Biological Characterization and in Vivo Assessment of the Activity of a New Synthetic Macrocyclic Antifungal Compound

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S *Supporting Information*
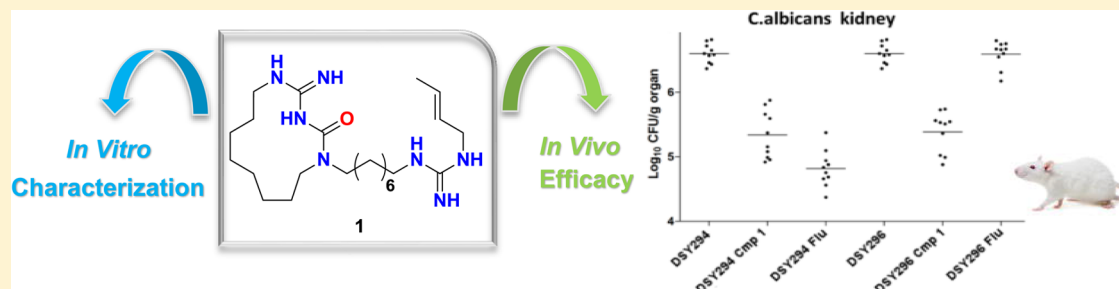
ABSTRACT: We recently identified a novel family of macrocyclic amidinoureas showing potent antifungal activity against *Candida* spp. In this study, we demonstrate the fungicidal effect of these compounds as well as their killing activity in a dose-dependent manner. Transcriptional analysis data indicate that our molecules induce a significant change in the transcriptome involving ATP binding cassette (ABC) transporter genes. Notably, experiments against *Candida albicans* mutants lacking those genes showed resistance to the compound, suggesting the involvement of ABC transporters in the uptake or intracellular accumulation of the molecule. To probe the mode of action, we performed fluorescence microscopy experiments on fungal cells treated with an ad-hoc synthesized fluorescent derivative. Fluorescence microscopy images confirm the ability of the compound to cross the membrane and show a consistent accumulation within the cytoplasm. Finally, we provide data supporting the in vivo efficacy in a systemic infection murine model setup with a drug-resistant strain of *C. albicans*.

INTRODUCTION

In the public opinion, mycoses are often associated with the development of external infections involving the skin or the nails. Less known, but more dangerous diseases, are those caused by systemic fungal infections. In those cases, fungi are systemically spread through the bloodstream and invade internal organs.[1] The large use of antifungal agents, most of them launched in the market more than 20 years ago, has led to the development of drug-resistant or even multi-drug-resistant fungi.[2−6] Multi-drug-resistant strains are responsible for life-threatening acquired infections, which are especially relevant in immunocompromised patients, such as AIDS patients, organ and bone marrow transplant recipients under immuno-suppressive therapy, or cancer patients treated with anti-proliferative drugs.[7] Among fungal pathogens, *Candida* species represent one of the most common causes of nosocomial bloodstream infections. The mortality rate associated with candidemia is significantly high, ranging between 25% and 50%. Furthermore, therapies for systemic candidosis commonly
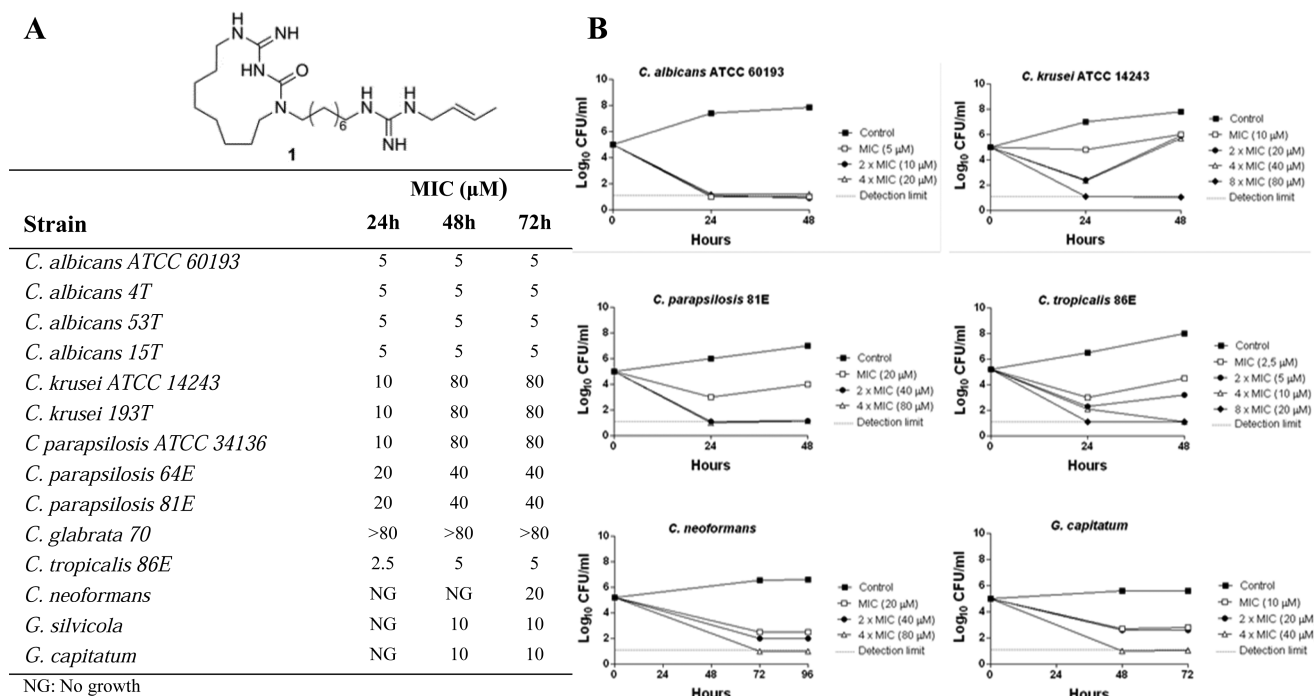
Figure 1. In vitro assays on compound **1**. (A) Table of antifungal activity of **1** against 14 yeast strains. (B) Time-kill activity of **1** on representative fungal species. Concentrations of **1** are expressed as multiples of the minimum inhibitory concentration (MIC). Fungal viability was determined by plate counts after different times of exposure to the drug.

treated with fluconazole and amphotericin B might be unsuccessful.[8] This situation is further aggravated by the emergence of drug-resistant *Candida* strains of both *albicans* and non-*albicans* species, whose treatment represents an increasingly worrisome challenge to clinicians.[9,10] We recently reported data on the in vitro efficacy of a novel class of macrocyclic amidinoureas,[11] which proved highly active against various *Candida* species, including drug-resistant strains, and showed a low cytotoxicity in vitro.[12−15] Further efforts to optimize the antifungal activity of the compounds allowed us to develop a new generation of compounds showing extremely promising biological activities and pharmacological properties.[16,17] One representative of such optimized molecules is compound **1** (Figure 1), whose synthesis and chemical characterization have been described elsewhere.[13,18,19] In this work, we provide an in-depth biological characterization of this compound, its impact on the fungal cell physiology, and a clear assessment of its in vivo efficacy against drug-resistant *Candida* clinical strains, using a murine model of systemic infection.

■ RESULTS AND DISCUSSION

In Vitro Antifungal Activity. In vitro experiments were conducted to investigate the biological activity of compound **1**. A total of 14 clinical isolates belonging to clinically relevant yeast species were tested, including strains of *Candida albicans*, *Candida parapsilosis*, *Candida krusei*, *Candida glabrata*, *Candida tropicalis*, *Cryptococcus neoformans*, *Geotrichum silvicola*, and *Geotrichum capitatum*. Strains were previously identified by standard morphological, cultural, and biochemical tests.[20] The minimum inhibitory concentration (MIC) of **1** ranged between 20 and 2.5 μM (Figure 1A). At 24 h, **1** was very active in inhibiting the growth of all strains of *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* (MIC = 2.5−20 μM). It also showed activity against isolates of *Cry. neoformans* (MIC = 20 μM) and *Geotrichum* spp. (MIC = 10 μM), though MICs for

these species could only be determined at 72 and 48 h, respectively, when growth in the control row could be seen. The lowest activity was observed toward *C. glabrata* (MIC > 80 μM). We also investigated the effect of incubation time on antifungal activity. Compound **1** showed a species-dependent increase of MICs during time, most strikingly for *C. krusei* and *C. parapsilosis*. At 48 h, the MIC increased 8-fold for *C. krusei* (ATCC 14243 and 193[T]) and *C. parapsilosis* ATCC 34136, and 2-fold for *C. parapsilosis* (64E and 81E) and *C. tropicalis* 86E. No significant effect of the incubation time was observed for *C. albicans*, *Cry. neoformans*, and *Geotrichum* spp., with unchanged MICs for up to 96 h. It is possible that extended incubation times allow resistant organisms to overgrow the initially susceptible subpopulation, or that the whole population requires an adaptation phase after exposure to the drug, resulting in higher MICs after prolonged incubation. On the other hand, during prolonged incubation, degradation of the antifungal compound may also occur, possibly by enzymes expressed only by certain species or strains. Indeed, the stability of **1** in RMPI-1640 medium was assessed in order to exclude spontaneous degradation of the compound during the antifungal susceptibility assay. These experiments confirmed the stability of **1** in RMPI-1640 for up to 72 h of incubation at 30 °C (Figure S1, Supporting Information).

To evaluate the fungicidal activity of **1**, yeast viability was assessed after 24 h of incubation at 37 °C by colony-forming unit (CFU) counts on Sabouraud dextrose agar. It was considered to have fungicidal activity if a decrease greater than or equal to 3·Log10 CFU/mL (99.9%) of the initial inoculum was observed at 24 h (Experimental Section). Figure 1B shows that **1** exerted the strongest fungicidal activity on *C. albicans*, resulting in 99.9% killing within 24 h at 5 μM (corresponding to the MIC value) of the drug. Compound **1** was fungistatic for *C. parapsilosis* at the MIC value and fungicidal at 2 times the MIC. The killing activity was also

observed on *Cry. neoformans* and *G. capitatum* at 2-fold and 4-fold the MICs determined at 72 and 48 h, respectively. *C. krusei* and *C. tropicalis* exhibited 99.9% CFU reduction for 8-fold the MICs of **1** determined at 24 h. The overall fungicidal effect observed has relevant clinical implications, since in vivo killing of fungi would greatly facilitate the immune-mediated eradication of the infection.

Transcriptional Analysis of *C. albicans* Exposed to Compound 1. To better understand the antifungal activity of **1** on *C. albicans*, whole genome transcriptional profile experiments were performed in the presence and absence of sub-inhibitory concentrations (3 μM) of this compound at two different time points (15 and 45 min). These conditions were chosen to identify the primary effects of the drug without inducing extensive cell damage. Transcriptional profiles were obtained with high-density microarrays and single-labeled cRNA. Data were analyzed with biological triplicates. Figure 2
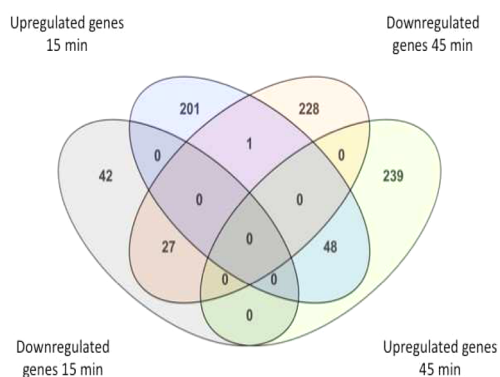

Figure 2. Venn diagram showing the results of the comparative transcriptome analysis and the impact of **1** on gene expression. Genes were at least 1.5-fold commonly up- and down-regulated between 15 and 45 min exposure to **1**. The diagram was obtained using Genespring Software Version 12 (Agilent).

shows Venn diagrams of up- and down-regulated genes (by at least 1.5-fold) at both time points. The diagram reveals that 48 and 27 genes were commonly up- and down-regulated between the two time points.

Details on these commonly expressed genes are given in Tables 1 (up-regulated genes) and S2 (down-regulated genes, Supporting Information). Strikingly, up-regulated genes exhibited a typical signature for *TAC1*-regulated genes (Table 1). *TAC1* is a transcription factor regulating several genes involved in drug resistance and particularly azole resistance. As summarized in Table 1, *TAC1* is itself up-regulated as well as its target genes such as *CDR1*, *CDR2*, *RTA3*, *LCB4*, and *IFU5*. *CDR1* and *CDR2* up-regulation was verified by separate real-time quantitative polymerase chain reaction (RT-qPCR) analysis. Results are given in Figure 3, and show that genes identified to be up-regulated by microarrays are regulated in a similar way by RT-qPCR analysis. *CDR1* and *CDR2* are ATP binding cassette (ABC) transporters and contribute to drug efflux and azole resistance.[21]

Susceptibility of *C. albicans* CDR1/CDR2 Mutants to Compound 1. We showed above that *CDR1* and *CDR2* were up-regulated by **1**. These genes have been reported to be up-regulated by a large number of drugs, including antifungal agents.[22] In the majority of cases, inducers themselves serve as substrates for transporters. In order to test this hypothesis, we

used *C. albicans* mutants lacking both *CDR1* and *CDR2* (DSY448, DSY654)[23] and addressed their susceptibility to **1** (Figure 4). Hypersusceptibility to **1** of the mutants as compared to a wild-type control (CAF2-1) would strongly suggest that **1** is a substrate for these ABC transporters. Surprisingly, as shown in Figure 4, the mutants became unexpectedly more resistant to **1** as compared to the wild type. The validity of the assay was confirmed, since the *C. albicans* *CDR1*/*CDR2* mutants were susceptible to fluphenazine, which is consistent with previously published results. The resistance of the *CDR1*/*CDR2* mutants to **1** indicates a unique feature of this antifungal substance, which probably requires the presence of ABC transporters in order to be active. A similar behavior has been recently observed by Sun and co-workers, in the case of the antifungal compound BMQ,[24] which proved to be more active in *C. albicans* isolates overexpressing multi-drug-resistant transporter Mdrp1. Those authors demonstrated that the transporter facilitates the uptake and increases the intracellular accumulation of the compound, rather than acting as an efflux pump. It is thus rational to assume that, in our case, the intracellular accumulation of **1** can be favored by the expression of ABC transporters. The reversed biological profile observed for this compound, compared to current antifungal drugs, enhances the potential of our class of macrocyclic amidinoureas for development as new antifungal agents to treat drug-resistant infections.

Fluorescence Microscopy Experiments. To test the aforementioned hypothesis of an intracellular accumulation of the compound, we performed fluorescence microscopy assays. The aim of our study is to investigate the interaction of macrocyclic amidinoureas with the fungal cell and to verify if the compounds are accumulated in the cytoplasm and/or they interact with other structures (membrane, nucleus). In order to perform fluorescence experiments, three different fluorescent probes were designed and synthesized. Two of them, namely compounds **2** and **3** (Figure 5), derived from **1**, with the fluorophore (dansyl group) directly attached on the side chain. In particular, **2** is linked to the fluorophore directly on the terminal amino group, while **3** has the dansyl group attached to the guanidine moiety. We used the dansyl group as first choice for two reasons: it is easily introduced by nucleophilic substitution of commercial dansyl chloride, and the small dimensions give good chances to retain the biological activity. Unfortunately, the introduction of the fluorophore on the guanidinium moiety in **2** and **3** resulted in a loss of antifungal activity in vitro (MIC > 125 μM), probably because the terminal guanidine does not allow for a bulky substituent. Fluorescence microscopy experiments were conducted on samples of *C. albicans* and *Cry. neoformans* cultures, but, although both compounds showed a clear cell-association, the resolution used did not allow for the discrimination between cell wall association and internalization (Figure S2, Supporting Information). In order to obtain better results, a new fluorescent probe was thus synthesized (compound **4**, Figure 5), bearing the fluorophore group far away from the guanidinium moiety. In this case an aromatic derivative (from Sanguinetti et al.[16]) was chosen as the parent compound, since it possess antifungal activity comparable to that of **1**, but the presence of a benzene ring offers a useful attachment point for fluorophores (fluorescein). Despite its chemical structure being bulkier than the dansyl group, we chose fluorescein as fluorophore because it has better performance in confocal microscopy and the distance from the active guanidine as well

Table 1. Selection of Genes Commonly Up-Regulated by Compound 1 in *C. albicans* SC5314 at 15 and 45 min Time Points[a]

orf19 name	gene name	15 min	45 min	description/annotation
orf19.5958	CDR2	71.84	134.38	multi-drug transporter, ATP-binding cassette (ABC) superfamily
orf19.4531		17.22	21.54	putative PDR subfamily ABC transporter
orf19.1438		6.13	17.28	protein with homology to NADH dehydrogenase
orf19.2726		5.86	6.56	putative plasma membrane protein; Plc1p-regulated
orf19.3378		5.32	3.52	predicted ORF in assemblies 19, 20, and 21
orf19.1027	PDR16	4.43	6.89	phosphatidylinositol transfer protein; increased transcription correlates with CDR1 and CDR2 overexpression and azole resistance
orf19.4907		3.90	5.30	putative protein of unknown function; Hap43p-repressed gene
orf19.24	RTA2	3.07	2.93	putative flippase required for sphingolipid long-chain base
orf19.6000	CDR1	3.07	3.75	multi-drug transporter of ABC superfamily
orf19.6817	FCR1	2.86	5.88	zinc cluster transcription factor; negative regulator of fluconazole resistance
orf19.7336		2.80	3.49	predicted membrane transporter; member of the drug:proton antiporter (14 spanner) (DHA2) family and major facilitator superfamily (MFS)
orf19.3188	TAC1	2.79	3.38	transcriptional activator of drug-responsive genes, including CDR1 and CDR2
orf19.6026	ERG2	2.61	2.83	C-8 sterol isomerase; enzyme of ergosterol biosynthesis pathway
orf19.3089		2.39	2.63	orthologue(s) have role in cardiolipin metabolic process and cristae formation
orf19.2356	CRZ2	2.28	2.66	zinc finger transcription factor; crz1, not crz2, null mutation suppresses fluconazole resistance of homozygous cka2 null (CK2 kinase defective)
orf19.1887		2.19	3.44	orthologue(s) have sterol esterase activity and role in sterol metabolic process, and are integral to membrane lipid particle localization
orf19.6873.1		2.16	1.90	orthologue(s) have electron carrier activity and iron and zinc ion-binding activity
orf19.2248	ARE2	2.11	2.88	acyl CoA:sterol acyltransferase (ASAT)
orf19.5257	LCB4	2.04	3.69	putative sphingosine kinase; expression is Tac1p-regulated
orf19.2568	IFU5	1.63	2.58	predicted membrane protein; estradiol-induced; increased transcription associated with CDR1 and CDR2 overexpression or fluphenazine treatment

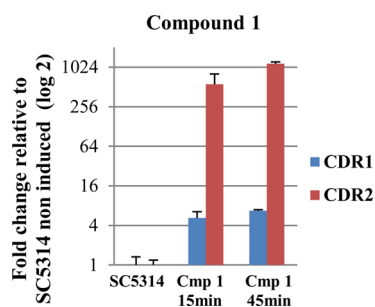[a]Complete data are given in Table S1 (Supporting Information).

Figure 3. Validation of microarray results with RT-qPCR on *C. albicans* SC5314.

Figure 4. Serial dilution assay of *C. albicans* with drug inhibitory substances. *C. albicans* isolates were 10-fold serially diluted starting from a density of 2×10^7 cells/mL. An aliquot (5 μL) was spotted onto YEPD agar containing the different drugs as indicated.

as the C3 spacer should avoid a pronounced loss of activity. As a result, **4** showed a retention of antifungal activity, even though with a reduced potency (MIC = 80 μM), and it was used in confocal microscopy experiments.

Confocal microscopy examination of *C. albicans* ATCC 60193 and *Cry. neoformans* exposed for 48 h to **4** showed strong cell-associated fluorescence which was absent in untreated cells (Figure 6). Fluorescent images were visually compared, and no significant difference was observed between the two fungal species treated with **4**. Fluorescence was mostly associated with the cytoplasm, confirming the intracellular accumulation of the compound, in accordance with the hypothesis of an ABC transporters-mediated accumulation. Moreover, fluorescence showed a patchy distribution in some cells, suggesting that the compound can interact with multiple and/or differently located targets within the fungal cell. No fluorescent signal was observed in cells presenting a damaged cell wall, suggesting release of the fluorescent compound due to cell lysis, in accordance with the previously demonstrated fungicidal effect of this class of compounds.
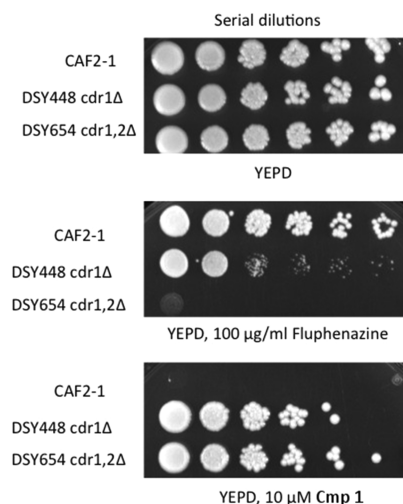
Chemistry. The synthesis of compounds **2** and **3** is depicted in Scheme 1, starting from primary amine **5**, which was prepared according to a procedure already described in a previous paper.[19] For the synthesis of **2**, amine **5** was reacted with dansyl chloride (**6**) in CH$_3$CN, furnishing the sulfonamide derivative **7**, which was deprotected with freshly distilled 10% trifluoroacetic acid (TFA) in anhydrous dichloromethane (DCM) solution, to give the desired fluorescent probe **2**. For the preparation of fluorescent probe **3**, it was necessary to synthesize the opportune guanylating agent **9**, which was obtained by reacting dansyl chloride (**6**) with *N*-Boc-1*H*-
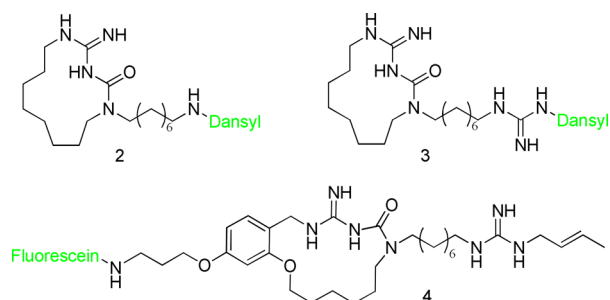
Figure 5. Structures of the fluorescent probes synthesized. Fluorophores are represented in green.

Candida Albicans ATCC 60193 (compound **4**, 40 μM)

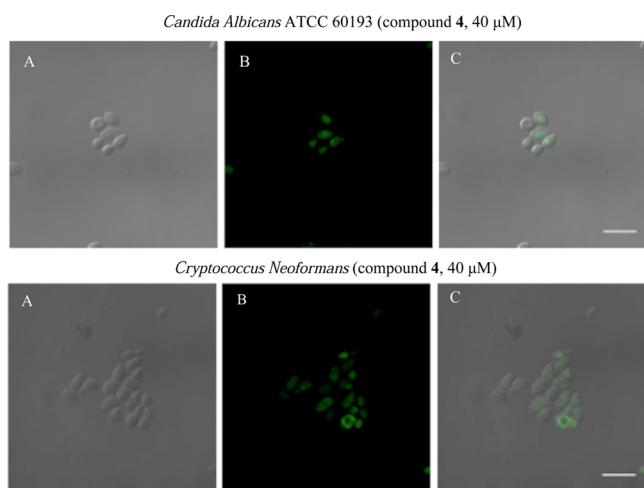*Cryptococcus Neoformans* (compound **4**, 40 μM)

Figure 6. Confocal microscope images of *C. albicans* ATCC 60193 and *Cry. neoformans* cells treated with compound **4** at 40 μM concentration. Panels A, B, and C show the visible, fluorescent, and merged images, respectively. Scale bar = 10 μm.

pyrazolecarboxamidine (**8**) in the presence of NaH as a base. The final guanylation reaction of amine **5** with **9** furnished, after Boc-cleavage, the desired fluorescent probe **3** (Scheme 1).

The synthesis of **4** is described herein. Starting from 2,4-dihydroxybenzaldehyde (**11**), we first functionalized the 4-OH position with *N*-(3-bromopropyl)phthalimide to afford intermediate **12**, which was then etherified with 5-bromo-1-pentene to give **13**. The aromatic aldehyde was converted to aldoxime **14** and then reduced to benzyl amine **15**. During this reaction one carbonyl group of the phthalimide moiety was reduced, giving a 3-hydroxyisoindolinone moiety. Intermediate **16** was obtained by reacting **15** with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine. The coupling reaction between the secondary amine **17**[16] and **16**, followed by ring-closing metathesis reaction, furnished the amidinoureic macrocycle **19** in good yields (Scheme 2).

While trying to remove the carboxybenzyl (Cbz) protecting group by hydrogenation over Pd/C, we observed the simultaneous reduction of the 3-hydroxyisoindolinone ring to isoindolin-1-ol; despite several attempts, we were not able to obtain selective Cbz removal. For this reason we decided to change the protecting group on the C3 lateral chain of **19**. To do so, we first oxidized the alcohol group of the 3-hydroxyisoindolinone to a carbonyl group with MnO$_2$, and then cleaved the phthalimide moiety with hydrazine monohydrate to afford the corresponding primary amine **20**, which was then protected using methyl trifluoroacetate, furnishing **21**.

Scheme 1[a]
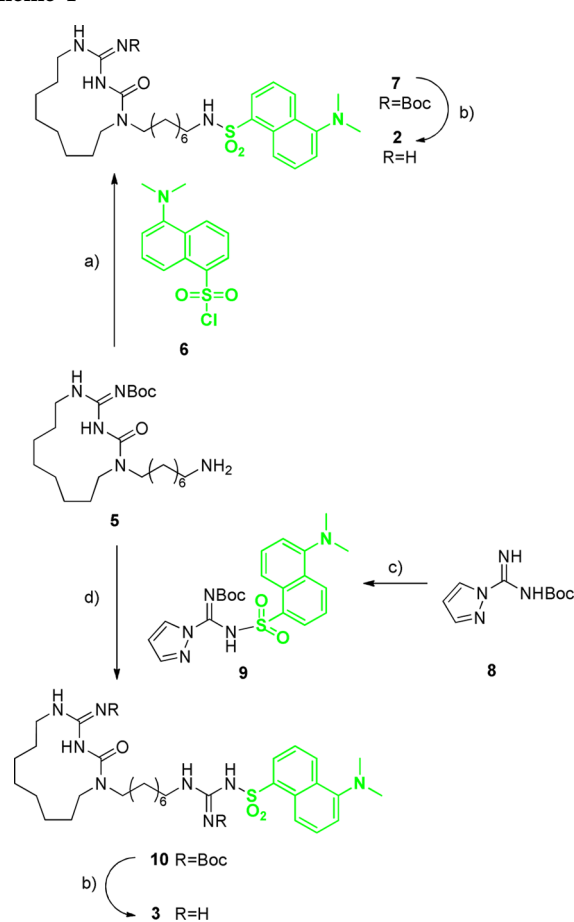
[a]Reagents and conditions: (a) TEA, CH$_3$CN, rt, 10 min; (b) TFA, DCM, rt, 3 h; (c) **6**, NaH, THF, rt, 3 h; (d) DIPEA, CH$_3$CN/MeOH, 50 °C, 24 h.

Hydrogenation over Pd/C allowed simultaneous reduction of the endocyclic double bond and removal of the Cbz group, affording derivative **22**. The following guanylation with *N*-crotylpyrazoleguanidine (**23**)[16] furnished **24**. The C3 spacer of **24** was deprotected to give amine **25**, which was reacted with fluorescein isothiocyanate (FITC) in dimethylformamide (DMF) to form the desired conjugate **26**. Finally, butyloxycarbonyl (Boc) deprotection performed in 20% TFA in dry DCM afforded the final product **4** in quantitative yield as a yellow powder (Scheme 3).

In Vivo Animal Studies. In order to test the potential antifungal therapeutic effect of **1** in vivo, experimental treatments of invasive candidiasis were carried out in immunocompetent mice. Two different drug regimens, i.e., 20 and 40 mg/kg/day, were applied, whereas fluconazole was used as a control at a dosage of 8 mg/kg/day. Figure 7A shows results of tissue burdens obtained after 7 days of experiments in spleen and kidneys with both 20 and 40 mg/kg/day regimens of **1**. Experiments showed that **1** significantly reduced the CFU counts of the organs (kidneys and spleen) of mice infected intravenously with *C. albicans* ATCC 90028 (a fluconazole-susceptible control strain). A similar effect was noticed in mice treated with fluconazole, as expected. Furthermore, experimental mouse infections were also done with two isogenic strains of *C. albicans*: DSY294 that is susceptible to azole drugs (fluconazole MIC = 0.25 μg/mL) and DSY296 that is azole-

Scheme 2[a]
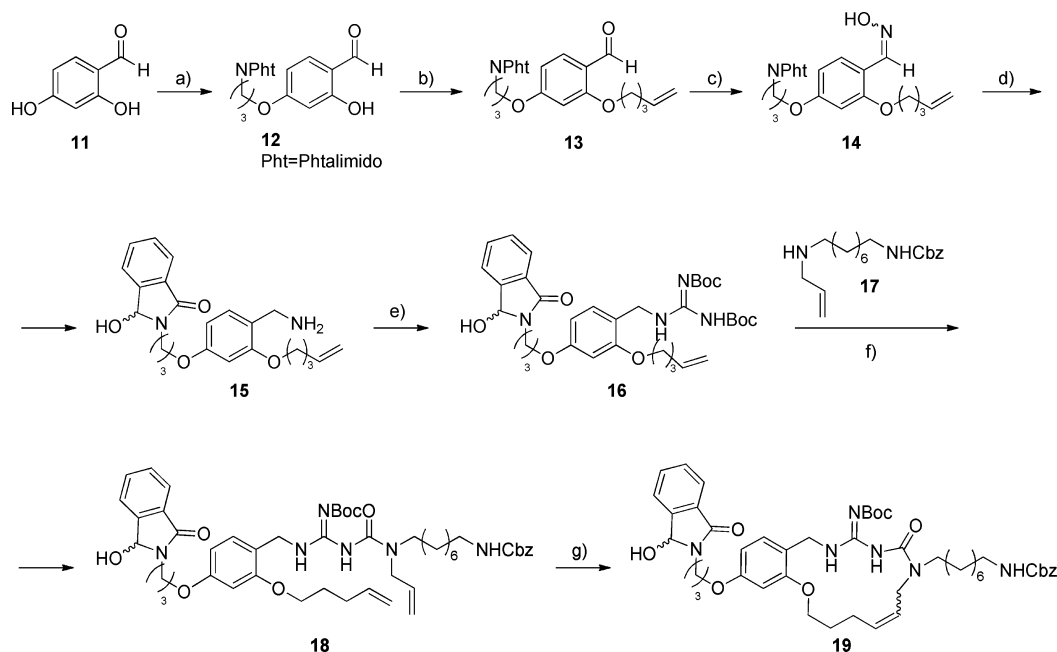
[a]Reagents and conditions: (a) N-(3-bromopropyl)phthalimide, K$_2$CO$_3$, CH$_3$CN, reflux, 6 h; (b) 5-bromo-1-pentene, K$_2$CO$_3$, CH$_3$CN, reflux, 6 h; (c) NH$_2$OH−HCl, pyridine, EtOH, reflux, 3.5 h; (d) Zn, HCl, THF, reflux, 2 h; (e) 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, rt, 18 h; (f) TEA, THF, reflux, 12 h; (g) Grubb's second generation catalyst, degassed DCM, reflux, 3 h.

resistant (fluconazole MIC = 64 μg/mL).[25] In these experiments, only the 40 mg/kg/day regimen of **1** was applied, whereas fluconazole was used at the same dosage (8 mg/kg/day) (Figure 7B). By contrast to fluconazole treatment, which was effective only against DSY294, **1** significantly reduced CFU counts of the selected organs of mice infected with both DSY294 and DSY296, and it is worth noting that the in vivo efficacy of **1** was thus achieved also with the azole-resistant isolate strain. With regard to DSY296, the reductions in CFU counts by **1** treatment were markedly higher than those observed in mice treated with fluconazole. Remarkably, the resistance to fluconazole of the isolate DSY296 is due to the overexpression of multi-drug transporters, triggered by an up-regulation of *CDR1* and *CDR2* genes.[25] The in vivo efficacy of **1** against DSY296 infection thus corroborates the assumption of the ABC transporters-mediated accumulation of the molecule inside the fungal cell.

Conclusions. To date, only three classes of antifungal agents are used for the treatment of systemic candidiasis, and the market calls for new drugs that would address a growing medical need, determined by the emergence of drug-resistant clinical isolates. In this work, we present a set of in vitro and in vivo data supporting the promising potential of the herein described novel antifungal agent, a macrocyclic amidinourea derivative. Our research highlights that the molecular target is new and not shared by any other antifungal compound on the market, since our compound preserves a potent activity also against drug-resistant strains. Indeed, compound **1** was proved particularly active on resistant isolates overexpressing multi-drug transporters of the ABC superfamily, a mutation usually associated with resistance to therapeutic antifungals. The involvement of ABC transporters in the mode of action of the compound was demonstrated with in vitro experiments on mutant strains and confirmed with fluorescence microscopy and in vivo assays. Our findings could represent the first step

toward the development of a new therapeutic class of antifungal agents, potentially useful to treat acquired infections, and might represent a suitable strategy to address the growing emergence of antifungal-resistant isolates in the clinical setting.

■ EXPERIMENTAL SECTION

Antifungal Activity in Vitro. *C. albicans* ATCC 60193, *C. krusei* ATCC 14243, and *C. parapsilosis* ATCC 34136 were purchased from the American Type Culture Collection (Manassas, VA, USA). The EUCAST susceptibility testing protocol, including the update for testing of non-fermenting yeasts (*Cryptococcus* spp. and *Geotrichum* spp.), was used for the determination of the minimal inhibitory concentrations (MICs) of antifungal agents.[26,27] Results were read after 24, 48, and 72 h of incubation at 37 °C. The MIC was defined as the lowest compound concentration which prevented visible growth. Aliquots of 10 μL were removed from the wells corresponding to the MIC, 2MIC, 4MIC, and 8MIC and serially 10-fold diluted in sterile saline, to minimize the drug carry-over. An aliquot of 10 μL of each dilution was plated on Sabouraud dextrose agar plates. The plates were incubated at 37 °C for 24/48 h, and the number of CFUs was counted. For *Cryptococcus* spp. and *Geotrichum* spp., incubation was for 48 h to facilitate colony counting.[28] When less than 100 CFU/mL was expected, 10 μL of sample was plated without dilution. The experiment was performed in duplicate. The minimum detection limit of this method was 10 CFU/mL. The Log10 CFU/mL was plotted on a graph as a function of time and used to compare the rate and extent of antifungal activity in the presence various concentrations of **1** and in its absence. Activity was considered fungicidal when there was a decrease greater than or equal to 3·Log10 CFU/mL (99.9%) of the initial inoculum in 24 h. Activity lower than 3·Log10 reduction in the number of CFU/mL of the initial inoculum was considered fungistatic.[29] *C. glabrata* was not tested since it is poorly susceptible to **1**.

RPMI Medium Stability Assay. To verify the chemical stability of **1** in RPMI-1640 medium, 0.4 mL of a stock solution in MeOH (10 mM) of the compound and 1.6 mL of RPMI-1640 (final concentration 2 mM) were mixed in a test tube. The solution was filtered through a 0.45 μm nylon filter (Acrodisc) before LC-UV-MS analysis. The tube

Scheme 3[a]
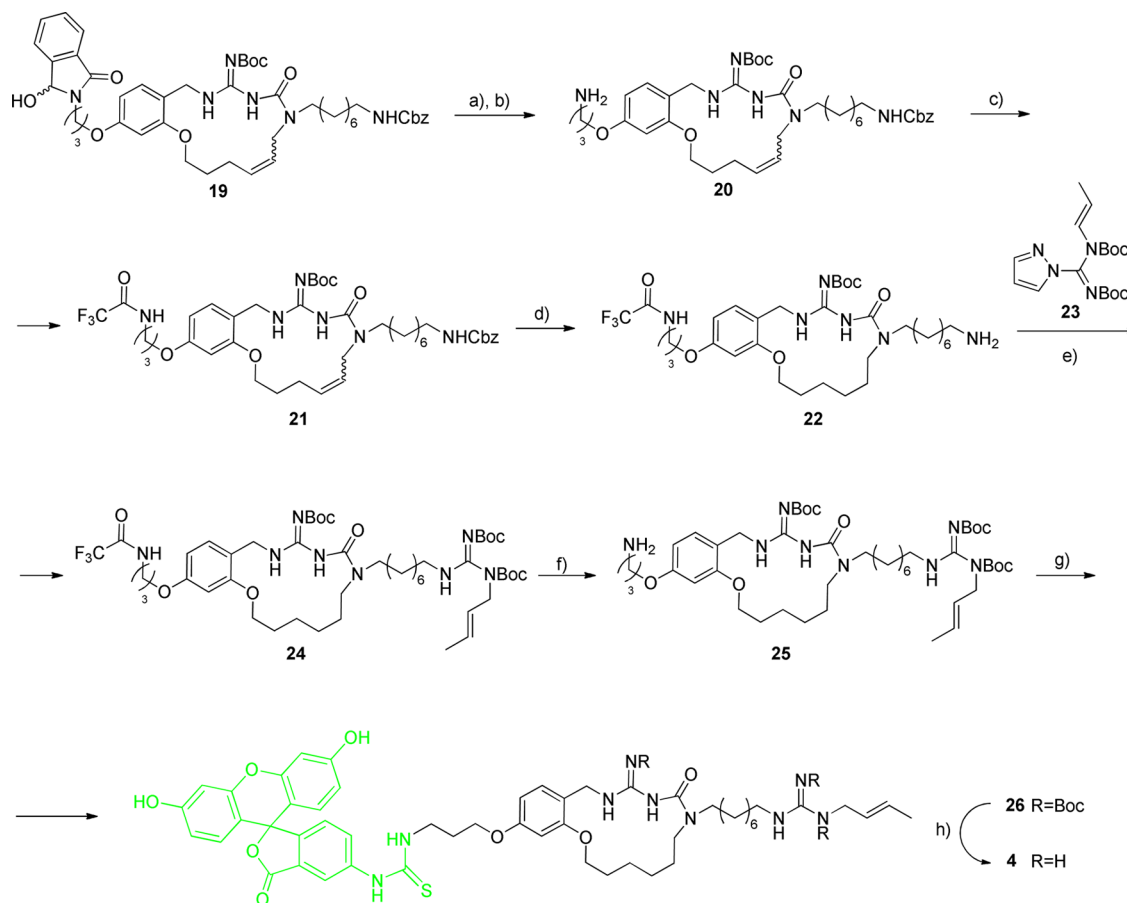
[a]Reagents and conditions: (a) MnO$_2$, DCM, rt, 18 h; (b) hydrazine monohydrate, MeOH sol., rt, 12 h; (c) methyl trifluoroacetate, TEA, THF, 0 °C to rt, 16 h; (d) H$_2$, Pd/C, cat. HCl, i-PrOH, rt, 3 h; (e) N-crotylpyrazoleguanidine (**23**), DIPEA, CH$_3$CN/MeOH, 60 °C, 16 h; (f) K$_2$CO$_3$, MeOH, H$_2$O, 70 °C, 2 h; (g) FITC, DIPEA, DMF, rt, 24 h; (h) TFA/DCM 20%, rt, 8 h.

was maintained at 30 °C, and four aliquots (20 μL each) were removed and analyzed by HPLC at predetermined time points (1, 24, 48, and 72 h). Each aliquot was analyzed in triplicate. The stability was followed by HPLC with an UV–MS detection method (Supporting Information). Quantitative analysis was performed using an appropriate calibration curve obtained by solubilizing **1** in MeOH.

Transcriptional Analysis. Sample preparation was performed on three biological triplicates. Total RNA was extracted from log phase cultures in liquid yeast extract peptone dextrose (YEPD) as previously described.[30] Compound **1** was added to an end concentration of 3 μM, and incubation points of 15 and 45 min were chosen. Control cells were taken at the same time points but without **1**. Briefly, after centrifugation of 5 mL of culture (corresponding to 10^8 cells), the yeast cell pellets were mixed with 0.3 g of glass beads, 300 μL of RNA extraction buffer (0.1 M Tris-HCl at pH 7.5, 0.1 M LiCl, 10 mM EDTA, 0.5% SDS), and 300 μL of phenol–chloroform–isoamyl alcohol (24:24:1). After 1 min of vortexing in a bead beater (Fastprep-24 Instrument, MP Biomedicals Switzerland, Zürich), the aqueous phase was re-extracted with phenol–chloroform–isoamyl alcohol, and RNA was precipitated with 600 μL of ethanol at −20 °C for 1 h. The RNA pellet was resuspended in 50 μL of diethyl pyrocarbonate-treated H$_2$O. The integrity of the input template RNA was determined prior to labeling/amplification, using an Agilent RNA 6000 Nano LabChip kit and 2100 bioanalyzer (Agilent Technologies). Agilent's One-Color Quick Amp Labeling Kit (Agilent Technologies) was used to generate fluorescent cRNA according to the manufacturer's instructions. Briefly, 1 μg of total RNA from each sample was used, to which a spike mix and T7 promoter primers were added, both of which are provided by the manufacturer. cDNA synthesis was promoted by MMLV-RT

(Moloney Murine Leukemia Virus Reverse Transcriptase) in the presence of dNTPs and RNaseOUT. Next, cRNA was produced from this first reaction with T7 RNA polymerase, which simultaneously amplifies target material and incorporates cyanine 3-labeled CTP. The labeled cRNAs were purified with RNeasy Mini Kit (Qiagen) and quantified using a NanoDrop ND-1000 UV–vis spectrophotometer. Next, 600 ng of Cy3-labeled cRNAs was fragmented and hybridized for 17 h at 65 °C to each array using the Gene Expression Hybridization Kit (Agilent Technologies) and a gasket slide with an 8 microarrays/slide format for sample hybridization to separate each sample in specific sub-arrays of the 8 × 15K format. The C. albicans microarray format was published by Synnott et al. (design ID 017942).[31]

Slides were washed and processed according to the Agilent 60-mer Oligo Microarray Processing protocol and scanned on a Agilent microarray scanner G2565BA (Agilent Technologies). Data were extracted from the images with Feature Extraction (FE) software (Agilent Technologies). FE software flags outlier features, and detects and removes spatial gradients and local backgrounds. Data were normalized using a combined rank consistency filtering with LOWESS intensity normalization. The gene expression values obtained from FE software were imported into GeneSpring 12 software (Agilent Technologies) for preprocessing and data analysis. For inter-array comparisons, a linear scaling of the data was performed using the 75th percentile signal value of all of non-control probes on the microarray to normalize one-color signal values. Probe sets with a signal intensity value below the 20th percentile were considered as absent and discarded from subsequent analysis. The expression of each gene was normalized by its median expression across all samples. Genes were
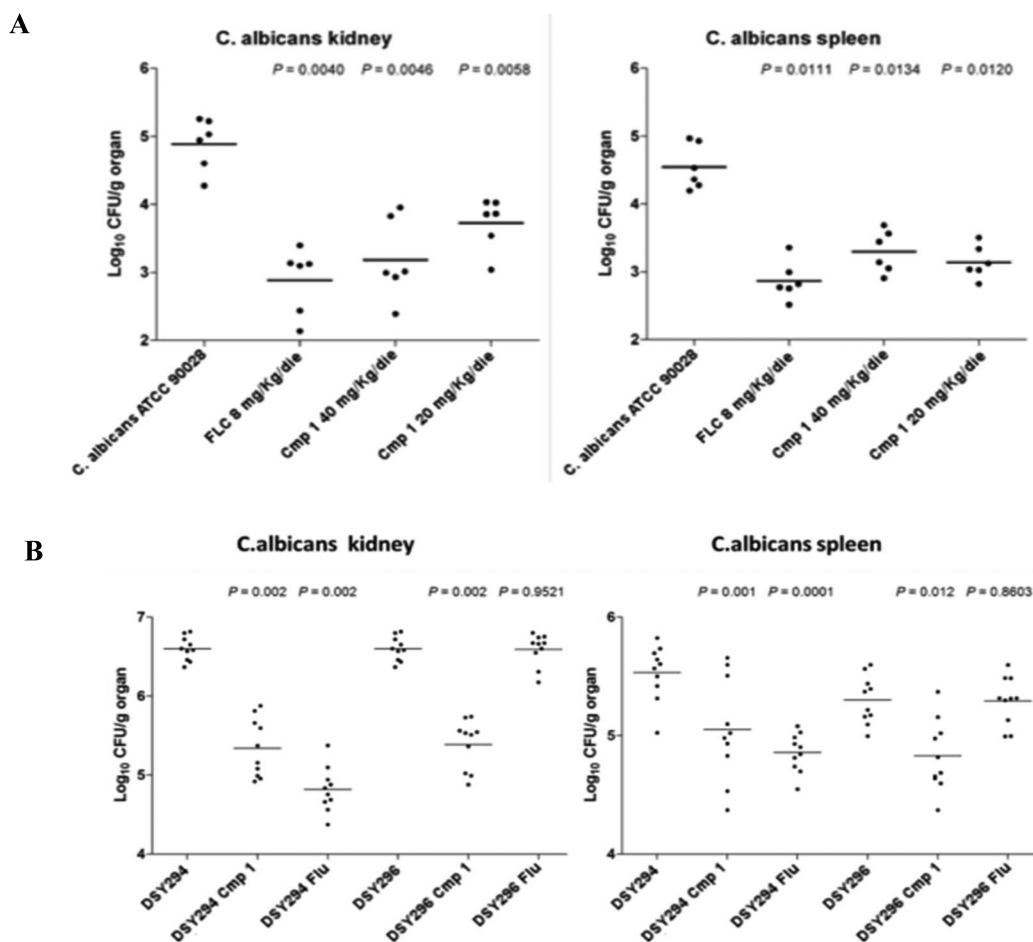
Figure 7. In vivo animal studies. The regimens for **1** and fluconazole were 40 and 8 mg/kg/day, respectively. Each data point corresponds to one animal, and the origin of tissue (kidney or spleen) is shown for each diagram. (A) Fungal tissue burdens of mice infected with *C. albicans* and treated with fluconazole (FLC) and **1**. (B) Fungal tissue burdens of mice infected with *C. albicans* DSY294 and DSY296, treated with fluconazole (Flu) and **1**.

included in the final data set if their expression changed by at least 1.5-fold as compared to **1**-unexposed cells. Corrected *P*-value <0.05 was chosen as the cutoff for significance. Microarray data have been uploaded to the NCBI GEO microarray repository.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted from log phase cultures with an RNeasy Protect Mini kit (Qiagen) by a process involving mechanical disruption of the cells with glass beads and an RNase-free DNase treatment step as previously described.[32] To remove contaminating DNA, 10 μg of each RNA preparation was treated with DNase using a DNA-Free DNA removal kit (Ambion). For the reverse transcription reactions, a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) was used on 1 μg of DNA-free RNA of each sample, and the manufacturer's instructions were followed. RNA samples were stored at −80 °C, and cDNA samples were stored at −20 °C. Quantitative expression of *CDR1* and *CDR2* was performed with the StepOne Real-time PCR System (Life Technologies). RT-qPCR was carried out in a 10 μL volume containing the following reagents: 5 μL of iTaq Supermix with Rox (BioRad), each primer pair, the Taqman probe at a final concentration of 200 nM for the primers (CDR2-ORF-F: TAGATATTTGAGCCACATG and CDR2-ORF-R: TTGGCA-TTGAAATTTTCG; CDR1-ORF-F: ATGACTCGAGATATTTTG-ATA; and CDR1-ORF-R: TTAACAGCAATGGTCTTTA) and 100 nM for the probes (CDR2-P2: TTAGTCCATTCAACGGCA-ACATTAG; CDR1-P2: CATTATGAGACCTGGTGAACTTACT), and 1% of the total cDNA sample produced as described above. Each reaction was run in triplicate, and data were analyzed with the StepOne software. For relative quantification of the target genes, each

set of primer pairs and the Taqman probe were used in combination with the *ACT1* primers (ACT1-RT-F: ATAACGGTTCTGGTATGT; ACT1-RT-R: CCTTGATGTCTTGGTCTA) and an *ACT1* probe (ACT1-RT-P: CGGTGACGACGCTCCAAG).

For data analysis and fold change calculations, the comparative CT method was used. For each experiment, a standard curve for the reference gene and the studied gene was included, and the amplification efficiency was determined for all genes. The CT values of the target genes were normalized to the endogenous reference. Fold changes were obtained from the mean normalized expression of the samples relative to the mean normalized expression of a selected control.

Confocal Fluorescence Microscopy Assays. Aliquots of 100 μL of *C. albicans* and *Cry. neoformans* treated for 48 h with a 40 μM DMSO solution of **4** were washed twice in phosphate-buffered saline (PBS) and fixed in 4% formaldehyde for 30 min at 4 °C. After a second PBS wash, the cells were suspended in 100 μL of PBS, and 3 μL of the suspension was spotted onto a glass slide, dried, and mounted with 6 μL of Vectashield mounting medium. Images were acquired with a Leica TCS SP5 inverted confocal microscope equipped with a 63×/1.40 OIL objective (Zeiss), set for fluorescein (FITC) (excitation 488 nm, emission window from 500 to 540 nm).

General Chemistry Directions. Reagents were obtained from commercial suppliers and used without further purification. DCM and CH$_3$CN were dried over sodium hydride. THF was dried over Na/benzophenone prior to use. Anhydrous reactions were run under a positive pressure of dry N$_2$ or argon. Degassed DCM was prepared by using the freeze−pump−thaw method. Silica gel 60 was used for flash

chromatography (23−400 mesh). ^{1}H NMR and ^{13}C NMR are reported in parts per million (δ scale) and internally referenced to the CDCl$_3$ or CD$_3$OD signal, respectively at δ 7.24 and 3.31 ppm. Chemical shifts for carbon are reported in parts per million (δ scale) and referenced to the carbon resonances of the solvent (CDCl$_3$ at δ 77.00 and CD$_3$OD at δ 49.00 ppm). Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet and/or multiplet resonances, br = broad), coupling constant in hertz (Hz), and integration. Mass spectrometry (MS) data were acquired on an Agilent 1100 LC/MSD VL system (G1946C) with a 0.4 mL/min flow rate using a binary solvent system of 95:5 methanol/ water. UV detection was monitored at 254 nm. Mass spectra were acquired in positive mode, scanning over the mass range. Purity of final products was assessed by HPLC/MS analysis, conducted using a Polaris C18 column (150−4.6 mm, 5 μm particle size) at a flow rate of 0.8 mL min^{-1}, with a mobile phase composed of 50% CH$_3$CN/50% H$_2$O−formic acid 0.1%. The purity of all final compounds was above 95%.

5-(Dimethylamino)-N-(8-(2-oxo-4-(tert-butoxycarbonylimino)-1,3,5-triazacyclotridecan-1-yl)octyl)naphthalene-1-sulfonamide (7). A solution of macrocyclic amine **5** (20 mg, 1 equiv) and TEA (0.01 mL, 1.5 equiv) in CH$_3$CN at room temperature under argon atmosphere was treated with a solution of dansyl chloride **6** (17 mg, 1.5 equiv) in CH$_3$CN. The mixture was stirred at room temperature for 10 min. Solvent was then evaporated, and the crude residue was purified with flash chromatography on silica gel, eluting with 10% AcOEt/Hex to give **7** (isolated yield 79%). ^{1}H NMR (400 MHz CDCl$_3$) 12.00 (1H, s); 8.50−8.48 (d, J = 8.4 Hz, 1H), 8.24−8.22 (d, J = 8.2 Hz, 1H), 8.19−8.17 (d, J = 8.1 Hz, 1H), 8.02 (t, J = 4.0 Hz, 1H), 7.52−7.44 (m, 2H), 7.14−7.12 (d, J = 8.2 Hz, 1H), 4.50 (s, 1H), 3.36 (m, 2H), 3.30 (m, 2H), 3.13−3.11(m, 2H), 2.81(s, 6H), 2.78 (m, 2H), 1.56 (m, 8H), 1.40 (s, 9H), 1.25−1.19 (m, 16H) ppm. ^{13}C NMR (100 MHz CD$_3$OD) 163.9; 154.0; 153.5;134.7; 130.2, 129.6; 128.2; 123.2; 118.8; 115.1; 81.9; 46.9; 46.2; 45.4; 43.2; 39.5; 31.8; 29.6; 29.3; 29.1; 28.8; 28.3, 28.0; 27.7; 26.1; 25.8; 25.2; 23.6; 23.4 ppm. LRMS m/z (ESI) = 695.0 [M + Na]$^+$, 673.0 [M + H]$^+$.

tert-Butyl ((5-(Dimethylamino)naphthalene-1-sulfonamido)(1H-pyrazol-1-yl)methylene)carbamate (9). To a suspension of NaH (27 mg, 3 equiv) in 2 mL of THF was added dropwise a solution of N-Boc-1H-pyrazole-1-carboxamidine **8** (78 mg, 1 equiv) in 0.5 mL of THF at 0 °C. The mixture was stirred at room temperature for 30 min, and then a solution of dansyl chloride **6** (300 mg, 3 equiv) in 1 mL of THF was added. After further stirring for 2 h at room temperature, water (5 mL) and DCM (10 mL) were added, and the layers were separated. The aqueous layer was extracted with DCM (2 × 10 mL), and the combined organic phases were washed with NaHCO$_3$, water, and brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude residue was purified with flash chromatography on silica gel, eluting with 20% AcOEt/Hex to give **9** (isolated yield 90%). ^{1}H NMR (400 MHz CDCl$_3$): δ 8.87 (s, 1H), 8.50−8.48 (d, J = 8.4 Hz, 1H), 8.37−8.35 (d, J = 8.4 Hz, 1H), 8.27−8.25 (d, J = 8.4 Hz, 1H), 7.85 (m, 1H), 7.62 (m, 1H), 7.52−7.44 (m, 2H), 7.13−7.11 (d, J = 8.3 Hz, 1H), 6.27 (m, 1H), 2.84 (s, 6H), 1.40 (s, 9H) ppm. LRMS m/z (ESI) = 466.2 [M + Na]$^+$, 443.2 [M + H]$^+$.

5-(Dimethylamino)-N-(N′-tert-butoxycarbonyl-N-(8-(4-(tert-butoxycarbonylimino)-2-oxo-1,3,5-triazacyclotridecan-1-yl)octyl)-carbamimidoyl)naphthalene-1-sulfonamide (10). To a stirred solution of guanylating agent **9** (21 mg, 1.1 equiv) in 5% MeOH/ CH$_3$CN (1 mL) was added a solution of the macrocyclic amine **5** (20 mg, 1 equiv) in 5% MeOH/CH$_3$CN (1 mL) dropwise, followed by the addition of catalytic diisopropylethylamine (DIPEA) (2 drops). The mixture was stirred at 50 °C overnight. After cooling, solvent was evaporated under reduced pressure, and the crude residue was purified with flash chromatography on silica gel, eluting with 10% AcOEt/Hex to give **10** (isolated yield 93%). ^{1}H NMR (400 MHz CDCl$_3$): δ 12.02 (s, 1H), 9.79 (s, 1H), 8.50−8.48 (d, J = 8.4 Hz, 1H), 8.37−8.35 (d, J = 8.4 Hz, 1H), 8.27−8.25 (d, J = 8.4 Hz, 1H), 7.49−7.41 (m, 2H), 7.13−7.11 (d, J = 8.2 Hz, 1H), 3.38 (m, 2H), 3.29 (m, 2H), 3.18 (m, 2H), 2.82 (s, 6H), 1.48(m, 8H), 1.40 (s, 9H), 1.37 (s, 9H), 1.25−1.19 (m, 16H) ppm. ^{13}C NMR (100 MHz CD$_3$OD) 163.9; 154.0; 153.5;

152.5; 150.6; 138.9; 129.7; 129.1; 127.2; 126.2; 123.3; 115.1; 84.1; 81.9; 46.9; 46.2; 45.5; 41.3; 39.5; 31.8; 30.7; 29.5; 29.3; 29.0; 28.7; 28.3, 28.0; 27.7; 26.1; 25.8; 25.2; 23.6; 23.4 ppm. LRMS m/z (ESI) = 837.5 [M + Na]$^+$, 814.5 [M + H]$^+$.

General Procedure for Boc Deprotection. The appropriate protected compound **7** or **10** (0.025 mmol) was dissolved in a 10% solution of freshly distilled TFA in dry DCM (1 mL). The resulting solution was stirred at room temperature under argon for 24 h. The reaction mixture was then concentrated under reduced pressure, affording the desired compound as the trifluoroacetate salt in quantitative yield.

5-(Dimethylamino)-N-(8-(4-imino-2-oxo-1,3,5-triazacyclotridecan-1-yl)octyl)naphthalene-1-sulfonamide (2). ^{1}H NMR (400 MHz CDCl$_3$): δ 9.79 (s, 1H), 8.50−8.48 (d, J = 8.4 Hz, 1H), 8.37−8.35 (d, J = 8.2 Hz, 1H), 8.27−8.25 (d, J = 8.4 Hz, 1H), 7.49−7.41 (m, 2H), 7.13−7.11 (d, J = 8.2 Hz, 1H), 3.56 (m, 2H), 3.46 (m, 2H), 3.23 (m, 2H), 3.05 (m, 2H), 2.80 (s, 6H), 1.48(m, 8H), 1.25−1.19 (m, 16H) ppm. ^{13}C NMR (100 MHz CDCl$_3$): δ 163.6, 153.7, 153.2, 135.1, 130.2, 129.7, 129.2, 124.0, 121.4, 81.6, 46.7, 46.0, 41.9, 39.3, 33.5, 29.2, 28.2, 27.8, 26.8, 25.8, 25.5, 24.9, 23.3 ppm. LRMS m/z (ESI) = 595.0 [M + Na]$^+$, 573.0 [M + H]$^+$.

5-(Dimethylamino)-N-(N-(8-(4-imino-2-oxo-1,3,5-triazacyclotridecan-1-yl)octyl)carbamimidoyl)naphthalene-1-sulfonamide (3). ^{1}H NMR (400 MHz CD$_3$OD): δ 12.00 (s, 1H); 8.44−8.42 (d, J = 8.4 Hz, 1H), 8.22−8.20 (d, J = 8.4 Hz, 1H), 8.19−8.17 (d, J = 8.4 Hz, 1H), 8.03 (m, 1H), 7.49−7.43 (m, 2H), 7.13−7.11 (d, J = 8.4 Hz, 1H), 3.38 (m, 2H), 3.31 (m, 2H), 3.12 (m, 2H), 2.80 (s, 6H), 2.77 (m, 2H), 1.56 (m, 8H), 1.25−1.19 (m, 16H) ppm. ^{13}C NMR (100 MHz CDCl$_3$): δ 206.8, 163.9, 154.05, 153.0, 152.5, 150.6, 138.9, 129.8, 129.1, 127.2, 126.2, 123.3, 116.2, 84.1, 82.0, 46.9, 46.2, 45.7, 41.3, 39.6, 31.8, 30.8, 29.5, 29.2, 29.0, 28.4, 27.8, 25.8, 25.2, 23.5, 22.6, 14.0 ppm. LRMS m/z (ESI) = 637.5 [M + Na]$^+$, 614.5 [M + H]$^+$.

4-(3-(Phthalimido)propoxy)-2-hydroxybenzaldehyde (12). A solution of 2,4-dihydroxybenzaldehyde **11** (0.500 g, 1 equiv), N-(3-bromopropyl) phthalimide (0.970 g, 1 equiv), and K$_2$CO$_3$ (0.500 g, 1 equiv) was refluxed in CH$_3$CN (20 mL) for 6 h. The reaction mixture was concentrated and extracted with DCM and water. The organic phase was dried over Na$_2$SO$_4$, filtered, and concentrated. After the purification of the crude by flash chromatography (SiO$_2$) using DCM as eluent, product **12** was obtained a white solid (isolated yield 34%). ^{1}H NMR (400 MHz CDCl$_3$): δ 11.42 (s, 1H, 2-OH); 9.69 (s, 1H); 7.84−7.81 (m, 2H); 7.74−7.71 (m, 2H); 7.37 (d, J = 8.8 Hz, 1H); 6.41 (d, J = 8.5 Hz, 1H); 6.32 (d, J = 1.2 Hz, 1H); 4.07 (t, J = 6.8 Hz, 2H); 3.90 (t, J = 6.8 Hz, 2H); 2.22 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): 194.3; 168.2; 165.8; 164.3; 135.1; 133.9; 132.0; 123.2; 115.2; 108.5; 101.0; 66.1; 35.1; 27.9 ppm. LRMS m/z (ESI) = 324 [M + H]$^+$.

4-(3-(Phthalimido)propoxy)-2-(pent-4-en-1-yloxy)benzaldehyde (13). A solution of **12** (0.200 g, 1 equiv), K$_2$CO$_3$ (0.255 g, 3 equiv), and 5-bromo-1-pentene (0.160 mL, 2.2 equiv) was refluxed in CH$_3$CN (10 mL) for 6 h. The reaction mixture was concentrated under vacuum and extracted with DCM. The organic phase was dried over Na$_2$SO$_4$, filtered, and concentrated. The reaction crude was purified by flash chromatography (SiO$_2$) using DCM as eluent to obtain **13** as a white solid (isolated yield 78%). ^{1}H NMR (400 MHz CDCl$_3$): δ 10.27 (s, 1H); 7.81−7.79 (m, 2H); 7.72−7.68 (m, 4H); 6.38 (d, J = 8.4 Hz, 1H); 6.27 (s, 1H); 5.87−5.76 (m, 1H); 5.04 (d, J = 17.2 Hz, 1H); 4.99 (d, J = 10 Hz, 1H); 4.06 (t, J = 6 Hz, 2H); 3.95 (t, J = 6.4 Hz, 2H); 3.88 (t, J = 6.8 Hz, 2H); 2.25−2.14 (m, 4H); 1.89 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): 188.1; 183.7; 177.5; 168.2; 165.1; 163.0; 137.3; 133.9; 132.0; 130.1; 123.2; 119.1; 115.4; 106.0; 98.9; 67.5; 66.0; 35.2; 29.9; 28.05; 28.00 ppm. LRMS m/z (ESI) = 394.1 [M + H]$^+$; 416.1 [M + Na]$^+$.

4-(3-(Phthalimido)propoxy)-2-(pent-4-en-1-yloxy)benzaldehyde oxime (14). A stirred solution of aldehyde **13** (0.120 g, 1 equiv) in EtOH (10 mL), hydroxylamine hydrochloride (0.053 g, 2.5 equiv), and pyridine (0.028 g, 1.2 equiv) was heated to 80 °C for 3.5 h. The reaction mixture was concentrated in vacuo and used with no further purification. ^{1}H NMR (400 MHz CDCl$_3$): δ 8.86 (br, 1H); 8.40 (s, 1H); 7.83−7.81 (m, 2H); 7.71 (m, 2H); 7.57 (d, J = 8.4 Hz, 1H); 6.36

(d, J = 8.6 Hz, 1H); 5.88−5.77 (m, 1H); 5.05 (d, J = 17.6 Hz, 1H); 4.99 (d, J = 10.4 Hz, 1H); 4.03 (t, J = 5.6 Hz, 2H); 3.91−3.87 (m, 4H); 2.24−2.14 (m, 4H); 1.86 (quint, J = 6.8 Hz, 2H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): 165.1; 163.2; 146.2; 137.3; 133.8; 132.1; 127.3; 123.2; 119.1; 115.3; 105.8; 98.9; 67.5; 66.0; 44.0; 35.4; 29.9; 28.1 ppm. LRMS m/z (ESI) = 409.0 [M + H]$^+$; 431.0 [M + Na]$^+$.

2-(3-(4-(Aminomethyl)-3-(pent-4-en-1-yloxy)phenoxy)propyl)-3-hydroxyisoindolin-1-one (15). To a stirred solution of aldoxime **14** (0.124 g, 1 equiv) in THF (10 mL) were added Zn^0 granules (0.198 g, 10 equiv) and aqueous 2 M HCl solution (1.51 mL, 10 equiv), and the resulting mixture was heated to 80 °C for 2 h. After the solution was cooled to room temperature, it was filtered to remove the residual Zn and extracted in DCM and 1 M NaOH solution. The organic layer was washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated to yield **15** as a colorless oil that was used with no further purification (isolated yield 66%). ^{1}H NMR (400 MHz CDCl$_3$): δ 7.65 (d, J = 7.2 Hz, 1H); 7.53−7.48 (m, 2H); 7.42−7.39 (m, 2H); 6.88 (d, J = 8.4 Hz, 1H); 6.29 (s, 1H); 5.85−5.73 (m, 1H); 5.74 (s, 1H); 5.03 (d, J = 17.6 Hz, 1H); 4.98 (d, J = 11.2 Hz, 1H); 4.00−3.80 (m, 6H); 3.72−3.67 (m, 1H); 3.62−3.55 (m, 1H); 3.48 (s, 1H); 2.17−2.10 (m, 4H); 1.81 (m, 2H) ppm. LRMS m/z (ESI) = 419.1 [M + Na]$^+$.

1-(4-(3-(1-Hydroxy-3-oxoisoindolin-2-yl)propoxy)-2-(pent-4-en-1-yloxy)benzyl)-2,3-bis(tert-butoxycarbonyl)guanidine (16). To a stirred solution of **15** (0.277 g, 1 equiv) in DCM (15 mL) Et$_3$N (0.486 mL, 5 equiv) was added 1,3-di-Boc-2-(trifluoromethylsulfonyl)-guanidine (0.300 g, 1.1 equiv), and the resulting mixture was stirred for 18 h at room temperature. Water was added, and after 30 min of stirring, the reaction mixture was extracted with DCM. The organic layer was washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The crude was purified by flash chromatography (SiO$_2$) (petroleum ether:EtOAc:MeOH = 7:2:0.5) to give the desired product **16** as an oil (isolated yield 46%). ^{1}H NMR (400 MHz CDCl$_3$): δ 11.44 (s, 1H); 8.64 (m, 1H); 7.52−7.44 (m, 3H); 7.34 (t, J = 7.2 Hz, 1H); 7.08 (d, J = 8.0 Hz, 1H); 6.28 (s, 1H); 6.26 (s, 1H); 5.82−5.76 (m, 1H); 5.73 (s,1H); 5.01 (d, J = 17.6 Hz, 1H); 4.94 (d, J = 10.0 Hz, 1H); 4.67 (br, 1H); 4.47 (d, J = 4.8 Hz, 2H); 3.93−3.87 (m, 2H); 3.84 (t, J = 6.4 Hz, 2H); 3.57−3.44 (m, 2H); 2.18 (q, J = 7.2 Hz, 2H); 2.03 (m, 2H); 1.85 (m, 2H); 1.47 (s, 9H); 1.42 (s, 9H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): 167.6; 163.5; 159.6; 158.0; 155.7; 152.8; 144.0; 137.7; 132.0; 131.4; 130.4; 129.4; 123.2; 122.9; 117.9; 115.1; 104.3; 99.6; 82.6; 81.9; 79.0; 67.1; 65.8; 40.6; 36.7; 30.0; 28.2; 28.1; 27.9 ppm. LRMS m/z (ESI) = 639.2 [M + H]$^+$; 661.2 [M + Na]$^+$.

N-(N′-(4-(3-(1-Hydroxy-3-oxoisoindolin-2-yl)propoxy)-2-(pent-4-en-1-yloxy)benzyl)-tert-butoxycarbonylcarbamimidoyl)-N′-prop-2-enyl-N′-(8-(benzyloxycarbonylamino)octyl)urea (18). To a stirring solution of **16** (0.276 g, 1 equiv) in dry THF (20 mL) was added amine **17** (0.207 g, 1.5 equiv)^{19} as a dry THF solution (5 mL). To the resulting mixture was added Et$_3$N (0.060 mL, 1 equiv). After 12 h of heating at 80 °C, the reaction mixture was cooled to room temperature and concentrated in vacuo. The reaction mixture was purified by flash chromatography (SiO$_2$) (petroleum ether:EtOAc MeOH = 7:3:1) to yield the desired compound **18** as an oil (isolated yield 60%). ^{1}H NMR (400 MHz CDCl$_3$): δ 12.21 (d, J = 4 Hz, 1H); 8.42 (m, 1H); 7.58 (t, J = 6.4 Hz, 1H); 7.53 (d, J = 7.6 Hz, 1H); 7.48 (t, J = 7.2 Hz, 1H); 7.37 (t, J = 7.6 Hz, 1H); 7.28 (m, 5H); 7.05 (d, J = 8.8 Hz, 1H); 6.31 (d, J = 7.6 Hz, 2H); 5.83−5.78 (m, 1H); 5.75 (s, 1H); 5.12−4.96 (m, 6H); 4.42 (dd, J1 = 5.6 Hz, J2 = 16 Hz, 2H); 4.12 (d, J = 5.2 Hz, 1H); 3.95−3.68 (m, 6H); 3.67−3.54 (m, 2H); 3.42 (t, J = 7.6 Hz, 1H); 3.21 (t, J = 7.2 Hz, 1H); 3.10−3.06 (m, 2H); 2.22 (q, J = 6.8 Hz, 2H); 2.09 (m, 2H); 1.89 m, 2H); 1.48 (m, 2H); 1.41 (s, 9H); 1.24 (m, 8H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): 167.5; 163.8; 163.7; 159.4; 157.9; 157.8; 156.4; 153.8; 153.6; 153.1; 144.1; 137.1; 136.5; 135.3; 134.7; 131.9; 131.6; 130.1; 129.6; 129.4; 128.4; 127.9; 123.1; 122.9; 118.9; 115.5; 115.1; 104.3; 99.6; 82.0; 81.9; 81.8; 67.1; 66.4; 65.8; 50.44; 48.4; 47.5; 45.6; 41.0; 36.9; 30.0; 29.8; 29.6; 29.4; 29.3; 29.1; 28.5; 28.2 ppm. LRMS m/z (ESI) = 883.3 [M + H]$^+$.

tert-Butyl (10-(8-(Benzyloxycarbonylamino)octyl)-11-oxo-18-(3-(1-Hydroxy-3-oxoisoindolin-2-yl)propoxy)-2,3,4,5,8,9,10,11,12,15-decahydrobenzo[b][1,5,7,9]oxatriazacycloheptadecin-13-yl)-

carbamate (19). In a 250 mL round-bottomed flask equipped with a stirring bar and a condenser, **18** (0.231 g, 1 equiv) was dissolved in 175 mL of dry DCM, and the resulting mixture was degassed three times. After the third degassing cycle, Grubbs's second-generation (0.024 g, 0.1 equiv) catalyst was added, and the mixture was degassed another time. The reaction mixture was refluxed for 3 h. The solvent was removed, and the reaction crude was purified by flash chromatography (SiO$_2$) (petroleum ether:EtOAc: = 7:3) to yield the desired compound as a mixture of two isomers (E/Z) (isolated yield 75%). ^{1}H NMR (400 MHz CDCl$_3$): δ 12.21 (s, 1H), 12.03 (s, 1H), 8.21 (s, 1H), 7.61 (d, J = 7.3 Hz, 3H), 7.57−7.47 (m, 6H), 7.39 (t, J = 7.1 Hz, 3H), 7.31 (s, 7H), 7.06 (d, J = 8.1 Hz, 3H), 6.33 (t, J = 7.6 Hz, 3H), 6.27 (s, 2H), 6.05−5.95 (m, 1H), 5.76 (s, 3H), 5.63−5.48 (m, 2H), 5.41−5.31 (m, 2H), 5.03 (s, 4H), 4.85 (s, 2H), 4.56 (s, 4H), 4.22 (d, J = 5.8 Hz, 2H), 3.95 (s, 9H), 3.87 (d, J = 3.3 Hz, 6H), 3.64 (m, 6H), 3.32−3.22 (m, 4H), 3.12 (s, 6H), 2.40 (d, J = 6.7 Hz, 2H), 2.13 (m, 10H), 1.52 (s, 8H), 1.41 (m, 14H), 1.38 (m, 12H), 1.24 (m, 28H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): δ 167.48, 164.05, 163.39, 159.81, 159.26, 158.29, 157.38, 156.36, 153.41, 153.21, 152.42, 144.01, 134.30, 132.13, 131.96, 131.57, 131.13, 129.51, 128.38, 127.93, 125.98, 125.03, 123.15, 123.03, 119.52, 118.06, 104.80, 104.29, 99.80, 99.43, 82.00, 81.82, 69.70, 66.42, 65.85, 60.29, 50.36, 47.11, 44.58, 40.98, 39.61, 36.85, 32.25, 29.77, 29.56, 29.31, 29.11, 28.66, 27.99, 27.68, 26.98, 26.90, 26.55, 14.06 ppm. LRMS m/z (ESI) = 855.3 [M + H]$^+$.

tert-Butyl (10-(8-(Benzyloxycarbonylamino)octyl)-11-oxo-18-(3-(amino)propoxy)-2,3,4,5,8,9,10,11,12,15-decahydrobenzo[b]-[1,5,7,9]oxatriazacycloheptadecin-13-yl)carbamate (20). To a stirred solution of **19** (0.160 g, 1 equiv) in 30 mL of DCM was added MnO$_2$ (0.244 g, 15 equiv) in one portion, and the resulting mixture was stirred for 18 h at room temperature. The reaction mixture was filtered through a pad of Celite and concentrated. The crude was purified by flash chromatography (SiO$_2$) (petroleum ether:EtOAc:MeOH = 7:2:1) to yield the desired phthalimido derivative (*tert*-butyl (10-(8-(benzyloxycarbonylamino)octyl)-11-oxo-18-(3-(1,3-dioxoisoindolin-2-yl)propoxy)-2,3,4,5,8,9,10,11,12,15-decahydrobenzo[b][1,5,7,9]oxatriazacycloheptadecin-13-yl)-carbamate) as a mixture of two isomers (E/Z) (isolated yield 85%). ^{1}H NMR (400 MHz CDCl$_3$): δ 12.28 (s, 1H), 12.11 (s, 1H), 8.21 (t, J = 5.3 Hz, 1H), 7.83−7.77 (m, 4H), 7.75 (t, J = 4.4 Hz, 1H), 7.67 (m, 4H), 7.31 (d, J = 3.8 Hz, 5H), 7.07 (d, J = 8.2 Hz, 2H), 6.32 (t, J = 7.8 Hz, 3H), 6.26 (s, 2H), 6.01 (m,1H), 5.64−5.48 (m, 2H), 5.06 (s, 4H), 4.86 (s, 2H), 4.57 (s, 4H), 4.22 (d, J = 6.6 Hz, 2H), 3.97 (m, 8H), 3.88 (dd, J = 8.1, 5.1 Hz, 10H), 3.35−3.22 (m, 4H), 3.15 (d, J = 5.7 Hz, 5H), 2.41 (dd, J = 14.3, 7.1 Hz, 2H), 2.15 (dd, J = 5.6, 3.5 Hz, 8H), 1.54 (s, 6H), 1.47−1.44 (m, 4H), 1.41 (s, 9H), 1.39 (s, 9H), 1.27 (m, 18H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): δ 168.22, 164.07, 163.40, 159.83, 159.29, 158.26, 157.35, 156.31, 153.43, 153.23, 152.47, 136.65, 134.26, 133.79, 132.08, 131.11, 129.46, 128.37, 127.95, 127.90, 126.03, 125.08, 123.12, 119.54, 118.08, 104.67, 104.18, 99.85, 99.47, 81.88, 81.73, 69.68, 66.58, 66.38, 65.68, 50.33, 47.09, 44.58, 42.49, 40.99, 39.61, 38.23, 35.40, 32.27, 29.81, 29.58, 29.32, 29.29, 29.12, 28.68, 28.14, 28.00, 27.70, 27.00, 26.91, 26.56, 24.30 ppm. LRMS m/z (ESI) = 853.3 [M + H]$^+$; 875.3 [M + Na]$^+$.

The protected intermediate (0.137 g, 1 equiv) was dissolved in 50 mL of 0.2 M hydrazine monohydrate methanol solution. The resulting mixture was stirred overnight at room temperature. The solvent was removed and the crude purified by flash chromatography (SiO$_2$) (petroleum ether:EtOAc:MeOH:Et$_3$N = 7:2:1:0.25) to yield the primary amine **20** as a mixture of two isomers (E/Z) as an oil (isolated yield 80%). ^{1}H NMR (400 MHz, CDCl$_3$): δ 12.36 (s, 1H), 12.28 (s, 1H), 8.21 (s, 1H), 7.98 (t, J = 5.1 Hz, 2H), 7.75 (s, 1H), 7.35−7.31 (m, 6H), 7.08 (dd, J = 8.2, 3.7 Hz, 2H), 6.39 (s, 4H), 6.36 (s, 1H), 6.01 (dd, J = 14.8, 7.4 Hz, 1H), 5.64−5.48 (m, 1H), 5.42−5.33 (m, 1H), 5.07 (s, 4H), 4.83 (s, 4H), 4.77 (s, 4H), 4.57 (d, J = 5.0 Hz, 4H), 4.23 (d, J = 6.4 Hz, 1H), 3.99 (d, J = 5.3 Hz, 4H), 3.95 (d, J = 5.2 Hz, 2H), 3.90 (s, 4H), 3.65 (t, J = 6.2 Hz, 2H), 3.35−3.28 (m, 1H), 3.28−3.20 (m, 4H), 3.15 (d, J = 6.0 Hz, 4H), 3.02 (s, 4H), 2.07−1.99 (m, 4H), 1.65 (s, 4H), 1.59−1.49 (m, J = 4.8 Hz, 6H), 1.47−1.42 (m, 6H), 1.41 (s, 5H), 1.40 (s, 9H), 1.38 (s, 4H), 1.27 (s, 18H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): δ 164.21, 163.40, 159.66, 158.09, 153.44, 153.39,

153.26, 138.00, 136.62, 134.27, 130.37, 128.38, 127.96, 127.93, 118.90, 104.55, 99.94, 81.93, 81.83, 67.99, 66.42, 65.59, 44.43, 40.99, 39.03, 38.44, 30.36, 29.80, 29.58, 29.31, 29.13, 28.29, 28.01, 27.85, 27.03, 26.56, 26.17, 24.49, 24.24 ppm. LRMS m/z (ESI) = 723.4 [M + H]$^+$.

tert-Butyl (10-(8-(Benzyloxycarbonylamino)octyl)-11-oxo-18-(3-(trifluoroacetylamino)propoxy)-2,3,4,5,8,9,10,11,12,15-decahydrobenzo[b][1,5,7,9]oxatriazacycloheptadecin-13-yl)-carbamate (21). Primary amine **20** (0.100 g, 1 equiv) was dissolved in dry THF (15 mL), and Et$_3$N was added (20 μL). To the resulting mixture, cooled to 0 °C, was added methyl trifluoroacetate dropwise, and the ice bath was removed. The reaction mixture was stirred for 16 h at room temperature. The reaction was diluted with MeOH, concentrated in vacuo, and purified by flash chromatography (SiO$_2$) (petroleum ether:EtOAc:MeOH = 7:2:1) to yield the desired product **21** as a mixture of two isomers (*E/Z*) as an amorphous white solid (isolated yield 90%). ^{1}H NMR (400 MHz, CDCl$_3$): δ 12.35−12.31 (m, 1H), 12.27−12.22 (m, 1H), 12.09−12.05 (m, 1H), 8.23 (t, J = 5.3 Hz, 1H), 7.99 (t, J = 5.1 Hz, 1H), 7.77 (t, J = 4.3 Hz, 1H), 7.40 (s, 2H), 7.32 (d, J = 3.9 Hz, 4H), 7.12 (dd, J = 7.9, 4.3 Hz, 2H), 6.42−6.33 (m, 4H), 6.07−5.97 (m, J = 14.7, 7.2 Hz, 1H), 5.64−5.48 (m, 1H), 5.42−5.33 (m, 1H), 5.06 (s, 3H), 4.86 (s, 1H), 4.62−4.57 (m, J = 5.1 Hz, 4H), 4.23 (d, J = 6.5 Hz, 1H), 4.04 (t, J = 5.2 Hz, 4H), 3.98−3.88 (m, J = 18.4, 8.2 Hz, 4H), 3.66 (t, J = 6.5 Hz, 2H), 3.54 (q, J = 5.7 Hz, 4H), 3.35−3.20 (m, 4H), 3.14 (d, J = 6.2 Hz, 4H), 2.07−1.99 (m, 4H), 1.67 (s, 4H), 1.54 (s, 6H), 1.48−1.43 (m, 5H), 1.42 (s, 4H), 1.40 (s, 9H), 1.39 (s, 3H), 1.30−1.23 (m, 17H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): δ 164.20, 164.06, 163.39, 159.39, 159.21, 158.82, 158.47, 158.44, 158.17, 157.54, 157.31, 156.94, 156.35, 153.45, 153.40, 153.26, 153.23, 152.42, 136.61, 134.23, 132.06, 131.27, 130.47, 129.63, 128.38, 127.92, 126.02, 125.11, 120.11, 119.33, 118.64, 117.30, 114.44, 104.55, 104.33, 104.05, 99.71, 99.56, 99.25, 82.02, 81.93, 81.88, 69.76, 67.95, 66.43, 60.28, 50.35, 47.10, 44.46, 42.52, 40.98, 39.03, 38.22, 32.23, 29.79, 29.57, 29.30, 29.11, 28.66, 28.26, 27.98, 27.84, 27.68, 27.01, 26.90, 26.55, 26.18, 24.48, 24.23 ppm. LRMS m/z (ESI) = 819.4 [M + H]$^+$.

tert-Butyl (10-(8-(Amino)octyl)-11-oxo-18-(3-(trifluoroacetylamino)propoxy)-2,3,4,5,6,7,8,9,10,11,12,15-dodecahydrobenzo[b][1,5,7,9]oxatriazacycloheptadecin-13-yl)-carbamate (22). To a solution of **21** (0.100 g, 1 equiv) in 2-propanol (20 mL) were added 10 μL of 36% HCl and a catalytic amount of 10% Pd/C (0.032 g, 0.2 equiv). The resulting mixture was stirred under H$_2$ atmosphere for 3 h. The reaction mixture was filtered through a pad of Celite and the filtrate concentrated in vacuo. The crude was used for the next reaction without any further purification. ^{1}H NMR (400 MHz, CD$_3$OD): δ 9.27 (s, 1H), 7.20 (s, 1H), 6.51 (s, 2H), 4.57 (s, 2H), 4.01 (m, 4H), 3.65 (s, 2H), 3.47 (s, 2H), 2.91 (s, 2H), 2.02 (s, 2H), 1.77−1.52 (m, 14H), 1.46 (s, 9H), 1.43−1.25 (m, 12H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$/ CD$_3$OD): δ 160.47, 158.37, 131.40, 104.92, 99.78, 68.03, 66.28, 45.22, 37.73, 28.58, 28.46, 28.42, 28.13, 27.72, 27.02, 26.01, 25.87, 25.78, 24.39 ppm. LRMS m/z (ESI) = 687.2 [M + H]$^+$; 344.1 [M+2H]$^{2+}$.

tert-Butyl (10-(8-(((N'-tert-Butoxycarbonyl)-N-tert-butoxycarbonyl-N-crotylcarbamimidoyl)amino)octyl)-11-oxo-18-(3-(trifluoroacetylamino)propoxy)-2,3,4,5,6,7,8,9,10,11,12,15-dodecahydrobenzo[b][1,5,7,9]oxatriazacycloheptadecin-13-yl)-carbamate (24). To a stirred solution of amine **22** (0.082 g, 1 equiv) in 20 mL of CH$_3$CN and MeOH (9:1) were added DIPEA (0.068 mL, 3 equiv) and N,N'-di-Boc-N-crotyl-1H-pyrazole-1-carboxamidine (23) (0.071 g, 1.5 equiv).[16] The reaction mixture was heated to 60 °C for 16 h. The solvent was removed in vacuo and the crude purified by flash chromatography (SiO$_2$) (petroleum ether:EtOAc:MeOH = 7:2:1) to yield the desired product **24** as an oil (isolated yield 70%). ^{1}H NMR (400 MHz, CDCl$_3$): δ 12.34 (s, 1H), 7.99 (t, J = 5.3 Hz, 1H), 7.25 (s, 1H), 7.12 (d, J = 8.1 Hz, 1H), 6.39 (d, J = 1.9 Hz, 1H), 6.36 (s, 2H), 5.67−5.41 (m, 2H), 4.59 (d, J = 5.3 Hz, 2H), 4.13 (d, J = 6.3 Hz, 2H), 4.05 (t, J = 5.5 Hz, 2H), 3.91 (s, 2H), 3.65 (t, J = 6.6 Hz, 2H), 3.55 (q, J = 6.0 Hz, 2H), 3.26−3.21 (m, 2H), 3.18 (t, J = 7.1 Hz, 2H), 2.09−2.02 (m, 2H), 1.71−1.62 (m, 8H), 1.58−1.50 (m, J = 5.5 Hz, 6H), 1.47 (s, 9H), 1.43 (s, 9H), 1.40 (s, 9H), 1.29 (s, 8H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): δ 164.20, 159.12, 158.18, 153.40, 153.27, 130.49, 126.08, 119.42, 104.30, 99.68, 81.89, 78.97, 67.94,

66.62, 44.46, 43.72, 39.01, 38.37, 29.28, 29.23, 29.12, 28.25, 28.13, 28.08, 27.99, 27.91, 27.88, 27.04, 26.75, 26.17, 24.46.

tert-Butyl (10-(8-(((N'-tert-Butoxycarbonyl)-N-tert-butoxycarbonyl-N-crotylcarbamimidoyl)amino)octyl)-11-oxo-18-(3-(amino)-propoxy)-2,3,4,5,6,7,8,9,10,11,12,15-dodecahydrobenzo[b][1,5,7,9]-oxatriazacycloheptadecin-13-yl)carbamate (25). K$_2$CO$_3$ (0.045 g, 5 equiv) was added to a solution (10 mL) of **24** (0.064 g, 1 equiv) in MeOH:H$_2$O (9:1). The resulting mixture was heated to 70 °C for 2 h, concentrated, extracted with DCM (3 × 10 mL), dried over Na$_2$SO$_4$, and concentrated. The crude was used without any further purification. ^{1}H NMR (400 MHz, CDCl$_3$): δ 12.36 (s, 1H), 7.99 (t, J = 4.9 Hz, 1H), 7.11 (d, J = 8.8 Hz, 1H), 6.40 (d, J = 6.3 Hz, 2H), 5.72−5.41 (m, 2H), 4.59 (d, J = 5.3 Hz, 2H), 4.14 (d, J = 6.3 Hz, 2H), 4.02 (t, J = 6.0 Hz, 2H), 3.93 (s, 2H), 3.66 (t, J = 6.5 Hz, 2H), 3.27−3.21 (m, 2H), 3.18 (t, J = 7.1 Hz, 2H), 2.91 (t, J = 6.2 Hz, 2H), 1.96−1.89 (m, 2H), 1.71−1.62 (m, 8H), 1.55 (d, J = 5.7 Hz, 8H), 1.47 (s, 9H), 1.44 (s, 9H), 1.41 (s, 9H), 1.29 (s, 12H), 1.23 (s, 6H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$): δ 164.23, 159.90, 158.11, 153.38, 153.28, 130.40, 126.13, 118.71, 104.56, 99.90, 81.81, 67.96, 65.85, 44.43, 43.74, 39.05, 32.48, 29.57, 29.28, 29.13, 28.39, 28.15, 28.10, 28.01, 27.89, 27.06, 26.77, 26.18, 24.50, 24.24, 17.59 ppm. LRMS m/z (ESI) = 887.5 [M + H]$^+$; 909.5 [M + Na]$^+$.

tert-Butyl (10-(8-(((N'-tert-Butoxycarbonyl)-N-tert-butoxycarbonyl-N-crotylcarbamimidoyl)amino)octyl)-11-oxo-18-(3-(5-fluorescein-amino)thiocarbonylaminopropoxy)-2,3,4,5,6,7,8,9,10,11,12,15-dodecahydrobenzo[b][1,5,7,9]-oxatriazacycloheptadecin-13-yl)carbamate (26). To a stirred solution of **25** (0.035 g, 1 equiv) in 1.5 mL of dry DMF were added sequentially DIPEA (0.047 mL, 7 equiv) and FITC isomer I (0.022 g, 1.5 equiv). The resulting mixture was stirred for 24 h at room temperature. The solvent was removed and the crude purified by flash chromatography (SiO$_2$) (DCM:MeOH = 9:1) to yield the desired product a yellow powder (isolated yield 52%). ^{1}H NMR (400 MHz, CDCl$_3$/ CD$_3$OD): δ 7.83 (d, J = 11.3 Hz, 1H), 7.03 (t, J = 7.1 Hz, 2H), 6.61 (s, 2H), 6.54 (d, J = 8.3 Hz, 2H), 6.43 (t, J = 10.1 Hz, 1H), 5.64−5.35 (m, 1H), 4.50 (s, 2H), 4.10−4.02 (m, 2H), 4.02−3.94 (m, 2H), 3.85 (s, 2H), 3.77 (s, 2H), 3.58 (s, 2H), 3.46−3.37 (m, 4H), 3.31 (d, J = 15.0 Hz, 4H), 3.13 (m, 6H), 2.13−2.04 (m, 2H), 1.58 (d, J = 4.7 Hz, 6H), 1.47 (d, J = 4.2 Hz, 6H), 1.40 (s, 9H), 1.38 (s, 9H), 1.34 (s, 9H), 1.23 (s, 10H) ppm. LRMS m/z (ESI) = 1276.6 [M + H]$^+$.

(10-(8-((N-Crotylcarbamimidoyl)amino)octyl)-11-oxo-18-(3-(5-fluorescein-amino)thiocarbonylaminopropoxy)-2,3,4,5,6,7,8,9,10,11,12,15-dodecahydrobenzo[b][1,5,7,9]-oxatriazacycloheptadecin-13-yl)amine (4). Boc-protected **26** (0.012 g, 1 equiv) was treated with a 20% CF$_3$COOH solution in DCM. The reaction mixture was stirred at room temperature for 8 h to yield the final product **4** as a yellow solid in quantitative yield. ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.95 (s, 1H), 7.10−7.01 (m, 2H), 6.64 (m, 4H), 6.51 (m, 3H), 5.69 (s, 1H), 5.46 (s, 1H), 4.14−3.75 (m, 5H), 3.70 (s, 3H), 3.20 (s, 4H), 3.11 (s, 3H), 2.97 (s, 2H), 2.83 (s, 2H), 1.67 (s, 4H), 1.51 (s, 9H), 1.34 (s, 7H), 1.26 (s,15H) ppm. ^{13}C NMR (100 MHz, CDCl$_3$/ CD$_3$OD): δ 180.77, 164.09, 159.54, 158.00, 153.12, 152.82, 152.74, 140.00, 130.27, 128.98, 125.69, 124.76, 118.90, 112.58, 110.03, 105.07, 102.75, 99.80, 82.34, 82.22, 79.34, 67.99, 66.37, 44.69, 44.52, 43.67, 38.96, 31.77, 29.54, 29.21, 29.08, 28.02, 27.88, 26.96, 26.72, 22.52, 17.50, 13.91 ppm. LRMS m/z (ESI) = 976.5 [M + H]$^+$; 488.5 [M+2H] $^{2+}$; 326.4 [M+3H] $^{3+}$.

Animal Experiments. Mouse experiments were performed under the approval of the Institutional Animal Use and Care Committee at the "Università Cattolica del Sacro Cuore", Rome, Italy, and authorized by the Italian Ministry of Health, according to the Legislative Decree 116/92, which implemented the European Directive 86/609/EEC on laboratory animal protection in Italy. Animal welfare was routinely checked by veterinarians of the Service for Animal Welfare.

Experimental animal infections were carried out according to protocols described previously.[33] Briefly, cell suspensions of *C. albicans* strains, namely the reference ATCC 90028 and the clinical isolates DSY294 (azole-susceptible) and DSY296 (azole-resistant), were prepared in sterile saline and used separately (each in a volume of 200 μL) to inject immunocompetent BALB/c mice into their lateral

vein. Groups of 10 mice were established for each strain and used in tissue burden experiments. Seven days after injection with 7×10^4 CFU of yeast cells, mice were sacrificed, and their target organs (spleen and kidneys) were excised aseptically, weighted individually, and homogenized in sterile saline. Organ homogenates were then diluted and plated onto yeast peptone dextrose agar, and plates were incubated for 2 days at 30 °C. After growth, yeast colonies were counted, and the numbers of CFU per gram of organ were calculated. CFU counts were analyzed with nonparametric Wilcoxon rank-sum tests. Statistical significance was set at a P-value of <0.05. An injectable solution of **1** was prepared by adding 1 volume of PEG 400 to 2 volumes of a DMSO solution of **1** (0.2 mg/mL) in order to obtain a 0.1 mg/mL solution with DMSO/PEG 400 ratio of 2:1. The DMSO/PEG solution was injected in a 100 μL volume intraperitoneally each day. Fluconazole was injected intraperitoneally at dosages of 8 mg/kg/day.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00018.

> Molecular formula strings (CSV)
> Figure S1, showing the result of RMPI stability assays; Tables S1 and S2, showing common up- and down-regulated genes resulting from transcriptional analysis; Figure S2, showing fluorescence microscopy images of **2** and **3**; and ¹H NMR and ¹³C NMR spectra of selected compounds (PDF)

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED

ABC, ATP binding cassette; ATCC, American type culture collection; CFU, colony-forming unit; cRNA, complementary RNA; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, dimethylformamide; FITC, fluorescein isothiocyanate; LRMS, low-resolution mass spectrometry; Mdrp1, multi-drug-resistant protein; RT-qPCR, real-time quantitative polymerase chain reaction; TEA, triethylamine; TFA, trifluoroacetic acid; YEPD, yeast extract peptone dextrose

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