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Preparation of phosphorylated bis-pyrazolyl–pyridine nonadendate ligands and their terbium complexes. Part 2

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ABSTRACT

A series of ligands has been constructed from a central bis-pyrazolyl–pyridine core and various deprotonable chelating pockets based on mixed carboxylate/phosphate or phosphate anionic functions and a central flexible pendent arm for subsequent grafting to biomaterials. For some of these ligands, the sequence of reactions incorporates an additional step introducing a substituted diethynylphenyl residue to increase significantly their solubility in polar organic solvents. The terbium(III) complexes of some of these ligands display outstanding spectroscopic properties with lifetimes ranging from 2.7 to 3.2 ms and quantum yields from 16% to 26% in water.

Current luminescence imaging techniques employing highly sensitive detection methods and sophisticated probes have been successively exploited for the development of analytical techniques. These include multiplexing assays for the simultaneous determination of an analyte in several samples and/or different analytes in the same sample.[1] Luminescence imaging has also been extensively used to access the spatial distribution of a given target molecule in chemical or biochemical processes in macro- and microsamples, and to conduct the in vivo evaluation of biological and pathological processes.[1,2]

All such studies depend fundamentally on the design and molecular architecture of the luminescence probe used to tackle the problem. It is generally considered that lanthanide (Ln) binding provides the underpinning for luminescent labels in fluoroimmunoassays.[3] The workhorses in the field are europium(III) and terbium(III) complexes which are luminescent in aqueous solution if certain conditions are fulfilled: (i) the ligand should include a highly absorbing chromophore (since f→f transitions of simple solvated lanthanide ions have extremely small absorption coefficients), (ii) energy transfer from the ligand-centred excited states to the Ln centre should be fast and efficient, (iii) water must be largely excluded from the first coordination sphere, since detrimental non-radiative deactivation pathways for the excited Ln atoms are promoted by the vibrational modes of any bound solvent molecules.[4] The antenna effect,[5] represented by a combination of requirements (i) and (ii), makes the ligand design crucial for the photophysical properties of the resulting complexes. Amongst the first systems satisfying these criteria to be investigated were the europium(III) and terbium(III) complexes of oligopyridine-derived ligands including cryptands, branched macrocycles and podands.[6]

If luminescent lanthanide chelates are to be used as tags in more advanced technologies such as homogeneous assays, fluorescence imaging, immuno-histochemistry, or in situ hybridization techniques, additional strict requirements arise: (iv) high thermodynamic and kinetic stability, (v) hydrophilicity, (vi) very efficient cation emission and high absorption at suitable wavelengths, (vii) a chemical structure allowing covalent linkage of the label to the target biomolecule. As a consequence of requirements (i) to (vi) for the optimal stability and luminescence properties, only a few viable labels have to date been developed and tested in biomedical analysis protocols.

It is usually considered that lanthanide coordination occurs predominantly via ionic bonding interactions,[7] leading to a strong preference for negatively charged donor groups. The advantages of using negatively charged donor groups are well-illustrated in the use of the tetraanionic ligand DOTA to form Gd(III) complexes for nuclear magnetic resonance imaging, this tetrakis(carboxylatomethyl) derivative of a macrocyclic tetramine providing not only a complex of the required kinetic and thermodynamic stability but also engendering hydrophilicity due to the overall negative charge of that complex. However, the synthesis of this kind of

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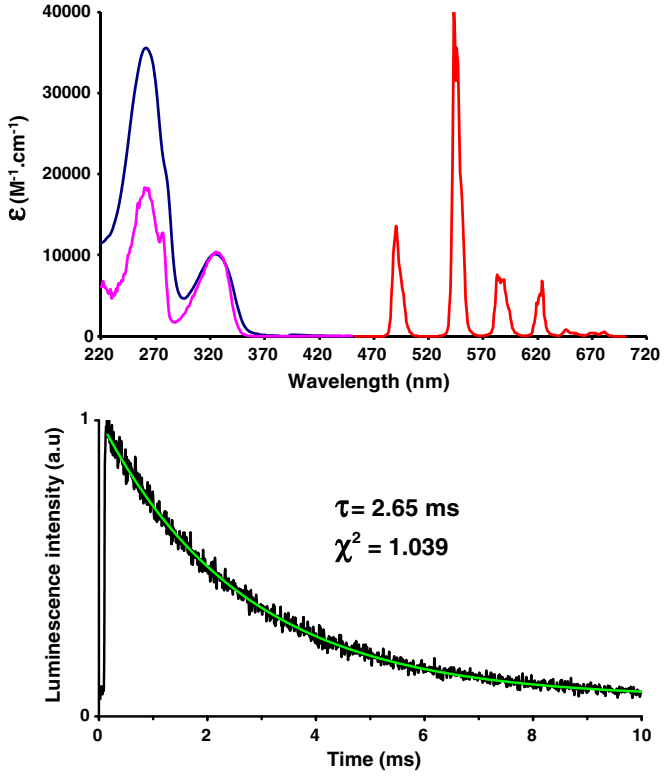


Figure 1. Top: absorption (blue line), emission (red line) excitation (plum-red line) of complex **18** ($c = 6.9 \times 10^{-5}$ M in buffer tris/HCl, 0.01 M at pH 7.2). Excitation wavelength 320 nm for the emission spectrum, and emission wavelength at 543 nm for the excitation wavelength. Bottom: deactivation decay of the Tb-excited state lifetime after excitation at 320 nm.

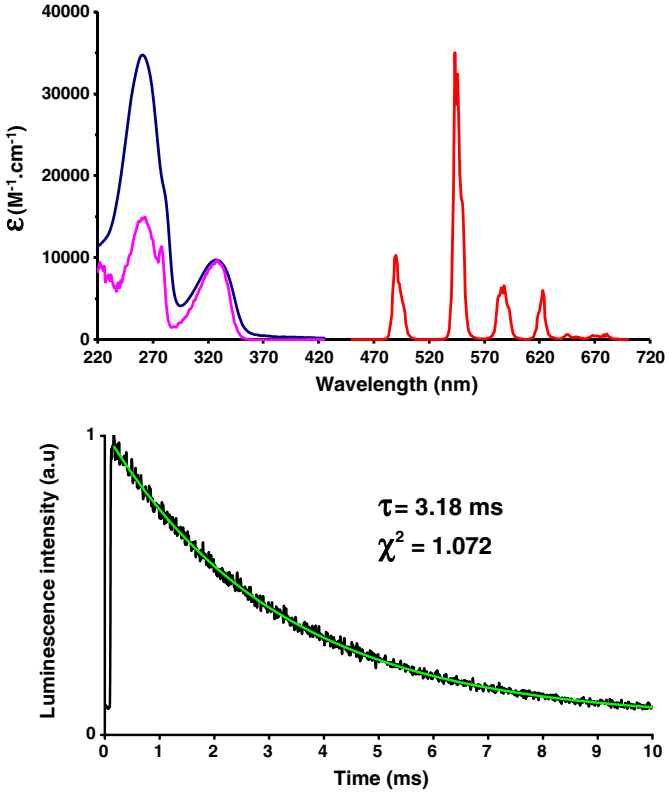
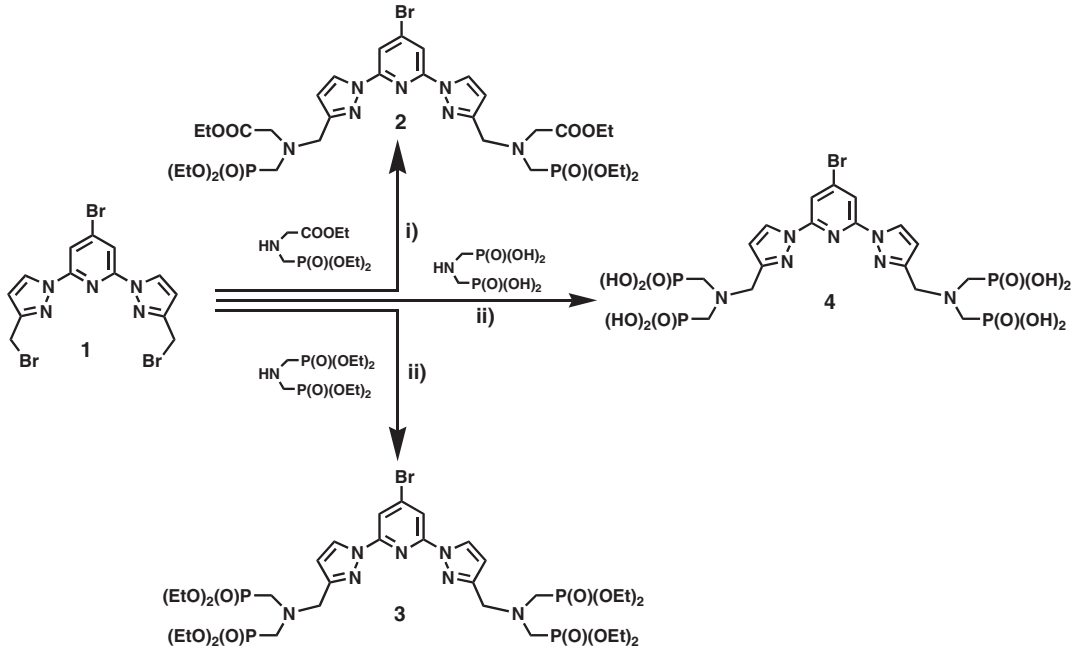


Figure 2. Top: absorption (blue line), emission (red line) excitation (plum-red line) of complex **19** ($c = 5.77 \times 10^{-5}$ M in buffer tris/HCl, 0.01 M at pH 7.2). Excitation wavelength 320 nm for the emission spectrum, and emission wavelength at 543 nm for the excitation wavelength. Bottom: Deactivation decay of the Tb-excited state lifetime after excitation at 320 nm.

molecule is challenging and often requires linear multi-step protocols,[8–10] so that a facile method for the introduction of charged substituents continues to be an attractive objective. The importance of phosphates for the stabilisation and solubilisation of numerous biomolecules suggested to us that the phosphate entity or its derivatives would be a desirable substituent of ligands designed for lanthanide ion exploitation in aqueous media. We have pursued this opinion through the synthesis of multiply



Scheme 1. Key: (i) anhydrous acetonitrile, anhydrous K_2CO_3, 60 °C overnight, 59%; (ii) anhydrous acetonitrile, anhydrous K_2CO_3, 80 °C, overnight, 65% yield for **3** and 60% for **4**.

phosphonated ligands, specifically avoiding an approach based on the functionalization of less readily accessible macrocyclic species.

Our synthesis began with nucleophilic substitution at both bromomethyl groups of **1**[11] with either glyphosate, amino-di(methylenediethylphosphite), or amino-di(methylenephosphinic acid) under conditions previously described for pyridine derivatives (Scheme 1).[12]

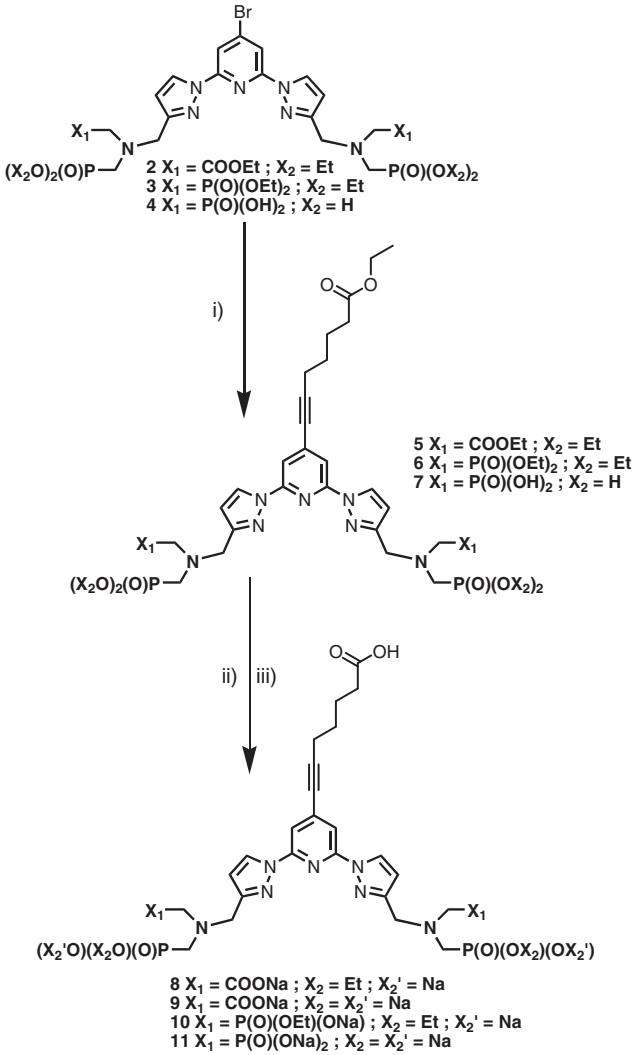
Cross-coupling of derivatives **2**, **3** and **4** with ethyl 6-heptynoate allows the linkage to the chelation pocket to a short flexible chain carrying a protected carboxylic acid for the possible use of this group in bioconjugation (Scheme 2). For derivatives **5** and **6**, simultaneous hydrolysis of both the carboxylate and one of the phosphonate ester groups proceeds using the appropriate amount of NaOH in a water/THF mixture leading, respectively, to compounds **8** and **9**. The ligands (as the sodium salts), could be isolated by precipitation with diethylether at neutral pH. For compound **7**, saponification of the ester is achieved in a methanol/water solution in the presence of NaOH (6 equiv).

Along these lines we succeeded on connecting 2,2′-(ethylenedioxy)bis(ethylamine) under the carboamidation conditions under a flux of CO in the para position of the central pyridine (Scheme 3).[13] The resulting derivatives **12** to **13** were allowed to react under Schotten-Bauman peptidic coupling reaction with D-(+)-biotin allowing to isolated the biotinylated derivatives **15**–**17** in acceptable yields.

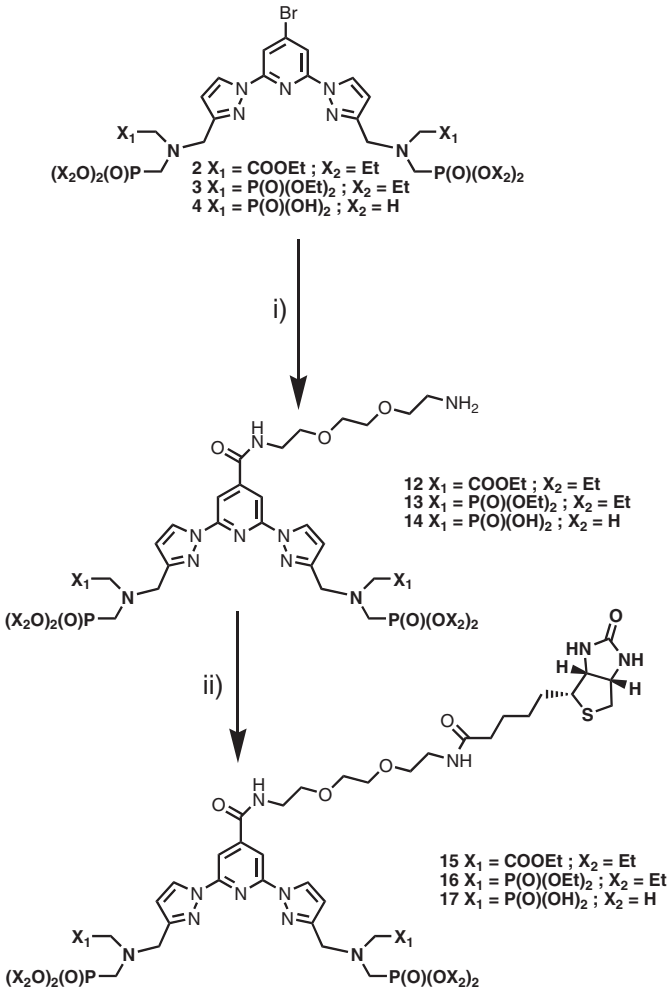
Terbium complexes of ligands **9** and **11** were prepared by first dissolving the metal-free ligand in water at pH 7.2 (about 1 mM), then titrating in an aqueous solution of $TbCl_3{\cdot}6H_2O$ (about 10 mM). Addition was stopped at the first sign of precipitate formation (near 1:1 composition). After stirring the mixture for 1 h, it was filtered and the complex was precipitated from the filtrate by addition of methanol and diethylether.

The UV–vis absorption spectra of complexes **11** and **12** are dominated by intense $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transitions centred on the pyridyl[14] and pyrazolyl[15] groups (Fig. 1). As would be expected by the lack of unsaturation in the pendent arms, the absorption spectra are not sensitive to the nature of the deprotonable donor groups (phosphate or carboxylate). The absorption band around 261 nm is attributed to a transition located on the pyrazolyl fragments. The high energy absorption band about 325 nm is assigned as a pyridyl-centered transition and its intensity is sensitive to the presence of substituents in the para position.

For both complexes (Chart 1), excitation at 320 nm resulted in the characteristic emission pattern of Tb(III) (Fig. 1 and 2).[16] The Tb emission spectrum consists of four intense emission bands with maxima at 490, 543, 583 and 624 nm, attributed to the $^5D_4 \rightarrow {}^7F_J$



Scheme 2. Key: (i) ethyl 6-heptynoate (1.2 equiv), THF/TEA, $[Pd(PPh_3)_4]$ (6 mol %), CuI (10 mol %), 60 °C, 74% for **5**, 78% for **6** and 69% for **7**; (ii) THF, NaOH (9 equiv), rt, overnight except for **9** for which key (iii) applies; (iii) TMSBr in anhydrous dichloromethane and anhydrous 2,6-lutidine, rt followed by THF/water, NaOH (excess), rt, overnight. In all cases, isolated yields were around 85–92%.



Scheme 3. Key: (i) 2,2′-(ethylenedioxy)bis(ethylamine) (2 equiv), $[Pd(PPh_3)_2Cl_2]$ (12 mol %), benzene, TEA, CO flux at 1 atm, 70 °C, one night, 62% for **5**, 58% for **6** and 55% for **7**; (ii) D-(+)-biotin (1.5 equiv), THF, EDCI, DMAP, 2h., rt, 75% for **8**, 71% for **9** and 65% for **10**.

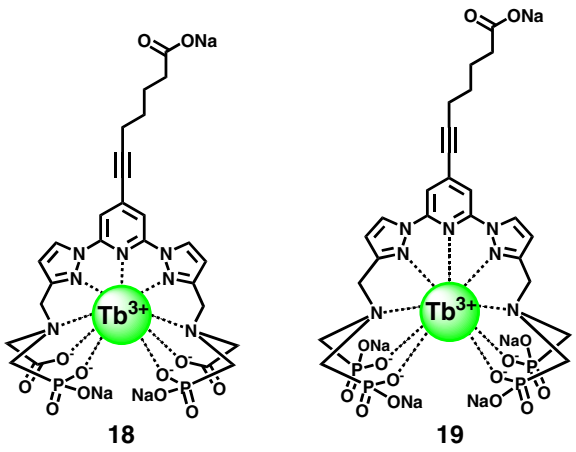


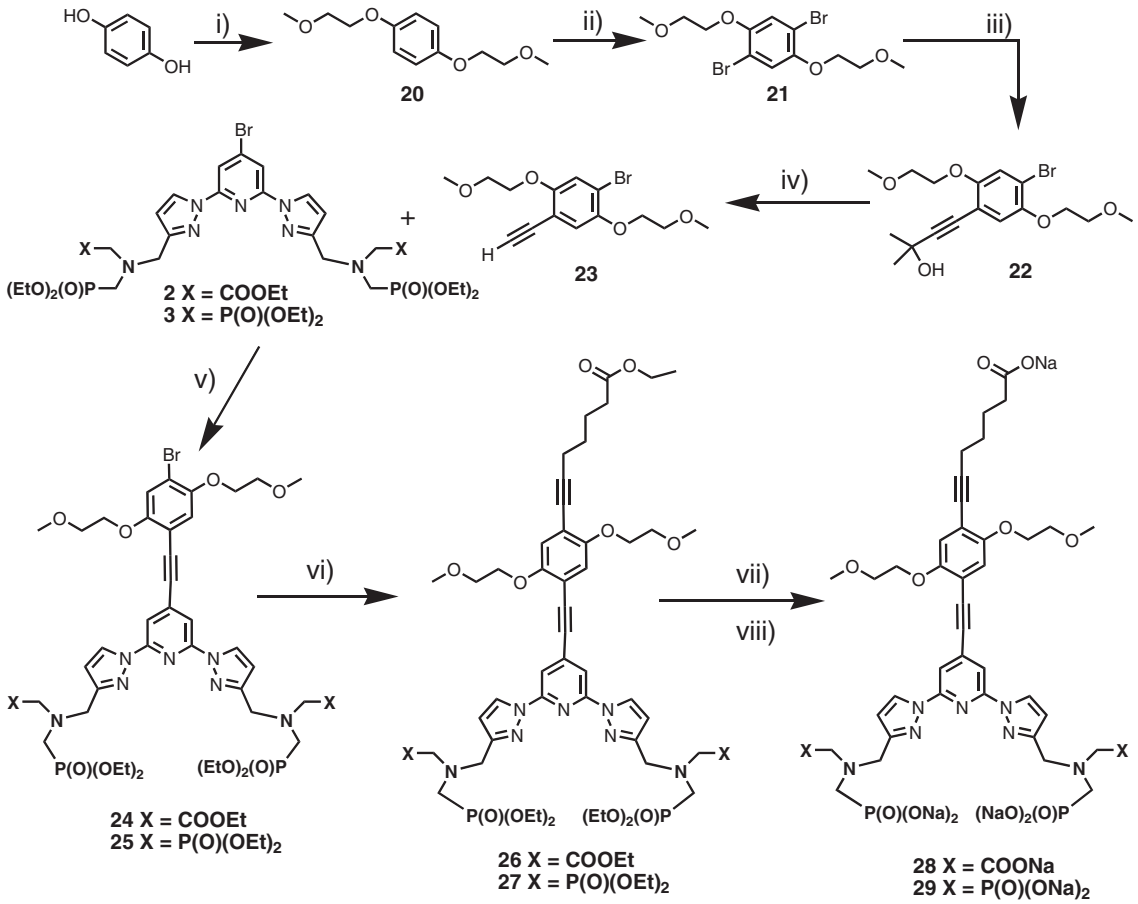
Chart 1. Chemical formula of the terbium complex highlighting the complexation pocket and the hanging side-arm.

transitions with $J = 6–3$, respectively,[17] and weaker bands between 643 and 681 nm corresponding to $J = 2–0$. As seen from the corresponding excitation spectra, the excitation in the UV domain can be directly correlated with the absorption spectra of the complexes as a result of an efficient ligand to metal energy transfer.[18] Note that the excitation matches the first absorption at 325 nm but not the second around 261 nm. This may indicate that in the excited state the pyridine–terbium interaction flattens the coordination sphere of the complex and distorts the pyrazole-terbium interaction in such a way that the energy transfer from the pyrazole part is not as efficient as from the pyridine part. For both complexes, the excited state decay can be fitted to a single exponential decay, with exceptionally long excited state lifetimes of 2.7 and 3.2 ms, respectively, for complexes **11** and **12**. The luminescence quantum yields are rather good at 26% and 16% in aqueous solution.

Again for both terbium complexes, the hydration number was estimated to be close to zero by determining the excited state lifetimes in D_2O and thus is indicative of a saturated coordination sphere lacking any water molecules in the first coordination sphere of the terbium. This is in keeping with a perfect shielding of the metal by the nonadentate ligand. The quantum yields increased to 35% and 30% in D_2O for complexes **18** and **19**, respectively.

Some difficulties emerged when we tried to prepare the succinic ester from the pendent carboxylic function. These complexes are not soluble in organic solvents or in mixtures of water and DMF or *N*-methyl-2-pyrrolidone (NMP). Despite the fact that the activation of carboxylic acids in water with NHS has been reported,[19] we were unable to prepare the targets under standard conditions.

We therefore, decided to construct a ligand with a phenylpolyoxoethylene chain spanning the pyridine bis-pyrazole complexation pocket and the anchoring function. The idea was to enhance the lipophilicity of the molecule by introducing a substituted diethynylphenyl module, thereby increasing the solubility of the complexes in DMF/water for the activation procedure. This is illustrated in Scheme 4.



Scheme 4. Keys: (i) $CH_3OCH_2CH_2OTs$, K_2CO_3, CH_3CN, 80 °C, overnight, 67%; (ii) Br_2, CCl_4, reflux, overnight, 64%; (iii) 2-methyl-3-butyn-2-ol (1.1 equiv), THF/TEA, $[Pd(PPh_3)_4]$ (6 mol %), CuI (10 mol %), 60 °C, 40%; (iv) NaOH (10 equiv), THF/methanol, rt, 93%; (v) THF/TEA, $[Pd(PPh_3)_4]$ (6 mol %), 80 °C, 74%; (vi) ethyl 6-heptynoate (1.2 equiv), THF/TEA, $[Pd(PPh_3)_2]$ (6 mol %), CuI (10 mol %), 50 °C, 80% for compound **18** and 75% for compound **19**; (vii) TMSBr, anhydrous dichloromethane and anhydrous 2,6-lutidine; (viii) THF, methanol, NaOH (8 equiv), 50 °C.

Alkylation of hydroquinone with a tosylate salt under basic conditions provides derivative **20** in 67% yield. Bromination in CCl_4 under reflux provided **21** in 64% isolated yield. The use of 2-methyl-3-butyn-2-ol in 1:1 stoichiometry enabled the preparation of derivative **22** in 40% yield through a statistical cross-coupling reaction promoted by Pd(0) generated in situ with Cu(I) salts. Chromatographic separation from the starting material and di-substituted derivative was facilitated by the presence of the polar alcohol function. After deprotection, the terminal alkyne **23** was cross-coupled to the preformed platforms **2** and **3** under our standard conditions, leading to derivatives **24** and **25** in acceptable yields. Grafting of ethyl 6-heptynoate generates ligands **26** and **27** containing pockets with at least 9 donor atoms (5N + 4O). A two step hydrolysis of the phosphonate with TMSBr and the ethylcarboxylate with NaOH provided the anionic ligands **28** and **29** in fair yields. Interestingly, these ligands as expected are soluble in water and partially soluble in organic solvents like DMF, NMP and DMSO. This solubility is sufficient for production of an activated *N*-hydroxysuccinimide (NHS) ester.

In summary, the present investigation describes the preparation of novel phosphonylated nonadentate ligands constructed around a bis-pyrazolyl–pyridine framework. One of their particular attributes, in addition to the generation of water soluble lanthanide ion complexes, is the exclusion of water from the coordination sphere of Tb(III) in aqueous media. The efficient and relatively simple syntheses offer many opportunities for subtle variations in the ligand structure which might be exploited for practical applications of their complexes. Full description of the spectroscopic properties and application of the complexes in bioanalysis will be disclosed in forthcoming papers.

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