Functionalized Linear Tetra-aza Ligands for Complexing Technetium: Potential Species for Antibody Labelling

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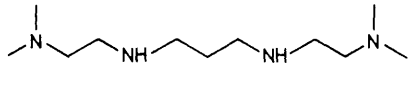
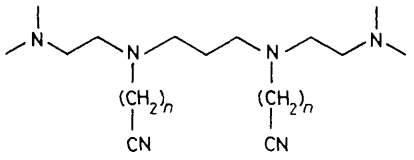
Functionalized tetra-aza ligands have been selectively attached to *p*-^{131}iodo-benzylamine, a model substance of lysine in monoclonal antibodies; p*K*-values and stability constants of the tetra-aza-dicarbonic acids have been determined.

There is a growing recognition of the potential of radioactive metal complexes which irreversibly bind to tumour localizing monoclonal antibodies for use in tumour imaging and radioimmunotherapy.[1,2,3] In such a discipline, it is essential that the radiolabel does not dissociate from the antibody conjugate over a period of days. In the special case of the γ-emitting radioisotope ^{99m}Tc (half life 6 h) sufficient stability is obtained if no decomposition products are observed within one day after preparation.[4] ^{99m}Tc is available as TcO_4^- and after reduction forms stable complexes with 2,12-dimethyl-2,5,9,12-tetra-azatridecane. This bifunctional ligand was chosen because it is easily coupled to proteins after activation

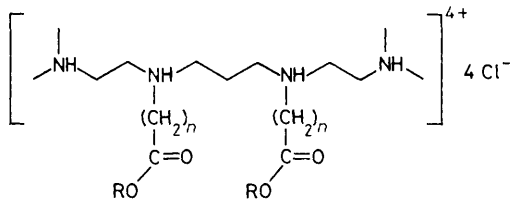


(1)



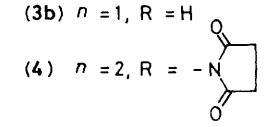
(2a) $n = 2$

(2b) $n = 1$



(3a) $n = 2$, R = H

(3b) $n = 1$, R = H



(4) $n = 2$, R = -N

of the acids by *N*-hydroxysuccinimide (NHS). Because of the difficulties in determining the coupling yield, *p*-^{131}iodobenzylamine (IBA) was chosen as a test substance. The aromatic ring is easily labelled and the molecule contains a primary aliphatic amine.

The concentration of IBA was chosen to be in the same order of magnitude as the amine groups of proteins, and physiological phosphate buffer was used as a solvent. Although the pK_a value of IBA is lower than that of lysine in proteins, the whole system provides a simple model in order to investigate the ability to couple this ligand to proteins.

Reaction of 1,3-dibromopropane with 1,1-dimethyl-ethylenediamine in ethanol and KOH afforded 2,12-dimethyl-2,5,9,12-tetra-azatridecane (**1**) (30%, b.p. 62 °C/0.01 mmHg) which was purified by 'Spaltrohrkolonnen'† distillation (a system for a very effective fractionated distillation). The addition of (**1**) to 2 equiv. of acrylonitrile in water during several days in the dark yielded (**2a**);‡ the reaction with hydroxyacetonitrile yielded the analogue (**2b**).[5,6] Hydrolysis of the nitriles with boiling hydrochloric acid afforded the related tetra-aza-dipropionic acid (**3a**) and diacetic acid (**3b**) respectively which could be isolated, by removal of the solvent, as extremely hygroscopic, white salts.‡ They are insoluble in nearly all organic solvents, slightly soluble in dimethylformamide (DMF) and methanol, and extremely soluble in water. All nitrogen atoms of (**3a**) are tertiary and are unreactive to activated carboxylic esters.

In order to link (**3a**) to an antibody, the active NHS-ester of (**3a**) was synthesized.[7] The NHS-derivate (**4**) was obtained by stirring (**3a**) with NHS and dicyclohexylcarbodiimide in dry DMF for several hours. It was isolated as a white, hygroscopic solid by precipitation from the filtered DMF solution with absolute ethyl acetate and stored under hexane.

Such activated compounds have been shown to undergo hydrolysis very slowly as compared with aminolysis[8] and thus have the potential of labelling the lysines in proteins in good yield, even in aqueous solutions. The general coupling conditions have been chosen as follows; ^{131}iodobenzylamine (IBA, 3.7×10^7 Bq/mg) was dissolved in a 0.2 M phosphate buffer of pH 7.4.

Hexane was eliminated under a stream of nitrogen before the activated ligand (**4**) was dissolved in the buffer and added to the IBA solution. The relative amounts are given in Table 1. Incubation was carried out over a period of 2 h at 25 °C. Product distribution was checked with t.l.c. (Kieselgel, EtOH/EtOAc 1 : 1). A sharp peak at $R_f = 0.20$ was attributed to unreacted IBA, a peak at $R_f = 0.05$ to the 1 : 1 product, and a third peak at $R_f = 0.55$ represented the 1 : 2 product. The 1 : 1 and 1 : 2 values represent the molar ratio, (**4**): IBA, of the products and can be attributed by variation of the molar ratio of educts. Table 1 gives the total percentage of coupled IBA as a function of the ratio (**4**): IBA.

It can be seen that IBA couples nearly quantitatively to (**4**) if the latter is present in large excess. Assuming an antibody with M_r of 150 000, a concentration of 3 mg/ml and 30 lysines present in the molecule, the conditions for connecting (**4**) to the antibody would be roughly the same as in our model system. In the best case, 3 to 20 molecules of ligand per antibody could be coupled. The tertiary and quaternary structure of the protein sometimes conceals the lysine amines, which are inaccessible for coupling reactions. In most cases, the exact structure of the antibody is not known and therefore the number of coupled ligands cannot be predicted. The tetra-aza ligand may be labelled with ^{99m}Tc subsequent to antibody conjugation but there is the inherent problem of non-specific binding of the metal to the protein.

The stability of the specific bound Tc in the protein is the same as found for the free complex. The nonspecific bound Tc is oxidized to TcO_4^- within two hours. The relative amounts of specific and nonspecific bound Tc vary considerably and therefore are still under investigation. Reaction of (**3a**) (2

Table 1. Percentage of coupled IBA to (**3a**), [IBA] = 6.0×10^{-4} M, pH = 7.4.

Molar ratio (**4**): IBA	Coupled IBA/%
3	10 (1 : 1 and 1 : 2 product)
8	18
16	25
23	42
37	85 (mainly 1 : 1 product)

Table 2. The stability constants of some metal complexes ML (log K_1) and the pK values of MHL$^+$ with L = (**3a**) and (**3b**) (potentiometric titration, $I = 0.1$ M, KNO_3, 25 °C), {K_1 = [ML]/[M][L]}.

	Ligand (**3a**)		Ligand (**3b**)	
	log K_1	pK of MHL$^+$	log K_1	pK of MHL$^+$
Ni^{2+}	7.23	8.2	17.23	5.50
Cu^{2+}	12.72	7.6	>20[a]	7.37
Zn^{2+}	7.32	7.1	13.60	6.48
Cd^{2+}	6.55	8.6	12.58	6.46
Pb^{2+}	7.00	7.3	10.03	7.5
Ca^{2+}	1.9		1.98	

[a] CuL [L = (**3b**)] is too stable for determination by direct titration.

† Spaltrohr-System: Fischer, Bonn - Bad Godesberg.

‡ (**2a**): ^{13}C n.m.r. ($CDCl_3$) δ 119.4, 58.0, 52.2, 52.2, 50.3, 46.1, 25.5, 16.6; (**3a**): ^{13}C n.m.r. (D_2O, pH 0) δ 175.0, 51.2, 50.7, 49.6, 47.8, 43.6, 29.0, 19.1; (**3b**): ^{13}C n.m.r. (D_2O, pH 0) δ 168.6, 54.7, 52.5, 51.4, 49.5, 43.6, 19.3. All new compounds showed no other n.m.r. signals and gave satisfactory microanalytical and mass spectrometry analyses.

μ mol, no protein present) with $^{99m}TcO_4^-$ (1.85×10^8 Bq) at pH 11 in phosphate buffer proceeds to >95% conversion within 2 min after reduction with Sn^{II} tartrate (0.1 μM) in HCl (100 μl, 0.01 M). Solutions of the Tc complexes produced are stable for at least three hours at pH 3—4 and ≥1 day at pH 7, as reported for other ligands containing four nitrogen donor atoms.[9]

Analyses of the Tc compounds with ligands (**3a**) and (**3b**) (with long living ^{99}Tc) suggest different structures. The u.v.–visible spectrum of the yellow Tc complex with (**3a**) is very similar to that of other *trans*-dioxo-Tc^V-tetra-aza complexes. The i.r. spectrum of the solid shows bands at 940 and 820 cm^{-1} which can be assigned to $Tc{=}O^{3+}$ and $O{=}Tc{=}O^+$ stretching frequencies.[10,11] The complex with (**3b**) is violet, has a very different u.v.-visible spectrum, and shows no Tc-oxo bands in the i.r. spectrum. Complex (**3a**) is more suitable for labelling antibodies because it has less tendency to co-ordinate the carboxylates to Tc. Moreover this complex is formed faster than the Tc compound of (**3b**).

The stability constants of Tc^V complexes cannot be determined because of the non-existence of stable aqueous Tc^V species. The stability constants of the given metal ions are important in judging possible metal exchange reactions after intravenous injection of these ligands. Generally the complexes of (**3b**) are more stable than those of (**3a**), because of the co-ordination of the carboxylic group in the former case (Table 2). The p*K*-values of the six-protonated acid H_6L (**3a**) are 1.85, 2.91, 3.97, 5.65, 9.14, and 9.85, those of (**3b**) are ≤0, 1.67, 3.39, 5.43, 9.61, and 10.54. (I = 0.1 M, KNO_3, potentiometric titration).

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