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THE SYNTHESIS OF β-D-GALACTOPYRANOSIDE ANALOGS THAT CHELATE LANTHANIDE METAL IONS*

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ABSTRACT

The analogs of *o*-nitrophenyl β-D-galactopyranoside and -fucopyranoside, namely, 3-hydroxy-2-nitrophenyl β-D-galactopyranoside (**5**) and -fucopyranoside (**6**), respectively, were synthesized by Koenigs–Knorr reaction of the *O*-acetylglycosyl bromides with 2-nitroresorcinol and characterized by u.v. and n.m.r. spectroscopy. They chelate La^{3+} ions, and their complexes with Gd^{3+}, Eu^{3+}, and Pr^{3+} were compared by 100-MHz n.m.r. spectrometry to those obtained with diamagnetic La^{3+}. The stability constants of the metal complexes in solution were measured by u.v. spectroscopy. Compound **5** is a substrate for β-D-galactosidase and both **5** and **6** bind specifically to *lac* repressor from *Escherichia coli*.

INTRODUCTION

Lanthanide ions (Ln^{3+}) have been used in the study of the structure of proteins by paramagnetic-induced, nuclear magnetic relaxation or as paramagnetic shift reagents[1–4]. Several proteins form strong complexes with Ln^{3+} ions, and others have to be chemically altered in order to obtain complex-formation (see review by Reuben[3]). An elegant method introduced by Marinetti *et al.*[5] uses nitrotyrosine as an amino acid analog capable of chelating rare-earth metal ions, and gives useful information on the distances between specific amino acid side-chains and these metal ions[6]. However, these studies gave information only on the environment of those tyrosine residues that are accessible to chemical nitration, and they may be at the surface of the protein or at some other sites not necessarily near an active part of the protein. We were interested in introducing paramagnetic centers in the region of the effector binding-site of the lactose-operon repressor of *E. coli*. The study of the

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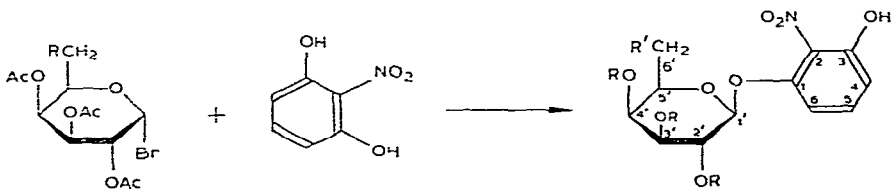
compounds described herein complements the work with organic spin-labels described in the accompanying paper[7].

Nuclear magnetic resonance studies of the *lac* repressor are currently in progress in several laboratories[8-10]. Thus, the synthesis of an inducer or an anti-inducer analog that selectively brings a paramagnetic ion into the binding site of the protein would be of value, and eventual measurements of enhanced relaxation or chemical shifts should yield useful information about the nature and geometry of the effector binding-site. Because of the success of Marinetti *et al.*[5,6] with 3-nitrotyrosine, in which the phenolic hydroxyl group and an oxygen atom of the nitro group chelate lanthanides, we synthesized compounds that are analogs of the anti-effectors, nitrophenyl β-D-galactopyranoside and -fucopyranoside. Koenigs–Knorr reaction[11]



1 R = OAc
2 R = H

3 R = Ac, R' = OAc
4 R = Ac, R' = H
5 R = H, R' = OH
6 R = R' = H

of poly-*O*-acetyl-α-D-glycopyranosyl bromides (**1**,**2**) with 2-nitroresorcinol and subsequent *O*-deacetylation, led to the corresponding 3-hydroxy-2-nitrophenyl β-D-galactopyranoside (**5**) and -fucopyranoside (**6**). These analogs have, overall, geometric similarity to the parent nitrophenyl compounds. Furthermore, both compounds **5** and **6** have a hydroxyl group in *ortho* position to the nitro group and are expected to chelate metal ions as well as nitrotyrosine. This was confirmed by the present data. The metal complexes of La^{3+}, Pr^{3+}, Eu^{3+}, and Gd^{3+} with **5** and **6** have a complex constant similar to those found for nitrotyrosine and lanthanide ions. Thus, a convenient introduction of the chelating function into other glycosides would give valuable structural information about other interesting proteins for which they are substrates or inhibitors. In addition to the study of the lactose repressor, the substrate specificity of **5** and **6** for the β-D-galactosidase of *E. coli* was investigated.

RESULTS AND DISCUSSION

The reaction of only one hydroxyl group on 2-nitroresorcinol with **1** and **2** was favored by use of a ten-fold excess of the phenol and by a short reaction-time. The reaction product gives only one isomer, regardless which of the two hydroxyl groups reacts. The compounds were easily separated, by silica gel chromatography, from unreacted starting-material and small proportions of side-products resulting from the

reaction of both hydroxyl groups of 2-nitroresorcinol. *O*-Deacetylation was achieved by treatment with methanol half-saturated with ammonia. Although compounds **5** and **6** were obtained pure, according to silica gel chromatography and paper chromatography, in 30% yield, they failed to crystallize. A small impurity of acetate derivatives (n.m.r.) could be removed by chromatography on DEAE-cellulose, for which the compounds had an affinity at pH 8.0 through the dissociated phenolic proton. Compounds **5** and **6** were eluted with a gradient of triethylammonium hydrogencarbonate, and subsequent exchange of the triethylammonium salts into the free acids by ion-exchange resin resulted in a spectroscopically pure compound that easily crystallized from water. The sodium salts could also be obtained in this way, followed by lyophilization of a concentrated water solution.

Both glycosides **5** and **6** exhibited identical u.v. spectra, as shown in Fig. 1. The phenol group was found to have a pK_a value of 6.2 $\pm$0.1. The titration of the phenol group with base caused a bathochromic shift in the absorption maximum accompanied by hyperchromicity of ~60% in that region. A new maximum appeared at 410 nm, while the shoulder at 315 nm formed a new minimum yielding two isosbestic points. The change in absorbance at 280 nm was plotted against pH, and the titration curve obtained is shown in the inset of Fig. 1. Postmus *et al.*[12] described a simple method for measuring the complex dissociation constants by u.v. spectroscopy for complexes where three absorbing species change concentration. This method was applied to evaluate the complex-stability constants, in aqueous and deuterium oxide solution, of **5** and **6** with La^{3+} and Gd^{3+}. Firstly, the maximum change in absorbance

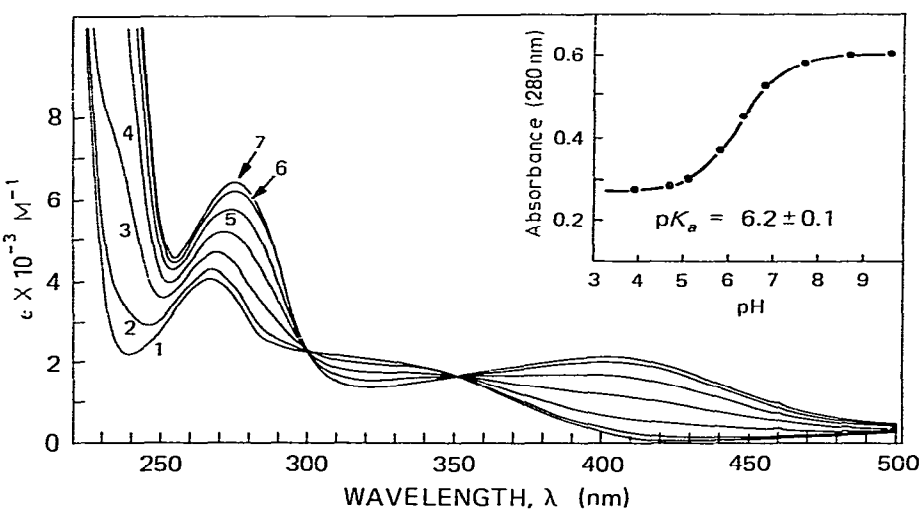


Fig. 1. U.v. absorption spectra of **5** and **6** at various pH values: (1) pH 3.9, (2) pH 5.1, (3) pH 5.8, (4) pH 6.3, (5) pH 6.8, (6) pH 7.7, and (7) pH 9.6. The inset is a plot of the change in absorbance at 280 nm *vs.* pH. The titration was performed in H_2O. The pH was recorded after each addition of hydrochloric acid or sodium hydroxide, before and after the spectrum was recorded. The change in total volume was less than 2%.

TABLE I

BINDING CONSTANTS OF 5 AND 6 WITH Gd^{3+} AND La^{3+}

Compound	*Solvent*	*log* K_1[a]	
		Gd^{3+}	La^{3+}
5	H_2O	1.87	1.65
5	D_2O	1.82	1.61
6	H_2O	1.79	1.62
6	D_2O	1.75	1.60

[a] K_1 = [Metal–ligand complex]/[Metal, free] · [Ligand, free]; approx. ±0.05.

was found by the u.v. difference-spectrum of the glycoside with an excess of metal ligand. Titrations with La^{3+} and Gd^{3+} solutions were performed at constant pH, at several wavelengths of maximum difference between the complex and the uncomplexed glycoside, *i.e.*, at 275, 310, and 420 nm, and the change in u.v. absorbance was measured in order to evaluate the various binding-constants (Table I).

The n.m.r. spectrum of **6** (middle, Fig. 2) shows two triplets in the aromatic region next to the glycosidic-ring-proton resonances. The triplet, integrating to two protons, is a superposition of the two doublets expected of either H-4 and H-6.

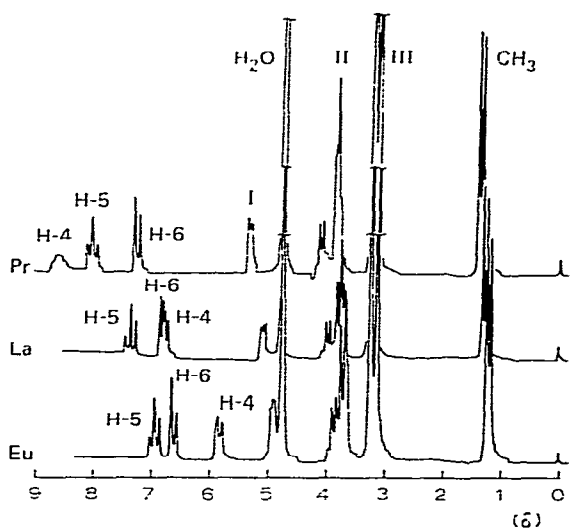


Fig. 2. ^{1}H-N.m.r. spectrum at 100 MHz of **6** in the presence of the paramagnetic shift reagents Pr^{3+} (upper line) and Eu^{3+} (lower line) as compared to the diamagnetic La^{3+} complex spectrum (middle line). The spectra were recorded in solutions in D_2O, 0.05M PIPES buffer (pH 6.8), and 0.1M KCl. The concentrations were: 0.05M **6**; 0.05M La^{3+} and 0.05M Pr^{3+}; 0.1M La^{3+}; 0.05M La^{3+} and 0.05M Eu^{3+}. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was the internal reference standard. The signals labeled I and II correspond to the anomeric and glycosidic ring-protons, respectively, and signal III was assigned to PIPES.

Because the largest perturbations of the spectrum, upon complex-formation with lanthanides, are expected in this region, the proton resonances of the aglycon were assigned first by a pD titration of **5** and **6** with NaOD in D_2O, assuming that dissociation of the deuterium-ion of the phenol group has its largest effect upon the nearest proton, *i.e.*, H-4 (see Fig. 3). During this titration, the doublet corresponding to δ 6.90 had a higher shift than the doublet corresponding to δ 6.82. At pH 6.7, the two doublets directly overlapped, giving rise to only one doublet. Upon further titration >pH 7.0, the curves crossed over. This titration also gave the pK of the compounds in deuterium oxide. The value of pK_D of 6.7 is, within experimental error, identical to the pK_H value when corrected for the deuterium effect on the glass electrode[13,14]. Thus, at low pH, δ 6.82 was assigned to H-6 and δ 6.90 to H-4. As shown later, t_1 measurements strongly reinforce this conclusion.

As the protons that interact with the relaxation or shift reagents were known, the complexes between **5** or **6** and the shift reagents, Pr^{3+} and Eu^{3+}, could be investigated by n.m.r. spectrometry. The advantage of many anisotropic Ln^{3+} ions lies in their ability to induce chemical shifts in opposite directions in a given magnetic field[5,15]. As shown in Fig. 2, Eu^{3+} induces an upfield shift (lower spectrum), whereas Pr^{3+} shifts downfield (upper spectrum). The shifts are most pronounced for the aromatic protons. These spectra were compared with that obtained in the presence of diamagnetic La^{3+} which, by its nature, had no effect on the chemical shifts of 3-hydroxy-2-nitrophenyl galactoside at the same concentration. This indicates that there are no shifts due to structural changes of the glycoside upon complex formation.

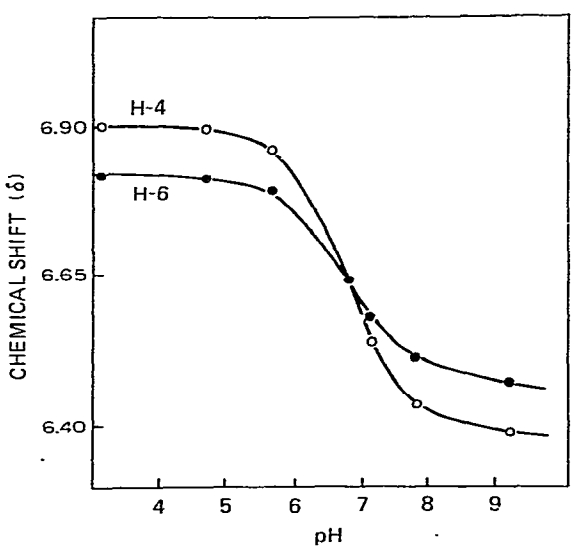


Fig. 3. N.m.r. spectroscopic titration of **5** in D_2O with NaOD and DCl. The t_1 values of H-4 and -6 are plotted *vs.* pH. The pH value is the uncorrected pH-meter reading of a glass micro-electrode introduced directly in the n.m.r. sample. The signal that shifts from δ 6.90 to 6.40 has been assigned to H-4, and the one that shifts from δ 6.82 to 6.50, to H-6.

A parallel experiment using either nitrophenyl β-D-galactopyranoside or fucopyranoside and this set of Ln^{3+} ions, on the other hand, showed no measurable shifts. Thus, the chelating function of the *o*-nitrophenol group was definitely necessary for complex-formation.

The dependence of the lanthanide-induced shifts upon the lanthanide concentration for the different cations leads to the evaluation of the stoichiometry of the sugar–lanthanide complexes[16]. From titrations, a 1 : 1 stoichiometry could be extrapolated, as found also by others for nitrophenol–lanthanide complexes[12]. As these analogs would be used for measurement of the distance from the binding site, in the *lac* repressor, by bringing a paramagnetic ion in or near the effector binding-site of the protein, the Gd^{3+} complexes were of special interest.

Owing to its high, electron-relaxation rate ($t \sim 10$ ns), the isotropic Gd^{3+} is an ideal n.m.r.-relaxation probe that causes no resonance shifts but resonance broadening[15]. Thus, it was possible to estimate electron–nucleus distances by the spin–lattice, relaxation enhancement induced by Gd^{3+} in various protons. It was also necessary to confirm the chelation of Gd^{3+} by the nitrophenol group, as selective effects of Gd^{3+} interactions with sugar acids have been reported[17]. The rates measured for the different protons of **5** are shown in Fig. 4. As the effect of Gd^{3+} upon the values t_1 of the aromatic regions was proportional to r^{-6} of the distance from the affected nuclei[18], it could be concluded that H-4 is closest to the ion. An interesting point is the noticeable effect of the relaxation enhancement of H-1'. Possibly, rotation around the glycosidic bond of the aromatic ring brings Gd^{3+} into the vicinity of this proton (see Fig. 5). Experiments with **6** indicated a much smaller relaxation-enhancement of the even more distant methyl protons of this compound (results not shown). The

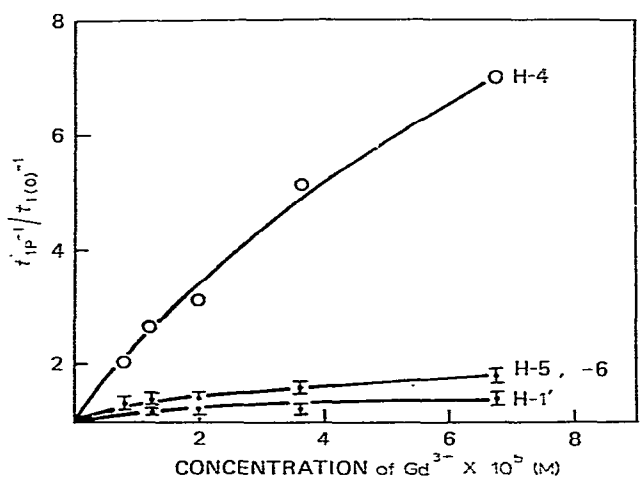


Fig. 4. Relaxation enhancement of the spin-lattice relaxation rates (t_1) of **5** on Gd^{3+} binding. The relaxation enhancement is defined as $t_{1P}^{-1}/t_{1(0)}^{-1}$, where t_{1P}^{-1} is the spin–lattice relaxation rate in the presence of Gd^{3+} at a constant La^{3+} concentration (0.05M), and $t_{1(0)}^{-1}$ the spin–lattice relaxation rate in the presence of 0.05M La^{3+}. The measurement of relaxation rates is described in the Experimental section.

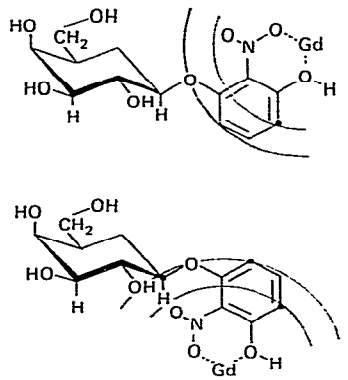


Fig. 5. Most likely structures for the complex between 5 and Gd^{3+}. The circles indicate the different spheres of interaction between Gd^{3+} and the protons of 5.

results clearly indicate a complex-formation between the analogs **5** and **6** and Ln^{3+} cations. The n.m.r. shifts induced by Pr^{3+} and Eu^{3+}, and the relaxation enhancements caused by Gd^{3+} are mainly due to pseudocontact interactions of the metals with protons in the spatial vicinity. As the perturbations are reflected mostly by the n.m.r. signal for H-4, complex-formation with the oxygen atoms of the nitro and hydroxyl groups of the rigid aromatic ring seems to be most likely for **5** and **6**. This is in agreement with the X-ray structure of nitrophenol and the isotropic relaxation-probe Gd^{3+} in complexes of nitrotyrosine analogs[5].

The close structural relationship of **5** and **6** to nitrophenyl β-D-galactopyranoside and -fucopyranoside, both effectors of the *lac* repressor[19,20], suggested their use for structural measurements of the inducer binding-site of *lac* repressor

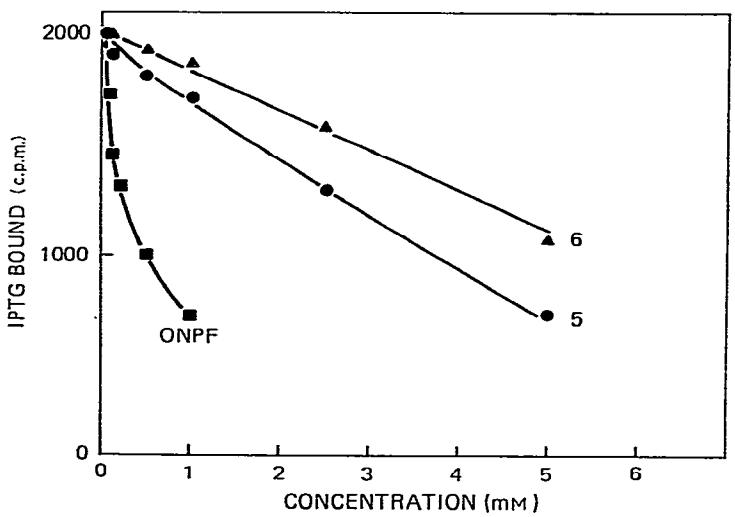


Fig. 6. Binding of **5**, **6**, and nitrophenyl β-D-fucopyranoside (ONPF) to *lac* repressor of *E. coli*, as indicated by competition with isopropyl 1-thio-β-D-galactopyranoside (IPTG) binding.

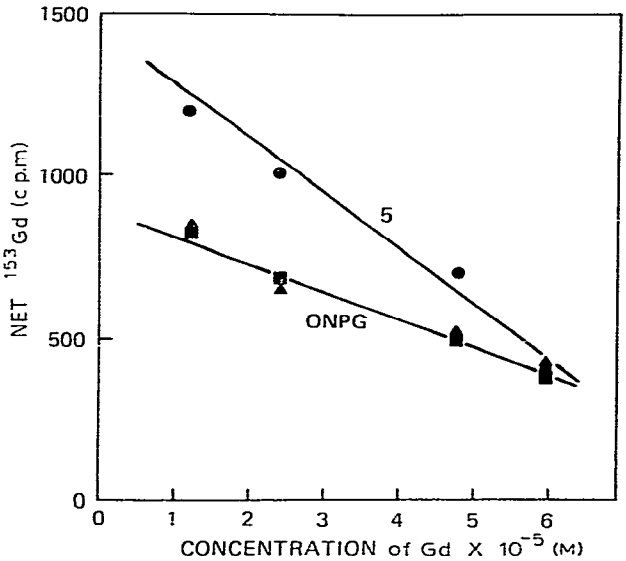


Fig. 7. Ternary complex between **5** (2.5mM), *lac* repressor, and Gd^{3+}. The amount of Gd bound to *lac* repressor in the presence of **5** *vs.* the nonchelating effector nitrophenyl β-D-galactopyranoside (ONPG, 2.5mM), and in the absence of any effector (■) was obtained by equilibrium dialysis against $^{153}Gd^{3+}$ solutions as indicated.

from *E. coli*. Both compounds were found to compete with isopropyl 1-thio-β-D-galactopyranoside for the inducer binding-site (see Fig. 6). Although the dissociation constant indicated by the 50%-competition value is about one order of magnitude larger than that for nitrophenyl β-D-fucopyranoside (~0.5mM), both analogs specifically bound to the lactose repressor almost equally well. In order to use the chelators as paramagnetic, effector analogs, it was necessary to demonstrate the presence of the ternary complex formed between *lac* repressor, effector, and Gd^{3+}. In a separate study, Gd^{3+} itself was found to bind to the *lac* repressor at a single, specific site per tetramer, which was not affected by the binding of the inducer[21]. When an equilibrium-dialysis experiment was performed with *lac* repressor inside the dialysis bag, and $^{153}Gd^{3+}$ in the presence of **5**, the amount of excess Gd^{3+} bound to the repressor was significantly higher than in the presence of nitrophenyl β-D-galactopyranoside (without chelating function), or in the absence of any effector (see Fig. 7). The excess of $^{153}Gd^{3+}$ bound to *lac* repressor when **5** was present allowed us to postulate the formation of a complex between Gd-*lac* repressor and **5**. Thus, **5** should be a useful tool for probing the structure of the effector binding-site of the protein.

As the use of **5** and **6** as specific reporter-groups for other biological molecules, such as permeases, transport systems, and β-D-galactosidases, would also be of interest, the hydrolysis of **5** by β-D-galactosidase from *E.* coli was investigated. The wavelength, where the maximum absorption-difference upon hydrolysis of **5** and **6** occurs, was determined from the spectrum of free nitroresorcinol at pH 7.5 and the

TABLE II

PROPERTIES OF COMPOUNDS **3–6**

Compound	*Yield* (%)	*M.p.* (°)	*Formula*	*Anal.*						R_F	*N.m.r. spectrum* (δ)
				Calc.			*Found*				
				C	*H*	*N*	*C*	*H*	*N*		
3	53		$C_{20}H_{23}NO_{13}$	49.5	4.8	2.9	49.1	4.4	3.0	1.0 (B) and 0.26 (C)	
4	43		$C_{18}H_{21}NO_{11}$	50.6	5.0	3.3	50.3	4.7	3.1	1.0 (B) and 0.28 (C)	
5	78	205	$C_{12}H_{15}NO_8 \cdot H_2O$	45.1	5.4	4.4	45.4	5.0	4.6	0.2 (A) and 0.37 (D)	D_2O: 7.3 (1 H) and 6.8 (2 H, aromatic), 5.1 (1 H, H-1′), and 4.0–3.6 (6 H, H-2′, -3′, -4′, -5′, and H_2-6′)
6	63	214	$C_{12}H_{15}NO_7 \cdot H_2O$	47.5	5.7	4.6	47.9	4.9	4.5	0.45 (A) and 0.47 (D)	D_2O: 7.4 (1 H) and 6.8 (2 H, aromatic), 5.1 (1 H, H-1′), 4.0–3.7 (4 H, H-2′, -3′, -4′, and -5′), and 1.3 (3 H, H_3-6′)

[a]Silica gel, 17:3 (v/v) chloroform–methanol (A), 19:1 (v/v) chloroform–methanol (B), chloroform (C); and paper chromatography, 7:1:2 (v/v) 2-propanol–25% ammonia–water (D).

spectra of **5** and **6**. The difference in the absorptivity (ε) at 320 nm gave a $\Delta\varepsilon$ 2300, and the hydrolysis of both **5** and **6** by β-D-galactosidase was measured at this wavelength. The initial rate of hydrolysis of **5** by β-D-galactosidase was almost the same as that found for the substrate normally used for this assay, nitrophenyl β-D-galactopyranoside[22]. The β-D-fucopyranoside, on the other hand, was not hydrolyzed by β-D-galactosidase, even after a reaction time of 1 h at 37°. Experiments to inhibit the hydrolysis of nitrophenyl-β-D-galactopyranoside with **6** indicated that **6** also is not an inhibitor of β-D-galactosidase, in agreement with the observation of Wallenfels and Weil[23] that the binding specificity of fucosides is reduced. If the specificity were reduced by a factor >1000, the hydrolysis of **6** would not be detected under the conditions described here. This is also consistent with the observation that **6** is not an inhibitor.

EXPERIMENTAL

General. — See ref. 7. 2-Nitroresorcinol was obtained from Pfaltz & Bauer Inc., Stamford, CT 06902. The metal ions were purchased as the corresponding oxides from Alfa Div., Ventron Corp., Danvers, MA 04923, and dissolved in hydrochloric acid. For n.m.r. experiments, the chlorides were treated by repeated additions and evaporations of deuterium oxide. The n.m.r. spectra were recorded either at 60 MHz with a continuous-wave Varian A-60 spectrometer or at 100 MHz with a JEOL-100 PFT spectrometer operated in the Fourier-transform mode. The longitudinal relaxation times were measured by the 180°–τ–90° pulse method and 25 accumulations were taken for each τ value. All spectra were recorded at room temperature. The binding of effector analogs to *lac* repressor was monitored by displacement of radioactively-labeled isopropyl 1-thio-β-D-galactopyranoside by the Millipore filter method[24]. The counts of the filter were determined in a PPO–1,4-dioxane solution with a Searle liquid-scintillation counter. The lactose repressor from *E. coli* was isolated according to the method of Platt *et al.*[25].

*3-Hydroxy-2-nitrophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside* (**3**) *and 3-hydroxy-2-nitrophenyl 2,3,4-tri-*O*-acetyl-β-D-fucopyranoside* (**4**). — A solution of bromide **1** or **2** (5 g) was introduced dropwise into a solution of 2-nitroresorcinol (10-fold molar excess) in M sodium hydroxide at 0°. The mixture was stirred at room temperature, and samples were withdrawn, and analyzed by t.l.c. (silica gel, 19:1 v/v, chloroform–methanol). The reaction was stopped between 1 and 2 h, longer times giving lower yields. The mixture was concentrated under diminished pressure to about half its volume, and extracted with chloroform (5 × 100 mL). The combined extracts were washed thrice with a small amount of M sodium hydroxide and twice with water, dried ($MgSO_4$), and evaporated. The residue in chloroform was applied to a preparative silica gel column and was eluted with 19:1 (v/v) chloroform–methanol. The faint-yellow fractions gave, upon evaporation, a residue that could be crystallized from ethanol to give chromatographically pure, yellow crystals, dec. 150–180° (see Table II).

3-Hydroxy-2-nitrophenyl β-D-galactopyranoside (**5**) *and -fucopyranoside* (**6**).

— Compounds **3** and **4** (2.0–2.5 g) were *O*-deacetylated by treatment with half-saturated ammonia in methanol (100 mL) for 12 h. The solvent was evaporated and the residue, dissolved in water, was applied to a DEAE cellulose column. The column was eluted with a linear gradient of triethylammonium hydrogencarbonate from 0 to 0.1M. Fractions containing **5** or **6** (orange color) were combined, concentrated, and separated from the buffer by repeated additions and evaporations of water, and then the water solution was passed through a short column of Dowex (H^+) cation-exchange resin. The free acids thus obtained crystallized easily as faint yellow needles from a small amount of water. In some cases, the sodium salts were prepared by passing the triethylammonium salts through a column of Dowex (Na^+) cation-exchange resin. In these cases, the sodium salts were lyophilized from water to give an orange powder (see Table II).

REFERENCES

1 E. Nieboer, *Struct. Bonding (Berlin)*, 22 (1975) 1–45
2 A. T. Morris and R. A. Dwek, *Q. Rev. Biophys.*, 10 (1977) 421–489.
3 J. Reuben, in K. A. Geschneidner, Jr., and L. Eyring (Eds.), *Handbook on the Physics and Chemistry of the Rare Earths*, North Holland, Amsterdam, 1979, in press.
4 K. Wüthrich, *NMR in Biological Research: Peptides and Proteins*, North Holland, Amsterdam, 1976.
5 T. D. Marinetti, G. H. Snyder, and B. D. Sykes, *J. Am. Chem. Soc.*, 97 (1975) 6562–6570.
6 T. D. Marinetti, G. H. Snyder, and B. D. Sykes, *Biochemistry*, 15 (1976) 4600–4608.
7 H.-R. Rackwitz, *Carbohydr. Res.*, 88 (1981) 223–232.
8 P. Lu, M. A. Jarema, K. Mosser, and W. E. Daniel, *Proc. Natl. Acad. Sci. U.S.A.*, 73 (1976) 3471–3475.
9 K. S. Matthews, N. G. Wade-Jardetzky, M. Graber, W. Conover, and O. Jardetzky, *Biochim. Biophys. Acta*, 490 (1977) 534–538.
10 F. Buck, H. Rüterjans, and K. Beyreuther, *FEBS Lett.*, 96 (1978) 335–338.
11 J. Conchie and G. A. Levvy, *Methods Carbohydr. Chem.*, 2 (1962) 335–336.
12 C. Postmus, Jr., L. B. Magnusson, and C. A. Craig, *Inorg. Chem.*, (1966) 1154–1157.
13 A. K. Covington, M. Paabo, R. A. Robinson, and R. G. Bates, *Anal. Chem.*, 40 (1968) 700–706.
14 L. P. Kalinchenko and U. P. Lobyshev, *Stud. Biophys.*, 58 (1976) 235–240.
15 C. M. Dobson and B. A. Levine, *New Tech. Biophys. Cell Biol.*, 3 (1976) 19–91.
16 See ref. 4, p. 278.
17 B. Casu, G. Gatti, N. Cyr, and A. Perlin, *Carbohydr. Res.*, 41 (1975) c6–c8.
18 I. Solomon and N. Bloembergen, *J. Chem. Phys.*, 25 (1956) 261.
19 B. Müller-Hill, H. V. Rickenberg, and K. Wallenfels, *J. Mol. Biol.*, 10 (1964) 303–318.
20 K. Jayaraman, B. Müller-Hill, and H. V. Rickenberg, *J. Mol. Biol.*, 18 (1966) 339–343.
21 H. R. Rackwitz, M. A. Jarema, and P. Lu, unpublished results.
22 G. R. Craven, E. Steers, Jr., and C. B. Anfinsen, *J. Biol. Chem.*, 240 (1965) 2468–2477.
23 K. Wallenfels and R. Weil, in P. D. Boyer (Ed.), *The Enzymes*, 3rd edn., Vol. VIII, Academic Press, New York, pp. 617–663.
24 A. D. Riggs and S. Bourgeois, *J. Mol. Biol.*, 34 (1968) 361–364.
25 T. Platt, J. G. Files, and K. Weber, *J. Biol. Chem.*, 248 (1973) 110–121.