

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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Abstract

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
15 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

20 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
25 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
30 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
35 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
40 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
45 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
50 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.

55 **Results and discussion**

Chemistry Scaffolds I–III were considered for library generation. Scaffold I was obtained from the combination of di methoxy acetaldehyde A and serine or L-threonine derivatives corresponding to building blocks B with R1 H or CH₃, respectively (Scheme 1).¹⁰ Scaffold II was obtained as a single stereoisomer in two steps from phenacylbromide C and threonine
60 derivative B consisting of a coupling in N-methylpyrrolidone, followed by cyclization in HCl/MeOH solution (see ESI data). 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 embed-ding 2,5-diketopiperazine, 2-oxopiperazine, morpholine and 1,4-dihydro-oxazine heterocycles was
65 generated (see Scheme 2 and Table S1 in ESI data for the complete Chart of compounds).

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
70 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

75 mixture, and the use of catalytic quantities of p-TSA in the second step 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
80 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). 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
85 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.

90 **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
95 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
100 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
105 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,
110 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
115 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,
120 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 BY4742 Δ snq2 and BY4742 Δ 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 BY4742Derg6strain (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 BY4742 Δ 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 BY4742 Δ erg6 strain, and an opposite effect on BY4742 Δ pdr3 and BY4742 Δ 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 BY4742 Δ erg6 and BY4742 Δ 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 myristoylated compound 44 induced a O.D._{st} decrease in all the tested strains, with the greatest effect on the BY4742 Δ 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 b-oxidation of fatty acids, can be involved in inactivation of toxic substrates (H₂O₂-based respiration), in the synthesis of ether phospholipids (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, b-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 4 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 BY4742 $\Delta erg6$, as observable by decreased Calcofluor White staining, confirming the hypothesis that this molecule target is the ergosterol biosynthesis pathway (Fig. 5). Our results indicate that the molecules 3 and 5 show bright activities. Compound 3, inducing the lowest peroxisomal proliferation and a growth increase in $\Delta snq2$ and $\Delta pdr3$ 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 $\Delta erg6$ 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,

170 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
175 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 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
180 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)
185 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 CH₃CN (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% NaHCO₃ and brine. The organic layers were dried over anhydrous Na₂SO₄, filtered and evaporated. A dark brown oil was
190 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 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-toluene sulfonic 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 CH₃CN (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% NaHCO₃ and brine. The organic layers were dried over anhydrous Na₂SO₄, 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 CH₂Cl₂ (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 CH₂Cl₂ and sequentially washed with 5% HCl, 5% NaHCO₃ and brine. The organic layers were dried over Na₂SO₄, filtered and evaporated to give a brownish foam. The crude product was then dissolved in toluene (5 mL mmol⁻¹) and p-toluene sulfonic 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% NaHCO₃ and brine. The organic layers were dried over anhydrous Na₂SO₄, filtered and evaporated to give a dark brown oil. The crude product was dissolved in 30% diethylamine in CH₃CN (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.

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

Bromoacetyl-lactoneIV7 was dissolved in MeOH (1 mL mmol⁻¹), then selected primary amine (5 eq) and Et₃N (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 CH₂Cl₂ and eluted through Amberlist A-21 Amberlist 15 resins. After solvent evaporation, pure compound was obtained
225 without further chromatographic purification. See ESI file for characterization data.

Yeast strains and media

The wild-type BY4742 (MAT_{ahis3} leu2 lys2 ura3) strain was used. The deletion strains used were BY4742Dsnq2 (MAT_{ahis3} leu2 lys2 ura3 snq2::kanMX), BY4742Dpdr3 (MAT_{ahis3} leu2 lys2 ura3 pdr3::kanMX4), and BY4742Derg6 (MAT_{ahis3} leu2 lys2 ura3 erg6::kanMX4). For
230 growth inhibition tests, yeast cells were inoculated at 1×10⁵ cell/ml in YPD medium (Yeast Peptone Dextrose, yeast extract 1% (w/v), peptone 2% (w/v) supplemented with 2% glucose). BY4742FOX3-GFP (MAT_a 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
235 proliferation assays YP added with various Carbon sources was used: 2% Glucose (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 were dispensed at equal volume onto 96-well
240 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 $D(t_2-t_1)/\{3.3 \cdot [\log_{10}(O.D.t_2/O.D.t_1)]\}$, where t₁ and t₂ 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 strains was assayed for selected 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.

255 **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 × 10⁶ cells ml⁻¹ in 10 mM HEPES buffer, pH 7.4, containing 5% glucose. Rhodamine B hexylester 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 at 555 nm, green/emission 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

265 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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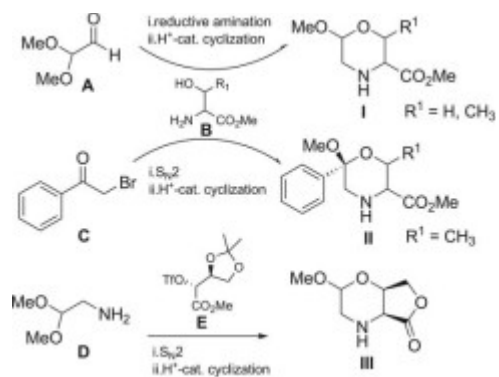
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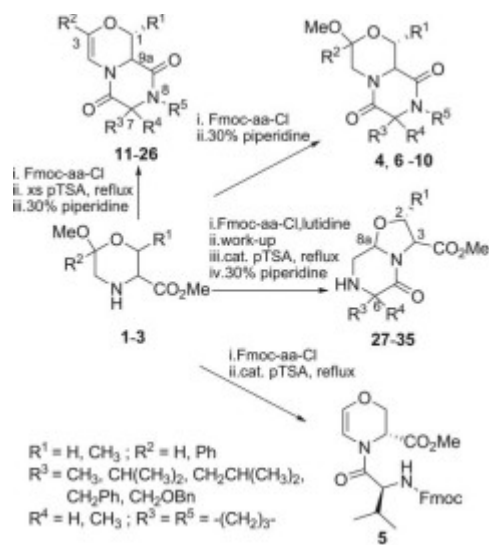
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Schemes

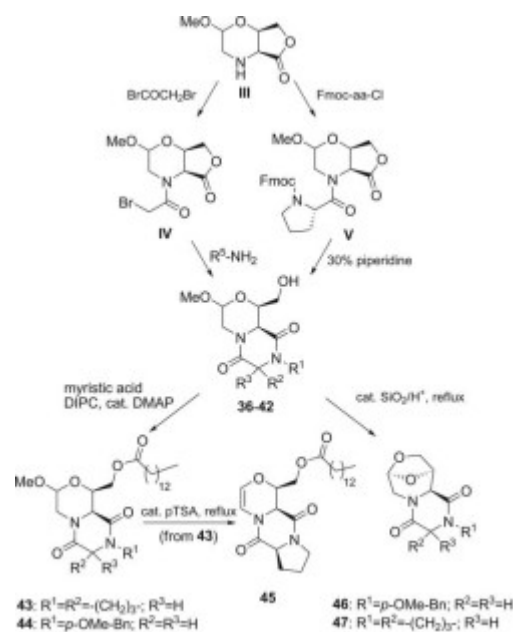
Scheme 1 Generation of morpholine scaffolds through three different pathways.



Scheme 2 Library members from scaffolds I–II.



Scheme 3 Library members from scaffold III.



320 **Figures**

Fig. 1 X-Ray structure of 34.

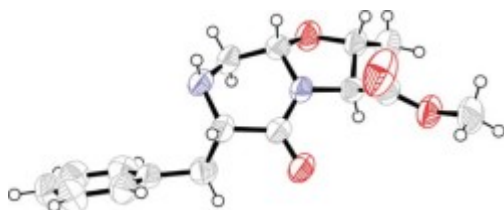
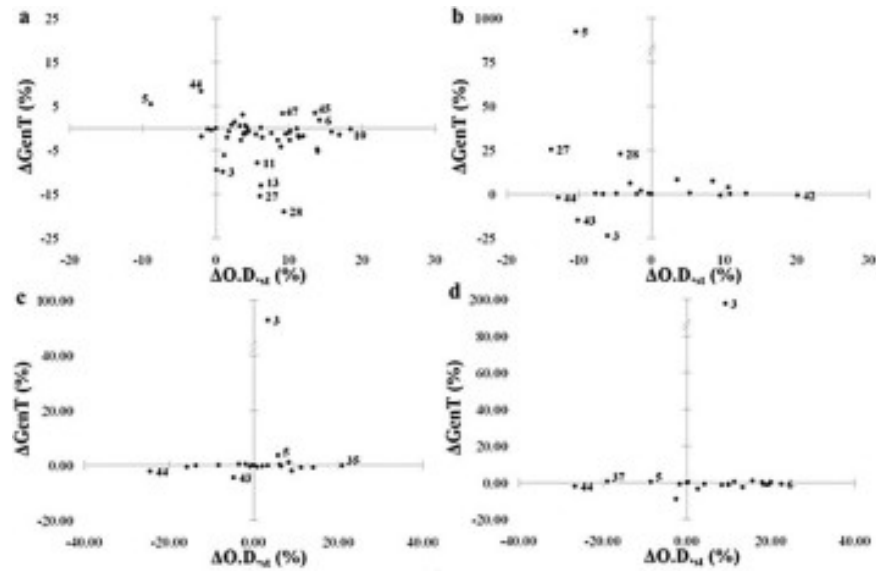


Fig. 2 Graphical representation of the effects of the molecules a) on the BY4742 wild-type strain b) on the BY4742Derg6 strain c) on the BY4742Dsnq2 strain and d) on the BY4742Dpdr3 strain. Percent values on both axes are referred to the untreated strain.



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

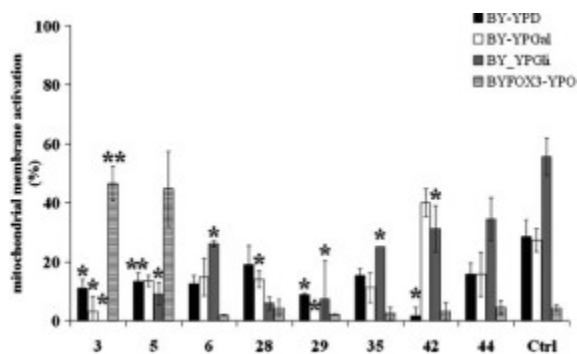


Fig. 4 Peroxisomal proliferation induced by the treatment of the strainBY4742-GFP/FOX3 with 330 the selected molecules. Values indicate the mean of tree measurements, error bars indicate the corresponding standard deviations values. Student's t test was used to evaluate the data significance, *, $p < 0.05$; **, $p < 0.01$

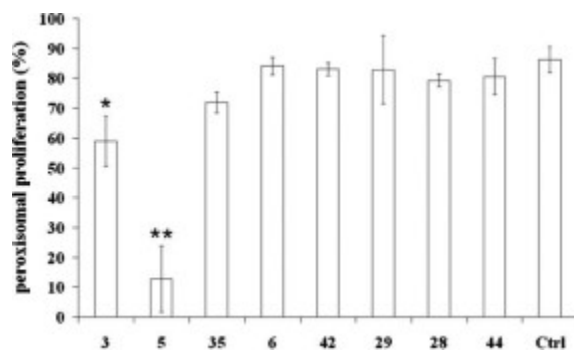


Fig. 5 Microscopic observation of the effects of eight selected molecules on the BY4742FOX3-GFP strain grown in YPO (0.2%). a) cells wall stained with Calcofluor White b) 335 mitochondrial membrane potential activation visualized with Rhodamine B hexyl ester c) peroxisomes individuated by fusion product FOX3-GFP expression, a 3-ketoacyl-CoA thiolase, having peroxisomal localization.

