

Hapten Design and Monoclonal Antibody to Fluoroacetamide, a Small and Highly Toxic Chemical

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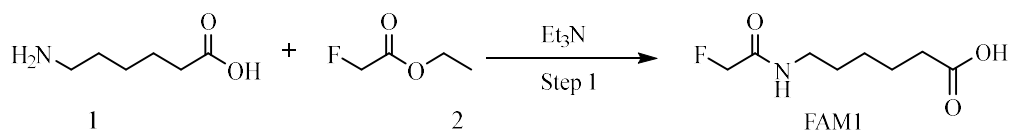
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1. Buffers

The following buffers were used in the work: (1) coating buffer, 0.05 M sodium carbonate buffer, pH 9.6; (2) blocking buffer, 0.01 M phosphate-buffered saline (PBS), pH 7.4, 0.5% casein (w/v); (3) washing buffer, 0.01 M PBS with 0.05% Tween-20 (v/v); (4) antibody dilution buffer, 0.01 M PBS containing 0.2% albumin; (5) enzyme labeled secondary antibody dilution buffer, antibody dilution buffer containing 5% albumin (w/v); (6) substrate, 0.1% TMB (w/v) and H₂O₂ in 0.05 M citrate buffer, pH 4.5; and (7) stopping reagent, 2 M H₂SO₄.

2. Synthesis and characterization of haptens

2.1. Synthesis of FAM1

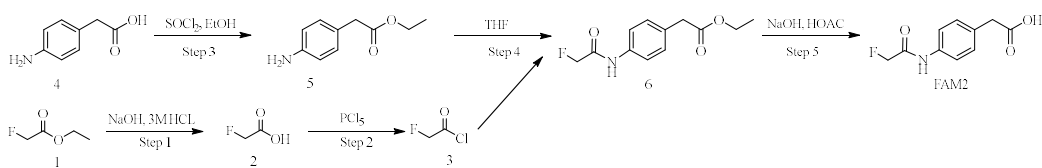


Scheme S1. The synthetic route of FAM1.

FAM1 was prepared according to the following scheme.

First, 6-aminocaproic acid (10.00 g, 76 mmol), triethylamine (Et₃N, 23.00 g, 227 mmol) and ethyl fluoroacetate (12.00 g, 113 mmol) in methanol (MeOH, 100 mL) were stirred at room temperature (RT) for 10 min, then refluxed for 24 h. The mixture was cooled to RT and adjusted pH to 5 with acetic acid and evaporated. The residue was re-dissolved in ethyl acetate (EtOAc) and washed by 30 mL of 0.9% NaCl, dried over Na₂SO₄, then filtered and evaporated to give the product FAM1 (7 g, 48%). HRMS (*m/z*) calc. for C₈H₁₄FN O₃ (-) 191.10, found 190.08, ¹H NMR (400 MHz, CDCl₃) δ 6.48 (bs, 1H, NH), 4.87 (s, 1H, CH₂), 4.75 (s, 1H, CH₂), 3.35 (q, 2H, CH₂, *J* = 6.8 Hz), 2.36 (t, 2H, CH₂, *J* = 8.4 Hz), 1.67 (m, 2H, CH₂), 1.59 (m, 2H, CH₂), and 1.40 (m, 2H, CH₂).

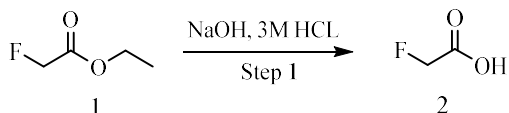
2.2. Synthesis of FAM2



Scheme S2. The synthetic route of FAM2.

FAM2 was prepared according to the following scheme in 5 steps.

Step 1 Synthesis of compound 2 (2-fluoroacetic acid)



A mixture of NaOH (13.50 g, 339 mmol) and compound 1 (30.00 g, 283 mmol) in ethanol (300 mL) was stirred at RT for 24 h; then the mixture was evaporated by rotary evaporation. After that, the residue was subject to 3 M HCl (200 mL), extracted with ether (4 × 50 mL), filtered, evaporated, dried over Na₂SO₄, and obtained the crude solid compound 2 (15.00 g).

Step 2 Synthesis of compound 3 (2-fluoroacetyl chloride)

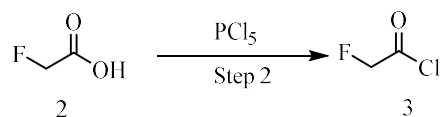
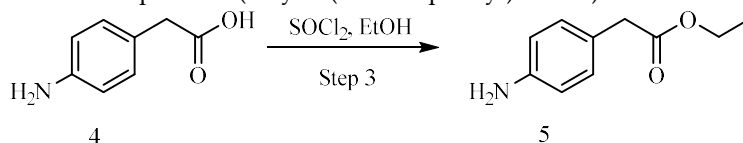


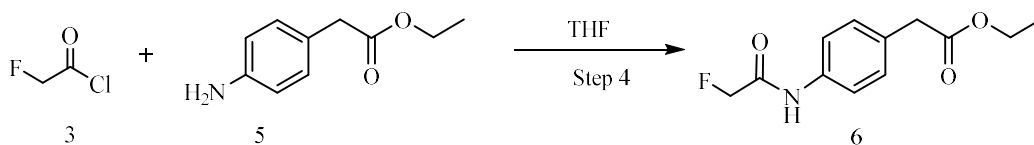
Table 5. 44.00 g, 211 mmol) was added to a three-neck round-bottom flask and cooled to 0°C, and the compound 2 (15.00 g) was slowly added and stirred at RT for 0.5 h. Then the mixture was heated in an oil bath until the effluent came out at 80°C for 1 h. The distillate at 70–72°C was collected to give a yield of 12.00 g of a colorless compound 3 (44%).

Step 3 Synthesis of compound 5 (ethyl 2-(4-aminophenyl)acetate).



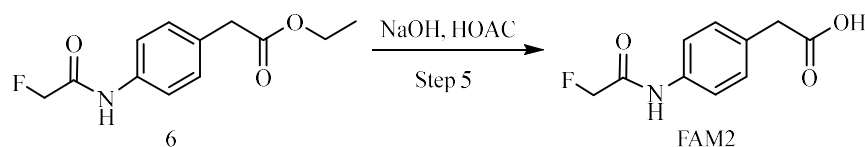
The mixture of thionyl chloride (SOCl₂, 17.3 g, 146 mmol) and compound 4 (11.00 g, 72.8 mmol) in ethanol (90 mL) was stirred at RT for 1 h. The mixture was adjusted pH to 8–9 with Na₂CO₃ and extracted with ethyl acetate (EtOAc, 3 × 100 mL). The combined extract was washed by 30 mL of 0.9% NaCl, dried over Na₂SO₄, filtered, and evaporated to give light-yellow liquid compound 5 (13.00 g, 100%).

Step 4 Synthesis of compound 6 (ethyl 2-(4-(2-fluoroacetamido)phenyl)acetate)



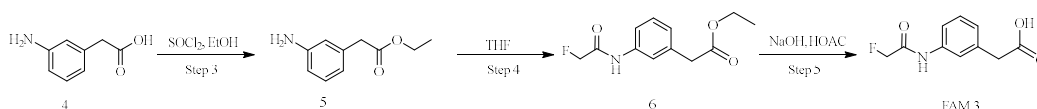
The solution of compound 3 (12.00 g, 44%) and compound 5 (13.00 g, 100%) in tetrahydrofuran (THF, 100 mL) was stirred at RT for 2 h. The combined organic layers were extracted with EtOAc (3 × 100 mL), washed by 30 mL of 0.9% NaCl, dried over Na₂SO₄. The residue was purified by silica gel chromatography separation (DCM: MeOH = 80: 1) to give a yellow solid compound 6 (12.50 g, 72%).

Step 5 Synthesis of FAM2



After that, NaOH (1.80 g, 45 mmol) in water (25 mL) was added in compound 6 (12.50 g, 72%) dissolved in ethanol (70 mL), stirred at RT for 1 h, and the pH was adjusted to 5 with acetic acid followed by filtering, washing, and drying steps. The filter residue was dried, and a yellow powder was obtained (3.70 g, 59%) FAM2, HRMS (*m/z*) calc. for C₁₀H₁₀FN₂O₃⁽⁺⁾ 211.06, found 212.07, ¹H NMR (DMSO-*d*₆, 400 MHz) δ 3.52 (s, 2H), 4.92 (s, 1H), 5.03 (s, 1H), 7.21 (d, *J* = 4.2 Hz, 2H), 7.57 (d, *J* = 4.2 Hz, 2H), and 10.07 (br, 1H).

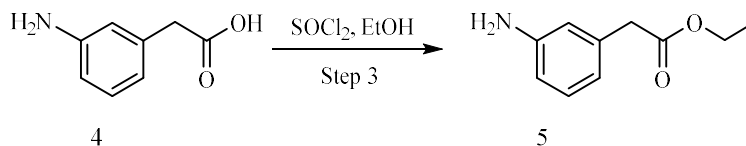
2.3. Synthesis of FAM3



Scheme S3. The synthetic route of FAM3.

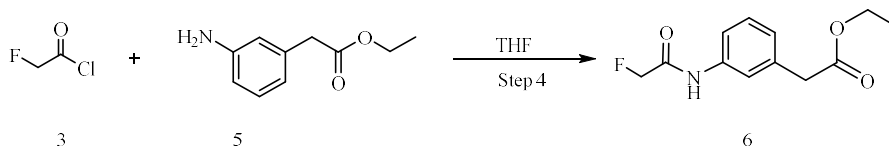
FAM3 was prepared according to the following steps. The first two steps were already described in the procedure of FAM2 preparation.

Step 3 Synthesis of compound 5 (ethyl 2-(3-aminophenyl)acetate).



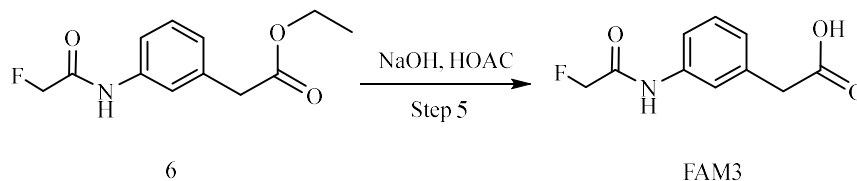
The mixture of thionyl chloride (SOCl_2 , 15.70 g, 132 mmol) and compound 4 (10.00 g, 66 mmol) in ethanol (80 mL) was stirred at RT for 1 h. The mixture was adjusted pH to 8–9 with Na_2CO_3 and extracted with EtOAc (3×100 mL). The combined extract was washed by 30 mL of 0.9% NaCl, dried over Na_2SO_4 , filtered, and evaporated to give yellow liquid compound 5 (11.86 g, 100%).

Step 4 Synthesis of compound 6 (ethyl 2-(3-(2-fluoroacetamido)phenyl)acetate)



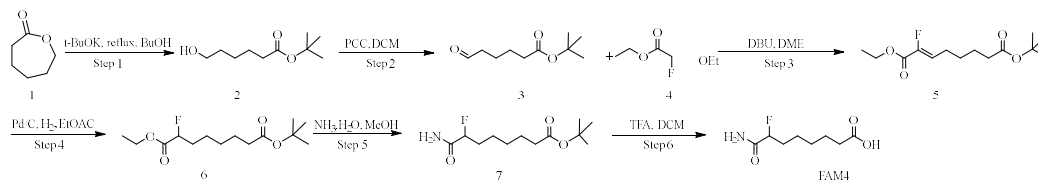
Next, the solution of compound 3 (1.91 g, 20 mmol) and compound 5 (3.14 g) in tetrahydrofuran (THF, 30 mL) was stirred at RT for 2 h. The combined organic layers were extracted with EtOAc (3×100 mL), washed by 30 mL of 0.9% NaCl, dried over Na_2SO_4 . The residue was purified by silica gel chromatography separation (PE: EA = 1: 3) to give a yellow solid compound 6 (2.73 g, 12 mmol, 65%).

Step 5 Synthesis of FAM3



After that, NaOH (1.80 g, 45 mmol) in water (25 mL) was added in compound 6 (2.73 g, 12 mmol, 65%) dissolved in ethanol (30 mL), stirred at RT for 1 h, and the pH was adjusted to 5 with acetic acid followed by filtering, washing, and drying steps. The filter residue was dried and a white powder of FAM3 was obtained (1.29 g, 7 mmol, 53%), HRMS (m/z) calc. for $\text{C}_{10}\text{H}_{10}\text{FNO}_3$ (+) 211.06, found 212.07, ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 12.31 (s, 1H, -COOH), 10.04 (s, 1H, -CONH), 7.53 (m, 2H, ArH), 7.22 (m, 1H, ArH), 6.07 (d, $J = 7.6$ Hz, 1H, ArH), 4.95 (d, $J = 46.8$ Hz, 2H, -FCH₂), and 3.52 (s, 2H, CH₂).

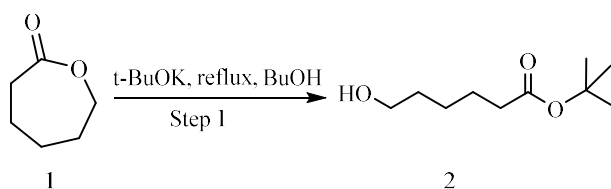
2.4. Synthesis of FAM4



Scheme S4. The synthetic route of FAM4.

FAM4 was prepared according to the following scheme in 6 steps.

Step 1 Synthesis of compound 2 (*tert*-butyl 6-hydroxyhexanoate)



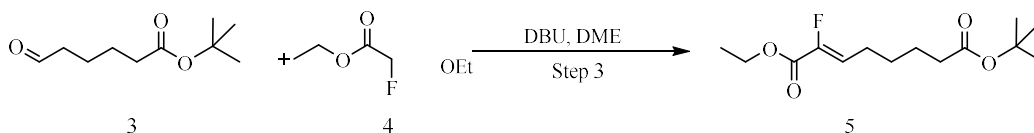
A mixture of compound 1 (10.96 g, 96 mmol) and *t*-BuOK (21.54 g, 192 mmol) in *t*-BuOH (300 mL) was heated under reflux for 8 h. The reaction mixture was cooled to RT and poured onto water (1000 mL). The mixture was extracted with toluene (2 × 500 mL). The combined organic layers were washed with water (2 × 1000 mL), brine (500 mL), dried over Na₂SO₄. Solvent removal gave a yellow oil (5.70 g, 32%) as a crude product.

Step 2 Synthesis of compound 3 (*tert*-butyl 6-oxohexanoate)



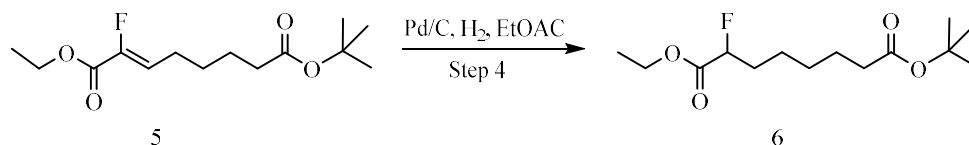
To a solution of compound 2 (5.70 g, 30.32 mmol) in DCM (100 mL) cooled to 0°C was added PCC (7.20 g, 33.4 mmol). The reaction mixture was then stirred at RT for 4 h. Celite (20.00 g) was added to the reaction mixture. The mixture was filtered and concentrated to give a residue. The residue was further purified by silica gel chromatography (silica gel, PE: EtOAc = 10: 1) to give a colorless oil (3.60 g, 59%) as compound 3.

Step 3 Synthesis of compound 5 (8-(*tert*-butyl) 1-ethyl (Z)-2-fluorooct-2-enedioate)



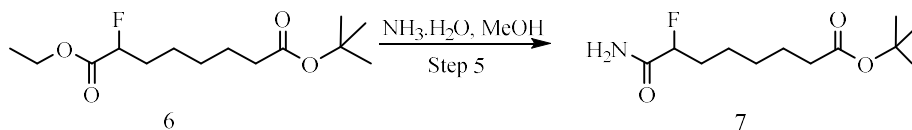
To a solution of compound 3 (3.60 g, 18 mmol) and compound 4 (6.54 g, 27 mmol) in THF (50 mL) was added DBU (4.10 g, 27 mmol). The resulting mixture was stirred at RT for 1 h. Saturated ammonium chloride (100 mL) was added to the reaction mixture and extracted with EtOAc (2 × 150 mL). The combined organic layers were washed with water (200 mL) and then brine (200 mL), and then they were dried over Na₂SO₄. Solvent removal gave a residue. The residue was purified with silica gel chromatography (PE: EtOAc = 10: 1) to afford a colorless oil (3.20 g, 65%) as compound 5.

Step 4 Synthesis of compound 6 (8-(*tert*-butyl) 1-ethyl 2-fluorooctanedioate)



To a solution of compound 5 (3.20 g, 11.7 mmol) in EtOAc (30 mL) was added palladium on carbon (10%, 0.30 g). The mixture was hydrogenated under a H₂ balloon for 16 h. The mixture was filtered to remove palladium on carbon. The filtrate was concentrated to afford a pale-yellow oil as compound 6 (2.90 g, 91%).

Step 5 Synthesis of compound 7 (*tert*-butyl 8-amino-7-fluoro-8-oxooctanoate)



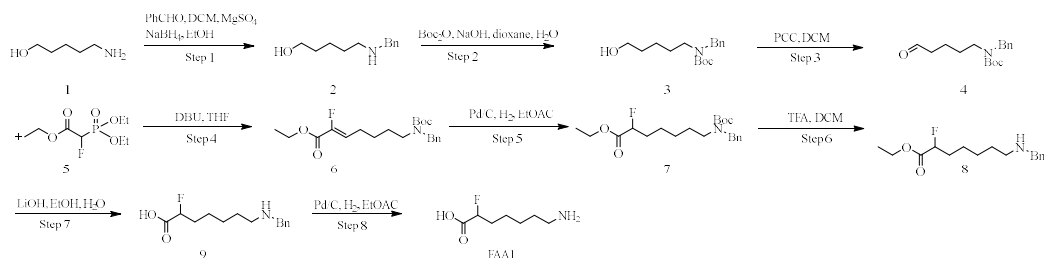
To a solution of compound 6 (5.88 g, 21 mmol) in MeOH (50 mL) was added ammonia (28% in water (5.88 g, 168 mmol)). The mixture was heated at 50°C for 16 h. The mixture was concentrated under vacuum to give compound 7 (4.12 g, 79%).

Step 6 Synthesis of FAM4



TFA (8 mL) was added to a solution of compound 7 (4.12 g, 16.6 mmol) in DCM (40 mL). The mixture was stirred at RT for 16 h. The reaction mixture was then concentrated. To the reaction mixture was added saturated NaHCO₃ to adjust pH to 9. To the reaction mixture was then added 6 M HCl to adjust pH to 1. A white precipitate appeared, which was collected via filtration. The solid was dried under vacuum to give FAM4 (1.92 g, 61%). HRMS (*m/z*) calc. for C₈H₁₄FN₃O₃ 191.10, found 190.08, ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.28–1.37 (m, 4H), 1.48–1.51 (m, 2H), 1.67–1.81 (m, 2H), 2.19 (t, *J* = 7.5 Hz, 2H), 4.74–4.86 (m, 1H), 7.39 (s, br, 1H), 7.54 (s, br, 1H), and 11.98 (s, 1H).

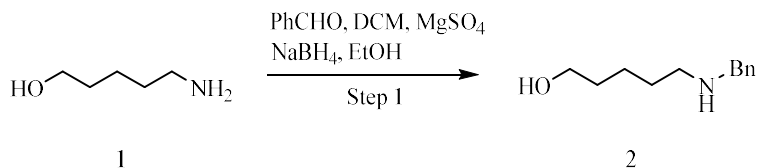
2.5. Synthesis of FAA1



Scheme S6. The synthetic route of FAA1.

FAA1 was prepared according to the following scheme in 8 steps.

Step 1 Synthesis of compound 2 (5-(benzylamino)pentan-1-ol)



To a solution of compound 1 (23.90 g, 231.67 mmol) in DCM (400 mL) was added benzaldehyde (29.50 g, 278 mmol), followed by the addition of anhydrous magnesium sulfate (70 g). The reaction mixture was stirred at RT for 16 h. The reaction mixture was filtered to remove the solid. The filtrate was concentrated under vacuum. To the residue was added EtOH (500 mL), followed by the addition of NaBH₄ (29.12 g, 462 mmol). The mixture was stirred at RT for 4 h. The organic solvent was removed under vacuum. To the residue was added DCM (500 mL). The mixture was washed with water (2 × 500 mL), dried over Na₂SO₄, and concentrated to give an oily residue (32.43 g). The residue was dissolved in DCM (200 mL). To the mixture was added 1M HCl (150 mL) and the mixture was stirred for 30 minutes. The organic layer was separated. The aqueous layer was treated with 1 N

NaOH to adjust pH to 10. The mixture was extracted with DCM (2 × 150 mL), the combined extracts were dried over anhydrous Na₂SO₄ and concentrated to give a light-yellow oil as compound 2 (22.32 g, 45%).

Step 2 Synthesis of compound 3 (*tert*-butyl benzyl(5-hydroxypentyl)carbamate)

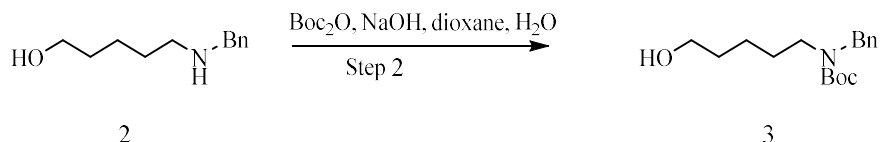
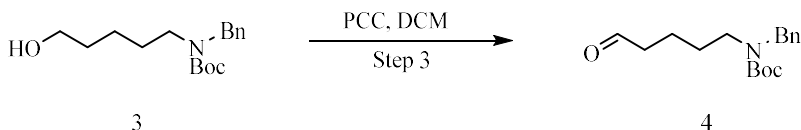


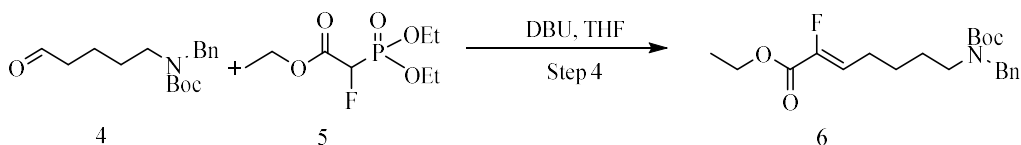
Table 2. (21.30 g, 110.6 mmol) in dioxane (100 mL) was added NaOH solution (21.30 g, 221.2 mmol in 100 mL of water), followed by the addition of Boc₂O (36.30 g, 166 mmol). The resulting mixture was stirred at RT for 16 h. To the mixture was added 1 N HCl (350 mL). The mixture was extracted with DCM (2 × 150 mL). The combined organic extracts were washed with 1 M HCl (2 × 100 mL), dried over Na₂SO₄. Concentration under vacuum gave an oily residue. The residue was subjected to silica gel column chromatography separation (PE: EtOAc = 5: 1 to 1: 1) to give a colorless oil as compound 3 (27.10 g, 84%).

Step 3 Synthesis of compound 4 (*tert*-butyl benzyl(5-oxopentyl)carbamate).



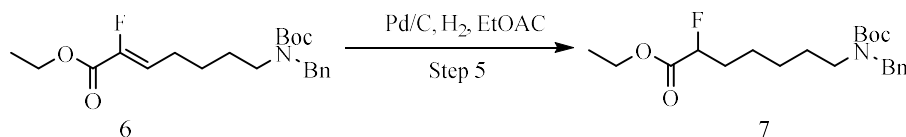
Silica gel (45 g) was added to a solution of compound 3 (27.10 g, 92 mmol) in DCM (300 mL), followed by the addition of PCC (30.00 g, 138 mmol). The mixture was stirred at RT for 16 h. The mixture was concentrated and subjected to silica gel column chromatography separation (PE: EtOAc = 10: 1 to 4: 1) to afford a colorless oil (19.80 g, 75%) as compound 4.

Step 4 Synthesis of compound 6 (ethyl (Z)-7-(benzyl(*tert*-butoxycarbonyl)amino)-2-fluorohept-2-enoate)



DBU (15.60 g, 64.4 mmol) was added to a solution of compound 4 (15.00 g, 51.5 mmol) and compound 5 (9.80 g, 64.4 mmol) in THF (150 mL). The mixture was stirred at RT for 16 h. Then, 1 M HCl (150 mL) was added to the mixture. The mixture was extracted with EtOAc (2 × 150 mL). The combined organic layers were washed with brine (150 mL), dried over Na₂SO₄ and concentrated. The residue was separated with silica gel column (PE: EtOAc = 10: 1) to give a colorless oil (13.72 g, 70%) as compound 6 (cis and trans mixture of double bond, ratio 1 to 1).

Step 5 Synthesis of compound 7 (ethyl 7-(benzyl(*tert*-butoxycarbonyl)amino)-2-fluoroheptanoate)



Palladium on carbon (10%, 2.00 g) was added to a solution of compound 6 (13.72 g, 36.2 mmol) in EtOAc (150 mL). The resulting mixture was hydrogenated under a hydrogen balloon for 18 h.

Palladium on carbon was removed with filtration. The filtrate was concentrated under vacuum to give a colorless oil (12.78 g, 92%) as compound 7.

Step 6 Synthesis of compound 8 (ethyl 7-(benzylamino)-2-fluoroheptanoate)

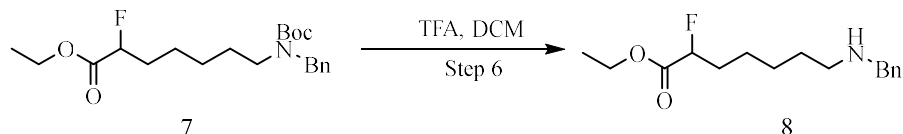
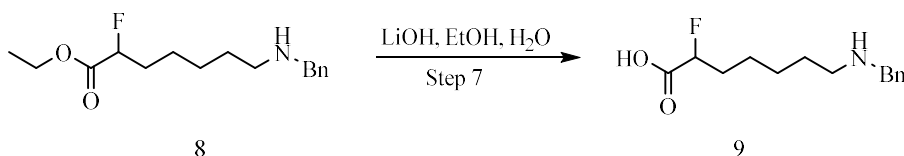


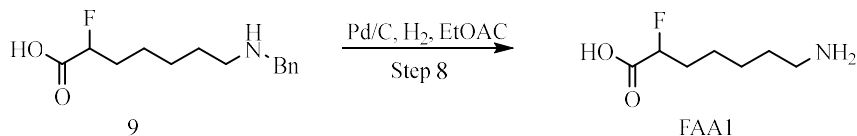
Table 30. mL) was added to a solution of compound 7 (11.87 g, 31.2 mmol) in DCM (100 mL). The mixture was stirred at RT for 16 h. The mixture was concentrated under vacuum to give a residue. The residue was dissolved in DCM (100 mL) and washed with saturated NaHCO_3 (100 mL), dried over Na_2SO_4 . The mixture was concentrated under vacuum to give a residue (8.20 g, 94%) as compound 8.

Step 7 Synthesis of compound 9 (7-(benzylamino)-2-fluoroheptanoic acid)



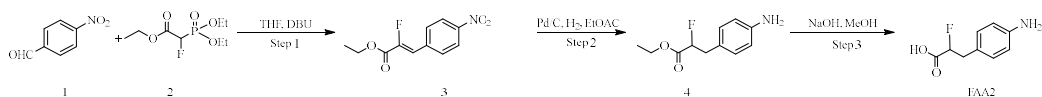
LiOH (2.52 g, 60 mmol) was added to a mixture of compound 8 (5.62 g, 20 mmol) in MeOH (30 mL) and water (30 mL). The mixture was then stirred at RT for 16 h. The mixture was concentrated. Water (20 mL) was added to the residue. The pH value of the mixture was adjusted to 4 with the addition of 1 N HCl. The white precipitate was collected by filtration and dried to afford a white solid (4.30 g, 85%) as compound 9.

Step 8 Synthesis of FAA1



To a mixture of compound 9 (4.30 g, 17 mmol) in MeOH (15 mL) and water (20 mL) was added palladium on carbon (10%, 2.00 g). The mixture was hydrogenated at 25°C under a hydrogen gas balloon for 96 h. The mixture was filtered to remove palladium on carbon. The filtrate was concentrated. Water was added to the residue to obtain a solution. The mixture was washed with DCM (2×20 mL). The aqueous layer was concentrated under vacuum to give a white solid (1.50 g, 56%) as FAA1. HRMS (m/z) calc. for $\text{C}_7\text{H}_{14}\text{FNO}_2$ (+) 163.10, found 164.11, ^1H NMR (300 MHz, DMSO- d_6) δ 1.33 (m, 4H), 1.57–1.75 (m, 4H), 2.72 (m, 2H), 4.51–4.68 (m, 1H), and 8.49 (s, br, 1H).

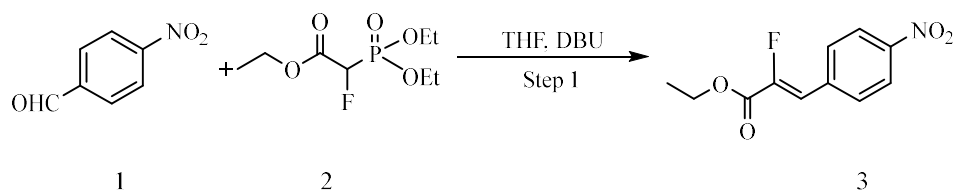
2.7. Synthesis of FAA2



Scheme S7. The synthetic route of FAA2.

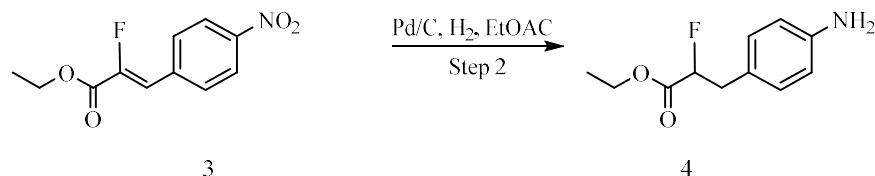
FAA2 was prepared based on the following scheme in 3 steps.

Step 1 Synthesis of compound 3 (ethyl (Z)-2-fluoro-3-(4-nitrophenyl)acrylate)



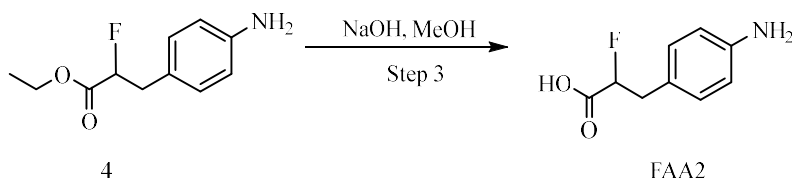
DBU (6.08 g, 40 mmol) was added to a solution of compound 1 (4.00 g, 26.47 mmol) and compound 2 (9.61 g, 39.7 mmol) in THF (70 mL). The mixture was stirred at RT for 2 h. To the reaction mixture was added 1 N HCl (30 mL). The mixture was extracted with EtOAc (3 × 50 mL). The extracts were combined and dried over Na₂SO₄. The solvent was removed, the residue was subject to silica gel column chromatography (PE: EtOAc = 20: 1) to give a yellow powder (4.30 g, 68%) as compound 3.

Step 2 Synthesis of compound 4 (ethyl 3-(4-aminophenyl)-2-fluoropropanoate)



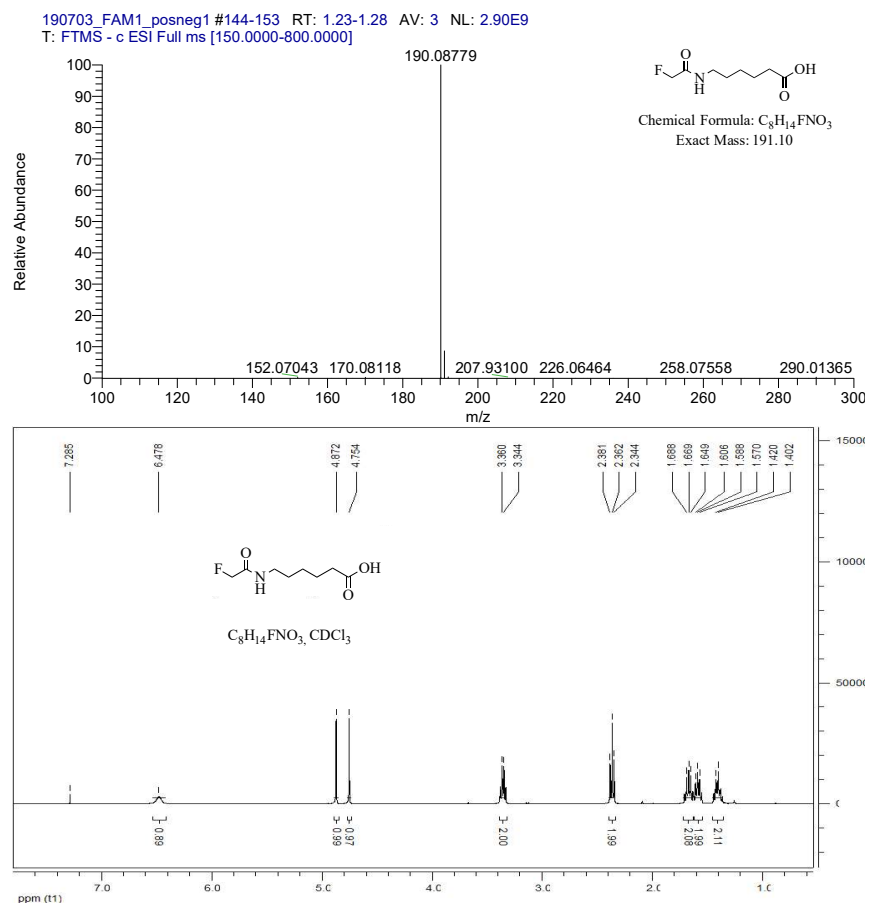
Palladium (10%, 1.00 g) was added to a solution of compound 3 (4.30 g, 18 mmol) in EtOAc (120 mL). The mixture was hydrogenated under a hydrogen balloon for 16 h. The mixture was filtered to remove palladium on carbon. The filtrate was concentrated. The residue was purified with silica gel column (PE: EtOAc = 20: 1) to give a red oil (1.90 g, 50%) as compound 4.

Step 3 Synthesis of FAA2



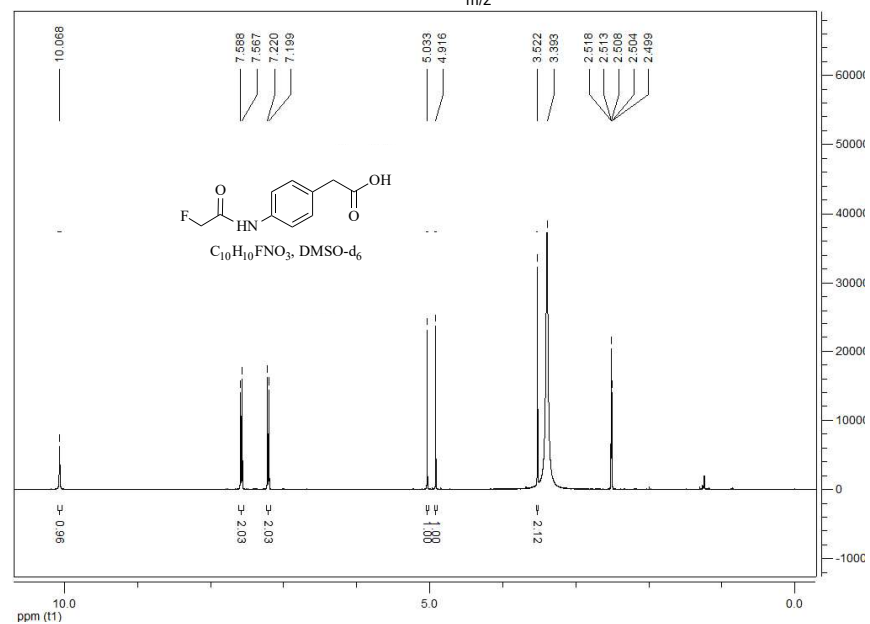
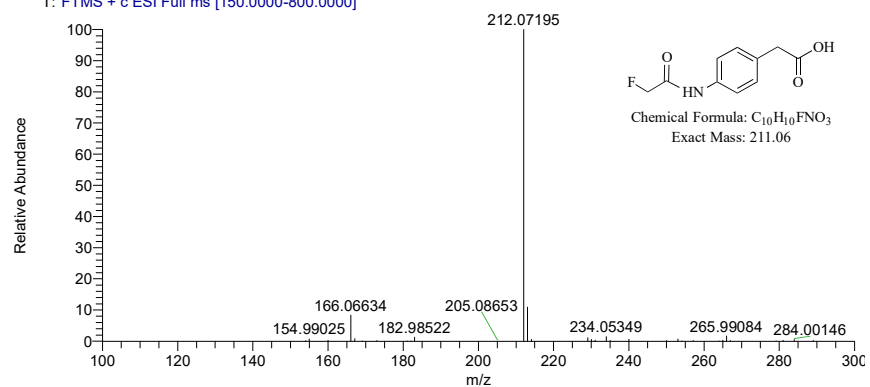
A solution of NaOH (1.10 g, 27.1 mmol in 10 mL of water) was added to a suspension of compound 4 (2.86 g, 13.55 mmol) in MeOH (30 mL). The reaction mixture was stirred for 16 h at RT. The pH value of the reaction mixture was adjusted to 4 with 2 M HCl. The precipitate was filtered and dried in vacuum to give FAA2 (2.00 g, 81%). HRMS (*m/z*) calc. for C₉H₁₀FNO₂⁽⁺⁾ 183.07, found 184.07, ¹H NMR (300 MHz, DMSO-*d*₆) δ 2.78–3.07 (m, 2H), 4.96–5.16 (m, 1H), 6.49 (d, *J* = 8.1 Hz, 2H), 6.89 (d, *J* = 8.1 Hz, 2H), and 7.45 (s, br, 2H).

A



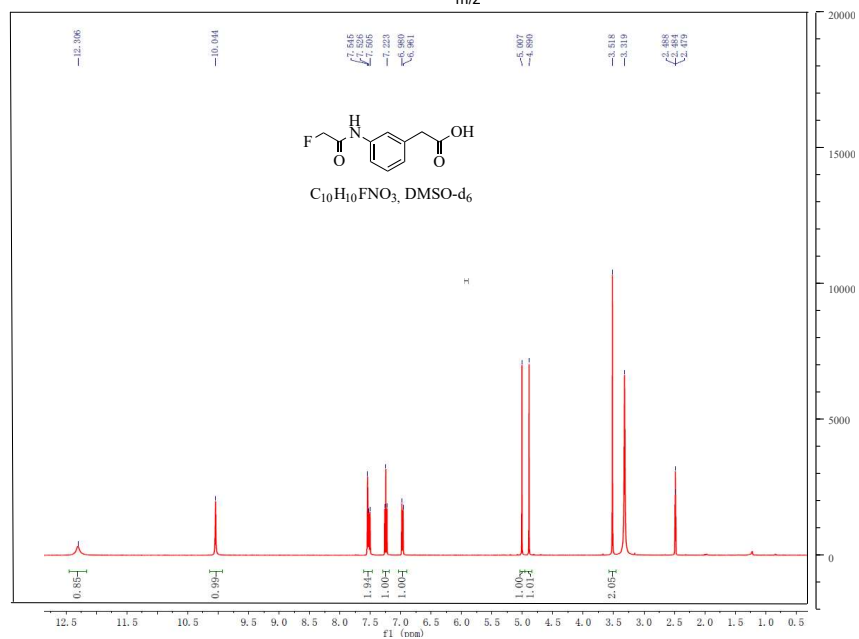
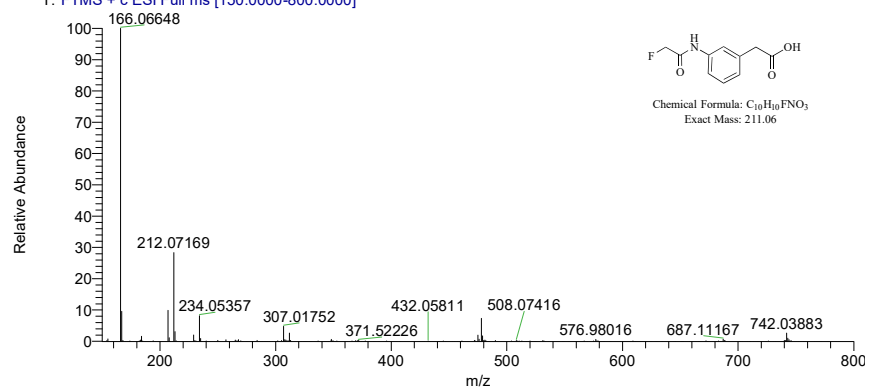
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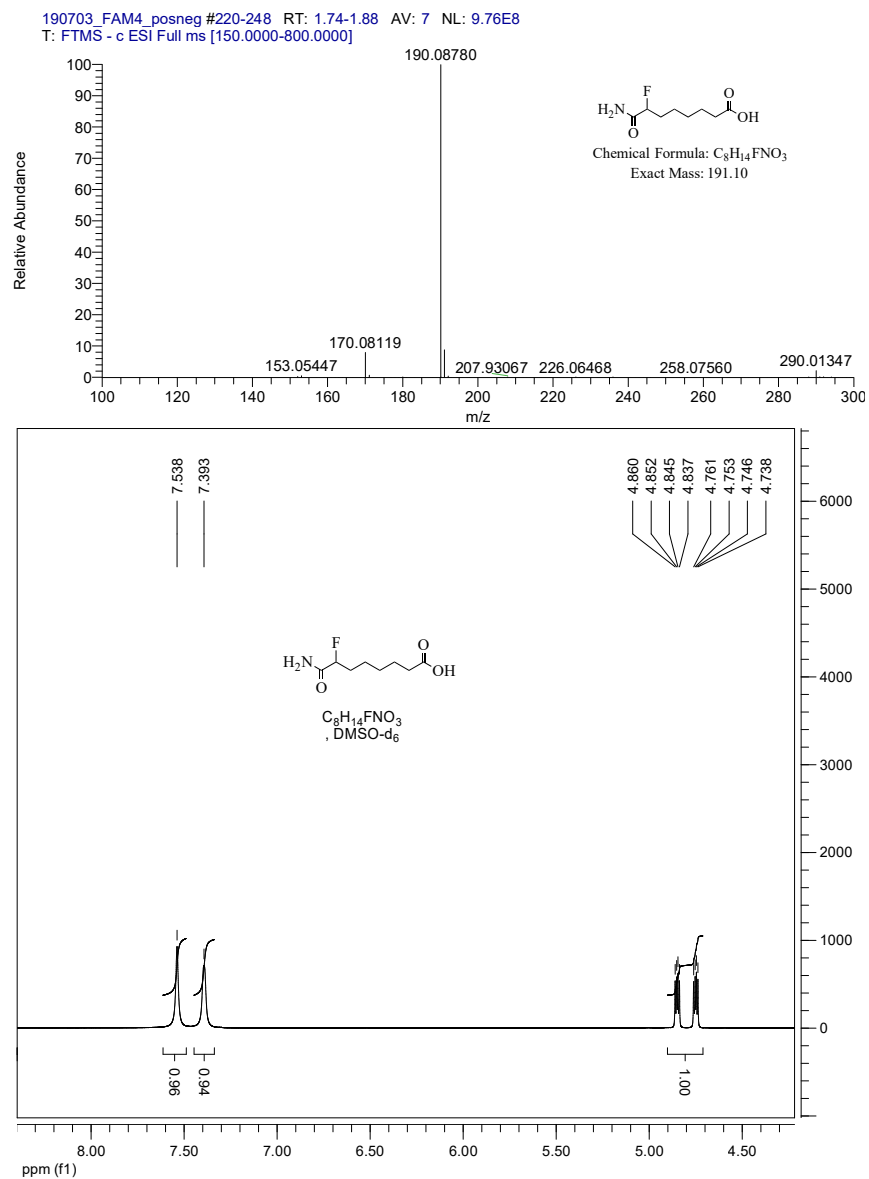


C

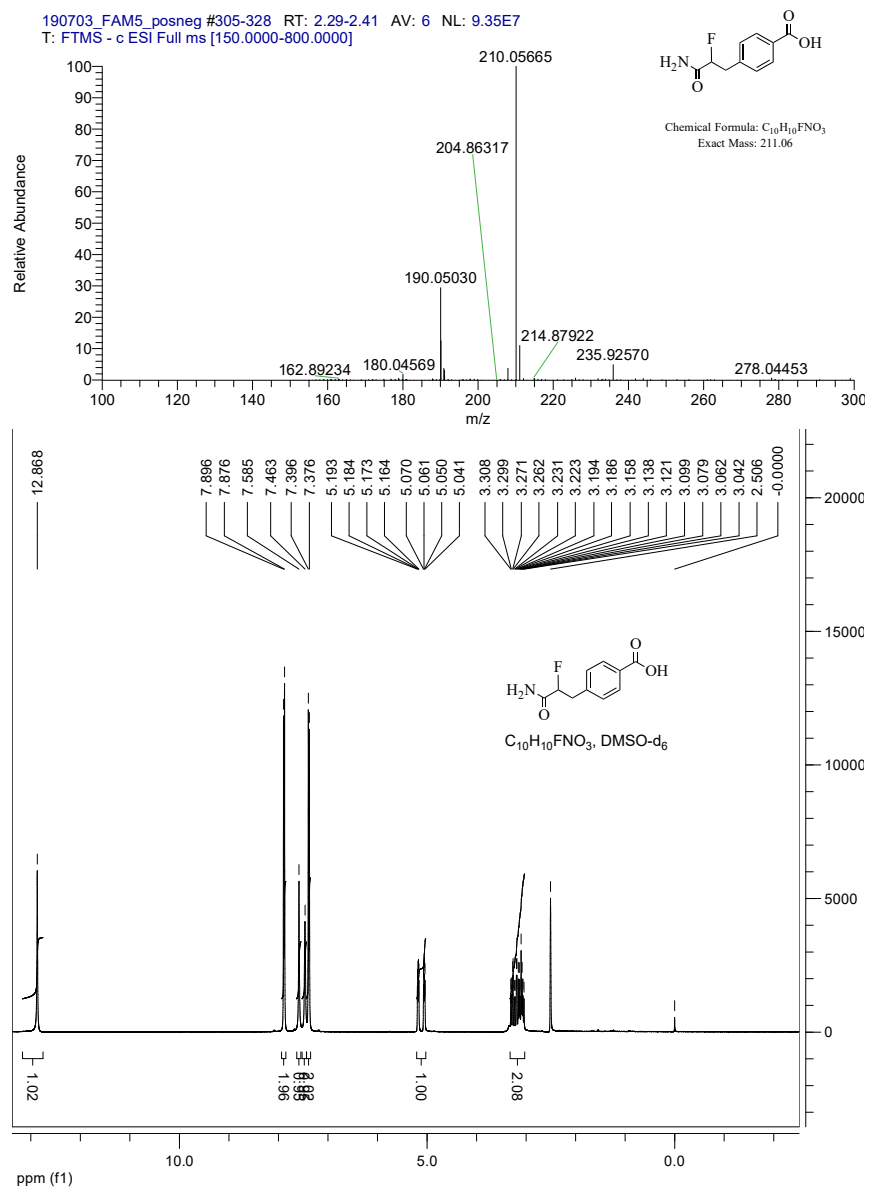
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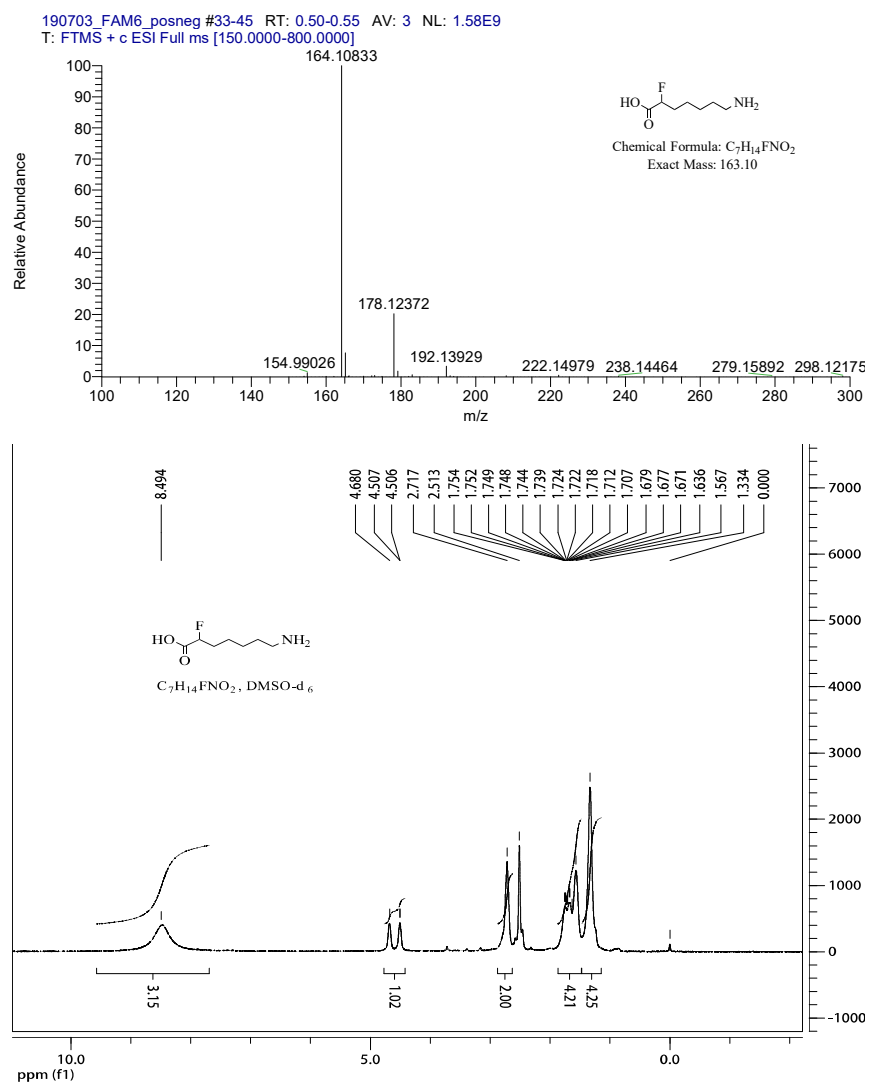
D



E



F



G

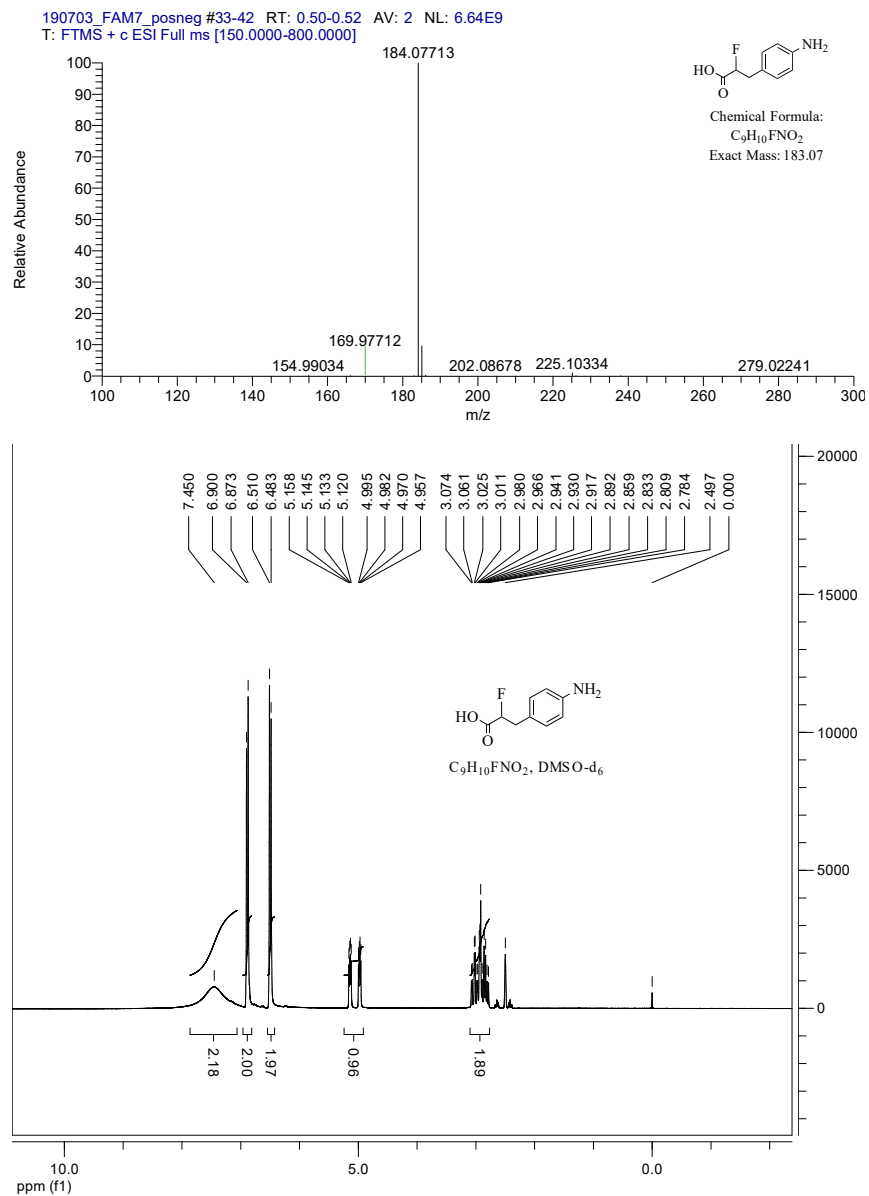


Figure S1. The mass spectra and 1H NMR spectra of (A) FAM1, (B) FAM2, (C) FAM3, (D) FAM4, (E) FAM5, (F) FAA1, and (G) FAA2.

3. Conjugates preparation and determination

3.1. Preparation of FAM1-FAM5 conjugates

Active ester method was used for the preparation of immunogens hapten-KLH and coating antigens hapten-BSA. Briefly, hapten (40 mg), DCC (80 mg), and NHS (80 mg) were sequentially added in 3 mL of DMF, and the mixture was kept magnetically stirred (400 rpm) at RT for 6 h in darkness. The activated haptens were added drop-wise to a 10 mL solution of 1 mg mL⁻¹ KLH or 10 mg mL⁻¹ BSA in PBS (pH 7.4). The reactive solution was kept stirred for another 12 h at RT, dialyzed against PBS for 3 d, and then stored at -20°C until use.

3.2. Preparation of FAA1 and FAA2 conjugates

The immunogens hapten-cKLH and the coating antigens hapten-cBSA were prepared by activated carboxylic acids of carrier proteins with carbodiimide modification methods. In the first step, EDC was added to a final concentration of 2 mg/mL to a solution of 1 mg mL⁻¹ KLH or 10 mg mL⁻¹ BSA in 0.1 M PBS (pH 7.4), followed by magnetically stirred (400 rpm) at RT for 2 h. Subsequently, the solution was dialyzed against PBS for 3 d. Activated KLH and BSA carrier proteins were defined as cKLH and cBSA. In the second step, each hapten was coupled to cKLH and cBSA through its aminol group. To each carrier, 15 mM hapten was added. The solution was kept stirred for 2 h at RT, followed by dialysis against PBS for 3 d, and stored at -20°C.

The all prepared BSA conjugates were identified using MALDI-TOF/MS and were calculated as follows:

$$\text{Conjugation ratio} = \frac{M(\text{conjugates}) - M(\text{BSA})}{M}(\text{haptens}) \quad (1)$$

4. Immunization procedure of mice

Eight female BALB/c mice (eight weeks old) were immunized subcutaneously with 1: 1 emulsified mixture (*v/v*, 200 µL) of Hapten-KLH conjugates (100 µg) in PBS and Freund's complete adjuvant for the primary immunization and Freund's incomplete adjuvant for every four weeks booster immunization. One week after each booster injection, antibody titers and affinity were determined by non-competitive ELISA (nc-ELISA) and indirect competitive ELISA (ic-ELISA), respectively. After a rest period of three weeks following the third injection, each mouse from the immunized FAM group with the highest antibody titers selected as the donors of spleen cells for hybridoma production received a final intraperitoneal injection with the same number of conjugates in PBS. Three days later, the mice were sacrificed, and splenocytes were obtained.

5. ELISA development and data analysis

An nc-ELISA was used to determine the titers of the mouse sera and for the initial screening of hybridomas. An ic-ELISA was used to determine the sensitivity and specificity of the mouse sera and monoclonal antibodies.

nc-ELISA: Wells of microplate were coated with 100 µL of antigens in coating buffer by overnight incubation at 4 °C. Nonspecific binding was decreased by blocking the wells with a blocking solution at 37 °C for 2 h with 150 µL/well and then washed three times with a washing solution. An amount of 50 µL/well of antibody or appropriate cell culture supernatant dilutions and 50 µL of PBS was allowed to bind to the coated microwells and incubated at 37 °C for 1 h. The unbound antibody was removed by washing three times with a washing solution. Next, 100 µL/well of HRP labeled goat anti-mouse IgG (1: 5000 dilution) was added and incubated at 37 °C for 30 min. The final washing step was followed by adding 100 µL of the substrate/chromogen solution. The solution was incubated for 15 min at 37 °C and then stopped by adding 2 M H₂SO₄ (50 µL/well). The OD values of each well were measured at 450 nm.

ic-ELISA. The procedure of ic-ELISA was similar to nc-ELISA. The difference was in the addition of the antibody step. In the ic-ELISA procedure, 50 μ L of antibody and 50 μ L of drug standard were added to the microwell instead of 50 μ L of PBS in the nc-ELISA procedure.

Triplicate experiments were performed for each concentration of competitors. Blank wells were treated in the same way as the test wells except that PBS replaced the competitor solutions. The absorbance values at 450 nm obtained in the presence of various competitor concentrations and without competitor (maximal signal) were referred to as B and B₀, respectively. The inhibition ratio was calculated as follows:

$$\text{Inhibition ratio} = (B / B_0) \times 100\% \quad (2)$$

6. Monoclonal antibody production

Splenocytes from immunized mice were fused with SP2/0 myeloma cells using PEG1500. Fused cells were resuspended in HAT medium (RPMI minimal media supplemented with 2 mM glutamine, 1% penicillin-streptomycin, and 20% fetal bovine serum) and pipetted into the wells of 96-well plates containing a feeder layer of mouse macrophages. After one week, hybridoma supernatants were monitored by nc-ELISA and ic-ELISA, as described above. Cells in fusion wells with supernatants exhibiting the highest antibody titers and affinity when both heterologous and homologous coating antigens were transferred from the fusion plates to 24-well plates and were cloned twice by limiting dilution. For each cloning procedure, the cells were plated on a layer of macrophage feeder cells, and the final clones were obtained from rows that had been seeded with one cell per well. Finally, one part of the hybridoma was cryopreserved, and another part was collected to produce ascites via intraperitoneal injection.

The ascites collected from mice were extracted and purified by the saturated ammonium sulfate. The isotypes of the purified mAbs were then characterized with a mice mAb isotyping kit obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Table 1. Summary of reports about antibodies for very small molecules found in the literature and described in this study.

Compounds	Structures	Haptens	MW (Da)	IC ₅₀ (ng/mL)	Reference
Xylitol			152.15	608000	[1]
3-methylindole			131.17	511.6~6834.3	[2]
L-hydroxyproline			131.13	160	[3]
Melamine			126.14	150000	[4]
Melamine			126.14	70.6	[5]
Melamine			126.14	22.6	[6]
Melamine			126.14	13	[7]
Melamine			126.14	6.0	[8]
Melamine			126.14	1.7	[9]

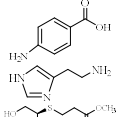
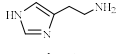
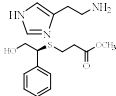
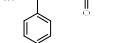
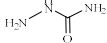
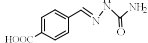
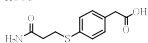
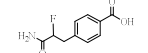
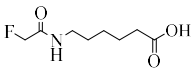
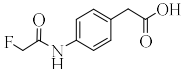
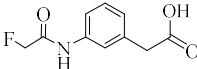
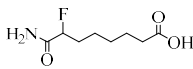
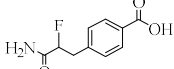
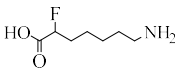
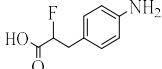
Benzoic Acid			122.12	2100	[10]
Histamine			111.15	333000	[11]
Styrene			104.15	5360	[12]
Semicarbazide			75.07	13000	[13]
Acrylamide			71.08	28600	[14]
Fluoroacetamide			77.06	97000	This study

Table 2. The detailed information on antibodies titers and inhibition ratios of mice at the third immunizations.

Haptens	FAM1	FAM2	FAM3	FAM4	FAM5	FAA1	FAA2
Hapten							
Titers (×10 ⁴) ^a	0.69	2.63	2.38	0.63	2.75	0.50	2.19
Inhibition ratios ^b	96.48	93.57	95.22	95.75	55.68	98.31	96.33

The antibody titer is the dilution of antiserum when furnishing OD_{max} between 1.5 and 2.0 determined by nc-ELISA. Data were averaged from values of 8 mice, and each mouse was tested in triplicate.

^b The inhibition ratio is calculated by using the following equation: *Inhibition ratio* = $(B / B_0) \times 100\%$, where B₀ and B are the responses, i.e., the OD values in the absence of FAM (B₀) and for a concentration of FAM of 500 µg/mL (B) determined by ic-ELISA. Data are averaged values of eight mice, and each mouse was tested in triplicate.

Table 3. The isotypes of mAbs.

Isotypes ^b	mAb name ^a				
	1B6	2C11	3F1	4E10	5D11
IgG ₁	2.056^c	1.915	2.920	2.089	0.190
IgG _{2a}	0.282	0.137	0.452	0.199	1.908
IgG _{2b}	0.206	0.083	0.335	0.156	0.068
IgG ₃	0.142	0.067	0.332	0.156	0.059
IgA	0.059	0.145	0.063	0.071	0.167
IgM	0.060	0.142	0.063	0.072	0.308
Kappa (κ)	0.927	0.249	0.753	1.394	0.562
Lambda (λ)	0.106	1.255	0.272	0.252	2.817

Note: ^aAll of the mAbs were 1 mg mL⁻¹ and diluted 1 × 10⁴ fold.

^b For wells with the highest response, the isotype and light chain composition are indicated.

^c The numbers are the OD values, and the bold font highlights the antibody isotype or subtype.

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