EFFECT OF METHYLATION INHIBITORS ON FERMENTATIONS OF *S. LINCOLNENSIS*

PRODUCTION OF N-DEMETHYLLINCOMYCIN

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The effects of several methylation inhibitors on fermentations of *Streptomyces lincolnensis* depend on the composition of the fermentation media, the levels of the metabolic inhibitors added, and the timing of the addition. Of all the compounds screened, it appears that sulfonamides and sulfanilamide in particular inhibit N-methylation, rather selectively, resulting in production of N-demethyllincomycin, in addition to the normally produced lincomycin and 4′-depropyl-4′-ethyllincomycin.

The effect of antimetabolites such as sulfa drugs[1], ethionine[2], aminopterin[3], D-methionine[4] and several homocysteine derivatives[5] has been examined by several workers studying the biosynthesis of tetracyclines. These antimetabolites added to fermentations of *Streptomyces aureofaciens* effected production of 7-chloro-6-demethyltetracycline in addition to the normally synthesized 7-chloro-tetracycline. Since the C-6 methyl group of tetracycline is derived from methionine, it has been concluded that the antimetabolites interfere either with methionine metabolism of *S. aureofaciens* or with the enzymes responsible for the methyl transfer[4]. Methylation inhibitors also decreased the production of streptomycin by *Streptomyces griseus* resulting in the formation of N-demethylstreptomycin[6].

In a continuing study on the effects of methylation-inhibiting antimetabolites on the biosynthesis of lincomycin by *S. lincolnensis* we have used DL-ethionine, several sulfonamides, folic acid antagonists, and analogs of both *p*-aminobenzoic acid and methionine. Results obtained with the use of DL-ethionine have been reported in a preceding communication[7] and are discussed briefly in a later section of this paper. We now report the effects of other methylation inhibitors on antibiotic production by *S. lincolnensis*.

Experimental

Fermentation Procedures

Seed cultures of *S. lincolnensis* var. *lincolnensis* were prepared in a medium consisting of glucose monohydrate (Cerelose), 25 g/liter and Pharmamedia (Trader's Oil Mill Co., Fort Worth, Texas, U.S.A.); presterilization pH 7.2. The cultures were incubated at 28°C for 48 hours on a rotary shaker. Fermentation media A or B (Table 1) were inoculated at a level of 5% (v/v) with the 48-hour seed medium and incubated at 28°C on a rotary shaker (250 rpm, 6-cm stroke). The metabolic inhibitors (Table 2) were added at the desired time at different levels. Fermentation beers were normally harvested after a total fermentation time of 96 hours.

Analysis of Fermentations

Fermentation beers were filtered using filter aid and analyzed by paper and thin-layer chromatographic procedures as described by ARGOUDELIS *et al.*[8] The antibiotics present in the fermentation beers were detected by bioautography on *Sarcina lutea*-seeded agar. A typical thin-layer

Table 1. Composition of fermentation media used for methylation inhibitor studies

Medium A (g/liter)		Medium B (g/liter)	
Cerelose	15.0	Glucose	30.0
Starch	40.0	Sodium citrate	3.0
Pharmamedia	25.0	$MgSO_4$	1.0
Blackstrap molasses	20.0	K_2HPO_4	2.5
$CaCO_3$	8.0	NaCl	0.5
		NH_4NO_3	2.0
		$ZnSO_4 \cdot 7H_2O$	0.001
		$FeSO_4 \cdot 7H_2O$	0.001

Fig. 1. Thin-layer chromatography of a mixture of: lincomycin (I); 4′-depropyl-4′-ethyllincomycin (II); *N*-demethyllincomycin (III); lincomycose (IV)

Thin-layer plates were prepared from silica gel G (Merck-Darmstadt). The solvent system consisted of methyl ethyl ketone-acetone-water (140 : 40 : 22 v/v). Detection systems used: periodate-permanganate spray and bioautography on *S. lutea*-seeded agar

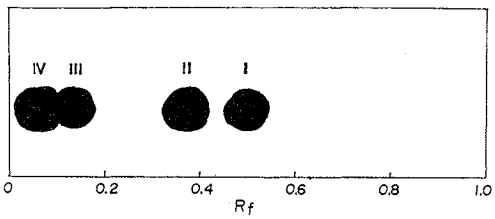


Table 2. Metabolic inhibitors* added in fermentations of *S. lincolnensis*

	Metabolic inhibitors
1	Sulfanilamide
2	Sulfaguanidine
3	Sulfadiazine
4	Sulfathiazole
5	Aminopterin
6	Amethopterin
7	4-Aminosalicylic acid
8	*p*-Nitrobenzoic acid
9	α-Methylmethionine
10	Selenomethionine

* For concentration of the inhibitors used, media, timing of addition and antibiotic production *see* text.

chromatography of the antibiotics produced in the presence of sulfonamides is shown in Fig. 1. Antibiotic titers were measured by a disc plate assay using *S. lutea* as an assay organism[9].

Fermentation Procedures for the Production of N-Demethyllincomycin (III) using Sulfanilamide as the Methylation Inhibitor

The cultures were seeded as described above and incubated at 28°C for 72 hours on a rotary shaker. Fermentation medium A (presteriliza-

Table 3. Antibiotic production by *S. lincolnensis* by addition of sulfanilamide[a] as methylation inhibitor

A. Shake flask fermentations

Bioactivity[c]			Antibiotics produced[d]	
Time[b]	Control	Plus sulfanilamide	Control	Plus sulfanilamide
24	0	—	—	—
48	86	118	—	—
72	338	296	—	—
96	792	461	**I, II**	**I, II, III,** traces of **IV**

B. Tank fermentations[e]

Bioactivity[c]		Antibiotics produced[d]
Time[b]	Plus sulfanilamide	
45	<4	—
68	89	—
89	280	**I, II, III,** traces of **IV**

[a] Five mg of sulfanilamide were added per ml of fermentation medium.
[b] Hours after inoculation.
[c] Antibiotic titers were measured by disc plate activity using *S. lutea* as assay organism, and are expressed in mcg of lincomycin present in 1 ml of filtrate.
[d] Tlc or paper chromatography was used for identification of antibiotics produced.
[e] Control tank fermentations produced *ca* 1,000 mcg/ml of bioactivity due to **I** and **II**.

tion pH, 6.8) was inoculated at a level of 5 %(v/v) with the 48-hour medium and incubated at 28°C. Twenty four hours after inoculation, 5 mg of sulfanilamide was added per ml of medium. Beers were harvested after total fermentation time of 96 hours, and tested for bioactivity against *S. lutea* and N-demethyllincomycin content, using thin-layer and paper chromatography[8]. Results obtained in shaken flasks and tank fermentations are shown in Table 3.

Extraction Procedures. Isolation of N-Demethyllincomycin (**III**)

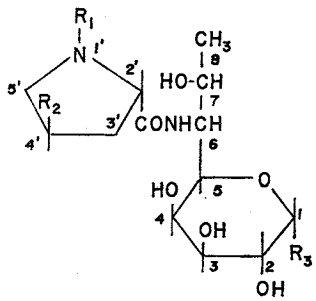
Fermentation beer (4 liters) was filtered using filter aid. The mycelial cake was washed with water and the cake was discarded. The filtrate was adjusted to pH 6.0 and extracted with 400 ml of a 9 % solution of sodium dinonylnapthalene sulfonate in Skellysolve B. The extract was first washed with 200 ml of water, then it was mixed with 200 ml of water and 160 ml of a 25 % solution of Aliquat-336* in Skellysolve B. The mixture was shaken well and then the two phases were allowed to separate. The upper phase (Skellysolve B-phase) was discarded. The aqueous phase (lower) was washed with 200 ml of Skellysolve B, adjusted to pH 10.0 and then extracted three times with 350 ml portions of methylene chloride. The methylene chloride extracts, concentrated to dryness, afforded material containing lincomycin and 4′-depropyl-4′-ethyllincomycin as the only bioactive components.

The spent aqueous solution (pH 10.0) was then extracted three times with 350 ml portions of 1-butanol. The butanol extracts were combined and concentrated to dryness. The residue crystallized after trituration with 95 percent aqueous ethanol. The crystalline material was isolated by filtration and recrystallized from water-acetone; yield 140 mg, $[\alpha]_D^{25}+150°$ (*c* 0.4, water) (reported for N-demethyl-lincomycin, +149°[8]). *Anal.* Calcd. for $C_{17}H_{32}N_2O_6S\cdot HCl\cdot H_2O$: C, 45.73; H, 7.90; N, 6.28; S, 7.18; Cl, 7.94; O, 25.08. Found: C, 45.62; H, 7.78; N, 6.23; S, 7.31; Cl, 7.82; O, 25.24 (by difference). I.R. spectrum and paper and thin-layer chromatographic patterns of the isolated material were identical to those reported for N-demethyl-lincomycin.

Results and Discussion

Fermentations of *Streptomyces lincolnensis* var. *lincolnensis* contain lincomycin (**I**)[10] and 4′-depropyl-4′-ethyllincomycin (**II**)[11] (Fig. 2). Biosynthetic studies have shown[12,13] that the $-NCH_3$, the $-SCH_3$ and the terminal $-C-CH_3$ group of the amino acid part of both antibiotics are derived from one carbon donor systems.

Fig. 2



Lincomycin (**I**):
$R_1=CH_3$; $R_2=CH_2CH_2CH_3$; $R_3=-SCH_3$
4′-Depropyl-4′-ethyllincomycin (**II**):
$R_1=CH_3$; $R_2=CH_2CH_3$; $R_3=-SCH_3$
N-Demethyllincomycin (**III**):
$R_1=H$; $R_2=CH_2CH_2CH_3$; $R_3=-SCH_3$
Lincomycose (**IV**):
$R_1=CH_3$; $R_2=CH_2CH_2CH_3$; $R_3=-OH$

Our previous work on the influence of DL-ethionine on antibiotic production by *S. lincolnensis*[7] showed marked differences depending on the medium used and the timing of the addition of methylation antagonist. Addition of the

Table 4. Relative bioactivity of lincosaminide antibiotics

Antibiotic	Bioactivity[a]
Lincomycin	1,000
4′-Depropyl-4′-ethyllincomycin	350
N-Demethyllincomycin	50
Lincomycose	10

[a] Biological activity was determined by disc plate assay using *S. lutea* as the assay organism and is expressed in mcg of lincomycin per mg of the antibiotic under consideration.

* Industrial grade tricaproyl methyl ammonium chloride supplied by General Mills, Chemical Division, Kankakee, Illinois, U.S.A.

aminoacid to fermentations of *S. lincolnensis* grown in a complex medium result in the production of S-demethyl-S-ethyllincomycin. However, in a synthetic medium, ethionine produces S-demethyl-S-ethyllincomycin and N, S-didemethyl-N, S-diethyllincomycin[7].

The metabolic inhibitors used in the present study (Table 2) included sulfonamides (compounds **1**~**4**), folic acid antagonists (compounds **5** and **6**), *p*-aminobenzoic acid analogs (compounds **7** and **8**) and methionine antagonists (compounds **9** and **10**). In contrast to the ethionine work we expected no replacement reactions to occur in this study. Therefore, we assumed that addition of metabolic inhibitors which effect transmethylation reactions to fermentations of *S. lincolnensis* could produce changes at one or more sites of the lincomycin molecule.

Since, as shown in Table 4, the relative bioactivities of the listed antibiotics differ considerably, we were required to use paper and thin-layer chromatography to evaluate the effect of each metabolic inhibitor on the *S. lincolnensis* fermentation.

Effect of Medium

In the absence of any inhibitor, levels of *S. lutea* activity of *ca* 1,000 mcg/ml were obtained when the complex medium A (Table 1) was used for the fermentation of *S. lincolnensis*. On the other hand the levels of antibiotics produced, in the absence of any inhibitor, using the chemically defined medium B (Table 1) were usually low (*ca* 300~400 mcg/ml). In both cases lincomycin and 4′-depropyl-4′-ethyllincomycin were the only antibacterials produced. The ratio of lincomycin to its 4′-ethyl analog was determined by gas liquid chromatography to be 95 : 5.

Addition of any of the metabolic inhibitors listed in Table 2 to cultures of *S. lincolnensis* grown in the synthetic medium B resulted in marked reduction of the growth of the organism and almost no production of antibiotics. These results were obtained whether the inhibitors were added at 0 time (inoculation time) or 24 or 48 hours after inoculation.

Effect of Timing of Addition

Addition of any of the compounds listed in Table 2 at 0 time in fermentations grown in the complex medium A resulted in inhibition of growth of the organism. Although only a slight effect on the growth of the organism was observed when the metabolic inhibitors were added 24 hours after inoculation, a significant effect on both the antibiotic levels and the antibiotics produced was observed. (See subsequent discussion). Addition of any of the inhibitors at 48 hours after inoculation did not effect the growth of the organism, the antibiotic titers or the antibiotics produced; lincomycin and 4′-depropyl-4′-ethyl lincomycin were the only compounds detected in such fermentations. We therefore determined the production of antibiotics in the presence of the methylation inhibitors by adding the inhibitors 24 hours after inoculation using the complex medium A (Table 1).

Production of N-Demethyllincomycin (III). Effect of Sulfonamides

The biological transfer system for one-carbon groups involves tetrahydrofolic acid (THFA). Originally sulfonamides were thought to act as competitive inhibitors of the enzymes involved in the formation of dihydropteroic acid, an intermediate in the biosynthesis of THFA. The action of sulfonamides is now believed to be due, in part, to their acceptance as substrates with the consequent formation of modified folic acids within the bacterium[14].

Addition of the sulfonamides listed in Table 2 at levels below 1 mg/ml of medium had no effect on the growth, the antibiotic titers, or the antibiotics produced. Lincomycin and 4′-depropyl-4′-ethyllincomycin were the only antibiotics detected. At concentrations of 1～5 mg/ml of medium a slight effect on the growth of the organism and decrease of the antibiotic titers were observed. For example, at 5 mg/ml (Table 5) a decrease of *ca* 30 % of antibiotic titers was observed. Examination of these fermentations showed the presence of one major and one minor bioactivity in addition to lincomycin and its 4′-ethyl analog. The minor activity was not isolated but its paper and tlc behavior

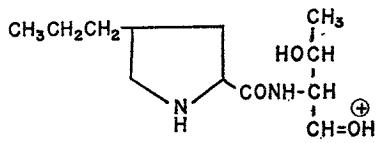
Table 5. Antibiotics[a] produced by *S. lincolnensis* in fermentations supplemented with sulfonamides[b]

Time[d]	Bioactivity[c] of fermentations containing the following inhibitors				
	None	Sulfanilamide	Sulfadiazine	Sulfaguanidine	Sulfathiazole
24	0	—	—	—	—
48	86	118	86	108	90
72	338	296	310	326	270
96	792	461	520	530	460
Antibiotics produced	I, II[e]	I, II[e], III, IV[e]	I, II[e], III, IV[e]	I, II[e], III, IV[e]	I, II[e], III, IV[e]

[a] Antibiotics were detected by paper and tlc chromatography.
[b] Five mg/ml of the sulfonamide were added 24 hours after inoculation.
[c] Bioactivity against *S. lutea* is expressed as mcg/ml of lincomycin.
[d] Hours after inoculation.
[e] 4′-Depropyl-4′-ethyllincomycin (**II**) and lincomycose (**IV**) were produced in small amounts.

was identical to that of lincomycose (**IV**), a compound described earlier as a product of prolonged fermentations of *S. lincolnensis*[15]. The major bioactivity behaved like N-demethyllincomycin in several chromatographic systems. This antibiotic has been described earlier[9] as the product of fermentations of *S. lincolnensis* when methyl α-thiolincosaminide (the aminosugar present in lincomycin) is added to the fermentation medium. N-Demethyllincomycin was extracted from the fermentation filtrate by the liquid ion-exchange procedure described in the experimental. This procedure proved to be an excellent method for the fast extraction and evaluation of fermentations. N-Demethyllincomycin was obtained as the crystalline hydrochloride and identified by comparison of specific rotation, IR and NMR spectra to those of authentic sample. In addition the mass spectrum of the product showed a molecular ion peak at *m/e* 392 (calcd. mol. weight, 392). A fragment peak at *m/e* 345 is assigned to M-SCH_3. A peak at *m/e* 243 is assigned to ion **V** (Fig. 3). Finally the base peak at *m/e* 112 is due to ion **VI** (Fig. 3) resulting from the amino acid moiety (4-*n*-propylproline). Amounts of 100～150 mcg of N-demethyllincomycin have been isolated from shake flask fermentations. Tank fermentations (Table 3) were run using sulfanilamide (5 mg/ml) as the metabolic inhibitor, since it appeared to yield larger amounts of N-demethyllincomycin than other sulfonamides.

Fig. 3



V *m/e* 243 VI *m/e* 112

Although the antibiotic titers in tank fermentations containing sulfonilamide were lower than those obtained in shake flasks (Table 3), the ratio of lincomycin and 4′-depropyl-4′-ethyllincomycin to N-demethyllincomycin favored the latter antibiotic. Yield of 150~200 mcg of N-demethyllincomycin were obtained routinely in large-scale fermentations.

Effect of Folic Acid Antagonists

Amethopterin and aminopterin have been shown to inhibit dihydrofolate reductase, the enzyme transforming dihydrofolic acid to tetrahydrofolic acid[14,16]. The results obtained with amethopterin and aminopterin fermentations of *S. lincolnensis* are presented in Table 6. At concentrations of 0.1 or 0.01 mg/ml amethopterin did not inhibit the growth of *S. lincolnensis* and good antibiotic titers, due to lincomycin only, were observed. When either compound was added at concentrations of 1 mg/ml or greater, poor or no growth of the organism was observed. The antibiotic titers were severely depressed and lincomycin again was the only antibiotic detected.

Table 6. Antibiotics[a] produced by *S. lincolnensis* in fermentations supplemented with folic acid antagonists[b]

Time[c]	Bioactivity[d] of fermentations containing					
	Aminopterin (1 mg/ml)	Amethopterin (1 mg/ml)	Amethopterin			Control[e]
			1 mg/ml	0.1 mg/ml	0.01 mg/ml	
24	—	—	—	—	—	0
48	0	7.7	1.9	68	86	86
72	5.8	12	<9.0	308	348	338
96	5.7	12.2	4.1	648	648	792
Antibiotics produced	traces of lincomycin	traces of lincomycin	traces of lincomycin	lincomycin	lincomycin	lincomycin

[a] Antibiotics were detected by paper and tlc chromatography.
[b] The folic acid antagonists used were added 24 hours after inoculation.
[c] Time is expressed in hours after inoculation.
[d] Bioactivity against *S. lutea* is expressed as mcg/ml of lincomycin.
[e] Control fermentation did not contain any metabolic inhibitor.

Effect of *p*-Aminobenzoic Acid Analogs

p-Aminosalicylic acid (PAS) is thought to act by the same biochemical mechanism as the sulfonamides. However, this substance has a specific action against the tubercle bacillus[14]. PAS added in fermentations of *S. lincolnensis* inhibit neither the growth of the organism, nor affected antibiotic titers. Furthermore, no production of any antibiotic expected to result from the inhibition of methylation, was observed.

In contrast to the action of *p*-aminosalicylic acid, *p*-nitrobenzoic acid had a profound effect on the fermentations of *S. lincolnensis* (Table 7). At 0.1 mg/ml concentrations no effect was observed on the growth of the organism or the antibiotics produced. At higher levels of the acid (1 mg/ml) a marked decrease (*ca* 65 %) of antibiotic titers was observed with concomitant production of small amounts of N-demethyllincomycin. At levels of 5 mg/ml *p*-nitrobenzoic acid inhibited antibiotic production without inhibiting the growth.

Table 7. Effect of *p*-nitrobenzoic acid[a] on fermentations of *S. lincolnensis*

Time[c]	Bioactivity[b] of fermentations containing *p*-nitrobenzoic acid (PNBA)			
	Control	PNBA (0.1 mg/ml)	PNBA (1 mg/ml)	PNBA (5 mg/ml)
24	0	0	0	0
48	68	70	0	0
72	776	692	140	0
96	1036	996	386	0
Antibiotics[d] produced	**I, II**	**I, II**	**I, II** traces of **III**	None

[a] *p*-Nitrobenzoic acid was added 24 hours after inoculation.
[b] Bioactivity against *S. lutea* is expressed as mcg/ml of lincomycin.
[c] Time is expressed in hours after inoculation.
[d] Antibiotics were detected by paper and thin-layer chromatography.

Effect of Methionine Analogs

α-Methyl-DL-methionine and selenomethionine were used in the present work as methionine antagonists. Inhibition of methylation by these compounds could result from competition with methionine for ATP to form the corresponding adenosyl aminoacids, and the subsequent antagonism of the unnatural nucleotides with S-adenoxyl methionine.

α-Methyl-DL-methionine had no effect on fermentations of *S. lincolnensis* when added 24 hours after inoculation at levels of 0.1 to 1 mg/ml. At 5 mg/ml a decrease of antibiotic titers (*ca* 40%) was observed. N-Demethyllincomycin was found to be among the antibiotics produced in the fermentation. However, much less N-demethyllincomycin was produced under the above conditions than was produced when sulfanilamide was the metabolic inhibitor.

Selenomethionine was found to interfere with the antibiotic production. When levels of 2.5 mg/ml were used, antibiotic titers of 13 mcg/ml were observed (control, 990 mcg/ml). No N-demethyllincomycin was found in the fermentation.

Conclusion

Most of the metabolic inhibitors used in the present study effect the fermentations of *S. lincolnensis* (growth of the organism, antibiotic composition) in varying degrees. Of all the compounds screened, it appears that sulfonamides and sulfanilamide in particular inhibit N-methylation rather selectively and result in the production of N-demethyllincomycin.

The present production of N-demethyllincomycin is the third method reported for the generation of this antibiotic by microbial means. The other two are the production of N-demethyllincomycin by *S. lincolnensis* in media supplemented with methyl α-thiolincosaminide[8], and the N-demethylation of lincosaminides by streptomycetes like *Streptomyces punipalus*[17].

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