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Biosynthesis of the Sulfonolipid 2-Amino-3-Hydroxy-15-Methylhexadecane-1-Sulfonic Acid in the Gliding Bacterium *Cytophaga johnsonae*

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The biosynthesis of the sulfonolipid 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid (capnine) was studied by measuring the incorporation of possible precursors into the lipid by cells grown in the presence of precursors which were labeled with stable isotopes. Cells grown on yeast extract in the presence of DL-[3,3-$^2H_2$]serine contained 40.1 mol% of the protein-bound serine and 5.0 mol% of the protein-bound cysteine derived from the labeled serine. Cells grown in the presence of DL-[3,3-$^2H_2$]cystine contained labeled L-cysteine in which 48.4 mol% of the protein-bound cysteine was derived from the exogenous cystine. In both cases, no label was found in the capnine. Cells grown in the presence of L-[*sulfonic*-$^{18}O_3$]cysteic acid, however, incorporated the cysteic acid into the sulfonolipid with little or no isotopic dilution. Capnine isolated from cells grown in the presence of DL-[3,3-$^2H_2$]cysteic acid contained 86.4 mol% of the molecules that had two deuteriums. These results are consistent with the possibility that biosynthesis of capnine occurs by the condensation of 13-methylmyristoyl-coenzyme A with cysteic acid, in a reaction analogous to the condensation of a palmitoyl-coenzyme A with serine to form 3-keto-sphinganine during the biosynthesis of sphingolipids.

Only a limited number of naturally occurring sulfonic acids have been described. Among these are coenzyme M, a coenzyme involved in the microbiological formation of methane (32); taurine, a component of the bile salt taurocholic acid (18); isethionate, a possible neurotransmitter (27) found in high concentrations (>100 mM) in cephalopod nerves (19, 28); sulfoacetaldehyde, an intermediate in the bacterial metabolism of taurine (21, 29); cysteic acid, an oxidative metabolite of cysteine produced by both bacteria (31) and mammals (30); a series of sulfonated carbohydrates, 3-sulfolactaldehyde, sulfolactate, and sulfopropanediol, believed to be involved in the biosynthesis of 6-sulfoquinovose (A. A. Benson and I. Shibuya, Fed. Proc. **20**:79, 1961), a sugar component of the sulfolipids present in chloroplasts (6), certain pseudomonads (36) and *Bacillus acidocaldarius* (23); and the sulfonolipids which have been described in the gliding bacteria (16, 17, 17a) and the nonphotosynthetic diatom *Nitzschia alba* (2). Although it is clear that these sulfonic acids serve a wide range of functions, they all may, in fact, be biosynthetically related (Fig. 1). The work presented in this paper was done to determine whether cysteic acid is involved in the biosynthesis of 2-amino-3 hydroxy-15-methylhexadecane-1-sulfonic acid (capnine). The results clearly indicate that cysteic acid is a direct precursor of the sulfonolipid capnine, which is produced by cultures of the bacterium *Cytophaga johnsonae*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. johnsonae* (ATCC 17061) was maintained at 30°C on agar slats containing 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, and 10 g of yeast extract per liter of tap water. The pH was adjusted to between 7.0 and 7.2. After 24 h of growth, cells were transferred from a slant directly to 200 ml of the same medium containing the labeled compounds at a concentration of 0.3 mg/ml.

Isolation of capnine. At the end of log-phase growth (~24 h), the cells were removed from the growth medium by centrifugation (10,000 × *g*) for 15 min, and the resulting cell pellet was extracted with methylene chloride-methanol (1:1) by using 4 ml of solvent per g (wet weight) of cells. After 30 min at room temperature, the suspended cells were removed by centrifugation, and the resulting cell pellet was extracted a second time with the same volume of solvent. The combined extracts were then shaken with 0.5 volume of water, and, after phase separation, the methylene chloride layer was removed and evaporated under a stream of nitrogen. The resulting lipids were heated at 70°C for 24 h with aqueous methanolic HCl (~0.5 ml of solvent per mg of lipid) to effect methanolysis of the *N*-acylaminosulfonates (17, 17a). The aqueous methanolic HCl was prepared by diluting a solution containing 8.6 ml of concentrated HCl and 9.4 ml of water to 100 ml with methanol as described by Gaver and Sweeley (15). After heating, the solution was cooled, extracted twice with 0.1 volume of pentane, and mixed with equal volumes of methylene chloride and water. The resulting emulsion was shaken and centrifuged to clarify the methylene chloride layer. The capnine was then isolated from the methylene chloride layer by evaporation with a stream of nitrogen at 90°C and was purified from the resulting residue by preparative thin-layer chromatography on Silica Gel G with a solvent system composed of chloroform–methanol–7 N aqueous ammonia (70:30:5 [vol/vol]), as described by Godchaux and Leadbetter (16). The thin-layer chromatography spot was identified by its positive ninhydrin color, its absorption of iodine vapor, and its R_f (16). Identification of the isolated material as capnine was based on a comparison of the mass spectrum of the *N,O*-diacetyl methyl ester derivative with the spectrum found for the same capnine derivative (17a).

Derivative formation and mass spectrometry of capnine. The purified capnine (see above) derived from 1 to 3 g (wet weight) of cells was treated with 100 μl of pyridine and 100 μl of acetic anhydride at 70°C for 2 h. After evaporation of the solvent with a stream of nitrogen at 70°C, the resulting *N,O*-diacetylated capnine was dissolved in a small volume of methanol and passed through a small column (0.8 by 8 mm)

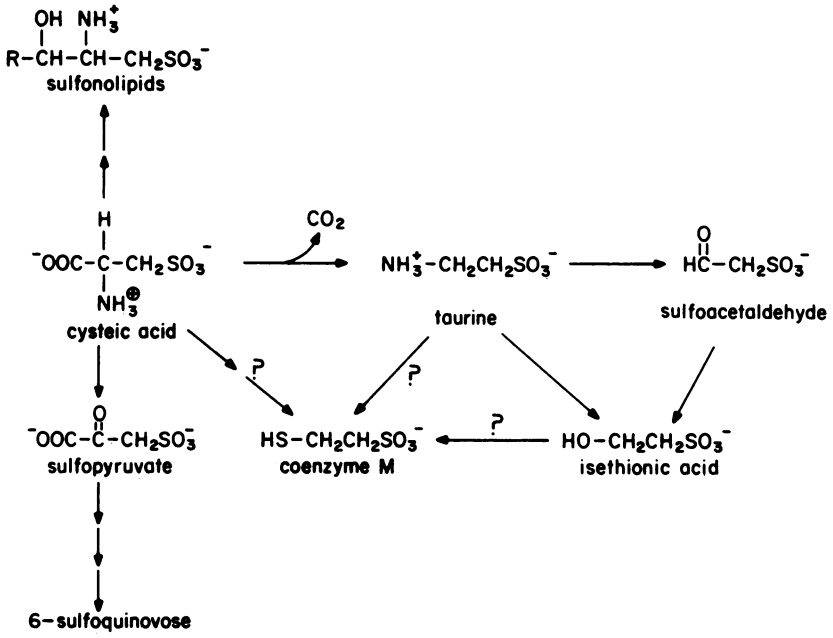


FIG. 1. Possible metabolic relationships between the naturally occurring sulfonic acids.

of Dowex 50 H^+ previously washed with methanol. The Dowex 50 H^+ treatment removed the yellow color generated during the acetylation reaction and converted the sulfonate to the free acid. Treatment of the resulting methanolic solution with a slight excess of diazomethane in diethyl ether converted the sulfonic acid to the methyl ester. The resulting solution containing the *N,O*-diacetyl methyl ester derivatives was then evaporated, and its mass spectrum was obtained by direct insertion with a Varian MAT 112 mass spectrometer operating at an ionization voltage of 70 eV and a source temperature of 250°C. The incorporation of label was measured with ions in the mass spectrum having mass-to-charge ratios *m/z* of 138, 163, 181, 223, and 294 (Godchaux and Leadbetter, in press). These ions were chosen because they were the most intense and they contained the labeled atoms of interest. The results of analyses of isotopic incorporation into each of these ions, averaged from three to five separate mass spectra, agreed to within 1 to 2%.

Isotopic analysis of the bound cellular serine and cysteine. The amount of deuterium incorporated into the bound cellular serine and cysteine was determined by the gas chromatographic-mass spectrometric analysis of volatile derivatives of the amino acids, which were prepared from the cell pellet after the methylene chloride-methanol extraction as previously described (34, 35). These procedures assured that any free cysteine or serine present in the medium or cells would not interfere with the analysis of the isotopic abundance of the cysteine and serine metabolized by the cells.

Synthesis of DL-[3,3-$^2H_2$]cystine. The labeled cystine was prepared as described by Atkinson et al. (4). The labeled formaldehyde solution was obtained by heating deuterated paraformaldehyde in dilute HCl.

Synthesis of DL-[3,3-$^2H_2$] cysteic acid. The above DL-[3,3-$^2H_2$]cystine was oxidized as described below for the preparation of L-[*sulfonic*-$^{18}O_3$]cysteic acid.

Synthesis of L-[*sulfonic*-$^{18}O_3$]cysteic acid. L-Cystine was oxidized with bromine in the presence of ^{18}O-labeled water (10.0 atom% ^{18}O), as described by Clarke and Inouye (12). After removal of the labeled water in vacuo, the resulting L-cysteic acid was purified by crystallization from ethanol-water. The extent of labeling of the sulfonic acid group of the cysteic acid was measured from the *m/z* 190 (M^+-$CO_2Si(CH_3)_3$ in the 70 eV mass spectrum of the *O,O*-ditrimethylsilyl derivative, which was prepared by reacting the cysteic acid with 1-(trimethylsilyl)imidazole as described by Eagles and Knowles (14). Since this fragment ion contains only the sulfonic acid oxygens of the cysteic acid, it specifically measures the ^{18}O incorporated into the sulfonic acid during the synthesis. Subjecting the labeled material to the methanolysis conditions used to cleave the *N*-acylated capnines resulted in no detectable exchange of ^{18}O.

Labeled compounds. DL-[3,3-$^2H_2$]serine and [$^2H_2$]paraformaldehyde, both labeled at 98 atom% $^2H_2$, were obtained from Merck & Co., Inc. ^{18}O-labeled water, labeled at 10 atom%, was obtained from Bio-Rad Laboratories, Richmond, Calif.

RESULTS

Growth of *C. johnsonae* with 0.3 mg of DL-[3,3-$^2H_2$]serine per ml resulted in the production of cells in which the bound serine was labeled to the extent that it contained 40.9 mol% of the molecules that had two deuteriums. The cysteine in these same cells contained 5.2 mol%, and the capnine contained <2 mol% of the molecules that had two deuteriums. The serine and capnine isolated from cells grown with DL-[3,3-$^2H_2$]cystine contained less than 0.5 mol%, and the cysteine contained 48.4 mol% of the molecules that had two deuteriums. Capnine isolated from cells grown in the presence of 0.3 mg of L-[*sulfonic*-$^{18}O_3$]cysteic acid per ml, however, showed extensive incorporation of ^{18}O into the molecule. This was observed as an increase in the intensity of the ion +2 *m/z* in all fragments containing the sulfonic acid portion of the molecule (Fig. 2). The molar ratio of the molecules containing one ^{18}O to those containing no ^{18}O was ~27% in both the labeled cysteic acid and the isolated capnine, indicating that the cysteic acid is incorporated into the capnine with no exchange of the oxygen label or dilution of the cysteic acid. The extent to which the capnine was

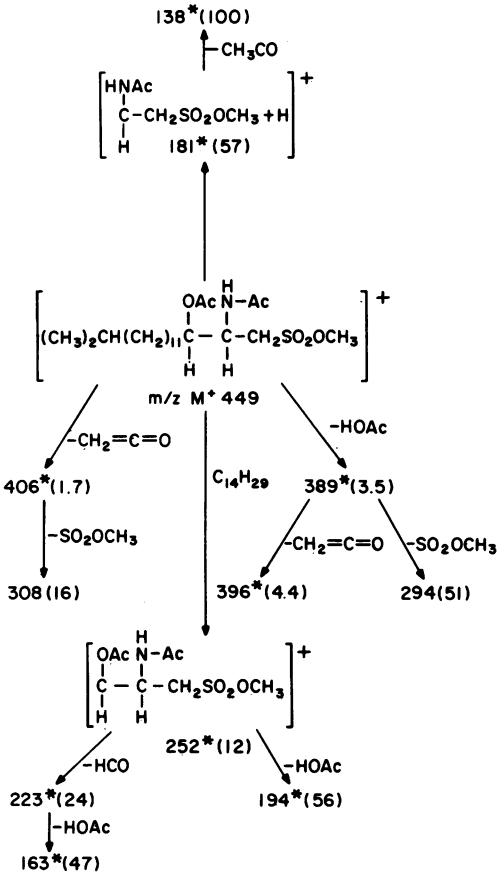
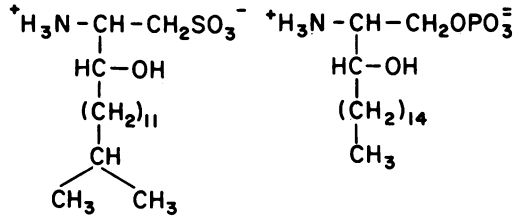


FIG. 2. Fragmentation of the *N,O*diacetylcapnine methyl ester upon electron impact ionization at 70 eV. Numbers represent the mass-to-charge ratios (*m/z*) of some of the principal fragments produced. The assignment of this fragmentation pattern is based on the observed nominal masses of the fragments, the presence of ^{34}S isotope ions in the fragments, the expected fragmentation of the derivative (10), and the retention of label in the capnine derivative prepared from biosynthetically produced capnine containing ^{18}O in the sulfonic acid and from biosynthetically produced capnine containing deuterium at C-1. Those fragments containing the sulfonic acid oxygens and ^{34}S are indicated by asterisks. The base peak for the ions observed above 100 *m/z* was at 128 *m/z* (data not shown).

labeled was clearly observable in all the fragments containing the sulfonic acid group (Fig. 2). Measurement of the intensities of the ion and ion +2 *m/z* for the most intense ions at 138, 163, 181, and 223 *m/z* gave an average value for the +2 ion of 26.7%. Ions at +4 *m/z* and +6 *m/z* were also observed to have the intensities expected for molecules containing two and three ^{18}O, respectively. Growth of the cells in the presence of 0.3 mg of DL-[3,3-$^2H_2$]cysteic acid per ml also led to the incorporation of label into the capnine. In this case, two deuteriums were incorporated into the ions with *m/z* values of 138, 163, 181, 223 and 294, with an average value for all the ions of 86.4 mol%. From the postulated fragmentation pattern and the occurrence of ions in the 181-*m/z* fragment, it is clear that the incorporated deuteriums reside on C-1 of the capnine.

DISCUSSION

The structure of capnine is remarkably similar to that of sphinganine phosphate (dihydrosphingosine phosphate), a central component of the sphingomyelins (16; Fig. 3). Their



capnine sphinganine phosphate

FIG. 3. Chemical structures of capnine and sphinganine phosphate.

structural similarity is augmented by the fact that both of these molecules exist naturally as their *N*-acylated derivatives of fatty or hydroxy fatty acids (16, 17, 25). The principal difference between these molecules is the substitution of an anionic sulfonic acid in capnine for the anionic phosphate ester in sphinganine phosphate.

Considering this structural similarity, it is very likely that capnine is biosynthesized by a reaction analogous to that in sphinganine biosynthesis (16). Sphinganine is known to be formed in both mouse brain tissue (8) and in yeasts (9, 22) by the pyridoxal phosphate-dependent condensation of serine with palmitoyl-coenzyme A to form 3-ketosphinganine. The 3-ketosphinganine is then reduced in an NADPH-dependent reaction to sphinganine. Using these reactions as a model, one can conceive of three possible reactions whereby the carbon framework of capnine could be formed. Each of these reactions would involve the condensation of 13-methylmyristoyl-coenzyme A with the pyridoxal complex which could be formed with either cysteine, serine, or cysteic acid. These are represented by reactions a, b, and c, respectively, in Fig. 4. The reaction products derived from cysteine or serine would require several steps to convert their initial condensation products, I and II, respectively, into capnine (Fig. 4). The cysteic acid condensation product III, however, would yield capnine by a simple reduction of its keto group.

The selection of reaction c, the most direct reaction, as the one most likely to be operating in *C. johnsonae* is based on the following observations. Reaction b is clearly eliminated from consideration because cells grown with the labeled serine have 40.9% of their protein serine residues labeled with deuterium, yet no deuterium was found in the capnine. Reaction a can also be eliminated since cells grown with DL-[3,3-$^2H_2$]cystine were found to contain 48.4 mol% of the molecules that had two deuteriums, but, again, the capnine was not labeled. These results confirm that cysteine and serine are readily utilized by these cells but that neither of the molecules is converted directly into capnine by this organism. The lack of involvement of cysteine as a precursor of capnine was first reported by Godchaux and Leadbetter based on experiments which showed that inorganic sulfate was incorporated into capnine but not into cellular protein (16). L-[*sulfonic*-$^{18}O_3$]cysteic acid was incorporated with no dilution of the ^{18}O label. Since sulfur dioxide readily exchanges its oxygen with water (7), this proves that the carbon-sulfur bond of cysteic acid remains intact during the biosynthesis and that the biosynthetic unit derived from cysteic acid is incorporated without dilution. In addition, the efficient incorporation of DL-[3,3-$^2H_2$]cystine clearly demonstrates that the C-2 and C-3 carbons and, most likely, the nitrogen of the cysteic acid are also incorporated. Thus,

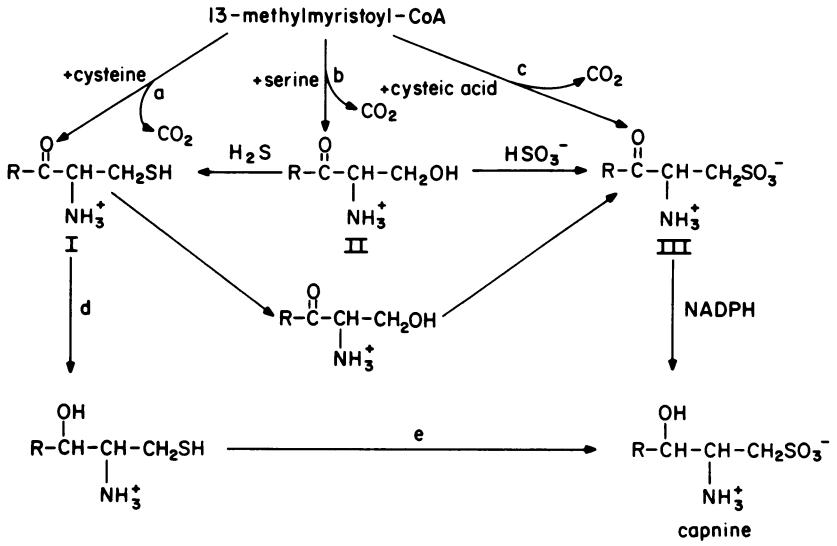


FIG. 4. Possible routes for the formation of capnine from cysteine, serine, and cysteic acid. The initial condensation reactions (a, b, and c) are patterned after the known biosynthesis of sphinganine (22).

reaction c most likely accounts for all of the capnine produced.

These results run counter to the pathway proposed by Anderson et al. (3) for the biosynthesis of the deoxyceramide sulfonate produced by the diatom *Nitzschia alba*. Based on L-[^{34}S]cysteine and L-[^{34}S]cystine incorporation studies, Anderson et al. proposed that cysteine condensed with palmitoyl-coenzyme A to form a 3-keto intermediate I (Fig. 4, reaction a). Reduction of the keto group and subsequent oxidation of the thiol finally gave the sulfonolipid (Fig. 4, reactions d and e). Their experimental data did not, however, exclude the oxidation of the cysteine or cystine to cysteic acid or the incorporation of the cysteine sulfur into cysteic acid by a less-direct route before the condensation of the cysteic acid with palmitoyl-coenzyme A. This leaves open the possibility that the biosynthesis of the deoxyceramide sulfonate in *Nitzschia alba* involves the condensation of cysteic acid with an acyl-coenzyme A derivative, which is basically the same as the pathway proposed herein for capnine biosynthesis.

Being aerobic, *C. johnsonae* could readily form cysteic acid from cysteine by a two-step reaction sequence. This possible route would involve the conversion of cysteine to cysteine sulfinic acid, catalyzed by the enzyme cysteine dioxygenase (26), followed by a subsequent oxidation of the cysteine sulfinic acid to cysteic acid. This biosynthetic route, however, can be eliminated in the case of *C. johnsonae* since the labeled cysteine present in these cells is not incorporated into capnine. The oxygen-dependent oxidation of cysteine to cysteic acid is also eliminated as a possible reaction involved in capnine biosynthesis in bacteria of the genus *Capnocytophaga* since these aerotolerant organisms are also able to produce capnine when grown under anaerobic conditions (16, 24). However, since oxygen-nondependent pathways in bacteria for the oxidation of sulfide have been described (1, 5), there is no chemical reason to prevent a similar anaerobic oxidation of cysteine to cysteic acid from operating in these organisms or in other organisms which produce sulfonolipids (20).

Alternatively, the cysteic acid could be formed by the condensation of sulfite with a suitable acceptor, the sulfite being generated either by the oxidation of sulfide or the reduction of sulfate. Suitable acceptors include pyruvate, which, in conjunction with glutamate, has been shown by Chapeville and Fromageot (11) to produce cysteic acid or phosphoenolpyruvate which may react with sulfite to form 2-phospho-3-sulfolactate (13). This product could then be converted into cysteic acid by undergoing a dephosphorylation and oxidation followed by a transamination of the resulting 3-sulfopyruvate. It is clear that the acceptor could not be cysteine, which is known to be converted into cysteic acid by cysteine lyase (33), or an α-aminoacrylic acid compound generated either from cysteine, serine, or a product derived directly from these, i.e., O-acetylserine.

Determining the exact pathway used by these bacteria to produce cysteic acid may not only establish a new pathway for cysteic acid biosynthesis but may also provide insight into how other bacteria produce sulfonic acid-containing compounds, i.e., coenzyme M in the methanogens.

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