DIRECTED BIOSYNTHESIS OF NEW INDOLMYCINS

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Tryptophan in a concentration of 0.4 μg/ml increased the production of indolmycin by 37%. The lipophilic character of indolmycin was reduced *via* directed biosynthesis by substituting the aromatic ring system with a methoxy or hydroxy group in the 5-position of the antibiotic. This substitution was achieved by the addition of the corresponding tryptophan and indole precursors to a growing culture of *Streptomyces griseus* ATCC 12648.

The more hydrophilic indolmycin derivatives displayed a moderate increase in antimicrobial activity as compared to indolmycin, but did not markedly change the Gram-positive/Gram-negative ratio of activity. Thin-layer chromatography and mass spectrometry showed that additives substituted in the 6-position were not incorporated into the molecule. Antibiotic titer was reduced by addition of the modified precursors, especially in the case of the precursors substituted in the 6-position.

Indolmycin, a tryptophan analog, inhibits tryptophanyl t-RNA ligase in cell-free systems from Gram-positive and Gram-negative bacteria[1]. Despite this, the spectrum of antibacterial activity is restricted mainly to Gram-positive bacteria[2,3,4].

In *Staphylococcus aureus* and *Bacillus subtilis*, indolmycin is transported by the uptake systems for tryptophan[5]. Because a comparable transport system is present in *Escherichia coli*, the poor activity of indolmycin against Gram-negative bacteria might be explained by the failure of this hydrophobic antibiotic to penetrate through the hydrophilic barrier of the Gram-negative outer membrane. The biosynthesis of indolmycin originates from tryptophan, arginine and methionine[6,7]. In an attempt to reduce the lipophilic character of the antibiotic, substitution of the aromatic ring system with a methoxy or a hydroxy group was investigated by means of directed biosynthesis.

Materials and Methods

Microorganisms

Streptomyces griseus ATCC 12648, the producer of indolmycin and the test organisms *Staphylococcus aureus* ATCC 13150 and *Escherichia coli* ATCC 11775 were obtained from the American Type Culture Collection. *Escherichia coli* DC 2, a permeability mutant was a gift of Prof. MARK H. RICHMOND, Department of Bacteriology, University of Bristol, Bristol, U.K.

Media

The fermentation broth consisted of 20 g peptone and 2 g yeast extract in 50 mM morpholinoethylsulfonic acid (MES) buffer, pH 6.8. For detection of antimicrobial activity during the fermentation and for the determination of the minimal inhibitory concentrations (MIC), the following medium was used: 2 g glucose, 1 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 7 g K_2HPO_4, 0.1 g $MgSO_4$, 20 mg of all amino acids except tryptophan and 5 ml vitamin solution (Flow Laboratories) in 1 liter distilled water pH 7.2. For preparing agar plates, a further addition of 15 g Difco-agar was made.

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Materials

The potential precursors 5-DL-methoxytryptophan, 5-DL-hydroxytryptophan, tryptophan and the amino acids used in the broth dilution test were obtained from Serva Feinbiochemica GmbH & Co, Heidelberg. 5-Methoxy-, 6-methoxy and 5,6-methylenedioxyindole were gifts from Hoffmann-La Roche, Nutley, New Jersey, U.S.A. Silica gel thin-layer chromatography (TLC) plates 60 F_{254} and all other chemicals were purchased from Merck, Darmstadt.

Production of Indolmycins

Streptomyces griseus ATCC 12648 was grown in 500 ml shake flasks containing 100 ml of fermentation broth. The flasks were incubated at 27°C and agitated at 120 rpm for 40 hours. All potential precursors were added in concentrations of 0.1, 0.2 and 0.4 μg/ml 20 hours after inoculation, before antibiotic synthesis starts. Antibiotic activity was analysed by a disc diffusion assay using *Staphylococcus aureus* ATCC 13150 as test organism. For analysis of the reaction products, the culture filtrate was adjusted to pH 3 with 1 N HCl and extracted three times with 50% (v/v) of ethyl acetate.

The combined organic phases were re-extracted by 5% (v/v) of a 10% (w/v) Na_2CO_3 solution, dried over Na_2SO_4 and concentrated under reduced pressure. After separation by TLC using chloroform - dimethylformamide - ethyl acetate (3: 1: 1) as developing solvent, the biologically active fractions were scraped from the plates and analyzed by mass spectrometry.

Minimal Inhibitory Concentrations (MIC)

The MIC values were determined by a broth dilution test in microtiter plates with *Staphylococcus aureus* ATCC 13150, *Escherichia coli* ATCC 11775 and the permeability mutant *Escherichia coli* DC 2 using an inoculum size of 10^4 viable cells/ml and an incubation period of 16 hours at 37°C.

Physico-chemical Analysis

The partition coefficient was determined in an octanol - H_2O (1: 1) solvent system. The concentration of antibiotic in the organic and aqueous phases was determined photometrically.

Mass spectra were taken with a Varian-Mat instrument model CH-5, high resolution mass spectra with an AEI-instrument, Model MS-903, equipped with a data system using a PDP-8 computer.

Results

Incorporation of tryptophan derivatives or indole derivatives led to an overall reduction in antibiotic titer. Supplementation with the indole compounds was limited to the highest non-toxic concentration of 0.1 μg/ml, whereas the tryptophan derivatives were tolerated in a concentration of 0.4 μg/ml. These concentrations resulted in normal growth curves. The inhibitory effect on antibiotic synthesis observed with the indoles substituted in the 5-position was greater than that of the tryptophan derivatives. In general, substitution of indole in the 6-position led to a stronger inhibitory effect. The production of indolmycin was stimulated by tryptophan even in a complex fermentation medium (Fig. 1). The production of antibiotics with the potential precursors is shown in Figs. 1 and 2.

Thin-layer chromatography and mass spectrometry demonstrated that only derivatives substituted in the 5-position are incorporated into the antibiotic (Fig. 3). The comparable indole and tryptophan derivatives yielded the same indolmycin compound. The amount of substituted indolmycins, determined by TLC is about 30% of the total biosynthesis of indolmycin. The only antibiotic synthesized in the presence of the derivatives substituted in 6-position was indolmycin. The methoxy and the hydroxyindolmycin derivatives, which are more hydrophilic due to their substitution, did not show a selectively increased Gram-negative activity (Table 1). However, these results do demonstrate that 5-substituted indole and tryptophan derivatives are incorporated into the indolmycin molecule and that an increase in hydrophilicity may increase the antimicrobial activity.

Fig. 1. Production of indolmycin and derivatives following addition of the corresponding tryptophan derivatives.

The arrow indicates the time of addition of 0.4 μg/ml of the precursors: (■) tryptophan, (▲) 5-methoxytryptophan and (▼) 5-hydroxytryptophan to the fermentation broth. (□) Control represents indolmycin biosynthesis with no addition; (●) growth and (○) pH of all the cultures.

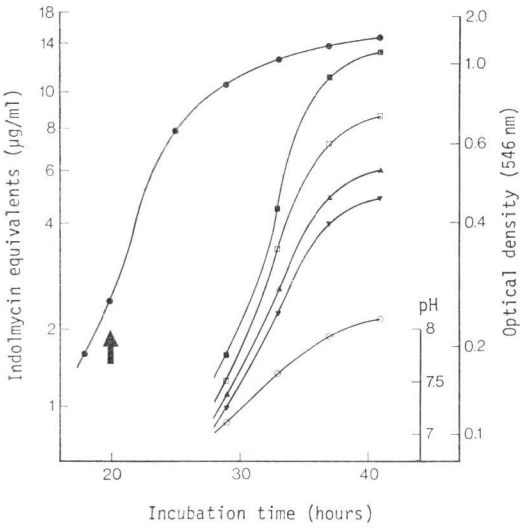


Fig. 2. Production of indolmycin and derivatives following addition of the corresponding indole derivatives.

The arrow indicates the time of addition of 0.1 μg/ml of the precursors: (■) 5-methoxyindole, (▲) 5-hydroxyindole, (▼) 6-methoxyindole and (▽) 5,6-methylenedioxyindole to the fermentation broth. (□) Control represents indolmycin biosynthesis with no addition; (●) growth and (○) pH of all cultures.

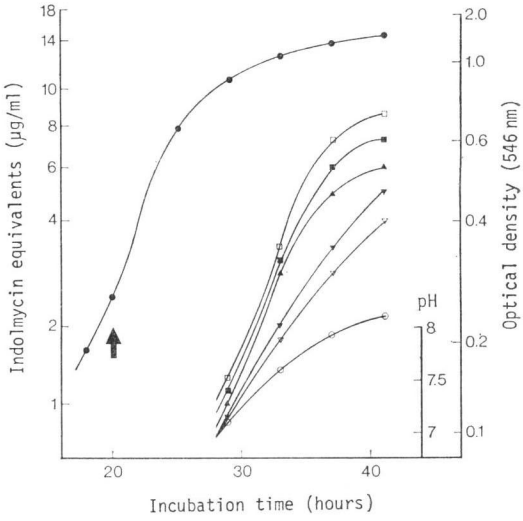
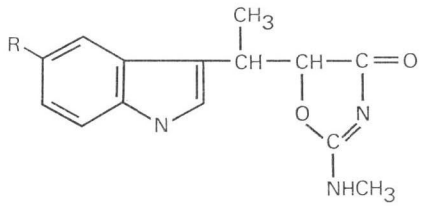


Table 1. Biological and physico-chemical properties of indolmycin and their derivatives.

	Indolmycin	5-Methoxy indolmycin	5-Hydroxy indolmycin
Molecular weight	257	287	273
Partition coefficient	1.02	0.98	0.34
Rf value	6.8	6.4	5.9
MIC (μg/ml)			
S. aureus ATCC 13150	0.3	0.3	0.15
E. coli ATCC 11775	30.0	25.0	10.0
E. coli DC 2	0.3	0.3	0.3

Fig. 3. Structural formula of indolmycin and its derivatives produced by directed biosynthesis.



R=H Indolmycin
R=OCH_3 5-Methoxyindolmycin
R=OH 5-Hydroxyindolmycin

Discussion

The fact that derivatives substituted in 5-position are incorporated into the antibiotic in contrast to derivatives substituted in 6-position indicates a stereoselectivity of the tryptophan synthase for the corresponding precursor. The stronger inhibitory effect of the indole derivatives with respect to the tryptophan derivatives on the growth of the producing strain might be explained by greater inhibition of tryptophan biosynthesis by the indole compounds. The overall reduction of the biosynthesis of indolmycin and its derivatives could be due to a lower affinity of the biosynthetic enzymes to the substituted precursors.

Similar to the biosynthesis of indolmycin, addition of tryptophan stimulates the production of pyrrolnitrin[8], whereas indole derivatives possess an inhibitory effect[9]. In the case of pyrrolnitrin biosynthesis, addition of indole to the fermentation broth led to the formation of an indole condensation product 2,2-bis(3'-indolyl)indoxyl[10].

The comparison of the MIC values of indolmycin against *Escherichia coli* ATCC 11775 and the permeability mutant *Escherichia coli* DC 2 clearly demonstrates that the lack of penetration into wild type *Escherichia coli* strains is the one deciding factor for the reduced effectiveness of the antibiotic. In *Escherichia coli* DC 2, the same MIC value is found as in *Staphylococcus aureus* ATCC 13150.

On the other hand, the results indicate that the more hydrophilic indolmycin derivatives are better able to penetrate the outer membrane of *Escherichia coli* ATCC 11775. This suggests that tryptophan derivatives with an even more hydrophilic substituent, such as 5-methylsulfinyl- or 5-methylsulfonyltryptophan, might further improve the activity. To increase the productivity of the indolmycin derivatives, tryptophan auxotroph mutants of *Streptomyces griseus* ATCC 12648[11] should be used in further studies.

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