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Synthetic Strategy to Prepare DOTA-Based Bifunctional Chelating Agent Ready to Bind Biomolecular Probes

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Abstract Due to the continued interest in new bifunctional chelating agents (BFCA), we focused on the development of a convenient synthesis of 1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid)-10-butyrate mono (*N*-hydroxysuccinimidyl ester). It consists in the macrocycle DO3A derivatized with an aliphatic linker containing an active ester that requires selective and mild conditions to react with the targeting biomolecule. It is important to underlay the versatility of the obtained BFCA, which can be conjugated both to a biomolecule (protein, Fab fragment) or to a synthetic molecule. In a subsequent step, the developed chelator was successfully conjugated to a peptide sequence.

Keywords DOTA · Bifunctional chelating agent · Biological probe · Peptide labeling

Introduction

DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) belongs to the family of cyclic chelating agents able to form metal ions complexes endowed with thermodynamic stability and kinetic inertness, for applications both as diagnostic and therapeutic agent. Indeed, this macrocycle is very useful for the complexation of β^--emitting radionuclides, like ^{90}Y, ^{177}Lu, widely employed in nuclear medicine protocols, and trivalent ions like Gd(III) for application in magnetic resonance imaging as contrast agent (Dischino et al. 1991; Mishra et al. 2006). Introduction of a reactive group into this macrocycle allows the preparation of the so-called bifunctional chelating agents which are commonly used to label biomolecules with radiometal or paramagnetic ions for biomedical applications (Lattuada et al. 2011). Many examples of DOTA-like bifunctional chelators have been published (Li et al. 2009; Jamous et al. 2012), most of them were obtained as activated esters of DOTA in order to react with an amino group, usually present in an antibody or peptide, to form an amide bond. Among the active esters, for instance, one of the most commonly prepared derived from *N*-hydroxysuccinimide (Mier et al. 2005).

DO3A-tris-*tert*-butyl ester is widely employed as a precursor to explore new protocols of synthetically useful DOTA-based BFCA, in fact the free secondary amino group can be derivatized in order to conjugate the macrocycle to a target-specific biomolecules (Yoo and Pagel 2006; Bernhard et al. 2012; Barge et al. 2009; Wangler et al. 2008; Mishra and Chatal 2001; Knor et al. 2007).

In addition, it is characterized by an accessible price, so that we decided to develop a convenient protocol to prepare a bifunctional chelating agent containing the ligand DO3A and a reactive group allowing the conjugation to a biological probe. An aliphatic spacer is present between the two moieties, in order to avoid that the bulky macrocycle could affect the interaction with the biological target.

Materials and Methods

DO3A-tris-*tert*-butyl ester was purchased from Macrocyclics (Dallas, TX); *N*-hydroxysuccinimide, 4-bromobutyric acid, potassium carbonate and *N,N′*-dicyclohexylcarbodiimide were purchased from Sigma-Aldrich (St Louis, MO). Fmoc protected amino acids, *N*-hydroxybenzotriazole

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(HOBT), benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium (PyBOP) were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland), piperidine and diisopropylethylamine (DIPEA) were purchased from Fluka (Milwaukee, WI), Rink Amide MBHA resin and all remaining solvents were purchased from Aldrich (St Louis, MO) or Fluka (Milwaukee, WI) and were used without further purification, unless otherwise stated.

Analytical RP-HPLC runs were carried out on a HP Agilent Series 1100 apparatus using a Phenomenex (Torrance, CA) C18 column, 4.6 × 250 mm with a flow rate of 1.0 mL min^{-1}. Preparative RP-HPLC was carried out on a Shimadzu 8A apparatus equipped with an UV Shimadzu detector using a Phenomenex (Torrance, CA) C18 column, 22 × 250 mm with a flow rate of 20 mL min^{-1}. The system solvent used was H_2O 0.1 % TFA (A) and CH_3CN 0.1 % TFA (B), with a linear gradient from 5 to 70 % B in 30 min. LC-ES-MS data were obtained using a Finnigan Surveyor MSQ single quadrupole electrospray ionisation mass spectrometer coupled with a Finnigan Surveyor HPLC (Finnigan/Thermo Electron Corporation San Jose, CA, USA).

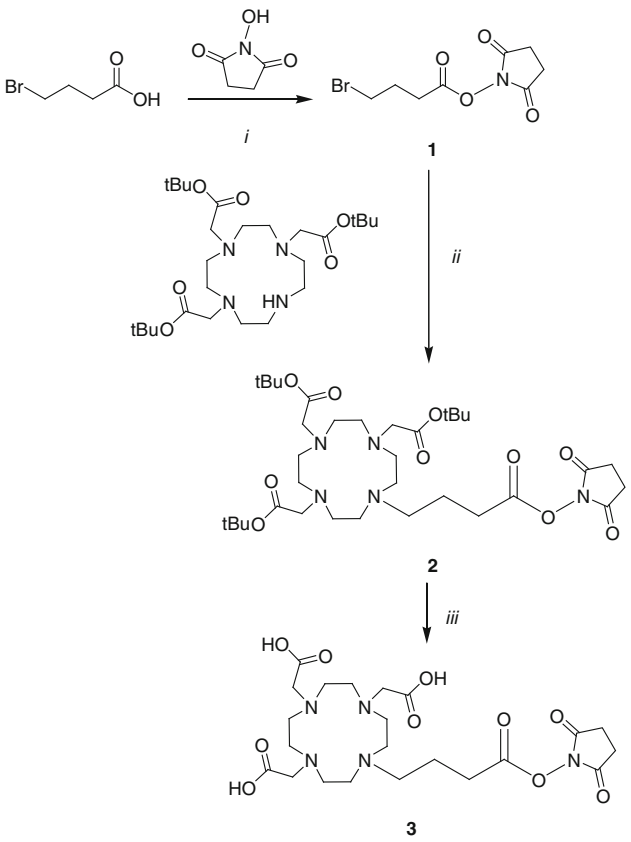
^{1}H NMR spectra were recorded on a Varian Unity Inova 400 MHz spectrometer equipped with z-axis pulsed-field gradients and a triple resonance probe. Samples were prepared in tubes with a diameter of 5 mm using 0.5 mL of deuterated solvent (CD_3OD). The experiments were acquired at 25 °C and the spectra were recorded with 256 scans and a relaxation delay of 1 s.

Synthesis

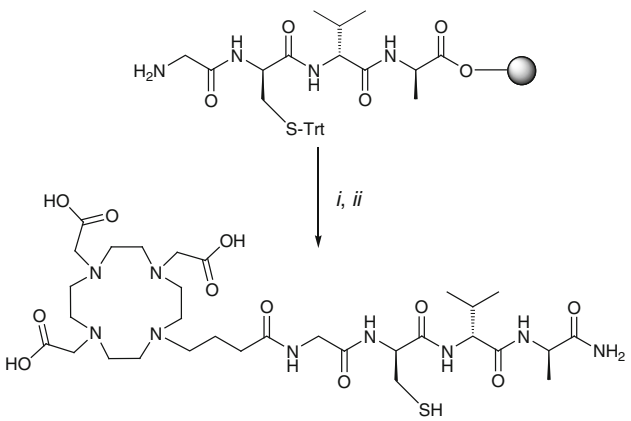
Synthesis of 4-bromobutyric acid *N*-hydroxysuccinimidyl ester (**1**). 4-bromobutyric acid (100 mg, 0.6 mmol) was dissolved in dry dichloromethane (anhydrous, ≥99.8 %, 2 mL) with 1 equiv. of *N*-hydroxysuccinimide (69 mg, 0.6 mmol). *N,N′*-Dicyclohexylcarbodiimide (1 equiv., 124 mg), previously dissolved in the minimum volume of the same solvent, was added dropwise. The solution was cooled in an ice-water bath and stirred vigorously for 4 h. The white solid *N,N′*-dicyclohexylurea was removed by filtration and the solvent was evaporated in vacuo to give the final product **1**, as assessed by analytical HPLC and mass spectrometry analysis. (Yield 80 %). ES-MS: calcd. $[M+H]^+$, 265.1; found, *m/z* 265.1.

^{1}H NMR: δ (ppm) 2.28 (BrCH2C***H***2CH2COO(NHS), p, $J = 6.37, 6.78$ Hz, 2H); 2.85 (BrCH2CH2C***H***2COO(NHS), t, 2H); 2.87 (C***H***2 NHS, broad signal, 4H); 3.59 (BrC***H***2CH 2CH2COO(NHS), t, $J = 6.49$ Hz, 2H).

Synthesis of 1,4,7,10-tetraazacyclododecane-1,4,7-tris (*tert*-butyl acetate)-10-butyrate mono (*N*-hydroxysuccinimidyl ester) (**2**). 1.2 equiv. of **1** (13 mg, 0.05 mmol) were placed in a round-bottom flask containing 20 mg of



Scheme 1 Synthesis of DO3A based-bifunctional chelator. Reagents and conditions: (*i*) DCC (1 equiv.), DCM, 0 °C, 4 h; (*ii*) K_2CO_3 (1 equiv.), DMF, 6 h, rt; (*iii*) TFA/TIS/EDT (96.5:1:2.5), 3 h



Scheme 2 Solid phase synthesis of DO3A-peptide conjugate. Reagents and conditions: (*i*) 1,4,7,10-Tetraazacyclododecane-1,4,7-tris (*tert*-butyl acetate)-10-butyrate mono (*N*-hydroxysuccinimidyl ester) **2** (1.5 equiv.), DIPEA (3 equiv.), DMF, 1 h; (*ii*) TFA/TIS/EDT/H_20 (93.5:2:2.5:2), 3 h

DO3A-tris-*tert*-butyl ester dissolved in 2 mL of DMF and in presence of 1 equiv. of K_2CO_3 (5 mg, 0.04 mmol). The mixture was stirred at room temperature for 6 h and the course of the alkylation reaction was followed by HPLC-ES-MS analysis. The mixture was concentrated under

vacuum and the crude product was purified by RP-HPLC to be fully characterized by mass spectrometry. (Yield 50 %). ES-MS: calcd. $[M+H]^+$, 698.9; found, *m/z* 698.4.

^{1}H NMR: δ (ppm) 1.51 (*tert*-butyl C***H***3), broad signal, 27H); 2.00 (NCH2C***H***2CH2COO(NHS), p, t, $J = 6.50$, 6.26 Hz, 2H); 2.77 (NCH2CH2C***H***2COO(NHS), t, $J = 6.97$ Hz, 2H); 2.69 (C***H***2 NHS, broad signal, 4H), 4.13 (NC***H***2CH2CH2COO(NHS), t, $J = 5.83$ Hz, 2H); 2.93, 3.06, 3.20, 3.30, 3.51, 3.63, 3.72, 3.98 (broad signals, NC***H***2 DOTA, 22H).

Synthesis of 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid 10-butyrate mono (*N*-hydroxysuccinimidyl ester) (**3**). 10 mg of compound **2** was treated with 200 µL of a solution of TFA/EDT/TIS (Trifluoroacetic acid/Triisopropylsilane/Ethanedithiol) (96.5:2.5:1) for 3 h. Then the solution was concentrated and the product **3** was isolated by precipitation into cold diethyl ether to be fully characterized by mass spectrometry. (Yield 85 %). ES-MS: calcd. $[M+H]^+$, 530.6; found, *m/z* 530.1.

^{1}H NMR: δ (ppm) 2.01 (NCH2C***H***2CH2COO(NHS), p, $J = 6.02$, 6.30 Hz, 2H); 2.73 (NCH2CH2C***H***2COO(NHS), t, J = 7.16 Hz, 2H); 2.69 (C***H***2 NHS, broad signal, 4H); 4.13 (NC***H***2CH2CH2COO(NHS), t, $J = 5.80$ Hz, 2H); 2.96, 3.05, 3.15, 3.31, 3.54, 3.57, 3.73, 3.98 ppm (broad signals, NC***H***2 DOTA, 22H).

Synthesis of DO3A-peptide conjugate. Peptide synthesis was carried out by solid phase method using the standard Fmoc procedure. Appropriate Fmoc-amino acid derivatives were employed (Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH) and a Rink Amide MBHA resin (0.28 mmol g^{-1} substitution) was used as solid support, as it releases peptide amidated at C-terminus upon acid treatment. All amino acid couplings were performed twice for 20 min by using an excess (4 equiv.) of each amino acid derivative. The amino acids were activated in situ by the standard HOBt/PyBOP/DIPEA protocol. Fmoc deprotection was performed with 20 % piperidine in DMF. The last coupling was performed using compound **2** (1.5 equiv.) and DIPEA (3 equiv.) in DMF. After 1 h the reaction was monitored by Kaiser test (Kaiser et al. 1970). The peptide cleavage from the solid support and the simultaneous removal of all protecting groups from the amino acid residues and from the BFCA was carried out by suspending the fully protected compound-resin in TFA/TIS/EDT/H_2O/(93.5:2:2.5:2) for 3 h followed by filtration. The solution was then concentrated and the crude product isolated by precipitation into cold diethyl ether. RP-HPLC and mass spectrometry analysis confirmed the presence of the desired compound which was isolated by HPLC preparative (yield 25 %). HPLC: $t_R = 11.1$; ES-MS: calcd. $[M+H]^+$, 761.9; found, *m/z* 762.4

Results and Discussion

A practical and easy synthetic protocol in order to prepare in good yield a versatile bifunctional chelating agent was developed. As shown in Scheme 1, the first synthetic step consisted in activating the carboxylic function of the 4-bromobutyric acid with NHS (*N*-hydroxysuccinimide) in

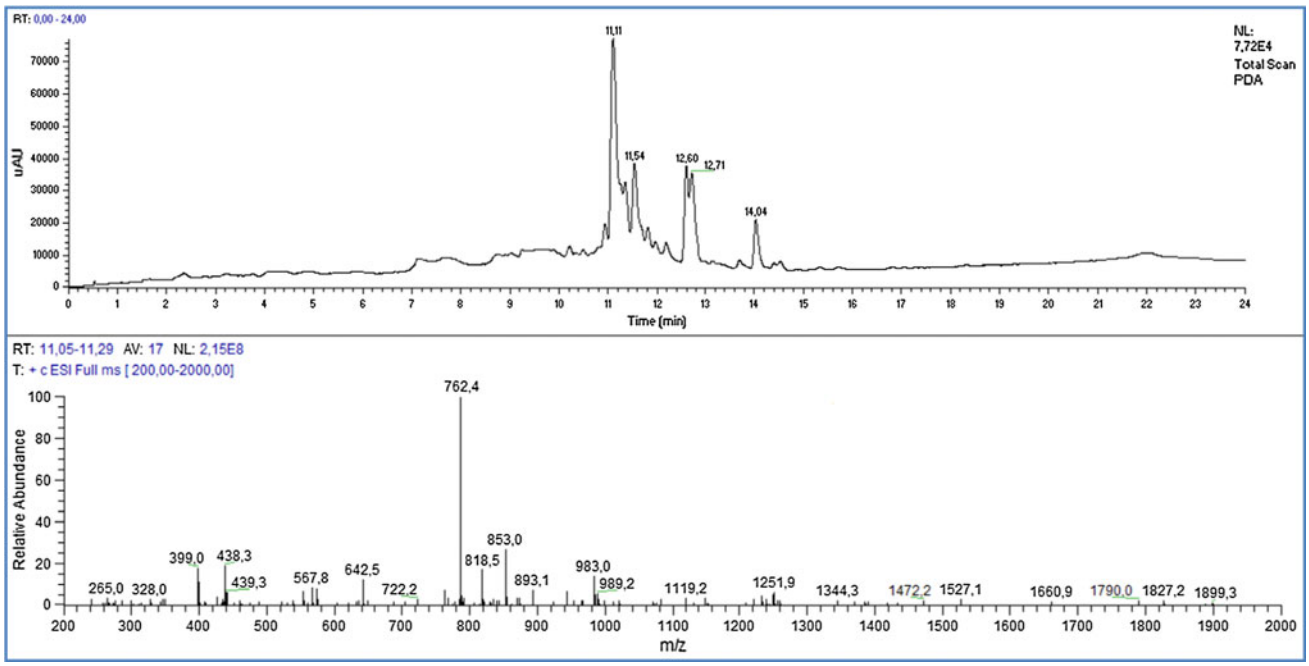


Fig. 1 LC–MS profile of the DO3A-peptide conjugate crude product

presence of a carbodiimide such as DCC (dicyclohexylcarbodiimide). The reaction was performed in DCM as solvent. Afterwards, **1** was reacted with DO3A-tris-*tert*-butyl ester, via N-alkylation reaction, in DMF and in presence of the inorganic base (K_2CO_3), the stirring was kept for 6 h. The amount of K_2CO_3 should not exceed 1 equivalent, in order to reduce to less than 5 % the hydrolysis of the NHS that can occur into the reaction mixture. Subsequently, the solvent was evaporated under reduced pressure and, after purification by RP-HPLC, an aliquot of **2** was treated with trifluoroacetic acid (TFA) in order to remove the *tert*-butyl groups of the chelator DO3A (Scheme 1). After precipitation in cold diethyl ether, the obtained BFCA (**3**) can readily react with an unprotected biomolecules, like protein or a Fab fragment.

Concerning with **2**, it can bind, in a selected site, biomolecules like peptides. Since most peptides are synthesized by solid phase method, it is convenient to perform the conjugation step during the peptide chain elongation. This approach, after completion of the whole synthetic process, allows the simultaneous removal of the *tert*-butyl esters of the BFCA, of all the protecting groups and the cleavage of the peptide from the resin (Scheme 2). Indeed, compound **2** was designed in order to be used in SPPS by its activated carboxylic function, while compound **3** was designed to directly bind biomolecules containing available amino group.

In order to prove the practical applicability of **2**, we performed the coupling of this bifunctional chelator to a generic peptide by using an excess of 1.5 equiv. (see Scheme 2).

The reaction was characterized by a high yield of functionalization, as showed by the HPLC profile of the conjugate crude product (Fig. 1).

Conclusion

In conclusion, starting from inexpensive and readily available materials, we have developed a fast and straightforward procedure to synthesize useful BFCA which can be conjugated to biological ligands, in order to label them with metal ions (radionuclides or paramagnetic metal) for targeted diagnostic imaging or therapy.

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