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Synthesis of a new hapten for generating catalytic antibodies that activate doxorubicin prodrugs

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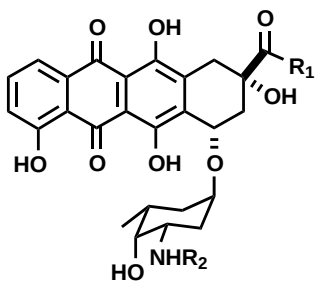
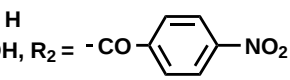
Abstract—In order to assess a novel strategy for catalytic activation of a new prodrug of doxorubicin, a new hapten has been designed and prepared, which can be used to induce an immune response from which catalytic antibodies (cAbs) may be produced.

Doxorubicin (adryamycin®) (**1**)[1] and its derivatives, such as daunomycin (**2**), within the family of the anthracyclines glycosides, are among the most potent and clinically useful of all the anticancer agents. The activity is due to inhibition of DNA and RNA synthesis.[2] Effective dosages in animal models are in the 1–10 mg/kg range, but prolonged treatment can result in serious cardiotoxicity[3] and so, its clinical efficacy is limited.

Targeting the drug to the affected tissue by means of a specific anti-tumor monoclonal antibody has been explored as a means to mitigate toxicity against normal tissue, but this approach has some limitations on the effective dose of drug that can be delivered per antibody molecule.[4] An interesting solution to this problem and the prevailing targeting approach is to chemically modify the drug into a less toxic derivative (prodrug) that can be converted back to the active form by the action of a specific enzyme delivered to the tumor cell, a process termed antibody-directed enzyme prodrug therapy (ADEPT).[5] The ideal enzyme for ADEPT should have high specific activity for the prodrug, should not elicit an immune response and should not be widely distributed in the body.

In principle, a catalytic antibody (cAb)[6] offers many advantages. The cAb could be selected for binding specificity or chemical specificity that identifies the prodrug. Examples of antibody-mediated prodrug activation by hydrolytic or retro-aldol reactions have been demonstrated.[7,8] The antibody can also be humanized to render it nonimmunogenic. A hybrid antibody or immune complex between the cAb and a tumor specific antibody could serve as a well-tolerated pre-targeting agent in ADEPT.



1 Doxorubicin $R_1 = CH_2OH$, $R_2 = H$
2 Daunomycin $R_1 = Me$, $R_2 = H$
3 Prodoxorubicin $R_1 = CH_2OH$, $R_2 =$ 

Here we describe the design and synthesis of a doxorubicin prodrug and the respective hapten that could be used to develop a custom cAb for the hydrolytic prodrug activation strategy.

Design and synthesis of the prodrug

The design of a prodrug required a simple, enzymatically reversible chemical modification, which masks the toxicity of the parent drug. The amino group within the sugar moiety of doxorubicin is important for activity of this drug because it is postulated to interact strongly

Keywords: anthracyclinone; antitumour compound; antibodies; catalysis; sulfonamide.

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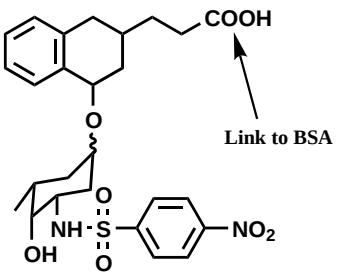
with the phosphate backbone of nucleic acids. This functionality served as a convenient site for modification (mainly acylation) to find a derivative to be tested for reduced toxicity in cytotoxicity assays.[9] Among several amide derivatives tested we found that compound **3** with a *N*-*p*-nitrobenzamide moiety at C-2 of the daunosamine sugar was 30 times less cytotoxic than doxorubicin itself. This compound was easily synthesized in high yield by nitrobenzoylation of doxorubicin with succinimidyl *p*-nitrobenzoate.[10]

*Chemical synthesis of the hapten **4***

In the design of a hapten for eliciting cAbs to catalyze hydrolysis of **3**, we proposed a sulfonamide group as the mimic for the transition state for amide hydrolysis. Compared to other derivatives used as hydrolytic transitions state analogues, the sulfonamide group lacks a net charge. However, the tetrahedral structure, chemical stability and S–O bond polarity are favorable features. Antibodies obtained against a sulfonamide hapten have been reported to accelerate hydrolysis of a heterocyclic amide.[11]Additional design concerns include the presentation of structural features of the substrate that are remote from the site of chemical activity in order to obtain cAbs with desirable substrate specificity. Consequently, we designed compound **4**, containing appropriate functionality to satisfy these requirements. In addition to the sulfonylated daunosamine and glycosidic group approximating the anthracyclin unit, a carboxylic group in the glycoside provides a site for covalent coupling for preparing an immunogenic conjugate.



Hapten Structure (4)

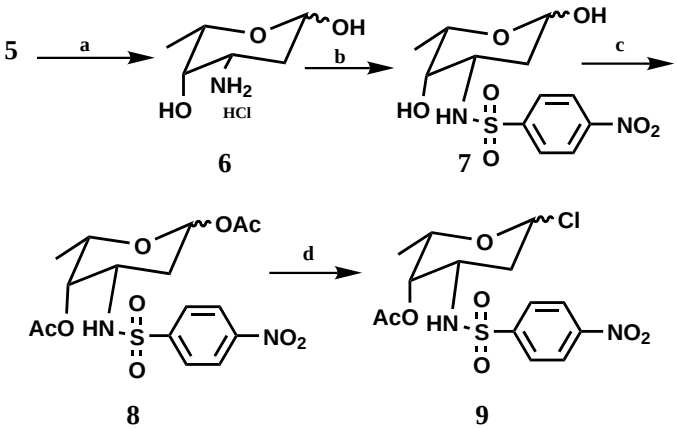
The hapten **4** was prepared by coupling of the chlorosugar **9** and the methylester alcohol **12**. The chlorosugar **9** was prepared from daunosamine (**6**), available in one-step from the commercially available β-methyl daunosaminide (**5**). The amine group of daunosamine was converted to the *N*-*p*-nitrobenzenesulphonyl derivative **7** in 64% yield from **5**.[12] This was subsequently acetylated and transformed to the chlorosugar **9** by treatment with dry HCl (Scheme 1).

On the other hand, the aglycon alcohol **12** was obtained from the commercially available tetrahydronaphthalene propionic acid **10** by esterification in methanolic HCl, followed by ketone reduction with $NaBH_4$ (78% yield overall). Glycosidation of alcohol **12** with the chlorosugar **9** by a Koneigs–Knorr procedure afforded compound **13** in a 27% yield from **8**.[13] Finally, hydrolysis of **13** with 0.5N NaOH removed both the acetyl group and the methyl ester to give **4** in a quantitative yield (Scheme 2).[14]

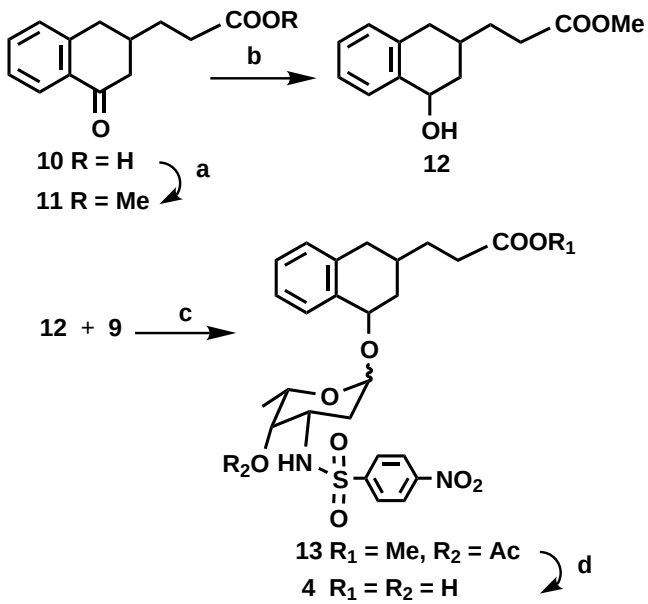
*Chemical synthesis of substrates **18** and **19***

Corresponding substrates 3-[4-*O*-(2,3,6-trideoxy-3-*N*-*p*-nitrobenzoyl - α - L - lyxohexopyranosyl) - 1,2,3,4 - tetrahydronaphthalen-2-yl]-propionic acid (**18**) and 1-*O*-(2,3,6-trideoxy-3-*N*-*p*-nitrobenzoyl-α-L-lyxohexopyranosyl)-1,2,3,4-tetrahydronaphthalene (**19**) were prepared in order to facilitate screening assays for the identification of hydrolytic antibodies elicited by **4**. Following a similar strategy used in the synthesis of hapten **4**, compounds **18** and **19** were prepared from **10** and 1,2,3,4-tetrahydro-1-naphthol, respectively, as shown in the Scheme 3. The use of the *p*-nitrobenzoyl group for protection of both hydroxyl and acetal groups afforded the α isomer in both cases.[15]

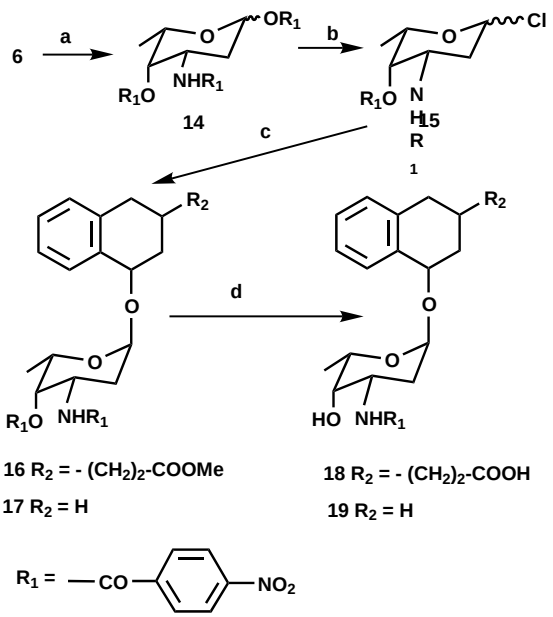
The sulfonamide hapten **4** was readily conjugated with carrier proteins using DCC/NHS in 4:1 water/DMF to obtain an immunogen for mouse immunization. The preparation and assay of monoclonal antibodies will be



Scheme 1. *Reagents and conditions*: (a) 1% HCl aqueous, reflux 2 h; (b) *p*-nitrosulfonyl chloride, 2:1 CH_3CN/0.1N CO_3Na_2, 2 h, 64% from **5**; (c) Ac_2O/Py, 65%; (d) bubbling HCl gas, 10 min in dry benzene.



Scheme 2. *Reagents and conditions*: (a) satd HCl in dry MeOH, reflux at 100°C, 48 h, 95%; (b) $NaBH_4$ in MeOH, 82%; (c) silver triflate in dry DMF, rt, 3 h, 27% from **8**; (d) 0.5N NaOH in THF, 94%.



Scheme 3. *Reagents and conditions*: (a) *p*-Nitrobenzoyl chloride in pyridine, 45%; (b) bubbling HCl anhyd. in dry CH_2Cl_2 for 10 min; (c) **12** or 1,2,3,4-tetrahydro-1-naphtol, silver triflate in dry DMF, rt, 3 h, 15% for **16**, 8% for **17** both from **14**; (d) 0.1N NaOH in THF, 52% for **18**, 77% for **19**.

performed by the standard methods.[16,17] The studies will establish whether the expected amidase cAbs can work as efficient reagents for an ADEPT strategy.

Acknowledgements

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10. Preparation of **3**: To a solution of 5 mg (0.009 mmol) of doxorubicin (**1**) in 0.5 mL of CH_3CN/0.1N Na_2CO_3 (3:1) was added 10 mg of succinimidyl *p*-nitro benzoate and stirred at rt for 1 h. The mixture was evaporated under reduced pressure, dissolved in H_2O, and extracted with $CHCl_3$. The organic extract was then chromatographed by preparative TLC (20% MeOH in CH_2Cl_2) to give 5 mg of *N*-*p*-nitrobenzoyldoxorubicin (**3**) in a 84% yield as an amorphous red powder; ^{1}H NMR ($CDCl_3$) δ: 8.25 and 7.89 (2H each, d, $J=8.6$ Hz, AB system of *p*-nitrobenzoyl moiety), 8.05 (1H, d, $J=7.6$ Hz) and 7.80 (1H, dd, $J=7.6$ and 8.3 Hz), and 7.39 (1H, d, $J=8.3$ Hz) corresponding to ABX system of D ring, 6.62 (1H, $J=8.0$ Hz, -NH-), 5.55 (1H, brd, $J=3.7$ Hz, H-1′), 1.33 (3H, d, $J=6.4$ Hz, Me-5′); IR ($CDCl_3$) ν_{max} 1780, 1750, 1645 (amide), 1535 cm^{-1}; (+)-LRFABMS *m*/*z* 677 (M+H)$^+$.
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12. Preparation of **7**: To a solution of daunosamine hydrochloride (**6**) in 3 mL of a 2:1 CH_3CN/0.1N Na_2CO_3 solution was added 300 mg of *p*-nitrobenzenesulphonyl chloride and the mixture was stirred at rt for 1 h, keeping the pH above 8.0 by adding several drops of a concentrated Na_2CO_3 solution. After 1 h, further 120 mg of *p*-nitrobenzenesulphonyl chloride was added to the reaction mixture and stirred for an additional 30 min. Then, 20 mL of water was added, and the solution was extracted first with $CHCl_3$ and then with *n*-BuOH saturated with water. The butanolic extract was concentrated under reduced pressure and separated by flash column chromatography (5% MeOH in CH_2Cl_2) to give 268 mg of pure compound **7** (64% yield from compound **5**). ^{1}H NMR (MeOH-d_4) δ: 8.30 and 8.04 (4H each, d, $J=6.9$ Hz, AB aromatic system), 5.01 (1H, brd, $J=2.9$ Hz, H-1α), 4.52 (1H, dd, $J=9.2$ and 2.6 Hz, H-1β), 3.96 (2H, q, H-5), 3.66 (2H, m, H-4), 1.73 and 1.42 (4H, m, H-2). (+)-LRFABMS *m*/*z* 333 (M+H)$^+$.

13. Preparation of **13**: To a stirred mixture of 247 mg of the alcohol **12** (1.055 mmol) and powered 4 Å molecular sieves in 1 mL of dry methylene chloride were added the previously obtained chlorosugar **9** (aprox. 200 mg) in 3 mL of dry methylene chloride and a solution of 166 mg of silver trifluormethanesulfonate in 1 mL of dry DMF under dry argon in the dark at 0°C. The ice-water bath was removed and the mixture was stirred at room temperature for 2 h. Then, the mixture was poured into a cooled brine solution, filtered, and the organic layer was separated, washed with water, dried ($MgSO_4$), and evaporated to give a residue which was flash chromatographed in a silica gel (18 g) column with EtOAc/hexanes (2:3) to yield 110 mg of a mixture of the α,β-glycoside **13** (27% of yield from compound **8**). ^{1}H NMR ($CDCl_3$) δ: 8.35 and 8.04 (4H each, d, J=8.9 Hz, AB aromatic system), 7.26–7.21 (8H, aromatic protons), 5.20 (1H, brd, H-1α), 5.13 (1H, brd, H-1β), 5.04 and 5.01 (1H each, d, J=8.6 Hz each, -NH-), 4.94 and 4.87 (1H each, m, H-4′), 4.79 and 4.67 (1H each, m, H-4), 4.12 (2H, m, H-5′), 4.00 (2H, q, H-3′), 3.68 (6H, OMe), 2.80 (4H, m, H-1), 1.06 (6H, J=6.6 Hz, Me-5′). ^{13}C NMR ($CDCl_3$) δ: 173.5 (COOMe), 170.2 (OCOMe), 149.6–124.0 (aromatics), 98.1 (C-1α), 92.6 (C-1β), 72.1, 70.7, 65.4 (C-4′, C-5′, C-4), 51.1 (OMe), 47.9 (C-3′), 20.2 (MeCO), 16.3 (Me-5). (+)-LRFABMS m/z 723 $[M+Cs]^+$; UV (MeOH) λ_{max} 268 nm.
14. Selected data for **4**: ^{1}H NMR ($CDCl_3$) δ: 8.32 and 8.04 (4H each, d, J=8.8 Hz, AB aromatic system), 7.30–7.19 (8H, aromatics), 5.14 (1H, brd, H-1α), 5.07 (1H, brd, H-1β), 4.80 and 4.67 (1H each, m, H-4), 4.11 (2H, m, H-5′), 3.80 (2H, q, H-3′), 3.59 and 3.49 (1H each, br d, H-4′), 1.20 (6H, J=6.6 Hz, Me-5′). ^{13}C NMR ($CDCl_3$) δ: 177.2 (COOH), 149.6–124.0 (aromatics), 98.8 (C-1α), 92.9 (C-1β), 72.1, 70.7, 66.7 (C-4′, C-5′, C-4), 49.5 (C-3′), 16.6 (Me-5). (+)-LRFABMS m/z 667 $[M+Cs]^+$; 799 $[M-H+2Cs]^+$. UV (MeOH) λ_{max} 268 nm. The α and β isomers can be separated by reverse phase HPLC using 60% CH_3CN/40% H_2O with 0.01 M TEA as eluent.
15. Selected data for substrates: **18**: ^{1}H NMR (C_5D_5N) δ: 8.40 and 8.23 (2H each, d, J=8.8 Hz, AB aromatic system), 7.32–7.19 (4H, aromatics), 5.45 (1H, brd, H-1α), 4.33 (1H, m, H-4), 3.73 (3H, s, OMe), 2.83 (2H, m, H-1), 1.52 (3H, J=5.9 Hz, Me-5′). ^{13}C NMR (C_5D_5N) δ: 174.7 (CO), 141.7–123.8 (aromatics), 100.6 (C-1α), 78.1 (C-4′), 69.6 (C-5′), 68.1 (C-4), 52.1 (OMe), 48.3 (C-3′), 18.0 (Me-5). (+)-LRFABMS m/z 645 $[M+Cs]^+$. **19**: ^{1}H NMR ($CDCl_3$) δ: 8.27 and 7.92 (2H each, d, J=8.8 Hz, AB aromatic system), 7.22–7.14 (4H, aromatics), 6.62 (1H, d, J=8.2 Hz, -NH-), 5.22 (1H, brd, H-1α), 4.75 (1H, m, H-4), 4.58 (1H, m, H-4′), 3.69 (1H, m, H-3′), 4.25 (1H, brq, J=6.7 Hz, H-5′), 2.81 (2H, m, H-1), 1.28 (3H, J=6.7 Hz, Me-5′). (+)-LRFABMS m/z 559 $[M+Cs]^+$.
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