Antibody Labelling with Functionalised Cyclam Macrocycles

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Functionalised 'cyclam' macrocyclic ligands have been selectively attached to thiol residues on a monoclonal antibody and form kinetically inert complexes with Cu^{2+} and Tc^{V}.

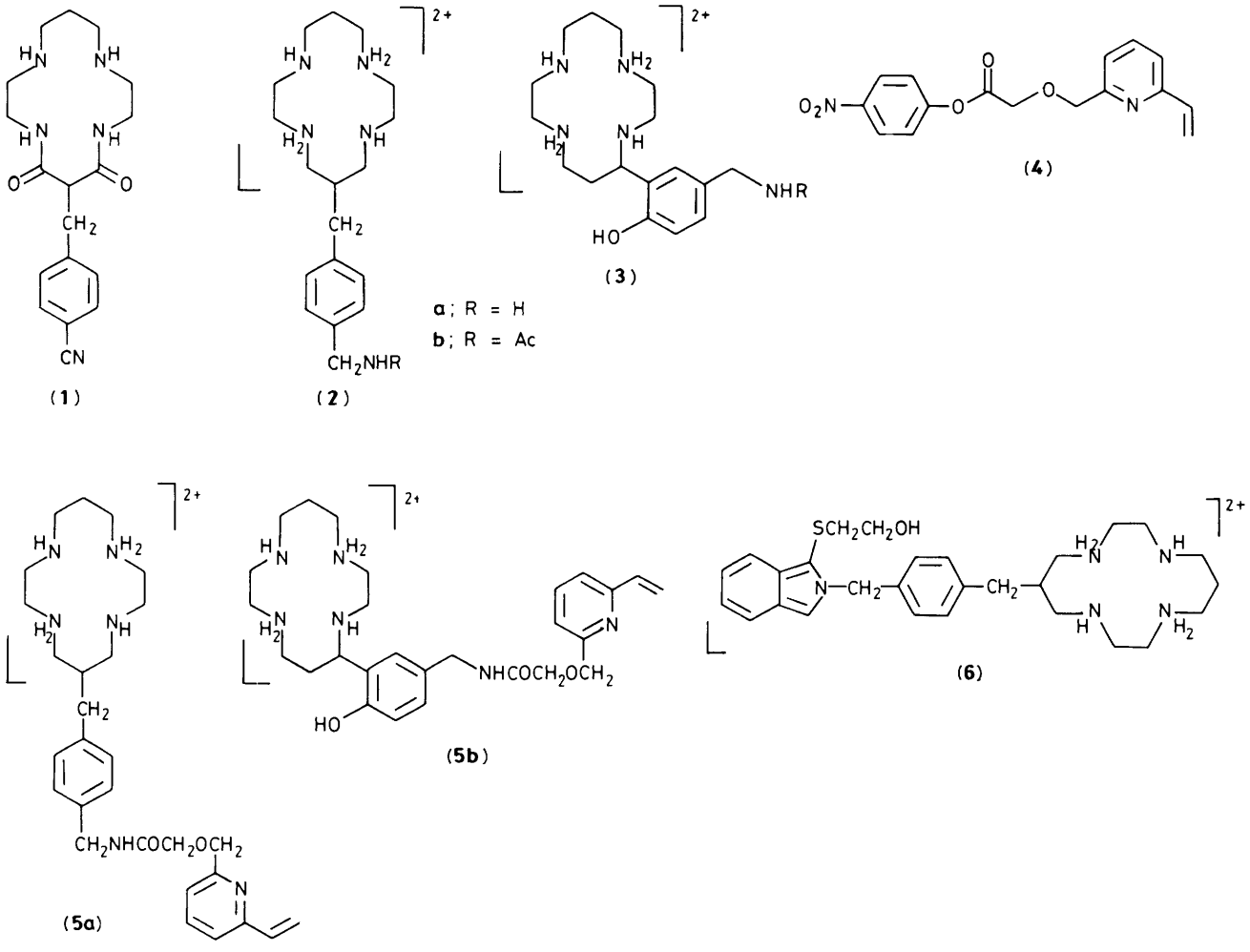
There is a growing recognition of the potential of irreversibly binding radioactive metal complexes to tumour-localising monoclonal antibodies for use in tumour imaging and radioimmunotherapy.[1,2] In such a challenging discipline it is essential that the radiolabel is not dissociated from the antibody conjugate over a period of days. The use of kinetically inert macrocyclic complexes is therefore attractive. For radioimmunoscintigraphy, the γ-emitting radioisotope ^{99m}Tc (available as TcO_4^-) is widely used in diagnostic nuclear medicine, while the positron emitter ^{64}Cu ($t_{\frac{1}{2}}$ 12.8 h) is of potential use in positron emission tomography.[3] Both of the elements form thermodynamically stable and kinetically inert complexes with 1,4,8,11-tetra-azacyclotetradecane (cyclam).[4,5] Our initial work has focused on selectively attaching appropriately functionalised macrocycles to the antibody B72.3 which binds to tumour-associated glycoprotein found in human breast and colorectal cancers.[6]

Reaction of 1,4,8,11-tetra-azaundecane with diethyl *p*-cyanobenzyl malonate in refluxing EtOH afforded the cyclic diamide (**1**) (19%, m.p. 210 °C) which was reduced with BH_3·THF (THF = tetrahydrofuran) to give the aminobenzyl derivative (**2a**) (83%, m.p. 149 °C). The related cycle (**3a**), bearing a pendant phenol, was made similarly using established methods,[7] involving condensation of 6-cyanocoumarin[8] with 1,4,8,11-tetra-azaundecane followed by reduction with BH_3·THF. The nitrogen atoms of the tetra-aza ring assist the ionisation of the exocyclic phenol which exhibits a pK_a of 8.8 (293 K, μ = 0.1 M), determined spectrophotometrically. In order to link (**2a**) and (**3a**) to an antibody, a bifunctional linker molecule was required which could *selectively* be attached to the aminobenzyl group. With this constraint in mind, the vinylpyridine derivative (**4**) was synthesised.† 2- and 4-Vinylpyridine derivatives were found to react selectively with thiols in the pH range 5—9 and were much less sensitive to attack by primary amines in this pH régime. Representative reaction profiles are shown in Figure 1, comparing the reactivity of 2- and 4-vinylpyridine with *N*-acetylcysteine as a function of pH. Under the same conditions, the simple dipeptide primary amine Ala-Pro-NH_2 was unreactive. It was therefore possible to form an amide bond selectively to the functionalised macrocycle, thereby generating a stable, isolable vinylpyridine intermediate which can be subsequently reacted selectively with thiol residues on the antibody (and not the numerous lysine primary amine residues) to give a stable conjugate.

The acylation of (**2a**) and (**3a**) proceeded smoothly under mild conditions (pH 6.8, aqueous dioxane, 2 h) to give the desired intermediates (**5a**) and (**5b**).‡ Under these reaction conditions the cyclam ring is diprotonated and effectively protected from electrophilic attack so that ring acylation does not compete. The ^1H n.m.r. spectrum of (**5a**) is shown in Figure 2 and exhibits three singlets [$\delta_H(D_2O)$ 4.13, 4.33, and

† Prepared by monoalkylation of pyridine-2,6-dimethanol (NaH, THF, $BrCH_2CO_2Me$), following by oxidation (MnO_2, CH_2Cl_2), olefination ($PPh_3{=}CH_2$, THF), ester hydrolysis (LiOH, H_2O, MeOH), and esterification (*p*-nitrophenol, dicyclohexylcarbodiimide, CH_2Cl_2).

‡ All new compounds gave satisfactory microanalytical (C,H,N), spectroscopic (^1H, ^{13}C n.m.r., i.r., chemical ionisation or fast atom bombardment mass spectrometry), and chromatographic (h.p.l.c.) analyses.



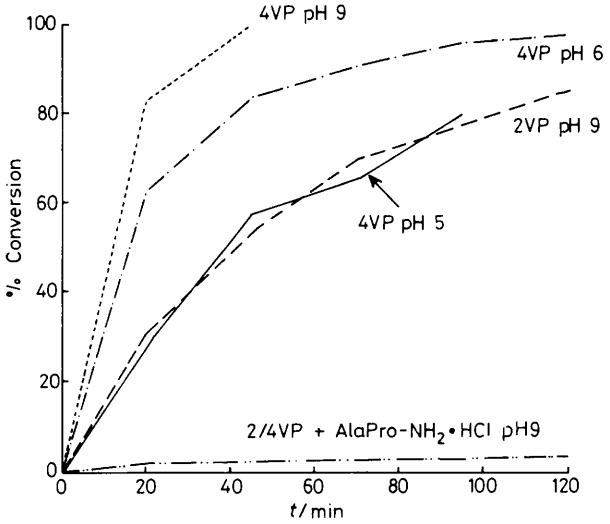


Figure 1. Reaction of 2- and 4-vinylpyridine (2VP, 4VP) with *N*-acetylcysteine (298 K, 5-fold excess of thiol, monitor by h.p.l.c.).

4.64] due to the heterotopic CH_2O groups and the CH_2NDCO group, consistent with selective acylation of the aminobenzyl group. The conjugates (**5a**) and (**5b**) may be attached to thiol residues on an antibody. Free thiol residues were generated by the reaction of B72.3 with 2-iminothiolane at pH 8 followed by gel filtration through Sephadex G-50. The modified antibody was incubated with (**5**) (pH 6, 15 h, 4 °C) and purified, by gel filtration through Sephadex G-50. At this stage it was important to measure the number of macrocycles linked to the antibody and this was achieved using a spectrofluorimetric assay rather than conventional ^{14}C radiolabelling methods.[1] Exhaustive hydrolysis of the antibody conjugate (6 M HCl for 18 h) gave a mixture of amino acid residues and the protonated cycle (**2a**) or (**3a**). Reaction of (**2a**), for example, with *o*-phthalaldehyde and 2-mercaptoethanol gave the isoindole (**6**) which may be detected and assayed spectrofluorimetrically[9] ($\lambda_{exc.}$ 334 nm, $\lambda_{fluor.}$ 453 nm). Separation of (**6**) from the excess of other *N*-alkylisoindoles was achieved using cation-exchange h.p.l.c., taking advantage of the dipositive charge of (**6**) at ambient pH. Typically up to 2 or 3 macrocycles per antibody were found (detection limit was 5×10^{-11} M), and at this level of conjugation, the modified antibody exhibited no diminution of immunoreactivity.

The parent macrocyclic ligand, (**2**) or (**3**), may be labelled with ^{64}Cu or ^{99m}Tc either prior or subsequent to antibody conjugation. The former route avoids the inherent problem of non-specific binding of the metal to the protein. Reaction of (**2a**) (100 μM) with $^{64}CuCl_2$ at pH 8 in phosphate buffer proceeds to ≥95% conversion within 15 min, in accord with the rapid complexation of Cu^{2+} by cyclam.[10] The binding of reduced technetium (derived from TcO_4^- by reaction with $SnCl_2$) is reported to occur rapidly, in the presence of citrate, at pH 8.[11] Such conditions appeared ineffective with (**2b**) and (**3b**). At pH 8.95 in the presence of phosphate, a 72% incorporation of ^{99m}Tc by (**3b**) (100 μM) was observed after 15

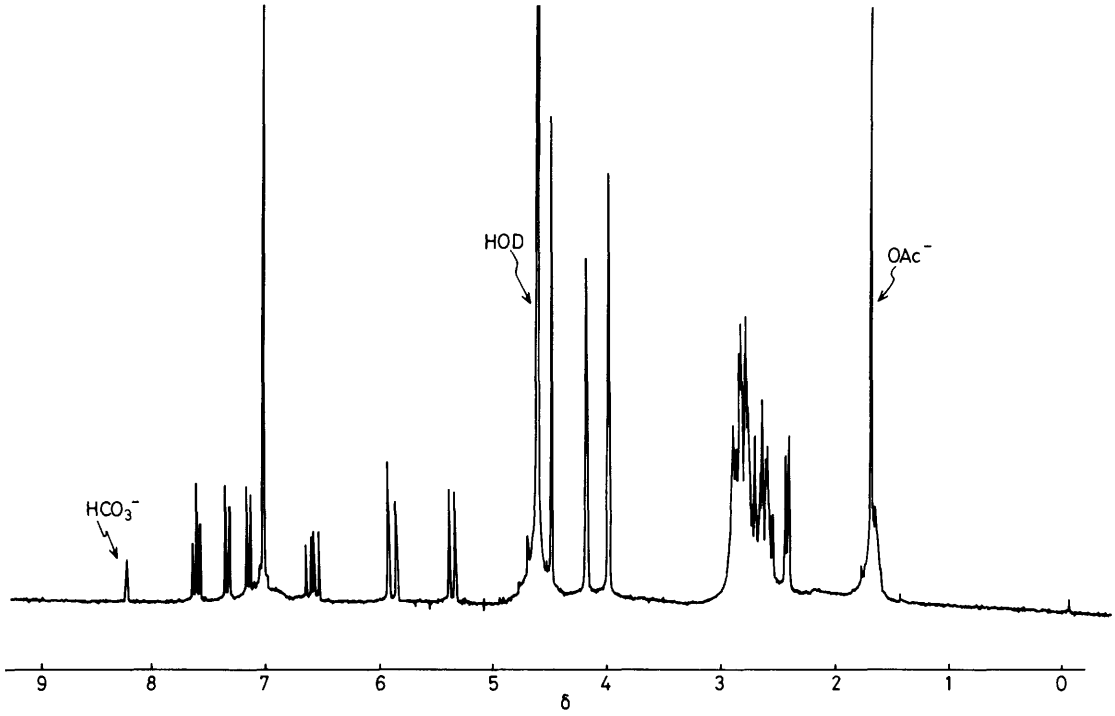


Figure 2. ^{1}H N.m.r. spectrum of (**5a**) (298 K, D_2O, 250 MHz).

min, whilst only 13% was incorporated by (**2b**) under identical conditions (h.p.l.c. radiometry). Evidently the phenolic group either is assisting deprotonation of the ring ammonium protons or is attracting the cationic 'reduced technetium oxo' species. In preliminary biodistribution studies in mice, the technetium complexes of (**2b**) and (**3b**) remain intact and are rapidly cleared with no build-up or retention of radiolabel in any of the major internal organs.

The labelling of antibodies with macrocyclic ligands using methods such as those outlined herein, has potential application particularly for *in vivo* radioimmunodiagnosis or therapy and in *in vitro* radioimmunoassay.[12]

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