Tetrahedron Letters,Vol.23,No.12,pp 1293-1296,1982 0040-4039/82/121293-04$03.00/0
Printed in Great Britain

BIOSYNTHESIS OF MALONOMICIN-III
ADVANCED PRECURSOR STUDIES USING ^{2}H NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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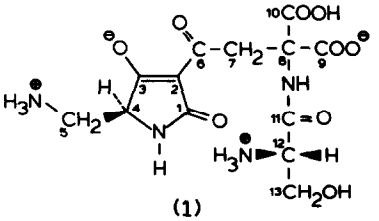
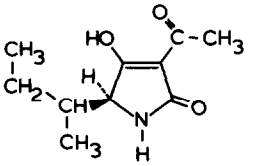
Abstract: ^{2}H-labelled precursors and high-field ^{2}H NMR spectroscopy have been used to demonstrate that the first tetramic acid derived intermediate in the biosynthesis of malonomicin is 5-aminomethyl-4-hydroxy-3-succinoyl-3-pyrrolin-2-one, formed by condensation of L-2,3-diaminopropanoic acid and 3-oxoadipic acid.

Investigations on the biosynthesis of the tetramic acid antibiotic malonomicin (1) by Streptomyces rimosus var. paramomycinus have shown that the tetramic acid nucleus is biosynthetically derived from one molecule each of acetate and L-2,3-diaminopropanoic acid (L-DAP), whereas succinic acid, carbon dioxide and L-serine are precursors of the acyl side-chain[1].

On the analogy of tenuazonic acid (2) for which further details of the tetramic acid ring formation have been obtained[2], the biosynthesis of malonomicin might be initiated by acylation of the α-amino group of L-DAP with acetyl-CoA or with an acetyl-CoA derivative in which the acyl side-chain of malonomicin is already elaborated (partly or completely) prior to formation of the tetramic acid ring structure.



(1)


(2)

The aim of the present study was to verify this hypothesis by incorporation experiments and to identify the first intermediate in the biosynthesis of malonomicin in which the heterocyclic ring is closed.
As the required hypothetical intermediates are quite polar, permeability barriers were expected to cause low incorporation. Although radioactivity incorporation studies could in principle overcome this problem, they were not applicable in this case due to lack of suitable degradative chemistry. Therefore, ^{2}H-labelling and high-field ^{2}H NMR spectroscopy[3] (61.42 MHz) were used to provide the necessary sensitivity.

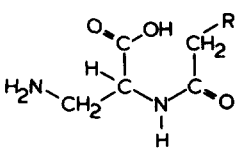
On the basis of the established basic precursors and the presumed involvement of a $N^{\alpha-}$

acylated L-DAP derivative, 2-acetamido-3-aminopropanoic acid (3a) and its ring closed tetramic acid derivative (5a) were considered to be most straightforward primary intermediates. However, after feeding (3a) (1.0 mmol) ([carbonyl-^{13}C]labelled in the acetyl group) and (5a) (D,L; 2,4 mmol) (labelled with ^{2}H at C(5))[4], no trace of isotopic enrichment[5] of malonomicin (in both cases, 0.6 mmol of (1) was isolated)[6] could be detected[7]. This implies[8], that the biosynthesis of (1) does not proceed by acylation of tetramic acid (5a) and that the supposed acetyl-CoA derivative which acylates DAP to furnish the two remaining carbon atoms of the tetramic acid ring must be more complex and must include at least part of the acyl side-chain of malonomicin.

The nature of this acetyl-CoA derivative was inferred from the fact that the carbon atoms C(1,2) and C(6,7,8,9) of (1) are derived from intact units of acetate and succinate, respectively[1].
Moreover, [2,3-$^{2}H_4$]succinic acid gives 5.9% deuterium enrichment at C(7) of (1) (4 mmol of perdeuteriated (>95%) succinic acid were administered to a 1 l culture of S. rimosus and 0.7 mmol of (1) was isolated; specific (probably stereospecific) incorporation of one deuteron at C(7) was concluded from ^{2}H-chemical shift determinations in dependence on pH[3] and from the effect of broadband proton coupling and decoupling; % enrichment was calculated by integration and comparison with a known internal amount of $^{2}H_6$-DMSO (≥99% ^{2}H)). A similar result is obtained with [2,3-$^{2}H_2$]fumaric acid, but, contrary to succinic acid, its incorporation is considerably lower (by a factor of 2.5) in the presence of 0.01 M malonate, which is a competitive inhibitor for the conversion of fumarate to succinate. Therefore, it is concluded again that succinic acid constitutes an integral and direct building block of the acyl side-chain of malonomicin. Based upon this evidence, the condensation product of acetic acid and succinic acid, i.e. 3-oxoadipic acid (4) was tested as a possible precursor of both the tetramic acid ring and its acyl group. Indeed, after feeding [4,5-$^{2}H_4$]-3-oxoadipic acid (prepared by hydrogenolysis of the corresponding benzyl ester[9]), 0.15% deuterium enrichment, specifically at C(7), was established by means of ^{2}H NMR spectroscopic methods[3] (5 mmol of deuteriated (4) (>95% ^{2}H) were administered to a 1.5 l culture and 0.7 mmol of (1) recovered). This low enrichment value may be due to the known strong permeability barriers in cell membranes to 3-oxoadipic acid[10] and/or to spontaneous decarboxylation under the incubation conditions which has also been suggested as a possible cause of the very low incorporation of 3-oxoadipic acid into the mould metabolite caldariomycin[11].

Hence, a prime candidate for consideration as a complex precursor of the heterocyclic nucleus of malonomicin is (3b), the acylation product of 3-oxoadipic acid and L-DAP, which by a Claisen-type ring closure *in vivo* could yield the possible intermediate tetramic acid (5b). The NMR spectrum of the isolated malonomicin (0.4 mmol) after feeding (3b) (0.6 mmol , fully deuteriated (>95% ^{2}H) at the succinoyl part and at the β-position of the L-DAP moiety), did not show, however, the distribution of deuterium label expected for intact incorporation of (3b): C(5) was significantly enriched while C(7) was not (the respective enrichments calculated from the enhancement of the signals at δ=3.25 and δ=3.82 ppm (measured at pH8) differed by a factor

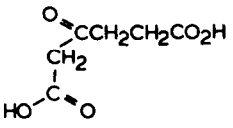
of at least five). Probably, enzymatic hydrolysis of the amide bond of (3b) had occurred to some extent and, given the high enrichment by L-[3-$^2H_2$]DAP (15-20%) and the low enrichment by 3-oxoadipic acid (0.15%, vide supra), this would lead to a distribution of label in malonomicin very different from that of the administered precursor (3b), even if only a very small amount of (3b) was hydrolyzed.



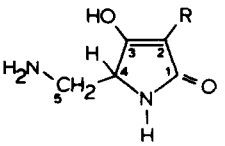
(a) R = H

(b) R = $C(=O)CH_2CH_2CO_2H$

(3)



(4)



(a) R=H

(b) R = $C(=O)CH_2CH_2CO_2H$

(c) R = $C(=O)CH_2C(CO_2H)(NH_2)CO_2H$

(5)

While the intermediacy of (3b) could thus be neither proved nor excluded directly, its ring closed tetramic acid derivative (5b) might be an intermediate in the following stage of the biosynthetic pathway to malonomicin. Therefore, in a first experiment, (5b) labelled with ^2H at C(7,8) (D,L; 0.87 mmol ; >95%^2H) was fed to S. rimosus. Enrichment of the isolated malonomicin (0.4 mmol) occurred specifically at C(7)as inferred from chemical shift determinations at low and high pH, although to a very low extent (0.03% in excess of natural abundance).

To exclude the possibility that this enrichment had its origin in (enzymatic) degradation of (5b) to (5a) and ^2H-labelled succinic acid, followed by incorporation of the latter, a similar experiment was performed with (5b) fully deuteriated at the DAP part (D,L; 1.1 mmol ; >95% ^2H), taking advantage of the fact that (5a) is not incorporated into malonomicin (vide supra) if cleavage of (5b) to ^2H-labelled (5a) and succinic acid should occur. Again, a very small but positively identified enrichment of 0.04%, specifically at the expected positions C(4,5) of malonomicin (0.53 mmol) was established by comparison of the chemical shift, shape, integrals and integral ratio of both ^2H-peaks with those of corresponding simulated spectra in dependence on pH (40,000 transients; S : N ratio 25 : 1).

Finally, the possibility that (5b) is incorporated not as an intact unit but via cleavage to (3b) (*retro-Claisen condensation*) followed by incorporation of its hydrolysis products 3-oxoadipic acid and DAP,was ruled out by the result of a feeding experiment with (5b) (D,L; 1.1 mmol) doubly-labelled with ^2H (>95%) at C(7,8) and at C(5). If degradation to (3b) would occur, a similar distribution of ^2H-label as obtained in the experiment with labelled synthetic (3b) (vide supra) can be expected. This was not the case. The ^2H NMR spectrum of 1 (0.53 mmol) clearly showed a very low but specific incorporation of ^2H at C(7) and C(5) (enrichment 0.02%), reasonably in agreement with the results of separate experiments with (5b) (as described above)

labelled at C(7,8) and at C(4,5), respectively, but completely different from the result obtained by administering synthetic (3b).

Thus we conclude that (5b) is incorporated intact and therefore is the first tetramic acid derived intermediate in the biosynthesis of malonomicin. At the same time, proof of (5b) being a precursor strongly supports the suggestion that (3b) is a real intermediate.

Further elaborated hypothetical (but plausible) intermediates such as (5c) were not incorporated, at least not to the minimum extent necessary for detection with ^{2}H NMR spectroscopy. A preliminary experiment where (5b) (labelled with tritium at C(4)) was added to a cell-free extract of S. rimosus (to overcome permeability barriers) provided evidence that radioactive malonomicin is produced, whereas none of the potential intermediates between (5b) and malonomicin are released into the reaction medium. Further investigations are in progress.

Acknowledgement: We thank Spectrospin A.G. Zürich for access to their Bruker WH400 spectrometer.

References and Notes

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3. D. Schipper, J.L. van der Baan, and F. Bickelhaupt, preceding paper.
4. All labelled compounds derived from DAP and tetramic acid were prepared by synthetic methods analogous to those used in the total synthesis of malonomicin: J.L. van der Baan, J.W.F.K. Barnick, and F. Bickelhaupt, Tetrahedron, 1978, 34, 223.
5. % Enrichment is defined as atom % of isotopic tracer in excess of natural abundance (natural abundance of ^{2}H: 0.016%).
6. For details of feeding experiments and isolation procedure of malonomicin, see ref. 1. Usually, ca. 75% of (1) present in the culture medium was obtained in a form suitable for ^{2}H NMR measurements.
7. For ^{13}C spectrum of (1) and NMR-measuring conditions, see ref. 1; the ^{1}H- and ^{2}H NMR spectra, and the general conditions for determining ^{2}H-incorporation into (1), are discussed in ref. 3.
8. The possibility that (3a) and (5a) are not incorporated as a consequence of permeability barriers can be excluded because of the observed incorporation of the much more polar and elaborated compound (5b).
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10. L. Ornston and D. Parke, J. Bacteriol, 1976, 125, 475.
11. J.R. Beckwith, R. Clark, and L.P. Hager, J.Biol.Chem., 1963, 238, 3086. Caldariomycin appears to be the only other case in which 3-oxoadipic acid has been considered as a (not readily incorporated) biosynthetic intermediate. We thank Professor R. Thomas, University of Surrey, England, for drawing our attention to this publication.

(Received in UK 26 November 1981)