Carbohydrate Research, 37 (1974) 321–329

THE INHIBITORY ACTIVITY OF 2-ACETAMIDO-2-DEOXY-D-GLUCONOLACTONES AND THEIR ISOPROPYLIDENE DERIVATIVES ON 2-ACETAMIDO-2-DEOXY-β-D-GLUCOSIDASE

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(Received June 6th, 1974; accepted June 27th, 1974)

ABSTRACT

2-Acetamido-2-deoxy-D-glucono-1,4-lactone (**1**) and 2-acetamido-2-deoxy-D-gluconic acid (**3**) have been examined for inhibitory activity against 2-acetamido-2-deoxy-β-D-glucosidase from bull epididymis. Crystalline **1** and **3** were compared with the known, crystalline 2-acetamido-2-deoxy-D-glucono-1,5-lactone (**2**), and a correlation of the activities of these compounds with various factors is presented. The inhibition constant of the 1,5-lactone **2** is lower (0.45μM) than that (4.43μM) of the 1,4-lactone **1**. The effect of time is the opposite; whereas the activity of solutions of **2** decreases with time, solutions of **1** show an increase in inhibitory power, but both reach an equilibrium after 5 h. The free acid **3** exhibits no inhibitory activity. 2-Acetamido-2-deoxy-5,6-*O*-isopropylidene-D-glucono-1,4-lactone (**4**) and 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-D-glucono-1,5-lactone (**5**), which are appropriately protected to prevent conversion into the other lactone isomer, were also tested; **4** has 1/1000th the activity of **5**.

INTRODUCTION

The inhibitory activity of 2-acetamido-2-deoxy-D-gluconolactones on 2-acetamido-2-deoxy-β-D-glucosidase (β-*N*-acetylglucosaminidase; 2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) has been extensively investigated[1–4], and it is generally considered[5] that the 1,5-lactone is more active as an inhibitor than its corresponding 1,4-lactone. Furthermore, it was sug-

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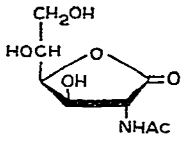
gested[4] that the weak activity ascribed to the 1,4-lactone might be a result of the formation of the 1,5-lactone during the process of equilibration in aqueous medium under the conditions of enzyme assay. All of those studies were conducted with heterogeneous and chemically impure materials, thereby creating ambiguity.

The isolation of pure, crystalline 2-acetamido-2-deoxy-D-glucono-1,4-lactone (**1**) and a preliminary examination of its inhibitory effect were reported earlier[6]. The present article describes the study of the 1,4-lactone **1** in comparison with 2-acetamido-2-deoxy-D-glucono-1,5-lactone (**2**) as inhibitors against 2-acetamido-2-deoxy-β-D-glucosidase from bull epididymis. However, the ease of interconversion of a lactone into its other ring form and the corresponding free acid (**3**) makes it virtually impossible to obtain a precise evaluation of the inhibitory effect of any lactone *per se*. The derivatives of lactones having fixed ring-size may be better models for investigation of the dependence of the inhibitory effect on the lactone ring-size. 2-Acetamido-2-deoxy-5,6-*O*-isopropylidene-D-glucono-1,4-lactone (**4**) and 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-D-glucono-1,5-lactone (**5**) were therefore investigated, and the results are also reported in this paper.



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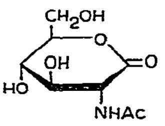

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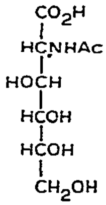

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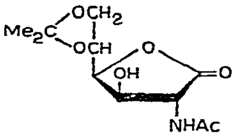

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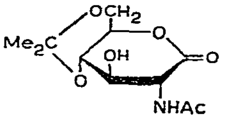

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RESULTS AND DISCUSSION

The availability of pure 2-acetamido-2-deoxy-D-glucono-1,4-lactone[6] (**1**) and of crystalline 2-acetamido-2-deoxy-D-gluconic acid[7] (**3**) has given us the opportunity to re-investigate their inhibitory activity and to obtain more reliable results.

The inhibitory activity of the 1,4-lactone **1**, the 1,5-lactone **2**, and the free acid **3** was each assayed against the 2-acetamido-2-deoxy-β-D-glucosidase extracted from bull epididymis. The enzyme assays were made by measuring colorimetrically the amount of *p*-nitrophenol liberated from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside as the substrate. Both of the lactones and the acid were readily distinguishable from each other by thin-layer chromatography (t.l.c.), and this was a great aid in monitoring the progress of formation of equilibrium mixtures. Several crystalline samples of 1,5-lactone **2** that were available from different sources were checked by t.l.c., and the purest samples were used in our studies. An interesting observation concerning these samples was the similarity in their inhibitory activity, regardless of their purity; this matter will be discussed later.

The activity of these compounds in freshly prepared, aqueous solutions of various concentrations was first considered (see Fig. 1). The 1,5-lactone **2** showed 50% inhibition at ~0.5μM concentration, whereas the 1,4-lactone **1** had far less potency, requiring a concentration of ~4μM to attain a similar activity. T.l.c. showed that even freshly prepared solutions of either lactone contained traces of the two other equilibrium products. The acid **3** initially showed no sign of lactone formation and was practically inactive, even at the higher concentrations. On standing at room temperature, however, lactone formation accompanied by inhibitory activity was observed, and these increased proportionally with time (see Fig. 2).

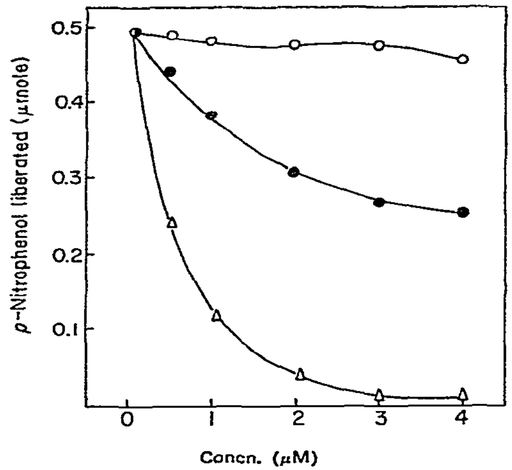

Fig. 1. Inhibitory activity of fresh, aqueous solutions of 2-acetamido-2-deoxy-D-glucono-1,5-lactone (△—△), 2-acetamido-2-deoxy-D-glucono-1,4-lactone (●—●), and 2-acetamido-2-deoxy-D-gluconic acid (○—○).

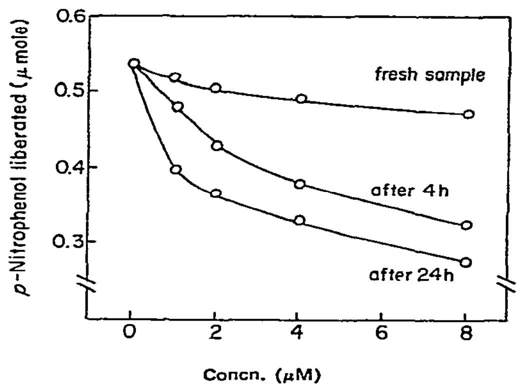

Fig. 2. Inhibitory activity of 2-acetamido-2-deoxy-D-gluconic acid in aqueous solution, with respect to time.

The effect of time on the inhibitory power of lactones **1** and **2** in aqueous solutions is given in Table I. According to the results obtained, the amount of the 1,4-lactone **1** needed to cause 50% inhibition decreased with time, indicating an increase in activity with increase in time; after 5 h, an equilibrium was reached, from an initial concentration of 3.6 to 1.8μM. On the other hand, solutions of the 1,5-lactone **2** decreased in activity with aging. The concentration necessary to give

50% inhibition increased from 0.85μM at 1 min to 1.02μM at equilibrium (5 h). The corresponding values of the inhibition constant (K_i) at equilibrium were 0.8μM for the 1,4-lactone and 0.65μM for the 1,5-lactone.

TABLE I

EFFECT OF TIME ON THE INHIBITION OF 2-ACETAMIDO-2-DEOXY-D-GLUCONO-1,4-LACTONE (**1**) AND 2-ACETAMIDO-2-DEOXY-D-GLUCONO-1,5-LACTONE (**2**) IN AQUEOUS SOLUTION AT ROOM TEMPERATURE

Time	*50% inhibition*[a] (μM)		K_i[a] (μM)	
	1	**2**	**1**	**2**
1–2 min	3.62	0.85	4.43	0.45
30 min	3.24	0.90	1.42	0.50
1 h	3.05	0.90	1.12	0.55
5 h	1.82	1.02	0.83	0.65
20 h	1.83	1.03	0.82	0.65

[a] Average values from three determinations.

Another variant considered with respect to time was pH (see Fig. 3). At pH values of 3.0 and 4.0, the activity of the 1,4-lactone **1** originally increased and then slowly fell. At pH values of 6.0 and 7.3, the activity immediately decreased, and, at pH 7.3, the rate was very high. Inhibition was also enhanced when the incubation temperature and the time of incubation were increased.

The assay of several samples of the 1,5-lactone **2** of various origins was undertaken. Interestingly, although their purity and chemical composition were very

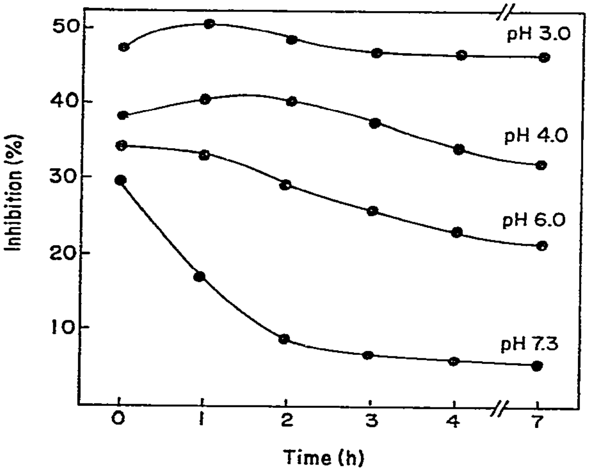

Fig. 3. Inhibitory activity of 2-acetamido-2-deoxy-D-glucono-1,4-lactone (4μM) in McIlvaine buffer at various pH values, with respect to time.

different, the values of K_i found for all of the samples lay close to 0.5μM, a value near that commonly reported in the literature[5,8]. A comparison of various samples of 1,5-lactone and related compounds is presented in Fig. 4. Samples 2 and 3 showed traces of 1,4-lactone **1** and free acid **3**. The proportions of these impurities in sample 4 were high; it also contained a faster-moving, fluorescent lactone having the same mobility as 2-acetamido-2,3-dideoxy-D-*erythro*-hex-2-enono-1,4-lactone[9] or 2-acetamido-2,3-dideoxy-D-*erythro*-hex-2-enono-1,5-lactone[10]. These unsaturated lactones have already been tested and found to be inhibitors of 2-acetamido-2-deoxy-β-D-glucosidase[11]. Sample 5, a commercial 2-acetamido-2-deoxy-D-gluconolactone (Koch & Light), was found to be a complex mixture. In addition to the aforementioned compounds, it contained two unidentified components. According to t.l.c. in a solvent system that distinguishes between the 1,5-lactone **2** and methyl 2-acetamido-2-deoxy-D-gluconate[12], this methyl ester was present in samples 4 and 5.

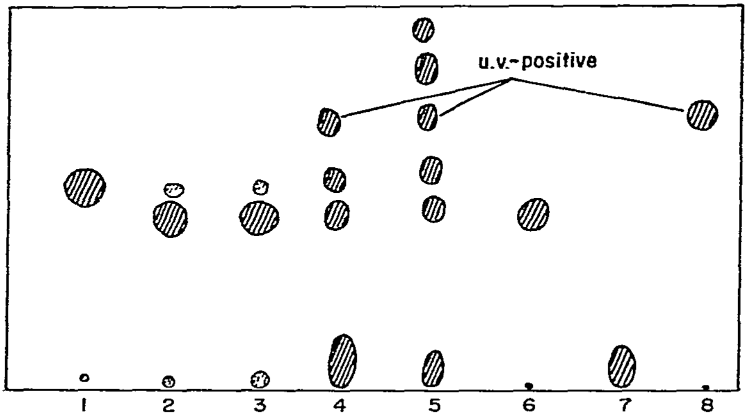

Fig. 4. T.l.c. in solvent system *A*. 1, 2-acetamido-2-deoxy-D-glucono-1,4-lactone[6]; 2, 2-acetamido-2-deoxy-D-glucono-1,5 lactone[12]; 3, 2-acetamido-2-deoxy D-glucono-1,5-lactone (prepared by Dr. N. M. Cross); 4, 2-acetamido-2-deoxy-D-glucono-1,5-lactone[13]; 5, 2-acetamido-2-deoxy-D-gluconolactone (Koch & Light Labs. Ltd.); 6, methyl 2-acetamido-2-deoxy-D-gluconate[12]; 7, 2-acetamido-2-deoxy-D-gluconic acid[7]; and 8, 2-acetamido-2,3-dideoxy-D-*erythro*-hex-2-enono-1,4-lactone[9], or 2-acetamido-2,3-dideoxy-D-*erythro*-hex-2-enono-1,5-lactone[10].

The presence of the impurities tentatively identified in sample 4 is not surprising, because the procedure[13] used for the preparation of 2-acetamido-2-deoxy-D-glucono-1,5-lactone (sample 4) employed (*a*) 10% sodium hydroxide solution in the acetylation of 2-amino-2-deoxy-D-gluconic acid, and (*b*) methanol for crystallization of the 1,5-lactone **2**. The ease of formation of the unsaturated lactones by the action of mild alkali has been pointed out[9]. Avoidance of methanol in the preparation of 2-acetamido-2-deoxyaldonolactones, in order to obviate the formation of methyl esters, has also been discussed[6,9]. However, in investigations of the inhibitory activity of 2-acetamido-2-deoxy-D-gluconolactones[1–4], the 1,5-lactone had been prepared by a procedure essentially the same as that just outlined[13]. In addition, our findings as to the heterogeneity of the 2-acetamido-2-deoxy-D-gluconolactone (sample 5) at present commercially available should be noted by those who wish to use it.

An authentic sample of methyl 2-acetamido-2-deoxy-D-gluconate was assayed for inhibitory activity while the solution was monitored by t.l.c. It was known[6,12] that esters may lactonize spontaneously on prolonged storage at room temperature, but this methyl ester was found to be very unstable in aqueous solution, even at 0°, being readily converted into the corresponding acid and lactones. Inhibitory activity was also noted, and the trends of increased activity and lactone formation ran in parallel (see Fig. 5).

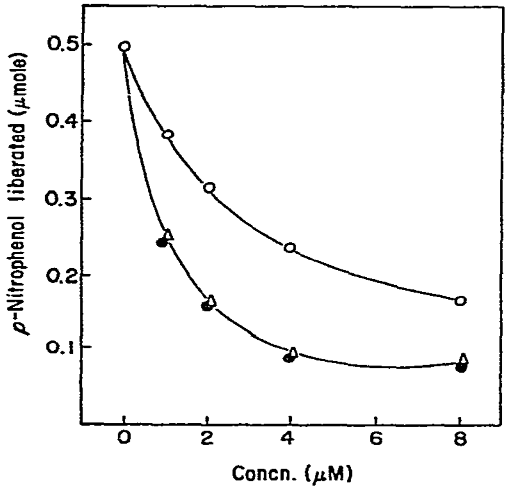

Fig. 5. Inhibitory effect of methyl 2-acetamido-2-deoxy-D-gluconate; fresh sample (○—○), 15 min (●—●), 15 min (pretreated with 0.05 E. U. of the enzyme) (△—△).

Leaback[14] recently reported the detection of esterase activity in a highly purified sample of 2-acetamido-2-deoxy-β-D-glucosidase. Had the appropriate enzyme been present in our incubation system, enzymic hydrolysis of the methyl ester into the acid, followed by equilibration of the acid into the lactones, would have been expected. However, in inhibition experiments performed simultaneously, there was no difference in the rate of activity of the methyl ester when it was pretreated with the enzyme preparation, implying that our enzyme preparation contained no appropriate esterase, as its presence would have enhanced the activity as a function of time. A more important point is the high activity of the methyl ester, which is very close to that of the 1,5-lactone **2** (50% inhibition at ~1μM), suggesting that formation of the acid is bypassed to a great extent, and that the methyl ester lactonizes directly, and mainly, to the lactone **2** having the six-membered ring.

Finally, 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-D-glucono-1,4-lactone (**4**) and 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-D-glucono-1,5-lactone (**5**) (both recently prepared crystalline[6]), derivatives suitably protected to prevent conversion into the other lactone, were also examined for inhibitory activity. They were adequately soluble and stable in water and in the enzyme assay mixture. Lineweaver–Burke plots, as well as Dixon plots (see Fig. 6), clearly showed competitive inhibition for both isopropylidene lactones. Nevertheless, the 4,6-acetal **5** (having a K_i value of 1.5μM is 1,000 times as active (as an inhibitor) as the corresponding 5,6-acetal **4** (K_i value, 1.5mM). The activity of both compounds in aqueous solution remained constant for two days at room temperature and seven days when refrigerated, indicating their stability. However, after that, their activity slowly began to change in the same direction as for the corresponding, unprotected lactones **1** and **2**. On comparing the isopropylidene derivatives to their parent lactones, decreasing activities were generally noted (see Table II); this may indicate that the presence of either a five- or a six-membered acetal ring results in some steric hindrance and, consequently, in a lower affinity for the active site of the enzyme.

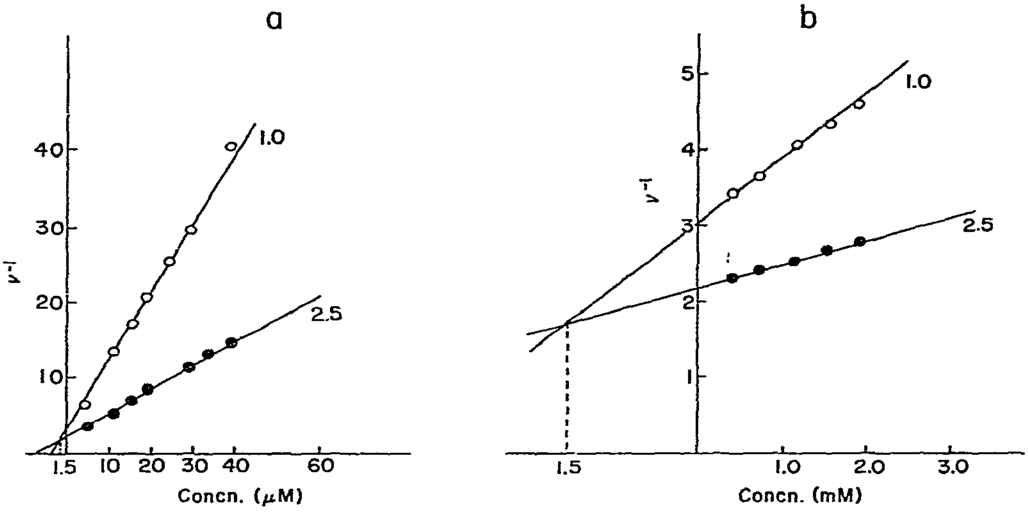

Fig. 6. Dixon plot showing competitive type of inhibition. (a) 2-Acetamido-2-deoxy-4,6-*O*-isopropylidene-D-glucono-1,5-lactone, and (b) 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-D-glucono-1,4-lactone.

In summary, 2-acetamido-2-deoxy-D-gluconic acid (**3**) has no inhibitory activity, whereas 2-acetamido-2-deoxy-D-glucono-1,5-lactone (**2**) is highly active. On the other hand, the weak activity of 2-acetamido-2-deoxy-D-glucono-1,4-lactone (**1**), increasing significantly with time, is rather difficult to evaluate quantitatively. Is the activity due solely to 1,5-lactone formed during the process of equilibration, or is the 1,4-lactone itself partly responsible? That the 1,4-lactone contributes to some extent to the activity observed seems reasonable, primarily because 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-D-glucono-1,4-lactone (**4**), whose conversion into the 1,5-lactone is impossible, shows some inhibition against 2-acetamido-2-deoxy-β-D-

TABLE II

INHIBITORY ACTIVITY OF 2-ACETAMIDO-2-DEOXY-D-GLUCONOLACTONES, THEIR ISOPROPYLIDENE ACETALS, AND RELATED COMPOUNDS

Compounds	*50% inhibition*	K_i
2-Acetamido-2-deoxy-D-glucono-1,4-lactone	3.6μM	4.4μM
5,6-isopropylidene acetal	3.2mM	1.5mM
2-Acetamido-2-deoxy-D-glucono-1,5-lactone	0.85μM	0.45μM
4,6-isopropylidene acetal	2.4μM	1.5μM
2-Acetamido-2-deoxy-D-glucose	10.2mM	5.1mM
4,6-isopropylidene acetal[15]	20.2mM	16.2mM
Methyl 2-acetamido-2-deoxy-β-D-glucofuranoside[16]	8.5mM	—
5,6-isopropylidene acetal[16]	>20mM	—

glucosidase. Furthermore, the decrease in activity noted for other isopropylidene derivatives in comparison to that of their parent compound (see Table II) might imply an inhibitory power higher for the free 1,4-lactone **1** than that found for the acetal **4**.

EXPERIMENTAL

General. — The following materials were commercially available: *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, 2-acetamido-2-deoxy-D-glucose, and bovine serum albumin (BSA) from Sigma Chemical Co.; frozen bull-epididymis from Pel Freeze Co.; and 2-acetamido-2-deoxy-D-gluconolactone from Koch & Light Labs. Ltd.

Silica Gel GF (250 μm; Analtech Inc.) was used for analytical t.l.c. in the following solvent systems: (*A*) 13:4 dichloromethane–methanol, (*B*), 4:1 ether–methanol, and (*C*) 99:1 acetone–water. Components were developed by charring with 10% sulfuric acid; in specific cases, sprays of Bromocresol Green and of hydroxylamine–ferric chloride were utilized.

Enzyme preparation. — 2-Acetamido-2-deoxy-β-D-glucosidase was extracted from frozen, bull epididymis according to the method described by Levvy and Conchie[17]. The fraction precipitated between 20 and 70% saturation with ammonium sulfate was retained. The precipitate was dissolved in 0.05M sodium citrate buffer (pH 4.5) and dialyzed against the same buffer, to give a stock preparation that contained 1.1 E.U. per mg of protein and had a K_m value of 1.1mM for *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside in McIlvaine buffer at the optimal pH of 4.8. The preparation also contained 2-acetamido-2-deoxy-β-D-galactosidase activity (0.2 E.U. per mg of protein, K_m 0.2mM for the corresponding *p*-nitrophenyl glycoside in McIlvaine buffer at the optimal pH of 4.3). This stock solution was quite stable on storage at 0–4°. When a weaker enzyme solution was needed, the stock solution was diluted with BSA buffer solution until the diluted solution contained 0.08% of BSA.

Enzyme assay. — The incubation mixture (2.0 ml) usually contained 1.0 mmole of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, 0.05 E.U. (10 μl of stock solution) of the enzyme, and various concentrations of the inhibitor in 0.2M McIlvaine buffer at pH 4.8. After incubation for 10 min at 38°, 5 ml of 0.4M glycine–sodium hydroxide buffer (pH 10.5) was added, and the *p*-nitrophenol liberated was determined at 430 nm by means of a Beckman DU spectrophotometer. The K_i values were obtained by plotting $1/v$ *versus* I for 1.0 and 2.5mM concentrations of substrate, where v is the μmoles of *p*-nitrophenol liberated and I is the concentration of the inhibitor; the intersection of the two lines plotted gives K_i. The amount of compound needed to cause 50% inhibition was determined by incubating various concentrations of inhibitor at 1.0mM concentration of the substrate.

ACKNOWLEDGMENTS

We are grateful to Mr. W. N. Jones, Lilly Research Centre, Erl Wood Manor, Windlesham, Surrey, England, for a sample of 2-acetamido-2-deoxy-D-glucono-1,5-lactone that had been prepared by the late Dr. N. M. Cross, and to Professor P. Sinaÿ, Laboratoire de Biochimie Structurale, U. E. R. de Sciences Fondamentales et Appliquées, 45045 Orleans Cédex, France, for a sample of methyl 2-acetamido-2-deoxy-β-D-glucofuranoside.

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