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Note

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NMR Tube Degradation Method for Sugar Analysis of Glycosides

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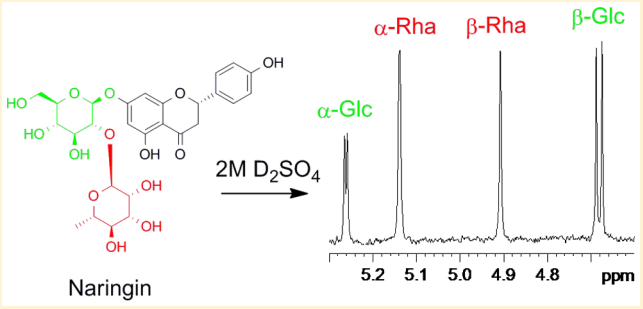
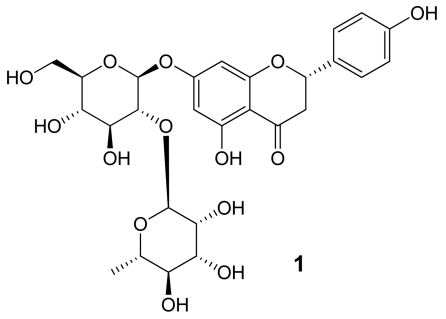
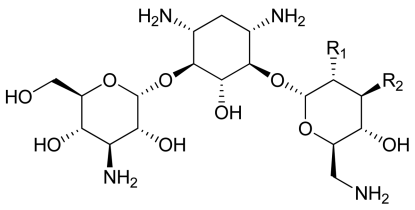
Supporting Information

ABSTRACT: The sugar subunits of natural glycosides can be conveniently determined by acid hydrolysis and ^{1}H NMR spectroscopy without isolation or derivatization. The chemical shifts, coupling constants, and integral ratios of the anomeric signals allow each monosaccharide to be identified and its molar ratio to other monosaccharides to be quantified. The NMR data for the anomeric signals of 28 monosaccharides and three disaccharides are reported. Application of the method is demonstrated with the flavonoid glycoside naringin (**1**), the aminoglycoside antibiotics kanamycin (**2**) and tobramycin (**3**), and the saponin digitonin (**4**).



Many natural products occur as glycosides. The combinatorial diversity generated by glycosylation is impressive, often overwhelming. Dozens of types of sugars can potentially be incorporated, each of which typically has multiple sites for the attachment of other sugars. NMR spectroscopy using 2D methods can be used to determine the structures of natural glycosides, but overlapping signals often make it challenging.[1] A common strategy used to delimit the component sugars is to hydrolyze a sample and to determine which sugars are present. This is generally accomplished by GC after TMS derivatization or LC, both of which require authentic standards.[2] An NMR method was developed that allows sugar determination without isolation or derivatization. Furthermore, once the NMR measurements have been made and the data recorded, authentic standards are no longer needed. This method has been used extensively in the analysis of polysaccharides from wood products[3,4] and has been applied to the analysis of glycolipids.[5] The method is general and can be advantageously applied to many glycosidic natural products. Herein we describe the method and demonstrate its application to some representative examples: the flavonoid glycoside naringin (**1**), the aminoglycoside antibiotics kanamycin (**2**) and tobramycin (**3**), and the steroidal saponin digitonin (**4**) (Figure 1).





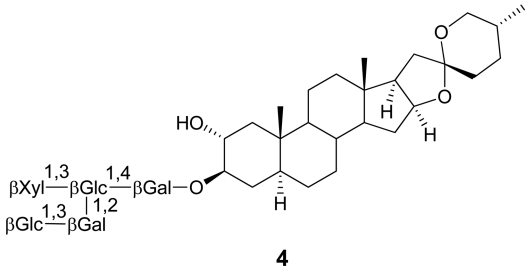


Figure 1. Natural product glycosides.

RESULTS AND DISCUSSION

Observation of the anomeric region by ^{1}H NMR in D_2O is usually difficult due to the overlapping water signal. However, this signal can be shifted by changing the pH. By measuring the spectra in 2 M sulfuric acid, the water peak is shifted downfield from ca. 4.7 ppm to ca. 6 ppm, where it does not interfere with the observation and integration of the anomeric protons (Figure S6, Supporting Information). The use of deuterated sulfuric acid helps to minimize the water signal, and presaturation can be used to greatly reduce its intensity, which improves the dynamic range of the signals of interest. In practice, when presaturation is used, it is often unnecessary to use deuterated solvents beyond what is

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Table 1. ^{1}H NMR Data for the Anomeric Signals of Reference Sugars

	δ (J in Hz; % total anomeric signals)		
Hexoses			
glucose (**5**)	5.26, d (3.6; 38%)	4.68, d (7.9; 62%)	
galactose (**6**)	5.30, d (3.8; 33%)	4.63, d (7.9; 67%)	
mannose (**7**)	5.21, s (62%)	4.94, s (38%)	
allose (**8**)	5.17, d (3.4; 18%)	4.90, d (8.3; 82%)	
allose (**8**)[a]	5.54,[b] d (2.2; 14%)		
	5.17, d (3.4; 16%)	4.90, d (8.3; 70%)	
altrose (**9**)	5.15, s (59%)	5.02, d (8.3; 41%)	
altrose (**9**)[a]	5.41,[b] d (2.2; 69%)		
5.15, s (18%)	5.02, d (8.3; 13%)		
gulose (**10**)	5.21, d (3.7; 14%)	4.90, d (8.4; 87%)	
gulose (**10**)[a]	5.48,[b] d (2.4; 62%)		
	5.21, d (3.7; 5%)	4.90, d (8.4; 33%)	
idose (**11**)	5.10, s (52%)	5.02, d (5.8; 48%)	
idose (**11**)[a]	5.38,[b] s (94%)		
	5.10, s (3%)	5.02, d (5.8; 3%)	
talose (**12**)	5.30, s (55%)	4.85, s (45%)	
Uronic Acids			
glucuronic acid (**13**)	5.32, d (3.7; 49%)	4.7, d (8.0; 51%)	
glucuronic acid (**13**)[a]	5.59,[c] v br s (6%)	5.51,[c] s (27%)	
	5.32, d (3.7; 32%)	4.75, d (8.0; 35%)	
glucuronolactone (**14**)	5.59, v br s (20%)	5.51, s (80%)	
galacturonic acid (**15**)	5.37, d (3.6; 44%)	4.68, d (8.0; 56%)	
Pentoses			
arabinose (**16**)	5.27, d (3.5; 34%)	4.56, d (7.8; 66%)	
ribose (**17**)	4.96, d (6.2; 69%)	4.88, s (31%)	
xylose (**18**)	5.22, d (3.4; 35%)	4.61, d (7.9; 61%)	
lyxose (**19**)	5.03, d (4.6; 69%)	4.90, d (1.3; 31%)	
Deoxy Sugars			
2-deoxyribose (**20**)	5.32, v br s (47%)	4.84, v br s (53%)	
2-deoxyglucose (**21**)	5.40, br s (47%)	4.96, br d (9.3; 53%)	
fucose (**22**)	5.23, d (3.7; 29%)	4.60, d (8.0; 71%)	
	1.26,[d] d (6.5; 71%)	1.22,[d] d (6.5; 29%)	
rhamnose (**23**)	5.14, s (57%)	4.91, s (43%)	
	1.31,[d] d (6.2; 43%)	1.29,[d] d (6.3; 57%)	
quinovose (**24**)	5.20, d (3.7; 31%)	4.68, d (8.0; 69%)	
	1.30,[d] d (6.2; 69%)	1.27,[d] d (6.2; 31%)	
Amino Sugars			
glucosamine (**25**)	5.49, d (3.4; 63%)	4.99, d (8.5; 37%)	
N-acetylglucosamine (**26**)	5.25, d (3.5; 55%)	4.80, d (8.4; 45%)	
galactosamine (**27**)	5.51, d (3.6; 59%)	4.93, d (8.5; 41%)	
kanosamine (**28**)	5.30, d (3.6; 50%)	4.76, d (7.7; 50%)	
6-glucosamine (**29**)	5.30, d (3.7; 41%)	4.72, d (8.0; 59%)	
nebrosamine (**30**)	5.40, d (3.4; 61%)	4.96, d (8.4; 39%)	
Disaccharides			
cellobiose (**31**)	5.26, d (3.7; 20%)	4.70, d (7.9; 30%)	4.53, d (8.0; 50%)
maltose (**32**)	5.40, d (3.8; 50%)	5.26, d (3.8; 20%)	4.69, d (8.0; 30%)
lactose (**33**)	5.26, d (3.6; 20%)	4.71, d (8.0; 30%)	4.48, d (7.9; 50%)
Methylated Sugars			
2,3,4,6-tetramethylglucose (**34**)	5.40, d (3.5; 60%)	4.64, d (8.0; 40%)	
2,3,6-trimethylglucose (**35**)	5.43, d (3.5; 60%)	4.68, d (7.9; 40%)	

[a]Sample was not heated. (All other samples were heated at 100 °C for 1 h.) [b]Signal from 1,6-anhydro sugar. [c]Signal from glucuronolactone (**14**). [d]Methyl signal.

required to maintain the lock signal. Since hydrolysis of glycosides to monosaccharides generally involves heating the sample in the presence of acid, the hydrolysis and analysis can be combined in the same NMR tube.

Every monosaccharide has a pair of characteristic chemical shifts and coupling constants for the anomeric (H-1) signals (Table 1). These signals are not concentration dependent (Figure S5, Supporting Information). Furthermore, they are found in a specific α/β ratio. Thus, glucose (**5**) can be identified by its signals at 5.26 ppm (J = 3.6 Hz, α-pyranoside, 38%) and 4.68 ppm (J = 7.9 Hz, β-pyranoside, 62%) (Figure 2d). However, for the purpose of identification, it is not necessary to know which

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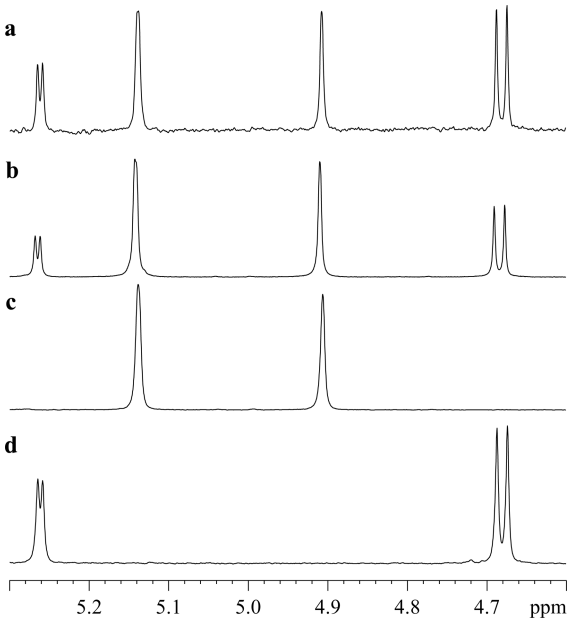

Figure 2. Anomeric protons of products from naringin (**1**) hydrolysis. (a) Hydrolysis of 250 μg of **1**, 1 h, 95 °C. (b) Hydrolysis of 12 mg of **1**, 15 min, 95 °C. (c) Rhamnose (**23**). (d) Glucose (**5**).

signals belong to the α- and β-anomers, respectively, as long as the pattern matches. Furthermore, knowing whether the signals originate from pyranose or furanose forms is unnecessary. Although the equilibrium between the furanoside and pyranoside forms could potentially double the number of anomeric signals,[6] in general, monosaccharides show only two predominant forms. It should be noted that sugars lacking anomeric hydrogens (e.g., ketoses) cannot be identified by this method.

After measuring the reference spectra, the sugars were heated to 100 °C in 2 M D_2SO_4/D_2O for 1 h to check their stability to conditions that would be sufficient to hydrolyze most glycosides. Perhaps somewhat surprisingly, the monosaccharides remain unchanged after heating in acid even for prolonged periods in almost all cases (Figure S4, Supporting Information). Changes were seen, however, for some sugars. The rare or unnatural hexoses allose (**8**), altrose (**9**), gulose (**10**), and idose (**11**) were found to equilibrate with their 1,6-anhydro forms upon heating.[7] These represented 14%, 69%, 62%, and 94% of the equilibrium mixtures, respectively, and their signals would have to be taken into account in a quantitative analysis. The more common glucuronic acid (**13**) also displayed new signals upon acid treatment. These signals were due to the formation of glucuronolactone (**14**), which was formed as 33% of the equilibrium mixture under these conditions. Again, this needs to be taken into account, but does not interfere with the analysis. Galacturonic acid (**15**) does not react under these conditions.

Although most sugars tolerated heating with acid, some sugars were unstable. The pentoses, arabinose (**16**), ribose (**17**), xylose (**18**), and lyxose (**19**), all displayed slow degradation to furfural (9.50, 7,94, 7.61, and 6.79 ppm). These were relatively minor amounts, representing 2%, 9%, 5%, and 4% of the mixtures, respectively, after heating for 1 h at 100 °C, and could be minimized by milder hydrolysis conditions.

Complete decomposition was observed for 2-deoxysugars (**20** and **21**). However, their glycosides are much more easily hydrolyzed than those of other sugars and do not require such harsh conditions. Stability tests showed that **21** was only 50% decomposed in the presence of 2 M D_2SO_4 at 37 °C for 4 days. This would allow its analysis by NMR in mixtures generated by the mild hydrolysis conditions typically used for 2-deoxysugars. It should be noted that the 2-deoxysugars **20** and **21** showed broad peaks, especially 2-deoxyribose (**20**), which may limit the utility of this method. The 6-deoxysugars fucose (**22**), rhamnose (**23**), and quinovose (**24**) did not pose any problems. Besides the anomeric signals, their methyl signals are also well separated from other signals and could also be diagnostic (Figure S2, Supporting Information).

As expected, the acetyl group of *N*-acetylglucosamine (**26**) was lost when heated for 1 h at 100 °C. However, at lower temperatures, **26** was relatively stable, undergoing only 43% deacetylation after 4.5 h at 65 °C.

As a demonstration of this method, a 250 μg sample of the flavonoid glycoside naringin (**1**) was hydrolyzed in the NMR tube. The spectrum (Figure 1a) was acquired over 30 min at 600 MHz using presaturation of the residual solvent peak. The sum of the integrals of the anomeric signals of glucose (**5**) is equal to the sum of the anomeric signals of rhamnose (**23**), indicating a 1:1 ratio. It was noticed that at early times during the hydrolysis of **1** the signals of the terminal α-rhamnose subunit predominated (Figure 1b). This is thought to be due to the more rapid hydrolysis of α-linkages and terminal glycosides.[8] The observation that one sugar is liberated before another might be of use for structure determination, but the extent of its generality remains to be demonstrated. Because of the insolubility of the flavonoid group, neither naringin, its monodeglycosylated product, nor its aglycone was observed in 2 M D_2SO_4. This simplifies the analysis of the free sugars, but if required, we found that DMSO-d_6 can be added to solubilize the nonpolar substances without interfering with either the hydrolysis or the NMR analysis (Figure S9, Supporting Information). However, the shifts and ratios of the anomeric signals do change in the presence of DMSO, and care must be taken in their assignment (Figure S10, Supporting Information).[9]

The data for three rare amino sugars were obtained through hydrolysis of the aminoglycoside antibiotics kanamycin (**2**) and tobramycin (**3**). Both of these antibiotics incorporate a kanosamine unit (3-amino-3-deoxyglucose, **28**) in their structures. The signals corresponding to **28** could therefore be assigned through their occurrence in the hydrolysis products of both **2** and **3** (Figure 2). The remaining signals from the hydrolysis of **2** and **3** therefore arise from 6-glucosamine (**29**) and nebrosamine (**30**), respectively. The hydrolyses of both aminoglycosides were slow, but that of the nebrosamine glycosidic

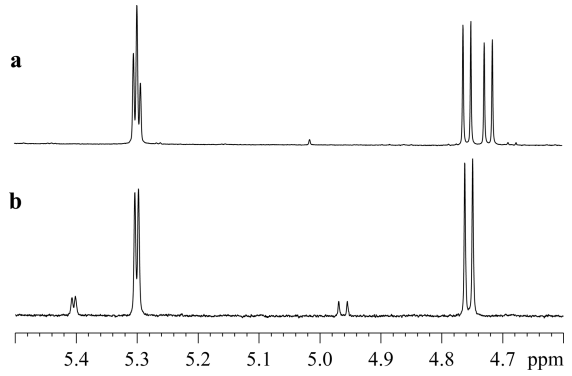

Figure 3. Anomeric protons of the hydrolysis products of kanamycin (**2**) and tobramycin (**3**). (a) Hydrolysis of 7.8 mg of **2**, 17 h, 95 °C. (b) Hydrolysis of 7.3 mg of **3**, 113 h, 95 °C.

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linkage in tobramycin (**3**) was extremely slow due to the 2-amino group. After more than 4 days at 95 °C, only 10% of this linkage had been hydrolyzed. This, however, was sufficient for the measurement of the anomeric signals of **30**.

The hydrolysis of the steroidal glycoside digitonin (**4**) gave a 2:2:1 mixture of glucose (**5**), galactose (**6**), and xylose (**18**) (Figure 4a). At an early point in the hydrolysis, it was noticed that

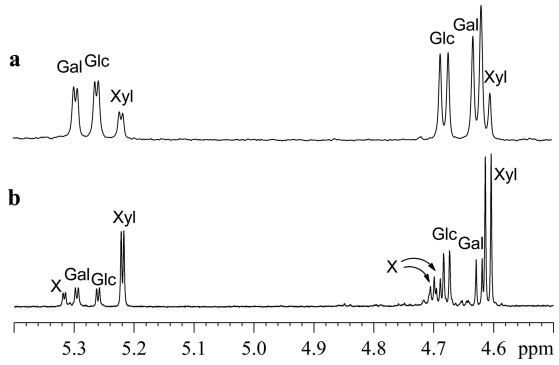

Figure 4. Anomeric protons of the hydrolysis products of digitonin (**4**). (a) Hydrolysis of 0.8 mg of **4**, 11 h, 100 °C. (b) Hydrolysis of 11.1 mg of **4**, 12 min, 90 °C (800 MHz).

the terminal xylose (**18**) group was liberated faster than the other sugars (Figure 4b). As was the case for naringin (**1**), neither digitonin (**4**), its aglycone, nor its intermediate hydrolysis products are soluble in water and are therefore not observed. In this case, unidentified anomeric signals were also detected early in the hydrolysis; these later vanished. The observed signals suggest a disaccharide fragment, having signals consistent with the α- and β-anomers of a terminal moiety, as well as a β-linkage, although additional anomeric signals indicative of a trisaccharide or higher oligomer may have been obscured. This observation should contain information about the linkage pattern and suggests that a library of disaccharide fragments might be useful in determining the sequence of sugars. The signals of three common disaccharides (**31**−**33**) are included in Table 1.

Methylation analysis has been a useful technique in classical carbohydrate analysis for determining linkage patterns.[2,10] Tests were carried out to ascertain whether this technique could be combined with the NMR method. Exhaustive methylation of glucose (**5**) was followed by hydrolysis in the NMR tube with 2 M D_2SO_4/D_2O at 100 °C for 1 h to give 2,3,4,6-tetramethylglucose (**34**). Repeating the process with maltose (**32**) yielded a 1:1 mixture of **34** and 2,3,6-trimethylglucose (**35**), the anomeric signals of which are well separated (Figure 5). This indicates the presence of a 1,4-linkage. Besides the anomeric signals, the methoxy signals of the different methylated sugars were also different, and, because they are strong signals found in a region of the spectrum without much overlap, these may also be diagnostically useful for sugar analysis by NMR.

Some drawbacks of the NMR tube hydrolysis method are that it is destructive and that NMR is less sensitive than GCMS. However, adequate amounts for analysis by this method are often available. In these cases, the experimental simplicity makes this a useful and convenient method.

EXPERIMENTAL SECTION

NMR spectra were obtained at 30 °C using a Bruker 600 Avance III instrument using either a 5 mm BBFO SmartProbe or a 5 mm BBFO Prodigy Cold probe, except for Figure 4b, which was obtained on a

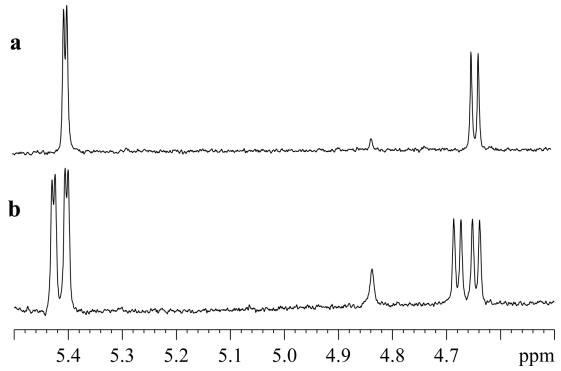

Figure 5. Anomeric protons of the hydrolysis products (1 h, 100 °C) of (a) fully *O*-methylated glucose and (b) fully *O*-methylated maltose.

Bruker Avance III HD with an Ascend 800 MHz magnet and a 5 mm TCI cryoprobe. All spectra were measured using 500 μL of 2 M D_2SO_4 as the solvent. Because of the high ionic strength of the solvent, it is not always possible to tune and match these samples in all NMR probes. To obtain quantitative data, the ^{1}H NMR spectra were acquired with a relaxation delay set to 10 s, 30° pulse angle, 2.7 s acquisition time, and spectral widths of 16 ppm. Since the T_1 of the anomeric signals of four representative sugars [galactose (**6**), glucose (**5**), mannose (**7**), and xylose (**18**)] were measured to be 1.3−2.0 s (Figure S8, Supporting Information), integration of the signals is quantitative with a 30° pulse according to the Ernst equation.[10] For discussion of quantitative NMR see Pauli et al.[11] Chemical shift calibration was with 0.05% sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, $(C\underline{H}_3)_3Si$ = 0.015 ppm). Spectra were acquired with 16K data points and processed using zero filling, exponential multiplication, and line broadening of 0.3 Hz. To prepare 2 M D_2SO_4/D_2O, 20 g of sulfuric acid-d_2 (99.5% D, Cambridge Isotope Laboratories) was diluted to a final volume of 100 mL with deuterium oxide (99.9% D, Cambridge Isotope Laboratories). Glycosides and sugars were obtained commercially. Typically, ca. 10 mg samples were analyzed. Heating of the reference sugars was carried out in a boiling water bath; otherwise, heating blocks were used. The anomeric ratios given in Table 1 were obtained by manual integration of the spectra shown in the Supporting Information. Methylation of glucose and maltose was carried out as described by Ciucanu and Costello.[12]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00180.

^{1}H NMR spectra of the anomeric regions of sugars **5**−**35** and the methyl regions of sugars **22**−**34**, a spectrum of furfural, spectra of **5**−**7** heated for varying lengths of time and different concentrations of **5**−**7**, spectra of **5** at different acid concentrations, T_1 measurements of **5**−**7** and **18**, hydrolysis of naringin (**1**) in the presence of DMSO, and spectra of glucose (**5**) in the presence of varying concentrations of DMSO (PDF)

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Notes

The authors declare no competing financial interest.

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