Supporting Information

Potent Agonists of the Protease Activated Receptor 2 (PAR_2)

Scott Boitano,[‡#] Andrea N. Flynn,[‡#], Stephanie M. Schulz, [‡#] Justin Hoffman,[‡#] Theodore J. Price, [†] and Josef Vagner[#]*

[‡]Arizona Respiratory Center and Department of Physiology, University of Arizona, 1501 N. Campbell Ave., Tucson, AZ 85724.

[#] The BIO5 Research Institute, University of Arizona, 1657 E Helen Street, Tucson, AZ 85721.

[†] Department of Pharmacology, University of Arizona, 1501 N. Campbell Ave. Tucson, AZ 85724.

Contents:

General methods:

Chemical Materials:

N-α-Fmoc-protected amino acids, HBTU, HCTU, HOCt and HOBt were purchased from SynPep (Dublin, CA) or from Novabiochem (San Diego, CA). Rink amide Tentagel S and R resins were acquired from Rapp Polymere (Tubingen, Germany). For the *N*α-Fmoc-protected amino acids, the following side chain protecting groups were used: Arg(N^g-Pbf); Lys(N^ε-Aloc). Reagent grade solvents, reagents, and acetonitrile for HPLC were acquired from VWR (West Chester, PA) or Aldrich-Sigma (Milwaukee, WI), and were used without further purification unless otherwise noted. *N*-terminal heterocyclic acids were obtained from Sigma-Aldrich or TCI. The solid-phase synthesis was performed in fritted syringes using a Domino manual synthesizer obtained from Torviq (Niles, MI).

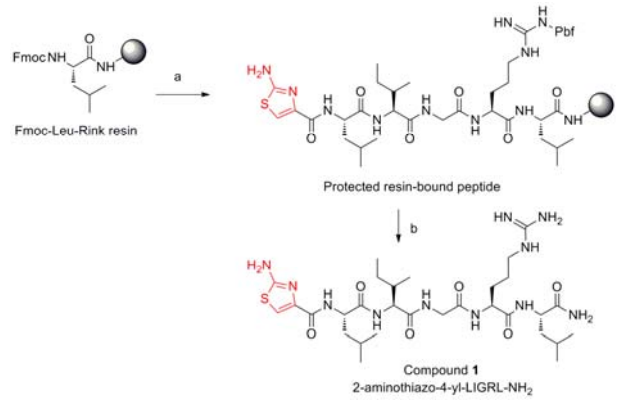
General Synthesis:

All reactions were conducted under Ar atmosphere using oven-dried glassware. All chemicals were obtained from commercial sources and used without further purification. ^{1}H NMR spectra were recorded on a Bruker-DRX-300 MHz instrument with chemical shifts reported relative to TMS (0.0 ppm) and residual DMSO (2.50 ppm). Proton-decoupled ^{13}C NMR spectra were referenced to $CDCl_3$ (77.0 ppm) as well as DMSO (39.51 ppm). Low resolution mass spectra were obtained on AGILENT (HP) MDS 1100 using AP-ESI. High resolution mass spectra (HRMS) were recorded on a JEOL HX110A instrument. Melting points were measured using a Thomas Hoover capillary melting point apparatus and are uncorrected.

Solid-phase Synthesis Fmoc-LIGRL-Rink resin:

Ligands were prepared as previously published by solid-phase synthesis as summarized in Scheme S1 on Rink Amide Tentagel resin (0.23 mmol/g) using a Fmoc/*t*Bu synthetic strategy and standard DIC activation.[1,2] N^{α}-Fmoc amino acid was double coupled using preactivated 0.3 M HOBt esters and HCTU/2,4,6-collidine couplings.

The Rink resin was washed with DMF, and the N^{α}-Fmoc protecting group was removed with 1:10 piperidine in DMF (1 × 2 min and 1 × 20 min). The resin was washed successively with DMF, DCM, DMF, a solution of 0.05 mM solution of Bromophenol Blue in 0.2 M HOBt in DMF, then DMF. The N^{α}-Fmoc amino acid was coupled using pre-activated 0.3 M HOBt or HOCt esters in DMF-DCM mixture (3 equiv of N^{α}-Fmoc amino acid, 3 equiv of HOBt or HOCt, and 3 equiv of DIC). The resin slurry was stirred for 2 h or until the bromophenol test became negative.[3] If the test failed, the resin was washed with DMF and the amino acid was coupled again by the HCTU/2,4,6-lutidine procedure (0.3 M solution of 3 equiv of N^{α}-Fmoc amino acid, 3 equiv of HCTU, and 6 equiv of 2,4,6-lutidine in DMF) for 3 h or by a preformed symmetric anhydride (3 equiv of N^{α}-Fmoc amino acid and 1.5 equiv of DIC in a 1:1 DMF-DCM mixture) until Kaiser test was negative. If the couplings did not result in a negative Kaiser test, the resin was washed with DMF, and the free amino groups were capped with 50% acetic anhydride in pyridine for 10 minutes. After all coupling sequentially to the Rink amide resin, the resin was washed with DCM, dried under vacuum, and then stored in refrigerator.



Scheme S1. Solid-phase synthesis of compound **1.**
(a) (i) Fmoc/*t*Bu synthetic strategy. (ii) 10% piperidine in DMF, 20 minutes. (iii) HBTU coupling of 2-aminothiazole-4-carboxylic acid (b) (i) 91 % TFA, 3% thioanisole, 3% 1,2-ethanedithiol, 3% water, 4 hrs (ii) ether extraction (iii) HPLC purification.

Solid-phase Synthesis of compound 1, (Scheme S1)

An aliquot of the dry protected Fmoc-LIGRL-Rink resin (10 μmol, from previous step) was placed in a 2 mL syringe reactor. The resin was swelled in DCM for an hour. The Fmoc protecting group was deprotected by 10% piperidine in DMF. The deprotected resin was washed with DMF (3X), 0.2 M

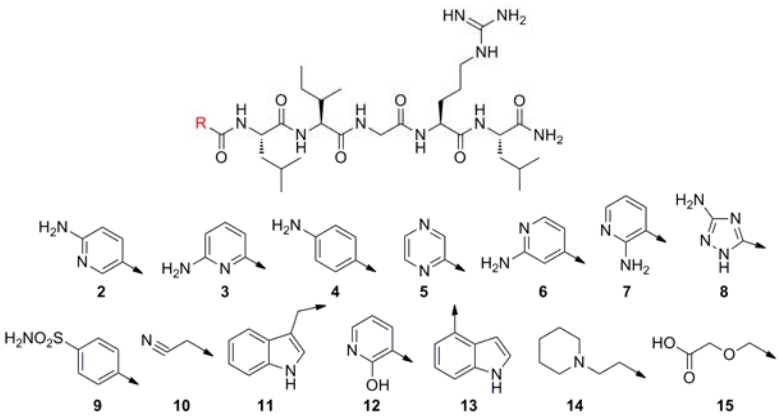
HOBt in DMF (2X), and DMF (2X0. The free amine N-terminus was coupled using 3 equiv of 2-amino-thiazole-4-carboxylic acid by the HBTU/2,4,6-lutidine procedure (0.3 M solution of 3 equiv of acid, 3 equiv of HBTU, and 6 equiv of 2,4,6-lutidine in DMF) overnight. The final resin was washed thoroughly with DMF (3x) and DCM (7x) then cleaved. A cleavage cocktail (10 mL per 1 g of the resin) consisting of CF_3CO_2H (91%), H_2O (3%), EDT (3%), and TA (3%) was injected into the resin and the mixture was agitated at room temperature for 4 h. The solution was filtered, the resin was washed with CF_3CO_2H (2 × 3 min), the liquid phases were collected and concentrated under a stream of nitrogen, and the product was precipitated using cold Et_2O. The crude product was washed three times with cold Et_2O, lyophilized, purified, and characterized as described above. The pure compounds were dissolved in DI water or DMSO at approximately 1-5 mM concentrations and concentration was determined by Trp-HPLC measurement[4,5].

QC and purification:

The purity of products was checked by analytical PR-HPLC using a Waters Alliance 2695 Separation Model with a Waters 2487 dual wavelength detector (220 and 280 nm) on a reverse phase column (Waters Symmetry C18, 4.6 × 75 mm, 3.5 μm). Peptides were eluted with a linear gradient of aqueous CH_3CN/0.1% CF_3CO_2H at a flow rate of 1.0 mL/min. Purification of ligands was achieved on a Waters 600 HPLC using a reverse phase column (Vydac C18, 15–20 μm, 22 × 250 mm). Peptides were eluted with a linear gradient of CH_3CN/0.1% CF_3CO_2H at a flow rate of 5.0 mL/min. Separation was monitored at 230 and 280 nm. Size exclusion chromatography was performed on a borosilicate glass column (2.6 × 250 mm, Sigma, St. Louis, MO) filled with medium sized Sephadex G-25 or G-10. The compounds were eluted with an isocratic flow of 1.0 M aqueous acetic acid. The pure compounds were dissolved in DI water or DMSO at approximately 1-5 mM concentrations. Accurate concentrations were determined by HPLC at 280 nm. A solution of D-Trp in water or DMSO, accordingly, was co-injected as an internal standard. Structures were characterized by ESI (Finnigan, Thermoquest LCQ ion trap instrument) or MALDI-TOF (Bruker Reflex-III, α-cyanocinnamic acid as a matrix). For internal calibration an

appropriate mixture of standard peptides was used with an average resolution of 8,000–9,000. High resolution mass measurements were carried out on a FT-ICR IonSpec 4.7T instrument.

Structures are provided in **Scheme S2.** Mass spectra and HPLC characterization data are provided in **Table S1.**



Scheme S2. Structures of PAR-2 agonists

Table S1: High resolution mass spectral data and HPLC retention times.

Compound	Structure	Rt (k')	Calculated [MH^+]	Exp. [MH^+]
	2-furoyl-Leu-Ile-Gly-Arg-Leu-Orn(Aloc)-HN_2 (**365**)	9.9	862.5145	862.5142
1	2-aminothiazo-4-yl-Leu-Ile-Gly-Arg-Leu-HN_2 (**13-4**)	7.71	696.3974	696.3978
2	6-aminonicotinyl-Leu-Ile-Gly-Arg-Leu-HN_2 (**383**)	6.50	690.4410	690.4407
3	6-aminopyridin-2-yl-Leu-Ile-Gly-Arg-Leu-HN_2 (**13-1**)	6.81	690.4410	690.4
4	4-aminophenyl-Leu-Ile-Gly-Arg-Leu-HN_2 (**7-05**)	7.63	689.4457	689.4
5	pyrazin-2-yl -Leu-Ile-Gly-Arg-Leu-HN_2 (**13-2**)	8.12	676.4253	676.4
6	6-aminopyridin-4-yl-Leu-Ile-Gly-Arg-Leu-HN_2 (**13-5**)	6.49	690.4410	690.5
7	6-aminopyridin-5-yl-Leu-Ile-Gly-Arg-Leu-HN_2 (**13-6**)	6.68	690.4410	690.5
8	3-amino-1H-1,2,4-triazol-5-yl-Leu-Ile-Gly-Arg-Leu-HN_2 (**13-7**)	6.5	680.4315	680.4
9	4-sulfonamidophenyl-Leu-Ile-Gly-Arg-Leu-HN_2 (**13-7**)	6.1	753.4076	753.4
10	2-cyanoacetyl-Leu-Ile-Gly-Arg-Leu-Orn(Aloc)-HN_2 (**365**)	6.7	637.4044	637.4

[a] Peptide was eluted with a linear MeCN/0.1% CF_3CO_2H aqueous gradient (10% to 90% in 30 min) at a flow rate of 0.3 mL/min); Waters XBridge C-18 column (3.0 x 150 mm, 3.5 µm); *HPLC* k' = (peptide retention time - solvent retention time)/solvent retention time. All the obtained purified peptides showed > 95% purity. [b] MS found molecular peaks MH^+; high resolution Bruker Reflex III MALDI-TOF instrument.

Table S2. Ca^{2+} mobilization and *in vitro* physiological (RTCA) assays

		$[Ca^{2+}]_i$ measurements				xRTCA[a]
	R-CO-Leu-Ile-Gly-Arg-Leu-NH_2[b]	% 100 µM	% 10 µM	% 2.5 µM	EC_{50} (µM)	EC_{50} (nM)
	2-furoyl-**LIGRL-Orn(Aloc)-NH_2 (365)**	100 ± 0	97.8 ± 1.0	93.6 ± 3.3	0.84 ± 0.08	138 ± 13
1	2-aminothiazol-4-yl (**13-4**)	100 ± 0	96.2 ± 1.4	67.5 ± 7.5	1.77 ± 0.24	142 ± 18
2	6-aminonicotinyl (**383**)	100 ± 0	93 ± 2.0	55 ± 11.6	2.60 ± 0.32	311 ± 26
3	6-aminopyridin-2-yl (**13-1**)	99.3 ± 0.7	94.4 ± 2.6	27.1 ± 6.9	3.50 ± 0.36	ND
4	4-aminophenyl (**13-3**)	93.0 ± 0.3	86.9 ± 4.1	16.5 ± 5.3	6.59 ± 1.7	ND
5	pyrazin-2-yl (**13-2**)	98.6 ± 1.4	31.5 ± 25.6	0	ND	ND
6	6-aminopyridin-4-yl (**13-5**)	96.1 ± 3.9	0	0	ND	ND
7	6-aminopyridin-5-yl (**14-1**)	98.7 ± 2.2	86.6 ± 12.1	0.6 ± 0.4	ND	ND
8	3-amino-1H-1,2,4-triazol-5-yl (**14-2**)	88.4 ± 6.4	6.0 ± 5.9	0	ND	ND
9	6-sulfonamidophenyl (**5-02I**)	88.8 ± 4.3	0	0	ND	ND

[a] EC_{50} = concentration of compound that was able to generate 50% maximal intracellular activity; values are expressed ± S.E.M. ($N \geq 2$ for 100 µM; N≥4 for all others in Ca^{2+} assay, data are expressed % response ± S.E.M; N≥8 in the RTCA assay).
[b] the structures do not contain C-terminal Orn as 2-furoyl-Leu-Ile-Gly-Arg-Leu-Orn(Aloc)-NH_2. ND = not determined.

References:

[1] J. Vagner, H. L. Handl, R. J. Gillies, V. J. Hruby, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 211-215.

[2] H. L. Handl, R. Sankaranarayanan, J. Josan, J. Vagner, E. A. Mash, R. J. Gillies, V. J. Hruby, *Bioconjug. Chem.* **2007**, 18, 1101-1109.

[3] V. Krchnak, J. Vagner, and M. Lebl, *Intl. J. Pept. Prot. Res.* **1988**, *32*, 415-421.

[4] J. Vagner, L. Xu, H.L. Handl, J.S. Josan, D.L. Morse, E.A. Mash, R.J. Gillies, and V.J. Hruby *Angew. Chem. Int. Ed.* 2008, 47, 1685 –1688

[5] J.S. Josan, J. Vagner, H.L. Handl, R. Sankaranarayanan, R.J. Gillies, V.J. Hruby *Int. J. Pept. Res. Ther.* (2008) 14, 93–300.