PLEUROMUTILINS

FERMENTATION, STRUCTURE AND BIOSYNTHESIS

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(Received for publication August 11, 1975)

Derivatives of pleuromutilin, formed during the fermentation of pleuromutilin, were isolated and their structure determined. 14-Acetyl-mutilin and mutilin as well as different unsaturated fatty acid esters of pleuromutilin were identified. The proportion of each derivative formed depends to a considerable degree on the conditions of the fermentation process. The possible biosynthetic pathways are shown.

During screening and testing for new antibiotics in a culture of *Clitopilus passeckerianus* (PIL.) SING., we found a new substance active against penicillin- and streptomycin-resistant staphylococci. We isolated the compound and determined its chemical and microbiological characteristics. During the investigation it was shown that *Clitopilus passeckerianus* (PIL.) SING.[1] is synonymous with *Pleurotus passeckerianus* PIL. and the compound isolated by us is identical with pleuromutilin.

In 1951 pleuromutilin was isolated by KAVANAGH *et al.*[2] who reported on its chemical and biological properties. ANCHEL[3] reported extensively on the chemical properties of the compound. KAVANAGH *et al.*[4] recognized the identity of pleuromutilin with drosophilin B, which was isolated from *Drosophila subatrata*. SZYBALSKI[5] as well as ASHESHOV *et al.*[6] defined further biological properties of pleuromutilin.

The structure of pleuromutilin was determined by ARIGONI[7] and BIRCH *et al.*[8] The biosynthesis was elucidated by BIRCH *et al.*[9] and ARIGONI's group.[10,11,12] During further studies a number of derivatives of pleuromutilin with antibacterial activity was synthesized by RIEDL.[13]

EGGER and REINSHAGEN[14] showed that the specific derivation of the acyl-group of the molecule not only produced compounds with an extended spectrum of activity, but also increased its activity up to the hundredfold.

Pleuromutilin as well as a series of pleuromutilin derivatives were produced during the fermentation, some, however, were produced only in very small quantities. The present paper deals with the structure, properties and presumed biosynthesis of these compounds.

Materials and Methods

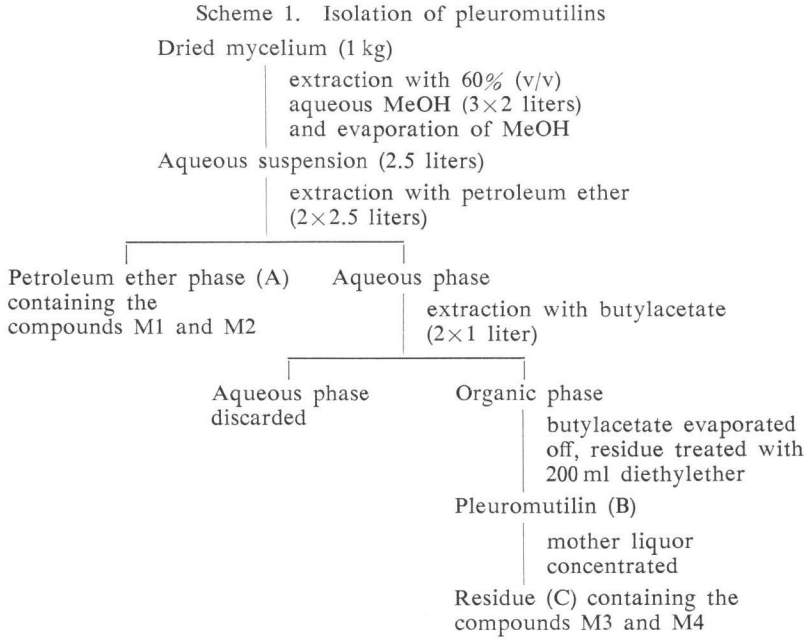
The antibiotic activity was determined by the agar diffusion method using *Sarcina lutea*. As a working standard a pleuromutilin preparation of high purity with an assumed microbiological activity of 1,000 μg/mg was used. For testing the content of pleuromutilin in the culture one aliquot of the broth was extracted with the same volume of ethylacetate for 15 minutes. The resulting ethylacetate extract was diluted (1:100) with sterile water and the antimicrobial activity was measured.

Fermentation: With a culture of *Clitopilus passeckerianus* NRRL 3100, grown on broken corn, shake-cultures were grown on the following culture medium (g/liter): autolysed brewer's yeast 75, glucose 30, dextrin 10, KH_2PO_4 4, $(NH_4)_2SO_4$ 5, pH 6.0. After cultivation for 72 hours

at 24°C the seed cultures were used as inoculum for a 100-liter fermenter. The composition of the medium was (g/liter): glucose 50, autolysed brewer's yeast 50, KH_2PO_4 50, $MgSO_4 \cdot 7H_2O$ 0.5, $Ca(NO_3)_2$ 0.5, NaCl 0.1, $FeSO_4 \cdot 7H_2O$ 0.5, pH 6.0. After 72 hours a 1,000-liter fermenter containing the same medium was inoculated with this seed culture. Foam was suppressed by the addition of sperm oil. After 6 days the dry-weight of mycelium was approximately 22 g/liter. The mycelium was separated from the whole broth by a vacuum rotary-filter, and after rinsing with water, dried at 40°C.

Isolation of pleuromutilins: Pleuromutilin and the compounds M1, M2, M3 and M4 were isolated from the dried mycelium as shown by Scheme 1.

Scheme 1. Isolation of pleuromutilins



Compounds M1 and M2: The petroleum ether phase (A) was concentrated *in vacuo* and the residue distributed between 100 ml methanol (containing 5 % of water) and 100 ml petroleum ether using a countercurrent distribution apparatus. After 10 cycles compound M2 was found in tubes 9 and 10, compound M1 in tubes 3~8. The content of tubes 3~8 were pooled and concentrated. For further purification, the residue was chromatographed on a silica-gel column (silica gel 0.063~0.2 mm, E. Merck) by developing and eluting with chloroform. Compound M1 was monitored by thin-layer chromatography (see Table 1). The corresponding eluate fractions were pooled and concentrated *in vacuo*. The residue was treated with diisopropyl ether and compound M1 precipitated as a white crystalline substance, m.p. 183°C. To purify compound M2 the petrol ether phases of tubes 9 and 10 were pooled and concentrated *in vacuo*. The residue was chromatographed as described for compound M1 and the corresponding fractions concentrated. Since on addition of diisopropyl ether no

Table 1. Comparative thin-layer chromatography of the compounds.

Substance	Rf		
	tlc+	tlc++	tlc+++
Pleuromutilin	0.34	0.30	0.48
Compound M1	0.47	0.45	0.54
Compound M2	0.56	0.55	—
Compound M3	0.44	0.40	0.54
Compound M4	—	—	0.20

+ Benzene-ethylacetate (1:1)
++ Toluene-ethylacetate-glacial acetic acid (50:50:1)
+++ Ethylacetate

precipitation was obtained. The resulting colorless oil was used for further investigations of compound M2.

Pleuromutilin (B): The crude antibiotic was dissolved in 20 ml of ethylacetate, treated with 2 % (w/v) of activated charcoal and concentrated *in vacuo*. After addition of 100 ml diethylether, the solution was chilled at +4°C. The precipitated crystalline antibiotic was separated, rinsed with cold diethylether and dried, m.p. 162~164°C.

Compounds M3 and M4: The residue (C) obtained from the mother liquor of the pleuromutilin-separation was chromatographed on a silica gel column (silica gel 0.063~0.2 mm, E. Merck) by developing with $CHCl_3$ and eluting with $CHCl_3$-EtOH (9:1). Separation of the compounds occured during elution and was monitored by thin-layer chromatography. The corresponding fractions of compound M3 were pooled. $CHCl_3$ and EtOH were removed by evaporation and the residue was recrystallized from ethylacetate, m.p. 192°C. The corresponding fractions of compound M4 were also pooled and further purified by repetitive silica gel column chromatography. After evaporating the eluents the residue was crystallized from diisopropylether. m.p. 115°C (decomposition).

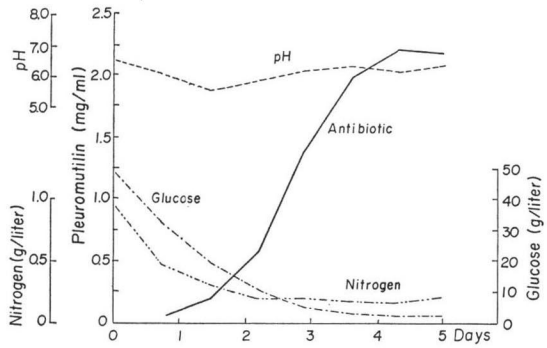
Thin-layer chromatography: The compounds were separated by thin-layer chromatography (tlc) on precoated silica gel 60-plates (E. Merck). The substances M1, M2, M3 and pleuromutilin were detected as greenish spot and compound M4 as a red spot by spraying with a reagent consisting of 0.1 % (w/v) $FeCl_3$, 1 % (v/v) CH_3COOH in concentrated sulfuric acid followed by heating for 3 minutes at 120°C (Table 1).

Results

Fermentation

The time-course of the fermentation in a 1,000-liter fermenter is shown in Fig. 1. The trophophase, *i.e.* the phase of active biomass synthesis, is completed within 48 hours. Antibiotic production begins toward the end of the trophophase and continues, though at diminishing rate during the idiophase. Since pleuromutilin is the main product, only the concentration of this antibiotic was measured during fermentation. At the end of fermentation the production of biomass was approximately 22 g/liter. The isolation and purification of pleuromutilin and compounds M1, M2, M3, M4 was started from dried mycelia.

Fig. 1. Production of antibiotics, residual sugar and nitrogen and pH change in fermentation of *Clitopilus passeck*. NRRL 3100.



Physico-chemical Properties and Structure

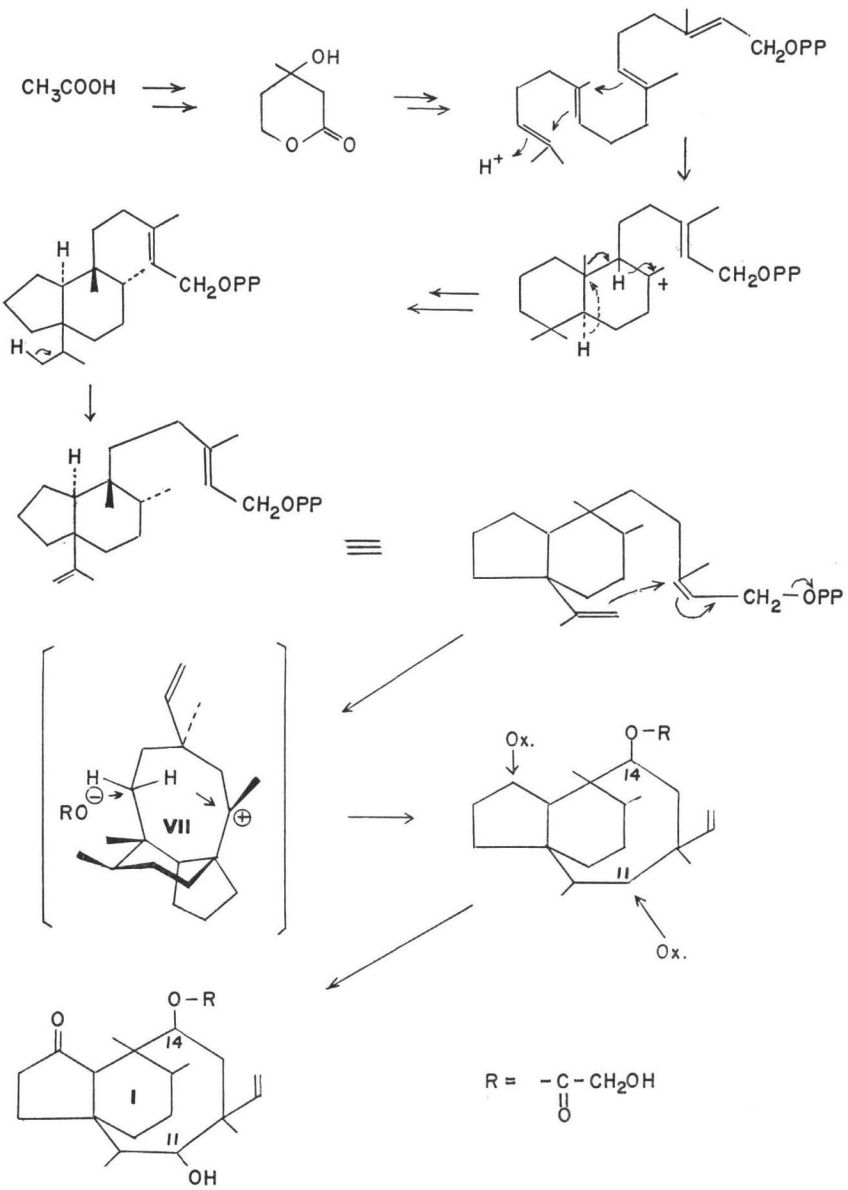
Pleuromutilin: As already mentioned pleuromutilin was isolated in 1951 by KAVANAGH *et al.*[2] The compound is a tricyclic diterpene having the molecular formula $C_{22}H_{34}O_5$ m.p. 167~168°C. The structure of the compound (**I**) was deduced by ARIGONI.[7] Alkaline hydrolysis of pleuromutilin leads to compound $C_{20}H_{32}O_4$, m.p. 192°C which was reported as mutilin (**II**) by ANCHEL *et al.*[2] and ARIGONI[7] (see Chart 2).

Compound M1: The compound was homogenous according to thin-layer chromatography. From elemental analysis an empirical formula of $C_{22}H_{34}O_4$ was calculated, m.p. 183°C. Mutilin (**II**) was formed by alkaline hydrolysis of the compound. By comparison of chromatographic

behavior and IR-spectra of 14-acetyl-mutilin, obtained by reduction of pleuromutilin,[18] with compound M1 identity of this compound with 14-acetyl-mutilin (**III**) could be established.

Compound M2: This metabolite was an oily liquid from which pleuromutilin was formed during mild alkaline hydrolysis. Transesterification of the compound M2 with methanol leads to a mixture of methylesters of fatty acids. By thin-layer chromatography using $AgNO_3$ impregnated silica gel plates[15] the esters were identified as the methylesters of oleic-, linoleic- and Δ^9 eicosenoic acids by comparison with reference substances. A quantitative separation of the methylesters by gas-chromatography yielded 40~45% (w/w) oleic acid, 45~50% (w/w) linoleic acid and 5% (w/w) Δ^9 eicosenoic acid methylester. Therefore it could be assumed that

Chart 1. Biosynthetic pathway of pleuromutilin.



compound M2 is a mixture of pleuromutilinesters (**IV**, **V** and **VI**) wherein the acyl-portions are long-chain fatty acids.

Compound M3: From elemental analysis an empirical formula of $C_{20}H_{32}O_3$ was calculated. Comparison of chromatographic behavior and IR-spectra of mutilin with compound M3 leads to the conclusion that compound M3 is identical with mutilin (**II**).

Compound M4: During isolation of pleuromutilins only traces of compound M4 were obtained. The material was homogenous according to thin-layer chromatography. By hydrolysis in weak alkaline media pleuromutilin (**I**) was produced. For further identification of the acyl-portion, not enough material was available.

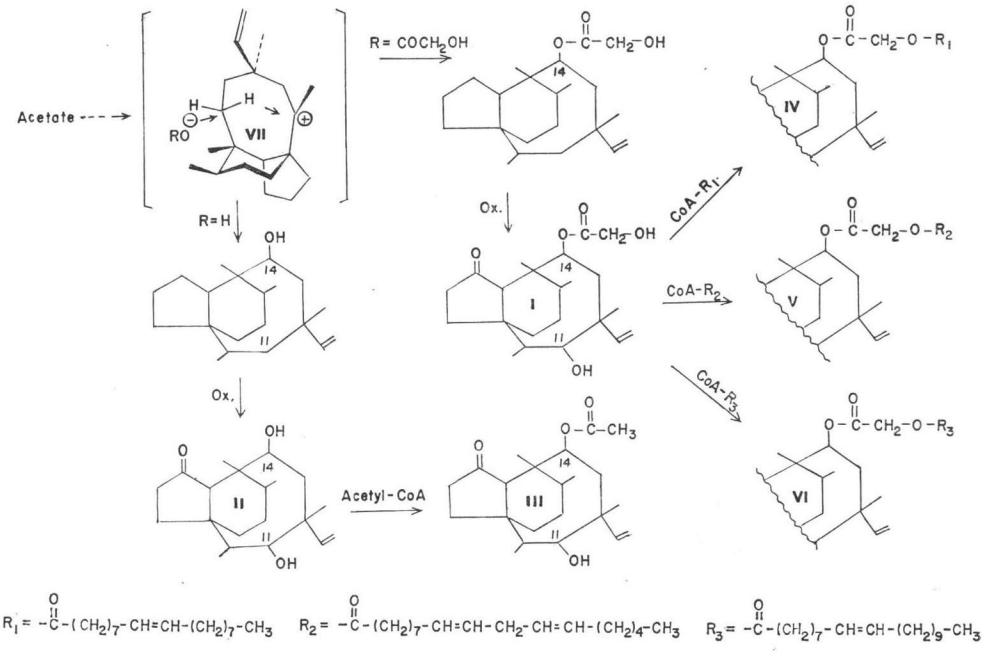
On average it was found that pleuromutilin was the main component formed during fermentation. The amounts of the compounds present calculated relative to pleuromutilin basis were: compound M1 2~5 %, M2 1~2 %, M3 0.5~1 % and M4<0.1 %. The composition depends on the fermentation medium as well as fermentation conditions.

Biosynthesis of Pleuromutilins

Pleuromutilin: The biosynthesis of pleuromutilin was studied by ARIGONI's group[10~12] and BIRCH.[8,9] The results of these investigations are shown in Chart 1. The postulated biosynthetic route to pleuromutilin (**I**) shows that during the last cyclisation step a transanular hydride displacement took place under formation of an intermediate cation (**VII**). This may occur by the attachment of a glycollate-ion as an external nucleophil. The C_2-unit originates from the glyoxylic-acid cycle.[11]

Compound M1 (**III**): The biosynthesis of 14-acetyl-mutilin (**III**) was stimulated strongly by addition of mutilin (**II**) together with sperm oil or corn oil as carbon source during fermentation. This observation leads to the presumption that compound M1 (**III**) originates from

Chart 2. Probable biosynthetic pathways of pleuromutilins.



acetylation of mutilin (**II**) as shown in Chart 2.

Compound M2: The compound consists of a mixture of various pleuromutilin esters (**IV**, **V**, **VI**). One can assume therefore that the biosynthesis results from acylation of the primary hydroxyl-group of pleuromutilin by CoA-activated acyl-portions (see Chart 2). The source of the long-chain acyl-portions may be derived from sperm oil. This hypothetical pathway was substantiated by the observation that Δ^9 eicosenoic acid is a component of sperm oil and was also detected in the acyl-portion in a pleuromutilin ester. The antibiotic-producing fungus *Clitopilus passeckerianus* is not able to synthesize Δ^9 eicosenoic acid.

Compound M3: The compound is identical with mutilin (**II**). Attempts at enzymatic cleavage of pleuromutilin by antibiotic-producing strains were not successful. Therefore we assume that the intermediate cation (**VII**) is stabilized by attaching a hydroxyl ion instead of a glycollate ion resulting in mutilin formation.

Discussion

It was demonstrated that during the fermentation of pleuromutilin, derivatives of this antibiotic were formed, the derivatives differing in the acyl-portions attached to the 14-OH-group of mutilin. Presumably these derivatives are endproducts of the secondary pathway of pleuromutilin.

Until now, no intermediate products carrying an acyl-portion in position 14, without a functional group in position 11 or position 3 were detected. Nevertheless further results for such compounds should make it possible to determine the sequence of oxidation after formation of the tricyclic ring-skeleton.

Acknowledgement

The authors wish to thank Miss G. ASCHABER for technical assistance.

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