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Microbial Desulfonation of Substituted Naphthalenesulfonic Acids and Benzenesulfonic Acids

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Sulfur-limited batch enrichment cultures containing one of nine multisubstituted naphthalenesulfonates and an inoculum from sewage yielded several taxa of bacteria which could quantitatively utilize 19 sulfonated aromatic compounds as the sole sulfur source for growth. Growth yields were about 4 kg of protein per mol of sulfur. Specific degradation rates were about 4 to 14 μkat/kg of protein. A *Pseudomonas* sp., an *Arthrobacter* sp., and an unidentified bacterium were examined. Each desulfonated at least 16 aromatic compounds, none of which served as a carbon source. *Pseudomonas* sp. strain S-313 converted 1-naphthalenesulfonic acid, 2-naphthalenesulfonic acid, 5-amino-1-naphthalenesulfonic acid, benzenesulfonic acid, and 3-aminobenzenesulfonic acid to 1-naphthol, 2-naphthol, 5-amino-1-naphthol, phenol, and 3-aminophenol, respectively. Experiments with $^{18}O_2$ showed that the hydroxyl group was derived from molecular oxygen.

Sulfonated aromatics (hard detergents) were the first compounds to impress on the general public the problems of the nonbiodegradability of some xenobiotic compounds (4). Sulfonated aromatics (e.g., naphthalenesulfonates) are still major pollutants, representing some 10% of the pollution load in the river Rhine (15).

Naphthalenesulfonates and their substituted analogs have been termed nonbiodegradable (2), and the xenobiotic character of the aromatic sulfonic group is known (14). However, selections in continuous culture have yielded organisms capable of quantitatively mineralizing 1-naphthalenesulfonate (NS-1) and 2-naphthalenesulfonate (NS-2) (3), and batch enrichment cultures yielded organisms capable of utilizing 2-amino-1-naphthalenesulfonate (17, 18) and some other substituted naphthalenesulfonates (16) as carbon sources. There are thus a few organisms able to desulfonate sulfonoaromatics, and these bacteria have narrow substrate ranges.

We observed that prometryne [*N*,*N*′-bis(1-methylethyl)-6-methylthio-1,3,5-triazine-2,4-diamine] is utilized by bacteria as a sole and growth-limiting source of sulfur (6, 8). By analogy, we decided to test the hypothesis that the sulfur of naphthalenesulfonates is available to microorganisms present in the environment. We now report that benzenesulfonates and naphthalenesulfonates serve readily and quantitatively as sulfur sources for the growth of bacteria with a broad substrate range and that oxygenolytic cleavage of the sulfonate group occurs.

MATERIALS AND METHODS

Chemicals. Sodium NS-1 and sodium NS-2 were purchased from Fluka (Buchs, Switzerland). Peri acid (as sodium 8-amino-1-naphthalenesulfonate), Laurent's acid (as sodium 5-amino-1-naphthalenesulfonate), Tobias acid (2-amino-1-naphthalenesulfonic acid), J acid (7-amino-4-hydroxy-2-naphthalenesulfonic acid), gamma acid (6-amino-4-hydroxy-2-naphthalenesulfonic acid), C acid (as disodium 3-amino-1,5-naphthalenedisulfonate), and H acid (as disodium 4-amino-5-hydroxy-2,7-naphthalenedisulfonate) were provided by CIBA-GEIGY AG (Basel, Switzerland). Most of the sulfonated naphthalenes were contaminated with sulfate ion and organics, both of which were removed by high-pressure liquid chromatography (HPLC). Compounds were made to 10 mM in 10 mM potassium phosphate buffer, the pH was readjusted to 7 (to bring some acids into solution), and the solutions were passed through membrane filters (0.2-μm pore diameter) to remove particles. Portions (250 μl) were chromatographed (11), and the peak of authentic material was collected, made to a concentration 1.25 mM, sterilized by passage through a membrane filter (0.2-μm pore diameter), and stored in the dark at 4°C. Samples of 2-naphthalenecarboxylic acid, 1,2-dihydroxy-1,2-dihydro-2-naphthalenecarboxylic acid and pure NS-1 were kindly supplied by H.-J. Knackmuss. 5-Amino-1-naphthol (technical grade) was a gift from U. Meyer. It was recrystallized from water. $^{18}O_2$ (98.6%) was from CEA (Gif-sur-Yvette, France). Oxiferm tubes (Roche, Basel, Switzerland) were used. The sources of other chemicals were described by Thurnheer et al. (21).

Apparatus and analytical methods. HPLC (1), gas chromatography (GC)(12), and mass spectral (MS) (7) analyses were done with apparatus described elsewhere. UV spectra were measured with a Beckman DU-40 spectrophotometer. GC-MS analyses were done with a Carlo Erba Fractovap 2150 GC coupled to a Varian MAT 112 MS (electron impact ionization at 70 eV) equipped with a Finnigan INCOS data system.

HPLC separation of aromatic sulfonates on reversed-phase columns (11) was used and extended to determine naphthols (and phenols) in the presence of aromatic sulfonates by modification of the gradient elution. The first eluent (20 mM potassium phosphate buffer, pH 6.7; 0.7 ml/min) was delivered for 5 min, and a linear gradient was made over 5 min to the second eluent (60% [vol/vol] methanol, 40% [vol/vol] 10 mM potassium phosphate buffer, pH 6.7), which was delivered for 20 min before the initial conditions were recreated. The spectrophotometer was set at 230 nm for analyses with NS-1, NS-2, and Laurent's acid, at 220 nm for NC-2, 1,2-dihydroxy-1,2-dihydro-2-naphthalenecarboxylic acid, benzenesulfonate, and 3-aminobenzenesulfonate, and at 212 nm for phenol and 3-aminophenol. The latter two compounds were eluted isocratically with 100 mM buffer containing 40 and 10% (vol/vol) methanol, respectively.

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Naphthols in ethanol were also analyzed by GC after separation on Tenax in glass columns (1.6 m by 2 mm; 15 ml of N_2 per min, 260°C), and MS of the compounds were examined after separation on SE-52 in a column (20 m by 300 μm; 1.2 ml of He per min, 240°C).

Growth of pseudomonads was quantified as protein in a Lowry-type reaction (8, 13). *Arthrobacter* sp. strain DZ-6 was not solubilized by this treatment, and a more complex workup was necessary (19a) in which, in addition, the NaOH solution contained 0.1% sodium dodecyl sulfate.

Media, isolation of organisms, and quantification of growth. Scrupulously clean glassware was required to prevent spurious growth on contaminant sulfur. The enrichment medium consisted of a simple salts solution (pH 7.2), three carbon sources, trace elements, and a single sulfur source at 30 μM (8). Isolation of strain DZ-6 required a vitamin (19) supplement to the medium. The inocula were from municipal sewage (Werdhölzli, Zürich, Switzerland), sewage pooled from 10 municipal sewage works (21), and industrial sewage (CIBA-GEIGY AG, Schweizerhalle, Switzerland). Enrichment procedures, cultivation, and isolation were done as previously described (8). Cultures were stored at −80°C in 50% glycerol.

Strain DZ-6 was grown in vitamin-containing enrichment medium containing 20 mM glycerol as the carbon source. The medium for strains S-313 and S-832 was 50 mM potassium phosphate buffer (pH 7.2)–19 mM ammonium chloride–0.5 mM magnesium chloride–25 mM succinate–sulfur-free trace elements (21)–30 μM sulfur source. Cultures were grown in screw-cap Erlenmeyer flasks. The ratio of medium/air volumes was 1:5. Inocula (2%, vol/vol) were from a culture grown with NS-2 as the sulfur source. Culture flasks containing substituted aromatic sulfonates were covered with aluminum foil because these compounds and their desulfonated products are light sensitive. Growth yield experiments were done as described by Cook and Hütter (8). Samples for aromatic sulfonates and their metabolites in growth media were centrifuged (20,000 × *g* for 15 min at 4°C), and the supernatant fluid was assayed immediately or after storage at −20°C. Samples were not filtered because naphthols and phenols sorbed on the filter.

Pseudomonas sp. strain A3 (3) was kindly supplied by H.-J. Knackmuss. The organism was grown as previously described (3).

Isolation of metabolites. The polarity of culture supernatant fluid was increased by the addition of analytical grade ammonium sulfate (0.5 g/ml) to improve phase separation. Any precipitate was removed by centrifugation (5,700 × *g*, 20 min). The naphthol (or phenol) in the supernatant fluid was extracted into peroxide-free diethyl ether (0.2 volume), taken to dryness, and recrystallized from water. Crystals were collected and recrystallized from water. Purification was monitored by HPLC, and pure samples were dried under vacuum (0.4×10^{-1} torr [5.33 Pa]) before MS were obtained. Partially purified preparations in ethanol could be used for GC or GC-MS analyses.

Incorporation of oxygen from $^{18}O_2$. Oxygen-free medium in 600-ml screw-cap bottles was prepared by boiling out gases and closing the bottles with gas-tight latex stoppers in screw caps. The gas phase was evacuated and flushed with oxygen-free nitrogen in six cycles. Evacuated bottles were then filled with 80% nitrogen (99.999%) and 20% $^{16}O_2$ or $^{18}O_2$ (98.6%) and autoclaved closed. The sulfur source (final concentration, 30 μM) and inoculum were added aseptically through the stopper with a syringe. Each metabolic product was partially purified and examined by GC-MS.

RESULTS AND DISCUSSION

Enrichment cultures. Three independent series of batch enrichment cultures were prepared. A culture was enriched from municipal sewage (Werdhölzli) to utilize benzenesulfonic acid as the sole source of sulfur, and it was observed to utilize NS-2 also. Two other inocula, one from mixed municipal sewage and one from industrial sewage, were used in media containing one of nine substituted naphthalenesulfonates. Of the 18 enrichments, 15 were positive through three subcultures. Growth of these cultures was complete within 1 week. Thus, it was clear that, however seldom these substances may serve as carbon and energy sources for growth (see Introduction), they are readily utilized as sulfur sources. The degradative microorganisms were present both in municipal sewage, where relatively low exposure to such sulfonates may be anticipated, and in industrial sewage, in which wastes (sulfonates) from dyestuff syntheses were treated.

Isolation and characterization of organisms. The enrichment for organisms to utilize benzenesulfonic acid yielded a coryneform bacterium, strain DZ-6, which was attributed to the genus *Arthrobacter* because of its eight (of eight)-character fit in cluster A of Seiler's key (20). The organism grew prototrophically in serial subculture with sulfate as the sulfur source, but serial subcultures with an aromatic sulfonate as the sulfur source required a vitamin supplement whose nature has not been defined. Strain DZ-6 utilized 18 of the 19 aromatic sulfonates tested as sole sulfur sources but did not utilize any of these compounds as a carbon source.

The enrichments for organisms to utilize substituted naphthalenesulfonates yielded 26 pure bacterial cultures on the basis of turbidity produced in the presence of a naphthalenesulfonate, but when supernatant fluids were examined by HPLC, only eight cultures were observed to catalyze quantitative disappearance of the substrates. Turbidity alone was obviously an inadequate measure of substrate utilization.

Three of the eight degradative organisms originated from municipal sewage, and five strains were derived from industrial sewage. All organisms were motile or nonmotile rods, seven of which were gram negative. Two presumed pseudomonads, strains Z-63 and S-313 (motile, obligately aerobic, oxidase-positive rods), and unidentified strain S-832 were choosen for further study. Strain S-313, which was used extensively, was assigned to the fluorescent pseudomonads because of the pattern of reactions in Oxiferm tubes (key = 2151) and because it contains ubiquinone Q9 (G. Auling, personal communication). Each of these three organisms was observed to have a broad substrate range (Table 1). None of the compounds listed in Table 1 served as a carbon source for these organisms.

The phenotype of utilizing aromatic sulfonates as sources of sulfur was not equally stable in all organisms. Whereas strain DZ-6 retained the character(s) for at least 4 years on nutrient agar plates, some organisms rapidly lost the character if stored under nonselective conditions. We lost the degradative characteristics of strain Z-63 in this manner and thereafter stored organisms for routine use in selective liquid culture. The apparently simultaneous loss of ability to utilize all aromatic sulfonates suggests that only one event was required to lose the phenotype, and we presume that one enzyme (complex) desulfonates many substrates.

Growth physiology. Strain DZ-6 had a molar growth yield of about 4 kg of protein per mol of sulfur (a normal value [6], whether it utilized sulfate or one of the aromatic sulfonates

TABLE 1. Utilization of aromatic sulfonates as sulfur sources by four bacteria

Sulfur source[a]	Quantitative (kg of protein/mol of substrate) or qualitative[b] utilization of sulfur compound for growth			
	DZ-6	S-313	S-832	Z-63
Sulfate	4.3 (0.12)	4.4 (0.35)	4.0	3.0
NS-1	4.6 (0.06)	5.1 (0.04)	+	2.4
NS-2	4.3 (0.07)	3.5 (0.28)	+	2.8
8-Amino-1-naphthalenesulfonate	4.3	+	+	3.6
5-Amino-1-naphthalenesulfonate (NS-4)	3.8	3.8 (0.07)	−	3.2
2-Amino-1-naphthalenesulfonate	3.9	−	−	3.5
7-Amino-4-hydroxy-2-naphthalenesulfonate	+	+	+	3.5
6-Amino-4-hydroxy-2-naphthalenesulfonate	3.9	+	+	−
3-Amino-1,5-naphthalenedisulfonate (NS-8)[c]	+	9.4	+	−
4-Amino-5-hydroxy-2,7-naphthalenedisulfonate (NS-9)[c]	+	4.0	+	−
4-Aminobenzenesulfonamide	−	−	−	ND[d]
Benzenesulfonate (BS)	+	4.0 (0.18)	+	ND
3-Aminobenzenesulfonate (MS)	+	4.0 (0.28)	+	ND
2-Aminobenzenesulfonate	+	+	+	ND
4-Aminobenzenesulfonate	+	+	+	ND
4-Methylbenzenesulfonate	+	+	+	ND
4-Hydroxybenzenesulfonate	+	+	+	ND
3-Nitrobenzenesulfonate	+	+	+	ND
4-Sulfobenzoate	+	+	+	ND
5-Sulfosalicylate	+	+	+	ND

[a] The aromatic sulfonates were stable under the experimental conditions used.

[b] Utilization (+) required both growth (development of turbidity) and substrate disappearance (HPLC). Failure to grow is shown by (−). Specific growth rates ($hour^{-1}$) are shown in parentheses.

[c] Disulfonic acids. These compounds chromatographed with several metabolic products also seen with sulfate as the sulfur source, and their behavior could not be monitored with this background.

[d] ND, Not determined.

tested (Table 1). Utilization of the sulfonate was quantitative (>99%), so sulfonate sulfur was as accessible to the organism as sulfate. Strain DZ-6 grew slowly: $\mu = 0.12$ and 0.07 h^{-1} in sulfate-glycerol salts medium and NS-2–glycerol salts medium, respectively. The specific degradation rate for NS-2 during growth was calculated to be about 4 μkat/kg of protein, which is some 2 orders of magnitude lower than the rate of *Pseudomonas* sp. strain A3 utilizing NS-2 as a carbon source (3). The advantage of a wide substrate range compared with that of strain A3 is accompanied by a low specific activity.

Strain S-313 grew more rapidly than strain DZ-6 (Table 1), with correspondingly higher specific degradation rates (14 μkat/kg of protein for NS-2). The organism could utilize at least two substituted naphthalenedisulfonates (NS-8 and NS-9; Table 1). The growth yields imply that both sulfono groups from NS-8 were utilized for growth, whereas only one sulfono group from NS-9 was available.

The growth medium supported a molar growth of 4 kg of protein per mol of sulfur up to about 80 μM sulfate or about 40 μM sulfonate. At higher concentrations of sulfonate, excess sulfonate was not degraded and remained in the growth medium. The enzyme (system) seemed to be active only in growing cells. If 0 to 80 μM sulfate and e.g., 30 μM NS-2 were present together in growth medium, the sulfate was obviously used preferentially (Fig. 1). At high sulfate concentrations, no NS-2 was metabolized; at intermediate sulfate levels, a portion of the sulfonate was degraded to product; while at low sulfate concentrations, all the sulfonate was degraded. We presume that the desulfonation system is repressed by sulfate.

Accumulation and identification of desulfonated metabolites. Each aromatic sulfonate was utilized as a source of sulfur only. Thus, at least one carbon-containing compound was presumed to accumulate in the culture concomitant with utilization of the carbon source.

Cultures of strain DZ-6, S-313, or S-832 were grown to the stationary phase with sulfate ion or one of several naphthalenesulfonates as the source of sulfur, and the supernatant fluid was subject to gradient elution from a reversed-phase column. Although some cases of release of many products were observed (e.g., strain DZ-6 utilizing NS-1 and NS-2), six cultures were found to contain a major product. Strains S-313 and S-832 each formed the same single product (cochromatography, identical UV spectra; Table 2) from NS-1, and each formed a different single product (cochromatography, UV spectra; Table 2) from NS-2. Strains S-313 and DZ-6 yielded yet another single product from 5-amino-1-naphthalenesulfonate (cochromatography, UV spectra; Table 2). Strain S-313 was then used for more detailed studies.

Each compound was rigorously identified (Table 2). Thus, 1-naphthol was formed from NS-1, 2-naphthol was formed from NS-2, and 5-amino-1-naphthol was made from 5-amino-1-naphthalenesulfonate. The same mechanism was observed when phenyl derivatives were utilized as sulfur sources: phenol was formed from benzenesulfonate, and 3-aminophenol came from 3-aminobenzenesulfonate (Table 2).

Each compound was formed concomitant with substrate utilization, as illustrated in the utilization of 5-amino-1-naphthalenesulfonate, for which the yield of product was close to 100% (Fig. 2). At the end of growth, however, the product was rapidly lost (Fig. 2). This loss was faster with the aminonaphthol (or the aminophenol) than with the unsubstituted naphthol (or phenol); this is presumably due to the chemical instability of these compounds (10, 22). This

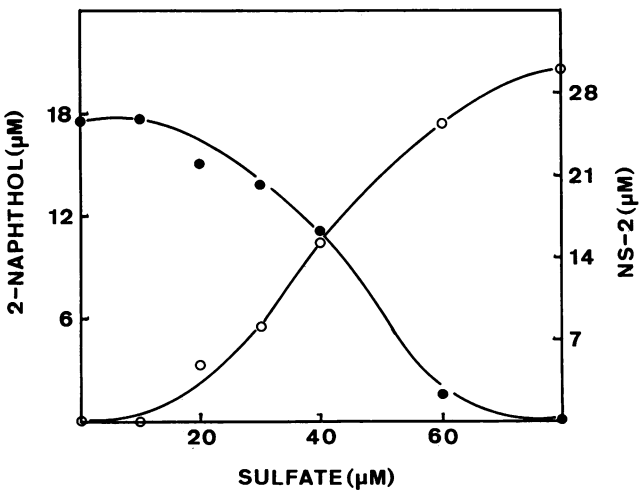

FIG. 1. Utilization of NS-2 by *Pseudomonas* sp. strain S-313 in succinate-salts medium in the presence of various amounts of sulfate. NS-2 (○) and its desulfonated product 2-naphthol (●) were determined after the end of growth (72 h).

TABLE 2. Identification of products released from substituted aromatic sulfonates during growth of *Pseudomonas* sp. strain S-313

Sulfur source[a]	Product	Yield[b] (%)	Cochromatography		UV maxima[e] (nm)	MS or GC-MS[e] (M^+)
			HPLC[c]	GC[d]		
NS-1	1-Naphthol	50	25.6	5.0	210, 292	144
NS-2	2-Naphthol	80	24.4	5.3	235, 272, 330	144
NS-4	5-Amino-1-naphthol	95	15.6	5.6	228, 307	159
BS	Phenol	100	14.6 (40%)	NA[f]	212, 270	NA
MS	3-Aminophenol	70	11.4 (10%)	NA	275	NA

[a] For abbreviations, see Table 1.
[b] The products were in variable degrees unstable. During long incubations the products obviously decayed.
[c] The data are retention times (minutes). Three data points are from gradient elutions, and two are from isocratic elutions for which the methanol content of the eluent is given in parentheses.
[d] The data are retention times (minutes).
[e] Identical results were obtained with authentic material.
[f] NA, Not assayed.

instability of the product is presumed to be one reason for the varying yields of products observed from the various substrates (Table 2).

Oxygenolytic cleavage of the C—S bond. The source of the oxygen atom in 1-naphthol, 2-naphthol, and 5-amino-1-naphthol was examined and found to be molecular oxygen. The molecular ion of each naphthol was increased by 2 units if $^{18}O_2$ was present during growth in place of $^{16}O_2$ (Fig. 3). There was 4 to 10% of the [^{16}O]naphthol in experiments with $^{18}O_2$ which we ascribe to the presence of $^{16}O_2$. Cleavage of the C—S bond was thus oxygenolytic.

The mechanism of this reaction has not been established. Brilon et al. (3) have evidence for dioxygenolytic cleavage of the C—S bond of NS-2 in strain A3, i.e., the formation of 1,2-dihydroxy-1,2-dihydro-2-naphthalenecarboxylic acid by whole cells from the substrate analog 2-naphthalenecarboxylic acid. We observed 1,2-dihydroxy-1,2-dihydro-2-naphthalenecarboxylic acid to be formed from 2-naphthalenecarboxylic acid by whole cells of the *Pseudomonas* sp. strain A3 of Brilon et al. (3), but strain S-313 did not transform 2-naphthalenecarboxylic acid, while utilizing NS-2 and releasing 2-naphthol. Specificity of transport in strain S-313 could explain the failure of the carboxylate to enter the cell and be metabolized. However, our ability to measure a known dihydrodiol and the stability of dihydrodiols at neutral pH values (9) suggest that our observation of naphthols and phenols is not due to the quantitative decay of dihydrodiols and that no dioxygenase is involved in C—S bond cleavage in strain S-313. A second potential mechanism of naphthol formation is via an epoxide intermediate, with which a mixture of 1-naphthol and 2-naphthol follows (5). We observed a single naphthol product (i.e., there was <0.1% 1-naphthol in 2-naphthol and <0.1% 2-naphthol in 1-naphthol), so we do not anticipate an epoxide intermediate in strain S-313. A third potential mechanism of naphthol formation is via a monooxygenase, and a toluene monooxygenase from *Pseudomonas* is known (G. M. Whited, L. D. Kwart, and D. T. Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K168, p. 199). We postulate desulfonation by a monooxygenase of broad substrate specificity, and we hope to obtain cell-free preparations of strain S-313 to test this hypothesis.

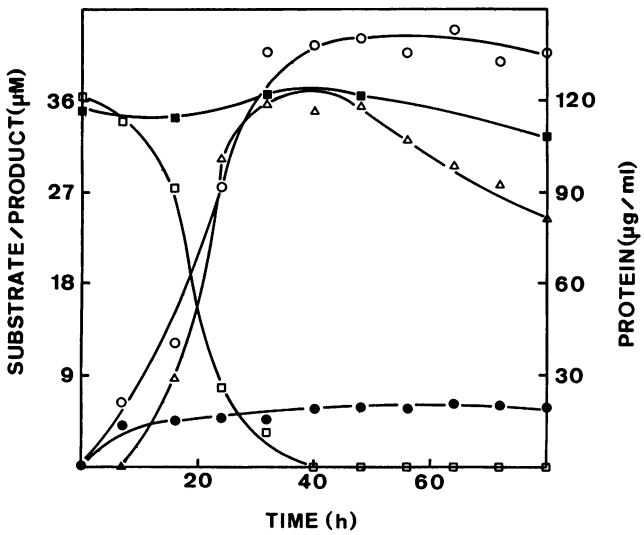

FIG. 2. Growth of *Pseudomonas* sp. strain S-313 with 5-amino-1-naphthalenesulfonate as the sulfur source in succinate-salts medium. Symbols: □, 5-amino-1-naphthalenesulfonate; ■, 5-amino-1-naphthalenesulfonate in sterile control; △, 5-amino-1-naphthol; ○, protein; ●, protein synthesis without added sulfur.

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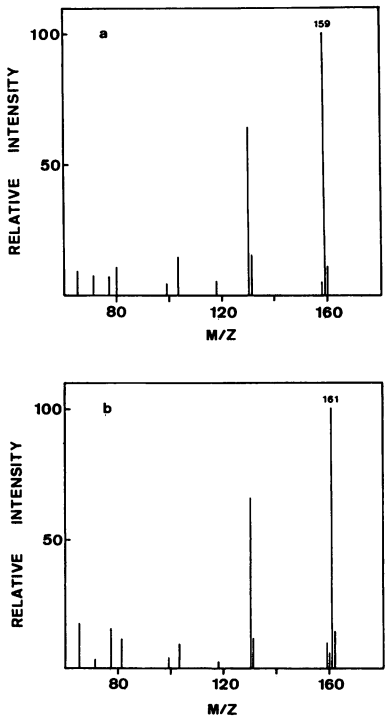

FIG. 3. MS identification of 5-amino-1-naphthol produced from 5-amino-1-naphthalenesulfonate by *Pseudomonas* sp. strain S-313. The product in panel a was obtained from a culture growing in the presence of $^{16}O_2$, and the spectrum is identical with that of authentic 5-amino-1-naphthol. The product in panel b was obtained from a culture growing in the presence of $^{18}O_2$, and the higher molecular weight indicates incorporation of one atom from molecular oxygen.

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