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NMR of All-Carbon-13 Sugars: An Application in Development of an Analytical Method for a Novel Natural Sugar, 1,5-Anhydrofructose

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Of the all-carbon-13 compounds, glucose is one of the most easily accessible, and therefore we applied ^{13}C-NMR technique to the metabolic study of glucose-related compounds, 1,5-anhydro-D-glucitol and 1,5-anhydro-D-fructose (AF). Applying an INADEQUATE method to the substitutes of these novel sugars fully labeled with carbon-13, we could trace out the entire carbon skeleton with high sensitivity and confirm the chemical structures of these sugars. The method also provided a much easier way to optimize the enzymatic oxidation for AF preparation: we selectively and continuously monitored the quantities, as well as their structures in aqueous solution, of the substrate and products in a noninvasive manner. Similarly relying upon information from the ^{13}C-NMR, we developed a valuable derivatization method of AF for its GC-MS application, which was so sensitive that we were able to demonstrate the natural occurrence of AF in rat liver.

Key words: 1,5-anhydro-D-glucitol, 1,5-anhydro-D-fructose, ^{13}C-NMR, GC-MS, polyol.

^{13}C-NMR technique has been widely used to study natural products, relying mostly on the natural abundance of carbon-13 in them (*ca.* 1%), or at most to study molecules partially substituted with carbon-13. We can, however, obtain added structural information with a dramatically increased sensitivity by application of the method to all-carbon-13 substitutes: we can trace out all the chains of carbon atoms in compounds in the 2D-INADEQUATE mode of measurement (*1, 2*). The present report deals with the effective use of this NMR technique in development of analytical methods for glucose-related sugars.

The cyclic polyol 1,5-anhydro-D-glucitol (AG) is known to be present in wide variety of animals and plants (*3*). We have demonstrated a fungal oxidation of AG to 1,5-anhydrofructose (AF) (*4*). Several reports have described fungal and algal production of AF from 1,4-glucans through lyase reactions (*5, 6*). These reports prompt the assumption of a metabolic connection between AG and AF also in mammalian cells, though no report has yet described AF in the animal kingdom. Evaluation of this assumption has been our persistent concern. Our preliminary studies indicated that AF may be present in minute amounts in animal tissues and that the development of a highly sensitive method for AF determination was needed for the study of AF metabolism in animal cells. Several microbial enzymes which oxidize glucose to glucosone can also oxidize AG to AF, but we have developed no satisfactory chromatographic method for AF isolation from the reaction mixture. The difficulty of obtaining authentic AF was overcome in this research by making use of [*U*-^{13}C]AG as the substrate in enzymatic preparation of AF. The reaction was optimized for AF production and, at the same time, its chemical structure in water was concretely determined by selectively monitoring the ^{13}C-NMRs of the all-carbon-13 substrate and products in the reaction mixture. Also, by selectively tracing chemical changes of the compounds maximally enriched with carbon-13, we have developed a convenient method for AF derivatization which allows application of GC-MS, one of the most sensitive methods, to AF determination in animal samples.

MATERIALS AND METHODS

Materials—[*U*-^{13}C]Glucose was a product of Isotec (Miamisburg, OH, USA). AG was given by Nippon Kayaku (Tokyo). [*U*-^{13}C]AG was prepared from [*U*-^{13}C]glucose in our laboratory according to an established method (*7*). Crude pyranose oxidase extracted from *Polyporus obtusus* (3.88 U/mg) and catalase of calf liver origin (1,920 U/mg) were purchased from Takara Shuzo (Kyoto) and Seikagaku Kogyo (Tokyo), respectively. *O*-Ethylhydroxylamine hydrochloride was obtained from Wako Pure Chemical Industries (Osaka) and purified with a Sep-pak C18 cartridge (Waters, Milford, MA, USA) before use. All other chemicals were from Wako Pure Chemical Industries and used without further purification.

NMR Spectroscopy—The 125 MHz ^{13}C-NMR spectra were recorded on a JNM-A500 spectrometer (JEOL, Tokyo), operated in Fourier transform mode with the probe

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Abbreviations: AF, 1,5-anhydro-D-fructose; AG, 1,5-anhydro-D-glucitol; DAF, dehydrated 1,5-anhydrofructose or 2*S*-(hydroxymethyl)-oxane-4,5-dione; EAF, 2-ethoxyimino derivative of 1,5-anhydrofructose or 3*S*,4*R*-dihydroxy-5-ethoxyimino-2*R*-(hydroxymethyl)-oxane; EDAF, 2,3-diethoxyimino derivative of dehydrated 1,5-anhydrofructose or 4,5-di(ethoxyimino)-2*S*-(hydroxymethyl)oxane; TMS-EAF, tri-(trimethylsilyl)-EAF; TMS-EDAF, trimethylsilyl-EDAF.

at room temperature, employing either a broad band decoupling method or an off-resonance decoupling method, both with 32,768 data points. Chemical shifts given in these cases were relative to [$^2H_6$]dimethylsulfoxide (δ: 39.7). For confirmation of assignments of ^{13}C-^{13}C couplings, ^{13}C-NMR was also measured in a 2D-INADEQUATE mode with 256×1,024 data points. In the case of ^{13}C-NMR monitoring of the enzymatic reactions described below, the chemical shifts were determined using methanol as the external standard.

Enzymatic AG Oxidation in NMR Tubes—Tubes contained the indicated amounts of [*U*-^{13}C]AG (6–8 mg) and pyranose oxidase (0.1 or 5 mg), and 0.1 mg of catalase dissolved in the mixture of 0.1 ml of deuterium oxide and 0.6 ml of 50 mM phosphate-Na^+ buffer (pH 5.9). These reaction mixtures were incubated at 37°C and the ^{13}C-NMR signals were recorded by the broad band decoupling method described above at the time points of 0, 3, and 6 h after the preparation of the mixture. The relative amounts of AG, AF, and dehydrated AF (DAF) were determined from the signal areas for respective C6 carbons.[2]

Derivatization of AF and Other Carbonyl Compounds with O-Ethylhydroxylamine—Portions of 10 μl of the reaction mixture were removed at the indicated incubation times and added to 200 μl of ethylhydroxylamine solution (200 mM *O*-ethylhydroxylamine hydrochloride in water). The mixtures were left to stand at room temperature in the dark for 12 h, then a 20-μl portion was directly applied onto a reverse-phase column (TSK gel ODS-120T, 4.6 mm I.D.φ×20 cm; Tosoh, Tokyo) mounted on a computer-aided LC-10A system (Shimadzu, Kyoto). The column was developed with a linear gradient from 100% water to acetonitrile/water (1:1 by volume) in 30 min at a flow rate of 1.0 ml/min. The elution was monitored by UV absorbance at 207 nm and the major peaks assigned to the reaction products were collected. The recovered fractions were then evaporated using a centrifugal evaporator (CC-100, Tomy, Tokyo) to which vacuum was applied with an oil-less scroll vacuum pump (Iwata Koki, Tokyo). Each residue thus obtained was dissolved in 20 μl of *N,O*-bis(trimethylsilyl)trifluoroacetoamide, a 1-μl portion was injected onto a capillary column (HiCap CBP1-M25; Shimadzu) in a GC-MS (QP-2000; Shimadzu) at 130°C, and the final separation was carried out at 230°C. The signal was recorded in a mass chromatography mode by scanning the mass number range from 100 to 600 every 3 s.

AF Identification in Rat Liver—A piece of rat liver (12 g) was homogenized in 20 ml of 150 mM NaCl with a Polytron homogenizer (Kinematica, Switzerland). The homogenate was mixed with 50 ml of ethanol and centrifuged at 5,000×*g* for 10 min. The resulting supernatant was then dried using the centrifugal evaporator. The residue thus obtained was dissolved in 30 ml of 10% *O*-ethylhydroxylamine hydrochloride and left to stand at room temperature in the dark for 12 h. The resulting solution was subjected to reverse-phase HPLC in essentially the same manner as was applied to the enzymatic oxidation mixture, and the chromatographic fractions which corresponded to the products of the enzymatic reaction were subjected to the same GC-MS analysis as that employed for the identification of the enzymatic oxidation products. For quantitative measurement, the liver homogenate was made in the presence of a known amount (0.2 μg/g wet tissue) of [*U*-^{13}C]AF, which was to serve as the internal standard in mass fragmentometry.

RESULTS

NMR Spectra of [*U*-^{13}C]*AG*—Table I shows the chemical shifts and signal multiplicities of [*U*-^{13}C]AG, as well as the coupling constant for each pair of adjoining carbon 13 s ($^1J_{C\text{-}C}$) in the molecule, which were obtained in [$^2H_6$]-dimethylsulfoxide by either the off-resonance decoupling method or the broad band decoupling method. The latter

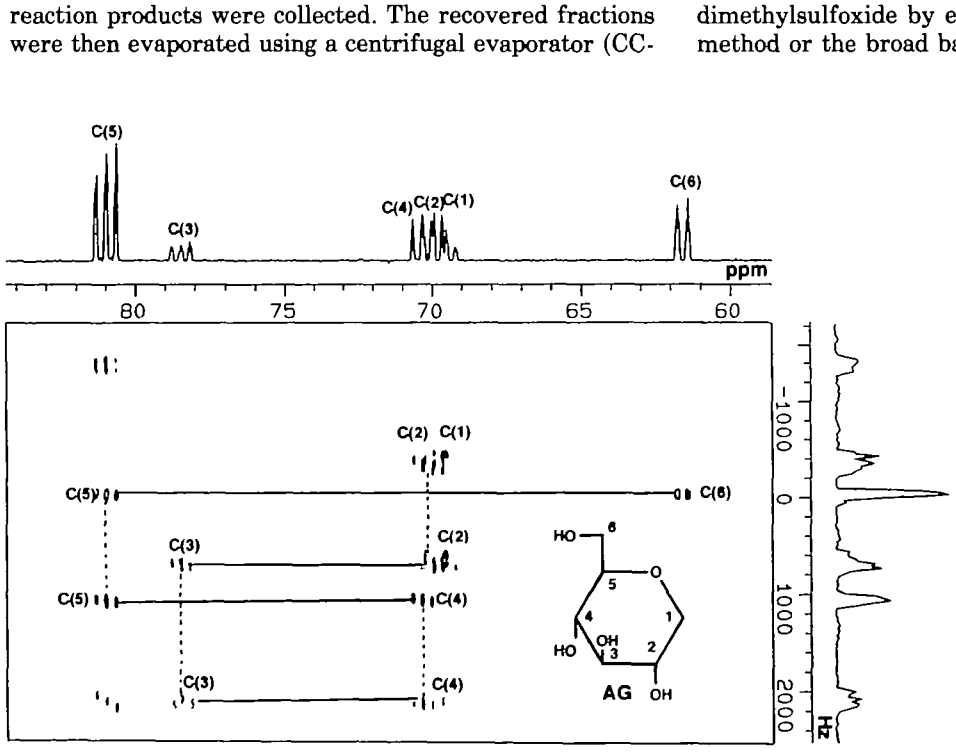

Fig. 1. 125 MHz 2D-^{13}C-INADEQUATE spectrum of authentic [*U*-^{13}C]AG in [$^2H_6$]dimethylsulfoxide.

[2] We use the positional numbering of carbon atoms based on the hexose backbones except when the systematic naming is given.

method revealed the signal splitting due to the direct coupling between adjoining carbon 13 s, while the former revealed that due to the coupling between directly bonded ^{13}C–H pairs. The coupling constants for any adjoining ^{13}C–^{13}C pair were more than 30 Hz in AG, while those for the long-range interaction were expected to be less than 4 Hz (*8*); and actually no such interaction was explicitly manifested in the ^{13}C-NMR spectra at the present spectral resolution. In the double quantum coherence NMR measured in 2D-INADEQUATE mode (Fig. 1), the AG's carbon skeleton was precisely traced out through the coupling of adjoining ^{13}C–^{13}C pairs, which facilitated the assignment of each resonance to the carbon atom at a specific position. The chemical shifts and ^{13}C–^{13}C adjoining coupling constants were almost the same when measured in either [$^{2}H_6$]-dimethylsulfoxide (this figure); 50 mM phosphate buffer, pH 5.9; 50 mM pyruvate buffer, pH 5.9; or [$^{2}H_4$]methanol.

Products in Enzymatic AG Oxidation—Figure 2 shows 2D-double quantum coherence NMR, accompanied by the broad band decoupling ^{13}C-spectra on the top, observed at 6 h after the preparation of the reaction mixture containing 5 mg of [*U*-^{13}C]AG and 0.1 mg each of pyranose oxidase and catalase. The signals assigned to C1 and C3 through C6 in AG shifted only marginally (within several ppm) in the 6 h of reaction but that assigned to C2 almost disappeared and a new doublet signal emerged at 93.14 ppm, which was due

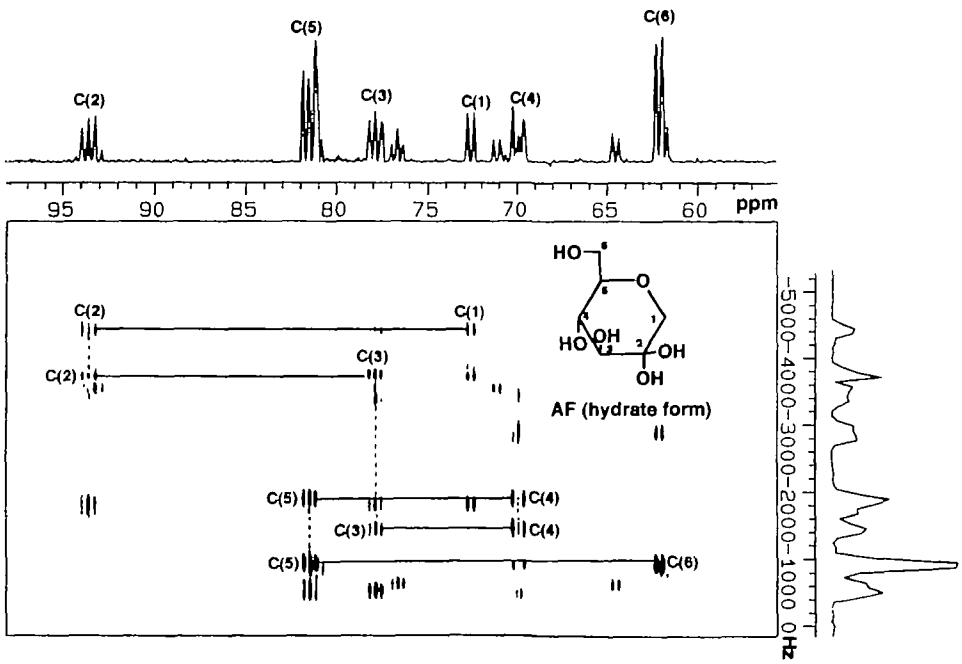

Fig. 2. **125 MHz 2D-^{13}C-INADEQUATE spectrum of enzymatic reaction solution containing 6 mg of [*U*-^{13}C]AG and 0.1 mg each of pyranose oxidase and catalase in 0.1 ml of deuterium oxide and 0.6 ml of 50 mM phosphate buffer (pH 5.9).** The chemical shifts were determined using an external standard of 1% (v/v) methanol in the same buffer (δ 49.9).

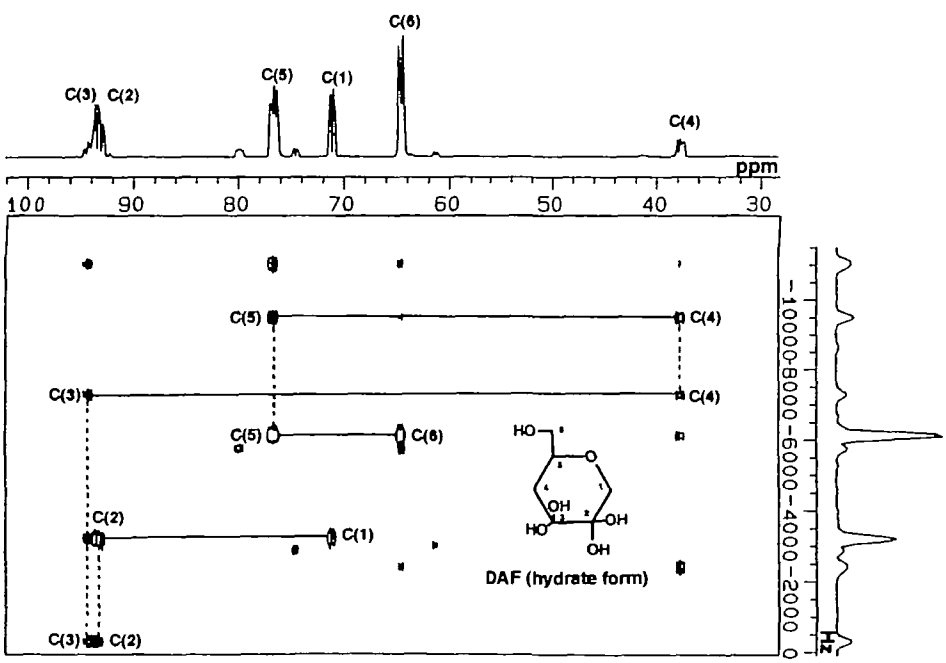

Fig. 3. **125 MHz 2D-^{13}C-INADEQUATE spectrum of enzymatic reaction solution containing 8 mg of [*U*-^{13}C]AG and 5.0 and 0.1 mg of pyranose oxidase and catalase in 0.1 ml of deuterium oxide and 0.6 ml of 0.150 mM phosphate buffer (pH 5.9).** *Chemical shifts* were determined in the same manner as in Fig. 2.

to a hydrated carbonyl carbon. The 2D-double quantum coherence NMR confirms the assignment of the major signals to their respective positions and the new *gem*-diol ^{13}C-signal to the C2 position. All these major signals also showed reasonable multiplicities in the broad band decoupling spectrum, which are summarized in Table I together with the respective coupling constants: the doublet signals were assigned to the two terminals, C1 and C6, and the triplets or double-doublets to C2 through C5. The multiplicities of the major signals in the off-resonance decoupling spectrum shown in Table I also indicated the number of protons attached to each carbon atom at the respective position: zero for C2, one for C3 through C5, and two for C1 and C6. Accordingly, the major product of the enzymatic oxidation of AG in 6 h was indicated to be hydrated AF or 3*S*,4*S*,5,5-tetrahydroxy-2*R*-hydroxymethyloxane. Figure 2 also shows a set of minor signals which were intensified in the reaction catalyzed by pyranose oxidase at a higher concentration. In the reaction shown in Fig. 3, a fivefold higher oxidase concentration was employed and the signals assigned to AG almost disappeared, while the signal set for DAF appeared. Namely, the chemical shifts of major signals were again assigned to the six carbons based on their signal multiplicities in the broad band decoupling spectrum (Fig. 3 and Table I) and on the 2D-double quantum coherence NMR (Fig. 3). The chemical shift for C3 shifted to 93.82 ppm, showing it to have been converted to a *gem*-diol bearing carbon; and that for C4 to 37.60 ppm, which has been shown to represent a methylene carbon (Table I). Other major signals were assigned likewise to specific chemical groups at their respective positions. These assignments showed definitely that the major product in the reaction mixture with the oxidase at the higher concentration was the dihydrated form (2*S*-hydroxymethyl-4,4,5,5-tetrahydroxyoxane) of DAF. This compound can arise from AF through dehydration, by removing the proton at C3 and the hydroxyl group at C4, which is followed by tautomeric isomerization from enol to keto form and then by hydration of the resulting two carbonyl groups.

Characterization of Enzymatic Reaction—The ^{13}C-NMR signals for the methylenic carbons at the C6 positions of AG, AF, and DAF (61.63, 62.03, and 64.65 ppm, respectively) were well separated from each other and from the signals for other positions: they formed isolated doublet signals in the broad band decoupling spectra for the mixtures of these glucose derivatives. Accordingly, the relative concentrations of these compounds may be most accurately determined from the signal areas for C6s of the respective compounds. The temporal concentration changes of AG, AF, and DAF determined from C6 signals are presented in Table II. These results suggest that the productions of AF and DAF are sequential: AG was converted first to AF and then to DAF.

The deduced structures of these products and their temporal changes in concentration indicated that the pyranose oxidase preparation also contained a hexose dehydratase, which was confirmed by the chromatographic separation of the oxidase and dehydratase activities on Superose 6 HR10/30 columns (Pharmacia, Uppsala, Sweden) eluted with 150 mM NaCl, 50 mM phosphate buffer, pH 7.0 (data not shown). However, the separated enzymes were both unstable and we have not yet developed a way to stabilize them. Since the dehydratase activity was selectively inhibited with a metallic ion chelator, EDTA, AF was predominantly produced in the presence of the chelator. We also observed another doublet signal in the hydroxymethyl group field and assumed it also represented one of the products of the enzymatic reaction. The amount of this unknown compound was also estimated from the peak area and listed in Table II.

Ethyloxyimino Derivatives of AF and DAF—The products in the enzymatic reaction were then made hydrophobic by indirectly alkylating them through alkoxyimino formation, which made reverse phase HPLC applicable to the target compounds. Optimization of the derivatization and identification of the products were again carried out by applying ^{13}C-NMR technique: the products of the enzymatic oxidation of the universally ^{13}C-labeled compound were analyzed by ^{13}C-NMR (data not shown), which indicated that either AF or DAF produced a single product. The two ethoxyimino derivatives (EAF and EDAF) were fully purified on a reverse phase column in a single applica-

TABLE I. **^{13}C-chemical shifts and other NMR properties of [*U*-^{13}C]AG, [*U*-^{13}C]AF, and [*U*-^{13}C]DAF.**

	Broad band decoupling		Off-resonance multiplicity (CH coupling)
	Assignment chemical shift (multiplicity) (ppm)	$^1J_{C-C}$ (Hz)	
[*U*-^{13}C]AG			
C1	69.62(d)		t (CH_2)
C2	70.06(dd)	$^1J_{C1\text{-}C2}=43$	d (CH)
C3	78.63(dd)	$^1J_{C2\text{-}C3}=37$	d (CH)
C4	70.46(dd)	$^1J_{C3\text{-}C4}=40$	d (CH)
C5	81.76(dd)	$^1J_{C4\text{-}C5}=41$	d (CH)
C6	61.63(d)	$^1J_{C5\text{-}C6}=40$	t (CH_2)
[*U*-^{13}C]AF			
C1	71.03(d)		t (CH_2)
C2	93.14(dd)	$^1J_{C1\text{-}C2}=46$	s (C)
C3	77.79(dd)	$^1J_{C2\text{-}C3}=46$	d (CH)
C4	69.86(dd)	$^1J_{C3\text{-}C4}=43$	d (CH)
C5	81.41(dd)	$^1J_{C4\text{-}C5}=40$	d (CH)
C6	62.03(d)	$^1J_{C5\text{-}C6}=42$	t (CH_2)
[*U*-^{13}C]DAF			
C1	72.54(d)		t (CH_2)
C2	93.51(dd)	$^1J_{C1\text{-}C2}=42$	s (C)
C3	93.82(dd)	$^1J_{C2\text{-}C3}=42$	s (C)
C4	37.60(dd)	$^1J_{C3\text{-}C4}=38$	t (CH_2)
C5	76.57(dd)	$^1J_{C4\text{-}C5}=38$	d (CH)
C6	64.45(d)	$^1J_{C5\text{-}C6}=42$	t (CH_2)

d: doublet, dd: double doublets, s: singlet, t: triplet.

TABLE II. **Enzymatic reactions of [*U*-^{13}C]AG with different amounts of crude pyranose oxidase.**

Incubation time	Pyranose oxidase (0.1 mg)			Pyranose oxidase (5 mg)			
	AG (%)	AF (%)	DAF (%)	AG (%)	AF (%)	DAF (%)	Unknown (%)
1 h	–	–		27	20	42	12
3 h	38	57	5	N.D.	N.D.	75	25
6 h	10	75	15	–	–	–	–

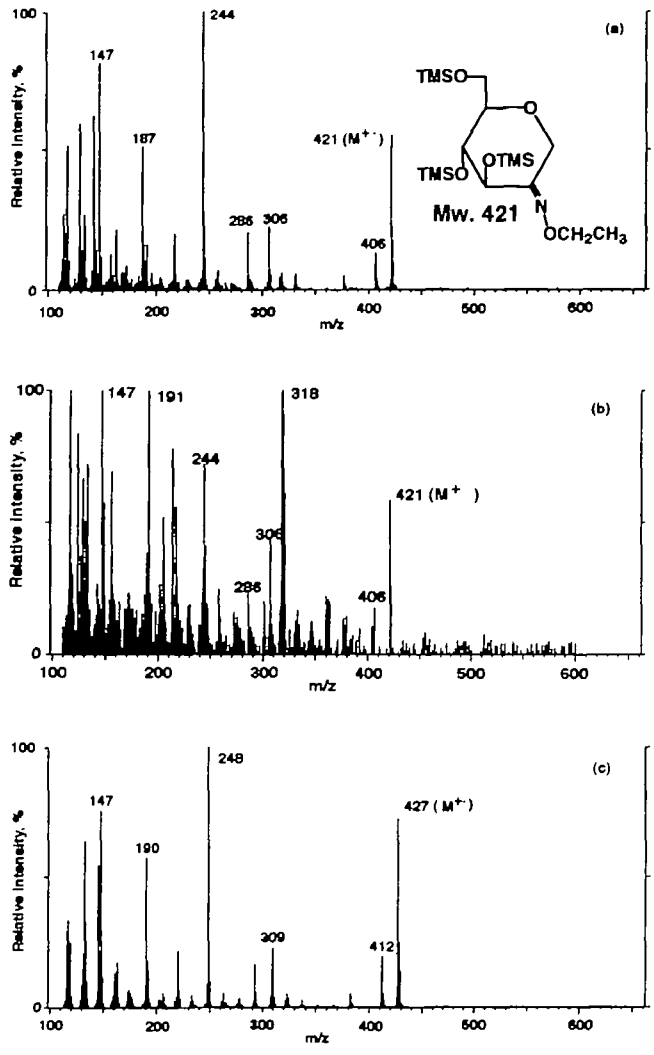

Fig. 4. **GC-MS spectra of pertrimethylsilyl derivatives of the authentic EAF (a), the corresponding material in the rat liver extract (b), and the authentic ethoxyimino derivative of [*U*-^{13}C]-AF (c).** The methods for GC-MS, cleaning-up, and derivatization were as described in the text.

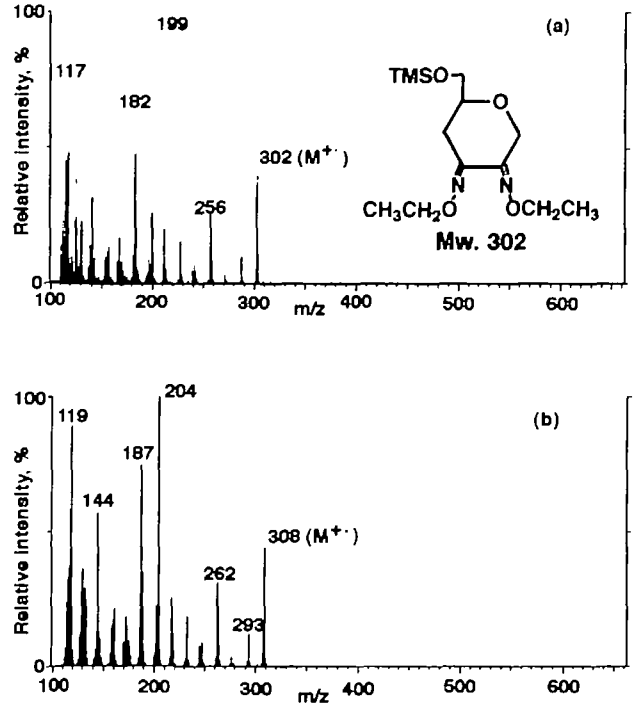

Fig. 5. **GC-MS spectra of permethylsilyl derivatives of authentic EDAF (a) and di(ethoxyimino) derivative of authentic [*U*-^{13}C]DAF (b).** The methods for GC-MS and derivatization were as described in the text.

tion of HPLC and the purified derivatives readily attained constant weight upon keeping *in vacuo*.

The mass spectra obtained by GC-MS of tri-(trimethylsilyl)-EAF (TMS-EAF) and trimethylsilyl-EDAF (TMS-EDAF) are shown in Figs. 4a and 5a. Figures 4c and 5b show the corresponding mass spectra for the corresponding derivatives from all-carbon-13 substitutes of the original sugars, which facilitated the assignment of each fragment because the corresponding fragments in Fig. 4, a and c, and Fig. 5, a and b, showed mass number differences which were equal to the numbers of backbone carbon atoms retained in each of the pair of corresponding fragments. All these figures showed distinct molecular ions whose ion mass coincided with their expected structures. The observation of the molecular ions can be regarded as added support for the structures of EAF and EDAF deduced from the ^{13}C-NMR analysis. These molecular ions seemed highly stable and showed high intensities in the fragmentograms: their intensities were more than 30% of the base ions, thus indicating that the ethoxyimino derivatives of these ketonic sugars may also be highly stable. The production of such a molecular ion of high intensity provides us with a highly selective and sensitive identification method by GC-MS, which was practically applied for detection of AF in the rat liver (Fig. 4b). A chromatographic peak corresponding to the authentic TMS-EAF was also observed in the total ion chromatogram for the rat liver extract, and the mass fragmentogram for the peak also contained all the signals for the molecular ion (m/z=421) and for other major fragments of TMS-EAF. On the other hand, no signal was observed at the elution time corresponding to that for TMS-EDAF. Accordingly, the AF occurrence was clearly demonstrated by this mass-spectroscopic method, while the existence of DAF was not supported in the rat liver. The AF content in the rat liver was roughly estimated to be 0.4 μg/g wet tissue.

DISCUSSION

The present study illustrates an effective use of compounds universally labeled with carbon-13 in the study of chemical reactions by allowing the "real time" recording of information on the structures of the products, as well as on the extent, of an on-going enzymatic reaction. The use of universally labeled reactants in the ^{13}C-NMR application had the following merits. First, the reactant and products are selectively monitored in the reaction mixture. This is because the natural abundance of carbon-13 is about 1%, and accordingly the reactant and products constituted purely of carbon-13 may mainly contribute to the formation of major signals in the ^{13}C-NMR spectra in many cases

and also in the present application. It was possible, therefore, to monitor the enzymatic reaction in the NMR tubes in a noninvasive manner. Second, a specific chemical shift is readily assigned to a specific backbone carbon in a particular product, thus the chemical groups indicated by their respective NMR signals are readily assigned to specific carbon atoms in the molecule and, accordingly, the chemical identities of the products are easily determined. With an all-carbon-13 molecule, a pair of chemical shifts can readily be selected and assigned to a pair of directly bonding carbons through identification of directly coupling signals, which made it possible to trace out the carbon backbone of the molecule. The coupling pairs are most conveniently and precisely identified in a 2D-double-quantum-coherence spectrum (*1, 2*), which is obtained by operating the spectrometer in 2D-INADEQUATE mode supported by the built-in operational program. Third, this ^{13}C-NMR method is considerably sensitive because the target molecules are maximally enriched with carbon-13: a sub-milligram order of quantity may be more than enough for a reliable result. Moreover, the all-carbon-13 compounds have a special application in mass spectroscopy. Comparison of the fragmentograms of mostly-carbon-12 and all-carbon-13 compounds can provide a persuasive basis for assignment of mass fragmentation, because the difference in mass number between the corresponding fragments, which is readily read from the general similarity of the fragmentation patterns, indicates the number of backbone carbons retained in those fragments. The mass spectroscopy is not only extremely sensitive, but such assignments are also helpful in the structural study.

Glucose is one of the most accessible and economical of the all-carbon-13 compounds available from commercial sources. Accordingly, it is quite feasible to apply to ^{13}C-NMR the all-carbon-13 compounds which are structurally related to glucose. Using [*U*-^{13}C]AG as the substrate, the accompanying paper demonstrates that *Escherichia coli* vigorously phosphorylates AG to AG 6-phosphate in the absence of glucose in the medium and releases the phosphate into the medium (*9*). Likewise, a number of such ^{13}C-NMR applications are expected in the field of metabolic study of glucose-related metabolites.

Animal cells can rapidly reduce AF to AG (M. Suzuki *et al.*, manuscript in preparation) and a 1,4-glucan lyase in the rat liver extract gives rise to AF from glycogen and other 1,4-glucans (S. Kametani *et al.*, manuscript in preparation). Thus these metabolic reactions make up an alternative pathway for glycogen degradation. The present study provided these studies with several basic pieces of information which were helpful for the development of qualitative and quantitative methods of AF analyses in the natural samples, as well as for establishment of AF preparation. The present study also demonstrated AF occurrence in the rat liver, which inevitably required an effective isolation method and a sensitive identification method applicable for such low abundance of AF in natural samples. Carbonyl compounds are apt to undergo reversible structural changes such as tautomeric isomerization and hydration. Possibly due to such slow and reversible changes on the columns, all the column separation systems we have developed for AG separation were not applicable for AF isolation. The treatment of the natural samples with *O*-ethyl hydroxylamine efficiently converted AF to its ethoxyimino derivative, which was stable and effectively purified on a reverse phase column to a sufficient extent for the subsequent GC-MS application. Consequently, the AF occurrence in a minute amount was concretely demonstrated in the rat liver.

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