

Synthesis of CCF2-AM for the Development of a Cytotoxin-Based Assay for High Throughput Screening

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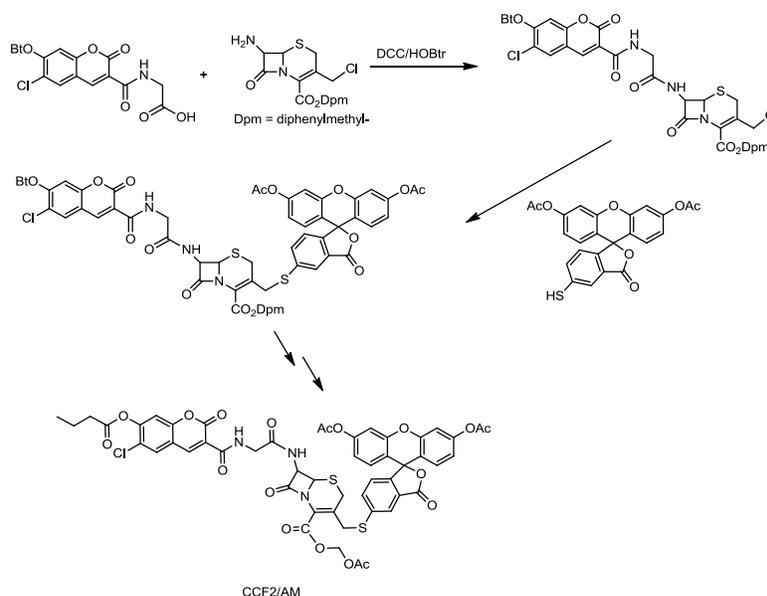
Background

Extracellular proteolysis in multicellular organisms is important for development, tissue homeostasis, tissue repair, and reproduction. Dysregulated extracellular proteolysis is critical for the genesis or progression of human diseases.^{1, 2} Despite its physiological and pathological importance, several fundamental aspects of extracellular proteolysis are still quite poorly understood and attempts to therapeutically manipulate extracellular proteolysis to treat complex diseases have not been always successful. The paucity of agents to visualize the activity of individual proteases *in situ* and monitor their pharmacological inhibition has been an impediment to basic research and to therapeutic targeting of proteases.^{3, 4} A simple and sensitive assay has been developed recently for imaging cell-surface proteolytic activity in single living cells using nontoxic, reengineered anthrax toxin- β -lactamase fusion proteins with altered protease cleavage specificity and a membrane-permeable fluorogenic β -lactamase substrate, coumarin cephalosporin fluorescein acetoxymethyl ester (CCF2-AM) imaging agent.^{5, 6, 7} The assay could be used to specifically image endogenous cell-surface furin, urokinase plasminogen activator and metalloprotease activity. In addition, the assay has been adapted for fluorescence microscopy, flow cytometry and fluorescent plate reader formats. The membrane-permeable fluorogenic lactamase substrate CCF2-AM can be used for the development of the cytotoxin-based assay for high throughput screening aimed at identifying novel inhibitors of tumor-associated cell surface proteolytic enzymes. Here, we described the synthesis of CCF2-AM modified from a method described by Tsien et al.⁷

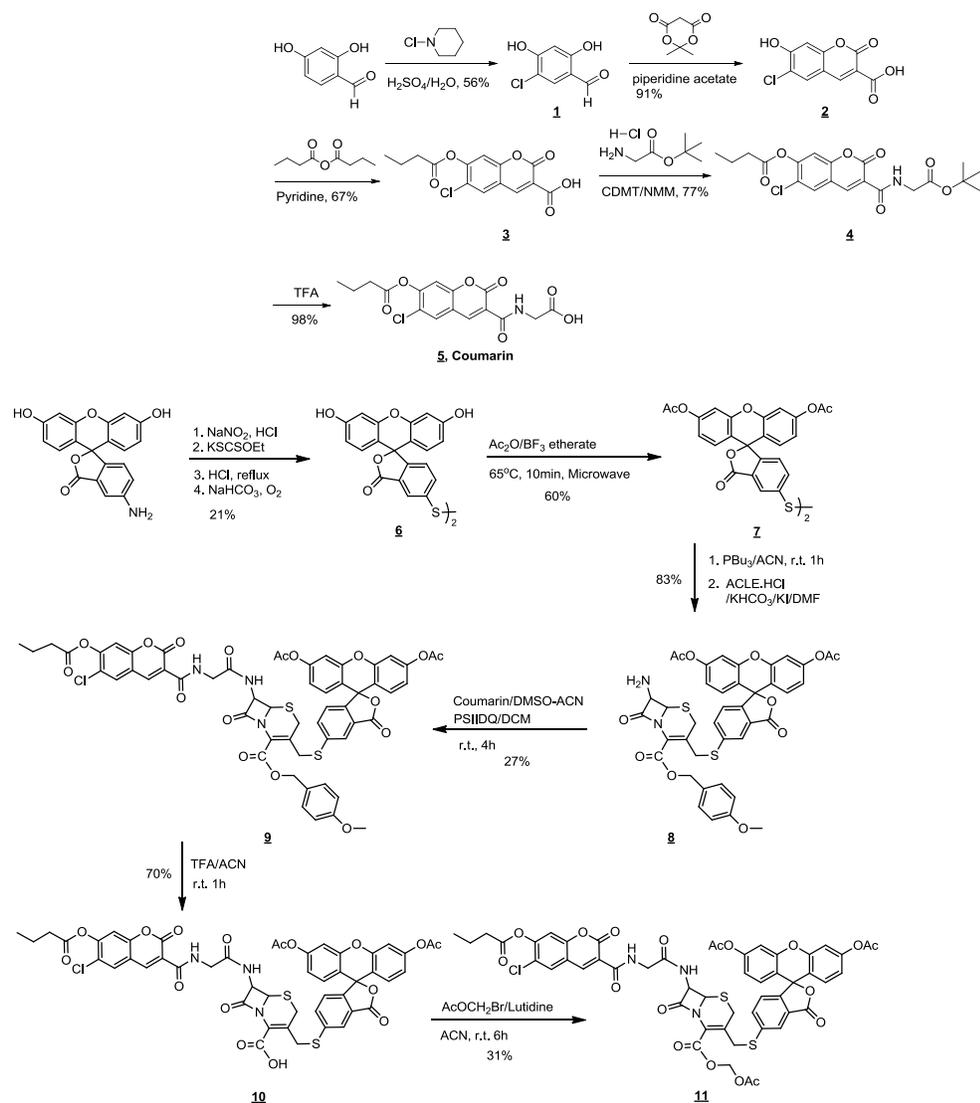
Chemistry

CCF2-AM is an expensive commercially available reagent (Invitrogen). Its synthesis has not been fully disclosed in the chemical literature except for a few of its analogs (**Scheme 1**).⁷ CCF2-AM molecule consists of three different protecting groups that are susceptible under either acidic or basic conditions. Modifications have been made to the reported synthesis route (**Scheme 2**).⁸ Firstly, the boron trifluoride catalysed acetylation of fluorescein disulfide, instead of pyridine, helped in maintaining the stability of the acetyl protecting groups and the lactone status of fluorescein for easier work-up. Secondly, the reactive chloromethyl group of 7-amino-3'-chloro cephalosporanic acid 4-methoxybenzyl ester (ACLE) in an intermediate was avoided by coupling ACLE with aminofluorescein prior to coupling with coumarin. Thirdly, the coupling of ACLE with coumarin was not very successful due to the poor solubility of coumarin. In the modified methodology, the polymer supported coupling reagent PS-IIDQ furnished an easier conjugation reaction and allowed for easier purification.⁹ In addition, the final product was found to decompose during work-up due to the basicity of lutidine catalyst. In the modified methodology, the product can be isolated by precipitation and then centrifuged in dry hexanes to reduce the contact of product with lutidine and moisture. A few modifications have also been made to the synthesis of coumarin to further increase the yield.

Scheme 1 Synthesis of CCF2-AM (patent literature route⁷):



Scheme 2 Synthesis of CCF2-AM



Experimental

General. 7-amino-3'-chloro cephalosporanic acid 4-methoxybenzyl ester hydrochloride (ACLE.HCl) was purchased from International Laboratory USA (San Bruno, CA). Polystyrene supported isobutoxy-1-isobutoxycarbonyl-1, 2-dihydroquinoline (PS-IIDQ) (1.3 mmol/g loading) was synthesized as described previously⁹ as was bromomethyl acetate.¹⁰ All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI). The ¹H NMR and the ¹³C NMR spectra were recorded on a Varian 400 MHz spectrometer operating at 400 MHz and 100 MHz

respectively. Chemical shifts were reported in part per million (δ) and referenced internally to deuterated solvents. HPLC analyses and mass measurements were performed on an Agilent 1200 series LC equipped with an Agilent G2440A LC/MSD-Trap-XCT ion trap mass spectrometer and a multi-wavelength detector. An Agilent C18 prep scalar column (4.6×50 mm, $5 \mu\text{m}$) with a flow of 1 mL/min were used for LC/MS. Product purification was performed on a preparative Agilent 1200 HPLC series instrument with either a preparative Zorbax XDB-C18 column (21.2 mm \times 150 mm, $5 \mu\text{m}$) and a flow of 40 mL/min, or a semi-preparative Eclipse XDB C18 column (9.4 mm \times 250 mm, $5 \mu\text{m}$) and a flow rate of 5 mL/min. The detectors were set at 280 nm. ESI-TOF high accuracy MS measurement was provided by The Scripps Research Institute (La Jolla, CA).

2, 4-Dihydroxy-5-chlorobenzaldehyde, 1.¹¹ *N*-chloro-piperidine (37.0 g, 310 mmol) was added drop-wise over a period of 2 h to a stirred solution of 2, 4-dihydroxybenzaldehyde (38.9 g, 282 mmol) in $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ (800 mL, 1:1 v/v). The mixture was stirred at room temperature under an argon atmosphere for 19 h and then filtered. The filtrate was washed with distilled water (5×400 mL). The solid was crystallized from 3 L of 10% ethanol in water and dried under high vacuum overnight to give the product as a pink powder (27 g, yield 56%). M.P. $147-9^\circ\text{C}$, ^1H NMR (CDCl_3 , 400 MHz): δ 11.27 (s, 1H), 9.71 (s, 1H), 7.53 (s, 1H), 6.63 (s, 1H), 6.21 (s, 1H).

3-Carboxy-6-chloro-7-hydroxy coumarin, 2.¹² A mixture of 2,4-dihydroxy-5-chlorobenzaldehyde (25.0 g, 145 mmol), Meldrum's acid (25.1 g, 174 mmol), piperidinium acetate (0.632 g, 4.35 mmol) and ethanol (75 mL) was stirred at room temperature for 30 min, and then refluxed for 2 h. The reaction mixture was allowed to chill in an ice-bath for 1 h. The yellow solid was filtered, washed with water (2×50 mL) and ethanol (2×50 mL), and dried under vacuum to give the product as a yellow powder (32 g, yield 91%). MS (APCI) (negative): calculated for $\text{C}_{10}\text{H}_5\text{ClO}_5$ 240.6; found 239.6 ($\text{M}-\text{H}^+$), ^1H NMR ($\text{DMSO}-d_6$, 400MHz): δ 12.95 (br, 1H, D_2O exchangeable), 11.85 (br, 1H, D_2O exchangeable), 8.59 (s, 1H), 7.94 (s, 1H), 6.84 (s, 1H).

7-Butyryloxy-3-carboxy-6-chloro-7-hydroxy coumarin, 3.⁷ 3-Carboxy-6-chloro-7-hydroxy coumarin (30 g, 125 mmol) was dissolved in dioxane (1000 mL). To the solution was added

butyric anhydride (41 mL, 250 mmol), pyridine (45 mL, 600 mmol) and dimethylaminopyridine (152 mg, 1.25 mmol). The reaction mixture was stirred at room temperature for 3 h and concentrated until the total volume in the flask was about 500 mL. Addition of heptanes (1500 mL) resulted in the formation of a white precipitate. The solid was recovered by filtration yielding the crude product **3** (37 g). The crude product was then dissolved in ethyl acetate (2000 mL) and washed with 1N HCl/brine (400 mL, v/v 1:1) and brine (2 × 500 mL). The organic phase was dried over Na₂SO₄, and then concentrated to give a light yellow powder (25 g, yield 67%). MS (APCI) (negative): calculated for C₁₄H₁₁ClO₆ 310.7; found 309.5 (M-H⁺), ¹H NMR (CDCl₃, 400 MHz): δ 12.00 (br, 1H, COOH), 8.86 (s, 1H, coumarin), 7.84 (s, 1H, coumarin), 7.37 (s, 1H, coumarin), 2.68 (t, 2H, *J* = 7.4 Hz, butyric methylene), 1.08 (t, 3H, *J* = 7.4 Hz, butyric methyl).

7-Butyryloxy-3-carbonyl-O-tert-butyl-glycine-6-chloro-coumarin, 4.¹³ 7-Butyryloxy-3-carboxy-6-chloro coumarin (7.1 g, 22.8 mmol), glycine *tert*-butyl ester hydrogen chloride (4.6 g, 27.4 mmol) and 6-chloro-2,4-dimethoxy-*s*-triazine (CDMT) (4.4 g, 25.1 mmol) were mixed together. To the mixture was added acetonitrile (100 mL). The slurry was stirred and *N*-methylmorpholine (NMM) (5.8 g, 57.1 mmol) was added over about 10 min. The slurry was stirred for 3 h at room temperature, and then water (120 mL) was added to the mixture, which initially dissolved the solids and then quickly generated a thick slurry as the product precipitated. The slurry was stirred for 1 h, and the solids were isolated by filtration and washed with 1N HCl (100 mL), water (3 × 100 mL), dried in air to give a white powder (7.42 g, yield 77%). MS (APCI) (negative): calculated for C₂₀H₂₂ClO₇ 423.8; found 366.8 [M-*t*Bu], ¹H NMR (CDCl₃, 400 MHz): δ 9.11 (t, 1H, *J* = 5.6 Hz, amide), 8.82 (s, 1H, coumarin), 7.77 (s, 1H, coumarin), 7.25 (s, 1H, coumarin), 4.14 (d, 2H, *J* = 5.6 Hz, glycine methylene), 2.66 (t, 2H, *J* = 7.2 Hz, butyric methylene), 1.84 (m, 2H, butyric methylene), 1.51 (s, 9H, *t*-Bu), 1.08 (t, 3H, *J* = 7.2 Hz, butyric methyl).

7-Butyryloxy-3-carbonylglycine-6-chloro-coumarin, 5. A solution of 7-butyryloxy-3-carbonyl-O-*tert*-butyl-glycine-6-chloro-coumarin (7.0 g, 16.5 mmol) and trifluoroacetic acid (100 mL) in anhydrous dichloromethane (250 mL) was stirred at room temperature for 2 h. The solution was concentrated to give a yellow powder (6.0 g, yield 98%). MS (APCI) (negative):

calculated for $C_{16}H_{14}ClO_7$ 367.7; found 366.8 $[M-H]^+$, 1H NMR (DMSO- d_6 , 400 MHz): δ 9.00 (t, 1H, $J = 5.6$ Hz, amide), 8.90 (s, 1H, coumarin), 8.35 (s, 1H, coumarin), 7.67 (s, 1H, coumarin), 4.07 (d, 2H, $J = 5.6$ Hz, glycine methylene), 2.70 (t, 2H, $J = 7.2$ Hz, butyric methylene), 1.73 (m, 2H, butyric methylene), 1.02 (t, 3H, $J = 7.4$ Hz, butyric methyl).

Mercaptofluorescein disulfide, 6.¹⁴ Fluoresceinamine, isomer I (2 g, 5.8 mmol) was stirred in aqueous 5M HCl (20 mL) while cooled in ice-bath. To the yellow suspension was slowly added a solution of 1g $NaNO_2$ dissolved in water (10 mL). After 2h of stirring at ambient temperature, the reaction mixture was neutralized with solid $NaHCO_3$. Potassium ethylxanthogenate (1 g, 6.3 mmol) was added to the dark-red solution and the mixture was stirred at ambient temperature for 4h and then concentrated to dryness. The residue was hydrolyzed by refluxing with concentrated HCl (10 mL) for 3 h and then concentrated to dryness *in vacuo*. The dark red residue was dissolved in aqueous saturated $NaHCO_3$ (50 mL) and the solution was bubbled with air for 1 h. The solution was acidified to pH 2 with 2N HCl and then extracted with ethyl acetate (2×50 mL), washed with water (2×50 mL) and brine (20 mL), and then dried over sodium sulfate. The organic solution was concentrated to dryness *in vacuo*. The residue was dissolved in the minimum amount of DMSO and purified by HPLC using an Agilent XDB-C18 (21.2×150 mm, 5 μ m) column at isocratic 50 % acetonitrile in water (with 0.1% TFA) and 40 mL/min flow rate. The product fraction ($t_R = 4.5$ min) was concentrated on rotary evaporator below 40 °C to remove majority of acetonitrile. The leftover traces of acetonitrile were extracted with ethyl acetate (2×20 mL) and the solution was dried over sodium sulfate. Evaporation of the solvents afforded an orange solid (460 mg, yield 21 %). 1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.14 (d, $J = 1.6$ Hz, 2H), 7.86 (d-d, $J = 2.0, 8.2$ Hz, 2H), 7.16 (d, $J = 8.2$ Hz, 2H), 6.75 (d, $J = 2.3$ Hz, 2H), 6.73 (d, $J = 8.9$ Hz, 2H), 6.57 (d, $J = 2.3, 8.9$ Hz, 4H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ (ppm) 170.0, 165.9, 161.8, 157.0, 141.6, 135.4, 132.2, 131.5, 129.2, 127.4, 117.1, 114.0, 104.4. MS (APCI) (positive): calculated for $C_{40}H_{22}O_{10}S_2$ 726.1; found 727.1 (MH^+).

5-mercaptofluorescein diacetate disulfide, 7. To a suspension of mercaptofluorescein disulfide (500 mg, 0.69 mmol) in dry acetic anhydride (10 mL) was added boron trifluoride etherate (0.1 mL). After stirring at ambient temperature for 12 h, a clear brown solution was formed. Evaporation of solvent under reduced pressure afforded a yellowish-brown solid (616 mg,

quantitative). The crude product was purified by isocratic HPLC using an Agilent XDB-C18 (21.2 × 150 mm, 5 μm) column and 0.1% TFA containing 75 % acetonitrile in water and a 40 ml/min flow rate. The colorless product fraction ($t_R = 5.2$ min) was concentrated at reduced pressure at 25°C and then lyophilized to afford an off-white product (370 mg, yield 60 %). TLC (Hexane: ethyl acetate : ethanol v/v/v 1:1:1 product $R_f = 0.38$). $^1\text{H NMR}$ (CDCl_3 , 400 MHz): δ (ppm) 8.12 (d, $J = 1.6$ Hz, 2H), 7.77 (d-d, $J = 1.6, 8.0$ Hz, 2H), 7.16 (d, $J = 8.0$ Hz, 2H), 7.08 (d, $J = 1.6$ Hz, 4H), 6.83 (m, 8H), 2.28 (s, 12H). $^{13}\text{C NMR}$ (CDCl_3 , 400 MHz): δ (ppm) 169.9, 168.2, 152.3, 151.9, 151.6, 139.7, 133.5, 129.0, 127.6, 125.0, 122.6, 118.0, 115.9, 110.6, 82.0, 60.5, 29.8, 21.2, 21.1, 14.3. MS(APCI) (positive): calculated for $\text{C}_{48}\text{H}_{30}\text{O}_{14}\text{S}_2$ 894.1 found 895.1 (MH^+).

3-[(5-diacetylfluorescein)thio]methyl)-7-amino-3-cephem-4-(4-methoxybenzyl)carboxylate,

8. 7 A solution 5-mercaptofluorescein diacetate disulfide (180 mg, 0.20 mmol) in acetonitrile (5 mL) was treated with tributyl phosphine (50 μl, 0.42 mmol) under nitrogen atmosphere at ambient temperature for 2 h until LC/MS showed a sole new peak with 449.1 mass (M^+). A solution of 7-amino-3'-chlorocephalosporanic acid 4-methoxybenzyl ester hydrochloride (ACLE·HCl) (178 mg, 0.44 mmol), potassium bicarbonate (48 mg, 0.40 mmol) and potassium iodide (66 mg, 0.40 mmol) in DMF (10 mL) was added. The mixture was stirred at ambient temperature for 3 h after which time LC/MS analysis showed a sole new peak with 781.3 mass (MH^+). The reaction mixture was concentrated at ambient temperature under reduced pressure. The residue was dissolved in a minimum amount of DMSO and purified by HPLC using an Agilent XDB C18 (21.2 × 50 mm, 5μm) column and 0.05 % formic acid added solvents with a gradient of 30% to 70 % acetonitrile in water in 3 min and a 40 mL/min flow rate. The product fraction ($t_R = 2.2$ min) was concentrated, extracted with ethyl acetate and dried over sodium sulfate. Removal of solvents afforded a yellow powdery product (258 mg, yield 83 %). LCMS analysis was carried out using an Agilent Zorbax XDB C18 (4.6 × 50 mm, 5μm) column and a gradient of 40% to 95% acetonitrile in water (containing 0.05% TFA) in 5 min at a 1 mL/min flow rate. The product $t_R = 3.35$ min. MS(ESI) (positive): calculated for $\text{C}_{40}\text{H}_{32}\text{N}_2\text{O}_{11}\text{S}_2$ 780.1; found 781.1 (MH^+). $^1\text{H NMR}$ (CD_3CN , 400 MHz): δ (ppm) 8.01 (m, 1H), 7.69 (t-t, $J = 8.0, 1.7$ Hz, 1H), 7.23 (m, 2H), 7.16 (m, 2H), 6.97 (m, 1H), 6.88 (s, 2H), 6.83(m, 2H), 5.09(m, 1H), 5.00(m, 2H), 4.99(m, 1H), 4.62(b, mH), 4.16(d-d, $J = 36.8, 13.3$ Hz, 2H), 3.73(m, 4H), 3.70 (s,

1H), 2.96(s, 1H), 2.83(s, 1H), 2.28(m, 6H). ¹³C NMR (CDCl₃, 400 MHz): δ (ppm) 169.0, 169.0, 168.7, 161.5, 160.1, 152.5, 152.3, 152.3, 151.7, 151.6, 140.3, 136.4, 131.0, 130.2, 129.6, 129.3, 128.8, 127.0, 126.9, 125.9, 124.4, 118.0, 118.0, 116.2, 114.2, 114.1, 114.1, 110.6, 110.4, 82.0, 68.0, 55.4, 37.8, 27.5, 21.3.

3-[(5'-diacetylfluorescein)thio]methyl)-7-(7"-butyryloxy-6"-chloro-coumarin-3"-carbonylglycinamido)-3-cephem-4-(4-methoxybenzyl)carboxylate (CCF2-MB)₂, 9. A

suspension of coumarin glycine compound, **5** (33 mg, 0.092 mmol, 1.2 equiv.) in anhydrous DMSO (1 mL), a suspension of PS-IIDQ (293 mg, 0.38 mmol, 5 equiv) in DCM (1.0 mL) and a solution of benzhydryl cephalosporanate fluorescein amine, **8** (60 mg, 0.076 mmol, 1 equiv.) in anhydrous acetonitrile (5 mL) were mixed at room temperature. The mixture was then shaken for 12 h until the LC/MS analysis showed one major peak with mass of 1130. The resin was then filtered off and washed with DCM (1 mL) and then MeOH (1 ml) for three cycles. The filtrate was concentrated *in vacuo*, diluted with ethyl acetate, washed with brine and dried over sodium sulfate. The solution was filtered through a silica pad in a sintered funnel and then eluted with ethyl acetate. Solvents were removed to afford an off-white solid product (70 mg, yield 81 % with 70% HPLC purity at 254nm). The crude product was applied to hydrolysis without further purification. A small amount was purified by HPLC for NMR analysis using an Agilent SB C18 (9.4 × 150 mm, 5μm) column using 85-95% acetonitrile (0.1% formic acid) in water (0.1% formic acid) in 5 min solvent system at a gradient of and 4 mL/min flow rate. The product fraction (*t_R* = 3.20 min) was concentrated to dryness below 40 °C. LC/MS (ESI) analysis carried out by using an Agilent prep scalar C18 (4.6 × 50 mm, 5 μm) column, 0.1 % TFA added water and acetonitrile as solvents at a gradient of 60-95% acetonitrile in 5 min and 1 mL/min flow rate. The product *t_R* = 4.06 min. MS(ESI) (positive): calculated for C₅₆H₄₄ClN₃O₁₇S₂ 1129.2; found 1130.3 (MH⁺). ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 9.25 (t, *J* = 5.6 Hz, 1H), 8.62(s, 1H), 7.97 (s, 1H), 7.90 (d, *J* = 8.6 Hz, 1H), 7.74 (d-d, *J* = 7.3, 1.6 Hz, 1H), 7.39 (s, 1H), 7.13 (d, *J* = 8.6 Hz, 1H), 7.01(m, 3H), 6.84 (m, 3H), 6.76 (d-d, *J* = 8.6, 2.2 Hz, 1H), 6.74 (s, 1H), 6.68 (d-d, *J* = 8.6, 2.2 Hz, 1H), 6.61 (d, *J* = 8.6 Hz, 1H), 5.86 (d-d, *J* = 8.6, 4.7 Hz, 1H), 5.11 (d, *J* = 4.7 Hz, 1H), 4.77 (s, 2H), 4.40 (d, *J* = 12.3 Hz, 1H), 4.36 (d-d, *J* = 15.5, 5.5 Hz, 1H), 4.14 (d-d, *J* = 17.1, 5.9 Hz, 1H), 3.98 (d, *J* = 17.4 Hz, 1H), 3.79 (s, 3H), 3.62 (d, *J* = 17.4 Hz, 1H), 3.04 (d, *J* = 12.3 Hz, 1H), 2.63 (m, 2H), 2.32 (s, 3H), 2.28 (s, 3H), 1.83 (q, *J* = 9.8 Hz, 2H), 1.07 (t, *J* = 14.9 Hz, 3H).

^{13}C NMR (CDCl_3 , 400 MHz): δ (ppm) 170.34, 169.99, 169.67, 168.91, 168.89, 164.97, 162.53, 160.82, 160.53, 160.12, 153.89, 153.21, 152.20, 152.11, 150.95, 150.79, 150.72, 146.55, 143.38, 135.11, 133.79, 130.64, 129.78, 128.83, 126.68, 126.21, 125.18, 124.01, 123.83, 123.69, 119.46, 118.07, 117.57, 115.59, 115.31, 114.23, 112.09, 110.70, 110.32, 82.15, 67.81, 59.96, 58.29, 55.53, 44.19, 41.49, 38.25, 36.06, 26.86, 21.29, 18.45, 13.88.

3-[(5'-diacetylfluoroscein)thio]methyl)-7-(7''-butyryloxy-6''-chloro-coumarin-3''-

carbonylglycinamido)-3-cephem-4-carboxylic acid (CCF2), 10. ⁷ The crude CCF2-MB (70 mg, 0.062 mmol) from the last step was treated with a mixture of TFA:DCM (1:1) (0.50 mL) and a drop of anisole at room temperature for 1h and checked by LC/MS to verify the completion of the reaction. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in minimum amount of DMF and then purified by HPLC using an Agilent Zorbax SB C18 (9.4 \times 150 mm, 5 μm) column and a gradient of 70-95% of acetonitrile (containing 0.1% TFA) in water (with 0.1% TFA) in 5 min at a 4 mL/min flow rate. The product fraction ($t_R = 3.29$ min) was concentrated at room temperature and then lyophilized to afford an off-white powdery product (44 mg, yield 70 %). LC/MS analysis of the reaction was carried out using an Agilent Prep C-18 Scalar (4.6 \times 50 mm, 5 μm) column and water and acetonitrile containing 0.1 % TFA as solvents. A gradient of 60-95 % acetonitrile in 5 min and 1 ml/min flow rate was used. The product $t_R = 2.56$ min (MS-ESI: 1010.0). ^1H NMR (CDCl_3 , 400 MHz, TMS = 0): δ (ppm) 9.26(t, $J = 10.6$ Hz, 1H), 8.59(s, 1H), 7.92(s, 1H), 7.71(m, 2H), 7.45(s, 1H), 6.95(d, $J = 2.4$ Hz, 1H), 6.92(d, $J = 7.8$ Hz, 1H), 6.89(m, 1H), 6.87(m, 1H), 6.69(m, 2H), 6.60(d, $J = 8.6$ Hz, 1H), 5.69(d-d, $J = 8.0, 4.7$ Hz, 1H), 5.00(d, $J = 4.7$ Hz, 1H), 4.20-4.12(m, 3H), 3.81(d, $J = 16.8$ Hz, 1H), 3.66(d, $J = 17.6$ Hz, 1H), 3.34(d, $J = 12.7$ Hz, 1H), 2.57(d-d, $J = 7.2, 6.3$ Hz, 2H), 2.23(s, 3H), 2.19(s, 3H), 1.77(q, $J = 7.4$ Hz, 2H), 1.18(s, 1H), 1.01(t, $J = 7.3$ Hz, 3H). ^{13}C NMR (CDCl_3 , 400 MHz): δ (ppm) 170.4, 170.2, 169.6, 169.1, 160.7, 158.1, 153.3, 152.2, 151.3, 151.1, 133.9, 130.2, 129.9, 129.0, 125.8, 124.2, 118.3, 118.1, 117.5, 115.7, 114.1, 112.3, 110.7, 110.1, 82.4, 55.5, 40.3, 38.2, 36.0, 29.9, 27.3, 21.3, 19.2, 18.5, 13.9. ESI-TOF HAMS: calculated for $\text{C}_{48}\text{H}_{37}\text{ClN}_3\text{O}_{16}\text{S}_2$ (MH) 1010.1298; found 1010.1294 (MH⁺).

3-[(5'-diacetylfluoroscein)thio]methyl)-7-(7''-butyryloxy-6''-chloro-coumarin-3''-

carbonylglycinamido)-3-cephem-4-(4-acetylmethoxy)carboxylate (CCF2-AM)₂, 11. ¹⁵ CCF2

(15 mg, 15 μ mol) was dissolved in warm anhydrous acetonitrile (1.5 mL) in a capped LC/MS vial. Bromomethyl acetate (90 μ L, 108 μ mol) and 2, 6-lutidine (18 μ L, 18 μ mol) were added sequentially *via* Hamilton syringes. The mixture was maintained at room temperature and monitored by LC/MS. HPLC analysis of the reaction was carried out by using an Agilent C18 prep scalar (4.6 μ 50 mm, 5 μ m) column and a gradient of 40% to 95% acetonitrile in water (both containing 0.1 % TFA) in 5 min and a 1 mL/min flow rate. The product t_R = 4.6 min. After 6 h, most of the starting material was consumed. Purification of the reaction mixture was accomplished by using an Agilent Eclipse XDB C18 (9.4 \times 250mm, 5 μ m) column and a gradient of 70% to 95 % acetonitrile in water (both with 0.5 % AcOH) in 5 min and a 5 mL/min flow rate. The product fraction (t_R = 3.65 min) was lyophilized to afford a white solid product (5.1 mg, 31 % in yield). Alternatively, the sample was precipitated from dry hexanes and centrifuged to obtain the solid product with relatively higher yield (54%). MS(ESI): 1082(M⁺). ¹H NMR (CDCl₃, 400 MHz, TMS=0): δ (ppm) 9.25(t, J = 10.6 Hz, 1H), 8.61(s, 1H), 7.98(s, 1H), 7.89(d, J = 8.8 Hz, 1H), 7.86(d-d, J = 8.0, 1.6 Hz, 1H), 7.39(s, 1H), 6.97(d-d, J = 5.6, 2.4 Hz, 2H), 6.93(d, J = 8.0 Hz, 1H), 6.90(s, 1H), 6.87(d, J = 2.0 Hz, 1H), 6.77(d-d, J = 8.8, 2.4 Hz, 1H), 6.74(s, 1H), 6.60(d, J = 8.8 Hz, 1H), 5.90(d-d, J = 8.8, 5.6 Hz, 1H), 5.22(d, J = 6.4 Hz, 1H), 5.19(s, 2H), 5.17(d, J = 6.0 Hz, 1H), 4.43(d, J = 12.8 Hz, 1H), 4.38(d-d, J = 16.8, 5.6 Hz, 1H), 4.14(d-d, J = 16.8, 6.4 Hz, 1H), 4.14(d, J = 17.6 Hz, 1H), 3.67(d, J = 17.2 Hz, 1H), 3.04(d, J = 12.4 Hz, 1H), 2.64(h, J = 3.8 Hz, 2H), 2.33(s, 6H), 2.06(s, 3H), 1.85(q, J = 7.4 Hz, 2H), 1.26(s, 1H), 1.10(t, J = 7.4 Hz, 3H). ESI-TOF HAMS: calculated for C₅₁H₄₁ClN₃O₁₈S₂(MH⁺) 1082.1515; found 1082.1520 (MH⁺).

References

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