STUDIES ON MYCOBACILLIN DERIVATIVES

I. ACETYL DERIVATIVES OF MYCOBACILLIN

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Di- and triacetyl derivatives of mycobacillin, a cyclic peptide, have been prepared. Acetylation lowers its antifungal activity, the inhibitory concentration (μg/ml) for the di- and triacetyl derivatives being 35~40 and 40~45 respectively as against 15~20 for mycobacillin; but acetylation gives complete protection against serum inactivation of the antibiotic whose inhibitory concentration is increased tenfold in its presence.

Mycobacillin is a cyclic peptide antibiotic consisting of 13 residues of 7 different amino acids whose structure has been reported earlier[1~4]. It is a broad-spectrum antibiotic and greatly inactivated in presence of serum which limits its possible use as a drug[5]. Now mycobacillin molecule (Fig. 1) contains many free reactive groups which may cause its inactivation by serum.

In the present work attempts were therefore made to study the effect of acetylation on its antifungal activity both in presence and absence of serum.

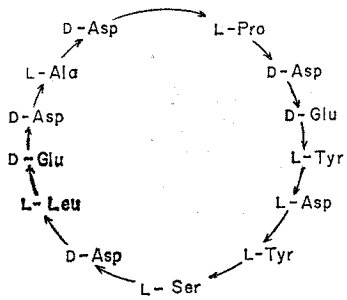


Fig. 1. Mycobacillin.
→CO-NH linkage.

Materials and Methods

Mycobacillin was isolated from the culture filtrate of *Bacillus subtilis* (B_3) according to the method used by MAJUMDER and BOSE[6].

A sensitive strain of *Aspergillus niger* (G_3Br) was used as the test organism. For testing antifungal activity in absence of serum CZAPEK broth (pH 6~6.5) containing 20 μg per ml each of penicillin and streptomycin was used and for the same in presence of serum was used CZAPEK broth (pH 6~6.5) containing all the ingredients in two-fold concentration and mixed with an equal volume of normal horse serum preincubated for 2 hours at 37°C with the antibiotics in two-fold concentration. Spore suspension (0.05 ml) from 7 days old culture was added per 5 ml of the medium. Mycobacillin and its derivatives were used in 60% ethanolic solution.

Preparation of diacetyl derivative: Diacetyl derivative at the two tyrosine hydroxyl groups of mycobacillin was prepared according to RIORDAN and VALLEE[7]. In practice, 88 mg of mycobacillin was dissolved in 22 ml of barbital sodium-HCl buffer (pH 7.4, 0.02 M). Fifty fold molar excess of redistilled acetic anhydride (0.5 ml) was added dropwise and the pH maintained within 7~7.5 by automatic addition of 2 N NaOH. Reaction was carried out at 1~2°C in a Radiometer pH-stat. Acetylation was complete within 45 minutes as observed from the automatic titration curve. Acetyl mycobacillin so obtained was extracted with *n*-butanol and the extract washed thrice with a small volume of water, distilled under reduced pressure to a small volume, precipitated with ether and the

precipitate washed twice with ether and centrifuged. The residue was dissolved in water, again centrifuged and the supernatant freeze-dried; yield 70 mg. Purity of the compound was tested by ascending paper chromatography in different solvent systems.

Preparation of triacetyl derivative: The tyrosine and serine hydroxyl groups were all acetylated by reaction of mycobacillin with acetic anhydride in presence of a basic catalyst pyridine. Mycobacillin (100 mg) was dissolved in 10 ml pyridine and then 15 ml acetic anhydride added to it. Acetylation was found to be extremely slow at 37°C. Only 20% of mycobacillin got acetylated in 24 hours at this temperature. But at 100°C (on boiling water bath) acetylation was almost complete within one hour. The reaction mixture was then cooled in ice-water bath to which was added excess of water. The derivative was extracted thrice with chloroform. Chloroform layer was washed thrice with water and then evaporated to a small volume and the concentrated extract precipitated with dry ether. The precipitate was dissolved in chloroform-methanol (8:2) and the solution precipitated with cold, dry ether. The process was repeated once more. Finally the derivative was crystallized from 95% methanol under reduced pressure over P_2O_5 in the cold. Purity of the derivative was tested by ascending paper chromatography using different solvent systems.

Paper chromatography: WHATMAN No. 1 paper was used for paper chromatography using the solvent systems:

1) *n*-Butanol-pyridne-water-acetic acid (60:40:30:3) and 2) 5% NH_4Cl in water for diacetyl derivative; and 1) *n*-butanol-pyridine-water-acetic acid (60:40:30:3), 2) *t*-butanol-acetic acid-H_2O (55:6:4) and 3) *n*-butanol-acetic acid-water (75:10:25) for triacetyl derivative.

Acid hydrolysis of mycobacillin and the triacetyl derivative: Acid hydrolysis was carried out at 105°C with conc. HCl for 18 hours (concentration 5 mg/ml).

Determination of the extent of acetylation: Number of acetyl groups in the derivatives was estimated both by colorimetric[8] and spectrophotometric[9] methods. By colorimetric method was determined the total number of hydroxyl groups acetylated, whereas by spectral method only the number of tyrosine hydroxyl groups acetylated. In practice, for colorimetric estimation, 2 ml of 90% methanol containing 1~5 mg of acetyl derivative was mixed with 0.6ml hydroxylamine reagent, kept for 30 minutes at room temperature and then 5 ml of ferric perchlorate reagent added. Readings were taken after 10 minutes at 520 mμ in a 'Bausch & Lomb' spectronic 20 colorimeter against a blank containing all the materials in which hydroxylamine reagent was added after the addition of ferric perchlorate reagent. Moles of acetyl groups present per mole of mycobacillin was estimated from a standard curve prepared with *p*-nitrophenylacetate.

Spectral analysis was carried out by treatment of 2 ml solution of the derivatives in 90% methanol with an equal volume of 2 M hydroxylamine in 90% methanol (pH 9.0) at room temperature for 2 hours and then the reaction mixture was made acidic with 1.0ml of 10% HCl. Readings were taken in the 'Uvispek' spectrophotometer. Moles of acetyl groups per mole of mycobacillin was calculated from the change in molar absorptivity with reference to O-acetyl tyrosine.

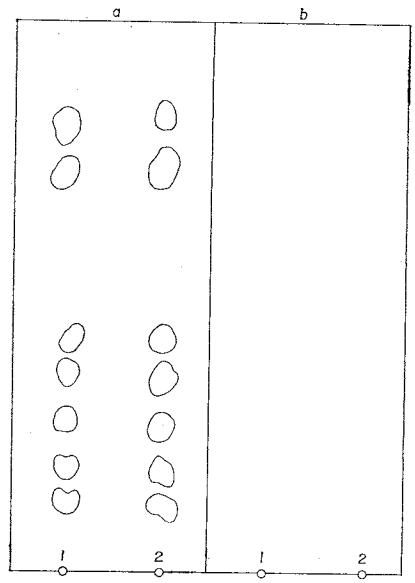
Results

Properties of the Derivatives

Both di- and triacetyl derivatives differ from mycobacillin in respect of solubility and absorption of light in the UV region. Mycobacillin is soluble in water at alkaline pH and almost insoluble in absolute alcohols, whereas the derivatives are readily soluble in absolute alcohols. Diacetyl derivative is also soluble in water both at acidic and alkaline pH, but the triacetyl derivative is insoluble in dil. acid or alkali, but soluble in strong alkali (0.4 N). Triacetyl derivative is readily soluble in nonpolar solvent chloroform. Mycobacillin absorbs light in the UV region and has a peak at 272 mμ. This peak disappears totally in case of acetyl derivatives, but appears again when the derivatives are treated with hydroxylamine in alkaline pH (Fig. 2).

The condition at which the triacetyl derivative has been prepared may lead to some keto group formation at the free α-carboxyl groups of glutamic acid according to DAKIN and WEST reaction[10,11]. The derivative does not however respond to the iodoform and nitroprusside tests for ketones. That the derivative does not contain any keto group was further confirmed by the observation that the paper chromatogram of the acid hydrolysate of the derivative contains all the ninhydrin-positive spots as in the chromatogram of mycobacillin hydrolysate but no DNP-positive spot showing the absence of keto group (Fig. 3).

Fig. 3. Descending paper chromatogram of acid hydrolysates of mycobacillin and its triacetyl derivative. Solvent system: *n*-butanol-pyridine-water-acetic acid (60:40:30:3)



1. Acid hydrolysate of mycobacillin.
2. Acid hydrolysate of triacetyl derivative.

a. Sprayed with 0.2% ninhydrin in acetone containing 1 ml glacial acetic acid per 100 ml.

b. Sprayed with 0.1% 2,4-DNP in ethanol containing 1 ml conc. HCl per 100 ml.

Extent of Acetylation.

Fig. 2. Effect of hydroxylamine on UV absorption spectra of O-acetyl derivatives of mycobacillin and tyrosine

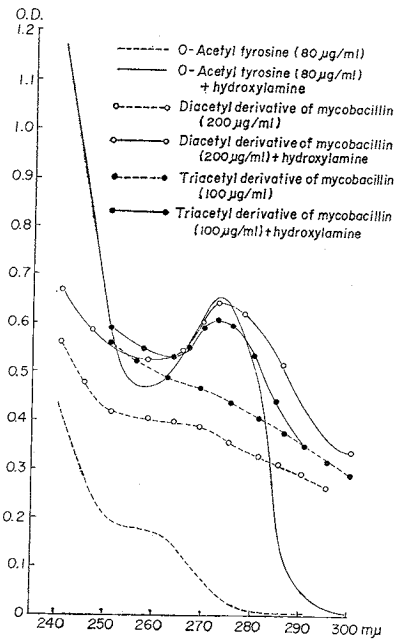


Table 1 shows that the number of acetyl groups per mole of mycobacillin derivatives as prepared in the pH-stat and in presence of pyridine catalyst are two and three respectively.

Biological Activity.

Table 2 shows the biological activity of mycobacillin and its acetyl derivatives against *A. niger* (G_3Br) spores in presence and in absence of serum. Acetylation of the antibiotic at two tyrosine hydroxyl groups adversely affects the antifungal activity. The minimum inhibitory concentration (MIC) is 35~40 μg/ml whereas for mycobacillin it is only 15~20 μg/ml. Interestingly the derivatives are not at all inactivated in presence of serum, if incorporated to the extent of 50% (v/v) in the medium whereas mycobacillin is under identical condition inactivated by serum to more than 90%. Triacetyl mycobacillin has almost the same biological activity as the diacetyl derivative and

Table 1. Extent of acetylation of mycobacillin derivatives

Acetylation by	Moles of O-acetyl/mole of mycobacillin derivative	
	Colorimetric analysis	Spectral analysis
pH stat	1.88	1.90
Pyridine catalysed	3.15	2.00

Table 2. Minimum inhibitory concentration (MIC) of mycobacillin and its derivatives in presence and in absence of serum against *A. niger* (G_3Br) spores

Compound	MIC (μg/ml)		Ratio (A/B)
	In presence of serum (A)	In absence of serum (B)	
Mycobacillin	180~200	15~20	10
Diacetyl derivative	40~45	35~40	1.1
Triacetyl derivative	40~50	40~45	1.1

its serum inactivation is also nil.

It is of interest to note that acetylation of hydroxyl groups, as reported by EBATA *et al.* grossly reduces the biological activity of siomycin A, a peptide antibiotic[12].

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