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Facile Synthesis of 7-Amino-4-carbamoylmethylcoumarin (ACC)-Containing Solid Supports and Their Corresponding Fluorogenic Protease Substrates

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Abstract—The bifunctional fluorophore, 7-amino-4-carbamoylmethylcoumarin (ACC) without any protection groups, was regioselectively attached to different solid supports functionalized with a primary amino group. The resulting resins were used to synthesize fluorogenic protease substrates with high yield and purity.

From the genomic data collected so far, it is estimated that more than 2% of the gene products are proteases.[1] Proteases are enzymes that hydrolyze the amide backbone of proteins. Many of them are vital to every aspect of an organism's life, being involved in a plethora of cellular processes. Consequently, a number of proteases are avidly investigated currently as major therapeutic targets for diseases such as AIDS, Alzheimer's disease and cancer.[2] With the completion of the human genome project recently, many new putative proteases have been identified. There is thus a pressing need to develop rapid and general methods for high-throughput determination of protease substrate specificity. To date, a number of methods have been developed, some of which are biochemical-based, such as those based on phage-display peptide libraries on the surface of filamentous phage particles,[3] whilst others are chemical-based, in which combinatorial chemical synthesis is used to generate libraries of potential protease substrates on suitable solid supports.[4]

The use of 7-amino-4-methyl coumarin (AMC) peptide substrates for the assay of protease activity is a well-established method.[5] AMC, a fluorogenic molecule, is highly fluorescent in its free form. Upon conjugation of its aromatic amino group to the C-terminal carboxyl group of a peptide, however, the fluorescence of the molecule is essentially quenched, thereby rendering the resulting peptide–AMC conjugate practically non-fluorescent. Incubation of an AMC-containing peptide substrate with a protease leads to specific cleavage of the anilide bond between AMC and the conjugated peptide, which liberates the fluorogenic AMC leaving group, allowing for the simple determination of cleavage rates for individual substrates. Libraries of fluorogenic AMC peptide substrates have also been synthesized to investigate the substrate specificity of proteases.[6,7] In particular, positional-scanning, synthetic combinatorial libraries (PS-SCLs) have been used to establish the substrate profiles of proteases in an extremely rapid fashion, as only a few libraries, as well as assays, are needed in order to provide information for all side chains at every position of the peptide. In an elegant demonstration of this approach, Thornberry et al. synthesized Ac-X-X-X-D-AMC positional-scanning libraries and used them to investigate caspases.[7] Their design of these libraries was made possible by the absolute specificity of caspases for an aspartic acid residue at the P1 position. By attaching the carboxylic acid side chain of the C-terminal residue Asp to the solid support, the authors were able to generate AMC-containing peptide libraries and used them to screen against different caspases. However, this method is not applicable for the screening of most other proteases, as libraries having other amino acids at the C terminus can not be generated this way.[8] Recently, a more efficient method

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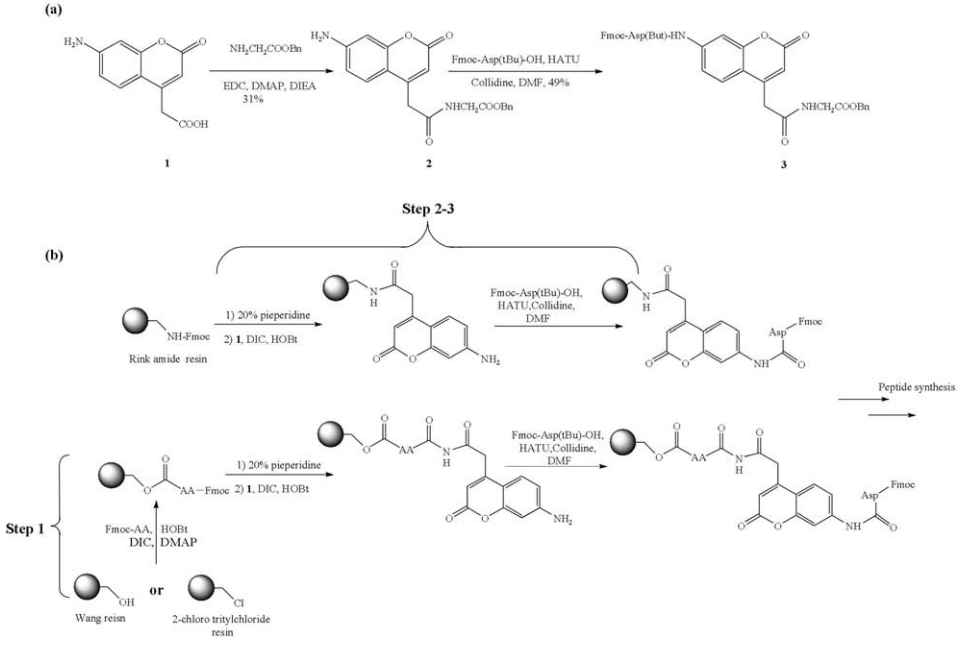
has been developed which allows for direct incorporation of any amino acid at all possible positions in a peptide substrate.[9–11] This was accomplished by the use of a bifunctional fluorophore, 7-amino-4-carbamoylmethylcoumarin (ACC), to replace the original AMC in the library synthesis.[10] With this method, the aromatic amine of ACC was first protected with a Fmoc group, followed by loading onto an amine-containing resin such as Rink amide resin, via the carboxyl group of ACC. This was then followed by piperidine deprotection of the Fmoc group and coupling of the first amino acid to the resin using Fmoc-based peptide chemistry. Compared with that of a regular aliphatic amine, the much lower nucleophilicity of an aromatic amine, such as that of ACC, has been well documented.[12] We reasoned that, given the obvious difference in reactivity, it should be possible to directly couple ACC, via its carboxyl group, to an aliphatic amine-containing resin without the need of protecting the aromatic amine on ACC. In this way, tedious steps needed to protect/deprotect the Fmoc group may be avoided. In addition, since no Fmoc group is used to protect ACC before its conjugation to a solid support, other types of peptide chemistry (i.e., Boc-based chemistry) may be readily adopted for the synthesis of ACC-containing peptides.

In this paper, we developed a facile method for the synthesis of ACC-containing solid supports and their corresponding fluorogenic peptide substrates. By conjugation of unprotected ACC directly to aliphatic amine-containing solid supports, we were able to successfully synthesize ACC-containing peptides with high yield and purity. Furthermore, we demonstrated that this strategy was also adaptable to non-amine-containing solid supports such as Wang and 2-chlorotrityl resins, which were conveniently functionalized with an amino acid linker, followed by ACC conjugation. The introduction of an extra amino acid linker between the resin and ACC is not expected to affect the protease profile of ACC-conjugated peptides.

The feasibility of this strategy was first examined and optimized in solution,[13] then applied to solid-phase reactions (Scheme 1a). Unprotected ACC, **1**, was coupled with glycine benzyl ester at room temperature to give the desired product **2** in moderate yield. Critically, no self-condensation of ACC was observed, indicating the feasibility of its direct conjugation to the solid support without protecting the aromatic amine. Following published protocols,[11] **2** was added to a mixture containing Fmoc-Asp(*t*Bu)-OH, HATU and collidine in DMF and the reaction was run at room temperature for three days, generating the desired product **3** with an yield comparable to reported data.[11] To demonstrate the generality of this strategy for solid-phase peptide synthesis, we next investigated the ACC coupling with different types of solid supports.[14] Wang, Rink amide and 2-chlorotrityl chloride resins were chosen because each contains a different functional group (Scheme 1b). For Rink amide resin, which contains an aliphatic amine, ACC was directly coupled to the resin. For Wang and 2-chlorotrityl chloride resins, which do not contain amine groups, an amino acid was first conveniently attached to the solid support to provide an amine handle for the subsequent ACC coupling. Five different Fmoc-protected amino acids were attached to each resin. Loadings of the resins were determined to be between 0.6–0.8 mmol/g. After deprotection of the Fmoc group, **1** was coupled directly to resins without any protection, followed by direct coupling of the first amino acid, Fmoc-Asp(*t*Bu)-OH using HATU coupling as previously reported.[11] The loading efficiency and purity of the steps were determined by both Fmoc UV analysis, as well as LC-MS analysis of the cleavage products.[15] Results are summarized in Table 1. Loadings of



Scheme 1.

Table 1. The efficient loading to generate Asp-ACC-AA-resins

Resin		Rink[a]	Wang					2-Chlorotrityl chloride				
Amino acid			Ala	Asp	Gly	Leu	Lys	Ala	Asp	Gly	Ile	Leu
Loading (mmol/g)	Step 1		0.67	0.75	0.72	0.67	0.76	0.63	0.9	0.80	0.75	0.69
	Step 2–3	0.62	0.62	0.68	0.68	0.62	0.68	0.20	0.18	0.21	0.16	0.15

[a]The loading efficiency of double coupling. See Scheme 1b and main text for detailed steps.

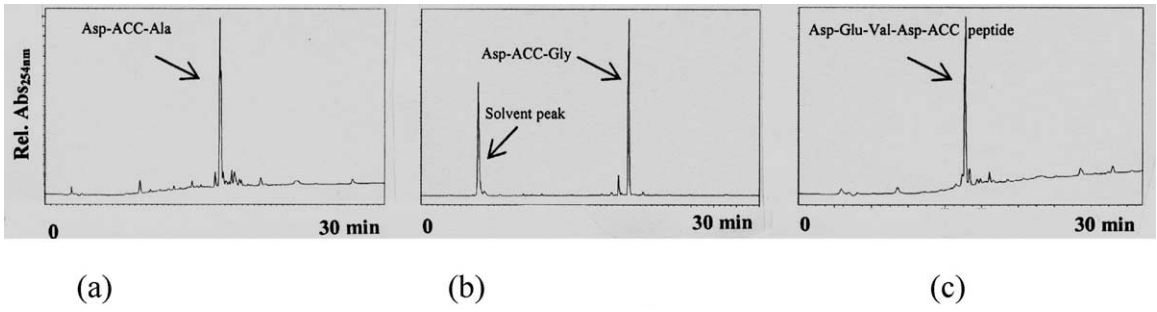


Figure 1. LC-MS analysis of products cleaved from: (a) Wang resin; (b) 2-chlorotrityl resin; (c) Rink resin.

1 with different resins, followed by first amino acid loading, were generally good, except in the case of 2-chlorotrityl resins, where loadings were ~0.2 mmol/g for Steps 2–3 combined. LC-MS analysis (vide infra) of the cleavage product, however, confirmed the formation of >90% of the desired product (Fig. 1b), indicating the low loading efficiency of ACC + first amino acid was not due to side product formation. The exact cause is still under investigation.

After three steps of ACC loading and amino acid couplings as indicated in Scheme 1b, the products were cleaved off the resins and analyzed by LC-MS in order to further assess the loading efficiency and purity.[15] All three resins produced >90% of the desired products (Fig. 1). One of them, the Asp-ACC-Rink resin was used further to synthesize a known ACC-conjugated tetrapeptide substrate of Caspase 1,[7] Ac-Asp-Glu-Val-Asp-ACC, by standard solid-phase peptide synthesis on an automatic peptide synthesizer.[16] Upon cleavage, the crude peptide product was analyzed by LC-MS (Fig. 1c), which indicated the desired product with >90% purity, further demonstrating the highly efficient nature of the ACC loading and our strategy.

In conclusion, we have developed a facile strategy that allows efficient conjugation of unprotected ACC directly on a number of resins with different types of functionalities. The strategy was simple and highly efficient, generating little or no side products. One of the ACC-conjugated resins was used to successfully synthesize a known fluorogenic tetrapeptide substrate of Caspase 1 with high efficiency and purity. This method should be amendable to the synthesis of other fluorogenic protease substrates with different types of solid-phase peptide chemistry. It should also be adaptable to the combinatorial synthesis of fluorogenic peptide libraries for potential high-throughput profiling of protease specificities.

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13. Glycine benzyl ester (305 mg, 1.1 mmol) was dissolved in DMF in an ice–salt bath under nitrogen. DIEA (500 μL, 2.88 mmol) was slowly added and the resulting mixture was stirred for 10 min, followed by addition of **1** (200 mg, 0.91 mmol), DMAP (56 mg, 0.46 mmol) and EDC (210 mg, 1.1 mmol). The reaction was allowed to warm up to room temperature and stirred overnight. The solvent was removed under reduced pressure and the residue was separated with ethyl acetate and water. The organic layer was washed with 10% hydrochloric acid and brine, dried (Na_2SO_4), and concentrated to afford a yellow solid, which was further purified by flash chromatography (silica gel, ethyl acetate/hexane = 3:1) to afford **2** as a yellow solid (120 mg, 36%). Mp 239–241; ^{1}H NMR (300 MHz, DMSO) δ 8.67 (t, J = 5.6 Hz, 1H), 7.43 (d, J = 8.7 Hz, 1H), 7.35 (s, 5H), 6.52 (d, J = 8.7 Hz, 1H), 6.41 (s, 1H), 6.13 (s, 2H), 6.00 (s, 1H), 5.12 (s, 1H), 3.93 (t, J = 5.9 Hz, 1H), 3.64 (s, 1H); HRMS (EI): calcd for $C_{20}H_{18}N_2O_5$ 366.1216, found 366.1216. Fmoc-Asp(*t*Bu)-OH (116 mg, 0.28 mmol) and HATU (212 mg, 0.56 mmol) dissolved in DMF, were added collidine (80 uL, 0.60 mmol) and the resulting solution was agitated for 10 min. Compound **2** (50 mg, 0.14 mmol) was then added and the mixture was agitated for three days. DMF was removed under reduced pressure and the residue was extracted with ethyl acetate and 10% HCl. The organic layer was washed with brine, dried (Na_2SO_4), and concentrated to afford a yellow oil, which was further purified by flash chromatography (silicon gel, ethyl acetate/hexane = 3:1) to afford **3** as a white plate (52 mg, 49%). Mp: 155–157. ^{1}H NMR (300 MHz, DMSO) δ 10.57 (s, 1H), 8.72 (t, J = 5.6 Hz, 1H), 7.90–7.88 (m, 3H), 7.78–7.72 (m, 4H), 7.50 (d, J = 8.7 Hz, 1H), 6.37 (s, 1H), 5.12 (s, 1H), 4.55–4.53 (M, 1 h), 4.33–4.20 (M, 3 h), 3.95 (t, J = 5.9 Hz, 1H), 3.77 (s, 1H), 2.79–2.71 (m, 1H), 2.61–2.50 (m, 1H), 1.37 (s, 9H); HRMS (FAB): calcd for $C_{43}H_{41}N_3O_{10}Na$ 782.2690, found 782.2685.

14. Representative synthesis of Asp-ACC-AA-resin. Wang resin (0.2 g, 1.0 mmol/g) was suspended in a 2 mL DCM/DMF (9:1) solution. Fmoc-Gly-OH (0.3 g, 1.0 mmol), HOBt (0.15 g, 1.0 mmol), DIC (0.156 mL, 0.2 mmol), DMAP (2.4 mg, 0.1 mmol) were added. After stirring for 4 h at room temperature, acetic anhydride (41 mg) and pyrindine (32 mg) were added and the mixture was reacted further for 30 min. Filtration and wash of the resin with 3×20 mL DMF (AR grade), 3×20 mL DCM, 3×20 mL MeOH give Fmoc-Gly-Wang resin. The substitution level of the resin was 0.72 mmol/g (72%) as determined by Fmoc UV analysis. To a 20 mL conical tube was added the resin (0.1 g) and 10 mL 20% piperidine in DMF. After shaking for 30 min, the resin was filtrated and washed with DMF (3×20 mL). Compound **1** (66 mg, 0.3 mmol), HOBt (46 mg, 0.3 mmol), DIC (38 mg, 0.3 mmol) in 2 mL DMF were subsequently added. The mixture was shaken for two days and then filtered, washed with DMF (3×20 mL), DCM (3×20 mL), MeOH (3×20 mL) and dried over P_2O_5 to give ACC-Gly-Wang resin. To a 20 mL conical test tube was added the above resin (0.1 g) and DMF (2 mL). Fmoc-Asp(*t*Bu)-OH (0.288 g, 0.7 mmol), HATU (0.266 g, 0.7 mmol), and 2,4,6-collidine (85 mg, 0.7 mmol) were added, followed by shaking for three days to give Fmoc-Asp(*t*Bu)-ACC-Gly-Wang resin. The resin was filtered and washed with DMF (3×20 mL). The substitution level of the resin was determined by Fmoc UV analysis to be 0.68 mmol/g (>95%).

15. LC was performed on a Waters™ 600 HPLC equipped with a Phenomenex RP-18 (5 μm, 4.6×250 mm) column using an acetontrile–water gradient (with 0.1% TFA). MS was run on an Electrospray mass spectrometer (Finnigan, USA).

16. Peptides were synthesized on Pioneer™ Peptide Synthesizer (PerSeptive Biosystems, USA) using standard Fmoc peptide chemistry with the HBTU/HOBt/DIEA coupling method.