

Supplementary Information

A novel strategy for the synthesis of enzymatically stable biotin-DOTA conjugates for *in vivo* use

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EXPERIMENTAL PROCEDURES

General. The used chemical were purchased from Sigma-Aldrich Corporation, St Louis, MA as synthetic grade and used without further purification except p-H₂NPhCH₂DOTA which was purchased from Macrocyclics (Dallas, TX). Solvents for HPLC analysis were obtained as HPLC grade and were filtered prior to use. Silica gel chromatography was conducted with 70-230 mesh silica gel purchased from Merck .

Spectral Analysis: ¹H and ¹³C NMR were obtained on a Bruker Avance 500 DRX instrument operating at 500 MHz and 126 MHz, respectively. Tetramethylsilane was used as internal standard for chemical shifts which are given in ppm. MALDI-MS spectra were obtained on Applied Biosystems QSTAR XL hybrid QTOF spectrometer using oMALDI ionization source (Applied Biosystems Inc., Foster City, CA) and α-cyano-4-hydroxycinnamic acid was used as the matrix.

Chromatography: The HPLC purifications were performed on a Shimadzu RP-HPLC system (Kyoto, Japan) equipped with semipreparative Waters xTERRA C₁₈ column (Waters, Milford, MA) using acetonitrile gradient (0.1%TFA in H₂O/0-85% ACN gradient for 60 min) and monitored at 220 nm. HPLC analyses were performed with analytical Waters xTERRA C₁₈ column using acetonitrile gradient (0.1%TFA in H₂O/0-85% ACN gradient for 30 min) and monitored at 240 nm.

Characterization of the prepared compounds: The intermediates to target compounds were identified by ¹H NMR spectroscopy and were comparable with those reported earlier in literature^{1,2}, but amide protons from biotin ring were not observed due to solvent (CD₃OD) used in measurements. The target DOTA derivatives **1** and **2** were identified only by mass spectrometric methods based on their [M+H]⁺ peaks due to their low isolated yield.

Synthesis of Biotin Derivatives

4-(5-Hydroxypentyl)tetrahydro-1*H*-thieno[3,4-*d*]imidazol-2(3*H*)-one (biotinol, 4): To a stirred suspension of LiAlH₄ (1.00 g, 26.35 mmol) in dry diethyl ether (100 ml) was added in several portions a hot (ca. 60 °C) solution of d-biotin (1.00 g, 4.09 mmol) in dry pyridine (25 ml). The reaction mixture was stirred for 30 min at room temperature followed by refluxing for 1 h. Excess of LiAlH₄ was quenched by drop wise addition of H₂O. The organic phase was separated from the solids; the solids were filtered off, dried *in vacuo*, acidified with 6 M HCl, evaporated to dryness and extracted with acetone (5 x 25 ml). The organic phase and combined acetone fractions were evaporated to dryness and purified on silica column chromatography by using a mixture of EtOAc/MeOH (7:3) as an eluent to afford biotinol **4** (772 mg, 82%) as a white solid: ¹H NMR [CD₃OD/CDCl₃ (1:1), 500 MHz] δ 4.51 (dd, 1H, ³J_{HH} = 7.8 and 4.7 Hz), 4.33 (dd, 1H, ³J_{HH} = 7.8 and 4.8 Hz), 3.58 (t, ³J_{HH} = 6.5 Hz, 2H), 3.20 (ddd, 1H, ³J_{HH} = 8.9, 6.0 and 4.7 Hz), 2.94 (dd, 1H, ²J_{HH} = -12.9, ³J_{HH} = 4.8 Hz), 2.75 (d, 1H, ²J_{HH} = -12.9 Hz), 1.78-1.69 (m, 1H), 1.67-1.52 (m, 3H), 1.50-1.38 (m, 4H).

5-(2-Oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentyl methanesulfonate (5): To a stirred solution of **4** (250 mg, 1.09 mmol) in dry pyridine (3 ml) was added freshly distilled mesyl chloride (100 µl, 1.29 mmol) and the reaction mixture was stirred for 45 min at 20°C. The residue after evaporation was purified on silica column chromatography by using a mixture of EtOAc/MeOH (8:2) as an eluent to give **5** (120 mg, 36%) as a white solid: ¹H NMR (CDCl₃, 500 MHz) δ 5.10 (s, 1H), 4.71 (s, 1H), 4.56-4.51 (m, 1H), 4.36-4.31 (m, 1H), 4.25 (t, 2H, ³J_{HH} = 6.4 Hz), 3.21-3.15 (m, 1H), 3.03 (s, 3H), 2.95 (dd, 1H, ²J_{HH} = -12.9, ³J_{HH} = 5.0 Hz), 2.75 (d, 1H, ²J_{HH} = -12.9 Hz), 1.84-1.64 (m, 4H), 1.54-1.43 (m, 4H).

Synthesis of 6-(5-Iodo-pentyl)-tetrahydro-thieno[3,4-*d*]imidazol-2-one (6): To a stirred solution of **5** (38 mg, 0.12 mmol) in dry acetone (2 ml) was added NaI (27 mg, 0.25 mmol, 2 eq) and the reaction mixture was stirred for 62 h. Volatiles were removed under reduced pressure and the residue was dissolved in CHCl₃, washed with H₂O, dried and concentrated under reduced pressure to give **6** (26 mg, 62%) as white solid: ¹H NMR (CD₃OD, 500 MHz) δ 4.49 (dd, 1H, ³J_{HH} = 7.8 and 5.1 Hz), 4.31 (dd, 1H, ³J_{HH} = 7.8 and 4.3), 3.24 (t, *J* = 7.0 Hz, 2H), 3.23-3.19 (m, 1H), 2.93 (dd, 1H, ²J_{HH} = -12.8, ³J_{HH} = 5.1 Hz), 2.71 (d, 1H, ²J_{HH} = -12.8 Hz), 1.86-1.79 (m, 2H), 1.78-1.70 (m, 1H), 1.63-1.54 (m, 1H), 1.52-1.41 (m, 4H).

2,2',2'',2'''-(2-(4-(5-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentylamino)benzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid tris(tetrabutylammonium) salt (1):

To a stirred mixture of H₂NPhCH₂DOTA tris(tetrabutylammonium) salt (36.2 mg, 0.029 mmol) and Na₂CO₃ (43 mg, 0.40 mmol) in dry DMF (0.5 ml) was added **6** (10.0 mg, 0.029 mmol) in dry DMF (1.0 ml). The reaction mixture was stirred for 22 h at 20°C. The residue after evaporation was dissolved in MeOH and the remaining solids were removed by centrifugation. The solution was evaporated to dryness under reduced pressure and purified by semi-preparative HPLC to give **1**: MALDI-MS calcd. for C₃₃H₅₂N₇O₉S [M+H]⁺: 722.35, found: 722.34.

4-[5-(6-Hydroxyhexylamino)pentyl]tetrahydro-1*H*-thieno[3,4-*d*]imidazol-2(3*H*)-one (7): To a stirred solution of **5** (30.8 mg, 0.10 mmol) in dry CH₃CN (2.5 ml) was added 6-amino-1-hexanol (23.5 mg, 0.20 mmol) and the mixture was stirred for 70 h at 50°C. The residue after evaporation was purified on semi-preparative HPLC to give **7** (25.5 mg 78%) as colourless syrup: ¹H NMR (CD₃OD, 500 MHz) δ 4.53 (dd, 1H, ³J_{HH} = 7.8 and 5.0 Hz), 4.33 (dd, 1H, ³J_{HH} = 7.8 and 4.5 Hz), 3.59 (t, 2H, ³J_{HH} = 6.4 Hz), 3.24 (ddd, 1H, ³J_{HH} = 9.1, 5.6 and 4.5 Hz), 3.05-2.99 (m, 4H), 2.96 (dd, 1H, ²J_{HH} = -12.8, ³J_{HH} = 5.0 Hz), 2.75 (d, 1H, ²J_{HH} = -12.8 Hz), 1.84-1.68 (m, 5H), 1.67-1.56 (m, 3H), 1.55-1.42 (m, 8H). ¹³C NMR (CD₃OD, 125 MHz) 166.2, 69.3, 63.5, 62.7, 61.7, 57.0, 48.9, 41.0, 33.2, 29.8, 29.6, 27.5, 27.4, 27.3, 27.1, 26.5. MALDI-MS calcd. for C₁₆H₃₂N₃O₂S [M+H]⁺: 330.22, found: 330.21.

2,2',2'',2'''-(2-(4-(6-(5-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentylamino)hexyl-amino)benzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid tris(tetrabutylammonium) salt (2): To a stirred mixture of **7** (27.4 mg, 83 μmol) and pyridine (6.6 μl, 83 μmol) in dry CH₃CN (1.5 ml) were added mesyl chloride (7.7 μl, 100 μmol). The reaction mixture was stirred at 20°C for 5 h and solvents were evaporated to dryness under high vacuum (0.1 mmHg) to give the intermediate **8**, which was used without further purification. The residue containing the intermediate **8** was dissolved into dry DMF (750 μl) followed by adding H₂NPhCH₂DOTA tris(tetrabutylammonium) salt (51.0 mg, 41 μmol), which was prepared from H₂NPhCH₂DOTA and tetrabutylammonium hydroxide. The reaction mixture was stirred for 20 h at 50°C, evaporated to dryness and purified by semi-preparative HPLC to give **2**: MALDI-MS calcd. for C₃₉H₆₅N₈O₉S [M+H]⁺: 821.46, found: 821.41.

Dimerization of compound 7

A rarely reported phenomenon was observed in the ^1H NMR spectrum for compound 7, since an unusual chemical shift at “ester” region (4.42 ppm) was observed for $-\text{CH}_2\text{-OH}$ fragment. The intensity of this signal (see Figure 1) was dependent on the concentration of the sample solution, since the same sample with dilution (ca. 10 times) afforded the same triplet at 3.58 ppm, which is a normal chemical shift for methylene group next to OH group. Moreover, calculating the sum of the integrals from these chemical shifts the value was ca. 2, as expected. The only rational explanation for this phenomenon is dimerization of compound 7 at higher concentrations due to hydrogen bonds formed between CH_2OH group and amide group(s) in biotin ring. The large 0.84 ppm downfield shift can be explained based on magnetic anisotropy effect caused by C=O bond. The effect is shielding above and below the C=O bond and deshielding in the plane of $\text{N}_2\text{C=O}$ structure³. In this case, it is expected that due to hydrogen bonds CH_2OH fragment lies at the plane with the urea unit.

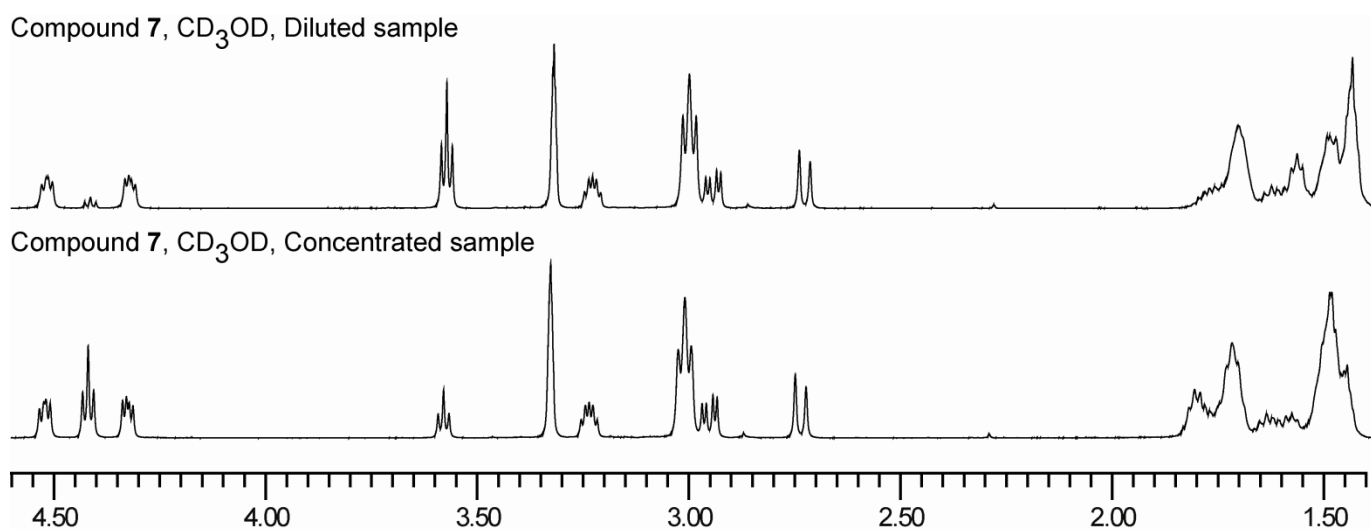


Figure 1. ^1H NMR spectra from compound 7 in CD_3OD . The lower spectrum is measured from concentrated sample (ca. 10 mg/ml) and the upper one from the same sample with 10 times dilution.

IC₅₀.

The relative affinity of the biotin-DOTA (**1**) or (**2**) were compared to free biotin using modified EIA method. Microtitration plate wells (Thermo Scientific, Finland) were coated with avidin (10 µg/ml in 100 mM carbonate buffer, pH 9.5, 100 µl / well) (Rockland, USA) over night at room temperature (RT). After coating, the wells were washed three times with 250 µl of PBST (Phosphate buffered saline + 0.1% Tween 20, pH 7.4) and saturated with 1% Bovine serum albumin (BSA) in PBST for 1 h at RT. After washing as above free biotin and biotin-DOTA molecules (0.25, 0.12, 0.06, 0.03 and 0.02 mM) were diluted with PBST containing 1% BSA and pipetted on avidin. After 30 minutes incubation wells were washed with PBST, biotin conjugated horseradish peroxidase (biot-HRP, 1:10 000 in PBST + 1%BSA) (Rockland, USA) was pipetted in every well and incubated for 15 minutes. After PBST washing the binding of the biot-HRP was visualized using hydrogen peroxide as substrate and tetramethylbenzidine (TMB, Sigma, USA) as chromogen. After 15 minutes incubation at room temperature colour reaction was stopped with 50 µl of 1 N H₂SO₄ and the wells were measured at 450 nm using Multiscan RC(Labsystems, Finland) (Figure 2.).

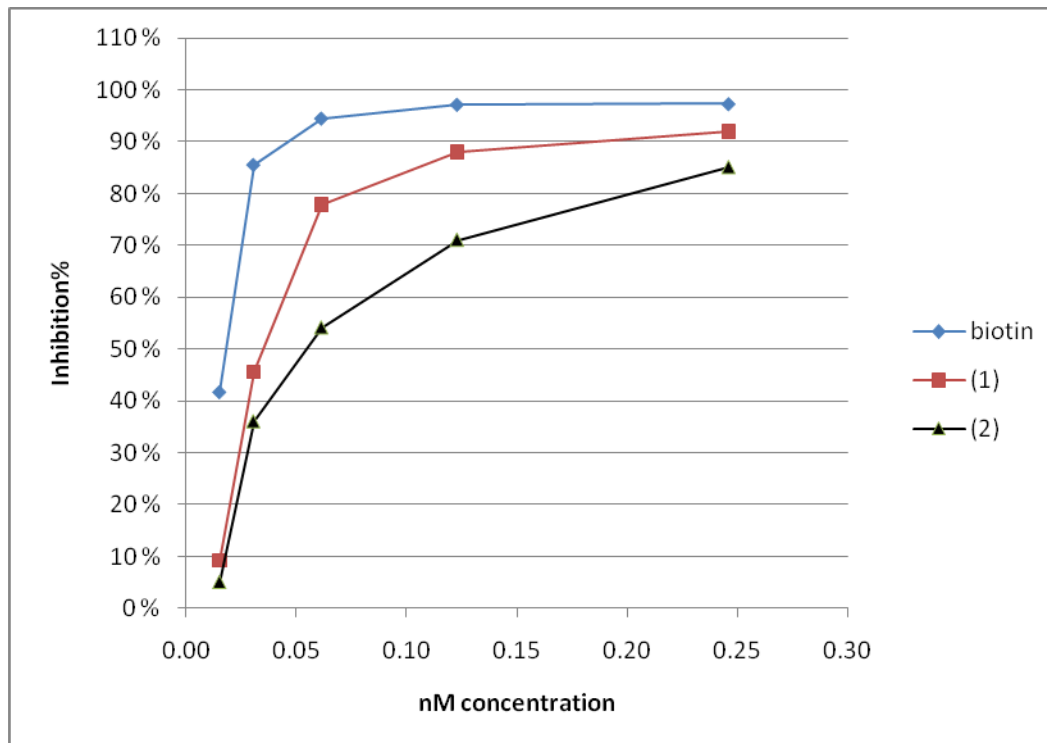


Figure 2. The blot of inhibition% against the concentration of free biotin and biotinylated DOTA molecules (1) and (2) and the estimation of concentrations that inhibit the binding of biotin-HRP by 50%.

Labeling with ^{111}In . 50 μl of free DOTA, biotin-DOTA (1) or (2) (1mg/ml in water) were mixed with 50 μl of 10 mM acetate buffer pH 4.5 and 1 MBq of ^{111}In in acetate buffer. Mixtures were incubated at 96°C in dry path for 30 minutes. The radiochemical purity was tested by using instant thin-layer chromatography (ITLC SG, Pall Corporation) in 13 x 1.5 cm strip and saline as the mobile phase.

Plasma stability. The stability of the ^{111}In labeled molecules 1 and 2 was tested in human plasma⁴. 20 μg of labeled molecules were mixed with 400 μl of human plasma. The first sample (100 μl) was taken immediately after mixing the rest 100 μl samples were taken after incubating at $+37^\circ\text{C}$ for 15, 40 and 60 minutes. After incubation the samples were moved to Microcon YM-10 filter (cut off 10 000 d) and centrifuged with Eppendorf centrifuge 14 000 rpm for 10 minutes. After centrifugation the filter was washed gently with 100 μl of PBS and centrifuged again for 10 minutes. 100 μl of filtered samples were moved to Eppendorf tube and mixed with 20 μl of avidin (10 mg/ml water). After 30 minutes incubation samples were moved to another Microcon YM-10 filters and centrifuged 14 000 rpm for 10 minutes. The activity of the all the filtrates were measured with 1277 Gammamster (Wallac, Turku, Finland). The amount (%) of radioactive biotinyl-DOTA bound to avidin at each time point was calculated from the CPM ratio of filtrate after avidin binding / filtrate after plasma incubation.

References

- 1 C. Corona, B.K. Bryant and J.B. Arterburn, *Org. Lett.*, 2006, **8**, 1883-1886.
- 2 I. Islam, Ng Ka-yun, K.T. Chong, T.J. McQuade, J.O. Hui, K.F. Wilkinson, B.D. Rush, M.J. Ruwart, R.T. Borchardt, J.F. Fischer, *J. Med. Chem.* 1994, **37**, 293.
- 3 H. Günther, *NMR Spectroscopy 2nd Ed.*, Wiley, p. 82.
- 4 M. Pakkala, C. Hekim, P. Soininen, J. Leinonen, H. Koistinen, J. Weisell, U.H. Stenman, J. Vepsäläinen and A. Narvanen, *J. Pept. Sci.*, 2007, **13**, 348-353.