was added and the mixture was stirred for 2 h. CH_2Cl_2 was evaporated and Et_2O (0.7 l) was added. After cooling, the precipitated product was filtered, washed with cold Et_2O, dried and recrystallised from EtOH. This material (13.73 g, 2.5 mmol) was dissolved in EtOH/Pr^iOH/H_2O (3 : 1 : 1; 200 ml). To the stirring solution $NaBH_4$ (1.89 g, 50 mmol) was added in portions over 30 min. Stirring was continued for 2 h, when excess borohydride was destroyed by addition of AcOH. The mixture was filtered and the filtrate was evaporated to near-dryness. The product was precipitated with Et_2O, filtered and recrystallised (12.07 g, 75%). This material (6.0 g, 1.1 mmol) was dissolved in dry CH_2Cl_2 (150 ml) and dichlorotriphenylphosphorane (4.66 g, 10.4 mmol) was added. The mixture was stirred under N_2 for 24 h, concentrated and triturated with Et_2O/hexane. The product was filtered, washed extensively on a sinter with the same solvent and

dried *in vacuo* to afford the title compound (6.47 g, quant.). Polymer loading (by ^{1}H-NMR): 81%.

Fmoc-Xaa-O-(4-carboxytrityl)-NH-PEG-OMe (6)

2-(4-Bromophenyl)-4,4-dimethyl-4,5-dihydrooxazole (7). 4-Bromobenzoyl chloride (5.49 g, 25.0 mmol) and 2-amino-2-methyl-1-propanol (2.45 g, 27.5 mmol) were dissolved in dry CH_2Cl_2 (100 ml). DIEA (13.1 ml, 75 mmol) was added and the mixture was stirred under N_2 for 1 h. Precipitated DIEA.HCl was filtered and the filtrate was evaporated to dryness. The residue was redissolved in $SOCl_2$ (18.2 ml, 0.25 mol) and the solution was stirred under N_2 for 1 h. The mixture was evaporated and the residue was partitioned between EtOAc (200 ml) and 1 M aq $NaHCO_3$ (200 ml). The aqueous phase was extracted twice more with EtOAc (50 ml each). The combined organic fractions were washed successively with 1 M aq $NaHCO_3$, H_2O, 10% aq citric acid and brine (2 × 50 ml each), followed by drying and decolourising ($MgSO_4$ and activated charcoal), filtration and evaporation to dryness. The residue was purified by flash chromatography (4.5 × 15 cm column; 95 : 5 CH_2Cl_2/Et_2O) to afford the title compound as an oil. Crystallisation was complete after keeping the product under high vacuum for several days and slightly yellow long soft needles (4.56 g; 72%) were obtained. TLC (95 : 5 CH_2Cl_2/Et_2O): R_f 0.22. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.39 (s, 6H, 2 Me), 4.11 (s, 2H, OCH_2), 7.44 (dm, *J* = 8.1 Hz, 2H, ArH), 7.95 (dm, *J* = 8.1 Hz, 2H, ArH).

(4-(4,4-Dimethyl-4,5-dihydrooxazol-2-yl)phenyl) diphenyl-methanol (8). Oxazoline 7 (4.56 g, 18.0 mmol) was dissolved in dry THF (25 ml), Mg turnings (0.52 g, 21.6 mmol) and resublimed I_2 (23 mg) were added. The mixture was sonicated under N_2 for 20 min. A solution of benzophenone (3.3 g, 18.0 mmol) in dry THF (25 ml) was added dropwise with stirring for 15 min. After addition was complete, the mixture was stirred for another 2.5 h. The reaction was quenched by addition of 5% aq $KHSO_4$ (50 ml). The mixture was extracted with EtOAc (3 × 50 ml), the combined extracts were washed with brine (30 ml), dried ($MgSO_4$), filtered and evaporated. The foamy residue was kept under high vacuum until crystallisation was complete (1 week). The discoloured crystalline mass was boiled in hexane (20 ml), cooled, filtered and dried to afford the title compound as a slightly yellow powder (4.94 g; 77%). This material was of sufficient purity for the subsequent step; an analytical

sample was obtained by flash chromatography (1 : 2 EtOAc/hexane). TLC (1 : 2 EtOAc/hexane): R_f 0.20. ^{1}H-NMR (300 MHz, CDCl$_3$) δ: 1.38 (s, 6H, 2 Me), 2.89 (br. s, 1H, OH), 4.10 (s, 2H, OCH$_2$), 7.23–7.32 (m, 10H, ArH), 7.35 (dm, $J = 8.5$ Hz, 2H, ArH), 7.89 (dm, $J = 8.5$ Hz, 2H, ArH).

Dicyclohexylammonium 4-(hydroxydiphenylme-thyl)benzoate (9).

Protected trityl alcohol **8** (0.89 g, 2.5 mmol) was suspended in 80% aq AcOH (10 ml) and the mixture was kept under reflux overnight. The clear solution was cooled and evaporated. The residual oil was dissolved in EtOH (10 ml) and 20% aq NaOH (10 ml) was added. The mixture was again kept under reflux for 3 h. After cooling, it was diluted with H$_2$O (25 ml), acidified to pH 2 with 6 M aq HCl, and extracted with EtOAc (3 × 25 ml). The combined extracts were washed (2 M aq HCl and brine; 2 × 25 ml each), dried/decolourised (MgSO$_4$ and activated charcoal), filtered and evaporated. The residue of crude product (0.66 g) was dissolved in EtOAc (20 ml). To the stirring solution dicyclohexy-lammonia (0.53 ml, 2.64 mmol) was added. Stirring was continued for 30 min, the mixture was cooled to ca. 0 °C, the precipitate filtered and washed with a little ice-cold EtOAc. After drying, the title com-pound (0.80 g; 65%) was obtained as a white amor-phous powder. TLC (98 : 2 CH$_2$Cl$_2$/AcOH): R_f 0.25.

4-(Hydroxydiphenylmethyl)benzoic acid succin-imido ester (10).

Salt **9** (728 mg, 1.50 mmol) was partitioned between 5% aq KHSO$_4$ (10 ml) and CH$_2$Cl$_2$ (25 ml). The organic phase was extracted successively with more 5% aq KHSO$_4$ and with brine (10 ml each), dried (MgSO$_4$), filtered, evap-orated and dried. The residue was redissolved in dry CH$_2$Cl$_2$ (25 ml) and N-hydroxysuccinimide (173 mg, 1.50 mmol) was added. The mixture was stirred and DIC (282 μl, 1.80 mmol) was added. Stirring was continued under N$_2$ overnight. Pre-cipitated diisopropylurea was removed by filtration through Celite and the filtrate was evaporated. The residue was purified by flash chromatography (2.5 × 10 cm column; 1 : 1 EtOAc/hexane) to afford the pure title compound (448 mg; 74%). TLC (1 : 1 EtOAc/hexane): R_f 0.28. ^{1}H-NMR (300 MHz, CDCl$_3$) δ: 2.81 (br. m, 4H, CH$_2$CH$_2$), 3.87 (br. S, 1H, OH), 7.20–7.34 (m, 10H, ArH), 7.47 (dm, $J = 8.1$ Hz, 2H, ArH), 8.03 (dm, $J = 8.1$ Hz, 2H, ArH). M.p. 155°–157 °C (Lit. [25] 151°–153 °C)

4-(Chlorodiphenylmethyl)-benzoic acid succin-imido ester (11).

Active ester **10** (401 mg, 1.00 mmol) was refluxed under N$_2$ in freshly distilled

acetyl chloride (10 ml) for 4 h. The mixture was con-centrated and rotary evaporated from dry CH$_2$Cl$_2$ (3 × 25 ml). The resulting foam was triturated with dry hexane, filtered and dried *in vacuo* to afford the pure title compound (322 mg; 77%). TLC (95 : 5 CH$_2$Cl$_2$/Et$_2$O): R_f 0.22, indistinguishable from start-ing material; R_f 0.36 for solvolysed (2 h treat-ment with 17 : 2 : 1 CH$_2$Cl$_2$/MeOH/DIEA) product. M.p. 171°–174 °C (Lit.[25] 173°–175 °C).

Fmoc-Xaa-O-(4-carboxytrityl)-NH-PEG-OMe (6a).

Fmoc-Xaa-OH (Xaa = Ala, βAla, Glu(Bu^t), Phe, Gly, Ile, Lys(Boc), Pro, Arg(Pmc), or Val; 1.00 mmol) and DIEA (697 μl, 4.00 mmol) were dissolved in dry CH$_2$Cl$_2$ (10 ml). Trityl chloride **11** (210 mg, 0.50 mmol) was added and the mixture was stirred under N$_2$ for 40 min. The solution was added to H$_2$N-PEG-OMe (1.314 g, 0.25 mmol) and HOBt (68 mg, 0.50 mmol). The mixture was sonicated for 30 min, concentrated to ca. 7.5 ml under a stream of N$_2$, and treated with Et$_2$O (40 ml). The mixture was cooled and the precipitated product was collected by centrifugation/decantation. Title compounds were obtained after recrystallisation from EtOH (5 ml) and drying *in vacuo*.

Chain Assembly Starting with Fmoc-amino acid-derivatised 1–5 and 6

Appropriate quantities of Fmoc-Xaa-[Linker]-NH-PEG-OMe were weighed into polypropylene tubes (typically 0.125 mmol or 0.25 mmol in 50 ml, 0.05 mmol in 12 ml Falcon tubes). Alternate Fmoc-deprotection and acylation steps, with isolation of the intermediates, were then carried out as follows: N^α-*Fmoc deprotection.* Fmoc-Xaa$_{(n)}$-[Linker]-NH-PEG-OMe (1 eq) was dissolved in TBAF.H$_2$O solution in DMF (0.1 M; 4 eq). 1-Octanethiol (10 eq) was added, the mixture was flushed with N$_2$ and was sonicated for 15 min. Cold Et$_2$O (160 ml/mmol) was then added and precipitated H-Xaa$_{(n)}$-[Linker]-NH-PEG-OMe was isolated by centrifugation at 4 °C (2 min at 4000 rpm) and decantation. The product pellet was triturated with cold Et$_2$O/petroleum ether (80 ml/mmol) and was again isolated by centrifugation and decantation. The air-dried pellet was then dissolved in EtOH (20 ml/mmol) with warming; once the product had crystallised, Et$_2$O (140 ml/mmol) was added, the mixture was cooled and centrifuged as before. The pellet was then dried, first under a stream of N$_2$ and then under high vacuum. *Acylation.* Fmoc-Xaa$_{(n+1)}$-OH was pre-activated with PyBOP, HOBt and DIEA (2 : 2 : 3 : 4 eq) in

DMF (25 ml/mmol). After 5 min, this mixture was added to H-Xaa$_{(n)}$-[Linker]-NH-PEG-OMe. The resulting solution was sonicated for 60 min. Fmoc-Xaa$_{(n+1)}$Xaa$_{(n)}$-[Linker]-NH-PEG-OMe was precipitated with Et$_2$O (160 ml/mmol), isolated, recrystallised and dried as above.

Deprotection/Cleavage Reactions

The peptide-PEGs were dissolved in acidolysis reagent (20 ml/mmol). The mixtures were stirred for 2 h and were concentrated to a small volume using a stream of N$_2$. Et$_2$O was added. After cooling, the precipitates were collected by centrifugation/decantation as described above. Peptides were isolated by preparative RP-HPLC. Protected peptides assembled on trityl polymer 6 were obtained after treatment with HFIP/CH$_2$Cl$_2$ (1:4; 50 ml/mmol) for 2 h, concentration, precipitation with cold Et$_2$O/hexane, and drying. PEG was removed by solid-phase extraction on silica gel cartridges (elution with appropriate CH$_2$Cl$_2$/Et$_2$O or CH$_2$Cl$_2$/MeOH mixtures).

Biochemical Assays

Kinase assays. Phosphorylation of pRb by CDK2/cyclin E, CDK2/cyclin A and CDK4/cyclin D1 was determined using 96-well format *in vitro* kinase assays. The phosphorylation reaction mixture (total volume 40 µl/well) consisted of 50 mM HEPES buffer, pH 7.4, 20 mM MgCl$_2$, 5 mM EGTA, 2 mM DTT, 20 mM β-glycerophosphate, 2 mM NaF, 1 mM Na$_3$VO$_4$, protease inhibitors cocktail, 0.5 mg/ml BSA, 1 µg purified recombinant CDK/cyclin enzyme complex, 10 µl of GST-pRb-Sepharose beads, 100 µM ATP, and 0.2 µCi [γ-^{32}P]-ATP. The reaction was carried out for 30 min at 30 °C with constant shaking of the reaction plate. At the end of this period, 100 µl of 50 mM HEPES, pH 7.4 and 1 mM ATP were added to each well and the total volume was transferred onto GFC filter plates (Whatman Polyfiltronics, Kent, UK). The plates were washed five times with 200 µl/well each of 50 mM HEPES, pH 7.4, 1 mM ATP. To each well was added 50 µl scintillant and the radioactivity of the samples was measured using a scintillation counter (TopCount, Packard Instruments, Pangbourne, Berks, UK).

Competitive binding assay. Biotinylated p21(149–159) peptide was immobilised on streptavidin-coated 96-well plates (Pierce Chemical Company, St Louis, MO, USA) at a concentration of 0.5 µM. Serial dilutions of test compounds were mixed with 5 µg cyclin A and then loaded onto the plate with immobilised peptide ligand. The amount of bound cyclin A was immunodetected and quantified by Turbo-ELISA reagent (Pierce Chemical Co., St Louis, MO, USA).

RESULTS

Synthesis of PEG-linker Constructs and Loading of the C-terminal Fmoc-amino acid

Starting polymers HMPA-NH-PEG-OMe 2, HMBA-NH-PEG-OMe 3, Fmoc-[Rink linker]-NH-PEG-OMe 4 and ABHL-NH-PEG-OMe 5 were obtained simply by carbodiimide-mediated amidation of H$_2$N-PEG-OMe with the appropriate carboxylic acid and HOBt or HOAt. In the case of 5, the benzophenone linker precursor was immobilised and the polymeric material was then elaborated by borohydride reduction to the benzhydrol, followed by chlorination to the final benzhydryl chloride form. Unlike the free benzhydrol linker precursor, the polymer-bound equivalent could not be chlorinated effectively using either conc. HCl or acetyl chloride [21]. However, chlorination using the reagent dichlorotriphenylphosphorane was successful [26].

The trityl linker precursor was prepared as follows (Figure 3): 4-Bromobenzoyl chloride was transformed to the dimethyloxazoline 7, whose Grignard derivative added cleanly to benzophenone to afford the key intermediate trityl alcohol 8. The oxazoline function was hydrolysed and 4-carboxyltrityl alcohol was isolated as the dicyclohexylammonium salt 9. The carboxyl function was re-protected/activated and the succinimido ester 10 was chlorinated to afford the linker precursor 11 in overall 20% yield. Although this intermediate could be immobilised on H$_2$N-PEG-OMe, the resulting chlorotrityl polymer was found to be unstable and chloride was lost upon isolation and storage. For this reason, a one-pot procedure was used firstly to acylate intermediate 11 with the desired Fmoc-amino acid, followed by reaction of the intermediate Fmoc-Xaa-O-carboxytrityl succinimido ester with H$_2$N-PEG-OMe to afford 6.

The appropriate *C*-terminal Fmoc-amino acids were attached to alcohol polymers 1a, 2 and 3 by carbodiimide/DMAP-mediated esterification, essentially as described [27]. H$_2$N-PEG-OMe 1b and Rink linker polymer 4 were used directly in the peptide chain assembly. Polymer 5, on the other
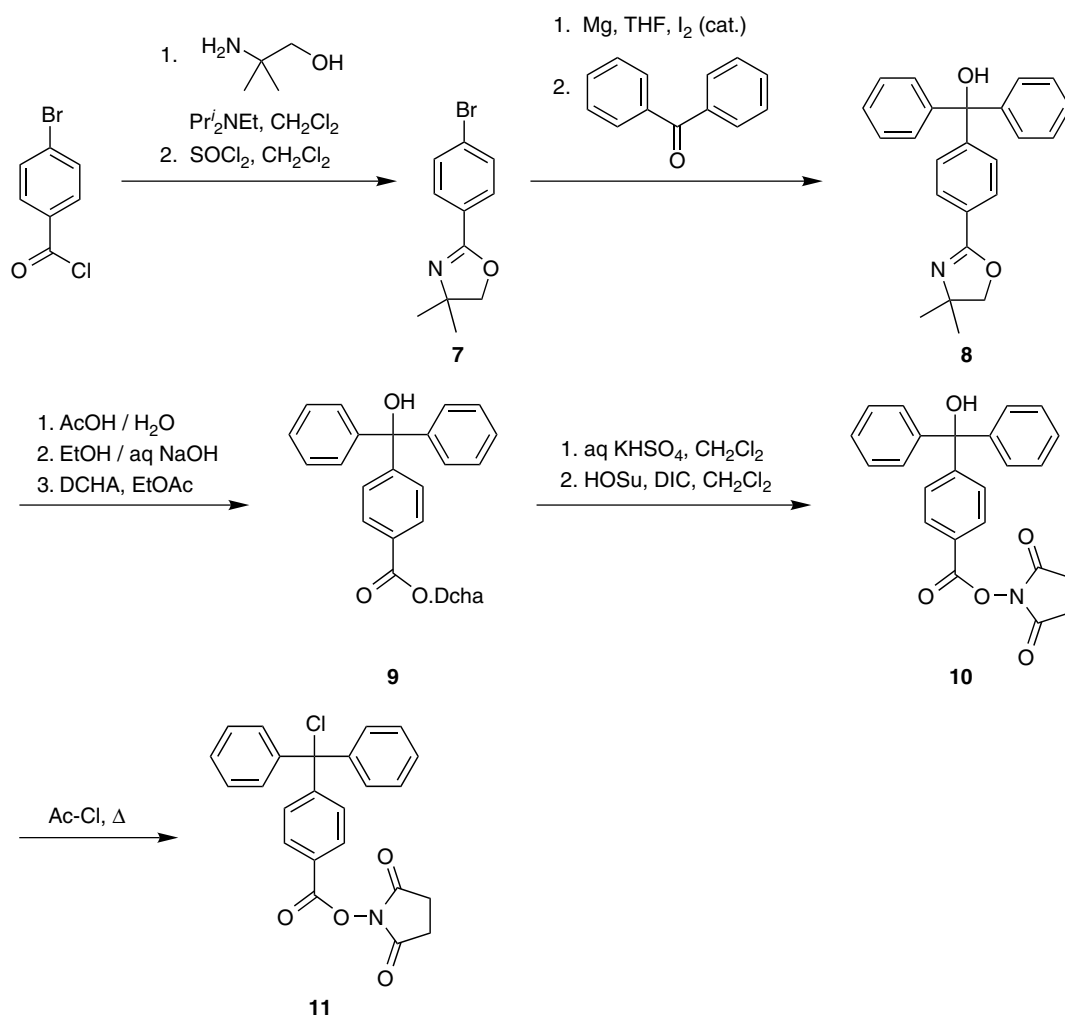
Figure 3   Synthesis of trityl linker precursor.

hand, could be acylated simply by reaction with Fmoc-amino acids and DIEA.

The extent of loading of the product polymers was assessed most readily using ^{1}H-NMR. PEG methyl ethers contain a single methoxy group ($\delta = 3.4$ ppm) [28], which serves as an internal standard for comparison of appropriate peak integrals (Figure 4). Depending on the quality of H_2N-PEG-OMe used, loadings of 80%–90% were thus determined. Polymers to which C-terminal Fmoc-amino acids had been attached were also subjected to quantitative ninhydrin [29] and spectrophotometric Fmoc [30] determinations, with similar results. In the case of amino acyl polymers **6**, loadings ranged from 40% to 70%, with the exception of Xaa = Arg(Pmc), where incorporation was very low. For example, loading of **6** (Xaa = Phe) was determined as 1.1 mmol/g (65%) after acidolysis (treatment with TFA for 30 min) by

anal. RP-HPLC quantitation of released Fmoc-Phe-OH and 0.9 mmol/g (53%) by ^{1}H-NMR (comparison of integrals over PEG-OCH_3 δ 3.41 and Fmoc ArH_2 δ 7.55 signals).

Peptide Chain Assembly

Peptide chains of up to 12 residues were assembled on polymers **1**–**6** using repetitive Fmoc-deprotection and acylation cycles. Removal of Fmoc groups was carried out using a 4-fold molar excess of TBAF.H_2O/DMF and with sonication of the reaction mixtures. Liberation of amino groups under these conditions is very efficient [31]. Although the Fmoc-deprotection product DBF and excess TBAF appear to be removed effectively during the precipitation/recrystallisation work-up, the scavenger
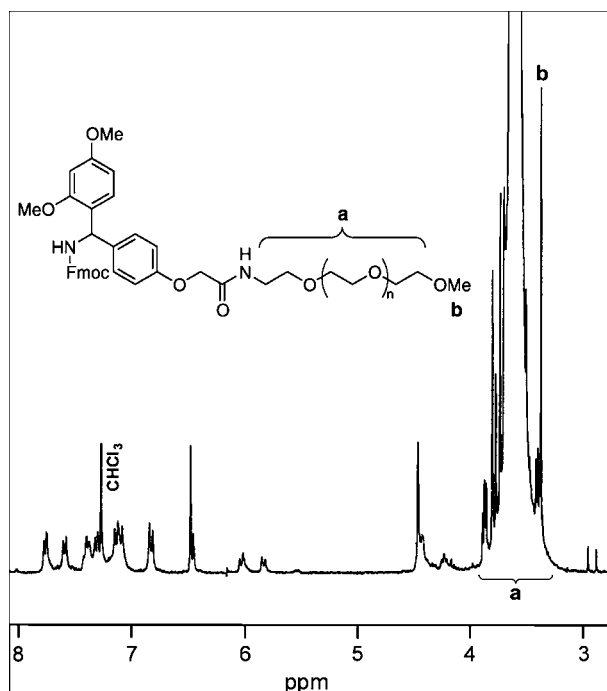
Figure 4   Excerpt from ^{1}H-NMR spectrum (300 MHz) of Fmoc-[Rink Linker]-NH-PEG-OMe **4**. The PEG methylene (**a**) and methoxy (**b**) resonances are indicated. Integration of appropriate signals permits calculation of the polymer loading, in the case shown ca. 80%.

1-octanethiol was included in the deprotection mixtures. Additionally, subsequent PyBOP-mediated acylation reactions were carried out with excess HOBt, which neutralises any remnant traces of fluoride ion [32]. The acylation reactions were also found to be accelerated significantly by sonication. Model peptides containing all common amino acid residues and side-chain protecting groups were assembled using the various linker-modified PEGs. It is also possible to synthesise peptides using our version of LPPS starting from HO-PEG-OMe **1a** or H$_2$N-PEG-OMe **1b** esterified or amidated with the appropriate *C*-terminal amino acids. However, side-chain deprotected PEGylated peptides are not obtained in good yield in the case of **1a** since the PEG ester bond is incompletely resistant to the acidolysis conditions required for the removal of the amino acid side-chain protecting groups employed in Fmoc-based chemistry. For our early work we isolated the peptide-PEG intermediates by filtration. This had the disadvantages that multiple syntheses could not easily be carried out concurrently and that sample losses were large. We found that carrying out each chain assembly in one polypropylene

centrifugation tube and isolating the intermediates by centrifugation and decantation of the solvents after precipitation/crystallisation minimised sample losses and was more practical for multiple simultaneous syntheses. Progress of the chain assemblies was routinely monitored using the ninhydrin test on small aliquots (ca. 0.1 mg) of the dried isolated intermediates after the acylation and Fmoc-deprotection cycles. Where necessary, acylations were repeated.

Using the procedures described above, e.g. an identical decamer sequence was assembled simultaneously on polymers **1b** and **2**–**5**. The overall isolated yields of the products H-Tyr(Bu^t)-His(Trt)-Ser(Bu^t)-Lys(Boc)-Arg(Pmc)-Arg(Pmc)-Leu-Ile-Phe-βAla-(O or NH)-[Linker]-NH-PEG-OMe were 86%, 81%, 85%, 84% and 83%, respectively. For example, starting with 722 mg (0.125 mmol) of Rink polymer **4**, 823 mg of peptidyl PEG product was obtained.

Cleavage/Deprotection and Analysis

After acidolysis of the five peptidyl PEGs referred to above, RP-HPLC analysis showed that correct free peptide H-Tyr-His-Ser-Lys-Arg-Arg-Leu-Ile-Phe-βAla-(OH or NH$_2$) had been liberated in the case of linkers **2**, **4** and **5**, as expected. The relative yields of desired product (obtained by integration of peptidic chromatogram peaks, $\lambda = 214$ nm) were 78%, 72% and 75%, respectively, whereas the isolated yields of pure peptide (relative to starting polymers **2**, **4** or **5**) after preparative RP-HPLC and lyophilisation were 48%, 39% and 45%, respectively. For example, the 823 mg of peptidyl PEG derived from polymer **4** thus afforded 62 mg of pure peptide. The anal. RP-HPLC profile of the crude peptide obtained from the peptidyl PEG derived from **2** is compared with that of crude peptide from conventional SPPS in Figure 5.

The particular utility of the base-labile HMBA linker polymer **3** lies in the fact that it permits preparation of side-chain deprotected peptidyl PEGs for biochemical assay purposes, while at the same time allowing liberation and characterisation of the peptide moiety. Thus the free peptide acid or amide could be obtained from the side chain-deprotected decapeptidyl PEG derived from polymer **3** referred to above by saponification or ammonolysis (not shown). Conversion of a model pentapeptidyl HMBA-PEG to the free peptide by treatment with alkali is demonstrated in Figure 6.

Preparation of side chain-protected peptides is demonstrated by the synthesis of Ac-Arg(Pmc)-Arg(Pmc)-Met-Lys(Boc)-Trp(Boc)-Lys(Boc)-Lys(Boc)-
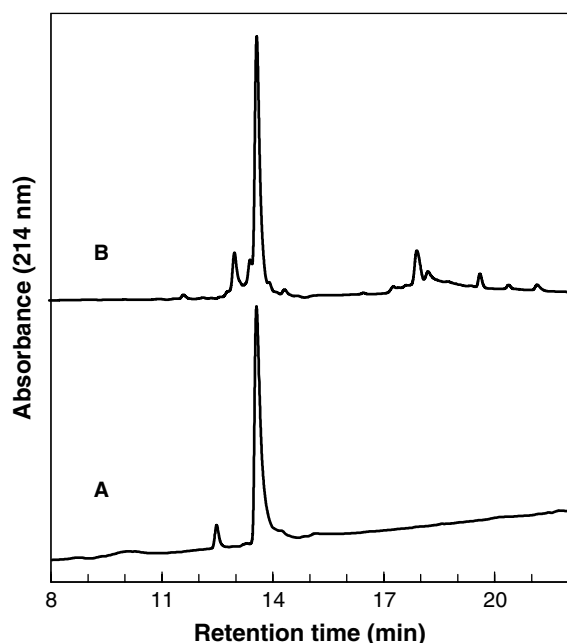
Figure 5 RP-HPLC analysis of crude synthetic decapeptide H-Tyr-His-Ser-Lys-Arg-Arg-Leu-Ile-Phe-βAla-OH after acidolysis. From automated SPPS ('FastMoc' chemistry) on Wang resin [54] (**A**) and from LPPS on HMPA-NH-PEG-OMe (**B**). Chromatographic conditions according to Materials and Methods section (10%–50% MeCN gradient over 20 min).

Figure 6 Saponification of H-Tyr-Ile-Lys-Gln-Phe-O-[HMBA]-NH-PEG-OMe. The deprotected PEGylated peptide was dissolved at 100 mg/ml in 2 M aq NaOH. Aliquots were quenched with excess AcOH and analysed by RP-HPLC (10%–20% MeCN gradient): $t = 0$ (**A**), after 30 min (**B**) and after 2 h (**C**). The peak eluting at ca. 16.1 min corresponded to the peptide H-Tyr-Ile-Lys-Gln-Phe-OH by MS and co-elution with an authentic sample.

OH starting from Fmoc-Lys(Boc)-O-[4-carboxytrityl]-NH-PEG-OMe (53% polymer loading) and using the standard chain assembly protocol. Treatment of the peptidyl PEG with HFIP afforded the protected peptide in approximately quantitative yield (relative to Lys content of starting polymer) after isolation by precipitation and solid-phase extraction (to remove polymer). The product showed the correct molecular ion upon DE-MALDI TOF MS and was homogeneous by TLC. Analysis of the deprotected peptide confirmed the success of the synthesis (Figure 7).

Biochemical Assays

The peptide we chose in order to demonstrate the applicability of PEGylated peptides in biochemical assays is derived from the *C*-terminus of the tumour suppressor protein p21^{WAF1}. We have previously shown that this peptide binds specifically to a macromolecular substrate recruitment site present on certain cyclins [33,34]. Complexation of cyclins with appropriate cyclin-dependent kinases (CDKs) is required for substrate recognition and phosphorylation and these activities are inhibited
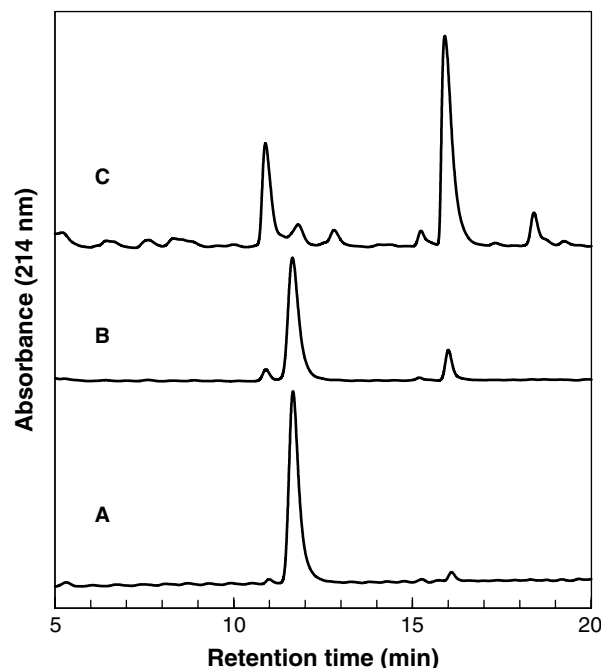
by the p21-derived peptides [35]. The synthesis of the decapeptide sequence in question using different polymer supports is described above. We compared the peptide H-Tyr-His-Ser-Lys-Arg-Arg-Leu-Ile-Phe-βAla-NH$_2$, its H$_2$N-PEG-OMe-bound equivalent, a control peptide polymer possessing the same amino acid composition but in a scrambled sequence (H-Arg-Ile-Ser-Phe-Arg-His-Lys-Tyr-Leu-βAla-NH-PEG-OMe), as well as free HO-PEG-OMe in both functional and binding assays. The former entails the phosphorylation of an immobilised retinoblastoma protein (pRb) fragment by CDK2 complexes with cyclin A or cyclin E, as well as by CDK4/cyclin D1 complexes. Protein kinase activity is quantitated in this assay by measurement of substrate-incorporated radioactive phosphate from [γ-^{32}P]-ATP. The latter is a competitive cyclin A binding assay using immobilised p21 peptide and an immunochemical detection system. A summary of the results is given in Table 1 and actual dose-response curves for two of the assay experiments are shown in Figure 8. Unmodified PEG itself had
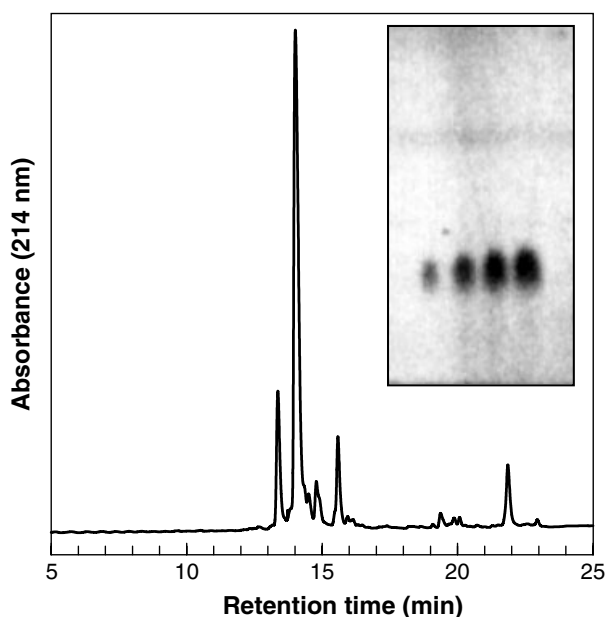
Figure 7   Analysis of peptide prepared using LPPS on trityl linker. Treatment of Ac-Arg(Pmc)-Arg(Pmc)-Met-Lys(Boc)-Trp(Boc)-Lys(Boc)-Lys(Boc)-O-[4-carboxytrityl]-NH-PEG-OMe with HFIP liberated the protected peptide. TLC (9 : 1 CHCl$_3$/MeOH, UV$_{254}$) analysis after isolation by solid-phase extraction (inset). RP-HPLC analysis (0–40% MeCN gradient) after treatment of protected peptide with acidolysis mixture and isolation by Et$_2$O-precipitation. The main peak eluting at 14.1 min corresponded to authentic Ac-Arg-Arg-Met-Lys-Trp-Lys-Lys-OH.

no significant effect in either assay system at concentrations up to 2.5 m$_M$. The control peptide-PEG did not inhibit kinase activity or cyclin A binding sufficiently in order to calculate IC$_{50}$ values, although some, presumably non-specific, inhibition was observed. Both the free and polymer-bound p21 peptides, on the other hand, potently inhibited substrate phosphorylation and cyclin A binding in a dose-dependent manner. Furthermore, inhibitory potency was similar for both forms of the peptide: approximately 2-fold differences in IC$_{50}$ values were observed, with the exception of the CDK4/cyclin D assay, where the apparent potency of the PEGylated peptide was about 10-fold lower than that of the free peptide.

DISCUSSION

The unique solubility properties of PEGs render them particularly suitable as dissolving polymer supports for step-wise synthetic assembly of peptides and oligonucleotides. PEGs (e.g. PEG 5000 as used in this study) are very soluble in halogenated and polar aprotic solvents (e.g. DMF, NMP, DMSO), i.e. the solvents favoured for peptide synthesis. On the other hand PEGs are practically insoluble in Et$_2$O or hexanes and these solvents can be used to precipitate quantitatively PEGylated synthesis intermediates. As far as protic solvents are concerned,

Table 1   Summary of Biological Activity of Free and Polymer-bound Peptides

Compound	Sequence	IC$_{50}$ (μM)[a]			
		Kinase assay			Competitive binding assay
		CDK2/ cyclin A	CDK2/ cyclin E	CDK4/ cyclin D1	Cyclin A
p21 peptide	H-Tyr-His-Ser-Lys-Arg-Arg-Leu-Ile-Phe-βAla-NH$_2$	2.5 ± 3.7	11 ± 4	30 ± 22	0.71 ± 0.05
p21 peptide-PEG	H-Tyr-His-Ser-Lys-Arg-Arg-Leu-Ile-Phe-βAla-NH-PEG-OMe	0.9 ± 0.4	24 ± 6	350 ± 45	0.86 ± 0.10
Control peptide-PEG	H-Arg-Ile-Ser-Phe-Arg-His-Lys-Tyr-Leu-βAla-NH-PEG-OMe	n/a[b]	n/a	n/a	n/a
PEG	HO-PEG-OMe	n/a	n/a	n/a	n/a

[a] Concentration at which 50% inhibition was determined (average $\pm$ standard deviation, $n = 3$);
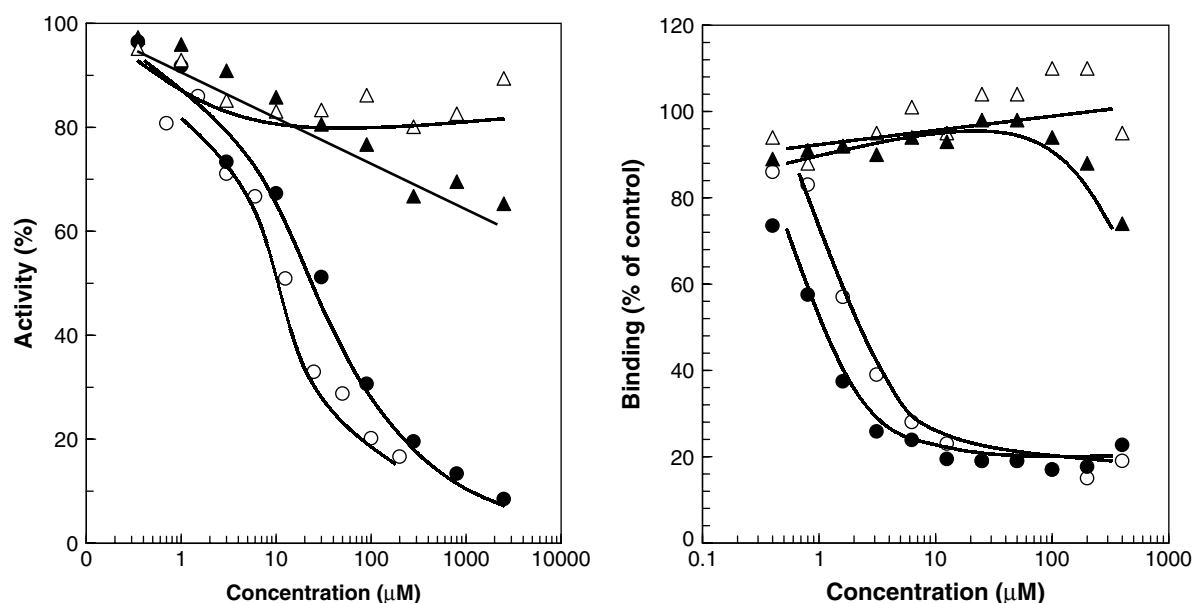[b] Not active.

Figure 8 Comparison of biological activity between the free p21[WAF1]-derived *C*-terminal peptide H-Tyr-His-Ser-Lys-Arg-Arg-Leu-Ile-Phe-βAla-NH$_2$ (●), its polymer-bound equivalent H-Tyr-His-Ser-Lys-Arg-Arg-Leu-Ile-Phe-βAla-NH-PEG-OMe (○), a scrambled version H-Arg-Ile-Ser-Phe-Arg-His-Lys-Tyr-Leu-βAla-NH-PEG-OMe (▲), and underivatised HO-PEG-OMe (△). Dose-response curves for the CDK2/cyclin A kinase assay and the competitive cyclin A binding assay are shown in the left- and right-hand panels, respectively.

the solubility of PEGs is extremely high in water, lower in MeOH and very low in EtOH. The fact that PEGs adopt ordered structures in alcohols and thus crystallise readily and cleanly from such solvents can be used for the purification of PEGylated peptide intermediates. The solubility in aqueous media can be taken advantage of for the manipulation and biochemical screening of PEGylated peptides or peptide libraries at high concentration (including hydrophobic sequences practically insoluble in free form).

The Fmoc protecting group has found only limited application in solution peptide syntheses because of the difficulties encountered in isolating the deprotected peptide products from the secondary organic bases used for deprotection (typically piperidine, Et$_2$NH or DBU), as well as the Fmoc degradation product DBF [36]. The situation is aggravated by formation of intractable adducts between the amines used and DBF, as well as polymers of the latter (Figure 9). It has been shown that these problems can be overcome in part by substituting certain organic bases, which can be extracted from the deprotection mixtures with aqueous buffer solutions [37,38]. However, due to the high water-solubility of PEG derivatives, these improvements are not applicable to LPPS. An alternative Fmoc-deprotection method uses fluoride ions in place of

the secondary amines [31,39,40]. We have found that this method is particularly suitable to LPPS procedures. This is due to the speed and efficiency of the Fmoc-deprotection itself, as well as the fact that fluoride ions can be used in modest excess and are removed effectively during the precipitation and recrystallisation steps employed for the isolation of the PEGylated deprotected peptide intermediates. It has been reported that during Fmoc-deprotection with both DBU [41] and TBAF [42] scavenging of DBF may be necessary in order to prevent formation of hard-to-remove DBF polymers as well as in order to suppress loss of deprotected amino component through adduct formation. The original report [31] on TBAF-mediated Fmoc deprotection advocated the use of a large molar excess of MeOH as a DBF scavenger. It was later found that addition of a moderate excess of certain thiols to the TBAF.H$_2$O/DMF deprotection mixtures improved both speed and extent of Fmoc removal [32] (Figure 9). In our hands, the addition of 10 eq of 1-octanethiol during deprotection gave superior results in terms of peptide yields and purity compared with omission of scavenger or addition of MeOH. Scavenging of DBF by 1-octanethiol appears to be very effective; furthermore, this thiol is readily deactivated and removed after the Fmoc-deprotection reaction through oxidation

to the disulphide in air, a process itself catalysed by fluoride ions. An extension of the thiol scavenger approach is simultaneous TBAF deactivation and oxidation of the thiol with bis(1-methyl-1H-tetrazol-5-yl)disulphide after deprotection, followed directly by the next acylation step procedure [32]. Similarly, deactivation of fluoride ions with e.g. HOAt immediately after Fmoc-deprotection, followed by carbodiimide-mediated acylations without isolation of the intermediate N-terminally deprotected peptide has been reported [43,44]. We have investigated the applicability of such one-pot peptide synthesis procedures to our liquid-phase approach. Although we have found (not shown) that in principle this is possible, we observe inferior product yields and purity, presumably due to accumulation of impurities. Nevertheless, closer investigation of such one-pot approaches is clearly of interest since they hold promise of further technical simplification of the synthesis assembly process.

Linkers based on derivatives of triphenylmethane have attained importance in peptide synthesis mainly for two reasons. Firstly, attachment of amino acyl groups to such linkers can be achieved very smoothly and without epimerisation using substitution reactions on trityl chloride derivatives with simple carboxylate anions. Secondly, trityl-type esters are very acid-labile and thus represent suitable
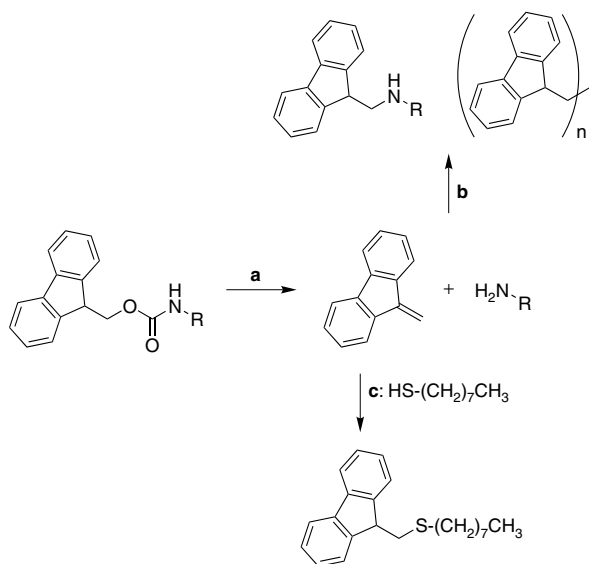
linkers for the preparation of protected peptides. Solid phase-bound trityl chloride derivatives are generally elaborated from immobilised benzophenones by alkylation, followed by chlorination [45]. We preferred a route using a pre-formed linker precursor, which could be attached to H_2N-PEG-OMe. 4-Carboxytrityl alcohol has been described as such a linker, although no synthetic details were reported [46]. The most direct route towards this compound appeared to be direct alkylation of benzophenone with lithiated 4-bromobenzoic acid, as described [47]. However, this procedure failed in our hands as a preparative method under a range of different reaction conditions. We therefore took recourse to a less direct route (Figure 3) previously shown to be feasible [25,48,49]. Protected peptides can be obtained by cleavage with dilute TFA, AcOH/TFE [46] or with HFIP [50], i.e. conditions that are used with the corresponding SPPS carboxytrityl supports.

It has previously been reported that MeO-PEG-peptide libraries are suitable for ELISA-type biochemical screening [4,9]. Here we compare directly the effect of free versus PEG-bound peptides not only in a simple binding assay, but also in multi-component functional assays. Our results confirm that it may be possible generally to use PEG-modified molecules from liquid-phase syntheses in biochemical screens. This is particularly attractive due to the powerful solubilising effect of PEG; it should therefore be possible to evaluate even very hydrophobic compounds. It is known that one of the main reasons for unreliable and false results in high-throughput screening in fact emanates from the unpredictable solubility of test compounds [51]. Modification of peptides [52] and proteins [53] with PEG is also advantageous because this prolongs circulatory life times *in vivo* and reduces immunogenicity.

Figure 9 Deprotection with e.g. TBAF.H_2O (**a**) of an Fmoc-amino acid or -peptide results in formation of DBF and the amine. DBF may polymerise and the amine can form adducts with DBF (**b**). In the presence of 1-octanethiol (**c**), DBF is effectively scavenged.

REFERENCES

1. Bayer E, Mutter M. Liquid phase synthesis of peptides. *Nature* 1972; **237**: 512–513.
2. Mutter M, Bayer E. The liquid phase method for peptide synthesis. In *The Peptides*. Academic Press: New York, 1979; 285–332.
3. Han H, Wolfe MM, Brenner S, Janda KD. Liquid-phase combinatorial synthesis. *Proc. Natl Acad. Sci. USA* 1995; **92**: 6419–6423.

4. Janda KD, Han H. Combinatorial chemistry: A liquid-phase approach. *Methods Enzymol.* 1996; **267**: 234–247.

5. Wentworth P Jr., Janda KD. Liquid-phase chemistry: Recent advances in soluble polymer-supported catalysts, reagents and synthesis. *Chem. Commun.* 1999; 1917–1924.

6. Han H, Yoon J, Janda KD. Azatides as peptidomimetics. Solution and liquid phase syntheses. *Methods Mol. Med.* 1999; **23**: 87–102.

7. Sieber F, Wentworth P Jr., Janda KD. Exploiting poly(ethylene glycol) as a matrix for liquid-phase organic synthesis. *Molecules* 2000; **5**: 1018–1032.

8. Bonara GM, Scremin CL, Colonna FP, Garbesi A. HELP (high efficiency liquid phase) new oligonucleotide synthesis on soluble polymeric support. *Nucleic Acids Res.* 1990; **18**: 3155–3159.

9. Gravert DJ, Janda KD. Organic synthesis on soluble polymer supports: Liquid-phase methodologies. *Chem. Rev.* 1997; **97**: 489–509.

10. Shey J-Y, Sun C-M. Liquid phase combinatorial synthesis of benzylpiperazines. *Bioorg. Med. Chem. Lett.* 1999; **9**: 519–522.

11. Gravert DJ, Janda KD. Soluble polyethylene glycol supports for liquid-phase combinatorial synthesis. *Drugs of the Future* 1997; **22**: 1147–1150.

12. Colombo R, Pinelli A. Liquid-phase synthesis of naturally occurring peptides. I. Syntheses of leucine-enkephalin and methionine-enkephalin on a p-alkoxybenzyl-modified soluble support. *Hoppe-Seyler's Z. Physiol. Chem.* 1981; **362**: 1385–1391.

13. Fields GB, Noble RL. Solid phase peptide synthesis utilizing 9-fluorenylmethoxy-carbonyl amino acids. *Int. J. Peptide Protein Res.* 1990; **35**: 161–214.

14. Pillai VNR, Mutter M, Bayer E, Gatfield I. New, easily removable poly(ethylene glycol) supports for the liquid-phase method of peptide synthesis. *J. Org. Chem.* 1980; **45**: 5364–5370.

15. Colombo R. New poly(ethylene glycol) supports for the liquid-phase synthesis of protected peptide hydrazides. *Tetrahedron Lett.* 1981; **22**: 4129–4132.

16. Tjoeng FS, Heavner GA. 4-(2-Chloropropionyl)phenylacetoxy-polyethylene glycol: A new photolabile support for liquid-phase peptide synthesis. *Tetrahedron Lett.* 1982; **23**: 4439–4442.

17. Colombo R. Liquid-phase synthesis of naturally occurring peptides. II. Syntheses of three mast cell degranulating tetradecapeptide amides from wasp venoms. *Hoppe-Seyler's Z. Physiol. Chem.* 1981; **362**: 1393–1403.

18. Arshady R, Atherton E, Clive DLJ, Sheppard RC. Peptide synthesis. Part 1. Preparation and use of polar supports based on poly(dimethylacrylamide). *J. Chem. Soc. Perkin Trans. 1* 1981; 529–537.

19. Atherton E, Logan CJ, Sheppard RC. Peptide synthesis. Part 2. Procedures for solid-phase synthesis using N^{α}-fluorenylmethoxycarbonylamino-acids on polyamide supports. Synthesis of substance P and acyl carrier protein 65–74 decapeptide. *J. Chem. Soc. Perkin Trans. 1* 1981; 538–546.

20. Rink H. Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin. *Tetrahedron Lett.* 1987; **28**: 3787–3790.

21. Yamashiro D, Li CH. New segment synthesis of α-inhibin-92 by the acyl disulfide method. *Int. J. Peptide Protein Res.* 1996; **31**: 322–334.

22. King DS, Fields CG, Fields GB. A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. *Int. J. Peptide Protein Res.* 1990; **36**: 255–266.

23. Beavis RC, Chaudhary T, Chait BT. α-Cyano-4-hydroxycinnamic acid as a matrix for matrix-assisted laser desorption mass spectrometry. *Org. Mass Spectrom.* 1992; **27**: 156–158.

24. Still WC, Kahn M, Mitra A. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 1978; **43**: 2923–2925.

25. Patel VF, Hardin JN, Mastro JM, Law KL, Zimmermann JL, Ehlhardt WJ, Woodland JM, Starling JJ. Novel acid labile col1 trityl-linked difluoronucleoside immuno-conjugates: Synthesis, characterization, and biological activity. *Bioconjugate Chem.* 1996; **7**: 497–510.

26. Atkinson GE, Fischer PM, Chan WC. A versatile polymer-supported 4-(4-methyl-phenyl(chloro)methyl)-phenoxy linker for solid-phase synthesis of pseudopeptides. *J. Org. Chem.* 2000; **65**: 5048–5056.

27. Zalipsky S, Gilon C, Zilkha A. Esterification of polyethylene glycols. *J. Macromolec. Sci.* 1984; **A21**: 839–845.

28. Douglas SP, Whitfield DM, Krepinsky JJ. Polymer-supported solution synthesis of oligosaccharides. *J. Am. Chem. Soc.* 1991; **113**: 5095–5097.

29. Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 1970; **34**: 595–598.

30. Chan WC, White PD. Fmoc solid phase peptide synthesis: A practical approach. In *The Practical Approach Series*, Hames BD (ed.). Oxford University Press: Oxford, 2000.

31. Ueki M, Amemiya M. Removal of 9-fluorenylmethyloxycarbonyl (Fmoc) group with tetrabutylammonium fluoride. *Tetrahedron Lett.* 1987; **28**: 6617–6620.

32. Ueki M, Nishigaki N, Aoki H, Tsurusaki T, Katoh T. One-pot deprotection and coupling of peptides by the Fmoc- strategy. *Chem. Lett.* 1993; 721–724.

33. Zhelev NZ, Zheleva DI, Fischer PM, Duff SV, Gavine A-L, Lane DP. Growth inhibitory effect of peptides derived from the C-terminal domain of p21[WAF] is mediated by cyclin D1/Cdk4 inhibition: PCNA binding is not essential. *Miami Nature Biotechnology Short Reports* 2000; **11**: 22.

34. Zheleva DI, McInnes C, Gavine A-L, Zhelev NZ, Fischer PM, Lane DP. P21[WAF1]-derived peptide inhibitors of CDK-mediated pRb phosphorylation: Delineation

and structural insights into their interactions with cyclin A. *J. Peptide Res* 2002; in press.

35. Fischer PM, Lane DP. Inhibitors of cyclin-dependent kinases as anti-cancer therapeutics. *Curr. Med. Chem.* 2000; **7**: 1213–1245.

36. Carpino LA. The 9-fluorenylmethyloxycarbonyl family of base-sensitive amino-protecting groups. *Acc. Chem. Res.* 1987; **20**: 401–407.

37. Beyermann M, Bienert M, Niedrich H, Carpino LA, Sadat-Aalaee D. Rapid continuous peptide synthesis via Fmoc amino acid chloride doupling and 4-(aminomethyl)piperidine deblocking. *J. Org. Chem.* 1990; **55**: 721–728.

38. Hoeg-Jensen T, Jakobsen MH, Holm A. A new method for rapid solution synthesis of shorter peptides by use of PyBop. *Tetrahedron Lett.* 1991; **32**: 6837–6390.

39. Carpino LA, Sau AC. Convenient source of 'naked' fluoride: Tetra-*n*-butylammonium chloride and potassium fluoride dihydrate. *J. Chem. Soc. Chem. Commun.* 1979; 514–515.

40. Jiang J, Li WR, Joullie MM. Selective removal of fluorenylmethoxycarbonyl (Fmoc) groups under mild conditions. *Synth. Commun.* 1994; **24**: 187–195.

41. Kates SA, Sole NA, Beyermann M, Barany G, Albericio F. Optimized preparation of deca(L-alanyl)-L-valinamide by 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase synthesis on polyethylene glycol-polystyrene (PEG-PS) graft supports, with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) deprotection. *Pept. Res.* 1996; **9**: 106–113.

42. Ueki M, Nishigaki N, Aoki H, Tsurusaki T, Katoh T. Rapid one-pot deprotection and coupling of Fmoc peptides. *Pept. Chem. 1992, Proc. Jpn. Symp., 2nd.* ESCOM: Leiden, 1993; 122–125.

43. Ueki M, Yanagihara T. A new one-pot method for Fmoc solution synthesis. *Pept. 1998, Proc. Eur. Pept. Symp., 25th (1999).* Akademiai Kiado: Budapest, Hungary, 1998; 252–253.

44. Ueki M, Tsurusaki T, Okumura J. Rapid solution phase synthesis of peptides by the Fmoc strategy. *Pept. Chem.* 1994; **32**: 213–216.

45. Fréchet JMJ, Nuyens LJ. Use of polymers as protecting groups in organic synthesis. Iii. Selective functionalization of polyhydroxy alcohols. *Can. J. Chem.* 1976; **54**: 926–934.

46. Bayer E, Clausen N, Goldammer C, Henkel B, Rapp W, Zhang L. New polymer and strategy for the solid-phase synthesis of protected peptide fragments. *Peptides: Chemistry, Structure and Biology.* ESCOM: Leiden, 1994; 156–158.

47. Zikos CC, Ferderigos NG. (R,S) 2-fluoro (chloro)-4′-carboxy-triphenyl methanol. Novel acid labile trityl type handles for solid phase peptide synthesis. *Tetrahedron Lett.* 1994; **35**: 1767–1768.

48. Gildea BD, Coull JM, Koester H. A versatile acid-labile linker for modification of synthetic biomolecules. *Tetrahedron Lett.* 1990; **31**: 7095–7098.

49. Quibell M, Packman LC, Johnson T. Synthesis of the 3-repeat region of human tau-2 by the solid phase assembly of backbone amide-protected segments. *J. Am. Chem. Soc.* 1995; **117**: 11656–11668.

50. Bollhagen R, Schmiedberger M, Barlos K, Grell E. A new reagent for the cleavage of fully protected peptides synthesised on 2-chlorotrityl chloride resin. *J. Chem. Soc. Chem. Commun.* 1994; 2559–2560.

51. Lipinski CA. Avoiding investment in doomed drugs. Is poor solubility an industry wide problem? *Current Drug Discovery* 2001; (April) 17–19.

52. Kawasaki K, Namikawa M, Yamashiro Y, Iwai Y, Hama T, Tsutsumi Y, Yamamoto S, Nakagawa S, Mayumi T. Amino acids and peptides. XXV. Preparation and fibronectin-related peptide(polyethylene glycol) hybrids and their inhibitory effect on experimental metastasis. *Chem. Pharm. Bull.* 1995; **43**: 2133–2138.

53. Gaertner HF, Offord RE. Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins. *Bioconjugate Chem.* 1996; **7**: 38–44.

54. Wang S-S. P-alkoxybenzyl alcohol resin and p-alkoxybenzyloxycarbonylhydrazide resin for solid phase synthesis of protected peptide fragments. *J. Am. Chem. Soc.* 1973; **95**: 1328–1333.