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Electronic Supplementary Information

Polythiazole Linkers as Functional Rigid Connectors. A new RGD Cyclopeptide with Enhanced Integrin Selectivity

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Supporting Information

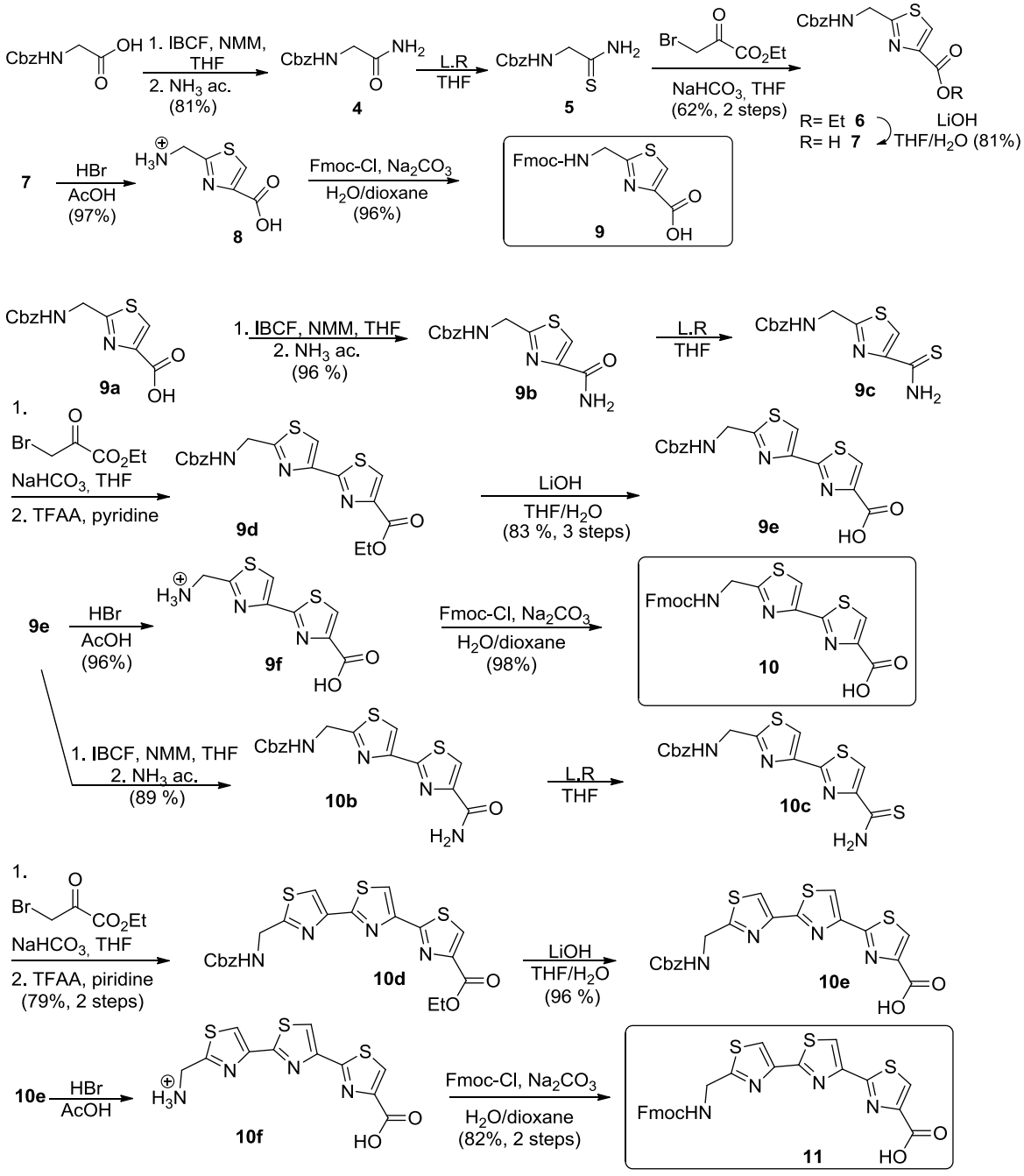
1. General experimental information.

Solid-phase synthesis: SPPS were performed in polypropylene syringes (10-50 mL), each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. The Fmoc group was removed with piperidine (2:8, v/v) (2x10 min). Washings after deprotection and coupling were performed with DMF (3x) and DCM (3x) using 10 mL/g resin each time.

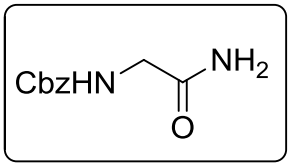
Peptide analysis. Peptides were analyzed by RP-HPLC and RP-HPLC-MS. HPLC reversed-phase columns C_{18} 4,6 x 150 mm, 5 µm (Column A) and Symmetry 300 C_{18} 30 x 150 mm, 5 µm (Column B) were used. Analytical HPLC was performed on an instrument comprising two solvent delivery pumps, automatic injector, dual wavelength detector and system controller (Breeze v.3.20). UV detection was at 220 and 254 nm, using linear gradients of MeCN (0,036% TFA) in H_2O (0,045% TFA). HPLC-MS was performed on an instrument comprising two solvent delivery pumps, automatic injector, dual wavelength detector, ZQ mass detector and system controller (Masslynx v.3.20). UV detection was at 220 and 254 nm, using linear gradients of MeCN (0,05% HCOOH) in H_2O (0,1% HCOOH). MALDI-TOF analysis of peptide samples was performed using ACH matrix.

2. Experimental procedures and characterization data for compounds.

Synthesis of the thiazole-based amino acids.



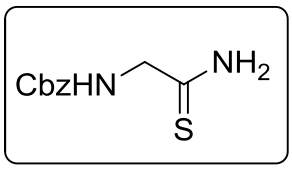
Benzyloxycarbonyl glycine amide (4)



Z-Gly-OH (10.0 g, 47.8 mmol) was dissolved in anhydrous THF (60 mL) at -20 ºC under N_2. Isobutyl chloroformate (6.5 mL, 50.2 mmol) and *N*-methylmorpholine (5.5 mL, 50.2 mmol) was added dropwise for 2 min at -20 ºC and the solution was stirred at 0 ºC for 15 min. Then aqueous ammonia (15.1 mL, 239.0 mmol) was added and the solution was stirred for 1 h. After that, the solvents were eliminated under vacuum and the residue was re-dissolved in 150 mL of ethyl acetate. The organic phase was washed with $NaHCO_{3sat}$ (4 x 20 mL), dried over Na_2SO_4, filtered and concentrated under vacuum to afford **4** (8.07 g, 81%) as a white solid. The product was used for the next step without purification. This product was identical to that reported in the literature.[1]

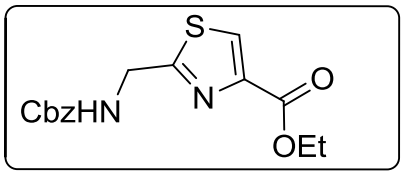
^{1}H-NMR (DMSO-d_6, 400 MHz): δ 7.43 – 7.26 (m, 6H), 6.99 (s, 1H), 5.01 (s, 2H), 3.54 (d, *J* = 5.7 Hz, 2H) ppm.

Benzyloxycarbonyl glycine thioamide (5)



To a mixture of **4** (8.07 g, 38.76 mmol) and Lawesson reagent (15.67 g, 38.76 mmol) was added 50 mL of anhydrous THF under N_2. After 18 h, 50 mL of $NaHCO_{3sat}$ was added and the mixture was stirred for 30 min. After that, the product was extracted with ethyl acetate (4 x 20 mL), the organic layers were collected, dried over N_2SO_4, filtered and concentrated under vacuum to obtain **5** as a white solid. The product was used for the next step without purification.

Ethyl 2-(*N*-benzyloxycarbonylaminomethyl)-4-thiazolecarboxylate (6)

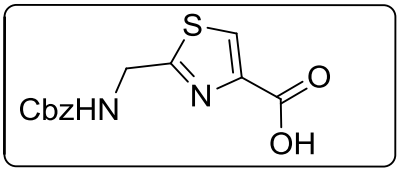


A mixture of **5** (38.76 mmol) and $NaHCO_3$ (9.77 g, 116.28 mmol) was suspended in 50 mL of anhydrous THF under N_2. Ethyl bromopyruvate (5.72 mL, 38.76 mmol) was added. After 18 h, the THF was removed under reduced pressure, the residue was re-dissolved in ethyl acetate and the organic phase was washed with H_2O (2 x 15 mL), brine (1 x 15 mL), dried over Na_2SO_4, filtered and concentrated under vacuum to obtain **6** as an oil. The crude was purified by flash

chromatography with hexane/ethyl acetate (1:1) to (3:7) to afford **6** (7.70 g, 62%, two steps) as a pale yellow solid.

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.41 (s, 1H), 8.24 (t, *J* = 6.0 Hz, 1H), 7.51 – 7.10 (m, 5H), 5.05 (d, *J* = 17.1 Hz, 2H), 4.48 (d, *J* = 6.2 Hz, 2H), 4.27 (q, *J* = 7.1 Hz, 2H), 1.28 (t, *J* = 7.1 Hz, 3H) ppm.

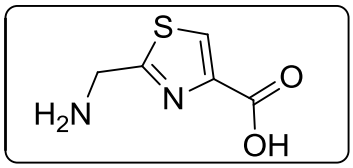
2-(*N*-Benzyloxycarbonylaminomethyl)-4-thiazolecarboxylic acid (7)



Compound **6** (7.70 g, 24.04 mmol) was dissolved in 50 mL of a mixture of THF/H_2O (4:1) and LiOH (1.73 g, 72.12 mmol) was added. The mixture was stirred for 3 h. After that, 30 mL of H_2O were added and the aqueous phase was washed with Et_2O (2 x 5 mL). The aqueous phase was acidified with HCl 6N and extracted with ethyl acetate to obtain **7** (5.73 g, 81%) as solid. The product was used for the next step without purification.

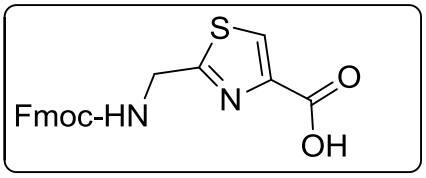
^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.34 (s, 1H), 8.23 (t, *J* = 6.1, 1H), 7.54 – 7.11 (m, 5H), 5.07 (s, 2H), 4.47 (d, *J* = 6.2, 2H) ppm.
^{13}C-NMR (DMSO-d_6, 100 MHz): δ 172.06, 163.37, 157.77, 148.21, 138.22, 130.01, 129.76, 129.26, 129.11, 67.14, 43.62 ppm.

2-(Aminomethyl)-4-thiazolecarboxylic acid (8)



Compound **7** (2.00 g, 6.84 mmol) was suspended in 10 mL of HBr (33% in acetic acid). The mixture was stirred overnight. The precipitate was filtered through a sinter funnel, washed with Et_2O (4 x 20 mL) and dried under vacuum to obtain **8** (1.50 g, 97%). The product was used for the next step without purification. This product was identical to that reported in the literature.

2-(*N*-9*H*-Fluoren-9-ylmethoxycarbonylaminomethyl)-4-thiazolecarboxylic acid (9)

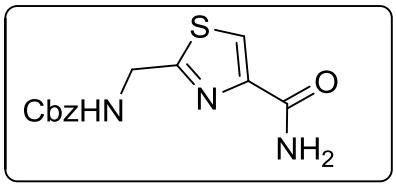


Compound **8** (1.50 g, 6.62 mmol) was dissolved in 20 mL of H_2O and the pH was adjusted to 8-9 with a solution of Na_2CO_{3sat}. The Fmoc-Cl (1.92 g, 7.18 mmol) was dissolved in 20 mL of dry 1,4-dioxane and added dropwise for 2 h. The pH was controlled during the addition and re-adjusted to 8-9 with a solution of Na_2CO_{3sat}. The mixture was stirred overnight. The product was filtered through a sinter funnel, washed with HCl 1N, re-filtered to obtain **9** (2.42 g, 96%) as a white solid. The product was used without purification.

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.32 (s, 1H), 8.27 (t, *J* = 5.9, 1H), 7.87 (d, *J* = 7.5, 2H), 7.68 (d, *J* = 7.3, 2H), 7.40 (t, *J* = 7.4, 2H), 7.31 (t, *J* = 7.4, 2H), 4.44 (d, *J* = 6.0, 2H), 4.37 (d, *J* = 6.8, 2H), 4.23 (t, *J* = 6.7, 1H) ppm.

^{13}C-NMR (DMSO-d_6, 100 MHz): δ 171.37, 162.86, 157.09, 150.68, 137.53, 129.10, 128.62, 128.46, 124.83, 66.47, 42.97 ppm.

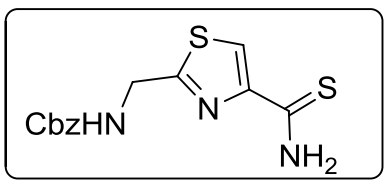
2-(*N*-Benzyloxycarbonylaminomethyl)-4-thiazolecarboxamide (9a)



Compound **9a** was prepared under the same conditions of compound **4**, with **7** (3.71 mg, 12.69 mmol), isobutyl chloroformate (1.78 mL, 13.32 mmol), *N*-methylmorpholine (1.46 mL, 13.32 mmol), aqueous ammonia (4.01 mL, 63.45 mmol) in 30 mL of dry THF to obtain **9a** (3.54 g, 96%). The crude was used without any purification.

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.23 (t, *J* = 6.0 Hz, 1H), 8.13 (s, 1H), 7.64 (s, 1H), 7.52 (s, 1H), 7.38 – 7.31 (m, 5H), 5.08 (s, 2H), 4.48 (d, *J* = 6.1 Hz, 2H) ppm.

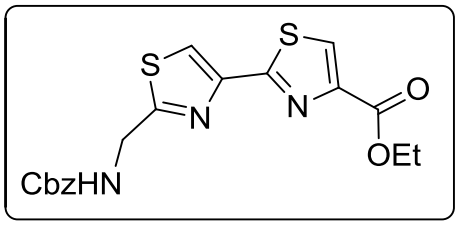
2-(*N*-Benzyloxycarbonylaminomethyl)-4-thiazolecarboxathioamide (9b)



Compound **9b** was prepared under the same conditions of compound **5**, with **9a** (3.54 g, 12.16 mmol), Lawesson reagent (4.91 g, 12.16 mmol) in 30 mL of dry THF to obtain **9b** as a yellow solid. The product was used without any purification.

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 9.95 (s, 1H), 9.42 (s, 1H), 8.34 (s, 1H), 8.27 (t, *J* = 6.0 Hz, 1H), 7.42 – 7.28 (m, 5H), 5.08 (s, 2H), 4.48 (d, *J* = 6.1 Hz, 2H) ppm.
^{13}C-NMR (DMSO-d_6, 100 MHz): δ 189.73, 171.26, 157.10, 154.02, 137.50, 129.10, 128.63, 128.47, 127.87, 66.50, 43.06 ppm.

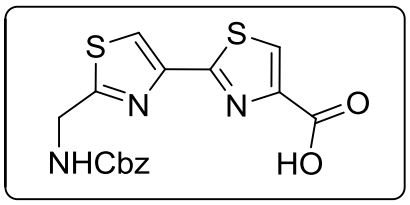
Ethyl 2'-(*N*-benzyloxycarbonylaminomethyl)-2,4'-bisthiazole-4-carboxylate (9c)



A mixture of **9b** (12.16 mmol) and $NaHCO_3$ (2.04 g, 24.32 mmol) was suspended in 30 mL of anhydrous THF under N_2. Ethyl bromopyruvate (1.97 mL, 13.38 mmol) was added. After 18 h, the solution was filtered and the THF was removed under reduce pressure. The residue was re-dissolved in 60 mL of dry THF at -15 ºC under N_2. TFAA (6.93 mL, 49.86 mmol) and Pyr (3.62 mL, 107.01 mmol) were added dropwise to the solution. The mixture was stirred at -15 ºC for 2 h and then 1 h at room temperature. Then, H_2O was added and the product was extracted with ethyl acetate (4 x 10 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduce pressure to obtain **9c** as a white solid. The product was used without any purification.

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.54 (s, 1H), 8.27 (s, 1H), 7.60 – 7.01 (m, 5H), 5.08 (s, 2H), 4.53 (d, *J* = 6.1, 2H), 4.32 (q, *J* = 7.1, 2H), 1.31 (t, *J* = 7.1, 3H) ppm.
^{13}C-NMR (DMSO-d_6, 100 MHz): δ 173.44, 163.70, 161.97, 157.74, 148.39, 148.29, 130.75, 129.71, 129.22, 129.05, 119.62, 67.11, 62.14, 43.53, 15.52 ppm.

2'-(*N*-Benzyloxycarbonylamino)methyl)-2,4'-bisthiazole-4-carboxylic acid (9d)

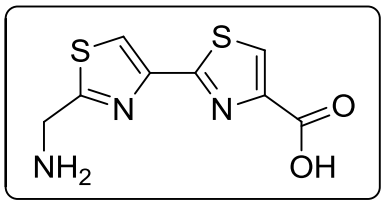


Compound **9c** (12.16 mmol) was dissolved in 60 mL of a mixture of EtOH/H_2O (3:1) and LiOH (559 mg, 24.32 mmol) was added. The mixture was stirred for 18 h. After that, the precipitate was filtered through a sinter funnel. The filtrate was concentrated under reduce pressure, re-dissolved in cold EtOH and re-filtered to obtain a white solid. The precipitate was suspended in HCl 1N, filtered through a sinter funnel and dried over reduce pressure to obtain pure **9d** (3.80 g, 83% three steps). The product was used for the next step without purification.

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.47 (s, 1H), 8.30 (t, *J* = 6.1, 1H), 8.23 (s, 1H), 7.34 (dt, *J* = 8.6, 4.1, 5H), 5.08 (s, 2H), 4.53 (d, *J* = 6.1, 2H) ppm.

^{13}C-NMR (DMSO-d_6, 100 MHz): δ 172.80, 162.76, 162.71, 157.12, 148.80, 147.96, 139.87, 137.54, 129.69, 129.10, 128.61, 128.44, 125.59, 118.74, 66.48, 42.90 ppm.

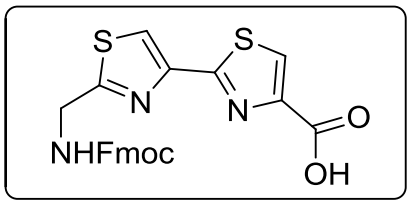
2'-(Aminomethyl)-2,4'-bisthiazole-4-carboxylic acid (9e)



The compound was prepared under the same conditions of compound **8**, with **9d** (2.00 g, 5.33 mmol) and 10 mL of 33% HBr in acetic acid to obtain **9e** (1.64 g, 96%).

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.55 (s, 2H), 8.50 (s, 1H), 8.41 (s, 1H), 4.55 – 4.49 (m, 2H) ppm.

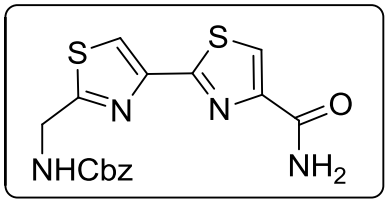
2'-(*N*-9*H*-Fluoren-9-ylmethoxycarbonylaminomethyl)-2,4'-bisthiazole-4-carboxylic acid (10)



The compound was prepared under the same conditions of compound **9**, with **9e** (1.63 g, 5.07 mmol) and Fmoc-Cl (1.42 g, 5.33 mmol) to obtain **10** (2.31 g, 98%) as a white solid. The product was used without any purification.

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.46 (s, 1H), 8.32 (t, *J* = 6.1 Hz, 1H), 8.22 (s, 1H), 7.88 (d, *J* = 7.3 Hz, 2H), 7.70 (d, *J* = 7.4 Hz, 2H), 7.41 (t, *J* = 7.3 Hz, 2H), 7.32 (t, *J* = 7.3 Hz, 2H), 4.51 (d, *J* = 5.9 Hz, 2H), 4.39 (d, *J* = 6.8 Hz, 2H), 4.25 (t, *J* = 6.6 Hz, 1H).

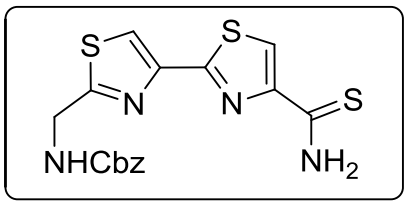
2'-(*N*-Benzyloxycarbonylaminomethyl)-2,4'-bisthiazole-4-carboxamide (10a).



The compound was prepared under the same conditions of compound **4**, with **9d** (1.80 g, 4.79 mmol), isobutyl chloroformate (671 μL, 5.03 mmol), *N*-methylmorpholine (553 μL, 5.03 mmol), aqueous ammonia (1.51 mL, 23.95 mmol) in 150 mL of dry THF to obtain **10a** (1.61 g, 89%). The product was used for the next step without any purification.

^{1}H-NMR (200 MHz, DMSO-d_6): δ 8.25 (s, 1H), 8.20 (s, 1H), 7.78 (s, 1H), 7.64 (s, 1H), 7.36 (m, 5H), 5.09 (s, 2H), 4.53 (d, *s* = 6.1, 2H) ppm.

2'-(*N*-Benzyloxycarbonylaminomethyl)-2,4'-bisthiazole-4-carboxathioamide (10b).

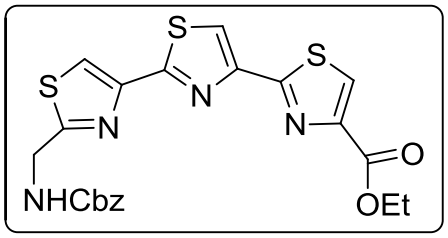


The compound was prepared under the same condition of compound **5**, with **10a** (1.60 g, 4.29 mmol) and Lawesson reagent (1.73 g, 4.29 mmol) in 70 mL of dry THF to obtain **10b**. The product was used for the next step without any purification.

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 10.06 (s, 1H), 9.54 (s, 1H), 8.44 (s, 1H), 8.27 (t, *J* = 6.0, 1H), 8.23 (s, 1H), 7.48 – 7.13 (m, 5H), 5.09 (s, 2H), 4.54 (d, *J* = 6.1, 2H) ppm.

^{13}C-NMR (DMSO-d_6, 100 MHz): δ 189.66, 172.90, 161.82, 157.13, 154.99, 148.14, 137.54, 129.09, 128.59, 128.42, 128.08, 118.80, 66.49, 42.93 ppm.

Ethyl 2''-(*N*-benzyloxycarbonylaminomethyl)-2,4'-2',4''-trithiazole-4-carboxylate (10c).

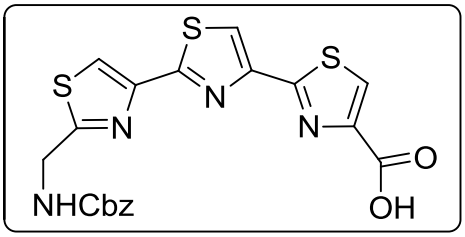


A mixture of **10b** (4.29 mmol) and $NaHCO_3$ (719 mg, 8.57 mmol) was suspended in 30 mL of anhydrous THF under N_2. Ethyl bromopyruvate (696 μL, 4.71 mmol) was added. After 18 h, the solution was filtered and the THF was removed under reduce pressure. The residue was re-dissolved in 30 mL of dry THF at -15 ºC under N_2. TFAA (2.44 μL, 17.58 mmol) and Pyr (3.04 μL, 37.73 mmol) were added

dropwise to the solution. The mixture was stirred at -15 ºC for 2 h and them 1 h at room temperature. Then, the solvent was removed under reduce pressure, the residue was suspended in 15 mL of cold EtOH and filtered through a sinter funnel to obtain **10c** (1.66 g, 79% of two steps) as a white solid. The product was used without any purification.

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.58 (s, 1H), 8.41 (s, 1H), 8.30 (s, 2H), 7.42 – 7.31 (m, 5H), 5.09 (s, 2H), 4.55 (d, *J* = 6.1, 2H), 4.34 (q, *J* = 7.1, 2H), 1.32 (t, *J* = 7.1, 3H) ppm.

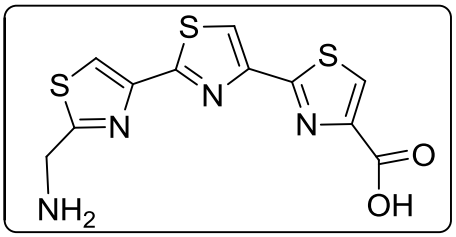
2''-(*N*-Benzyloxycarbonylamino)methyl)-2,4'-2',4''-trithiazole-4-carboxylic acid (10d).



The compound was prepared under the same conditions of compound **7**, with **10c** (1.66 g, 4.31 mmol), LiOH (2.98 mg, 12.94 mmol) in 30 mL of EtOH/H_2O (3:1) to obtain **10d** (1.76 g, 96%). The product was used without any purification.

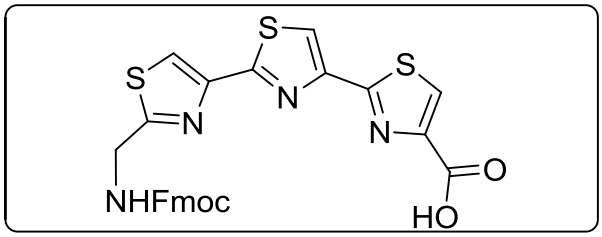
^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.65 (s, 1H), 8.44 (s, 1H), 8.32 (s, 1H), 8.22 (s, 1H), 7.37 – 7.09 (m, 5H), 4.99 (s, 2H), 4.43 (d, *J* = 4.7 Hz, 2H) ppm.

2''-(Aminomethyl)-2,4'-2'-4''-trithiazole-4-carboxylic acid (10e)



The compound was prepared under the same conditions of compound **8**, with **10d** (1.76 g, 4.08 mmol) and 10 mL of 33% HBr in acetic acid to obtain **10e**. The product was used for the next step without any purification.

2'-(*N*-9*H*-Fluoren-9-ylmethoxycarbonylaminomethyl)- 2,4'-2'-4''-trithiazole-4-carboxylic acid (11)



The compound was prepared under the same condition of compound **9**, with **10e** (4.31 mmol) and Fmoc-Cl (1.21 g, 4.53 mmol) to obtain **11** (1.94 g, 82%) as a white solid. The product was used without any purification.

^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.50 (s, 1H), 8.36 (m, 2H), 8.29 (s, 1H), 7.88 (d, J = 7.5, 2H), 7.70 (d, *J* = 7.3, 2H), 7.41 (t, *J* = 7.4, 2H), 7.32 (t, *J* = 7.3, 2H), 4.52 (d, *J* = 5.8, 2H), 4.40 (d, *J* = 6.8, 2H), 4.25 (t, *J* = 6.6, 1H) ppm.

^{13}C-NMR (DMSO-d_6, 100 MHz): δ 173.00, 163.57, 162.68, 162.65, 157.09, 149.25, 148.85, 147.71, 144.40, 141.44, 129.88, 128.34, 127.79, 125.81, 120.82, 119.39, 118.98, 66.46, 47.38, 42.88 ppm.

General procedure A for the synthesis of the linear peptides.

The synthesis of the linear peptides was performed using Fmoc-based SPPS with 2-chlorotrityl chloride resin (2,0 g, 3,2 mmol).

Fmoc-NH-Asp(tBu)-(Thiazole)$_n$-Arg(Pbf)-Gly-OH

Resin loading: Fmoc-Gly-OH (594 mg, 2.0 mmol) was attached to the resin with DIPEA in DCM at room temperature for 1.5 h. The remaining trityl groups were capped adding 0.5 mL of MeOH for 30 min. After that, the resin was filtered and washed with DCM (2x), DMF (2x). The loading of the resin was determined by titration the Fmoc group.[2] The final loading was 2.0 mmol/g. The Fmoc group was eliminated by treatment with 20% piperidine in DMF (2 x 10 min). The resin was washed with DMF (3x) and DCM (3x).

Peptide coupling: Fmoc-Arg(Pbf)-OH (5.19 g, 8.0 mmol), DIPCDI (1.23 mL, 8.0 mmol) and HOBt (1,08 g, 8,0 mmol) were dissolved in DMF and added to the resin for 1.5 h. The end of the coupling was monitored by ninhydrin test (free amine group). The resin was filtered and washed with DMF (3x) and DCM (3x). The Fmoc group was eliminated with 20 % piperidine in DMF (2x10 min). The coupling of the thiazole module was carried out with **9** (1.14 g, 3.0 mmol), PyAOP (1.56 g, 3.0 mmol) and DIPEA (1.02 mL, 6.0 mmol) in DMF for 1.5 h. The completion of the reaction was checked with the ninhydrin test.
Finally the deprotection of the amine and coupling of the Fmoc-Asp(^{t}Bu)-OH were carried out under the same conditions of second amino acid.

Peptide cleavage: The resin bound peptide was treated with 2% TFA in DCM (6 x 30 sec.) The resin was washed with DCM and the combined solution was evaporated under vacuum with Et_2O several times, furnishing the linear peptide **12** as a white solid. The peptide was used for the next step without purification.

Fmoc-NH-Asp(tBu)-Thiazole-Arg(Pbf)-Gly-OH (12)

The linear peptide **12** was prepared following the procedure A, from thiazole amino acid **9**.

HPLC (gradient 20 to 80% of CH_3CN in 15 min): t_R= 8.33 min.
HPLC-MS (ES(+)): m/z 795.3.

Fmoc-NH-Asp(tBu)-Bisthiazole-Arg(Pbf)-Gly-OH (13)

The linear peptide **13** was prepared following the procedure A, from bisthiazole amino acid **10** in place of **9**.

HPLC (gradient 0 to 100% CH_3CN in 15 min.): t_R = 10.34 min.
HPLC-MS (ES(+)): m/z 877.81.

Fmoc-NH-Asp(tBu)-Tris thiazole-Arg(Pbf)-Gly-OH (14)

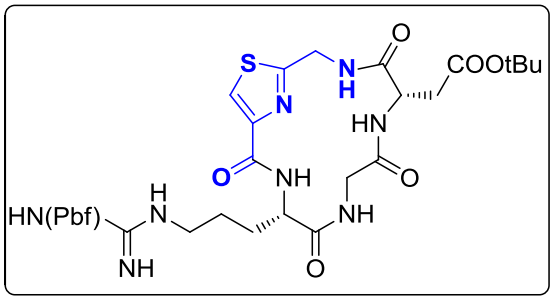
The linear peptide **14** was prepared following the procedure A, from tristhiazole amino acid **11** in place of **9**.

HPLC (gradient 20 to 80% of CH_3CN in 15 min.): t_R = 7.60 min.
HPLC-MS (ES(+)): m/z 961.23.

General procedure B for the cyclization.

Cyclization: The linear peptide (0,25 mmol) was dissolved in anhydrous DMF (50 mL, 5 mM), PyAOP (262 mg, 0,50 mmol) and DIPEA (213 μL, 1,25 mmol) were added. The reaction was monitored by HPLC. Once the reaction was finished, the DMF was evaporated under vacuum. The crude was dissolved in EtOAc and the solution was washed with NH_4Cl_{sat} and $Na_2CO_{3\ sat.}$ The organic layer was collected, dried over Na_2SO_4, filtered and concentrated under vacuum. The peptide was purified by flash chromatography furnishing the desired protected cyclized peptide.

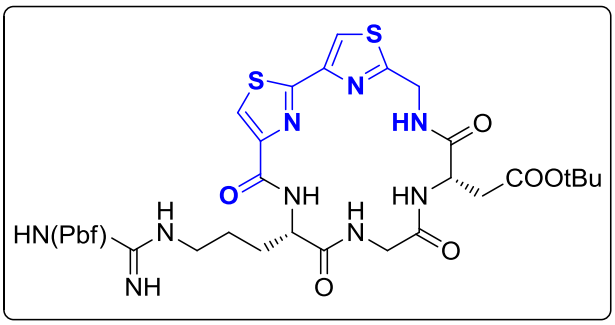
***Cyclo*-[Arg(Pbf)-Gly-Asp(tBu)-Thz-] (15)**



Cyclopeptide **15** was prepared following the procedure B, from **12** (200 mg, 0,25 mmol). The residue was absorbed onto silica and then subjected to flash chromatography (SiO_2, $CHCl_3$:MeOH, 8:2), obtaining the protected cyclic peptide **15** as a white solid.

HPLC (gradient 40 to 90% of CH_3CN in 15 min): t_R= 8.86 min.
HPLC-MS (ES(+)): m/z 778.2.

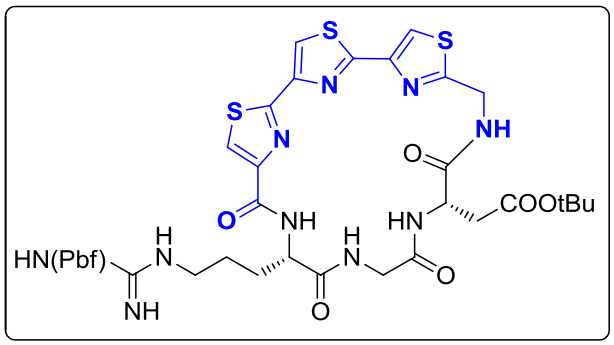
***Cyclo*-[Arg(Pbf)-Gly-Asp(tBu)-Thz-Thz-] (16)**



Cyclopeptide **16** was prepared following the procedure B, from **13** (447 mg, 0,51 mmol). The residue was absorbed onto silica and then subjected to flash chromatography (SiO_2, $CHCl_3$:MeOH, 8:2), obtaining the protected cyclic peptide **16** as a white solid.

HPLC (gradient 0 to 100% CH_3CN in 15 min.): t_R = 13.91 min.
HPLC-MS (ES(+)): m/z 860.54.

***Cyclo*-[Arg(Pbf)-Gly-Asp(tBu)-Thz-Thz-Thz-] (17)**



Cyclopeptide **17** was prepared following the procedure B, from **14** (400 mg, 0,42 mmol). The residue was absorbed onto silica and then subjected to flash chromatography (SiO_2, $CHCl_3$:MeOH, 8:2), obtaining the protected cyclic peptide **17** as a

white solid.

HPLC (gradient 20 to 80% of CH_3CN in 15 min.): t_R = 13.13 min.

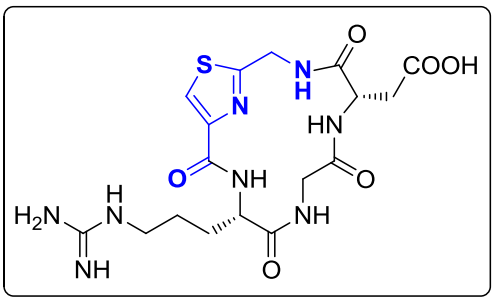
HPLC-MS (ES(+)): m/z 944.3.

General procedure C for the side deprotection in solution.

Side chain deprotection: The protected peptide (0.16 mmol), was treated with 25 mL of a solution of TFA/H_2O (95:5). After 3 h, the solvent was evaporated under vacuum and the residue was precipitated with Et_2O (4x). The Et_2O solution was discarded and the white solid was lyophilized to afford 55 mg of **1** (73% yield).

Peptide purification: The final product **1** was dissolved in 5 mL MilliQ water and filtered with 0,2 μm filter. The cyclic peptide was purified by semipreparative RP-HPLC using acetonitrile (0,05% TFA)/water (0,1% TFA). The HPLC sample was concentrated under vacuum and transformed into the hydrochloride salt lyophilized in water with HCl 0,05%.

***Cyclo*-[Arg-Gly-Asp-Thz-] (1)**



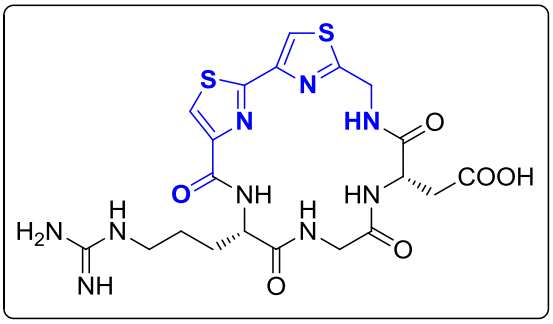
Compound **1** was prepared following the procedure C, from cyclopeptide **15**. The residue was purified by semipreparative RP-HPLC, obtaining the cyclic peptide **1** as a white solid (156 mg, 80% yield, 2 steps).

^{1}H-NMR (500 MHz, H_2O:D_2O 9:1, 278 K): δ 9,29 (t, N*H* Gly), 9,20 (d, *J* = 7,24 Hz, N*H* Asp), 8,90 (t, *J* = 5,89/5,89 Hz, N*H* Thz), 8,46 (d, *J* = 8,93 Hz, N*H* Arg), 7,79 (s, C*H* Thz), 7,22 (t, *J* = 5,39/5,39 Hz, NH_ε Arg), 4,75 (m, CH_α Arg), 4,63 (m, CH_α Asp), 4,04 (dd, *J* = 3,35/14,90 Hz, CH_α Gly), 3,82 (dd, *J* = 6,69/14,96 Hz, CH_α Gly), 3,17 (m, C$H_{2\delta}$ Arg), 2,89 (m, C$H_{2\beta}$ Asp), 1,92 (m, CH_β Arg), 1,82 (m, CH_β Arg), 1,63 (m, C$H_{2\gamma}$ Arg) ppm.

HRMS (EIS) m/z calculated for ($C_{17}H_{24}N_8O_6S$): 468.1540 $(M+H)^+$; found 469.16099.

HPLC (gradient 0 to 20% of CH_3CN in 15 min): t_R= 10.52 min.

***Cyclo*-[Arg-Gly-Asp-Thz-Thz-] (2)**



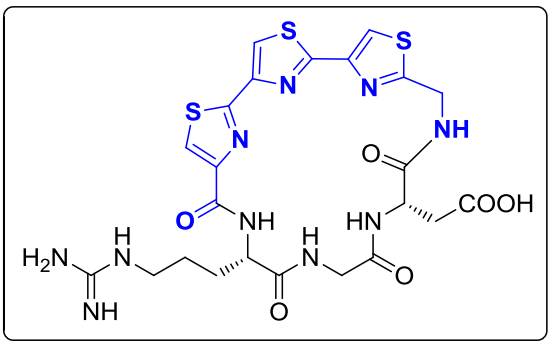
Compound **2** was prepared following the procedure C, from cyclopeptide **16**. The residue was purified by semipreparative RP-HPLC, obtaining the cyclic peptide **2** as a white solid (271 mg, 47% yield, 2 steps).

^{1}H-NMR (500 MHz, H_2O:D_2O 9:1, 298 K): δ 8,93 (s_{broad}, N*H* Gly), 8,82 (d, *J* = 7,62 Hz, N*H* Asp), 8,75 (t, *J* = 5,69/5,69 Hz, N*H* Thz), 8,51 (d, *J* = 7,62 Hz, N*H* Arg), 8,05 (s, C*H* Thz^1), 7,50 (s, C*H* Thz^2), 7,19 (t, *J* = 5,38/5,38 Hz, NH_ε Arg), 4,13 (dd, *J* = 5,82/14,24 Hz, C*H* Gly), 3,87 (dd, *J* = 5,96/15,69 Hz, C*H* Gly), 3,21 (m, C$H_{2\delta}$ Arg), 2,94 (m, C$H_{2\beta}$ Asp), 1,95 (m, CH_β Arg), 1,87 (m, CH_β Arg), 1,68 (m, C$H_{2\gamma}$ Arg) ppm.

HPLC (gradient 10 to 25% of CH_3CN in 15 min): t_R = 8.73 min.

HRMS (EIS) m/z calculated for $C_{20}H_{25}N_9O_6S_2$: 551.1369 $(2M+2H)^+$, found 552.14392.

***Cyclo*-[Arg-Gly-Asp-Thz-Thz-Thz-] (3)**



Compound **3** was prepared following the procedure C, from cyclopeptide **17**. The residue was purified by semipreparative RP-HPLC, obtaining the cyclic peptide **3** as a white solid (86 mg, 33% yield, 2 steps).

^{1}H-NMR (500 MHz, DMSO-d_6, 298 K): δ = 9.21 (t, *J* = 5.4, N*H* Gly), 8.72 (m, N*H* Asp + N*H* Thz), 8.37 (s, C*H* Thz^1), 7.96 (d, *J* = 9.2, NH_α Arg), 7.77 (s, C*H* Thz^2), 7.68 (t, *J* = 6.0, NH_ε Arg), 7.23 (s, C*H* Thz^3), 4.83 (dd, *J* = 14.3, 8.5, CH_α Arg), 4.72 (dd, *J* = 16.3, 6.6, C*H* Thz), 4.59 (m, C*H* Thz + CH_α Asp), 3.89 (d, *J* = 11.5, C*H* Gly), 3.59 (d, *J* = 9.7, C*H* Gly), 3.13 (dd, *J* = 12.6, 6.3, C$H_{2\delta}$ Arg), 2.81 (dd, *J* = 16.3, 4.3, CH_β Asp), 2.58 (dd, *J* = 16.5, 8.7, CH_β Asp), 1.82 (m, CH_β Arg), 1.71 (m, CH_β Arg), 1.49 (m, C$H_{2\gamma}$ Arg) ppm.

HRMS (EIS) m/z calculated for $C_{23}H_{26}N_{10}O_6S_3$: 634.1199 $(2M+2H)^+$, found 635.12683.

HPLC (gradient 10 to 30% CH_3CN in 15 min): t_R = 8.26 min.

3. Spectroscopic data.

Structural characterization by NMR

	Residue	NH	Δδ/ΔT	H/H$_2$ (α)	H$_2$ (β)	H$_2$ (γ)	H$_2$ (δ)	NH (ε)	$^3J_{NH-H\alpha}$
1	**Arg**	8,46	-4,5	4,75	1,92/1,82	1,63	3,17	7,22	8,93
	Gly	9,29	-7,5	4,04/3,82					
	Asp	9,20	-8,5	4,63	2,89				7,24
	1xThz	8,90	-4,5						
2	**Arg**	8,51	0,0	*	1,95/1,87	1,68	3,21	7,19	7,62
	Gly	8,93	-6,0	4,13/3,87					
	Asp	8,82	-5,5	*	2,94				
	2xThz	8,75	-6,0						7,62
3	**Arg**								
	Gly								
	Asp								
	3xThz								
Cilengitide	**Arg**	8,55	-7,5	3,95	1,91	1,57	3,20	7,22	7,28
	Gly	8,37	-11,0	4,12/3,54					
	Asp	8,13	-5,5	4,56	2,84/2,63				8,06
	d-Phe	7,97	-6,0	5,19	3,02				9,19
	MeVal			4,34	2,06	0,88/0,56			

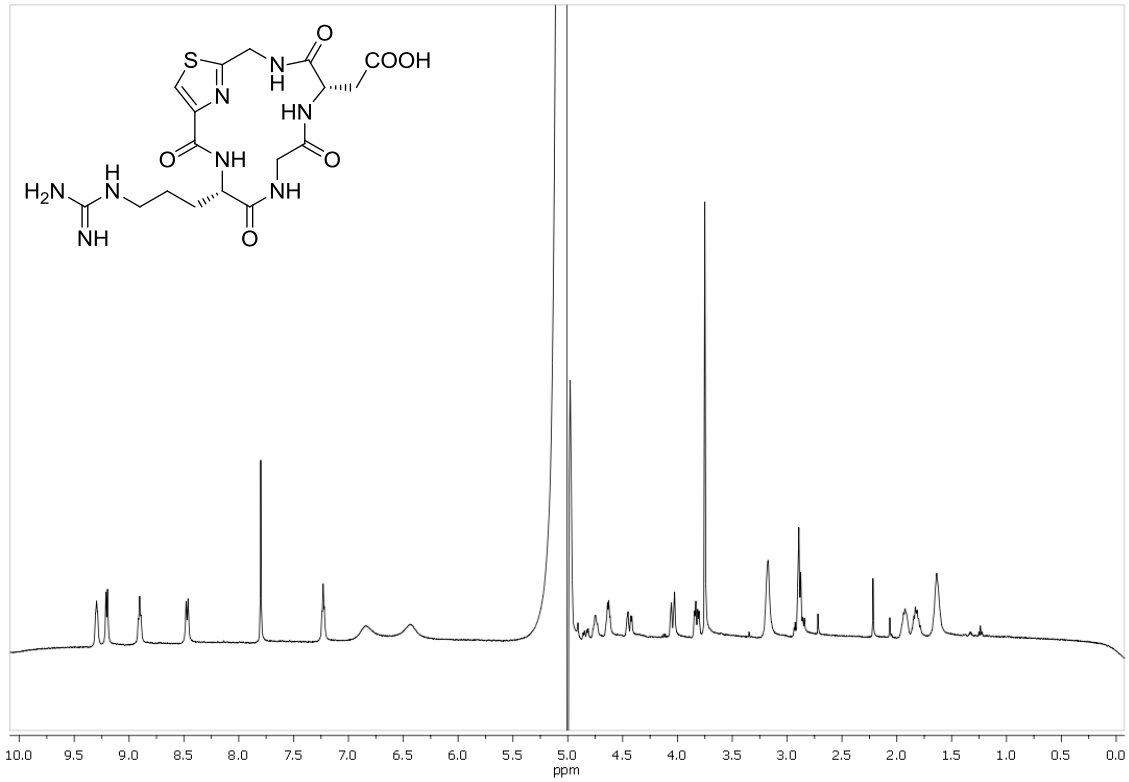
* the chemical shift is overlapped by the water.

Cilengitide			Correction H_2O:D_2O (90:10)		
NOE	Area	distance	Correction 1XNH	Correction 2XNH	Atoms
NHGly-CH2bArg	0,05203	2,91	2,62		H9-H7,H25
NHGly-CHaArg	0,16623	2,40	2,16		H9-H26
NHAsp-CHGly	0,16623	2,40	2,16		H10-H8
NHAsp-CHGly	0,07656	2,73	2,46		H10-H11
NHAsp-NHPhe	0,04233	3,02		2,44	H10-H18
NHArg-CHaVal	0,30322	2,17	1,95		H1-H36
NHArg-NCH3Asp	0,03622	3,09	2,79		H1-H3,H2,H6
NHArg-CHbVal	0,05742	2,87	2,58		H1-H33
NHArg-CH3Val	0,04783	2,95	2,66		H1-H35,H37,H38
Nharg-NHGly	0,03707	3,08		2,50	H1-H9
NHPhe-CHaAsp	0,21174	2,31	2,08		H18-H14

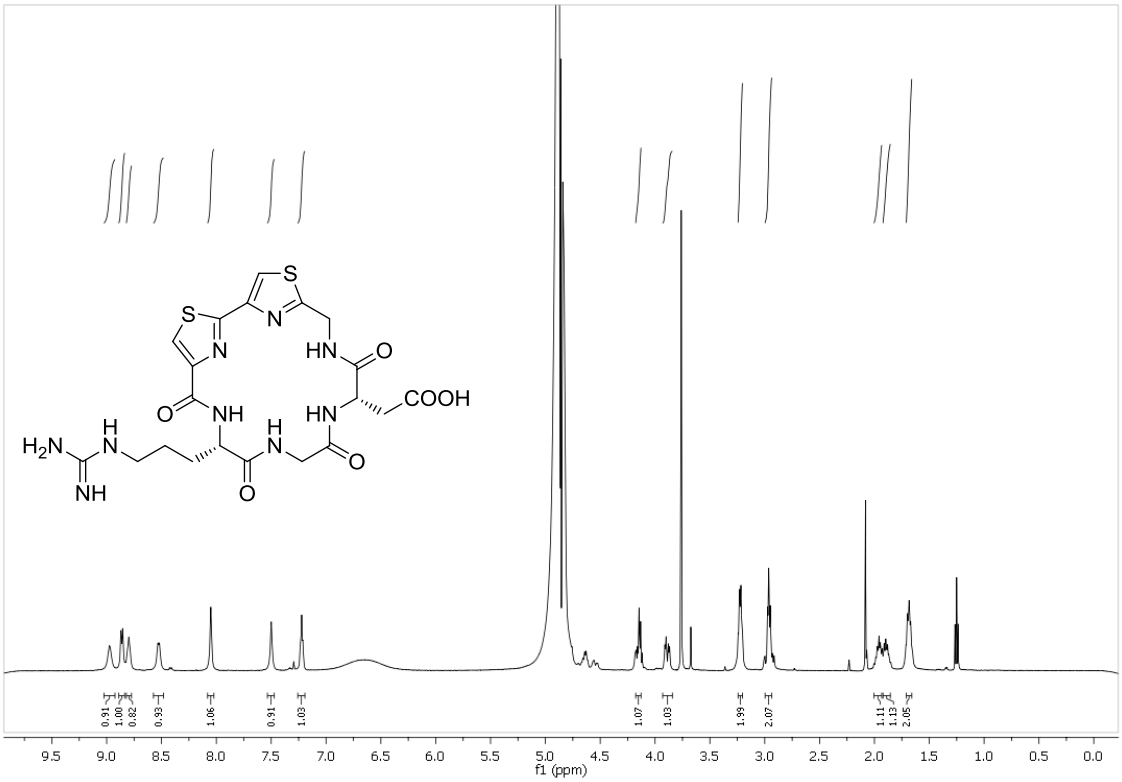
1			Correction H_2O:D_2O (90:10)		
NOE	Area	distance	Correction 1XNH	Correction 2XNH	Atoms
NHGly-CHbArg	0,05463	2,89	2,60		H9-H7
NHGly-CHbArg	0,01734	3,50	3,15		H9-H4
NHGly-CHaArg	0,34486	2,13	1,91		H9-H5
NHAsp-CHGly	0,35052	2,12	1,91		H10-H11
NHAsp-CHGly	0,09216	2,65	2,38		H10-H8
NHAsp-NHThz	0,17131	2,39		1,93	H10-H18
NHThz-CHaAsp	0,03482	3,11	2,80		H18-H14
NHThz-CHGly	0,01738	3,50	3,15		H18-H11
NHThz-CH2bAsp	0,01497	3,59	3,23		H18-H16,H13
NHThz-NHArg	0,00341	4,59		3,72	H18-H1
NHArg-CHbArg	0,02128	3,38	3,04		H1-H7
NHArg-CHbArg	0,09253	2,65	2,38		H1-H4
NHArg-CH2gArg	0,02566	3,28	2,95		H1-H6,H15
NHArg-CHThz	0,03106	3,17	2,86		H1-H2

2			Correction H_2O:D_2O (90:10)		
NOE	Area	distance	Correction 1XNH	Correction 2XNH	Atoms
NHGly-CH2bArg	0,04150	3,03	2,72		H9-H7,H14
NHGly-CHaArg	0,46116	2,03	1,82		H9-H5
NHGly-NHArg	0,02697	3,25		2,63	H9-H1
NHAsp-CHGly	0,18823	2,35	2,12		H10-H11
NHAsp-CHGly	0,45636	2,03	1,83		H10-H8
NHThz-CHaAsp	0,03387	3,13	2,82		H18-H14
NHThz-CH2Asp	0,05808	2,86	2,57		H18-H13,H16
NHArg-CHThz ring a	0,07550	2,74	2,46		H1-H17

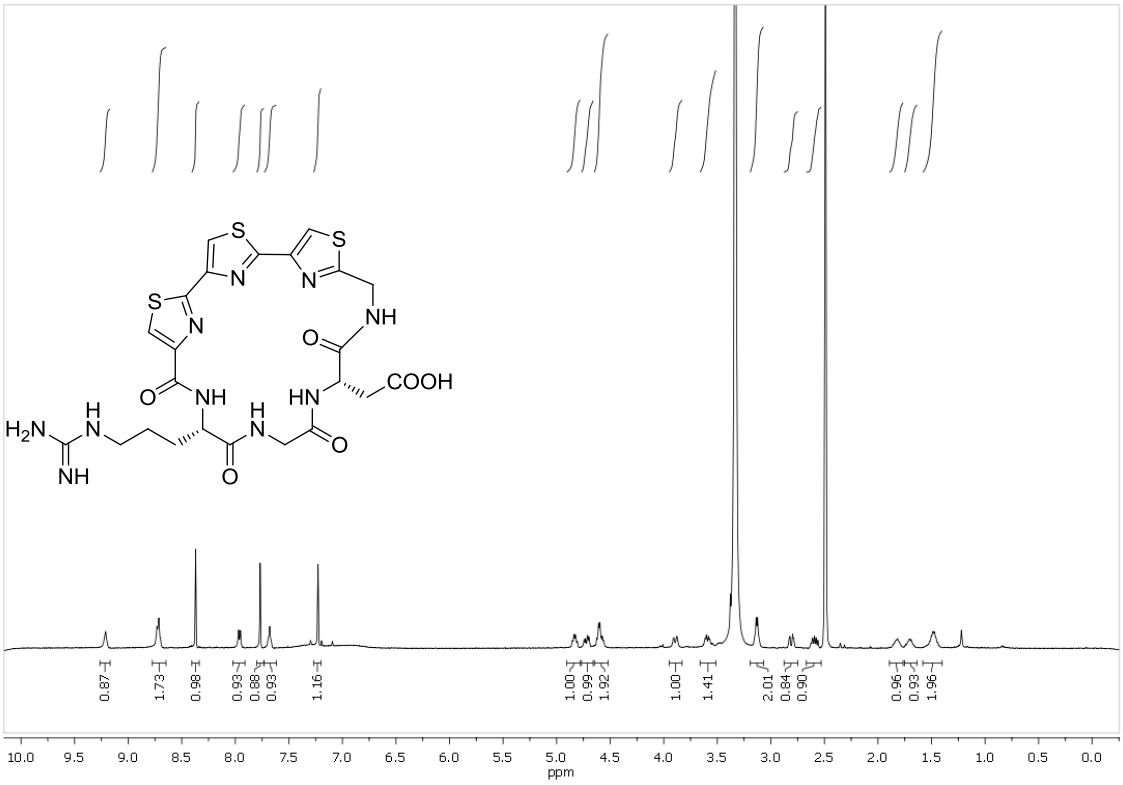
***Cyclo*-[Arg-Gly-Asp-Thz-] (1)**



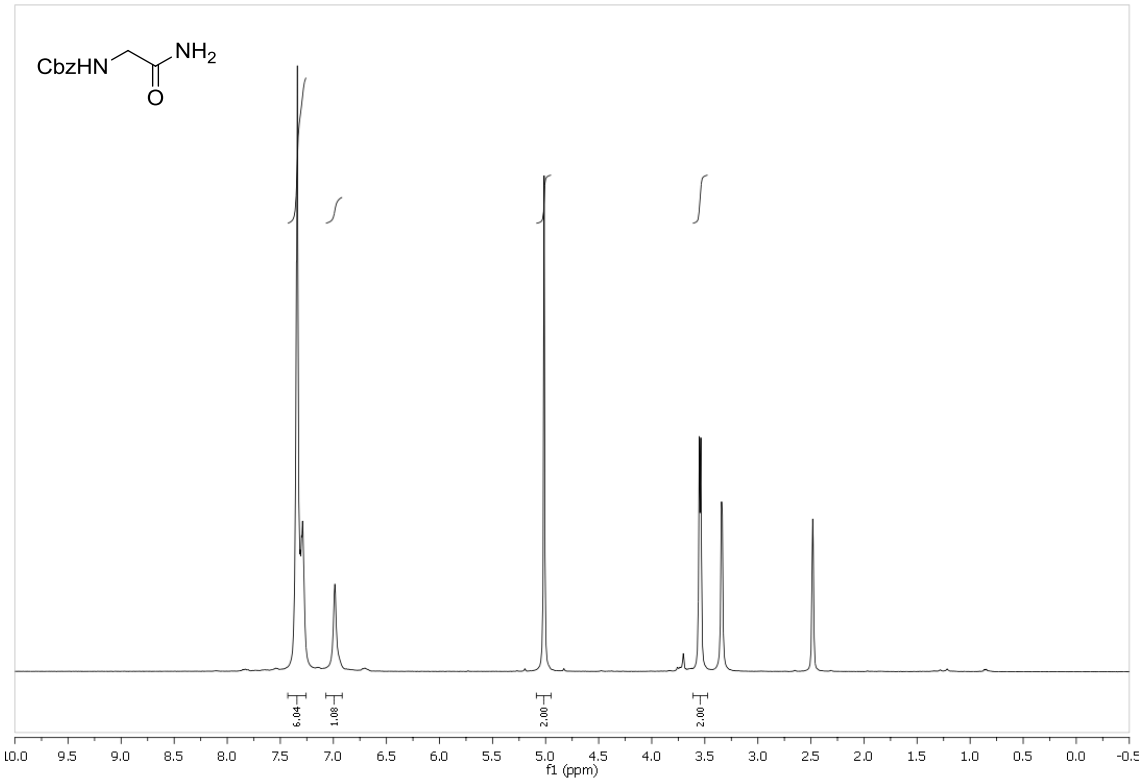
***Cyclo*-[Arg-Gly-Asp-Thz-Thz-] (2)**



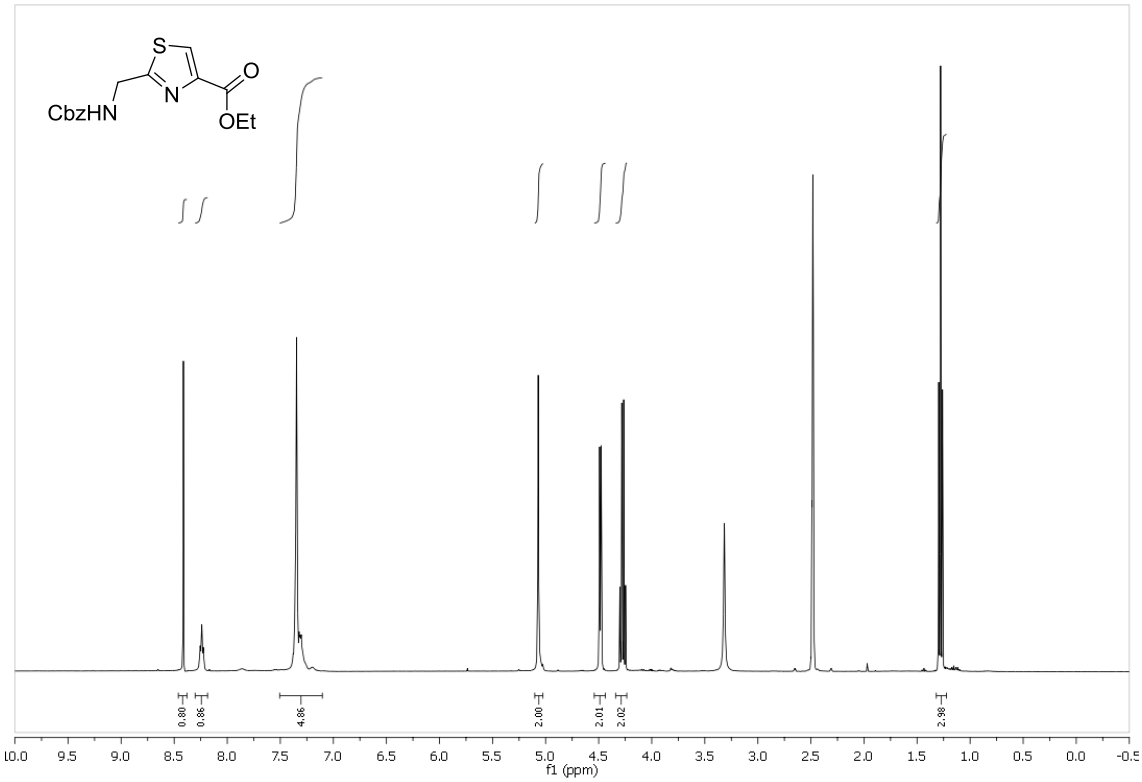
***Cyclo*-[Arg-Gly-Asp-Thz-Thz-Thz-] (3)**



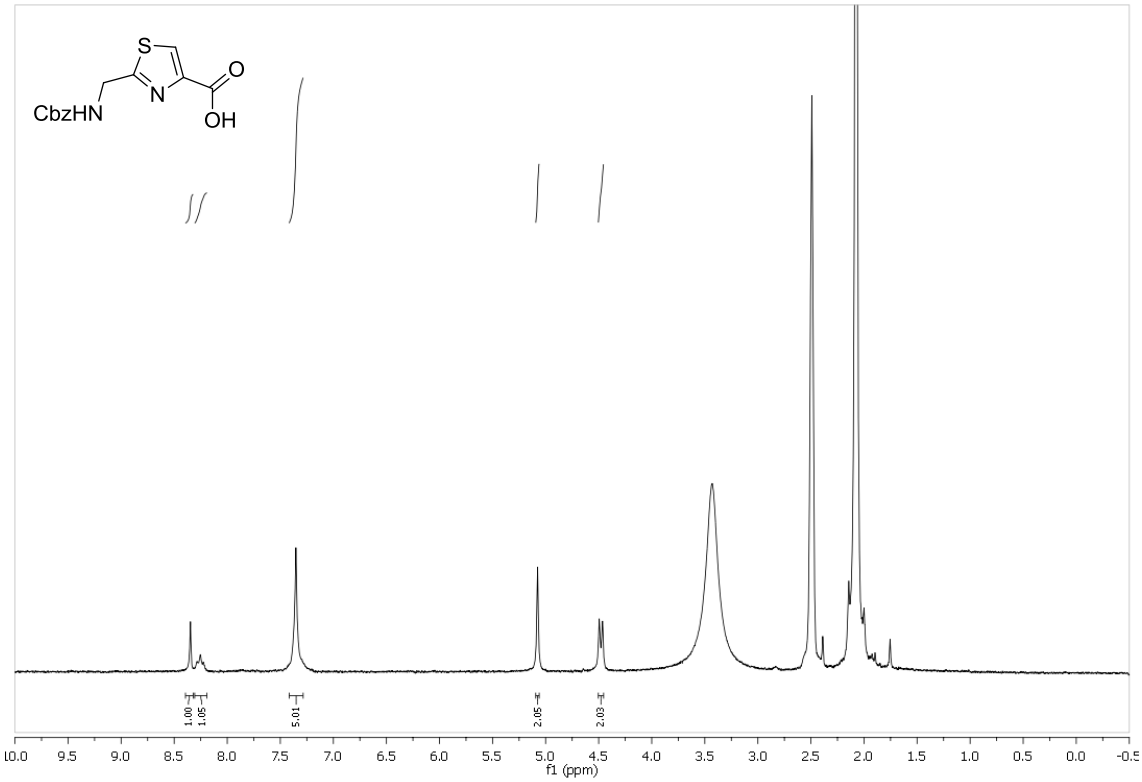
Benzyloxycarbonyl glycine amide (4)

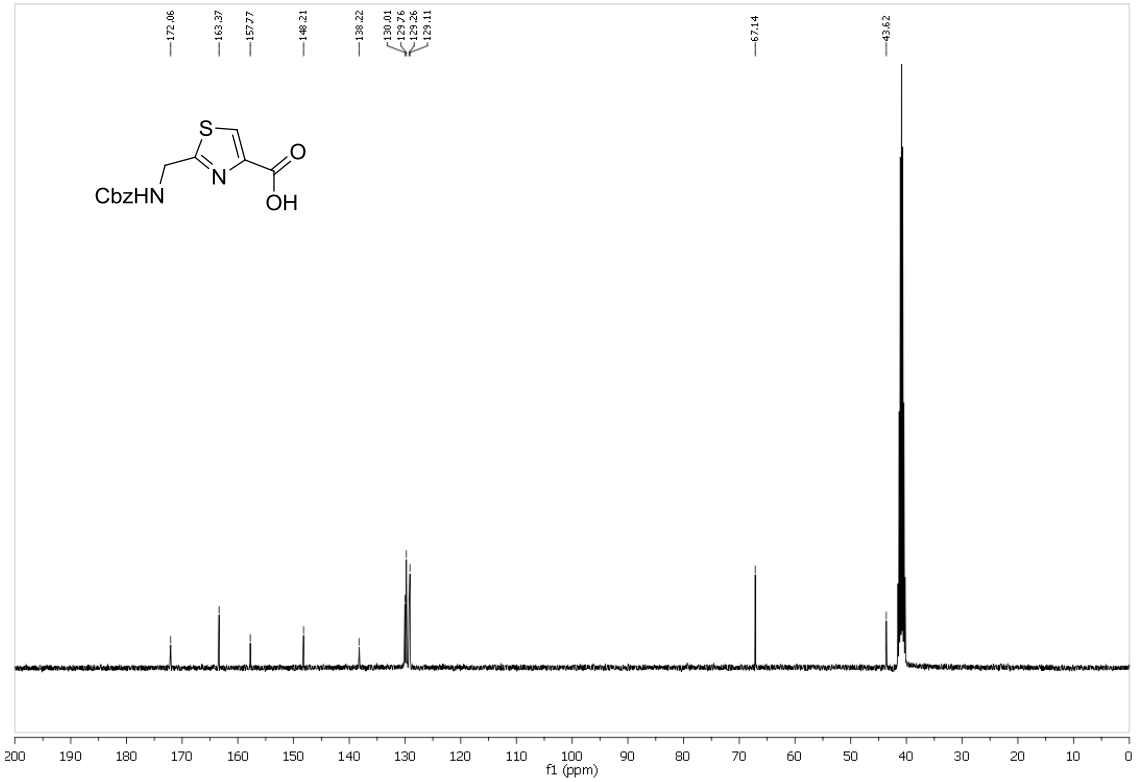


Ethyl 2-(*N*-benzyloxycarbonylaminomethyl)-4-thiazolecarboxylate (6)

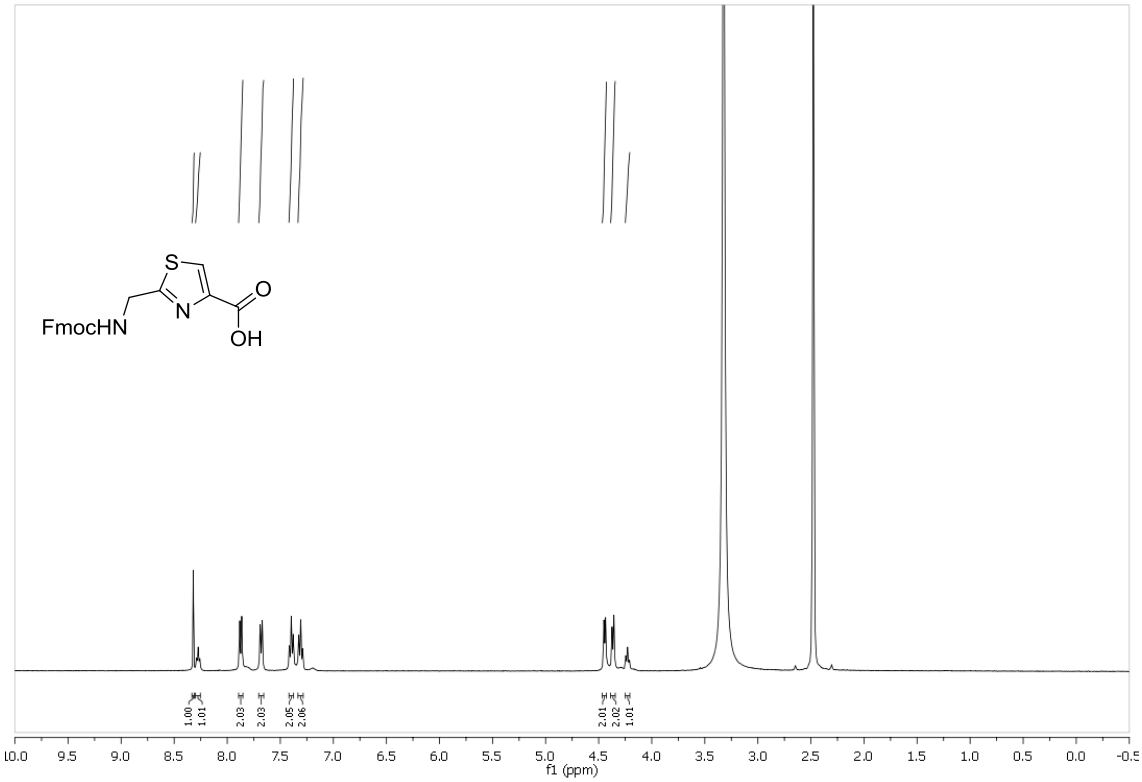


2-(*N*-Benzyloxycarbonylaminomethyl)-4-thiazolecarboxylic acid (7)

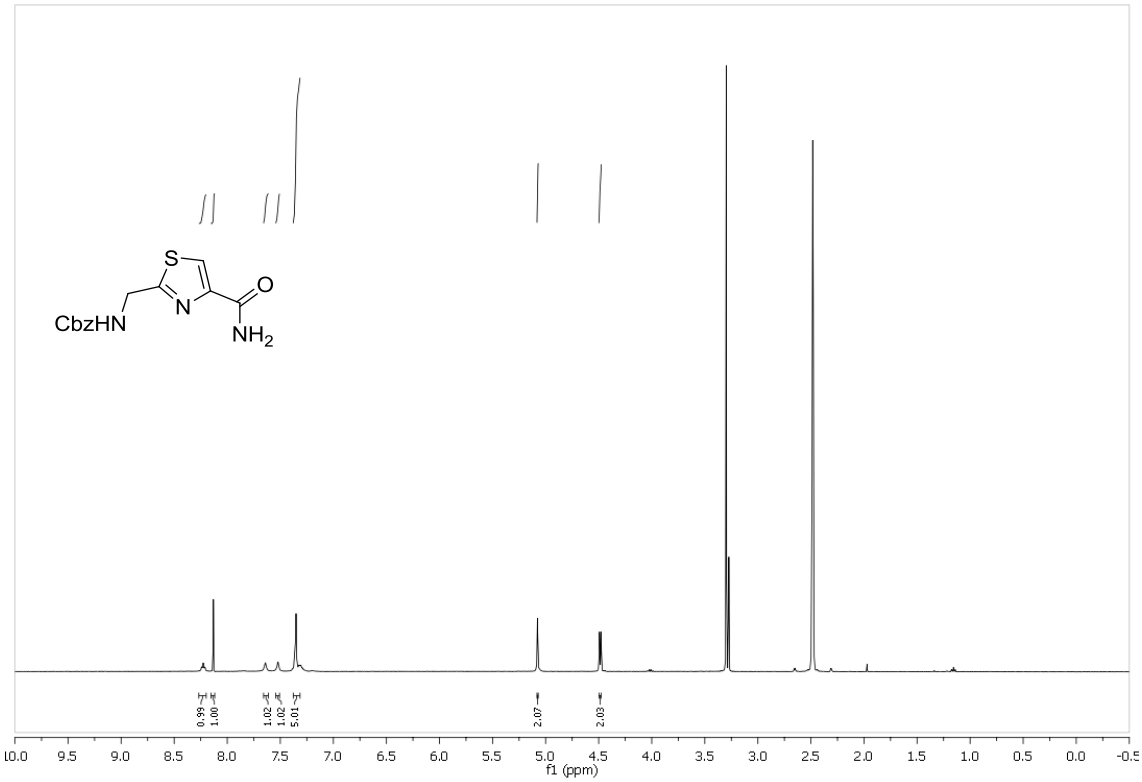




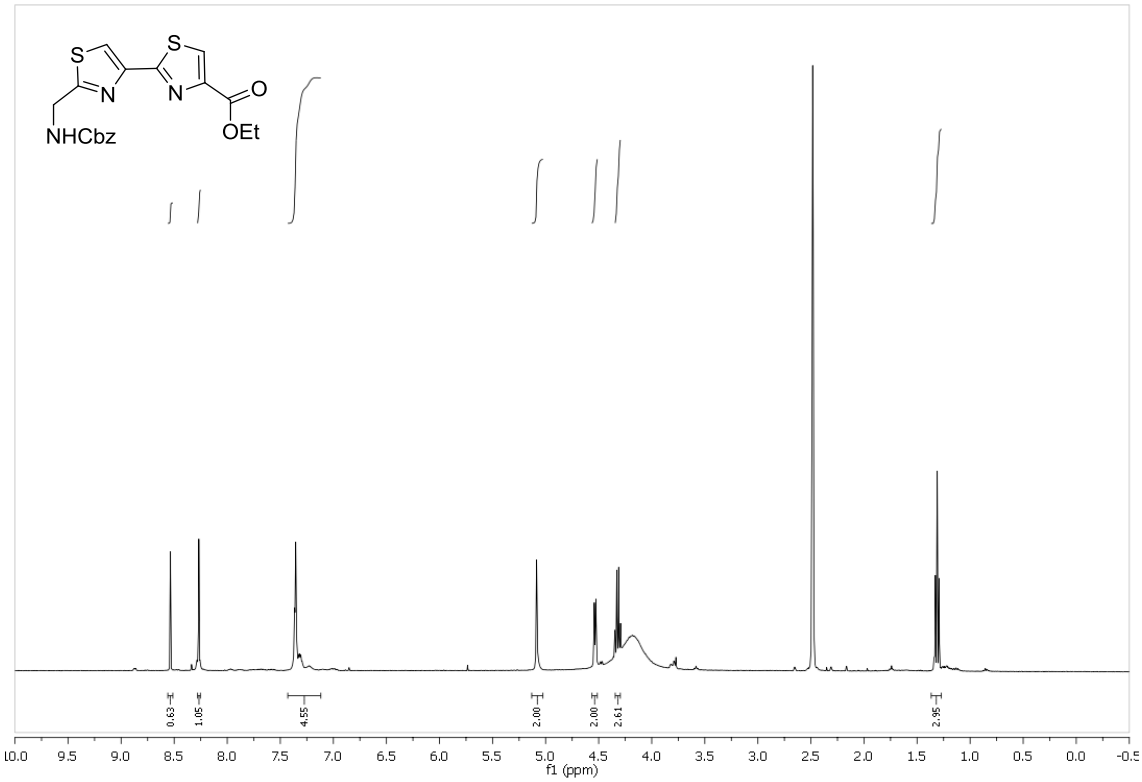
2-(*N*-9*H*-Fluoren-9-ylmethoxycarbonylaminomethyl)-4-thiazolecarboxylic acid (9)



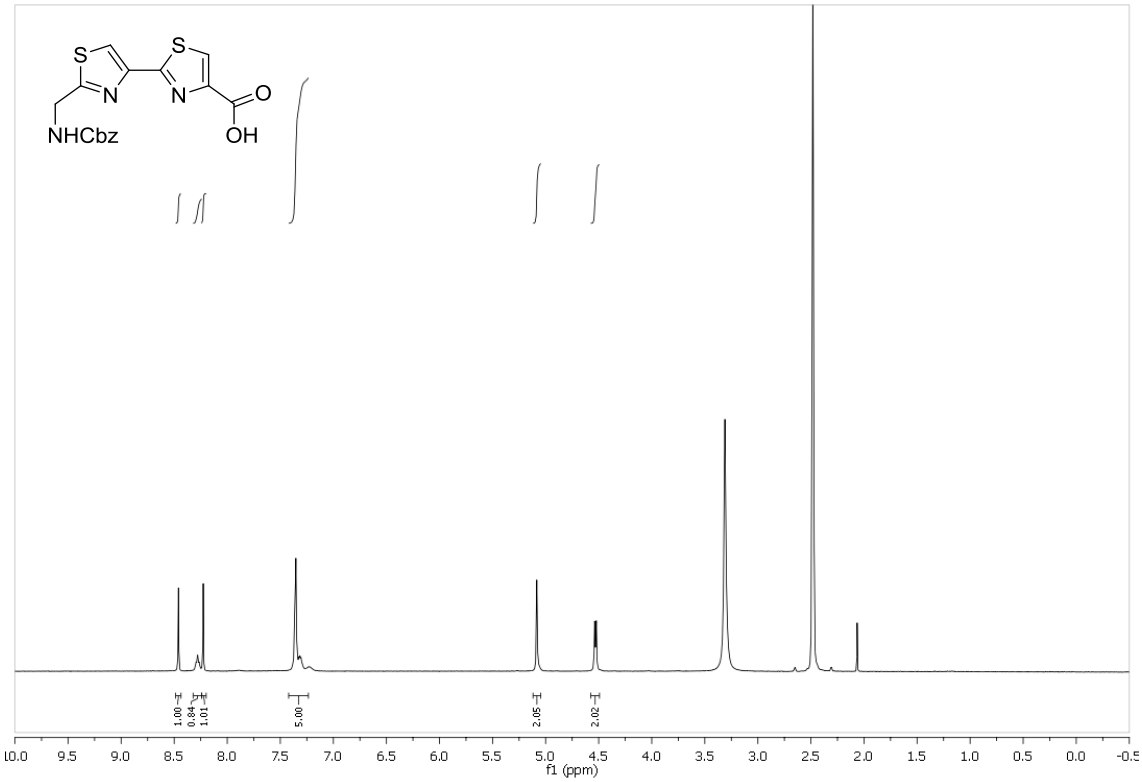
2-(*N*-Benzyloxycarbonylaminomethyl)-4-thiazolecarboxamide (9a)

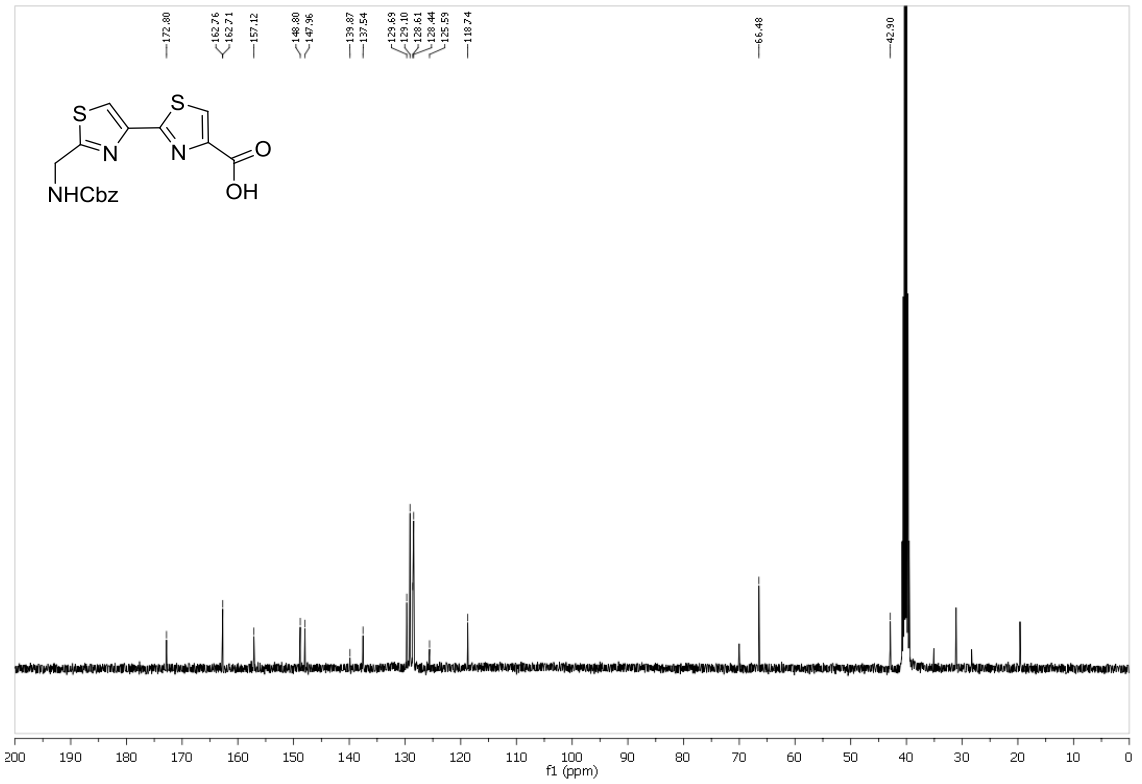


Ethyl 2'-(*N*-benzyloxycarbonylaminomethyl)-2,4'-bisthiazole-4-carboxylate (9c)

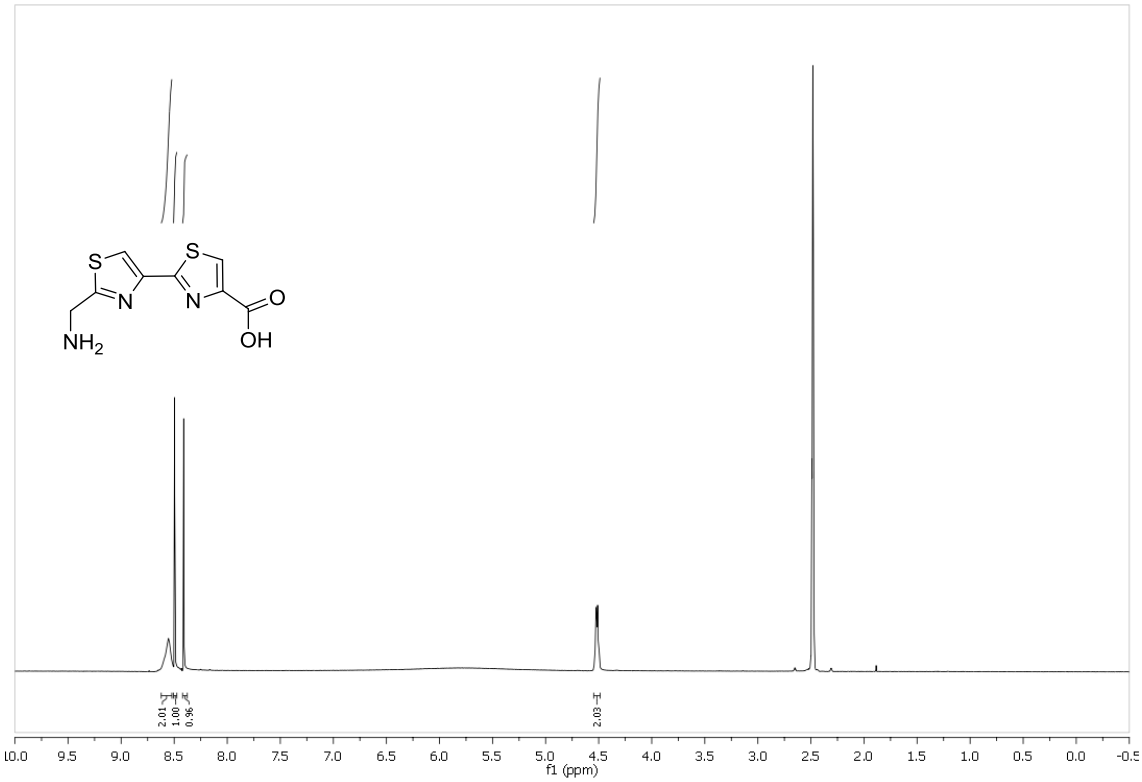


2'-(*N*-Benzyloxycarbonylamino)methyl)-2,4'-bisthiazole-4-carboxylic acid (9d)

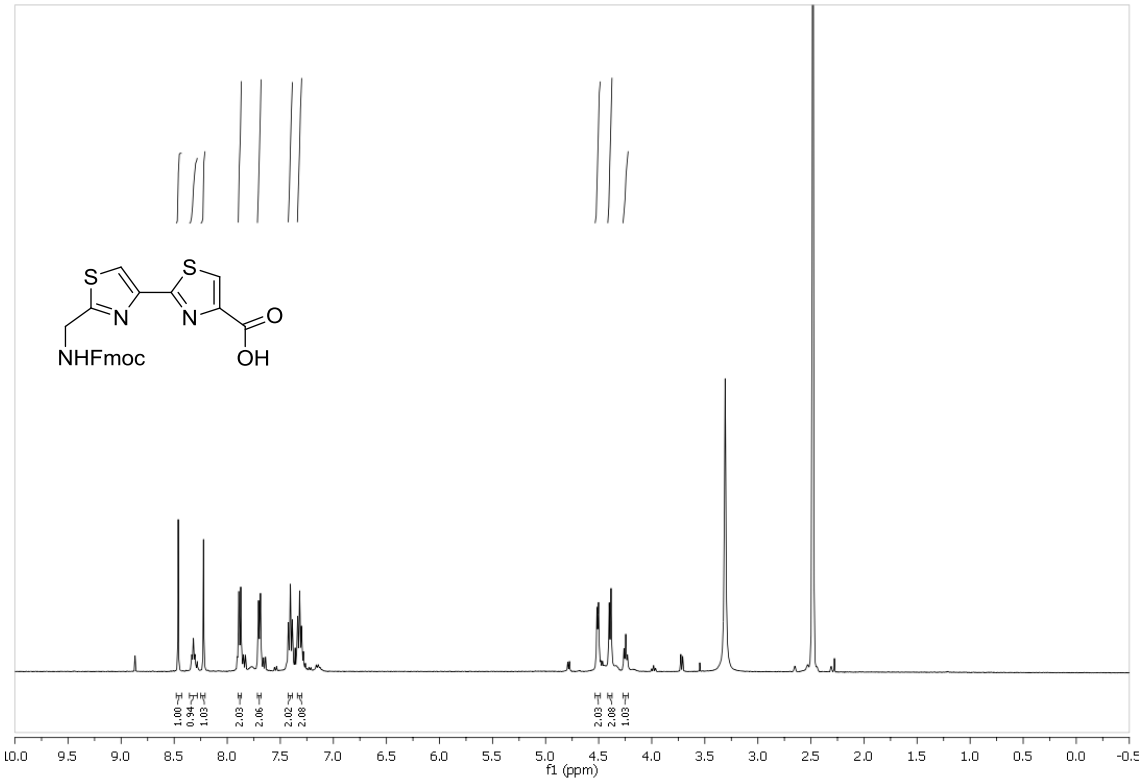




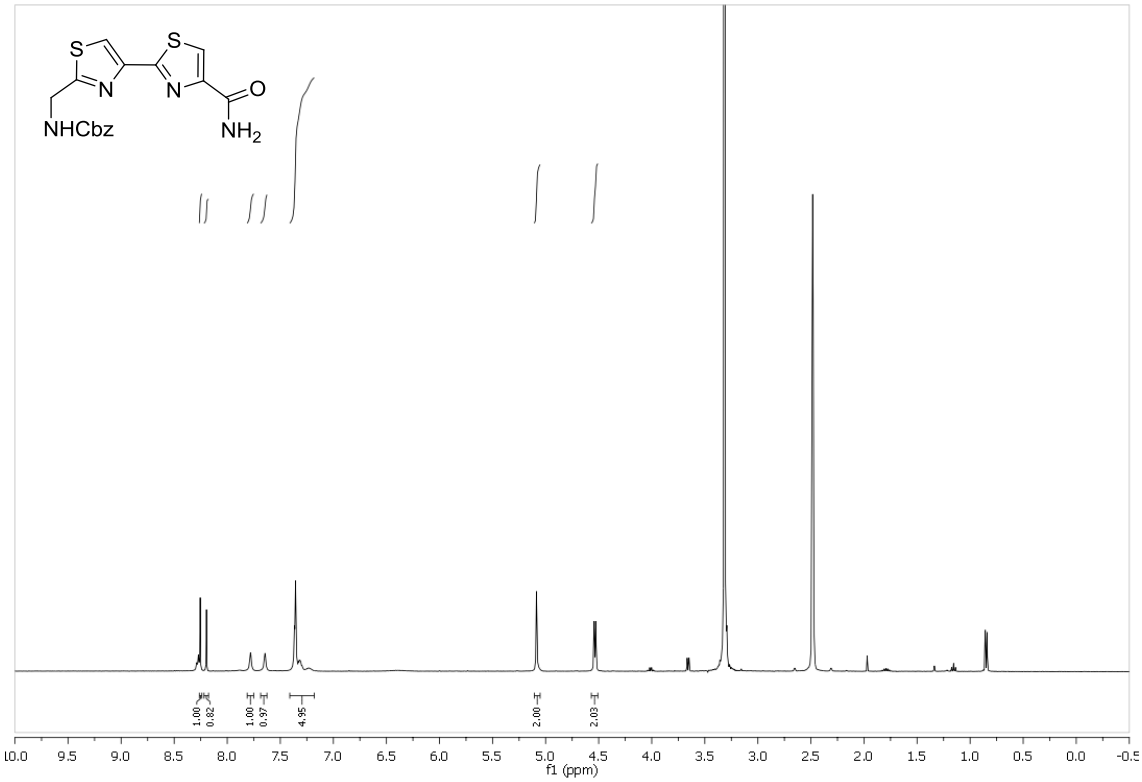
2'-(Aminomethyl)-2,4'-bisthiazole-4-carboxylic acid (9e)



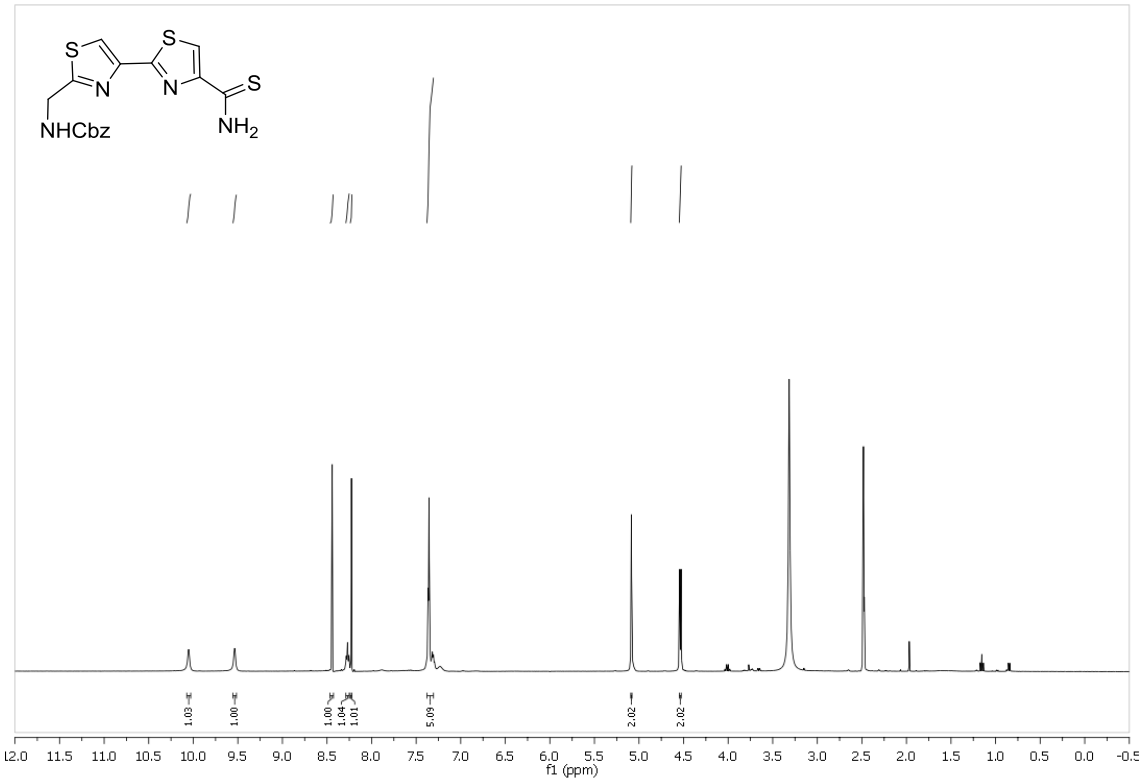
2'-(*N*-9*H*-Fluoren-9-ylmethoxycarbonylaminomethyl)-2,4'-bisthiazole-4-carboxylic acid (10)



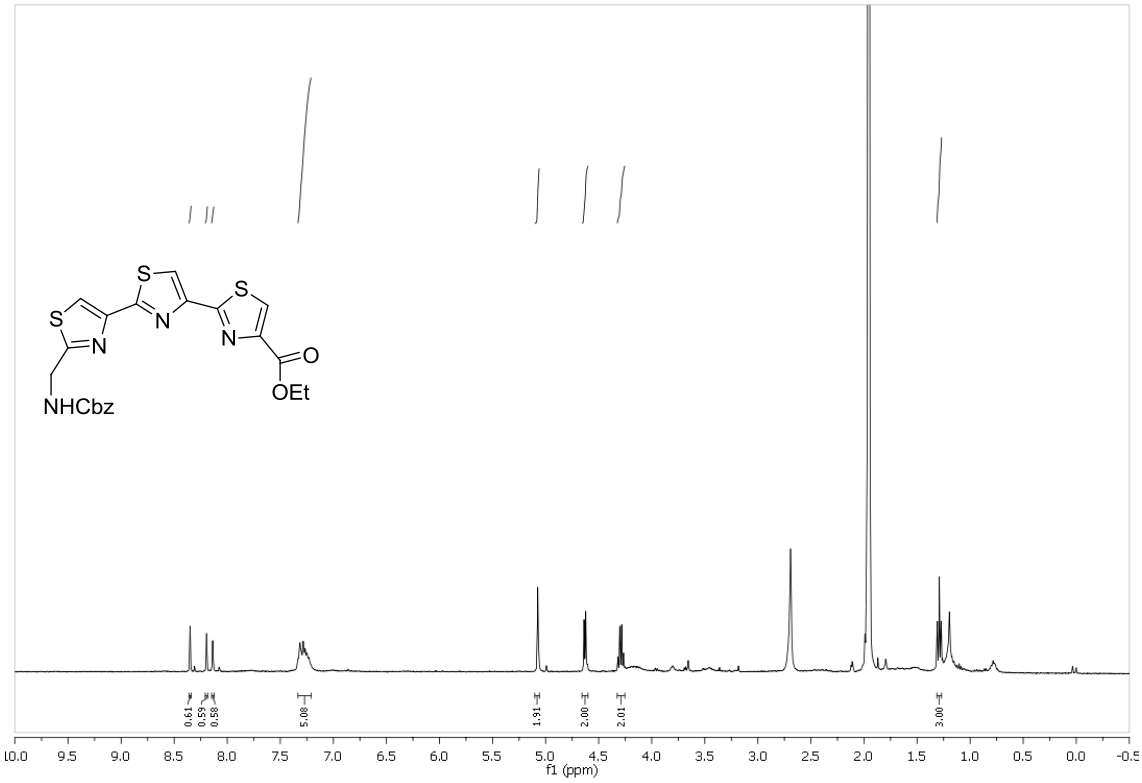
2'-(*N*-Benzyloxycarbonylaminomethyl)-2,4'-bisthiazole-4-carboxamide (10a)



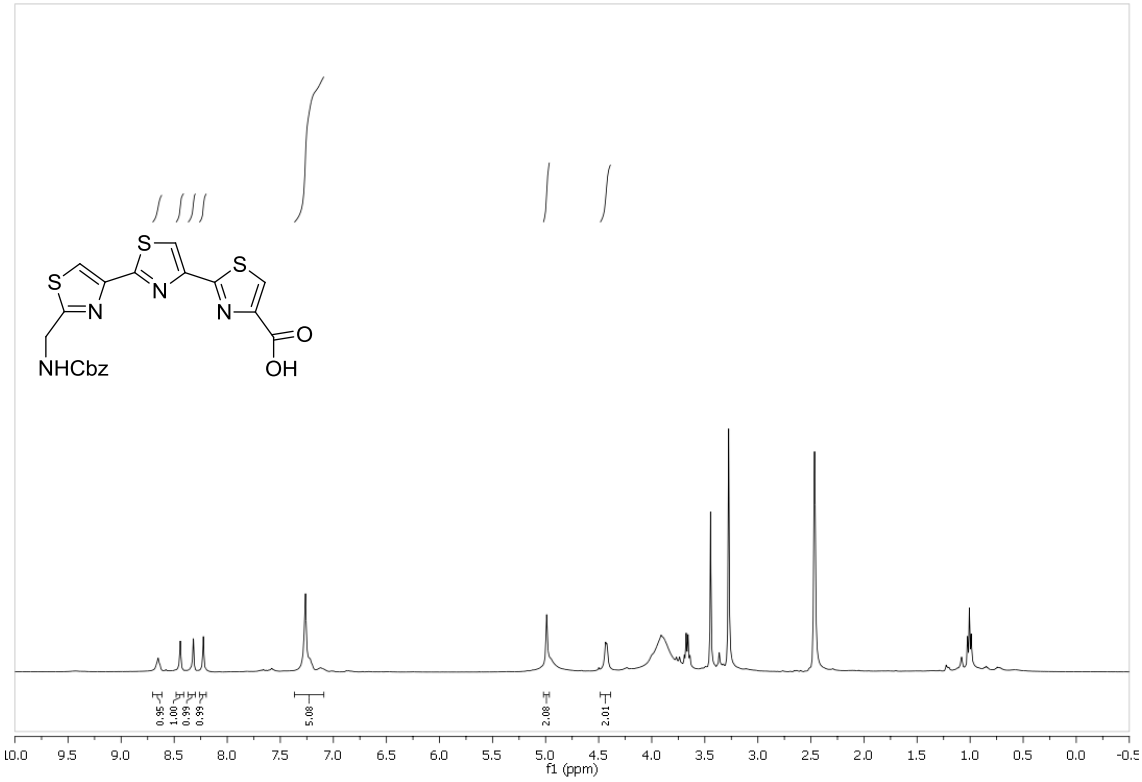
2'-(*N*-Benzyloxycarbonylaminomethyl)-2,4'-bisthiazole-4-carboxathioamide (10b)



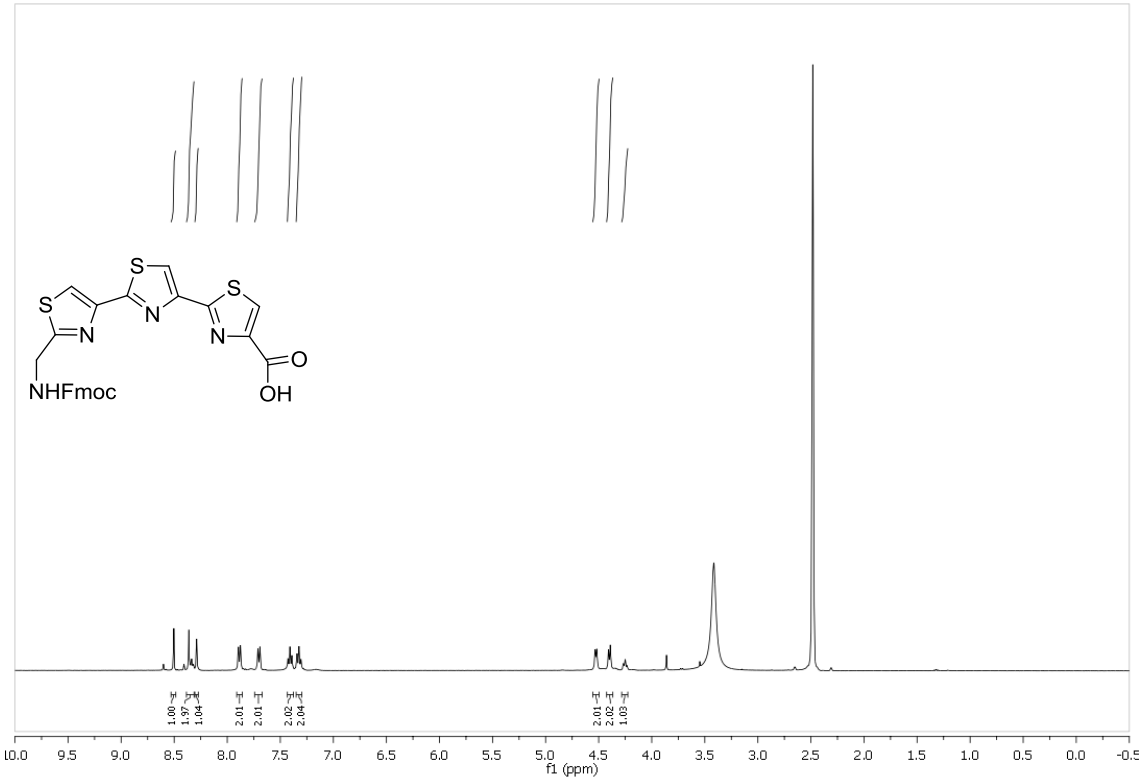
Ethyl 2''-(*N*-benzyloxycarbonylaminomethyl)-2,4'-2',4''-trithiazole-4-carboxylate (10c)

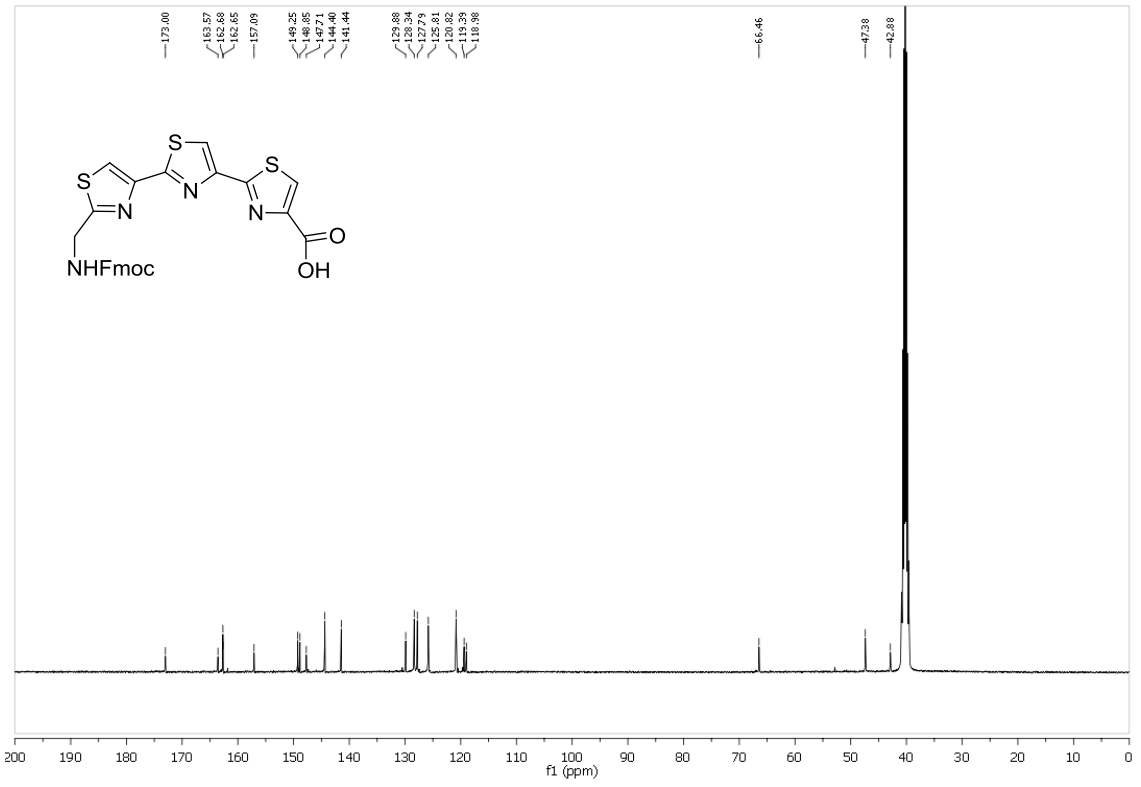


2''-(*N*-Benzyloxycarbonylamino)methyl)-2,4'-2',4''-trithiazole-4-carboxylic acid (10d)



2'-(*N*-9*H*-Fluoren-9-ylmethoxycarbonylaminomethyl)- 2,4'-2''-4'''-trithiazole-4-carboxylic acid (11)





4. Biological assays

Ethical Animal Procedures

All procedures involving experimental animals were approved by the "Ethical Committee of Animal Experimentation" of the animal facility place at Science Park of Barcelona (Platform of Applied Research in Animal Laboratory). Once approved by the Institutional ethical committee, these procedures were additionally approved by the ethical committee of the Catalonian authorities according to the Catalonian and Spanish regulatory laws and guidelines governing experimental animal care.

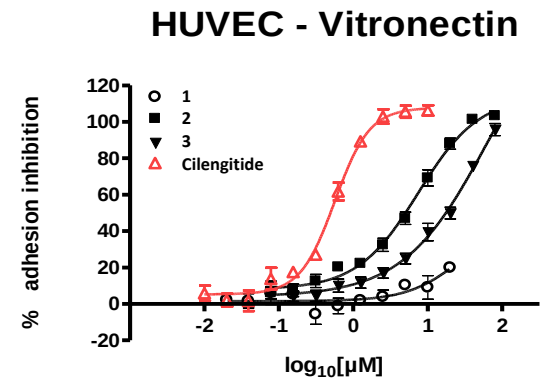
Along the procedures using experimental animals, there was established a continuous supervision control of the animals that evaluated the degree of suffering of the animals and if it was the case to sacrifice them according to the defined end point criteria [D. B. Morton, P. H. Griffiths, Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Vet. Rec.* 1985, **116**, 431–436]. The euthanasia applied was by CO_2 saturated atmosphere.

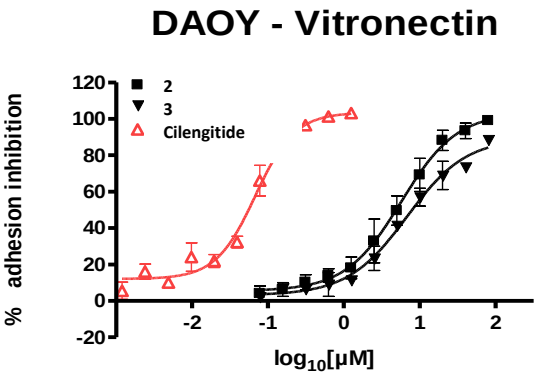
Cell adhesion assays:

Non-tissue culture treated ELISA plates [NUNC, Maxisorp 442404] were coated ON at 4 ºC with the specific concentration of the ligand. Coating solution was discarded and wells were blocked with blocking solution (BSA 1.5%; 60 minutes at 37 ºC). Blocking solution was discarded by flicking and serial dilutions of the compounds were plated in quadruplicates. Immediately, harvested cells are plated at a given concentration (20000-25000 / well for HUVEC and DAOY and 50000 for HT-29) to the same plate. Plates were incubated for 90 minutes at 37 ºC/5%CO_2 to allow cell adhesion on the ligand. After then, non-adhered cells were removed and hexosaminidase substrate (N-acetyl-β-D-glucosaminide) was added to each well and incubated for 3 hours. Optical density was read at 405 nm. The proliferation inhibition EC50 was calculated using the Prism-4 software based on the sigmoidal dose-response (variable slope) equation.

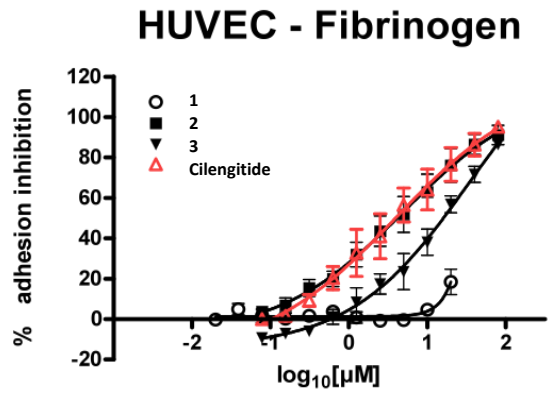
Each plate contains positive and negative controls and peptides are tested as duplicates. Each assay has been repeated at least twice and the adhesion inhibition EC50 is calculated, when possible, using the Prism-4 software based on the sigmoidal dose-response (variable slope) equation.

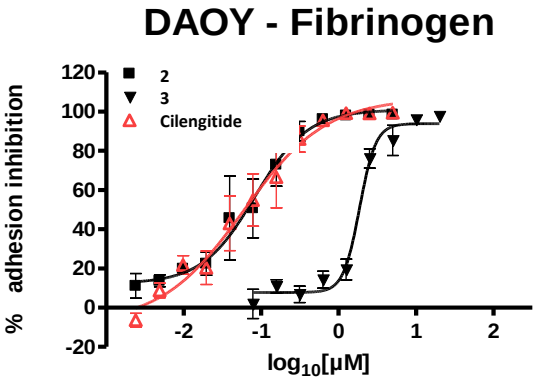
Cell adhesion inhibition curves for compounds **1-3** using VN as ligand in HUVEC and DAOY cell lines.



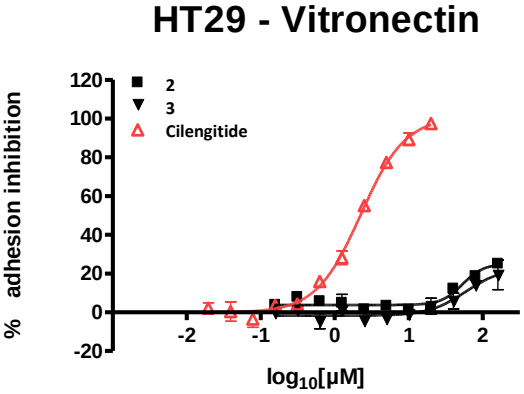


Cell adhesion inhivition curves for compounds **1**-**3** using FB as ligand in HUVEC and DAOY cell lines.

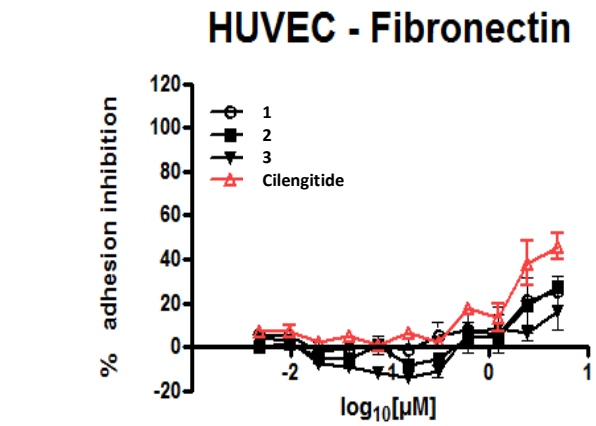


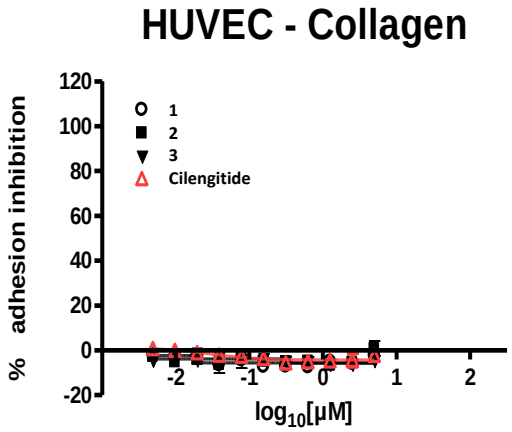


Cell adhesion inhibition curves for compounds **2-3** using VN as ligand in HT29 cell line.



Cell adhesion inhivition curves for compounds **1-3** using FN and COL as ligands.





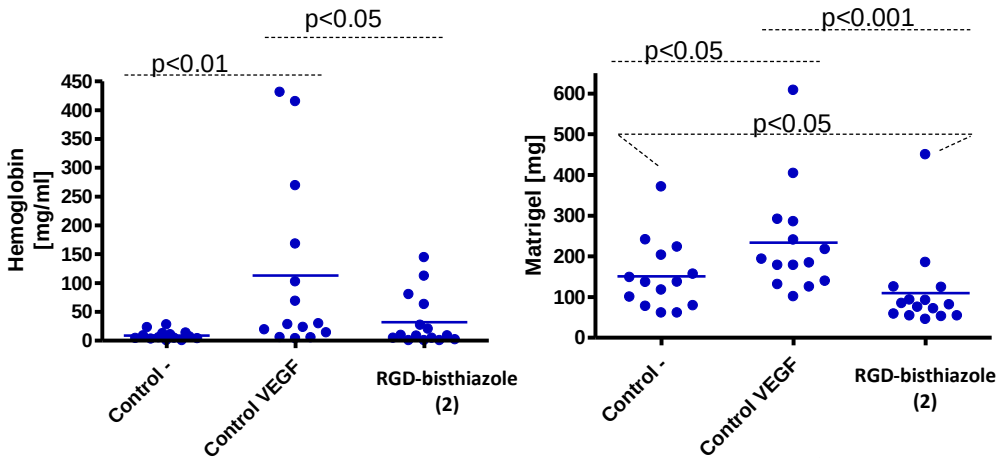
Matrigel Assay: Matrigel Growth Factor reduced (Beckton&Dickinson, 356231) was thawed on ice or overnight at 4 °C. 4 mL of Matrigel was added and mixed by pippeting to 150mg/ml VEGF (100 µg/ml, Isokine 01-AA010) and 25 u/ml heparin (1000 U/ml, Lab ROVI), that were previously incubated at room temperature for 3 min. Then, RGD-bis-Thiazole **2** was added at a 2 µg/mL final concentration; finally, 4 mL matrigel were added to complete 8 mL of solution. Negative and positive controls were included (minus-plus VEGF). Matrigel was kept always in ice.

C57/BL6 mice immunocompetent mice were used. Each treatment group consisted on 15 mice. Housing: 5 mice were maintained in sterile room in micro-isolator cages, and were given sterilized food and water ad libitum at the “Servei d’Experimentació Animal”, Scientific Park of Barcelona. Mice were injected subcutaneously with corresponding

500 µL solution into the ventral area of each mouse. After 7 days, mice were killed and plugs were removed and frozen until haemoglobin quantification.

Each plug was homogenized in 500 µL of DRABKIN reagent (Sigma D5941), centrifuged at 4000 g, and the supernatant was recovered for haemoglobin quantification. Absorbance at 540 nm was measured in a Multiskan (Thermo Scientific). For curve calibration, a standard solution was prepared using a serial dilution (1:1) of haemoglobin, starting at 180 mg/mL.

The results obtained in the matrigel plug assay showed a significant antiangiogeneic effect of the RGD-bis-Thiazole 2 ($p<0.05$). Moreover, the median weight of the plug obtained was also significantly lower than both positive (VEGF) and negative controls.



5. Computational methods.

Methods. Homology modelling, docking and molecular dynamics (MD) simulations were used to perform structural and binding studies to unravel at the atomic level the differences in selectivity for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ between cilengitide and the bis-thiazole based peptide. The X-ray structure of cilentigide bound to $\alpha_v\beta_3$ (PDB ID: 1L5G[3,4]) was used to build up the 3D model of the complex with the bis-thiazole based peptide. Furthermore, a 3D model of the $\alpha_v\beta_5$ integrin-propeller complex was built with the SWISS-MODEL server (http://swissmodel.expasy.org)[5,6] using the X-ray structure 1L5G as a template. To validate the model, a second homology modelling experiment was run using MODELLERv9.1[7] with the same template. The results obtained only differed in some side-chain orientations of surface exposed residues, thus giving us confidence in the predicted model.

Stable conformations of bis-thiazole peptide was obtained using NOE distances from NMR experiments as distance restraints in MD simulations. To this end, the ligand was simulated in an octahedral box of water molecules at 298 K. After heating, an NMR-restrained MD simulation was run for 0.5 μs. Then, the simulation was further continued without restraints up to 1.2 μs, which was used to identify the most populated structure opf compound **2**.

The Glide[8] module of the Schrodinger Suite[9] was then used to build the protein-ligand complexes using the position of cilengitide in the crystal structure as a template to set-up the grid box size and position. The best predicted models of cilengitide-$\alpha_v\beta_5$, bis-thiazole-RGD-$\alpha_v\beta_3$ and bis-thiazole-RGD-$\alpha_v\beta_5$, as well as the X-ray based 3D model of cilengitide-$\alpha_v\beta_3$, were studied by MD simulations, which in turn were used to estimate the binding affinity with the MM/PBSA methodology. The PMEMD module of the AMBER[10] package was used to perform the MD simulations with the parm99SBildn[11,12] force field. The complexes were immersed in an octahedral box of

water and the system was neutralized with sodium ions. A cut-off of 9 Å was used for the nonbonding energy computation with a grid space of 1 Å for the electrostatic computation with particle mesh Ewald method. For each complex, a 50 ns trajectory was obtained and the last 20 ns were used for the MM/PBSA analysis. In these computations, a dielectric constant of 80 was used for the solvent, while three different values were considered for the solute dielectric constant (i.e., 4, 10 and 20) for the sake of comparison.

Structural model of compound 2 in solution. Table 1 shows the mean distances related to NMR data as determined from the snapshots sampled during restrained and unrestrained windows of the MD simulation. The only differences were found in the distances from hydrogen atoms of the side chains of Arg and Asp to the N-amide hydrogens of Gly and thiazole (Thz). These differences reflect reorganizations of the side-chains of the natural amino acids, which can be attributed to the different polarity of the solvents used in NMR (DMSO) and MD simulations (water). Importantly, no significant difference was found in the backbone of the cyclopeptide in the restrained and unrestrained windows of the trajectory.

Table 1. Mean distances for selected atoms derived from MD simulations.

	Mean distances (Å)	
	Restrained	**Unrestrained**
HN-Gly-HB2-Arg	3.1	4.3
HN-Gly-HB3-Arg	3.2	4.4
HN-Gly-HA-Arg	1.7	2.9
HN-Gly-HN-Arg	3.3	2.8
HN-Asp-HA2-Gly	2.6	2.8
HN-Asp-HA3-Gly	2.3	2.4
HN-Thz-HA-Asp	2.3	2.2
HN-Thz-H2B-Asp	2.9	4.3
HN-Thz-H3B-Asp	2.8	4.3
HN-Arg-H-Thz ring	2.2	2.1

Predicted binding affinities. Table 2 shows the binding affinities estimated from MM/PBSA calculations carried out with the module in AMBER by assigning distinct dielectric constants to the interior of the protein. In all cases the results consistently point out the larger selectivity of cyclopeptide *2* for the binding to the $\alpha_v\beta_3$ integrin.

Table 2. Binding affinities (kcal mol^{-1}) determined for different internal permittivities in MM/PBSA calculations.

Compound	**Integrin**	**Binding affinity**		
		$\varepsilon = 4$	$\varepsilon = 10$	$\varepsilon = 20$
Cilengitide (**A**)				
	$\alpha_v\beta_3$	-11.3	-10.8	-10.3
	$\alpha_v\beta_5$	-13.1	-11.5	-10.8
	$\Delta\Delta G$	+1.8	+0.7	+0.5
Cyclopeptide **2**				
	$\alpha_v\beta_3$	-8.7	-8.1	-7.6
	$\alpha_v\beta_5$	-8.0	-6.1	-5.3
	$\Delta\Delta G$	-0.7	-2.0	-2.3

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