Biosynthesis of Marcfortine A

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This report describes the results of a biosynthesis study of marcfortine A (MA). We report here that MA is derived from methionine, tryptophan, lysine and two isoprene units, the latter two being derived from acetic acid. From the ^{13}C enrichment pattern of the pipecolic acid moeity we further conclude that this unit is derived from lysine *via* α-ketoglutarate. Therefore, we have accounted for the biogenesis of every carbon atom of MA and established the biosynthetic pathway for the pipecolic acid moiety of MA.

Marcfortine A (MA) was isolated from the culture of *Penicillium roqueforti* by POLONSKY *et al.* in 1980[1]. We have recently encountered this fungal metabolite during our screening of microbial fermentations for bioactive compounds[2]. Our producing culture, UC 7780, was found to belong to the genus *Penicillium* with species yet to be determined. Structurally, marcfortines belong to a class of alkaloids which includes the paraherquamides[3] (PH) and brevianamides[4] (BV). Interest in the biosynthetic pathway of this class compounds has been focused on the formation of the novel bicyclo(2.2.2) ring system[5,6]. Biogenetically, it was reported that the basic skeletons of BV and PH are derived from dioxopiperazine formed by cyclization of tryptophan and proline[7]. The two isoprene units were presumed to derive from mevalonate for the biogenesis of BV. No experimental results regarding biogenetic precursors have ever been reported for the marcfortines. We therefore initiated a series of studies with the aim of identifying all biogenetic precursors of MA. This report describes the details of the incorporation of ^{13}C labeled acetate, D,L-lysine, L-methionine and L-tryptophan into MA.

Materials and Methods

Microbiological Methods

Penicillium sp. UC 7780 was grown in a minimal salts medium with sucrose as the sole carbon source (with the exception of labeled substrates). The composition of this medium was 20 g sucrose, 100 ml basal salts, 5 g MOPS, 50 mg K_2HPO_4, and 5 mg $CuSO_4$ per liter of tap water. The pH of this medium was adjusted to 7.0 using NaOH. The basal salts solution contained 46.7 g NaCl, 10.7 g NH_4Cl, 4.26 g Na_2SO_4, 2.03 g $MgCl_2 \cdot 6H_2O$, 290 mg $CaCl_2 \cdot 2H_2O$ and 3 mg $ZnCl_2$ per liter of deionized water. The complete minimal medium was sterilized by autoclaving 100 ml volumes contained in 500 ml large mouth fermentation flasks. The minimal medium was inoculated using seed cultures grown in GS-7F medium. GS-7F contained 25 g Pharmamedia, 25 g Cerelose and 5 g malt extract per liter of tap water with its pH adjusted to 7.2 using NH_4OH. GS-7F was sterilized in the manner of the minimal salts medium. The seed medium was inoculated using 3 agar plugs of UC 7780 stored over liquid nitrogen per flask . The seed culture was shaken at 200~250 rpm at 24°C for 36 hours and was used to inoculate the minimal salts medium at a 1~2% rate. The inoculated minimal salts medium was shaken in the manner of the seed culture for 24 hours before the labeled substrates were added as filter sterilized solutions. The fermentations were continued for an additional 2~3 days before harvest.

Methods for [1-^{13}C] Acetate (SIC, 90%) Addition

One gram of filter sterilized potassium [1-^{13}C] acetate (SIC, 90%) (pH 7.0) in 10 ml of water was added aseptically to 1 liter of UC7780 at 24 hours of fermentation. The fermentation was continued another 48 hours before harvest.

Methods for [2-^{13}C] Acetate (CIL, 99%) Addition

One and one half gram of filter sterilized potassium [2-^{13}C] acetate (CIL, 99%)acetate-2-C13 (pH 7.0) in 15 ml of water was added aseptically to 1.5 liters of UC7780 at 24 hours of fermentation. The fermentation was continued another 48 hours before harvest.

Methods for [1,2-^{13}C] Acetate (CIL, 99%) Addition

One gram of filter sterilized potassium [1,2-^{13}C] acetate (CIL, 99%) (pH 7.0) in 10 ml of water was added aseptically to 2.0 liters of UC 7780 at 24 hours of fermentation. The fermentation was continued for another 72 hours before harvest.

Methods for [Indole 2-^{13}C]-L-Tryptophane (CIL, 99%) Addition

Five hundred and seventy mg of filter sterilzed [indole-2-^{13}C]-L-tryptophane (CIL, 99%) in 113 ml of water were added aseptically to 1.5 liters of UC 7780 at 24 hours of fermentation. The fermentation was continued another 72 hours before harvest.

Methods for [1-^{13}C]-DL-Lysine (CIL, 99%) Addition

Five hundred mg of filter sterilized [1-^{13}C]-DL-lysine in 100 ml of water were added aseptically to 2.0 liters of UC7780 at 24 hours of fermentation. The fermentation was continued another 72 hours before harvest.

Methods for [*methyl*-^{13}C]-L-Methionine-(CIL, 99%) Addition

One gram of filter sterilized [*methyl*-^{13}C]-L-methionine (99%) in 2 ml of water was added aseptically to 2 liters of UC7780 at 24 hours of fermentation. Fermentation was continued another 72 hours before harvest.

Methods for Purification of MA

Two liters of harvested fermentation broth was centrifuged to obtain the mycelial portion which was homogenized with 3 × 330 ml of CH_2Cl_2. The combined solvent extracts were dried over $MgSO_4$ and evaporated to dryness *in vacuo* to give 123.1 mg of crude product. This was purified over 30 gm silica gel 60 (0.04~0.063 mm from EM Science) prepared with CH_2Cl_2 in a 2 × 25 cm Michel-Miller column. Stepwise gradient elution began with 0.5 liter CH_2Cl_2, then 1.9 liters of 0.5% MeOH in CH_2Cl_2; 1.6 liters of 1% MeOH in CH_2Cl_2 and finally 2 liters of 2% MeOH in CH_2Cl_2. Twenty ml fractions were collected and evaluated by TLC and UV quenching. Fractions containing MA were pooled, and on evaporation *in vacuo* gave 17 mg of labeled MA of sufficient purity for NMR study.

Methods of NMR Spectroscopy

Nuclear Magnetic Resonance [NMR] Analysis: Proton NMR (HMR) spectra were recorded in methylene chloride (d_2, 99.9 %D, Cambridge Isotope Laboratories, Cambridge, MA) solutions at 300 K (27°C) using a Bruker AM-300 spectrometer (Billerica, MA) operating at 300.13 MHz. Sample solution volumes were *ca.* 500 ·l. One-dimensional proton spectra were recorded as free induction decays (fids) of 32K complex points and Fourier transformed. A polynomial baseline correction was applied after phasing and before peak integration. Residual methylene chloride solvent peak was used as proton reference at 5.23 ppm.

The 2D COSY and ^{1}H-^{13}C heterocorrelation spectra were performed using standard acquisition methods. Sweep widths were typically 7~9 ppm for proton and 200 ppm for ^{13}C dimensions. Relaxation delays typically were 1.0 second. For long range C-H correlations, the COLOC[8] method was used. The D2 and D3 delays were 27 msec and 37 msec, respectively.

One dimensional ^{13}C NMR (CMR) spectra were obtained on a Bruker AM 300 spectrometer. In experiments to obtain the shift assignments, a delay of 1 second was used to obtain the broad-band and DEPT spectra. The center of the quintet of deuterated methylene chloride at 53.7 ppm was used as the carbon signal reference. For experiments where the relative intensities are measured, the reverse gated-decoupling pulse sequence was used in order to minimize NOE and relaxation effects. A delay of 15 seconds was used on all reverse gated-decoupling experiments. Under such experimental conditions, the control sample (unenriched MA) gave essentially equal peak intensity for all signals.

Results and Discussion

From the viewpoint of biosynthetic investigation, MA can be envisioned as an adduct of one tryptophan, one pipecolic acid, one methionine and two isoprenes. It is known that the isoprene is derived from acetate *via* mevalonate pyrophosphate while pipecolic acid derives from lysine. The carbon-13 labeled varieties of these four presumed biosynthetic precursors were therefore dispensed individually to the cultures at 24 hours after inoculation, and the cultivation was continued for an additional 48~72 hours. The resulting MA was purified and studied by NMR spectroscopy in six different experiments. The incorporation of these molecules are discussed below separately.

Assignments of HMR and CMR Spectra

Since complete and rigorous determinations of NMR spectral signals was a prerequisite for this study, the 300 MHz proton and 75 MHz carbon spectra were unambiguously assigned based on one-dimensional DEPT and two-dimensional ^{1}H-^{1}H homonuclear, ^{1}H-^{13}C heteronuclear one bond and multiple-bond correlation spectroscopy methods. The results are summarized in Table 1.

Incorporation of ^{13}C-Labeled Acetate

A) [1-^{13}C] Acetate

The first labeling experiment was the incubation of the UC 7780 culture with [1-^{13}C] acetate. The reverse gated proton decoupled CMR spectrum of purified MA showed that 5 of the 29 carbons are enhanced by 20-fold, one by 12-fold and another carbon by 3-fold. With the carbon assignments already determined for MA, the positions of ^{13}C-enriched carbons by [1-^{13}C] acetate can be located readily (see Table 1). The enrichment of carbons at positions 19, 21, 24 and 26 suggests that both isoprene units are derived *via* the mevalonate pathway[9].

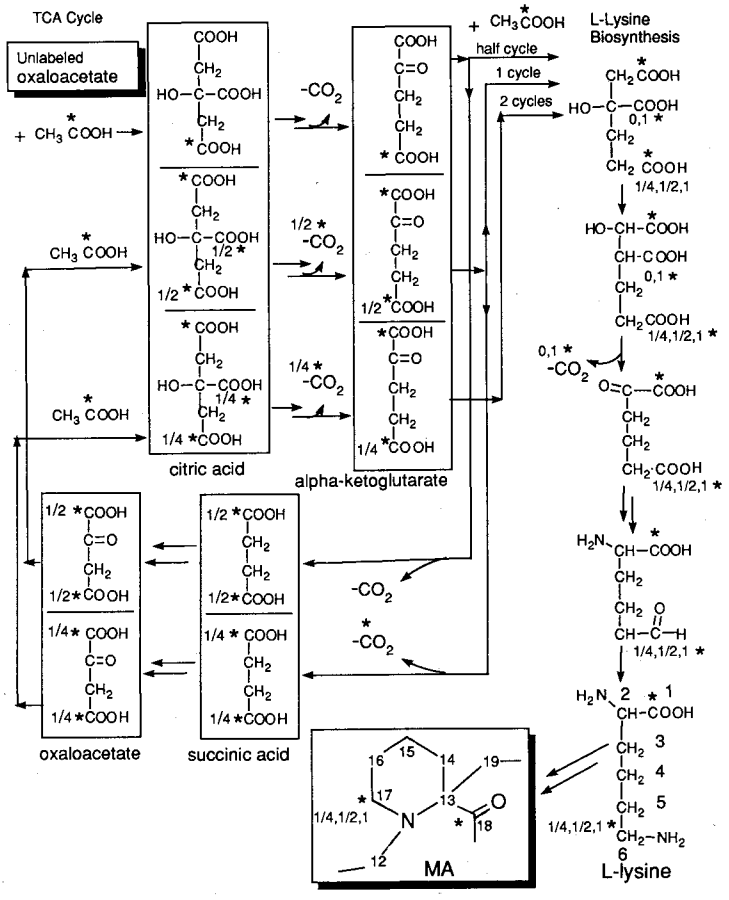
Within the pipecolic acid moiety only carbon atoms

Table 1. NMR assignments and results of C-13 labeled acetate additions during marcfortine production[a].

Carbon number (Chemical shift)	Enhancement by C-1 acetate	Enhancement by C-2 acetate	Carbon number	Enhancement by C-1 acetate	Enhancement by C-2 acetate
2 (182.9)	1	1	16 (26.3)	1	10
3 (63.3)	1	1	17 (54.7)	12	1.5[b]
4 (120.8)	1	1	18 (174.0)	20	1
5 (117.4)	1	1	19 (32.0)	20	1
6 (146.4)	1	1	20 (53.1)	1	20
7 (135.7)	1	1	21 (46.8)	20	1.5
8 (133.1)	1	1	22 (20.7)	1	22
9 (125.5)	1	1	23 (24.0)	1	22
10 (37.5)	1	1	24 (139.7)	20	1.5
11 (64.4)	1	1.5	25 (115.4)	1	20
12 (61.7)	3	1	26 (80.1)	20	1.5
13 (60.6)	1	20	27 (29.9)	1	22
14 (32.0)	1	5	28 (30.1)	1	22
15 (21.3)	1	4	29 (26.3)	1.5	1.5

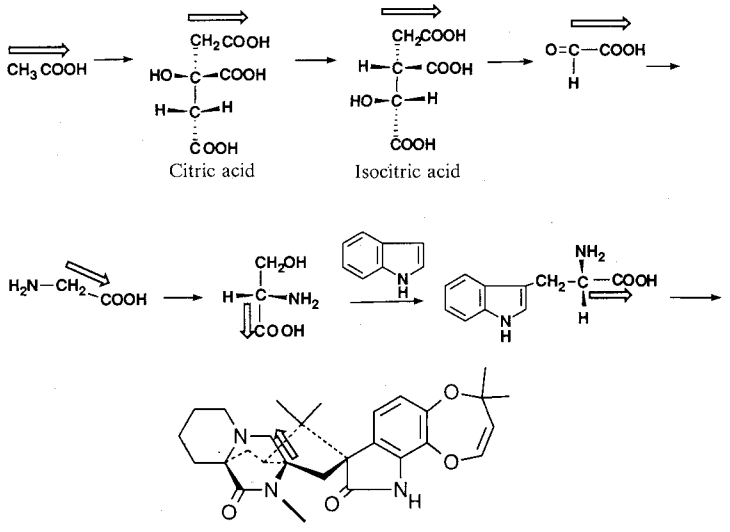
[a] The smallest peak is arbitrarily defined as 1 unit.
[b] Obtained by repeating the experiment in $CDCl_3$.

Scheme 1. Biosynthetic pathway of the pipecolic acid moiety of MA: Fate of acetate C-1.



17 and 18 are labeled. The 20-fold enrichment of carbon atom 18 implies that this carbon is derived from an acetate unit directly. The 12-fold enrichment of carbon atom 17 suggests that this carbon is also derived from acetate. However, this acetate unit had probably undergone a dilution process before being utilized. This enrichment pattern is consistent with the TCA cycle-α-ketoglutarate-lysine metabolic sequences[10,11] as shown in Scheme 1 in which the α-ketoglutarate unit is generated by the TCA cycle and shunted to the lysine

Scheme 2. Biosynthesis of tryptophan *via* glycine.



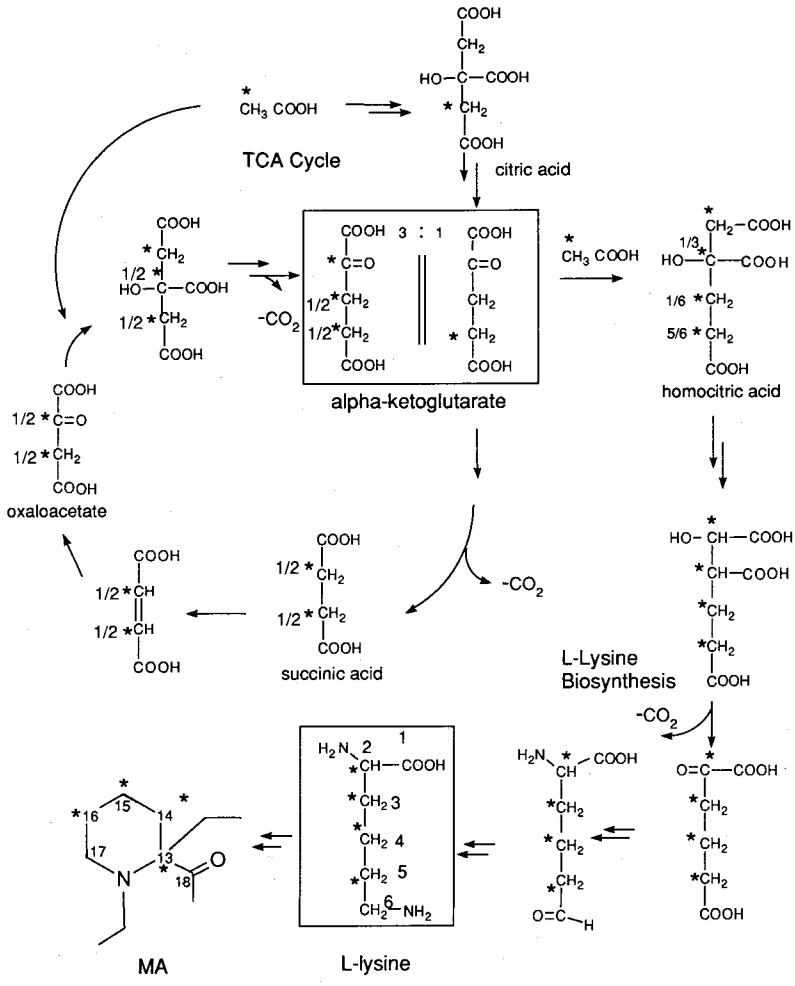
biosynthetic pathway. Carbon atom 17 therefore is derived sequentially from [1-^{13}C] acetate, α-ketoglutarate C-5, and lysine C-6, while atom 18 of MA is derived from [1-^{13}C] acetate *via* lysine C-1. The difference between the enrichment levels of atom number 17 and 18 is due to the dilution effect of the TCA cycle as shown in Scheme 1. The acetate unit added to the α-ketoglutarate unit to form homocitric acid, a precursor of lysine, would not be subjected to any isotope dilution. Thus, the pipecolic acid moiety formed through the combined pathways would be labeled more intensively at atom number 18 compared to 17. Equally significant, this scheme also explains the *lack of labeling* of the rest of the carbon atoms of the pipecolic acid moiety. Note that every complete TCA turn reduces the labeling of carbon atom 17 of MA by a factor of 2. Since we observed a relatively high enrichment level for this carbon, it can be concluded that the α-ketoglutarate unit is shunted from the TCA cycle rather efficiently. This phenomenon probably reflects the fact that the feeding of labeled acetate occurs at the late phase of the primary growth period.

Finally, the moderate enrichment of carbon at position 12 is due most likely to the combined dilution effects of the TCA cycle (or acetyl-CoA-glyoxylate cycle) and alternative amino acid synthesis for serine (from D-glyceric acid-3-phosphate)[12] on the biosynthesis of tryptophan from acetate (Scheme 2). The C-1 labeled acetate invariably labels isocitric acid at the carboxylic position which then sequentially labels the carboxylic carbon of glyoxylic acid, glycine, serine and tryptophan[13]. The carboxylic acid moiety of the tryptophan ultimately becomes the methylene group at the position 12 of MA.

B) [2-^{13}C] Acetate

The CMR spectrum of MA resulting from incorporation of [2-^{13}C] acetate showed that 7 of the 29 peaks are enhanced at least by 20-fold, 1 peak by 10-fold, and 8 peaks by factors between 1.5 and 5, as summarized in Table 1. The 20- to 22-fold enrichment of carbons at position 20, 22, 23, 25, 27 and 28 (all isoprene carbons) obtained by this experiment agrees reasonably well with that of the [1-^{13}C] acetate experiment. The same observation can also be made for carbon atom number 13, further supporting the hypothesis that the 13/18 pair of carbons are derived directly from acetate. Before we try to rationalize these results further in the context of the TCA cycle-α-ketoglutarate-lysine sequence, it is important to confirm our previous conclusion about the efficiency of the lysine shunt. As indicated in Scheme 3, the TCA turn would scramble the [2-^{13}C] acetate more extensively as opposed to the [1-^{13}C] acetate if the labeled material travels more than one cycle. As a result, the exiting α-ketoglutarate can be labeled quite differently both in terms of the location of the labels and the enrichment levels, depending on the number of the TCA turns the [2-^{13}C] acetate travels. Nontheless, it can be predicted from Scheme 3 that every complete TCA cycle would increase the relative enrichment of carbon number 17 of MA (the terminal carboxylic acid carbon of α-ketoglutarate). However, we observed very little labeling of this carbon by [2-^{13}C] acetate, indicating little scrambling of isotopes and therefore is in agreement with

Scheme 3. Biosynthetic pathway of the pipecolic acid moiety of MA: Labeling by C-2 acetate after half and single TCA cycle.



the [1-^{13}C] results. By assuming that α-ketoglutarate exits at and/or before two complete cycles, every carbon on the pipecolic acid ring would be labeled with carbon number 13 being the most enriched followed by carbon number 16, 14, 15 and 17, as we have observed. To summarize, the relative levels observed for the pipecolic acid moiety can be totally rationalized by the lysine pathway as indicated in Scheme 3. Finally, the small enrichments of carbon atoms 11, 21, 24, and 26 are probably due to the glyoxylate shunt where the isocitrate is shunted to form glyoxylate-malate-pyruvate-acetate in series. Thus, through the TCA cycle and the glyoxylate shunt the [2-^{13}C] acetate would label both acetate carbons.

C) [1,2-^{13}C] Acetate

To further confirm above findings and to show that the labeling represents a direct conversion of acetate into those carbons, dual labeled [1,2-^{13}C] acetate was added to the fermentation culture at 24 hours. The fermentation beer was harvested at day 4 and processed similarly to other feeding experiments (*vide supra*). The resulting pure MA was analyzed by CMR spectroscopy. The integration ratios of the signals agree fairly well with results from singly labeled incorporation experiments. The coupling constants are also measured for each signal (Table 2). These coupling constants, along with the multiplicity information, provide us a picture of the statistical distribution of isotopes. For example, the signal of carbon atom 13 is comprised of a singlet (4% of total area), a doublet (65%), a doublet of doublets (30%), and a doublet of triplets (1%). These data indicate that carbon atom number 13 and 18 are derived from one acetate unit with very little scrambling as the majority of signal intensity is derived from the doublet and doublet of doublets. The appearance of a doublet of doublets at 30% level reflects the fact that carbon atom number 19 is also significantly enriched. Another example of useful information provided by these coupling constants are the small satellites observed for carbon atom number 11 and

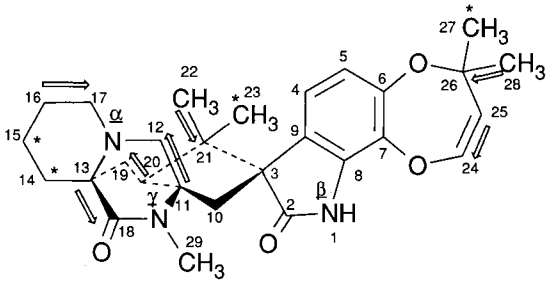
Table 2. CMR coupling pattern and constants resulted from dual labeled acetate incorporation.

Carbon number (Chemical shift)	Coupling pattern[a] (Coupling constant in Hz)	Carbon number	Coupling pattern (Coupling constant in Hz)
2 (182.9)	s	16 (26.3)	d (35), t (35)
3 (63.3)	d (30)	17 (54.7)	d (36)
4 (120.8)	s	18 (174.0)	d (47)
5 (117.4)	s	19 (32.0)	d (35), t (35)
6 (146.4)	s	20 (53.1)	d (35), t (35)
7 (135.7)	s	21 (46.8)	d (35), t (35), q (35)
8 (133.1)	s	22 (20.7)	d (35)
9 (125.5)	s	23 (24.0)	d (35)
10 (37.5)	s	24 (139.7)	d (78)
11 (64.4)	d (34)	25 (115.4)	d (78), dd (78, 47)
12 (61.7)	d (34)	26 (80.1)	d (40), t (40), dtd (47, 40, 4), ddd (47, 40, 4)
13 (60.6)	d (47), dd (47, 36)	27 (29.9)	d (40)
14 (32.0)	[b]	28 (30.1)	d (40)
15 (21.3)	d (34), t (34)	29 (26.3)	s

[a] s: Represents singlet only, d: represents doublet, t: represents triplet, q: represents quartet, d,t: doublet and triplet, dd: represents doublet of doublet, ddd: represents doublet of doublet of doublet, dtd: represents doublet of triplets of doublets, and qd: represents quartet of doublets. Singlets are not noted where multiplet appears.

[b] Coupling constants not measurable due to overlapping signals.

Fig. 1. Labeling pattern of marcfortine A by dual labeled acetate.



⟹ : Derived from an Intact CH_3COOH

* : Derived from a split acetate

12. The latter two have the same coupling constants and peak intensities, suggesting that the enrichment of these two carbons are derived from the same acetate unit. The much lower level is a result of isotope dilution as the tryptophan biosynthesis pathway utilizes acetate in an indirect fashion (*vide supra*). Fig. 1 and Scheme 4 are summaries of the information obtained from the analysis of the coupling patterns of each signal.

Incorporation of [*methyl*-^{13}C]-L-Methionine

To confirm that the *N*-methyl (C-29) is derived from the methyl group of methionine, we added [*methyl*-^{13}C]-L-methionine into the culture of the producing organism. The resulting MA sample produced a 10-fold enriched signal (Table 3) at the *N*-methyl position in the CMR spectrum. The results thus establish that the the *N*-methyl of MA is derived from the methionine methyl group.

Incorporation of [2-^{13}C]-L-Tryptophan

As suggested by the results from the C-1 labeled acetate experiment, C-12, -11, -10, and the indole moeity are most likely derived from tryptophan. To confirm this hypothesis, we incorporated [C-2 ^{13}C] tryptophan to the culture and harvested MA. The resulting CMR spectrum indicated that the C-2 signal is enriched by 70-fold and the C-29 signal is enriched by 10-fold (Table 3). As a result of the heavy enrichment of the C-2 signal, the neighboring C-3 signal is partially split into a doublet. The peak integration ratio of the doublet to the unsplit singlet is 2 : 1, agreeing well with the 70-fold enrichment of its neighboring carbon. The 10-fold enrichment of the C-29 signal can be attributed to the tryptophan catabolic pathway[14] where the C-2 carbon of the tryptophan is detached from the indole ring by the action of a dioxygenase. The C-2 carbon enters the one-carbon metabolite pool in the form of formate (Scheme 5) and is incorporated into the biosynthesis of methionine. As a result, its metabolic fate is same as that of methionine. It is of interest to note that the enhancement level of the *N*-methyl carbon obtained in this experiment is same as that in the methionine incorporation experiment. This suggests that the methyl addition happens at a relative late stage and utilizes a rather small endogeneous one-carbon metabolite pool.

Scheme 4. Biosynthetic pathway of the pipecolic acid moiety of MA: Labeling by dual labeled acetate.

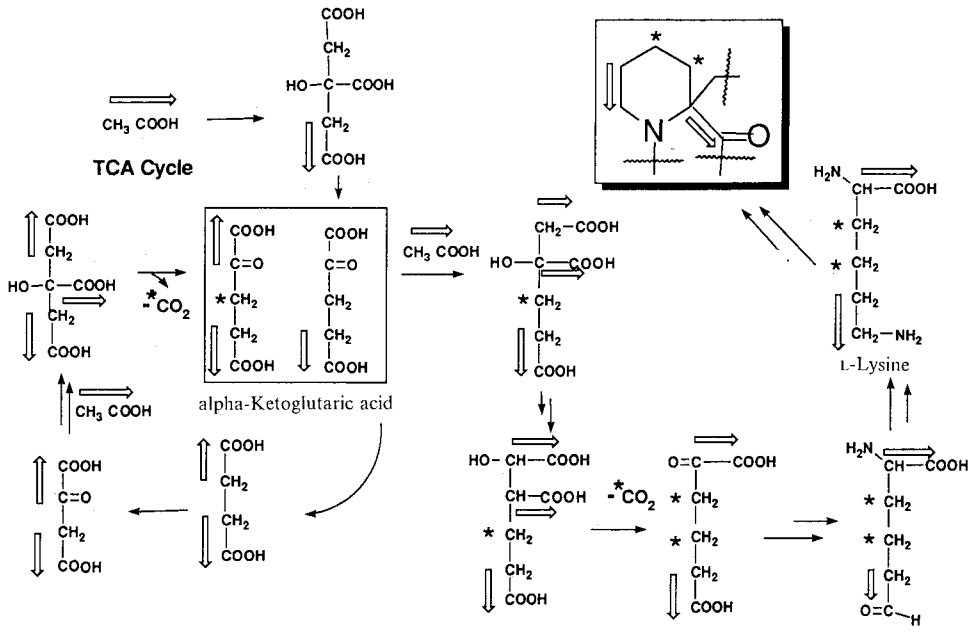


Table 3. Summary of carbon enrichment of MA by incorporation of C-13 labeled amino acids.

Labeled amino acid	Carbon number	Relative enhancement[a]
[*methyl*-^{13}C]-L-Methionine	29	10-fold
[2-^{13}C]-L-Tryptophan	2	70-fold
	29	10-fold
[1-^{13}C]-DL-Lysine	18	60-fold

[a] Obtained by comparing the peak intensities of signals of interest to the aromatic carbon signals. The absolute aromatic carbon intensities were shown to be comparable to the control sample.

Incorporation of [1-^{13}C]-DL-Lysine

The data from the acetate incorporation experiment suggested that the pipecolic acid moiety is lysine derived. To provide more definitive data to confirm this conclusion the incorporation of [C-1 ^{13}C]-D,L-lysine into MA was investigated. The CMR spectrum of the isotopically enriched MA shows that carbon atom number 18 is enriched by 60-fold (Table 3). This is reflected also in the signals of its neighboring carbons C-14 and -19, as each of these two carbons has a doublet flanking a singlet with approximately 1 : 1 : 1 ratio. This evidence clearly supports the conclusion drawn from the acetate incorporation experiments.

Scheme 5. Tryptophan catabolic pathway.

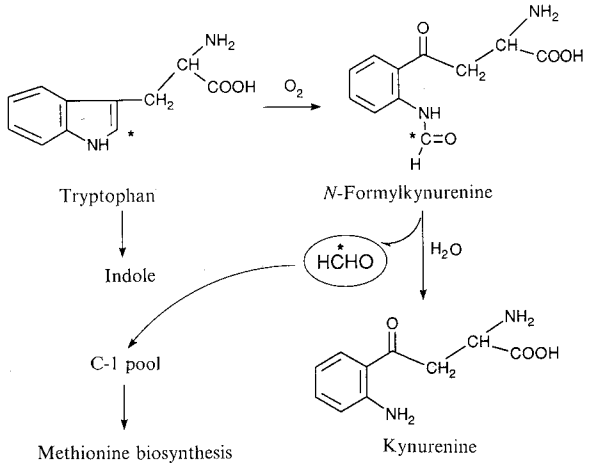
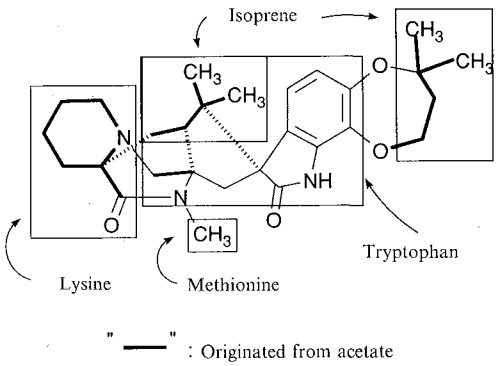


Fig. 2. Biogenesis of MA.



Conclusion

The ^{13}C-labeling studies reported here demonstrate quite clearly that MA is derived from methionine, tryptophan, lysine and two isoprenes. Furthermore, we have demonstrated that the isoprenes and lysine moieties are derived from acetic acid. Therefore, we have accounted for the biogenesis of every carbon of MA as shown in Fig. 2. By implication, we deduced that the three nitrogen atoms are derived from tryptophan and lysine. In an upcoming report, we will address the question of which of the two nitrogen atoms of the lysine is specifically incorporated into the pipecolic acid moiety and its implication to the overall biosynthesis of MA.

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