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Studies on the Metabolism of Unsaturated Fatty Acids. I. Synthesis and Identification of 6-Hydroxydodec-3-*cis*-enoic Acid, the Final Metabolite of Ricinoleic Acid by *Escherichia coli* K-12

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6-Hydroxydodec-*cis*-3-enoic acid was synthesized to identify a metabolite of ricinoleic acid by *Escherichia coli* and to use it as substrate in the investigation on unsaturated fatty acid metabolism.

In a total synthesis of the 6-hydroxy acid, the key step involved the reaction of the trialkynylborane with ethyl diazoacetate. Dec-1-yn-4-ol was lithiated after protection of the hydroxyl group with dihydropyran, and was transformed into the trialkynylborane by treating with boron trifluoride etherate. The subsequent reaction with ethyl diazoacetate afforded the 6-hydroxy-3-ynoate, which was hydrogenated over Lindlar catalyst to yield 6-hydroxydodec-3-*cis*-enoic acid.

The results obtained by gas chromatography–mass spectrometry (GC–MS) showed that the final metabolite of ricinoleic acid by *E. coli* was identical with the synthetic 6-hydroxy acid.

Keywords——metabolism of ricinoleic acid; *Escherichia coli*; 6-hydroxydodec-3-*cis*-enoic acid; ethyl 6-hydroxydodec-3-ynoate; 4-(tetrahydro-2-pyranyloxy)-1-decyne; GC–MS

It has been shown that C_2 cleavages of hydroxy fatty acids by *Escherichia coli* ceased when the total chain length of a hydroxy acid becomes twelve to ten and, at the same time, the hydroxyl group is located on the sixth, fifth or fourth carbon atom of the acid.[2] When ricinoleic (12-hydroxyoctadec-9-*cis*-enoic) acid, the main component of castor oil, was added in a medium containing meat extracts as exclusive nutrients, this acid was converted by *E. coli* successively to 10-hydroxyhexadec-7-*cis*-enoic (I), 8-hydroxytetradec-5-*cis*-enoic (II) and 6-hydroxydodec-3-*cis*-enoic (III) acids as shown in Chart 1.[3] Compounds II and III accumulated in the medium and the further degradation of III by *E. coli* was negligible. The structure of III was proposed by elemental analysis and by some chemical means such as catalytic hydrogenation and the degradation according to the method of von Rudloff,[4] however, its identification with the synthetic sample has not been tried. In order to elucidate the discontinuance of the degradation, III is a valuable substance as substrate, although it is not easy to isolate a considerable amount of III from a mixture of the metabolites of

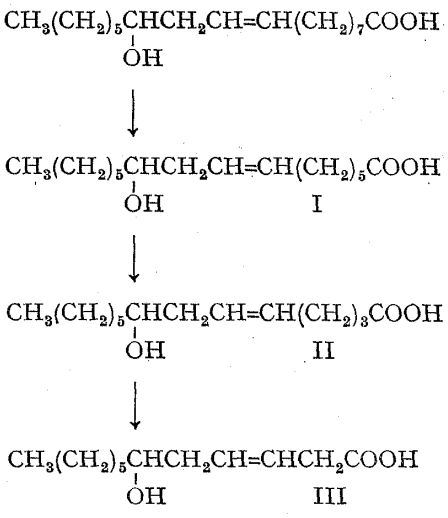

Chart 1

1) Location: *Aobayama, Sendai 980, Japan.*
2) M. Mizugaki, M. Yonaha, M. Uchiyama, and S. Okui, *J. Biochem.*, **63**, 390 (1968).
3) S. Okui, M. Uchiyama, and M. Mizugaki, *J. Biochem.*, **53**, 265 (1963).
4) E. von Rudloff, *J. Am. Oil Chemists' Soc.*, 33, 126 (1956).

ricinoleic acid. Therefore, the synthesis of III is desirable for the identification and for the utilization as substrate in our investigation on the fatty acid metabolism.

Experimental

Ethyl Dodec-3-ynoate (IV)——To a solution of 1-decynyllithium (from 1-decyne (6.21 g, 45 mmol) and *n*-butyllithium (45 mmol)) in tetrahydrofuran (35 ml) was gradually added an ethereal solution of boron trifluoride etherate (60 mmol) at a temperature not exceeding −20°. The resulting solution was stirred an additional 1 hr at −20°, then ethyl diazoacetate (2.21 g, 19.2 mmol) was slowly added into this solution. After stirring the mixture for 1.5 hr at −20°, and for 0.5 hr below 0°, the mixture was poured into ice-water. The aqueous phase was extracted with ether and the organic extract was dried over Na_2SO_4. The solvent was removed and the residue was distilled under reduced pressure to afford a colorless liquid, bp 110—115° (3 mmHg), yield 2.45 g (73%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1735. NMR (CCl_4, δ): 0.88 (3H, broad t, J=6.0 Hz), 1.14—1.85 (15H, m), 1.95—2.31 (2H, m), 3.07 (2H, t, J=2.5 Hz), 4.10 (2H, q, J=7.0 Hz). *Anal.* Calcd. for $C_{14}H_{24}O_2$: C, 74.95; H, 10.78. Found: C, 74.96; H, 10.61.

Ethyl 6-(Tetrahydro-2-pyranyloxy)dodec-3-ynoate (VI)——According to a similar manner as above, ethyl diazoacetate (0.74 g, 6.4 mmol) was added to a tetrahydrofuran (20 ml) solution of 4-(tetrahydro-2-pyranyloxy)-1-decyne (V) (3.51 g, 15 mmol), *n*-butyllithium (15 mmol) and boron trifluoride etherate (2.84 g, 20 mmol). The crude product was purified by SiO_2 column chromatography using 0—30% ether in *n*-hexane as eluants to give a colorless liquid, yield 1.13 g (70%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740. NMR (CCl_4, δ): 0.88 (3H, broad t, J=6.0 Hz), 1.04—2.08 (19H, m), 2.15—2.60 (2H, m), 3.10 (2H, t, J=2.5 Hz), 3.40—3.90 (3H, m), 4.12 (2H, q, J=7.2 Hz), 4.40—4.90 (1H, m).

Methyl 6-Hydroxydodec-3-ynoate (VII)——A mixture of VI (2.0 g, 6.3 mmol), 20% H_2SO_4 (10 ml) and methanol (45 ml) was refluxed for 1.5 hr, concentrated under reduced pressure, extracted with ether, and the solution was dried over Na_2SO_4. The solvent was removed and the residue was purified by SiO_2 column chromatography using 0—30% ether in *n*-hexane as eluants to give a colorless liquid, yield 0.92 g (65%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3700—3200, 1740. NMR (CCl_4, δ): 0.88 (3H, broad t, J=6.0 Hz), 1.30 (10H, broad s), 2.10—2.40 (3H, m), 3.15 (2H, t, J=2.5 Hz), 3.40—3.80 (1H, m), 3.68 (3H, s).

Methyl 6-Hydroxydodec-3-*cis*-enoate (VIII)——A mixture of Lindlar catalyst (0.3 g), quinoline (3 drops) and methanol (50 ml) was shaken in a stream of hydrogen until absorption of hydrogen ceased. To this mixture VII (0.92 g, 4.1 mmol) was added and the mixture was shaken in a stream of hydrogen, until one molar equivalent of hydrogen was absorbed. After the catalyst was filtered, the filtrate was concentrated to dryness under reduced pressure. The residue was dissolved in ether, washed with 3 N HCl, and dried over Na_2SO_4. The solvent was removed to give a pale yellow liquid which was purified by SiO_2 column chromatography, yield 0.89 g, (97%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3700—3280, 1745. NMR (CCl_4, δ): 0.92 (3H, broad t, J=6.0 Hz), 1.30 (10H, broad s), 1.90—2.50 (3H, m), 3.00—3.20 (2H, m), 3.40—3.90 (1H, m), 3.70 (3H, s), 5.40—5.90 (2H, m).

6-Hydroxydodec-3-*cis*-enoic Acid (IX)——To a chilled solution of VIII (0.89 g, 3.9 mmol) and methanol (1 ml), was added 3 N NaOH (6 ml) and the mixture was allowed to stand overnight at room temperature. The methanol was removed and the aqueous solution was washed with ether, neutralized with 3 N HCl, extracted with ether and the solution was dried over Na_2SO_4. The solvent was removed to give a pale yellow liquid which was purified by SiO_2 column chromatography, yield 0.84 g (98%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3420, 1720. NMR (CCl_4, δ): 0.92 (3H, broad t, J=6.0 Hz), 1.30 (10H, broad s), 2.10—2.50 (2H, m), 2.90—3.30 (2H, m), 3.50—3.80 (1H, m), 5.40—5.80 (2H, m), 6.00—6.40 (2H, broad s).

High Resolution Mass Spectrometry——A JMS-01 SG-2 was employed. The current was 250 μA, the ionizing voltage was 75 eV, the accelerating voltage was 9.0 kV, the sample temperature was 55° and the chamber-temperature was 60°.

Gas Chromatography-Mass Spectrometry (GC-MS)——A Shimadzu-LKB-9000 system was employed. A glass coil column (1 m) packed with 1.5% OV-101 on 80/100 mesh chromosorb W-HP was used. The column temperature was 150°, the temperature of the ion source, injection port and separator was 230°, the ionizing current was 60 μA, the ionizing voltage was 20 eV and the accelerating voltage was 1.75 kV.

Results and Discussion

Synthesis of 6-Hydroxydodec-3-*cis*-enoic Acid

An introduction of a C_2 group into an ω-acetylenic compound is a common method in the stereoselective preparation of 3-*cis*-alkenoic acids. For instance, the Grignard reaction of 1-alkynylmagnesium bromide with ethylene oxide affords 3-alkyn-1-ol. Subsequent oxidation of the unsaturated primary alcohol with chromium trioxide gives the corresponding alkynoic

acid, which is readily converted to the 3-*cis*-alkenoic acid by catalytic hydrogenation over Lindlar catalyst.

In our case, however, the Grignard reagent prepared from 4-(tetrahydro-2-pyranyloxy)-1-decyne (V) with ethylmagnesium bromide did not react smoothly with ethylene oxide. Consequently, it is not suitable to employ this method for the preparation of III and its homologues.

On the other hand, Hooz *et al.*[5] have reported a convenient synthesis of ethyl 3-alkynoates *via* trialkynylboranes. 1-Alkynyllithium was treated with boron trifluoride etherate and the resulting trialkynylborane reacted with ethyl diazoacetate to provide ethyl 3-alkynoate.

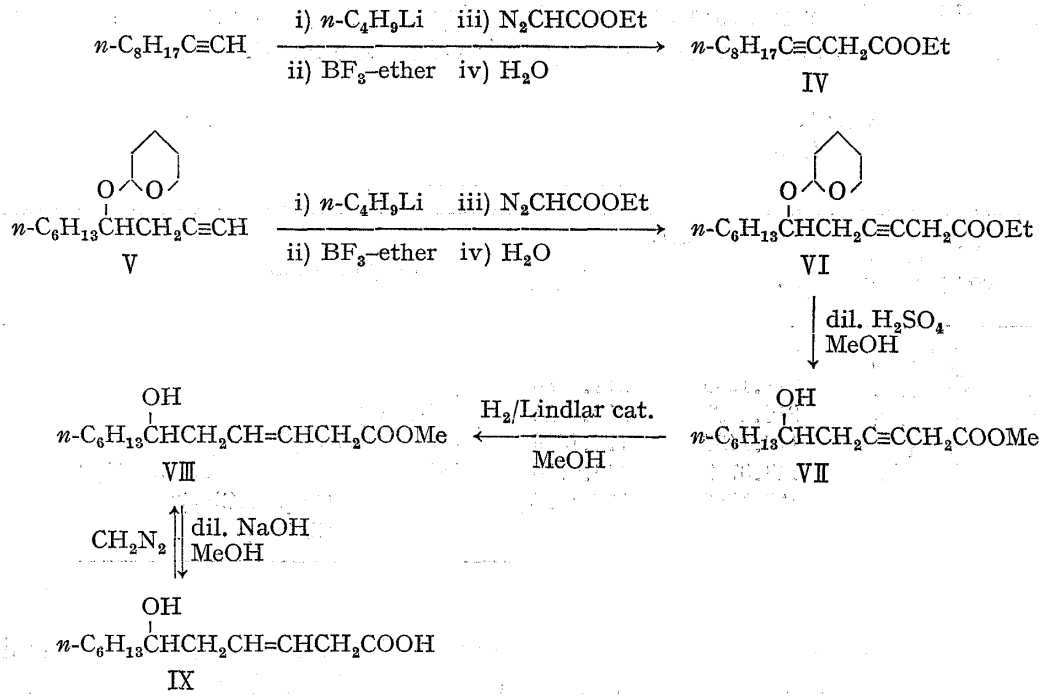

Chart 2

To apply this method to the preparation of III, as a preliminary experiment, 1-decyne was employed as a starting material, which was lithiated with *n*-butyllithium, and the resulting 1-alkynyllithium was treated with boron trifluoride etherate, then with ethyl diazoacetate to afford ethyl 3-dodecynoate (IV) finally in a good yield. Then 4-tetrahydropyranyloxy-1-decyne (V) prepared according to the method reported by Crombie *et al.*[6] was treated in a similar manner as above to give a colorless liquid (VI) which showed a single peak on a gas chromatogram (stationary phase: diethyleneglycol succinate polyester). The infrared (IR) spectrum of VI exhibits an ester carbonyl band at 1740 cm^{-1}, and its nuclear magnetic resonance (NMR) spectrum indicates the presence of an α-methylene group (3.10 ppm, t, J=2.5 Hz) and an ethoxyl group along with a tetrahydropyranyloxy group. Based on these observations, the structure of ethyl 6-tetrahydropyranyloxydodec-3-ynoate has been assigned to VI.

By treatment with dilute sulfuric acid in methanol at room temperature VI was transformed into the methyl ester (VII). The IR spectrum of VII shows a broad band around 3400 cm^{-1} due to a hydroxyl group as well as an ester carbonyl band at 1740 cm^{-1}. In the NMR spectrum of VII, signals due to a tetrahydropyranyloxy group are not observed and multiplet signals at 2.10—2.40 ppm decreased their intensity by one proton by treatment with heavy water. Subsequently VII was partially hydrogenated in the presence of Lindlar

5) J. Hooz and R.B. Layton, *Can. J. Chem.*, **50**, 1105 (1972).
6) L. Crombie and A.G. Jacklin, *J. Chem. Soc.*, **1955**, 1974.

catalyst. There was observed a decrease in the rate of the catalytic hydrogenation after one molar equivalent of hydrogen was absorbed and methyl 6-hydroxydodec-3-*cis*-enoate (VIII) was obtained in a good yield. The NMR spectrum of VIII exhibits signals at 5.40—5.90 ppm due to two olefinic protons.

Hydrolysis of VIII with dilute sodium hydroxide at room temperature gave the corresponding carboxylic acid (IX) which was quantitatively returned to VIII by treatment with diazomethane. This indicated that during the alkaline hydrolysis of VIII to IX there was no isomerization on the *cis* double bond.

High Resolution Mass Spectrometry of the Synthetic Sample

The results of high resolution mass spectrometry of VIII are consistent its structure as shown in Table I, in which several important *m/e* values are listed.

Table I. Several Typical Fragment Ions of Synthesized Methyl (±)-6-Hydroxydodec-3-*cis*-enoate obtained by High Resolution Mass Spectrometry

Elemental composition	*m/e* Calculated	*m/e* Observed	Fragment ion
$C_{13}H_{24}O_3$	228.1727	228.1717	M^+
$C_{13}H_{23}O_2$	211.1699	211.1684	$[M-OH]^+$
$C_{13}H_{22}O_2$	210.1621	210.1621	$[M-H_2O]^+$
$C_{12}H_{19}O$	179.1437	179.1429	$[M-H_2O-OCH_3]^+$
$C_7H_{11}O_3$	143.0709	143.0710	$[HOCHCH_2CH{=}CHCH_2COOCH_3]^+$
$C_8H_{17}O$	129.1280	129.1293	$[CH_3(CH_2)_5CH(OH)CH_2]^+$
$C_7H_{15}O$	115.1124	115.1137	$[CH_3(CH_2)_5CHOH]^+$

GC-MS for Identification

A gas chromatogram of methyl esters of the metabolic mixtures of ricinoleic acid by E. *coli* is illustrated in Fig. 1. Four remarkable peaks A, B, C and D corresponded to methyl

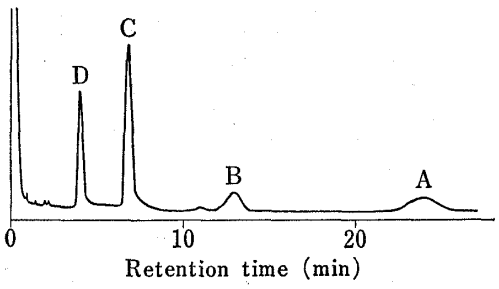

Fig. 1. A Gas Chromatogram of Methyl Esters of the Metabolic Mixtures of Ricinoleic Acid by *E. coli*

A: methyl ricinoleate, B: methyl 10-hydroxyhexadec-7-*cis*-enoate, C: methyl 8-hydroxytetradec-5-*cis*-enoate, D: methyl 6-hydroxydodec-3-*cis*-enoate. A Shimadzu GC-6A gaschromatograph (FID) was employed. A glass column (1 m) packed with 10% diethyleneglycol succinate polyester was used. The column temperature was 190°; the temperature of the injection port and detector was 250°, the carrier gas (N_2) flow rate was 60 ml/min.

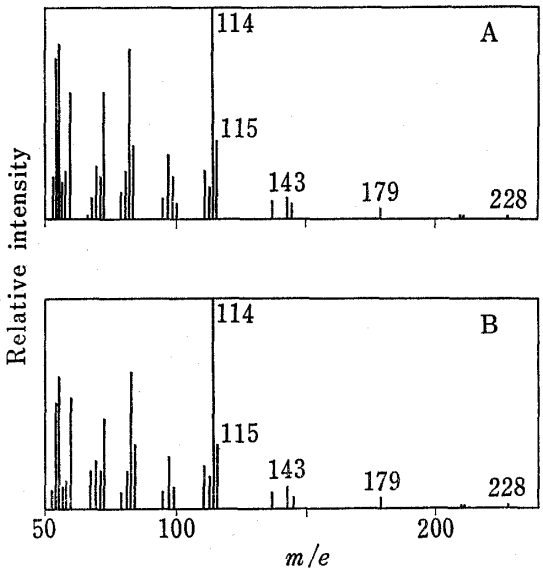

Fig. 2. GC-MS Spectra of Methyl Ester of the Metabolite III (A) and the Authentic Sample VIII (B)

Operating conditions are described in the experimental part.

esters of ricinoleic acid, I, II and III, respectively. A mass spectrum of Peak D obtained by GC-MS with chemical ionization (reactant gas: isobutane) showed the quasi-molecular ion $(M+1)^+$ at *m/e* 229. The synthetic sample VIII was shown to have the same retention time as that of peak D under the same conditions by gas chromatography.

Finally identity of the metabolite III and the synthetic sample was extablished by comparison of mass spectra. GC-mass spectra of methyl ester of III and the authentic sample (VIII) are shown in Fig. 2-A and -B, respectively. The concordant results obtained by GC-MS exhibited clearly that both are identical, except the metabolite III is optically active,[2] whereas the synthetic sample is optically inert.

Although IX is racemic 6-hydroxydodec-3-*cis*-enoic acid, the use of IX as substrate does not give rise to any disturbance in our plan to elucidate the phenomenon that a medium chain fatty acid containing a hydroxyl group on a special position is not degraded by *E. coli*, because our earlier investigation has shown that this phenomenon was similarly observed not only in the case of optically active metabolites but also in the case of optically inactive metabolites.[2,3,7]

A new favorable method has been established for preparations of 3-unsaturated hydroxy fatty acids in this experiment.

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7) M. Mizugaki, M. Uchiyama, and S. Okui, *J. Biochem.*, **58**, 273 (1965).