Supporting Information

Improved synthesis and biological evaluation of chelator-modified α-MSH analogues prepared by copper-free click chemistry

Nicholas J. Baumhover[a,b], Molly E. Martin[c], Sharavathi G. Parameswarappa[d], Kyle C. Kloepping[a,b], M. Sue O'Dorisio[c], F. Christopher Pigge[d]*, and Michael K. Schultz[a,b]*

a Department of Radiology, Carver College of Medicine, The University of Iowa, 500 Newton Road, ML B180, Iowa City, IA 52242 USA. Fax: +1 (319) 335-8039; Tel: +1 (319) 355-8017; E-mail:michael-schultz@uiowa.edu.

b Department of Radiation Oncology (Free Radical Radiation Biology Program), Carver College of Medicine, The University of Iowa, 500 Newton Road, ML B180, Iowa City, IA 52242 USA. Fax: +1 (319) 335-8039; Tel: +1 (319) 355-8017; E-mail:michael-schultz@uiowa.edu.

c Department of Pediatric Hematology/Oncology, Carver College of Medicine, The University of Iowa Research Park, B145 MTF, 2501 Crosspark Road, Coralville, IA 52241 USA. +1 (319) 335-8668; Tel:+1(319)335-6506; E-mail:molly-martin@uiowa.edu.

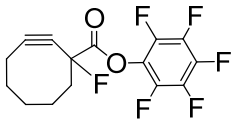
d Department of Chemistry, The University of Iowa, E557 CB, Iowa City, IA 52242 USA. Fax: +1 (319) 335-1270; Tel: +1 (319) 335-3805; E-mail:chris-pigge@uiowa.edu.

Contents:

Chemicals and Reagents

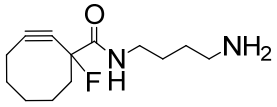
Fmoc-protected amino acids, *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate, 1-hydroxybenzotriazole, benzotriazole-1-ly-oxy-tris-pyrrolidinophosphonium hexafluorophosphate, and Rink amide resin for peptide synthesis were obtained from Advanced ChemTech (Louisville, KY). DOTA-tris (*t*-Bu ester) was purchased from Macrocyclics (Dallas, TX). *N,N*-dimethylformamide, *N,N*-diisopropylethylamine, methanol, trifluoroacetic acid, anisole, acetonitrile, $PhSiH_3$, $Pd(PPH_3)_4$, and 2,6- lutidine were purchased from Thermo Fisher Scientific (Waltham, MA). Dichloromethane, triisopropylsilane, sodium ascorbate, copper(I) bromide, BSA, 1,10-phenanthroline, HEPES, human serum, and citric acid were from Sigma Aldrich (St. Louis, MO). DMEM, MEM, FBS, and penicillin/streptomycin were from Invitrogen Life Technologies (Carlsbad, CA). B16/F10 murine melanoma cells were a gift from Dr. Michael Anderson at the University of Iowa (Iowa City, IA). DOTA-NHS ester was acquired from Macrocyclics (Dallas, TX). NOTA-NHS ester was custom prepared and obtained from Chematech (Dijon, France).

Synthesis of MFCO-PFP (2).



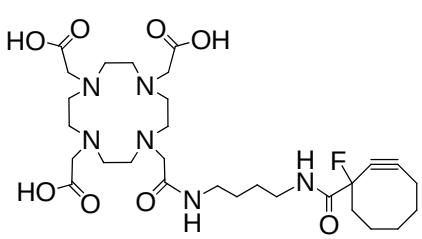
See Schultz, M. K.; Parameswarappa, S. G.; Pigge, F. C. *Org Lett*. **2010**, *12*, 2398. Pentafluorophenyltrifluoroacetate (0.49 mL, 2.82 mmol) was added to the stirred solution of freshly prepared MFCO-acid (**1**) (0.40 g, 2.35 mmol) in dichloromethane (10 mL) at 0 ^{o}C. Diisopropylethylamine (0.47 mL, 2.82 mmol) was then added and the reaction was stirred at 0 ^{o}C for 30 min, then allowed to warm to rt and maintained an additional 2 h. The reaction mixture was then filtered through a short silica gel column eluted with 2-5% ethyl acetate in hexanes. Column fractions containing product were combined and concentrated *in vacuo* to yield the desired PFP ester (**2**) as a yellow oil (0.58 g, 73%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm) 2.58-2.34 (m, 4H), 2.13-1.89 (m, 4H), 1.87-1.76 (m, 1H), 1.58-1.48 (m, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm) 165.0 (d, $J_{C\text{-}F}$ = 31.4 Hz), 142.8, 141.9, 139.6, 138.4, 136.4, 124.8, 110.7 (d, $J^{3}_{C\text{-}F}$ = 10.0 Hz), 91.7 (d, $J_{C\text{-}F}$ = 188.2 Hz), 85.4 (d, $J^{2}_{C\text{-}F}$ = 31.4 Hz), 46.8 (d, $J^{2}_{C\text{-}F}$ = 24.6 Hz), 33.9, 29.2, 25.6, 20.8. The signals corresponding to the pentafluorophenyl carbons (142.8-124.8) exhibited considerable broadening due to extensive C-F coupling.

Synthesis of MFCO-Amine (3).



A solution of MFCO-PFP ester **2** (0.50 g, 1.49 mmol) in dichloromethane (20 mL) was added to a stirred solution of 1,4-diaminobutane (0.66 g, 7.44 mmol) and triethylamine (2.0 mL, 14.9 mmol) in dichloromethane (20 mL) at 0 ^{o}C. The reaction was allowed to warm to rt and was stirred for 20 min. The reaction was then diluted with water (50 mL) and extracted with dichloromethane (3 x 20 mL). The combined organic layer was washed with water (5 x 10 mL) and brine (10 mL), and dried over anhydrous Na_2SO_4. Filtration and evaporation of the solvent gave the desired MFCO amine **3** as a pale yellow colored gummy solid (0.33 g, 92%, crude yield). This crude material was found suitable for use in subsequent coupling reactions directly without further purification. ^{1}H NMR (300 MHz, CD_3OD), δ (ppm): 3.24 (t, J = 6.5 Hz, 2H), 2.72 (t, J = 7.0 Hz, 2H), 2.35-2.23 (m, 4H), 2.10-1.81 (m, 4H), 1.68-1.44 (m, 6H). ^{13}C NMR (75MHz, CD_3OD), δ (ppm): 171.2 (d, $J^{2}_{C\text{-}F}$ = 24.1 Hz), 110.1 (d, $J^{3}_{C\text{-}F}$ = 10.0 Hz), 95.3 (d, $J_{C\text{-}F}$ = 185.3 Hz), 88.5 (d, $J^{2}_{C\text{-}F}$ = 31.4 Hz), 48.2 (d, $J^{2}_{C\text{-}F}$ = 24.9 Hz), 41.8, 40.2, 35.1, 30.3, 29.7, 27.8, 26.9, 21.2. HRMS (ESI): calculated for $C_{13}H_{22}FN_2O$ $[M + H]^{+}$, 241.1716; found 241.1724.

Synthesis of DOTA-MFCO (6) and NOTA-MFCO (7).



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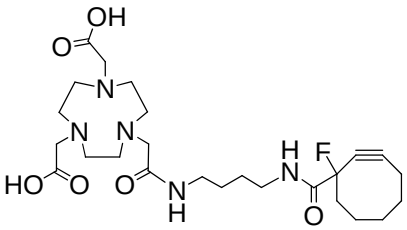

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DOTA-MFCO **6** was prepared by reacting DOTA-NHS ester **4** (33.9 mg, 61 μmol) with MFCO-amine **3** (15 mg, 62 μmol) for 2 h at rt in a solvent mixture of DMF (0.5 mL), DMSO (1 mL), and triethylamine (88 μL). Similarly, NOTA-MFCO **7** was prepared by reacting NOTA-NHS ester **5** (25 mg, 62.5 μmol) with MFCO-amine **3** (15 mg, 62 μmol) for 2 h at rt in the previously mentioned solvent mixture (Scheme 2). The MFCO modified chelators were purified on an Agilent 1200 Series RP-HPLC by injecting the reaction mixture on an Agilent semi-preparative C18 analytical column (9.4 x 25 cm) eluted at 3 mL/m with 50 mM triethylamine acetate buffer (pH 7.0) with 0.1 v/v % acetonitrile and acetonitrile gradient of 1-30 v/v % over 20 m while monitoring Abs at 214 nm. The major peak was collected and pooled from multiple runs, lyophilized, and stored at -20°C. The products were characterized by ESI-MS in the positive and negative mode by preparing samples in a solution of acetonitrile/water (50/50 v/v %). Chelator MFCO's were analyzed by RP-HPLC using an Agilent C18 analytical column (4.6 x 15 cm) eluted at 1 mL/m with 50 mM triethylamine acetate buffer (pH 7.0) with 0.1 v/v % acetonitrile and acetonitrile gradient of 1-30 v/v% over 20 m while monitoring Abs at 214 nm.

Solid Phase Peptide Synthesis

General Peptide Synthesis

Linear peptides α-MSH (Ac-SYSMEHFRWGKPV-NH_2) and NDP-α-MSH (Ac-SYS-Nle-EHfRWGKPV-NH_2) were synthesized on Rink amide resin at a 0.1 mmol scale following standard Fmoc procedures on an AAPPTEC Apex 396 automated multiple peptide synthesizer. DOTA-tris (*t*-Bu ester) was coupled to the *N*-terminus of the protected peptide on-resin. Each peptide-resin (1 equiv peptide) was suspended in *N,N*-dimethylformamide (DMF); and DOTA-tris (*t*-Bu ester) (5 equiv), *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU; 5 equiv), 1-hydroxybenzotriazole (HOBt; 5 equiv), and *N,N*-diisopropylethylamine (DIPEA; 10 equiv) were added and the reaction was mixed at 37°C overnight. Completeness of coupling was checked by Kaiser test for free amines (*37*). The peptide-resin was filtered, washed with DMF, dichloromethane (DCM), and methanol, and allowed to dry.

DOTA-amide-NDP-α-MSH

The linear DOTA-conjugated peptide DOTA-NDP-α-MSH was prepared in the following way: DOTA-tris (*t*-Bu ester) was coupled to the *N*-terminus of α-MSH and NDP-α-MSH by conjugating the resin-bound peptide with a 5-fold excess each of DOTA-tris (*t*-Bu ester), HATU, HOBt, and a 10-fold excess of DIPEA in DMF. The reaction was mixed overnight, then the resin was washed with DMF, DCM, and MeOH. Peptides were cleaved and deprotected with 95:2.5:2.5 trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/water for 2 h at room temperature, then precipitated with ice-cold ether. The peptides were purified on RP-HPLC by injection onto a Vydac C_{18} semipreparative column (10 x 250 mm, 10 μm, 300 Å; Grace, Deerfield, IL) eluted at 5 mL/min with 0.1% TFA and a 20-30% gradient of acetonitrile over 30 minutes while monitoring absorbance at 280 nm. The peak of interest was collected, concentrated by rotary evaporation, and lyophilized. Purified peptides were characterized on an Agilent 1100 LC-ESI-ion trap mass spectrometer.

Azido-Hex-NDP-α-MSH (8).

Hex-NDP-α-MSH was prepared by standard Fmoc procedures employing HBTU and 9-hydroxybenzotriazole single couplings on a 100 μmol scale using an AAPPTEC Apex 396 solid phase peptide synthesizer (Louisville, KY). Azido-Hex-NDP-α-MSH (**8**) was prepared by coupling 6-azido-hexanoic acid to the N-terminus of the fully side-chain protected peptide on resin utilizing a five-fold excess of 6-azido-hexanoic acid, 4.9-fold excess of HBTU, and 5-fold excess DIPEA. Following synthesis, the resin was washed with DMF, DCM, and methanol. The peptide was removed from the resin and side chain deprotected using a cleavage cocktail of TFA/TIS/H_2O (95:2.5:2.5 v/v/v) for two h followed by precipitation in ice cold diethyl-ether. The precipitate was centrifuged at 3000 rpm for 20 m at 4°C and the supernatant decanted. The crude peptide was reconstituted in water and purified to homogeneity with a Dionex Ultimate 3000 RP-HPLC system by injecting the crude material onto a Vydac C18 column (2 x 25 cm) eluted with 0.1 v/v % TFA using a 25-30 % (v/v) acetonitrile gradient at 5 mL/min over 30 min while monitoring tryptophan and tyrosine absorbance (Abs 280 nm). The major peak was collected and pooled from multiple runs, lyophilized, and stored at -20°C.

The peptide was prepared as the acetate salt by TFA salt exchange by employing multiple freeze drying cycles with 10 v/v % acetic acid. Purified peptides in the acetate salt form were reconstituted in water and quantified by tryptophan + tyrosine Abs (ε 280 nm = 6930 M^{-1} cm^{-1}) to determine isolated yield. Purified Azido-Hex-NDP-α-MSH (**8**) was characterized by LC-MS on an Agilent 1100 series LC-ESI-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 x 25 cm) eluted at 0.7 mL/m with 0.1 % (v/v) TFA and an acetonitrile gradient of 5-45 % (v/v) over 30 min (Supplemental Figure 3). Mass spectral data was obtained in the positive ion mode.

Synthesis of DOTA-Click-Hex-NDP-α-MSH (9) and NOTA-Click-Hex-NDP-α-MSH (10).

DOTA and NOTA-Click-Hex-NDP-α-MSH were synthesized by reacting 100 nmol of Azido-Hex-NDP-α-MSH (**8**) with a 10-fold excess of DOTA-MFCO (**6**) or a 20-fold excess of NOTA-MFCO (**7**) in 0.5 mL of H_2O (Scheme 3). Reaction progress was monitored by observing the disappearance of starting material using RP-HPLC (214 nm). The click reaction between DOTA-MFCO (**6**) and azido-Hex-NDP-α-MSH (**8**) was complete in 2 h while the NOTA-Click-Hex-NDP-α-MSH **(10)** required 5.5 h. The peptide conjugates were purified on an Agilent 1200 Series RP-HPLC by injecting the reaction mixture on an Agilent semi-preparative C18 analytical column (9.4 x 25 cm) eluted at 3 mL/min with 0.1 v/v % TFA and acetonitrile gradient of 5-45 v/v % over 20 min while monitoring tryptophan + tyrosine absorbance at 280 nm. The major peak was collected and pooled from multiple runs, lyophilized, and stored at -20°C. The peptides were prepared as the acetate salt by TFA salt exchange by employing multiple freeze drying cycles with 10 v/v % acetic acid. Purified peptides in the acetate salt form were reconstituted in water and quantified by tryptophan + tyrosine Abs (ε 280 nm = 6930 M^{-1} cm^{-1}) to determine isolated yield. Purified peptides were characterized on an Agilent 1100 series LC-ESI-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 x 25 cm) eluted at 0.7 mL/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 v/v% over 30 min (Supplemental Figure 4).

Radiochemistry (Figure 1).

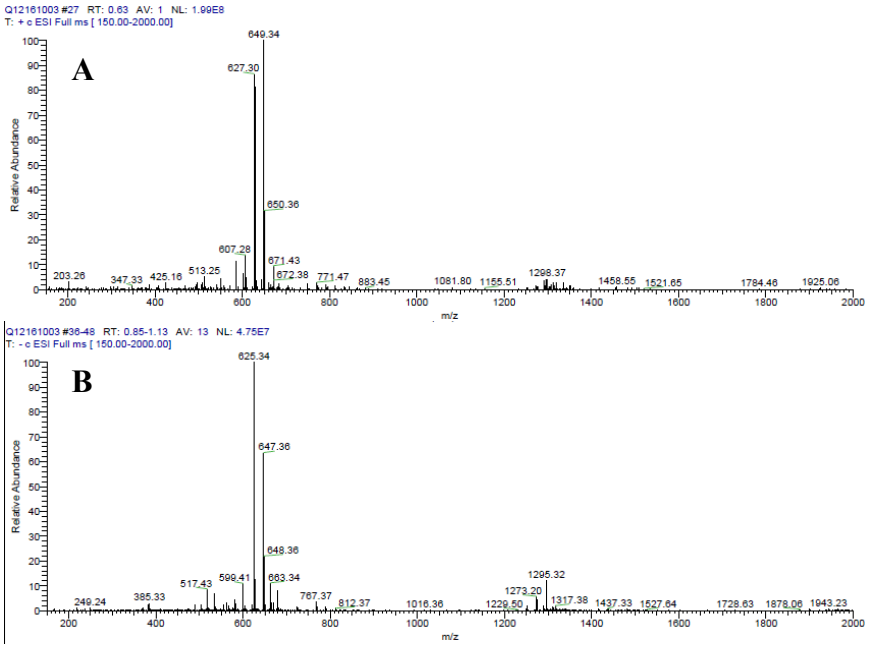
Copper-64: Copper-64 was obtained as ^{64}CuCl2 in mildly acidic solution from the College of Medicine, Washington University in Saint Louis (WashU, St. Louis, MO USA). Radiolabeling of DOTA-peptides with ^{64}Cu was carried out as described previously. Briefly, ^{64}Cu was received from WashU in approximately 10-40 µL of mildly acidic solution in a screw-top plastic "v-vial", enclosed within appropriate lead shielding. To this vial was added ultra-pure water or pH 6.0 sodium acetate buffer solution to a final volume of 200 µL and the solution was mixed. The 200 µL solution was withdrawn and transferred to a 1.5 mL plastic v-vial (SealRite, Natural microcentrifuge tube, Cat# 1615-5500, USA Scientific) and a measurement of the total activity was determined by dose calibrator measurement. Aliquots of appropriate radioactivity amounts could then be transferred volumetrically to appropriate radiolabeling solutions. Generally, between 5 and 10 nmoles of peptide were suspended in 50 uL of pH 6.0 acetate buffer. To this solution was added an appropriate aliquot of ^{64}Cu master solution and the solution was incubated for 45 m at 70°C. This solution was used without further purification for in vitro stability testing and in vivo imaging experiments. The identity of radiolabeled species was confirmed by radioHPLC retention time comparison to UV traces of the unlabeled species at 254 nm (Figure 1). An aliquot (~ 20 µL) was injected for HPLC mediated radioactivity detection by in-line radioHPLC using an in-line liquid scintillation counting (LSC) system (β-RAM, IN/US Systems, Tampa, FL USA) equipped with a 20 µL internal mixing loop and a 4:1 liquid scintillant:mobile phase mixing ratio. Liquid scintillant was In-Flow™ 2:1 (IN/US). RadioHPLC traces were collected using the software program ScintFlow® (IN/US), which integrates direct data collection via Microsoft Excel®. Radiochemical purity and the stability of ^{64}Cu-coupling to chelator-modified peptide bioconjugates was determined by radioHPLC using the analytical column and gradient described above or radioTLC using iTLC-SG glass microfiber chromatography paper impregnated with silica gel (Varian, Lake Forest, CA USA); mobile phase 0.1 M citric acid; 2 µL spotting, migration time 3 m, followed by strip drying with a common hairdryer. iTLC paper was cut into 5 cm x 25 cm strips and heated at 100 °C for at least 30 m prior to measurements.

Gallium-68: Radiolabeling with ^{68}Ga was carried through the use of a ^{68}Ga/^{68}Ge generator system IGG100 (Eckert Ziegler, GmBH, Berlin, De) with a total ^{68}Ge activity of approximately 900 MBq at the time of experiments presented here. The system was aged between 5-10 months for experiments conducted for this investigation. The methodology involves a single step purification of 68Ga eluted from the generator that is evaluated in detail elsewhere. Briefly, ^{68}Ga is eluted from the generator with 10 mL of 0.1 M HCl with a flow rate of approximately 2 mL per minute directly to a cation exchange (Strata™-X-C , 33µ, Strong Cation, 30 mg/mL #8B-S029-TAK, Phenomenex) column mounted by supports fabricated in house. Following generator elution, the generator outflow line is disconnected and the Strata-X-C column is air dried using a 20 mL plastic syringe (2x). Once dry, pure ^{68}Ga is eluted from the Strata-X-C column with 400 µL of 98% acetone/0.05 M HCl (prepared weekly and stored at 4°C) directly to a glass vial containing 5-10 nmoles DOTA-peptide conjugate dissolved in 5 mL of pure water that had been preheated to approximately 80 °C. This solution was then heated (open vessel) to 100°C and 68Ga and the DOTA-peptide are incubated for 15 m. Acetone is evaporated to less than 300 ppm in the final purified drug product by this method (data not shown). Following the radiolabeling incubation period, the reaction mixture containing the [^{68}Ga]-peptide and a small amount of free ^{68}Ga was drawn up through a Strata™-X cartridge (33µ Polymeric Reversed Phase, 30 mg/1mL, #8B-S100-TAK, Phenomenex® Inc., Torrance, CA USA) that had been preconditioned by passing 1 mL of 95% ethanol (USP for Injection) and 2.5 mL of pure water. The cartridge was then rinsed with 2 mL of water to remove any residual free ^{68}Ga and finally the purified ^{68}Ga-peptide conjugate was eluted in 500 µL of 1:1 ethanol (95%):isotonic saline solution. Radiochemical purity was determined by radioHPLC as described for ^{64}Cu.

In Vitro Competitive Binding Assays (Figure 2).

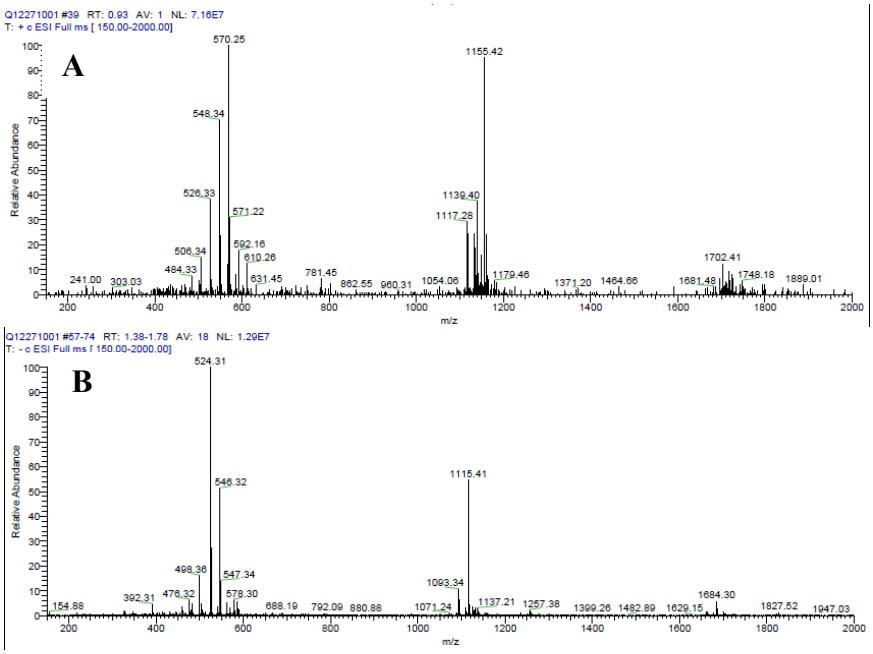
B16-F10 murine melanoma cells were cultured in high glucose DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin in 150 cm2 culture flasks at 37oC in a humidified 5% CO2 incubator. Cells were grown to 70% confluency and used in competitive binding assays similar to the methods developed by Eberle et al., *J Recept Res.* **1991**, *11*, 311. Cells were scraped from the culture flasks, counted on a Beckman Coulter cell counter, and resuspended in binding media (MEM containing 25 mM HEPES, 0.2% BSA, and 0.3 mM 1,10-phenanthroline). For binding assays, a total volume of 500 µL was used in 1.5 mL Eppendorf tubes. The cell suspension (50 µL containing approximately 4 million cells) was incubated with approximately 30,000 cpm of [^{125}I]-[Nle^{4},D-Phe^{7}]-α-MSH and increasing concentrations of α-MSH or peptide analog ranging from 10^{-6} to 10^{-11} M. The tubes were mixed at 25 °C, 600 rpm on an Eppendorf Thermomixer for 1.5 h. Following incubation, cells were pelleted by centrifugation, the media was aspirated, pellets were transferred to 12 x 75 mm glass tubes, and the radioactivity of the cell pellet was determined using a PerkinElmer Cobra II gamma counter (PerkinElmer, Freemont, CA). All handling of radioactive materials was conducted using procedures and protocols approved through the University of Iowa Environmental Health and Safety Committee and Health Protection Office, adhering to ALARA principles. Each experiment was performed in quadruplicate and binding curves were plotted; IC50 values and their associated standard errors were calculated with GraphPad Prism 5 curve-fitting software (GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego, CA).

Mass spectrum of DOTA-MFCO (6).



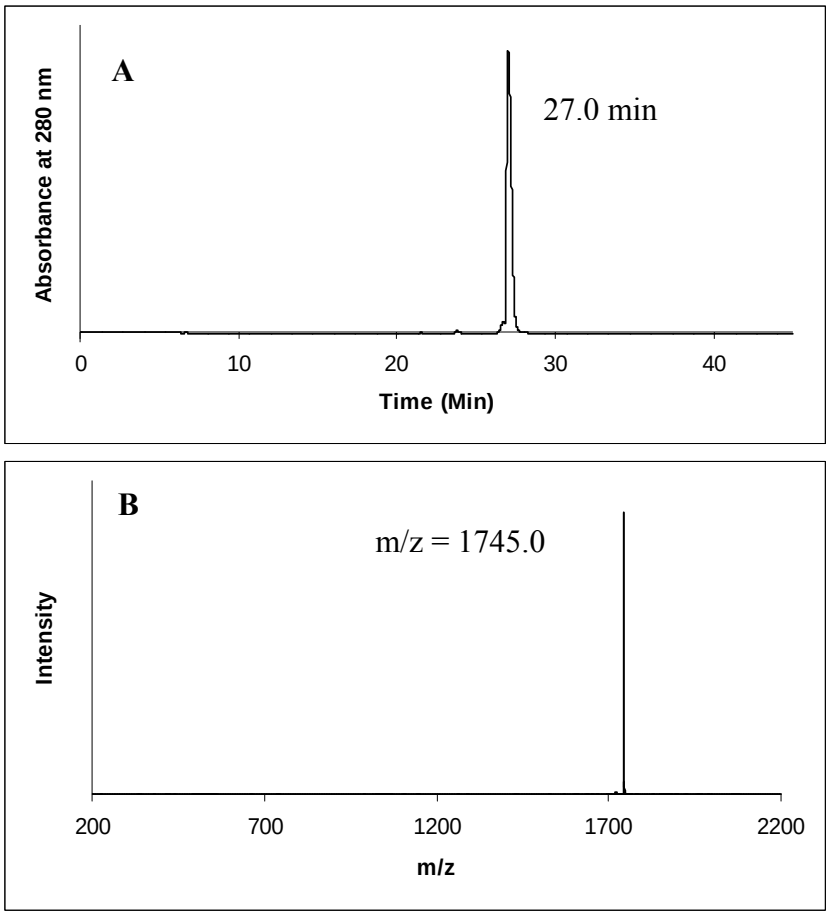
Supplemental Figure 1. ESI-MS of purified DOTA-MFCO **6** (calcd. for $C_{29}H_{47}FN_6O_8$, 626.3 amu). Panel A: positive-ion ESI-MS. $[M+H]^+$ *m/z* 627.30; $[M+Na]^+$ *m/z* 649.34. Panel B: negative-ion ESI-MS. $[M-H]^-$ *m/z* 625.34; $[M+Na-2H]^-$ *m/z* 647.36.

Mass spectrum of NOTA-MFCO (7).



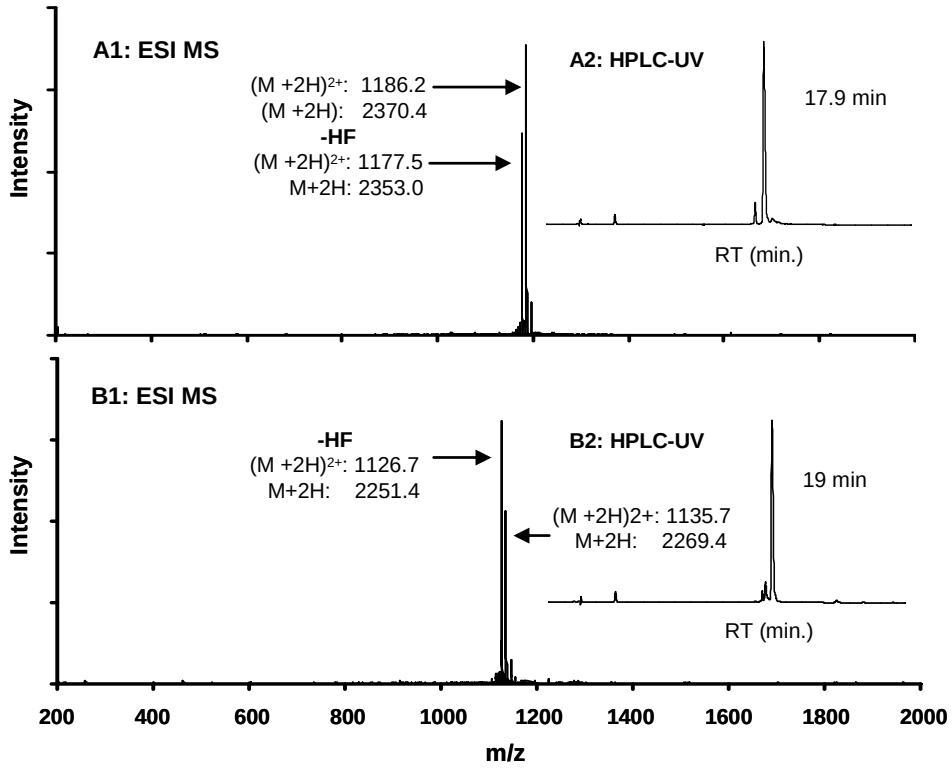
Supplemental Figure 2. ESI-MS of purified DOTA-MFCO **7** (calcd. for $C_{25}H_{40}FN_5O_6$, 525.3 amu) and observed ESI peak identifications. Panel A: positive-ion ESI-MS. $[M+H]^+$ *m/z* 526.33; $[M+Na]^+$ *m/z* 548.34; $[M+2Na-H]^+$ *m/z* 570.25; $[2M+Triethylamine]^+$ *m/z* 1155.42; $[2M+Triethylamine-HF]^+$ *m/z* 1139.4; $[2M+Triethylamine-2(HF)]^+$ *m/z* 1117.29 Panel B: negative-ion ESI-MS. $[M-H]^-$ *m/z* 524.31; $[M+Na-H]^-$ *m/z* 546.32; $[2M+3Na-H]^-$ *m/z* 1115.41; $[2M+3Na-HF]^-$ *m/z* 1093.34.

HPLC trace and mass spectrum of azido-hex-NDP-α-MSH (8).

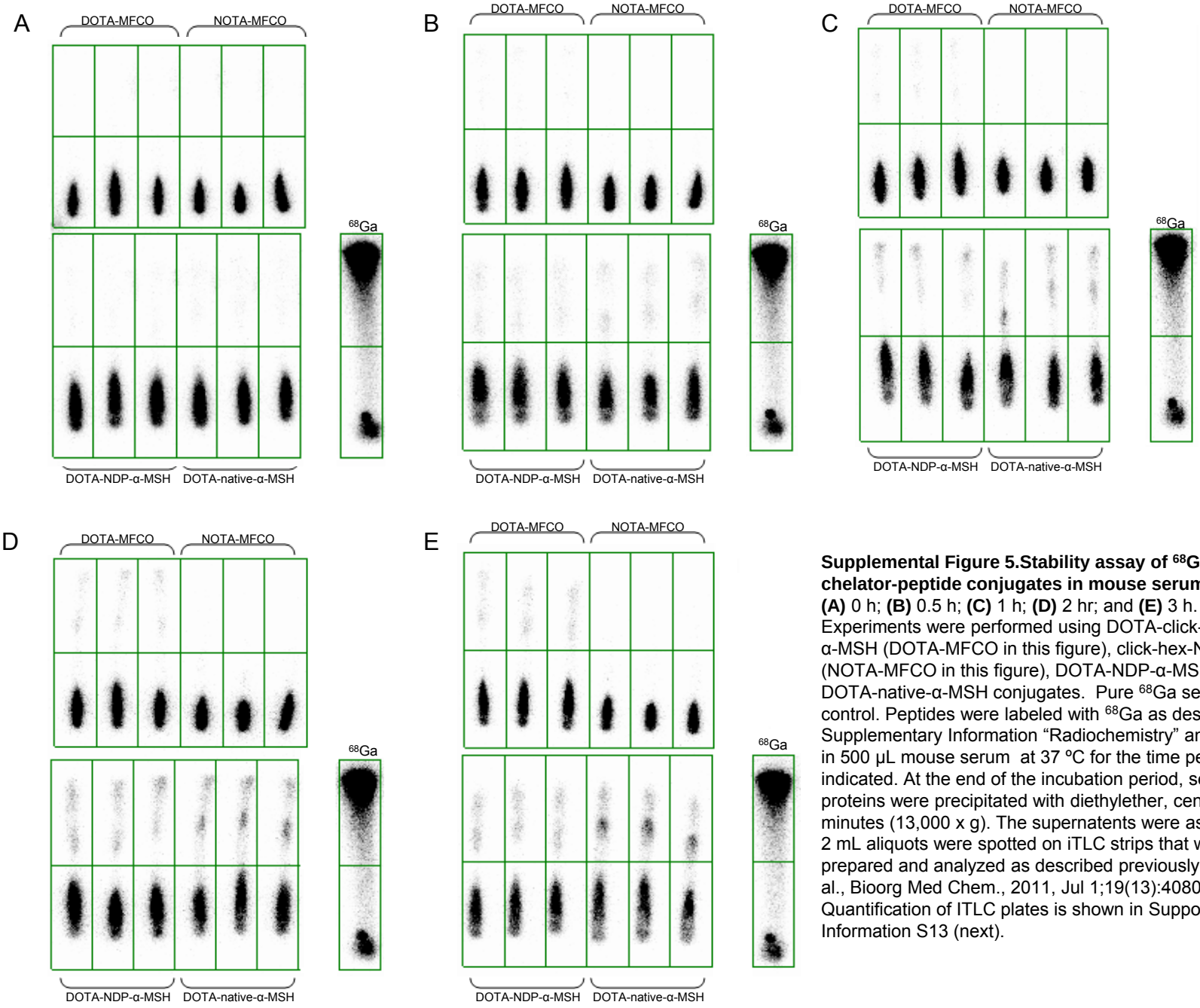


Supplemental Figure 3. Analytical RP-HPLC and ESI-MS of purified Azido-Hex-NDP-α-MSH **8**. Following preparative HPLC purification, Azido-Hex-NDP-α-MSH was rechromatographed by injecting 2 nmol onto an Agilent analytical C18 analytical column (4.6 x 25 cm) eluted at 0.7 mL/min with 0.1 v/v % TFA and an acetonitrile gradient of 5-45 v/v% over 30 min while monitoring absorbance at 280 nm (Panel A). ESI-MS of the purified peptide produces a singly charged positive ion of 1745.0 *m/z* which agrees closely with the calculated mass of 1744.0 amu (Panel B).

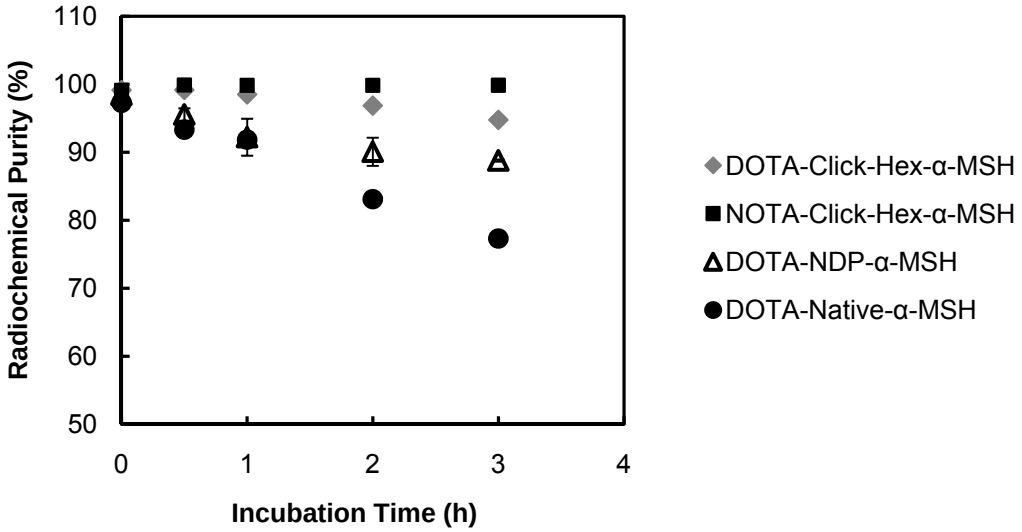
HPLC traces and mass spectra of DOTA and NOTA-click-hex-NDP-α-MSH (9 & 10).



Supplemental Figure 4. RP-HPLC-UV and ESI-MS analysis of purified DOTA and NOTA peptide conjugates: (A1) DOTA-click-Hex-NDP-α-MSH **(9)** produces a doubly charged ion of 1186.2 *m/z* corresponding to an observed mass of 2370.4 amu; theoretical 2370.7 amu and loss of HF from the triazole ring is observed as a doubly charged ion of 1177.5 *m/z*: observed 2353.0; theoretical 2352.7. (B1) observed masses of desired NOTA-click-Hex-NDP-α-MSH **(10)** produces a doubly charged ion of 1135.7 *m/z* producing an observed mass of 2269.4; theoretical 2370.7 amu; and mass corresponding to loss of HF from the triazole ring interestingly produces the major doubly charged ion of 1126.7 *m/z*, producing an observed mass of 2251.4; agreeing with the theoretical mass of 2251.6 amu. (A2 and B2 insets); HPLC-UV traces of purified compounds.



Supplemental Figure 5.Stability assay of ^{68}Ga labeled chelator-peptide conjugates in mouse serum over time. **(A)** 0 h; **(B)** 0.5 h; **(C)** 1 h; **(D)** 2 hr; and **(E)** 3 h. Experiments were performed using DOTA-click-hex-NDP-α-MSH (DOTA-MFCO in this figure), click-hex-NDP-α-MSH (NOTA-MFCO in this figure), DOTA-NDP-α-MSH and DOTA-native-α-MSH conjugates. Pure ^{68}Ga served as the control. Peptides were labeled with ^{68}Ga as described in Supplementary Information "Radiochemistry" and incubated in 500 µL mouse serum at 37 ºC for the time period indicated. At the end of the incubation period, serum proteins were precipitated with diethylether, centrifuged 10 minutes (13,000 x g). The supernatents were aspirated and 2 mL aliquots were spotted on iTLC strips that were prepared and analyzed as described previously. (Rockey et al., Bioorg Med Chem., 2011, Jul 1;19(13):4080-90. Quantification of ITLC plates is shown in Supporting Information S13 (next).



Supplemental Figure 6. Stability of chelator modified peptide conjugates (analysis of raw data shown in Supplemental Figure 5, S12). Experiments were performed using DOTA-click-hex-NDP-α-MSH, NOTA-click-hex-NDP-α-MSH, DOTA-NDP-α-MSH and DOTA-native-α-MSH conjugates and 0 h, 0.5 h, 1 h, 2 h and 3 h time points. Pure ^{68}Ga served as the control (see previous). ITLC strips (Supplemental Figure 5) were scanned using a GE phosphor imager. ^{68}Ga-labeled peptides (1 nmole; specific activity ~10 MBq nmole^{-1}) were incubated in human serum at 37 ºC for incubation time periods shown in the x-axis. At the end of the incubation period, serum proteins were precipitated with diethylether and the supernatent was analyzed by ITLC as shown in (Supplemental Figure 5). Radiochemical purity of the chelator-modified peptides was calculated by dividing the average signal intensity over the bottom half of the TLC strips by average signal intensity over the entire length of the TLC strips. Experiments were performed in triplicate and uncertainty bars represent the standard deviation of the triplicate measurements. The DOTA-click-hex-NDP-α-MSH and NOTA-click-hex-NDP-α-MSH prepared by copper free click chemistry appear to have a stability advantage over the DOTA-amide-NDP-α-MSH shown. As expected the native peptide is less stable in serum than the NDP-α-MSH analogs. The high stability of the NOTA variant in comparison to the DOTA variant may reflect superior kinetic stability of the ^{68}Ga-NOTA coupling and is the subject of future investigations in our laboratories.