Supplementary Information (SI)

for

Arg-Thz is a minimal substrate for the N^{α},N^{α}-arginyl methyltransferase involved in the biosynthesis of plantazolicin

by

Natalia A. Piwowarska, Srinivas Banala, Herman S. Overkleeft and Roderich D. Süssmuth*

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1. Reagents and solvents

If not otherwise stated, reagents and solvents were purchased from Carl Roth GmbH & Co (Karlsruhe, Germany)

2. Primers

#	Primer Name	Primer Sequence (5' – 3')
1	FW-SUMO-pznL	AGCGAAATTGAAACAATTGTCAGAG
2	RW-SUMO-pznL	TACTTTCTTCACGTATACCTTTTG

3. PznL expression and purification

E. coli Rosetta-gami™ 2(DE3) were transformed with Champion™ pET-SUMO-*pznL* construct. Starter cultures were grown at 37 °C over night in 10 ml LB medium with kanamycin (50 µg/ml), tetracyclin (15 µg/ml) and chloramphenicol (34 µg/ml) with shaking at 160 rpm. Main stage culture (200 ml, TB medium) was inoculated with 4 ml starter culture and induced with 0.25 mM isopropyl-β-D-thiogalactoside (IPTG) when its OD_{600} reached a value of 0.5-0.6. Incubation was continued at 16 °C for an additional 16 h, and subsequently cells were harvested by centrifugation. The cell pellet was stored at -80 °C until purification. Cells were re-suspended in lysis buffer (50 mM Tris, 500 mM NaCl, 20 mM imidazole, 0.5 mM DTT, pH 8.0) including the protease inhibitors, benzamidine and phenylmethanesulfonyl fluoride. The cells were lysed using homogenizer Emulsiflex (Avestin, Inc., Ottawa, ON, USA) for two runs, and centrifuged for 40 min at 12,000g to remove cell debris. His_6-SUMO-PznL protein was first purified by immobilized metal ion affinity chromatography (IMAC) with a HisTrap column (GE Healthcare) attached to an AKTA 10 purifier system (GE Healthcare). Protein was eluted using an imidazole gradient. Elution buffer contained 50 mM Tris, 500 mM NaCl, 300 mM imidazole, and 0.5 mM DTT, pH 8.0. Eluted fractions containing target protein were re-buffered (50 mM Tris, 300 mM NaCl, 0.5 mM DTT, pH 8.0) by use of an Amicon ultrafiltration device (Milipore). The progress of purification was monitored by SDS-PAGE and Coomassie staining. Protein concentrations were determined photospectrometrically resulting in 15 mg of protein / L of bacterial culture.

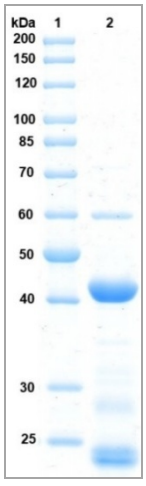


Figure S1. Coomassie stained SDS-PAGE gel of His_6-SUMO-PznL (44.2 kDa). Lane 1: molecular weight marker, Lane 2: His_6-SUMO-PznL after purification.

4. Experimental conditions for *in vitro* PznL-mediated methylations

For all the experiments conducted the assay mixtures consisted of 25 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM dithiothreitol and 4.5 µM His_6-SUMO-PznL. The concentrations of substrate and SAM as well as reaction time were varied and optimised reaction conditions were as follows: for the PznL activity tests against different truncated plantazolicin analogues **1**-**3** the substrate concentration was 60 µM, *S*-adenosyl-L-methionine (SAM) concentration was 500 µM and the reaction time was 180 min. For competition experiments the concentration of each substrates was 30 µM, SAM concentration was limited to 60 µM and reaction time was 60 min. For detection of mono-methylated substrates the substrate concentration was 60 µM, SAM concentration limited to 60 µM and reaction carried out in a time-dependent fashion over 2, 10 and 60 min.

5. HPLC-ESI-MS analysis of *in vitro* reactions

All HPLC-ESI-MS measurements were performed using an Agilent 1200 HPLC System (Agilent Technologies, Waldbronn, Germany) coupled to a HR-ESI-Orbitrap Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was carried out on a GromSil 120 ODS-4 HE column (50 x 2.0 mm; Grace, Deerfield, IL, USA) by using a mobile phase system that consisted of formic acid (0.1%) in H_2O (A) and formic acid (0.1%) in acetonitrile (B), with a gradient from 5% - 100% B over 10 min. All LC-MS measurements were carried out in positive-ionisation mode.

6. Bioactivity assay

LB agar (20 ml) was mixed with 0.5 ml of *Bacillus megaterium* (source: laboratory stock) overnight culture. 6 µg of each compound tested was spotted on the filter-paper disc, left for drying and placed on the agar plate with indicator strain (volumes of each compound are stated in Table S1). As positive control 10 µg of Apramycin (10 µl of 1 mg/ml stock solution) was used. Two negative controls were made (control 1: 10 µl of 50% DMSO in water; control 2: 10 µl of Millipore water), as compounds **1** and bismethylated **1** were dissolved in 50% DMSO and compounds **2**, **3**, and bismethylated **3** were dissolved in Millipore water. Plates were incubated for 22 h at 30°C. The growth inhibition was observed as a clear zone around the paper disk.

Table S1. Volumes and amounts of substrates and positive control (apramycin) used in bioactivity assay against *Bacillus megaterium*.

Compound	Volume spotted on disc [µl]	Concentration of stock solution [µg/µl]	Amount spotted on disc [µg]
1	10.3	0.582	6
bismethylated **1**	9.8	0.610	6
2	7.1	0.840	6
3	11.7	0.512	6
bismethylated **3**	21.0	0.284	6
apramycin (positive control)	10.0	1.0	10

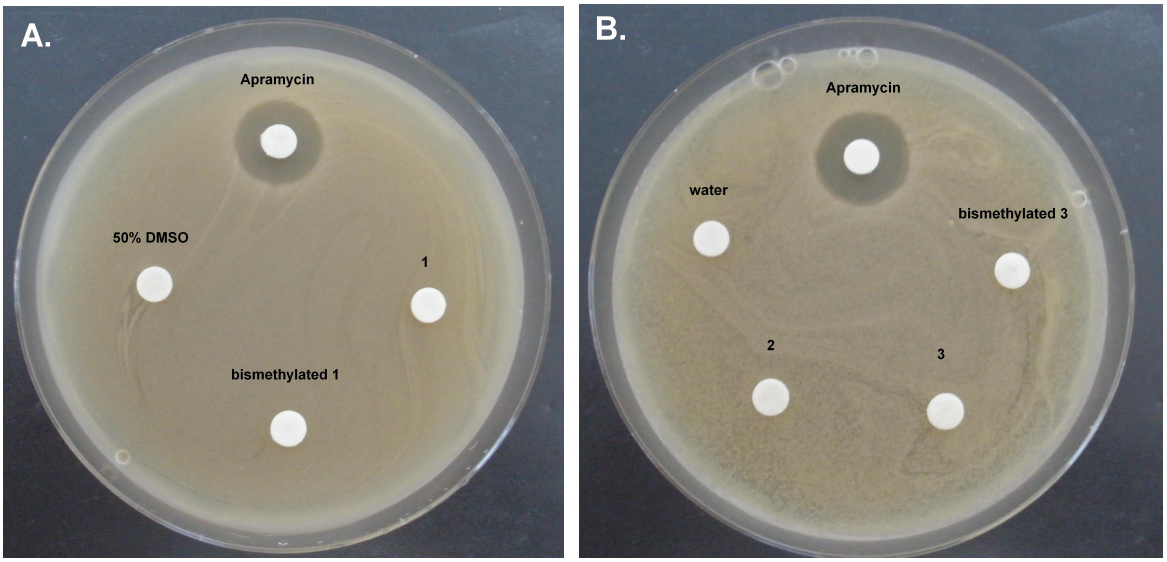
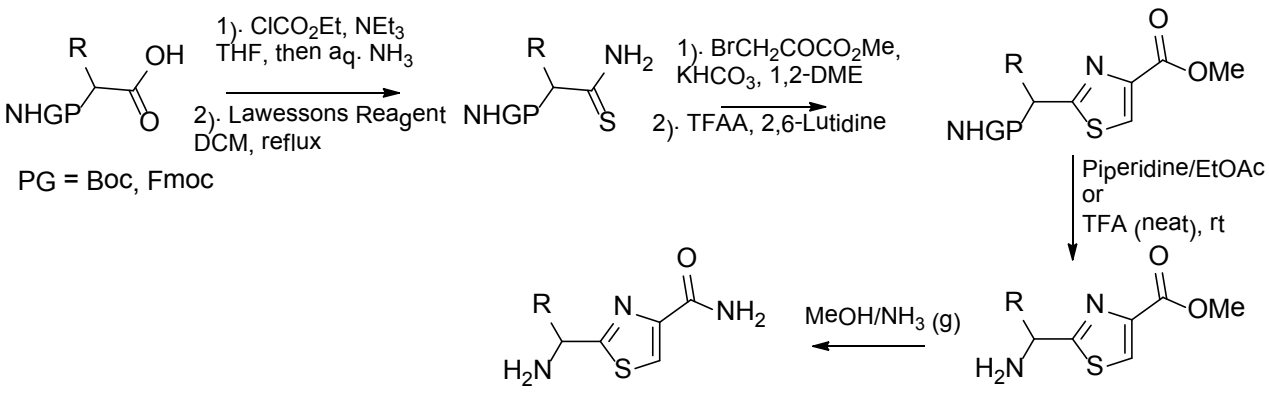


Figure S2. Antibacterial activity of **1**, **2**, **3** and bismethylated **1** and **3** in an agar disk diffusion bioassay against *Bacillus megaterium*. All tested compounds were prepared by chemical synthesis. A. upper: 10 µg control of Apramycin (positive control), left: 10µl of 50% DMSO (negative control; compound **1** and its bismethylated variant were dissolved in 50% DMSO to a final concentration of 0.6 µg/µl), bottom: 6 µg of bismethylated **1**, right: 6 µg of **1**; B. upper: 10 µg control of Apramycin (positive control), upper left: 10µl of water (negative control), bottom left: 6 µg of **2**, bottom right: 6 µg of **3**, upper right 6 µg of bismethylated **3**.

7. Peptide synthesis and purification: general experimental details.

Commercially available reagents were used throughout, without further purification unless otherwise stated. Unless otherwise specified, reactions were performed under dry nitrogen using absolute solvents, either freshly taken over a PureSolv purification system (Innovative Technologies, USA) or purchased from Acros. Amino acids and condensation reagents were obtained from IRIS (Marktredwitz, Germany), Novabiochem (Darmstadt, Germany) or Bachem (Basel, Switzerland). Reactions were routinely carried out under a nitrogen atmosphere in an oven dried flasks. Analytical thin layer chromatography was carried out using aluminium-backed plate coated with Merck silica gel 60 GF_{254}. Plates were visualized under UV light (at λ = 254 and/or 360 nm) and stained with $KMnO_4$ solution or ninhydrin solution. Flash chromatography was carried out using silica gel 60 (Merck, Darmstadt, Germany). LCMS/HRMS (high-resolution mass spectra) were recorded on an Orbitrap high resolution mass spectrometer using electrospray ionization (ESI) in positive mode.

8. Synthesis of H-AA-Thz-OMe and H-AA-Thz-NH_2 (3 – 12)



General procedure for synthesis of:

PG-AA-$CONH_2$

Fmoc- or Boc-protected amino acid (5 mmol, 1.0 eq) was dissolved in dry THF (10 mL) and NEt_3 (15 mmol, 2.6 eq) was added. The solution was cooled in an ice bath, followed by addition of ethyl chloroformate (10 mmol, 2 eq). The solution turned into a white thick suspension within few minutes, which was stirred for another 30 min in an ice bath, followed by addition of 30% aqueous ammonia solution (1 mL). The suspension was then allowed to warm up to room temperature, and stirred for another 30 min. Then solvent was removed *in vacuo* and the resulting precipitate was washed with 10% $NaHCO_3$ and extracted with ethyl acetate to obtain the amide (>98%) as a colourless powder, which was used without further purification.

PG-AA-$CSNH_2$

Amide (4.5 mmol, 1.00 eq) and Lawesson's reagent (2.5 mmol, 0.55 eq) were suspended in dry CH_2Cl_2 (15mL) and the reaction mixture was heated to reflux for 3 h. After completion of the reaction, the mixture was cooled to room temperature, sat. $NaHCO_3$ (50 mL) was added and the mixture stirred overnight. The mixture was extracted with CH_2Cl_2 (3 x 50 mL) and washed with 10% $NaHCO_3$ (50 mL), after which the organic layer was concentrated *in vacuo*. Purification by column chromatography on silica gel (ethyl acetate/hexane) gave the product (up to 70%) as light yellow solid.

PG-AA-Thz-OMe

Thioamide (2.5 mmol, 1.0 eq) and $KHCO_3$ (10 mmol, 4.0 eq) were suspended in 1,2-dimethoxyethane (10 mL) and cooled to -40 °C under nitrogen, followed by addition of bromopyruvate methylester (7.5 mmol, 3.0 eq). The reaction mixture was warmed to -17 °C and stirring continued overnight. The reaction mixture was filtered through celite under nitrogen flow, the precipitates were washed with 1,2-dimethoxyethane (3 x 3 mL), and the orange filtrate re-cooled to - 17 °C under nitrogen. Trifluoroacetic anhydride (8 mmol, 3.2 eq) and 2,6-lutidine (15 mmol, 6.0 eq) were added sequentially at -17 °C. The reaction mixture was stirred for 30 min at -17 °C, warmed to 0 °C and stirring continued for 3 h. The reaction was quenched with 10% $NaHCO_3$ (100 mL), extracted with ethyl acetate (3 x 50 mL) and the combined organic phases were washed with 5% HCl (50 mL), brine, dried over Na_2SO_4 and the solvent was removed *in vacuo* to obtain an orange oil. Purification by column chromatography on silica gel (ethyl acetate/hexane) gave the product (up to 80%) as pale yellow solids.

H-AA-Thz-OMe

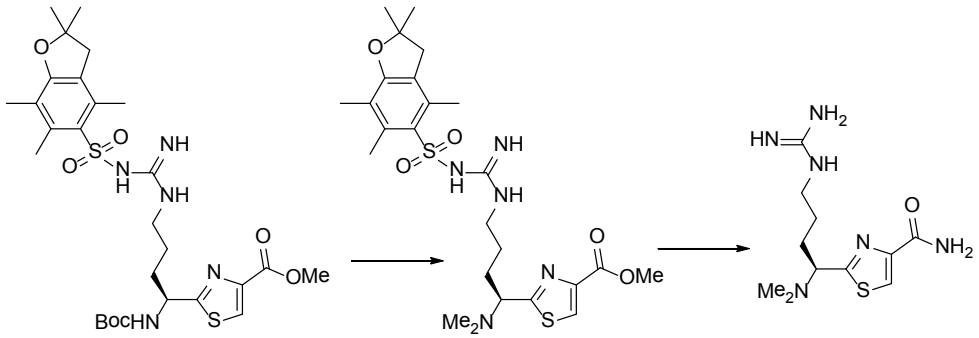
A). Fmoc-deprotection: Fmoc-AA-Thz-OMe (0.1 mmol) was dissolved in ethyl acetate (3 mL), and to the resulting solution was added piperidine (0.5 mL), and the mixture stirred at room temperature until disappearance of starting material on TLC was completed. Then the solvent was removed under reduced pressure, the residue re-suspended in toluene and evaporated under reduced pressure. The crude product was purified on preparative HPLC [C18 reversed phase, methanol/water, with 0.1% TFA] and lyophilisation yielded the products.

B). Boc-deprotection: Boc-AA-Thz-OMe (0.1 mmol) was dissolved in TFA (2 mL) and H_2O (0.05 mL) and the mixture was stirred at room temperature for 1-4 h, until disappearance of starting material was observed on TLC. Then the solvent was removed under reduced pressure, the residue co-evaporated with toluene and the product was purified on preparative HPLC [C18 column, methanol/water, with 0.1% TFA]. Lyophilisation yielded the products.

H-AA-Thz-NH_2

H-AA-Thz-OMe (0.1 mmol) was dissolved in MeOH (3 mL) and cooled in liquid N_2. Liquid ammonia was generated by purging NH_3 gas into a flask cooling in liquid nitrogen and added to the above cold solution. The mixture was placed in an autoclave, left to warm to room temperature and stirred overnight (14 h to 24 h). All solvent was removed under reduced pressure, and the residue was purified on preparative HPLC [C18 column, methanol/water, with 0.1% TFA] . Lyophilisation afforded the colourless products.

9. Synthesis of Me_2N-Arg-Thz-NH_2 (21)



Me_2-Arg(Pbf)-Thz-NH_2 (20)

The Boc-Arg(Pbf)-Thz-OMe (15 mg, 0.024mmol) was dissolved in 4M HCl/dioxane (0.5 mL) and the reaction stirred at room temperature for 35 min (TLC control) and after complete conversion MTBE (5 mL) was added to the reaction. Then all the solvents were removed under reduced pressure to obtain the dry product which was used further.

The above product was dissolved in THF (2 mL), H_2O (1 mL), and NaOAc $3H_2O$ (35 mg) was added. The solution was placed in an ice bath and then to the cold solution 37% HCHO in water (50 µl) was added and stirred for 5 min, followed by addition of $NaCNBH_3$ (10 mg) and the reaction continued to stir in an ice bath for 1 h. Then the mixture was poured into sat. $NaHCO_3$ (5 mL), extracted with ethyl acetate (3 x 10 mL) and dried over Na_2SO_4. The solvent was removed and the mixture lyophilized to obtain the product **20** as white solid (15 mg, 99%).

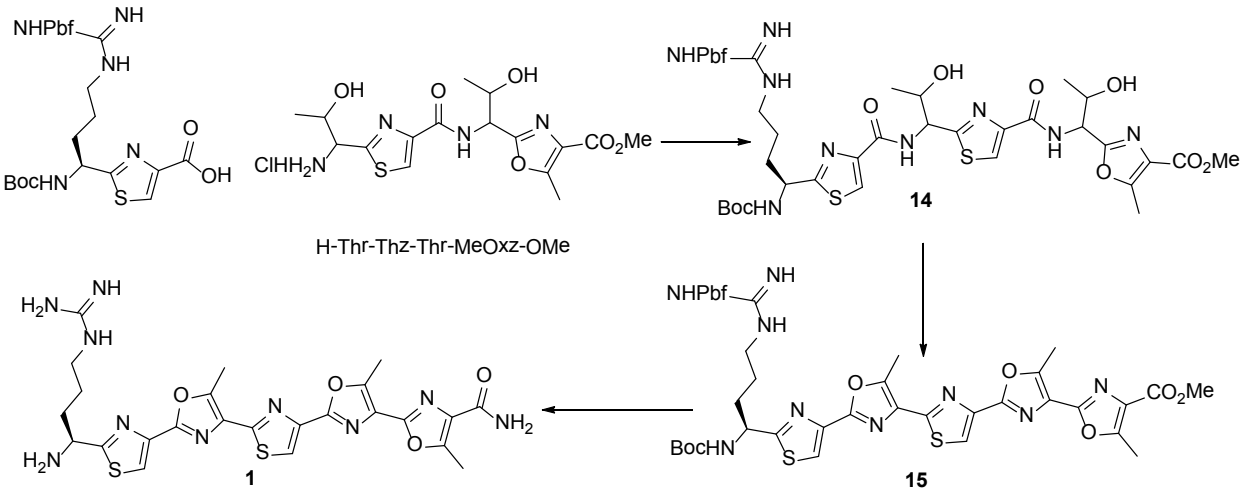
Me_2N-Arg-Thz-NH_2 (21)

The above product **20** (15 mg, 0.024 mmol) was dissolved in MeOH/THF (1:1, 3 mL) and the solution was cooled in liquid N_2. To this liquid ammonia (generated by purging the NH_3 gas into a flask cooling in liquid nitrogen), about 2 ml was added and the mixture was placed in autoclave and left to warm up to room temperature and stirred for 48h. Then all the solvent was removed under reduced pressure, and used the product for further reaction.

The above product dissolved in TFA (2 mL) and H_2O (0.02 mL), and stirred the reaction over 3 h, until the disappearance of educt. Then all the solvent was removed under reduced pressure, and then the residue purified the product on preparative HPLC [C18 column, methanol/water, with 0.1% TFA] and lyophilized to obtain colourless powder 5.2 mg (75%).

10. Synthesis of H-Arg-Thz-MeOxz-Thz-MeOxz-MeOxz-NH_2 (1)

The synthesis was performed according to synthesis procedures published previously.[1]



Boc-Arg(Pbf)-Thz-Thr-Thz-Thr-MeOxz-OMe (14)

Boc-Arg(Pbf)-Thz-OH (310 mg, 0.522 mmol, 1.1 eq) and H-Thr-Thz-Thr-MeOxz-OMe (200 mg, 1.0 eq) were suspended in DMF and cooled in an ice bath, followed by addition of HATU (160 mg, 1.5 eq) and DIPEA (0.15 ml, 3.0 eq). The mixture was left to stir at room temperature for 14 h. Then the reaction was quenched with 10% aqueous $NaHCO_3$ solution (10 mL), extracted with CH_2Cl_2 (3 x 10 mL), followed by washing the organic phases with 5% aqueous HCl solution (10 mL) and drying over Na_2SO_4. The solvent was removed under reduced pressure and the crude product purified by column chromatography on silica [ethyl acetate/hexane] to obtain compound **14** as a white solid (244 mg, 48%).

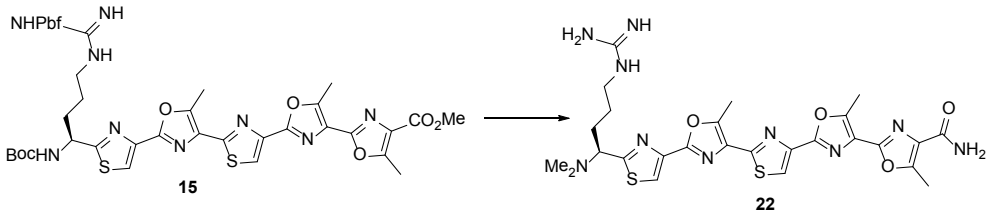
Synthesis of Boc-Arg(Pbf)-Thz-MeOxz-Thz-MeOxz-MeOxz-OMe (15)

Hexapeptide **14** (230 mg, 0.232 mmol, 1.0 eq) was dissolved in CH_2Cl_2 (15 mL) and the solution was cooled to -78 °C. To the reaction was added dimethylamino sulfurtrifluoride (DAST, 0.220 mL, 6.0 eq) and the reaction was stirred at the same temperature for 1 h and then at -20 °C for 3h. After all starting material (**14**) was consumed (as observed by TLC), to the mixture was added sat. $NaHCO_3$ (10 mL) and the reaction mixture was left to warm up to room temperature. The product was extracted with CH_2Cl_2 (3 x 10 mL) and dried over Na_2SO_4. Then the solvent was removed under reduced pressure and used further. The crude product (0.232 mmol) was dissolved in CH_2Cl_2 (15 mL) and cooled in an ice bath. To this mixture

DBU (0.4 mL) was added followed by addition of $CBrCl_3$ (0.3 mL). The reaction mixture was stirred at room temperature for 24 h. Another portion of DBU (0.4 mL) and $CBrCl_3$ (0.3 mL) were added, and the reaction was stirred for another 24 h at room temperature. Then to the reaction was added sat. $NaHCO_3$ (15 mL) and product was extracted with CH_2Cl_2 (3 x 10 mL). The organic phases were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (ethyl acetate/CH_2Cl_2, 15%) to obtain compound **15** as white solid (84 mg, 38%).

The above product **15** (20 mg, 0.021 mmol) was dissolved in MeOH/THF (1:1, 3 mL) and the solution was cooled in liquid N_2. To this about 2 mL liquid ammonia (generated by purging the NH_3 gas into a flask cooling in liquid nitrogen), was added and the mixture was placed in autoclave and left to warm to room temperature and stirred for 48 h. Then all the solvent was removed under reduced pressure. The crude product was dissolved in TFA (2 mL) and H_2O (0.02 mL), and the mixture was stirred for 3 h, until disappearance of starting material was observed by TLC. Then all the solvent was removed, the residue purified on preparative HPLC [C18 column, methanol/water, with 0.1% TFA] and lyophilized to obtain **1** as a colourless powder (8 mg, 65%).

11. Synthesis of Me_2N-Arg-Thz-MeOxz-Thz-MeOxz-MeOxz-NH_2 (22)



The heterocyclic peptide **15** (20 mg, 0.021 mmol) was taken in a flask and 4 M HCl/dioxane (0.5 mL) was added. The reaction mixture stirred at room temperature for 35 min (TLC control) and after complete conversion MTBE (5 mL) was added to the reaction. Then all the solvents were removed under reduced pressure to obtain the dry product which was used further.

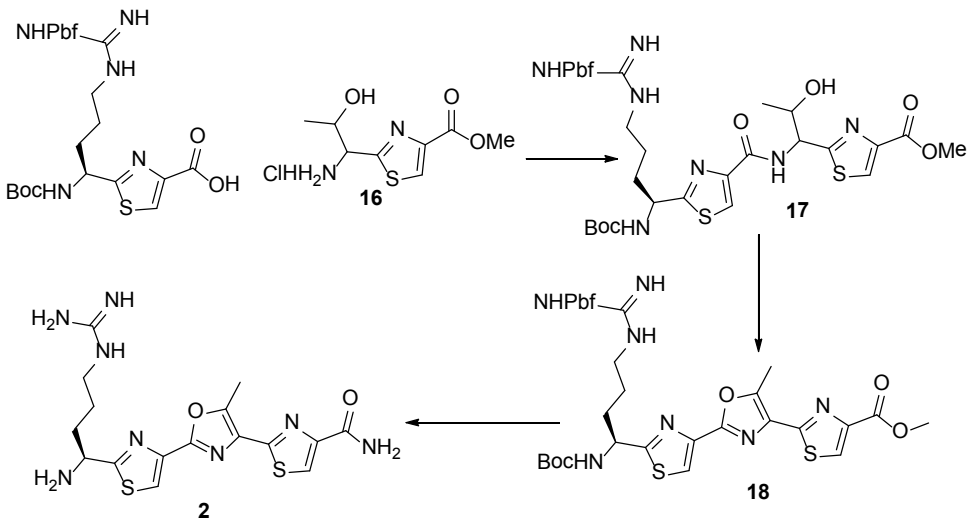
The above product was dissolved in THF (4 mL), H_2O (2 mL), and NaOAc $3H_2O$ (65 mg) was added. The solution was placed in an ice bath and then to the cold solution 37% HCHO in water (50 µl) was added and stirred for 5 min, followed by $NaCNBH_3$ (15 mg) was added and the reaction continued to stir in an ice bath for 1 h. Then the mixture was poured into sat. $NaHCO_3$ (5 mL), extracted with ethyl acetate (3 x 10 mL) and dried over Na_2SO_4. The solvent was removed and the mixture lyophilized to obtain the methylated product **19** as white solid (19 mg, 99%).

Me_2N-Arg-Thz-MeOxz-Thz-MeOxz-MeOxz-NH_2 (22)

The above N-terminal product **19** (19 mg, 0.020 mmol) was dissolved in MeOH/THF (1:1, 3 mL) and the solution was cooled in liquid N_2. To this liquid ammonia (generated by purging the NH_3 gas into a flask cooling in liquid nitrogen), about 2 ml was added and the mixture was placed in autoclave and left to warm to room temperature and stirred for 48h. Then all the solvent was removed under reduced pressure, and used the product for further reaction.

The above product dissolved in TFA (2 mL) and H_2O (0.02 mL), and the reaction was stirred over 3 h, until the disappearance of educt. Then all the solvent was removed under N_2 flow, and then the residue co-evaporated with toluene, and purified the product on preparative HPLC [C18 column, methanol/water, with 0.1% TFA] and lyophilized to obtain colourless powder 5.5 mg (45%).

12. Synthesis of H-Arg-Thz-MeOxz-Thz-NH_2 (2)



Boc-Arg(Pbf)-Thz-Thr-Thz-OMe (17)

Boc-Arg(Pbf)-Thz-OH (200mg, 0.327 mmol, 1 eq) and H-Thr-Thz-OMe (90 mg, 1.1 eq) were suspended in DMF (6 ml) and cooled in an ice bath, followed by addition of HATU (150 mg, 1.5 eq), DIPEA (0.15 ml, 3.0 eq). The mixture was stirred at room temperature for 14 h. Then the reaction was quenched with 10% aqueous $NaHCO_3$ solution (10 mL), extracted with CH_2Cl_2 (3 x 10 mL), followed by washing the organic phases with 5% aqueous HCl solution (10 mL) and drying over Na_2SO_4. The solvent was removed under reduced pressure and the crude product purified by column chromatography on silica [ethyl acetate/hexane] to obtain compound **17** as a white solid (110 mg, 55%).

Synthesis of Boc-Arg(Pbf)-Thz-MeOxz-Thz-OMe (18)

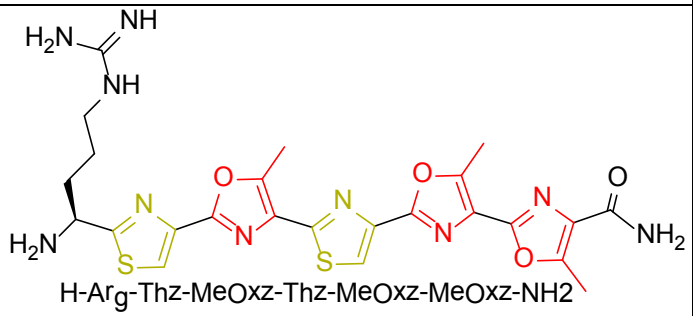
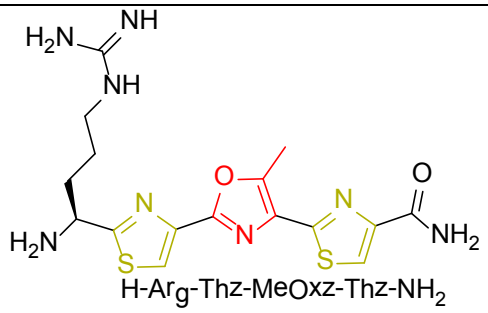
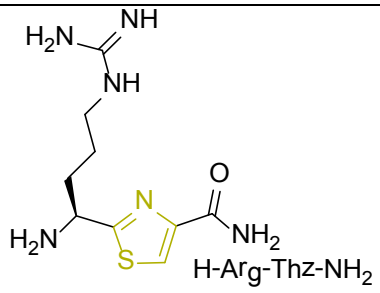
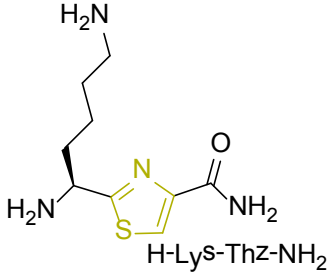
Tetrapeptide **17** (36 mg, 0.044 mmol, 1.0 eq) was dissolved in CH_2Cl_2 (12 mL) and the solution was cooled to -78 °C. To the reaction mixture was added dimethylamino sulfurtrifluoride (DAST, 0.10 mL, 6.0 eq) and the mixture was stirred at the same temperature for 1 h and at -20 °C for 3h. After all starting material **17** was consumed (TLC), to the mixture was added sat. $NaHCO_3$ (10 mL) and the reaction was left to warm up to room temperature. The product was extracted with CH_2Cl_2 (3 x 10 mL), and dried over Na_2SO_4. Then the solvent was removed under reduced pressure. The residue (0.044 mmol) was dissolved in CH_2Cl_2 (15 mL) and cooled in an ice bath. To this mixture DBU (0.4mL) was added followed by addition of $CBrCl_3$ (0.3 mL). The reaction mixture was stirred at room temperature for 24 h. Another portion of DBU (0.4 mL) and $CBrCl_3$ (0.3 mL) were added, and the reaction was stirred for further 24 h at room temperature. Then to the reaction was added sat. $NaHCO_3$ (15 mL) and product was extracted with CH_2Cl_2 (3 x 10 mL). The organic phases were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was used for the subsequent reaction.

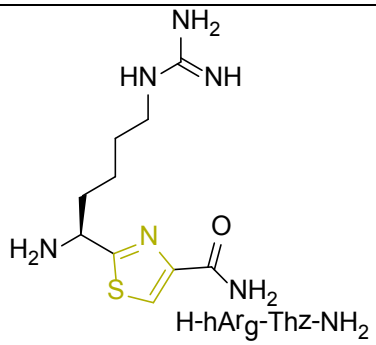
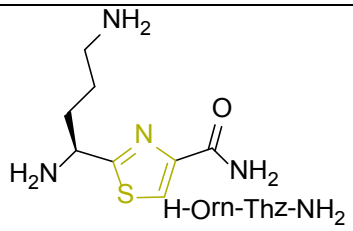
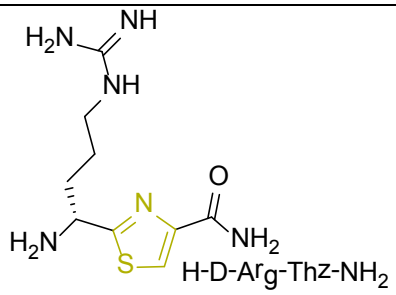
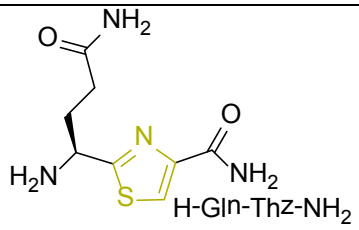
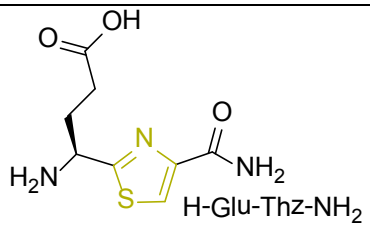
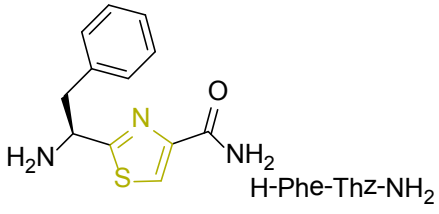
Synthesis of H-Arg-Thz-MeOxz-Thz-NH_2 (2)

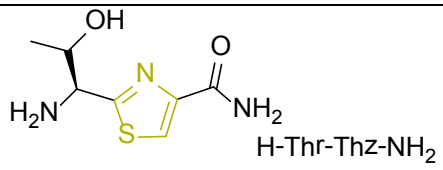
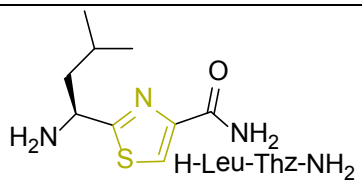
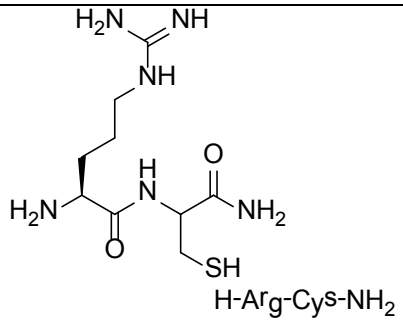
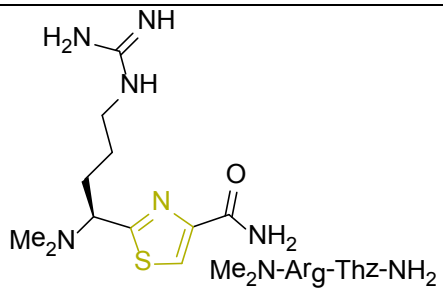
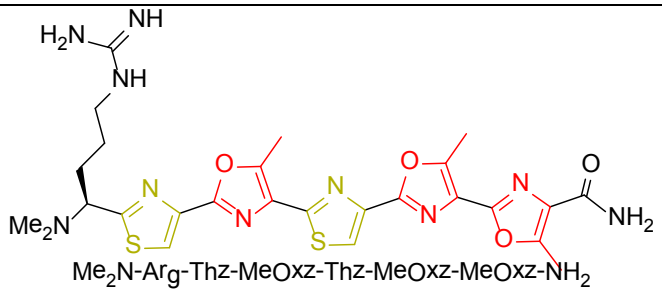
The above product **18** (0.044 mmol) was dissolved in MeOH/THF (1:1, 3 mL) and the solution was cooled in liquid N_2. To this about 2 ml liquid ammonia (generated by purging the NH_3 gas into a flask cooling in liquid nitrogen) was added and the mixture was placed in autoclave and left to warm to room temperature and stirred for 48 h. Then all the solvent was removed under reduced pressure. The residue was dissolved in TFA (2 mL) and H_2O (0.02 mL), and stirred for 3 h, until the disappearance of starting material was observed by TLC. Then all the solvent was removed, the residue purified on

preparative HPLC [C18 column, methanol/water, with 0.1% TFA] and lyophilized to obtain the target compound as a colourless powder (12.4 mg, 60%).

13. High resolution mass spectrometry (HRMS) data of substrates 1-13:

No.	Compound formula	Molecular formula, Calculated Exact Mass	HRMS found $[M+H]^+$
1	 H-Arg-Thz-MeOxz-Thz-MeOxz-MeOxz-NH2	C24H26N10O4S2; 582.15799	583.16625
2	 H-Arg-Thz-MeOxz-Thz-NH_2	C16H20N8O2S2; 420.11506	421.12290
3	 H-Arg-Thz-NH_2	C9H16N6OS; 256.11063	257.11819
4	 H-Lys-Thz-NH_2	C9H16N4OS; 228.10448	229.11154

5		C10H18N6OS; 270.12628	271.13379
6		C8H14N4OS; 214.08883	215.09581
7		C9H16N6OS; 256.11063	257.11724
8		C8H12N4O2S; 228.06810	229.07568
9		C8H11N3O3S; 229.05211	230.05925
10		C12H13N3OS; 247.07793	248.08467

11	 H-Thr-Thz-NH_2	$C_7H_{11}N_3O_2S$; 201.05720	202.06426
12	 H-Leu-Thz-NH_2	$C_9H_{15}N_3OS$; 213.09358	214.10037
13	 H-Arg-Cys-NH_2	$C_9H_{20}N_6O_2S$ 276.13684	277.14390
21	 Me_2N-Arg-Thz-NH_2	$C_{11}H_{20}N_6OS$ 284.14193	285.15228
22	 Me_2N-Arg-Thz-MeOxz-Thz-MeOxz-MeOxz-NH_2	$C_{26}H_{30}N_{10}O_4S_2$ 610.71100	611.19775

14. Mass spectrometric data

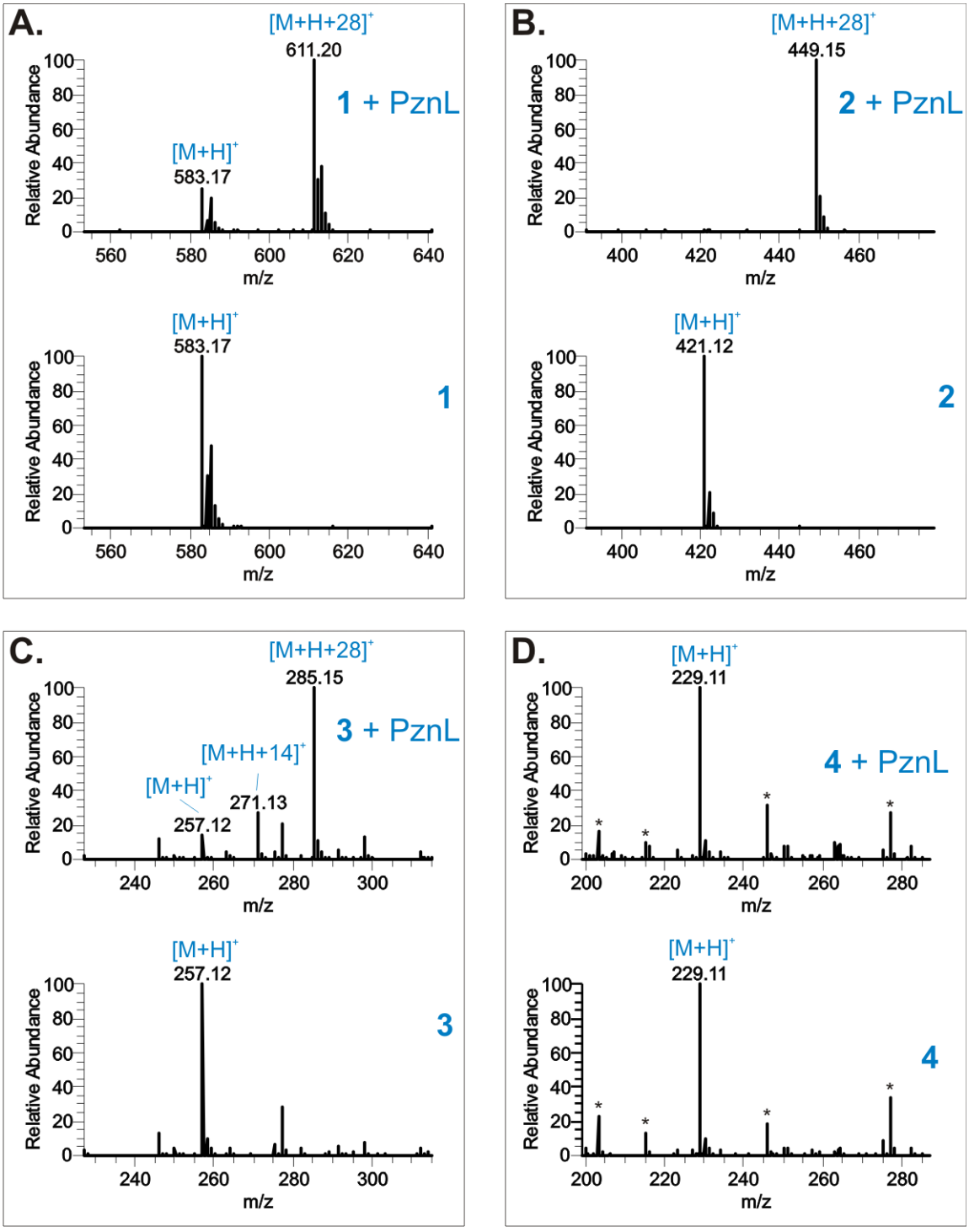


Figure S3 A-D. LC-MS spectra of His_6-SUMO-PznL mediated *in vitro* reactions of substrates: **A)** H-Arg-Thz-MeOxz-Thz-MeOxz-MeOxz-NH_2 (**1**), **B)** H-Arg-Thz-MeOxz-Thz-NH_2 (**2**), **C)** H-Arg-Thz-NH_2 (**3**), **D)** H-Lys-Thz-NH_2 (**4**). In each frame the upper spectrum shows results of a 3 h reaction of substrate with PznL, the lower spectrum shows the same reaction carried out without PznL. Asterisks correspond to molecules in the samples unrelated to the substrate (impurities in the starting material or fragmentation products).

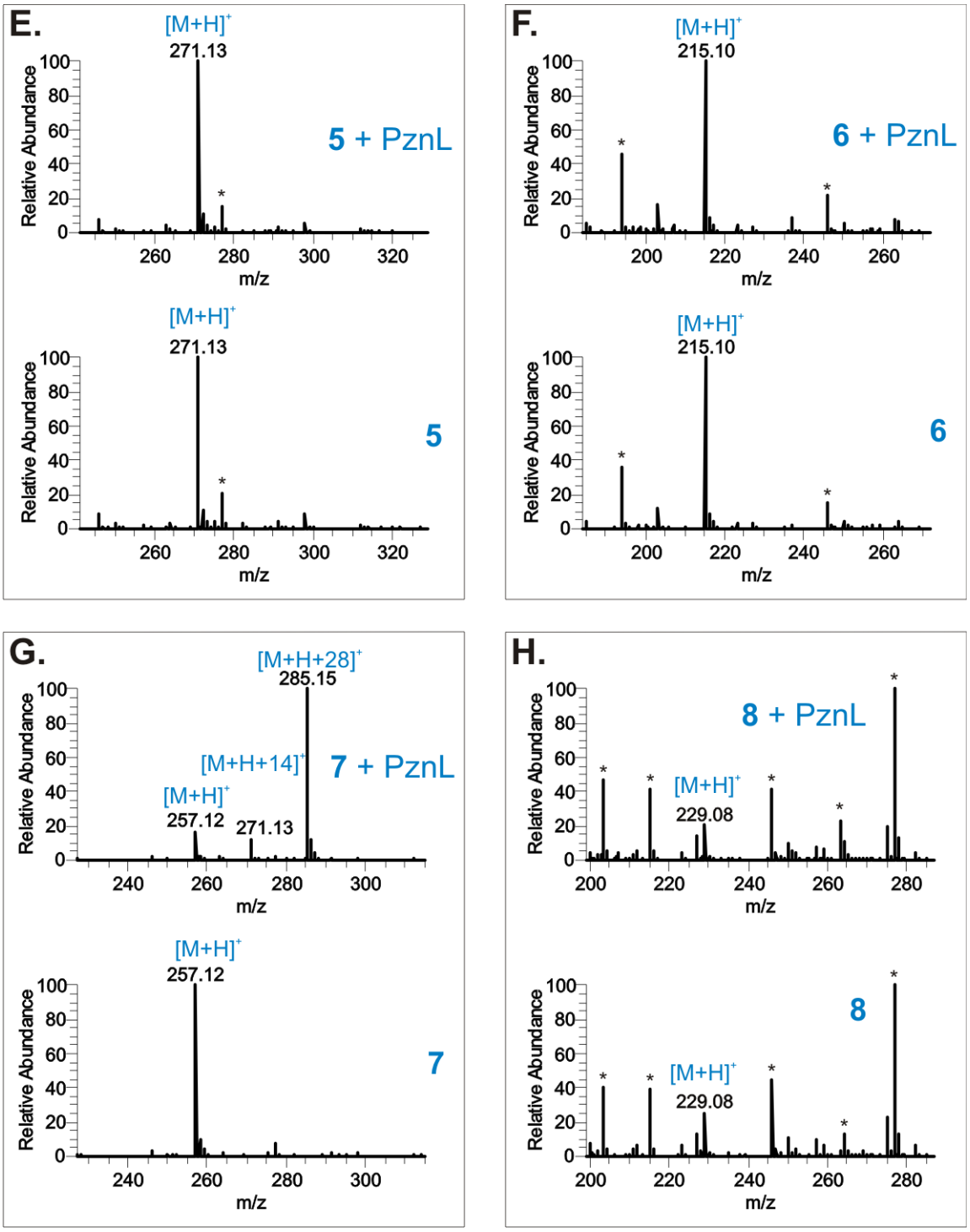


Figure S3 (continuation) E-H. LC-MS spectra of His_6-SUMO-PznL mediated *in vitro* reactions of substrates: **E)** H-hArg-Thz-NH_2 (**5**), **F)** H-Orn-Thz-NH_2 (**6**), **G)** H-D-Arg-Thz-NH_2 (**7**), **H)** H-Gln-Thz-NH_2 (**8**). In each frame the upper spectrum shows results of a 3 h reaction of substrate with PznL, the lower spectrum shows the same reaction carried out without PznL. Asterisks correspond to molecules in the samples unrelated to the substrate at hand (impurities in the starting material or fragmentation products). The low intensity of the peaks corresponding to **8** is due to reduced ionization efficiency.

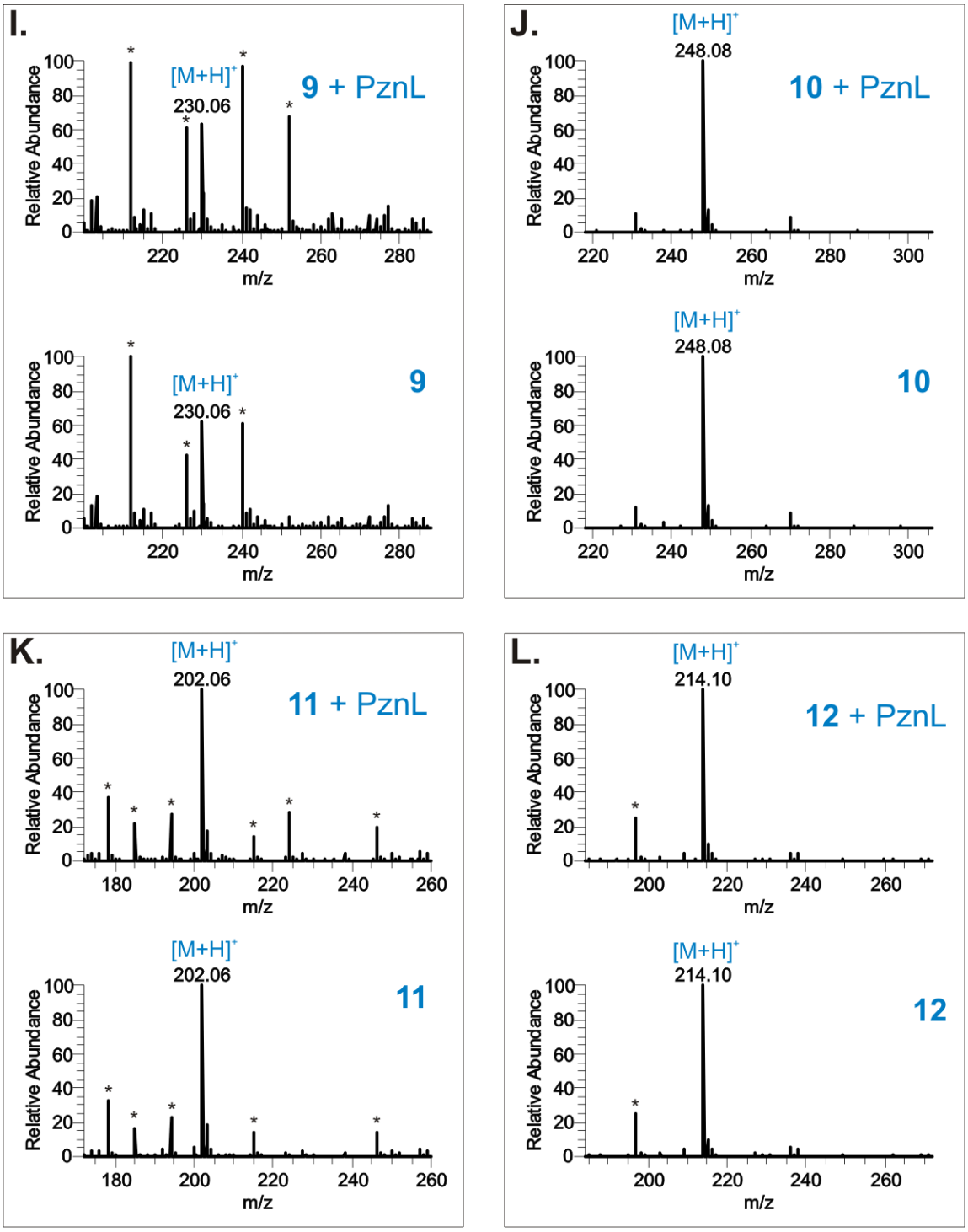


Figure S3 (continuation) I-L. LC-MS spectra of His_6-SUMO-PznL mediated *in vitro* reaction of substrates: **I)** H-Glu-Thz-NH_2 (**9**), **J)** H-Phe-Thz-NH_2 (**10**), **K)** H-Thr-Thz-NH_2 (**11**), **L)** H-Leu-Thz-NH_2 (**12**). In each frame the upper spectrum shows results of a 3 h reaction of substrate with PznL, the lower spectrum shows the same reaction carried out without PznL. Asterisks correspond to molecules in the samples unrelated to the substrate at hand (impurities in the starting material or fragmentation products). The low intensity of the peaks corresponding to **9** is due to reduced ionization efficiency.

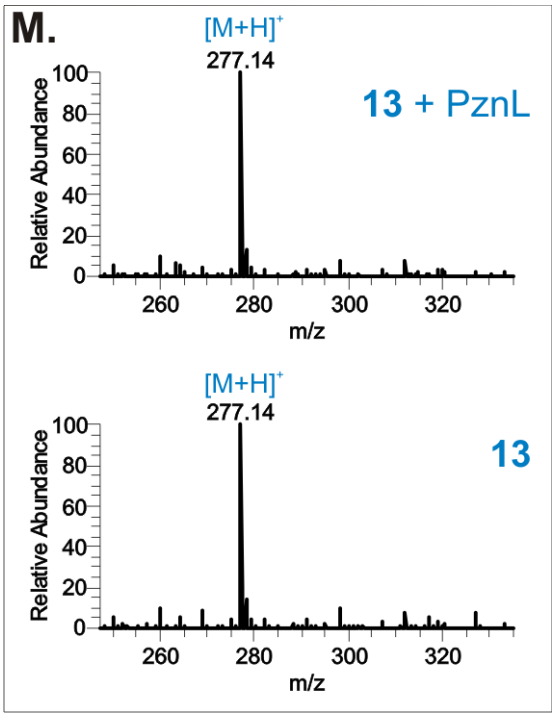


Figure S3 (continuation) M. LC-MS spectrum of His_6-SUMO-PznL mediated *in vitro* processing of substrate **M)** H-Arg-Cys-NH_2 (**13**). In the frame the upper spectrum shows result of a 3 h reaction of substrate with PznL, the lower spectrum shows the same reaction carried out without PznL.

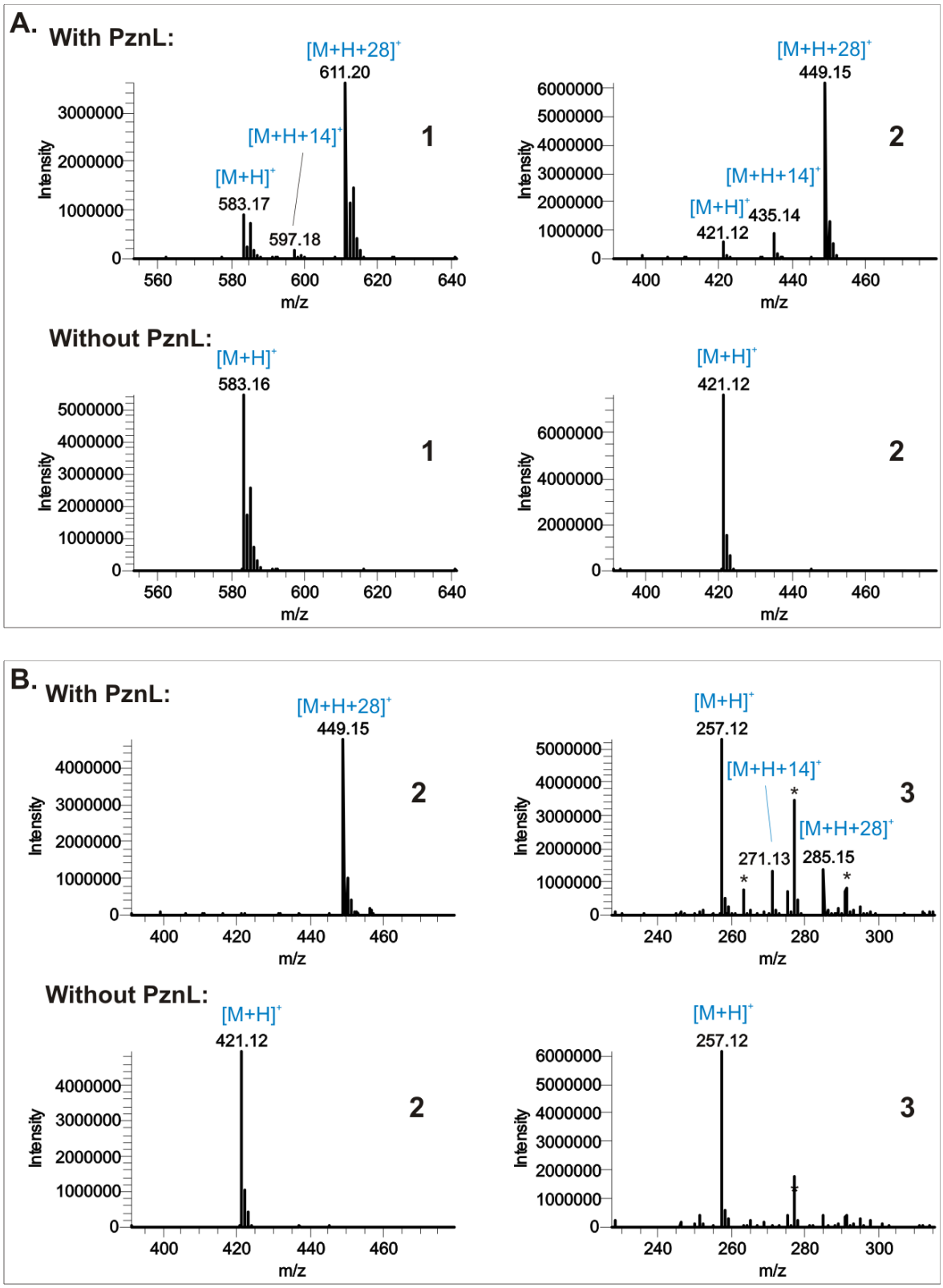


Figure S4 A-B. LC-MS spectra of PznL competition experiments. A) Competition experiments carried out with **1** and **2**. B) Competition experiments carried out with **2** and **3**. Extracted ion chromatograms (EIC) are shown separately due to differences in retention times of all tested substrates. The intensity of the peaks is indicated at the y-axis. Asterisks correspond to molecules in the samples unrelated to the substrate at hand (impurities in the starting material or fragmentation products).

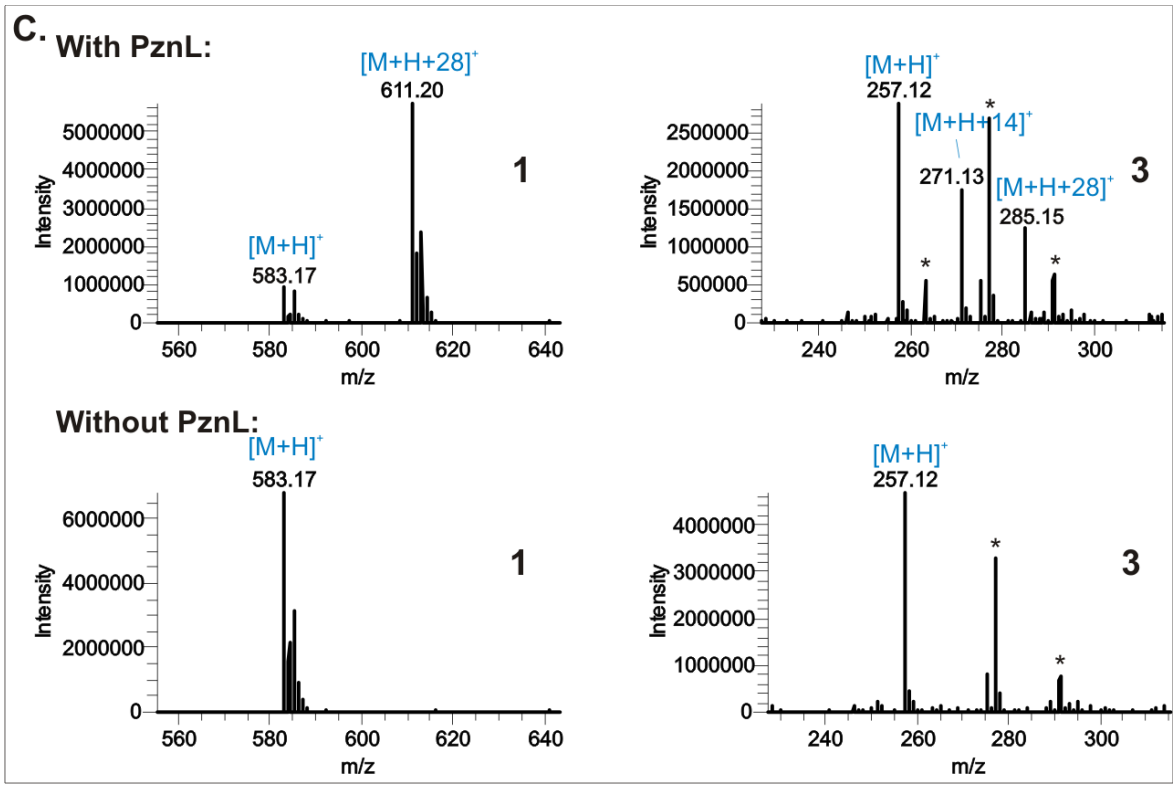


Figure S4 (continuation) C. LC-MS spectra of PznL competition experiments. C) Competition experiments carried out with **1** and **3**. Extracted ion chromatograms (EIC) are shown separately due to differences in retention times of all tested substrates. The intensity of the peaks is indicated at the y-axis. Asterisks correspond to molecules in the samples unrelated to the substrate at hand (impurities in the starting material or fragmentation products).

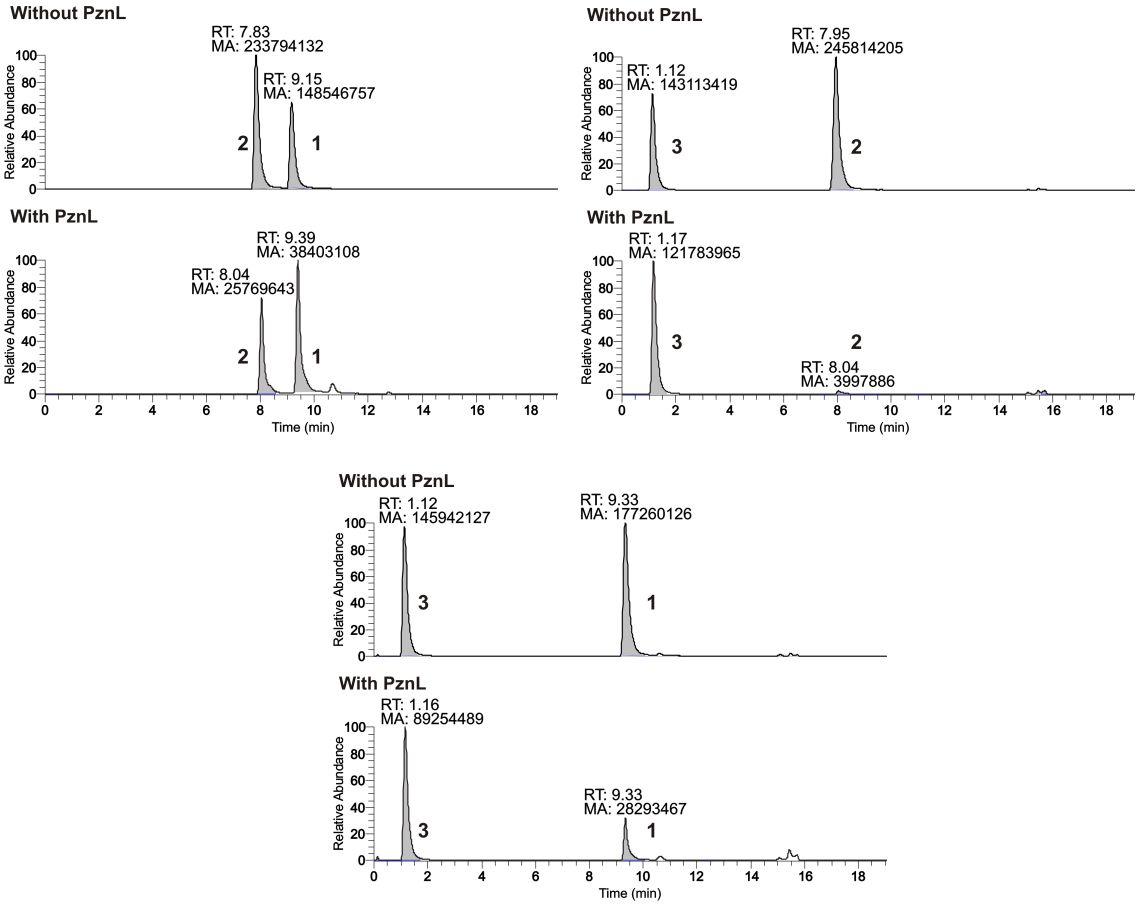


Figure S5. Extracted ion chromatograms (XIC) of PznL competition experiments used for calculations of substrate depletion after treatment with plantazolicin methyltransferase. XIC are shown for following substrate m/z ranges: **1**: 583.16 – 584.16, **2**: 421.12 – 422.12, **3**: 257.11 – 258.11. RT – retention time, MA: area under the peak. Reaction conditions see Table S2.

Table S2. Amount of substrate left after methylation of substrate during competition experiments in which equimolar mixtures of two substrates (30 µM each) and PznL (4.5 µM) were incubated with limiting concentration of *S*-adenosyl-L-methionine (60 µM) for 60 min. The %-values were calculated as a ratio of area under the peak corresponding to substrate in the presence of enzyme to the area under the peak of substrate peak in the negative control (without enzyme).

Competitive substrates	Amount of substrate after methylation [%]		
	1	**2**	**3**
1, **2**	26	11	-
2, **3**	-	2	85
1, **3**	16	-	61

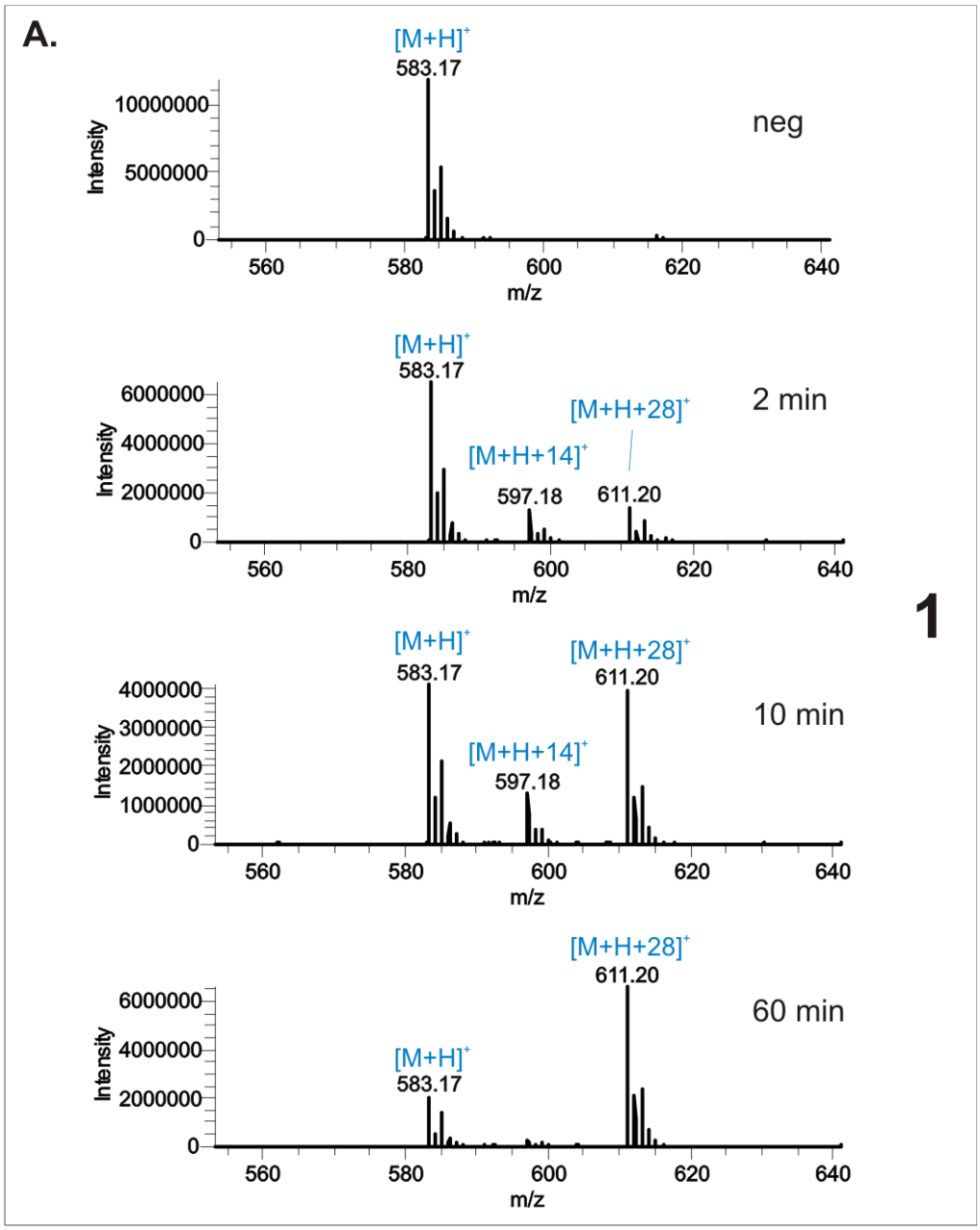


Figure S6 A. MS spectra of time-dependant reactions of PznL with **1** in equimolar concentration together with SAM and substrate (60 µM each).

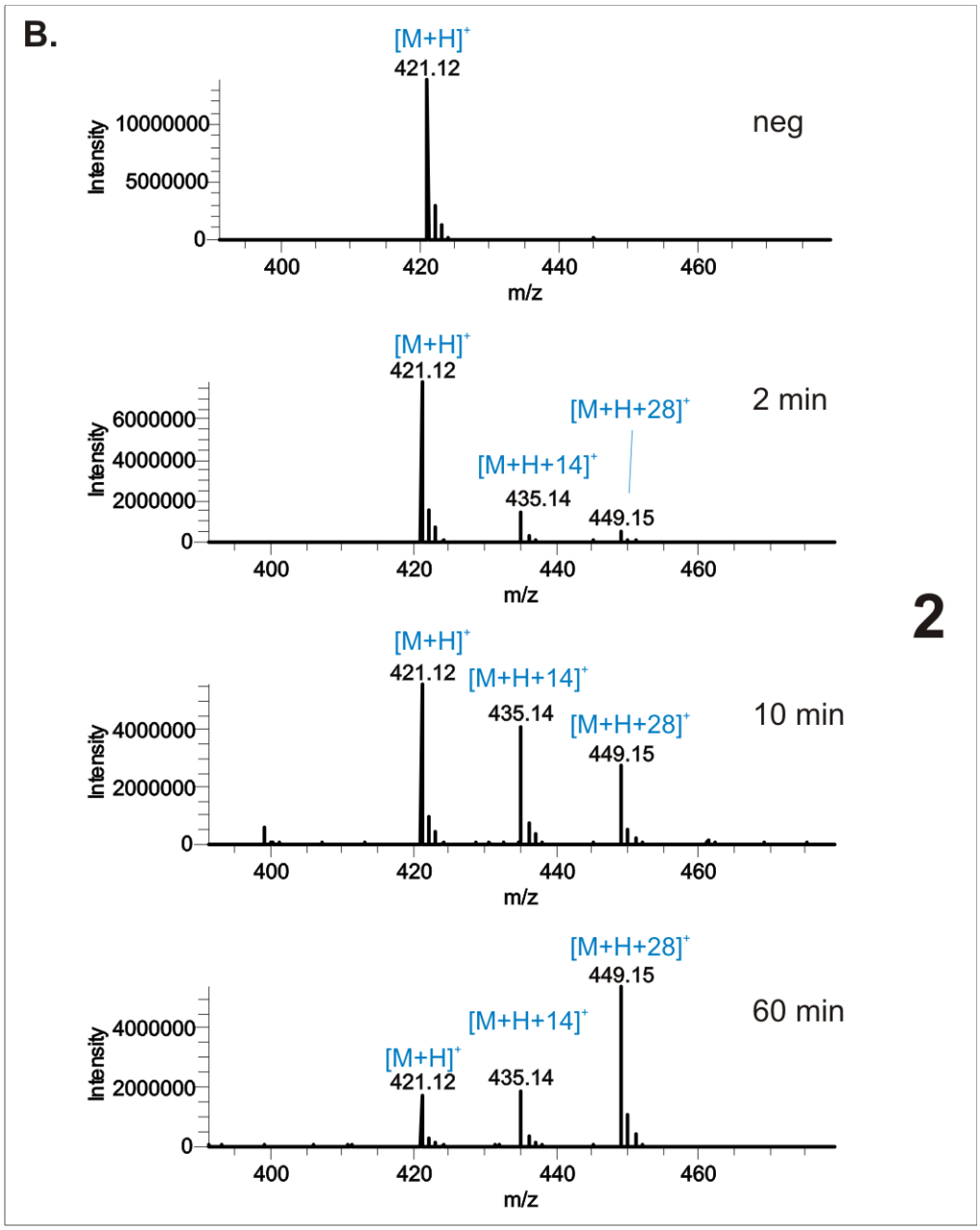


Figure S6 (continuation) B. MS spectra of time-dependant reactions of PznL with **2** in equimolar concentration together with SAM and substrate (60 μM each).

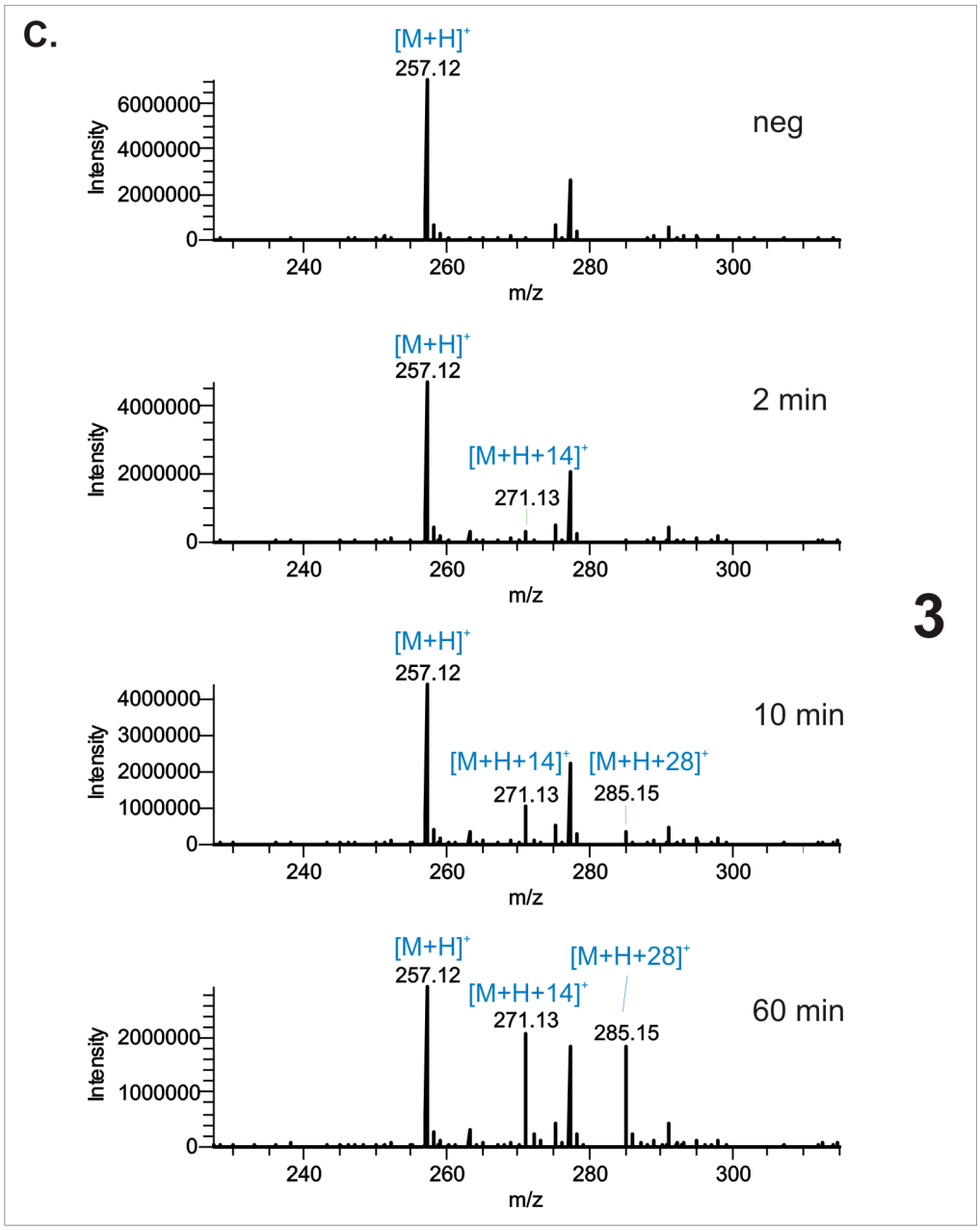


Figure S6 (continuation) C. MS spectra of time dependant reactions of PznL with **3** in equimolar concentration together with SAM and substrate (60 μM each).

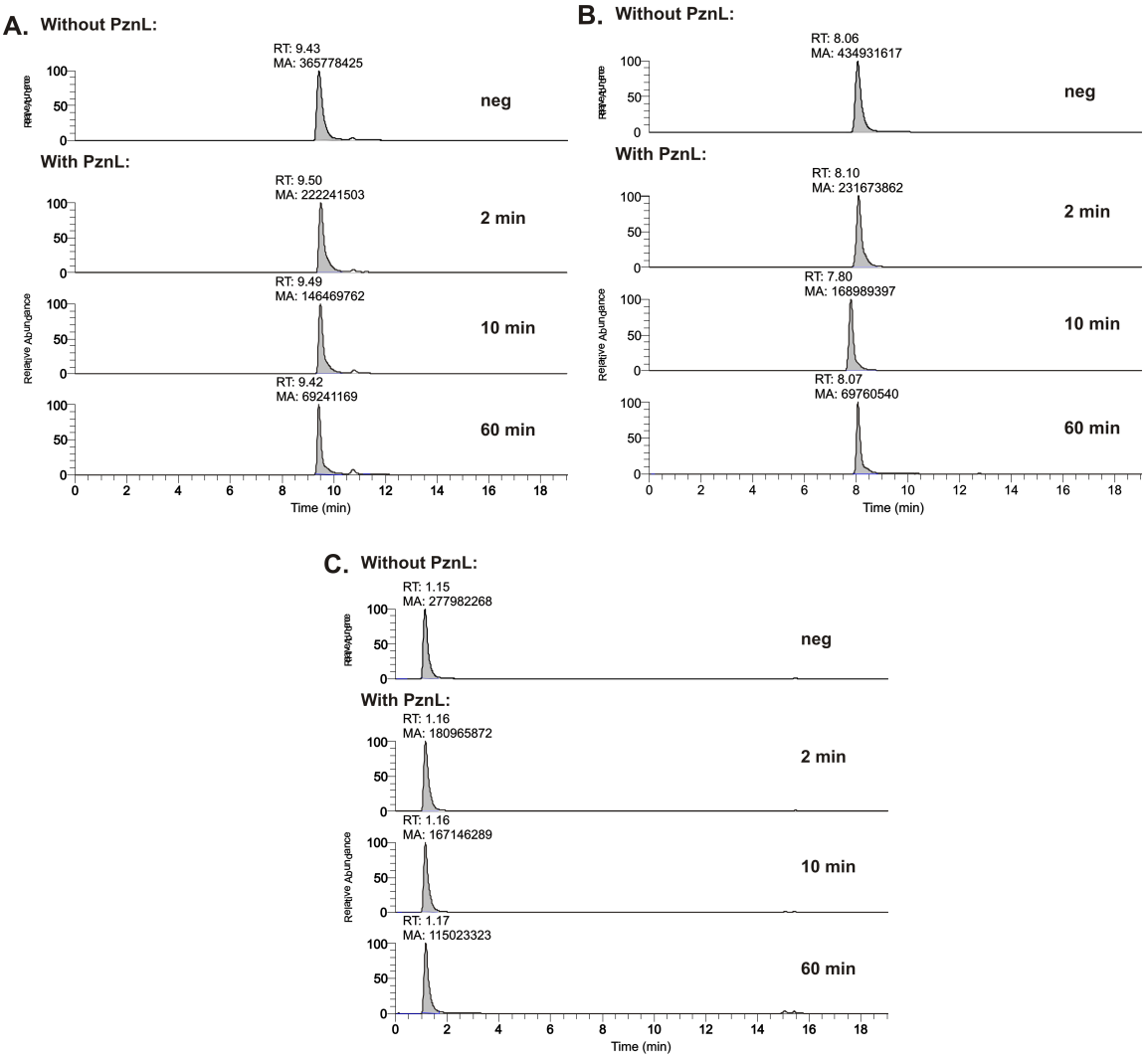


Figure S7. Extracted ion chromatograms (XIC) of PznL time dependant reactions. XIC are shown for the following substrate *m/z* ranges: **1**: 583.16 – 584.16 (A), **2**: 421.12 – 422.12 (B), **3**: 257.11 – 258.11 (C). RT – retention time, MA: area under the peak.

Table S3. Amount of substrate left after methylation of substrate during time dependant reactions. Substrate **1**, **2** and **3** (60 µM) were incubated with PznL (4.5 µM) and S-adenosyl-L-methionine (60 µM) for 2, 10 or 60 min. The %-values were calculated as a ratio of area under the peak corresponding to substrate in the presence of enzyme to the area under the peak of substrate peak in the negative control (without enzyme).

Time [min]	Amount of substrate after methylation [%]		
	1	**2**	**3**
0 (neg)	100	100	100
2	61	53	65
10	40	39	60
60	19	16	41

15. Manual docking of minimal substrate in PznL

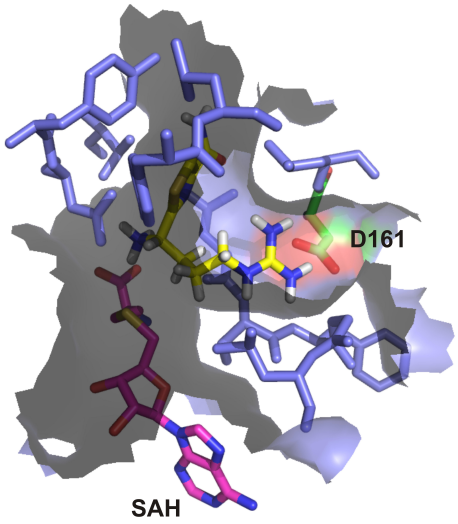


Figure S8. Manual docking of H-Arg-Thz-NH_2 in the putative substrate-binding pocket using previously published crystal structure.[2] As shown in this model, the change of the character of the N-terminal side chain residue might lead to disorder the putative stabilizing forces of D161 and improper positioning toward *S*-adenosyl-L-homocysteine (SAH), which inhibits methylation. Depicted are PznL residues (blue colour) forming the putative substrate-binding pocket, D161 of PznL (green colour), *S*-adenosyl-L-homocysteine (SAH) (pink colour) and the minimal substrate H-Arg-Thz-NH_2 (yellow colour). This figure was generated using computer programs: PyMol, WinCoot, The GlycoBioChem PRODRG2 Server, and CorelDraw.

16. Supporting Information references

1. S. Banala, P. Ensle, and R. D. Süssmuth, *Angew. Chem. Int. Ed.*, 2013, **52**, 9518-9523.
2. J. Lee, Y. Hao, P. M. Blair, J. O. Melby, V. Agarwal, B. J. Burkhart, S. K. Nair and D. A. Mitchell, *Proc. Natl. Acad. Sci. U. S. A*, 2013, **110**, 12954-12959.