MICROBIAL OXIDATION OF AMINOIMIDAZOLES TO NITROIMIDAZOLES

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The structural requirements for the microbial transformation of 2-aminoimidazoles to 2-nitroimidazoles by *Streptomyces* strain LE/3342 have been investigated. Substrates carrying an alkyl group in position 4 (or 5) were oxidized, the efficiency decreasing with the increase of the length of the chain. Lower yields in the oxidation were observed with the 4,5-dimethyl derivative. When a methyl group in position 1 or a phenyl group in position 4 (or 5) was present no transformation product could be detected. The influence of the substrate concentration and the time course of the transformation are reported and discussed.

Studies on the biogenesis of nitro compounds of biological origin have led to the discovery of microorganisms able to oxidize aromatic amines to the corresponding nitroderivatives.

The oxidation of *p*-aminobenzoate to *p*-nitrobenzoate by *S. thioluteus* has been observed by KAWAI, OSHIMA and EGAMI[1]. It was then found that the same microorganisms oxidizes other aromatic amines bearing substituents in *para* position[2].

During our studies on the biogenesis of azomycin (2-nitroimidazole) we were able to demonstrate[3] that a *Streptomyces* strain which produces this antibiotic (*Streptomyces* sp. LE/3342) is capable to oxidize 2-aminoimidazole and 4(5)-methyl-2-aminoimidazole to azomycin and 4(5)-methylazomycin respectively.

We wish now to report the microbial oxidation to nitroimidazoles of other aminoimidazoles. This process provides an useful method for the preparation of some of these compounds which could not be obtained by the synthetic approach and which are of interest because azomycin and several derivatives of this antibiotic possess a notable antiprotozoal activity[4,5]. Data on the substrate specificity, time course and concentration dependence of this microbial reaction are reported and discussed.

Materials and Methods

Culture and Transformation Conditions

Throughout all this work *Streptomyces* strain LE/3342 was used. The strain was maintained on slants of oatmeal agar at 4°C.

For the fermentations the complete medium AZ/2, whose composition has been previously reported[3], was used. Fermentations were carried out aerobically at 28°C in 500 ml Erlenmeyer flasks containing 100 ml of the medium, agitated on a rotary shaker at 200 r.p.m. The growth estimated as p.m.v. (percent mycelium value) generally reached a stationary phase after about 48 hours. When the transformation experiments were performed with growing cultures the substrate was added in hydroalcoholic solution to

48-hour cultures and the fermentation was continued in the same conditions for a further 72 hours. In transformation experiments with resting cells, the mycelium of 48-hour cultures was harvested, washed with 0.07 M phosphate buffer (pH 6.5) and suspended in buffer of the same composition to give a cell suspension corresponding approximately to 10 mg of dry cells per ml. The substrate was added and the mixture incubated again at 28°C on a rotary shaker for different periods of time.

Extraction and Purification

As a rule the nitroimidazoles were extracted from the filtered acidified broth with ethyl acetate. A partial purification from other antibiotic substances produced by the strain was obtained by reextraction in borate buffer at pH 10 (solution A), acidification of the buffer solution and extraction in ethyl acetate (solution B). Evaporation of the solvent yields a crude mixture of the nitroimidazoles. The final separation and purification was performed by counter current distribution in an O. Post 200 tube apparatus (volume of each phase 10 ml per tube). The solvent systems used were: Ethyl acetate/phosphate buffer (pH 6.5) or ethyl acetate/phosphate buffer (pH 7.38).

At the end of the distribution process the fractions which on spectrophotometric analysis showed the presence of nitroimidazoles were collected and the products recovered by extraction in ethyl acetate, evaporation of the solvent and crystallization from a suitable solvent. In the attempts to convert 1-methyl-2-aminoimidazole, the reextraction with borate buffer (pH 10) was replaced by reextraction with 5 N hydrochloric acid, since the expected product of the reaction, the 1-methyl-2-nitroimidazole, is a weak base[6].

Analyses

An estimate of the total amount of nitroimidazoles extracted was given by the light absorption of the borate buffer solution A (see above under "extraction and purification"), in the region of 370~410 mμ. For this estimate an approximate log ε of 4.2 was assumed for the C-alkyl-2-nitroimidazoles. Quantitative determinations were performed by paper chromatography of aliquots of the ethyl acetate extracts (solution B). Solvent system: phosphate buffer (pH 7.38) as a stationary phase, ethyl acetate as a mobile phase, paper Whatman 3MM. The areas containing the nitroimidazoles, visualized by ultraviolet light, were eluted separately with borate buffer (pH 10) and the amounts determined spectrophotometrically. The λ_{max} and the log ε values of the single compounds are given under "Results".

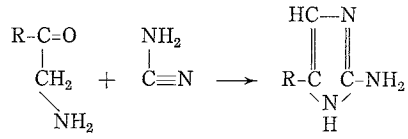
Synthesis of the Substrates

The following products: 1-methyl-2-aminoimidazole·HCl, 4(5)-methyl-2-aminoimidazole·HCl, 4,5-dimethyl-2-aminoimidazole·HCl, 4(5)-phenyl-2-aminoimidazole, were prepared as previously described[7].

The 4(5)-ethyl-, 4(5)-*n*-propyl-, 4(5)-isopropyl-, and 4(5)-*n*-butyl-2-aminoimidazoles were prepared (Fig. 1) by condensation of the appropriate α-aminoketones and cyanamide by means of the following procedure:

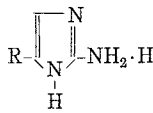
To a solution of 0.1 mole of α-aminoketone hydrochloride in 50 ml of water, brought to pH 4.5 with 1 N sodium hydroxide, 0.2 mole of cyanamide is added and the resulting mixture is heated on a steam bath for one and half hours. By evaporation of the water under reduced pressure a syrupy residue is obtained which on treatment with anhydrous ethyl ether solidifies into a brownish powder. The product is taken up in 100 ml of a 5% ethanolic solution of hydrogen chloride, the insoluble inorganic salts removed by filtration and the solvent evaporated to dryness. Recrystallization of the residue from methylethylester gives the pure 2-aminoimidazole.

Fig. 1. Synthesis of 4(5)-alkyl-2-aminoimidazoles



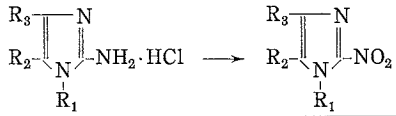
In the following table (Table 1) are given the m. p. of the starting aminoketones, prepared by the GABRIEL synthesis from chloroketones, the melting points and analyses of the 2-aminoimidazoles obtained and the reaction yields.

Table 1. Synthesis of alkyl-2-aminoimidazoles

R-CO-CH_2-NH_2·HCl[1]							
R	m. p.	% yield	m. p.	Analyses			
				C	H	N	Cl
C_2H_5	152°	98	151~152°[2]	calcd. 40.68 found 40.51	6.83 6.82	28.45 28.60	24.02 23.80
n-C_3H_7	163~164°	84	128~129°	calcd. 44.58 found 44.66	7.48 7.78	26.00 25.86	21.94 21.72
i-C_3H_7	164°	90	122~123°	calcd. 44.58 found 44.57	7.48 7.40	26.00 26.07	21.94 21.60
n-C_4H_9	112°	95	174~175°[3]	calcd. 42.39 found 42.45	4.38 4.60	22.82 22.99	

1) These α aminoketones have previously been prepared by others by different methods. The melting points found, shown above, coincide with those reported in the literature.
2) This substance had been obtained previously by chemical degradation of roseothricin[8].
3) The product was identified as picrate.

Table 2. Biological oxidation of 2-aminoimidazoles to 2-nitroimidazoles by *Streptomyces* LE/3342



Substrate added	mg	Transformation			Nitroimidazole obtained[4]		
		Conditions	Volume	Incubation period (hours)	mg	m. p.	Electronic spectrum (borate buffer pH 10)
R_1=R_2=H, R_3=C_2H_5	405	Washed mycelium	2,700	24	148	153~155°[1]	λ_{max} 395 mμ, log ε=4.18
R_1=R_2=H, R_3=n·C_3H_7	400	Washed mycelium	2,000	20	115	140~141°[2]	λ_{max} 395 mμ, log ε=4.22
R_1=R_2=H, R_3=iso C_3H_7	600	Washed mycelium	4.000	20	150	138~140°[2]	λ_{max} 395 mμ, log ε=4.21
R_1=R_2=H, R_3=C_4H_9	735	Washed mycelium	4,200	24	25	126~128°[2]	λ_{max} 395 mμ, log ε=4.18
R_1=H, R_2=R_3=CH_3	30	Growing cultures	300	72	2.4[3]		
R_1=CH_3, R_2=R_3=H	15~60	Growing culture and washed mycelium	300	12~72	No transformation		
R_1=R_2=H, R_3=C_6H_5	15~60	Growing culture and washed mycelium	300	12~72	No transformation		

1) After recrystallization from ethyl acetate.
2) After recrystallization from isopropyl ether.
3) Determined by quantitative chromatographic analysis. The product had been previously prepared by synthesis[4~5].
4) All the nitroimidazoles obtained gave correct elemental analyses with exception of the butyl derivative.

For the transformation either 48-hour growing cultures of *Streptomyces* LE/3342 or washed mycelium suspended in buffer (pH 6.5) were employed. After the incubation periods indicated the nitroimidazoles were extracted from the filtered broth, partially purified and finally separated from the azomycin spontaneously produced by means of counter-current distribution and crystallized from the solvent indicated.

For details see under Materials and Methods.

Results

Streptomyces strain LE/3342 produces[9], besides azomycin, two other antibiotic substances, possibly eurocidin and tertiomycin. Since these compounds could interefere with the analysis and the purification of the nitroimidazoles produced by transformation of aminoimidazoles, the transformations were first attempted with washed mycelium suspended in buffer solution. In these conditions only small amounts of the interfering substances were produced.

For the first experiments concentrations and times of incubation similar to those adopted for the conversion of 2-aminoimidazole into azomycin were employed[3]. In instances in which negative results were obtained (as for 4(5)-phenyl-2-aminoimidazole and 1-methyl-2-aminoimidazole) further attempts were made by varying the transformation time and employing growing cultures instead of washed mycelium.

In Table 2 are given the results obtained and the analytical characteristics of the nitroimidazoles. The transformation yields obtained for the lower alkyl compounds can be considered satisfactory, when compared with the low yields reported for the chemical conversion of 4(5)-methyl-2-aminoimidazole to 4(5)-methyl-2-nitroimidazole[4,5]. In addition to the experiments summarized in Table 2, the conversion of the 4,5-dimethyl-2-aminoimidazole and of the (4)5-butyl-2-aminoimidazole was attempted in several other conditions (substrate concentration from 50 to 200 μg/ml, incubation period from 6 to 72 hours) but no higher transformation yields were observed. In all the experiments the counter-current distribution of the crude products and the paper chromatography revealed the presence of azomycin, produced in concentrations of about 15 μg/ml by resting cells, and of about 100 μg/ml by growing cultures of the microorganism. Similar concentrations were generally detected in control flasks.

Fig. 2. Time course for the conversion of alkylaminoimidazoles to alkyl-2-nitroimidazoles by washed mycelium of *Streptomyces* LE/3342.

The substrates were added as hydrochlorides at the concentration of 150 μg/ml to Erlenmeyer flasks containing 100 ml of mycelial suspension.

At the times indicated the mixture was filtered and the culture liquids extracted and analysed as described under Materials and Methods. Each result is the average of three experiments.

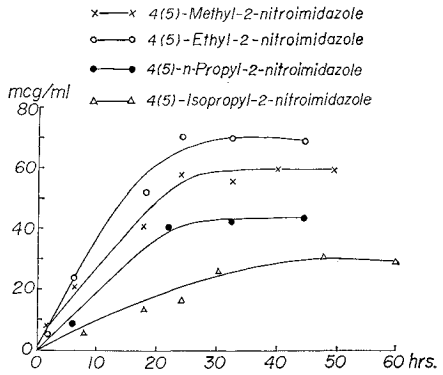
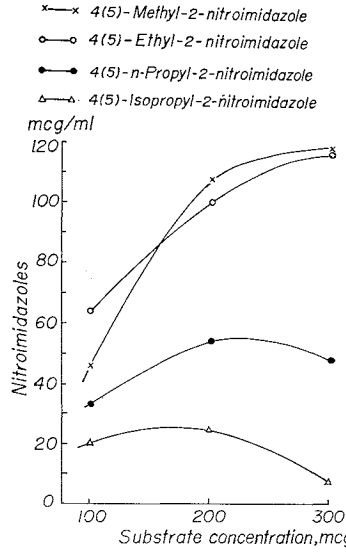


Fig. 3. Influence of the substance concentration on the microbial oxidation of 2-aminoimidazoles to 2-nitroimidazoles.

The substrates were added at the concentrations indicated to washed mycelium of *Streptomyces* LE/3342 suspended in phosphate buffer (pH 6.5). After an incubation period of 24 hours the filtrate was treated and analyzed as described under Materials and Methods.



Influence of the Incubation Time and the Substrate Concentration on the Yields of Transformation

The time course of the microbial conversion of aminoimidazoles to nitroimidazoles was studied using as starting products the 4(5)-methyl, 4(5)-ethyl, 4(5)-*n*-propyl and 4(5)-isopropyl derivatives. In Fig. 2 are given the results obtained by adding 150 μg/ml of the substrate to suspensions of washed mycelium in phosphate buffer (pH 6.5). The amounts of the alkyl nitroimidazoles were determined spectrophotometrically after separation from azomycin and other interfering substances by paper chromatography.

An incubation period of about 22 hours is generally needed to reach the maximum transformation. The isopropyl derivative appears to be oxidized at a slower rate, reaching the maximum in more than 30 hours.

The influence of the concentration of the substrate on the yields of the reaction was examined by adding to standard suspensions of the mycelium different amounts of the alkyl aminoimidazoles. After an incubation period of 24 hours the alkylnitroimidazoles concentration was determined by quantitative chromatographic analysis. The results are reported in Fig. 3 where the concentrations of nitroimidazoles produced are plotted against the concentrations of the starting aminoimidazoles. The yields of the reaction generally decrease with the increase of the substrate concentration. Moreover the data indicate, at least in the case of the *n*-propyl and of the iso-propyl derivatives, that a negative effect on the course of the reaction is obtained when the concentration of the substrate is superior to 200 μg/ml. In fact the absolute amounts of *n*-propyl-2-nitroimidazole and more evidently isopropyl-2-nitroimidazole decrease when the concentration of the corresponding aminoimidazole is increased from 200 to 300 μg/ml. These findings conform to the previously reported[3] effect of the 2-aminoimidazole concentration on the production of azomycin.

Attempts at Microbial Oxidation of *p*-Aminobenzoic Acid

The conversion of this product to *p*-nitrobenzoic acid was attempted in order to compare the substrate specificity of our microorganism and of *S. thioluteus*. To standard suspensions of washed mycelium *p*-aminobenzoic acid was added in concentrations from 50 to 200 μg/ml. Samples were taken out after different times of incubation and worked up as described by KAWAI *et al.*[2] The chromatographic analysis did not reveal the presence of *p*-nitrobenzoic acid. Spectrophotometric examination of the ethyl acetate extracts showed only the presence of small amounts of the starting material.

Discussion

The results reported provide further examples of the possibility, when the biogenesis of an antibiotic is known, of producing some derivatives of the antibiotic by supplying the microorganism with modified precursors. The ability of strain LE/3342 to oxidize amino groups to nitro groups is, however, limited by severe structural requirements for the substrate. It fact, good transformations were obtained only with imidazoles bearing a lower alkyl group in position 4(5). When a longer chain (butyl) or a double substitution (4,5-dimethyl) was present the yields were very low. No reaction at all was observed

when the substituent in position 4(5) was aromatic and the same negative results were obtained when the hydrogen atom in position 1 was replaced by an alkyl group.

A comparison with the microbial oxidation carried on by *S. thioluteus* reveals noteworthy differences. The substrate requirements of the two microorganism are different. With our strain it was not possible to oxidize *p*-aminobenzoic acid, which is easily converted into *p*-nitrobenzoic acid by *S. thioluteus*. Moreover the optimal time for the conversion of *p*-aminobenzoate by washed mycelium of *S. thioluteus*, as reported by KAWAI *et al.*[2], is about 2 hours and other aromatic amines are oxidized in a few hours. The oxidation of aminoimidazoles with *Streptomyces* LE/3342 in similar conditions is much slower, since at least 20 hours of incubation are required to reach the maximum of the transformation. It has been suggested[10] that azomycin may occur naturally in the form of a riboside. If this is the case it is possible that alkylaminoimidazoles are transformed into nitroimidazoles only after a preliminary conversion to the corresponding ribosides. The finding that 1-methyl-2-aminoimidazole is not oxidized and the relative slowness of the reaction for the other aminoimidazoles is in agreement with this suggestion. At the moment, however, no experimental support for such a hypothesis has been provided.

Acknowledgement

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