OUDEMANSIN, STROBILURIN A, STROBILURIN B AND MYXOTHIAZOL: NEW INHIBITORS OF THE bc_1 SEGMENT OF THE RESPIRATORY CHAIN WITH AN E-β-METHOXYACRYLATE SYSTEM AS COMMON STRUCTURAL ELEMENT

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1. Introduction

The inhibitory action and binding characteristics of the new anti-fungal antibiotic myxothiazol has been described in [1,2]. Like antimycin, myxothiazol binds very tightly, inhibiting the electron flow completely at a titer of ~1 mol inhibitor/mol complex III. However, myxothiazol binds to a site other than that of antimycin, as indicated by a red shift of the ferrocytochrome *b* spectrum. The myxothiazol shift is independent of and differs from the antimycin red shift.

Myxothiazol contains a bis-thiazol system as a predominant structural element. Besides it contains an E-β-methoxyacrylate system as a terminal segment. The rest of the molecule is linked in β′-position to acrylic acid, present as amide [3,4] (fig.1).

This study describes the inhibitory action and binding behavior of three further new antifungal antibiotics: oudemansin, strobilurin A and strobilurin B, and compares them with myxothiazol and antimycin. Like myxothiazol, all three molecules comprise an E-β-methoxyacrylate system (moa-system) as a structural element, but in these three antibiotics the rest of the molecule is linked in α-position to the acrylic acid, present here as methylester. The four antibiotics containing the moa-systems are termed moa-inhibitors in the following. Oudemansin has been isolated from *Oudemansiella mucida* [5], the strobilurins from *Strobilurus tenacellus* [6]. The strobilurins differ from each other in their substituents at the benzyl ring; oudemansin, when compared to strobilurin A, has an added methanol to $\Delta^{9,10}$. The relative configuration of the crystalline oudemansin has been elucidated by X-ray analysis [7], permitting the discussion of a probable structure–function relationship.

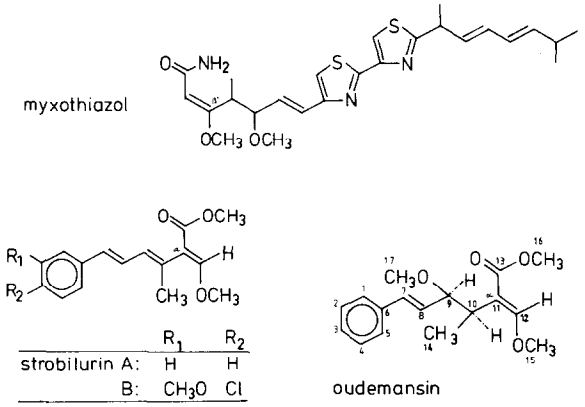

Fig.1. Structural formulae of the moa-inhibitors of the bc_1 segment of the respiratory chain containing the E-β-methoxyacrylate system as common structural element.

2. Materials and methods

Myxothiazol was a generous gift from Dr H. Reichenbach (Gesellschaft für Biotechnologische Forschung, Braunschweig). Oudemansin, strobilurin A and strobilurin B were isolated at the Lehrbereich Spezielle Botanik (University of Tübingen). Antimycin and all other biochemical reagents were purchased from Boehringer (Mannheim).

The inhibitors were dissolved in ethanol. The absorbance coefficients used were 4.8 $mM^{-1} \cdot cm^{-1}$ at 320

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nm for antimycin [2], 10.5 $mM^{-1} . cm^{-1}$ at 313 nm for myxothiazol [2], 30.2 $mM^{-1} . cm^{-1}$ at 245 nm for oudemansin [5], 21.8 $mM^{-1} . cm^{-1}$ at 294 nm for strobilurin A and 28.4 $mM^{-1} . cm^{-1}$ at 304 nm for strobilurin B [6].

Submitochondrial particles from beef heart were prepared as in [2]. Complex III was isolated according to [11], and the bc_1-complex devoid of iron–sulfur protein according to [12].

3. Results

3.1. *Inhibition of electron flow*

Oudemansin and the strobilurins inhibit electron flow in a similar fashion, as demonstrated by the inhibition of the NADH oxidation of submitochondrial particles (fig.2). Oudemansin binds with low affinity. Titrations were performed at 56 μg protein/ml, corresponding to 18 nM complex III. The oudemansin titration curve can be simulated by a mass action plot if it is assumed that the concentration of the free inhibitor roughly equals that of the added inhibitor. The best fit of the titration curve is obtained with an app. $K_d = 5 \times 10^{-7}$ M. The strobilurins bind more tightly than oudemansin; the K_d-values have not yet been estimated.

As is well known, antimycin inhibits in amounts nearly stoichiometric to complex III, since it binds with $K_d \sim 10^{-11}$ M [8]. The myxothiazol binding is not much lower than that of antimycin. Concerning the K_d-values one must remember that they may be influenced substantially by a partition of the hydrophobic antibiotics between the phospholipid phase of the particles and the aqueous phase of the incubation medium; the actual K_d-values may in fact be higher [9].

3.2. *Characterization of the primary site of action of the various inhibitors by difference spectrophotometry*

The site of action can be traced by difference spectrophotometry: the redox components before the block are in the reduced state, while those behind the block are in the oxidized state. In these experiments a spectrum where the cytochromes are completely reduced by dithionite on the sample side and fully oxidized by ferricyanide on the reference side, serves as reference (fig.3). In the subsequent spectra the

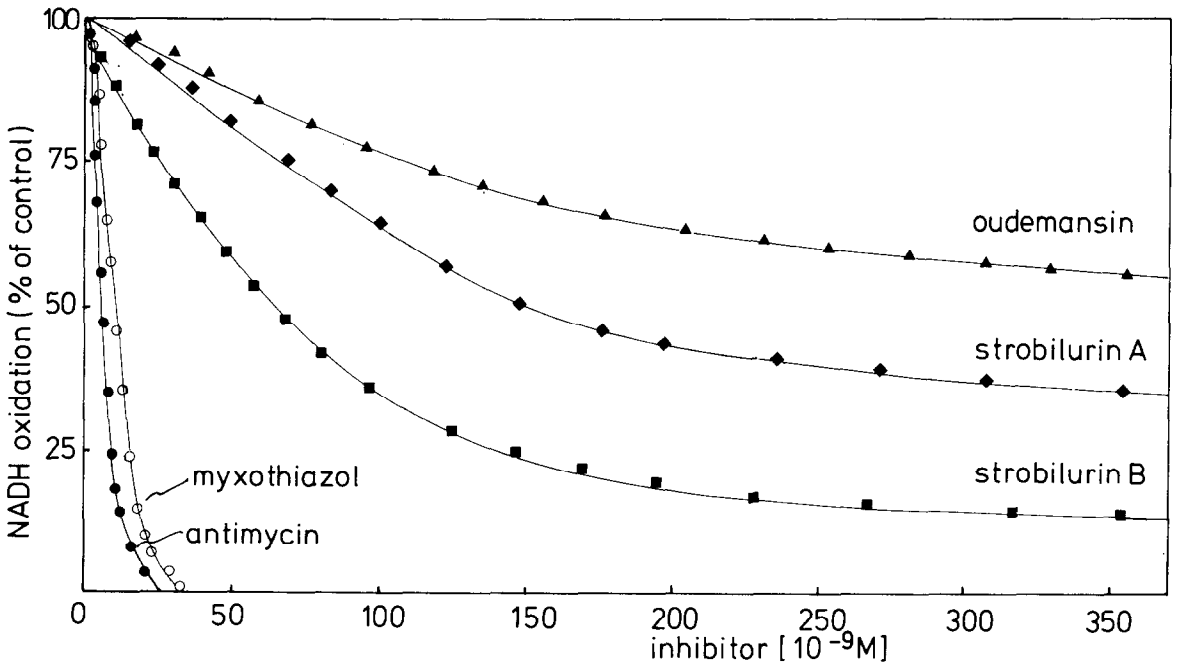

Fig.2. Inhibition of the NADH oxidation of beef heart submitochondrial particles by the various moa-inhibitors and by antimycin. The NADH oxidation was observed by difference spectrophotometry with 340 nm as measuring and 420 nm as reference wavelength. The rates were measured with 1 mM NADH, $V_{max} = 1.05 \times 10^3$ μmol/min × g protein at 25°C.

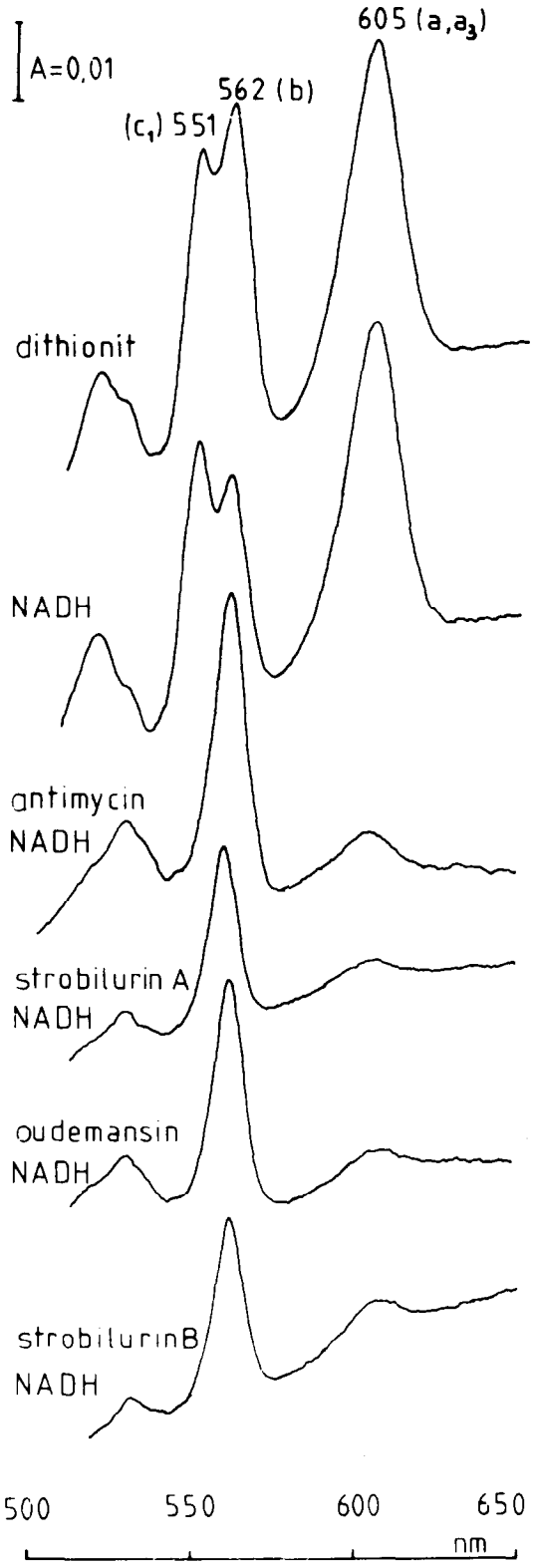

Fig.3. Difference absorbance spectra of beef heart submitochondrial particles in the dithionite- and NADH-reduced anaerobic state and in the NADH-reduced aerobic states blocked by the various inhibitors. The particle concentration was 1.7 mg protein/ml, corresponding to 1.02 μM heme *b*. The additions were: 1 mM NADH, 15 μM antimycin, 160 μM strobilurin A, 44 μM strobilurin B and 280 μM oudemansin.

reference side is in the oxidized state. In the anaerobic state with NADH as electron donor, cytochromes c_1 and aa_3 are nearly completely reduced, while cytochrome *b* is reduced to ~60%. With oudemansin and the strobilurins, cytochrome *b* preserves its degree of reduction, while cytochromes c_1 and aa_3 change to a completely oxidized state. The antimycin spectrum differs from the oudemansin and strobilurin spectra in its complete degree of cytochrome *b* reduction.

The experiments show that all three inhibitors block the electron transfer between cytochrome *b* and cytochrome c_1 but, in contrast to antimycin, the moa-inhibitors cause a reduction of only ~50% of cytochrome *b* (myxothiazol not shown, cf. [1]). This difference in the degree of cytochrome *b* reduction provides a hint that antimycin and the moa-inhibitors may act differently.

3.3. *The different bathochromic shifts of the absorbance spectrum of ferrocytochrome* b *induced by the binding of the various inhibitors*

As described for antimycin and myxothiazol, the binding of the inhibitors causes a bathochromic shift of the α-absorbance spectrum of reduced cytochrome *b*. The maximum–minimum shift spectra can be analyzed quantitatively. In the case of antimycin [10] and myxothiazol [2] the analysis indicates a linear binding and saturation of the binding site at 1 mol inhibitor/mol complex III. The red-shifts of the moa-inhibitors were tested with complex III isolated in Triton X-100 by means of hydroxyapatite chromatography followed by gel chromatography [11]. Experiments with the isolated complex give signals at a height suitable for quantitative analysis, even when the shift is very minor as in the case of myxothiazol. The experiments were performed with completely reduced complex in both sample and reference cuvettes, and addition of inhibitor to the sample cuvette. The red-shifts of the various moa-inhibitors compete with each other (not shown) whereas the red shift of antimycin is independent from and additive to those of the moa-

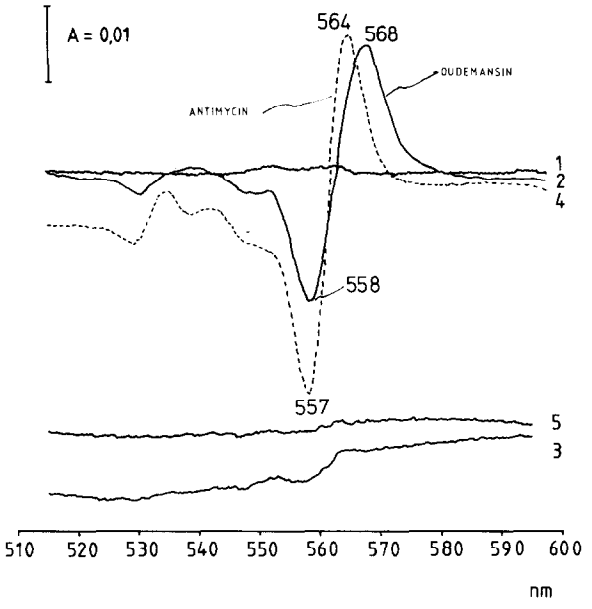

Fig.4. Independence of the antimycin- and oudemansin-induced red shifts of isolated beef heart complex III, reflecting the independent binding of the two inhibitors. The complex concentration was 1.6 mg protein/ml, corresponding to 11 μM heme *b*; 280 μM oudemansin and 15 μM antimycin were added. Trace: (1) base line before addition of inhibitors; (2) oudemansin-induced red shift; (3) base line with oudemansin in both cuvettes; (4) antimycin-induced red shift; (5) base line with oudemansin plus antimycin in both cuvettes.

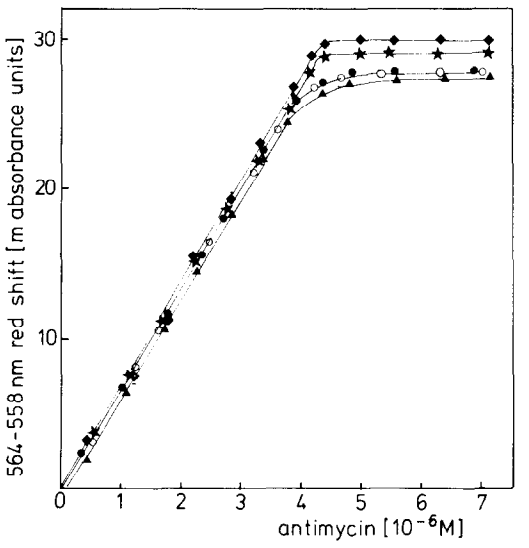

Fig.5. Antimycin titrations of the red shift of ferrocytochrome *b* of isolated beef heart complex III in the presence and absence of the moa-inhibitors. The concentration of isolated complex III was 1.2 mg protein/ml, corresponding to 8.4 μM heme *b*: (*——*) no addition; (○——○) 140 μM oudemansin; (●——●) 280 μM oudemansin; (▲——▲) 160 μM strobilurin A; (◆——◆) 44 μM strobilurin B.

inhibitors. Fig.4 shows the independence of the antimycin red shift from that of oudemansin. Trace 1 gives the base line originating from a difference spectrum of the dithionite-reduced complex. Trace 2 shows the symmetric spectrum of the red shift as induced by saturating concentrations of oudemansin. The absorbance maximum lies at 568 nm, the minimum at 558 nm. Subsequent addition of oudemansin to the reference side abolishes the spectrum (trace 3). The red shift spectrum of antimycin can now be induced by addition of antimycin to the sample side (trace 4). The same spectra can be reached by the reverse sequence of additions. Finally a base line can be re-established by addition of antimycin to the reference cuvette (trace 5). The antimycin red shift can also be obtained by adding antimycin after myxothiazol or the strobilurins. The spectral data of the red shifts caused by the other moa-inhibitors will be published later.

Fig.5 gives binding curves of antimycin in the presence and absence of the moa-inhibitors. The signal height of the antimycin red shift is only slightly changed by the binding of the other inhibitors, while the titer for full saturation and the binding affinity for antimycin are not altered. The experiments demonstrate that antimycin and the moa-inhibitors bind to different sites. In addition to the α-red shift, the inhibitors induce alterations in the γ-absorbance bands of ferri- and ferrocytochrome *b*, as will be described later. The complex III preparation containing bound antimycin and lacking the iron–sulfur-carrying peptide [12] still binds the moa-inhibitors as indicated by the respective red shifts (unpublished).

4. Discussion

The recent elucidation of the DNA sequence of the mitochondrial cytochrome *b* gene [13,14] together with the partial amino acid sequence of the isolated cytochrome *b* peptide [15] give strong support to the existence of only one cytochrome *b* molecule in complex III, possessing an M_r of ~43 000. This molecule shelters two different heme *b* centers [13,15]. Absorbance and EPR data give some indication that antimycin influences mainly the ligand field of the b_K center

In view of the independence of the antimycin-induced and the moa-inhibitor-induced red shifts shown here, it is tempting to speculate that the moa-inhibitors have a pronounced influence on the other heme center, namely the b_T center. Further experiments will have to elucidate this question, just as it will have to be clarified whether the moa-inhibitors act competitively with ubiquinol or semiquinone, or whether they react at a site not identical with the catalytic center(s) of cytochrome *b*.

Preliminary inhibition experiments reveal that the moa-segment is essential for the biological action, e.g., dimethyl myxothiazol has only 1% of the inhibitory action of myxothiazol [2]. The relative configuration, i.e., the three-dimensional form of the oudemansin molecule when it is in crystalline array has been elucidated and shown in an ORTEP stereoplot [5]. The molecule is arranged along two planes which form an angle of 78°. One plane is formed by the terminal styryl moiety, the other by the terminal E-β-methoxyacrylate system. Due to the steric hindrance, the rotation around the 9,10 single bond is restricted, therefore the configuration in the aqueous phase may be very similar to the crystalline state. Thus the low binding affinity of oudemansin may be induced by a hindrance of the interaction of the moa-system with the binding site due to an impeded accessibility of the system in the relatively rigid molecule. The strobilurins may bind more tightly, since all 7 double bonds are in a conjugated state, leading to a planar configuration of the molecule. Myxothiazol finally, the most tightly binding inhibitor, possesses a moa-system substituted in β′-position with an unconjugated double bond of the acrylic acid. In this way the moa-system achieves a relatively high mobility, facilitating the adaption of the key (moa-system) to the lock (the specific binding site).

Acknowledgements

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