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ISOLATION AND CHARACTERIZATION OF A NOVEL VITAMIN-K FROM *EUBACTERIUM LENTUM*

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SUMMARY: A novel fat-soluble vitamin K like molecule was isolated from the prokaryote, *Eubacterium lentum*, and its structure investigated by mass spectrometry and proton nuclear magnetic resonance spectrometry. On the basis of these studies the novel quinone is shown to be 2,5 and 6- or 2,7 and 8-trimethyl-3-farnesylfarnesyl-1,4-naphthoquinone.

One of the major groups of bacterial respiratory quinones are the naphthoquinones of which there are two major types, phylloquinone (vitamin K_1) and menaquinone (vitamin K_2). Phylloquinone, 2-methyl-3-phytyl-1, 4-naphthoquinone (Fig. 1a), is normally associated with the green tissues of plants and its presence within the prokaryotes seems to be limited to the cyanobacteria (1). In contrast, menaquinones are more widely distributed amongst the prokaryotes and are constituents of the plasma membranes of many Gram-positive and negative bacteria (1). Menaquinones, 2-methyl-3-polyisoprenyl-1, 4-naphthoquinones (Fig. 1b), form a large class of molecules in which the length of the C-3 isoprenyl side-chain varies from 1 up to 15 isoprene units. In addition to differences in the number of isoprene units varying degrees of saturation or hydrogenation of the side-chain are found (1,2).

Abbreviations: MK-n, menaquinone containing n isoprene units; MMK-n, methyl-menaquinone containing n isoprene units; DMMK-n, dimethyl-menaquinone containing n isoprene units.

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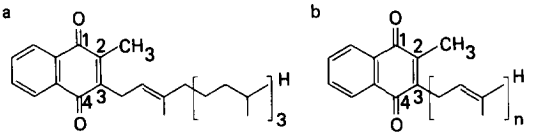

Fig. 1. Structure of (a) Phylloquinone (vitamin K_1) and (b) menaquinone (vitamin K_2).

Recently a new group of vitamin K molecules containing an additional methyl residue in the ring system (methyl-menaquinones) have been isolated from several prokaryotes (e.g. *Campylobacter*, *Thermoplasma*, *Wolinella*) (3,4,5,6). In this article we report the isolation of a hitherto unknown vitamin K containing two additional ring methyl groups. The results of mass spectrometry and proton nuclear magnetic resonance spectrometry show the quinone corresponds to 2,5 and 6 or 2,7 and 8-trimethyl-3-farnesylfarnesyl-1, 4-naphthoquinone.

MATERIALS AND METHODS

Culture and cultivation: *Eubacterium lentum* DSM 2243 (type strain) was obtained from the Deutsche Sammlung von Mikroorganismen, Gottingen, F.R.G. *Eubacterium lentum* was grown under anaerobic conditions on blood agar plates at 37°C for 5d. The strain was sub-cultured into a starter of 100ml Todd Hewitt (Oxoid) broth supplemented with L-arginine (0.5%) and incubated under an anaerobic atmosphere (90% H_2, 10% CO_2) at 37°C. After 48h the starter culture was transferred to 2L volumes of the above broth and incubated for 5d at 37°C. Culture purity was checked by microscopic examination and cells harvested by centrifugation (8000g). Cells were washed in physiological saline, recentrifuged and freeze-dried.

Extraction and analysis of quinones: Lipids were extracted from lyophilised cells by stirring with chloroform-methanol (2:1 v/v) for 2h. The cell-solvent mixture was filtered (to remove cell debris) and the lipid extract evaporated to dryness under reduced pressure using a rotary-evaporator at 40°C. Isoprenoid quinones were initially separated from other lipid and non-lipid components by preparative thin-layer chromatography (tlc) using Merck Kieselgel $60F_{254}$ plastic-backed sheets (10x10cm) with hexane-diethyl ether (85:15 v/v) as developing solvent (2). Quinones were revealed by brief irradiation under ultraviolet light (254nm) and eluted from the gel with chloroform. The separation of methylmenaquinone from the unknown quinone was achieved by tlc using triple development in a single dimension with hexane-diethyl ether (20:1 v/v) as developing solvent. Purified quinones were examined by high performance liquid chromatography (hplc) using a Laboratory Data Control liquid chromatograph fitted with a Merck Lichrosorb RP-18 (5μ) column (250mm x 4.6mm i.d.). Quinones were monitored at 269nm and eluted with methanol-1-chlorobutane (20:1 v/v) at 2ml/min (2). Ultraviolet spectra of the quinones were recorded on a Pye Unicam SP8-150 spectrophotometer using isooctane as solvent. Mass spectral analyses were performed using an AEI MS9 instrument using a direct insertion probe, an ionizing voltage of 70eV, and a temperature of 180°C. Proton nuclear magnetic resonance spectra were recorded on a Bruker WH40 spectrometer. Samples were dissolved in deuterated chloroform with tetramethylsilane as an internal standard.

RESULTS AND DISCUSSION

Thin layer chromatographic analysis of the lipid extract of *Eubacterium lentum* using hexane-diethyl ether (85:15 v/v) revealed the presence of two ultraviolet-absorbing bands at R_f approx. 0.7 and 0.8. On examination by ultraviolet spectroscopy, the lower component (R_f 0.7) displayed absorption maxima at 238 (shoulder), 242, 248, 259, 269 and 325nm in accordance with published data on menaquinones (1,2). This result was confirmed by mass spectrometry. The mass spectrum showed intense peaks at *m/z* 225 (base peak) and 187 derived from the naphthoquinone nucleus whereas the presence of a strong peak at *m/z* 580 attributable to molecular ions (M^+) demonstrated the quinone corresponded to MK-6.

The upper band (R_f 0.8) on examination by reverse-phase partition hplc revealed the presence of two quinones with equivalent chain lengths (2) of 6.6 and 6.9 (Fig. 2). Although neither of these quinones co-chromatographed

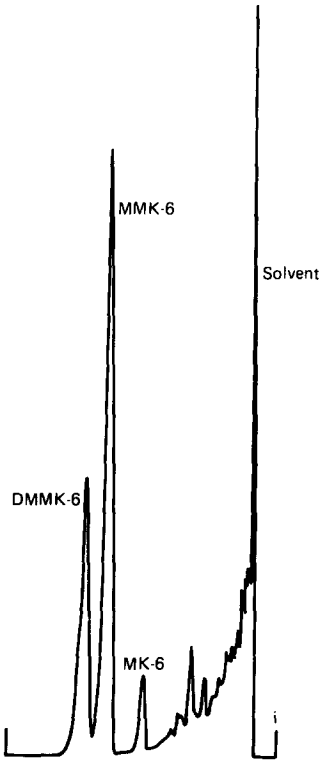

Fig. 2. Separation of MK-6, MMK-6 and DMMK-6 by reverse-phase partition hplc.

with reference menaquinones, the retention time of the faster eluting component was identical to methyl-substituted MK-6. The ultraviolet absorption spectrum (λmax 242, 248, 259, 269 and 339nm) of the quinone was consistent with this assignment. Upon mass spectrometry the quinone produced intense peaks at *m/z* 239 (base peak) and 201 in accordance with published data on methyl-menaquinones (3,4,5). The presence of a strong peak in the high mass region at *m/z* 594 (M^+) confirmed the quinone corresponded to MMK-6.

The third quinone possessed ultraviolet absorption characteristics quite distinct from those of menaquinone and methyl-menaquinone (2) with λmax at 245 (shoulder), 253, 262 (shoulder), 272 and 343nm. Upon mass spectrometry this unknown quinone produced a similar fragmentation pattern to that of menaquinones except that the base peak occurred at *m/z* 253 with a second intense peak at *m/z* 215 (Fig. 3). The most intense peak in the high mass region occurred at *m/z* 608 and was attributable to M^+ with a peak of lower intensity at *m/z* 593 due to the loss of CH_3 from M^+. Peaks of low intensity at *m/z* 539, 471, 403, 335 and 267 corresponded to C-3 side-chain fragmentation and were due to the sequential loss of one terminal (M^+-69) and four internal (68 mass units each) isoprene units, respectively (Fig. 3).

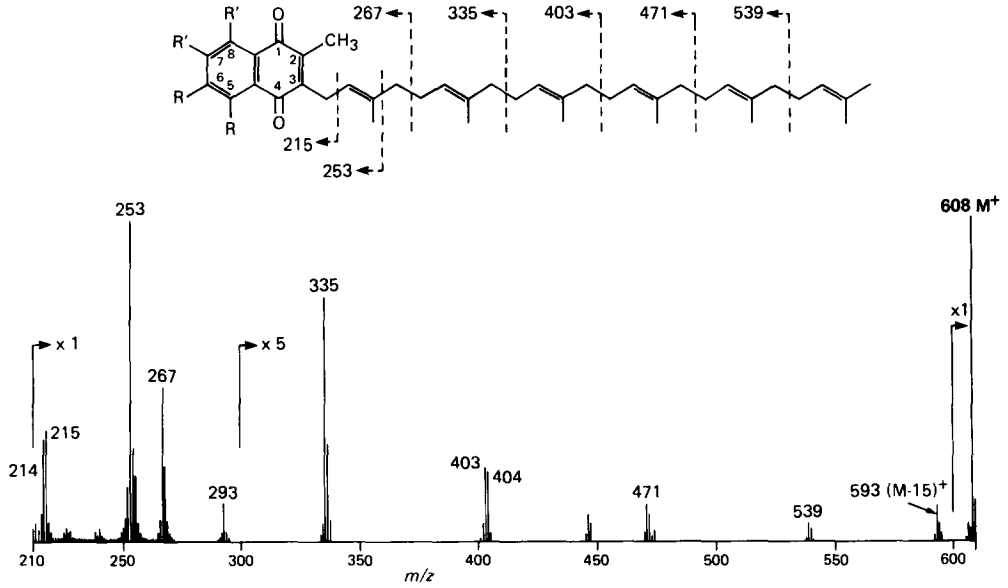

Fig. 3. Mass spectrum of DMMK-6 from *Eubacterium lentum* R and R' = CH_3 and H (not necessarily respectively).

High resolution analysis showed that *m/z* 608 corresponded to $C_{43}H_{60}O_2$ (accurate experimental mass 608.4594; theoretical mass 608.4593), whereas *m/z* 253 corresponded to $C_{17}H_{17}O_2$ (accurate experimental mass 253.1227, theoretical mass 253.1228). These data clearly indicate that the quinone is MK-6 containing two additional $-CH_2$ residues. Furthermore, the presence of intense peaks at *m/z* 253 and 215 (derived from the naphthoquinone nucleus) (Fig. 3) indicate that the two $-CH_2$ residues are in the ring system and not in the polyprenyl side-chain

The proton NMR spectrum of the unknown quinone revealed some major differences to that of menaquinones (Table 1). Menaquinones normally exhibit very complex absorption in the δ7.6 to 8.1 region due to the presence of four adjacent aromatic protons (2,7). The unknown quinone however revealed a relatively simple absorption in this region consisting of two doublets centred at δ7.893 (J = 7.8 Hz) and δ7.433 (J = 7.4 Hz). This absorption pattern is consistent with the presence of two ring protons (on adjacent carbon atoms) with the signal at δ7.895 corresponding to a proton at C-5 or C-8 whereas that at δ7.433 is due to a proton at position C-6 or C-7. In

Table 1. Comparison of the chemical shifts (δ) and splitting patterns for ^1H NMR of dimethylmenaquinone and menaquinone.

Assignment	DMMK	MK
Aromatic hydrogens	7.893 (doublet) (C-5 or C-8)	8.09-8.05 (multiplet) (C-5 and C-8)
	7.433 (doublet) (C-6 or C-7)	7.69-7.66 (multiplet) (C-6 and C-7)
Olefinic hydrogens	5.2-4.85 (multiplet)	5.2-4.85 (multiplet)
Allylic methylene next to ring	3.310 (doublet)	3.34 (doublet)
Ring methyls		
C-5 or C-8	2.663 (singlet)	absent
C-6 or C-7	2.400 (singlet)	absent
C-2	2.141 (singlet)	2.16 (singlet)
Allylic methylenes	2.1-1.9 (multiplet)	2.1-1.9 (multiplet)
trans-methyl first isoprene unit	1.765 (singlet)	1.77 (singlet)
cis-end of chain methyl	1.665 (singlet)	1.67 (singlet)
trans-internal methyl groups	1.6-1.5 (broad signals)	1.6-1.5 (broad signals)

addition, the quinone produced two singlets at δ2.663 and δ2.400 corresponding to the two additional ring methyl groups. The signal at δ2.663 clearly corresponded to the presence of CH_3 at C-5 or C-8 of the ring whereas that at δ2.400 is attributable to CH_3 at positions C-6 or C-7. These assignments were confirmed by nuclear Overhauser enhancement difference spectroscopy, although these experiments were not able to distinguish the 5,6-dimethyl compound from the 7,8-dimethyl alternative. The remaining signals in the spectrum of the quinone were essentially similar to those of menaquinones, and correspond to the C-2 ring methyl (δ2.141) and contributions from the C-3 hexaprenyl side-chain (δ4.85 - 5.2, olefinic protons; δ3.31,-CH_2-adjacent to ring; δ1.9 - 2.1,-CH_2-allylic; δ1.765, *trans*-CH_3 next to ring; δ1.665, *cis*-CH_3 end of chain; δ1.5 - 1.6, *trans*-CH_3) (Table 1). Thus the ^1H absorption due to ring-methyls together with the splitting patterns of the aromatic protons indicate the novel quinone corresponds to either 2,5 and 6-trimethyl-3-farnesylfarnesyl-1, 4-naphthoquinone or 2,7 and 8-trimethyl-3-farnesylfarnesyl-1, 4-naphthoquinone (Fig. 4). Unfortunately ^1H NMR does not facilitate the distinction of these two closely related structures and although this could be achieved by degradative studies the latter would require large amounts of quinone.

It is now well established that vitamin K and related compounds play an important role in aerobic and anaerobic electron transport and other biochemical reactions (8,9,10). For example MK acts as a low potential (E'_o = -74mV) reversible redox component of electron transport chains, shuttling between dehydrogenases and iron-sulphur proteins, cytochromes and reductases. Although the function of the novel dimethyl-menaquinone from *Eubacterium lentum* is not known it seems probable that this also may have a

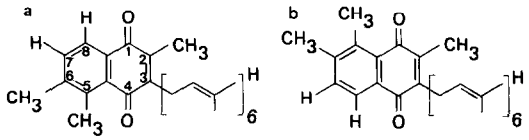

Fig. 4. Structure of DMMK-6 from *Eubacterium lentum*.

respiratory or proton porter function. Furthermore, the redox potential of this quinone would be expected to be different from that of menaquinone due to the presence of two additional ring methyl substituents. Electron-donating methyl groups would tend to stabilize the oxidized form of the quinone relative to the reduced (hydroquinone) form and result in a lower redox potential for DMMK. It is interesting to speculate that this difference in redox potential may enable DMMK to participate in different cellular reactions.

ACKNOWLEDGEMENTS

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