On the Use of Hydrophobic Probes in the Chromatographic Purification of Solid-phase-synthesized Peptides

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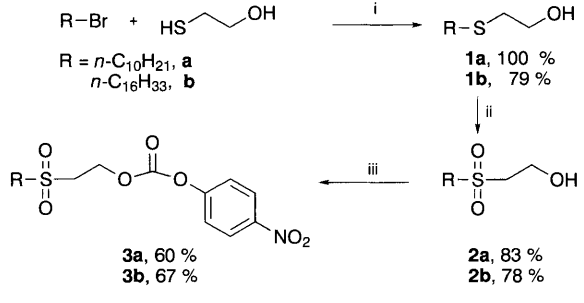
A strategy for the reversed-phase chromatographic purification of solid-phase-synthesized peptides is described and illustrated by the synthesis of a 23-mer model peptide; the target peptide is distinguished from terminated by-products by the attachment of a hydrophobic probe to the resin-bound peptide.

In solid-phase peptide synthesis, the crude compound is found to contain undesired terminated peptides originating from incomplete incorporation of an amino acid and subsequent capping with acetic anhydride to minimize the formation of deletion sequences.[1] Terminated peptides often have physical and chemical properties similar to the target peptide, making the purification step a tedious and laborious process. This communication shows that the purification difficulties caused by the presence of terminated peptides in the crude mixture can be overcome by introducing an *n*-alkyl group into the resin-bound peptide at the last coupling step. The hydrophobic probe, which must be quantitatively incorporated and removed, increases the lipophilicity of the target peptide in relation to that of the terminated sequences and thus simplifies the reversed-phase chromatographic purification process.† The reagents used for incorporating two such hydrophobic probes have been synthesized (Scheme 1) and their applications evaluated in the solid-phase synthesis of a 23-mer model peptide, H-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Gly-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Lys-Ser-NH_2 (Magainin 1).[3,4]

The starting thioethers **1a,b** were prepared in high yields by treating $HSCH_2CH_2OH$ with the corresponding 1-bromo-*n*-alkyl reactant (Scheme 1). Hydrogen peroxide oxidation of **1a,b** in the presence of a catalytic amount of sodium tungstate dihydrate gave the sulfones **2a,b**, which were purified by crystallization from dichloromethane. The desired active alkoxy carbonates **3a,b** were obtained from the reaction of **2a,b** with *p*-nitrophenylchloroformate in anhydrous pyridine. Compounds **3a,b** were characterized by elemental analysis, NMR and mass spectrometry. They can be stored for seven months at 4 °C without detectable deterioration.

Magainin 1[3] was synthesized on a Milligen 9050 automated peptide synthesizer (continuous-flow), employing the fluorenylmethoxycarbonyl (Fmoc) strategy. The required Fmoc-amino acids (3 equiv.; single coupling) were incorporated using their 2,4,5-trichlorophenyl esters with minimum reaction times of 30 min. A capping procedure with acetic anhydride (5 min) was performed after the amino acid coupling step to prevent the formation of deletion sequences. Side chains were protected with the following groups: *tert*-butyl for glutamic acid and serine; *tert*-butyloxycarbonyl for lysine; and trityl for histidine. The hydrophobic probes **3a,b** (5 equiv.) were coupled manually to the *N*-terminal residue of the protected peptide resins in the presence of an equimolar amount of 1-hydroxybenzotriazole in *N*-methylpyrrolidinone. Once incorporation of **3a,b** was completed (usually after 3 h), the peptide resins were simultaneously cleaved/deprotected with trifluoroacetic acid–water (95 : 5, *v*/*v*) for 3 h at room temperature. In the crude mixtures, the methionine residue of the target peptide was partially oxidized or alkylated.[5] These side reactions could be reverted by



Scheme 1 *Reagents and conditions*: i, dicyclohexylamine (1.0 equiv.), DMF, 20 °C, 15 h; ii, 30% H_2O_2, AcOH–H_2O (1.5 : 1; *v*/*v*), 70 °C, 5 h, Na_2WO_4; iii, *p*-nitrophenyl chloroformate (1.0 equiv.), pyridine, 0 °C, 5 h

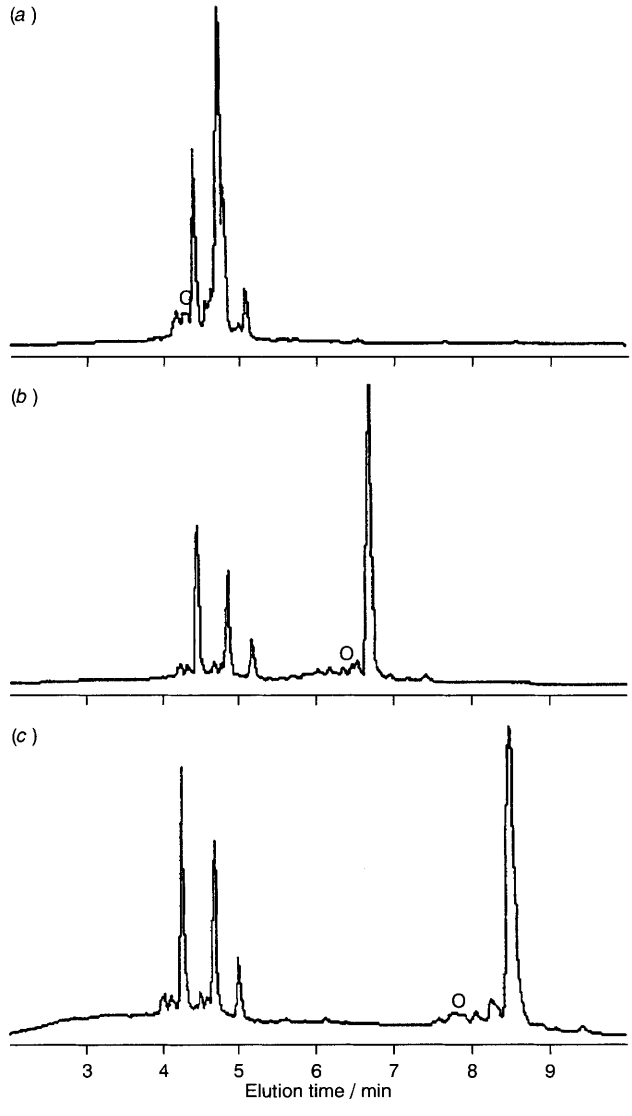


Fig. 1 Analytical reversed-phase HPLC chromatograms of the crude compounds after reversal of methionine by-products: (*a*) underivatized magainin; (*b*) **3a**-derivatized magainin; and (*c*) **3b**-derivatized magainin. Linear gradient over 8 min of MeCN–0.09% TFA and H_2O–0.1% TFA from 15 : 85 to 1 : 0 and 2 min isocratic at 1 : 0, flow rate 2.0 ml min^{-1}, detection at 215 nm. The symbol O denotes where the methionine by-products emerged. Chromatographic analysis was performed on a Nucleosil C_{18} analytical column (25 × 0.4 cm, 100 Å, 5 μm).

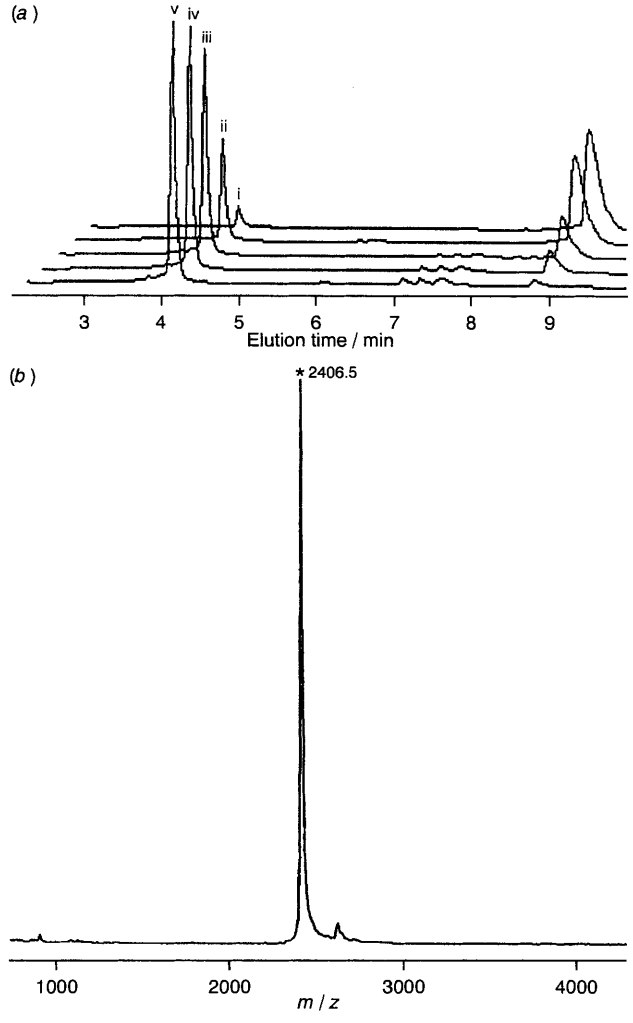


Fig. 2 (*a*) Time-line analytical reversed-phase HPLC chromatograms of the removal of the lipophilic moiety from the **3b**-derivatized magainin intermediate: i, 5; ii, 10; iii, 60; iv, 90 min; and v, 2.5 h. Linear gradient over 7 min of MeCN–0.09% TFA and H_2O–0.1% TFA from 2:8 to 1:0 and 3 min isocratic at 1:0, flow rate 2.0 ml min^{-1}, detection at 215 nm. Chromatographic analysis was performed on a Nucleosil C_{18} analytical column (25 × 0.4 cm, 100 Å, 5 μm). Conditions: 10% of NH_4OH in 2,2,2-trifluoroethanol, T = 45° C; (*b*) MALDI-TOF mass spectra (negative ion mode) of free magainin: Calc. 2406.3 [M − H], found 2406.5.

treatment of the crude compounds with ammonium iodide (reduction of the methionine sulfoxide) and mild heating in 1 mol dm^{-3} AcOH (removal of the *tert*-butyl groups from the sulfonium salts). Fig. 1 shows the analytical reversed-phase HPLC chromatograms of the underivatized and derivatized peptides. The lipophilic character of the linear alkyl moiety substantially changes the chromatographic profile of the target peptide, allowing its separation from closely related terminated peptides by medium-pressure liquid chromatography. The identity of the purified *N*-terminal alkyl peptides was confirmed by correct amino acid and mass spectral (matrix-assisted laser-desorption ionization time-of-flight mass spectrometry, MALDI-TOF) analyses.

One of the requirements in the use of hydrophobic probes is that they must be quantitatively and cleanly removed to retrieve the target peptide in its free form. Removal of the hydrophobic probes under conditions reported for an identical linkage,[6] 30 min at room temperature with 5% NH_4OH, was a sluggish reaction in our hands. Complete and clean removal of the lipophilic moieties was accomplished by treatment of the derivatized peptides with a 10% solution of NH_4OH in 2,2,2-trifluoroethanol. The reaction was followed by analytical HPLC, and complete conversion to the free peptide in its pure form was observed after 18 h at room temperature or 2.5 h at 45 °C (Fig. 2).

The results reported above show that the introduction of certain *n*-alkyl groups at the *N*-terminus of resin-bound peptides is an effective approach to facilitate the separation of the target peptide from terminated sequences. The purification method, however, will not be effective in the separation of the desired product from other peptidic by-products; *e.g.* modified, addition or deletion peptides. For this reason, we think that the use of *n*-alkyl probes will be better applicable for the purification of small to medium-size solid-phase-synthesized peptides for which there is a high capping efficiency. The low cost and easy synthesis of the hydrophobic probes **3a,b**, are also important factors to consider in the general applicability of the method.

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Footnote

† Representative reversible chromatographic probes currently published: (*i*) tetrabenzo[*a*,*c*,*g*,*i*]fluorenyl-17-methoxycarbonyl [ref. 2(*a*)]; (*ii*) 4-carboxylate Fmoc derivatives containing either hydrophobic or charged groups [ref. 2(*b*)].

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