Electronic Supplementary Material (ESI) for ChemComm.

Additional Figures

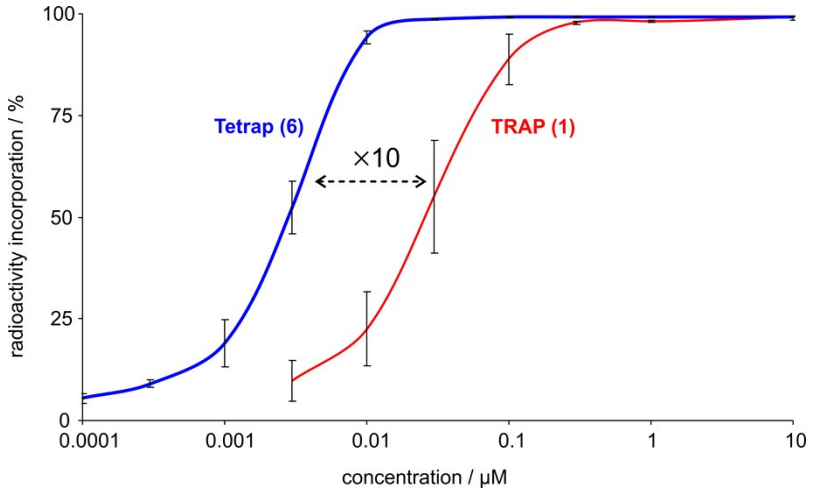

Figure S1: Incorporation of the gallium isotope $^{68}Ga^{III}$ by Tetrap (**6**, see Figure 3**)** and TRAP (**1**, data taken from the literature).[2] Labelling was done for 5 min at 95 °C and pH 2. Despite Tetrap features only four TRAP monomers, the Tetrap concentration required to achieve the same extent of radioactivity incorporation is about one order of magnitude lower, pointing at a synergistic, cooperative effect of the linked chelator cages.

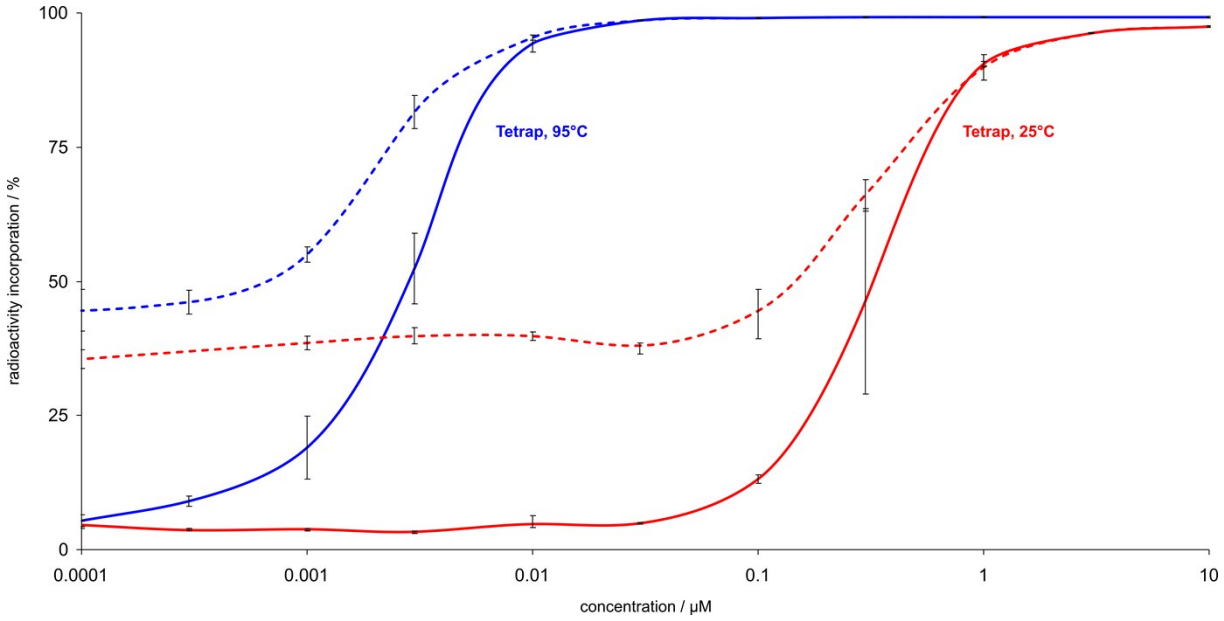

Figure S2: Incorporation yields of $^{68}Ga^{III}$ according to radio-TLC (see Experimental Section) for radiolabelling of Tetrap (**6**) (5 min at pH 2), before (dashed lines) and after (solid lines) subsequent reaction with 100 µL of 0.1 M Na_2EDTA (r.t., 30 min). Initially, a substantial amount of $^{68}Ga^{III}$ complexation in a kinetically labile fashion (out-of-cage complexes) is observed. This activity fraction can be removed by quenching with excess disodium EDTA. The residual Tetrap-bound activity is assumed to reflect only $^{68}Ga^{III}$ coordinated in an in-cage fashion (details on coordination environment and its molecular structure have been reported before).[1]

Experimental Section

Materials and Methods

Unless otherwise noted, all reagents and solvents were of analytical grade. 3-azidopropylamine was purchased from Sigma-Aldrich. Compounds **1** (TRAP, 1,4,7-triazacyclononane-1,4,7-tris[methylene(2-carboxyethyl)phosphinic acid], formerly named PrP9)[1] and **2** (TRAP(AHX)$_3$)[2] and (*t*Bu)$_3$-KuE[3] were synthesized as described previously. NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) was purchased from CheMatech (Dijon, France).

Analytical and preparative HPLC were performed on Shimadzu gradient systems with a SPD-20A dual wavelength UV/Vis detectors (220 nm, 254 nm). Eluents were purified water (from Millipore system, **A**) and acetonitrile (J.T.Baker® Ultra Gradient HPLC grade, supplemented with 5% H_2O, **B**), each containing 0.1% trifluoroacetic acid. Analytical HPLC was done on a Nucleosil 100-5 C18 column (125×4.6 mm), flow 1.0 mL min^{-1}. Preparative HPLC purification was done on a Multospher 100 RP 18-5µ column (250×10 mm), flow 5.0 mL min^{-1}, with eluents as above. Mass spectra (ESI) were measured on a 500-MS Ion Trap spectrometer (Varian, by Agilent Technologies). pH values were meassued with a SevenEasy pH-meter (Mettler Toledo, Gießen, Germany). Gel permeation chromatography (GPC) was done on Sephadex GP-10 (100 g, bed size approx. 30×3 cm) with water as eluent, separating the eluate in 20 mL fractions.

Cultivation of PSMA-expressing LNCaP cells, their use for determination of PSMA activity, generation of respective tumor xenografts in SCID mice as well as small animal PET have been carried out as described previously.[3]

Improved synthesis of TRAP(azide)$_3$ (3)

TRAP·2H_2O (40 mg, 65.0 µmol,) was added to a mixture of dry DMSO (135 µL) and DIPEA (132 µL, 780 µmol). Subsequently, 3-azido-1-propylamine (39.0 mg, 390 µmol) and HATU (380 mg, 585 µmol) were added in one portion with stirring. After 1 h at room temperature, the orange reaction mixture was quenched with water (200 µL) and the crude product was purified by twofold size exclusion chromatography with intermediate lyophilization (solid phase: Sephadex G-10, column size: 40×3 cm, eluent: water, adjusted to pH 3 with HCl). After lyophilization, TRAP(azide)$_3$ was obtained as a yellow solid (49.0 mg, 59.4 µmol, 91 %). MS (ESI, positive): *m/z:* 826.9 [*M*+H$^+$]. HPLC (15–65 % **B** in 20 min): t_R = 8.5 min.

Synthesis of monopropargyl-TRAP (4)

TRAP·2H_2O (**1**, 110 mg, 178 µmol), diisopropylethylamine (DIPEA, 397 µL, 295 mg, 2.28 mmol), and propargyl amine (14.0 µL, 12.0 mg, 218 µmol were dissolved in DMSO (400 µL). Then HATU (217 mg, 570 µmol) was added in several portions over 10 min. After 1 h at room temperature, water (600 µL) was added, pH 3 was adjusted by addition of 0.1 M aq. HCl, and the crude product was subjected to GPC purification. Fractions containing the product were identified by MS, pooled and

concentrated in vacuo. Final purification was done by preparative HPLC, affording 60.2 mg (82.4 µmol, 46 %) of **4** as a colorless solid. MW (calcd. for $C_{21}H_{39}N_4O_{11}P_3$): 616.49 MS (ESI, positive): *m/z* = 617.5 [*M*+H$^+$]. HPLC (2–40 % **B** in 20 min): t_R = 6.0 min.

^{1}H-NMR (300 MHz, D_2O, 300 K) δ = 1.96–2.08 (m, 6H, C(O)-C**H**$_2$), 2.46–2.55 (m, 2H, P^B-C**H**$_2$-C), 2.59–2.70 (m, 4H, P^A-C**H**$_2$-C), 3.36 (d, 2H, $^2J_{PH}$ = 6 Hz, P^B-C**H**$_2$-N), 3.41 (d, 4H, $^2J_{PH}$ = 6 Hz, P^A-C**H**$_2$-N), 3.47–3.48 (m, 12H, ring-C**H**$_2$), 3.95 (d, 2H, C**H**$_2$-C≡CH, $^4J_{HH}$ = 3 Hz) ppm*. ^{13}C{^{1}H}-NMR (101 MHz, D_2O, 300 K) δ = 24.61 (d, $^1J_{PP}$ = 95 Hz, P^B-**C**-C), 25.07 (d, $^1J_{PP}$ = 94 Hz, P^A-**C**-C), 26.71 (d, $^2J_{PP}$ = 4 Hz, P^B-C-**C**), 27.82 (d, $^2J_{PP}$ = 3 Hz, P^A-C-**C**), 28.89 (**C**-C≡C), 51.29 / 51.41 / 51.50 (three different ring-C), 53.66 (d, $^1J_{PP}$ = 91 Hz, N-**C**-P^A), 53.66 (d, $^1J_{PP}$ = 90 Hz, N-**C**-P^B), 71.79 (C-C≡**C**), 79.65 (C-**C**≡C), 174.46 (d, $^3J_{PP}$ = 14 Hz, N(H)-**C**=O^B), 177.24 (d, $^3J_{PP}$ = 13 Hz, **C**=O^A) ppm*. ^{31}P{^{1}H}-NMR (162 MHz, D_2O, 300 K) δ = 37.99 (P^A), 38.68(P^B) ppm*. *: indices ^{A} and ^{B} indicate P and O atoms belonging to the undecorated^A and decorated^B side arm, respectively.

Synthesis of KuE-pentynoic acid (9)

(*t*Bu)$_3$-KuE (1.2 g, 2.46 mmol), 4-pentynoic acid (290 mg, 2.96 mmol), and HOAt (503 mg (3.70 mmol) are dissolved in dimethyl formamide (DMF, 1.5 mL). Then diisopropylcarbodiimide (570 µL, 3.70 mmol) and diisopropylethylamine (DIPEA, 1.90 µL, 11.1 mmol) were added and the mixture was allowed to react for 20 h. Then the mixture was poured into brine (100 mL), the product was extracted with dichloromethane and concentrated in vacuo. Purification by column chromatography on silica gel 60 with ascending concentrations of ethyl acetate (40–70%) in petrolether yielded 1.31 g (2.31 mmol, 94 %) of crude *t*Bu$_3$-**9** as a colorless solid. Deprotection with trifluoroacetic acid (3 mL) for 1 h, followed by precipitation in diethyl ether and final purification by preparative HPLC yielded compound **9** (614 mg, 1.53 mmol, 66 %) as a colorless solid.

^{1}H-NMR (300 MHz, D_2O, 300 K): δ = 1.26–1.37 (m, 2H), 1.40–1.50 (m, 2H), 1.55–1.65 (m, 1H), 1.67–1.79 (m, 1H), 1.81–1.94 (m, 1H), 2.02–2.14 (m, 1H), 2.26–2.44 (m, 7H), 3.09 (t, 2H), 4.07 (q, 1H), 4.15 (q, 1H) ppm. ^{13}C-NMR (101 MHz, D_2O, 300 K): δ = 14.69, 22.26, 26.25, 27.79, 30.03, 30.60, 34.56, 38.97, 159.25, 174.48, 176.18, 177.07, 177.21 ppm. MW (calcd. for $C_{17}H_{25}N_3O_8$): 399.40. MS (ESI, positive): *m*/*z* = 400.1 [*M*+H$^+$].

Synthesis of dendrimer 5

Compound **2** (50 µmol, 44 mg, prepared as described before)[2], TRAP (**1**) dihydrate (0.5 mmol, 308 mg) and diisopropylethylamide (3.5 mmol, 452 mg, 595 µl) were dissolved in DMSO (3 mL). Then HATU (1.5 mmol, 571 mg) was added in portions within 10 min. After 30 min, the reaction mixture was poured into water (50 mL) and the crude product separated off by ultrafiltration as described previously[1] (Amicon/Millipore 50 mL stirred cell, regenerated cellulose membrane with 500 Da molecular weight cut off, eluent: 300 mL water). The retentate was lyophilized and purified by preparative HPLC (gradient: 14–29% **B** in 20 min), yielding 27.3 mg (8 µmol, 16 %) of **5** as a colorless solid. MW (calcd. for $C_{90}H_{180}N_{18}O_{42}P_{12}$): 2558.22. MS (ESI, positive): m/z = 1706.4 [$2M$+3H$^+$], 1280.2 [M+2H$^+$], 853.9 [M+3H$^+$].

Addition of 4 equivalents of 0.1 M aq. $Ga(NO_3)_3$ to an aqueous solution of **5** delivered the respective tetragallium(III) complex (vide infra); MS (ESI, positive): m/z = 1413.8 [M+2H$^+$], (ESI, negative): m/z = 1411.8 [M–2H$^+$].

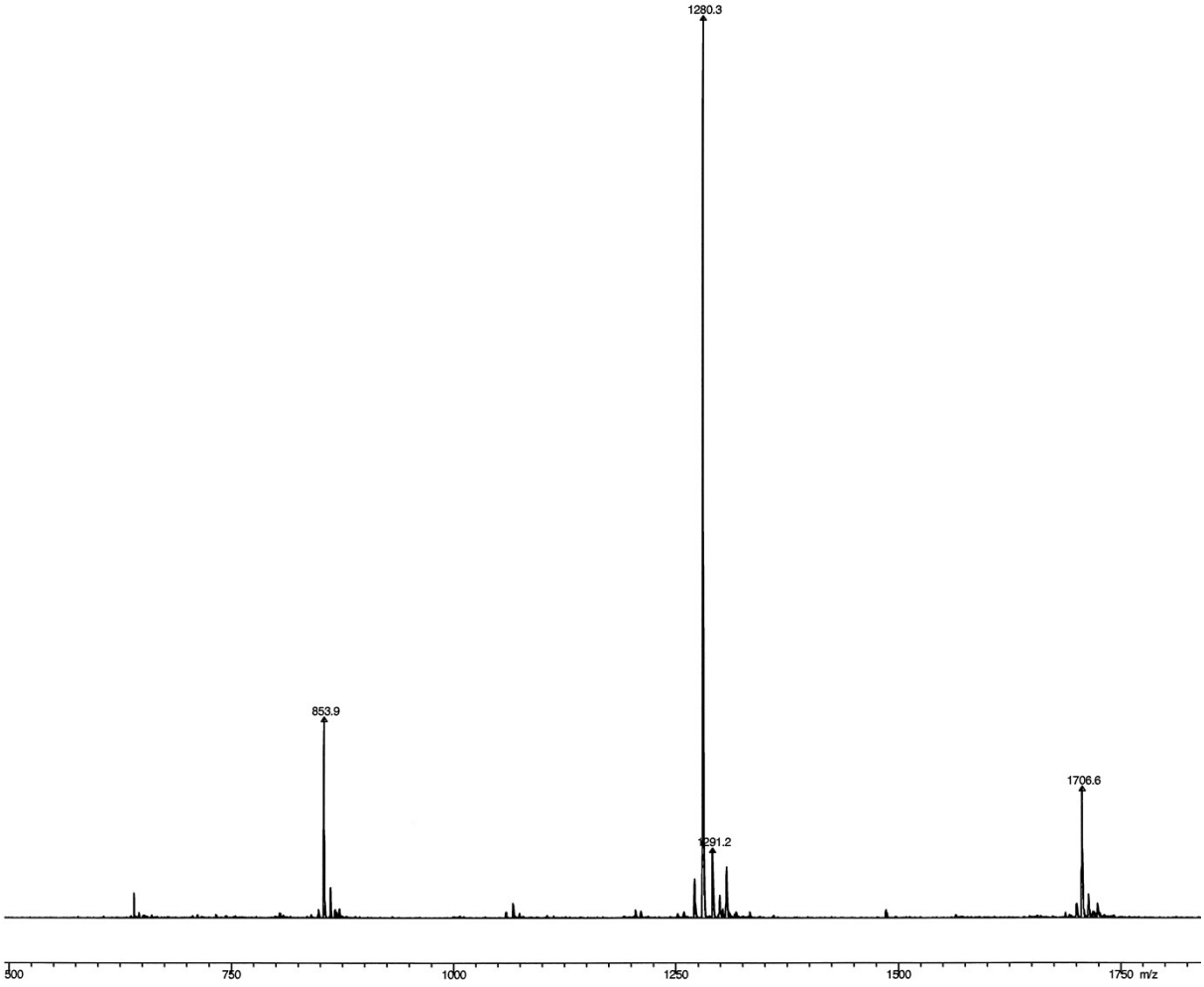

Figure S3: ESI-MS (positive mode) for compound **5**

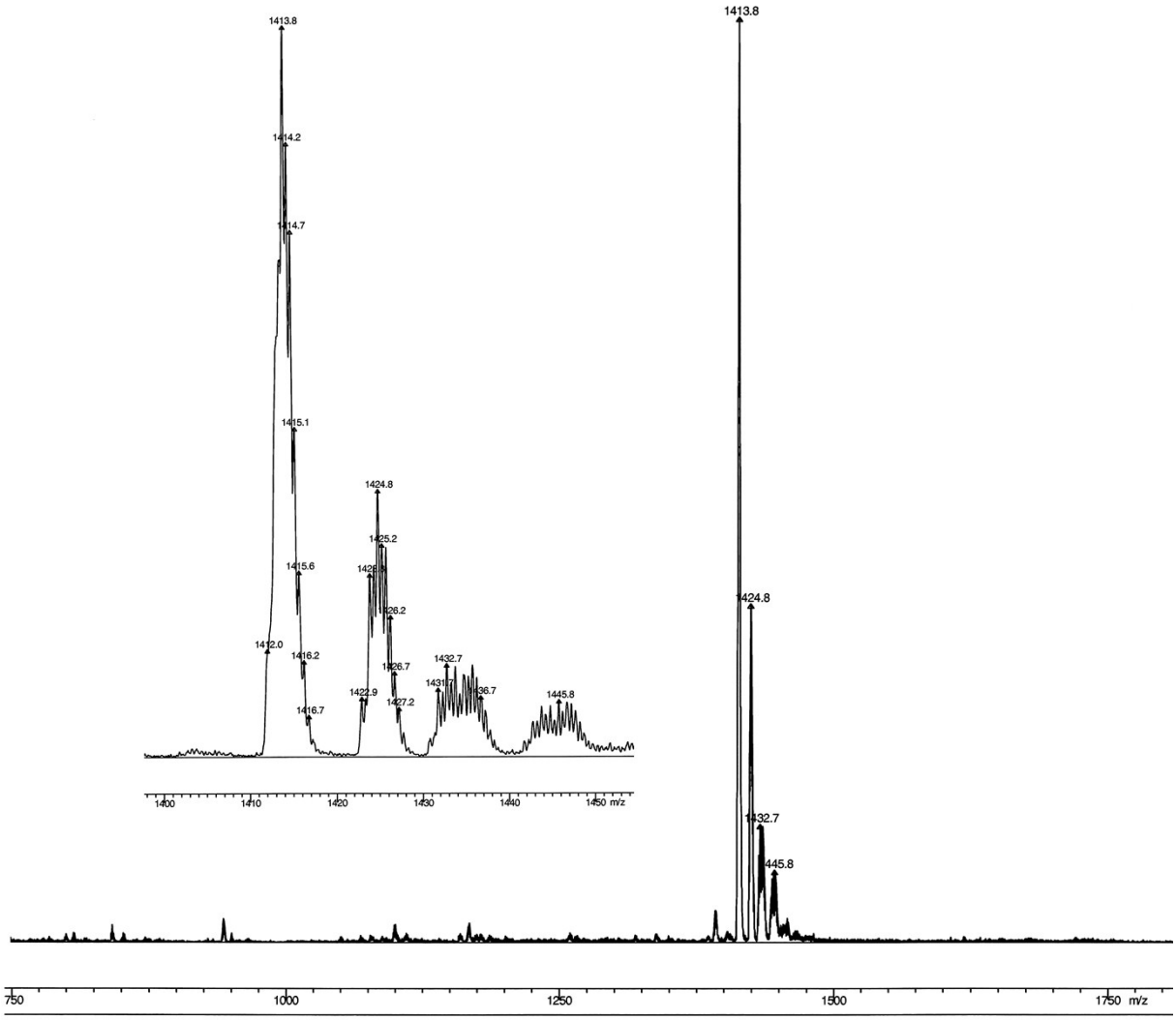

Figure S4: ESI-MS (positive mode) for the tetragallium(III) complex of compound **5**. The signals at m/z = 1424.8 and 1432.7 correspond to the sodium- and potassium adducts M+Na^++H^+ and M+K^++H^+, respectively; further signals originate from mixed multisodium / potassium species (M+2Na^+; +Na^++K^+; +3Na^+–H^+; +2Na^++K^+–H^+).

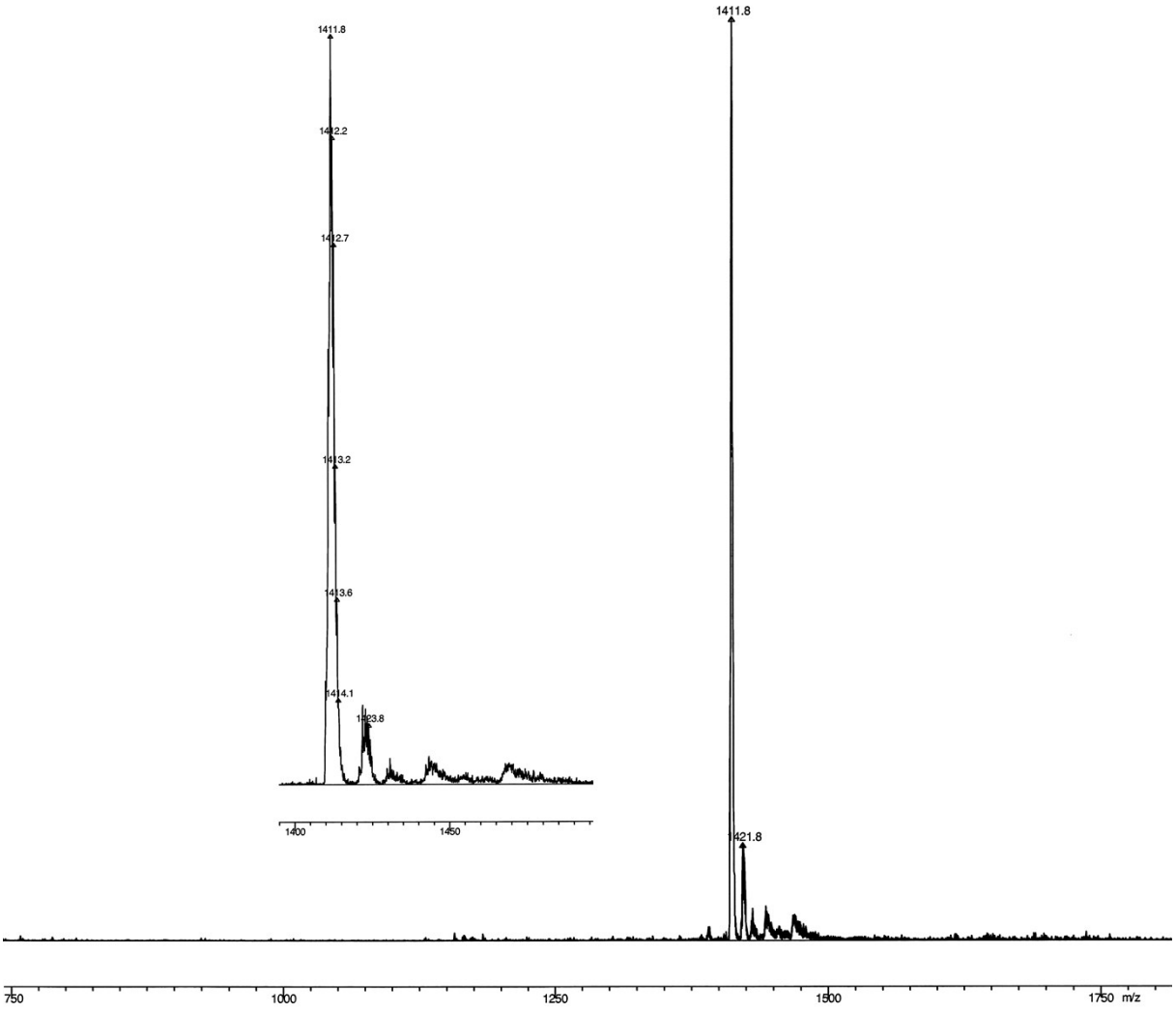

Figure S5: ESI-MS (negative mode) for the tetragallium(III) complex of compound **5**

Synthesis of Tetrap (6)

TRAP(azide)$_3$ (**3**, 9.4 mg, 9.0 µmol), propargyl-TRAP (**4**, 24.8 mg, 34.0 µmol) and sodium ascorbate (159 mg, 800 µmol) were dissolved in a mixture of water (550 µL) and methanol (70 µL), Addition of a solution of $Cu(OAc)_2 \cdot H_2O$ (10.0 mg, 50 µmol) in water (230 µL) yielded a clear green solution. After 1 h reaction time, the mixture was directly subjected to purification by preparative HPLC , yielding Cu_4-**6** (14.5 mg, 4.9 µmol 55 %) as a blue solid. MW (calcd. for $C_{90}H_{163}Cu_4N_{27}O_{42}P_{12}$): 2921.28; MS (ESI, positive): m/z = 1461.9 [M+2H$^+$], (ESI, negative): *m*/*z* = 1459.9 [M–2H$^+$]. HPLC (Gradient: 5–55 % **B** in 20 min): t_R = 8.0 min. Such material was directly used for synthesis of Compound **7** (see below).

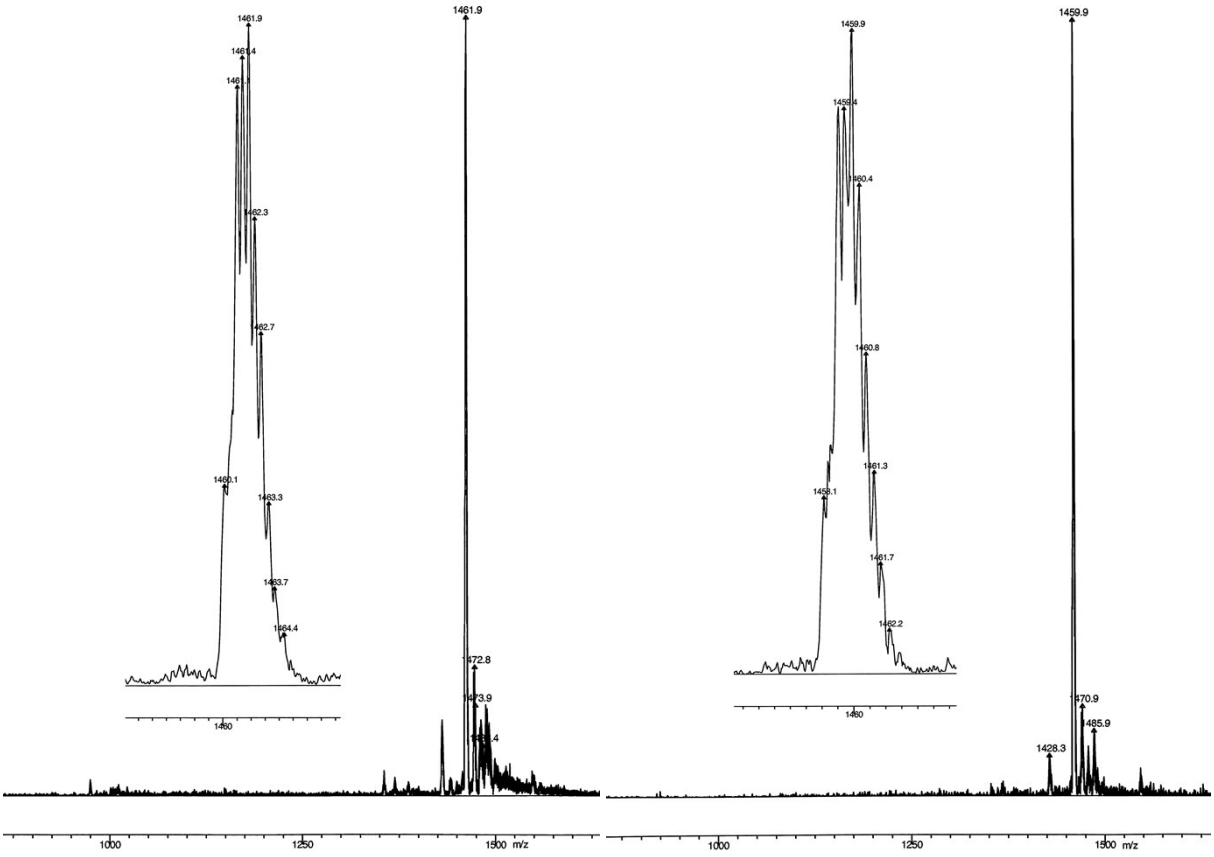

Figure S6: ESI-MS for Cu_4-**6** (left: positive; right: negative mode)

For demetallation, a solution of NOTA (30.3 mg, 100 µmol) in H_2O (2.0 mL) was added. The pH was adjusted to 2.2 using 1 N aq. HCl and the mixture was heated to 60°C for 60 min. After purification by preparative HPLC, Tetrap was obtained as a colorless solid (8.4 mg, 3.0 µmol, 61 %). MW (calcd. for $C_{90}H_{171}N_{27}O_{42}P_{12}$): 2675.21. MS (ESI, positive): m/z = 1784.4 [$2M$+3H$^+$], 1338.7 [M+2H$^+$], 892.9 [M+3H$^+$], 669.9 [M+4H$^+$]. HPLC (gradient: 5–55 % **B** in 20 min): t_R = 7.0 min.

Addition of 4 equivalents of 0.1 M aq. $Ga(NO_3)_3$ to an aqueous solution of **6** delivered the respective tetragallium(III) complex (vide infra); MS (ESI, positive): m/z = 1472.2 [M+2H$^+$], (ESI, negative): m/z = 1470.2 [M–2H$^+$].

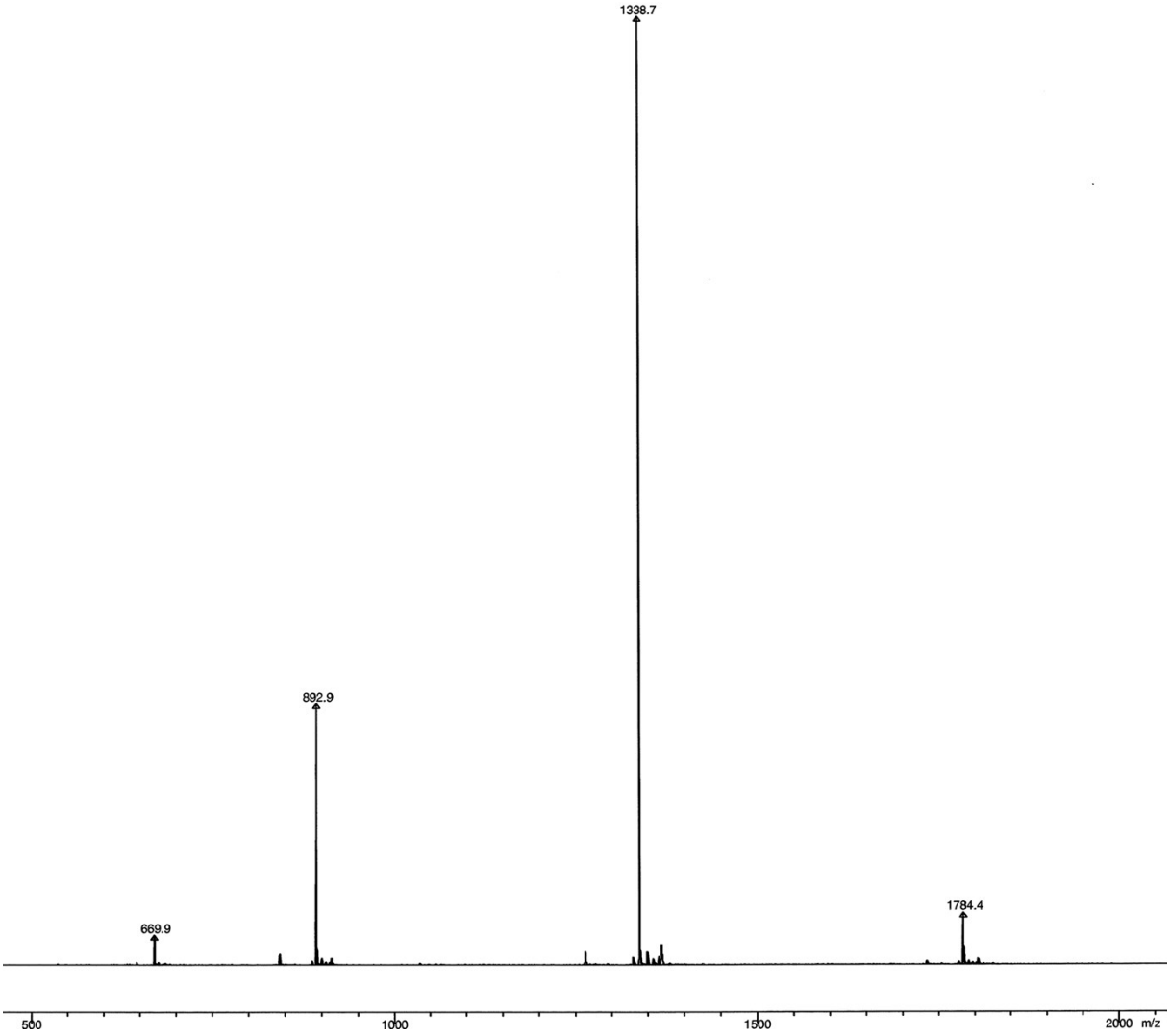

Figure S7: ESI-MS (positive mode) for compound **6**

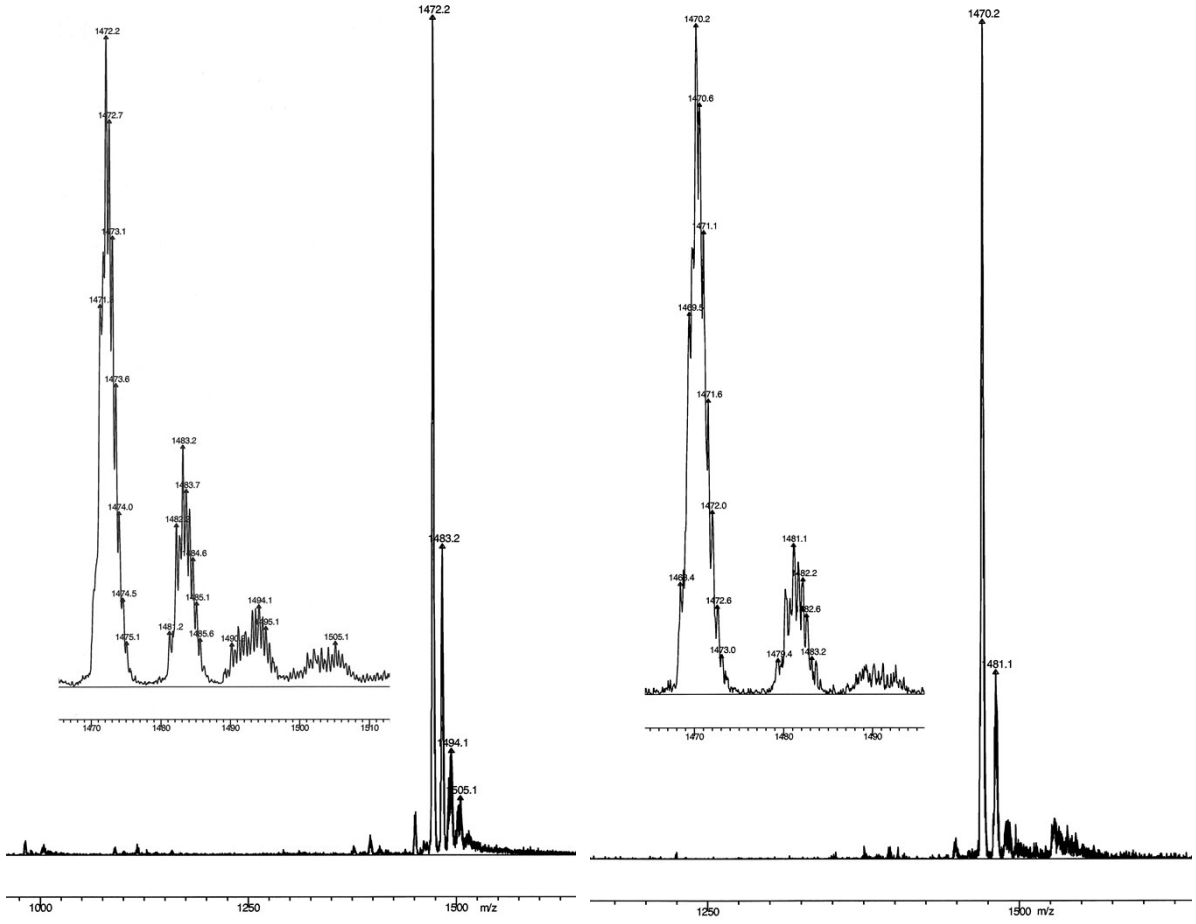

Figure S8: ESI-MS for Ga_4-**6** (left: positive; right: negative mode). Additional minor peaks originate from sodium- and potassium adducts, see explanations for Figure S4.

Synthesis of Tetrap(azide)$_6$ (7)

Synthesis of **7** was carried out using the Cu_4 complex of **6** (see above). Cu_4-**6** (14.5 mg, 4.95 µmol) and diisopropylamide (104 µL, 77 mg, 600 µmol) were dissolved in DMSO (700 µL). Then, 3-azidopropylamine (9.7 µL, 10 mg, 100 µmol) and HATU (60 mg, 158 µmol) were added with stirring. After 1 h, water (800 µL) was added and the mixture was purified by GPC. Lyophilization yielded 17.1 mg (4.8 µmol, 97 %) of Cu_4**7** as a blue solid. MS (ESI, positive): m/z = 1708.7 [M+2H$^+$], 1139.8 [M+3H$^+$]. HPLC (gradient: 5–55 % **B** in 20 min): t_R = 8.0 min. Such material was directly used for synthesis of Compound **8** (see below).

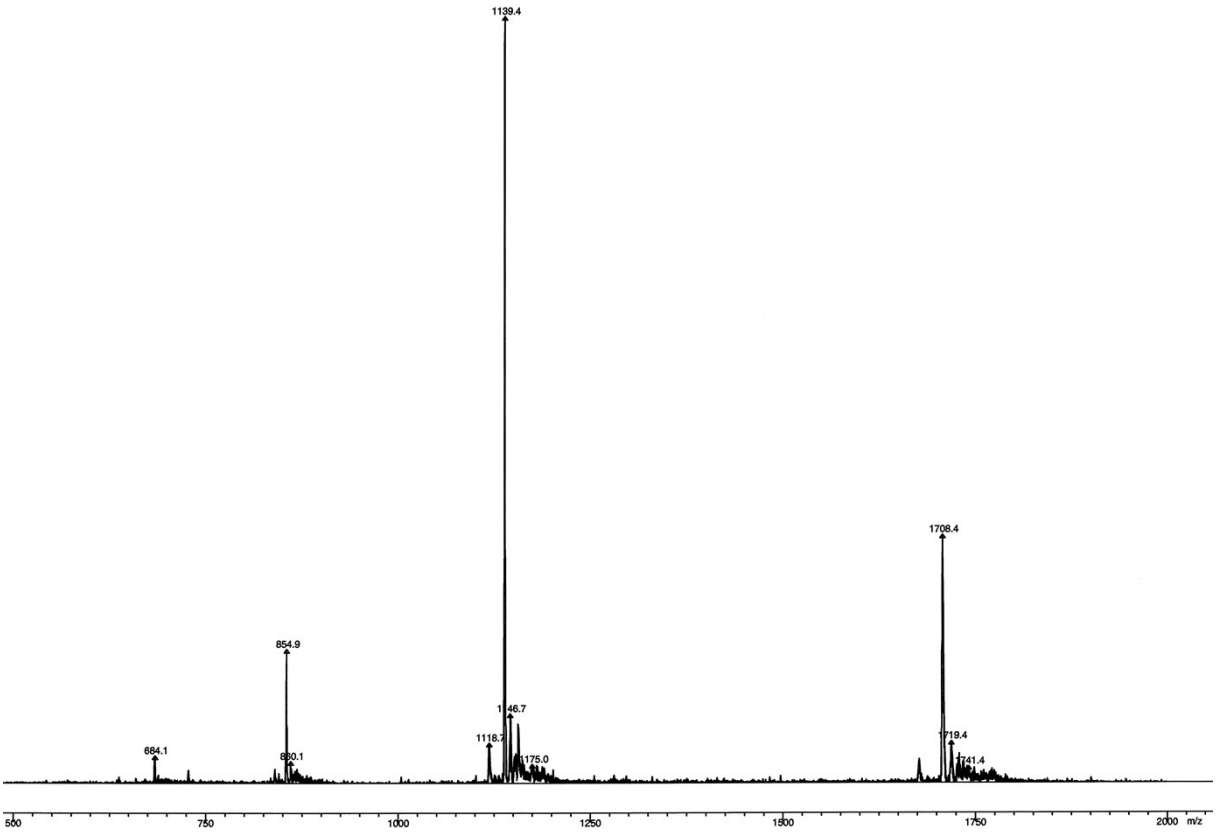

Figure S9: ESI-MS (positive mode) for Cu_4-**7**

For demetallation, a solution of NOTA (30.3 mg, 100 µmol) in H_2O (2.0 mL) was added. The pH was adjusted to 2.2 with 1 N aq. HCl and the mixture was heated to 60°C for 60 min. This solution was directly subjected to preparative HPLC, affording the copper-free product Tetrap(azide)$_6$ (**7**, 9.2 mg, 2.8 µmol, 56 %) as a colorless viscous oil. MW (calcd. for $C_{108}H_{207}N_{51}O_{36}P_{12}$): 3167.86. MS (ESI, positive): *m*/*z* = 1584.6 [*M*+2H$^+$], 1056.8 [*M*+3H$^+$], 792.9 [*M*+4H$^+$]. HPLC (gradient: 5–55 % **B** in 20 min): t_R = 15.0 min.

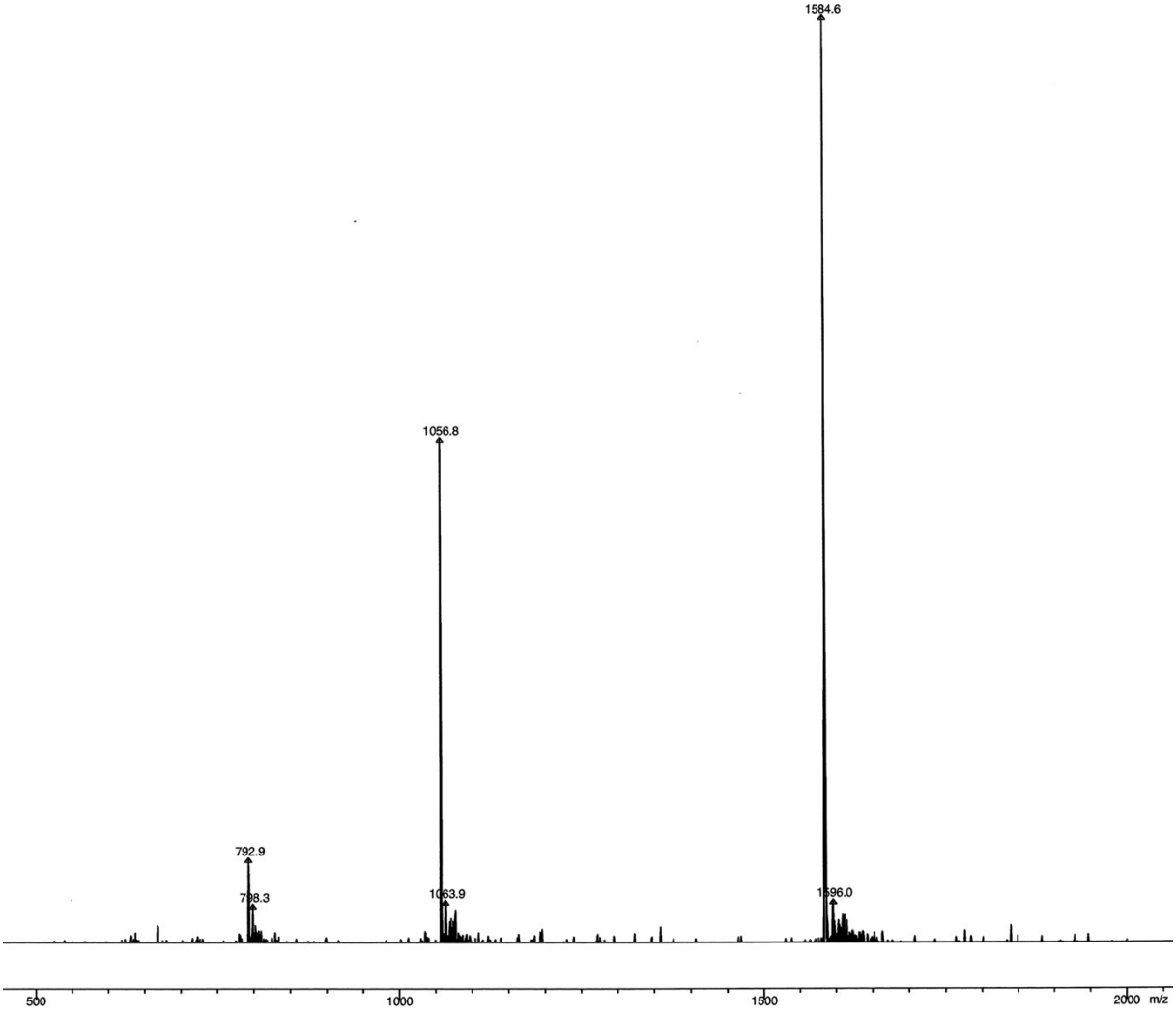

Figure S10: ESI-MS (positive mode) for compound **7**.

Synthesis of Tetrap(KuE)$_6$ (8)

Cu_4-**7** (4.0 mg, 1.17 µmol) and KuE-pentynoic acid (**9**, 5.4 mg, 10.5 µmol) were dissolved in a mixture of water (300 µL) and DMF (100 µL). After the addition of sodium ascorbate (11.6 mg, 58.5 µmol) in water (200 µL), the reaction was started by the addition of an aqueous solution of $Cu(OAc)_2 \cdot H_2O$ (0.23 mg, 1.17 µmol). The dark green reaction mixture was then stirred for 1 hour at room temperature. In order to remove Cu^{2+}, complexed by the TRAP chelator system, $Na_2S \cdot 9\,H_2O$ (11.24 mg, 46.8 µmol) dissolved in water (1 mL) was added, which resulted in a black precipitate of copper sulfide. After centrifugation, the supernatant was directly purified by preparative HPLC, yielding Tetrap(KuE)$_6$ (4.8 mg, 0.85 µmol, 73 %) as a colourless oil. MW (calcd. for $C_{210}H_{357}N_{69}O_{84}P_{12}$): 5564.28. MS (ESI, positive): m/z = 1854.3 [M+3H$^+$], 1391.0 [M+4H$^+$], 1112.9 [M+5H$^+$]. HPLC (gradient: 5–55 % **B** in 15 min): t_R = 8.7 min.

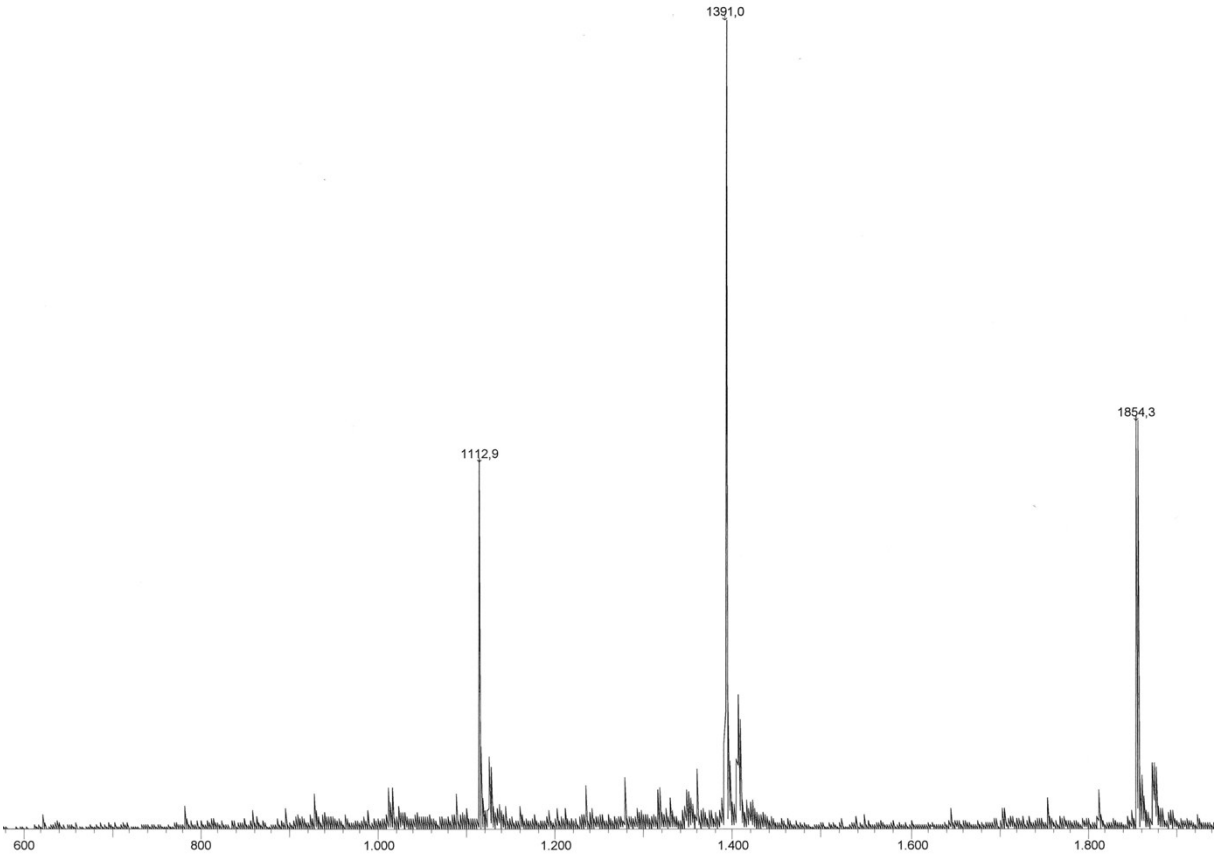

Figure S11: ESI-MS (positive mode) for compound **8**.

^{68}Ga / ^{67}Ga radiochemistry

^{68}Ga-labelling was done using neat eluate (1.25 mL, 1 M HCl) of a ^{68}Ge/^{68}Ga generator with SnO_2-matrix (manufactured by IThemba LABS, SA, distributed by IDB Holland, NL), adjusted to pH 2 by

addition of 2.7 M aq. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (450 µL). The resulting solutions possessed activity concentrations in the range of 200–240 MBq/mL. ^{67}Ga labelling was done using $^{67}Ga^{III}$ in 0.1 M HCl (manufactured by Mallinckrodt Inc., NL), adjusted to pH 2 with HEPES and slightly diluted to achieve a final activity concentration of approx. 450 MBq/mL. pH values were monitored using a Seven-Easy pH-meter (Mettler-Toledo, Germany).

Aliquots of these activity solutions containing ≈ 20 MBq (90 µL for ^{68}Ga, 45 µL for ^{67}Ga) were mixed with Tetrap (**6**) stock solutions of different concentrations (10 µL for ^{68}Ga, 5 µL for ^{67}Ga) in eppendorf cups, resulting in final Tetrap concentrations ranging from 0.1 nM – 10 µM (^{68}Ga) or 30 nM – 3 µM (^{67}Ga). These were left to react for 5 min at room temperature (25 °C, ^{68}Ga) or heated to 95 °C in a water bath. In order to remove any out-of-cage Ga^{III} complexes (where Ga^{III} is associated to the dendrimeric framework but not complexed by triazacyclononane chelator cages in a kinetically inert fashion), Na_2EDTA (20 µL of a 0.1 M aq. solution, pH 4.5) was added and solutions left to react at r.t. for 30 min. Analysis of the samples was done by radio-TLC (stationary phase: ITLC silica impregnated chromatography paper by Agilent Technologies; mobile phase: water), where the dendimer-bound activity is deposited at the start and the $[Ga(edta)]^-$ complex is eluted (R_f = 0.6–0.8). Analysis of the TLC was done using a radio-TLC scanner.

^{68}Ga-labelling of Tetrap(KuE)$_6$ (**8**) was done as described previously,[2] using the same generator as mentioned above in connection with a fully automated synthesis module (GallElut$^+$ by Scintomics, Germany). Briefly, 0.2 nmol of **8** were reacted with ^{68}Ga at pH 2 (HEPES-buffered generator eluate) for 5 min at 95 °C, followed by workup by means of solid phase extraction: The reaction solution was passed over a SepPak C8 light cartridge, followed by purging with water (10 mL) and elution of ^{68}Ga-Tetrap(KuE)$_6$ with 50% aq. ethanol (2 mL). The product was formulated by addition of 1 mM aq. $GaCl_3$ (10 µL) and phosphate-buffered saline (PBS, 2 mL) and concentrated in vacuo to 2 mL in order to remove the ethanol. After sterile filtration, this solution was used for small-animal PET imaging as described.[3]

For determination of the octanol-water distribution coefficients at pH 7.4 ($\log D_{OW}$), aliquots of approx. 0.5 MBq of the radiolabelled compound were added to eppendorf cups containing *n*-octanol and PBS (500 µL each). These were shaken vigorously for 2 min, centrifuged for 10 min at 11.500 g to separate phases, whereafter samples of *n*-octanol (200 µL) and water (20 µL) were taken. The activity in each aliquot was quantified in a gamma-counter and log *D* values were calculated from the quotients by correcting for the different sample volumes. Experiments were repeated 8 times. For the carrier-free ^{68}Ga-Tetrap(KuE)$_6$ (without addition of $^{69/71}GaCl_3$ after labelling), a log *D* of –5.55 ± 0.04 was measured. For the radiolabelled compound saturated with $^{69/71}GaCl_3$ as described above (i.e., the carrier-added [^{68}Ga]Ga_4-Tetrap(KuE)$_6$), a log *D* of –4.44 ± 0.10 was found.

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