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Simultaneous detection of different glycosidase activities by ^{19}F NMR spectroscopy☆

Martin Albert [a], Werner Repetschnigg [a], Jörg Ortner [a], Joseph Gomes [b], Bernhard J. Paul [a], Carina Illaszewicz [a], Hansjörg Weber [a], Walter Steiner [b], Karl Dax [a,*]

[a] *Institute of Organic Chemistry, Technical University Graz, Stremayrgasse 16, A-8010 Graz, Austria*
[b] *Institute of Biotechnology, Technical University Graz, Petersgasse 12, A-8010 Graz, Austria*

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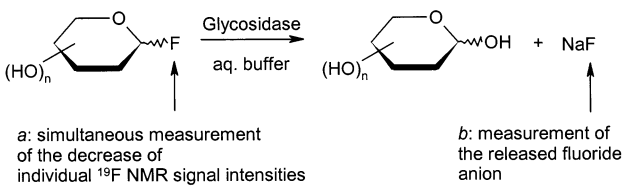
Abstract

A fast method for the simultaneous detection of different glycosidolytic activities in commercially available enzyme preparations and crude culture filtrates was found in using, as substrate, a mixture of different glycosyl fluorides and ^{19}F NMR spectroscopy as a screening technique. Accompanying studies regarding the hydrolytic stability of these fluorides in various buffer systems, as well as conditions of their long-term storage, were carried out. A simple procedure for the preparation of β-D-mannopyranosyl fluoride in gram quantities is given.

Keywords: Glycosyl fluorides; Glycosidases; ^{19}F NMR spectroscopy; β-D-Mannopyranosyl fluoride

1. Introduction

Increasing evidence of the essential role that carbohydrates play in biological systems [2,3] stimulates the search towards efficient methods for the synthesis of these compounds. For di- and oligosaccharides, as an alternative to the chemical glycosylation, the enzyme-catalysed glycoside synthesis constitutes a promising option. Besides the powerful, but expensive and often hardly accessible glycosyl transferases [EC 2.4], the cheaper glycosidases [EC 3.2] have also been successfully used in many instances [4–7]. The search for such enzymes by screening crude enzyme preparations is time consuming since most methods for determining glycosidase activities are based on photometric measurements which allow the testing of just one activity at a time [8]. In the course of an ongoing study towards the enzymatic synthesis of di- and oligosaccharides employing glycosidases from new sources, a method for the simultaneous detection of individual glycosidolytic activities in crude culture filtrates was sought.



Scheme 1.

☆ See Ref. [1].

* Corresponding author. Tel.: +43-316-8738244; fax: +43-316-8738740.
E-mail address: dax@orgc.tu-graz.ac.at (K. Dax).

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Table 1
^{19}F chemical shift values [a] (δ) of glycosyl fluorides used and of some fluorine containing salts in aq NaOAc–HOAc buffer (50 mmol, pH 5)

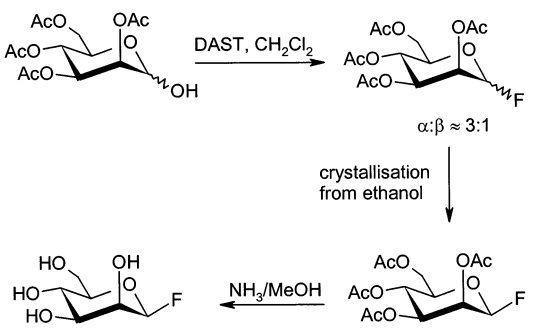
Glycosyl fluoride	δ (ppm)	Glycosyl fluoride	δ (ppm)	Salts	δ (ppm)
α-D-Man-F	−138.6	β-D-Man-F	−146.6	CF_3COONa	−75.4
α-D-Glc-F	−150.1	β-Cell-F	−143.8	$NaHF_2$	−122
α-D-GlcNAc-F	−146.0	β-D-Gal-F	−143.1	NaF	−133
α-L-Fuc-F	−151.8	β-Lac-F	−143.5		

[a] Dependence (± 0.5 ppm) on the pH value was observed.

2. Results and discussion

Glycosyl fluorides are known to be accepted as substrates by glycosidases [9] and methods for the determination of a distinct glycosidase activity, by monitoring the release of fluoride with an ion-sensitive electrode, are elaborated (Scheme 1(b)) [10,11]. As an extension of this principle, an approach for the simultaneous detection of different glycosidase activities by means of ^{19}F NMR spectroscopy was investigated. The resonance signals of the individual glycosyl fluorides differ sufficiently in their ^{19}F chemical shift (see Table 1), consequently allowing the detection of any glycosidolytic activity by comparing the integral values over the corresponding signals from a 'blank' and an incubated sample (Scheme 1(a)).

The individual glycosyl fluorides were prepared according to known methods [12], i.e., either by treatment of the corresponding peracetates with pyridine–HF followed by deacetylation with a 0.01 M NaOMe–MeOH solution to give the more thermodynamically stable α-fluorides or by the reaction of per-O-acetylated glycosyl bromides with KHF_2 in CH_3CN under reflux followed by treatment with a saturated solution of NH_3 in MeOH to yield the corresponding β anomers. Nonetheless, β-D-mannopyranosyl fluoride was not accessible by this method. A published procedure [13] for the synthesis of 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl fluoride using collidine-HF as fluorinating agent could not be reproduced. However, fluorination of 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose [14] with diethylaminosulfur trifluoride (DAST) led to an anomeric mixture (α:β ratio approximately 3:1), from which the pure β anomer was isolated in 21% yield by simple crystallisation; the same product had been obtained previously in 7% yield by a similar strategy [15]. Subsequent deacetylation with NH_3–MeOH furnished the desired β-D-mannopyranosyl fluoride (Scheme 2).



Scheme 2.

Considering the lability of β-glycosyl fluorides towards hydrolysis [16], the influence of the buffer system (nature, concentration, pH value) in this respect was examined. For this purpose, a mixture of different glycosyl fluorides (plus CF_3COONa as an internal standard) was dissolved in the respective buffer system (containing 10% D_2O for NMR-locking purposes) and the decrease in concentration of the individual fluorides was determined in appropriate time intervals by ^{19}F NMR spectroscopy. Half-life periods calculated therefrom are summarised in Table 2.

Based on these hydrolysis rates as well as on general enzyme stability, we chose the NaOAc–HOAc buffer (System 1 in Table 2) for subsequent experiments. Similar studies were carried out with regard to the long-term storage of these fluorides. From the various conditions tested for this purpose, solutions in absolute methanol, stored at −80 °C, gave the best results, e.g., β-D-galactopyranosyl fluoride (the most labile) had a half-life period of approximately 2 years.

Table 2
Half-life periods ($t_{1/2}$ in h) of some glycosyl fluorides in aqueous buffer systems

Glycosyl fluoride	System 1 [a]	System 2 [b]	System 3 [c]	System 4 [d]
α-D-Man-F	580	190	290	290
α-D-Glc-F	290	230	290	290
β-D-Gal-F	7	7	6	8
β-Cell-F	39	29	39	39
β-D-Man-F	14	13	14	11
β-Lac-F	14	13	17	11
β-D-Glc-F	29	39	29	29

[a] NaOAc–HOAc, 90 mM, pH 5.
[b] Tris–HCl, 90 mM, pH 7.5.
[c] Phosphate–citrate, 90 mM, pH 7.0.
[d] Phosphate–citrate, 180 mM, pH 7.0.

For screening experiments, defined solutions containing comparable amounts of different glycosyl fluorides (and sodium trifluoroacetate as internal standard) in dry methanol were prepared. Therefrom, two identical samples, containing approximately 3 mg of each fluoride, were taken and evaporated to dryness. The samples were then redissolved in aqueous NaOAc–HOAc buffer (containing 10% D_2O for NMR referencing). One sample was either incubated directly with an aqueous solution of a commercially available glycosidase or a defined volume of a crude culture filtrate and the other — to take into account the non enzymatic rate of hydrolysis — was diluted with the same volume of neat buffer solution. The decrease of the concentration of the individual glycosyl fluorides (GF) was determined by the measurement of the relative intensities of their ^{19}F NMR signals (I_{GF} (sample) and I_{GF} (blank)) after an appropriate interval of time (4–7 h). By forming fractions (V (blank) and V (sample)) with the respective intensities of the corresponding internal standard (I_{ST} (blank) and I_{ST} (sample)) the proportion of unhydrolysed glycosyl fluorides was obtained. From this, the percentage of the enzyme catalysed hydrolysis (EH) was calculated.

$$V(\text{blank}) = \frac{I_{GF}(\text{blank})}{I_{ST}(\text{blank})}; \quad V(\text{sample}) = \frac{I_{GF}(\text{sample})}{I_{ST}(\text{sample})};$$

$$EH = \frac{V(\text{blank}) - V(\text{sample})}{V(\text{blank})} \times 100$$

As a general drawback, the accuracy in quantitative determination of individual glycosidolytic activities is limited by the fact that certain glycosidases, in particular 'inverting' ones, are able to hydrolyse not only the stereochemically corresponding glycosyl fluoride but also its anomer; however, the hydrolysis rates for these two reactions were found to differ by two orders of magnitude [17,18]. Furthermore, due to the high number of substrates present in the sample, matrix and inhibition effects may also affect the results.

An initial series of experiments using commercially available glycosidases was carried out. As an example, the results obtained from a mixture of four glycosyl fluorides after incubation (4 h) with a β-galactosidase from *Aspergillus oryzae* are summarised in Table 3.

As expected, the corresponding β-D-galactosyl fluoride was completely hydrolysed. The hydrolysis of α-L-fucosyl fluoride and α-D-glucosyl fluoride was attributed to side activities in the enzyme preparation. The considerable β-glucosidase activity, responsible for the partial disappearance of the β-lactosyl fluoride, could be confirmed by the conventional *p*-nitrophenyl method (using *p*-nitrophenyl β-D-glucopyranoside as a substrate).

The practical usability of this NMR method was studied with crude culture filtrates of nine different microorganisms. In each case, a solution of selected fluorides was incubated and then analysed by means of ^{19}F NMR spec-

Table 3
Enzyme catalysed hydrolysis (%) of the individual fluorides by an enzyme preparation [a] from *Aspergillus oryzae*, with a β-galactosidase as the main activity, after 4 h

	α-D-Glc-F	α-L-Fuc-F	β-Lac-F	β-D-Gal-F
Enzyme catalysed hydrolysis (%)	17	8	66	100

[a] 10 u β-galactosidase; NaOAc–HOAc buffer, 50 mM, pH 5.

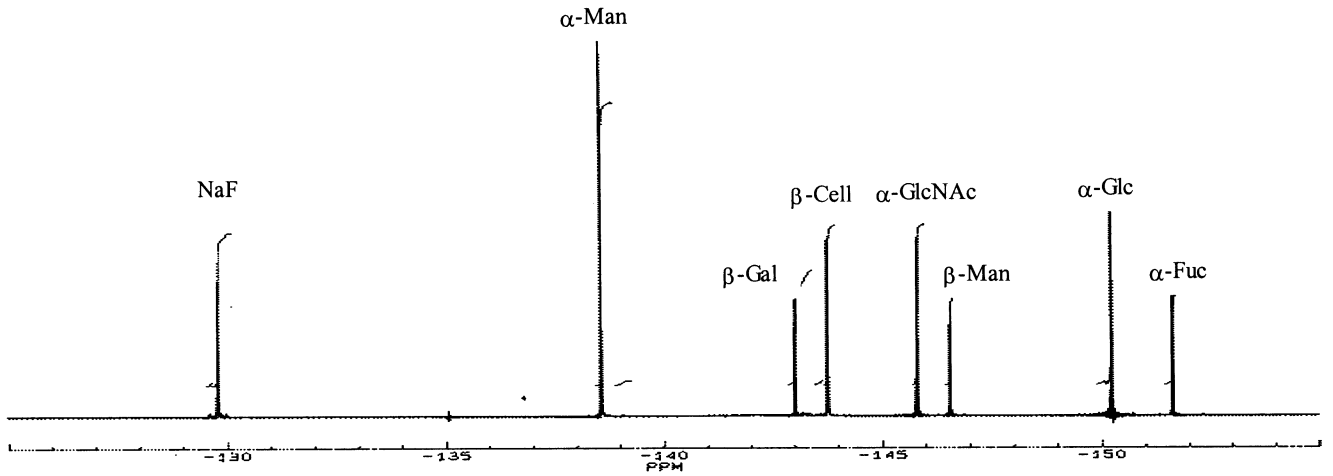


Fig. 1. Blank sample. This spectrum was obtained 7 h after dissolving the set of glycosyl fluorides in acetate buffer (50 mmol, pH 5). Reference signal (sodium trifluoroacetate) at −75.4 ppm is not shown.

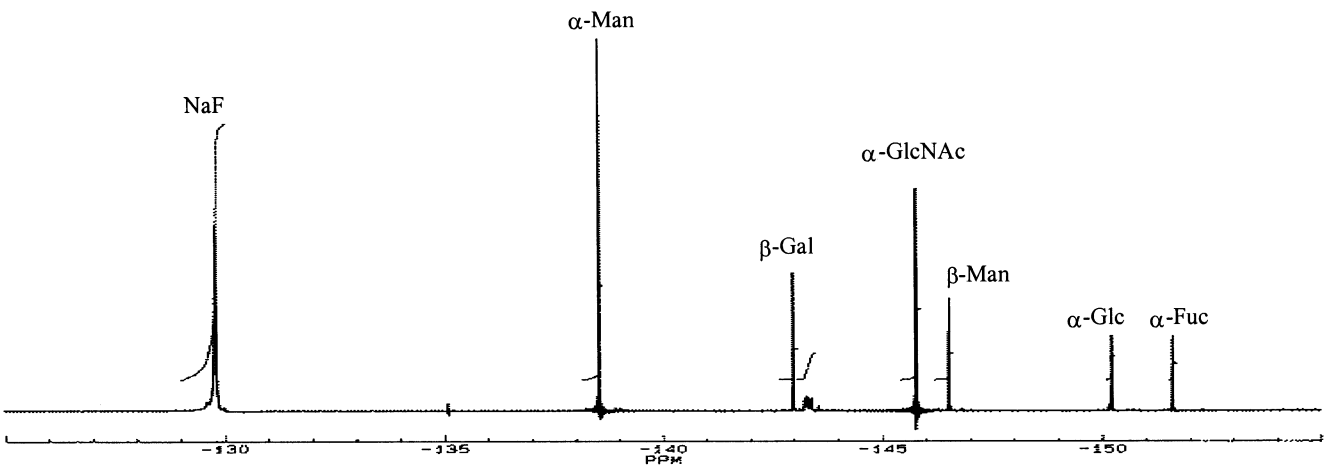


Fig. 2. Study of a culture filtrate from *Thermoascus aurantiacus*. The spectrum was recorded 7 h after incubation with the crude enzyme preparation. Reference signal (sodium trifluoroacetate) at −75.4 ppm is not shown.

Table 4
Individual rate of hydrolysis (%) of the glycosyl fluorides in the presence of a crude culture filtrate [a] from *Thermoascus aurantiacus* after 4 h

	α-D-Man-F	β-D-Gal-F	β-Cell-F	α-D-GlcNAc-F	β-D-Man-F	α-D-Glc-F	α-L-Fuc-F
Enzyme catalysed hydrolysis (%)	36	25	100	7	26	43	16

[a] Addition of 5 vol% culture filtrate; NaOAc–HOAc buffer, 50 mM, pH 5.

troscopy. The results obtained with the thermophilic fungus *Thermoascus aurantiacus*, after 7 h of incubation of a mixture of seven glycosyl fluorides, are shown in Figs. 1 and 2 (Fig. 1 corresponds to the blank sample, Fig. 2 to the incubated one). The interpretation is given in Table 4.

In most cases, the results obtained from these experiments matched those obtained from the photometric method. Due to mechanistic differences in the hydrolysis of glycosidases over glycosyl fluorides and *p*-nitrophenyl glycosides, a comparison can be undertaken only qualitatively (low, medium or

high activity). In some rare instances, photometrically detected glycosidase activities could not be verified with the ^{19}F NMR method (and vice versa), which might be explained by substrate specificities of the corresponding glycosidases or attributed to matrix effects. Deviations were also observed when coloured culture filtrates were used.

Studies on the transglycosylation potential of the crude culture filtrate of *T. aurantiacus* are presented in a following paper [19].

3. Experimental

General procedures.—Melting points were determined with a Tottoli apparatus (Büchi 300) and are uncorrected. Optical rotations were measured with a Jasco DIP-360 digital polarimeter at 589 nm at ambient temperature. NMR spectra were recorded at 300.13 or 199.98 MHz (^1H), 75.47 or 50.29 MHz (^{13}C) and 282.4 MHz (^{19}F), using a Bruker MSL 300 and a Varian Gemini 200 apparatus, respectively. As reference standards Me_4Si (^1H and ^{13}C NMR) and trichlorofluoromethane (^{19}F NMR) were used. TLC was performed on Silica Gel 60 F 254 precoated aluminum plates (E. Merck 5554) with detection by charring after spraying with vanillin–H_2SO_4 (1%). For column chromatography, Silica Gel 60, 230–400 mesh (E. Merck 9385) was used.

The crude culture filtrate used in this study was that of the strain *Thermoascus aurantiacus* Miehe, isolated from decomposed jute in Bangladesh. It has been identified by Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, and deposited in the collection of the Institute of Biotechnology, Technical University Graz, Austria, under the stock number BT 2079. The basic mineral medium containing (g L^{-1} tap water) 5, KH_2PO_4; 0.5, $MgSO_4 \cdot 7H_2O$; 0.05, $CaCl_2 \cdot 2H_2O$; 40, Solka floc 40; 5, NH_4NO_3 and 1 mL trace element solution (g L^{-1} tap water: 1.6, $MnSO_4 \cdot H_2O$; 1.4, $ZnSO_4 \cdot 7H_2O$; 2, $CoSO_4 \cdot 7H_2O$) was used for growth. The initial pH of the medium was adjusted to 5 prior to sterilisation. The sterilised culture medium (100 mL) in 300 mL Erlem neyer flasks was inoculated with a 1 cm^2 PDA (potato dextrose agar) piece of an actively growing 4-day-old PDA culture of *T. aurantiacus* and cultivated at 47 °C on an orbital shaker (150 rpm) for 10 days. The crude broth was centrifuged and the supernatant was used for further experiments.

2,3,4,6-Tetra-O-*acetyl-β*-D-*mannopyranosyl fluoride.*—To a solution of 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (20.0 g, 57.4 mmol) in abs CH_2Cl_2 (300 mL) were added 10.5 mL DAST (80 mmol) at rt within 10 min. After 30 min the excess of DAST was quenched with MeOH (20 mL) at 0 °C. The organic phase was washed with water and satd aq $NaHCO_3$ soln, then dried over Na_2SO_4. Concentration under reduced pressure led to a brown oil which was filtered through silica gel (100 g, 1:1 EtOAc–cyclohexane eluent). The slightly brown residue was redissolved in boiling EtOH. 2,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranosyl fluoride precipitated as colourless rhombic crystals. These were separated by filtration (4.5 g, 21%) as soon as colourless needles (the corresponding α anomer) started to deposit; mp 106–106.5 °C, lit. 105.5–107 °C [15]; $[\alpha]_D^{20}$ +5.1° (*c* 1.1, $CHCl_3$), lit. −1.0° (*c* 1.5, $CHCl_3$) [15]; ^1H NMR (199.98 MHz, $CDCl_3$): δ 5.53 (dd, 1 H, $J_{1,F}$ 50.9 Hz, $J_{1,2}$ 1.9 Hz, H-1), 5.45 (ddd, 1 H, $J_{2,F}$ 9.5 Hz, $J_{2,3}$ 2.9 Hz, H-2), 5.1–5.3 (m, 2 H, H-3, H-4), 3.90 (m, 1 H, $J_{5,6}$ 5.1 Hz, H-5), 4.2–4.4 (m, 2 H, H-6a, H-6b); ^{13}C NMR (50.29 MHz, $CDCl_3$): δ 104.0 (d, $J_{1,F}$ 223 Hz, C-1), 66.6 (d, $J_{2,F}$ 20 Hz, C-2), 68.3 (d, $J_{3,F}$ 6 Hz, C-3), 66.1 (C4), 72.5 (d, $J_{5,F}$ 4 Hz, C-5), 62.5 (C-6); ^{19}F NMR (282.4 MHz, $CDCl_3$): δ −142.6 ($J_{F,H1}$ 50.1 Hz, $J_{F,H2}$ 9.5 Hz).

β-D-*Mannopyranosyl fluoride.*—2,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranosyl fluoride (1.0 g, 2.85 mmol) was dissolved in a satd soln of NH_3 in abs MeOH (50 mL). After 15 h at 4 °C, the solvent was removed under reduced pressure at rt and β-D-mannopyranosyl fluoride was obtained as a colourless oil in quantitative yield (0.52 g); $[\alpha]_D^{20}$ −4.2° (*c* 2.15, MeOH); ^1H NMR (199.98 MHz, methanol-d_4): δ 5.38 (dd, 1 H, $J_{1,F}$ 50.1 Hz, $J_{1,2}$ 1.0 Hz, H-1), 3.76 (bd, 1 H, $J_{2,F}$ 11.8 Hz, H-2), 3.92 (bs, 1 H, H-3), 3.5–4.5 (m, 3 H, H-4, H-6a, H-6b), 3.33 (m, 1 H, H-5); ^{13}C NMR (50.29 MHz, methanol-d_4): δ 108.4 (d, $J_{1,F}$ 210 Hz, C-1), 71.2 (d, $J_{2,F}$ 18 Hz, C-2), 74.1 (d, $J_{3,F}$ 9

Hz, C-3), 68.1 (C4), 78.4 (d, $J_{5,F}$ 4 Hz, C-5), 62.7 (C-6); ^{19}F NMR (282.4 MHz, aq NaOAc–HOAc buffer, 50 mM, pH 5): δ −146.6 (d, $J_{F,H1}$ 48.3 Hz).

^{19}F NMR enzyme assay.—A total of 0.33 mmol per glycosyl fluoride and 20 mg of the internal standard sodium trifluoroacetate were dissolved in abs MeOH (20 mL) for a series of 20 experiments. For one sample, 1 mL of this solution was taken out and the solvent was removed under reduced pressure at 20 °C. The samples were redissolved in the corresponding buffer (0.5 mL, 80 mM, 10 vol% D_2O) at the same time. A solution of the enzyme preparation (200 μL) or the crude culture filtrate (200 μL) was then added. The blank samples were diluted with buffer (200 μL). In order to minimise errors, two identical samples were prepared in each case. The ^{19}F NMR spectra were recorded 4–7 h later. The evaluation was made after averaging the data of the corresponding samples.

Measurement of half lives for spontaneous hydrolysis of glycosyl fluorides.—The samples were prepared as described above. They were dissolved in the appropriate buffer system (4 mL, 10 vo1% D_2O) and the ^{19}F NMR spectra were recorded after 5, 40, and 80 min and 2.5, 4, 18, and 40 h. The obtained hydrolysis curves were in accordance to a first-order expression over this period of time.

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