

Electronic Supplementary Information for

Rapid Identification of the Pharmacophore in a Peptoid Inhibitor of the Proteasome Regulatory Particle

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General Remarks.

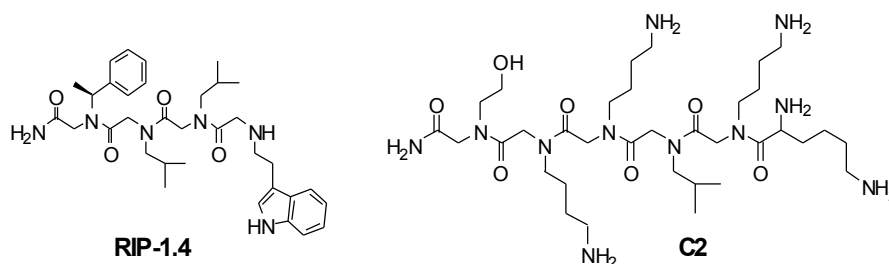
All chemicals and solvents were purchased from commercial suppliers and used without further purification. Synthesis of the compounds was performed on Rink Amide MBHA resin purchased from Nova Biochem (San Deigo, CA). Preparative HPLC was performed on a Waters Breeze HPLC system with a Vydac C18 preprative column. Flow rate was 10 ml/min. HPLC runs used linear gradients of 0.1% TFA and 90% acetonitrile plus 0.1% TFA. Mass spectrometry was performed on all synthesized compounds with the MALDI-Voyager DE Pro instrument.

Synthesis of RIP-1.4 and C2.

After removing the Fmoc protecting group on Rink Amide MBHA resin, the peptoid residues involved in RIP-1.4 and C2 were conjugated using a microwave-assisted protocol in a scale of 0.1 mmol as described previously [S1,S2]. The resulting conjugates were cleaved from the resin by 2 mL of 95% TFA, 2.5% water, and 2.5% triisopropylsilane (cleavage cocktail) at room temperature for 2 h. TFA was removed and the crude product was purified by C18 RP-HPLC.

MALDI-TOF: RIP-1.4: observed 605.4 $[M+H]^+$, expected 605.3 for $C_{34}H_{48}N_6O_4 + H$.

C2: observed 743.5 $[M+H]^+$, expected 743.5 for $C_{34}H_{69}N_{11}O_7 + H$.

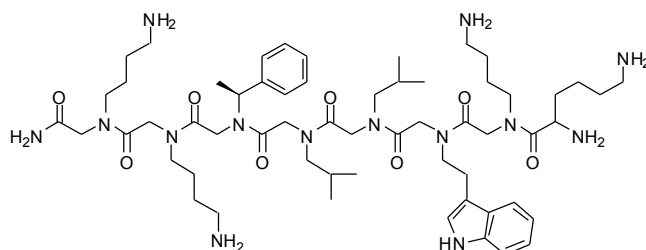


Synthesis of purine-deleted compound.

At the end of the peptoid synthesis, Boc-Lys(Fmoc)-OH (Novabiochem) was attached to the N-terminal of the peptoids by standard peptide synthesis using Fmoc chemistry. The Fmoc protecting group was removed by 20% piperidine in DMF. The resulting compound was cleaved from the resin by 2 mL of the cleavage cocktail at room

temperature for 2 h. TFA was removed and the crude product was purified by C18 RP-HPLC.

MALDI-TOF: observed 989.2 $[M+H]^+$, expected 989.6 for $C_{52}H_{84}N_{12}O_7 + H$.

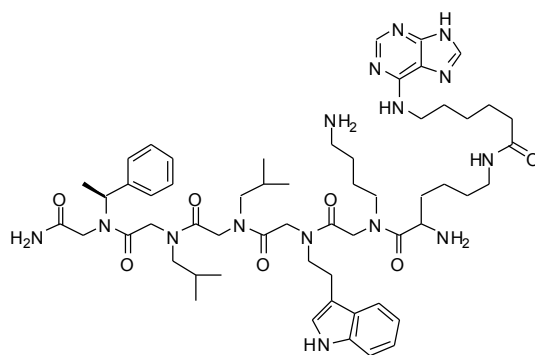


Purine-deleted compound

Synthesis of ICT-deleted compound.

The peptoid residues involved in the ICT-deleted compound and subsequently Boc-Lys(Fmoc)-OH were conjugated as described above. The Fmoc protecting group was removed by 20% piperidine in DMF. The resulting free amine was coupled with 6-(9H-purin-6-ylamino)hexanoic acid as described previously. The resulting conjugate was cleaved from the resin by cleavage cocktail and purified by C18 RP-HPLC.

MALDI-TOF: RIP-1.1: observed 1092.7 $[M+H]^+$, expected 1092.6 for $C_{57}H_{85}N_{15}O_7 + H$.



ICT-deleted compound

Synthesis of glycine-scanned compounds (R1 – R5).

Glycine-scanned compounds were synthesized as described above except for the glycine conjugation step. The coupling reaction to the corresponding peptoids was performed with a mixture of Fmoc-Gly-OH (Novabiochem), HBTU and N-methylmorpholine in

DMF. Following conjugation of the remaining peptoids, Boc-Lys(Fmoc)-OH, and 6-(9H-purin-6-ylamino)hexanoic acid, deprotection and cleavage from the resin was performed with cleavage cocktail. The crude products were purified by C18 RP-HPLC.

MALDI-TOF analysis: R1-deleted compound: observed 988.3 [M+H]⁺, expected 988.6 for C₄₉H₇₇N₁₅O₇ + H. R2-deleted compound: observed 1036.5 [M+H]⁺, expected 1036.6 for C₅₃H₇₇N₁₅O₇ + H. R3-deleted compound: observed 1036.3 [M+H]⁺, expected 1036.6 for C₅₃H₇₇N₁₅O₇ + H. R4-deleted compound: observed 949.4 [M+H]⁺, expected 949.6 for C₄₇H₇₆N₁₄O₇ + H. R5-deleted compound: observed 1021.3 [M+H]⁺, expected 1021.6 for C₅₃H₇₆N₁₄O₇ + H.

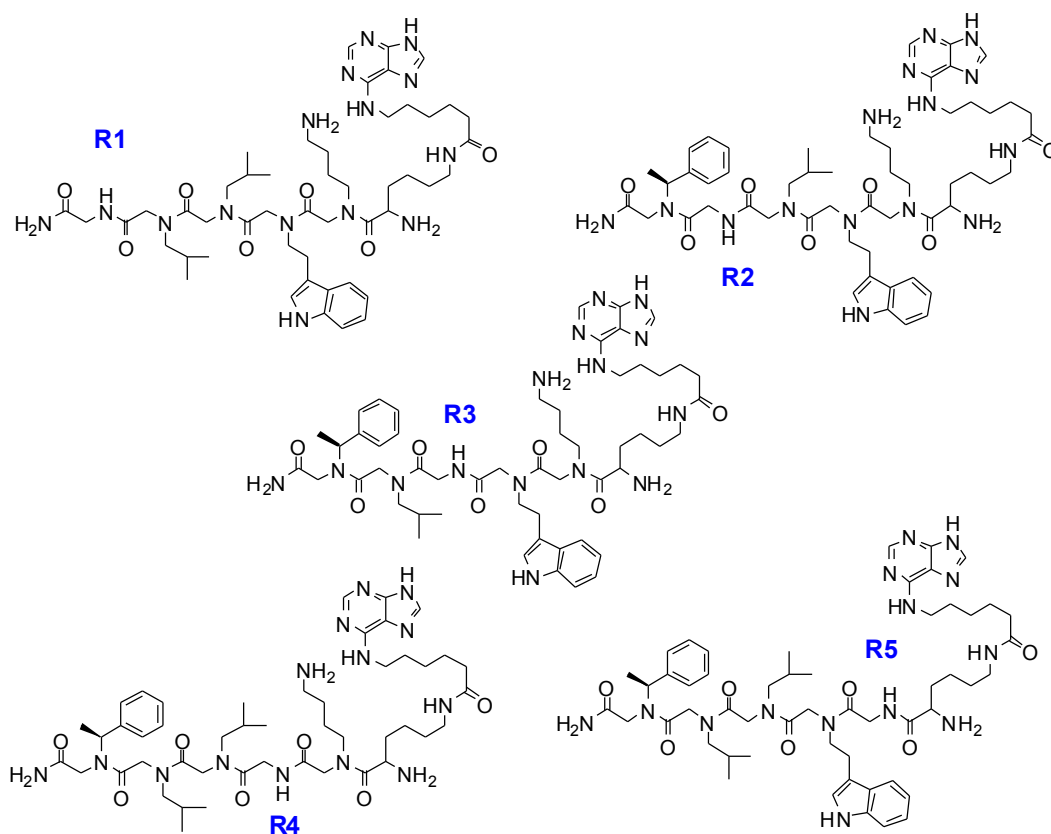
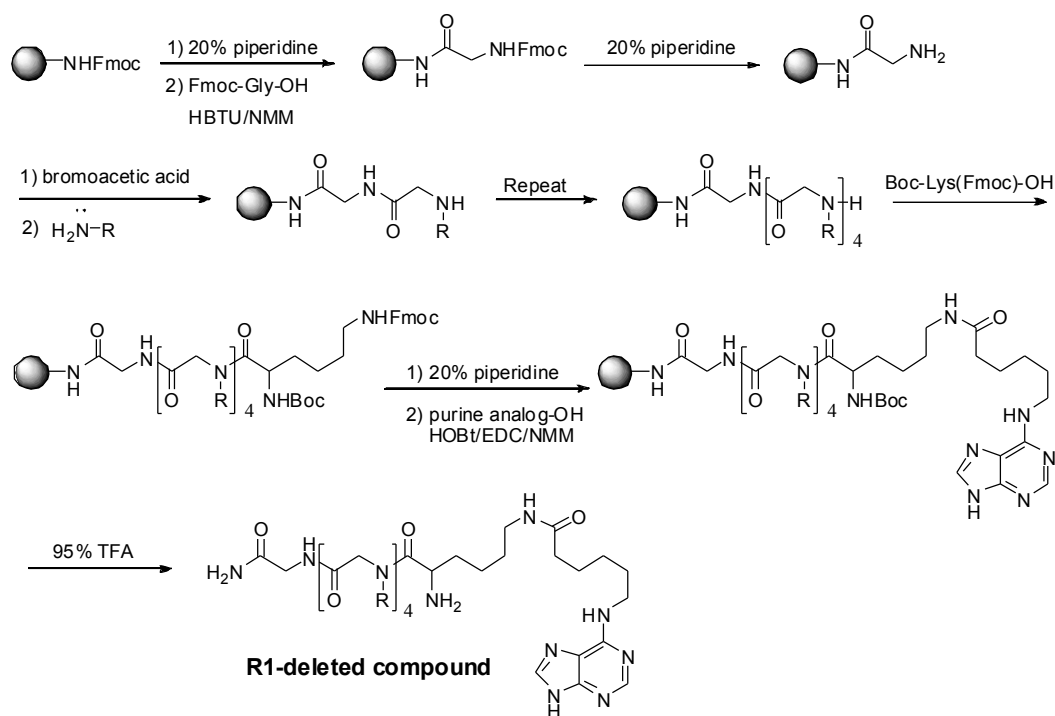


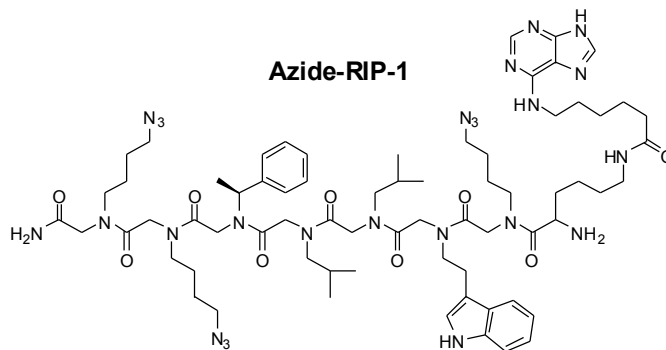
Figure S1. Chemical structures of glycine-scanned compounds (R1-R5).



Scheme S1. Synthesis of R1-deleted compound as a representative of glycine-scanned compounds.

Synthesis of azide-RIP-1.

Azide-RIP-1 was prepared as described previously for the synthesis of RIP-1 [S3] by using 4-azidobutyl-1-amine [S4] instead of mono-Boc-protected 1,4-diaminobutane. MALDI-TOF analysis: observed 1427.0 $[\text{M}+\text{H}]^+$, expected 1426.8 for $\text{C}_{69}\text{H}_{103}\text{N}_{25}\text{O}_9 + \text{H}$.



Affinity purification of the 20S proteasome and the 26S proteasome. Affinity-purified 26S proteasome was prepared from *S. cerevisiae* strain RJD1144 (MATa his3 Δ 200 leu2-3, 112 lys2-801 trp Δ 63 PRE1 FLAG::Ylplac211 (URA3) as described previously [S5] with minor modification [S6]. The protocol involves purification of the 26S proteasome carrying a FLAG tag on the C-terminus of the Pre1 protein (α 4 subunit) by immunoaffinity chromatography and FLAG[®] peptide (Sigma) elution. To purify the 20S proteasome, the entire procedure described above was carried out in the absence of ATP and 1 \times ARS (ATP regenerating system).

Peptidase Assay. The proteasome peptidase activity was measured using Suc-LLVY-amc (Bachem) to analyze the chymotrypsin-like activities of the 20S proteasome or the 26S proteasome. The 20S proteasome (2 nM) or the 26S proteasome (2 nM) was incubated at 25°C for 10 min with or without MG132 (1 μ M), C2 (10 μ M), or RIP-1 derivatives (10 μ M) in the reaction buffer containing 50 mM Tris (pH 8.0) and 20 μ M β -mercaptoethanol (100 μ L of final reaction volume). After adding Suc-LLVY-amc (50 μ M), the peptidase activities were monitored for 20 min by measuring the fluorescence of the released 7-amido-4-methylcoumarin (Ex: 365 nm, Em: 460 nm)

Chaperonin Activity Assay. The chaperonin activity assay has been described previously [S7] with the following changes. The activator-DNA complex was added at a 60 nM final concentration of activator-DNA complex to the reaction mix containing 25 nM 26S proteasome pre-incubated for 15 minutes at room temperature with the indicated amount of RIP-1.4 or C2, 3 mM ATP, and 1 μ M non-biotinylated DNA containing Gal4 binding sites in TR reaction buffer (10 mM HEPES (pH 7.8) 50 mM KCl, 6.25 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT). After the assay, the samples were run on SDS-PAGE and western blotted with an antibody raised against the Gal4 DBD. The bands were analyzed by “ImageJ” software for quantification.

Cell Culture and Western Blot. C2C12 cells (3×10^4) were placed in 6-well plate and grown in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 20% fetal calf serum (Invitrogen) for 48 h. After incubation with the indicated concentrations of

RIP-1.4, DMSO itself and MG132, cells were lysed in 1% SDS/10 mM Tris-HCl, pH 7.5. Protein concentration was measured using BCA protein assay reagent (Pierce). Equal amount of protein samples (25 μ g) were subjected to 10% SDS-PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore). The membranes with transferred proteins were blocked with 1 \times TBST containing 5% non-fat, dried milk and then incubated with anti-p27^{Kip1} antibody (Cell Signaling) and subsequently secondary antibody labeled with horseradish peroxidase. Signals were visualized by enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

References

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