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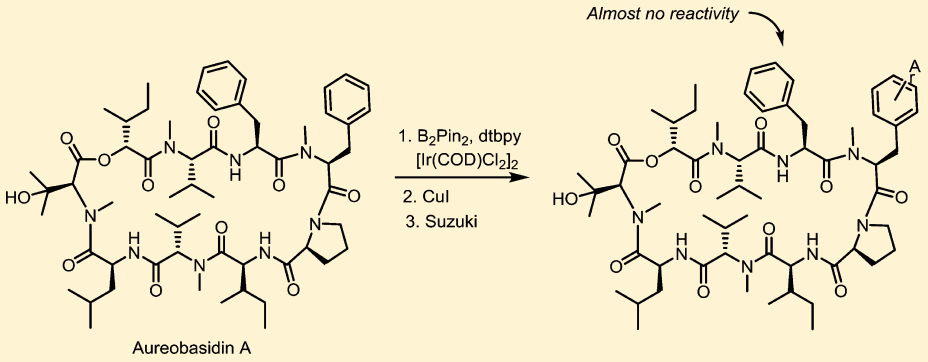
Generation of Broad-Spectrum Antifungal Drug Candidates from the Natural Product Compound Aureobasidin A

Peter G.M. Wuts,[†] Lloyd J. Simons,[†] Brian P. Metzger,[‡] Rachel C. Sterling,[‡] Jerry L. Slightom,[‡] and Ake P. Elhammer*[,‡]

[†]Kalexsyn, Inc., 4502 Campus Drive Kalamazoo, Michigan 49008, United States

[‡]AureoGen Biosciences, Inc., Southwest Michigan Innovation Center, 4717 Campus Drive, Suite 2300, Kalamazoo, Michigan 49009, United States

Supporting Information



Aureobasidin A

ABSTRACT: The natural product aureobasidin A (AbA) is a potent, well-tolerated antifungal agent with robust efficacy in animals. Although native AbA is active against a number of fungi, it has little activity against *Aspergillus fumigatus*, an important human pathogen, and attempts to improve the activity against this organism by structural modifications have to date involved chemistries too complex for continued development. This report describes novel chemistry for the modification of AbA. The key step involves functionalization of the phenylalanine residues in the compound by iridium-catalyzed borylation. This is followed by displacement of the pinacol boron moiety to form the corresponding bromide or iodide and substitution by Suzuki biaryl coupling. The approach allows for synthesis of a truly wide range of derivatives and has produced compounds with *A. fumigatus* minimal inhibitory concentrations (MIC) of <0.5 μg/mL. The approach is readily adaptable to large-scale synthesis and industrial production.

KEYWORDS: *SAR, antifungal, aureobasidin A, C−H borylation*

An increasing number of cancer, transplantation, abdominal surgery, and other immuno-compromised patients that need treatment for fungal infections together with a drug inventory limited to only three classes of therapeutics, all with significant limitations, has created an urgent need for new and better antifungal drugs (e.g., refs 1−3).

The *Aureobasidium pullulans* strain BP-1938 produces the cyclic depsipeptide, aureobasidin A (AbA; Scheme 1). This compound is a potent, fungicidal drug that is well tolerated in animals.[4] AbA has a mode of action (MoA) that is distinct from all currently used therapeutics. It is a specific, time dependent, inhibitor ($K_i \sim 0.2$ nM) of inositol phosphorylceramide (IPC) synthase, an enzyme in the fungal sphingolipid biosynthesis pathway.[5,6] IPC synthase is essential in fungi, and attempts to develop spontaneous resistance mutants have to date been unsuccessful, suggesting that development of resistance to AbA, in clinical settings, will be slow.[7] Unfortunately, although native AbA is quite active against a number of fungi, including several clinically important pathogens such as *Candida spp.* and *Crytococcus neoformans*, it shows little activity against *Aspergillus fumigatus*, another important pathogen[4] that has an efflux pump(s) capable of efficiently clearing the drug.[4,8] However, since broad-spectrum antibiotics are preferred in the clinic, this lack of (*A. fumigatus*) activity has to date prevented the development of AbA into a marketable drug[9] and a derivative capable of avoiding or blocking the pump(s) would have significantly improved development potential.

Published structure activity relationship (SAR) studies have demonstrated that AbA's pharmacological properties, including the activity against *A. fumigatus*, can be altered by modifying and/or exchanging amino acids in the sequence (reviewed in ref 9). Substitution of an *N*-methyl-D-Ala, or a sarcosine, for the *N*-methyl-L-Phe residue at position #4 (in the molecule), results in a compound with significantly improved activity, and

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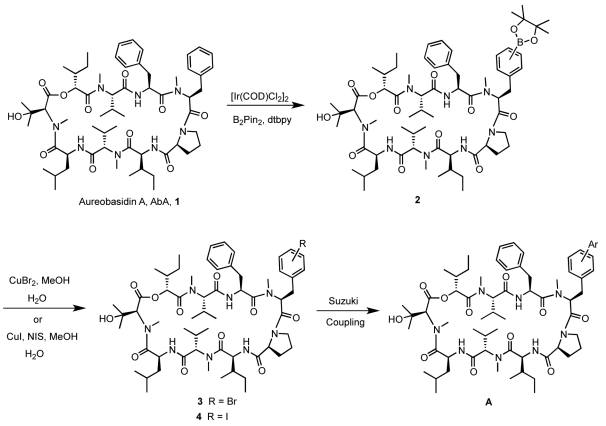
Scheme 1



combining this substitution with modifications to the side chain of the L-Phe residue in position #3 produces compounds with *A. fumigatus* MIC values in the low single digit microgram/mL range.[9] Importantly, these derivatives retain their potent activities against other fungi. Nonetheless, the complexity and low (<1%) overall yield of the 21 step synthesis used to produce these derivatives have to date represented a considerable barrier against continued development. Several groups have tried to develop more tractable approaches,[9] but none has (to date) produced a chemistry compatible with development. This report describes an approach to produce AbA derivatives with *A. fumigatus* activity that involves no more than three synthesis steps and that currently has overall yields (without any directed effort for improvement) in the 50−70% range.

In a recent report Meyer et al.[10] described the specific functionalization of aromatic side chains, such as the Phe residues in AbA by iridium catalyzed borylation. This suggested the possibility of preparing AbA derivatives with improved *A. fumigatus* activity with just two or three synthetic steps. Nonetheless, at the initiation of the research reported here, it was not known whether this chemistry could be used on a large and complex molecule such as AbA, without modifying other parts of it. It was also not known if one, and if so which, or if both of the phenylalanine side chains in AbA would be modified. X-ray crystallographic data suggested that most of the polar functionalities in the AbA structure are buried in the central core of the molecule and that the side chain on mPhe[4] may be more accessible than that on Phe[3].[9,11]

Initial results clearly indicated that the boronate chemistry outlined in Scheme 1 can be applied to the Phe residues in AbA without modifying other parts of the molecule. Silica gel chromatography yielded one well-defined spot, and LC-MS analysis showed that the majority of the reaction products had the mass of native AbA conjugated to one boronate moiety, indicating that one of the Phe residues may be more accessible than the other and that derivatives with both phenyl side chains substituted were formed only to a minor extent (data not shown).

To identify the amino acid(s) functionalized by the borylation reaction, the bromide derivative **3** (prepared from **2;** Scheme 1) was hydrolyzed in 12 M hydrochloric acid/TFA (Supporting Information, Scheme S2). The resulting amino acids were converted to the corresponding acetamides, separated on HPLC, and identified by MS and NMR (Supporting Information Figure S1, panels A and B). To allow positive identification of all bromated species in the hydrolysate, authentic standards **8** and **9** were prepared, characterized (by HPLC-MS and NMR), and cochromatographed with the hydrolysis products (Scheme S2). This allowed both identification of the substituted amino acids and determination of the regiochemistry on the substituted phenyl groups. Ninety percent of the brominated amino acids in the hydrolysate were recovered as the brominated *N*-methyl-Phe isomers **6** and **7** (Figure S1, panel A). Since the phenylalanine residue at position 3 in AbA is not *N*-methylated, this suggests, consistent with the crystallography data discussed above, that primarily the *N*-methyl-Phe residue at position 4 was brominated. The vast majority of the remaining 10% of the brominated amino acids was phenylalanine, indicating that a smaller portion of the compound was instead borylated on Phe[3]. As discussed above, LC-MS analysis of the intact brominated compound suggests <1% of dibrominated AbA (likely modified on both mPhe[4] and Phe[3]). The analysis also revealed that bromine atoms linked to the phenyl group of both amino acids were located in the meta and para positions of the phenyl ring, in a 2-to-1 ratio, suggesting essentially random borylation of these two positions, under the conditions used. Consistent with a sterically driven process, no substitution at the ortho position was found (Figure S1, panels A and B). MS-MS analysis of the intact brominated compound produced three diagnostic fragment ions confirming the preferential borylation of mPhe[4] (Data not shown). The preferential borylation of the mPhe[4] residue has been a consistent and repeatable result in several experiments, suggesting that the conformation of AbA, in the solvent (hexane/MTBE) used for the borylation reaction, is similar to that adopted upon crystallization and that the reaction is quite specific for mPhe[4].

The boronate **2** could be hydrolyzed to the boronic acid **10** without affecting the rest of the molecule. More importantly, however, following substitution of the pinacol boron moiety with bromine or iodine, the resulting halogenated derivatives **3** and **4** can be used as a starting material for the addition of a wide range of larger, more complex structures (biaryls A) to the mPhe[4] side chain, using the Suzuki biaryl coupling.[12] A large number of the precursor boronic acids required for this type of substitution are available commercially. This allows for extensive SAR work, which was not possible with previous chemistries. The use of the boronate **2** in the Suzuki coupling does give the expected products but since, in general, the boronates are used in excess it was more efficient to convert the boronate **2** to the bromide **3** or iodide **4** and use this in the coupling reaction.

The meta and para isomers could not be separated by silica gel chromatography and were thus used as a mixture in (most of) the biological evaluation of the compounds. Moreover, evaluation of derivatives synthesized to date suggests that the isomeric mixture likely will have sufficient activity to allow development of a clinically useful drug. Currently ongoing development efforts are pursued according to this strategy. However, should a derivative be identified for which separation of the two isomers is considered necessary, or advantageous, this could be accomplished by simulated moving bed (SMB) chromatography. SMB chromatography is readily scaleable.

Additional experiments showed that Br-mPhe[4]-AbA **3** can also be used for selective modification of the Phe[3] residue in AbA. RuO_4 oxidation[13] selectively oxidizes the more electron rich Phe[3] group of Br-mPhe[4]-AbA **3** to give **11** (Scheme 2). Notably, while Phe[3] was converted to an aspartic acid, the Br-

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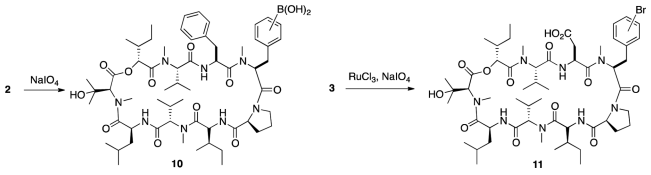
Scheme 2



mPhe[4] residue as well as the rest of the AbA molecule remained intact. The correct structure of the Asp[3]-Br-mPhe[4]- AbA compound was verified with MS and MS-MS analysis (data not shown). The use of a halogenated AbA derivative as starting material for the generation of novel compounds **A** (Scheme 1) was verified and to some extent explored by the synthesis of compounds **12** through **30** in Table 1. The coupling with halides **3** or **4** was found to proceed quite efficiently with no side reactions, but using I-mPhe[4]-AbA **4** instead of Br-mPhe[4]-AbA **3** in the biaryl coupling gave improved yields of biaryls A.

Table 1. *In Vitro* Activities of AbA Derivatives Synthesized with the Borylation Chemistry

		MIC (μg/mL)	
Cmpd	Modification	*C. albicans*	*A. fumigatus*
1	AbA (native)	<0.05	>25
2	boronate-mPhe4	<0.5	10
3	Br-mPhe[4]	0.031	2.5
4	I-mPhe[4]	2.5	>5
10	B(OH)2-mPhe4	<0.05	>100
11	Asp[3]-Br-mPhe[4]	5	10
12	4-MeO-Phe-mPhe[4]	5	ND
13	Br-Phe[3]-Br-mPhe[4]	5	>100
14	Asp[3]-mAsp[4]	10	>100
15	phenyl-mPhe[4]	<0.05	<0.94
16	5-methyl-furan-1-yl-mPhe[4]	ND	>5
17	4-*N*-methylamino carboxyphenyl-mPhe[4]	ND	>5
18	5-methylthiophen-1-yl-mPhe[4]	ND	>5
19	5-pyrimid-1-yl-mPhe[4]	ND	>5
20	3-pyrid-1-yl-mPhe[4]	<0.05	<0.94
21	1-cyclohexenyl-mPhe[4]	ND	>5
22	4-acetamidophenyl-mPhe[4]	ND	>5
23	3-Cl-phenyl-mPhe[4]	ND	>5
24	1-octene-1-yl-mPhe[4]	ND	>5
25	2-Cl-phenyl-mPhe[4]	<0.5	<1.25
26	4-Cl-phenyl-mPhe[4]	<0.5	<2.5
27	4-pyridyl-mPhe[4]	<0.025	<1.25
28	3-biphenyl-mPhe[4]	<5	>5
29	4-biphenyl-mPhe[4]	<5	>5
30	2-chloropyridin-5-yl-mPhe[4]	<0.025	<1.25

[a]ND, not determined.

The impact of the modifications discussed above, on the antifungal activity of the respective AbA derivatives, was investigated by determining their antifungal activities against *C. albicans* and *A. fumigatus* (Table 1). While the SAR information summarized in ref 9 suggest that both Phe residues in AbA should be modified to gain activity against *Aspergillus spp*., Table 1 shows that modification of mPhe[4] alone provides an alternative approach to accomplish this. It is notable that even the boronate-mPhe[4]-AbA (Cmpd **2**) and Br-mPhe[4] -AbA (Cmpd **3**) synthetic intermediates exhibited significantly improved *A. fumigatus* activity, while retaining robust activity against *C. albicans* and that the improved activity of these compounds was achieved with a completely unmodified Phe[3] residue and a retained L configuration at mPhe[4]. Clearly, AbA derivatives with considerable *Aspergillus spp.* activity can be generated by modifying the mPhe[4] residue only.

Table 1 also shows that comparatively large functionalities can be added to the mPhe[4] phenyl group without any deleterious impact on the antifungal activity. Compounds **2**, **3**, **10**, **15**, **20**, **25**, **26**, **27**, and **30** all retain *C. albicans* MIC values very close to that of native AbA. By contrast, other modifications, in particular the addition of polar moieties, produced significant increases in *C. albicans* MIC values. Nonetheless, several of the derivatives synthesized to date have gained significant activity against *A. fumigatus*. Compounds **15**, **20**, **25**, **26**, **27**, and **30** are of particular interest because they all retain essentially unaltered activities against *C. albicans* (MIC values in the sub 50 ng/mL range) while their *A. fumigatus* MICs have dropped more than an order of magnitude (Table 1). The SAR data reported in ref 9 suggest that adding a hydrophilic (−OH) functionality, to the phenyl ring or to the β-carbon, of the mPhe[4] residue on AbA has no impact on the MIC against *A. fumigatus*, while the MIC against *C. albicans* remains unchanged (phenyl ring) or increases by about an order of magnitude (β-carbon). A possible explanation for the improved activity of **2** could be that the pinacol portion of the boronate masks the underlying hydrophilic components of the compound. It is notable that the SAR reported in ref 9 indicates that additions of fluorine to the mPhe[4] phenol ring, in combination with the same addition to Phe[3], do not impact the activity against *C. albicans*, or improve the activity against *A. fumigatus*. This suggests that halogenation of Phe[3] may counteract the improved *A. fumigatus* MIC, provided by halogenation of mPhe[4], and is consistent with the observation that compound **13** (which is brominated at both Phe[3] and mPhe[4]) has less potency than the wild type compound against both organisms. Still, there is a considerable difference in mass and size between fluorine and bromine and a determination of the MIC values for F-mPhe[4]-AbA (yet to be made) must be considered essential before any firm conclusions can be made.

The poor performance of compounds **11** and **14**, which both contain an Asp residue at position 3, is consistent with previous observations[9] that the addition of a hydrophilic functionality at this location increases the *C. albicans* MIC to >5 μg/mL. The free acid group on the side chain of the mAsp[4] residue of **14** further increases the deleterious impact on the antifungal activity. The significantly increased polarity of the side group at position 4 (and/or 3) may influence the compound's capacity to partition through the cell wall and/or membrane—alternatively it may directly impact the interaction with the target enzyme. Both substrates for IPC synthase are lipids, and the enzyme is an integral membrane protein and as such is located in a very hydrophobic environment.[14] It is notable that the methoxy-biphenyl compound (**12**) lost a considerable amount of activity. The added bulkier side chain on mPhe[4] may impact the compound's interaction with the target enzyme, rather than (or more than) the *A. fumigatus* drug pump(s). In addition, although the side chain extension on this compound is largely hydrophobic, the methoxy group does add significant hydrogen bonding capacity to the structure

Considering that the overall objective of the SAR studies reported here is to identify an AbA derivative with improved *A. fumigatus* activity, most of compounds **16** to **30** were evaluated against this organism first and if insufficient improvement was

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found, the compound was not evaluated further. To date, compounds **15** and **20** clearly have the lowest *A. fumigatus* MICs. And as can be seen in Table 1, the activities of both compounds against *C. albicans* are very similar to that of the wild type compound. Nonetheless, of the two, compound **20** appears to provide a more complete eradication of the pathogen cells (in the assays). Consequently, this compound was (tentatively) chosen for a more detailed evaluation against a panel of fungal pathogens. The results, shown in Tables 2 and 3, are in good agreement with the initial evaluation. Compound **20**'s MICs against both evaluated *A. fumigatus* strains are 1−2 μg/mL, i.e. only about one dilution step higher than against the reference strain used in Table 1.

Table 2. *In Vitro* Activity of Compound 15 against Filamentous Fungi

Organism	ATCC #	MIC (μg/mL)	
		Cmpd 20	Am B[a]
Aspergillus fumigatus	20435	1	0.25
Aspergillus fumigatus	MYA-32626	2	0.25
Aspergillus flavus	204304	1	0.25
Aspergillus flavus	22546	2	0.25
Aspergillus flavus	64025	1	0.5
Aspergillus candidus	13686	0.008	0.03
Aspergillus clavatus	10058	0.03	0.004
Aspergillus niger	16888	0.125	0.03
Aspergillus niger	64028	0.03	0.015
Aspergillus ochraceus	96919	0.25	0.25
Emericella nidulans	96921	0.125	1
Fusarium oxysporum	48112	>64	1
Rhizopus oryzae	11886	>64	0.03
Sporothrix schenkii	14284	>64	0.5
Trichophyton mentagrophytes	MYA-4439	0.5	0.06
Trichophyton mentagrophytes	28185	1	0.06
Trichophyton rubrum	MYA-4438	2	0.03

[a]Am B, amphothericin B.

Table 3. *In Vitro* Activity of Compound 20 against Yeasts

Organism	ATCC #	MIC (μg/mL)	
		Cmpd **20**	Am B
Candida albicans	90028	0.015	0.03
Candida albicans	90029	0.5	0.03
Candida albicans	10231	1	0.03
Candida albicans	204276	0.03	0.06
Candida albicans	MYA-2732	0.03	0.06
Candida albicans	24433	0.125	0.03
Candida guilliermondii	34134	0.03	0.008
Candida krusei	14243	0.06	0.125
Candida lusitaniae	66035	0.125	0.015
Candida parapsilosis	90018	0.03	0.06
Candida parapsilosis	22019	0.06	0.125
Candida glabrata	90030	0.03	0.03
Candida tropicalis	750	0.03	0.06
Candida tropicalis	90874	0.015	0.06
Cryptococcus neoformans	901142	0.03	0.015
Issatchenkia orientalis	6258	0.06	0.125
Saccharomyces cerevisiae	7754	0.03	0.06

[a]Am B, amphothericin B.

Tables 2 and 3 also show that compound **20** has improved activity, against all other evaluated aspergilli, as well as against *Crytococcus neoformans* (>10×) and *Trichophyton spp.*,[4] while the activity against a range of other fungal pathogens remains similar to that of native AbA.[4] In addition, preliminary *in vivo* data generated with compound **20** suggest a significantly improved plasma half-life, as compared to native AbA.

Since the MIC values of the AbA derivatives described in this report all were obtained using a mixture of the two isomers produced by the borylation reaction, it was of interest to investigate whether individual isomers could differ in their antifungal activities. Thus, MIC values were determined for the purified para and meta isomers of **20** (separated by reverse-phase HPLC; data not shown). This revealed that while the *A. fumigatus* MIC for the meta isomer was approximately 0.6 μg/mL, the corresponding value for the para isomer was >2.5 μg/mL. Both isomers were equally active against *C. albicans*. As discussed above, (the degree of) interaction with pumps in *A. fumigatus* likely is a major contributor to the MIC value against this organism. Analysis of the properties of derivatives synthesized to date suggests that, to acquire significant *A. fumigatus* activity, substituents added to mPhe[4] should overall be hydrophobic, with no or few polar functionalities. This is consistent both with the general properties of the derivatives discussed in ref 9 and with published observations on the apparent substrate specificities of ABC transporters (e.g., refs 15−17) which suggest that the bulk (size) of added substituents (to a compound) can significantly impact its interaction with (ABC type) pump(s).[18] Native AbA is a substrate for MDR pumps, as well as other ABC transporters,[9,19,20] and it is notable, that a computational analysis of AbA's interactions with Pgp-type transporters has revealed that mPhe[4] is a key contributor in this regard.[21] In addition, experimental work has shown that modifications of residues 3 and 4, alone, can convert AbA from a (human) MDR substrate to a nonsubstrate.[22] However, since the specificities of drug pumps, both in *A. fumigatus* and in general, are only partly understood, the exact reason for the difference (in pump interaction) between the meta and para isomers of **20** is currently not clear. Additional SAR data and/or modeling studies may help clarify this issue.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, compound synthesis procedures, and (MIC) assay protocols. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00029.

■ AUTHOR INFORMATION

Corresponding Author

* Phone: +1 (269) 353-3805. Fax: +1 (269) 585-6083. Email: ake.p.elhammer@aureogen.com.

Notes

The authors declare no competing financial interest.

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