# Expedient Solid-Phase Synthesis of Fluorogenic Protease Substrates Using the 7-Amino-4-carbamoylmethylcoumarin (ACC) Fluorophore

Dustin J. Maly,[†] Francesco Leonetti,[†] Bradley J. Backes,[†] Deborah S. Dauber,[§] Jennifer L. Harris,[§] Charles S. Craik,[§] and Jonathan A. Ellman*,[†]

*Department of Chemistry, University of California, Berkeley, California 94720 and Department of Pharmaceutical Chemistry, Program in Chemistry and Chemical Biology, University of California, San Francisco, California 94143*

*jellman@ucink4.berkeley.edu*

*Received September 23, 2001*

A highly efficient solid-phase synthesis method for the preparation of fluorogenic protease substrates based upon the bifunctional leaving group 7-amino-4-carbamoylmethylcoumarin (ACC) is reported. Methods for the large-scale preparation of the novel fluorogenic leaving-group ACC are provided (Scheme 1). Detailed procedures are also provided for loading a diverse set of amino acids to support-bound ACC in good yields and with minimal racemization. Finally, procedures are included for the preparative synthesis of optimized ACC substrates for HIV-1 protease and plasmin.

## Introduction

Proteases, a family of enzymes that catalyze the hydrolysis of amide bonds in proteins and peptides, are ubiquitous in nature and constitute approximately 2% of all known gene products.[1] These enzymes play a critical role in virtually all biological processes and represent important drug targets in numerous therapeutic areas,[2] including cancer,[3] AIDS,[4] cardiovascular disease,[5] neurodegenerative disease,[6] and osteoperosis.[7]

Fluorogenic substrates that provide a spectrophotometric readout upon proteolysis are one of the most important chemical tools for the characterization of proteases.[8] Fluorogenic substrates allow for the continuous kinetic analysis of proteases and are useful reagents in the screening of potential protease inhibitors. In addition, these substrates can serve as powerful tools for determining protease substrate specificity,[9] which can provide valuable insight into biological function and also aid in the design of potent and selective substrates and inhibitors.

Fluorogenic 7-amino-4-methylcoumarin (AMC) peptide substrates **1** (Figure 1) are extensively utilized in the study of proteases.[10] However, general solid-phase synthesis methods are not available for the preparation of these substrates because the coumarin lacks the necessary functionality for linkage to a solid support. Attachment to solid support through the side chain functionality of the *C*-terminal amino acid has been employed,[11] but this method is difficult to apply for a majority of amino acids.[12] Therefore, we have recently developed a modified aminocoumarin leaving group, 7-amino-4-carbamoylmethylcoumarin (ACC), that can be directly attached to a

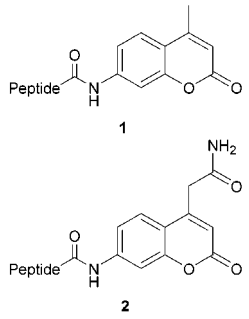



**Figure 1.** Fluorogenic substrates peptide−AMC, **1**, and peptide−ACC, **2**.

[†] University of California, Berkeley.

[§] University of California, San Francisco.

(1) Barrett, A. J.; Rawlings, N. D.; Woessner, J. F. *Handbook of Proteolytic Enzymes*; Academic Press: London, 1998.

(2) Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 305.

(3) Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing A. J. H. *Chem. Rev.* **1999**, 99, 2735.

(4) (a). Molla, A.; Granneman, G. R.; Sun, E.; Kempf, D. J. *Antiviral Res.* **1998**, *39*, 1. (b) Kempf, D. J.; Sham, H. L. *Curr. Pharm. Des.* **1996**, *2*, 225.

(5) Ripka, W. C. *Curr. Opin. Chem. Biol.* **1997**, 1, 242.

(6) Vassar, R.; Bennett, B. D.; Babu-Kahn, S.; Kahn, S.; Mendiaz, E. A.; Denis, P.; Teplow, D. B.; Ross, S.; Amarante, P.; Loeloff, R.; et al. *Science* **1999**, *286*, 735.

(7) Drake, F. H.; Dodds, R. A.; James, I. E.; Connor, J. R.; Debouck, C.; Richardson, S.; LeeRykaczewski, E.; Coleman, L.; Rieman, D.; Barthlow, R.; Hastings, G.; Gowen, M. *J. Biol. Chem.* **1996**, *271*, 12511.

(8) Knight, C. G. *Methods Enzymol.* **1995**, *248*, 18.

(9) (a) Harris, J. L.; Backes, B. J.; Leonetti, F.; Mahrus, S.; Ellman, J. A.; Craik, C. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7754. (b) Backes, B. J.; Harris, J. L.; Leonetti, F.; Craik, C. S.; Ellman, J. A. *Nat. Biotechnol.* **2000**, *18*, 187. (c) Thornberry, N. A.; Rano, T. A.; Peterson, E. P.; Rasper, D. M.; Timkey, T.; Garcia-Calvo, M.; Houtzager, V. M.; Nordstrom, P. A.; Roy, S.; Vaillancourt, J. P. et al. *J. Biol. Chem.* **1997**, *272*, 17907. (d) Meldal, M.; Svendsen, I.; Breddam, K.; Auzanneau, F. I. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3314. (e) St. Hilaire, P. M.; Willert, M.; Juliano, M. A.; Juliano, L.; Meldal, M. *J. Comb. Chem.* **1999**, *1*, 509.

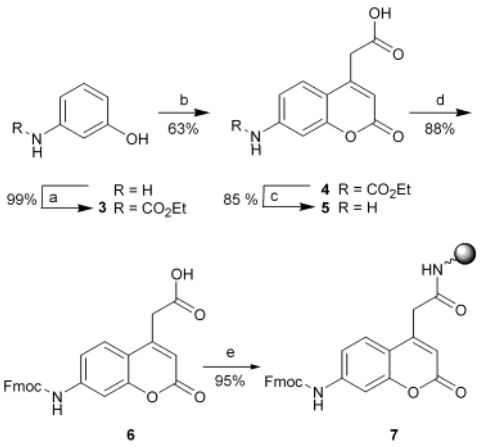
(10) Zimmerman, M.; Ashe, B.; Yurewicz, E.; Patel, G. *Anal. Biochem.* **1977**, *78*, 47.

(11) (a) Rano, T. A.; Timkey, T.; Peterson, E. P.; Rotonda, J.; Nicholson, D. W.; Becker, J. W.; Chapman, K. T.; Thornberry, N. A. *Chem. Biol.* **1997**, *4*, 149. (b) Edwards, P. D.; Mauger, R. C.; Cottrell, K. M.; Morris, F. X.; Pine, K. K.; Sylvester, M. A., Scott, C. W., Furlong, S. T. *Biorg. Med. Chem. Lett.* **2000**, *10*, 2291.

(12) Safety-catch linkers can be used to access virtually any aminomethyl coumarin substrate by nucleophilic attack of an aminocoumarin amide derivative of the relevant amino acid upon a support-bound peptide substrate. However, this method requires additional effort to scavenge the aminocoumarin amide nucleophile. See ref 9b.

10.1021/jo016140o CCC: $22.00 
Published on Web 01/12/2002

**Scheme 1**[a]




[a] (a) Ethyl chloroformate (0.5 equiv), EtOAc, reflux; (b) 1,3-acetonedicarboxylic acid (1.1 equiv), 70% $H_2SO_4$; (c) 10 M NaOH, reflux; (d) i. TMSCl (2.1 equiv), *i*-$Pr_2EtN$ (2.1 equiv); ii. FmocCl (1.1 equiv); (e) Rink amide AM resin (0.5 equiv), HOBt (1 equiv), DICI (1 equiv), DMF.

solid support, enabling solid-phase synthesis of peptide−ACC substrates **2**. Notably, for a number of proteases, including thrombin and plasmin, fluorogenic peptide substrates based upon ACC **2** were shown to have similar kinetic parameters to the corresponding AMC peptide substrates **1**.[9a] Furthermore, the substrate specificities of numerous proteases have been determined using positional-scanning libraries of ACC fluorogenic peptide substrates **2**. These results were validated by the high level of correlation between substrate specificity profiles obtained with ACC **2** and AMC **1** substrate libraries for thrombin and plasmin and by the consistency with previously determined specificity information.

Herein, we report a method for the efficient large-scale preparation of the novel fluorogenic leaving group ACC. Detailed procedures are also provided for loading a diverse set of Fmoc-amino acids to support-bound ACC in good yields and with minimal racemization. Finally, in previous work we identified optimized fluorogenic ACC peptide substrates for numerous therapeutically important proteases.[9a] Procedures are provided for the preparative synthesis of optimized ACC substrates for HIV-1 protease and plasmin.

## Results and Discussion

**Synthesis of ACC−Resin.** Three criteria were deemed necessary for the preparation of the support-bound ACC. First, the synthesis sequence must be efficient and allow for the preparation of multigram quantities of compound without the need for column chromatography. Second, the ACC must be loaded to support in an efficient manner without the formation of byproducts. Finally, the resin-bound ACC should be amenable to standard solid-phase Fmoc-peptide synthesis. To meet these criteria, *N*-Fmoc-7-amino-4-carboxymethylcoumarin **6** was prepared as a bifunctional molecule to be loaded onto solid support via the acid-labile Rink linker (Scheme 1). Starting from commercially available 3-aminophenol, treatment with ethyl chloroformate provides carbamate **3**. Coumarin **4** was formed via a Pechmann reaction of carbamate **3** with 1,3-acetonedicarboxylic acid in 70% $H_2SO_4$. An extensive study of experimental parameters was undertaken to optimize conditions for this reaction. The two most significant factors were the choice of $\beta$-keto electrophile and the concentration of the reaction mixture. Using a $\beta$-ketodiester (e.g., diethyl 1,3-acetonedicarboxylate) or running the reaction at a concentration greater than 0.2 M caused significantly increased formation of byproducts. However, by using 1,3-acetonedicarboxylic acid[13] at a concentration of 0.2 M the reaction can be run on 100 g scale to afford pure **4** in 63% yield after precipitation and crystallization from $CH_3CN$. The carbamate of **4** was hydrolyzed using 10 M NaOH with heating to reflux, and the resulting free amine **5** was protected with FmocCl utilizing the Meienhofer procedure to provide acid **6**.[14] ACC−resin **7** was then prepared by coupling acid **6** to Rink amide AM resin using standard DICI and HOBt coupling conditions. The substitution level of the resin was 0.58 mmol/g (>95%) as determined by Fmoc analysis.[15]

**Loading of the First Amino Acid.** Due to the poor nucleophilicity of the support-bound aminocoumarin, specialized coupling conditions were necessary to efficiently load the first amino acid. Specifically, the use of the in situ activating agent HATU (*N*-[(dimethylamino)-1-*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide) in the presence of an equimolar amount of the hindered organic base 2,4,6-collidine gave the best substitution levels with minimal racemization.[16] The level of racemization during the loading of the first amino acid was evaluated for the representative amino acids Fmoc-Asn-(Trt)-OH and Fmoc-Leu-OH (Scheme 2). ACC−resin loaded with Fmoc-L-Asn(Trt)-OH **8a** and Fmoc-L-Leu-OH **8b** was coupled with Fmoc-L-Phe-OH under standard solid-phase conditions. No DL epimer (<1.0%) was observed by reverse-phase HPLC anaysis of the resulting dipeptide products **10a** and **10b**. Similarly, Fmoc-L-Phe-OH was coupled to ACC−resin loaded with Fmoc-D-Asn-(Trt)-OH **9a** and Fmoc-D-Leu-OH **9b**. No LL-epimer (<1.0%) was observed for the dipeptide products **11a** and **11b**.

To demonstrate the generality of our coupling conditions, a diverse set of Fmoc-amino acids were coupled to **7** and the loading efficiencies were determined (Scheme 3, Table 1). For the majority of the amino acids tested, good yields were achieved with a single coupling step. For those amino acids that provided lower coupling yields (Pro, Thr, Ile, Val, and Arg), a second coupling was carried out to increase the loading level.

Although our chosen coupling conditions gave high coupling yields for most amino acids, the coupling of Fmoc-His(Trt)-OH (Table 1, entry **8i**) was accompanied by an unexpected competitive transfer of the side chain trityl group to the support-bound ACC.[17] To efficiently load His onto the support-bound coumarin without trityl

(13) It is important that the 1,3-acetonedicarboxylic acid used in this step is pure. Contamination with the decarboxylation product, acetoacetic acid, leads to the formation of 7-carbethoxyamido-4-methylcoumarin, which is difficult to remove at this step.

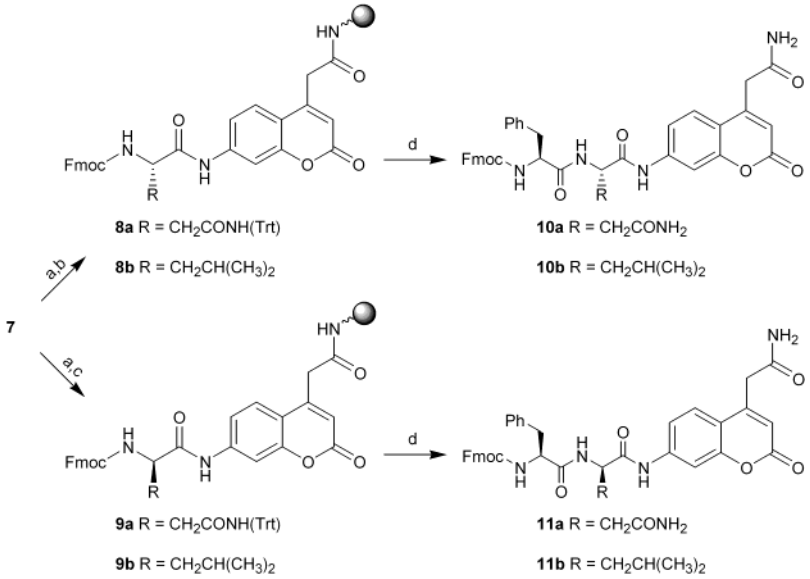
(14) Bolin, D. R.; Sytwu, I.-I.; Humiec, F.; Meienhofer, J. *Int. J. Pept. Protein Res.* **1989**, *33*, 353.

(15) Bunin, B. A. *The Combinatorial Index*; Academic Press: San Diego, 1998; pp 213−236.

(16) Carpino, L. A.; Ionescu, D.; El-Faham, A. *J. Org. Chem.* **1996**, *61*, 2460.

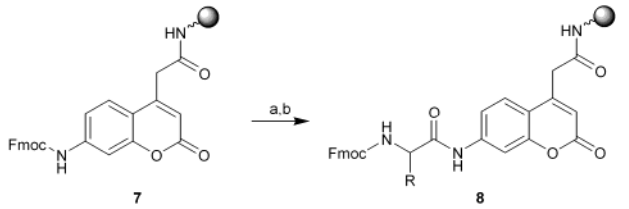
(17) Trityl transfer was established by attempted acetylation to cap the unreacted coumarin. However, after acidic cleavage from support only His-ACC and free ACC were detected without any presence of acetyl-ACC.

**Scheme 2**[a]




[a] (a) 20% Piperidine/DMF; (b) Fmoc-L-Asn(Trt)-OH (5 equiv) or Fmoc-L-Leu-OH (5 equiv), HATU (5 equiv), 2,4,6-collidine (5 equiv), DMF, 24 h; (c) Fmoc-D-Asn(Trt)-OH (5 equiv) or Fmoc-D-Leu-OH (5 equiv), HATU (5 equiv), 2,4,6-collidine (5 equiv), DMF, 24 h; (d) i. 20% piperidine/DMF; ii. Fmoc-L-Phe-OH, DICI, HOBt, DMF; iii. TFA/*i*-$Pr_3SiH/H_2O$ (95:2.5:2.5).

**Scheme 3**[a]




[a] (a) 20% Piperidine/DMF; (b) HATU (5 equiv), 2,4,6-collidine (5 equiv), Fmoc-amino acid (5 equiv), DMF, 24 h.

**Table 1. Loading Efficiencies for a Diverse Set of Amino Acids to Support-Bound ACC**

| resin product | Fmoc-amino acid | yield,[a] % |
|---|---|---|
| **8c** | Fmoc-Ala-OH | 80 |
| **8d** | Fmoc-Arg(Pbf)-OH | 50[b] |
| **8a** | Fmoc-Asn(Trt)-OH | 85 |
| **8e** | Fmoc-Asp(O-*t*-Bu)-OH | 92 |
| **8f** | Fmoc-Glu(O-*t*-Bu)-OH | 82 |
| **8g** | Fmoc-Gln(Trt)-OH | 81 |
| **8h** | Fmoc-Gly-OH | 81 |
| **8i** | Fmoc-His(Trt)-OH | 78 |
| **8j** | Fmoc-His(Boc)-OH | 90 |
| **8k** | Fmoc-Ile-OH | 57[b] |
| **8b** | Fmoc-Leu-OH | 83 |
| **8l** | Fmoc-Lys(Boc)-OH | 84 |
| **8m** | Fmoc-Met-OH | 94 |
| **8n** | Fmoc-Nle-OH | 83 |
| **8o** | Fmoc-Phe-OH | 98 |
| **8p** | Fmoc-Pro-OH | 75[b] |
| **8q** | Fmoc-Ser(O-*t*-Bu)-OH | 92 |
| **8r** | Fmoc-Thr(O-*t*-Bu)-OH | 70[b] |
| **8s** | Fmoc-Trp(Boc)-OH | 94 |
| **8t** | Fmoc-Tyr(O-*t*-Bu)-OH | 98 |
| **8u** | Fmoc-Val-OH | 57[b] |

[a] Loading efficiency determined by Fmoc analysis. [b] Yield for double coupling.

transfer, several different coupling conditions were explored (Table 2). Trityl transfer could be completely suppressed by using 10 equiv of base to HATU. However, under all of these conditions the use of a large excess of base resulted in extensive epimerization of this racemization-prone amino acid (Table 2).[18] We then explored the use of alternative side-chain protecting groups. When Fmoc-His(Boc)-OH was used with our standard coupling conditions, we obtained a high loading level of **8j**, with a significant decrease in racemization (4.0% DL-epimer). Thus, by using Fmoc-His(Boc)-OH rather than Fmoc-His(Trt)-OH for the coupling of the first amino acid, uniform coupling conditions can be employed to load all tested Fmoc-amino acids.

**Table 2. Optimization of Reaction Conditions to Load Histidine to Support-Bound ACC**

| | collidine/ HATU[a] | DL-epimer, %[b] | trityl transfer, %[c] |
|---|---|---|---|
| Fmoc-His(Trt)-OH | 1:1 | 17 | 15 |
| Fmoc-His(Trt)-OH | 5:1 | 27 | 4 |
| Fmoc-His(Trt)-OH | 10:1 | 40 | <1 |
| Fmoc-His(Boc)-OH | 2:1 | 4.7 | – |
| Fmoc-His(Boc)-OH | 1:1 | 4.0 | – |

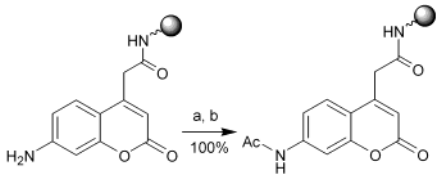
[a] Equivalent of 2,4,6-collidine/equivalent of HATU. [b] Percentage of DL-epimer was determined by reverse-phase HPLC analysis of Fmoc-L-Phe-L-His-ACC dipeptide. [c] Trityl transfer determined by HPLC quantitation of unacylated ACC.[17]

**Capping of Unreacted Aminocoumarins.** When purification of fluorogenic substrates is not straightforward, (e.g., when combinatorial libraries of peptide substrates are generated), a method for completely acetylating all unreacted support-bound aminocoumarin is essential. Quantitative capping of unacylated coumarin is necessary because the presence of unreacted ACC creates a high background fluorescence that reduces the sensitivity of the assay. A number of conditions were explored for the complete acylation of the support-bound aminocoumarin. The optimal results were obtained using the nitrotriazole ester of acetic acid.[19] Treating the resin

(18) Benoitin, N. L. Quantitation and Sequence Dependence of Racemization in Peptide Synthesis. In *The Peptides: Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1983; vol. 5, p 260.

(19) Blankemeyer-Menge, B.; Nimtz, M.; Frank, R. *Tetrahedron Lett.* **1990**, *31*, 1701.

**Scheme 4**[a]




[a] (a) AcOH (10 equiv), 3-nitro-1,2,4-triazole (10 equiv), DICI (10 equiv), DMF, 24 h; (b) *i*-$Pr_2EtN$, 3 h.

with AcOH, 3-nitro-1,2,4-triazole, and DICI in DMF, followed by addition of *i*-$Pr_2EtN$ after 24 h, quantitatively acetylated any unreacted ACC (Scheme 4).

**Synthesis and Evaluation of Fluorogenic Peptide Substrates of HIV-1 Protease and Plasmin.** To illustrate the facility of the solid-phase synthesis method for the production of useful quantities of fluorogenic substrates, ACC−peptide substrates Ac-Lys-Gln-Trp-Lys-ACC **12** for plasmin and Ac-Arg-Lys-Ser-Leu-Val-Nle-ACC **13** for HIV-1 protease were prepared. These ACC−peptides were determined to be optimal substrates, as reported previously, using positional scanning combinatorial substrate libraries.[9a,20] Substrates **12** and **13** were prepared using the appropriately substituted Fmoc-amino acid ACC−resins (**8l** and **8n**) and standard HOBt and DICI coupling conditions. After purification by preparative HPLC, the substrates were obtained in 53% and 55% overall yield, respectively. The kinetic parameters for each substrate were determined as shown in Table 3. The cleavage of ACC substrate **13** by HIV-1 protease is particularly noteworthy since AMC substrates have not previously been reported for HIV-1 protease. Because the ACC fluorogenic substrate is more straightforward to prepare and is significantly more stable than the standard fluorescence-quenched peptide substrates for HIV-1 protease,[21] fluorogenic substrate **13** may be of utility for inhibitor characterization and screening efforts.

## Conclusion

An efficient strategy has been developed for the straightforward solid-phase synthesis of single fluorogenic substrates. This methodology allows for the straightforward extension to fluorogenic-substrate libraries. A large number of diverse amino acids can be efficiently coupled to the ACC−resin with acceptable loading levels and minimal racemization. A capping protocol has also been developed to quantitatively acetylate any unreacted ACC. As previously reported, the resulting ACC−peptides can be utilized to rapidly identify preferred peptide substrates using positional scanning libraries. Furthermore, fluorogenic substrates prepared with ACC should be of considerable use for biochemical studies, as demonstrated by the straightforward preparation of the fluorogenic substrates **12** and **13** for plasmin and HIV-1 protease, respectively.

## Experimental Section

**Reagents and General Methods.** Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Rink Amide AM resin and Fmoc-amino acids were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). The amine substitution level of the Fmoc-substituted Rink resin was determined (0.63 mmol/gram) by a spectrophotometric Fmoc-quantitation assay.[15] Anhydrous, low-amine content DMF was purchased from EM Science. HATU was purchased from Perseptive Biosystems (Framingham, MA). DICI, HOBt, AcOH, FmocCl, TFA, 2,4,6-collidine, and *i*-$Pr_3SiH$ were purchased from Aldrich and used without further purification. TMSCl was freshly distilled under $N_2$ from $CaH_2$. Prior to use, 1,3-acetonedicarboxylic acid, which was purchased from Aldrich, was assessed by $^1H$ NMR to be free of acetoacetic acid.[13] Thin-layer chromatography was carried out on Merck 60 $F_{254}$ 250-$\mu$m silica gel plates. IR spectra were recorded as KBr pellets. NMR chemical shifts are reported in ppm downfield from an internal solvent peak and *J* values are in hertz. All HPLC analyses were performed utilizing a Rainin Dynamax Microsorb C18 reverse-phase analytical column (4.6 mm × 25 cm). All HPLC purifications were carried out using a Varian Microsorb C18 reverse-phase preparatory column (21.4 mm × 25 cm). The human enzyme plasmin was purchased from Haematologic Technologies Inc. (Essex Jct., VT) and used as received.

**3-*N*-(Carbethoxy)aminophenol (3).** To a 2 L round-bottom flask fitted with a stir bar were added 3-aminophenol (150 g, 1.37 mol) and EtOAc (500 mL). The flask was fitted with a condenser, and the mixture was heated to reflux for 30 min. Ethyl chloroformate (74.6 g, 0.687 mol) was added dropwise over a 1 h period. The reaction mixture was allowed to cool to room temperature at which time a white precipitate formed. The precipitate was removed by filtration and washed with EtOAc (3 × 300 mL) and petroleum ether (3 × 300 mL). The combined filtrate was concentrated to afford 123 g (99%) of **3** as a white solid. The product was sufficiently pure by NMR analysis to be taken on to the next step without further purification. The analytical data are in agreement with literature values.[22] $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 1.19 (t, 3, $J$ = 7.1), 4.06 (quartet, 2, $J$ = 7.1), 6.32−6.36 (m, 1), 6.75−6.82 (m, 1), 6.95−6.99 (m, 2), 9.29 (br s, 1), 9.44 (s, 1).

**7-*N*-(Carbethoxy)aminocoumarin-4-acetic Acid (4).** To a 5 L round-bottom flask fitted with a stir bar were added **3** (100 g, 0.552 mol) and 70% $H_2SO_4$ (2.75 L). The rapidly stirred reaction mixture was cooled in an ice bath, and 1,3-acetonedicarboxylic acid (88.7 g, 0.607 mol) was added in portions. The reaction mixture was allowed to warm to room temperature and stirred for 8 h, after which the reaction mixture was poured onto ice (4 kg) and stirred for 30 min. The resultant white precipitate was collected by filtration and washed with $Et_2O$ (3 × 2 L). The crude material was then partially dissolved in hot $CH_3CN$ (700 mL). Upon cooling to room temperature, the precipitate was collected to afford 101 g (63%) of **4** as a white solid. mp 194−195 °C. $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 1.21 (t, 3, $J$ = 7.1), 3.81 (s, 2), 4.11 (quartet, 2, $J$ = 7.1), 6.28 (s, 1), 7.31−7.35 (m, 1), 7.52−7.58 (m, 2), 10.12 (s, 1). $^{13}C$ NMR (125 MHz, DMSO-$d_6$): $\delta$ 14.4, 37.1, 60.8, 104.5, 113.7, 113.7, 114.3, 126.1, 143.0, 149.8, 153.4, 154.1, 160.1, 170.7. Anal. Calcd for $C_{14}H_{13}NO_6$: C, 57.73; H, 4.50; N, 4.81. Found: C, 57.57; H, 4.57; N, 4.70.

If the 1,3-acetonedicarboxylic acid used in this reaction is contaminated with acetoacetic acid, the formation of 7-carbethoxyamido-4-methylcoumarin can result. This byproduct can be removed in the next step.

**7-Aminocoumarin-4-acetic Acid (5).** To a 2 L round-bottom flask fitted with a condenser were added **4** (101 g, 0.345 mol), NaOH (138 g, 3.45 mol), and $H_2O$ (900 mL). The reaction mixture was stirred at reflux for 16 h. After cooling to room temperature, the pH of the reaction mixture was adjusted to 2, by the dropwise addition of $H_2SO_4$. The resultant yellow precipitate that formed was collected by filtration to afford 64.3 g (85%) of **5** as a fluffy yellow powder. The product was sufficiently pure by NMR analysis to be taken on to the next step without further purification. The analytical data are in

(20) Dauber, D. S.; Ziermann, R.; Parkin, N.; Maly, D. J.; Mahrus, S.; Harris, J. L.; Ellman, J. A.; Petropoulos, C.; Craik, C. S. *J. Virol.* **2002**, *76*, 1359.
(21) Krafft, G. A.; Wang, G. T. *Methods Enzymol.* **1994**, 241, 70.
(22) Atkins, R. L.; Bliss, D. E. *J. Org. Chem.* **1978**, *43*, 1975.

**Table 3. Kinetic Parameters for Fluorogenic Substrates**

| substrate | yield, % | $k_{cat}$, $s^{-1}$ | $K_m$, mM | $k_{cat}/K_m$, $M^{-1}$ $s^{-1}$ |
|---|---|---|---|---|
| Ac-Lys-Gln-Trp-Lys-ACC (**12**) | 53 | 11.0 ± 0.4 | 0.040 ± 0.005 | 280000 ± 10000 |
| Ac−Arg-Lys-Ser-Leu-Val-Nle-ACC (**13**) | 55 | nd[a] | nd[a] | 296 ± 83.4 |

[a] Not determined.

agreement with literature values.[23] $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 3.73 (s, 2H), 5.97 (s, 1), 6.16 (br s, 2), 6.41 (d, 1, $J$ = 0.7), 6.54 (dd, 1, $J$ = 8.6, 2.1), 7.33 (d, 1, $J$ = 8.6), 12.65 (s, 1).

To remove any 7-amino-4-methylcoumarin, which is formed by the hydrolysis of the 7-carbethoxyamido-4-methylcoumarin byproduct formed in the last step, the following procedure was followed. The yellow precipitate is dissolved in a minimal volume of 2 M NaOH. The brown solution is then filtered to remove any insoluble 7-amino-4-methylcoumarin. The pH of the filtrate is then adjusted to 2, and the yellow precipitate that forms is collected by filtration to afford pure **5**. This procedure generates product **5** free of any contaminants with a minimal loss in yield (∼10%).

**7-*N*-(Fluorenylmethoxycarbonyl)aminocoumarin-4-acetic Acid (6).** To a 1 L round-bottom flask fitted with a condenser and heating mantle were added **5** (20.0 g, 91.2 mmol) and $CH_2Cl_2$ (150 mL). To this stirring suspension was added freshly distilled TMSCl (21.8 g, 201 mmol) and *i*-$Pr_2$-EtN (25.9 g, 201 mmol). *It is important that the TMSCl is freshly distilled*. The reaction mixture was stirred and heated to reflux for 3 h, followed by cooling in an ice bath. FmocCl (26.0 g, 100 mmol) was then added in portions, and the reaction mixture was allowed to warm to room temperature, followed by stirring for an additional 11 h. The reaction mixture was then stirred rapidly, and MeOH (500 mL) was added. The resultant white precipitate that formed was collected by filtration and washed with MeOH (2 × 250 mL) and $Et_2O$ (2 × 500 mL) to afford 35.4 g (88%) of **6**. mp 273−276 °C. IR 3305, 3038, 1735, 1701 $cm^{-1}$. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 3.86 (s, 2), 4.33 (t, 1, $J$ = 6.2), 4.55 (d, 2, $J$ = 6.2), 6.34 (s, 1), 7.33−7.44 (m, 5), 7.56 (s, 1), 7.61 (d, 1, $J$ = 8.6), 7.76 (d, 2, $J$ = 7.3), 7.91 (d, 2, $J$ = 7.4) 10.23 (s, 1), 12.84 (s, 1). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 37.1, 46.6, 65.9, 104.7, 113.8, 113.8, 114.4, 120.2, 125.1, 126.1, 127.1, 127.7, 140.8, 142.7, 143.6, 149.8, 153.2, 154.0, 160.0, 170.6. Anal. Calcd for $C_{26}H_{19}NO_6$: C, 70.74; H, 4.34; N, 3.17. Found: C, 70.56; H, 4.18; N, 3.17.

**ACC−Resin Synthesis (7).** To a 5 L round-bottom flask were added Rink Amide AM resin (95 g, 60 mmol) and DMF (1 L). The mixture was gently stirred with an overhead mechanical stirring apparatus for 30 min and then filtered using a filter cannula (Pharmacia, Uppsala, Sweden). A 20% solution of piperidine in DMF (1 L) was then added, and the mixture was agitated for 30 min. The resin was filtered and washed with DMF (3 × 1 L). ACC (53 g, 120 mmol), HOBt (18.5 g, 120 mmol), and DMF (1 L) were added to the flask followed by DICI (19 mL, 120 mmol). The mixture was agitated overnight and then filtered, washed with DMF (3 × 1 L), THF (3 × 1 L), and MeOH (3 × 1 L), and dried over $P_2O_5$. The substitution level of the resin was 0.53 mmol/g (>95%) as determined by Fmoc-analysis.[15]

**Procedure for Coupling Fmoc-Amino Acids to the ACC−Resin (8a−u).** To a 100 mL round-bottom flask were added ACC−resin (5.0 g, 2.6 mmol) and DMF (50 mL). The mixture was stirred using a magnetic stir bar for 30 min and filtered with a filter cannula. A 20% solution of piperidine in DMF (50 mL) was added followed by agitation for 30 min. The resin was filtered and washed with DMF (3 × 50 mL). An Fmoc-amino acid (13 mmol), DMF (30 mL), HATU (5.0 g, 13 mmol), and 2,4,6-collidine (1.8 mL, 13 mmol) were added to the flask followed by agitation for 24 h. The resin was then filtered and washed with DMF (3 × 50 mL). To efficiently load Fmoc-Arg(Pbf)-OH, Fmoc-Ile-OH, Fmoc-Thr(O-*t*-Bu)-OH, Fmoc-Val-OH, and Fmoc-Pro-OH, a second coupling was carried out.

**General Procedure for Capping Unreacted Aminocoumarin with an Acetyl Group.** In a separate 50 mL round-bottom flask were added 3-nitro-1,2,4-triazole (3.0 g, 26 mmol), AcOH (1.6 mL, 26 mmol), DMF (18 mL), and DICI (4.2 mL, 26 mmol). After stirring for 5 min, the mixture was added to the resin and allowed to react for 24 h, whereupon *i*-$Pr_2$EtN (0.90 mL, 5.3 mmol) was added followed by stirring for an additional 3 h. The resin was filtered and washed with DMF (3 × 50 mL).

**Representative Synthesis of Single Peptide−ACC.** As a representative example, the synthesis of Fmoc-L-Phe-L-His-ACC is reported. To a 6 mL syringe cartridge were added Fmoc-L-His-ACC (0.06 mmol) and DMF (2 mL). The mixture was agitated with $N_2$ for 30 min and filtered. A 20% solution of piperidine in DMF (2 mL) was added, followed by agitation for 30 min. The resin was filtered and washed with DMF (3 × 2 mL). In a separate scintillation vial were added Fmoc-L-Phe-OH (112 mg, 0.290 mmol), HOBt (44 mg, 0.29 mmol), DMF (1.2 mL), and DICI (45 $\mu$L, 0.29 mmol). After a preactivation time of 5 min, the mixture was added to the resin, followed by agitation for 3 h. The resin was filtered, washed with DMF (3 × 2 mL), THF (3 × 2 mL), and MeOH (3 × 2 mL), and then dried in vacuo over $P_2O_5$. The crude products were subjected to reverse-phase HPLC preparatory chromatography followed by lyophilization. For the tetrapeptide **12**, as well as the hexapeptide **13**, the amino terminus was capped as the acetyl derivative. This was accomplished by premixing AcOH (5 equiv), DICI (5 equiv), and HOBt (5 equiv) in DMF and adding the resulting mixture to the resin. After agitation for 4 h the resin was filtered, washed, and dried. The purification of all ACC−peptides was performed using reverse-phase HPLC preparatory chromatography ($CH_3CN/H_2O$−0.1% TFA, 10−60% for 20 min, 5 mL/min, 254 nm detection for 60 min). Finally, all ACC−peptides have been analyzed by analytical reverse-phase HPLC analysis ($CH_3CN/H_2O$−0.1% TFA, 10−60% for 15 min, 0.8 mL/min, 254 nm detection for 60 min).

**Fmoc-L-Phe-L-Asn-ACC (10a).** Analytical reverse-phase HPLC analysis of crude material according to General Conditions ($t_R$ = 23.3 min, 83% purity). $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.51 (m, 1) 2.62 (m, 1), 2.76 (m, 1), 3.0 (m, 1), 3.62 (s, 2), 4.11 (m, 3), 4.28 (m, 1), 4.68 (m, 1), 6.28 (s, 1), 6.95 (s, 1), 7.05−7.30 (m, 8), 7.34−7.41 (m, 3), 7.47 (dd, 1, $J$ = 9.0, 1.8), 7.50−7.70 (m, 5), 7.77 (d, 1, $J$ = 1.8), 7.84 (d, 2, $J$ = 7.5), 8.45 (d, 1, $J$ = 7.7), 10.39 (s, 1). MS (ESI), $m/z$ calcd for $C_{39}H_{35}N_5O_8$: 701.3. Found: $m/z$ 702.2 $(M + H)^+$.

**Fmoc-L-Phe-D-Asn-ACC (11a).** Analytical reverse-phase HPLC analysis of crude material according to General Conditions ($t_R$ = 22.7 min, 85% purity). $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.40−2.60 (m, 2), 2.70−2.85 (m, 1), 2.90−3.00 (m, 1), 3.61 (s, 2), 4.00−4.20 (m, 3), 4.25−4.35 (m, 1), 4.70−4.80 (m, 1), 6.28 (s, 1), 6.92 (s, 1), 7.10−7.40 (m, 11), 7.49 (d, 1, $J$ = 8.9), 7.55−7.65 (m, 4), 7.72 (d, 1, $J$ = 8.2), 7.76 (s, 1), 7.82 (t, 2, $J$ = 7.1), 8.54 (d, 1, $J$ = 8.3), 10.23 (s, 1). MS (ESI), $m/z$ calcd for $C_{39}H_{35}N_5O_8$: 701.3. Found: $m/z$ 702.2 $(M + H)^+$.

**Fmoc-L-Phe-L-Leu-ACC (10b).** Analytical reverse-phase HPLC analysis of crude material according to General Conditions ($t_R$ = 26.1 min, 86% purity). $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 0.70−0.95 (m, 6), 1.40−1.75 (m, 3), 2.75 (m, 1), 3.00 (m, 1), 3.62 (s, 2), 4.12 (m, 3), 4.30 (m, 1), 4.47 (m, 1), 6.29 (s, 1), 7.10−7.33 (m, 8), 7.37 (t, 2, $J$ = 7.4), 7.45 (d, 1, $J$ = 8.9), 7.55−7.65 (m, 4), 7.69 (d, 1, $J$ = 8.5), 7.78 (s, 1), 7.85 (d, 2, $J$ = 7.5), 8.29 (d, 1, $J$ = 7.9), 10.50 (s, 1). MS (ESI), $m/z$ calcd for $C_{41}H_{40}N_4O_7$: 700.3. Found: $m/z$ 723.3 $(M + Na)^+$.

**Fmoc-L-Phe-D-Leu-ACC (11b).** Analytical reverse-phase HPLC analysis of crude material according to General Condi-

(23) (a) Kanaoka, Y.; Kobayashi, A.; Sato, E.; Nakayama, H.; Ueno, T.; Muno, D.; Sekine, T. *Chem. Pharm Bull.* **1984**, *32*, 3926. (b) Besson, T.; Joseph, B.; Moreau, P.; Viaud, M. C.; Coudert, G.; Guillaumet. *Heterocycles* **1992**, *34*, 273.

tions ($t_R$ = 24.8 min, 83% purity). $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 0.76 (d, 3, $J$ = 6.3), 0.79 (d, 3, $J$ = 6.5), 1.32 (m, 1), 1.35−1.60 (m, 2), 2.75−3.00 (m, 2), 3.62 (s, 2), 4.00−4.20 (m, 3), 4.25−4.60 (m, 2), 6.28 (s, 1), 7.10−7.40 (m, 10), 7.50 (d, 1, $J$ = 8.7), 7.55−7.68 (m, 4), 7.72−7.85 (m, 4), 8.49 (d, 1, $J$ = 8.2), 10.29 (s, 1). MS (ESI), $m/z$ calcd for $C_{41}H_{40}N_4O_7$: 700.3. Found: $m/z$ 723.2 $(M + Na)^+$, 739.2 $(M + K)^+$.

**Fmoc-L-Phe-L-His-ACC.** Analytical reverse-phase HPLC analysis of crude material according to General Conditions ($t_R$ = 20.3 min, 82% purity). $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.65−2.82 (m, 1), 2.92−3.16 (m, 2), 3.17−3.50 (m, 1), 3.63 (s, 2), 4.10−4.40 (m, 4), 4.74 (m, 1), 6.31 (s, 1), 7.10−7.50 (m, 13), 7.55−7.80 (m, 6), 7.85 (d, 2, $J$ = 7.5), 8.62 (d, 1, $J$ = 7.5), 8.91 (s, 1), 10.48 (s, 1). MS (ESI), $m/z$ calcd for $C_{41}H_{36}N_6O_7$: 724.3. Found: $m/z$ 725.3 $(M + H)^+$.

**Fmoc-L-Phe-D-His-ACC.** Analytical reverse-phase HPLC analysis of crude material according to General Conditions ($t_R$ = 19.7 min, 83% purity). $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.65−2.8 (m, 1), 2.81−2.91 (m, 1), 2.92−3.05 (m, 1), 3.10−3.20 (m, 1), 3.63 (s, 2), 4.05−4.20 (m, 3), 4.21−4.35 (m, 1), 4.70−4.85 (m, 1), 6.31 (s, 1), 7.10−7.40 (m, 12), 7.46 (dd, 1, $J$ = 8.8, 1.8), 7.58 (d, 2, $J$ = 7.4), 7.66 (d, 2, $J$ = 8.7), 7.75 (d, 1, $J$ = 1.8), 7.76−7.85 (m, 3), 8.73 (d, 1, $J$ = 8.2), 8.90 (s, 1), 10.36 (s, 1). MS (ESI), $m/z$ calcd for $C_{41}H_{36}N_6O_7$: 724.3. Found: $m/z$ 725.3 $(M + H)^+$.

**Ac-Lys-Gln-Trp-Lys-ACC (12).** Resin **8l** (150 mg, 0.057 mmol) was used to construct peptide **12** (32 mg, 0.030 mmol, 53% yield) according to General Procedures. Analytical reverse-phase HPLC analysis according to General Conditions ($t_R$ = 9.2 min). $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 1.15−1.35 (m, 5), 1.40−1.75 (m, 9), 1.84 (s, 3), 2.05−2.11 (m, 2), 2.60−2.80 (m, 4), 2.90−3.05 (m, 1), 3.06−3.20 (m, 1), 3.64 (s, 2), 4.10−4.25 (m, 2), 4.32−4.41 (m, 1), 4.46−4.60 (m, 1), 6.31 (s, 1), 6.78−6.82 (m, 1), 6.88 (t, 1, $J$ = 7.30), 6.99 (t, 1, $J$ = 7.30), 7.10−7.22 (m, 2), 7.20−7.30 (m, 2), 7.44 (dd, 1, $J$ = 8.9, 1.9), 7.54 (d, 1, $J$ = 7.7), 7.55−7.75 (m, 8), 7.80 (d, 1, $J$ = 1.8), 7.95 (d, 1, $J$ = 6.9), 8.00−8.10 (m, 2), 8.25−8.28 (m, 1), 10.46 (s, 1). MS (ESI), $m/z$ calcd for $C_{41}H_{54}N_{10}O_9$: 830.4. Found: $m/z$ 831.4 $(M + H)^+$.

**Ac-Arg-Lys-Ser-Leu-Val-Nle-ACC (13).** Resin **8n** (32 mg, 0.012 mmol) was used to construct peptide **13** (8.0 mg, 0.0068 mmol, 55% yield) according to General Procedures. Analytical reverse-phase HPLC analysis according to General Conditions ($t_R$ = 11.6 min). $^1$H NMR (300 MHz): $\delta$ 0.70−0.90 (m, 15), 1.13−1.33 (m, 6), 1.35−1.55 (m, 8), 1.55−1.75 (m, 5), 1.81 (s, 3), 1.91−1.97 (m, 1), 2.65−2.75 (m, 2), 3.02−3.10 (m, 2), 3.50−3.54 (m, 2), 3.62 (s, 2), 4.12−4.33 (m, 6), 5.02 (br s, 1), 6.28 (s, 1), 7.16 (br s, 2), 7.41 (dd, 1, $J$ = 8.4, 1.8), 7.51 (t, 1, $J$ = 5.7), 7.60−7.80 (m, 8), 7.92−8.20 (m, 7), 10.45 (s, 1). MS (ESI), $m/z$ calcd for $C_{45}H_{72}N_{12}O_{11}$: 956.5. Found: $m/z$ 957.4 $(M + H)^+$.

**Acknowledgment.** We thank Cleo Salisbury for helpful suggestions and review of the manuscript. This work was supported by National Institutes of Health Grants GM54051 (to J.A.E.) and CA72006 and AI35707 (to C.S.C.)

JO016140O