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ARYL-2-HALOGENOALKYLAMINES—XXVI*
GLUCURONIC, SULPHURIC AND PHOSPHORIC ESTERS OF *p*-DI-2-CHLOROETHYLAMINOPHENOL

M. A. BUKHARI, J. L. EVERETT and W. C. J. ROSS
Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London SW3 6JB

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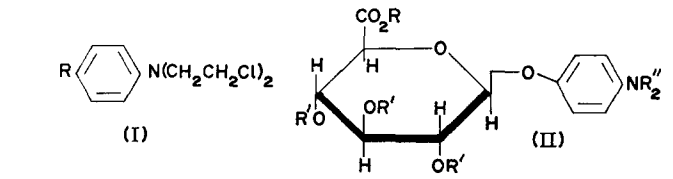
Abstract—It has been proposed that the high activity of *NN*-di-2-chloroethylaniline as an inhibitor of the growth of a mouse plasma cell tumour is mediated by the formation of the ethereal glucuronide of its *p*-hydroxy derivative. To test this hypothesis this conjugate and also the corresponding ethereal phosphate and sulphate have been synthesised and submitted to preliminary antineoplastic assays. The glucuronic acid derivative and its methyl ester exhibit high carcinostatic activity against the mouse plasma cell tumour (ADJ/PC6A). The ethereal phosphate also shows significant activity against this tumour but the sulphate is considerably less active. The significance of these results for the design of tissue specific anticancer agents is discussed.

CONNORS and Whisson[1,2] showed that the mouse plasma cell tumour (PC5), though resistant to most alkylating agents, was highly sensitive towards *NN*-di-2-chloroethylaniline (I, R=H). It was suggested that this specificity of action arose from the initial conversion of the aniline derivative into the less toxic *o*-glucuronide (II, R=R′=H, R″=CH_2CH_2Cl) of its *p*-hydroxy derivative (I, R=OH).[3,4] As a consequence of the high β-glucuronidase activity of the plasma cell tumour it was considered that this conjugate would be selectively transformed into the much more toxic *p*-di-2-chloroethylaminophenol (I, R=OH) within the cells of this neoplasm.

This concept suggested that an approach to the chemotherapy of malignancies could be based on their ability to potentiate the cytotoxic effect of conjugates of the *p*-hydroxy derivative. Accordingly the synthesis of three such conjugates, the *o*-glucuronide, (II, R=R′=H, R″=CH_2CH_2Cl), the ethereal sulphate (III) and the ethereal phosphate (IV) has been undertaken.



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MATERIALS AND METHODS

Methyl (*p*-nitrophenyl-2,3,4-tri-*o*-acetyl-β-D-glucopyranosid) uronate[5] (II, R=Me, R′=Ac, R″=O) was catalytically reduced to give the corresponding *p*-amino derivative[6] (II, R=Me, R′=Ac, R″=H) which on treatment with ethylene oxide in glacial acetic acid afforded the *p*-di-2-hydroxyethyl derivative (II, R=Me, R′=Ac, R″=CH_2CH_2OH). This diol yielded the di-2-chloroethyl derivative (II, R=Me, R′=Ac, R″=CH_2CH_2Cl) when allowed to react with methanesulphonyl chloride in pyridine solution. On hydrolysis in methanolic ammonium hydroxide the di-2-chloroethyl derivative afforded methyl (*p*-di-2-chloroethylaminophenyl-2,3,4-trihydroxy-β-D-glucopyranosid) uronate (II, R=Me, R′=H, R″=CH_2CH_2Cl). Complete hydrolysis to the uronic acid (II, R=R′=H, R″=CH_2CH_2Cl) was achieved by the use of tetra-*n*-butyl ammonium hydroxide in methanol–toluene solution.*

p-Di-2-chloroethylaminophenyl sulphate (III) was obtained by the action of chlorosulphonic acid in lutidine solution on *p*-di-2-chloroethylaminophenol (I, R=OH). It was characterised as its lutidine and sodium salts.

Treatment of the phenol (I, R=OH) with phosphoryl chloride and hydrolysis of the product afforded the ethereal phosphate (IV) which was characterised by the preparation of the cyclohexylamine and sodium salts.

Methyl (p-*di*-2-*hydroxyethylaminophenyl*-2,3,4-*tri*-o-*acetyl*-β-D-*glucopyranosid*) *uronate* (II, R=Me, R′=Ac, R″=CH_2CH_2OH). Methyl (*p*-aminophenyl-2,3,4-triacetyl-β-D-glucopyranosid) uronate (4·2 g, m.p. 160°, $[\alpha]_D^{20}$ −33° (c = 1 in $CHCl_3$); lit.[6] m.p. 154–156°, $[\alpha]_D^{15}$ −24° (c=5 in $CHCl_3$)), obtained by catalytic reduction of the corresponding nitro compound, was dissolved in glacial acetic acid (20 ml) and after the addition of ethylene oxide (1 ml) the mixture was left at room temperature overnight. After removal of the solvent under reduced pressure aqueous ammonium hydroxide was added to adjust the pH to 7 and the solution was evaporated to dryness under reduced pressure. The residue crystallised from isopropyl alcohol giving the *di-2-hydroxyethyl* derivative (2·5 g), m.p. 75°, $[\alpha]_D^{20}$ −26° (c = 1 in $CHCl_3$). (Found: C, 53·5%; H, 6·0%; N, 2·9%. Calc. for $C_{23}H_{31}NO_{12}$: C, 53·8%; H, 6·1%; N, 2·7%.)

Methyl(p-*di*-2-*chloroethylaminophenyl*-2,3,4-*tri*-o-*acetyl*-β-D-*glucopyranosid*)*uronate.* Methanesulphonyl chloride (1·3 ml) was added to a solution of the dihydroxyethyl derivative (1·8 g) in dry pyridine (10·6 ml). After the initial exothermic reaction had subsided the mixture was heated at 80° for 20 min. Benzene was added to the cooled solution and pyridine was removed by repeatedly washing with water. After the addition of light petroleum (2 vol. b.p. 60–80°) to the dried ($MgSO_4$) benzene layer the solution was passed through a column of activated alumina (Spence Type H). The eluates contained an oil which solidified in contact with pentane. On crystallization from ethanol *methyl* (p-*di*-2-*chloroethylaminophenyl*-2,3,4-*tri*-o-*acetyl*-β-D-*glucopyranosid*) *uronate* was obtained as needles, m.p. 148° $[\alpha]_D^{25}$ −25° (c = 1 in $CHCl_3$). Yield 1 g. (Found: C, 50·5%; H, 5·4%; Cl, 12·5%; N, 2·5%. Calc. for $C_{23}H_{29}Cl_2NO_{10}$: C, 50·2%; H, 5·3%; Cl, 12·8%; N, 2·5%.)

Hydrolysis of methyl (p-*di*-2-*chloroethylaminophenyl*-2,3,4-o-*acetyl*-β-D-*glucopyranosid*) *uronate.* (a) The triacetyl methyl ester (500 mg) was added to a solution of tetra-*n*-butylammonium hydroxide (40 ml 0·1 N in toluene–methanol, B.D.H.) and heated under reflux for 1 hr. The cooled solution was passed through a column of

* The authors thank Dr. M. Jarman for advice regarding the hydrolysis of the glucuronic acid derivatives.

amberlite resin IR 120 H^+) and eluted with methanol. Evaporation of the eluates gave a resin which crystallized from isopropyl alcohol. The product, m.p. 137–138°, $[\alpha]_D^{25}$ −60° (c = 1 in $CHCl_3$) proved to be the *tetra*-n-*butylammonium salt* of (p-*di*-2-*chloroethylaminophenyl*-β-D-*glucopyranosid*) *uronic acid.* (Found: C, 58·7%; H, 8·7%; Cl, 10·7%; N, 4·6%. Calc. for $C_{32}H_{56}Cl_2N_2O_7$: C, 58·9%; H, 8·7%; Cl, 10·8%; N, 4·3%.)

This salt (500 mg) was dissolved in water (5 ml) and the pH adjusted to 2 by the addition of dilute aqueous hydrochloric acid (0·1 N). On evaporation, a dried ethyl acetate extract of this solution yielded (p-*di*-2-*chloroethylaminophenyl*-β-D-*glucopyranosid*) *uronic acid.* (II, R=R′=H, R″=CH_2CH_2Cl), m.p. 91–92°, $[\alpha]_D^{22}$ −61° (c = 1 in $CHCl_3$). (Found: C, 46·4%; H, 5·5%; Cl, 17·0%; N, 3·3%. Calc. for $C_{16}H_{21}Cl_2NO_7$: C, 46·8%; H, 5·2%; Cl, 17·3%; N, 3·4%.)

(b) A methanolic solution of ammonia (9 ml, 2·8 N) was added to the triacetyl methyl ester (5 g) dissolved in methanol (600 ml) and the mixture was left at room temperature overnight. The residue obtained on evaporating the solution to dryness under reduced pressure was placed on the top of a silica gel column (300 g Merck 7734) and eluted with methanol–chloroform (6 : 94). Early eluates contained unchanged material and this was followed by the *methyl ester* of (p-*di*-2-*chloroethylaminophenyl*-β-D-*glucopyranosid*) *uronic acid* (II, R=Me, R′=H, R″=CH_2CH_2Cl) (2·5 g) which after crystallization from nitromethane had m.p. 96°, $[\alpha]_D^{25}$ −80° (c = 1 in $CHCl_3$). (Found: C, 48·3%; H, 5·5%; Cl, 16·6%; N, 3·1%. Calc. for $C_{17}H_{23}Cl_2NO_7$: C, 48·1%; H, 5·5%; Cl, 16·7%; N, 3·3%.)

Final eluates from the column contained (p-*di*-2-*chloroethylaminophenyl*-β-D-*glucopyranosid*) *uronamide*, m.p. 190°, $[\alpha]_D^{24}$ −65° (c = 1 in $CHCl_3$) which was crystallized from a large volume of ethanol. (Found: C, 47·2%; H, 6·0%; Cl,17·0%; N, 6·7%. Calc. for $C_{16}H_{22}Cl_2N_2O_6$: C, 47·0%; H, 5·4%; Cl, 17·3%; N, 6·8%.)

p-*Di*-2-*chloroethylaminophenyl sulphate* (III). A solution of chlorosulphonic acid (2 ml) in dry ethanol-free chloroform (10 ml) was added dropwise during 10 min to a cooled (20°) solution of *p*-di-2-chloroethylaminophenol hydrochloride[7] (5·4 g) and 2,6-lutidine (11·6 ml) in dry chloroform (50 ml). After heating under reflux for 1 hr most of the solvents were removed under reduced pressure. The residue was triturated with ice-cold water (300 ml) for 1 hr and the solid was collected by filtration, washed with water and a little benzene and finally dried *in vacuo*. The product (7·9 g), m.p. 149–151°, on crystallization from methanol gave the *lutidine salt* of p-*di*-2-*chloroethylaminophenyl sulphate* as prismatic needles, m.p. 150–152°. (Found: C, 48·1%; H, 5·3%; Cl, 16·8%; N, 6·5%; S, 7·8%. Calc. for $C_{17}H_{22}Cl_2N_2O_4S$: C, 48·5%; H, 5·2%; Cl, 16·9%; N, 6·7%; S, 7·6%.)

A dry methanolic solution (47 ml) of sodium methoxide (20 m-mole) was added to a solution of the above lutidine salt (8·42 g, 20 m-mole) in warm dry methanol (100 ml). After keeping in a dark place for 3 hr the solvent was removed under reduced pressure and the solid was washed with benzene and then ether to remove residual lutidine. Addition of dry ether to a methanolic solution of the product afforded the crystalline *sodium salt* which was collected by filtration and dried *in vacuo* over phosphorus pentoxide. Yield: 5·0 g. (Found: C, 33·8%; H, 4·1%; Cl, 20·9%; N, 3·9%; Na, 7·0%; S, 9·1%. Calc. for $C_{10}H_{12}Cl_2NNaO_4S.H_2O$: C, 33·9%; H, 4·0%; Cl, 20·1%; N, 4·0%; Na, 6·5%; S, 9·0%.)

p-*Di*-2-*chloroethylaminophenyl phosphate* (IV). Phosphoryl chloride (16 ml) was

added at room temperature to stirred *p*-di-2-chloroethylaminophenol hydrochloride (11 g). After heating under reflux for 2 hr the mixture was added dropwise to stirred ice-cold water (40 ml). The resultant solution was heated at 60° for 45 min and then evaporated under reduced pressure with a trap of sodium hydroxide pellets in the vacuum line. Most of the residual water was removed by azeotropic distillation with benzene under reduced pressure and the syrupy product was finally dried over phosphorus pentoxide. Cyclohexylamine was added to a solution of the syrup (28·1 g) in methanol (200 ml) until the pH recorded was 9. After the addition of more methanol the crystalline precipitate was collected by filtration, washed with methanol and dried. This solid (60·9 g), m.p. 200–211° (decomp.) was extracted with water (300 ml), filtered and washed with a little benzene giving a product (23·7 g), m.p. 162–165°. Repeated extraction of this solid with boiling methanol and final concentration of the extracts to 300 ml under reduced pressure caused the separation of solid, m.p. >200°. Addition of ethyl acetate to the filtered solution afforded needles, m.p. 156–158°. Recrystallization from methanol–ethyl acetate gave the *cyclohexylamine salt* of p-*di*-2-*chloroethylaminophenyl phosphate*, m.p. 159–160°. (Found: C, 51·5%; H, 8·3%; Cl, 13·3%; N, 8·0%; P, 6·4%. Calc. for $C_{22}H_{40}Cl_2N_3O_4P$: C, 51·6%; H, 7·8%; Cl, 13·9%; N, 8·2%; P, 6·1%.) By concentrating the initial methanolic filtrate and processing the solid obtained as described above a further quantity of the *cyclo*hexylamine salt was obtained; total yield, 15·1 g.

On treating the *cyclo*hexylamine salt (1·02 g) in methanol solution with two equivalents of methanolic sodium methoxide as described above the *di-sodium salt* of *di*-2-*chloroethylaminophenyl phosphate* (597 mg) was obtained. (Found: C, 33·2%; H, 4·2%; Cl, 18·9%; N, 4·0%; Na, 13·2%; P, 8·1%. Calc. for $C_{10}H_{12}Cl_2NNa_2O_4P.\frac{1}{2}H_2O$: C, 32·7%; H, 3·5%; Cl, 19·4%; N, 3·8%; Na, 12·5%; P, 8·5%.)

Antineoplastic assay

The protocol for this assay is given by Wade *et al.*[8] Compounds were administered as a single intraperitoneal injection in the vehicle indicated. In Table 1 the results are reported as median lethal dose (LD_{50}), the dose required to produce 90 per cent inhibition of tumour growth (ED_{90}) and the chemotherapeutic index (C.I. = LD_{50}/ED_{90}).

TABLE 1. SCREENING AGAINST THE ADJ/PC6A MOUSE PLASMA CELL TUMOUR

Compound	Vehicle	LD_{50} (mg/kg)	ED_{90} (mg/kg)	C.I.
Glucuronic acid (II, R=R′=H,R″=CH_2CH_2Cl)	water	270	8·2	32·9
sodium salt	water	305	15·5	19·5
methyl ester (II, R=Me, R′=H, R″=CH_2CH_2Cl)	arachis oil	35	0·38	92
methyl ester triacetate (II, R=Me, R′=Ac, R″=CH_2CH_2Cl)	arachis oil	850	19	44·7
Sulphate (III), sodium salt	water	230	74	3·1
Phosphate (IV), sodium salt	water	113	3·8	29·7
(I, R=H)	arachis oil	70	1·0	70
(I, R=OH)	arachis oil	7	0·5	14

RESULTS

The results of the antineoplastic assay against the mouse plasma cell tumour, ADJ/PC6A, are given in Table 1. Appreciable activity is shown by the glucuronic acid derivatives and the sodium salt of the ethereal phosphate. Considerably lower activity is exhibited by the sodium salt of the ethereal sulphate. Highest activity is shown by the methyl ester of the glucuronic acid derivative which has a chemotherapeutic index somewhat higher than that of the parent aniline mustard (I, R=H), and six times that of the much more toxic unconjugated phenolic derivative (I, R=OH).

DISCUSSION

These conjugates were designed for use against those tumours possessing high glucuronidase, sulphatase or phosphatase activity. For a preliminary assessment of their tumour growth inhibitory potential they were submitted for assay against one of the standard alkylating-agent-sensitive mouse tumours. As a general rule highly ionised molecules such as the ethereal sulphate and phosphate will not readily pass through cellular and nuclear membranes to react with the presumed target site of the cytotoxic action of alkylating agents, namely nuclear DNA. The lower activity of the sulphate is probably due to this lack of penetration. However, the ethereal phosphate shows much higher activity which could indicate the presence of extracellular phosphatases. Significant proportions of the ethereal glucuronic acid will be in the unionized form at physiological pH and prior deconjugation need not be invoked to account for its considerable anti-tumour activity. Nevertheless, the neutral methyl ester and its triacetate show higher activity and these would be expected to reach the cell nucleus more readily than the free acid.

A range of animal tumours is now being screened for glucuronidase, sulphatase and phosphatase activity and the appropriate conjugate will be assayed against those tumours possessing the relevant deconjugase activity. The ultimate intention is to screen human tumours for deconjugase activity so as to guide the selection of agents for clinical application.

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