Biosci. Biotechnol. Biochem., **63** (2), 390–394, 1999

Convenient Preparation and Quantification of 5,5′-Diferulic Acid

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Received September 16, 1998; Accepted October 28, 1998

5,5′-Diferulic acid (5,5′-DFA), which is one of the cross-linking residues in plant cell walls, was prepared by using a facile procedure. The phenol oxidation of vanillin with Fe Cl_3 gave divanillin, which was further devoted to a Perkin reaction to give the desired product. It was found on ^{13}C-NMR that the chemical shift of C-5 of ferulic acid (FA) clearly shifted downfield, when this carbon is quaternarized by the oxidative dimerizaton to 5,5′-DFA, while those of other carbons of 5,5′-DFA are fundamentally same as those of FA. Also prepared was [9,9′-$^{13}C_2$]-5,5′-DFA, which was proved to be a good internal standard on GC-MS quantification of endogenous 5,5′-DFA from plant tissues.

Key words: diferulic acid; ferulic acid; Perkin reaction; phenol oxidation; divanilin

The biological effects of ferulate (1a), which is esterified to cell wall polymers such as arabinoxylans, are of particular interest from the perspective of the formation of chemically cross-linked structures (Fig. 1).[1] It is widely believed that the phenol oxidation of 1a mediated by cell wall peroxidases and hydrogen peroxide produces polysaccharide esters 2a, which are cross-linked by dehydrodimers of ferulic acid (FA, 1b).[2] Besides extensive work on isolation of 5,5′-diferulic acid (5,5′-DFA, 2b) from various plant cell walls via saponification,[3] Ishii *et al.* have isolated oligosaccharide esters such as 1c[4] and 2c[5] from bamboo shoot cell walls by treating cell walls with Driselase. The 5,5′-coupled dehydroferulate, commonly referred to as diferulic acid, had been the only dehydrodimer of ferulate. However, Ralph *et al.* identified other isomers such as 5,8′-, 4,8′-*O*-, and 4-*O*-,5′-DFA in grass cell walls.[6a] The isomeric composition of ferulate dehydrodimers seems depends on plant species.[6] Photo-induced [2+2] cycloaddition has also been reported in monocotyledonous and dicotyledonous plants as a mechanism for cross-linking polysaccharide chains.[7] In addition to the biological role as cell wall extensibility regulators,[8] formation of these dehydrodimers or cyclodimers of ferulate can be an important factor affecting the digestibility of graminaceous fibrous material, such as cereal straw, in ruminants.[9]

In spite of these biological points of significance, conventional preparation of 2b can not supply enough authentic specimens.[10,11] A convincing assignment of 2b on ^{13}C-NMR, which might have been very informative for the identification of natural sugar esters such as 2c,[5] is yet to be done. In this paper, we present a facile preparation of 2b, which can be manipulated by very simple procedures. We also describe unequivocal assignments of authentic 2b compared with 1b on ^{13}C-NMR. We also mention the preparation and derivatization of [8,8′-$^{13}C_2$]-2b, which was proved to be a good internal standard for GC-MS quantification of endogenous 2b in plant tissues.

Richitzenhain has reported the first chemical synthesis of 2b by four steps.[10] To date, most works in the area of plant physiology and phytochemistry have used this conventional method to obtain an authentic standard.[3] Nevertheless, this method is often not reproducible and can not be used to prepare a substantial amount of 2b. One of the main obstacles is the coupling of vanillin 3 to divanillin 4a (Fig. 2) in an aqueous system, which is composed of peroxidase and H_2O_2. This reaction is not suitable for a large scale experiment due to the poor water solubility of 3 and the difficulty of controlling the unstable enzymatic reaction.[12] Another task is the elongation of two C_2-units on aldehyde by the Knoevenagel reaction which requires 2 weeks for its completion.[10] To overcome those difficulties, we examined the phenol coupling of 3 by a chemical method (Fig. 2). A stoichiometric amount of $FeCl_3$ was used for the oxidation of the *ortho* position of the phenol group of 3.[13] Compared with the enzymatic method, a high concentration of 3 and a short reaction time could be adopted to obtain 4a efficiently. The resulting 4a was precipitated out during the reaction as an insoluble sediment, and harvested by filtration. The typical yield of crude 4a from 3 was 57%. Next, we examined a Perkin reaction using acetic anhydride and sodium acetate[14] to obtain the desired product 2b directly from crude 4a without further purification[12] or preliminary acetylation.[10] Although it took 2 days for the completion of this reaction, this was a significant improvement over the conventional method.[10] When the reaction was monitored before its completion, an intermediate 5 was identified as a major component in the reaction mixture. This indicates that one elongation of a C_2 unit on 4a is fairly smooth to give 5, while introduction of another C_2 unit on 5 is rate-limiting due to steric hindrance. In order to remove pig-

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Abbreviations: COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; DFA, diferulic acid; FA, ferulic acid; GC-EI-MS, gas chromatography electron impact ionization mass spectrometry; NOESY, nuclear Overhauser enhancement and exchange spectroscopy; TMS, trimethylsilyl

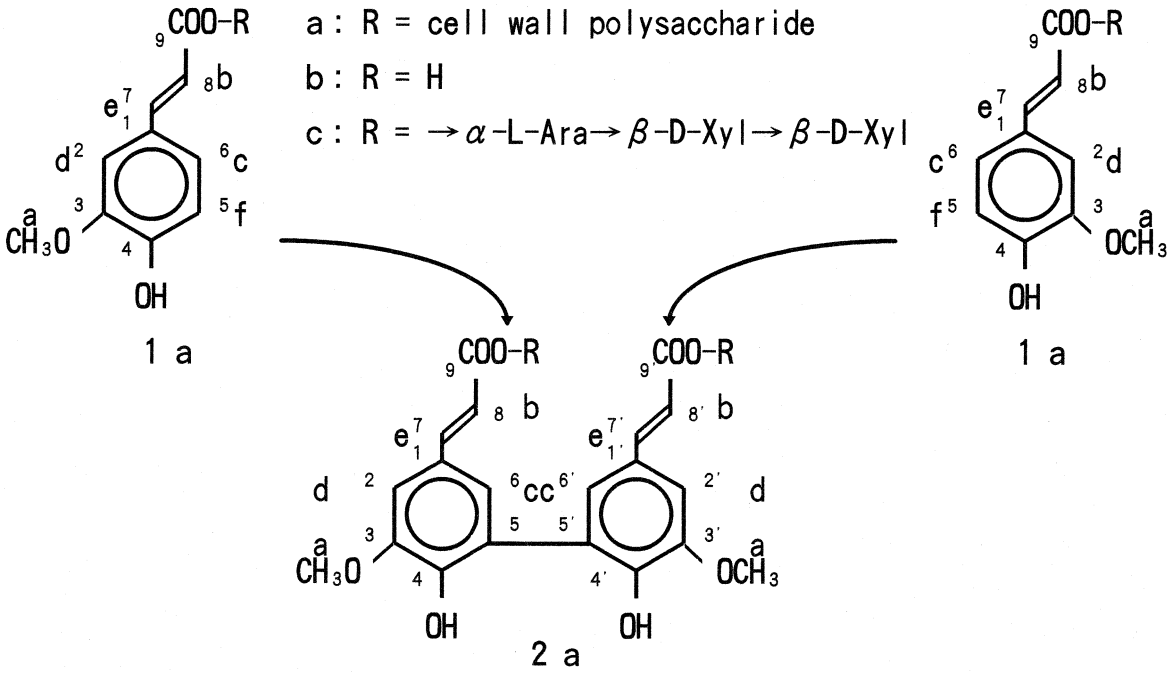

Fig. 1. Proposed Mechanism for the Formation of Chemical Cross-Linkage in Plant Cell Walls.
Alkaline saponification or driselase digestion of cell wall polysaccharide ester (a) gives free acid (b) or oligosaccharide ester (c), respectively.[1)]

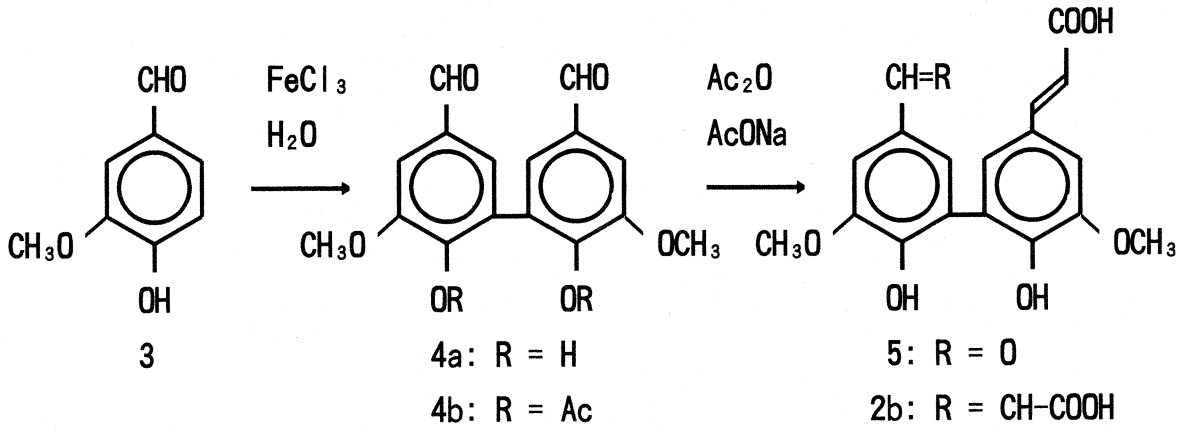

Fig. 2. Facile Preparation of 5,5′-Diferulic Acid 2b.

mented by-products other than 2b after the reaction, the resulting mixture was treated with active carbon at a slightly acidic pH. Under these conditions, 2b, which has two carboxyl and two phenolic hydroxyl groups in its molecule, was exempted from absorption. The stepwise acidification of the reaction mixture before and after decoloration gave 2b in a high purity. The typical yield of 2b from crude 4a was 63%. Although the yield of this Perkin reaction is rather modest, its simple procedures are a great practical merit.

Richitzenhain found that the treatment of 1b with peroxidase/H_2O_2 failed to give 2b.[10)] We also examined the $FeCl_3$ oxidation of 1b in an attempt to obtain 2b in one step. The lack of formation of 2b from 1b by these treatments suggests that the sugar esterification of the carboxyl group of 1a might be important for the cross linkage that is mediated by peroxidase in plant cell walls. This is probably due to the increase of water solubility and the electron density at C-5[12,13)] of the substrate.

Hartley and Jones obtained authentic 2b by using the conventional method and first reported its ^{1}H-NMR data.[3a)] To date, nevertheless, the ^{13}C-NMR of 2b has not yet appeared. We did a series of measurements of 2b on ^{1}H- and ^{13}C-NMR in 10% NaOD-D_2O due to its very poor solubility in any solvent, while Ishii isolated and characterized 2c as a D_2O soluble sugar ester.[5)] To assign all five chemical shifts of 2b on ^{1}H-NMR, we measured the ^{1}H-^{1}H COSY and NOESY spectra. A coupling constant (16.6 Hz) between H-b and H-e confirmed that 2b

is the (8*E*, 8′*E*)-isomer. An obvious cross peak between H-a and H-d was also recognized on the NOESY spectrum, indicating that H-d is a phenyl proton on C-2 while H-c is on C-6. On the other hand, to assign all ten chemical shifts on ^{13}C-NMR unequivocally, we measured the HMQC and HMBC spectra (Table).

In the same manner as 2b, all of the ^{13}C-signals for 1b could also be assigned. 2D-Heteronuclear shift-correlated spectroscopy, which was measured by Ishii and Hiroi,[4c] indicated that previous assignments of C-5 and C-8 of 1c[4a,b] had been incorrect. Our assignment of authentic 1b also confirmed that the chemical shift of C-5 is definitely upfield from that of C-8 in spite of their close chemical shifts.

We found that all of the carbon chemical shifts of 2b except for C-5 were almost completely coincident with those of 1b. This indicated that the chemical shift of C-5 is obviously shifted downfield when this carbon was quaternarized by oxidative dimerization. Our data for 1b and 2b were also compared with those of their natural sugar esters reported previously. The larger chemical shifts for C-4 and C-9 of authentic 1b and 2b as compared with 1c[4c] and 2c[5] were thought probably due to the alkaline condition we used for ^{13}C-NMR measurement. However, all other carbon chemical shifts of authentic 1b and 2b were very similar to those found in the corresponding sugar esters 1c and 2c, respectively. Lacking authentic data, Ishii have tentatively adopted the ^{13}C-NMR assignment of 1c to assign the chemical shifts of 2c.[5] In conclusion, our assignment presented here suggests that the chemical shifts for C-5, C-1, and C-6 in this tentative assignment should be transposed into C-1, C-6, and C-5, respectively.

Previously, endogenous 2b in various plant sources has been quantified using HPLC with fluorescence detection.[6] The GC-MS analysis with an internal standard can be a new technique, which has greater accuracy due to the ihherent correction for the variable losses occuring during sample purification.[15] As a stable internal standard for this purpose, We chose [9,9′-$^{13}C_2$]-2b, which is to be prepared by the Horner reaction using [1-^{13}C]-methyl diethylphosphonoacetate. Coupling of diacetate 4b and 2 equivalent of [1-^{13}C]-phosphonoacetate anion followed by one pot hydrolysis gave [9,9′-$^{13}C_2$]-2b in a 90% yield. Authentic [9,9′-$^{13}C_2$]-2b was converted to TMS or methyl derivatives.[16] Molecular ion cluster of TMS derivative of 2b or [9,9′-$^{13}C_2$]-2b on GC-EI-MS showed a typical composition of [M]:[M+1]:[M+2]:[M+3]=100:56:29:10 while that of the methyl derivative was [M]:[M+1]:[M+2]=100:27:5. These ratios were identical with those theoretically calculated from natural isotopic compositions of Si and/or C atoms. Consequently, the level of natural 2b can be quantified by equation 1 and 2 (experimental) for TMS and methyl derivatives, respectively. Compared with TMS derivatization, methylation of 2b using etheral diazomethane is facile and the isotopic compostion of the product is simple. We recomend methyl derivatization when the natural 2b to be quantified is free or separated from 5,5′-dicaffeic acid, which would give the same product as the methyl derivative of 2b when treated together with diazomethane. Application of our method for various plant materials will be presented in due course.

Table. Cross Peaks Observed in HMQC and HMBC Experiments, and Assignment of Chemical Shifts on ^{13}C-NMR [ppm]

		1b[a]			2b[a]	
HMQC		^{13}C – ^{1}H			^{13}C – ^{1}H	
		2	d		2	d
		5	f		5	
		6	c		6	c
		7	e		7	e
		8	b		8	b
		CH_3	a		CH_3	a
HMBC	^{13}C	[ppm]	^{1}H	^{13}C	[ppm]	^{1}H
	1	121.8[b]	b, f	1	119.8[b]	b, e[c]
	2	112.1	c, e	2	110.3	c, e
	3	152.7[b]	a, f, d[c]	3	153.0[b]	a, d[c]
	4	161.5[b]	c, d	4	160.3[b]	c, d
	5	119.9		5	131.8[b]	c[c]
	6	125.6	d, e	6	128.2	d, e
	7	144.0	d	7	144.5	c, d
	8	118.7		8	118.0	e[c]
	9	178.3[b]	e, b[c]	9	178.4[b]	e, b[c]
	CH_3	57.4		CH_3	57.5	

[a] Measured in 10% NaOD–D_2O. See Structures in Fig. 1.
[b] Disappeared on DEPT 45 spectrum.
[c] Interpreted for $^{13}C-X-^{1}H$.

Experimental

Authentic 1b and all chemical agents were purchased from Aldrich. ^{1}H- and ^{13}C-NMR spectra were measured on a Bruker DPX 400 spectrometer at 27°C. Chemical shifts on ^{1}H-NMR were locked by D_2O (4.70 ppm) as a solvent, while those on ^{13}C-NMR were corrected by MeOH (49.30 ppm) as an internal standard. GC-EI-MS spectra were measured on a Jeol SX102 spectrometer (ion source temp. at 200°C, ionization voltage of 70 eV) with DB-1 column (30 m × 0.32 mm, J & W Scientific, 250–300°C at 6°C/min, under 40 μl/min of He flow). IR spectra were recorded on a Jasco IR-810 infrared spectrometer. Flush column chromatography was done by using silica gel 60 (230–400 mesh, Merck)

Preparation of 5,5′-Diferulic acid 2b. To a solution of $FeCl_3 \cdot 6H_2O$ (29.7 g, 110 mmol) in water (500 ml) in a round-bottomed flask equipped with a magnetic stirrer, an oil bath, and a condenser, 3 (15.2 g, 100 mmol) was added and suspended. The oil bath was gradually warmed to 50°C, and the stirring was kept for 4 h. After cooling with ice bath, the resulting precipitate was harvested by vacuum filtration, rinced with water and then MeOH, and oven-dried to give crude 4a (8.6 g, 29 mmol, 57% yield) as a brown powder. Next, crude 4a (8.6 g) and anhydrous sodium acetate (15 g) were powdered together in a mortar, and suspended in acetic anhydride (100 g, 490 mmol). The suspension was refluxed with vigorous stirring for 2 d. Water (250 ml) and NaOH (50 g, 1.25 mol) were carefully added to the mixture, and the whole was refluxed for 2 h. After cooling, conc. H_2SO_4 and the active carbon (3.0 g) were added to a dark brown mixture, and this suspension was stirred at

pH 5–6 for 1 h. Then, the active carbon was removed by vacuum filtration to give a yellow filtrate, which was further acidified to pH 1 by adding conc. H_2SO_4. The white precipitate of 2b was recovered by vacuum filtration at 30°C and rinsed with water to give 2b (7.1 g, 18.5 mmol, 37% overall yield from 3). Recrystallization from MeOH gave an amorphous powder of 2b, which was decomposed above 280°C.[10] IR_{max} (KBr) cm^{-1}: 3490, 1790, 1630, 1600, 1490, 1450, 1420, 1280, 1270, 1200, 1140, 1040. ^{1}H-NMR (400 MHz, 10% NaOD-D_2O): δ 3.49 (3H, s, H-a), 5.81 (1H, d, J=16.0 Hz, H-b), 6.58 (1H, s, H-c), 6.72 (1H, s, H-d), 6.93 (1H, d, J=16.0 Hz, H-e). Although this data was very different from those reported previously (270 MHz, acetone-d_6),[3a] the following measurement was fundamentally in agreement. ^{1}H-NMR (400 MHz, MeOH-d_4): δ 4.05 (3H, s), 6.43 (1H, d, J=16.0 Hz), 7.18 (1H, s), 7.31 (1H, s), 7.72 (1H, d, J=16.0 Hz). ^{13}C-NMR (100 MHz, 10% NaOD-D_2O) was measured and assigned as shown in Table 2. GC-EI-MS as a *tetra*-TMS derivative (t_R: 11.4 min, rel. area on GC: 100%) m/z (rel. int.): 674 (M^+, 100), 659 (M^+-Me, 18), 73 (45). HREIMS. Found 674.2583; calcd. for $C_{32}H_{50}O_8Si_4$, 674.2583. GC-EI-MS as a *tetra*-methyl derivative (t_R: 9.2 min, rel. area on GC: 100%) m/z (rel. int.): 442 (M^+, 100), 207 (30). HREIMS. Found 442.1628; calcd. for $C_{24}H_{26}O_8$, 442.1628.

Detection of 5 as a reaction intermediate. An aliquot of the reaction mixture, which was composed of 4a, acetic anhydride, and sodium acetate as prepared above, was sampled after 6 h and subjected to TMS derivatization. Three peaks were identified on GC-EI-MS. 4a (t_R: 4.1 min, rel. area on GC: 4%) m/z (rel. int.): 446 (M^+, 58), 431 (M^+-Me, 100), 73 (72); 5 (t_R: 7.4 min, rel. area on GC: 81%) m/z (rel. int.): 560 (M^+, 100), 545 (M^+-Me, 29), 383 (39), 73 (70); 2b (t_R: 11.4 min, rel. area on GC: 15%). HREIMS for a *tri*-TMS derivative of 5. Found 560.2081; calcd. for $C_{27}H_{40}O_7Si_3$, 560.2081.

Preparation of [9,9′-$^{13}C_2$]-5,5′-Diferulic acid 2b. To a solution of crude 4a (3.0 g, 10 mmol) in pyridine (100 ml), acetic anhydride (20 g, 98 mmol) and 4-dimethylaminopyridine (50 mg) were successively added. After stirring for 24 h at room temperature, the whole was evaporated *in vacuo*. The residue was flash-chromatographed over SiO_2 (100 g). Elution with hexane-ethyl acetate (5:1) gave 4b (2.9 g, 7.6 mmol, 76% yield). Recrystallization from hexane-ethyl acetate (5:1) gave a needle crystal of 4b; mp 131°C, IR_{max} (KBr) cm^{-1}: 2840, 2800, 2740, 1770, 1700, 1590, 1470, 1270, 1200, 1180, 1140. ^{1}H-NMR (400 MHz, MeOH-d_4): δ 2.11 (3H, s), 3.96 (3H, s), 7.39 (1H, s), 7.55 (1H, s), 9.95 (1H, s). ^{13}C-NMR (100 MHz, MeOH-d_4): δ 20.2, 56.2, 110.2, 126.4, 131.1, 134.5, 142.7, 152.2, 167.7, 190.6. GC-EI-MS (t_R: 3.5 min, rel. area on GC: 100%) m/z (rel. int.): 386 (M^+, 1), 344 (29), 302 (100) 43 (12). HREIMS. Found 386.1003; calcd. for $C_{20}H_{18}O_8$, 386.1002. According to the similar manner reported previously,[15a] [1-^{13}C]-methyl diethylphosphonoacetate was prepared by the thermal condensation of [1-^{13}C]-bromoacetic acid methyl ester (99 atom % ^{13}C) and triethylphosphite. To a suspension of NaH (0.20 g, 5.0 mmol, 60% suspension in oil) in THF (20 ml), [1-^{13}C]-methyl diethylphosphonoacetate (1.05 g, 5.0 mmol) was added dropwise at room temperature. After this was stirred for 30 min, a solution of 4b (869 mg, 2.25 mmol) in THF (3 ml) was added. After the whole mixture was refluxed for 8 h, 25% aq. NaOH solution (40 ml) was added and stirred for 12 h at room temperature. In a similar manner to the precipitation of 4b after the Perkin reaction, the resultant mixture was treated to give [9,9′-$^{13}C_2$]-2b (787 mg, 2.03 mmol, 90% yield). ^{13}C-NMR (100 MHz, 10% NaOD-D_2O): δ 178.4. GC-EI-MS as a *tetra*-TMS derivative (t_R: 11.4 min, rel. area on GC: 100%) m/z (rel. int.): 676 (M^+, 100), 661 (M^+-Me, 18), 73 (45). HREIMS. Found 676.2663; calcd. for $^{12}C_{30}{}^{13}C_2H_{50}O_8Si_4$, 676.2660. GC-EI-MS as a *tetra*-methyl derivative (t_R: 9.2 min, rel. area on GC: 100%) m/z (rel. int.): 444 (M^+, 100), 209 (30). HREIMS. Found 444.1697; calcd. for $^{12}C_{22}{}^{13}C_2H_{26}O_8$, 444.1695.

GC-MS quantification. To an alkaline solution for cell wall saponification (or containing authentic 2b), a proper amount of [9,9′-$^{13}C_2$]-2b was added as an internal standard. It was desirable that the amount of the internal standard should be almost equimolar to the endogenous product. After general extraction and HPLC purification,[6] the recovered mixture of 2b and [9,9′-$^{13}C_2$]-2b was converted to TMS or methyl derivatives by the standard methods.[16] On GC-EI-MS analysis, the level of natural 2b can be calculated by the following equation 1 and 2 for TMS and methyl derivatives, respectively.

$$\left[\frac{\text{intensity of } m/z\ 674}{\text{intensity of } m/z\ 676-(\text{intensity of } m/z\ 674\times 0.29)}\right] \times [9,9'\text{-}{}^{13}C_2]\text{-2b added } [\mu g] \quad -\text{eq. 1}$$

$$\left[\frac{\text{intensity of } m/z\ 442}{\text{intensity of } m/z\ 444-(\text{intensity of } m/z\ 442\times 0.05)}\right] \times [9,9'\text{-}{}^{13}C_2]\text{-2b added } [\mu g] \quad -\text{eq. 2}$$

To avoid partial fractionation of isotopic isomers on GC, a slightly broader width than a peak was measured and averaged. Normaly, several μg of natural 2b was unequivocally identifed and reproducibly quantified on full GC-EI-MS.

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