Synthesis and characterization of a highly stable dendritic catechol-tripod bearing technetium-99m†

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The synthesis and preliminary biological tests (*in vitro* toxicity, *in vitro* stability) of new Tc(III)-radiolabelled dendro-chelates are presented. A dendritic ^{99m}Tc chelate **1** derived from a pre-organized tripodal tris-catecholamide exhibits a kinetic stability by far more important than its corresponding diethylenetriamine pentaacetic acid (DTPA) homologue **2**. This permitted an assessment of the real impact of the pre-organized tripodal structure on kinetic inertness (and thus toxicity), an important issue to address when considering *in vivo* applications. Radiolabelling was performed using the stannous chloride reduction method; while DTPA-homologue **2** showed a high radiolabelling efficiency (96% radiolabelling yield after 30 min), tripodal complex **1** induced a 93% complexation yield after 45 min. In contrast, radiocomplex **1** derived from the most rigid and organized structure has a higher kinetic stability than **2**. Indeed, while dissociation of **2** reached 50% after 1 h 30 min in physiological media like phosphate buffer saline (PBS) and bovine serum albumin (BSA), over 80% of **1** remained stable during the half-life of the radionucleide (6.02 h for ^{99m}Tc). Measurements of the cell leakage resulting from membrane damage of neuronal cells treated with increasing concentrations of dendritic ligand **16**, together with pictures of treated neurons after staining, showed no detectable toxicity.

Introduction

The principle of scintigraphy, a powerful medical imaging method, is based on the detection of electromagnetic radiation emitted by a body-injected radioactive element. Such a technique provides dynamic and, above all, functional information and can explore the intimate mechanisms of life, hence its interest in the early detection of certain neurodegenerative (Alzheimer's) and proliferative (cancers) diseases.[1]

Two organ systems, namely the brain and heart, continue to dominate much in the field of nuclear medicine today and nuclear medicine's future is significantly linked with developments in radiopharmaceuticals (radiotracers), since their uptake, metabolism and release provide the relevant information about the functional status and structural changes of any diseased organ.

^{99m}Technetium (metastable) complexed to an appropriate ligand (chelation),[2] is regarded as a choice radioisotope in nuclear medicine, mainly due to its availability at low operating costs[3] and its negligible radiotoxicity. Indeed, it is a pure photoemitter (140 keV γ-ray with 89% abundance) with a short period (6 h), which poses no radioactive waste storage problem.

Scintigraphy based on ^{99m}Tc radiopharmaceuticals brings great hopes in medicine, and particularly in neurology (dementia, Alzheimer's disease), cardiology and oncology.[4]

In this context, the coordination chemistry of technetium is a determining factor at the crossroads of chemistry, pharmacology and medical biology. Indeed, use of this technique in the form of pharmaceutical kits requires an effective one-step complexation method providing thermodynamically and kinetically stable complexes in physiological conditions. We therefore sought to develop a functional tris-catecholamide chelate in order to increase the kinetic stability of the corresponding technetium complexes. Indeed, as far as functional imaging is concerned, kinetic inertness is a key point since longer medical examination times are required. Moreover, the use of dendrimers or dendritic compounds for biomedical applications is a flourishing area of research, mainly because of their precisely defined structure and high tunability, leading to biocompatible, polyfunctionnal and water-soluble systems.[2–6] Considering *in vivo* applications, water solubility is required but it is generally claimed that access to the cell through biological membranes depends on the lipophilicity of the chelator or of its metal complex. This led us to graft an oligoethyleneglycol dendrimer at the focal point of the triscatecholamide coordination sphere through a diamine linker. This not only allows fine tuning of the complex's hydro/lipophilic balance based on the dendron generation, but also brings the possibility of multi-functionalization to target

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organs, of combining diagnostic imaging and treatment by grafting of a therapeutic agent, or using complementary imaging modalities (MRI and optical imaging) through the connection of a fluorescent dye, and all of this on a low molecular weight molecule (Fig. 1). The use of dendrimers for the complexation of ^{99m}Tc is scarcely reported in the literature so far: in 2001, F. Vögtle *et al.*[7] reported host–guest properties of multi-crown dendrimers of four different generations towards sodium pertechnetate. Extraction studies performed showed that the guest molecules are mainly bound in the interior of the poly-amine skeleton. The same year, H. Mukhtar and coworkers[8] reported the synthesis and *in vivo* distribution of water-soluble ^{99m}Tc-labeled dendritic porphyrins for tumor imaging and diagnosis; these dendritic systems were administered to a C6-glioma tumor-bearing Wistar rats and scinti-imaging studies showed their potential for early-stage tumor detection. Finally, A. Adronov, J. F. Valliant *et al.*[9] very recently published a paper dealing with the use of high-generation polyester dendrimers to complex ^{99m}Tc and their use for SPECT imaging. It was found that all three dendrimer generations (G5 to G7) were rapidly and efficiently removed from the bloodstream *via* the kidneys and excreted through the bladder within 15 min, post injection. The SPECT-CT data were corroborated with a quantitative biodistribution study involving *ex vivo* harvesting of various organs and determining the radioactivity within the organs as a function of time.

Experimental

Materials and synthesis

Reagents and solvents were purchased as reagent grade and used without further purification. Compounds **5**,[10] **10**[11] and **13**[12] were prepared according to the literature. All reactions were performed in standard glassware under Ar and solvents were, if necessary, purified by standard procedures prior to use. Evaporation and concentration were done at water-aspirator pressure and drying *in vacuo* at 10^{-2} Torr. Column chromatography: silica gel 60 (230–400 mesh, 0.040–0.063 mm) from E. Merck; LH20 column (Sephadex) from Sigma-Aldrich. NMR spectra: Bruker AM-300 (^{1}H: 300 MHz; ^{13}C: 75 MHz); solvent peaks as reference; δ in ppm. **Caution**: ^{99m}Tc is a γ-emitter (E_γ = 140 eV, $t_{\frac{1}{2}}$ = 6 h), which should only be used in a licensed and appropriately shielded facility. Sodium pertechnetate was obtained from a ^{99}Mo/^{99m}Tc generator (Cisbio International) and the labelling experiments were performed in a licensed and shielded facility at the Nuclear Medicine service of the Hôpital Universitaire—Hospices Civil de Strasbourg.

3: a solution of 2,3-dihydroxybenzoïc acid (10.00 g, 64.89 mmol) and K_2CO_3 (33.20 g, 240.07 mmol) in acetonitrile (140 mL) was heated at 80 °C for 1 h. The reaction mixture was then cooled to room temperature and a solution of allyl bromide (20.80 mL, 240.07 mmol) in acetonitrile (80 mL) was added dropwise. The resulting mixture was heated for 17 h at 70 °C and filtered. The filtrate was evaporated to dryness and diluted in EtOH (100 mL). After adding a solution of NaOH (7.40 g, 184.92 mmol) in 12 mL water, the reaction mixture was refluxed for 23 h and evaporated to dryness. The residue obtained was dissolved in CH_2Cl_2 (100 mL) and 150 mL of HCl 1 M was added. The aqueous sub-phase was extracted twice by using CH_2Cl_2 (200 mL) and the organic phases combined, washed twice with water (200 mL), dried ($MgSO_4$), filtered and evaporated. After recrystallisation in hexane–ether (50/50 mL), compound **3** (9.88 g, 42.18 mmol) was obtained in

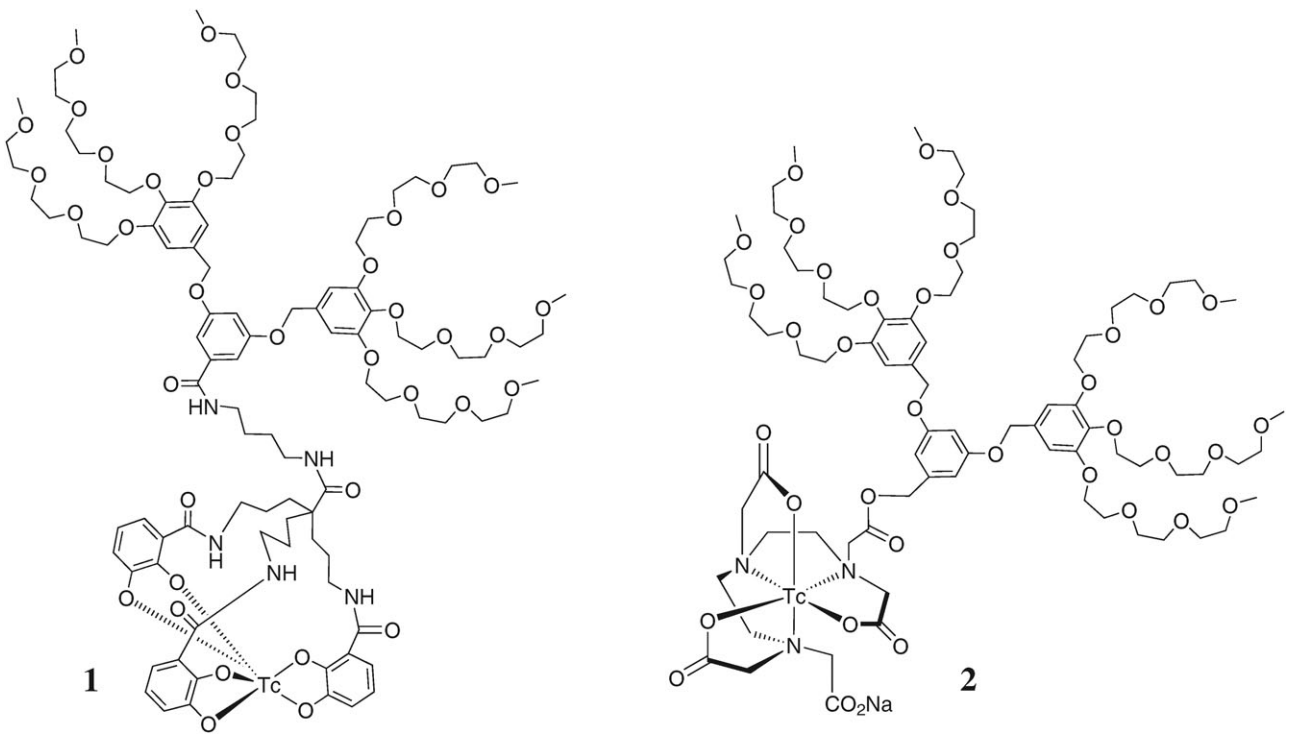


Fig. 1 Proposed structures for dendritic ^{99m}Tc chelates **1** and **2** at pH 7.4.

65% yield. White powder. M.p. 138 °C. ^{1}H NMR ($CDCl_3$): 4.62 (d, ^3J = 5 Hz, 2H, OCH_2), 4.82 (d, ^3J = 6 Hz, 2H, OCH_2), 5.32–5.50 (m, 4H, allyl H), 6.01–6.15 (m, 2H, allyl H), 7.16 (m, 2H, ArH), 7.75 (dd, ^3J = 6 Hz, 1H, ArH); ^{13}C NMR ($CDCl_3$): 75.6 (OCH_2 allyl), 114.8 (Ar), 117.0 (CH_2 allyl), 120.2 (Ar), 121.5 (Ar), 123.4 (Ar), 137.2 (CH allyl), 147.3 (Ar), 149.1 (Ar), 171.8 (C═O); $C_{13}H_{14}O_4$: calc. C 66.66, H 6.02, O 27.32; found C 66.58, H 6.02, O 27.40.

4: 0.51 mL (5.977 mmol) of trifluorotriazine was added to a stirred solution of **3** (2.00 g, 8.54 mmol) in dry CH_2Cl_2 (100 mL) cooled to 0 °C. The mixture was stirred for 10 min before addition of an anhydrous CH_2Cl_2 (30 mL) solution of pyridine (0.76 mL, 9.39 mmol). After 17 h at room temperature, the mixture was washed with cold water (2 × 75 mL) and NaCl-saturated water (75 mL). The organic sub-phase was then dried ($MgSO_4$) filtered and evaporated. Crude compound **4** was obtained as a brown oil (1.82 g, 7.69 mmol) with 90% yield and used in the next step without further purification. ^{1}H NMR ($CDCl_3$): 4.60–4.66 (m, 4H, OCH_2), 5.23–5.48 (m, 4H, allyl H), 6.00–6.19 (m, 2H, allyl H), 7.09–7.22 (m, 2H, ArH), 7.49 (d, ^3J = 9 Hz, 1H, ArH); ^{13}C NMR ($CDCl_3$): 75.02 (OCH_2 allyl), 117.95 (Ar), 118.42 (CH_2 allyl), 120.32 (Ar), 123.99 (Ar), 124.10 (Ar), 132.46 (CH allyl), 150.50 (Ar), 152.76 (Ar), 157.46.

6: **4** (4.96 g, 21.00 mmol) in freshly distilled CH_2Cl_2 (100 mL) was added dropwise over 90 min to a stirred solution of *N*,*N*-diisopropylethylamine (4.52 mL, 27.36 mmol) and **5**[10] (2.11 g, 6.36 mmol) in freshly distilled CH_2Cl_2 (100 mL) kept under nitrogen. After 60 h stirring at room temperature, the crude mixture was filtered, washed with water, dried ($MgSO_4$), filtered and then evaporated to dryness. Column chromatography (SiO_2, CH_2Cl_2/1% MeOH) gave compound **6** (6.05 g, 6.17 mmol) in 97% yield. Pale yellow oil. ^{1}H NMR ($CDCl_3$): −0.01 (s, 6H, CH_3), 0.82 (s, 9H, tBu), 1.25–1.48 (m, 12H, CH_2), 3.29 (s, 2H, CH_2O), 3.34–3.38 (m, 6H, CH_2NH), 4.57 (d, 12H, OCH_2 allyl), 5.24–5.46 (m, 12H, allyl H), 6.00–6.12 (m, 6H, allyl H), 6.99 (d, ^3J = 13 Hz, 3H, ArH), 7.03 (d, ^3J = 9.7 Hz, 3H, ArH), 7.68 (d, ^3J = 9 Hz, 3H, ArH), 8.02 (t, 3H, NH); ^{13}C NMR ($CDCl_3$): −5.67 ($SiCH_3$), 18.06 (SiC), 23.35 (CH_2), 25.76 (C(CH_3)), 31.48 (CH_2), 39.38 (C), 40.54 (CHNH), 66.51 (OCH_2), 69.77 (OCH_2), 74.59 (OCH_2), 116.75 (Ar), 117.67 (Ar), 118.76 (Ar), 123.17 (Ar), 124.23 (Ar), 127.50 (Ar), 132.80 (Ar), 133.19 (Ar), 146.24 (Ar), 151.48 (Ar), 164.98 (C═O); $C_{56}H_{77}N_3O_{10}Si$. $\frac{1}{2}$ H_2O: calc. C 68.09, H 7.90, N 4.26; found C 68.23, H 7.83, N 4.12.

7: compound **6** (735 mg, 0.75 mmol) was dissolved in 10 mL THF at 0 °C. Tetra-*n*-butylammonium fluoride (2.21 mmol) was slowly added and the reaction mixture was heated at reflux for 3 h. The solution was evaporated and the obtained residue was dissolved in CH_2Cl_2 (100 mL), washed with water, dried ($MgSO_4$), filtered and evaporated. Column chromatography (SiO_2, CH_2Cl_2/3% MeOH) afforded **7** (610 mg, 0.71 mmol) in 94% yield. Yellow oil. ^{1}H NMR ($CDCl_3$): 1.27–1.63 (m, 12H, CH_2), 3.39 (m, 8H, CH_2OH + CH_2NH), 4.58 (d, 12H, OCH_2 allyl), 5.26–5.46 (m, 12H, allyl H), 6.00–6.12 (m, 6H, allyl H), 7.02 (d, ^3J = 8 Hz, 3H, ArH), 7.12 (t, ^3J = 8 Hz, 3H, ArH), 7.68 (d, ^3J = 9 Hz, 3H, ArH), 8.08 (t, ^3J = 5 Hz, 3H, NH); ^{13}C NMR ($CDCl_3$): 23.27 (CH_2), 31.14 (CH_2), 39.41 (C), 40.43 (CHNH), 66.39 (OCH_2), 69.76 (OCH_2), 74.63 (OCH_2), 116.82 (CH), 117.73 (CH), 118.87 (Ar), 123.13 (Ar), 124.37 (Ar), 127.39 (Ar), 132.78 (Ar), 133.21 (Ar), 146.24 (Ar), 151.48 (Ar), 158.25 (Ar), 165.18 (C═O); $C_{50}H_{63}N_3O_{10}$: calc. C 69.34, H 7.33; found C 69.04, H 7.78.

8: a solution of anhydrous dimethyl sulfoxide (144 µL, 2.03 mmol) in 300 µL dry dichloromethane was added to a solution of oxalyl chloride (89 µL, 1.02 mmol) in freshly distilled dichloromethane (1 mL) kept at −60 °C. The mixture was stirred for 15 min and then a solution of **7** (0.80 g, 0.92 mmol) in dry dichloromethane (3 mL) was added over a 10 min period; the solution obtained was stirred for an additional 1 h and the reaction mixture was allowed to warm to −30 °C. *N*,*N*-Diisopropylethylamine (10 equiv.) was added and the reaction mixture was allowed to warm to room temperature. Water (50 mL) and dichloromethane (50 mL) were added and the organic layer was washed with brine, dried ($MgSO_4$), filtered and evaporated to dryness. Compound **8** was used in the next step without further purification. Colourless oil. ^{1}H NMR ($CDCl_3$): 1.26–1.58 (m, 12H, CH_2), 3.40 (m, 6H, CH_2NH), 4.58 (d, 12H, OCH_2 allyl), 5.26–5.47 (m, 12H, allyl H), 6.02–6.11 (m, 6H, allyl H), 7.01 (d, ^3J = 9 Hz, 3H, ArH), 7.11 (t, ^3J = 8 Hz, 3H, ArH), 7.68 (d, ^3J = 7 Hz, 3H, ArH), 8.08 (t, 3H, NH), 9.42 (s, 1H, CHO); ^{13}C NMR ($CDCl_3$): 23.72 (CH_2), 29.30 (CH_2), 39.93 (C), 51.29 (CHNH), 69.75 (OCH_2), 74.49 (OCH_2), 116.86 (Ar), 117.71 (Ar), 118.79 (Ar), 123.09 (Ar), 124.27 (Ar), 127.20 (Ar), 132.74 (Ar), 133.15 (Ar), 146.25 (Ar), 151.46 (Ar), 165.13 (C═O), 206.59 (CHO).

9: sulfamic acid (466 mg, 4.80 mmol) and then sodium chlorite (434 mg, 4.80 mmol) were added to a stirred solution of **8** (3.19 g, 3.69 mmol) in a THF–water (1/1) mixture. After 13 h stirring at room temperature, 200 mL CH_2Cl_2 and 200 mL water were added and the resulting organic layer was washed with brine, dried ($MgSO_4$), filtered and evaporated to dryness. Column chromatography (SiO_2, CH_2Cl_2/3% MeOH) afforded **9** (0.47 g, 0.53 mmol) in 58% yield over the two steps. Pale yellow oil. ^{1}H NMR ($CDCl_3$): 1.53–1.66 (m, 12H, CH_2), 3.40 (d, 6H,CH_2NH), 4.57 (d, 12H, OCH_2 allyl), 5.24–5.45 (m, 12H, allyl H), 5.99–6.12 (m, 6H, allyl H), 6.98 (d, ^3J = 9 Hz, 3H, ArH), 7.09 (t, ^3J = 8 Hz, 3H, ArH), 7.68 (d, ^3J = 9 Hz, 3H, ArH), 8.08 (t, 3H, NH); ^{13}C NMR ($CDCl_3$): 24.16 (CH_2), 31.92 (CH_2), 40.02 (C), 48.06 (CHNH), 69.76 (OCH_2), 74.64 (OCH_2), 116.80 (Ar), 117.72 (Ar), 118.96 (Ar), 123.12 (Ar), 124.25 (Ar), 127.34 (Ar), 132.79 (Ar), 133.16 (Ar), 146.25 (Ar), 151.48 (Ar), 165.19 (CO), 178.96 (CO_2H); $C_{49}H_{61}N_3O_{11}$. 1,5 H_2O: calc. C 66.23, H 7.06, N 4.63, O 22.08; found C 66.43, H 6.83, N 4.31, O 22.43.

11: a mixture of DCC (112 mg, 0.55 mmol) and DMAP (11 mg, 0.09 mmol) in dry CH_2Cl_2 (5 mL) and a solution of **10**[11] (85 mg, 0.46 mmol) in dry CH_2Cl_2 (5 mL) were added to **9** (400 mg, 0.46 mmol) dissolved in freshly distilled dichloromethane (10 mL). A catalytic amount of HOBt was then added and the mixture obtained was stirred for 60 h at room temperature before filtration and evaporation to dryness. Column chromatography (SiO_2, CH_2Cl_2/3% MeOH) afforded **11** (455 mg, 0.44 mmol) in 97% yield. Pale yellow oil. ^{1}H NMR ($CDCl_3$): 1.41–1.55 (m, 25H, *t*Bu + CH_2 tren + CH_2 alkyl), 3.07 (d, ^3J = 5 Hz, 2H, CH_2NHBoc), 3.23 (d, ^3J = 5 Hz, 2H, $CONHCH_2$), 3.37 (d, 6H, CH_2NH tren), 4.56

(d, 12H, OCH_2 allyl), 5.24–5.45 (m, 12H, allyl H), 5.98–6.11 (m, 6H, allyl H), 6.98 (d, ^3J = 9 Hz, 3H, ArH), 7.09 (t, ^3J = 8 Hz, 3H, ArH), 7.66 (d, ^3J = 9 Hz, 3H, ArH), 8.04 (t, ^3J = 11 Hz, 3H, NH); ^{13}C NMR ($CDCl_3$): 24.28 (CH_3), 25.57 (CH_2), 26.83 (CH_2), 27.53 (CH_2), 28.40 (CH_2), 32.21 (C), 40.00 (C), 47.87 (CHNH), 69.77 (OCH_2), 74.65 (OCH_2), 116.82 (Ar), 117.73 (Ar), 118.96 (Ar), 123.06 (Ar), 124.25 (Ar), 127.36 (Ar), 132.77 (Ar), 133.19 (Ar), 146.26 (Ar), 151.49 (Ar), 156.14 (C═O), 165.21 (C═O), 175.60 (C═O); $C_{59}H_{79}N_5O_{12}$. CH_3OH: calc. C 66.58, H 7.73, N 6.47, O 19.22; found C 66.77, H 7.75, N 6.63, O 18.85.

12: TFA (68 mL, 0.89 mmol) was added to a solution of **11** (465 mg, 0.44 mmol) in dry CH_2Cl_2 (10 mL). After 6 h stirring at reflux, the reaction mixture was evaporated and filtered on a silica pad (SiO_2, CH_2Cl_2/5%MeOH). Compound **12** (376 mg, 0.40 mmol) was obtained as a white foam in 90% yield. ^{1}H NMR ($CDCl_3$): 1.46–1.73 (m, 16H, CH_2), 2.99 (d, 2H, CH_2NH_2), 3.26–3.32 (m, 8H, CH_2NH), 4.56 (d, 12H, OCH_2 allyl), 5.23–5.43 (m, 12H, allyl H), 5.96–6.09 (m, 6H, allyl H), 6.98 (d, ^3J = 9 Hz, 3H, ArH), 7.06 (t, ^3J = 8 Hz, 3H, ArH), 7.56 (d, 3H, ArH), 7.80 (br s, 2H, NH_2), 8.34 (br s, 4H, NH); ^{13}C NMR ($CDCl_3$): 24.19 (CH_2), 24.60 (CH_2), 26.10 (CH_2), 31.84 (CH_2), 38.65 (CH_2), 39.87 (C), 40.20 (CHNH), 47.94 (CHNH), 69.77 (OCH_2), 74.67 (OCH_2), 117.16 (Ar), 117.76 (Ar), 119.03 (Ar), 122.64 (Ar), 124.33 (Ar), 126.76 (Ar), 132.68 (Ar), 133.06 (Ar), 146.33 (Ar), 151.52 (Ar), 165.81 (C═O), 176.71 (C═O); $C_{54}H_{71}N_5O_{10}$. 2,5 CH_2Cl_2: calc. C 58.59, H 6.56, N 5.99; found C 58.25, H 6.35, N 5.67.

14: a solution of NaOH (38 mg, 0.95 mmol) in H_2O (10 mL) was added to a stirred solution of **13**[12] (1.00 g, 0.76 mmol) in THF (40 mL). After 2 h reflux, the solvent was evaporated. Column chromatography (SiO_2, CH_2Cl_2/10% MeOH) afforded **14** (0.87 g, 0.67 mmol) in 88% yield. Pale yellow oil. ^{1}H NMR ($CDCl_3$): 3.38 (s, 18H, OCH_3), 3.53–3.87 (m, 60H, OCH_2), 4.13–4.19 (m, 12H, OCH_2), 4.99 (s, 4H, OCH_2 Ar), 6.67 (s, 1H, ArH), 6.78 (br t, 4H, ArH), 7.29 (d, ^4J = 2 Hz, 2H, ArH); ^{13}C NMR ($CDCl_3$): 58.90 (OCH_3), 68.78 (OCH_2), 69.83 (OCH_2), 70.22 (OCH_2), 70.31 (OCH_2), 70.36 (OCH_2), 70.45 (OCH_2), 70.53 (OCH_2), 71.76 (OCH_2), 71.87 (OCH_2), 72.31 (OCH_2), 103.47 (Ar), 106.39 (Ar), 108.10 (Ar), 133.17 (Ar), 137.06 (Ar), 142.74 (Ar), 152.36 (Ar), 158.86 (Ar), 171.12 (C═O); $C_{63}H_{102}O_{28}$. 2 H_2O: calc. C 56.32, H 7.95, O 35.73; found C 56.56, H 7.86, O 35.58.

15: a mixture of DCC (31 mg, 0.15 mmol) and DMAP (3 mg, 0.03 mmol) in dry CH_2Cl_2 (5 mL) and a solution of **12** (130 mg, 0.14 mmol) in dry CH_2Cl_2 (5 mL) were added to a solution of **14** (190 mg, 0.14 mmol) dissolved in freshly distilled dichloromethane (5 mL). A catalytic amount of HOBt was added and the mixture was stirred 43 h at room temperature. The resulting solution was then filtered and evaporated to dryness. The crude mixture was filtered on a silica pad (SiO_2, CH_2Cl_2/5% MeOH) to afford **15** (210 mg, 0.10 mmol) in 72% yield. Pale yellow oil. ^{1}H NMR ($CDCl_3$): 1.46–1.54 (m, 16H, CH_2), 3.30–3.36 (m, 28H, OCH_3 + CH_2NH), 3.48–3.82 (m, 60H, OCH_2), 4.10 (m, 12H, OCH_2), 4.53 (d, ^3J = 5 Hz, 12H, OCH_2 allyl), 4.85 (s, 4H, OCH_2 Ar), 5.21–5.42 (m, 12H, allyl H), 5.95 –6.08 (m, 6H, allyl H), 6.57–6.64 (m, 4H, ArH), 6.94–7.17 (m, 6H, ArH), 7.17 (s, 2H, ArH), 7.60 (d, ^3J = 7 Hz, 3H, ArH), 7.71 (t, 1H, ArH), 8.09 (t, ^3J = 5 Hz, 3H, NH); ^{13}C NMR ($CDCl_3$): 24.33 (CH2), 32.21 (CH2), 40.00 (CHNH), 47.92 (CHNH), 58.94 (OCH_3), 68.84 (OCH_2), 69.67 (OCH_2), 69.73 (OCH_2), 70.18 (OCH_2), 70.48 (OCH_2), 70.62 (OCH_2), 70.75 (OCH_2), 71.87 (OCH_2), 71.89 (OCH_2), 72.27 (OCH_2), 74.62 (OCH_2), 106.05 (Ar), 107.22 (Ar), 116.87 (Ar), 117.73 (Ar), 118.93 (Ar), 122.87 (Ar), 124.27 (Ar), 127.19 (Ar), 131.94 (Ar), 132.74 (Ar), 133.18 (Ar), 138.16 (Ar), 146.26 (Ar), 151.50 (Ar), 152.68 (Ar), 152.86 (Ar), 159.73 (Ar), 165.26 (C═O), 175.67 (C═O); MALDI-MS (negative mode): $[M]^-\cdot Na^+$ 2262.29 obtained for $C_{117}H_{170}N_5O_{37}Na$ (2261.65).

16: $Pd(PPh_3)_4$ (23 mg, 0.02 mmol) was added to a solution of **15** (75 mg, 0.033 mmol) in freshly distilled THF (2 mL). The solution was stirred for 1 h before addition of $NaBH_4$ (11 mg, 0.30 mmol). Then, after 1 h stirring at room temperature, HCl 1 M was added and the resulting mixture was filtered on celite. The solvent was evaporated and the residue was purified by LH 20 column (Sephadex) (CH_2Cl_2) to afford **16** (70 mg, 0.035 mmol) in 95% yield. Pale green oil. ^{1}H NMR (CD_3OD): 1.34–1.63 (m, 16H, CH_2), 3.30–3.36 (m, 28H, OCH_3 + CH_2NH), 3.50–3.89 (m, 60H, OCH_2), 4.10–4.18 (m, 12H, OCH_2), 4.96 (br s, 4H, OCH_2Ar), 6.57–7.23 (m, 12H, ArH), 8.06–8.18 (m, 3H, ArH), 8.73 (br s, 1H, ArH), 8.95 (br s, 3H, NH); ^{13}C NMR (CD_3OD): 23.17, 26.13, 29.82, 30.97, 35.17, 38.94, 41.88, 57.25, 67.92, 68.85, 69.42, 69.48, 69.59, 69.65, 69.71, 70.56, 71.00, 71.04, 71.61, 71.69, 105.71, 105.95, 114.82, 116.80, 117.76, 126.95, 132.28, 136.70, 141.04, 145.44, 146.57, 148.40, 151.98, 159.39, 169.58; MALDI-MS (negative mode): $[M]^-\cdot K^+$ 2036.76 obtained for $C_{99}H_{146}KN_5O_{37}$ (2035.93).

Radiolabelling

Radiolabelling was achieved through the stannous chloride reduction method. The reduction reaction was carried out in water using stannous chloride as the reducing agent. One must then consider that the following ratios [Red]/[TcO_4^-] and [L]/[TcO_4^-] have to be very high in order to observe the complete reduction of pertechnetate and reach the complexation of the reduced form with a notable performance. Indeed, the lower the ratio [Red]/[TcO_4^-], the higher the percentage of reduced but not complexed nor reduced technetium. Reducing TcO_4^- by Sn^{2+} in the presence of DTPA involves a four electron mechanism and a +3 predominant valence state for technetium. Note that the net charge of such a Tc-DTPA complex, for pH values between 4.6 and 7.0, is −2. In our case, as one of the DTPA-carboxylate functions is engaged in the connection with the dendritic part, the net charge of the complex 2 is therefore −1.

The catecholamide tripod studied here, possessing hard donor atoms (oxygen) could stabilize technetium(III) into hexacoordinated compounds with more or less distorted octahedral geometries by analogy with iron.[13]

Ligands **16** (1.7 mg) and **17** (4.5 mg) were dissolved in water (2 mL). A solution (250 μL) of stannous chloride (1 mg mL^{-1}, 1.3 μmol) in 0.1M hydrochloric acid was first added to the above solution and then the $^{99m}Tc(VII)O_4^-$ solution (220 MBq mL^{-1}). 25 μL of a 1 N aqueous solution of NaOH (25 μmol) and 200 μL of an aqueous solution of sodium ascorbate (150 mM) were also added in order to buffer the

reaction mixture at pH ≅ 7 and to keep a low redox potential. The resulting mixture was then stirred at room temperature for 15 min.

Determination of the radiochemical yield

Radiochemical yield was determined by instant thin layer chromatography (ITLC): the reaction mixture was spotted at 2 cm from the lower end of the paper and then the strips were developed up to 9.5 cm using methylethylketone (MEK), dried, and counted in a Bioscan Miniscan counter linked to a Bioscan flow count and to a radiochromatogram (LKB Broma 2210, 2-channel recorder, 1 mm s^{-1}). The amount of free pertechnetate was calculated following the equation below.

$$\%\ \text{of free}\ ^{99m}TcO_4^- = (\text{activity at}\ R_f\ 1.0/\text{total activity})*100$$

Stability tests

After 45 min radiolabelling in warm water (35 °C), 600 μL of the resulting mixtures was divided into 2 samples: 300 μL poured into 1 mL PBS; 300 μL poured into 1 mL BSA.

In vitro toxicity studies

Rat pups were sacrificed by decapitation at 7 d of age and neurons were isolated from the cerebellum and plated in multi-well plates coated with poly-D-lysine. The plates were incubated for 3 d to allow cell maturation to continue to some extent. At DIV 3 (day *in vitro* 3), the cells were exposed to 0.0001–10 μM of the test products, to 0.001–1% TritonX100 or to 0.001–100 nM of β-bungarotoxin. β-Bungarotoxin is a known neurotoxin that is not expected to decrease cell counts but has a striking negative effect on neuron specific parameter. The cells were treated with the compounds for 2 d and then analysed as follows. *Measurement of the cell leakage resulting from membrane damage*: lactate dehydrogenase (LDH) is normally a cytoplasmic enzyme. When the integrity of the plasma membrane is compromised, it leaks into the culture medium, where it can be measured by a colorimetric assay (Roche Applied Science, cytotoxicity detection kit (LDH Assay), cat. No 11 644 793 001). As a positive control for the test, a few test wells in the plate were exposed to a strong detergent, 1% Triton X100, for 30 min (thus causing immediate and measurable LDH release), then 120 μl of culture media were collected from all the wells and analysed. *Immunostaining of the cells*: the cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X100, blocked with 5% BSA (bovine serum albumin) and incubated with an antibody for neuron specific beta III tubulin (RandD). The secondary antibody used is a TRIC-labelled anti-mouse antibody. The nuclei were stained with DAPI or Hoechst 33342. *High cell content analysis* (Array Scan system, Cellomics Thermofisher): an imaging technique that uses fluorescent probes and automated microscopy to quantify multiple cellular markers at the single-cell level. Fluorescence microscopy coupled to electronic imaging allows quantification of many cell specific parameters, such as nucleus counts, intensity, size or shape of nuclei, quantification of signals in the cytoplasm or specific organelles (*e.g.* neuritis). The Array Scan system is based on an automated fluorescence microscope that acquires images from cells plated in multi-well plates. This instrument measures intensity and localization of fluorescence signals associated to single cells, over a wide cell population. The image analysis is performed for up to 6 fluorescence channels. The advantage of the approach resides in the possibility to acquire data from either individual cells or from population of cells within a single analysis.

Ligands **16** and **17** were suspended in 5% DMSO at a stock concentration of 500 mM. A 10-fold serial working dilution down to 0.05 mM was performed in 5% DMSO. 4 μl of each working dilution was applied to the cells covered by 200 μl culture medium, *i.e.* a 50× further dilution. In summary, the cells were exposed to the following final concentrations of product: 10, 1, 0.1, 0.01 and 0.0001 μM. As a control, β-bungarotoxin (neurotoxic) was included on each plate of cells at concentrations ranging from 100 down to 0.001 nM. In addition, cells were also exposed to a dilution range of TritonX100 (1% down to 0.0001%), a detergent expected to deteriorate cell membranes.

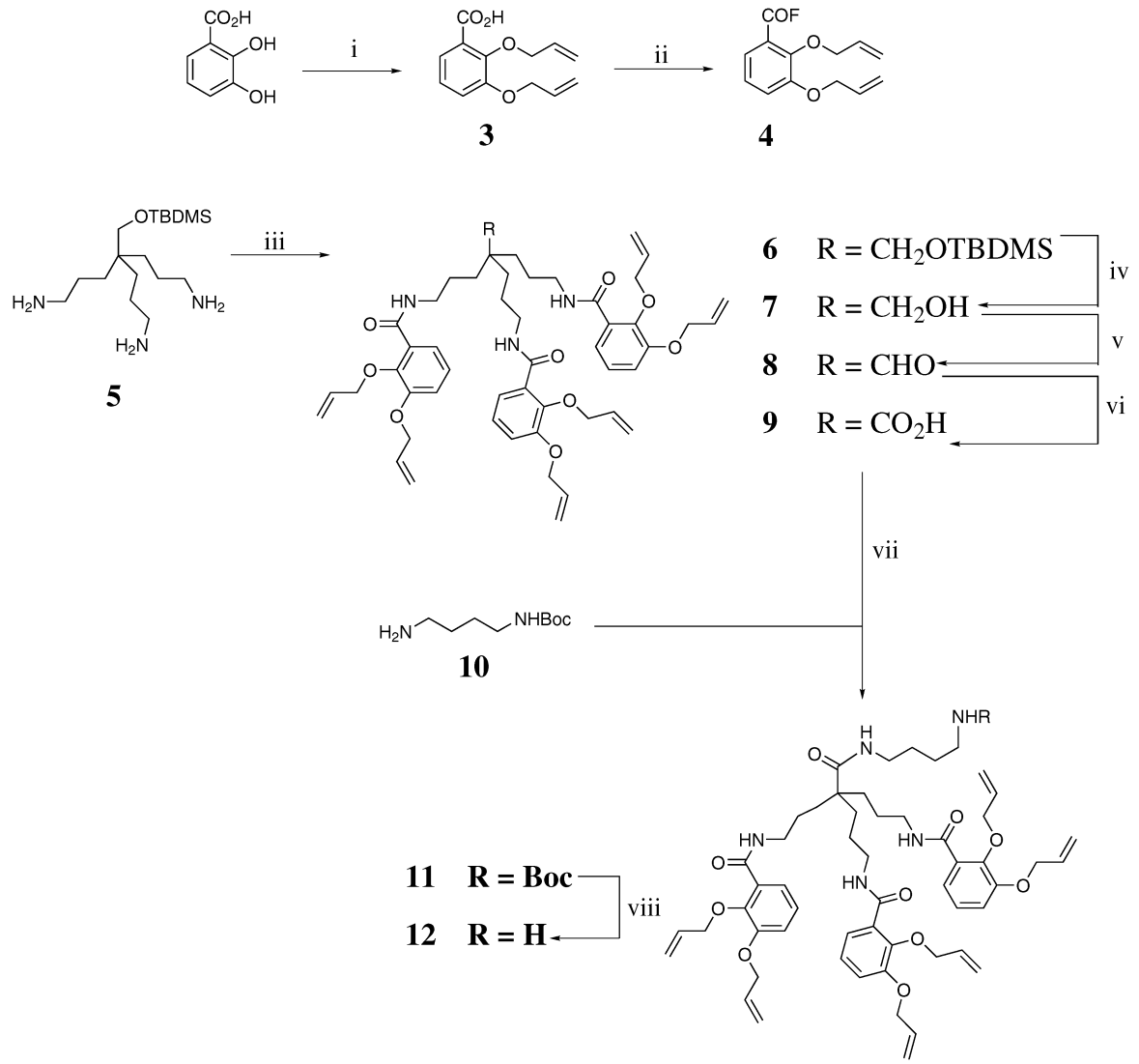
Results and discussion

Synthetic strategy

J.-L. Pierre and co-workers previously reported hydrosoluble TRENCAM[14] derivatives based on three catechol subunits connected to a tris(2-aminoethyl)amine (TREN) framework *via* an amide linkage in the *ortho* position of their catechol groups, in which the three catechol subunits were sulfonated in position 5.[15] However, we wanted to avoid any problems resulting from a highly ionic environment associated with sulfonate groups around the coordination sphere and the inability to cross over the lipophilic membranes. It seemed that grafting a water-soluble oligoethyleneglycol dendron at the focal point of the tripodal structure could be a means to obtain highly hydrophilic compounds. CacCAM,[16] synthesized by attachment of three catecholamide subunits to a CO_2H-functionalized triamine backbone, then allowing a functionalization of the quaternary carbon located at the focal point of the tripod precursor, is more adapted to our study than TRENCAM.[13] Moreover, CacCAM[16] allows a greater flexibility all around the coordination sphere thanks to the TREN spacer length used.

The synthesis of the first basic synthon, namely tripod **5**, displaying a protected alcohol and three amine functions has already been reported in the literature.[10] We then prepared the second basic synthon **4** derived from catechol (Scheme 1).

At first, a specific protecting group for the phenol functions of the catechol has to be chosen since its deprotection conditions should not induce any degradation of oligoethyleneglycol dendron introduced subsequently. The alcohol functions of the catechol groups cannot be protected as methyl esters in oligoethyleneglycol-based dendrimers. This is also the case for protections such as O-benzyl, MOM or equivalent (deprotection conditions lead to dendron degradation), acetal (deprotection problems and generation of many secondary products), silyl (has been considered with OTBDMS but remains unusable since TBDMS protection is already used



Scheme 1 Synthetic pathway to allyl-protected catecholamide. Reagents and conditions: (i) 1. K_2CO_3, allyl bromide, CH_3CN, reflux then 2. NaOH, EtOH; (ii) trifluorotriazine, pyridine, dry CH_2Cl_2, 0 °C, rt; (iii) *N*,*N*-diisopropylethylamine, CH_2Cl_2, rt; (iv) TBAF, THF, rt; (v) 1. oxalyl chloride, dry DMSO, dry CH_2Cl_2, −60 to −40 °C then 2. *N*,*N*-diisopropylethylamine, −30 °C to rt; (vi) H_2NSO_3H, $NaClO_2$, THF–water, rt; (vii) DCC, DMAP, HOBt, CH_2Cl_2, rt; (viii) TFA, CH_2Cl_2, rt.

with the amine scaffold) and acetate (not strong enough in the conditions of the subsequent synthetic steps). Thus, allyl protection was undertaken; the treatment of 2,3-dihydroxybenzoic acid in the presence of an excess of potassium carbonate, allyl bromide in acetonitrile led to a tris-allylation. The allylated acid function was then hydrolyzed by NaOH in ethanol to obtain compound **3** in 65% yield. The acid function of **3** was activated by treatment with cyanuric fluoride in the presence of pyridine to give **4** in 90% yield. The reaction of **4** with amino-tripod **5**[10] in the presence of *N*,*N*-diisopropylethylamine in dichloromethane gave compound **6** in 97% yield. After this coupling, the *tert*-butyldimethylsilyl (TBDMS) protecting group of tripod **6** was cleaved by tetra-*n*-butylammonium fluoride (TBAF) in refluxing tetrahydrofuran (THF) to obtain corresponding alcohol **7** in 94% yield.

Then, alcohol **7** was oxidized, leading to intermediate aldehyde **8** through a Swern oxidation. Treated by sulfamic acid and sodium hypochlorite in THF, crude aldehyde **8** led to the carboxylic acid **9** with 58% yield over the two last steps. Note at this point that we decided to introduce a symmetric and flexible spacer between the chelating and the dendritic parts in order to avoid steric effects from the bulky dendritic part on the complexation ability of the tripodal chelate. Thus, monoprotected 1,4-butanediamine **10**[11] was grafted on tripod **9** under routine peptide coupling (DCC, DMAP, HOBt) to give **11** in 97% yield. Finally, the amine function of the spacer was regenerated by the action of trifluoroacetic acid to get **12** with 90% yield.

We previously described the synthesis of highly hydrophilic oligoethyleneglycol dendron **13** and DTPA-derivative **17**[12] (Scheme 2). Tripod **12** was then connected to dendron **14** displaying a carboxylic acid group at the focal point and obtained in 88% yield by hydrolysis of the corresponding methyl ester **13**. Thus, the allyl-protected dendrochelate **15** was

obtained in 72% yield. Finally, the effective total allyl deprotection was achieved in the presence of [tetrakis(triphenyl)-phosphine]palladium ($Pd(PPh_3)_4$), sodium borohydride ($NaBH_4$) and hydrochloric acid, and allowed preparation of ligand **16** with 95% efficiency. All prepared compounds were fully characterized by NMR (^1H and ^{13}C), elemental analysis and mass spectrometry (final ligand). All of those studies were consistent with the proposed molecular structures.

Complexation and *in vitro* stability of the corresponding technetium complexes

In vivo stabilisation of the metal complex is an important issue to address. Indeed, it is well known that besides the thermodynamic stability of a complex, its kinetic stability or inertness is of equal, and sometimes greater, importance considering *in vivo* applications.[17]

Complexation of ligands **16** and **17** to $^{99m}Tc(III)$ was achieved through the tin chloride reduction method starting from a radioactive solution of technetium pertechnetate ($Tc(VII)O_4^-$) in the presence of ascorbic acid. A comparative kinetic monitoring of the two dendritic complexes **1** and **2** has been performed (Fig. 2): at $t = 0$, a radiolabelling yield as high as 96% was reached for the DTPA-derived radiocomplex **2**. Nevertheless, the kinetic marking of radiocomplex **2** showed fast dissociation into $^{99m}Tc(IV)O_2$, reaching 50% after 90 min. Indeed, a commonly admitted advantage of

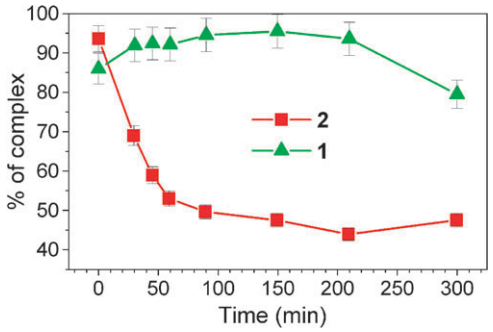
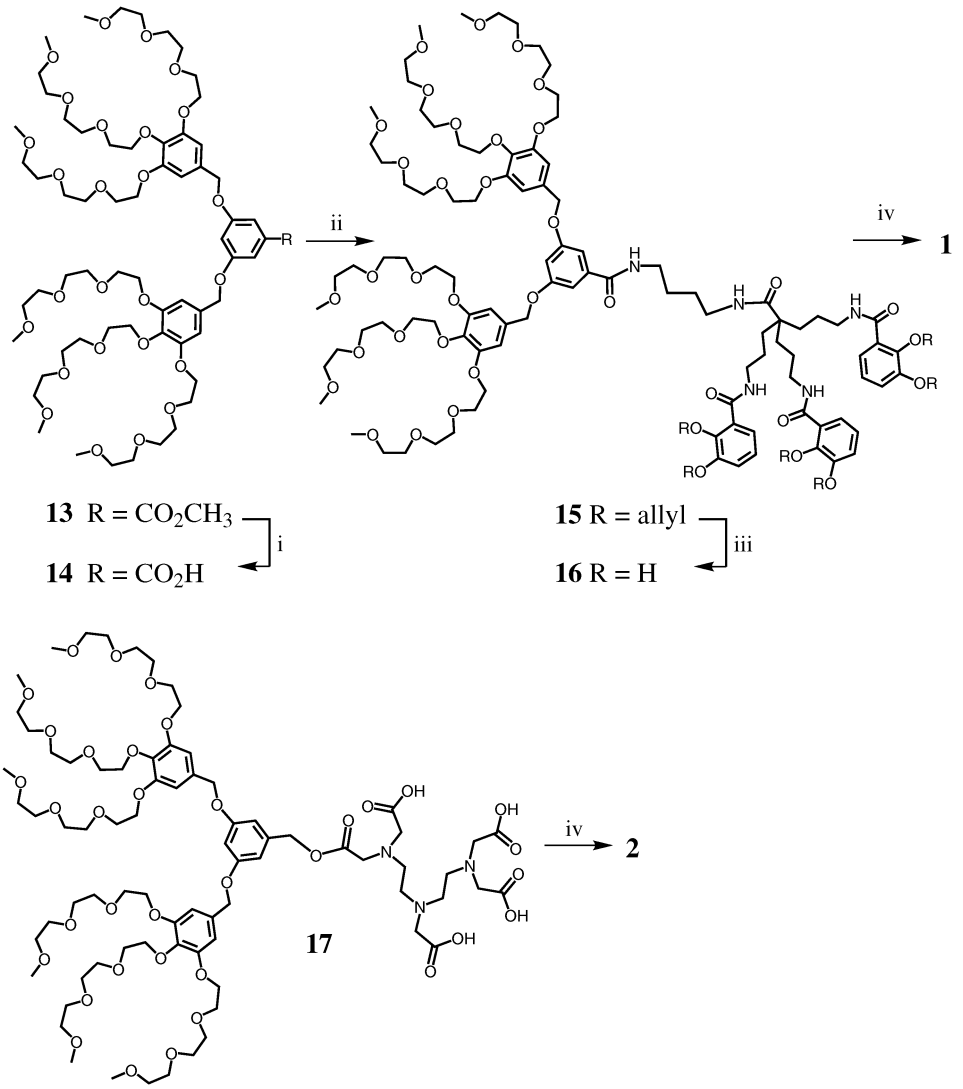


Fig. 2 Stability as a function of time of radiolabelled complexes **1** and **2** measured in buffer PBS (pH 7.4) at 25 °C; error bars of 5%.



Scheme 2 Synthetic pathways to dendritic complexes **1** and **2**. Reagents and conditions: (i) NaOH, THF–water; (ii) **12**, DCC, DMAP, HOBt, dry CH_2Cl_2, rt; (iii) $Pd(PPh_3)_4$, THF, $NaBH_4$, HCl, rt; (iv) $^{99m}TcO_4^-$, $SnCl_2$, ascorbic acid, H_2O, rt.

diethylenetriamine pentaacetic acid (DTPA) analogs is their extremely high radiolabelling efficiency due to high radiolabelling yield under mild conditions, but one must then point out that the kinetic instability of their metal chelates often results in fast dissociation and leads to radiation toxicity towards non-targeted organs such as bone marrow.[18] In contrast to DTPA, compound **16**, displaying a tripodal frame, is a candidate of choice for obtaining stable technetium complexes. Indeed, the inclusion of aromatic rings in the chelation sphere provides a better structural rigidity and allows a pre-organization of the hexadentate ligand.[13]

As shown on Fig. 2, at $t = 0$, the radiolabelled tripodal complex **1** induced a complexation rate as high as 88% increasing over time before stabilizing at around 93% and it then seems reasonable to say that complexation of **1** was effective after 45 min. Thus, the radiolabelling yield induced by radiocomplex **1** was not significantly lower than that of radiocomplex **2**, but the key result is that kinetic stability of **1** was, as expected, much more important than **2**. Indeed, over 80% of **1** remained stable during the half-life of the radionucleide (6.02 h for ^{99m}Tc).

Taking into account that the complexation of **1** is effective (reaches the highest value of 93%) after 45 min in PBS (Fig. 2), we performed stability tests of complex **1** in PBS and BSA at room temperature after this complexation period and showed that the complex stays stable in both physiological media during almost 3 h (Fig. 3).

In vitro toxicity

Cells used in our *in vitro* toxicity tests were rat cerebellar neurons isolated from 7 day-old pups. These cells are useful tools for toxicity tests as they are primary cells, unmodified and without any genomic aberration. They represent a great intermediate between cell lines and tests on tissue sections or *in vivo* tests. The assay is proposed to be used for analysing compounds aimed at treatment of any disease, in case of possible brain penetration, or in case the target protein or a protein closely related to the target is expressed in the central nervous system (CNS). More generally, the assay can be proposed if there is a concern about potential neurotoxic effects.

In terms of *in vitro* toxicity, ligand **16** showed no detectable toxicity when tested in LDH release assays (Fig. 4); applied to cultured rat neurons at concentrations ranging from 10 μM to

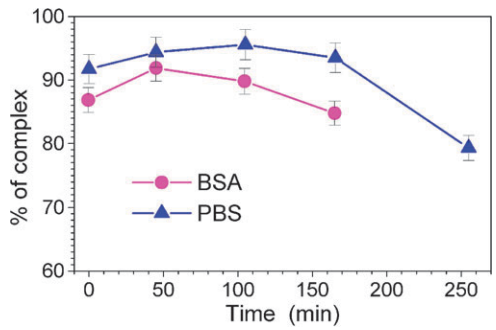


Fig. 3 Evolution of the stability of complex **1** as a function of time, in phosphate buffer saline (PBS) and in Bovine serum albumin (BSA) at 25 °C; error bars of 5%.

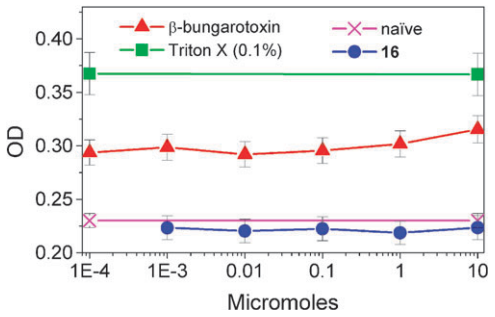


Fig. 4 LDH efflux. Measurements of the cell leakage resulting from membrane damage of neuronal cells treated with increasing concentrations of dendritic ligand **16** compared to venom-treated (β-bungarotoxin and Triton X) and untreated cells (naïve).

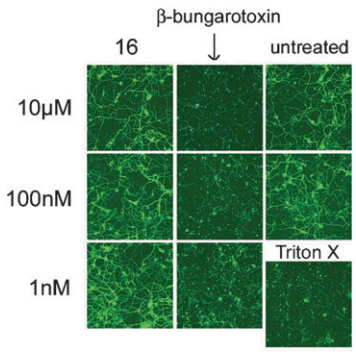


Fig. 5 Pictures (1024 μm^2) of treated neurons after staining for dendritic ligands **16**, **17**, together with venom- (β-bungarotoxin, Triton X) and untreated neurons.

0.1 nm, **16** did not elicit LDH release, suggesting that the compound does not compromise cell membrane integrity.

Moreover, analysis of the neuritic network by staining revealed that treatment with dendritic ligand **16** did not alter neuron's ability to differentiate (Fig. 5): even at the highest concentration of **16**, neurites are similar in their features to untreated neurons.

Conclusions

In conclusion, these data show that a more rigid and pre-organized dendrochelate **1** derived from a tris-catecholamide core possess a higher kinetic stability in physiological media and can therefore be considered as a viable alternative to the DTPA-derived technetium complexes currently used for single photon emission computed tomography (SPECT). Further investigation concerning *in vivo* stability are under way in our laboratory. Moreover, extension of the dendritic approach by grafting L-dopamine to the dendron periphery will allow the elaboration of brain-targeting radiopharmaceuticals. Work along these lines is also currently ongoing in our laboratory.

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