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Note

6-Deoxy-6-fluoro-L-ascorbic acid: crystal structure and oxidative degradation

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Abstract

Ascorbic acid and its oxidation products have been implicated in non-enzymatic modification of proteins in aging and diseases of oxidative stress. We have studied the feasibility of using 6-deoxy-6-fluoroascorbic acid (**6**) for identification of ascorbic acid degradation products by ^{19}F NMR spectroscopy. Crystals of compound **6** from nitromethane belonged to the space group $P2_1$ with $a = 5.547(2)$, $b = 6.769(3)$, $c = 9.302(2)$ Å, $\beta = 91.80(3)°$ and $Z = 2$. Atomic coordinates, bond lengths and angles, hydrogen coordinates, anisotropic and isotropic displacement parameters were similar if not identical with those of native ascorbic acid. Similarly, UV properties and oxidation kinetics by $CuCl_2$ at different pH values were essentially identical with ascorbic acid. Using 750 MHz ^{19}F NMR spectroscopy, five to six new fluorinated products were detected after overnight oxidation of **6** with Cu^{2+}, suggesting that **6** may be a powerful and sensitive tool for assessment of its catabolism in vivo.

Keywords: Ascorbic acid; Glycation; Maillard reaction; Aging; Diabetes

1. Introduction

Ascorbic acid (vitamin C) is one of the major water-soluble antioxidants. Under physiological conditions, most of the intracellular ascorbic acid is in a reduced form and less than 10% is present in form of L-*threo*-hex-2,3-diulosono-1,4-lactone (dehydro-L-ascorbic acid) or its delactonized form, L-*threo*-hex-2,3-diulosonic acid (2,3-diketo-L-gulonic acid). The biology governing the tissue levels of these three compounds is fairly well understood because both L-*threo*-hex-2,3-diulosono-1,4-lactone and L-*threo*-hex-2,3-diulosonic acid are unequivocally related to ascorbic acid and can be assayed relatively easily in biological specimens. However, in selected tissues, it has been hypothesized that fragmentation products of ascorbic acid, L-*threo*-hex-2,3-diulosono-1,4-lactone and L-*threo*-hex-2,3-diulosonic acid may participate in Maillard reaction with proteins and form advanced glycation products, which are in

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part responsible for browning and crosslinking of proteins in aging and in diseases of accelerated aging, such as diabetes and end stage renal disease [2,3].

Extensive studies have been carried out by various groups on the nature of ascorbate degradation products formed in vitro. A dozen or so degradation compounds have been found, which include L-xylose [4], L-*threo*-pentos-2-ulose (L-xylosone) [5], 3-deoxy-L-*glycero*-pentos-2-ulose (3-deoxy-L-xylosone) [6], L-lyxonic and L-xylonic acid [7–9], L-threose [10], L-threonic, glyceric acid and oxalic acid [8,11]. Recently, while this work was in progress, L-erythrulose and not L-threose was found to be the major degradation product of ascorbic acid under non-oxidative conditions [12].

On the other hand, various advanced ascorbylation products have been characterized both in vitro and in vivo and found to accumulate in the aging eye lens, or to be present in plasma proteins. Examples include carboxymethyl-lysine [13], pentosidine [14], oxalic acid monoamide (HO_2CCONH_2), vesperlysine A [15] to name just a few. In spite of these accomplishments little is yet known of the in vivo nature and catabolic pathways of ascorbate degradation products beyond 2,3-diketo-L-gulonic acid because many of the degradation products do not substantially differ from reducing sugars. Similarly, most ascorbylation products found so far in vivo, such as carboxymethyl-lysine and pentosidine are also advanced glycation products, and even the recently described oxalic acid monoamide is not entirely specific for ascorbate, as it can also form from reducing sugars [16,17].

Searching for ways to label ascorbate at a position that would allow detection of most of its degradation products, we hypothesized that ascorbic acid, when labeled with fluorine in position 6, should be suitable for identification of ascorbate catabolites in conjunction with ^{19}F NMR spectroscopy. Here we describe the synthesis for 6-deoxy-6-fluoroascorbic acid (**6**), which was modified from the procedure of Kiss et al. [1], its crystal structure and oxidation kinetics in comparison with native ascorbic acid, and demonstrate the feasibility of detecting several oxidation products with ^{19}F NMR spectroscopy. It should be noted that a synthesis for 6-deoxy-6-^{18}F-ascorbic acid was also proposed, which, however, did not lead to the crystalline product [18].

*Chemical synthesis of 6-deoxy-6-fluoro-*L*-ascorbic acid* (**6**).—The synthetic scheme is outlined in Scheme 1.

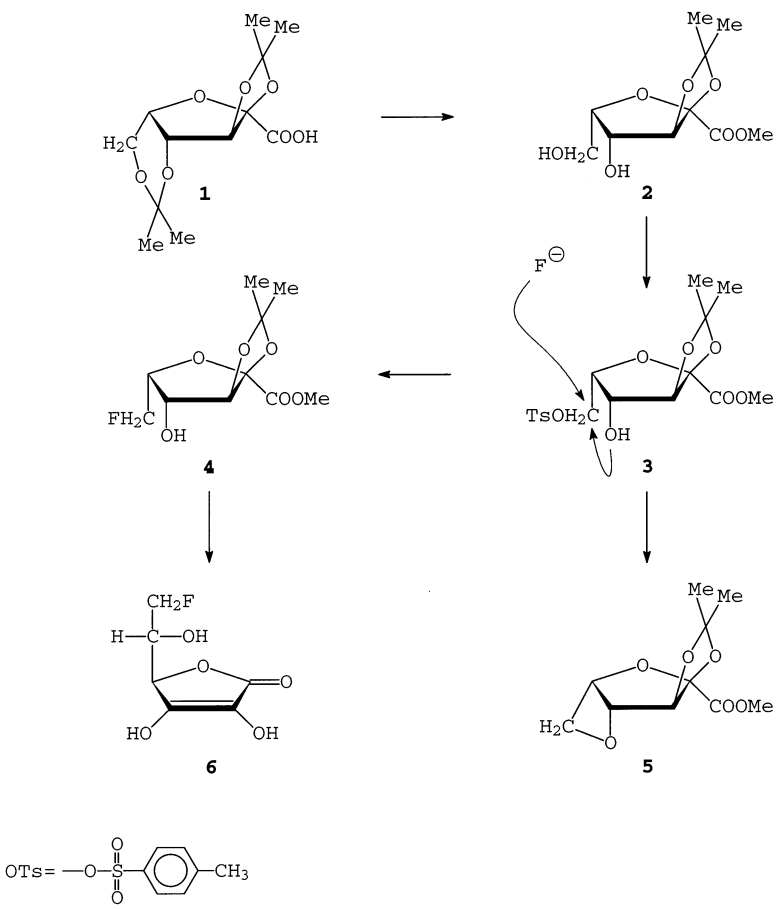
In contrast to the method of Kiss et al., methyl 2,3-*O*-isopropylidene-L-*xylo*-hex-2-ulofuranosonate (**2**) was obtained in a one-pot reaction from commercially available 2,3:4,6-di-*O*-isopropylidene-L-*xylo*-hex-2-ulofuranosonic acid (2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid) (**1**). During heating of the substrate in methanol in the presence of sulfuric acid, the carboxyl group was converted into methyl ester and the 4,6-*O*-isopropylidene protecting group was selectively cleaved. The crude by-product was, without any purification, directly O-tosylated with *p*-toluenesulfonyl chloride in pyridine. Crystals of the mono *O*-tosyl derivative (**3**) were obtained with good yield. The next step, in which the *O*-tosyl group is replaced with a fluoride ion, is the bottleneck of this method. Because the fluoride anion is a weak nucleophile, the replacement was carried out with KF in dry *N*,*N*-dimethylformamide at 150 °C. In order to obtain methyl 6-deoxy-6-fluoro-2,3-*O*-isopropylidene-L-*xylo*-hex-2-ulofuranosonate (**4**) we tested CsF and KF from many sources, but satisfactory results were obtained only with dry 99.99% KF packed under argon in vials (Aldrich). In addition to the fluoro derivative, methyl 4,6-anhydro-2,3-*O*-isopropylidene-L-*xylo*-hex-2-ulofuranosonate (**5**) was formed as the side-product of intramolecular substitution during heating at relatively high temperatures (Scheme 1). Both the weak nucleophilicity of the fluoride anion and the formation of this product caused the yield to drop. In the last step of the synthesis, the 2,3-*O*-isopropylidene group was cleaved and the product underwent isomerization to 6-deoxy-6-fluoro-L-ascorbic acid (**6**). For this step we attempted to use ion-exchange resin (H^+ form) according to Kiss et al., but better results were obtained by refluxing **4** in 40% sulfuric acid aqueous solution, with acetonitrile as a co-solvent (1:1 ratio).

The crystalline product was obtained from nitromethane.

Determination of the X-ray structure of 6-deoxy-6-fluoro-ascorbic acid (**6**).—The crystal and X-ray diffraction analyses data of **6** are shown in Table 1. The molecular structure of 6-deoxy-6-fluoroascorbic acid is shown in Fig. 1. All bond lengths and angles are illustrated in Table 2, which includes ascorbic acid data from Hvoslef [19]. The crystal and molecular structures of **6** are very close to those of ascorbic acid. In particular, the planar structure on the lactone ring and the structure of the enediol group on 6-deoxy-6-fluoro-L-ascorbic acid was almost equal to that of ascorbic acid. This characteristic structure is considered most important in the reducing activity of ascorbic acid, thus **6** is also expected to have almost the same reducing activity. Although the planar structure in the lactone ring and the enediol group of both structures are similar, the replacement of the OH group in the side chain of ascorbic acid by a fluorine atom in the 6-deoxy position might introduce some slight difference in both chemical characteristics and biological functions. This assumption needs to be confirmed.

It should be noted that Kiss et al. determined the crystal structure of 6-deoxy-6-chloroascorbic acid. Their *R* value was only 0.187 and could not be improved through anisotropic refinement. Thus, the precise position of the atoms in the crystal could not be determined except for the chloride atom, which could be assigned to the end of the side chain. The question of whether **6** was present as dienol-γ-lactone or γ-keto-enol acetal could not be resolved.

UV spectroscopic and oxidant properties of (**6**).—As expected based on the X-ray structure, the UV spectrum of **6** showed a maximum at 260 nm, which was indistinguishable from that of ascorbic acid (Fig. 2). When 0.216 mM solutions were incubated with 10 μM $CuCl_2$ in 0.1 mM sodium phosphate at various pH values, initial oxidation rates for



Scheme 1.

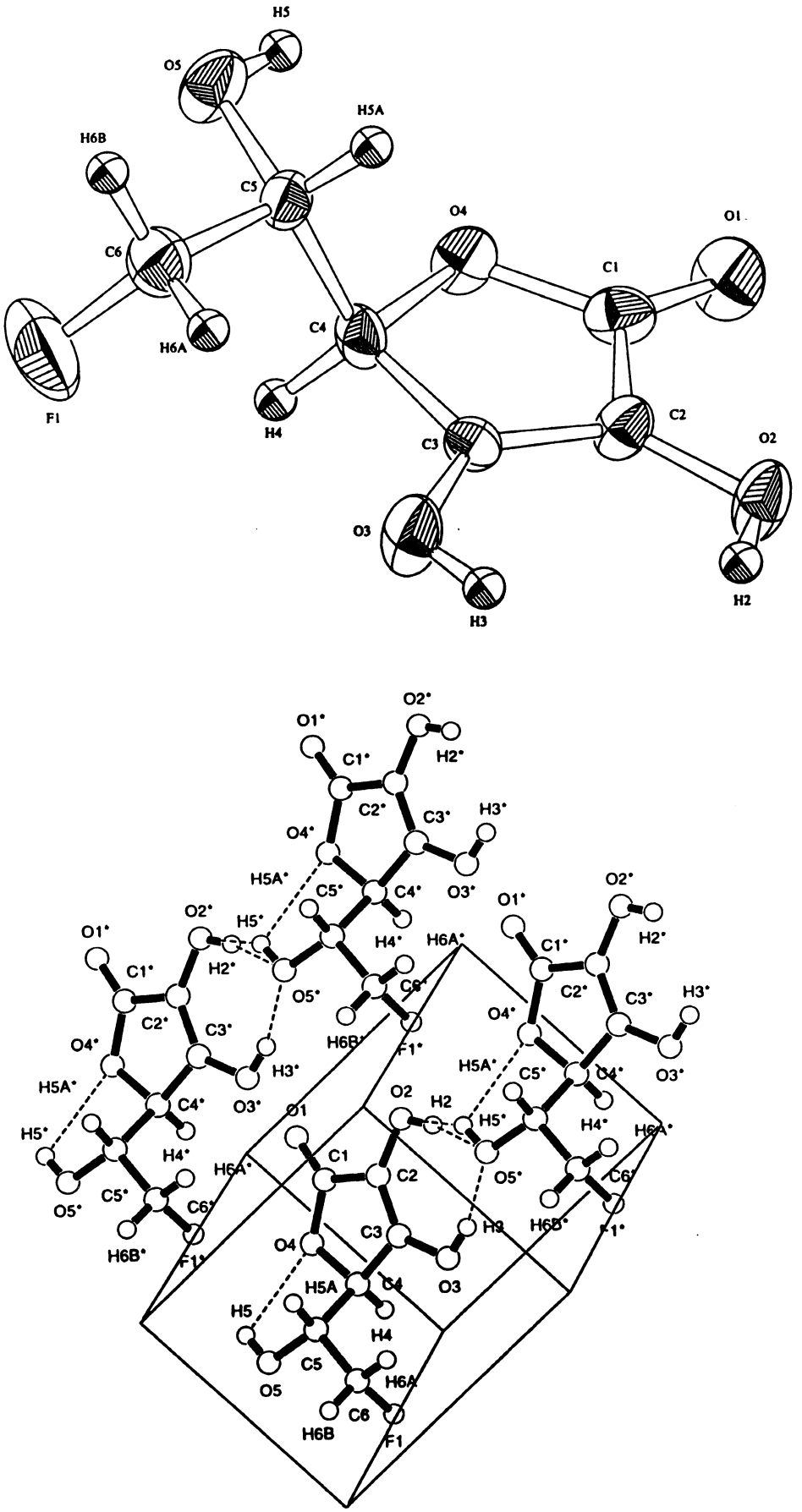


Fig. 1. X-ray structure of 6-deoxy-6-fluoro-L-ascorbic acid. Below: the arrangement of 6-deoxy-6-fluoroascorbic acid in the crystal structure.

Table 1
Crystal data and structure refinement parameters for **6** [a]

Crystal data	
Molecular formula	$C_6H_7FO_5$
Molecular weight	178.12
Crystal system	monoclinic
Space group	$P2_1$
Z	2
Cell dimensions	
a (Å)	5.547(2)
b (Å)	6.769(3)
c (Å)	9.302(2)
α (°)	90.00
β (°)	91.80(3)
γ (°)	90.00
V (Å^3)	349.1(2)
D_{calcd} (mg cm^{-3})	1.695
Structure determination and refinement data	
Crystal dimensions (mm)	$0.52\times0.32\times0.12$
Temperature (K)	293
Radiation	Cu K_α ($\mu = 14.7$ cm^{-1}, $\lambda = 1.5418$ Å)
No. of reflections measured	689
No. of unique reflections	675 ($I>2\sigma$)
Range of h, k, l	$0<h<6$, $0<k<8$, $-11<l<11$
θ_{max} (°)	67.47
Largest residual electron density peaks (e Å^3)	0.488 and −0.398
Final agreement factors	$R = 0.0519$, $R_w = 0.2152$
Function minimized	$R = [w(F_o^2 - F_c^2)^2$, using least B square weights, $w = 1/[\sigma^2(F_o^2) + 0.1000P^2]$, where $P = (F_o^2 + 2F_c^2)/3$, 109 parameters were refined

[a] Intensity data were processed using the program TEXSAN. A few cycles of refinement were carried out using SHELXL-93.

both compounds were virtually identical (Fig. 3). Slower and more complex kinetics were apparent at higher pH (pH 9) with **6**, suggesting that enolization of ascorbate hydroxyls in positions 5 and 6 can influence the delactonization rate. However, it is unlikely that this phenomenon would be of importance for the in vivo application of **6** since its initial oxidation rate and half-life was not different from that of ascorbic acid at physiological pH values.

^{19}F NMR properties of oxidation products of **6**.—The fluorine NMR of pure **6** in deuterium oxide recorded with a 750 MHz Varian instrument is shown in Fig. 4 (top). A triplet split into a doublet of triplets by the protons at C-6 was observed at −227.64 ppm relative to

Table 2
Bond lengths (Å) and bond angles (°) for 6-deoxy-6-fluoro-ascorbic acid and ascorbic acid (non-dissociated form[19])

	6-Deoxy-6-fluoro-ascorbic acid	Ascorbic acid non-dissociated form[19]
F–C-6	1.398	1.431 (O-6–C-6)
O-1–C-1	1.201	1.216
O-2–C-2	1.348	1.361
O-3–C-3	1.337	1.326
O-4–C-1	1.373	1.355
O-4–C-4	1.466	1.444
O-5–C-5	1.415	1.427
C-1–C-2	1.461	1.452
C-2–C-3	1.337	1.338
C-3–C-4	1.495	1.493
C-4–C-5	1.535	1.521
C-5–C-6	1.511	1.521
C-1–O-4–C-4	108.6	109.1
O-1–C-1–C-2	128.8	129.1
O-4–C-1–C-2	109.0	109.5
C-3–C-2–O-2	129.5	127.5
C-3–C-2–C-1	108.5	107.8
O-2–C-2–C-1	122.2	124.6
O-3–C-3–C-2	132.0	133.5
O-3–C-3–C-4	118.4	117.1
C-2–C-3–C-4	109.9	109.5
O-4–C-4–C-3	103.9	104.0
O-4–C-4–C-5	106.8	110.4
C-3–C4–C-5	115.1	114.8
O-5–C-5–C-6	108.0	106.9
O-5–C-5–C-4	110.3	111.7
C-6–C-5–C-4	111.8	112.7
F–C-6–C-5	109.2	108.0 (O-6–C-6–C5)

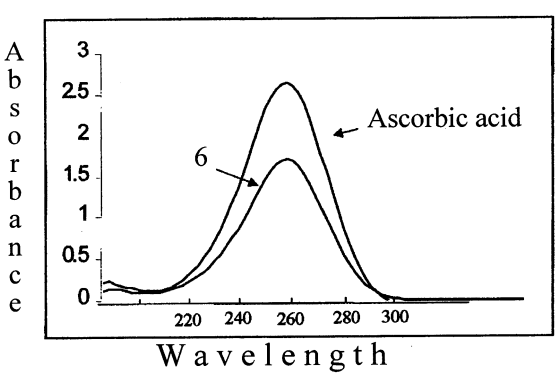


Fig. 2. UV spectrum of ascorbic acid and 6-deoxy-6-fluoro-L-ascorbic acid. Concentrations were arbitrarily chosen to fit on the absorbance scale. For both compounds a 15.6 μM solution of the compound had 0.95 absorbance units at 264 nm.

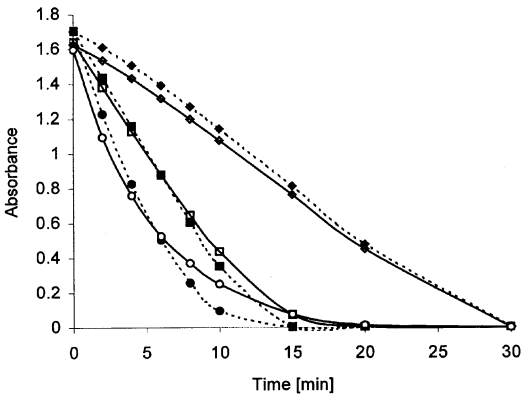


Fig. 3. Comparative oxidative degradation kinetics at various pH values measured by absorbance at 260 nm of ascorbic acid and 6-deoxy-6-fluoro-L-ascorbic acid (each about 10 μM) in presence of 10 μM $CuCl_2$ in 0.1 M sodium phosphate buffer at pH 6.0, 7.4 and 9.0. (Ascorbic acid: pH 6.0 --◆--, pH 7.4 --■--, pH 9.0 --●--; compound **6**: pH 6.0 –◇–, pH 7.4 –□–, pH 9.0 –○–).

$CFCl_3$ as the internal standard. Further splitting due to long-range coupling with H-5 is also evident, with additional fine splittings most likely from H-4. Following 30 min of oxidation at room temperature with 10 μM $CuCl_2$, the ascorbic acid peak at −227.64 ppm completely disappeared (not shown). The signal shown in Fig. 4 (bottom) for **6** was pasted into the Figure in order to illustrate the position of unoxidized **6** relative to the five new signals (U-1–U-5). Importantly, similar results were obtained when fresh **6** was added to an already oxidized mixture of ascorbic acid (not shown). The structures of the compounds corresponding to the new signals remain to be elucidated.

In summary, we have presented here a partially modified synthesis of **6** and demonstrated that its structure, UV properties, and behavior toward pro-oxidant conditions do not differ from those of native ascorbic acid. Furthermore, we have shown that sufficiently well resolved new signals are detectable in the ^{19}F NMR spectrum recorded with a 750 MHz instrument, suggesting that 6-deoxy-6-fluoro-L-ascorbic acid will be a powerful tool to monitor ascorbic acid catabolism in vivo and identify its major degradation products.

2. Experimental

General methods

NMR spectroscopy. ^1H 400 MHz and ^{13}C 100 MHz NMR spectra were determined on a Varian Mercury 400 spectrometer with chloroform-*d* as solvent and $(CH_3)_4Si$ as the internal standard. ^{19}F NMR spectra were obtained with a Varian UnityPlus-750 MHz spectrometer equipped with a Nalorac 5 mm $^1H/^{13}C/^{19}F$ PFG triple resonance probe (optimized for ^{19}F detection). The spectra were obtained from samples in D_2O solution with the temperature regulated at 25 ± 0.1 °C. All data processing was performed on a Sun SPARCstation-10 using Varian's Vnmr software. The one-dimensional ^{19}F spectra were obtained with the following parameters: a 5.0 μs ($\sim 30°$) ^{19}F pulse width, 25,000 Hz spectral window, 0.5 s acquisition time, 250 transients and no relaxation delay. The data were weighted with 0.20 Hz exponential line broadening and zero-filled before Fourier transformation.

X-ray measurement.—Single crystals of 6-deoxy-6-fluoroascorbic acid crystallized from nitromethane having a size about $0.52 \times 0.32 \times 0.12$ mm were mounted on a glass fiber. Intensity data for 6-deoxy-6-fluoroascorbic acid were collected with graphite-monochromated Cu K_α radiation ($\mu = 14.7$ cm^{-1}, $\lambda = 1.5418$ Å) on a Rigaku AFC-7R four-circle diffractometer at 293 K. The initial positions of 12 atoms without hydrogen atoms were obtained by the direct method with the SHELXS-86 [20] program for space group $P2_1$. These atomic positions, together with anisotropic temperature factors, were refined by the full-matrix least-squares procedure after several refinement cycles with isotropic temperature factors. Computations were performed on an Iris Indy workstation with the help of TEXSAN [21] and the SHELXL-93 [22] package program. Further refinement of the atomic parameters were carried out using the package SHELXL-93.

Methyl 2,3-O-isopropylidene-6-O-tosyl-L-xylo-hex-2-ulofuranosonate (**3**).—2,3:4,6-Di-*O*-isopropylidene-L-*xylo*-hex-2-ulofuranosonic acid (**1**) (10 g) was dissolved in MeOH (20 mL) and carbon tetrachloride (20 mL) and five drops of concd H_2SO_4 were added. The

mixture was refluxed for 30 min. After cooling to room temperature (rt), $CaCO_3$ (1.5 g) was added and the mixture was stirred for 20 min. After filtration, inorganic salt solvents were evaporated off and methyl 2,3-*O*-isopropylidene-L-*xylo*-hex-2-ulofuranosonate (**2**) was

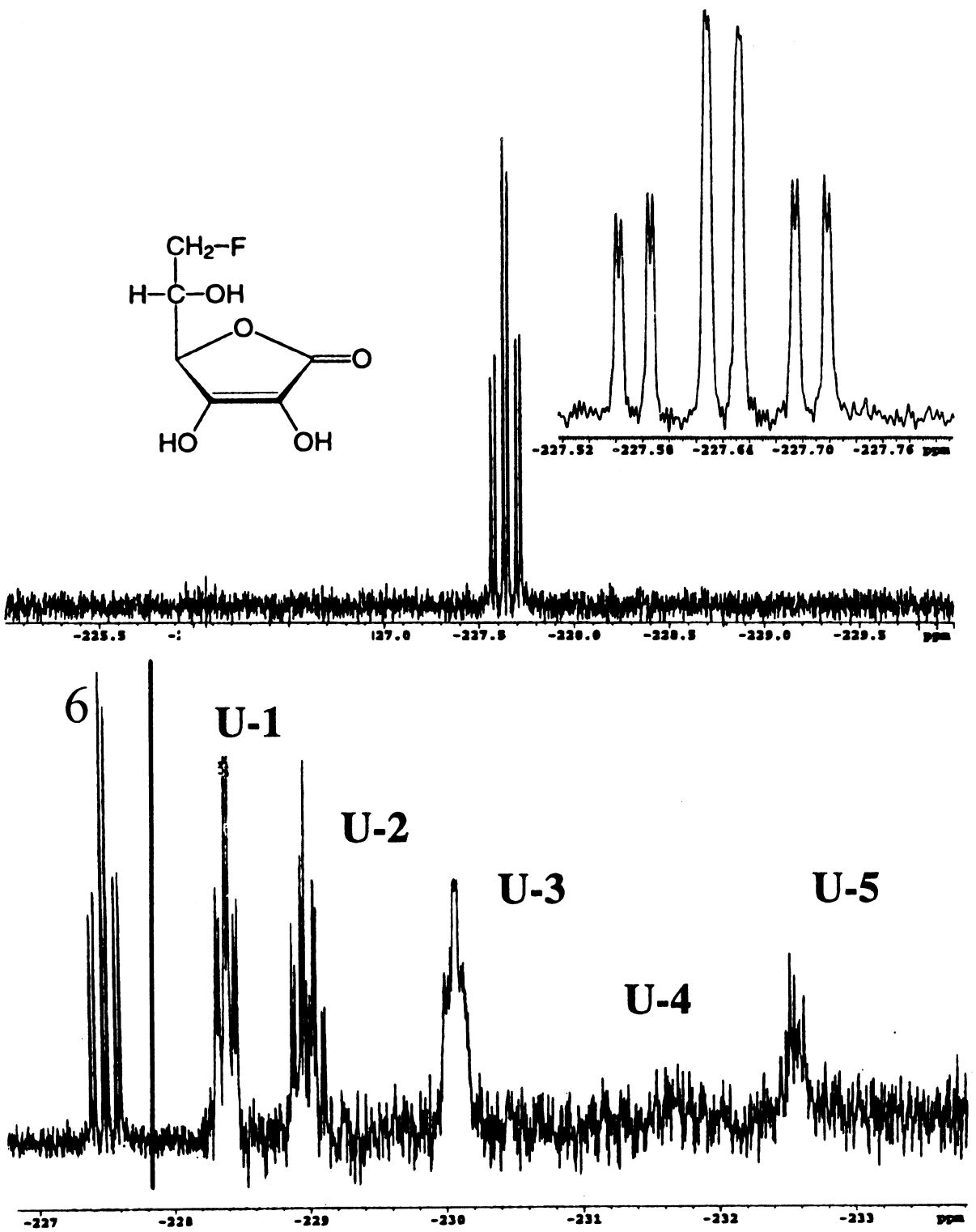


Fig. 4. 750 MHz ^{19}F NMR spectra of **6** before (top) and after oxidation with 100 μM $CuCl_2$ (bottom). In the bottom spectrum, the signal corresponding to **6** at −227.64 ppm was absent in the original spectrum. It was pasted from the spectrum to illustrate the position of **6** relative to its degradation products U1–U5.

obtained as a thick yellow oil (9.5 g). The oil was dissolved in freshly distilled pyridine (135 mL), and TsCl (8.1 g) was added at 0 °C. After 16 h at rt, pyridine was evaporated and the residue was dissolved in 1 L of cold water. After 1 h white crystals were removed by filtration. The product, recrystallized from EtOH and water, yielded methyl 2,3-*O*-isopropylidene-6-O-tosyl-L-*xylo*-hex-2-ulofuranosonate (**3**) (7.5 g, yield 38%); ^{1}H NMR ($CDCl_3$): 7.70–7.35 (m, 4 H, aromatic ring); 4.70 (s, 1 H, H-3); 4.48 (m, 1 H, $J_{5,6}$ 6.1 Hz, H-5); 4.38 (dd, 1 H, $J_{6,6'}$ 10.4 Hz, H-6); 4.28 (d, 1 H, $J_{4,5}$ 2.2 Hz, H-4); 4.18 (dd, 1 H, H-6′); 3.80 (s, 3 H, OCH_3); 2.45 (s, 3 H, $PhCH_3$); 1.50–1.35 (2 × s, 6 H, $C(CH_3)_2$); ^{13}C NMR ($CDCl_3$): 145 (C1); 140–160 (aromatic ring); 115 ($C(CH_3)_2$); 110 (C-2); 87 (OCH_3); 81 (C-6); 74 (C-3); 67 (C-4); 54 (C-5); 27–26 ($C(CH_3)_2$); 22 ($PhCH_3$).

Methyl 6-deoxy-6-fluoro-2,3-O-*isopropylidene*-L-xylo-*hex-2-ulofuranosonate* (**4**).—To a stirred suspension of KF (5 g, 99.99%, Aldrich Co.) in 100 mL dry of *N*,*N*-dimethylformamide (distilled over P_2O_5) was added methyl 2,3-*O*-isopropylidene-6-*O*-tosyl-L-*xylo*-hex-2-ulofuranosonate (**3**) (5 g) and the mixture was stirred and heated for 5 h at 150 °C (glycerol bath). After cooling to rt, inorganic salts were filtered off and solvents were evaporated (bath temperature 70 °C). To the residue, EtOAc (100 mL) was added and undissolved salts were filtered off. The mixture was concentrated to a yellow oil, which was purified twice by column chromatography (Silica Gel, E. Merck 70–230 mesh, 3:7 acetone–hexane) yielding pure crystals of methyl 6-deoxy-6-fluoro-2,3-*O*-isopropylidene-L-*xylo*-hex-2-ulofuranosonate (**4**) (650 mg, yield 21%); ^{1}H NMR ($CDCl_3$): 4.82 (m, 1 H, H-6); 4.70 (d, 1 H, H-3); 4.59 (m, 2 H, H-5 and H-6′); 4.30 (d, 1 H, H-4); 3.87 (s, 3 H, OCH_3); 1.54–1.38 (2 × s, 6 H, $C(CH_3)_2$); ^{13}C NMR ($CDCl_3$): 168.89 (C1); 114.83 ($C(CH_3)_2$); 109.82 (C-2); 87.53 (C-3); 81.58–82.02 (d, J_{CF} 22.1 Hz, C-5); 79.57–82.90 (d, J_{CF} 167.4 Hz, C-6); 74.85–74.97 (d, J_{CF} 5.9 Hz, C-4); 53.66 (OCH_3); 26.92–25.76 ($C(CH_3)_2$).

6-Deoxy-6-fluoro-L-*ascorbic acid* (**6**).—Methyl 6-deoxy-6-fluoro-2,3-*O*-isopropylidene-L-*xylo*-hex-2-ulofuranosonate (**4**) (500 mg) was refluxed under nitrogen in a mixture of 40% aq soln H_2SO_4 (25 mL) and MeCN (25 mL) for 1.5 h. After cooling to rt, MeCN was evaporated off and the residue was extracted with EtOAc (4 × 20 mL). The organic layers were combined, washed with cold water (< 10 mL), dried with Na_2SO_4 and concentrated by evaporation to a solvent-free yellow oil (290 mg). The oil was dissolved in $MeNO_2$ (3 mL) and after conditioning overnight at −18 °C white crystals of 6-deoxy-6-fluoro-L-ascorbic acid (**6**) were isolated (130 mg, yield 36%, mp 139–141 °C, $[\alpha]_D$ +19.5° (0.5, water); ^{1}H NMR (D_2O): 4.89 (d, 1 H, H-4); 4.65 (m, 1 H, H-6); 4.56 (m, 1 H, H-6′); 4.25 (m, 1 H, H-5); ^{13}C NMR (Me_2SO): 170.39 (C-1), 152.06 (C-3), 118.26 (C-2); 85.56–82.20 (d, J_{CF} 168.7 Hz, C-6); 74.65–74.51 (d, J_{CF} 7.0 Hz, C-4); 66.51–66.11 (d, J_{CF} 20.2 Hz, C-5); ^{19}F NMR (D_2O, $CFCl_3$ as a reference peak): dt −227.64 ppm.

Oxidative degradation of **6** *with* $CuCl_2$.—Compound **6** (1.9 mg, 10.7 μmol) was dissolved in 1 mL of water (concentration 10.7 mM) and 20 μL of this solution was added to 1 mL of 10 μM aq $CuCl_2$. The solution containing 0.216 mM of **6** was kept at rt and the spectrum was recorded 30 min later.

Acknowledgements

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