Synthesis and Biological Activity of Derivatives of Ubiquinone: Photoaffinity Analogues Containing the 4-Azido-2-nitroanilino Group

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ABSTRACT: The photoaffinity analogues of ubiquinone 2,3-dimethoxy-5-methyl-6-[2-[1-oxo-3-(4-azido-2-nitroanilino)propoxy]-3-methylbutyl]-1,4-benzoquinone (2′-ANAP-Q-1) and 2,3-dimethoxy-5-methyl-6-[3-[1-oxo-3-(4-azido-2-nitroanilino)propoxy]-3-methylbutyl]-1,4-benzoquinone (3′-ANAP-Q-1) have been synthesized. The required intermediate alcohols 2,3-dimethoxy-5-methyl-6-(2-hydroxy-3-methylbutyl)-1,4-benzoquinone and 2,3-dimethoxy-5-methyl-6-(3-hydroxy-3-methylbutyl)-1,4-benzoquinone were prepared in good yield from ubiquinone 1 by hydration of the side-chain double bond via hydroboration or acid catalysis, respectively. These alcohols were then coupled with 3-(4-azido-2-nitroanilino)propanoic acid, with *p*-toluenesulfonyl chloride in dry pyridine, to give 2′- and 3′-ANAP-Q-1. The synthetic methods presented should be of general utility in the preparation of derivatives of ubiquinone in which a reactive or reporter group is relatively close to the ubiquinone ring. By use of membrane vesicles prepared from a *ubi⁻men⁻* strain of *Escherichia coli* described previously [Wallace, B., & Young, I. G. (1977) *Biochim. Biophys. Acta 461*, 84-100], it has been shown that 2′- and 3′-ANAP-Q-1 substitute for ubiquinone 8 in the NADH, succinate, and D-lactate oxidase systems. Thus, these compounds may be of value in labeling respiratory chain proteins that interact with ubiquinone.

Recently there has been increased interest in the role of ubiquinone (Q)[1] in the aerobic respiratory chain. Controversy has developed over the nature of the involvement of Q in electron transfer between the primary dehydrogenases and other components of the respiratory chain. Yu & Yu (1981) have proposed that Q bound to various "Q-binding proteins" acts as the actual electron carrier. The more classical view that the bulk of the Q functions as a free, mobile carrier in mitochondria (Kröger & Klingenberg, 1973a,b) or *Escherichia coli* (Wallace & Young, 1977a,b) has recently received strong support from studies involving fusion of phospholipid liposomes containing different levels of ubiquinones with mitochondrial inner membranes (Schneider et al., 1982).

In an attempt to facilitate definition of the Q binding site(s) at the structural level, we have designed and synthesized analogues of Q incorporating the 4-azido-2-nitroanilino group, which, on photolysis, generates a highly reactive nitrene capable of C-H bond insertion (Bayley & Knowles, 1977, Chowdhry & Westheimer, 1979).

In the present paper we report simple, high-yield procedures for synthesis of Q derivatives containing the 4-azido-2-nitroanilino group close to the ubiquinone nucleus. These procedures should be of general utility for synthesis of biochemically interesting derivatives of Q. In addition, we report on the activity of the synthesized analogues as electron carriers in the NADH, succinate, and D-lactate oxidase systems, by use of a mutant strain of *E. coli* completely lacking Q, MK, and DMK (Wallace & Young, 1977b).

Experimental Procedures

Materials. Q-0 and Q-1 were prepared by reported methods (Mayer & Isler, 1971) or were generous gifts from Hoffman-La Roche and Co., Basel. Q-0 is commercially available from Pfaltz and Bauer. The purity of Q-0 and Q-1 samples was monitored by TLC and by ultraviolet, proton magnetic resonance, and mass spectrometries.

4-Fluoro-3-nitroaniline and DMAP were from Ega-Chemie. NaN_3 (purum) was from Fluka, who also supplied boron trifluoride diethyl etherate, triethylamine, DMF, and DCCD (puriss). Freshly opened THF from Mallinckrodt was dried over $MgSO_4$ or, for coupling reactions, in small volumes over CaH_2. Ethanol and methanol (Merck) were redistilled. Purified, dry pyridine was a gift from Dr. D. Magrath and was stored over molecular sieve 4A. *N,N′*-Carbonyldiimidazole (mp 114-119 °C) was from Sigma, and imidazole (Merck) was recrystallized from benzene. Silver oxide and *p*-toluenesulfonyl chloride (98.5% minimum, mp 67-70 °C) were from Ajax Chemicals Ltd., Sydney.

3-Aminopropanoic acid was from Sigma, and [1-^{14}C]-3-aminopropanoic acid (54.7 Ci/mol) was from New England Nuclear. Sodium borohydride and silica gel 60 TLC plates (F_{254}) were from Merck. All solvents used were of analytical grade, and all other chemicals used were of analytical grade or the best grade available.

Melting Points. Melting points were recorded on an Electrothermal melting point apparatus and are uncorrected.

Spectrometry. Proton magnetic resonance spectra were recorded on a JEOL FX-90Q Fourier-transform nuclear magnetic resonance spectrometer at 30 °C. Samples (~1 mg)

[1] Abbreviations: Q, ubiquinone; MK, menaquinone; DMK, demethylmenaquinone; Q-*n*, ubiquinone isoprenologue containing *n* isoprene units in the side chain; Q-0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; 2′-hydroxy-Q-1, 2,3-dimethoxy-5-methyl-6-(2-hydroxy-3-methylbutyl)-1,4-benzoquinone; 3′-hydroxy-Q-1, 2,3-dimethoxy-5-methyl-6-(3-hydroxy-3-methylbutyl)-1,4-benzoquinone; ANAP, 3-(4-azido-2-nitroanilino)propanoic acid; FNPA, 4-fluoro-3-nitrophenyl azide; 2′-ANAP-Q-1, 2,3-dimethoxy-5-methyl-6-[2-[1-oxo-3-(4-azido-2-nitroanilino)-propoxy]-3-methylbutyl]-1,4-benzoquinone; 3′-ANAP-Q-1, 2,3-dimethoxy-5-methyl-6-[3-[1-oxo-3-(4-azido-2-nitroanilino)propoxy]-3-methylbutyl]-1,4-benzoquinone; THF, tetrahydrofuran; DMF, *N,N*-dimethylformamide; DCCD, *N,N′*-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; Me_4Si, tetramethylsilane; $NaDodSO_4$, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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were dissolved in $CDCl_3$ (~0.5 mL), and chemical shifts are expressed relative to the internal standard Me_4Si. Typically, 100–500 pulses were accumulated. Electron-impact (70 eV) and chemical-ionization (NH_3) mass spectra were recorded on a VG-Micromass 7070 F mass spectrometer fitted with a direct-insertion probe. Visible and ultraviolet spectra were recorded on a Cary 118 recording spectrophotometer or, for routine spectra, on a Varian series 634 scanning spectrophotometer equipped with a chart recorder. Liquid scintillation spectrometry was performed on a Packard Tri-Carb 460 CD liquid scintillation spectrometer, with a xylene/Triton X-100 based scintillant.

Determination of Quinone Concentrations. The concentration of stock solutions of ubiquinones and ubiquinone derivatives was determined by borohydride reduction in ethanol as described previously (Crane & Barr, 1971).

Determination of Protein. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard, as described previously (Campbell, 1983; Campbell & Young, 1983). Values were corrected according to the results of amino acid analysis (Jaworowski et al., 1981b).

Enzyme Preparation. Highly purified *E. coli* NADH: ubiquinone oxidoreductase was prepared as described previously (Jaworowski et al., 1981a,b).

Enzyme Assays. NADH:ubiquinone-1 oxidoreductase activity was measured at 30 °C, pH 7.5, as described previously (Jaworowski et al., 1981a).

Reconstitution of Oxidases in ubi⁻men⁻ Membranes. Preparation of membranes from the *E. coli* K12 derivatives AN384 (*ubi⁻men⁻*) and AN387 (*ubi⁺men⁺*) was accomplished as described previously (Wallace & Young, 1977b). AN387 and AN384 are isogenic transductants.

Polarographic assay of NADH, succinate, and D-lactate oxidases was performed as previously described (Wallace & Young, 1977b). Quinones were added as a solution in ethanol (stock concentration 1–1.5 mM).

Synthesis of 2′-Hydroxy-Q-1. To 37 μmol of Q-1 in 400 μL of THF was added 8 mg of $NaBH_4$. The mixture was incubated at room temperature for 10 min, during which time the yellow color of the quinone disappeared, indicating reduction to the quinol. Boron trifluoride diethyl etherate (64 μL) plus 236 μL of THF were added, and after 2-h incubation at room temperature protected from light, 120 μL of water was added. When effervescence had ceased (5 min), 20 μL of NaOH (30% w/v) followed by 20 μL of H_2O_2 (30% w/v) was added to oxidize the organoborane. The mixture was then acidified with 75 μL of 2 N H_2SO_4, and the quinol was extracted with 3 × 1 mL of $CHCl_3$. Silver oxide (15 mg) was added to the chloroform extract, which was shaken in the absence of light for 3 h, and then filtered through Celite. The Celite was washed with further $CHCl_3$. The combined filtrates were rotary evaporated and dissolved in 1 mL of $CHCl_3$, for loading onto two silica gel TLC plates (20 cm × 20 cm × 0.25 mm), which were developed in $CHCl_3$. The 2′-hydroxy-Q-1 band (golden yellow, R_f ~0.30) was eluted with ethanol. The yield was 30–50% on the basis of Q-1.

Synthesis of 3′-Hydroxy-Q-1. A 3-mL tube containing 20.5 μmol of solvent-free Q-1 was chilled to −10 °C, and 0.5 mL of 67% (v/v) H_2SO_4 (−10 °C) was added with rapid stirring. The mixture was stirred for 60 s at −10 °C, and then ~1-mL of ice and water was added. The yellow quinone was then extracted 4 times with ~1 mL of diethyl ether (total 4 mL). The ether extract was washed 3 times with saturated NaCl solution, rotary evaporated, and redissolved in a small volume of diethyl ether. The solution was loaded onto a silica gel TLC plate (20 cm × 20 cm × 0.25 mm), which was developed in diethyl ether. The golden yellow hydroxyquinone band was eluted with ethanol. The yield was 80–90% on the basis of Q-1.

Preparation of ANAP. FNPA was prepared from 4-fluoro-3-nitroaniline as described (Fleet et al., 1972). After one recrystallization from petroleum ether (40–60 °C), FNPA had a mp of 53–53.5 °C [lit. mp 52 °C (Fleet et al., 1972) and 52–52.5 °C (Guillory & Jeng, 1977)] and appeared homogeneous by TLC on silica (1-butanol/water, 86:14 v/v, R_f 0.83).

ANAP was synthesized as described previously (Guillory & Jeng, 1977; Jeng & Guillory, 1975). The compound began to soften at 142 °C with sudden decomposition at 147 °C (lit. mp 142.5–145 °C, Guillory & Jeng, 1977). It appeared homogeneous by TLC on silica (1-butanol/water, 86:14 v/v, R_f 0.51). Syntheses and experimental procedures involving compounds containing the azide group were carried out in subdued light or in the dark.

Coupling of 2′- and 3′-Hydroxy-Q-1 with ANAP. Typically, to 13 μmol of solvent-free 2′-hydroxy-Q-1 in a 3-mL test tube was added 1 equiv of ANAP and pyridine (100 μL), followed by 2–2.5 equiv of *p*-toluenesulfonyl chloride. The tube was sealed, briefly warmed (50 °C), and shaken to achieve a homogeneous appearance and then incubated at room temperature overnight. Pyridine was thoroughly removed from the reaction mixture by rotary evaporation (50 °C), and the sample was taken up in 200 μL of CH_2Cl_2 for loading onto a silica gel TLC plate (20 cm × 20 cm × 0.25 mm). The plate was developed 10 cm in $CHCl_3$, dried, and then redeveloped 20 cm in $CHCl_3$. 2′-ANAP-Q-1 was identified as the major orange band at the center of the plate.

The product band was eluted with ethanol. The yield was ~55% on the basis of 2′-hydroxy-Q-1 or ANAP. 2′-ANAP-Q-1 appeared to be a viscous red oil at room temperature, which solidified on ice. It was stored as a solution in ethanol, protected from light, at −20 °C.

3′-Hydroxy-Q-1 was also esterified by this method. 3′-Hydroxy-Q-1 (69 μmol) was reacted for 48 h with 1 equiv of ANAP and 2.5 equiv of *p*-toluenesulfonyl chloride in 500 μL of dry pyridine. After TLC as described above (on a 20 cm × 20 cm × 1 mm plate), a number of colored bands were observed. These were eluted, and their ultraviolet spectra in ethanol (±$NaBH_4$) were examined. One had the expected ultraviolet spectral characteristics and was further purified by silica gel TLC (20 cm × 20 cm × 0.25 mm) with $CHCl_3$/diethyl ether, 95:5 v/v, as solvent (R_f 0.50). The overall yield was 3% from 3′-hydroxy-Q-1. A large amount of unreacted 3′-hydroxy-Q-1 was recovered (61% final recovery after further purification by silica gel TLC with diethyl ether as solvent).

Other Methods of Esterification. The first samples of 2′-ANAP-Q-1 were obtained with *N,N′*-carbonyldiimidazole as coupling agent, with catalysis by sodium imidazolide (Staab & Mannschreck, 1962; Paul & Anderson, 1960). Typically, 1 equiv of 2′-hydroxy-Q-1, 2 equiv of ANAP and *N,N′*-carbonyldiimidazole, and 0.2 equiv of sodium imidazolide were employed, with THF as solvent. Reagents were dried in vacuo over P_2O_5 before use. Yields varied from 2 to 12%.

Esterification of 2′-hydroxy-Q-1 was also achieved with DCCD as coupling agent, with catalysis by DMAP (Neises & Steglich, 1978). Typically, 1 equiv each of 2′-hydroxy-Q-1 and ANAP was used, with 1.5 equiv DCCD and 0.1 equiv of DMAP in dry DMF as solvent. The yield was ~10%.

Although the secondary alcohol group of 2′-hydroxy-Q-1 could be esterified with ANAP by these methods, both

methods failed with the more hindered tertiary alcohol group of 3′-hydroxy-Q-1.

The coupling products from 2′-hydroxy-Q-1 obtained by the three different esterification methods cochromatographed on silica gel TLC ($CHCl_3$) and exhibited identical visible and ultraviolet spectral properties ($\pm NaBH_4$).

Preparation of [^{14}C]ANAP. [1-^{14}C]-3-Aminopropanoic acid (50–100 μCi) was reacted with FNPA as described previously (Guillory & Jeng, 1977) except that a 6-fold molar excess of FNPA was employed, and the reaction was carried out at 55 °C for 15 h (yield based on ^{14}C = 57–67% after thin-layer chromatography).

Coupling of 2′-Hydroxy-Q-1 with [^{14}C]ANAP. Initial samples of the ^{14}C-labeled azidoquinone were prepared by the DCCD coupling method described above (yield 1.5–8%). Much higher yields (56–72%) were obtained by the *p*-toluenesulfonyl chloride coupling method described above. In these syntheses, the ^{14}C-labeled ANAP was diluted 5-fold with unlabeled material prior to coupling. With the *p*-toluenesulfonyl chloride method, from 50 μCi of [1-^{14}C]-3-aminopropanoic acid, 12 μmol of [^{14}C]-2′-ANAP-Q-1 with a specific radioactivity of 3×10^6 dpm/μmol was typically obtained (overall radiochemical yield from [1-^{14}C]-3-aminopropanoic acid, 36%). The radioactive quinone was stored as a solution in ethanol or methanol at −20 °C, protected from light.

Labeling of E. coli Respiratory NADH Dehydrogenase with [^{14}C]-2′-ANAP-Q-1. In a typical experiment, 2 mL of purified NADH dehydrogenase [5.8 mg of protein/mL dialyzed vs. 5 mM potassium phosphate buffer, pH 7.5, containing 0.1% (w/v) potassium cholate, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 20 μM FAD] was placed in a 3-mL stoppered quartz cuvette and after addition of 15 μL of an ethanol solution of [^{14}C]-2′-ANAP-Q-1 (61 mM, sp. act. 3×10^6 dpm/μmol) was irradiated at 4 °C for 3 min as described below. After addition of a further 15 μL of [^{14}C]-2′-ANAP-Q-1, irradiation was continued for 3 min. Finally, a further 20 μL of [^{14}C]-2′-ANAP-Q-1 was added, and irradiation was continued for 6 min. For photoirradiation, cuvettes containing samples were held horizontally, 20 cm above an ultraviolet light source (C-63 Mineralight, 302 nm, Ultraviolet Products Inc.). Under the conditions used, the cuvette contents remained at 4 °C. Control experiments showed only a small loss of NADH:Q-1 oxidoreductase activity (<20%).

The enzyme was then precipitated and washed with acetone (Poulis et al., 1981), and the dried pellet was dissolved in 0.7 mL of 5 mM potassium phosphate buffer, pH 7.5, containing 10% (w/v) $NaDodSO_4$ and 5 μL of 2-mercaptoethanol, by heating for 10 min at 100 °C. The protein was immediately chromatographed at room temperature on a column (25 × 1.6 cm) of Sephacryl S-300 equilibrated with 5 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 2% (w/v) $NaDodSO_4$, and 0.1% (v/v) 2-mercaptoethanol (Poulis et al., 1981). The radioactivity and protein concentration of the effluent fractions were determined. In experiments where the effect of added Q-1 was examined, two samples of enzyme were treated in parallel in a fashion similar to that described above, except that one sample contained 0.6 mM Q-1, added before irradiation.

RESULTS

Preparation of 2′- and 3′-Hydroxy-Q-1. Both 2′- and 3′-hydroxy-Q-1 were prepared from the readily available starting material Q-1 (Figure 1). In order to synthesize the anti-Markownikoff (Markownikoff, 1890) addition product 2′-hydroxy-Q-1, the quinone nucleus of Q-1 was reduced to the quinol with sodium borohydride in dry THF. Hydroboration

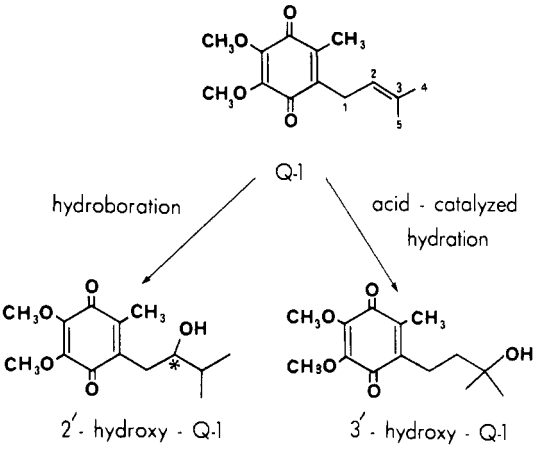

FIGURE 1: Preparation of 2′- and 3′-hydroxy-Q-1 from Q-1. The numbering of the carbon atoms of the isoprenoid side chain is indicated on the structure of Q-1. The asymmetric carbon atom in the side chain of 2′-hydroxy-Q-1 is indicated by an asterisk.

of the side-chain double bond was then accomplished with diborane generated in situ from boron trifluoride and sodium borohydride. The resulting alkylborane was oxidized with alkaline hydrogen peroxide to yield the quinol form of 2′-hydroxy-Q-1. The quinol was extracted into chloroform and oxidized with silver oxide to give the quinone, which was purified by preparative TLC. The yield was 30–50%. Markownikoff addition of H_2O to the side-chain double-bond to give 3′-hydroxy-Q-1 was catalyzed by treatment with cold 67% (v/v) H_2SO_4, followed by dilution with water. The crude hydroxyquinone was extracted with ether and purified by preparative thin-layer chromatography. The yield was 80–90%.

3′-Hydroxy-Q-1 has been prepared previously by ferric chloride oxidation of the 6-chromanol derived from Q-1 (Wagner et al., 1963), although no details of the preparation were given.

The structures of 2′- and 3′-hydroxy-Q-1 were verified by several means. The synthetic methods used were simple and were expected to yield the anti-Markownikoff and Markownikoff addition products 2′- and 3′-hydroxy-Q-1, respectively. The two compounds obtained appeared pure and could be separated from each other by TLC on silica [(diethyl ether) 2′-hydroxy-Q-1, R_f 0.85, 3′-hydroxy-Q-1 R_f 0.70; ($CHCl_3$/ethanol, 90:10 v/v) 2′-hydroxy-Q-1, R_f 0.74; 3′-hydroxy-Q-1, R_f 0.68], where both appeared considerably more polar than Q-1. Other solvent systems tested did not resolve the two hydroxyquinones, although both appeared homogeneous [acetone, R_f 0.57; 1-butanol/H_2O (86:14 v/v) R_f 0.72].

Ultraviolet spectra of the two compounds in ethanol were very similar, with λ_{max} = 279 nm, slightly shifted from the λ_{max} of Q-1 (275 nm). Both spectra showed typical borohydride reducibility (Crane & Barr, 1971) to yield the spectra of the quinols.

Proton magnetic resonance spectra of the compounds were consistent with their proposed structures. For 2′-hydroxy-Q-1, NMR showed the following: 0.96 and 1.04 (d, 4′,5′-CH_3), 2.06 (s, 5-CH_3), 2.57 and 2.60 (d, 1′-CH_2), 2.65 (s, 1′-CH_2), 3.46 (m, 2′-CH), and 3.99 and 4.00 ppm (d, 2,3-OCH_3). For 3′-hydroxy-Q-1, NMR showed the following: 1.28 (s, 4′,5′-CH_3), 1.52 (m, 2′-CH_2), 2.03 (s, 5-CH_3), 2.59 (m, 1′-CH_2), and 3.99 ppm (s, 2,3-OCH_3).

In proton magnetic resonance spectra of 3′-hydroxy-Q-1, the terminal methyl groups of the side chain appear as a singlet, whereas in 2′-hydroxy-Q-1 they are split into a doublet by interaction with the hydrogen on C-3′. Additionally, in

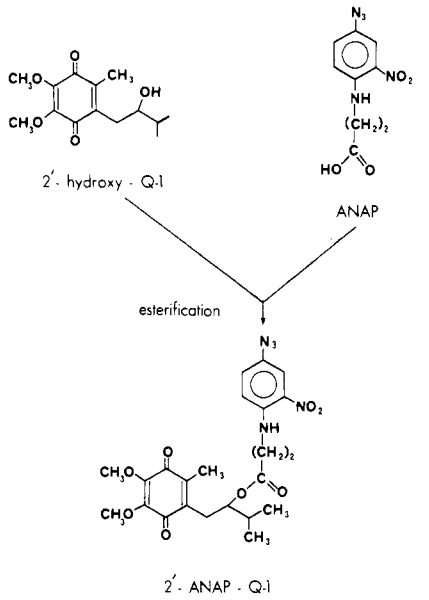

FIGURE 2: Preparation of 2′-ANAP-Q-1 from 2′-hydroxy-Q-1 and ANAP. This procedure should be of general utility in the coupling of carboxylic acids containing desired functional groups with 2′-hydroxy-Q-1 (see text). 3′-Hydroxy-Q-1 is coupled with ANAP in the same way.

3′-hydroxy-Q-1 the terminal methyl groups of the side chain appear further downfield due to the inductive effect of the 3′-hydroxyl group. The pattern of an apparent singlet and doublet from the 1′-CH_2 of 2′-hydroxy-Q-1 may arise as a result of the chirality of C-2′, combined with some degree of restricted rotation about the 2′–3′ carbon–carbon bond brought about by intramolecular hydrogen bonding of the 2′-hydroxyl group to the nearby quinone carbonyl oxygen. This could also account, at least in part, for the difference in R_f values of the two hydroxyquinones on TLC. Note, however, that 2′- and 3′-ANAP-Q-1 can also be separated by TLC in appropriate solvent systems (see below).

Mass spectra (electron impact) of the two hydroxyquinones each showed a prominent pair of peaks at m/e 268 and 270, attributed to the quinone and quinol forms (M and M + 2, respectively). The measured mass of the m/e 268 peak in the high-resolution mass spectrum of 2′-hydroxy-Q-1 was 268.1306 (cf. 268.1311 calcd. for $C_{14}O_5H_{20}$).

Both hydroxyquinones were capable of ester formation with a carboxylic acid and, as expected, it proved easier to esterify the secondary hydroxyl group of 2′-hydroxy-Q-1 than the tertiary hydroxyl group of 3′-hydroxy-Q-1 (see below).

Preparation of 2′- and 3′-ANAP-Q-1. The strategy for synthesis of the required derivatives of ubiquinone involved esterification of the carboxyl group of a carboxylic acid containing desired substituents with the side-chain hydroxyl group of 2′- or 3′-hydroxy-Q-1. Figure 2 illustrates the preparation of 2′-ANAP-Q-1 from 2′-hydroxy-Q-1 and ANAP. Several methods of esterification were tested (see Experimental Procedures).

High yields (~55%) of 2′-ANAP-Q-1 were obtained by using *p*-toluenesulfonyl chloride as coupling agent in dry pyridine (Brewster & Ciotti, 1955). This method proved the key to the synthesis of ^{14}C-labeled 2′-ANAP-Q-1 of high specific radioactivity and in good yield. In this connection, we modified the conditions of reaction of FNPA with [^{14}C]-3-aminopropanoic acid (Guillory & Jeng, 1977) to obtain a considerably higher radiochemical yield of [^{14}C]ANAP. Thus, the overall radiochemical yield of [^{14}C]-2′-ANAP-Q-1 from [^{14}C]-3-aminopropanoic acid was >30%.

Although the tertiary alcohol group of 3′-hydroxy-Q-1 was successfully esterified with ANAP by the method of Brewster & Ciotti (1955), the yield of 3′-ANAP-Q-1 was low (3%). This route to ubiquinone derivatives containing desired substituents on the isoprenoid side-chain could probably be improved by inclusion of DMAP or 4-pyrrolidinopyridine as acylation catalyst (Höfle et al., 1978), although we have not pursued this further at this stage.

Characterization of 2′- and 3′-ANAP-Q-1. Ultraviolet spectra of 2′- and 3′-ANAP-Q-1 in ethanol (±$NaBH_4$) were similar to the spectra of the 3-(4-azido-2-nitroanilino)propoxy-substituted ubiquinone reported by Yu & Yu (1982). The 4-azido-2-nitroanilino group was quantitated from the 450-nm absorption peak, which is independent of the quinone absorption, as well as the 260-nm absorption peak after $NaBH_4$ reduction, with a small correction for absorption by the quinol moiety. The ubiquinone moiety was quantitated from the absorbance change on $NaBH_4$ reduction (Crane & Barr, 1971). These analyses showed that for both 2′- and 3′-ANAP-Q-1 1.0 mol of the 4-azido-2-nitroanilino group is present per mole of ubiquinone moiety.

Both 2′- and 3′-ANAP-Q-1 appeared homogeneous by TLC on silica with a variety of different solvent systems, most of which ($CHCl_3$, R_f 0.50; acetone, R_f 0.87; ethyl acetate, R_f 0.77; acetonitrile, R_f 0.81) did not resolve the 2′- and 3′-compounds. Interestingly, it was found that the two esters could be resolved on silica gel TLC with diethyl ether as the mobile phase, although the R_f values were high (~0.95). Good separation was achieved by use of diethyl ether/petroleum ether (40–60 °C), 2:1 (v/v), as the mobile phase (2′-ANAP-Q-1, R_f 0.55; 3′-ANAP-Q-1, R_f 0.49). The order of migration of the two esters is the same as that of the parent alcohols (see above).

Chemical ionization mass spectra of 2′-ANAP-Q-1 showed prominent peaks at m/e 504 and 502, attributed to the quinol and quinone forms of the protonated ester (M + 3 and M + 1, respectively). Electron impact mass spectra of 2′-ANAP-Q-1 showed strong peaks at m/e 475 and 473, attributed to the quinol and quinone forms after loss of N_2 from the azido group (M − 16 and M − 18, respectively).

2′- and 3′-ANAP-Q-1 as Substrates for Purified NADH Dehydrogenase. Both 2′- and 3′-ANAP-Q-1 acted as acceptors for the purified respiratory NADH dehydrogenase of *E. coli*. Assays were performed as described previously for the NADH:Q-1 activity of the enzyme (Jaworowski et al., 1981a), except that various amounts of 2′- or 3′-ANAP-Q-1 in ethanol replaced the Q-1. In both cases, the maximum activity was 30–60% of that observed with Q-1. The nature of the velocity vs. substrate concentration plots suggested some form of substrate inhibition, although the kinetics have not been further characterized.

Covalent Labeling of Purified NADH Dehydrogenase with [^{14}C]-2′-ANAP-Q-1. After photoirradiation of purified *E. coli* NADH dehydrogenase in the presence of [^{14}C]-2′-ANAP-Q-1, the extent of covalent labeling was assessed by gel filtration of the treated protein in the presence of $NaDodSO_4$. This procedure has been shown to completely separate endogenous FAD, phospholipid (Jaworowski et al., 1981b), and ubiquinone 8 (Campbell & Young, 1983) from the enzyme polypeptide. On chromatography of [^{14}C]-2′-ANAP-Q-1-treated enzyme, two peaks of radioactivity are observed, the first of which is coincident with the protein peak. The extent of incorporation of label was typically 0.1–0.15 mol of [^{14}C]-2′-ANAP-Q-1/mol of protein (47 200 g). However, the presence of Q-1 during photoirradiation did not cause any reduction in the observed incorporation, suggesting that the labeling is nonspecific.

Table I: Restoration of Oxidase Activities in *ubi⁻men⁻* Membranes by Ubiquinone Analogues

membrane	quinone added[a]	oxidase activity[b] NADH	succinate	D-lactate
AN384 (*ubi⁻men⁻*)	Q-3	(100)	(100)	(100)
	Q-1	71	93	100
	2′-ANAP-Q-1	65	71	53
	3′-ANAP-Q-1	59	91	87
	nil	4	4	8
AN387 (*ubi⁺men⁺*)	Q-3	(100)	(100)	(100)
	Q-1	64	104	75
	2′-ANAP-Q-1	76	100	88
	3′-ANAP-Q-1	ND[c]	ND	ND
	nil	76	96	81

[a] A total of 50 nmol of quinone added per reaction (2.5 mL). [b] Activities are expressed as a percentage of the activity observed with Q-3. Absolute rates with Q-3 were similar to those reported previously (Wallace & Young, 1977b). [c] Not determined.

Control experiments showed that there was no detectable incorporation in the absence of photoirradiation.

Reconstitution of Oxidase Activities in ubi⁻men⁻ Membranes by 2′- and 3′-ANAP-Q-1. Membranes prepared from AN384, which are completely deficient in both ubiquinone and menaquinones (Wallace & Young, 1977b), were used to test the effectiveness of 2′- and 3′-ANAP-Q-1 in various oxidase systems. The NADH, succinate, and D-lactate oxidase systems are all quinone-dependent, so that the activity of these systems in unsupplemented AN384 membranes is very low. Previously, it was shown that Q-3 was effective in restoration of the oxidase activities in AN384 membranes (Wallace & Young, 1977b); i.e., in these oxidase systems, Q-3 could substitute for the Q-8 normally present in wild-type membranes.

In the present work, we have used this system to test the biological activity of 2′- and 3′-ANAP-Q-1. Both 2′-ANAP-Q-1 and 3′-ANAP-Q-1 showed good restoration of NADH, succinate, and D-lactate oxidases (Table I). This shows that in all three oxidase systems, the two photoaffinity analogues can effectively substitute for Q-8 at all points involved in electron transfer between substrate and molecular oxygen. We have also shown that the same is true of Q-1 (Table I).

Discussion

In the present work, we have prepared two derivatives of Q containing the photoactivatable 4-azido-2-nitrophenyl group. The azidophenyl group is located the equivalent of seven or eight methylene units from the benzoquinone ring. Recently, Yu & Yu (1982) have reported the preparation of an analogous derivative of Q, in which the azidophenyl group is located more distantly, the equivalent of 15 methylene units, from the quinone ring. The syntheses developed here should be of value in the preparation of other biochemically interesting derivatives of Q in which the reactive or reporter group is relatively close to the ubiquinone ring. The required intermediate alcohols are prepared in good yield effectively by hydration of the side-chain carbon–carbon double bond of the readily available starting material Q-1. The procedures employed would readily permit ^{3}H-labeling of the synthesized hydroxyquinones by use of [^{3}H]$NaBH_4$ in the 2′-hydroxy-Q-1 synthesis or ^3H_2O in the 3′-hydroxy-Q-1 synthesis.

2′-Hydroxy-Q-1 contains an asymmetric carbon atom at C-2′. As chemically synthesized here, the compound and esters derived from it are racemic mixtures. It should be borne in mind that the *R* and *S* isomers of these compounds could react differently with an enzyme. 3′-Hydroxy-Q-1 does not contain an asymmetric carbon atom, so this problem does not arise with esters derived from it, unless the carboxylic acid moiety contains a center of asymmetry. However, the tertiary alcohol group of 3′-hydroxy-Q-1 is considerably more difficult to esterify in good yield than is the secondary alcohol group of 2′-hydroxy-Q-1.

The coupling of the secondary alcohol group of 2′-hydroxy-Q-1 with the carboxyl group of ANAP is accomplished on a submicroscale in good yield with *p*-toluenesulfonyl chloride as coupling agent. This coupling method has previously been shown to be effective in the synthesis of a wide variety of esters from secondary and tertiary alcohols (Brewster & Ciotti, 1955; Hennion & Barrett, 1957; Blotny et al., 1963). It should be readily possible to link other carboxylic acids containing particular functional groups of interest to 2′-hydroxy-Q-1 by this method.

Thus, the possibility exists of using the photoaffinity analogues described here, or ubiquinone derivatives containing other suitable reactive groups prepared by the method developed in this paper, to label respiratory chain components that interact with ubiquinone. Such studies could be carried out with intact membranes, perhaps containing genetically amplified levels of components of interest, or with purified proteins. In the present work we have shown that 2′- and 3′-ANAP-Q-1 act as electron acceptors for purified *E. coli* NADH dehydrogenase. However, attempts to demonstrate specific labeling of *E. coli* NADH dehydrogenase have been unsuccessful. Although covalent binding of 2′-ANAP-Q-1 to the protein was detected on photolysis, we have been unable to show protection by Q-1.

We have used membrane vesicles prepared from a double quinone mutant strain of *E. coli* to test the biological activity of the two photoaffinity analogues of ubiquinone. These membrane vesicles are completely lacking Q, MK, and DMK (Wallace & Young, 1977) and thus provide a good test system for investigating the effectiveness of quinone derivatives in respiratory chain reactions. The two photoaffinity analogues of Q were both effective at low concentrations in restoration of NADH, succinate, and D-lactate oxidase activity in the *ubi⁻men⁻* membrane vesicles. This provides good evidence that these Q analogues are capable of functioning at all sites that normally involve Q-8 in these respiratory oxidase systems.

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Registry No. Q-1, 727-81-1; (±)-2′-hydroxy-Q-1, 99440-93-4; 3′-hydroxy-Q-1, 960-95-2; ANAP, 58775-35-2; FNPA, 28166-06-5; [^{14}C]FNPA, 99440-94-5; NADH oxidase, 9032-21-7; (±)-2′-ANAP-Q-1, 99440-95-6; 3′-ANAP-Q-1, 99440-96-7; (±)-[^{14}C]-2′-ANAP-Q-1, 99440-97-8; [^{14}C]$HO_2C(CH_2)_2NH_2$, 36724-63-7; NADH dehydrogenase, 9079-67-8; succinate, 9014-35-1; D-lactate oxidase, 61461-65-2.

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Evidence for Nystatin Micelles in L-Cell Membranes from Fluorescence Photobleaching Measurements of Diffusion†

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ABSTRACT: Diffusion of a nitrobenzoxadiazole derivative of the polyene antibiotic nystatin in the membranes of L cells is found to depend on the concentration of nystatin in the membrane. Its diffusion coefficient measured by fluorescence photobleaching decreases hyperbolically as the concentration of nystatin is increased. This behavior is reproduced when the concentration of the derivative is increased. In contrast, diffusion of a nitrobenzoxadiazole derivative of a phospholipid is insensitive to the nystatin concentration under these conditions. The nystatin-specific diffusion changes can be understood if nystatin exists in a monomer–micelle equilibrium within the membrane but cannot be accounted for by binding or phase partitioning.

Nystatin is one of several polyene antibiotics used clinically as an antifungal agent (Hammond, 1977; Medoff et al., 1983). These drugs are known to be membrane-active agents which can induce K^+ leakage and cell lysis in yeast, erythrocytes, and mammalian cells including murine L cells (Gale, 1974). A large body of work with model membranes [reviewed by Hammond (1977)] has led to the suggestion that polyene antibiotics, such as nystatin and amphotericin B, associate with sterols in the membrane to form transmembrane pores (de Kruijff & Demel, 1974; van Hoogevest & de Kruijff, 1978).

The polyene antibiotics produce a variety of effects on mammalian cells in vivo and in culture (Medoff et al., 1983) perhaps involving more than one mechanism. Accordingly, we have become interested in characterizing in more detail the interactions of polyene antibiotics with membranes of living cells. Measurements of diffusion of membrane components represent one approach to studying their dynamic interactions through, for example, binding to slowly moving structures (Elson & Reidler, 1979). We have recently reported the synthesis and characterization of nitrobenzoxadiazole derivatives of amphotericin B (Petersen, 1983), nystatin, and pimaricin (Petersen, 1985) and have characterized an interesting and potentially useful intramolecular fluorescence energy transfer process in these derivatives (Petersen, 1985). In this paper, we present diffusion data which provide evidence for

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