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THE MODE OF ACTION OF STIGMATELLIN, A NEW INHIBITOR OF THE CYTOCHROME *b*-c_1 SEGMENT OF THE RESPIRATORY CHAIN *

GEORG THIERBACH [a,**], BRIGITTE KUNZE [a], HANS REICHENBACH [a] and GERHARD HÖFLE [b]

GBF, Gesellschaft für Biotechnologische Forschung mbH, [a] Abteilung Mikrobiologie and [b] Abteilung Naturstoffchemie, Mascheroder Weg 1, D-3300 Braunschweig (F.R.G.)

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The new antibiotic stigmatellin, obtained from the myxobacterium *Stigmatella aurantiaca*, was found to block the electron flow in the respiratory chain of bovine heart submitochondrial particles at the site of the cytochrome *b*-c_1 segment. Its inhibitory potency was identical with that of antimycin and myxothiazol, and like these antibiotics, stigmatellin caused a shift in the spectrum of reduced cytochrome *b*. Difference spectroscopic studies with the three inhibitors in various combinations indicated that the binding site of stigmatellin was different from that of antimycin, but more or less identical with that of myxothiazol. Experiments with 14 synthesized derivatives of stigmatellin showed that good inhibitory activity can be expected only if the side chain was kept relatively lipophilic, and the keto and the hydroxy groups of the chromone system were left intact.

Introduction

Stigmatellin (Fig. 1) is a new antifungal antibiotic produced by the myxobacterium, *Stigmatella aurantiaca* strain Sg a15. The production and isolation of the compound and the elucidation of its chemical structure have been described [1,2]. Since stigmatellin was not active against the yeast, *Saccharomyces cerevisiae*, under fermentative growth conditions, it was concluded that the antibiotic might interfere with respirative energy metabolism [1]. In this paper we report on experiments with isolated yeast mitochondria showing that stigmatellin indeed inhibits respiration, and with bovine heart submitochondrial particles allowing a more precise determination of the inhibition site of stigmatellin.

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** Present address: Depts. of Biochemistry and Genetics, Leeds University, Leeds, LS2 9JT, U.K.

Materials and Methods

Myxothiazol was kindly supplied by Dr. W. Trowitzsch (GBF). Stigmatellin, myxothiazol and antimycin A were dissolved in methanol. Their exact concentrations were determined photometrically using the absorption coefficients: 65.4 $mM^{-1} \cdot cm^{-1}$ at 267 nm for stigmatellin [2]; 10.5 $mM^{-1} \cdot cm^{-1}$ at 313 nm for myxothiazol [3]; and 4.8 $mM^{-1} \cdot cm^{-1}$ at 320 nm for antimycin A [4]. The synthesis of the derivatives of stigmatellin is described elsewhere [2]. All chemicals were of the highest purity available. The preparation of submitochondrial particles from bovine heart and of yeast mitochondria from *S. cerevisiae* strain D6,

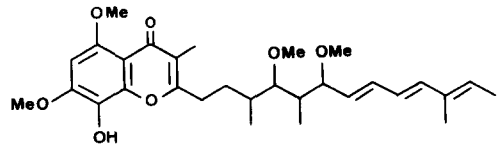

Fig. 1. Chemical structure of stigmatellin [2].

and the performance of the experiments have been described previously [5,6]. The heme *b* content of submitochondrial particles was calculated from difference spectra (dithionite reduced minus ferricyanide oxidized) using an absorption coefficient of 28 $mM^{-1} \cdot cm^{-1}$ [7] for the optical density difference at 563 minus 577 nm.

Results

Stigmatellin inhibited NADH oxidation in isolated mitochondria of the yeast, *S. cerevisiae* D6 (Table I), as was to be expected from earlier experiments [1]. Mitochondria were isolated from several mutant strains resistant to the new electron transport inhibitor myxothiazol [5,6,8,9]. When tested for cross resistance to stigmatellin, NADH oxidation proved as sensitive to stigmatellin as in the wild strain mitochondria.

For a more detailed study on the site of action of the antibiotic, bovine heart submitochondrial particles were chosen as the experimental system. Fig. 2 shows that stigmatellin strongly inhibited NADH oxidation in submitochondrial particles. The titration curve had a moderately sigmoidal shape. At a dose of 0.24 nmol stigmatellin/mg protein, corresponding to 0.36 mol stigmatellin per mol heme *b*, NADH oxidation was inhibited by 50%. This ratio was obtained with different particle densities (1-, 2- and 4-fold: Fig. 2). For comparison, NADH oxidation was titrated with myxothiazol. As can be seen in Fig. 2, the inhibitory efficiencies of the two antibiotics were identical.

The site of inhibition within the electron transport chain was determined by difference spectroscopy. Upon reduction with a physiological substrate, e.g. NADH, the cytochromes in front of the block become reduced, while those behind it remain oxidized. Upon addition of NADH to oxidized submitochondrial particles treated with stigmatellin, cytochrome *b* was reduced, while cytochromes $(c + c_1)$ and aa_3 remained in the oxidized state (Fig. 3). This result showed that stigmatellin, like myxothiazol (Fig. 3) [5], inhibited the electron flow within the cytochrome b-c_1 segment of the respiratory chain.

When stigmatellin was added to a suspension of dithionite-reduced submitochondrial particles, a spectral shift with a maximum at 569 and a minimum at 563 nm was produced (Fig. 4). This indicated an interaction of stigmatellin with cytochrome *b*. In contrast to stigmatellin and in agreement with previous results [5], myxothiazol produced a spectral effect with a maximum at 564 nm, but without a minimum (Fig. 4).

TABLE I

THE EFFECT OF STIGMATELLIN ON NADH OXIDATION BY YEAST MITOCHONDRIA

The protein concentration in the test was 0.163 mg/ml. The rate of NADH oxidation in the control without the antibiotic was 384 nmol/mg protein per min.

Concentration of stigmatellin (nM)	Rate of NADH oxidation (%)
0	100
15.2	74
21.3	54
27.4	23
45.7	6

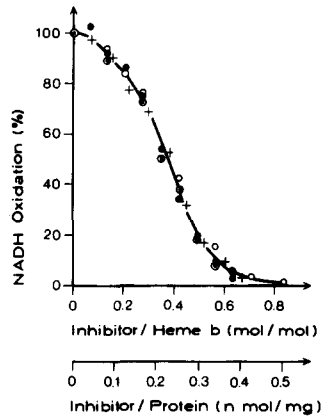

Fig. 2. The effect of stigmatellin on the rate of NADH oxidation in bovine heart submitochondrial particles at different particle densities. The protein concentrations in the tests were: 0.034 mg/ml corresponding to 22 nM heme *b* (●———●), 0.068 mg/ml corresponding to 44 nM heme *b* (○———○) and 0.136 mg/ml corresponding to 88 nM heme *b* (⊙———⊙). For comparison, the effect of myxothiazol on NADH oxidation was titrated (at a protein concentration of 0.068 mg/ml, or 44 nM heme *b*: +———+). The suspensions were preincubated for 4 min with the inhibitor before the reaction was started by the addition of NADH. The rate of NADH oxidation in the control was 1.6 μmol/mg protein per min.

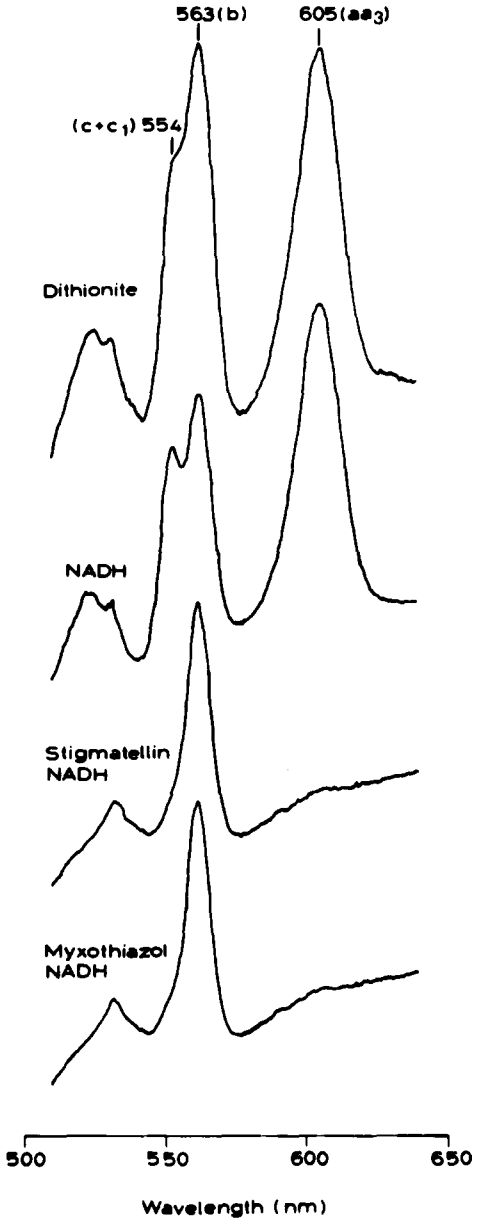

Fig. 3. Difference spectra (reduced minus oxidized) of bovine heart submitochondrial particles reduced either with NADH in presence or absence of stigmatellin or myxothiazol, or with dithionite. Submitochondrial particles were diluted with air-saturated buffer at room temperature at a concentration of 1.9 mg protein/ml corresponding to 1.25 μM heme *b*. The cuvettes (optical pathway 1 cm) contained 2.5 ml of the suspension. Volume corrections were made in the reference cuvette for all additions to the sample cuvette. Full reduction of the cytochromes was achieved by adding a few grains of solid dithionite to the sample cuvette. The concentration of NADH was 2 mM, that of the inhibitors 5 μM. The suspensions were preincubated for 2 min with the respective inhibitor before the reduction with NADH was started.

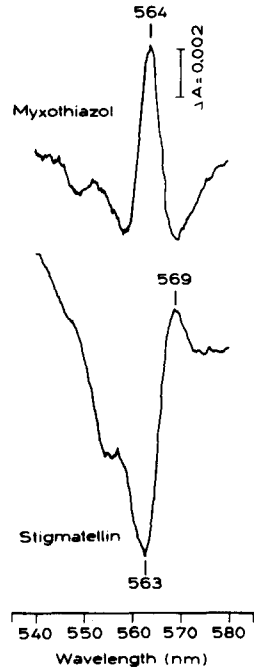

Fig. 4. Spectral shift induced by stigmatellin in dithionite reduced bovine heart submitochondrial particles. For comparison, the spectral effect induced by myxothiazol is shown. A suspension of submitochondrial particles (15.5 mg protein/ml corresponding to 10 μM heme *b*) was reduced with dithionite and filled (2.5 ml) into sample and reference cuvettes with a 1 cm light path. After adjustment of the baseline either stigmatellin or myxothiazol (final concentrations, 10 μM) was added to the sample cuvette, and the volume was corrected in the reference cuvette. The difference spectrum was recorded with a bandwidth of 1.5 nm and a scanning speed of 0.2 nm/s.

The spectral shift induced by stigmatellin was used to follow the binding of the inhibitor to its binding site. The height of the spectral shift, measured as difference of the optical densities at 569 and 563 nm, increased linearly with the dose of stigmatellin up to a ratio of 0.4–0.5 mol stigmatellin/mol heme *b* (Fig. 5). This indicated that the number of stigmatellin binding sites was approximately half the number of the heme *b* centers of the respiratory chain, and that the binding of stigmatellin occurred in a linear way. When the same type of experiment was done with myxothiazol, the binding curve was almost identical to that of stigmatellin (Fig. 6).

To see whether the binding sites of stigmatellin and myxothiazol were the same, we studied the

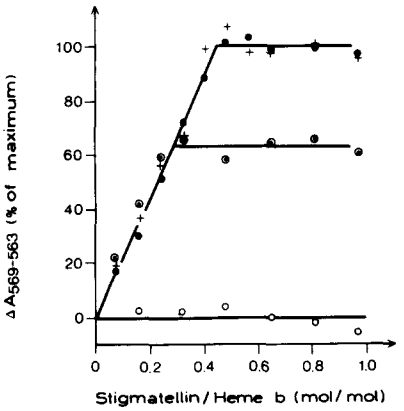

Fig. 5. Titration of the stigmatellin-induced spectral shift in absence or presence of antimycin A or myxothiazol. Dithionite reduced bovine heart submitochondrial particles (16.7 mg protein/ml corresponding to 10 μM heme *b*) were treated in the sample cuvette with increasing amounts of stigmatellin, and the resulting absorption difference at the indicated wavelengths was recorded (●———●); a difference of 100% corresponded to a ΔA value of 0.0091. The experiment was repeated in the presence of either 9.5 μM antimycin A (+ ——— +) (100% $\Delta A = 0.0109$) or 2.5 μM myxothiazol (⊙——— ⊙) (100% $\Delta A = 0.0091$) or 10.5 μM myxothiazol (○——— ○) (100% $\Delta A = 0.0091$). For further experimental details, see Fig. 4.

binding of stigmatellin in presence of myxothiazol and vice versa. As Fig. 5 shows, the stigmatellin-induced shift could not be produced with submitochondrial particles pretreated with an excess of myxothiazol, and Fig. 6 shows that there was no myxothiazol effect when stigmatellin was present in excess. When dithionite-reduced submitochondrial particles were treated with an amount of myxothiazol just enough to fill half of the myxothiazol binding sites, the stigmatellin shift reached the saturation point already at 0.28 mol/mol heme *b* (Fig. 5), which suggested that stigmatellin could bind only to sites not occupied by myxothiazol.

The antibiotic antimycin A is another strong inhibitor interacting with cytochrome *b* and producing a red shift in the absorption spectrum of reduced cytochrome *b* [10]. Recently, evidence was produced that the antimycin and the myxothiazol binding sites are different [5]. As can be seen in Figs. 5 and 6, the presence of antimycin neither affected the titration curve of the stigmatellin shift, nor of the spectral effect of myxothiazol. Fig. 7 shows the complementary experiment: excessive amounts of stigmatellin did not affect the titration curve of the antimycin-induced red shift. These results strongly indicated that the binding site of our new inhibitor, stigmatellin, was identical with, or very close to, that of myxothiazol, but different from that of antimycin.

The difference spectra in Fig. 3 show that in presence of stimatellin only part of the heme *b* centers were reduced by NADH. This fact could

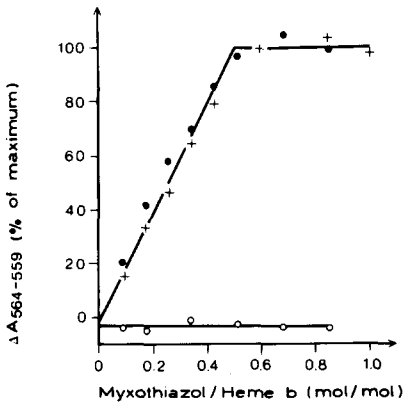

Fig. 6. Titration of the myxothiazol-induced spectral effect in absence and presence of antimycin A or stigmatellin. The experiment was performed as described in Fig. 5. In the titration curve with myxothiazol alone (●———●) a 100% difference in ΔA corresponded to 0.0062. Titration in presence of 9.1 μM antimycin A (+ ——— +) (100% $\Delta A = 0.0086$) and in presence of 10 μM stigmatellin (○——— ○) (100% $\Delta A = 0.0062$).

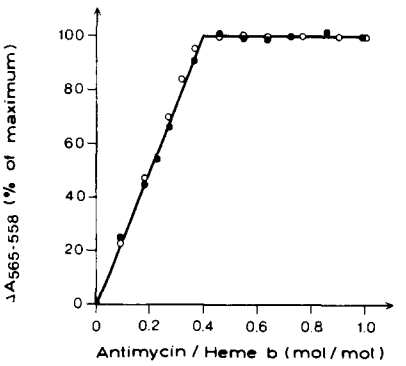

Fig. 7. Titration of the antimycin A-induced spectral shift in absence and presence of stigmatellin. The experiment was performed as described in Fig. 5 with the exception that the protein concentration was 13.6 mg/ml corresponding to 8.8 μM heme *b*. Titration with antimycin A alone (●———●) (100% $\Delta A = 0.0366$) and in presence of 10 μM stigmatellin (○——— ○) (100% $\Delta A = 0.0349$).

be demonstrated more clearly by measuring the reduction kinetics of heme *b* in the presence of the inhibitor (Fig. 8). As a control, all heme *b* centers of the submitochondrial particles were reduced artificially with dithionite (trace 1). Trace 2 shows the reduction of cytochrome *b* by NADH in presence of antimycin A. The difference in the signal height between the dithionite and the antimycin/NADH kinetics is probably due to cytochrome *b* of complex II of the respiratory chain [11,12]. After inhibition by stigmatellin (trace 3), only 60% of all heme *b* centers, or 70% of the heme *b* centers of complex III, were reduced. The same reduction level was obtained in the myxothiazol-inhibited state (trace 5), which is in agreement with earlier results [12]. Recently, it was shown that myxothiazol and antimycin when applied together almost completely inhibit the reduction of cytochrome *b* [12,13]: the reduction was slowed down to 1/10000 of its normal rate. This is shown by trace 6. When this type of experiment was performed with a mixture of antimycin and stigmatellin (trace 4), a very fast initial reduction rate was observed, similar to that with antimycin or stigmatellin alone, followed after a short time by a slow reduction rate. After a few minutes, a constant reduction level comprising 20% of all heme *b* centers, or 30% of the heme *b* centers of complex III, was reached. No such synergistic effect was observed with the combination of stigmatellin and myxothiazol (trace 7). These results are consistent with the view that the site of action of stigmatellin is different from the antimycin site and identical with, or at least related to, the myxothiazol site. In spite of the similarities in the behaviour of stigmatellin and myxothiazol, the different reduction kinetics of heme *b* seen in the two combinations of either stigmatellin or myxothiazol with antimycin A (traces 4 and 6) suggested differences in their precise mode of action.

To investigate which parts of the molecules are essential for the inhibitory activity of stigmatellin,

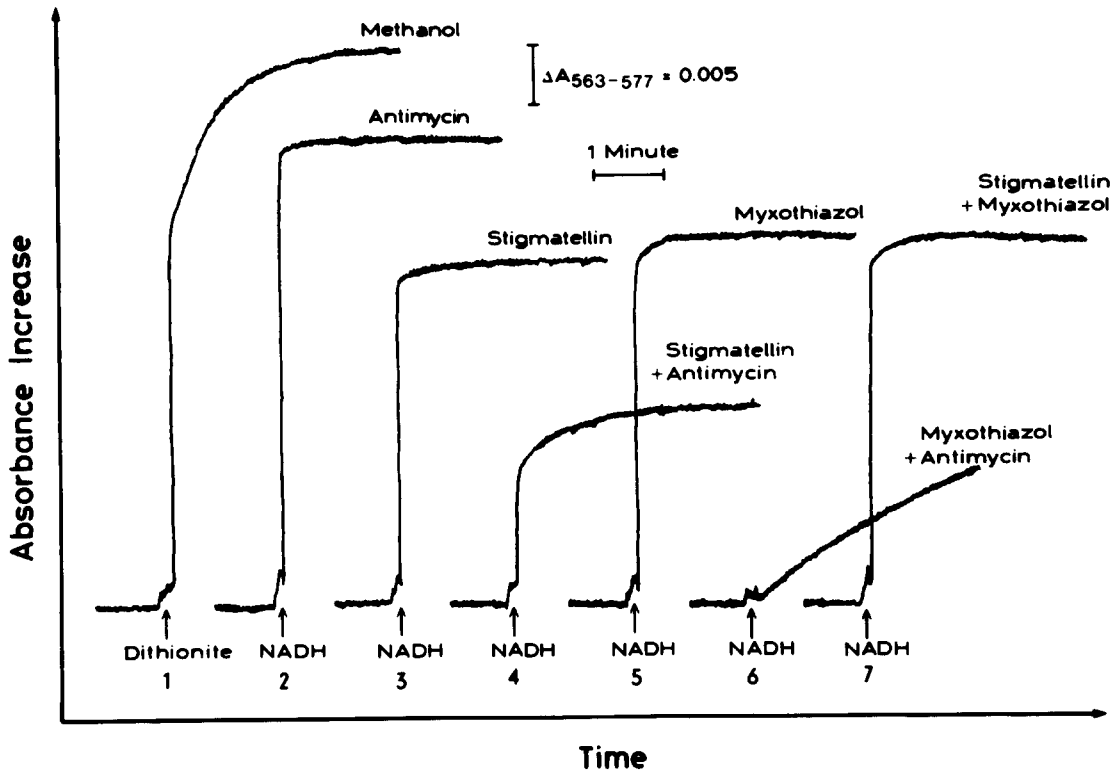

Fig. 8. Reduction kinetics of cytochrome *b* in bovine heart submitochondrial particles under various conditions. The protein concentration in the tests was 2.9 mg/ml corresponding to 1.9 μM heme *b*. All other experimental details were as described in Fig. 3.

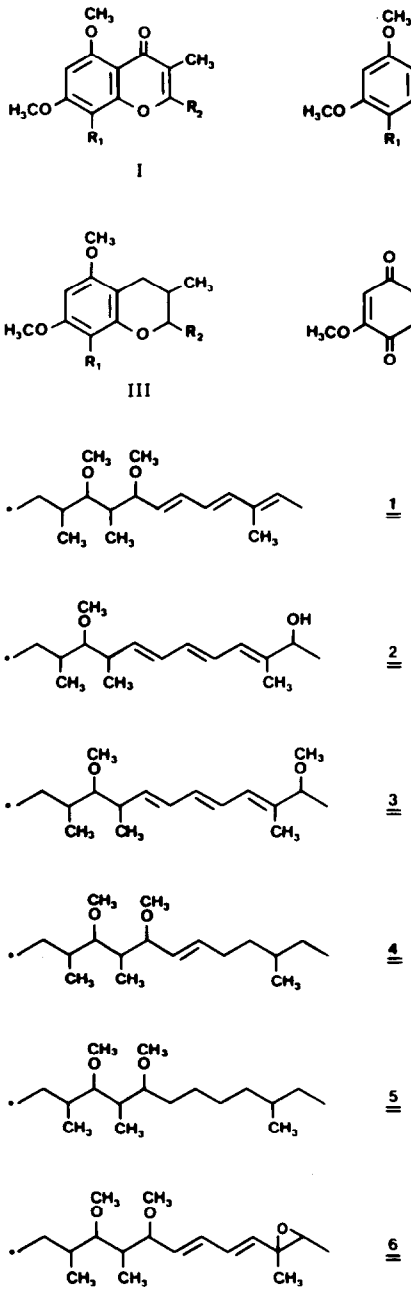

Fig. 9. Structural elements of the derivatives of stigmatellin listed in Table II.

a number of derivatives were synthesized [2] and tested for their effect on NADH oxidation in bovine heart submitochondrial particles (Table II, Fig. 9). Their minimal inhibitory concentration for *S. cerevisiae* was also determined. As can be seen, in the experiments with submitochondrial particles most modifications of the side chain, viz. reduction of double bonds (derivatives *Ig, Ih*), a shift in the position of a methoxy group (*If*), or elimination of methyl and methoxy groups (*Ik*) had no effect on the inhibitory activity of the drug. Likewise, the reduction of the chromone to a chromanone system (II) did not interfere significantly with the activity of the antibiotic. On the other hand, introduction of hydrophilic groups into the side chain (*Ie, Il*), a shortening of the side chain (*Ij*), a modification of the hydroxyl group at the site of R_1 in the chromone system (*Ib, Ic, Id, Ii*), or elimination of the keto group of the chromone (III) resulted in a more or less substantial loss of inhibitory activity. In almost all cases, the minimal inhibitory concentration values corresponded reasonably to the biochemical data. The only exception was derivative *Ik* which was very little water soluble and probably simply did not reach a concentration in the medium to elicit a response by the yeast.

Discussion

Our data clearly demonstrate that the new antibiotic stigmatellin blocks the electron transport within the cytochrome *b*-c_1 segment of the mitochondrial respiratory chain (Fig. 3). The inhibitory potency of stigmatellin was identical with that of myxothiazol [5] (Fig. 2) and antimycin A [14,15], which both act on the same segment.

Addition of stigmatellin to dithionite-reduced submitochondrial particles induced a shift in the spectrum of cytochrome *b* (Fig. 4), which was neither identical with the red shift caused by antimycin A [10,11] nor with the spectral effect produced by myxothiazol [5]. This is not surprising, for the chemical structures of the three antibiotics are completely different [2,8,17]. The stigmatellin-induced effect had a maximum at 569 and a minimum at 563 nm, and could be taken to indicate that stigmatellin binds to cytochrome *b*. The myxothiazol effect has a single maximum at 565 nm [5]. Other investigators reported that in isolated complex III myxothiazol induces a red shift with a maximum at 568 and a minimum at 558–560 nm [12,13]. When we repeated the experiment with

TABLE II

THE EFFECT OF DIFFERENT DERIVATIVES OF STIGMATELLIN ON NADH OXIDATION IN BOVINE HEART SUBMITOCHONDRIAL PARTICLES

The chemical structures of the various derivatives are shown in Fig. 9. *Ia* is stigmatellin. The protein concentration in the tests was 0.068 mg/ml corresponding to 44 nM heme *b*. The rate of NADH oxidation in the control was 1.6 μmol/mg protein per min. As the inhibition of NADH oxidation by derivatives *Ib* and *Id* showed a pronounced time dependence, the rates for the first ([1]) and second minute ([2]) after addition of NADH are given separately. The inhibition index of each compound was calculated by dividing its concentration required for a 50% inhibition by the corresponding figure of stigmatellin. The minimum inhibitory concentration values were determined with *S. cerevisiae* GT 66 [6] by serial dilution assay in a medium containing 1% Bacto-peptone (Difco), 1% yeast extract (Difco), 2% glycerol, and 50 mM phosphate buffer (pH 6.3).

Derivative number	R_1	R_2	Concentration required for a 50% inhibition (nM)	Inhibition index	Minimum inhibitory concentration (μg/ml)
Ia	-OH	1	15	1	0.1
Ib	$-OCH_3$	1	60 [1]	4.0 [1]	0.5–1
			54 [2]	3.6 [2]	
Ic	$-OCH_2COOH$	1	7000	470	> 50
Id	$-OCH_2COOC_2H_5$	1	65 [1]	4.3 [1]	7.5
			46 [2]	3.1 [1]	
Ie	-OH	2	240	16	> 25
If	-OH	3	17	1.1	1.25
Ig	-OH	4	13	0.9	0.1
Ih	-OH	5	15	1.0	0.5
Ii	-β-glucose	1	19000	1300	50
Ij	-OH	$-C_5H_{11}$	800	53	5 –20
Ik	-OH	$-C_{13}H_{27}$	13	0.9	–
Il	-OH	6	550	37	2.5–25
II	-OH	5	17	1.1	1 –5
III	-OH	5	1700	110	>100
IV	=O	5	160	11	1 –5

submitochondrial particles, we obtained the same result as before, with a minor difference in the position of the maximum (Fig. 4). Apparently, the kind of interaction of myxothiazol with cytochrome *b* is somewhat different in submitochondrial particles and in isolated complex III.

Taking the height of the stigmatellin-induced signal as a measure of the amount of antibiotic bound, we were able to study the stoichiometry of this binding. Like myxothiazol and antimycin, stigmatellin was bound, presumably to cytochrome *b*, in a strictly linear concentration dependence (Figs. 5–7) [5]. Apparently, the binding constants of the three antibiotics are extremely high, so that our method did not allow to detect differences in the binding behaviour. The number of binding sites for stigmatellin was half the number of heme *b* centers in the respiratory chain, and was thus identical with the number of myxothiazol and antimycin binding sites. These experiments strongly suggested that, virtually every molecule of antibiotic added was bound exclusively to its specific site of action.

Difference spectroscopy [10,16,18] as well as genetic data [8,19] indicate that the binding sites of antimycin and of myxothiazol are on the cytochrome *b* of the cytochrome b-c_1 segment of the respiratory chain. Experiments with isolated complex III suggest that myxothiazol binds in the vicinity of the heme b_T center (*b*-566), and antimycin in the vicinity of the heme b_K center (*b*-562) [12,13]. These two sites are supposed to be ubiquinone binding sites. The titration curves of the stigmatellin-induced spectral effect in presence of either myxothiazol or antimycin (Fig. 5) showed that myxothiazol interferes with the binding of stigmatellin, but that antimycin A does not. In

agreement with this result, stigmatellin interfered with the binding of myxothiazol (Fig. 6), but not with the binding of antimycin A (Fig. 7). The binding site of stigmatellin seemed thus identical with, or at least very close to the myxothiazol site, i.e., in the vicinity of the heme b_T center of complex III.

When antimycin and myxothiazol are applied together, they almost completely block the reduction of the heme *b* centers of complex III [12,13]. Therefore, we tested whether a mixture of antimycin and stigmatellin had a similar effects. The kinetics of the heme *b* reduction in presence of the two inhibitors consisted of a very fast initial reaction which was followed by a much slower reduction (Fig. 8). The reduction level finally reached was 20% of the total heme *b* centers, or 30% of the heme *b* centers of complex III, i.e., considerably below the reduction level obtained with stigmatellin alone. The reduction kinetics in the presence of a mixture of stigmatellin and myxothiazol showed no difference to the kinetics observed with either inhibitor alone. Although the reduction kinetics with stigmatellin plus antimycin A was different from that with myxothiazol plus antimycin A, the synergistic effect still indicated that the site of action of antimycin A and that of stigmatellin were different. The different shape of the two reduction kinetics further suggested that the precise modes of action of stigmatellin and myxothiazol are different. This assumption was supported by the fact that mitochondrial NADH oxidation in mitochondrial mutants of *Saccharomyces cerevisiae* resistant to myxothiazol [6] was not cross-resistant to stigmatellin.

Our experiments with derivatives of stigmatellin allow us to draw certain conclusions with respect to the chemical groups essential for the inhibitory activity of the antibiotic (Fig. 9, Table II). It seems that the chromone system is responsible for the inhibition reaction, while the significance of the side chain is to give the molecule the proper polarity. This was clearly demonstrated by derivatives *Ij* and *Ik* which differ only in the length of the side chain: the pentyl group in the case of *Ij* resulted in a much reduced inhibitory activity, while the tridecyl group in the case of *Ik* left the activity of the compound intact. Provided a suitable polarity was maintained, the side chain could be modified without loss of activity (derivatives *If, Ig, Ih*). When the side chain was made more hydrophilic (*Ie, Il*), this resulted in a considerable loss of inhibitory activity, which was not surprising, because the target site of stigmatellin is within the lipophilic membranes of the submitochondrial particles. While reduction of the chromone to a chromanone system (derivative *II*) had no effect on the inhibitory activity, elimination of the keto group (derivative *III*) drastically reduced the activity of the compound. Replacement of the hydroxyl group in the chromone system by a methoxy group (*Ib*) resulted in a 3- to 4-fold decrease in activity. It seems unlikely that this effect is due to the larger volume of the methoxy group, because derivative *Id* bearing an even bulkier ligand had about the same inhibitory activity as *Ib*. Perhaps the hydroxyl function must interact with the binding site via a hydrogen bond for stigmatellin to become fully active. Attachment of a carboxyl methyl group (*Ic*) or of glucose (*Ii*) to the hydroxyl in the chromone caused the compound to become more water soluble and reduced its activity. It is remarkable that no derivative was more efficient than the natural compound produced by the myxobacterium.

We feel that the new inhibitor stigmatellin might be another useful probe for studies into the molecular mechanism of electron flow within complex III of the respiratory chain.

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