

Identification of a Carboxylic Acid Metabolite from the Catabolism of Fluoranthene by a *Mycobacterium* sp.

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A *Mycobacterium* sp. previously isolated from oil-contaminated estuarine sediments was capable of extensively mineralizing the high-molecular-weight polycyclic aromatic hydrocarbon fluoranthene. A carboxylic acid metabolite accumulated and was isolated by thin-layer and high-pressure liquid chromatographic analyses of ethyl acetate extracts from acidified culture media. The metabolite reached a maximum concentration of approximately 0.65% after 24 h of incubation. On the basis of comparisons with authentic compound in which we used UV and fluorescence spectrophotometry and R_f values, as well as mass spectral and proton and carbon nuclear magnetic resonance spectral analyses, the metabolite was identified as 9-fluorenone-1-carboxylic acid. This is the first report in a microbial system of a fluoranthene metabolite in which significant degradation of one of the aromatic rings has occurred.

Fluoranthene (FA), a tetracyclic polycyclic aromatic hydrocarbon (PAH), is commonly found as a principal component in PAH-contaminated sediments, which can originate from petrogenic and pyrogenic sources (9). FA occurs in high concentrations in urban environments as a product of incomplete combustion of fossil fuels (9, 18). Since PAHs such as FA have been shown to be cytotoxic, mutagenic, and potentially carcinogenic (4-6, 18-21, 23, 27-30) and are ubiquitous in the environment, there is interest in the environmental fate and bioremediation of PAH-contaminated sediments.

Many microorganisms capable of degrading PAHs containing up to three rings have been isolated (8, 12); however, considerably less is known about the ability of microorganisms to metabolize the larger, more recalcitrant molecules. Studies of FA metabolism in eukaryotic systems have been described previously (1, 2, 26). Bacteria capable of PAH degradation, including some bacteria that are able to oxidize or co-oxidize FA, have been isolated (3, 7, 10, 11, 13-15, 24, 25, 31). Mueller and colleagues isolated a strain of *Pseudomonas paucimoblis*, strain EPA505, which can utilize FA as a sole source of carbon and energy (24) from a bacterial consortium previously enriched from creosote-contaminated soil (25).

Previously, we described the isolation of a *Mycobacterium* sp. with the ability to co-oxidize high-molecular-weight PAHs (11, 13-15). The metabolism of pyrene by this *Mycobacterium* sp. has been studied in detail (15). Recently, we reported that this bacterium extensively mineralizes FA (22). In addition, incubation of the *Mycobacterium* sp. with soil and river water enhanced mineralization of FA by 92.7% compared with mineralization by the indigenous microbiota (22). Until now, no metabolites from bacterial degradation of FA have been identified. In this paper, we report the isolation and characterization of a carboxylic acid metabolite resulting from the oxidation of FA by the *Mycobacterium* sp.

MATERIALS AND METHODS

Chemicals. [3- 14 C]FA (54.8 mCi/mmol) with a radiochemical purity of >98% was purchased from Chemsyn Science

Laboratories, Lenexa, Kans. Unlabeled FA was purchased from Fluka AG, Buchs, Switzerland. 9-Fluorenone-1-carboxylic acid was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Bacterial media and reagents were purchased from Difco Laboratories, Detroit, Mich.; all of the solvents and chemicals used were of the highest purity available.

Mineralization of FA. The organism and growth conditions used have been described in detail previously (22). FA mineralization (14 CO₂ evolution) by the *Mycobacterium* sp. was monitored in eight replicate flowthrough microcosm test systems (13, 17, 22) containing 200 ml of nutrient-supplemented minimal basal salts medium (22) supplemented with [3- 14 C]FA (0.021 μ Ci/ml) and 2.75 μ g of unlabeled FA per ml dissolved in 2.75 μ l of *N,N*-dimethyl-formamide. Volatile organic compounds were trapped with columns containing polyurethane foam and 500 mg of Tenax GC. The 14 CO₂ was trapped in 50 ml of monoethanolamine-ethylene glycol (3:7, vol/vol). Two microcosms without FA and two sterile microcosms (autoclaved) were used as controls to correct for abiotic decomposition and adsorption of FA to cells and glass. Each microcosm was shaken manually three times daily. The methods used for analyses of the trapping agents, culture medium, and bacteria have been described previously (22).

Physical and chemical analyses. Reversed-phase high-pressure liquid chromatographic (HPLC) analysis was performed with two model 100A HPLC pumps (Beckman Instruments Co., Berkeley, Calif.), a 5- μ m C₁₈ Ultrasphere octyldecylsilane column (4.6 mm by 25 cm), and a model 100-40 spectrophotometer (Hitachi Scientific Instruments, Mountain View, Calif.). The mobile phase was a methanol-water (35 to 95% [vol/vol] in methanol, 40 min) linear gradient solvent system maintained at a flow rate of 1.0 ml/min. For the separation of the acidic metabolites, 1% acetic acid was added to the mobile phase. UV absorbance was measured at 254 nm, and peak areas were integrated with an Altex-Shimadzu model C-R1A integrator (Shimadzu Scientific Instruments, Columbia, Md.).

Fractions of the HPLC effluent were collected at 30-s intervals in scintillation vials, mixed with 7.5 ml of Scintisol (Isolab Inc., Akron, Ohio), and analyzed by liquid scintillation spectrometry, using a model 2000CA Tri-Carb liquid

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scintillation analyzer (Packard Instrument Co., Downers Grove, Ill.). UV-visible absorption spectra were determined in methanol by using a Beckman model DU-7 spectrophotometer. HPLC on-line absorbance spectra were obtained by using a model 1040A diode array detector system (Hewlett-Packard Inc., Palo Alto, Calif.) attached to the HPLC system described above. Fluorescence spectroscopy was performed with a model RF5000U spectrophotometer (Shimadzu Scientific, Plano, Tex.) at an excitation λ of 293 nm and an emission λ of 200 to 600 nm by using methanol as the solvent.

Isolation of FA metabolites. For chemical and physical analyses of metabolites, batch cultures were grown in 1,000-ml Erlenmeyer flasks containing 250 ml of nutrient-supplemented mineral basal salts medium (22) and 3 mg of FA. Cultures were maintained in the dark at 24°C on a rotary shaker at 150 rpm. Approximately 6 to 7 liters of medium was required to yield 0.75 to 1 mg of the major acidic metabolite. Metabolites and residual FA were extracted with 3 equal volumes of ethyl acetate, dried with anhydrous Na_2SO_4 , and evaporated to dryness under reduced pressure at 37°C. The aqueous phase was acidified to pH 2.5 with concentrated H_2SO_4 and extracted in the same manner. The residue was dissolved in a small amount of methanol and enriched for metabolites by thin-layer chromatography (500- μm Silica Gel GF plates [20 by 20 cm]), using a benzene-hexane (1:1, vol/vol) solvent system to separate FA metabolites from FA, benzene-ethanol (9:1, vol/vol) for further separation of polar and nonpolar compounds, and a benzene-acetone-acetic acid (85:15:5, vol/vol) solvent system to isolate acidic FA metabolites. The R_f values of the major acidic metabolite and the standard were determined by using Baker-flex Silica Gel IB2-F (J. T. Baker Inc., Phillipsburg, N.J.) analytical (200- μm) plates and the benzene-acetone-acetic acid solvent system.

Thin-layer plates were scanned with a Varian Aerograph model 6000 2-pi thin-layer scanner to locate the bands containing ^{14}C metabolites. The resolved radioactive bands on the thin-layer chromatography plates were isolated and extracted with methanol. The methanol was then evaporated under a gentle stream of argon gas.

For mass spectral analysis, the acid-extractable compound of interest was derivatized by acetylation with acetic anhydride and pyridine and by methylation with diazomethane (16).

The isolated FA metabolite and authentic compound were analyzed by electron impact mass spectrometry, using a direct exposure probe, and by gas chromatography-mass spectrometry, using a model 4023 quadrupole mass spectrometer (Finnigan Mat, San Jose, Calif.). The mass spectrometer was operated at 70 V of electron energy and 270°C for all analyses. In the direct exposure probe electron impact mass spectrometry analyses we used a platinum wire probe and a current programmer (Vacumetrics, (Ventura, Calif.)). In the gas chromatography-mass spectrometry analyses we used a type DB-1 capillary column (0.25 mm [diameter] by 15 m; J&W Scientific, Rancho Cordova, Calif.) and 10 lb/in² of He head pressure. The column temperature was held isothermally at 50°C for 1 min and then programmed to increase to 250°C at a rate of 10°C/min. All analyses were performed with the samples dissolved in acetone.

^1H nuclear magnetic resonance (NMR) spectra were obtained at 500 MHz with a Bruker model AM500 spectrometer at 29°C by using a sweep width of 7 or 1.8 kHz, a data size of 32K, a flip angle of 80°, and a relaxation delay of 0 s, except for spectra recorded under quantitative conditions,

when a 10-s relaxation delay was used. An estimated 1 mg of metabolite was dissolved in 0.6 ml of deuterated methanol. The data were processed with Lorentzian-to-Gaussian resolution enhancement by using Bruker parameters of -0.5 and 0.17. ^1H resonance assignments were based on chemical shift and coupling constant measurements, integrations, the results of selective decoupling experiments, and nuclear Overhauser effect measurements.

^{13}C NMR measurements were recorded with the same instrument at 126 MHz by using the following acquisition conditions: data size, 32K; flip angle, 50°; sweep width, 32 kHz; relaxation delay, 2 s; and number of scans, 28,000. Data were acquired with proton decoupling by using a WALTZ-16 sequence and were processed with an exponential function producing line broadening of 1 Hz. For ^{13}C NMR measurements each sample was dissolved in 150 μl of deuterated methanol and placed in a cylindrical microcell, which was inserted into a 5-mm NMR tube. ^{13}C resonance assignments were made by direct comparison with the authentic standard.

Approximately 5 mg of authentic 9-fluorenone-1-carboxylic acid dissolved in 0.6 ml of deuterated methanol was converted to the carboxylate anion by dropwise addition of 1 M sodium hydroxide until no further change was evident in the ^1H NMR spectrum. ^{13}C resonance assignments were based on the results of direct and long-range two-dimensional heteronuclear chemical shift correlation experiments, which will be described elsewhere. ^1H and ^{13}C chemical shifts are reported below in parts per million; these values were determined by assigning the methanol resonances to 3.30 and 49.0 ppm, respectively.

RESULTS AND DISCUSSION

Isolation and accumulation of metabolites. Mineralization of FA up to 65.5% of the total amount of radioactivity recovered was observed after 9 days of incubation, as discussed previously (22). Accumulation of acid-extractable ethyl acetate-soluble metabolites and water-soluble metabolites increased steadily up to 48 h (22). The radioactivity in neutral extracts decreased rapidly in the first 48 h because of the rapid degradation of FA. Although several FA metabolites were detected in the HPLC analyses (22), one metabolite was isolated from the acidified ethyl acetate-extractable fraction, and its HPLC retention time was 23.8 min. The maximum accumulation of the metabolite occurred at 24 h (Fig. 1) and amounted to 0.65% of the total radioactivity recovered. The level of the metabolite decreased below the level of detection by 336 h. Although this compound was rapidly metabolized by the *Mycobacterium* sp., it was the predominant metabolite and was chemically stable enough for isolation and purification. The metabolite was isolated and purified by preparative thin-layer chromatography and HPLC. The other metabolites were not formed in sufficient quantities for further isolation and structural characterization.

Characterization of the metabolite. The metabolite had UV absorption maxima at λ of 258, 254, and 207 nm (Fig. 1, inset) and showed only slight fluorescence. The UV and fluorescence spectra of the FA metabolite compared with the spectra of FA indicated that considerable metabolism and ring fission of the FA molecule had occurred. The metabolite had an R_f value of 0.71.

The structure of the metabolite was determined by ^1H and ^{13}C NMR spectroscopy. The 500-MHz ^1H NMR spectrum

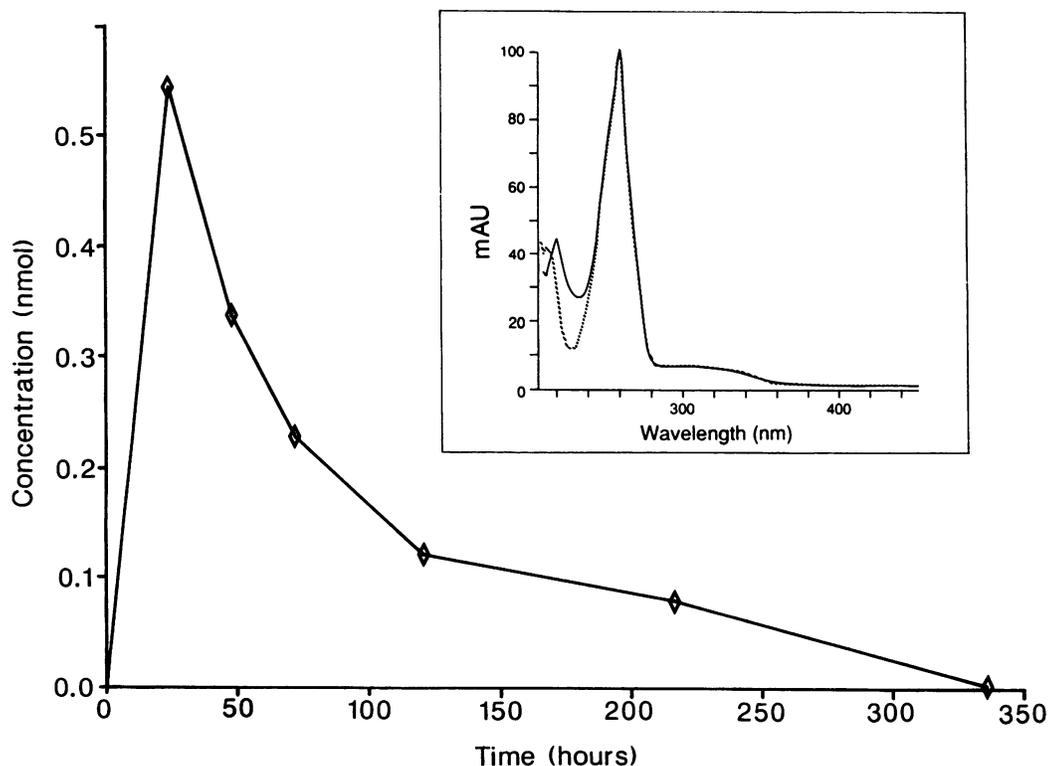


FIG. 1. Formation of an acidic metabolite from FA by cells of the *Mycobacterium* sp. (Inset) UV absorption spectra of the metabolite (—) and authentic 9-fluorenone-1-carboxylic acid (---).

(Fig. 2a) consisted of only seven aromatic resonances, which in conjunction with the results of decoupling experiments indicated the presence of two rings with aromatic protons. A nuclear Overhauser effect for H-4 as a result of selective saturation of H-5, along with the results of selective decoupling experiments, provided evidence that one of the aromatic rings of the FA structure had been substantially altered, but did not by itself enable identification of the metabolite. The ^1H spectrum also showed that the sample was free of aromatic impurities. Since no other information on the carbon skeleton was available and the amount of material was sufficient for conventional ^{13}C NMR measurements, the proton-decoupled ^{13}C NMR spectrum was obtained. The 126-MHz ^{13}C spectrum revealed the seven protonated carbons along with six detectable unprotonated carbons, one of which was well downfield at 194 ppm (Fig. 2b). Two additional relatively large resonances at 180 and 24 ppm were characteristic of the acetate anion, which had been used in the purification process. From this information and a consideration of the effect of possible substituents on chemical shifts, we concluded that the compound may be 9-fluorenone-1-carboxylate. The acid form of this chemical was commercially available. Subsequent analysis of the commercial standard, converted to the anionic form, revealed a virtually identical ^{13}C NMR spectrum. The carboxylic acid resonance (C-10) was not detected in the spectrum of the metabolite because of an unfavorable relaxation time, but this was not unexpected nor did it interfere with the interpretation since the presence of the 1-carboxylate group was confirmed indirectly through all of the other chemical shifts.

Direct exposure probe electron impact mass spectral

analysis of the free acid standard produced a spectrum that was consistent with the structure of 9-fluorenone-1-carboxylic acid, with a molecular ion (M^+) at m/z 224 and fragment ions at m/z 180 ($\text{M}^+ - 44$) and m/z 152 (base peak at m/z 180), corresponding to probable losses of CO_2 and $\text{CO}_2\text{-CO}$, respectively (data not shown). Ions at m/z 180 and 152 were also observed for the underivatized metabolite. Because of the anionic nature of the sample, a good mass spectrum was not obtained, possibly because of the amount, purity, and/or thermal decomposition of the sample.

The methylated metabolite and methylated authentic 9-fluorenone-1-carboxylic acid produced two compounds that were separated by gas chromatography-mass spectrometry. The compound with a molecular ion at m/z 238 was produced at a level that was approximately 1 order of magnitude higher than the level of the compound with a molecular ion at m/z 252. The mass spectrum of the first compound (Fig. 3a) was consistent with methylation of the acid moiety (molecular weight, 238), with fragment ions at m/z 223, 207, 180, and 151; the probable losses to produce these ions were CH_3 , OCH_3 , CO_2CH_3 and $\text{CO}_2\text{CH}_3\text{-CO}$, respectively. The mass spectrum of the second compound (data not shown) had an apparent molecular ion at m/z 252, indicating that there was further methylation of the compound, probably the keto moiety. All of the mass spectra obtained were consistent with each other with respect to the fragment ions observed and differed only in the relative intensities of these ions. The fact that the UV spectrum, the ^{13}C spectrum, the ^1H NMR spectrum, and the mass spectra (Fig. 3b) of the standard were identical to the spectra of the metabolite confirmed that this compound was 9-fluorenone-1-carboxylate.

The results of this study and previous results from our

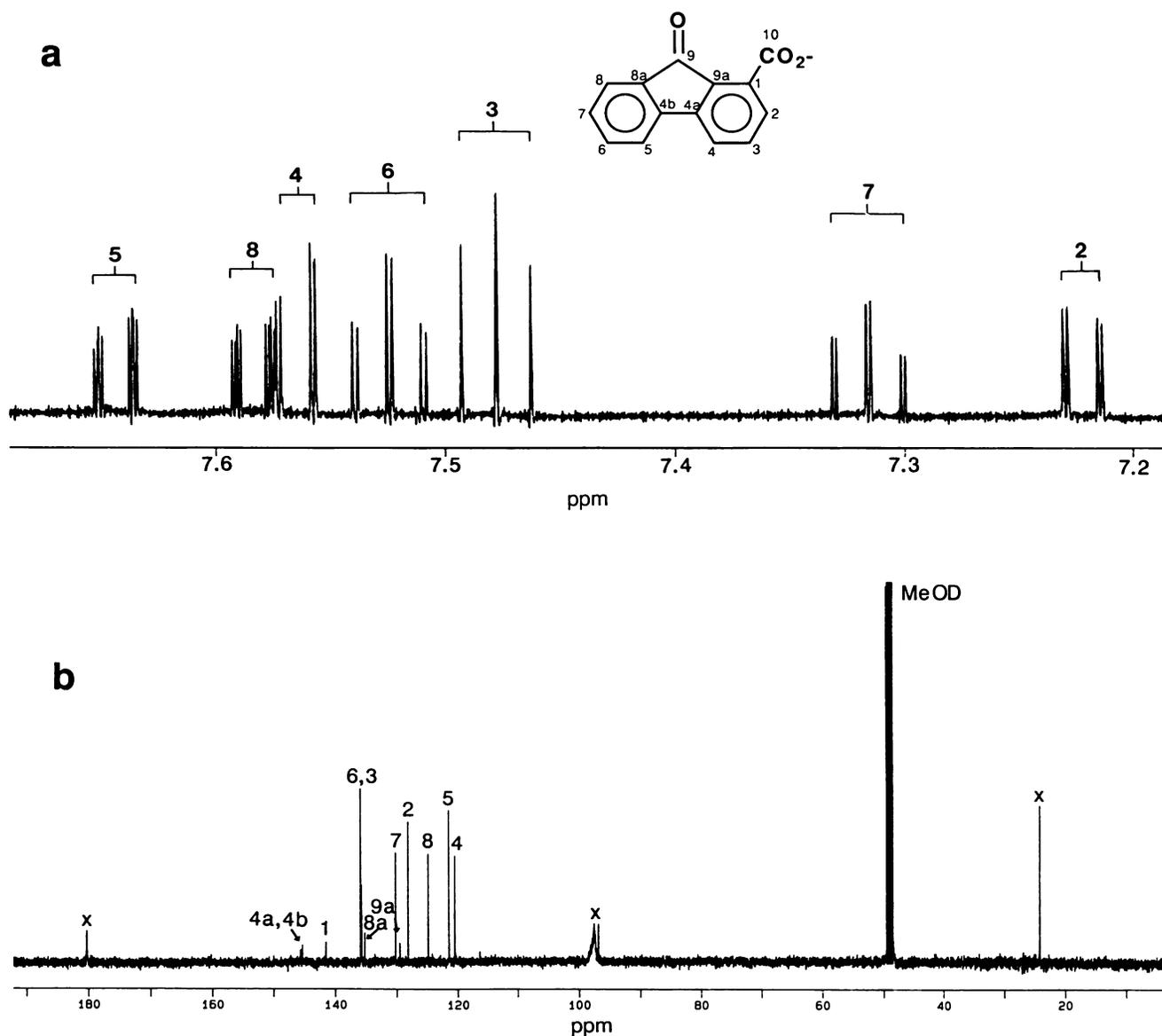


FIG. 2. (a) Resolution-enhanced 500-MHz ¹H NMR spectrum of 9-fluorenone-1-carboxylate derived from the metabolism of FA. (b) 126-MHz ¹³C NMR spectrum of 9-fluorenone-1-carboxylate derived from the metabolism of FA. The sample was dissolved in methanol and is shown with the structure and resonance assignments; X indicates resonances due to impurities.

laboratory indicated that FA is rapidly degraded to CO₂. However, a small amount of ring cleavage metabolite does accumulate, and its structure was rigorously characterized. The formation of 9-fluorenone-1-carboxylic acid is consistent with the results of analogous studies on the initial degradative steps for bacterial oxidation of aromatic hydrocarbons (8) and with the results of previous studies on the degradation of pyrene (15) by the *Mycobacterium* species. The isolation and identification of 9-fluorenone-1-carboxylic acid indicate that the *Mycobacterium* sp. can initially attack FA in the fused aromatic ring portion of the molecule, presumably via a dioxygenase in the 1,2 or 2,3 positions, to form dihydroxylated FA intermediates. Oxidative cleavage of the dihydroxylated intermediates at the 1,2 or 2,3 positions of FA and subsequent metabolism could result in 9-fluorenone-1-carboxylic acid. The metabolite appears to be

a chemically stable molecule. However, it is apparently easily metabolized and does not accumulate in significant amounts. Further research on the identification of the minor ring fission products, as well as research on the identification of initial oxygenated metabolites, is warranted to provide a scheme for the degradation of FA by the *Mycobacterium* species.

The ability of the *Mycobacterium* sp. to mineralize FA rapidly, with a relatively small, transient accumulation of intermediate compounds, strengthens the hypothesis that this organism is a potentially useful agent for bioremediation of PAHs. From a practical standpoint, since the bacterium was able to degrade FA and pyrene, hydrocarbons containing four fused aromatic rings, it might be useful in the biodegradation of higher-molecular-weight PAHs and their substituted derivatives.

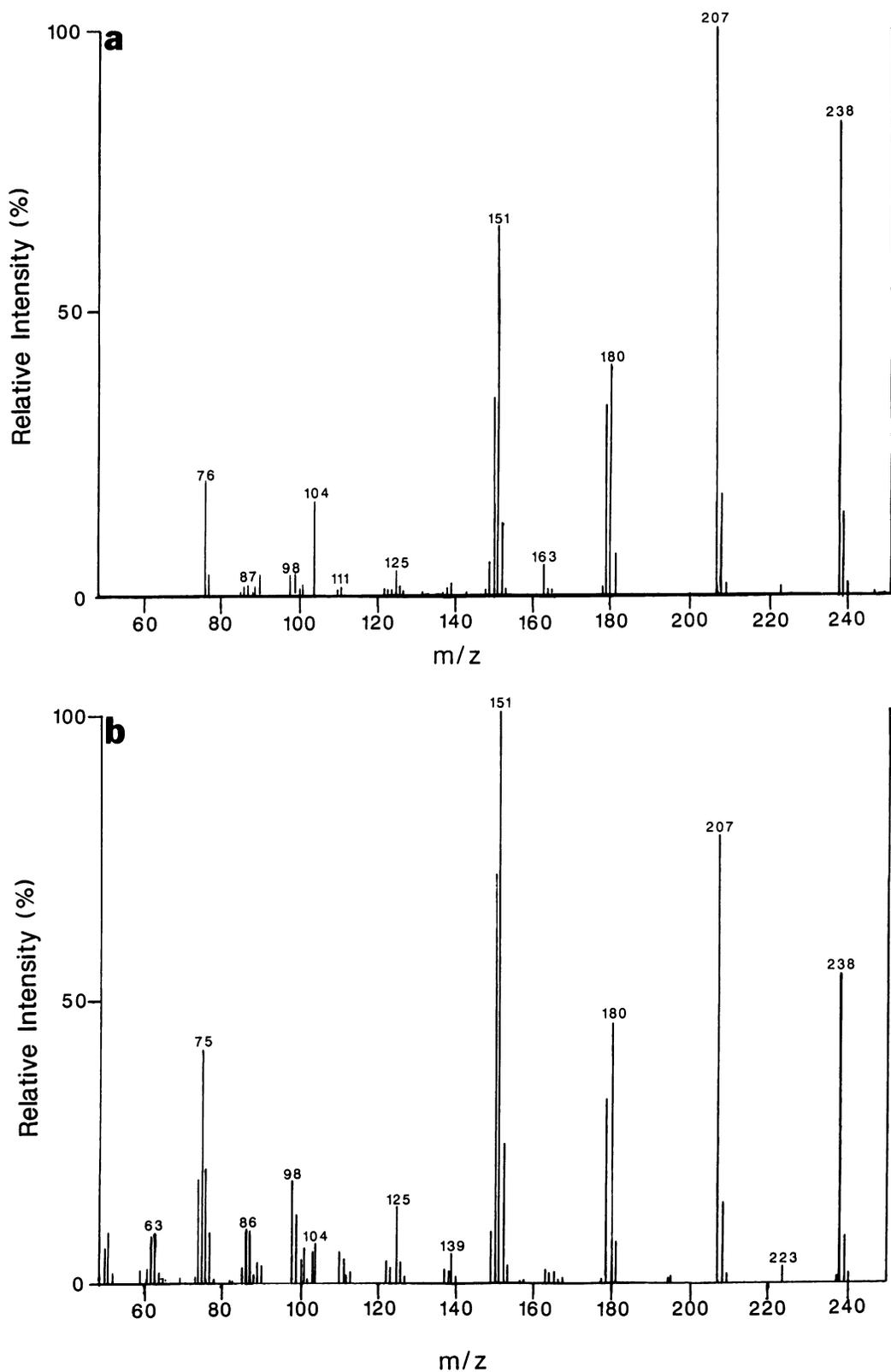


FIG. 3. Mass spectra of the predominant product formed during the methylation of the metabolite (a) and authentic 9-fluorenone-1-carboxylic acid (b).

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