A dimeric bicyclic RGD ligand displays enhanced integrin binding affinity and strong biological effects on U-373 MG glioblastoma cells

Electronic Supplementary Information (ESI)

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MATERIALS AND METHODS

All manipulations requiring anhydrous conditions were carried out in flame-dried glassware, with magnetic stirring and under nitrogen atmosphere. All commercially available reagents were used as received. The reaction outcome was controlled by analytical Thin Layer Chromatography (TLC) using Macherey-Nagel 0.20 mm silica gel 60 with fluorescent indicator pre-coated polyester sheet (40 x 80 mm). Spot visualization was accomplished by irradiation of UV lamp and/or staining with ninhydrin. The resin and the amino acids used in the SPPS were purchased from Merck KGaA Novabiochem®. Dry *i*Pr_2NEt was distilled over CaH_2 and transferred under nitrogen. Dry DMF (over molecular sieves in bottles with crown cap) was purchased from Sigma Aldrich, stored under nitrogen, and withdrawn from the container by syringe, under a slight positive pressure of nitrogen. High-resolution Mass Spectrometry (HRMS) spectra were recorded on the ion trap mass spectrometer Finnigan LCQ Advantage analysing an aqueous solution of the analyte.

GENERAL PROCEDURE FOR SYNTHESIS OF BICYCLIC PEPTIDES

LOADING ANALYSIS OF FIRST AA ON 2CTC RESIN

Fmoc deprotection of loaded residue was performed manually. The Fmoc group was removed treating the resin twice with 20% (v/v) piperidine in DMF (3 mL per each step, first cycle lasted 1 min and the second one lasted 10 min). The liquid phase was collected using a nitrogen flow inside the vessel and the total volume is carefully measured. The beads were washed with DMF, DCM and again with DMF (3 mL per each step, 5 x 30 s per each solvent). A fraction of deprotection solution was diluted 1:1000 with 20% (v/v) piperidine in DMF in order to respect the linearity of the Lambert-Beer law at λ=301 nm (if necessary, make further dilutions). 20% (v/v) piperidine in DMF solution was the blanc. The loading was calculated using this formula:

$$X = \frac{A(301\ mn) * V * F_d}{\varepsilon(301\ mn) * m * b}$$

Where:

X = loading on the resin (mmol/g);

A (301 nm) = absorbance of the solution measured at 301 nm;

V = total volume of collected deprotection solution (6 mL in this case);

F_d = dilution factor;

ε (301 nm) = 7800 M^{-1} cm^{-1};

m = mass of the resin (g);

b = length of the cell (cm).

RP-HPLC PURIFICATION

The HPLC purifications were performed using a Dionex Ultimate 3000 instrument equipped with a Dionex RS Variable Wavelength Detector (column: Atlantis® Prep T3 OBDTM 5 µm 19 × 100 mm). The crude reaction mixture was dissolved in H_2O and/or acetonitrile (ACN). The solution so obtained was filtered over HPLC syringe filters (Sigma Aldrich, polypropylene, 0.45 µm, 13 mm ø, PK/100) and injected in the HPLC in order to isolate the purified products.

HPLC ANALYSIS

Purity analysis was carried out on a Waters HPLC instrument (2 Waters 515 HPLC pumps equipped with 996 photodiode array detector and Waters Atlantis® T3 - 5 µm - 4.6 x 100 mm column). 0.10 mg of analyte was dissolved in 500 µL of H_2O and 200 µL of this solution are injected. The analysis was performed using the same gradient used in the purification step. Chromatograms were recorded at 210 nm. Analysis of peak integrals and the relative percentage of purity was performed with Empower software.

FREEZE-DRYING

The product was dissolved in H_2O and frozen with dry ice: the freeze-drying was carried out at least for 48 h at -50 °C using the instrument 5Pascal Lio5P DGT, equipped with a Edwards rotary vacuum pump.

Synthesis of side-chain protected peptide via semi-automatic SPPS

The semi-automatic SPPS was carried out using a Biotage® Initiator™ synthesizer, assisted by microwave (MW) irradiation; Fmoc/tBu strategy and 2-ClTrt resin were used for bicyclic derivative **1, 2** and **3**. The monocyclic RGD peptide **7** was prepared starting from a Rink amide 4-methylbenzhydrylamine (MBHA) resin with the same strategy of the bicyclic peptides.

Each coupling step consisted in:

1) activation of the Fmoc-protected amino acid;

2) addition of the activated amino acid to the resin at the synthesizer to start the coupling;

3) steps of washing, deprotection and washing again.

Before these steps, when the first AA of the peptide was not the Gly, it needed a coupling step of the first AA (in our case β-Ala) to the 2-ClTrtCl resin.

SOLUTIONS AND SOLVENTS

To perform the automated SPPS, one solution and four solvents were prepared: 20% piperidine in DMF (v/v), DCM, DMF, MeOH and Et_2O. DCM was used both as swelling and washing solvent, DMF was necessary either for washings and as solvent for the reactions of coupling and deprotection, MeOH is used as washing solvent and capping agent, Et_2O is used as drying solvent for resin storage.

RESIN PREPARATION

The resin was weighted in a 10 mL Teflon vial (Biotage) and processed with the swelling task. The resin was then ready for peptide synthesis.

ACTIVATION OF FMOC-AA-OH

Activation of Fmoc-AA-OH took place as follows: a solution of Fmoc-AA-OH (4.0 eq) in DMF dry (3 mL) was cooled to 0°C in an ice bath. HOAt (4.0 eq), DIPEA (8.0 eq) and DIC (4.0 eq) were added to the solution and the mixture was stirred for 15 min at 0°C. After that, the reaction mixture was added to the swollen resin (1.0 eq) in order to start the coupling reaction.

BIOTAGE® INITIATOR™ PROGRAMS

The Biotage® Initiator™ programs ("Tasks") used for the semi-automatic SPPS are reported below. Each task can be modified in every parameter and it is performed under vortex mixing at 800 rpm.

Swelling task: 3 mL of DCM were added to the vial and the mixture was stirred for 20 min at RT. It followed the removal of DCM and 3 mL of DMF were added to the vial. The mixture was then stirred at RT for 5 min and the DMF was removed in order to obtain swollen beads.

Coupling of Fmoc-β-Ala-OH on resin – capping with MeOH: a solution of Fmoc-β-Ala-OH (398.5 mg, 1.28 mmol, 4.0 eq) and DIPEA (446 µL, 2.56 mmol, 8.0 eq) in 3 mL of a mixture DCM:DMF 1:1 was prepared. The resulting solution was added to the swollen resin (200 mg, 1.0 eq) and the coupling was made at RT for 1 hour. It followed the capping with MeOH (3.0 mL, 15 min at RT). The beads were washed with DMF, DCM, MeOH and DMF (6x3.0 mL, 2x3.0 mL, 2x3.0 mL, 2x3.0 ml, respectively. 20 sec for each wash step).

Coupling MW - Fmoc deprotection: The activated Fmoc-AA-OH residue was added to the 2-ClTrt resin in the reaction vessel of the synthesizer and the coupling reaction assisted by microwaves was carried out at 75 °C under inert atmosphere for 10 min. At the end of the reaction, the beads were washed twelve times with DMF (3 mL × 20 s for every wash). Two deprotection steps were then performed by adding the deprotection solution (20% v/v piperidine in DMF, 3.0 mL for each step) to the beads: the reaction was performed at r.t. under inert atmosphere for 2 min and 10 min for the first and the second deprotection step, respectively. The beads were washed six times with DMF, twice with DCM and twice with DMF (3.0 mL × 20 s for every wash). At the end of each step, the resin was rinsed and ready for the next coupling reaction.

Coupling MW – Capping - Fmoc deprotection: The activated Fmoc-AA-OH residue was added to the Rink Amide MBHA resin in the reaction vessel of the synthesizer and the coupling reaction assisted by microwaves was carried out at 75 °C under inert atmosphere for 10 min. At the end of the reaction, the beads were washed twelve times with DMF (3 mL × 20 s for every wash). To the washed beads, a capping step was performed adding 3 mL of an acetic anhydride solution (20% Ac_2O in DMF). The beads were stirred for 15 min at r.t. and then they were washed six times with DMF. It followed two deprotection steps that were carried out adding the deprotection solution (20% v/v piperidine in DMF, 3.0 mL for each step) to the beads: the reaction was performed at r.t. under inert atmosphere for 2 min and 10 min for the first and the second deprotection step, respectively. The beads were washed six times with DMF, twice with DCM and twice with DMF (3.0 mL × 20 s for every wash). At the end of each step, the resin was rinsed and ready for the next coupling reaction.

Resin drying with Et_2O: 3 mL of Et_2O were added to the vessel and the mixture is stirred for 1 min at RT. Then the beads were washed six times with Et_2O (3.0 mL per each wash, 5x 45 sec and, for the last one, 1x30 sec with a 2 min draining).

RESIN STORAGE

The 2-ClTrt attached peptides were stored at -20 °C after the drying task. The next SPPS cycle always starts with a swelling step before continuing the peptide synthesis. The Rink amide MBHA attached peptide were stored swollen at -20°C in DMF dry with the N-terminus equipped with Fmoc protecting group.

Peptide cleavage from resin

The cleavage reactions from the resin were performed manually, under inert atmosphere and vortex mixing. The resin was swollen as reported before. In the meantime, 12 mL of cleavage cocktail were prepared mixing 1.2 mL of glacial acetic acid, 2.4 mL of 2,2,2-trifluoroethanol (TFE) and bringing the total volume to 12 mL with DCM. The resin was washed 4 times for 20 minutes with 3 mL of cleavage cocktail. Each liquid fraction was collected by flushing nitrogen into the vessel. Cold Et_2O was added to the cleaved peptide solution in the cleavage cocktail, in order to precipitate the peptide. The solid was isolated by centrifugation, dissolved in DCM:MeOH mixture and concentrated in vacuo. The crude product was used as starting material for the next synthetic steps.

Synthesis of cyclic protected peptide via intramolecular coupling reaction (macrolactamization)

In a two-neck round-bottom flask, under inert atmosphere and flame-dried, the protected peptide (1.0 eq) was dissolved in dry DMF (C = 1.4 mM referred to the protected peptide). The solution was cooled to 0°C and HATU (4.0 eq), HOAt (4.0 eq) and DIPEA (6.0 eq) were added in the order reported before. The reaction mixture changed color form colorless to yellow. The mixture was stirred at 0°C for 1 hour and after it was stirred at RT overnight. The end of the reaction was monitored by TLC (eluent: DCM:MeOH 9:1). The solvent was removed at the high-vacuum pump. The resulting solid was dissolved in AcOEt (40 mL), the organic phase was washed 3 times with $KHSO_4$ 1 M (3x15 mL) and one time with brine (20 mL). The resulting organic phase was dried with sodium sulfate, the solid was filtered and the solvent was removed in vacuo. The resulting crude was purified by flash-chromatography (eluent DCM:MeOH 95:5) obtaining the desired product.

Synthesis of cyclic deprotected peptide via acid cleavage

For this synthetic step, a deprotection cocktail made by TFA:TIS:H_2O 95:2.5:2.5 must be prepared in a quantity necessary to obtain a 0.02 M solution referred to the starting material. In a round-bottom flask, the cleavage cocktail was added to the cyclic protected peptide (1.0 eq) at 0 °C. The reaction mixture was stirred at RT for 2 hours, giving different colors from yellow to violet passing through orange. The reaction mixture was concentrated in vacuo using a NaOH trap and cold Et_2O was added to the resulting mixture in order to precipitate the product. The crude was isolated by centrifugation and the resulting solid was used in the next synthetic steps.

Synthesis of bicyclic peptide via disulfide bridge formation

In a round-bottom flask, the cyclic deprotected peptide (1.0 eq) was dissolved in a mixture H_2O/ACN 1:1 (C= 2.17 mM, the solvents must be HPLC grade). I_2 (20.0 eq) was added to the solution and the mixture is stirred for 30 minutes at RT. The reaction mixture is concentrated in vacuo and the resulting crude was purified in RP-HPLC and the isolated product is freeze-dried.

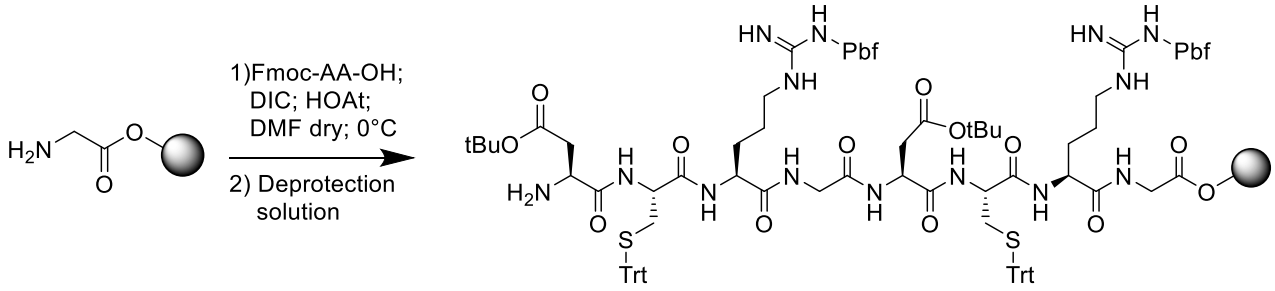
Peptide cleavage from the resin and simultaneous deprotection

The cleavage reactions from the resin were performed manually, under inert atmosphere and vortex mixing. The protected on-beads peptide was swollen first with DMF (3 mL), then with dichloromethane (3 mL). Under stirring and nitrogen atmosphere, the beads were treated three times with the cleavage cocktail (3.0 mL per 0.1 mmol of resin) 95:2,5:2,5 TFA /TIS/H_2O (v/v/v). After 1 h, the liquid phase was filtered off under nitrogen flow and collected in a round bottom flask: the beads were washed with neat TFA (1.0 mL) that was collected. The combined filtered fractions were concentrated and poured in cold diethyl ether, provoking precipitation of the product. Diethyl ether was removed with a centrifuge affording the crude product that underwent to oxidation step.

SYNTHESIS OF BICYCLIC PEPTIDES 4-6

Synthesis of the dimeric RGD bicyclic peptide 1

The synthesis was accomplished following the procedures reported before. The followed synthetic pathway is reported below starting from commercially available H-Gly-2-ClTrt resin (450 mg, 0.234 mmol).



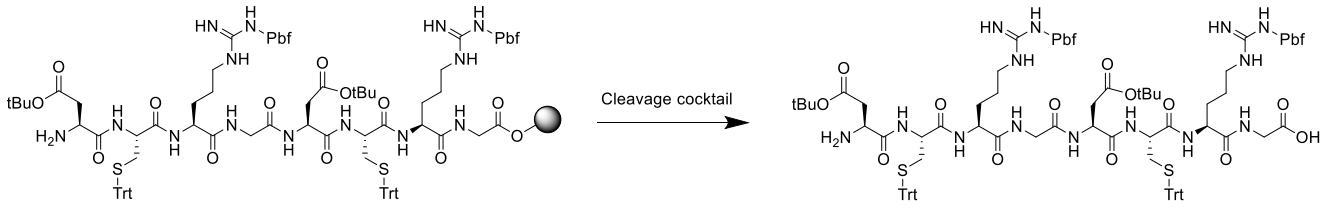
5 **6**

The order and the exact quantity of Fmoc-AA-OH used for each coupling step is reported before:

Table 1. Amounts of amino acid derivatives used in the SPPS of compound **6**

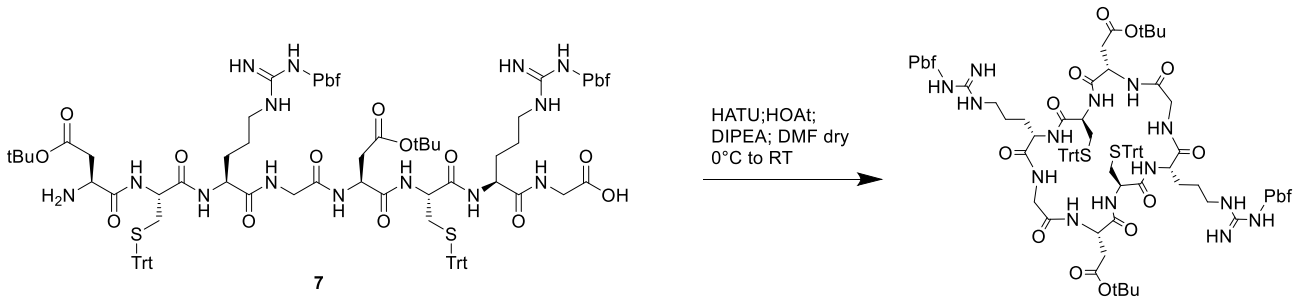
Fmoc-AA-OH	Molecular Weight (g/mol)	Amount (mg)
Fmoc-Arg(Pbf)-OH	648.78	607.2
Fmoc-Cys(Trt)-OH	585.71	548.2
Fmoc-Asp(OtBu)-OH	411.15	384.8
Fmoc-Gly-OH	297.31	278.3
Fmoc-Arg(Pbf)-OH	648.78	607.2
Fmoc-Cys(Trt)-OH	585.71	548.2
Fmoc-Asp(OtBu)-OH	411.15	384.8

Peptide cleavage:



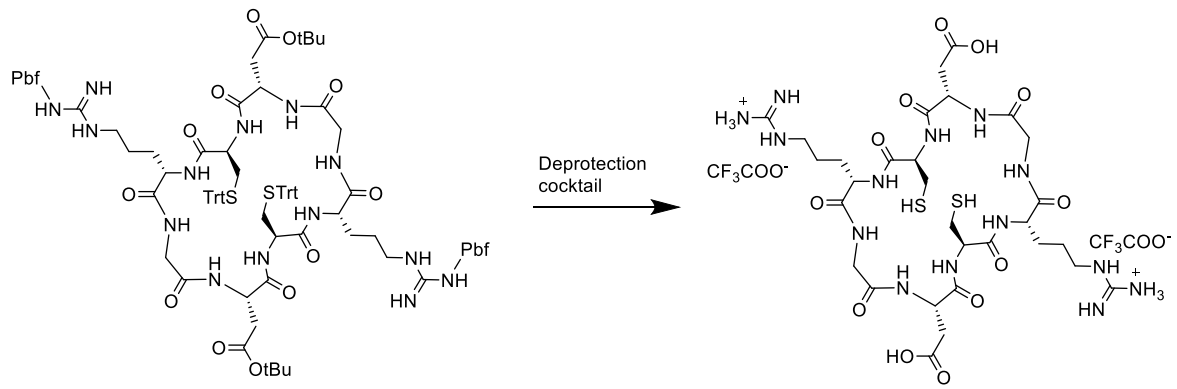
6 7

After the last step of the cleavage, 91.5 mg of solid was obtained and used as starting material for the cyclization step without further purifications:



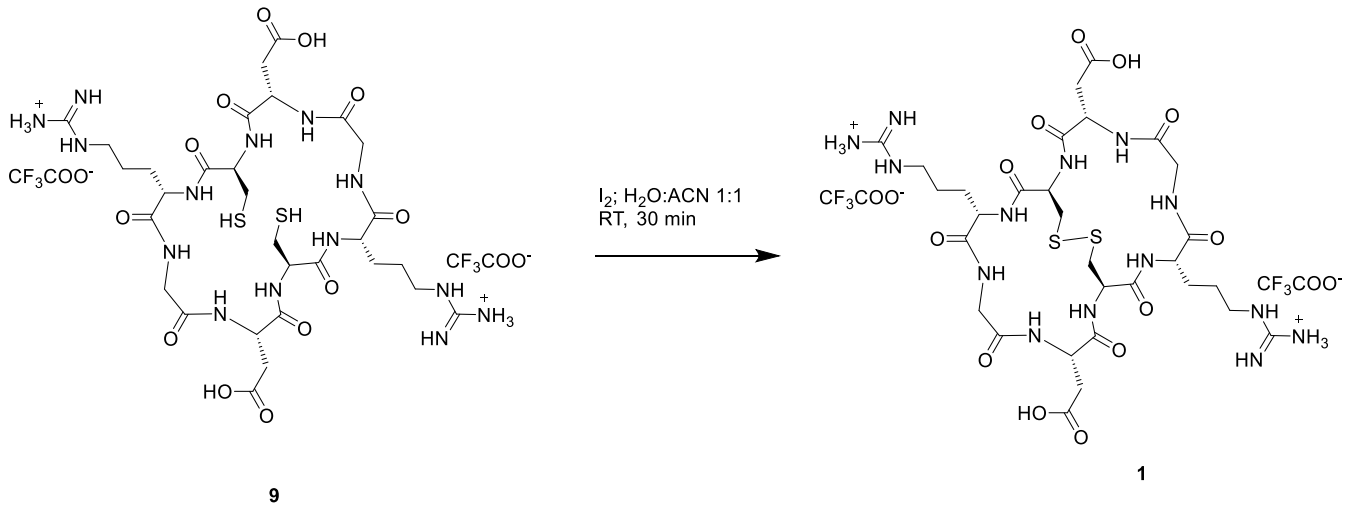
7 8

After flash-chromatography (eluent DCM:MeOH 9:1, Rf = 0.56), 67.3 mg of product were obtained (Y = 14,64 % over three steps). It followed the deprotection step:



8 9

After this step, 30.1 mg of crude were obtained and used as starting material for the oxidation step without further purifications:



9 1

The crude of this reaction was purified in RP-HPLC using the separation conditions reported below:

Flow: 10 mL/min; UV channels:210 nm; 221 nm; solvents: H_2O + 0.1% TFA, ACN without TFA

Table 2. Gradient program used for RP-HPLC purification of compound **1**

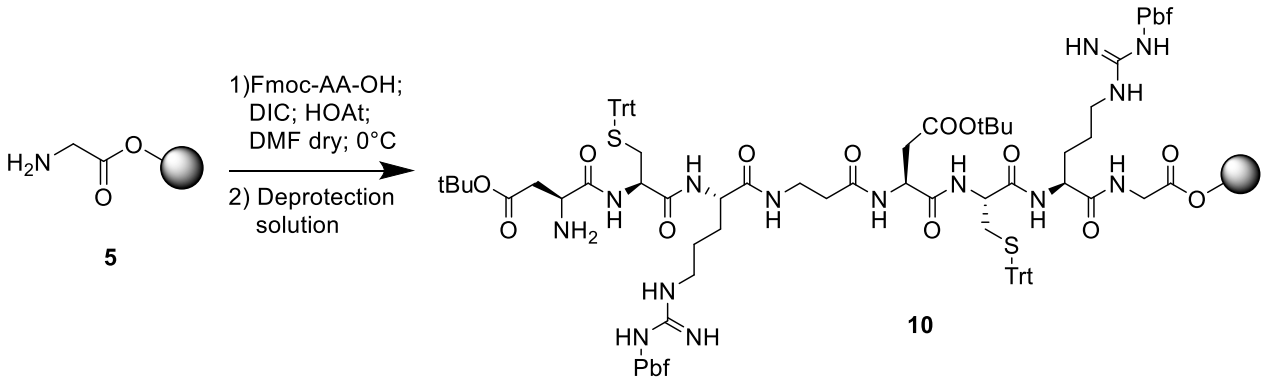
Time (min)	% ACN
0	0
2	0
15	40
16	100
18	100
19	0
20	0

t_r of the product = 9.25 min. The desired product is freeze-died, obtaining its trifluoroacetate salt that appears as a white solid (7.6 mg, yield = 20,54 % over two steps).

MS (ESI) *m/z* calculated for $[C_{30}H_{49}N_{14}O_{12}S_2]^+$: 861.3105; found: 861.3096 $[M+H]^+$

Synthesis of the monomeric RGD bicyclic peptide 2

The synthesis was accomplished following the procedures reported before. The followed synthetic pathway is reported below starting from commercially available H-Gly-2-ClTrt resin (300 mg, 0.156 mmol).

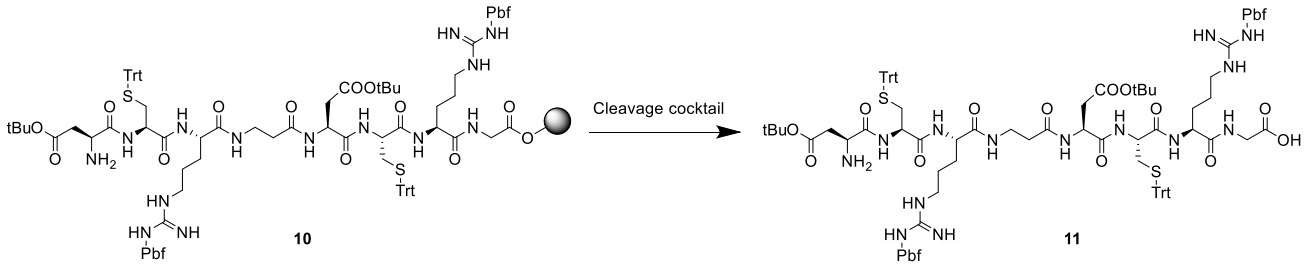


The order and the exact quantity of Fmoc-AA-OH used for each coupling step is reported before:

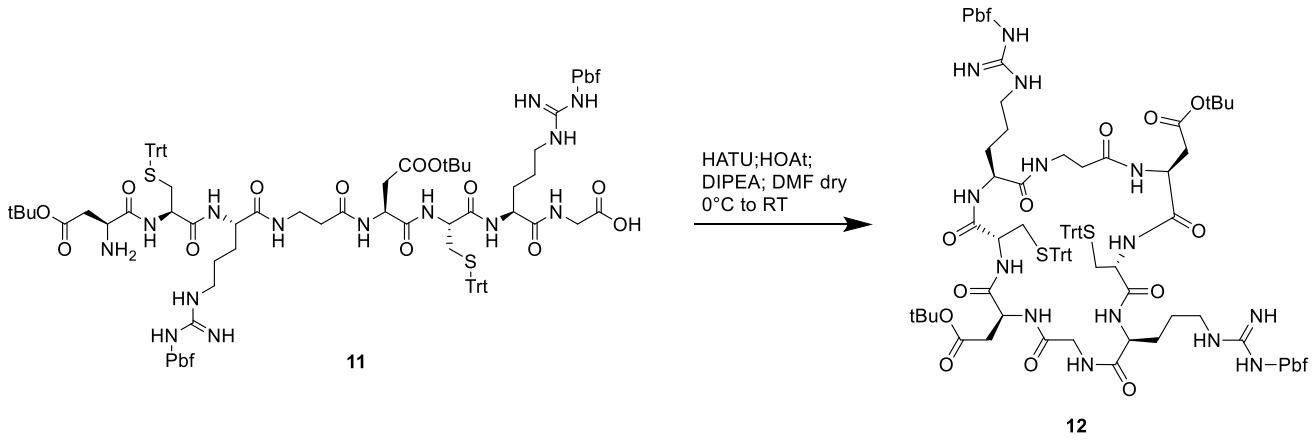
Table 3. Amounts of amino acid derivatives used in the SPPS of compound **10**

Fmoc-AA-OH	Molecular Weight (g/mol)	Amount (mg)
Fmoc-Arg(Pbf)-OH	648.78	404.8
Fmoc-Cys(Trt)-OH	585.71	365.5
Fmoc-Asp(OtBu)-OH	411.15	256.7
Fmoc-β-Ala-OH	311.33	194.3
Fmoc-Arg(Pbf)-OH	648.78	404.8
Fmoc-Cys(Trt)-OH	585.71	365.5
Fmoc-Asp(OtBu)-OH	411.15	256.7

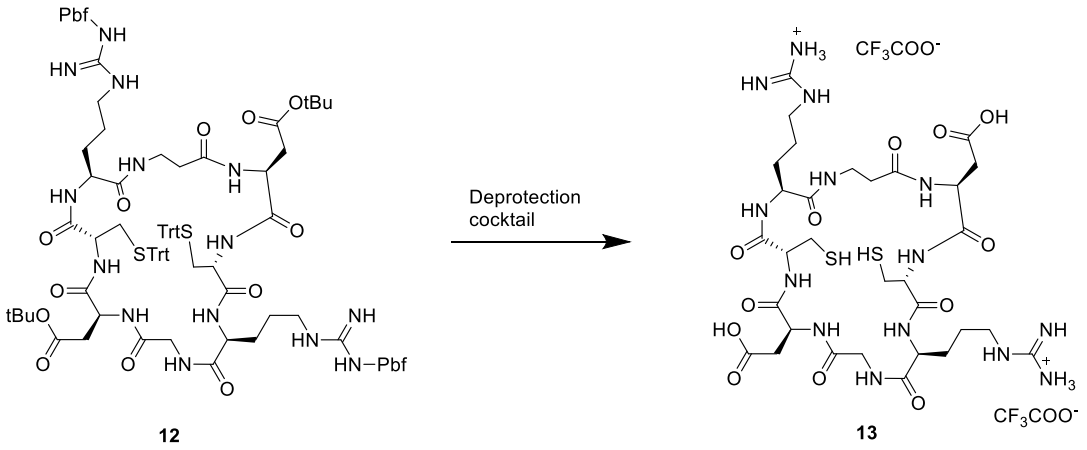
Peptide cleavage:



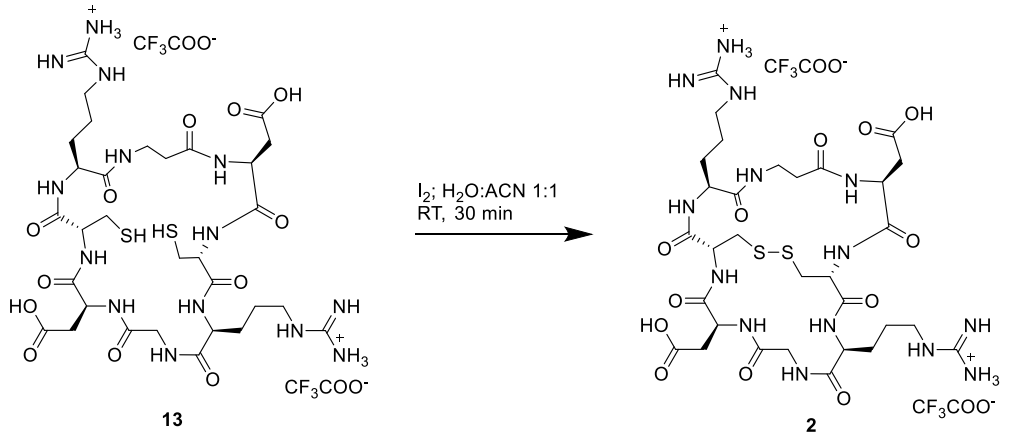
After the last step of the cleavage, 95.4mg of solid was obtained and used as starting material for the cyclization step without any further purifications:



After flash-chromatography (eluent DCM:MeOH 95:5, Rf = 0.092), 59.6 mg of product were obtained (Y = 19,31% over three steps). It followed the deprotection step:



After this step, 29.2 mg of crude were obtained and used as starting material for the oxidation step:



13 2

The crude of this reaction was purified in RP-HPLC using the separation conditions reported below:

Flow: 10 mL/min; UV channels:210 nm; 221 nm; solvents: H_2O + 0.1% TFA, ACN without TFA

Table 4. Gradient program performed for RP-HPLC purification of compound **2**

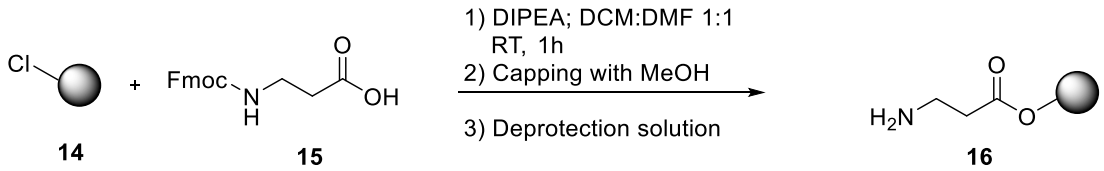
Time (min)	% CAN
0	0
2	0
15	40
16	100
18	100
19	0
20	0

t_r of the product = 9.75 min. The desired product is freeze-died, obtaining its trifluoroacetate salt that appears as a white solid (6.2 mg, Y = 18.67% over two steps).

MS (ESI) m/z calculated for $[C_{31}H_{51}N_{14}O_{12}S_2]^+$: 875.3252; found:875.3244 $[M+H]^+$

Synthesis of the dimeric RβAD bicyclic peptide 3

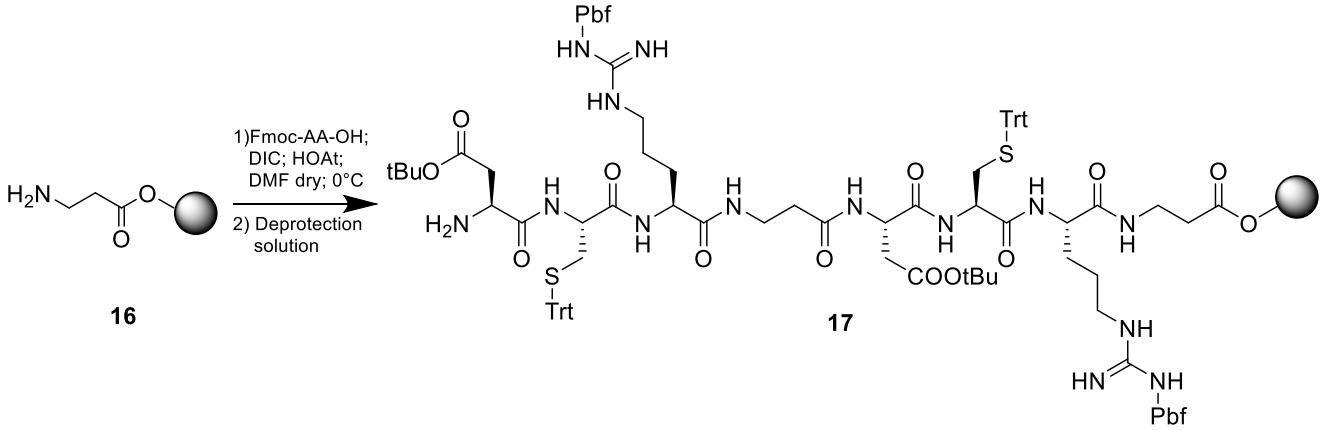
The synthesis was accomplished following the procedures reported before. The followed synthetic pathway is reported below starting from 2-ClTrt resin (200 mg) and coupling Fmoc-β-Ala-OH (398.5 mg, 1.28 mmol) to the resin:



14 15 16

The loading was determined as reported before. X = 1.74 mmol/g, obtaining a quantitative coupling because the biggest loading reported on the commercially available resin is 1.6 mmol/g.

SPPS step:

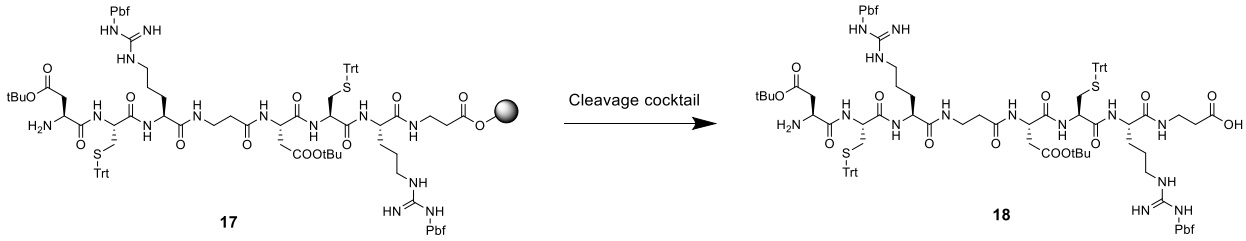


The order and the exact quantity of Fmoc-AA-OH used for each coupling step is reported before:

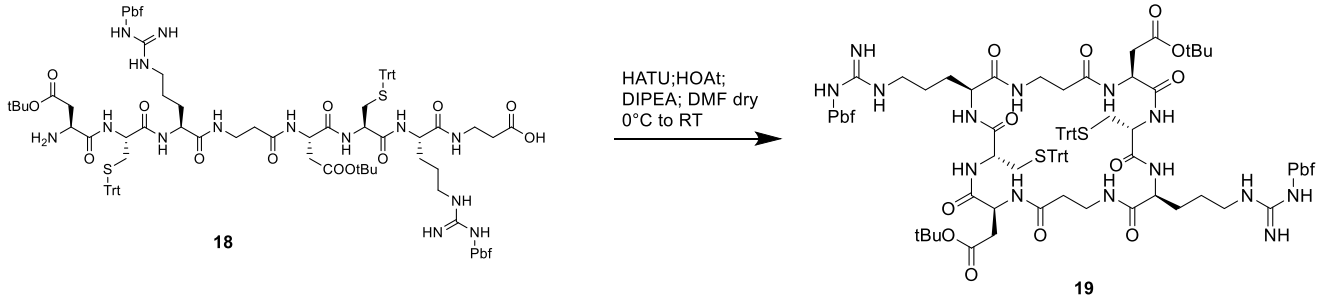
Table 5. Amounts of amino acid derivatives used in the SPPS of compound **17**

Fmoc-AA-OH	Molecular Weight (g/mol)	Amount (mg)
Fmoc-Arg(Pbf)-OH	648.78	892.7
Fmoc-Cys(Trt)-OH	585.71	805.9
Fmoc-Asp(OtBu)-OH	411.15	565.7
Fmoc-β-Ala-OH	311.33	428.3
Fmoc-Arg(Pbf)-OH	648.78	892.7
Fmoc-Cys(Trt)-OH	585.71	805.9
Fmoc-Asp(OtBu)-OH	411.15	565.7

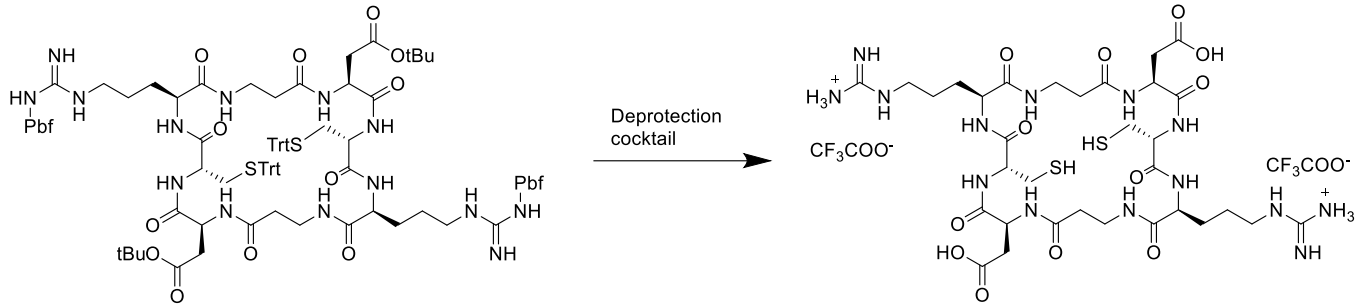
Peptide cleavage:



After the last step of the cleavage, 393.1 mg of solid was obtained and used as starting material for the cyclization step:

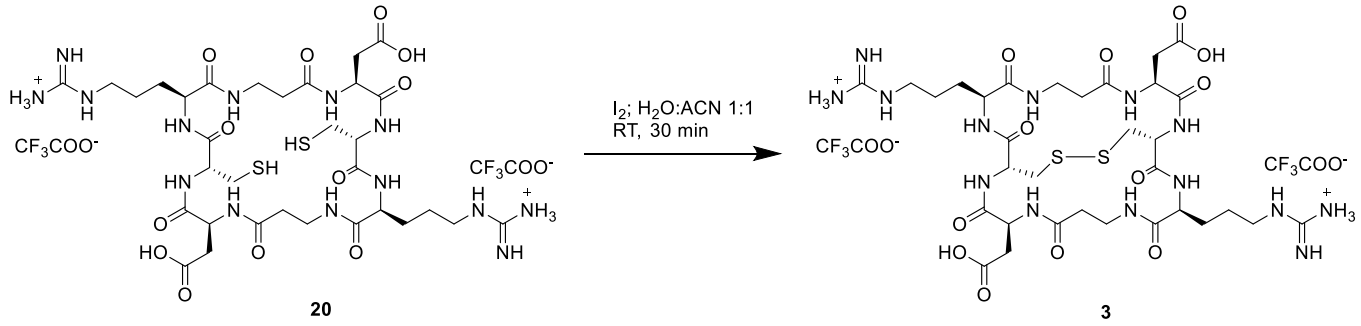


After flash-chromatography (eluent DCM:MeOH 9:1, Rf = 0.53), 134.8 mg of product were obtained (Y = 19.66% over three steps). It followed the deprotection step:



19 20

After this step, 64.7 mg of crude were obtained and used as starting material for the oxidation step:



20 3

The crude of this reaction was purified in RP-HPLC using the separation conditions reported below:

Flow: 10 mL/min; UV channels:210 nm; 221 nm; solvents: H_2O + 0.1% TFA, ACN without TFA

Table 6. Gradient program performed for RP-HPLC purification of compound **3**

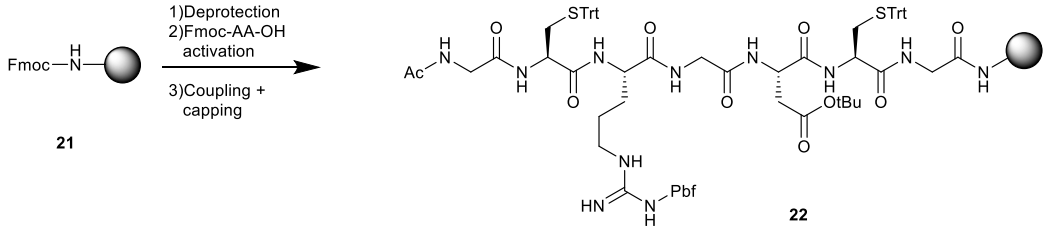
Time (min)	% CAN
0	0
2	0
15	40
16	100
18	100
19	0
20	0

t_r of the product = 10.4 min. The desired product is freeze-died, obtaining its trifluoroacetate salt that appears as a white solid (26.1 mg, Y = 34,59% over two steps).

MS (ESI) m/z calculated for $[C_{32}H_{53}N_{14}O_{12}S_2]^+$: 889.3409; found:889.3401 $[M+H]^+$

Synthesis of the monocyclic RGD peptide 4

The synthesis was accomplished using a Rink amide MBHA resin (loading = 0.5 mmol/g). The followed synthetic pathway is reported below starting from SPPS step (100 mg, 0.05 mmol).

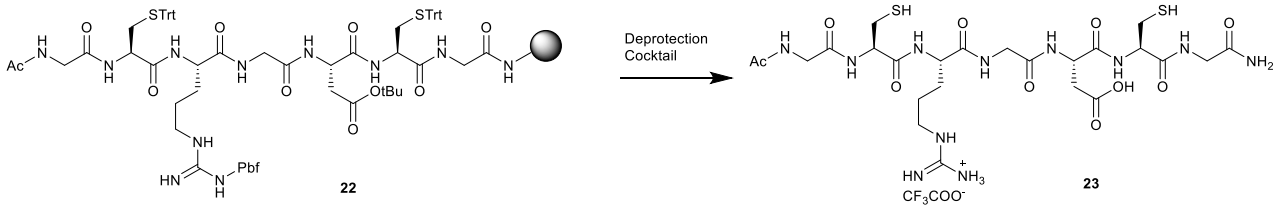


The order and the exact quantity of Fmoc-AA-OH used for each coupling step is reported before:

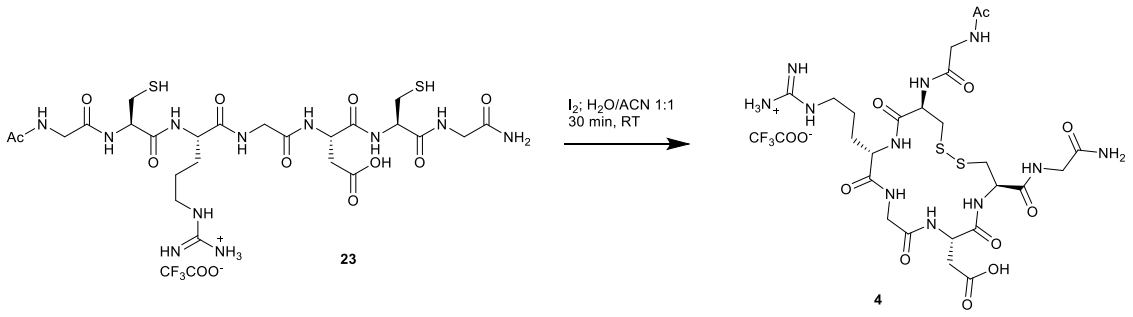
Table 7. Amounts of amino acid derivatives used in the SPPS of compound **22**

Fmoc-AA-OH	Molecular Weight (g/mol)	Amount (mg)
Fmoc-Arg(Pbf)-OH	648.78	129.7
Fmoc-Cys(Trt)-OH	585.71	2 x 117.1
Fmoc-Asp(OtBu)-OH	411.15	82.3
Fmoc-Gly-OH	297.31	3 x 59.5

It followed the simultaneous cleavage and deprotection step step:



After the cleavage and deprotection step, 80,9 mg of solid was obtained and used as starting material for the oxidation step without any further purifications:



The crude of this reaction was purified in RP-HPLC using the separation conditions reported below:

Flow: 10 mL/min; UV channels:210 nm; 221 nm; solvents: H_2O + 0.1% TFA, ACN without TFA

Table 8. Gradient program performed for RP-HPLC purification of compound **4**

Time (min)	% ACN
0	0
2	0
12	20
13	100
15	100
16	0
17	0

t_r of the product = 9 min. The desired product is freeze-died, obtaining its trifluoroacetate salt that appears as a white solid (31,7 mg, Y = 76,6% over three steps).

MS (ESI) m/z calculated for $[C_{24}H_{40}N_{11}O_{10}S_2]^+$: 706,2401; found: 706,2394 $[M+H]^+$

PROTOCOLS FOR BIOLOGICAL EVALUATION

Solid Phase Receptor Binding Assays

Recombinant human integrin receptors $\alpha_v\beta_3$ or $\alpha_5\beta_1$ (R&D Systems, Minneapolis, MN, USA) were diluted to 0.5 mg mL^{-1} in coating buffer containing 20 mm Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. An aliquot of diluted receptors (100 µL/well) was added to 96-well microtiter plates (Nunc MaxiSorp) and incubated overnight at 4 °C. The plates were then incubated with blocking solution (coating buffer plus 1% bovine serum albumin) for 2 h at room temperature to block nonspecific binding. After washing 2 times with blocking solution, plates were incubated shaking for 3 h at room temperature, with various concentrations (10^{-5}-10^{-12} M or 10^{-4}-10^{-11}) of test compounds in the presence of 1 µg/mL biotinylated vitronectin (Molecular Innovations, Novi, MI, USA) in the case of $\alpha_v\beta_3$ integrin or biotinylated fibronectin (Sigma, St. Luis MO, USA) in the case of integrin $\alpha_5\beta_1$. Biotinylation was performed using an EZ-Link Sulfo-NHS-Biotinylation kit (Pierce, Rockford, IL, USA). After washing 3 times, the plates were incubated shaking for 1 h, at room temperature, with streptavidin-biotinylated peroxidase complex (Amersham Biosciences, Uppsala, Sweden). After washing 3 times with blocking solution, plates were incubated with 100 µL/well of Substrate Reagent Solution (R&D Systems, Minneapolis, MN, USA) for 30 min, in the dark, with shaking. After stopping the reaction with the addition of 50 µL/well 2N H_2SO_4, absorbance at 415 nm was read in a SynergyTM HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). Each data point represents the average of triplicate wells; data analysis was carried out by nonlinear regression analysis with GraphPad Prism software (GraphPad Prism, San Diego, CA, USA). Each experiment was repeated in triplicate.

Cell Culture

The U-373 MG human glioblastoma cell lines were purchased from Istituto Zootecnico Regione Lombardia (Brescia, Italy). The cell lines were grown in DMEM supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, penicillin-streptomycin (10000 u/ml) and cells were grown at 37 °C in controlled atmosphere (5% CO_2/95% air). Confluent cells were split (1:5-1:10 ratio) by trypsinization and used at the third-fourth passage after thawing. For all the experiments the cells were plated at a density of 10000 cells/cm^2. The reagents used for the cell cultures were from Euroclone, Italy.

Real time quantitative RT-PCR (qRT-PCR)

For mRNA expression analysis, RNA was extracted from U-373 MG cells with Quiazol (Qiagen), followed by a DNAse digestion step. RNA quality was assessed by measuring the 260/280 ratio and concentration was estimated at 260 nm. The primers were designed using the Primer3 Input software; and the specificity of each primer was checked by BLAST analysis. Primers used for integrin subunits and for the housekeeping gene RPL6 in quantitative real time RT-PCR reactions have been reported in Table 9.

Table 9. qRT-PCR analysis of gene expression in U-373 MG cells

Gene	Ct	ACCESSION NUMBER	PRIMER SEQUENCE
α_v	18,32	NM_002210	F: actggcttaagagagggctgtg R: tgccttacaaaaatcgctga
α_5	25,71	NM_000212	R: tcctcaggaaaggtccaatg R: tcctcaggaaaggtccaatg
β_1	29,06	NM_002213	F: agcctatctccacgcacact R: cctcggagaaggaaacatca
β_3	16,05	NM_002205	F: cctgctgtccaccatgtcta R: ttaatggggtgattggtggt
β_5	19,96	NM_133376	F: tccaatggcttaatttgtgg R: cgttgctggcttcacaagta
GAPDH	14,78	NM_002046.5	R: cagcaagagcacaagaggaag F: caactgtgaggaggggagatt

At the end of the reaction, a melting curve analysis was carried out to check for the presence of primer-dimers. Experiments were performed on three different cell preparations and each run was analyzed in duplicate. Data are expressed as Ct (Table 9), defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample.

Cell Detachment Assays

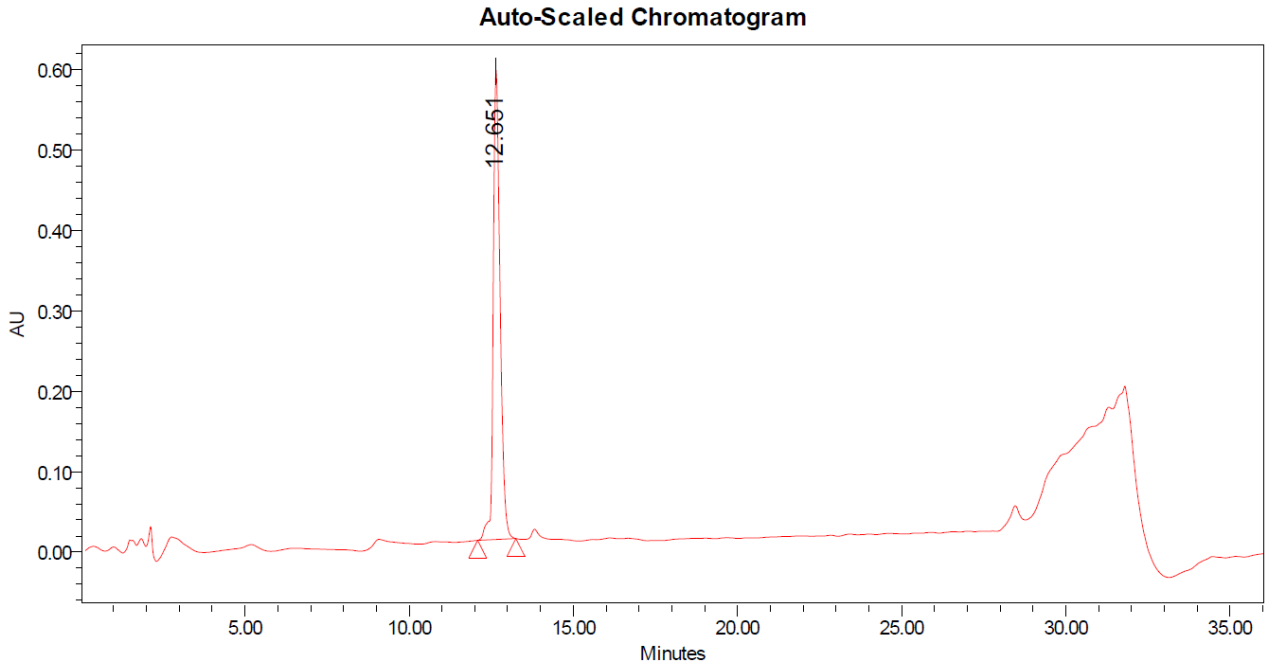
Cells plated in 96 multiwells in the growth medium (10.000 cells/100 µl per well) were treated with cell culture medium containing 5, 10, 20 50 µM concentrations of compounds **1**-**3** for 48 hours. Compound **1**, **2** and **3** stock solutions (200 mM in PBS) were diluted in the growth medium and added to the wells. In control wells only the growth medium was added. At the end of treatments, wells were rinsed three times with PBS and floating cells were removed; cell viability was therefore measured in adherent cells only. 20 µl of MTS reagent (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega) were added to each well. After incubation for 3 hours under standard conditions, the absorbance was read in a multiwell plate reader at 450 nm. Six wells were used for each experimental point and each independent experiment was performed three times in quadruplicate.

Western Blot Analysis

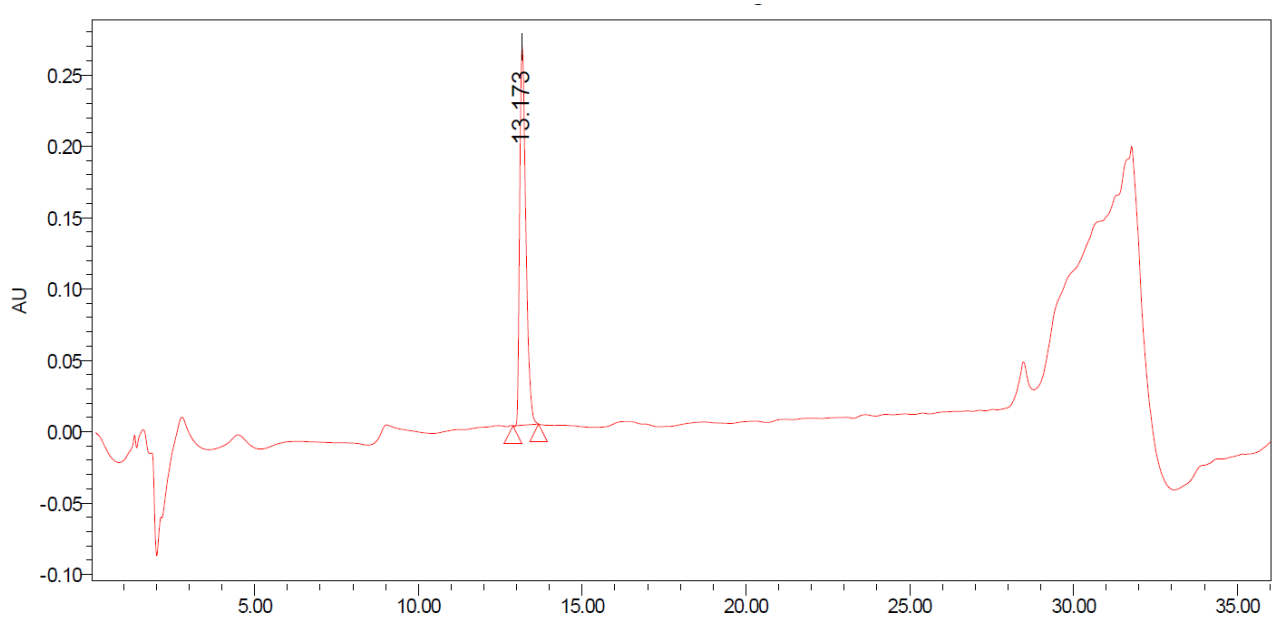
Cells grown in 60 mm dishes were treated for 48 hours with 50 µM compounds **1-3**. The cells were then rinsed twice in ice-cold PBS, and 200 ml of cell lysis buffer were added to the dishes (composition: 50mM Tris-HCl pH 7.4, 1% v/v NP40, 0.25% w/v sodium deoxycholate, 1 mM phenylmethylsulphonyl-fluoride (PMSF), 1mM Na_3VO_4, 1 mM EDTA, 30 mM sodium pyrophosphate, 1 mM NaF, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mg/ml aprotinin and 1 mg/ml microcystin). After scraping, the cells were sonicated for 10 seconds, centrifuged at 12.000xg for 5 min at 4 °C. The amount of proteins in the supernatant was then measured by the BCA protein Assay Kit (Pierce). For Western blot analysis, 30 µg of proteins were separated by 10% SDS-PAGE at 150 V for 2 hours and blotted onto 0.22 mm nitrocellulose membranes at 50 mA for 16 hours. The membranes were first blocked for 2 hours in Tris buffered saline solution (TBST composition: Tris 10 mM, NaCl 150 mM, 0.1% Tween 20) plus 5% low fat dry milk (TBSTM) and then incubated with the appropriate antibody diluted to 1:1000 in 5% albumin (pFAK) or TBSTM (FAK), for 16 hours at 4 °C under gentle agitation. The membranes were rinsed three times in TBST and then incubated for 2 hours at 21 °C with a goat anti-rabbit IgG horseradish-peroxidase conjugate secondary antibody (Upstate Biotechnology), diluted to 1:10 000 in TBSTM. The membranes were rinsed three times in TTBS and the luminescent signal was captured with the Image Quant LAS4000, General Electric. Experiments were repeated three times in quadruplicate.

HPLC PROFILES OF FINAL PRODUCTS

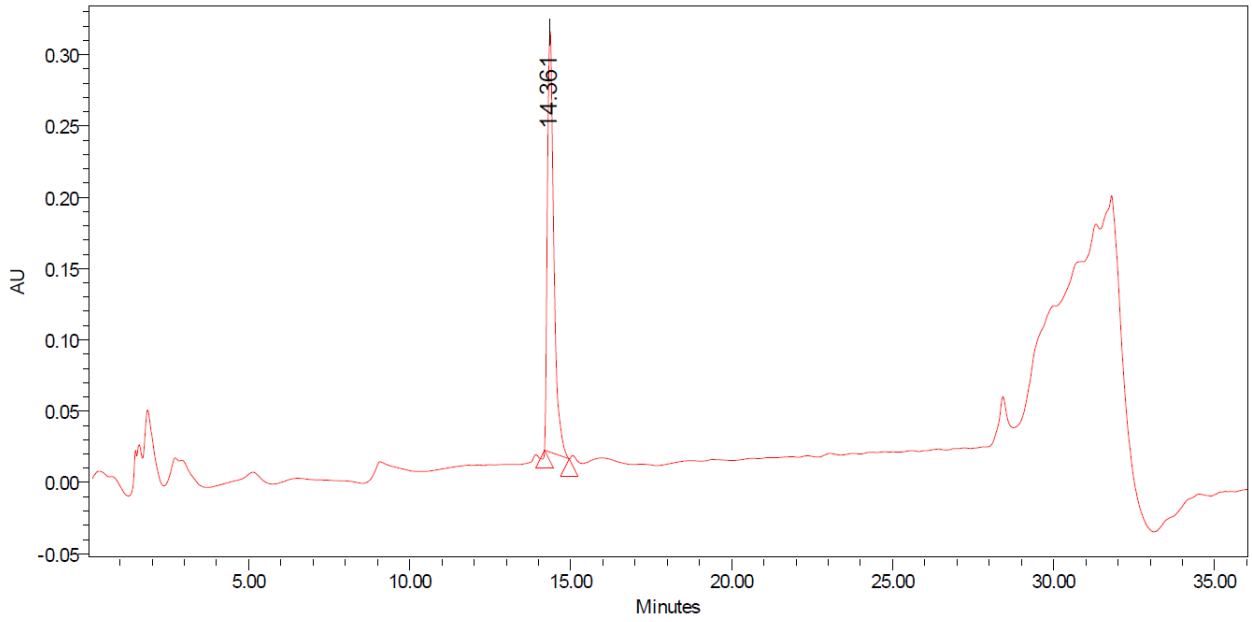
Compound 1



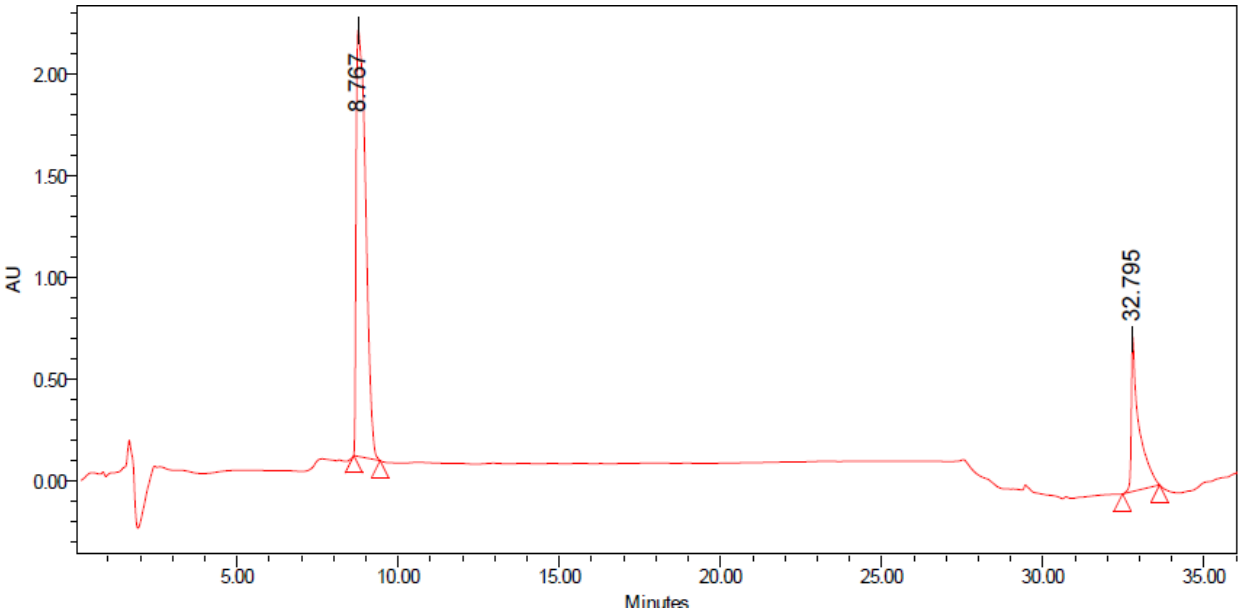
Compound 2



Compound 3



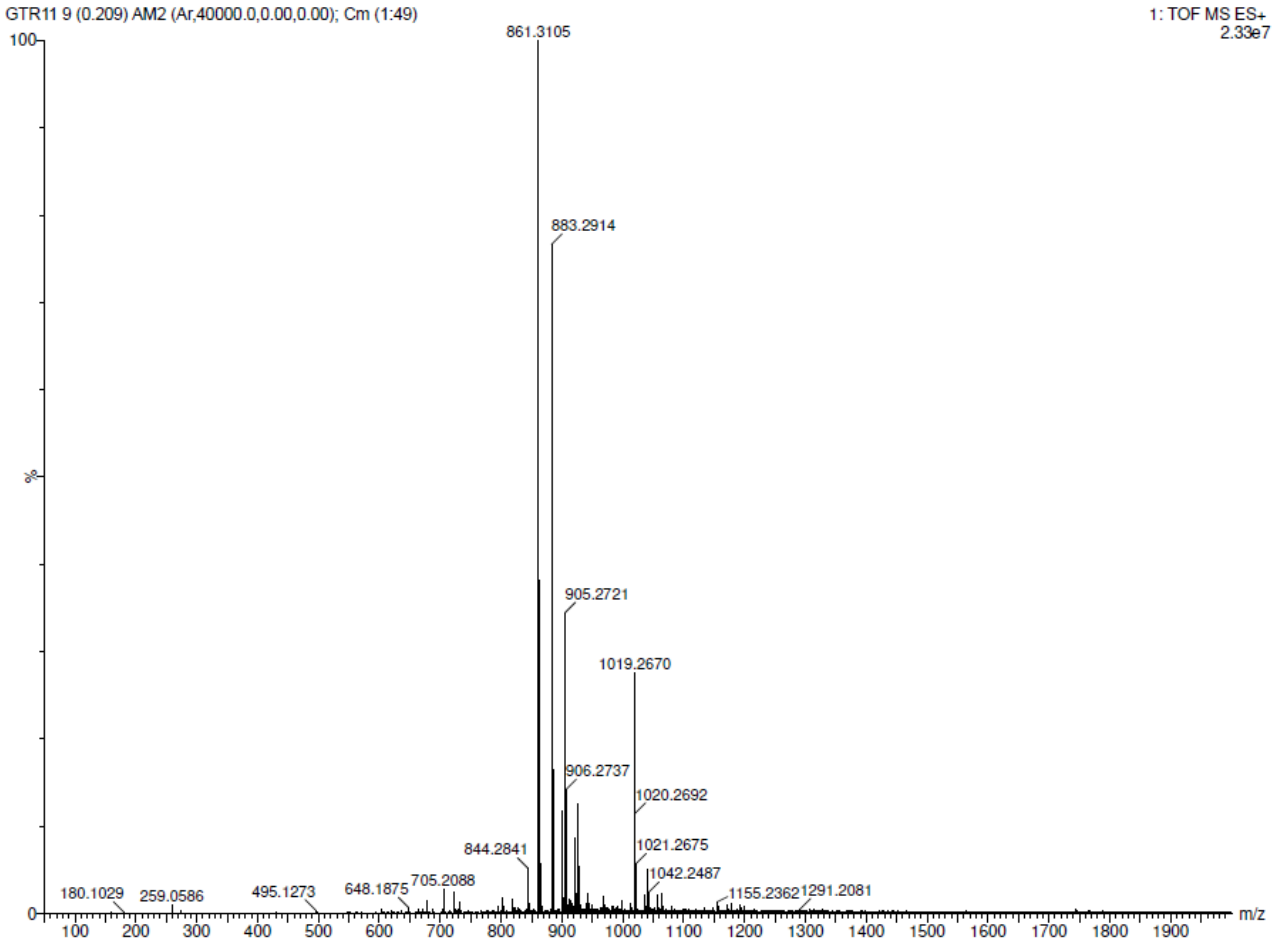
Compound 4



HRMS SPECTRA OF FINAL PRODUCTS

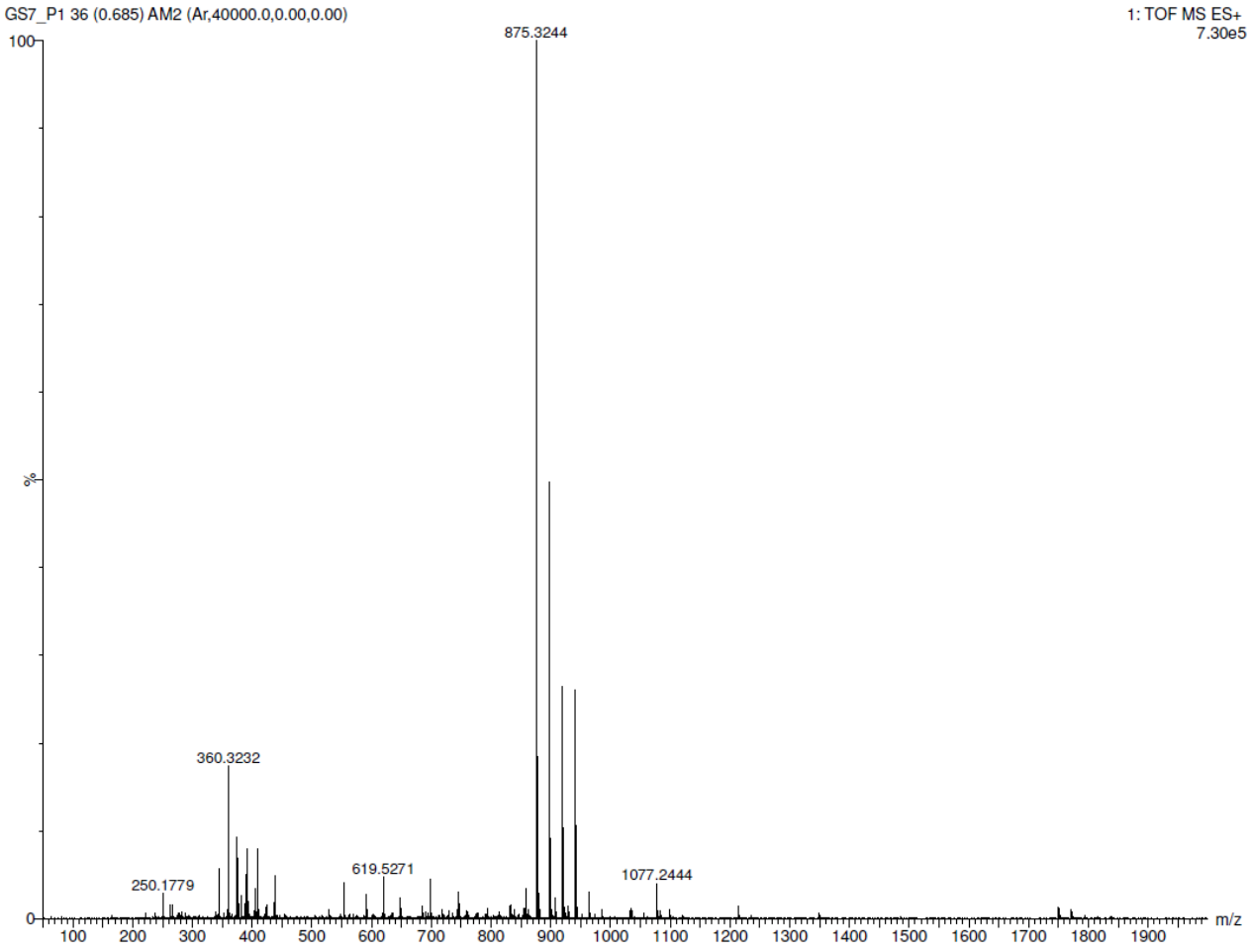
Compound 1

(+) ESI – MS



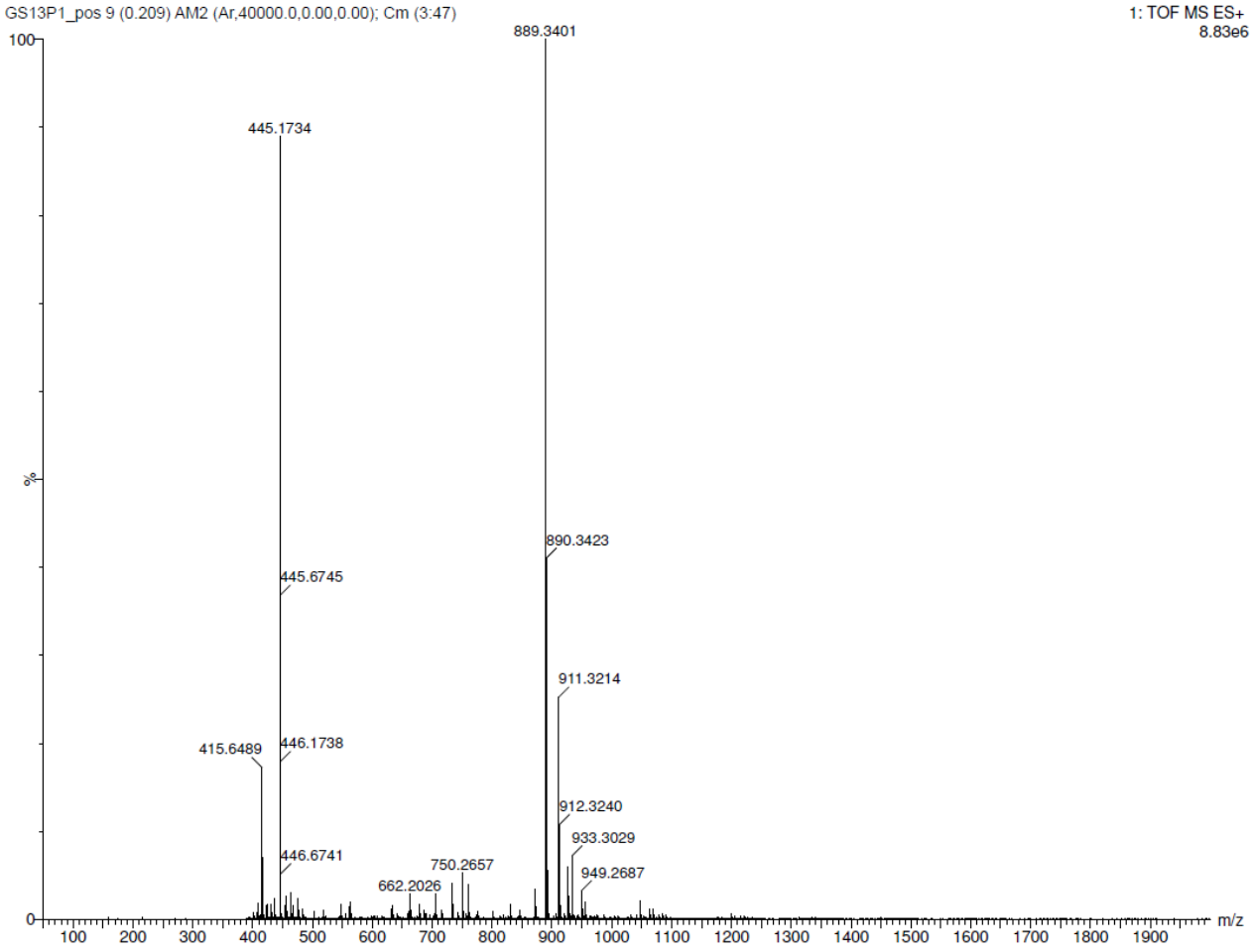
Compound 2

(+) ESI – MS



Compound 3

(+) ESI – MS



Compound 4

(+) ESI – MS