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Application of Affinity Selection/Mass Spectrometry to Determine the Structural Isomer of Parnafungins Responsible for Binding Polyadenosine Polymerase

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Abstract: To discover antifungal treatments that possess the desired characteristics of broad spectrum activity, a strong safety profile, and oral bioavailability, new discovery strategies must be implemented to identify structural classes of molecules capable of combating these microorganisms. One such technique that has been implemented is the *Candida albicans* Fitness Test, a whole cell screening platform capable of delineating the mechanism of action of compounds that demonstrate activity against the clinically relevant pathogenic fungus, *C. albicans*. Screening crude natural product extracts with this technology has resulted in the identification of a novel family of antifungal natural products, named the parnafungins, which inhibit the enzyme polyadenosine polymerase (PAP), a key component of the mRNA cleavage and polyadenylation complex. Owing to the rapid interconversion of the structural and stereoisomers of the parnafungins at neutral pH, the determination of the structural isomer with the highest affinity for PAP with standard biochemical assays has not been possible. Herein, we present an application of affinity-selection/mass spectrometry (AS−MS) to determine that the "straight" parnafungin structural isomer (parnafungin A) binds preferentially to PAP compared to the "bent" structural isomer (parnafungin B).

Introduction

With the higher prevalence of invasive medical procedures as well as an increased number of immuno-compromised patients due to factors such as cancer and organ transplant chemotherapy and diseases such as AIDS, life-threatening fungal infections have become a more prominent clinical issue.[1] The current arsenal of antifungal agents could be enhanced by discovering new structural classes that possess broad spectrum activity, have a strong safety profile, and are orally bioavailable. New screening methods have been developed to identify novel chemical scaffolds that specifically target unexploited biochemical pathways in fungi. One such method is the *Candida albicans* Fitness Test (*Ca*FT), a whole cell screening platform that can be used to define the mechanism of action of compounds that demonstrate activity against the clinically relevant pathogenic fungus *C. albicans*.[2,3] In the *Ca*FT, a pool of 2868 molecularly bar coded *C. albicans* strains have been genetically engineered to be heterozygous for a unique gene. These genes have been selected based on their documented importance for normal growth and/or viability in *Saccharomyces cerevisiae*[4] and/or *C. albicans*.[3,5] This pool is screened for hypersensitivity against sublethal concentrations of chemical agents. Compound-specific growth effects on individual mutant strains provide crucial information to the identity of the inhibitor's cellular target and/or the affected biochemical pathway.

Traditionally, the *Ca*FT assay had been applied to screening purified libraries and collections of synthetic compounds with antifungal activity.[5,6] Recently, however, the *Ca*FT technology has been extended to the analysis of crude fermentation extracts to discover natural products with novel modes of action.[7] Historically, natural products screening has provided a wealth of structurally diverse antifungal agents from which two major classes have become commercial drugs. These include the enchinocandin lipopeptides (caspofungin,[8] micafungin,[9] anidulafungin[10]), which target fungal cell wall biosynthesis,[11] and the macrocyclic polyenes (amphotericin), which target membrane ergosterol.[12] From the screening of a large set of fermentation broth extracts, the *Ca*FT identified a sample derived

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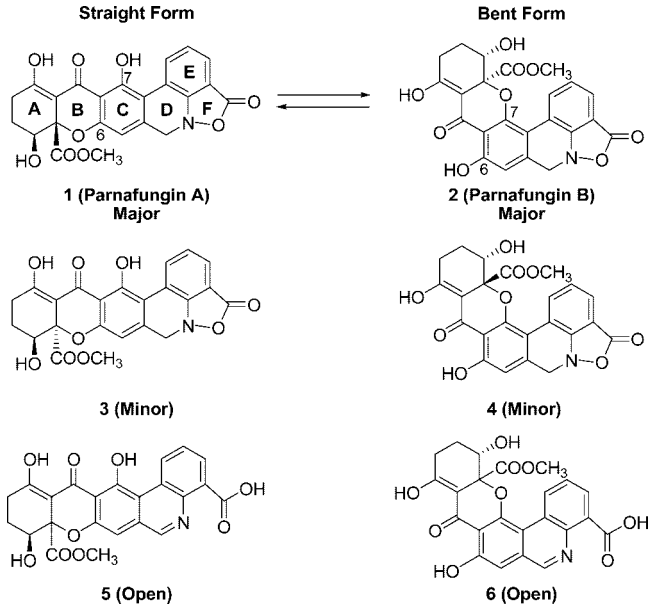

Figure 1. Structures of parnafungins. Compounds **1** and **2** are the major diastereomers.

from lichenolous strains of *Fusarium larvarum* (Ascomycota, Hypocreales) that demonstrated potent antifungal activity.[7] Isolation efforts resulted in the purification and structural determination of a novel class of natural products, parnafungin A and B. These compounds are the first examples of natural products which include an isoxazolidinone ring system containing an N−O bond.[7,13] Profiling of both the crude extract and the purified parnafungins in the *Ca*FT provided a selective and reproducible pattern of hypersensitive heterozygous *C. albicans* strains.[7] This set of sensitive strains included multiple members of the mRNA cleavage and polyadenylation complex. After further biochemical and genetic studies, the fungal polyadenylate polymerase (PAP) was confirmed as the enzyme inhibited by the parnafungins.[7] PAP functions as a template independent nucleotransferase that catalyzes the processive addition of adenosine to the 3′-end of mRNA to form a poly(A) tail.[14] The formation of poly(A)-tails is essential to the maturation of mRNA and plays a role in both translation and mRNA stability.[15] The parnafungins also showed broad spectrum in vitro antifungal activity and in vivo efficacy against *C. albicans* in a mouse model of infection. Despite being equipotent in vitro inhibitors of fungal and mammalian PAP activity, the parnafungins presented no observable toxicity in the mouse.[7]

Extensive isolation efforts and structural characterization of parnafungins A and B resolved the structures of these isoxazolidinone-containing natural products (Figure 1).[13] The parnafungins were isolated as a mixture of four components, two major (**1**, **2**) and two minor (**3**, **4**), all with an identical molecular formula of $C_{23}H_{17}NO_9$. The parnafungins can exist in either a "bent" or "straight" topology with the major diastereomers having the *S*-hydroxyl group adopt an equatorial orientation in the half-chair conformer of the A ring. Interconversion of the various parnafungin isomers occurs by a retro-Michael opening of the B ring of the xanthone of **1** with subsequent ring closure by Michael addition of either the C6 or C7 phenol to the enone of the A ring of the intermediate. Facial selectivity of the ring closure provides a mixture of diastereomers for both the "straight" and "bent" structures. All forms rapidly interconvert at neutral or basic pH with slow equilibration observed over a few hours at acidic pH.[13] The nitrogen−oxygen bond of the isoxazolidinone ring was found to be highly labile, generating the benzoquinoline open form of the natural product, as in structures **5** and **6**.[13] Ring opening of the isoxazolidinone was more rapid at neutral or basic pH then at acidic pH. The opening of the isoxazolidinone ring resulted in a loss of antifungal activity. Since the formation of **5** and **6** involved both the addition and loss of a molecule of water, these degradation products have the same molecular formula as the parnafungins. There was no evidence that **5** and **6** were generated directly by the producing organism.

With the need for additional antifungal agents, there is considerable interest in converting a natural product scaffold like the panafungins into a commercial drug. In designing a synthetic program around this scaffold, it is important to determine whether the "bent" or the "straight" form of the parnafungins binds to PAP and is responsible for the observed potent inhibition. A second mechanistic question is whether the potency of the natural product is resultant from covalent binding of the enzyme to parnafungin through nucleophilic attack of the isoxazolidinone. Because of the fast interconversion of the natural product, traditional activity assays are not amenable to determining which form of parnafungin is the active species. Samples enriched for one isomer at pH 3 will rapidly equilibrate under the physiological pH of the reaction conditions and thus the relative activities of the various forms will be difficult to determine.

Affinity selection (AS) screening presents an attractive alternative to standard assays for determining which isomer of parnafungins binds to PAP. This strategy has emerged as a complementary approach to traditional high throughput screening for small molecule lead discovery because of the ability, once purified protein is obtained, to screen large mixtures of compounds in one experiment.[16−18] Multiple forms and activation states of proteins and protein complexes[19] as well as multiple binding pockets on each protein[20,21] can be screened utilizing AS techniques. To date, several AS technologies have emerged that combine mass spectrometry (MS) as a detector with various separation strategies to isolate protein-bound ligands from free small molecules. Frontal affinity chromatography,[19,21] ultrafiltration,[22,23] affinity capillary electrophoresis,[24,25] and size

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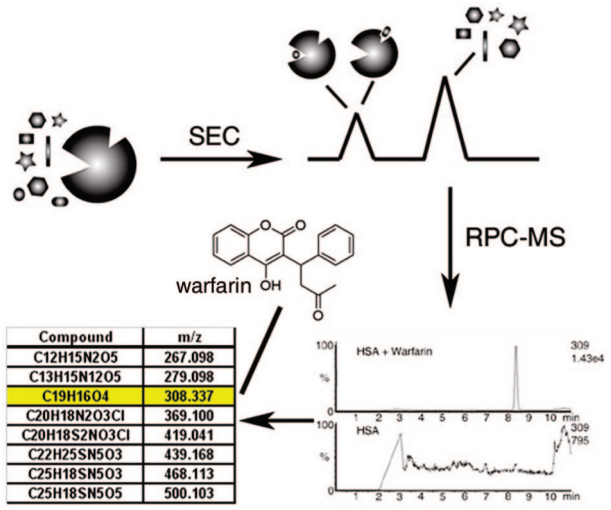

Compound	m/z
C12H15N2O5	267.098
C13H15N12O5	279.098
C19H16O4	308.337
C20H18N2O3Cl	369.100
C20H18S2NO3Cl	419.041
C22H25SN5O3	439.168
C25H18SN5O3	468.113
C25H18SN5O5	500.103

Figure 2. Diagram of AS−MS method.

exclusion chromatography (SEC)[26−28] have been applied as separation strategies for affinity selection experiments. Recently, automated systems that integrate SEC separation with LC-MS detection into a single platform provide a technology capable of rapidly screening large compound libraries against a given target.[29−33]

For a standard affinity selection experiment applying an integrated SEC-LC-MS platform, anywhere from one to a mixture of several hundred compounds is preincubated with the protein target at 4 °C, as demonstrated with human serum albumin and warfarin in Figure 2. A portion of this mixture is injected into an integrated two-step LC−MS system where a fast SEC column rapidly separates the protein and protein-bound ligands from unbound compounds in 15 s. The excluded protein peak containing the bound small molecules is then trapped and diverted to a reverse phase column (RPC) while unbound compounds are diverted to waste. On the RPC, the protein is exposed to low pH (∼pH 2) in order to dissociate the ligands from the protein. The free ligands are subsequently separated with a standard H_2O/CH_3CN gradient and the unique binders in the mixture are detected by MS.

Several characteristics of this platform make it ideal for determining which parnafungin structure binds to PAP. A mixture of ligands, in this case the equilibrium mixture of parnafungins, can be incubated at a physiologically relevant pH with the protein. Because of the potency of the inhibitor, the active structural form of the parnafungins will be trapped upon protein binding, carried through the SEC column with the protein, and diverted to the RPC. Dissociation from PAP will occur at pH ∼2. This will effectively stop the isomerization of the natural product, freeze out the active conformation of parnafungin responsible for binding to the enzyme, and allow this form to be detected by analytical LC−MS. Herein, we will present studies with AS−MS that led to the identification of the "straight" parnafungin A (**1**) as the form enriched for binding to PAP and demonstrate the ability of this approach to detect ligands present in natural product broths.

Results and Discussion

To identify whether the "bent" or "straight" conformation of the parnafungins binds with higher affinity to PAP, it was imperative to determine first that the protein was amenable to screening with affinity selection. Recombinant human PAP 1-513 (hPAP) was expressed as an N-terminally tagged 6xHis fusion protein and purified by sequential affinity and size exclusion chromatographies.[7] In an in vitro assay employing a poly(A) substrate, parnafungins inhibited hPAP with an IC_{50} of 114 nM which is similar to the IC_{50} determined for recombinant *S. cerevisiae* PAP1p (145 nM) and recombinant *C. albicans* PAPα (22 nM).[7] Considering the similar activity of the parnafungins for the various PAP isoforms and the ready availability of large quantities of crystallographic grade hPAP, we selected the human form of the protein for subsequent studies. For AS−MS, the protein must produce a sufficient UV absorbance peak to trigger an in-line valve responsible for trapping and diverting the excluded fraction of protein and protein-bound small molecules to the RPC-MS in the AS−MS system. After screening several concentrations of protein, it was determined that 9 μM hPAP provided a large enough UV signal for reliable detection and peak trapping with the in-line valve (Figure 3A). This is consistent with the historical concentrations we have utilized for a wide range of proteins for high throughput screens with AS−MS (5 to 20 μM). The protein (9 μM) was preincubated in the presence and absence of an equilibrium mixture of parnafungins (10 μM) in binding buffer (25 mM HEPES, pH 7.9, 0.1 mM $CaCl_2$) containing 0.5 mM $MnCl_2$ and then injected into the AS−MS system. Comparing the extracted ion chromatogram (XIC) for MW 452 ($(M + H^+)$ for the parnafungins), a distinct cluster of peaks at time 6.0 to 6.5 min was detected with hPAP in the presence of ligand (Figure 3B,C, top), but not in the absence of ligand (Figure 3B,C, middle). If the ligand covalently modified the protein, it is unlikely that it would have been detected; therefore, under these conditions, the parnafungins appear to be reversibly bound to hPAP. Because of the potential of ligands to aggregate or form higher order species that may be falsely detected as hits on the AS−MS system, all samples were run with at least one other protein to identify nonspecific binding resultant from physical properties. As a system control, 10 μM parnafungins were screened against 10 μM β-lactoglobulin, an unrelated protein known to bind hydrophobic compounds such as retinol[34] and palmitic acid.[35] As evident in the XIC (Figure 3B,C, bottom), the parnafungins did not bind to the system-control protein indicating the binding detected to hPAP was specific.

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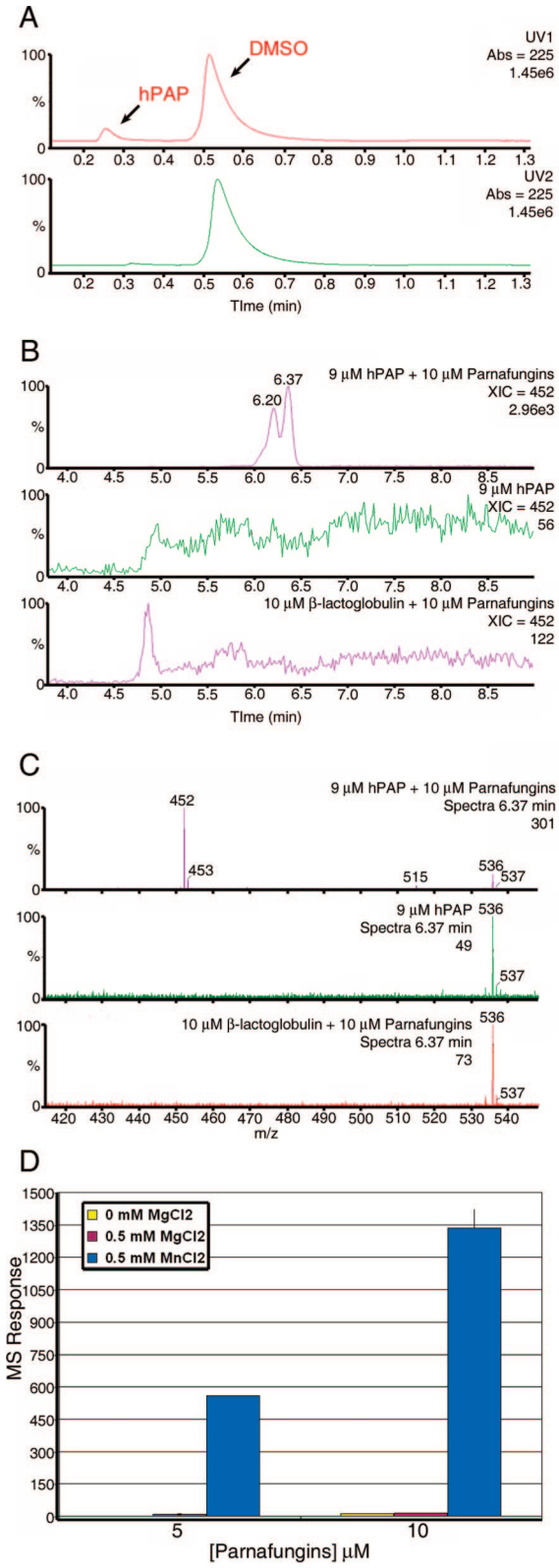

Figure 3. Selective binding of parnafungins to hPAP is detected by AS−MS. (a) Trace of abs_{225} indicated detection and exclusion of hPAP from SEC column on AS−MS system (top panel). Lack of a peak corresponding to hPAP in waste stream indicated proper diversion of protein fraction to RPC-MS (bottom panel). (b) XIC = 452 and (c) spectra at time 6.37 min demonstrated parnafungins bind to hPAP (top panel) but not to β-lactoglobulin (bottom panel). (d) Binding of parnafungins to hPAP enhanced in the presence of $MnCl_2$ relative to $MgCl_2$ or the absence of divalent cations.

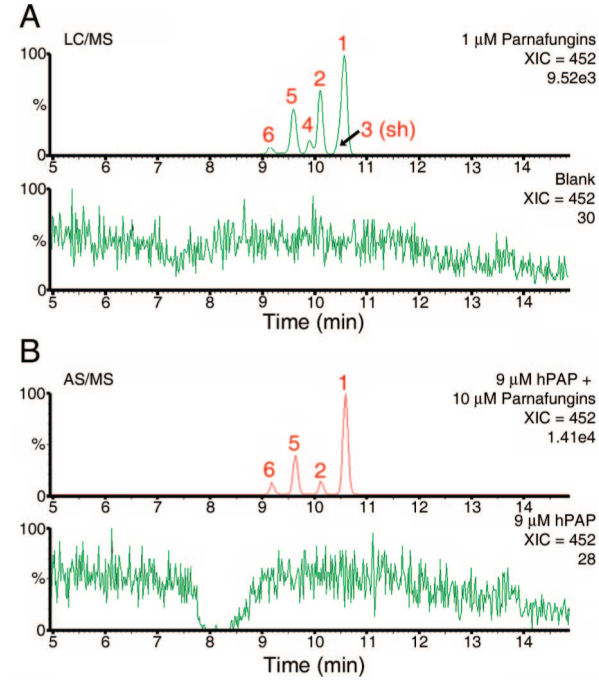

Figure 4. AS−MS analysis with improved elution conditions showed enrichment in parnafungin A (**1**) binding to hPAP. (a) MS^+ XIC = 452 for LC−MS detection of parnafungins from a mixture diluted in methanol. Minor diastereomer (**3**) appears as shoulder. (b) MS^+ XIC = 452 for AS−MS detection of **1** enriched for binding to hPAP.

Divalent metal cations, either Mg^{2+} or Mn^{2+}, are required for the catalytic activity of hPAP with one ion serving as a cosubstrate and the other involved in the coordination of both ATP and the 3′-hydroxyl group of the poly(A) substrate.[14] To test the effect of metal ions on the binding of parnafungins to hPAP, 5 and 10 μM parnafungins were incubated both in the absence of divalent cations or in the presence of 0.5 mM $MgCl_2$ or $MnCl_2$ with 9 μM hPAP and analyzed by AS−MS (Figure 3D). From comparing the integrated peak area for XIC = 452, the relative MS response was significantly greater in the presence of Mn^{2+} relative to Mg^{2+} or the absence of added metal ion. This result was consistent with previous studies that have shown that substrate analogues demonstrate tighter binding to hPAP in the presence of Mn^{2+} relative to Mg^{2+}.[36] Other studies have shown that the catalytic activity of hPAP increases with Mn^{2+}.[37,38] All subsequent AS−MS studies described herein were performed in the presence of 0.5 mM $MnCl_2$.

Because of mass overlap of the various parnafungin isomers and the degradation products, LC conditions that separate each component on the basis of retention time were developed. For LC−MS experiments, the SEC was bypassed and the ligands were not incubated with protein. An improved LC gradient (35% CH_3CN, 0.2% formic acid to 95% CH_3CN, 0.2% formic acid over 10 min at 10 μL/min) similar to conditions utilized for the initial purification and characterization of the natural product was adopted.[13] Utilizing the improved gradient, components **1**−**6** from a 1 μL mixture dissolved in 70% MeOH/30% DMSO (Figure 4A) could be resolved. Similar to prior observations, compound **1** eluted last at 10.56 min with **3** and **4** appearing as a shoulder and a small peak, respectively, just prior to the peaks

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correlating to **1** and **2**. The degradation products **5** and **6** were more polar and eluted at 9.60 and 9.16 min, respectively, prior to compounds with an intact isoxazolidinone ring. The equilibrium mixture of parnafungins (10 μM) was then incubated with 9 μM purified hPAP at 25 °C for 30 min. After 2 h at 4 °C in the HPLC autosampler, the sample was analyzed by AS−MS. Compared to a control injection of protein with DMSO (Figure 4B, bottom panel), four peaks appeared in the XIC = 452 for the sample indicating binding to hPAP (Figure 4B, top panel). The retention time of the peaks correlated to the retention times observed for parnafungins **1** and **2** and degradation products **5** and **6** in the LC spectra. In the LC−MS spectra, the integrated peak area for the signal in the XIC at 10.59 min correlating to parnafungin A (**1**) was 1.5 times the size of the integrated peak area for the peak at 10.12 min correlating to parnafungin B (**2**). In the AS−MS spectra, the ratio of **1** to **2** increased to 8:1 indicating that the straight form of the parnafungins binds preferentially to the protein. Again, the recovery of the natural product enriched for the straight form versus the bent form indicated that the compounds dissociate from the protein at low pH and thus reversibly bind to hPAP.

Because of the presence of degradation products in the compound mixture, a fresh sample of parnafungins was isolated and purified from fermentation broth and a solution in methanol was then analyzed by LC−MS. Compounds **1** and **2** were isolated at acidic pH and used immediately to minimize any possible re-equilibration. A 1:1 mixture of **1** and **2** was prepared and analyzed by LC−MS. This sample gave a 1:1 relative integrated MS response for XIC = 452 at times 10.09 and 9.62 min, respectively (Figure 5A). After preincubation of a 10 μM mixture of **1** and **2** with 9 μM hPAP, the sample was subjected to AS−MS analysis and 4 peaks in the XIC = 452 were detected (Figure 5B). The integrated MS response for the peak correlating to **1** at a retention time of 10.06 min is 4 times larger than the integrated MS response for **2** at 9.6 min again indicating hPAP preferentially bound to the "straight" form of the parnafungins relative to the "bent" form. Small peaks correlating to the degradation products **5** and **6** at 9.11 and 8.69 min were also recovered. To determine whether the observed degradation of the parnafungins in the AS−MS buffer conditions was dependent on the presence of protein, the parnafungin mixture (1 μM) was preincubated at 25 °C for 30 min then at 4 °C for 2 h in either methanol or the standard binding buffer with 0.5 mM $MnCl_2$. These conditions mimicked the typical treatment of the compounds under AS−MS, but the protein was excluded. In methanol, compounds **1** and **2** retained about a 1:1 ratio in integrated MS response and minor hydrolysis was observed (Figure 5C, bottom). However, in buffer, the hydrolysis products **5** and **6** were detected as predominant species in the mixture and the ratio of **1** to **2** changed from 1:1 to 1:3 favoring the bent form of the natural product (Figure 5C, top). This is indicative of previous observations that exposing the parnafungins to neutral or basic conditions resulted in both degradation of the isoxazolidinone ring and rapid isomerization.[13] At pH 7.9, either the equilibrium between **1** and **2** favored the bent structure or the decomposition of **1** occurred at a faster rate than **2.** In AS−MS analyses, however, **1** was still detected as preferentially binding to hPAP providing further evidence that the "straight" form is the relevant structure of the parnafungins responsible for inhibiting the enzyme.

To gain further insight into the preference of PAP for binding to the different structural forms of these natural products, freshly purified samples of either the bent or the straight structural form

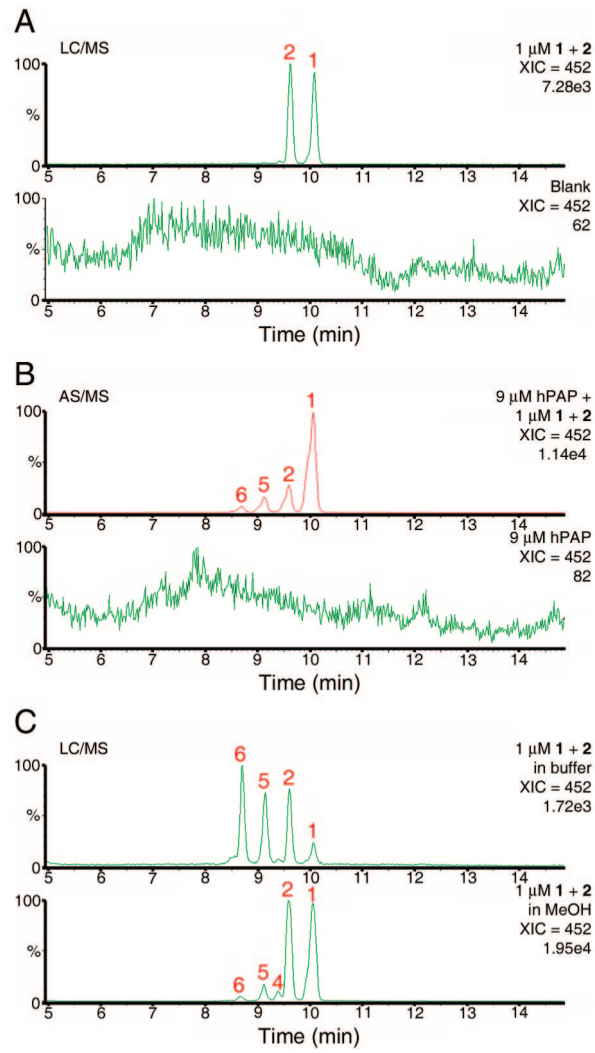

Figure 5. AS−MS analysis of a purified mixture of **1** + **2** with improved elution conditions showed isomerization of natural products in buffer and preferential binding of parnafungin A (**1**) to hPAP. (a) MS^+ XIC = 452 for LC−MS in methanol and (b) MS^+ XIC = 452 for AS−MS with hPAP of a purified mixture of parnafungins A and B. (c) MS^+ XIC = 452 for LC−MS of purified mixture of parnafungins show higher amounts of degradation products **5** and **6** when incubated in buffer (top panel) compared to incubation in MeOH (bottom panel).

of the parnafungins were analyzed by AS−MS. A solution enriched for **1** was first analyzed by LC−MS in methanol (Figure 6A, middle). After preincubation with hPAP under standard conditions, the sample was analyzed by AS−MS (Figure 6A, top). As expected, the straight form of the parnafungins was detected at 10.06 min by MS at XIC = 452 as the predominant species bound to PAP. The experiment was repeated with a sample enriched for the "bent" form **2,** which is detected at 9.62 min in the LC−MS XIC (Figure 6B, middle). The sample enriched for compound **2** was then incubated with hPAP and analyzed by AS−MS. In comparison to the LC−MS spectra of the equilibrium mixture of **1** and **2** (Figure 6B, bottom), surprisingly, the compound recovered as bound to PAP was distinctly enriched for **1** at 10.06 min. Upon exposure to buffer, either enriched fraction will rapidly isomerize to an equilibrium mixture with all components present. Because of the strong inhibition of PAP by the parnafungins (hPAP $IC_{50} \approx 114$ nM),[7] the active component should tightly bind to PAP thereby sequestering the molecule from buffer conditions. The

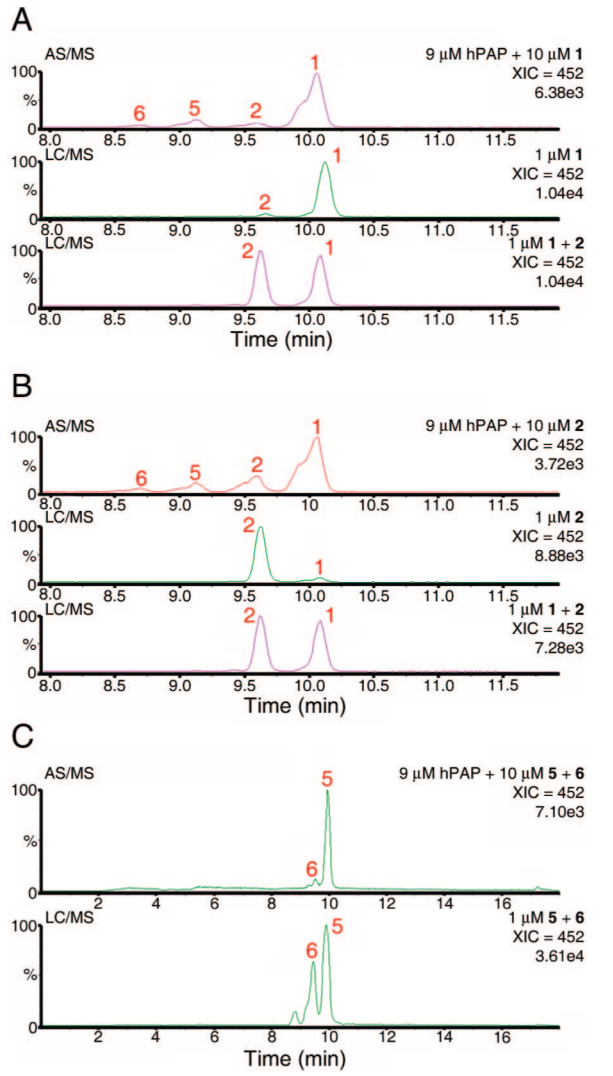

Figure 6. Analysis of each purified parnafungin isoform confirmed preferential binding of the "straight" forms, parnafungin A (**1**) and the open isoxazolidinone (**5**), to hPAP. (a) MS^+ XIC = 452 for AS−MS detection (top panel) of purified **1** (LC−MS in MeOH, middle panel) bound to hPAP. (b) MS^+ XIC = 452 for AS−MS detection (top panel) of **1** binding to hPAP from purified **2** (MS^+ XIC = 452 for LC−MS in MeOH, middle panel). (c) MS^+ XIC = 452 for AS−MS detection of open isoxazolidinone **5** predominantly binding to hPAP (hPAP) from a mixture of **5** and **6** (MS^+ XIC = 452 for LC−MS in MeOH, bottom panel).

removal of the active form from solution through protein binding should shift the compound mixture further toward the bound form. In addition, binding of parnafungin to the enzyme may protect the molecule from rapid degradation of the isoxazolidinone ring.

Previous studies have determined that opening of the isoxazolidinone ring, as in structures **5** and **6**, resulted in loss of antifungal activity;[7] however, varying levels of these compounds were detected as binding to hPAP in the AS−MS experiments. Under screening conditions, the isoxazolidinone ring is readily opened as demonstrated in Figure 5C. To determine whether the open forms of the isoxazolidinones are capable of binding to hPAP or if the experiments are detecting isomerization and ring-opening after dissociation from the protein in the RPC column, a mixture of compounds **5** and **6** was purified and analyzed by LC−MS (Figure 6C, bottom). Subsequent AS−MS analysis of a mixture of 9 μM hPAP with 10 μM **5** and **6**, detected binding of primarily the straight form **5** at a retention

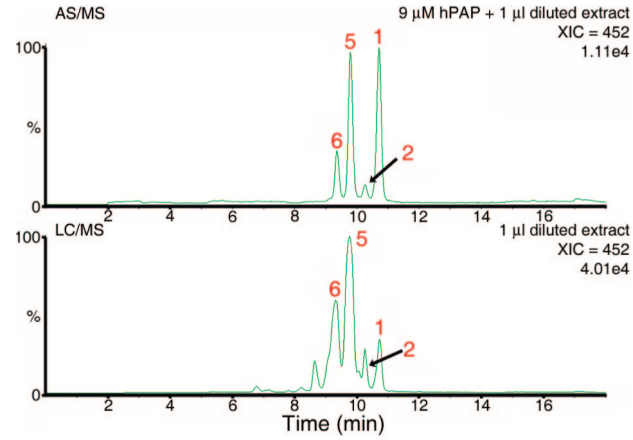

Figure 7. Detection and enrichment of **1** and **5** binding to hPAP from the natural product extract of *F. larvarum* with antifungal activity. (Top panel) MS^+ XIC = 452 for AS−MS detection of parnafungins from extract binding to hPAP. (Bottom panel) MS^+ XIC = 452 for LC−MS analysis of extract containing antifungal activity.

time of 9.95 min in the XIC = 452 (Figure 6C, top). Despite a lack of inhibitory activity for the open form analogues, the preference of PAP for binding the "straight" form **5** over the "bent" form **6** provides further confirmation that parnafungin A (**1**) is the relevant structural form for inhibiting PAP.

Natural products chemistry has provided a rich history of novel structural classes that have led to drugs that aid human health. To find biologically relevant natural products, complex broths are typically screened in assays to find samples that have the desired phenotypic or biologic response. Numerous steps of fractionation and purification are required to isolate and identify these natural products. Recently, more sophisticated approaches to detect active compounds in natural product extracts have been developed.[39−41] Since AS−MS detects bound compounds by mass and retention time with RPC, this technology could provide a complementary method for screening natural product broths for active compounds. From a complex broth, the ligand responsible for binding to a target and potentially producing the desired biological response can be detected and partially characterized by both mass and retention time. These data can be used for the dereplication of known inhibitors of the target of interest. To demonstrate whether AS−MS can be utilized to detect a natural product from a clarified broth, we analyzed acetone extracts of fermentation broths from several fungal strains, including *Fusarium larvarum* from which the parnafungins were originally identified in the *Ca*FT assay.[7] LC−MS analysis from one of the *F. larvarum* extracts indicated a cluster of peaks with XIC = 452 (Figure 7, bottom) similar to the purified mixtures of parnafungins previously analyzed but with a higher ratio of open isoxazolidinone forms **5** and **6** compared to **1** and **2**. hPAP (9 μM) was preincubated with 1 μL of a 6-fold dilution of each extract and then analyzed by AS−MS (Figure 7, top). Four peaks correlating with **1**, **2**, **5**, and **6** were detected in the XIC = 452 (Figure 7, top) with a notable enrichment in signal for the straight forms, parnafungin A (**1**) and the open isoxazolidinone form (**5**).

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(40) Belenky, A.; Hughes, D.; Korneev, A.; Dunayevskiy, Y. *J. Chromatogr. A* **2004**, *1053*, 247–251.
(41) Cummins, L. L.; Chen, S.; Blyn, L. B.; Sannes-Lowery, K. A.; Drader, J. J.; Griffey, R. H.; Hofstadler, S. A. *J. Nat. Prod.* **2003**, *66*, 1186–1190.

Through scanning from mass 250 to 800, no other signals were detected significantly above background for binding to PAP from the broth indicating that the compounds with mass 451 bind and potentially inhibit PAP. The ability to detect binding of a natural product from a broth highlights the potential of this strategy to aid in the identification of unknown natural products that bind to and affect the activity of protein targets of interest. Because of the relative generality of method development for AS−MS screening, multiple purified protein targets can be readily screened against a library of natural product broths.

In summary, affinity selection techniques have been applied to the identification of the structural form of the parnafungins responsible for binding to PAP. This has further confirmed the mechanism of action of this potent, broad spectrum antifungal agent. Owing to the inherent instability of the natural product, traditional activity assays were incapable of determining the structural isoform responsible for the observed activity. Although typically utilized as a tool for high throughput screening, the characteristics of the AS−MS platform were ideal for addressing this mechanistic question. With the ability to incubate compounds with a protein at physiological pH, and then analyze the compounds bound to the protein at a low pH, this method was particularly suited to tackle the relatively facile chemical interconversions of the parnafungins and identify the straight form as the species bound to the protein. Future chemistry efforts will focus on the design of stable analogues of **1** that are potent inhibitors of PAP enzyme activity and *C. albicans* growth in vitro and in animal models of infection. Parnafungin binding to PAP was also detected by AS−MS analysis of a clarified fermentation broth from which the natural products were originally isolated. This study indicates the potential of applying affinity selection to the identification of novel natural products that bind to a biologically relevant protein as well as to solve intriguing problems in chemical biology.

Experimental Section

General Methods. All HPLC grade solvents (Fisher) and reagent grade chemicals (Fisher) were used without further purification. Water was deionized and purified (Millipore Milli Q synthesis). Warfarin, human serum albumin, and β-lactoglobulin were purchased from Sigma-Aldrich.

Identification and Fermentation of *Fusarium Larvarum.* Fungal strains were isolated from an unidentified lichen thallus collected in Miraflores de la Sierra, Madrid, Spain. The fungi, designated MF7022 (ATCC PAT 7894) and MF7023 (ATCC PAT 7895), are maintained in the culture collection of Merck Research Laboratories and the American-type Culture Collection. The identification and fermentation of these strains have been described previously.[13]

Isolation and Characterization of Parnafungins and Ring-Opened Parnafungins. Isolation and characterization of the parnafungins (**1**, **2**, **3**, **4**) and the ring-opened parnafungins (**5**, **6**) have been described previously.[13] Samples of purified parnafungin A (**1**) and parnafungin B (**2**) were obtained by preparative C18 HPLC (gradient elution of CH_3CN in 0.1% formic acid) and assayed in AS−MS immediately after isolation.

Cloning and Expression of Poly-A Polymerase Enzymes. The cloning and expression of the C-terminal histidine-tagged truncated (amino acids 1−513) human PAP has been described previously.[7]

Analysis of Ligand Binding by AS−MS. A 2× solution of ligand in DMSO, unless otherwise specified, was diluted in binding buffer (25 mM HEPES, pH 7.9, 0.1 mM $CaCl_2$, 1 mM $MnCl_2$) with 10% DMSO, and centrifuged for 10 min at 17933*g* at 25 °C. An equal volume of the 2× ligand solution was added to 18 μM hPAP and transferred to a 96-well plate for a final concentration of 9 μM protein in 25 mM HEPES, pH 7.9, 0.1 mM $CaCl_2$, 0.5 mM $MnCl_2$, 5% DMSO. The samples were incubated at 25 °C for 30 min followed by centrifugation at 1266*g* for 30 min at 4 °C. Samples were then transferred to a 4 °C autosampler of an Agilent 1100 series HPLC.

AS−MS analysis was performed on an integrated SEC-LC-MS platform. A 1.5 μL portion of each sample was injected into a mobile phase of NaH_2PO_4, pH 7.5, at a 300 μL/min flow rate for fast SEC separation with a polyhydroxyethyl aspartamide column (Poly LC, 2.1 mm × 33 mm, 5 μm particle size, 60 Å pore size, The Nest Group). UV-based detection triggered a valve to trap and divert the excluded protein peak to the C18 analytical RPC (0.5 mm × 50 mm, 5 μm particles size, 100 Å pore size, Higgens Analytical) at a flow rate of 10 μL/min. Small molecules were dissociated from the protein with 5% CH_3CN/95% H_2O in 0.2% formic acid for 3 min, then, under standard conditions, separated with a gradient of 50% CH_3CN/50% H_2O, 0.2% formic acid to 95% CH_3CN/5% H_2O, 0.2% formic acid over 4 min. For separation of the parnafungins, the RPC gradient was changed to 35% CH_3CN/65% H_2O, 0.2% formic acid to 95% CH_3CN/5% H_2O, 0.2% formic acid over 10 min. The samples flowed directly into the LCT Premiere electrospray time-of-flight mass spectrometer (Waters). Data was analyzed with MassLynx (Waters).

LC−MS Ligand Analysis. Ligands dissolved in DMSO were diluted in either MeOH or binding buffer following the same protocol as AS−MS samples. Samples were then injected on the integrated LC−MS system utilized for AS−MS experiments. For LC−MS analysis, protein was not added to samples and the SEC column was bypassed.

Supporting Information Available: Complete refs 3, 4, 6, 7, 11, and 13. This material is available free of charge via the Internet at http://pubs.acs.org.

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