THE STRUCTURE OF THE HEXAHYDROGENATED ISOPRENOID SIDE-CHAIN MENAQUINONE WITH NINE ISOPRENE UNITS ISOLATED FROM *ACTINOMADURA MADURAE*¹

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A new menaquinone homologue was isolated from a nocardioform bacterium, *Actinomadura madurae*. The isolated compound had ultra-violet and infrared absorption spectra very similar to those of menaquinone. The mass spectrum of the compound showed the molecular ion peak at m/z 790 with the coexistence of two intense fragment ion peaks at m/z 225 and 187. However, two weak fragment ion peaks were not found at m/z 721 ($M-69$) and 653 ($M-69-68$) due to the successive loss of isoprene units. The ¹H-NMR spectrum represented the signals of the following groups: $4 \times \phi\text{-H}$, δ 8.02–7.60; $6 \times \text{-CH=C-}$, δ 5.04; $\text{Q-CH}_2\text{-}$, δ 3.32 (doublet, $J=6.7$ Hz); Q-CH_3 , δ 2.16; $10 \times \text{=C-CH}_2\text{-}$, δ 1.98; =C-CH_3 (*trans*, 1st unit from quinone ring), δ 1.78; =C-CH_3 (*cis*, 9th unit), δ 1.66; $5 \times \text{=C-CH}_3$ (*trans*, internal and 9th units), δ 1.57; $3 \times \text{-CH}_2\text{-CH}_2\text{-CH-CH}_2\text{-}$, δ 1.28; $3 \times \text{-C-CH}_3$, δ 0.90 (ϕ , benzene ring; Q, quinone ring). The chromenyl acetate was synthesized from the menaquinone, and was subjected to ozonolysis. The ozonolysis product had a mass spectrum showing ion peaks at m/z 464 (M^+), 449 ($M-15$), 422 ($M-42$), 407 ($M-15-42$), 267, and 225. The results obtained indicated that the isolated menaquinone is formulated as 2-methyl-3-II, III, VIII-hexahydro-multiprenyl⁹-1, 4-naphthoquinone.

It is known that the molecular species of respiratory quinones are useful for classifying gram-positive and gram-negative bacteria (2, 3). In coryneform and nocardioform bacteria, several polyhydrogenated isoprenoid side-chain me-

¹ Significance of the ubiquinone and menaquinone systems in the classification of gram-positive and gram-negative bacteria. Part IX. Abbreviations: Menaquinone- n , with n denoting a specified number of isoprene units in the side chain; menaquinone- n (H_m), with m indicating the number of hydrogen atoms saturating the isoprenoid side-chain. The nomenclature and terminology used follow IUPAC-IUB recommendations (1).

naquinones have been reported (2, 3). However, the chemical structures of the menaquinones have been elucidated to only a very small extent. In previous papers (4-6), it was recognized in the coryneform and nocardioform bacteria that several of menaquinones had migration patterns on paper chromatograms quite different from normal unsaturated isoprenologues of menaquinone. Of the quinones, the chemical structures of the tetrahydrogenated isoprenoid side-chain menaquinones isolated from *Oerskovia turbata* and "*Brevibacterium lipolyticum*" were formulated as 2-methyl-3-II, III-tetrahydromultiprenyl-1, 4-naphthoquinones (7-9). The present paper deals with a hexahydrogenated isoprenoid side-chain menaquinone with nine isoprene units isolated from a nocardioform bacterium, *Actinomadura madurae*. The preliminary report has been published (10).

MATERIALS AND METHODS

Microorganism. A nocardioform bacterium, *Actinomadura madurae*, was used for the isolation of menaquinone-9 (H_9), which was sent with the kindness of Drs. I. Komura and K. Yamada, the Central Research Laboratories, Ajinomoto Co., Kawasaki, Japan; it was of strain AJ 9136, originating from NRRL B-2127. Since the name of *Brevibacterium lipolyticum* is not on the Approved Lists of Bacterial Names, 1980 (11), the bacterial name is enclosed within quotation marks.

Culture of microorganism. The microorganism was grown with continuous rotary agitation in 5-l conical flasks containing 1 l of medium composed of 7 g glucose, 3 g glycerol, 3 g peptone (Kyokuto), 3 g meat extract (Kyokuto), and 1 g brain-heart infusion (Eiken) at pH 7.0 for 24 hr at 30°. The seed cultures were prepared in 500-ml conical flasks which contained 100 ml of the same medium, for 24 to 48 hr at 30°.

Reagents and chemicals. Vitamin K_1 used in the experiment was a commercial preparation (Nakarai). Vitamin K_2 was kindly supplied by Dr. N. Seto, Eisai Co., Tokyo, Japan.

Mass spectroscopy. The mass spectra of the isolated menaquinone homologues were measured on a Hitachi Model RMU-6M single-focusing mass spectrometer at 200° of the chamber temperature with a direct-inlet system heated to 135°. The ozonolysis product of chromenyl acetate derived from the isolated menaquinone-9 (H_9) was analyzed by the same method. However, the sample was vaporized at 130°.

1H -Nuclear magnetic resonance spectrometry. The 1H -NMR spectrum of the isolated menaquinone-9 (H_9) was taken at 100 MHz with a JEOL Model JNM-MH-100 spectrometer. The material was dissolved in carbon tetrachloride, and was measured using tetramethyl silane as an internal reference standard.

Extraction and purification of menaquinone. The bacterial cells of *A. madurae* AJ 9136 harvested from the cultured media by centrifugation were immediately lyophilized. Menaquinone was extracted from the lyophilized cells with a mixture

of acetone-ether (4:1, by volume), and was purified by preparative, combined thin-layer chromatography, as described in a previous paper (7). The lipid extract from the extraction was dissolved in a small volume of acetone, and the solution was spotted onto silica gel (Kieselgel GF₂₅₄ nach Stahl, Type 60, Merck) plates. The loaded plates were developed with a solvent system of benzene-hexane-chloroform (2:5:1, v/v). Then, the menaquinone preparation obtained was further purified by thin-layer chromatography with a solvent system of ether-hexane (1:9, v/v). The menaquinone homologues were separated from each other on silica gel plates impregnated with 9% silver nitrate (w/v) using a solvent system of 2-butanone-hexane (1:9, by volume).

RESULTS

General properties of isolated menaquinone

When the purified menaquinone preparation was chromatographed on the silica gel plates containing silver nitrate with the solvent system of 2-butanone-hexane, one intense and three weak band(s) were observed at the R_f values of 0.50, 0.63, 0.59, and 0.28, respectively, under ultraviolet light. The separated compounds were eluted with small volumes of acetone, and were purified by thin-layer chromatography with the solvent system of benzene-hexane-chloroform (2:5:1,

Fig. 1. The UV spectrum of the menaquinone isolated from *Actinomadura madurae* AJ 9136.

The spectrum was taken on a Shimadzu Model UV-200 spectrophotometer, in ethanol solution.

Fig. 2. The IR spectrum of the menaquinone isolated from *Actinomadura madurae* AJ 9136.

The spectrum was taken on a JASCO Model IRA-1 spectrophotometer, in carbon tetrachloride solution.

v/v). The compounds obtained from the three weak bands were analyzed by mass spectroscopy; they were menaquinone-9 (H_8), menaquinone-9 (H_8), and menaquinone-9 (H_4), respectively. The compound from the intense band at the R_f value of 0.50 was subjected to analysis as follows.

A yellowish orange-colored oil, weighing 12.3 mg, was obtained from 583 g (dry weight) of the bacterial cells harvested from the total volume of 217 l of the cultured media. The substance was analyzed by ultraviolet and infrared absorption spectrophotometry. The ultraviolet and infrared absorption spectra were very similar to those of menaquinone-9 (H_4) in *O. turbata* and of menaquinone-8 (H_4) in "*B. lipolyticum*" (7). The ultraviolet absorption maxima in ethanol were observed at 247, 263, and 328 nm in the spectrum as measured on a Shimadzu Model UV-200 double-beam spectrophotometer (Fig. 1). The infrared absorption spectrum on a JASCO Model IRA-1 spectrophotometer showed absorption bands at 1300 and 1330 cm^{-1} and at 718, 1600, 1620, and 1660 cm^{-1} , suggesting the presence of a quinone nucleus (Fig. 2). An intense C-H stretching band appeared at 2900 cm^{-1} with a shoulder at 2840 cm^{-1} , whereas C-C stretching bands due to C-CH₂ and C-CH₃ groups were exhibited at 1450 and 1380 cm^{-1} , respectively. Weak bands at 840 and 890 cm^{-1} suggested the presence of an isoprenoid chain. The above-mentioned data indicated that the isolated compound is a member of the menaquinone family.

Mass spectroscopy of menaquinone

The isolated menaquinone was analyzed by mass spectroscopy. As shown in Fig. 3, the mass spectrum showing the molecular ion peak at m/z 790 and intense fragment ion peaks at m/z 225 and 187 was typical of a menaquinone, having a hexahydrogenated isoprenoid side-chain with nine isoprene units (7). Besides the two intense fragment ion peaks, several weak fragment ion peaks were found at m/z 775 ($M-15$) and at m/z 583–447, indicating the expulsion of a methyl group and the successive loss of the isoprene units in the side chain, respectively. However, the menaquinone preparation obtained from the *A. madurae* strain did not exhibit any of the fragment ion peaks at m/z 721 ($M-69$) and 653 ($M-69-68$). The weak fragment ion peaks were not observed at m/z 379 ($447-68$), 311 ($447-68-68$), and 239 ($225+14$), as shown in a previous paper (7).

1H -Nuclear magnetic resonance spectroscopy of menaquinone

The 1H -NMR spectrum of the menaquinone-9 (H_8) isolated from the bacterial cells was measured at 100 MHz. The 1H -NMR spectrum shown in Fig. 4 represented the presence of all the signals observed in the menaquinone-9 (H_4) of *O. turbata* and the menaquinone-8 (H_4) of "*B. lipolyticum*" (7). The spectrum showed the following groups: $4 \times \phi-H$, δ 8.02–7.60; $6 \times -CH=C-$, δ 5.04; $Q-CH_2-$, δ 3.32 (doublet, $J=6.7$ Hz); $Q-CH_3$, δ 2.16; $10 \times =C-CH_2-$, δ 1.98; $=C-CH_3$ (*trans*, 1st unit from quinone ring), δ 1.78; $=C-CH_3$ (*cis*, 9th unit²), δ 1.66; $5 \times =C-CH_3$ (*trans*, internal and 9th units²), δ 1.57; $3 \times -CH_2-CH_2-CH-CH_2-$, δ 1.28; $3 \times -C-CH_3$, δ 0.90 (ϕ , benzene ring; Q, quinone ring) (12). The integrations of the proton signals at δ 1.28 and δ 0.90 due to the methylene-methine and the methyl groups of the hydrogenated isoprene units, respectively, were estimated to be in a

Fig. 3. The mass spectrum of the menaquinone isolated from *Actinomadura madurae* AJ 9136.

The spectrum was taken as described in the text. The weak fragment ion peaks were not found at m/z 721 ($M-69$) and 653 ($M-69-68$).

² Concerning the two methyl groups in the 9th isoprene unit, *cis* or *trans* is expressed on the basis of the olefinic proton in this paper.

Fig. 4. The ^1H -NMR spectrum of the menaquinone isolated from *Actinomadura madurae* AJ 9136.

The spectrum was taken as described in the text. The relative numbers of protons are based on the calibration value of the 4 protons attached to the benzene ring which represent the δ 8.02–7.60 signals.

Fig. 5. Saturation of the three double bonds of isoprene units in an isoprenoid side-chain of menaquinone.

The menaquinone isolated from *A. madurae* AJ 9136 was confirmed to have a side chain with three double bonds saturated. (a) When one double bond is saturated at the ω -terminal of the chain, the relative numbers of protons in the methylene-methine and the methyl groups are calculated to be in a ratio of 19:12. (b) In contrast, the numbers are in a ratio of 7:3 when all the three saturated units are located inside the chain. ○, methylene group; □, methine group; ●, methyl group.

ratio of 7:3 (Fig. 5). The data obtained above indicated that the menaquinone-9 (H_6) has all the three saturated units inside the isoprenoid side-chain.

Synthesis and ozonolysis of chromenyl acetate from menaquinone

The menaquinone-9 (H_6) (5.0 mg) obtained from the *A. madurae* strain was dissolved in 5.0 ml of benzene. The solution was refluxed in the presence of sodium hydride (10.0 mg) on a boiling water-bath for 2 hr under a nitrogen atmosphere, as described in a previous paper (9). The mixture was treated with 0.02 ml of acetic anhydride for 1 hr at room temperature. The reaction mixture, neutralized with 0.02 ml of acetic acid, was filtered, and the filtrate was concentrated to dryness under a reduced pressure. The residue was dissolved in a very small amount of acetone, and the solution was applied to chromatography on a silica gel plate using a solvent system of benzene-hexane-ether (2:4:1, by volume). An ultraviolet-absorbing and very slight yellow-colored band was found at the R_f value of 0.82, and the compound was eluted with a small volume of acetone. The material (2 mg) had in the ultraviolet spectrum absorption maxima at 222, 266, 276, 333, 347, and 366 nm, which were identical with those of the chromenyl acetates derived from vitamins K_1 and K_2 . The mass spectrum of the material synthesized from the menaquinone-9 (H_6) of the *A. madurae* strain showed the molecular ion peak at m/z 832 and fragment ion peaks at m/z 817 ($M-15$), 790 ($M-42$), 775 ($M-15-42$), 267, and 225. The spectral pattern was identical with those of the chromenyl acetates from the menaquinone-9 (H_4) of *O. turbata* and the menaquinone-8 (H_4) of "*B. lipolyticum*" (9).

The chromenyl acetate (1 mg) derived from the menaquinone-9 (H_6) of the *A. madurae* strain was dissolved in 4.0 ml of a mixture of chloroform-methanol (1:7, by volume), and was subjected to ozonolysis at -70° , as described previously (9). The ozone, which had passed through the solution, amounted to 0.28 mg (flow rate, 0.020 mg/sec; for 14 sec). The ozonide was reduced with 0.02 ml of dimethyl sulfide under a nitrogen atmosphere to give an aldehyde (9). The ozonolysis product was purified by thin-layer chromatography on a silica gel plate using a solvent system of benzene-hexane-ether (14:4:3, by volume). The ultraviolet-absorbing, hydrazine-reacting band was found on the plate at the R_f value of 0.60. The compound was eluted with a small volume of acetone, and was measured by mass spectroscopy. As shown in Fig. 6, the mass spectrum of the compound showed ion peaks at m/z 464 (M^+), 449 ($M-15$), 422 ($M-42$), 407 ($M-15-42$), 267, and 225. The spectrum was identical with those of the ozonolysis products derived from the menaquinone-9 (H_4) of *O. turbata* and the menaquinone-8 (H_4) of "*B. lipolyticum*" (9). The molecular ion peak at m/z 464 indicated that the two saturated isoprene units are located at the second and the third positions in the isoprenoid side-chain starting from the quinone ring. The third saturated unit was assumed to be at the eighth or the ninth position, because the weak fragment ion peaks were not found at m/z 721 ($M-69$) and 653 ($M-69-68$) due to

Fig. 6. The mass spectrum of the ozonolysis product of the chromenyl acetate derived from the menaquinone isolated from *Actinomadura madurae* AJ 9136.

The spectrum was taken as described in the text. The ultraviolet-absorbing, hydrazine-reacting band on the silica gel plate contained a very small amount of another aldehyde to give ion peaks at m/z 532 (M^+ , $464+68$), 517 ($M-15$), 490 ($M-42$), 475 ($M-15-42$), 267, and 225, as seen in a previous paper (9).

Fig. 7. The presumed structure of the menaquinone, having a hexahydrogenated isoprenoid side-chain with nine isoprene units, isolated from *Actinomadura madurae* AJ 9136.

the successive loss of isoprene units in the mass spectrum of the menaquinone-9 (H_6) of the *A. madurae* strain (Fig. 3). However, the 1H -NMR spectrum shown in Fig. 4 excluded the possibility that the saturated unit would be located at the ninth position. The results described above indicate that the structure of the menaquinone-9 (H_6) isolated from *A. madurae* AJ 9136 is formulated as shown in Fig. 7.

DISCUSSION

Our findings described above and in a previous paper (9) suggest that the saturation of the isoprenoid side-chain in the 2nd and the 3rd units from the quinone ring is characteristic of such polyhydrogenated menaquinones.

PHILLIPS et al. (13) pointed out, by use of 1H -NMR spectrometry, that one of the three saturated units in the hexahydrogenated isoprenoid side-chain of a menaquinone-9 isolated from *Streptomyces* species was located at the position distal

from the quinone ring. The positions of the remaining two saturated units were unresolved.

It was found in this study that the hexahydrogenated isoprenoid side-chain menaquinone-9 isolated from *Actinomadura madurae* AJ 9136 has a different chemical structure from that of the *Streptomyces* hexahydrogenated isoprenoid side-chain menaquinone-9; the former has a saturated unit not at the 9th but at the 8th position starting from the quinone ring. Thus, we obtained a new menaquinone homologue from a nocardioform bacterium, *A. madurae*, which can be designated as 2-methyl-3-II, III, VIII-hexahydromultiprenyl⁹-1, 4-naphthoquinone (I).

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