Maduramicin: Rat Metabolism of a Highly Potent Polyether Anticoccidial Examined by Carbon-13 NMR

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Maduramicin is unique among the agriculturally important polyether ionophore antiobiotics in that its structure contains a sugar moiety. Exclusively the parent compound (36%) and a single metabolite (64%) are detected in rat liver tissues at 0-day withdrawal after administering carbon-14-labeled maduramicin in feed at 5.2 ppm for 1 week. A single oral dose of 1 mg to 12 rats provided 3 mg of the metabolite in a pure form. Although this material was not the same as the major metabolite found in chicken liver (produced by the O-demethylation of one of two methoxy groups not attached to the sugar moiety), mass spectroscopy shows that it is an isomer. Carbon-13 NMR spectroscopy shows that it is an isomer of the major chicken metabolite in which O-demethylation occurs selectively at the sugar moiety.

INTRODUCTION

Maduramicin [ammonium (2*R*,3*S*,4*S*,5*R*,6*S*)-tetrahydro-2-hydroxy-6-[(*R*)-1-[(2*S*,5*R*,7*S*,8*R*,9*S*)-9-hydroxy-2,8-dimethyl-2-[(2*S*,2′*R*,3′*S*,5*R*,5′*R*)-octahydro-2-methyl-3′-[[(2*R*,4*R*,5*S*,6*S*)-tetrahydro-4,5-dimethoxy-6-methyl-2*H*-pyran-2-yl]oxy]-5′-[(2*S*,3*S*,5*R*,6*S*)-tetrahydro-6-hydroxy-3,5,6-trimethyl-2*H*-pyran-2-yl][2,2′-bifuran]-5-yl]-1,6-dioxaspiro[4.5]dec-7-yl]ethyl]-4,5-dimethoxy-3-methyl-2*H*-pyran-2-acetate], a polyether carboxylic ionophore antibiotic produced by the bacterium *Actinomadura yumaensis*, is a potent anticoccidial compound that is currently under development as a feed additive for chickens to prevent costly losses from the internal parasitic disease coccidiosis. It is a unique anticoccidial polyether ionophore in that its structure includes a sugar moiety with two methoxy groups (the G-ring, Figure 1). Previous investigations on the metabolism of monensin, a structurally similar and less potent polyether anticoccidial, using mass spectroscopy, in the steer, rat, and chicken have shown that O-demethylation and/or hydroxylation reactions predominate, although exact structures of metabolites could not always be assigned (Donoho et al., 1978 and 1982). Earlier investigations at the laboratories of American Cyanamid on the metabolism of maduramicin in the chicken have identified the major metabolite isolated from chicken liver (CLM) as the product arising from O-demthylation of one of two *O*-methyl groups on the A-ring of the parent, (Figure 1) (unpublished results). CLM has also been isolated as a minor product in the production of maduramicin by *A. yumaensis* and thus is available as an analytical standard. This study was initiated to compare metabolism of maduramicin in the rat.

MATERIALS AND METHODS

Carbon-14-Radiolabeled Maduramicin. Carbon-14-labeled maduramicin was prepared as the sodium salt from sodium propionate (C-1 labeled) in fermentation with *A. yumaensis*. The radiopurity (lot no. AC 3505:121, sp act. 8.43 mCi/mmol) was approximately 70% by thin-layer chromatography (TLC), and thus a sample was purified by column chromatography (13.5 mg on a silica gel column 2 × 20 cm, Woelm activity grade III/30; eluting with chloroform–ethyl acetate (1:1) to recover 8.92 mg (66%) between 80- and 120-mL elution volume). Material purified in this manner was shown to have >99% radiopurity by TLC.

Dosing Rats. Rats were treated with maduramicin either by mixing it into their feed or by gavage, using male Sprague–Dawley rats (approximately 210 g each) held individually in metabolism cages for 24-h prior to treatment. In feeding studies, four rats were administered maduramicin at 5.2 ppm in feed for 7 days (1000 g of rat chow treated with 1.2 mg of carbon-14-labeled maduramicin with a specific activity of 8.43 mCi/mmol plus 4.0 mg of unlabeled maduramicin in 20 mL of acetone, evaporated under vacuum and shaken for 4 h to achieve uniform mixing, to give a final specific activity of 1.94 mCi/mmol). Under these conditions rats consumed approximately 30 g of feed/day per rat.

In studies involving a single oral dose, rats were treated by gavage with maduramicin in a single oral dose (1 mg each), giving each rat an aqueous formulation of [^{14}C]-maduramicin (diluted to a specific activity of 0.0493 mCi/mmol). Rats treated by gavage were fed ad libitum until sacrifice by heart puncture and blood withdrawal.

Tissue Analysis. The liver and kidneys and portions of fat and muscle were removed, weighed, and ground after freezing with dry ice in a Waring blendor. After evaporation of the dry ice, aliquots of each tissue were weighed into combustion cones and combusted on a TRI-CARB Model 306 oxidizer with CARBO-SORB II (9 mL) and PERMAFLUOR V (12 mL) (Packard Instrument Co. Downers Grove, IL). Radioactivity was measured on a Searle Mark III Model 6880 liquid scintillation spectrometer (Searle Analytical, Inc., Des Plaines, IL).

Isolation and Purification of Liver Metabolite. Rat livers were homogenized in water (10 g of liver containing 40 mL of water and 250 mg each of sodium chloride and sodium bicarbonate) at 0 °C in a POLYTRON (Brinkmann Instruments, Westbury, NY). This aqueous homogenate was extracted three times with ethyl acetate (50-mL portions) and the organic layer separated by centrifugation (500*g* for 8 min). The combined organic extracts were evaporated under vacuum, and the residue was extracted with acetonitrile (25 mL). The acetonitrile portion was combined with hexane (5 mL), which was extracted with three portions of acetonitrile. The combined acetonitrile extracts were evaporated under vacuum, and the residue was purified by TLC (1 plate/20-g liver equivalent).

Thin-Layer Chromatography. TLC was performed with commerical precoated plates (silica gel 60 F-254, 20 × 20 cm × 0.25 mm, EM Science, Cincinnati, OH) either

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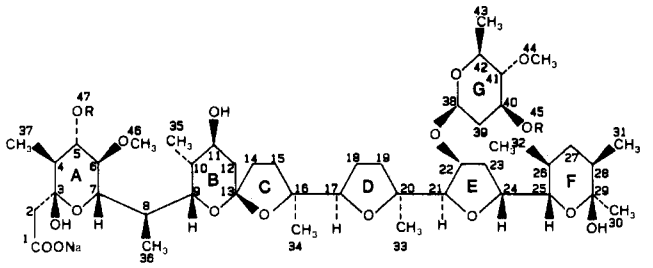

Figure 1. Structures of maduramicin and the rat and chicken liver metabolites: maduramicin, R_{45} = CH_3, R_{47} = CH_3; rat liver metabolite, R_{45} = H, R_{47} = CH_3; chicken liver metabolite, R_{45} = CH_3, R_{47} = H.

Table I. R_f Values[a] for Maduramicin and the Rat Liver and Chicken Liver Metabolites

		metabolites	
solvent system	maduramicin	rat liver	chicken liver
hexane–acetone	0.67	0.56	0.52
ethyl acetate–chloroform	0.47	0.24	0.20
ethyl acetate–ether–ammonium hydroxide	0.69	0.40	0.35
ethyl acetate–toluene–ammonium hydroxide	0.43	0.17	0.17

[a] Solvent systems and conditions given in Materials and Methods.

in two dimensions for cochromatography or by one-dimensional strip chromatography for isolation of metabolites. The following solvent systems were developed to separate metabolites from parent compound: ethyl acetate–ether–ammonium hydroxide (50:50:1); hexane–acetone (1:1); ethyl acetate–chloroform (7:3); ethyl acetate–toluene–ammonium hydroxide (66:33:1). Elution times for these solvent systems were between 90 and 120 min at room temperature (approximately 22 °C). Systems containing ammonium hydroxide were only used in two-dimensional TLC and were always used first. R_f values for maduramicin, CLM, and the rat liver metabolite (RLM) with these solvent systems are given in Table I. Maduramicin and its metabolites were visualized on two-dimensional TLC by autoradiography and by using a spray reagent (4 g of vanillin, 4 mL of concentrated sulfuric acid, 100 mL of methanol) followed by heating at 100 °C until the spot turned a characteristic purple color (about 2 min) (method modified from Donoho et al. (1978)). Isolation of larger amounts of RLM by TLC used ethyl acetate–chloroform on precleaned plates (washed by repeated elutions with chloroform–methanol (4:1) to remove any potential interfering contaminants.

Spectroscopy. Mass spectroscopy was performed with a Kratos MS 50 using fast atom bombardment (FAB) with the sample in a thioglycerol matrix using xenon as a source gas and an ion potential of 8 kV. Although metabolites were isolated and analyzed as Na^+ salts, substitution of the cation with K^+ and NH_4^+ was carried out directly in the thioglycerol matrix.

NMR spectra were recorded on a Bruker CPX-300 wide-bore superconducting NMR spectrometer. Proton (300 MHz) and carbon-13 (75 MHz) NMR spectra were obtained using 5-mm sample tubes with benzene-d_6 as a solvent. The deuterium signal from the solvent served as an internal field frequency lock. For proton NMR, 1500 transients were accumulated and the time domain signal was Fourier transformed to give the conventional frequency domain NMR spectra.

Proton-decoupled carbon-13 NMR experiments were carried out using a 5-mm tube in a 10-mm carbon-13 probe (3 mg of metabolite in 0.3 mL of solvent). A 30° flip angle pulse was used with an acquisition time of 1.032 s. Approximately 150K transients were accumulated in a 50-h period. A line-broadening parameter of 3 Hz was used in processing the data using 32K data points. The base-line distortions resulting from this weak sample were removed by using a convolution difference technique. Peak assignments for the carbon-13 spectrum of maduramicin were taken from Rajan et al. (1984).

RESULTS AND DISCUSSION

Metabolism of Maduramicin in Rats. At 0-time withdrawal after administering maduramicin at 5.2 ppm in feed for 7 days, tissue residues were as follows (ppm, ± standard error): muscle, 0.09 ± 0.01; liver, 26.1 ± 0.9; kidney, 0.34 ± 0.07; fat, 0.55 ± 0.03; blood, 0.01 ± 0.00. Only 4% of the total activity remained in the liver marc after extraction. TLC of the extractable residue and analysis by autoradiography and visualization with the vanillin/H_2SO_4 spray reagent showed that it consisted almost exclusively of two components. The higher R_f compound (total recovery of 110 μg, 36%) (R_f values in Table I) was shown to be the parent compound by cochromatography in four solvent systems with unlabeled authentic standard (ethyl acetate–toluene–ammonium hydroxide, ethyl acetate–ether–ammonium hydroxide, hexane–acetone, ethyl acetate–chloroform). The lower R_f metabolite (total recovery of 195 μg, 64%) did not cochromatograph with either CLM or maduramicin (R_f values in Table I).

In another experiment, three rats given a single dose of maduramicin by gavage with 1.0 mg each and sacrificed after 1, 2, and 6 days yielded recoveries of 530, 520, and 360 μg equiv in liver tissue, respectively, of which 15, 28, and 63% was the unknown rat liver metabolite (RLM), respectively. Cochromatography confirmed that the metabolite was identical with the one from the previous experiment. Similarly, the only other material recovered was parent. Thirteen rats dosed in this manner and sacrificed after 7 days yielded 3.0 mg of the pure RLM.

Mass Spectroscopy and NMR. Mass spectroscopy using FAB indicated that RLM was an isomer of CLM (the major metabolite isolated from chicken liver). Thus, in addition to a parent ion at m/z 925 (for the Na^+ salt), treatment with Na^+, K^+, or NH_4^+ of RLM directly in the thioglycerol matrix gave the corresponding Na^+, K^+, or NH_4^+, parent ions at m/z 925, 941, and 920, respectively. These results are consistent with CLM; however, since TLC data conclusively demonstrate that RLM is not CLM (see Table I), the conclusion is that it is an isomer that involves the O-demethylation of an alternate methoxy group.

Proton NMR spectra of maduramicin, CLM, and RLM are very complex. However, comparison of the methoxy region between 3.1 and 3.6 ppm of the three compounds (Figure 2) indicates that both CLM and RLM have lost one of four methoxy groups from different regions relative to the parent compound. Figure 2 shows the expected four singlets from the four methoxy groups of maduramicin, but only three singlets for CLM and RLM in the corresponding region of the spectra. The two most upfield methoxy singlets of CLM have chemical shifts nearly identical with those of two singlets in the parent, presumably arising from the two methoxy groups on the unaffected portion of the molecule, i.e. the sugar moiety (G-ring, Figure 1). With RLM the opposite effect is observed; the two most downfield singlets have nearly identical chemical shifts relative to the most downfield singlets observed for the methoxy groups of the parent. Presum-

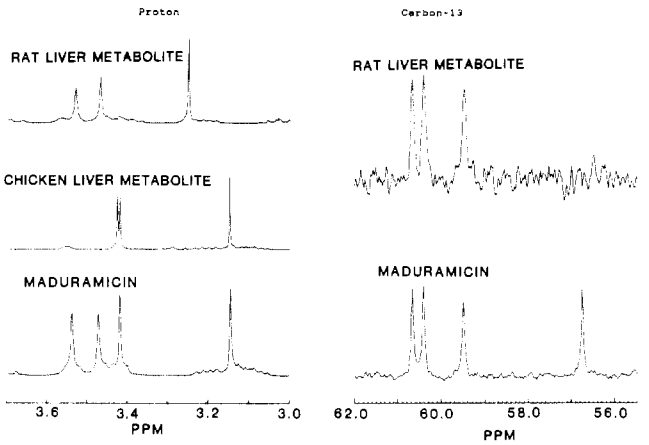

Figure 2. Comparison of the methoxy resonances of maduramicin and the rat liver metabolite in proton and carbon-13 NMR and of the chicken liver metabolite in proton NMR.

Table II. Selected Carbon-13 NMR Chemical Shifts[a] of Maduramicin[b] vs. Its Rat Liver Metabolite

carbon atom[c]	maduramicin	rat liver metabolite	change[d]
5	86.0	86.5	−0.5
6	82.5	82.5	0.0
39	37.4	40.2	−2.8
40	81.3	71.6	+9.7
41	86.6	87.7	−1.1
42	71.7	71.6	+0.1
44	60.6	60.7	−0.1
45	56.8	*e*	*e*
46	59.5	59.5	0.0
47	60.5	60.5	0.0

[a] In ppm relative to Me_4Si in deuteriobenzene. [b] Taken from Rajan et al. (1984). [c] See Figure 1 for carbon atom assignments. [d] (−) = downfield shift; (+) = upfield shift. [e] C-45 was absent from the metabolite spectrum.

ably these two singlets arise from the two methoxy groups on the unaffected portion of RLM, i.e. the A-ring (Figure 1).

Carbon-13 NMR provides conclusive evidence that the O-demethylation reaction producing the rat liver metabolite occurs at C-45 on the sugar moiety (Figures 1 and 2; Table II). In addition to the disappearance of the C-45 methyl carbon peak in the spectrum of the metabolite, the carbon atom on the other side of the relevant oxygen (C-40) is shifted upfield 9.7 ppm from 81.3 to 71.6 ppm (Table II). Also the two nearest neighbors to C-40 (C-39 and C-41) in the sugar moiety are shifted from 37.4 and 86.6 to 40.2 and 87.7 ppm, respectively (Table II). These observations are completely consistent with the changes in chemical shifts seen in the analogous carbon atoms in the comparison of maduramicin with CLM (Tsou et al., 1984) or in the comparison of the carbon-13 NMR spectra of cyclohexanol vs. cyclohexane methyl ether (Wehrli and Withlin, 1976). All other carbon nuclei in the metabolite give chemical shifts that are essentially indistinguishable from their equivalent nuclei in maduramicin.

Thus, rats treated orally with maduramicin either in feed at 5.2 ppm or by gavage with a single 1.0-mg dose efficiently produce a single major metabolite in the liver, which has been shown by carbon-13 and proton NMR and mass spectroscopy to represent an O-demethylation reaction at one of two methoxy groups of the sugar moiety. This is the first report of the involvement of a sugar moeity in the metabolism of a polyether carboxylate ionophore antibiotic. In contrast, metabolism of maduramicin in chickens involves O-demethylation of the A-ring. Metabolism examined to date of polyether anticoccidial compounds in mammals and chickens appears to primarily involve O-demethylation. In the case of maduramicin only the site of O-demethylation is different in rats vs. chickens.

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Registry No. Maduramicin, 79356-08-4; rat liver metabolite, 100702-75-8.

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Thin-Layer Bioautographic Assay for Salinomycin in Chicken Liver

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A thin-layer bioautographic method was developed for the determination of salinomycin in chicken liver. An aliquot of liver homogenate equivalent to 2 g of liver was extracted with isooctane. The extract was then purified on a silica gel column and chromatographed on silica gel thin-layer plates. The salinomycin zones were quantitated by a bioautography technique that makes use of *Bacillus subtilis*. The limit of detection was 25 ppb, and the assay response was linear from 25.0 to at least 200 ppb. The mean recovery of salinomycin from spiked samples was 108.2 ± 15.8% ($\bar{x}$ + SD).

INTRODUCTION

Sodium salinomycin is a polyether antibiotic that functions as an anticoccidial (Miyazaki et al., 1974) and that promotes growth (McClure et al., 1980). Its chemistry and biological activity have been previously described by Miyazaki et al. (1974).

Thin-layer bioautographic techniques that make use of *Bacillus subtilis* for the detection of monensin in animal tissues have been described by Donoho et al. (1967) and

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