Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 24, pp. 2975-2980, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00523-4

SYNTHESIS AND EVALUATION OF NOVEL DAUNOMYCIN-PHOSPHATE -SULFATE -β-GLUCURONIDE AND -β-GLUCOSIDE PRODRUGS FOR APPLICATION IN ADEPT.

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Abstract: The synthesis, antiproliferative effect and enzymatic hydrolysis of daunomycin-3'-*N*- and -4'-*O*-phosphate and -sulfate derivatives and of daunomycin-3'-*N*-CO-β-glucuronide and -β-glucoside, designed to be prodrugs in ADEPT are described. The phosphate derivatives were almost as toxic as the parent drug whereas the sulfates were not hydrolyzed by aryl sulfatases. Glucuronyl and glucosyl prodrugs were found to be useful for application in ADEPT.

Anthracycline antitumor antibiotics are among the most potent and widely used anticancer agents. Their use is mainly limited by myelosuppression and by cumulative and irreversible cardiotoxicity[1]. Therefore, it would be of great value in chemotherapy to target these drugs specifically against tumor cells. The use of monoclonal antibodies (mAb) as carriers to deliver cytotoxicity to tumor tissue is a relatively new approach in chemotherapy research programmes[2]. The classical strategy is to covalently link drug molecules to mAb's prior to use, these conjugates are then systemically administered and will mainly localize into tumor sites. Several drawbacks however are encountered in this approach. Because of the limited number of antigens present on a tumor cell surface it is often impossible to deliver cytotoxic concentrations of a drug at the tumor site, besides that only a limited number of drug molecules can be bound to a mAb[3]. In an alternative approach, enzymes are localized at the target tumor cells making use of a mAb, these targeted enzymes are chosen to convert a relatively non-toxic prodrug to the parent drug. This ADEPT (Antibody Directed Enzyme-Prodrug Therapy)[4] or ADC (Antibody Directed Catalysis)[5] concept which has extensively been reviewed in the last few years[6], possesses several advantages over the mAb-drug conjugate approach. These advantages lie in the catalytic nature of ADEPT; one mAb-enzyme conjugate can generate many drug molecules, and in the fact that a higher dose of a prodrug is tolerated when compared with a normal drug dosage. A prodrug must fulfil a number of conditions to be applicable in ADEPT. Important features for prodrugs are a reduced toxicity, stability *in vivo*, an efficient enzymatic hydrolysis only by the targeted enzymes, and a low cellular uptake.

To explore the applicability of anthracycline prodrugs in ADEPT, we synthesized six polar daunomycin analogues and subjected them to a series of cytotoxicity- and enzyme-hydrolysis assays. Inactivation of a cytotoxic drug can be achieved by coupling a polar moiety to it, resulting in a poor cell-membrane penetration of the resulting polar drug derivative. As a consequence the derivative should display a reduced cytotoxicity. Moreover, when the polar group is attached to the drug *via* a functional group which is important for the antitumor activity, the cytotoxicity of the prodrug will be further reduced. Promising *in vivo* experiments employing phosphate based prodrugs of etoposide[4], mitomycin[7] and of phenol mustard[8] in combination with a mAb-alkaline phosphatase (AP) conjugate have been described in literature. To date studies on the potential of sulfate based prodrugs in ADEPT have never been reported. A β-glucuronide group as a pro-moiety in prodrugs

of *p*-hydroxyaniline mustard[9] or directly coupled to an anthracycline[10], and a β-glucuronide and other glycosides coupled to compounds in the anthracycline series *via* an *o*- or *p*-hydroxy benzyl alcohol spacer were also reported in literature[11]. In our laboratories, β-glycosyl-carbamate based anthracycline prodrugs possessing a spacer moiety between drug and sugar entity have already been synthesized[12]. However, to prevent side effects of an expelled immolative spacer after activation of a spacer containing prodrug, prodrugs without a spacer entity between drug and polar group are preferred. To prevent undesired side effects, the polar moiety in prodrugs should preferably be a compound occurring in the human body. Together with the novel phosphate- and sulfate based prodrugs, we now present β-glycosyl-carbamate based prodrugs without a spacer entity. The sulfate prodrug/mAb-aryl sulfatase (AS), β-glucuronide prodrug/mAb-β-glucuronidase (GUS) and β-glucoside prodrug/mAb-β-glucosidase systems are promising for application in ADEPT as alkaline sulfatase, β-glucuronidase and β-glucosidase enzyme blood concentrations in blood are very low[13]. This in contrast to the alkaline phosphatase enzyme concentration.

Chemistry

The synthesis of the daunomycin 4'-*O*-phosphate and 4'-*O*-sulfate analogues required a new protective group approach because it was infeasible to remove the 3'-*N*-trifluoroacetyl group, which is frequently used to protect the 3'-amino group in the anthracycline series[14], without hydrolysis of the 4'-*O*-phosphate and 4'-*O*-sulfate groups respectively. The use of the 2-(methylsulfonyl)ethoxy (MSE) group as a base labile protecting moiety allowed a relatively mild and fast deprotection *via* β-elimination[15]. In a typical procedure, an MSE protecting group is removed by vigorously stirring the protected substrate for 30 - 60 sec in a mixture of dioxane, MeOH and 4 N NaOH 14/5/1 (v/v/v), 10 equivalents in NaOH per MSE group.

Daunomycin-3'-*N*-phosphate **4** (scheme 1) was obtained in 40% overall yield after reaction of daunomycin hydrochloride **1** with the bis-MSE-protected phosphorochloridate **2**[16], immediately followed by deprotection of the phosphate group and purification on a reversed phase C-18 column. The cyclic phosphate **19** (chart 1) was isolated as a major side product of the phosphorylation reaction. Prior to phosphorylation at the 4'-hydroxyl function of daunomycin, the 3'-amino group was protected employing *N*-hydroxy succinimide carbonate **5**[15] resulting in MSE-carbamate **6**. The protected intermediate **6** was not phosphorylated at the 4'-hydroxyl group using the phosphorochloridate **2**. Phosphorylation using the phosphoroamidite **7**[17] however, followed by *m*-CPBA mediated oxidation of the phosphorus atom yielded the tris-MSE-protected intermediate **8**. Simultaneous removal of all three MSE groups afforded daunomycin-4'-*O*-phosphate **9** in 74% overall yield. Daunomycin-3'-*N*-sulfate **10** was easily obtained in 71% yield by refluxing daunomycin-HCl with sulfur trioxide-triethylamine complex in chloroform followed by conversion of the resulting triethyl ammonium salt to the sodium salt using sodium bicarbonate (scheme 1). Daunomycin-4'-*O*-sulfate **12** was prepared in an overall yield of 51% by sulfatation of the 4'-hydroxyl group of **6** with sulfur trioxide-pyridine complex followed by removal of the MSE group. The daunomycin analogues **4**, **9** and **10** thus obtained are considerably soluble in water whereas the daunomycin-4'-*O*-sulfate **12** moderately dissolved in water and partly precipitated on standing.

Scheme 1

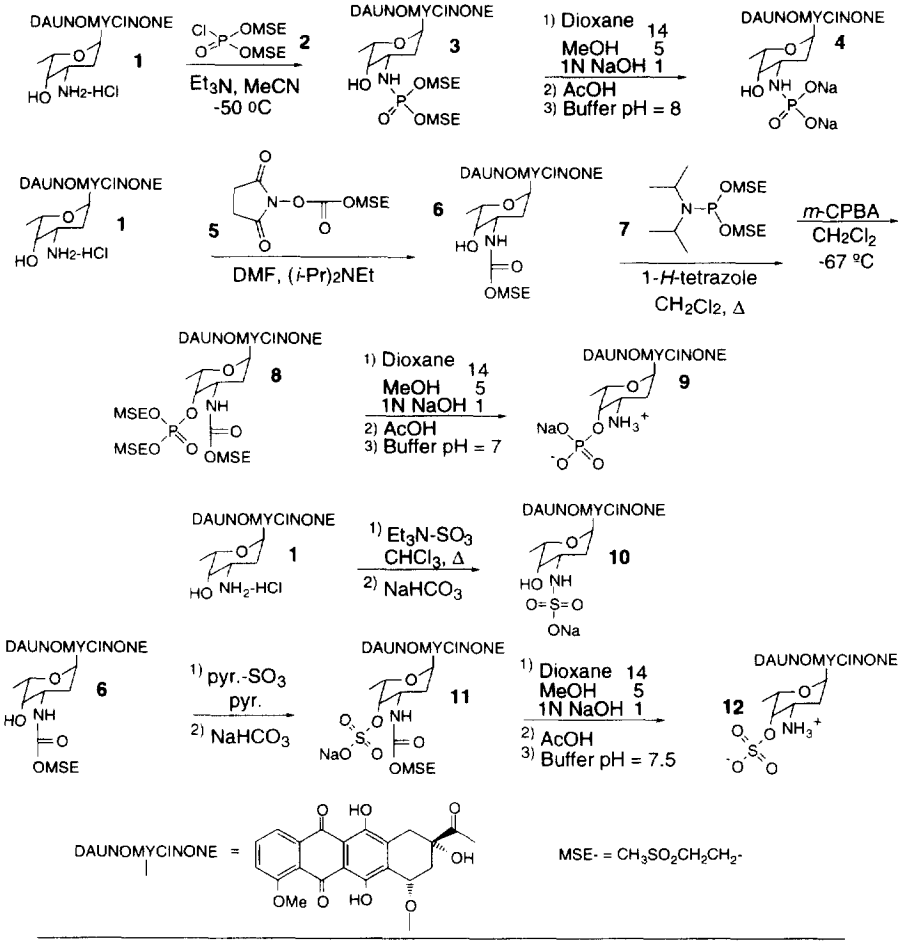
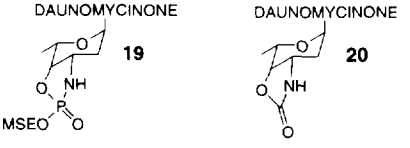


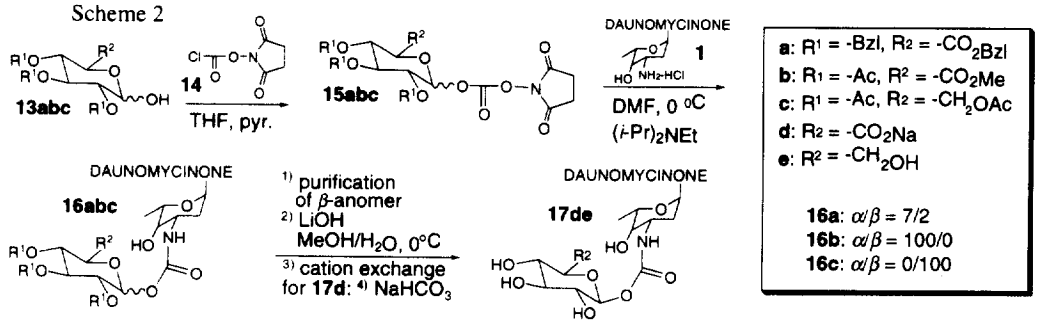
Chart 1



For the synthesis of daunomycin β-glucuronyl-carbamate **17d**, the protected intermediates **16a** and **16b** were prepared by condensation of the anomerically unprotected glucuronide **13a** and **13b** respectively with chloroformate **14** (scheme 2). In a further transformation, the resulting mixed carbonates **15a** and **15b** reacted with the 3'-amino group of daunomycin yielding the protected prodrugs **16a** or **16b** respectively. When excess of chloroformate **14** was used, cyclic carbamate **20** (chart 1) was also obtained. The anomeric mixture of the benzyl protected glucuronide **16a** was separated by preparative centrifugal thin layer chromatography and afforded the anomerically pure products in a ratio $\alpha/\beta = 7/2$. Using a number of different hydrogenolysis

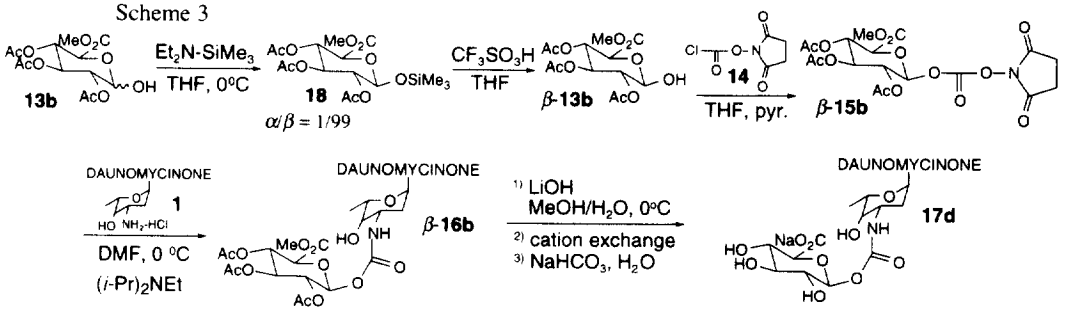
procedures to remove the benzyl groups, we were not able to deprotected β-**16a** without decomposition of daunomycin. In a reaction of the acyl protected glucuronic acid **13b** with **14**, followed by a reaction of the resulting mixed carbonate **15b** with daunomycin leading to **16b**, the β-anomer was not detected viz. the reaction only afforded the α-anomer in 75% yield.

Scheme 2



Yet the desired β-anomer **17d** was prepared with 99% β-diastereoselectivity (scheme 3) in 38% overall yield by hydrolysis of the trimethylsilyl group of **18**, followed by immediate condensation of the resulting anomeric β-hydroxyl group of the product β-**13b** with the chloroformate **14** *in situ*. During the transformation of **18** to β-**15b** and further to β-**16b**, no anomerization was observed using ^{1}H-NMR. Simultaneous removal of all four protective groups on the glucuronide moiety in β-**16b** was conveniently achieved in 93% in a one step procedure with LiOH in MeOH-H_2O at 0°C furnishing **17d**[18]. In the key step of the synthesis, the 1-*O*-trimethylsilyl β-glucuronide **18** was prepared 99% β-diastereoselectively using *N*,*N*-diethyl trimethylsilyl amine as silylating agent[19] in tetrahydrofuran at 0 °C[20]. Daunomycin-3'-*N*-CO-β-glucoside **17e**[18] (scheme 2) was conveniently prepared in 60% overall yield by a reaction of the anomerically pure glucose tatraacetate-1-β-OH (β-**13c**), which selectively crystallized from the anomeric mixture of **13c**, with chloroformate **14** followed by deprotection of the resulting intermediate β-**16c**.

Scheme 3



Pharmacological properties[21]

The antiproliferative effects of the novel daunomycin-phosphate and -sulfate analogues (table 1) were evaluated using a panel of 5 human tumor cell lines consisting of rhabdomyosarcoma cells (A-204), mammary carcinoma cells (MCF-7), bladder carcinoma cells (T-24), melanoma cells (IgR-37) and colon carcinoma cells (WiDr). For the β-glucuronyl and β-glucosyl prodrugs (table 2), a panel consisting of MCF-7 and WiDr Cells (*vide supra*), mammary carcinoma cells (EVSAT), ovarian carcinoma cells (IGROV), melanoma cells (M-19) and

renal cancer cells (A-498) was used. ID_{50} values were calculated from a dose-response curve which was obtained from cell-kill values at 12 different concentrations performed twice for each drug. Table 1 shows a highly decreased cytotoxicity for the sulfate prodrugs, especially the 3'-*N*-derivative **10** which is 1,600 - 230,000 times less toxic than daunomycin. On the other hand, the phosphate prodrugs **4** and **9** exhibit only a slightly diminished cytotoxicity on the cell lines which can be explained by the presence of endogenous phosphatase enzymes in the culture medium. Furthermore, the 4'-*O*-sulfate prodrug shows a higher residual activity when compared to the 3'-*N*-sulfate analogue. This can probably be ascribed to the unoccupied 3'-amino group which is present in the 4'-*O*-sulfate derivative so that cytostatic activity of this sulfate ester is not completely blocked. In comparison with the sulfate based prodrugs **10** and **11**, the cytotoxicity of the β-glucosyl based prodrug **17e** (table 2) is less strongly reduced whereas the β-glucuronyl based prodrug **17d** is at least 2,000 times less toxic than the parent drug. The difference in cytotoxicity between the β-glucuronyl and β-glucosyl based prodrugs can probably be explained by their large difference in polarity and hence difference in cellular uptake.

table 1. antiproliferative effect and enzyme hydrolysis of phosphate and sulfate prodrugs

	IC_{50} (μM)					enzyme activation
Compound	A204	MCF-7	T24	WiDr	IgR-37	$t_{1/2}$ (min)
Daunomycin **1**	3	2	2	7	11	
4	17	370	2	10	96	n.d.+
9	17	89	3	58	45	n.d.+
10	4.900	10.400	>180.000	>1.600.000	>1.600.000	0.0*
12	1.300	870	5.500	>1.600.000	3.000	0.0*

+ Not determined. Enzymes: * aryl sulfatase

table 2. antiproliferative effect and enzyme hydrolysis of β-glycosyl prodrugs

	IC_{50} (μM)						enzyme activation
Compound	MCF-7	WiDr	EVSAT	IGROV	M-19	A-498	$t_{1/2}$ (min)
Daunomycin **1**	2	7	2	18	2	3	
17d	11200	39500	13500	36900	15800	12100	8200#
17e	125	370	280	520	200	250	> 8000‡

β-glucuronidase, ‡ β-glucosidase

Enzymatic activation of the prodrugs was determined (table 1 and 2) incubating 100 mM prodrug with 1 μg/mL aryl sulfatase, 0.03 U/mL β-glucuronidase or 0.3 U/mL β-glucosidase respectively, in PBS at pH = 6.8 at 37 °C. The hydrolysis half lives under standard conditions of the phosphate prodrugs **4** and **9** were not determined because these results are not indicative in *in vivo* and *in vitro* situations were phosphatase enzymes are endogenous. Whereas the novel daunomycin phosphate prodrugs were hydrolyzed rapidly by endogenous enzymes, the daunomycin sulfates were not activated to the parent drug by aryl sulfatase. The β-glycosyl based prodrugs **17d** and **17e** (table 2) were hydrolyzed at a rate which should be considered too low to be applicable in ADEPT. It was shown in literature[6b, 22] that prodrugs having an enzymatic hydrolysis rate comparable to those of prodrugs **17d** and **17e** are less suitable to be used in ADEPT.

These results indicate that prodrugs consisting of a β-glucuronyl-carbamate moiety connected to the drug *via* a spacer to facilitate enzyme hydrolysis, are potential candidates for application in ADEPT. In our laboratories β-glururonyl carbamate based prodrugs having an immolative spacer are subject of current research[12].

Acknowledgment
This work was performed as a Dutch Cancer Foundation grant (no. VU 93-655).
Daunomycin was kindly provided by Dr. D. de Vos, Pharmachemie b.v. Haarlem, The Netherlands.
We thank Prof. Dr. G.I. Tesser and P.J.H.M. Adams for their inspiration concerning the MSE protection-deprotection strategy.

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18 Some characteristic signals in the 400 mHz ^{1}H-NMR spectrum (d^6-DMSO) of **17d**: N-*H* δ = 6.93 ppm, J = 8.0 Hz. Glucuronyl-H-1 δ = 5.09 ppm, J = 8.1 Hz. **17e**: N-*H* δ = 6.92 ppm, J = 7.8 Hz. Glucosyl-H-1 δ = 5.15 ppm, J = 8.1 Hz.
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(*Received in Belgium* 1 *September* 1995; *accepted* 8 *November* 1995)