Pre-clinical efficacy and selectivity of vaccines targeting fentanyl, alfentanil, sufentanil, and acetylfentanyl in rats.

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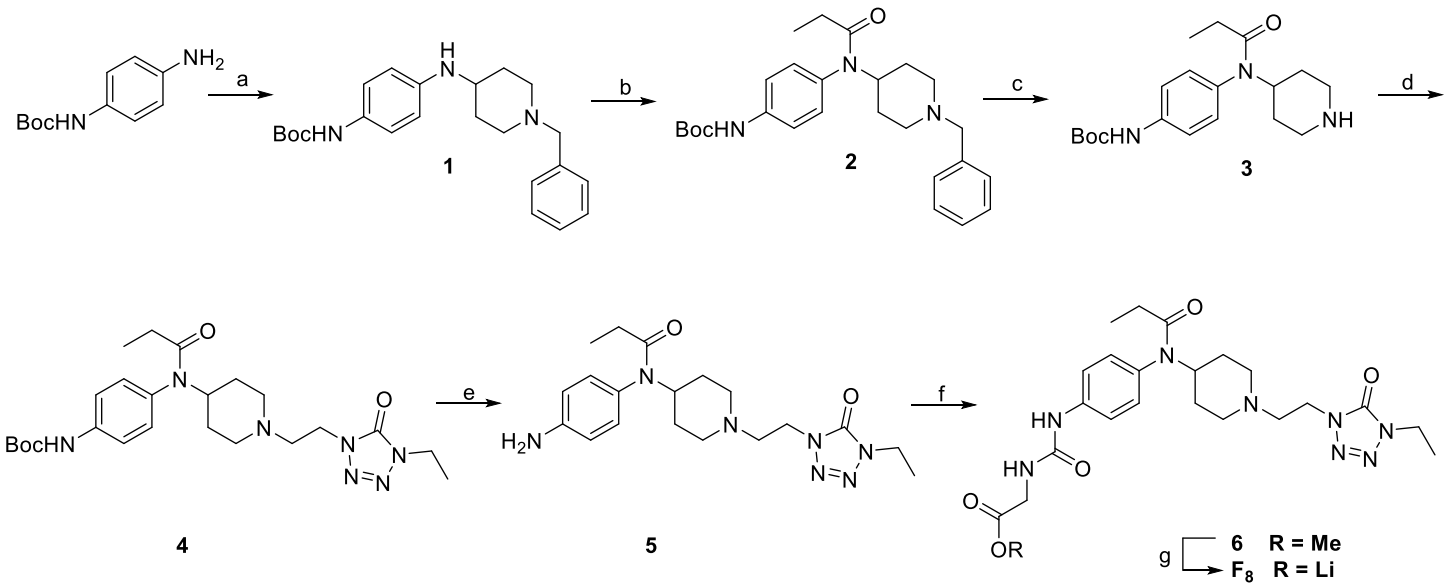
SUPPLEMENTAL MATERIAL

Supplemental Methods

Scheme I. Synthesis of F_8 hapten



Reagents and conditions: a) *N*-Benzyl-4-piperidone, AcOH, $Na(OAc)_3BH$, DCM:THF, 0 °C→rt, 14 h; b) propionic anhydride, DIPEA, DCM, 0 °C→rt, 20 h; c) ammonium formate, Pd (10% on C), MeOH, rt, 3 h; d) 1-(2-Bromoethyl)-4-ethyl-1,4-dihydro-5H-tetrazol-5-one, Na_2CO_3, 4-Methyl-2-pentanone, reflux, 4 h; e) HCl (4 M in dioxane), DCM, 0 °C→rt, 20 h; f) 4-nitrophenyl chloroformate, DIPEA, glycine methyl ester, THF, 0 °C→rt, 2 h; g) $LiOH·H_2O$, THF/MeOH/H_2O, 36 h.

***tert*-Butyl (4-((1-benzylpiperidin-4-yl)amino)phenyl)carbamate (1).** A solution of *N*-Boc-*p*-phenylenediamine (2.75 g, 13.21 mmol) in DCM:THF (100 mL, 1:1, v/v) was cooled to 0 °C and acetic acid (0.76 mL, 13.21 mmol) was added dropwise to the above solution. *N*-Benzyl-4-piperidone (2.50 g, 13.21 mmol) was then added, followed by sodium triacetoxyborohydride (4.20 g, 19.82 mmol) in three portions at 0 °C. The reaction was warmed and stirred at room temperature for 14 h. Upon completion, the reaction was quenched with saturated aq. $NaHCO_3$ (30 mL). The organic layer was separated, and the aqueous layer was extracted with DCM (3 x 30 mL). The combined organic layers were washed with brine (3 x 30 mL), and dried (Na_2SO_4). The solvent was removed under reduced pressure and the residue was subjected to chromatography on silica gel using 0–50% CMA80 in DCM to furnish amine **1** (4.58 g, 91%) as a yellow solid. ^{1}H NMR

(300 MHz, $CDCl_3$) δ 7.35–7.21 (m, 5H), 7.11 (d, *J* = 8.3 Hz, 2H), 6.61–6.44 (m, 2H), 6.31 (s, 1H), 3.51 (s, 2H), 3.42–3.13 (m, 2H), 2.89–2.75 (m, 2H), 2.12 (td, *J* = 11.6, 2.3 Hz, 2H), 2.05–1.94 (m, 2H), 1.52–1.34 (m, 11H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.5, 143.6, 138.4, 129.1, 128.6, 128.2, 127.0, 121.4, 113.9, 79.9, 63.2, 52.4, 50.5, 32.6, 28.4; MS (ESI) *m/z*: calculated for $C_{23}H_{31}N_3O_2$ 381.51, found 382.4 $[M + H]^+$.

***tert*-Butyl (4-(*N*-(1-benzylpiperidin-4-yl)propionamido)phenyl)carbamate (2).** To a solution of amine **1** (4.30 g, 11.27 mmol) in dry DCM (60 mL), DIPEA (3.85 mL, 22.54 mmol) and propionic anhydride (5.75 mL, 45.08 mmol) was added at 0 ºC. The reaction which resulted, was stirred for 20 h at room temperature. At this point, the reaction was cooled to 0 ºC and quenched with saturated aq. $NaHCO_3$ (40 mL). The layers were separated, and the aqueous layer was extracted with additional DCM (3 x 50 mL). The combined organic layers were washed with brine (3 x 50 mL), dried (Na_2SO_4), and concentrated under reduced pressure to give a yellowish residue. The residue was subjected to chromatography on silica gel using 0–50% CMA80 in DCM to furnish amide **2** (4.58 g, 93%) as a yellow solid. ^1H NMR (300 MHz, $CDCl_3$) δ 7.65 (s, 1H), 7.40 (d, *J* = 8.6 Hz, 2H), 7.34–7.14 (m, 5H), 6.92 (d, *J* = 8.5 Hz, 2H), 4.70–4.53 (m, 1H), 3.53 (s, 2H), 3.06–2.91 (m, 2H), 2.31–2.10 (m, 2H), 1.94 (q, *J* = 7.4 Hz, 2H), 1.80–1.67 (m, 2H), 1.57–1.35 (m, 11H), 0.99 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.0, 152.9, 138.9, 136.6, 133.0, 130.5, 129.5, 128.2, 127.4, 118.9, 80.6, 62.3, 52.5, 51.9, 29.8, 28.4, 28.3, 9.6; MS (ESI) *m/z*: calculated for $C_{26}H_{35}N_3O_3$ 437.57, found 438.6 $[M + H]^+$.

***tert*-Butyl (4-(*N*-(piperidin-4-yl)propionamido)phenyl)carbamate (3).** To a solution of benzylamine **2** (4.58 g, 10.47 mmol) in MeOH (60 mL), ammonium formate (3.30 g, 52.33 mmol) and Pd (2.23g, 2.09 mmol, 10% on C) were added. The resulting reaction was stirred at room temperature for 3 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated to dryness. The residue was re-dissolved in DCM (50 mL), washed with saturated aq. $NaHCO_3$ (30 mL), brine (3 x 50 mL), dried (Na_2SO_4), and concentrated *in vacuo* to provide amine **3** (3.12 g, 86%) as a white solid. ^1H NMR (300 MHz, $CDCl_3$) δ 7.41 (d, *J* = 8.7 Hz, 2H), 7.03–6.90 (m, 3H), 4.80–4.62 (m, 1H), 3.15–2.98 (m, 2H), 2.85–2.61 (m, 3H), 1.93 (q, *J* = 7.4 Hz, 2H), 1.84–1.71 (m, 2H), 1.53 (s, 9H), 1.34–1.16 (m, 2H), 1.00 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.7, 152.6, 138.6, 133.5, 130.8, 118.9, 80.9, 52.2, 45.8, 31.6, 28.4, 28.3, 9.6; MS (ESI) *m/z*: calculated for $C_{19}H_{29}N_3O_3$ 347.45, found 348.4 $[M + H]^+$.

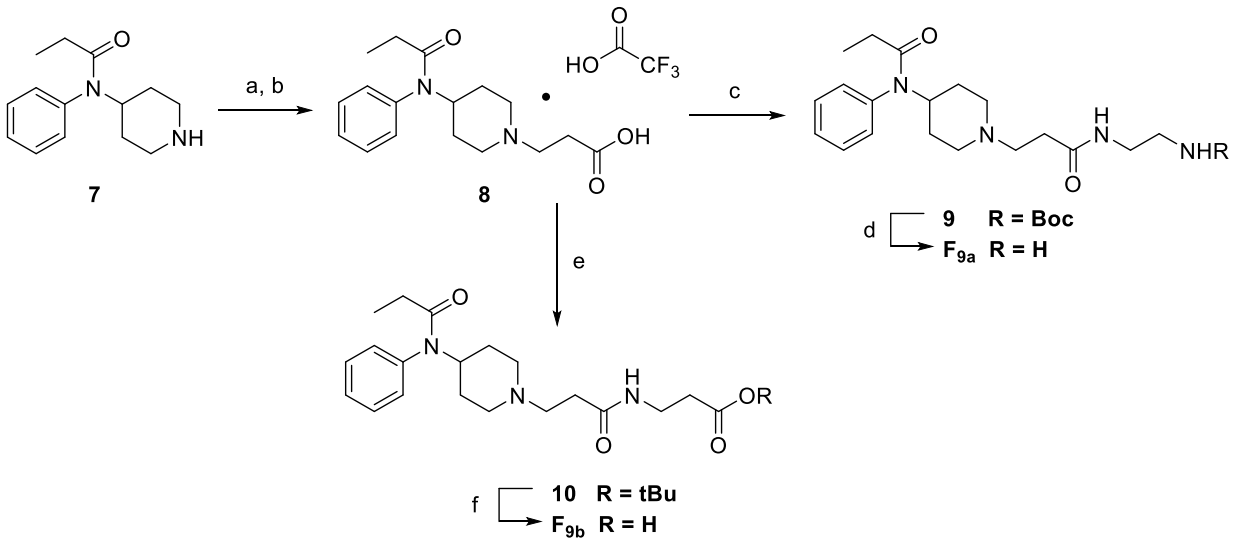
***tert*-Butyl (4-(*N*-(1-(2-(4-ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl)piperidin-4-yl)propion-amido)phenyl) carbamate (4).** A suspension of amine **3** (0.80 g, 2.30 mmol), 1-(2-Bromoethyl)-4-ethyl-1,4-dihydro-5H-tetrazol-5-one (0.29 mL, 2.30 mmol) and Na_2CO_3 (0.49 g, 4.60 mmol) in 4-Methyl-2-pentanone (15 mL) was refluxed for 4 h. After that, the solution was cooled to room temperature and diluted with EtOAc (20 mL). The solution was then washed with H_2O (2 x 30 mL), brine (2 x 20 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The residue was subjected to chromatography on silica gel using 0–50% CMA80 in EtOAc to furnish tetrazolone **4** (0.89 g, 80%) as a white solid. ^1H NMR (300 MHz, $CDCl_3$) δ 7.41 (d, *J* = 8.6 Hz, 2H), 6.96 (d, *J* = 8.6 Hz, 2H), 6.81 (s, 1H), 4.67–4.52 (m, 1H), 4.07–3.86 (m, 4H), 3.04–2.83 (m, 2H), 2.79–2.65 (m, 2H), 2.27–2.11 (m, 2H), 2.01–1.84 (m, 3H), 1.78–1.67 (m, 2H), 1.53 (s, 9H), 1.40–1.30 (m, 4H), 0.99 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.8, 152.6, 150.7, 138.5, 133.4, 130.8, 118.9, 80.9, 55.5, 52.9, 52.0, 42.4, 40.0, 30.4, 28.4, 28.3, 13.7, 9.6; MS (ESI) *m/z*: calculated for $C_{24}H_{37}N_7O_4$ 487.60, found 488.6 $[M + H]^+$.

***N*-(4-aminophenyl)-*N*-(1-(2-(4-ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl)piperidin-4-yl)propion-amide (5).** To an ice-cold solution of carbamate **4** (0.89 g, 1.83 mmol) in dry DCM (20 mL), HCl (4.58 mL, 4 M solution in dioxane) was added. The reaction, which resulted was stirred at room temperature for 20 h. The solvent was removed under reduced pressure and the residue was subjected to chromatography on silica gel using 0–100% CMA80 in DCM to furnish amine **5** (0.59 g, 84%) as a white solid. ^1H NMR (300 MHz, $CDCl_3$) δ 6.88–6.71 (m, 2H), 6.70–6.56 (m, 2H), 4.66–4.50 (m, 1H), 4.05–3.89 (m, 4H), 3.80 (br s, 2H), 2.99–2.85 (m, 2H), 2.71 (t, *J* = 6.9 Hz, 2H), 2.27–2.10 (m, 2H), 1.95 (q, *J* = 7.5 Hz, 2H), 1.76–1.64 (m, 2H), 1.41–1.23 (m, 5H), 0.99 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.2, 150.7, 146.3, 131.0, 129.3, 115.1, 55.6, 53.0, 51.9, 42.4, 40.0, 30.4, 28.3, 13.7, 9.6; MS (ESI) *m/z*: calculated for $C_{19}H_{29}N_7O_2$ 387.48, found 388.4 $[M + H]^+$.

Methyl 2-(3-(4-(*N*-(1-(2-(4-ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl)piperidin-4-yl)propionamido) phenyl) ureido)acetate (6). To a suspension of amine **5** (376 mg, 0.97 mmol) in THF (6 mL), DIPEA (0.42 mL, 2.43 mmol) was added. The reaction was then cooled to 0 ºC, and a solution of 4-nitrophenyl chloroformate (0.24 g, 1.16 mmol) in THF (3 mL) was added dropwise. After that, the reaction was stirred at 0 ºC for 30 min. At this point, a solution of glycine methyl ester (0.17 g, 1.94 mmol) and DIPEA (0.42 mL, 2.43 mmol) in THF (10 mL) was added to the above reaction. The reaction was then stirred for 30 min at 0 ºC and an additional 1.5 h at room temperature. The reaction was then quenched with MeOH (5 mL) and concentrated to dryness under reduced pressure. The residue was subjected to chromatography on silica gel using 0–100% CMA80 in EtOAc to furnish urea **6** (314.5 mg, 64%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.04–7.83 (m, 1H), 7.42 (d, *J* = 8.7 Hz, 2H), 6.95 (d, *J* = 8.5 Hz, 2H), 6.14–5.95 (m, 1H), 4.73–4.47 (m, 1H), 4.08 (d, *J* = 5.2 Hz, 2H), 4.04–3.90 (m, 4H), 3.76 (s, 3H), 2.92 (d, *J* = 10.9 Hz, 2H), 2.71 (t, *J* = 6.7 Hz, 2H), 2.17 (t, *J* = 11.3 Hz, 2H), 1.95 (q, *J* = 7.4 Hz, 2H), 1.73 (d, *J* = 11.3 Hz, 2H), 1.43–1.22 (m, 5H), 1.00 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.3, 171.6, 155.4, 151.0, 139.4, 133.4, 130.9, 120.0, 55.7, 53.1, 52.5, 52.4, 42.6, 42.1, 40.3, 30.6, 28.7, 13.9, 9.8; MS (ESI) *m/z*: calculated for $C_{23}H_{34}N_8O_5$ 502.57, found 503.6 $[M + H]^+$. HPLC (280 nm) t_R = 10.10 min.

Lithium 2-(3-(4-(N-(1-(2-(4-ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl)piperidin-4-yl)propionamido)phenyl) ureido)acetate (F_8). To a solution of the ester **6** (314 mg, 0.62 mmol) in THF/MeOH/H_2O (5 mL, 1: 1: 0.5, v/v/v), LiOH·H_2O (33 mg, 0.78 mmol) was added and the resulting reaction was stirred at room temperature for 36 h. The solvent was then removed under nitrogen flow to provide lithium salt **F_8** (310 mg, quant.) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.86 (br s, 1H), 7.86 (br s, 1H), 7.61 (d, *J* = 8.5 Hz, 2H), 6.93 (d, *J* = 8.6 Hz, 2H), 4.45–4.28 (m, 1H), 3.93 (t, *J* = 6.1 Hz, 2H), 3.84 (q, *J* = 7.2 Hz, 2H), 3.71–3.61 (m, 2H), 2.83 (d, *J* = 10.3 Hz, 2H), 2.58 (t, *J* = 6.2 Hz, 2H), 2.02 (t, *J* = 11.2 Hz, 2H), 1.84 (q, *J* = 7.4 Hz, 2H), 1.60 (d, *J* = 9.7 Hz, 2H), 1.27–1.05 (m, 5H), 0.86 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.3, 172.3, 155.4, 150.1, 141.7, 130.5, 129.9, 117.9, 54.9, 52.2, 51.5, 45.0, 41.7, 39.4, 30.0, 27.6, 13.4, 9.5; MS (ESI) *m/z*: calculated for $C_{22}H_{32}N_8O_5$ 488.54, found 489.4 $[M + H]^+$. HPLC (220 nm) t_R = 9.35 min.

Scheme II. Synthesis of F_{9a}, F_{9b} haptens



Reagents and conditions: a) *tert*-Butyl acrylate, CH_3CN, rt, 20 h; b) TFA, rt, 1.5 h; c) *N*-Boc-ethylenediamine, EDC, DMAP, CH_2Cl_2, 0 °C→rt; 20 h; d) 4 M HCl in dioxane, THF, rt, 1 h; e) β-Alanine HCl *tert*-butyl ester, EDC, DMAP, CH_2Cl_2, 0 °C→rt; 20 h; f) TFA, rt, 1.5 h.

3-(4-(N-phenylpropionamido)piperidin-1-yl)propanoic acid TFA salt (8). (29) A mixture of norfentanyl (**7**, 3.0 g, 12.9 mmol, 1.0 equiv) in 18 mL of anhydrous CH_3CN was treated with *tert*-butyl acrylate (2.4 mL, 16.1 mmol, 1.25 equiv) via syringe at ambient temperature. The reaction was maintained for 24 h. TLC analysis (5% MeOH/CH_2Cl_2) showed the reaction was mostly complete. The resulting solution was concentrated to afford a viscous, yellow oil. The crude product

was purified by filtering through a plug of SiO_2 (150 mL fritted glass funnel) eluting with CH_2Cl_2 (250 mL) then 5% MeOH/CH_2Cl_2 (400 mL) to afford 4.2 g (90 %) of a white solid. ^{1}H NMR (500 MHz, DMSO-d_6): MS: m/z 305.16 [M-^{t}Bu+2H]$^+$. The resulting *tert*-Butyl ester (0.863 g, 2.39 mmol, 1.0 equiv) was then treated with TFA (11 mL, 60 equiv) at ambient temperature. The reaction was maintained for 1.5 h. LC-MS analysis showed that the reaction was complete. The reaction was concentrated to afford a viscous, yellow oil. Trituration from dry Et_2O resulted in the formation of a white precipitate. The solid was filtered and washed with Et_2O then dried under reduced pressure to afford 960 mg (96%) of a white solid. ^{1}H NMR (500 MHz, DMSO-d6): δ 12.75 (brs, 1H), 8.97 (brs, 1H), 7.54-7.48 (m, 2H), 7.48-7.42 (m, 1H), 7.26 (d, J = 7.3 Hz, 2H), 4.80-4.64 (m, 1H), 3.45 (d, J = 11.8 Hz, 2H), 3.18 (t, J = 7.5 Hz, 2H), 3.12 (brt, J = 12.6 Hz, 2H), 2.66 (t, J = 7.5 Hz, 2H), 1.93 (brd, J = 13.0 Hz, 2H), 1.83 (q, J = 7.4 Hz, 2H), 1.57-1.40 (m, 2H), 0.88 (t, J = 7.4 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d6): δ 172.2, 171.5, 138.2, 130.5 (2C), 129.5 (2C), 128.6, 51.5, 51.4 (2C), 48.5, 28.7, 27.8, 27.3 (2C), 9.4; MS: m/z calculated for $C_{17}H_{24}N_2O_3$ 304.18, found 305.12 [M+H]$^+$.

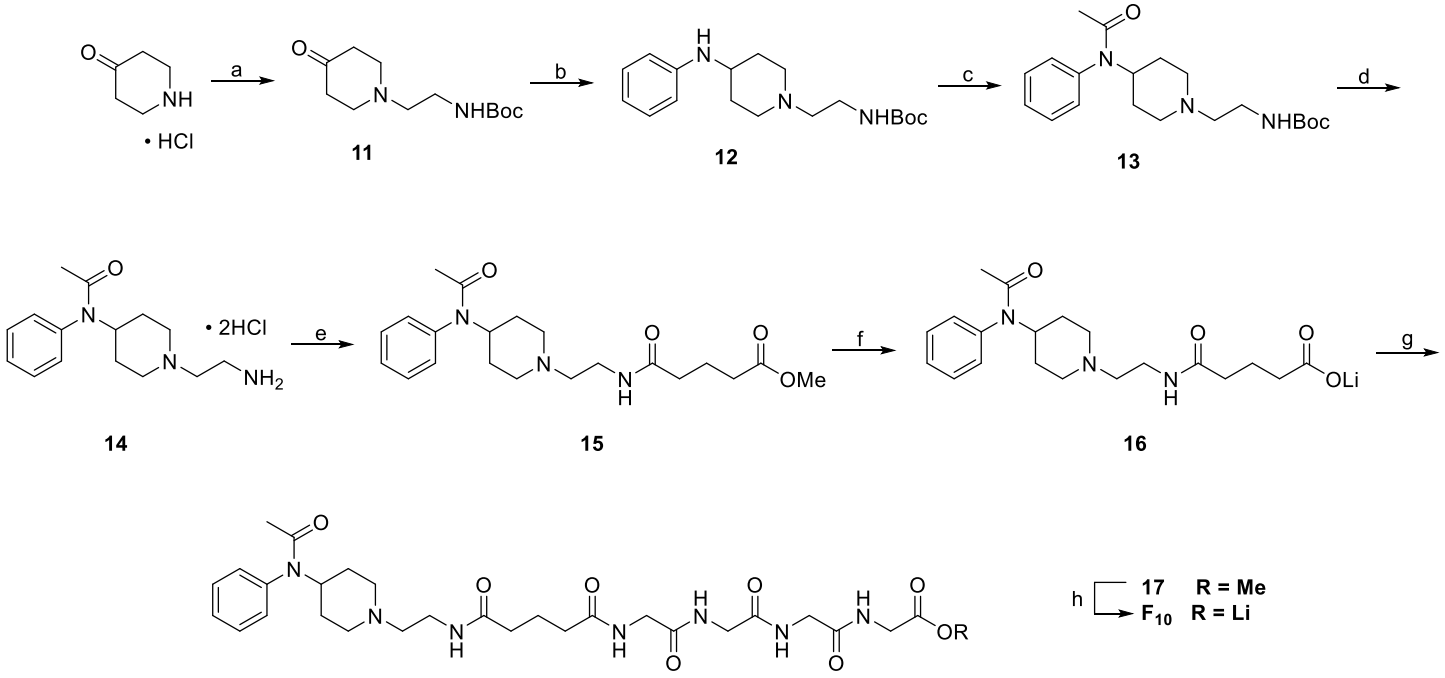
***tert*-Butyl (2-(3-(4-(N-phenylpropionamido)piperidin-1-yl)propanamido)ethyl)carbamate (9).** To a 0°C suspension of acid **8** (0.300 g, 0.717 mmol, 1.0 equiv) in 7 mL of anhydrous CH_2Cl_2 was added *N*-Boc ethylenediamine (0.170 mL, 1.08 mmol, 1.5 equiv) via pipette. The suspension became a clear solution. EDC (0.344 g, 1.79 mmol, 2.5 equiv) and DMAP (9 mg, 0.0717 mmol, 10 mol%) were added and the reaction was maintained at ambient temperature for 22 h. LC-MS analysis showed the reaction was complete. The reaction mixture was diluted with CH_2Cl_2 (60 mL) and washed with 1 M NaOH (2 x 40 mL), H_2O (2 x 40 mL), and brine. The organics were dried over anhydrous $MgSO_4$, filtered, and concentrated. Purification by flash chromatography on SiO_2 (45 g, 8% MeOH/CH_2Cl_2) afforded 0.285 g (89%) of a white foam. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.87-7.75 (m, 1H), 7.51-7.35 (m, 3H), 7.18 (d, *J* = 7.4 Hz, 2H), 6.72 (t, *J* = 5.5 Hz, 1H), 4.50-4.30 (m, 1H), 3.05-2.95 (m, 2H), 2.93-2.85 (m, 2H), 2.85-2.75 (m, 2H), 2.49-2.38 (br, 2H), 2.19-2.06 (m, 2H), 2.05-1.89 (br, 2H), 1.81 (q, *J* = 7.4 Hz, 2H), 1.66 (d, *J* = 11.6 Hz, 2H), 1.36 (s, 9H), 1.23-1.10 (m, 2H), 0.87 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 171.7, 171.1, 155.6, 138.8, 130.3 (2C), 129.3 (2C), 128.2, 77.6, 53.8, 52.3 (2C), 51.7, 38.6, 33.4, 30.0 (2C), 28.2 (4C), 27.8, 9.5; MS: m/z calculated for $C_{24}H_{38}N_4O_4$ 446.29, found 447.27 [M+H]$^+$, 469.25 [M+Na]$^+$.

***N*-(2-Aminoethyl)-3-(4-(*N*-phenylpropionamido)piperidin-1-yl)propanamide dihydrochloride (F_{9a}).** *N*-Boc amine **9** (0.126 g, 0.282 mmol, 1.0 equiv) was treated with 5.6 mL of 4 M HCl in dioxane (22.6 mmol, 80 equiv) at 0 °C. The resulting reaction mixture was then removed from the ice bath and maintained at ambient temperature. After 1 h, the reaction was concentrated under reduced pressure and triturated with anhydrous Et_2O. The resulting precipitate was filtered and dried to yield 83 mg (70 %) of a tacky, off-white solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.1 (br s, 1H), 8.48 (t, *J* = 5.5 Hz, 1H), 8.16 (br s, 3H), 7.54-7.39 (m, 3H), 7.23 (d, *J* = 7.4 Hz, 2H), 4.78-4.62 (m, 1H), 3.30 (q, *J* = 6.0 Hz, 2H), 3.24-3.16 (m, 2H), 3.15-3.03 (m, 2H), 2.84 sextet, *J* = 5.9 Hz, 2H), 2.62 (t, *J* = 7.5 Hz, 2H), 1.90 (br d, *J* = 13 Hz, 2H), 1.82 (q, *J* = 7.4 Hz, 2H), 1.61 (qd, *J* = 3.5, 13 Hz, 2H), 0.88 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.2, 169.4, 138.1, 130.4 (2C), 129.5 (2C), 128.5, 51.9, 51.2 (2C), 48.9, 38.4, 36.5, 29.6, 27.7, 27.2 (2C), 9.4; MS: m/z calculated for $C_{19}H_{30}N_4O_2$ 346.24, found 347.20 [M+H]$^+$, 369.16 [M+Na]$^+$.

***tert*-Butyl 3-(3-(4-(*N*-phenylpropionamido)piperidin-1-yl)propanamido)propanoate (10).** To a 0 °C suspension of carboxylic acid **8** (0.300 g, 0.717 mmol, 1.0 equiv) in 7 mL of anhydrous CH_2Cl_2 was added β-alanine *tert*-butyl ester hydrochloride (0.195 g, 1.08 mmol, 1.5 equiv) followed by diisopropylethylamine (0.250 mL, 1.43 mmol, 2.0 equiv). The resulting clear solution was then treated with EDC (0.344 g, 1.79 mmol, 2.5 equiv) and DMAP (9 mg, 0.0717 mmol, 10 mol%). The reaction was maintained at ambient temperature for 20 h. LC-MS analysis showed the reaction was complete. The mixture was diluted with CH_2Cl_2 (60 mL) then washed with 1 M NaOH (2 x 40 mL), H_2O (2 x 40 mL), and brine. The organics were dried over anhydrous $MgSO_4$, filtered, and concentrated. Purification by flash chromatography on SiO_2 (42 g, 5% MeOH/CH_2Cl_2) afforded 286 mg (92%) of a viscous, pale yellow oil. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.91 (t, *J* = 5.6 Hz, 1H), 7.50-7.37 (m, 3H), 7.19 (d, *J* = 7.4 Hz, 2H), 4.48-4.32 (m, 1H), 3.16 (q, *J* = 6.7 Hz, 2H), 2.79 (br d, *J* = 11 Hz, 2H), 2.42 (br t, *J* = 6.8 Hz, 2H), 2.26 (t, *J* = 6.8 Hz, 2H), 2.12 (t, *J* = 7.2 Hz, 2H), 1.95 (br t, *J* = 11 Hz, 2H), 1.80 (q, *J* = 7.4 Hz, 2H), 1.66 (br d, *J* = 11 Hz, 2H), 1.37 (s, 9H), 1.16 (qd, *J* = 3.2, 12 Hz, 2H), 0.87 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 171.7, 171.1, 170.6, 138.8, 130.3 (2C), 129.2 (2C), 128.2, 79.8, 53.7, 52.3 (2C), 51.7, 35.0, 34.7, 33.2, 30.1 (2C), 27.8, 27.7 (3C), 9.5; MS: m/z calculated for $C_{24}H_{37}N_3O_4$ 431.28, found 432.23 [M+H]$^+$.

3-(3-(4-(*N*-phenylpropionamido)piperidin-1-yl)propanamido)propanoic acid (F_{9b}). *tert*-Butyl ester **10** (0.188 g, 0.436 mmol, 1.0 equiv) was treated with TFA (2 mL, 60 equiv) at ambient temperature. The reaction was maintained for 1.5 h. LC-MS analysis showed that the reaction was complete. The reaction mixture was concentrated under reduced pressure to afford a viscous, yellow oil. Trituration from dry Et_2O afforded a sticky, white oil. The Et_2O was decanted and the residue was washed with Et_2O then dried under reduced pressure to yield 204 mg (96%) of a tacky, pale yellow residue. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.95 (br s, 1H), 8.17 (t, *J* = 5.5 Hz, 1H), 7.54-7.40 (m, 3H), 7.25 (d, *J* = 7.3 Hz, 2H), 4.78-4.66 (m, 1H), 3.43 (d, *J* = 12 Hz, 2H), 3.28-3.15 (m, 4H), 3.14-3.02 (m, 2H), 2.48 (t, *J* = 7.2 Hz, 2H), 2.37 (t, *J* = 6.8 Hz, 2H), 1.93 (d, *J* = 13 Hz, 2H), 1.83 (q, *J* = 7.4 Hz, 2H), 1.49 (qd, *J* = 13, 3.6 Hz, 2H), 0.88 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.3, 172.6, 169.1, 138.6, 130.9 (2C), 130.0 (2C), 129.0, 52.6, 51.7 (2C), 49.0, 35.3, 34.1, 29.9, 28.2, 27.8 (2C), 9.8; MS: m/z calculated for $C_{20}H_{29}N_3O_4$ 375.22, found 376.15 $[M+H]^+$.

Scheme III. Synthesis of F_{10} hapten



Reagents and conditions: a) *N*-Boc-2-aminoacetaldehyde, $Na(OAc)_3BH$, 1,2-DCE, 0 °C→rt, 4 h; b) $PhNH_2$, AcOH, $Na(OAc)_3BH$, DCM, 0 °C→rt, 14 h; c) Ac_2O, DIPEA, DCM, 0 °C→rt, 24 h; d) HCl (4 M in dioxane), DCM, 0 °C→rt, 3 h; e) Methyl glutaryl chloride, TEA, DCM, 0 °C→rt, 1 h; f) $LiOH·H_2O$, THF/MeOH/H_2O, 6 h; g) gly_4OMe, BOP, TEA, DMF, 0 °C→rt, 8 h; h) $LiOH·H_2O$, THF/MeOH/H_2O, 30 h.

***tert*-Butyl (2-(4-oxopiperidin-1-yl)ethyl)carbamate (11).** 4-Piperidone monohydrate hydrochloride (5 g, 32.55 mmol) was dissolved in 1,2-DCE (50 mL). *N*-Boc-2-aminoacetaldehyde (6.2 g, 39.06 mmol) was dissolved in 1,2-DCE (10 mL) and added dropwise to the above solution at 0 °C. After that, sodium triacetoxyborohydride (10.3 g, 48.82 mmol) was added to the above reaction in three portions at 0 °C. The reaction was warmed to room temperature and stirred for 4 h. After completion of the reaction, as indicated by LCMS, the reaction was quenched with saturated aq. $NaHCO_3$ (30 mL). The organic layer was separated, and the aqueous layer was extracted with DCM (3 x 50 mL). The combined organic layers was washed with brine (3 x 50 mL), and dried (Na_2SO_4). The solvent was removed under reduced pressure to furnish compound **11** as a yellowish residue (7.83 g, 99% crude yield). This material was used for the next transformation without purification. MS (ESI) *m/z*: calculated for $C_{12}H_{22}N_2O_3$ 242.31, found 243.2 $[M+H]^+$.

***tert*-Butyl (2-(4-(phenylamino)piperidin-1-yl)ethyl)carbamate (12).** A solution of aniline (2.9 mL, 32.31 mmol) in DCM (50 mL) was cooled to 0 °C. Acetic acid (1.8 mL, 32.31 mmol) was added dropwise to the above solution at 0 °C. After that, piperidone **11** (7.83 g, 32.31 mmol) dissolved in DCM (20 mL) was added slowly, followed by sodium

triacetoxyborohydride (10.27 g, 48.47 mmol) in three portions at 0 ºC. The reaction was stirred at room temperature for 14 h. The reaction was then quenched with saturated aq. $NaHCO_3$ (30 mL). The layers were separated, and the aqueous layer was extracted with additional DCM (3 x 30 mL). The combined organic layers was washed with brine (3 x 30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The residue was subjected to chromatography on silica gel using 0–100% CMA80 in DCM to furnish amine **12** (3.72 g, 36%) as a waxy white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.18 (dd, *J* = 8.4, 7.4 Hz, 2H), 6.70 (t, *J* = 7.3 Hz, 1H), 6.61 (d, *J* = 7.7 Hz, 2H), 5.08 (br s, 1H), 3.54 (br s, 1H), 3.41–3.08 (m, 3H), 2.88 (d, *J* = 11.6 Hz, 2H), 2.50 (t, *J* = 5.9 Hz, 2H), 2.19 (t, *J* = 11.3 Hz, 2H), 2.12–1.95 (m, 2H), 1.62–1.32 (m, 11H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.9, 147.1, 129.3, 117.3, 113.3, 79.2, 57.2, 52.2, 49.8, 37.4, 32.4, 28.4; MS (ESI) *m/z*: calculated for $C_{18}H_{29}N_3O_2$ 319.44, found 320.4 $[M+H]^+$.

***tert*-Butyl (2-(4-(*N*-phenylacetamido)piperidin-1-yl)ethyl)carbamate (13).** To a solution of amine **12** (1.00 g, 3.13 mmol) in dry DCM (30 mL), DIPEA (1.1 mL, 6.26 mmol) and acetic anhydride (1.3 mL, 12.52 mmol) was added at 0 ºC. The reaction which resulted, was stirred at room temperature for 24 h. The reaction was cooled to 0 ºC and quenched with saturated aq. $NaHCO_3$ (30 mL). The layers were separated, and the aqueous layer was extracted with additional DCM (3 x 30 mL). The combined organic layers was washed with brine (3 x 50 mL), dried (Na_2SO_4), and concentrated under reduced pressure to give a yellowish residue. The residue was subjected to chromatography on silica gel using 0–100% CMA80 in DCM to furnish acetamide **13** (1.10 g, 97%) as a yellowish oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40–7.28 (m, 3H), 7.07–6.97 (m, 2H), 4.87 (br s, 1H), 4.64–4.48 (m, 1H), 3.14–3.00 (m, 2H), 2.81 (d, *J* = 10.7 Hz, 2H), 2.32 (t, *J* = 5.9 Hz, 2H), 2.05 (t, *J* = 11.7 Hz, 2H), 1.76–1.64 (m, 5H), 1.37–1.27 (m, 11H;. ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.1, 155.8, 139.4, 130.1, 129.3, 128.3, 79.0, 57.1, 52.9, 52.2, 37.4, 30.3, 28.4, 23.4; MS (ESI) *m/z*: calculated for $C_{20}H_{31}N_3O_3$ 361.48, found 362.4 $[M+H]^+$.

***N*-(1-(2-Aminoethyl)piperidin-4-yl)-*N*-phenylacetamide hydrochloride (14).** To an ice cold solution of carbamate **3** (0.08 g, 0.21 mmol) in dry DCM (6 mL), HCl (0.6 mL, 4 M solution in dioxane) was added. The reaction was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and excess HCl was removed by flash evaporation with DCM (3 x 10 mL) to provide the HCl salt of amine **14** (0.05 g, 76%) as a white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 7.59–7.46 (m, 3H), 7.29 (d, *J* = 6.9 Hz, 2H), 4.87–4.76 (m, 1H), 3.72 (d, *J* = 11.7 Hz, 2H), 3.52–3.41 (m, 4H), 3.36–3.28 (m, 2H), 2.21–2.11 (m, 2H), 1.95–1.82 (m, 2H), 1.78 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 173.1, 139.9, 131.2, 131.1, 130.3, 54.5, 54.0, 51.3, 35.3, 28.9, 23.4; MS (ESI) *m/z*: calculated for $C_{15}H_{23}N_3O$ 261.36, found 262.4 $[M+H]^+$.

Methyl 5-oxo-5-((2-(4-(*N*-phenylacetamido)piperidin-1-yl)ethyl)amino)pentanoate (15). To a solution of amine·HCl **14** (0.05 g, 0.17 mmol) in dry DCM (5 mL), TEA (0.13 mL, 0.96 mmol) and glutaric acid monomethyl ester chloride (0.03 mL, 0.24 mmol) was added at 0 ºC. The reaction was then stirred at room temperature for 1h. After the completion of the reaction, as indicated by TLC, the reaction was quenched with cold aq. $NaHCO_3$ (20 mL). The layers were separated, and the aqueous layer was extracted with DCM (3 x 10 mL). The combined organic layers was washed with brine (3 x 20 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The residue was subjected to chromatography on silica gel using 0–100% CMA80 in DCM to furnish amide **15** (48 mg, 69%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51–7.34 (m, 3H), 7.18–7.03 (m, 2H), 6.07 (s, 1H), 4.69–4.56 (m, 1H), 3.63 (s, 3H), 3.29 (dd, *J* = 11.1, 5.5 Hz, 2H), 2.91 (d, *J* = 11.1 Hz, 2H), 2.44 (t, *J* = 5.7 Hz, 2H), 2.33 (t, *J* = 7.2 Hz, 2H), 2.24–2.12 (m, 4H), 1.98–1.85 (m, 2H), 1.84–1.76 (m, 2H), 1.74 (s, 3H), 1.52–1.33 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 172.0, 170.2, 139.4, 130.1, 129.4, 128.4, 56.6, 52.9, 52.0, 51.5, 36.0, 35.3, 33.1, 30.2, 23.4, 20.8; MS (ESI) *m/z*: calculated for $C_{21}H_{31}N_3O_4$ 389.49, found 390.4 $[M+H]^+$.

Lithium 5-oxo-5-((2-(4-(*N*-phenylacetamido)piperidin-1-yl)ethyl)amino)pentanoate (16). To a solution of the ester **15** (1.18 g, 3.03 mmol) in THF/MeOH/H_2O (50 mL, 1: 1: 0.5, v/v/v), $LiOH·H_2O$ (0.16 g, 3.79 mmol) was added and the reaction, which resulted, was stirred at room temperature for 6 h. The solvent was removed under nitrogen flow to provide lithium salt **16** (1.02 g, quant.) as a white solid. This material was used for the next transformation without any purification. ^{1}H NMR (300 MHz, CD_3OD) δ 7.57–7.42 (m, 3H), 7.24 (d, *J* = 6.8 Hz, 2H), 4.62–4.49 (m, 1H), 3.26 (t, *J* = 6.7 Hz, 2H), 2.98 (d, *J* = 11.3 Hz, 2H), 2.43 (t, *J* = 6.7 Hz, 2H), 2.24–2.08 (m, 6H), 1.88–1.78 (m, 4H), 1.76 (s, 3H), 1.43 (dt, *J* = 12.0,

9.4 Hz, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 181.8, 176.0, 172.6, 140.4, 131.3, 130.7, 130.0, 58.1, 54.1, 54.0, 38.4, 37.7, 37.0, 31.3, 24.1, 23.6; MS (ESI) *m/z*: calculated for $C_{20}H_{29}N_3O_4$ 375.47, found 376.4 $[M+H]^+$.

Methyl 4,7,10,13,17-pentaoxo-20-(4-(*N*-phenylacetamido)piperidin-1-yl)-3,6,9,12,18-pentaazai-cosan-1-oate (17). The lithium salt **16** (0.62 g, 1.62 mmol) and BOP (1.07 g, 2.43 mmol) were dissolved in DMF (5 mL) and cooled to 0 °C. A solution of TEA (0.34 mL, 2.43 mmol) and Gly_4OMe (0.53 g, 2.03 mmol) in DMF (5 mL) was added dropwise to the above reaction at 0 °C. The reaction was stirred at room temperature for 8 h. The solvent was removed under nitrogen and the residue was subjected to chromatography on silica gel using 0–100% CMA80 in DCM to furnish amide **17** (413 mg, 41%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.27 (t, *J* = 5.8 Hz, 1H), 8.22–8.06 (m, 3H), 7.94 (t, *J* = 5.3 Hz, 1H), 7.55–7.42 (m, 3H), 7.27 (d, *J* = 6.8 Hz, 2H), 4.68 (t, *J* = 11.9 Hz, 1H), 3.85 (d, *J* = 5.9 Hz, 2H), 3.79–3.69 (m, 6H), 3.63 (s, 3H), 3.49–3.34 (m, 3H), 3.34–3.24 (m, 2H), 3.03–2.87 (m, 3H), 2.10 (dt, *J* = 14.5, 7.4 Hz, 4H), 1.92 (d, *J* = 12.2 Hz, 2H), 1.77–1.67 (m, 2H), 1.64 (s, 3H), 1.46 (dd, *J* = 23.5, 12.0 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.5, 172.3, 170.1, 169.5, 169.3, 169.1, 168.9, 138.8, 130.2, 129.5, 128.5, 55.2, 51.6, 51.5, 49.0, 42.0, 41.7, 40.5, 34.5, 34.3, 33.9, 27.4, 23.0, 21.0; MS (ESI) *m/z*: calculated for $C_{29}H_{43}N_7O_8$ 617.69, found 618.6 $[M + H]^+$. HPLC (220 nm) t_R = 9.05 min.

Lithium 4,7,10,13,17-pentaoxo-20-(4-(*N*-phenylacetamido)piperidin-1-yl)-3,6,9,12,18-pentaazai-cosan-1-oate (F_{10}). To a solution of ester **17** (144 mg, 0.23 mmol) in THF/MeOH/H_2O (5 mL, 1: 1: 0.5, v/v/v), $LiOH·H_2O$ (14 mg, 0.35 mmol) was added, and the resulting reaction was stirred at room temperature. After complete consumption of starting material, as indicated by TLC, the reaction was evaporated to dryness under nitrogen flow to provide the lithium salt **F_{10}** (145 mg, quant.) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.41–8.30 (m, 2H), 8.21 (t, *J* = 5.7 Hz, 1H), 7.77 (t, *J* = 5.5 Hz, 1H), 7.51–7.39 (m, 3H), 7.32 (t, *J* = 4.5 Hz, 1H), 7.20 (d, *J* = 6.6 Hz, 2H), 4.48–4.34 (m, 1H), 3.79–3.62 (m, 6H), 3.35 (s, 2H, merged with DMSO-H_2O), 3.07 (dd, *J* = 12.3, 6.2 Hz, 2H), 2.83 (d, *J* = 11.4 Hz, 2H), 2.25 (t, *J* = 6.6 Hz, 2H), 2.10–1.90 (m, 6H), 1.72–1.63 (m, 4H), 1.61 (s, 3H), 1.27–1.10 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.3, 171.6, 170.4, 169.8, 169.1, 168.5, 167.6, 139.3, 130.2, 129.2, 128.2, 56.8, 52.6, 51.6, 43.9, 42.2, 42.2, 42.0, 36.1, 34.6, 34.4, 30.0, 23.1, 21.4; MS (ESI) *m/z*: calculated for $C_{28}H_{41}N_7O_8$ 603.67, found 604.6 $[M+H]^+$. HPLC (220 nm) t_R = 8.57 min.

Conjugation of haptens to carrier proteins.

Conjugation of F_8, F_{10} to CRM carrier protein.
F_8 hapten (3.6 mg) was dissolved in 0.1M MES buffer pH 5.0 containing 80% DMSO and 250 mM sucrose. F_{10} hapten (4.46 mg) was dissolved in 0.1M MES buffer pH 5.0 containing 10% DMSO. Haptens were activated with 57.2 mg *N*-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Sigma). Carrier protein (4 mg) was added (EcoCRM, FinaBiosolutions, MD; CRM, Pfenex, or BSA, Sigma), and reaction was allowed to proceed for 3 h at rt. The reaction mixture was purified by ultrafiltration using Amicon filters with PBS pH 7.2 to remove unreacted reagents.

Conjugation of F_{9a} to CRM carrier protein.
To a solution of CRM carrier protein (5 mg/mL) in 2 mL of pH 8.0 HEPES buffer was added 2 mL of 1X PBS buffer, pH 6. The protein solution was treated with hapten (20 mg), EDC (18 mg), and sulfo-NHS (210 µL of 10 mg/mL solution in pH 6 buffer, 2.1 mg) in 1.0 mL of pH 6 1X PBS buffer via syringe pump (50 µL/min). The reaction was maintained at ambient temperature for 2 h. At the end of the reaction, the crude reaction mixture became turbid and was purified by dialysis (50K MWCO dialysis membrane) against 1X PBS (pH 7.4) to remove unreacted reagents.

Conjugation of F_{9b} to CRM carrier protein.
To a solution of CRM carrier protein (5 mg/mL) in 1.8 mL of pH 8 HEPES buffer was added 1.8 mL of pH 8.3 1X PBS buffer. The protein was then treated with a solution of hapten (18 mg), EDC (14 mg), and sulfo NHS (160 µL of 10 mg/mL solution in pH 8.3 buffer, 1.6 mg) in 1.0 mL of pH 8.3 1X PBS buffer via syringe pump (50 µL/min). The reaction was maintained at ambient temperature for 2 h. The reaction became turbid. The crude reaction mixture was purified by dialysis (50K MWCO dialysis membrane) against 1X PBS (pH 7.4) to remove unreacted reagents.

Table SI. $F_{8\text{-}10}$ haptens and F_1 as control were conjugated to EcoCRM (Fina Biosolutions) or CRM_{197} (Pfenex) depending on product availability. Molecular weight (MW) was determined by MALDI-TOF, and haptenation ratio was calculated as ((Conjugate MW – Carrier Protein MW) / Hapten MW).

Conjugate	Conjugate MW (Da)	Calculated Haptenation Ratio	Precipitate/ Aggregate
F_1-CRM	69,378	17.0	No
F_8-CRM	58,897	14.0	Yes
F_{9a}-CRM	60,394	4.2	No
F_{9b}-CRM	62,143	8.5	No
F_{10}-CRM	59,055	16.0	No

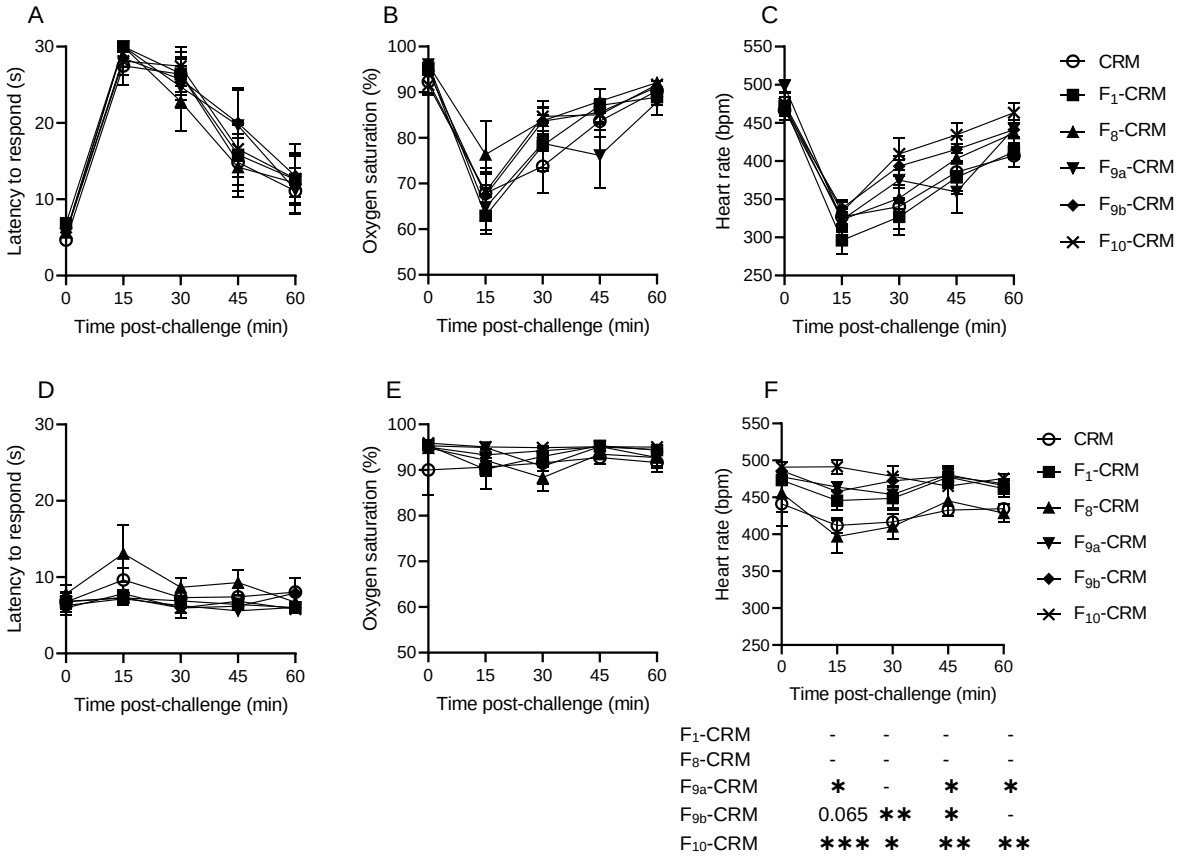


Figure S1. Efficacy of F_x-CRM conjugate vaccines against alfentanil (A-C) and acetylfentanyl (D-F). Sprague Dawley rats (n=6, each group) were vaccinated i.m. on days 0, 21, 42, and 63 with conjugate vaccines containing the F_1 and $F_{8\text{-}10}$ haptens or with CRM control, and were challenged with 0.5 mg/kg alfentanil or acetylfentanyl s.c. Rats were monitored at 15-minute intervals for A,D) antinociception by latency to respond on a hot plate, and for B,E) oxygen saturation (%) and C,F) heart rate measured by oximetry. Data are expressed as mean ± SEM. Panel A-E, no significant differences among groups, $0.10 \leq p$. Panel F (below), significance of each vaccine group vs CRM control is indicated at each time point. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to control; exact p-values are listed for $0.05 \leq p \leq 0.1$; and – indicates no significant difference, $0.10 \leq p$.

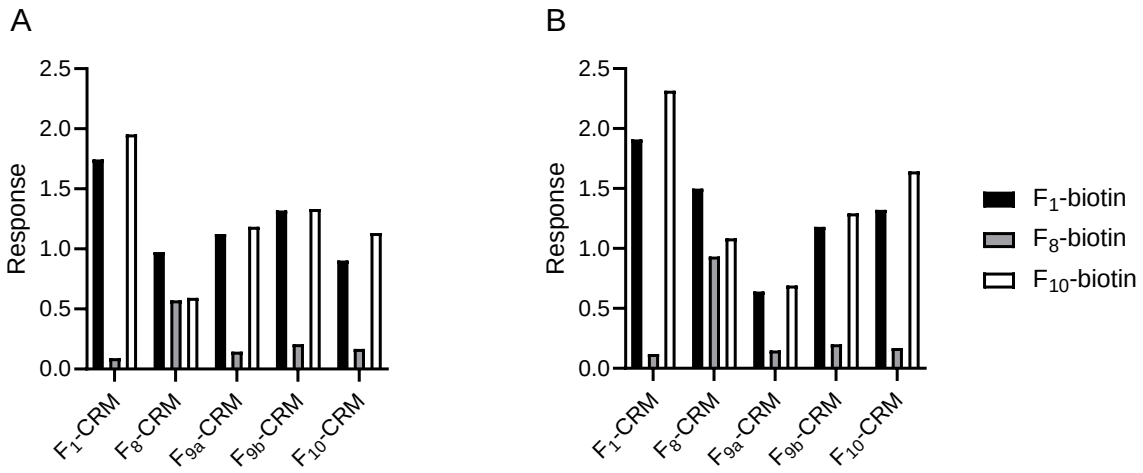


Figure S2. Evaluation of polyclonal antibody binding to F_1-, F_8-, and F_{10}-biotin by biolayer interferometry (BLI) on Octet Red96e (Sartorius). Serum was collected on day 49 (A) and after all challenges on day 105 (B), and serum from all rats in each vaccine group was pooled for analysis. Biotinylated F_1, F_8, and F_{10} were loaded onto streptavidin-coated biosensors, and association of serum diluted 1:200 in PBS + 0.05% Tween-20 was evaluated against each biotinylated hapten. Response values after 3-min association time were recorded as a correlate of cross-reactive polyclonal antibody concentration.

Table SII. Summary of F_1-CRM and $F_{8\text{-}10}$-CRM efficacy against fentanyl and fentanyl analogs in rats. For each target, significance vs CRM control is indicated by hot plate antinociception (latency), respiratory depression (SaO_2), and heart rate (HR). Asterisks indicate a significant difference vs CRM control at one or more time points; *p≤0.05, **p≤0.01.

		Fentanyl			Sufentanil			Acetylfentanyl		
Conjugate	Target Opioid	Latency	SaO_2	HR	Latency	SaO_2	HR	Latency	SaO_2	HR
F_1-CRM	Fentanyl	*	*	**	n.s.	**	n.s.	n.s.	n.s.	n.s.
F_8-CRM	Alfentanil	n.s.	*	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.
F_{9a}-CRM	Fentanyl	*	*	**	n.s.	**	n.s.	n.s.	n.s.	*
F_{9b}-CRM	Fentanyl	*	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	*
F_{10}-CRM	Acetylfentanyl	n.s.	*	*	n.s.	*	n.s.	n.s.	n.s.	**