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Quantification of Deuterium Isotopomers of Tree-Ring Cellulose Using Nuclear Magnetic Resonance

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Stable isotopes in tree rings are important tools for reconstruction of past climate. Deuterium (D) is of particular interest since it may contain climate signals and report on tree physiology. Measurements of the D/H ratio of tree-ring cellulose have proven difficult to interpret, presumably because the D/H ratio of the whole molecule blends the abundances of the seven D isotopomers of cellulose. Here we present a method to measure the abundance of the D isotopomers of tree-ring cellulose by nuclear magnetic resonance spectroscopy (NMR). The method transforms tree-ring cellulose into a glucose derivative that gives highly resolved, quantifiable deuterium NMR spectra. General guidelines for measurement of D isotopomers by NMR are described. The transformation was optimized for yield and did not alter the original D isotopomer abundances, thus, conserving the original signals recorded in wood cellulose. In the tree-ring samples tested, the abundances of D isotopomers varied by approximately ±10% (2% standard error). This large variability can only be caused by biochemistry processes and shows that more information is present in D isotopomer abundances, compared to the D/H ratio. Therefore, measurements of the D isotopomer distribution of tree rings may be used to obtain information on long-term adaptations to environmental changes and past climate change.

Variations in the natural abundance of stable isotopes ($^2H/^1H$ or D/H, $^{13}C/^{12}C$, and $^{18}O/^{16}O$ ratios) are important tools to constrain biogeochemical fluxes[1,2] and to reconstruct past climate from paleological archives.[3−5] To forecast global climate change, records of past climate with broad geographic coverage are needed, as well as an understanding of long-term interactions between the biosphere and climate. Tree-ring series are attractive archives for this purpose, because they can span thousands of years[6] and may record variation in climate over these lengths of time. While the D/H isotope ratio is a well-established tool for climate reconstruction from ice cores,[3] attempts to correlate the D/H ratio of tree-ring cellulose with climate parameters have led to ambiguous results.[7,8] We assume that this ambiguity reflects the fact that the factors influencing the D/H ratio of tree-ring cellulose have not been properly taken into account: (1) the D/H ratio of precipitation, which depends on temperature,[9,10] (2) D enrichment of leaf water by transpiration, (3) kinetic isotope effects (KIE) of enzymes,[11] and (4) hydrogen isotope exchange between source water and carbohydrates during cellulose synthesis in the trunk.[12−14]

The influences of the above factors could not be differentiated from each other, because until now only the average D/H ratio of all C−H groups of cellulose (commonly expressed as the δD value)[15] has been measured. Such measurement ignores the fact that enzyme isotope effects and enzyme-catalyzed hydrogen exchange modulate the D abundance of specific C−H groups; that is, they influence abundances of specific D isotopomers. The D abundance of tree-ring cellulose must therefore be described by a D isotopomer distribution (DID) if information about each of the four factors is of interest. DIDs have been measured previously by deuterium nuclear magnetic resonance spectroscopy (NMR),[11,13] but DIDs of tree-ring cellulose have never been studied. Here we describe a method for routine measurement of DIDs of tree-ring cellulose by NMR. Use of the method reveals that DIDs of tree-ring cellulose are nonrandom and retain signals originating from regulation of photosynthesis. DIDs also allow tracking of sources

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of compounds or reconstruction of mechanistic information in other research areas such as food control,[16] environmental chemistry,[17] and human physiology.[18] For these purposes, general experimental guidelines for DID measurement by NMR are presented.

EXPERIMENTAL SECTION

Material. To test the sample preparation on varied materials, four groups of samples were used: group I, commercial α-cellulose from spruce (*Picea abies*; Fluka catalog no. 22181); group II, α-cellulose of known δD purified from oak tree rings (*Quercus petraea*) from Northern Ireland, U.K.[19] (only the cellulose extracted from the early and late wood of the years 1945 and 1951 and early, middle, and late wood of the years 1946 and 1948 were used); group III, a trunk disk of a 50 year old Douglas fir (*Pseudostuga menzeisii*) from Santa Catalina Mountains, Arizona (early and late wood from 1983 and 1984 were used); group IV, two trunk disks from 41 year old spruce trees (*Picea abies*) from the Flakaliden experimental forest, Sweden[20] (the 1998−2000 tree rings were used). For comparison, leaf soluble sugars were extracted from sunflower plants (*Helianthus annuus*) grown in a greenhouse (summer 2001, Umeå University) following Augusti et al.[14] All solvents and reagents were of analytical grade or higher. Chloroform, stabilized with amylene instead of ethanol, was chosen to avoid sample contamination with ethanol.

Wood Preparation. Approximately 500 mg of material was used from each wood sample. A microtome was used to separate early and late wood (80−120-μm wood chips) for group III. The wood chips were reduced to a powder (approximately 200-μm grain size) using a ball mill. Part of the sample was used for α-cellulose extraction.[21] For group IV, the three outer rings of the trunk disks were ground to a fine powder using a hand file. All wood powder samples were transferred to glass funnels and washed with warm water and warm acetone to remove soluble compounds. The samples were left to dry overnight under a fume hood.

Cellulose Hydrolysis. Acid hydrolysis of wood and cellulose samples (step A in Figure 1) was performed according to Saeman et al.[22] except that for each 100 mg of sample (regardless of source), 1 mL of 72% H_2SO_4 was used and the glucose solution obtained after autoclaving was cooled on ice and neutralized with $CaCO_3$. Salts were then removed by vacuum filtration, and the filtrate was thoroughly dried using a rotary evaporator followed by an oil pump.

Glucose Derivatization. The derivatization process described by Schleucher et al.[11] was optimized for yield and reproducibility.

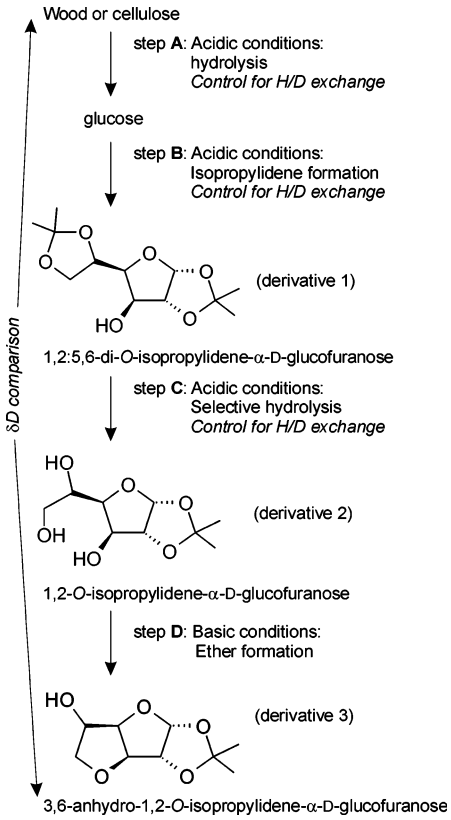

Figure 1. Reaction scheme for preparation of glucose derivatives from wood or cellulose. Reaction type and reaction conditions are summarized for each step. Control experiments are indicated in italics.

All of the following steps are described for samples containing 250 mg of glucose. Glucose was converted to 1,2−5,6-di-*O*-isopropylidene-α-D-glucofuranose (derivative 1; step B in Figure 1) by reaction with 30 mL of acetone and 1.26 mL of 96% H_2SO_4 for 4 h. The acid was added as three portions: 180 μL at the beginning, 540 μL after 1 h, and 540 μL after 3 h. The solution was vacuum-filtered into a flask containing a mixture of 75 g of ice and 45 mL of water. This solution was left at 0 °C for 17 h (overnight) to selectively hydrolyze derivative 1 to 1,2-*O*-isopropylidene-α-D-glucofuranose (derivative 2; step C in Figure 1). The hydrolysis was stopped by neutralizing the solution with $CaCO_3$. The solution was filtered and evaporated and the residue dried under vacuum. The residue was extracted three times with 10 mL of warm ethyl acetate (EA) and the solution filtered. The solvent was evaporated, leaving a mixture of derivatives 1 and 2 as solids. Derivative 2 was purified using flash chromatography on a 20-mm inner-diameter column packed with 10 cm of silica gel (Matrex, 60 Å, 35−70 μm, Grace Amicon, Beverly, MA) soaked with EA. The sample was applied in a minimum volume of EA−ethanol (9:1, v:v) and eluted with 120 mL of EA−ethanol (19:1; v:v). The chromatography fractions were analyzed by thin-layer chromatography (TLC; silica gel 60 F_{254}; Merck) with EA−ethanol (9:1, v:v) as solvent. Pure derivative 2 was obtained after evaporation of the solvent. Derivative 2 was converted to 3,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose (derivative 3; step D in Figure 1) according to previously published work.[11] Derivative 3 was purified by flash chromatography using silica gel and diethyl ether as solvent. Fractions were checked using TLC (as above, diethyl ether as solvent). Pure fractions were carefully evaporated to avoid any loss of the slightly volatile derivative 3. Dry derivative

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3 (colorless oil) was washed by evaporation with chloroform and weighed. This ensures the complete evaporation of diethyl ether which has overlapping NMR signals.

Yields were monitored for samples from groups I−IV. The yield of the wood or cellulose hydrolysis (step A) was quantified by mixing an aliquot of the reaction mixture with a sodium acetate solution of known concentration (50 mM) and integrating ^{1}H NMR spectra of the mixture. The total glucose content of tree rings, which depends on wood species, was taken from the literature.[23] The yield of derivative 2 (steps B and C) was quantified using the ratio between derivative 2, derivative 1, and glucose after step C. The yield of derivative 3 (step D) was calculated by measuring the amount of nonreacted derivative 2 in an aliquot mixed with a glucose solution of known concentration (50 mM) using ^{1}H NMR.

Deuterium NMR Measurements. Glucose derivative 3 was dissolved in a solvent mixture containing 83% acetonitrile, 17% C_6F_6, and 0.1% (v:v) C_6D_6. A filter column was prepared using a Pasteur pipet with a cotton plug at the bottom, followed by layers of $NaHCO_3$ (0.5 cm) and dry Na_2SO_4 (3 cm). The sample was dissolved in 200 μL of the solvent mixture and pushed with slight overpressure through the column into a 5-mm NMR tube (with PTFE valve; J. Young Scientific Glassware Ltd., Windsor, U.K.). The column was washed with more solvent until the sample reached a suitable height (26−31 mm) in the NMR tube. The PTFE valves of the NMR tubes prevent solvent evaporation at the measurement temperature of 50 °C. After the sample purity was checked by ^{1}H NMR, deuterium NMR spectra were measured using a DRX600 spectrometer (Bruker, Switzerland) equipped with a 5 mm broadband-observe probe with ^{19}F lock. On a representative sample, longitudinal relaxation times (T_1) of the D signals were measured by the inversion−recovery technique. DIDs were measured using a 90° excitation pulse, and ^{1}H decoupling[24] was applied during the acquisition time of 2.5 s. Typically, five spectra of 4000 scans each were recorded for a 200-mg sample, resulting in a total measurement time of 14 h. The NMR raw data (FIDs) were Fourier-transformed with 0.7 Hz line broadening, phase-corrected using the strong signals of C_6D_6 and acetonitrile, and baseline-corrected. Spectra were then integrated using the Lorentzian line shape fit of the XWINNMR program (Bruker, Switzerland), which yielded integrals for all D isotopomers of derivative 3.

Equations and Calculations. No reference compounds exist for deuterium isotopomer abundances. Therefore, we propose three ways of quantifying D isotopomers, depending on the question to be answered. (1) If changes in D isotopomer abundance ratios are of interest, it is preferable to define the DID as $DID_x = I_x/\overline{I}_x$, where I_x is the integral of the NMR signal of the Dx isotopomer ($x = 1, 2, 3, 4, 5, 6^R$, or 6^S) and $\overline{I}_x$ is the average of all the I_x. (2) If the D isotopomer abundances of two or more samples must be compared, a reference has to be used. The methyl groups of derivative 3, introduced during the derivatization, can be used as internal reference if the samples have been derivatized using the same acetone (as we did for groups II−IV). The relative abundance of each Dx isotopomer (RA_x) of derivative 3 can be expressed as $RA_x = I_x/I_{CH_3}$, where I_{CH_3} is the average integral of both CH_3 groups, scaled to one-third. (3) If the D isotopomer abundances are to be compared with samples measured by IRMS, the abundance of the D isotopomers must be translated to the δD scale, which compares the D/H ratio of a sample to the D/H ratio of the international reference Vienna Standard Mean Ocean Water (VSMOW).[15] The abundance of the Dx isotopomer equals the D abundance (D/(D + H)) at the position x. At natural abundance, D is extremely rare (0.15‰) and consequently the D abundance is almost identical to the D/H ratio.[13] Therefore, the D/H ratio of each of the seven positions in the glucose molecule (R_x) can be calculated from the DID and the D/H ratio of the whole sample (R_s) as $R_x = DID_x R_s$. From that, and standard equations,[13,15] the seven position-specific δD (δD_x) of the glucose molecule can be calculated using the following equation:

$$\delta D_x = \left\{\left[DID_x\left(\frac{\delta D_s}{1000} + 1\right)\right] - 1\right\} \times 1000 \qquad (1)$$

where δD_s is the δD of the glucose part. In the same way, the δD of each of the seven positions in the glucose molecule can be calculated from the RA and the δD of the methyl groups (δD_{CH_3}) as

$$\delta D_x = \left\{\left[RA_x\left(\frac{\delta D_{CH_3}}{1000} + 1\right)\right] - 1\right\} \times 1000 \qquad (2)$$

Control Experiments. Control experiment 1 aimed to detect possible primary kinetic isotope effects caused by C−H bond breakage in each step of the sample preparation, by testing for epimer formation using ^{1}H-NMR.

Control experiment 2 was set up to detect possible hydrogen isotope exchange between glucose derivatives and solvent in acid-catalyzed reactions (steps A−C). A commercial cellulose sample (group I) was converted to derivative 2, once with normal solvents (containing around 0.015% of D) and once with solvents containing 1% of D. Deuterium NMR spectra of both samples were compared to quantify possible hydrogen exchange.

Control experiment 3 tested for all kinds of D isotope effects during sample preparation. The δD values of cellulose samples (group II) should be compared to the δD values of the glucose part of derivative 3 obtained from those samples. However, no method is readily available to measure the δD of derivative 3. To overcome this problem, isotope balance calculations using the δD of the methyl groups were used. To estimate the δD of the methyl groups, commercial cellulose (group I) was nitrated and its δD measured using standard methodology.[25] The same cellulose was converted into derivative 1. The δD of the nonexchangeable H of derivative 1 was measured using a technique based on sample equilibration with water of known δD.[26] From a comparison of both measurements, an estimate of the δD of the methyl groups of derivative 1 was obtained. This estimate was used to recalculate the δD_s of derivative 3 using eq 2.

Statistical Analysis. Statistics were performed using the data analysis tool from Microsoft Excel (Microsoft Corp., USA) and Minitab 14 (Minitab inc., USA). The correlation between IRMS

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Table 1. Average Yields and Reproducibility of Sample Preparation Steps

	step	yield[a] (%)	SE	*n*
A	cellulose hydrolysis	92.0	1.2	13
A	wood hydrolysis	87.0	1.6	8
B, C	formation of derivative 2 via derivative 1	90.7[b]	0.7	17
D	formation of derivative 3	95.6	0.7	12

[a] Yields are calculated as described in the Experimental Section. [b] Steps B and C are carried out as a one-pot reaction; thus, the combined yield is stated. The standard error (SE) is calculated on *n* samples.

and NMR results was assessed using Pearson's Product Moment Correlation Coefficient and least-squares linear regression. When comparing D isotopomer abundances in wood and cellulose (Table S-1 of the Supporting Information), unpaired *t*-tests were used. When testing for DID pattern similarity between two Douglas fir wood samples and a sunflower glucose sample (Figure 3), a general linear model was used with isotopomers and samples as factors. This was followed by a one-way analysis of variance (ANOVA) to test for nonrandomness of the DID within each sample.

RESULTS AND DISCUSSION

Yields. The average yields of the sample preparation steps for groups I−IV along with the standard errors are shown in Table 1. Yields are high and well-reproducible, which minimizes the possible influence of isotope effects on isotopomer distributions. The high reproducibility of each reaction step (Table 1) shows that the sample preparation was not influenced by the four different origins of the starting material (groups I−IV). In spite of the high yields for each step of the sample preparation, the optimal sample size is still 0.5 g of dry wood or 0.3 g of α-cellulose, which will yield around 200 mg of pure derivative 3. At least 50 mg is currently required for deuterium NMR analysis. The yield of wood hydrolysis is slightly lower than the yield of cellulose hydrolysis (step A), which is in agreement with the literature.[27−29] Indeed, wood is harder to hydrolyze than extracted cellulose, due to the presence of other components such as lignin, resins, and hemicellulose, which hinder the access of the acid to the cellulose fibers. Formation of derivative 2 is the result of the addition of two isopropylidene groups (step B) followed by the selective cleavage of the 5,6-*O*-isopropylidene group (step C). Therefore, step C was optimized to minimize re-formation of glucose (1.3 ± 0.2% re-formation) by cleavage of the 1,2-*O*-isopropylidene group.

Derivative Formation for Deuterium NMR. Sugars (and many other compounds) must be converted into derivatives for DID measurements by NMR. Here, we define general requirements for such derivatives and for quantitative deuterium NMR measurements. First, the derivative should not be composed of a mixture of isomers (such as anomers), as this would reduce resolution and sensitivity. Second, any protecting groups added during derivatization should increase molecular weight as little as possible and should improve solubility in low-viscosity solvents such as acetonitrile. Minimal molecular weight of the derivative and low viscosity of the sample greatly contribute to obtain narrow, well-resolved signals. Third, protecting groups with simple structures and few, but intense, NMR signals are preferable, because their signals can serve as an intramolecular reference for D isotopomer abundances, allowing comparison of D isotopomer abundances of several samples. For quantification of D abundance, an intramolecular reference is preferable to an added reference compound because a reference compound must be added in an extremely well-defined molar ratio. Finally, the signals of the derivative should have good dispersion and similar NMR relaxation times, essential to ensure that the signals can be integrated and compared. Derivative 3 fulfills the criteria, because (1) it does not have anomers, (2) isopropylidene formation and ether formation increase molecular weight only slightly and derivative 3 is highly soluble in acetonitrile, (3) the isopropylidene methyl groups are a suitable intramolecular reference, and (4) all signals of derivative 3 are well-separated and have similar longitudinal relaxation times (180−330 ms). Note that the formation of derivative 3 occurs with inversion at carbon 6, implying that the $D6^R$ and $D6^S$ isotopomer abundances are interchanged in derivative 3 compared to glucose.

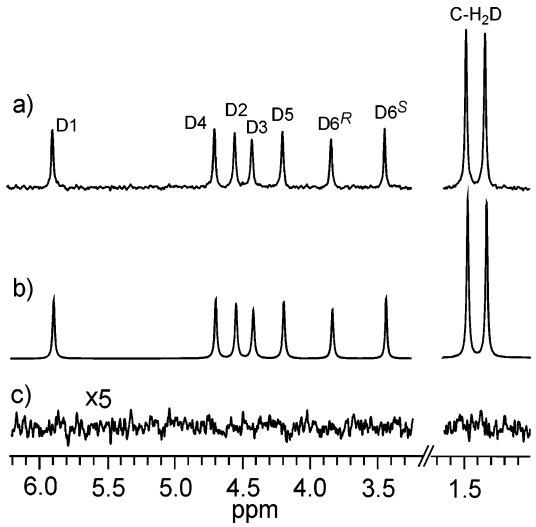

Figure 2. Line shape fit of a deuterium NMR spectrum: (a) deuterium NMR spectrum of derivative 3 showing one signal for each D isotopomer, (b) line shape fit calculated by deconvolution, and (c) difference between the spectrum and the fit, magnified five times.

NMR parameters were chosen to guarantee quantitative measurements. Because the longest relaxation time was 330 ms, the acquisition time of 2.5 s assured that all signals relaxed completely between pulses, so that the NMR spectra could be quantified. A deuterium NMR spectrum of derivative 3 is shown in Figure 2a. All signals of derivative 3 are about 1 Hz wide, while the signal of C_6D_6 (not shown) is sharper (0.6 Hz) and was used to optimize line shapes before recording spectra to be integrated. Together with a line broadening of 0.7 Hz applied during processing, this ensures that the signals can be fitted as Lorentzian line shapes (Figure 2b). As shown in Figure 2c, the difference between fitted and experimental spectra was within the noise level, indicating that the Lorentzian fit yielded reliable integrals. The precision of the NMR measurements (for 200 mg of derivative 3 and 14-h measurement time) was around 2% and was limited by the signal-to-noise ratio.

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Theoretical Considerations and Control Experiments for Isotope Effects. No reference compounds with defined DID exist. During the sample preparation, the DID could be influenced by isotope effects and hydrogen exchange with the solvent. A straightforward way to test for such undesired effects would be to follow the δD of a glucose sample through the whole protocol. However, no established protocols exist for δD measurements of the glucose derivatives, and the variable number of hydroxyl and methyl groups is difficult to account for. Therefore, three control experiments were performed to rule out isotope effects and isotope exchange.

Primary kinetic isotope effects (KIE) occur when an atom is directly involved in a chemical reaction and can strongly influence isotopomer abundances. In the sample preparation, primary KIEs could occur if C−H bonds were broken, which would be detectable as hydrogen exchange with the solvent and epimer formation. In control experiment 1, the reaction mixtures were analyzed after each step by ^{1}H NMR for epimer formation. Epimers were only found after wood hydrolysis (sample groups III and IV), but these are naturally present in wood (mannose, arabinose, galactose, and xylose). This agrees with previous results that acid hydrolysis of cellulose occurs without epimer formation.[29] Breakage of C−H bonds could occur in acid-catalyzed reactions (steps A−C) and would give rise to hydrogen exchange with the solvent. In control experiment 2, steps A−C were carried out with D-enriched solvent, to test for hydrogen exchange. Hydrogen exchange was below the 0.2% detection limit (data not shown). From the absence of epimers and of hydrogen exchange, it follows that C−H bonds are not broken during the sample preparation. Therefore, D isotopomer abundances cannot be affected by primary KIEs.

Secondary kinetic isotope effects occur when a hydrogen atom is in close proximity to an atom implied in a chemical reaction. They are generally small (<1.1) but could affect any reaction step. For cellulose hydrolysis, the greatest expected secondary KIE is of α type (during C1−O bond breakage) and should be 1.09 at the C1−H position.[30] For 92% yield (Table 1), this KIE could deplete the product by 2% in the D1 isotopomer.[31] However, we did not observe any byproducts (such as oligomers of glucose or its decomposition product, hydroxymethyl furfural)[32] in the reaction. This indicates that the hydrolysis of dissolved cellulose was complete and that the 92% yield simply reflected incomplete physical dissolution of cellulose, which is not subject to isotope effects. This agrees with the lower yield for wood hydrolysis as compared to cellulose, because other polymers present in wood hinder the access of acid to cellulose fibers. Thus, no significant secondary KIE during the hydrolysis of cellulose is expected. During the formation of derivative 2, the isopropylidene groups are formed and hydrolyzed via protonation of the carbonyl oxygen atom of acetone and attack on OH groups of glucose. Secondary KIEs on the C−H groups of the glucose would therefore be of β type (concerning an H bound to the carbon next to the reacting OH). However, β secondary KIEs range only up to 1.04 and have only been observed in reactions involving carbonium ions.[33] Secondary KIEs on the C−H groups of glucose should therefore be smaller than 1.04, which—for 90% overall yield of steps B and C—leads to a discrimination of less than 1%, which is below the precision of DID measurements by NMR. The mechanism of formation of derivative 3 is a series of trans-esterifications, initiated by deprotonation of glucose hydroxyl groups. Secondary KIEs have not been described for such reactions and are most likely small. The very high yield of ether formation (>95%) further minimizes any D discrimination. In summary, D discrimination by isotope effects is negligible during sample preparation. Note that for reproducible yields (Table 1), even a substantial secondary KIE cannot reduce the *precision* of DID measurements, because the induced D discrimination would be constant. However, the constant D discrimination would degrade the *accuracy* of the measurements.

In control experiment 3, independent δD measurements on cellulose samples were compared with NMR measurements on derivative 3. From the δD values of cellulose (−52‰ vs VSMOW) and derivative 1 (−87.7‰), δD of the methyl groups of derivative 1 was calculated via isotope mass balance to be −108‰ vs VSMOW. Because the methyl groups of derivative 3 originate from derivative 1, this value was used as the δD value of the methyl groups of derivative 3. The cellulose samples of group II (with published δDs which varied by 45‰)[19] were converted into derivative 3 and measured by deuterium NMR. From the NMR measurements and the δD value of the methyl groups, δD of the glucose moiety of derivative 3 for each sample was calculated using eq 2 (see Experimental Section). The δD values calculated in this way correlated linearly with the published δD values measured by IRMS ($R = 0.95$, $P < 0.0001$, $n = 9$). The slope and intercept of this relationship were not significantly different from 1 and 0 (values within 90% confidence interval), respectively, indicating that the δD values calculated from NMR measurements and measured by IRMS agreed within the measurement accuracies.

Influence of Purifying α-Cellulose on the Measured DID. The standard method for δD measurements of tree rings requires purification of α-cellulose for nitration. Because our protocol yields a pure glucose derivative, prior purification of cellulose is not necessary. This assumes that the glucose units incorporated into cellulose and other wood polymers come from a common glucose pool with a common DID. To verify this, we compared the DID of derivative 3 obtained from whole wood (sample group III) and from α-cellulose extracted from the same wood. There was no significant difference in the relative abundance of each isotopomer (RA_x), expressed relative to the methyl groups, between the two samples (unpaired *t*-test, $n = 4$ spectra, $P > 0.1$; Table S-1 of the Supporting Information). The minimum difference between whole wood and cellulose that was detectable was around 0.06, based on an average standard error of 0.02. Thus, the tedious purification of α-cellulose can be omitted in analyses of DIDs of tree rings, especially if samples to be compared are processed identically. Starting the sample preparation from whole wood results in increased glucose recovery as it includes all glucose units present in wood and avoids loss of material during the purification of α-cellulose.

Variation in Tree-Ring DID and Interpretation. DIDs of all compounds analyzed to date were nonrandom; therefore, it is likely that the difficulties in interpreting tree-ring δD arise from nonrandom DIDs. Measurements of DIDs of tree-ring cellulose are presented in Figure 3, which compares DIDs of early and late wood of the same tree ring of a Douglas fir (group III sample) as

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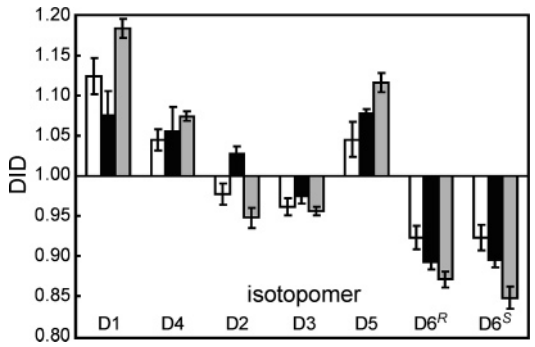

Figure 3. DIDs of early wood (white bars) and late wood (black bars) of Douglas fir tree-ring cellulose and of sunflower leaf glucose (gray bars). Error bars correspond to the SE of the NMR measurements ($n = 4-8$ spectra).

well as the DID of glucose from sunflower leaf sucrose. Analysis of the DIDs using a general linear model revealed that there was significant variation in the D isotopomer abundances of the three samples. This variation was mostly explained (>90%) by abundance differences between isotopomers ($P < 0.0001$), while variation between samples was not significant ($P = 1$). Therefore, we can conclude that there is a common pattern in the DID between samples. Using a one-way ANOVA, we found that the DID of each sample was highly nonrandom ($n = 4-8$ spectra; $P < 0.0001$), in agreement with results of previous studies.[11,13] The D isotopomer abundances of both wood samples vary by over 10% around the average D abundance within each sample. In comparison, variation in δD encountered in tree-ring cellulose studies is typically $\pm$5% between samples. Thus, it is likely that information that is present in the DID is lost in δD measurements. The common DID pattern of the samples shows highest abundance for the D1 isotopomer; the D4 and D5 isotopomers had similar above-average abundances; the abundances of the D2 and D3 isotopomers were similar and in the region of the average; the abundances of the $D6^R$ and $D6^S$ isotopomers were similar and lower than the average. Because the Douglas fir and sunflower have the same photosynthetic pathways, the common pattern indicates that the DID of tree-ring cellulose reflects isotope discriminations during photosynthesis, which depend on regulation of enzyme activity.[11] Thus, the DID of tree-ring glucose retains information about the regulation of photosynthesis. However, during cellulose synthesis, the DID is partly modified by hydrogen exchange with source water. In a previous report, we identified C−H groups in cellulose that exchange largely or little with the hydrogen from source water.[14] We proposed that the D2 isotopomer should carry source water signals (especially temperature), while other isotopomers should carry physiological signals from leaf-level processes. As an example, we have observed in annual plants that the abundance ratio $D6^R/D6^S$ responds to changing atmospheric CO_2 levels.[34] Thus, physiological responses to changing CO_2 levels may be detected from DIDs. In the present work, we cannot test directly if the D2 isotopomer carries a source water signal, because we do not have samples for which the source water δD is known. However, the samples of group II encompass early and late wood cellulose of different δD. Assuming that variation in source water δD is the origin of the δD variation between the samples, this variation should be about 2-fold more pronounced for D2, because C2−H exchanges about twice as strongly during cellulose synthesis than the whole glucose moiety.[14] In agreement with this hypothesis, we find that the abundance of the D2 isotopomer correlates with δD with a slope of nearly two (slope $= 1.8$, $R^2 = 0.789$, $P = 0.0014$, $n = 9$). This supports our inference that the abundance of the D2 isotopomer reflects the D/H ratio of source water, which contains a climate signal. Interestingly, other isotopomer abundances do not correlate with δD, which can explain why δD measurements of tree-ring cellulose have led to ambiguous results in the past.[7,8] In contrast, DID measurements open a new way of accessing both physiological and climate data from tree-ring cellulose.

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CONCLUSION

In this paper, a method to measure DIDs of glucose moieties of tree-ring cellulose is described. Moreover, we present general experimental guidelines for DID measurements. This method, combined with recent improvements in the sensitivity of NMR equipment (in particular cryogenic probes), greatly increases the applicability of DID measurements in environmental sciences and other research areas.

For the first time, DIDs of tree-ring cellulose are shown to be nonrandom and to retain a pattern originating from isotope effects during photosynthesis. The variation in tree-ring DIDs suggests that the abundance of each D isotopomer of tree-ring cellulose may represent a separate signal. DID measurements on tree-ring series may therefore allow parallel reconstruction of several signals, related to climate and tree physiology. That way, information about high-frequency climate variation in the past could be retrieved. Moreover, these parallel signals may be a unique tool to unravel long-term responses of trees to environmental changes, information which is crucial to forecast the role of vegetation as a sink for CO_2 in the coming century.

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SUPPORTING INFORMATION AVAILABLE

Statistical comparison of isotopomer abundances, comparing whole-wood hydrolysis and cellulose purification. This material is available free of charge via the Internet at http://pubs.acs.org.

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