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Improved solid-phase peptide synthesis of 'difficult peptides' by altering the microenvironment of the developing sequence

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Abstract—Three types of resins, related to the spacer, environmental and microenvironmental models were prepared by grafting commercial AMP polymer with 2-[2-(2-aminoethylamino)-2-oxoethoxy]acetic acid. All resins were highly loaded and functionalized with Rink-amide linker. A comparative synthesis of the classic difficult sequence ACP (65–74) on the prepared resins by Fmoc/*t*-Bu chemistry is presented. The 'microenviromental' model resin afforded the crude peptide in the highest purity (98%).

Solid-phase[1] methodologies have been successfully used for the preparation of a wide range of peptides[2,3] and oligonucleotides[4] as well as for the development of new drugs via combinatorial chemistry.[5] The reagents and protocols for the solid-phase technique have been well optimized to produce extremely pure crude products. Despite these continuous efforts to optimize the protocol of peptide synthesis, serious shortcomings have intriguingly persisted. They are mainly related to incomplete reactions at various stages of the synthesis and occur in a class of relatively short peptide sequences, termed 'difficult sequences'.[6–8] This phenomenon is sequence specific and is recognized as the most serious difficulty in solid-phase peptide synthesis (SPPS).

The synthetic difficulty has been mechanistically attributed to the occlusion of the growing peptide chain within the polymeric network and is due to either (both) steric effects or (and) rigid secondary structures. The steric effects are related to amino acids with bulky side chains (or protecting groups), and can occur anywhere along the peptide chain and are frequently correctable by repeated couplings or capping. On the other hand, the secondary structures resulting from the intermolecular aggregation of the resin-bound peptide chains by β-sheet hydrogen bonding are often irreversible and are responsible for the major difficulties encountered in the syntheses of these peptides. To overcome the latter problem, a large number of refinements have been reported, namely: optimized Boc chemistry for SPPS,[9] backbone protection with reversibly N^{α}-blocked amino acids;[10] the use of pseudo-prolines as a temporary protection technique for serine, threonine, and cysteine;[11] the addition of a chaotropic salt such as KSCN or $NaClO_4$;[12] more reactive coupling agents;[13–17] and changing the polymer backbone as well as the cross-linkers, leading to styrene- and nonstyrene-based solid supports.[18–24] However, a general solution to difficult peptide syntheses, would require inhibition of the intermolecular backbone hydrogen bonding, a process which has not yet been developed. To this end, we report the synthesis of three PS-grafted resins with 2-[2-(2-aminoethylamino)-2-oxoethoxy]acetic acid (H-G-OH) (Fig. 1) and the study of their ability to overcome the synthetic problems of 'difficult sequence' peptides.

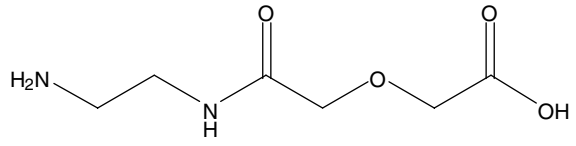

Figure 1. The structure of the grafting molecule H-G-OH.

Keywords: Solid-phase peptide synthesis; Difficult sequences; Grafting aminomethylpolystyrene resins.
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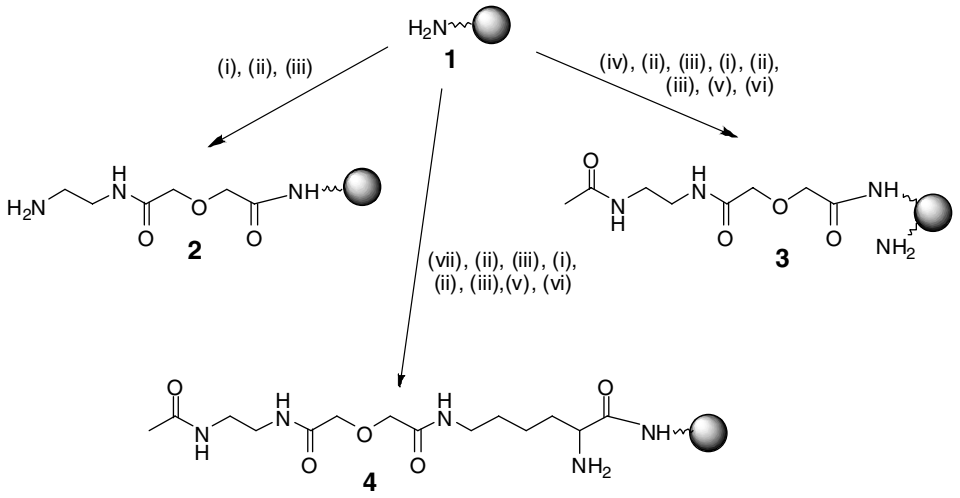

H-G-OH=2-[2-(2-aminoethylamino)-2-oxoethoxy]acetic acid

Scheme 1. Reagents and conditions: (i) Boc-G-OH/DIC/HOBt (3 equiv), DMF; (ii) TFA/CH_2Cl_2; (iii) DIPEA/CH_2Cl_2; (iv) Boc-Ala-OH (0.55 equiv), Fmoc-Ala-OH (0.55 equiv), DIC/HOBt (1.1 equiv); (v) Ac_2O/DIPEA (10 equiv), CH_2Cl_2; (vi) piperidine/DMF (20%); (vii) Fmoc-Lys(Boc)-OH/DIC-HOBt (3 equiv).

Three types of novel grafted model co-polymers were prepared based on the commercially available aminomethyl-functionalized resin **1** (AMPS, 1 mmol/g NOVAbiochem): (a) the 'spacer' model,[22,24] whereby the grafting molecule separates the starting point for solid-phase synthesis from the PS core (**2**, Scheme 1); (b) the 'environmental' model,[22,24] where the starting point for synthesis is alternated with the pendant grafting molecule (**3**, Scheme 1); and (c) the 'microenvironmental' model,[24] where the starting point for synthesis is a bifunctional group situated between the pendant molecule and the PS matrix (**4**, Scheme 1).

The 'spacer' model **2**, was prepared by loading Boc-G-OH[25] via DIC/HOBt mediated coupling, with the AMPS resin (1 mmol/g), followed by Boc removal and neutralization with DIPEA/CH_2Cl_2.

The synthesis of the 'environmental' model **3** was achieved by following the Adams protocol,[24] specifically, a (1:1) mixture of Boc- and Fmoc-alanine, activated by DIC/HOBt, was anchored to high loaded AMPS resin (2 mmol/g). After TFA-mediated removal of the N^{α}-Boc-protecting group and neutralization with DIPEA, Boc-G-OH was attached. Finally, the Boc group was again removed, and after neutralization and acetylation, the Fmoc group was cleaved, by piperidine in DMF, to yield the free amino starting point. The resin **4** was synthesized as follows: first, coupling of Fmoc-Lys(Boc)-OH to AMPS (1 mmol/g) was followed by Boc removal and neutralization. Then, attachment of Boc-G-OH to the ε-amino group of lysine, Boc removal, neutralization, and acetylation was completed by Fmoc removal with piperidine/DMF.

The resins **1**–**4** were further functionalized by incorporating [4-[(*R*,*S*)-α-[1-(9*H*-fluoren-9-yl)methoxycarbonylamino]-2,4-dimethoxybenzyl]phenoxyacetic acid (Rink-amide linker). The capacities of the resulting resins, were 0.64, 0.58, 0.53, and 0.50 mmol/g, respectively, as confirmed by quantification of the Fmoc group.[26]

The well known difficult sequence from the acyl carrier protein (ACP), residues 65–74 (**5**) was chosen in order to examine the synthetic efficacy of supports **1**–**4**. ACP (65–74) is known to aggregate significantly on deprotection of the penultimate glutamine residue. Incorporation of the final amino acid valine is often incomplete, typically reaching 80–90% via traditional solid phase procedures.[27]

H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2 **5**

The peptides were synthesized in parallel using manual batchwise protocols for Fmoc/*t*-Bu chemistry. The mild activating DIC/HOBt procedure was chosen in order to expose the synthetic difficulties. All syntheses employed the same batches of amino acids, resins, and other reagents and all procedures, from the initial linker loading step until the final cleavage work-up, were performed identically. The protected peptides were cleaved from the resin by treatment with TFA/H_2O/TIPS/CH_2Cl_2 (170:5:5:20) at room temperature for 2 h. After filtration of the exhausted resins, ether was added to the filtrates and the residues were removed by centrifugation. The purities of the obtained crude peptides from resins **1**, **2**, **3**, and **4** were 82%, 79%, 77%, and 98%, respectively, as estimated by analytical RP-HPLC chromatography (Fig. 2A). On the other hand, after purification by semi-preparative RP-HPLC, the overall yields of the peptides synthesized on resins **1**, **2**, and **3** were 60%, 54%, and 57%, respectively. The peptide prepared on resin **4** gave an HPLC profile containing a single major peak (Fig. 2A) corresponding to the target peptide, which was isolated in 92% yield after semi-preparative RP-HPLC. Semi-preparative RP-HPLC was performed using a gradient of 0–50% B over 50 min. The solvent

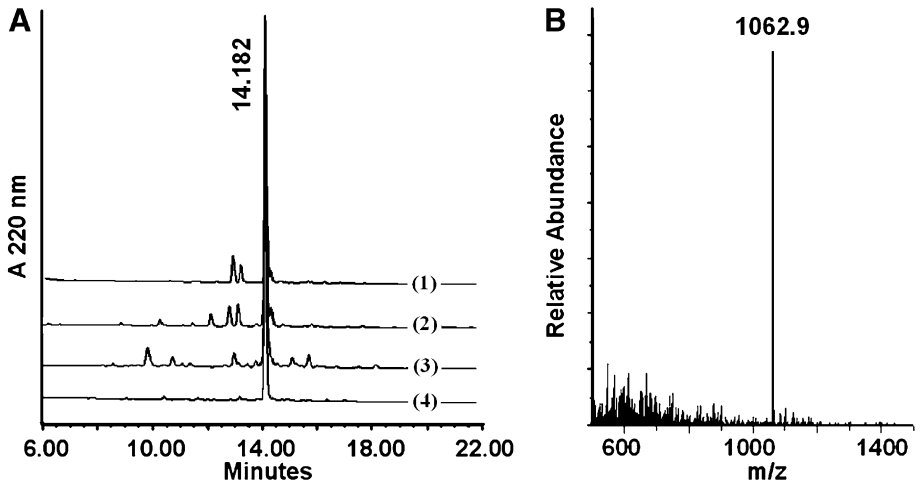

Figure 2. (A) Analytical RP-HPLC of unpurified peptides isolated from resins **1** to **4**, and (B) ESI-MS analysis of the peptide isolated from resin **4**. For HPLC analysis, elution of injected material was carried out using a gradient of 10–40% B over 20 min (solvent A, 0.05% TFA in 0.1 M $NaCl_{(aq)}$; solvent B, 0.05% TFA in 90% acetonitrile/10% water). Mass spectrometry was performed on a ThermoFinnigan Surveyor MSQ spectrometer using ESI in positive ionization mode.

system consisted of 0.05% TFA in water (solvent A) and 0.05% TFA in 60% acetonitrile/40% solvent A (solvent B). In the ESI mass spectrum of the crude peptide prepared on resin **4**, a major ion was observed at *m*/*z* 1062.9, corresponding to the protonated molecular ion $[M+H]^+$. The molecular mass of the truncated *des*-Val65 nonapeptide (MW = 962.0) contaminant was not present (Fig. 2B).

Swelling studies have been performed by studying the solvent uptake of the resin by the centrifuge method.[28] The swelling ratio was determined by the increase in net weight gain after swelling and was converted into the volume of solvent incorporated per weight of dry resin (swelling ratio, mL/g). Data from the swelling studies are summarized in Table 1. All the resins swelled satisfactorily with the solvents commonly employed in peptide synthesis. As can be seen from Table 1, the swelling behaviour in the solvent used in this study (DMF) was not proportional to the purities obtained.

In conclusion, we have synthesized three new resins by grafting commercial AMP resin with 2-[2-(2-aminoethylamino)-2-oxoethoxy]acetic acid. The resins were evaluated as supports for syntheses of the difficult sequence peptide ACP (65–74). The 'microenvironmental' model **4** provided the target product in excellent purity, while the others afforded the normally expected purities. We should point out that, contrary to the previously published procedures which rely on the 'microenvironmental' model, a small molecular weight grafting molecule was used in this study. The increased hydrophilicity of the resin is not a sufficient explanation for the improved results obtained, since there is no difference in hydrophilicity between resins **1**–**4**. A possible explanation of the improved behavior of resin **4** could be related to the presence of hydrogen bonding donor and acceptor groups in the pendant molecule. These groups being in the vicinity of the growing peptide chain could interact preferentially with it through hydrogen bonding, thus inhibiting β-sheet formation.

Table 1. Swelling ratio in mL/g

Resin	DCM	DMF	NMP	THF	MeOH
Commercial AMP (**1**)	6.29	2.37	3.61	3.55	0.63
Spacer (**2**)	3.86	2.75	3.37	2.07	0.75
Environmental (**3**)	3.60	1.99	2.52	1.87	0.52
Microenvironmental (**4**)	3.21	2.75	3.34	1.50	0.73

Acknowledgements

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25. To a solution of Boc-ethylene diamine[29] (1.6 g, 10 mmol) and triethylamine (1.2 g, 12 mmol) in ethyl acetate (30 mL), 1,4-dioxane-2,6-dione (1.4 g 12 mmol) was added. After 18 h, the reaction mixture was diluted with ether (50 mL), and was washed successively with saturated $KHSO_4$ and brine. The organic phase was dried over anhydrous Na_2SO_4 and the solvents were evaporated in vacuo. The residue was crystallized from ethyl acetate/petroleum ether (40–60) (1:5) to yield the title compound: 2.37 g (85%). Mp 122–124 °C. MS (ESI): m/z $[M-H]^-$ calcd for $C_{11}H_{20}N_2O_6$: 276.13; found: 275.4. ^1H NMR (200 MHz, DMSO-d_6): $\delta = 1.34$ (s, 9H), 2.95–3.01 (m, 2H), 3.07–3.12 (m, 2H), 3.91 (s, 2H), 4.06 (s, 2H), 6.79 (br, 1H), 7.83 (br 1H). ^{13}C NMR (75 MHz, DMSO-d_6): 172.02, 169.64, 156.35, 78.33, 70.68, 68.46, 40.20, 39.08, 28.85.
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