Biosynthesis of malonomicin

Schipper, D.

1980

BIOSYNTHESIS of MALONOMICIN

DIRK SCHIPPER

VRIJE UNIVERSITEIT TE AMSTERDAM

BIOSYNTHESIS OF MALONOMICIN

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van
doctor in de wiskunde en natuurwetenschappen
aan de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
dr. H. Verheul,
hoogleraar in de faculteit der wiskunde en natuurwetenschappen,
in het openbaar te verdedigen
op vrijdag 2 mei 1980 te 13.30 uur
in het hoofdgebouw der universiteit,
De Boelelaan 1105

door

DIRK SCHIPPER

geboren te Oldenzaal

Rodopi

AMSTERDAM 1980

Promotor : Prof. dr. F. Bickelhaupt
Copromotor : Dr. J.L. van der Baan

De voltooiing van dit proefschrift biedt mij een welkome gelegenheid om allen te bedanken, die aan de totstandkoming hebben meegewerkt.

aan mijn ouders
voor Kristine

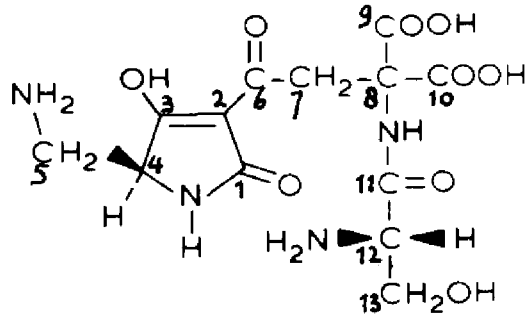
Contents

Chapter 1

Malonomicin

1.1. Introduction

Cultures of Streptomyces rimosus forma paramomycinus characteristically develop UV absorptions at 240 and 230 nm after about 24 hours of growth together with biological activity against trypanosoma species. Three metabolites could be isolated, of which the major one (named K16) was responsible for the anti-trypanosoma activity [1]. The two other minor metabolites have been shown to be identical with paramomycin and streptimidon. Extensive degradation studies have established structure (1) for the major bioactive compound, which could be confirmed by ^{1}H NMR spectroscopy [2].



(1)

Recently, total synthesis, involving acylation of the heterocyclic nucleus with an activated side-chain, provided the final proof for this structure [3]. The most

striking structural feature of K16 is the aminomalonic acid moiety, and because of this the antibiotic has been named malonomicin.

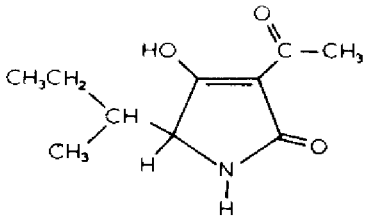
From a biosynthetic point of view, malonomicin has several interesting features. In the first place, there is the presence of a heterocyclic ring, a pyrrolidin-dione or tetramic acid, substituted at C-2 with an acyl group and at C-4 with an aminomethyl group (the numbering used throughout this thesis is shown in (1)) (described in chapter 1.2.). The origin of the characteristic UV absorptions is the 1,3 keto-enol system. According to the biosynthesis of tetramic acids (vide infra), this particular tetramic acid can be thought of as derived from 2,3-diaminopropanoic acid (DAP), which is a rather uncommon amino acid (1.3). The third and maybe most conspicuous feature in malonomicin is the presence of an aminomalonic acid moiety which rapidly looses CO_2 when heated in water, thereby being converted into a bio-inactive compound (previously named K 16A)(1.4).

1.2. Tetramic acid

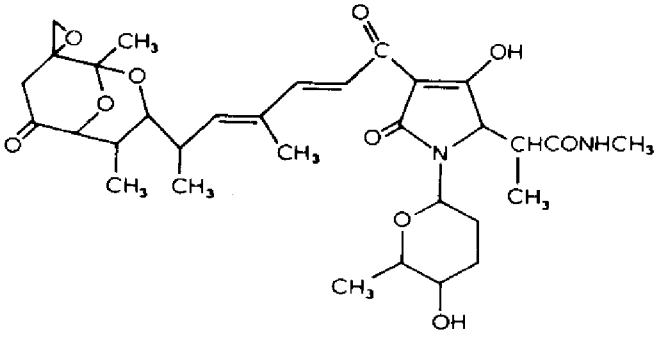
Since the discovery of tenuazonic acid (2) in 1959 [4] the number of tetramic acids, isolated from natural sources, is steadily growing. Enolmethylethers derived from tetramic acids also occur in nature. Most of them are produced by Streptomyces and Penicillium species and they all exhibit biological activity, e.g. tenuazonic acid has an anti-tumour [5] and insecticide-activity [6], and streptolydigin shows an inhibitory activity on RNA polymerase [7]. They show great variation in substitution patterns at N, C-2 and C-4; if

substituted at C-2, this is always an acyl group. A survey of the many different structures is shown below.

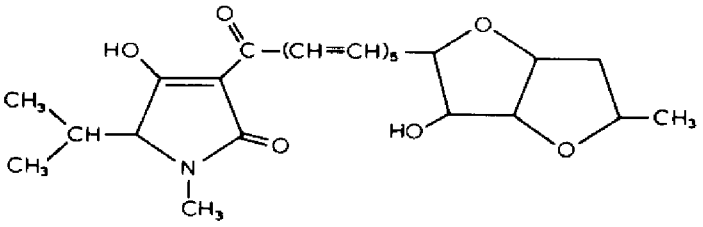
tenuazonic acid[4] (1959) (2)



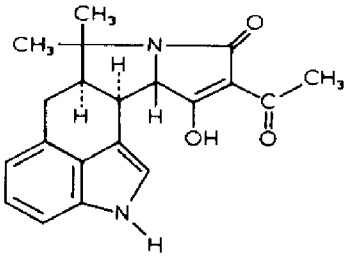
streptolydigin[5] (1963) (3)



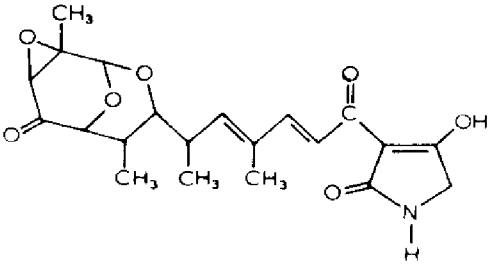
erythroskyrine[6] (1964) (4)



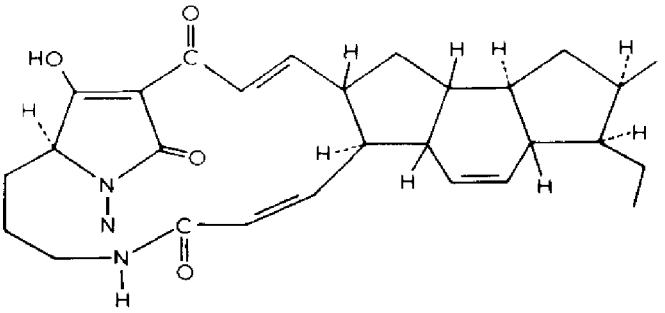
cyclopiazonic acid[7] (1968) (5)



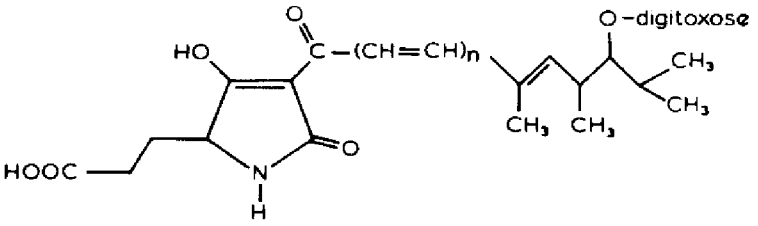
tirandamycine[8] (1971) (6)



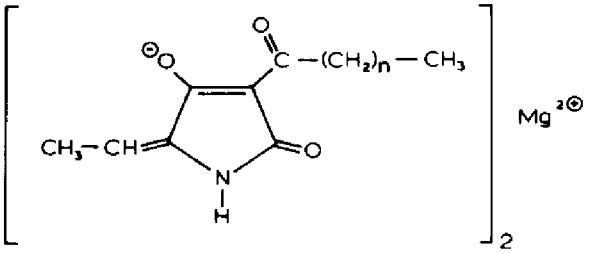
ikarugamycin[9]
(1972) (7)



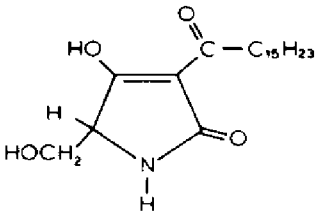
lipomycin[10]
(1973 ; n = 4 (8)
oleficin[11]
(1978 ; n = 5 (9)



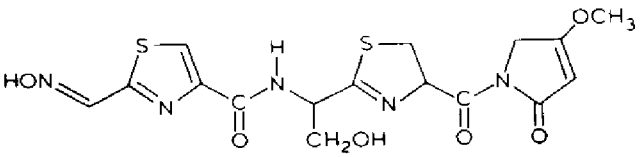
magnesidin[12]
(1974 ; n = 4,6) (10)



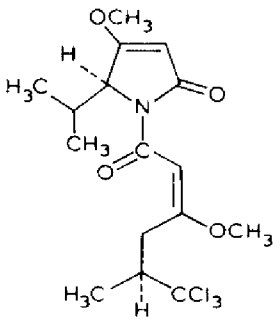
equisetin[13]
(1974) (11)



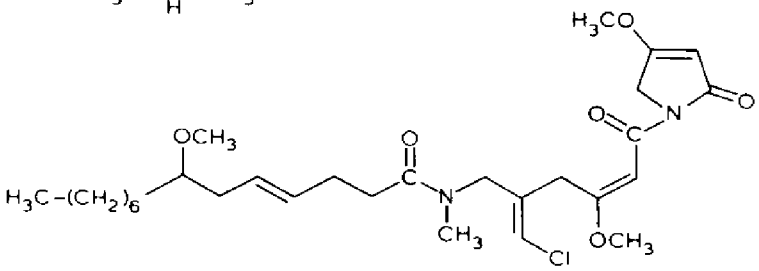
althiomycin[14]
(1974) (12)



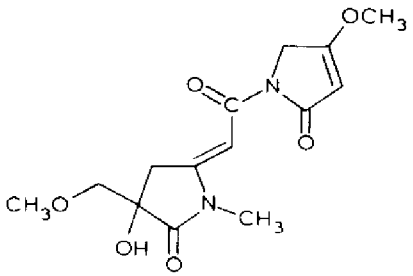
dysidin[15]
(1977) (13)



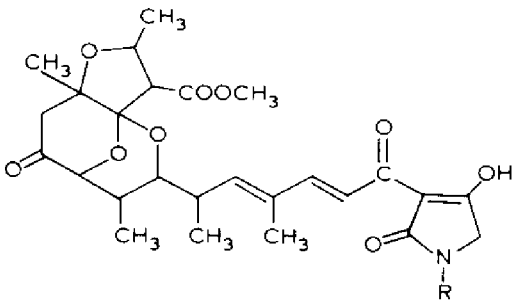
malyngamide A[16]
(1979) (14)



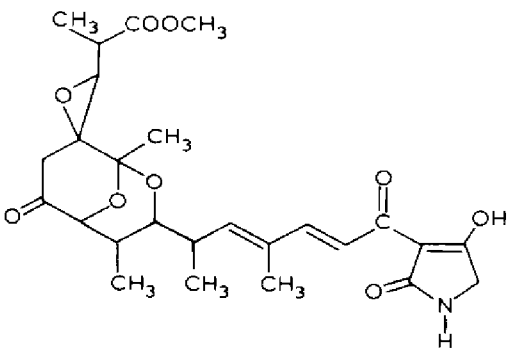
pukeleimide C[17]
(1979) (15)



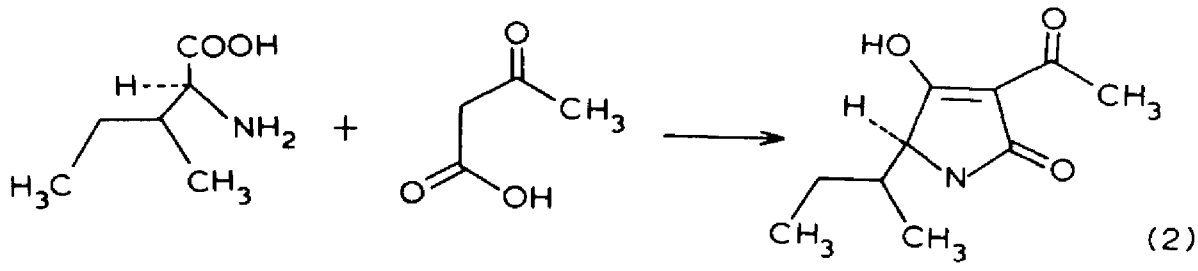
B4 2313A[18]
(1979, R=H) (16)
B4 2313B[18]
(1979; R=CH_3) (17)



nocamycin[19]
(1979) (18)



Biosynthetic studies on tenuazonic acid (2) [23], erythroskyrin (3) [24] and cyclopiazonic acid (4) [25] have revealed that the tetramic acid nucleus originates from the condensation of an acetate derived precursor and an α-amino acid i.e. isoleucine, valine and tryptophane, respectively (see e.g. figure 2).



(2)

figure 2

An analogous pathway has been suggested for ikarugamycin (5) [26], but no tracer experiments were performed to prove the hypothesis.

A tetramic acid has also been postulated as an intermediate in the biosynthesis of tenellin and bassianin [27]. Here as well, the origin of the nucleus, which isomerizes to a N-hydroxy-dihydropyridone (figure 3), is an α-amino acid and an acetate derived precursor.

1.3. Diaminopropanoic acid

The 2,3-diaminopropanoic acid (DAP) moiety occurs in several secondary metabolites with strongly divergent structures. Some of them are shown on page 8 and 9. DAP and simple DAP derivatives (20, 22) are used for chemotaxonomic purposes in Acacia species [39]. Secondary metabolites containing DAP can exert biological

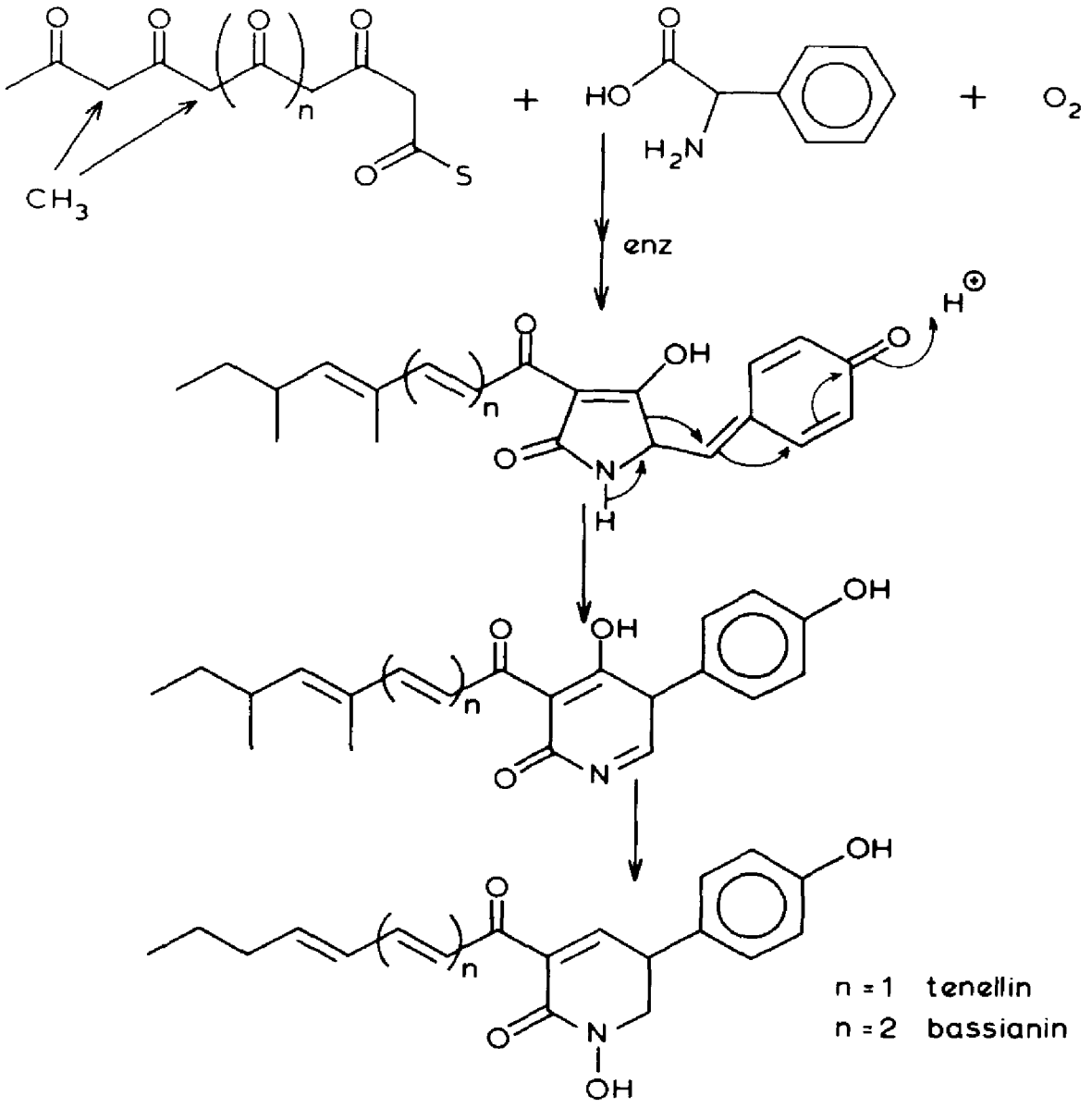
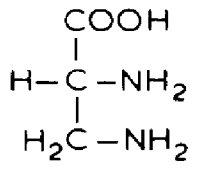


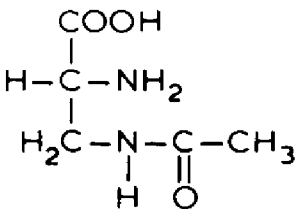
figure 3

activity, e.g. N^3-oxalyl DAP is a strong neurotoxin [31] and the bleomycins (29) are used as antineoplastic agents in the treatment of a wide variety of human carcinomas and lymphomas [40]. In addition, bleomycin labelled with radioactive isotopes such as ^{57}Co, ^{64}Cu etc., has been employed as a tumour scanning agent

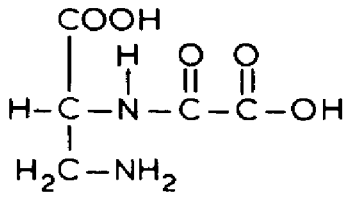
[41]. It has been proven that the presence of DAP in bleomycin is essential for the biological activity, involving a bleomycin (Fe) complex [42].



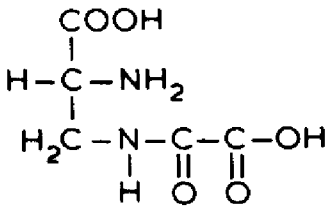
L-DAP
(19)



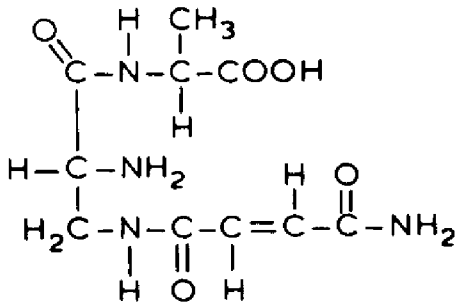
N^3-acetyl DAP
(20)



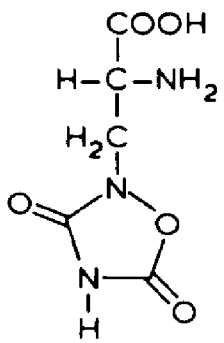
N^2-oxalyl DAP
(21)



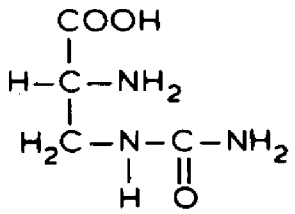
N^3-oxalyl DAP
(22)



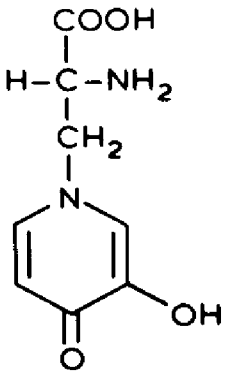
N^3-fumarylcarboxyamido-L-2,3-diaminopropanoyl-L-alanine
(23)



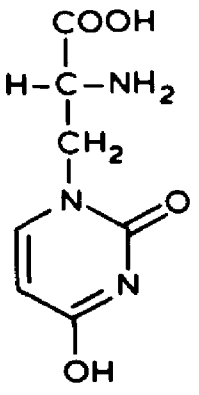
quisqualic acid
(24)



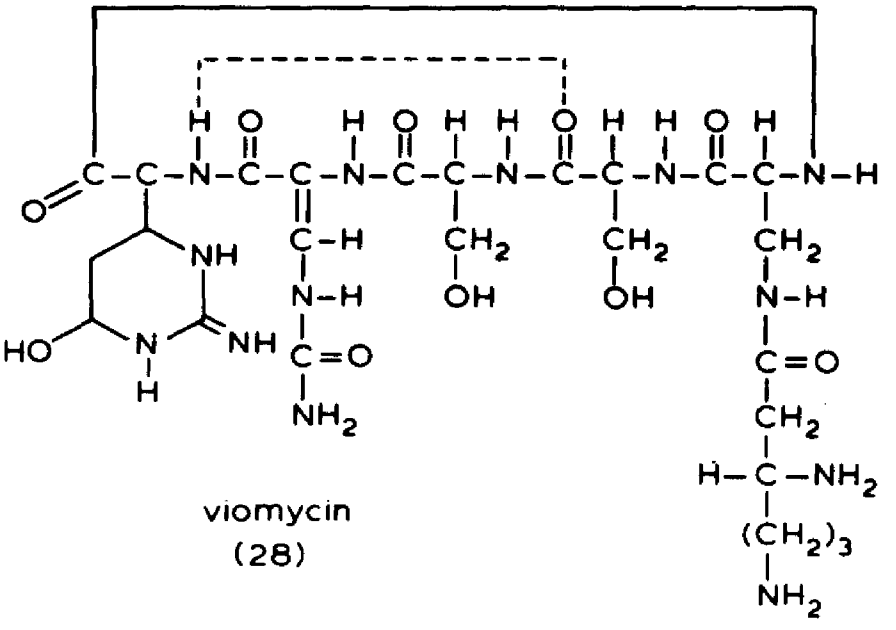
albiziine
(25)



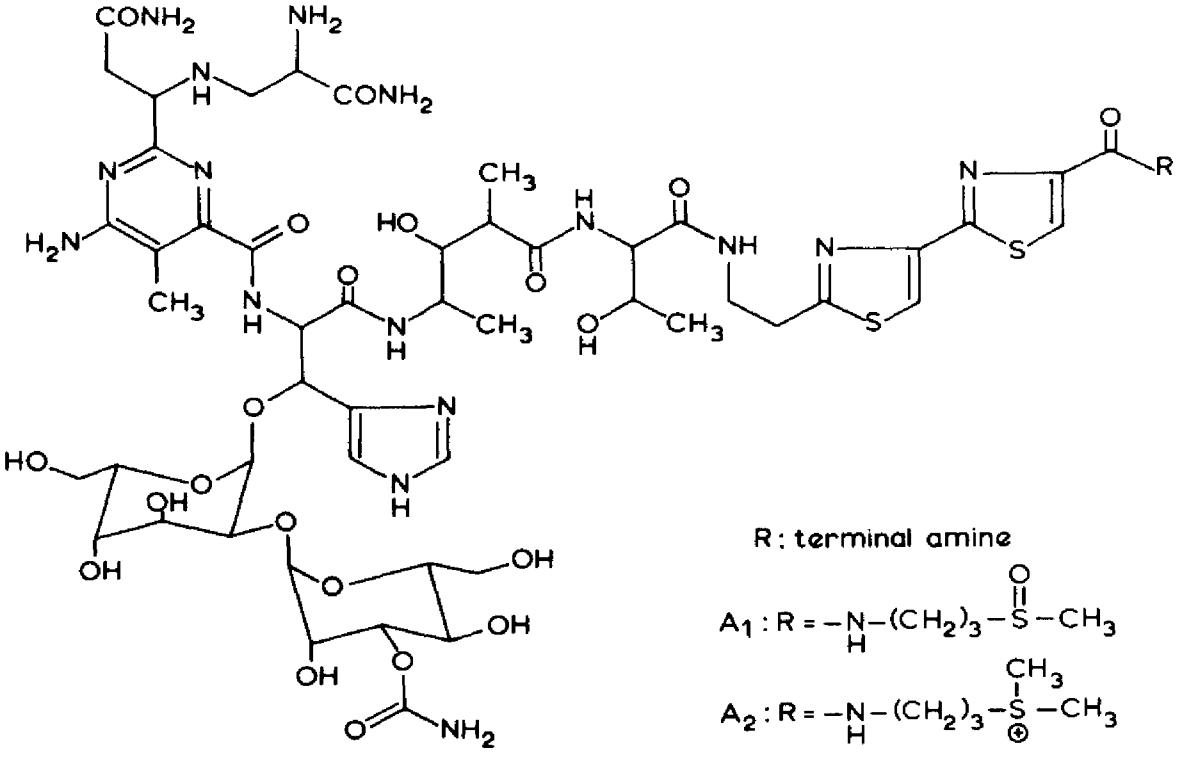
mimosine
(26)



willardiine
(27)



viomycin
(28)



R : terminal amine

A_1 : R = $-\underset{H}{N}-(CH_2)_3-\overset{O}{\overset{\|}{S}}-CH_3$

A_2 : R = $-\underset{H}{N}-(CH_2)_3-\overset{CH_3}{\overset{|}{S}}\overset{\oplus}{}-CH_3$

B_2 : R = $-\underset{H}{N}-(CH_2)_4-NH-\overset{NH}{\overset{\|}{C}}-NH_2$

The metabolic origin of DAP is equivocal for different organisms. Reinbothe studied the biosynthesis of albiziine (25) in Albizzia lophantha Bent. and found a good incorporation of [1-^{14}C] and [3-^{14}C] serine [43]. He suggested a pathway for the biosynthesis of diaminopropanoic acid involving oxidation of serine to aminomalonic acid semialdehyde and subsequent transamination (figure 4).

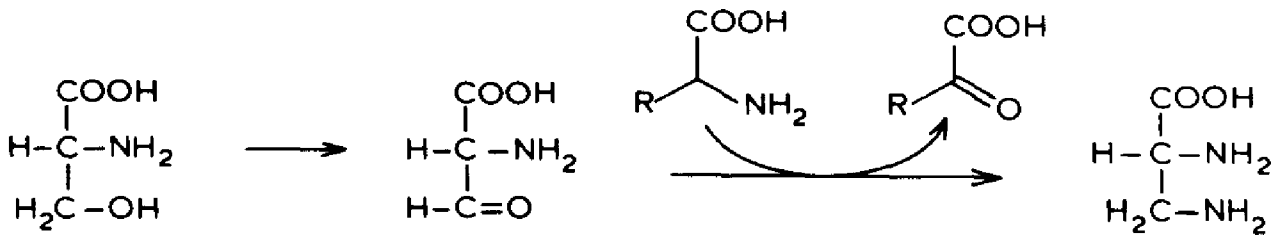


figure 4

Seviratne and Fowden however, found no incorporation of [U-^{14}C]serine and only a small incorporation of [U-^{14}C]DAP in albiziine in Acacia podalyriaefolia [44].

Also in N^3-acetyl DAP (20) no incorporation of [U-^{14}C] serine was found, but on the other hand a highly efficient incorporation of 95.4% of [U-^{14}C] DAP. The N^3-disubstituted DAP derivatives e.g. quisqualic acid (24), mimosin (26) and willardiine (27) appear to be formed by condensation of a serine derivative with a heterocyclic ringsystem like pyrazole. In a crude extract from Quisqualis indica var. villosa, quisqualic acid is synthesized in vitro from O-acetyl-serine and 3,5-dioxo-1,2,4-oxadiazolidine (30) (figure 5) [45].

Carter et al. (1974) [46] studied the biosynthesis of viomycin (tuberactinomycin B) (28) and found

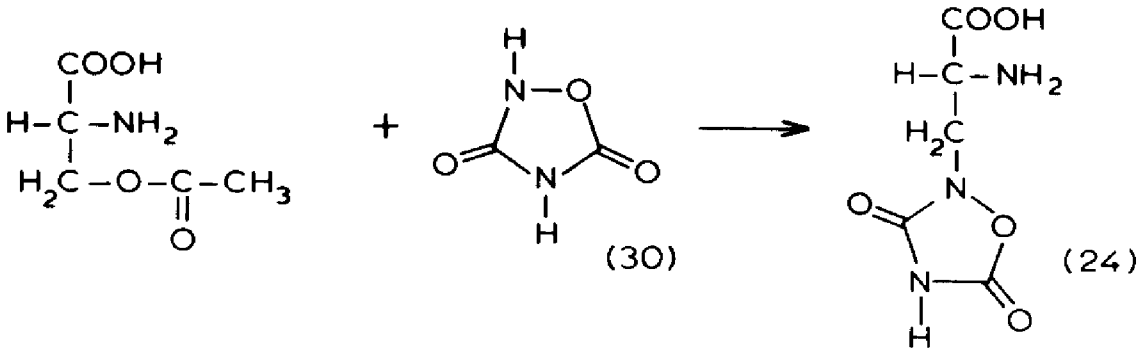


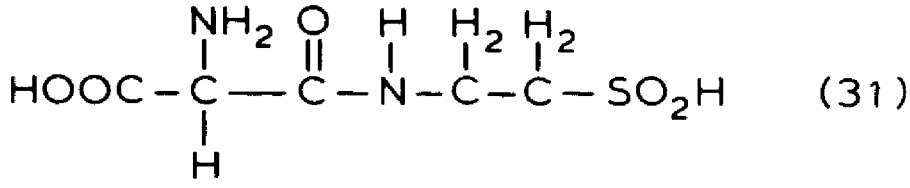
figure 5

incorporation of [1-^{14}C] and of [3-^{14}C] serine in the serine part as well as in the DAP part of the molecule. [U-^{14}C] DAP was incorporated almost exclusively in the DAP part and only slightly in the serine part. This finding was not consistent with that of Tam and Jordan (1972) [47] who could not prove incorporation of serine into viomycin. Carter et al., like Reinbothe, suggested an oxidation of serine followed by a transamination for the biosynthesis of DAP, but preliminary investigation of the incorporation of [3-$^{2}H_2$] serine could not prove this hypothesis for both deuterons were present in the product. Since then, no further details have been reported and, as far as we know, no other evidence on the mechanism of the serine to 2,3-diaminopropanoic acid interconversion has been presented.

1.4. Aminomalonic acid

At the time of its structural elucidation (1972), malonomicin was the first natural occurring derivative of aminomalonic acid. In 1975 the structure of the fish attractant arcamine (31) from Arca zebra was published

which is also an aminomalonic acid derivative although the evidence presented for this structure is not firm [48].



(31)

Aminomalonic acid itself is not stable in solution and decarboxylation gives glycine which led Knoop to suggest that aminomalonic acid might be a precursor of glycine in living organisms and an intermediate in the route from serine to glycine [49] (figure 6).

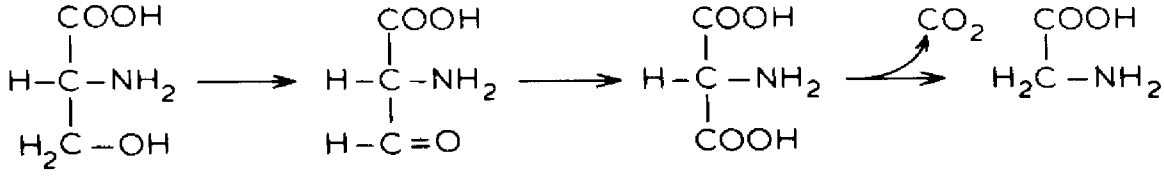


figure 6

In 1956 Shimura et al. [50] discovered in the posterior silk-gland of silkworm and also in rat liver an enzyme that catalyzed the decarboxylation of aminomalonic acid to glycine. Thanassi and Fruton found that the addition of pyridoxal phosphate stimulated the decarboxylation [51]. Recent investigation showed the decarboxylation by rat liver not to be stereospecific and also the aminomalonic acid decarboxylase activity to be identical with serine hydroxymethylase [52]. This fitted the findings of Matthew and Neuberger that aminomalonic acid is an inhibitor of serine hydroxymethylase [53] However, formation of glycine from serine by serine hydroxymethylase does occur stereospecifically;

therefore, aminomalonic acid cannot be an intermediate in this reaction.

Apart from the fact that aminomalonic acid can be transformed to glycine in living organisms, there is the question of how it might be biosynthesized. The possibility of formation from serine is not probable (vide supra). In 1958 Shimura et al. [54] discovered an enzyme (again in the posterior silk-gland of silk-worms) that catalyzed the transamination of oxomalonic acid (33) to aminomalonic acid; alanine proved to be the best NH_2-donor (figure 7).

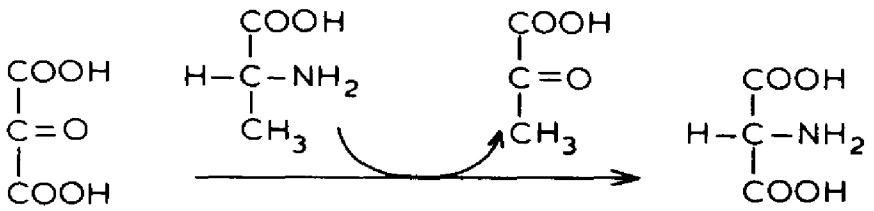


figure 7

Vedel and Guitton also found a transaminase in Catalpa bignoniodes that catalyzed the formation of amino-malonic acid from oxomalonic acid and glutamic acid as the NH_2-donor [55]. In its turn, the origin of oxomalonic acid was found to be hydroxymalonic acid (tartronic acid) [56], a degradation product of 2-oxo-D-gluconic acid [57].

Although there have been found some enzymes that catalyze the formation of aminomalonic acid in vitro, the amino acid itself has never been found in nature, except for its derivatives malonomicin and arcamine.

1.5. The scope of the investigation

The investigation described in this thesis has the aim to elucidate the biological pathways and mechanisms by which malonomicin is formed, in particular with respect to the construction of the tetramic acid nucleus, the mechanism of DAP formation and the construction of the aminomalonic acid moiety. This was expected to answer the question whether aminomalonic acid is a natural amino acid or not.

In chapter 2 the methodology is described with special attention to the use of stable isotopes in biosynthetic studies. In chapter 3 the interpretation and assignment of the various NMR spectra of malonomicin will be given. Incorporation experiments with simple precursors are described in chapter 4 and with more complex precursors in chapter 5. Finally in chapter 6 an evaluation and the results of some preliminary experiments will be given.

1.6. References

1 H. Ottens and C. Vos, U.S.P. 3,536,811.

2 J.G. Batelaan, J.W.F.K. Barnick, J.L. van der Baan, and F. Bickelhaupt, Tetrahedron Letters, 1972, 3103, 3107.

3 J.L. van der Baan, J.W.F.K. Barnick, and F. Bickelhaupt, Tetrahedron, 1978, 34, 223.

4 C.E. Stickings, Biochem. J., 1959, 72, 332.

5 H.T. Shigeura, Antibiotica, 1967, 1, 360.

6 M. Cole and G.N. Rolinson, Appl. Microbiol., 1972, 24, 660.

7 K. von der Helm and J. Krakow, Nature New Biology, 1972, 235, 32.

8 K.L. Rinehart, Jr., J.R. Beck, D.B. Borders, T.H. Kinstle, and D. Kraus, J. Amer. Chem. Soc., 1963, 85, 4038.

9 J. Shoji and S. Shibata, Chem. Ind., 1964, 419.

10 C.W. Holzapfel, Tetrahedron, 1968, 24, 2101.

11 F.A. MacKellar, M.F. Grostic, E.C. Olson, R.J. Wnuk, A.R. Branfman, and K.L. Rinehart, Jr., J.Amer. Chem. Soc., 1971, 93, 4943.

12 S. Itoh and Y. Hirata, Tetrahedron Letters, 1972, 1131.

13 a) B. Kunze, K. Schabacher, H. Zähner, and A. Zeeck, Arch. Microbiol., 1972, 86, 147.
b) K. Schabacher, and A. Zeeck, Tetrahedron Letters, 1973, 2691.

14 J.G. Gyimesi, Zs. Mehesfalvi-Vajna, and Gy. Horvàth, J. Antibiotics 1978, 31, 626.

15 H. Kohl, S.V. Bhat, J.R. Patell, N.M. Ghandi, J. Nazareth, P.V. Divekar, N.J. de Souza, H.G. Berscheid, and H.W. Fehlhaber, Tetrahedron Letters, 1974, 983.

16 a) H.R. Burmeister, U.S.P. 3,959,468.
b) R.F. Vesonder, L.W. Tjarks, W.K. Rohwedder, H.R. Burmeister, and J.A. Langal, J. Antibiotics, 1979, 32, 759.

17 B.W. Bycroft and R. Pinchin, J. Chem. Soc. Chem. Comm., 1975, 121.

18 W. Hofheinz and W.E. Oberhänsli, Helv. Chim. Acta, 1977, 60, 660.

19 J.H Cardellina II, F.-J. Marner, and R.E. Moore, J. Amer. Chem. Soc., 1979, 101, 240.

20 C.J. Simmons, F.-J. Marner, J.H.Cardellina II, R.E. Moore, and K. Seff, Tetrahedron Letters, 1979, 2003.

21 Dutch Patent 7807570.

22 Gy. Horvàth, M.G. Brazhnikova, N.V. Konstantinova, I.V. Tolstykh, and N.P. Potapova, J. Antibiotics, 1979, 32, 555.

23 a) C.E. Stickings and R.J. Townsend, Biochem. J., 1961, 78, 412.
b) S. Gatenbeck and J. Sierankiewicz, Acta Chem. Scand., 1973, 27, 1325.

24 S. Shibata, and U. Sankawa, H. Taguchi, and K. Yamasaki, Chem. and Pharm. Bull. (Japan), 1966, 14, 474.

25 R.M. McGrath, P.S. Steyn, N.P. Ferreira, and D.C. Neethling, Bioorg. Chem., 1976, 5, 1, and references cited therein.

26 S. Itoh and Y Hirata, Tetrahedron Letters., 1972, 2557.

27 A.G. McInnes, D.G. Smith, J.A. Walter, L.C. Vining, and J.L.C. Wright, J. Chem. Soc. Chem. Comm., 1974, 282.

28 R. Gmelin, G. Strauss, and G. Hasenmaier, Z. Physiol. Chem., 1959, 316, 164.

29 A.S. Seviratne and L. Fowden, Phytochemistry, 1968, 7, 1039.

30 E.A. Bell and J.P. O'Donovan, Phytochemistry, 1966, 5, 1211.

31 S.L.N. Rao and E.A. Bell, Biochemistry, 1964, 3, 242.

32 R.M. Gale and D.H. Lively, U.S.P. 3,832,287.

33 T. Takemoto, N. Takagi, S. Arihara, and K. Koike, 16th Symposium on the Chemistry of Natural Products, Osaka, 1972, Symposium Papers, p. 256.

34 R. Gmelin, G. Hasenmaier, and G. Strauss, Z. Naturforschung, 1958, 136, 252.

35 J. Renz, Z. Physiol. Chem., 1936, 244, 153.

36 R. Gmelin, G. Strauss, and G. Hasenmaier, Z. Physiol. Chem., 1959, 314, 28.

37 B.W. Bycroft, J. Chem. Soc. Chem. Comm., 1972, 660.

38 H. Umezawa, K. Maeda, T. Tageuchi, and Y. Okami, J. Antibiotics, 1966, 19A, 200.

39 C.S. Evans, M.Y. Qureshi, and E.A. Bell, Phytochemistry, 1977, 16, 565.

40 For a review see: S.T. Crooke and W.T. Bradner, J. Med. (Basel), 1977, 7, 333.

41 For a review see: E. Silberstein, Amer. J. Med., 1976, 60, 226.

42 T. Takita, Y. Muraoka, T. Nakatani, A. Fujii, Y. Iitaka, and H. Umezawa, J. Antibiotics, 1978, 31, 1073.

43 H. Reinbothe, Flora, 1962, 152, 545.

44 A.S. Seviratne and L. Fowden, Phytochemistry, 1968, 7, 1047.

45 I. Murakoshi, F. Kato, J. Haginiwa, and T. Takemoto, Chem. Pharm. Bull. (Japan), 1974, 22, 473.

46 J.H. Carter II, R.H. Du Bus, J.R. Dyer, J.C. Floyd, K.C. Rice, and P.D. Shaw, Biochemistry, 1974, 13, 1221.

47 A.H.-K. Tam and D.C. Jordan, J. Antibiotics, 1972, 25, 524.

48 A.W. Sangster, S.E. Thomas, and N.L. Tingling, Tetrahedron, 1975, 31, 1135.

49 F. Knoop, Z. Physiol. Chem., 1914, 89, 151.

50 K. Shimura, H. Nagayama, and A. Kikuchi, Nature, 1956, 177, 935.

51 J.W. Thanassi and J.S. Fruton, Biochemistry, 1962, 1, 975.

52 A.G. Palekar, S.S. Tate, and A. Meister, J. Biol. Chem., 1973, 248, 1158.

53 M. Matthew and A. Neuberger, Biochem. J., 1963, 87, 601.

54 H. Nagayama, M. Muramatsu, and K. Shimura, Nature, 1958, 181, 417.

55 F. Vedel and Y. Guitton, Compt. Rend., 1965, 260, 1733.

56 H.A. Stafford, Plant Physiol., 1956, 31, 135.

57 K. Kondo, M. Arneyama, and T. Yamaguchi, Nippon Nogei-kagaku Kaishi, 1956, 30, 419.

Chapter 2

Methodology

2.1. Introduction

Though the term "biosynthesis" suggests formation of any substance by a living organism, it is mostly used in the more restricted anabolic (constructive) sense. Biosynthesis in the still more restricted sense used in this thesis comprises the elaboration of molecules from less complex precursors by endergonic reactions [1]. Before biosynthetic studies were performed, the term biogenesis was used in considering more or less obvious relations between classes of secondary metabolites, which were postulated to imply actual reaction sequences, but in most cases they were not experimentally verified by tracer studies.

Apart from the study in biosynthesis as a fundamental occupation with its own justification, studies in biosynthesis are also useful for many purposes. One of them is to derive possible structures of natural products, for which other methods of structure determination have yielded incomplete evidence. Historically, the isoprene rule, though more an empirical than a biosynthetic principle, has been one of the most conclusive factors in determining terpene structures. The polyketide hypothesis by Birch [2] is useful in

limiting the number of possible formulae for natural products derived from acetate units. However, it has to be mentioned that too much reliance on biogenetic principles can lead to error; Robinson for instance, suggested a wrong structure for strychnine based on the biogenetical relationship between Strychnos and Cinchona alkaloids [3].

Considering the biosynthesis of natural products (see for example Art in Biosynthesis [4]) will show the superiority of nature over the synthetic chemist and maybe induce a stronger interest and insight in the fascinating possibilities which nature offers. Another application of biosynthetic studies is in biomimetic synthesis, an area to which more and more attention is paid in recent years. In this field, non-enzyme catalyzed laboratory analogies of natural pathways are carried out which in several instances have succeeded in producing moderate yields of the expected products. However, in the absence of the corresponding enzyme-controlled conversions, this apparent biosynthetic evidence in support of a particular speculative scheme is at best circumstantial.

With knowledge of the biosynthesis of certain antibiotics, metabolism sometimes can be guided by adding compounds, related to the original precursors, which are incorporated instead of them [5]. The obtained new antibiotics may have some other qualities than the original antibiotic, which can alter the mode of action or can circumvent resistance.

2.2. Biosynthesis and natural product chemistry

Interest in the biosynthetic origin of natural products developed parallel with the elucidation of more and more diverse structures. The observation of Wöhler in 1828, that the natural product urea could be formed by the thermal rearrangement of ammonium cyanate, meant a landmark in chemistry as it made chemists realize that these compounds were formed in nature by basically unexceptional processes. This important finding served to remove the mystique of natural products which until then had been regarded rather as supernatural products, the formation of which required an unknown vital force.

The structural elucidation of new natural products could not always keep pace with the isolation, and sometimes it took over 100 years before the correct structure was assigned. In the period since 1940 the natural product chemistry developed rapidly, and the classical trio of natural product chemistry, i.e. isolation, structure elucidation and total synthesis was accomplished in progressively shorter times. The main factors for these rapid advances were:

- the development of new chromatographic techniques, by which even microquantities of chemically similar substances could be separated;

- the marked progress in spectroscopic aids to structure determination like UV, IR, nuclear magnetic resonance and mass spectroscopy and also X-ray analysis;

- the elaboration and application of sophisticated synthetic methods.

From the biosynthetic point of view, it was particularly fortunate that these advances also coincided with the development of nuclear technology, which led to the commercial availability of isotopic tracers. Their application to the investigation of metabolic pathways has been so successful that biosynthetic studies can now be regarded as an integral part of natural product chemistry.

2.3. Approaches to the study of biosynthesis

The study of biosynthesis with tracer methods is almost invariably performed with isotopic tracers. These studies fall into two general categories, concerned respectively with pathways and reaction mechanisms. It is only natural that the former have greatly predominated, since one must establish the identity of at least some of the intermediates in a reaction sequence before mechanisms of the conversion can be seriously considered.

By tracer methods, precursors and intermediates can be identified with a good degree of confidence in intact organisms, and considerable information on mechanisms is also obtainable by this approach. But the ideally systematic approach would be identification of intermediates in a pathway by tracers, followed by purification of the enzymes mediating the individual steps and finally the use of these enzymes in a detailed study of the reaction mechanisms with the aid of position-specifically or stereospecifically labelled compounds.

This last step is of course not different in principle from the study of chemical reaction mechanisms in general.

The choice of candidate precursors is normally based on considerations of potential biosynthetic schemes. These schemes are generally arrived at by preliminary structural analysis of a natural product in terms of possible simple precursors, and also by comparison with products of related structure of which the mode of biosynthesis is known. If meaningful results are to be obtained, a labelled compound administered to an organism obviously must not only penetrate to the synthetic site, but must also do so at a time when the enzyme systems mediating the synthetic reactions are present and active. This seems trivial, but permeability problems can sometimes obscure the obtained results and addition of the same precursor at different moments to a microbial culture can sometimes give contradictory results. For micro-organisms, incorporation of precursors is normally effected by their addition to the culture-medium during the phase of maximum production of the required metabolite. The optimum incorporation efficiency is determined following exploratory experiments, in which the right moment of addition, the right concentration of the labelled precursor and the best moment for working up are determined by use of a radioactive precursor, e.g. [1-^{14}C]acetate, that with high certainty will be incorporated.

An ideal biosynthetic incorporation experiment should meet the following criteria according to McDonald [6]:

- The precursor should be specifically labelled
- The precursor should be pure (at least isotopically)
- Relatively small quantities should be "fed"
- The metabolite should be extensively purified
- The level of incorporation should far exceed that of a blank experiment*
- The site(s) and quantity of label should be determined

In the past, biosynthetic experiments were (and some still are) done with precursors labelled with radioactive isotopes. Detection occurred usually by scintillation counting. Therefore it was mostly necessary that the labelled metabolite was degraded under controlled conditions to small fragments, e.g. to one-carbon fragments for ^{14}C labelling, to locate the site and to determine the quantity of labelling. The specific degradation reactions were often elaborated during the preceding structure determination.

More recently, in particular since the late sixties, non-destructive methods of locating labelled atoms were developed based on nuclear magnetic resonance spectroscopy, following a suggestion by Lauterbur [7]; mass spectrometry was already used before and still is, especially for oxygen and nitrogen labelled compounds

---

*) Of course, in a blank experiment, there is no level of incorporation, but the meaning of this sentence will be clear.

but its use is limited by its low sensitivity due to the appearance of all natural abundance isotopes in the mass spectrum. This direct approach, which has met with considerable success, was applied initially to ^{13}C and subsequently to ^{2}H, ^{3}H, ^{15}N and ^{17}O. Though the use of radioactive isotopes in certain cases offers advantages such as low detection limit and low-cost detection, the disadvantages, especially the extensive and time-consuming degradations with often cumulative errors, are so severe that, if possible, one nowadays prefers the use of stable isotopes. Moreover, of recently discovered metabolites, the chemical knowledge necessary to degrade them unambiguously is rather meagre. This is due to the tendency to use almost exclusively spectroscopic methods for structure elucidation.

Of course, the use of stable isotopes has its disadvantages too, like low sensitivity (e.g. ^{13}C has a relatively high natural abundance), expensive equipment, and the fact that, because of low sensitivity, high amounts of precursor must be "fed", which sometimes can lead to metabolic distortion. Although the latter warning is often sounded , in practice there are only a very few examples known. In some cases the obtained result could be interpreted from a knowledge of intermediary metabolism [8] (see also chapter 4).

2.4. Application of NMR-spectroscopy in biosynthetic studies

2.4.1. Carbon-13

Several excellent reviews on the use of ^{13}C in biosynthetic studies are available [9], so comments here are limited to those aspects that are of importance for the present study (see table 1 for the relevant properties of ^{13}C compared to ^{1}H).

Table 1

	^{1}H	^{13}C
Nuclear spin I[a]	1/2	1/2
Resonance frequency[b]	90	22.63
Magnetic moment[c]	2.792	0.70216
Natural abundance[d]	99.9844	1.108
Relative sensitivity[e]	1.00	0.016
Receptivity[f]	1.00	0.000175
Normal chemical shift range[g]	10	225

a) in multiples of $\frac{h}{2\pi}$
b) at 2.11 Tesla in MHz
c) in multiples of the nuclear magneton
d) %
e) for equal number of nuclei at constant field
f) sensitivity at natural abundance
g) ppm

The quality of biosynthetic information depends almost entirely on the unequivocal assignment of the ^{13}C resonances and ideally these should be made independent of biosynthetic assumptions and experiments. The intensities of ^{13}C resonances increase after incorporation of ^{13}C labelled precursors. Labelled sites may be

identified by the differences between signal intensities in the p.n.d. (proton noise decoupled) spectra of the enriched and the unenriched material (see e.g. chapter 4, fig. 2). The subtraction necessary causes obvious difficulties at small enrichments, where errors are of the same order as the differences. To assign the observed intensity enhancements to incorporation, the enrichment factor (vide infra) must usually be larger than 0.5%.

Relative intensities (peak-heights) of ^{13}C resonances are mainly determined by the spin-lattice relaxation time (T_1) and nuclear Overhauser enhancement (NOE). Many factors can influence the intensities, e.g. pulse width, pulse delay, concentration, temperature, homogeneity of the static magnetic field etc.; therefore, enrichments measured by a difference method are only meaningful if all these variables are constant for the two spectra. Uncontrolled intensity variations, too large to be attributed to noise, can occur between successive Fourier transform (FT) measurements and are attributed to instrumental fluctuations [9a], although the use of large data blocks can circumvent these variations to a certain extent. Further errors can be introduced if the samples contain different trace quantities of paramagnetic impurities which can decrease both T_1 and the heteronuclear Overhauser enhancement, particularly for quaternary carbons. This is of special concern in aqueous solutions, for in organic solvents these impurities can easily be removed by washing with an aqueous solution of a chelating agent such as ethylenediaminetetraacetic acid. Although in FT experiments peak-heights are usually measured, integrals are preferable as they compensate for instrumental line-

broadening and unresolved couplings.

If incorporation is low, special effort is necessary to assign the labelled sites unambiguously and to calculate the enrichment factor. In these cases, it is advisable to normalize the peak-intensities, for which various methods have been developed. In this thesis, we have used the method of Hanson and coworkers [10], except that we use integrals instead of intensities. This method consists of dividing all the peak-intensities in both, the natural abundance spectrum and the spectrum of the enriched metabolite, by the respective intensity of a reference line, belonging to a carbon atom that with great certainty has not been labelled or affected by scrambling of the tracer:

$$i_n \text{ (normalized int.)} = \frac{\text{observed int.}}{\text{observed int. of ref. line}} \quad (2.1)$$

The total ^{13}C content X at a specific site can be obtained as X at a centre with ^{13}C at natural abundance only is 1.1% :

$$X = \frac{i_n \text{ labelled}}{i_n \text{ nat. abund.}} \times 1.1\% \quad (2.2)$$

The enrichment factor*, EF, for a specific site, i.e. the excess label above natural abundance, can be calculated with the following formula:

$$EF = X - 1.1\% \quad (2.3)$$

---

*) The term enrichment factor is not correct, for it is defined as a difference, not a factor.

The low probability (10^{-4}) of two adjacent carbon atoms being ^{13}C isotopes usually precludes the observation of ^{13}C-^{13}C satellites due to spin-spin coupling in the p.n.d. ^{13}C spectrum of a compound at natural abundance. The incorporation of precursors containing ^{13}C-^{13}C units will increase satellite intensities and this can be effectively used to measure enrichment factors at lower levels, e.g. 0.1% . 0.5% for singly-labelled precursors. An EF of 0.1% for a singly-labelled precursor means that the intensity of a resonance will increase by 10%, which is within the accuracy limit, so this cannot be assigned to an incorporation of a labelled precursor. An EF of 0.1% of a doubly-labelled precursor, (81% doubly-labelled*), will give satellites of 4% of the centre peak. For observation a signal-to-noise ratio of over 100:1 is desirable and the spinning-rate of the tube should be adjusted in such a way that the spinning side bands do not coincide with the satellites. This can be deduced from the known or estimated ^{13}C-^{13}C spin-spin coupling constant.
Another important advantage of using doubly-labelled precursors in enrichment studies, is that evidence can be obtained for the incorporation of <u>intact</u> units. Moreover, rearrangements will be visualized by the loss of ^{13}C-^{13}C coupling, indicating bond scission. On the other hand, using suitable nonadjacent labelled precursors, observation of ^{13}C-^{13}C couplings can also indicate rearrangements as used effectively in the study of porphyrin biosynthesis [11].

---

*) Nowadays almost all commercial available ^{13}C enriched compounds are enriched to 90%.

2.4.2. Deuterium and tritium

The use of ^{2}H NMR in biosynthetic studies has been reported only recently*. In the excellent comprehensive 1977 review of Mantsch, Saito and Smith [14] only two applications are mentioned [15]. This number has grown to about 15 at this moment and most of them deal with stereochemical problems [16]. This rise is in particular due to the increasing availability of cryomagnets with high field strength and the improved sensitivity of modern spectrometers. The application of NMR spectroscopy of the other hydrogen isotope tritium in biosynthesis has also been reported in recent years [17]**.

In table 2 the nuclear properties of ^{1}H, ^{2}H and ^{3}H are shown. The deuteron, in contrast to the other hydrogen isotopes, has a quadrupole moment which, in spite of its weakness, constitutes the major source of relaxation. This relaxation behaviour is responsible for the absence of a nuclear Overhauser enhancement, which theoretically could be a factor of 4.25. Under the same conditions deuteron chemical shifts are essentially the same as proton chemical shifts, since there is no primary isotope effect. These shifts are scaled down by a factor of 6.51 due to the smaller magnetogyric ratio of ^{2}H. Together with the broader lines, this considerably lowers the chemical shift dispersion

---

*) The use of deuterium as a label in biosynthetic studies has also been used in combination with ^{1}H NMR [12] and ^{13}C NMR [13] as a detection probe.

**) Very recently, the use of protium as a label in the study of biosynthesis has been reported, using $H_3{}^{13}C-{}^{13}CO_2H$ as a substrate in a culture in D_2O [18].

Table 2

	^{1}H	^{2}H	^{3}H
Nuclear spin I[a]	1/2	1	1/2
Resonance frequency[b]	90	13.82	96.0
Magnetic moment[c]	2.7927	0.8574	2.9788
Natural abundance[d]	99.9844	0.0156	-
Relative sensitivity[e]	1.00	0.00965	1.21
Receptivity[f]	1.00	$1.5.10^{-6}$	-
Chemical shift range[g]	10	10	10
Quadrupole moment[h]	-	$2.77.10^{-3}$	-

h) in units of e x 10^{-24} cm^2
For other units see Table 1.

relative to that of the proton. The demand for higher fields in deuteron NMR is therefore almost imperative. Also, the (scalar) spin-spin coupling constant $^{n}J(X-^{1}H)$ is reduced by a factor of 6.51 upon replacement of the proton by a deuteron. In comparison to $^{n}J(^{1}H-^{1}H)$, $^{n}J(^{2}H-^{2}H)$ is scaled down by a factor of 42.4. $^{2}H-^{2}H$ coupling constants in perdeuteriated compounds can therefore hardly be detected. Because of the low probability of two deuterons in adjacent positions at natural abundance, these couplings are not observed in unenriched compounds. The absence of $^{2}H-^{2}H$ couplings and the use of broadband proton decoupling result in an extremely useful simplification of the spectra with regard to the proton spectra, provided the individual resonances are resolved. Unfortunately, this is not the case in more complex molecules with similar functional groups, e.g. in peptides or steroids. Except

for the use of higher field strengths* (which is rather limited) attempts at narrowing the resonance-lines might improve the resolution. Apart from narrowing techniques by data-manipulation, which are accompanied by a loss in signal-to-noise ratio, one should consider the following theoretical possibilities. The linewidth at half height, $\Delta\nu_{1/2}$ is given by equation (2.4) (only valid under extreme narrowing conditions)

$$\Delta\nu_{1/2} = \frac{1}{T_2^*} = \frac{1}{T_2} + \delta\nu_{1/2} \qquad (2.4)$$

where T_2^* is the effective spin-spin relaxation time, T_2 is the spin-spin relaxation time which equals T_1 under these conditions and $\delta\nu_{1/2}$ the linewidth contribution from inhomogeneity in the static magnetic field. In modern superconducting magnets the contribution of field inhomogeneity is almost negligible (<0.1 Hz). The possibilities for line-narrowing can be extracted from equation (2.5) and (2.6) where $\frac{e^2qQ}{h}$ is the quadrupole coupling constant, τ_c the correlation time, η the viscosity and a the radius of a spherical molecule

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{3}{3}\left|\frac{e^2qQ}{\hbar}\right|^2 \tau_c \qquad (2.5)$$

---

*) The tendency to use increasingly higher fieldstrenghts, can give unexpected problems. Recently, line splittings have been observed which are caused by a small alignment induced by the interaction of the magnetic field with the diamagnetic susceptibility [19]. These splittings are proportional to the anisotropy in the diamagnetic susceptibility and to the square of the magnetic field.

$$\tau_c = \frac{4\pi\eta a^3}{3kT} \qquad (2.6)$$

From equation (2.5) and (2.6) it follows that T_1 ($=T_2$) is inversely proportional to the viscosity coefficient and directly proportional to the absolute temperature. Thus, with solvents of low viscosity (e.g. acetone or benzene) and with increasing temperatures, T_1 (and T_2) become longer and so ^2H resonances rapidly become narrower, e.g. the relaxation times of the enolic =CD-deuteron in acetylacetone-d8 are 0.346 sec at 0 °C, 0.615 sec at 35 °C and 0.914 sec at 63 °C, corresponding with a linewidth of 0.92 Hz, 0.52 Hz and 0.35 Hz, respectively [20]. However, one should not forget that the detection sensitivity decreases by increasing the temperature for the Boltzmann distribution is changed unfavourably.

Application of ^2H NMR in biosynthetic studies has the following advantages compared to ^{13}C:

- An enrichment factor with deuterium of only 1%* leads to a sixty-fold enhancement over the natural abundance signal.
- Shift assignments can be made from the corresponding proton spectra.

---

*) The calculation of enrichment factors in ^2H spectra (and also in ^{15}N spectra) is identical to ^{13}C spectra; equation (2.2) and (2.3) are changed in so far that 1.1% becomes 0.016% (0.365%).

- Integrals can be measured without difficulties (no NOE, no partial saturation as the the T_1's are short).

- Not only the construction of a skeleton can be studied but also the fate of the more peripheral hydrogens, and thus the stereochemical course of a reaction, can be followed.

- Deuteriated substrates and reagents are relatively inexpensive and deuterium can often be introduced into the molecule in one of the last steps of a reaction sequence by exchange reactions.

The use of tritium NMR-spectroscopy in biosynthesis seems most promising. The much higher sensitivity, the larger chemical shift separation, the absence of a quadrupole moment, the observability of spin-spin couplings and the absence of any background signals makes tritium in theory superior to deuterium. But the concomitant radioactivity makes its use limited to those cases where the precursor can be labelled in one of the last steps or where the incorporation is rather high. If one assumes a NMR detection limit of 0.5 mCi and an enrichment corresponding with an enrichment factor of 0.02% for deuterium as described in chapter 5, we would have needed in the first step of the ten-step synthesis of labelled tetramic acids (overall yield 5%) the huge amount of 9000 Ci of radioactivity.

2.4.3. Nitrogen-15

Since the skeleton of many natural products is largely composed of carbon and nitrogen, the application of carbon and nitrogen NMR to structural problems

is potentially even more powerful than proton NMR techniques. Table 3 gives some of the important nuclear properties of both nitrogen isotopes compared to ^{1}H.

Table 3

	^{1}H	^{15}N	^{14}N
Nuclear spin[a]	1/2	1/2	1
Resonance frequency[b]	90	9.12	6.5
Magnetic moment[c]	2.7927	-0.28304	0.40357
Natural abundance[d]	99.9844	0.365	99.635
Relative sensitivity[e]	1.00	0.001	0.001
Receptivity[f]	1.00	$3.8.10^{-6}$	0.001
Chemical shift range[g]	10	1000	1000
Quadrupole moment[h]	-	-	$7.1.10^{-2}$

For units see Table 1 and 2.

Although the ^{14}N isotope has the advantage of a relatively high overall sensitivity, it is unsuitable for high resolution NMR because the relatively large electric quadrupole moment gives rise to too broad lines ($\Delta\nu_{1/2}$ > 100 Hz) Despite some of its unfavourable nuclear magnetic properties the ^{15}N isotope has received increasing attention in the last few years. Undoubtedly, the advent of the modern high field spectrometers has been the major impetus for the application of ^{15}N NMR to divergent structural problems. In biosynthetic studies only a few examples of ^{15}NMR have yet been reported [21]; however, more mass spectroscopic studies have been performed, as ^{15}N is the only means available to study nitrogen metabolism with tracer methods.

In measuring ^{15}N, attention should be paid to the following more or less characteristic features:

- The heteronuclear Overhauser effect is large but leads to negative enhancements because of the negative magnetogyric ratio of ^{15}N

$$\frac{M_z}{M_z^o} = 1 + \frac{\gamma_H}{2\gamma_N}\frac{T_1}{T_1 dd} = 1 - 4.93\frac{T_1}{T_1 dd} \simeq -3.9 \quad (2.7)$$

  $T_1 dd$ is the dipole-dipole relaxation time which is mostly dominant in nitrogen relaxation, at least for those nitrogens directly bonded to hydrogen. However, dipolar relaxation is not the exclusive relaxation mechanism and hence NOE's between 0 and -2 have to be avoided for these lead to lower intensities or even to a nulling of a resonance line on continuous irradiation of the proton frequencies. In these cases gated decoupling will eliminate this problem.

- Long spin-lattice relaxation times (T_1 up to 170 sec. have been reported [22]) can be shortened by addition of paramagnetic relaxation reagents; this method is particularly suitable to tertiary nitrogens. However, the absence of broadening effects of these reagents is a prerequisite.

- For measurement of nitrogens in solvents where exchange of protons attached to nitrogens is possible, attention should be paid to circumvent conditions where the exchange rate is comparable to the time scale of the NMR experiment, otherwise signals will broaden or will hardly be detectable.

When these features are taken into account, sharp lines can be observed, (often presented like negative lines (with NOE)), and ^{15}N NMR can be a powerful tool in natural product chemistry.

2.5. References

1 T. Swain, in Biosynthetic Pathways in Higher Plants, J.B. Pridham and T. Swain, eds., Academic Press, London, 1965, p. 9.

2 For a review see: A.J. Birch, Interdiscip. Sci. Rev., 1976, 1, 215.

3 R. Robinson, Nature, 1947, 159, 263.

4 D. Ranganathan and S. Ranganathan, Art in Biosynthesis, Vol. 1, Academic Press, London, 1976.

5 a) S. Gatenbeck and J. Sierankiewicz, Antimicrob. Agents Chemother., 1973, 3, 308.
b) H. Umezawa, Pure and applied Chem., 1971, 28, 665.
c) Y. Okumura, T. Takei, M. Sakamoto, T. Ishikura, and Y. Fukagawa, J. Antibiotics, 1979, 32, 1002.

6 E. McDonald, Pharm. Weekblad, 1976, 111, 941.

7 P.C. Lauterbur, Ann. New York Acad. Sci., 1958, 70, 841.

8 E.F. Elstner, R.J. Suhadolnik, and A. Allerhand, J. Biol. Chem., 1973, 248, 5385.

9 a) J.B. Grutzner, Lloydia, 1972, 35, 375.
b) G. Lukacs, Bull. Soc. Chim. Fr., 1972, 351.
c) M. Tanabe, Biosynthesis, Vol. 2, T.A. Geissman, ed., The Chemical Society, London, 1973, p. 241.
d) U. Sèquin and A.I. Scott, Science, 1974, 186, 101.
e) A.G. McInnes and J.L.C. Wright, Acc. Chem. Res., 1975, 8, 313.
f) T.J. Simpson, Chem. Soc. Rev.,1975, 4, 497.
g) N. Neuss, Methods in Enzymology, Vol. 43, J.H. Hash, ed., Academic Press, New York, 1975, p. 404.
h) A.G. McInnes, J.A. Walter, J.L.C. Wright, and L.C. Vining, Topics in Carbon-13 NMR Spectroscopy, Vol. 2, G.C. Levy, ed., 1976, p. 123.
i) U. Sèquin and A.I. Scott, Heterocycles, 1976, 5, 525.
j) G. Kunesch and C. Poupat, Isotopes in Organic Chemistry, Vol. 3, E. Buncel and C.C. Lee, eds., Elsevier, Amsterdam, 1977, p. 105.

10 J.R. Hanson, T. Marten, and M. Siverns, J. Chem. Soc. Perkin I, 1974, 1033.

11 For a review see: A.R. Battersby and E. McDonald, Acc. Chem. Res., 1979, 12, 14.

12 L.M. Jackman, I.G. O'Brien, G.B. Cox, and F. Gibson, Biochim. Biophys. Acta, 1967, 141, 1.

13 U. Sankawa, H. Shimada, T. Sato, T. Kinoshita, and K. Yamasaki, Tetrahedron Letters, 1977, 483.

14 H.H. Mantsch, H. Saitô, and I.C.P. Smith, Progress in Nuclear Magnetic Resonance Spectroscopy, Vol. 11, J.W. Emsley, J. Feeney, and L.H. Sutcliffe, eds., Pergamon Press, Oxford, 1977, p. 211.

15 a) B.W. Bycroft, C.M. Wels, K. Corbett, and D.A. Lowe, J. Chem. Soc. Chem. Comm., 1975, 123.
b) Y. Sato, T.Oda, and H. Saitô, Tetrahedron Letters 1976, 2695.

16 a) Y. Sato, T. Oda, and H. Saitô, J. Chem. Soc. Chem. Comm., 1977, 415.
b) D.E. Cane and S.L. Buchwald, J. Amer. Chem. Soc., 1977, 99, 6132.
c) D.E. Cane and P.N. Murthy, J. Amer. Chem. Soc., 1977, 99, 3327.
d) P.M. Dewick and D. Ward, J. Chem. Soc. Chem. Comm., 1977, 338.
e) Y. Sato, T. Oda, and H. Saitô, J. Chem. Soc. Chem. Comm., 1978, 135.
f) D.J. Aberhart, S.P. Jindal, and E. Caspi, J. Chem. Soc. Chem. Comm., 1978, 333.
g) B.F. Chumpitazi-Hermoza, D. Gagnaire, and F. Taravel, Biopolymers, 1978, 17, 2361.
h) Y. Sato, T. Oda, E. Miyata, and H. Saitô, FEBS Letters, 1979, 93, 271.
i) D.J. Aberhart and E. Caspi, J. Amer. Chem. Soc., 1979, 101, 1013.
j) J.R. Hanson and H.J. Wadsworth, J. Chem. Soc. Chem. Comm., 1979, 360.
k) C.R. Hutchinson, A.H. Heckendorf, J.L. Straughn, P.E. Daddona, and D.E. Cane, J. Amer. Chem. Soc., 1979, 101, 3358.
l) S. Damtoft, S.R. Jensen, and B.J. Nielson, J. Chem. Soc. Chem. Comm., 1980, 42.
m) D.E. Cane and R.B. Nachbar, Tetrahedron Letters, 1980, 437.

17 For a review see: J.A. Elvidge, J.R. Jones, V.M.A. Chambers, and E.A. Evans, Isotopes in Organic Chemistry, Vol. 4, E. Buncel and C.C. Lee, eds., Elsevier, Amsterdam, 1978, p. 1.

18 J. Barber and J. Staunton, J. Chem. Soc. Chem. Comm., 1979, 1098.

19 J.A.B. Lohman and C. MacLean, Mol. Phys., 1979, 38, 1225.

20 W. Egan, G. Gunnarson, T.E. Bull, and S. Forsèn, J. Amer. Chem. Soc., 1977, 99, 4568.

21 a) H. Booth, B.W. Bycroft, C.M. Wels, K. Corbett, and A.P. Maloney, J. Chem. Soc. Chem. Comm., 1976, 110.
b) J.A. Hörig, P. Renz, and G. Heckmann, J. Biol. Chem., 1978, 253, 7410.

22 D. Schweitzer and H.W. Spiess, J. Magn. Resonance, 1974, 16, 243.

Chapter 3

Interpretation of ^{13}C, ^{1}H, ^{2}H and ^{15}N NMR spectra of malonomicin

3.1. Introduction

A prerequisite for the unambiguous interpretation of the results from biosynthetic studies using NMR methods, is the complete and unequivocal interpretation of the NMR spectra involved. Furthermore, the solubility and stability in solution of the compound to be measured have to be such that a NMR spectrum can be obtained in a time, during which degradations occur to only a minor extent.

Malonomicin is not soluble in organic solvents; in water it is soluble only by addition of acid or base. Since malonomicin is not very stable in acidic solution, and at high pH the protons at C-4 and C-7 are exchanged at a measurable rate, the NMR spectra of malonomicin are measured mostly in aqueous solutions of pH 7-10. However, two of the pK_a's fall in this region, pK_a= 7.8 of the serine amino group and pK_a=9.2 of the aminomethyl group [1]; therefore, chemical shifts of most resonances in ^{13}C, ^{1}H, ^{2}H and ^{15}N spectra of malonomicin will be pH dependent. At the beginning of this investigation, only the ^{1}H spectrum of the decarboxylation product of malonomicin (K16A) had been interpreted [1] at several pH's, showing the

considerable pH-dependency of the chemical shifts in the pH region 7-10.

In ^{13}C spectra of malonomicin, on changing the pH, the rather small chemical shift differences in comparison with the chemical shift range will give no special problems in determining the site(s) of labelling and calculating the enrichment factors. Moreover, the pH dependency of some chemical shifts can be used effectively for the assignment of the NMR spectrum.

^{1}H chemical shifts (and thus ^{2}H chemical shifts) are relatively more sensitive to changes in pH due to the much smaller chemical shift range. This is reinforced by the fact that all stable protons of malonomicin resonate within 0.8 ppm; thus, control of the pH and the use of high field strength is necessary for complete analysis of the proton spectra. From this analysis automatically the deuterium chemical shifts are obtained, as there is no primary isotope effect for the chemical shifts of protons and deuterons.

In the proton broadband decoupled ^{15}N spectrum, only four resonances are to be expected, which will also show a strong pH-dependency. Moreover, in the pH region 7-10, broadening of those resonances of nitrogen atoms, that are involved in protonation-deprotonation processes at a rate comparable to the NMR time scale, can be expected. However, given the large chemical shift range, this will not raise insuperable problems.

3.2. Carbon-13 NMR spectra

The natural abundance ^{13}C spectrum of malonomicin in H_2O at pH 8.5 (proton noise decoupled; p.n.d.) is shown in figure 1.

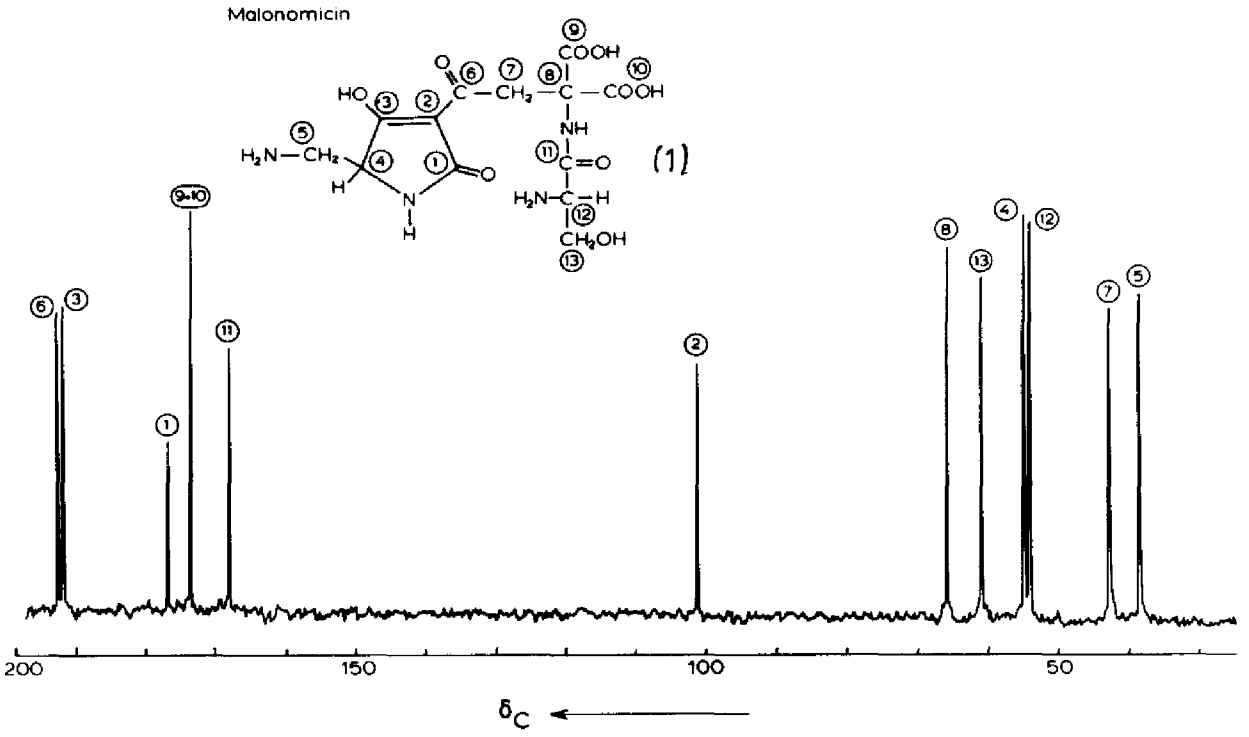
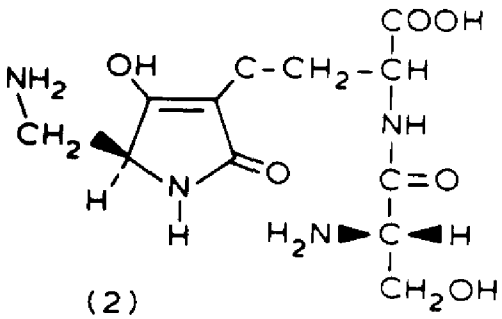
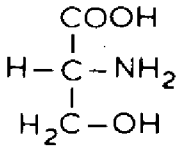


figure 1 Natural abundance proton noise decoupled ^{13}C spectrum of malonomicin in H_2O at pH 8.5.

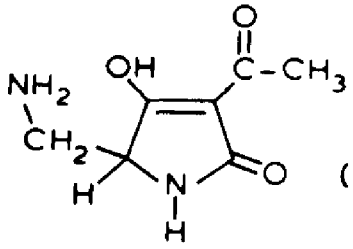
The resonances were assigned by comparison with resonances of the decarboxylation product of malonomicin (2), the model compound 2-acetyl-4-aminomethyltetramic acid (3) [1], serine (4), 2-carboxyaspartic acid (5) and its decarboxylation product D,L-aspartic acid (6). Use was also made of single frequency off resonance decoupling (s.f.o.r.d.) to determine the number of attached protons at each carbon atom, and of the results of selective deuteriation studies. ^{13}C resonances of these compounds at pH 8.5 are summarized in table 1 and in table 2 the resonances of malonomicin at



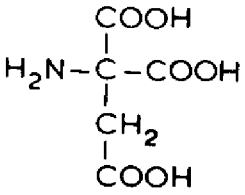
(2)



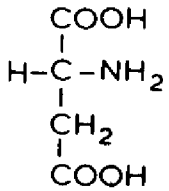
(4)



(3)



(5)



(6)

different pH's are given. In the ^{13}C spectrum of malonomicin, the resonances of five carbonyl carbon atoms, one olefinic carbon atom and six sp^3 hybridised carbon atoms are visible. However, in malonomicin six carbonyl carbon atoms are present. The carbonyl carbon resonance at δ 175.6 with about twice the intensity of the other carbonyl carbon atoms is assigned to the coincident resonances of the two carboxylic carbon atoms C-9 and C-10. Evidence for this assignment is provided by decarboxylation of malonomicin to give (2), which causes a decrease of signal intensity of this particular resonance accompanied by a downfield shift of 3.0 ppm in agreement with similar phenomena observed with the model compounds 2-carboxyaspartic acid (5) and D,L-aspartic acid (6).

Decarboxylation of malonomicin also causes a strong upfield shift of the resonance at δ 67.4, assigned to C-8, and a smaller upfield shift (2.4 ppm) of the resonance at δ 44.5 (which is a triplet in the s.f.o.r.d. spectrum), which can be assigned to C-7, the carbon atom in the β position.

Table 1

^{13}C chemical shifts of malonomicin (1) and model compounds in H_2O at pH 8.5 (in ppm downfield from TMS (δ = 0), calculated from internal dioxan (δ 66.7); multiplicities in the s.f.r.o.d. spectra are indicated with s, d, t and q.

carbon*	(1)	(2)**	(3)+(4)	(5)	(6)
1	178.4 s	178.4 s	177.3 s		
2	103.0 s	103.1 s	102.8 s		
3	194.0 s	194.5 s	194.9 s		
4	57.2 d	57.6 d	58.6 d		
5	40.8 t	40.7 t	41.7 t		
6	194.3 s	194.0 s	195.9 s	177.8 s	177.9 s
7	44.5 t	42.1 t	14.0 q	39.3 t	37.0 t
8	67.4 s	52.4 d		67.1 s	52.4 d
9/10	175.6 s	178.6 s		172.2 s	174.3 s
11	170.4 s	173.2 s	173.9 s		
12	56.0 d	56.2 d	57.5 d		
13	63.3 t	63.5 t	63.9 t		

*) for numbering system see figure 1

**) for all carbons except C-4, C-7 and C-13 two resonances could be observed ($\Delta\delta$ < 0.3 ppm) due to the formation of diastereoisomers on decarboxylation; in the table the average chemical shift is given.

Assignment of the resonances at δ 57.2 and δ 56.0 (both are doublets in the s.f.o.r.d. spectrum) could be made by selective deuteriation. Proton-deuteron exchange at

Table 2

^{13}C chemical shifts of malonomicin at different pH's.

pH	7.6	8.0	8.5	9.0	9.5	10.0
1	178.4	178.4	178.4	178.4	178.4	178.4
2	102.9	102.9	103.0	103.1	103.3	103.5
3	193.8	193.8	194.0	194.4	194.9	195.2
4	56.7	56.8	57.2	57.9	58.7	59.1
5	40.7	40.7	40.8	41.0	41.3	41.5
6	194.3	194.3	194.3	194.3	194.3	194.3
7	44.5	44.5	44.5	44.5	44.5	44.5
8	67.6	67.5	67.4	67.4	67.4	67.4
9/10	175.5	175.5	175.6	175.7	175.7	175.8
11	168.4	169.7	171.1	171.9	172.3	172.5
12	55.5	55.7	56.0	56.3	56.4	56.5
13	62.0	62.6	63.3	63.7	63.9	64.0

C-4 in D_2O at pD 14 (monitored also by following the simultaneous racemization by optical rotation measurement) caused a more than ten-fold decrease in intensity of the δ 57.2 resonance; the expected splitting by $^{13}C-^{2}H$ spin-spin coupling is not clearly observed, probably due to the fast quadrupolar relaxation rate. Proton-deuteron exchange at C-12 by means of pyridoxal phosphate in D_2O at pD 9.6 (which notably affects the exchange of the α-proton of α-amino acids) has a pronounced effect on the δ 56.0 resonance. Prolonged treatment of malonomicin with NaOD at pD 14 also caused a relatively slow proton-deuteron exchange of the

protons at C-7 (44.5 ppm) which provided corroboration for its assignment.

The resonances at lowest field with normal values for keto- or enolic carbon atoms are attributed to C-3 and C-6. The C-3 resonance was expected to show a titration shift in the pH region 7-10, as a consequence of the proximity of the NH_2 group at C-5* which has a pK_a of 9.2 (see table 2). The behaviour of the δ 194.0 resonance in this pH region ($\Delta\delta$ 1.4) is in agreement with this expectation. The nearby carbonyl resonance at δ 194.3 is not pH dependent in this region and is therefore assigned to C-6.

The behaviour of the highest field carbonyl group at different pH's, ($\Delta\delta$ 4.1 β(pH 7.6-10)), points to a position of an amino group with a pK_a in this region. This agrees with the position of C-11, which is β to the amino group of the serine part of the molecule (pK_a = 7.8).

The remaining resonances at δ 178.4, δ 103.0 and δ 63.3 can be readily assigned; δ 178.4 to C-1 (normal amide resonance) , δ 103.0, (the only olefinic carbon atom) to C-2 and δ 63.3 to the hydroxymethyl group of serine at C-13. The enrichment studies (chapter 4) provide ample confirmatory evidence for all assignments made. From spectra of malonomicin, labelled with ^{13}C, coupling constants have been determined and they are given

---

*) Normally a value of 3-4 is found for the lowest pK_a of tetramic acids [2] and the lowest pK_a for malonomicin (0.9) had been explained by the presence of an amino group at C-5 by which a hydrogen bond can be formed with the oxygen at C-3 [1]. This can only explain the observed low pK_a in part, for the pK_a of model compound (3) is found to be 1.9 [1].

in table 3. They all agree with literature values.

Table 3

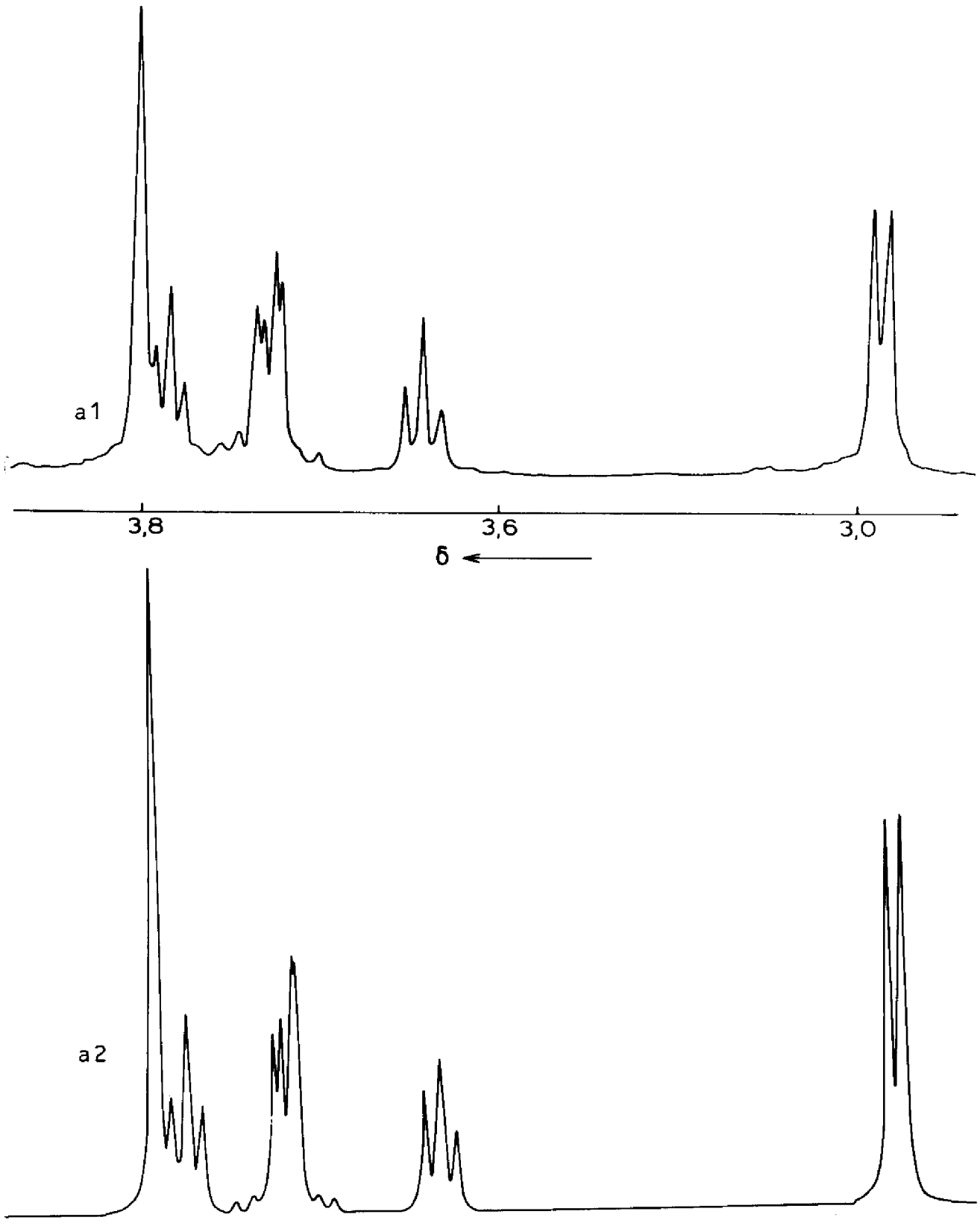
Coupling constants observed in [1,2-$^{13}C_2$]acetate-, [2-^{13}C]acetate-, DL-[1-^{13}C]DAP-, and [1,4-$^{13}C_2$]succinate-derived malonomicin.

Carbons	^1J(C-C)	^2J(C-C)	^3J(C-C)
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		

3.3. Proton and deuteron spectra

3.3.1. Proton spectra

Proton spectra of malonomicin were measured in D_2O at different pD's and analyzed by simulation. In figure 2 the measured and simulated spectra are given at pD 8.37 and pD 9.64. The coupling constants are tabulated in table 4. At high field strength, the proton spectrum can easily be assigned. Depending on the pH, three spin systems can be distinguished, an ABX or A_2X system, an ABX, ABM or ABC system, and an AB or A_2 system. On the basis of chemical shifts and decoupling


a1
3,8
3,6
3,0
δ
a2

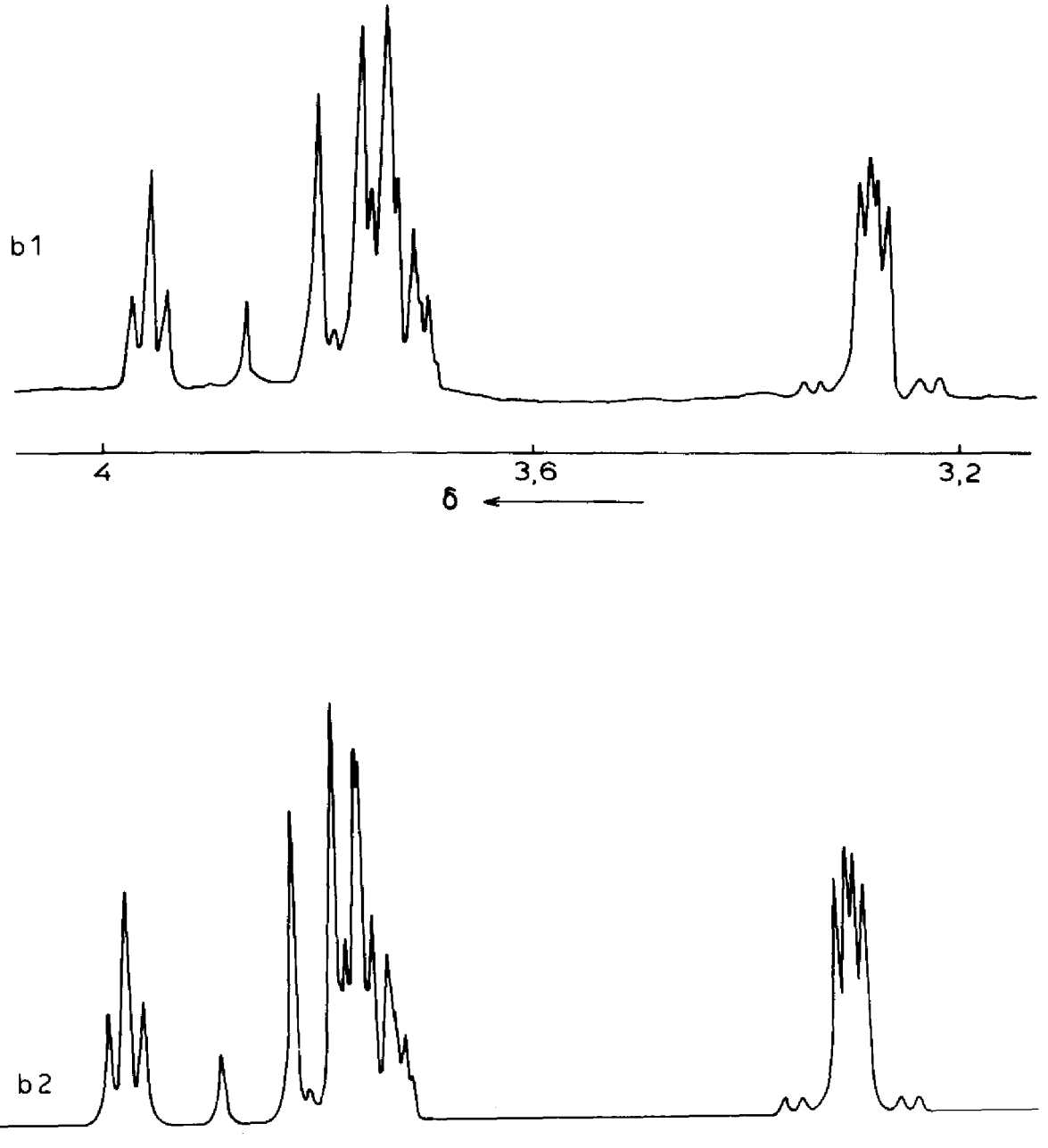


figure 2 Proton spectra of malonomicin (250 MHz) at pD 9.64 (a) and pD 8.37 (b); measured (1), simulated (2).

Table 4

Coupling constants observed in the proton spectrum of malonomicin at pD 8.5 (the different protons bonded to one carbon have been designated by a and b).

^nJ(H-H)	Hz	^nJ(H-H)	Hz
4 -5a	4.2	12 -13a	4.7
4 -5b	4.5	12 -13b	5.8
5a-5b	13.2	13a-13b	-12.4
7a-7b	17.4		

experiments, the ABX or A_2X system can be assigned to the methylene protons of the aminomethyl group (C-5) and the proton at C-4; the ABX, ABM or ABC system is formed by the protons of the serine moiety, and the AB or A_2 system is formed by the protons at C-7. These assignments agreed with the behaviour of the chemical shifts on changing the pH. In figure 3, the chemical shifts of all resonances are plotted as a function of the pD. From these data it can be concluded that nearly all chemical shifts are strongly pH dependent. Furthermore, the protons at C-5 are equivalent at high pD (>10.5), but not at lower pD. This is also found for the protons at C-7.

The non-equivalence of the aminomethyl protons at C-5 is probably due to a hydrogen bridge between the protonated amino group at C-5 and the oxygen at C-3 (see footnote at p. 46). The reason for the increased chemical shift difference of the C-7 protons at lower pD's is not clear; probably the serine amino group is

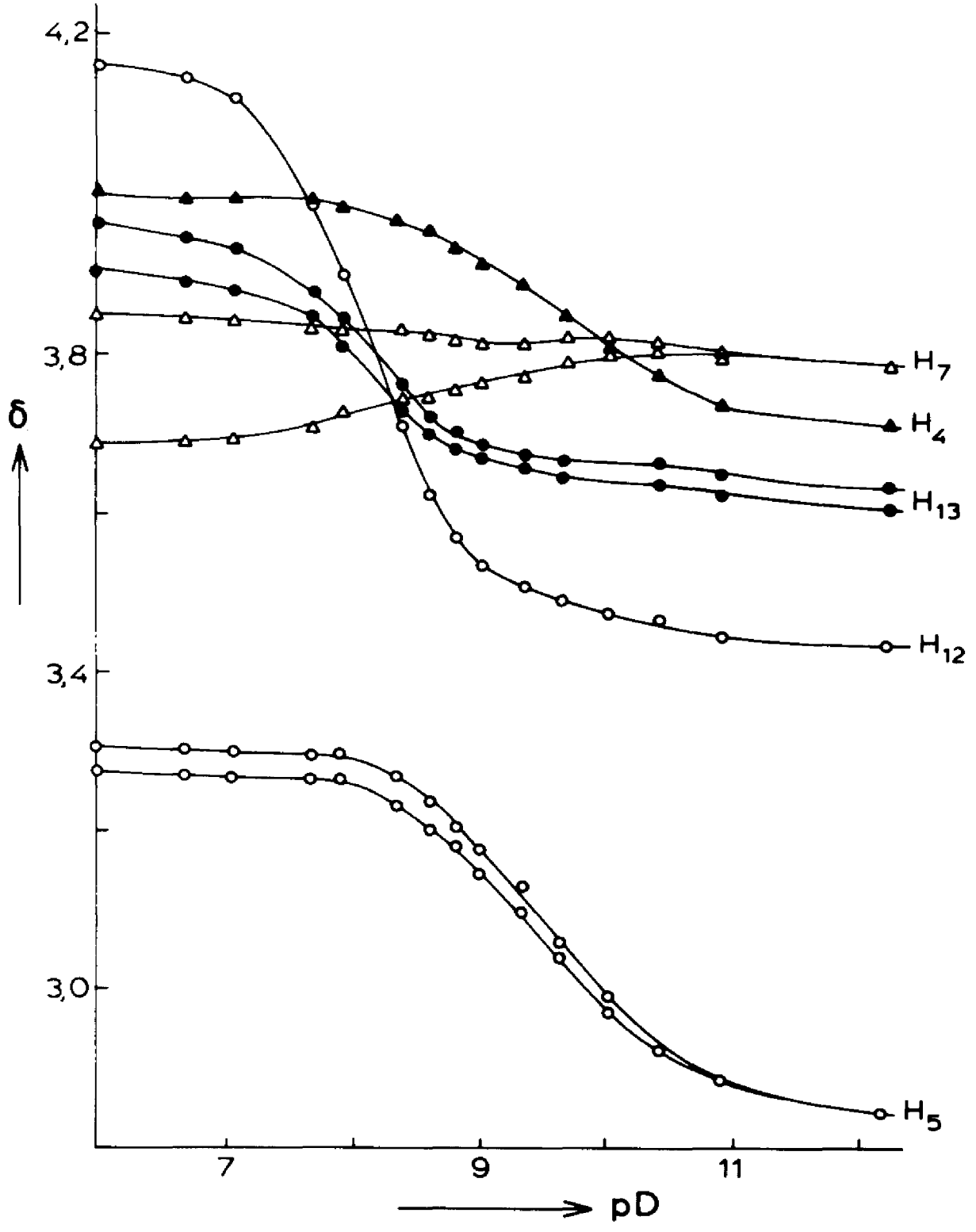


figure 3 Proton chemical shifts of malonomicin as a function of the pD.

involved in an intramolecular hydrogen bond, fixing the molecule in a rather rigid conformation. Evidence for this is provided by the fact that the difference in chemical shift between the protons at C-7 develops parallel with the degree of protonation of the amino group of the serine part (see figure 3).

3.3.2. Deuteron spectra

In general, with high field spectrometers it is not difficult to obtain a deuteron spectrum at natural abundance of solutions of >0.3 M. Malonomicin however, has several characteristics which make the observation of deuteron spectra very unfavourable (cf. chapter 2.4.2.):

- Malonomicin is only soluble in aqueous acid and base, which are relative viscous solvents; this will broaden the resonances considerably (for example by a factor of 3 compared to solutions in acetone).

- Malonomicin is not stable at high temperatures.

- Chemical shift dispersion in the proton spectra of malonomicin is small; at pH 8 all protons resonate between δ 4.0-3.6 except for the protons at C-5 which resonate at δ 3.2. This is felt the more strongly in the deuteron spectra, where the differences in chemical shift (in Hz) are reduced by a factor of 6.5 (at constant field).

- The resonances are at high field of the solvent peak, δ 4.7 at room temperature. The high molarity of the solvent vs 0.4 M malonomicin will cause severe overlap of almost all resonances by the solvent peak.

Moreover, at higher temperatures, the solvent peak shifts to higher field and aggravates the overlap, though the linewidth will be smaller.

As a consequence of these disadvantages, special measures have to be taken for measuring deuteron spectra of malonomicin at natural or low abundance (EF <0.5%). The only way of lowering the viscosity is raising the temperature, but for stability and detection-sensitivity reasons this is limited to a small temperature range and a short period. In practice, this means that measurements were made at 323 K and for 1-2 hours.

To overcome the problem of small chemical shift dispersion and to observe spectra at low enrichments, spectra were measured at high field, 9.3 Tesla (61.42 MHz) which also provided the necessary sensitivity. Samples with rather high enrichments (EF >1%) were usually measured at 27.64 MHz.
By the use of deuteron-depleted water (to 1% of the natural abundance) the overlap of resonances with the solvent peak could be avoided, even at higher temperatures.
In figure 4a the natural abundance ^{2}H spectrum of malonomicin is given. In this spectrum, except for the solvent peak at 4.5 ppm, only two deuteron resonances are visible; the C-5 deuterons at high field (3.25 ppm) and the other deuterons at 3.82 ppm. Apparently, the linewidth is too large to observe single resonances for each deuteron. From spectra of malonomicin, specifically enriched at positions 4, 7 or 12, linewidths could be measured which appeared to be in the range of 14-18 Hz (at 323 K). Low level enrichments (EF <0.1%)

can only be made visible by comparison of the spectrum with the natural abundance spectrum. From the integral ratio between the two groups of resonances, an enrichment can be indicated, although the determination of the exact position of the label is not easily accomplished, because the position of the resonances is strongly pH-dependent (see figure 3).
When the pH of the enriched sample solution is known accurately, the natural abundance spectrum at the same pH can be simulated from the chemical shifts of the corresponding proton spectrum (see figure 3). When the pH is not accurately known (the pH of the samples was usually adjusted using a pH indicator strip), the positions of all other resonances can be read from figure 3 when the chemical shift of the deuterons at C-5 is known. The resonance of the deuterons at C-5 is the only resolved resonance and the chemical shift of this resonance is pH dependent in the region in which the spectra are usually measured.

In figure 4 the natural abundance ^{2}H spectrum of malonomicin is compared with a simulated spectrum. The simulation of the spectrum was done by calculating the proton-stick spectrum and adding a lorenztian linewidth of 19 Hz.

In this manner, also enrichments can be determined at very low levels (chapter 5).

3.4. Nitrogen spectrum

With modern high field spectrometers it is possible to obtain nitrogen-15 NMR spectra of solutions down to 0.05 M when the spectral parameters are carefully

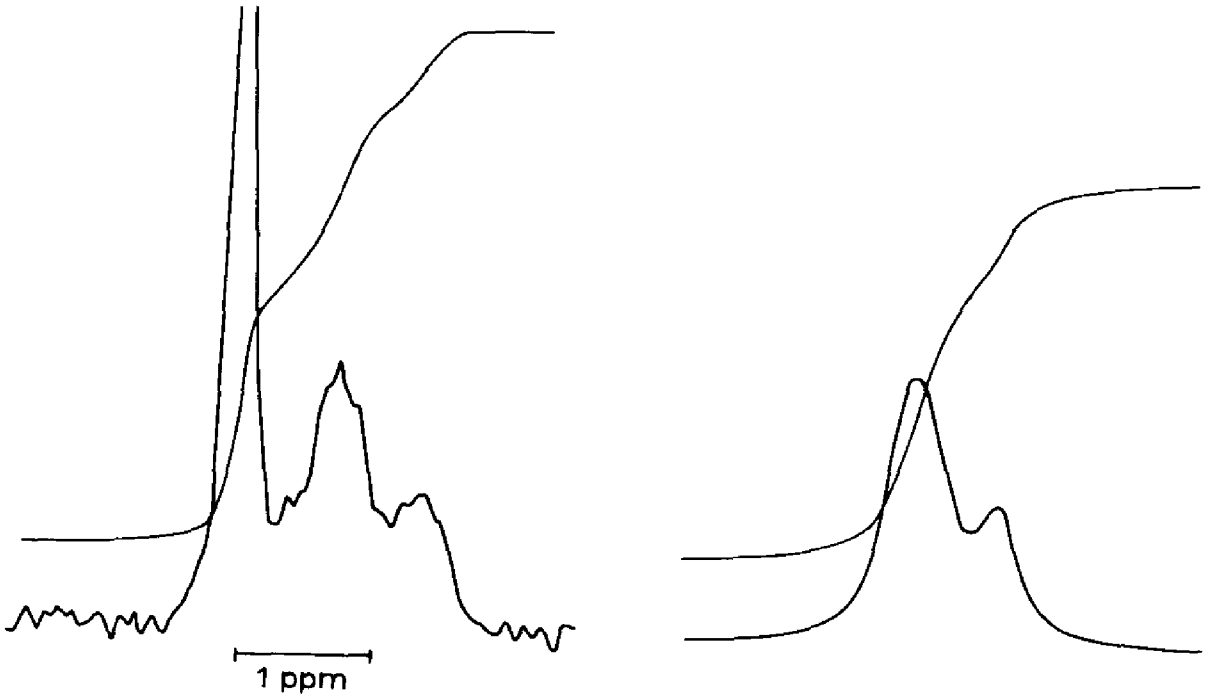


figure 4 Natural abundance ^{2}H spectrum of malonomicin (61.42 MHz) (a) and simulated spectrum (b).

chosen (cf. chapter **2.2.3.**). The ^{15}N spectrum of malonomicin at pH 8.5 is shown in figure 5. The expected four resonances are observed, all of them with a considerable NOE (all nitrogens are proton bearing). The assignment of the resonances is straightforward. The lowest field resonance is assigned to the amide resonance (N-1). The chemical shift (δ 132.8) agrees very well with the observed chemical shift of the α-nitrogen of DAP in viomycin (δ 132.5) [4]. The other low field resonance can then be assigned to the aminomalonic acid nitrogen (N-3). The two other high field resonances belong to primary amino nitrogens. In model compound L-**2,3**-diaminopropanoic acid the chemical shifts are δ 31.3 for the 2-amino group and δ **24.8** for the **3**-amino

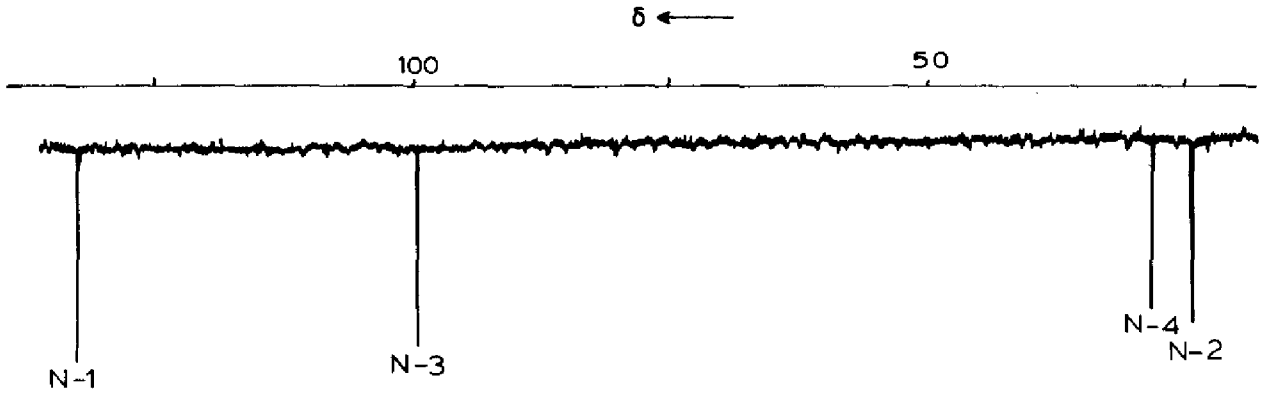


figure 5 ^{15}N spectrum of malonomicin. Chemical shifts are in ppm from anhydrous liquid ammonia and have been converted from the originally measured $NH_4{}^{15}NO_3$ (5M in 2 N-HNO_3) reference with conversion constant 375,6 ppm [3].

group, which is expected as increasing alkyl substitution at the nitrogen-bonded carbon will cause downfield shifts [3]. Therefore, the resonance at δ **28.4** is assigned to the serine amino group (N-4) and the resonance at δ 24.7 to the amino group at C-5 (N-2).

In a labelling experiment with $^{15}NH_4Cl$ (500 mg/l), uniformally enriched malonomicin (ca 20% ^{15}N) was obtained. By means of ^{13}C NMR, ^{13}C-^{15}N coupling constants could be measured, which are tabulated in table 5. They all agree with literature values [3].

Table 5

Coupling constants observed in ^{15}N enriched malonomicin (absolute values)

$^1J(^{13}C-^{15}N)$	Hz	$^1J(^{13}C-^{15}N)$	Hz
$^{13}C_1-^{15}N_1$	12.8	$^{13}C_8-^{15}N_3$	11.6
$^{13}C_4-^{15}N_1$	10.0	$^{13}C_{11}-^{15}N_3$	16.0
$^{13}C_5-^{15}N_2$	4.9	$^{13}C_{12}-^{15}N_4$	6.5

3.5. Experimental

3.5.1. Carbon-13 spectra

^{13}C spectra were recorded on a Varian XL-100 at 25.16 MHz or a Bruker WH 90 at 22.63 MHz. Typically, malonomicin was dissolved in H_2O-D_2O (1.2 ml; 10 :1 v/v) and after adjusting the pH to 8.5, filtered into a 10 mm tube; teflon anti vortex plugs were used. The spectral width was 5 KHz (XL-100) or 6 KHz (WH 90). The pulse delay was 5 sec., 8K data points were recorded and 45 ^{o} pulses were used; ca 12.000 transients were accumulated under proton broadband decoupling. For determination of enrichments, spectra were recorded during the weekend and always a reference spectrum of unlabelled malonomicin was run under identical conditions.

3.5.2. Proton spectra

^{1}H spectra were recorded on a Bruker WH 90, a Bruker WM 250, a Bruker WH 270, and a Bruker HX 360. Spectral width and number of data points were adjusted in such a way as to determine chemical shifts with an accuracy of at least 0.002 ppm. The pD of the samples (0.15 M) in D_2O was measured using a pH meter with a semi-micro glass electrode. Simulations were performed using the ITRCAL program on the Bruker WH 90.

3.5.3. Deuteron spectra

^{2}H spectra were recorded on a Bruker WH 130 (27.64 MHz), a Bruker WM 250 (33.39 MHz), and a Bruker WH 400 (61.42 MHz). Typically, malonomicin (125-300 mg) was dissolved in 1.2 ml H_2O and filtered into a 10 mm tube; for measurements of samples with low enrichments, deuteron-depleted water (Aldrich) was used. The pH was adjusted to ca 8.5 with 2N-KOH using a pH indicator strip. The spectral width was usually 1000 Hz; 256 data points were used for accumulation and 1K data points for Fourier transform. 90^{o} pulses were used without delay. An exponential line-broadening of 3 Hz was applied.

3.5.4. Nitrogen-15 spectra

^{15}N spectra were recorded on a Bruker WP 200 (20.28 MHz), a Bruker WH 270 (27.36 MHz), and a Bruker WH 400 (40.55 MHz). Typically, malonomicin (300 mg) was dissolved in 3.5 ml H_2O-D_2O (10 : 1) and filtered into a 15 mm NMR tube after adjusting the pH to 8.5. Spectral width 3000 Hz (WP 200), pulse width 20 µsec. (45 deg.),

acquisition time 2.64 sec., 16 K data points. 1000 scans were accumulated under proton broadband decoupling. Exponential line-broadening of 0.3 Hz was applied. The reference line was external $^{15}NO_3^-$ in 5 M HNO_3. The accuracy in chemical shift determination is about 2 Hz.

Acknowledgement

Some of the spectra have been measured at the laboratories of Shell Amsterdam, University of Groningen and Bruker Spectrospin Karlsruhe. I wish to thank all the people involved with these measurements. I wish to thank Bruker Spectrospin ZÜrich for the hospitality and assistance at the measurement of the high-field deuteron spectra.

3.5. References

1 J.G. Batelaan, J.W.F.K. Barnick, J.L. van der Baan, and F. Bickelhaupt, Tetrahedron Letters, 1972, 3103, 3107.

2 a) C.E. Stickings, Biochem. J., 1959, 72, 332.
b) S. Ito and Y. Hirata, Tetrahedron Letters, 1972, 1181, and references cited therein.

3 G.C. Levy and R.L. Lichter, Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy, John Wiley, New York, 1979.

4 G.E. Hawkes, E.W. Randall, W.E. Hull, D. Gattegno, and F. Conti, Biochemistry, 1978, 17, 3986.

Chapter 4

Feeding experiments with simple precursors

4.1. Introduction

Consideration of the possibilities for the biosynthesis of malonomicin from simple precursors, and comparison with the known mode of biosynthesis of other tetramic acids like tenuazonic acid (chapter 1, refs. 23-25) lead to the working-hypothesis that the heterocyclic nucleus of malonomicin could be derived from L-2,3-diaminopropanoic acid (L-DAP) and acetate (or malonate), whereas the origin of DAP itself and the serine part of the molecule could be L-serine [1]. The origin of the rest of the side-chain (C-6, C-7, C-8, C-9 and C-10) however, is far less obvious. As already mentioned in chapter 1, the occurrence of aminomalonic acid as such in nature is still unknown; only the enzymatic conversion (in vitro!) of oxomalonic acid to aminomalonic acid [2] and the natural occurrence of two derivatives give reason not to exclude the possibility of aminomalonic acid being a natural amino acid. Aminomalonic acid itself as a precursor for this moiety in malonomicin seems not likely from a reaction mechanistic point of view, especially if acetate or an acetyl group at C-2 of the tetramic acid nucleus would be the source for C-6 and C-7 (as in tenuazonic acid); this would imply without any precedent, condensation of a

methyl group with the central carbon of aminomalonic acid. On the other hand, oxomalonic acid could theoretically condense easily with acetate or an acetyl group, and after dehydration and amination yield the carbon atoms of the side-chain in question (figure 1). Besides, in this context, the involvement of iminomalonic acid can not be excluded.
The abovementioned considerations formed the starting point for the investigation.

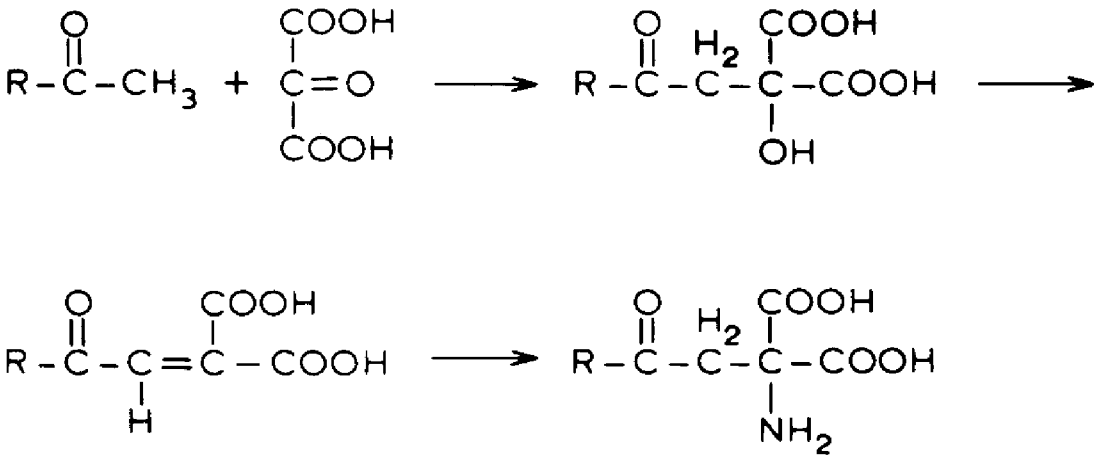


figure 1

4.2. Exploratory experiments

Before starting biosynthetic studies, the right moment for the addition of the right amount of labelled precursors has to be determined (see chapter 2). This is especially difficult when the organism is growing slowly or when the required metabolite has no special physical or physiological features. Fortunately, Streptomyces rimosus is a rather fast growing organism in which the beginning of the malonomicin production can easily be visualized by measuring the UV spectrum of

the culture at regular intervals, the emerging absorption at 280 nm indicating this moment. Usually this will be after 24 hrs of incubation and production will continue for about 16 - 20 hrs.

Exploratory feeding experiments for determining the optimum addition parameters were performed with [1-^{13}C]acetate. Addition of 200 mg/l gave good incorporation, almost independent of the mode of addition. Addition in one portion at the beginning of the production, or addition in two portions, one at the beginning of the production and the second after 8 hours, or working up before the end of the malonomicin production (after 8 hours) did not change the enrichment factors significantly. Henceforward, addition of all precursors was performed in the same way, i.e. in one portion at the beginning of the production. The yield of malonomicin was about 200-300 mg/l; thus, for experiments with two synchronous cultures, the capacity of the incubator (2 x 4 flasks of 500 ml with 110-160 ml each) was sufficient to give high enough yields of each culture for accurate incorporation measurements.

The exploratory experiments established also that the presence of different trace quantities of paramagnetic ions (from the culture medium or from the NMR-sample solvent) which can form stable complexes with malonomicin, causes rather strong intensity variations for the quaternary carbons. This behaviour can explain some of the different enrichment factors for carbon atoms for which, on biosynthetic arguments, equal labelling was expected. This is also the reason that the accuracy of the enrichment factors for the quaternary carbon atoms is not better than 0.5%.

4.3. Results and discussion

Addition of D,L-[1-^{13}C]DAP to a culture of Streptomyces rimosus (for details see Experimental 4.4) caused a high incorporation at C-3 (an enrichment factor of ca 16.5, (table 1, figure 2)).

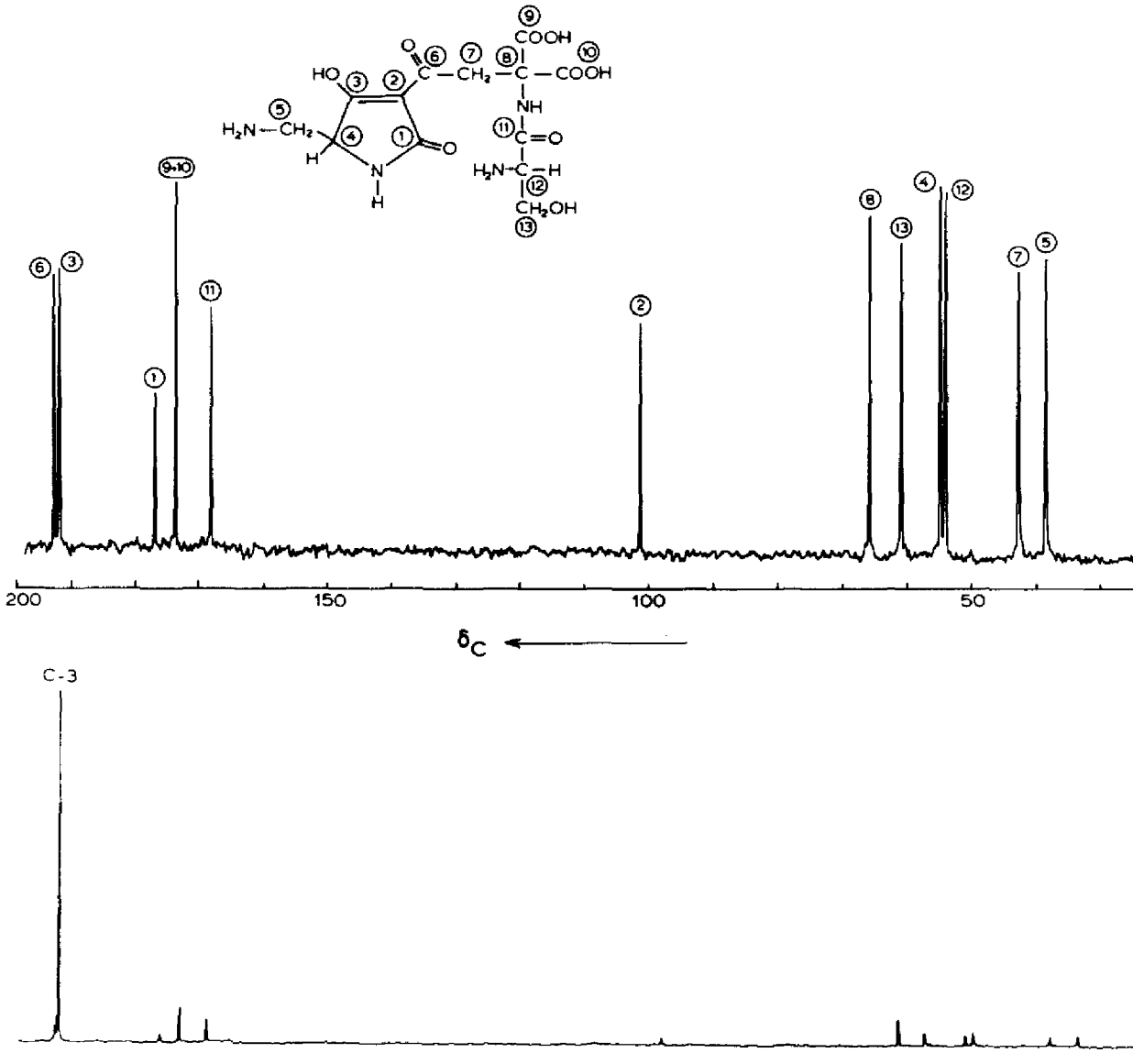


figure 2 ^{13}C spectrum of malonomicin a) at natural abundance; b) after incorporation of D.L-[1-^{13}C]DAP.

The label from D,L-[1-^{13}C]serine was incorporated at

Table 1

Enrichment factors at individual positions in ^{13}C-enriched samples of malonomicin.

Carbon	[1-^{13}C]NaOAc	[2-^{13}C]NaOAc	[1,2-$^{13}C_2$]NaOAc	[1-^{13}C]Malonate	[1,4-$^{13}C_2$]Succinate
	200 mg/0.5 l[a]	200 mg/0.5 l	200 mg/0.5 l	270 mg/l	150 mg/0.5 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	4.5
7		3.5	3.5		
8		4.5	3.0		
9/10[d]	6.0	5.0	9.0	0.5	6.5
11	0.5	0.5	1.0		
12					
13					

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

a) Amount of labelled precursor b) Single addition c) Continuous addition

d) Enrichment calculated for one carbon atom

C-11 and only slightly less at C-3, which suggests a rather direct pathway from serine to DAP (table 1; figure 3)*.

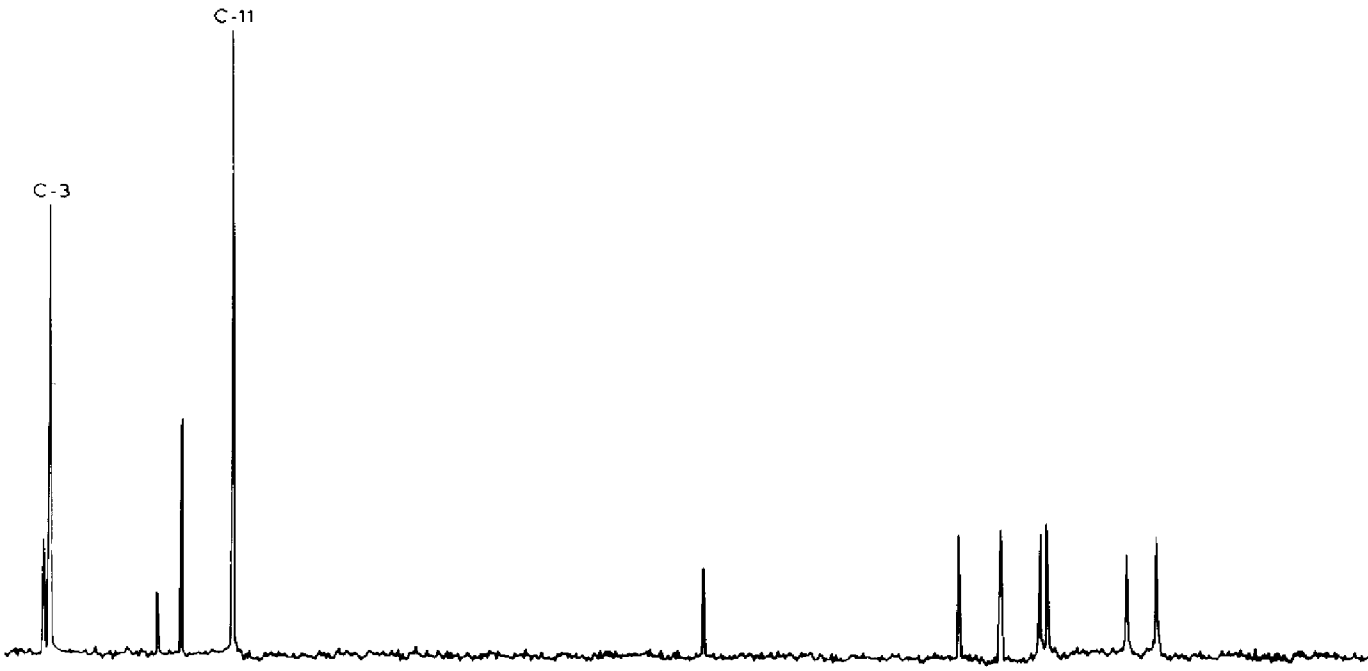


figure 3 ^{13}C spectrum of malonomicin after incorporation of D,L-[1-^{13}C]serine.

Addition of L-[3-$^{2}H_2$]DAP and D-[3-$^{2}H_2$]DAP and measurement of the ^{2}H NMR spectrum (figure 4) demonstrated that L-DAP is incorporated efficiently, while D-DAP is almost not incorporated. The small incorporation in the experiment with D-DAP might have been originated from a minor quantity of L-DAP present in D-DAP (the detection limit from measurement of the optical rotation is 1-2% L-DAP). Concomitant with this low incorporation, there was the fact that the yield of malonomicin after addition of D-DAP was considerably lower (ca 1/3 of the

---

*) The small quantity of label on C-9/10 can be explained by assuming degradation of [1-^{13}C]amino acids to $^{13}CO_2$, which can be incorporated (vide infra).

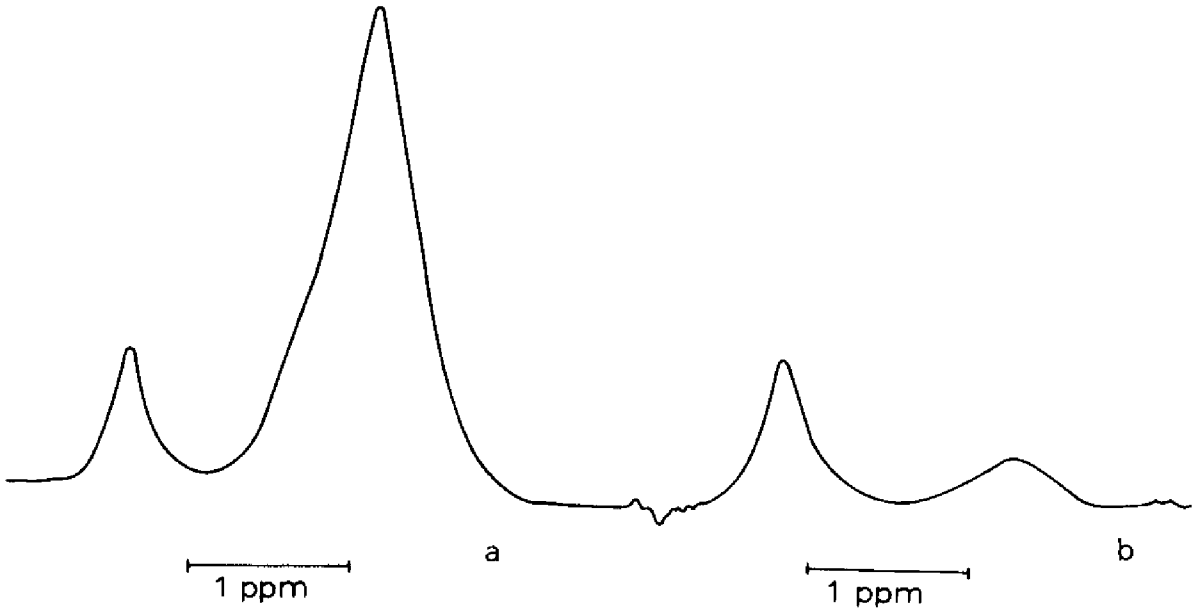


figure 4 ^{2}H spectrum of malonomicin (27.64 MHz) a) after incorporation of L-[3-$^{2}H_2$]DAP; b) after incorporation of D-[3-$^{2}H_2$]DAP.

normal production) which was reproducible in a second experiment. From these experiments it can be concluded that D-DAP is probably an inhibitor of one of the enzyme systems, mediating the biosynthesis of malonomicin. The experiments with labelled DAP and serine prove that serine is the origin of DAP and that L-DAP is incorporated as such in the tetramic acid nucleus. Experiments on the mechanism of the transformation from serine to DAP are described in the next chapter.

Addition of [1-^{13}C]oxomalonic acid to a growing culture of S. rimosus did not give the expected result. Incorporation could not be demonstrated and therefore oxomalonic acid will probably not be direct precursor for the carbon atoms C-8, C-9 and C-10. The involvement of aminomalonic acid as a direct precursor is all the more unlikely. This was established by the fact that at least one of the carboxyl groups (C-9 and/or

C-10, which are coincident in the ^{13}C spectrum) is CO_2-derived. After feeding $NaH^{13}CO_3$, only C-9/10 showed an enrichment (table 1; figure 5), with an enrichment factor of ca 3.5, calculated for one carbon atom. This might implicate that the aminomalonic acid moiety is constructed via a carboxylation reaction in some stage of the biosynthesis.

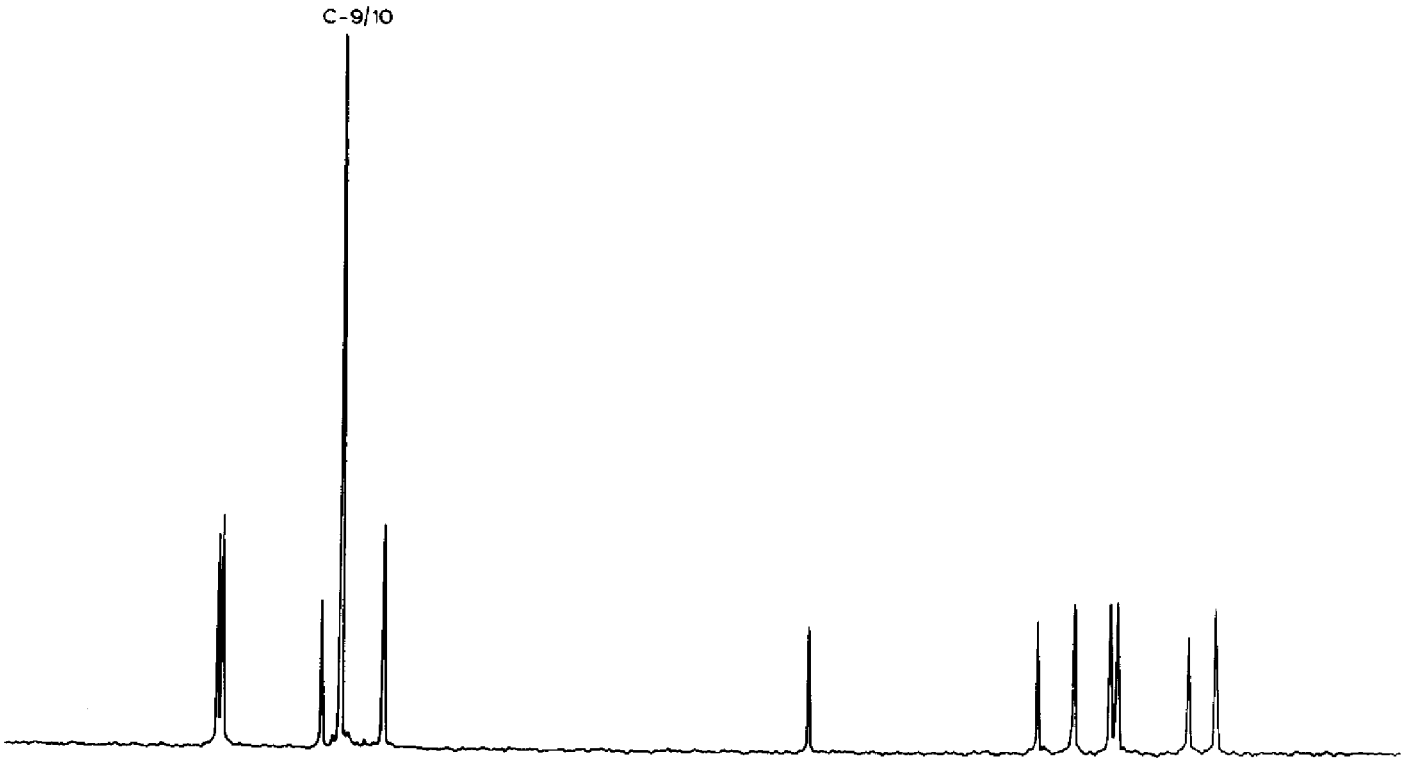


figure 5 ^{13}C spectrum of malonomicin after incorporation of $NaH^{13}CO_3$.

Complicated and surprising, but of decisive significance for the successful elucidation of the biosynthesis of the side chain, was the outcome of the experiments with labelled acetate (and malonate). The aim of these experiments was initially only to determine the origin of C-1 and C-2 and perhaps C-6 and C-7. After addition of [1-^{13}C]acetate, enrichments were

observed not only at C-1 and C-6, as expected, but also at C-9/10 (table 1; figure 6)*.

figure 6 ^{13}C spectrum of malonomicin after incorporation of [1-^{13}C]acetate.

The ^{13}C spectrum obtained after addition of [1-^{13}C]malonate showed essentially the same labelling pattern, though with a much lower enrichment (corrected for loss of label by scrambling over C-1 and C-3 of the singly-labelled malonate) suggesting that acetate is incorporated without conversion to malonate. The ratio of incorporation at positions 1, 6, and 9/10 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-9/10 by [1-^{13}C]acetate originated directly from the carboxyl group of an acetate

---

*) The labelling of C-11 and C-3 after feeding labelled acetate is probably due to the synthesis of serine from pyruvic acid, which is formed by decarboxylation of oxaloacetic acid in the TCA cycle (vide infra).

unit or from $^{13}CO_2$ (vide supra) generated by degradation of acetate in the tricarboxylic acid (TCA) cycle (figure 7) or even from both sources. This last possibility became plausible by feeding [2-^{13}C]acetate (table 1; figure 8).

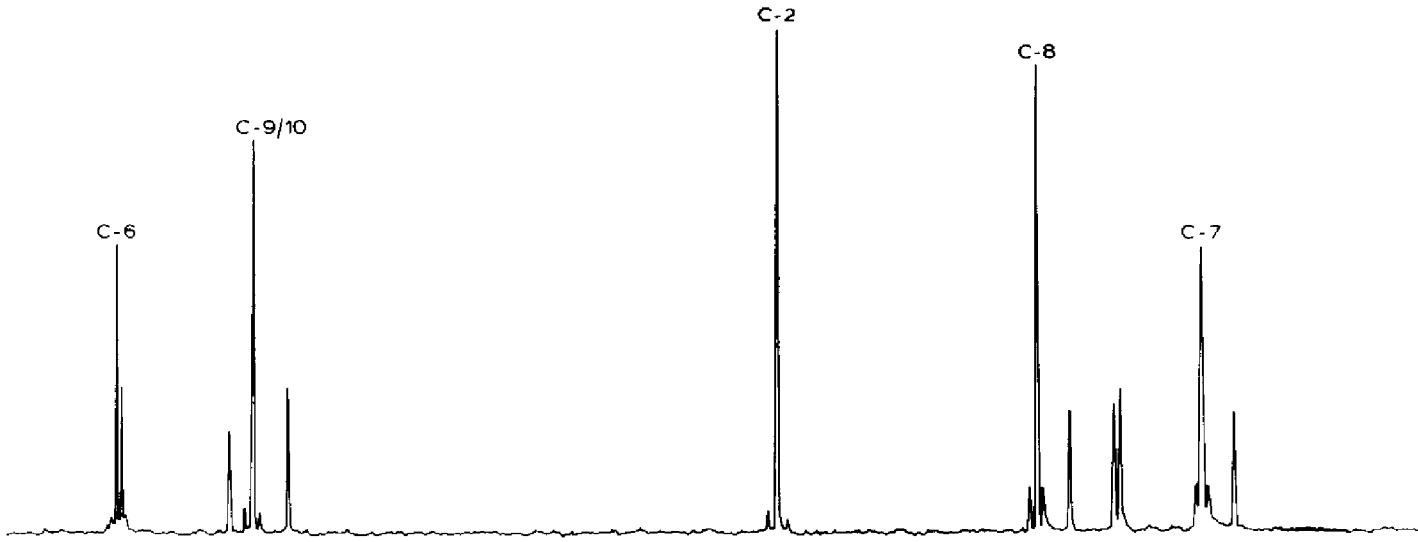


figure 8 ^{13}C spectrum of malonomicin after incorporation of [2-^{13}C]acetate.

Not only C-2 and C-7 were labelled as expected, but also C-8, C-9/10 and even C-6. The more or less equal labelling of C-7 and C-8, accompanied by comparable labelling of C-6 and C-9/10 (with the latter in excess, which is probably due to $^{13}CO_2$ 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, the label of [2-^{13}C]acetate is distributed equally over the central carbon atoms of some C_4-dicarboxylic acids after formation of the symmetrical succinic acid in a first turn, leading to label on C-7 and C-8. In a second turn, these labels are transposed partially to the carboxyl groups (labelling of C-6 and

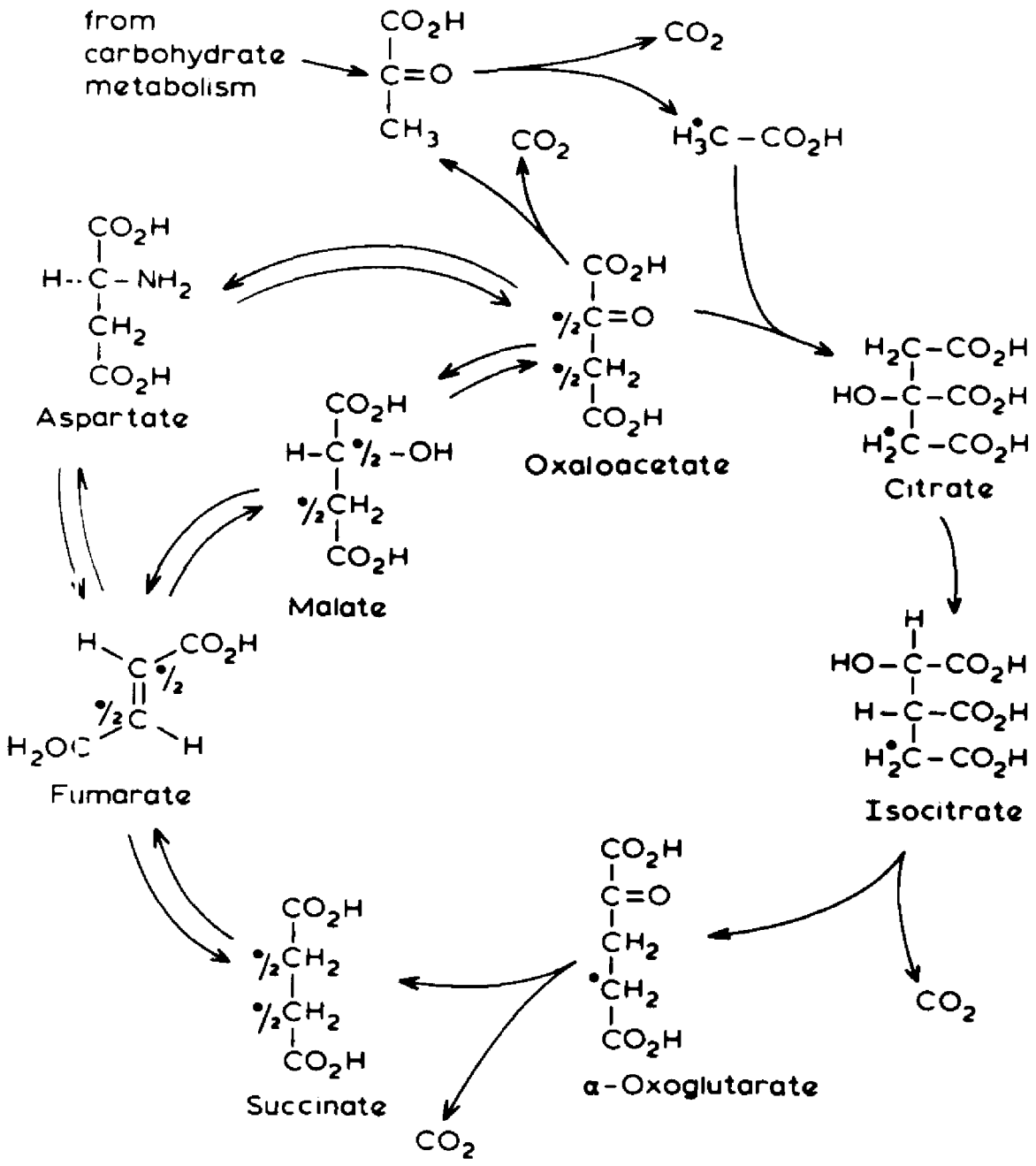


figure 7

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 is not two carbon atoms from acetate which are immediately removed as CO_2 but two atoms from oxaloacetate. Only after several turns of the cycle are the carbon atoms of acetate completely converted into CO_2.

C-9/10); finally the label is extruded as $^{13}CO_2$ (extra label on C-9/10).

To prove the incorporation of <u>intact</u> acetate units, an additional experiment with [1,2-$^{13}C_2$]acetate was performed. This 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-9/10 (table 1; figure 9).

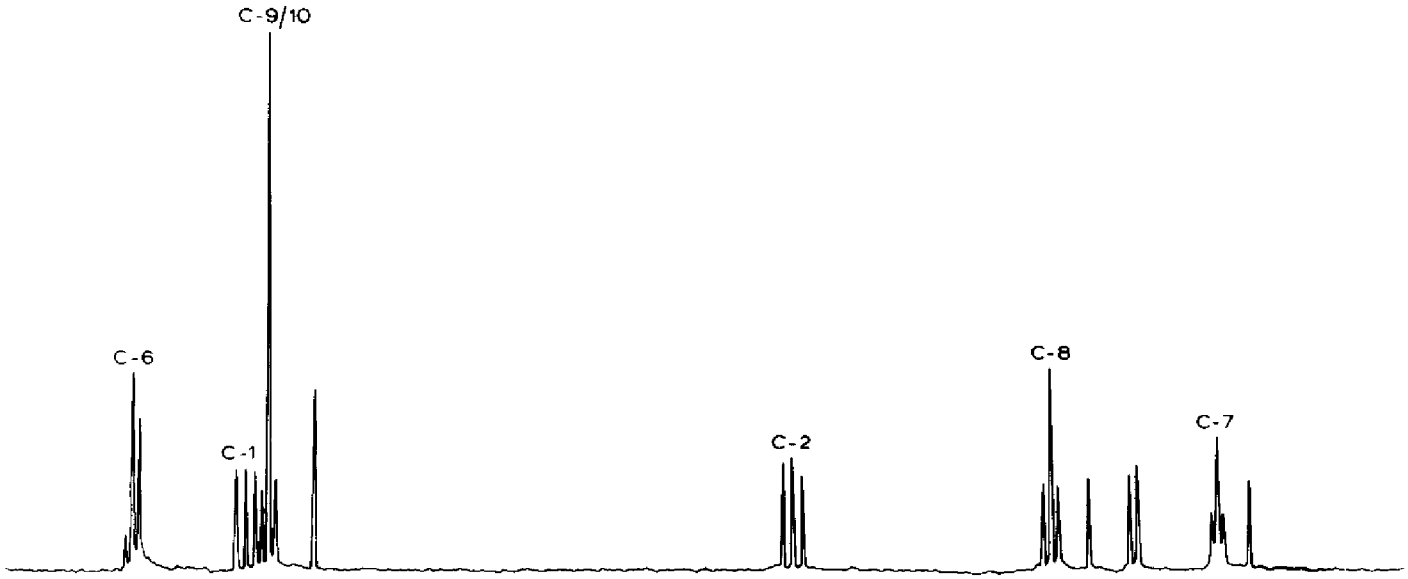


figure 9 ^{13}C spectrum of malonomicin after incorporation of [1,2-$^{13}C_2$]acetate.

Of these, C-1 + C-2 were fully coupled in the ^{13}C NMR spectrum, demonstrating that exclusively an intact acetate unit is used for the biosynthesis of the heterocyclic nucleus of malonomicin. The increased intensity of the satellites of C-1 and C-2 originates from the incorporation of the doubly-labelled precursor (81% doubly-labelled); the increased intensity of the centre peak is due to incorporation of the singly-labelled precursor (9% singly-labelled). C-6 +C-7 and C-8 + C-9/10 were only partially coupled, in accordance with the assumed conversion to a C_4-dicarboxylic acid in the

TCA cycle prior to incorporation into malonomicin. The satellites of C-6, C-7, C-8 and C-9/10 originate from the incorporation of a C_4-dicarboxylic acid in which the intact acetate is present. The labelling, visible in the increase of the intensities of the centre peaks, originates from the incorporation of a C-4 dicarboxylic acid in which the coupling is lost by one or more turns in the TCA cycle; this leads to excess of label on the carboxyl groups of the C_4-dicarboxylic acid (see figure 7).

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-9/10 was enriched (figure 10). But by measuring peak integrals, an enrichment at C-6 also was clearly indicated. The observed line-broadening of the C-6 and C-9/10 resonances is caused by a three bond ^{13}C-^{13}C coupling which is not resolved in the normal 5000 Hz spectrum on 4K data points. However, by measuring a 1200 Hz spectrum on 4K data points, both C-6 and C-9/10 were partially resolved into two doublets with a coupling constant of 2.6 Hz. This is a clear demonstration of the problems in enrichment studies which are associated with the use of intensities instead of integrals (see chapter 2). The higher extent of labelling at C-9/10 may also be caused by incorporation of $^{13}CO_2$, generated by one turn in the TCA cycle.

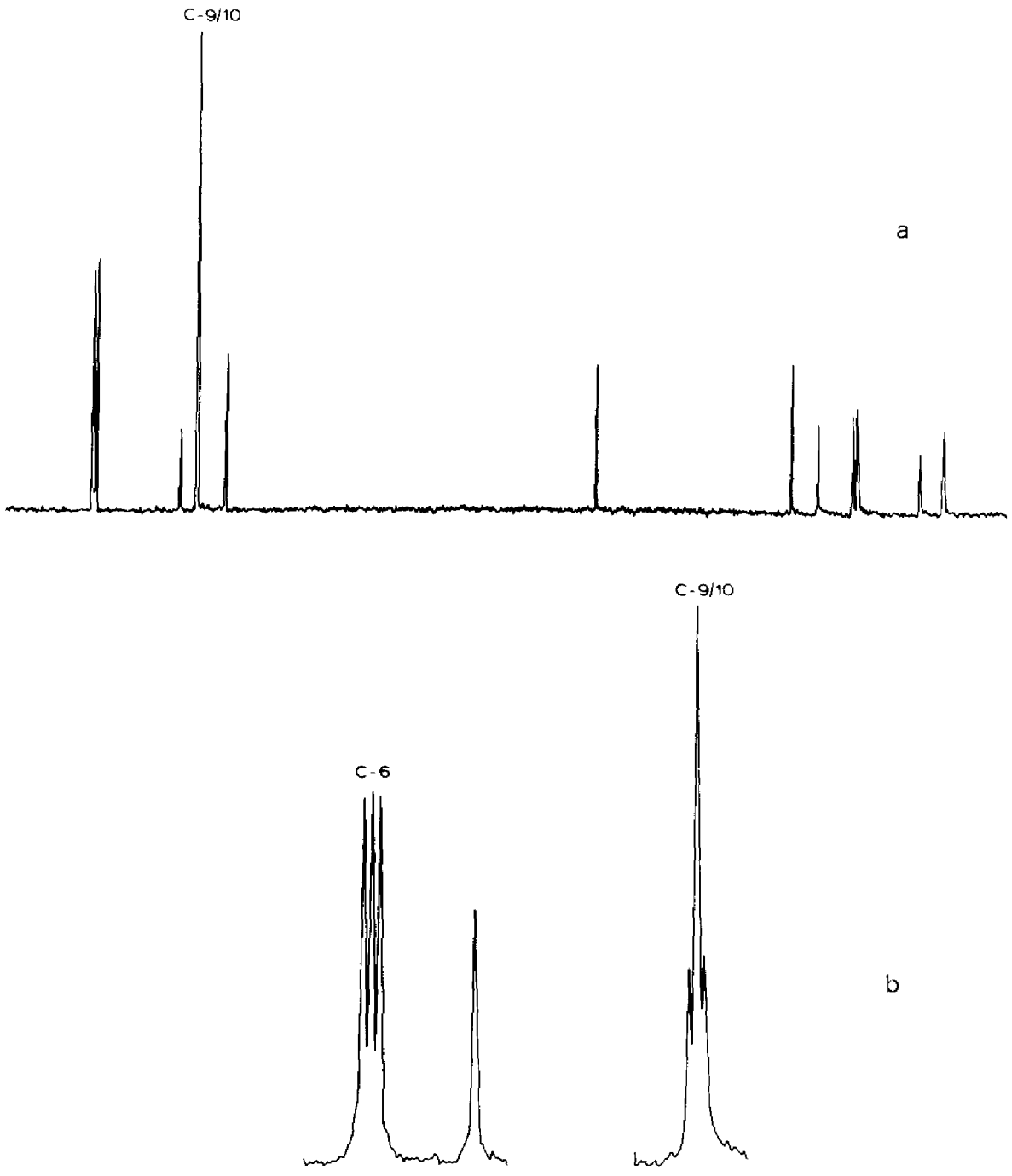


figure 10 ^{13}C spectrum of malonomicin after incorporation of [1,4-$^{13}C_2$]succinic acid; a) spectrum recorded with a spectral width of 6000 Hz; b) spectrum recorded with a spectral width of Hz.

On the basis of this result, oxaloacetic acid was regarded as an attractive direct precursor of malonomicin. This because of the presence of the keto group, which can provide the necessary activation for both central carbon atoms (cf. figure 1). However, this possibility was ruled out by the result of a feeding experiment with [3-^{13}C]oxaloacetic acid. Instead of carrying a considerable excess of label at C-7 or C-8, the malonomicin isolated showed a similarly complex pattern of enrichment as was obtained with [2-^{13}C]acetate. The ratio and extent of enrichment of C-2, C-6, C-7, C-8 and C-9/10 was dependent on the rate of oxaloacetic acid addition to the culture medium (table 1; figure 11). When adding the labelled oxaloacetic 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 oxaloacetic acid to acetic acid via pyruvic acid. When the exogenic oxaloacetic acid concentration was kept low by continuous feeding during the malonomicin production (ca. 16 hrs), the labelling of C-2 (demonstrated to be directly acetate derived, vide supra) was significantly less; apparently, degradation to acetic acid had been considerably suppressed by this method. However, the labelling pattern still resembled that obtained with [2-^{13}C]acetate, with the conspicuous feature of almost equal labelling of C-7 and C-8, regardless of the feeding method. The unexpected and complicated incorporation pattern after addition of oxaloacetic acid demonstrates that in the stud, of biosynthesis by means of ^{13}C labelled precursors the large amount necessary can indeed cause metabolic distortion (see chapter 2), especially when precursors are added, which are pivotal

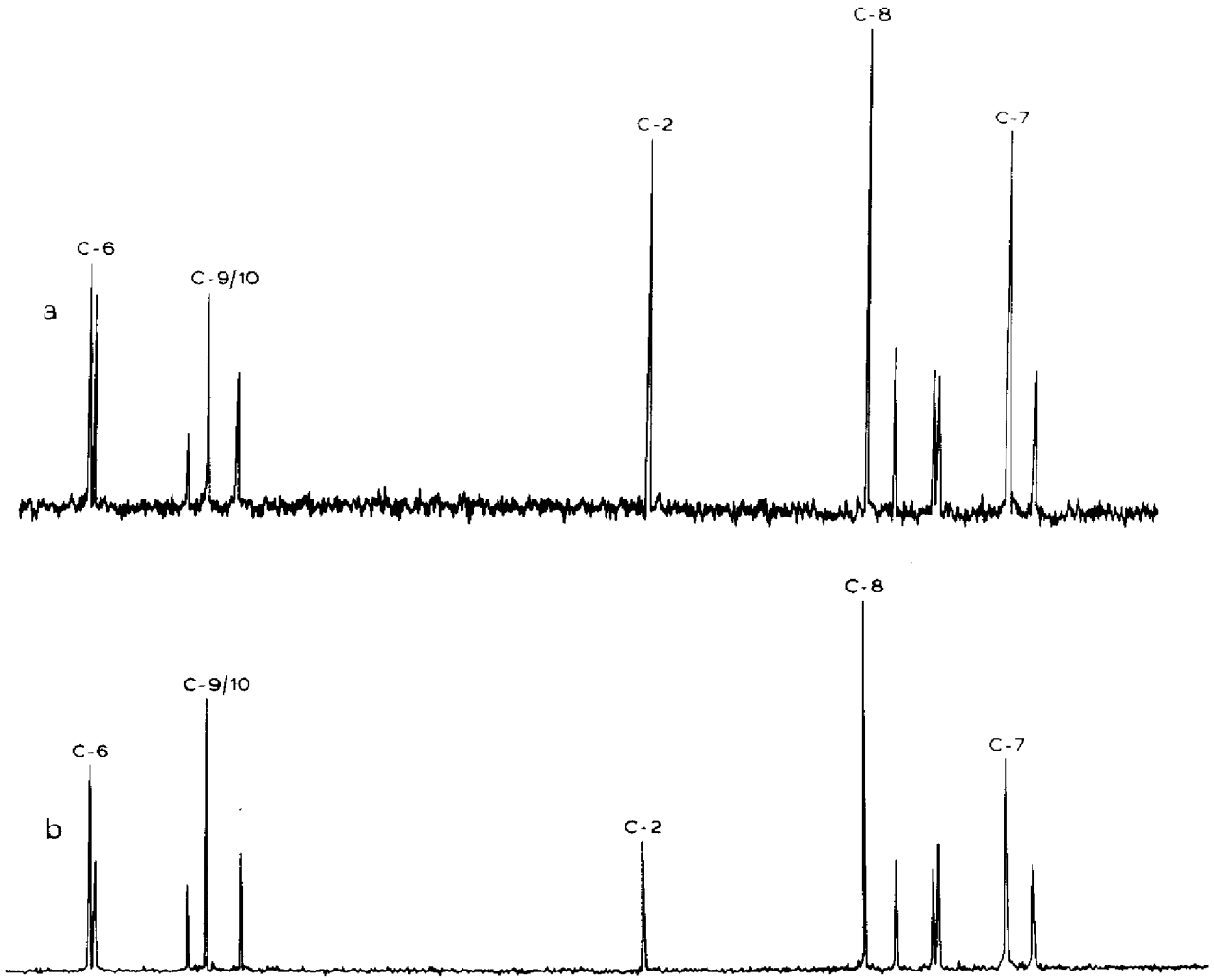


figure 11 ^{13}C spectrum of malonomicin after incorporation of [3-^{13}C]oxaloacetate; a) after addition of precursor at the beginning of the malonomicin production; b) after continuous feeding during the malonomicin production.

intermediates in intermediary metabolism.

The scrambling of the label of [3-^{13}C]oxaloacetic acid over C(6-10), the equal labelling of C-7 and C-8 both from [2-^{13}C]acetate and from [3-^{13}C]oxaloacetic acid and the incorporation of intact succinic acid point to a <u>symmetrical</u> 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]oxaloacetic acid over C-7 and C-8 can be explained as the result of a metabolic equilibrium existing between oxaloacetic acid and the symmetrical succinic or fumaric acid via malic and/or aspartic acid (figure 7). Similarly, the incorporation of label on C-6 and C-9/10 is a consequence of the irreversible conversion of [3-^{13}C]oxaloacetic acid into carboxy-labelled succinic or fumaric acid via decarboxylation of oxoglutaric acid in the TCA cycle.

From an experiment with L-[3-$^{2}H_2$]aspartic acid it turned out that deuterium was incorporated. However, aspartic acid is in equilibrium with both oxaloacetic acid and fumaric acid (figure 7) and an experiment with L-^{15}N aspartic acid (100 mg/l) proved that aspartic acid is not a direct precursor of malonomicin, for enrichment could not be demonstrated in the ^{15}N NMR spectrum. Therefore, aspartic acid is probably incorporated via oxaloacetic acid and/or fumaric acid.
Addition of 500 mg/l $^{15}NH_4Cl$ to a growing culture resulted in a high enrichment (enrichment factor ca 20% as measured by determining the heights of the ^{15}N satellites in the ^{13}C spectrum). All four nitrogens were labelled to an almost equal extent, so this experiment gives no further information*.
Whether succinic or fumaric acid is the direct

---

*) In this experiment, another (known [3]) metabolite of Streptomyces rimosus, diaminosuccinic acid, could be isolated. The ^{15}N content was 50%; probably the high exogenous ammonium concentration had induced its synthesis.

precursor cannot easily be concluded only from experiments with the labelled acids, because of the rapid equilibrium between these two acids. And indeed, incorporation of [2,3-$^2H_4$]succinic acid and [2,3-$^2H_2$]fumaric acid to two synchronous cultures did show no significant difference (figure 12).

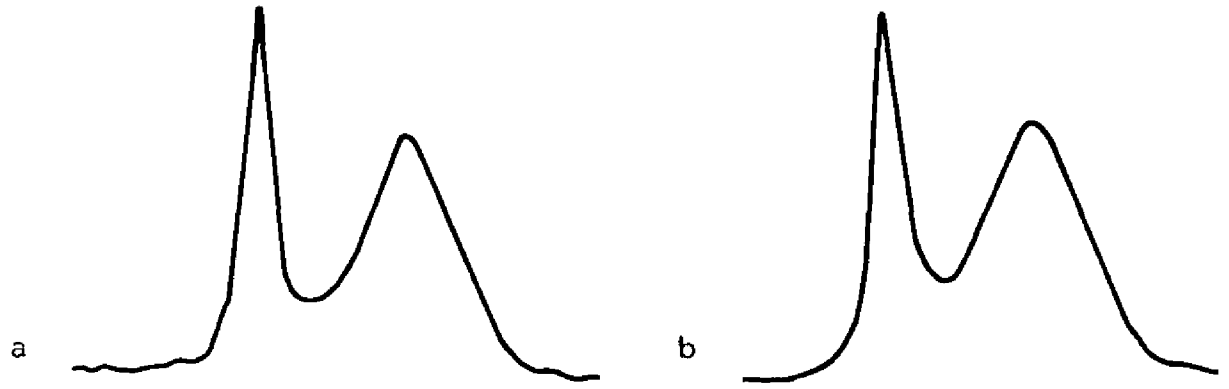


figure 12 ^2H spectrum of malonomicin (27.64 MHz), a) after incorporation of [2,3-$^2H_4$]succinic acid; b) after incorporation of [2,3-$^2H_2$]fumaric acid.

However, if this equilibrium can be influenced by means of a competitive inhibitor, discrimination between these two precursors might be possible. Malonate is such an inhibitor (on the basis of its close structural analogy), which, at moderate concentrations (0.01M), does not influence the malonomicin production. Experiments with the deuteriated acids performed with synchronous cultures with and without added of malonate (figure 13) showed that the incorporation of fumaric acid is considerably lower in the presence of malonate, while malonate had no effect on the incorporation of succinic acid. Therefore, it is concluded that succinic acid is the most direct precursor from the TCA

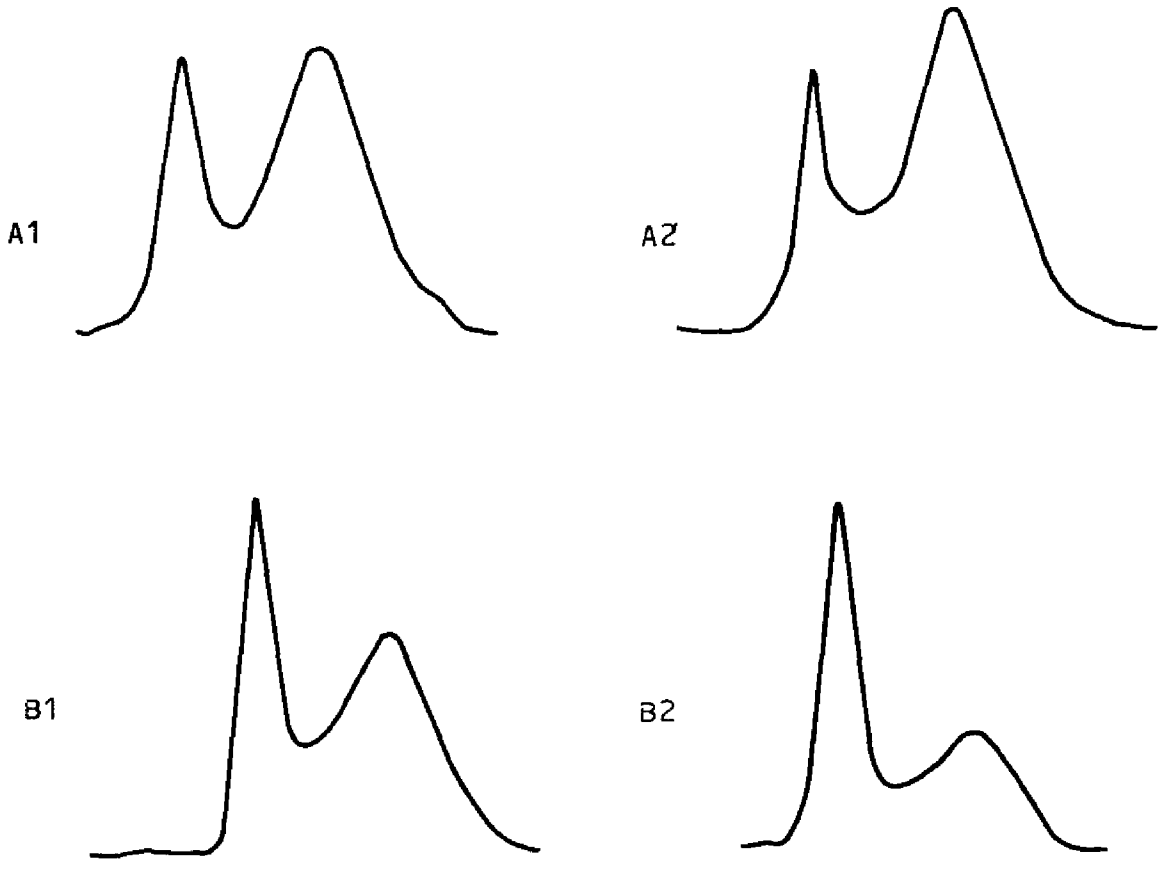


figure 13 ^{2}H spectrum of malonomicin (27.64 MHz) after incorporation of a) deuteriated succinic acid (A) and fumaric acid (B), with added malonate (A1 and B1) and without added malonate (A2 and B2).

cycle for C-6, C-7, C-8 and C-9 or C-10

The effect of broadband proton decoupling on the deuterium spectrum after incorporation of [2,3-$^{2}H_{4}$]succinic acid is shown in figure 14. Due to the large linewidths of the deuteron resonances, ^{1}H-^{2}H couplings do not result in observable splittings; however, on broadband proton decoupling, the linewidth will decrease to the extent of the value of the coupling constant. The coupling constant $^{2}J(^{1}H_{7}-^{1}H_{7})$ is 17.4

Hz, thus the corresponding proton-deuteron coupling constant will be 2.7 Hz. In figure 14 the predicted line narrowing on decoupling with the concomitant increase of intensity is clearly visible (the integral remains constant); the solvent peak serves as an internal reference.

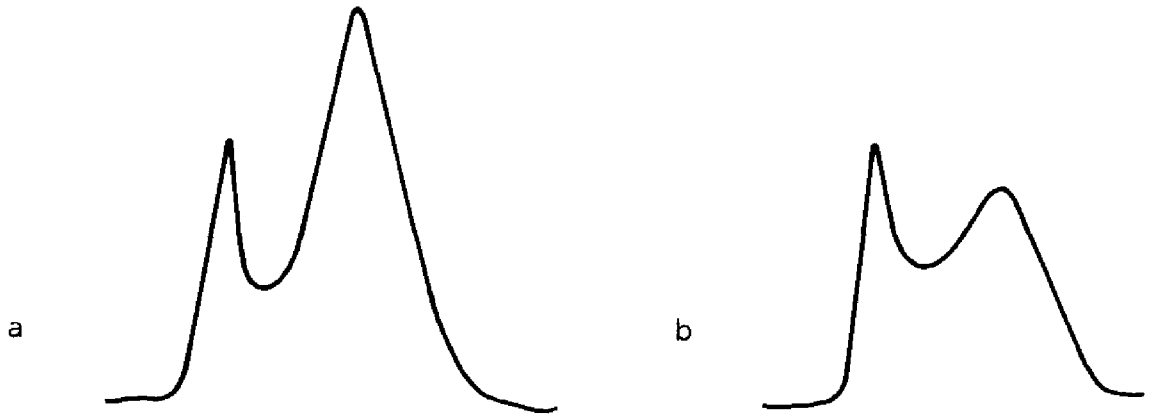


figure 14 ^{2}H spectrum of malonomicin (27.64 MHz) after incorporation of [2,3-$^{2}H_4$]succinate a) decoupler on, b) decoupler off.

From this, it can be concluded that only one hydrogen of succinic acid is incorporated into malonomicin. This is in agreement with the observation that the extent of labelling from deuteriated succinic acid and fumaric acid does not differ much, though the deuterium content in succinic acid is twice that in fumaric acid. A ^{2}H spectrum of malonomicin after incorporation of deuteriated succininic acid was measured at pH 6.5; at this pH (and lower) the chemical shift difference between the two protons at C-7 is largest (see figure 3, p. 51). It appears that only at 3.82 enrichment is observed. The small resonance at high field originates from incorporation of deuterium at C-5; the

large quantity fed (1g/1.5 l) may lead to incorporation in the serine- and DAP part via pyruvic acid. This means that the step from succinic acid to the aminomalonic acid moiety in which one of the protons is lost, is a stereospecific process.

From the abovementioned results the origin of all the carbon atoms in malonomicin can be explained from incorporation of acetate, succinic acid, CO_2 and serine. However, there is no evidence yet with respect to the order in which the building blocks are assembled and to the intermediates which are involved. This will be the subject of the next chapter where some mechanisms and intermediates are discussed.

4.4. Experimental

4.4.1. General method for feeding experiments

A high yielding strain of Streptomyces rimosus forma paramomycinus*, maintained on malt extract agar {malt extract (Oxoid L 39; 15 g), bacteriological pepton (Oxoid L 34; 10 g), NaCl (5 g),agar (Oxoid L 11; 20 g) and tapwater (1 l)}, was inoculated into 500-ml baffled conical flasks with wadding closure containing ca 130 ml of medium of the following composition: a) malt extract (Oxoid CM 57; 18 g), pepton (Difco 0118-01; 7 g), NaCl (5 g) and tapwater (1 l), or b) the abovementioned agarmedium (without agar). The pH of the medium was adjusted to 9 with 4N-ammonia (or 2N-NaOH) and the

---

*) A generous gift of malonomicin and a high yielding strain of S. rimosus from Gist-Brocades N.V. is greatfully acknowledged.

medium was sterilized in a pressure cooker at 125 °C for 25 minutes. After sterilization the pH of the medium was ca 8.5. When cultures were grown without long intervals, an aliquot of a 24 hr old culture was used to inoculate the next culture. Incubations were performed at 28°C on a rotary shaker (300 rpm; 3.8 cm eccentricity). Isotopically-labelled substrates in dilute aqueous solution of pH 8 were added as single additions at the the beginning of the malonomicin production, (usually at ca 24 h after start of the incubation). Only [3-^{13}C]oxaloacetic acid was added continuously during the production by means of a multichannel peristaltic pump. The amounts of labelled precursor are given in table 1. In the study of the incorporation of two precursors in two synchronous cultures, the contents of all the flasks were pooled together at the start of the malonomicin production and divided again over the flasks before addition of the labelled substrate.

4.4.2. 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 two hours at -20 °C. The brown residue 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 x 2

cm; adsorbent swollen in 0.5M-NH_4OAc; pH adjusted to 8.5 with 4N-NH_4OH). 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 a 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.

4.4.3. Chemicals

Sodium [1-^{13}C]acetate, [2-^{13}C]acetate, [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 [4]. [1-^{13}C]Oxomalonic acid was synthesized from [1-^{13}C]malonic acid via dibromomalonic acid [5]. [1,4-$^{13}C_2$]Succinic acid was synthesized from potassium [^{13}C]cyanide and 1,2-dibromoethane [6], [1-^{13}C]malonic acid from sodium [^{13}C]cyanide and monochloroacetic acid [7]. [1-^{13}C]-2,3-Diaminopropanoic acid was synthesized from [1-^{13}C]acrylic acid (from barium [^{13}C]carbonate and vinylmagnesium bromide) via bromine addition [8] and amination [9] with NH_4OH in ca 30% overall yield. [1-^{13}C]Serine was prepared [10] from methyl [1-^{13}C]-2,3-dibromopropanoate in 30% overall yield. The protecting O-benzylgroup 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 [11]. 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. L-[3-$^{2}H_2$]DAP and D-[3-$^{2}H_2$]DAP were synthesized from L- and D-[3-$^{2}H_2$]aspartic acid [12], which had been prepared by deuteriation (>95%) of L- and D-aspartic acid (Fluka) with 4N-DCl according to [13]. 2,3-$^{2}H_4$ Succinic acid and 2,3-$^{2}H_2$ fumaric acid were prepared from the unlabelled acids with 2N-KOD/D_2O using a modified procedure of Atkinson et al. [14]: succinic acid 15 h at 150 °C and fumaric acid 2 h at 150 °C and subsequently 2 h at 100°C in a Teflon coated stainless steel autoclave. After acidification and evaporation to dryness, the acids were purified by sublimation (140 °C, 10^{-3} mm Hg), yield >95% for succinic acid and 50% for fumaric acid (deuterium content >95%).

All isotopically- labelled substrates were pure by t.l.c. and ^{1}H and ^{13}C NMR spectroscopy.

4.5. References

1 a) H. Reinbothe, Flora, 1962, 152, 545.
b) I. Murakoshi, F. Kato, J. Haginiwa, and T. Takemoto, Chem. Pharm. Bull. (Japan), 1974, 22, 473.
c) J.H. Carter II, R.H. Du Bus, J.R. Dyer, J.C. Floyd, K.C. Rice, and P.D. Shaw, Biochemistry, 1974, 13, 1221.

2 a) H. Nagayama, M. Muramatsu, and K. Shimura, Nature, 1958, 181, 417.
b) F. Vedel and Y. Guitton, Compt. Rend., 1965, 260, 1733.

3 F.A. Hochstein, J. Org. Chem., 1959, 24, 679.

4 D.B. Melville, J.R. Rachele, and E.B. Keller, J. Biol. Chem., 1947, 169, 419.

5 M. Conrad and H. Reinbach, Chem. Ber., 1902, 35, 1313.

6 B. Bak, J.T. Nielsen, and M. Schottländer, Acta Chem. Scand., 1962, 16, 123.

7 M. Calvin, C. Heidelberger, J.C. Reid, B.M. Tolbert, and P.E. Yankwich, Isotopic Carbon, Wiley, New York, 1949, p. 191.

8 L. Pichat, M. Herbert, and F. Aubert, J. Labelled Compounds, 1965, 1, 66.

9 E. Winterstein and A. Küng, Z. Physiol. Chem., 1909, 59, 141.

10 W. Grassmann, E. Wünsch, P. Duefel, and A. Zwick, Chem. Ber., 1958, 91, 538.

11 C. Heidelberger and R.B. Hurlbert, J. Amer. Chem. Soc., 1950, 72, 4704.

12 S.L.N. Rao, Biochemistry, 1975, 14, 5218.

13 I. Putter, U.S.P. 3,699,158.

14 J.G. Atkinson, J.J.Csakvary, G.T. Herbert, and R.S. Stuart, J. Amer. Chem. Soc., 1968, 90, 498.

Chapter 5

Mechanistic investigation

5.1. Introduction

After the determination of the simple precursors that form the basis for the construction of malonomicin, the questions arise of the sequence in which the skeleton is built up and of the mechanisms involved. Starting point for this investigation was the known mode of biosynthesis of some other tetramic acids, particularly tenuazonic acid (1). For this simple antibiotic, it had been established that the first step is the condensation of L-isoleucine (2) with an acetylCoA derivative as depicted in figure 1 [1]. Analogously, it seemed obvious that the biosynthesis of malonomicin should start with the condensation of L-2,3-diaminopropanoic acid (3) and a more or less substituted acetylCoA derivative, after which cyclisation to (5) occurs and eventually the rest of the side-chain is attached (figure 2). In figure 2, R is H in one extreme; in the other extreme, the rest of the basic precursors including serine is already attached before the ring closure. Between the two extremes, in principle several other possibilities exist.

Diaminopropanoic acid is not a common amino acid and in S. rimosus it is probably formed directly from serine

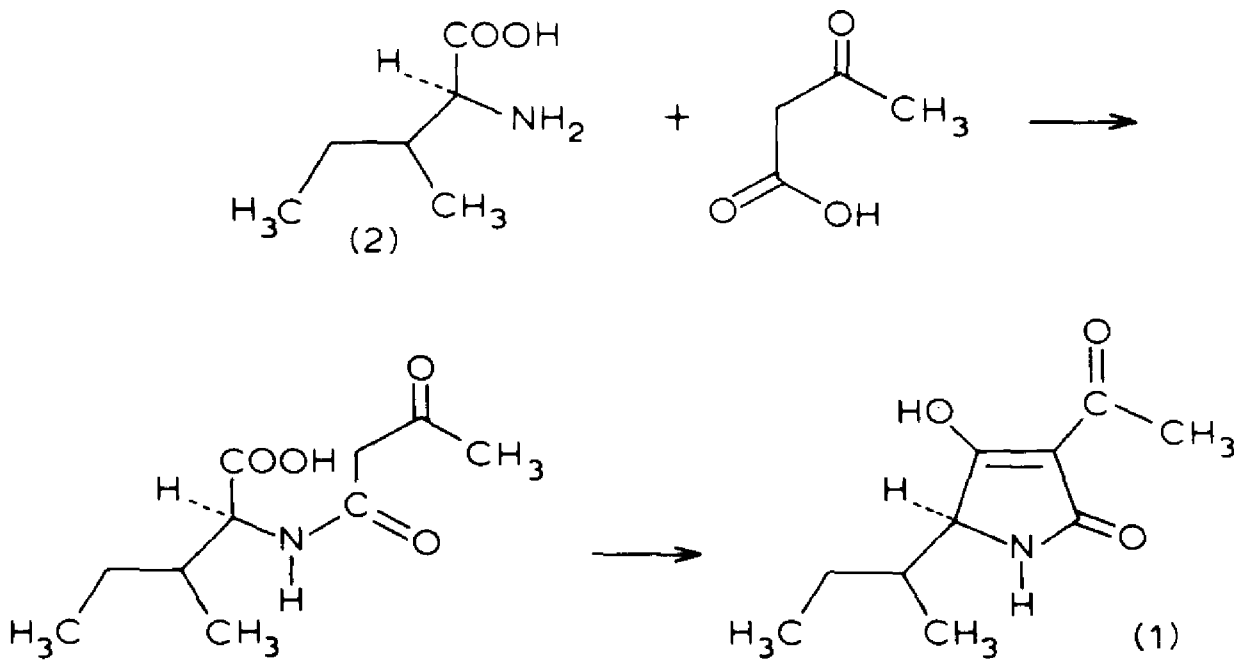


figure 1

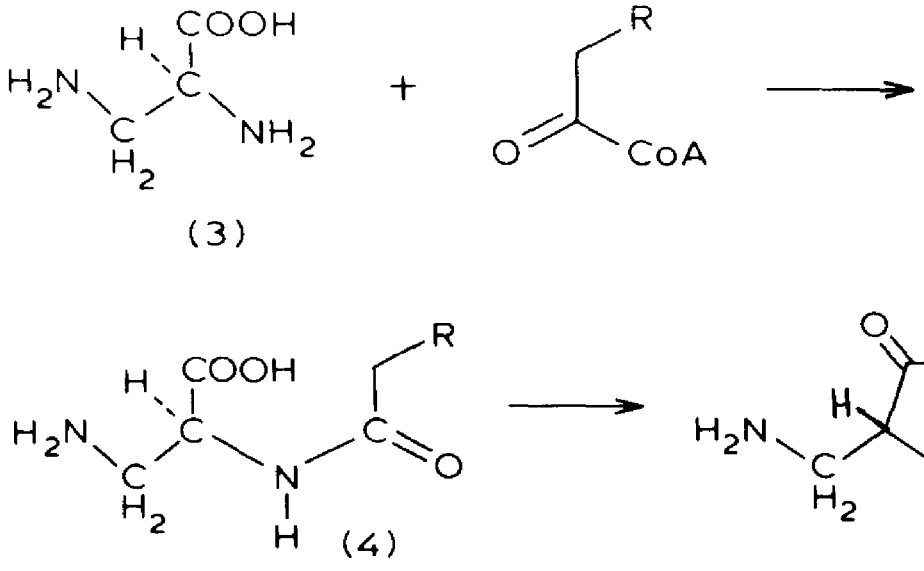


figure 2

as demonstrated in chapter 4. On the basis of normal reaction pathways in primary metabolism, two mechanisms for this conversion seem likely. These can be studied using serine, deuteriated at the 2- and 3-positions (figure 3 and 4).

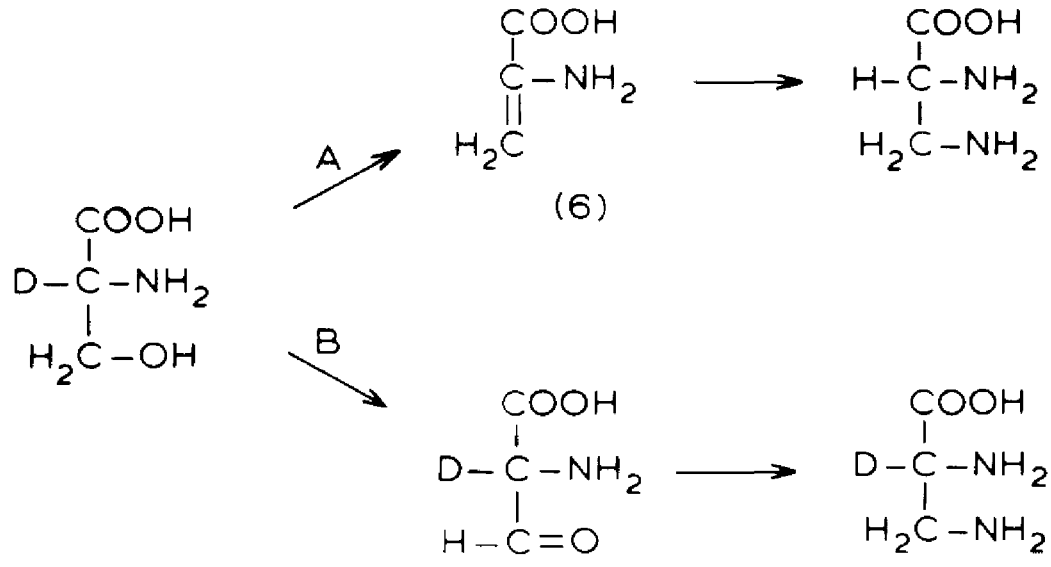


figure 3

Mechanism A involves dehydration of serine to α-amino acrylic acid (6) and addition of ammonia to the double bond. In mechanism B, serine is oxidized to aminomalonic acid semialdehyde and transformed to DAP by a transamination.

In mechanism A, the deuteron in the α-position will disappear, but both deuterons in the β-position will remain in DAP. The fate of the α-deuteron in mechanism B is different from mechanism A and it will be retained; however, of the β-deuterons, one will be lost in the oxidation step.

A third possible mechanism, which is excluded here, for it has not been demonstrated in in vivo experiments for

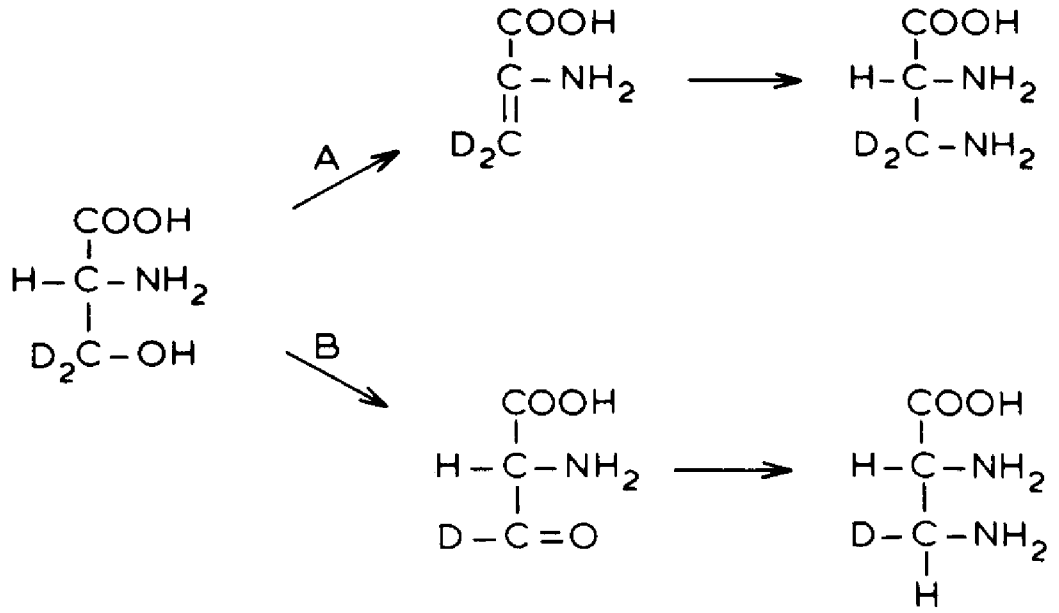


figure 4

serine, is the direct substitution of the hydroxyl or activated hydroxyl group* of serine by an amino group. In this case, both α- and β-deuterons would be retained in the DAP molecule.
The results of these experiments are described in chapter 5.2.1.

The investigation of the nature of the acetylCoA derivative that is coupled to the α-amino group of DAP has been carried out using ^{13}C- and ^{2}H-labelled precursors (5.2.2.).

Using deuterium labelled precursors, it could be established, in which stage of the biosynthesis of

---

*) The hydroxyl group of serine is often activated by an acetyl, sulphate or phosphate group (see e.g. chapter 1.3.).

malonomicin cyclization occurs. The enrichment factors in these experiments were very low (0.02% and 0.05%); however, by doubly labelling, the identity of the first ringclosed precursor could be established with certainty.
In experiments with more elaborate precursors the detection limit of NMR methods was probably reached, since it was not possible to detect incorporation of substrates, in which the aminomalonic acid moiety had been either completely or partly constructed (5.3.3.) and (5.3.4.).

5.2. Results

5.2.1. Formation of 2,3-diaminopropanoic acid from serine

In figure 5 the ^2H spectra obtained after feeding D,L-[2-^2H]serine [2] and D,L-[3-$^2H_2$]serine [3] are given. In spectrum a), only one resonance is visible with the same chemical shift as observed in the spectrum of malonomicin where the proton at C-12 is exchanged with deuterium by means of pyridoxal phosphate in D_2O at pD 9.2 [2]. If there also would have been a resonance of the deuteron at C-4, these two resonances would probably not have been resolved, but the resulting chemical shift of the broad resonance would have been intermediate between the resonances of the deuterons at C-4 and C-12.
Incorporation of D,L-[3-$^2H_2$]serine gave two resonances in the deuteron spectrum with the expected chemical shifts. An advantage in studying these mechanisms with the aid of NMR is that the incorporation of serine can be observed as well as the incorporation of DAP. This

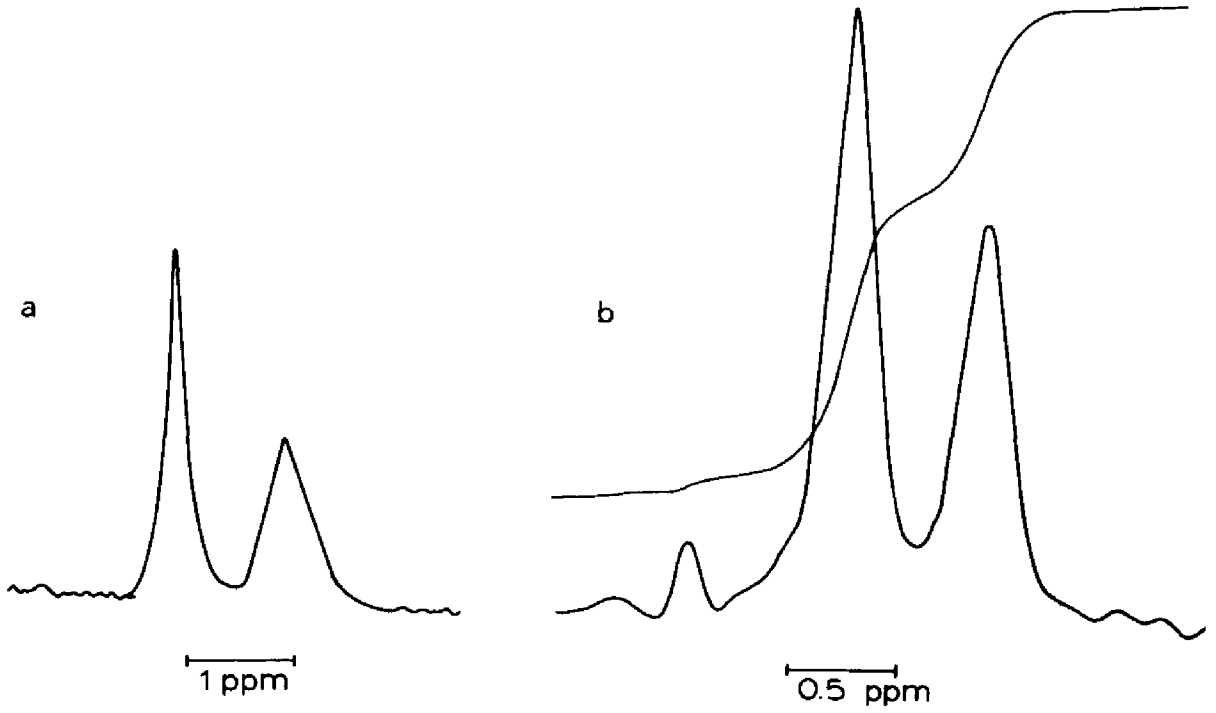


figure 5 ^{2}H spectra of malonomicin after incorporation of a) D,L-[2-^{2}H]serine (27.64 MHz) and b) D,L-[3-$^{2}H_2$]serine (38.39 MHz)

provides an internal check, since it allows a comparison of the integrals from the serine and the DAP deuterons with respect to the ratio of ^{13}C incorporation from [1-^{13}C]serine, which is about 1 : 1.5 (cf. chapter 4.2.). The integral ratio of the two resonances in figure 5b is 1 : 1.4, which means that both deuterons from D,L-[3-$^{2}H_2$]serine are incorporated in the diaminopropanoic acid part of malonomicin.
Additional evidence for the incorporation of both β deuterons of serine in the β-position of DAP can be derived from the effect of proton decoupling on the linewidth of the deuteron resonances. Proton broadband decoupling narrows both lines by about 1 Hz. This is expected for both resonances when no deuterium is lost, for the coupling constants $^{3}J(H_{12}-H_{13})$ and $^{3}J(H_4-H_5)$

are 4.3-5.8 Hz (chapter 3.3); this would result in a theoretical line narrowing of 0.7-0.9 Hz. The loss of one deuteron in the β-position of DAP would have resulted in an additional line narrowing of 2 Hz on irradiating the proton frequencies (the geminal coupling constant is 13.2 Hz).
On the basis of these results, mechanism B can be rejected and it seemed obvious that mechanism A was the correct mechanism.
For comparison, D,L-[2,3-$^{2}H_{3}$]DAP was added to a culture and the isolated malonomicin was analyzed. Unexpectedly, however, the deuteron spectrum (figure 6a) showed only enrichment at the β-position and not at all in the α-position.

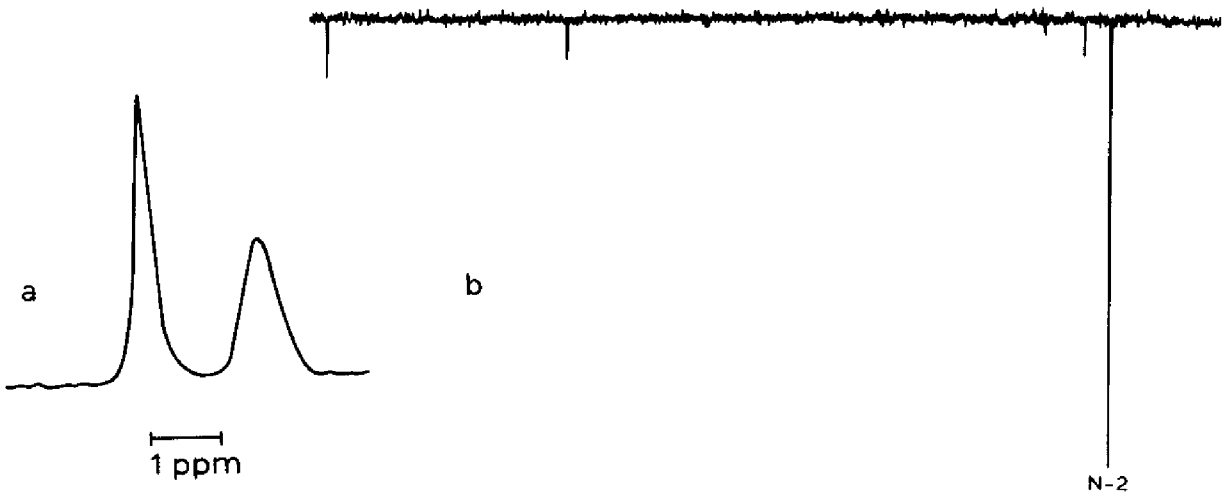


figure 6 a) ^{2}H spectrum of malonomicin (27.64 MHz) after incorporation of D,L-[2,3-$^{2}H_{3}$]DAP (300 mg/l); b) ^{15}N spectrum of malonomicin after incorporation of [3-^{15}N]DAP (100 mg/l)

There are at least two possible reasons for the deuteron (proton) loss at the α-position of DAP.
In the first place it is possible that serine is not

converted into DAP and thereupon incorporated into malonomicin, but that there is a joint intermediate from both serine and DAP which is further modified to form the tetramic acid nucleus. It is known that serine can be dehydrated enzymatically to α-aminoacrylic acid (6), e.g. in the formation of tryptophane [4]. This intermediate, which is hydrolysed to pyruvic acid and ammonia, could also arise from DAP by elimination of ammonia [5]. This hypothesis was tested by feeding [3-^{15}N]DAP, and analysis of malonomicin by ^{15}N NMR (figure 6b). Only the resonance at δ 24.7, which is assigned to the nitrogen at C-5, showed a strong enrichment; the enrichment factor is about 7%. From this result it can be concluded that the β-NH_2 group of DAP is incorporated into malonomicin at C-5 and that therefore α-aminoacrylic acid is not an intermediate between DAP and malonomicin.

The second explanation for the α-proton loss from DAP is that in one of the following stages of the biosynthesis the α-proton is stereospecifically exchanged*. One of these stages could be the ring closure reaction (which almost always proceeds with racemization at the 4-position in non-enzymatic systems [6a]). In section 5.2.4. a possible mechanism is drawn.

---

*) The most self-evident explanation for the absence of the resonance from the α-deuteron would be the loss of this deuteron by racemization during work-up. It is known from malonomicin and also from other tetramic acids substituted at C-4, that the C-4-proton is rather labile [6]; it is easily exchanged by both acid and base (accompanied by racemization). Therefore, the optical rotation of the malonomicin solution was measured after the recording of the deuteron spectrum, but no significant racemization was observed.

These results make it impossible to verify mechanism A, although the retainment of both β deuterons does support it strongly.

5.2.2. Acylation of DAP

As established in chapter 4, acetate is incorporated intact at positions 1 and 2 and therefore the first step after the formation of DAP might be the N^2-acetylation to form N^2-acetyl-DAP ((4); R=H). This hypothesis was tested using N^2-acetyl DAP, labelled at the amide-carbon with ^{13}C. However, incorporation could not be demonstrated. This was also the case with the derived ring-closed precursor 4-aminomethyltetramic acid ((5); R=H), labelled at C-5 with deuterium. Although it cannot be excluded that the incorporations are below the detection limit, there seems to be no special reason why these precursors would give a so much lower incorporation than DAP itself.

From these experiments, it was concluded that the formation of the tetramic acid nucleus occurs only after at least part of the side-chain has been built up. Therefore, we decided to investigate intermediates in which succinic acid is already involved. As both succinic acid and acetate are incorporated next to each other, their condensation product 3-oxoadipic acid (7) might seem a plausible intermediate.
3-Oxoadipic acid is known in nature as the degradation product of several aromatic compounds like catechol and protocatechuate to finally form acetic acid and succinic acid (figure 7).
As deuterium-labelled 3-oxoadipic acid had been synthesized from [2,3-$^2H_4$]succinic acid and malonic acid

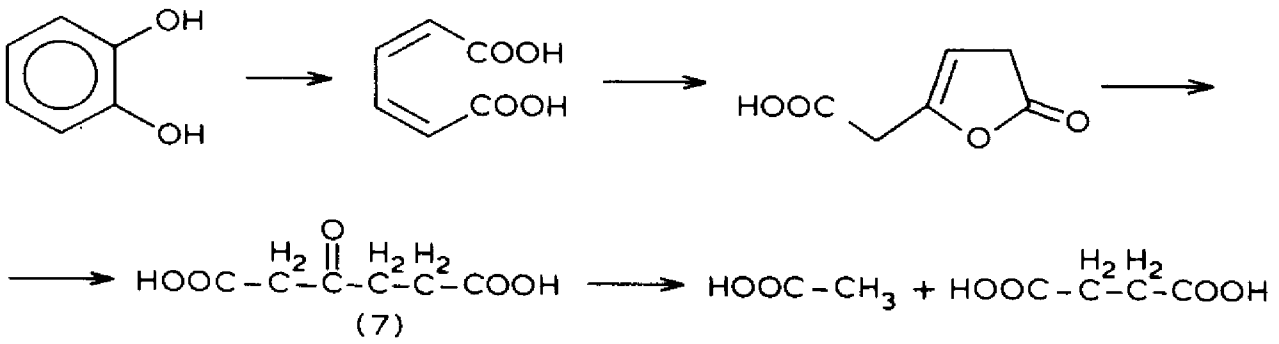
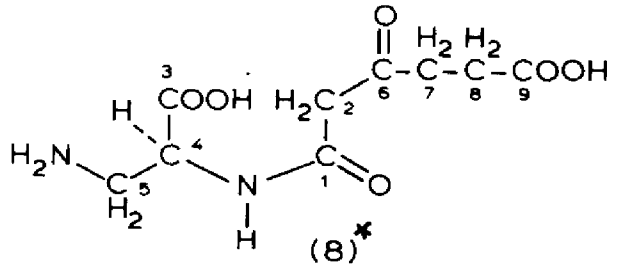


figure 7

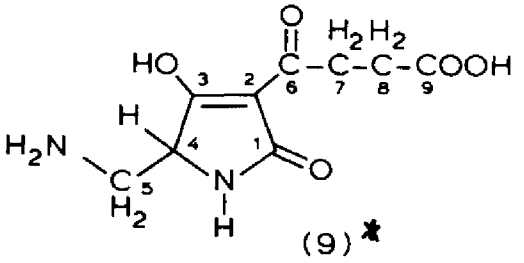
[7], special attention was paid to be sure that no labelled succinic acid was present in the precursor sample. If present, [2,3-$^{2}H_2$]succinic acid would certainly have been incorporated (cf. chapter 4.3.) and false conclusions could have been drawn.
After these precautions, a rather large dose (800 mg/ 1.5 l) was fed and incorporation of deuterium could be established (figure 8a). The incorporation was not high; it was estimated to be in the range of 0.15-0.2%. Compared to the high incorporations of the basic precursors this might seem almost insignificant, but it is well known in the literature that more complex precursors are often incorporated to a low extent (0.1-0.005%), especially when they are polar [8]. It is also known that 3-oxoadipic acid cannot easily permeate the cytoplasma membrane in bacteria [9]. Given this low permeability, incorporation to 0.1-0.15% can be considered to be significant. However, <u>enzymatic</u> degradation of 3-oxoadipic acid to succinic acid prior to incorporation (see figure 7) cannot be excluded in this experiment. To establish whether the 3-oxoadipate pathway is operative in Streptomyces rimosus (in the

literature the pathway is not known for Streptomyces species), deuteriated catechol was added to a culture to see whether 3-oxoadipic acid can be generated by biological degradation. However, we could not detect any incorporation of deuterium into malonomicin and it seems plausible that there are no enzyme systems present in Streptomyces rimosus, which can degrade 3-oxoadipic acid to succinic acid.
Although the evidence for the incorporation of 3-oxoadipic acid is not conclusive, the evidence presented is at least circumstantial. As far as we know, this would be the first time, that 3-oxoadipic acid is demonstrated to be involved in an anabolic pathway.

A logical next step in the biosynthesis might be the N-acylation of L-DAP with 3-oxoadipic acid to form (8).



(8)*



(9)*

The risk of adding (8) to a culture when labelled in position 5 or 7 (positions 2,4 and 8 are not suitable), is that by hydrolysis both products, L-DAP and 3-oxoadipic acid, can be incorporated separately which can lead to erroneous conclusions. To avoid this, doubly labelling at position 5 and 7 was used, and the

*) The numbering has been adapted to the numbering in malonomicin for convenience. In the experimental part the IUPAC nomenclature is used.

absence of L-DAP in the precursor sample was checked by t.l.c. The resulting deuteron spectrum after addition of the doubly-labelled compound is shown in figure 8b.

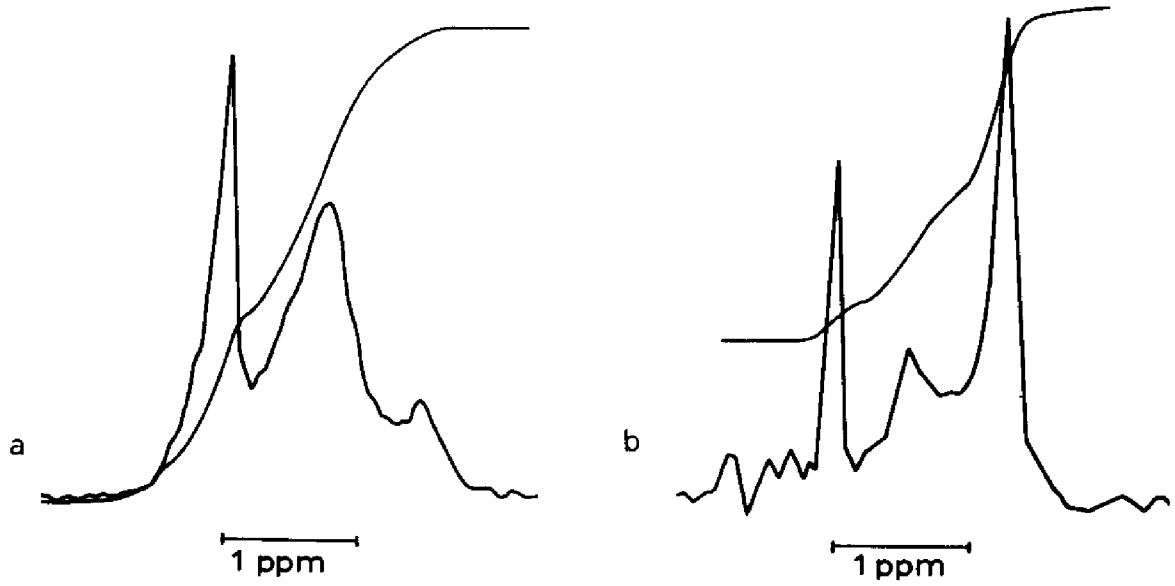


figure 8 ^{2}H spectra of malonomicin after incorporation of a) [4,5-$^{2}H_4$]-3-oxoadipic acid (300 mg/1.5 l) and b) [5,7,8-$^{2}H_6$]-N^{2}-(3-oxoadipoyl)-DAP (300 mg/l)

It is clearly visible that position-5 is significantly enriched. However, position-7 (which should be enriched to only half of the labelling at position-5, because one deuteron is lost; see chapter 4) is not enriched to the corresponding extent. Probably, hydrolysis has occurred for a fraction only, and given the high incorporation of DAP (300 mg gives an enrichment factor of 16.5%; hydrolysis of 300 mg of (8) for 2% would result in an enrichment factor of 0.17%, which is more than ten times the natural abundance of deuterium. From this experiment we cannot conclude whether (8) is a precursor or not.

5.2.3. Ring closure

When 3-oxoadipic acid is a precursor in the biosynthesis of malonomicin, ring closure could occur after its reaction with DAP. The involvement of this reaction product, N^2-3-oxoadipoyl DAP, could not be established, but its involvement seems mechanistically plausible. Therefore, its ring closed product (9) labelled at C-7 with deuterium was fed to a culture and enrichment could be demonstrated, though to a low extent. However, in this case too, we could not exclude degradation to labelled succinic acid prior to incorporation. Therefore, we labelled (9) in two positions, at C-7 and C-5. If degradation occurs, only label at C-7 of malonomicin is expected, for 4-aminomethyltetramic acid is not incorporated (vide supra). From figure 9 it can be concluded, that both positions are enriched (compare the simulated natural abundance spectrum at the same pH; cf. chapter 3). The ratio of the integrals of both signals has been changed from the normal value 3 : 1 to about 1.5 : 1. This can be expected, taking into account that only one deuteron at C-7 is incorporated into malonomicin v. two deuterons at C-5. From the integral ratio 1.5 : 1, an enrichment factor of 0.02% can be calculated. The observed chemical shift of the resonance at lower field deviates from the simulated one and corresponds more with the chemical shift of the deuterons at C-7; this is expected when more deuterium is present at C-7 than at the other constituting positions. This means that (9) is incorporated intact and thus is an intermediate in the biosynthesis of malonomicin.

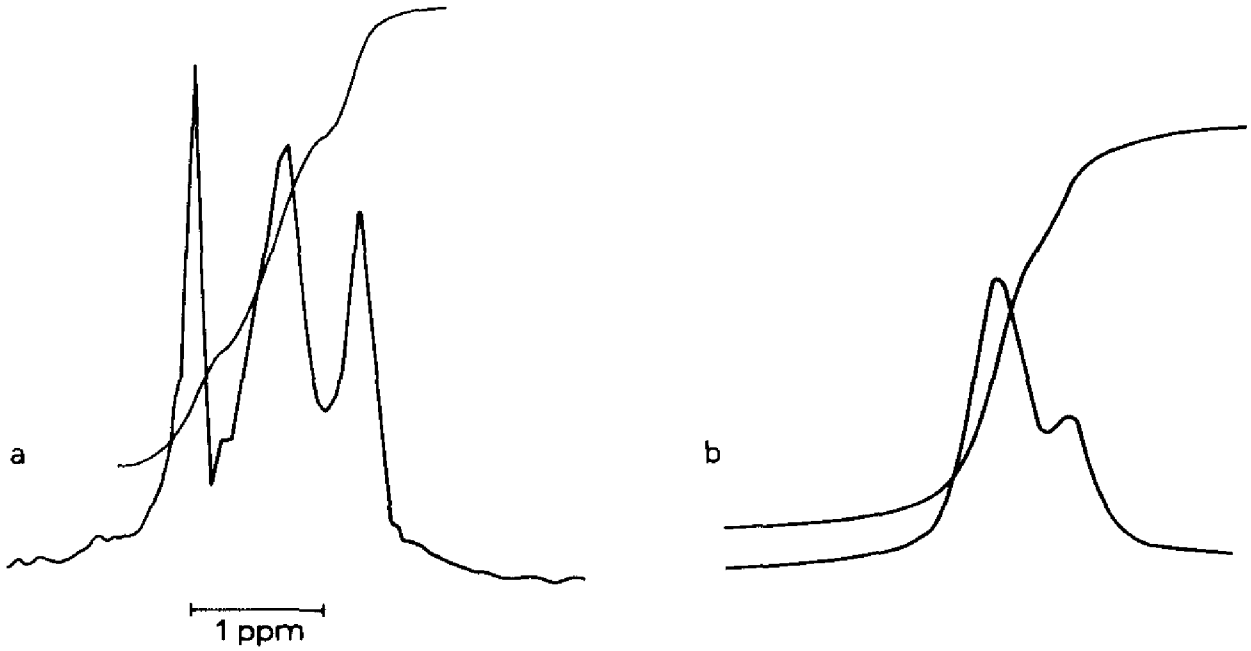


figure 9 ^{2}H spectrum of malonomicin (61.42 MHz) a) after incorporation of [5,7,8-$^{2}H_{6}$]-4-aminomethyl-2-succinoyltetramic acid; b) simulated spectrum

If the second explanation, given in section 5.2.1., for the proton loss at the α-position of DAP is correct, the proton at C-4 in (11) is not exchanged any more at this stage of the biosynthesis. When (9), labelled with deuterium at positions 4 and 5, was added, incorporation into malonomicin (figure 10) and indeed label at C-4 was unequivocally demonstrated by comparing the spectrum and integral with the simulated natural abundance spectrum and its integral. The integral ratio of 1.12 : 1 implicates an enrichment factor of 0.047%. The expected difference in chemical shift for the enriched and the unenriched compound is clearly observed (0.1 ppm) and is too large to be explained by experimental errors. Thus, there is no doubt that the C-4 proton is retained after ring closure.

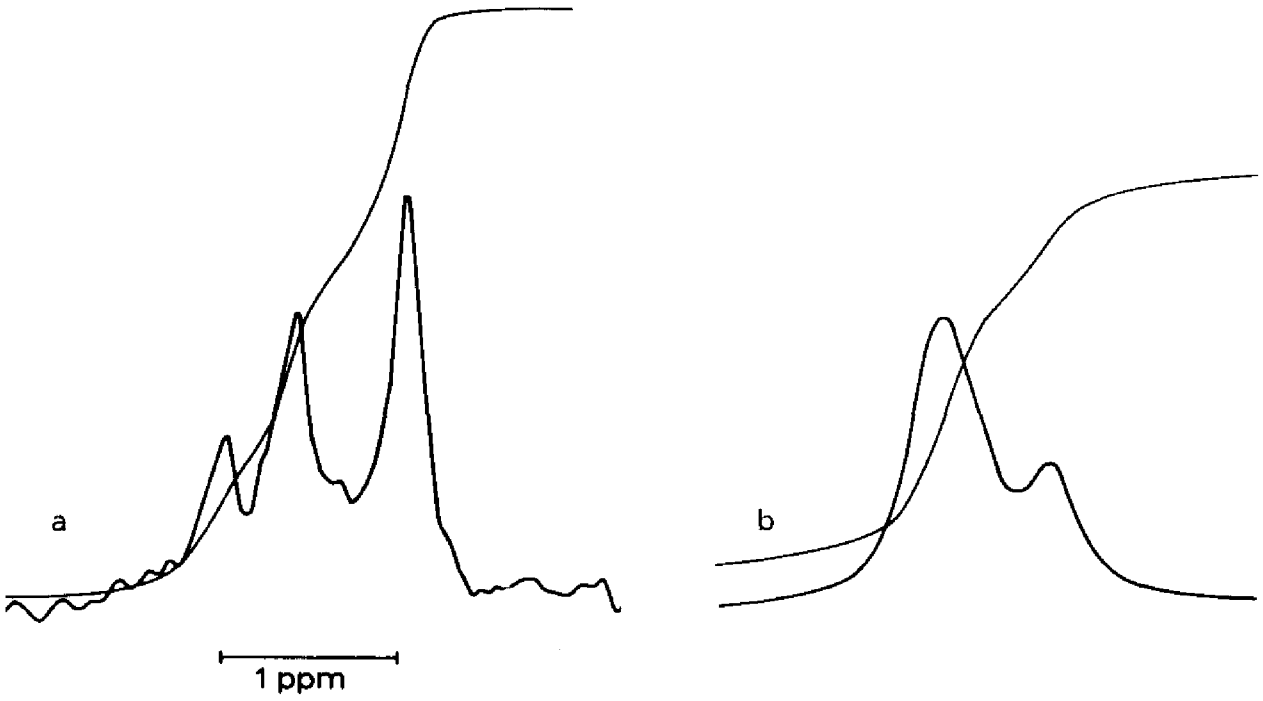


figure 10 ^{2}H spectrum of malonomicin (61.42 MHz), a) after incorporation of [4,5-$^{2}H_3$]-4-aminomethyl-2-succinoyltetramic acid; b) simulated spectrum

A tentative mechanism for the stereospecific exchange of the α-proton of DAP is drawn in figure 11. In this mechanism, the first step is a Claisen type condensation mediated by an enzyme (complex). In a following step, the activating group can be removed from the tetrahedral intermediate by an elimination via proton abstraction at C-4. Finally, the resulting enolate-anion could be stereospecifically protonated at C-4 by the enzyme.

5.2.4. Construction of the aminomalonic acid moiety

CO_2, ammonia and serine are the basic precursors, which together with (9) will form malonomicin. It is not easy however, to derive a mechanism for the

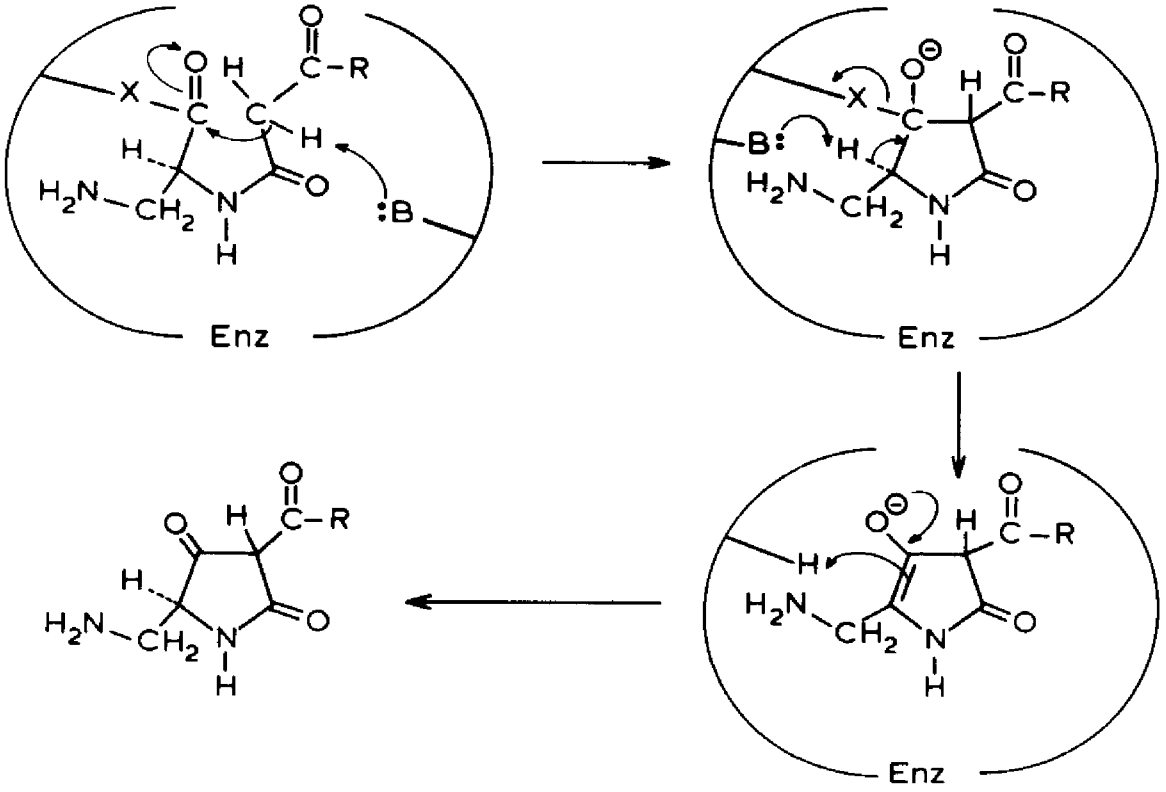
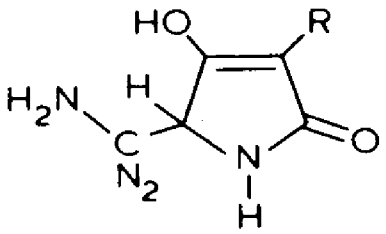
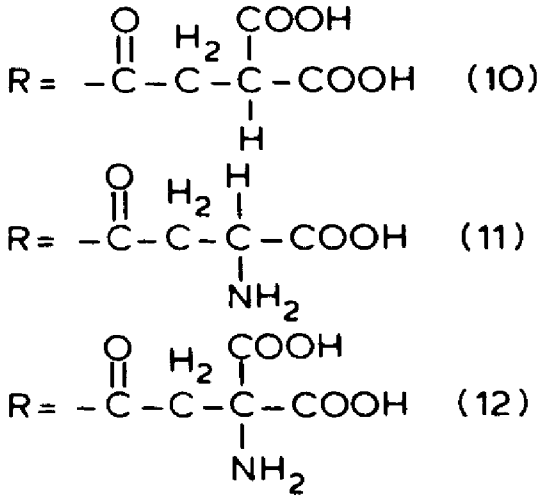


figure 11

construction of the aminomalonic acid moiety starting from (9). Given the incorporation of CO_2 and ammonia, (10), (11) and (12) can be postulated as intermediates, although a mechanism for the direct formation of (12) from (10) or (11) is without precedent, since carboxylation of an α-amino acid at the α-position and amination at the central carbon atom of a malonic acid derivative are both unknown processes in nature. However, (10), (11) and (12), (labelled with deuterium at C-5), were not incorporated, at least not to the minimum extent necessary for detection with ^2H NMR (the detection limit can assumed to be 0.5 x natural abundance = 0.008%*). Low permeability could be the reason





for not detecting any enrichment since the incorporation of polar (9) is low and (10), (11) and (12) are still more polar and so their permeability will be still lower. This means that for deuterium NMR, which has certainly proven to be an excellent method for demonstrating incorporations at a low level, the lower limit of its application has been reached. Other methods, that can circumvent the potential permeability problems or that can establish incorporations at lower levels or a combination of both will have to be used to elucidate the mechanism of the last steps of the biosynthesis of malonomicin. In chapter 6 some preliminary experiments in this direction are described.

*) This can probably only be achieved in spectra with well resolved resonances which can provide the necessary internal reference. Up to now the lowest level of incorporation of deuterium reported in the literature is 0.08% [10].

5.3. Experimental

5.3.1. Synthesis of labelled precursors

D,L-[2,3-$^{2}H_3$]-2,3-diaminopropanoic acid

This compound was synthesized from L-aspartic acid [11], that had been deuteriated using the modified method of Atkinson et al. (cf. chapter 4.4.3.).

[3-^{15}N]-2,3-diaminopropanoic acid

Nitrogen-15 labelled DAP was prepared from L-3-chloroalanine (250 mg) by amination in 1 ml conc $^{15}NH_4OH$ in a small Teflon coated stainless steel autoclave (2 h at 120 ^{o}C). Purification was performed by ion-exchange chromatography [11]. Yield 50%.

^{13}C NMR: $^{1}J(^{13}C-^{15}N)$ 5.9 Hz.

N^2-[1-^{13}C]acetyl-2,3-diaminopropanoic acid (4)

(4) was synthesized from methyl N^3-benzyloxycarbonyl-2,3-diaminopropanoate [12] and [1-^{13}C]acetyl chloride (MSD Holland) using the method described in [13]. After basic hydrolysis of the methyl ester, the protecting benzyloxycarbonyl group was removed by catalytic hydrogenation with H_2-Pd/C in methanol/aqueous HCl. Yield ca 60%.

^{13}C NMR (H_2O, pH 8.5): δ 174.5 (C-1)*, 22.3 (C-2), 177.0 (C-3), 54.2 (C-4), 42.0 (C-5), $^{1}J(^{13}C-1-^{13}C-2)$ 49.8 Hz.

---

*) The numbering in the spectral data corresponds with the numbering in the figures

[5-$^{2}H_{2}$]5-aminomethyl-4-hydroxy-Δ^{3}-pyrrolin-2-one (5)(R=H)

(5)(R=H) was synthesized from L-[3-$^{2}H_{2}$]DAP (see chapter 4.4.3.) according to [6a].

^{13}C NMR (H_2O,pH 8.5): δ 170.0 (C-1), 44.0 (C-2), 210.2 (C-3), 63.9 (C-4), 42.3 (C-5).

[4,5-$^{2}H_{4}$]-3-hexanedioic acid (7)

(7) was prepared from [2,3-$^{2}H_{4}$]succinic acid and bis trimethylsilyl malonate according to [7]. The benzyl-group of the intermediate O^{6}-benzyl O^{1}-hydrogen 3-oxohexanedioate was removed by catalytic hydrogenation with H_2-Pd/C in methanol.

^{13}C NMR (non-labelled compound; H_2O, pH 8.5): δ 174.5 (C-1), 50.2 (C-2), 206.7 (C-3), 37.7 (C-4), 29.27 (C-5), 177.7 (C-6).

[2,3,4,5-$^{2}H_{4}$]catechol

Catechol (2.5 g) was dissolved in 1.5 N DCl (20 ml), heated for 2 h at 120 ^{o}C in 5 Teflon coated stainless steel autoclaves and evaporated to dryness. Deuterium content >90%.

N^{2}-1-[4,5-$^{2}H_{4}$]-3-oxohexanedioyl-2,3-diaminopropanoic acid (8)

The synthesis of (8) was carried out from benzyl [3-$^{2}H_{2}$]-N^{3}-benzyloxycarbonyl-2,3-diaminopropanoate and O^{6}-benzyl O^{1}-hydrogen 3-oxohexanedioate using the HOBT/DCC coupling method as described in [6a]. The protecting groups were removed by catalytic hydrogenation with H_2-Pd/C in methanol. Overall yield from L-[3-$^{2}H_{2}$]DAP

30%.

^{13}C NMR (non-labelled compound; H_2O, pH 8.5): δ 170.1 (C-1), 49.6 (C-2), 171.3 (C-3), 50.5 (C-4), 40.2 (C-5), 207.0 (C-6), 37.9 (C-7), 27.9 (C-8), 177.0 (C-9).

Ring closed compounds (9), (10), (11) and (12)

Synthesis of these compounds was accomplished using the same methods as used for the total synthesis of malonomicin [6a]. The tetramic acids had been labelled using labelled (5) (vide supra). The identity of the compounds was established by ^{13}C NMR. In some compounds a minor quantity of the starting material was present. This was mainly the (unlabelled) sidechain, which cannot disturb the incorporation experiment. The yields are calculated starting from (5).

[5-$^2H_2$]-5-aminomethyl-4-hydroxy-3-succinoyl-Δ^3-pyrrolin-2-one (9)

(9) was prepared from (5) and monobenzyl succinate. Yield 40%. The [5,7,8-$^2H_6$] labelled precursor was synthesized from labelled monobenzyl succinate and (5). The [4,5-$^2H_3$] labelled precursor was obtained by exchange of the C-4 proton in D_2O/1N-DCl for one hour.

^{13}C NMR (H_2O, pH 8.5): δ 178.3 (C-1), 103.1 (C-2), 196.0 (C-3), 59.6 (C-4), 42.0 (C-5), 197.0 (C-6), 36.8 (C-7), 32.5 (C-8), 180.5 (C-9).

[5-$^2H_2$]-5-aminomethyl-3-(3-carboxysuccinoyl)-4-hydroxy-Δ^3-pyrrolin-2-one (10)

This compound was synthesized from (5) and O^1-benzyl O^4-hydrogen 2-benzyloxycarbonylsuccinate (13) which was prepared from dibenzyl malonate and t-butyl chloroace-

tate using the method described for the synthesis of the aminomalonic acid derivatives as described in [6a]. The monoalkylated malonate was separated from the dialkylated malonate by selective extraction from water, buffered at pH 6.0 (after removal of the t-butyl group). Yield of (10) 35%.

^{13}C NMR (H_2O, pH 8.5): δ 178.7 (C-1), 102.7 (C-2), 193.6 (C-3), 56.5 (C-4), 40.1 (C-5), 195.7 (C-6), 41.8 (C-7), 53.5 (C-8), 179.7 (C-9).

[5-$^2H_2$]-5-aminomethyl-3-(4-aspartyl)-4-hydroxy-Δ^3-pyrrolin-2-one (11)

The synthesis of (11) was carried out from (5) and O^1-benzyl O^4-hydrogen N-benzyloxycarbonylaspartate [14]. Yield 40%.

^{13}C NMR (H_2O, pH 8.5): δ 177.7 (C-1), 102.7 (C-2), 192.7 (C-3), 59.4 (C-4), 41.25 (C-6), 196.0 (C-6), 40.3 (C-7), 52.4 (C-8), 175.9 (C-9).

[5-$^2H_2$]-5-aminomethyl-3-(2-carboxy-4-aspartyl)-4-hydroxy-Δ^3-pyrrolin-2-one (12)

This compound was prepared from (5) and O^1-benzyl O^4-hydrogen 2-benzyloxycarbonyl-N-benzyloxycarbonyl-aspartate (14). Yield 30%. The synthesis of (14) was performed according to [6a] from dibenzyl N-benzyloxycarbonylaminomalonate and t-butyl chloroacetate.

^{13}C NMR (H_2O, pH 8.5): δ 177.8 (C-1), 102.8 (C-2), 192.6 (C-3), 59.4 (C-4), 42.4 (C-5), 195.9 (C-6), 43.4 (C-7), 67.1 (C-8), 173.2 (C-9/10).

5.4. References

1 S. Gatenbeck and J. Sierankiewicz, Acta Chem. Scand., 1973, 27, 1825.

2 E.W. Miles and P.McPhie, J. Biol. Chem., 1974, 249, 2852.

3 J.A. King, J. Amer. Chem. Soc.,1947, 69, 2738.

4 H. Kumagai and E.W. Miles, Biochem. Biophys. Res. Commun., 1971, 44, 1271.

5 D. Rajagopal Rao, K.R. Vijayalakshmi, and K. Hariharan, Biochem. J., 1970, 119, 113.

6 a) J.L. van der Baan, J.W.F.K. Barnick, and F. Bickelhaupt, Tetrahedron, 1978, 34, 223.
b) W. Hofheinz and W.E. Oberhänsli, Helv. Chim. Acta, 1977, 60, 660.

7 J.W.F.K. Barnick, J.L. van der Baan, and F. Bickelhaupt, Synthesis, 1979, 787.

8 See E. Leete, Biosynthesis, Vol. 1, T.A. Geissman, ed., The Chemical Society, London, 1972, p. 158.

9 a) R.Y. Stanier, Bacteriol. Rev., 1950, 14, 179.
b) L.N. Ornston and D. Parke, J. Bacteriol., 125, 475.

10 S. Damtoft, S.R. Jensen. and B.J. Nielson, J. Chem. Soc. Chem. Comm., 1980, 42.

11 S.L.N. Rao, Biochemistry, 1975, 14, 5218.

12 a) K. Poduska. J. Rudinger, and F. Sorm, Coll. Czech. Chem. Comm., 1955, 20, 1174.
b) F. Schneider, Liebigs Ann. Chem., 1937, 529, 1.
c) S. Takagi, H. Tsukatani, and K. Hayashi, Chem. Pharm. Bull. (Japan), 1959, 7, 616.

13 W. Kerp and K. Unger, Chem. Ber., 1897, 30, 584.

14 A.K. Bose and R.E. Strube, J. Pharm. Sci., 1963, 52, 847.

Chapter 6

Evaluation and some preliminary experiments

6.1. Introduction

The results of the biosynthetic experiments with simple and more elaborate precursors described in chapter 4 and 5 are summarized in figure 1 and 2. Serine is converted into diaminopropanoic acid, which is probably N-acylated with the condensation product of acetate and succinate (1) to form (2). After ringclosure to (3), the remaining precursors CO_2, ammonia and serine are attached to ultimately form malonomicin.
As shown in this figure, not all questions have been solved. In the first place, the mechanism of the conversion of serine into diaminopropanoic acid is not definitely known. Also the involvement of 3-oxoadipic acid as a direct precursor is not absolutely sure and the involvement of the N-acylated product (2) could not be established. Furthermore, the reactions that take place after the formation of 2-succinoyl-4-aminomethyltetramic acid (3) could not be determined. Whether the failure of incorporation of some potential intermediates such as (4), (5) and (6) was caused by the fact that these compounds are not real intermediates, or by the inability to penetrate to the site of synthesis of malonomicin, is an open question. Permeability barriers do pose serious problems in

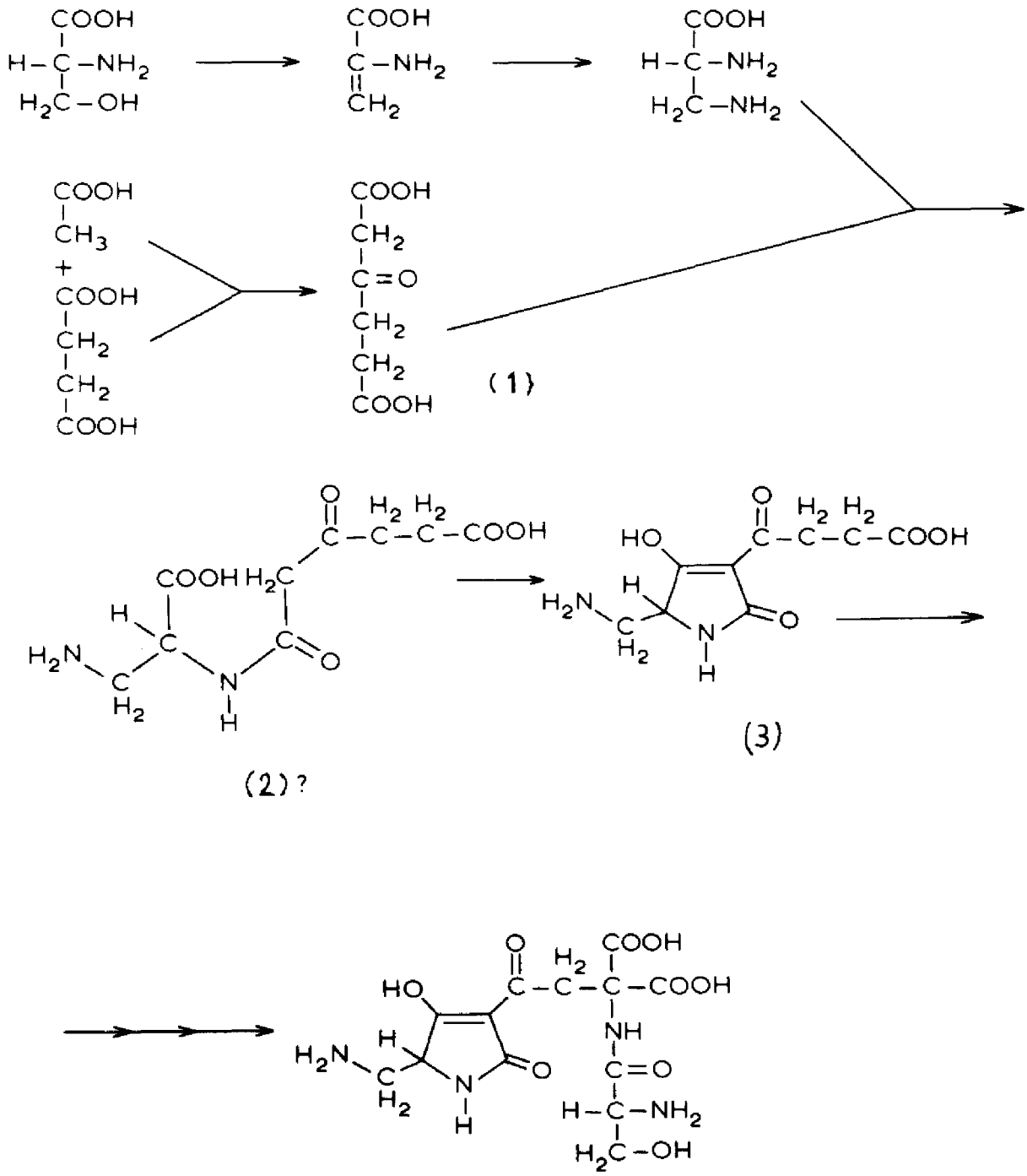


figure 1

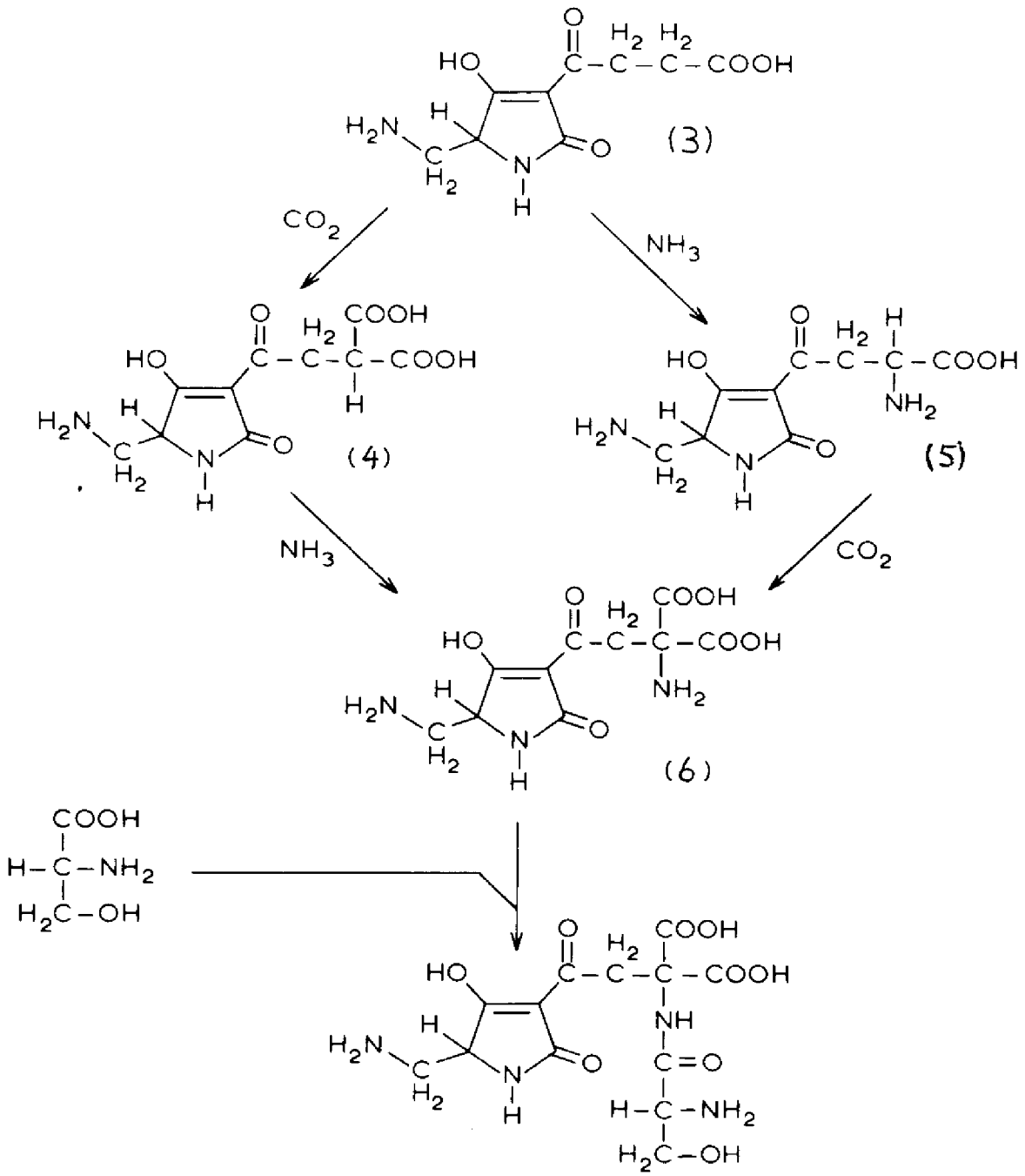


figure 2

biosynthetic studies. Indeed, it has been reported that even simple precursors like mevalonate and acetate cannot cross the permeability barriers in some organisms [1].
In looking for a solution to these problems, several approaches will be discussed and also some preliminary experiments described.

6.2. Conversion of serine into diaminopropanoic acid

As a consequence of the loss of the -proton of DAP in one of the subsequent steps of the biosynthesis of malonomicin (presumably in the ringclosure of (2) to (3), see chapter 5.2.3.), the exact mechanism of the serine to DAP conversion could not be established with certainty. One mechanism, which, although less likely, still cannot be excluded on the basis of the performed experiments, is the direct substitution of the (activated) hydroxyl group of serine by ammonia. This mechanism can be tested by the use of serine, stereospecifically labelled with deuterium at C-3; in the case of direct substitution by ammonia, the configuration at C-3 will be inverted and in the case of an elimination-addition reaction, the configuration will remain the same [2]. By investigating other DAP containing metabolites in which the DAP part is incorporated without further modification, circumstantial evidence for the conversion mechanism in malonomicin can be obtained. Therefore, experiments with Streptomyces collinus lindenbein, which produces N^3-fumarylcarboxyamido-L-2,3-diaminopropanoyl-L-alanine [3] are under way. Labelling experiments with [2-^{2}H]- and [3-$^2H_2$]serine could give the following possible results:

- in the case of direct substitution, label will be found at both the α- and β-positions and in the original ratio.
- β-oxidation followed by transamination will give loss of one deuteron in the β-position.
- the occurrence of an elimination-addition reaction will yield deuterium at the β-position only.

This last possibility, involving α-aminoacrylic acid or dehydroalanine, is the most likely one. Dehydroaminoacids have also frequently been implicated as biochemical reaction intermediates. It has been suggested for example, that dehydrocysteine- and dehydrovaline-containing peptides could be intermediates in penicillin and cephalosporin biosynthesis [4]. In addition, enzyme-bound dehydroalanine has been proposed as an intermediate in the desulphuration of cysteine catalyzed by S-alkyl-L-cysteine lyase [5], in the dehydration of serine by serine dehydrolase [6], and in the metabolism of O-acetylserine [7]. Dehydroalanine occurs in some peptide antibiotics, e.g. siomycin [8], thiostrepton [9], and berninamycin [10]. In berninamycin, which contains five dehydroalanine residues, it has been demonstrated that serine is the best precursor for these residues [11], though it is not known whether the dehydration occurs at the individual amino acid level or following incorporation into a peptide structure.

An experiment which can give more detailed insight in the dehydration step is addition of serine and O-acetylserine, both labelled at C-3, to two synchronous cultures of Streptomyces rimosus. O-acetyl-L-serine is involved in the biosynthesis of many DAP-containing plant metabolites [12] (cf. chapter 1) and has been

detected recently in Citrullus vulgaris (water-melon) [13], which also produces a DAP-containing metabolite [14]. If the dehydratation step follows the activation of serine by conversion to O-acetyl-L-serine, the ratio of incorporation into the serine and the DAP part of malonomicin will probably be changed in favour of the incorporation into the DAP part. If O-acetyl-L-serine is not an intermediate, the ratio of incorporation will probably be the same as in the experiment with labelled serine.

6.3. 3-Oxoadipic acid

Although circumstantial evidence has been presented for the incorporation of 3-oxoadipic acid into malonomicin, degradation of 3-oxoadipic acid to succinic acid prior to incorporation cannot be excluded with certainty. Double labelling of 3-oxoadipic acid could definitely prove the incorporation of the intact molecule. There,fore we synthesized [2,3,6-$^{13}C_3$]3-oxoadipic acid from [2-^{13}C]malonate and [1,4-$^{13}C_2$]succinic acid (see chapter 5.4.). When incorporated intact, satellites are expected to be visible in the ^{13}C spectrum at the resonances of C-2 and C-3. However, enrichment could not be detected. Unfortunately, only 100 mg of the doubly labelled precursor was available for the incorporation experiment (vs 300 mg in the deuterium experiment), and thus the detection of incorporation was much more difficult. Unexpectedly, the yield of malonomicin in the ^{13}C experiment was twice as high as usual, which caused an extra dilution of label. Assuming incorporation proportional to the concentration of labelled precursor(which in the case of good permeability is certainly not true),and given the enrichment factor of

0.16 for the deuterium experiment, the expected incorporation in the ^{13}C experiment is 0.01%. The detection limit with doubly labelled precursors can be estimated to be 0.025%, which will give satellites of 1.5% of the centre peak (the natural abundance satellites have an intensity of 0.55% of the centre peak).
An experiment with a larger quantity of precursor doubly labelled with ^{13}C (see however 6.5.3.) or, much less expensive, the use of radioactive double labelling with ^{14}C and ^{3}H, might give the definite proof for the incorporation of intact 3-oxoadipic acid. With radioactive labelling, the ^{3}H : ^{14}C ratio should be reduced to one fourth of the original value as only one proton of 3-oxoadipic acid is incorporated (cf. chapter 4.2.).

The experiment with deuteriated catechol (cf. chapter 5) cannot be used as a definite proof for the 3-oxoadipate pathway not being operative in Streptomyces rimosus. It is possible that the stereospecific loss of deuterium (cf. chapter 4) is the explanation for the absence of deuterium in malonomicin after feeding the deuteriated precursor. Therefore, an experiment with catechol, labelled with ^{13}C or ^{14}C can give extra information on the occurrence of this pathway.

6.4. N^2-(3-oxoadipoyl)-2,3-diaminopropanoic acid (2)

The only way to obtain a definite proof for the incorporation of the N-acylated DAP molecule (2) is a double labelling experiment. The double labelling has to be such that hydrolysis of this compound to DAP and 3-oxoadipic acid will not disturb the detection of incorporation of the intact precursor. This can be

accomplished by labelling the -nitrogen and the amide carbonyl, by which a coupling of 12.3 Hz (see chapter 3.4) will be visible in both the ^{13}C and the ^{15}N spectrum. The enrichment can best be detected in the ^{15}N spectrum since with the smaller linewidth there is less risk of obscuring the satellites in the base of the resonance. Moreover, the theoretical detection limit is lower because of the lower natural abundance of nitrogen-15.

6.5. Approaches to the elucidation of the final steps in the biosynthesis of malonomicin

6.5.1. Permeabilization

An obvious method to study the incorporation of precursors, for which the cytoplasma membrane is poorly permeable, is to permeabilize the cells. This method is rather mild and fast and avoids the risk of mechanical disruption methods which destroy the organizational integrity of the cell and may lead to inactivation of enzymes, dissociation of enzyme complexes, and other artifacts. In the literature several procedures are known. With Saccharomyces cerevisiae the application of nystatin [15], dimethylsulfoxide (DMSO) [16], and a mixture of toluene and ethanol [17] have been shown to be successful for specific purposes. Recently, a permeabilization method using 0.05% Triton X-100 has been reported which has also been used in Streptomyces species [18].

Permeabilized cells were used to study the involvement of (2) in the biosynthesis of malonomicin. This molecule is the probable precursor of the ring-closed

product (3) and other intermediate tetramic acids. As a sensitive detection method of (3) UV spectroscopy was chosen, for acyltetramic acids have an ϵ of about 10,000 at 240 and 280 nm.
However, preliminary experiments using Triton X-100 (0.05 and 1%) and DMSO could not establish the ringclosure from (2) to (3). Possibly, known methods for testing the permeability of the cells have to be used to establish the most favourable conditions, before these permeabilized cells can be successfully applied in the study of the biosynthesis of malonomicin.

6.5.2. Use of cell-free extracts

Cell-free extracts have been used in biosynthetic studies with considerable success, e.g. in the biosynthesis of porphyrins [18]. The absence of any permeability barrier might favour this method in its application to our problems, but the disintegration of the organized enzyme complexes can inhibit its successful application. In one preliminary experiment we prepared a cell-free extract using sonication and lysozyme. Two biosynthetic reactions were used to test this method: a) the serine to DAP conversion using [U-^{14}C]serine and b) the incorporation of (3) (labelled with tritium at C-4) into one of the next intermediates. Detection was performed with preparative HPTLC, and liquid scintillation counting. Although no quantitative data are available yet, some tentative conclusions may be drawn.

- In the experiment with ^{14}C-labelled serine, two radioactive zones were found with the r_f values of serine and DAP. The activity of the latter fraction was very low and isolation of the DAP formed and an

independent identification will be necessary for the definite proof of this conversion in cell-free extracts.

- In the experiment with tritium labelled (3), two radioactive zones could also be measured with the r_f values of the substrate and an unknown compound. The activity of the unknown compound was also very low and its identity could not be established with certainty. None of the potential intermediates between (3) and malonomicin (cf. chapter 5) could be detected. Surprisingly, the r_f value of the unknown compound agreed very well with the r_f value of malonomicin itself. Assuming that the radioactive compound is malonomicin, it can tentatively be concluded that the synthesis of malonomicin from (3) is accomplished in an enzyme complex without release of intermediates. Isolation of the unknown compound and recrystallization to constant specific activity after addition of carrier malonomicin can establish whether this product is identical to malonomicin or not. When the method for obtaining cell-free extracts has been optimized, this method can also be used in experiments with tritium-labelled (4), (5), and (6) to establish their involvement in the biosynthesis of malonomicin.

6.5.3. "Tracer" studies

When permeability problems are not the reason that incorporation of the most elaborate precursors could not be detected, another explanation could be that after the formation of 2-succinoyl-4-aminomethyl tetramic acid (2) the subsequent intermediates in the

biosynthesis are enzyme-bound. In that case, addition of 300 mg precursor will be sufficient to block equilibration of the exogenous material with the biosynthetic intermediates. It is known in the literature [20,21], that the use of levels of exogenous substrate in vast excess over endogenous substrate is liable to generate misleading results. It has been emphasized [21], that the use of labelled precursors in testing biosynthetic hypotheses is a tracer technique; therefore, repetition of the experiments with labelled (8), (9) and (10) at lower level of addition (e.g. 25 mg/l) may give positive results.

6.5.4. The possible involvement of a plasmid in malonomicin synthesis by Streptomyces rimosus

Numerous investigations with Streptomyces species have suggested the role of plasmids as genetic determinants of a number of phenotypic properties (e.g. fertility [22,23], aerial mycelium formation [24,25], antibiotic production [22,24-32], antibiotic resistance [25,28], and melanin synthesis [33,34]. The elimination of these characteristics by treatment with acridinium or phenantrium dyes has been used to demonstrate the involvement of a plasmid. Of particular interest is the finding that antibiotic production may depend on the presence of a plasmid. The transfer of antibiotic producing ability by conjugation has also been reported [32] and transfer of this ability from a microorganism with poor permeability properties to an organism with better permeability properties, could make possible the study of the biosynthesis of this antibiotic.
Therefore, we tried to detect plasmids in Streptomyces rimosus, and, if present, to investigate whether the

plasmid is responsible for the malonomicin production. Two different methods were used to detect the plasmid DNA: the "cleared lysate" method [35] and the "salt out" method [36]. Detection was performed using ethidium bromide and CsCl gradient centrifugation, followed by agarose-gel electroforesis.
However, with both methods no plasmid DNA could be detected.

6.5.5. Mutation of Streptomyces rimosus

The use of microbial mutants has been of enormous value in the study of biosynthetic pathways [37]. The basis of the method is the production through mutagenic agents, of mutants which are deficient in an enzyme mediating one reaction of the biosynthetic process under investigation. The intermediate immediately preceding the deleted enzyme in the pathway may accumulate in sufficient quantity to be isolated and identified. This method has been successfully applied in the study of the biosynthesis of, for instance, tetracyclines [38], prodigiosin [39] and erythromycin [40].
As mutant production is a random process, the success of this approach clearly hinges on the feasibility of treatment of large populations. Another requirement is a practical procedure for selecting the desired mutants from the treated population. As a selection criterion the mutants are sometimes tested biologically.
Malonomicin is active against Pseudomonas sp. 257 [41] and related molecules such as the decarboxylation product of malonomicin and a number of synthetic analogues are not [42]. Therefore, after treatment with N-methyl-N'-nitro-N-nitrosoguanidine (killing factor 90%), potential mutants (10,000) were plated and tested

against Pseudomonas. The non-active mutants were transferred to and grown in a shaken culture and the UV spectrum was measured since we were in search of acyltetramic acids, which have characteristic UV absorptions at 240 and 280 nm. The cultures themselves were also biologically tested against Pseudomonas as the biological test on agar-grown cultures was not always reliable. However, no culture of the inactive mutants showed the characteristic UV spectrum; on the other hand some mutants could be isolated which produced malonomicin at a much lower level than normal (the identity of malonomicin was checked by t.l.c.). The development of a biological test which is more reliable and the use of other mutagenic agents should be tried before this method can be abandoned.

6.6. Conclusion

In this chapter the remaining problems in the biosynthesis of malonomicin and several approaches to solve them have been discussed. From the preliminary experiments, it can be concluded that the use of cell-free extracts seems promising for the solution of some of the problems. Also, experiments using doubly labelled precursors may give some additional information.

Acknowledgement

I wish to thank Dr. E. Veltkamp, dep. of Molecular Genetics, for advice and encouragement.

6.7. References

1 C.J. Coscia, R. Guarnaccia, and L. Botta, Biochemistry, 1969, 8, 5036.

2 C. Fuganti, D. Ghiringhelli, D. Giangrasso, P. Grasselli, A. Santopietro Amisano, La Chimica e l' Industria, 56, 424.

3 R.M. Gale and D.H. Lively, U.S.P. 3,832,287.

4 For a comprehensive review, see D.J. Aberhart, Tetrahedron, 1977, 33, 1545.

5 A. Kjaer and P.O. Larsen, Biosynthesis, Vol. 5, J.D. Bu'Lock, ed., The Chemical Society, London, 1977, p. 121.

6 H. Mahler and E. Cordes, Biological Chemistry, 2nd ed., Harper and Row, New York, 1971, p. 394.

7 A. Kjaer and P.O. Larsen, Biosynthesis, Vol. 2, T.A. Geissman, ed., The Chemical Society, London, 1973, p. 74.

8 M. Ebata, K. Miyazaki, and H. Otsuka, J. Antibiotics, 1969, 22, 423.

9 M. Bodansky, J.A. Scozzie, and I. Muramatsu, J. Antibiotics, 1970, 23, 9.

10 J.M. Liesch, D.S. Millington, R.C. Pandey, I.C. Paul, K.L. Rinehart, Jr., and F. Reusser, J. Amer. Chem. Soc., 1976, 98, 8237.

11 C.J. Pearce and K.L. Rinehart, Jr., J. Amer. Chem. Soc., 1979, 101, 5069.

12 I. Murakoshi, F. Kato, J. Haginiwa, and T. Takemoto, Chem. Pharm. Bull. (Japan),1974, 22, 473.

13 I. Murakoshi, F. Ikemagi, T. Ariki, K. Harada, and J. Haginiwa, Chem. Phar. Bull. (Japan), 1979, 27, 2484.

14 a) S. Shinano and T. Kaya, J. Agric. Chem. Soc. Japan, 1957, 31, 759.
b) F.F. Noe and L. Fowden, Biochem. J., 1960, 77, 543.

15 J. Bechet and J.M. Wiame, Biochem. Biophys. Res. Comm., 1965, 21, 226.

16 B.G. Adams, Analyt. Biochem., 1972, 45, 137.

17 R. Serrano, J.M. Gancedo, and C. Gancedo, Eur. J. Biochem., 1973, 34, 479.

18 G.F. Miozzari, P. Niederberger, and R. HÜtter, Anal.Biochem., 1978, 51, 220.

19 A.R. Battersby and E. McDonald, Acc. Chem. Res., 1979, 12, 14.

20 J.C. MacDonald and G.P. Slater, Canad. J. Biochem., 1975, 53, 475.

21 J.D. Bu'Lock and C. Leigh, J. Chem. Soc, Chem. Comm., 1975, 628.

22 A. Vivian and D.A. Hopwood, J. Gen. Microbiol., 1970, 64, 101.

23 A. Vivian, J. Gen. Microbiol., 1971, 69, 353.

24 M. Okanishi, T.Ohta, and H. Umezawa, J. Antibiotics, 1970, 23, 45.

25 P.A. Redshaw, P.A. McCann, L. Sankaran, and B.M. Pogell, J. Bacteriol., 1976, 125, 693.

26 R. Kahler and D. Noack, Z. Alg. Mikrobiol., 1974, 14, 529.

27 R. Kirby, L.F. Wright, and D.A. Hopwood, Nature, 1975, 254, 265.

28 H. Akagawa, M. Okanishi, and H. Umezawa, J. Gen. Microbiol., 1976, 90, 336.

29 L.F. Wright and D.A. Hopwood, J. Gen. Microbiol., 1976, 95, 96.

30 P.D. Shaw and J. Piwowarski, J. Antibiotics, 1977, 30, 404.

31 K. Hotta, Y. Okami, and H. Umezawa, J. Antibiotics, 1977 30, 1146.

32 H. Umezawa, Y. Okami, and K. Hotta, J. Antibiotics, 1973 31, 99.

33 K.F. Gregory and J.C.C. Huang, J. Bacteriol., 1964, 87, 1281.

34 K.F. Gregory and J.C.C. Huang, J. Bacteriol., 1964, 87, 1287.

35 a) D.B. Clewell and D.R. Helinsky, Proc. Natl. Acad. Sci. USA, 1969, 62, 1159.
b) E. Veltkamp and H.J.J. Nijkamp, Molec. gen. Genet., 1973, 125, 329. P. Guerry, P.J. Le Blanc, ans S. Falkow, J. Bacteriol., 1973, 116, 1064.

37 A.L. Demain, Advances in applied Microbiology, Vol. 16, D. Perlman, ed., Acad. Press, New York, 1973, p. 177.

38 J.R.D. McCormick, Antibiotics, Vol. 2, D. Gottlieb and P.D. Shaw, eds, Springer Verlag, Berlin and New York, 1967, p. 113.

39 R.P Williams and W.R. Hearn, Antibiotics, ibid., p. 410 and p. 449.

40 J.R. Martin and A.W. Goldstein, Progress in Antimicrobial and Anticancer Chemotherapy, Vol. 2, University Park Press, Baltimore, 1970, p. 1112.

41 Gist-Brocades N.V., Internal Report.

42 J.L. van der Baan, J.W.F.K. Barnick, and F. Bickelhaupt, unplublished results.

Samenvatting

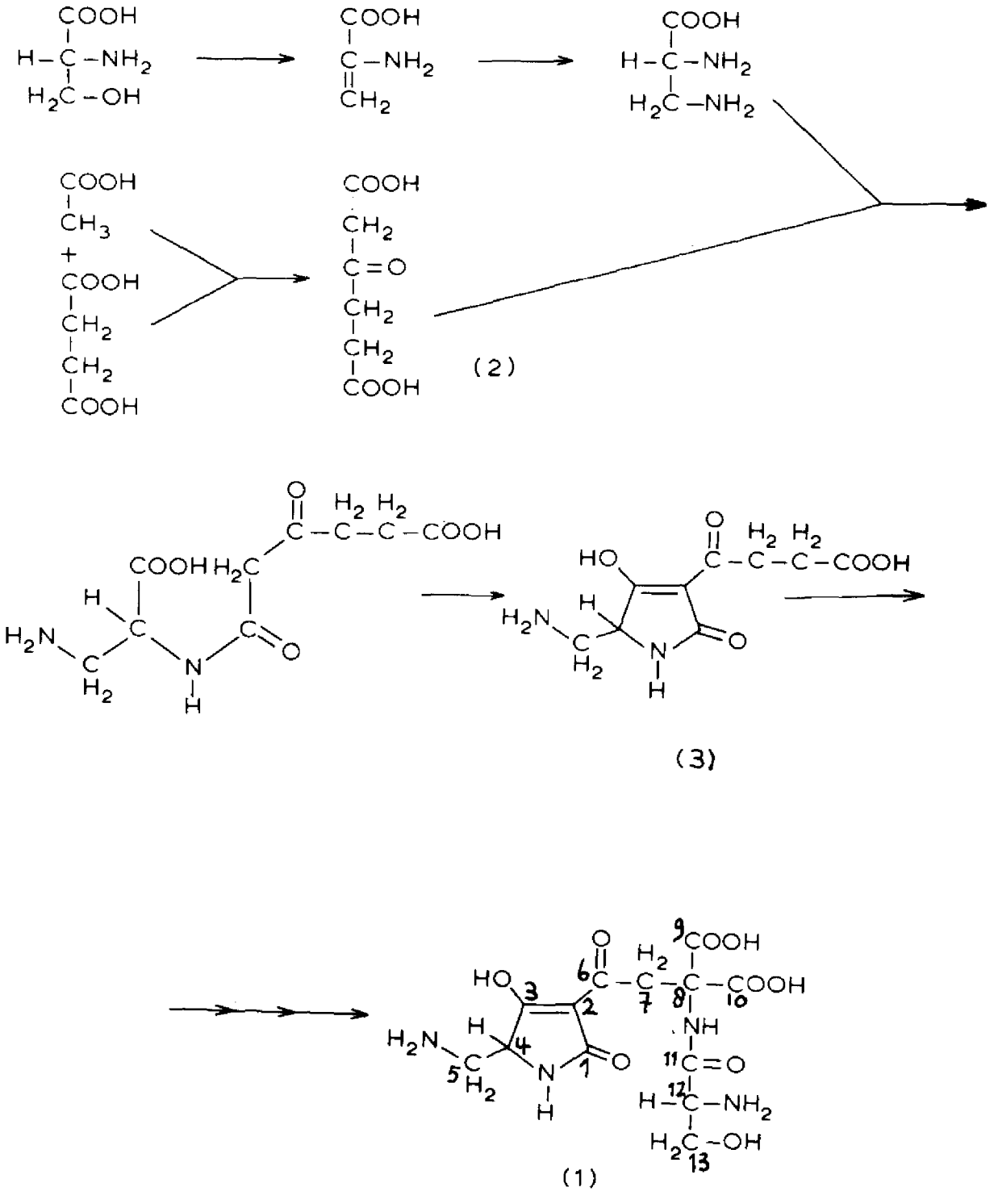
In dit proefschrift wordt het onderzoek beschreven naar de biosynthese van het antibioticum malonomicine (1), dat actief is tegen de verwekkers van de slaapziekte. De bijzondere kenmerken van dit antibioticum zijn de aanwezigheid van de tetramzuurkern (C_1-C_5), het aminozuur diaminopropionzuur (C_3-C_5) en met name het aminomalonzuur gedeelte (C_8-C_{10}).
Ten tijde van de structuuropheldering van malonomicine (toen nog K16 geheten), was dit de eerste natuurstof waarin aminomalonzuur voorkwam. Sindsdien is er nog een natuurlijke verbinding ontdekt met hetzelfde kenmerk.
Voordat de structuur van malonomicine was opgehelderd, waren er reeds enzymen bekend die in staat waren aminomalonzuur om te zetten in glycine en aminomalonzuur te maken uit oxomalonzuur. Aminomalonzuur zelf kon echter niet in vivo worden aangetoond.
Om na te gaan hoe malonomicine wordt opgebouwd en of aminomalonzuur een natuurlijk voorkomend aminozuur is dat betrokken is bij de opbouw van malonomicine , werd het hiervoor beschreven onderzoek verricht (hoofdstuk 1).

In hoofdstuk 2 wordt de methodologie van biosynthetisch onderzoek beschreven met de nadruk op het gebruik van stabiele isotopen in kombinatie met kernspinresonantie. Hiermee is het mogelijk inbouw van bepaalde bouwstenen te bestuderen zonder dat de gelabelde verbinding tot kleine fragmenten moet worden afgebroken om de positie en mate van labelling te kunnen bepalen.

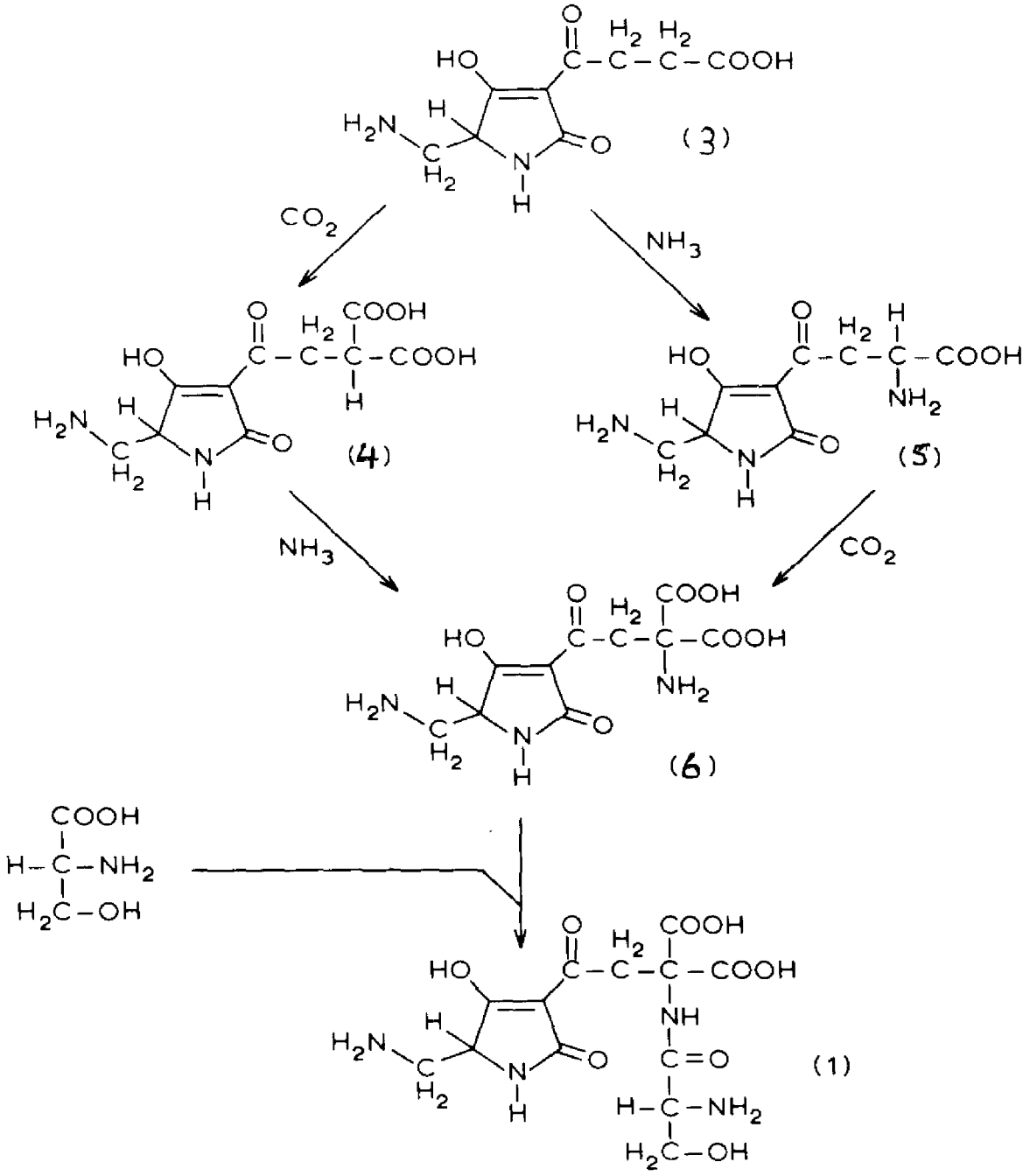
In hoofdstuk 3 worden de interpretaties van koolstof-13, proton, deuteron en stikstof-15 NMR spektra van malonomicine besproken. Een juiste en volledige interpretatie is noodzakelijk om tot eenduidige resultaten bij biosynthetisch onderzoek te komen.

De resultaten van experimenten met eenvoudige bouwstenen worden beschreven in hoofdstuk 4. Hieruit kon worden gekonkludeerd dat malonomicine wordt opgebouwd uit azijnzuur, barnsteenzuur, CO_2, ammonia en serine (waarvan diaminopropionzuur afkomstig is); aminomalonzuur en oxomalonzuur bleken niet in malonomicine te worden ingebouwd.

In hoofdstuk 5 worden experimenten beschreven om de volgorde van opbouw en het mechanisme van de omzetting van deze eenvoudige bouwstenen tot malonomicine te bepalen. De biosynthese begint waarschijnlijk met de omzetting van serine naar diaminopropionzuur. Aangetoond werd dat deze omzetting waarschijnlijk verloopt via afsplitsing van water en additie van ammonia. Hierdoor verdwijnt het α-proton van serine. Echter, ook het α-proton van diaminopropionzuur wordt niet teruggevonden in malonomicine. Mogelijk wordt dit proton vervangen bij een van de volgreacties, b.v. de ringsluiting.
De volgorde van opbouw verloopt waarschijnlijk volgens schema A. Niet alle stappen konden met zekerheid worden vastgesteld, maar met name het optreden van het eerste ringgesloten intermediair (3) kon worden bewezen. Het blijkt dat na de ringsluiting het proton op C-4 behouden blijft wat een ondersteuning is voor de gegeven verklaring.
Welke intermediairen na (3) optreden kon niet worden



schema A



schema B

vastgesteld, omdat (4), (5) en (6) niet werden ingebouwd. Een mogelijke oorzaak zou kunnen zijn dat de inbouw onder de detectiegrens ligt, of dat de aangeboden bouwstenen geen echte intermediairen zijn. Een lage inbouw zou verklaard kunnen worden door aan te nemen dat deze bouwstenen het cytoplasma membraan niet of zeer moeilijk kunnen passeren (voor (2) en (3) wordt ook een lage inbouw gevonden).

Om deze verklaring te kunnen testen worden in hoofdstuk 6 enkele methoden beschreven om ook de laatste stappen in de opbouw van malonomicine te kunnen bestuderen. De resultaten van een aantal voorlopige experimenten toonde aan dat het gebruik van cel-vrije extracten van Streptomyces rimosus hierbij een belangrijke rol kan vervullen.

VRIJE UNIVERSITEIT TE AMSTERDAM

BIOSYNTHESIS OF MALONOMICIN

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van
doctor in de wiskunde en natuurwetenschappen
aan de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
dr. H. Verheul,
hoogleraar in de faculteit der wiskunde en natuurwetenschappen,
in het openbaar te verdedigen
op vrijdag 2 mei 1980 te 13.30 uur
in het hoofdgebouw der universiteit,
De Boelelaan 1105

door

DIRK SCHIPPER

geboren te Oldenzaal

Rodopi

AMSTERDAM 1980

Promotor : Prof dr. F. Bickelhaupt
Copromotor : Dr. J.L. van der Baan

STELLINGEN

1. De toekenning van het ^{13}C spectrum van ostreogrycine A is behalve onvolledig, ook voor verscheidene resonanties onjuist.
   B.W. Bycroft, J. Chem. Soc. Perkin I, 1977, 2464.

2. Het gebruik van massaspectrometrie als enige methode voor de structuurbepaling van de vetzure zijketen van tunicamycine leidt tot onbetrouwbare conclusies.
   A. Takatsuki, K. Kawemura, M. Okina, Y. Kodama, T. Ito en G. Tamura, Agr. Biol. Chem., 1977, *41*, 2307.

3. Door onjuiste vooronderstellingen en waarschijnlijk door experimentele fouten komen Fazakerley en Reid tot een onjuiste structuur voor het Cu(II)-bleomycine A_2 complex.
   G.V. Fazakerley en D.G. Reid, S. Afr. J. Chem., 1979, *32*, 89.

4. Door inconsequent gebruik van NMR parameters bij het onderzoek naar de biosynthese van formycrine is de betrouwbaarheid van de resultaten gering.
   K. Ochi, S. Yashima, Y. Eguchi en K. Matsushita, J. Biol. Chem., 1979, *254*, 8819.

5. De door Dabrowiak et al. gegeven verklaring voor de sterke lijnverbreding die wordt waargenomen in de ^{13}C spectra van Fe-bleomycine A_2 complexen is onjuist.
   J.C. Dabrowiak, F.T. Greenaway, F.S. Santillo en S.T. Crooke, Biochem. Biophys. Res. Comm., 1979, *91*, 721.

6. Het verschil tussen artsen en verloskundigen voor wat betreft het honorarium voor gelijke verrichtingen dient te komen vervallen.

7. Uit het oogpunt van energiebesparing dienen de reiskostenaftrek- en verhuiskostenregeling herzien te worden.

8. Wetenschappelijke ketterijen kunnen zeer waardevol zijn voor de ontwikkeling van de wetenschap en dienen niet verworpen te worden omdat ze niet in overeenstemming zijn met het gangbare wereldbeeld.

9. Niet alleen het posttarief, maar ook de postbezorging is aan ernstige inflatie onderhevig.