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An improved procedure for the preparation of aminomethyl polystyrene resin and its use in solid phase (peptide) synthesis

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ABSTRACT

2-Aminoethanol was used to successively replace hydrazine in the preparation of aminomethyl polystyrene resin thereby facilitating purification and by-product removal. The syntheses of the polypeptides ACP (65–74) and oxytocin demonstrated that the use of aminomethyl polystyrene resin prepared in this manner was equal to or better than that prepared using the hydrazine method.

The pioneering work of Merrifield on peptide synthesis using an insoluble solid support has revolutionized the field of peptide chemistry.[1] Solid phase peptide synthesis (SPPS) is arguably the first choice method for synthesizing a polypeptide of any length as simple, robust protocols can be easily executed under standard laboratory conditions. While SPPS has undergone many refinements, such as the development of coupling reagents and microwave enhancement, the critical factor to successful syntheses is arguably the properties of the solid support.[2–5]

One of the most popular solid matrices used for SPPS is a co-polymer of polystyrene (PS) and 1% divinylbenzene (DVB) abbreviated as 1% DVB–PS. This resin is commonly functionalized with either a suitable handle, such as an aminomethyl (AM) (NH_2CH_2–) group or preloaded with the desired linker and first amino acid as required for Fmoc- or Boc-SPPS. We and others have shown, however, that batch-to-batch variation of commercially supplied resins is a significant problem and ultimately results in poor quality peptides.[6–9] We and others have also previously established that polypeptides prepared using 'in house synthesized' AM-PS resin dramatically improved the quality of the synthetic polypeptides.[6,8]

Several methods have been previously reported for introducing an aminomethyl handle (or masked form thereof) on PS resin including phthalimidomethylation,[10–15] chloromethylation,[16] amidomethylation,[16] acetamidomethylation,[17] and co-polymerization[18] although all of these methods suffer from drawbacks, such as prolonged reaction times and difficulty in by-product removal. In the case of the widely used phthalimidomethylation procedure, the ensuing dephthalimidomethylation uses hydrazine to unmask the aminomethyl handle, and is accompanied by formation of the highly insoluble by-product, 2,3-dihydrophthalazine-1,4-dione (**3a**), and consequently requires extensive washing with large volumes of organic solvents (hot or boiling) to ensure its complete removal.[19]

From an environmental and safety viewpoint this procedure is undesirable. Furthermore, replacement of the toxic and potentially explosive hydrazine with a more benign reagent is advantageous.

We herein describe a significant improvement in the preparation of aminomethyl polystyrene resin (AM-PS resin) whereby the phthalimidomethylation–dephthalimidomethylation reaction times have been reduced, by-products are easily removed with minimal washing steps, and hydrazine has been substituted by less toxic reagents. We also demonstrate that AM-PS synthesized using this new procedure enables the preparation of several polypeptides with good recovery and in comparable purity to those obtained using AM-PS prepared by the existing methods using hydrazine.

The *N*-phthalimidomethyl resin **2** was prepared in multigram (5 g) amounts using *N*-(hydroxymethyl)phthalimide (**1**) and methanesulfonic acid (MsOH) as catalyst in dichloromethane.[6] Incorporation of the phthalimide was confirmed by IR spectroscopic analysis of resin **2** showing a C=O absorption at 1717 cm^{-1}.

For introduction of the phthalimide moiety use of the inexpensive methanesulfonic acid is preferred as it is easier to handle than hydroscopic trifluoromethanesulfonic acid.[10] Furthermore the use of a strong acid scavenges any undesired pre-existing functionality

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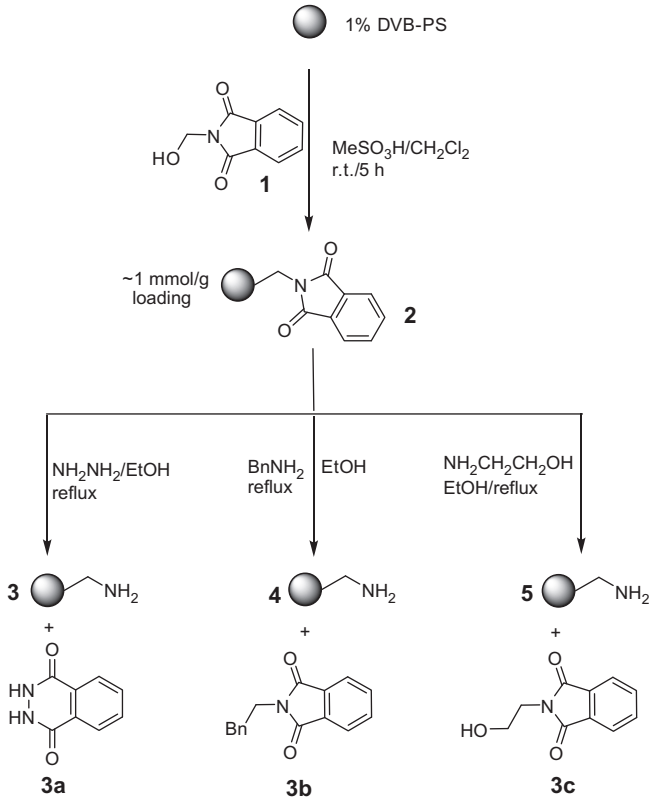
Table 1
Aminolysis of *N*-phthalimidomethyl PS resin **2**

Entry	Reaction conditions	Aminolysis %	Resin washing
1[a]	5% NH_2NH_2, ethanol, reflux, 5 h	100	Hot EtOH (400 mL), hot MeOH, (400 mL), DMF (200 mL), CH_2Cl_2 (200 mL), CH_2Cl_2–TFA (100 mL, 1:1, v/v), CH_2Cl_2 (200 mL), 10% DIPEA in DMF (200 mL), DMF (200 mL), CH_2Cl_2 (200 mL)
2[b]	5% $PhNHNH_2$, ethanol, reflux, 7 h	None	n/a
3[b]	5% $BnNH_2$, ethanol, reflux, 3 h	50	n/a
4[b]	5% $BnNH_2$, ethanol, reflux, 6 h	60	n/a
5[b]	20% $BnNH_2$, ethanol, reflux, 7 h	100	n/a
6[a]	20% $HOCH_2CH_2NH_2$, ethanol, reflux, 7 h	100	1 × EtOH, 1 × DMF, 1 × CH_2Cl_2 (100 mL each)

[a] 5 g of resin **2** used.
[b] 1 g of resin **2** used.

on the starting polystyrene resin. The level of substitution is estimated by the amount (mmol) of *N*-(hydroxymethyl)phthalimide used per gram of resin and proceeds to completion in ~5 h. Importantly, no decrease in swelling properties of the *N*-phthalimidomethyl resin **2** is noticed[20] indicating that no additional cross links have been introduced which would hinder diffusion of reagents during SPPS.

We next examined the reaction of resin **2** with various amine nucleophiles such as phenylhydrazine, benzylamine, or 2-aminoethanol in refluxing ethanol. These reagents were chosen as they are expected not only to react rapidly with the *N*-phthalimidomethyl group, but the resultant by-products containing aromatic or alkoxy groups were expected to be more soluble in organic solvents facilitating removal by filtration. The results of this study are listed in Table 1 and illustrated in Scheme 1.[21] For a direct comparison to existing protocols, hydrazinolysis using hydrazine was also undertaken.



Scheme 1. Preparation of aminomethyl resin by hydrazinolysis or aminolysis.

Using 5% (v/v) hydrazine to affect hydrazinolysis was rapid, but the resultant by-product, 2,3-dihydrophthalazine-1,4-dione (**3a**), required extensive washing to completely remove it from the resin (entry 1). Substitution of hydrazine with phenylhydrazine failed to give any reaction as judged by analysis of the resin by IR spectroscopy (entry 2). Replacing hydrazine with benzylamine required a more concentrated solution (20%, v/v) to enable complete aminolysis within 7 h (entries 3–5). However, benzylamine, a known lachrymator, was deemed to be unsuitable hence it was replaced with 2-aminoethanol resulting in complete aminolysis (entry 6) in a similar timeframe (7 h). Moreover, following completion of the reaction, the resin was only washed minimally with ethanol, dimethylformamide, and finally dichloromethane effecting total removal of the highly soluble by-product **3c**. The absence of **3c** was confirmed by IR analysis of a dried resin sample which did not exhibit a carbonyl (C=O) stretch. The loading capacities of resins **3**, **4**, and **5** were 0.86, 0.75, and 0.87 mmol/g, respectively, as determined by elemental analysis.

The suitability of each aminomethyl resin prepared using either benzylamine or 2-aminoethanol was evaluated using Fmoc SPPS. The so-called 'difficult' peptide sequence ACP (65–74), containing a C-terminal acid and the peptide hormone oxytocin, containing a C-terminal amide were chosen as representative polypeptides for this purpose (Fig. 1). As control experiments, both ACP (65–74) and oxytocin, were concurrently synthesized using aminomethyl PS resin derived from the hydrazine protocol.

Each resin (0.1 g), functionalized with [4-[(*R,S*)-α-[1-(9*H*-fluoren-9-yl]methoxycarbonylamino]-2,4-dimethoxy]phenoxyacetic acid (Rink linker)[22] or Fmoc-Gly-hydroxymethylphenoxy propionic acid (HMPP linker)[23] to deliver a C-terminal amide or acid, respectively, was elongated using 20% piperidine (v/v in DMF) as the Fmoc deblocking reagent and HBTU as the coupling agent in the presence of DIPEA. Identical batches of reagents and identical protocols for deprotection and coupling were utilized and for uniformity, machine-assisted synthesis was employed.[24] Upon completion of the synthesis, cleavage with trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5, v/v) for ACP (65–74) or trifluoroacetic acid/triisopropylsilane/water/ethanedithiol (95:1:2.5:2.5, v/v) for oxytocin was performed for 2 h. Crude products were precipitated using ether, isolated by centrifugation, dissolved in aqueous

ACP (65-74) :
NH_2-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH

Oxytocin :
NH_2-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

Figure 1. Amino acid sequences of test peptides oxytocin and ACP (65–74).

Table 2
Preparation of ACP (65–74) or oxytocin using amino methyl resins **3**, **4,** or **5**

Resin	Scale (mmol)	Peptide	Calculated weight of peptide resin (mg)	Weight of peptide resin (mg)	Calculated weight of crude product (mg)	Weight of crude product (mg)	Purity (%)
3[a]	0.086	ACP (65–74)	256	248	91.4	74.5	86
4[b]	0.075	ACP (65–74)	236	225	79.9	66.1	89
5[c]	0.087	ACP (65–74)	258	252	92.5	82.5	86
3[a]	0.086	Oxytocin	299	276	86.7	75.3	79
4[b]	0.075	Oxytocin	274	263	75.6	65.3	75
5[c]	0.087	Oxytocin	301	288	87.8	82.2	86

[a] Loading 0.86 mmol/g.
[b] Loading 0.75 mmol/g.
[c] Loading 0.87 mmol/g.

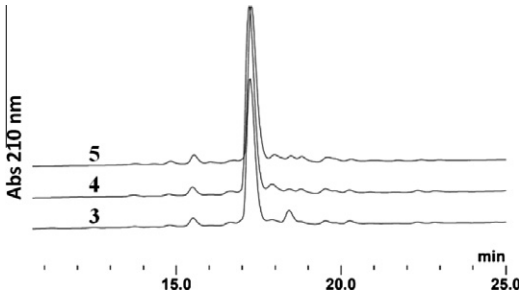


Figure 2. RP-HPLC of crude ACP (65–74) synthesized on aminomethyl resins **3**, **4,** or **5**. Column C18 Gemini 2.0 × 50, gradient 5–30% B (1% B/min), *A* = 0.1% TFA–H_2O, *B* = 0.1% TFA–MeCN.

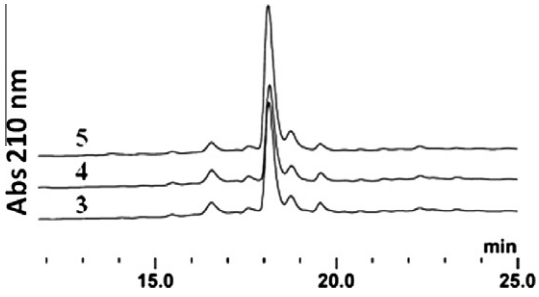


Figure 3. RP-HPLC of crude oxytocin synthesized on aminomethyl resins **3**, **4,** or **5**. Column C18 Gemini 2.0 × 50, gradient 5–30% B (1% B/min), *A* = 0.1% TFA–H_2O, *B* = 0.1% TFA–MeCN.

acetonitrile, and lyophilized. The results are given in Table 2 and Figures 2 and 3.

For aminomethyl resins **3**, **4**, and **5** the weight of the peptide resin following assembly by SPPS was comparable to that anticipated. The quantities recovered and the purities of the crude products, obtained after cleavage from the resin, as estimated by analytical HPLC (Figs. 2 and 3), were also similar although the highest recoveries were found for aminomethyl resin **5**. Therefore, based on the data presented in Table 1, it is evident that either of the dephthalimidomethylation methods using benzylamine or 2-aminoethanol can be used confidently for the preparation of AM-PS resin.

In conclusion, we have demonstrated that for the preparation of AM-PS resin by phthalimidomethylation–dephthalimidomethylation on polystyrene resin, the common dephthalimidomethylation reagent hydrazine can be replaced effectively by 2-aminoethanol or benzylamine. Significantly, the use of 2-aminoethanol or benzylamine enabled the resultant aminomethyl-functionalized polystyrene resin to be easily separated from highly soluble by-products by filtration using minimal solvent washing. Fmoc-SPPS of aminomethyl resins **4** or **5** for the synthesis of the peptides ACP (65–74) and oxytocin afforded crude products in excellent purity and good recovery which compared well to resin **3** obtained from the hydrazine protocol. We have used resin **5** exclusively for the preparation of many (>50) polypeptides by Fmoc- or Boc-SPPS, the results of which will be communicated in the subsequent publications.

Acknowledgment

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19. Ref. 9 used $MeNH_2$ for de-phthalimidomethylation resulting in a soluble by-product but requiring 16 h to complete.
20. Swelling (in CH_2Cl_2) of 1% DVB–PS is 8 mL/g, swelling of **2** was 8.1 mL/g.
21. *N*-Phthalimidomethyl resin **2** was prepared as outlined in Ref. 5 A 250 mL round-bottomed flask was charged with *N*-phthalimidomethyl resin **2** (6.02 g), 20% (v/v) ethanolamine in absolute EtOH (AR, 100 mL) and the mixture is refluxed by stirring for 7 h. The resulting mixture was cooled to room temperature and washed successively with EtOH (100 mL), DMF (100 mL), and CH_2Cl_2 (100 mL) and dried in vacuo to afford resin **5** (5.01 g, 94%) as a white solid. IR analysis showed an absence of carbonyl absorptions. Elemental analysis gave 1.22% N corresponding to a loading of 0.87 mmol/g.
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23. Albericio, F.; Barany, G. *Int. J. Pept. Prot. Res.* **1985**, *26*, 92–97.
24. *General procedure for peptide synthesis with aminomethyl resins*: For ACP (65–74); resin **5** (0.1 g) was swelled in CH_2Cl_2 and reacted with Fmoc-Gly-$OCH_2PhOCH_2CH_2CO_2H$ (2 equiv) and diisopropylcarbodiimide (DIC) (2 equiv) in CH_2Cl_2 for 1 h. The Kaiser test was negative. For oxytocin; resin **5** (0.1 g) was swelled in DMF and reacted with Rink linker (3 equiv), HOBt (3 equiv), and diisopropylcarbodiimide (DIC) (3 equiv) in DMF for 1 h. The Kaiser test was negative. All Fmoc-SPPS was performed using a Tribute 2 channel peptide synthesizer (Protein Technologies, Tucson, Az) using a 2 × 5 min deprotection with 20% (v/v) piperidine/DMF, followed by 5 × 30 s DMF washes. Coupling was performed for 30 min with Fmoc-Aa (5 equiv), HBTU (4.6 equiv) and DIPEA (10 equiv) followed by a 5 × 30 s DMF wash.