Isolation of 6-, 8-, and 10-Gingerol from Ginger Rhizome by HPLC and Preliminary Evaluation of Inhibition of *Mycobacterium avium* and *Mycobacterium tuberculosis*

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Diseases caused by *Mycobacterium avium* (*M. avium*) and *Mycobacterium tuberculosis* (*M. tuberculosis*) have reached pandemic proportions with some strains being resistant to existing chemotherapies. Complex therapies requiring four to six drugs are sometimes required to prevent the emergence of resistant strains. There is a need for the discovery of new drugs or compounds that are potential drug templates that can be used to treat diseases caused by these bacteria. The research reported in this paper describes the isolation of 6-, 8-, and 10-gingerol from fresh ginger rhizome and the identification of 10-gingerol as the most active inhibitor of *M. avium* and *M. tuberculosis* in vitro. The gingerols were isolated by fractionation of a crude methylene chloride extract of fresh ginger rhizome by normal phase HPLC. Identification was based on mass spectral data. The identification of 10-gingerol was confirmed by synthesis.

Keywords: *Zingiber officinale; ginger; 6-gingerol; 8-gingerol; 10-gingerol; AIDS; Mycobacterium avium; Mycobacterium tuberculosis*

INTRODUCTION

Ginger (*Zingiber officinale*) is grown throughout the tropics. It is primarily used as a spice and is valued for its pungency which is due to a class of compounds called gingerols.

Mycobacterium avium (*M. avium*) is a common environmental pathogen found in soil, water, and foodstuffs. It is a slow growing, aerobic, facultative intracellular parasite. It is estimated that 70% of people worldwide are exposed to *M. avium* at some time during their lives but never develop disease because healthy individuals are protected by their immune response. However, people with AIDS are especially susceptible to infection with 43−70% of AIDS patients developing infections (Bensen and Ellner, 1993; Bermundez, 1994; Garcia-Rodriguez and Gomez Garcia, 1993).

Respiratory infections caused by *M. avium* were first diagnosed in Europe and North America in the middle of this century. The symptoms were similar to tuberculosis. However, people who developed *M. avium* infections usually had another underlying lung disease present, and the infection rarely disseminated to sterile parts of the body (Bermundez, 1994; Bermundez and Kaplan, 1995; Crawford, 1994). *M. avium* was therefore seen as an opportunistic pathogen.

Disseminated disease was first diagnosed in AIDS patients in 1982. It is now the most common form of bacterial infection in patients with advanced immunosuppression (Barradell et al., 1993). Disseminated disease refers to the spread of infection from the initial areas of colonization, which are the respiratory tract or, in AIDS patients, usually the gastrointestinal tract, to sterile parts of the body such as the liver, spleen, lymph nodes, bone marrow, and blood. This results in a general deterioration of the body, loss of organ function due to high bacterial burden, and low life expectancy (Barradell et al., 1993; Hoffner et al., 1992).

Mycobacterium tuberculosis (*M. tuberculosis*) is a contagious pathogen causing the disease tuberculosis. In areas of the world where the disease is prevalent, people with suppressed immune systems, such as those who are HIV+, are especially prone to infection. It is estimated that by the year 2000 there will be 15 million new cases in sub-Saharan Africa. Many strains of *M. tuberculosis* are resistant to at least one of the drugs available for treatment of tuberculosis. The W-strain, which was isolated in New York City, is resistant to virtually all commonly used drugs which means potentially toxic drugs must be relied on to inhibit this strain of the bacterium. To effectively fight this disease and to decrease the likelihood of *M. tuberculosis* strains developing resistance to drugs, therapy involves the use of more than one drug. Standard therapy takes 1 year and must be strictly adhered to in order to prevent drug resistant strains. This protocol has been reduced to 6 months with the introduction of the drug rifampicin, but, as with the standard therapy, strict adherence is very important (Enarson et al., 1995). Clearly, there is a need for new, inexpensive, nontoxic drugs for the inhibition of *M. avium* and *M. tuberculosis*.

It is generally believed that foods, especially herbs and spices, are a source of many pharmacologically active compounds. Evidence gathered at Gillis W. Long Hansen's Disease Center at Louisiana State University, which screens a large number of plant extracts, found

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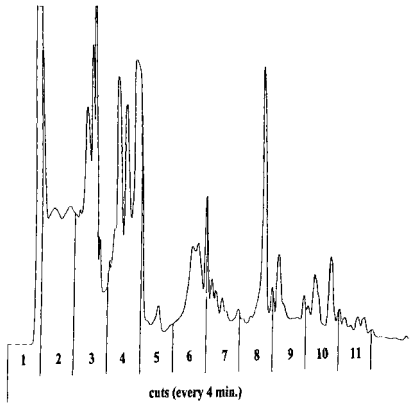

Figure 1. HPLC chromatogram of crude residue showing location of cuts. Cuts 1−3 were combined to form semicrude fraction 1, cuts 4 and 5 were combined to form semicrude fraction 2, cuts 6 and 7 were combined to form semicrude fraction 3, and cuts 8−11 were combined to form semicrude fraction 4.

that ginger contained a compound or compounds that inhibited the growth of *M. avium* and *M. tuberculosis* (Franzblau et al., 1994). This study was initiated to identify the compounds responsible for this activity.

This paper will describe the procedure for the isolation of 6-, 8-, and 10-gingerol by normal phase HPLC. 10-Gingerol, identified as the most active compound against the mycobacteria tested, was identified by APcI-LC-MS and direct probe EI-MS and the identity confirmed by synthesis via an Aldol reaction.

MATERIALS AND METHODS

Isolation of Crude Extract. A 100 g sample of fresh ginger rhizome was purchased locally and shredded in a food processor (Vita-Mix Corp., Cleveland, OH). This was extracted with 300 mL of methylene chloride for 5 min using a Brinkmann PT 10/35 homogenizer (Brinkmann Instruments, Westbury, NY). An ice bath was used to prevent the decomposition of thermally labile compounds. The methylene chloride extract was filtered through a plug of glass wool and centrifuged for 30 min at 10 000 rpm (14 476 g.) at 4 °C using a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Instruments, Wilmington, DE). The aqueous layer was removed and the methylene chloride layer filtered through a 0.45 μm Nylon 66 membrane. The crude residue was isolated by concentrating the methylene chloride extract to 10 mL using a Buchi rotary evaporator at 40 °C (Brinkmann). This was quantitatively transferred to a tared culture tube and the residue isolated by concentrating to dryness using a stream of nitrogen at room temperature. The crude residue was dissolved initially in methylene chloride and then diluted with methylene chloride/hexanes (50:50) so the final concentration was 17.0 mg/mL in methylene chloride/hexanes (65:35). The compounds in this solution were fractionated by normal phase HPLC.

Fractionation of the Crude Extract by HPLC. The HPLC system consisted of a Kratos Spectroflow 400 solvent delivery system, a Kratos Spectroflow 783 programmable absorbance detector at 282 nm and 0.7 AUFS (Perkin-Elmer Corp., Norwalk, CT), and a Varian 4290 integrator (Varian, Sugarland, TX). Eleven cuts were taken, one every 4 min, during the separation using a Retriever II fraction collector (ISCO Inc., Lincoln, NE). Figure 1 contains a chromatogram for the crude extract showing the location of the cuts. The column used was a Spherisorb CN (Phase Separations, Inc., Norwalk, CT), $dp = 5$ μm, 250 mm × 4.6 mm (i.d.). Mobile phase A was hexanes, and mobile phase B was chloroform/methanol (95:5). The flow rate was 0.8 mL/min, and the injection volume was 400 μL. The following linear gradient was used:

time (min)	mobile phase A	mobile phase B
0	80%	20%
10	80%	20%
45	20%	80%

Cuts 1−3 were combined to form semicrude fraction 1, cuts 4 and 5 were combined to form semicrude fraction 2, cuts 6 and 7 were combined to form semicrude fraction 3, and cuts 8−11 were combined to form semicrude fraction 4. The residue from each semicrude fraction was isolated by concentrating to 10 mL using a rotary evaporator at 40 °C. This was quantitatively transferred to a tared culture tube and the residue isolated by concentrating to dryness using a stream of nitrogen at room temperature.

Purification of 6-, 8-, and 10-Gingerol by HPLC. The residue from semicrude fraction 2 was dissolved in methylene chloride and diluted with hexanes so the final concentration was 15 mg/mL in methylene chloride/hexanes (50:50). Purified 6-, 8-, and 10-gingerol were isolated from semicrude fraction 2 using the instrument conditions outlined in the above section "Fractionation of the Crude Extract by HPLC" but with the following changes: isocratic elution, 20% mobile phase B; flow rate, 1.0 mL/min; injection volume, 250 μL; wavelength, 282 nm (0.5 AUFS). Fractions were collected manually. The purified gingerols were isolated by concentrating each to 10 mL using a rotary evaporator at 40 °C. Each was quantitatively transferred to a tared culture tube and isolated by concentrating to dryness using a stream of nitrogen at room temperature.

Identification of Gingerols by APcI-LC-MS. A VG Platform II quadrupole mass spectrometer (Micromass, Beverly, MA) operating in the positive ion mode was interfaced to a Varian 9012 solvent delivery system (Varian) and a Varian 9050 variable wavelength UV−vis detector at 282 nm (0.1 AUFS). The column used was a Spherisorb CN (Phase Separations) $dp = 5$ μm, 250 mm × 4.6 mm (i.d.). Mobile phase A was hexanes; and mobile phase B was chloroform/methanol (95:5). The components were separated using isocratic elution with 20% mobile phase B. The flow rate was 1.0 mL/min, and 10 μL was injected. Masses were scanned from 150 to 500 amu in 3 s. The other mass spectrometer conditions used were as follows: corona discharge, +3.2 kV; cone voltage, +10 V; probe temperature, 450 °C; source temperature, 150 °C.

Identification of Gingerols by Direct Probe EI-MS. EI-mass spectra were obtained for purified fractions containing 6-, 8-, and 10-gingerol using a Finnigan MAT 8230 double focusing mass spectrometer (Finnigan Corp., San Jose, CA) with the following instrument conditions: ionization mode, positive ion (70 eV); ion source temperature, 250 °C; filament emission current, 1 mA; direct probe temperature ramp, 35−350 °C; mass range, 35−600 amu at 1 s/decade.

Synthesis of 10-Gingerol (Denniff and Whiting, 1976). A 10 g sample of zingerone was weighed into a three necked round-bottomed flask and dissolved in 200 mL of tetrahydrofuran (THF). The middle neck was fitted with a rubber septum. The other two necks were fitted with tube connector adapters so the system could be purged with nitrogen. The system was vented through a mineral oil bubbler to maintain positive nitrogen pressure within the system. It was purged with nitrogen for 30 min prior to addition of 1,1,1,3,3,3-hexamethyldisilazane (HMDS). A 10 mL aliquot of HMDS was added through the septum using a syringe and the derivatization reaction allowed to proceed for 2 h at room temperature while being magnetically mixed. The reaction was cooled to −78 °C using a dry ice-acetone bath which was maintained throughout the reaction. A 55 mL aliquot of lithium bis(trimethylsilyl)amide was added over a 10 min period, and then 10 mL of decyl aldehyde was added over a 5 min period. The reaction was allowed to proceed for 1 h at −78 °C with magnetic mixing. At the end of the reaction the flask was warmed to room temperature and the nitrogen disconnected. The pH was adjusted to 5.0 with 25% HCl while

Table 1. Percent Inhibition of *M. avium* for Crude Extract and Semicrude Fractions of Fresh Ginger Rhizome

	% inhibition at given concn (μg/mL)			
residue ID	100 (μg/mL)	50 (μg/mL)	25 (μg/mL)	12.5 (μg/mL)
crude	87	58	13	0
semicrude 1	85	0	0	0
semicrude 2	95	95	73	42
semicrude 3	65	0	48[a]	0
semicrude 4	0	0	0	0

[a] Outlier.

Table 2. Percent Inhibition of *M. tuberculosis* $H_{37}Rv$ for Crude Extract and Semicrude Fractions of Fresh Ginger Rhizome

	% inhibition at given concn (μg/mL)			
residue ID	100 (μg/mL)	50 (μg/mL)	25 (μg/mL)	12.5 (μg/mL)
crude	85	0	0	0
semicrude 1	93	27	0	0
semicrude 2	95	0	0	0
semicrude 3	30	0	0	0
semicrude 4	0	0	0	0

magnetic mixing. The crude 10-gingerol was purified by normal phase HPLC.

Antimycobacterial Activity. Antimycobacterial activity was determined using the BACTEC 460 system expressed either as percent inhibition of drug-free controls (Cantrell et al., 1996, 1998) or as the minimum inhibitory concentration (MIC; Collins and Franzblau, 1997).

Solvents and Reagents. Hexanes, chloroform, methanol, and methylene chloride, used for the preparation of mobile phases and sample dissolution, were HPLC grade and purchased from Fisher Scientific (Springfield, NJ). Zingerone was purchased from Penta International Corp. (Fairfield, NJ). Lithium bis(trimethylsilyl)amide (1.0 M in THF), HMDS (99.9%), decyl aldehyde (95%), and THF (HPLC grade, inhibitor free) were purchased from Aldrich Chemical Co. (St. Louis, MO).

RESULTS AND DISCUSSION

The crude residue isolated from the methylene chloride extract of fresh ginger rhizome was a clear golden oil that was 0.55% of the fresh ginger weight. It had a characteristic ginger aroma and consisted of a complex mixture of components that were fractionated by normal phase HPLC. Samples of the crude residue from the initial methylene chloride extract and residues from semicrude fractions 1−4 were analyzed to determine the percent inhibition of *M. avium* and *M. tuberculosis*. At 100 μg/mL the crude residue was inhibitory for both *M. avium* (Table 1) and *M. tuberculosis* (Table 2) in vitro. This activity was increased in semicrude fraction 2 and provided the justification for further fractionating semicrude fraction 2 to purified 6-, 8-, and 10-gingerol. Since the residues from semicrude fractions 1, 3, and 4 did not show an increase in activity, nothing further was done with these residues.

The residue from semicrude fraction 2, which was 0.20% of the fresh ginger weight, was analyzed by APcI-LC-MS and was found to consist of three major compounds. The area percent and LC-MS data are listed in Table 3. APcI is a soft ionization technique which generates protonated molecular ions in the positive ion mode. The mass spectra for the three main components in semicrude fraction 2 showed weak protonated molecular ions, at 5% relative intensity, and indicated the molecular weights for the three components to be 350, 322, and 294 amu in the order of the retention time.

Table 3. APcI-LC-MS and Area Percent Data for the Analysis of Gingerols in Semicrude Fraction 2[a]

gingerol	retention time (min)	area % at 282 nm	$(M + H)^+$ (amu)	base peak (amu)
10-gingerol	9.8	15	351	333
8-gingerol	10.5	11	323	305
6-gingerol	11.6	72	295	277

[a] Other minor compounds = 2%.

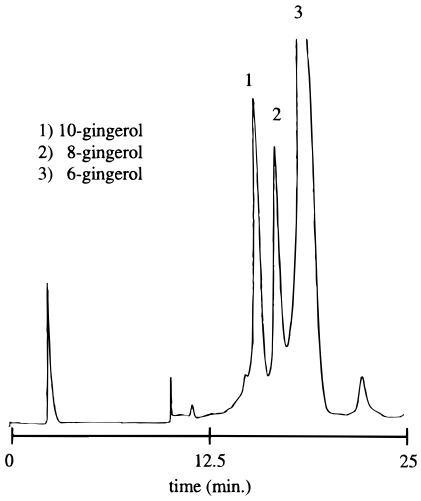

Figure 2. HPLC chromatogram of semicrude fraction 2.

Table 4. Area Percent Purity of Isolated Gingerols[a]

	area % at 282 nm		
gingerol	10-gingerol	8-gingerol	6-gingerol
10-gingerol	*84*	6	10
8-gingerol	2	*86*	12
6-gingerol	nd	nd	*99*

[a] nd = none detected.

The base peak in each mass spectra was 18 amu less than the molecular ion which indicated the three components each lost a molecule of water. Additionally, the molecular weights decreased, with retention time, by 28 amu or two methylene units, which indicated the three compounds were part of a homologous series. On the basis of these data and data from the literature (Chen et al., 1986a,b), the three compounds in semicrude fraction 2 were tentatively identified as 6-, 8-, and 10-gingerol. The APcI data did not indicate the presence of coeluting impurities with 6-, 8-, and 10-gingerol in semicrude fraction 2

Semicrude fraction 2 was fractionated to purified 6-, 8-, and 10-gingerol using essentially the same chromatographic conditions used to fractionate the residue from the crude extract, the major difference being that isocratic elution rather than gradient elution was used to achieve the separation (Figure 2). The area purities for 6-, 8-, and 10-gingerol isolated from semicrude fraction 2 are listed in Table 4. The effect of peak fronting, which is the result of operating under overload conditions, is evident from the area purity of 10- and 8-gingerol. The yields of gingerols based on the fresh ginger weight are listed in Table 5.

The minimum inhibitory concentration (MIC) was determined for 6-, 8-, and 10-gingerol for the in vitro inhibition of *M. avium* and *M. tuberculosis*. The minimum inhibitory concentration is defined as the lowest concentration that will inhibit 99% of an initial inoculum. These results are listed in Table 6. The data show

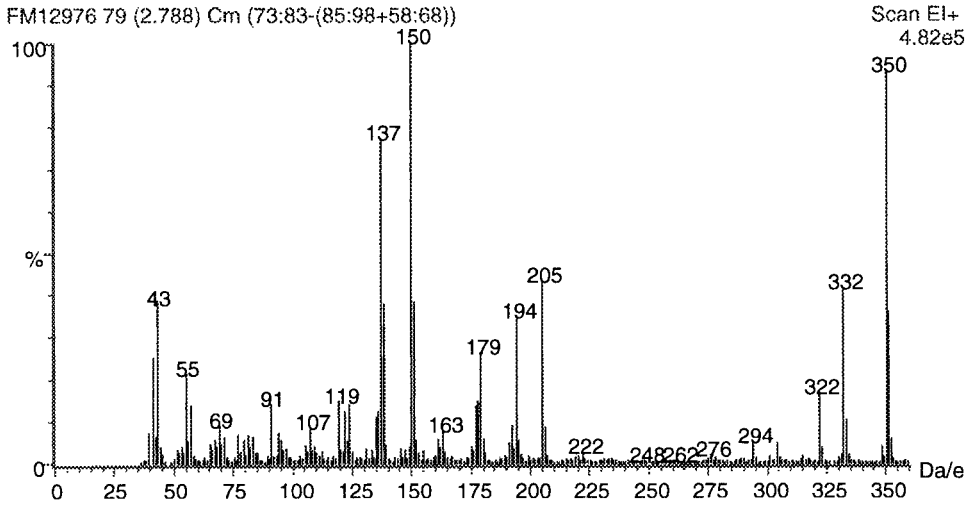

Figure 3. EI-mass spectrum of 10-gingerol isolated from fresh ginger rhizome.

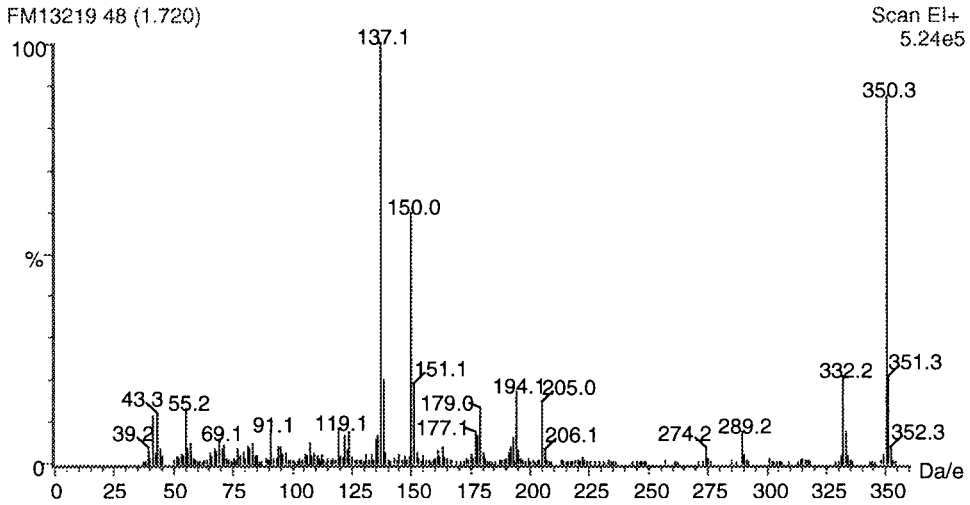

Figure 4. EI-mass spectrum of 10-gingerol synthesized via the Aldol reaction.

Table 5. Yields of Gingerols Isolated from Fresh Ginger Rhizome

gingerol	yield[a] (ppm)
10-gingerol	120
8-gingerol	93
6-gingerol	880

[a] Based on weight of fresh ginger.

Table 6. Minimum Inhibitory Concentration (MIC) for *M. avium* and *M. tuberculosis*

	MIC (μg/mL)	
gingerol	*M. avium*	*M. tuberculosis*
10-gingerol	25	50
8-gingerol	50	50
6-gingerol	>100	>100

10-gingerol to be the most active compound for inhibition of *M. avium* with a MIC of 25 μg/mL. This represents a significant increase in activity over the crude residue which by definition was greater than 100 μg/mL. 10-Gingerol was less active for inhibition of *M. tuberculosis* with a MIC of 50−100 μg/mL.

Direct probe EI-mass spectra were obtained for 6-, 8-, and 10-gingerol which confirmed the APcI-LC-MS data. The EI-mass spectrum for 10-gingerol is shown in Figure 3. The ions at $m/z = 322$ and 294 are due to the presence of 8- and 6-gingerol as impurities in this sample.

To confirm the identity of 10-gingerol, it was synthesized using an Aldol reaction. The direct probe EI-mass spectra for synthesized 10-gingerol (Figure 4) correlated well with the direct probe EI-mass spectra for 10-gingerol extracted from fresh ginger rhizome which confirmed the identification.

CONCLUSION

A systematic approach was developed for screening methylene chloride extracted ginger compounds for inhibition of *M. avium* and *M. tuberculosis*, and 10-gingerol was found to inhibit the growth of these mycobacteria in vitro. The mechanism of inhibition may be related to lipophilicity since the inhibition of *M. avium* increased from 6-gingerol (MIC > 100 μg/mL) to 10-gingerol (MIC = 25 μg/mL). The significance of this research lies in the recognition of 10-gingerol as a lead compound for more active homologues and analogues. Some of these compounds have been synthesized and are being tested for inhibition of *M. avium* and *M. tuberculosis* by Gillis W. Long Hansen's Disease Center.

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