# MECHANISM OF ACTION OF MONOKETO-ORGANOMYCIN, CYSTAURIMYCIN AND THEIR PERFORMIC ACID-OXIDIZED MODIFICATIONS

## I. EFFECTS ON BACTERIAL GROWTH AND RIBOSOMAL PEPTIDYL TRANSFERASE ACTIVITY

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(Received for publication April 16, 1979)

The response of 15 bacterial cultures to the inhibitory effects of the above mentioned compounds was investigated. These varied considerably. *Escherichia coli* cultures resistant to the compounds tested showed that monoketo-organomycin and cystaurimycin inhibit bacterial growth by what appears to be the one and same mechanism which is not the same as that exhibited by their performic acid-oxidized modifications. This was confirmed by using cell free extracts of *E. coli*. In light of the puromycin reaction, using chloramphenicol and chlorotetracycline as control inhibitors, monoketo-organomycin and cystaurimycin were found to inhibit protein synthesis *in vitro* by inhibiting peptidyl transferase of ribosomes. In marked contrast, this enzyme was activated, as also was protein synthesis, by their performic acid-oxidized modifications. It was thus suggested that the growth inhibitory effects of the latter compounds might be due to their interference in other metabolic activities of the above test organism. The results obtained are discussed in light of the chemical similarities or differences existing between the compounds investigated.

Studies on monoketo-organomycin (MKOM)[1] revealed that this antibiotic is, through partial enzymic digestion, converted to the broad spectrum substance cystaurimycin (CYST) and that performic acid oxidation of these compounds appreciably improved their antimicrobial activities[2]. In spite of the close chemical similarities between these compounds, they showed differences in their chromatographic and electrophoretic behaviour as well as their antimicrobial activities[2].

The present study was made to obtain more information about the functional relationship and mode of action of these compounds. Puromycin with its known mode of action[3~5] was used to elucidate the mechanisms of action of the present compounds. Chlorotetracycline and chloramphenicol were used as control inhibitors.

### Materials and Methods

Preparations of MKOM, CYST and their performic acid oxidation products are those reported by IMAM[1] and by IMAM and KÜNTZEL[2].

#### 1. Growth Inhibitory Studies

In growth inhibitory experiments each substance was dissolved in phosphate buffer, pH 7.9, and serially diluted in 1 ml aliquots. To these volumes 4-ml portions of brain heart infusion broth (Difco), previously inoculated with the test organism, were added and the cultures were incubated at 37°C for 2 days. The maximum tolerated concentration (MTC) of each substance was recorded. The bacterial growth was related to the concentration of a given compound and was determined by measuring the

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amount of 2-day bacterial mass spectrophotometrically at 660 nm using a Zeiss spectrophotometer.

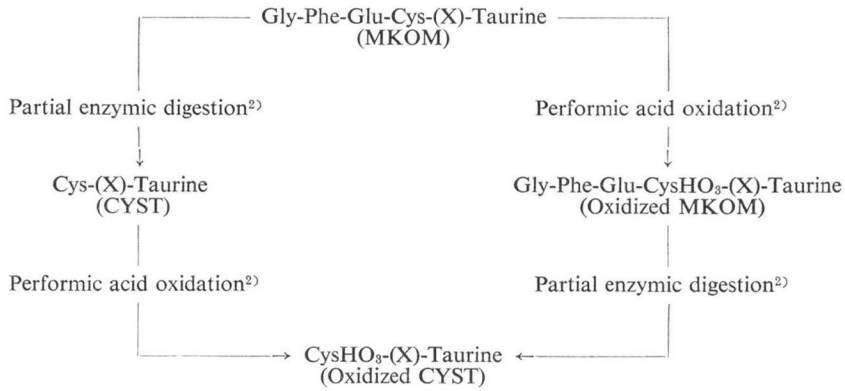
Separate resistant cultures of *Escherichia coli* NCTC 6820 to each of the 4 compounds were made by a series of transfers in brain heart infusion broth to which increasing amounts of the respective material was added. At each transfer, each mutant was tested for the simultaneous development of resistance to the other substances.

2. Effects on Ribosomal Peptidyl Transferase Activity

*Escherichia coli* NCTC 6820 was grown on DAVIS minimal growth medium[6]. The cultures were incubated at 37°C with shaking (250 r.p.m.) and the cells were harvested during the exponential phase, washed twice with 5 mM Tris-HCl of pH 7.0 containing 10 mM magnesium acetate and stored frozen until required. Crude extracts were prepared[4] and diluted to 12 mg/ml ribosomes. The techniques adopted for the preparation of ribosome-free supernatant fraction and the reaction mixture for radioactive amino acid incorporation in protein and the conditions used for the puromycin reaction were those described by CANNON[4].

## Results and Discussion

Conversion of MKOM into CYST and their performic acid oxidation was performed as described[2] according to the following scheme:




(X) is the internal region of the peptide where aliphatic, keto and all of the amino acids constituting the molecule, except for Cys and taurine, are located[1,2]. The sequence of the first four amino acids forming the N-terminal portion of MKOM was determined by using leucine aminopeptidase[2,7] and EDMAN degradation[8].

It is obvious that MKOM and its oxidized (ox-)-form include in their molecules the CYST- and ox-CYST-moieties respectively. These moieties are, however, masked by the N-terminal portion formed by the tripeptide Gly-Phe-Glu with subsequent establishment of Gly as the N-terminal residue instead of Cys and $CysHO_3$ in the forecited moieties respectively.

Despite the close chemical similarities, particularly those existing between each given compound and its ox-form, previous results showed differences in their growth inhibitory activities[2]. The concept of biological specificity suggested that bacteria might exist in nature whose growth could be inhibited by one of these substances but not the others. Such a property should thus be associated with those parts of the molecule which are structurally different. Thus an attempt was made to find organisms which could possess such a specificity and the responses of 15 bacterial cultures to MKOM, CYST and their performic acid oxidation products were determined.

Table 1. Response of test organisms to MKOM, CYST and their performic acid-oxidized modifications.

| Test organism | MKOM | | ox-MKOM | | CYST | | ox-CYST | |
|---|---|---|---|---|---|---|---|---|
| | MIC (mcg/ml) | MTC (mcg/ml) | MIC (mcg/ml) | MTC (mcg/ml) | MIC (mcg/ml) | MTC (mcg/ml) | MIC (mcg/ml) | MTC (mcg/ml) |
| **Escherichia coli* B-2346 | 0.1 | 0.075 | 0.05 | 0.03 | 0.01 | 0.005 | 0.005 | 0.004 |
| **Escherichia coli* NCTC 6820 | 3.0 | 2.5 | 1.5 | 1.0 | 0.1 | 0.075 | 0.01 | 0.005 |
| **Escherichia coli* O-127, NCTC 9707 | 5.0 | 4.0 | 3.5 | 3.0 | 0.1 | 0.075 | 0.05 | 0.03 |
| **Proteus rettgeri* 1163- CDC | R | R | R | 100.0 | 75.0 | 65.0 | 50.0 | 40.0 |
| **Proteus mirabilis* | 20.0 | 15.0 | 15.0 | 12.5 | 10.0 | 7.5 | 6.5 | 5.5 |
| **Salmonella typhosa* NRRL B-210 | R | R | R | R | 50.0 | 40.0 | 35.0 | 30.0 |
| **Salmonella typhi* | 100.0 | 90.0 | 100.0 | 90.0 | 30.0 | 25.0 | 15.0 | 12.5 |
| **Klebsiella pneumoniae* NCTC 5046 | 1.2 | 0.8 | 0.6 | 0.5 | 0.1 | 0.075 | 0.05 | 0.03 |
| **Pseudomonas aeruginosa* ATCC 9027 | 10.0 | 8.0 | 10.0 | 7.5 | 5.0 | 3.5 | 5.0 | 3.5 |
| **Staphylococcus aureus* D6 | 10.0 | 8.0 | 10.0 | 7.5 | 5.0 | 3.5 | 1.5 | 1.25 |
| **Bacillus subtilis* D161 (Staphylomycin R) | 1.5 | 1.25 | 1.5 | 1.0 | 0.5 | 0.35 | 0.1 | 0.075 |
| **Bacillus subtilis* D161 (Chloramphenicol R) | 20.0 | 17.5 | 20.0 | 15.0 | 10.0 | 8.0 | 3.5 | 2.5 |
| **Bacillus cereus* NRRL B-569 | 10.0 | 8.0 | 10.0 | 7.5 | 7.5 | 5.0 | 5.0 | 3.5 |
| *Bacillus mycoides* | 100.0 | 90.0 | 80.0 | 70.0 | 75.0 | 65.0 | 30.0 | 25.0 |
| *Shigella equi* | 100.0 | 90.0 | 100.0 | 90.0 | 75.0 | 70.0 | 50.0 | 40.0 |
| *Streptomyces* AS-400 (MKOM-producing) | R | R | R | R | 60.0 | 55.0 | 25.0 | 20.0 |

* Pathogenic organisms, R=Resistant
MIC=Minimum inhibitory concentration, MTC=Maximum tolerated concentration.

The results of this survey (Table 1) show that the responses of the bacterial cultures to the inhibitory effects of the 4 compounds tested varied considerably and that none of the test organisms was susceptible to the four compounds to the same extent. For example, while the growth of *E. coli* NCTC 6820 was inhibited by 0.1 mcg CYST, only 0.01 mcg ox-CYST was required to bring about the same effect but neither 1.0 mcg ox-MKOM nor 2.5 mcg MKOM could exert the same degree of inhibition exhibited by the two former compounds. Identical responses to the inhibitory effects of CYST and ox-CYST in case of *Escherichia coli* O-127, NCTC 9707 and *Klebsiella pneumoniae* O-2 NCTC 5046 and to those of MKOM, ox-MKOM and CYST in case of *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* D6 could be, however, observed but the latter organism was more sensitive to ox-CYST than the former.

The results obtained in case of *E. coli* NCTC 6820 are of especial interest because of the large differences in its response to the inhibitory effects of the compounds tested (Table 1). Thus separate resistant cultures of this organism to each of the 4 compounds were prepared and tested for the simultaneous development of resistance to the other substances by use of the cross-resistance technique. The basis of this method lies in the assumption that related antibiotics or those with similar chemical composition exert their growth inhibitory action through the same or closely similar groupings in their

Table 2. Response of *Escherichia coli* NCTC 6820 and its resistant mutants to MKOM, CYST and their performic acid-oxidized modifications.

| Mutant of *E. coli* resistant to | Compound tested against resistant mutant | MTC (mcg/ml) Transfer No. (days) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Parent | 1 | 2 | 3 | 6 | 12 |
| MKOM | MKOM | 2.5 | 5.0 | 7.5 | 15.0 | 60.0 | 175.0 |
| | ox-MKOM | 1.0 | 0.075 | 0.5 | 0.25 | 0.125 | 0.1 |
| | CYST | 0.075 | 0.15 | 0.35 | 0.6 | 2.5 | 7.5 |
| | ox-CYST | 0.005 | 0.005 | 0.005 | 0.003 | 0.001 | 0.0008 |
| CYST | CYST | 0.075 | 0.15 | 0.25 | 0.45 | 1.75 | 5.25 |
| | ox-CYST | 0.005 | 0.008 | 0.008 | 0.004 | 0.003 | 0.001 |
| | MKOM | 2.5 | 3.0 | 7.5 | 15.0 | 60.0 | 250.0 |
| | ox-MKOM | 1.0 | 0.75 | 0.5 | 0.35 | 0.25 | 0.15 |
| ox-MKOM | ox-MKOM | 1.0 | 2.0 | 3.0 | 4.0 | 9.0 | 30.0 |
| | MKOM | 2.5 | 2.5 | 2.5 | 2.5 | 5.0 | 7.5 |
| | CYST | 0.075 | 0.075 | 0.075 | 0.075 | 0.075 | 0.15 |
| | ox-CYST | 0.005 | 0.015 | 0.025 | 0.03 | 0.15 | 0.20 |
| ox-CYST | ox-CYST | 0.005 | 0.01 | 0.015 | 0.02 | 0.05 | 0.15 |
| | CYST | 0.075 | 0.075 | 0.075 | 0.075 | 0.075 | 0.15 |
| | MKOM | 2.5 | 2.5 | 2.5 | 2.5 | 5.0 | 10.0 |
| | ox-MKOM | 1.0 | 2.0 | 3.0 | 5.0 | 12.5 | 30.0 |

MTC=Maximum tolerated concentration.

respective molecules[9]. Using this method, presumptive evidence for identity or for a close similarity of unknown antibiotics may also be obtained[10,11]. Cross-resistance technique was thus used to demonstrate the relationship between the inhibitory properties of the investigated compounds and their chemical nature.

The results of Table 2 show that in 12 successive transfers in the presence of MKOM and CYST, the bacterium increased its resistance to these compounds by 70 times compared with only 30 times in case of their corresponding ox-forms. The resistance to MKOM and CYST proceeded at identical rates. Similar results, though with different rates and magnitudes, were also recorded with ox-MKOM and ox-CYST. Cultures resistant to MKOM showed high resistance to CYST. The inverse relationship holds true. Similar behaviour was recorded in case of cultures resistant to ox-MKOM and ox-CYST. When the cultures resistant to the latter compounds were tested against their nonoxidized forms, the resistance increased by only 2 times to CYST and by 3~4 times to MKOM depending on the resistant culture used. On the other hand, cultures resistant to MKOM or CYST showed more sensitivity to the inhibitory effects of the oxidized compounds compared with the parent culture *E. coli* NCTC 6820.

The aquired resistance to all levels of the oxidized compounds together with their characteristic response to MKOM and CYST was retained after 30 daily transfers in inhibitor-free medium. In contrast, organisms resistant to MKOM and CYST lost their resistance to these compounds as well as the increased sensitivity to the oxidized compounds after being transfered for 6~8 days in inhibitor-free medium. None of the compounds tested was either destroyed or inactivated as indicated through measuring their titers after 7-day incubation with the organisms.

Since the only difference between the molecule of MKOM and that of CYST as well as between

those of ox-MKOM and ox-CYST is the presence or absence of the N-terminal portion (see the above scheme) and since those lacking this portion, namely CYST and ox-CYST, are still biologically active (Table 1), it is thus logical to conclude that this portion constitutes neither a structural nor a fundamental part of the biologically active moiety in MKOM or its ox-form. Furthermore, that CYST and ox-CYST were toxic to the growth of MKOM-producing culture (*Streptomyces* AS-400) and that this toxic effect was abolished in MKOM[2], and which contains the N-terminal portion, may indicate that the latter might have been synthesized by MKOM-producing culture as a possible mechanism for self protection against the toxic effect of the CYST-moiety in the molecule. This toxicity masking effect of the N-terminal portion could also be seen from the MTCs and MICs of the various compounds (Table 1).

That the 4 compounds tested, although very closely related, showed different inhibitory activities is not an isolated phenomenon. WELCH *et al*[12] and REEDY *et al*[13] observed marked differences in the growth inhibitory activities of three closely related antibiotics, chlorotetracycline, oxytetracycline and tetracycline.

Due to the probability that the mutants obtained might have been permeability ones, the 4 inhibitors mentioned above were tested for their ability to inhibit the puromycin reaction with the ribosome-bound nascent peptides formed under the direction of natural mRNA. Chlorotetracycline, known to inhibit the codon-directed binding of amino acyl-tRNA to ribosomes[14], and chloramphenicol, known to bind to peptidyl transferase and directly inhibits the enzyme in puromycin reaction[4,15], were used as control inhibitors.

The results obtained (Table 3) show that neither chlorotetracycline nor ox-MKOM and ox-CYST inhibited the puromycin reaction while MKOM and CYST were potent inhibitors (71.82% and 88.2%

Table 3. Effect of MKOM, CYST, ox-MKOM and ox-CYST on puromycin reaction.

| Reaction system | % | | | |
|---|---|---|---|---|
| | Protein released from ribosomes by puromycin* | Inhibition of puromycin reaction | Stimulation of puromycin reaction | Reduction in inhibition of puromycin reaction |
| Ribosomes+puromycin (control=C) | 55 | — | — | — |
| C+MKOM | 15.50 | 71.82 | — | — |
| C+CYST | 6.49 | 88.20 | — | — |
| C+ox-MKOM | 64.90 | — | 18.0 | — |
| C+ox-CYST | 71.50 | — | 30.0 | — |
| C+chlorotetracycline | 54.60 | — | — | — |
| C+chloramphenicol | 11.30 | 79.46 | — | — |
| C+chloramphenicol+CYST | 1.54 | 97.20 | — | — |
| C+chloramphenicol+MKOM | 2.10 | 96.18 | — | — |
| C+chloramphenicol+ox-MKOM | 38.40 | 30.19 | — | 49.20 |
| C+chloramphenicol+ox-CYST | 31.50 | 42.73 | — | 36.73 |
| C+MKOM+ox-MKOM | 32.70 | 40.55 | — | 31.27 |
| C+MKOM+ox-CYST | 39.06 | 28.98 | — | 42.84 |
| C+CYST+ox-MKOM | 20.66 | 62.44 | — | 25.76 |
| C+CYST+ox-CYST | 26.89 | 51.11 | — | 37.09 |

*=None of the compounds tested, except for puromycin, could release any protein when used alone. All the figures given are the mean values of three independent estimations.

Table 4. Effect of MKOM, CYST, ox-MKOM and ox-CYST on *in vitro* protein synthesis by isolated *Escherichia coli* ribosomes.

| Reaction system | c.p.m. of protein/2 mg ribosomes | % | | |
|---|---|---|---|---|
| | | Inhibition of protein synthesis | Stimulation of protein synthesis | Reduction in inhibition of protein synthesis |
| Ribosomes+amino acids (control=C) | 21,000 | — | — | — |
| C+MKOM | 5,000 | 76.19 | — | — |
| C+CYST | 3,000 | 85.71 | — | — |
| C+ox-MKOM | 25,620 | — | 22.00 | — |
| C+ox-CYST | 28,000 | — | 33.33 | — |
| C+MKOM+ox-MKOM | 12,000 | 42.86 | — | 33.33 |
| C+MKOM+ox-CYST | 12,500 | 40.48 | — | 38.09 |
| C+CYST+ox-MKOM | 10,000 | 52.38 | — | 33.33 |
| C+CYST+ox-CYST | 11,000 | 47.62 | — | 35.71 |

inhibition respectively). Under the experimental conditions adopted, peptidyl-tRNA would have been bound to either site A or P on to ribosomes[5]. Puromycin can only react with peptidyl-tRNA bound at site P. Inhibition of this reaction would thus indicate an inhibition of the enzyme peptidyl transferase[3,16]. Based on these results it is logical to conclude that MKOM and CYST exert their inhibitory action through inhibiting peptidyl transferase activity. This enzyme is an integral part of 50S subunit of ribosomes[3]. That chlorotetracycline failed to inhibit the puromycin reaction was not unexpected since this antibiotic acts on the 30S subunit and thus would be unable to block the puromycin reaction.

The results obtained with ox-MKOM and ox-CYST (Table 3) are of particular interest. Addition of each of these compounds to the puromycin-containing system stimulated the release of peptides from the prelabelled ribosomes by 18% and 30% respectively. Furthermore, addition of these compounds to MKOM- as well as to CYST-containing systems resulted in lowering the inhibitory effects of MKOM and CYST on the puromycin reaction by 25~42%, depending on the compound used, compared with the inhibitory effects in controls with subsequent increase in the amounts released of peptides. This would indicate that ox-MKOM and ox-CYST might have stimulated the puromycin reaction through stimulating peptidyl transferase activity which catalyzes the puromycin reaction with peptidyl-tRNA[3]. It is unlikely, however, that these compounds might have stimulated the translocation reaction in the light of the fact that the reaction system used was lacking in GTP which is essential for translocation to occur[17]. In addition, the possibility that ox-MKOM and ox-CYST might have exerted their effect in a similar way to that of puromycin is ruled out in the light of the failure of these compounds to release the ribosome-bound peptides under the same conditions used for puromycin reaction. Using the methods of NATHANS[18], the peptides released in the systems containing puromycin and each of these compounds were found all of peptidyl-puromycin and none as peptidyl-derivatives of these compounds. Hydrolysis of the released peptides with 6N HCl at 110°C for 20 hours released no taurine which is a main component of the compounds tested.

Addition of chloramphenicol (Table 3), the second control inhibitor, resulted in the inhibition of puromycin reaction by 79.46% compared with the controls. Almost complete inhibition of puromycin reaction could be recorded when a mixture of chloramphenicol and either MKOM or CYST

was present together with puromycin in the reaction system. Since chloramphenicol inhibits peptidyl transferase[4,15] and that its action was intensified by MKOM and CYST, it is thus logical to assume that these compounds might have the same mode of action. Addition of ox-MKOM or ox-CYST to the chloramphenicol-containing system had reduced its inhibitory action on puromycin reaction from 79.46% to 30.19% and to 42.73% respectively. Similar results were obtained when the oxidized compounds were added to the MKOM- or CYST-containing systems (Table 3). These results would indicate that the oxidized compounds might have affected the inhibitory action of chloramphenicol, MKOM and CYST by probably competing for binding on the sensitive site(s) in the molecule of peptidyl transferase and/or through unknown mechanism.

The mode of action of the various compounds was further investigated by studying their effects on the *in vitro* protein synthesis. The amino acids incorporation mixture was prepared[4] and incubation was conducted for 5 minutes at 37°C in the presence and absence of the compounds investigated. Incorporation of amino acids in protein was directed by the natural mRNA of the extract. The ribosomes were then sedimented from the incorporation mixture, washed and the released nascent peptides from puromycin-treated ribosomes were estimated. From the corresponding results (Table 4) it is clear that MKOM and CYST are potent inhibitors for the *in vitro* protein synthesis by ribosomes while their ox-forms have activated this process. Addition of ox-MKOM or ox-CYST to MKOM- or CYST-containing systems had reduced the inhibitory action of the latter compounds by 33~38%, depending on the compound added.

The results obtained with the performic acid-oxidized substances were rather unexpected in light of the fact that the concentrations used were those of the minimum inhibitory ones. Using half, twice and three times these concentrations no change in the results could be observed. This would indicate that these compounds do not act on ribosomes. The bacterial growth *in vivo* might have been inhibited by interfering of these compounds with one or more of the other metabolic activities of the test organism. The exact mechanism of action of ox-MKOM and ox-CYST, and which differs from that of MKOM and CYST, was identified and described in a separate communication[19].

Finally, it is worth pointing out that despite the efforts made to determine the full structural configuration of the part (X) in the compounds tested only limited information could be obtained but the exact location of the aliphatic acids[1,2] could not yet been achieved.

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