# Metabolically stable Apelin-analogues, incorporating cyclohexylalanine and homoarginine, as potent apelin receptor activators

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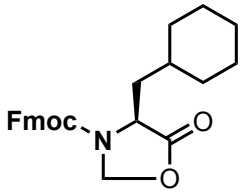
## Analysis table of all included compounds

| | Substitution | Molecular Formula | Purity | Detected Ion | Theoretical *m/z* | HRMS |
|---|---|---|---|---|---|---|
| **Small Molecules** | | | | | | |
| **20** | (4*S*)-3-(9-Fluorenylmethyloxy carbonyl)-4-cyclohexylalanine-5-oxo-oxazolidinone | $C_{25}H_{27}NO_4$ | Pure | $(M+Na)^+$ | 405.1940 | 428.1831 |
| **21** | Fmoc-*N*-Me-Cha-OH | $C_{25}H_{29}NO_4$ | Pure | $(M+H)^+$ | 407.2097 | 408.2165 |
| **22** | Fmoc-*N*-Me-Cha-OBn | $C_{32}H_{35}NO_4$ | Pure | $(M+H)^+$ | 497.2566 | 498.2646 |
| **23** | Fmoc-Lys(Boc)-*N*-Me-Cha-OBn | $C_{43}H_{55}N_3O_7$ | Pure | $(M+H)^+$ | 725.4055 | 726.4129 |
| **24** | Fmoc-hArg(Boc)$_2$-*N*-Me-Cha-OBn | $C_{49}H_{65}N_5O_9$ | Pure | $(M+H)^+$ | 867.4776 | 868.4849 |
| **25** | Fmoc-hArg(Boc)$_2$-*N*-Me-Cha-OH | $C_{42}H_{59}N_5O_9$ | Pure | $(M+H)^+$ | 777.4313 | 778.4376 |
| **26** | Fmoc-Lys(Boc)-*N*-Me-Leu-OBn | $C_{40}H_{51}N_3O_7$ | Pure | $(M+H)^+$ | 685.3727 | 683.3791 |
| **27** | Fmoc-hArg(Boc)$_2$-*N*-Me-Leu-OBn | $C_{46}H_{61}N_5O_9$ | Pure | $(M+H)^+$ | 827.4469 | 828.4535 |
| **28** | Fmoc-hArg(Boc)$_2$-*N*-Me-Leu-OH | $C_{39}H_{55}N_5O_9$ | Pure | $(M+H)^+$ | 737.4000 | 738.4062 |
| | | | | | | |
| **Apelin analogues** | | | | | | |
| **1** | Apelin13A2 | $C_{69}H_{109}BrN_{22}O_{16}$ | 98.5 % | - | - | - |
| **2** | NMeLeu13A2 | $C_{70}H_{111}BrN_{22}O_{16}$ | 96.3 % | - | - | - |
| **3** | hArg13A2 | $C_{70}H_{111}BrN_{22}O_{16}$ | 96.8 % | $(M+2H)^{2+}$ | 811.4017 | 811.4019 |
| **4** | Cha13A2 | $C_{72}H_{113}BrN_{22}O_{16}$ | 98.2 % | $(M+2H)^{2+}$ | 798.3939 | 798.3916 |
| **5** | hArgCha13A2 | $C_{73}H_{115}BrN_{22}O_{16}$ | 98.4 % | $(M+2H)^{2+}$ | 818.4095 | 818.4074 |
| **6** | Apelin17A2 | $C_{96}H_{157}BrN_{34}O_{20}$ | 97.5 % | - | - | - |
| **7** | NMeLeu17A2 | $C_{97}H_{159}BrN_{34}O_{20}$ | 97.0 % | - | - | - |
| **8** | hArg17A2 | $C_{97}H_{159}BrN_{34}O_{20}$ | 96.8 % | $(M+3H)^{3+}$ | 742.7343 | 742.7329 |
| **9** | Cha17A2 | $C_{99}H_{161}BrN_{34}O_{20}$ | 98.8 % | $(M+3H)^{3+}$ | 734.0624 | 734.0605 |
| **10** | hArgCha17A2 | $C_{100}H_{163}BrN_{34}O_{20}$ | 97.0 % | $(M+3H)^{3+}$ | 747.4062 | 747.4050 |
| **11** | NMeCha17A2 | $C_{100}H_{163}BrN_{34}O_{20}$ | 96.8 % | $(M+3H)^{3+}$ | 747.4076 | 747.4062 |
| **12** | hArgNMeCha17A2 | $C_{101}H_{165}BrN_{34}O_{20}$ | 95.2 % | $(M+4H)^{4+}$ | 564.3025 | 564.3103 |
| **13** | hArgNMeLeu17A2 | $C_{98}H_{161}BrN_{34}O_{20}$ | 98.4 % | $(M+4H)^{4+}$ | 555.3025 | 554.2950 |

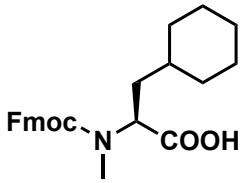
## 1. Experimental procedures for the synthesis of Fmoc-hArg(diBoc)-*N*-Me-Cha-OH

### 20. (4*S*)-3-(9-Fluorenylmethyloxycarbonyl)-4-cyclohexylalanine-5-oxo-oxazolidinone



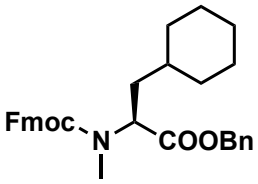

This reaction was adapted from a literature procedure.[1] Fmoc-L-Cha-OH (2.00 g, 5.08 mmol) was suspended in 100 mL of toluene and paraformaldehyde (1.06 g, 33.8 mmol) and *p*-toluenesulfonic acid (0.12 g, 0.55 mmol) were subsequently added. The mixture was refluxed at 120 °C for 2 h with azeotropic water removal, giving a pale-white solution. The solution was cooled to room temperature and subsequently washed with 1M NaHCO3 (3 x 50 mL) and brine (3 x 50 mL). The organic layer was dried over $Na_2SO_4$ and concentrated *in vacuo.* The product was purified by flash chromatography (silica gel, 25% EtOAc in hexanes), yielding **20** as a white solid (1.90 g, 95%). ($R_f$ = 0.29 on $SiO_2$, 25% EtOAc in hexanes); $[\alpha]_D^{26}$ 63.36 (*c* 0.65 $CH_2Cl_2$); IR ($CH_2Cl_2$ cast) 3067, 3041, 3019, 2924, 2851, 1801, 1716, 1478, 1450, 1417, 1056 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) $\delta$ 7.78 (dd, J = 3.3 Hz, 2H, Fmoc Ar-H), 7.54 (d, J = 7.3 Hz, 2H, Fmoc Ar-H), 7.42 (dt, J = 7.5 Hz, 2H, Ar-H), 7.33 (dt, 7.39 Hz, 2H, Fmoc Ar-H), 5.34 (app. s, 1H, Fmoc-CH), 5.07 (d, J = 4.61 Hz, 2H, Fmoc-$CH_2$), 4.68 (app. s, 2H, oxazolidinone $CH_2$), 4.23, (t, J = 4.74, 1H, Cha-CH$\alpha$), 1.80 – 1.60 (app. s, 6H, Cha-$CH_2$), 1.45 – 1.35 (app. s, 2H, Cha-$CH_2\beta$), 1.25 – 1.15 (app. s, 4H, Cha-$CH_2$), 1.00 – 0.60 (app. s, 1H, Cha-CH$\gamma$); $^{13}C$ ($CDCl_3$, 100 MHz) $\delta$ 172.5, 141.5, 128.0, 127.3, 127.2, 124.6, 120.2, 65.9, 52.9, 47.3, 37.8, 33.2, 26.3, 26.1, 26.0, 15.3; HRMS (ESI) Calculated for $C_{25}H_{27}NO_4$ 405.1940, found 428.1831 $(M+Na)^+$.

### 21. Fmoc-*N*-Me-Cha-OH



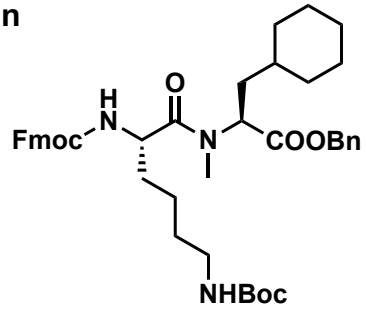

This reaction was adapted from a literature procedure.[1] The oxazolidinone intermediate **20** (1.90 g, 4.67 mmol) was dissolved in $CHCl_3$ and trifluoroacetic acid (30 mL) and triethylsilane (2.35 mL, 14.67 mmol) were added. The solution was stirred at room temperature for 22 h followed by concentration *in vacuo,* affording a sticky yellow solid. The product was purified by flash chromatography (silica gel, 5% MeOH in $CH_2Cl_2$), yielding **21** as an off-white solid (1.50 g, 80%). ($R_f$ = 0.54 on $SiO_2$, 5% MeOH in $CH_2Cl_2$); $[\alpha]_D^{26}$ -22.05 (*c* 1.16 $CH_2Cl_2$); IR ($CH_2Cl_2$ cast) 3067, 3043, 3019, 2924, 2851, 1742, 1706, 1478, 1451 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) $\delta$ 8.68 (bs, 1H, COOH), 7.75 (d, J = 7.6 Hz, 2H, Fmoc Ar-H), 7.65 – 7.52 (t, J = 6.6 Hz, 2H, Fmoc Ar-H), 7.39 (dt, J = 7.4 Hz, 2H, Fmoc Ar-H), 7.37 – 7.27 (dt, 6.89 Hz, 2H, Fmoc Ar-H), 5.01 – 4.50 (d, 4.8 Hz, 2H, Fmoc-$CH_2$), 4.46 (t, J = 7.6 Hz, Cha-CH$\alpha$), 4.34 – 4.19 (t, J = 7.0 Hz, 2H, Fmoc-CH), 2.88 (s, 3H, N-$CH_3$), 1.92-1.48 (m, 6H, Cha-$CH_2$), 1.34 – 1.08 (m, 4H, Cha-$CH_2$), 1.05 – 0.91 (m, 2H, Cha-$CH_2\beta$), 0.83 – 0.71 (m, 1H, Cha-CH$\gamma$); $^{13}C$ ($CDCl_3$, 100 MHz) $\delta$ 177.7, 157.2, 144.0, 141.4, 127.7, 127.1, 125.1, 124.8, 120.0, 67.9, 56.1, 47.3, 36.0, 33.9, 31.9, 31.8, 30.5, 26.4, 26.3, 26.0; HRMS (ESI) Calculated for $C_{25}H_{29}NO_4$ 407.2097, found 408.2165 $(M+H)^+$.

**22. Fmoc-*N*-Me-Cha-OBn**




This reaction was adapted from a literature procedure.[2] A solution of **21** (1.50 g, 3.68 mmol) in DMF (100 mL) was cooled to 0 °C. $K_2CO_3$ (0.67 g, 4.86 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. Subsequently, benzyl bromide (0.58 mL, 4.86 mmol) was added and the reaction was stirred at 0 °C for 30 min and then reacted at room temperature overnight to afford an off-white solution. The solution was poured into $H_2O$ (200 mL) and extracted with hexane (4 x 200 mL). The organic layers were combined and washed with cold $H_2O$ (2 x 200 mL) and brine (2 x 200 mL), then dried over $Na_2SO_4$ and concentrated *in vacuo* to afford a yellow oil. The product was purified by flash chromatography (silica gel, 6:1 hexane:EtOAc), yielding **22** as white crystals (1.33 g, 89%). ($R_f$ = 0.41 on $SiO_2$, 6:1 hexane:EtOAc); $[\alpha]_D^{26}$ -13.47 (*c* 1.24 $CH_2Cl_2$); IR ($CH_2Cl_2$ cast) 3089, 3066, 3037, 2924, 2851, 1740, 1703, 1149 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) $\delta$ 7.80 – 7.58 (d, J = 7.5 Hz, 2H, Fmoc Ar-H), 7.76 – 7.49 (t, J = 6. 8 Hz, 2H, Fmoc Ar-H), 7.43 – 7.34 (dt, J = 7.4 Hz, 2H, Fmoc Ar-H), 7.33 – 7.27 (m, 2H, Fmoc Ar-H), 7.33 – 7.27 (m, 5H, Ar-H), 5.15 (s, 2H, Ar-$CH_2$), 5.03 – 4.57 (d, 4.9 Hz, 2H, Fmoc-$CH_2$), 4.41 (t, J = 7.3 Hz, Cha-$CH\alpha$), 4.28 (t, J = 7.1 Hz, 1H, Fmoc-CH), 2.86 (s, 3H, N-$CH_3$), 1.74 – 1.60 (m, 6H, Cha-$CH_2$), 1.29 – 1.17 (m, 4H, Cha-$CH_2$), 1.17 – 1.08 (m, 2H, Cha-$CH_2\beta$), 0.98 – 0.89 (m, 1H, Cha-$CH\gamma$); $^{13}C$ (CDCl3, 100 MHz) $\delta$ 156.5, 143.6, 140.9, 135.3, 128.2, 127.9, 127.8, 127.7, 127.3, 126.6, 124.6, 123.4, 119.6, 67.2, 66.4, 55.6, 46.9, 35.9, 35.7, 33.5, 31.6, 29.8, 26.0, 25.9, 25.6; HRMS (ESI) Calculated for $C_{32}H_{35}NO_4$ 497.2566, found 498.2646 $(M+H)^+$.

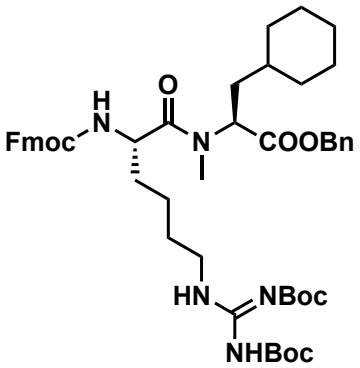
**23. Fmoc-Lys(Boc)-*N*-Me-Cha-OBn**




This reaction was adapted from a literature procedure.[3] **22** (0.67 g, 1.35 mmol) was dissolved in a 1:1 solution of DEA:DCM (2 mL) and stirred at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure affording a sticky yellow oil. The free amine was used as the crude for the next step. Fmoc-L-Lys(Boc)-OH (1.71 g, 3.65 mmol), PYBOP (1.39 g, 2.67 mmol), HOBt (0.36 g, 2.67 mmol) and DIPEA (3.5 mL, 15.55 mmol) were dissolved in DMF (20 mL) and allowed to pre-activate for 10 min. The free amine was dissolved in dry $CH_2Cl_2$ (30 mL) and DIPEA (0.47 mL, 2.67 mmol) was added to it, where the solution was then added to the previous step. The reaction was stirred at room temperature for 22 h. Subsequently, the reaction was washed with saturated $NaHCO_3$ (50 mL) and EtOAc was added to dissolve the resulting precipitate (50 mL). The organic layer was washed with 10% citric acid (50 mL), cold $H_2O$ (50 mL) and brine (50 mL), then dried over $Na_2SO_4$ and concentrated under reduced pressure to give a sticky yellow oil. The product was purified by flash chromatography (silica gel, 33% EtOAc in hexane), yielding **23** as a white powder (0.43 g, 64%). ($R_f$ = 0.43 on $SiO_2$, 33% EtOAc in hexane);

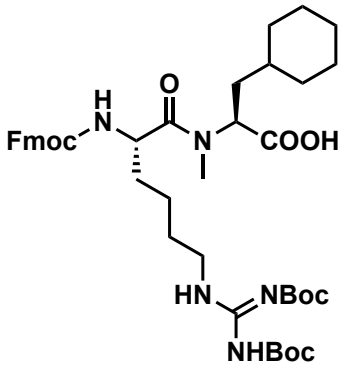
$[\alpha]_D^{26}$ -15.59 (*c* 0.88 $CH_2Cl_2$); IR ($CH_2Cl_2$ cast) 3333, 3289, 3066, 3043 2854, 2979, 2928, 1721, 1640, 1619, 1499 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) $\delta$ 7.76 (d, J = 7.4 Hz, 2H, Fmoc Ar-H), 7.59 (dd, J = 7.2, 4.0 Hz, 2H, Fmoc Ar-H), 7.40 – 7.37 (m, 2H, Fmoc Ar-H), 7.36 – 7.28 (m, 2H, Fmoc Ar-H), 7.36 – 7.28 (m, 5H, Ar-H), 5.68 – 4.59 (d, 8.4 Hz, 2H, Ar-$CH_2$), 5.39 (dd, 10.8, 5.0 Hz, 1H, Lys-CH$\alpha$), 5.13 (dd, J = 12.2, 10.8 Hz, Cha-CH$\alpha$), 4.36 (d, J = 7.0 Hz, 2H, Fmoc-$CH_2$), 4.22 (t, 7.1 Hz, 1H, Fmoc-CH), 3.07 (m, 2H, Lys-$CH_2\varepsilon$), 2.94 (s, 3H, N-$CH_3$), 1.88-1.74 (m, 2H, Lys-$CH_2\beta$), 1.73 – 1.56 (m, 2H, Lys-$CH_2\delta$),1.73 – 1.56 (m, 6H, Cha-$CH_2$), 1.43 (s, 9H, Boc-$(CH_3)_3$), 1.37 (m, 2H, Lys-$CH_2\gamma$), 1.18 – 1.07 (m, 1H, Cha-CH$\gamma$), 1.18 – 1.07 (m, 4H, Cha-$CH_2$),1.04 – 0.78 (q, 10.4 Hz, 1H, Cha-$CH_2\beta$), 1.04 – 0.78 (q, 11.4 Hz, 1H, Cha-$CH_2\beta$); $^{13}C$ (CDCl3, 100 MHz) $\delta$ 172.3, 171.2, 155.6, 143.4, 140.9, 135.4, 128.2, 128.1, 127.9, 127.3, 126.7, 124.7, 119.6, 109.6, 66.7, 53.7, 50.5, 46.8, 43.0, 35.1, 33.8, 33.7, 32.0, 31.7, 30.7, 28.8, 28.1, 26.0, 25.8, 25.6, 21.8; HRMS (ESI) Calculated for $C_{43}H_{55}N_3O_7$ 725.4055, found 726.4129 $(M+H)^+$.

**24. Fmoc-hArg(Boc)$_2$-*N*-Me-Cha-OBn**




This reaction was adapted from a literature procedure.[3] Dipeptide **23** (0.43 g, 0.59 mmol) was dissolved in dry $CH_2Cl_2$ (20 mL) and TFA (10 mL) was added to the solution. The reaction was stirred at room temperature for 1.5 h and concentrated *in vacuo* giving a sticky yellow foam. The foam was resuspended in dry $CH_2Cl_2$ (20 mL) with the addition of N,N'-Di-Boc-N"-guanidine triflate (0.254 g, 0.65 mmol) and $Et_3N$ (0.18 mL, 1.3 mmol) and stirred at room temperature for 1.5 h. The reaction was washed with 2M $NaHSO_4$ (20 mL), 10% $NaHCO_3$ (20 mL) and brine (20 mL), then dried over $Na_2SO_4$ and concentrated *in vacuo* to give an off-white foam. The product was purified by flash chromatography (silica gel, 33% EtOAc in hexane), yielding **24** as a white powder (0.28 g, 65%). ($R_f$ = 0.57 on $SiO_2$, 33% EtOAc in hexane); $[\alpha]_D^{26}$ -12.70 (*c* 0.63 $CH_2Cl_2$); IR ($CH_2Cl_2$ cast) 3333, 3066, 2978, 2929, 2854, 1721, 1640, 1134 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) $\delta$ 7.76 (d, J = 7.4 Hz, 2H, Fmoc Ar-H), 7.59 (dd, J = 7.2, 4.0 Hz, 2H, Fmoc Ar-H), 7.43 – 7.35 (m, 2H, Fmoc Ar-H), 7.35 – 7.25 (m, 2H, Fmoc Ar-H), 7.35 – 7.25 (m, 5H, Ar-H), 5.60 (d, J = 8.2 Hz, 1H, Cha-CH$\alpha$), 5.38 (dd, J = 10.6, 5.0 Hz, 1H, Lys-CH$\alpha$), 5.10 (dd, J = 12.7, 12.0 Hz, Ar-$CH_2$), 4.64 (app. s, 1H, Lys-NH$\alpha$), 4.35 (m, 2H, Fmoc-$CH_2$), 4.19 (t, J = 7.0 Hz, 1H, Fmoc-CH), 3.37 (m, 2H, Lys-$CH_2\varepsilon$), 2.92 (s, 3H, N-$CH_3$), 1.90 – 1.74 (m, 2H, Lys-$CH_2\beta$), 1.74 – 1.55 (m, 6H, Cha-$CH_2$), 1.74 – 1.55 (m, 2H, Lys-$CH_2\delta$), 1.50 (s, 9H, Boc-$(CH_3)_3$), 1.47 (s, 9H, Boc-$(CH_3)_3$), 1.37 (m, 2H, Lys-$CH_2\gamma$), 1.26 – 1.07 (m, 4H, Cha-$CH_2$), 1.26 – 1.07 (m, 1H, Cha-CH), 0.97 (q, J = 10.4 Hz, 1H, Cha-$CH_2\beta$), 0.85 (q, J = 16.4 Hz, 1H, Cha-$CH_2\beta$); $^{13}C$ (CDCl3, 100 MHz) $\delta$ 172.7, 171.4, 156.1, 143.9, 143.8, 141.3, 135.4, 128.6, 128.5, 128.4, 127.7, 127.1, 125.2, 120.0, 67.1, 54.1, 50.1, 47.2, 35.5, 34.1, 33.8, 32.4, 32.1, 28.3, 28.1, 26.4, 26.2, 25.4, 22.4; HRMS (ESI) Calculated for $C_{49}H_{65}N_5O_9$ 867.4776, found 868.4849 $(M+H)^+$.

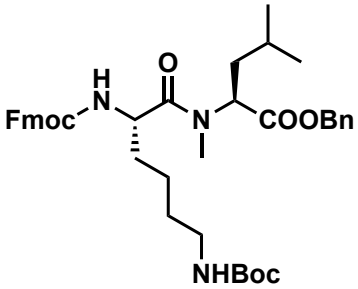
**25. Fmoc-hArg(Boc)$_2$-*N*-Me-Cha-OH**




This reaction was adapted from a literature procedure.[3] A solution of **24** (0.28 g, 0.32 mmol) was dissolved in MeOH (25 mL) and 10% Pd/C (20 mg) was added to it. The suspension was stirred under $H_2$ gas overnight, filtered through a pad of celite and concentrated *in vacuo* to give a white powder. The product was purified by flash chromatography (silica gel, 2% MeOH in EtOAc), yielding **25** as a white powder (0.20 g, 71%). ($R_f$ = 0.31 on $SiO_2$, 2% MeOH in EtOAc); $[\alpha]_D^{26}$ -5.20 (*c* 0.75 $CH_2Cl_2$); IR ($CH_2Cl_2$ cast) 3332, 3283, 3138, 3057, 2979, 2928, 2853, 1721, 1640, 1577, 1158 cm$^{-1}$; $^1$H ($CDCl_3$, 400 MHz) $\delta$ 7.75 (d, J = 7.6 Hz, 2H, Fmoc Ar-H), 7.62 (t, J = 8.1 Hz, 2H, Fmoc Ar-H), 7.38 (dt, J = 7.3, 4.8 Hz, 2H, Fmoc Ar-H), 7.29 (m, 2H, Fmoc Ar-H), 5.39 (dd, J = 10.6, 4.7 Hz, 1H, Lys-CH$\alpha$), 4.74 (d, J = 5.4 Hz, 1H, Cha-CH$\alpha$), 4.32 (app. d, J = 7.1 Hz, 2H, Fmoc-$CH_2$), 4.20 (t, J = 7.4 Hz, 1H, Fmoc-CH), 3.90 (t, J = 6.0 Hz, Lys-$CH_2\varepsilon$), 2.92 (s, 3H, N-$CH_3$), 2.06 – 1.94 (m, 2H, Lys-$CH_2\beta$), 1.74 – 1.66 (m, 6H, Cha-$CH_2$), 1.74 – 1.66 (m, 2H, Lys-$CH_2\delta$), 1.50 (s, 9H, Boc-$(CH_3)_3$), 1.49 (s, 9H, Boc-$(CH_3)_3$), 1.37 (m, 2H, Lys-$CH_2\gamma$), 1.33 – 1.06 (m, 4H, Cha-$CH_2$), 1.33 – 1.06 (m, 1H, Cha-CH), 1.04 – 0.81 (m, 2H, Cha-$CH_2\beta$); $^{13}$C (CDCl3, 100 MHz) $\delta$ 168.0, 165.9, 152.9, 126.6, 124.9, 119.5, 59.5, 55.4, 46.8, 41.0, 35.2, 33.7, 33.5, 32.6, 28.2, 27.9, 27.7, 25.9, 25.7, 25.5, 22.7; HRMS (ESI) Calculated for $C_{42}H_{60}N_5O_9$ 777.4313, found 778.4376 $(M+H)^+$.

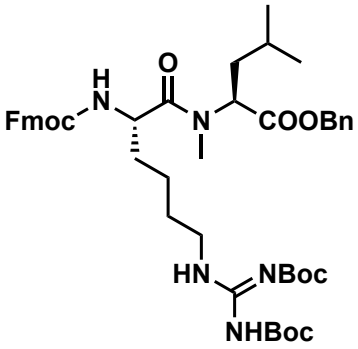
## 2. Experimental procedures for the synthesis of Fmoc-hArg(diBoc)-*N*-Me-Leu-OH

### 26. Fmoc-Lys(Boc)-*N*-Me-Leu-OBn



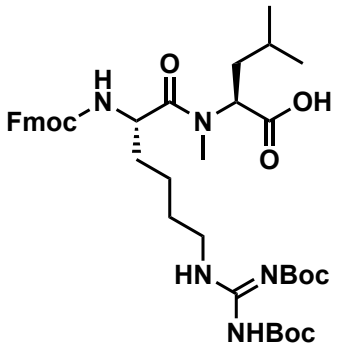

This reaction was adapted from a literature procedure.[3] Fmoc-*N*-Me-Leu-Bn (0.60 g, 1.63 mmol) was dissolved in a 1:1 solution of DEA:DCM (2 mL) and stirred at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure affording a white powder. The free amine was used as the crude for the next step. Fmoc-L-Lys(Boc)-OH (1.71 g, 3.65 mmol), PYBOP (1.39 g, 2.67 mmol), HOBt (0.36 g, 2.67 mmol) and DIPEA (3.5 mL, 15.55 mmol) were dissolved in DMF (20 mL) and allowed to pre-activate for 10 min. The free amine was dissolved in dry $CH_2Cl_2$ (30 mL) and DIPEA (0.47 mL, 2.67 mmol) was added to it, where the solution was then added to the previous step. The reaction was stirred at room temperature for 22 h. Subsequently, the reaction was washed with saturated $NaHCO_3$ (50 mL) and EtOAc was added to dissolve the resulting precipitate (50 mL). The organic layer was washed with 10% citric acid (50 mL), cold $H_2O$ (50 mL) and brine (50 mL), then dried over $Na_2SO_4$ and concentrated under reduced pressure to give a pale yellow oil. The product was purified by flash chromatography (silica gel, 10% EtOAc in $CH_2Cl_2$), yielding **26** as a white powder (0.39 g, 65%). ($R_f$ = 0.23 on $SiO_2$, 10% EtOAc in $CH_2Cl_2$); $[\alpha]_D^{26}$ -17.03 (*c* 0.91 $CH_2Cl_2$); IR ($CH_2Cl_2$ cast) 3321, 3065, 3038, 2956, 2869, 1713, 1645, 1511, 1248, 1172 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) $\delta$ 7.76 (d, J = 7.5 Hz, 2H, Fmoc Ar-H), 7.59 (d, J = 7.4 Hz, 2H, Fmoc Ar-H), 7.45 – 7.35 (m, 2H, Fmoc Ar-H), 7,.45 – 7.35 (m, 5H, Ar-H), 5.36 (dd, J = 10.7, 5.0 Hz, 1H, Lys-CH$\alpha$), 5.14 (t, J = 10.0 Hz, 2H, Lys-$CH_2\varepsilon$), 4.65 (m, 1H, Leu-CH$\alpha$), 4.36 (d, J = 7.2 Hz, 2H, Fmoc-$CH_2$), 4.21 (t, J = 6.9 Hz, 1H, Fmoc-CH), 3.07 (m, 2H, Ar-$CH_2$), 2.93 (s, 3H, N-$CH_3$), 1.89 – 1.63 (m, 2H, Leu-$CH_2\beta$), 1.60 – 1.49 (m, 2H, Lys-$CH_2\beta$), 1.49 – 1.46 (m, 1H, Leu-CH$\gamma$), 1.43 (s, 9H, Boc-$(CH_3)_3$), 1.41 – 1.36 (m, 2H, Lys-$CH_2\delta$), 1.30 – 1.26 (m, 2H, Lys-$CH_2\gamma$), 0.94 (d, J = 6.7 Hz, 3H, Leu-$CH_3$), 0.90 (d, J = 6.5 Hz, 3H, Leu-$CH_3$); $^{13}C$ (CDCl3, 100 MHz) $\delta$ 171.0, 155.7, 143.4, 140.9, 135.0, 128.3, 128.1, 127.9, 127.3, 126.7, 124.8, 119.6, 66.7, 54.4, 50.5, 46.8, 36.4, 32.0, 28.1, 24.5, 22.8, 21.7, 21.0; HRMS (ESI) Calculated for $C_{40}H_{51}N_3O_7$ 685.3727, found 683.3791$(M+H)^+$.

### 27. Fmoc-hArg(Boc)$_2$-*N*-Me-Leu-OBn



This reaction was adapted from a literature procedure.[3] Dipeptide **26** (0.39 g, 0.57 mmol) was dissolved in dry $CH_2Cl_2$ (20 mL) and TFA (10 mL) was added to the solution. The reaction was stirred at room temperature for 1.5 h and concentrated *in vacuo* giving a sticky yellow foam. The foam was resuspended in dry $CH_2Cl_2$ (20 mL) with the addition of N,N'-Di-Boc-N"-guanidine triflate (0.254 g, 0.65 mmol) and $Et_3N$ (0.18 mL, 1.3 mmol) and stirred at room temperature for 1.5 h. The reaction was washed with 2M $NaHSO_4$ (20 mL), 10% $NaHCO_3$ (20 mL) and brine (20 mL), then dried over $Na_2SO_4$ and concentrated *in vacuo* to give an off-white foam. The product was purified by flash chromatography (silica gel, 25% EtOAc in hexane), yielding **27** as a white powder (0.29 g, 74%). ($R_f$ = 0.26 on $SiO_2$, 25% EtOAc in hexane); $[\alpha]_D^{26}$ -12.00 (*c* 0.84 $CH_2Cl_2$); IR ($CH_2Cl_2$ cast) 3333, 3065, 3038, 2957, 2869, 1720, 1640, 1575, 1156, 1134 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) $\delta$ 7.76 (d, J = 7.5 Hz, 2H, Fmoc Ar-H), 7.59 (d, J = 7.4 Hz, 2H, Fmoc Ar-H), 7.43 – 7.37 (m, 2H, Fmoc Ar-H), 7,.44 – 7.37 (m, 5H, Ar-H), 5.36 (dd, J = 10.7, 4.8 Hz, 1H, Leu-CH$\alpha$), 5.13 (t, J = 12.2 Hz, 2H, Lys-$CH_2\varepsilon$), 4.64 (m, 1H, Lys-CH$\alpha$), 4.36 (d, J = 7.2 Hz, 2H, Fmoc-$CH_2$), 4.21 (t, J = 6.9 Hz, 1H, Fmoc-CH), 3.35 (m, 2H, Ar-$CH_2$), 2.93 (s, 3H, N-$CH_3$), 1.85 – 1.74 (m, 2H, Leu-$CH_2\beta$), 1.72– 1.58 (m, 2H, Lys-$CH_2\beta$), 1.57 – 1.52 (m, 1H, Leu-CH$\gamma$), 1.50 (s, 9H, Boc-$(CH_3)_3$), 1.48 (s, 9H, Boc-$(CH_3)_3$), 1.43 – 1.33 (m, 2H, Lys-$CH_2\delta$), 1.31 – 1.16 (m, 2H, Lys-$CH_2\gamma$), 0.93 (d, J = 6.7 Hz, 3H, Leu-$CH_3$), 0.90 (d, J = 6.5 Hz, 3H, Leu-$CH_3$); $^{13}C$ (CDCl3, 100 MHz) $\delta$ 170.9, 155.7, 143.5, 140.1, 135.0, 128.3, 128.1, 128.0, 127.3, 126.7, 124.8, 119.5, 66.7, 54.4, 50.5, 46.8, 36.4, 32.1, 28.0, 27.7, 24.5, 22.8, 22.0, 21.0; HRMS (ESI) Calculated for $C_{46}H_{61}N_5O_9$ 827.4469, found 828.4535 $(M+H)^+$.

**28. Fmoc-hArg(Boc)$_2$-*N*-Me-Leu-OH**




This reaction was adapted from a literature procedure.[3] A solution of **27** (0.29 g, 0.35 mmol) was dissolved in MeOH (25 mL) and 10% Pd/C (20 mg) was added to it. The suspension was stirred under $H_2$ gas overnight, filtered through a pad of celite and concentrated *in vacuo* to give a clear oil. The product was purified by flash chromatography (silica gel, 25% EtOAc in hexane), yielding **28** as a white powder (0.18 g, 62%). ($R_f$ = 0.72 on $SiO_2$, 25% EtOAc in hexane); $[\alpha]_D^{26}$ -6.00 (*c* 0.26 $CH_2Cl_2$); IR ($CH_2Cl_2$ cast) 3325, 3287, 3065, 2957, 2924, 2870, 1721, 1641, 1415, 1368, 1137 $cm^{-1}$; $^1H$ ($CDCl_3$, 400 MHz) $\delta$ 8.36 (bs, 1H, COOH), 7.76 (d, J = 7.7 Hz, 2H, Fmoc Ar-H), 7.60 (dd, J = 13.8, 7.6 Hz, 2H, Fmoc Ar-H), 7.41 – 7.34 (m, 2H, Fmoc Ar-H), 7.34 – 7.28 (m, 2H, Fmoc Ar-H), 5.35 (dd, J = 10.8, 4.8 Hz, 1H, Leu-CH$\alpha$), 4.75 (dd, J = 13.9, 6.1 Hz, 2H, Lys-$CH_2\alpha$), 4.32 (d, J = 7.6 Hz, 2H, Fmoc-$CH_2$), 4.19 (t, J = 7.5 Hz, 1H, Fmoc-CH), 3.40 (m, 2H, Leu-$CH_2\varepsilon$), 3.00 (s, 3H, N-$CH_3$), 1.89 – 1.74 (m, 2H, Leu-$CH_2\beta$), 1.73 – 1.58 (m, 2H, Lys-$CH_2\beta$), 1.54 – 1.52 (m, 1H, Leu-CH$\gamma$), 1.50 (s, 9H, Boc-$(CH_3)_3$), 1.49 (s, 9H, Boc-$(CH_3)_3$), 1.46 – 1.35 (m, 2H, Lys-$CH_2\delta$), 1.31 – 1.24 (m, 2H, Lys-$CH_2\gamma$), 0.97 (d, J = 6.7 Hz, 3H, Leu-$CH_3$), 0.89 (d, J = 6.5 Hz, 3H, Leu-$CH_3$); $^{13}C$ (CDCl3, 100 MHz) $\delta$ 171.0, 155.7, 128.3, 128.2, 127.9, 127.3, 126.7, 124.8, 119.5, 54.4, 50.5, 46.8, 36.4, 32.2, 28.1, 27.7, 24.5, 22.8, 21.9, 21.0; HRMS (ESI) Calculated for $C_{39}H_{55}N_5O_9$ 737.4000, found 738.4062 $(M+H)^+$.

### 3. *In vitro* neprilysin (NEP) stability

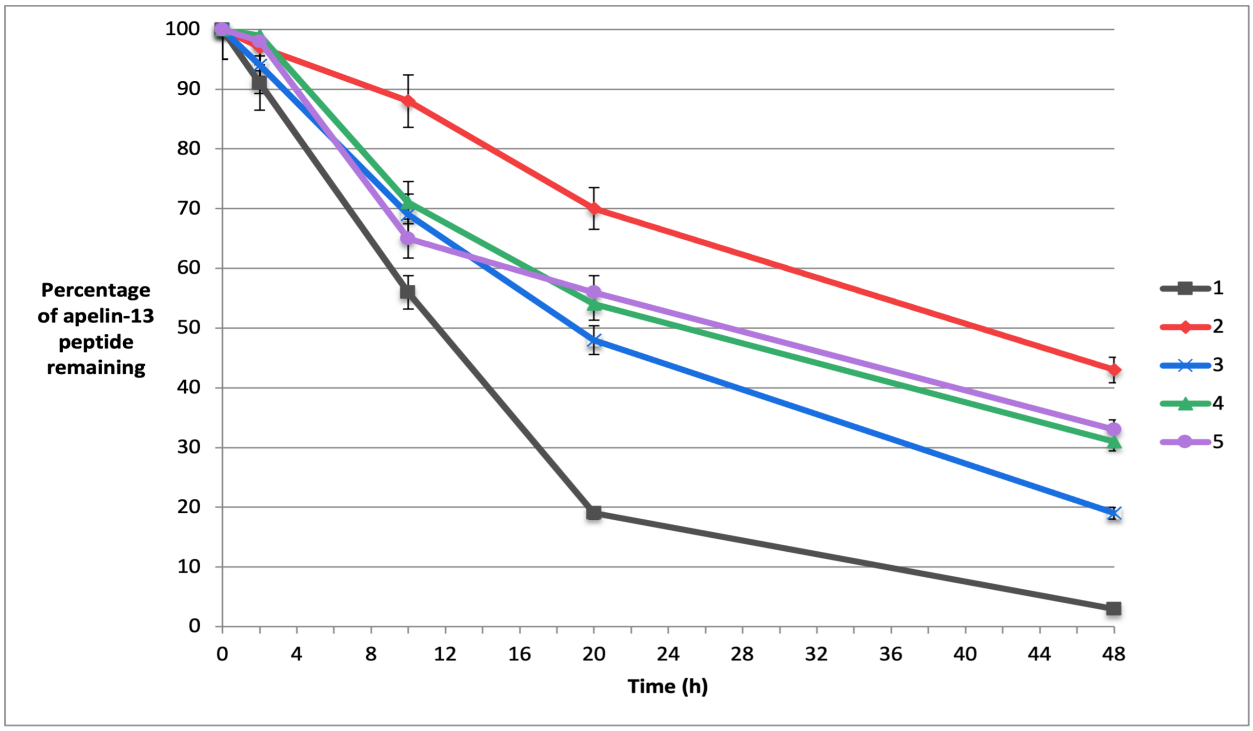



**Figure S1**. *In vitro rh*NEP degradation trends for pyr-1-apelin-13 analogues, comparing (**1**) ACE2-resistant, (**2**) *N*-Me-Leu13A2, and (**3-5**) novel Arg/Leu substituted analogues. Experiments done in triplicate.

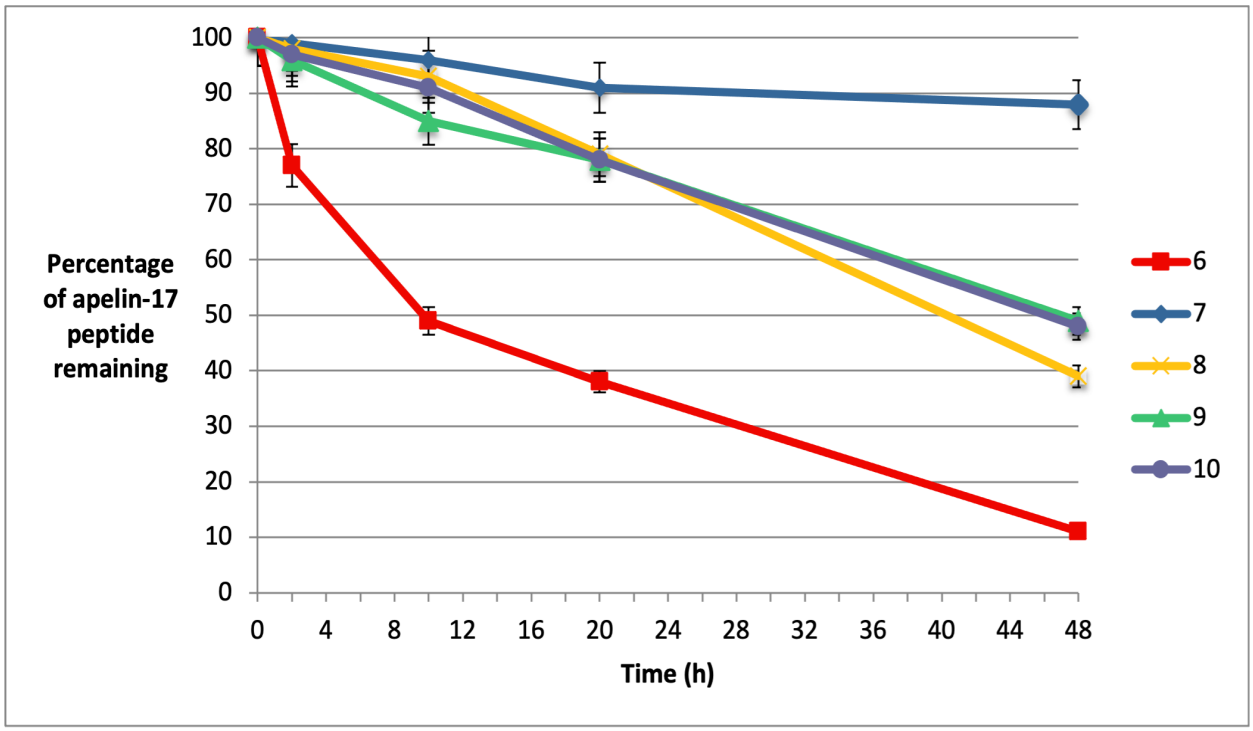



**Figure S2.** *In vitro rh*NEP degradation trends for apelin-17 analogues, comparing (**6**) ACE2-resistant, (**7**) *N*-Me-Leu17A2, and (**8-10**) novel Arg/Leu substituted analogs. Experiments done in triplicate.

## 4. *In vitro* human plasma stability of novel 13-mers

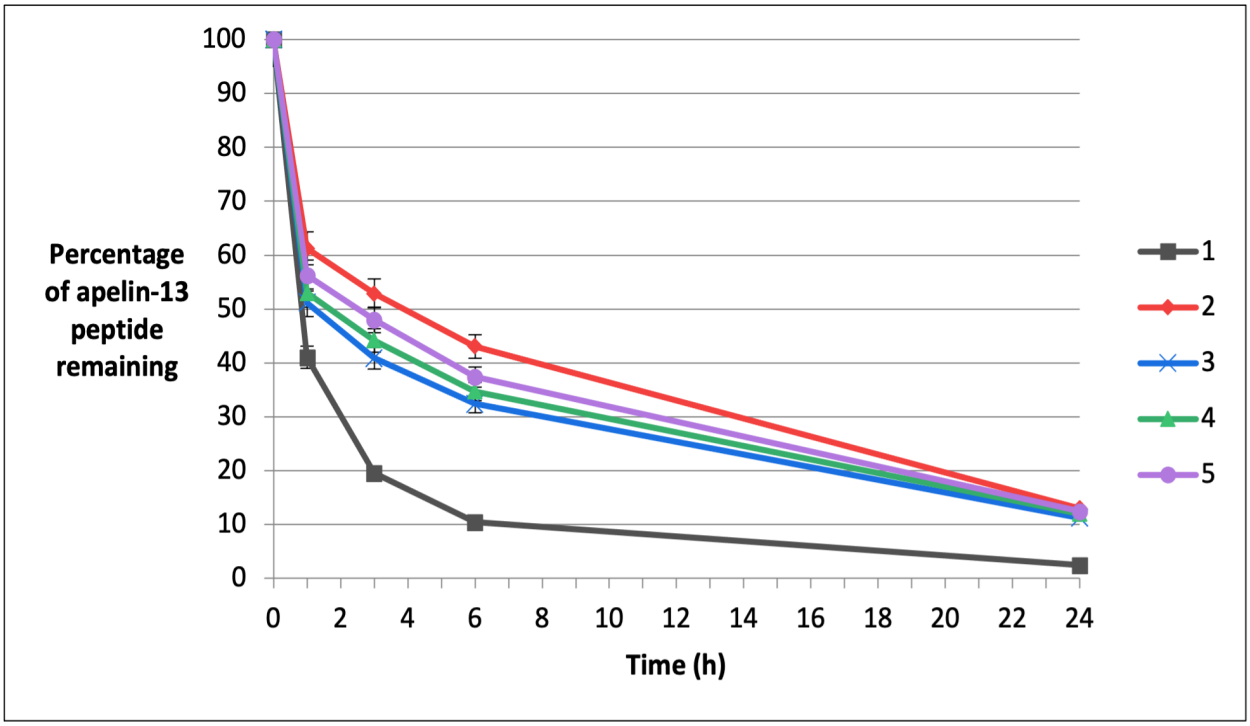



**Figure S3.** *In vitro* human plasma degradation trends for pyr-1-apelin-13 analogues, comparing (**1**) ACE2-resistant, (**2**) *N*-Me-Leu13A2, and (**3-5**) novel Arg/Leu substituted analogues. Experiments done in triplicate.

## 5. *In vitro* mice plasma stability of novel 17-mers

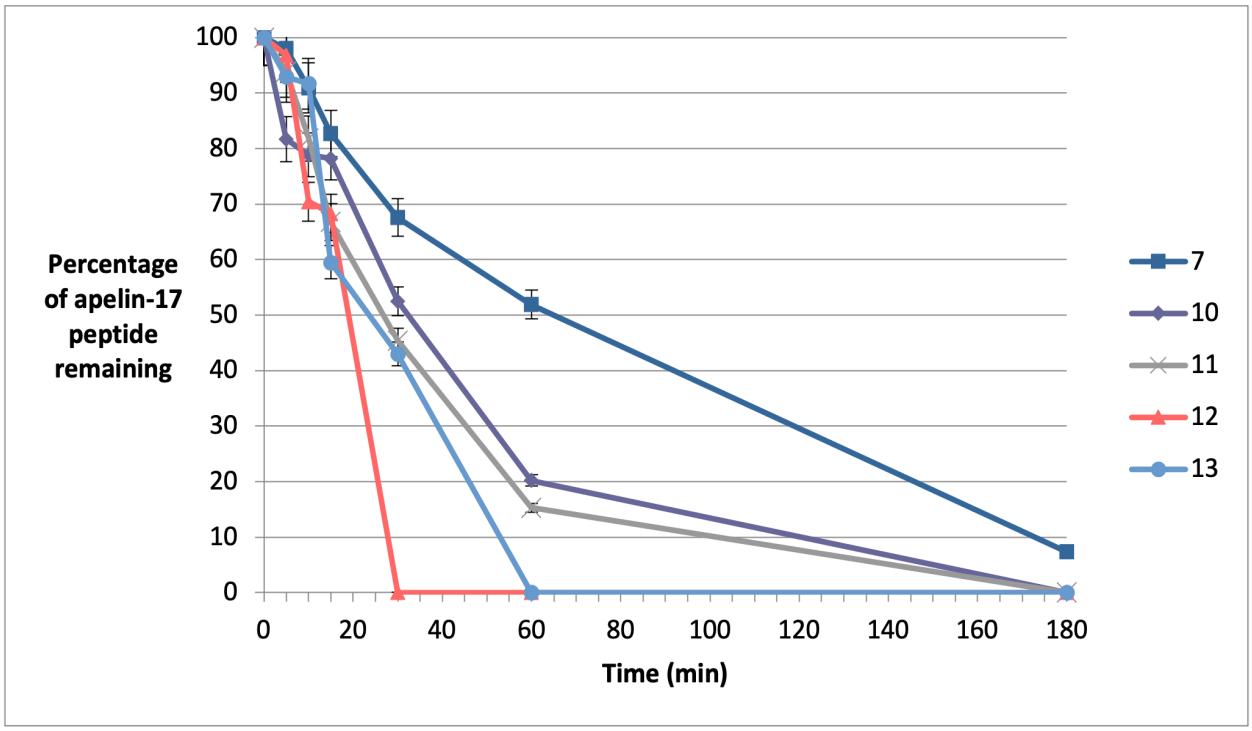
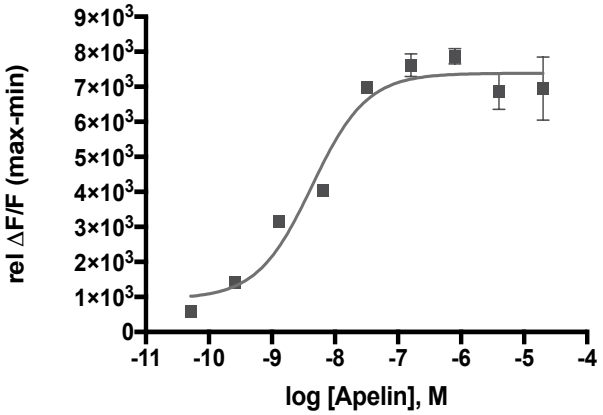



**Figure S4**. *In vitro* murine plasma degradation trends for apelin-17 analogues, comparing (**7**) *N*Me-Leu-17A2, and (**10, 11-13**) novel Arg/Leu substituted analogues. Experiments done in triplicate.

## 6. $Ca^{2+}$-mobilization: concentration response curves for novel analogues

1

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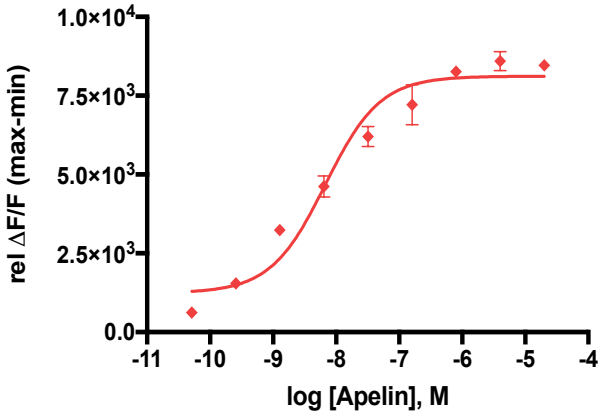


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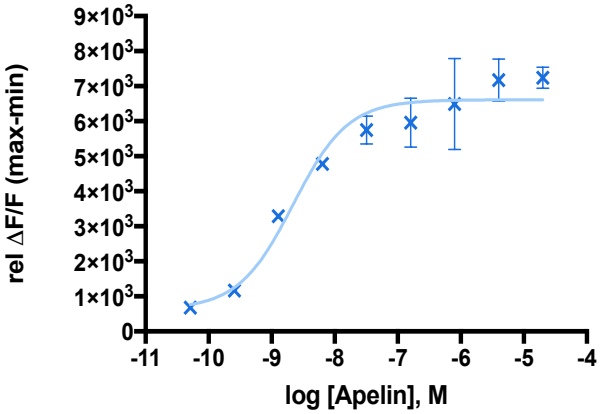


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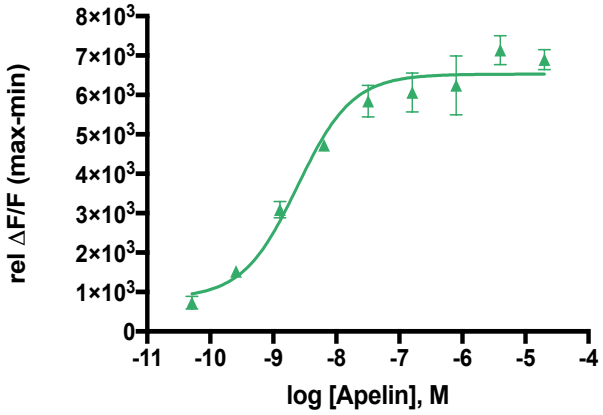


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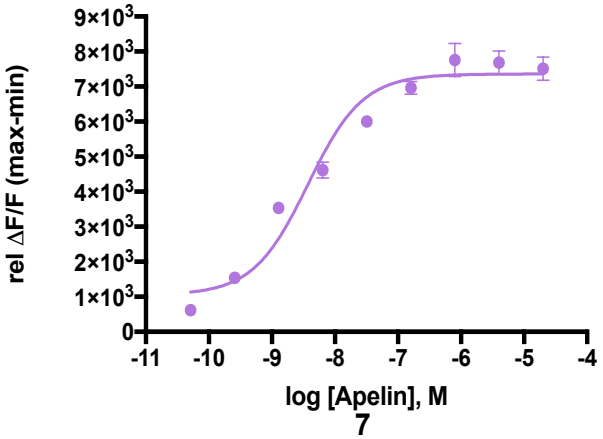


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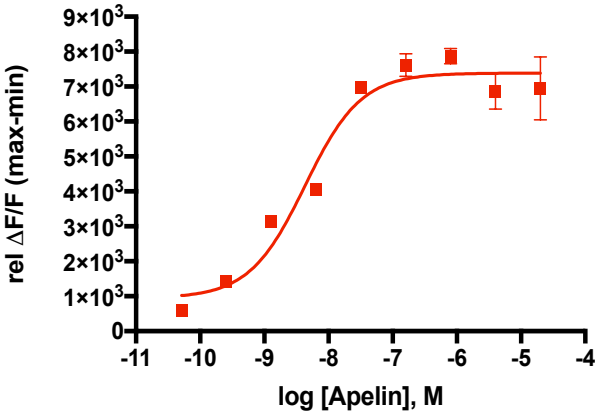


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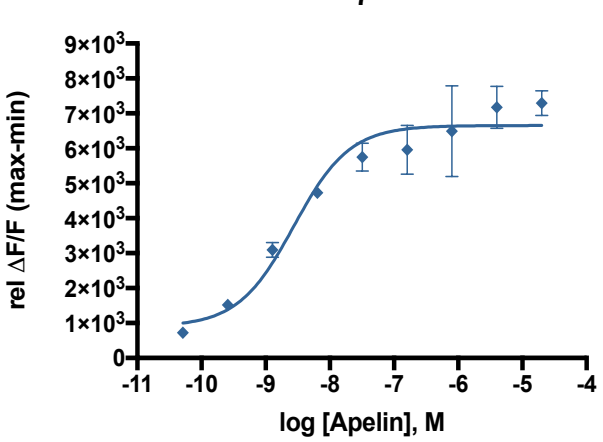


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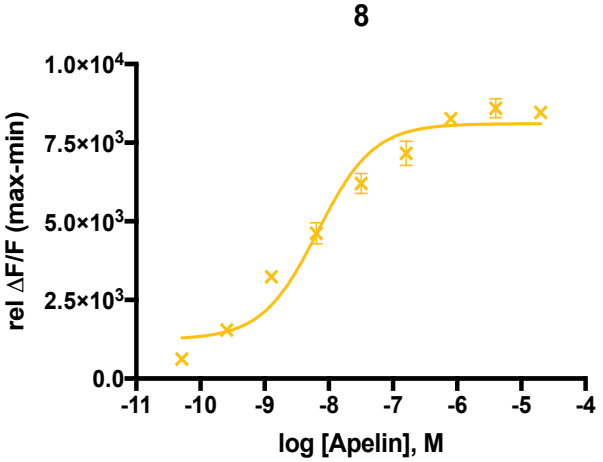

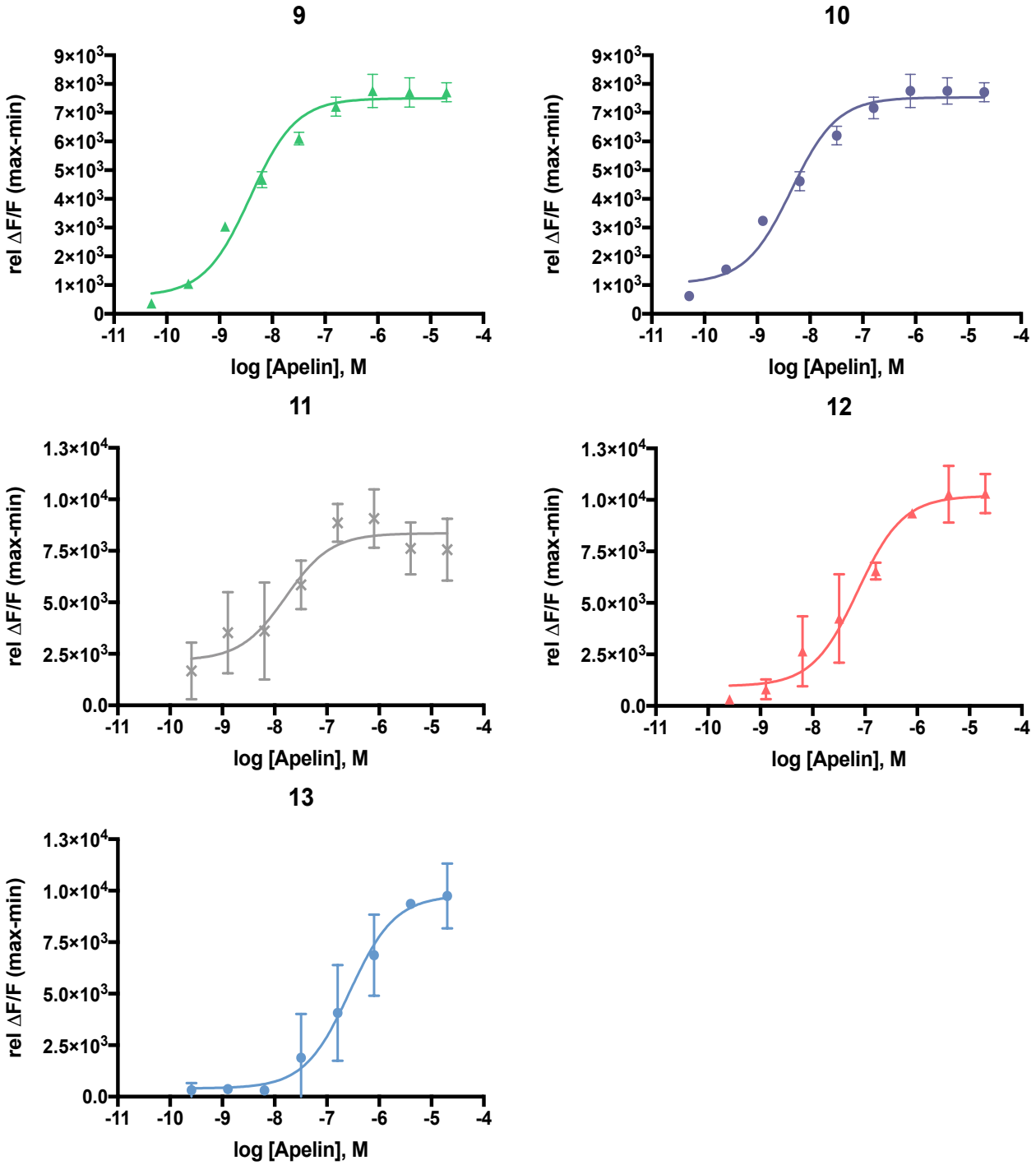


**Figure S5.** Concentration response (fluorescence) curves of ACE2-resistant (**1,6**), NEP-stabilized (**2, 7**) and title (**3-5** and **8-13**) analogues.

## 7. Blood pressure data for novel 13-mers

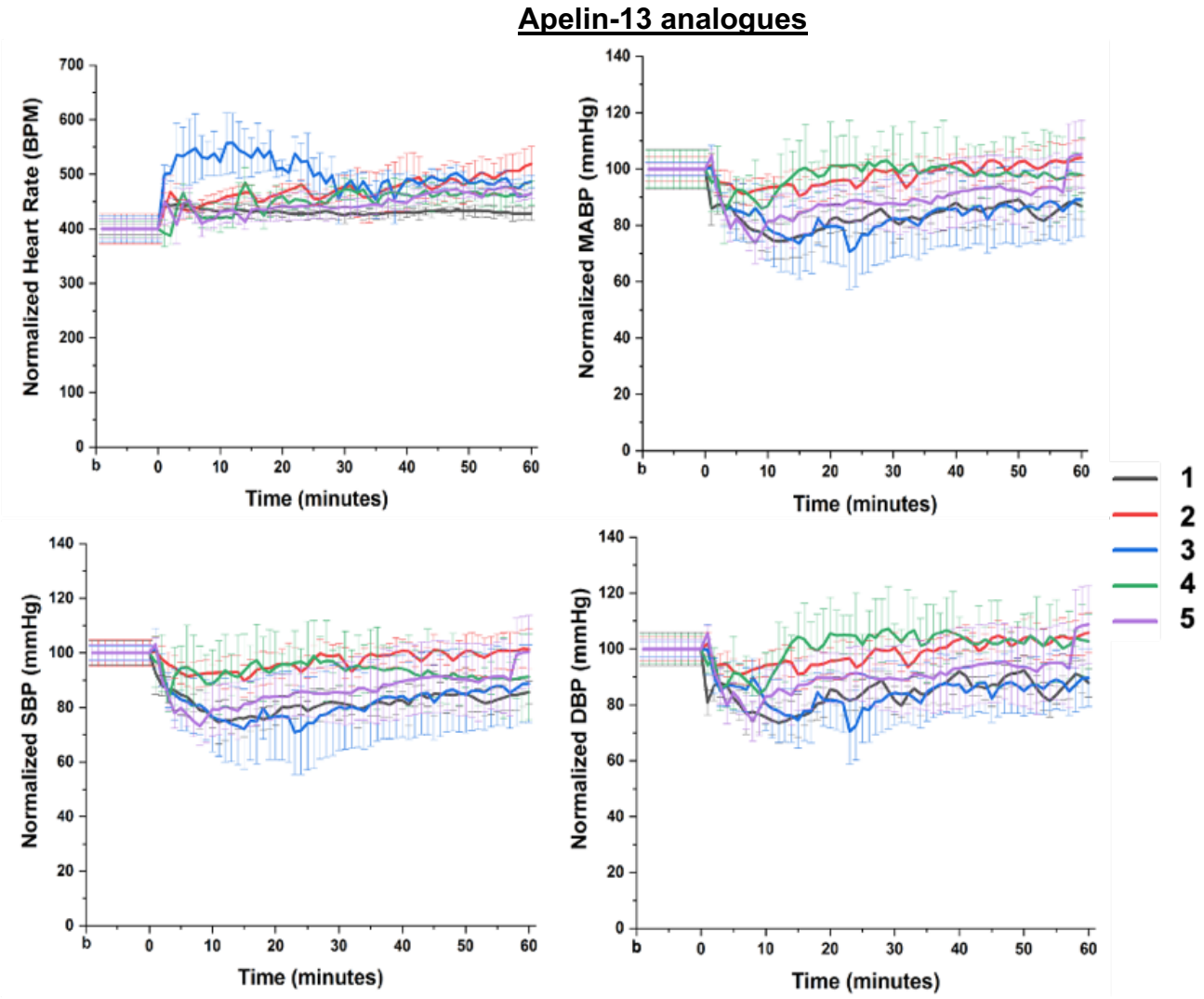


**Figure S6.** *In vivo* heart rate (HR), mean arterial (MABP), systolic (SBP), and diastolic blood pressure (DBP) analyses following injection of apelin analogs **1-5** in anesthetized mice (n=3). Values represent mean ± S.E.M.

**8. LCMS/MS ions for synthesized analogues**

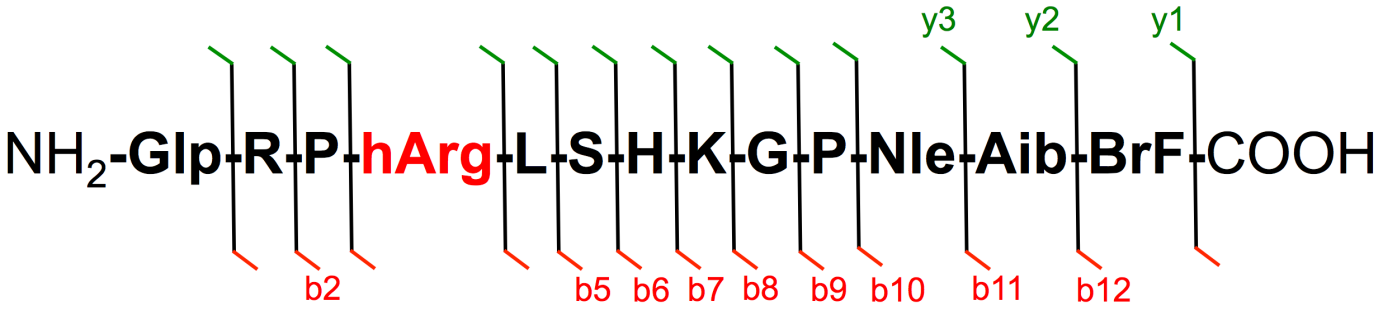


**Figure S7.** LCMS/MS ion characterization for analogue **3**.

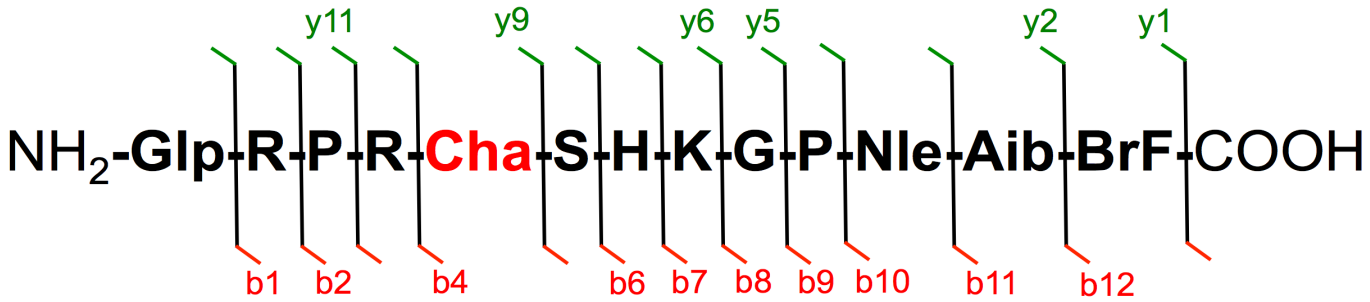


**Figure S8.** LCMS/MS ion characterization for analogue **4.**

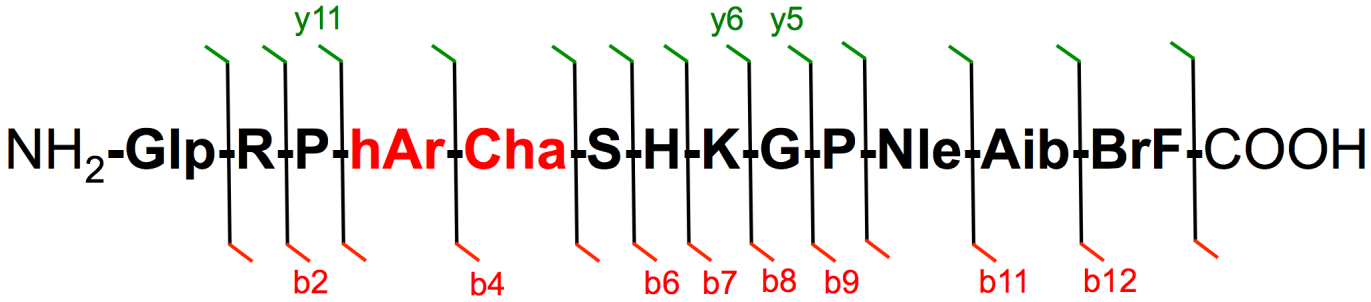


**Figure S9.** LCMS/MS ion characterization for analogue **5**.

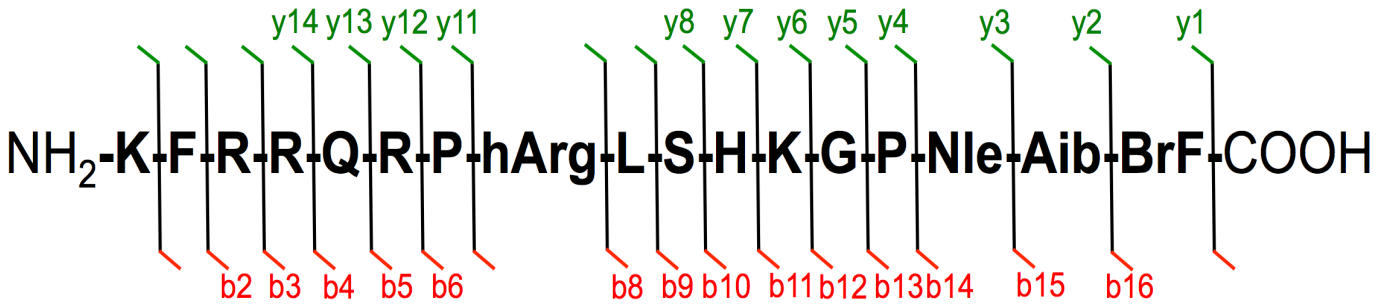


**Figure S10.** LCMS/MS ion characterization for analogue **8**.

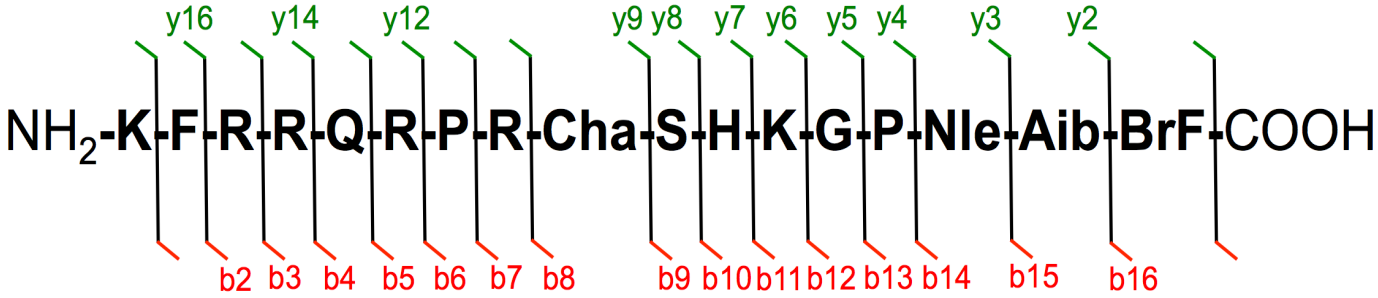


**Figure S11.** LCMS/MS ion characterization for analogue **9**.

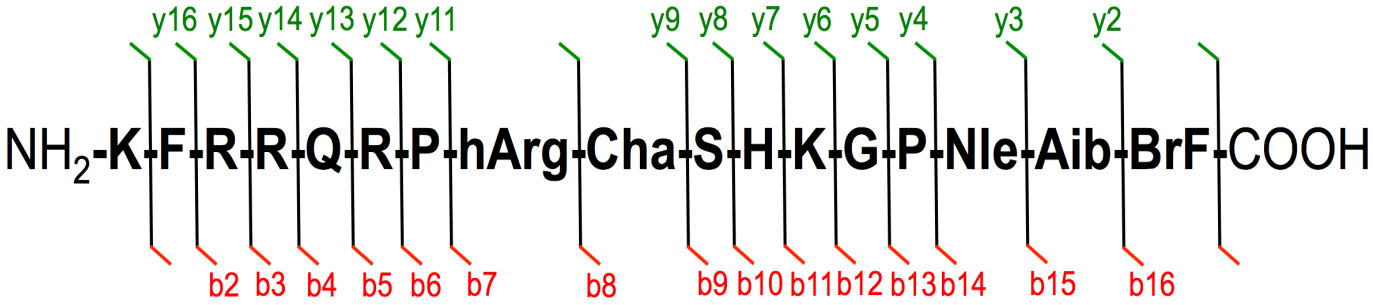


**Figure S12.** LCMS/MS ion characterization for analogue **10**.

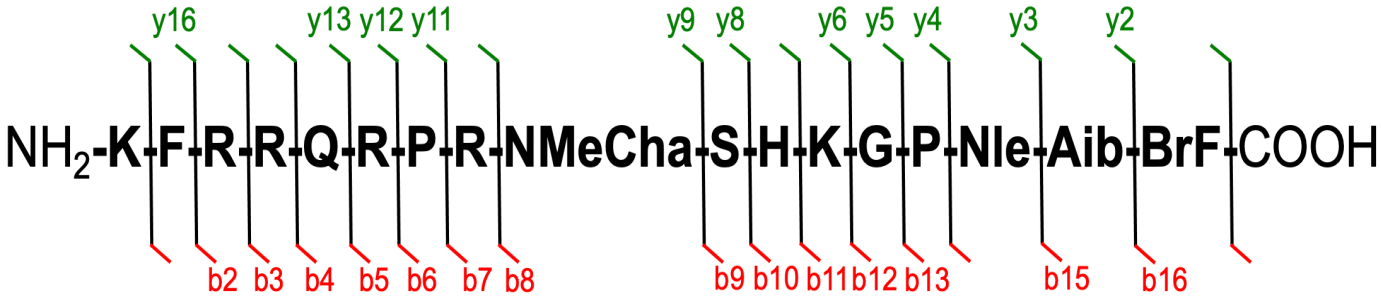


**Figure S13.** LCMS/MS ion characterization for analogue **11**.

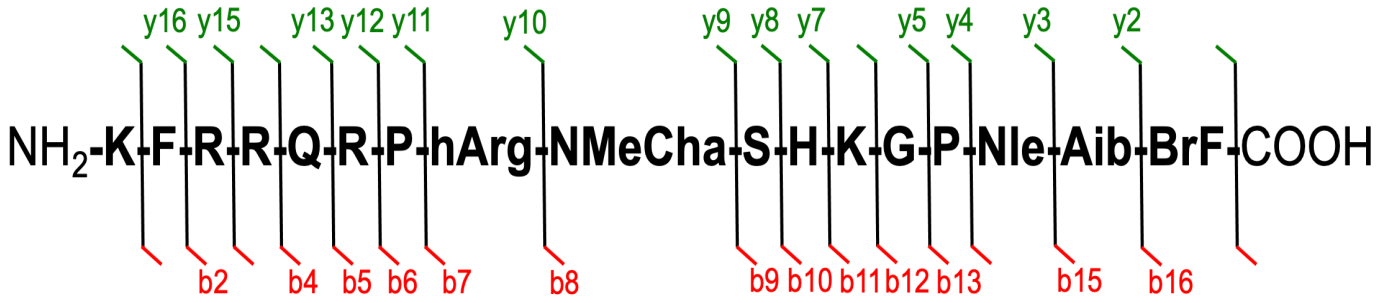


**Figure S14.** LCMS/MS ion characterization for analogue **12**.

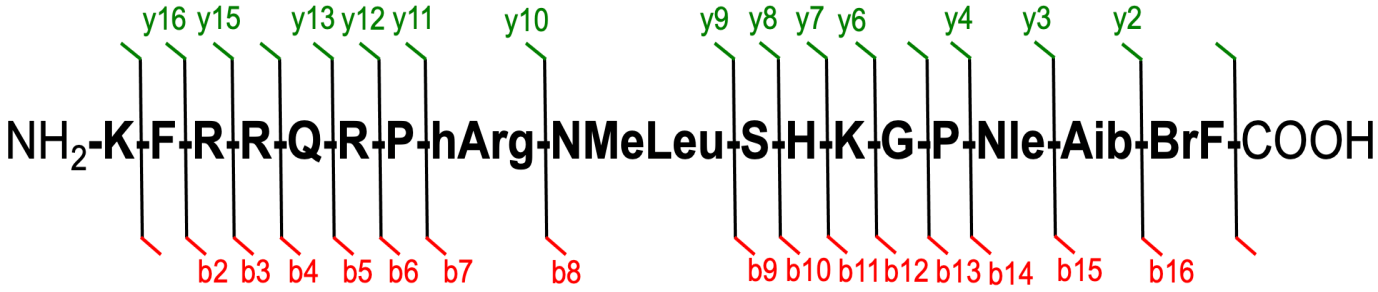


**Figure S15.** LCMS/MS ion characterization for analogue **13**.

**9. HPLC traces for novel analogues**

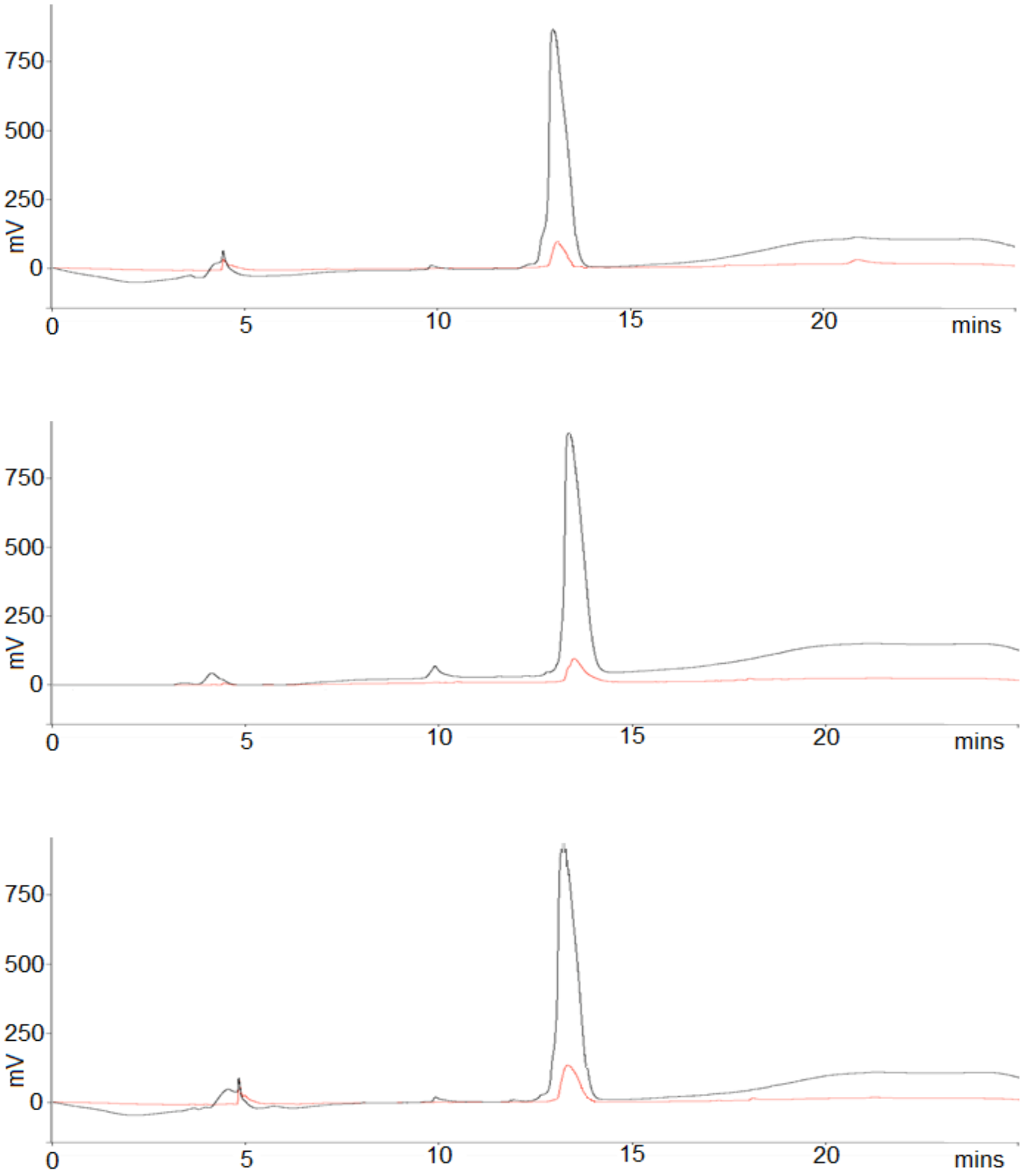


**Figure S16**. Exemplary HPLC trace of analogues **3-5** after HPLC purification (black line = 220 nm, red line = 280 nm). An average purity of >95% was obtained after repeated HPLC runs.

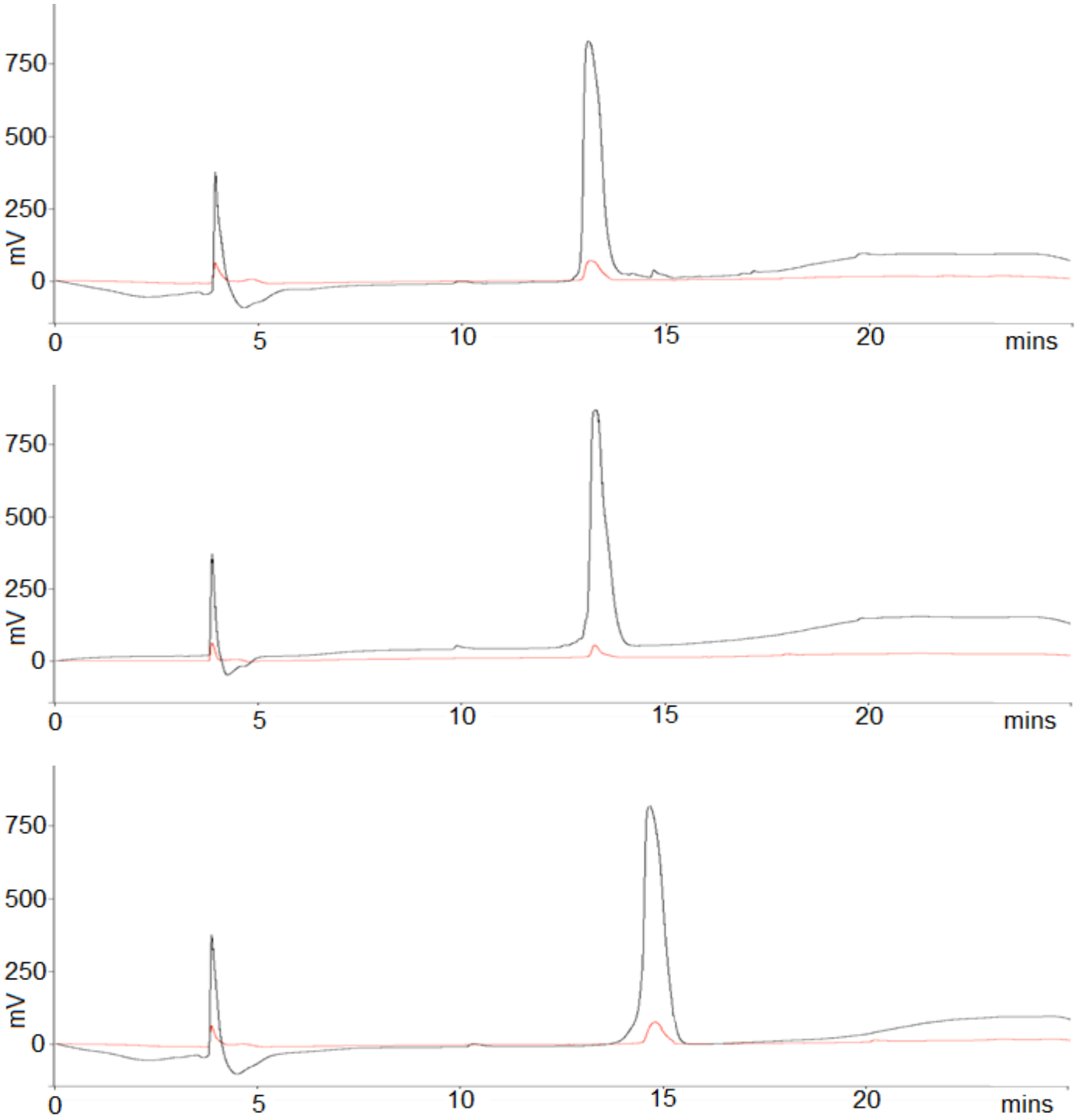


**Figure S17.** Exemplary HPLC trace of analogues **8-10** after HPLC purification (black line = 220 nm, red line = 280 nm). An average purity of >95% was obtained after repeated HPLC runs.

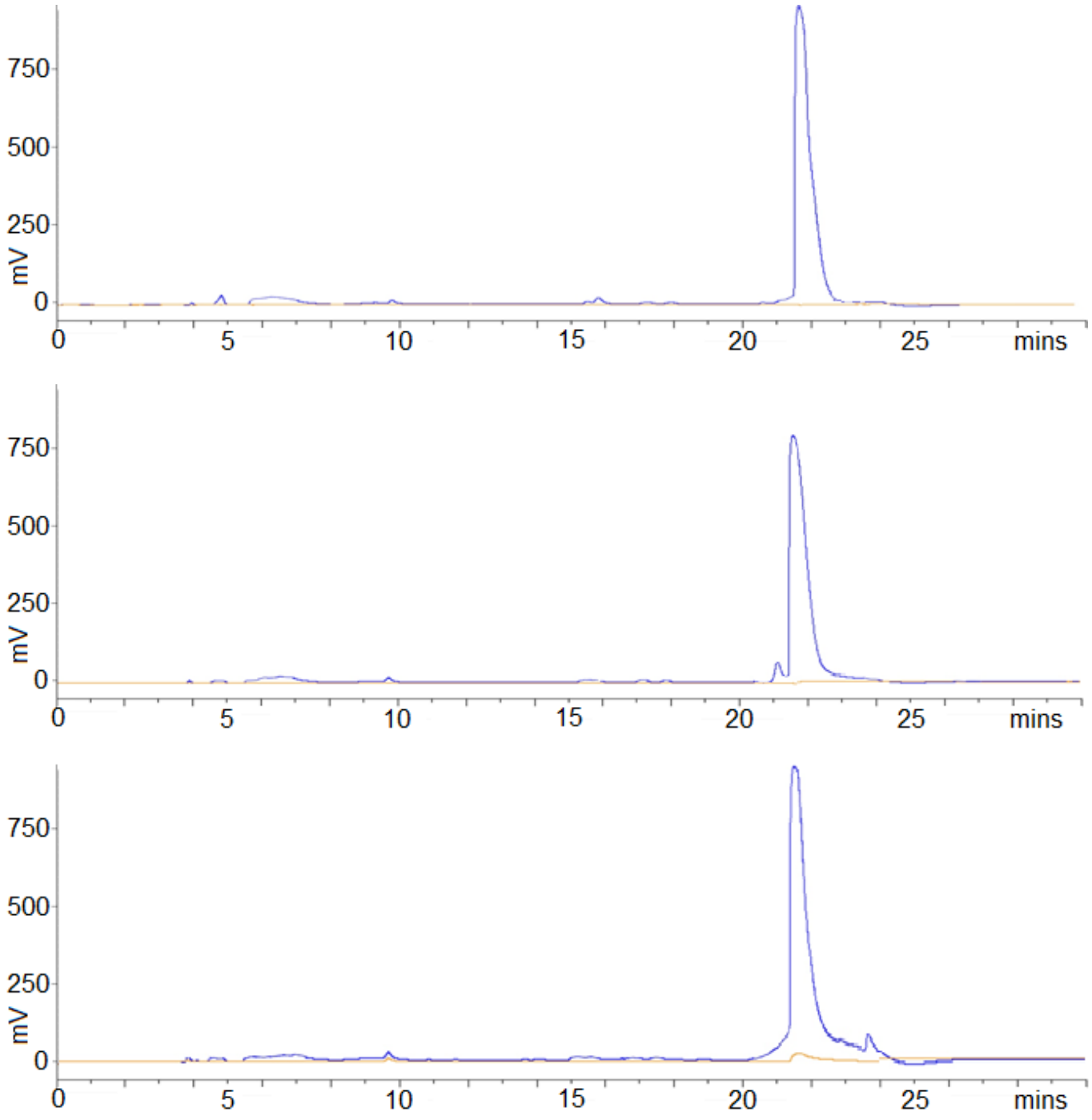


**Figure S18.** Exemplary HPLC trace of analogues **11-13** after HPLC purification (blue line = 220 nm, orange line = 280 nm). An average purity of >95% was obtained after repeated HPLC runs.

**10. References**

[1]R.M. Freidinger, J.S. Hinkle, D.S. Perlow, B.H. Arison, Synthesis of 9-fluorenylmethyloxycarbonyl-protected N-alkyl amino acids by reduction of oxazolidinones, J. Org. Chem. 48 (1983), 77-81.

[2]F.E. Dutton, B.H. Lee, S.S. Johnson, E.M. Coscarelli, P.H. Lee, Restricted conformation analogues of an anthelmintic cyclodepsipeptide, J. Med. Chem. 46 (2003), 2057-2073.

[3]S.M.K. McKinnie, W. Wang, C. Fischer, T. McDonald, R.K. Kalin, X. Iturrioz, C. Llorens-Cortes, G.Y. Oudit, J.C. Vederas, Synthetic modification with the “RPRL” region of apelin peptides: impact on cardiovascular activity and stability to neprilysin and plasma degradation, J. Med. Chem. 60 (2017), 6408-6427.