# Biosynthesis of Malonomicin. Part 1. $^{13}C$ Nuclear Magnetic Resonance Spectrum and Feeding Experiments with $^{13}C$-Labelled Precursors

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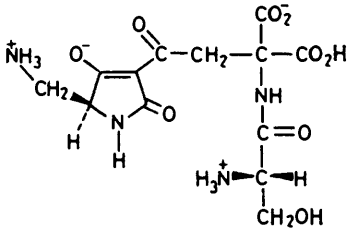
The $^{13}C$ n.m.r. spectrum of malonomicin (1) has been completely assigned. The biosynthesis of malonomicin was studied in *Streptomyces rimosus* by feeding experiments with $^{13}C$-labelled precursors. Evidence is presented that malonomicin is derived from 2,3-diaminopropanoic acid, one molecule of acetate, a $C_4$-dicarboxylic acid (presumably succinic acid), $CO_2$, and serine.

CULTURES of *Streptomyces rimosus var. paramomycinus* characteristically develop u.v. absorptions at 240 and 278 nm, shown to be associated with a metabolite active against *Trypanosoma* species, which cause sleeping sickness in cattle.[1] Chemical and spectral investigations,[2] and recently total synthesis [3] have established structure (1) for this bioactive compound which has been named malonomicin (previously K16).

Malonomicin was unique among natural products in containing an aminomalonic acid moiety. Recently a second aminomalonic acid derivative was reported to have been isolated from natural sources.[4] There has been some speculation about the occurrence of aminomalonic acid itself as a natural amino-acid.[5] L-Serine [6] has been mentioned as a possible precursor, and enzymes have been isolated which catalyse the transamination of oxomalonic acid to aminomalonic acid.[7] However, free aminomalonic acid has never been detected in any organism.

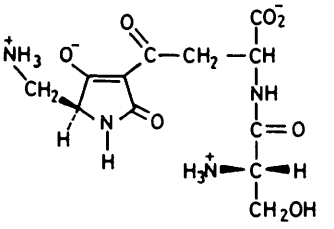
Although relatively uncommon, the tetramic acid nucleus present in malonomicin does occur in several natural products which usually possess biological activity.† The biosynthesis of three tetramic acids has been studied; in all cases the participation of an α-amino-acid and an acetate-derived precursor was demonstrated.[8]



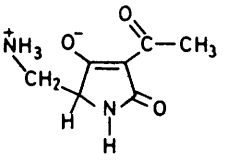
(1)

In the present paper, $^{13}C$ n.m.r. studies of malonomicin and biosynthetic investigations in *Streptomyces rimosus* are reported which provide additional evidence for the structure of malonomicin and indicate the mode of biogenesis of the tetramic acid nucleus and of the aminomalonic acid containing side-chain.

*Assignments of* $^{13}C$ *Resonances.*—The $^{13}C$ nuclear magnetic resonances of malonomicin (1) were assigned by comparison with resonances of the decarboxylation product of malonomicin (2), the model compound (3),[2] and serine (4), by use of single-frequency off-resonance decoupling (s.f.o.r.d.) to determine the number of attached protons at each carbon atom, and by selective



(2)



(3)

deuteriation studies. The natural-abundance $^{13}C$ n.m.r. spectrum of malonomicin in $H_2O$ at pH 8.5 (proton noise-decoupled; p.n.d.) is shown in Figure 1. $^{13}C$ Resonances of (1)—(4) are summarized in Table 1.

Roughly three different groups of resonances are observed in the natural-abundance p.n.d. $^{13}C$ n.m.r. spectrum of malonomicin. A group of six resonances is due to the $sp^3$-hybridised carbon atoms, one resonance is seen in the olefinic carbon atom region, and the position of a group of five resonances is typical for carbonyl carbon atoms. In malonomicin, six carbonyl groups are present. However, the carbonyl carbon resonance at δ 175.4 p.p.m. with about twice the intensity of the other

TABLE 1

$^{13}C$ Chemical shifts (at 22.63 MHz) of malonomicin (1) and model compounds (2)—(4) in $H_2O$ at pH 8.5 [in p.p.m. downfield from $Me_4Si$ ($\delta_C$ 0), calculated from internal dioxan ($\delta_C$ 66.5); multiplicities are indicated with s, d, t, and q]

| Carbon atom * | (1) | (2) | (3) | (4) |
|---|---|---|---|---|
| 1 | 178.2 (s) | 178.0 (s) | 177.8 (s) | |
| 2 | 102.8 (s) | 102.2 (s) | 102.8 (s) | |
| 3 | 193.8 (s) | 193.7 (s) | 194.9 (s) | |
| 4 | 57.0 (d) | 56.5 (d) | 58.6 (d) | |
| 5 | 40.6 (t) | 39.7 (t) | 41.7 (t) | |
| 6 | 194.1 (s) | 193.1 (s) | 195.9 (s) | |
| 7 | 44.3 (t) | 41.3 (t) | 14.0 (q) | |
| 8 | 67.2 (s) | 51.5 (d) | | |
| 9/10 | 175.4 (s) | 177.5 (s) | | |
| 11 | 170.2 (s) | 172.3 (s) | | 178.9 (s) |
| 12 | 55.8 (d) | 55.3 (d) | | 57.5 (d) |
| 13 | 63.1 (t) | 62.4 (t) | | 63.9 (t) |

* For numbering system of malonomicin, see Figure 1.

† For a survey, see ref. 3.

carbonyl carbons, is assigned to the coincident resonances of the two carboxylic carbon atoms C-9 and -10. Evidence for this assignment is provided by decarboxylation of malonomicin to give (2),[2] which causes a decrease in signal intensity of this particular resonance, accompanied by a downfield shift of 2.1 p.p.m., in agreement with similar phenomena observed in α-carboxyaspartic acid and its decarboxylation product DL-asp.[9]

Decarboxylation of malonomicin also causes a strong upfield shift of the signal at δ 67.2 p.p.m., assigned to C-8, a smaller upfield shift of the resonance at δ 44.3 p.p.m. (which is a triplet in the s.f.o.r.d. spectrum), and a downfield shift of the δ 170.2 p.p.m. carbonyl $^{13}C$ $D_2O$ at pD 9.6 (which notably effects the exchange of the α-proton of α-amino-acids)[11] had a pronounced collapsing effect on the δ 55.8 p.p.m. resonance. Prolonged treatment of malonomicin with NaOD at pD 14 also caused a relatively slow proton–deuterium exchange at C-7 which provided corroboration for its assignment.

The C-3 resonance was expected to show a titration shift at pH 8—10, as a consequence of the proximity of the $\overset{+}{N}H_3$-group at C-5 which has $pK_a$ 9.2.[2] The behaviour of the δ 193.8 p.p.m. resonance in this pH region is in agreement with this expectation (Δδ 1.4). The nearby carbonyl resonance at δ 194.1 p.p.m. is not pH-dependent and is assigned to C-6.

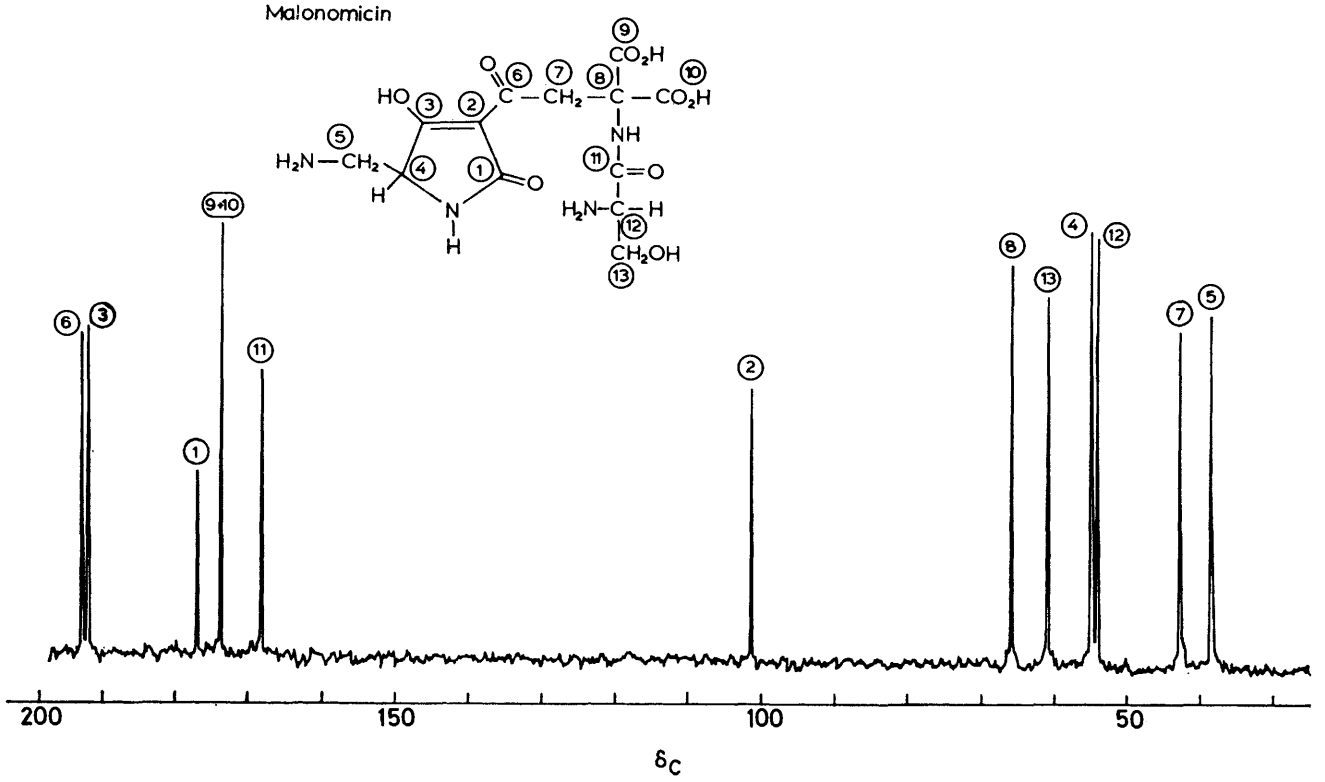



FIGURE 1 Natural-abundance proton noise-decoupled 22.63 MHz $^{13}C$ spectrum of malonomicin (1) in $H_2O$ at pH 8.5; spectral width 6 000 Hz; pulse delay 5 s; radiofrequency pulse 45°; transients 8 000; data points 4 K after Fourier transform

resonance. Therefore, the two latter resonances are assigned to C-7 and -11, respectively, in agreement with the known effects due to removal of a carboxy-group, *i.e.* downfield shift of the carbon atom in the β-position and upfield shift of the carbon atom in the γ-position, respectively.[10]

Assignment of the resonances at δ 57.0 and 55.8 p.p.m. (both are doublets in the s.f.o.r.d. spectrum) could be made by selective deuteriation. Proton–deuterium exchange at C-4 * in $D_2O$ at pD 14 (monitored also by following the simultaneous racemization by means of optical rotation measurement) caused a collapse of the resonance at δ 57.0 p.p.m., while proton–deuterium exchange at C-12 by means of pyridoxal phosphate in

* The fast racemization of C-4 substituted tetramic acids has been noticed before, *e.g.* ref. 8*c*.

The remaining resonances at δ 178.2, 102.8, and 63.1 p.p.m. can be readily assigned to C-1, -2, and -13, respectively, on the basis of their chemical shifts and multiplicities, and by comparison with the $^{13}C$ spectra of (2)—(4). The enrichment studies (see below) provide ample confirmatory evidence for all the assignments made.

*Incorporation Studies.*—Biosynthetic studies on tenuazonic acid,[8a] erythroskyrine,[8b] and cyclopiazonic acid,[8c] three other acyltetramic acids derived from micro-organisms, have revealed that the heterocyclic nucleus originates from the condensation of an acetate-derived precursor and an α-amino-acid, *i.e.* isoleucine, valine, and tryptophan, respectively. By analogy, the corresponding α-amino acid in malonomicin was expected to be 2,3-diaminopropanoic acid (DAP). Indeed, addition

of DL-[1-$^{13}$C]DAP to a culture of *Streptomyces rimosus* (for details, see Experimental section) caused a high incorporation at C-3 (*ca.* 16.5% of $^{13}$C label in excess over natural abundance, Table 2). DAP is not a common amino-acid, though a number of secondary metabolites containing it have been found, *e.g.* the peptide antibiotic viomycin.[12] The origin of DAP in viomycin has been proven to be L-serine. Likewise, DL-[1-$^{13}$C]serine, added to a culture of *S. rimosus*, was incorporated into malonomicin, and the labelling pattern showed enrichments at C-11 and (only slightly less) at C-3, which suggests a rather direct pathway from serine to DAP. The mechanism of this transformation is under further investigation.

TABLE 2

Excess $^{13}$C abundance (%) at individual positions in $^{13}$C-enriched samples of malonomicin

| Carbon | [1-$^{13}$C]NaOAc 200 mg/0.5 l [a] | [2-$^{13}$C]NaOAc 200 mg/0.5 l | [1,2-$^{13}C_2$]NaOAc 200 mg/0.5 l | [1-$^{13}$C]Malonate 270 mg/l |
|---|---|---|---|---|
| 1 | 7.0 | 0.5 | 5.0 | 1.0 |
| 2 | 0.5 | 9.0 | 4.0 | |
| 3 | 0.5 | | 0.5 | |
| 4 | | | | |
| 5 | | | | |
| 6 | 4.0 | 4.0 | 4.5 | 0.5 |
| 7 | | 3.5 | 3.5 | |
| 8 | | 4.5 | 3.0 | |
| 9/10 | 6.0 | 5.0 | 9.0 | 0.5 |
| 11 | 0.5 | 0.5 | 1.0 | |
| 12 | | | | |
| 13 | | | | |

| Carbon | DL-[1-$^{13}$C]DAP 300 mg/l | DL-[1-$^{13}$C]Ser 300 mg/l | [1,4-$^{13}C_2$]Succinate 150 mg/0.5 l | [$^{13}$C]$NaHCO_3$ 300 mg/l | [3-$^{13}$C]Oxalacetate [b] 200 mg/0.5 l | [3-$^{13}$C]Oxalacetate [c] 150 mg/0.5 l |
|---|---|---|---|---|---|---|
| 1 | | | | | 0.5 | |
| 2 | | | | | 3.0 | 0.5 |
| 3 | 16.5 | 5.0 | | | | |
| 4 | | | | | | |
| 5 | | | | | | |
| 6 | | | 4.5 | | 2.0 | 0.5 |
| 7 | | | | | 3.5 | 2.0 |
| 8 | 0.5 | 0.5 | | | 3.0 | 1.5 |
| 9/10 | 0.5 | 1.0 | 6.5 | 3.5 | 2.5 | 1.0 |
| 11 | 0.5 | 7.5 | | | | |
| 12 | | | | | | |
| 13 | | | | | | |

[a] Amount of addition per volume. [b] Single addition. [c] Continuous addition.

The source of at least one of the carboxy-groups (C-9 or -10, which are coincident in the $^{13}$C n.m.r. spectrum) was found to be $CO_2$-derived. After feeding with $NaH^{13}CO_3$, only C-9 or -10 showed an enrichment, *ca.* 3.5% of $^{13}$C label in excess over natural abundance (Table 2).

The origin of C-1, -2, and part of the acyl substituent attached to the heterocyclic nucleus, was expected to be acetate-derived, analogous to the other acyltetramic acids investigated,[8] and this was clearly shown by feeding [1-$^{13}$C]acetate. Enrichments were observed at C-1 and -6, and also at C-9 or -10 (Table 2). The $^{13}$C n.m.r. spectrum obtained on feeding [1-$^{13}$C]malonate showed the same labelling pattern, though with a lower degree of enrichment (corrected for loss of label by scrambling over C-1 and -3 of the singly labelled malonic acid), suggesting that acetate is incorporated without conversion to malonate. The *ratio* of incorporation at positions 1, 6, and 9 or 10 is about the same for acetate and malonate, which indicates that malonate is decarboxylated to acetate prior to incorporation into malonomicin.

At this stage of the investigation it was not clear whether the labelling of C-9 or -10 by [1-$^{13}$C]acetate originated directly from the carboxy-group of an acetate unit or from $^{13}CO_2$ generated by degradation of acetate in the tricarboxylic acid (TCA) cycle (Figure 2) or even from both sources. The latter possibility became plausible by feeding [2-$^{13}$C]acetate. Not only C-2 and -7 were labelled, as expected, but also C-8, C-9 or -10, and even C-6 (see Table 2). The more or less equal labelling of C-7 and -8, accompanied by comparable labelling of C-6 and C-9 or -10 (with the latter in excess, which is probably a consequence of $^{13}CO_2$ formation and incorporation) could be accounted for by assuming a $C_4$-dicarboxylic acid derived from the TCA cycle as a source of these carbon atoms. Indeed, in the TCA cycle (Figure 2) the label of [2-$^{13}$C]acetate is distributed equally over the central carbon atoms of a number of $C_4$-dicarboxylic acids after formation of the symmetrical succinic acid in a first turn, leading to labelling of C-7 and -8. In a second turn, these labels are transposed partially to the carboxy-groups (labelling of C-6 and C-9 or -10); finally, they are extruded in the form of $^{13}CO_2$ (extra labelling of C-9 or -10).

An additional experiment with [1,2-$^{13}C_2$]acetate furnished a clear proof of the incorporation of three acetate units, *i.e.* C-1 + C-2, C-6 + C-7, and C-8 + C-9 or -10. Of these, C-1 + C-2 were fully coupled, demonstrating that an *intact* acetate unit is used exclusively for the biosynthesis of the heterocyclic nucleus of malonomicin. C-6 + C-7 and C-8 + C-9 or -10 were only partially

coupled, in accordance with the assumed conversion to $C_4$-dicarboxylic acids in the TCA cycle prior to incorporation into malonomicin. Coupling constants $^1J_{C-C}$ and $^2J_{C-C}$ were determined and are given in Table 3. They all agree with literature values.[10]

Straightforward evidence for the incorporation of an *intact* $C_4$-dicarboxylic acid, either directly from the TCA cycle or in equilibrium with it, was obtained in a feeding experiment with [1,4-$^{13}C_2$]succinic acid. By measuring *intensities* (the technique mostly used in biosynthetic studies with $^{13}C$-labelled precursors), it seemed at first that only C-9 or -10 was enriched. But by measuring *peak integrals*, an enrichment at C-6 also was clearly indicated. The observed line broadening of the C-6 and C-9 or -10 resonances is caused by a three-bond $^{13}C-^{13}C$ coupling which is not resolved in the normal 6 000 Hz spectrum on 4 K data points. However, by measuring a 1 200 Hz spectrum on 4 K data points, both C-6 and C-9 or -10 were partially resolved into two doublets with a coupling constant of 2.6 Hz. Thus, in order to avoid errors in enrichment studies caused by unresolved couplings it is more reliable to use integrals than intensities of resonances. The higher extent of labelling at C-9 or -10 than at C-6 may again be caused by incorporation of $^{13}CO_2$, generated by one turn of [1,4-$^{13}C_2$]succinic acid in the TCA cycle.

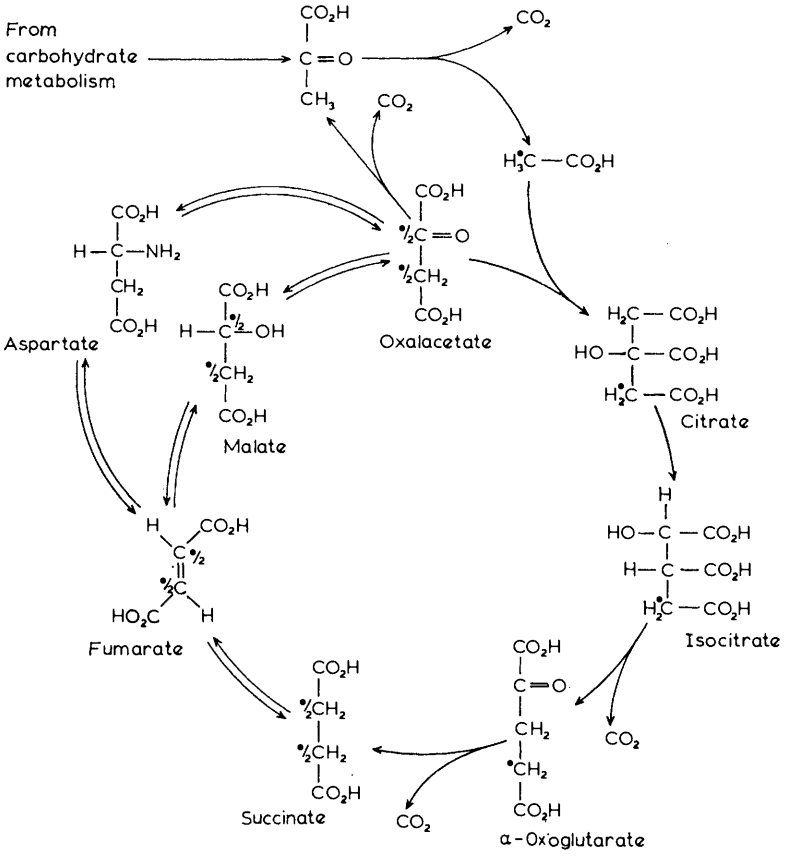



FIGURE 2 Reactions of the tricarboxylic acid cycle. The symbol ● designates positions of label from entrance of $CH_3$-labelled acetate into the cycle (one turn). Note that it is *not* two carbon atoms from acetate which are immediately removed as $CO_2$ but two atoms from oxalacetate. Only after several turns of the cycle are the carbon atoms of acetate completely converted into $CO_2$

TABLE 3

Couplings observed in [1,2-$^{13}C_2$]acetate-, [2-$^{13}C$]acetate-, DL-[1-$^{13}C$]DAP-, and [1,4-$^{13}C_2$]succinate-derived malonomicin

| Carbons | $^1J(^{13}C-^{13}C)$/ Hz | $^2J(^{13}C-^{13}C)$/ Hz | $^3J(^{13}C-^{13}C)$/ Hz |
|---|---|---|---|
| 1–2 | 68.8 | | |
| 2–3 | 65.0 | | |
| 2–6 | 65.5 | | |
| 2–7 | | 11.7 | |
| 3–4 | 40.0 | | |
| 6–7 | 41.1 | | |
| 6–9 | | | 2.6 |
| 7–8 | 42.5 | | |
| 8–9/10 | 50.1 | | |

On the basis of this result, oxalacetic acid was regarded as an attractive direct precursor of malonomicin because of the presence of functional groups considered suitable for the construction of the substituted aminomalonic acid moiety at a certain stage of the biosynthesis. However, this possibility was ruled out by the result of a feeding experiment with [3-$^{13}C$]oxalacetic acid. Instead of only carrying a considerable excess of label at C-7 or -8, the malonomicin isolated showed nearly the same complex pattern of enrichment as obtained with [2-$^{13}C$]-acetate, the ratio and extent of enrichment of C-2, -6, -7,

-8, and C-9 or -10 being dependent on the rate of oxalacetic acid addition to the culture medium. When adding the labelled oxalacetic acid all at once, the result was almost the same as with [2-$^{13}$C]acetate which can be explained by assuming a rapid degradation of oxalacetic acid to acetic acid *via* pyruvic acid. When the exogenic oxalacetic acid concentration was kept low by continuous feeding during the malonomicin production (*ca.* 16 h), the labelling of C-2 (demonstrated to be directly acetate-derived, see above) was significantly less; apparently, degradation to acetic acid had been suppressed considerably by this method. However, the labelling pattern still closely resembled that obtained with [2-$^{13}$C]acetate feeding, with the conspicuous feature of almost equal labelling of C-7 and -8, regardless of the feeding method.

Although the evidence presented is not yet conclusive, the scrambling of the label of [3-$^{13}$C]oxalacetic acid over C-6, -7, -8, and C-9 or -10, the equal labelling of C-7 and -8 both from [2-$^{13}$C]acetate and from [3-$^{13}$C]oxalacetic acid, and the incorporation of intact succinic acid, in our view, point to a *symmetrical* TCA-cycle $C_4$-dicarboxylic acid, *i.e.* succinic or fumaric acid, as a direct precursor in the biosynthesis of malonomicin. The distribution of label from [3-$^{13}$C]oxalacetic acid over C-7 and -8 can be explained as the result of metabolic equilibrium existing between oxalacetic acid and the symmetrical succinic or fumaric acid *via* malic acid and/or aspartic acid (see Figure 2). In the same train of thought, the considerable quantity of label on C-6 and C-9 or -10 is a consequence of the irreversible conversion of [3-$^{13}$C]oxalacetic acid into carboxy-labelled succinic or fumaric acid *via* decarboxylation of oxoglutaric acid in the TCA cycle. Whether succinic or fumaric acid (which also is incorporated as tested with [1,4-$^{13}C_2$]fumaric acid *) is this direct precursor of C-6, -7, -8, and C-9 or -10, cannot be inferred yet, but preliminary results obtained with deuteriated precursors (monitored by $^2$H n.m.r.) can be interpreted in favour of succinic acid.

The order in which the simple precursors of malonomicin are assembled and the mechanism of the carboxylation and amination steps (aminomalonic acid moiety) are under active investigation.

## EXPERIMENTAL

*Measurement of* $^{13}$C *N.M.R. Spectra.*—$^{13}$C N.m.r. spectra were recorded on a Bruker WH90 at 22.63 MHz or on a Varian XL-100 at 25.16 MHz. Typically, malonomicin (100—200 mg) was dissolved in $H_2O$–$D_2O$ (1 ml; 10 : 1 v/v) in a 10-mm tube. The pH was adjusted with 40% NaOH to pH 8.5. The spectral width was 5 kHz (XL-100) or 6 kHz (WH90). The pulse delay was 5 s, 8 K data points were recorded, and 45° pulses were used; *ca.* 12 000 transients were accumulated.

*General Method for Feeding Experiments.*—A high-yielding strain of *Streptomyces rimosus forma paramomycinus*, maintained on oat-meal agar, was inoculated into 500-ml baffled conical flasks with wadding closure containing 110 ml of sterilized medium (adjusted to pH 8.5 with 2N-NaOH) of the following composition: malt extract broth (Oxoid CM57; 18 g), pepton (Difco 0118–01; 7 g), NaCl (5 g), and tap water (1 l). Incubations were performed at 28 °C on a rotary shaker (300 r.p.m.; 3.8-cm eccentricity). Isotopically labelled substrates in dilute aqueous solution of pH 8 were added as single additions at the start of the malonomicin production (usually at *ca.* 24 h after start of the incubation). This moment was determined by measuring the u.v. spectrum of the culture, the emerging absorption at 280 nm indicating the appearance of malonomicin. Only [3-$^{13}$C]oxalacetic acid was added continuously during the production by means of a multichannel peristaltic pump. The amounts of additions are given in Table 2.

*Isolation of* (*Labelled*) *Malonomicin.*—Mycelium was centrifuged off 16—20 h after the start of the production of malonomicin. The supernatant (*ca.* 1 l) was concentrated to *ca.* 75 ml by evaporation *in vacuo* at 40 °C and acidified to pH 2 with concentrated HCl. The precipitate was centrifuged off and the pH of the supernatant was adjusted to the isoelectric point of malonomicin (pH 2.7). MeOH (four volumes) was added and the precipitate was collected by centrifugation after cooling for 2 h at −20 °C. The yellow-brown solid obtained was dissolved in $H_2O$ (150 ml), the pH was adjusted to 8.5, and the filtered solution was adsorbed on DEAE–Sephadex A-25 (column 35 × 2 cm; adsorbent swollen in 0.5M-$NH_4$OAc; pH adjusted to 8.5 with $NH_4$OH). After washing with $H_2O$, malonomicin was eluted with a linear gradient of $H_2O$–1N-HOAc (total 900 ml; flow rate 60 ml h$^{-1}$). The fractions containing the antibiotic were concentrated to small volume (5—10 ml), after which crystallization occurred. After cooling, the crystals were collected by suction filtration, washed with cold $H_2O$ and MeOH, and dried over $P_2O_5$ *in vacuo*, yielding 200—300 mg of malonomicin.

*Chemicals.*—Sodium [1-$^{13}$C]acetate, [2-$^{13}$C]acetate, and [1,2-$^{13}C_2$]acetate, barium [$^{13}$C]carbonate, potassium [$^{13}$C]-cyanide, and sodium [$^{13}$C]cyanide (each 90% enriched) were obtained from Merck, Sharp and Dohme, Holland. Sodium hydrogen[$^{13}$C]carbonate was prepared from barium [$^{13}$C]-carbonate and sodium hydroxide.[13] [1,4-$^{13}C_2$]Succinic acid was synthesized from potassium [$^{13}$C]cyanide and 1,2-dibromoethane,[14] [1-$^{13}$C]malonic acid from sodium [$^{13}$C]-cyanide and monochloroacetic acid.[15] [1-$^{13}$C]-2,3-Diaminopropanoic acid was synthesized from [1-$^{13}$C]acrylic acid (from Ba$^{13}CO_3$ and vinylmagnesium bromide) *via* bromine addition [16] and amination [17] with $NH_4$OH in *ca.* 30% overall yield. [1-$^{13}$C]Serine was prepared [18] from methyl [1-$^{13}$C]-2,3-dibromopropanoate [16] in 30% overall yield. The protecting *O*-benzyl group was removed by catalytic hydrogenation over Pd–C. [3-$^{13}$C]Oxalacetic acid was synthesized *via* ester condensation of t-butyl [2-$^{13}$C]acetate and di-t-butyl oxalate, according to the method of Heidelberger and Hurlbert.[19] The required t-butyl [2-$^{13}$C]acetate was prepared by reacting finely powdered sodium [2-$^{13}$C]acetate (500 mg) with liquid isobutene (10 ml) in diethyl ether (2 ml) and concentrated $H_2SO_4$ (0.5 ml) in a well-stoppered glass tube at room temperature under vigorous shaking during 15 h. All isotopically labelled substrates were pure by t.l.c. and $^1$H and $^{13}$C n.m.r. spectroscopy.

* Due to contamination of the culture which resulted in too low a yield of malonomicin, no quantitative data are yet available for this experiment.

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