Electronic Supplementary Material (ESI) for Reaction Chemistry & Engineering.

Supporting Information

A general [^{18}F]AlF radiochemistry procedure on two automated synthesis platforms

L. Allott‡, C. Da Pieve‡, D. R. Turton and G. Smith*

Division of Radiotherapy and Imaging, The Institute of Cancer Research, 123 Old Brompton Road, London, SW7 3RP, UK

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Materials and Methods

Section 1 - General

All the reagents and solvents were purchased from commercial sources and used without further purification unless otherwise stated. NOTA-octreotide and NOTA-RGDfK were purchased from ABX GmbH (Radeberg, Germany). HPLC grade acetonitrile and trifluoroacetic acid (TFA), dichloromethane (DCM), ethyl acetate (EtOAc), ethanol (EtOH), hexane, were purchased from Fisher Scientific (Loughborough, UK). Triethylamine, p-toluenesulfonyl chloride, and *N,N*-diisopropylethylamine (DIPEA) were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$, 99.9995%) was purchased from Alfa Aesar (Heysham, UK). Sodium acetate (AnalR Normapur) and silica gel (40-63 μm, 60 Å) were purchased from VWR International. di-*tert*-Butyl 2,2'-(1,4,7-triazacyclononane-1,4-diyl)diacetate (NO2AtBu) was purchased from CheMatech (Dijon, France). Oasis HLB (1 mL, 30 mg) SPE cartridges were purchased from Waters (Elstree, UK) and were conditioned using EtOH (3 mL) and water (6 mL). [^{18}F]-Fluoride was produced by a GE PETrace cyclotron by 16 MeV irradiation of enriched [^{18}O]H_2O target, supplied by Alliance Medical Radiopharmacy Ltd (Warwick, UK) and delivered to a dispensing hotcell in *ca* 2 mL of water. [^{18}F]Fluoride was used without further purification. The automated radiosynthesis platforms used in the study were: GE Tracerlab FX FN™ (GE Healthcare, UK) and Trasis AllInOne™ (Trasis, Belgium). Analytical RP-HPLC were carried out on an Agilent Infinity 1260 quaternary pump system equipped with a 1260 Diode array (Agilent Technologies). Elution profiles were analysed using Laura software (Lablogic, UK). Peptide radioconjugates (NOTA-octreotide and NOTA-RGDfK) were analysed on a Zorbax-300SB-C18 column, 7.8 × 250 mm, 5 μm (Agilent Technologies) using gradient **1**: 0 – 2 min, 95% - 5% B isocratic, 2.1 – 20 min, 35% - 65% B; 20.1 – 22 min, 95% - 5% B with 0.1% TFA in water as eluent A and 0.1% TFA in acetonitrile as eluent B at a flow rate of 1 mL/min. NODA-Tz (Compound **4**) was analysed on a Zorbax Eclipse XDS C18 column, 4.6 × 150 mm, 5 μm (Agilent Technologies) using gradient **2**: 0 – 20 min, 3% - 90% B, 20.1 – 21 min, 90 % - 3% B with 0.1% TFA in water as eluent A and 0.1% TFA in acetonitrile as eluent B at a flow rate of 1 mL/min. NODA-Tz (**4**) was purified by semi-preparative RP-HPLC using a Gemini C18 column, 10 × 250 mm, 10 μm (Phenomenex) using gradient **3**: 0 – 30 min, 3% - 90% B, 30.1 – 31 min, 90% - 37% with 0.1% TFA in water as eluent A and 0.1% TFA in

acetonitrile as eluent B at a flow rate of 3 mL/min. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker 500 MHz spectrometer operating at room temperature. Chemical shifts (δ) are reported in parts per million (ppm) and residual solvent peaks have been used as an internal reference. Peak multiplicities have been abbreviated as follows: s (singlet), d (doublet), m (multiplet). Liquid Chromatography Electrospray ionisation high resolution mass spectrometry (LC-ESI-HRMS) was performed using an Agilent 1200 series LC pump with a 6210 time-of-flight (TOF) mass analyser.

Section 2 - Synthesis of NOTA-tetrazine (4)

4-((6-Hydroxyhexyl)oxy)benzonitrile (1). A solution of 4-cyanophenol (655 mg, 5.5 mmol), 6-bromohexanol (1 g, 5.5 mmol) and K_2CO_3 (760 mg, 5.5 mmol) in acetone (40 mL) was refluxed for 24 h and a precipitate formed. The reaction was filtered while hot, the filtrate was concentrated *in vacuo* and the residue was purified by silica column chromatography (30% EtOAc/hexane) to yield the product as a clear oil (750 mg, 62%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57 – 7.59 (d, 2H, *J* = 9.1 Hz), 6.93 – 6.95 (d, 2H, *J* = 9.1 Hz), 4.01 (t, 2H, *J* = 6.2 Hz), 3.68 (t, 2H, *J* = 6.2 Hz), 1.80 – 1.86 (m, 2H), 1.59 – 1.65 (m, 2H), 1.42 – 1.54 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 134.0, 119.3, 116.3, 115.1, 103.6, 68.2, 62.8, 32.6, 29.0, 25.8, 25.5. HRMS-ESI: $[M + H]^+$ (m/z) calcd. for $C_{13}H_{18}NO_2$: 220.1338, found 220.1341

6-(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenoxy)hexan-1-ol (2). To a pressure tube, a mixture of **1** (219 mg, 1 mmol), $Ni(OTf)_2$ (178 mg, 0.5 mmol), acetonitrile (525 µL, 10 mmol) and hydrazine hydrate (3.1 mL, 50 mmol) was added. The tube was sealed and incubated at 60 °C for 48 h. The reaction mixture was allowed to cool and poured over an aqueous sodium nitrite solution (690 mg in 5 mL). The mixture was adjusted to pH 3 with 1M HCl. The reaction mixture was extracted with DCM (3 × 100 mL) and the organic layer was dried over $MgSO_4$. The bulk solvent was removed *in vacuo* and the residue was purified by silica column chromatography (50% EtOAc/hexane) to yield a pink powder (176 mg, 30%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.41 (d, 2H, *J* = 9.0 Hz), 7.19 (d, 2H, *J* = 9.0 Hz), 4.36 (t, 1H, *J* = 5.1 Hz), 4.10 (t, 2H, *J* = 6.5 Hz), 3.40 (q, 2H, *J* = 6.5 Hz), 2.96 (s, 3H), 1.73 – 1.79 (m, 2H), 1.41 – 1.48 (m, 4H), 1.33 – 1.40 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.95, 163.42, 162.65, 129.65,

124.30, 115.80, 68.31, 61.09, 32.94, 29.09, 25.84, 25.74, 21.18. HRMS-ESI: $[M + H]^+$ (m/z) calcd. for $C_{15}H_{21}N_4O_2$: 289.1665, found 289.1657

6-(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenoxy)hexyl 4-methylbenzenesulfonate (3). To a solution of **2** (176 mg, 0.6 mmol) in DCM (10 mL), tosyl chloride (171 mg, 0.9 mmol) was added followed by trimethylamine (100 µL, 0.6 mmol). The reaction was stirred for 16 h at ambient temperature. Solvent was removed *in vacuo* and the residue was purified by silica column chromatography (50% EtOAc/hexane) to yield the product as a pink powder (172 mg, 65%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.41 (d, 2H, J = 9.0 Hz), 7.79 (d, 2H, J = 8.3 Hz), 7.48 (d, 2H, J = 8.3 Hz), 7.17 (d, 2H, J = 9.0 Hz), 4.03 (q, 4H, J = 6.3 Hz), 2.96 (s, 3H), 2.41 (s, 3H), 1.69 (quint, 2H, J = 6.5 Hz), 1.59 (quint, 2H, J = 6.5 Hz), 1.25 – 1.38 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.96, 163.41, 162.59, 145.30, 132.98, 130.62, 129.64, 128.03, 124.33, 115.77, 71.33, 68.16, 28.76, 28.55, 25.22, 24.97, 21.54, 21.18. HRMS-ESI: $[M + H]^+$ (m/z) calcd. for $C_{22}H_{27}N_4O_4S$: 443.1748, found 443.1722

2,2'-(7-(6-(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenoxy)hexyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (4). To a mixture of **3** (33 mg, 0.074 mmol) and NO2AtBu (30 mg, 0.082 mmol) in acetonitrile (5 mL) *N*,*N*-diisopropylethylamine (40 µL, 0.222 mmol) was added. The reaction was stirred for 16 h at ambient temperature. The solvent was removed *in vacuo*. TFA (3 mL) was added to the residue and the mixture was stirred for 2 h at ambient temperature. After the solvent was removed *in vacuo*, the product was obtained by semi-preparative RP-HPLC purification (Gradient 3) as a pink oil (4.3 mg, 11%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.42 (d, 2H, J = 9.0 Hz), 7.19 (d, 2H, J = 9.0 Hz), 4.12 (t, 2H, J = 6.5 Hz), 3.49 (q, 4H, J = 18 Hz), 3.23 – 3.28 (m, 2H), 3.16 – 3.19 (m, 4H), 3.02 – 3.07 (m, 2H), 2.69 (s, 3H), 2.75 – 2.87 (m, 4H), 2.62 – 2.67 (m, 2H), 1.80 (quint, 2H, J = 6.5 Hz), 1.71 (quint, 2H, J = 6.5 Hz), 1.49 (quint, 2H, J = 7.5 Hz), 1.38 (quint, 2H, J = 7.5 Hz). ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.36, 166.99, 163.41, 162.59, 129.68, 124.39, 115.78, 105.88, 99.99, 73.21, 68.20, 55.15, 54.78, 51.32, 49.86, 46.46, 28.84, 26.14, 25.59, 24.25, 21.19. HRMS-ESI: $[M + H]^+$ (m/z) calcd. for $C_{25}H_{38}N_7O_5$: 516.2929, found 516.2907

Section 3 - GE Tracerlab FX FN platform setup and radiosynthesis of radioconjugates

Modification of the GE Tracerlab™ FX FN was required to bypass the semi-preparative HPLC capabilities in favour of a simple HLB-SPE purification (Figure 3). Junction V12 (right) was routed directly to junction V17 (left) which permitted direct connection between the holding vessel (V12) and the HLB-SPE cartridge (V17). A glass reactor was used for the radiolabelling of the peptides, while a glassy carbon reactor was used for NODA-Tz. Non-purified [^{18}F]-fluoride (300-380 μL, *ca* 1000 MBq) was transferred directly to the radioisotope vial. Only when the larger volume of [^{18}F]-fluoride was used, a small amount of NaOAc 0.5 M (10 μL) was added to it in order to control the final reaction pH. Vial 3 contained the solution of the azamacrocycle substrate (30, 40 or 60 nmol), $AlCl_3$ 2 mM in NaOAc 0.5 M pH 4 (13, 19 or 27 μL), aqueous NaOAc 25 mM pH 4 (100 μL), and acetonitrile (100 μL). Only in the case of peptides radiolabelling, a solution of ascorbic acid 9.5 M in NaOAc 25 mM pH 4 (21, 30 or 45 μL) was added to the above mentioned solution. Vial 4 contained acetonitrile (434-594 μL, to have a total 1:1.2 v/v aqueous to organic solvent ratio). The buffered [^{18}F]-fluoride solution was transferred into the pre-warmed (50 °C) reactor (22 sec), followed by the addition of vial 3 and vial 4 (10 sec). The reactor was sealed, heated to 105°C for 15 min, and then cooled with compressed air to 50 °C (typically over 3 min). Water (5 mL) was added to the reactor from vial 6 (20 sec). The contents were transferred from the reactor into the holding reservoir and then loaded into the HLB-SPE cartridge (8 min). Another portion of water (5 mL from vial 6) was used to further rinse the reactor and, after being transferred to the holding reservoir, it was loaded to the HLB-SPE cartridge (4 min). Water (5 mL from vial 9) was used to wash the cartridge to remove unreacted [^{18}F]-fluoride and un-chelated [^{18}F]AlF (4 min). The process was complete in *ca* 35 min. The HLB cartridge was removed from the automated platform for further product elution by hand. Exclusively for our study, a small sample for the RP-HPLC analysis of the crude reaction mixture (*ca* 80 μL) was kept in the holding reservoir and collected at the end of the synthesis. This step can be omitted for routine production.

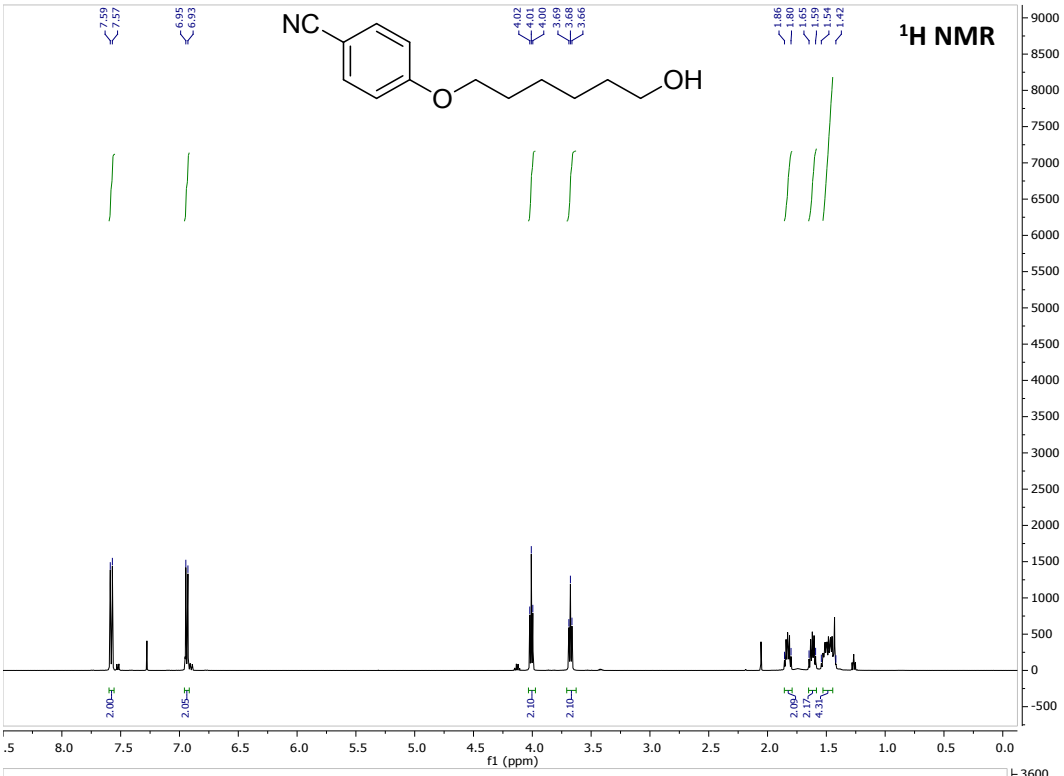
Section 4 - Trasis AIO platform setup and radiosynthesis of radioconjugates

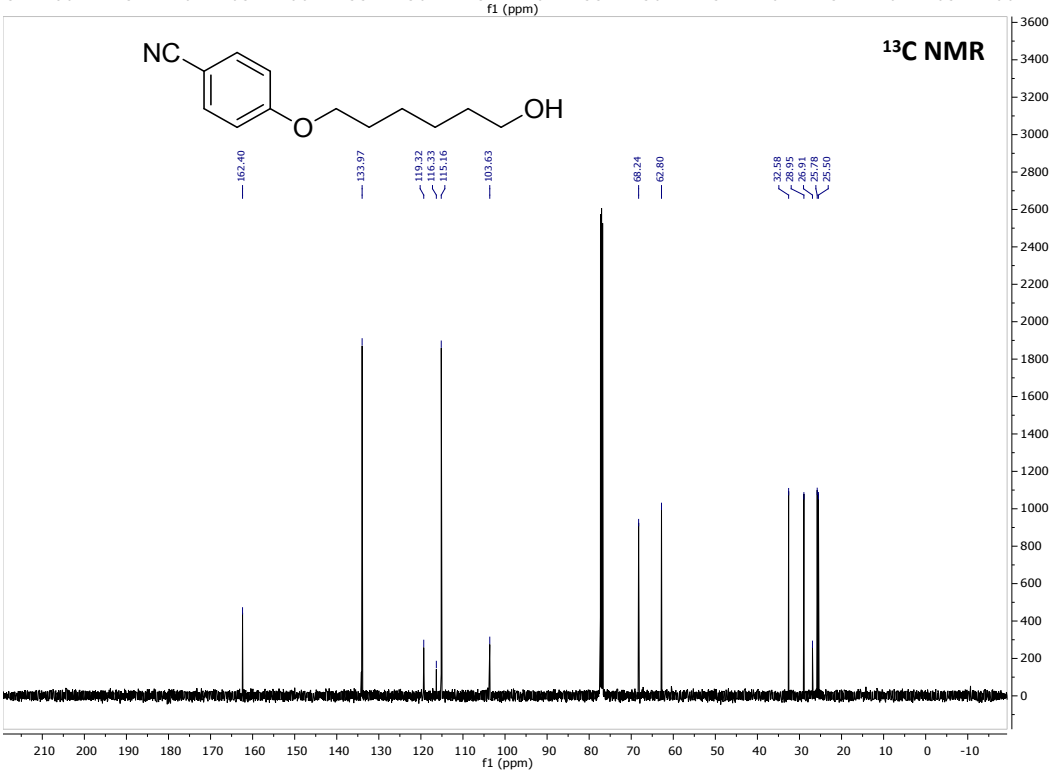
The cassette for [^{18}F]AlF radiochemistry was assembled as follows: two manifolds were joined and placed onto the system from position 1 to 12 (Figure 4). The standard activity plunger, usually connected to position 6, was bypassed in this procedure in favour of a more efficient method of transferring small volumes (<1 mL) of radioactivity. Non-purified [^{18}F]-fluoride was mixed with acetonitrile in the "^{18}F vial" and introduced into the cartridge a syringe (3 mL) at position 3. The substrate solution is stored in the syringe (1 mL) at position 8. A water reservoir is attached to position 12 for loading the radioconjugate solution onto the HLB cartridge at position 10/11. Non-purified [^{18}F]-fluoride (300-380 µL, *ca* 1000 MBq) was added directly to the radioisotope vial with acetonitrile (434-594 µL, to have a final 1:1.2 v/v aqueous to organic solvent ratio). Only when the larger volume of [^{18}F]-fluoride was used, a small amount of NaOAc 0.5 M (10 µL) was added to it in order to control the final reaction pH. A syringe (1 mL) containing the azamacrocycle substrate (60 nmol), $AlCl_3$ 2 mM in NaOAc 0.5 M pH 4 (27 µL), aqueous NaOAc 25 mM pH 4 (100 µL), and acetonitrile (100 µL) is located in position 8. Only in the case of peptides radiolabelling, ascorbic acid 9.5 M in NaOAc 25 mM pH 4 (45 µL) was added to the above mentioned solution. The [^{18}F]-Fluoride/acetonitrile mixture was taken up into the syringe (3 mL) at position 3 and expelled into the reaction vessel followed by the substrate solution (position 8). The reaction vessel was then sealed and heated to 105 °C for 15 min. The reaction was cooled with compressed air to 50 °C (typically over 3 min). Water (7 mL) from position 12 was taken up into the syringe (20 mL) at position 9 and expelled into the reaction vessel. The reaction vessel was rinsed with the diluted reaction mixture and slowly loaded onto the HLB cartridge through position 10. The processed was repeated with a further aliquot of water to ensure the maximum transfer of the reaction vessel contents to the HLB cartridge. Syringe 9 was then used to flush the cassette with water (7 mL) and a flow of nitrogen dried the line. Water (7 mL) was used to wash the HLB cartridge (to remove unreacted [^{18}F]-fluoride and un-chelated [^{18}F]AlF) and it was then dried under a flow of nitrogen for 30 seconds.

The process was complete in *ca* 26 min. The HLB cartridge was removed for further elution by hand. Exclusively for our study, a small aliquot (typically *ca* 80 μL) of the crude reaction mixture for RP-HPLC analysis was obtained *via* position 5 before the SPE cartridge loading. This step can be omitted for routine production.

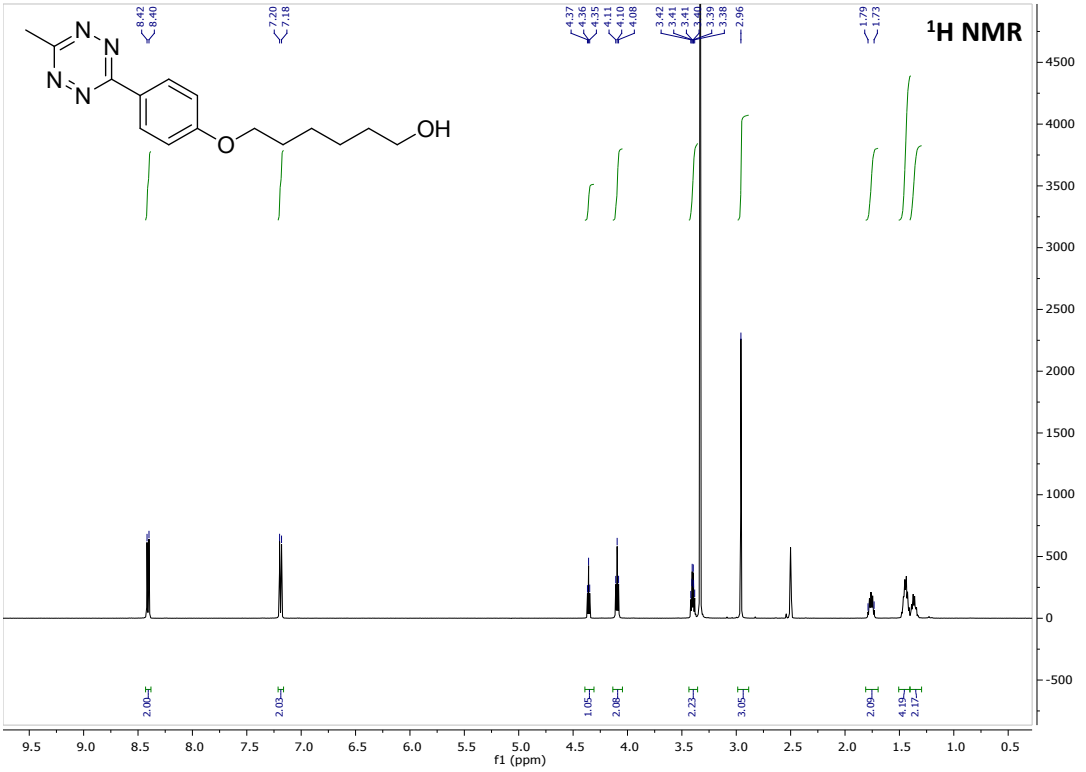
NMR spectra

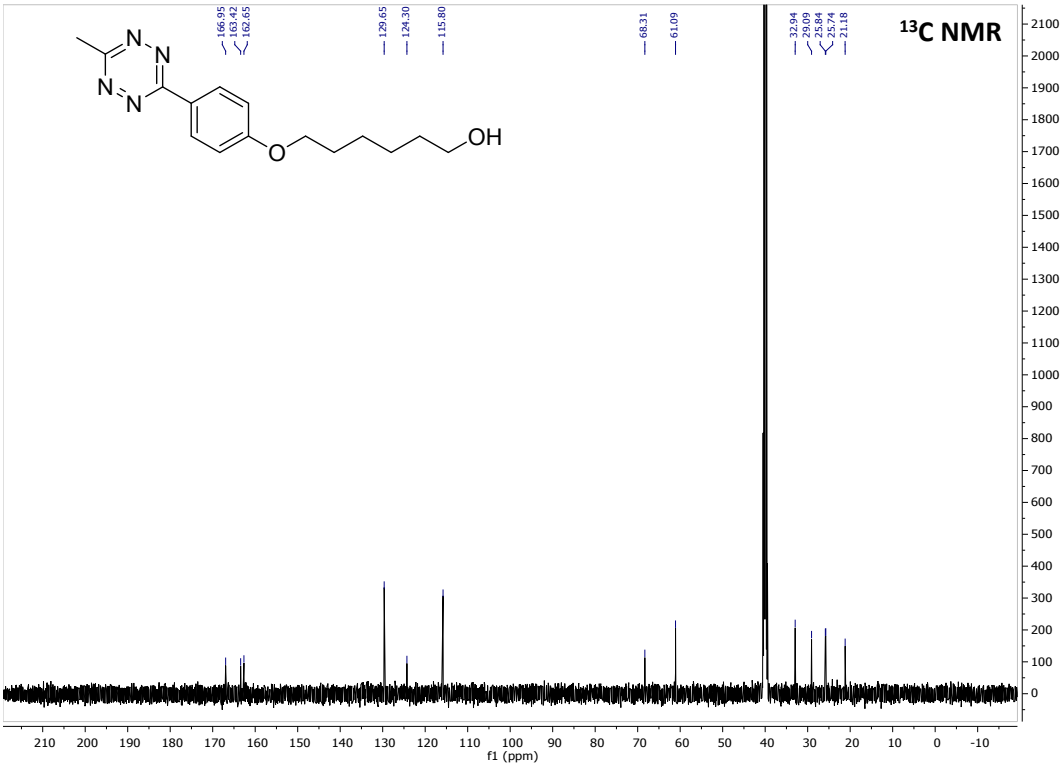
4-((6-Hydroxyhexyl)oxy)benzonitrile (**1**).



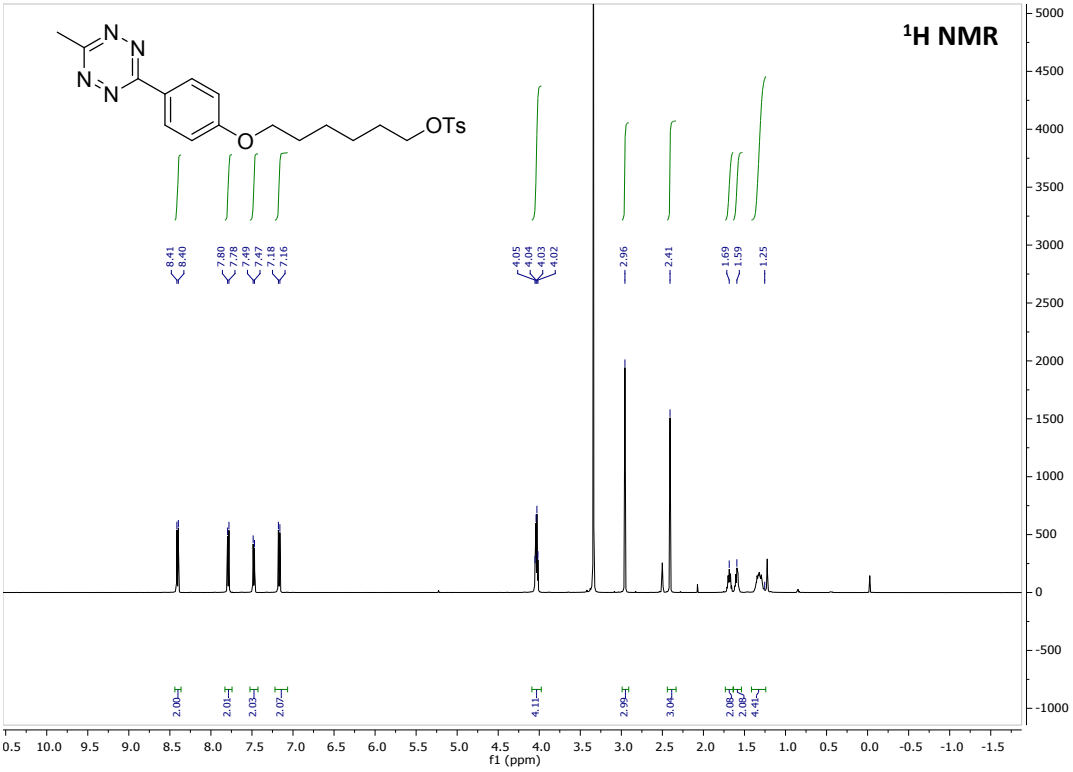


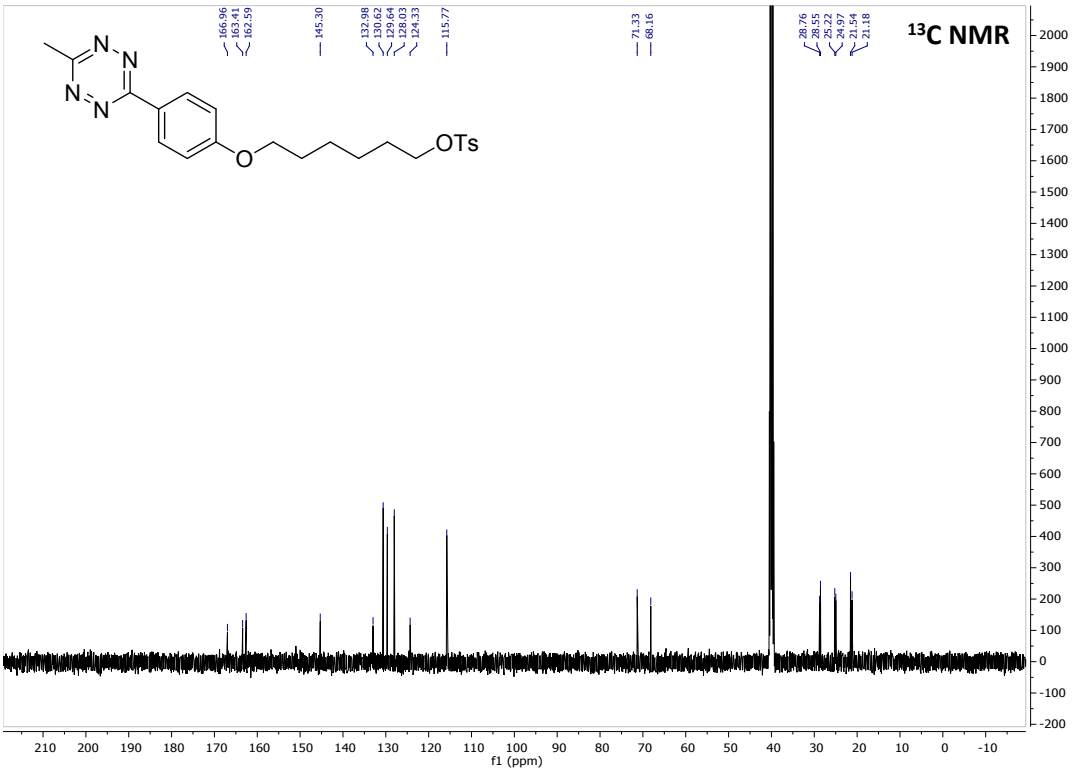
6-(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenoxy)hexan-1-ol (**2**).



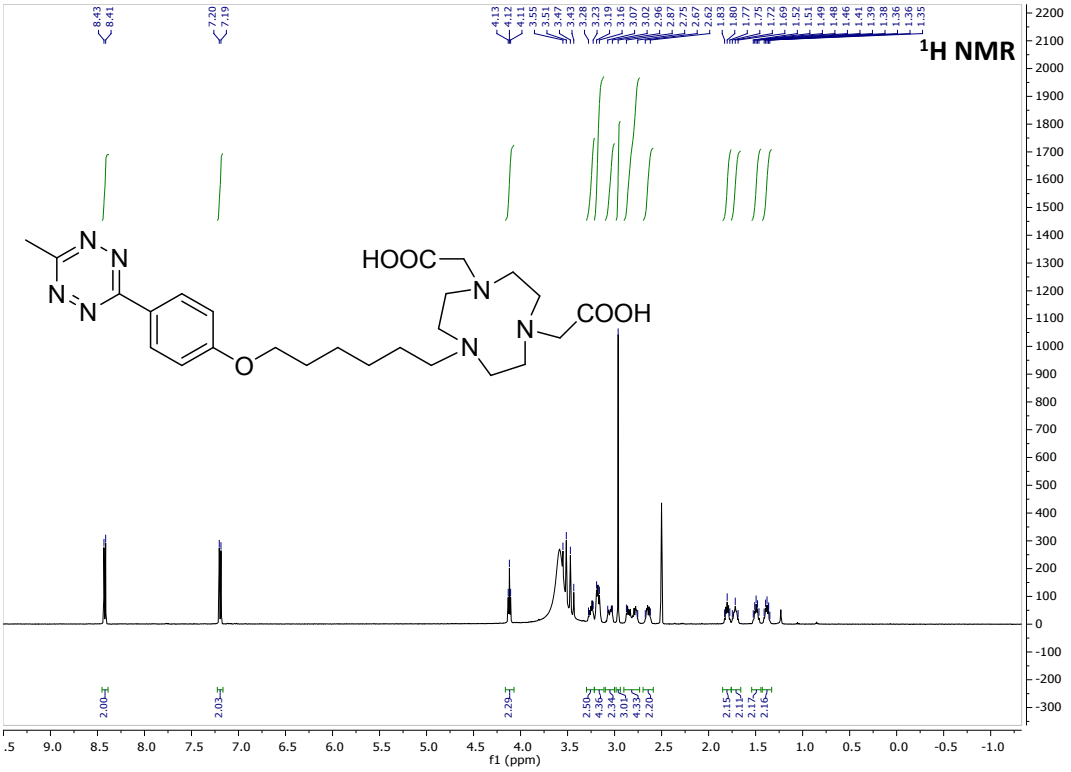


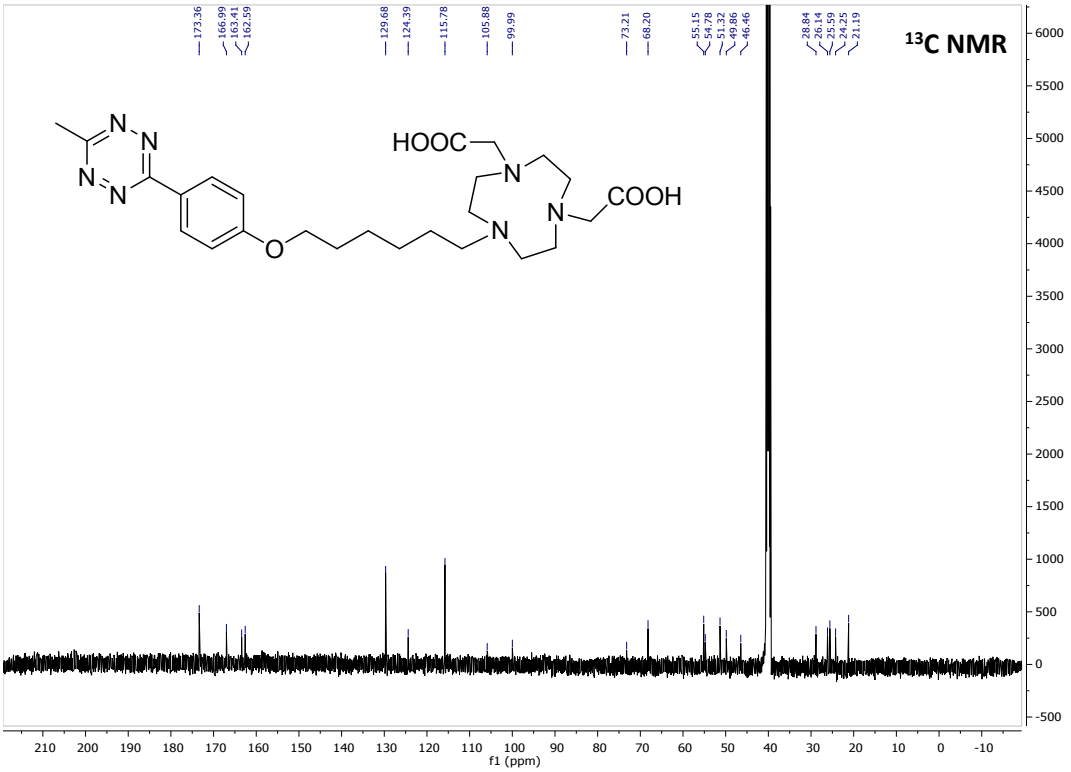
6-(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenoxy)hexyl 4-methylbenzenesulfonate (**3**).





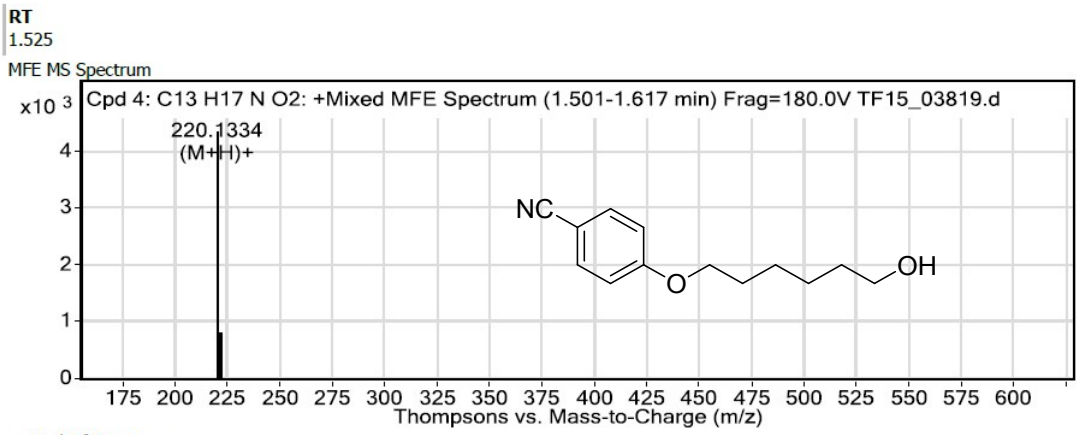
2,2'-(7-(6-(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenoxy)hexyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (**4**).



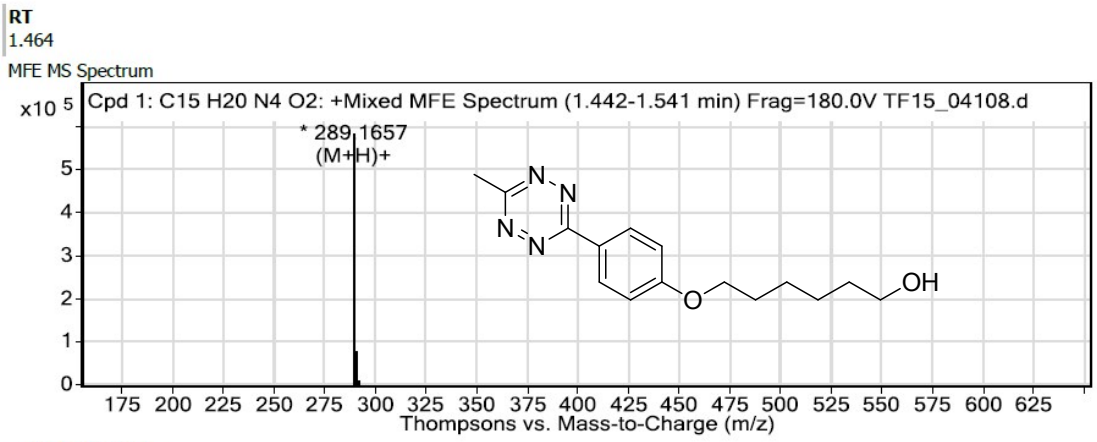


MS data

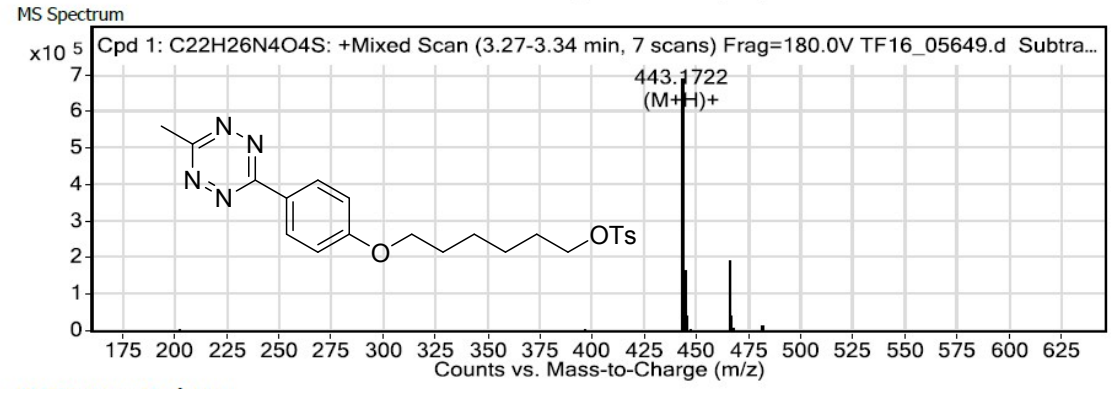
4-((6-Hydroxyhexyl)oxy)benzonitrile (**1**).



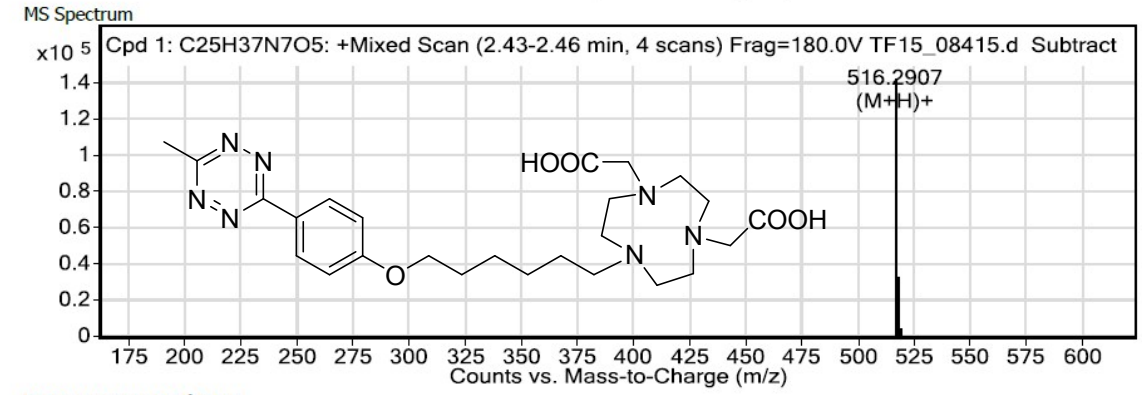
6-(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenoxy)hexan-1-ol (**2**).



6-(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenoxy)hexyl 4-methylbenzenesulfonate (**3**).



2,2'-(7-(6-(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenoxy)hexyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (**4**).



HPLC Radiochromatograms

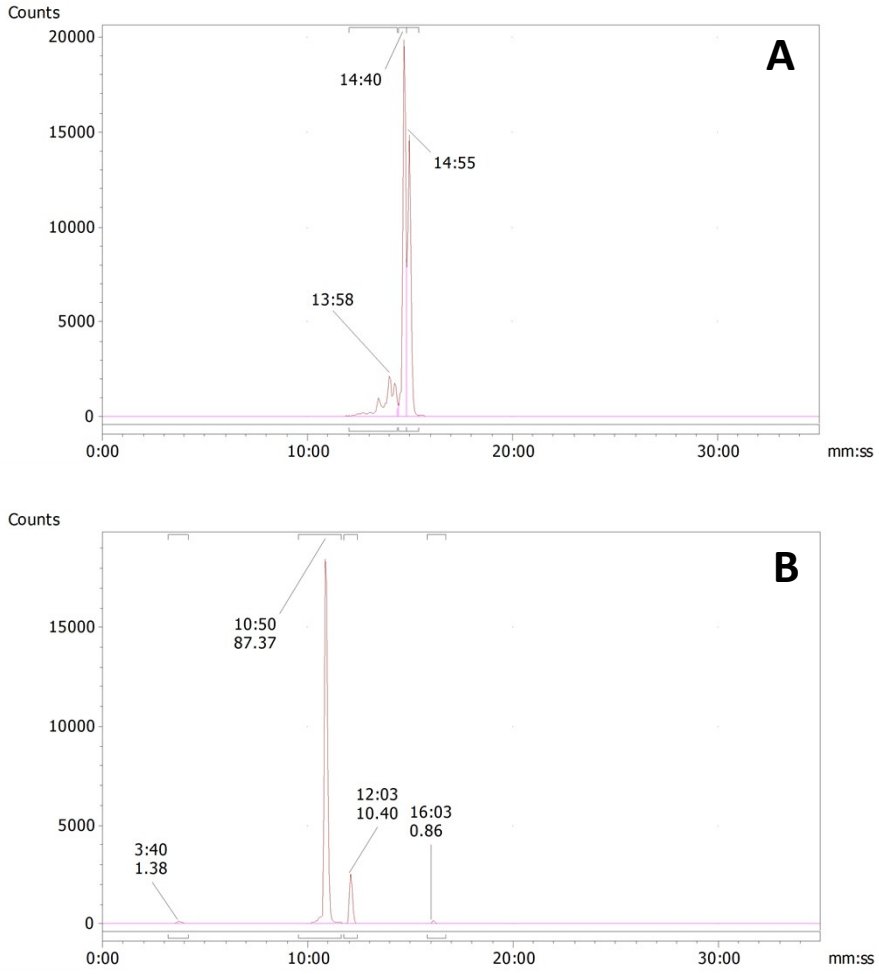


Figure S1. Representative HPLC radiochromatograms of the purified [^{18}F]AlF-NOTA-Octreotide (**A**) and [^{18}F]AlF-NOTA-RGDfK (**B**) in the absence of ascorbic acid as a radioprotector using Gradient **1**. The [^{18}F]AlF-NOTA-Octreotide radiolysis products elute at *ca* 12 min. The [^{18}F]AlF-NOTA-RGDfK radiolysis products elute at 12:03 and 16:03 (min:sec). Each peak was also expressed as %ROI (region of interest, bottom value).

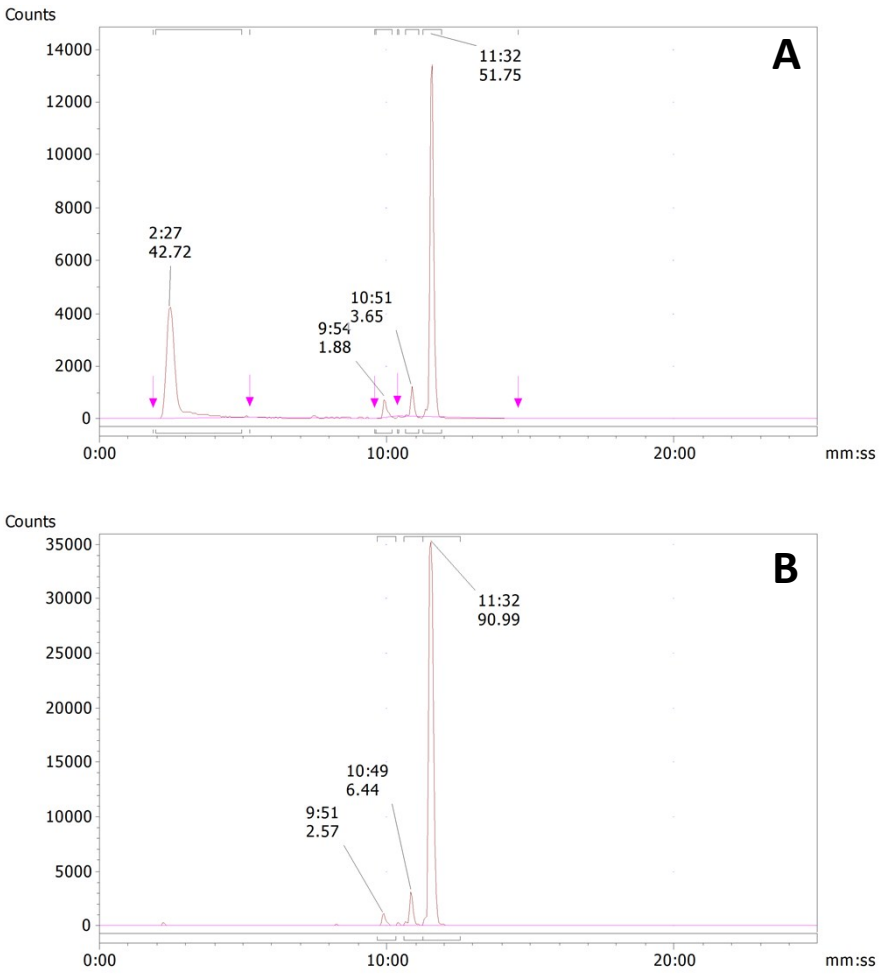


Figure S2. Representative HPLC radiochromatogram of the NODA-Tz radiolabelling reaction mixture (**A**) and purified [^{18}F]AlF-NODA-Tz (**B**) using Gradient **2**. As a similar [^{18}F]AlF radiolabelled molecule previously reported in the literature, [^{18}F]AlF-NODA-Tz is a mixture of products.[1] The retention time of the main product is 11:32 while free [^{18}F]-fluoride elutes at 2:27 (min:sec). Each peak was also expressed as %ROI (region of interest, bottom value).

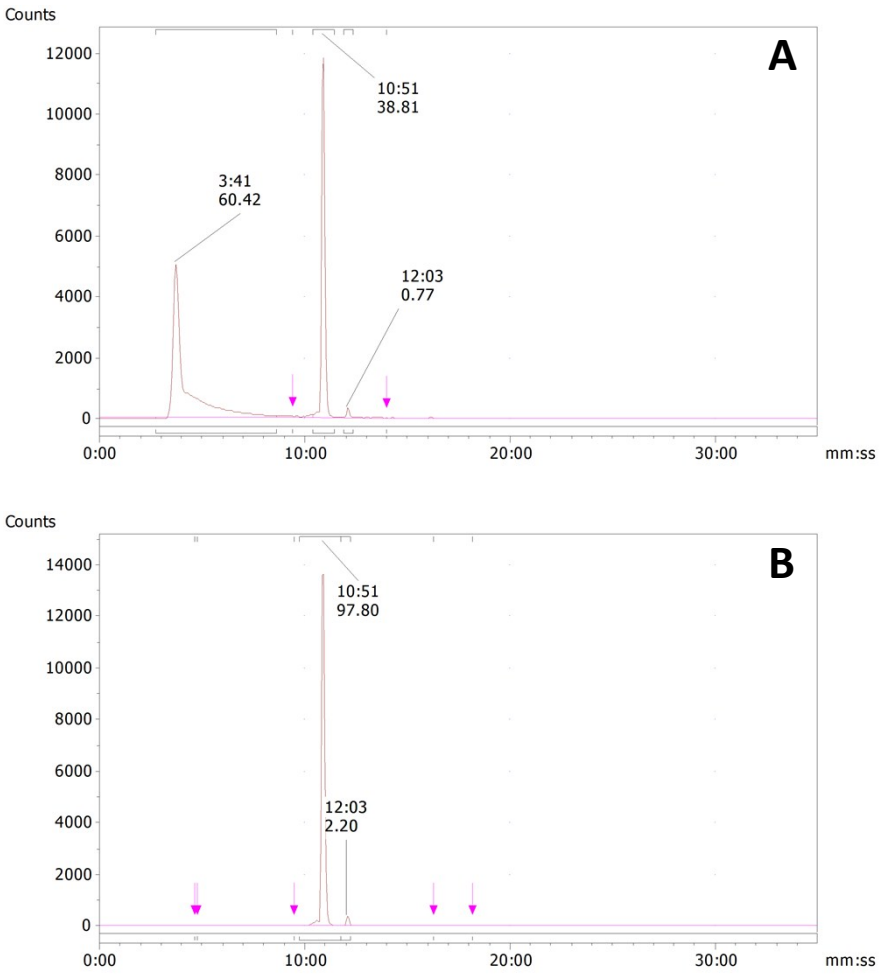


Figure S3. Representative HPLC radiochromatogram of the NOTA-RGDfK radiolabeling reaction mixture (**A**) and of purified [^{18}F]AlF-NOTA-RGDfK (**B**) using Gradient **1**. The retention time of [^{18}F]AlF-NOTA-RGDfK is 10:51 while free [^{18}F]-fluoride elutes at 3:41 (min:sec). Each peak was also expressed as %ROI (region of interest, bottom value).

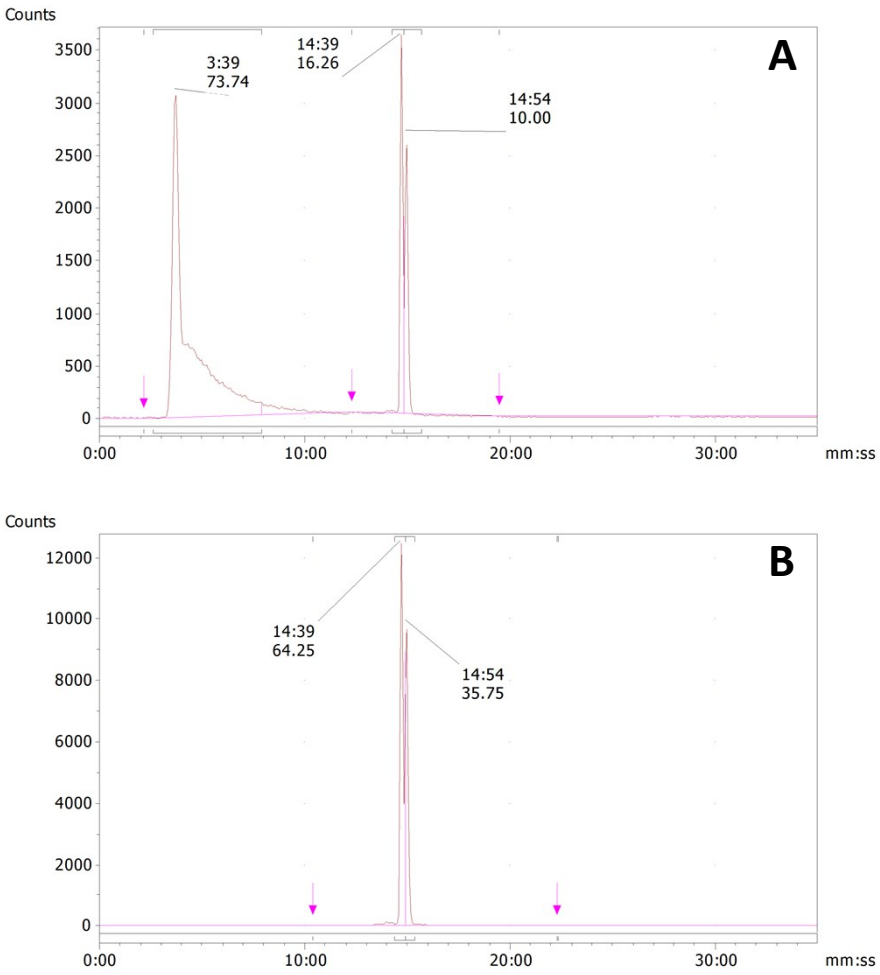


Figure S4. Representative HPLC radiochromatogram of the NODA-Octreotide radiolabeling reaction mixture (**A**) and of purified [^{18}F]AlF-NODA-Octreotide (**B**) using Gradient **1**. As reported in the literature, [^{18}F]AlF-NOTA-Octreoide is present as a mixture of two isomers.[2] The retention time of the product is 14:39-14:54 while free [^{18}F]-fluoride elutes at 3:39 (min:sec). Each peak was also expressed as %ROI (region of interest, bottom value).

GE Tracer Lab FX FN radiolabelling efficiencies

Substrate	Quantity (nmol)	Incorporation (%)	RCY (%)	SA (MBq/nmol)
NODA-Tz	30	14.8 ± 1.6	12.7 ± 1.3	4.0 ± 0.5
	40	21.8 ± 1.1	15.1 ± 2.7	4.6 ± 0.7
	60	51.2 ± 5.6	27.5 ± 6.8	6.9 ± 0.1
NOTA-RGDfK	30	17.3 ± 3.9	11.5 ± 0.8	4.6 ± 1.8
	40	34.2 ± 4.0	9.4 ± 0.8	5.2 ± 1.2
	60	37.9 ± 1.6	17.9 ± 3.9	5.4 ± 0.8
NOTA-Octreotide	30	21.1 ± 4.2	18.1 ± 6.5	5.2 ± 1.6
	40	20.4 ± 4.5	17.2 ± 3.0	5.4 ± 1.5
	60	40.1 ± 6.5	29.4 ± 4.6	6.2 ± 0.5

Table S1. Incorporation degree, radiochemical yield (RCY) and specific activity (SA) in correlation with the quantity of substrate on the GE Tracerlab FX FN system. The reactions were performed in triplicate (n=3) and the results are presented as mean ± SD. The incorporation percentages were derived from the HPLC traces of the reaction mixture (Figure S1A, S2A, and S3A). RCYs were decayed corrected to time of the fluorine-18 delivery. SAs were calculated from the HPLC traces and decay corrected to the end of reaction.

References

1. C. Da Pieve, L. Allott, C. D. Martins, A. Vardon, D. M. Ciobota, G. Kramer-Marek and G. Smith, *Bioconj. Chem.*, 2016, **27**, 1839 - 1849.
2. P. Laverman, W. J. McBride, R. M. Sharkey, A. Eek, L. Joosten, W. J. Oyen, D. M. Goldenberg and O. C. Boerman, *J. Nucl. Med.*, 2010, **51**, 454-461.