Biosynthesis of Caldariellaquinone in *Sulfolobus acidocaldarius*

by

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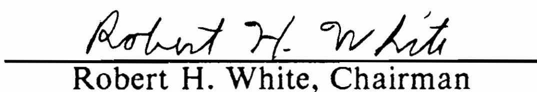

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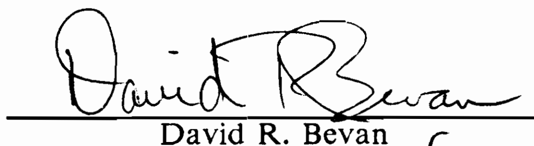

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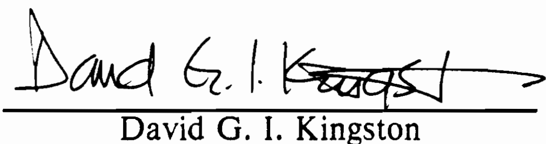

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(ABSTRACT)

The biosynthesis of caldariellaquinone (CQ) has been studied in *Sulfolobus acidocaldarius* using a variety of methods. By growing cells with a series of tyrosines labeled with deuterium or ^{13}C and measuring the extent and position at which label was incorporated into the CQ by mass spectrometry, it was concluded that the benzo[*b*]thiophen-4,7-quinone ring of CQ is derived as an intact unit from all of the carbons of tyrosine except C-1. Additional work, using (3S)-L-[2-^{2}H, 3-^{2}H]-, (3R)-D-[2-^{2}H, 3-^{2}H]-, (3S)-D-[3-^{2}H]-, and (3R)-L-[3-^{2}H]-tyrosine, demonstrated that the pro-3S hydrogen of either D- or L- tyrosine is the origin of the C-3 proton of the benzo[*b*]thiophene ring.

Considering the above information and the structure of CQ, it was concluded that CQ was most likely biosynthesized by the condensation of farnesylfarnesyl pyrophosphate with homogentisic acid (HA) in a reaction analogous to that found in the biosynthesis of ubiquinone. The possibility of this reaction being involved in the biosynthesis of CQ was supported by the identification of farnesylfarnesol, a hydrolytic breakdown product of farnesylfarnesyl pyrophosphate, by gas chromatography-mass spectrometry (GC-MS) of purified lipid extracts. The possible involvement of HA in CQ

biosynthesis, however, could not be confirmed by five independent methods. The possible formation of CQ by the condensation of benzo[*b*]thiophen-4,7-quinone with farnesylfarnesyl pyrophosphate was eliminated by the inability to detect benzo[*b*]thiophen-4,7-quinone in *S. acidocaldarius*.

Attempts to identify the tyrosine metabolites leading to CQ by studing the metabolisms of tyrosine, 2-fluorotyrosine, and 3-fluorotyrosine in *S. acidocaldarius* lead to the identification of two previously undescribed pathways for tyrosine metabolism. These two pathways branch after the conversion of tyrosine to 4-hydroxyphenylacetic acid (*p*HPA). The ability of labeled *p*HPA to be incorporated into these metabolites, but not into CQ, indicates that the first committed step in the biosynthesis of CQ occurs at either tyrosine or a metabolite very closely related to tyrosine, e.g., 4-hydroxyphenylpyruvate (*p*HPP). Analysis of the extract of the cells grown with 3-fluorotyrosine showed two fluorine-containing compounds, which are likely to be fluoro-analogues of the intermediates in the biosynthesis of CQ. However, because of the small amount of these two compounds found (24 nmoles/g of wet weight), structural characterization was not possible.

Both the methyl and sulfur groups of the methylthio portion of CQ were shown to arise from methionine. Mass spectral analysis of the CQ isolated from cells grown in the presence of [^{34}S-*methyl*-$^{2}H_3$]-L-methionine clearly showed, however, that the methylthio group of CQ is not derived as an intact unit from the methylthio group of methionine. Additional work supported the theory that the methionine sulfur first undergoes transsulfuration to cysteine, which then

supplies the sulfur for both the methylthio and the benzo[*b*]thiophene moieties of CQ. This represents the first example of transsulfuration from methionine to cysteine occurring in archaebacteria.

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I would like to thank my advisor, Dr. Robert H. White for his intelligent guidance and support throughout my work in his laboratory. The knowledge and techniques I learned from him and from this project will lay a solid ground for my scientific career in the future.

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Finally, I would like to thank my husband, Weiping Jiang, for his help, understanding, and support.

List of Abbreviations

A	absorbance
Ac_2-CQ	diacetate derivative of CQ
Ac_3-HA	triacetate derivative of 2,5-dihydroxyphenylethanol
Ac_3-phy-HA	triacetate derivative of phytyl 2,5-dihydroxyphenylethanol
CPM	counts per minute
CQ	caldariellaquinone
DHBA	dihydroxybenzoic acid
DHBAD	dihydroxybenaldehyde
DHBOH	dihydroxybenzylalcohol
DHMA	dihydroxymandelic acid
DHPA	dihydroxyphenylacetic acid
DHPG	dihydroxyphenylglyoxylic acid
DHPP	dihydroxyphenylpyruvate
F-HA	fluorohomogentisic acid

F-HL	fluorohomogentisic acid lactone
F-*p*HBA	fluoro-*p*-hydroxybenzoic acid
F-*p*HBAD	fluoro-*p*-hydroxybenzaldehyde
F-*p*HMA	fluoro-*p*-hydroxymandelic acid
F-*p*HPA	fluoro-*p*-hydroxyphenylacetic acid
F-Tyr	fluorotyrosine
HMG-CoA	3-hydroxy-3-methyl-glutaryl CoA
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HA	homogentisic acid
HL	homogentisic acid lactone
M	molecular ion
MS	mass spectrometry
NMR	nuclear magnetic resonance
***p*HBA**	*p*-hydroxybenzoic acid
***p*HBAD**	*p*-hydroxybenzaldehyde
***p*HPG**	*p*-hydroxyhpenylglyoxylic acid
***p*HPA**	*p*-hydroxyphenylacetic acid
***p*HMA**	*p*-hydroxymandelic acid
***p*HPP**	*p*-hydroxyphenylpyruvate
R	distance of TLC spot center from starting point
SAM	S-adenosylmethionine
SQ	sulfolobusquinone

THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
TQ	tricycloquinone
tyr	tyrosine
UV	ultraviolet
vol	volume

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INTRODUCTION

ARCHAEBACTERIA

Archaebacteria are considered to form the so-called third kingdom of life, distinct from eubacteria and eucaryotes based upon the 16 S rRNA sequence (Woese et al., 1978). Archaebacteria are generally found in rather harsh and extreme environments on all continents of the world and consist of three main phenotypes, the extreme halophiles which live in high salt environment, the methanogens which live in rumen fluids or sewage sludge, and the sulfur-dependent extreme thermoacidophiles which live in hot acid springs. Whether these extreme conditions represent the harsh conditions where life began and where the archaebacteria have remained since life first evolved, is presently unknown (Woese and Fox, 1978), as is the evolutionary relationship among eubacteria, eucaryotes, and archaebacteria (Woese, 1987; Gouy and Li, 1990). One hypothesis that archaebacteria and eucaryotes are derived from eubacteria,

is based on their marked molecular and cellular similarities. These similarities include the presence of the histone-like proteins (Reddy and Suryanarayana, 1989; Green et al., 1983), introns in the tRNA genes (Kaine, 1987), the nature of their translational systems (Schmid et al., 1982), ribosomal proteins (Matheson et al., 1990), sequence of 5S rRNA (Hori et al., 1979), the properties of DNA-dependent RNA polymerase (Pühler et al., 1989), and ATPase (Mukohata et al., 1990; Gotarten et al., 1989). Under this hypothesis, extreme thermophiles branched off shortly after the divergence between archaebacteria and eucaryotes, and methanogens branched off shortly after that (Figure 1, Woese et al., 1990). This proposal also explains the closer relationship between thermophilic archaebacteria and eucaryotes (Woese, 1990). Despite the close relationship between archaebacteria and eucaryotes, archaebacteria also have some features that are typically eubacterial, such as the absence of a nuclear membrane, organelles, 5.8 S rRNA, and a low deoxyribonucleic acid content (Schleifer et al., 1982; De Rosa et al., 1986).

There are other unique biochemical features of archaebacteria which are distinct from both eucaryotes and eubacteria. Archaebacteria possess diverse cell envelopes and do not have the universal cell wall polymers like most eubacteria. The characteristic eubacterial murein is not present in archaebacteria (König, 1989). The most common archaebacterial cell envelope is composed of a regular crystalline array of protein or glycoprotein subunits (König, 1989; Phipps et al., 1990). In contrast to the straight chain of fatty acyl ester-linked glycerolipids (with sn-1,2-glycerol) of eubacterial and eucaryotic membranes, archaebacterial

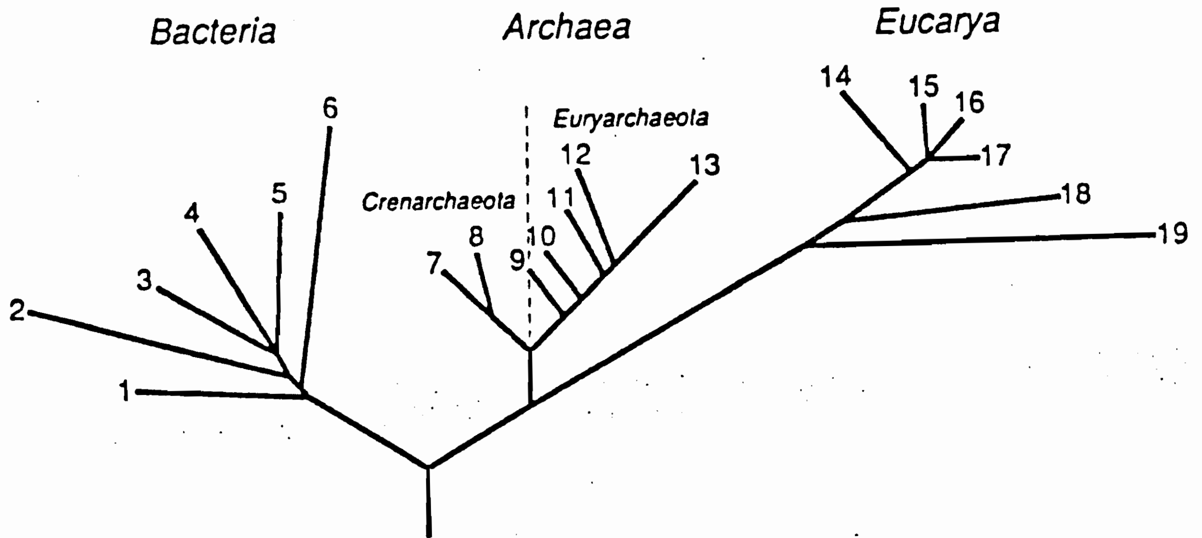


Figure 1. Universal phylogenetic tree in rooted form, showing the three domains (from Woese et al., 1990): The numbers on the branch tips correpond to the following groups of organisms. Bacteria: 1, the Thermotogales; 2, the Flavobacteria and relatives; 3, the Cyanobacteria; 4, the purple bacteria; 5, the Gram(+) bacteria; and 6, the green nonsulfur bacteria. Archae: the kingdom Crenarchaeota: 7, the genus *Pyrodictium*; and 8, the genus *thermoproteus*; and the kingdom Euryarchaeota: 9, the Thermococcales; 10, the Methanococcales; 11, the Methanobacteriales; 12, the methanomicrobiales; and 13, the extreme halophiles. Eucarya: 14, the animals; 15, the ciliates; 16, the green plants; 17, the fungi; 18, the flagellates; and 19, the microsporidia.

membranes contain isopranyl ether-linked glycerolipids with sn-2,3-glycerol configuration (Langworthy, 1985; De Rosa et al., 1986; 1989). The glycerolipids from the thermoacidophilic and some methanogenic archaebacteria also contain tetraethers, which allows for the formations of lipid "monolayer" membranes. The glycerolipids from the thermoacidophiles have the additional feature that the biphytanyl chains may contain 1-4 linked cyclopentyl rings. The unique isopranyl lipid membranes and cell envelope of these cells may be related to the extreme conditions in which these organisms live. The tRNA sequence TΨCG, which is nearly universal in eubacteria and eucaryotes, is not present in archaebacteria. Instead, the sequence 1-methyl ΨΨCG is present in most archaebacterial tRNAs (Gupta and Woese, 1980).

Some of the major pathways of glucose catabolism have been investigated in the heterotrophic archaebacteria (Danson, 1988). These cells do not use the Embden-Meyerhof glycolytic pathway, which is used in eucaryotic cells and a large number of anaerobic eubacteria, to metabolize glucose. Instead, they use a modified Entner-Doudoroff pathway (Figure 2), which is used in strictly aerobic eubacteria. In halobacteria, glucose is oxidized and dehydrated to 2-keto-3-deoxygluconate prior to the kinase and aldolase reaction. A similar pathway has also been found during the fermentation of gluconate by *Clostridium aceticum* and a few other eubacteria (Danson, 1988). In *Sulfolobus* and *Thermoplasma* spp., glucose is metabolized via an additional modification of the Entner-Doudoroff pathway (Danson, 1988). In this pathway glucose is

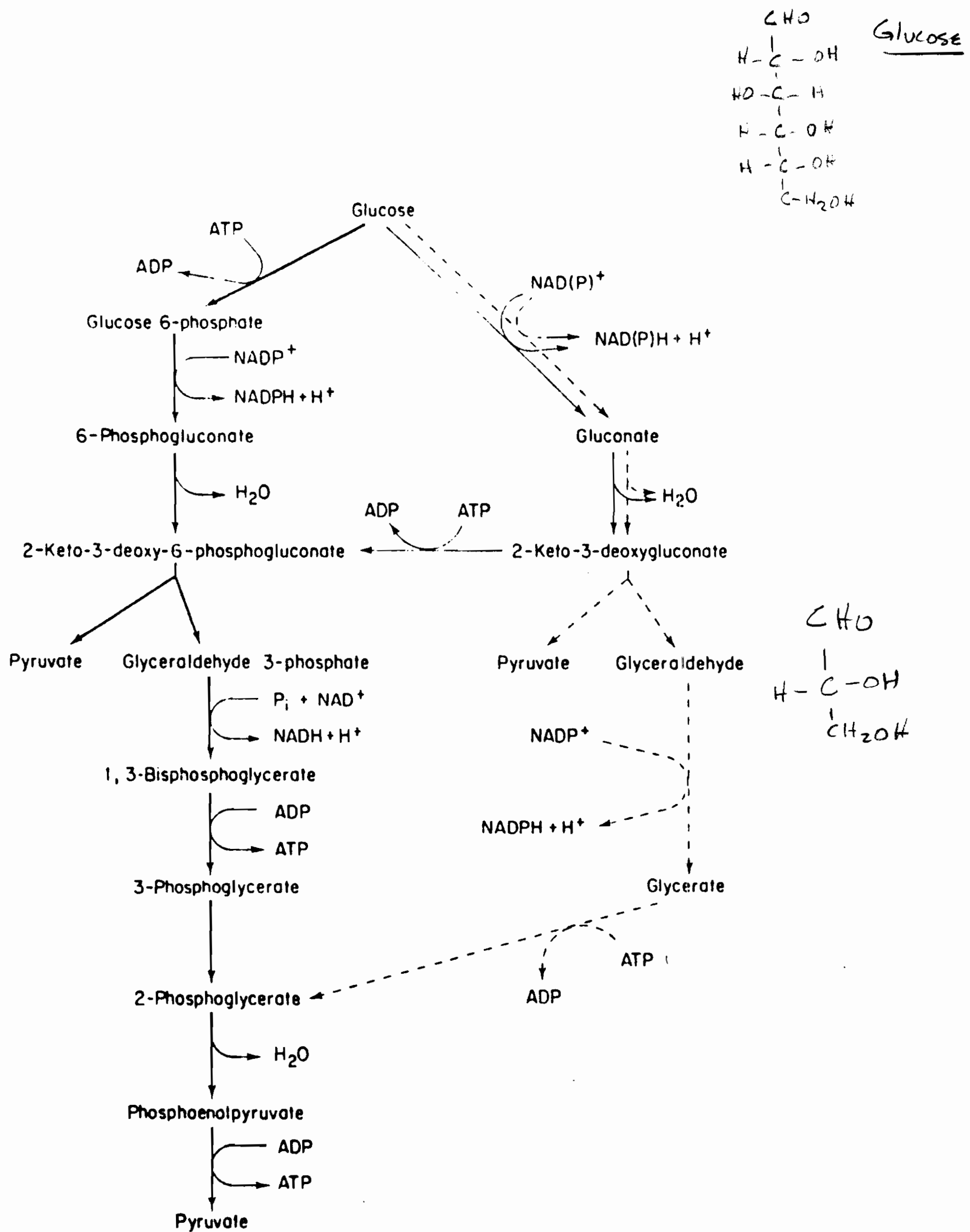


Figure 2. Pathways of glucose catabolism in halophilic and thermophilic archaebacteria (from Danson, 1988): The modified Entner-Doudoroff pathway of halophiles (→) and the non-phosphorylated Entner-Doudoroff pathway of *S. solfataricus* and *Thermoplasms acidophilum* (--→) are shown alongside the classic Entner-Doudoroff pathway of eubacteria (→). Conversion of glyceraldehyde into pyruvate via glycerate (--→) has been demonstrated only in *Thermoplasms acidophilum.*

oxidized to pyruvate and glyceraldehyde via 2-keto-3-deoxygluconate without the formation of any phosphorylated intermediates.

The enzymes of the citric acid cycle are present in most heterotrophic and autotrophic archaebacteria. The complete citric acid cycle is probably present in species of *Halobacteria, Sulfolobus*, and *Thermoplasma* (Danson, 1988). *Sulfolobus* may also use a reductive (reverse) citric acid cycle for autotrophic growth (Danson, 1988). A number of enzymes from the citric acid cycle have been characterized, for example, citrate synthase from halobacteria, *Sulfolobus*, and *Thermoplasma* resembles the enzyme from eucaryotes and the gram-positive eubacteria (Löhlein-Werhahn et al., 1988).

Several specific enzymes in archaebacteria have been isolated, characterized, and compared with the same enzymes present in eucaryotes and eubacteria (Danson, 1988). A interesting observation from this work is that, unlike eucaryotic and eubacterial nicotinamide nucleotide-dependent dehydrogenases which are characteristically specific for either NAD^+ or $NADP^+$, a number of dehydrogenases in thermoacidophilic archaebacteria have been found to lack specificity toward NAD^+ or $NADP^+$. The best studied of these include isocitrate dehydrogenase, malate dehydrogenase, and glucose dehydrogenase from *Sulfolobus acidocaldarius* (Danson, 1988).

There is little known about the biosynthetic pathways in archaebacteria. The biosynthesis of a few amino acids in methanogenic archaebacteria has been studied (Ekiel et al., 1983). The results are in agreement with most of the amino acids being produced by the familiar pathways found in eubacteria and

eucaryotes, e. g., arginine is biosynthesized via the ornithine acetyltransferase pathway and lysine is biosynthesized via diaminopimelic pathway (Jones et al., 1987). In contrast, isoleucine is not biosynthesized from threonine, as it is in eubacteria and eucaryotes. Instead, isoleucine is biosynthesized from pyruvate and acetyl-CoA via citramalate (Ekiel et al., 1984). The biosynthesis of nucleosides in methanogenic bacteria is also consistent with established pathways (Ekiel et al., 1983). The biosynthesis of archaebacterial glycerolipids has been studied and reviewed (Langworthy, 1985; De Rosa et al., 1986; 1989). This work has demonstrated that isoprenyl lipids are derived from acetate via the mevalonate pathway by the same general biosynthetic pathway operating in eucaryotes (Beytía and Porter, 1976). How this biosynthesis compares with the isoprenoid biosynthesis in eubacteria is not clear since free acetate and mevalonic acid were found not to be involved in the biosynthesis of isoprenoid chain of ubiquinone in *E. coli* (Zhou and White, 1991). The situation is further complicated by the observation that in halobacteria, *H. cutirubium* and *H. halobium*, the methyl and methine carbons in phytanyl chain were derived from lysine (Ekiel et al., 1986). The mechanisms responsible for this unexpected incorporation are far from clear.

Many unique coenzymes have also been found in archaebacteria, these include coenzyme M, methanofuran, F_{420}, F_{430}, and methanopterin in the methanogenic archaebacteria (DiMarco et al., 1990) and caldariellaquinone (CQ) in species of *Sulfolobus* (De Rosa et al., 1977). Each of the coenzymes found in methanogenic bacteria is involved in methanogenesis in these bacteria. CQ, on

the other hand, as is the case for other naturally occuring quinones, most likely plays a role in the electron transport system in *Sulfolobus*. This idea is further supported by the absence of menaquinone and ubiquinone in *Sulfolobus* (Collins and Langworthy, 1983; De Rosa et al., 1977). The half-reduction potential of CQ in micelles at pH 6.5 for a two-electron transfer between the fully reduced and fully oxidized state is 100 mV, slightly higher than that of regular ubiquinones or menaquinones (Schäfer et al., 1990a). It was proposed that CQ functions as a central pool for reducing equivalent delivered from the flavin/iron-sulfur protein, and reoxidized by two alternate cytochrome complexes, a cytochrome o and a cytochrome aa_3.

PHENOTYPICAL CHARACTERIZATION OF GENUS *SULFOLOBUS*

Members of the genus *Sulfolobus*, together with several other organisms, comprise the group of sulfur-dependent thermophilic archaebacteria (Woese, 1987). Most organisms in this group, except for the genera of *Sulfolobus* and *Thermoplasma*, are strictly anaerobic. Only two species of *Sulfolobus* have been validly described to date, *S. acidocaldarius* and *S. solfataricus* (Grogan, 1989). Both of these species grow optimally at high temperature (70-80°C) and low pH (2-4) and display an irregular coccoid shape. Unlike most of the other thermophilic archaebacteria, most *Sulfolobus* spp. can grow either autotrophically or heterotrophically on a number of organic compounds including amino acids,

yeast extract, and sugar. During autotrophic growth, oxidation of elemental sulfur to sulfate, which is repressed by yeast extract (Brock, 1972), serves as the sole energy source for the cells. The high growth temperature and acidic growth conditions of the *Sulfolobus* spp. may be responsible for many unique biochemical properties that makes these cells different from other organisms. Although *Sulfolobus* spp. grows in extremely acidic environments, they maintain a neutral cytoplasmic pH (Schäfer et al., 1990a) and all the cytoplasmic enzymes examined so far from these cells have all been found to have neutral pH optima (Bartolucci et al., 1987; Puchegger et al., 1990; Löhlein-warhahn, 1988; Salhi et al., 1989). This large pH gradient across the membrane is most likely maintained by energetically linked respiration, using a proton-dependent ATP synthetase as is found in eucaryotes and eubacteria (Mitchell, 1969). This is supported by observations that species of *Sulfolobus* transduce and conserve respiratory energy by the classical proton-driven chemiosmotic mechanism (Shäfer et al., 1990a; 1990b; Lübben and Shäfer, 1989; Moll and Shäfer, 1988; Anemüller et al., 1985) where in fact the high proton gradient may actually facilitate the energy generation via the proton pump (Matin, 1990; Cobley and Cox, 1983). The existence of a membrane-bound ATPase has been established in species of *Sulfolobus* but it is not typical of the F_0F_1-type ATPase found in eucaryotes (Shäfer et al., 1990a; 1990b; Mukohata et al., 1990; Gogarten et al., 1989) although it shares some similarities with the F_0F_1-type ATPase.

Some unusual and at present unknown phenotypical characteristics of *Sulfolobus* spp. may also occur in these cells which allow them to grow at such

high temperatures (Brock, 1985; Wiegel, 1990). One group of these characteristics would have to be concerned with the increased rate of the chemical reactions occurring in the cells. These increased reaction rates could have several consequences for the cells. These could include the increased hydrolytic decomposition of biomolecules, the increased production of undesirable side reaction products and the increase in the rate of metabolic reactions (White, 1984).

The principal result of the hydrolytic decomposition of biomolecules would be the breakdown of peptide bonds in proteins and phosphodiester bonds in nucleic acids. In addition, many small molecules, such as coenzymes and metabolites, would also be subjected to hydrolytic decompositions. Species of *Sulfolobus* may have evolved several mechanisms to deal with these decompositions. The easiest solution would be to simply produce the required biomolecules at a faster rate in order to replace the hydrolyzed molecules. This, however, does not address the problem of what the cell does with the decomposition products. If these are protein derived peptides, they could be broken down by the proteases present in these cells (Hanner et al., 1990). If the increased production of cellular byproducts is a problem for these cells, they may have evolved mechanisms to break down and transport these undesirable products from the cells. An alternative solution for the cells would be to produce biomolecules which are resistant to these hydrolytic reactions. This may in fact be one of the reasons for the increased thermal stability of the enzymes isolated from these cells, although the biochemical and molecular basis for the thermostability of proteins as well as other macromolecules is still not fully understood (Sharp and Munster, 1986).

Alterations in the structure of cofactors to generate thermally stable structures are also possible, which may explain why the members of the genus *Sulfolobus*, as well as other thermophiles, contain modified folates (White, unpublished results).

Changes in the rates of metabolic reactions at high temperatures could also have profound effects on either the regulation of metabolic pathways or the actual alteration of metabolic pathways. This could be one possible reason for the differences in the metabolic pathways described for species of *Sulfolobus* (Danson, 1988).

DISTRIBUTION OF ISOPRENOID QUINONES

Isoprenoid quinones are present in bacteria, yeast, higher plants, and animals. They play important roles in electron transport systems and oxidative phosphorylation (Lenaz, 1981). Two major types of isoprenoid quinones have been found in nature, the benzoquinones and the naphthoquinones. Benzoquinones can be further divided into ubiquinones and plastoquinones, and naphthoquinones can be divided into menaquinones (formerly coenzyme K_2) and phylloquinones (formerly coenzyme K_1) (Figure 3). Ubiquinones and menaquinones are present in microorganisms, higher plants, and animals; whereas plastoquinones and phylloquinones are exclusively found in higher plants and some in algae and cyanobacteria. The distribution of isoprenoid quinones in bacteria has been reviewed by Collin and Jones (1981) and Collin (1985a).

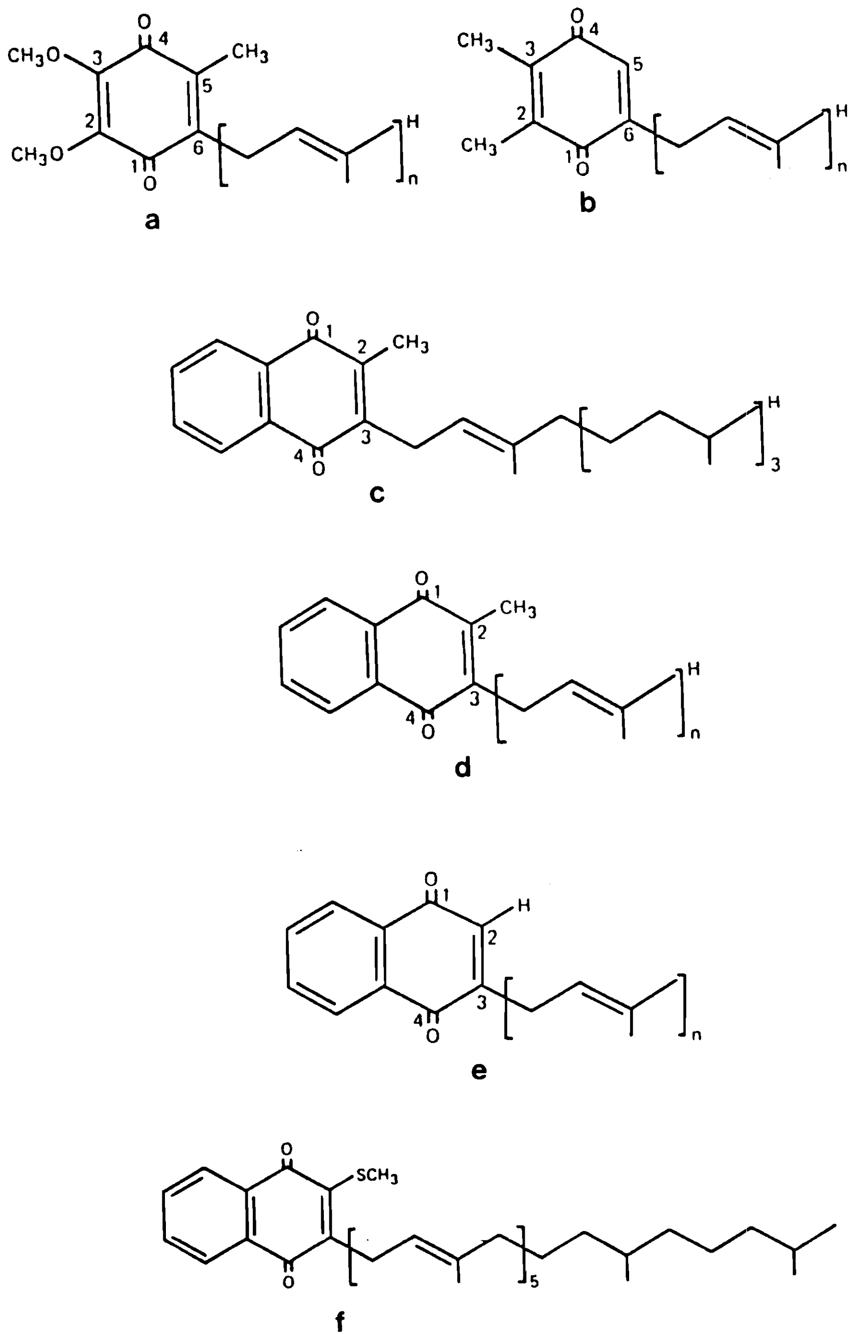


Figure 3. Structures of biological quinones: a) ubiquinone; b) plastoquinone; c) phylloquinone; d) menaquinone; e) demethylmenaquinone; f) methiomenaquinone.

Within procaryotes, ubiquinones have a more restricted distribution than menaquinones. Although ubiquinones are present in Gram(-) eubacteria, they have not yet been reported in Gram (+) eubacteria nor in archaebacteria (Collin, 1985a). Various degrees of saturation of the side chain have been found in bacterial menaquinones, whereas bacterial ubiquinones with a partially saturated side chain and bacterial quinones with completely saturated side chain have never been found. Replacement of the methyl group at C-2 of menaquinones with a hydrogen atom leads to demethylmenaquinones, which are found in many Gram (-) eubacteria and one species of Gram (+) eubacteria (*Streptococcus faecalis*), but appear to be absent from archaebacteria (Collins, 1985a). Another modification on menaquinones is the substitution of the C-2 methyl group with a thiomethyl group to form methiomenaquinone, which is found in a thermophilic hydrogen bacterium (Ishii et al., 1983) (Figure 3).

Generally speaking, aerobic bacteria contain ubiquinones, anaerobic bacteria contain menaquinones, and facultative anaerobic bacteria contain both ubiquinones and menaquinones. For example, *E. coli* produces only menaquinone when grown anaerobically, but produces both ubiquinone and menaquinone when grown aerobically. The actual ratio of ubiquinones and menaquinones produced was found to change with the oxygen availability (Polgase et al., 1966; Whistance and Threlfall, 1968c).

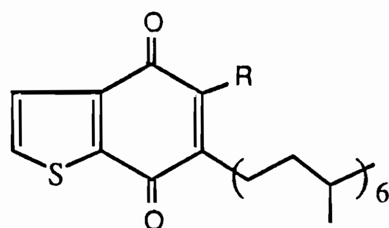
The respiratory lipoquinone composition has also been studied in archaebacteria, which have generally been found to contain menaquinones, but have never been found to contain ubiquinones. Aerobic archaebacteria, such as

Thermoplasma and members of *Halobacteriaceae*, contain menaquinones with unsaturated side chain as the major lipoquinone (Collins, 1985a; 1985b; Collins and Jones, 1981; Collins and Langworthy, 1983). Some anaerobic thermophilic archaebacteria, such as *Pyrococcus islandicum* and *Archaeoglobus fulgidus*, contain menaquinones with fully saturated isoprenoid side chains (Tindall, 1989; Tindall et al., 1989). Other anaerobic thermophilic archaebacteria, such as *Thermococcus celer, Desulfurococcus mucosus*, and *Desulfurococcus mobilis*, are devoid of lipoquinones (Thurl et al., 1985; 1986). Despite the lack of published information on the methanogenic archaebacteria there is no evidence to date to suggest that lipoquinones are present in any of these organisms (Tindall et al., 1989). In contrast, all known members of the genera *Sulfolobus* synthesize an unusual series of lipoquinones containing benzo-[*b*]-thiophen-4,7-quinone and a fully saturated isoprenoid side chain. In *Sulfolobus solfataricus*, the major lipoquinone component is caldariellaquinone (CQ) which makes up 83% of total lipoquinone (Figure 4, a) (Collins and Langworthy, 1983; Thurl et al., 1986; Lanzotti et al., 1986; Tricone et al., 1989). This *Sulfolobus* spp. also contains a small amount of sulfolobusquinone (SQ) (Figure 4, b, 5% of total lipoquinone) and a trace amount of tricycloquinone (TQ) (Figure 4, c), as well as other homologous benzothiophen quinones with different side chain lengths and/or a different number of double bonds. This same series of benzothiophenquinones have also been found in other thermophilic archaebacteria including three strains of *Acidianus* and *Desulfolobus ambivalens* (Segerer et al., 1986; Trincone et al., 1989). It is interesting to note that *Desulfolobus ambivalens* contains 80% of its

total lipoquinones as CQ and 20% as SQ when grown aerobically, but contains only SQ when grown anaerobically. Changes in the distribution of the quinones present in *Sulfolobus* when grown aerobically as opposed to anaerobically appears to have never been examined. This is probably because of the slow growth rate of these cells when grown under anaerobic conditions (Brock, 1972). This shift of quinone distribution with oxygen may be related to the sulfur and energy metabolisms of these sulfur-dependent archaebacteria, since the only difference between CQ and SQ is one sulfur atom.

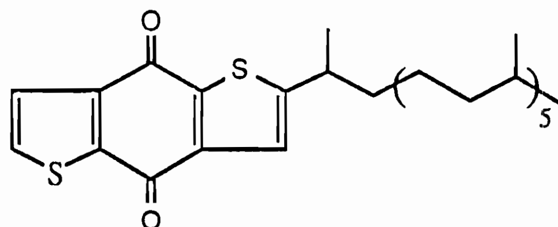
BIOSYNTHESIS OF UBIQUINONES AND PLASTOQUINONES IN EUCARYOTES AND EUBACTERIA

The biosynthesis of biological quinones (Figure 5) has been extensively studied in both eucaryotes and eubacteria (Bentley and Meganathan, 1982; Olson and Rudney, 1983; Ramasarma, 1985; Threlfall et al., 1971). The biosynthetic pathways have been determined using several different methods including the use of isotope tracer methodology, the isolation and characterization of mutants, and the preparation of enzyme extracts capable of performing the individual reactions. Since higher animals have lost their ability to synthesize aromatic rings from simple precursors, the quinone ring must be derived from dietary sources of aromatic compounds, such as tyrosine and phenylalanine. In contrast, microorganisms and many plants are able to synthesize aromatic compounds from such simple precursors as acetate or glucose via the polyketide pathway or



(a) $R=SCH_3$ Caldariellaquinone (CQ)

(b) $R=CH_3$ Sulfolobusquinone (SQ)



(c) Tricycloquinone (TQ)

Figure 4. Structures of CQ (a), SQ (b), and TQ (c).

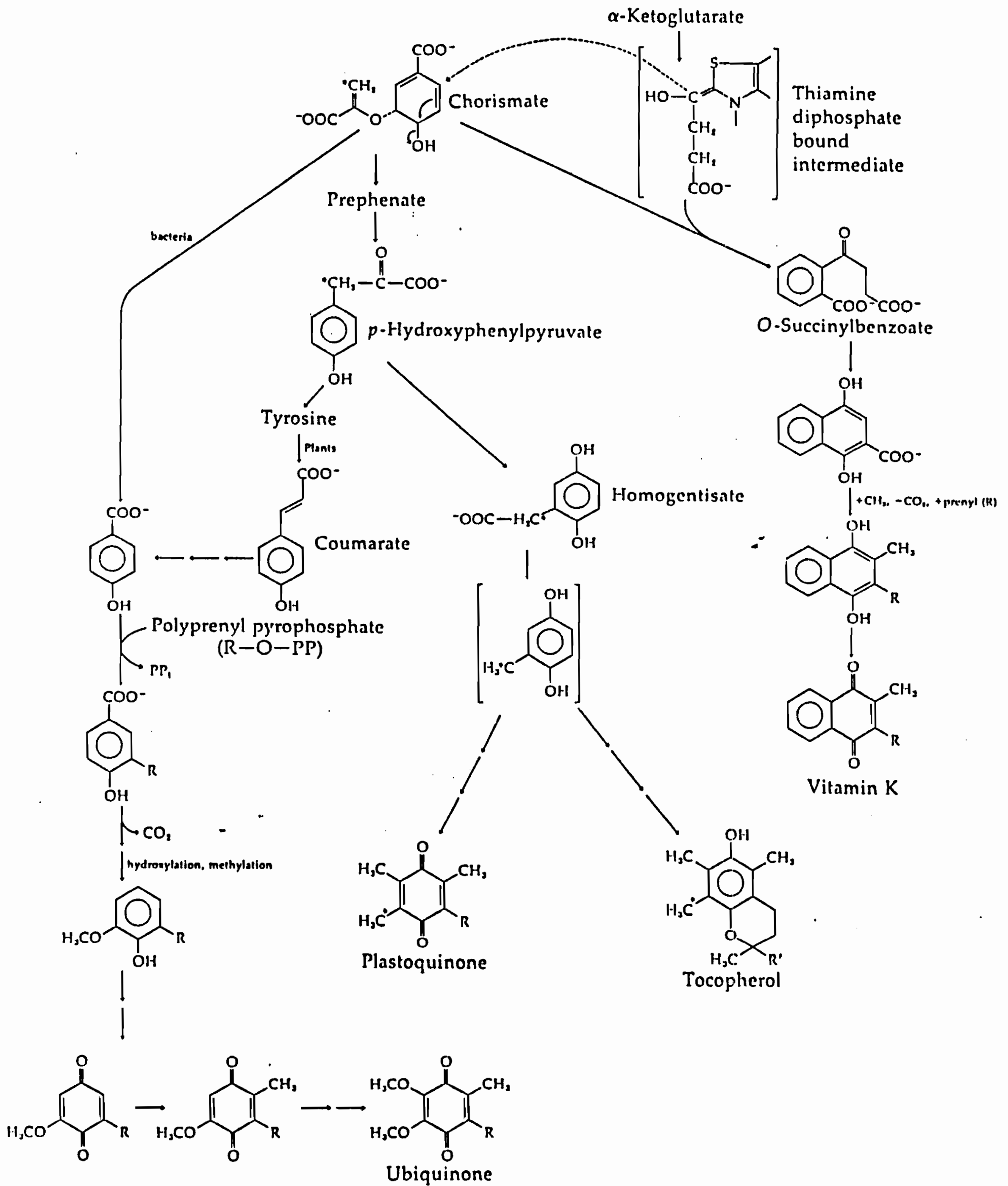


Figure 5. Biosynthesis of quinones in eucaryotes and eubacteria: (from Metzler, 1977).

shikimic pathway. Tyrosine has been determined to be the precursor of ubiquinones in animals, fungi, and higher plants and of plastoquinones in higher plants. In the biosynthesis of ubiquinones in eucaryotes, tyrosine is metabolized to 4-hydroxybenzoic acid which is then converted to the quinone ring of ubiquinone with only the aromatic carbons of tyrosine being incorporated into the final product (Figure 5). In the biosynthesis of plastoquinones, both the C-3 and aromatic carbons of the tyrosine are incorporated into quinones via homogentisic acid (Whistance and Threlfall, 1968a; 1968b; 1970). In most eubacteria, 4-hydroxybenzoic acid, the precursor of ubiquinones in eucaryotes, is formed directly from chorismic acid without the involvement of tyrosine (Gibson and Gibson, 1962; Gibson and Pittard, 1968; Lawrence et al., 1974). In one strain of *Aerobacter aerogenes*, it was found that tyrosine could serve as a source of 4-hydroxybenzoic acid with 4-hydroxyphenylpyruvate as a likely intermediate (Gibson and Pittard, 1968; Lawrence et al., 1974).

In both eubacteria and eucaryotes, polyprenylation on the ring occurs at an early stage in the biosynthesis, i.e., at 4-hydroxybenzoic acid (Daves et al., 1967; Olson and Rudney, 1983). Many polyprenylated intermediates have been identified, and the corresponding nonpolyprenylated compounds failed to be incorporated into ubiquinones (Olson and Rudney, 1983). The pathway for the conversion of polyprenylated 4-hydroxybenzoic acid to ubiquinone is slightly different in eubacteria and eucaryotes (Figure 6). In eubacteria, polyprenylated 4-hydroxybenzoic acid was decarboxylated, hydroxylated, and then methylated to form the substituted quinone ring found in compound 6 of Figure 6, whereas

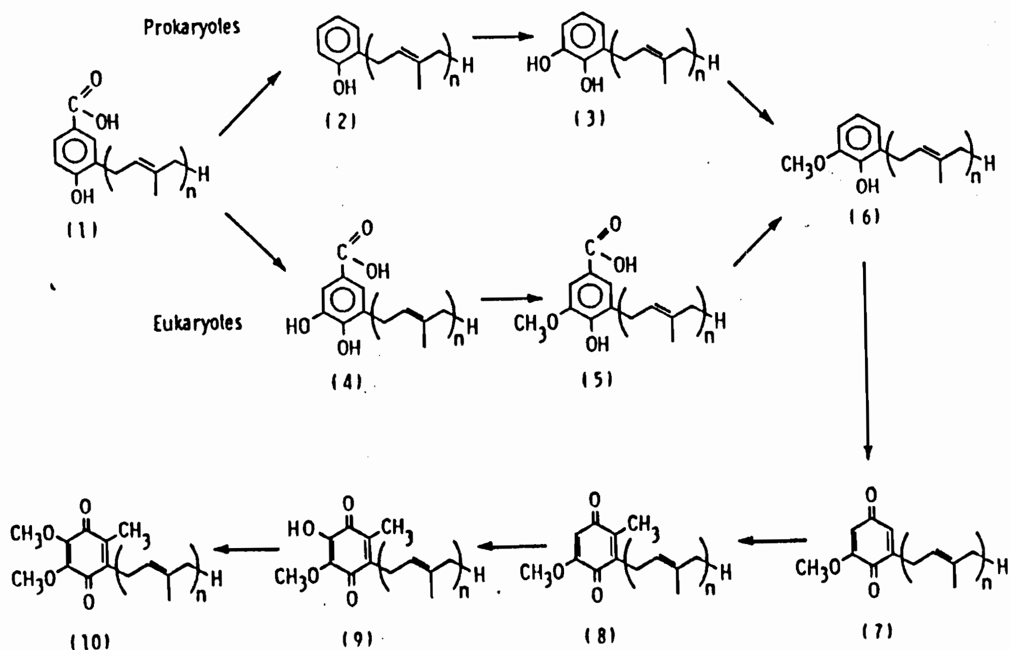


Figure 6. Reaction routes for the biosynthesis of ubiquinone in eucaryotes and eubacteria: (from Olson and Rudney, 1983.)

in eucaryotes, the polyprenylated 4-hydroxybenzoic acid was first hydroxylated, then methylated, and finally decarboxylated to form the substituted quinone ring. During the biosynthesis of plastoquinone, the isoprenoid chain is also attached to homogentisic acid at an early stage (Soll et al., 1980; Marshall et al., 1985; Thomas and Threlfall, 1975). Gentisate, gentisaldehyde, gentisyl alcohol, and toluquinol which were considered likely intermediates, were not found to be involved in the biosynthesis (Whistance and Threlfall, 1968a; 1968b; 1970). It was demonstrated that in broken chloroplasts homogentisic acid reacts with solansyl pyrophosphate or solansol plus ATP to form C_{45}-2-methylquinol, which could lead to the formation of plastoquinone-9 (Soll, 1980).

One of the objectives of this thesis is to determine the biosynthesis of CQ and establish if there are any relationships to the biosynthesis of isoprenoid quinones in eucaryotes and eubacteria.

BIOSYNTHESIS OF BENZOTHIOPHENE QUINONE RING OF CQ

INTRODUCTION

In a preliminary study on the biosynthesis of CQ by De Rosa et al. (1977), both [1-^{13}C]- and [2-^{13}C]-acetate were found to be incorporated exclusively into the isoprenoid side chain of the CQ. This indicates that the quinone ring is not biosynthesized from acetate via the polyketide pathway. On the basis of the structural similarities between CQ and plastoquinone and ubiquinone, It was postulated that CQ was biosynthesized from tyrosine, with homogentisic acid serving as likely intermediate (Zhou and White, 1989).

The biosynthesis of CQ in *Sulfolobus* was thus studied by feeding a series of tyrosines labeled with ^{18}O, $^{2}H_2$, or ^{13}C and measuring the extent and position at which label was incorporated into the CQ by mass spectrometry. In addition

to tyrosine, several other labeled compounds which were considered to be likely intermediates to CQ were synthesized, fed to growing *S. acidocaldarius* cells, and their incorporation into CQ was measured by mass spectrometry. This was carried out in the hope that one or more of the intermediates between tyrosine to CQ could be identified.

MATERIALS AND METHODS

Isotopically labeled compounds. L-[*aromatic*-^{18}O]Tyrosine (79.1 atom% ^{18}O) was prepared from L-*p*-aminophenylalanine using ^{18}O water (2.8 atom% ^{16}O, 1.8 atom% ^{17}O, and 95.4 atom% ^{18}O) as described by Eckert and Fiat (1986). The labeled water was obtained from the Monsanto Research Corp. Mound Facility, Miamisburg, Ohio. DL-[3,3-$^{2}H_2$]Tyrosine (94 atom% $^{2}H_2$) was prepared by the condensation of the sodium salt of ethyl acetamidocyanoacetate with [*methylene*-$^{2}H_2$]-4-methoxybenzyl bromide as previously described (White, 1979). DL-[2-^{13}C]Tyrosine (99 atom% ^{13}C) was prepared in a similar manner but using diethyl [2-^{13}C]acetamidocyanoacetate obtained from Sigma Chemical Co., St. Louis, Mo. L-[*aromatic*-$^{13}C_6$]Tyrosine (99 atom% ^{13}C) was obtained from Cambridge Isotope Laboratories, Woburn, Mass. (The labeled tyrosine was diluted with unlabeled L-tyrosine in order to prepare a tyrosine sample in which 20 mole% of the tyrosines was labeled.)

[U-$^{2}H_5$]Homogentisic acid was prepared by the acid-catalyzed exchange of homogentisic acid lactone with $^{2}H_3PO_4$ as follows. Homogentisic acid lactone

(69.2 mg) was dissolved in 4 ml of 85% $^{2}H_3PO_4$ (99% atom% deuterium) obtained from Sigma Chemical Co. and the mixture was heated at 90°C for 3 h. After the solution was cooled, 5 ml of ice water was added and most of the [U-$^{2}H_5$]homogentisic acid lactone precipitated and was collected by filtration. The remaining [U-$^{2}H_5$]homogentisic acid lactone was extracted with diethyl ether and was purified by preparative thin layer chromatography (TLC) using ethyl acetate : acetic acid (9:1 [vol/vol]). The combined yield of the product was 58.1 mg. Analysis by mass spectrometry showed 75.6 atom% with 5 deuterium and 17.1 atom% with 4 deuterium. The [U-$^{2}H_5$]homogentisic acid lactone (58.1 mg) was hydrolyzed in 0.3 ml of 1% sodium bisulfite and 0.3 ml of 1% sodium hydroxide solution for 1 h at 100°C and the resulting homogentisic acid was extracted by diethyl ether after acidification with acetic acid. (Homogentisic acid lactone is later found to be partially hydrolyzed to homogentisic acid in the bacterial growth medium due to the acidity of the medium and the high temperature in which the cells were grown. Thus, hydrolysis of the [U-$^{2}H_5$]homogentisic acid lactone to the acid is not required.)

DL-[3,3-$^{2}H_2$]-2′,5′-Dihydroxyphenylalanine was prepared by the reaction of [*methylene*-$^{2}H_2$]-2′,5′-dimethoxybenzyl bromide with diethyl acetamidomalonate followed by hydrolysis of the product as described by Shulgin and Gal (1953). The [*methylene*-$^{2}H_2$]-2′,5′-dimethoxybenzyl bromide was prepared from the benzyl alcohol which had been prepared by $LiAl^{2}H_4$ reduction of 2′,5′-dimethoxybenzoic acid (Adams et al., 1949).

[$\alpha,\alpha,2',3',5',6'-{}^2H_6$]-4-Hydroxyphenylacetic acid was synthesized by exchanging 4-hydroxyphenylacetic acid with DCl at 100°C for 24 hr. After removal of the DCl by evaporation, the deuterium on the carboxylic acid and hydroxy groups was exchanged with hydrogens by dissolving the sample in water and evaporating the water. The deuterium distribution of this labeled 4-hydroxyphenylacetic acid was determined by mass spectrometry and NMR. It contained ~60 atom % of molecules with 6 deuterium and ~40 atom % of molecules with 5 deuterium.

[1-^{13}C]-Tyramine was prepared by the hydrogenation of [1-^{13}C]-2-(*p*-benzyloxyphenyl)acetonitrile as described by Russo et al., (1981). The labeled nitrile was prepared by the reaction of *p*-benzyloxybenzyl chloride with $Na^{13}CN$ (99 atom % ^{13}C, Cambridge Isotope Laboratories, Woburn, MA).

[2,2-$^2H_2$]-2-(4-Hydroxyphenyl)ethanol was prepared by the $LiAl^2H_4$ reduction of 4-hydroxyphenylacetic acid. Thus 1.52 g of 4-hydroxyphenylacetic acid was suspended in 40 ml of diethyl ether and 0.42 g of $LiAl^2H_4$ in 5 ml of diethyl ether was added. After stirring the thick grey suspension overnight the reaction mixture was worked up to give 0.64 g of an oil which turned solid after a few hours. This solid product was crystalized from methylene chloride and showed an intense molecular ion at m/z 140 in its mass spectrum. From the intensities of isotope ions of the molecular ion and the m/z 107 fragment ion, the molecule was labeled to an extent of at least 95% with two ^2H on C-2. This compound was converted into the thiol analogue, [2,2-$^2H_2$]-2-(4-hydroxyphenyl)-ethanethiol, by heating it in 57% HI containing thiourea (Segre et al., 1957).

$[2,2-^2H_2]$-2-(2,5-Dihydroxyphenyl)ethanol was prepared by reduction of homogentisic acid lactone with $LiAl^2H_4$. Thus to homogentisic acid lactone dissolved in 5 ml of THF was added $LiAl^2H_4$ (0.126 g) dissolved in 10 ml of diethyl ether. After refluxing the reaction mixture for 4 h, sample workup afforded 135 mg of crude product which was purified by preparative TLC. The final product gave the expected M^+ at *m/z* 156. This compound was converted into the thiol analogue, $[2,2-^2H_2]$-2-(2,5-dihydroxyphenyl)ethanethiol, as described above. The final product was purified by TLC and gave the expected molecular ion at *m/z* 172.

Growth of bacterial strains. *Sulfolobus acidocaldarius* (ATCC 33909) were grown at 70°C for 2 to 3 days in a pH 3.0 medium consisting of 2.0 g of yeast extract, 1.3 g of $(NH_4)_2SO_4$, 0.28 g of KH_2PO_4, 0.25 g of $MgSO_4$, 70 mg of $CaCl_2 \cdot 2H_2O$, and 10 ml of trace mineral solution (Brock et al., 1972) per liter of medium. The labeled compounds were added at the concentrations shown in Table 1, the pH was adjusted to 3.0 with sulfuric acid, and the medium was autoclaved.

The labeled tyrosine and the deuteriated 2,5-dihydroxyphenylalanine were all stable when incubated in the growth medium at 70°C and when autoclaved in the growth medium. Homogentisic acid, however, was found to undergo a slight decomposition when incubated in the growth medium at 70°C for 2 to 3 days and even more decomposition when autoclaved in the growth medium. This was readily observed by the development of a brown color in the medium. The decomposition resulting from autoclaving was eliminated by adding the

homogentisic acid to the previously autoclaved medium at the time of inoculation. Because of the high temperature and low pH of the medium, which prevented the growth of the other bacteria, no contamination was ever observed using this procedure. Analysis, by TLC, of extracts of the medium after the growth of the cells showed that most of the homogentisic acid added to the medium was still present.

The deuteriated 4-hydroxyphenylacetic acid was added to the growing culture after the cell density had reached an absorbance at 540 nm of 0.3 to 0.4, since 4-hydroxyphenylacetic acid inhibited the cell growth at lower cell densities.

Isolation of CQ. The cells were harvested from the growth medium by centrifugation (10,000 x g) for 15 min, and the cell pellet was extracted for 30 min with CH_2Cl_2-CH_3OH (1:1 vol/vol) at 50°C. After centrifugation to remove the insoluble material, 0.5 volume of water was added to the supernatant. After shaking and centrifugation, the CH_2Cl_2 layer was separated and dried over anhydrous Na_2SO_4, and the CQ was purified by TLC (silica gel 60 F254, Merck) using the solvent system hexane : diethyl ether (8:2 vol/vol). The orange CQ spot (R_f=0.38) was eluted with CH_2Cl_2 and analyzed with mass spectrometry by direct probe insertion.

Derivatization of CQ. The crude CQ fraction was derivatized by treatment with acetic anhydride and zinc dust as described by Campbell et al. (1971) to produce the diacetate derivative of dihydrocaldariellaquinone. The diacetate derivative of CQ was then purified on TLC with the same solvent system as described above.

Mass spectrometry of CQ. The mass spectrum of the isolated CQ shows an intense molecular ion at *m/z* 630, which allows for the measurement of the incorporation of stable isotopically labeled precursors into the entire molecule. The mass spectrum of CQ also has fragment ions at *m/z* 225 (Figure 7, b) and *m/z* 212, which allow for the specific measurement of the label incorporated into the benzo[*b*]thiophenquinone portion of the molecule. Thus, the difference between the label incorporated into the entire molecule and that incorporated into the benzo[*b*]thiophenquinone-containing fragments represents the label incorporated into the isoprenoid portion of the molecule.

Two problems, however, interfered with this straightforward measurement of label incorporation. The first was the occurrence of varying amounts of CQ in the cells that contained one site of unsaturation in the side chain. Since the procedure used for the isolation of CQ is unable to separate unsaturated from saturated CQ, a small amount of this unsaturated CQ was present in the samples from which the mass spectral data were obtained. The presence of unsaturated CQ was confirmed by the occurrence of its molecular ion at *m/z* 628 in the mass spectrum of the CQ. Since this molecule contains $M^+ + 1$, $M^+ + 2$, $M^+ + 3$, and $M^+ + 4$ isotope peaks which interfere with the measurement of the intensities of the required ions, their occurrence must be taken into consideration when the isotopic distributions are calculated. This was accomplished by assuming the normalized intensities of $M^+ + 1$, $M^+ + 2$, $M^+ + 3$, and $M^+ + 4$ to be the same for the unsaturated and saturated CQ.

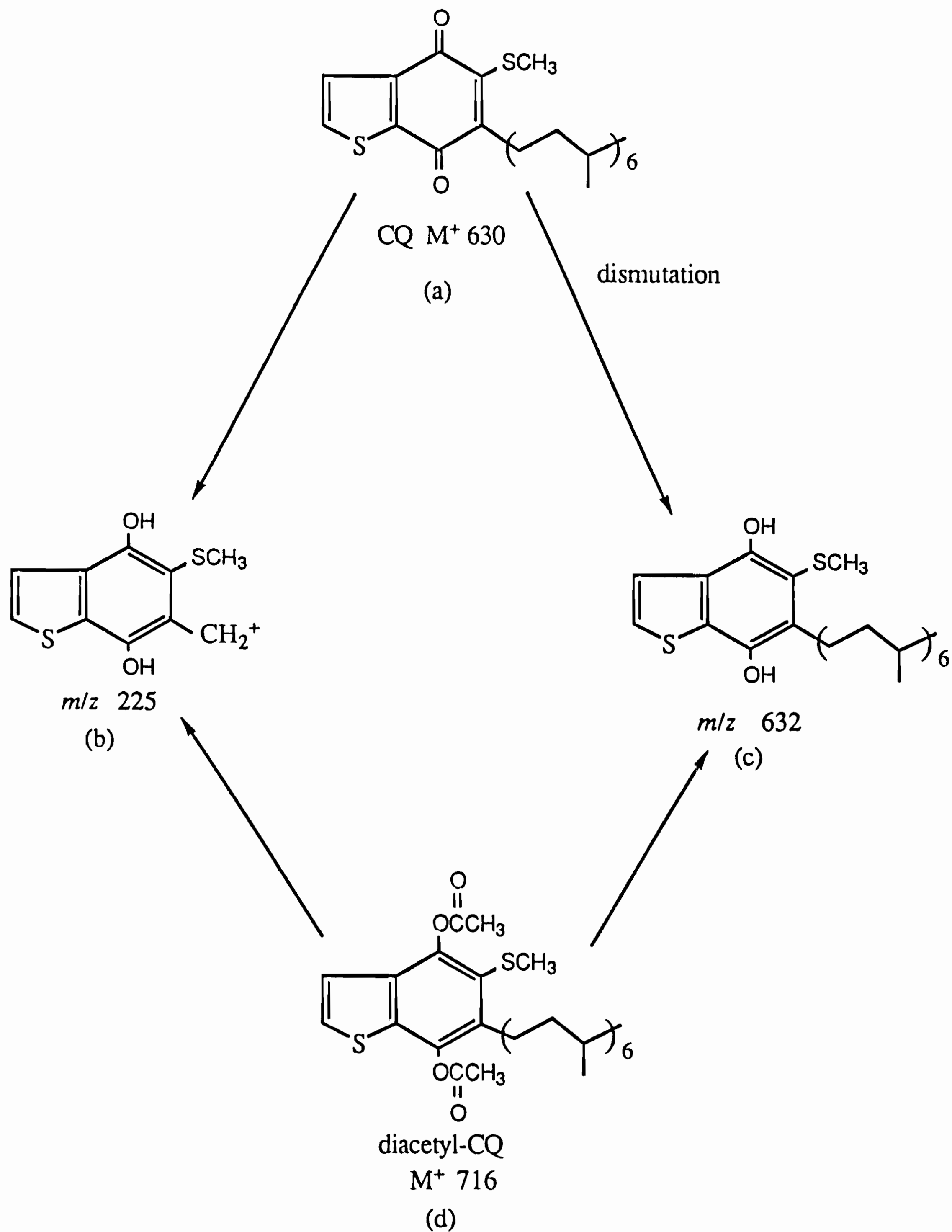


Figure 7. The mass spectral fragmentation of CQ.

A more serious problem in measuring the incorporation of more than one ^2H or ^{13}C was the reduction (dismutation) of CQ in the mass spectrometer, resulting in the generation of the hydroquinone of CQ which has a molecular ion at *m/z* 632 (Figure 7, 1c). This reaction, which has been studied in other quinones, leads to variations in the intensity of the $M^+ + 2$ ions (Aplin and Pike, 1966; Das et al., 1965; Ukai et al., 1967). The intensity of the $M^+ + 2$ ion in the mass spectra of CQ varied from sample to sample in a seemingly random fashion and caused an ~20% error in the measurement of the intensity of the $M^+ + 2$ ion of CQ. This problem was solved by converting CQ to the hydroquinone diacetate derivative (Figure 7, d). The mass spectrum of this diacetate shows a molecular ion at *m/z* 716, an intense fragment ion at *m/z* 632, which corresponds to the hydroquinone, and a fragment ion at *m/z* 225 (Figure 7). The intense *m/z* 632 ion allows for the accurate measurement of isotope incorporation into the entire molecule of CQ without the interference caused by reduction in the mass spectrometer. Using this method with DL-[3,3-$^2H_2$]tyrosine it was shown (data not shown) that no multiple labeling of the CQ was observed, therefore tyrosine was not metabolized and incorporated into the side chain of CQ. Since the experiments reported in this thesis required the measurement of the incorporation of only a single ^2H or ^{13}C, all of the data were obtained from the mass spectra of the labeled CQ.

Analysis of the isotopic distribution of amino acids in cellular proteins. The isotopic distribution of ^2H or ^{13}C in the protein-bound amino acids present in the cells was determined by gas chromatography-mass spectrometry (GC-MS) of

their N-(trifluoroacetyl) and N, O-bis(trifluoroacetyl) *n*-butyl esters as previously described (White, 1985). Important ions in the mass spectrum of the tyrosine derivative are shown in Figure 8.

Detection of homogentisic acid in cells grown with tyrosine. The media after cell growth was extracted with ethyl acetate under acidic condition. The extracted phenol compounds were separated by TLC and identified by spraying with phenol reagent (Folin-Cioclateau reagent) (Keith et al., 1958). The crude media extract was also treated with TMS reagent and the resulting TMS derivatives were analyzed by GC-MS. (For more details see the section on the analysis of fluorotyrosine metabolites.)

Enzymatic analysis of homogentisic acid degradation. The degradation of homogentisic acid (Figure 9) in cell extracts was examined by assaying spectrophotometrically the homogentisic acid oxygenase and maleylacetoacetate isomerase as described by Crawford et al. (1977). The assay of the homogentisic acid oxygenase is easily accomplished since its ring fusion product maleylacetoacetate has a characteristic intense UV-visible absorption spectrum. Thus the production of maleylacetoacetate from homogentisic acid can be monitored by following the increase in the absorbance of the product maleylacetoacetate. The degradation of the maleylacetoacetate then proceeds via isomerization to fumarylacetoacetate, by either a glutathione-dependent or glutathione-independent reaction. For the assay of the glutathione-dependent isomerization, the absorbance of the maleylacetoacetate produced by the oxidation of homogentisic acid is first measured in the absence of added

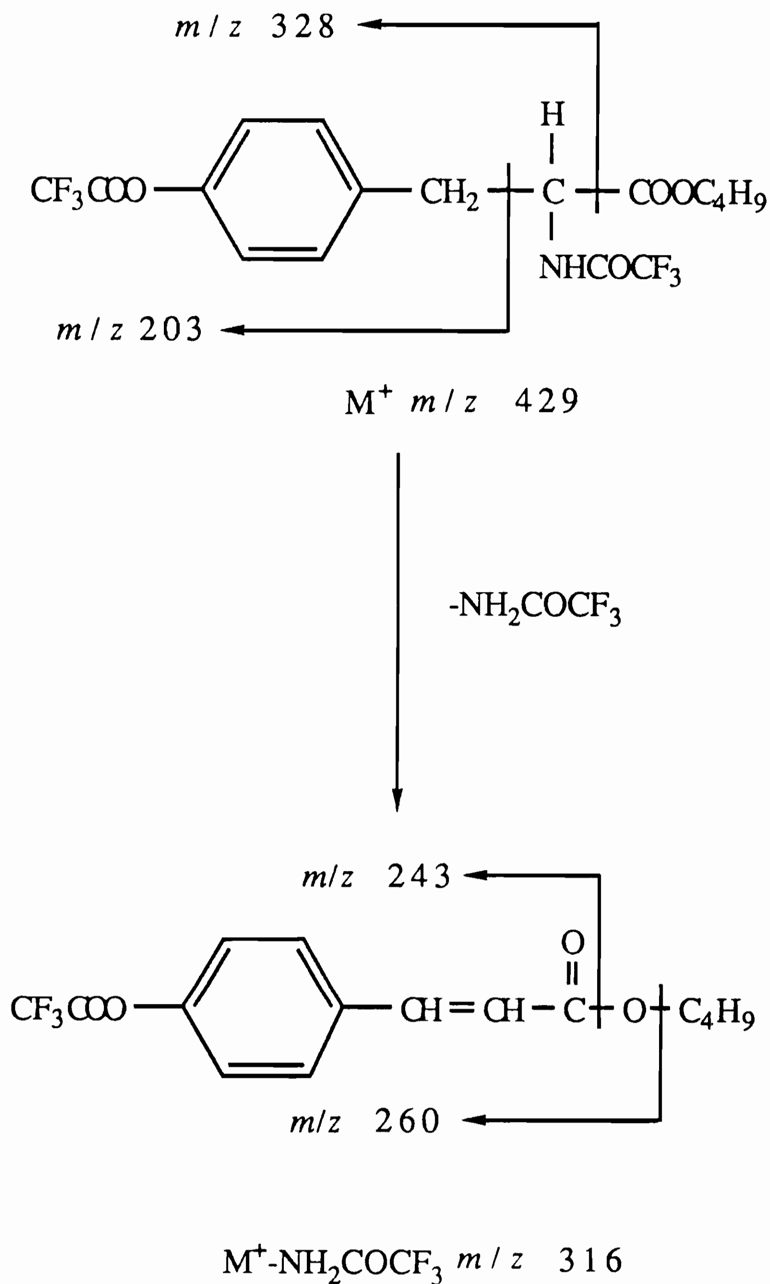


Figure 8. **Mass spectral fragmentation of N,O-bis(trifluoroacetyl) *n*-butyl ester derivative of tyrosine.**

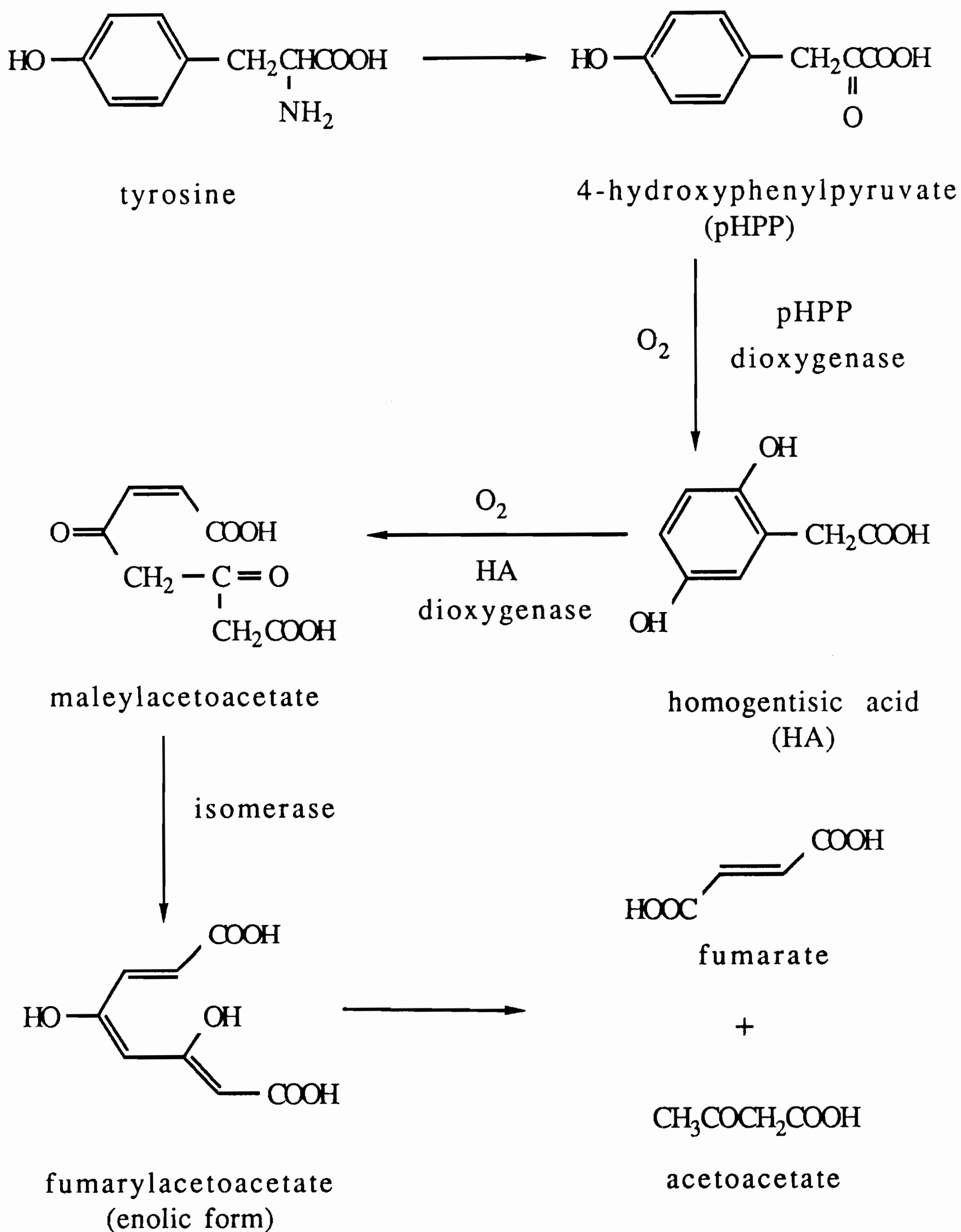


Figure 9. Metabolism of homogentisic acid.

glutathione. The decrease in the absorbance is then measured after the addition of glutathione to determine the activity of the glutathione-dependent isomerase. The glutathione-independent reaction, on the other hand, is measured by following the decrease in the absorbance of extracts to which only homogentisic acid has been added.

The enzymatic degradation of homogentisic acid was assayed by incubating homogentisic acid (0.05 μM) in 1 ml 0.1 M phosphate buffer (pH 7.0) with cell extract at 70°C and following the absorbance change at 320 nm with time. After several minutes, glutathione (0.1 μM) was added and the decrease in A_{320} was monitored. The crude enzyme extract was prepared by sonicating the cells of *S. acidocaldarius* suspended in 0.1 M phosphate buffer (pH 7.0) at 0°C for 4 x 30 sec followed by centrifugation (10,000 x g) to remove the insoluble materials. Cells extracts used in these experiments were derived from cells grown with tyrosine. The protein present in the cell extracts was measured by BIO-RAD protein assay and found to contain 0.9 mg/ml.

RESULTS AND DISCUSSION

Incorporation of tyrosine. The first indication that tyrosine was a precursor to the benzo[*b*]thiophene ring of CQ was the demonstration that DL-[2-^{13}C]tyrosine was readily incorporated into CQ. As can be seen from the data reported in Table 1 on page 34 (experiment 1a), a single ^{13}C was incorporated from DL-[2-^{13}C]tyrosine into the *m/z* 225 fragment ion of the CQ to an

Table 1. Incorporation of labeled tyrosine into CQ and cellular tyrosine.

Expt.	Precursors (mg, ml of medium)[a]	Distribution of ^{2}H or ^{13}C[b]: caldariellaquinone, m/z 225[d] 0	1	6	caldariellaquinone, m/z M-630[d] 0	1	6	tyrosine, m/z 203 or 260[c] 0	1	2	6
1a	DL-[2-^{13}C]tyrosine (47 mg, 100 ml)	33.7 (45.2	66.3 100)		30.6 (44.0	69.4 100)		18.8 (22.3	81.1 100	0 18.0)	
b	DL-[2-^{13}C]tyrosine, (47 mg, 100 ml) + homogentisic acid, (50 mg, 100 ml)	44.0 (65.7	56.0 100)		39.3 (50.3	60.7 100)		30.1 (39.9	69.9 100)		
2	L-[*aromatic*-$^{13}C_6$]tyrosine[e] (25 mg, 50 ml)	83.3 (100	0.0 20.2	16.7 26.6)	85.0 (100	0.8 45.2	14.2 16.7)	82.5 (100	1.0 19.6	0 2.5	16.4 20.1)
3	[U-$^{2}H_5$]homogentisic acid (40 mg, 80 ml)	98.1 (100	1.9 25.6)		98.7 (100	1.3 45.6)		ND[f]			
4a	DL-[3,3-$^{2}H_2$]tyrosine (100 mg, 200 ml)	80.2 (100	19.8 49.3)		79.4 (100	20.6 70.2)		49.3 (100	27.6 66.2	23.1 53.3)	
b	DL-[3,3-$^{2}H_2$]tyrosine (100 mg, 200 ml) + homogentisic acid (50 mg, 200 ml)	80.3 (100	19.7 49.2)		77.3 (100	22.7 73.7)		47.7 (100	28.9 71.2	23.7 57.0)	

[a]The cells used were *S. solfataricus* in expt. 4a, b and *S. acidocaldarius* in all other experiments.

[b]The numbers in parenthesis are the observed normalized ion intensities.

[c]The intensity data were obtained from m/z 260 of the N,O-bistrifluoroacetyl *n*-butyl derivative of the tyrosine in all experiments except 4, in which case, intensitities of the m/z 203 were used. The measured normalized ion intensities for an unlabeled tyrosine derivative sample were 100%, 18.4%, 2.95%, and 0.0% for the m/z 260, 261, 262, and 266 and 100%, 10.2%, 0.8%, and 0.0% for the m/z 203, 204, 205, and 208 ions, respectively.

[d]The measured normalized ion intensities for an unlabeled sample of CQ were 100%, 24.6%, and 0.3% for the m/z 225, 226, and 231 and 100%, 44.3%, and 0.0% for the m/z 630, 631, and 636 ions, respectively.

[e]Sample contained 20% of the molecules with [*aromatic*-$^{13}C_6$]tyrosine (99% of ^{13}C).

[f]ND, Not determined.

extent of 66.3% and into the M^+ *m/z* 630 ion to an extent of 69.4%. (On the basis of the biosynthetic argument presented below, it is assumed that this carbon is incorporated into the C-2 of the benzo[*b*]thiophene ring, however, this cannot be determined unequivocally by the mass spectroscopic method used here.) The difference between the label found in the intact molecule and that found in the aromatic portion of the ring can be explained by the incorporation of a small amount of ^{13}C from the tyrosine into the side chain.

That the aromatic ring of the tyrosine is incorporated as a single, intact unit into the six-member ring of the quinone was confirmed by growing cells in the presence of L-tyrosine in which 20% of the molecules contained L-[*aromatic*-$^{13}C_6$]tyrosine. The reason for using tyrosine labeled in this manner is to allow for the detection of the degradation and resynthesis of the aromatic ring from its degraded products. If this was to occur, no intact C_6 unit would be observed. That the tyrosine was incorporated as a unit into both the cellular protein and the benzo[*b*]thiophene ring of CQ was confirmed by the data (Table 1 on page 34, experiment 2), which showed, from both the *m/z* 225 and M^+ *m/z* 630 ions of the CQ and the *m/z* 260 ion of the N,O-bis(trifluoroacetyl) *n*-butyl derivative of the cellular tyrosine, that L-[*aromatic*-$^{13}C_6$]tyrosine is incorporated as an intact C_6 unit into both the benzo[*b*]thiophenquinone ring of the CQ and the cellular protein. Since the extent of label incorporation for the *m/z* 225 ion of the CQ and the *m/z* 260 ion of the tyrosine derivative were both ~16% , and since only 20% of the tyrosine molecules were labeled, then ~80% of the tyrosine present in the cells must have been derived from the fed tyrosine.

Furthermore, since no significant incorporation of ^{13}C units other than $^{13}C_6$ was observed, there must have been no significant degradation of tyrosine and reincorporation of the carbons of the tyrosine into CQ.

The only hydrogen of tyrosine which could be retained in the benzo[*b*]thiophenquinone of CQ would have to arise from one of the C-3 hydrogens of the tyrosine since all of the other hydrogens are displaced in the transformation. This single hydrogen would have to be incorporated at C-3 of the CQ. That this hydrogen is, in fact, retained was confirmed by the data (Table 1 on page 34, experiment 4a), which showed the incorporation of a single deuterium into the m/z 225 fragment and M^+ m/z 630 of the CQ isolated from cells grown with DL-[3,3-$^2H_2$]tyrosine. The extent of incorporation of deuterium originating from the deuteriated tyrosine, however, was much less than the extent of incorporation of ^{13}C originating from any of the ^{13}C-labeled tyrosines. This would indicate that, at some stage in the transformation of tyrosine to CQ, the hydrogens originally on the C-3 of the tyrosine must exchange with the hydrogens of the water. One intermediate which would be expected to exchange protons would be *p*-hydroxyphenylpyruvate, which can be derived reversibly from tyrosine by a number of different reactions. That this exchange does, in fact, occur was confirmed in that the tyrosine in the cellular protein was shown to contain only ~23% of the molecules with two deuterium and ~27% of the molecules with a single deuterium (Table 1 on page 34, experiment 4a). If we assume that *p*-hydroxyphenylpyruvate has the same label distribution as the cellular tyrosine, and that the deuteriated tyrosine is incorporated to the same extent as the

^{13}C-labeled tyrosines, then the decreased incorporation of deuterium from the deuteriated tyrosine can be completely accounted for by the exchange from the *p*-hydroxyphenylpyruvate.

Feeding of ^{18}O tyrosine resulted in no observed incorporation of ^{18}O into the CQ. Since the tyrosine in the cellular protein was found to be labeled to an extent of 56% with ^{18}O, the ^{18}O from the tyrosine must be lost either during or after the formation of the CQ. Exchange of the ^{18}O directly from the quinone, as has been observed in other quinones (Samuel, 1962; Snyder and Rapoport, 1970), is the most plausible explanation for this result, especially when the required high growth temperature for these cells is considered.

The logical conclusion from these experiments is that all of the carbons of tyrosine, except C-1, are incorporated as a unit into the benzo[*b*]thiophenquinone. This could occur by many different pathways as outlined in Figure 10. In each of these pathways, C-2 and C-3 of the tyrosine supply the C-2 and C-3 of the benzo[*b*]thiophenquinone and the aromatic carbons supply the carbons of the six-member ring. An important consideration in determining which of the pathways is correct is whether or not homogentisic acid is an intermediate. Homogentisic acid is an established intermediate in both the degradation of tyrosine (Walsh, 1979; Yoshizako et al., 1985) in microorganisms and in the biosynthesis of plastoquinones and tocopherols (Whistance and Threlfall, 1968a; 1968b; 1970) in plants, but its function or occurrence in species of *Sulfolobus* is unknown. On the basis of the pattern of incorporation of tyrosine into CQ and on the established biosynthetic pathway of plastoquinone (Whistance and

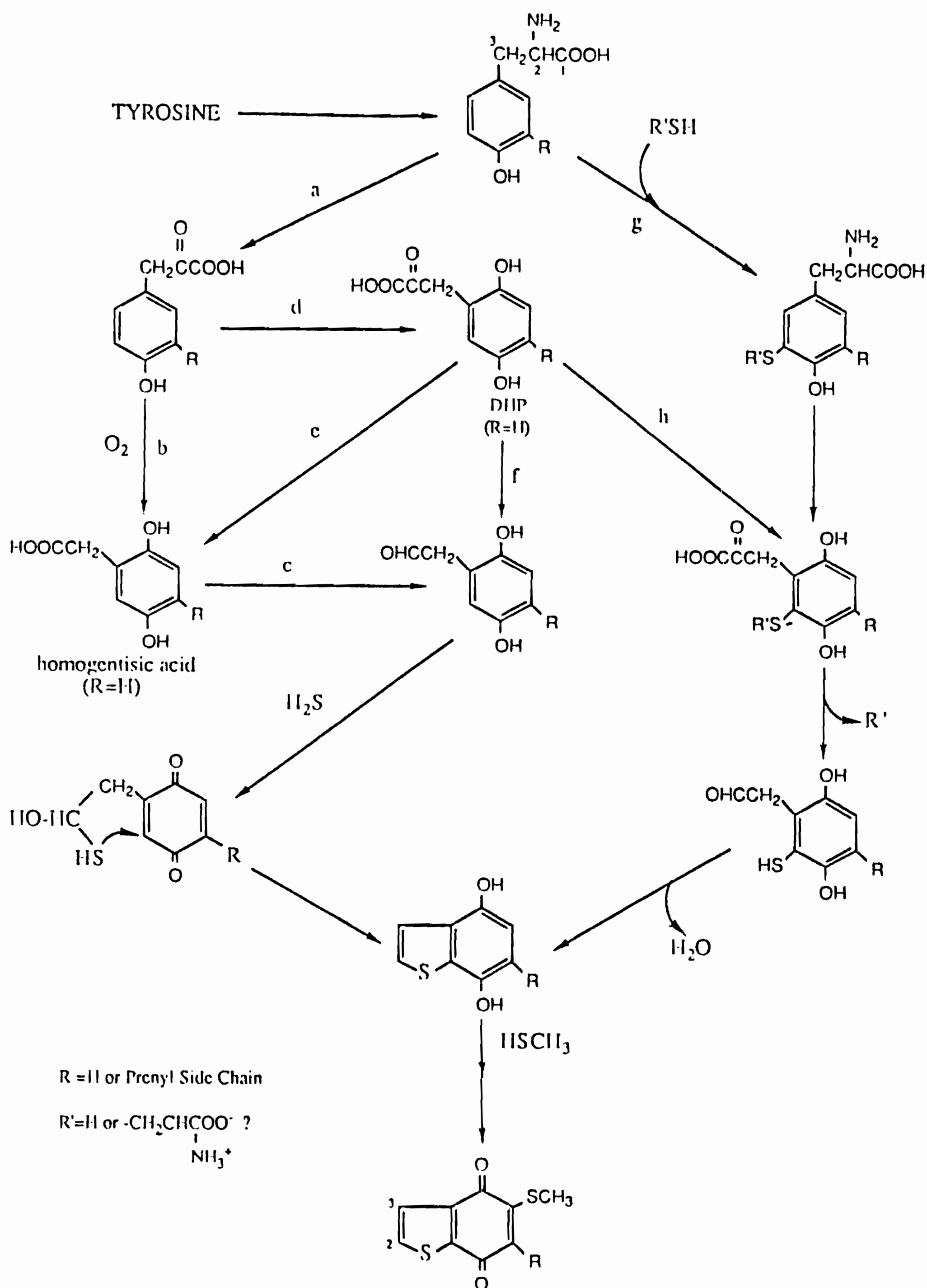


Figure 10. Possible pathways for the biosynthesis of CQ: DHP, 2,5-dihydroxyphenylpyruvate.

Threlfall, 1968a; 1968b; 1970), homogentisic acid was considered to be a likely intermediate in the biosynthesis of CQ from tyrosine. After transamination to 4-hydroxyphenylpyruvate (Figure 10, reaction a), the tyrosine would be metabolized to homogentisic acid by a well-known rearrangement catalyzed by *p*-hydroxyphenylpyruvate dioxygenase (Figure 10, reaction b) (Walsh, 1979). Further reactions leading toward CQ could proceed by the conversion of the carboxylic acid of the homogentisic acid to an aldehyde, reaction with sulfide, and subsequent cyclization to form benzo[*b*]thiophen-4,7-quinone (Figure 10, reaction c).

Incorporation of labeled potential intermediates. The possible involvement of homogentisic acid in the biosynthesis of CQ in *Sulfolobus* spp. was tested both by measuring the suppression of ^{2}H- or ^{13}C-labeled tyrosine incorporation into CQ by growing cells in the presence of homogentisic acid and by measuring the incorporation of [U-$^{2}H_5$]homogentisic acid into CQ. The results of these experiments are reported in Table 1 on page 34 (experiments 1b, 4b, and 3). No suppression of [3,3-$^{2}H_2$]tyrosine incorporation into CQ by homogentisic acid was observed in experiment 4b, whereas about 10% suppression of [2-^{13}C]tyrosine incorporation by homogentisic acid was observed in experiment 1b. In experiment 3, label from the [U-$^{2}H_5$]homogentisic acid was not incorporated into the CQ. Measurement of the incorporation of deuterium from the homogentisic acid into the cellular amino acids by GC-MS showed less than 1% incorporation into alanine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, phenylalanine, proline, serine, threonine, and valine. Thus, the 10% reduction in tyrosine in-

corporation into CQ by homogentisic acid observed in experiment 1b, did not appear to result from the conversion of homogentisic acid to CQ but from the decreased utilization of the labeled tyrosine. This was confirmed by the decrease in % ^{13}C in the tyrosine present in the cells from 81.1 to 69.9. These negative results do not prove or disprove that homogentisic acid is an intermediate in CQ biosynthesis as the cell may simply be unable to take up the homogentisic acid.

Another method that could be used to establish the possible involvement of homogentisic acid in CQ biosynthesis is to determine if homogentisic acid is in fact present in the cells or the medium from cells which have been grown with tyrosine. Thus, cells of *S. acidocaldarius* were grown in a medium supplemented with L-tyrosine (50 mg/100 ml) and after separation of the cells from the medium both were acidified and extracted with ethyl acetate to recover any homogentisic acid that may have been present. After TLC purification, homogentisic acid was assayed by GC-MS of its $(TMS)_3$ derivative. Homogentisic acid, however, could only be detected in these extracts when it was added as an internal standard to the analytical procedure.

Another approach which was used to try to establish if homogentisic acid was involved in tyrosine metabolism by *Sulfolobus* was to establish if cell extracts could metabolize homogentisic acid. This was accomplished by assaying for the homogentisic acid oxygenase and isomerase activity in cell extracts. Attempts to measure the conversion of homogentisic acid to maleylacetoacetate in cell extracts by measuring changes in A_{320} showed insignificant changes (less than 0.005 A/min). The addition of glutathione, however, did not have much effects on

absorbance change (increased the rate of the absorbance change by 0.002/min). The same results, however, were observed by adding β-mercaptoethanol instead of glutathione and by incubating homogentisic acid, glutathione, and a heat denatured cell extract. These results thus indicate that the observed small increase in absorbance was not a result of the enzymatic degradation of homogentisic acid. The small increase in absorbance in the absence of glutathione indicated either that no significant enzymatic activity for homogentisic acid degradation was present in the cell extract, or that the degradation of maleylacetoacetate is a glutathione-independent nonenzymatic process and occurs faster than its formation from homogentisic acid. Since this last option has not been observed in other extracts, this would indicate that homogentisic acid is not metabolized by these cells. [It is possible that HA oxygenase is cold labile thus accounting for the lack of activity, this seems unlikely since the previous work for the extraction of this enzyme from other cells was done at 0°C (Crawford, 1977).]

The inability to obtain evidence for the involvement of homogentisic acid as an intermediate in CQ biosynthesis prompted the testing of several other possible precursors for CQ. Since all the carbons of tyrosine except C-1 are incorporated into CQ, the rearrangement of the carbon structure that occurs during the conversion of *p*-hydroxyphenylpyruvate to homogentisic acid is a critical step in CQ biosynthesis. However, if homogentisic acid is not an intermediate, this rearrangement must occur in some other manner. In an attempt to determine just how this rearrangement could occur, It was reconsidered that the mechanism of homogentisic acid synthesis from *p*-hydroxyphenylpyruvate. The enzyme

catalyzing this reaction has been found to act as a dioxygenase with one of the oxygens of dioxygen being incorporated into the 2 position of the homogentisate and the other into the carboxylic acid portion of the molecule (Jefford and Cadby, 1981; Leinberger et al., 1981; Lindblad et al., 1970). This reaction, however, could easily be broken down into two separate steps. In the first step (Figure 10 on page 38, reaction d), an oxidative rearrangement would convert *p*-hydroxyphenylpyruvate into 2,5-dihydroxyphenylpyruvate. This compound, in turn, could be converted into homogentisate by an oxidative decarboxylation (Figure 10 on page 38, reaction e). This two-step oxidation has been in fact observed by Yuasa et al. (1978) in tyrosine metabolism in *Aspergillus sojae*. If the first oxidative rearrangement is followed by a nonoxidative decarboxylation (Figure 10 on page 38, reaction f), 2,5-dihydroxyphenylpyruvate could be converted to the 2,5-dihydroxyphenylacetylaldehyde. This resulting aldehyde would then be converted to a thiol by a reaction sequence recently discovered in the biosynthesis of coenzyme M and 7-mercaptoheptanoic acid (White, 1985; 1986; 1989). Oxidation of the hydroquinone to the quinone would allow for the addition of the thiol into the quinone to generate a dihydrothiophene ring. Dehydrogenation of the C-2, C-3 bond of the hydrothiophene would then produce the desired benzothiophenquinone nucleus. Addition of the isoprenoid side chain and the thiomethyl group would complete the biosynthesis of CQ (Figure 10 on page 38).

Growing cells in the presence of [3,3-$^{2}H_2$]-2′,5′-dihydroxyphenylalanine, which would be expected to undergo a transamination to the required ketoacid

did not lead to incorporation of deuterium into CQ. Possible explanations for this negative result are that either 2,5-dihydroxyphenylpyruvate is not a precursor to homogentisic acid, that the labeled compound was not taken up by the cells, or that the desired ketoacid was not produced.

There are, of course, many other possible pathways whereby the required carbons of tyrosine could be incorporated into CQ. Some of these pathways are outlined in Figure 10 on page 38. Each pathway requires an oxidative rearrangement of the original tyrosine carbon structure in order to generate a compound that can be transformed into the benzo[*b*]thiophen-4,7-quinone structure of CQ. Other possible pathways (Figure 11) were tested by growing cells with labeled compounds that could be intermediates in the biosynthesis, and by measuring the incorporation of label into the CQ using mass spectrometry. The compounds tested were [β-$^{2}H_{2}$]-2-(4-hydroxyphenyl)-ethanethiol (compound 1, Figure 11), [β-$^{2}H_{2}$]-2-(2,5-dihydroxyphenyl)-ethanethiol (compound 2, Figure 11), [1-^{13}C]tyramine (compound 4, Figure 11), [β-$^{2}H_{2}$]-2-(2,5-dihydroxyphenyl)ethanol (compound 5, Figure 11), [β-$^{2}H_{2}$]-2-(4-hydroxyphenyl)ethanol (compound 6, Figure 11), and [β, β, 2′, 3′, 5′, 6′-$^{2}H_{6}$]- 4-hydroxyphenylacetic acid (compound 7, Figure 11). In each case, the thiol compounds were found to completely inhibit cell growth at a concentration of 0.5 mg/ml, a concentration which prevented them from being tested as precursors to CQ. The alcohols, on the other hand, had little or no effect on cell growth at a concentration of 0.5 mg/ml, but were not found to be incorporated into the CQ. Deuteriated 4-hydroxyphenylacetic acid was not incorporated into CQ but was metabolized

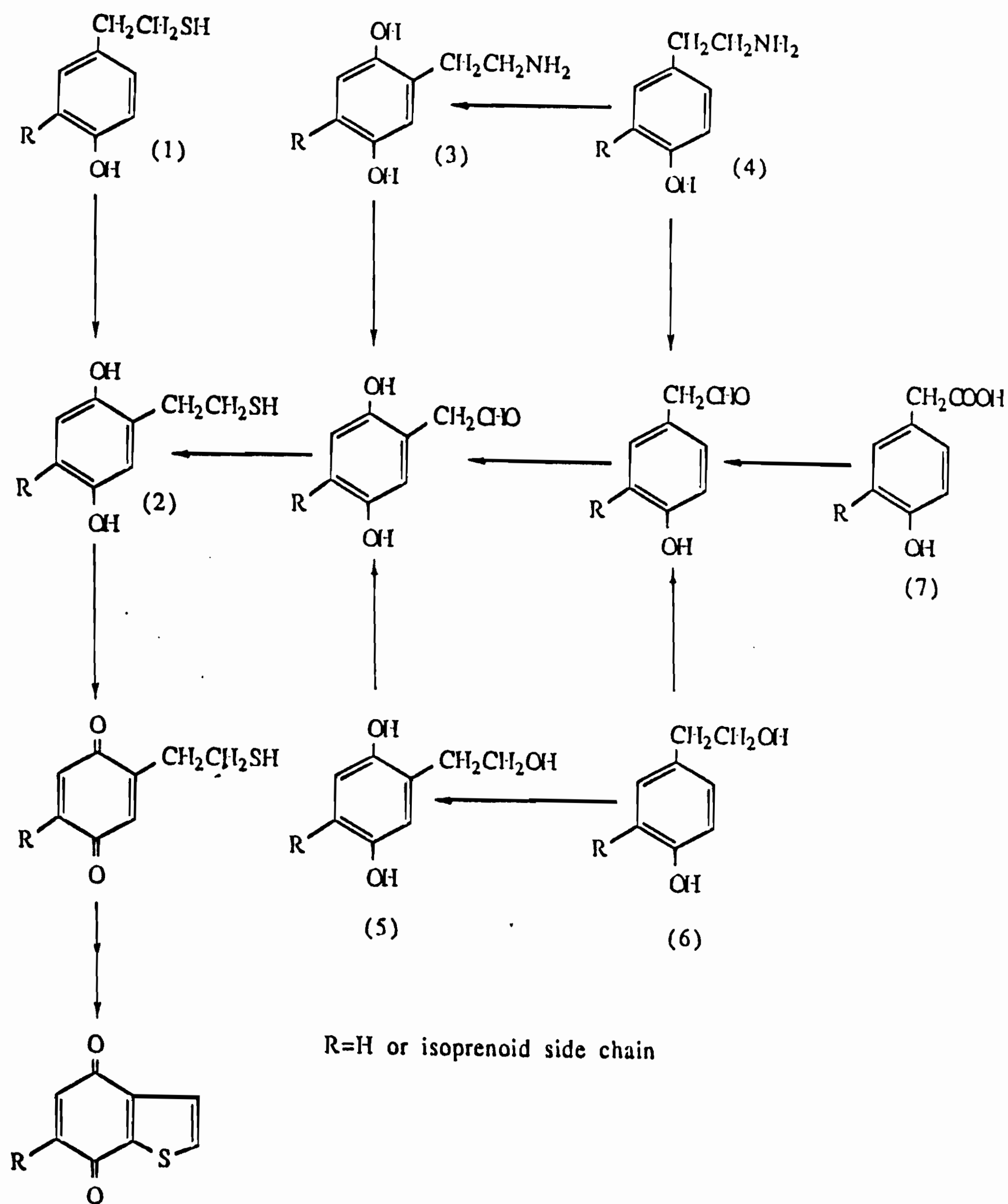


Figure 11. Other possible pathways for CQ biosynthesis.

by the cells to 3,4-dihydroxyphenylacetic acid. [1-^{13}C]Tyramine did not affect the cell growth but was not found to be incorporated into CQ. Attempts to synthesize labeled 2,5-dihydroxyphenylacetaldehyde, a critical intermediate in the proposed pathways, in order to test its incorporation to CQ, were not successful because of its instability (Bruce and Creed, 1970).

A possible explanation for why none of the above intermediates, including homogentisic acid, are incorporated into CQ, is that the isoprenoid side chain is introduced at an early step in the biosynthesis and that the formation of the benzo[*b*]thiophene ring occurs at a later step in the biosynthesis. This early addition of the side chain does, in fact, occur during the biosynthesis of ubiquinones where *p*-hydroxybenzoic acid reacts with isoprenyl pyrophosphate to form 2-polyprenylphenol in procaryotes and 3,4-dihydroxy-5-polyprenylbenzoate in eucaryotes. This also happens in the biosynthesis of plastoquinone where homogensitic acid reacts with polyprenyl pyrophosphate to form polyprenylated homogensitic acid in higher plants (Soll et al., 1980; Marshall et al., 1985; Thomas and Threlfall, 1975). The resulting prenylated phenols are then subsequently modified to the final ubiquinones or plastoquinones (Olson and Rudney, 1983; Davis et al., 1967).

The formation of any sulfur-containing derivative of tyrosine early in the biosynthesis would also explain why none of the labeled compounds tested were incorporated. This would occur if the sulfur is introduced at an early stage of the biosynthesis as shown in reaction g or h of Figure 10 on page 38. This idea is based on the biosynthesis of melanins, where cysteine sulfur is introduced into the

aromatic ring of tyrosine at an early stage in melanin formation (Hack and Helmy, 1983; Thomson, 1974). Attempts were made to synthesize some of the possible sulfur derivatives of tyrosine, such as 3′-S-cysteinyl-tyrosine and 3′-thiol-tyrosine. The S-cysteinyl-tyrosine (product of reaction g in Figure 10 on page 38, where R′SH = cysteine and R = H) was synthesized by heating tyrosine and cystine (1:2 mole ratio) in 40% HBr at reflux for 4 hr (Ito and Prota, 1977). The crude product mixture was concentrated under nitrogen and purified on a Dowex-50 column eluted with a HCl gradient. The product was detected by absorbance at 295 nm and identified by MS as its trifluoroacetyl, *n*-butyl derivative. However, the yield of the compound was so low that the synthesis of the stable isotopically labeled compound was impractical. The synthesis of 3′-thiol-tyrosine (product of reaction g in Figure 10 on page 38, R, R′ = H) has been described by Lutz et al. (1972). However, in several attempts to synthesize this compound, a suitable yield of the final product was never obtained. If both of these sulfur containing modified tyrosines could be made in a good yield, the labeled compound could be synthesized, fed to cells, and their incorporation into CQ could be readily established by MS. Having these compounds available would also allow for the development of sensitive methods that could be used to detect small amounts of these compounds in the cell.

BIOSYNTHESIS OF THE ISOPRENOID CHAIN OF CQ

INTRODUCTION

The biosynthesis of isoprenoids via the mevalonate pathway in eucaryotes is well established (Beytía and Porter, 1976). In this pathway, three molecules of acetate are condensed to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) which is then transformed into mevalonic acid. The mevalonic acid is then converted by a series of enzymatic reactions to isopentenyl pyrophosphate. The head-to-tail condensation of this active isoprene unit produces farnesyl pyrophosphate, which is further elongated with isopentenyl pyrophosphate to form a series of higher polyprenoids. Condensation of polyprenyl pyrophosphate with the quinone precusor, 4-hydroxybenzoic acid for ubiquinones and homogentisic acid for plastoquinones, leads to the formation of ubiquinones and

plastoquinones. The enzyme responsible for this reaction, polyprenyl pyrophosphate transferase, has been studied in several organisms, including rat liver and a few microorganisms (Olson and Rudney, 1982). It was found that the enzyme lacks specificity toward the isoprene chain length, which suggests that the length of the side chain of ubiquinone, within any given species, is regulated at the level of polyprenyl pyrophosphate biosynthesis.

The early steps of isoprenoid biosynthesis in eubacteria have recently been found to be different from those in eucaryotes. This finding was based on the observation that free acetate and free mevalonic acid were not involved in the biosynthesis of the isoprenoid side chain of ubiquinone in *E. coli* (Zhou and White, 1991). The incorporation pattern of [1-^{13}C]- and [2-^{13}C]-acetate into the isoprenoid side chain of CQ (De Rosa et al., 1977), however, indicates that the isoprenoid side chain is biosynthesized from acetate via the mevalonate pathway as it is in eucaryotes, which is also consistent with the biosynthesis of isoprenoid lipids in archaebacteria (De Rosa et al., 1989). Labeled mevalonate has also been shown to be incorporated into the archaebacterial lipids (De Rosa et al., 1975).

Considering the mechanism of formation of long chain isoprenoids and the mechanism of the reaction used to couple the isoprenoid chain to the aromatic ring, the long chain unsaturated polyprenyl pyrophosphate must be formed before the polyprenyl unit is attached to the aromatic ring. This of course is what occurs during the biosynthesis of isoprenoid quinones in both eucaryotes and eubacteria (Olson and Rudney, 1983; Threlfall and Whistance, 1971). A similar reaction is also likely to occur during the biosynthesis of CQ. However, since the

isoprenoid chain in CQ is completely saturated and the isoprenoid chain produced via the mevalonate pathway is completely unsaturated, the hydrogenation of six double bonds must occur at some stage during the formation of the saturated isoprenoid chain of CQ. One can envision two different pathways whereby this hydrogenation could occur (Figure 12). In the first pathway (a, Figure 12), the hydrogenation occurs after the isoprenoid chain has been attached to the ring. In the second pathway (b, Figure 12), the hydrogenation of all the double bonds except the one next to the pyrophosphate occurs before the isoprenoid chain is attached to the ring. This limited hydrogenation is required since an allylic double bond next to the pyrophosphate is required for the coupling of side chain to the aromatic ring. The final step in pathway b would be the hydrogenation of the double bond remaining in the side chain of the coupled product. Since pathway b requires the cells to have a mechanism to hydrogenate all of the double bonds except the one next to the pyrophosphate, more steps are required to accomplish the synthesis of isoprenylated aromatic compounds. Pathway a would appear to be the pathway more likely to operate in the cells. Evidence for these pathways was obtained by establishing the presence of farnesylfarnesol, a decomposition product of farnesylfarnesyl pyrophosphate, in cell extracts.

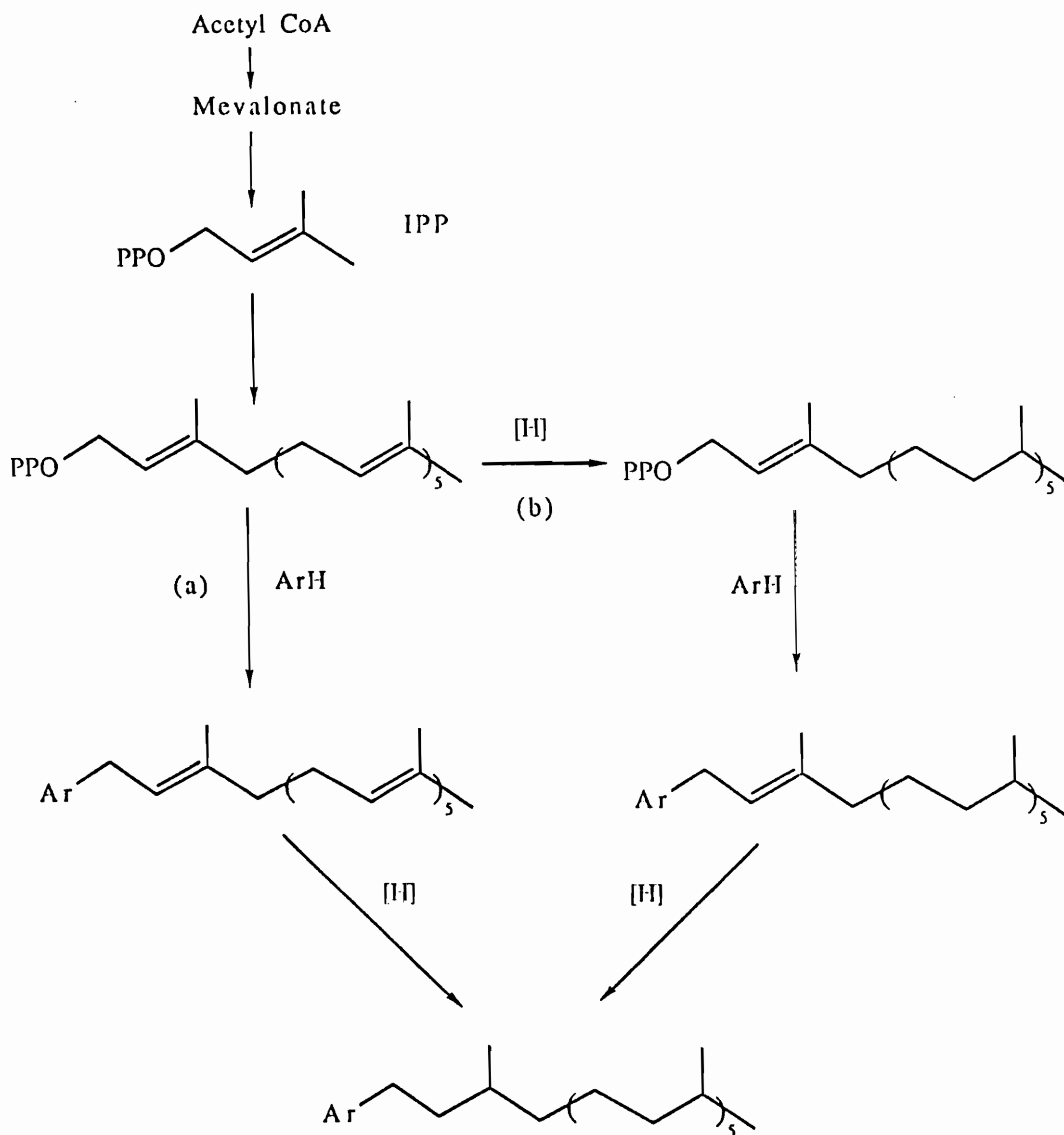


Figure 12. Possible mechanisms for the formation of the isoprenoid side chain of CQ.

MATERIALS AND METHODS.

Chemicals. Farnesylfarnesol was obtained from Takasago International Corporation, Tokyo, Japan. The sample was found to contain other minor contaminants by separating the sample with TLC and spraying with molybdenum spray. A portion of the sample was thus purified by TLC and the resulting pure sample was used as a marker to identify the compound in the cell. The farnesylfarnesol has a R_f of 0.24 in the solvent system hexane/diethyl ether (15:5).

Farnesylfarnesyl acetate. A portion of the TLC purified farnesylfarnesol was converted to its acetate derivative by treatment with acetic anhydride and pyridine and the resulting acetate was purified on TLC (R_f=0.58 in 15:5 hexane/diethyl ether).

GC-MS of farnesylfarnesol and its acetate derivative. The TLC purified farnesylfarnesol and its acetate derivative were dissolved in methylene chloride solvent and analyzed on GC-MS using a HP5 capillary column (25 mm x 0.5 mm) programmed from 70°C at 10°C /min. A sample of squalene was used as an internal standard for comparison of the retention time of these samples.

Analysis of C_{30} isoprenyl alcohol in cells. Lipid components present in *S. acidocaldarius* were extracted with methylene chloride and methanol (1:1) as described earlier in this thesis and separated by preparative TLC. The individual compounds present in the TLC separated samples were detected on the TLC plates either by spraying a portion of the plate with molybdenum/sulfuric acid spray followed by heating or by exposing the plates to iodine vapor. The area

of the plate with the same R_f as standard farnesylfarnesol was removed from the plate, and the compounds contained therein were eluted with methylene chloride. After reaction with acetic anhydride and pyridine, the resulting acetate was purified by TLC. Both nonacetylated and acetylated samples purified from TLC were analyzed by GC-MS.

RESULTS AND DISCUSSION

GC analysis of the TLC purified farnesylfarnesol gave a single broad GC peak, a characteristic of compounds with a free hydroxyl group. As expected, the acetate derivative gave a much sharper peak, presumely because of conversion of the polar hydroxyl group to an acetate. The retention times of farnesylfarnesol, its acetate derivative, and squalene under the GC conditions used were 0.5, 0.7, and 0.4, respectively. The mass spectrum of farnesylfarnesol showed a molecular ion at m/z 426 and a fragment ion at m/z 408 (M^+-18). The acetate derivative showed a molecular ion at m/z 468 and a fragment ion at m/z 408 (M^+-60).

The acetylated sample of the crude cell extract was the first to be analyzed by GC-MS and showed the presence of several peaks, two of the major peaks were identified as indole-3-acetic acid and a $C_{30}H_{50}$ isoprenoid, possibly squalene. The occurrence of indole-3-acetic acid (White, 1987) and the $C_{30}H_{50}$ isoprenoid (Holzer and Oró, 1979) in species of *Sulfolobus* has been previously described. Acetylated farnesylfarnesol, however, was not detected. This acetylated crude sample was further purified on TLC and the analysis by GC-MS

was repeated. The results of this analysis showed squalene and a very small amount of a compound which had the same retention time as farnesylfarnesyl acetate. The mass spectrum of this peak had a molecular ion at *m/z* 468 and a fragment ion at 408 (M^+-60), indicating the presence of farnesylfarnesyl acetate. Two other unknown peaks in the GC-MS were also found. One of the peaks had mass spectral ions at *m/z* 586 and 383, the other peak had mass spectral ions at *m/z* 386 and 368.

The inability to detect C_{30}-isoprenyl alcohols with either no double bonds or one double bond in lipid extracts, in which farnesylfarnesol was detected, can be taken as supporting evidence that the biosynthesis of CQ proceeds as in pathway a, Figure 12 on page 50. In this pathway, farnesylfarnesyl pyrophosphate is the source of the isoprenyl side chain of CQ.

SEARCH FOR THE INTERMEDIATES IN CQ BIOSYNTHESIS

INTRODUCTION

The intermediates in the biosynthesis of ubiquinones in rat liver were first identified by labeling the cells with [U-^{14}C]4-hydroxybenzoic acid, a precursor of ubiquinone, and isolating the resulting radioactive compounds (Trumpower et al., 1972). The identification of the intermediates involved in the biosynthesis in microorganisms was somewhat more difficult because of the small amount of the intermediates that were present in the cells (Young et al., 1973). This problem was solved by using mutations that specifically block the different steps of ubiquinone biosynthesis. Many of these mutants accumulated intermediates of ubiquinone biosynthesis to a high enough level that they could be isolated and identified (Bentley and Meganathan, 1987; Young et al., 1973). One result of this

work was that all the intermediates leading to ubiquinone after 4-hydroxybenzoic acid were found to be polyprenylated.

The inability to show the incorporation of a series of labeled compounds including homogensitic acid into CQ compelled one to try another experimental approach. This approach involved an attempt to isolate and identify lipid soluble intermediates in CQ biosynthesis directly from the cells. The idea behind this approach was that the early attachment of the isoprenoid side chain to the aromatic ring was the reason that none of the intermediates tested was found to be incorporated into CQ. If this is the case, then a series of polyprenylated intermediates maybe present in the cell. The most logical polyprenylated compound would be polyprenylated homogentisic acid or its lactone. Thus an attempt has been made to find the farnesylfarnesyl homogentisic acid or lactone in a lipid cell extract of *S. acidocaldarius*. Since tyrosine is a precursor of CQ, [U-^{14}C]tyrosine was used to label these intermediates so that they could be isolated and identified.

MATERIALS AND METHODS

Chemicals. [U-^{14}C]Tyrosine and phytol were obtained from Sigma Chemical Company. Benzo[*b*]thiophen-4,7-quinone was synthesized according to Fieser and Kennelly's method (1935) which is outlined in Figure 13. The benzo[*b*]thiophen-4,7-quinone product was purified by TLC and analyzed by mass spectrometry and GC-MS. The mass spectral data showed the expected

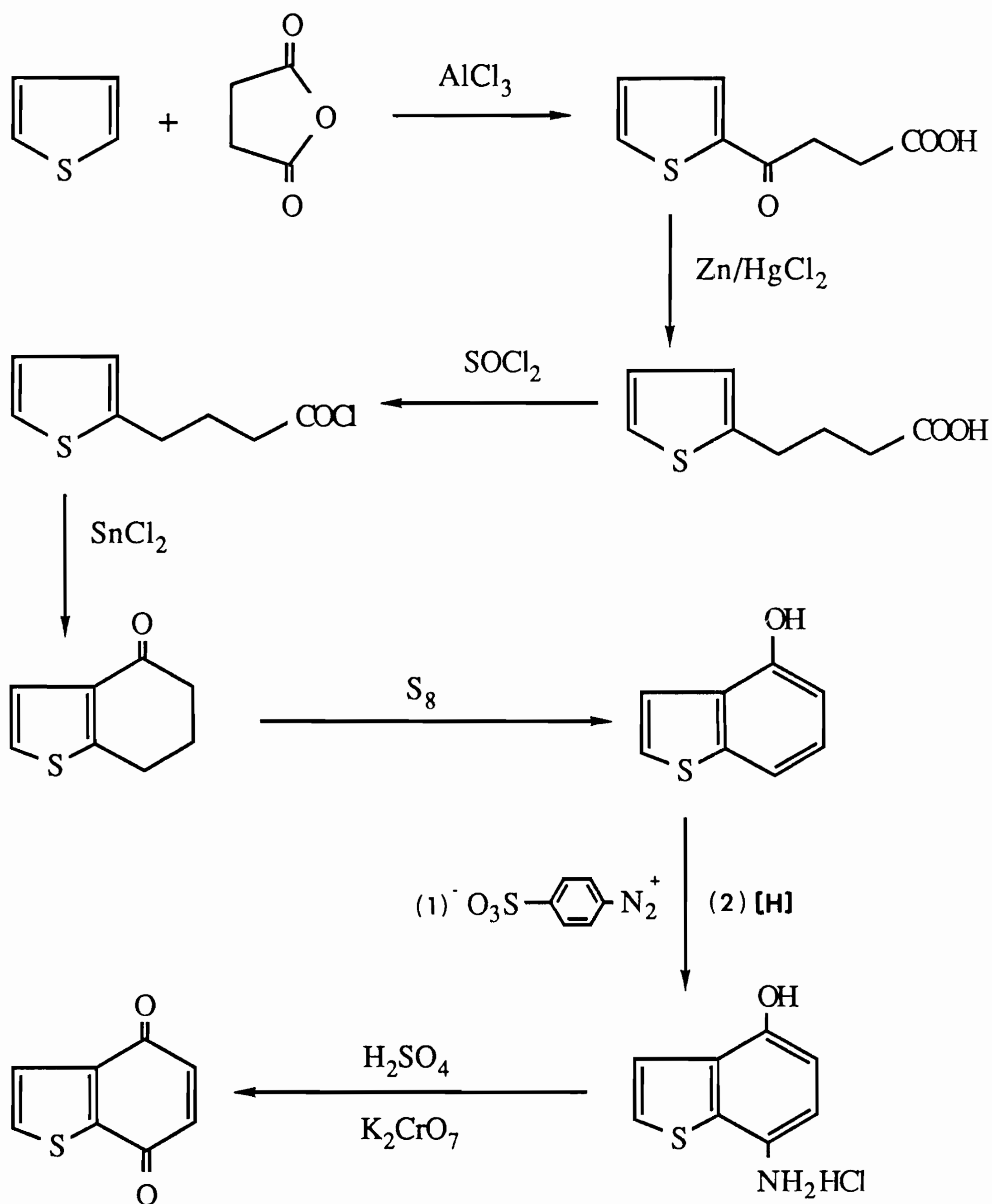
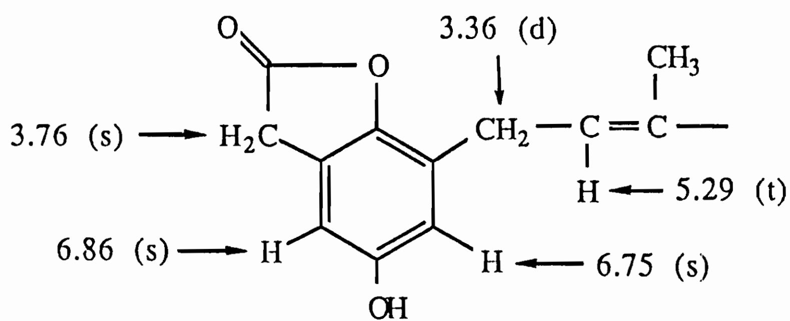


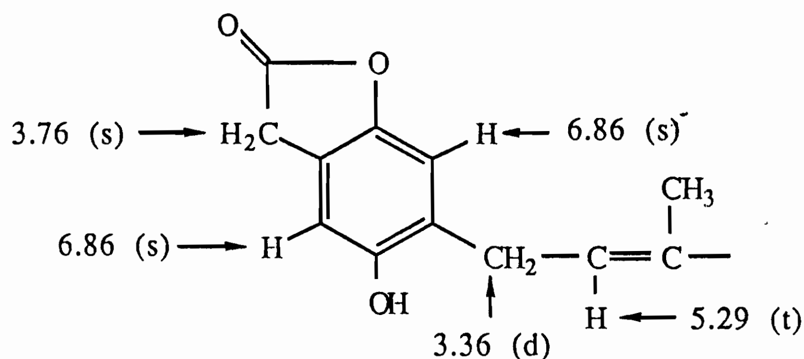
Figure 13. Chemical synthesis of benzothiophene quinone.

molecular ion at *m/z* 164. The R_f value of this compound upon TLC analysis, the retention time in GC, and its mass spectrum were consistant with a known sample of 5-methylbenzothiophene quinone provided by Dr. John S. Swenton, Department of Chemistry, The Ohio State University.

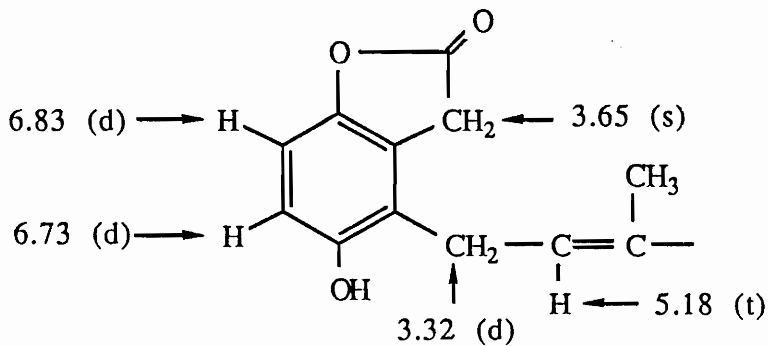
Phytyl homogentisic acid lactone was synthesized from homogentisic acid lactone and phytol using boron trifluoride etherate as catalyst (Hirschmann et al., 1954). Homogentisic acid lactone (150 mg, 1.0 mmole) was dissolved in 0.6 ml dioxane to which was added 0.2 mmol of $BF_3 \bullet Et_2O$ (30 μl) followed by 88.3 mg (0.3 mmol) of phytol. The solution was stirred at 60°C for 30 min. The phytyl homogentisic acid lactones were extracted with ether, washed with water and 5% of $NaHCO_3$ solution, and dried over anhydrous Na_2SO_4. A portion of the products of the reaction were purified by preparative TLC using methylene chloride as solvent, to give 7.4 mg of a lower spot (R_f=0.27) and 3.4 mg of a upper spot (R_f=0.39). Mass spectral analysis of both spots showed the expected molecular ion at M^+ *m/z* 428. NMR spectra of these two bands (Figure 14) showed the lower R_f band to contain the 3 and 6 substituted isomers, and the upper band to contain the 4 substituted isomer. Thus the minor component of the lower TLC spot, which represented 22% of the materials based on the intensities of the aromatic protons, was the desired isomer. The UV spectrum of the phytyl homogentisic acid lactone was obtained using either a spectrophotometer for samples dissolved in methylene chloride or a TLC/gel scanner for samples separated on a TLC plate (Figure 15, the spectrum recorded with gel scanner is not



major component of lower TLC spot



minor component of lower TLC spot (desired isomer)



upper TLC spot

Figure 14. Chemical shift assignments and structures of the synthetic phytyl homogentisic acid lactones.

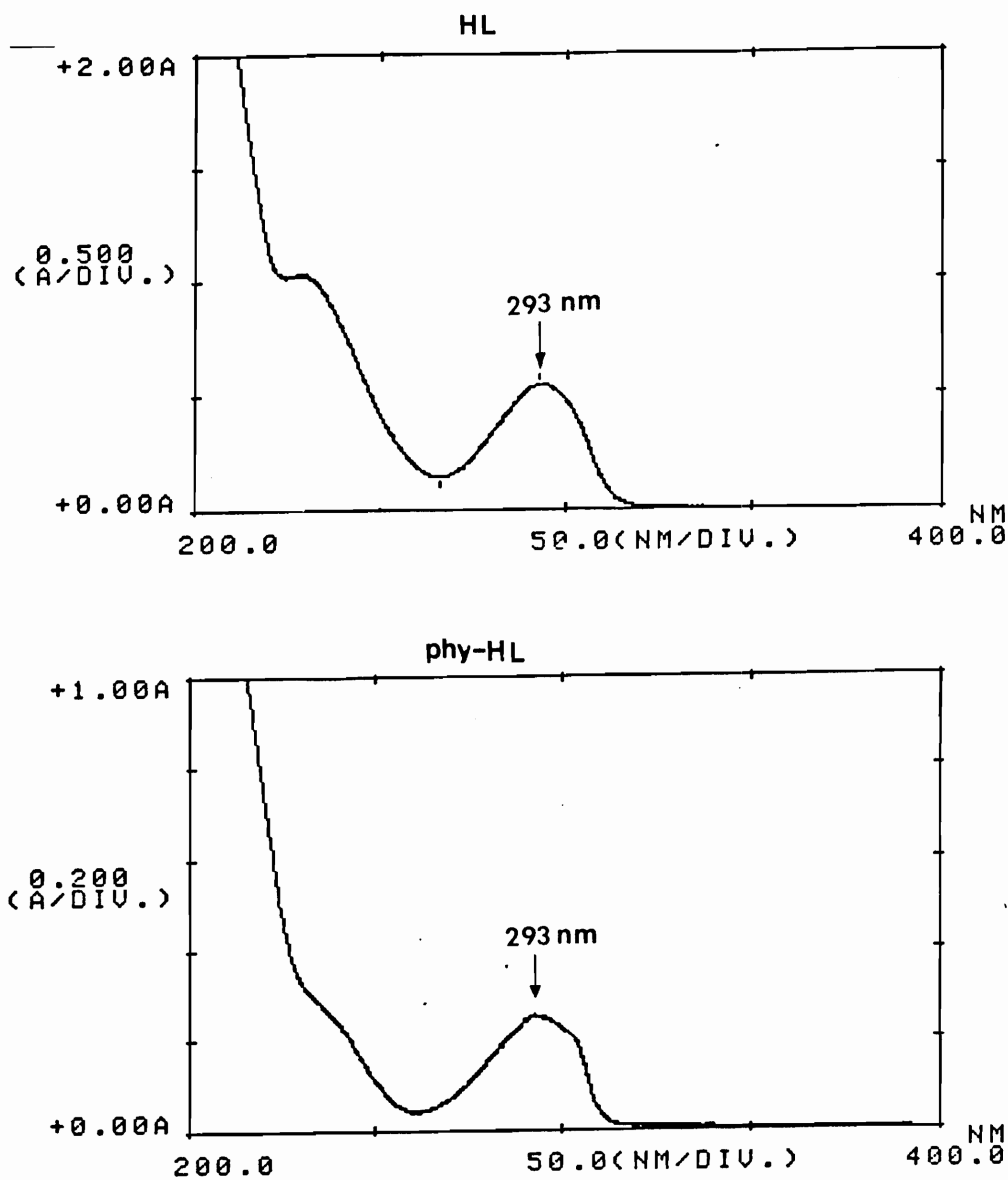


Figure 15. UV spectrum of synthetic phytyl homogentisic acid lactone.: The upper pannel is the spectrum of HL in CH_2Cl_2. The lower panel is the spectrum of phytyl-HL in the same solvent.

reported here). The λ_{max} obtained from the TLC scanner was found to be about 10 nm shorter than that obtained for samples dissolved in methylene chloride.

Farnesylfarnesyl homogentisic acid lactone was synthesized using the same methods as described for the phytyl isomer, but the reaction was run in a much smaller scale because of the small amount of farnesylfarnesol available. Since the R_f value for phytyl homogentisic acid lactone was almost identical to the farnesylfarnesyl homogentisic acid lactone, the phytyl homogentisic acid lactone was used as a marker for the identification of farnesylfarnesyl homogentisic acid lactone from cell extracts.

$LiAlH_4$ reduction of homogentisic acid, homogentisic acid lactone, and phytyl homogentisic acid lactone and acetylation of the products. A solution of homogentisic acid, homogentisic acid lactone, or phytyl homogentisic acid lactone in tetrahydrofuran was treated with excess $LiAlH_4$ at 50°C for 30-60 min, respectively. After the addition of 2 N HCl and water, the products of each of the reactions were extracted with ethyl acetate. TLC analysis of each of the reactions showed three major UV spots (Figure 16). The very top spot in each reaction was relatively nonpolar and was believed to be the 2,5-dihydroxystyrene. The lowest spot of each reaction was found to turn brown when the developed TLC plates were exposed to ammonia vapor. The mass spectral data of this spot from homogentisic acid lactone sample (panel a, Figure 16) and homogentisic acid sample (panel b, Figure 16) showed the molecular ion at *m/z* 154, and the fragmentation ions at *m/z* 136, 123, and 107, which is consistent with the compound being 2,5-dihydroxyphenylethanol. The lowest spot in the phytyl homogentisic

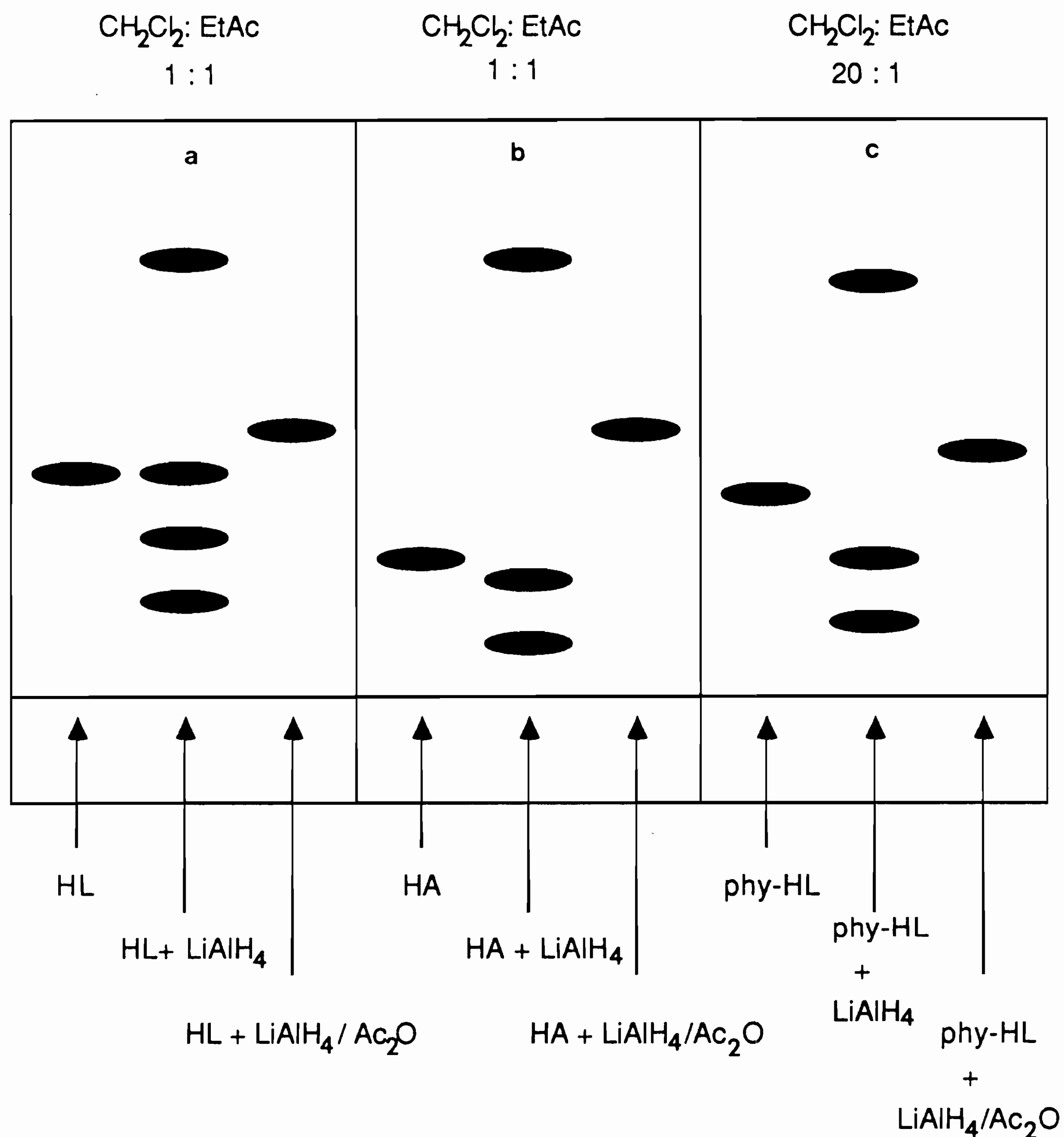


Figure 16. TLC of reductive acetylation of HL (a), HA (b), and phy-HL (c): HL = homogentisic acid lactone, HA = homogentisic acid, phy-HL = phytyl homogentisic acid lactone.

acid lactone sample (panel c, Figure 16), which was considered to be phytyl 2,5-dihydroxyphenylethanol, was found to slowly convert to the middle spot. This spot did not turn brown when exposed to ammonia vapor. The mass spectrum of this middle spot showed the molecular ion at *m/z* 430, which is consistent with the quinone form of phytyl 2,5-dihydroxyphenylethanol. TLC purified samples of 2,5-dihydroxyphenylethanol, phytyl 2,5-dihydroxyphenylethanol and its quinone, were acetylated to the corresponding triacetyl derivatives (Ac_3-HA and Ac_3-phy-HA) with acetyl anhydride, zinc dust, and triethylamine as described earlier. Their separation by TLC is shown in Figure 16. The TLC purified triacetyl derivatives were evaluated by mass spectrometry.

Search for benzothiophene quinone. Cells of *S. acidocaldarius* were grown on 1 liter of yeast extract containing medium, and after separation from the medium by centrifugation, were extracted with methylene chloride and methanol (1:1 vol/vol) as described earlier for the isolation of CQ. The cell extract was purified on TLC using methylene chloride as solvent. The area of the plate with the same R_f as synthetic benzo[*b*]thiophen-4,7-quinone was scraped from the plate and eluted with methylene chloride and the concentrated sample was analyzed by GC-MS.

Search for polyprenylated homogentisic acid. The cells (2 g of wet weight) were extracted with ethyl acetate (2 x 10 ml) under acidic condition and the resulting extract was chromatographed on TLC using methylene chloride-ethyl acetate (2:1) as solvent. The UV spots near the R_f of phytyl homogentisic acid

lactone were removed from the plate and eluted with methylene chloride. The mass spectrum of eluted compounds were obtained by direct inlet.

Tracing the intermediates by using [U-^{14}C]tyrosine. *S. acidocaldarius* was grown in the yeast extract media (200 ml) containing 10 mg of [U-^{14}C]tyrosine (2 μCi). The growth curve was recorded by measuring the cell density at 540 nm. Aliquots of culture (40 ml) were taken out at 5 different time points during the growth, the cells were removed by centrifugation and extracted under slightly acidic condition with methylene chloride and methanol (1:1 vol/vol). A portion of this total lipid extract was counted with a scintillation counter to determine the total amount of ^{14}C tyrosine incorporated into lipid soluble materials. CQ was purified on TLC from the remaining cell extract as described above. According to the ratio of (radioactivity in CQ)/(radioactivity in the total lipid), the time point in the growth curve at which the intermediates are likely to be labeled to their maximal extent was determined.

The experiment was repeated once with the [U-^{14}C]tyrosine (5 μCi/4.5 mg) being added to the growing culture (200 ml) at the beginning of log-phase growth. The lipid and CQ were isolated and counted as in the first experiment. The compounds in the total lipid extract were separated on TLC and the radioactive areas were localized by removing equally divided areas of the plate and determining the radioactivities present by scintillation counting. The areas of the plate containing radioactivity should be lipid soluble metabolites derived from tyrosine and would thus likely be intermediates between tyrosine and CQ.

After the radioactive spot(s) is(are) located, a large portion of the *S. acidocaldarius* (4 liter) was grown with non-labeled tyrosine and a lipid extract was obtained as described before. This was done in the hope of obtaining enough of the purified intermediates for their characterization. Because of the large amount of lipid extract obtained, it was separated into three fractions by chromatography on a silica gel column (1 x 5 cm). Fraction 1 contained compounds eluted with hexane-ether (8:2), fraction 2 contained compounds eluted with methylene chloride, and Fraction 3 contained compounds eluted with ethyl acetate. Each of those fractions was then further purified by preparative TLC.

RESULTS AND DISCUSSION

Benzothiophene quinone in cell extract. Benzo[*b*]thiophen-4,7-quinone is the aromatic core in the structure of CQ. The attachment of the isoprenoid side chain and thiomethyl group to a pre-formed benzo[*b*]thiophen-4,7-quinone ring would be one route in which CQ could be biosynthesized. This route to CQ would be analogous to the biosynthesis of menaquinones where the fully formed quinone ring reacts with isoprenyl pyrophosphate to form menaquinones (Bentley and Meganathan, 1982). Thus the detection of benzothiophene quinone in *S. acidocaldarius* could be taken as evidence that it may be an intermediate in CQ biosynthesis. The benzothiophene quinone, however, could not be detected in cell extracts using the procedure outlined in the experimental section. The reason for this lack of detection could be either that this compound is an intermediate in the

biosynthesis, but occurs in the cells at a concentration too low to be detected or that it is not an intermediate in the biosynthesis of CQ. However, since the sensitivity to detect this compound was very high, this finding argues that the compound is not an intermediate in the biosynthesis and that the isoprenoid side chain is added to the aromatic portion of the molecule before the formation of the benzothiophene ring.

Search for farnesylfarnesyl homogentisic acid lactone. If the isoprenoid side chain is attached to the ring at an early stage, for instance at tyrosine, this isoprenylated tyrosine could be transaminated to isoprenylated 4-hydroxyphenylpyruvate, which could then be oxidatively rearranged to isoprenylated homogentisic acid. This pathway and others are consistent with the fact that homogentisic acid was not incorporated into CQ because the cells may not have the enzyme to isoprenylate homogentisic acid. The isoprenylated homogentisic acid can then proceed to form CQ by the pathways discussed before. If this is the pathway in *S. acidocaldarias*, then a series of polyprenylated compounds including the farnesyl-farnesyl derivative of homogentisic acid may be present in the cells. In order to determine if this compound was present in the cells, the phytyl homogentisic acid lactone was synthesized and used as a marker to develop methods to extract and purify the farnesyl-farnesyl homogentisic acid lactone which could be present in the cells. The final purified material could be identified by mass spectrometry and other physical methods. Unfortunately, this compound was not detected in the cells using the methods outlined in the experimental section. As discussed before, this negative result may be due to either the

absence of the compound in the cells, or its presence in a very small amounts. This latter explanation was apparently the reason for the failure to identify the intermediates in the biosynthesis of ubiquinone in *E. coli* (Young et al., 1973). This problem was later solved by the isolation of *E. coli* mutants which over-produced the required intermediates. At present, however, no successful attempt to isolate mutants from any of the species of *Sulfolobus* has been described.

Labeling the intermediates to CQ with [U-^{14}C]-L-tyrosine. An alternate method to identify and isolate possible intermediate in CQ biosynthesis would be to label these intermediates with a radioactive precursor and monitor their isolation and purification by measuring radioactivity. Since tyrosine has been previously demonstrated to be readily incorporated into CQ, growth of cells with [U-^{14}C]-DL-tyrosine would be expected to label all the intermediates leading to CQ and thus facilitate their isolation.

In the first experiment to use this method, the cells were grown with [U-^{14}C]-L-tyrosine (1.99 μCi/10 mg) and a portion of cells were withdrawn at five different times during the growth. The extract from the cells was then purified by TLC to separate the CQ from the other compounds. The results showed no significant incorporation of ^{14}C into any compound other than CQ. The material remaining at the origin of the plate which contained some radioactivity was recovered and purified on a second TLC plate using methylene chloride and ethyl acetate (1:1 vol/vol) as the developing solvent system. Only the very top UV spot showed any radioactivity. The radioactivity incorporated from the labeled tyrosine into total lipid fraction, CQ, and the UV spot as a function of time is

shown in Figure 17. The figure shows the maximum incorporation of radioactivity into CQ and the UV spot occurred at the end of cell growth.

In order to obtain intermediates labeled with a higher specific activity, [U-^{14}C]-L-tyrosine with a higher radiospecific activity was fed to cells (5 μCi/4 mg in 100 ml culture) and the resulting cell extract was purified by TLC. The distribution of radioactivity on the TLC plate is shown in Figure 18 and Figure 19. The major radioactive spot on the TLC plate shown in Figure 19 was eluted from the plate and purified on a third plate in methylene chloride solvent (Figure 20). The R_f of this radioactive spot (0.5) is higher than that of farnesyl-farnesyl homogentisic acid lactone (0.25) indicating that this tyrosine derived spot is not farnesyl-farnesyl homogentisic acid lactone. It was also found that the spot decomposed on TLC plate, probably due to air oxidation. This was confirmed by the inability to isolate the material from developed TLC plates that had been exposed to air for several hours. This oxidative decomposition was also observed by the spots turning brown upon exposure to air, a characteristic observed for TLC plates with spots of homogentisic acid or homogentisic acid lactone. This information indicated that the spot is likely a compound with structural features like homogentisic acid. However, because of this instability, it has been impossible thus far to identify the compound.

In an attempt to get around this decomposition, a series of chemical reactions were used in order to convert the compound to a more stable compound that could be more easily characterized. Various derivatization methods were tested, with homogentisic acid, homogentisic acid lactone, and phytyl

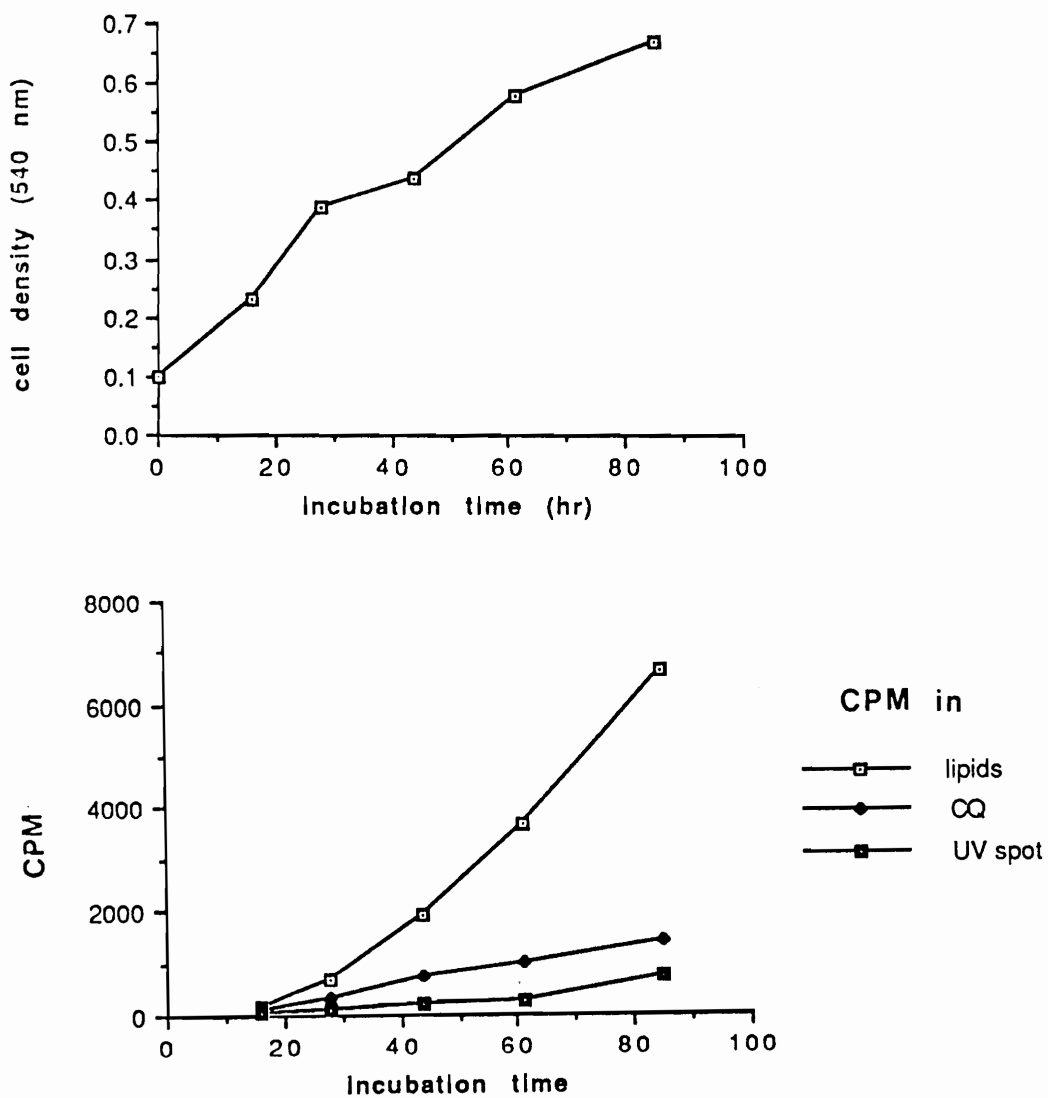


Figure 17. Growth curve of cells (upper) and the incorporation of ^{14}C-tyrosine into lipids, CQ and the UV spot.

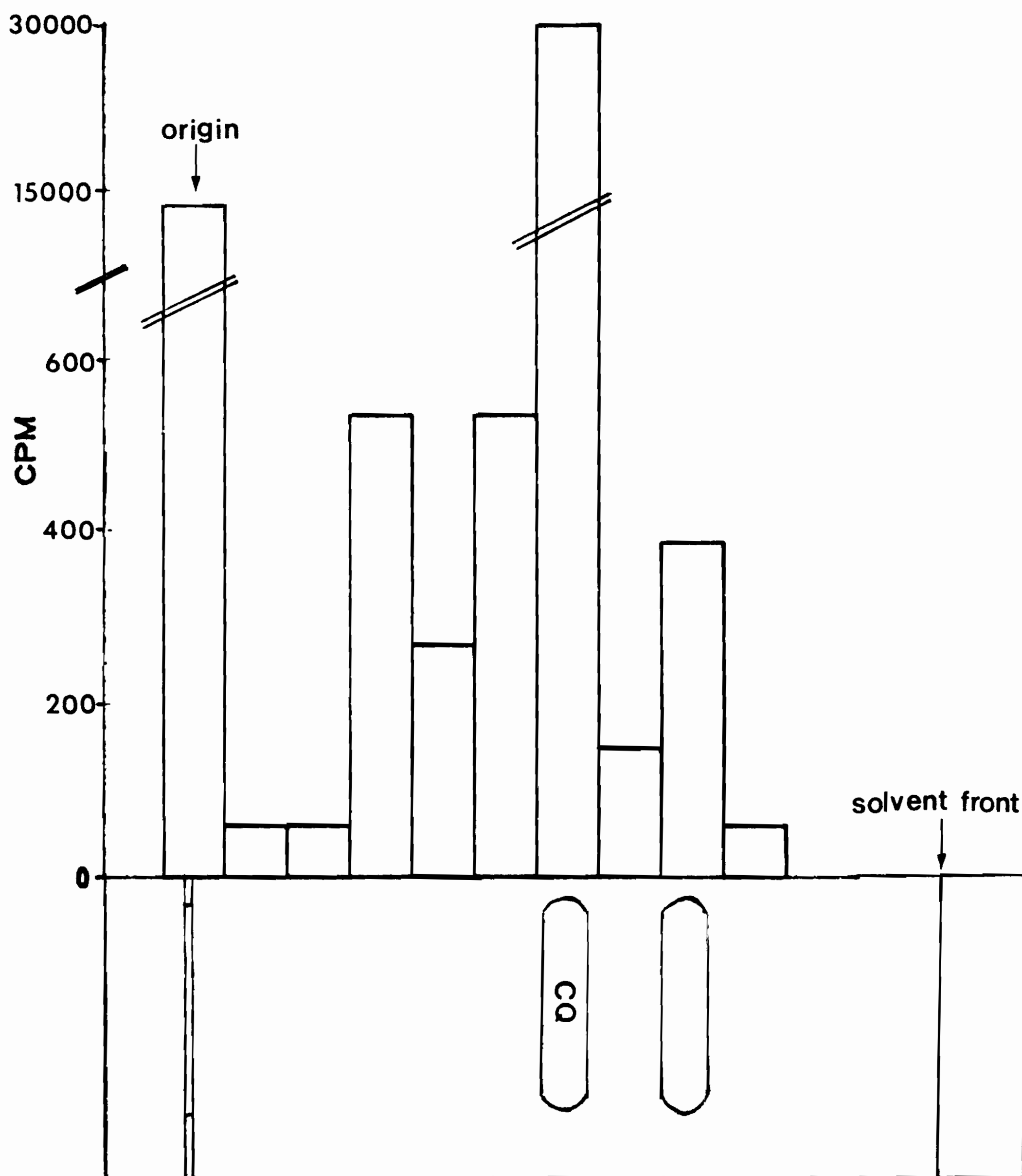


Figure 18. Radioactive profile of a cell extract separated by TLC using hexane and ether (8:2 vol/vol) as solvent: The origin and the solvent front of the TLC are indicated in the figure.

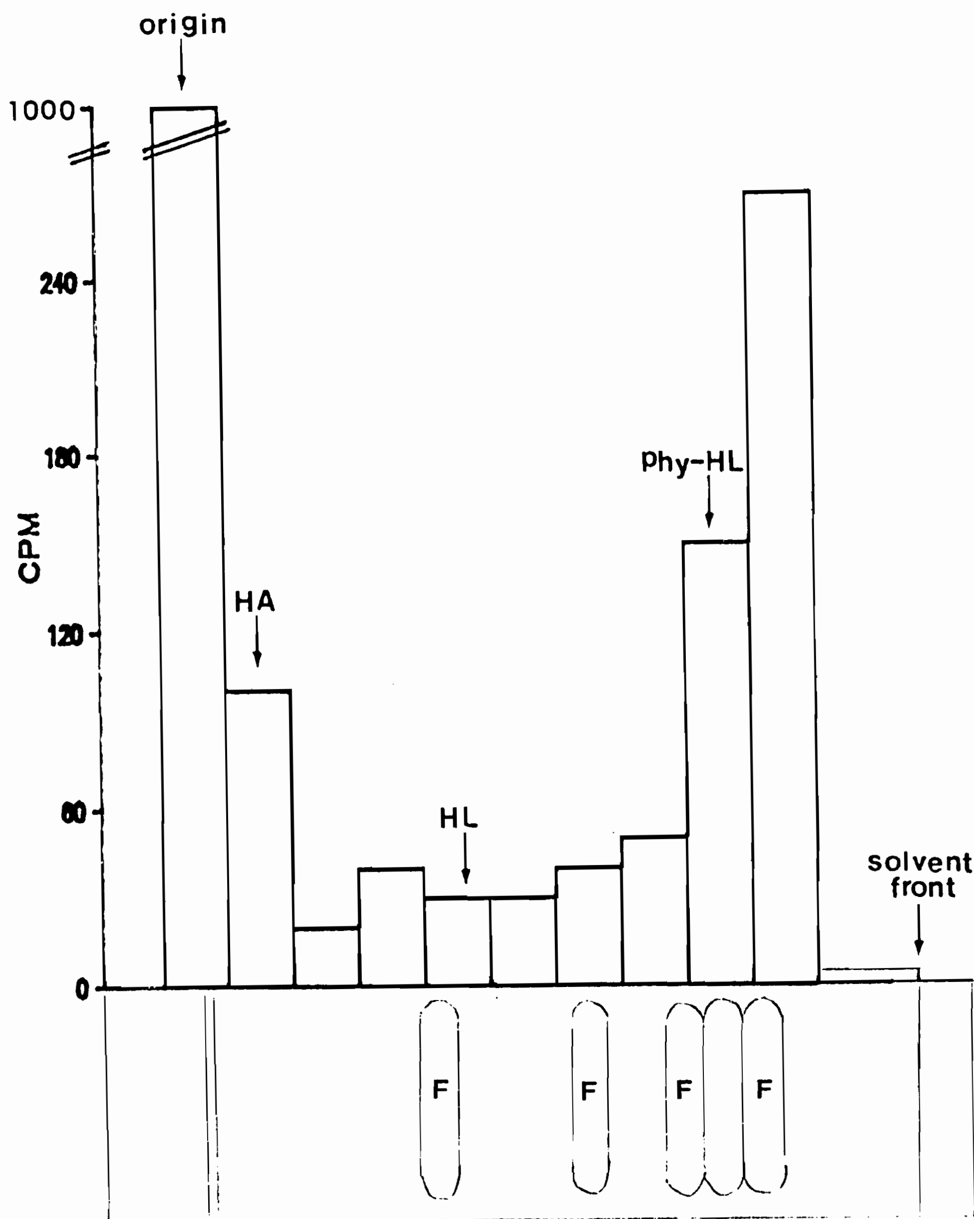


Figure 19. Radioactive profile of the origin material from Figure 18 separated by TLC: The TLC was developed in methylene chloride and ethyl acetate (1:1 vol/vol) solvent. The positions of known homogentisic acid, homogentisic acid lactone, and phytyl homogentisic acid lactone on TLC are shown in the figure. The spot labeled F indicates the fluorescent spots. Others are UV spots.

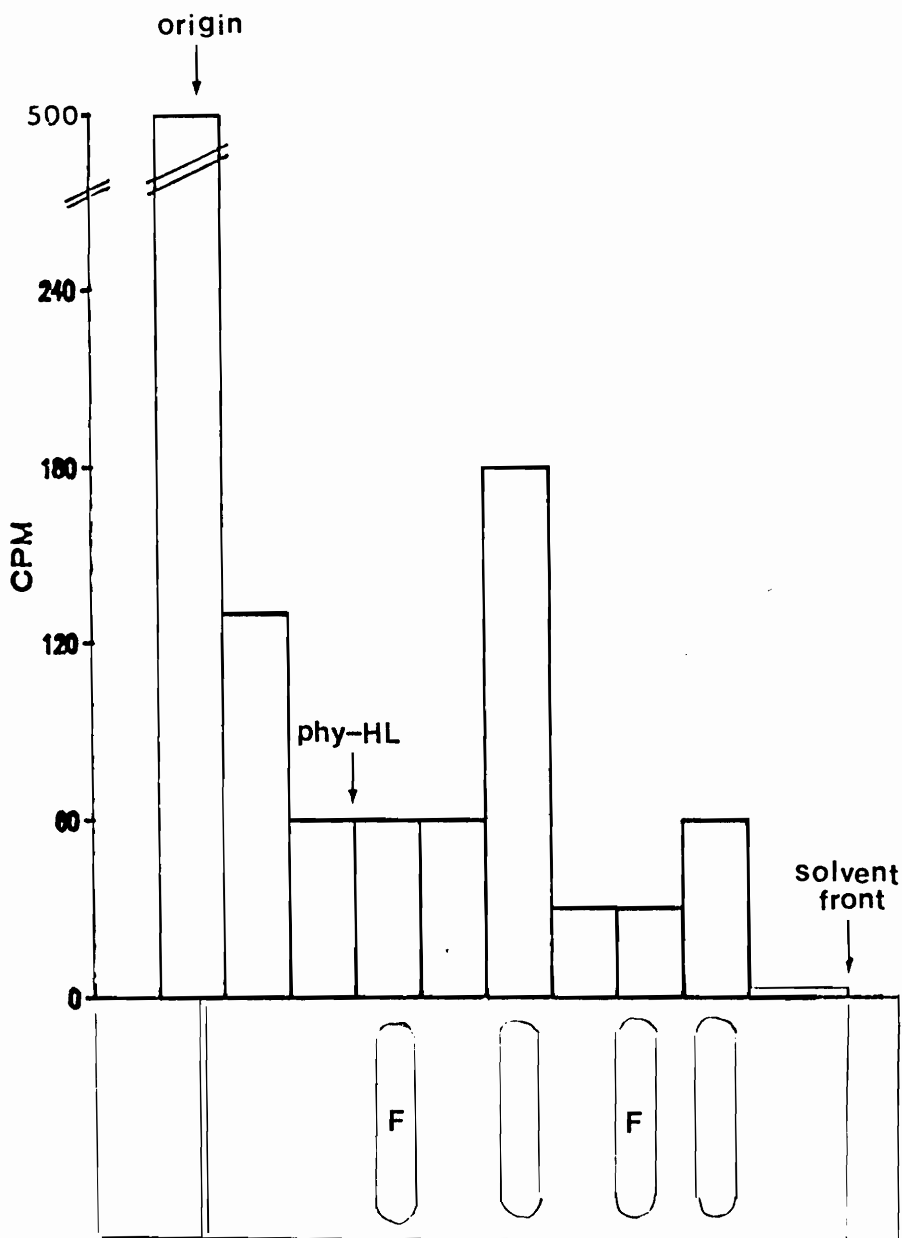


Figure 20. **Radioactive profile of the radioactive spot from Figure 19 separated by TLC:** The TLC was developed in methylene chloride solvent. The position of known phytyl homogentisic acis lactone is indicated in the figure. Spots labeled F are fluorescent, others are UV spots.

homogentisic acid lactone serving as model compounds. These methods included methylation with different methylation reagents (diazomethane, methyl iodide, dimethyl sulfate, and HCl in methanol) and acetylation of the products formed by $LiAlH_4$ reduction. The best method found was the acetylation of the products formed by $LiAlH_4$ reduction with acetic anhydride.

Thus, *S. acidocaldarius* was grown in 100 ml of yeast extract medium containing [U-^{14}C]-L-tyrosine (11 μCi/5.7 mg). Two thirds of the lipid extract was reduced with $LiAlH_4$ and the resulting product was acetylated. The radioactivity recovered in these acetylated products was 70% of that in the original lipid extract. The product mixture was then purified by TLC using methylene chloride as the developing solvent. The distribution of radioactivity on the TLC plate is shown in Figure 21. As can be seen in the figure, most of the radioactivity did not have a R_f corresponding to the acetylation products generated by the $LiAlH_4$ reduction of phytyl homogentisic acid lactone. Only a small amount of radioactivity was associated with a spot that had a R_f close to acetylated product of phytyl homogentisic acid lactone. To further resolve this spot, it was removed and purified on a second plate with methylene chloride and ethyl acetate (2:1 vol/vol) as the developing solvent (Figure 22). The distribution of radioactivity on this second TLC plate was found to be low and to be spread over most of the plate with no clear maximum radioactivity being observed at the R_f of the expected compound. Thus it appears that farnesylfarnesyl homogentisic acid is not an intermediate in CQ biosynthesis.

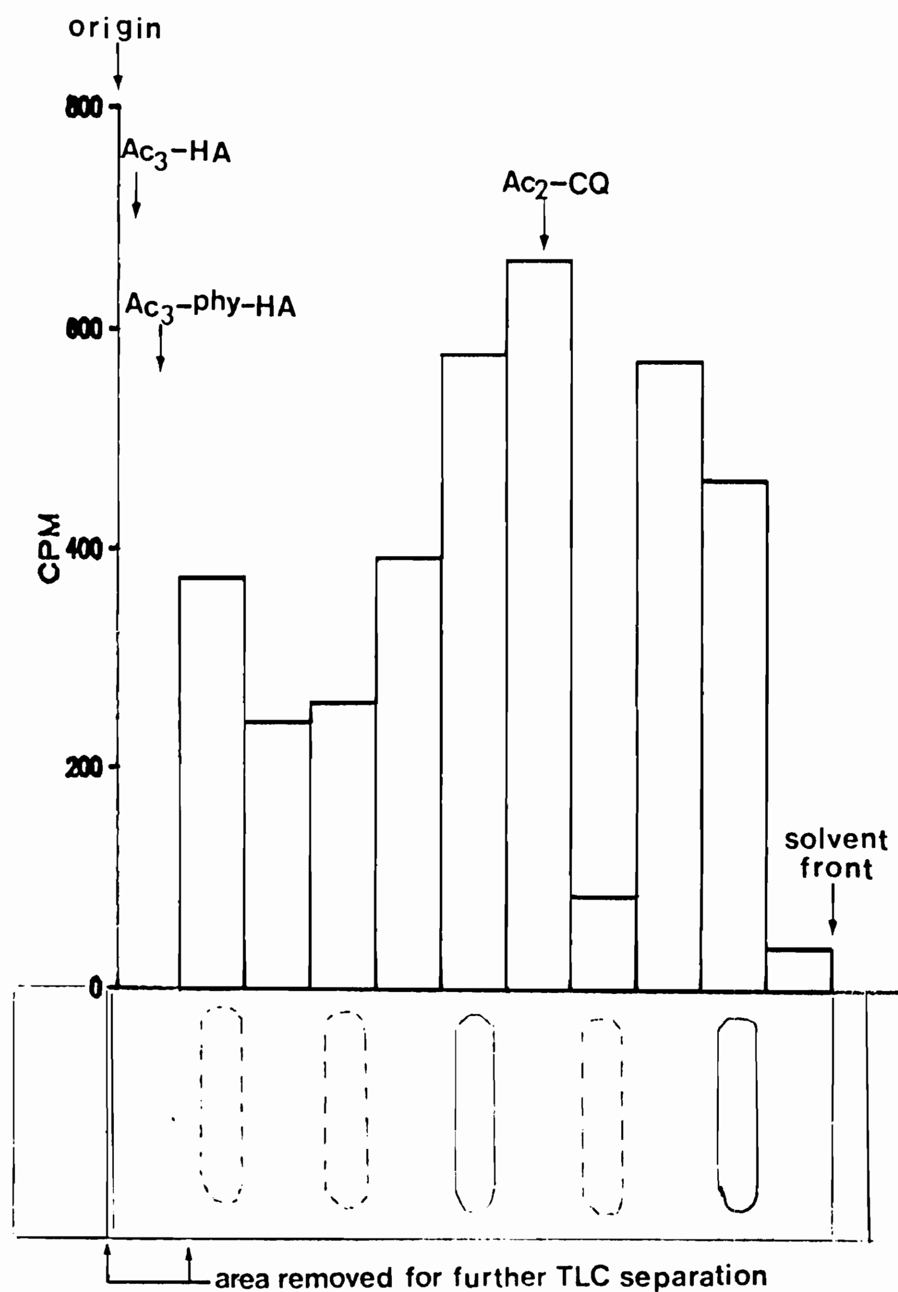


Figure 21. **Radioactive profile of acetylated cell extract separated on TLC with methylene chloride as solvent:** The positions of known samples of acetyl phytyl dihydroxyphenylethanol (Ac_3-HA) and acetyl CQ (Ac_2-CQ) were indicated in the figure. Spots with dashed circle were weak UV spots.

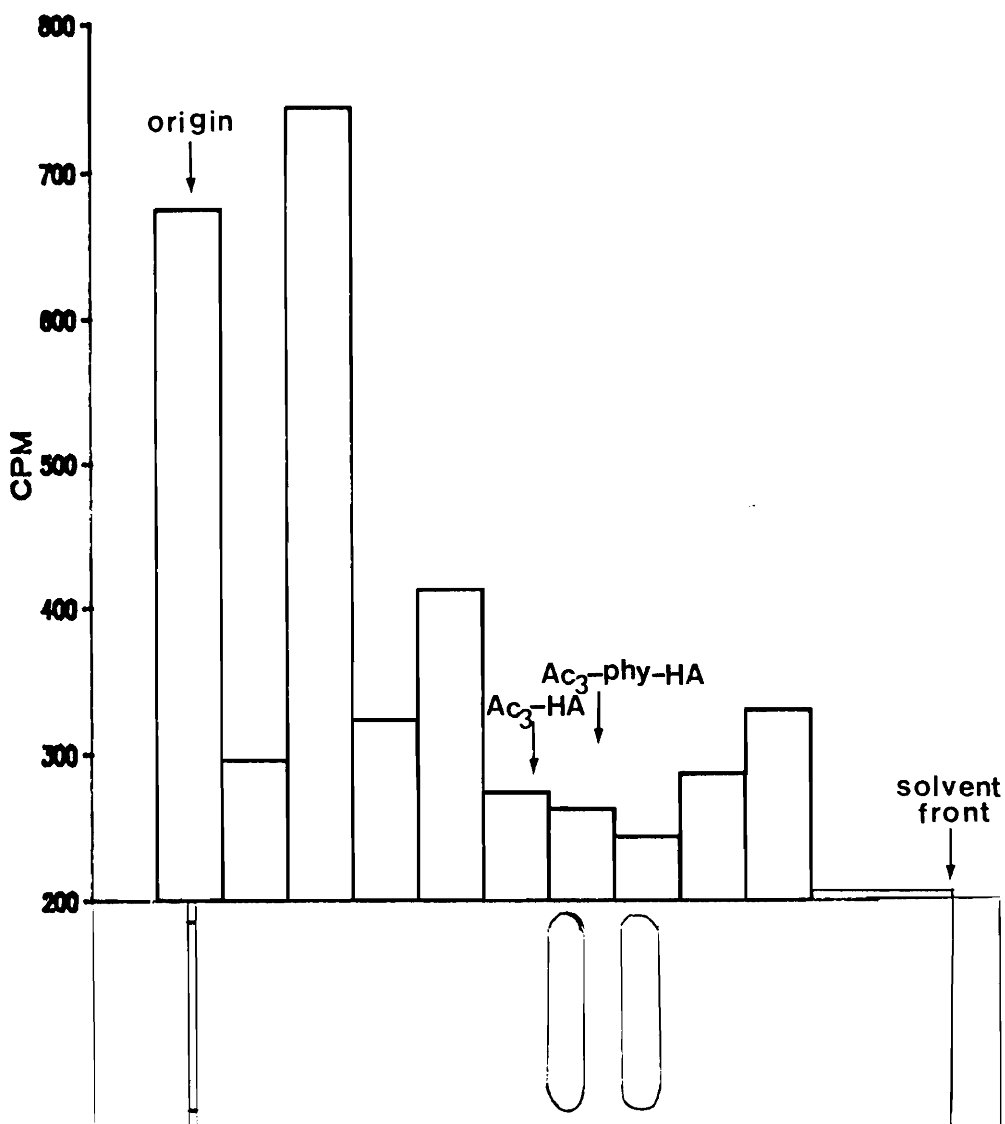


Figure 22. Radioactive profile of the origin area from figure 21 with methylene chloride and ethyl acetate as solvent (2:1): The positions of acetyl phytyl dihydroxyphenylethanol (Ac_3-phy-HA) and acetyl dihydroxyphenylethanol (Ac_3-HA) were indicated in the figure.

In order to obtain more materials for the identification of the compound responsible for the radioactive peaks observed, another portion of the radioactive cell extract was mixed with the methylene chloride fraction of the cell extract purified by a silica gel column chromatography. The TLC purified spot which had the radioactivity (R_f 0.45 in methylene chloride solvent) was analyzed by UV spectroscopy (Figure 23) and the spectrum was found not to match the UV spectrum of phytyl homogentisic acid lactone. The UV spectrum did not change when measured in 0.1 N NaOH solution, indicating the absence of a phenol. This was confirmed by the inability of the compound dissolved in ethyl acetate to be extracted into 0.1 N NaOH. These data also indicated that the compound is not an acid. When the compound was reduced with $LiAlH_4$ and acetylated with acetic anhydride, a series of different products were generated as evaluated by TLC. When the compound was acetylated with acetic anhydride without the reduction with $LiAlH_4$, however, two major UV spots were detected. One of these spots had the same R_f as the nontreated compound, the other has a lower R_f (0.23 in CH_2Cl_2), but higher than that of acetylated phytyl homogentisic acid lactone (R_f=0.13 in CH_2Cl_2). The UV spectra of these two spots are recorded in Figure 24. Based on all of the information discussed above, this compound appears to be a polyprenylated quinone but its exact structure could not be established from the available data.

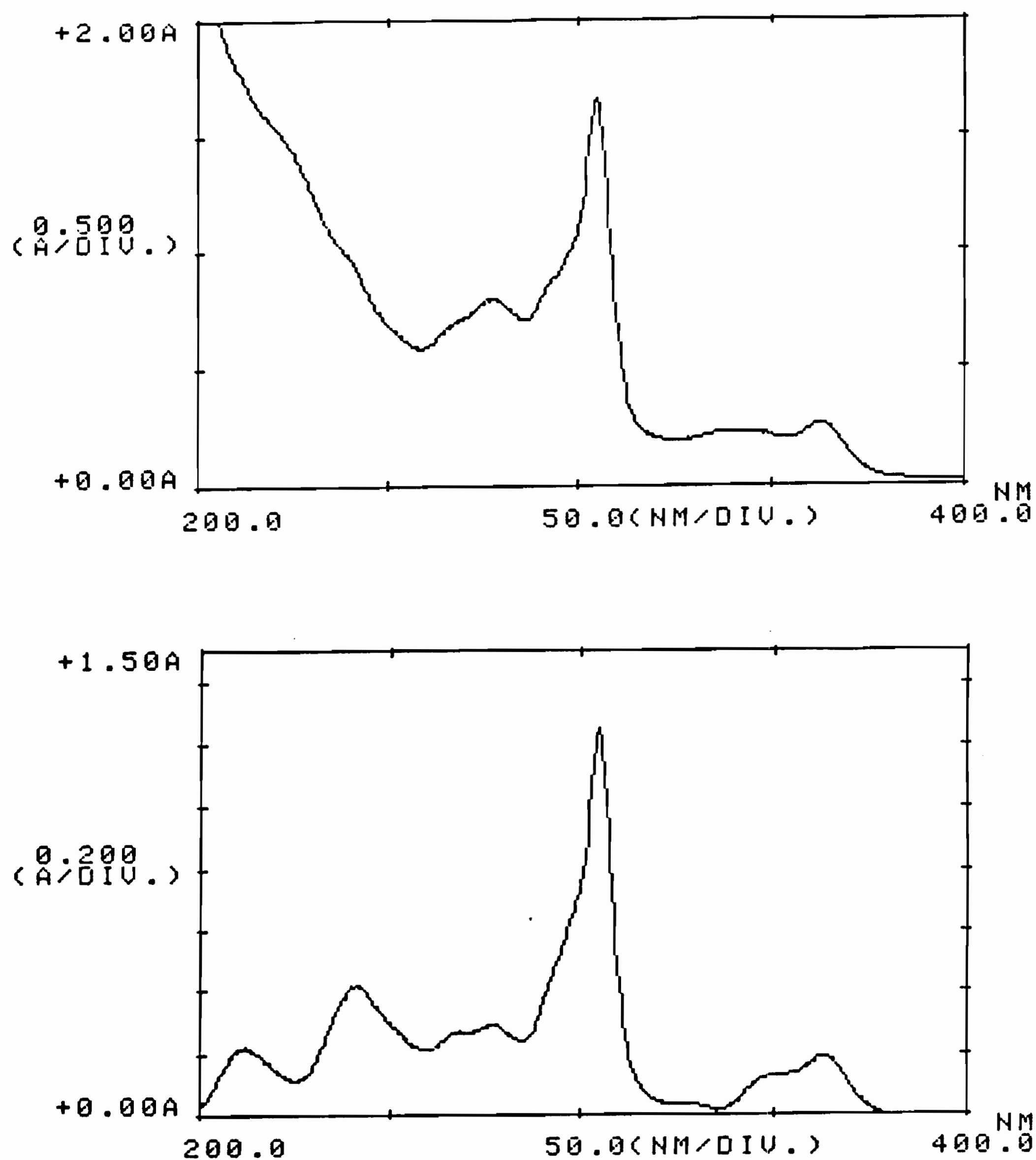


Figure 23. UV spectrum of the nonacetylated compound: The UV spectrum was taken in pentane solvent with a Shimadzu spectrophotometer. The upper panel is the spectrum in pentane and the lower panel is the spectrum recorded with pentane as a reference.

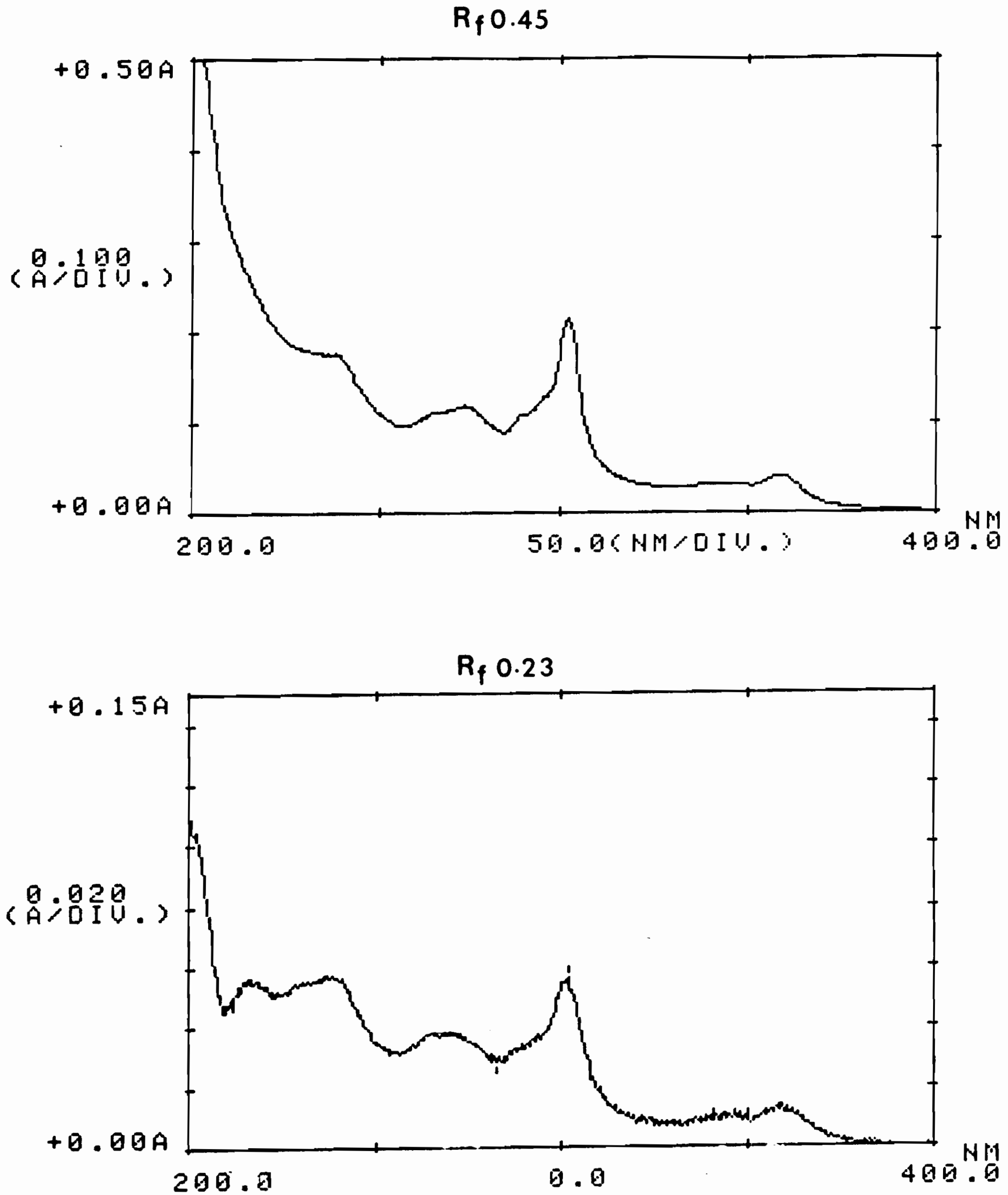


Figure 24. UV spectra of acetylated compounds: The conditions for the UV spectrum were the same as described in figure 23. The upper panel is the spectrum of the Rf 0.45 spot and the lower panel is the spectrum of the Rf 0.23 spot. The spectrum of the lower spot was recorded with pentane as a reference.

INCORPORATION OF STEREOSPECIFICALLY LABELED TYROSINES INTO CQ

INTRODUCTION

Earlier in this thesis, it was shown that only one of the two deuteriums of [3,3-$^{2}H_{2}$]-DL-tyrosine was incorporated into CQ by *S. acidocaldarius*. The stereospecificity for the incorporation of this deuterium, however, was not established. Thus, four stereospecifically deuteriated isomers of tyrosines (Figure 25) were synthesized and used to study the stereospecificity of the incorporation of the C-3 hydrogen. This was accomplished by growing cells of *S. acidocaldarius* in a medium containing each of the four tyrosine isomers and measuring the incorporation of these labeled tyrosines into caldariellaquinone (CQ) by mass spectrometry.

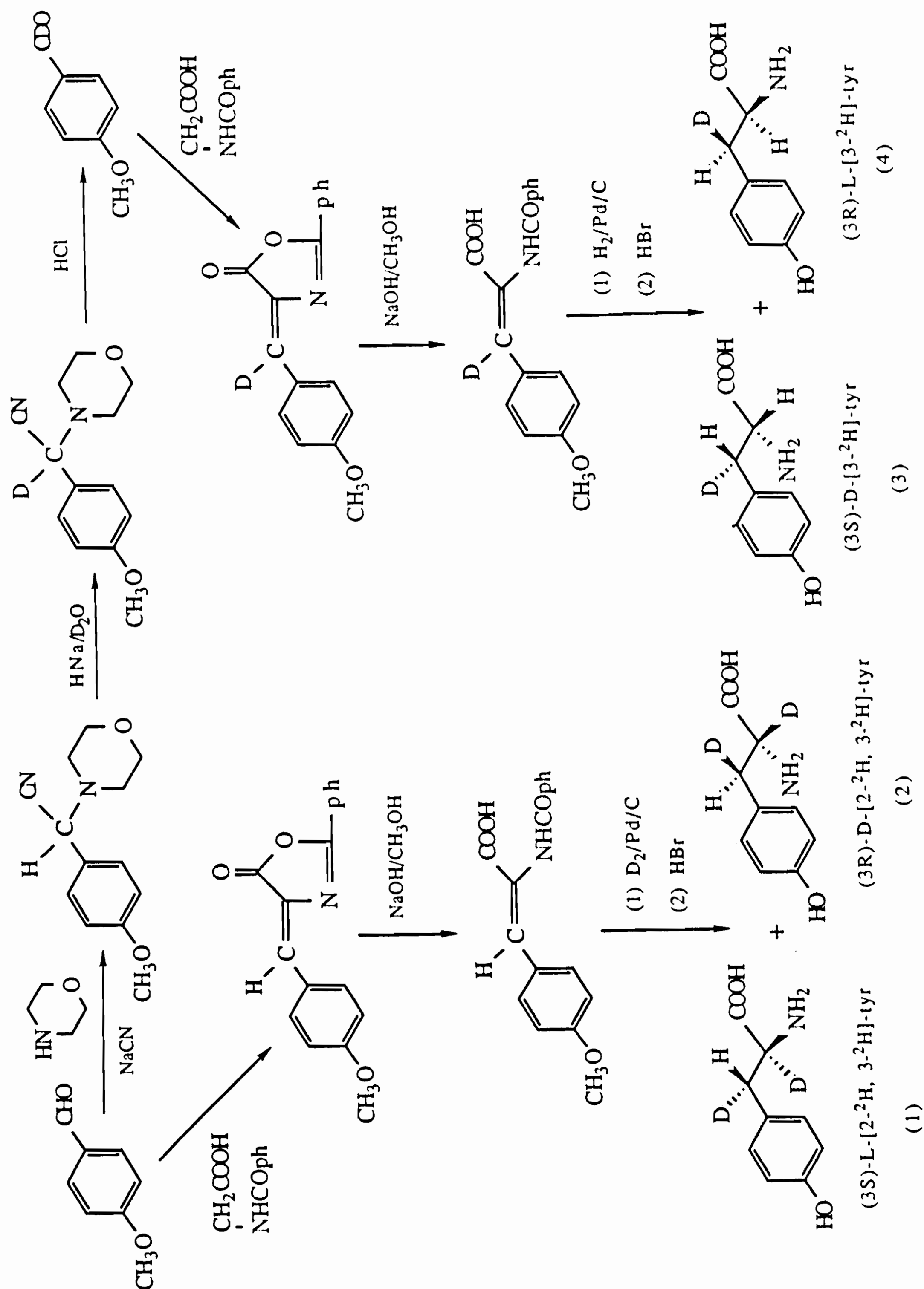


Figure 25. Synthesis of stereospecifically labeled tyrosines.

MATERIALS AND METHODS

Synthesis of the stereospecifically deuterium labeled tyrosines. The four stereospecifically deuteriated isomers of tyrosine, (3S)-L-[2-^{2}H, 3-^{2}H]- (1), (3R)-D-[2-^{2}H, 3-^{2}H]- (2), (3S)-D-[3-^{2}H]- (3), and (3R)-L-[3-^{2}H]-tyrosine (4), were synthesized using the procedure described by Kirby and Michael (1973) (Figure 25). The simultaneous synthesis of isomers (3) and (4) began with the condensation of [1-^{2}H]-*p*-anisaldehyde and N-benzylglycine to form an oxazolinone derivative, which was hydrolyzed with alkaline-methanol to (Z)-α-benzoylamino-4-methoxy-[3-^{2}H]-*p*-cinnamic acid. The cinnamic acid derivative was reduced by hydrogenation in the presence of palladium to N-benzoyl-O-methyl[3-^{2}H]-tyrosine, which was hydrolyzed by 40% hydrogen bromide to give a mixture of tyrosine isomers (3) and (4). Isomers (1) and (2) were synthesised in an analogous manner except that nonlabeled (Z)-α-benzoylamino-4-methoxycinnamic acid was hydrogenated with deuterium gas. Each pair of D-L isomers was resolved by treatment of its chloroacetyl derivative with carboxypeptidase A from bovine pancreas (Sigma) (Kirby and Michael, 1973). [1-^{2}H]-*p*-Anisaldehyde was prepared from the nonlabeled *p*-anisaldehyde as described by Kirby and Michael (1973) (Figure 25).

Analysis of the labeled tyrosines. The deuterium content of each sample was analyzed from the mass spectral data of its N,O-bistrifluoroacetyl, *n*-butyl ester derivative (White, 1985). Total deuterium content of the tyrosine was measured from the isotopic enrichment of the *m/z* 328 fragment ion (M^+-$COOC_4H_9$) and

deuterium specifically at C-3 was measured from the isotopic enrichment of the *m/z* 203 fragment ion ($CF_3COOC_6H_4CH_2^+$). The enantiomeric purity of each sample was determined using the phenylalanine ammonium lyase assay (Ogata et al., 1967). In this assay, L-tyrosine is deaminated to *p*-coumaric acid with the specific removal of the pro-3S hydrogen of the tyrosine. Thus, each stereospecifically labeled L-tyrosine sample was incubated with phenylalanine ammonium lyase from *Rhodotorula glutinis* (Sigma) in Tris buffer (65 mM, pH 8.6) and the appearance of *p*-coumarate was measured by the increase in absorbance at 333 nm. The *p*-coumaric acid was then isolated by extracting the acidified reaction mixture with ethyl acetate and its deuterium content was calculated from the isotopic enrichment of the M^+ *m/z* 164 ion. The difference between the deuterium content of the *p*-coumaric acid and the total deuterium content in the starting L-tyrosine was used to determine the stereochemical purity for the C-3 hydrogen of tyrosine. Since the enzyme only acts on L-tyrosine, the enantiomeric purity of the two D-isomers was assumed to be the same as the L-isomers since the synthesis and resolution methods used were the same. The two D-isomers treated with the enzyme did not produce any detectable amount of *p*-coumaric acid, indicating no significant amount of L-isomer contamination in the samples.

Data on the deuterium content and enantiomeric purity of each sample are shown in Table 2 on page 83. Compounds (3) and (4) contain about 18% unlabeled molecules, which is consistent with the extent of labeling of the [1-^{2}H]-*p*-anisaldehyde used in the synthesis. The labeled molecules were about

85% enantiomerically pure. This stereoimpurity resulted from racemization at the C-2 position of tyrosine during its chemical conversion to the chloroacetyl derivative prior to its resolution with carboxypeptidase (Wightman et al., 1972).

Growth of *S. acidocaldarius* with the labeled tyrosine and measurement of their incorporation into CQ and cellular proteins. Four cultures of *S. acidocaldarius* were grown for 3 days at 70°C in the yeast extract medium (100 ml) as described earlier, each was supplemented with 45 mg of one of the stereospecifically deuteriated tyrosines. The distribution of ^2H in the CQ and in the protein-bound tyrosine in the cells was analyzed by mass spectrometry as described earlier.

Measurement of tyrosine utilization by growing cells. The utilization of DL-tyrosine by the cells was quantitated by measuring the amount of free tyrosine present in the medium before and after cell growth using an amino acid analyzer.

RESULTS AND DISCUSSION

The results (Table 2) show that the C-3 deuterium from all four tyrosine isomers was readily incorporated into the cellular protein, but only isomers (1) and (3) contributed a significant amount of deuterium into the CQ. Thus it is the pro-3S hydrogen of either L- or D-tyrosine that is retained during the biosynthesis of CQ (Figure 26).

Table: Incorporation of stereospecifically labeled tyrosines into CQ and cellular protein by *S. acidocaldarius*

Expt. Tyr. fed[c]	distribution of ^2H in CQ[a]		% incorp.[d] into CQ	distribution of ^2H in protein tyrosine[b]			% incorp.[e] into tyr.
	$^2H_0$	$^2H_1$		$^2H_0$	$^2H_1$	$^2H_2$	
1. (3S)-L-[2-^{2}H,3-^{2}H]-	56.7 (82.9	43.4 100)[f]	55.2	47.6 (82.9	49.1 100	3.3 20.9)	52
2. (3R)-D-[2-^{2}H,3-^{2}H]-	90.5 (100	9.5 54.8)	7.7	67.3 (100	32.5 65.9	0.1 8.7)	34
3. (3S)-D-[3-^{2}H]-	66.8 (100	33.2 94.0)	47.0	61.0 (100	38.8 98.2	0.2 9.8)	47
4. (3R)-L-[3-^{2}H]-	94.0 (100	6.0 52.0)	7.8	77.0 (100	22.6 47.0	0.4 5.6)	27

[a]The M^+ *m/z* 630 and the M^+ +1 *m/z* 631 ions were used for the measurement of the isotopic distribution in the CQ. The measured normalised ion intensity for an unlabeled CQ sample was 100% and 44.3% for M^+ *m/z* 630 and 631, respectively. [b]The intensity data were obtained from the M^+ -101 *m/z* 328 fragment ion of the N,O-bis(trifluoroacetyl)*n*-butyl derivative of the tyrosine. The measured normalized ion intensity for an unlabeled tyrosine derivative sample was 100%, 17.6%, and 0.0% for the *m/z* 328, 329, and 330, respectively. [c] Sample (1) contains 78.6% of (3S)-L-form and 16.4% of (3R)-L-form, sample (2) contains 78.6% of (3R)-D-form and 16.4% of (3S)-D-form, sample (3) contains 68.0% of (3S)-D-from and 14.2% of (3R)-D-form, and sample (4) contains 68.0% of (3R)-L-form and 14.2% of (3S)-L-form. [d] The % incorporation of the indicated isomer into CQ. [e] The % incorporation of deuterium of C-3 tyrosine into protein-bound tyrosine. [f] Numbers in parentheses are the observed normalised ion intensities.

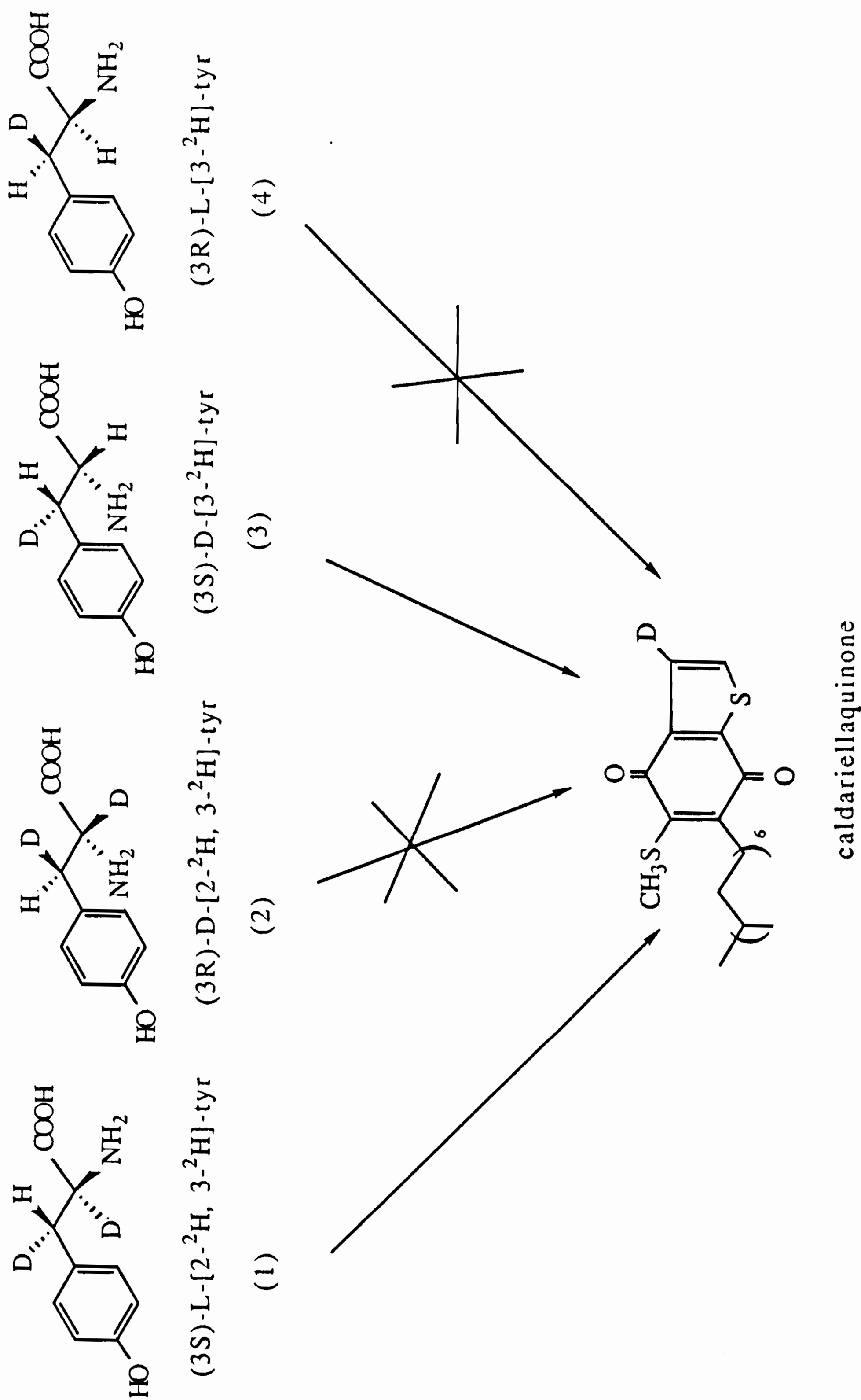


Figure 26. Incorporation of stereospecifically labeled tyrosines into CQ.

The quantitative incorporation of pro-3R or pro-3S hydrogen of the tyrosine into CQ was calculated from the deuterium incorporated in CQ and the enantiomeric purity of each isomer. Thus for experiments 1 and 4, it was shown that the pro-3S deuteriated isomer of L-tyrosine was incorporated into CQ to an extent of 55.8% and the pro-3R deuteriated isomer was incorporated to an extent of less than 0%. Similarly, it was shown from the incorporation data reported for experiments 2 and 3, that the pro-3S isomer of D-tyrosine was incorporated to an extent of 48.4% and the pro-3R isomer was incorporated to an extent of less than 2%. (The calculation of an incorporation of less than 0% are likely a result of small errors present in mass spectral data.) Thus all of the deuterium incorporated into CQ were derived from the pro-3S hydrogen.

The stereospecificity of a number of biosynthetic processes involving the loss or retention of the prochiral C-3 centers of phenylalanine and tyrosine has been investigated (Hill, 1978). Depending upon the specific reaction involved, either the pro-R or pro-S hydrogen could be retained during the process. The retention of the pro-3R hydrogen of phenylalanine or tyrosine is known to occur during their nonoxidative deamination to their respective cinnamic acid (Ogata et al., 1967; Wightman et al., 1972; Hill, 1978; Ife and Haslam, 1971; Strange et al., 1972) and during the dehydrogenation in the biosynthesis of securinine (Parry, 1978), mycelianamide (Kirby and Marayanaswamin, 1973), and dhurrin (Rosen et al., 1975). On the other hand, the pro-3S hydrogen is retained during the biosynthesis of haemanthamine (Kirby and Michael, 1973), taxiphylin (Rosen et al., 1975), and tuberin (Herbert and Mann, 1984). Since no clear pattern has

emerged between the stereochemistry of the proton lost in these different reactions and a specific reaction type or mechanism, it is impossible to relate the observed loss of the pro-S hydrogen of tyrosine during the formation of the benzothiophene ring of CQ with a specific reaction pathway. Thus, the true importance of this stereochemical information on CQ biosynthesis from tyrosine will only become apparent when the specific steps in the reaction are established.

Another interesting observation on the metabolism of tyrosine by *S. acidocaldarius* is that both D- and L-tyrosine are readily utilized by the cells. The utilization of D-tyrosine by living organisms is well known (Stegink, 1977) and D-tyrosine can serve as the sole carbon source for some halobacteria (Tanaka et al., 1989). The utilization of D-tyrosine by *S. acidocaldarius* is apparent from the data presented in Table 2 on page 83 which shows that both isomers are incorporated efficiently into both CQ and the cellular proteins. The efficient utilization of both D- and L-tyrosine was also confirmed by the observation that the cells grown on the yeast extract medium supplemented with 15 mg DL-tyrosine consume 87% of the added tyrosine. If we assume that all tyrosine in proteins is L-tyrosine, then some mechanism must be in place to convert the D-tyrosine to L-tyrosine. At present, there are two mechanisms known to accomplish this conversion. One is the direct conversion of D-amino acids to L-amino acids via a racemase. Amino acid racemases have been found in *E. coli* (Raunio et al., 1978), *Pseudomonas* spp. (Asano et al., 1988), many other microoganisms (Bodanszky and Perlman, 1969), and higher plants (Robinson, 1976), but no racemase activity has been found in mammalian systems (Friedman and

Gumbmann, 1989). The other mechanism is the oxidation of D-amino acids to keto acids via D-amino acid oxidase, D-amino acid dehydrogenase, or transamination. The resulting optically inactive keto acids can then be reaminated to L-amino acids. D-Amino acid oxidase is the major enzyme responsible for the metabolism of D-amino acids in most living organisms. It is widely present in microorganisms (Raunio et al., 1978; Simonetta et al., 1982; Kubicek-pranz and Röhr, 1985a; 1985b; Manohara and Jayaraman, 1978), higher plants (Robinson, 1976), and animals (Friedman and Gumbmann, 1989; Konna and Yasumura, 1984). D-Amino acid dehydrogenases have been found in only a few microorganisms, e.g., *Salmonella* spp., *E. coli,* and *Pseudomonas* spp. (Wild et al., 1978; Wild and Obrepalska, 1982). Since other work presented in this thesis has shown that the first step in the metabolism of D- and L-tyrosine by *S. acidocaldarius* is its conversion to *p*-hydroxyphenylpyruvate (*p*HPP), this finding would support the involvement of this keto acid in the conversion of D- to L-tyrosine by these cells. The ready reversibility of the L-tyrosine into *p*-hydroxyphenylpyruvate is also apparent from the data presented in Table 2 on page 83 which shows that the (3S)-L-[2-^{2}H, 3-^{2}H]-tyrosine is incorporated into the cellular proteins with almost complete loss of the C-2 deuterium, suggesting that all of the cellular tyrosine equilibrates with this keto acid. The involvement of *p*HPP in the conversion between D- and L-tyrosine is also supported by the exchange of deuterium from [3,3-$^{2}H_2$]tyrosine (see page 36).

It should be noted that the incorporation of both D- and L-tyrosine was also observed during the biosynthesis of plastoquinones and tocopherol in higher

plants (Threlfall and Whistance, 1968a; 1968b; Whistance and Threlfall, 1968; 1970). It was suggested that *p*HPP was the first intermediate of the pathway and that it was generated from either D- or L-tyrosine by either a dehydrogenase or an oxidase. The *p*HPP was then oxidatively converted to homogentisic acid, which serves as a common intermediate to plastoquinones, tocopherols, and probably CQ.

Although I have shown that *S. acidocaldarius* readily metabolizes D-tyrosine, the reason that it has this ability is not clear. One possibility is that D-tyrosine and other D-amino acids, either as free amino acids or protein-bound amino acids, are generated by the thermal racemization of the L-amino acids that are present in the high growth temperature environment of *Sulfolobus*. Available data, however, indicate that little if any D-amino acids would be produced at the temperature and pHs used for the growth of *Sulfolobus* because of the slow thermal racemization rates (Man and Bada, 1987). However, since D-amino acids occur at low levels in many different biological materials (Man and Bada, 1987), the enzymes required for the metabolism of the D-tyrosine, as well as other D-amino acids, may simply be present to allow cells to metabolize the naturally occurring D-amino acids. This would also offer an explanation for the widespread occurrence of the D-amino acid-metabolizing enzymes found in nature.

SULFUR METABOLISM IN *S. ACIDOCALDARIUS* AND THE ORIGIN OF SULFUR IN CQ

INTRODUCTION

The methylthio group is found in a wide assortment of natural products. The most important of these is methionine, an essential amino acid. Other natural products that contain the methylthio group include dimethylthiomethane and the methyl and methylthio esters of 3-thiomethyl propionate which represent the odourous components of the truffle (Fiecchi et al., 1967), the pineapple (Rodin et al., 1966), and human urine after the ingestion of asparagus (White, 1975), respectively. Important metabolites which contain the methylthio group include urothion, a metabolite of the molybdenum cofactor (Goto et al., 1967), and the S-methylcytokinins (Burrows et al., 1969), which are modified bases in

transfer RNAs. The methylthio group is found in a metabolite of the fungicide pentachloronitrobenzene (Fall and Murphy, 1984) and it is increasingly being found in metabolites of drugs (Bergman et al., 1980; Kaul et al., 1981; Pal and Spiteller, 1982). It also forms part of the structure of CQ (De Rosa et al., 1977).

The methylthio group in a metabolite could be generated by one of two completely different mechanisms. In the first mechanism, sulfur is transferred to an acceptor molecule to generate a thiol which is subsequently methylated to the methylthio group, whereas, in the second mechanism, the methylthio group is transferred either from methionine or some other donor molecule as an intact unit to an acceptor molecule. The first route is used in the biosynthesis of methionine where the sulfur of cysteine is transferred via cystathionine to homocysteine which is methylated to methionine in a methyltetrahydrofolate-dependent reaction (Flavin, 1975). A mechanism in many ways analogous to that used to generate the methylthio group of methionine also appears to operate in the methylthiolation of the drug 1-allyl-3,5-diethyl-6-chlorouracil in rabbits (Pal and Spitellor, 1981) and in the methylation of the fungicide pentachloronitrobenzene in *Tetrahymena thermophila* (Bergman et al., 1980). In both cases, evidence indicates that these compounds are first converted to a glutathione or cysteine conjugate (Tateishi et al., 1978; Tomisawa et al., 1978) which is then cleaved by a cysteine conjugate β-lyase to generate the free thiol. Methylation of the resulting thiols by S-adenosylmethionine (SAM) generates the methylthio-containing metabolites.

Evidence for the transfer of the intact methylthio group of methionine to an acceptor molecule is weak, however, several authors have proposed it as a mechanism in the methylthiolation of drugs (Calder et al., 1974). This could occur by the cleavage of the methionine to methanethiol followed by the addition of the methanethiol to an acceptor molecule. The enzymatic cleavage of methionine to methanethiol is well documented and is known to occur by two separate metabolic pathways. In the first, the methionine is deaminated and then dethiomethylated with the release of methanethiol (Ruiz-Herrera and Starkey, 1969; Segal and Starkey, 1969). In the second, the methionine is deaminated and dethiomethylated simultaneously to methanethiol by the enzyme L-methionine γ-lyase (Tanaka et al., 1977). No evidence for the addition of methanethiol to an acceptor molecule is presently available, however.

After considering the above points, it was decided to determine the metabolic origin of the methylthio group in CQ and to determine whether this group comes from methionine as an intact unit. This was accomplished by growing cells of *S. acidocaldarius* with [^{34}S-*methyl*-$^{2}H_3$]-L-methionine and measuring the incorporation of label into CQ as described earlier in this thesis.

An additional aspect of these experiments is the ability to test for the occurrence of transsulfuration in the archaebacteria. Transsulfuration is the transfer of sulfur from homocysteine to cysteine via cystathionine, and represents an important series of reactions in the metabolism of reduced sulfur (Delavin-Kulch and Flavin, 1965). At present, three patterns of transsulfuration have been described in different organisms, each using a different series of reactions. In the

mammalian type of transsulfuration, homocysteine derived from methionine is converted unidirectionally to cysteine, whereas in bacteria cysteine is converted to homocysteine which is then converted into methionine. The fungi appear to be able to interconvert cysteine sulfur to methionine sulfur. The transfer of sulfur from methionine to cysteine in *S. acidocaldarius* as well as other organisms can be established by measuring the incorporation of ^{34}S into protein-bound cellular cysteine and methionine in the cells grown with the [^{34}S-*methyl*-$^{2}H_3$]-L-methionine.

MATERIALS AND METHODS

Isotopically labeled compounds. [*methyl*-$^{2}H_3$]Methionine (98 atom % ^{2}H) was obtained from Merck Sharp & Dohme Canada Limited, Montreal, Canada. [^{35}S]-Methionine (98 μCi) was kindly supplied by Dr. Timothy Larson in the Department of Biochemistry and Nutrition at Virginia Polytechnic Institute and State University. Sodium [^{34}S] sulfate (92.6 atom % ^{34}S) was obtained from Prochem Isotopes, U.S. Services, Inc., Summit, New Jersey. Elemental sulfur (^{32}S, 2.48 atom%; ^{33}S, 2.23 atom%; ^{34}S, 92.59 atom%; and ^{36}S, 1.7 atom%) was obtained from Monsanto Research Corp.'s Mound facility in Miamisburg, OH. [^{34}S-*methyl*-$^{2}H_3$]-L-Methionine was prepared by reacting ethyl L-α-benzamido-γ-chlorobutyrate (1 mmole) with [^{34}S-*methyl*-$^{2}H_3$]methanethiol (1 mmol) dissolved in ethanol (5 ml) containing sodium ethoxide (1 mmol) (Figure 27). After heating the mixture at 80°C for 3 h, the resulting ester was

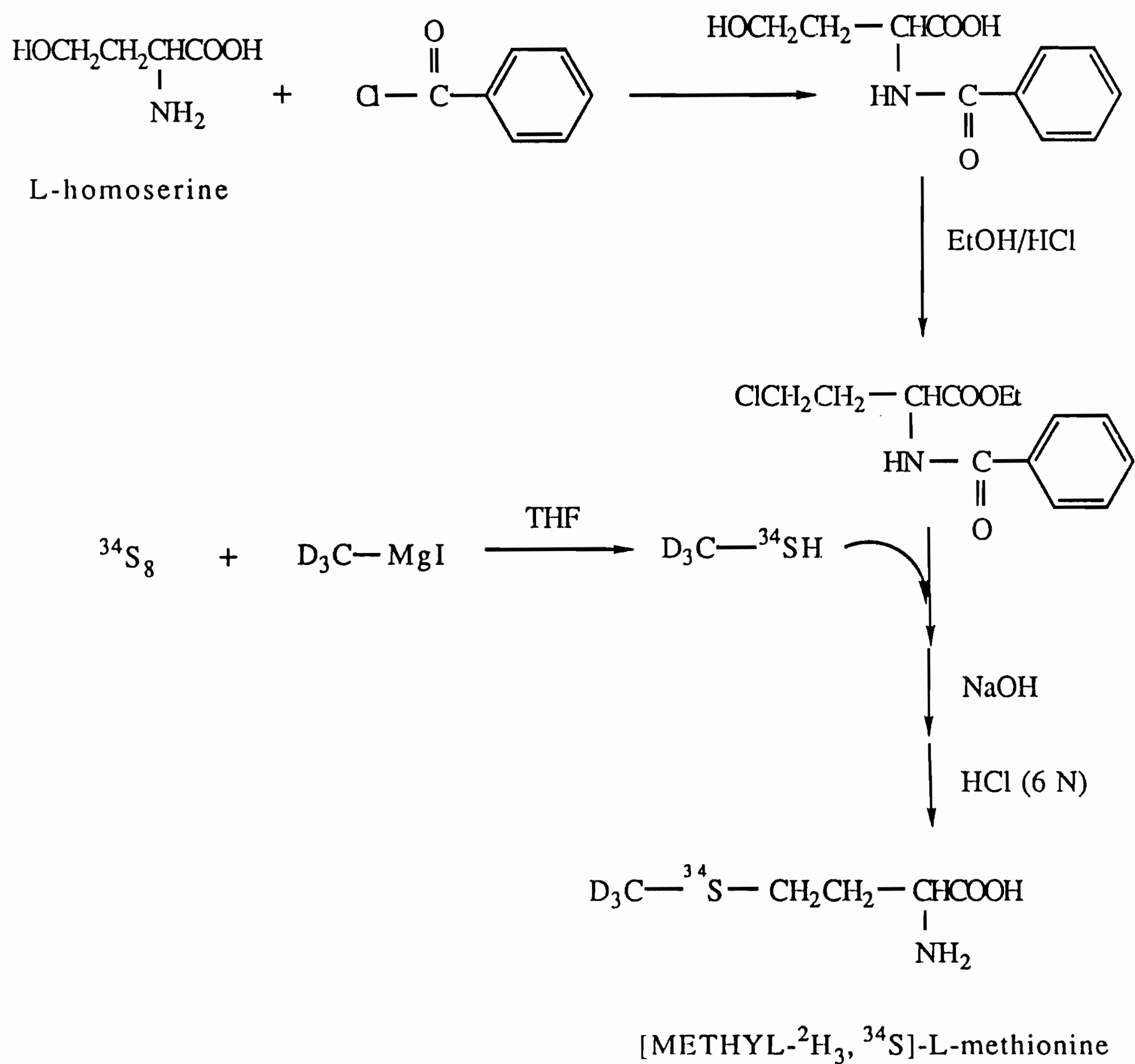


Figure 27. Synthesis of double-labeled methionine.

saponified with aqueous sodium hydroxide and the amide was hydrolyzed with 6 N HCl. The resulting [^{34}S-*methyl*-^{2}H$_3$]-L-methionine was isolated from the acid hydrolysis mixture by absorption onto Dowex-50 and elution with aqueous ammonia. The resulting labeled methionine was mixed with unlabeled methionine and the final product was crystallized from water-ethanol. Mass spectral analysis of the final product, as the *n*-butyl trifluoroacetyl derivative, showed the methionine from the first and second synthesis to contain, respectively, 34.15 % and 42.0 % of the molecules with [^{34}S-*methyl*-^{2}H$_3$]-L-methionine. (The isotopic incorporation of the methionine was measured from the M$^+$ *m/z* 301 and the *m/z* 61 fragment [CH_3SCH_2]$^+$ ions in the mass spectrum of the methionine as previously described.)

Ethyl L-α-benzamido-γ-chlorobutyrate was prepared by reacting a solution of N-benzoyl L-homoserine lactone in ethanol and HCl as described by Hill and Robson (1936). N-Benzoyl-L-homoserine was prepared by reacting L-homoserine lactone with benzoyl chloride in basic aqueous solution. [^{34}S-*methyl*- ^{2}H$_3$]Methanethiol was prepared in a 73% yield by reacting a solution of $^{34}S_8$ dissolved in tetrahydrofuran (1.5 ml) with a 1 M solution of C^2H_3MgI (99+ atom % ^{2}H) in diethyl ether (2 ml). After stirring at room temperature for 30 min, the solvents were removed by evaporation and the resulting Mg salt was dissolved in ethanol for reaction with ethyl L-α-benzamido-γ-chlorobutyrate.

Archaebacterial growth. Cells of *S. acidocaldarius, Halobacterium marismortui,* and *Methanobacterium formicicum*, were grown on the medium in-

dicated in Table 3 on page 98 to which the indicated amount of the different labeled methionine was added.

Incorporation of [^{35}S]methionine into CQ. The cells were grown with [^{35}S]-L-methionine (10 mg/100 ml, 1.16 x 10^9 cpm/mmol of ^{35}S) and the CQ was isolated and purified by TLC as described earlier. The amount of CQ produced was quantitated by UV absorbance and the radioactivity was measured on a scintillation counter.

CQ analysis. CQ was isolated and analyzed by mass spectrometry as either CQ or diacetyl CQ. The quantitation of CQ produced by the cell was done by measuring the the UV spectrum of CQ at 325 nm using a molar extinction coefficient of 5 x 10^3 reported by De Rosa (1977).

Analysis of label incorporated into protein-bound cysteine and methionine. The protein-bound cysteine and methionine present in the cell pellet after the extraction of the CQ were isolated and converted to the S-methyl *n*-butyl trifluoroacetyl derivative for cysteine and *n*-butyl trifluoroacetyl derivative for methionine as previously described (White, 1979). GC-MS analysis of these derivatives was used to measure the isotopic incorporation into each of these molecules. (The GC-MS conditions were the same as described in the analysis of protein-bound amino acids in the section on biosynthesis of the benzothiophene quinone ring. GC-MS analysis of the cysteine and methionine derivatives was done immediately after the derivatization, since decomposition of these derivatives was observed.) Because the procedure used (White, 1979) involved dialysis of cell pellets solublized in 6 M urea, all the non-protein amino acids were re-

moved before acid hydrolysis was performed. This ensures that no contamination of the samples with free labeled methionine was occuring.

The ^{34}S content of the cysteine was calculated from the isotopic enrichment of either the molecular ion at *m/z* 267 or the *m/z* 61 fragment ion $[CH_3SCH_2]^+$ as previously described (White, 1979). Measurement of the ^{34}S enrichment in the methionine was made more difficult because of the presence of a trideuteriated methyl group in some of the methionine resulting from the direct incorporation of the fed labeled methionine into cellular protein without cleavage of the methyl-sulfur bond of the methionine. The extent of the incorporation of the intact labeled methionine can be calculated from the relative intensities of the isotope-containing ions 5 *m/z* higher than the principle ion. Thus, the extent of incorporation of the intact methionine can be measured from either the $[CH_3SCH_2]^+$ fragment ion (intensity of *m/z* 66)/(intensity of *m/z* 61 + intensity of *m/z* 66) or the molecular ion (intensity of *m/z* 306)/(intensity of *m/z* 301 + intensity of *m/z* 306). In addition, the metabolic cleavage of the methionine to ^{34}S-homocysteine, followed by its subsequent remethylation, would produce methionine containing only ^{34}S. The extent of this reaction can be calculated from the relative intensities of the ion 2 *m/z* higher than the principle ion after the ion intensity was corrected for the natural occurrence of ^{34}S (White, 1979). Thus, the total incorporation of ^{34}S from the labeled methionine into protein-bound methionine will be the sum of the methionine molecules containing a $CH_3{}^{34}S$ and a $CD_3{}^{34}S$ unit.

RESULTS AND DISCUSSION

Mechanism of the formation of the methylthio group. The mass spectral analysis of the CQ isolated from *S. acidocaldarius* grown with [*methyl*-$^{2}H_3$]methionine (98 atom % ^{2}H, 50 mg/100 ml) after the end of log phase growth showed that 74 % of the molecules had incorporated a $C^{2}H_3$ group (Expt. 1, Table 3). Since the *m/z* 225 fragment ion in the mass spectrum of the CQ contains only one methyl group and this fragment was found to be labeled, then the methionine methyl must be specifically incorporated into the methylthio group of the CQ. In a similar experiment, the cells were grown with [^{35}S]-L-methionine (1.16 x 10^{9} cpm/mmol, 10 mg/100 ml). From the specific activity of the isolated CQ (7.1 x 10^{8} cpm/mmol), it was calculated that 0.61 mol of sulfur from the methionine was incorporated per mole of CQ. As CQ contains two sulfur atoms, these results show that, on average, the sulfur from the methionine was incorporated to an extent of 30% into each sulfur site. (Since the incorporation into the individual sulfur positions was not determined, one cannot determine from this data what the actual incorporation was at each site.)

These observations show that the methyl and the sulfur of the methylthio group and the sulfur are derived from methionine and suggest the possibility that the methylthio group of CQ may be derived as an intact unit from the methionine.

In order to test this idea, *S. acidocaldarius* was grown in a medium (Expt. 2, Table 3) containing L-methionine (50 mg/100 ml) in which 34.1% of the mol-

Table 3. Incorporation of labeled methionine into CQ and cellular protein.

Exp. Compounds fed[5]	Distribution of ^{2}H or ^{34}S in[1]										
	CQ[2]						cysteine[3]		methionine[4]		
	m/z 632						m/z 61		m/z 301		
	+0	+1	+2	+3	+4	+5	+0	+2	+0	+2	+5
1. [methyl-$^{2}H_3$]-Met.(98%)	26.0 (34.4			74.0 100)							
2. [methyl-$^{2}H_3$, ^{34}S]-L-Met.(34.1%)	53.2 (100	3.0 50.4	21.5 62.7	12.6 48.7	1.8 22.5	7.4 22.5)	88.1 (100	11.9 19.1)	63.4 (100	10.4 21.2	21.2 36.0)
3. [methyl-$^{2}H_3$, ^{34}S]-L-Met.(42%)	52.6 (100	0 44.5	10.2 39.1	31.0 73.5	0 29.8	6.1 24.4)	90.9 (100	9.1 15.5)	64.9 (100	13.0 25.0	22.1 33.9)
4. [methyl-$^{2}H_3$, ^{34}S]-L-Met.(34.1%) + Cys.	76.3 (100	1.3 46.4	4.9 26.8	15 28.6	2.2 13.0	0.3 5.9)	ND				
5. $Na_2{}^{34}SO_4$ (98%)[6]	95.0 (100	1.0 45.8	1.9 22.1	0.5 7.4	0.5 2.3)		ND				

1. Numbers in parentheses are the observed normalized ion intensities.

2. The m/z 632 ion of the acetate derivative of CQ was used for the measurement of the isotopic distribution in the CQ in Expt. 2-5. The measured normalised ion intensities for an unlabeled acetate-CQ sample were 100%, 44.7%, 19.6%, 5.7%, 0%, and 0% for m/z 632, 633, 634, 635, 636, and 637, respectively.

3. The incorporation of labled methionine into cellular cystein was measured from the m/z 61 fragment ion (CH_3S^+) of the S-methyl, N-trifluoroacetyl, n-butyl derivative of the cystein. The measured normalised ion intensity for an unlabeled cystein derivative was 100%, 7.5%, and 5.5% for the m/z 61, 62, and 63, respectively.

4. The incorporation of labeled methionine into cellular methionine was measured from the M^+ m/z 301 ion of the N-trifluoroacetyl n-butyl derivative of the methionine. The measured normalised ion intensity for an unlabeled methionine derivative was 100%, 13.5%, 6.0%, 0%, 0%, and 5.0% for the m/z 301, 302, 303, 304, 305, 306, respectively. The intensities of m/z 302, 304, 305 ions were used in calculation but not listed in Table.

5. Carbon source used is yeast extract in expt. 1-2 and 4-5, and tryptone in expt. 3.

6. A slightly modified medium was used. $(NH_4)_2SO_4$ (1.3 g/l) and $MgSO_4 \bullet 7H_2O$ (0.25 g/l) were replaced by $(NH_4)_2HPO_4$ (1.3 g/l), $MgCl_2 \bullet 6H_2O$ (0.21 g/l), and $Na_2{}^{34}SO_4$ (1.5 g/l).

ecules contained [^{34}S-*methyl*-$^{2}H_3$]-L-methionine. If the methylthio group was incorporated into CQ as an intact unit and if no sulfur was incorporated into benzothiophene ring from the methionine, then CQ molecules with an intact $^{34}SCD_3$ unit would be the only labeled product observed. This labeled product would be detected by an increase in the intensity of the $M^+ + 5$ ion in the mass spectrum of the CQ. Alternatively, if the sulfur and methyl groups of the methionine were incorporated as separated units, then the CQ would be found to contain a ^{34}S or a trideuteriated methyl group as the major labeled species. These labeled products would be detected by an increase in the intensities of the $M^+ + 2$ and $M^+ + 3$ ions of the CQ. The CQ isolated from these cells was observed to contain 21.5% of the molecules with ^{34}S, 12.6% with a deuteriated methyl group, 1.8% with two ^{34}S, and 7.4% with both a ^{34}S and a trideuteriated methyl group. Since the abundance of molecules containing an intact $CD_3{}^{34}S$ unit is lower than those containing a ^{34}S or a trideuteriated methyl group, this indicates that the most of the thiomethyl group was not derived as an intact unit from the methionine. However, since a significant portion of the molecules (7.4%) were found to contain a $CD_3{}^{34}S$ unit it is important to establish if this population of labeled ,olecules arose from the partial incorporation of an intact $CD_3{}^{34}S$ unit or is simply a result of the probability of having both a ^{34}S and CD_3 group in the same molecule. Assuming that CD_3 and the ^{34}S were incorporated as separate units, the abundance of molecules containing a $CD_3{}^{34}S$ unit can be calculated from the abundance of molecules containing a ^{34}S and a trideuteriated methyl group by solving for the values in the equation $(a + b)^2(x + y)$ that generate the

observed isotopic pattern. (In this equation, $b/(a+b)$ is the atom % labeling at sulfur sites with ^{34}S and $y/(x+y)$ is the mole % labeling of the thiomethyl group with CD_3.) From this calculation, it was found that the two sulfur sites are labeled to an extent of 18.4% (b) with ^{34}S and that the methyl group is labeled to an extent of 25.1% (y). (In this calculation, the two sulfur sites were originally found to be labeled to the different extents, 10.6% and 22.3% respectively, which will be discussed later in this thesis.) From the calculated percent incorporation of ^{34}S (18.4%) and deuterium (25.1%), it was calculated that 7.5% of the molecules would be found to contain both a ^{34}S and a trideuteriated methyl group in the same molecule, if the sulfur and methyl groups were incorporated into CQ as separate units. Considering that the difference between 7.5% and 7.4% is within the error present in the mass spectral data measurement and calculation, it is clear that the thiomethyl group was not derived as an intact unit from the methionine.

In order to ensure that the methionine in the cells still contained an intact methylthio group, the isotopic distribution of the cellular methionine was measured (White, 1981). The results of this measurement revealed that 10.43% of the molecules contained one ^{34}S and that 21.2% of the molecules contained both an ^{34}S and a deuteriated methyl group, thereby indicating that some of the cellular methionine had been metabolized to ^{34}S-homocysteine and resynthesized from this labeled homocysteine using a nonlabeled methyl group. These results do show, however, that the major portion of the methionine was utilized by the cells without scrambling of the methyl-sulfur bond.

The above results prove that the methylthio group of CQ is not incorporated as a unit from methionine even though both the sulfur and methyl group are found to be derived from methionine. The above evidence indicates that the first step in the formation of the methylthio group in CQ is most likely the generation of a thiol which is then subsequently methylated, mostly likely by SAM, to CQ. This pathway is consistent with all known examples for the formation of thiomethyl group. The intermediacy of this thiol is also supported by the recent discovery and characterization of a new quinone in *S. solfataricus* that contains a benzo[1,2-b:4,5-b′]dithiophen-4,8-quinone ring (structure c in Figure 4 on page 16, Lanzotti et al., 1986). The second thiophene ring would be formed by the addition of a 5′ thiol into a 2-3 unsaturated demethylated CQ precursor.

The origin of sulfur in CQ. In the experiment described in the preceding section, it was calculated that the two sulfurs of CQ were each labeled from [^{34}S-*methyl*-$^{2}H_3$]-L-methionine to a different extent, 10.6% and 22.3%, respectively. (The determination of which sulfur was labeled to which extent could not be established by this method.) The incorporation of the labeled methionine into protein-bound cysteine and methionine showed that cysteine was labeled to an extent of 11.9% and methionine to an extent of 31.6% of ^{34}S. Had cysteine been the sole source of one of the sulfurs and methionine the sole source of the other sulfur, we would have expected the calculated incorporation of sulfur into these two positions to be 11.9% and 31.6%. Since both of these values are higher than the observed values for the cellular cysteine and methionine, each of these sulfur sources must be mixed with unlabeled sulfur before being incorporated into CQ.

However, because of the low ^{34}S incorporation into CQ, resulting from the low percentage of label in the fed methionine, and the low intensities of the ions used to generate the labeling data for the cysteine and methionine, there was a potential for significant errors in the measurement of isotope enrichment.

To solve this problem, a sample of [^{34}S-*methyl*-$^{2}H_3$]-L-methionine which was more highly enriched in $^{34}SCD_3$ (42 atom % of $^{34}SCD_3$) was synthesized. In addition, the cells were grown with this newly synthesized labeled methionine in a medium containing tryptone as carbon source instead of yeast extract because the cells grow better in tryptone than in yeast extract. The results showed that CQ, cellular cysteine and methionine were all labeled with ^{34}S to a lower extent (Expt. 3, Table 3 on page 98), indicating that there was more sulfur supply to the cells from tryptone than that from yeast extract. By solving for the values in the above equation that best generate the observed ion intensity data, it was found that both of the sulfurs in CQ were labeled to an extent of 8.9% per sulfur. The cellular protein-bound cysteine was labeled to an extent of 9.0% ^{34}S and methionine, as well as homocysteine, to 35% of ^{34}S. This indicates that homocysteine is not the sulfur source for CQ. Therefore, it appears that both of the sulfurs of CQ could come from either cysteine or another sulfur source which was labeled to the same extent with ^{34}S as the cellular cysteine.

To test the involvement of cysteine as the sulfur source for CQ, the above experiment was repeated with the addition of nonlabeled cysteine (50 mg/100 ml) to the medium. The idea behind this experiment was that if one of the sulfur atoms originated from cysteine, the incorporation of ^{34}S from the ^{34}S-methionine

into one of the sulfur sites of CQ would be diluted. Comparison of the distribution of ^{34}S in CQ with the cellular protein-bound cysteine and methionine would then tell if any of the sulfur came from cysteine. The results (Expt. 4, Table 3 on page 98) showed that the total ^{34}S incorporated into CQ was greatly reduced. By solving the equation discussed above, the two sulfur atoms of CQ were found to be labeled to an extent of 3.1% and 5.6% respectively, indicating that cysteine suppresses the incorporation of ^{34}S from the labeled methionione into both of the sulfurs of CQ. However, the fact that the incorporation of ^{34}S of methionine into CQ was also greatly diluted by cysteine, indicates that cysteine supplies the sulfur for both sulfurs of CQ either directly or indirectly.

One other sulfur source present in the medium in which the cells were grown is sulfate. To test if sulfate could be a source of the sulfur, the cells were grown in a medium in which nonlabeled sulfate was replaced with $Na_2{}^{34}SO_4$ (15 mg/100 ml). The CQ isolated from the cells was found not to be labeled (Expt. 5, Table 3 on page 98). This indicates that *S. acidocaldarius* can not utilize sulfate as sulfur source.

Transsulfuration in archaebacteria. The transsulfuration from methionine to cysteine in *S. acidocaldarius* and *Halobacterium marismortui* was studied by growing the cells with [^{34}S-*methyl*-$^{2}H_3$]-L-methionine and measuring the incorporation of ^{34}S into protein-bound cysteine and methionine. Measuring a ^{34}S enrichment in both of these amino acids would demonstrate that they have the ability to convert methionine sulfur into cysteine sulfur.

In the case of *Halobacterium marismortui*, 89 % of the protein-bound methionine and 87% of the protein-bound cysteine were found to be derived from the labeled methionine (Table 4). The same high atom percent excess ^{34}S in the cysteine and methionine indicates that essentially all of the cysteine sulfur was derived from the methionine. In addition, from the atom percent of the methionine containing a $CH_3{}^{34}S$ and a $CD_3{}^{34}S$ group, it can be concluded that 59% of the methionine must have been resynthesized from homocysteine that was derived from the labeled methionine. The fact that the ^{34}S incorporated into both the cysteine and methionine was less than 100% can be explained by the uptake by the cells of a small amount of labeled cysteine/cystine and methionine present in the peptone of the medium.

The derivation of nearly all of the cysteine and methionine sulfur from the fed methionine by *H. marismortui* is to be compared to the incorporation observed in *S. acidocaldarius* grown on yeast extract containing medium plus labeled methionine. In these cells, 93% of the protein-bound methionine was found to be derived from the fed methionine, whereas only 38% of the cysteine was derived from the methionine sulfur (Table 4). Thus, an additional source of sulfur must be present to account for the reduced amount of ^{34}S incorporated into the cysteine. This sulfur would have to arise from unlabeled cysteine which is either present in the medium or newly biosynthesized in the cells from an unlabeled sulfur source. Since these cells did not incorporate $^{34}SO_4{}^{2-}$ into the protein-bound cysteine or methionine, the most likely explanation is that the unlabeled cysteine or cystine in the rich medium is being used as a source of

Table 4. Distribution of labeled methionine in protein-bound cysteine and methionine.

Organism	Level of methionine fed and (atom % excess of $CD_3{}^{34}S$)	Atom % excess of ^{34}S in cysteine	methionine
Halobacterium marismortui [a]	22.6 mg/95mL (34.15%)	29.6	30.5 [b]
Sulfolobus acidocaldarius [c]	50.0 mg/100mL (34.15%)	11.9	31.6 [d]
Sulfolobus acidocaldarius [e]	25.0 mg/100ml (42.0%)	9.0	35.0 [f]

[a] Cells were grown on a defined salts medium containing 1 g/l peptone.

[b] The methionine was found to contain 18.1 atom % excess of the molecules with ^{34}S and 12.4 atom % excess of the molecules with $C^2H_3{}^{34}S$.

[c] The media and the growth conditions used were the same as previously described and contained 2 g/L of yeast extract.

[d] The methionine was found to contain 10.4 atom % excess of the molecules with ^{34}S and 21.2 atom % excess of the molecules with $C^2H_3{}^{34}S$.

[e] The media and the growth conditions used were the same as in [c] but contained 2 g/L of tryptone instead of yeast extract.

[f] The methionine was found to contain 13.0 atom % excess of the molecules with ^{34}S and 22.0 atom % excess of the molecules with $C^2H_3{}^{34}S$.

cysteine. [This statement regarding the lack of $^{34}SO_4^{2-}$ incorporation into cysteine and methionine is based on the lack of incorporation of $^{34}SO_4^{2-}$ into CQ, a sulfur-containing metabolite of *Sulfolobus* spp., that derives its sulfur from cysteine and/or methionine (Table 4).] As in the Halobacteria, about half of the methionine incorporated by the *S. acidocaldarius* was broken down to homocysteine and remethylated to methionine.

In comparison, when *S. acidocaldarius* cells were grown with tryptone as the carbon source, the labeled methionine was found to be incorporated into the cellular methionine to the extent of 83%, 59% of which was metabolized to homocysteine and remethylated to methionine by the cells. In this experiment, however, only 26% of the cysteine was derived from the methionine sulfur as compared to 38% when the cells were grown with yeast extract. This depression of incorporation into both the cysteine and methionine most likely results from the greater amount of cysteine and methionine in the tryptone than in the yeast extract (Orr and Watt, 1957). The ability of *S. acidocaldarius* to derive more of its cysteine sulfur from the medium than *H. marismortui* must reflect a species-specific difference in the cell's ability to take up cysteine from the medium.

In contrast to the above examples, where transsulfuration from methionine to cysteine was readily determined, establishing the occurrence of transsulfuration in the methanogenic archaebacteria by using the methods described has a number of problems. The major problem is that all of the media used for the growth of methanogens contain high levels of cysteine and/or cysteine and sulfide in order to maintain the media in a strongly reducing condition (Balch et al., 1979). Even

a recently developed medium with titanium (III) citrate as a substitute for sulfide still contains cysteine (Zehnder and Wuhrmann, 1976). One would expect this cysteine be taken up by the cells and block the conversion of the methionine sulfur to cysteine sulfur. An attempt to grow *Methanobacterium formicicum* in a cysteine-free titanium (III) citrate medium (Zehnder and Wuhrmann, 1976) with methionine as the sole sulfur source, showed growth only when sulfide was added to the medium. Under these growth conditions, however, none of the methionine sulfur was found to be incorporated into the protein-bound methionine, making it impossible to determine if transsulfuration had occurred.

The ready transfer of methionine sulfur to cysteine in members of the archaebacteria is a characteristic previously believed to occur only in eucaryotes. Its demonstration in the archaebacteria adds further support for the closer association of the Domain Archaea with the Domain Eucarya than with the Domain Bacteria as recently outlined by Woese et al. (1990). One must, however, be careful about these conclusions since the original work of Delavier-Klutchko and Flavin (1965), which first demonstrated the inability of bacteria to convert methionine to cysteine, was tested on only two closely related bacteria, *Escherichia coli* and *Salmonella typhimurium*, and has been extended to only one other bacteria, *Synechaococcus leopoliensis* (Lawry and Jensen, 1986). Since these examples represent such a small sample of all the known bacteria, and since the archaebacteria can readily convert methionine sulfur to cysteine, one must seriously consider that this type of transsulfuration may, in fact, occur in at least some eubacteria.

METABOLISM OF TYROSINE AND FLUOROTYROSINES IN *S. ACIDOCALDARIUS*

INTRODUCTION

Studies on the biosynthesis of CQ have clearly shown that the benzo[*b*]thiophene-4,7-quinone moiety of the structure is derived as an intact unit from all of the carbons of tyrosine except C-1 (Zhou and White, 1989) and that the side chain is derived via an isoprenyl unit (De Rosa et al., 1977). On the basis of the transformation that is required to change the carbon structure of tyrosine into the benzo[*b*]thiophene ring of CQ, it was proposed that homogentisic acid was an intermediate in the biosynthesis of CQ. However, despite an extensive effort, homogentisic acid could neither be shown to be a precursor to CQ nor be found in the cells or the growth media after the completion of growth. This ina-

bility to demonstrate that homogentisic acid was an intermediate prompted the testing of several other possible intermediates to CQ, none of which could be shown to be precursors (see page 43). Thus one was left the critical problem of identifying exactly which of several possible pathways was used to generate the CQ structure. In an effort to establish the specific biochemical route used by the cells to produce CQ, the metabolites generated by the cells grown in the presence of 2- and 3-fluorotyrosines have been analyzed. The rationale behind these experiments was that either one or both of the fluorotyrosines, acting as an analogue of tyrosine, would be converted into specific fluorine-containing intermediates leading to CQ. In one or more of these intermediates, however, the position of the fluorine in the molecule would specifically block its conversion into CQ (Figure 28). This, in turn, would likely lead to a buildup of this fluorine-containing intermediate which could then be isolated and identified. On the basis of the structure(s) of these intermediate(s), the correct pathway for the biosynthesis of CQ could then be established.

Two approaches have been used in an attempt to detect the desired intermediates so that they could be isolated and structurally characterized. In the first approach, phenols produced by the cells grown with the fluorotyrosines were detected after TLC separation of the cell extracts using a phenol specific spray. The rationale behind this approach was that since tyrosine is the precursor to CQ, any intermediate on the pathway between tyrosine and CQ would also have to be a phenol. The second approach was to detect the desired compounds by assaying fractionated cell extracts for the presence of organo-fluorocompounds. In either

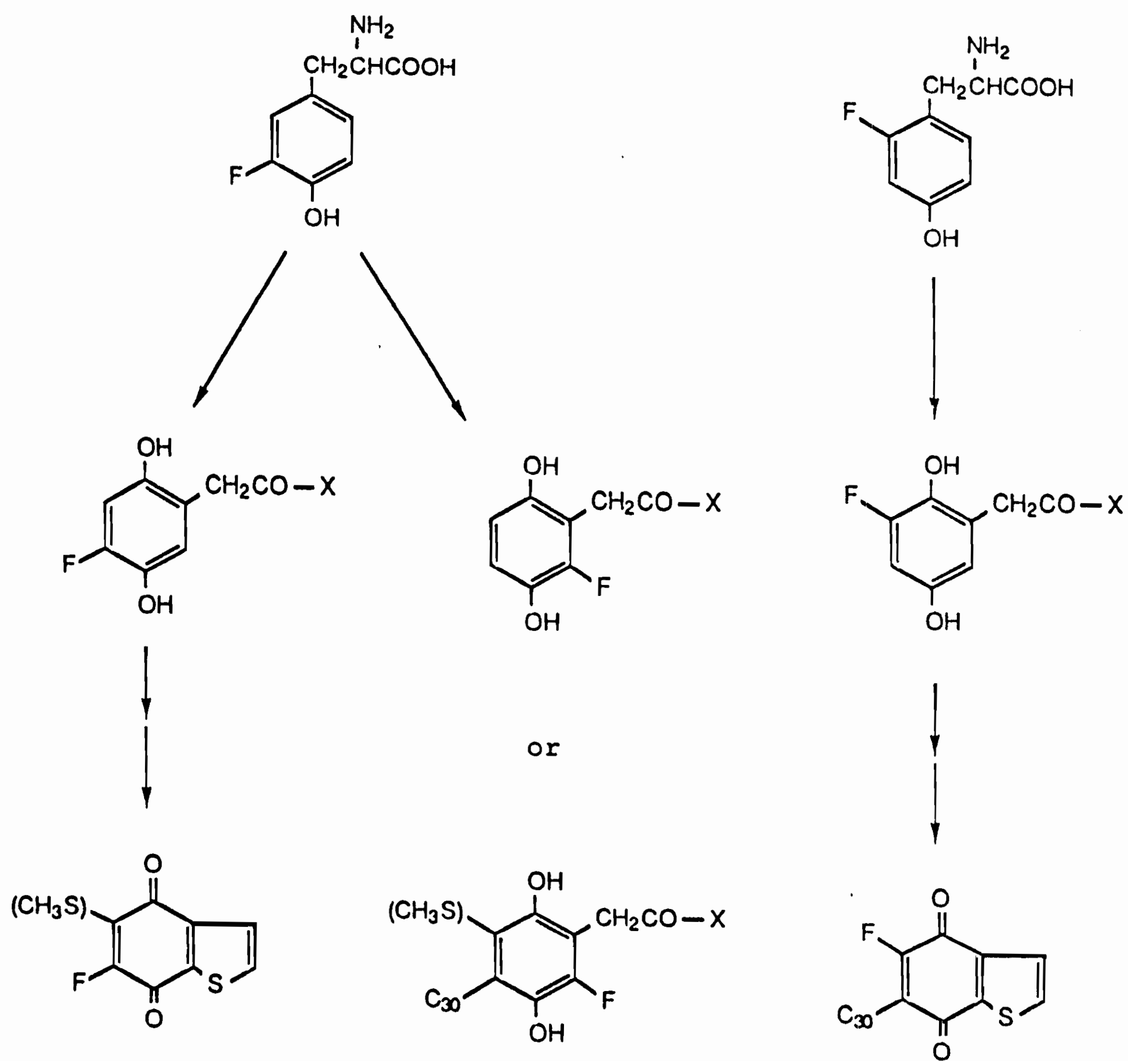


Figure 28. Possible fluoro-analogues of CQ intermediates.

case, UV/visible spectroscopy and mass spectrometry of the desired fractions were used to establish the identities of the detected compounds.

MATERIALS AND METHODS

Chemicals and supplies. DL-2-Fluorotyrosine (2-F-tyr) was supplied by Dr. Robert A. Pascal, Princeton University (Pascal et al., 1985). DL-3-Fluoro-tyrosine (3-F-tyr), 3-fluoro-4-hydroxyphenylacetic acid (3-F-*p*HPA), 4-hydroxy-phenylacetic acid (pHPA), 4-hydroxybenzoic acid (*p*HBA), 4-hydroxy-benzaldehyde (*p*HBAD), 4-hydroxymandelic acid (*p*HMA), 3,4-dihydroxyphenylacetic acid (DHPA), 3,4-dibenzyloxybenzaldehyde, homogentisic acid (HA), homogentisic lactone (HL), hexamethyldisilazane and trimethylsilyl chloride were obtained from either Sigma Chemical Company or Aldrich Chemical Company. 3,4-Dihydroxybenzyl alcohol (DHBOH) was pre-pared from 3,4-dibenzyloxybenzaldehyde (DHBAD) as described by Bristow (1957) and Reimann (1971). The phenol reagent (Folin-Cioclateau) used for spraying the TLC plates was obtained from Fisher Scientific Company (product SO-P-24) (Keith et al., 1958). The trimethylsilyl derivatization reagent contained pyridine-hexamethyldisilazane-trimethylsilyl chloride (3:3:1,vol/vol/vol).

TLC was performed on Kieselgel 60 F254 thin-layer chromatography plates (E. Merck AG, Darmstadt, Germany). Ultraviolet absorption spectra of the separated compounds were recorded either directly from these TLC plates using a Shimadzu CS-9000 dual-wavelength flying-spot TLC scanner or the spots were

scraped from the plate and eluted with appropriate solvent and the UV spectra were recorded using a Shimadzu UV-16A UV-visible recording spectrophotometer. Mass spectrometry and GC-MS were performed on a VG 70-70EHF mass spectrometer operating at 70 eV.

Synthesis of [α, α, 2′, 3′, 5′,6′-$^2H_6$]- 4-hydroxyphenylacetic acid. Deuteriated *p*HPA was prepared by reacting *p*HPA (50 mg) with DCl (2 ml, 35% DCl in 99 atom % ^2H_2O) at 100°C for 24 h. After the reaction mixture was evaporated to dryness under nitrogen, water was added to exchange the deuterium incorporated into the carboxylic acid and hydroxyl groups with protons. Evaporation of the water gave the deuteriated *p*HPA. The deuterium distribution of this labeled compound was determined both by mass spectrometry and NMR and was found to contain about 60 atom % of molecules with six deuteriums and about 40 atom % with five deuteriums.

Bacterial strain and growth conditions used. *S. acidocaldarius* (ATCC 33909) cells were grown for 3 days at 70°C in 100 ml of a yeast extract-containing media as described earlier in this thesis. When the growing culture reached an absorbance of from 0.3 to 0.4 at 540 nm (approximately one day of growth) 15 mg of either DL-tyrosine, DL-2-fluorotyrosine, DL-3-fluorotyrosine, deuteriated *p*HPA, or 3-F-*p*HPA was added.

Extraction of cells and TLC purification and analysis of the cell extracts. At the end of cell growth, the cells were harvested by centrifugation (15,000 x g, 15 min) and the cell pellet was extracted with methanol-methylene chloride (1:1, vol/vol) under acidic conditions at 40°C for 30 min as previously described in this

thesis. After partitioning of the extract with water, the resulting methylene chloride-soluble material was purified by preparative TLC. A solvent mixture consisting of hexane-diethyl ether (9:1, vol/vol) was used to separate the CQ and other materials of similar polarity. The materials at the origin of TLC were further purified by TLC with methylene chloride as the solvent. Spots present in the fluorotyrosine samples and not present in the tyrosine samples were possible fluorinated intermediates involved in CQ biosynthesis. However, since the fluorinated compounds had the same R_f as the nonfluorinated compounds and would appear at the same place on the TLC plate, all spots were analyzed. After elution of the individual TLC spots from the TLC plates, they were partially characterized by their UV and mass spectra. Phenols present in the cell extracts were separated as described below for the medium extract and analyzed by direct-inlet mass spectrometry.

Organic fluoride assay. The total methanol-methylene chloride extraction of 1 g wet weight of cells grown with 3-fluorotyrosine (from one liter of medium) was purified on TLC plates as described above. Each individual band was eluted from the plates, and half of each sample was assayed for organic fluoride. The procedure used was patterned after that described by Fresen and Cox (1968) and consisted of evaporating the sample to be assayed for organic fluoride on a 1 x 1 cm piece of filter paper and performing an oxygen flask combustion of the sample in pure oxygen using a 500 ml plastic bottle equipped with a rubber stopper, a platinum sample holder, and 10 ml of 0.1 N NaOH. After the combustion was complete, the sealed flask was set aside for 20 min to allow for the absorption of

the combustion gases into the NaOH solution. The resulting NaOH solution was then transferred into a plastic centrifuge tube, 1 ml of concentrated HCl was added followed by 0.5 ml of a 0.06% solution of trimethylsilyl chloride in benzene. After shaking for 5 min, the benzene layer was transferred into a vial and analyzed by GC using a 1/8 in x 6 ft glass column operating at 60°C containing 10% SP-2100 on Supelcoport. Under these conditions the extracted trimethylsilyl fluoride had a retention time of about one min. Analysis of known amount of 3-F-*p*HPA was used to standardize the procedure which used GC peak heights for quantitation. Recoveries of organic fluorine as fluoride in the combustion and absorption procedures were greater than 90%.

TLC purification and analysis of phenols extracted from the medium. After separating the cells from the medium (100 ml) and adjusting the medium to pH ~1.7 with HCl, the medium was extracted twice with 25 ml of ethyl acetate. The combined extracts were dried with anhydrous Na_2SO_4, evaporated with a stream of nitrogen gas to dryness and the resulting sample was dissolved in 100 μl of ethyl acetate for further analysis.

A portion of the extract from the medium was separated by TLC using the solvent mixture methylene chloride-ethyl acetate-formic acid (88%) (15:4:1, vol/vol/vol) and the UV absorption spectra of the individual spots were obtained using the TLC scanner. Individual spots were identified as phenols by the appearance of blue spots after spraying the TLC plates with the phenol reagent. Homogentisic acid, homogentisic lactone, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxybenzylalcohol, all of which contain dihydroxylated rings, showed

dark blue spots immediately after spraying, whereas simple phenols such as *p*-hydroxyphenylacetic acid developed color only after exposing the plate to ammonia vapor either before or after spraying.

The spots corresponding to phenols were purified by preparative TLC and those phenols containing carboxylic acid group were converted into methyl esters by reacting with 3 N HCl in methanol for 3 h at 60°C. After evaporation of the solvent, the resulting methyl esters were purified by preparative TLC using the methylene chloride-ethyl acetate-formic acid (88%) solvent. Mass spectra of these samples were obtained by direct inlet analysis and compared to knowns prepared and analyzed in the same manner. The methyl esters were further identified by comparing their TLC R_f's with those of the knowns.

In addition, the compounds were also analyzed by GC-MS of the TMS derivatives of the phenols. The TMS derivatives were prepared by reacting a known amount of the medium extract with the TMS reagent for 5 min at 100°C. After cooling the reaction mixture and evaporating the solvent, the resulting TMS derivatives were dissolved in a known amount of methylene chloride and a known amount was assayed by GC-MS. The GC separation was carried out using a Hewlett-Packard PH5 capillary column (0.32 mm x 25 m) programmed from 70°C at 10°C/min. Since some of the compounds including 3,4-DHPA, 3,4-DHBOH, and their corresponding fluorocompounds were relatively minor components in the total medium extract, obtaining clean mass spectra of these compounds was impossible because of the large amount of interfering compounds. To eliminate this problem, a portion of the crude extract was purified

by preparative TLC and the TMS derivative was prepared from the individual fraction prior to GC-MS analysis. In this way unambiguous mass spectra of these minor compounds were obtained.

Identification of all compounds except the fluorinated analogues of 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxybenzylalcohol, 3,4-dihydroxybenzoic acid, and *p*-hydroxybenzoic acid was confirmed by demonstrating that each had the same R_f on TLC, UV spectrum, mass spectrum, and GC retention time as the knowns. Since no knowns of the fluorinated compounds of 3,4-DHPA, 3,4-DHBOH, 3,4-DHBA, and *p*HBA were available they were identified by the mass spectra of their TMS derivatives, which showed M+ ions 18 m/z higher than the nonfluorinated compounds. In addition, the TMS derivatives of all the fluorinated compounds had GC retention times similar to the TMS derivatives of the nonfluorinated compounds.

Quantitation of the phenols in the medium. The amount of each metabolite in the medium was quantitated by GC-MS of the TMS derivatives of the unpurified medium extract. This was accomplished by comparison of the peak areas of single ion plots for each of the compounds generated from a known amount of the medium extract with the same single ion plots generated from a mixture of knowns. The known mixture was prepared by adding a known amount of *p*HPA, 3-F-*p*HPA, *p*HBA, HA, *p*HBAD, *p*HMA, and HL, to 100 ml fresh medium and extracting it with ethyl acetate in the same manner as for the sample. By knowing what proportion of the known and unknown were injected and the areas under the single ion plots for both samples, the amount of each of com-

pound present in the medium could be calculated assuming that a linear relationship between the peak area and the amount of sample present. In general the intensities of the molecular ion and/or the base peak ion for each of compound were used to calculate the amount of the compound in the samples.

Quantitation of the utilization of DL-tyrosine and 3-fluorotyrosine by *S. acidocaldarius*. The total amount of DL-tyrosine or DL-3-fluorotyrosine used by the cells was determined by measuring the amount of free tyrosine or 3-fluorotyrosine remained in the medium at the end of cell growth. Thus the cells grown with 15 mg/100 ml of DL-tyrosine or DL-3-fluorotyrosine were separated from the medium by centrifugation and the free tyrosine or 3-fluorotyrosine remained in the medium was measured by amino acid analysis. The yeast extract medium without tyrosine or fluorotyrosine was measured in the same manner in order to determine the amount of free tyrosine present in the yeast extract.

RESULTS AND DISCUSSION

Only when *S. acidocaldarius* was grown in the presence of tyrosine or the fluorotyrosines were phenols produced in amounts that could be readily detected by the TLC detection methods used. These phenols were tentatively identified on the TLC plate by their UV absorbance and confirmed by the appearance of a blue color after spraying the plates with the phenol spray reagent. The individual phenols, as well as their corresponding methyl esters, were purified by preparative TLC and were identified as specific compounds by comparing their

R_f's, their UV spectra, and their mass spectra, with those of known samples. The R_f's and absorbance maximam of these metabolites and their methyl esters are reported in Table 5. These compounds, as well as 3,4-DHBOH, *p*HMA, *p*HBAD, 3,4-DHBA, and their fluorinated analogues were also detected and quantitated by GC-MS analysis of the TMS derivatives (Table 6).

The presence of the metabolites, *p*HPA, *p*HMA, *p*HBAD, *p*HBA, and the corresponding fluorinated analogues in the media from the cells grown with 2- or 3-fluorotyrosine, indicates a pathway of tyrosine metabolism shown in the left side of Figure 29 on page 123. In this pathway, tyrosine is converted into *p*-hydroxyphenylpyruvate which after oxidative decarboxylation to *p*HPA is alpha-hydroxylated to *p*HMA. Two mechanisms for the conversion of *p*HMA into *p*HBA are possible. In the first, the *p*HMA is oxidized to 4-hydroxyphenylglyoxylic acid (*p*HPG) which is then either oxidatively decarboxylated directly to *p*HBA or converted to *p*HBA via *p*HBAD. In the second mechanism, *p*HMA is decarboxylated directly to *p*HBOH which is converted into *p*HBA via *p*HBAD. At present, the data do not allow for the specific pathway to be chosen because no intermediate specific to either pathway was identified. Evidence supporting this second mechanism comes from the identification of DHBOH, an intermediate involved in the metabolism of tyrosine by a second pathway to be discussed below. The conversion of *p*HPA to *p*HMA by the cells was also confirmed by the production of F-*p*HMA by the cells grown with 3-F-*p*HPA and the production of $[^2H_5]$-*p*HMA by the cells grown with $[^2H_6]$-*p*HPA. The presence of $[^2H_4]$-*p*HBA in the cells grown with $[^2H_6]$*p*HPA

Table 5. Analytical data on the medium extractable phenol.

compound	max (nm)	R_f[a]	R_f of methyl ester[a]
p HPA	219, 272	0.15	0.51
2-F-*p* HPA	215, 269	0.15	0.51
3-F-*p* HPA	217, 268	0.17	0.55
p HBA	255	0.18	0.52
2-F-*p* HBA	254	0.18	0.52
3-F-*p* HBA	254	0.18	0.52
3,4-DHPA	215, 288	0.05	0.21
2- or 6-F-DHPA	219, 279	0.09	0.26
5-F-DHPA	219, 277	0.09	0.26

[a] The solvent used for the separation consisted of methylene chloride-ethyl acetate-formic acid (88%) (15:4:1, vol/vol/vol).

Abbreviations: *p*HPA, *p*-hydroxyphenylacetic acid; *p*HBA, *p*-hydroxybenzoic acid; DHPA, dihydroxyphenylacetic acid.

Table 6. Quantitation of some of the medium extractable phenols.

assayed compounds	control	µmol of compound/100mL of medium for cells grown with 100 µmoles of tyr.	2-F-tyr.	3-F-tyr.	3-F-*p*HPA[a]
*p*HPA	ND	56.6	1.4		6.4
F-*P*HPA	ND		18.5	24.2	D
*P*HMA[c]	ND	8.9	0.12	D	D
F-*P*HMA[c]	ND		D	D	D
*P*HBAD	ND	0.03	0.005	D	ND
F-*P*HBAD	ND	0.007	D	ND	
*P*HBA	ND	0.6	0.11	0.25	ND ?[b]
F-*P*HBA	ND		D	0.12	ND ?[b]
DHPA[c]	ND	0.01	0.002	0.16	D
F-DHPA[c]	ND		0.002	0.05	D
DHBOH	ND	?	D	D	ND
F-DHBOH	ND		D	D	ND
DHBA	ND	?	D	D	ND
F-DHBA	ND		ND	D	ND
OHPA	0.13	1.8	3.1	1.4	D
total tyr. or 3-F-tyr. metabolized		76.8		72.9	

[a] The compounds produced by the cells grown with 3-F-*p*HPA and deuteriated *p*HPA were not quantitated, but only identidied by GC-MS.

[b] An important factor in not being able to identify *p*HBA and F-*p*HBA in this extract was the fact that both of these compounds had very similar retention times which was also similar to the retention time of the 3-F-*p*HPA. Since a large amount of the 3-F-*p*HPA added to the medium was not metabolized and large amounts was recovered in the extract, its presence obscured the detection of the *p*HBA and F-*p*HBA peaks.

[c] The quantitation of *p*HMA/F-*p*HMA and DHPA/F-DHPA was very not accurate because they occurred in relatively low amount in the extract compared to the other metabolites and their TMS derivatives proved not to be stable. Abbreviations: *p*HPA, *p*-hydroxyphenylacetic acid; *p*HMA, *p*-hydroxymandelic acid; *p*HBA,: *p*-hydroxybenzylaldehyde; *p*HBA, *p*-hydroxybenzoic acid; DHPA, dihydroxyphenylacetic acid; DHBOH, dihydroxybenzylalcohol; DHBA, dihydroxybenzoic acid; *o*HPA, *o*-hydroxyphenylacetic acid; ND, none detected; D, detected but not quantitated.

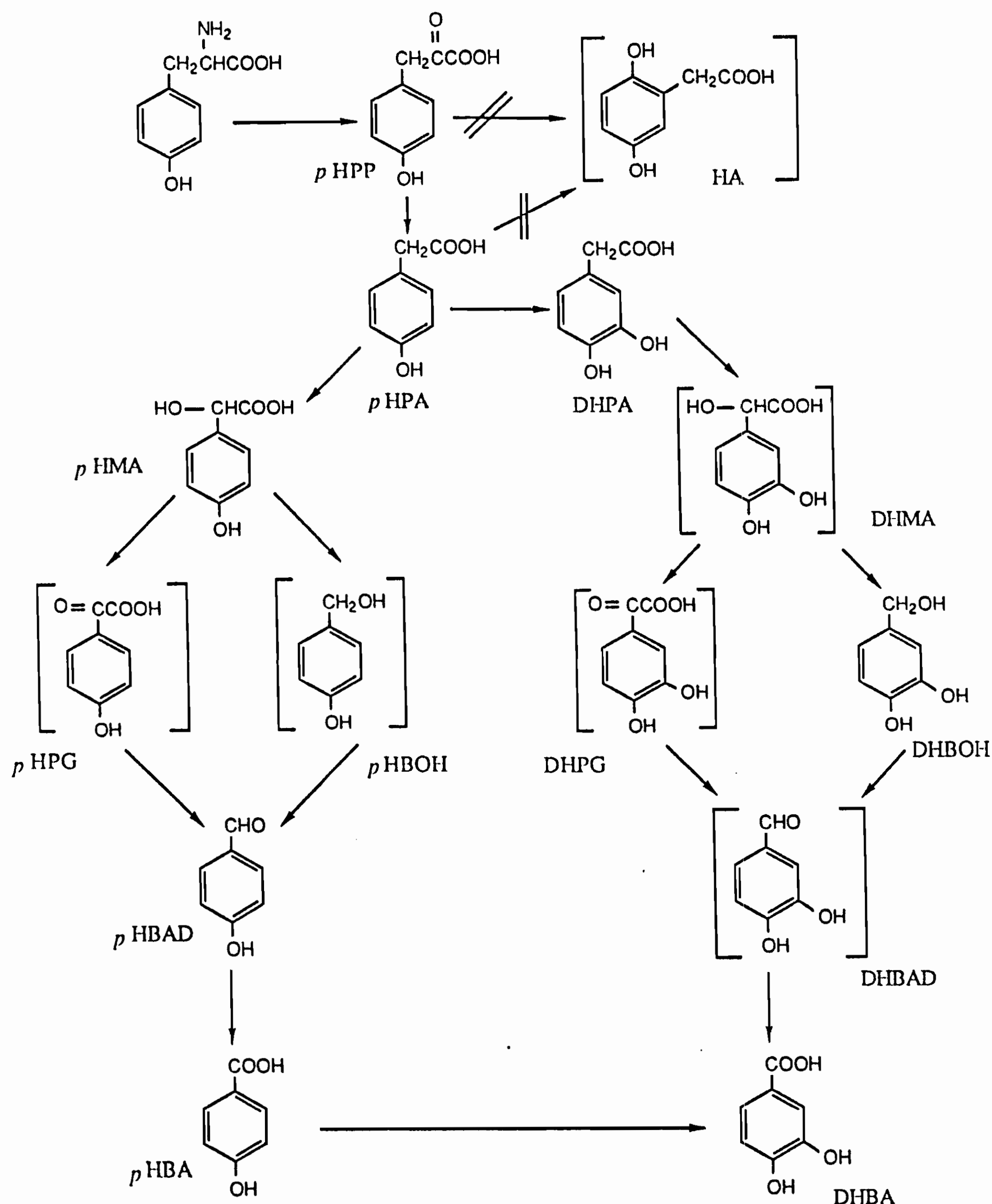


Figure 29. Proposed pathways of tyrosine metabolism in *S. acidocaldarius*: The metabolites that are not in [ ] were detected in the medium extracts. Abbreviations: *p*HPA, *p*-hydroxyphenylacetic acid; *p*HMA, *p*-hydroxymandelic acid; *p*HBA, *p*-hydroxy- benzylaldehyde; *p*HBA, *p*-hydroxybenzoic acid; DHPA, dihydroxyphenylacetic acid; DHBOH, dihydroxybenzyl alcohol; DHBA, dihydroxybenzoic acid.

also indicates that *p*HPA is metabolized to *p*HBA. *p*HBA is generally metabolized via its conversion to 3,4-DHBA (Johnson and Stanier, 1971; Kishore et al., 1977). This appears to occur in *S. acidocaldarius* since 5-F-3,4-DHBA was detected in the medium extracts of the cells grown with 3-fluorotyrosine (Table 6 on page 121). This pathway for the degradation of the side chain of tyrosine appears to have been proposed in the bracket fungus *Polyporus hispidus* (Perrin and Towers, 1973). The conversion of *p*HPA to *p*HMA has been characterized as a portion of the pathway for the metabolism of phenylalanine in *Aspergillus niger* (Kishore et al., 1974). The conversion of *p*HMA to *p*HBA via *p*-hydroxyphenylglyoxylic acid has been described in bacteria (Kennedy and Fewson, 1966; 1968). The conversion of tyrosine to *p*HPA has also been identified in an organism tentatively identified as a *Micrococcus* (Sparnins and Chapman, 1976).

The presence of a second pathway for tyrosine metabolism (right side of Figure 29) was indicated by the detection of 3,4-DHPA, 3,4-DHBOH, 3,4-DHBA, and the corresponding fluorinated analogues in the medium extract from cells grown with tyrosine or fluorotyrosines. This pathway would begin by the aromatic hydroxylation of *p*HPA to 3,4-DHPA as has been observed to occur in *Micrococcus* (Sparnins and Chapman, 1976) and *E. coli* (Cooper and Skinner, 1980) and was confirmed by the production of fluoro-3,4-DHPA and $^{2}H_{5}$-labeled 3,4-DHPA by the cells grown 3-F-*p*HPA and $^{2}H_{6}$-*p*HPA, respectively. The identification of 3,4-DHPA and the F-3,4-DHPA's were made by comparison of their R_f values on TLC, their UV spectra, and their mass spectra

with known 3,4-DHPA. The differentiation between 3,4-DHPA and 2,5-DHPA (HA) was established both by their slightly different GC retention times when chromatographed as the $(TMS)_3$ derivatives and differences in the mass spectra of their $(TMS)_3$ derivatives. Although the $(TMS)_3$ derivatives of the two isomers have the same molecular ion and generally the same fragment ions, the relative intensities of some of the ions were different enough to readily distinguish the two isomers. The most important of these were the *m/z* 179 ion which was present in 3,4-isomer but not in 2,5-isomer, and the *m/z* 341 ion which was present in 2,5-isomer but not in 3,4-isomer. Using these criteria only 3,4-DHPA and its fluorinated compounds were identified, no HA or fluoro-HA were ever detected.

The presence of 3,4-DHBOH and 3,4-DHBA as well as their fluorinated analogs in the cells grown with 2-fluorotyrosine and 3-fluorotyrosine indicates that 3,4-DHPA is probably metabolized via DHBOH to DHBA as shown in Figure 29 on page 123. Two of the proposed compounds in the pathway, 3,4-dihydroxymandelic acid (3,4-DHMA) and 3,4-dihydroxyphenylglyoxylic acid (3,4-DHPG), however, were probably not detected either because they occurred at such a low concentration in the medium extracts or that they failed to form TMS derivatives that were suitable for analysis by GC. This pathway is thus analogous to the pathway for the conversion of *p*HMA to *p*HBA via 4-hydroxybenzyl alcohol (*p*HBOH).

Whether DHBA, which is known to be readily metabolized in other microorganisms (Johnson and Stanier, 1971; Kishore et al., 1977), is further metabolized by the *S. acidocaldarius* can not be clearly established from the

present data. The uncertainty about the metabolism of DHBA arises when one attempts to equate the amount of tyrosine and 3-fluorotyrosine metabolized by the cells with the amount of metabolic products identified. In the case of tyrosine, 92% of the tyrosine (77 μmoles) was metabolized and of this metabolized tyrosine, 86.4% could be accounted for the metabolites found in the medium and reported in Table 6. This leaves only 14% of the tyrosine that would have to be metabolized via DHBA assuming that no tyrosine was generated by the cells. Since this 14% could be accounted by the metabolites not searched for, this leaves very little DHBA to be further metabolized. The metabolic balance for the metabolism of 3-fluorotyrosine leads to a similar conclusion. The interesting observation about these results is that so much of the metabolic intermediates actually appear in the medium.

Homogentisic acid has been found to be the metabolite of tyrosine in many organisms (Kishore et al., 1977), and to be an intermediate in the biosynthesis of plastoquinones and tocopherols (Whistance and Threlfall, 1970). The formation of HA from tyrosine can be accomplished in two different manners, the oxidative rearrangement of *p*HPP by a dioxygenase (Jefford and Cadby, 1981; Lindblad et al., 1970), or the rearrangement of *p*HPA, formed by decarboxylation of *p*HPP, via a 4-hydroxyphenylacetate 1-hydroxylase (Blakley, 1972; Hareland et al., 1975; van den Tweel, 1988). The fact that HA, F-HA or deuteriated HA were not detected in any of the medium extracts indicates that these cells either do not produce enough HA for it to be detected by the methods used or that HA is not involved in the metabolism of these cells. Since *p*HPA is a central

metabolite in tyrosine metabolism and also a possible precursor to HA, feeding [$^2H_6$]-*p*HPA to the cells would label all the intermediates derived *p*HPA. Thus if any of these intermediates were involved in CQ biosynthesis, this could be established by measuring the incorporation of label into CQ. When these experiments were carried out, *p*HBA, 3,4-DHPA, and *p*HMA in the medium extract were all found to be deuteriated yet CQ was not labeled. This result indicate that none of the metabolites derived from *p*HPA was involved in CQ biosynthesis. This result taken together with the fact that tyrosine is a known precursor to CQ and that HA could neither be detected in the cells nor demonstrated to be a precursor to CQ, indicates that the pathway for the biosynthesis of CQ starts from either tyrosine or *p*HPP.

The blockage of the biosynthesis of CQ by 2- or 3-fluorotyrosine was examined by quantitating the amount of CQ produced by the cells grown with tyrosine, 2-fluorotyrosine, and 3-fluorotyrosine. The results (Table 7) showed that fluorotyrosine did not reduce the production of CQ, although fluorotyrosines were metabolized by the cells.

Extract of the cells grown with 3-fluorotyrosine was also analyzed for the presence of fluorine-containing compounds and phenols. Two fluorine-containing compounds with R_f's of 0.14 and 0.59 were detected by organic fluoride analysis after TLC separation of the cell extract using the solvent system, hexane-ether (9:1) (Figure 30). The only phenol(s) detected remained at the origin. Quantitation of the amount of organic fluorine present in these two peaks showed that each contained only about 24 nmoles of organic fluorine per gram wet weight

Table 7. Quantitation of CQ in the cells grown with 2- or 3-F-tyrosine.

	control	2-F-tyr	3-F-tyr
A_{318} of CQ	0.216	0.133	0.191
amount of CQ (ug)	28	17.2	24.7
dry cell weight (mg)	27.3	18.4	25.8
A_{318}/dry weight (10^{-3} mg^{-1})	7.9	7.2	7.4

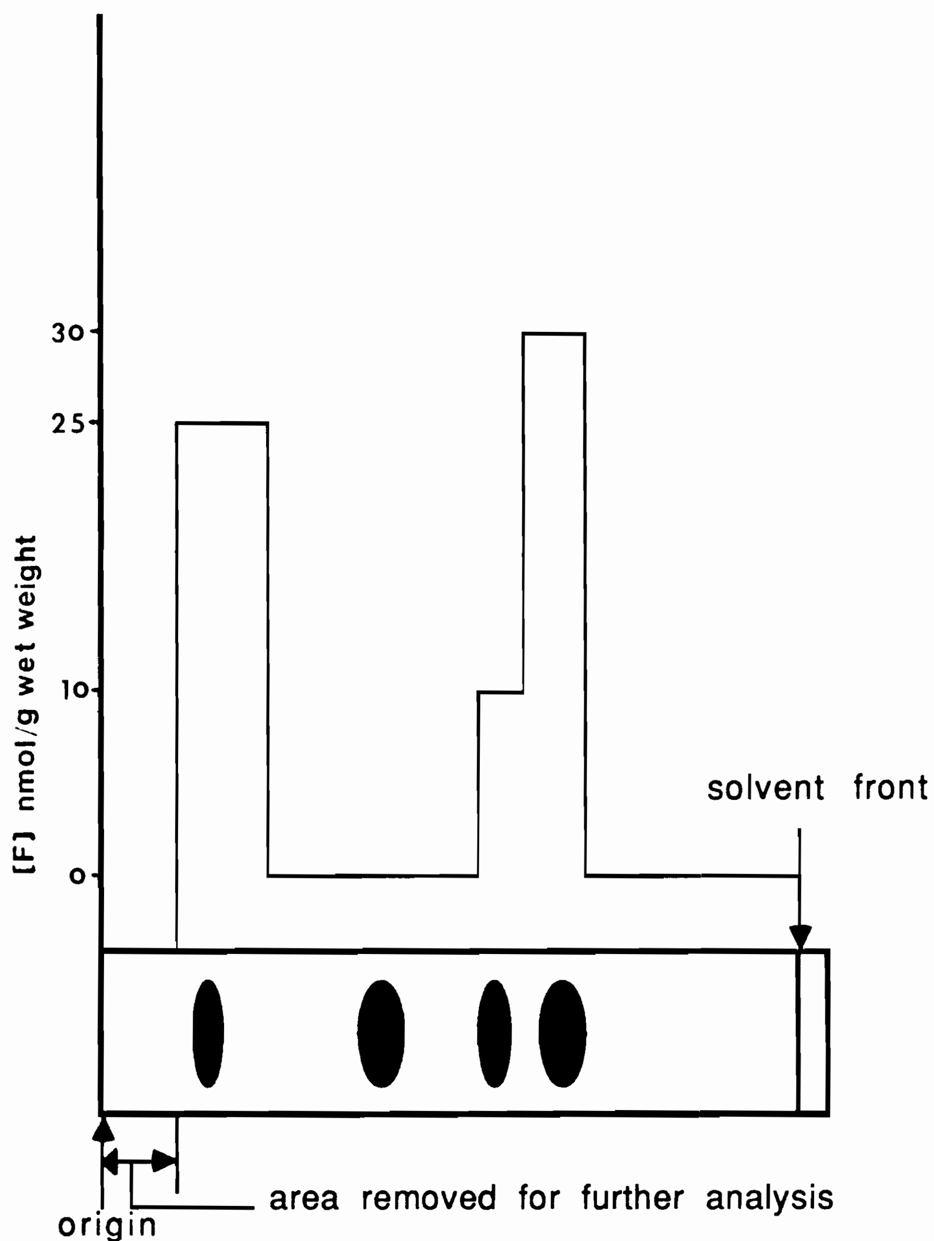


Figure 30. TLC of extract from cells grown with 3-fluorotyrosine and assay of fractions for organic fluorine.

of cells. UV and mass spectral analysis of the top R_f spot showed the major component of this spot to be elemental sulfur. Thus, in agreement with the amount of organic fluorine detected, the desired compound must be only a small portion of the total sample. However, since the R_f of this compound was greater than that of CQ, it is hard to rationalize how the compound could be an intermediate in CQ biosynthesis, which would be expected to be more polar and thus have a lower R_f than CQ. The UV and mass spectral data on the lower R_f spot also failed to give any useful information as to the nature of the compound. The R_f of this spot also failed to match any of the known compounds such as the farnesylfarnesol derivative of homogentisic acid lactone, as well as the radioactive spot found in the extract of the cells grown with [U-^{14}C]-tyrosine. In addition to these two fluorocompounds, two phenols were detected by the separation of the material remaining at the origin after the first separation. These compounds had a R_f of 0.10 and 0.65 when separated by TLC using methylene chloride as the solvent. These compounds were analyzed by their R_f's on TLC, UV spectra, and mass spectra but the chemical structures of the compounds have yet to be established. However, from the observed molecular weights of these compounds (data not shown), it appears that these compounds are not C_{30}-isoprenylated aromatic compounds.

The above data support the view that the first committed step in the biosynthesis of CQ occurs at either tyrosine or a metabolic product very closely related to tyrosine, e.g. *p*HPP. The most probable reaction to occur at this branch point would likely involve the introduction of either the isoprenyl side

chain or the sulfur into the aromatic ring of the intermediate. This, however, will only be established when the chemical structure of the product of this reaction is established.

CONCLUSIONS

From the data presented in this thesis, the pathway for the biosynthesis of CQ shown in Figure 31 is proposed. From the experiments with labeled tyrosines it has been clearly established that all of the carbons of the benzothiophene quinone ring of CQ arises as an intact unit, from all of the carbons of tyrosine except for C-1. This incorporation of **all** the tyrosine carbons except C-1 is to be compared with the limited incorporation of the tyrosine hydrogen, where it was found that only the pro-3S hydrogen of tyrosine was incorporated into CQ.

The detection by GC-MS of farnesylfarnesol, a hydrolytic product of farnesylfarnesyl pyrophosphate, but none of its hydrogenated products, in lipid extracts of *S. acidocaldarius* indicates that farnesylfarnesyl pyrophosphate is the most likely source of the C_{30} isoprenyl side chain of CQ. Assuming this to be correct, then this data indicates that the formation of the completely hydrogenated side chain must occur at a later step in the biosynthesis. Consid-

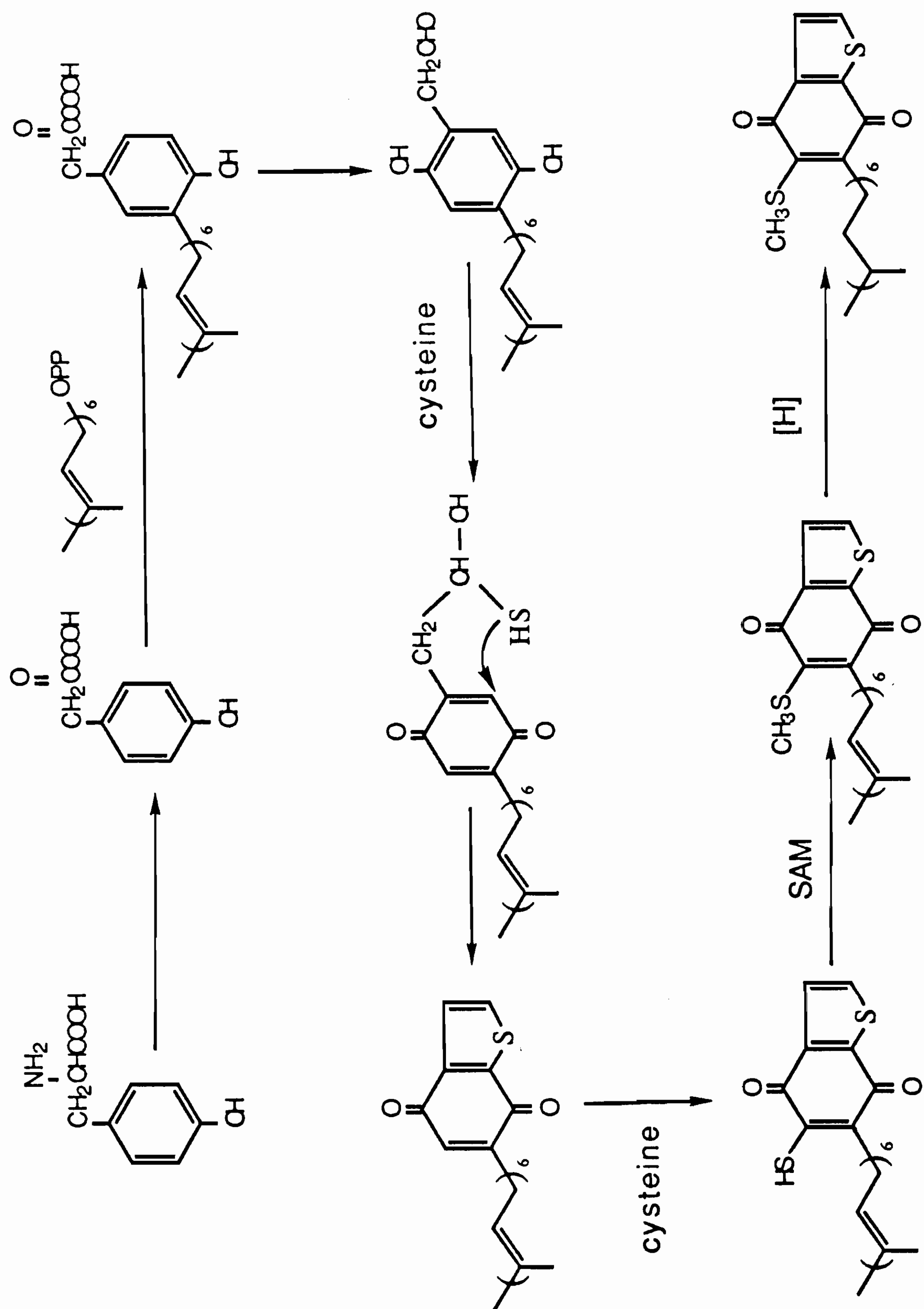


Figure 31. Proposed biosynthetic pathway of CQ.

ering that the biosynthesis of the tricycloquinone requires not only the presence of at least one double bond in the side chain but also a preformed benzothiophene quinone ring containing a thiol, hydrogenation of the side chain, as shown in Figure 31, must occur as the last step in the biosynthetic pathway. This could be confirmed by the isolation of CQ from cells containing one or more unsaturated sites in the side chain.

The exact step at which the farnesylfarnesyl pyrophosphate condenses with either the aromatic ring of tyrosine or one of its metabolites, was not firmly established by the experiments described in this thesis. Indirect evidence, however, indicated that this condensation occurs at an early stage in the biosynthesis. This idea is based primarily on the lack of incorporation of a series of non-isoprenylated precursors, which based on their structures would be expected to be precursors to CQ. The most important of these was homogentisic acid, a well known metabolite of tyrosine, and the compound that was considered to be the most logical precursor to CQ.

On the basis of the characterized metabolism of tyrosine and fluorotyrosine in these cells, and the demonstration through labeling experiments, that only tyrosine or the first intermediate in its metabolism, *p*-hydroxyphenylpyruvate, were precursors to the benzothiophene quinone ring of CQ. It was concluded that the aromatic compound that condenses with the farnesylfarnesyl pyrophosphate was either tyrosine or *p*-hydroxyphenylpyruvate. Confirmation of this step in the pathway as well as the other steps indicated in Figure 31 will require the isolation and identification of all the proposed intermediates.

The introduction of the thiomethyl group is proposed to occur at next to the last step in the biosynthesis of CQ. This idea is based primarily on the occurrence of tricycloquinone in *Sulfolobus* and the consideration that its biosynthesis would most likely require the intermediacy of a thiolated isoprenylated benzothiophene quinone with an unsaturated side chain. This intermediate could then either be cyclized to the dithiophene quinone ring present in the tricycloquinone or be methylated and reduced to CQ. However, regardless of exactly which steps are involved in the biosynthesis, the data clearly indicates that the thiomethyl group is generated in two steps and that the sulfur originates from cysteine. This important thiolated intermediate could possibly be traped and identified in lipid extracts using the wide range of thiol specific reagents which are currently available.

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1. D. Zhou and R. H. White (1989) Biosynthesis of caldariellaquinone in *Sulfolobus* spp. **J. Bacteriol.** 171:6610-6616.

2. D. Zhou and R. H. White (1990) Biosynthesis of the methylthiol side chain of caldariellaquinone. **J. Chem. Soc. Perkin Trans I.** 1990:2346-2348.

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6. D. Zhou and R. H. White. The metabolisms of DL-tyrosine and DL-2- and 3-fluoro-tyrosine in *Sulfolobus*. Submitted for publication.