Towards Tumour Imaging with Indium-111 Labelled Macrocycle–Antibody Conjugates

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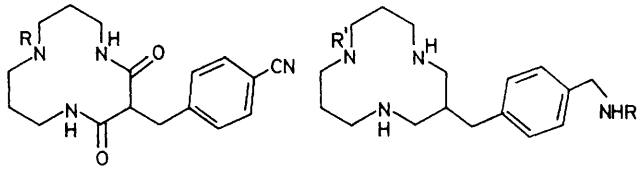
C-Functionalised triazacyclododecane and triazacyclononane triacid macrocycles have been covalently attached to a monoclonal antibody and may be labelled with ^{111}In to form kinetically inert radiolabelled complexes.

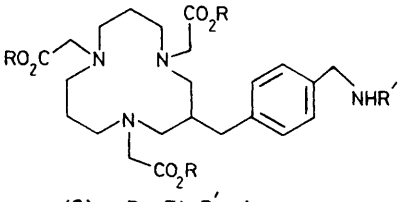
When tumour-localising monoclonal antibodies are radiolabelled for use in tumour imaging, it is essential that the radiolabel does not dissociate from the antibody conjugate over a period of several days. We have therefore sought to use macrocyclic complexes to bind the radiolabel,[1] taking advantage of their slow rate of metal dissociation.[2] For radioimmunoscintigraphy, the γ-emitting radioisotope ^{111}In ($t_{1/2}$ 2.83 days) has been promulgated for use in diagnostic nuclear medicine.[3] In order to bind it effectively, we sought a hexaco-ordinating ligand with three ionisable groups to reduce the effective nuclear charge at the tripositive indium centre. The overall complex is then electrically neutral and is thereby much less sensitive to acid-catalysed dissociation.[4] This is in contrast to In complexes of the modified chelates ethylenediaminetetra-acetic acid (EDTA) and diethylenetriaminepenta-acetic acid (DTPA). Although stable in serum at pH 7.2, they protonate at lower pH (*e.g.* in liver, stomach) and are more susceptible to dissociation, either acid or metal catalysed. The indium may then be bound by transferrin, and transported to the liver. Our first approach was to synthesise a *C*-functionalised derivative of 1,5,9-triazacyclododecane triacetic acid and covalently attach it to the antibody B72.3 which selectively binds to tumour associated glycoprotein (TAG-72) found in human breast and colorectal cancers.[5]

Reaction of 1,5,9-triazanonane with diethyl *p*-cyanobenzyl malonate in refluxing methanol afforded the cyclic diamide (**1a**) (16%, m.p. 264—265 °C). In order to obviate formation

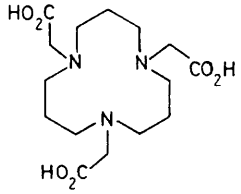


(**1**) **a**; R = H
b; R = Ts = *p*-MeC$_6$H$_4$SO$_2$

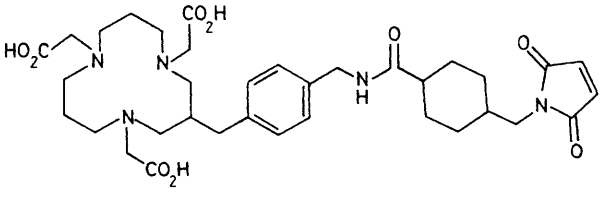
(**2**) **a**; R = H, R′ = Ts
b; R = Ac, R′ = H



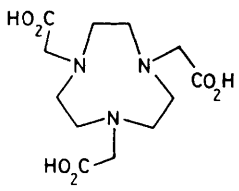
(**3**) **a**; R = Et, R′ = Ac
b; R = R′ = H



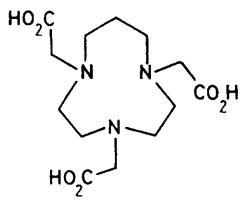
(**4**)



(**5**)



(6)



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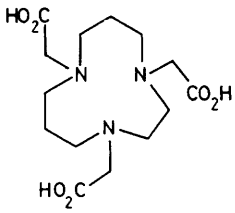

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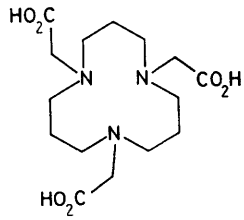

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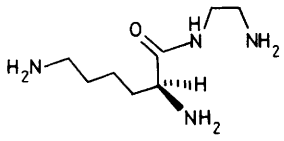

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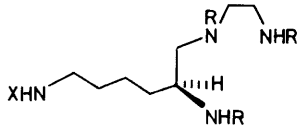
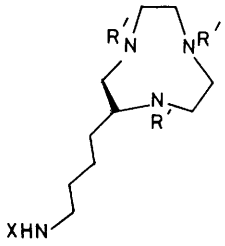


(11) R = X = H

(12) R = H, X = COPh

(13) R = Ts, X = COPh

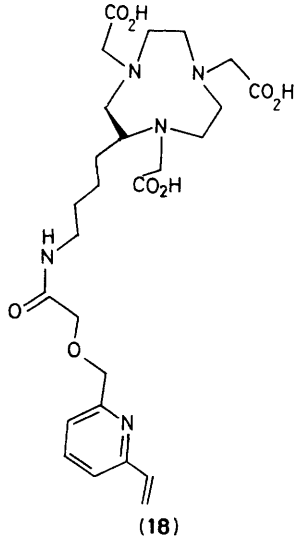


(14) R′ = Ts, X = COPh

(15) R′ = H, X = COPh

(16) R′ = CH_2CO_2Et, X = COPh

(17) R′ = CH_2CO_2H, X = H



(18)

of the stable aminoborane complex of the reduced [12]-N_3 ligand,[6] tosylation of N-1 was effected (TsCl, Et_3N, CH_2Cl_2) (Ts = *p*-$OSO_2C_6H_4Me$) prior to borane reduction [BH_3·THF (tetrahydrofuran) 36 h, 80 °C; followed by 6 M HCl, 3 h, 110 °C], to yield the triamine (**2a**). Detosylation (HBr/AcOH/PhOH) followed by selective exocyclic acetylation of the benzylamine moiety[1] (pH 6.8, *p*-nitrophenylacetate, aqueous dioxan 1 : 1) gave the acetamide (**2b**). *N*-Alkylation of the three nitrogens in the twelve-membered ring is rather slow in aqueous media because the monoprotonated acid is slow and difficult to deprotonate (pK_a is 13.2).[7] Accordingly alkylation of (**2b**) was effected in ethanol ($BrCH_2CO_2Et$, Cs_2CO_3, EtOH) to yield the triester (**3a**) which was acid-hydrolysed (6 M HCl, 18 h) to give the desired amino-triacid (**3b**).† The parent cycle (**4**) was prepared in a similar manner.

† New compounds gave satisfactory microanalytical, spectroscopic (i.r., ^{13}C and ^{1}H n.m.r. mass spectrometry) and chromatographic (h.p.l.c., t.l.c.) analyses in accord with the proposed structures.

Linkage to the antibody in a stepwise manner was effected by reacting the *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl)maleimide[8] with (**3b**) [pH 6.8, aqueous-dioxan (1 : 1)] to give the maleimide (**5**) which was purified by reverse phase h.p.l.c. Incubation of (**5**) with $^{111}InCl_3$ in aqueous acetic acid (pH 5, 55 °C, 1 h) gave the desired indium labelled intermediate which was purified by h.p.l.c. (radiometric detection). This complex was incubated (4 °C, 0.3 M phosphate, pH 8) with B72.3 antibody, which had previously been modified with 2-iminothiolane to yield 5.7 thiols per antibody, and the antibody-conjugate subsequently purified by PD-10 gel filtration chromatography. The modified antibody exhibited no diminution of immunoreactivity. The conjugate was injected in mice and the tissue distribution of the ^{111}In label examined after 4 h and 24 h (Table 1). The maintenance of a high level of activity in the blood after 24 h (a 50% blood level is expected after 24 h from the diffusion from blood to extra-cellular tissue) and the relatively low values found in the liver, kidneys, and spleen (liver is ~30% perfused

Table 1. Tissue distribution of B72.3-macrocycle conjugate.

	4 h		24 h	
Tissue	% Dose g m^{-1}	% Total Injected dose	% Dose g m^{-1}	% Total Injected dose
Blood	32.4	60.9	21.3	47.5
Kidneys	12.3	2.88	8.92	2.49
Liver	8.61	12.4	6.55	9.95
Lungs	15.2	2.21	9.31	1.45
Spleen	6.07	0.41	6.44	0.53
Stomach	—	0.7	—	0.65

with blood) suggest that the ^{111}In label is remaining firmly bound to the antibody *in vivo*. Indeed when the ^{111}In complex of the parent macrocycle (**4**) was injected into mice, all radioactivity was cleared from the tissues in a few hours.

The limiting feature of this approach is that ^{111}In labelling of the macrocycle is required before antibody conjugation. Indium binding by (**4**) [10 μM, pH 5→6] is insufficiently fast at 37 °C for efficient radiolabelling. The rate-determining step in the binding of In by (**4**) is likely to be associated with the last deprotonation of the [12]-N_3 cycle. Other tribasic triaza-macrocyclic ligands were screened therefore for their ability to bind indium rapidly under mild conditions (20 °C, pH 5, < 1 h), yet still form a kinetically stable complex *in vivo*. The forward rate of indium binding by ligands (**6**) to (**9**) was compared under standard conditions [20 °C, pH 5 (0.1 M OAc^- buffer), 10 to 100 μM in ligand], monitoring the binding by h.p.l.c. with radiometric detection. At 50 μM in ligand, the order of the rate of indium uptake (30 mins) was (**6**) > (**7**) >> (**9**) > (**8**). However only (**6**) proved effective when the ligand concentration was 10 μM, and under these conditions a 96% radiolabelling yield was determined (30 min, pH 5, 20 °C). When the ^{111}In complex of (**6**) was injected into normal mice, all radioactivity was cleared from the tissues in a few hours. Perturbed angular correlation spectroscopic measurements for the ^{111}In complex of (**6**) revealed a quadrupole frequency (78 K), ω_Q of 60 MHz, (compared to 14 MHz for ^{111}In–transferrin and 9.8 MHz for ^{111}In–DTPA) consistent with high complex stability in solution.[9] It was appropriate thereafter to prepare a *C*-functionalised derivative of (**6**), bearing an aminoalkyl side-chain so that linkage to the antibody could be effected.[1]

Reaction of the methyl ester of (2*S*)-lysine with neat ethylenediamine afforded the amide (**10**) (96%), which was reduced with borane–THF in THF (70 °C, 21 h) to yield the tetra-amine (**11**) (75%). The diethylenetriamine sub-unit of (**11**) was protected by complexation with copper(II) in aqueous solution {log K[Cu^{2+}·diethylenetriamine] = 16.1 (H_2O)}[10] thereby permitting selective acylation of the remote primary amine group with PhCOCl. Treatment with H_2S removed the copper giving the benzamide (**12**) (58%), which was reacted with tosyl chloride (Et_3N, CH_2Cl_2) to yield the tritosylamide (**13**) (63%). Condensation of (**13**) with ethylene glycol ditosylate [Cs_2CO_3, dimethylformamide (DMF), 65 °C, 18 h] afforded the cyclic tritosylamide (**14**) (71%). Removal of the tosyl groups was effected either using Li/NH_3/MeOH/THF or more simply using 98% H_2SO_4 (18 h, 110 °C) to yield the triamine (**15**) (64%). Ethoxycarbonylmethylation of (**15**) ($BrCH_2CO_2Et$, Cs_2CO_3, EtOH) afforded the triester (**16**) (77%) and acid hydrolysis (6 M HCl, 18 h) yielded the amino-functionalised triacid (**17**) quantitatively.§

Antibody linkage was effected in a stepwise manner by reacting the *p*-nitrophenyl ester of 2-vinyl-6-(4′-carboxy-3′-oxa-butyl)pyridine[1] with (**17**) in DMF to yield (**18**) which was purified by reverse phase h.p.l.c. Incubation of (**18**) with B72.3 antibody (previously modified with 2-iminothiolane to generate *ca.* 3 free thiols per antibody)[1] gave a macrocycle–antibody conjugate which was purified by h.p.l.c. Direct labelling of this macrocycle–antibody conjugate with ^{111}In was effected (pH 5, 20 °C, 0.1 M OAc^-, 1 h) giving the indium labelled antibody with a 95% radiolabelling yield. In summary, macrocycle–antibody conjugates of this type which may be labelled directly and efficiently with ^{111}In (or ^{67}Ga)‡ offer considerable promise for tumour imaging in human patients.

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§ An alternative synthesis has also been effected starting from lysinamide: a, BH_3·THF in THF; b, Cu^{2+}/H_2O then PhCOCl followed by H_2S; c, TsCl/Et_3N/CH_2Cl_2; d, condensation with $TsN(CH_2CH_2OTs)_2$/DMF/Cs_2CO_3 yielding (**14**) in 6% overall yield from lysinamide. Step a was very slow (180 h).

‡ *Note added in proof:* The gallium complex of (**6**) is resistant to acid dissociation ($t_{1/2}$ > 2 weeks, pH 0) as observed by ^{71}Ga n.m.r. spectroscopy [$\delta_{GA}(D_2O)$ = 170 ($\omega_{1/2}$ = 210 Hz)].