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Note

A (1→3)-α-D-mannan from a water extract of *Dictyophora indusiata* Fisch.*

CHIHIRO HARA, YOSHINAKA YOKOMORI,
Shotoku Gakuen Women's Junior College, Nakauzura, Gifu 500 (Japan)

TADASHI KIHO, KATSUYUKI NAGAI, AND SHIGEO UKAI†
Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502 (Japan)

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Previously, we reported[2–7] the structural features of the polysaccharides isolated successively from several extracts, *i.e.*, hot 70% aqueous ethanol, hot water, 2% sodium carbonate, and M sodium hydroxide, of the fruit bodies of *Dictyophora indusiata* Fisch. The hot-water extract contained some polysaccharides; in order to fractionate them, it was subjected to ethanol precipitation, and then to the freeze–thawing procedure[8]. The gelatinous, cold-water-insoluble fraction (T-3-GM) obtained by this procedure contained two polysaccharides, as previously reported[4]. Attempts to isolate each polysaccharide in native form from fraction T-3-GM by fractional precipitation and gel filtration were unsuccessful.

By treatment of T-3-GM with alkali, these polysaccharides could be separated from each other. One (T-3-G) is[4] a water-soluble, (1→6)-branched, (1→3)-β-D-glucan; the other polysaccharide (T-3-M) was isolated as a water-insoluble compound. When fraction T-3-GM was treated with exo-(1→3)-β-D-glucanase to remove the branched, (1→3)-β-D-glucan (T-3-G), an undigested polysaccharide (T-3-M′) was obtained as water-soluble material. The present article deals with the structural analysis of these polysaccharide (T-3-M and T-3-M′).

The brownish-tinged white polysaccharide T-3-M was insoluble in water or dimethyl sulfoxide (Me_2SO), but could be dissolved in >2M sodium hydroxide. By elementary analysis, T-3-M contained a trace of ash (~0.9%; Mg, Ca, and Na were detected by emission spectrography of T-3-M), but no nitrogen, sulfur, or phosphorus. T-3-M showed a single sedimentation pattern in ultracentrifugal analysis in 2.5M sodium hydroxide (see Fig. 1), and gave a symmetrical elution peak in gel filtration on Sepharose CL-2B with 2M sodium hydroxide as the eluant, as shown in Fig. 2. T-3-M was composed of mannose (~98%), with a trace of glucose, as shown

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†To whom correspondence should be addressed.

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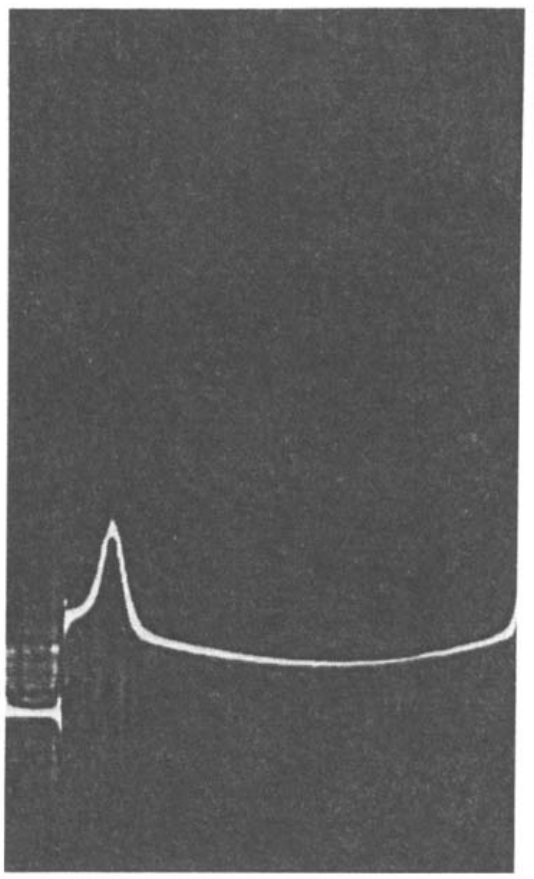

Fig. 1. Ultracentrifugal pattern of T-3-M (5 mg/mL in 2.5M sodium hydroxide) after 50 min at 60,000 r.p.m.

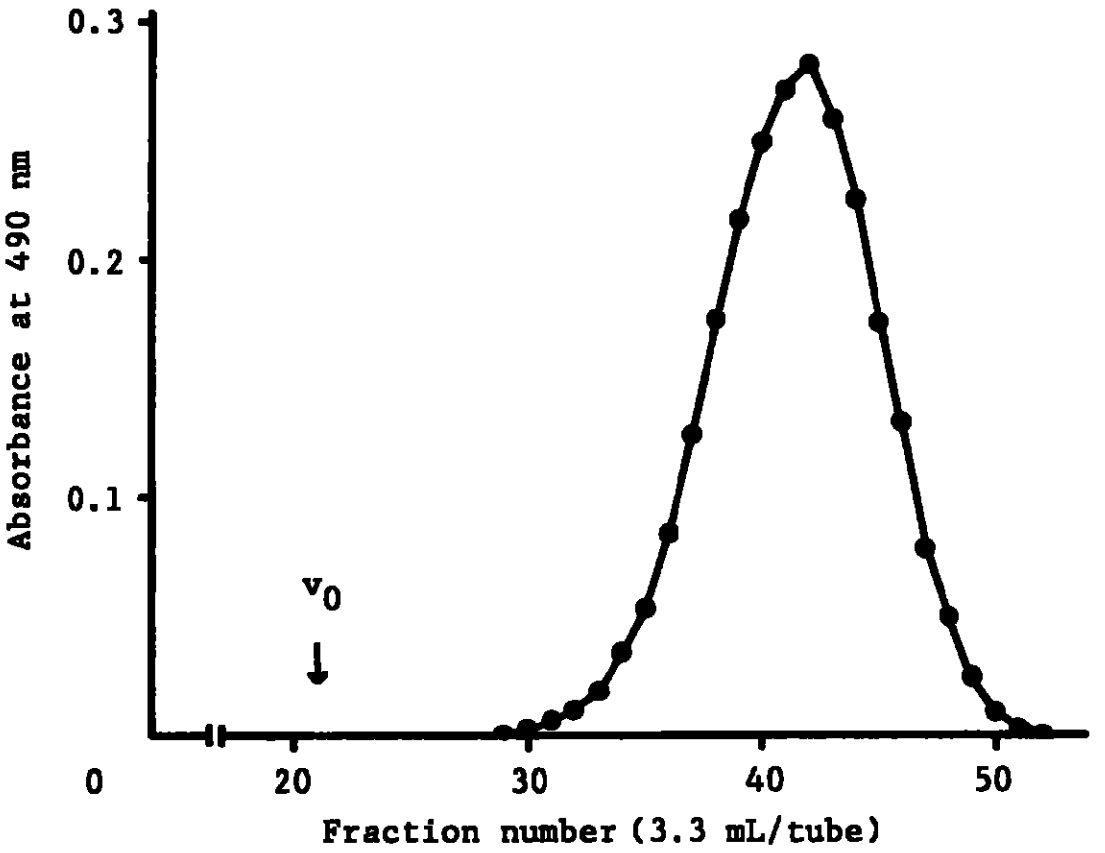

Fig. 2. Chromatogram of T-3-M on Sepharose CL-2B. [The column (1.5 × 98 cm) was irrigated with 2M sodium hydroxide.]

by paper chromatography (p.c.) of the acid hydrolyzate, and by gas–liquid chromatography (g.l.c.) of the alditol acetates[9] prepared from the hydrolyzate. The absolute configuration of the sugar was determined to be D by the specific rotation of the hydrolyzate. The polysaccharide had a highly positive, specific rotation, $[\alpha]_D^{17}$ +119.2° (*c* 0.073, 2M sodium hydroxide), and showed characteristic absorbance at 825 cm^{-1} in the infrared (i.r.) spectrum, indicating the presence of α-D-glycosidic linkages[10]. The molecular weight ($\overline{M}_w$)was estimated to be ~1.9 × 10^5, by use of the calibration curve obtained by gel filtration of standard dextrans on Sepharose CL-2B.

As T-3-M was insoluble in Me_2SO, it was dissolved in 4-methylmorpholine *N*-oxide[5,11] and the solution, after being diluted with Me_2SO, was treated with methylsulfinyl carbanion and methyl iodide, according to the method of Hakomori[12]. The acid hydrolyzate of the methylated T-3-M was analyzed as the alditol acetate derivatives[9] by g.l.c. and g.l.c.–mass spectrometry (g.l.c.–m.s.). The methylation analysis showed the presence of 2,4,6-tri-*O*-methyl-D-mannopyranose (*m/z*: 43, 45, 87, 101, 117, 126, 161, and 233)[13] with a negligible proportion of 2,3,4,6-tetra-*O*-methyl-D-mannose. An attempt was made to oxidize T-3-M with 2.5mM sodium metaperiodate for 19 days, but the periodate consumption per hexosyl residue was negligibly small (<0.03 mol.). The foregoing data indicated that the mannan T-3-M has a linear chain composed of α-(1→3)-linked D-mannopyranosyl residues.

On the other hand, the polysaccharide (T-3-M′), obtained by treatment of fraction T-3-GM with exo-(1→3)-β-D-glucanase (from Basidiomycetes QM-806), was composed of mannose (~96%) and traces of glucose and galactose (by g.l.c.). T-3-M′ showed characteristic absorbance at 825 cm^{-1} in its i.r. spectrum, suggesting the presence of α-D-glycosidic linkages, as in T-3-M, in addition to absorption bands at 1250 and 1725 cm^{-1} that indicated the presence of *O*-acyl groups in the molecule. (Such absorption bands were not observed in the i.r. spectrum of T-3-M, because it was prepared by alkali treatment of T-3-GM.) The acidic compound arising from the acyl groups in T-3-M′ was identified as acetic acid by g.l.c. of the saponification product in a procedure similar to that previously described[2], and the total acetyl content was determined to be ~1.8%. Furthermore, from the results of carbon-13 nuclear magnetic resonance (^{13}C-n.m.r.) spectroscopy of T-3-M′ (in Me_2SO-d_6), the presence of *O*-acetyl groups was confirmed by signals at 20.5 (methyl carbon) and 170.3 p.p.m. (carbonyl carbon) in the spectrum[3], as shown in Fig. 3. In addition, the resonance at 101.6 p.p.m. would correspond to the anomeric carbon atoms of D-mannopyranosyl residues in T-3-M′, and the $^1J_{CH}$ value of 171 Hz indicated that the mannosyl residues had the α configuration (see ref. 14). The signals at 78.5, 73.6, 69.4, 65.9, and 61.4 p.p.m. could be assigned to C-3, C-5, C-2, C-4, and C-6 of α-(1→3)-linked D-mannopyranosyl residues. Signals at 63.4 [due to C-6 atoms of 6-*O*-acetylated, α-(1→3)-linked D-mannopyranosyl residues] and 70.6 p.p.m. [C-5 of 6-*O*-acetylated, α-(1→3)-linked D-mannopyranosyl residues] influenced by 6-*O*-substitution[3,15,16] were observed (see Fig. 3). On addition of

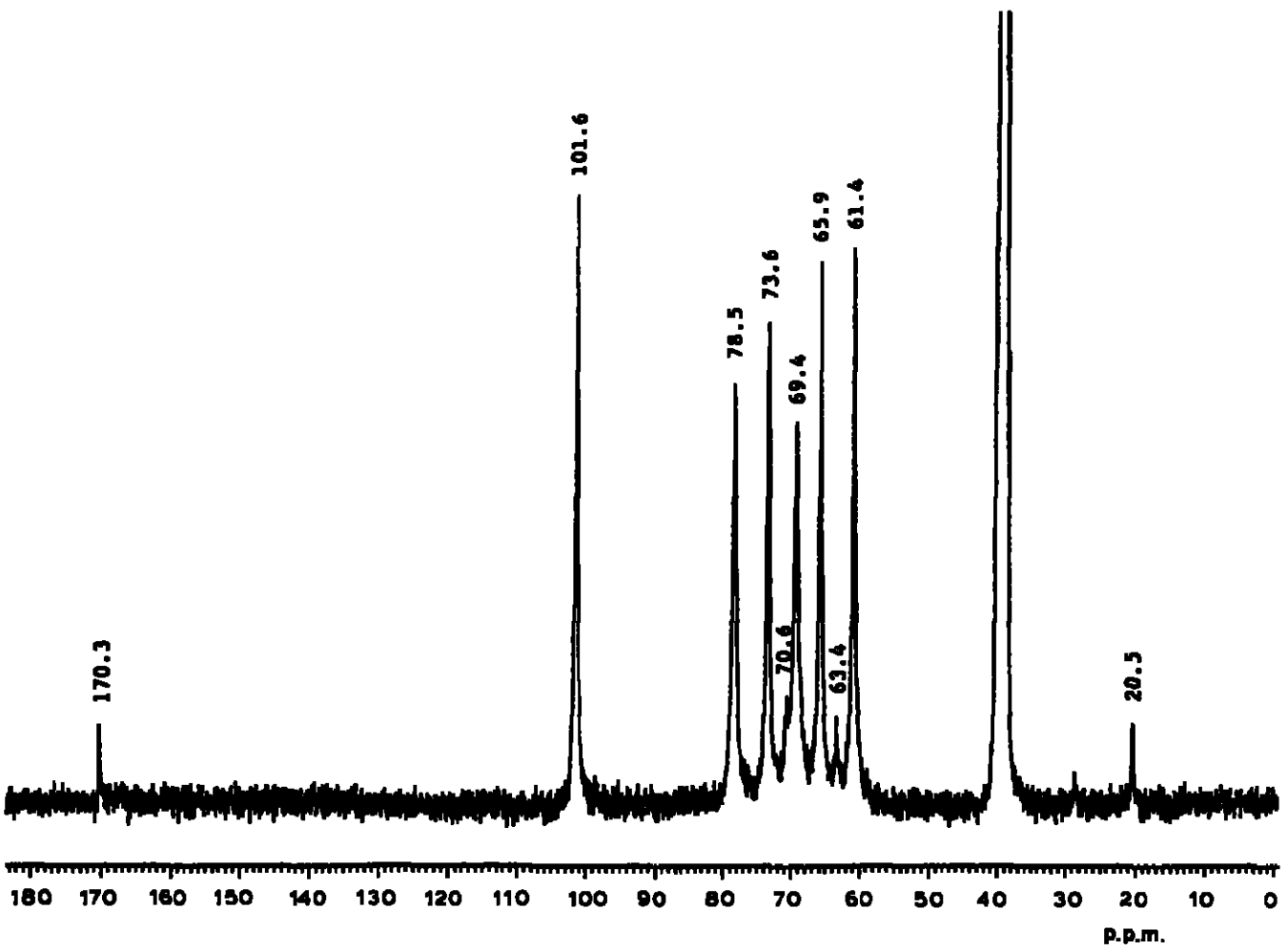

Fig. 3. ^{13}C-N.m.r. spectrum of T-3-M′ in Me_2SO-d_6 at 60°.

NaOD, these signals at 63.4 and 70.6 p.p.m. disappeared, and the signals at 20.5 (CH_3 of *O*-acetyl group) and at 170.3 p.p.m. (CO of *O*-acetyl group) shifted to lower magnetic field, at 25.0 (CH_3 of CH_3CO_2Na) and 175.1 p.p.m. (CO of CH_3CO_2Na), respectively.

From the results just mentioned, the α-(1→3)-linked D-mannan (T-3-M) isolated by alkali treatment of fraction T-3-GM from a hot-water extract of the fruit bodies of *D. indusiata* can be regarded as the deacetylated form of T-3-M′. Therefore, it is considered that T-3-M′ having *O*-acetyl groups in the molecule is close to being the naturally occurring product.

Previously, we reported the structural features[2,3] and the chain conformation (by the X-ray diffraction method)[17] of a new type of partially *O*-acetylated α-D-mannan (T-2-HN) isolated, prior to extraction with hot water, from a hot, 70% aqueous ethanol extract of this fungus, and reported that, on deacetylation of the native mannan T-2-HN (water- or Me_2SO-soluble) with alkali, the product was insoluble in these solvents. T-3-M′ gradually dissolved in water or Me_2SO, but T-3-M did not. The solubility of T-3-M′ in these solvents is attributable to the presence of *O*-acetyl groups, as already described[2,3] in the case of T-2-HN. The proportion (~1.8%) of *O*-acetyl groups in T-3-M′ is, however, less than that (11.4%)[2] in T-2-HN. Furthermore, the earlier work indicated that T-2-HN is composed of a linear chain of α-(1→3)-linked D-mannopyranosyl residues (~97%). Accordingly, as may

be seen from the results of the methylation analysis of T-3-M, it has almost the same chemical structure as that of the sugar units in T-2-HN, although T-2-HN contains small proportions of internal, (1→6)-linked D-mannosyl residues and branching points at O-2 or O-4 on each of the (1→3)-linked D-mannosyl residues[2]. Moreover, the ^{13}C-n.m.r. data, as in the case[3] of T-2-HN, might suggest that the *O*-acetyl groups are located partly at O-6 of the α-(1→3)-linked D-mannopyranosyl residues in T-3-M′. In addition, the value of the specific rotation ($[\alpha]_D^{17}$ +119.2°, 2M sodium hydroxide) of T-3-M was almost the same as those of T-2-HN ($[\alpha]_D^{20}$ +116°, water) and the deacetylated T-2-HN ($[\alpha]_D^{27}$ +119°, 2M sodium hydroxide)[2]. However, the molecular weight ($\overline{M}_w$: ~1.9 × 10^5) of T-3-M was smaller than those[3] of T-2-HN (~6.2 × 10^5) and its deacetylated form (~5.5 × 10^5). Thus, it is suggested that a partially *O*-acetylated (1→3)-α-D-mannan (T-3-M′) isolated from a hot-water extract had failed to be extracted with hot, 70% aqueous ethanol because of the difference in the acetyl content between T-3-M′ and T-2-HN, although the chemical structures are similar to each other, except for their respective molecular weight.

EXPERIMENTAL

Materials. — The dried fruit-bodies of *D. indusiata* are commercially available in Hong Kong. A solution of exo-(1→3)-β-D-glucanase from Basidiomycetes (166 μmol of D-glucose produced/min/mL) was donated by Taito Co. Ltd. 4-Methylmorpholine *N*-oxide was purchased from ICN Pharmaceuticals, Inc. Sepharose CL-2B and standard dextrans were purchased from Pharmacia Fine Chemicals.

General methods. — Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. I.r. spectra were recorded with a JASCO A-102 spectrometer. Ultracentrifugal analysis was conducted at 20° in 2.5M sodium hydroxide with a MOM 3170/b analytical ultracentrifuge at 60,000 r.p.m. P.c. was performed by the double-ascending method, using Toyo Roshi No. 51A filter-paper and the following solvent systems (v/v): 6:4:3 1-butanol–pyridine–water and 10:4:3 ethyl acetate–pyridine–water. Sugars were detected with an alkaline silver nitrate reagent. G.l.c. was performed in a Shimadzu GC-4CM apparatus equipped with a flame-ionization detector. For sugar analyses, a glass column (0.3 × 200 cm) packed with 3% of ECNSS-M on Gaschrom Q (100–120 mesh) was used, with nitrogen as the carrier gas at a flow rate of 45 mL/min, at 183° (for alditol acetates) or 168° (for methylated alditol acetates), and, for acetic acid analyses, a column (0.3 × 150 cm) packed with Chromosorb 101 (80–100 mesh), at 170° and a flow rate of nitrogen of 40 mL/min. Peak areas were measured with a Shimadzu E1A Chromatopac. G.l.c.–m.s. was conducted with a JEOL JMS-D 300 apparatus equipped with a glass column (0.2 × 100 cm) packed with 3% of ECNSS-M, at 185°, at a pressure of helium of 127.5 kPa. The mass spectra were recorded under the conditions previously reported[3].

Isolation of the polysaccharide. — A water extract of the fruit bodies (100 g) of *D. indusiata*, after proteins had been removed, was subjected to ethanol precipitation. The resulting precipitate was dissolved in water, and the solution was frozen and allowed to thaw[8] at ~5°. Gelatinous, cold-water-insoluble material (T-3-GM) was formed in the solution. The material was collected by centrifugation, dissolved in hot water, reduced with sodium borohydride, and then treated, under a nitrogen atmosphere, with 0.1M sodium hydroxide. The mixture was dialyzed, and the water-insoluble material (T-3-M) formed in the inner solution was collected, and dried *in vacuo* (yield, ~1 g). These processes were reported in detail in a preceding paper[4].

A portion (115 mg) of T-3-GM was dissolved in 0.1M McIlvaine buffer (36 mL, pH 4.0), and the solution was treated with exo-(1→3)-β-D-glucanase (0.5 mL) for 46 h at 38°. After being heated for 10 min at 100°, the mixture was dialyzed, centrifuged to remove a small proportion of insoluble materials formed in the inner solution, and then lyophilized, to afford the undigested polysaccharide (T-3-M′) as colorless flakes (yield, 71 mg).

Gel filtration and estimation of molecular weight. — Gel filtration of T-3-M (1.5 mg/0.7 mL) and standard dextrans on a column of Sepharose CL-2B, with 2M sodium hydroxide as the eluant, was performed as previously reported[3]. A calibration curve was constructed by use of Dextran T-500 (mol. wt., 495,000), T-250 (253,000), and T-150 (154,000), and therefrom, the molecular weight was estimated.

Analysis of component sugars. — The polysaccharide fractions (T-3-M and T-3-M′) were hydrolyzed with 90% formic acid (9 h, 100°), and then with 0.25M sulfuric acid (15 h, 100°). The specific rotation of the hydrolyzate of T-3-M was $[\alpha]_D^{21}$ +12.8° (*c* 0.082 as mannose, 0.05M sulfuric acid). Authentic D- and L-mannose gave the following results: D-mannose, $[\alpha]_D^{16}$ +14.0° (equil., *c* 0.139, 0.05M sulfuric acid); L-mannose, $[\alpha]_D^{21}$ −13.9° (equil., *c* 0.215, 0.05M sulfuric acid). Then the hydrolyzate was made neutral with barium carbonate in the usual way, and analyzed by p.c., and by g.l.c. as the corresponding alditol acetates[9].

Methylation analysis. — T-3-M (5 mg) in 4-methylmorpholine *N*-oxide (500 mg) was heated, with stirring, for 3 h at 120° in a tightly stoppered tube under nitrogen[5,11]. After complete solubilization of the sample, Me_2SO (4 mL) was added, the mixture was cooled to room temperature, and the polysaccharide was methylated twice by the Hakomori procedure[12]. The fully methylated product was hydrolyzed with 90% formic acid and 0.25M sulfuric acid, as already described, and the partially methylated sugars thus obtained were analyzed by g.l.c. and g.l.c.–m.s. as the corresponding alditol acetates[9].

Periodate oxidation. — T-3-M (7 mg) was suspended in 2.5 mM sodium metaperiodate (40 mL) and oxidized, with stirring, for 19 days at 3–6° in the dark. The periodate consumption was measured by the spectrophotometric method (290 nm) used by Ikenaka[18].

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra were recorded with a

JEOL-FX 270 spectrometer in the Fourier-transform mode, with complete proton-decoupling, for a solution in Me_2SO-d_6 (~40 mg/mL) in a 4-mm tube at 60°. The chemical shifts were obtained by the use of tetramethylsilane as an external standard.

Identification of the acetyl group. — T-3-M′ (20 mg) was suspended in methanol (3 mL) containing 0.14M sodium methoxide, and allowed to stand for 5 h at room temperature. The resulting supernatant liquor and washings obtained by centrifugation were combined, and evaporated to dryness *in vacuo*. The residue was dissolved in water (0.3 mL), and the solution was made acid by the addition of 4M hydrochloric acid (20 μL), and then analyzed by g.l.c.[2]. Only one peak was observed, and the retention time (2.5 min) was identical with that of acetic acid.

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