# STUDIES ON MYCOBACILLIN DERIVATIVES. III. REDUCTION OF CARBOXYL GROUPS OF THE ANTIBIOTIC

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We previously reported some of the biological activities of acetyl[1,2] and ester[2,3] derivatives of mycobacillin, a cyclic peptide antibiotic produced by *Bacillus subtilis* $B_3$[4]. The present communication deals with the effect of reducing the free carboxyl groups of mycobacillin on its antifungal and hemolytic activities, as well as on its activity in presence of serum.

Mycobacillin in its cyclic tridecapeptide chain contains seven free carboxyl groups, two from $\gamma$-linked glutamic acid and five from $\alpha$-linked aspartic acid residues[5]. Attempts were made to reduce these free carboxyl groups and to study the biological activities of the reduced derivatives. For reduction of free carboxylic groups to primary alcoholic groups of the cyclic peptide, the methods found to be applicable are (i) the direct reduction of free carboxyl groups by diborane, and (ii) the lithium borohydride reduction of carboxylic esters.

Reduction of mycobacillin with diborane was done according to ATASSI and ROSENTHAL[6] with the omission of the trifluoroacetylation step, since the antibiotic does not contain any free amino group. A suspension of mycobacillin (100 mg/5 ml) in tetrahydrofuran was made by precipitating the antibiotic from methanolic solution with dry ether, washing the precipitate once with the same solvent, then with dry tetrahydrofuran, and finally suspending the material in 5 ml of the latter solvent. Twenty ml of a saturated diborane solution in tetrahydrofuran[7] were added dropwise with stirring to the mycobacillin suspension and the mixture was kept for 16 hours at room temperature. The reduced derivative separated as a solid mass and the supernatant was drawn out as completely as possible. The product was then dried in a flow of nitrogen. To this dry solid 20 ml of 10% (v/v) glycerol in 0.15 M potassium phosphate buffer (pH 7.5) were added, the mixture stirred for 15 minutes, then kept for 1 hour at 5°C. Reduced mycobacillin adhered to the glass surface; it was washed twice with water, once with ethanol, then dissolved in 95% methanol, and centrifuged; the solvent was evaporated and the precipitate dried over $P_2O_5$ *in vacuo*. Reduced mycobacillin was obtained as glistening yellow crystals. The product was found to be homogeneous in various TLC solvent systems and designated as derivative I (Table 1).

Reduction of the heptamethyl ester[3] of mycobacillin by lithium borohydride ($LiBH_4$) was done according to CHIBNALL and REES[8] using different stoichiometric proportions of the reducing agent to the ester. In practice, reduction of the ester under two conditions was found to give two different products. These conditions were: (i) 2.5 molar excess of $LiBH_4$ for each mole of carboxyl group, 4 hours reflux, and (ii) 10.0 molar excess of $LiBH_4$ per mole of carboxyl group, 6 hours reflux. The products were designated as derivative II and derivative III respectively, purified according to SENGUPTA

Table 1. Rf values of mycobacillin and its derivatives in TLC systems

| Solvent systems | Adsorbent | Rf values | | | |
|---|---|---|---|---|---|
| | | Mycobacillin | Derivative I | Derivative II | Derivative III |
| A* | Silica gel G | 0.1 | 0.82 | 0.54 | 0.94 |
| B* | | 0.08 | 0.56 | 0.28 | 0.75 |
| A | Silica gel G plated with 1% $NaHCO_3$ solution | 0.04 | 0.24 | 0.13 | 0.32 |
| B | | 0.05 | 0.28 | 0.16 | 0.40 |

* System A: *n*-Butanol - acetic acid - water (23: 1: 1, v/v).
System B: Methyl ethyl ketone - pyridine (7: 3, v/v).

Table 2. Partial amino acid composition and biological activities of mycobacillin derivatives

| Compound | Moles of amino acid per mole of compound | | | | Minimum inhibitory concentrations against *A. niger* ($\mu$g/ml) | | Minimum concentration for complete hemolysis ($\mu$g/ml) |
|---|---|---|---|---|---|---|---|
| | Glu | HAV | Asp | HAB | In absence of serum | In presence of serum | |
| Derivative I | 0.84 | 0.78 | 1.05 | 3.45 | 45~50 | >200 | 45 |
| Derivative II | 1.12 | 0.81 | 2.83 | 1.65 | >200 | —* | 180 |
| Derivative III | 0.08 | 1.70 | 0.16 | 4.28 | >200 | —* | >200 |
| Mycobacillin | 2.08 | — | 4.85 | — | 16~20 | 190~200 | 25 |

—* "not tested, since they have no antifungal activity".

*et al.*[5] and found to be chromatographically (TLC) pure (Table 1).

Derivative I is soluble in chloroform, but insoluble in alcohol at room temperature; it slowly solubilizes on heating. On the other hand, derivatives II and III are readily soluble in alcohol, insoluble in chloroform; they are highly hygroscopic when crystallized.

The extent of reduction under the conditions above described was determined by hydrolysing the derivatives with 10 M HCl-formic acid (1: 1, v/v) for 18 hours at 105°C and determining the molar proportions of unreacted aspartic and glutamic acids, as well as their reduced products in the hydrolysates. On reduction, $\gamma$-hydroxy-$\alpha$-amino butyric acid (HAB) and $\gamma$-amino-$\delta$-hydroxy valeric acid (HAV) arose respectively from $\alpha$-linked aspartic acid (Asp) and $\gamma$-linked glutamic acid (Glu). Aspartic and glutamic acids were first separated from the hydrolysates on alumina column according to KUHN and WIELAND[9]; then the hydroxy acids (HAB, HAV) were isolated on a Dowex-50 ($H^+$) column according to CHIBNALL *et al.*[10]. Amino acids were estimated by the ninhydrin method of ROSEN[11]. The partial amino acid composition of the derivatives revealed the extent of carboxyl group reduction and is given in Table 1. The analyses showed that the derivatives I, II, III and mycobacillin contain respectively 0.84, 1.12, 0.08 and 2.08 moles of glutamic acid in their molecules. Thus it appears that one glutamic acid residue in derivatives I and II, and two in derivative III have been reduced; this is also supported by the concomitant formation of 0.78, 0.81 and 1.70 moles of HAV respectively. Similarly, the molar proportions of aspartic acid in derivatives I, II, III and in mycobacillin are found to be 1.05, 2.83, 0.16 and 4.85 respectively, indicating the reduction of four, two and five aspartic acid residues of mycobacillin in these derivatives, with the concomitant formation of 3.45, 1.65 and 4.28 moles of HAB as estimated in the hydrolysates. Thus, it has been possible to reduce five (one glutamyl and four aspartyl), three (one glutamyl and two aspartyl), and seven (two glutamyl and five aspartyl) carboxyl groups of mycobacillin to obtain derivatives I, II and III. Antifungal activity of the compounds was tested against *Aspergillus niger* spores in absence and in presence of 50% horse serum as described earlier[1]. Haemolytic activity was measured according to DIMICK[12], using freshly prepared erythrocyte suspension in normal saline[2]. Biological activities of mycobacillin and its reduced derivatives are presented in Table 2. Of the three products, only derivative I retains the considerable biological activity of mycobacillin. This derivative possesses antifungal and haemolytic activities that are 37% and 55% respectively of those of the parent compound. Neither derivative II nor derivative III exhibited antifungal activity, even at the concentration of 200 $\mu$g/ml. Derivative II is slightly haemolytic (14% of the original activity), whereas derivative III has no such activity, even at the concentration of 200 $\mu$g/ml. Since derivatives II and III have no antifungal activity, serum inactivation property of these derivatives was not tested. It was also observed that in presence of serum the antifungal activity of derivative I was not expressed, even at a concentration of 200 $\mu$g/ml indicating inactivation of the molecule by serum.

Thus it appears from the results that the derivative containing more carboxyl groups reduced (derivative I) is more active than that having less (derivative II). This indicates that probably

the carboxyl groups, in the expression of biological activity of the peptide, are not involved quantitatively but rather in a qualitative manner according to their positions in the molecules. Thus the vital carboxyl group or groups might have been reduced in less reduced product resulting in almost complete inactivation. But, with the limitations of the methods available for the reduction of specific carboxyl groups of the peptide, their individual role could not be assessed.

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