IDENTIFICATION OF MYCOLUTEIN AND PULVOMYCIN AS AUREOTHIN AND LABILOMYCIN RESPECTIVELY

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Two antibiotics isolated from a culture MA-2465 were identified as aureothin and labilomycin, the chemical structures of which had been reported previously. It was also concluded that mycolutein and pulvomycin isolated as antibiotics in 1955 and 1957 respectively are also identical with aureothin and labilomycin. A cursory study of MA-2465 indicates that it is distinctly different from the culture which was first observed to produce labilomycin and is probably different from the organism first noted to produce aureothin.

In 1955 and 1957, two paper appeared on the isolation of the antibiotics mycolutein and pulvomycin from cultures of an identified species of streptomyces.[1,2] While some of the physical, chemical and microbiological properties of these antibiotics were reported, no chemical structure was proposed for either substance.

In the course of a search for new antibiotics elaborated by soil actinomycetes, a culture of an identified species designated by us as MA-2465 was found to produce two antibiotics which we established as mycolutein and pulvomycin. We should like to report our work with these compounds and their chemical identification.

Isolation and Purification

The antibiotic broth was obtained from the fermentation of MA-2465 in shake flasks containing a medium of 1.5% soya bean meal, 1.5% dextrose, 0.5% NaCl, 0.03% KH_2PO_4 and 0.1% $CaCO_3$ in tap water. The two antibiotics were present in the mycelium and were separated from it by extraction with methanol and concentration of the extracts to dryness under reduced pressure. The residue was extracted exhaustively with chloroform until no more microbial activity was found in the extracts. The combined chloroform extracts was concentrated to a small volume on a flash evaporator and a mixture of the crude antibiotics (established as a mixture of two antibiotics by TLC using silicic acid plates) was obtained as a precipitate on addition of excess petroleum ether. As the antibiotic mixture was contaminated by an oily,

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microbially inactive fraction, it was dissolved in ethylene glycol and the solution was extracted with petroleum ether. The ethylene glycol solution was then extracted with benzene whereupon most of the mycolutein and small amounts of pulvomycin appeared in the benzene layer. The major portion of pulvomycin was then removed from the ethylene glycol solution by extraction with ethyl acetate.

Final purification of both antibiotics was accomplished by silicic acid column chromatography using chloroform-methanol (20:1) as the eluting medium. We found that mycolutein eluted first, followed quickly by pulvomycin. The separation, however, was rather sharp. Based on the dry weights of the mycelium, the approximate yields of mycolutein was about 15 mg/g and of pulvomycin between 2~4 mg/g.

Structure of Mycolutein

The mycolutein obtained by us as yellow crystals from methanol recrystallization (m.p. 157~158°C) was identical with the product described in the SCHMITZ and WOODSIDE paper.[1] The identity of our material with mycolutein was established by comparing melting points, specific rotations, IR and UV spectra. Our material also showed antifungal activity in the dilution assay against *Candida albicans*, *Cryptococcus neoformans* and *Trichophyton rubrum* as was reported previously for mycolutein.

The IR spectrum of our mycolutein (Fig. 1) exhibited the typical absorptions of an aromatic nitro group at 1505 cm^{-1} and 1321 cm^{-1}, which belong to the antisymmetric and symmetric stretching vibrations of $-NO_2$ respectively.[3] The UV spectrum of the antibiotic in methanol exhibiting two peaks, one at 257 nm ($E^{1\%}_{1cm}$ 345), also suggested benzenoid and aromatic nitro group absorptions.[4] Accordingly, we compared the properties of our mycolutein with the antibiotics known to contain an aromatic nitro group such as chloramphenicol,[5] pyrrolnitrin,[6] azomycin[7] and aureothin.[8,9] While no mention was made as to whether aureothin had anti-fungal activity, its physical properties as reported by MAEDA[8] and by HIRATA *et al.*[9] corresponded to those reported for mycolutein[1] and to those observed in our laboratories (Table 1). In addition, the NMR spectrum we obtained with mycolutein (Fig. 2) indicated

Fig. 1. IR spectrum of mycolutein (KBr pellet)

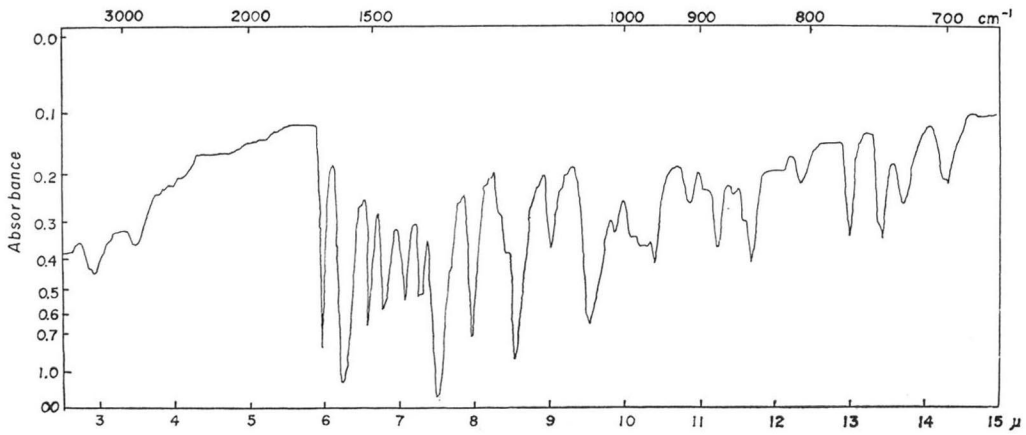


Fig. 2. 60 MHz NMR spectrum of mycolutein in $CDCl_3$

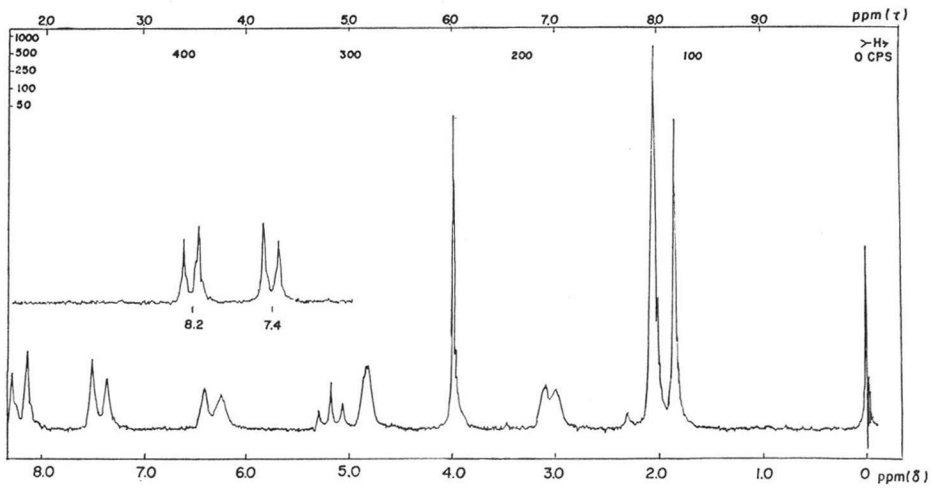


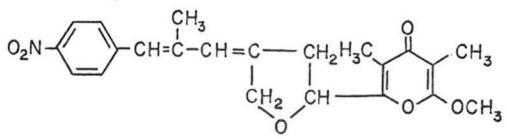
Table 1. Physical properties of mycolutein and aureothin

	Mycolutein	Aureothin[9]
Melting	157~158°C	158C°
$[\alpha]_D^{25}$	+53° ($CHCl_3$)	+51° ($CHCl_3$)
Empirical formula found	$C_{22}H_{23}NO_6$*	$C_{22}H_{23}NO_6$
UV spectrum (methanol)	257 nm ($E_{1cm}^{1\%}$ 680)	257 (log ε 4.39)
	345 nm ($E_{1cm}^{1\%}$ 345)	346 (log ε 4.27)

* Based on our analytical data (Found: C, 66.65; H, 5.89; N, 3.57) and mass spectrograph which showed a peak at *m/e* 397 attributable to the molecular ion.

that the integrated proton numbers and the assigned respective peaks completely coincided with the chemical structure of aureothin **I** as established by HIRATA *et al.*[9] in 1961.

The identity of our mycolutein with aureothin was confirmed further by heating a solution of it in ethanol containing concentrated hydrochloric acid (10:1). Desmethylisoaureothin, prepared previously by HIRATA from aureothin[9] was obtained from our reaction mixture. Its melting point (196°C) and specific rotation ($[\alpha]_D^{20}$+116°) (EtOH) agree with the published values.



I

Structure of Pulvomycin

The pulvomycin obtained by us after silicic acid chromatography appeared to be identical with the material described by ZIEF and coworkers as judged by IR spectra, UV spectra and microbial activity. Like the published material, our sample is readily soluble in chloroform, benzene, ethanol, acetone, ethylacetate and dioxan but insoluble in water, ethyl ether, carbon

tetrachloride, dilute hydrochloric acid and sodium carbonate solution. Paper chromatography studies carried out on our product with water-saturated butanol containing 2% *p*-toluene sulfonic acid for 6 hours at 25°C by descending techniques gave an Rf of 0.92 while under identical conditions oxytetracycline showed an Rf value of 0.58. These data (Rf values of 0.94 and 0.55 respectively) are essentially identical with the published data for pulvomycin. However, our product did not show a distinct melting point but rather sintered with decomposition over a range of 110~120°C in contrast to the fairly sharp m.p. 94~97°C reported for pulvomycin. Our data also differed from the published data in one other but major aspect, namely in elemental composition. Pulvomycin was reported to contain 1.86% nitrogen; we found none in our samples. We are inclined to believe that this difference is due to impurities present in the pulvomycin samples of ZEIF and coworkers since our samples initially contained small quantities of nitrogen which were removed by careful elution in the final column chromatography step.

We have now identified pulvomycin as labilomycin, a very labile antibiotic isolated in 1963 by H. UMEZAWA and co-workers,[10] the chemical structure of which was proposed as **II** in the

Fig. 3. IR spectrum of pulvomycin (KBr pellet)

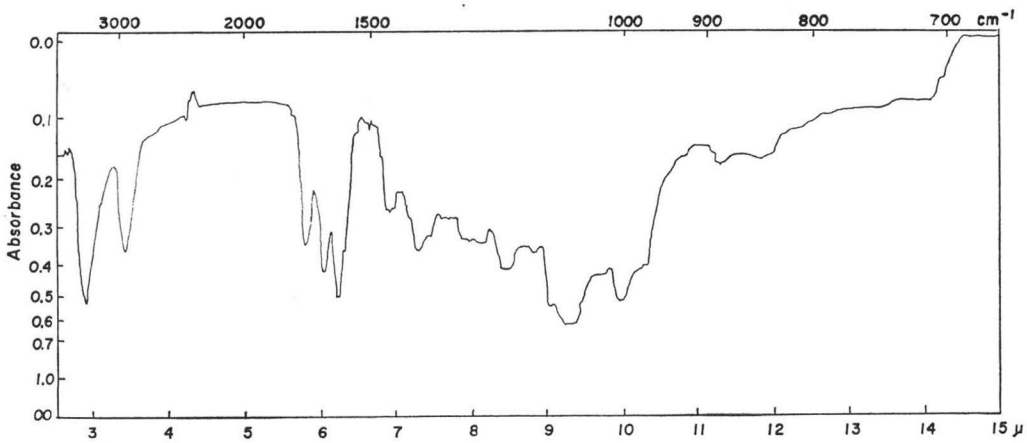
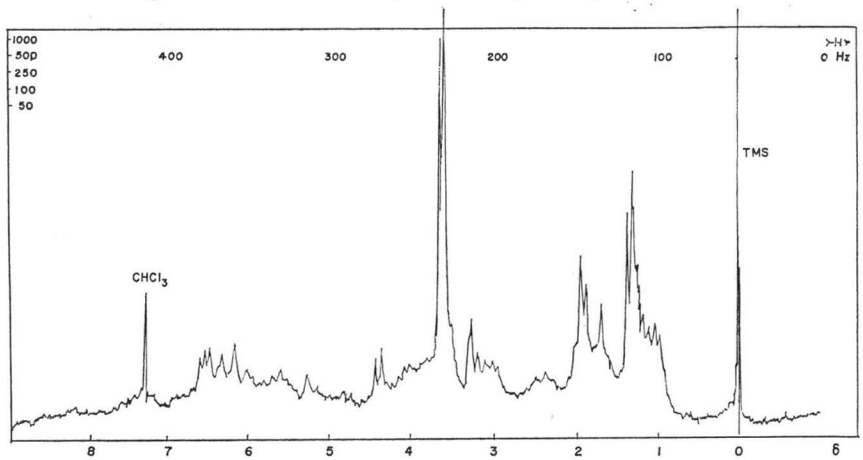


Fig. 4. 100 MHz NMR spectrum of pulvomycin in $CDCl_3$



following year.[11] The physical data obtained from our studies are in some instances almost identical with those reported for labilomycin. Some small differences in data were observed but considering the sensitive nature of labilomycin these differences are not unexpected. UV spectra in methanol revealed three distinct peaks at 265 nm, 276 nm and 320 nm. Our melting point renge with decomposition corresponds with the reported value of 112~120°C. The observed analytical figures and specific rotations for our samples were C, 64.7; H, 7.69 and $[\alpha]_D^{25}$ −188° (*c* 1, methanol); for labilomycin: C, 64.3, 63.4; H, 7.67, 7.61 and $[\alpha]_D^{20}$ −237° (*c* 1, methanol). The IR spectrum of our sample is shown in Fig. 3 and it closely resembles that of labilomycin. Our pulvomycin also resembles labilomycin with respect to its antibiotic activity against Gram-positive organisms and its inactivity against Gram-negatives. Like labilomycin, its microbial activity disappears when solutions are kept at pH 3.0 and at pH 10.0 for several hours. Pulvomycin possesses a marked degree of cytotoxicity against a variety of malignant tissue culture lines. The ED_{50} against L-cells has been found to be 250 ng/ml.[12]

Considering the lability of labilomycin and our product, it is not surprising that a good NMR spectrum (Fig. 4) was not obtained. Nevertheless, NMR indicated that both materials are identical or at the very least are very closely related. It is suspected that the minor differences in NMR spectra may be due to differences in impurities present in the antibiotic samples. This probability is borne out by the overly complicated spectra suggesting that several species are contributing. As a whole, however, both studies show the same distribution of the number and types of hydrogen. Table 2 contains a comparison of a few of the proton types.

Table 2. NMR comparsion of labilomycin and pulvomycin

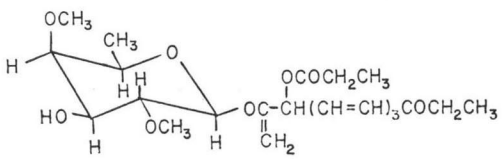
Proton type	Labilomycin[a,b]	Pulvomycin[c]
Anomeric H	4.37 (J=8.0~8.5)	4.38 (J=8.0)
CH_3O	3.61	3.63
CH_3O	3.58	3.59
Unassigned	1.91	1.93
Unassigned	1.85	1.87
Unassigned	1.69	1.69
CH_3C	1.4~0.9	1.36~0.9

[a] estimated error in chemical shift, ±0.02~0.03.

[b] in $CDCl_3$ using 60 MHz spectrometer with TMS.

[c] in $CDCl_3$ using 100 MHz spectrometer with TMS.

With respect to the published structure **II** for labilomycin,[11] it should be noted that signals corresponding to the terminal methylene group are not seen. Protons of the type $CH_2=C\langle^{O}_{R}$ have been shown recently to absorb near δ 4.6[13] and should be readily detected since they would appear as two weakly coupled peaks ($J\approx1-2$ Hz) or less likely as a two proton singlet. The apparent absence of these protons coupled with the presence of three unsplit peaks at δ 1.39, 1.87 and 1.69 suggests a facile migration of the terminal double bond resulting in the formation of species all characterized by an allylic methyl group.



II

Taxonomical Study of MA-2465

A brief study of the morphological and culture description of MA-2465 was carried out.

MA-2465 appears to resemble the description of *Streptomyces netropsis* now being called *Streptoverticillium netropsis* as given by BALDACCI, FARINA and LOCCI.[14] However, a taxonomical assignment cannot be made since a side-by-side comparison study with the type culture was not performed. The description of MA-2465 differs considerable from *Streptomyces albosporeus* var. *labilomyceticus*, the organism producing labilomycin,[15] in several important respects, namely the morphology of the sporophores, the color of the sporulating aerial mycelium, the color of the vegetative growth and the production of melanin pigments. MA-2465 has the same verticillate morphology of the sporophorus as *Streptomyces thioluteus*, the organism which produces aureothin[9] but differs from it in color of sporulating aerial mycelium. Thus we believe that MA-2465 differs from the organisms known to produce labilomycin and aureothin. The culture description of the WOODSIDE and SCHMITZ organism[1] is too incomplete for a comparison with MA-2465. However, the description of the color of the sporulating aerial mycelium differs from the color of MA-2465. No further work on these cultures is contemplated.

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References

1) SCHMITZ, H. & R. WOODSIDE: Mycolutein, a new antifungal antibiotic. Antibiot. & Chemoth. 5: 652~657, 1955
2) ZIEF, M.; R. WOODSIDE & H. SCHMITZ: Pulvomycin. Antibiot. & Chemoth. 7: 384~386, 1957
3) BELLAMY, L. J.: "Infrared spectra of complex molecules". 2nd ed. p. 297 Methuen & Co. Ltd., London, 1958
4) DOUB, L. & L. M. VANDEBELT: Ultraviolet absorption spectra of simple unsaturated compounds. I, Mono- and disubstituted benzene derivatives. J. Amer. Chem. Soc. 69: 2714~2723, 1947
5) EHRLICH, J.; O. R. BARTZ, R. M. SMITH, A. A. JOSLYN & P. R. BURKHOLDER: Chloromycetin, a new antibiotic from a soil actinomycete. Science 106: 417, 1947
6) ARIMA, K.; H. IMANAKA, M. KOUSAKA, A. FUKUDA & G. TAMURA: Studies on pyrrolnitrin, a new antibiotic. I. Isolation and properties of pyrrolnitrin. J. Antibiotics, Ser. A 18: 201~204 1965
7) NAKAMURA, S.: Structure of azomycin, a new antibiotic. Chem. Pharm. Bull. 3: 379~383, 1955
8) MAEDA, K.: Chemical studies on antibiotic substances. IV. A crystalline toxic substance of *Streptomyces thioluteus* producing aureothricin. J. Antibiotics, Ser. A 6: 137~138, 1953
9) HIRATA, Y.; H. NAKATA, K. YAMADA, K. OKUHARA & T. NAITO: The structure of aureothin, a nitro compound obtained from *Streptomyces thioluteus*. Tetrahedron 14: 252~274, 1961
10) AKITA, E.; K. MAEDA & H. UMEZAWA: Isolation and characterization of labilomycin, a new antibiotic. J. Antibiotics, Ser. A 16: 147~151, 1963
11) AKITA, E.; K. MAEDA & H. UMEZAWA: Chemistry of labilomycin. J. Antibiotics, Ser. A 17: 200~215, 1964
12) Private Communication. Professor DAVID PERLMAN, School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706
13) YAMAMOTO, O.; M. YANAGISAWA, H. TOMITA, T. OGAWA, Y. SENGA, K. HAYAMIZU & F. TAKA: Graphic representation of nuclear magnetic resonance chemical shifts for the olefinic protons. Anal. Chem. 44: 2180~2186, 1972
14) BALDACCI, E.; G. FARINA & R. LOCCI: Emendation of the genus *Streptoverticillium* BALDACCI (1958) and revision of some species. G. Microbiol, 14: 153~171, 1966
15) OKAMI, Y.; M. SUZUKI & H. UMEZAWA: Taxonomical studies of a streptomyces strain producing labilomycin. J. Antibiotics, Ser. A 16: 152~154, 1963