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PURIN-6-YL 6-DEOXY-1-THIO-β-D-GLUCOPYRANOSIDE. ENZYMOLOGY, CHEMISTRY, AND CYTOTOXICITY

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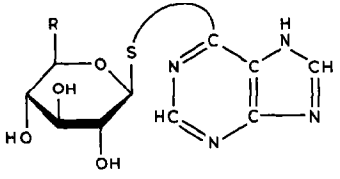
ABSTRACT

Purin-6-yl 6-deoxy-1-thio-β-D-glucopyranoside (**4**) is a substrate for almond β-glucosidase and a weak competitive inhibitor of bovine liver β-D-glucuronidase (K_i ~20mM). Both **4** and purine-protonated **4** undergo hydrolysis catalyzed by dilute acid in the pH range 0.17–2.59. These results are compared with those previously obtained with ammonium (purin-6-yl 1-thio-β-D-glucopyranosid)uronate, (purin-6-yl 1-thio-β-D-glucopyranosid)uronamide, purin-6-yl 1-thio-β-D-glucopyranoside, and purin-6-yl 2-deoxy-1-thio-β-D-glucopyranoside, and it is concluded that the data support an involvement of substituents at C-5 in producing productive Michaelis-complex conformers. The 6-deoxyglucoside is more active than the D-glucosiduronic acid in an L1210 mouse screen.

INTRODUCTION

During a study aimed at developing selective cytotoxic agents[1], some purin-6-yl 1-thio-β-D-glucopyranosides (**1–3**) were synthesized and found to be substrates



1 R = CO_2NH_4
2 R = $CONH_2$
3 R = CH_2OH
4 R = CH_3
5 R = CO_2Me

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for bovine liver β-D-glucuronidase[1,2]. The relative V_{max} values for the D-glucopyranosyluronate **1**, D-glucopyranosyluronamide **2**, and D-glucopyranoside **3** were 575, 1.5, and 1 respectively, at pH 4.7. A plot of log V_{max} *vs.* σ_I values[3], the comparative inductive substituent constant for the *R* groups, gave[2] ρ_I −5 (r 0.971). This was interpreted to mean that the electron-releasing *R* groups primarily stabilize an electron-deficient transition state[2,3]. Alternatively, the result could reflect the different abilities of the *R* groups to participate in the formation of reactive Michaelis-complex conformers[4,5].

To distinguish between these possibilities, we synthesized purin-6-yl 6-deoxy-1-thio-β-D-glucopyranoside (**4**) for testing as a substrate for β-D-glucuronidase. If the reactivity differences among **1–3** were due to the different abilities of the *R* groups to stabilize transition states by releasing electrons, then **4** [σ_I (CH_3) −0.04] would be approximately as reactive[3] as **3** [σ_I (CH_2OH) 0.1]. If, on the other hand, polar *R* groups are necessary to produce reactive complexes, then **4** would not be a substrate for β-D-glucuronidase. We report herein the hydrolysis of **4** catalyzed by almond β-D-glucosidase and dilute aqueous acid, and its activity in a L1210 mouse screen.

RESULTS AND DISCUSSION

β-D-Glucuronidase (3000 u/mL) did not catalyze the hydrolysis of purin-6-yl 6-deoxy-1-thio-β-D-glucopyranoside (**4**) at pH 4.7 in the range 0.5–4mM. Thus, the rates of hydrolysis in the absence or presence of enzyme were within ±15% of each other. However, **4** was a weak competitive inhibitor of the enzyme (K_i ~20mM) when [(α-4:4′-trihydroxytriphenylmethane-2-carboxylic acid-4-yl) (phenolphthalein) β-D-glucopyranosid]uronic acid was used as a substrate. This result may be compared with the apparent dissociation constants (K_m) for **1** (0.13mM), **2** (9.3mM), and **3** (0.32mM) obtained under comparable conditions. The result obtained with **4** indicated that the relative reactivities of **1–3** are not solely due to different electron-releasing capabilities of the CO_2^-, $CONH_2$, and CH_2OH groups and accompanying electronic, transition-state stabilization. The result does support the involvement of the *R* group in productive binding of substrates to the enzyme. In the series **1–4**, the groups at C-6 of **1–3** are able to form polar bonds with the enzyme that lead to more (for **1**) or less (for **2** and **3**) productive Michaelis complexes, whereas the 5-methyl group of **4** cannot form a polar bond and a productive complex. For glucosyluronic derivatives, Wang and Touster[4] suggested that a cationic group of rat liver β-D-glucuronidase, probably a histidinium ion, is involved in binding. This inferrence was based on the effect of ionic strength on V_{max} and K_m, the failure of *p*-nitrophenyl β-D-glucopyranosiduronamide to bind to the enzyme, and the greater inhibitory effect of an isoxazolium salt bearing a sulfonate group than one that did not bear this group.

Although these results suggest a requirement for a polar 6-substituent for activity, they do not establish that glucosyluronic derivatives induce formation of a

catalytically active Michaelis conformer. However, this possibility is supported by the results of a comparison of K_m and V_{max} values for **1, 2,** and **3**. The values of K_m (**1**, 0.13mM, **2**, 9.3mM, and **3**, 0.32mM) do not differ by more than 70-fold, yet the values of V_{max} differ by 500-fold. This difference of reactivity, especially that between **1** and **3** whose K_m values are very similar, points to a different kind of binding, ranging from productive for **1** to barely productive for **2** and **3** having polar 6-groups. In the usual way, productive binding here refers to alignment of the reacting groups and flattening of the pyranose ring to resemble the transition state. That β-D-glucuronidase can, in fact, undergo a change in conformation upon binding the sugar derivatives is supported by the demonstration of Keller and Touster[6] that D-glucoro-1,4-lactone, a putative, transition-state, analog inhibitor having K_i 40μM, induces a conformation change in preputial gland β-D-glucuronidase.

D-Glucosidases are known to catalyze the hydrolysis of various 6-deoxy-6-substituted D-glucopyranosides, including *p*-nitrophenyl 6-deoxy-β-D-glucopyranoside[7]. Compound **4** was a substrate for almond β-D-glucosidase. At pH 4.7, in acetate buffer at 30°, V_{max} was 0.8 μmol/min/enzyme unit, and K_m 10mM. The relative V_{max} values of **3** (refs. 1, 2), **4**, and the 2-deoxy derivative[8] were 95, 2.5 and 1. It should be noted that this reactivity is not that predicted for nonenzymic thioacetal hydrolysis. In fact, the predicted order of reactivity would be the reverse of that given for the enzyme reactions (see below). Here, we suggest that the order of reactivity obtained with β-D-glucosidase could be rationalized if the glucosides effected a degree of conformation change in the enzyme, as suggested for β-D-glucuronidase and glycosidases generally, that promoted catalysis. Optimal conformation change is effected by glucosides, with the 2-hydroxyl group playing a prominent role[9].

$$\mathbf{4}(H^+) \underset{K_a}{\rightleftharpoons} \mathbf{4} + H^+ \qquad (1)$$

$$\mathbf{4} + H_2O \xrightarrow{k_a, H^+} \text{6-MP} + \text{6-deoxy-D-glucose}$$

$$\mathbf{4}(H^+) + H_2O \xrightarrow{k_b, H^+} \text{6-MP} + \text{6-deoxy-D-glucose}$$

Previously, we determined the kinetics of hydrolysis of **3** and its 2-deoxy derivative[8,10]. The simplest mechanism of hydrolysis is that shown for **4** (Eq. *1*) where **4**(H⁺) is purine-protonated **4**, and k_a and k_b are mixture constants that report a pretransition-state, equilibrium protonation of thioacetals and a rate-determining bond-breaking step. The kinetic solution of this mechanism leads to the rate law (Eq. *2*), where for **4**, k_a = 2.21 L/mol/min, k_b = 0.11 L/mol/min, and pK_a = 2.1, as determined from a least-squares, nonlinear regression analysis. From these data and those previously reported, it may be shown that the relative reactivities of the

2-deoxy derivative of **3**, **4**, and **3** are 2000, 12, 1 (k_a term) and 100, 2, 1 (k_b term), which order is the reverse of that for β-D-glucosidase-catalyzed hydrolysis (see above).

$$\text{Rate} = \{k_a K_a [H^+] + k_b [H^+]^2\}\{[\mathbf{4}] + [\mathbf{4}(H^+)]\}/\{K_a + [H^+]\} \quad (2)$$

Goodman *et al.*[13,14] synthesized several potentially selective 6-mercaptopurine-glycosides (6-MP), including **3**, and found that **3** was active against sarcoma 180 and adenocarcinoma 755, and that it had relatively little toxicity compared to 6-MP. However, **3** was rapidly cleared and it did not penetrate cells well. The 1-thio-D-glucopyranosiduronic acid **1** also was designed to be a potentially selective prodrug of 6-MP. This was based on work of Bicker[11] who showed that phenolphthalein accumulated in tumor tissue in rats administered (phenolphthalein β-D-glucopyranosid)uronic acid. The 1-thio-β-D-6-deoxyglucopyranoside **4**, a potential substrate for β-D-glucosidase, was designed to be a more lipophilic prodrug of 6-MP. This was based on the report by Arakawa *et al.*[12] that 5-fluoro-4-(methyl β-D-glucopyranosyluronate)-1*H*-2-oxopyrimidine increased the life span of mice with experimental tumors, it exhibited very low toxicity, and the prodrug was apparently hydrolyzed by β-D-glucosidase. Table I gives the results obtained with **1** and **4** in the L1210 mouse screen employed by the National Cancer Institute. It can be seen that **1** was virtually inactive by the NCI criterion. The methyl ester was scarcely more active, and it may be suggested that these compounds are also rapidly cleared

TABLE I

BIOLOGICAL TESTING OF 6-MERCAPTOPURINYL GLYCOSIDE PRODRUGS IN MICE

Compound	*Dose (mg/kg)*	*T/C (%)*	*BWD (g)*
1	400	115	−2.7
	200	112	−0.8
	100	109	0
5	400	135	−0.8
	200	122	−0.7
	100	109	−0.2
4	400	170	−1.8
	200	150	−0.5
	100	131	−0.5
6-MP[c]	160	142	−4.5
	80	151	−3.0
	40	130	−1.7
	20	116	−1.1

[a]Test evaluation expressed as a percent of the control evaluation, providing a measure of effectiveness of the compound. Survival systems indicate a degree of success when T/C percents exceed 125. These data are the results of screening performed under the auspices of the Developmental Therapeutics Program, Division Cancer Treatment, National Cancer Institute, Bethesda, MD. [b]Body-weight difference = test-group, body-weight change minus control-group, body-weight change. [c]Data provided by the NCI for 6-MP are median values based on 37 separate tests.

and may not penetrate cells sufficiently. The more lipophilic **4** was active by the NCI criterion, but not as active as 6-MP.

We have no information concerning whether **4** penetrates cancer cells or is hydrolyzed *in situ*, either chemically or enzymically; it may be active in its own right. However, there is evidence that **1** has moderate selectivity for leukemic cells[1], and **1** and **4** appear to be less toxic (more selective?) than 6-MP, when body-weight loss is used as a criterion of toxicity (Table I).

EXPERIMENTAL

General methods. — Melting points (uncorrected) were taken with a Mel-Temp apparatus on open capillary tubes. Optical rotation was measured with a Perkin–Elmer 141 polarimeter. U.v. spectra were recorded with a Cary 118 C spectrophotometer, i.r. spectra with a Nicolet FTIR spectrophotometer, and ^{1}H-n.m.r. spectra with a Varian A-60 spectrometer. pH was measured with a Radiometer PHM 26 with a GK 2301 B electrode and enzyme rates were recorded with a Gilford 2400 spectrophotometer.

The syntheses of ammonium (purin-6-yl 1-thio-β-D-glucopyranosid)uronate (**1**) and purin-6-yl 1-thio-β-D-glucopyranosiduronamide (**2**) were previously described[1]. Purin-6-yl 1-thio-β-D-glucopyranoside (**3**) was synthesized by the method of Goodman *et al.*[14]. 1,2,3,4-Tetra-*O*-acetyl-6-deoxy-6-iodo-β-D-glucopyranose (**5**) was synthesized by the method of Hardegger and Montavon[15]. Bovine liver β-D-glucuronidase and almond β-D-glucosidase were purchased from Sigma Chem. Co. (St. Louis, MO 63 178).

1,2,3,4-Tetra-O-acetyl-6-deoxy-β-D-glucopyranose (**6**). — Compound **5** (13.1 g, 29 mmol) was dissolved in dichloromethane (60 mL). Triethylamine (10 mL) was added, followed by 10% Pt–C (0.4 g). The mixture was placed in a Parr hydrogenator and shaken in the presence of H_2 for 16 h (325 kPa). The mixture was filtered through Celite, washed with 0.1M HCl (50 mL), water (2 × 50 mL), and dried (Na_2SO_4). Evaporation on the rotary evaporator gave a syrup which crystallized from ethanol to give **6** (7 g, 74%), m.p. 146–147°; lit. m.p. 151° (ref. 15), 146° (ref. 16); ^{1}H-n.m.r. ($CDCl_3$): δ 5.8 (d, 1 H, H-1), 2.2–2.0 (12 H, CH_3CO), and 1.3 (d, 3 H, H_3-6).

Purin-6-yl 2,3,4-tri-O-acetyl-6-deoxy-1-thio-β-D-glucopyranoside (**7**). — 6-Mercaptopurine monohydrate (0.857 g, 6.86 mmol) was dissolved in warm (steam bath), dry *N,N*-dimethylformamide (25 mL), benzene (15 mL) added, and the benzene–water azeotrope removed on the rotary evaporator. Anhydrous K_2CO_3 (0.825 g, 5.97 mmol) was added with stirring, and to this mixture was added 2,3,4-tri-*O*-acetyl-6-dideoxy-α-D-glucopyranosyl bromide[16] (2 g, 5.66 mmol) dissolved in dry *N,N*-dimethylformamide (10 mL). The mixture was stirred for 18 h, during which time it became dark. Chloroform (250 mL) was added, and the solution was washed with cold, saturated $NaHCO_3$ (2 × 10 mL), cold water (2 × 100 mL), and dried (Na_2SO_4). The solution was reduced to 50 mL, decolorized with charcoal,

filtered through Celite, and dried (Na_2SO_4). It was reduced further to 10 mL and layered evenly on the top of a silica gel H column (9 × 4 cm). Fractions (10 × 200 mL) were collected and evaporated, and the residue crystallized from methanol, m.p. 265–270°; ^{1}H-n.m.r. ($CDCl_3$): δ 8.8 (s, 1 H, purine), 8.2 (3, 1 H, purine), 2.2–2.0 (18 H, CH_3CO), and 1.3 (2 d, 6 H, CH_3). This compound, which was presumed to be the diglycoside[17], was not further investigated. The column was further eluted with 4:1 chloroform–acetone (10 × 200 mL), the fractions were pooled, and the solvent was evaporated to give **7** (0.6 g, 21% after crystallization from chloroform–methanol), m.p. 219–221°, $[\alpha]_D^{25}$ +43.4° (*c* 2, chloroform); λ_{max}^{EtOH} 279 nm, log ε 4.17; ν_{max}^{KBr} 1767, 1710, 1569, and 3253 cm^{-1}; ^{1}H-n.m.r. ($CDCl_3$): δ 8.8 (s, 1 H, purine), 8.3 (s, 1 H, purine), and 1.3 (d, 3 H, CH_3-6).

Anal. Calc. for $C_{17}H_{20}N_4O_7S \cdot 0.5\ H_2O$: C, 47.11; H, 4.88; N, 12.93. Found: C, 47.35; H, 4.83; N, 12.80.

Purin-6-yl 6-deoxy-1-thio-β-D-glucopyranoside (**4**). — In a 50-mL flask fitted with a drying tube and a gas-inlet tube was placed **7** (0.5 g, 1.18 mmol) dissolved in methanol (25 mL). The flask was partially immersed in an ice bath and the solution saturated with NH_3 during 0.5 h, after which the solution was removed from the bath and allowed to stand at room temperature for 15 h. Removal of methanol and NH_3 on the rotary evaporator gave a white solid which was washed with chloroform, crystallized from pentane, was suspended in water, and lyophilized to give **4** (0.33 g, 94%), m.p. 160–162°, $[\alpha]_D^{25}$ −20° (*c* 0.5, dimethyl sulfoxide); λ_{max}^{EtOH} 279 nm log ε 4.09; ν_{max}^{KBr} 1570 and 3500 cm^{-1}; ^{1}H-n.m.r. [($^2H_6$)Me_2SO]: δ 8.7 (s, 1 H, purine), 8.4 (s, 1 H, purine), 5.9 (d, 1 H, H-1), and 1.2 (d, 3 H, CH_3-6).

Anal. Calc. for $C_{11}H_{14}N_4O_4S \cdot 1.5\ H_2O$: C, 40.61; H, 5.27; N, 17.22. Found: C, 40.52; H, 5.27; N, 17.18.

Kinetics. — In this study, one unit of β-D-glucuronidase activity is defined as the amount of enzyme required to liberate 1 μg/h of phenolphthalein from (phenolphthalein β-D-glucopyranosid)uronic acid at 30° and pH 5. One unit of β-D-glucosidase activity is defined as the amount of enzyme required to liberate 1 μg/h of *p*-nitrophenol from *p*-nitrophenyl β-D-glucopyranoside at 30° at pH 4.7. Kinetics methods have been previously described[1]. Stock solutions of **2**, **3**, and **4** in dimethyl sulfoxide were used. The maximum concentration of dimethyl sulfoxide (1.7%) introduced in reactions of these compounds had no affect on rates. Hydrolysis of **2** was not catalyzed by 1% bovine serum albumin, a model protein used to test for nonspecific protein catalysis. Compound **2** was not hydrolyzed by β-D-glucuronidase in the presence of D-glucaro-1,4-lactone, a competitive inhibitor (K_i 0.1 μM) of the enzyme. Hydrolysis of **2** and **3**, catalyzed by β-D-glucuronidase, was not affected by D-glucaro-1,4-lactone, an inhibitor (K_i 0.1 μM) of β-D-glucosidase. Acid-catalyzed hydrolysis of **4** was carried out under pseudo-first-order conditions at 30° and ionic strength 1 M (KCl). Production of 6-mercaptopurine was monitored at 325 nm, and pseudo-first-order rate constants were the average of 2 or 3 experiments at each of 12 different hydrogen-ion concentrations that ranged from 3.5 mM to 1.0 M.

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