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2-Acetamido-2-deoxy-D-mannono-1,5-lactone: Synthesis and Inhibition of 2-Acetamido-2-deoxy-β-D-glucosidase

*M. Pokorny and N. Pravdić**

*»Krka« Pharmaceutical and Chemical Works, 68000 Novo Mesto, Slovenia, and *Department of Organic Chemistry and Biochemistry, »Ruđer Bošković« Institute, 41000 Zagreb, Croatia, Yugoslavia*

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2-Acetamido-2-deoxy-D-mannono-1,5-lactone (I) was prepared by mild hydrolysis of 2-acetamido-2-deoxy-4,6-*O*-isopropylidene--D-mannono-1,5-lactone (IV).

The inhibitory activity of I and of the isomeric 1,4-lactone II, as well as of 2-acetamido-2-deoxy-D-mannonic acid (III), was assayed against 2-acetamido-2-deoxy-β-D-glucosidase from bull epididymis. The free acid III exhibits no inhibitory activity. The two lactones act as competitive inhibitors against *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (inhibition constants K_i 33 μmol/dm³ and 8.3 mmol/dm³ for I and II, respectively), whereas against *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside only lactone I exhibits weak inhibitory activity (K_i, 9.3 mmol/dm³).

INTRODUCTION

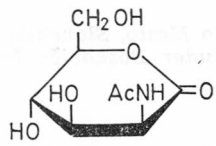
It is well known that various glycosidases are inhibited by aldonolactones corresponding in configuration to the same carbohydrate as the substrate[1,2]. The inhibition by lactones may be due to conformational and electrostatic resemblance of the lactone and the hypothetical transition state in an enzyme--catalyzed hydrolysis[3,4]. Thus, 2-acetamido-2-deoxy-β-D-glucosidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase; β-*N*-acetylglucosaminidase, EC 3.2.1.30), one of the key enzymes associated with the metabolism of glycoproteins, which catalyses the hydrolysis of 2-acetamido-2-deoxy-β-D--glucosyl and -β-D-galactosyl residues, is most powerfully inhibited by 2-acetamido-2-deoxy-D-glucono-1,5-lactone[5–7].

In the course of our earlier studies[8,9], the inhibitory activities of the two 2-acetamido-2-deoxy-D-gluconolactones and some of their derivatives towards 2-acetamido-2-deoxy-β-D-glucosidase from bull epididymis were examined and quantitatively evaluated. Further, we found[10] that the same enzyme was also inhibited by some unsaturated 2-acetamido-2,3-dideoxy-D-hex-2-enonolactones as well as by 2-acetamido-2-deoxy-D-glycals. In addition, it was established[11] that this enzyme could be successfully purified by affinity chromatography using 2-acetamido-2-deoxy-D-glucono-1,4-lactone and the corresponding D-galactono- or D-mannono-lactones as affinity ligands. By far the best results, with regard to the yield and purification of the enzyme, were achieved with 2-acetamido-2-deoxy-D-mannono-1,4-lactone, i. e., the lactone different in configuration at C-2. This fact stimulated an extension of these studies, and a detailed

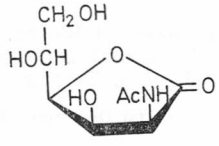
examination of the inhibitory power of the lactones of the D-*manno* configuration was undertaken. The results are reported in the present paper.

RESULTS AND DISCUSSION

The inhibitory activity of the two lactones: 2-acetamido-2-deoxy-D-mannono-1,5-lactone (I) and the -1,4-lactone (II), as well as of the 2-acetamido-2--deoxy-D-mannonic acid (III), was each assayed against the 2-acetamido-2-deoxy--β-D-glucosidase extracted from bull epididymis. Pure samples of compounds



I



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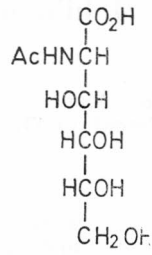

III

II and III were available in our laboratories[12,13]. The synthesis of the unknown 1,5-lactone I was elaborated, and will be described here.

As the source for the preparation of the 1,5-lactone I, two corresponding 4,6-acetals (benzylidene and isopropylidene), prepared recently[14] in our laboratory by selective oxidation of partially protected 2-acetamido-2-deoxypyranoses, could be used. As catalytic hydrogenolysis of the 4,6-*O*-benzylidine derivative resulted in the isolation of the free lactone changed in ring-size[14], 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-D-mannono-1,5-lactone (IV) was chosen as the starting material. Hydrolysis of the isopropylidene group from IV was carried out under very mild conditions, using a dry, ion-exchange resin in the H^+ form[8] in a nonaqueous solvent at room temperature. Pure, crystalline lactone I was thus obtained in 18% yield.

It should be emphasized that, in the course of the preparation of the 2-acetamido-2-deoxy-D-mannono-1,5-lactone (I), special care was given to testing its purity with regard to the presence of the D-*gluco* epimer, because 2-acetamido-2--deoxy-D-glucono-1,5-lactone is known to be an extremely powerful inhibitor (K_i 0.45 μmol/dm³)[9], and even a minor quantity present in samples of I can create ambiguous results. On the other hand, it was evident[14] that the formation of the 4,6-*O*-isopropylidene derivative IV was accompanied by *manno*-to-*gluco* epimerization. In purified samples of IV, the content* of the D-*gluco* acetal was 5—20%, varying from batch to batch. Samples of IV with the lower content of the D-*gluco* epimer were used for the removal of the isopropylidene group. The crude product, obtained in 97% yield, clearly showed in its NMR spectrum the presence of the axial as well as of the equatorial *N*-acetyl group. Crystalline lactone I obtained in low yield by trituration, was shown to be homogenous:

* The most reliable method for distinguishing acetal IV from its corresponding D-*gluco* epimer was found to be nuclear magnetic resonance spectroscopy (spectra recorded in methyl sulfoxide; see Experimental for details), whereas chromatographic methods (tlc and glpc) were unsuccessful.

the NMR spectrum revealed the presence of only one acetyl group, at τ 8.07, and its infrared spectrum showed carbonyl absorption at 1745 cm^{-1}, characteristic of the 1,5-lactone structure[15]. Samples of this pure, D-*gluco*-free lactone I were used for the inhibitory activity examination.

In an attempt to isolate an additional amount of the lactone I from the mother liquor, the latter was chromatographed on silica gel, using a methanol-containing solvent mixture (solvent *D*) for the elution. However, a mixture of two components was obtained: methyl 2-acetamido-2-deoxy-D-gluconate and 2-acetamido-2-deoxy-D-mannono-1,4-lactone. The first was formed by methanolysis from the D-gluco-1,5-lactone, as already observed[8,12], and the second, through transformation of the 1,5-lactone on the silica gel[14]. It is interesting that these two structurally similar lactones react differently under identical conditions.

The enzyme assays were carried out in the standard manner, described earlier[9], using *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside or -D--galactopyranoside as the substrate, and measuring colorimetrically the amount of *p*-nitrophenol liberated.

The results obtained with lactones I and II, and with the free acid III are given in Figure 1. As expected, acid III was practically inactive; the 1,5--lactone I in fresh, aqueous solution shows a considerable inhibitory activity against 2-acetamido-2-deoxy-β-D-glucosidase, whereas the 1,4-lactone II is less active. The 1,4-lactone II required a concentration of 7 mmol/dm^3 to cause 50% inhibition, the concentration of the 1,5-lactone I needed to achieve the same activity, was 22 μmol/dm^3.

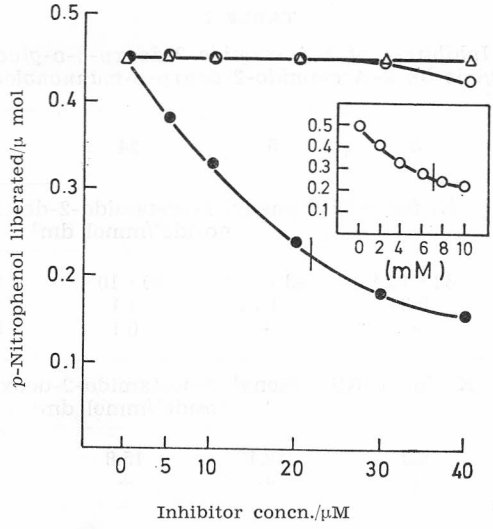

Figure 1. Inhibition of 2-acetamido-2-deoxy-β-D-glucosidase from bull epididymis by 2-acetamido--2-deoxy-D-mannono-1,5-lactone (I) (●—●), 2-acetamido-2-deoxy-D-mannono-1,4-lactone (II) (○—○). 2-acetamido-2-deoxy-D-mannonic acid (III) (△—△). Substrate: *p*-nitrophenyl 2-acetamido-2-deoxy--β-D-glucopyranoside in concentration of 2.0 mmol/dm^3. Vertical interrupted lines, 50% inhibition.

Using *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside as the substrate, the activity of compunds I—III as inhibitors of the ability of the enzyme to hydrolyse the corresponding β-D-galactosyl residues was tested: only the 1,5-lactone I was found to act as a weak inhibitor. In sharp contrast stands the fact that 2-acetamido-2-deoxy-D-glucono-1,5-lactone is known[16-18] to be a potent inhibitor of 2-acetamido-2-deoxy-β-D-galactosidase activity.

It may be noted, in passing, that the isopropylidene acetal IV, in fresh solution, showed a low activity (K_i = 0.46 mmol/dm³) which differs by a factor of 14 from the activity of the free lactone I. This is in agreement with the effect of all acetals previously examined (from the D-*gluco*[9] and from the unsaturated[10] series), which also show diminished activities. Due to the fast hydrolysis of the isopropylidene group in IV, the inhibitory activity of an aqueous solution of IV kept at room temperature corresponded, when measured after 24 hours, to the activity of the free lactone. The hydrolysis was readily recognizable by thin-layer chromatography.

The effect of time on the inhibitory activity of samples I—III in aqueous solutions, measured against 2-acetamido-2-deoxy-β-D-glucosidase and 2-acetamido-2-deoxy-β-D-galactosidase, is summarized in Table I. Again, as with the D-*gluco* lactones[9], the effect of time is the opposite for I and II: solutions of the 1,4-lactone II show an increase in inhibitory activity, whereas the activity of solutions of the 1,5-lactone I decreases with time. For both lactones equilibrium is achieved in the period between 24 and 48 h: at equilibrium, the inhibition constant (K_i) measured against 2-acetamido-2-deoxy-β-D-glucosidase, was 1 mmol/dm³ for lactone II and 0.1 mmol/dm³ for lactone I. The samples containing acid III (after aging for 24 h) showed low activity. All these results indicate that the ease of interconversion of a D-*manno* lactone into its other ring form and into the free acid, as well as in the reverse direction, is much lower than in the D-*gluco* series, where the equilibrium was reached after 5 h.

TABLE I

Effect of Time on Inhibition of 2-Acetamido-2-deoxy-β-D-glucosidase from Bull Epididymis by 2-Acetamido-2-deoxy-D-mannonolactones

Age of solution[a]/h	0	5	24	48	72
	K_i for *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside[b]/mmol dm³				
1,5-lactone I	$33 \cdot 10^{-3}$	$43 \cdot 10^{-3}$	$50 \cdot 10^{-3}$	0.11	0.22
1,4-lactone II	8.3	1.7	1.1	1.0	—
acid III	+	+	0.1	0.1	0.1
	K_i for *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside[b]/mmol dm³				
1,5-lactone I	9.3	12.1	15.8	—	—
1,4-lactone II	+	+	+	+	+
acid III	+	+	+	—	—

[a] Aqueous solutions were kept at room temperature; —, it was not measured; +, no significant inhibition could be observed up to 10 mmol/dm³ concentration; [b] Average values from three determinations.

Khorlin et al.[19] reported the isomerization of one lactone form into the other as the result of the action of the enzyme. The data given in Table I suggest that the change of the lactone ring-size, 1,5-lactone⇄1,4-lactone, is spontaneous and that it occurs in aqueous solutions.

The opening of the lactone ring to give the free acid could be easily detected by thin-layer chromatography (solvent *D*); however, the two lactones were not distinguishable from each other. The lactone⇄acid interconversion was quantitatively monitored by a colorimetric method based on the reactivity of lactones with hydroxylamine[20,7]. The procedure applied here was that described by Boxer and Everett[21] for the determination of the β-lactam ring in penicillin. It is practically identical with the method[7] used for determination of a mixture of 2-acetamido-2-deoxy-D-gluconolactones, except that hydroxylamine—ferric complexes are more stable, and reproducible results could be obtained. Figure 2 shows the relationship of hydroxylamine assay of compounds I—III to time. The acid⇄lactone interconversion is obvious, the equilibrium being achieved after ca. 40 h. These results fit well with the data for the enzyme assay, described earlier in this paper.

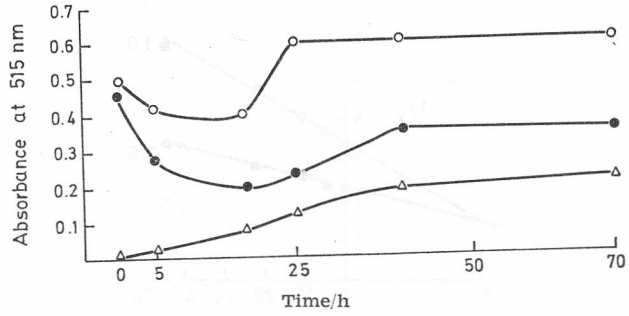

Figure 2. Stability of 2-acetamido-2-deoxy-D-mannono-1,5-lactone (I) (●—●), 2-acetamido-2-deoxy-D-mannono-1,4-lactone (II) (○—○) and 2-acetamido-2-deoxy-D-mannonic acid (III) (△—△) as revealed by the hydroxamic acid test.

The inhibition constant, $K_i = 33$ μmol/dm³, of the D-mannono-1,5-lactone I, in comparison with that of the corresponding D-*gluco* lactone[9] is 73 times lower. In the 1,4-lactone series, the difference is even more evident: the activity differs by a factor of ~2000. The low inhibitory activity of the D-mannono-lactones may be due to the lower affinity these lactones exhibit towards the 2-acetamido-2-deoxy-β-D-glucosidase. The low inhibitory activity of the 1,4-lactone II against 2-acetamido-2-deoxy-β-D-glucosidase is the quality that should be especially appreciated. This lactone was shown to be very suitable as the affinity ligand for the purification of the same enzyme[11]. Most probably because of the low inhibitory activity of the 1,4-lactone II, the enzyme did not bind very strongly to the inhibitor.

The results of the inhibition of 2-acetamido-2-deoxy-β-D-glucosidase and -β-D-galactosidase activities by free 2-acetamido-2-deoxy-D-aldopyranoses (Table II) also indicate that the lowest inhibitory power is exhibited by the compound of the D-*manno* configuration; this is in accordance with similar data previously reported[22].

TABLE II

Inhibition of 2-Acetamido-2-deoxy-β-D-glucosidase from Bull Epididymis by 2-Acetamido-2-deoxy-D-aldopyranoses

2-Acetamido-2-deoxy-D-aldopyranose	K_i[a]/mmol dm^{-3} for p-Nitrophenyl 2-acetamido-2-deoxy-β-D-	
	-glucopyranoside	-galactopyranoside
-*gluco*	5.1	4.5
-*galacto*	0.12	0.30
-*manno*	18	17

[a] Average values from three determinations.

Lineweaver-Burke plots, as well as Dixon plots, indicate that D-mannono--1,5-lactone I (Figure 3) and -1,4-lactone II act as competitive inhibitors; this is in agreement with the behavior of the aldonolactones as inhibitors in general.

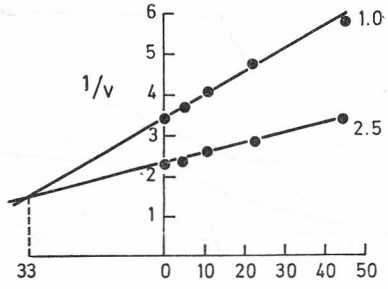

Figure 3. Dixon plot showing competitive inhibition of 2-acetamido-2-deoxy-β-D-glucosidase from bull epididymis by 2-acetamido-2-deoxy-D-mannono-1,5-lactone (I). Substrate: p-nitrophenyl 2--acetamido-2-deoxy-β-D-glucopyranoside in concentrations of 1.0 mmol/dm^3 and 2.5 mmol/dm^3.

Of particular interest is the evidence that the inhibitory activity shown by lactone I could be ascribed to 2-acetamido-2-deoxy-D-mannono-1,5-lactone. As stated earlier in this paper, 2-acetamido-2-deoxy-D-glucono-1,5-lactone — an extremely powerful inhibitor, if present as an impurity in samples of lactone I, could be responsible for the inhibitory activity found. If the inhibitory activities of tested lactone I are completely due to the D-*gluco* analog, the content of the latter should be ca 2% (calculated from 50% inhibition data; 22 and 0.5 μmol/dm^3 for I and D-*gluco*[9] lactone, respectively). Since the homogeneity of samples of lactone I was tested by the NMR at 60 MHz (there are no other reliable chemical criteria), the presence of only 2% of the D-*gluco* lactone might possibly be unobserved. Therefore, we turned our attention to the difference shown by lactone I and its D-*gluco* analog towards p-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside as the substrate. As given in Table

I, K_i of lactone I is 9.3 mmol/dm³. On the other hand, 2-acetamido-2-deoxy-D-glucono-1,5-lactone[23] was now found to inhibit the same activity of the enzyme with $K_i \sim 1$ μmol/dm³. The results obtained with *a*) lactone I; *b*) lactone I containing 2% of D-*gluco* analog, and c) with D-*gluco* lactone are given in Figure 4. It could be clearly seen that the curve of *b*) follows that one of c), i. e., that the presence of 2% of the D-*gluco* lactone in the sample of lactone I remarkably increases the inhibitory activity shown by the intact lactone I as represented by curve *a*). Thus, it could be concluded that the sample of lactone I certainly does not contain its D-*gluco* epimer in the amount of 2%. Whether it is completely D-*gluco*-free or not is the question that still remains. Figure 4 shows

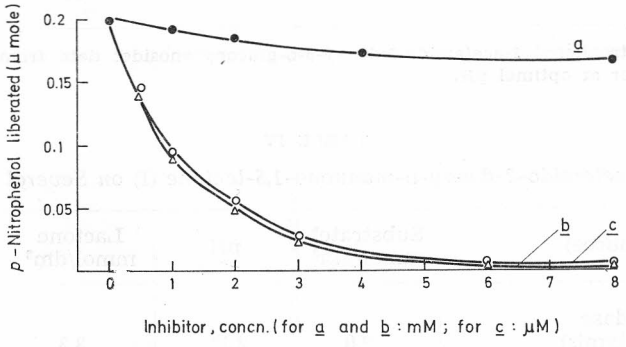

Figure 4. Inhibition of 2-acetamido-2-deoxy-β-D-glucosidase from bull epididymis by *a*) 2-acetamido-2-deoxy-D-mannono-1,5-lactone (I) (●—●); *b*) the mixture of 2-acetamido-2-deoxy-D-mannono-1,5-lactone (I) with 2% of 2-acetamido-2-deoxy-D-glucono-1,5-lactone (△—△); c) 2-acetamido-2-deoxy-D-glucono-1,5-lactone (○—○). Substrate: *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside in concentration of 0.25 mmol/dm³.

that 100% inhibition is achieved with D-*gluco* lactone at a concentration of 6μmol/dm³, whereas 6 mmol/dm³ of lactone I is required to cause 10% inhibition. It means that lactone I might contain 1/10,000 parts of the D-*gluco* lactone. A similar ratio is obtained by comparing the K_i values of the two lactones for *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside.

In addition, it should be pointed out that if the inhibitory activity shown by lactone I is due to D-*gluco* lactone, the ratio of K_i for the two substrates should be the same or at least similar. However, it proved not to be the case: with D-*gluco* lactone, K_i for *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside is 0.45 μmol/dm³ and for the corresponding -galactopyranoside 1 μmol/dm²; with lactone I, K_i values are 33 μmol/dm³ and 9.3 mmol/dm³ respectively (Table I).

In conclusion it might be suggested that 2-acetamido-2-deoxy-D-mannono-1,5-lactone (I) itself is mostly responsible for the inhibitory activity shown against 2-acetamido-2-deoxy-β-D-glucosidase from bull epididymis.

The inhibitory activity of the 1,5-lactone I towards 2-acetamido-2-deoxy-β-D-glucosidase from different sources was determined, and the results are given in Table III. The inhibitory action is lower towards the enzyme of plant origin, but it appeared to be competitive in all cases.

Finally, the effect of lactone I on various glycosidases was tested under comparable conditions. The results presented in Table IV clearly show that neither of the enzymes examined was significantly inhibited by 2-acetamido-2-

TABLE III

Inhibition of 2-Acetamido-2-deoxy-β-D-glucosidase from Different Sources by 2-Acetamido-2-deoxy-D-mannono-1,5-lactone (I)

Source	K_m^a/mmol dm^{-3}	K_i μmol dm^{-3}	pH[b]
Bull epididymis	1.10	33	4.8
Horse liver	1.11	39	4.3
Almond emulsin	0.39	1500	4.5
Oat coleoptiles	0.41	1600	4.9
Aspergillus oryzae	0.85	105	4.5

[a] Substrate: *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside; data from ref. 10; [b] McIlvaine buffer at optimal pH.

TABLE IV

Effect of 2-Acetamido-2-deoxy-D-mannono-1,5-lactone (I) *on Several Glycosidases*

Enzyme (source)	Substrate[a] mmol/dm^3	pH	Lactone mmol/dm^3	Inhibition/%
β-D-galactosidase (bull epididymis)	2.0	3.1[b]	3.3	0
α-D-mannosidase (bull epididymis)	2.0	4.5[c]	3.3	9
α-D-galactosidase (oat coleoptiles)	1.0	4.9	3.3	0
β-D-glucosidase (almond emulsin)	1.0	4.8[b]	6.6	0
β-D-galactosidase (almond emulsin)	1.0	4.5[b]	6.6	2
α-D-mannosidase (almond emulsin)	1.0	4.5[c]	6.6	15
β-D-glucosiduronase *(Helix pomatia)*	1.0	4.5[c]	3.3	5

[a] Corresponding *p*-nitrophenyl glycopyranoside; [b] McIlvaine buffer; [c] sodium acetate buffer.

-deoxy-D-mannono-1,5-lactone, indicating the specificity of this lactone for 2-acetamido-2-deoxy-β-D-glucosidase. It has recently been established[22,24,25] that hydrolysis by β-*N*-acetylglucosaminidase could be effective only if a 2-acetamido group is present in the substrate. Our results, on the other hand, show that 2-acetamido-2-deoxy-β-D-glucosidase is specifically inhibited by 2-acetamido--2-deoxy-D-gluconolactones[9], 2-acetamido-2-deoxy-D-mannonolactones, and certain unsaturated lactones[10] containing a 2-acetamido group. It seems reasonable to assume that one of the essential requirements for the inhibitory activity of a lactone towards a given glycosidase in the presence of the same substituent

at C-2 position in the lactone and in the substrate. Surprisingly, the configuration at C-2 seems not to be fully decisive; in the unsaturated lactones the 2-acetamido group is attached to the vinyl carbon, i. e., it is in the plane defined by the double bond. The results presented in this paper suggest that 2-acetamido-2-deoxy-β-D-glucosidase appears not to be dependent upon the configuration at C-2, in addition to the already known data[2] that it shows no specificity for C-4. The fact that 2-acetamido-2-deoxy-β-D-glucosidase is unable to distinguish the configuration at C-2 of the inhibitor allows us to speculate upon the possible mannosaminidase activity that this enzyme may also possess.

EXPERIMENTAL

General Methods

Specific rotations were measured at 20—24 °C. Column chromatography was performed on silica gel (E. Merck; particle size 0.05—0.20 mm), with the following solvent systems, all ratios being *v/v: A,* éther-chloroform-acetone (1 : 1 : 2); *B,* ether-acetone (2 : 1); *C,* chloroform-acetone (1 : 1); *D,* dichloromethane-methanol (13 : 4). *TLC* was conducted on silica gel in the solvent system specified, the components being detected by spraying with 10% sulfuric acid. Infrared spectra were recorded with a Perkin-Elmer Model 137 spectrometer. The NMR spectra of solutions in methyl sulfoxide-d_6 were recorded with a Varian A-60*A* spectrometer, with tetramethylsilane as the internal standard. Optical absorptions were measured by means of a Perkin-Elmer spectrophotometer 139.

The following materials were commercially available: 2-acetamido-2-deoxy-D-mannose, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-galactose from Pfanstiehl Labs. Inc.; *p*-nitrophenyl D-glycopyranosides from Sigma Chem. Co. Bull epididymis and horse liver were obtained from a neighborhood slaughterhouse, and were kept at — 15 °C until used. Jack-bean meal, almond emulsin, β-D-glucosiduronase *(Helix pomatia),* and α-amylase *(Aspergillus oryzae)* were obtained from Sigma Chem. Co. Oat coleoptiles (3—5 cm in length) were prepared in our laboratory[18].

2-Acetamido-2-deoxy-4,6-O-isopropylidene-D-mannono-1,5-lactone (IV) was prepared as described[14], and isolated (ca. 30%) as one of the products formed in the oxidation of 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-D-mannopyranose[26]. Fractionation of the components was carried out by repeated chromatography on columns of silica gel, using solvent systems *A* and *B*. The title compound (contaminated by a minor amount of the corresponding D-*gluco* isomer) was obtained in the form of a stable foam, $[\alpha]_D$ in the range + 115—123° (acetone).

NMR signals were noted at τ 2.14 (doublet, *J* 8.5 Hz, removed by D_2O exchange, NH), 4.12 (doublet, *J* 5.5 Hz, removed by D_2O exchange, OH), 8.06 (NAc, *ax*), 8.17 (NAc, *eq*), 8.52 and 8.63 (CMe_2). From the intensities of the NAc signals, the amount of the impurity could be estimated (5—20%). An authentic sample of 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-D-glucono-1,5-lactone[14] showed *N*-acetyl signal at τ 8.17.

2-Acetamido-2-deoxy-D-mannono-1,5-lactone (I)

Into a solution of IV (146 mg) in 2-methoxyethanol (10 ml) was added dry Dowex-50 X-8 (H^+) ion-exchange resin (1.5 g), and the mixture was kept for 2 h at room temperature with occasional stirring. The progress of the reaction was monitored by TLC with solvent *C*; at the termination, the presence of the starting material was barely detectable. The resin was filtered off and washed several times with acetone, and the filtrate and washing were combined and evaporated in vacuo to an oily residue, which was dried first under high vacuum and finally in a desiccator over sodium hydroxide: reddish syrup, 120 mg (97%). The NMR spectrum showed signals for an *N*-acetyl group at τ 8.07 (*ax*) and at 8.14 (*eq*).

The crude product was triturated with acetone, and the solvent removed by evaporation in vacuo; this treatment was repeated several times until crystallization was induced. The crystals were filtered off: 22 mg (18%), m. p. 158—160 °C $[\alpha]_D$ + 107.7° (*c* 0.31, water, 3 min after dissolution). The optical rotation was observed (1-dm tube) over a period of 24 h, and found to change, from an initial value of + 0.334, to + 0.414, and then to a final (and constant) rotation of + 0.232. Samples of

the lactone I in admixture with the isomeric 2-acetamido-2-deoxy-D-mannono-1,4-lactone[12] or with 2-acetamido-2-deoxy-D-glucono-1,5-lactone[23] give a definite depression in melting points.

The infrared spectrum showed absorption at 3400 (OH), 3300 (NH), 1745 (C=O), 1650 and 1550 cm^{-1} (Amide I and II). In the NMR spectrum, only the signal at τ 8.07 (NAc, *ax*) was present.

Anal. $C_8H_{13}NO_6$ (219.20) calc'd.: C 43.84; H 5.98; N 6.39%
found: C 43.77; H 5.93; N 6.34%.

The acetone mother liquor was evaporated to dryness, and the residue was chromatographed on a column of silica gel (10 g) using solvent *D* as the eluant. Fractions containing homogeneous material were pooled to give semicrystalline material: 62 mg. Its NMR spectrum, showing signals at τ 6.38 (OCH_3), 8.06 (NAc, *ax*), and 8.15 (NAc, *eq*), and its infrared spectrum with carbonyl absorption at 1780 cm^{-1}, identified this material as a mixture of two components: 2-acetamido-2-deoxy-D-mannono-1,4-lactone[12] and methyl 2-acetamido-2-deoxy-D-gluconate[23]. The approximate ratio of the *gluco* : *manno* component was 1 : 4.

Enzyme Preparation

Following the general procedure of Levvy and Conchie[27] and the details given in our previous paper[9], the glycosidases were extracted with 0.05 mol/dm^3 citric acid containing 0.1 mol/dm^3 NaCl, or with 0.1 mol/dm^3 acetate buffer. The fraction that was precipitated between 20 and 70% saturation with ammonium sulphate was retained; this was dissolved in a small volume of 50 mmol/dm^3 sodium phosphate buffer, or 50 mmol/dm^3 sodium acetate buffer. Dialysis against the same buffer gave the stock solution containing 2—5 E. U. per mg of protein. One enzyme unit (E. U.) was defined as the amount of the enzyme that hydrolyzed 1 μmol of the corresponding *p*-nitrophenyl glycopyranoside as the substrate per min at 38 °C.

Enzyme Assay

The incubation mixtures contained: *a*) 1.0 ml of either 0.2 mol/dm^3 McIlvaine buffer or 0.1 mol/dm^3 acetate buffer at the optimal pH of the enzyme tested, *b*) 0.05 E. U. of enzyme preparation (usually 10 μl of stock solution), *c*) the substrate (corresponding *p*-nitrophenyl glycopyranoside in final concentrations of 1.0 mmol/dm^3 or 2.0 mmol/dm^3, whereas for *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside the final concentrations were 0.25 mmol/dm^3 and 0.5 mmol/dm^3, and *d*) inhibitors in various concentrations. After dilution with water to a volume of 2.0 ml, the mixtures were incubated for 10 min at 38 °C, treated with 4 ml of 0.4 mol/dm^3 glycine—sodium hydroxide buffer (pH 10.4), and the liberated *p*-nitrophenol determined spectrophotometrically at 430 nm.

The K_i values were obtained by plotting $1/v$ versus I for 1.0 mmol/dm^3 and 2.5 mmol/dm^3 concentrations of the 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 (Dixon plot). The amount of compound needed to cause 50% inhibition was determined by incubating various concentrations of inhibitor at 1.0 mmol/dm^3 concentration of the substrates.

Hydroxylamine Assay

The procedure described by Boxer and Everett[21] was applied. To an aqueous solution of the lactone (1 mg/ml), was added 3 ml of freshly prepared, neutralized hydroxylamine (1 part of 5 mol/dm^3 hydroxylamine hydrochloride, 1 part of sodium acetate buffer pH 11.9, and 4 parts of 96% ethanol). After standing for 3 min at room temperature, 1 ml of a 20% solution of $Fe(NH)_4SO_4 \cdot 12H_2O$ in 1.75 mol/dm^3 sulfuric acid was added, and the absorption of the complex was read between 3 and 10 min (spectrophotometrically at 515 nm).

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SAŽETAK

2-Acetamido-2-deoksi-D-manono-1,5-lakton: sinteza i studij inhibicije 2-acetamido-2-deoksi-β-D-glukozidaze

M. Pokorny i N. Pravdić

2-Acetamido-2-deoksi-D-manono-1,5-lakton (I) pripravljen je hidrolizom 2-acetamido-2-deoksi-4,6-*O*-izopropiliden-D-manono-1,5-laktona (IV) pod blagim uvjetima. Ispitivana je inhibitorska aktivnost laktona I, izomernog 1,4-laktona II i 2-acetamido-2-deoksi-D-manonske kiseline (III) prema enzimu 2-acetamido-2-deoksi-β-D-glukozidazi iz nadspolne žlijezde goveda. Slobodna kiselina III ne pokazuje inhibitorsku aktivnost. Oba laktona djeluju kao kompetitivni inhibitori prema *p*-nitrofenil 2-acetamido-2-deoksi-β-D-glukopiranozidu (konstante inhibicije K_i: 33 μmol/dm³ za lakton I, 8.3 mmol/dm³ za lakton II), dok prema *p*-nitrofenil 2-acetamido-2-deoksi-β-D-galaktopiranozidu samo lakton I pokazuje slabo inhibitorsko djelovanje (K_i, 9.3 mmol/dm³).

»KRKA«, TVORNICA FARMACEUTSKIH
I KEMIJSKIH PROIZVODA
NOVO MESTO
I
INSTITUT »RUĐER BOŠKOVIĆ«
ZAGREB

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