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Straightforward Synthesis of DFO* - An Octadentate Chelator for Zirconium-89

Xabier Guarrochena[+],[a, b, c, d] Julia Kronberger[+],[a, b, c, d] Manuel Tieber,[a] Philipp Ciesielski,[f] Thomas L. Mindt,*[a, c, d, e] and Irene V. J. Feiner*[a, c, d]

DFO* is an octadentate chelator able to form highly stable chelates with Zirconium-89 (^{89}Zr) for nuclear medicinal applications in Positron Emission Tomography (PET).[1,2] The synthesis of DFO* and its scale-up remains challenging by reported synthetic protocols. For this reason, we set out to develop a *de novo* synthesis of a hydroxamate-containing building block suitable for the coupling to the commercially available DFO (desferrioxamine B, mesylate salt) yielding, after deprotection, the desired chelator DFO* in a more efficient procedure. Highlights of the new synthesis of DFO* reported herein are less synthetic steps and the isolation of the desired product DFO* by using solid phase extraction (SPE), thus avoiding tedious HPLC purification. DFO* is obtained in excellent purity (92-98%) and an overall yield of approximately 29%. In addition, the isolated trifluoroacetic acid (TFA)-salt of DFO* displays an improved solubility in organic solvents (DMSO, DMF, methanol), which will facilitate its use for the preparation of structurally diverse derivatives suitable for bioconjugation chemistry and the development of ^{89}Zr-labeled radiotracers.

Introduction

Zirconium-89 (^{89}Zr) is a metallic radioisotope used in nuclear medicine for diagnostic imaging by positron emission tomography (PET).[3] The long physical half-life of ^{89}Zr ($t_{1/2}$ = 78.4 h) makes it an ideal radioisotope for the labelling of (bio)molecules which exhibit slow pharmacokinetics, such as antibodies (immuno-PET).[4] To do so, an appropriate chelator is required that provides ^{89}Zr-complexes of sufficient stability *in vivo*. For the chelation of ^{89}Zr, DFO (desferrioxamine B) is currently the state-of-the-art chelator used in nuclear medicine. However, preclinical animal studies have shown unspecific uptake of ^{89}Zr in bone material suggesting insufficient stability of the ^{89}Zr-DFO complex leading to potential bone marrow toxicity, unnecessary radiation dose to the patient and potentially false-positive imaging results.[5,6] A plausible explanation for the undesired demetallation *in vivo* is the unsaturated coordination sphere of ^{89}Zr with the hexadentate DFO, which needs two molecules of water to complete the favored octadentate coordination state of the radiometal.[6] One of the most promising alternatives to DFO is represented by the commercially available octadentate chelator DFO*.[2,7] ^{89}Zr-DFO* showed a remarkable *in vivo* stability in several studies,[8–10] and recently started the first clinical trial conjugated to the monoclonal antibody trastuzumab (^{89}Zr-DFO*-trastuzumab, NCT05955833[11]). The reported synthesis of DFO* makes use of the commercial DFO mesylate salt, which is elongated by the coupling to an additional unit containing a benzyl-protected (Bn) hydroxamate group.[1] This synthetic approach is *a priori* straight forward, however entails some challenges. We and others observed the formation of dehydroxylated side products as the result of the final removal of the Bn-protecting group by Pd-catalyzed hydrogenation.[12] In addition, the limited solubility of the obtained DFO*NH_2 in conventional high-performance liquid chromatography (HPLC) solvents makes its purification arduous.

With the goal to achieve a more efficient synthesis of DFO* devoid of HPLC purification of the final product, we envisioned an alternative synthesis of the key intermediate, the hydroxamate containing building block for coupling to DFO, utilizing a different protective group, namely the acid-labile *tert*-butyl (*t*Bu) group. Adaption of the synthesis of the hydroxamate building block (Boc-bb(*t*Bu)), the use of microwave-assisted

[a] X. Guarrochena,[+] J. Kronberger,[+] M. Tieber, T. L. Mindt, I. V. J. Feiner
Bioinorganic Radiochemistry, Institute of Inorganic Chemistry, Faculty of Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria
E-mail: thomas.mindt@univie.ac.at

[b] X. Guarrochena,[+] J. Kronberger[+]
Vienna Doctoral School in Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

[c] X. Guarrochena,[+] J. Kronberger,[+] T. L. Mindt, I. V. J. Feiner
Ludwig Boltzmann Institute Applied Diagnostics, AKH Wien c/o Sekretariat Nuklearmedizin, Waehringer Guertel 18–20, 1090 Vienna, Austria
E-mail: Irene.feiner@lbiad.lbg.ac.at

[d] X. Guarrochena,[+] J. Kronberger,[+] T. L. Mindt, I. V. J. Feiner
Department of Biomedical Imaging and Image Guided Therapy, Division of Nuclear Medicine, Medical University of Vienna, Waehringer Guertel 18–20, 1090 Vienna, Austria

[e] T. L. Mindt
Joint Applied Medicinal Radiochemistry Facility, University of Vienna and Medical University of Vienna, Vienna, Austria

[f] P. Ciesielski
ABX Advanced Biochemical Compounds, Heinrich-Glaeser-Strasse 10–14, 01454 Radeberg, Germany

[+] These authors contributed equally to this work

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reactions, and the purification of the final product DFO* by solid-phase extraction (SPE) led to significant improvements.

Experimental part

Materials and methods

Reagents were purchased from Sigma-Aldrich (Buchs, Switzerland), Merck (Darmstadt, Germany), Acros Organics (Geel, Belgium), Fluorochem (Hadfield, United Kingdom), Novabiochem (Darmstadt, Germany), TCI (Zwijndrecht, Belgium) and Fluka (Buchs, Switzerland) and were used without further purification. DFO* was purchased from ABX (Radeberg, Germany). The TLC-plates used for the monitoring of the reactions were purchased from Macherey-Nagel (DC-Fertigfolien ALUGRAM® Xtra SIL G/UV$_{254}$) Solid-Phase Extraction cartridges from Waters™, Sep-Pak C18 Plus Long Cartridge, 820 mg Sorbent per Cartridge, 55–105 μm were used for purification of the chelator. RP-HPLC-MS analyses were conducted at 220 nm on an Agilent 1260 Infinity II system equipped with a Flexible pump, a 1260 VWD UV-Vis detector and the LC–MSD system using the Acquity UPLC® BEH C18 column (300 Å, 1.7 μm, 2.1 mm x 100 mm). Mobile phase A is H_2O (0.1% formic acid (FA)) and mobile phase B is acetonitrile (ACN) (0.1% FA). Flowrate was 0.6 mL/min. For preparative RP-HPLC a Waters XBridge C18 column was used on an Agilent 1200 Series system. The methods used are described in the respective protocol. HRESI-MS spectra (m/z 50–1900) were obtained on a maXis UHR ESI–Qq-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in the positive-ion mode by direct infusion. The sum formulas of the detected ions were determined using Bruker Compass DataAnalysis 4.1 based on the mass accuracy ($\Delta m/z \leq 5$ ppm) and isotopic pattern matching (SmartFormula algorithm). CHNS-O elemental analyses were performed at the Microanalytical Laboratory of the University of Vienna using an Eurovector EA 3000 Elemental Analyser (built 2009). In the case of Oxygen, the device was equipped with a high temperature pyrolysis furnace (HT, Hekatech, Germany, 2009). Ionic components, such as TFA, were measured using capillary electrophoresis. Analysis was done using a 7100 CE (Agilent) combined with a conductivity detector TraceDec (Innovative Sensor Solutions). TFA was quantified by calibrating a mixed anion standard which, among others, contained acetate and TFA (SI, FT-S2). Nuclear magnetic resonance (NMR) measurements were recorded on a Bruker FT-NMR Avance III 500 MHz spectrometer at 500.10 (1H) and 125.75 (13 C) MHz at 298 K in deuterated methanol (MeOD) or deuterated dimethyl sulfoxide (DMSO-d6). The respective residual solvent peak was used as internal reference for the chemical shifts (ppm). Spin multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad signal. The values of the coupling constants (*J*) are given in Hertz (Hz). For centrifugation a ROTINA 380/380R from Hettich was used. Microwave reactions were performed with a Biotage Initiator+ system. The device has a range of power up to 400 W and needed about 1 min to heat to the desired temperature (method report available in SI, F-S21).

Syntheses

Amino aldehyde 2. To 32.5 mL of a 1 M solution of DMSO (32.5 mmol, 2.2 eq.) in anhydrous dichloromethane, 8.8 mL of 2 M solution of oxalyl chloride (17.6 mmol, 1.2 eq.) in anhydrous dichloro-methane was added dropwise over 5 min at −45 °C (dry ice/acetonitrile). The reaction was stirred for 10 min and 11.5 mL of a 1.3 M solution of the amino alcohol **1**, (15 mmol, 1.0 eq., 3.05 g) in anhydrous dichloromethane was slowly added at −45 °C. The reaction was stirred for another 30 min at −45 °C, followed by the addition of 7.7 mL N,N-diisopropylethylamine (DIPEA, 44.1 mmol, 3 eq.), after which the temperature was allowed to increase to −20 °C and the mixture was stirred for 1.5 h. The reaction was monitored by thin layer chromatography (TLC, hexane/ethyl acetate 6:4), stained with $KMnO_4$ solution. Upon completion of the reaction indicated by TLC, the solvent was removed in vacuum, the crude product was dissolved in dichloromethane (100 mL) and washed three times each with 60 mL 1 M $KHSO_4$ and 80 mL brine. The organic phase was dried over Na_2SO_4, filtered, and the solvent was removed under reduced pressure to yield amino aldehyde **2** as a yellow oil ($R_f = 0.56$). Crude amino aldehyde **2** was used for the next step without further purification.

Hydroxylamine 3. A 0.1 M solution of the amino aldehyde **2** (15 mmol, 1.0 eq.) was prepared in methanol. *O*-*t*Bu-hydroxylamine-hydrochloride (45.0 mmol, 3.0 eq., 5.65 g) was added and the mixture stirred at room temperature overnight. The completion of the reaction was verified by TLC (hexane/ethyl acetate 6:4) and the solvent was evaporated under reduced pressure. The crude mixture was dissolved in methanol (15 mL) and the reduction was conducted by adding $NaBH_3CN$ (60 mmol, 4.2 eq., 3.77 g) and acetic acid (75 mmol, 5.2 eq., 4.3 mL) at room temperature. The reaction was stirred for 3.5 h at room temperature and the conversion to the desired hydroxylamine **3** was confirmed by TLC (hexane/ethyl acetate 6:4). After evaporation of methanol, the crude product was dissolved in dichloromethane (60 mL) and washed three times each with sat. $NaHCO_3$ (40 mL) and brine (50 mL). The organic phase was dried over Na_2SO_4, filtered, and the solvent was removed under reduced pressure to yield hydroxylamine **3** as a colorless oil. The crude product was analyzed by NMR and MS (see SI, F-S1 and F-S12) and matched reported data.[13] Hydroxylamine **3** was used in the next step without further purification.

Boc-bb(*t*Bu) 4. Hydroxylamine **3** (15 mmol, 1.0 eq., 3.0 g) was dissolved in 12 mL dimethyl-formamide (DMF). Succinic anhydride (75 mmol, 5.0 eq., 7.5 g) and 4-(dimethylamino)pyridine (DMAP, 7.5 mmol, 0.5 eq., 0.9 g) were added at room temperature. The reaction was stirred for 90 min at 50 °C and the conversion to the desired Boc-bb(*t*Bu) **4** was monitored by LC–MS. Therefore, 10 μL of the reaction solution were diluted with 190 μL 5% ACN/H_2O with 0.1% FA. The sample was filtered, 5 μL of sample were injected into the LC–MS system. The gradient for the measurement was 5–95% ACN/H_2O over 6 min with 0.1% FA. The DMF was removed under high vacuum at 40 °C and the crude product was dissolved in methanol to yield 200 mg/mL of crude product for its subsequent purification *via* preparative-HPLC (flow rate: 17 mL/min, mobile phase A = H_2O + 0.1% FA, mobile phase B = ACN + 0.1% FA, gradient: 0–3.5 min 45% B, 14.6 min-57% B, 14.8 min-95% B, 18.8 min-95% B, 19.0 min-45% B). The fractions containing the product were pooled and lyophilized to yield Boc-bb(*t*Bu) **4** as yellow oil (1.46 g, 26% overall yield for 3 steps including a single purification step).

ESI-HR-MS m/z found (calculated) for $C_{18}H_{34}N_2O_6$: $[M+Na]^+$ 397.2322 (397.2309).

^{1}H NMR (500.10 MHz, MeOD) δ = 3.27 (m, 2H), 2.98 (t, *J* = 6.9 Hz, 2H), 2.72 (s, 2H), 2.56–2.49 (m, 2H), 1.62 (m, 2H), 1.45 (d, *J* = 6.5 Hz, 2H), 1.39 (s, 9H), 1.31 (s, 9H), 1.23 (t, *J* = 7.8 Hz, 2H) ppm.

^{13}C-NMR (151 MHz, DMSO-d6, DEPTq135) δ = 176.36, 158.54, 94.22, 79.80, 53.70, 52.18, 49.85, 41.16, 30.56, 29.61, 28.79, 28.07, 24.91 ppm.

DFO*(*t*Bu)-NHBoc 6. In a 5 mL microwave reactor equipped with a stirring bar, Boc-bb(*t*Bu) **4** (0.7 mmol, 2.0 eq., 262 mg) was dissolved in 4 mL DMF. O-(7-Azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium-hexafluorphosphat (HATU, 0.63 mmol, 1.8 eq., 239 mg) and DIPEA (1.05 mmol, 3.0 eq., 178 μL) were added and stirred for

10 min at room temperature. DFO mesylate **5** (0.35 mmol, 1.0 eq., 230 mg) was added, followed by DIPEA (0.525 mmol, 1.5 eq., 89 μL). The reaction was conducted in the microwave reactor for 2 h at 45 °C with magnetic stirring. Completion of the reaction confirmed by LC–MS (10-95 % B over 5.5 min). The solvent was removed under high vacuum, the crude product was suspended in 7 mL of ice-cold acetone and transferred to a 15 mL falcon tube. The mixture was vortexed, sonicated, and centrifuged (5000 rpm for 10 min at 4 °C) and the supernatant was discarded. This process was repeated one more time. The precipitate was dissolved in 5 mL methanol and transferred to a glass vial, where the solvent was removed under reduced pressure. DFO*(*t*Bu)-NHBoc **6** was obtained as a white solid (311 mg).

[DFO*NH$_2$]TFA salt 7. In a 5 mL microwave reactor equipped with a stirring bar, DFO*(*t*Bu)-NHBoc **6** (0.35 mmol, 311 mg) was mixed with 3 mL TFA and 100 μL H_2O. The reaction was conducted in the microwave for 1 h at 40 °C. Full deprotection was confirmed by LC–MS (10-95 % B over 5.5 min). Solvents were evaporated under a stream of argon and the chelator DFO*NH$_2$ precipitated as its TFA salt **7** in 8 mL ice-cold tert-Butyl methyl ether (MTBE). The suspension was centrifuged (5000 rpm for 5 min at 4 °C) and the supernatant discarded. The crude product was lyophilized and obtained as a white solid (178 mg).

For the solid-phase extraction (SPE) purification, 30 mg of the crude [DFO*NH$_2$]TFA salt **7** was dissolved in 200 μL of DMSO. The solution was diluted with 1.8 mL of 10 % ACN/H_2O. The SPE cartridge (Sep-Pak® Plus Long C18, Waters) was pre-conditioned with 10 mL ethanol followed by 20 mL H_2O and [DFO*NH$_2$]TFA salt **7** was loaded. The cartridge was then washed with 10 mL of water followed by 5 mL 5 % ACN/H_2O. [DFO*NH$_2$]TFA **7** was eluted with 5 mL 10 % ACN/H_2O and 5 mL 15 % ACN/H_2O, which were collected in 1 mL fractions. Each fraction was then subjected to LC–MS analysis (10-95 % B over 5.5 min, LC–MS measurements of each fraction can be found in SI, F-S20 and T-S1). The samples exceeding purities of >90 % were collected and lyophilized. Typically, recoveries of 10–13 mg (33-43 %) of [DFO*NH$_2$]TFA salt **7** with purities of 92–98 % were achieved. Overall yields of the chelator, starting with the coupling reaction of DFO **5** with Boc-bb(*t*Bu) **4**, were 22–29 %. Full characterization, including LC–MS, HR-MS, NMR and elemental analysis was performed confirming the identity and purity of [DFO*NH$_2$]TFA salt **7** (SI, F-S4-S11).

ESI-HR-MS m/z found (calculated) for $C_{34}H_{64}N_8O_{11}$: $[M+H]^+$ 761.4761 (761.4767).

^{1}H-NMR (500.10 MHz, DMSO-d6) δ = 7.79 (q, *J* = 5.1 Hz, 3H), 3.52–3.42 (m, 8H), 2.99 (q, *J* = 6.6 Hz, 6H), 2.75 (t, *J* = 7.5 Hz, 2H), 2.58 (dt, *J* = 10.3, 5.0 Hz, 6H), 2.36–2.20 (m, 6H), 1.96 (s, 3H), 1.50 (q, *J* = 7.9 Hz, 10H), 1.37 (m, 6H), 1.31–1.12 (m, 8H) ppm.

^{13}C-NMR (151 MHz, DMSO, DEPTq135) δ = 171.93, 171.25, 170.09, 47.04, 46.80, 46.74, 40.03, 39.89, 39.75, 38.87, 38.39, 29.85, 29.75, 28.78, 27.52, 27.42, 26.92, 25.99, 25.71, 23.46, 22.86, 20.32 ppm.

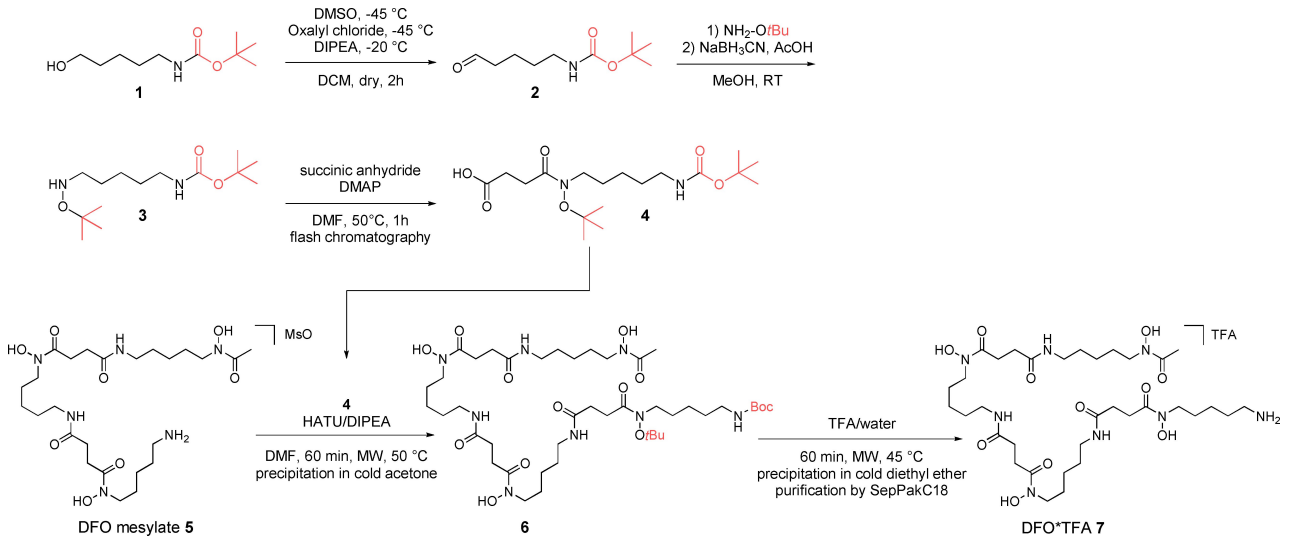
Elemental analysis: Found % (calc. %) for DFO*·TFA salt $C_{34}H_{64}N_8O_{11}\cdot 1\ C_2HF_3O_2$: C 48.88 (48.96), H 7.37 (7.41), F 7.13 (7.06), N 12.64 (12.62), O 23.98 (23.96).

Determination of solubility. Solubility was determined by weighing 5 mg of freshly purified [DFO*NH$_2$]TFA **7** or the commercial DFO* followed by consecutive addition of the solvent of interest until a visually clear solution was obtained.

Results and discussion

In analogy to previously described methods for the preparation of DFO* analogues,[1,13–15] our new synthesis of the DFO* presented herein is based on the combination of commercial DFO mesylate salt **5** with a protected hydroxamate-containing building block (Boc-bb(*t*Bu) **4**). The choice of the protective groups was based on the premise of their simultaneous removal under acidic conditions, therefore avoiding the need of hydrogenation as in the case when reported Bn/Cbz protective groups are employed. The use of hydrogen and palladium on carbon in the final deprotection step can lead to the formation of dehydroxylated side products, as observed by us and others.[12]

As depicted in Scheme 1, the commercial Boc-protected 5-amino-1-pentanol (amino alcohol **1**) was oxidized to the corresponding amino aldehyde **2** *via* Swern-oxidation followed by a reductive amination using *t*Bu-protected hydroxylamine to



Scheme 1. Synthesis of the hydroxamate containing building block Boc-bb(tBu) **4** and the chelator [DFO*NH$_2$]TFA salt **7**.

yield intermediate hydroxylamine **3**. The synthesis of Boc-bb(*t*Bu) **4** was completed by the reaction of the hydroxylamine **3** with succinic anhydride in the presence of DMAP. Noteworthy, all intermediates could be carried forward in the synthesis as crude products and only the final Boc-bb(*t*Bu) **4** was purified by preparative HPLC. In comparison to previously reported syntheses of related compounds, the reaction sequence leading to the key intermediate Boc-bb(*t*Bu) **4** could be shortened from 6 steps[16] to 3 steps yielding the desired building block **4** in overall yield of 26%.

The synthesis of DFO* was accomplished by coupling of commercial DFO mesylate salt (**5**, HPLC chromatogram see Figure 1A) with Boc-bb(*t*Bu) **4** followed by deprotection. Unlike previously reported methods, where the coupling reaction took up to 48 h,[1,14] the coupling reaction was performed by microwave heating (45 °C) for only 2 h in DMF using HATU as activation agent. The use of conventional heating or employment of other activation reagents (e.g., Oxyma®, N,N′-Diisopropylcarbodiimid (DIC)) in varying stoichiometries, higher temperature, or elongated reaction times did not result in further improvements. Under the optimized conditions quantitative conversion of DFO **5** to the partially protected DFO* (DFO*(*t*Bu)-NHBoc **6**) could be achieved as determined by LC–MS analysis (Figure 1B). Conveniently, precipitation of the product from ice-cold acetone provided intermediate **6** in sufficient purity (> 85%) for the next step (Figure 1C).

The overall deprotection of intermediate **6** by the simultaneous cleavage of the *t*Bu and Boc protective groups was conducted in a microwave reactor at 40 °C within 60–90 min. As in the previous step, the use of microwave provided better results than conventional heating in a heating block or oil bath. Applying higher temperatures or longer reaction times resulted in the formation of more side products. The use of TFA/H_2O (97.5/2.5) in the absence of additional scavenger (e.g., triisopropylsilane (TIPS)) was preferred as it led to a cleaner crude product [DFO*NH_2]TFA **7** (purity > 73%, Figure 1D) upon precipitation from ice-cold diethyl ether.

However, for further use of [DFO*NH_2]TFA **7**, e.g., for the synthesis of bifunctional chelating agents (BFCAs) useful in radiotracer development, higher purities are needed. It is known to the community that the poor solubility of DFO* (and as a matter of fact also other DFO and DFO* derivatives) can impose challenges from a synthesis as well as from a purification point of view. To avoid HPLC purification altogether, we set out to explore the use of C18-SPE cartridges as a convenient and efficient alternative.[17] Indeed, the use of SPE purification yielded [DFO*NH_2]TFA **7** in purities of 92–98% (Figure 1E, full LC–MS characterization can be found in SI F-S15-S19). The method also represents an improvement in terms of yield compared to previously reported syntheses of DFO* (29% *versus* 12%).[14] Also, the fractions with product of purities < 90% can be pooled and purified *via* SPE in an iterative manner. Full characterization, including HPLC, LC–MS, HR-MS, NMR and elemental analysis was performed to confirm the identity and purity of [DFO*NH_2]TFA **7**.

We were also pleased to observe a much-improved solubility for [DFO*NH_2]TFA **7**. For example, [DFO*NH_2]TFA **7** exhibited a solubility in anhydrous DMSO of 250 mg/mL at room temperature whereas its commercial counterpart DFO*NH_2 remained insoluble at concentrations of < 5 mg/mL.

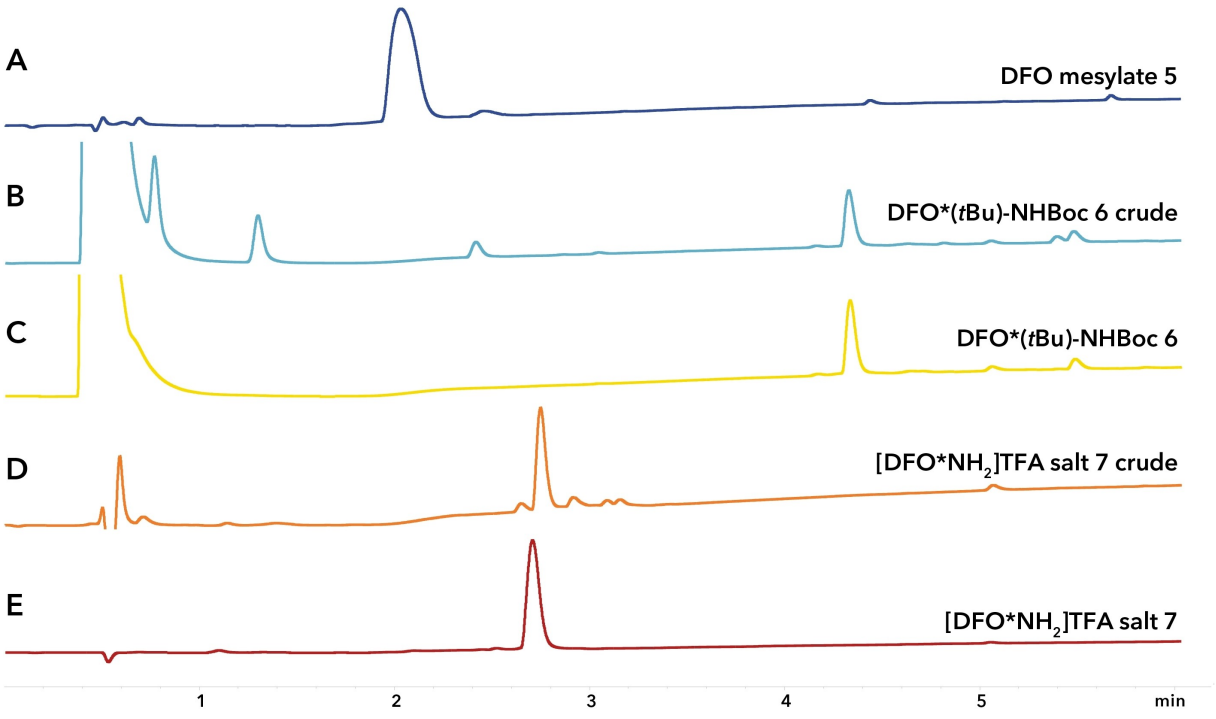


Figure 1. HPLC-Chromatograms (10-95% B over 5.5 min) of each step of the DFO* synthesis. A) Commercial DFO mesylate **5** (t_R 2.4 min), B) DFO*(tBu)-NHBoc **6** crude, after the coupling of DFO with the hydroxamate containing building block **4** (t_R 4.4 min), C) DFO*(tBu)-NHBoc **6** after Acetone precipitation (t_R 4.4 min), D) [DFO*NH_2]TFA **7** crude after deprotection (t_R 2.8 min), E) [DFO*NH_2]TFA salt **7** after Sep-Pak purification (t_R 2.8 min)

[DFO*NH_2]TFA **7** was also found soluble in DMF (9,0 mg/mL), methanol (7,0 mg/mL) and ethanol (1,5 mg/mL), however, to a lesser extent. As expected, its solubility in dichloromethane remained low (< 1 mg/mL). The difference in solubility originates from the TFA counter ion, the presence of which was later confirmed by ^{19}F-NMR and elemental analysis verified a one-to-one ratio of TFA and DFO*.

Conclusions

In this work, we describe the simplified synthesis and purification of the chelator DFO* in form of its TFA salt, [DFO*NH_2]TFA **7**. The strategic choice of using acid-labile protecting groups has contributed to the preparation of the main building block, Boc-bb(*t*Bu) **4**, in less synthetic steps and without the need of purifications of intermediates. Application of microwave-assisted heating has considerably improved both the coupling of DFO **5** with Boc-bb(*t*Bu) **4** and the overall deprotection of the resulting product DFO*(*t*Bu)-NHBoc **6**. The use of SPE cartridges has proven a practical alternative to HPLC purification affording the desired chelator [DFO*NH_2]TFA **7** in remarkable purities in good recovery rates. The obtained TFA salt is shown to have enhanced solubility in various solvents and is potentially an advantage for the bifunctionalization of DFO* and its conjugation to biomolecules for radiotracer development.

Author Contributions

X.G., J.K., and I.V.J.F. contributed equally to the design of the study and execution of the synthesis. M.T. assisted in the synthesis. P.C. contributed with scientific discussions. X.G., J.K. and I.V.J.F. wrote the manuscript. T.L.M., P.C. and I.V.J.F. reviewed and edited the manuscript. All authors have agreed on the final version of the manuscript.

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Conflict of Interests

Thomas L. Mindt is co-inventor of a patent applications describing DFO* and its derivatives. The patent is owned by the University of Zurich and the University of Basel, Switzerland (WO 2015/140212 Al). The patent owners have granted an exclusive license to the company ABX Advanced Biochemicals Compounds (Radeberg, Germany). The inventors have no connections to this company. The author Philipp Ciesielski works for ABX advanced biochemical compounds GmbH.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Synthesis · Chelator · DFO* · Positron Emission Tomography (PET) · Zirconium-89 (^{89}Zr)

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