NOJIRIMYCIN AND D-GLUCONO-1,5-LACTONE AS INHIBITORS OF CARBOHYDRASES

E. T. REESE AND F. W. PARRISH,
U. S. Army Laboratories, Natick, Massachusetts 01760 (U. S. A.)

AND M. ETTLINGER
University of Copenhagen, Copenhagen (Denmark)

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SUMMARY

Nojirimycin and D-glucono-1,5-lactone are powerful inhibitors of glucosidases, but poor inhibitors of exo-glucanases, endo-glucanases, and related enzymes. Nojirimycin can be used to differentiate between α-glucosidase and exo-α-D-glucanase, and between β-glucosidase and exo-β-D-glucanase. The structural similarity of the two inhibitors is discussed in relation to the mechanism of action of the enzyme types. Some data on D-glucal are included.

INTRODUCTION

Nojirimycin is an antibiotic that differs from D-glucose only by the substitution of an NH group for oxygen in the ring[1,2]. As a result, it is an interesting compound for physiological and biochemical investigations. In previous work, we[3,4] and others[5–8] have investigated the ability of D-glucono-1,5-lactone to inhibit glucosidases and related enzymes. Niwa *et al.*[2] have shown that nojirimycin is an even more potent inhibitor of glucosidases than D-glucono-1,5-lactone, and that both compounds show the competitive type of inhibition. In this report, we have extended that work, paying particular attention to effects on exo-glucanases as compared with those on glucosidases.

EXPERIMENTAL

The amount of a compound required to obtain 50% inhibition of enzyme was determined by incubating various concentrations of inhibitor (0.5 ml) with substrate (0.5 ml) and enzyme (0.5 ml). The substrates were used at concentrations $5–10 \times 10^{-3}$ M in a reaction mixture buffered with a citrate solution at pH 4.5 (25.7 ml of 50mM citric acid and 23.3 ml of 50mM sodium citrate). These concentrations are slightly higher than those used by Niwa *et al.*[2], but less than required for saturation of the enzyme. Our work with nojirimycin (courtesy of S. Inouye) was limited to use of the bisulfite complex, a more stable form than nojirimycin itself[2], (although both are much more

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TABLE I

EFFECT OF NOJIRIMYCIN HYDROGEN SULFITE, D-GLUCONO-1,5-LACTONE, AND D-GLUCAL ON GLUCOSIDASES AND EXO-GLUCANASES

Enzyme	*Source of enzyme*	*Substrate*	*Concentration of substrate* 10^{-3} *M*	*Molar concentration required to give 50% inhibition*		*R I/S 50*		
				Nojirimycin hydrogen sulfite,	D-*Glucono-1,5-lactone*	*Nojirimycin hydrogen sulfite*	D-*Glucono-1,5-lactone*	D-*Glucal*
α-Glucosidase (EC 3.2.1.20)	*Aspergillus niger* QM 877	Maltotriitol	6.0	2.2×10^{-5}	2.2×10^{-3}	0.004	0.37	1.0
		Maltohexaitol	6.0	1.3×10^{-5}	1.8×10^{-3}	0.002	0.30	
		Methyl α-D-glucopyranoside	30.9	0.5×10^{-5}	1.7×10^{-3}	0.0002	0.05	
		Phenyl α-D-glucopyranoside	5.0	5.0×10^{-5}	28×10^{-3}	0.01	6.[a]	
	Paecilomyces varioti QM 10a	Maltotriitol	6.0	3.1×10^{-5}	2.2×10^{-3}	0.005	0.37	0.5
		Phenyl α-D-glucopyranoside	5.0	0.9×10^{-5}	28×10^{-3}	0.002	6[a]	
	Penicillium parvum QM 1878	Maltotriitol	6.0	1.6×10^{-5}	0.8×10^{-3}	0.003	0.13	
		Maltohexaitol	6.0	1.1×10^{-5}	0.9×10^{-3}	0.002	0.15	
	Trichoderma viride QM 6a	Maltotriitol	6.0	8.0×10^{-5}	0.8×10^{-3}	0.013	0.13	
Exo-α-glucanases (glucoamylase, EC 3.2.1.3)	*Aspergillus niger* (Diazyme 455, Miles Co.)	Maltotriitol	6.0	3.0×10^{-3}	4.2×10^{-3}	0.50	0.70	30[a]
		Maltohexaitol	6.0	19×10^{-3}	6.0×10^{-3}	4.8	1.5	50[a]

	Endomyces sp. (Matulase, Matsutani Co.)	Maltotriitol	6.0	1.7×10^{-3}	0.7×10^{-3}	0.28	0.12	30[a]
		Maltohexaitol	6.0	29×10^{-3}	4.5×10^{-3}	4.8	0.75	
β-Glucosidase (EC 3.2.1.21)	Almond emulsin (gift of Dr. Richtmyer)	Laminaratriitol	6.0	2.5×10^{-6}	2.6×10^{-5}	0.00042	0.0043	
		Salicin	10.0	4.6×10^{-6}	1.6×10^{-5}	0.00046	0.0016	1.7
	Aspergillus luchuensis QM 873	Laminaratriitol	6.0	1.1×10^{-6}	2.6×10^{-5}	0.00018	0.0043	
		Salicin	10.0	4.0×10^{-6}		0.00040		0.32
	Aspergillus niger QM 877	Laminaratriitol	6.0	0.6×10^{-6}	2.8×10^{-5}	0.00010	0.0046	
	Aureobasidium pullulans QM 72c	Salicin	10.0	1.9×10^{-6}	2.2×10^{-5}	0.00019	0.0026	0.4
	Penicillium melinii QM 1931	Laminaratriitol	6.0	7.0×10^{-6}	8.4×10^{-5}	0.0012	0.014	
		Salicin	10.0	1.4×10^{-6}	1.6×10^{-5}	0.00014	0.0016	
Exo-β 1,3-glucanase (EC 3.2.1.–)	*Basidiomycete* sp. QM 806	Laminaratriitol	6.0	1.9×10^{-4}	56×10^{-3a}	0.03	9[a]	30[a]
		Laminaran	(5 mg/ml)	3.4×10^{-3}	100×10^{-3a}	1.7[b]	50[ab]	
	Chrysosporium pruinosum QM 826	Laminaratriitol	6.0	2.7×10^{-4}	56×10^{-3a}	0.04	9[a]	30[a]
		Laminaran	(5 mg/ml)	4.2×10^{-4}	37×10^{-3a}	0.2[b]	18[ab]	

[a]The concentration of inhibitor required to give 50% inhibition was not reached, and the values shown are extrapolations based on the data obtained.
[b]R values assuming a degree of polymerization of 20 for laminaran.

stable than the sugar lactones). The nojirimycin complex, at 2 mg/ml of citrate buffer, was stable for at least two weeks at 4°. D-Glucono-1,5-lactone solutions were prepared just prior to usage, as their half-life at pH 4.5 is only about 60 min. The hydrolysis times were kept as short as possible, usually 15–30 min.

The enzymes used were previously described[3,4]. Hydrolysis was measured as the rate of production of reducing sugar at 35° per unit time. The extent of hydrolysis did not exceed 25% of the theoretical under these conditions. The R I/S 50 values (Table 1) are ratios of the molar concentration of inhibitor (required for 50% inhibition) to the molar concentration of substrate. The most potent inhibitors have the lowest R values.

RESULTS

The current work is an extension of that previously reported[3,4], in which it was shown that D-glucono-1,5-lactone is much more inhibitory to β-glucosidases than to α-glucosidases, and that exo-glucanases are relatively unaffected by the lactone. Our data (Table I) support those of Niwa[2] in showing that nojirimycin is a more powerful inhibitor than D-glucono-1,5-lactone. We further find that nojirimycin (like D-glucono-1,5-lactone) is more inhibitory to β-glucosidases than to α-glucosidases (nojirimycin by a factor of 10; the lactone by a factor of 100).

In previous work[3,4], we showed that the lactone strongly inhibited β-glucosidases but not exo-β-1,3-glucanases, thus serving as a means of differentiating between the two. It could not, however, differentiate between the two types of α-enzyme. Now it appears that nojirimycin can be used for this purpose. It is a very good inhibitor of α-glucosidase, much better than D-glucono-1,5-lactone, and it is relatively ineffective against the exo-enzyme glucoamylase. Perhaps the best substrate to be used for characterization of α-enzymes based on nojirimycin inhibition is maltohexaitol. This compound is a substrate for both enzymes, and with it the difference between α-glucosidase and exo-α-D-glucanase is greatest (2000 times as much nojirimycin is needed to inhibit the latter enzyme as to inhibit the former). More readily available substrates, however, can also be used for this purpose.

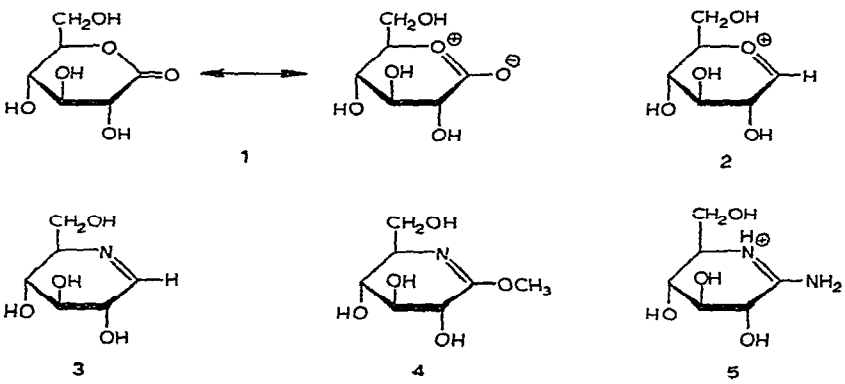
α,α-Trehalase (a Neurospora preparation kindly supplied by Dr. A. Sussman) is relatively insensitive to the inhibitors tested (Table II). In this, and in its high linkage specificity, it behaves more nearly like exo-glucanases than like α-glucosidases.

The effect of the two inhibitors has been tested on a variety of other enzymes (Table II). First, there are other enzymes with a specific requirement for the glucopyranose ring: (a) a thioglucosidase active against mustard oil glucosides, and (b) glucose oxidase. Secondly, there are β-glycosidases where the difference between the glycosidic sugars and D-glucose is in the position of a single hydroxyl group, (a) β-mannosidase and (b) β-galactosidase. All of the above, invertase and β-xylosidase, were inhibited relatively little by nojirimycin, and only rarely by D-glucono-1,5-lactone. Other enzymes, acting on chains of two or more D-glucosyl residues (*e.g.* amylase, cellulase, etc.), are scarcely affected by the inhibitors.

D-Galactal was found to be an excellent competitive inhibitor of β-galactosidase by Lee[9], K_i/K_m ratios being 0.05–0.006 for experiments done at pH 4.0. It was much less effective against α-galactosidases and other glycosidases. Our results with D-glucal show it to be a much less effective inhibitor of the corresponding glucosidases. (Table I; R I/S 50 values approximate K_i/K_m ratios).

DISCUSSION

Although Conchie, Levvy, and co-workers have investigated the inhibition of glycopyranosidases by D-aldono-1,5-lactones in numerous examples, they have not discussed the mechanism of inhibition, except to suggest[5] that the carbonyl groups of enzyme-bound lactones might be hydrated. Leaback[10] pointed out that D-glucono-1,5-lactone (1) can assume, as in fact[11] it does in the crystal, a half-chair conformation similar to that of a D-glucopyranosyl cation (2). He went on to postulate that the high affinity of certain glycosidases for the corresponding 1,5-lactones reflects steric similarity between the lactones and glycopyranosyl



portions of transition states in enzymic hydrolyses and that these hydrolyses involve intermediates having half-chair conformations (the differences and relations between transition states and intermediates were not discussed). No significance was ascribed to the common electrophilic character of C-1 in the lactones and glycosyl ions.

A half-chair conformation can extend not only to transition states and intermediates during enzymic hydrolyses of pyranosides, but also to the enzyme-bound substrates[12], so the assumption of intermediates having the same conformation as that of lactone inhibitors is not verified. There are additional possible reasons, however, to support an analogy between the binding of lactones and that of glycopyranosyl ions. One difference between glucosidases that are inhibited by D-glucono-1,5-lactone and those, such as exo-glucanases, that are not, is that the former cleave substrates with net retention of anomeric configuration, the latter with inversion[3]. Hydrolyses with net retention are the ones that can be expected to be two-step

reactions having an enzyme-bound glucosyl intermediate. The attachment of the D-glucopyranosyl ion to, and its stabilization by, the enzyme may be thought of as a intermediate 1 state between electrostatic and covalent bonding to, for example, a carboxylate group[13–15], in terms vividly expounded by Jencks[12]. If D-glucono-1,5-lactone combines analogously with an enzyme, the presence of a partial positive charge at C-1 of the lactone is important.

TABLE II

EFFECT OF NOJIRIMYCIN HYDROGEN SULFITE AND OF D-GLUCONO-1,5-LACTONE ON VARIOUS ENZYMES

Enzyme	*Source*	*Substrate*	*Concentration of substrate* ($\times 10^{-3}$ M)	*Inhibition*[a], %	
				Nojirimycin (1.9×10^{-3} M)	D-*Glucono-1,5-lactone* (17×10^{-3} M)
α, α-Trehalase (EC 3.2.1.28)	*Aureobasidium pullulans* QM 72c	α,α-Trehalose	5.3	37	71
	Penicillium brefeldianum QM 1872	α,α-Trehalose	5.3	11	3
	P. parvum QM 1878	α,α-Trehalose	5.3	22	37
Myrosinase	Yellow mustard	Sinigrin	7.5	18	27
(EC 3.2.3.1)	*Aspergillus sydowi* QM 31c	Sinigrin	7.5	17	93
Glucose oxidase (EC 1.1.3.4)[b]	Worthington Biochemical Corp.	D-Glucose	5.6	5	
β-Mannosidase (EC 3.2.1.25)	*Penicillium funiculosum* QM 474	Mannotriitol	6.0	17	22
	P. ochro-chloron. QM 477	Mannotriitol	6.0	17	79
	Snail (*Helix pomatia*)	Mannotriitol	6.0	27	29
β-Galactosidase (EC 3.2.1.23)	*Aspergillus niger* QM 877	Methyl β-D-galactoside	30	0	0
	Penicillium melinii QM 1931	Methyl β-D-galactoside	30	22	3
β-Xylosidase (EC 3.2.1.37)	*Penicillium wortmanni* QM 7323	Methyl β-D-xyloside	12	0	
	P. islandicum QM 7571	Methyl β-D-xyloside	12	0	
	Botryodiplodia QM 7092	Methyl β-D-xyloside	12	0	
β-Fructosidase (sucrase EC 3.2.1.26)	*Aureobasidium pullulans*[c] QM 72c	Raffinose	5.8	0	13
	Aureobasidium pullulans[c] QM 72c	Raffinose	5.8	10	30
	Saccharomyces (Invertase, N.B.C.)	Raffinose	5.8	12	23
α-Amylase (EC 3.2.1.1)	*Aspergillus oryzae* (Clarase, Miles Co.)	β-Schardinger dextrin	(5 mg/ml)	7	62
	Aspergillus oryzae (Clarase, Miles Co.)	Maltohexaitol	6.0	0	27
β-Amylase (EC 3.2.1.2)	Wallerstein Lab.	Maltohexaitol	6.0	5	7

TABLE II (*continued*)

EFFECT OF NOJIRIMYCIN HYDROGEN SULFITE AND OF D-GLUCONO-1,5-LACTONE ON VARIOUS ENZYMES

Enzyme	*Source*	*Substrate*	*Concentration of substrate* ($\times 10^{-3}$ M)	*Inhibition*[a], %	
				Nojirimycin (1.9×10^{-3} M)	D-*Gluconono-1,5-lactone* (17×10^{-3} M)
Cellulase (EC 3.2.1.4)	*Basidiomycete* QM 806	Carboxymethyl cellulose	(3 mg/ml)	24	0
		Cellotetraitol	6.0	14	35
	Chrysosporium pruinosum QM 826	Carboxymethyl cellulose	(3 mg/ml)	41	38
		Cellotetraitol	6.0	53	50
	Trichoderma viride QM 6a	Carboxymethyl cellulose	(3 mg/ml)	23	44
		Cellotetraitol	6.0	14	35
Endo-β-1,3-glucanase (EC 3.2.1.6)	*Basidiomycete* sp. QM 806 (S353CA fr 7-11)	Laminaratriitol	6.0	60	68

[a]The inhibition values are those for the highest concentration of inhibitor tested. It should be noted that the lactone was tested at much higher concentration than the nojirimycin hydrogen sulfite, because of lack of sufficient nojirimycin. [b]Hydrogen sulfite alone inhibits the glucose oxidase system. The figure for nojirimycin hydrogen sulfite is corrected for the hydrogen sulfite effect. Assay at pH 7.0. [c]The two preparations from *A. pullulans* differed in their relative activities on sucrose and on raffinose.

Nojirimycin, 5-amino-5-deoxy-D-glucopyranose, is believed to exist in solution largely as a mixture of anomers of the cyclic form[1]. The pK_a of its conjugate acid[16] is 5.3, near the pH of glucosidase assays, so we do not known whether the active inhibitor is neutral, or cationic, or both. Nojirimycin solutions have a potential complexity that can be appreciated from study of Paulsen's review[17] and subsequent papers[18–21] dealing with cyclic sugars having nitrogen in the ring. Let us assume that, under our conditions, the hydrogen sulfite adducts, acyclic or cyclic, were completely dissociated. The monomeric forms of nojirimycin that might be present[1] include, besides the pyranose, its 1,6-anhydro derivative[17], the 5-amino-5-deoxy-D-glucofuranose, and the imine (3). One can estimate[16,22] pK_a values of 8.5 for the furanose and 3.5–4 for the imine, and so the effect of pH on equilibrium composition. We know, however neither equilibrium constants, nor rates of interconversion, nor the rate of irreversible decomposition[17] through deprotonation of iminium ion, with shift of the double bond, to enolamine.

The inhibitory effect of nojirimycin can be attributed[2] to the pyranose anomers. The neutral bases or their cations would then be construed as D-glucose analogs having enhanced affinity for glucosidases. An alternative possibility is that the active inhibitor is the imine (3) or its conjugate acid. These molecules, especially the iminium ion, are direct analogs of the D-glucopyranosyl ion (2).

Development of additional, effective glucosidase inhibitors, preferably lacking

the instability of nojirimycin or D-glucono-1,5-lactone[23], would offer practical and theoretical benefits. 5-Amino-5-deoxy-D-glucono-1,5-lactam is reported[2] to be nearly as inhibitory as D-glucono-1,5-lactone to apricot emulsin, but less active than the lactone by a factor of 50 towards *Trichoderma viride* β-glucosidase. Further analogs, such as the *O*-methyl lactim (**4**) and cyclic amidinium ion (**5**) [24,25] or the thiolactam and *S*-methyl derivatives, are attractive goals for study.

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