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Synthesis of analogs of the Gwt1 inhibitor Manogepix (APX001A) and *in vitro* evaluation against *Cryptococcus* spp

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Abstract: Fosmanogepix (APX001) is a first-in-class prodrug molecule that is currently in Phase 2 clinical trials for invasive fungal infections. The active moiety manogepix (APX001A) inhibits the novel fungal protein Gwt1. Gwt1 catalyzes an early step in the GPI anchor biosynthesis pathway. Here we describe the synthesis and evaluation of 292 new and 24 previously described analogs that were synthesized using a series of advanced intermediates to allow for rapid analoging. Several compounds demonstrated significantly (8- to 32-fold) improved antifungal activity against both *Cryptococcus neoformans* and *C. gattii* as compared to manogepix. Further *in vitro* characterization identified three analogs with a similar preliminary safety and *in vitro* profile to manogepix and superior activity against *Cryptococcus* spp.

Keywords: APX001, APX001A, Gwt1, GPI anchor biosynthesis, *Cryptococcus*, antifungal, fosmanogepix, manogepix, MGX

Cryptococcus neoformans is a major cause of illness in people living with HIV/AIDS and it is uniformly fatal unless treated. Widespread availability of Antiretroviral Therapy (ART) reduced the estimated cases of cryptococcal meningitis (CM) to 200,000-300,000 cases per year, but mortality is still >50% when inferior therapeutic regimens are used.[1] A recent report from Botswana illustrated that the incidence of CM has continued to be high and stable for the last 5 years despite ART use.[2] Even in resource-available regions such as North America and Europe, CM still occurs with a mortality rate in some risk groups of up to 30%.[3, 4] The morbidity and long-term health care costs (5 years post-infection) remain substantial.[5] Thus there is an urgent need for new treatment options for CM.

In August 2019, the FDA added CM to its priority review voucher program to encourage the development of new treatment options in this area of unmet medical need, and a few companies have entered this development space. Viamet Pharmaceuticals received Fast Track designation

for VT-1129 for the treatment of CM. This orally available inhibitor of fungal CYP51 additionally received orphan drug designation for the treatment of CM and has been designated a Qualified Infectious Disease Product (QIDP) by the FDA. Similarly, Amplyx Pharmaceuticals has received Orphan drug and QIDP designations for fosmanogepix (APX001), a first-in class broad spectrum antifungal agent. The active moiety of fosmanogepix inhibits the fungal enzyme Gwt1.

The Gwt1 enzyme catalyzes an early step in the glycosylphosphatidylinositol (GPI)-anchor biosynthesis pathway and was explored by Eisai as an antifungal target[6]. It has been shown that inhibition of Gwt1 prevents the appropriate localization of fungal cell wall mannoproteins, thus compromising cell wall integrity, germ tube formation, biofilm formation and fungal growth.[7, 8] Multiple series of Gwt1 inhibitors have been optimized through extensive medicinal chemistry efforts at Eisai and other groups.[9-12] E1210 [now manogepix (MGX), APX001A] is one of the most potent inhibitors and the phosphonooxymethyl prodrug (fosmanogepix, APX001, previously E1211) is currently in clinical development for the treatment of invasive fungal infections. While optimization efforts at Eisai focused on improving the activity against *Candida albicans* and *Aspergillus fumigatus*, little was known about the SAR of these compounds against *Cryptococcus* spp.[13]

In this study we synthesized a number of analogs of manogepix and evaluated their activity against two species of *Cryptococcus*. Compounds with minimum inhibitory concentration (MIC) values less than or equal to 0.016 µg/mL against both *C. neoformans* and *C. gattii* were assessed for stability in human liver microsomes (HLM) and cytotoxicity using a HepG2 assay. The best performing compounds were then evaluated in a Gwt1 overexpression assay to confirm the mechanism of action (MoA). The final selection criterion was lack of activity against PigW[7, 14] (the closest human homolog of Gwt1) to decrease the risk of mechanism-based toxicity in humans.

Data on Gwt1 inhibitors published by Eisai suggest that compounds with potent antifungal activity against *Aspergillus* and *Candida* are composed of four structural elements (**A** to **D**), which are interconnected as shown in **Figure 1**.[11]

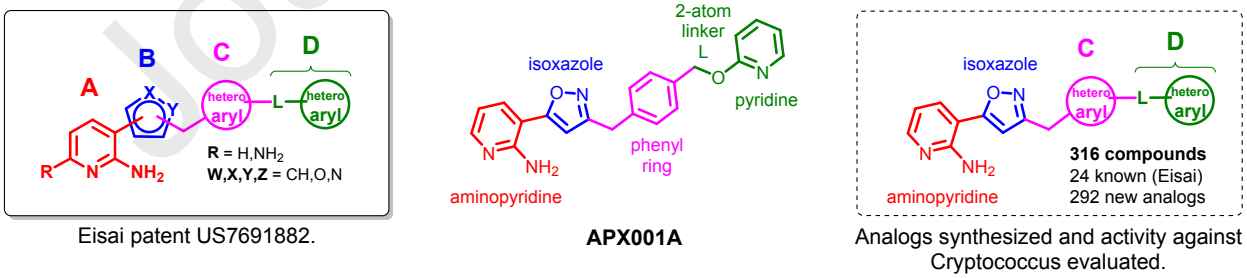
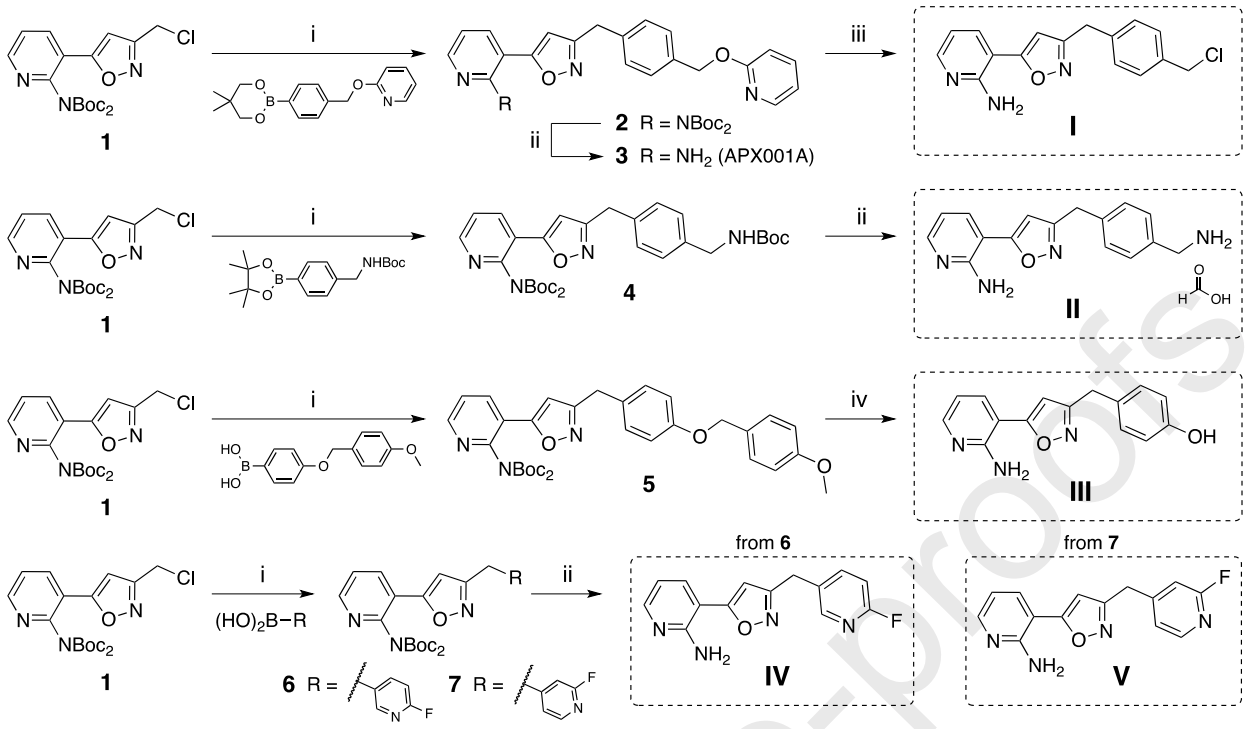


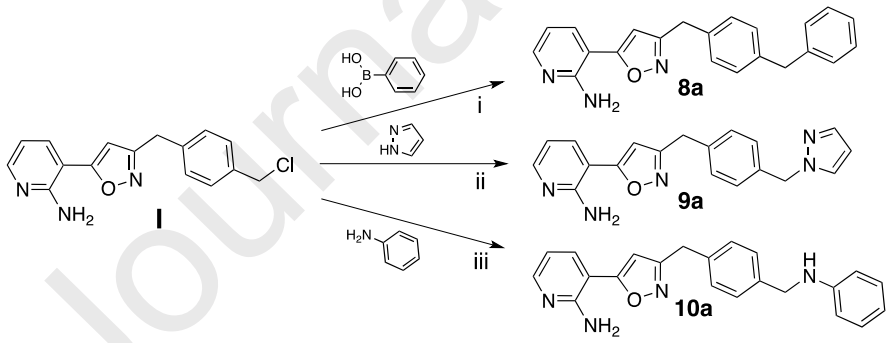
Figure 1. Structural elements of Gwt1 inhibitors and rationale for designing novel analogs.

Within the described compounds, most potent analogs have an aminopyridine or diaminopyridine head group **A** directly connected to a 5-membered aromatic heterocycle **B**, preferentially an isoxazole. This two-ring system is connected *via* a -CH_2-group to a 6-membered (hetero-)aryl ring **C**, preferably a phenyl ring, which is substituted at the *meta*- or *para*-position with group **D**. Group **D** consists of a one or two atom linker attached to a 5- or 6-membered (hetero-)aryl ring. A large variety of different linkers and aryl groups are tolerated at position **D** while maintaining potent antifungal activity against *Aspergillus* and *Candida*.

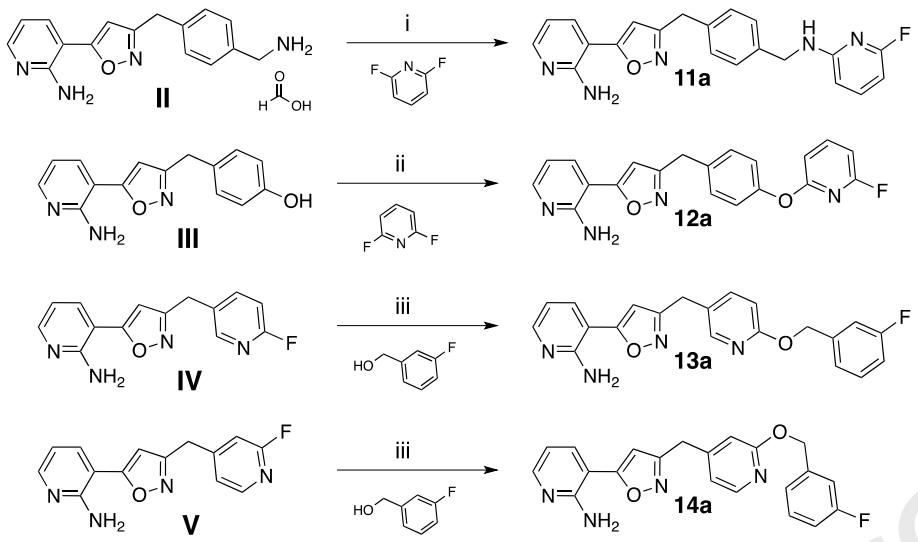
In order to establish SAR for manogepix based inhibitors of Gwt1 against *Cryptococcus*, we synthesized a collection of analogs of manogepix and evaluated their activity against *C. neoformans* and *C. gattii*. Based on the analysis above, we opted to hold constant the aminopyridine as head group **A** and the isoxazole as heterocycle **B** and focused on modifying groups **C** and **D**. Eisai has published several patents outlining the syntheses of manogepix related compounds.[11, 15] While the synthetic routes were highly convergent, they did not allow for rapid exploration of group **D** analogs for various **A**-**B**-**C**-ring scaffolds. Therefore, we adopted Eisai's procedures for the syntheses of the advanced intermediates **I** to **V**. From these intermediates, final compounds were accessible in only one synthetic step. The synthesis of the intermediates is outlined in **Scheme 1**. Eisai introduced the Boc-protected chloromethylisoxazole **1**[15], which was used in a Suzuki coupling reaction in the synthesis of manogepix (previously known as E1210), compound **3**. We observed that the hydroxypyridyl group in compound **3** (manogepix) would substitute quantitatively with chloride when refluxed in dioxane in presence of concentrated HCl. Thus, we used this method for the synthesis of the advanced intermediate **I** after following Eisai's procedure for the synthesis of compound **3**. The Boc-protected chloromethylisoxazole **1** also reacted smoothly with a variety of other boronic acids and boronic esters. Direct Boc-deprotection of the coupling products **4**, **6** and **7** in formic acid gave access to the intermediates **II**, **IV** and **V**. The global deprotection of coupling product **5** was achieved by using 4 M aq. HCl in dioxane to form advanced intermediate **III**.



Scheme 1. Synthesis of advanced intermediates **I** to **V**. *Reagents and conditions:* (i) 2 M sodium carbonate in water, palladium(0)tetrakis(triphenylphosphine), dimethoxyethane, 90 °C; (ii) formic acid, 21-25 °C; (iii) 12 M aq. HCl, dioxane, reflux; (iv) 4 M aq. HCl, dioxane, 50 °C.

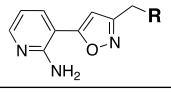
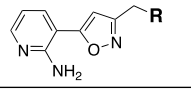
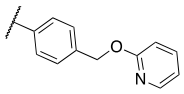
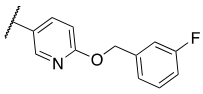
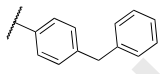
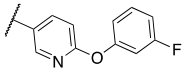
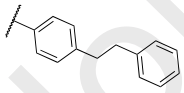
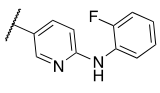
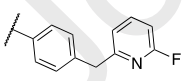
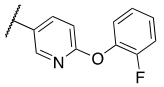
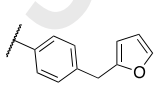
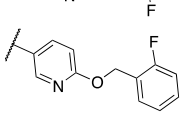
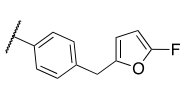
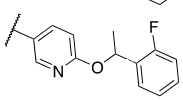
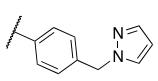
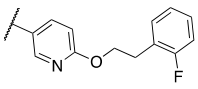


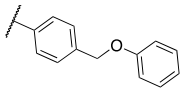
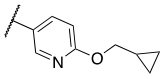
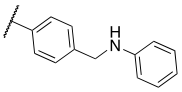
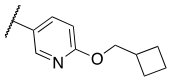
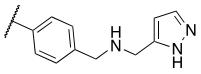
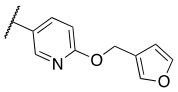
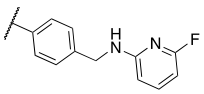
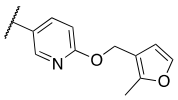
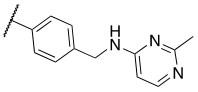
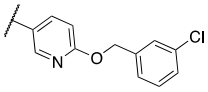
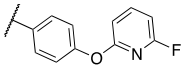
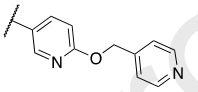
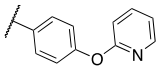
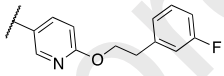
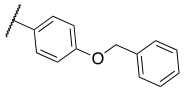
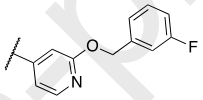
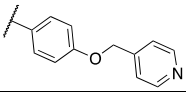
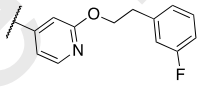
Scheme 2. Representative examples for the synthesis of analogs from intermediate **I**. *Reagents and conditions:* (i) 2 M sodium carbonate in water, palladium(0)tetrakis(triphenylphosphine), dimethoxyethane, 90 °C, analogous procedures for **8b-8e**; (ii) sodium hydride (60% in mineral oil), NMP, 21-25 °C then 50 °C, analogous procedure for **9b**; (iii) DMF, 90 °C, analogous procedure for **10b**.



Scheme 3. Representative examples for the synthesis of analogs from intermediates **II** to **V**. *Reagents and conditions:* (i) N-ethyl-N-isopropylpropan-2-amine, DMSO, 120 °C, analogous procedure for **11b**; (ii) potassium *tert*-butoxide (1M in THF), DMF, 21-25 °C then 90 °C, analogous procedures for **12b-12d**; (iii) potassium *tert*-butoxide (1 M in THF), analogous procedures for **13b-13n** and **14b**.

Table 1. Antifungal activity of compounds targeting Gwt1 against *Cryptococcus* spp.

Compd[a]		MIC (μg/mL)[b]		Compd[a]		MIC (μg/mL)[b]	
		C. neoformans	*C. gattii*			*C. neoformans*	*C. gattii*
MGX		0.25	0.125	**13a**		**0.016**	**0.016**
8a		0.031	**0.008**	***13b***		**0.008**	**0.016**
8b		0.5	0.5	**13c**		**0.016**	**0.016**
8c		0.031	**0.016**	***13d***		**0.016**	0.031
8d		**0.016**	**0.016**	**13e**		0.031	0.031
8e		**0.016**	**0.016**	**13f**		0.25	0.5
9a		0.25	0.063	**13g**		0.031	0.031

9b		0.125	0.063	**13h**		0.031	**0.016**
10a		0.125	0.063	**13i**		**0.016**	**0.008**
10b		> 4	> 4	**13j**		**0.016**	0.031
11a		0.25	0.25	**13k**		2	2
11b		> 4	> 4	**13l**		**0.016**	**0.016**
12a		**0.008**	**0.004**	**13m**		**0.008**	**0.008**
12b		**0.016**	**0.008**	**13n**		0.063	0.063
12c		0.031	0.031	**14a**		0.031	0.031
12d		**0.016**	**0.008**	**14b**		0.5	1

[a] Italicized compound numbers indicate a compound that has been previously synthesized by Eisai.
[b] MIC values were determined according to the methods under ***a) Antifungal susceptibility testing***. Values in bold fulfill the selection criteria.
[c] Abbreviation: MGX, manogepix

With the advanced intermediates **I** to **V** in hand, we synthesized 24 previously described Gwt1 inhibitors and 292 novel compounds. Intermediate **I** was most versatile and underwent Suzuki coupling reactions to form either 1- or 2-carbon atom linked analogs (**8a** and **8b**), as well as substitution reactions with a variety of nucleophiles, such as pyrazoles, phenols, anilines or amines. In that manner we were able to synthesize -CH_2-linked pyrazole **9a**, -CH_2O-linked aryloxy analog **9b** and the -CH_2NH-linked derivatives **10a** and **10b** (**Scheme 2**). The free amino group in intermediate **II** allowed for nucleophilic aromatic substitution reactions with a variety of activated 5- and 6-membered aromatic heterocycles forming the -CH_2NH-linked analogs **11a** and **11b**. In a similar fashion we reacted the free phenol in intermediate **III** to furnish the aryl ethers **12a** and **12b**. Phenol **III** also reacted smoothly with several benzyl or alkyl halides delivering -OCH_2-linked compounds **12c** and **12d**. The fluoropyridine intermediates **IV** and **V** were equally reactive and underwent nucleophilic aromatic substitutions with a large variety of alcohols, phenols, amines and anilines to give analogs **13a-n** and **14a-b**, respectively (**Scheme 3**). **Schemes 2** and **3** exemplify representative reactions for each of the intermediates, while additional analogs were synthesized in the same manner. **Table 1** summarizes the activities for selected compounds against *C. neoformans* and *C. gattii*. MIC values for manogepix were of

0.25 μg/mL and 0.125 μg/mL, respectively. More than half of the synthesized compounds had significantly improved MIC values against both strains, demonstrating the great potential for optimization of this compound series towards the activity against *Cryptococcus*. The obtained MIC values result from interactions of our compounds with whole fungal cells and thus are a summary of multiple processes, such as cellular penetration (active/passive transport), efflux and target engagement. Each of these events reacts differently to structural changes within our compounds. This inherent drawback of phenotypical assays made it difficult to extract sharp SAR, however, we identified several trends. We did not observe any strong preference for the type (-CH_2-, -O-, -NH-) or the length of the linker (1-, 2- or 3-atoms) between the **C**- and **D**-ring of the compounds with respect to their activity against *Cryptococcus*, though there was some interplay between various heteroaryl rings and linker types that affected activity. *Meta* fluoro- and chloro-substituents on the **D**-ring were well tolerated or improved activity, while any *ortho*-substituents larger than a fluorine atom decreased potency. The *ortho*-methyl group in **13k**, for example, showed about 100-fold weaker antifungal activity compared to the unsubstituted analog **13j**. Highly polar **D**-rings (4-amino-2-methylpyrimidine in **11b**) or basic amines (**10b**) led to inactive compounds. Decreased antifungal activity was also observed when substituents were introduced to the linker between ring **C** and group **D**. For example, the addition of a methyl-group to the -OCH_2-linker reduced the activity by 8- to 16-fold (**13e** vs. **13f**). Compounds derived from intermediate **V** (linker to **D**-ring is *meta* substituent on the **C**-ring) were generally less active than the corresponding analogs derived from intermediate **IV**. A total of 11 compounds demonstrated MIC values less than or equal to 0.016 μg/mL against *both* strains and were advanced to *in vitro* metabolic stability studies. The HLM half-life ($T_{1/2}$) for these compounds was determined according to the methods described under ***b) HLM half-life***. In this experiment, the half-life of manogepix was about 50 min and we decided to advance compounds with a half-life of at least 40 min. Compound **8d** with an unsubstituted furan as ring **D** had an extremely short half-life of about 3 min. To increase metabolic stability, a fluorine atom was introduced. The half-life of fluoro-analog **8e** increased to 14 min and the compound maintained its antifungal activity. For five compounds with a half-life of 40 min or longer, we assessed the cytotoxicity in a HepG2 viability assay as described under ***c) Cytotoxicity assay***. Only analog **12b** showed significant liability at a concentration of 3 μM and was not further evaluated. The remaining four compounds were, alongside manogepix, subjected to MoA studies. Overexpression/resistance assays are a valuable tool for confirming the target-based activity of inhibitors.[16] Overexpression of a target in bacteria or fungi often leads to enhanced resistance to compounds that inhibit that enzyme target. We evaluated the MIC of compounds against *S. cerevisiae* strains overexpressing high and low levels of Gwt1. MIC values of the compounds were determined for each strain followed by determining the ratio of the MIC values. An overexpression ratio of ≥8 is consistent with on-target activity. **12a**, **13a** and **13b** showed ratios of 8-fold or greater against the overexpression strain, which are comparable to manogepix and are consistent with Gwt1 as the target of these inhibitors.

Table 2. Metabolic stability, Cytotoxicity, and On-target activity of selected compounds.

Compd[a]	$T_{1/2}$ HLM (min)[b]	HepG2 cytotoxicity[c]	Gwt1 Overexpression ratio[d]	PigW Inhibition[e]
MGX	**49.9**	**98**	**8**	**0**
8d	3.15	-	-	-
8e	14.4	-	-	-
12a	**43.0**	**93**	**16**	**0**
12b	**61.5**	77	-	-
12d	17.3	-	-	-
13a	**62.3**	**97**	**8**	**5**
13b	**93.9**	**102**	**8**	**0**
13c	37.1	-	-	-
13i	4.65	-	-	-
13l	**68.2**	**98**	4	80
13m	20.3	-	-	-

[a] Italicized compound numbers indicate a compound that has been previously synthesized by Eisai. MGX, manogepix. Values were determined according to the methods for [b] under ***b) HLM half-life***, for [c] under ***c) Cytotoxicity assay*** (% viability at 3 μM)), for [d] under ***d) Overexpression assay*** and for [e] under ***e) PigW assay*** (% inhibition at 16 μM). Values in bold fulfill the selection criteria.

Interestingly, compound **13l**, which only showed a 4-fold shift in MIC values in the overexpression assay, was also the only compound that showed significant activity against the human homolog PigW[7, 14] and was deprioritized. Compounds **12a**, **13a** and **13b** are currently under further investigation.

In summary, we have synthesized over 300 analogs of manogepix and evaluated their activity against of *C. neoformans* and *C. gattii*. We were able to improve the antifungal activity up to 32-fold against both strains while maintaining a preliminary PK and toxicity profile similar to manogepix. We have selected three compounds for further *in vivo* studies.

Conflict of interest statement. The authors are current employees of Amplyx Pharmaceuticals which has Gwt1 antifungal programs.

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Methods and Experimental details for the synthesis of key intermediates and compounds are available in the Supporting Information document.

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