Synthesis of Ubiquinone-7 Metabolites*

Masazumi Watanabe, Isuke Imada, and Hiroshi Morimoto

ABSTRACT: XI, XIX, XXVI, XXIX, and XXXI were synthesized to confirm the structures of ubiquinone metabolites. 3,4,5-Trimethoxytoluene (I) was condensed with methylsuccinic anhydride (II) to give III, which was then subjected to the Clemmensen reduction. The resulting X was oxidized to DL-2,3-dimethoxy-5-methyl-6-(3′-carboxy-3′-methylpropyl)-1,4-benzoquinone (XI). The acid chloride XIV was converted into the acylmalonate (XV or XVI), then they were decarboxylated to DL-2,3-dimethoxy-5-methyl-6-(3′-methyl-4′-oxopentyl)-1,4-benzoquinone (XIX). 6-Acetoxy-4-methyl-4-hexenal (XXI) was oxidized with argentic oxide to the carboxylic acid XXII. The methyl ester XXIII was condensed with 2,3-dimethoxy-5-methyl-1,4-benzohydroquinone (XXIV) in the presence of boron trifluoride to give *trans,cis*-2,3-dimethoxy-5-methyl-6-(3′-methyl-5′-methoxycarbonyl-2′-pentenyl)-1,4-benzoquinone (XXV), which was then carefully hydrolyzed to *trans,cis*-2,3-dimethoxy-5-methyl-6-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-benzoquinone (XXVI). 2,3-Dimethoxy-5-methyl-6-carboxymethyl-1,4-benzoquinone (XXIX) and 2,3-dimethoxy-5-methyl-6-(3′-carboxy-3′-methylpropyl)-1,4-benzohydroquinone disulfuric acid (XXXI) were derived from ubiquinone-7 and XI, respectively.

New ubiquinone metabolites (XI, XIX, XXVI) and one of their conjugates (XXXI) have been isolated by the authors (Imada *et al.*, 1970) from the excrements and tissues of animals (rats, rabbits) dosed with ubiquinone-7 and their structures have been presumed from physicochemical data. In the present paper, confirmation of their structures by syntheses is described.

DL-2,3-Dimethoxy-5-methyl-6-(3′-carboxy-3′-methylpropyl)-1,4-benzoquinone (XI) and Its Ester XII. 3,4,5-Trimethoxytoluene (I) was allowed to react at room temperature with methylsuccinic anhydride (II) in nitrobenzene in the presence of aluminum chloride by the Friedel–Crafts condensation (Mitter and De, 1939). In this reaction, 2-methyl-3-(3′,4′-dimethoxy-2′-hydroxy-6′-methylbenzoyl)propionic acid (III) was obtained in 26% yield. The position of the hydroxyl in III was confirmed by nuclear magnetic resonance and infrared spectra, which showed the presence of a hydrogen bond between the hydroxyl and the carbonyl. In addition to III, IV was obtained as pale yellow crystals in 7% yield by this reaction. IV was confirmed to be 2-methyl-3-(2′,3′-dihydroxy-4′-methoxy-6′-methylbenzoyl)propionic acid by spectral data and its derivatives (V to IX). 2-Methyl-4-(3′,4′-dimethoxy-2′-hydroxy-6′-methylphenyl)butanoic acid (X) was obtained by the Clemmensen reduction of III. X was then oxidized by the method of Teuber and Jellinek (1952) with potassium nitrosodisulfonate in aqueous alkali to give DL-2,3-dimethoxy-5-methyl-6-(3′-carboxy-3′-methylpropyl)-1,4-benzoquinone (XI) after silicic acid column chromatography. Oxidation of X with hydrogen peroxide in acetic acid also gave XI, but the yield was not better than the former due to the formation of by-products. Changing the reaction order, III was first oxidized with potassium nitrosodisulfonate to give 2,3-dimethoxy-5-methyl-6-(3′-carboxy-3′-methyl-1′-oxopropyl)-1,4-benzoquinone (XIII) and then reduced by amalgamated zinc. Oxidation of the resulting hydroquinone afforded XI. Thus, the desired compound was synthesized by two different routes, of which the latter resulted in a lower yield. XI was treated with diazomethane to give DL-2,3-dimethoxy-5-methyl-6-(3′-methyl-3′-methoxycarbonylpropyl)-1,4-benzoquinone (XII). XII thus obtained was coincided with the methyl ester of a ubiquinone metabolite by thin-layer chromatographic R_F values, gas chromatographic retention time, and spectral data. The structure of the metabolite was thus established but the metabolite is optically active with regard to the 3′ position as described in the previous report (Imada *et al.*, 1970).

DL-2,3-Dimethoxy-5-methyl-6-(3′-methyl-4′-oxopentyl)-1,4-benzoquinone (XIX). When XI was treated with oxalyl chloride, the reaction proceeded with the evolution of gas to yield the acid chloride XIV. XIV was added to a solution of magnesium methoxy- or ethoxymalonate in benzene without purification, and then heated for 30 min to give acylmalonate (XV or XVI). When a solution of the acylmalonate (XV or XVI) in acetic acid was heated in the presence of 0.22% sulfuric acid for 7 hr (Bowman, 1950), DL-2,3-dimethoxy-5-methyl-6-(3′-methyl-4′-oxopentyl)-1,4-benzoquinone (XIX) was obtained from XV in 14% yield and from XVI in 11% yield accompanied by the keto esters (XVII in 49% or XVIII in 33%) which were formed by partial decarboxylation of XV or XVI, respectively. When a solution of XV in toluene was heated with hydrochloric acid, XIX was formed in one step in a satisfactory yield. XIX was also obtained from XVII under the same conditions. XIX exhibited an infrared absorption due to a carbonyl and a nuclear magnetic resonance signal due to a methyl ketone. In its mass spectrum, an intense molecular ion peak (M) and a fragment peak (M − $COCH_3$) were observed. These data, together with an ultraviolet absorption maximum and thin-layer chromatographic R_F values, coincided with those of a ubiquinone metabolite. Therefore, the structure of the metabolite was established as XIX except that the metabolite is an optically active compound with regard to the 3′ position, like XI.

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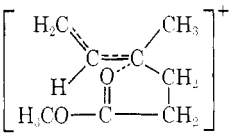
trans,cis-2,3-Dimethoxy-5-methyl-6-(5'-carboxy-3'-methyl-2'-pentenyl)-1,4-benzoquinone (XXVI). The hydrolysis of the conjugate of a ubiquinone metabolite to the acid XXVI is very difficult since lactonization of the acid to 2,3-dimethoxy-5-methyl-6-(5'-carboxy-3'-hydroxy-3'-methylpentyl)-1,4-benzoquinone lactone (XXVII) readily occurs, as described in the previous report (Imada *et al.*, 1970). Accordingly the authors obtained the methyl ester by treatment of the rats' urine with methanolic hydrochloric acid and attempted to identify the metabolite as 2,3-dimethoxy-5-methyl-6-(3'-methyl-5'-methoxycarbonyl-2'-pentenyl)-1,4-benzoquinone (XXV). From the nuclear magnetic resonance spectrum of the methyl ester of the metabolite, it was determined to be the *trans* isomer with regard to the double bond. Stork *et al.* (1969) and Corey *et al.* (1969) ozonolyzed selectively an isopropenyl group of easily obtainable geranyl acetate XX to the aldehyde XXI. This is suitable as a starting material for the synthesis of *trans* XXV. Ozonolysis of XX was carried out by bubbling ozonized oxygen through the methanolic solution under cooling at −78°. The resulting ozonide was then reductively cleaved with dimethyl sulfide. The reaction mixture was distilled under reduced pressure under nitrogen to give XXI.

In general, γ,δ-unsaturated carboxylic acids are known to be lactonized readily by heating or by treating with mineral acid (reviewed by Kröper, 1963). On the other hand, the alcohol formed by hydrolysis of XXI may be susceptible to allylic rearrangement or to oxidation of the alcohol moiety. Therefore, XXI should be oxidized carefully in a neutral medium under as mild conditions as possible. Using the procedure which Corey *et al.* (1968) used for the oxidation of dodecanal and 3-cyclohexenylcarboxaldehyde, XXI was oxidized in tetrahydrofuran–water (9:1) at room temperature with argentic oxide which was prepared from silver oxide and potassium permanganate. The yield of the desired compound was, however, not satisfactory because of the slow rate of reaction, but it was improved by heating at 70° for 6 hr. The resulting mixture was carefully acidified with hydrochloric acid under ice cooling, extracted with ethyl acetate, and then purified by silicic acid column chromatography to give 6-acetoxy-4-methyl-4-hexenoic acid (XXII). Hydrolysis of XXII with ethanolic potassium hydroxide under ice cooling gave *trans*-6-hydroxy-4-methyl-4-hexenoic acid in good yield. Since this compound was assumed to readily undergo lactonization, its carboxylic acid was immediately methylated with diazomethane, and then purified by silicic acid column chromatography to obtain methyl 6-hydroxy-4-methyl-4-hexenoate (XXIII). XXIII exhibited a nuclear magnetic resonance signal of a 4-methyl (τ 8.38, singlet), in a position *trans* to a 5 hydrogen (Bates and Gale, 1960; Bates *et al.*, 1962). The *trans,cis* isomerization, therefore, does not occur in the series of reactions starting from XX. Condensation of XXIII with XXIV in dioxane in the presence of boron trifluoride, was followed by oxidation to the quinone with ferric chloride and by purification using paraffin-impregnated layer chromatography to give XXV. XXV exhibited nuclear magnetic resonance signals of a *trans* methyl (τ 8.28, singlet) and *cis* methylene (τ 7.70, broad) in *trans* XXV. But it also exhibited signals of *cis* methyl (τ 8.34, singlet) and *trans* methylene (τ 7.61, broad) in *cis* XXV. The ratio of *trans*:*cis* was 3:1 from the areas of the nuclear magnetic resonance signals at τ 8.28 and 8.34. This showed that XXV was a mixture of *trans* and *cis* isomers and that partial isomerization took place during the reaction. An example of a similar reaction is seen in the synthesis of vitamin K_1 in which condensation of a hydroquinone with phytol in the presence of boron trifluoride gives the *trans* isomer in a yield of over 90% (Jackman *et al.*, 1965). The relatively high ratio of the *cis* isomer in the case of XXV may be interpreted by stabilization of the allylic cation by the carbonyl as shown in A. The methyl ester of a ubi-



A

quinone metabolite was identified as *trans* XXV by thin-layer chromatographic R_F values, gas chromatographic retention time, and spectral data. Thus, the structure of the metabolite was confirmed.

Ubiquinone is known to be unstable with alkali under air and this can be avoided in the presence of pyrogallol under nitrogen (Crane and Lester, 1962). According to this procedure, XXV was hydrolyzed with methanolic potassium hydroxide to obtain XXVI. XXVI is also a mixture of *cis* and *trans* isomers.

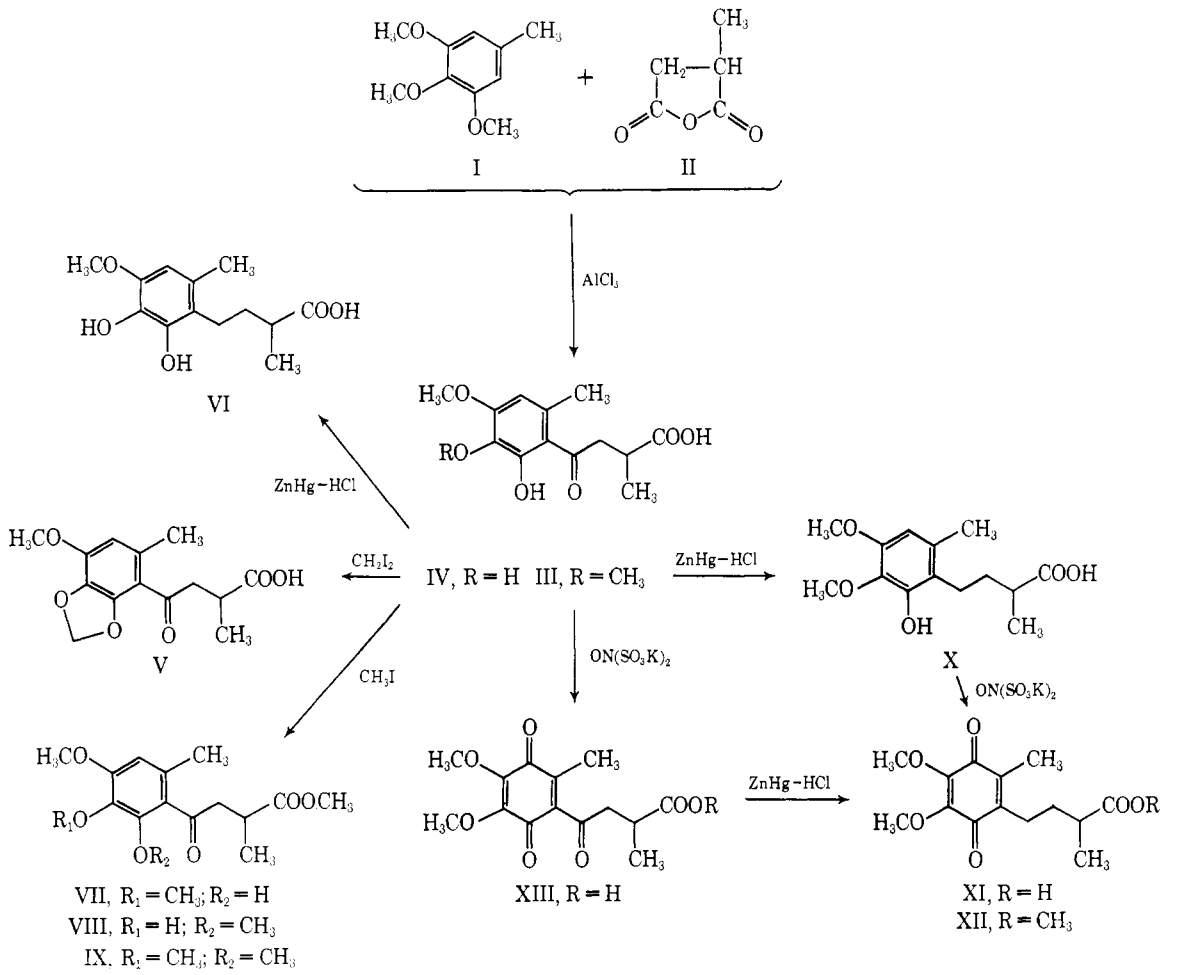
Treatment of XXVI with hydrochloric acid in tetrahydrofuran resulted in lactonization as expected and gave XXVII.

2,3-Dimethoxy-5-methyl-6-carboxymethyl-1,4-benzoquinone (XXIX). β Oxidation of XI to XXIX in the metabolic process would be formally possible. In an attempt to synthesize authentic XXIX, 3',6'-diacetoxy-4',5'-dimethoxy-2'-methylphenylacetic acid (XXVIII) derived from ubiquinone-7 by the procedure of Morton *et al.* (1958) was hydrolyzed, followed by oxidation. XXIX gave the methyl ester XXX by methylation with diazomethane.

DL-2,3-Dimethoxy-5-methyl-6-(3'-carboxy-3'-methylpropyl)-1,4-benzohydroquinonedisulfuric Acid (XXXI) and Ubihydroquinone-7-disulfuric Acid (XXXII). Treatment of the hydroquinone of XI with sulfur trioxide–triethylamine complex gave the disulfate XXXI. XXXI not only showed the same behavior on treatment with acid and alkali as the conjugate isolated from the rabbits' urine but showed the same thin-layer chromatographic R_F values, ultraviolet, and nuclear magnetic resonance spectra. Because ubihydroquinone-7 and ubiquinone-7 were detected in the rats' urine only after acid treatment, it was thought that ubiquinone-7 is excreted as such the conjugate XXXII. The authors synthesized XXXII in a similar manner to XXXI. XXXII exhibited the same ultraviolet absorption maximum and methoxy signals in nuclear magnetic resonance as XXXI.

Experimental Section

General Comments. Samples were dried *in vacuo* over P_2O_5 for 5 hr at room temperature for the measurement of physicochemical data. Melting points were capillary melting points and were uncorrected. Mass spectra were recorded by Hitachi RMU-6D double-focusing and RMS-4 mass spectrometers. The nuclear magnetic resonance signals are abbreviated as follows: s, singlet; d, doublet; t, triplet;



m, multiplet; b, broad. The number of protons is shown as the number in parentheses. Chemical shifts are expressed in τ values relative to tetramethylsilane as an internal standard. Other general experimental procedures were described in the previous report (Imada *et al.*, 1970). Thin-layer chromatography was carried out using a plate (20 × 20 cm) of silica gel GF_{254} (Merck A.G.) (10 g). Silicic acid for column chromatography was purchased from Mallinckrodt Co.

2-Methyl-3-(3',4'-dimethoxy-2'-hydroxy-6'-methylbenzoyl)propionic Acid (III) and 2-Methyl-3-(2',3'-dihydroxy-4'-methoxy-6'-methylbenzoyl)propionic Acid (IV). A mixture of methylsuccinic anhydride (II) (3.5 g) and 3,4,5-trimethoxytoluene (I) (Goodwin and Witkop, 1957) (5 g) in nitrobenzene (10 ml) was slowly added to a solution of $AlCl_3$ (10 g) in nitrobenzene (30 ml) with stirring under N_2 and ice cooling. The mixture became brown and viscous and was then kept for 20 hr at room temperature. The mixture was acidified with ice-cold 10% HCl and extracted with ether. The ether extracts were washed with water and evaporated. The residue was freed of nitrobenzene by steam distillation. The aqueous layer was extracted with ethyl acetate. The extracts were washed with water, dried over Na_2SO_4, and the solvent was evaporated *in vacuo* (usual manner hereafter) to give a viscous oil. The oil was separated by column chromatography on silicic acid (90 g) containing 6% of water, eluting with chloroform–ethanol (19:1) into two fractions. Fraction 1 (300 ml) and fraction 2 (200 ml) were evaporated to give III and IV, respectively.

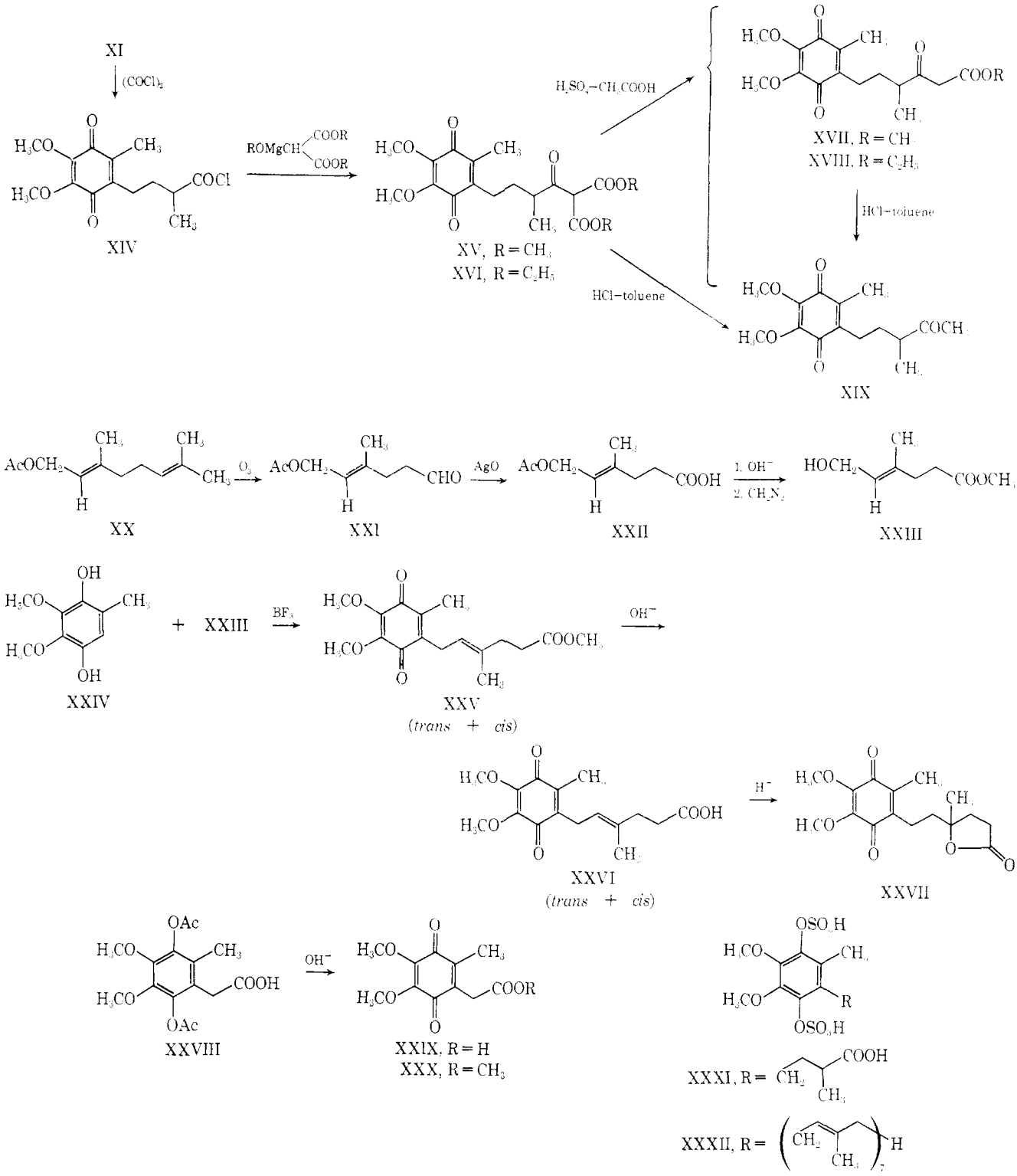
Fraction 1 was recrystallized from ethyl acetate to give III as pale yellow needles: mp 142.5°; yield 2 g (25.8%); infrared spectrum (KBr) ~2600, 1710 (COOH), 1610 cm^{-1} (CO); nuclear magnetic resonance spectrum (CD_3SOCD_3) 8.90 (d, 3, side chain CH_3), 7.91 (s, 3, ring CH_3), 7.22 (m, 1, CHCOO), 7.05 (d, 2, $COCH_2$), 6.37, 6.25 (s, 6, OCH_3), 3.65 (s, 1, ring H), 0.66 (s, 1, OH), −2.03 (b, 1, COOH).

Anal. Calcd for $C_{14}H_{18}O_6$: C, 59.56; H, 6.43. Found: C, 59.55; H, 6.52.

Fraction 2 was recrystallized from ethyl acetate to give IV as pale yellow needles: mp 170–171°; yield 525 mg (7.1%); infrared spectrum (KBr) 3450 (OH), ~2600, 1705 (COOH), 1630 cm^{-1} (CO); nuclear magnetic resonance spectrum (CD_3SOCD_3) 8.87 (d, 3, side chain CH_3), 7.90 (s, 3, ring CH_3), 7.18 (m, 1, CHCOO), 7.02 (d, 2, $COCH_2$), 6.22 (s, 3, OCH_3), 3.66 (s, 1, ring H), 1.52 (b, 1, OH), 1.03 (b, 1, OH), −1.90 (b, 1, COOH).

Anal. Calcd for $C_{13}H_{16}O_6$: C, 58.20; H, 6.01. Found: C, 58.01; H, 6.17.

Methylation of IV. K_2CO_3 (200 mg) and methyl iodide (53 mg) were added to a solution of IV (100 mg) in acetone (5 ml), and then refluxed for 5 hr under N_2. Water was added to the reaction mixture and it was extracted with ether. The ether extracts were washed with K_2CO_3 solution and worked up in the usual manner to recover IV (61 mg). The K_2CO_3 and water washings were acidified with 10% HCl. The separated oil was extracted with ether and worked up



in the usual manner to give an oil, followed by thin-layer chromatography using ether–petroleum ether (3:2).

METHYL 2-METHYL-3-(3′,4′-DIMETHOXY-2′-HYDROXY-6′-METHYLBENZOYL)PROPIONATE (VII). From the band of the middle R_F value, VII was obtained as a colorless oil: yield 14 mg (12.7%); infrared spectrum (liquid film) 3400 (OH), 1730, 1340 ($COOCH_3$), 1680 cm^{-1} (CO); nuclear magnetic resonance spectrum (CCl_4), 6.42 (s, 3, $COOCH_3$), 6.26, 6.22 (s, 6, OCH_3), 1.40 (s, 1, OH).

METHYL 2-METHYL-3-(2′,4′-DIMETHOXY-3′-HYDROXY-6′-METHYLBENZOYL)PROPIONATE (VIII). From the band of the lower R_F value, VIII was obtained as a colorless oil: yield 14 mg (12.7%); infrared spectrum (liquid film) 3400 (OH), 1730, 1340 ($COOCH_3$), 1690 cm^{-1} (CO); nuclear magnetic

resonance spectrum (CCl_4) 6.40 (s, 3, $COOCH_3$), 6.22, 6.19 (s, 6, OCH_3), 4.66 (b, 1, OH).

METHYL 2-METHYL-3-(6′-METHYL-2′,3′,4′-TRIMETHOXYBENZOYL)PROPIONATE (IX). From the band of the upper R_F value, IX was obtained as a colorless oil: yield 19 mg (16.4%); infrared spectrum (liquid film) 1730, 1330 ($COOCH_3$), 1690 cm^{-1} (CO); nuclear magnetic resonance spectrum (CCl_4) 6.39 (s, 3, $COOCH_3$), 6.26 (s, 3, OCH_3), 6.22 (s, 6, OCH_3).

2-Methyl-3-(2′,3′-methylenedioxy-4′-methoxy-6′-methylbenzoyl)propionic Acid (V). Methylene iodide (240 mg) and 45% KOH (0.1 ml) were added to a solution of IV (200 mg) in methanol (10 ml) and heated at 105–110° for 28 hr. The reaction mixture was poured into ice–water, acidified with 10% HCl, and extracted with ether. The ether extracts were worked up in the usual manner to give a brown oil. The oil was purified by thin-layer chromatography using ether–petroleum ether–acetic acid (15:15:1) to give V as colorless needles, which were recrystallized from a mixture of acetone–petroleum ether: mp 143°; yield 35 mg (16.8%); infrared spectrum (KBr) ~2650, 1700 (COOH), 1670 cm^{-1} (CO); nuclear magnetic resonance spectrum ($CDCl_3$) 4.02 (s, 2, OCH_2O).

2-Methyl-4-(2′,3′-dihydroxy-4′-methoxy-6′-methylphenyl)butanoic Acid (VI). Amalgamated zinc prepared from mossy zinc (400 mg), water (0.5 ml), acetic acid (0.5 ml), and 35% HCl (0.5 ml) were added to a solution of IV (200 mg) in toluene (2 ml). The mixture was refluxed for 14 hr. After separation of the toluene layer, the aqueous layer was extracted with ether. The ether extracts and the above toluene layer were combined and worked up in the usual manner to give VI as colorless needles, which were recrystallized from a mixture of ether–petroleum ether: mp 109–110°; yield 152 mg (80.2%); infrared spectrum (KBr) 3400, 3350 (OH), ~2600, 1700 cm^{-1} (COOH); nuclear magnetic resonance spectrum (CD_3SOCD_3) 8.8–8.2 (m, 2, CH_2), 7.8–7.5 (m, 3, CHCOO, ring CH_2), 2.10 (b, 1, OH), 1.90 (s, 1, OH).

Anal. Calcd for $C_{13}H_{18}O_5$: C, 61.40; H, 7.14. Found: C, 61.13; H, 7.12.

2-Methyl-4-(3′,4′-dimethoxy-2′-hydroxy-6′-methylphenyl)butanoic Acid (X). A mixture of amalgamated zinc prepared from mossy zinc (3 g), water (0.2 ml), 35% HCl (3 ml), toluene (3 ml), and III (1.5 g) was refluxed for 9 hr. The reaction mixture was similarly treated to give X as colorless needles: mp 104–105°; yield 1.3 g (91.2%); infrared spectrum (KBr) 3450 (OH), ~2600, 1700 cm^{-1} (COOH); nuclear magnetic resonance spectrum ($CDCl_3$) 8.72 (d, 3, side chain CH_3), 8.5–7.9 (m, 2, CH_2), 7.74 (s, 3, ring CH_3), 7.4 (m, 1, CHCOO), 7.34 (t, 2, ring CH_2), 6.18, 6.14 (s, 6, OCH_3), 4.01 (b, 1, OH), 3.71 (s, 1, ring H), −0.60 (b, 1, COOH).

Anal. Calcd for $C_{14}H_{20}O_5$: C, 62.67; H, 7.51. Found: C, 62.94; H, 7.72.

DL-*2,3-Dimethoxy-5-methyl-6-(3′-carboxy-3′-methylpropyl)-1,4-benzoquinone (XI).* $ON(SO_3K)_2$ (3 g) in water (60 ml) was added to a solution of X (1.4 g) in 2% NaOH (12 ml) and stirred for 2 hr at room temperature. The reaction mixture was acidified with 10% HCl and extracted with ether. The ether extracts were worked up in the usual manner to give an orange oil (1.38 g). The oil was purified by column chromatography on silicic acid (50 g) containing 3% of water eluting with chloroform to afford XI as a red oil: yield 1.36 g (92.3%); λ_{max}^{EtOH} ($E_{1\,cm}^{1\%}$) oxidized form 278 (505), reduced form 291 mμ (112), $\Delta E_{1\,cm}^{1\%}$ at 278 mμ 432; infrared spectrum (CCl_4) ~2600, 1700 (COOH), 1660, 1650, 1610 cm^{-1} (quinone); nuclear magnetic resonance spectrum (CCl_4) 8.75 (d, 3, side chain CH_3), 8.6–8.1 (m, 2, CH_2), 8.03 (s, 3, ring CH_3), 7.54 (t, 2, ring CH_2), 7.46 (b, 1, CHCOO), 6.10 (s, 6, OCH_3), −1.04 (b, 1, COOH); mass spectrum *m/e* 282 (M), 249, 236, 221, 208, 195, 149, 74.

Anal. Calcd for $C_{14}H_{18}O_6$: C, 59.56; H, 6.43. Found: C, 59.54; H, 6.52.

OXIDATION WITH H_2O_2–ACETIC ACID. H_2O_2 (30%) (0.5 ml) and H_2SO_4 (5%) (0.1 ml) were added to a solution of X (500 mg) in acetic acid (5 ml) and stirred for 67 hr at room temperature. Ice–water was added to the reaction mixture and it was extracted with ether. The ether extracts were washed with 1% $FeSO_4$ solution and worked up in the usual manner to give an orange oil. This oil was purified by column chromatography on silicic acid (10 g) containing 6% of water, eluting with chloroform to give XI as an orange oil: yield 200 mg (38.0%).

METHYL ESTER (XII). XI (15 mg) was methylated with diazomethane and purified by column chromatography on silicic acid (6 g) to give XII as an orange oil: yield 15 mg (95.3%); λ_{max}^{EtOH} ($E_{1\,cm}^{1\%}$) oxidized form 277 (500), reduced form 291 mμ (125), $\Delta E_{1\,cm}^{1\%}$ at 277 mμ 439; infrared spectrum (liquid film) 1745, 1270 ($COOCH_3$), 1665, 1650, 1615 cm^{-1} (quinone); nuclear magnetic resonance spectrum (CCl_4) 8.83 (d, 3, side chain CH_3), 8.7–8.2 (m, 2, CH_2), 8.05 (s, 3, ring CH_3), 7.8–7.4 (m, 3, CHCOO, ring CH_2), 6.39 (s, 3, $COOCH_3$), 6.10 (s, 6, OCH_3); mass spectrum *m/e* 296 (M), 265 (M − OCH_3), 249, 236, 221, 208, 195, 88.

Anal. Calcd for $C_{15}H_{20}O_6$: C, 60.80; H, 6.80. Found: C, 60.81; H, 6.87.

2,3-Dimethoxy-5-methyl-6-(3′-carboxy-3′-methyl-1′-oxopropyl)-1,4-benzoquinone (XIII). $ON(SO_3K)_2$ (150 mg) in water (3 ml) was added to a solution of III (50 mg) in 1% NaOH (1.5 ml) and stirred for 2 hr at room temperature. The reaction mixture was acidified with 10% HCl and extracted with ether. The ether extracts were worked up in the usual manner to give an orange oil. This oil was purified by column chromatography on silicic acid (5 g), eluting with chloroform. The eluate was evaporated to give XIII as an orange oil: yield 39 mg (74.3%); λ_{max}^{EtOH} ($E_{1\,cm}^{1\%}$) 267 mμ (381); infrared spectrum ($CHCl_3$) ~2600, 1700 (COOH), 1700 (CO), 1650, 1610 cm^{-1} (quinone); nuclear magnetic resonance spectrum ($CDCl_3$) 8.70 (d, 3, side chain CH_3), 8.05 (5, 3, ring CH_3), 7.40–6.73 (m, 1, CHCOO), 7.03 (d, 2, $COCH_2$), 6.00 (s, 6, OCH_3), 1.34 (b, 1, COOH).

Anal. Calcd for $C_{14}H_{16}O_7$: C, 56.75; H, 5.44. Found: C, 56.61; H, 5.74.

SYNTHESIS OF XI FROM XIII. A mixture of amalgamated zinc prepared from mossy zinc (100 mg), water (0.1 ml), 35% HCl (0.1 ml), toluene (1 ml), and XIII (20 mg) was refluxed for 3 hr. After cooling, the excess of amalgamated zinc was removed and the reaction mixture was poured into 10% $FeCl_3$ solution (1 ml). After shaking, the organic phase was separated and the aqueous phase was extracted with ether. The above organic solution and the ether extracts were combined, and worked up in the usual manner to give an orange oil. This oil was purified by column chromatography on silicic acid (3 g), eluting with chloroform. The eluate was evaporated to give XI as an orange oil: yield 13 mg (68.2%).

2,3-Dimethoxy-5-methyl-6-(3'-chloroformyl-3'-methyl-propyl)-1,4-benzoquinone (XIV). Oxalyl chloride (0.5 ml) was added to XI (165 mg) with stirring at room temperature. After the gas evolution ceased, the mixture was refluxed for 30 min. XIV was obtained by removal of excess oxalyl chloride using azeotropical distillation with benzene as an orange oil: yield 175 mg (quantitative); infrared spectrum (liquid film) 1790, 1740 cm^{-1} (COCl).

2,3-Dimethoxy-5-methyl-6-(5',5'-dimethoxycarbonyl-3'-methyl-4'-oxopentyl)-1,4-benzoquinone (XV). Methanol (0.1 ml) and carbon tetrachloride (1 drop), and then a solution of methylmalonate (330 mg) in a mixture of methanol (0.5 ml) and benzene (0.5 ml) were added to a suspension of magnesium (60 mg) in benzene (0.1 ml). The resulting mixture was refluxed to dissolve magnesium and excess methanol was distilled off with benzene azeotropically (Lund, 1934). A solution of XIV (491 mg) in benzene (5 ml) was added to a solution of the residue in benzene (5 ml) under ice cooling with stirring. The reaction mixture was refluxed for 30 min, water and 3 N H_2SO_4 were added and then it was extracted with ether. The ether extracts were washed with 3 N H_2SO_4 and worked up in the usual manner to give an orange oil. The oil was purified by silicic acid column chromatography to give XV as an orange oil: yield 562 mg (86.8%); λ_{max}^{EtOH} ($E_{1\,cm}^{1\%}$) 260 (436), 272 (493), 280 (455); $\lambda_{max}^{0.1\,N\,KOH-EtOH}$ ($E_{1\,cm}^{1\%}$) 275 mμ (900); infrared spectrum (liquid film) 1750, 1740–1710, 1270, 1240 ($COOCH_3$), 1710 (CO), 1660, 1640, 1610 cm^{-1} (quinone); nuclear magnetic resonance spectrum (CCl_4) enol form 8.80 (d, 3, side chain CH_3), 8.6–8.2 (m, 2, CH_2), 8.06 (s, 3, ring CH_3), 7.61 (m, 2, ring CH_2), 7.24 [m, 1, CHC(O) = C], 6.28, 6.20 (s, 6, $COOCH_3$), 6.09 (s, 6, OCH_3), −3.33, −3.31 (s, 1, OH), keto form 8.83 (d, 3, side chain CH_3), 8.03 (s, 3, ring CH_3), 7.24 (m, 1, CHCO), 6.30, 6.23 (s, 6, $COOCH_3$), 5.44 [s, 1, $COCH(COO)_2$].

Anal. Calcd for $C_{19}H_{24}O_9$: C, 57.57; H, 6.10. Found: C, 57.85; H, 6.28.

2,3-Dimethoxy-5-methyl-6-(5',5'-diethoxycarbonyl-3'-methyl-4'-oxopentyl)-1,4-benzoquinone (XVI). XIV (176 mg) and ethylmalonate (133 mg) similarly gave XVI as an orange oil: yield 180 mg (72.5%).

Anal. Calcd for $C_{21}H_{28}O_9$: C, 59.42; H, 6.65. Found: C, 59.15; H, 6.63.

Decarboxylation of XV and XVI with H_2SO_4–Acetic Acid. XV (50 mg) was dissolved in acetic acid (38 mg) containing 0.22% H_2SO_4 and the mixture was heated for 7 hr at 110°. Water was added to the reaction mixture and it was extracted with ether. The ether extracts were worked up in the usual manner to give the residue. A solution of the residue in chloroform was purified by thin-layer chromatography using chloroform–hexane–ether (6:5:3).

2,3-DIMETHOXY-5-METHYL-6-(3'-METHYL-5'-METHOXYCARBONYL-4'-OXOPENTYL)-1,4-BENZOQUINONE (XVII). The yellow band of the upper R_F value was extracted with ether to give XVII as an orange oil: yield 21 mg (49.2%); λ_{max}^{EtOH} ($E_{1\,cm}^{1\%}$) 278 (377), $\lambda_{max}^{0.1\,N\,KOH-EtOH}$ 282 mμ (634); infrared spectrum (liquid film) 1750, 1270 ($COOCH_3$), 1710 (CO), 1660, 1650, 1610 cm^{-1} (quinone); nuclear magnetic resonance spectrum (CCl_4) enol form 8.81 (d, 3, side chain CH_3), 8.7–8.2 (m, 2, CH_2), 8.06 (s, 3, ring CH_3), 7.8–7.5 (m, 2, ring CH_2), 7.5–7.2 [m, 1, CHC(O)═C], 6.31 (s, 3, $COOCH_3$), 6.09 (s, 6, OCH_3), 5.05 (s, 1, C═CHCOO), −2.06 (s, 1, enolic OH), keto form 8.85 (d, 3, side chain CH_3), 8.03 (s, 3, ring CH_3), 7.5–7.2 (m, 1, CHCO), 6.60 (s, 2, $COCH_2COO$); mass spectrum *m/e* 338 (M), 306 (M − CH_3OH), 237 (M − $COCH_2COOCH_3$), 221, 208, 196, 195, 101 ($COCH_2COOCH_3$).

Anal. Calcd for $C_{17}H_{22}O_7$: C, 60.34; H, 6.55. Found: C, 60.50; H, 6.45.

2,3-DIMETHOXY-5-METHYL-6-(3'-METHYL-4'-OXOPENTYL)-1,4-BENZOQUINONE (XIX). The yellow band of the lower R_F value was extracted with ether. The oil obtained from the ether extracts was then purified by reversed-phase-layer chromatography on paraffin-impregnated silica gel plates using acetone–water (2:3) to give XIX as an orange oil: yield 5 mg (14.1%); λ_{max}^{EtOH} ($E_{1\,cm}^{1\%}$) oxidized form 277 (460), reduced form 291 mμ (138), $\Delta E_{1\,cm}^{1\%}$ at 277 mμ 369; infrared spectrum (liquid film) 1710 (CO), 1665, 1650, 1610 cm^{-1} (quinone); nuclear magnetic resonance spectrum (CCl_4) 8.92 (d, 3, side chain CH_3), 8.8–8.1 (m, 2, CH_2), 8.06 (s, 3, ring CH_3), 7.94 (s, 3, $COCH_3$), 7.8–7.4 (m, 3, CHCO, ring CH_2), 6.13 (s, 6, OCH_3); mass spectrum *m/e* 282 (M + 2), 280 (M), 237 (M − $COCH_3$), 205, 197, 149, 43 ($COCH_3$).

Anal. Calcd for $C_{15}H_{20}O_5$: C, 64.27; H, 7.19. Found: C, 63.96; H, 7.24.

2,3-Dimethoxy-5-methyl-6-(5'-ethoxycarbonyl-3'-methyl-4'-oxopentyl)-1,4-benzoquinone (XVIII). XVI (40 mg) was worked up in a similar manner to XV. The resulting residue was purified by thin-layer chromatography using benzene–ethyl acetate (7:3). From the band of R_F 0.65, XVI (6 mg) was recovered.

XVIII. From the band of R_F 0.59, XVIII was obtained as an orange oil: yield 11 mg (33.1%).

Anal. Calcd for $C_{18}H_{24}O_7$: C, 61.35; H, 6.86. Found: C, 61.11; H, 7.06.

XIX. From the band of R_F 0.55, XIX was obtained as an orange oil: yield 3 mg (11.4%).

Decarboxylation of XV and XVII with HCl–Toluene. XVII and XIX. HCl (10%) (0.1 ml) was added to a solution of XV (10 mg) in toluene (1 ml) and the mixture was refluxed for 6 hr. The products were purified by thin-layer chromatography into XVII (0.9 mg, 10.5%) and XIX (4.5 mg, 63.6%).

XIX. XVII (20 mg) was decarboxylated to XIX (9 mg, 54.3%).

6-Acetoxy-4-methyl-4-hexenal (XXI). Ozonized oxygen was bubbled through a solution of geranyl acetate (XX) (40 g) in methanol (300 ml) at −78° until the disappearance of XX, checking by thin-layer chromatography. Dimethyl sulfide (20 ml) was added to the reaction mixture and it was stirred at −10° for 1 hr, at 0° for 1 hr, and then at room temperature for 1 hr to perform the reductive cleavage of the resulting ozonide. After evaporation of methanol, the residue was distilled under reduced pressure to give XXI as a pale yellow oil: bp 80–85° (0.4 mm); yield 19 g (54.8%); infrared spectrum (liquid film) 2720, 1730 (CHO), 1730, 1235 ($OCOCH_3$), 1670 cm^{-1} (C═C); nuclear magnetic resonance spectrum (CCl_4) 8.26 (s, 3, C═CCH_3), 8.03 (s, 3, $OCOCH_3$), 7.57 (m, 4, $COCH_2$, CH_2C═C), 5.51 (d, 2, CH_2O), 4.68 (t, 1, C═CH), 0.30 (s, 1, CHO).

6-Acetoxy-4-methyl-4-hexenoic Acid (XXII). AgO (400 mg), which was prepared from Ag_2O and $KMnO_4$, was added to a solution of XXI (100 mg) in 90% tetrahydrofuran (5 ml) and stirred for 14 hr at room temperature, and then 6 hr at 70°. After removal of excess AgO by filtration, the

filtrate was washed with water. The combined solution of the filtrate and the washings was acidified with 10% HCl, and then extracted with ethyl acetate. The ethyl acetate extracts were worked up in the usual manner to give a pale yellow oil. The oil was purified by silicic acid column chromatography to give XXII as a colorless oil: yield 83 mg (75.9%); infrared spectrum (liquid film) ~2650, 1710 (COOH), 1740, 1230 cm^{-1} ($OCOCH_3$); nuclear magnetic resonance spectrum (CCl_4) 8.28 (s, 3, $C{=}CCH_3$), 8.04 (s, 3, $OCOCH_3$), 7.62 (b, 4, $C{=}CCH_2$, CH_2COO), 5.53 (d, 2, OCH_2), 4.69 (t, 1, C=CH), −0.71 (b, 1, COOH).

Anal. Calcd for $C_9H_{14}O_4$: C, 58.05; H, 7.58. Found: C, 57.76; H, 7.69.

Methyl 6-Hydroxy-4-methyl-4-hexenoate (*XXIII*). A solution of KOH (180 mg) in 90% ethanol (1 ml) was added to a solution of XXII (200 mg) in ethanol (2 ml) under ice cooling with stirring for 1 hr. Water was added to the reaction mixture, it was acidified with 10% HCl, and then extracted with ethyl acetate. The ethyl acetate extracts were worked up in the usual manner to give 6-hydroxy-4-methyl-4-hexenoic acid (150 mg) as a pale yellow oil. The oil was methylated with diazomethane and purified by column chromatography on silicic acid (15 g) eluting with chloroform to give XXIII as a colorless oil: yield 118 mg (69.4%); infrared spectrum (liquid film) 3400 (OH), 1740, 1165 ($COOCH_3$), 1675 cm^{-1} (C=C); nuclear magnetic resonance spectrum (CCl_4) 8.38 (s, 3, $C{=}CCH_3$), 7.70 (m, 4, $C{=}CCH_2$, CH_2COO), 6.67 (s, 1, OH), 6.43 (s, 3, $COOCH_3$), 6.04 (d, 2, CH_2O), 4.71 (t, 1, C=CH).

Anal. Calcd for $C_8H_{14}O_3$: C, 60.74; H, 8.92. Found: C, 60.55; H, 9.05.

trans,cis-2,3-Dimethoxy-5-methyl-6-(3'-methyl-5'-methoxycarbonyl-2'-pentenyl)-1,4-benzoquinone (*XXV*). BF_3–ether complex (0.3 ml) was added to a solution of XXIV (Kawamatsu *et al.*, 1969) (100 mg) and XXIII (87 mg) in dioxane (5 ml) over 1.5 hr under N_2 with stirring and stirred for further 4.5 hr at room temperature. Water was added to the reaction mixture and it was extracted with ether. The ether extracts were washed with water and oxidized by being shaken with a solution of $FeCl_3$ (1 g) in 30% methanol. The ether layer was worked up in the usual manner to give an orange oil. The oil was purified by thin-layer chromatography on paraffin-impregnated silica gel plates using acetone–water (2:3) as the developing solvent system. From the band of the upper R_F value, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (15 mg) was obtained. The oil (89 mg) obtained from the band of the lower R_F value was purified by thin-layer chromatography using benzene–ethyl acetate (4:1) to give XXV as an orange oil: yield 67 mg (38.3%); λ_{max}^{EtOH} ($E_{1\,cm}^{1\%}$) oxidized form 275 (449), reduced form 292 mμ (121), $\Delta E_{1\,cm}^{1\%}$ at 275 mμ 397; infrared spectrum (liquid film) 1750, 1270, 1200 ($COOCH_3$), 1660, 1650, 1615 cm^{-1} (quinone); nuclear magnetic resonance spectrum (CCl_4) *trans* compound 8.28 (s, 3, $C{=}CCH_3$), 8.08 (s, 3, ring CH_3), 7.70 (b, 4, $C{=}CCH_2$, CH_2COO), 6.90 (d, 2, ring CH_2), 6.48 (s, 3, $COOCH_3$), 6.08 (s, 6, OCH_3), 5.08 (t, 1, C=CH); *cis* compound 8.34 (s, 3, $C{=}CCH_3$), 8.04 (s, 3, ring CH_3), 7.61 (b, 4, $C{=}CCH_2$, CH_2COO), 6.38 (s, 3, $COOCH_3$); mass spectrum *m/e* 322 (M), 307 (M − CH_3), 291 (M − OCH_3), 275, 247, 235, 197.

Anal. Calcd for $C_{17}H_{22}O_6$: C, 63.34; H, 6.88. Found: C, 63.41; H, 6.75

trans,cis-2,3-Dimethoxy-5-methyl-6-(5'-carboxy-3'-methyl-2'-pentenyl)-1,4-benzoquinone (*XXVI*). Methanolic KOH (10%) (2 ml) was added to a solution of XXV (20 mg) and pyrogallol (200 mg) in methanol (2 ml) and the mixture was heated at 70° for 1 hr under N_2. Water was added and the reaction mixture was acidified with 10% HCl and extracted with ether. The ether extracts were shaken with a solution of $FeCl_3$ (1 g) in 30% methanol (10 ml) and the ether layer was separated. The residue of the ether layer was purified by thin-layer chromatography using chloroform–ethanol (9:1) to give XXVI as an orange oil: yield 10 mg (52.4%); λ_{max}^{EtOH} oxidized form 275, reduced form 290 mμ; infrared spectrum (CCl_4) ~2650, 1700 (COOH), 1660, 1650, 1610 cm^{-1} (quinone); nuclear magnetic resonance spectrum (CCl_4) *trans* compound 8.27 (s, 3, $C{=}CCH_3$), 8.09 (s, 3, ring CH_3), 7.72 (b, 4, $C{=}CCH_2$, CH_2COO), 6.92 (d, 2, ring CH_2), 6.12 (s, 6, OCH_3), 5.06 (t, 1, C=CH), −0.06 (b, 1, COOH). *cis* compound 8.35 (s, 3, $C{=}CCH_3$), 7.60 (b, 4, $C{=}CCH_2$, CH_2COO); mass spectrum *m/e* 308 (M), 293 (M − CH_3), 247, 235.

2,3-Dimethoxy-5-methyl-6-(5'-carboxy-3'-hydroxy-3'-methylpentyl)-1,4-benzoquinone Lactone (*XXVII*). HCl (10%) (0.5 ml) was added to a solution of XXVI (1 mg) in tetrahydrofuran (0.5 ml) and the mixture heated at 75° for 1.5 hr under N_2. Water was added and the reaction mixture extracted with ether. The ether extracts were worked up in the usual manner to give an oil. The oil was purified by thin-layer chromatography using ether–chloroform–ethanol (3:2:1) to give XXVII (Morimoto *et al.*, 1969) as an orange oil: yield 0.2 mg (20.0%).

2,3-Dimethoxy-5-methyl-6-carboxymethyl-1,4-benzoquinone (*XXIX*). Methanolic KOH (0.1 N) (1 ml) was added to a solution of XXVIII (2.8 mg) (Morton *et al.*, 1958) in methanol (5 ml) and the mixture kept for 10 min at 70° under N_2. The mixture was treated in the usual manner to give the residue. The residue was oxidized with 10% methanolic $FeCl_3$ and the reaction mixture was treated in the usual manner to give XXIX as an orange oil: yield 1 mg (43.1%); λ_{max}^{EtOH} 275 mμ; infrared spectrum (liquid film) 2800–2500, 1710 (COOH), 1665, 1615 cm^{-1} (quinone); nuclear magnetic resonance spectrum (CCl_4) 8.06 (s, 3, ring CH_3), 6.62 (s, 2, ring CH_2), 6.11, 6.08 (s, 6, OCH_3); mass spectrum *m/e* 240 (M), 239, 197.

2,3-Dimethoxy-5-methyl-6-methoxycarbonylmethyl-1,4-benzoquinone (*XXX*). A solution of XXIX in ether was methylated with diazomethane to give XXX as an orange oil: λ_{max}^{EtOH} 274 mμ; infrared spectrum (liquid film) 1740, 1270 ($COOCH_3$), 1665, 1615 cm^{-1} (quinone); nuclear magnetic resonance spectrum (CCl_4) 8.06 (s, 3, ring CH_3), 6.60 (s, 2, ring CH_2), 6.37 (s, 3, $COOCH_3$), 6.09, 6.07 (s, 6, OCH_3); mass spectrum *m/e* 254 (M), 195.

DL-*2,3-Dimethoxy-5-methyl-6-(3'-carboxy-3'-methylpropyl)-1,4-benzohydroquinonedisulfuric Acid* (*XXXI*). The hydroquinone (36 mg) prepared from XI (40 mg) was dissolved in dimethylformamide–tetrahydrofuran (1:1) (1 ml) and SO_3–triethylamine complex (reviewed by Gilbert, 1962) (107 mg) was added, and then stirred for 14 hr at room temperature under N_2. After evaporation, the residue was purified by thin-layer chromatography using 1-propanol–NH_4OH (9:1). The band of the desired compound (R_F 0.7) was detected by an ultraviolet lamp (254 mμ), and then extracted with methanol. The extracts were evaporated to dryness to give XXXI as a pale yellow oil: yield 10 mg (17.8%); λ_{max}^{EtOH} 276, 282 mμ; nuclear magnetic resonance spectrum (CD_3OD)

8.8 (d, 3, side chain CH_3), 8.7–8.2 (b, 2, CH_2), 7.8 (s, 3, ring CH_3), 7.6–7.4 (b, 3, CHCOO, ring CH_2), 6.22, 6.12 (s, 6, OCH_3).

Ubihydroquinone-7-disulfuric Acid (XXXII). A solution of ubihydroquinone-7 (99 mg) (Morimoto *et al.*, 1965) in pyridine (1 ml) and SO_3–triethylamine complex (51 mg) was similarly worked up, and then the band of the desired compound (R_F 0.85) was extracted with methanol. The extracts were evaporated to dryness to give XXXII as a yellow oil: yield 62 mg (50.4%); λ_{max}^{EtOH} 276, 282 mμ; nuclear magnetic resonance spectrum (CD_3SOCD_3) 8.46 (s, 18, *trans* C=CCH_3), 8.39 (s, 3, *cis* C=CCH_3), 8.30 (s, 3, *trans* C=CCH_3), 8.04 (b, 24, C=CCH_2), 7.92 (s, 3, ring CH_3), 6.33, 6.21 (s, 6, OCH_3) 4.95 (b, 7, C=CH).

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Biosynthesis of Echinulin by *Aspergillus amstelodami* from Cyclo-L-alanyl-L-tryptophyl-^{14}C*

George P. Slater, James C. MacDonald, and Ruka Nakashima†

ABSTRACT: *Aspergillus amstelodami* PRL 1698 synthesized echinulin when it was grown on molasses medium to which was added cyclo-L-alanyl-L-tryptophyl or cyclo-L-alanyl-D-tryptophyl (both labeled with ^{14}C in the methylene carbon of the tryptophyl moiety). The organism incorporated radioactivity from cyclo-L-alanyl-L-tryptophyl-^{14}C into echinulin in a specific way, and to a greater extent than from cyclo-L-alanyl-D-tryptophyl-^{14}C. There was greater incorporation of radioactivity from cyclo-L-alanyl-L-tryptophyl-^{14}C into echinulin than into mycelial tryptophan, and the incorporation into echinulin was not significantly lessened by adding unlabeled tryptophan to the medium. Therefore, cyclo-L-alanyl-L-tryptophyl-^{14}C was not hydrolyzed to tryptophan-^{14}C before incorporation of radioactivity into echinulin, and the cyclic dipeptide itself appears to be an intermediate in the biosynthesis of echinulin.

Studies with ^{14}C-labeled tryptophan, alanine, and mevalonic lactone have shown that these compounds are precursors of echinulin (Figure 1a) synthesized by *Aspergillus amstelodami*. Tryptophan-^{14}C was incorporated exclusively into the echinin (Figure 2) moiety of the diketopiperazine ring of echinulin (Birch and Farrar, 1963; MacDonald and Slater, 1966). Mevalonic lactone-^{14}C and alanine-^{14}C were primarily

* From the Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, Saskatchewan, Canada. *Received February 24, 1970.* Issued as N.R.C. No. 11361.

† NRCC Postdoctorate Fellow, 1966–1968.