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# Discovery of uracil-based histone deacetylase inhibitors able to reduce acquired antifungal resistance and trailing growth in *Candida albicans*

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**Abstract**—Among fungal pathogens such as *Candida albicans*, acquired drug resistance has not been associated with plasmids or other transferable elements, but it is thought to involve primarily mutations and genetic or epigenetic phenomena. This prompted us to test some histone deacetylase inhibitors (HDACi) from our library, in combination with fluconazole, against *C. albicans* strains in vitro. Among the tested compounds, the two chloro-containing uracil-hydroxamates **1c** and **1d** showed a strong reduction of the MIC values on *Candida* strains that show the trailing growth effect. In this assay, **1c**,**d** were more potent than SAHA, a well-known HDAC inhibitor, in reducing the *Candida* growth. More interestingly, **1c**,**d** as well as SAHA were able to inhibit the fluconazole-induced resistance induction in *Candida* cultures.


*Candida albicans* is an opportunistic human fungal pathogen that causes mucosal, cutaneous, and systemic infections, including oropharyngeal candidiasis (OPC), particularly in immunocompromised, cancer, and transplant patients.[1–5] Fluconazole and other antifungal azoles have proven to be effective in the management of OPC; however, they lack fungicidal activity, and treatment failures are common with severely compromised patients. Such failures have been associated with the emergence of azole-resistant strains of *C. albicans* during treatment, and recently resistance was shown to be a direct consequence of the daily cumulative doses of azoles.[6] In vitro, azoles not only fail to kill but, with a number of clinical isolates, also fail to truly suppress growth, resulting in trailing growth in broth microdilution assays even at concentrations well above the MIC.[7–12] Among fungal pathogens, in vivo and in vitro acquired resistance to azoles and other drugs was shown to be inducible and rapid but transient,[13,14] and has not been associated with plasmids or other transferable genetic elements, but is thought to involve primarily mutations and genetic or epigenetic rearrangements.

In eukaryotic cells, some classes of proteins are involved in the repression or silencing of developmentally regulated genes. Among them, histone deacetylases (HDACs) regulate chromatin structure through selective histone tail deacetylation, which in turn affects chromatin folding and interactions between DNA and DNA-binding proteins. In particular, the accessibility of transcription factors to DNA depends on the acetylation extent of core histones: hypoacetylated histones condense the chromatin structure restricting the access of transcription factors to DNA and often leading to transcriptional repression, whereas histone acetylation has been associ-

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ated with relaxed chromatin structure and active gene transcription.[15–18] Aberrant regulation of this epigenetic mechanism has been shown to cause inappropriate gene expression, a key event in the pathogenesis of many forms of cancer.[19–22] Moreover, HDAC inhibition is related with growth arrest, differentiation, and/or apoptosis in many types of tumor cells by reactivating the transcription of a small number of genes.[23–31]

In *C. albicans*, the HDAC enzymes HDA1 and RPD3 are known to play distinct roles in regulation of high-frequency phenotypic switching, a relevant virulence trait. In particular, HDA1 suppresses the basic switch from the white to the opaque but not from the opaque to the white phenotypes, and RPD3 suppresses the basic switch events in both directions.[32] In addition, HDAC inhibitors such as trichostatin A (TSA) have been reported to increase the frequency of switching in the white-to-opaque transition,[33] and to reduce the azole trailing through reduction in azole-dependent upregulation of *CDR* and *ERG* genes.[34]

Recently, we described several classes of HDAC inhibitors as antiproliferative and cytodifferentiating agents in tumor cells.[35–46] Among them, we reported some uracil-based hydroxamates (UBHAs) active at nanomolar concentrations in inhibiting HDAC enzymes, and able to induce cell cycle arrest, apoptosis, antiproliferative effect, and granulocytic differentiation in human leukemia U937 cells at low micromolar range.[45,46] Since UBHAs are the most potent HDAC inhibitors in our library, we decided to evaluate their effects on *C. albicans*, and on sensitivity of *C. albicans* to azole antifungals in the presence of HDAC inhibitors. We selected four highly active UBHAs from our library (**1a**,**b** and the two chloro-containing **1c**,**d**, Fig. 1) and tested them against a number of *C. albicans* strains, alone and in combination with fluconazole. Afterward, we determined the effect of UBHAs on the induction of resistance to fluconazole in *C. albicans*. Finally, the effect of the combination treatment of fluconazole and UBHAs on the only *C. albicans* strains showing the trailing growth phenomenon was studied.

Compounds **1c**,**d** were prepared starting from the 6-(3- and 4-chlorophenyl)-2-thiouracils **2a**,**b**,[47] which were treated with ethyl 5-bromopentanoate in the presence of potassium carbonate to afford the ethyl 5-(6-(3- and 4-chlorophenyl)-3,4-dihydro-4-oxopyrimidin-2-ylthio) pentanoates **3a**,**b**. Alkaline hydrolysis of **3a**,**b** furnished the related acids **4a**,**b**, which were in turn converted into the corresponding hydroxamates **1c**,**d**[48] through reaction with (i) ethyl chloroformate and triethylamine, (ii) *O*-(2-methoxy-2-propyl)hydroxylamine,[49] and (iii) Amberlyst 15 ion-exchange resin in methanol (Scheme 1).

The new UBHA derivatives **1c**,**d** were tested against three maize HDAC enzymes, namely HD2,[50] HD1-B (class I),[51] and HD1-A (class II),[52] and against mouse HDAC1, where they were active at low concentrations (Table 1). Suberoylanilide hydroxamic acid (SAHA),[53,54] a well-known HDAC inhibitor, was also tested as reference drug.

Afterward, both the UBHA compounds **1a**–**d** and SAHA (all at 4 μg/mL) were tested against a number of *C. albicans* strains (ATCC 10231, ATCC 24433,

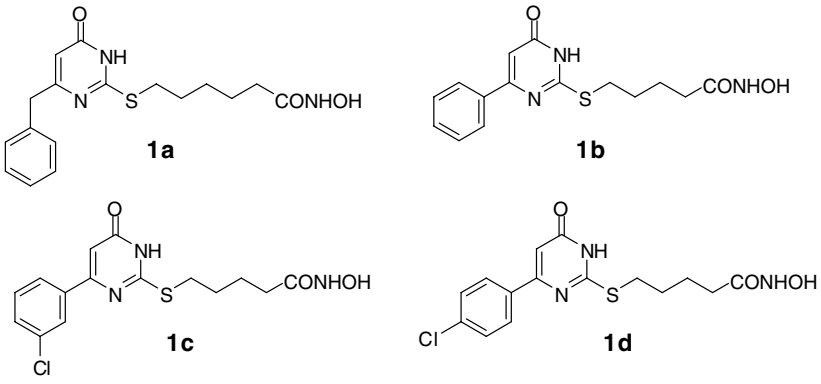
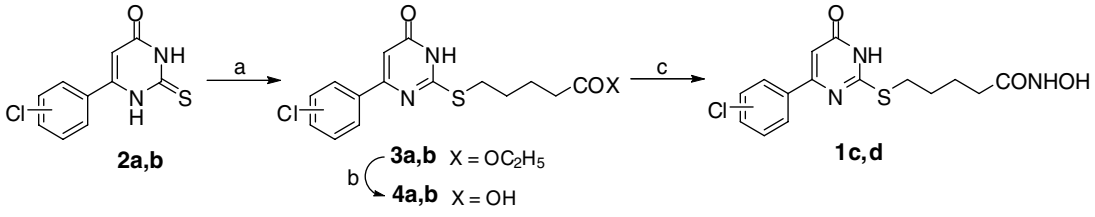



**Figure 1.** Uracil-based hydroxamic acids (UBHAs) tested on *Candida* cultures.




**Scheme 1.** Reagents and conditions: (a) Br-$(CH_2)_4$-$COOC_2H_5$, $K_2CO_3$, DMF, rt; (b) KOH, $C_2H_5OH$, $H_2O$, rt; (c) i—$ClCOOC_2H_5$, $(C_2H_5)_3N$, THF, 0 °C; ii—$NH_2OC(CH_3)_2OCH_3$, rt; iii—Amberlyst 15, $CH_3OH$, rt.

**Table 1.** HDAC (mouse HDAC1 and maize HD2, HD1-B, and HD1-A) inhibiting activity of compounds **1a–d**[a]

| Compound | $IC_{50}$ (μg/mL) | | | |
|---|---|---|---|---|
| | Mouse HDAC1 | HD2 | HD1-B | HD1-A |
| **1a**[b] | 0.036 | 0.006 | 0.006 | 0.010 |
| **1b**[b] | 0.014 | 0.003 | 0.003 | 0.003 |
| **1c** | 0.013 | 0.006 | 0.004 | 0.002 |
| **1d** | 0.018 | 0.006 | 0.004 | 0.001 |
| SAHA | 0.030 | 0.013 | 0.007 | 0.047 |

[a] Data represent mean values of at least three separate experiments.
[b] Refs. 45 and 46.

ATCC 20891, AIDS 32, AIDS 68, AIDS 37, AIDS 167, 3153, CO 23, 868) and *Candida parapsilosis*, alone and in combination with fluconazole (concentration range 0.125–128 μg/mL), to study the effects of these additions on the *Candida* strains' growth.[55] In both cases, the HDAC inhibitors did not show any significant anti-*Candida* effect: tested alone, they showed MIC values in the range of 73–124 μg/mL (24 h) and 96–128 μg/mL (48 h), and in combination with fluconazole they were just slightly effective in reducing the MIC values with respect to the fluconazole-alone treatment both at 24 and 48 h (Table 2).

Furthermore, we explored the effect of the HDAC inhibitors on the induction of resistance by fluconazole in *C. albicans*. To this aim, cultures of *C. albicans* were passed in increasing concentrations of fluconazole (up to 64 μg/mL), and fluconazole (up to 64 μg/mL) plus the HDAC inhibitors **1a–d** and SAHA (4 μg/mL). Then the MIC end points were determined following 24 and 48 h of incubation (fluconazole concentration range 0.125–512 μg/mL).[56]

As depicted in Figure 2, the two chloro-containing UBHAs **1c** and **1d**, respectively, gave a 83- and 196-fold reduction ($p < 0.01$) of the MIC values with respect to fluconazole alone at 24 h, and a 1003- and 143-fold reduction ($p < 0.01$) at 48 h. In the same assay, SAHA showed a strong inhibition of fluconazole-resistance induction similar to that obtained with **1c**,**d**, whilst **1a** and **1b** were less effective.

When the combined treatment of fluconazole (concentration range 0.125–128 μg/mL) plus UBHAs **1a–d** or SAHA (4 μg/mL) was repeated on the only *C. albicans* strains showing the trailing growth phenomenon (ATCC 10231, ATCC 20891, and 3153), after 48 h a strong reduction of the MIC values with respect to that of the control (fluconazole alone, MIC = 124.4 μg/mL) was observed. Compounds **1c**,**d** were the most potent in inhibiting the trailing growth in *C. albicans*, with MIC values of 7.0 (**1c**) and 5.2 (**1d**) μg/mL ($p < 0.01$), while the related UBHAs **1a**,**b** were less effective (MIC values = 68.7 (**1a**) and 71.0 (**1b**) μg/mL). In this assay, SAHA showed an intermediate behavior between the two groups of UBHA derivatives (MIC = 24 μg/mL) ($p < 0.05$) (Fig. 3).

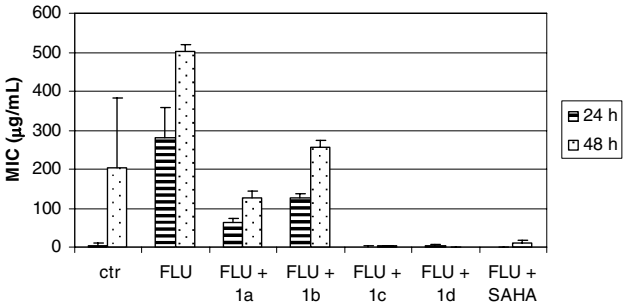



**Figure 2.** Effects of **1a–d** and SAHA on the fluconazole-induced resistance induction in *C. albicans*.

The data reported here show that the two chloro-containing UBHAs **1c**,**d** as well as SAHA are able to enhance the activity of fluconazole against the *C. albicans* strains displaying the trailing growth phenomenon, and to inhibit the induction of resistance to fluconazole.

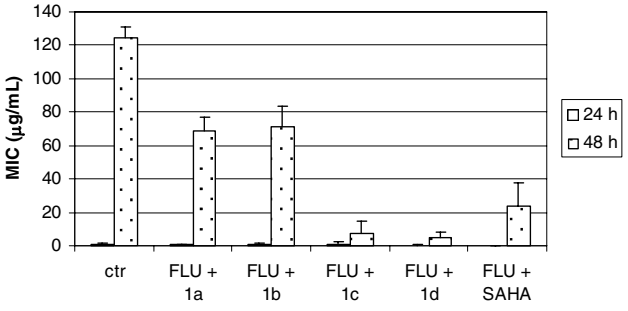



**Figure 3.** Effects of fluconazole plus **1a–d** or SAHA on the trailing growth in *C. albicans* strains.

**Table 2.** Effects of **1a–d** and SAHA on *C. albicans* sensitivity to fluconazole[a]

| Compound | 24 h, MIC (μg/mL)[b,c] | | | 48 h, MIC (μg/mL) | | |
|---|---|---|---|---|---|---|
| | 50% | 90% | Range | 50% | 90% | Range |
| Fluconazole (FLU) | 1.45 | 3.6 | 0.25–4.8 | 2.5 | 11 | 0.45–13.7 |
| FLU + **1a** | 0.5 | 4 | 0.09–5 | 0.625 | 12 | 0.09–16 |
| FLU + **1b** | 0.5 | 4 | 0.25–4 | 0.5 | 12 | 0.25–16 |
| FLU + **1c** | 0.44 | 3.42 | 0.06–8 | 1 | 8 | 0.06–18.7 |
| FLU + **1d** | 0.5 | 2 | 0.15–2.5 | 1 | 16 | 0.37–16 |
| FLU + SAHA | 0.5 | 2 | 0.06–8 | 1 | 8 | 0.06–32 |

[a] Data represent mean values of at least three separate experiments.
[b] Results are MIC minus 2 (prominent growth reduction or a 50% reduction in optical density).
[c] MIC50 and MIC90 values were calculated as concentrations that were able to inhibit the growth of 50% and 90% of the isolates.

Further studies are going on to clarify the molecular mechanisms of the described effects, and the reasons underlying the different behavior observed for the two groups of UBHAs (the unsubstituted phenyl-uracils **1a**,**b** and the chloro-containing **1c**,**d**). Different activities are probably due to a different susceptibility of the *Candida* HDACs to the inhibitors or, most likely, to a diverse penetration capability of the various UBHAs through the fungal membrane because of a change in lipophilicity (*C*log*P* of **1a**,**b**: 0.753; *C*log*P* of **1c**,**d**: 1.481).

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## References and notes

1. Wisplinghoff, H.; Bischoff, T.; Tallent, S. M.; Seifert, H.; Wenzel, R. P.; Edmond, M. B. *Clin. Infect. Dis.* **2004**, *39*, 309.
2. Ascioglu, S.; Rex, J. H.; de Pauw, B.; Bennett, J. E.; Bille, J.; Crokaert, F.; Denning, D. W.; Donnelly, J. P.; Edwards, J. E.; Erjavec, Z.; Fiere, D.; Lortholary, O.; Maertens, J.; Meis, J. F.; Patterson, T. F.; Ritter, J.; Selleslag, D.; Shah, P. M.; Stevens, D. A.; Walsh, T. J. *Clin. Infect. Dis.* **2002**, *34*, 7.
3. Stover, B. H.; Shulman, S. T.; Bratcher, D. F.; Brady, M. T.; Levine, G. L.; Jarvis, W. R. *Am. J. Infect. Control* **2001**, *29*, 152.
4. Wilson, L. S.; Reyes, C. M.; Stolpman, M.; Speckman, J.; Allen, K.; Beney, J. *Value Health* **2002**, *5*, 26.
5. Wisplinghoff, H.; Seifert, H.; Tallent, S. M.; Bischoff, T.; Wenzel, R. P.; Edmond, M. B. *Pediatr. Infect. Dis. J.* **2003**, *22*, 686.
6. Clancy, C. J.; Staly, B.; Nguyen, M. H. *Antimicrob. Agents Chemother.* **2006**, *50*, 3496.
7. Marr, K. A.; Rustad, T. R.; Rex, J. H.; White, T. C. *Antimicrob. Agents Chemother.* **1999**, *43*, 1383.
8. Revankar, S. G.; Kirkpatrick, W. R.; McAtee, R. K.; Fothergill, A. W.; Redding, S. W.; Rinaldi, M. G.; Patterson, T. F. *J. Clin. Microbiol.* **1998**, *36*, 153.
9. Rex, J. H.; Nelson, P. W.; Paetznick, V. L.; Lozano-Chiu, M.; Espinel-Ingroff, A.; Anaissie, E. J. *Antimicrob. Agents Chemother.* **1998**, *42*, 129.
10. Ruhnke, M.; Schmidt-Westhausen, A.; Engelmann, E.; Trautmann, M. *J. Clin. Microbiol.* **1996**, *34*, 3208.
11. Barker, K. S.; Crisp, S.; Wiederhold, N.; Lewis, R. E.; Bareither, B.; Eckstein, J.; Barbuch, R.; Bard, M.; Rogers, P. D. *J. Antimicrob. Chemother.* **2004**, *54*, 376.
12. Mi-Kyung, L.; Williams, L. E.; Warnock, D. W.; Arthington-Skaggs, B. A. *J. Antimicrob. Chemother.* **2004**, *53*, 217.
13. Calvet, H. M.; Yeaman, M. R.; Filler, S. G. *Antimicrob. Agents Chemother.* **1997**, *41*, 535.
14. Marr, K. A.; Lyons, C. N.; Ha, K.; Rustad, T. R.; White, T. C. *Antimicrob. Agents Chemother.* **2001**, *45*, 52.
15. Luger, K.; Mader, A. W.; Richmond, R. K.; Sargent, D. F.; Richmond, T. J. *Nature* **1998**, *389*, 251.
16. Urnov, F. D.; Wolffe, A. *Emerg. Ther. Targets* **2000**, *4*, 665.
17. Fischle, W.; Wang, Y.; Allis, C. D. *Curr. Opin. Cell Biol.* **2003**, *15*, 172.
18. Akey, C. W.; Luger, K. *Curr. Opin. Struc. Biol.* **2003**, *13*, 6.
19. Jones, P. A.; Baylin, S. B. *Nat. Rev. Genet.* **2002**, *3*, 415.
20. Momparler, R. L. *Oncogene* **2003**, *22*, 6479.
21. Huang, C.; Sloan, E. A.; Boerkoel, C. F. *Curr. Opin. Genet. Dev.* **2003**, *13*, 246.
22. Verma, M.; Srivastava, S. *Lancet Oncol.* **2002**, *3*, 755.
23. Weidle, U. H.; Grossmann, A. *Anticancer Res.* **2000**, *20*, 1471.
24. Kramer, O. H.; Göttlicher, M. G.; Heinzel, T. *Trends Endocrinol. Metab.* **2001**, *12*, 294.
25. Marks, P. A.; Richon, V. M.; Breslow, R.; Rifkind, R. A. *Curr. Opin. Oncol.* **2001**, *13*, 477.
26. Marks, P. A.; Rifkind, R. A.; Richon, V. M.; Breslow, R.; Miller, T.; Kelly, W. K. *Nat. Rev. Cancer* **2001**, *1*, 194.
27. Vigushin, D. M.; Coombes, R. C. *Anti-Cancer Drugs* **2002**, *13*, 1.
28. Johnstone, R. W. *Nat. Rev. Drug Disc.* **2002**, *1*, 287.
29. Kelly, W. K.; O'Connor, O. A.; Marks, P. A. *Expert Opin. Investig. Drugs* **2002**, *11*, 1695.
30. Miller, T. A.; Witter, D. J.; Belvedere, S. *J. Med. Chem.* **2003**, *46*, 5097.
31. Mai, A.; Massa, S.; Rotili, D.; Cerbara, I.; Valente, S.; Pezzi, R.; Simeoni, S.; Ragno, R. *Med. Res. Rev.* **2005**, *25*, 261.
32. Srikantha, T.; Tsai, L.; Daniels, K.; Klar, A. J. S.; Soll, D. R. *J. Bacteriol.* **2001**, *183*, 4614.
33. Klar, A. J. S.; Srikantha, T.; Soll, D. R. *Genetics* **2001**, *158*, 919.
34. Lamar Smith, W.; Edlind, T. D. *Antimicrob. Agents Chemother.* **2002**, *46*, 3532.
35. Massa, S.; Mai, A.; Sbardella, G.; Esposito, M.; Ragno, R.; Loidl, P.; Brosch, G. *J. Med. Chem.* **2001**, *44*, 2069.
36. Mai, A.; Massa, S.; Ragno, R.; Esposito, M.; Sbardella, G.; Nocca, G.; Scatena, R.; Jesacher, F.; Loidl, P.; Brosch, G. *J. Med. Chem.* **2002**, *45*, 1778.
37. Mai, A.; Massa, S.; Ragno, R.; Cerbara, I.; Jesacher, F.; Loidl, P.; Brosch, G. *J. Med. Chem.* **2003**, *46*, 512.
38. Mai, A.; Massa, S.; Pezzi, R.; Rotili, D.; Loidl, P.; Brosch, G. *J. Med. Chem.* **2003**, *46*, 4826.
39. Mai, A.; Massa, S.; Cerbara, I.; Valente, S.; Ragno, R.; Bottoni, P.; Scatena, R.; Loidl, P.; Brosch, G. *J. Med. Chem.* **2004**, *47*, 1098.
40. Ragno, R.; Mai, A.; Massa, S.; Cerbara, I.; Valente, S.; Bottoni, P.; Scatena, R.; Jesacher, F.; Loidl, P.; Brosch, G. *J. Med. Chem.* **2004**, *47*, 1351.
41. Mai, A.; Massa, S.; Pezzi, R.; Simeoni, S.; Rotili, D.; Nebbioso, A.; Scognamiglio, A.; Altucci, L.; Loidl, P.; Brosch, G. *J. Med. Chem.* **2005**, *48*, 3344.
42. Mai, A.; Massa, S.; Lavu, S.; Pezzi, R.; Simeoni, S.; Ragno, R.; Mariotti, F. R.; Chiani, F.; Camilloni, G.; Sinclair, D. A. *J. Med. Chem.* **2005**, *48*, 7789.
43. Mai, A.; Massa, S.; Pezzi, R.; Valente, S.; Loidl, P.; Brosch, G. *Med. Chem.* **2005**, *1*, 245.
44. Mai, A.; Massa, S.; Valente, S.; Simeoni, S.; Ragno, R.; Bottoni, P.; Scatena, R.; Brosch, G. *ChemMedChem* **2006**, *1*, 225.
45. Mai, A.; Massa, S.; Rotili, D.; Pezzi, R.; Bottoni, P.; Scatena, R.; Meraner, J.; Brosch, G. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4656.
46. Mai, A.; Massa, S.; Rotili, D.; Simeoni, S.; Ragno, R.; Botta, G.; Nebbioso, A.; Miceli, M.; Altucci, L.; Brosch, G. *J. Med. Chem.* **2006**, *49*, 6046.
47. Clark, J.; Munawar, Z.; Timms, A. W. *J. Chem. Soc., Perkin Trans. 2* **1972**, *3*, 233.
48. Chemical and physical data of new compounds **3a**,**b**, **4a**,**b**, and **1c**,**d** are reported as Supporting Information.
49. Mori, K.; Koseki, K. *Tetrahedron* **1988**, *44*, 6013.
50. Lusser, A.; Brosch, G.; Loidl, A.; Haas, H.; Loidl, P. *Science* **1997**, *277*, 88.

51. Kölle, D.; Brosch, G.; Lechner, T.; Pipal, A.; Helliger, W.; Taplick, J.; Loidl, P. *Biochemistry* **1999**, *38*, 6769.
52. Brosch, G.; Goralik-Schramel, M.; Loidl, P. *FEBS Lett.* **1996**, *393*, 287.
53. Richon, V. M.; Emiliani, S.; Verdin, E.; Webb, Y.; Breslow, R.; Rifkind, R. A.; Marks, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3003.
54. Mai, A.; Esposito, M.; Sbardella, G.; Massa, S. *Org. Prep. Proced. Int.* **2001**, *33*, 391.
55. Broth microdilution susceptibility test method: MICs of fluconazole and HDAC inhibitors were determined by the NCCLS broth microdilution method (National Committee for Clinical Laboratory Standards. 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard—second edition. Document M27-A2 National Committee for Clinical Laboratory Standards, Wayne, Pa). The yeast inoculum was adjusted to a concentration of $0.5 \times 10^3$ to $2.5 \times 10^3$ CFU/mL in RPMI medium, and an aliquot of 0.1 mL was added to each well of the microdilution plate. The plates were incubated at 35 °C. the MIC end points were read visually following 24 and 48 h of incubation and were defined as the lowest concentration that produced a prominent decrease in turbidity compared with that of the drug-free growth control. In addition to visual end point readings, the optical density of each microplate well was measured with a microplate spectrophotometer set at 405 nm. Spectrophotometric MICs were calculated based on the density of the growth control and were the lowest drug concentrations that resulted in a 50% reduction in growth compared with that of the drug-free growth control.
56. Development of FLU resistance: cultures of *C. albicans* were passed in increasing concentrations of fluconazole (up to 64 μg/mL) and fluconazole (up to 64 μg/mL) plus HDAC inhibitors (4 μg/mL). Specifically, the cultures were grown at 30 °C in YPD (1% yeast extract, 2% peptone, and 2% dextrose) in an environmental shaking incubator, and cells were passed when cultures were turbid (1–3 days between passages). At each passage, a 1 mL aliquot of the suspension was mixed with 0.5 mL of 50% glycerol, and the mixture was frozen at −70 °C for antifungal susceptibility testing as described above.