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EXPLORING STRUCTURE–ACTIVITY RELATIONSHIPS AROUND THE PHOSPHOMANNOSE ISOMERASE INHIBITOR AF14049 VIA COMBINATORIAL SYNTHESIS

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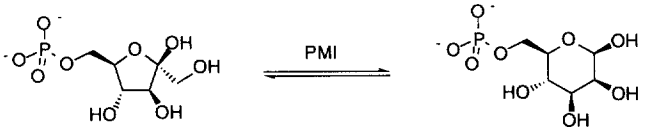
Abstract: Phosphomannose Isomerase (PMI) has been shown by genetic methods to be an essential enzyme in fungal cell wall biosynthesis. The PMI inhibitor AF14049 was discovered as an unanticipated side product from high-throughput library screening against the enzyme from *C. albicans*. Solid-phase synthetic methods were developed and a series of libraries and discrete analogs synthesized to explore SAR around AF14049.

Phosphomannose isomerase (PMI, EC 5.3.1.8) is a 48 kDa zinc metalloenzyme that catalyzes the reversible isomerization of mannose-6-phosphate (M6P) and fructose-6-phosphate (F6P).



F6P M6P

This reaction is the first committed step in the synthesis of mannosylated glycoproteins from glycolytic intermediates and the enzyme plays an essential role in yeast cell wall biosynthesis.[4] Temperature-sensitive mutations in the gene encoding PMI in *S. cerevisiae* lead to the production of mannose-deficient cell walls and cell lysis at the restrictive temperature (37 °C).[5] Cell-permeant inhibitors of yeast PMI could therefore have potential as fungicidal agents. A comparison of the protein encoded by the yeast gene with the PMI genes cloned from *C. albicans*,[6] *Aspergillus nidulans*, and other species (including human)[7] indicates a high level of sequence identity (>40%), and biochemical studies reveal very similar kinetic parameters for both the fungal and mammalian enzymes.[8] Although the requirement for PMI in mammalian cells is not entirely clear, it seems likely that fungal selectivity would be an important characteristic of any pharmacologically useful PMI inhibitor.

Sugar phosphates, such as erythrose-4-phosphate and arabinose-5-phosphate, have been shown to be weak competitive inhibitors of yeast, porcine and human PMI (IC_{50} ~ 50–100 μM) but show no evidence for species selectivity.[7] Two inhibitory metal binding sites have been identified in yeast PMI:[6] Zn^{2+}, Hg^{2+}, and Ag^{+} each inhibit the enzyme with potencies in the low micromolar range, the mercury and silver ions interacting irreversibly at a site (*viz.* Cys-150) distinct from the zinc inhibitory site, as demonstrated by site-directed mutagenesis[9] and X-ray crystallographic studies.[10] None of these inhibitors represent viable leads for developing antifungal agents, however, and we have recently been engaged in high-throughput screening of a variety of compound sources against the PMI enzyme from *C. albicans* (CaPMI) to identify an alternative starting point for lead optimization. In this report we describe the discovery of the 2-aminoindane-2-carboxamide derivative AF14049, **1**, as a weak PMI inhibitor from a combinatorial library of acylated dipeptides. Several new solid-

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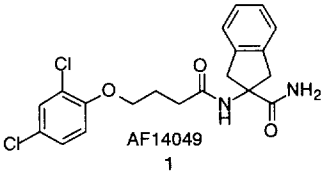
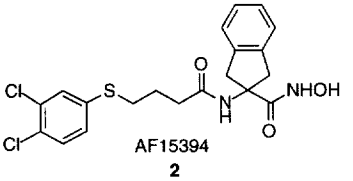
phase synthetic methods were developed in an attempt to analog and optimize the potency of **1**, resulting in the identification of AF15394, **2**, as a modestly selective PMI inhibitor with weak antifungal activity.

In screening a large number of combinatorial libraries (totalling >300,000 compounds) only one library, ACL0921, showed reproducible inhibition of CaPMI.[11] ACL0921 was designed as a collection of 1296 N-capped dipeptides containing 2-aminoindane-2-carboxylic acid as a conserved central building block. The library was generated via solid-phase split synthesis by first coupling 36 different amino acids (including natural and unnatural amino acids) to a photolabile amino resin (Novabiochem), pooling the resin, coupling the conserved amino acid and then dividing the resin into 36 aliquots for coupling to one of 27 carboxylic acids and 9 isocyanates. After photolytic cleavage from the solid support each pool (containing 36 compounds) was screened against CaPMI at an estimated concentration of ~ 130 μM. One pool, derived from acylation with 4-(2,4-dichlorophenoxy)butyric acid, showed a weak but significant level of enzyme inhibition (~30%) and was selected for deconvolution.



1



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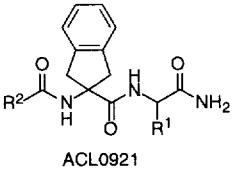


Resynthesis of the 36 discrete components and assay as crude samples indicated that inhibitory activity was associated with two structurally unrelated building blocks (3-pyridylalanine and citrulline). Intriguingly, HPLC and MS analysis of the active discretes indicated the presence of a common byproduct whose molecular mass (407.3) was consistent with the primary carboxamide, **1**, a deletion adduct formed through incomplete coupling of the first amino acid to resin (further investigation revealed that the resin had been successfully acylated under standard amino acid coupling conditions by the remaining 34 building blocks used in the preparation of ACL0921). This was confirmed by an authentic resynthesis of **1** (AF14049). **1** was found to be a weak, time-dependent inhibitor of CaPMI (IC_{50} = 40 μM), while neither of the purified acylated dipeptides showed any inhibitory activity at 200 μM.[12] Thus the product of an unanticipated side-reaction from a library synthesis afforded a potentially tractable lead having a simple, modular structure that appeared amenable to analoging via combinatorial methods (see Figure 1).

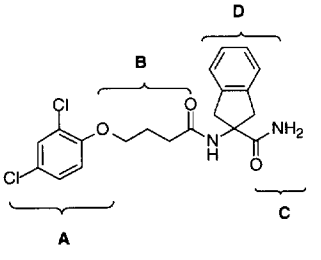
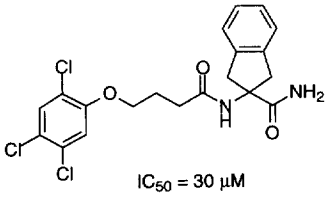
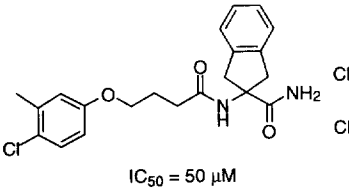


Figure 1. Dissection of **1** into modular subunits suitable for combinatorial exploration

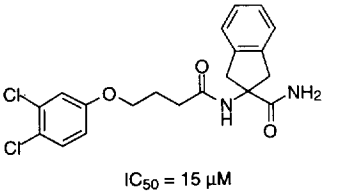
To ensure that we had not overlooked any SAR available from ACL0921, a resynthesis of N-acyl 2-aminoindane-2-carboxamides using the original 36 N-capping groups plus 30 additional substituted phenoxybutyric acid, phenoxyacetic acid and phenylacetic acid building blocks was undertaken. Up to concentrations of 200 μM (the limit of solubility for most of these compounds) a significant level of inhibition was observed for only 3 analogs of **1**, which were purified for accurate analysis:



IC_{50} = 30 μM



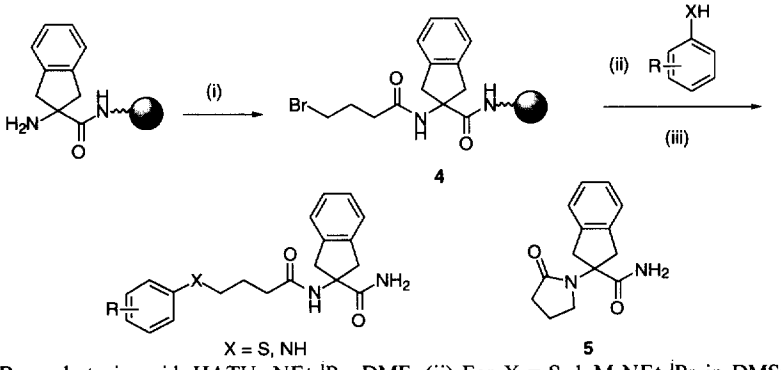
IC_{50} = 50 μM



IC_{50} = 15 μM

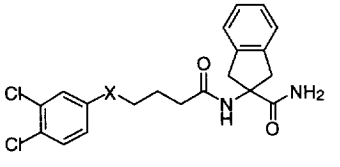
3

A. Modification of aryl ether moiety: A solid-phase methodology was established to modify the aryl ether capping moiety of **1** based on a nucleophilic displacement reaction of the Rink amide resin-bound 4-bromobutyramide, **4** (Scheme 1). This displacement proceeded reliably with thiophenols and anilines as nucleophiles, though in the latter reactions competitive lactamization to yield pyrrolidinone **5** was observed with less reactive anilines. Phenols were ineffective nucleophiles here, and the addition of several bases exclusively afforded the undesired **5**.

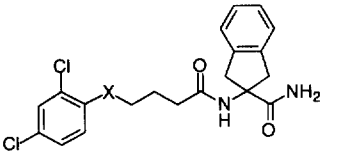


Scheme 1. (i) 4-Bromobutyric acid, HATU, $NEt_2{}^iPr$, DMF; (ii) For X = S, 1 M $NEt_2{}^iPr$ in DMSO; For X = NH, DMSO; (iii) TFA, CH_2Cl_2.

From a selection of 60 nucleophiles (comprising 30 thiophenols, 25 anilines, and 5 aminopyridines from commercial sources) HPLC/MS analysis of the crude reaction mixtures indicated clean conversion to the expected products in 51 instances (the aminopyridines proved uniformly unreactive). These crude products were assayed against CaPMI and 13 samples found to have estimated IC_{50}'s less than 25 μM. Haloanilino- or halothiophenoxy- substitutents were invariably present in the most active compounds, with the 3,4-dihaloaromatic motif being especially favored in both series. This was confirmed upon resynthesis, purification and assay of several individual compounds, and the 3,4-dichlorothiophenoxy derivative, **6**, found to be the most potent inhibitor characterized. A consistent trend was seen across the 3,4-dichloro- and 2,4-dichloro-capped series, with the rank order of potencies being thiophenoxy > phenoxy > anilino. While more detailed biological investigations of **6** were precluded by its poor aqueous solubility, modifications at the carboxy-terminus did provide soluble analogs (*vide infra*).

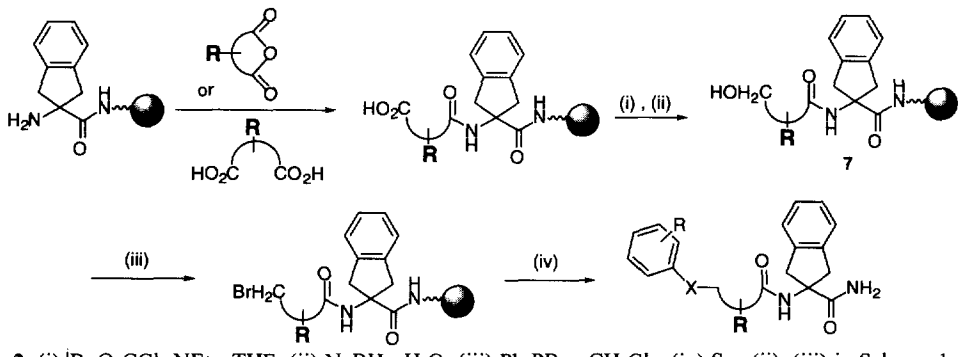


X = S **6** IC_{50} = 6 μM
X = O **3** IC_{50} = 15 μM
X = NH IC_{50} = 21 μM



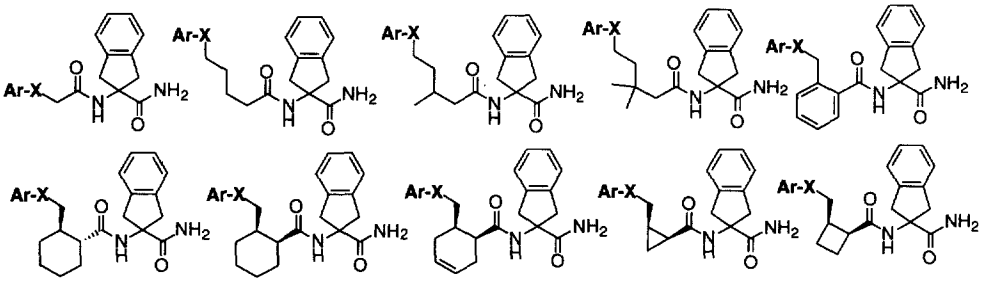
X = S IC_{50} = 14 μM
X = O **1** IC_{50} = 40 μM
X = NH IC_{50} = 50 μM

B. Modification of the linker moiety: Previous library work exploring substitutions for the 4-(2,4-dichlorophenoxy)butyryl group suggested that some activity could be retained in analogs where the butyl tether was replaced by other aliphatic chains. To enable a versatile approach to modification of this linker we developed the solid-phase reaction sequence in Scheme 2, wherein acylation of the immobilized 2-aminoindane-2-carboxamide with a symmetrical anhydride or diacid was followed by acid activation and reduction to the primary alcohol, **7**. Triphenylphosphine dibromide treatment afforded the corresponding bromide, which could be displaced as before by thiophenols and anilines.



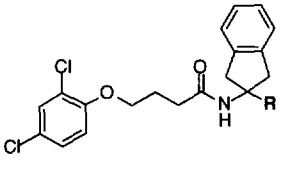
Scheme 2: (i) $^tBuO_2CCl$, NEt_3, THF; (ii) $NaBH_4$, H_2O; (iii) Ph_3PBr_2, CH_2Cl_2; (iv) See (ii), (iii) in Scheme 1.

This chemistry was used to prepare a library of 600 analogs as 60 pools of 10 compounds/pool (generic structures shown below) using the thiophenol and aniline nucleophiles from the previous experiment.



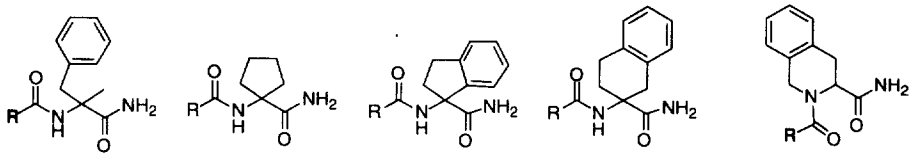
Flow injection MS analysis of several pools chosen at random revealed the 9 different molecular ions expected. Unfortunately none of these pools appeared to include inhibitors as potent as those in the butyryl series (e.g., in the 3,4-dichlorothiophenol pool the most active compound was that derived from the cyclopropyl dicarboxylate, with an $IC_{50} \sim 100$ μM).

C. Modification of the carboxy terminus: A series of simple analogs of AF14049, **1**, were prepared by solution chemistry to probe the role of the C-terminal carboxamide moiety. A free carboxylic acid at the C-terminus abolished PMI inhibitory activity, as did removal of the carboxamide fragment completely (R = H). Activity was recovered upon esterification of the acid (with $Me_3SiCH_2N_2$), while reduction of the ester with $NaBH_4$ provided the primary alcohol, having a fourfold loss in affinity. Displacement of the ester with small nitrogen nucleophiles (methylamine, hydrazine, hydroxylamine) afforded compounds that were equipotent with **1**. Of these the hydroxamate, **8**, was specially significant since it showed substantially enhanced solubility relative to amide derivatives.[13] Incorporation of the hydroxamate modification in the 3,4-dichlorothiophenoxy series gave compound **2** having an $IC_{50} = 6$ μM with good aqueous solubility to ~ 300 μM. Twelve additional secondary amide analogs of **1** derived from a diverse set of amines were synthesized but all were inactive when screened at 100 μM, emphasizing the strict steric requirements at the carboxy terminus for enzyme inhibition.



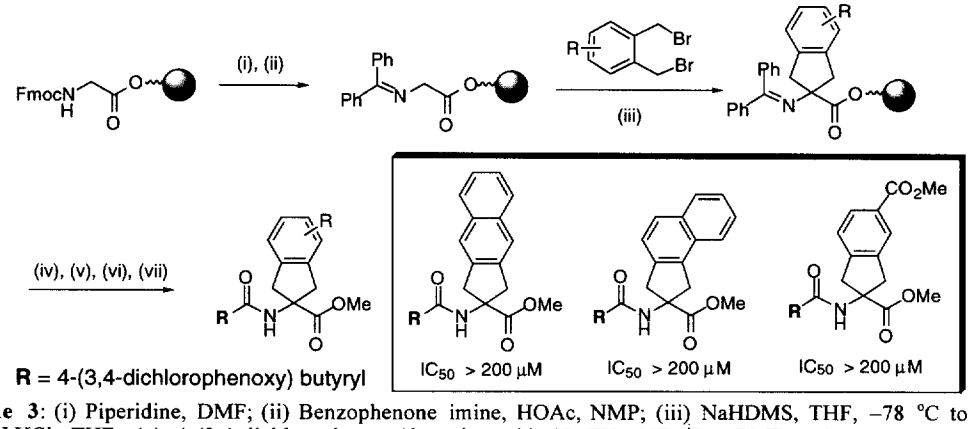
R	IC_{50} (μM)	R	IC_{50} (μM)
CO_2H	> 500	CONHMe	34
CO_2Me	34	$CONHNH_2$	40
CH_2OH	140	CONHOH **8**	39
H	> 200		

D. Modification of the 2-aminoindane moiety: Attempts were made to identify a better replacement for the 2-aminoindane 2-carboxylic acid residue in **1** by generation of a small library of discrete analogs, incorporating a variety of aromatic, α,α-dialkylated and cyclic amino acids.[14] Several of the more notable library members are shown below with their bioactivities:



R = 4-(2,4-dichlorophenoxy) butyryl

Remarkably, only the 2-aminotetralone 2-carboxamide retained any activity (IC_{50} = 93 μM), with even the most subtle modifications abrogating the PMI inhibition in every other case. We next looked to achieve a *de novo* solid-phase synthesis of 2-aminoindane 2-carboxamide analogs by employing substituted 2-indanones in both Ugi or Strecker type condensations with resin-bound amines or isocyanides as synthetic inputs. While in neither instance were any of the desired amino acid derivatives formed, a more successful outcome was obtained by adapting O'Donnell's "unnatural peptide synthesis" approach to permit dialkylation of a glycine enolate with various *o*-dibromoxylenes (see Scheme 3).[15] Optimized reaction conditions required a 20-fold excess of dibromide and a similar excess of NaHDMS as base, and permitted elaboration of one carbomethoxy analog and two naphthalene derivatives with purities of 50–70% upon cleavage from resin. Again, these modifications were not tolerated, indicating that only exceptionally restricted SAR that could be built up around this lead.



R = 4-(3,4-dichlorophenoxy) butyryl

Scheme 3: (i) Piperidine, DMF; (ii) Benzophenone imine, HOAc, NMP; (iii) NaHDMS, THF, −78 °C to rt; (iv) $NH_2OH.HCl$, THF; (v) 4-(3,4-dichlorophenoxy)butyric acid, HATU, $NEt_2{}^iPr$, DMF; (vi) TFA, CH_2Cl_2; (vii) $Me_3SiCH_2N_2$, THF. Wang resin was used in this synthesis.

Biological Activity of AF15394 (2)

Kinetic studies of the inhibition of CaPMI by hydroxamate compound **2** suggested a complex mode of interaction with the enzyme. Compound **2** appeared to undergo multiphasic binding, with the slowest component leading to a conformational change in the enzyme (as assessed by protein Trp fluorescence) and irreversible inhibition in a manner reminiscent of inhibition by silver ions.[9] Analysis at time points < 3 h were consistent with non-competitive binding to CaPMI with an inhibition constant K_i ~ 4 μM. Inhibition studies with human PMI indicated that **2** was weakly selective for fungal enzyme (K_i [huPMI] = 26 μM). Compound **2** inhibited overnight growth (MIC = 80 μM) of a *S. cerevisiae* strain harboring a temperature sensitive mutation

in the PMI gene in mannose-deficient media at the permissive temperature.[5] Supplementation of the media with 10 mM mannose relieved this growth inhibition, consistent with a PMI-dependent cellular effect. Moreover, a structurally related hydroxamate compound, 1-[4-(2,4-dichlorophenoxy)butyrylamino]-indane-1-carboxylic acid hydroxyamide, that was inactive in the enzymatic assay showed no effect on yeast growth at 300 μM. Compound **2** also inhibited growth of *C. albicans* (at very low inoculum densities) and Invsc 1, a wild-type strain of *S. cerevisiae* with MIC's of ~ 80–100 μM. Interestingly, inhibition of these strains was not rescuable by mannose addition, and further studies will be necessary to understand these phenotypes.[16]

References and Notes

1. These authors contributed equally to this study.
2. Present address: MetaXen, 3910 Trust Way, Hayward, CA 94545.
3. Present address: Versicor, Inc., 34790 Ardentech Court, Fremont, CA 94555.
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11. Inhibition of CaPMI was determined in a coupled enzyme assay using phosphoglucose isomerase (0.3 U/mL) and glucose-6-phosphate dehydrogenase (0.3 U/mL) as coupling enzymes. PMI (1.6 nM) was preincubated with inhibitor and coupling enzymes in 50 mM Tris buffer, pH 8.0, for 30 min. at ambient temperature prior to the addition of mannose-6-phosphate as substrate to 0.2 mM. Formation of the coupled product NADPH was monitored over time using a SpectraMax 250 microtiter platereader. Putative PMI inhibitors were checked for inhibition of the coupling enzymes in control incubations.
12. Compound **1** was a slow-binding inhibitor of CaPMI, with equilibrium being established after ~ 3 h preincubation with the enzyme. Under these conditions an inhibition constant K_i = 27 μM was obtained for **1**. For routine screening, a 30 min preincubation of PMI and inhibitor was found to generate significant and reproducible levels of enzyme inhibition and these conditions were typically used to compare the activities of different compounds.
13. Although hydroxamic acids are well known as zinc chelators, the equivalent PMI inhibitory potency of the ester, methyl amide, and hydrazide analogs of **8** suggests that interaction with the active site zinc is unlikely to be important here.
14. 1-Aminoindane 1-carboxylic acid was prepared from 1-indanone via the hydantoin according to the Bucherer-Bergs protocol.
15. O'Donnell, M. J.; Zhou, C.; Scott, W. L. *J. Am. Chem. Soc.* **1996**, *118*, 6070.
16. We gratefully acknowledge Dr. T. N. C. Wells (Geneva Biomedical Research Institute) and Dr. J. J. Martin (Glaxo-Wellcome, Tres Cantos, Madrid) for reagents and helpful discussions.