MICROBIAL HYDROXYLATION AND GLUCURONIDATION OF THE ANGIOTENSIN II (AII) RECEPTOR ANTAGONIST MK 954

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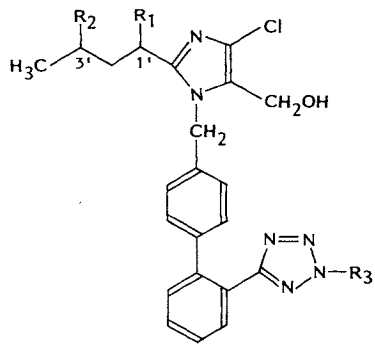
The microbial metabolism of MK 954 (Fig. 1), a novel nonpeptide angiotensin II receptor antagonist, was investigated using 40 microorganisms in an initial screen for cultures that will produce metabolites similar to those produced in the mammalian liver. The microbial transformation occurred under aerobic conditions in shake flasks incubated at 27°C. Three metabolites of MK 954 were isolated and identified as the 1'-hydroxy M_2, 3'-hydroxy M_1, and glucuronic acid conjugated M_3 derivatives. The structures of the metabolites were established by UV, ^{1}H-NMR spectroscopy and FAB-MS spectrometry and are identical to metabolites produced by incubation of MK 954 with mammalian liver slices.

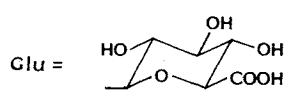
The renin-angiotensin system (RAS) plays a key role in the regulation of blood pressure and the etiology of hypertension. Angiotensin II (AII) is the primary effector molecule of RAS. MK 954 (Fig. 1) is an effective nonpeptidic anatagonist of the AII receptor AT1, currently in Phase III clinical trials[1~4]. In the course of studying the metabolism of MK 954, two hydroxylated compounds M_1 and M_2 and a glucuronic acid conjugated metabolite M_3 were isolated as major metabolites[5] from incubation with human liver slices.

To facilitate further pharmacological and toxicological studies on these metabolites, we screened for microorganisms capable of metabolizing MK 954 in the hope that compounds M_1, M_2 and M_3 could be produced microbiologically. The present communication describes the microbial production of three mammalian metabolites of MK 954 using three microorganisms: *Actinoplanes* sp. MA 6559 (ATCC 53771), *Streptomyces* sp. MA 6966 (ATCC 55293), and *Streptomyces* sp. MA 6751 (ATCC 55043).

Fig. 1. The structure of MK 954 and the metabolites M_1, M_2, and M_3.



MK 954	R_1=H	R_2=H	R_3=H
M_1	R_1=H	R_2=OH	R_3=H
M_2	R_1=OH	R_2=H	R_3=H
M_3	R_1=H	R_2=H	R_3=Glu



Materials and Methods

Chemicals

Phosphoric acid (Baker; Philipsburg, NJ) and all organic solvents (EM Science; Gibbstown, NJ) were HPLC grade. Water was purified in a Millipore Milli-Q system (Bedford, MA). Solvent for NMR analysis (CD_3OD) was purchased from Aldrich Chemical (Millwaukee, WI). MK 954 was prepared at DuPont/Merck Research Laboratories (Wilmington, DE).

NMR and MS Spectroscopy

^1H NMR experiments were performed on a Bruker 250 MHz instrument. The spectra were recorded in CD_3OD at ambient temperature using the solvent peak at 3.30 ppm as internal reference downfield of TMS at 0 ppm. MS measurements were obtained on a Finnigan MAT TSQ 70 instrument.

Microbiological

Biotransformation experiments were performed by shake culture technique utilizing a two-stage fermentation. Frozen vegetative mycelium was used to inoculate a 250-ml baffled flask containing 50 ml seed medium consisting of (in g/liter) dextrin 10.0, glucose 1.0, beef extract 3.0, Ardamine PH (Yeast Products, Inc.) 5.0, N-Z Amine type E 5.0, $MgSO_4 \cdot 7H_2O$ 0.05, KH_2PO_4 0.37, and $CaCO_3$ 0.5. The pH of the seed medium was adjusted to 7.1 before autoclaving. The seed flasks were incubated on a rotary shaker (220 rpm) at 27°C for 1~2 days. A 1 ml aliquot of the seed medium was used to inoculate a 50-ml non-baffled shake flask containing 10 ml of transformation medium. Transformation medium consisted of (in g/liter) glucose 10.0, Hy-case SF 2.0, beef extract 1.0, corn steep liquor 3.0. The pH was adjusted to 7.0 before autoclaving with 1 N NaOH. MK 954 was added to the fermentation as a sterile aqueous solution (pH 7.0) at 0 hour. The shake flasks were subsequently incubated for 1~4 days. A culture control consisted of the microorganism without substrate addition. Following incubation, the fermentations were sampled by the addition of 0.5 ml acetonitrile to 0.5 ml whole broth. After vortexing for 1 minute, the resulting solution was centrifuged and subjected to HPLC analysis for biotransformation products as described below.

Isolation and Purification of Metabolites

Preparative scale fermentations were conducted in 250-ml non-baffled Erlenmeyer shake flasks containing 50 ml of transformation medium. Seed culture (10%) was used as inoculum. Substrate (MK 954) was added at 0 hour to achieve a final concentration of 0.1 mg/ml. Transformation continued for 2~5 days. The whole broth was adjusted to pH 3.5 and centrifuged. The mycelial cake was washed with water, then discarded. The clear filtrate and washings were pooled and passed through a Spe-ed octadecyl cartridge (14% carbon load, Applied Separations, Bethlehem, PA) under vacuum. The cartridge was washed with 10% aqueous methanol. Column effluent and wash did not contain microbial product when tested by HPLC. The cartridge was eluted with methanol. Methanol was evaporated to dryness under reduced pressure at 30°C. The resulting oil was dissolved in methanol and subjected to purification by HPLC. HPLC was carried out on a Whatman Magnum 20 Partisil 10 ODS-3 column (C18, 22.1 mm i.d. × 25 cm) at room temperature and monitored at 250 nm. The column was developed at 6 ml/minute with linear gradient from 15% to 80% acetonitrile in 0.1% aqueous phosphoric acid 80 minutes. The metabolite fractions were pooled, adjusted to pH 3.5 and evaporated to remove acetonitrile. Desalting was carried out using a C18 Sep-Pak (Waters Associates) and methanol - water elution solvent to yield pure compounds.

HPLC Analysis

HPLC analysis was performed with a Beckman Method Development system consisting of a model 126 solvent delivery module and a 167 detector. Chromatograms were monitored by UV detection at 250 nm. For detection of microbial transformation metabolites the whole broth was mixed with an equal volume of methanol. The mixture was centrifuged and the clear supernatant was analyzed by HPLC. Samples (50 μl) were loaded onto a Whatman Partisil 10 ODS-3, 4.6 mm × 25 cm column. The separation was achieved with a gradient from 20% to 80% acetonitrile in water containing 0.1% phosphoric acid over 35 minutes. The elution rate was 1 ml/minutes.

Metabolite M_1. Obtained from transformation of MK 954 by *Actinoplanes* sp. MA 6559 (ATCC 53771) in 90% yield: ^1H NMR (250 MHz, CD_3OD, δ ppm), 1.12 (3H, d, J=6.1 Hz), 1.70 (2H, m), 2.65 (2H, m), 3.70 (1H, m), 4.48 (2H, s), 5.50 (2H, s), 7.04 (2H, d, J=8.2 Hz), 7.14 (2H, d, J=8.2 Hz), 7.55 (2H, m), 7.70 (2H, m); FAB-MS: m/z 439 $(M+1)^+$

Metabolite M_2. This metabolite was obtained in 20% yield from transformation of MK 954 by *Streptomyces* sp. MA 6966 (ATCC 55293): ^1H NMR (250 MHz, CD_3OD δ ppm), 0.85 (3H, t, J=7.3 Hz), 1.35 (2H, m), 1.80 (2H, q, J=7.5 Hz), 4.48 (2H, s), 4.60 (1H, t, J=7.0 Hz), 5.50 (2H, s), 7.04 (2H, d, J=8.2 Hz), 7.14 (2H, d, J=8.2 Hz), 7.55 (2H, m), 7.70 (2H, m); FAB-MS: m/z 439 $(M+1)^+$

Metabolite M_3. Produced from transformation of MK 954 by *Streptomyces* sp. MA 6751 (ATCC

55043) in 45% yield: ^{1}H NMR (250 MHz, CD_3OD, δ ppm), 0.86 (3H, t, J=7.3 Hz), 1.30 (2H, m), 1.53 (2H, m), 2.60 (2H, m), 3.62 (2H, m), 4.05 (2H, m), 4.49 (2H, s), 5.31 (2H, s), 5.78 (1H, d, J=9.1 Hz), 7.00 and 7.13 (4H, AA'XX', J=8.0 Hz), 7.50 (3H, m), 7.83 (1H, d, J=6.6 Hz); FAB-MS: m/z 599 $(M+1)^+$

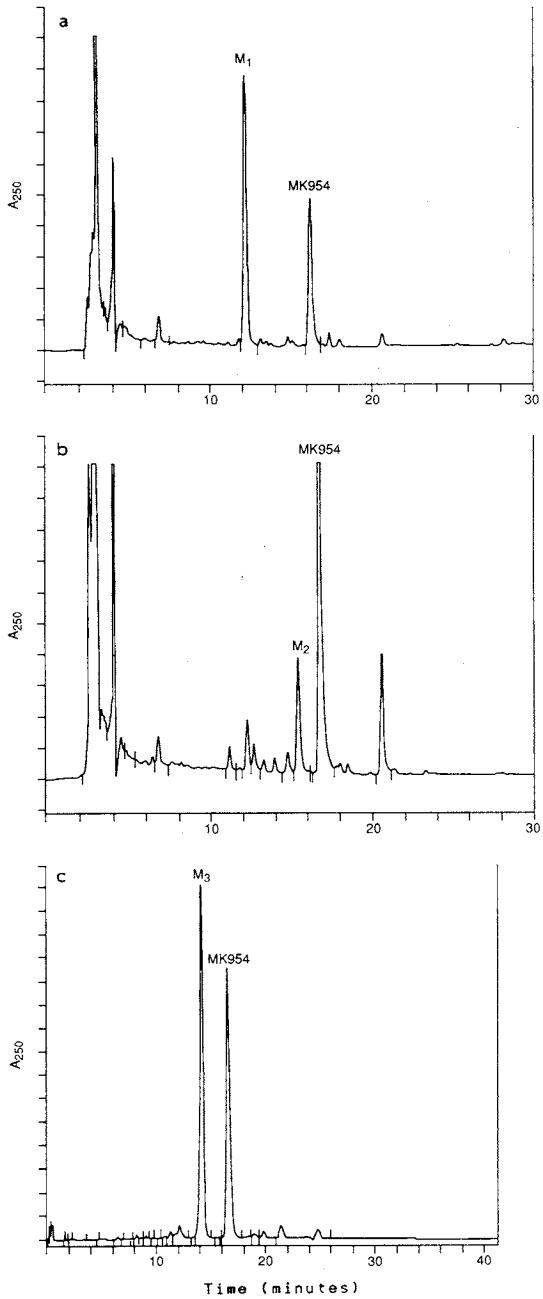
Results and Discussion

Forty microorganisms were screened for their ability to produce metabolites identical to metabolites produced by rat, monkey and human liver slices. Of the active cultures obtained from the screen three were used in large-scale preparation of the metabolites.

Actinoplanes sp. MA 6559 (ATCC 53771) was chosen for large-scale preparation of metabolite M_1 due to low chromatographic background. The HPLC chromatogram of the acetonitrile extract of the whole broth 48 hours after addition of MK 954 was obtained and presented in Fig. 2. Authentic sample of M_1 eluted with the same retention time. The positive FAB mass spectrum gave an $(M+H)^+$ ion at m/z 439, indicating a monohydroxylated derivative. The ^{1}H NMR spectrum showed that the terminal methyl group of the butyl side chain, observed as a triplet at 0.85 ppm in the NMR spectrum of MK 954, appeared as a doublet at 1.12 ppm and a new multiplet of one proton was observed at 3.70 ppm. The ^{1}H NMR in conjunction with MS data confirmed the assignment as a derivative of MK 954 hydroxylated on the penultimate methylene carbon (C-3') of the butyl side chain.

Preparative incubation of MK 954 with *Streptomyces* sp. MA 6966 (ATCC 55293) gave one metabolite M_2 (Fig. 1). A display of a molecular ion at m/z 439 $(M+H)^+$ in the positive FAB-MS indicated that the metabolite is a monohydroxylated MK 954. In the ^{1}H NMR spectrum, the triplet at 2.60 ppm corresponding to the C-1' side chain methylene protons of MK 954 was absent and a new triplet of one proton appeared at 4.60 ppm. The metabolite M_2 was assigned as a derivative hydroxylated at C-1' carbon of the butyl side chain.

Fig. 2. HPLC chromatogram from an incubation of MK 954 with a) *Actinoplanes* sp. MA 6559 (ATCC 53771), b) *Streptomyces* sp. MA 6966 (ATCC 55293), c) *Streptomyces* sp. MA 6751 (ATCC 55043).



Metabolite M_3 was obtained from preparative incubation of MK 954 with *Streptomyces* sp. MA 6751 (ATCC 55043). A molecular ion at *m/z* 599 $(M+H)^+$ in the positive FAB-MS and the doublet at 5.78 ppm in the ^1H NMR spectrum displayed by metabolite M_3, suggested that it is a glucuronic acid conjugate. The final assignment of M_3 as the tetrazole-N_2-β-glucuronide of MK 954 was based on ^1H NMR comparison with the same material isolated from an incubation with liver slices.[5]

STEARNS[5] has reported that M_1, M_2, and M_3 are major metabolites of MK 954 by rat, monkey, and human liver slices. Our observations, which indicate that microorganisms carry out hydroxylation and formation of glucuronic acid conjugate, reinforce the concepts reviewed by SMITH and ROSAZZA[6] on the parallelism between mammalian and microbial metabolism of drugs. The synthesis of a tetrazole-N_2-β-glucuronide by a *Streptomyces*, to our knowledge, is unprecedented, although the formation of tetrazole derivatives to tetrazole-*N*-β-glucuronides represents a common metabolic process in mammalian organisms to facilitate detoxification and excretion of xenobiotic compounds. The three metabolites have been assayed as AII receptor antagonist and show much lower activity than MK 954[5].

Acknowledgments

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