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Sulfonylureas Have Antifungal Activity and Are Potent Inhibitors of *Candida albicans* Acetohydroxyacid Synthase

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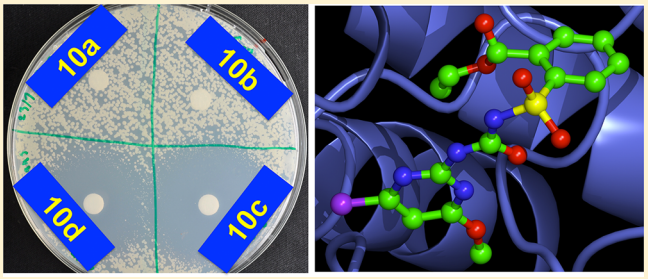
Supporting Information

ABSTRACT: The sulfonylurea herbicides exert their activity by inhibiting plant acetohydroxyacid synthase (AHAS), the first enzyme in the branched-chain amino acid biosynthesis pathway. It has previously been shown that if the gene for AHAS is deleted in *Candida albicans*, attenuation of virulence is achieved, suggesting AHAS as an antifungal drug target. Herein, we have cloned, expressed, and purified *C. albicans* AHAS and shown that several sulfonylureas are inhibitors of this enzyme and possess antifungal activity. The most potent of these compounds is ethyl 2-(*N*-((4-iodo-6-methoxypyrimidin-2-yl)carbamoyl)sulfamoyl)benzoate (**10c**), which has a K_i value of 3.8 nM for *C. albicans* AHAS and an MIC_{90} of 0.7 μg/mL for this fungus in cell-based assays. For the sulfonylureas tested there was a strong correlation between inhibitory activity toward *C. albicans* AHAS and fungicidal activity, supporting the hypothesis that AHAS is the target for their inhibitory activity within the cell.



INTRODUCTION

Invasive fungal infections such as candidiasis, cryptococcosis, and aspergillosis are among the most serious health problems in the world today.[1] They are a major cause of mortality and morbidity, particularly among those with AIDS, cancer patients, and organ transplant recipients, and particularly in third-world countries where the costs associated with current medications are prohibitive.[2] Defense against such infectious agents has relied on the use of a limited number of chemotherapeutic agents, including azoles, such as fluconazole, and polyenes, such as amphotericin B.[3] Of concern is that mortality is on the rise due to resistance to these medications and that the high dosages and long-term treatments that are required can result in severe side effects. There is therefore a pressing need to validate new antifungal drug targets and discover new compounds that can be developed as safe and effective antifungal agents.

A potential target for the development of new antifungal agents is acetohydroxyacid synthase (AHAS; E.C. 2.2.1.6; also referred to as acetolactate synthase, or ALS). AHAS is the first enzyme in the branched-chain amino acid biosynthesis (BCAA) pathway, which is absent in humans. It has been shown that when the gene for AHAS is mutated in *Cryptococcus neoformans*, this pathogen is avirulent in a murine mouse infection and that these mutants die upon isoleucine and valine starvation.[4] *Candida albicans* AHAS mutants are also significantly attenuated in virulence and are starvation-cidal, with a >100-fold reduction in viability after isoleucine and valine has been removed for 4 h.[5]

The reactions performed by AHAS are either the synthesis of (*S*)-acetolactate from two molecules of pyruvate or the synthesis of (*S*)-2-aceto-2-hydroxybutyrate from pyruvate and 2-ketobutyrate.[6] The end-point products of the metabolic pathway for (*S*)-acetolactate are leucine and valine, while isoleucine is synthesized from (*S*)-2-aceto-2-hydroxybutyrate. The catalytic activity of AHAS requires three cofactors: thiamine diphosphate (ThDP), Mg^{2+}, and flavin adenine dinucleotide (FAD). In the first part of the chemical reaction, ThDP reacts with pyruvate to form the enzyme-bound hydroxyethyl-ThDP intermediate and to release CO_2. This intermediate then reacts with the second molecule, 2-ketobutyrate or pyruvate, to form (*S*)-2-aceto-2-hydroxybutyrate or 2-acetolactate, respectively.

The sulfonylurea family of herbicides were developed in the late 1970s.[7] However, it was not until the mid-1980s that it was demonstrated that AHAS was the target for their herbicidal activity[8] and subsequently that the K_i values for the most potent sulfonylureas against plant AHAS are in the low nanomolar range.[9] The commercial success of these compounds has been profound, with well over 50 different AHAS inhibitors in worldwide use as herbicides (e.g., chlorsulfuron, sulfometuron, and metsulfuron).[10] Although some resistant weed strains

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(a)

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                                                  bbh h
C. albicans    QLMDDSFIGLT--GGEIFHEMMLRHKVDTVFGYAGGAILPVFDAIYNSDKFKFVLPRHEQGAGHMAEGYARASGKPGVVLVTSGPGATNVITPMADALMDGVPLV 181
S. cerevisiae  PDMDTSFVGLT--GGQIFNEMMSRQNVDTVFGYPGGAILPVYDAIHNSDKFNFVLPKHEQGAGHMAEGYARASGKPGVVLVTSGPGATNVVTPMADAFADGIPMV 185
C. neoformans  NGLDYSFIGLS--GGQIFQEMMLRHDVKQVFGYPGGAILPVFDAIYNSPHFEFVLPRHEQGAGHMAEGYARVSGKPGVVLVTSGPGATNVITPMQDALSDGVPMV 219
A. thaliana    TFISRFAPDQPRKGADILVEALERQGVETVFAYPGGASMEIHQALTRSSSIRNVLPRHEQGGVFAAEGYARSSGKPGICIATSGPGATNLVSGLADALLDSVPLV 190
                     hh  h     bbhh                                  h
C. albicans    VFSGQVPTTAIGTDAFQEADIVGISRSCTKWNVMVKNVAELPRRINEAFEIATTGRPGPVLVDLPKDVTASILRESIPINTT-------LPSNALSQITKKAVSE 279
S. cerevisiae  VFTGQVPTSAIGTDAFQEADVVGISRSCTKWNVMVKSVEELPLRINEAFEIATSGRPGPVLVDLPKDVTAAILRNPIPTKTT-------LPSNALNQLTSRAQDE 283
C. neoformans  VFCGQVATNLIGSDAFQEADVVGISRSCTKWNVMVKDIAELPRRINEAFKIATTGRPGPVLVDLPKDVTAAILRTPIPAKSVQPGHSPYLPSNPLNPSSQPSDPL 324
A. thaliana    AITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVMDVEDIPRIIEEAFFLATSGRPGPVLVDVPKDIQQQLAIPNWEQAMR-------LPGY-MSRMPKPPEDS 287
                                                                    ba                  hb
C. albicans    F-TSEAIKRAANILNKAKKPIIYAGAGILNNEQGPKLLKELADKANIPVTTTLQGLGAFDQRDPKSLDMLGMHGSAAANTAIQNADCIIALGARFDDRVTGNISK 383
S. cerevisiae  F-VMQSINKAADLINLAKKPVLYVGAGILNHADGPRLLKELSDRAQIPVTTTLQGLGSFDQEDPKSLDMLGMHGCATANLAVQNADLIIAVGARFDDRVTGNISK 387
C. neoformans  PGDADLITEAAQMINKAKRPIIFAGNGVLSSPEGPKLLKELSDKGRIPVTTTLQGLGAFDERDEKSLHMIGMHGSAYANFAMQEADVLIALGVRFDDRVTGKVDT 429
A. thaliana    H-----LEQIVRLISESKKPVLYVGGGCLNSSDELGRFVELTG---IPVASTLMGLGSYPCDDELSLHMLGMHGTVYANYAVEHSDLLLAFGVRFDDRVTGKLEA 384

C. albicans    FAPEAKLAASEGRGGILHFEISPKNINKVVEATEAIEGDVTANLQSFIPLVDSIEN-----RPEWFNKINEWKKKYPYSYQLETPGSLIKPQTLIKEISDQAQTY 483
S. cerevisiae  FAPEARRAAAEGRGGIIHFEVSPKNINKVVQTQIAVEGDATTNLGKMMSKIFPVKE-----RSEWFAQINKWKKEYPYAYMEETPGSKIKPQTVIKKLSKVANDT 487
C. neoformans  FAPAAKAAAAEGRGGIIHFEIQPKNINKIVEGQIPVLGDVVASLGELVPQIEAVD------RSAWIGRCKATKERYPFTYTPSQEGQKLKPQEVVQELDRQAEAL 528
A. thaliana    FASRAK---------IVHIDIDSAEIGKNKTPHVSVCGDVKLALQGMNKVLENRAEELKLDFGVWRNELNVQKQKFPLSFK--TFGEAIPPQYAIKVLDELTD-- 476
                          aa  a              aaaaaaa                                          bb  h
C. albicans    NK-EVIVTTGVGQHQMWAAQHFTWTQPRTMITSGGLGTMGYGLPAAIGAQVAKPDAIVIDIDGDASFNMTLTELSSAVQAGAPIKVCVLNNEEQGMVTQWQSLFY 587
S. cerevisiae  GR-HVIVTTGVGQHQMWAAQHWTWRNPHTFITSGGLGTMGYGLPAAIGAQVAKPESLVIDIDGDASFNMTLTELSSAVQAGTPVKILILNNEEQGMVTQWQSLFY 591
C. neoformans  GKEKFVISTGVGQHQMWACQYYRWTEPRSWVSSGGLGTMGFGLPSAIGAKVAAPEKYVIDIDGDASFSMTAMELATASQYDIGVKVLLFNNEFQGMVEQWQDLFY 633
A. thaliana    GK--AIISTGVGQHQMWAAQFYNYKKPRQWLSSGGLGAMGFGLPAAIGASVANPDAIVVDIDGDGSFIMNVQELATIRVENLPVKVLLLNNQHLGMVMQWEDRFY 579
                                                                                  hhh
C. albicans    EHRYSHTHQSN---------PDFMKLAESMNVKGIRITNQQELKSGVKEFLDATE--PVLLEVIVEKKVPVLPMVPAGKALDDFILWDAEVEKQQNDLRKERTGGKY 683
S. cerevisiae  EHRYSHTHQLN---------PDFIKLAEAMGLKGLRVKKQEELDAKLKEFVSTKG--PVLLEVEVDKKVPVLPMVAGGSGLDEFINFDPEVERQQTELRHKRTGGKH 687
C. neoformans  ENRYSRTRMTN---------PDFVKLSESMGAKGLRCTKLEDLPRMMKEFLEYDGKRPIVLECLVSSEH-VYPMIPAGKALHEQLLHP-----------LLRNGSE- 718
A. thaliana    KANRAHTFLGDPAQEDEIFPNMLLFAAACGIPAARVTKKADLREAIQTMLDTPG--PYLLDVICPHQEHVLPMIPSGGTFNDVITEG-----------DGRIKY 670
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(b)

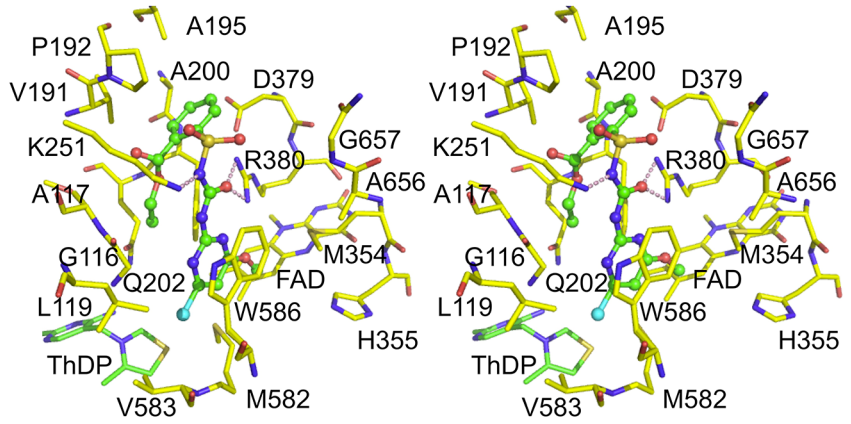

Figure 1. (a) Comparison of the sequences for three fungal AHASs and a plant AHAS. Residues that belong to the active site alone and the herbicide binding site alone and those that belong to both are labeled *a*, *h*, and *b*, respectively. Active site residues are defined as those that are at least partially accessible to the C2 carbon of ThDP and are residues that could interact with pyruvate or 2-ketobutyrate. (b) Amino acid residues and regions of the cofactors, FAD and ThDP, that are close to CE when it is in complex with *S. cerevisiae* AHAS. The image is based on the X-ray structure coordinates with PDB code 1N0H. The oxygen, nitrogen, carbon, sulfur, and chlorine atoms on CE are colored red, blue, green, yellow, and cyan, respectively.

initially emerged upon the use of these herbicides, it is now 30 years since they were introduced to the market, and today they still remain among the most popular choices for crop farmers and home-gardeners worldwide.[11]

The three-dimensional crystal structure of *Saccharomyces cerevisiae* AHAS has been determined as the free enzyme and in complex with six different sulfonylureas.[12] These studies have elucidated the molecular basis for the inhibition of the sulfonylureas toward fungal AHASs, with these compounds binding to what is described as the herbicide binding site, a location situated directly above the active site. These studies show that, as a consequence of herbicide binding, a tunnel is created on the surface of the enzyme that leads to the active site and this tunnel is blocked by the herbicide, thus preventing the substrates from gaining access to ThDP and catalysis from occurring.

The sulfonylureas have previously been shown to be good inhibitors of *S. cerevisiae* AHAS.[13] However, to date, no data have been obtained to determine the inhibitory effect that the sulfonylureas have on AHAS from human fungal pathogens such as *C. albicans*. Furthermore, no systematic analysis of this class of compounds as antifungal agents in cell-based assays has previously been reported. Herein, we report that the catalytic subunit of recombinant *C. albicans* AHAS has been expressed in bacterial cells, purified, and characterized. K_i values against *C. albicans* AHAS and MIC_{90} data for *C. albicans* grown in culture for a series of commercial sulfonylurea inhibitors and second-generation, newly synthesized sulfonylureas have also been determined. The results further demonstrate that AHAS is an excellent target for the development of new antifungal agents and that the sulfonylureas could be promising chemotherapeutic leads.

RESULTS AND DISCUSSION

Enzymes and Sequences. The catalytic subunit of recombinant *C. albicans* AHAS was cloned into the pET30a(+) vector and expressed in *Escherichia coli* cells. The resultant enzyme exhibits greater than 95% purity on SDS−PAGE after Ni^{2+} affinity chromatography. The specific activity and k_{cat} and k_m values for pyruvate for this enzyme are 3.0 ± 0.1 U/mg, 3.75 ± 0.13 s^{-1}, and 2.6 ± 0.1 mM, respectively. These values are similar to those observed for the catalytic subunit of *S. cerevisiae* AHAS (cf. 1.95 ± 0.2 U/mg, 2.4 ± 0.25 s^{-1}, and 3.6 ± 0.6 mM).[14] A sequence alignment of the catalytic subunits of *C. albicans*, *S. cerevisiae*, *Cr. neoformans*, and *Arabidopsis thaliana* AHAS is presented in Figure 1a. Overall, there is 84% identity between the catalytic subunits of *C. albicans* and *S. cerevisiae* AHAS. This identity drops to 41% when *C. albicans* AHAS is compared with *A. thaliana* AHAS. Thus, the overall sequences of the catalytic subunits of AHAS are highly conserved across the different fungal and plant species. More significantly, the amino acid residues that constitute the surface of the active sites

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of *C. albicans* and *S. cerevisiae* AHAS are completely conserved. This is consistent with the observation that the kinetic constants for these two enzymes are similar to each other.

Inhibition of Fungal AHASs by Commercial Sulfonylurea Herbicides. Eight commercial sulfonylurea herbicides were tested as inhibitors of the catalytic subunit of *C. albicans* AHAS (Table 1, Figure 2). Of these, chlorimuron ethyl (CE) is the most potent inhibitor with a K_i value of 7 nM. It is also a potent inhibitor of *S. cerevisiae* AHAS with a K_i of 4 nM. These values are similar to the observed K_i value of 8 nM for CE binding to *A. thaliana* AHAS,[15] suggesting that the modes of binding of CE are similar in all three enzymes. The crystal structures of *A. thaliana* and *S. cerevisiae* AHAS in complex with CE confirm that this is the case for these two enzymes.[16] The potent binding of CE (compared to the other sulfonylureas in this study) suggests that a simple sulfonylurea bridge linking the aromatic and heterocyclic groups and small substituents attached to the aromatic and heterocyclic rings are favorable characteristics for binding to the fungal enzymes.

Table 1. Commercial Herbicide Sulfonylurea Inhibition of *C. albicans* and *S. cerevisiae* AHASs and MIC_{50} Data for Fungal Growth in Cell Culture[a]

	C. albicans		*S. cerevisiae*	
	K_i (nM)	MIC_{50} (μM)	K_i (nM)	MIC_{50} (μM)
CE	7 ± 1	2	3.3[b]	19.5
ES	20 ± 2	2	64 ± 14	13
BSM	31 ± 4	62	4.4 ± 2	20.8
PSE	66 ± 8	62	32 ± 7	41.7
CS	434 ± 50	156	127[b]	117.0
SM	634 ± 80	NI	50.8[b]	19.5
ESM	656 ± 68	NI	346 ± 15	NI
TBM	1637 ± 113	NI	400 ± 20	NI

[a]NI = no inhibition detected. The MIC_{50} values are the average of three measurements. [b]Values taken from Duggleby et al.[13]

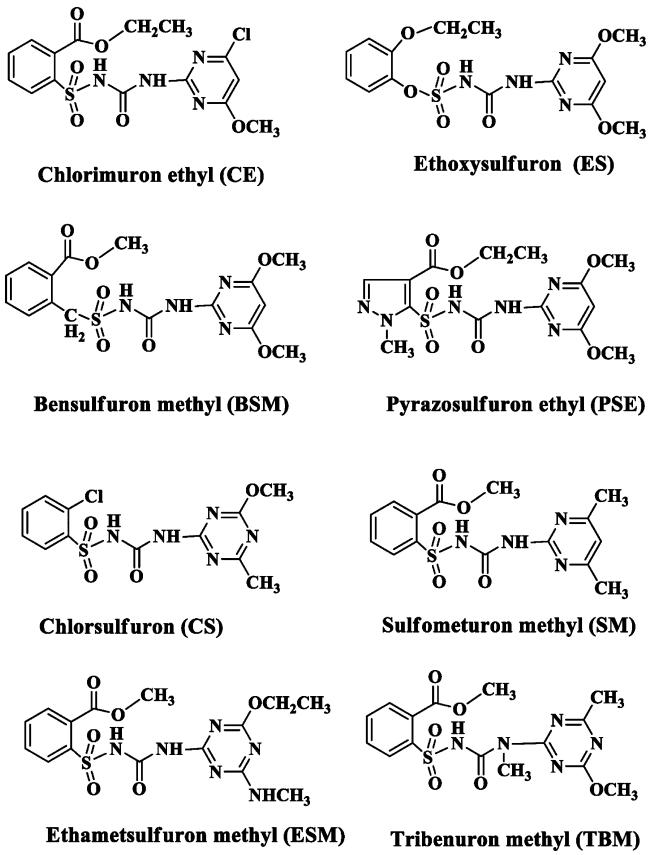

Figure 2. Chemical structures of the eight commercial sulfonylureas tested for their inhibition against fungal AHASs and for their antifungal activity in cell-based assays.

Bensulfuron methyl (BSM) is a potent inhibitor of *S. cerevisae* AHAS with a K_i value of 4 nM. However, with a K_i value of 31 nM, it does not bind as tightly to *C. albicans* AHAS (Table 1). Compared to CE, BSM has an additional methyl group between the sulfonylurea bridge and the aromatic ring. Thus, the spacing between the two rings of the sulfonylurea is longer by one atom in this molecule, an extension that appears to be responsible for the reduction in binding affinity to the *C. albicans* enzyme. A similar pattern of K_i values is observed for ethoxysulfuron (ES), which, instead of possessing a methyl group between the aromatic ring and sulfonylurea bridge, has an oxygen atom in this location. The remaining five compounds (Table 1) are good inhibitors of *C. albicans* and *S. cerevisiae* AHAS with K_i values in the ranges of 66−1367 and 32−400 nM, respectively, but none of these are as potent as CE. Thus, on the basis of these data, the CE scaffold appears to be optimal for binding to *C. albicans* AHAS.

Analysis of the Sulfonylurea Herbicide Binding Site in the Fungal AHASs. The sulfonylurea herbicide binding site is a region forming a tunnel leading to the active site of the enzyme (Figure 1b). The crystal structure of *S. cerevisiae* AHAS in complex with CE shows the general mode of binding of the sulfonylureas to the fungal enzymes. The sequence alignment (Figure 1a) shows that the amino acids that line the herbicide binding sites in *S. cerevisiae* and *C. albicans* AHAS are largely conserved. In particular, the amino acid residues that interact with the urea portion of the sulfonylurea bridge, K251 (interacting with the sulfonyl nitrogen atom), R380 (interacting with the carbonyl oxygen), W586, M354, and M582, and the residues that border these regions are preserved. In the *S. cerevisiae* AHAS−CE complex, W586 forms a π stacking arrangement with the heterocyclic ring, while the side chains of the two methionine residues flank the side chain of W586. Thus, many of the critical interactions that stabilize CE in the herbicide binding site are expected to be maintained upon the binding of this molecule to *C. albicans* AHAS.

The sequence alignment and crystal structure (Figure 1) show that there are four residues, P192, A195, A656, and G657, that can interact with the sulfonylureas, but these are not completely conserved across the fungal species. In the ascomycetes *C. albicans* and *S. cerevisiae*, P192 and A195 are identical, but in the basidiomycete *Cr. neoformans*, these two residues are alanine and leucine, respectively. The *S. cerevisiae* AHAS−CE crystal structure (Figure 1b) shows that the side chain atoms of P192 form van der Waals interactions with the aromatic ring of the sulfonylurea. These interactions may be important in allowing the sulfonylurea to adopt its customary U-shaped conformation that is observed in all of the crystal structures of these compounds with the AHAS enzymes. A substitution from proline to alanine at this site may therefore result in a steric clash between the enzyme and the inhibitor. The substitution of A195L that occurs in *Cr. neoformans* AHAS may also exert an effect on the binding of the sulfonlyureas. In *S. cerevisiae* AHAS the side chain carbon atom of alanine is only 4.0 Å away from one of the carbon atoms in the aromatic ring

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of CE and within 4.8 Å of two other such carbon atoms. Thus, substitution of the side chain to leucine could result in unfavorable steric clashes between the carbon atoms of the leucine side chain and the aromatic ring of CE. Thus, if CE were to bind to *Cr. neoformans* AHAS, its overall position in the sulfonylurea herbicide binding site would likely be perturbed compared to its position in *S. cerevisiae* AHAS.

The two other locations in the sulfonylurea herbicide binding site where differences in sequence between the fungal enzymes are observed are at positions 656 and 657. In *S. cerevisiae* these residues are alanine and glycine, while in both *C. albicans* and *Cr. neoformans* they are proline and alanine, respectively. When mutations using computer modeling are made at this site, it is apparent that side chain atoms for proline and alanine point away from the sulfonylurea binding site. Thus, these differences should have little, if any, effect on the binding affinity of the sulfonylureas to the fungal enzymes.

Ethametsulfuron methyl (ESM) is a moderate inhibitor of *S. cerevisiae* AHAS and *C. albicans* AHAS with K_i values of 656 and 346 nM, respectively. This molecule has large substituents on the heterocyclic ring, and if positioned in the herbicide binding site in the same way as CE, it is likely to make steric clashes at the bottom of this site, leading to a reduction of binding affinity.

Tribenuron methyl (TBM) is a moderate inhibitor of *C. albicans* AHAS with a K_i of 2 μM but is a potent inhibitor of *S. cerevisiae* AHAS (K_i of 400 nM). Structurally, this compound most closely resembles sulfometuron methyl (SM), but binds more weakly to both enzymes than does SM. The major difference between this compound and the other sulfonylureas in this study is the attachment of a methyl group to the sulfonylurea bridge. A crystal structure of TBM in complex with *S. cerevisiae* AHAS has been determined and shows that the methyl group can fill the site leading toward G116 (Figure 1b), but a water molecule that normally forms hydrogen bonds to the sulfonylurea bridge is not present in this structure, and there is no interaction between K251 and the nitrogen of the sulfonylurea group (also absent in the chlorsulfuron (CS) and SM complexes with *S. cerevisiae* AHAS).

Effect of the Sulfonylureas on the Growth of Fungal Cells. The eight commercial sulfonylureas were tested for their ability to inhibit the growth of *C. albicans* in cell culture and in disk diffusion assays (Table 1, Figures 2 and 3). The most active compound, CE, has an MIC_{50} of 2 μM against *C. albicans*. ES is another sulfonylurea that is an excellent inhibitor of *C. albicans* AHAS with a K_i value of 20 nM. It also exhibits good activity against *C. albicans* in cell culture with an MIC_{50} of 2 μM. These results (Table 1) show that there is a good correlation between the K_i values for the sulfonylurea against *C. albicans* AHAS and their ability to reduce the growth of *C. albicans* in cell-based assays. On the basis of these results, the CE structure was chosen as a starting point for the design and synthesis of new sulfonylurea compounds.

Second-Generation Sulfonylureas. A total of 20 sulfonylureas based on the structure of CE were designed, synthesized, and tested as inhibitors of *C. albicans* AHAS and to determine their effect on the growth of *C. albicans* in cell-based assays. Among them, **10c**,[17] **10e**,[17] **10f**,[18] and **10r**[19] were reported but have not been fully chemically characterized before. The basis for the design was to modify substituents X and R on the heterocyclic ring and aromatic rings, respectively (Figure 4) so that the new compounds would have improved shape complementarity for the herbicide binding site and

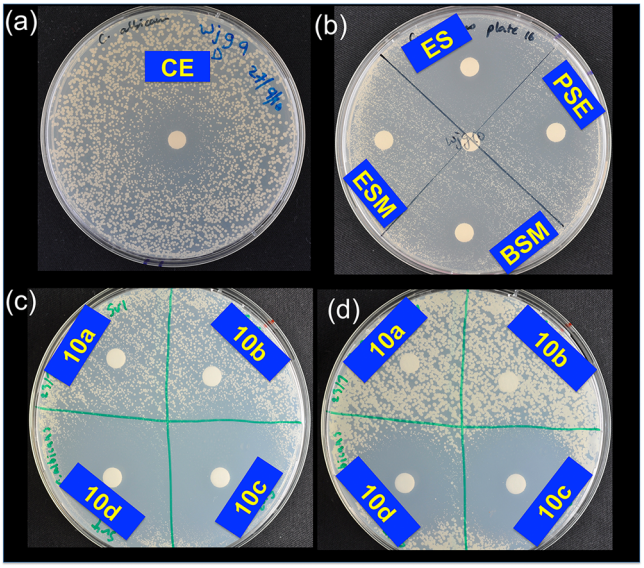

Figure 3. Disk diffusion assay images showing the zones of inhibition caused by the sulfonylureas on the growth of *C. albicans*: (a) CE after 72 h; (b) ES, PSE, ESM, and BSM after 72 h; (c, d) **10a**, **10b**, **10c**, and **10d** at 24 and 72 h. The inhibition zones for CE, ES, and **10c** were 25, 30, and 35 mm, respectively.

therefore have improved affinity. Substitutions in the X position were restricted to replacement of the chlorine atom with either a bromine or an iodine atom. It was reasoned that there would not be any additional space in this pocket to accommodate an extra atom, but increasing the size of the ionic radius compared to that of chlorine may be beneficial to binding. Site R was also modified so that a hydrogen atom on the terminal methyl group was replaced by iodine, bromine, or hydroxyl, or alternatively, this group was made shorter by one methyl group. The addition of the hydroxyl group was expected to make a favorable hydrogen bond with the side chain of Q202. Three compounds were also made that had a hydrophobic group attached to the hydroxyl to attempt to facilitate delivery of the sulfonylurea into the fungal cell. The K_i values for these 20 compounds against *C. albicans* AHAS are presented in Table 2. **10c**−**10e** and **10m** are all potent inhibitors of *C. albicans* AHAS with K_i values in the 3−4 nM range, approximately twice as potent as observed for CE, while all of the other compounds were less potent inhibitors of the enzyme. A plot of the inhibition data for **10c** is given in Figure 5. Compounds **10a**, **10b**, **10g**−**10l**, and **10n** all have a halogen attached to both the X and R groups and show reduced affinity for *C. albicans* AHAS (26−1199 nM). Thus, expansion of the inhibitor in both sites is detrimental to binding. Addition of a hydroxyl group to R slightly decreases the affinity for *C. albicans* AHAS (K_i values of 15−27 nM). Thus, if the new hydrogen bond is created, it is not strong enough to overcome the hydrophobic effect when only a methyl group is attached at this location. Nonetheless, such molecules are still potent inhibitors, thereby creating a site where a drug delivery system can be attached that could be cleaved by an endogenous esterase once it enters the fungal cell. **10n**−**10p** have a silicon-containing hydrophobic group attached through such a linkage. Not surprisingly, **10n** showed no inhibitory activity for *C. albicans* AHAS with this bulky group attached. However, **10o** and **10p**, where X is Br or I instead of Cl, are good inhibitors with K_i values of 49.8 and 13.0 nM, respectively. This suggests that as long as the

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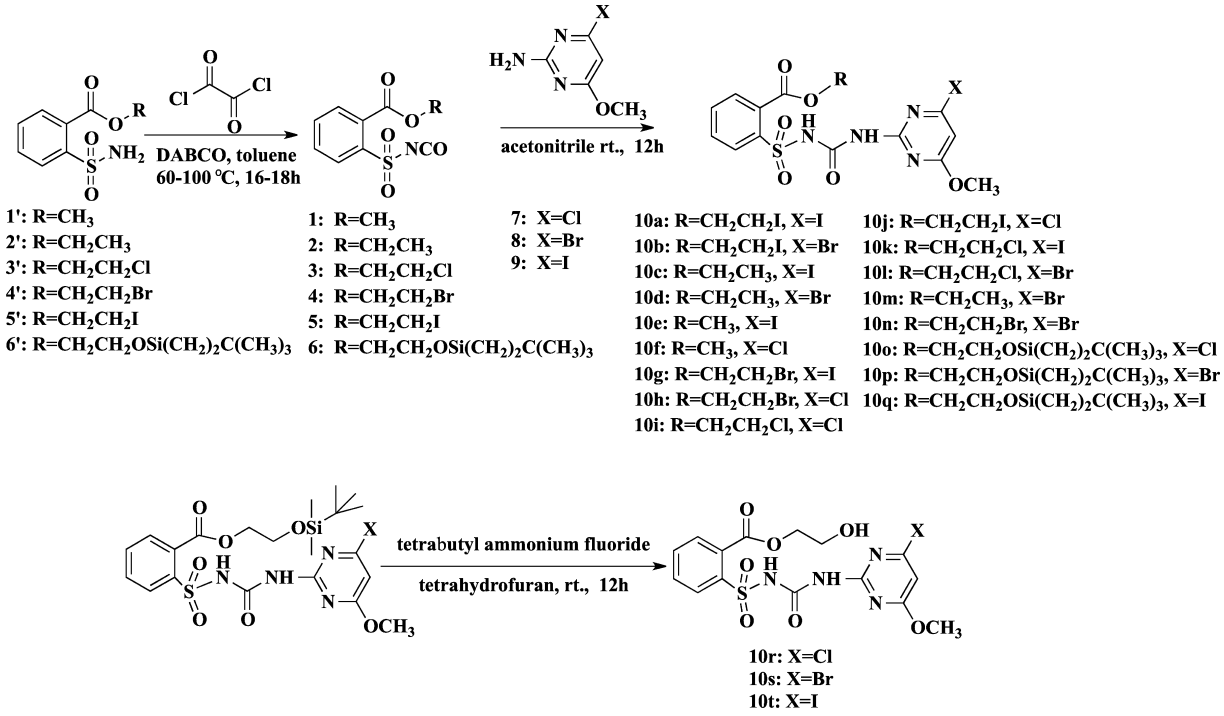

Figure 4. Scheme for the chemical synthesis of compounds **10a**−**10t**.

Table 2. Inhibition of *C. albicans* AHAS and *C. albicans* Growth in Cell Culture for the Second-Generation Sulfonylureas[a]

	K_i (nM)	MIC_{50} (μM)	MIC_{50} (μg/mL)	MIC_{90} (μM)	MIC_{90} (μg/mL)
10a	93 ± 9	12 ± 3.1	8.0	25.0	16.0
10b	270 ± 27	>25	>15.0	>25	>15.0
10c	3.8 ± 0.5	0.6 ± 0.2	0.30	1.56	0.78
10d	4.1 ± 0.6	0.7 ± 0.2	0.36	1.56	0.72
10e	3.9 ± 0.8	1 ± 0.5	0.78	3.13	1.56
10f	7.1 ± 0.6	3 ± 1.0	1.25	6.25	2.5
10g	26 ± 4.4	12 ± 3.1	7.3	>25	>14.6
10h	257 ± 25	>25	>12.3	>25	>12.3
10i	62 ± 6.2	15 ± 4.0	8.6	>25	>11.2
10j	1199 ± 112	>25	>13.5	>25	>13.5
10k	13.3 ± 1.0	4 ± 2.0	2.53	12.7	6.75
10l	±1.8	15 ± 3.3	9.22	>25	>12.3
10m	3.8 ± 0.4	1 ± 0.5	1.03	6.25	2.78
10n	65 ± 6	>25	>13.4	>25	>13.4
10o	NI	>25	>13.6	>25	>13.6
10p	49.8 ± 2.8	>25	>14.7	>25	>14.7
10q	13.0 ± 1.1	>25	>15.9	>25	>15.9
10r	65 ± 4	>25	>10.7	>25	>10.7
10s	27 ± 1.9	>25	>11.8	>25	>11.8
10t	15 ± 1.4	>25	>13	>25	>13.0

[a]The MIC_{50} values are the average of three measurements. The MIC_{50} for fluconazole was 0.93 μg/mL after 24 h. Incubation longer than 24 h leads to variability in the MIC for fluconazole.[31]

heterocyclic ring is firmly anchored it may be possible to induce conformational changes to accommodate this bulky group in the herbicide binding site in this location. Alternatively, these molecules bind to *C. albicans* AHAS in a mode that has yet to be described. All 20 of the compounds were subsequently tested for their ability to inhibit *C. albicans* growth. The most potent of these were **10c**−**10f** and **10m** with MIC_{90} values in the range of 0.72−2.0 μg/mL (Table 2). In general, compounds that exhibited K_i values much greater than ∼20 nM did not exhibit measurable inhibition of *C. albicans* in our assays, while those compounds with K_i values below 20 nM all exhibited good activity.

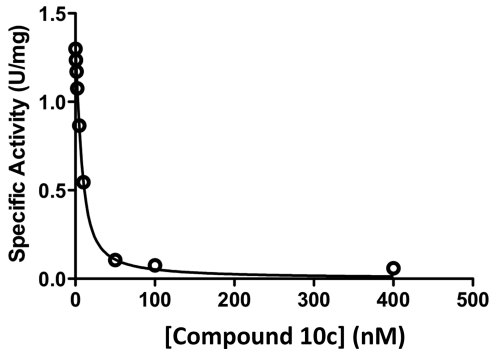

Figure 5. Inhibition of *C. albicans* AHAS by compound **10c**. Data are plotted as specific activity versus molar inhibitor concentration. The line of best fit represents the equation for tight-binding inhibition (see the Experimental Section).

Sulfonylureas as Potential Antifungal Drug Leads. CE is a potent inhibitor of *C. albicans* AHAS with a K_i value of 7 nM, but it is less effective as an inhibitor of *C. albicans* growth with an MIC_{50} value of 2 μM. There are two likely reasons for this difference, including restricted permeability through the cell membrane or hydrolysis of the compounds during the course of the assay. Difficulties in cell permeability could arise due to the fact that the nitrogen on the sulfonylurea bridge is likely to carry a charge under physiological conditions ($pK_a \approx 4.2$ for CE). The pK_a for the same atom on ES is elevated to 5.2, and this may explain why the MIC_{50} values are similar for the two inhibitors, but the K_i value for ES is about 3 times higher than that for CE when bound to *C. albicans* AHAS. To begin to overcome the problem of delivery into the cell, it may

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be necessary to design and synthesize prodrugs that have the ability to mask the charge on these molecules. Due to CE's development as a herbicide, its toxicity properties have been widely tested. The oral LD_{50} for this compound is >4000 mg/kg in rats, while the dermal LD_{50} is >2000 mg/kg,[20] which suggests that this compound does not, to any significant extent, inhibit any other key mammalian proteins. Furthermore, no evidence of neurotoxicity, mutagenicity, or carcinogenicity has been observed for CE,[21] while a 90 day feeding study of CE showed a no observable effect level of 18.75 mg/kg/day.[22]

Kingsbury and co-workers[4] showed that AHAS is required for successful infection by *Cr. neoformans* in a murine model. They also showed that SM is ineffective in reducing the growth of this fungus at concentrations as high as 550 mM. Two reasons for this lack of activity were suggested: the inhibitor cannot penetrate the fungal membrane, or SM is not a potent inhibitor of *Cr. neoformans* AHAS. Replacing the *Cr. neoformans* AHAS gene with the *S. cerevisiae* AHAS gene reduced the growth of the fungus in the presence of SM with an MIC_{80} of 17.1 mM, suggesting that SM does have minimal antifungal activity. Our studies show that SM is a very weak inhibitor of *C. albicans* AHAS but has no effect on the growth of *C. albicans* in culture. Thus, it is likely that SM would also be a weak inhibitor of *Cr. neoformans* AHAS and therefore possess only a minimal amount of anticryptococcal activity.

Resistance can be a serious problem associated with the overuse of drugs or herbicides. In the case of the commercial sulfonylureas, strong resistance has been observed to occur in weeds when these compounds are used as herbicides. The sites that are most commonly mutated are P197, A205, W574, and S653 (*A. thaliana* AHAS numbering, Figure 1). Thus, these are also potential locations where resistance could occur in the fungal enzymes in response to the use of the sulfonylureas as antifungal agents. Therefore, in the future design of antifungal sulfonylureas, care should be taken to minimize contact with these residues or alternatively to provide structural flexibility in the compound such that there is room for the sulfonylurea to adjust its conformation/location so that potent inhibition toward a mutated form of the enzyme can be maintained. Some success using this approach has been achieved with the development of the next-generation sulfonylurea herbicides monosulfuron and monosulfuron ester, which are effective herbicides against *Puccinella distans*.[23] For this weed none of the other commercial sulfonylureas are effective as herbicides.[23] The likely reason for the success of these new compounds as herbicides and the failure of the other herbicides lies in the fact that they have only one functional group attached to the heterocyclic ring, allowing them more freedom to fit in the herbicide binding sites in response to mutation. It should also be pointed out that, although sulfonylurea-resistant weed strains have been observed in the field, these herbicides have continued to be in high demand for ~30 years. Encouragingly, improved stewardship in monitoring the use of these herbicides has seen a marked decline in the number of new resistant biotypes to have emerged since 2008.[24]

CONCLUSIONS

The studies of Kingsbury and co-workers[4] have demonstrated the importance of AHAS in the life cycle of *C. albicans* and *Cr. neoformans*. This, together with the fact that the BCAA pathway is present in plants, bacteria, and fungi but not animals, makes AHAS an attractive target for the development of new antimicrobial agents. Here we have shown that CE, ES, and several second-generation sulfonylureas are inhibitors of *C. albicans* AHAS and that these compounds also possess antifungal activity. A number of sulfonylureas have been developed as herbicides, but not all of them have antifungal activity (Table 1). This suggests that there are significant differences in the three-dimensional structure between the plant and fungal enzymes at the sulfonylurea binding site. It also suggests that with rational drug design aimed specifically at the fungal enzymes more potent fungal AHAS inhibitors could be developed. Due to its low cytotoxicity and potency for AHAS, the sulfonylurea framework provides an excellent starting point for the development of new antifungal drug leads. On the basis of the sequence similarities, inhibitors of *C. albicans* AHAS are also likely to be inhibitors of other fungal AHASs.

EXPERIMENTAL SECTION

Chemical Synthesis. The starting chemical materials were purchased from Tianjin Alfa-Aesar or Shanghai Aladdin Regeants. All solvents and liquid reagents were dried by standard methods in advance and distilled before use. The synthesis procedures for the intermediates **1′−6′** and **7−9** (Figure 4) are detailed in the Supporting Information. Melting points were determined using an X-4 melting apparatus (Beijing Tech Instruments Co., Beijing, China) and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were obtained using a 400 MHz Bruker spectrometer in DMSO-d_6 or $CDCl_3$ with TMS as an internal standard. HRMS data were obtained on an FTICR-MS instrument (Ionspec 7.0T) or Agilent LC/MS instrument (6520 Q-TOF). All of the newly synthesized sulfonylureas in the study were of 95−99% purity as assessed by HPLC in an Agilent 1200 series system (ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm) eluted at 1 mL/min with CH_3OH/H_2O).

2-Iodoethyl 2-(*N*-((4-Iodo-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10a). DABCO (triethylenediamine; 0.26 g) was added to a stirred mixture of sulfonamide **5′** (8.17 g, 23 mmol) and oxalyl dichloride (10 mL, 105 mmol) in dry toluene (35 mL) at rt (room temperature). The reaction proceeded for 6 h at 60 °C and then was warmed to 90−100 °C slowly. After 10−12 h of reflux, the resulting precipitate was removed by filtration when the reaction was cooled to room temperature. After that the solution was condensed in vacuo to give the crude sulfonyl isocyanate (brown oil), a portion of which (1.07 g, 2.8 mmol) was further dissolved in dry acetonitrile (11 mL). This sulfonyl isocyanate solution was then added to a solution of pyrimidine **9** (0.70g, 2.8 mmol, in 11 mL of dry acetonitrile), and the reaction was kept at rt overnight with continuous stirring. Finally, the precipitate was filtrated and further purified by column chromatography on silica gel to give 1.5 g (88% yield) of **10a** as a white solid. Mp: 149−151 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.197 (s, 1H, SO_2NH), 10.857 (s, 1H, CONH), 8.212−7.822 (m, 4H, ArH), 7.234 (s, 1H, Prim-H), 4.534 (t, J = 6.4 Hz, 2H, OCH_2), 3.963 (s, 3H, OCH_3), 3.468 (t, J = 6.4 Hz, 2H, CH_2I). ^{13}C NMR (400 MHz, DMSO-d_6): δ 166.790, 163.936, 153.671, 146.369, 134.332, 132.374, 129.717, 129.570, 129.450, 127.765, 127.153, 110.510, 64.135, 52.869, 0.0000. HRMS (ESI, m/z): $C_{15}H_{15}I_2N_4O_6S$ ($M + H^+$) calcd 632.8796, found 632.8785.

2-Iodoethyl 2-(*N*-((4-Bromo-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10b). Yield: 0.86 g (60%), starting from 2.44 mmol of **5** and 2.44 mmol of **8**. White solid. Mp: 148−150 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.021 (s, 1H, SO_2NH), 10.929 (s, 1H, CONH), 8.219−7.830 (m, 4H, ArH), 7.045 (s, 1H, Prim-H), 4.526 (t, J = 6.4 Hz, 2H, OCH_2), 4.005 (s, 3H, OCH_3), 3.469 (t, J = 6.4 Hz, 2H, CH_2I). ^{13}C NMR (400 MHz, DMSO-d_6): δ 168.101, 164.042, 154.195, 149.457, 146.320, 134.357, 132.509, 129.793, 129.709, 129.467, 127.904, 103.515, 64.203, 53.303, 0.0000. HRMS (MALDI, m/z): $C_{15}H_{15}IBrN_4O_6S$ ($M + H^+$) calcd 584.8935, found 584.8935.

Ethyl 2-(*N*-((4-Iodo-6-methoxypyrimidin-2-yl)carbamoyl)-sulfamoyl)benzoate (10c). Yield: 1.1 g (55%), starting from 3.98 mmol of **2** and 3.98 mmol of **9**. White solid. Mp: 164−166 °C. ^{1}H

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NMR (40 MHz, DMSO-d_6): δ 12.182 (s, 1H, SO_2NH), 10.875 (s, 1H, CONH), 8.206−7.755 (m, 4H, ArH), 7.224 (s, 1H, Prim-H), 4.343 (q, J = 6.8 Hz, 2H, OCH_2), 3.978 (s, 3H, OCH_3), 1.287 (t, J = 6.8 Hz, 3H, CH_2CH_3). ^{13}C NMR (400 MHz, DMSO-d_6): δ 168.555, 166.377, 155.468, 148.165, 135.846, 134.112, 132.075, 131.326, 130.935, 129.293, 129.014, 112.318, 61.983, 54.616, 13.836. HRMS (MALDI, m/z): $C_{15}H_{16}IN_4O_6S$ (M + H^+) calcd 506.9830, found 506.9836.

Ethyl 2-(*N*-((4-Bromo-6-methoxypyrimidin-2-yl)carbamoyl)-sulfamoyl)benzoate (10d). Yield: 0.85 g (64%), starting from 2.89 mmol of **2** and 2.89 mmol of **8**. White solid. Mp: 167−169 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.996 (s, 1H, SO_2NH), 10.932 (s, 1H, CONH), 8.206−7.766 (m, 4H, ArH), 7.048 (s, 1H, Prim-H), 4.333 (q, J = 6.8 Hz, 2H, OCH_2), 4.024 (s, 3H, OCH_3), 1.290 (t, J = 6.8 Hz, 3H,CH_2CH_3). ^{13}C NMR (400 MHz, DMSO-d_6): δ 169.776, 166.408, 155.925, 151.215, 148.067, 135.847, 134.136, 132.006, 131.327, 130.984, 129.361, 105.221, 61.978, 54.976, 13.778. HRMS (MALDI, m/z): $C_{15}H_{16}BrN_4O_6S$ (M + H^+) calcd 458.9968, found 458.9963.

Methyl 2-(*N*-((4-Iodo-6-methoxypyrimidin-2-yl)carbamoyl)-sulfamoyl)benzoate (10e). Yield: 0.30 g (52%), starting from 1.19 mmol of **1** and 1.19 mmol of **9**. White solid. Mp: 150−152 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.178 (s, 1H, SO_2NH), 10.846 (s, 1H, CONH), 8.190−7.768 (m, 4H, ArH), 7.220 (s, 1H, Pyrim-H), 3.965 (s, 3H, $COOCH_3$), 3.857 (s, 3H, Pyrim-OCH_3). ^{13}C NMR (400 MHz, DMSO-d_6): δ 168.597, 166.884, 155.483, 148.126, 135.810, 134.142, 131.787, 131.400, 131.008, 129.356, 128.962, 112.299, 54.642, 53.216. HRMS (MALDI, m/z): $C_{14}H_{14}IN_4O_6S$ (M + H^+) calcd 492.9673, found 492.9665.

Methyl 2-(*N*-((4-Chloro-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10f). Yield: 0.44 g (58%), starting from 1.88 mmol of **1** and 1.88 mmol of **7**. White solid. Mp: 159−161 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.983 (s, 1H, SO_2NH), 10.914 (s, 1H, CONH), 8.200−7.760 (m, 4H, ArH), 6.895 (s, 1H, Pyrim-H), 4.023 (s, 3H, $COOCH_3$), 3.841 (s, 3H, Pyrim-OCH_3). ^{13}C NMR (400 MHz, DMSO-d_6): δ 170.507, 166.918, 159.914, 156.206, 148.041, 135.815, 134.191, 131.722, 131.384, 131.080, 129.447, 101.295, 55.091, 53.090. HRMS (MALDI, m/z): $C_{14}H_{14}ClN_4O_6S$ (M + H^+) calcd 401.0317, found 401.0311.

2-Bromoethyl 2-(*N*-((4-Iodo-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10g). Yield: 1.0 g (54%), starting from 3.17 mmol of **4** and 3.17 mmol of **9**. White solid. Mp: 165−167 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.201 (s, 1H, SO_2NH), 10.843 (s, 1H, CONH), 8.212−7.807 (m, 4H, ArH), 7.222 (s, 1H, Pyrim-H), 4.602 (t, J = 5.6 Hz, 2H, $COOCH_2$), 3.959 (s, 3H, OCH_3), 3.773 (t, J = 5.6 Hz, 2H,CH_2Br). ^{13}C NMR (400 MHz, $CDCl_3$): δ 168.545, 165.844, 155.441, 148.143, 136.118, 134.185, 131.489, 131.383, 131.187, 129.561, 129.016, 112.287, 65.328, 54.632, 30.156. HRMS (MALDI, m/z): $C_{15}H_{15}IBrN_4O_6S$ (M + H^+) calcd 584.8930, found 584.8935.

2-Bromoethyl 2-(*N*-((4-Chloro-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10h). Yield: 0.7g (56%), starting from 2.51 mmol of **4** and 2.51 mmol of **7**. White solid. Mp: 170−172 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.013 (s, 1H, SO_2NH), 10.920 (s, 1H, CONH), 8.226−7.821 (m, 4H, ArH), 6.893 (s, 1H, Pyrim-H), 4.594 (t, J = 5.6 Hz, 2H, $COOCH_2$), 4.023 (s, 3H, OCH_3), 3.775 (t, J = 5.6 Hz, 2H, CH_2Br). ^{13}C NMR (400 MHz, DMSO-d_6): δ 170.430, 165.882, 159.998, 156.179, 148.054, 136.114, 134.219, 131.510, 131.445, 131.109, 129.646, 101.278, 65.309, 55.074, 30.079. HRMS (MALDI, m/z): $C_{15}H_{15}ClBrN_4O_6S$ (M + H^+) calcd 492.9579, found 492.9573.

2-Chloroethyl 2-(*N*-((4-Chloro-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10i). Yield: 1.07 g (85%), starting from 2.80 mmol of **3** and 2.80 mmol of **7**. White solid. Mp: 167−169 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.019 (br, 1H, SO_2NH),10.927 (s, 1H, CONH), 8.240−7.832 (m, 4H, ArH), 6.870 (s, 1H, Pyrim-H), 4.545 (t, J = 5.2 Hz, 2H, $COOCH_2$), 4.028 (s, 3H, OCH_3), 3.930 (t, J = 5.2 Hz, 2H, CH_2Cl). ^{13}C NMR (400 MHz, DMSO-d_6): δ 170.410, 165.986, 160.008, 156.175, 148.057, 136.099, 134.211, 131.489, 131.417, 131.138, 129.622, 101.271, 65.579, 55.063, 42.098. HRMS (MALDI, m/z): $C_{15}H_{15}Cl_2N_4O_6S$ (M + H^+) calcd 449.0084, found 449.0082.

2-Iodoethyl 2-(*N*-((4-Chloro-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10j). Yield: 1.42 g (70%), starting from 3.75 mmol of **5** and 3.75 mmol of **7**. White solid. Mp: 148−150 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.997 (s, 1H, SO_2NH), 10.907 (s, 1H, CONH), 8.224−7.833 (m, 4H, ArH), 6.896 (s, 1H, Pyrim-H), 4.528 (t, J = 6.4 Hz, 2H, $COOCH_2$), 4.025 (s, 3H, OCH_3), 3.474 (t, J = 6.4 Hz, 2H, CH_2I). ^{13}C NMR (400 MHz, DMSO-d_6): δ 168.795, 164.081, 158.281, 154.509, 146.370, 134.411, 132.531, 129.823, 129.744, 129.483, 127.947, 99.609, 64.231, 53.410, 0.0000. HRMS (ESI, m/z): $C_{15}H_{15}ClIN_4O_6S$ (M + H^+) calcd 540.9440, found 540.9439.

2-Chloroethyl 2-(*N*-((4-Iodo-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10k). Yield: 1.06 g (70%), starting from 2.79 mmol of **3** and 2.79 mmol of **9**. White solid. Mp: 165−167 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.210 (s, 1H, SO_2NH), 10.843 (s, 1H, CONH), 8.221−7.806 (m, 4H, ArH), 7.228 (s, 1H, Pyrim-H), 4.554 (t, J = 4.2 Hz, 2H, $COOCH_2$), 3.971 (s, 3H, OCH_3), 3.936 (t, J = 4.2 Hz, 2H, CH_2Cl). ^{13}C NMR (400 MHz, DMSO-d_6): δ 168.552, 165.982, 155.465, 148.211, 136.175, 134.154, 131.456, 131.348, 131.236, 129.522, 128.975, 112.283, 65.605, 54.630, 42.163. HRMS (ESI, m/z): $C_{15}H_{15}ICl N_4O_6S$ (M + H^+) calcd 540.9440, found 540.9451.

2-Chloroethyl 2-(*N*-((4-Bromo-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10l). Yield: 0.66 g (67%), starting from 2.00 mmol of **3** and 2.00 mmol of **8**. White solid. Mp: 152−154 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.031 (s, 1H, SO_2NH), 10.933 (s, 1H, CONH), 8.221−7.804 (m, 4H, ArH), 7.042 (s, 1H, Pyrim-H), 4.535 (t, J = 5.2 Hz, 2H, $COOCH_2$), 4.006 (s, 3H, OCH_3), 3.926 (t, J = 5.2 Hz, 2H, CH_2Cl). ^{13}C NMR (400 MHz, DMSO-d_6): δ 169.770, 165.990, 155.909, 151.229, 148.051, 136.112, 134.216, 131.477, 131.416, 131.172, 129.608, 105.217, 65.597, 54.993, 42.126. HRMS (ESI, m/z): $C_{15}H_{15}BrClN_4O_6S$ (M + H^+) calcd 492.9579, found 492.9579.

Methyl 2-(*N*-((4-Bromo-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10m). Yield: 1.26 g (84%), starting from 3.28 mmol of **2** and 3.28 mmol of **8**. White solid. Mp: 141−143 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.008 (br, 1H, SO_2NH), 10.926 (s, 1H, CONH), 8.219−7.765 (m, 4H, ArH), 7.032 (s, 1H, Pyrim-H), 4.017 (s, 3H, $COOCH_3$), 3.858(s, 1H, Pyrim-OCH_3). ^{13}C NMR (400 MHz, DMSO-d_6): δ 168.703, 165.778, 154.802, 150.017, 146.890, 134.667, 133.063, 130.610, 130.274, 129.939, 128.305, 104.100, 53.884,52.003. HRMS (ESI, m/z): $C_{14}H_{14}BrN_4O_6S$ (M + H^+) calcd 444.9812, found 444.9820.

2-Bromoethyl 2-(*N*-((4-Bromo-6-methoxypyrimidin-2-yl)-carbamoyl)sulfamoyl)benzoate (10n). Yield: 0.87 g (60%), starting from 2.69 mmol of **4** and 2.69 mmol of **8**. White solid. Mp: 154−156 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.031 (s, 1H, SO_2NH), 10.899 (s, 1H, CONH), 8.226−7.817 (m, 4H, ArH), 7.026 (s, 1H, Pyrim-H), 4.602 (t, J = 5.6 Hz, 2H, $COOCH_2$), 4.008 (s, 3H, OCH_3), 3.780 (t, J = 5.6 Hz, 2H, CH_2Br). ^{13}C NMR (400 MHz, DMSO-d_6): δ 169.766, 165.894, 155.926, 151.211, 148.110, 136.191, 134.163, 131.477, 131.409, 131.130, 129.595, 105.176, 65.309, 54.984, 30.107. HRMS (ESI, m/z): $C_{15}H_{15}Br_2N_4O_6S$ (M + H^+) calcd 536.9074, found 536.9066.

2-((*tert*-Butyldimethylsilyl)oxy)ethyl 2-(*N*-((4-Chloro-6-methoxypyrimidin-2-yl)carbamoyl)sulfamoyl)benzoate (10o). Yield: 0.99 g (48%), starting from 3.80 mmol of **6** and 3.80 mmol of **7**. White solid. Mp: 154−156 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.019 (s, 1H, SO_2NH), 10.945 (s, 1H, CONH), 8.236−7.772 (m, 4H, ArH), 6.916 (s, 1H, Pyrim-H), 4.343 (t, J = 4.4 Hz, 2H, $COOCH_2$), 4.044 (s, 3H, OCH_3), 3.867 (t, J = 4.4 Hz, 2H, CH_2OSi), 0.805 (s, 9H, $C(CH_3)_3$), 0.000 (s, 6H, $Si(CH_3)_2$). ^{13}C NMR (400 MHz, DMSO-d_6): δ 170.445, 166.222, 159.991, 156.206, 148.059, 136.063, 134.127, 131.593, 131.564, 131.257, 129.464, 101.247, 66.935, 60.594, 55.052, 25.594, 17.806, −5.476. HRMS (ESI, m/z): $C_{21}H_{30}ClN_4O_7SSi$ (M + H^+) calcd 545.1288, found 545.1289.

2-((*tert*-Butyldimethylsilyl)oxy)ethyl 2-(*N*-((4-Bromo-6-methoxypyrimidin-2-yl)carbamoyl)sulfamoyl)benzoate (10p).

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Yield: 1.68 g (51%), starting from 5.59 mmol of **6** and 5.59 mmol of **8**. White solid. Mp: 147−149 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.035 (s, 1H, SO_2NH), 10.947 (s, 1H, CONH), 8.237−7.770 (m, 4H, ArH), 7.062 (s, 1H, Pyrim-H), 4.350 (t, J = 4.8 Hz, 2H, $COOCH_2$), 4.030 (s, 3H, OCH_3), 3.866 (t, J = 4.8 Hz, 2H, CH_2OSi), 0.805 (s, 9H, $C(CH_3)_3$), 0.000 (s, 6H, $Si(CH_3)_2$). ^{13}C NMR (400 MHz, DMSO-d_6): δ 169.770, 166.207, 155.923, 151.226, 148.026, 136.036, 134.136, 131.610, 131.578, 131.250, 129.455, 105.183, 66.952, 60.611, 54.973, 25.595, 17.805, −5.474. HRMS (ESI, m/z): $C_{21}H_{30}BrN_4O_7SSi$ (M + H$^+$) calcd 589.0782, found 589.0784.

2-((*tert*-Butyldimethylsilyl)oxy)ethyl 2-(*N*-((4-Iodo-6-methoxypyrimidin-2-yl)carbamoyl)sulfamoyl)benzoate (10q). Yield: 0.44 g (48%), starting from 1.32 mmol of **6** and 1.32 mmol of **9**. White solid. Mp: 130−132 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.997 (s, 1H, SO_2NH), 8.353−7.611 (m, 4H, ArH), 7.205 (s, 1H, CONH), 6.895 (s, 1H, Pyrim-H), 4.334 (t, J = 5.2 Hz, 2H, $COOCH_2$), 4.010 (s, 3H, OCH_3), 3.863 (t, J = 5.2 Hz, 2H, CH_2OSi), 0.818 (s, 9H, $C(CH_3)_3$), 0.000 (s, 6H, $Si(CH_3)_2$). HRMS (ESI, m/z): $C_{21}H_{30}IN_4O_7SSi$ (M + H$^+$) calcd 637.0644, found 637.0651.

2-Hydroxyethyl 2-(*N*-((4-Chloro-6-methoxypyrimidin-2-yl)carbamoyl)sulfamoyl)benzoate (10r). A solution of **10o** (1.65 mmol) in dry THF (15 mL) was treated with n-Bu_4NF (3 mL, 1 M in THF) at rt for 12 h. The reaction mixture was quenched with water and extracted with Et_2O (3 × 30 mL). The combined organic layers were dried over $MgSO_4$ and evaporated, after which the residue was purified by column chromatography on silica gel (hexanes/EtOAc, 2:1) to give 0.50 g (71%) of **10r** as a white solid. Mp: 154−155 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.934 (br s, 1H, SO_2NH), 10.844 (s, 1H, CONH), 8.203−7.805 (m, 4H, ArH), 6.871 (s, 1H, Pyrim-H), 5.003 (br s, 1H, CH_2OH), 4.273 (t, J = 4.8 Hz, 2H, $COOCH_2$), 4.019 (s, 3H, OCH_3), 3.659 (t, J = 4.8 Hz, 2H, CH_2OH). ^{13}C NMR (400 MHz, DMSO-d_6): δ 170.434, 166.423, 160.013, 156.204, 148.100, 136.009, 134.111, 131.809, 131.447, 131.112, 129.630, 101.289, 67.507, 58.673, 55.020. HRMS (ESI, m/z): $C_{15}H_{16}ClN_4O_7S$ (M + H$^+$) calcd 431.0422, found 431.0420.

2-Hydroxyethyl 2-(*N*-((4-Bromo-6-methoxypyrimidin-2-yl)carbamoyl)sulfamoyl)benzoate (10s). Yield: 0.32 g (62%), starting from 1.09 mmol of **10p**. White solid. Mp: 119−121 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.968 (br s, 1H, SO_2NH), 10.860 (s, 1H, CONH), 8.213−7.816 (m, 4H, ArH), 7.029 (s, 1H, Pyrim-H), 5.026 (br s, 1H, CH_2OH), 4.289 (t, J = 4.8 Hz, 2H, $COOCH_2$), 4.016 (s, 3H, OCH_3), 3.672 (t, J = 4.8 Hz, 2H, CH_2OH). HRMS (ESI, m/z): $C_{15}H_{16}BrN_4O_7S$ (M + H$^+$) calcd 474.9918, found 474.9921.

2-Hydroxyethyl 2-(*N*-((4-Iodo-6-methoxypyrimidin-2-yl)carbamoyl)sulfamoyl)benzoate (10t). Yield: 0.10 g (56%), starting from 0.34 mmol of **10q**. White solid. Mp: 134−135 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.119 (br s, 1H, SO_2NH), 10.786 (br s, 1H, CONH), 8.193−7.791 (m, 4H, ArH), 7.210 (s, 1H, Pyrim-H), 4.972 (br, 1H, CH_2OH), 4.285 (t, J = 4.2 Hz, 2H, $COOCH_2$), 3.960 (s, 3H, OCH_3), 3.654 (t, J = 4.2 Hz, 2H, CH_2OH). ^{13}C NMR (400 MHz, DMSO-d_6): δ 168.543, 166.402, 155.445, 148.177, 135.996, 134.085, 131.895, 131.426, 131.049, 129.548, 129.019, 112.320, 67.539, 58.727, 54.595. HRMS (ESI, m/z): $C_{15}H_{16}IN_4O_7S$ (M + H$^+$) calcd 522.9779, found 522.9780.

Cloning, Expression, and Purification of the Catalytic Subunit of *C. albicans* AHAS. The open reading frame for the catalytic subunit of the AHAS gene was amplified from *C. albicans* cDNA. The forward primer was 5′-ACGTCCATGGCTTTTAATACTGCTGATACATC-3′. The first 46 amino acids (containing the mitochondrial transit peptide) were removed, and an *Nco*I site was introduced at the N-terminus to facilitate cloning into the pET30a(+) vector. The reverse primer was 5′-ACGTCTCGAGCTAATATTTACCACCAGTTCTTTC-3′ and contained an *Xho*I site after the stop codon. The plasmid was transformed into *E. coli* strain Rosetta cells (to overcome the codon bias of *E. coli* leading to enhanced protein expression), and the cells were grown at 37 °C in LB medium containing 50 μg/mL kanamycin and 34 μg/mL chloramphenicol. When the OD_{600} reached 0.9, expression was induced with 0.1 mM IPTG, and cells were incubated for 4 h at room temperature (22 °C). Bio-Rad Profinity IMAC resin was used for purification following the manufacturer's instructions. Cells were harvested by centrifugation, and the cell paste was resuspended in 20 mL of 50 mM KPO_4, pH 8, 300 mM NaCl, and 5 mM imidazole per liter of culture and stored at −70 °C. All subsequent operations were performed at 4 °C, excluding light where possible. The cell paste was thawed, and 10 μM FAD was added. For lysis, 10 mg of lysozyme per gram of cells was added. This suspension was incubated on ice for 30 min and then sonicated in a Branson Sonifier 250 for 5 × 20 s at constant duty cycle and output control of 5, with 40 s rest intervals. After sonication the lysate was clarified by centrifugation and filtered through a 0.45 μm membrane. The hexahistidine-tagged catalytic subunit of AHAS was purified by immobilized nickel affinity chromatography using Bio-Rad Profinity IMAC resin. After the clarified lysate was applied, the column was washed with at least 5 volumes of 10 mM imidazole, pH 8, in buffer A (50 mM potassium phosphate, pH 8, 300 mM NaCl, and 10 μM FAD). The enzyme was then eluted with 6 volumes of 300 mM imidazole, pH 8, in buffer A. The enzyme was eluted in 2 mL fractions. A 1 mL volume of 1 M potassium phosphate, pH 8, 10 μM FAD, and 10% glycerol was added to each fraction prior to elution. Fractions containing the enzyme were pooled, concentrated using Amicon, stirred in an ultrafiltration cell, and desalted on a PD-10 column (Pharmacia) equilibrated with 0.5 mM potassium phosphate, pH 8, 10% glycerol, 10 μM FAD, and 1 mM DTT. The purified enzyme was stored in 30 μL aliquots at −70 °C.

AHAS Assay and Protein Analyses. AHAS activity was measured by the colorimetric single-point method of Singh et al.[25] in a 250 μL reaction mixture containing 250 mM potassium phosphate, pH 6.4, 50 mM pyruvate, 10 mM $MgCl_2$, 1 mM ThDP, 10 μM FAD, and enzyme. The reaction mixture was incubated at 30 °C for 30 min, and the reaction was stopped by adding 25 μL of 10% H_2SO_4 and heated at 60 °C for 15 min to convert the enzymatic product of AHAS activity, acetolactate, into acetoin. The reaction formed was quantified by incubation with 250 μL of 0.5% creatine and 250 μL of 5% α-napththol for 15 min at 60 °C, and the A_{525} was measured (ε_M = 22 700 M^{-1}·cm^{-1}). One unit of enzyme activity is defined as the production of 1 μmol of acetolactate/min. Protein concentration was measured by the bicinchoninic acid assay.[26] The kinetic constants K_m and k_{cat} were calculated by measuring the enzyme activity in a range of substrate concentrations (0.5−20 mM) and by fitting to the equation of Michaelis−Menten. The *S. cerevisiae* AHAS used in this study was expressed, purified, and assayed as described previously.[27]

Because the inhibition of AHAS by sulfonylureas is noncompetitive[27a] and therefore independent of the substrate concentration, measurement of K_i values was carried out at a single pyruvate concentration according to the standard activity assay. Initial test experiments using a wide range of inhibitor concentrations were undertaken to initially establish a suitable concentration range for testing. Subsequently, AHAS activity was measured at a series of inhibitor concentrations to cover the full range of inhibition. Typically, a total of 10−12 concentrations were used (including no inhibitor), and these were done in duplicate. The data were analyzed by nonlinear regression using eq 1 (low to medium binding inhibitors) or eq 2 (tight binding inhibitors) where ν_i is the inhibited rate, $[I]_o$ is the total inhibitor concentration, and $[E]_o$ is the concentration of enzyme.

$$\nu_i = \nu_o/(1 + [I]_o/K_i^{app}) \quad (1)$$

$$\nu_i^2[E]_o + \nu_o\nu_i([I]_o - [E]_o + K_i^{app}) - \nu_o^2K_i^{app} = 0 \quad (2)$$

Measurement of Antifungal Activity. To initially screen compounds for antifungal activity, a disk diffusion method analogous to the fluconazole susceptibility assay was used.[28] For these experiments, 10 μL of compound (sulfonylurea) dissolved in DMSO was added to the disk (Difco) and spotted onto Petri plates containing *C. albicans* or *S. cerevisiae*. The inoculated plates were incubated at 35 °C, and the diameters of the inhibition zones (IZs) were measured at 24, 48, and 72 h and compared against a DMSO control.

Antifungal susceptibility testing was performed using the broth microdilution method according to a standard procedure.[29] The compounds tested were dissolved in 100% dimethyl sulfoxide, and

dilutions (final range 0.0195−10 mM/mL for CE, CS, and SM and 0.00195−1 mM/mL for ES, BSM, PSE, ESM, and TBM) were prepared in yeast nitrogen base (YNB) medium (Difco). The YNB broth was supplemented with 0.5% glucose and 100 mM ammonium sulfate. The inoculum was prepared by picking yeast single colonies from 48 h culture plates and suspending them in 5 mL of sterile water. The turbidity of each mixed suspension was measured at 530 nm and adjusted to 1×10^6 to 5×10^6 CFU/mL using the 0.5% McFarland standard. The adjusted stock yeast suspensions were then diluted to an inoculum size of approximately 10^4 CFU/mL; 100 μL of each diluted inoculum was dispensed into the wells. The microdilution plates were incubated at 35 °C over a period of 72 h. Visual readings of the cultures were taken at 24, 48, and 72 h. $MIC_{50/90}$ values defined as the concentration that produced 50%/90% growth inhibition were then calculated. All of the assays were performed in triplicate.

Sequence Alignment. The amino acid sequences for the catalytic subunits of AHAS were obtained from NCBI and were aligned using ClustalW.[30]

■ ASSOCIATED CONTENT

Supporting Information

Synthetic steps for the production of the intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ThDP, thiamine diphosphate; FAD, flavin adenine dinucleotide; CE, chlorimuron ethyl; ES, ethoxysulfuron; BSM, bensulfuron methyl; PSE, pyrazosulfuron ethyl; CS, chlorsulfuron; SM, sulfometuron methyl; ESM, ethametsulfuron methyl; TBM, tribenuron methyl; MIC, minimum inhibitory concentration; AHAS, acetohydroxyacid synthase; ALS, acetolactate synthase; BCAA, branched-chain amino acid

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