Supporting Information

Design and Synthesis of Peptide YY Analogues with C-terminal Backbone Amide-to-Ester Modifications

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Experimental. Chemistry.

General. Unless otherwise stated, starting materials were obtained from commercial suppliers and were used without further purification. The 4-Methylbenzhydrylamine (MBHA) LL (100-200 mesh) (0.67 mmol/g) hydrochloride and rink amide ChemMatrix (0.5 mmol/g) resins were purchased from Novabiochem and PCAS BioMatrix, respectively. For 9-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids, the side chain protecting groups were: *tert*-butyl (tBu) for Tyr, Ser, and Thr; *tert*-butyloxycarbonyl (Boc) for Asp, Glu, and Lys; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg; trityl for Asn, Gln, His, and Cys. For Boc amino acids, the side chain protecting groups were: benzyl (Bzl) for Thr; 2-bromobenzyloxycarbonyl (2-Br-Z) for Tyr; 4-methylbenzyl (4-MeBzl) for Cys; tosyl (Tos) for Arg; xanthyl (Xan) for Gln. The L-Arg(Pbf) α-hydroxy acid equivalent was purchased from Apigenex s.r.o. (Prague, Czech Republic), L-α-hydroxyisovaleric acid was from Sigma-Aldrich, and other α-hydroxy acids suitably protected for Boc solid-phase synthesis were generated with inspiration from previously described conditions.[1] Following precipitation of synthetic peptides using diethyl ether, the crudes were spun down on a Sigma 3-18K centrifuge (3000 RCF, 5 min). The recombinant PYY(3-29) α-thioester (**7**) was prepared by intein technology according to previously described conditions.[2] For ligations a 65 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8) was used. For desulfurizations a 20% (w/v) sodium 2-mercaptoethanesulfonate (MESNa) stock in water, a 0.1 M solution of 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] (VA-61, AOKChem) in 0.2 M HCl, and a 0.5 M Bond-Breaker tris(2-carboxyethyl)phosphine (TCEP, Thermo Scientific) solution was used. Preparative HPLC was performed on a Waters Prep LC 4000 preparative HPLC system using C18 reverse phase columns with a linear gradient of the binary solvent system of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) and UV detection at 214 nm. Semi-preparative HPLC was performed on an Agilent 1200 series system using a Phenomenex Luna C18(2) 100 Å column (5 μm; 10 × 250 mm, 40 ˚C) and a linear gradient of the binary solvent system of 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B) at a flow of 4 mL/min. The purity of peptides was determined by analytical RP-UPLC on Waters Acquity UPLC System using 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B). Unless otherwise specified, the peptides were eluted from a Waters C18 Acquity UPLC BEH column (1.7 μm; 2.1 × 150 mm, 40 ˚C) using a linear gradient of 0-45% B in 16 min at a flow of 0.4 mL/min. Characterization of peptides was performed by LC-MS on a setup consisting of Waters Acquity UPLC system connected to a LCT Premier XE mass spectrometer from Micromass. LC-MS method A involved same column, solvents, and linear gradient as used on the analytical RP-UPLC system. LC-MS method B involved a Waters C18 Acquity UPLC BEH column (1.7 μm; 2.1 × 50 mm, 40 ˚C, flow: 0.4 mL/min) and a linear gradient of 5-95% solvent B in 4 min with solvent A and B being 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Analyses at intermediate stages of the synthesis were performed by quenching ligation and desulfurization samples with a 0.1% TFA in water solution. The samples were analyzed by RP-UPLC and LC-MS method A.

Preparation of full synthetic analogues.

Synthesis of PYY(3-36). The peptide was synthesized on a rink amide ChemMatrix resin (0.65 mmol) using a Prelude peptide synthesizer from Protein Technologies according to instructions of the manufacture. The protected amino acid derivatives used were standard Fmoc amino acids

supplied from Advanced ChemTech or Novabiochem. The amino acids (2.2 mmol) were activated with diisopropylcarbodiimide (DIC, 2.0 mmol), ethyl (hydroxyimino)cyanoacetate (oxyma, 2.2 mmol), and collidine (3.0 mmol) and couplings were allowed for 80 min. Fmoc-Arg(Pbf)-OH was double coupled using a reaction time of 55 min for the first coupling and 45 min for the second coupling. The coupling reagents and amino acids were prepared to desired concentrations in N-methylpyrrolidone (NMP). Fmoc deprotections were performed using 20% (v/v) piperidine in NMP. After final synthesis, the peptidyl-resin was washed three times in dichloromethane (DCM). The peptides were cleaved from the resin in TFA/triisopropylsilane (TIPS)/water (92.5/5/2.5 (v/v)) for 2 h. The crude products were precipitated by ice-cold diethyl ether, spun down, dissolved in 10% acetic acid in water, and purified by preparative HPLC on a Waters C18 Xbridge OBD prep column (5 μm; 50 × 250 mm) using a linear gradient of 20-40% B over 40 min at a flow of 60 mL/min. Characterization was performed with LC-MS method B. Yield: 82.0 μmol (12.4%). Purity (UPLC): 98%. Mw_{calc}: 4049.55 Da. Found by ESI-MS (m/z): $[M + 3H]^{3+}$, 1350.65; $[M + 4H]^{4+}$, 1013.24; $[M + 5H]^{5+}$, 810.59; $[M + 6H]^{6+}$, 675.66; $[M + 7H]^{7+}$, 579.44.

Synthesis of PYY(3-36), A30 (8a). PYY(3-36), C30 was prepared according to PYY(3-36) on a 0.25 mmol scale. A linear gradient of 15-35% B over 40 min was used for purification of PYY(3-36), 30C. Freeze-dried PYY(3-36), 30C (3.72 μmol) was dissolved in 65 mM HEPES buffer and treated with MESNa (244 eq., 1.2 mmol), VA-61 (16 eq., 79 μmol), and TCEP (500 eq., 2.5 mmol) at 37 ˚C over night. The product (**8a**) was purified by preparative HPLC using a Waters C18 Xbridge OBD prep column (5 μm; 30 × 150 mm) and a gradient of 20-40% B over 40 min at a flow of 20 ml/min. Fractions containing the desired product was freeze-dried, dissolved in 65 mM HEPES buffer (1.7 mL), and further purified by semi-preparative HPLC using a gradient of 21-29% B or 21-32% B in 40 min. Characterization was performed with LC-MS method B. Yield: 12.3 μmol (5%). Purity (UPLC): >99%. Mw_{calc}: 4007.44 Da. Found by ESI-MS: $[M + 3H]^{3+}$ = 1350.4, $[M + 4H]^{4+}$ = 1002.76, $[M + 5H]^{5+}$ = 802.41, $[M + 6H]^{6+}$ = 668.84, $[M + 7H]^{7+}$ = 573.43.

Preparation of semi-synthetic analogues.

PYY(3-36), A30 (8b).

Fmoc-based synthesis of PYY(30-36), 30C.

The heptapeptide was synthesized manually on a rink amide ChemMatrix resin (0.25 mmol), which was swollen in DCM for 30 min and subsequently washed thoroughfully with NMP. The amino acids (4 eq.) were pre-activated with oxyma (4 eq.) and DIC (4 eq.) for 15 min before addition to the resin along with diisopropylethylamine (DIPEA, 8 eq.). Fmoc deprotections were performed with two consecutive treatments with 20% piperidine in NMP (2 min, 18 min). After final synthesis, the peptidyl-resin was washed five times in DCM. The peptide was cleaved from the resin in TFA/TIPS/water/mercaptoethanol (90/5/2.5/2.5 (v/v)) for 3-4 h. The crude product was precipitated in ice-cold diethyl ether, spun down, and dissolved in 5-10% acetonitrile in water prior to purification by preparative HPLC using a Waters C18 Xbridge OBD prep column (5 μm; 30 × 150 mm) and a gradient of 0-15% B over 40 min at a flow of 20 ml/min. A purity and yield of 99% and 19%, respectively, was obtained as summarized in Table S1.

Ligation and desulfurization.

The heptapeptide (*PYY(30-36), C30*, 1.8 eq.) and recombinant PYY(3-29) α-thioester (**7**, 1 eq.) were each dissolved in 65 mM HEPES (pH 8) buffer. After combining the two fragments, TCEP (5 mM) and thiophenol (0.6% (v/v)) were added, which resulted in a final concentration of 0.15 mM and 0.28 mM of 7 and heptapeptide, respectively. Final pH during ligation was around 7.5. Reaction was allowed over night at 5 ˚C. Then, thiophenol was removed by extraction with ice-cold diethyl ether. Desulfurization was initiated by addition of MESNa (244 eq.), VA-61 (16 eq.), and TCEP (500 eq.) and reaction was allowed at 37 ˚C overnight. Final pH during desulfurization was measured to 7.0-7.5. The desulfurization solution was concentrated in a Millipore Centriprep 3K centrifugal filter (MWCO 3000 Mw) using a Sigma Laboratory centrifuge 4K15 (3000 × *g*, 4 × 12 min, 4 °C). The product (**3**) was purified by semi-preparative HPLC using a linear gradient of 21-32% B over 40 min. Yield: 0.42 μmol (39% compared to **7**). Purity (UPLC): 99%. Mw_{calc}: 4007.44 Da. Found by ESI-MS: $[M + 4H]^{4+}$ = 1002.76, $[M + 5H]^{5+}$ = 802.40, $[M + 6H]^{6+}$ = 668.84, $[M + 7H]^{7+}$ = 573.44.

PYY(3-36)[COO$^{30-31}$], A30 (9)

Boc-based synthesis of PYY(30-36)[COO$^{30-31}$], C30 (**1**).

The peptide was synthesized manually on a MBHA LL resin (0.25 mmol), which was swollen in DCM for 1 h prior to neutralization using 10% (v/v) DIPEA in DCM and washing in NMP. Couplings were performed using the previously described *in situ neutralization* strategy for Boc-SPPS[3] with minor modifications. The amino acid (4 eq.) was preactivated with 1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholino)] uranium hexafluorophosphate (COMU) (4 eq.) and DIPEA (8 eq.) in NMP for 3 min. All amino acids were double coupled using coupling times of 30-60 min. The L-α-hydroxyisovaleric acid (4 eq.) was introduced by a single coupling involving pre-activation with DIC (4 eq.) and 1-hydroxybenzotriazole (HOBt, 4.8 eq.) in 50% (v/v) DCM/NMP at 0 ˚C for 15 min and addition to the resin along with *N*-ethylmorpholine (NEM, 1.6 eq.) Coupling was allowed to proceed at room temperature for 60 min. Ester bond formation was performed by pre-activation of the following amino acid in 50% (v/v) DCM/NMP with DIC (4 eq.) at 0 ˚C for 15 min, and addition of this solution to the resin along with 4-dimethylaminopyridine (DMAP, 0.05 eq.) and NEM (1.6 eq.). Double couplings of 1 h at room temperature were performed. Boc deprotections were performed by neat TFA (2 × 1 min). Before and after Boc deprotection of glutamine, the peptidyl-resin was washed in DCM to avoid chain termination by cyclization to form a pyrrolidone carboxylic acid.[3] The peptide was cleaved from the resin with hydrogen fluoride (HF, 10 mL)/*p*-cresol/*p*-thiocresol (93/3.5/3.5 (v/v)) for 2 h at 0 °C on a Peptide Institute HF-reaction apparatus. The crude peptide was precipitated with ice-cold diethyl ether, spun down, dissolved in 50% acetonitrile in water (0.1% TFA), and lyophilized. The dry peptide was dissolved in 10% acetonitrile in water and purified by preparative HPLC using a Waters C18 Xbridge OBD prep column (5 μm; 30 × 150 mm) and a gradient of 0-25% B over 40 min at a flow of 20 ml/min. A purity and yield of 99% and 14%, respectively, was obtained as summarized in Table S1.

Ligation and desulfurization.

Ligation and desulfurization involving **1** was performed as for peptide **8b** without modifications. The resulting product **9** was purified by semi-preparative HPLC using a gradient of 21-32% B over 40 min. Yield: 0.49 μmol (28% compared to **7**). Purity (UPLC): 98%. Mw_{calc}: 4008.43 Da. Found by ESI-MS: $[M + 4H]^{4+}$ = 1003.00, $[M + 5H]^{5+}$ = 802.61, $[M + 6H]^{6+}$ = 669.01, $[M + 7H]^{7+}$ = 573.58.

PYY(3-36)[COO$^{31-32}$], A30 (10). 10 was synthesized according to PYY(3-36)[COO$^{30-31}$], A30. Introduction of the ester bond in **2** was performed by single coupling of α-hydroxy-L-Thr(Bzl) acid followed by triple coupling of the following Boc-Val-OH (5 eq.) for 45 min with 1-(2-mesitylene-sulfonyl)-3-nitro-1,2,4 triazole (MSNT, 5 eq.) and *N*-methylimidazole (3.75 eq.) in dry DCM. During coupling the reaction vessel was covered with para-film. Semi-preparative gradient: 21-32% B over 40 min. Yield: 0.23 μmol (11%). Purity (UPLC): 98%. Mw_{calc}: 4008.43 Da. Found by ESI-MS: $[M + 4H]^{4+}$ = 1003.02, $[M + 5H]^{5+}$ = 802.82, $[M + 6H]^{6+}$ = 669.18.

PYY(3-36)[COO$^{32-33}$], A30 (11). 11 was synthesized according to semi-synthetic PYY(3-36), A30. Introduction of the ester bond in **3** was performed by double coupling of α-hydroxy-L-Arg(Pbf) acid (4 eq.) for 1 h using benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate (PyBOP, 3.8 eq), HOBt (4 eq.), and DIPEA (4 eq.) followed by coupling the following Fmoc-Thr(tBu)-OH (5 eq.) for 45 min with MSNT (5 eq.) and *N*-methylimidazole (3.75 eq.) in dry DCM twelve times. During coupling the reaction vessel was covered with para-film. Before coupling the next amino acid, the peptidyl-resin was capped with 9.5% (v/v) acetic anhydride and 2% (v/v) DIPEA in NMP for 15 min. During desulfurization pH was adjusted to around 5 and reaction was allowed for 3 h at 37 ˚C followed by incubation at 5 ˚C over night. Semi-preparative gradient: 21-32% B over 40 min. Yield: 0.23 μmol (9%). Purity (UPLC): 98%. Mw_{calc}: 4008.43 Da. Found by ESI-MS: $[M + 4H]^{4+}$ = 1003.00, $[M + 5H]^{5+}$ = 802.61, $[M + 6H]^{6+}$ = 669.00, $[M + 7H]^{7+}$ = 573.57.

PYY(3-36)[COO$^{33-34}$], A30 (12). 12 was synthesized according to PYY(3-36)[COO$^{30-31}$], A30. α-hydroxy-L-Gln(Trt)-OH (1 eq.) was used for incorporation of the ester bond in heptapeptide **4**. After ligation and desulfurization 35% hydrolyzed PYY(3-33), A30 was observed, but this was removed upon purification. Semi-preparative gradient: 21-32% B or 24-29% B over 40 min. Yield: 0.19 μmol (9%). Purity (UPLC): 97%. Mw_{calc}: 4008.43 Da. Found by ESI-MS: $[M + 4H]^{4+}$ = 1003.01, $[M + 5H]^{5+}$ = 802.59, $[M + 6H]^{6+}$ = 669.00, $[M + 7H]^{7+}$ = 573.57

PYY(3-36)[COO$^{34-35}$], A30 (13). 13 was synthesized according to PYY(3-36)[COO$^{32-33}$], A30. The ester bond in heptapeptide **5** was formed by coupling of Fmoc-Gln(Trt)-OH (5 eq.) for 40 min using MSNT (5 eq.) and *N*-methylimidazole (3.75 eq.) in dry DCM four times. The reaction vessel was sealed with para-film during these couplings. The following Fmoc-Arg(Pbf)-OH was coupled similar to the α-hydroxy-L-Arg(Pbf) acid using PyBOP, HOBt, and DIPEA. When using the standard ligation and desulfurization protocols, extensive degradation into hydrolyzed (50%) and dehydrated (40%) PYY(3-34), A30 was observed. The protocols were optimized accordingly. During ligation, pH was adjusted to 6.8. Desulfurization was performed with a higher concentration of VA-61 (76 eq.) at pH 4.9 and 37 ˚C for 3 h followed by incubation at 5 ˚C over night. Using this protocol, the degradation was limited to 5% dehydration product that could be removed upon purification. Semi-preparative gradient: 21-32% B over 40 min. Yield: 1.1 μmol (35%). Purity (UPLC): 99%. Mw_{calc}: 4008.43 Da. Found by ESI-MS: $[M + 4H]^{4+}$ = 1003.00, $[M + 5H]^{5+}$ = 802.60, $[M + 6H]^{6+}$ = 669.00, $[M + 7H]^{7+}$ = 573.58.

PYY(3-36)[COO$^{35-36}$], A30 (14). 14 was synthesized according to PYY(3-36)[COO$^{30-31}$], A30. α-hydroxy-L-Tyr(Bzl)-OH was used for incorporation of the ester bond in hepta-depsipeptide **6**. After ligation and desulfurization 5% hydrolyzed PYY(3-35), A30 was observed, but this was

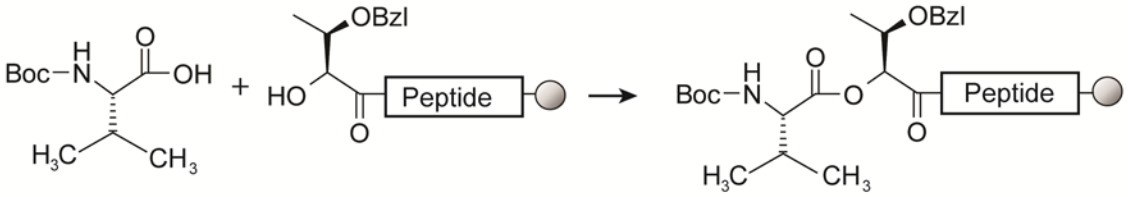
removed upon purification. Semi-preparative gradient: 21-32% or 23-32% B over 40 min. Yield: 0.27 μmol (18%). Purity (UPLC): 98%. Mw_{calc}: 4008.43 Da. Found by ESI-MS: $[M + 5H]^{5+}$ = 802.61, $[M + 6H]^{6+}$ = 669.02, $[M + 7H]^{7+}$ = 573.58.

Table S1. Summary of synthesis and purification of PYY(30-36), 30C heptapeptides.

No.	Heptapeptide	SPPS	Prep-HPLC gradient (%)	Monoisotopic masses		Purity (%)	Yield (%)
				Expected	Observed		
	PYY(30-36), 30C	Fmoc	0-15[a]	923.48	$[M + 2H]^{2+}$: 462.73 $[M + 3H]^{3+}$: 308.82	99	19
1	PYY(30-36)[COO^{30-31}], 30C	Boc	0-25[a]	924.46	$[M + 2H]^{2+}$: 463.27 $[M + 3H]^{3+}$: 309.19	99	14
2	PYY(30-36)[COO^{31-32}], 30C	Boc	5-30[a]	924.46	$[M + 2H]^{2+}$: 463.22 $[M + 3H]^{3+}$: 309.17	98	25
3	PYY(30-36)[COO^{32-33}], 30C	Fmoc	0-15[b]	924.46	$[M + 2H]^{2+}$: 463.37 $[M + 3H]^{3+}$: 309.25	99	2
4	PYY(30-36)[COO^{33-34}], 30C	Boc	8-18[a]	924.46	$[M + 2H]^{2+}$: 463.23 $[M + 3H]^{3+}$: 309.15	96	25
5	PYY(30-36)[COO^{34-35}], 30C	Fmoc	5-20[b]	924.46	$[M + 2H]^{2+}$: 463.22 $[M + 3H]^{3+}$: 309.16	85	7
6	PYY(30-36)[COO^{35-36}], 30C	Boc	0-25[a]	924.46	$[M + 2H]^{2+}$: 463.28 $[M + 3H]^{3+}$: 309.20	98	7

[a]Waters C18 Xbridge OBD prep column (5 μm; 30 × 150 mm; 40 min gradient at 20 mL/min).
[b]Phenomenex Luna C18(2) column, 100Å AXIA (5 μm; 21.2 × 250 mm; 40 min gradient at 20 mL/min).

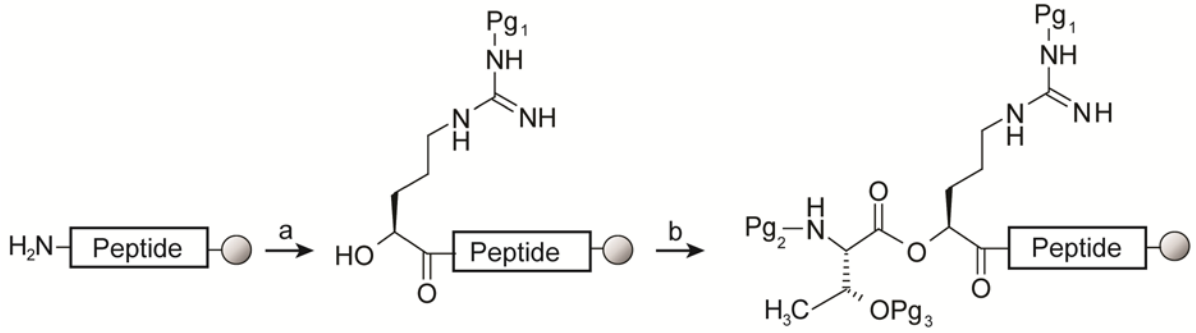
Table S2. Examination of conditions for ester coupling of Boc-Val-OH in heptapeptide **2** (analogue **10**).[a]



Entry	Conditions	Product formation [%] (Epimerization ratio)
1	Boc-Val-OH (4 eq.)/DIC (4 eq.)/DMAP (0.05 eq.) (2 × 1 h)	10 (2 diastereomers, 1:1)
2	Boc-Val-OH (10 eq.)/DIC (5 eq.)/DMAP (0.1 eq.) (3 × 2 h) + MSNT/MeIm (2 × 3 h)	48 (2, diastereomers, 3:7)
3	Boc-Val-OH (5 eq.)/MSNT (5 eq.)/MeIm (3.75 eq.) (2 × 3 h + 2 × overnight)	~100 (4 diastereomers, 1:1:1:1)
4	Boc-Val-OH (5 eq.)/MSNT (5 eq.)/MeIm (3.75 eq.) (3 × 1 h)	95 (2 diastereomers, 1:9)

[a]Product formation of hexapeptide Vψ(COO)TRQRY-NH_2 was monitored by UPLC and LC-MS. DMAP: 4-dimethylaminopyridine, MeIM: *N*-methylimidazole, MSNT: 1-(2-mesitylene-sulfonyl)-3-nitro-1,2,4 triazole.

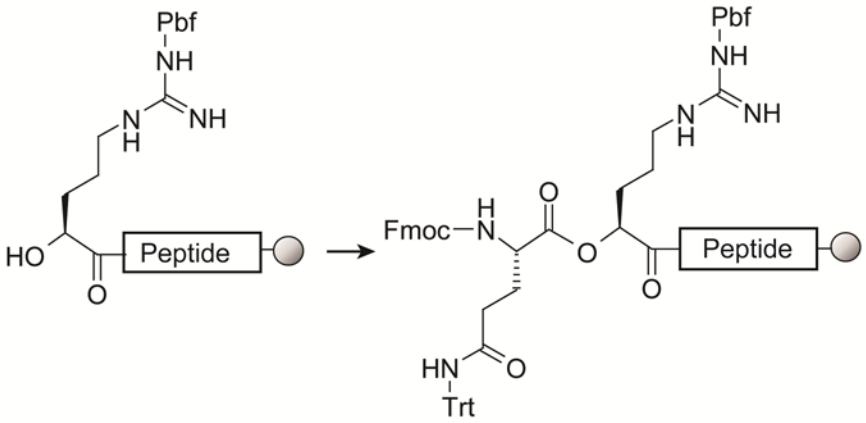
Table S3. Examination of coupling of L-Arg(Pg_1) α-hydroxy acid (a) and the following Pg_2-Thr-OH for preparation of heptapeptide **3** (analogue **11**).[a]



Entry	Conditions	Product formation [%]
1[b]	**a.** HO-Arg(Pg_1)-OH (4 eq.)/HOBt (4 eq.)/DIC (4 eq.) (1 h) **b.** Pg_2-Thr(Pg_3)-OH (4 eq.)/DIC (4 eq.)/DMAP (0.05 eq.) (2 h) + Pg_2-Thr(Pg_3)-OH (5 eq.)/MSNT (5 eq.)/MeIm (3.75 eq.) (overnight)	~5%[e]
2[b]	**a.** HO-Arg(Pg_1)-OH (4 eq.)/PyBOP (3.8 eq.)/HOBt (4 eq.) (1 × 1.5 h) **b.** Pg_2-Thr(Pg_3)-OH (5 eq.)/MSNT (5 eq.)/MeIm (3.75 eq.) (3 × 1 h)	15%[f]
3[c]	**a.** HO-Arg(Pg_1)-OH (4 eq.)/PyBOP (3.8 eq.)/HOBt (4 eq.) (2 × 1 h) **b.** Fmoc-Val-Thr(psi(Me,Me)pro)-OH (5 eq.)/MSNT (5 eq.)/MeIm (3.75 eq.) (3 × 1 h)	None
4[d]	**a.** HO-Arg(Pg_1)-OH (4 eq.)/PyBOP (3.8 eq.)/HOBt (4 eq.) (2 × 1 h) **b.** Pg_2-Thr(Pg_3)-OH (5 eq.)/MSNT (5 eq.)/MeIm (3.75 eq.) (6 × 45 min)	30%
5[d]	**a.** HO-Arg(Pg_1)-OH (4 eq.)/PyBOP (3.8 eq.)/HOBt (4 eq.) (2 × 1 h) **b.** Pg_2-Thr(Pg_3)-OH (5 eq.)/MSNT (5 eq.)/MeIm (3.75 eq.) (12 × 45 min)	55%

[a] Product formation of pentapeptide Tψ(COO)RQRY-NH_2 was monitored by UPLC and LC-MS. DMAP: 4-dimethylaminopyridine, MeIM: *N*-methylimidazole, MSNT: 1-(2-mesitylene-sulfonyl)-3-nitro-1,2,4 triazole.
[b]Boc-SPPS: Pg_1 = Tos, Pg_2 = Boc, Pg_3 = Bzl.
[c]Fmoc-SPPS: Fmoc-Val-Thr(psi(Me,Me)pro)-OH was used instead of Pg_2-Thr(Pg_3)-OH.
[d]Fmoc-SPPS: Pg_1 = Pbf, Pg_2 = Fmoc, Pg_3 = tBu.
[e]Major product is L-Arg(Pg_1) α-hydroxy acid deletion product. Approximately 5% Thr deletion.
[f]Major side product: α-hydroxy L-Arg(Pg_1) acid deletion product (~50%).

Table S4. Examination of ester coupling of Fmoc-Gln(Trt)-OH for preparation of heptapeptide **5** (analogue **13**).[a]



Entry	Conditions	Product formation [%] (Epimerization ratio)
1	Fmoc-Gln(Trt)-OH (5 eq.)/MSNT (5 eq.)/MeIm (3.75 eq.) (3 × 1 h)	70%[b] (2 diastereomers, 4:6)
2	Fmoc-Gln(Trt)-OH (5 eq.)/MSNT (5 eq.)/MeIm (3.75 eq.) (4 × 40 min)	50%[c]

[a]HO-Arg(Pbf)-OH (4 eq.) was coupled using PyBOP (3.8 eq.)/HOBt (4 eq.) (2 × 1 h). MeIM: *N*-methylimidazole, MSNT: 1-(2-mesitylene-sulfonyl)-3-nitro-1,2,4 triazole.
[b]Product formation of Qψ(COO)RY-NH_2 was monitored by UPLC and LC-MS. Gln deletion (10%) and *N*-terminal pyroglutamate (10%) products were major impurities.
[c]Product formation of CVTRQψ(COO)RY-NH_2 was monitored by UPLC and LC-MS. Major impurity was an Pbf adduct (40%).

Stability studies.

The stability of depsipeptide analogues at the conditions used during preparation and execution of the functional Actone assays were examined. Analogues **3-8** were dissolved in acetic acid/DMSO/water storage solution (2/80/18, v/v) to a final concentration of approximately 200 µM. The samples were incubated at 5 °C and aliquots were collected at 0 h, 24 h and 48 h and analysed by RP-UPLC and LC-MS method A (stability test 1). At 0 h of incubation, an aliquot of each sample was diluted to approximately 30 µM with Hank's balanced salt solution (HBSS) containing 20 mM HEPES, 0.1% Ovalbumin, 0.005% Tween 20 and 30% DMSO (pH 7.40). The diluted samples were frozen at -20 °C overnight, thawed, and stored at room temperature for 5 h prior to analysis by RP-UPLC and LC-MS method A (stability test 2). After both stability tests, analogues **3-8** had purities of 98-99% and no hydrolysis product was detected for any of the analogues (exemplified for analogue **4**, stability test 1).

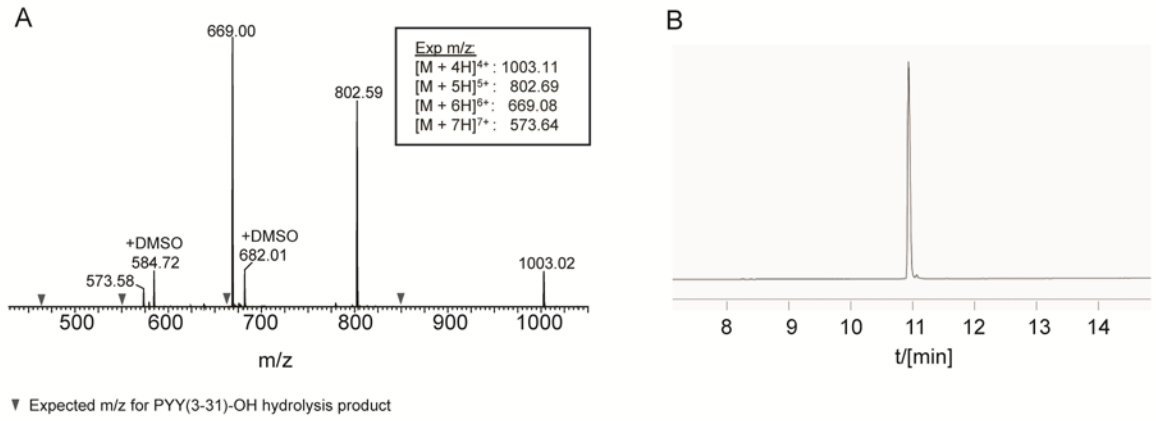


Figure S1. ESI-MS spectra (A) and UPLC (B) trace of analogue **10** after stability test 1 (48 h in acetic acid/DMSO/water storage solution at 5 °C). Expected average mass of **10**: 4008.43 Da.

Experimental. Biophysics.

Circular dichroism spectroscopy. Far-UV CD spectra were recorded over the 190-260 nm range on a JASCO J-815 circular dichroism system in a 2 mm path length cell at 20 ˚C using a scan speed of 50 nm/min, a time constant of 2 s and a bandwidth of 2 nm. Five scans were accumulated. The peptide solutions were approximately 15 μM in 5 mM sodium acetate buffer, pH 4.6. The absolute concentrations were determined by chemiluminescence nitrogen detection.[4] Spectra are represented as the mean from two independent experiments and expressed as mean difference as molar circular dichroism, Δε [$cm^{-1} \times M^{-1}$], after normalising to the molar concentration of peptide bonds. The reduction in helicity was measured as decrease in Δε at 222 nM compared to native PYY(3-36).

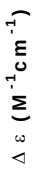


Figure S2. Circular dichroism of PYY(3-36), A30 (**8**) generated by SPPS or semi-synthesis. Spectra are represented as mean of two independent experiments.

Experimental. Biology.

Preparation of analogues. The analogues were dissolved in acetic acid/DMSO/water storage solution (2/80/18, v/v) to approximately 200 µM and diluted to the desired concentrations using HBSS solution containing 20 mM HEPES, 0.1% Ovalbumin, 0.005% Tween 20 and 30% DMSO (pH 7.40). The samples were stored at -20 °C overnight.

Actone functional potency assay. The neuropeptide Y (NPY) receptors are G_i-coupled seven trans-membrane receptors that mainly signal through the cAMP dependent pathway by inhibiting adenylate cyclase activity which results in a decrease of cAMP production from ATP. The Actone assay is based on a modified calcium channel that has a selective binding for cAMP, resulting in cellular calcium influx, detected by a calcium responsive dye. In order to measure decreased levels of cAMP, as result of NPY receptor activation, the β1/β2-adrenoreceptor agonist isoproterenol is added to activate adenylate cyclase and increase cAMP levels in the cell. Decreased cellular calcium concentrations, reflecting a decrease of cAMP levels due to NPY receptor activation, is detected as a decrease in fluorescence from the calcium sensitive dye. HEK-293 cells expressing the cAMP sensitive calcium channel and one of the NPY receptors, Y_1, Y_2, Y_4 or Y_5 (Codex Biosolution, Gaithersburg, MD, USA), were seeded into poly lysine coated 384 well plates (BD Biosciences, Franklin Lakes, NJ, USA) at a density of 14,000 cells/well in a volume of 25 µL in DMEM medium (Lonza, Verviers, Belgium) containing 10% FCS (Invitrogen, Carlsbad, CA, USA), 1% Penicillin-Streptomycin (Lonza), 250 µg/mL G418 (Invitrogen), 1 µg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were incubated over night at 37 °C in a humidified milieu in 5% CO_2 followed by addition of 25 µL calcium dye buffer containing: 1 vial Calcium 5 dye (part nr: 5000625 Molecular Devices, Sunnyvale, CA, USA) solved in 100 mL buffer containing 20 mM Hepes, 0.1% Ovalbumin, 0.005% Tween 20, 1.5 mM probenecid (Sigma-Aldrich), 250 µM cAMP phosphodiesterase inhibitor (4-(3-Butoxy-4-methoxybenzyl)imidazolidin-2-one, Sigma-Aldrich) and 8 mM $CaCl_2$ and pH was adjusted to 7.40. Cells were incubated for 1 h with the calcium dye buffer and then placed in a FLIPR Tetra System (Molecular Devices) where the liquid handling system added analogue and isoproterenol (Sigma-Aldrich, 0.05 µM final concentration) simultaneously directly followed by fluorescence signal measurement (Ex540/Em590) for 360 sec. with 30 sec. intervals. All measurements were performed in duplicates and EC_{50} values were calculated by nonlinear regression analysis of sigmoidal dose response curves using the GraphPad Prism v. 5.02 (Graph Pad software, La Jolla, CA, USA) with set hill slope of -1. All data presented represent the mean value of at least three individual assays performed at separate occasions ± SEM. The assay ranges from 1-1000 nM in agonist concentrations and reported EC_{50} values below or above these concentrations should be regarded as approximations. However, the PYY(3-36) reference peptide was examined at a range of 0.03-30 nM.

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