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Chemical genetics approach to identify new small molecule modulators of cell growth by phenotypic screening of *Saccharomyces cerevisiae* strains with a library of morpholine-derived compounds†‡

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A chemical genetics approach has been applied in the screening of yeast deletants strains with a pool of morpholine-derived compounds in order to identify candidate small molecules able to produce phenotypic effects on yeast cells. The analysis of the effects of structurally diverse molecules towards cell growth rate in both exponential and stationary phases provides a tool to select candidate compounds for subsequent assays to identify new chemical entities as chemical probes for drug discovery.

Introduction

A central challenge in chemical genetics is the mapping of 'biological-activity space', which involves analysing both protein binding and phenotypic responses with respect to small molecules.[1] A number of high-throughput phenotypic assays have been developed, including assays that measure cell viability or proliferation.[2] After collecting a large amount of data on the ability of the members of a chemical library to bind to a set of proteins and affect a set of phenotypes, the data can be analysed to determine the relationship between chemical structure and biological activity. Each compound can be assigned a vector that describes the quantitative level of binding to each protein, and the quantitative effect this has on each phenotype.

Saccharomyces cerevisiae is particularly suited for the high-throughput screening of new chemical entities on whole cell systems due to key features connected to easy manipulation and the versatility of its genome.[3] The life cycle is particularly rapid, as it divides in about two hours, it is possible to manipulate both haploid and diploid yeast systems. Moreover, it shows high degree of conservation with human cells concerning main biological processes.

Yeast cells can be used first of all to identify novel compounds showing an inhibitory effect on growth. Afterwards, functional information about the mode-of-action of selected compounds can be obtained from scoring ~5,000 viable yeast haploid deletion mutant strains for hypersensitivity or hyper-resistance to each specific drug, thus identifying pathways that influence cellular response to the toxic compound.[4]

We recently turned our attention to morpholine-containing molecules, as the relevance of such heterocycle in medicinal chemistry is remarkable, being present in several bioactive molecules, such as TACE (TNF-α converting enzyme),[5] MMP (matrix metalloproteinase), and TNF (tumour necrosis factor) inhibitors.[6] Accordingly, we reported on the diversity-oriented synthesis of morpholine scaffolds taking advantage of a two steps process involving the combination of amino acid derivatives as building blocks.[7] Also, the conformational analysis of peptides containing morpholine-3-carboxylic acid (Mor) suggested this molecule to act as an unexplored proline surrogate.[8]

We applied the reactivity of some morpholine acetals to give access to a library of morpholine-derived molecules, some of which also contained the 2,5-diketopiperazine nucleus, which in turn is considered a privileged scaffold in medicinal chemistry owing to the wide number of bioactive natural products and drugs containing such chemical entity.[9] The library of new chemical entities was successively applied for the systematic exploration of yeast deletants, so as to classify deletants as a function of bioactive molecules. Accordingly, a scatter diagram plotting the effect of the molecules towards yeast cell growth in both the exponential and stationary phases was assessed as a criterion for hit selection. Further experiments allowed for subsequent selection of candidate compounds as chemical probes to be applied in Systems Biology investigations of cell cycle mechanisms.

Results and discussion

Chemistry

Scaffolds **I–III** were considered for library generation. Scaffold **I** was obtained from the combination of dimethoxyacetaldehyde **A** and serine or L-threonine derivatives corresponding to building blocks **B** with R^1=H or CH_3, respectively (Scheme 1).[10] Scaffold **II** was obtained as a single stereoisomer in two steps from phenacyl bromide **C** and threonine derivative **B** consisting of a coupling in *N*-methylpyrrolidone, followed by cyclization in HCl/MeOH solution (see ESI data‡).

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‡ Electronic supplementary information (ESI) available: Table of library members, experimental procedures and characterization data for compounds **3–47**, crystallographic data for **34**, and tabular data of the screening of selected yeast strains with library members. CCDC reference number 769053. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c0ob00357c

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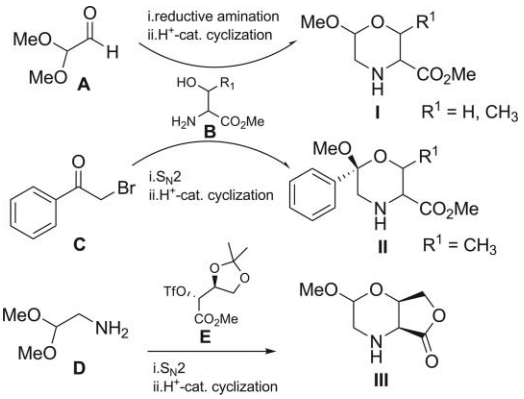
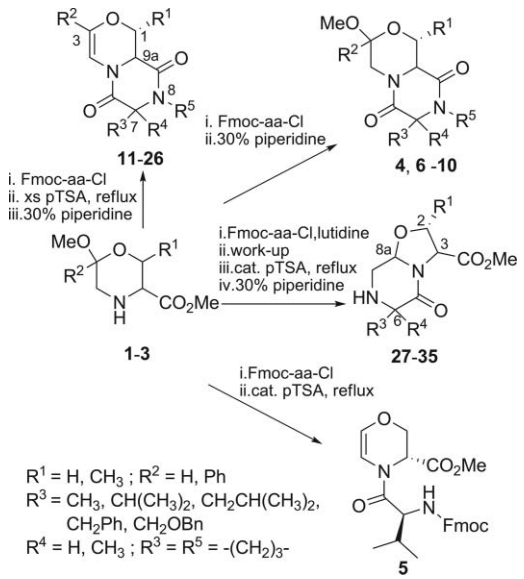


Scheme 1 Generation of morpholine scaffolds through three different pathways.

Scaffold **III** embedding a bicyclic lactone was obtained from aminoacetaldehyde dimethylacetal **D** and protected methyl threonate derivative **E**, as reported.[7]

Starting from scaffolds **I–II**, a library of compounds embedding 2,5-diketopiperazine, 2-oxopiperazine, morpholine and 1,4-dihydro-oxazine heterocycles was generated (see Scheme 2 and Table S1 in ESI data for the complete Chart of compounds‡).



Scheme 2 Library members from scaffolds **I–II**.

Compounds **4** and **6–10** were obtained from a two-steps process consisting of a coupling with Fmoc-aa-Cl, followed by Fmoc deprotection with concomitant formation of the 2,5-diketopiperazine ring. Library members **11–26** were obtained by a three-steps one-pot process consisting of 1) coupling of Fmoc-aa-Cl, 2) 1,4-dihydro-oxazine formation through acid-mediated double bond formation, 3) 2,5-diketopiperazine ring formation by treatment with 30% piperidine as the Fmoc-deprotecting agent. The stepwise process consisting of an acid–base workup after amino acid coupling allowed for the achievement of the bicyclic structure of compounds **27–35** embedding 2-oxo-piperazine and oxazolidine rings, as evinced by crystallographic data obtained for compound **34** (Fig. 1). The absence of 2,6-lutidine in the mixture, and the use of catalytic quantities of *p*-TSA in the second step

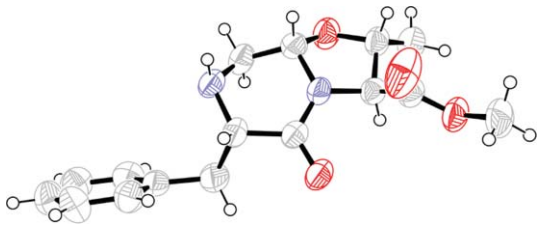
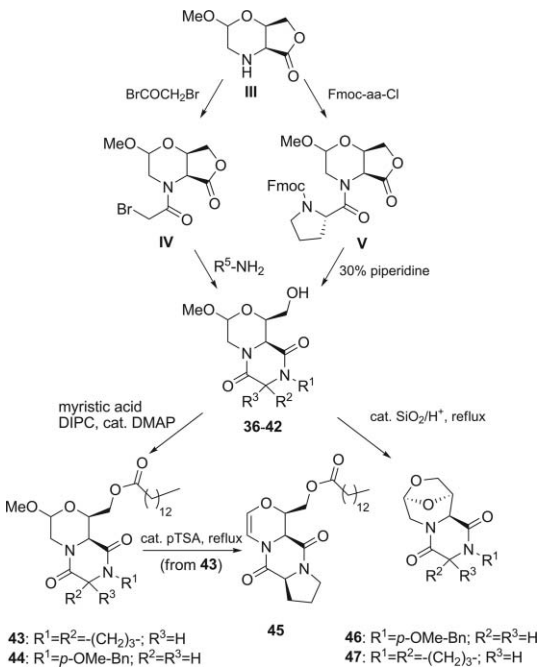


Fig. 1 X-Ray structure of **34**.

were found beneficial for the intermediate oxonium species to undergo an attack by the urethane nitrogen atom, followed by a rearrangement towards the new bicyclic species.

Finally, compound **5** was considered in the library to assess the effects of a monocyclic intermediate with respect to parent bicyclic scaffolds.

Bicyclic lactone **III** was employed for the construction of bicyclic diketopiperazines **36–42** by coupling with bromoacetyl bromide (scaffold **IV**) or Fmoc-L-Pro-Cl (scaffold **V**), and subsequent cyclization by treatment with selected primary amines or 30% piperidine as the Fmoc-deprotection agent (Scheme 3 and Table S1 in ESI data‡).



Scheme 3 Library members from scaffold **III**.

The hydroxymethyl group was successively processed to access further chemical diversity. Esterification of compounds **43–44**, coming from scaffold **V** and **IV** respectively, with myristic acid allowed for the generation of lipidated scaffolds to envisage potential interactions across the cell membrane.

Also, acid-catalyzed processes were applied to myristoyl-L-proline-derived compound **43** to obtain the corresponding 1,4-dihydro-oxazine derivative **45**, and to **41–42** possessing the free hydroxy group to obtain the corresponding polycyclic scaffolds **47** and **46**, respectively.

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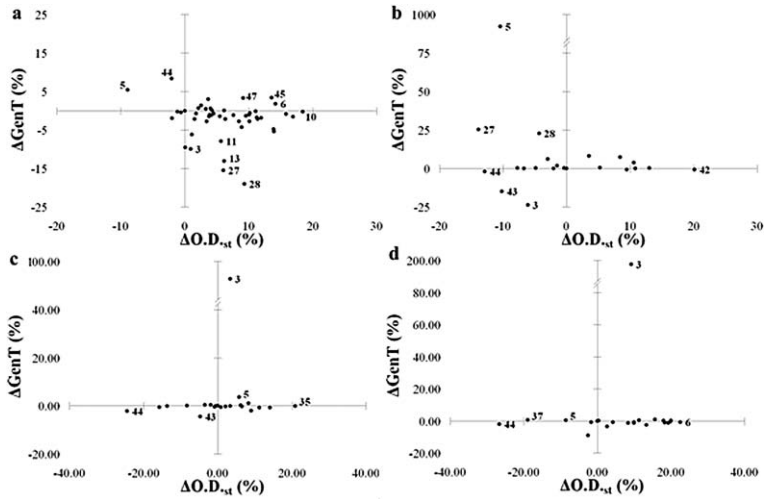

Fig. 2 Graphical representation of the effects of the molecules a) on the BY4742 wild-type strain b) on the BY4742Δ*erg6* strain c) on the BY4742Δ*snq2* strain and d) on the BY4742Δ*pdr3* strain. Percent values on both axes are referred to the untreated strain.

Biology

The effects of 48 compounds were initially tested at 0.3 mM concentration on the BY4742 wild-type strain (Fig. 2a). Compounds responsible for the variation in cell generation time, which is the time interval required for a yeast cell to divide, or of the $O.D._{650}$ value of the stationary phase ($O.D._{st}$), or both, were selected for further characterization. Most of the molecules induced higher fitness on the wild-type strain, as shown by higher $O.D._{st}$ and lower GenT values, some of which inducing a more intense effect, *e.g.* **11**, **13**, **27**, and **28**. Only two molecules, **5** and **44**, induced a decrease in the yeast fitness, assumed from both an $O.D._{st}$ decrease and a GenT increase. Other molecules, such as **6**, **45** and **47**, induced an $O.D._{st}$ and GenT increase. In this case the increase of the $O.D._{st}$ can be ascribed to an increase of the cellular volume, rather than to an increase in cell concentration, which should be associated to a lower GenT.

The screening on the wild-type strain allowed for the selection of 21 molecules inducing an $O.D._{st}$ and/or GenT variation in absolute value higher than 10% or others interesting effects (*e.g.* decrease of the yeast fitness).

Some structural determinants could be drawn for this first run of screening (see Table S1 in ESI file‡). Activity of compound **3** with respect to **2** highlighted the importance of the phenyl substituent at position 6 of the morpholine ring.

The role of such substituent was also evident by comparing L-leucine-derived hit compound **11** with respect to the corresponding scaffold **15** lacking of the phenyl substituent. Remarkable activity of **5** with respect to parent bicyclic compound **26** emphasized the importance of the 1,4-dihydro-oxazine ring with respect to the 2,5-diketopiperazine moiety, and also the influential role of the bulky hydrophobic Fmoc group. The effect of **42** with respect to **36–40** showed the role of the *p*-methoxybenzyl group in the pool of compounds originated from scaffold **IV**. Activity of compound **44** also put evidence on the role of lipidated derivatives. Such compounds were thus tested for effects on the BY4742Δ*erg6*, BY4742Δ*snq2* and BY4742Δ*pdr3* deletion strains bearing deficiencies in genes involved in cell wall and MDR (Multi Drug Resistance) (Fig. 2b, 2c, and 2d). The use of a pool of strains bearing deletions of genes involved in membrane assembly or function enables the identification of effects that could be concealed by membrane permeation incapability of the molecule. Moreover it allows for the identification of pathways that influence the cellular response to tested compounds (if the deleted gene is the gene coding the compound target, the compound stops inducing the effects showed on the wild-type strain). Erg6p is a methyl transferase, having a downstream role in the ergosterol biosynthesis with respect to one of the morpholine target (Erg24p), and upstream with respect to the other morpholine target (Erg2p); Snq2p is drug-efflux pump ABC (ATP-Binding Cassette) transporter conferring resistance to drugs and oxygen radicals; Pdr3 is a transcriptional co-activator of genes encoding ABC transporters.

Compound **3** induced dramatic effects on GenT of BY4742Δ*snq2* and BY4742Δ*pdr3* (102.6% and 157.6%, respectively), with a proportional $O.D._{st}$ increase (3.4% and 9.2%, respectively), whereas it induced an opposite effect on the BY4742Δ*erg6* strain (Gent -23.7%, $O.D._{st}$ –6.1%).

These results allowed us to hypothesize that **3** targets the ergosterol biosynthesis pathway downstream Erg6p. Much more intense was the effect of molecule **5** on GenT of the deletant strain BY4742Δ*erg6* (921.5%). Interestingly, several molecules showed opposite effects on deletant strains with respect to the wild-type strain. Specifically, molecules **3**, **6** and **35**, inducing an $O.D._{st}$ decrease in the wild-type strain, induced the same, though weaker, effect on BY4742Δ*erg6* strain, and an opposite effect on BY4742Δ*pdr3* and BY4742Δ*snq2* strains, showing that the activity of the molecule is affected by cell MDR (Multi Drug Resistance) systems. Compound **42**, inducing an $O.D._{st}$ decrease in the BY4742 (–10.8%), had opposite skills on BY4742Δ*erg6* and BY4742Δ*pdr3* strains (20.1% and 4.2%, respectively), indicating a possible transcriptional target. Molecule **5** induced an $O.D._{st}$ decrease on wild-type, Δ*erg6* and Δ*pdr3* strains (–28.1%, –10.5% and –8.5%, respectively), but induced an increase on the Δ*snq2* strain (5.8%).

Compound **29** induced an $O.D._{st}$ increase on every deletant strain, differently from the effect on the wild-type strain. The

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myristoylated compound **44** induced a $O.D._{st}$ decrease in all the tested strains, with the greatest effect on the BY4742Δ*pdr3* strain.

The eight molecules (**3**, **5**, **6**, **28**, **29**, **35**, **42**, and **44**) inducing $O.D._{st}$ and/or GenT variations higher than 10% on at least two of the deletant strains were tested for mitochondrial membrane potential activation (Fig. 3) and peroxisomal proliferation (Fig. 4). Mitochondrial membrane activation and peroxisomal proliferation are indicators of the metabolic state of the cell, disclosing respiratory and fatty acid metabolism, respectively. The variation in mitochondrial membrane potential is also caused by ion-channel permeability and drug binding.[11] Peroxisomes are organelles containing enzymes for the β-oxidation of fatty acids, can be involved in inactivation of toxic substrates (H_2O_2-based respiration), in the synthesis of etherphospholipids (in mammals) and in the breakdown of purines and amino acids.[12] Moreover, the peroxisome function and assembly are controlled by ABC proteins.[13] The mitochondrial activation skill of each molecule was assessed in various metabolic conditions, growing cells in YP supplemented with different carbon sources: glucose and galactose (both fermentable and non fermentable carbon source), glycerol (non fermentable) and oleate (fatty acid, β-oxidable by peroxisomes). All the selected molecules induced lower percentage of mitochondrial activation with respect to the control treatment (with DMSO), in particular the **3** molecule induced the lowest mitochondrial activation in all the media assayed (Fig. 3). The only molecule inducing an increase in mitochondrial activation was **42** in YPGal. The peroxisomal proliferation was unaltered in every treatment but in the treatment with **3** and **5** molecules, in which it was decreased in a statistically significant level. Interestingly, the molecule **3** induced an apparent wall modification on the strain BY4742Δ*erg6*, as observable by decreased Calcofluor White staining, confirming the hypothesis that this molecule target is the ergosterol biosynthesis pathway (Fig. 5).

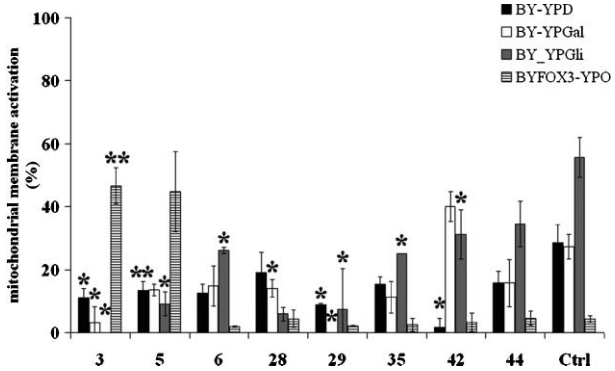

Fig. 3 Mitochondrial activation induced by the selected molecules on the BY4742 strain grown in various media. Values indicate the mean of tree measurements, error bars indicate the corresponding standard deviations values. Student's t test was used to evaluate the data significance, *, $p < 0.05$; **, $p < 0.01$.

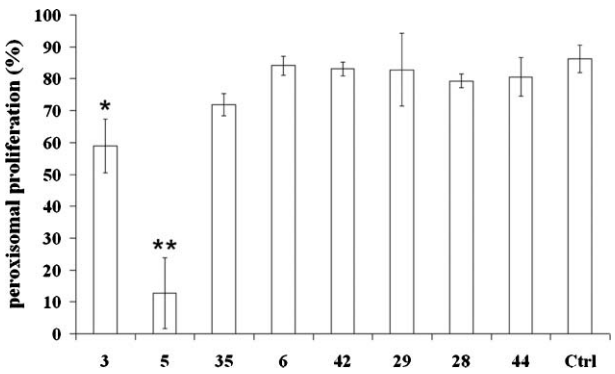

Fig. 4 Peroxisomal proliferation induced by the treatment of the strain BY4742-GFP/FOX3 with the selected molecules. Values indicate the mean of tree measurements, error bars indicate the corresponding standard deviations values. Student's t test was used to evaluate the data significance, *, $p < 0.05$; **, $p < 0.01$.

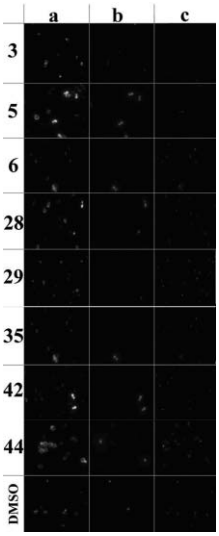

Fig. 5 Microscopic observation of the effects of eight selected molecules on the BY4742*FOX3*-GFP strain grown in YPO (0.2%). a) cells wall stained with Calcofluor White b) mitochondrial membrane potential activation visualized with Rhodamine B hexyl ester c) peroxisomes individuated by fusion product FOX3-GFP expression, a 3-ketoacyl-CoA thiolase, having peroxisomal localization.

Our results indicate that the molecules **3** and **5** show bright activities. Compound **3**, inducing the lowest peroxisomal proliferation and a growth increase in Δ*snq2* and Δ*pdr3* strains, is a promising powerful instrument to gain further insight into the drug resistance mechanism and acquisition in mammal and yeast cells. On the other hand, compound **5**, showing killer activity only toward wild-type and Δ*erg6* strains, may be further explored as a suitable chemical probe for assessing the mechanisms underlying the multidrug resistance of pathogenic cells with respect to normal cells.

The likely function of molecule **5** on the cells is a starvation induction, induced by respiration and β-oxidation decrease (observable by the mitochondrial activity and peroxisomal proliferation reduction).

Conclusions

The process of generating libraries of new chemical entities carrier of chemical diversity, and the screening towards suitable living systems such as *Saccharomyces cerevisiae* through a chemical genetics approach is a promising and powerful tool to identify candidate small molecules for drug discovery purposes. Accordingly, the graphical inspection of the effects of structurally diverse molecules towards cell growth rate in both exponential and stationary phases

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through scatter plots provides a rapid and efficient tool to evaluate the effects of library members with respect to different yeast deletant strains, thus enabling the classification of phenotype responses as a function of the chemotype, and to provide new chemical tools for Systems Biology.

Experimental

General procedure A for the synthesis of compounds 4,6–10 from scaffold I

Morpholine acetal **I** (1 eq) was dissolved in toluene (10 mL mmol^{-1}), then 2,6-lutidine (2 eq) and Fmoc-aa-Cl (1 eq) were sequentially added. The reaction mixture was brought to 60 °C and stirred for 2 h. The reaction mixture was brought back at r.t. and 30% diethylamine solution in CH_3CN (5 mL mmol^{-1}) was added. The reaction mixture was stirred for 2 h at r.t., then diluted with EtOAc (40 mL mmol^{-1}), washed with 5% $NaHCO_3$ and brine. The organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated. A dark brown oil was obtained and purified by flash chromatograhy (EtOAc–petroleum ether 2 : 1), giving the title 2,5-diketopiperazine compounds. See ESI file for characterization data.‡

General procedure B for the synthesis of compounds 11–26 from scaffold I or II

Morpholine acetal **I** or **II** (1 eq) was dissolved in toluene (10 mL mmol^{-1}), then 2,6-lutidine (2 eq) and Fmoc-aa-Cl (1 eq) were sequentially added. The reaction mixture was brought to 60 °C and stirred for 2 h, then allowed to return at r.t. Additional toluene (5 ml mmol^{-1}) and *p*-toluenesulfonic acid monohydrate (5 eq) were added, then the mixture was placed in a single-necked round-bottomed flask equipped with a reflux condenser and a dropping funnel containing 4 Å molecular sieves and it was refluxed for 2 h. The reaction mixture was brought back at r.t. and 30% diethylamine solution in CH_3CN (5 mL mmol^{-1}) was added. The reaction mixture was stirred for 2 h at r.t., then diluted with EtOAc (40 mL mmol^{-1}), washed with 5% $NaHCO_3$ and brine. The organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated. A dark brown oil was obtained and purified by flash chromatography (EtOAc–petroleum ether 2 : 1), giving the title 2,5-diketopiperazine compounds. See ESI file for characterization data.‡

General procedure C for the synthesis of compounds 27–35 from scaffold I

Morpholine acetal **I** (1 eq) was dissolved in anhydrous CH_2Cl_2 (4 mL mmol^{-1}), then 2,6-lutidine (2 eq) and Fmoc-aa-Cl (1 eq) were sequentially added. The reaction mixture was brought to 60 °C and stirred for 2 h under a nitrogen atmosphere. The reaction mixture was then diluted with CH_2Cl_2 and sequentially washed with 5% HCl, 5% $NaHCO_3$ and brine. The organic layers were dried over Na_2SO_4, filtered and evaporated to give a brownish foam. The crude product was then dissolved in toluene (5 mL mmol^{-1}) and *p*-toluenesulfonic acid monohydrate (1 eq) was added. The reaction mixture was placed in a single-necked round-bottomed flask equipped with a reflux condenser and a dropping funnel containing 4 Å molecular sieves and refluxed for 2 h, then it was allowed to return to r.t. EtOAc was added, and the organic solution was washed with 5% $NaHCO_3$ and brine. The organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated to give a dark brown oil. The crude product was dissolved in 30% diethylamine in CH_3CN (5 mL mmol^{-1}) and allowed to react for 2 h at r.t. The reaction mixture was then evaporated to obtain a pale brown solid, which was purified by flash chromatography (EtOAc-petroleum ether 3 : 1), to give the title bicyclic 2-oxopiperazine compounds as dark orange oils. See ESI file for characterization data.‡

General procedure D for the synthesis of compounds 36–40 and 42 from scaffold IV

Bromoacetyl-lactone **IV**[7] was dissolved in MeOH (1 mL mmol^{-1}), then selected primary amine (5 eq) and Et_3N (5 eq) were added, and the mixture was left reacting in a microwave synthesizer at 100 °C for 20 min. The mixture was then diluted with CH_2Cl_2 and eluted through Amberlist A-21 Amberlist 15 resins. After solvent evaporation, pure compound was obtained without further chromatographic purification. See ESI file for characterization data.‡

Yeast strains and media

The wild-type BY4742 (*MATα his3 leu2 lys2 ura3*) strain was used. The deletion strains used were BY4742Δ*snq2* (*MATα his3 leu2 lys2 ura3 snq2*::*kanMX*), BY4742Δ*pdr3* (*MATα his3 leu2 lys2 ura3 pdr3*::*kanMX4*), and BY4742Δ*erg6* (*MATα his3 leu2 lys2 ura3 erg6*::*kanMX4*). For growth inhibition tests, yeast cells were inoculated at 1×10^5 cell/ml in YPD medium (Yeast Peptone Dextrose, yeast extract 1% (w/v), peptone 2% (w/v) supplemented with 2% glucose). BY4742*FOX3-GFP* (*MATa his3 leu2 met15 ura3 YIL160c-GFP*), a strain bearing the fusion product GFP-FOX3, 3-ketoacyl-CoA thiolase, having peroxisomal localization) was used to follow peroxisomal proliferation. For mitochondrial membrane activation and peroxisomal proliferation assays YP added with various Carbon sources was used: 2% Glucsose (YPD), 2% Galactose (YPGal), 2% Glycerol (YPGly) and 0.2% Oleate (YPO, supplemented with 0.2% Tween80 to emulsify oleate).

Biological assays

BY4742 strain was grown in YPD. The cultures was dispensed at equal volume onto 96-well plates, each well containing a different compound at a concentration of 0.3 mM. The assay plates were incubated at 28 °C with shaking, and yeast growth was scored by measurement of $O.D._{650}$ with a Victor high-throughput microplate spectrophotometer equipped with a 96-well plate reader every 2 h for the first 24 h, and then once a day for 4 days. Compounds responsible for the variation in cell generation time (the time interval required for a yeast cell to divide), or of the $O.D._{650}$ value of the stationary phase ($O.D._{st}$), or both, were selected for further characterization. Cell generation time (GenT, the time interval required for a yeast cell to divide) was calculated as $\Delta(t_2\text{-}t_1)/\{3.3*[\log_{10}(O.D._{t2}/O.D._{t1})]\}$, where t_1 and t_2 are time points flanking the inflection point of the growth curves. The percent effect of each molecule was calculated as a function of the GenT or $O.D._{st}$ value of treated cells and the GenT or $O.D._{st}$ of untreated cells. The effects on deletant stains was assayed for selected

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compounds. Deletion strains BY4742Δ*snq2*, BY4742Δ*pdr3*, and BY4742Δ*erg6* were treated conformingly with the wild-type strain treatment, with 0.3 mM molecules. Compounds responsible for the variation in deletant strain cells generation time, or of the $O.D._{st}$ value, or both, were selected for mitochondrial and peroxisomal investigations.

Fluorescence microscopy

Effects at the mitochondrial level of selected compounds were observed by mitochondrial membrane potential investigation. Effects at the peroxisomal level were investigated by GFP-FOX3 microscopic observation. After 4 h of treatment with selected compounds at 0.3 mM concentration, or without them as a control, cultured cells were dissolved at 1×10^6 cells ml^{-1} in 10 mM HEPES buffer, pH 7.4, containing 5% glucose. Rhodamine B hexyl ester was added to a final concentration of 100 nM. After 15–30 min of incubation, the mitochondrial membrane potential was visualized by fluorescence microscopy (excitation λ at 555 nm, green/emission λ at 579 nm, red). Each aliquot was treated with Calcofluor White (M2R) (Blue) to evidence the cell wall in order to count total cells. Mitochondrial membrane potential activation was calculated as percentage of red-fluorescent cells (rhodamine B hexyl ester-labelled cells) toward total blue-cells (Calcofluor White-labelled cells) of the same treated culture.

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