THE UTILIZATION OF MOLECULAR OXYGEN DURING THE BIOSYNTHESIS OF MADURAMICIN

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Maduramicin sodium α ($C_{47}H_{79}O_{17}Na$) is a polyether ionophore antibiotic (Fig. 1) possessing a polyoxygenated carbon backbone and a 2,6-dideoxysugar[1,2]. It is a potent coccidiostat effective at a level of 5 ppm. We have established recently that the aglycone carbon skeleton of maduramicin is derived from eight acetate and seven propionate units and that the methoxy carbons are derived from methionine by feeding ^{13}C labeled precursors to cultures of *Actinomadura yumaensis*[3]. Also we have assigned unambiguously the ^{13}C NMR spectrum of this antibiotic[3,4]. The bio-origin of oxygen atoms in maduramicin has been studied in our laboratory by incorporation of [1-^{13}C, $^{18}O_2$]-acetate and [1-^{13}C, $^{18}O_2$]propionate in the fermentation cultures. It was concluded that five oxygen atoms O-(1), O-(6), O-(8), O-(9) and O-(14) are derived from acetate while three oxygen atoms O-(3), O-(4) and O-(7) originate from propionate. Based on these results, a biosynthetic scheme of maduramicin was proposed and it was postulated that at least three oxygen atoms O-(10), O-(11) and O-(13) in maduramicin were derived from molecular oxygen[3]. In this report, incorporation of $^{18}O_2$ gas into maduramicin is described[5].

In the literature, biosynthesis in $^{18}O_2$ gas resulted usually in poor yields of antibiotics. In order to increase the fermentation yield and simplify the experimental procedure, we have designed a closed system shaker flask, wherein the liberated CO_2 gas is absorbed in KOH solution as it diffuses through a sterile porous plug. The oxygen gas is supplied continuously as needed from a rubber balloon or, preferably, through a low pressure regulator connected to a cylinder of oxygen gas under pressure. With this equipment, it is convenient to switch from $^{16}O_2$ gas to $^{18}O_2$ gas after the fermentation has proceeded to the stage where vegetative growth is near completion and meaningful antibiotic production begins. The concentration of the dissolved oxygen in the fermentation can be increased by pumping out some of the air with a hand pump and then by replacing with oxygen gas. The details of the design (the apparatus was custom-made by ACE Glass Incorporated, Vineland, New Jersey) are shown in Fig. 2.

The fermentation using $^{18}O_2$ gas was carried out as follows. Five milliliters of the inoculum were transferred through the side arm to a 500-ml shaker flask containing 100 ml of the following medium (g/liter): glucose (30), soy flour (15), Mississippi lime $CaCO_3$ (1) and NaCl (2). Fifty milliliters of 30% KOH solution were added to the CO_2 gas trap. After half of the air inside

Fig. 1. The structure of maduramicin sodium α.

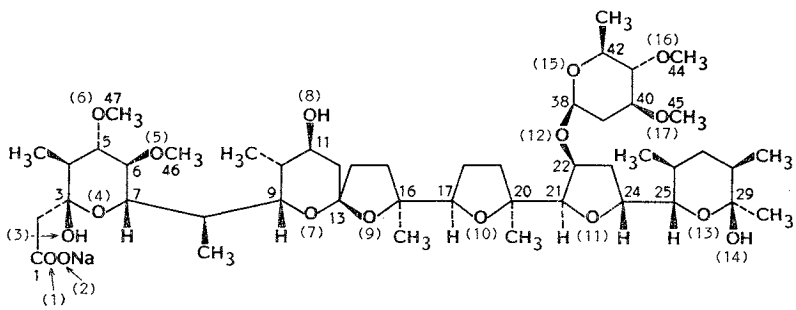

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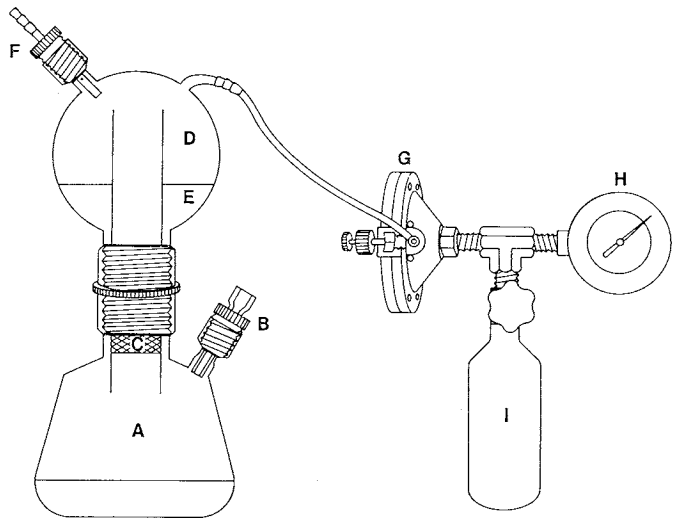
Fig. 2. Schematic of apparatus for the production of maduramicin in $^{18}O_2$ gas.

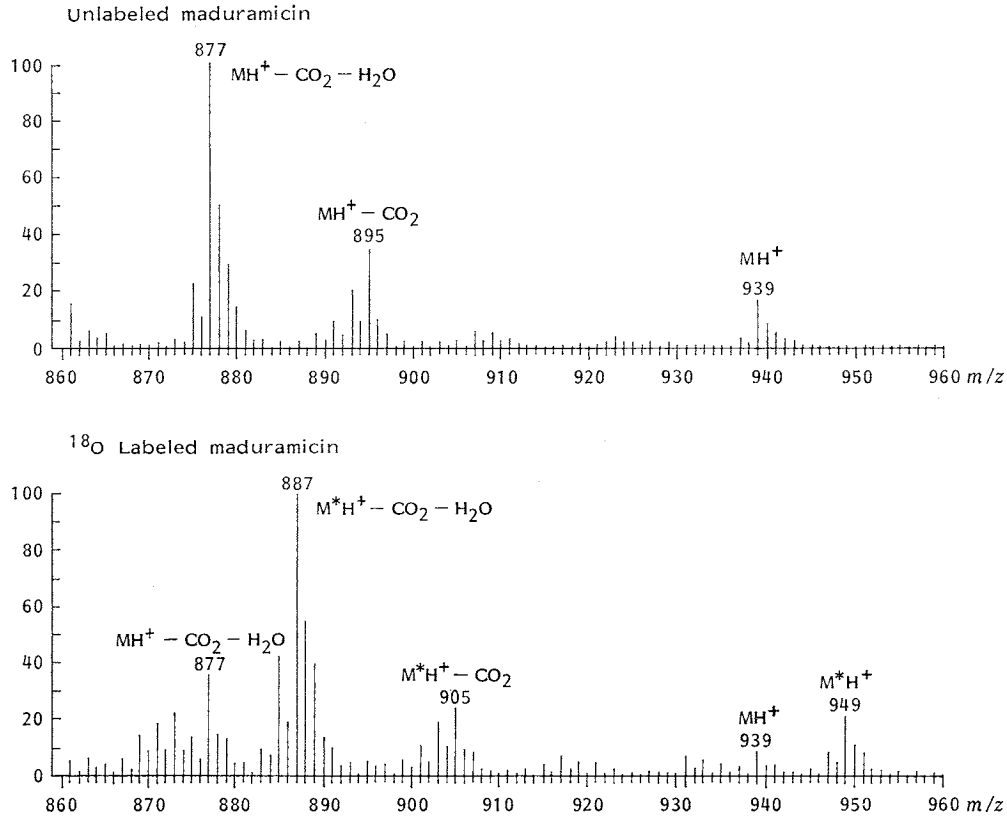
(A) 500-ml filter flask with wide mouth, 100 ml of culture medium; (B) side arm with septum for the addition or withdrawal of samples; (C) sterile porous plug; (D) 250-ml CO_2 gas trap; (E) 50 ml of 30% KOH solution; (F) gas outlet for the removal of air; (G) low pressure regulator; (H) pressure gauge; (I) lecture bottle containing oxygen gas.



the fermentation flask and the gas trap was removed by a hand pump (Nalge Company, Rochester, New York), a stainless-steel lecture bottle filled with $^{16}O_2$ gas was connected to the inlet of the gas trap through a pressure regulator (Model 3331, Matheson Gas Product, East Rutherford, New Jersey). The pressure of the regulator was set at 2.5-cm water drop below 1 atm. The fermentation was carried out in this closed system on a rotary shaker for 48 hours at 32°C with $^{16}O_2$ gas and then for further 120 hours under $^{18}O_2$ gas (95 atom % ^{18}O, Isotec Incorporated, Centerville, Ohio) at 32°C. At all times, oxygen gas was replenished continously to maintain 2.5-cm water drop below 1 atm with an approximately 1:1 ratio of oxygen and nitrogen gases in the closed fermentation apparatus. The rate of $^{18}O_2$ consumption was rapid (90 ml/hour) for the initial 9 hours and then remained steady at approximate 8 ml/hour. The antibiotic maduramicin was subsequently isolated by extraction of the whole mash with 300 ml of ethyl acetate after the addition of 0.8 g of sodium bicarbonate to yield 95 mg of crude product. Further purification by column chromatography on silica gel with methylene chloride - ethyl acetate (3:1), and recrystallization from ether - hexane afforded 28 mg of maduramicin sodium α, which was about one half of the yield of a normal run in open systems. Optimization of the fermentation yield in the presence of oxygen gas was not carried out. Although optimal dissolved oxygen tension for the biosynthesis of maduramicin has not been determined, we have established that fermentation yield of maduramicin in this closed system was decreased by one half when oxygen content in the gas phase was reduced from 50% to 20% at 1 atm.

An analysis of this ^{18}O labeled maduramicin sample by fast atom bombardment mass spectrometry (FAB-MS)[6] showed that the protonated molecular ion (MH^+) of the unlabeled maduramicin sodium α was shifted from *m/z* 939 to 949 as shown in Fig. 3. Similarly, two major fragment ions, *m/z* 895 (MH^+-CO_2) and 877 ($MH^+-CO_2-H_2O$), also shifted 10 daltons to *m/z* 905 and 887, respectively, indicating the major enriched product molecules contained five ^{18}O atoms. No detectable amount of the oxygen atoms in the CO_2 lost (O-(1) and O-(2)) or the (CO_2-H_2O) lost (O-(1), O-(2) and O-(3)) were ^{18}O labeled. Using the high-resolution FAB peak matching technique, the MH^+ ion of ^{18}O labeled maduramicin, *m/z* 949, was determined as 949.5467, which further confirmed

Fig. 3. Partial fast atom bombardment (FAB) mass spectra of unlabeled and ^{18}O labeled maduramicin.



the presence of five ^{18}O atoms in the structure. (Calculated value of $C_{47}H_{80}O_{12}{}^{18}O_5Na$= 949.5502, deviation=3.7 ppm.) Twenty FAB-MS spectra were recorded and averaged under the same operational condition for each of the control sample and labeled sample. Using the fragment pattern around *m/z* 877 ($MH^+ - CO_2 - H_2O$) of the unlabeled maduramicin, we have estimated that among the ^{18}O labeled molecules, 77% of the molecules contain five ^{18}O atoms, while 15% and 8% of the molecules contain four and six ^{18}O atoms, respectively (Table 1). In the averaged FAB-MS spectrum of the ^{18}O labeled maduramicin, comparison of the peak height of *m/z* 877 contributed from the unlabeled maduramicin and the portions of the peak heights of *m/z* 885, 887 and 889 contributed from the major fragment ions of $^{18}O_4$, $^{18}O_5$ and $^{18}O_6$ containing maduramicin, respectively, indicates that 77% of the product molecules are ^{18}O labeled and 23% contained no ^{18}O label and were presumably produced before the introduction of $^{18}O_2$ gas. The ^{18}O labeled maduramicin permitted the determination of fragmentation pattern and the locations of the ^{18}O atoms, which will be published separately[7].

The exact locations of the labeled ^{18}O atoms in maduramicin were established by ^{13}C NMR. The ^{18}O labeled maduramicin sodium α (22.4 mg) was diluted with equal amount of unlabeled maduramicin sodium α in C_6D_6 in a 5-mm NMR tube, and the broad band proton decoupled ^{13}C NMR spectrum was then recorded at 10°C on a Bruker CXP 300 spectrometer at 75.47 MHz. The spectrum (Fig. 4) showed distinct pairs of signals for C-17, C-20, C-21, C-29 and C-46, due to the presence of $^{13}C^{18}O$ resonances upfield by 0.020, 0.035, 0.028, 0.025 and 0.026 ppm, respectively, from the normal $^{13}C^{16}O$ resonances. The resonances for C-6, C-24 and C-25 were not resolved, however their half-height peak widths are about twice of those from the control sample (Table 2). Therefore, it is clear that O-(5), O-(10), O-(11) and O-(13) are ^{18}O enriched. Also two peaks at 96.33 ppm separated by 0.019 ppm are assigned to C-38

Table 1. The relative contribution of peak intensities from ^{18}O labeled maduramicin.

m/z[a]	Total relative peak intensity	Peak intensities contributed from maduramicin containing[b]			
		No ^{18}O label	$^{18}O_4$	$^{18}O_5$	$^{18}O_6$
877	35.3	35.3	—	—	—
883	10.7	—	4.2	4.3	—
885	40.0	—	17.5	22.1	0.4
887	100.0	—	5.1	92.1	2.2
889	37.0	—	1.0	26.1	9.4
891	10.5	—	—	5.0	2.7

[a] The major fragment ions ($MH^+ - CO_2 - H_2O$) of the unlabeled maduramicin and $^{18}O_4$, $^{18}O_5$ and $^{18}O_6$ containing maduramicin occur at m/z 877, 885, 887 and 889, respectively.

[b] The fragment patterns around $MH^+ - CO_2 - H_2O$ ions are estimated according to the fragment pattern around m/z 877 ($MH^+ - CO_2 - H_2O$) in the unlabeled maduramicin. In the unlabeled maduramicin, the relative peak intensities of m/z 873, 875, 877, 879 and 881 are 4.7, 24, 100, 29 and 5.4, respectively.

Fig. 4. Partial ^{13}C NMR spectrum of maduramicin sodium α showing the signals of carbons bearing ^{18}O.

The broad band proton decoupled ^{13}C NMR spectrum of a mixture (44.8 mg, 0.12 M) of 1:1 ratio of ^{18}O labeled and unlabeled maduramicin in 0.4 ml of C_6D_6 was recorded at 10°C in a 5 mm-sample tube on a Bruker CXP 300 spectrometer at 75.47 MHz.

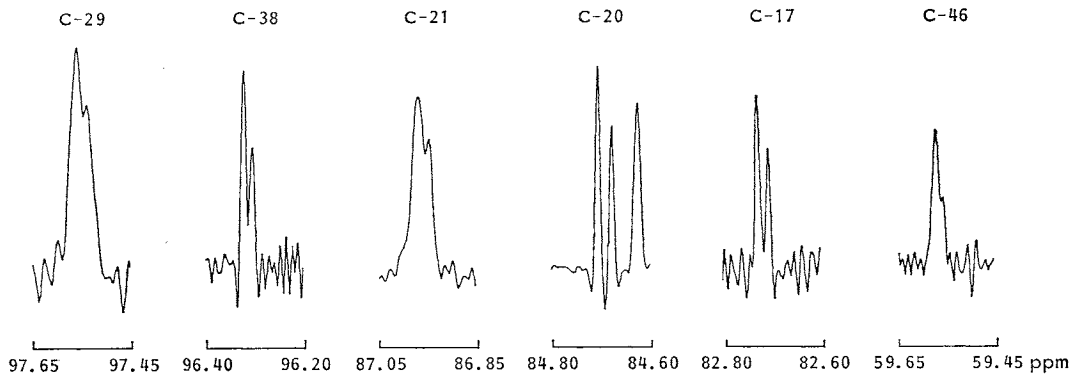


Table 2. The half-height peak widths.

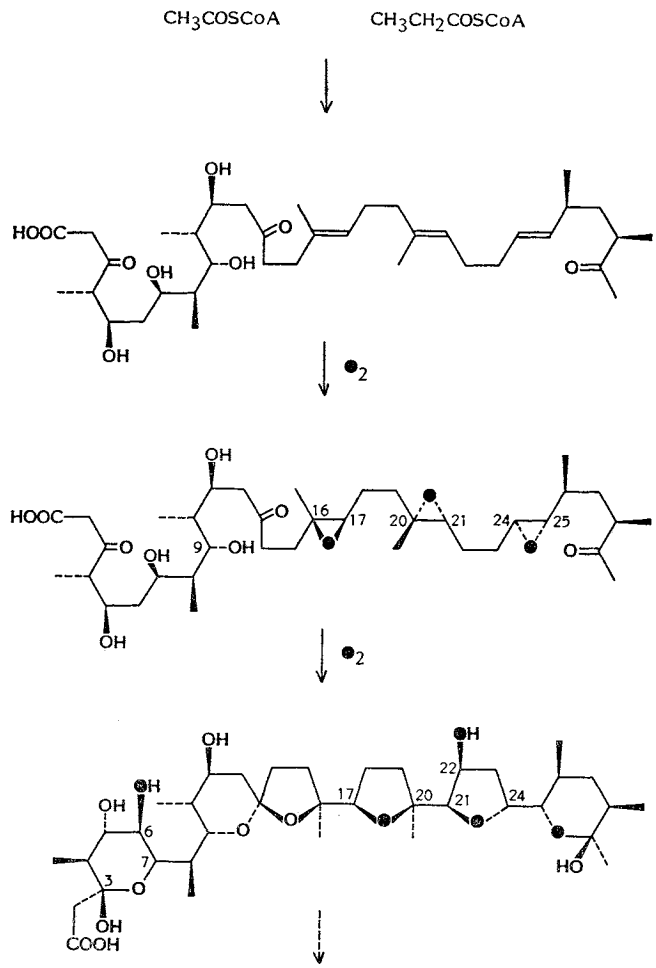
Carbon number	Chemical shift (ppm)[a]	Half-height peak width	
		Control sample (Hz)[b]	Labeled sample (Hz)[c]
6	82.53	3.2	6.0
24	80.34	3.1	10.0
25	73.29	3.4	5.6
42	71.73	1.7	1.7

[a] Bruker CXP 300, 75.47 MHz; spectral width 7,936 Hz; 45° pulse; 2.1 s pulse delay; resolution enhanced by Lorentz-Gauss multiplication of FID prior to Fourier Tansformation with line broadening −2 Hz and gaussian broadening parameter 0.35; 18,000~24,000 transients; 64 K data points; 0.242 Hz/data point.

[b] Unlabeled maduramicin (22.4 mg) was dissolved in 0.4 ml of C_6D_6 and the ^{13}C-H NMR spectrum was recorded at 10°C.

[c] An equal amount of ^{18}O labeled maduramicin (22.4 mg) and unlabeled maduramicin (22.4 mg) was dissolved in 0.4 ml of C_6D_6 and the ^{13}C-H NMR spectrum was recorded at 10°C.

Scheme 1. Postulated mechanism for the biosynthesis of maduramicin.



Maduramicin

and could be attributed to the presence of ^{18}O label in either O-(12) or O-(15). The fact that C-42 gives a sharp singlet and C-22 shows a shoulder with half-height peak width twice-wide as compared to the one in the control sample, indicates that O-(12) is enriched with ^{18}O label instead of O-(15). In the small fraction of molecules that contain six ^{18}O atoms, the extra ^{18}O atom is believed to be distributed among the oxygen atoms of the aglycone and originated from normal glucose metabolism with $^{18}O_2$ gas which would be used to give some ^{18}O labeled acetate and propionate as well as some ^{18}O labeled water. These low labeled precursors can then be incorporated into the antibiotic.

These data confirmed our previous prediction[3] that at least three oxygen atoms O-(10), O-(11) and O-(13) were derived from molecular oxygen (Scheme 1). In addition, O-(5) and the glycosidic O-(12) were shown to be derived from oxygen gas. As expected, none of the oxygen atoms derived from acetate or propionate was enriched by $^{18}O_2$ gas. This mechanism (Scheme 1) and the one for monensin[8,9] support the generalized triene-triepoxide pathway for acetate-propionate-propionate-acetate (APPA) polyethers proposed by CANE *et al.*[10] However, this is the first example to prove that the glycosidic oxygen, O-(12), comes from molecular oxygen. So far the origin of all the carbon and oxygen atoms in maduramicin has been determined except for those in the sugar backbone, which are neither enriched by acetate or propionate nor derived from molecular oxygen.

In the bacterial cell wall biosynthesis, D- and L-6-deoxypyranosides are derived from D-glucose through a common intermediate, *i.e.*, nucleotide-bound 4-keto-6-deoxy-α-D-glucose[11,12]. It is also known that in bacterial cells 3,6-dideoxy-L-mannose is biosynthesized from D-glucose through cytidine diphosphate-D-glucose (CDP-D-glucose), CDP-4-keto-6-deoxy-D-glucose and CDP-3,6-dideoxy-L-mannose consecutively[12]. It is conceivable that in the biosynthesis of maduramicin, D-glucose might be converted to nucleotide-bound 2,6-dideoxy-α-L-arabinopyranoside, which then proceeds through a transferase mechanism to form a glycoside of inverted configuration[13], *i.e.*, 2,6-dideoxy-β-L-arabinopyranoside. The anomeric center of this β-L-glycoside has the unusual chirality compared with β-D- and α-L-configurations from most of the polyether glycosides or macrolide glycosides[10,13] as predicted empirically by KLYNE's rule[14]. According to this postulate that the carbohydrate moiety of maduramicin originates from D-glucose unit, none of the oxygen atoms in the carbohydrate should be labeled by $^{18}O_2$ gas, except the glycosidic oxygen, O-(12). Work is continuing to clarify the bio-origin of the carbohydrate moiety of maduramicin.

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