CROATICA CHEMICA ACTA CCACAA 58 (4) 537—546 (1985)

CCA-1603

YU ISSN 0011-1643
UDC 547.965
Original Scientific Paper

Biosynthesis of the Cytochalasans. The Mode of Incorporation of L-Methionine into the Cytochalasins A and B, Protophomin and Proxiphomin*

Ronald Wyss and Christoph Tamm

Institut für Organische Chemie der Universität, St. Johannsring 19, CH-4056 Basel, Switzerland

Received April 8, 1985

Incorporation of [^{13}C, $^{2}H_3$—CH_3]-L-methionine into cytochalasin A (I), cytochalasin B (II), proxiphomin (III) and protophomin (IV) by *Phoma exiqua var. exiqua* demonstrated that the two C-methylations occur with retention of all three H-atoms of the methyl group. Conclusions are drawn concerning the stage of the methylation reaction during the biogenetic pathway. On the basis of the results a more detailed general scheme of the cytochalasan biogenesis is proposed.

INTRODUCTION

The cytochalasans are a still growing family of microbial metabolites which exhibit a variety of interesting biological activities[1]. They have become important as tools in cell biology. Previous biosynthetic studies have clearly demonstrated that the building blocks of the cytochalasan skeleton are 8 to 9 units of acetate/malonate, several C_1-units originating from methionine, and an α-amino acid, such as phenylalanine, tryptophan or leucine[2–4]. On the basis of these results a general scheme of the cytochalasan biogenesis has been proposed[1,2]. Key steps are the formation of a polyketide, followed by combination with the amino acid, C-methylations and the closing of the rings. However, the exact sequence of these reactions has not yet been established. Some steps are interchangeable. In order to clarify these points additional experimental data are required. In this communication evidence is presented concerning the mechanism of the C-methylation by L-methionine. The results obtained also lead to an answer to the question in which stage of the biosynthesis the C_1-units are being introduced.

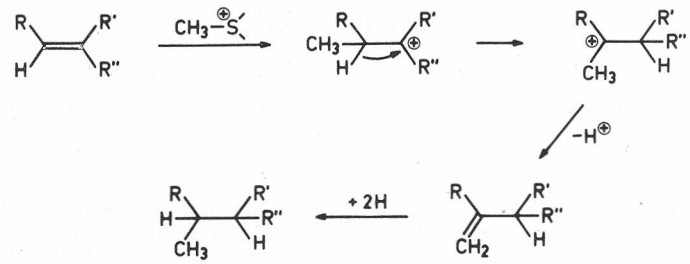
INCORPORATION OF L-METHIONINE

Biological transfers of methyl groups are frequent in nature. One of the known pathway makes use of S-adenosyl methionine which is formed from methionine. The methyl group is transferred as CH_3^+ from the sulfonium ion to a nucleophilic centre[5]. As shown by Lederer et al.[6], the reaction can proceed via two different mechanisms.

* Dedicated to Professor Mihailo Lj. Mihailović at the occasion of his 60th anniversary.

(1) The CD_2-Mechanism

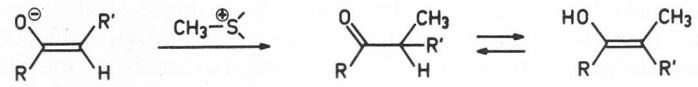
One of the three hydrogen atoms of the methyl group is lost in the course of the transfer as demonstrated by the use of [^{13}C, $^2H_3$—CH_3]-L-methionine as precursor for incorporation experiments. The transfer of the methyl cation is followed by a 1,2-hydride shift and subsequent stabilization of the resulting carbenium ion by abstraction of a proton (Scheme I). Final hydrogenation of the olefinic double fond restores the methyl group. This mechanism is in operation if the substrate to be methylated contains a weak nucleophilic C, C double bond.



Scheme I

(2) CD_3-Mechanism

Addition of the methyl group to a strong nucleophilic double bond, e. g. to an enolate, occurs with retention of all three hydrogen atoms of the methyl group (Scheme II).

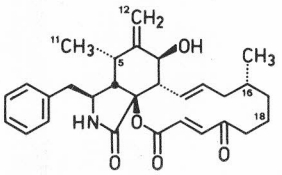


Scheme II

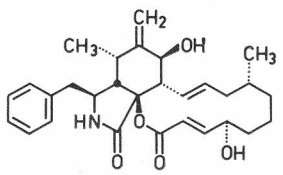
Many studies have been reported concerning the problem of the stereochemistry of the biological C-methylation[7,8] and of the possible pathways[9,10,11]. In solution, sulfonium salts are weak electrophiles. Therefore, Akhtar and Jones[12] have postulated a charge separation process to be the driving force of the enzymatic activation of S-adenosyl methionine.

A distinction between the two mechanisms is possible by ^{13}C-NMR spectroscopy after incorporation of the $^{13}C/^2H$-doubly-labelled precursor and measurement of the spectra of the isolated secondary metabolites[13]. The ^2H--atoms directly bound to ^{13}C are easily detected indirectly in ^1H-decoupled ^{13}C-NMR spectra. We have therefore administered [^{13}C, $^2H_3$—CH_3]-L-methionine (isotopic purity 91.5% C, 98% ^2H) to growing cultures of *Phoma exigua var. exigua*. The precursor was prepared using L-S-Benzyl homocysteine by known procedures[14,15]. The Figure shows the ^1H-decoupled ^{13}C-NMR-spectrum of the resulting cytochalasin B (II) after addition of 300 mg/Liter of [^{13}C, $^2H_3$—CH_3]--L-methionine. Next to the natural signals of C(18) at 20.0 ppm and of the methyl group at C(16) at 20.2 ppm, a septet appears which is shifted to a higher field by ca 0.93 ppm. The intensities of the lines approximately cor-

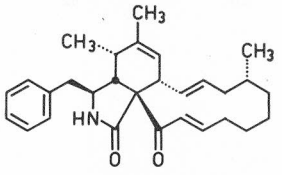
respond to the expected ratio of 1 : 3 : 6 : 7 : 6 : 3 : 1 with a coupling constant of ^1J (^{13}C, ^{2}H) = 19 Hz. A quintet for $^{13}C\,^2H_2\,^1H$-containing molecules cannot be unambiguously detected.* The appearence of C(12) is more complex. Next to the natural signal of CH_2 at 112.1 ppm a peak is found which is shifted to a higher field by 0.49 ppm. It is very likely the centre of a quintet with an intensity ratio of 1 : 2 : 3 : 2 : 1. The coupling constant ^1J (^{13}C, ^{2}H) = 24 Hz is as expected. In addition, a 1 : 1 : 1 triplet ($^{13}C\,^2H\,^1H$) cannot be excluded. It could explain the dominant signal at 111.0 ppm.



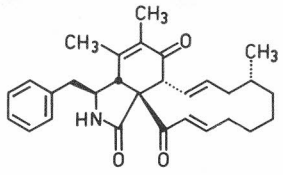
I Cytochalasin A



II Cytochalasin B



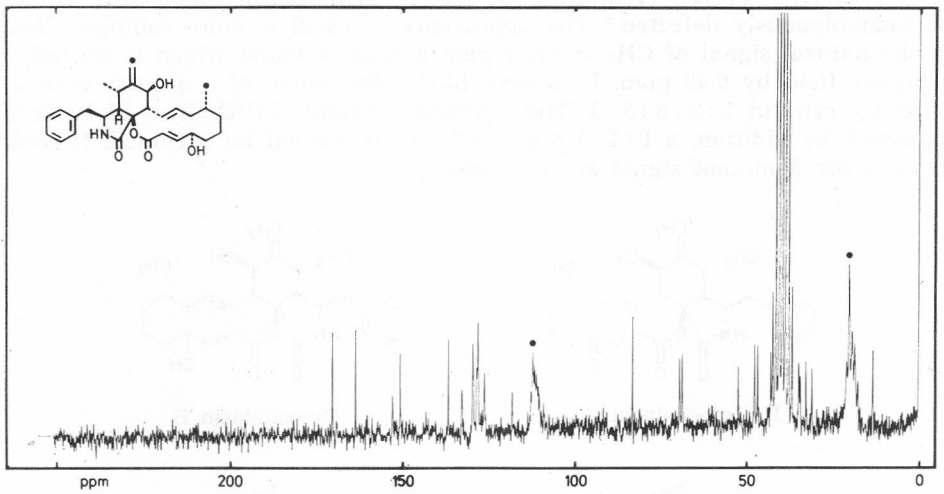
III Proxiphomin



IV Protophomin

It is also possible that both H-atoms at C(12), which are chemically not equivalent, give rise to a multiplet which is not of first order in the ^{13}C-NMR spectrum[16]. However, in the off-resonance ^{13}C-NMR-spectrum of II, C(12) appears only as a triplet (J_R = 50 Hz) instead of the expected double doublet. This was even the case when pyridin-d_5 was used as solvent instead of dimethylsulfoxide-d_6. A 100 MHz-^{13}C-NMR spectrum confirmed these findings. A ^{1}H-decoupled 30 MHz-^{2}H-NMR-spectrum gave a doublet for the methyl group at C(16). It confirms the value of ^1J (^{13}C, ^{2}H) = 10 Hz. The mass spectrum of the enriched sample of cytochalasin B (II) shows clearly that intact incorporation of the methyl group at C(16) had taken place and that C(12) contains 2 deuterium atoms. Because the intensities of the isotope peaks of the molecular ion (*m/z* 479) were weak, the peaks at *m/z* 461 (M^+—H_2O), 388 (M^+—91) and 370 (M^+—H_2O—91) were subjected to further investigation. All these peaks showed a significant enhancement by +3 ($^{13}C\,^2H_2$ and $^{12}C\,^2H_3$) and +4 ($^{13}C\,^2H_3$) mass units, which are not present in the mass spectra of the natural compound. By comparison with the corresponding intensities in the mass spectrum of natural cytochalasin B (III), the percentage of the molecules with additional labels can be estimated on the basis of the peak *m/z* 370 (Table I). The relative contribution of molecules with three and four additional labels are

* According to the content of 91.5% ^{13}C and 98% ^{2}H of the methyl group of [^{13}C, $^2H_3$—CH_3]-L-methionine only 86% are present as $^{13}C^2H_3$. However, 8.3% account for $^{12}C^2H_3$ and 5.3% for $^{13}C^2H_2{}^1H$.



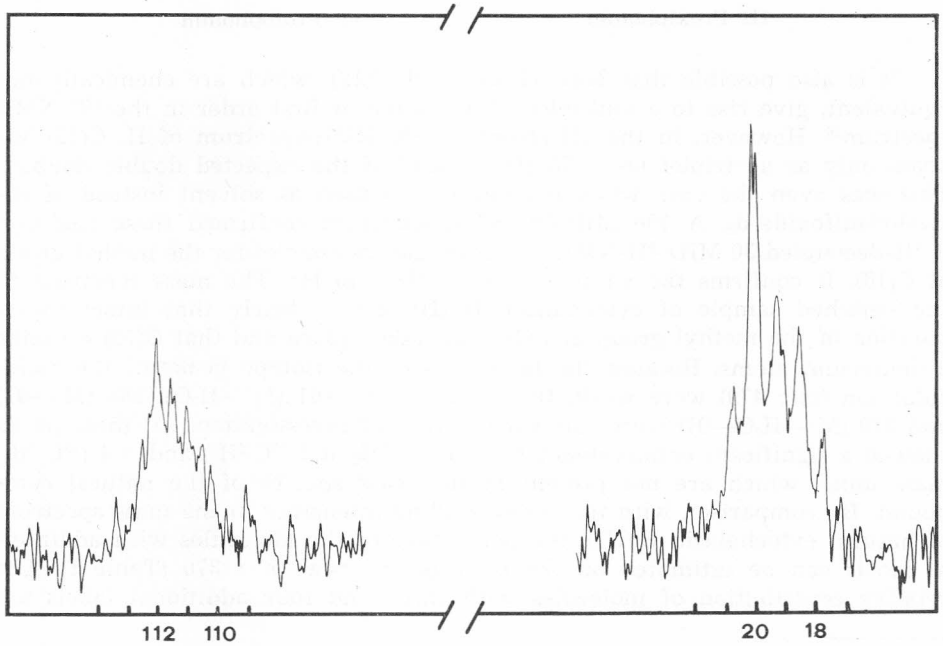


Figure. Noise decoupled ^{13}C-NMR-Spectrum of Cytochalasin B (II) after Incorporation of [^{13}C, $^{2}H_3$—CH_3]-L-Methionine, in DMSO-d_6.

of the same order, also in consideration of the ratios listed for the precursor*. These values permit the calculation of a specific incorporation rate of 0.3%. The results of the incorporation experiment clearly demonstrate that the methyl transfer proceeds via the »CD_3-mechanism«.

TABLE I

Percentage of Additionally Labelled Molecules of Cytochalasin B (II)

Additional Labelling	I_{370}	I_{371}	I_{372}	I_{373}	I_{374}	Percent Abundance
0	100	22	10	0	0	74.2
1		4	1	0.5	0	3.1
2			3	0.5	0	0.2
3				16	4	11.2
4					17	9.5
	100	26	14	17	21	100.0

In addition to cytochalasin B (II) the minor metabolites cytochalasin A (I), protophomin (III) and proxiphomin (IV) were isolated from the culture broth of the same incorporation experiment. For cytochalasin A (I) the same results are to be expected as for cytochalasin B (II). The 100 MHz-^{13}C-NMR--spectrum of I contains, next to the natural signals of C(18) and CH_3—C(16), a septet which is shifted to a higher field by 0.94 ppm. The ratio of the intensities is again approximately 1 : 3 : 6 : 7 : 6 : 3 : 1; ^1J (^{13}C ^{2}H) = 19 Hz). The pattern of the C(12) signal is the same as observed for cytochalasin B (II) in the high resolution spectrum. The mass spectrum shows isotope peaks of +3 and +4 at the peaks *m/z* 459 (M^+—H_2O and 386 (M^+—91). It therefore can be concluded that also in the case of cytochalasin A (I) the »CD_3-mechanism« operates for the methyl transfer.

In this connection the situation of protophomin (III) and proxiphomin (IV) is of special interest, because it is very likely that these minor metabolites are biogenetic precursors of the cytochalasin A (I) and B (II). Both precursors contain a methyl instead of the methylidene group. In addition, it should be possible to conclude at which stage of the biosynthetic pathway the methylation reaction takes place from its mechanism. Because III and IV are produced only in minute amounts only the mass spectra could be measured. Besides the isotope peaks of the molecular ions (*m/z* 445 for III and 431 for IV respectively), again the fragments M^+—91 (*m/z* 354 and 340 respectively) were compared with those of the spectra of the natural compounds. The isotope peaks at + 4 are 75—87% and at + 8, 25—60% of the main peaks. By comparing these intensities on the basis of fragment *m/z* 354 with those of naturally occurring III, the percentage of molecules having additional labels can be estimated (Table II). The specific incorporation rate for proxiphomin (III) resulting from these data amounts to 1.16%. This value is significantly higher than that calculated for cytochalasin B (II). This difference can be interpreted by the fact that the administered [^{13}C $^2H_3$—CH_3]-L-methionine is less diluted at the beginning of the incorporation and protophomin as a bio-

* See footnote on page 539.

TABLE II

Estimation of the Percentage of Additionally Labelled Molecules in Protophomin (III)

Additional Label	I_{354}	I_{355}	I_{356}	I_{357}	I_{358}	I_{359}	I_{360}	I_{361}	I_{362}	Per Abundance
0	100	22	5	0	0	0	0	0	0	38.1
1		6	1	0	0	0	0	0	0	2.1
2			15	3	1	0	0	0	0	5.7
3				5	1	0	0	0	0	1.8
4					76	17	4	0	0	29.2
5						1	0	0	0	0.3
6							11	2	1	4.2
7								12	3	4.5
8									47	14.0
	100	28	21	8	78	18	15	14	51	100

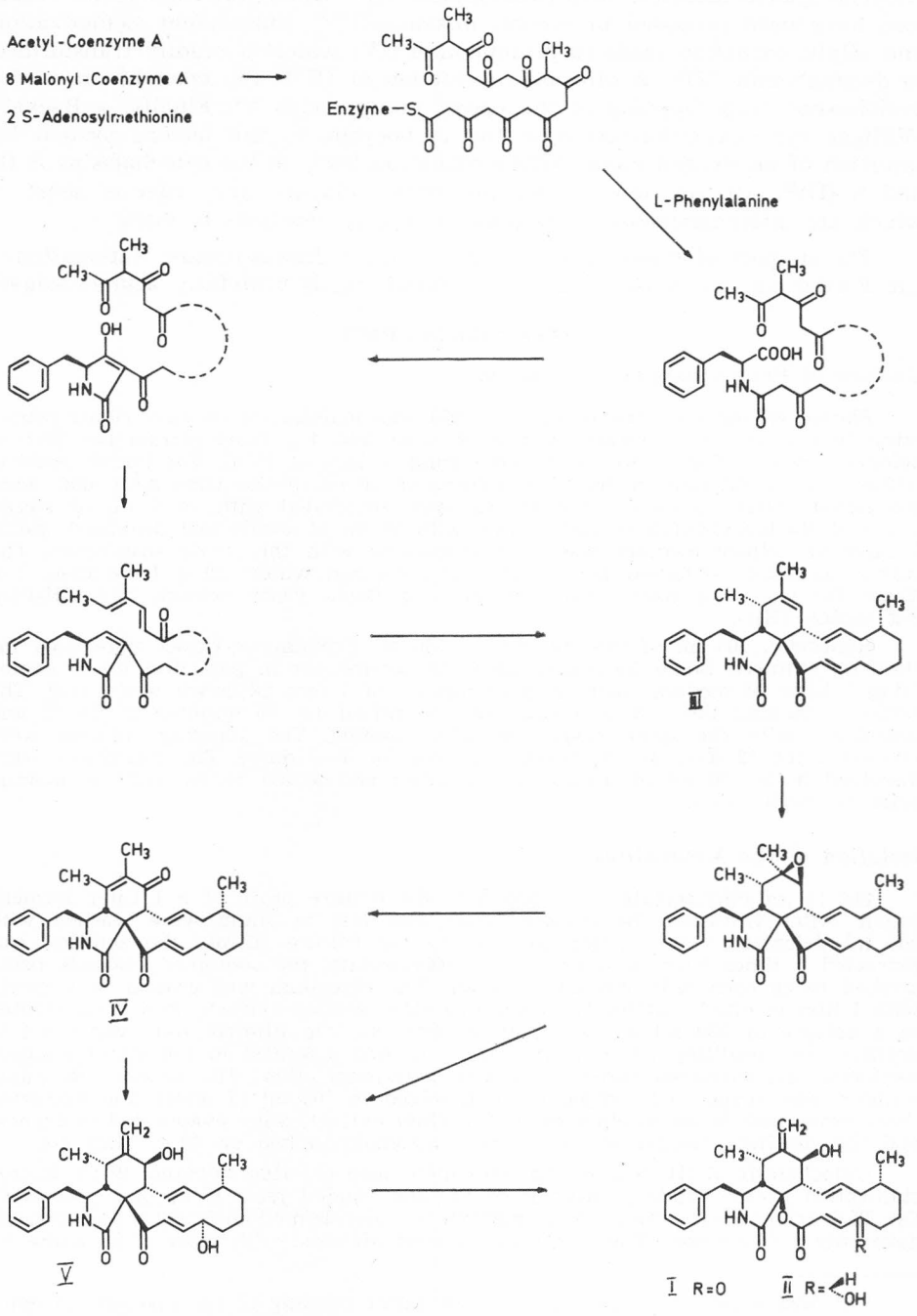
genetic intermediate of cytochalasin B (II) is secreted at a higher rate. Later, when the dilution of the precursor is growing, the transformation of III into II is enhanced.

The findings that the biological methylation of the cytochalasins A (I) and B (II), as well as of protophomin (III) and proxiphomin (IV), occur with retention of all deuterium atoms agree with the assumption that the two C-methylations take place at an early stage of the biosynthesis, when S-adenosylmethionine can react with the enolic double bonds of the polyketide chain. These results are also in agreement with those found for the methylation of chaetoglobosin A and 19-O-acetylchaetoglobosin A[4]. Several other cases of non-aromatic systems are known which demonstrate that the methylation takes place before the polyketide chain is stabilized[17].

General Biogenetic Scheme for the Cytochalasans

On the basis of these results a more detailed general biogenetic scheme for the cytochalasans is proposed. It is outlined for cytochalasin B (II) (Scheme III). It is also appliable to other cytochalasans by varying the length of the polyketide, the number of C-methylations and the amino acid. However, not all the postulated intermediates and only part of the reaction sequence are based on experimental proof.

Initially, acetyl-coenzyme A acts as starter unit. It is condensed in successive reactions with eight malonyl-coenzyme A units to form a C_{18}-polyketide. The C-methylations occur already at this stage, i. e. before stabilization of the polyketide; in any event before the formation of the perhydroisoindolinone system or its separation from the enzyme takes place, perhaps even during its formation. In the next steps the polyketide is combined with L-phenylalanine, probably forming first the amide linkage and subsequently closing the lactam ring to yield a tetramic acid derivative. For the formation of the isoindolone unit the tetramic acid derivative is transformed to a substituted pyrrolinone by reduction and dehydrations. The latter intermediate can under-


Acetyl-Coenzyme A
8 Malonyl-Coenzyme A
2 S-Adenosylmethionine
Enzyme—S
L-Phenylalanine
III
IV
V
I R=O
II R=

go an intramolecular electrocyclic reaction of the Diels-Alder type to form a tricyclic system identical with proxiphomin (III). Biological Diels-Alder reactions have been proposed in several instances[18,19,20]. Subsequent isomerization and allylic oxidation leads to protophomin (IV) which is readily transformed to deoxaphomin (V)[21]. A alternative reaction of III is the epoxidation of the cyclohexene ring. Opening of the epoxy group yields V*. Finally, a Baeyer-Villiger type oxidation converts the carbocyclic to the lactone system by insertion of an oxygen atom. Allylic oxidation leads to the cytochalasins A (I) and B (II)[22]. All the steps of the biogenetic sequence, the order of some of which are interchangeable, correspond to known reactions in vitro.

The support of these investigations by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung is gratefully acknowledged.

EXPERIMENTAL PART

Culture of Phoma exigua var. exigua

Phoma exigua var. exigua strain S 298 was maintained on agar slants containing 20 g Bacto malt extract, 20 g Bacto agar and 4 g yeast extract per liter of deionized water. They were incubated during 8 days at 18 °C. For larger fermentations 100 ml Erlenmeyer flasks containing ca 40 ml of the same malt agar were inoculated. After incubation the spores were suspended with ca 5 ml of sterile 0.01% of Na-laurylsulfonate and diluted with 50 ml of sterile and deionized water. 1 Liter of culture medium was than inoculated with this spore suspension. The culture medium contained per 1 Liter of deionized water: 20 g D-Glucose, 2 g Bacto Peptone, 2 g Bacto malt extract, 2 g Bacto yeast extract, 2 g KH_2PO_4, 2 g $MgSO_4 \cdot 7H_2O$.

Routinely, 100 ml of the medium in 500 ml Erlenmeyer flasks were used for standing cultures. Some fermentations were carried out in penicillin flasks containing 1 Liter of medium each. A pH-value of ca 5 (not adjusted) was found. The flasks containing the culture media were sterilized for 30 minutes at 120 °C and inoculated with the spore suspension after cooling. The standing cultures were incubated for 12 days at 18° (shake cultures for 4—5 days). The precursors were dissolved in 30—50 ml of demineralized water and added to the culture medium prior to sterilization.

Isolation of the Metabolites

800 ml of ethylacetate were added to the culture broth of a 1 Liter fermentation. After filtration the culture flasks and the mycelium were washed with 200 ml deionized water. After addition to the culture filtrate the solution was extracted 4 times each with 800 ml of ethylacetate, the combined extracts being washed twice each with 200 ml of water. The mycelium was ground in a mixer with 1 liter of ethyl acetate. The combined ethyl acetate extracts were concentrated to a volume of 500 ml in vacuo, dried with Na_2SO_4, filtered and evaporated to dryness. The resulting crude extract (ca 1 g) was dissolved in 100 ml of aqueous methanol and extracted three times with petroleum ether. The aqueous methanol solution was evaporated completely, redissolved in 100 ml of water and extracted three times with 50 ml of ether each. The ether extracts were evaporated to dryness and the resulting residue (ca 0.4 g) was chromatographed on 40 g silica gel.

Cytochalasin B (II) was eluted with methylene chloride/methanol 96 : 4. Recrystallization of the crude product from acetone yielded pure II of m. p. 218—220°. On TLC (chloroform/acetone 3 : 1, methylene chloride/methanol 95 : 5) the crystals gave only a single spot. The spectral data were identical with those of an authentic

---

* It was shown, that the oxygen atom (after opening of the epoxide) in cytochalasin B does not originate from acetate. It is therefore introduced at a later stage[22].

specimen. — Cytochalasin A (I) was found in the fractions which were eluted with methylene chloride containing 2 or 4% of methanol. I crystallized directly in the fractions obtained from larger fermentations. Sometimes purification was necessary by preparative TLC (chloroform/acetone 3 : 1 and methylene chloride/methanol 95 : 5) and subsequent recrystallization from methylene chloride/petroleum ether. M. p. 183—185 °C. The spectral data corresponded to those of authentic material. — Protophomin (IV) and proxiphomin (III) were eluted by methylene chloride containing 1 or 2% of methanol. These metabolites were isolated only from larger fermentations. Additional chromatography on silica gel was necessary. Whereas III was thus obtained in pure form, the fractions containing IV had to be subjected to further purification by preparative TLC (methylene chlorid/methanol 98 : 2 and benzene/methanol 95 : 5). The R_f-values in TLC and the mass spectra corresponded to the data of authentic specimens. — In the feeding experiments with [^{13}C, $^{2}H_3$—CH_3]-L-methionine the fractions which were eluted with methylene chloride/methanol 99 : 1 yielded, after subsequent precipitation with hexane and methylene chloride/hexane a mixture of III and IV which was used for the analysis by mass spectrometry.

Synthesis of [^{13}C, $^{2}H_3$—CH_3]-L-Methionine

Ca 0.5 g sodium was added to 1.58 g of L-S-benzylhomocysteine in ca 30 ml of liquid ammonia until the blue colour remained. Then 450 µl (1 g) [^{13}C, $^{2}H_3$—CH_3]--methyl iodide (91.4% ^{13}C, 98% ^{2}H) was added. After working up and recrystallization from ethanol 0.98 g [^{13}C, $^{2}H_3$—CH_3]-L-methionine of m. p. 79—80% and $[\alpha]_D^{20} = -4.5°$ ($c = 0.96$ in H_2O) was obtained. The product gave a single spot in TLC with the same R_f-value as natural L-methionine. (Solvent: butanol/acetic acid/water 8 : 2 : 2). ^{1}H-NMR spectrum (D_2O): signals at 3.8 (m, 1 H, H—C(2)); 2.5—2.7 (m, 2 H, H_2—C(4)); 1.95—2.2 (m, 2 H, H_2—C(3)). Mass spectrum: peaks at *m/z* 153 (M^+), 135, 108, 101, 91, 88, 83, 79, 74, 65, 56, 28.

REFERENCES

1. *Cytochalasins, Biochemical and Cell Biological Aspects,* S. W. Tanenbaum (Ed.), Elsevier/North Holland Press, Amsterdam 1978.
2. Ch. Tamm in P. S. Steyn (Ed.), *The Biosynthesis of Mycotoxins,* Academic Press, New York, London, Toronto, Sydney, San Francisco, 1980.
3. R. Wyss, Ch. Tamm, and J. C. Vederas, *Helv. Chim. Acta* **63** (1980), 1538.
4. A. Probst, and Ch. Tamm, *Helv. Chim. Acta* **64** (1981), 2065.
5. L. W. Parks, *J. Am. Chem. Soc.* **80** (1958), 2023.
6. E. Lederer, *Quart. Rev. Chem. Soc.* **23** (1969), 453.
7. H. F. Hegazi, R. T. Borchardt, and R. L. Schowen, *J. Am. Chem. Soc.* **98** (1976), 3048.
8. L. Mascaro, R. Höhrhammer, S. Eisenstein, L. K. Sellers, K. Mascaro, and H. G. Floss, *J. Am. Chem. Soc.* **99** (1977), 273.
9. M. Imfeld, C. A. Townsend, and D. Arigoni, *J. Chem. Soc. Chem. Commun.* (1976), 541.
10. A. Battersby, R. Hollenstein, E. McDonald, and D. C. Williams, *J. Chem. Soc. Chem. Commun.* 1976, 543.
11. A. I. Scott, M. Kajiwara, T. Takahashi, I. M. Armitage, P. Demon, and D. Petrocine, *J. Chem. Soc. Chem. Commun.* 1976, 544.
12. M. Akhtar, and C. Jones, *Tetrahedron* **34** (1978) 813.
13. M. J. Garson, and J. Staunton, *Chem. Soc. Rew.* **8** (1979) 539.
14. D. B. Melville, J. R. Rachele, and E. B. Keller, *J. Biol. Chem.* **169** (1947) 419.
15. P. Mohr, and Ch. Tamm, *Tetrahedron* **37** (1981) 201.
16. F. W. Wehrli and T. Nishida, *Progr. Chem. Org. Nat. Prod* **36** (1979) 1.
17. J. Barber, R. H. Carter, M. J. Garson, and J. Staunton, *J. Chem. Soc. Perkin I* 1981, 2577.
18. J. P. Kutney, T. Inaba, and D. L. Dreyer, *Tetrahedron* **26** (1970) 3171.
19. S. Seo, U. Sankawa, Y. Ogibara, Y. Iitaka, and S. Shibata, *Tetrahedron* **29** (1973) 3721.

20. A. C. Bazan, J. M. Edwards, and U. Weiss, *Tetrahedron* **34** (1978), 3005.
21. M. Binder and Ch. Tamm, *Helv. Chim. Acta* **56** (1973) 2287.
22. J. C. Vederas, T. T. Nakashima, and J. Diakur, *Planta Medica* **39** (1980) 201.
23. J.-L. Robert and Ch. Tamm, *Helv. Chim. Acta* **58** (1975) 2501.

IZVOD

Biosinteza citohalazana. Način inkorporacije L-metionina u citohalazin A i B, protofomin i proksifomin

Ronald Wyss i Christoph Tamm

Inkorporacijom [^{13}C, $^{2}H_3$-CH_3]-L-metionina u citohalazin A (I), citohalazin B (II), proksifomin (III) i protofomin (IV) pomoću *Phoma exiqua var. exiqua* određeno je da se oba C-metilovanja vrše sa retencijom sva tri H-atoma metil-grupe. Izvedeni su zaključci u pogledu faze odigravanja reakcije metilovanja u toku biogenetskog puta. Na osnovu dobivenih rezultata predložena je detaljnija opšta shema biogeneze citohalasana.