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A Rapid *in Vivo* Colorimetric Library Screen for Inhibitors of Microbial Respiration

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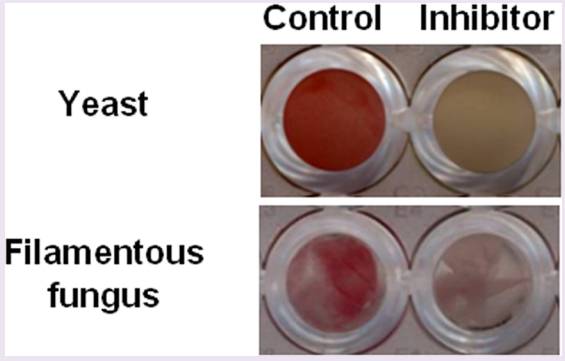
ABSTRACT: A number of fungicides that target the respiratory chain enzymes complexes II and III are used in agriculture. They are active against a large range of phytopathogens. Unfortunately, the evolution of fungicide resistance has quickly become a major issue. Resistance is often caused by mutations in the inhibitor binding domains of the complexes, and new molecules are required that are able to bypass such resistance mutations. We report here on a rapid *in vivo* high-throughput method, using yeast and the redox dye TTC to screen chemical libraries and identify inhibitors of respiratory function. We applied that screening process, followed by a series of tests, to a diverse library of 4,640 molecules and identified a weak inhibitor of complex III without toxic effect on the cell. Interestingly, that drug (D12) is fully active against the mutant enzyme harboring the G143A mutation that confers a high level of resistance toward most of the fungicides targeting complex III but is not active against bovine complex III. Using a collection of yeast strains harboring mutations in the inhibitor binding sites (Q_o and Q_i sites), we showed that D12 targeted the Q_o site and that its inhibitory activity was weakened by the mutation L275F. A phenylalanine is naturally present at position 275 in mammalian complex III, which could explain the differential sensitivity toward D12. The molecule is not structurally related to commercial inhibitors of complex III and could potentially be used as a lead compound for the development of antimicrobial agents.



The mitochondrial respiratory chain is an effective target for antimicrobial agents. In medicine, atovaquone, an inhibitor of complex III, is used for drug therapy against different parasitic diseases (malaria, toxoplasmosis, and *Pneumocystis* pneumonia caused by the opportunistic pathogen fungus *Pneumocystis jirovecii*). A range of fungicides are used in agriculture that inhibit complex II (succinate dehydrogenase) or complex III (bc_1 complex, quinol:cytochrome *c* reductase) of plant pathogenic fungi. Inhibitors of complex III (QIs) and of complex II (SDHIs) interfere with the substrate quinol binding sites. Complex III has two substrate quinol binding pockets called Q_o and Q_i that are provided by the mitochondrially encoded subunit cytochrome *b*. Most of the fungicides inhibiting complex III target the Q_o site (Q_oIs) and are synthetic analogues of the natural compounds strobilurins (for instance azoxystrobin). They were introduced in the market in the mid-1990s and are active against a wide spectrum of fungal diseases.[1] Only two Q_iIs (fungicides targeting the Q_i site of complex III) are available. They are sulfonamide compounds (cyazofamid[2] and amisulbrom) and are active only against oomycetes.

The overall structures of these respiratory complexes (especially the electron-transferring catalytic cores) are highly conserved between species. There are, however, some differences in sequence facilitating the search for inhibitors with differential reactivity, active against pathogens with low reactivity toward other organisms. Respiratory inhibitors developed as fungicides are effective against a broad range of important pathogens. Unfortunately, the problem of acquired resistance has rapidly emerged. A growing number of pathogen isolates resistant to inhibitor treatment have been reported, and this resistance is often linked to mutation within the active sites of the complexes.[3−5] For instance, the resistance mutation G143A located in the Q_o site of complex III has been reported in an increasing number of phytopathogenic fungi and causes a high level of resistance to all Q_o targeted fungicides.[3] New molecules targeting complex III are needed that are capable of bypassing such resistance.

Here we describe a rapid test to screen chemical libraries for drug inhibiting the respiratory function. The method uses the redox dye triphenyltetrazolium chloride (TTC). TTC is a colorless electron acceptor that can be reduced to a red formazan precipitate in microorganisms. It has been used as a means of screening for respiratory deficient mutants of yeast

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and *Chlamydomonas reinhardtii*:[6,7] colonies grown on agar plates were overlaid with TTC in soft agar; respiratory competent colonies became red, whereas respiratory deficient colonies did not accumulate the red dye. As previously reported,[8] TTC is reduced by the dehydrogenases of the respiratory chain (complex I, NADH- and succinate dehydrogenases). The reduction is faster when the mitochondrial membrane is coupled because of the accumulation in the mitochondrial matrix of the positively charged TTC^+ cations.[8] The accumulation of stable red formazan is possible only in anaerobiosis since the initial product of TTC reduction is rapidly reoxidized by oxygen. Cytochrome oxidase, the terminal enzyme of the respiratory chain, has a very high affinity for oxygen. Colonies of cells with a functioning cytochrome oxidase become anaerobic or hypoxic (inside the colonies) and accumulate the red formazan. Cells with a defective or otherwise inhibited respiratory chain do not reduce oxygen efficiently and therefore do not generate sufficient anaerobiosis for the red dye to form. Thus lack of formazan formation could act as a screen for respiratory deficient mutants, as previously reported,[6,7] but could also act as a screen to identify drugs that inhibit the respiratory function.

We applied this screening method to a collection of compounds of natural and synthetic origin, using the yeast *Saccharomyces cerevisiae* as a model organism. The screening, followed by biochemical and mutational analysis, identified a new compound inhibiting complex III, which was structurally unrelated to known inhibitors.

RESULTS AND DISCUSSION

Identification of Respiratory Inhibitors. Our goal was to develop a robust *in vivo* high-throughput methodology for the identification of inhibitors of a metabolic choke point, the mitochondrial respiratory complex III (cytochrome bc_1 complex). This enzyme is of considerable interest as a fungicidal and chemotherapeutic target. The colorimetric method we used is based on the reduction of the redox dye triphenyltetrazolium chloride (TTC) by the mitochondrial respiratory chain. This methodology is of general applicability for the identification of inhibitors of other complexes within the respiratory chain of aerobic microbes. The redox dye TTC accumulated inside the cells as a red formazan precipitate when reduced by the dehydrogenases of the respiratory chain under anaerobic conditions.[8] Compounds that inhibit the respiratory chain block the formation of the red precipitate. As described in Methods and illustrated in Figure 1, after addition of TTC to yeast (*S. cerevisiae*) cell suspensions in microwells, the red dye accumulated in cells with active respiratory chain but not in cells treated with azoxystrobin, a fungicide targeting complex III. This method can also be used with other organisms, such as the filamentous fungus *Podospora anserina* (Figure 1).

The chemical library of the ICSN, composed of 4,640 compounds of very large chemical diversity (natural products, molecules derived from natural substances, and synthetic compounds) was screened against yeast using the TTC test. Twenty molecules appeared to inhibit the accumulation of the red formazan, indicating that these drugs could potentially inhibit the respiratory chain enzymes. These 20 molecules then were retested manually using the same test (this was done twice). Six molecules were excluded as their inhibitory effect on the TTC reaction was not confirmed or was very weak. The 14 selected molecules were tested for their inhibitory effect on respiratory growth. Six molecules affected the respiratory

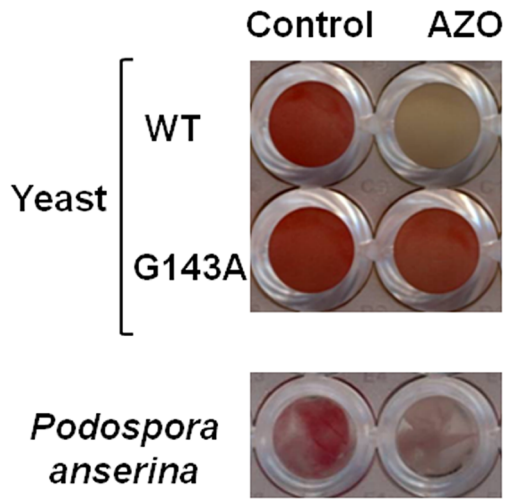

Figure 1. TTC test (as described in Methods) applied on yeast cells and on mycelium of a filamentous fungus. DMSO, solvent used as control; AZO, azoxystrobin, a potent inhibitor of the respiratory chain.

growth at 1 μg mL^{-1}: D5, D6, D9, D10, D11, and D12 (Table 1). The effect of these 6 drugs on the oxygen

Table 1. Effect of Drugs on Respiratory Growth and Oxygen Uptake by Cells

compound	respiratory growth (% control)[a]	oxygen uptake (% control)[b]
D5	61	65−70
D6	75	50
D9	54	70−80
D10	22	25
D11	44	100
D12	55	30

[a]WT cells were inoculated in 5 mL of ethanol medium with 1 μg mL^{-1} of the drugs and cultivated at 28 °C with agitation. Optical density ($A_{600\ nm}$) of the cultures was recorded at the end of the exponential phase and presented as % of the control cell density in the absence of drug. The measurements were done twice and averaged. [b]Oxygen uptake by cells was recorded in an oxygraph. Approximately 10^8 cells grown in ethanol medium were added to 1 mL of water with 3 μM CCCP. After 2−3 min recording of the control O_2 uptake rate, drugs were added at 5 μg mL^{-1}, and the oxygen uptake rates were recorded. The drugs have similar effect in the absence and in the presence of the uncoupling agent CCCP (not shown).

consumption of cells was monitored (Table 1). D11 had no effect. D5, D6, and D9 exhibited a weak inhibitory effect. A more severe inhibition was observed with D10 and D12. The study of these two drugs was pursued.

We checked their impact on the fermentative growth. For that end, we used a strain depleted of mitochondrial genome (rho^0). In this strain, the respiratory chain function is completely abolished as the mitochondrial genes encoding the main components of respiratory complexes III and IV and of complex V (ATP synthase) are absent. The rho^0 mutation (similarly to other mutations causing respiratory deficiency) is not lethal in *S. cerevisiae* as yeast can rely on fermentation to generate energy when grown on a suitable carbon source (such as glucose). The fermentative growth test was done as in Table 1, glucose replacing ethanol (not shown). We found that D10 was toxic to the cell proliferation and inhibited the fermentative growth at low dose (5 μg mL^{-1}). In addition the molecule seemed unstable in the culture medium since after >30 h

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incubation, the inhibition was lessened and the cell started to proliferate, both in fermentative and respiratory conditions. Thus D10 was excluded from further *in vivo* experiment. D12, on the contrary, was stable in culture media over 4 days of incubation. The drug had no effect on the *rho*^{0} fermentative growth at 30 μg mL^{-1}, whereas it inhibited the respiratory growth at lower concentration. The dose-dependent inhibition of the respiratory growth was monitored (performed as in Table 1, with increasing concentration of D12) (not shown). An IC_{50} of approximately 1 μg mL^{-1} was obtained.

Thus the target(s) of D12 are likely to be restricted to the respiratory function.

Identification of the Target of the Respiratory Inhibitors D10 and D12. We first tested the inhibitory effect of D10 and D12 on NADH oxidase activity of mitochondria as described in Methods. NADH, when added to the sample, directly delivers electrons to the NADH dehydrogenases (there is no protonmotive, metazoan-like complex I in yeast) bypassing the Krebs cycle. This was done in presence of the uncoupling agent carbonyl cyanide *m*-chloro phenyl hydrazone (CCCP). Therefore the electron flux through the respiratory chain enzymes − from the NADH dehydrogenases, *via* complex III and complex IV to oxygen − was not controlled by complex V activity. From the inhibitor titration curve (Figure 2a), an IC_{50} of 45 nM μg^{-1} protein was estimated for D10. D12 has similar inhibitory effect: at 125 nM μg^{-1} protein, a 90% inhibition of oxygen uptake was observed. Thus it is clearly apparent that both drugs target one or more enzymes of the respiratory chain.

In order to determine the enzyme(s) inhibited by D10 and D12, we monitored their effect on the different segments of the respiratory chain. The results are presented in Table 2 and illustrated in Figure 2b−d.

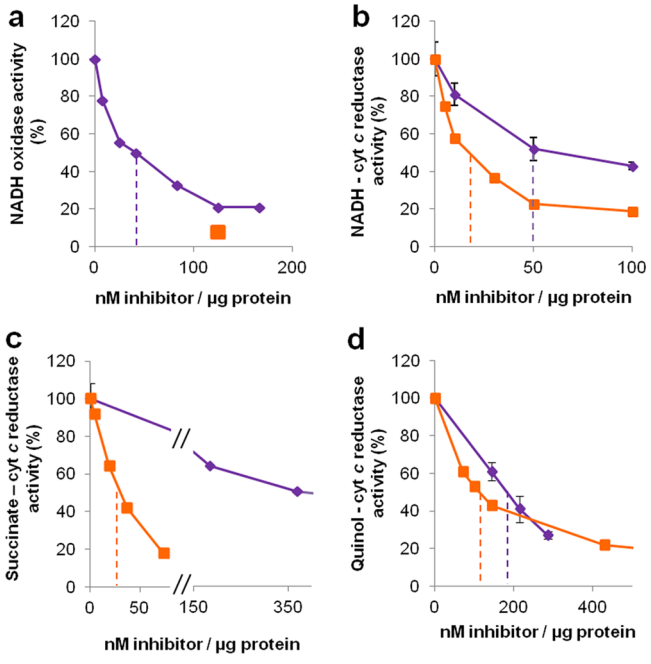

Figure 2. Effect of the compounds D10 and D12 on the respiratory chain activities. The measurements of the various activities were performed using mitochondria as described in Methods. NADH-oxidase activity (a) was monitored in a Clark-type oxygen electrode. Oxygen uptake was initiated by addition of 500 μM NADH. Oxygen uptake rate in the absence of drugs was 0.20 μmol O_2 min^{-1} mg protein $^{-1}$. Measurements of the NADH:-, succinate:-, and quinol:cytochrome *c* reductase activities (b−d) were done by following the reduction of cytochrome *c* spectrophotometrically. The activities in the absence of drugs are presented in Table 2. The measurements were done at least twice and averaged. The error bars are shown in the figures. IC_{50} values were estimated from the plot of % activity *vs* drug concentration per μg protein.

Table 2. Effect of D10 and D12 on Respiratory Chain Activities

activity[a]	D10 IC_{50} per μg protein	D12 IC_{50} per μg protein
Cytochrome *c* reductase (complex IV)	>3000	>450
NADH:cytochrome *c* reductase (NADH dehydrogenases + complex III)	50	15
Succinate:cytochrome *c* reductase (complexes II + III)	>350	30
NADH:quinone reductase (NADH dehydrogenases)	>280	>350
Quinol:cytochrome *c* reductase (complex III)	170	120

[a]Measurements were performed using mitochondria as described in Methods. The activities in the absence of drugs were as follows: NADH:cytochrome *c* reductase: 1.4 μmol cytochrome *c* min^{-1} mg^{-1}; Succinate:cytochrome *c* reductase: 0.85 μmol cytochrome *c* min^{-1} mg^{-1}; NADH:quinone reductase: 0.14 μmol quinol min^{-1} mg^{-1}; quinol:cytochrome *c* reductase: 3.2 μmol cytochrome *c* min^{-1} mg^{-1} (TN = 125 s^{-1}); cytochrome *c* oxidase activity: 1.2 μmol cytochrome *c* min^{-1} mg^{-1}.

D10 and D12 had no effect of cytochrome oxidase activity (complex IV), as expected since the only known inhibitors of mitochondrial complex IV are small molecules competitors of oxygen such as CO, CN, and azide. Both drugs inhibited the NADH:cytochrome *c* reductase activity (NADH dehydrogenase + complex III) with IC_{50}'s of 50 and 15 nM μg^{-1} protein for D10 and D12, respectively. They had no or little effect on NADH:quinone reductase (NADH dehydrogenase) but inhibited the quinol:cytochrome *c* reductase activity (complex III) with IC_{50}'s of 170 and 120 nM μg^{-1} protein for D10 and D12, respectively. Their reactivity toward complex III was low compared to commercially available complex III inhibitors such as the fungicide azoxystrobin: the IC_{50} (estimated as moles drugs per moles complex III) was around 8 for azoxystrobin,[4] whereas it was around 400 for D10 and 280 for D12. D10 and D12 differed by their effect on the succinate:cytochrome *c* reductase activity (complexes II + III). D10 had little effect on the activity (IC_{50} >350 nM μg^{-1} protein). Thus D10, while inhibiting complex III, had no effect on complex II + III activity, which may appear as a discrepancy. However, this has been previously explained by Brasseur *et al.*[9] Briefly, the authors demonstrated that complex III was rate-limiting in the NADH:cytochrome *c* reductase reaction, but was not rate-limiting in the succinate:cytochrome *c* reaction. Therefore a decrease in complex III activity impacted the NADH:cytochrome *c* reductase activity to a greater extent than the succinate:cytochrome *c* activity, which is what we observed: the IC_{50} for NADH:cytochrome *c* reductase is 10-fold lower that the IC_{50} for succinate:cytochrome *c* reaction. D12, on the contrary, inhibited the succinate:cytochrome *c* reaction with an IC_{50} of 30 nM μg^{-1} protein. This indicated that D12 had also a weak inhibitory effect on complex II.

We then compared the effect of D10 and D12 on the quinol:cytochrome *c* reductase activity (complex III) of

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mitochondrial membranes extracted from bovine heart (kindly provided by P. Rich, UCL, London). The activities were measured as for the yeast mitochondria. Interestingly, bovine complex III was not sensitive to D10 or D12. The IC_{50} values (presented as moles drugs per moles complex III) were >4000 with bovine mitochondria compared to 400 and 280 for D10 and D12, respectively, with yeast mitochondria. Similarly, we found that D12 IC_{50} for NADH:- and succinate:cytochrome *c* reductase activities were >1000 (reported as moles drugs per moles complex III) with the bovine mitochondria compared to 35 and 60 with the yeast mitochondria.

Finally, we checked the inhibitory effect of D10 and D12 on the NADH:cytochrome *c* reductase activity of mutant complexes III harboring mutations in the inhibitor binding pockets: the mutation G143A in the Q_o site that causes a high level of resistance toward the Q_o inhibitors myxothiazol (>5,000-fold) and azoxystrobin (65-fold);[10] and the mutation K228M, in the Q_i site that confers moderate level of resistance toward the Q_i inhibitor HQNO[11] and was first reported as antimycin-resistant mutation.[12] We found that D10 and D12 were fully active against the mutant enzymes: the same IC_{50} were obtained with WT and mutant complexes III. Thus, interestingly, mutation G143A causing acquired resistance to all Q_o site fungicides and detected in many plant pathogen fungi does not confer cross-resistance to D10 and D12.

Identifying the Binding Site of D12 in Complex III. As mentioned above, complex III has two inhibitor binding pockets, Q_o and Q_i, formed by the core subunit cytochrome *b*. In order to indentify the target site of D12 within complex III, we monitored the effect of a series of Q_o and Q_i site mutations on the inhibitory effect of the drug. The test was performed on the respiratory growth of the mutant strains. The 20 strains tested were identical to control strain, differing only by the amino acid substitutions in cytochrome *b*. D10 was excluded from that test since the molecule was found to be toxic to cells, inhibiting both respiratory and fermentative growth, and unstable upon incubation in the culture medium.

The Q_i site mutations were chosen on the basis of the structure[13,14] and/or on previous reports of their effect on the sensitivity toward the Q_i inhibitors HQNO and/or antimycin.[15] The Q_o site mutations, T127I, I147V, T148I, L150F, S152A, P226L, and L275F, were found in the human pathogenic fungus *P. jirovecii*, after atovaquone treatment and introduced into yeast.[16] The mutation F278A conferred resistance to atovaquone (not shown). An alanine at position 278 is naturally present in human cytochrome *b*, whereas it is replaced by a phenylalanine in yeast. G143A, as mentioned above, causes high level of resistance toward Q_o site fungicides in plant pathogenic fungi, while F129L confers moderate level of resistance to these fungicides (for a review of cytochrome *b* mutations see ref 10). The positions of the mutated Q_o site residues in the structure of the complex III and in the cytochrome *b* sequence are presented in Figure 3a and c, respectively. None of the 10 Q_i mutations tested conferred resistance to D12. Similarly, eight of the 10 Q_o site mutants studied were susceptible to the drug: T127I, F129L, G143A, T148I, L150F, S152A, P266L, and F278A. The sensitivity of the respiratory growth of G143A was thus in agreement with the enzymatic data.

Two Q_o site mutations, namely, I147V and L275F, caused a marked resistance to D12 as shown in Figure 3b. This finding indicates that D12 targets the Q_o site of complex III and that residues I147 and L275 are likely to be involved in the binding

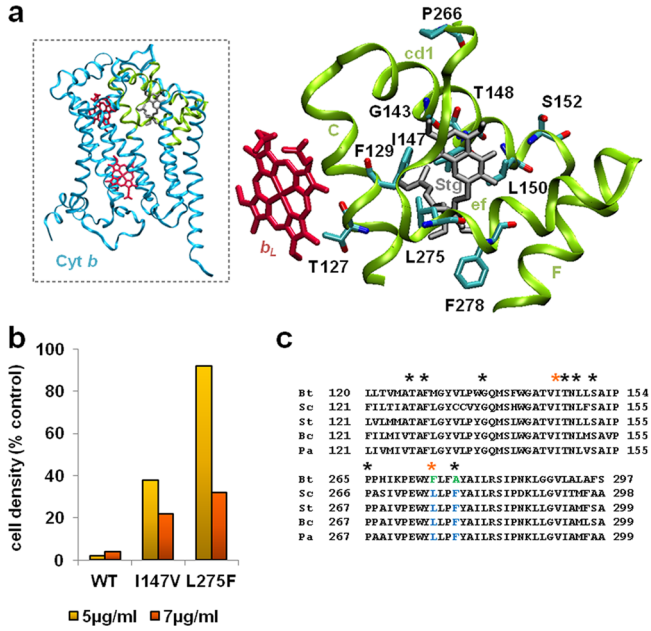

Figure 3. (a) Location of the mutated residues within the Q_o pocket of yeast cytochrome *b*. This figure was prepared using PDB coordinates 3CX5.[23] Regions encompassing the Q_o site (helices C, cd1 and the ef loop) are represented in green. The bound Q_o site inhibitor stigmatellin is labeled as "Stg". (b) Effect of Q_o mutations I147V and L275F on drug susceptibility. WT and mutant cells were inoculated in 5 mL of ethanol medium with 5 and 7 $\mu g\ mL^{-1}$ of the D12 and cultivated at 28 °C with agitation. Optical density ($A_{600\ nm}$) of the cultures was recorded after 48 h and presented as % of the control cell density in the absence of drug. The measurements were done at least twice. (c) Comparison of cytochrome *b* sequences. Only cytochrome *b* sequences that form the Q_o site are shown. Bt, *Bovus taurus*; Sc, *Saccharomyces cerevisiae*; St, *Septoria tritici*; Bc, *Botrytis cinerea*; Pa, *Podospora anserina*. The mutated residues studied here are marked by an asterisk. The variant residues at positions 275 and 278 are in color.

of the drug. As this mutational analysis shows a restricted overlap between residues involved in resistance toward atovaquone, azoxystrobin and D12, we hypothesize that the binding site and/or binding mode of D12 would be different from that of these known inhibitors.

Interestingly, a phenylalanine at position 275 is naturally present in the cytochrome *b* of the majority of organisms (including man). Fungi are unusual in having a leucine at that position. That variation could explain the differential sensitivity of the bovine and yeast complex III to D12 that we observed. By contrast, the mammalian variant F278A had no effect on the susceptibility to D12.

Chemical Structures of D10 and D12. Compounds D10 and D12 were found to be structurally different to the previously known complex III inhibitors. D10 is a carbazole while D12 is a pregnan. The chemical structure of D10, 8-(benzyloxy)-2-methyl-6,11-dihydro-5*H*-pyrido[3,2-*a*]carbazole, was determined as described in Methods. The structure of D12, 3*β*,5*α*,6*β*-triacetoxy-5*α*-pregnan-20-one ethylene ketal, has been previously reported in ref 17.

The chemical library screened in this study contained two other compounds structurally close to D12 (Figure 4). In our assay conditions, these molecules were found to be inactive, showing that the test could discriminate between closely related structures in series. From the comparison of molecules **2**, **3** and

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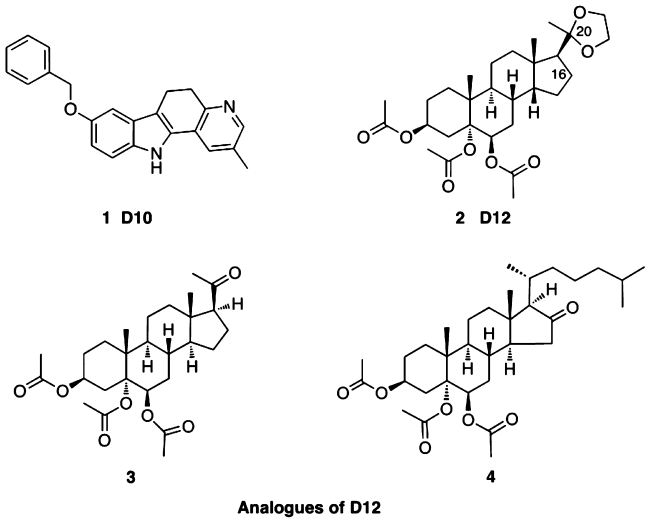

Figure 4. Chemical structures of D10, D12, and analogues of D12.

4, it appears that a dioxolane function in position 20 and no function in position 16 are important for the inhibitory effect of D12. The presence of a ketone function (*i.e.*, in compound **3**) or a 2-methylpentane chain (*i.e.*, in compound **4**) at position 20 seems detrimental for the inhibitory activity. Modifying the methylene group at position 16 by the introduction of a ketone function leads also to inactive compound (*i.e.*, compound **4**). Further work on the structure–activity relationship needs to be performed to determine the active site of the molecule.

Conclusion. We have described a rapid and easy-to-use *in vivo* screening method to identify inhibitors of respiratory function. In a collection of 4,640 molecules, we identified two new complex III inhibitors: D10, a carbazole, and D12, a pregnan. We found that D12, stable in culture medium and nontoxic to cells, targeted the Q_o site of complex III. Many inhibitors bind to the Q_o site. The Q_o pocket forms a large, bifurcated volume that is "loosely stitched" by inter sidechain H-bonds between discontinuous stretches of cytochrome *b* (mainly helices C, cd1, and the ef loop (Figure 3a)). This "loose stitching" may allow the expansion of the site upon binding of inhibitors.[18] That property presumably allows the site to accommodate diverse molecules and could explain the development of specific Q_o inhibitors active against pathogens. Interestingly, mammalian complex III was not susceptible to D12. In addition, D12 was unaffected by the Q_o site mutations G143A and F129L, responsible for acquired resistance to fungicides in phythopathogenic fungi. Related compounds with higher reactivity need to be synthesized that would have the same mode of action but tighter binding to complex III. Drugs developed on the scaffold of D12 may be a valuable tool and could circumvent Q_oI resistance caused by cytochrome *b* mutation.

METHODS

Yeast Strains and Media. The yeast strains used for TTC screening, for growth analysis, and oxygen uptake analysis were derived from AD1-9 (kindly given by M. Ghislain, UCL, Belgium). That strain harbors multiple deletions of the cell membrane transporter genes, which render the cells more sensitive to drugs than standard yeast strains.[16] AD1-9 [WT] contains a wild type mitochondrial genome. AD1-9 *rho*^{0} is missing the entire mitochondrial genome (obtained after ethidium bromide treatment of the WT strain). A series of strains derived from AD1-9 [WT] contain point mutations in the cytochrome *b* gene. They have been obtained by random- or by side-directed mutagenesis. The mutants are as follows: Q_i site mutations G33A, G37S,[11] H204Y, S206T/V,[20] N208V, R218K, F225L, K228 M,[11] and M221Q;[21] Q_o site mutations T127I,[16] F129F,[22] G143A,[4] I147V, T148I, L150F, S152A, P226L, and L275F.[16] F278A was constructed for this study by microprojectile bombardment-mediated mitochondrial transformation as described previously.[16] Respiratory medium contained 1% yeast extract, 2% peptone, 0.2% glucose, and 2% ethanol. Fermentative medium contained 1% yeast extract, 2% peptone, and 3% glucose.

TTC Screening. The yeast strain AD1-9 [WT] was cultivated in ethanol medium until mid- to late-log phase (optical density of 2 measured at 600 nm), with vigorous agitation for a good aeration of the cultures. The test was performed in 96-microwell plates using a pipetting robot. Culture (150 μL) and 5 μg of drugs (5 μL of a DMSO solution) were added, and the cell suspensions were aerated by pipetting; 7 mM TTC was then added (55 μL of TTC in a 2% ethanol solution), and the cell suspensions were again aerated by pipetting. The plates were then left at RT without agitation for a few minutes. The yeast cells sediment quickly. Cells with an active respiratory chain generate sufficient anaerobiosis in the cuvette for the red formazan precipitate to accumulate inside the cells. The red color appears after 10–15 min. Drugs inhibiting the accumulation of red formazan were identified.

Measurement of the Components of the Respiratory Chain in Yeast Mitochondria. Mitochondria were prepared as in ref 19. Protein concentration was determined by Bradford method (Protein Quantification Kit-rapid, Fluka). Complex III concentration was determined from dithionite-reduced optical spectra, using ε = 28.5 mM^{-1} cm^{-1} at 562 nm minus 575 nm. Activity measurements were performed at RT. The measurements were repeated at least twice and averaged. IC_{50} values (inhibitor concentration required to obtain 50% inhibition of the activity) were determined by inhibitor titration.

NADH-oxidase activities were measured using a Clarke-type oxygen electrode. Mitochondria were added at 120 μg of protein to 1 mL of 0.7 M sorbitol, 50 mM Tris-HCl pH7.5, 0.2 mM EDTA, 3 μM CCCP. Oxygen uptake activity was initiated by the addition of 0.5 mM NADH. After 2–3 min of recording, inhibitors were added at various concentrations, and the rate of oxygen consumption was recorded.

The quinol and/or cytochrome *c* oxidoreductase activities were measured spectrophotometrically in a Beckmann DU 640 spectrophotometer. Measurements were performed in 1 mL of 10 mM potassium phosphate pH 7.0. For quinol:cytochrome *c* reductase activity (complex III activity), 0.01% lauryl maltoside was added. Initial rates after addition of the substrate were recorded.

NADH:-, succinate:-, and quinol:cytochrome *c* reductase activities was determined by measuring the reduction of cytochrome *c* (20 μM) at 550 nm versus 540 nm over a 1-min time-course and in the presence of 1 mM KCN. Mitochondria were added at 10–40 μg of protein per mL. Activity was initiated by the addition of 20 μM decylubiquinol, a synthetic analogue of ubiquinol, for quinol:cytochrome *c* reductase activity and by the addition of 600 μM NADH for the NADH:cytochrome *c* reductase activity. The succinate:cytochrome *c* reductase activity was initiated by the addition of oxidized cytochrome *c* after 10 min of incubation of mitochondria with 10 mM succinate.

Cytochrome c oxidase activity. Mitochondria added at 15 μg of protein per milliliter. Activity was initiated by the addition of 20 μM reduced cytochrome *c*. Cytochrome *c* oxidation was recorded at 550 nm versus 540 nm over a 1-min time-course.

NADH:quinone Reductase Activity. Mitochondria added at 40 μg of protein per mL and incubated with 200 μM NADH for 10 min. Activity was initiated by the addition of 100 μM decylubiquinone. Decylubiquinone reduction was recorded at 280 nm versus 325 nm over a 1-min time-course.

Determination of the Structure of Compounds D10 and D12. ^{1}H NMR and ^{13}C NMR spectra were determined on Bruker Avance-500. ^{1}H NMR spectra are reported in parts per million (δ) relative to residual solvent peak. Data for ^{1}H are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, sxt = sextet, dd = double-doublet, m = multiplet),

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coupling constant in Hz, and integration. ^{13}C NMR spectra are reported in parts per million (δ) relative to the residual solvent peak. Optical rotations were measured with an Anton Par MCP 300 polarimeter in chloroform solutions. IR spectra were recorded with a Perkin-Elmer Spectrum 100 spectrophotometer. The mass spectra were determined with a Waters Acquity liquid chromatograph equipped with a Photodiode Array Detector and a LCT Premier XE mass spectrometer. A reverse-phase BEH C18 column, 1.7 μm, 2.1 mm × 50 mm, was used for the UPLC work with a mixture of acetonitrile/water as the solvent system.

Compound D10. 8-(Benzyloxy)-2-methyl-6,11-dihydro-5H-pyrido[3,2-a]carbazole. ^1H NMR (500 MHz, $CDCl_3$ δ (ppm) 2.36 (3H, s, H_{23}); 3.07 (2H, t, J = 10 Hz, H_{13}); 3.25 (2H, t, J = 10 Hz, H_{14}); 5.15 (2H, s, H_{16}); 6.96 (1H, dd, J_1 = 2.0 Hz, J_2 = 9.0 Hz, H_7); 7.11 (1H, d, J = 1.5 Hz, H_{10}); 7.29 (1H, d, J = 9.0 Hz, H_8); 7.34−737 (1H, m, H_{20}); 7.40−7.44 (3H, m, H_{19}, H_{21}, H_{16}); 7.50−7.52 (2H, m, H_{18}, H_{22}); 8.19 (1H, s, H_1); 8.24 (1H, s, NH). ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 18.2 (1C, C_{23}); 19.3 (1C, C_{13}); 31.6 (1C, C_{14}); 70.9 (1C, C_{16}); 100.0 (1C, C_6); 102.4 (1C, C_{10}); 111.9 (1C, C_8); 113.1 (1C, C_{12}), 113.7 (1C, C_7); 124.3 (1C, C_4); 127.1 (1C, C_3); 127.5 (2C, C_{18} C_{22}); 127.8 (1C, C_{20}); 128.5 (2C, C_{19} C_{21}); 131.1 (1C, C_2); 132.2 (1C, C_{15}); 133.0 (1C, C_{11}); 137.5 (1C, C_{17}); 146.6 (1C, C_1); 153.7 (1C, C_9); 154.5 (1C, C_{15}). ν (cm^{-1}): 3144, 2828, 1626, 1605,1583, 1558, 1377, 1242; MS (ESI, m/z): 341.2 $[M + H]^+$. HRMS (ESI, m/z): calculated for $C_{23}H_{21}N_2O$ 341.1654, found 341.1670.

Compound D12. 3β,5α,6β-Triacetoxy-5α-pregnan-20-one Ethylene Ketal. ^1H NMR (300 MHz, CH_3OD) δ (ppm) 0.83 (3H, s, H_{18}); 1.12−1.15 (1H, m, H_{16a}); 1.28 (3H, s, H_{19}); 1.29 (3H, s, H_{21}); 1.36−1.38 (1H, m, H_{7a}); 1.45−1.46 (1H, m, H_{15a}); 1.47−1.49 (1H, m, H_9); 1.50−1.52 (1H, m, H_{15b}); 1.54−1.59 (2H, m, H_2); 1.62−1.65 (2H, m, H_{11}); 1.66−1.67 (1H, m, H_{7b}); 1.68−1.72 (1H, m, H_{14}); 1.73−1.82 (2H, m, H_{12}); 1.81−1.83 (1H, m, H_{17}); 1.84−1.87 (1H, m, H_8); 1.88−1.93 (1H, m, H_{4a}); 2.02 (3H, s, Ac); 2.10 (3H, s, Ac); 2.12 (3H, s, Ac); 2.12 (2H, s, H_{12}); 2.69 (1H, t, J = 5.4 Hz, H_{17}); 2.78 (1H, ddd, J = 1.5 Hz, 4.8 and 13.2 Hz, H_{4b}); 3.83−4.05 (4H, m, H_{22}, H_{23}); 4.72 −4.83 (1H, m, H_3); 5.91 (1H,dd, J = 2.4 and 3 Hz, H_6). ^{13}C NMR (75 MHz, CH_3OD): δ (ppm) 13.2 (1C, C_{18}); 17.6 (1C, C_{19}); 21.1 (2C, Ac); 21.9 (1C, Ac); 24.0 (1C, C_{12}); 24.6 (1C, C_{11}); 24.8 (1C, C_{14}); 24.4 (1C, C_{15}); 31.0 (1C, C_8); 31.3 (1C, C_4); 32.3 (1C, C_7); 33.0 (1C, C_2); 40.8 (1C, C_{16}); 41.0 (1C, C_{10}); 43.3 (1C, C_{13}); 46.7 (1C, C_9); 57.0 (1C, C_1); 59.5 (1C, C_{17}); 64.2 (1C, C_{22}); 66.1 (1C, C_{23}); 70.7 (1C, C_6); 71.2 (1C, C_3); 88.1 (1C, C_5); 113.0 (1C, C_{20}); 171.4 (1C, C═O); 171.5 (1C, C═O); 172.2 (1C, C═O). $[\alpha]_D$ −27° (c 0.5). ν (cm^{-1}) 1732. MS (ESI, m/z) 543.3 [M + Na]. HRMS (ESI, m/z): calculated for $C_{29}H_{44}O_8Na$ 543.2934, found 543.2910.

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Notes

The authors declare no competing financial interest.

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