FEEDBACK INHIBITION OF THE SYNTHESIS OF AN ANTIBIOTIC: AURODOX (X-5108)†

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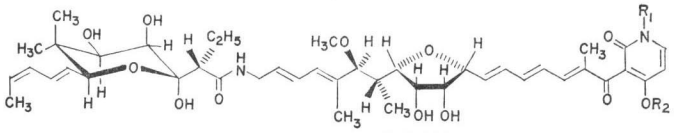
(Received for publication September 10, 1976)

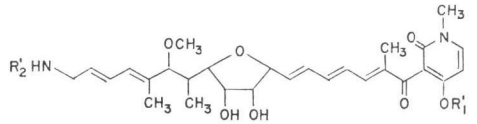
The effect of aurodox on its own biosynthesis by *Streptomyces goldiniensis* was studied. It was found that addition of exogenous aurodox inhibits further accumulation of aurodox by the antibiotic-producing culture. Both long term fermentation studies with aurodox-^{14}C and precursor incorporation studies over short time periods indicated that aurodox synthesis was regulated by feedback inhibition. The concentration of aurodox required to completely block further synthesis of the antibiotic was about 400 μg/ml. This is the same as the maximum concentration of aurodox normally accumulated by the culture used in this study. Antibiotic synthesis was inhibited not only by aurodox but also by some structural analogs of aurodox including several having no antibacterial activity. This effect was immediate and readily reversible, indicating that it could be due to inhibition of an enzyme(s) involved in the biosynthesis of aurodox.

The antibiotic aurodox (X-5108) first described by BERGER *et al.*[1] is produced by *Streptomyces goldiniensis* ATCC 21386. The antibiotic is mainly active against gram-positive bacteria and is an effective poultry growth promotant[1,11]. *S. goldiniensis* also produces an N-demethyl homolog of auro-

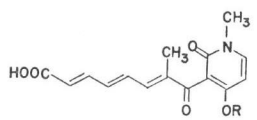
Fig. 1

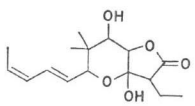


1. $R_1 = CH_3$, $R_2 = H$ Aurodox (Antibiotic X-5108)
2. $R_1 = H$, $R_2 = H$ Mocimycin (Kirromycin)
3. $R_1 = CH_3$, $R_2 = CH_3$
4. $R_1 = CH_3$, $R_2 = C_2H_5$



5. $R_1' = CH_2$–C$_6$H$_3$(Br), $R_2' = H$
6. $R_1' = CH_2$–C$_6$H$_3$(Br), $R_2' = CH_3CO$





7. $R = CH_2$–C$_6$H$_4$–Br

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† The generic name for antibiotic X-5108 is aurodox; the names, goldinodox and goldinomycin are no longer in use.

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dox[6] which was later found to be identical to kirromycin and mocimycin[12,14]. Aurodox and N-demethyl aurodox are structurally unique microbial products; their chemical structures including stereochemistry have been elucidated by MAEHR *et al.*[7,8] (Fig. 1).

During our earlier work on the biosynthesis of aurodox (LIU, C-M., H. MAEHR, T. HERMANN, M. LEACH, M. LIU and P. A. MILLER, Abstr. Ann. Meeting, American Society for Microbiology, p. 19, 1974), we had investigated the cellular and environmental factors affecting the production of aurodox by *S. goldiniensis*. While certain fermentation conditions and nutrients were found to be factors limiting the synthesis of aurodox, it was also found that the addition of aurodox to a culture interfered with further accumulation of the antibiotic. In this report we show that the biosynthesis of aurodox by *S. goldiniensis* is self-limiting, and that this limiting effect is a result of feedback inhibition. A preliminary account of this work has been reported elsewhere (LIU, C-M., T. HERMANN and P. A. MILLER, Abstr. Ann. Meeting, American Society for Microbiology, p. 194, 1975).

Materials and Methods

Organism:

A mutant of *Streptomyces goldiniensis* ATCC 21386 was used. Vegetative inoculum was developed by transferring 1 ml of a liquid nitrogen-stored stock culture into 100 ml of inoculum medium in a 500-ml Erlenmeyer flask. The inoculum medium contained (g/liter): Ardamine Z (Yeast Products Corp., Paterson, N. J.), 5; tomato pomace (Seaboard Supply Company, Philadelphia, Pa.) 5; cornstarch, 10; calcium carbonate (precipitated chalk), 1; potassium phosphate, dibasic (anhydrous), 1; and soybean oil, 10. The pH was adjusted to 7.0 before autoclaving. The culture was grown for 72 hours at 28°C on a rotary shaker running at 245 rpm. This culture was used either to inoculate a fermentation medium (3%, vol./vol.), or preserved as a stock culture in liquid nitrogen.

Fermentation:

The fermentation medium contained (g/liter): Phytone (pepsin digest of soybean meal, Baltimore Biological Laboratory Inc., Baltimore, Maryland), 15; soluble starch, 20; yeast extract (Difco), 1; potassium phosphate dibasic (anhydrous), 1; calcium carbonate (precipitated chalk), 1; $CoCl_2 \cdot 6H_2O$, 0.0238; and soybean oil, 30. The pH was adjusted to 7.5 with NaOH before sterilization. Fermentations were carried out in either 250-ml or 500-ml Erlenmeyer flasks containing 50 or 100 ml of medium respectively. Flasks capped with double layers of milk filter closures (Rapid-Flo, $6\frac{1}{2}$ inch single gauze faced disk, Filter Fabrics Inc., Goshen, Ind.) were incubated on a rotary shaker (245 rpm, 1-inch stroke) in a constant temperature incubation room maintained at 28°C.

Aurodox assay:

The standard used was 95% pure aurodox sodium salt. Aurodox in fermentation broth was assayed by an agar diffusion disc method with *Bacillus megaterium* ATCC 8011 as the test organism. Aurodox derivatives and derivatized fragments of aurodox were all assayed turbidimetrically with *Streptococcus faecalis* ATCC 8043 as the test organism. These assays have a standard error of about 15%. Aurodox was also determined by an isotope dilution technique where a measured quantity of aurodox-^{14}C (prepared by fermentation from propionate-1-^{14}C or butyrate-1-^{14}C) with known specific activity was added to an incubating culture of *S. goldiniensis*. At specific times thereafter, samples of broth were removed, adjusted to pH 7.0 with HCl, and then extracted with an equal volume of chloroform. The antibiotic in the chloroform extract was then purified by thin-layer chromatography (Merck silica gel 60 F–254) in a solvent system composed of chloroform - methanol - concentrated ammonia (80: 20: 2, vol/vol/vol). After chromatography, aurodox (Rf 0.23) was eluted from the TLC plate with methanol, and assayed spectrophotometrically at 325 nm ($E^{1\%}_{1cm}$ sodium salt 435). The radioactivity of the purified antibiotic was measured by an Intertechnique liquid scintillation spectrometer by counting 1 ml of the methanol eluant in 10 ml Aquasol (New England Nuclear, Boston, Mass.) at a counting efficiency of 71.5%. The amount of aurodox present in the culture broth can be calculated

from the ratio of the specific activity of the aurodox-^{14}C added to the broth to that isolated from the broth.

Stability of Aurodox during Fermentation:

Aqueous solutions of aurodox (including ^{14}C-labelled) added to the fermentations were sterilized by membrane filtration (0.22 μ filter unit, Millipore Corp., Bedford, Mass.). The stability of aurodox-^{14}C added to *S. goldiniensis* fermentations was determined by quantitative recovery of labelled aurodox from broth samples. An aliquot of broth adjusted to pH 9.0 was applied on a TLC plate together with unlabelled aurodox as a carrier. After chromatography (solvent system described previously) the aurodox band was located by U.V. illumination and then scraped from the TLC plate and transferred to a scintillation vial. One ml of methanol was added to extract the aurodox, and then the entire sample was diluted with 10 ml Aquasol and counted for radioactivity as described before.

Incorporation of Methionine-methyl-^{14}C into Aurodox by *S. goldiniensis* Washed Mycelium:

Washed mycelium was prepared from cultures grown for 3 days in the fermentation medium. After washing with distilled water, the mycelium was suspended in distilled water (6~30 mg dry mycelium per ml water). This suspension was incubated at 28°C, 250 rpm, in a water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, N. J.). An aqueous solution of L-methionine-methyl-^{14}C (unsterilized, 11.5 mCi/mM, New England Nuclear, Boston, Mass.) was added to the mycelial suspension to a final concentration of 1 μCi/ml (87 μM). At specified intervals, 1 ml portions of the suspension were transferred to 13×10 mm test tubes containing 2 ml chloroform and the mixture immediately mixed by vigorous shaking on a vortex mixer. TLC analysis of the chloroform extract indicated that all of the incorporated label was associated with the aurodox fraction. Quantitative determinations of aurodox-^{14}C were therefore made by simply transferring measured portions of the chloroform extract to scintillation vials, evaporating the chloroform, redissolving the residue in 1 ml methanol, diluting with 10 ml Aquasol, and counting the resulting solution in a liquid scintillation spectrometer.

Protein Synthesis:

Incorporation of valine-^{14}C by washed mycelium preparations was used as a measure of protein synthesis. Procedures for the preparation of washed mycelium and conditions for incubation were generally the same as those described in the previous section. Valine-U-^{14}C (0.25 mCi/mM, New England Nuclear Corp., Boston, Mass.) was used at a concentration of 39.4×10^{-6} M. At the specified times, 1 ml samples were removed, diluted with 1 ml 10% trichloroacetic acid (TCA) containing 0.1 mg/ml unlabelled L-valine and heated at 95°C for 45 minutes. The heated sample was then filtered through a 5-μ membrane filter (Millipore Corp., Bedford, Mass.). The filter was washed with the TCA-valine solution, 80% ethanol and finally air dried. The dried filter was transferred to a scintillation vial where it was dissolved in 1 ml methanol. Ten ml of Aquasol was added and the radioactivity determined in a liquid scintillation spectrometer.

Cell Growth:

A 5-ml portion of fermentation broth was centrifuged, the insoluble fraction washed with 5 ml *n*-hexane then twice with distilled water. The washed mycelium was dried at 100°C for 24 hours and then weighed.

Results

Stability of Aurodox

The stability of aurodox was determined under actual fermentation conditions. Aurodox-^{14}C was added to a growing culture of *S. goldiniensis* 3 days after inoculation. During the subsequent 4 days of incubation, samples were removed at 24-hour intervals and assayed quantitatively for aurodox-^{14}C. No losses in labelled aurodox occurred. In another experiment where aurodox-^{14}C was added at the time of inoculation, a loss of 15% was observed over the first 2 days of incubation (Fig. 2). However no further losses occurred, confirming the previous result on the stability of aurodox-^{14}C

Fig. 2. Stability of aurodox during fermentation.
Experimental procedure is described in Materials and Methods. Five mg aurodox-^{14}C with specific radioactivity of 2.7×10^{6} dpm/mg were added to 100 ml medium at the time of inoculation.

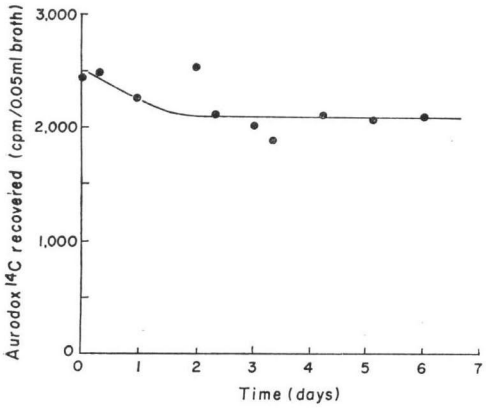
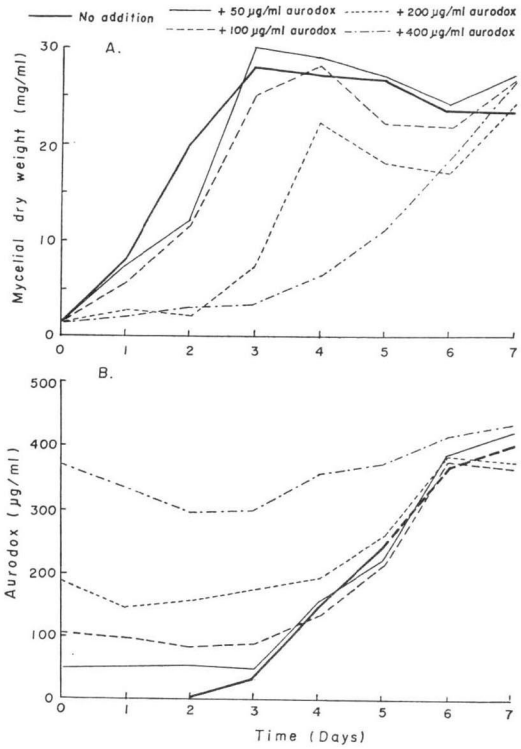


Fig. 3. Effect of various concentrations of aurodox on the growth of *S. goldiniensis* (A) and on antibiotic production (B).
The fermentation was run in 500-ml Erlenmeyer flasks containing 100 ml medium. Aurodox was added aseptically to the culture at the time of inoculation. Cell growth during fermentation was determined by measuring the mycelial dry weight. The potency of aurodox in broth was determined by a turbidimetric bioassay. Details of these measurements are given in Materials and Methods.



added at 3 days. Since no further mycelial growth took place 3 days after inoculation (Fig. 3A), these results show that aurodox is generally stable under fermentation conditions but that minor losses can occur during the growth phase of *S. goldiniensis*. The possibility that the added aurodox might be converted into its demethyl homolog (mocimycin) by the culture was also investigated. However, all attempts to demonstrate such transformation, using either cell-free extracts or intact cells, were unsuccessful.

Inhibition of *de novo* Antibiotic Synthesis

A typical time course for aurodox production in shake flask fermentations is shown in Fig. 3B. Antibiotic synthesis occurred at a linear rate from 72 hours after inoculation through the 6th day of incubation, with a final concentration of aurodox produced at about 400 μg/ml. Addition of aurodox to the fermentation at the time of inoculation reduced its production by a corresponding amount, so that the total concentration of aurodox never exceeded 400 μg/ml. Similar results were obtained when aurodox was added later in the fermentation.

This self-inhibition of aurodox synthesis was confirmed by an experiment in which the isotope dilution of labelled aurodox was monitored (Fig. 4). The specific radioactivity of aurodox-^{14}C (50 μg/ml) added at the time of inoculation was followed during the course of the fermentation. A linear decrease in specific radioactivity occurred over the period of antibiotic production. However, addition of 400 μg/ml of unlabelled aurodox at 3 days prevented any further change in specific radioactivity, presumably by blocking further synthesis of antibiotic.

Inhibition of Incorporation of Methionine-methyl-^{14}C

In the previous experiments, the self-limiting feature of aurodox synthesis was measured over relatively long time periods. By using the incorporation of label from methionine-methyl-^{14}C into aurodox (LIU, C-M., H. MAEHR, T. HERMANN, M. LEACH, M. LIU and P. A. MILLER: Abstr. Ann. Meeting, ASM, p. 19, 1974) as a measure of antibiotic synthesis, it was possible to determine changes in antibiotic levels over much shorter time intervals. Fig. 5 shows a dose response of graded concentrations of aurodox on the synthesis of aurodox-^{14}C by washed cell preparations. A typical mycelial preparation produced about 100~160 ng aurodox per mg mycelium in 1 hour. Within the first 30-minute period examined, it was clear that aurodox had a pronounced effect. Full inhibition of aurodox synthesis occurred at an antibiotic concentration of 300 μg/ml, a result which is in close agreement with previous data showing that 400 μg/ml fully blocked the *de novo* synthesis of aurodox.

Fig. 4. Effect of exogenously added aurodox on the biosynthesis of aurodox by *S. goldiniensis*.
The fermentation was started in a 500-ml Erlenmeyer containing 5 mg aurodox-^{14}C (1.95×10^6 dpm/mg) in 100 ml fermentation medium (B). At the time indicated by an arrow, 50 ml of the fermentation broth was transferred into another flask containing 20 mg unlabelled aurodox (A); incubation of both flasks was continued. Specific activities of the aurodox-^{14}C were determined on 1.0 ml portions taken from each of the two flasks; in the case of the samples from the control flask (B), 400 μg unlabelled aurodox was added to each 1 ml sample in order to facilitate isolation and determination of specific activities. Experimental procedures are described in Materials and Methods.

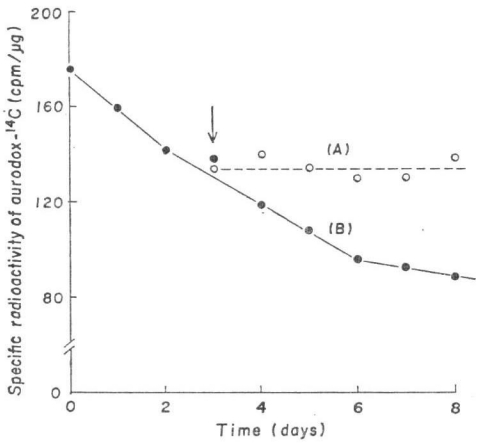
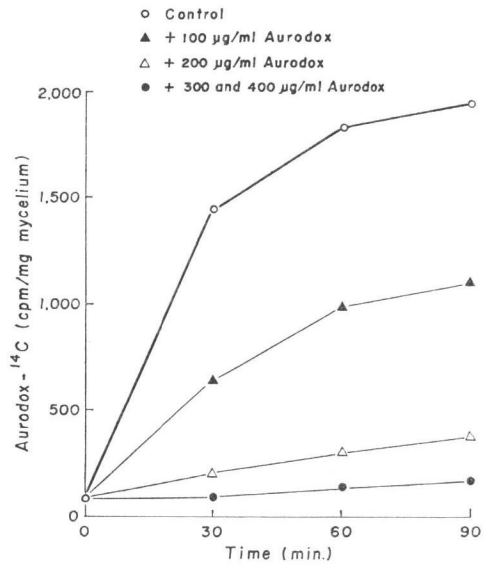


Fig. 5. Effect of aurodox on the incorporation of methionine-methyl-^{14}C into aurodox by *S. goldiniensis*.
The reaction was started by adding 3-day old washed mycelia suspended in distilled water to a mixture of methionine-methyl-^{14}C (0.2 μCi/ml) and various amounts of aurodox. Aurodox was dissolved in dimethyl sulfoxide (DMSO) before addition. Final DMSO concentration in all reaction mixtures was 8.8 mg/ml. Up to 10.0 mg/ml DMSO did not appreciably affect the assay. Incorporation of methionine-methyl-^{14}C into aurodox was measured as described in Materials and Methods.



Reversibility of Aurodox Effect

The incorporation of label from methionine-methyl-^{14}C was also a useful guide in determining the reversibility of the aurodox effect (Fig. 6). Washed cells of *S. goldiniensis* were resuspended in a buffer containing either methionine-methyl-^{14}C (control) or methionine-methyl-^{14}C plus 500 μg/ml aurodox. The suspensions were incubated and the incorporation of label into aurodox-^{14}C monitored.

Fig. 6. Reversal of aurodox effect on the incorporation of methionine-methyl-^{14}C into aurodox by *S. goldiniensis*.

All cultures were incubated in 10 ml of a medium containing washed mycelium, 295 mg dry weight; aurodox, 500 μg/ml; and methionine-methyl-^{14}C, 0.2 μCi/ml. Aurodox was omitted from the control. At the times indicated by arrows, aurodox was removed from the cultures by centrifuging and washing the mycelial pellet. The washed cultures were resuspended in a medium containing the labelled methionine at 0.2 μCi/ml. Other experimental procedures are given in the legend for Fig. 5.

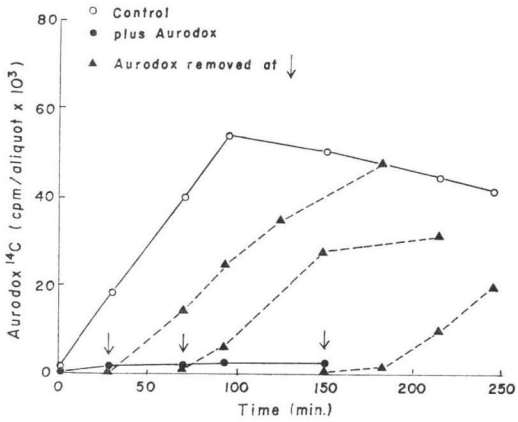
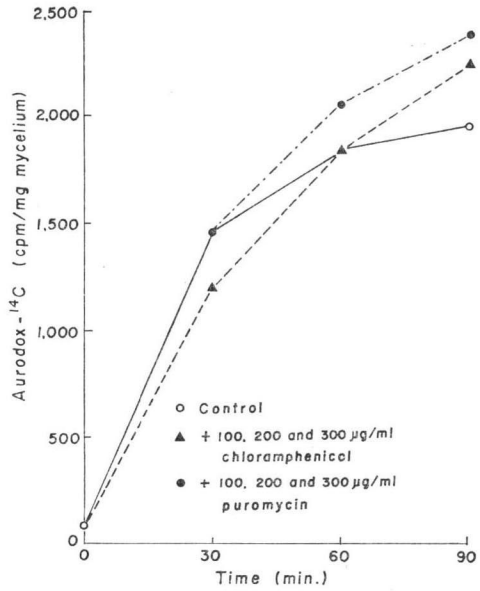


Fig. 7. Effect of chloramphenicol and puromycin on the incorporation of methionine-methyl-^{14}C into aurodox by *S. goldiniensis*.

The experimental procedure was essentially the same as that described in Fig. 5 except that mycelia were preincubated with various amounts of antibiotics for 40 minutes before the reaction was initiated by the addition of methionine-methyl-^{14}C.



At the specified times, aurodox was removed by washing, and the cells resuspended in buffer containing only methionine-methyl-^{14}C. The results clearly show that the inhibitory effect of aurodox is relieved by removing the aurodox up to 150 minutes after its introduction.

Table 1. Effect of various antibiotics on valine-^{14}C incorporation into the protein fraction of *S. goldiniensis*

Antibiotic	Concentration (μg/ml)	Valine-^{14}C incorporated (cpm/mg in 30 min)	Percent inhibition
Control	—	1,000	0
Chloramphenicol[1)]	100	45	95.5
Puromycin·HCl[2)]	100	185	81.5
Mocimycin	100	400	60.0
Aurodox	50	600	40.0
Aurodox	100	185	81.5
Aurodox	200	25	97.5
Trichloroacetic acid killed cells	—	0	—

Washed mycelia from a 2-day old culture grown in the fermentation medium were used. The mycelia were preincubated with the antibiotics for 30 minutes before the reaction was initiated by the addition of valine-^{14}C. All of the antibiotics were dissolved in an aqueous solution containing 20 percent dimethyl sulfoxide (DMSO) and 10 percent ethanol. The final DMSO and ethanol concentrations in the reaction mixture were 0.6 and 0.3% respectively. Incorporation of valine-^{14}C into the protein fraction of *S. goldiniensis* was determined by the procedure described in Materials and Methods.

1) Choramphenicol, Lot #H705230, Parke Davis and Company, Detroit, Michigan.
2) Puromycin HCl, Lot #3241B–77A, Lederle Laboratories, a Division of American Cyanamid Co., Pearl River, N.Y.

Specificity of the Aurodox Effect

Aurodox and its N-demethyl homolog are both inhibitors of bacterial protein synthesis[13] (Table 1). If the effect of aurodox on its own biosynthesis is mediated through a general inhibition of protein synthesis, then it would be expected that puromycin and chloramphenicol would similarly block aurodox accumulation. We found that the incorporation of valine-^{14}C into *S. goldiniensis* cellular protein was blocked by less than 200 μg/ml of either puromycin or chloramphenicol (Table 1). Nevertheless, neither of these inhibitors had any effect on aurodox biosynthesis when added to a washed cell preparation of *S. goldiniensis* (Fig. 7). Furthermore, several aurodox derivatives which are devoid of antibacterial activity (and therefore presumably not inhibitors of protein synthesis), were effective in blocking aurodox biosynthesis (Table 2). These results taken together suggest that the aurodox effect is a specific one depending on a structural characteristic of the aurodox molecule.

Table 2. The effect of aurodox derivatives and derivatized fragments of aurodox on the biosynthesis of aurodox

Compound*	Concentration (μg/ml)	% Inhibition of aurodox biosynthesis	% Bioactivity against *Streptococcus faecalis* ATCC 8043
1	200	40	100
	500	100	100
2	200	40	100
	500	100	
3	200	35	
	1,000	95	20
4	200	5	
	1,000	90	10
5	200	40	
	500	80	<1
6	200	65	<1
	500	90	
7	200	25	
	500	90	<1
8	200	30	
	500	45	<1

* The structures of these compounds are shown in Fig. 1.
The assay was essentially the same as described in Fig. 5. All of the compounds tested were dissolved in DMSO. Final DMSO concentration in the reaction mixtures was 7.5 mg/ml.
Bioactivity was determined by a turbidimetric bioassay described in Materials and Methods. The aurodox derivatives were provided by Dr. H. MAEHR of Hoffmann-La Roche Inc., Nutley, N. J.

Discussion

Control of the synthesis of secondary metabolites has been studied for only a limited number of products. LEGATOR and GOTTLIEB[5] and later MALIK and VINING[10] found that chloramphenicol production by *Streptomyces venezuelae* and *Streptomyces* 3022A is controlled by the antibiotic itself. Similarly, the production of penicillin and cycloheximide were also found to be self-limiting[3,4]. In each of these examples, however, the results were complicated by simultaneous production and degradation of antibiotic. In contrast, we have shown in this study that aurodox is stable during the period of its accumulation. Consequently, it was possible to directly demonstrate that aurodox production is self-limiting.

Two independent approaches were taken in studying the effect of aurodox on its own production. In one, the net synthesis of aurodox was measured under normal fermentation conditions. In the other, washed cells were used and antibiotic production was measured as methionine-methyl-^{14}C in-

corporated into aurodox. Both experimental approaches led to essentially identical conclusions. Namely that aurodox limits its own production by an amount equal to that added, and that 300~400 μg/ml of the antibiotic is sufficient to fully block further synthesis.

The effect of aurodox on its own biosynthesis cannot be attributed to a gross inhibition of protein synthesis, since neither puromycin nor chloramphenicol caused a similar effect (Fig. 7 and Table 1). Furthermore, the reversibility of the aurodox effect suggests that this effect does not involve repression of the synthesis of an enzyme(s) required in aurodox biosynthesis (Fig. 6). In another experiment not reported here, aurodox synthesis was immediately and fully restored by removing the antibiotic from a culture grown from the start in a medium containing sufficient aurodox to fully block its synthesis. While only indirect proof for a feedback inhibition mechanism for the aurodox effect has been given, the data presented are all consistent with such a mechanism.

Aurodox inhibits the growth of *S. goldiniensis* (Fig. 3A), presumably by blocking protein synthesis. During the course of a fermentation, *S. goldiniensis* gradually develops resistance to aurodox; the time required to acquire this resistance depends on the concentration of aurodox added to the culture. Development of resistance is similar to that described by MALIK for *Streptomyces* 13S[9], and appears to be due to a phenotypic change rather than a genetic modification (unpublished data). DEMAIN[2] suggests that a cell-membrane permeability change can lead to this type of resistance.

Feedback control is a general mechanism for regulation of the biosynthesis of primary metabolites. Results presented here suggest that a similar type of mechanism may occur in the regulation of the biosynthesis of a secondary metabolite, the antibiotic aurodox.

Acknowledgements

We wish to thank Dr. J. UNOWSKY for the mutant of *S. goldiniensis* used in this study, Mr. A. DOWNEY for his technical assistance and Dr. N. PALLERONI for useful discussions.

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