

Supplementary Material (ESI) for Chemical Communications:

**Novel DOTA-based prochelator for divalent peptide vectorization:
synthesis of dimeric bombesin analogues for multimodality tumor
imaging and therapy**

Materials and Methods:

All chemicals were obtained from commercial sources and used without further purification. Rink amide 4-methylbenzhydrylalanine (MBHA) resin (0.34 mmole/g; 100-200 mesh) and all the Fmoc-protected amino acids are commercially available from NovaBiochem (Laefelfingen, Switzerland). 1,4,7,10-Tetraazacyclododecane and 1,4,7,10-Tetraazacyclododecane-1,7-bis(t-butyl acetate) [**01**] was purchased from Macrocyclics, Dallas, Texas, USA. $^{177}\text{LuCl}_3$ was purchased from I.D.B. (Petten, the Netherlands). Reactions were carried out at ambient temperature, unless otherwise noted, and were monitored by thin-layer chromatography (TLC) on Merck plates precoated with silica gel 60 F-254 (0.25 mm). Spots were visualized either by UV light or iodine. Flash column chromatography was performed on silica gel 60 (Fluka). Electrospray ionisation (ESI) mass spectroscopy was carried out with a Finnigan SSQ7000 (Bremen, Germany) and MALDI-MS measurements on a Voyager sSTR equipped with an Nd:YAG laser (Applied Biosystems, Framingham, USA). Analytical high-performance liquid chromatography (HPLC) was performed on a Hewlett Packard 1050 HPLC system with a multiwavelength detector and a flow-through Berthold LB 506 Cl γ -detector using a Macherey-Nagel Nucleosil 120 C18 column. Preparative HPLC was performed on a Metrohm HPLC system LC-CaDI 22–14 with a Macherey-Nagel VP 250/21 Nucleosil 100–5 C18 column. Both analytical and preparative columns were eluted with a gradient system of mixtures of H_2O with 0.1% TFA (solvent A) and acetonitrile (solvent B). Quantitative γ -counting was performed on a COBRA 5003 γ -system well counter from Packard Instruments. The NMR spectra were recorded on a Bruker DRX 500 spectrometer using a triple inverse probe. ^1H shifts were referenced to DMSO- d_6 at 2.49 ppm. The 1D- ^1H , ^1H - ^1H -COSY, 1D- ^{13}C and ^1H - ^{13}C -COSY (HSQC) experiments were conducted.

Synthesis:

The (*R/S*) α -bromoglutaric acid 1-*tert*-butyl ester 5-benzyl ester (**02**) was synthesized starting from the commercially available L-glutamic acid 5-benzyl ester in two steps as described before.¹ The prochelator, 1,4,7,10-tetraazacyclododecane-1,4,7-tris(*tert*-butyl acetate)-10-(glutaric acid 1-*tert*-butyl ester) (**05**), was synthesized from 1,4,7,10-tetraazacyclododecane in three steps using the reported procedure.¹

1,4,7,10-tetraazacyclododecane-1,7-bis(*tert*-butyl acetate)-4,10-bis(glutaric acid 1-*tert*-butyl ester-5-benzyl ester) [03]: To a stirred solution of **01** (0.8 g, 2 mmol) in dry acetonitrile (10 mL) was added K₂CO₃ (1.146 g, 8 mmol) followed by the drop wise addition of racemic **02** (1.43 g, 4 mmol). After stirring for 18 h at room temperature, the mixture was filtered over Celite and concentrated under reduced pressure. Flash chromatography on silica gel 60 (CH₂Cl₂:EtOH 9:1 followed by EtOH:NH₃ 95:5) yielded **03** (1.21 g, 63%) as a pale yellow oil. R_f: 0.54 (CH₂Cl₂:EtOH 9:1); MS-ESI: *m/z* (%): 953.7 [*M*+H]⁺ (11), 975.6 [*M*+Na]⁺ (100); ¹H NMR (d⁶-DMSO): δ (ppm) = 7.35 (m, 10H, Ar-*H*), 5.1 (m, 4H, CH₂-Ar), 3.45 (m, 4H, -N-CH₂-CO₂C(CH₃)₃), 3.4 (m, 2H, -N-CH(R)-CH₂-CH₂-COOBzl), 3.2-2.0 (m, 16H, -N-CH₂-CH₂-N-), 2.41 (m, 4H, -N-CH(R)-CH₂-CH₂-COOBzl), 1.9 (m, 4H, -N-CH(R)-CH₂-CH₂-COOBzl), 1.46 (m, 36H, -C(CH₃)₃); ¹³C NMR (d⁶-DMSO): δ (ppm) = 172.8 (2C, -N-CH(R)-CH₂-CH₂-COOBzl), 172.2 (4C, -N-CH₂-CO₂C(CH₃)₃), 136.1 (2C, -CH₂-C(Ar)), 128.4, 128.1, 128.0 & 127.9 (10C, -C(Ar)), 81.4 & 81.6 (4C, -C(CH₃)₃), 64.5 (2C, -CH₂-Ar), 63.2 (2C, -N-CH(R)-CO₂C(CH₃)₃), 56.5 (2C, -N-CH₂-CO₂C(CH₃)₃), 50-44 (8C, -N-CH₂-CH₂-N-), 32.0 (2C, -N-CH(R)-CH₂-CH₂-COOBzl), 27.4, 27.5, 27.6 & 27.8 (12C, -C(CH₃)₃), 23.5 (2C, -N-CH(R)-CH₂-CH₂-COOBzl).

1,4,7,10-tetraazacyclododecane-1,7-bis(*tert*-butyl acetate)-4,10-bis(glutaric acid 1-*tert*-butyl ester) [04]: To a solution of **03** (1 g, 1.05 mmol) in methanol (100 mL) was added 100 mg of 10% Pd-C suspended in 5 mL of H₂O and H₂ was bubbled through the solution under normal pressure. After completion of hydrogenation (as monitored by TLC), the catalyst was removed by filtration through Celite. The solvent was evaporated under reduced pressure and the residue obtained was triturated with diethyl ether. The solid crude product thus obtained was subjected to flash chromatography on silica gel 60 (EtOH:NH₃ 98:2 to 95:5) to yield **04** (695 mg, 86%) as a white solid. R_f: 0.38 (EtOH:NH₃ 95:5); mp 206-208°C; MS-ESI: *m/z* (%): 773.5 [*M*+H]⁺ (18), 795.6 [*M*+Na]⁺ (100); ¹H NMR (d⁶-DMSO): δ (ppm) = 3.42 (m, 2H, -N-CH(R)-CH₂-CH₂-COOH), 2.74 & 3.46 (m, 4H, -N-CH₂-CO₂C(CH₃)₃), 2.5 (m, 4H, -N-

CH(R)-CH₂-CH₂-COOH), 2.14 & 2.64 and 2.0 & 3.1 (m, 8H, -N-CH₂-CH₂-N-), 2.1 & 2.5 and 2.35 & 3.0 (m, 8H, -N-CH₂-CH₂-N-), 1.63 & 1.9 (m, 4H, -N-CH(R)-CH₂-CH₂-COOH), 1.45 (m, 36H, -C(CH₃)₃); ¹³C NMR (d⁶-DMSO): δ (ppm) = 174.9 & 174.7 (2C, -N-CH(R)-CH₂-CH₂-COOH), 173.1 (4C, -N-CH₂-CO₂C(CH₃)₃), 81.8 (2C, -N-CH(R)-CO₂C(CH₃)₃), 79.6 (2C, -N-CH₂-CO₂C(CH₃)₃), 59.8 (2C, -N-CH(R)-CO₂C(CH₃)₃), 56.2 (2C, -N-CH₂-CO₂C(CH₃)₃), 52.9 & 44.0 (4C, -N-CH₂-CH₂-N-), 48.5 & 47.2 (4C, -N-CH₂-CH₂-N-), 32.6 (2C, -N-CH(R)-CH₂-CH₂-COOH), 28.2, 27.8 & 27.7 (12C, -C(CH₃)₃), 19.4 (2C, -N-CH(R)-CH₂-CH₂-COOH).

Proton and Carbon assignments were confirmed by ¹H-¹H COSY and ¹H-¹³C-COSY (HSQC) experiments.

Peptide Synthesis:

The SPPS was performed on a semiautomatic peptide synthesizer (RinkCombichem, Bubendorf, Switzerland) employing standard Fmoc strategy.² The required peptides were assembled on Rink amide MBHA resin. The Trt was used as protecting group for Gln, Asn and His, ivDde for Lys and Boc for Trp. The coupling reactions were achieved with 3 fold excess of Fmoc-amino acids, using DIC/HOBt as activating agents in NMP. After a coupling time of 2 h, the completeness of the reaction was monitored by standard ninhydrin test. Fmoc removal was achieved with 20% piperidine in DMF in three successive 10 min treatments. After assembling the desired amino acids, cleavage from the resin and simultaneous side-chain deprotection of the peptides was accomplished by incubating for 3.5h in a cleavage cocktail comprising TFA:thioanisole:H₂O:triisopropylsilane 95:3:1:1. The resin was then filtered and washed with the above mixture, evaporation of the filtrate followed by trituration with diethyl ether yielded the crude peptide. The yields of the crude peptides ranged from 70-80% and were further purified by semi-preparative HPLC.

General procedure for monovalent conjugation; synthesis of 06 and 07: After assembling the desired amino acids on the resin, the final Fmoc group was removed. The prochelator **05** (3 equivalents) was coupled to the N-terminal of the peptides using HATU (3.3 equivalents) and DIPEA (7 equivalents) for 4h. The monovalent peptide-chelator conjugates were then cleaved from the resin as described above. The conjugates were further subjected to deprotection for 3h using the same cleavage cocktail, triturated with diethyl ether, filtered and dried. This deprotection and trituration steps were repeated until the complete removal of ^tBu was observed (by analytical HPLC). The monovalent peptide-chelator conjugates were further

purified by semi-preparative HPLC (0 min, 80% A, 20 min 40% A) and characterized by ESI-MS.

Compound	Molecular Formula	Molecular Weight	Yield ^a (%)	R _t ^b (min)	MS-ESI: <i>m/z</i> (%)
06	C ₈₀ H ₁₂₆ N ₂₂ O ₂₃ S	1796.06	44	22.14	1835.1 [M+K] ⁺ (100) 918.0 [M+K] ⁺⁺ (74)
07	C ₉₀ H ₁₄₀ N ₂₆ O ₂₇ S	2050.30	38	20.85	2089.4 [M+K] ⁺ (22) 1044.9 [M+K] ⁺⁺ (100)

^aYields were based on the first Fmoc cleavage.

^bAnalytical HPLC (0 min, 95% A; 30 min 55% A).

General procedure for divalent conjugation; synthesis of 08 and 09: To a solution of prochelator **04** (10 mg, 13 μmol) in dry DMF (2 mL) was added purified peptide (28.6 μmol, 2.2 equivalents) and HATU (11 mg, 28.6 μmol) and adjusted the pH to 8 using DIPEA. The mixture was stirred at room temperature overnight and DMF was evaporated under reduced pressure. The residue was triturated with diethyl ether, dried and subjected to ^tBu deprotection as described above. The divalent peptide-chelator conjugates were purified by semi-preparative HPLC (0 min, 80% A, 30 min 40% A) and characterized by ESI-MS.

Compound	Molecular Formula	Molecular Weight	Yield ^a (%)	R _t ^b (min)	MS-ESI: <i>m/z</i> (%)
08	C ₁₄₄ H ₂₂₄ N ₄₀ O ₃₈ S ₂	3188.08	55%	22.88	1613.33 [M+K] ⁺⁺ (35) 1076.12 [M+K] ⁺⁺⁺ (100)
09	C ₁₆₄ H ₂₅₂ N ₄₈ O ₄₆ S ₂	3696.18	48%	21.99	1867.1 [M+K] ⁺⁺ (18) 1245.2 [M+K] ⁺⁺⁺ (100)

^aYields refer to the final divalent conjugation and deprotection steps.

^bAnalytical HPLC (0 min, 95% A; 30 min 55% A).

General procedure for the synthesis of Gd (III) complexes of peptide-chelator conjugates: A mixture of peptide-chelator conjugate (5 μmol) in 4 mL of sodium acetate buffer (0.4M, pH 5) was incubated with 10 μmol Gd(OAc)₃·6H₂O at 95°C for 25 min, cooled to room temperature, and purified over a SepPak C₁₈ cartridge preconditioned with 10 mL of methanol and 10 mL of water. The cartridge was eluted with 30 mL of sodium acetate buffer followed by 20 mL of methanol. The absence of free Gd(III) ions in methanol solution was

verified by using xylenol orange indicator. The methanol was evaporated to afford the corresponding Gd(III) complexes (purity >96% on HPLC), which were further characterized by MALDI-MS. The well resolved MALDI-MS spectra of gadolinium conjugated peptides showed broad isotopic distribution. The broad isotope pattern is due to the fact that gadolinium has five main isotopes (14.80% of ^{155}Gd , 20.47% of ^{156}Gd , 15.65% of ^{157}Gd , 24.84% of ^{158}Gd , 21.86% of ^{160}Gd) with similar abundance plus two isotopes of minor abundance.³

Compound ^a	Molecular Formula	Molecular Weight	Yield (%)	R _t ^b (min)
Gd-06	C ₈₀ H ₁₂₂ GdN ₂₂ O ₂₃ S	1949.27	88	24.23
Gd-07	C ₉₀ H ₁₃₆ GdN ₂₆ O ₂₇ S	2203.52	82	21.82
Gd-08	C ₁₄₄ H ₂₂₀ GdN ₄₀ O ₃₈ S ₂	3340.95	85	25.78
Gd-09	C ₁₆₄ H ₂₄₈ GdN ₄₈ O ₄₆ S ₂	3849.40	80	23.43

^aMALDI-MS spectra are given in **Figure 1-4**.

^bAnalytical HPLC (0 min, 95% A; 30 min 55% A).

Relaxivity measurements:

Longitudinal ^1H relaxation rates times T_1 were measured at 25 and 37 °C with Bruker Minispecs mq30 (30 MHz, 0.71T), mq40 (40MHz, 0.94T) and mq60 (60 MHz, 1.41 T). About 1 mM solutions of gadolinium-peptide conjugates were used for the measurements and the exact concentration of Gd(III) was determined by the measurement of the bulk magnetic susceptibility shifts of a $^t\text{BuOH}$ signal.⁴ The water proton relaxation times T_1 were acquired by the standard inversion recovery method. The temperature was stabilized by pumping a thermostated liquid through the probe and measured by a substitution technique

In Vitro Studies:

Radiolabeling: Radiolabeling was performed as described previously.⁵ To an aliquot of 10 μg of the corresponding peptide-chelator conjugate dissolved in 300 μL of sodium acetate buffer (0.4 mol/L, pH 5) was added $^{177}\text{LuCl}_3$ (1-2 mCi) and the solution was incubated for 25 min at 95°C. A 1.5 molar excess of $\text{Lu}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ was added and incubated again for 20 min at 95°C. Subsequently, the radiolabeled conjugate was purified utilizing SepPak C₁₈ cartridge preconditioned with 10 mL of methanol and 10 mL of water. The radiolabeled conjugate was

immobilized on the cartridge; excess of $\text{Lu}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ was removed by washing with water and finally the pure product (radiochemical purity >97%) was eluted with methanol, evaporated to dryness and reconstituted in water. Oxidation of C-terminal Met-NH₂ was suppressed by the addition of large excess of methionine. Quality control after radiolabeling was performed using analytical HPLC (0 min, 90% A; 20 min 50% A).

Cell Culture:

The PC-3 (human prostate adenocarcinoma) cell line was maintained by serial passage in mono-layers in Dulbecco's modified Eagle medium (DMEM; Cambrex Bio Science), supplemented with 10% fetal bovine serum, amino acids, vitamins, and penicillin–streptomycin, in a humidified 5% CO₂ atmosphere at 37°C.

Internalization and Cellular Retention Studies:

Internalization and cellular retention experiments were conducted following the protocols described earlier.⁶ Briefly, the PC-3 cells were seeded at a density of 0.8 –1.1 million cells per well in 6-well plates and incubated overnight with internalization buffer to obtain good cell adherence. On the day of the study, cells were washed once with 2 mL of internalization buffer (DMEM, 1% fetal bovine serum, amino acids, and vitamins, pH 7.4), incubated with fresh medium (1.3 mL) for 1h at 37°C. Approximately, 3 kBq (0.25 pmol) of radiolabeled peptide per well was added and the cells were incubated at 37°C for the indicated time periods in triplicates. To determine nonspecific membrane binding and internalization, cells were incubated with radioligand in the presence of large excess of Tyr⁴-Bombesin.

For internalization studies, the plates were removed at different time points (0.5h, 1h, 2h and 4h) and the cellular uptake was stopped by removing medium from the cells and by washing twice with 1 mL of ice-cold PBS. Acid wash for 5 min with a pH 2.8 glycine buffer on ice was also performed twice. This procedure was performed to distinguish between membrane-bound (acid releasable) and internalized (acid resistant) radiopeptide. Finally, the cells were treated with 1N NaOH. The culture medium, the receptor-bound fraction, and the internalized fraction were measured radiometrically in a γ –counter (Cobra II).

For cellular retention studies, all the plates were removed after 2h of incubation at 37°C. The medium was removed and the wells were washed twice with 1 mL of ice-cold PBS. In each experiment, an acid wash for 5 min on ice with a pH 2.8 glycine buffer was performed twice

to remove the receptor bound ligand and a PBS wash was performed quickly afterwards to restore the physiologic pH. Cells were then incubated again at 37°C with fresh internalization buffer (DMEM containing 1% fetal bovine serum, pH 7.4). Plates were removed at different time points, medium was removed and the cells were washed twice with 1 mL of ice cold PBS. Thereafter, the cells were solubilized in 1N NaOH. The culture medium (amount of radiopeptide externalized) and NaOH fractions (amount of radiopeptide retained in the cell) were measured radiometrically in a γ -counter (Cobra II).

References:

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Figure 1: MALDI-MS Spectrum of Gd-06

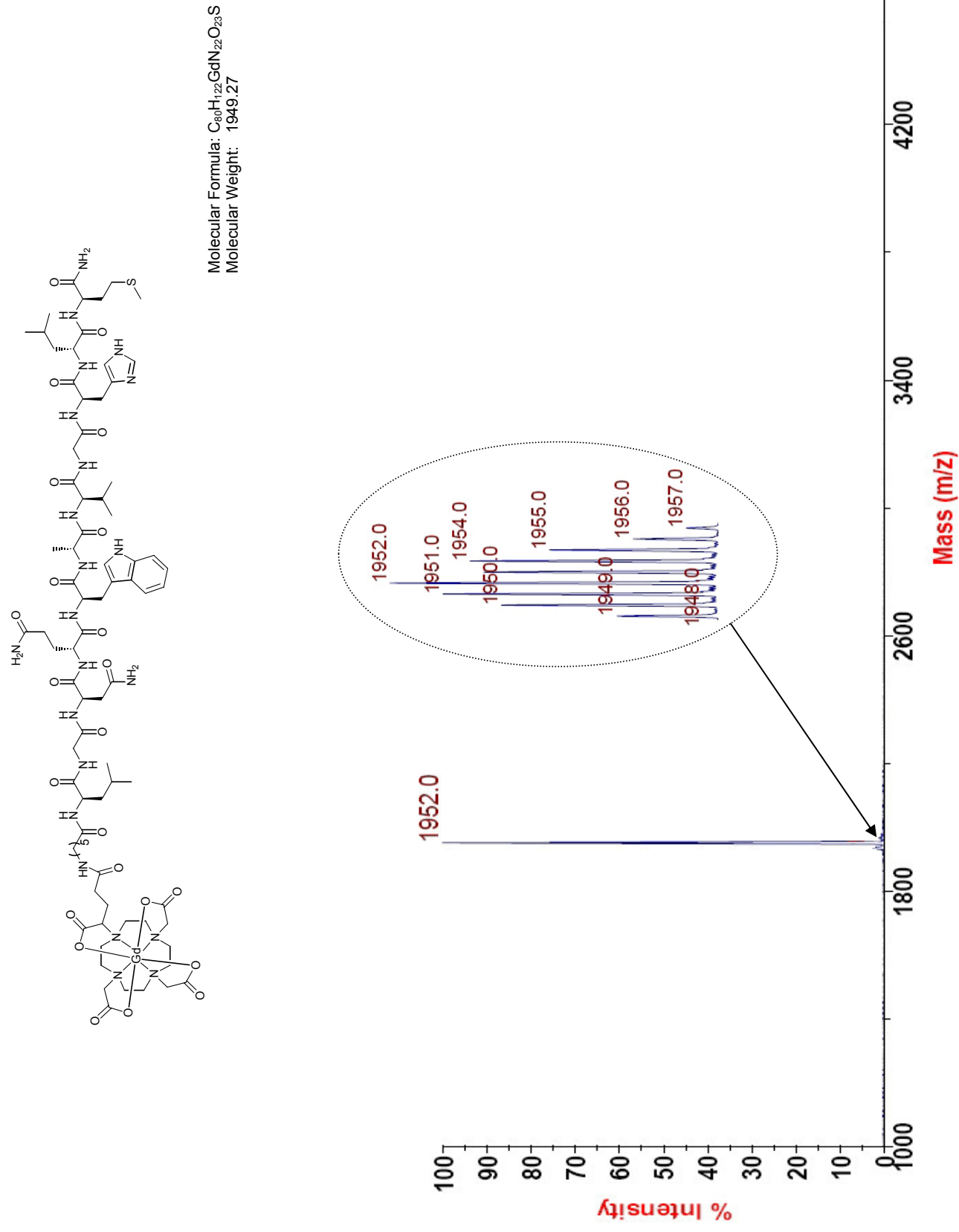
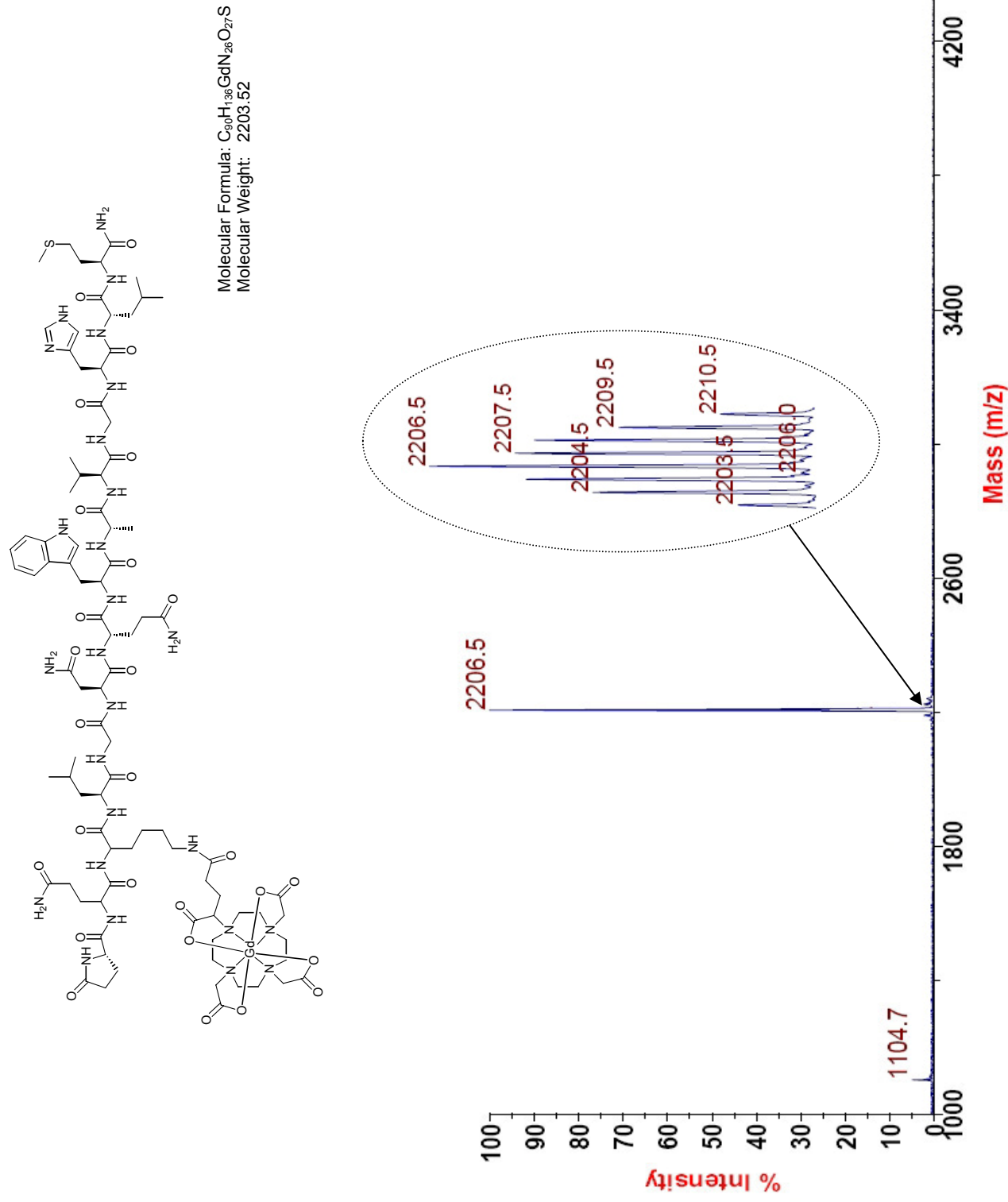


Figure 2: MALDI-MS Spectrum of Gd-07



10

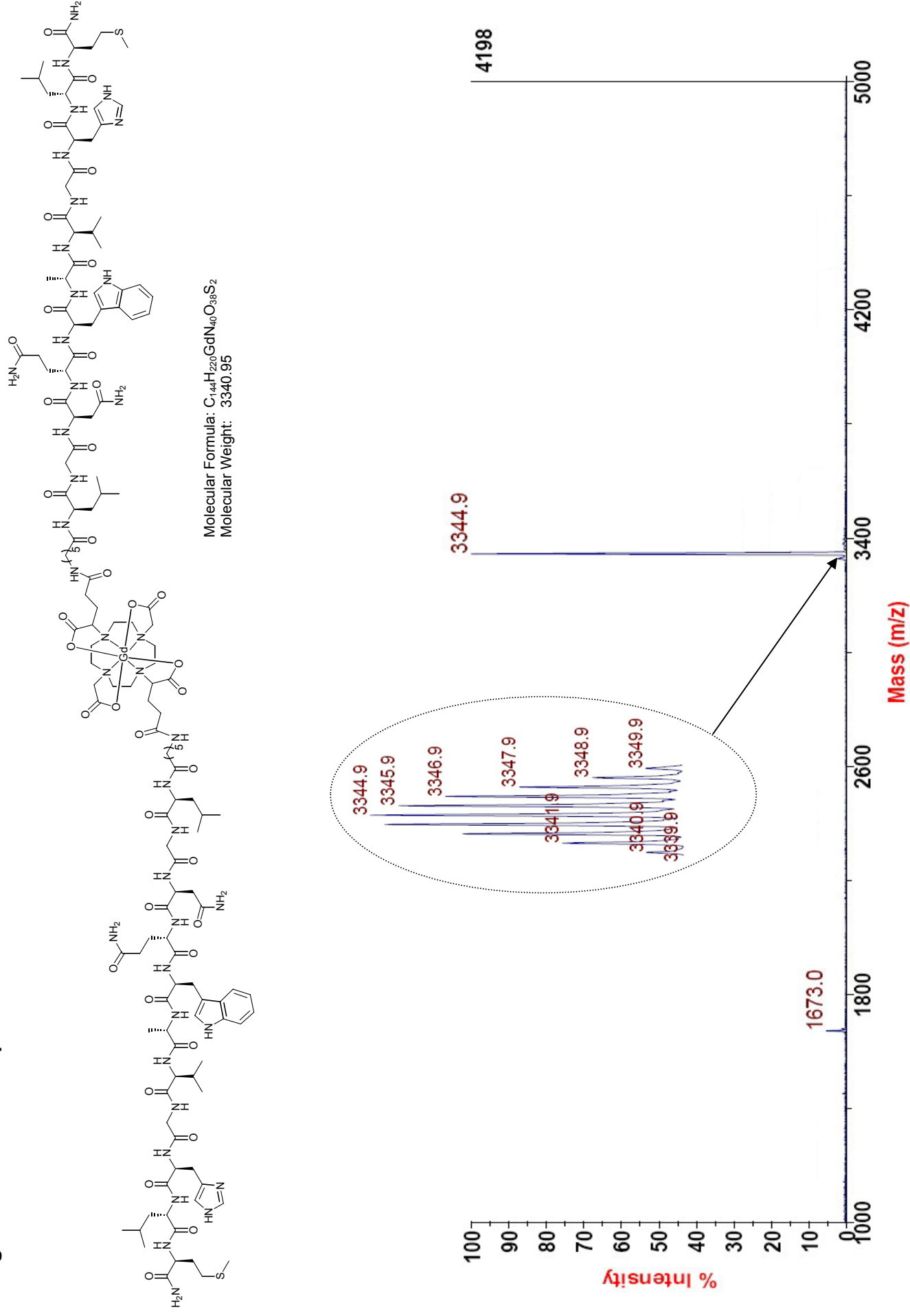


Figure 4: MALDI-MS Spectrum of Gd-09

