J. Biochem., 73, 749–753 (1973)

N-Acyl Specificity of Taka-N-acetyl-β-D-glucosaminidase Studied by Synthetic Substrate Analogs

II. Preparation of Some *p*-Nitrophenyl 2-Halogenoacetylamino-2-deoxy-β-D-glucopyranosides and Their Susceptibility to Enzymic Hydrolysis

Kazuhiko YAMAMOTO

Department of Chemistry, Osaka University College of Science, Toyonaka, Osaka

Received for publication, September 4, 1972

Some of *p*-nitrophenyl 2-acylamino-2-deoxy-β-D-glucopyranosides with monofluoroacetyl, monochloroacetyl, monobromoacetyl, difluoroacetyl, dichloroacetyl, and trifluoroacetyl group as N-substituent were prepared in order to investigate the N-acyl specificity of Taka-N-acetyl-β-D-glucosaminidase [EC 3.2.1.30].

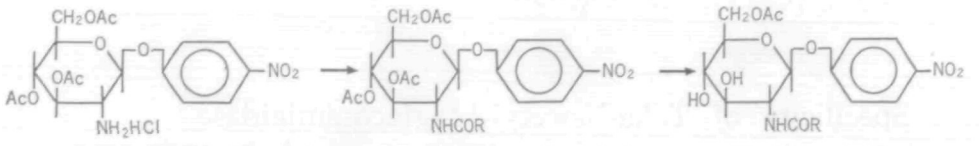
All of the substrate analogs prepared in this experiment, except the N-dichloroacetyl derivative, were hydrolyzed by this enzyme.

Comparison of the hydrolytic rate of each substrate analog to the N-acetyl derivative led to the conclusion that monohalogen substitution on the N-acetyl group of the substrate, even di-, and tri-substitution in the case of fluorine atom, is permissible in the N-acyl specificity and the specificity is predominantly controlled by the steric factor of the N-substituent of substrate.

During studis on the N-acyl specificity of N-acetyl-β-D-glucosaminidase [EC 3.2.1.30] obtained from Taka-diastase (Sankyo), investigation of the N-acyl specificity has been undertaken using the synthetic substrate analogs with several N-acyl substituent. In a previous report (*1*), some of *p*-nitrophenyl 2-acylamino-2-deoxy-β-D-glucopyranosides containing formyl, propionyl, *n*-butyryl, iosbutyryl, and benzoyl group as N-acyl substituent were synthesized, and the enzymatic hydrolysis of these substrate analogs was investigated. It was concluded that the N-acetyl derivative was the most reactive among the other N-acylated analogs, but the N-acyl specificity of this enzyme was not limited to N-acetyl group because the substrate containing N-formyl, N-propionyl, and N-*n*-butyryl group could be hydrolyzed in a measurable rate. Another effect of α-alkyl substitution in the N-acetyl group was also observed since the substrate containing N-propionyl group was hydrolyzed about three times faster than that containing N-formyl group.

It seems very interesting to study the in-

Taka-N-acetyl-β-D-glucosaminidase was used as abbreviation of the enzyme obtained from Taka-diastase (Sankyo) 2-amino-2-deoxy-D-glucose (IUPAC nomenclature); D-glucosamine 2-acylamino-2-deoxy-D-glucose (IUPAC nomenclature); N-acyl-D-glucosamine.



[100]

[107] R,—CH_2F
[108] R,—CH_2Cl
[109] R,—CH_2Br
[110] R,—CHF_2
[111] R,—$CHCl_2$
[112] R,—CF_3

[007] R,—CH_2F
[008] R,—CH_2Cl
[009] R,—CH_2Br
[010] R,—CHF_2
[011] R,—$CHCl_2$
[012] R,—CF_3

Fig. 1

fluence of replacing the α-hydrogen of the N-acetyl group with another atom on the enzymatic action, especially halogen atom which exhibited electronic effect opposite to alkyl group. A further study using the substrate analogs containing N-(haloacetyl) substituent was attempted in order to investigate the influence of the substitution of halogen atom on the N-acyl specificity.

The present report describes the preparation of *p*-nitrophenyl 2-acylamino-2-deoxy-β-D-glucopyranosides containing monofluoroacetyl, difluoroacetyl, trifluoroacetyl, monochloroacetyl, dichloroacetyl, and monobromoacetyl group as N-acyl substituent and the hydrolytic action of Taka-N-acetyl-β-D-glucosaminidase on these compounds. The process for preparing substrates used which was developed in the previous work (*1*) is shown in Fig. 1. For convenience, these compounds are numbered as indicated.

EXPERIMENTAL PROCEDURE

Melting points were determined by a micro-melting point apparatus (Yanagimoto MFG.) and uncorrected. Optical rotation were measured by a recording spectropolarimeter (Yanagimoto, ORD-185) at 25°C. Absorbance was measured by a spectrophotometer (Shimadzu, D-40S).

Preparation and Purification of N-acylating Reagent — Monofluoroacetic acid: Distillation from sulfuric acid solution of crude sodium monofluoroacetate gave a raw free acid, which was purified by twice repeated distillation. bp 168–170°C (lit. (*3*), bp 167–168.5°C).

Trifluoroacetic anhydride: Dehydration of trifluoroacetic acid over phosphorus pentoxide followed by distillation afforded the pure anhydride. bp 39–39.5°C (lit. (*4*), bp 39°C).

Monochloroacetic anhydride and monobromoacetyl bromide: Commercial products (CP-grade) were purified by distillation. bp 203–204°C (lit. (*5*), bp 203°C), and bp 149°C (lit. (*6*), bp 147°C), respectively.

Difluoroacetic acid, dichloroacetyl chloride, and dicyclohexyl carbodiimide: Commercial products (GR-grade) were used without further purification.

Preparation of p-Nitrophenyl 2-Acylamino-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside ([*107*], [*108*], [*109*], [*110*], [*111*], *and* [*112*])—*Procedure* (*A*): To a dimethylformamide (DMF) solution (30 ml) of [100] (0.50 g) (*7*) containing 0.3 ml of triethylamine and 10 ml of pyridine, was added 0.60 g of free acid dissolved in 5 ml of chloroform, and subsequently 1.6 g of DCC. After the reaction mixture was allowed to stand for 20 hr at 0°C, the residue obtained from the reaction mixture by evaporation was dissolved in 300 ml of chloroform. The chloroform solution freed from insoluble materials was washed with 0.1 N HCl, saturated sodium bicarbonate solution, and finally water under cooling, and then evaporated *in vacuo* to dryness after dried over anhydrous magnesium sulfate. Twice repeated crystallization of the residue gave a crystalline compound ([107] or [110]).

Procedure (*B*): Five hundred milligrams of [100] were treated by the same manner as in the previous work (*1*). Compounds ([108], [109], [111], and [112]) were obtained by crys-

tallization from suitable solvents.

Preparation of p-Nitrophenyl 1-Acylamino-2-deoxy-β-D-glucopyranosides ([007]–[012])—3, 4, 6-Tri-O-acetyl derivatives prepared above were deacetylated in methanol containing a catalytic amount of sodium methoxide at 0°C. After removing sodium ion with cation exchange resin (Dowex-50, acidic form), the methanol solution was evaporated *in vacuo* to dryness, and crystallization of the residue afforded white crystalline compounds ([007]–[012]).

p-Nitrophenyl 2-acetylamino-2-deoxy-β-D-glucopyranoside [002] was prepared by the method of Leaback (*8*).

Enzymic Hydrolysis of p-Nitrophenyl 2-Acylamino-2-deoxy-β-D-glucopyranosides ([002], [007]–[012])—N-Acetyl-β-D-glucosaminidase was extracted from Taka-diastase (Sankyo) and purified by Sephadex G-100 gel-filtration and CM-cellulose column chromatography (*9*). The most purified specimen used for experiments exhibited a single band on Disc electrophoresis. *p*-Nitrophenyl glycoside (1.0 mM) in 0.1 M citrate buffer, pH 4.5, was incubated with enzyme at 37°C. Aliquots of 1.5 ml were removed from the reaction mixture at suitable time intervals and made basic with 0.5 ml of 0.5 N NaOH, and its absorbance at 400 mμ was measured. For assays, the time course of the reaction was usually followed over a period of 5–10 min, and it was followed for more than 10 hr when the less susceptible substrates were examined. It was confirmed that, even in the long period incubation, enzyme activity was not altered and the non-enzymatic liberation of *p*-nitrophenol from the substrate used was negligible. The initial velocity was calculated from the linear portion of time course. The hydrolytic rate for each substrate was conveniently expressed as the value relative to that of the N-acetyl derivative [002].

RESULTS AND DISCUSSION

The N-acylation was undertaken by the reaction of the corresponding acyl halide or acid anhydride on *p*-nitrophenyl 2-amino-3, 4, 6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside [100] except for N-monofluoroacetyl and N-difluoroacetyl compounds. Transformation of monofluoroacetic, and difluoroacetic acids to the acyl chloride was difficult because of their toxicity. Therefore, the dicyclohexylcarbodiimide (DCC)-method was chosen for preparing those N-fluoroacetyl derivatives. Amide formation by DCC-method, which had been a most promising one for peptide synthesis, was applied to N-acylation of hexosamine by Greig *et al.* (*2*) and reported to afford insufficient yields. In this experiment, dimethylformamide (DMF) was used as a reaction solvent instead of chloroform because dipolar aprotic solvent seemed to be suitable for this reaction.

All of the compounds synthesized were crystallized, and the physical properties and the results of elemental analyses of *p*-nitrophenyl 2-acylamino-3, 4, 6-tri-O-acetyl-2-deoxy-β-D-glucopyranosides and *p*-nitrophenyl 2-acylamino-2-deoxy-β-D-glucopyranosides are listed in Tables I and II, respectively.

All of *p*-nitrophenyl glycosides could be prepared in good yield. The N-acylation process in which compound [100] were used as an intermediate under the conditions developed in the previous work (*1*) was found to be suitable in this case. However, since the amide formation by DCC-method was not so satisfactory owing to some difficulty to remove contaminant, the active ester method, as in the case of N-formylation (*10*), seemed to be preferred for this purpose.

The hydrolytic rate of substrate analogs, expressed as a value relative to the N-acetyl compound [002], is shown in Table III. Most of the compounds prepared in this experiment were hydrolyzed by Taka-N-acetyl-β-D-glucosaminidase except the N-dichloroacetyl derivative [011]. The N-monofluoroacetyl derivative [007] was especially well hydrolyzed, ten times faster than the N-propionyl derivative (*1*). In the case of the pig epididymal enzyme (*11*), the activity to the N-monofluoroacetyl derivative was reported to be also higher than that to the N-propionyl derivative, but by a factor of two.

Activity of the Taka-enzyme toward N-monohalogenoacetyl derivatives ([007], [008], and [009]) increases in the order of F>Cl>Br. This indicates that steric factor, that is, the

TABLE I. Physical properties and elemental analysis of *p*-nitrophenyl 2-acylamino-3, 4, 6-tri-O-acetyl-2-deoxy-*β*-D-glucopyranosides.

	N-Substituent	Formula	mp (°C)	$[\alpha]_{589}^{25°}$ (in acetone)
107	$-COCH_2F$	$C_{20}H_{23}N_2O_{11}F$	242–243	−33.4
108	$-COCH_2Cl$	$C_{20}H_{23}N_2O_{11}Cl$	244–245 (dec)	−29.8
109	$-COCH_2Br$	$C_{20}H_{23}N_2O_{11}Br$	235–236 (dec)	−36.7
110	$-COCHF_2$	$C_{20}H_{22}N_2O_{11}F_2$	209–210	−29.3
111	$-COCHCl_2$	$C_{20}H_{22}N_2O_{11}Cl_2$	219–220 (dec)	−28.5
112	$-COCF_3$	$C_{20}H_{21}N_2O_{11}F_3$	201–202	−25.1

	Elemental analysis							
	Calcd (%)				Found (%)			
	C	H	N	X†	C	H	N	X†
107	49.38	4.77	5.76	3.91	49.41	4.78	5.73	
108	47.77	4.61	5.57	7.05	47.88	4.52	5.49	6.88
109	43.89	4.24	5.12	14.60	43.88	4.17	5.00	14.72
110	47.62	4.40	5.55	7.53	47.67	4.43	5.56	
111	44.70	4.13	5.21	13.20	44.51	4.11	5.20	13.20
112	45.98	4.05	5.36	10.91	45.98	3.98	5.40	

† X=Halogens.

TABLE II. Physical properties and elemental analysis of *p*-nitrophenyl 2-acylamino-2-deoxy-*β*-D-glucopyranosides.

	N-Substituent	Formula	mp (°C)	$[\alpha]_{589}^{25°}$ (in DMF)
007	$-COCH_2F$	$C_{14}H_{17}N_2O_8F$	217–218 (dec)	−11.8
008	$-COCH_2Cl$	$C_{14}H_{17}N_2O_8Cl$	171–172 (dec)	−8.76
009	$-COCH_2Br$	$C_{14}H_{17}N_2O_8Br$	186–188 (dec)	−21.0
010	$-COCHF_2$	$C_{14}H_{16}N_2O_8F_2$	232–233 (dec)	−11.3
011	$-COCHCl_2$	$C_{14}H_{16}N_2O_8Cl_2$	225–226 (dec)	−14.4
012	$-COCF_3$	$C_{14}H_{15}N_2O_8F_3$	244–245 (dec)	−10.8

	Elemental analysis							
	Calcd (%)				Found (%)			
	C	H	N	X†	C	H	N	X†
007	46.67	4.76	7.78	5.27	46.34	4.71	7.71	9.78
008	44.63	4.55	7.44	9.41	44.37	4.57	7.31	9.78
009	39.92	4.07	6.65	18.97	40.07	4.03	6.58	18.38
010	44.45	4.26	7.41	10.12	44.49	4.38	7.36	
011	40.89	3.92	6.81	17.25	40.82	3.91	6.78	17.17
012	42.43	3.82	7.07	14.38	42.39	3.90	7.06	

† X=Halogens.

TABLE III. The relative rate of hydrolysis of *p*-nitrophenyl 2-acylamino-2-deoxy-β-D-glucopyranosides catalyzed by Taka-N-acetyl-β-D-glucosaminidase.

	N-Substituent	Hydrolytic rate
002	$-COCH_3$	1.0
007	$-COCH_2F$	0.50
008	$-COCH_2Cl$	0.012
009	$-COCH_2Br$	0.0040
010	$-COCHF_2$	0.0032
011	$-COCHCl_2$	0
012	$-COCF_3$	0.00033

space occupied with the halogen atom is responsible for the hydrolytic rate. The result that N-dichloroacetyl derivative [011] could not be hydrolyzed by this enzyme, differing from the N-difluoroacetyl [010] and N-trifluoroacetyl derivative [012], may be explained in the same way.

It was concluded from the above evidence (Table III) that the N-acyl specificity of this enzyme is not strictly limited to N-acetyl group, similarly to the conclusion from α-alkyl substitution of N-acetyl group (*1*). α-Monohalogen substitution of the N-acetyl group of substrate was permissible in the N-acyl specificity and, in the case of fluorine atom, even di- and tri- substitution was permitted in the specificity. Furthermore, that the specificity was predominantly controlled by steric factor of the N-acyl group is suggested. Contribution of steric factor to N-acyl specificity seems to be explained by considering the existence of a space fitted to methyl of the N-acetyl group in the specificity-determining site of enzyme and the flexibility of the space adapted to more bulky N-substituent than N-acetyl group.

As the factors corresponded to N-substituent was unable to be quantitatively evaluated, roles of hydrophobic and electronic factor is indistinguishable from that of steric factor.

The author wishes to thank Prof. Y. Matsushima (Faculty of Science, Osaka University) for his kind advice and support in this work and Dr. K. Shingu (Faculty of Science, Osaka University) for his kind operation of an ORD instrument.

Thanks of the author are also due to Dr. T. Mega for his generous gift of Taka-N-acetyl-β-D-glucosaminidase without which this work would not be possible.

REFERENCES

1. K. Yamamoto, *J. Biochem.*, **73**, 631 (1973).
2. C.G. Greig, D.H. Leaback, and P.G. Walker, *J. Chem. Soc.*, 879 (1961).
3. B.C. Saunders and G.J. Stacey, *J. Chem. Soc.*, 1773 (1948).
4. E.J. Bourne, M. Stacey, J.C. Tatlow, and J.M. Tedder, *J. Chem. Soc.*, 2977 (1947).
5. The "Merck Index" (eighth edition), Merck & Co. Inc. Rahway. U.S.A., p. 239 (1968).
6. J.F. Arens and D.A. Van Dorp, *Rec. Trav. Chem.*, **66**, 409 (1947).
7. E.L. May and E. Mosettig, *J. Org. Chem.*, **15**, 890 (1950).
8. D.H. Leaback, "Biochemical Preparation," **10**, 118 (1963).
9. T. Mega, T. Ikenaka, and Y. Matsushima, *J. Biochem.*, **68**, 109 (1970).
10. K. Yamamoto, *Bull. Chem. Soc. Japan*, **45**, 1253 (1972).
11. D.H. Leaback and P.G. Walker, *Biochem. J.*, **104**, 70p (1967).