BIOSYNTHESIS OF MUCIDIN, AN ANTIFUNGAL ANTIBIOTIC FROM BASIDIOMYCETE *Oudemansiella mucida* ^{2}H-, ^{13}C- AND ^{14}C-LABELLING STUDY

František NERUD[a], Petr SEDMERA[a], Zdeňka ZOUCHOVÁ[a], Vladimír MUSÍLEK[a] and Miloslav VONDRÁČEK[b]

[a] *Institute of Microbiology, Czechoslovak Academy of Sciences 142 20 Prague 4 and*

[b] *Research Institute of Antibiotics and Biotransformations, 252 63 Roztoky near Prague*

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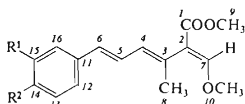
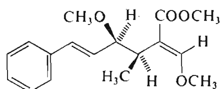
Incorporation of ^{2}H-, ^{13}C- and ^{14}C-labelled precursors into mucidin (*I*) was studied by mass and ^{13}C NMR spectroscopy and chemical degradations. Biosynthesis of *I* is connected with shikimate and acetate-malonate pathways. Methionine participates on the C- and O-methylation.

Antifungal antibiotic mucidin (*I*) belongs according to its structure to ω-arylhexatrienes[1-3]. It is identical to strobilurin A (refs.[4,5]) and related to strobilurin B (ref.[4]) (*II*) and oudemansin[6] (*III*). Compounds of this type were found in basidiomycetes *Strobilurus tenacellus*[4], *Oudemansiella mucida*[1,4], *Mycena zephira*[4], *Mycena fagetorum*[4], and in some species of *Cyphelopsis*[4]. This paper deals with the incorporation pattern of some precursors labelled both by radioactive and stable isotopes.



I, R¹ = R² = H

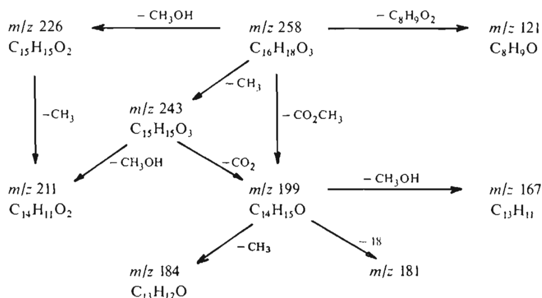
II, R¹ = CH₃O, R² = Cl



III

Ozonolysis of mucidin yields benzaldehyde, glyoxal, methyl formate and methyl 2,3-dioxobutanoate[5]. This reaction is therefore suitable mainly for the study of labelling of the benzene ring and the carbon atom next to it. It is not particularly good for the study of the isotope distribution in the side chain. In the mass spectroscopic fragmentation of mucidin (Scheme 1), the loss of $C_2H_3O_2$ from the molecular ion that gives rise to the *m/z* 199 ion is important. This loss corresponds to splitting off the $COOCH_3$ group. The following loss of CH_3OH leading to the ion *m/z* 176, cor-

responds to the loss of enolic methoxyl. Intense ions *m/z* 121 and 75, formed by a rearrangement process[4], can be used for the discussed purpose in the limited extent only. Among remaining fragments the ion *m/z* 77 corresponds to the phenyl group and the *m/z* 91 ion (tropylium ion) contains besides the atoms of this group also the neighbour atom of the side chain. Thus, both those fragments can be used in the case of nonspecific labelling of the aromatic ring. Studying the biogenesis, the assignment of ^{13}C NMR spectrum is of primary importance for the nuclear magnetic resonance represents a non-destructive method of following the fate of the labelled atoms.



SCHEME 1

Starting point for the assignment of all protonated carbon atoms is the ^1H NMR spectrum[5]. The assignment of proton signals follows from the chemical shifts, magnitudes of coupling constants and double resonance experiments (100 MHz, $CDCl_3$, 25°C): 1·97 d (J = 1·2 Hz, 3 H, $H_{(8)}$); 3·69 s (3 H, $COOCH_3$, see below); 3·74 s (3 H, $C_{(7)}$—OCH_3); 6·23 dqd (J = 9·4 Hz, 1·2 Hz and 0·8 Hz, $H_{(4)}$); 6·44 dd (J = 15·0 and 0·8 Hz, $H_{(6)}$); 6·67 dd (J = 15·0 and 9·4 Hz, $H_{(5)}$); 7·10 – 7·36 mt (5 H, phenyl); 7·40 s ($H_{(7)}$). Except for both methoxyl singlets, this assignment is unambiguous. The signals of $C_{(4)}$, $C_{(7)}$, $C_{(8)}$, $C_{(9)}$ and $C_{(10)}$ were assigned by selective heteronuclear decoupling. By the same method, the phenyl group methines were differentiated from the olefinic methines resonating in the same region. The signals of *ortho*- and *meta*-phenyl carbons are of double intensity; they can be distinguished using their chemical shifts. The remaining olefinic methines were assigned using off-resonance experiments[9]. This procedure was also used to verify the assignment of all protonated carbon atoms. The ester methoxyl that has no protons in its immediate vicinity, exhibits in the proton-coupled ^{13}C NMR spectrum (Table I) a neat quartet at 51·1 ppm. On the contrary, the signal of enol ether methoxyl resonating at 61·4 ppm is further split to a doublet as a result of its coupling to $H_{(7)}$. The direct couplings between the

signals δ_H 3·69 and δ_C 51·1 and δ_H 3·74 and δ_C 61·4 proved by selective decoupling (see above) requires interchange of the assignments in the proton spectrum made in the ref.[4]. This revision also changes the interpretation of the nuclear Overhauser effect among the low-field methoxyl and the one-proton singlet at 7·40 ppm; it is in fact an interaction of two geminal groups that cannot serve as an evidence for the *E*-configuration of the substituents on the corresponding double bond. The most downfield signal was assigned on the basis of its chemical shift to the ester carbonyl. The signal at 113·4 ppm which is enhanced upon irradiation of the phenyl protons was therefore assigned to $C_{(11)}$. Remaining signals of quaternary carbons at 131·0 and 110·3 ppm were assigned to carbons $C_{(3)}$ and $C_{(2)}$ on the basis of known effects of substituents on the chemical shifts and using the smaller width of the former multiplet in the proton-coupled ^{13}C NMR spectrum. The last mentioned spectrum also provides addi-

TABLE I
^{13}C NMR data of mucidin (*I*) and the results of experiments with ^{13}C-labelled precursors

Atom	δ_C	mult.[a]	J[b]	% of isotopic enrichment[c]		
				d	*e*	*f*
1	167·3	Sqd	4·9, 2·0	2·0	—	—
2	110·3	Smt		1·8	—	—
3	131·0	Smt		—	0·7	—
4	129·4	Dqd	[g], 5·5, 1·8	1·4	—	—
5	126·2	D[g]	[g]	—	0·7	—
6	130·7	Dmt		—	—	6·2
7	158·5	Dq	180·2, 4·9	—	0·9	—
8	23·3	Qd	127·0, 5·9	—	—	—
9	51·1	Q	146·5	—	—	—
10	61·4	Qd	145·5, 6·9	—	—	—
11	137·4	Smt		—	—	—
12,16	125·9[h]	D[g]	[g]	—	—	—
13,15	128·1[h]	D[g]	[g], 160·5	—	—	—
14	126·8	Dt	160·5, 7·3	—	—	—

[a] Upper case letters in the column for signal multiplicity denote ^1J, lower case ones mean ^2J and ^3J; s singlet, d doublet, t triplet, q quartet, mt multiplet; [b] magnitude of coupling constants in Hz, order according to the preceding column; [c] calculated from the signal intensity ratio in the spectra of labelled and unlabelled compound, I and I_0, measured under identical conditions (sample volume, concentration, FT experiment parameters) from the expression $E = 1·1\ (I/I_0 - 1)$; [d] after [1-^{13}C]acetate; [e] after [2-^{13}C]acetate; [f] after [1-^{13}C]benzoic acid; [g] not determined because of signal overlap; [h] double intensity.

tional arguments supporting the structure *I*. Quartet splitting of the $C_{(7)}$ signal and doublet splitting that of $C_{(10)}$ (Table I) are evidence for their geminal arrangement. The doublet splitting (2 Hz) of the ester carbonyl resonance is consistent with the mutual *cis*-arrangement of $H_{(7)}$ and $COOCH_3$ only. Therefore, the corresponding double bond has *E*-configuration. The splitting of olefinic methyl $C_{(8)}$, caused by $H_{(4)}$ is equal to 5·9 Hz; this is slightly more than the common value (3·5 – 5·5 Hz) for the *cis*-coupling in similar systems[10]. Since the *trans*-couplings are usually larger than the *cis*-couplings[11], the former possibility is more likely in our case.

Isotope Experiments

In preliminary experiments with ^{14}C-labelled precursors[7,8], we proved by autoradiography the incorporation of sodium formate, sodium [1-^{14}C]-acetate, sodium [2-^{14}C]acetate, [methyl-^{14}C]methionine, uniformly labelled cinnamic acid and phenylalanine and uniformly ring-labelled benzoic acid. Negative results were obtained with sodium [1-^{14}C]pyruvate, [1-^{14}C]mevalonic acid, [7-^{14}C]tryptophan and uniformly labelled valine. Incorporations of sodium [1-^{14}C]acetate, sodium [2-^{14}C]-acetate, DL-[Me-^{14}C]methionine, L[U-^{14}C]phenylalanine and [2-^{14}C] mevalonic acid found by scintillation method were 5·8, 6·9, 9·7, 10·9, 1·1 and 1·8%.

Methionine labelled by deuterium in the S-methyl group was added in four portions starting the sixth day of the fermentation. Mucidin, isolated after stopping the fermentation on the tenth day, was analyzed by mass spectrometry. Observed M + 9 peak is an evidence for the incorporation of three methyl groups. Also the peaks *m/z* 243 ($M-CH_3$), 226 ($M-CH_3OH$), 199 ($M-COOCH_3$) and 167 ($M-COOCH_3-CH_3OH$) are accompanied by +6 and +3 satellites. Following distribution of the individual species was calculated from the mass spectrum: 83·7% d_0, 5·8% d_3, 5·8% d_6, and 4·7% d_9. The decrease in intensity of the methoxyl and methyl signals in the ^1H NMR spectrum corresponding to an average incorporation of 8·1 ± 2% agrees well with those results. The retention of all three deuterium atoms shows that the methylation of a mucidin precursor undergoes probably by the S_N2 mechanism.

Large incorporation (10·9%) of [U-^{14}C]phenylalanine indicates a specific precursor. The activity found in benzaldehyde dinitrophenylhydrazone (77·8%) obtained by ozonolysis of mucidin is close to the theoretical value (78%) for the phenyl group and its vicinal carbon atom. Mucidin isolated from the cultivation to which DL-[$^2H_{11}$]phenylalanine was added, exhibited a M + 5 peak in its mass spectrum. Only one signal – 130·7 ppm, $C_{(6)}$, was enriched (6%) after incorporation of [1-^{13}C]-benzoic acid. Benzaldehyde dinitrophenylhydrazone from the experiments with sodium [1-^{14}C] and [2-^{14}C]acetates exhibited a residual activity only. The values of isotopic enrichment found by ^{13}C NMR are given in Table I. Every type of acetate labels three carbon atoms. The precursor labelled in carboxyl is incorporated more efficiently. Though according to our results $C_{(1)}$ and $C_{(2)}$ arise from the same source,

no $^{13}C—^{13}C$ coupling was observed. The results of all incorporation experiments are summarized in the Scheme 2. The formation of aromatic part of mucidin is connected

○ $CH_3\overset{*}{C}OONa$ △ $\overset{*}{C}H_3SCH_2CH(NH_2)COOH$
● $\overset{*}{C}H_3COONa$ □ $C_6H_5\overset{*}{C}OOH$
■ $[U]C_6H_5CH_2CH(NH_2)COOH$

SCHEME 2

with shikimate pathway; main evidences are specific incorporation of phenylalanine and benzoic acid. The side chain is built up by condensation of acetate units. The labelling of $C_{(1)}$ by sodium $[1\text{-}^{13}C]$ acetate is not clear. It is probable that the primary source of this carbon is some one-carbon precursor. Methyl groups are introduced by additional methylation by methionine. Further experiments will be directed to clarify the role of C-1 pool in the biosynthesis of mucidin, time consecutivity of C- and O-methylations and to the folding pattern of the side chain.

EXPERIMENTAL

Chemicals. All ^{14}C-labelled compounds were obtained from The Radiochemical Centre, Amersham, England. Sodium $[1\text{-}^{13}C]$ acetate and $[2\text{-}^{13}C]$ acetate (90% enrichment) were from Merck, Sharp and Dohme, Canada, $[1\text{-}^{13}C]$ benzoic acid (90%) was purchased from Prochem, England and DL-$[^2H_{11}]$phenylalanine (70%) was made by Chemie, Berlin, GDR. DL-$[C^2H_3]$-methionine was prepared from homocysteine through methylation by labelled bromomethane according to ref.[12].

Methods. Mass spectrum was measured on a Varian MAT 311 instrument (70 eV, ionizing current 1 mA, direct inlet at 150°C, ion source temperature 200°C). ^1H NMR spectra were measured on a Varian HA-100 spectrometer (100 MHz, CW mode, deuteriochloroform, tetramethylsilane as an internal standard, 25°C). Chemical shifts and coupling constants were read out by an electronic counter or from the calibrated expanded spectra (1 Hz/cm) with accuracy 0·001 ppm (0·1 Hz). ^{13}C NMR spectra were also measured at 25°C in deuteriochloroform containing tetramethylsilane as an internal standard. FT NMR spectrometer Jeol FX-60 was used. Chemical shifts and coupling constants were calculated from the digitally obtained address differences with accuracy 0·03 ppm (0·97 Hz). To attain the maximum accuracy of the % incorporation of ^{13}C-labelled precursors, the heights of corresponding peaks were compared in noise decoupled spectra of the labelled and unlabelled compounds measured with samples of the same volume and concentration under identical instrument settings. Radioactivity of mucidin was measured by the scintillation method on a Isocap 300, Liquid Scintillation System, Nuclear Chicago (U.S.A.) instrument, using a liquid scintillator SLT-42 (Spolana, Neratovice, Czechoslovakia). The activity

of benzaldehyde dinitrophenylhydrazone was measured by the method of infinitely thin layer on a Frieseke & Hoepner instrument, Erlangen, GFR (GM-tube, mica window, 1 mg/cm^2).

The experiments were carried out with the strain of basidiomycete ***Oudemansiella mucida*** (SCHRAEDER ex FR.) HÖHNEL maintained in the collection of fungi in the Institute of Microbiology, Czechoslovak Academy of Sciences, Prague. Cultivation was performed in 0·5 l flasks containing 80 ml of the synthetic medium[1] on a reciprocal shaker (1·9 Hz) at 23°C. ^{14}C-labelled precursors (740 Bq/flask) were added during the sixth day of cultivation. ^{2}H- and ^{13}C-labelled precursors (10 mg/flask) were added every 24 hours from the sixth to the ninth day of cultivation. After stopping the cultivation on the tenth day, mucidin was isolated according to the described procedure[3]. Benzaldehyde 2,4-dinitrophenylhydrazone, obtained after the reductive cleavage of ozonides, was isolated by thin-layer chromatography on silica gel (Kieselgel G, Merck, GFR) in the benzene–ethyl acetate 19 : 1 system.

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