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Discovery and Characterization of a Peptoid with Antifungal Activity against *Cryptococcus neoformans*

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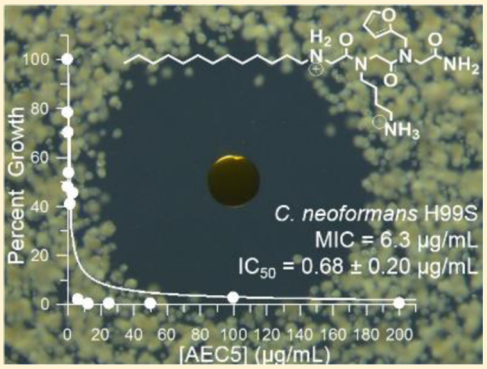
Supporting Information

ABSTRACT: Studies show there is an increasing rate of fungal infections, especially in immunocompromised patients and treatments for fungal genera, such as *Aspergillus*, *Candida*, and *Cryptococcus*, carry significant cytotoxicity with an increasing prevalence of antifungal resistance. We have previously reported a high-throughput assay for identifying peptoids with antimicrobial properties from combinatorial libraries. Here we report the application of this assay in identifying a peptoid with antifungal properties against *Cryptococcus neoformans*. Termed AEC5, this peptoid has comparable potency to existing clinical antifungal agents, excellent stability, and minimal cytotoxicity in mammalian cells.



KEYWORDS: *Peptoids, Cryptococcus neoformans, fungal infections, high-throughput screening*

Recent studies show an increase in fungal infections compared to previously seen data.[1] For example, diseases due to *Candida* species are now the fourth most common nosocomial fungal infection,[2] and infections of the central nervous system by *Cryptococcus neoformans* are the most common form of fungal meningitis.[3] Theories regarding the rise of fungal infections include the increased prevalence of these illnesses in patients with immunocompromising diseases, the growth in international travel, changes in land use, and incorporation of antifungal drugs in agriculture.[1] Currently, nearly 75% of fungal infections isolated from patients in hospitals are of the species *Candida*. The remaining 13% and 6% of fungal infections are due to *Aspergillus* spp. and *Cryptococcus* spp., respectively.[4]

Cryptococcus spp. is a yeast-like fungus with a thick polysaccharide capsule that results in infections located in the pulmonary system and central nervous system.[3] One species, *C. neoformans*, is divided into two serotypes (A and D) and is the most common infectious agent among transplant, and HIV/AIDS patients due to the compromised immune system of these individuals.[5] The most recent estimates indicate that roughly 1 million HIV/AIDS patients contract cryptococcal meningitis each year and that over 600,000 of these cases will be fatal.[6] The greatest prevalence of these infections is in Sub-Saharan Africa, where deaths due to cryptococcal meningitis exceed those due to tuberculosis.[6] Another *Cryptococcus* species is *C. gattii*, which is capable of infecting immunocompetent people.[5] An outbreak of *C. gattii* infections has been observed recently in the Pacific Northwest regions of Canada and the United States.[7]

Treatment for infections due to organisms such as *Aspergillus*, *Candida*, and *Cryptococcus* vary depending upon the patient and species of the infectious organism. Current therapeutics, such as amphotericin B, flucytosine, and fluconazole, carry their own risks, including high mammalian cytotoxicity that results in gastrointestinal complications, vomiting, QT prolongation, and hepatitis.[8−10] Drug resistance has also been observed against these therapeutics in several fungal species and strains.[11] Because of this resistance, current treatments usually include a cocktail or extended regimen of these drugs and possible surgery if there is no response to medication, necessitating new therapeutic options for patients dealing with fungal infections.[9]

One alternative to combat high toxicity levels and drug resistance is the use of antimicrobial peptides (AMPs), which can display minimal toxicity to mammalian cells and may be able to specifically target fungal cells. Antimicrobial peptides used against fungi are characterized by their ability to disrupt the cell membrane structure or form pores that cause intracellular compounds to leak out of the cell, leading to cellular death.[12] While fungal and mammalian cells share many similar features, AMPs are able to target key differences, such as ergosterol, a principal sterol only present in fungal cell membranes.[10] Unfortunately, while showing potency against pathogenic fungi, AMPs are also easily degraded within the human body by proteases.[13] Their short half-life renders AMPs

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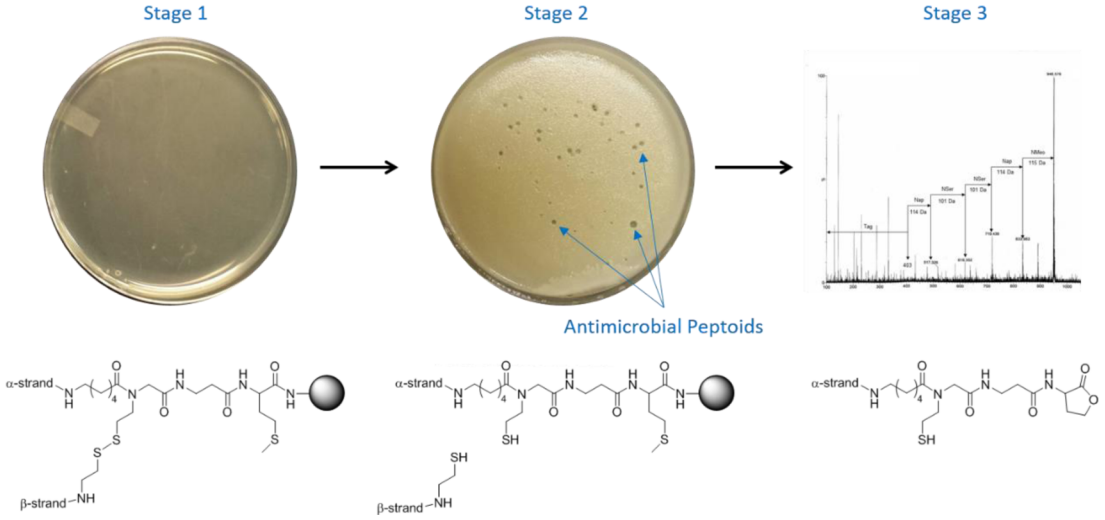

Figure 1. Application of the PLAD assay to screen against *Cryptococcus neoformans*. (Stage 1) PLAD linked peptoids immobilized on beads are imbedded into soft agar containing a small amount of reducing reagent and inoculated with *C. neoformans* H99S. (Stage 2) Overnight incubation resulted in growth of a *C. neoformans* lawn and release of the *beta*-strand peptoid from the bead. Peptoids with antifungal properties result in a zone of inhibition around the bead. (Stage 3) Beads showing zones of inhibition were removed from the plate, *alpha*-strand peptoids cleaved from the bead, and the peptoid structure determined by MS/MS.

unsuitable as clinical therapeutics for combating *in vivo* fungal infections.

One alternative to this problem is the employment of peptide mimics called peptoids. Peptoids, often termed N-substituted glycines, have the side chain attached at the nitrogen in the amide backbone, as opposed to the α-carbon in peptides.[14] This slight structural difference provides peptoids with increased *in vivo* stability and an extended half-life compared to peptides, while presumably maintaining the same or very similar modes of actions.[14,15] Although there are significant studies on antibacterial peptoids,[16−21] little research has been performed using peptoids as therapeutic agents against fungi. Nonetheless, studies have successfully demonstrated that peptoids may be used against some fungal pathogens such as *Candida albicans*,[22] *Fusarium virguliforme*, and *Fusarium lateritium*.[23]

The need in this field of research is for the rapid identification of antifungal peptoids via high-throughput screening of combinatorial libraries. Combinatorial libraries allow for a large cohort of unique compounds to be synthesized and screened in a relatively short period of time.[24−26] Combinatorial libraries produced by split-and-pool synthesis result in a one-bead−one-compound library, where each bead contains thousands of copies of a unique peptoid that is different from any other bead.[24−26]

We have recently reported a technique for the high-throughput screening of peptoid combinatorial libraries known as the Peptoid Library Agar Diffusion (PLAD) assay.[27] This assay relies on a branched linker immobilized on a solid-phase bead that displays parallel strands of identical peptoids. Each strand can be released from the bead orthogonally in response to different chemical stimuli. One peptoid strand, termed the beta-strand, is released during screening to identify peptoids with antimicrobial properties, while the other strand, termed the alpha-strand, is released after screening to identify the peptoid structure by mass spectrometry (MS). Herein we report the optimization and application of this assay to identify a tripeptoid, termed AEC5, with antifungal properties against *Cryptococcus neoformans*. Further characterization of this compound revealed that it has no observable unwanted cytotoxicity against mouse fibroblasts, human hepatocytes, human peripheral lung epithelial cells, or human erythrocytes at the minimum inhibitory concentration for *C. neoformans* H99S.

The PLAD assay was easily amendable to screening for antifungal peptoids against *C. neoformans*, given that this organism forms a robust lawn when grown in inoculated soft agar (Figure 1). *C. neoformans* H99S,[28] a lab strain of serotype A, was chosen for these studies. One requirement of the PLAD assay is that the microorganism being screened must tolerate a small amount of reducing reagent, which is required to cleave the disulfide on the PLAD linker and release the beta-strand peptoid. The tolerance to reducing reagent (dithiothrietol; DTT) and optimal cell count that would produce a robust, even lawn was determined experimentally. This was done by inoculating yeast extract peptone dextrose (YPD) soft agar containing 0 or 10 mM DTT with varying cell counts, solidifying the mixture onto a hard agar YPD plate, and incubating at 37 °C for 20 h. Evaluation of lawn density indicated that a robust lawn was easily formed at the lowest cell count used (1×10^6 cells/mL) (Figure S1). Encouragingly, the addition of 10 mM DTT, which was shown previously to be sufficient for release of the beta-strand during PLAD screening,[27] had no deleterious effects on *C. neoformans* growth regardless of the cell count used. The aforementioned DTT concentration and cell count were used for all subsequent PLAD screenings of *C. neoformans* H99S.

A diverse library of pentapeptoids was synthesized on the PLAD linker system to give a first generation antifungal peptoid library (Figure 2A). Comprising 10 different submonomers, this library had a theoretical diversity of 10^5 compounds. Split-and-pool synthesis on a 0.6 g scale using TentaGel Macrobeads (65,550 beads/g) gave an actual library diversity of 39,300 compounds, which is a moderate but adequate sampling of the theoretical diversity. The submonomers chosen for this library (Figure 2B) were aliphatic (isopropylamine (NVal), isobutylamine (NLeu), benzylamine (NPhe)), cationic (1,2-diaminoethane (Nae), 1,3-diaminopropane (Nap), 1,4-diaminobutane

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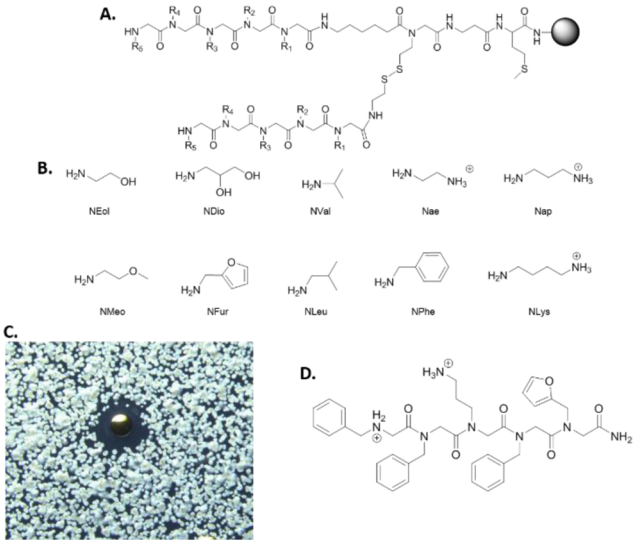

Figure 2. (A) General structure for the PLAD linked first generation antifungal library. (B) Submonomers randomly incorporated into the library were chosen to display polar, hydrophobic, and cationic side chains. (C) Image of AEC89 as identified during PLAD screening against *C. neoformans* H99S. (D) Structure of antifungal peptoid AEC89.

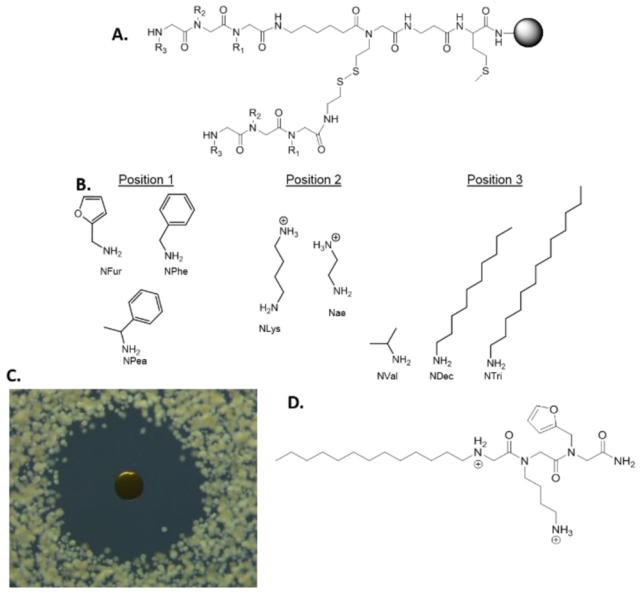

Figure 3. (A) General structure for the previously reported PLAD linked lipopeptoid library. (B) Submonomers incorporated into specific positions of the tripeptoid library to give hydrophobic and cationic side chains. (C) Image of AEC5 as identified during PLAD screening against *C. neoformans* H99S. (D) Structure of antifungal peptoid AEC5.

(NLys)) and polar uncharged (ethanolamine (NEol), 3-amino-1,2-propanediol (NDio), 2-methoxyethylamine (NMeo), and furfurylamine (NFur)). In general, these submonomers were chosen to provide a diverse set of hydrophobic, hydrophilic, and electrostatic properties while maintaining the overall cationic nature of antimicrobial peptides. Library quality was evaluated by MS/MS sequencing of peptoids from randomly chosen beads, which indicated that the alpha-strand of the PLAD linked peptoid was easily fragmented during MS/MS analysis, providing the submonomer sequence of the compound (Figure S2). This entire library was screened via the PLAD assay against *C. neoformans* H99S, evaluating 6 mg of library (~400 beads) per plate. Unfortunately, screening yielded only one "hit" or bead displaying a measurable zone of inhibition. Termed AEC89, the zone of inhibition for this compound was modest (0.270 mm); however, it did indeed appear to inhibit fungal growth (Figure 2C).The sequence of AEC89 was determined by MS/MS to be NPhe-NPhe-Nap-NPhe-NFur (Figure 2D). This peptoid was synthesized via solid-phase submonomer methods on Rink Amide resin, purified by HPLC, and evaluated by a standard broth dilution assay to determine the minimum inhibitory concentration (MIC) against *C. neoformans* H99S. Having an MIC of >200 μg/mL, no further characterization of this compound was done.

We hypothesized that the lack of potent antifungal compounds identified from our first generation antifungal peptoid library might be due to the library's relatively low lipophilicity. Previous studies have demonstrated the utility of long alkyl tails in improving peptoid antimicrobial potency.[16] To evaluate this hypothesis, we screened a previously reported[27] PLAD linked semicombinatorial library of 18 hydrophobic lipopeptides against *C. neoformans* H99S (Figure 3A,B). Several replicates of the library were screened and resulted in several hits with zones of inhibition much larger than those observed for the first generation antifungal peptoid library. Although sequencing of all of the hits identified proved to be problematic, a bead displaying one of the largest zones of inhibition (0.729 mm; Figure 3C) was successfully sequenced by MS/MS. The sequence of this tripeptoid, termed AEC5, is NTri-NLys-NFur (Figure 3D). Given the long 13 carbon alkyl tail and positively charged side chain, this compound exhibits the cationic/hydrophobic nature of many naturally occurring antimicrobial peptides.

To properly characterize the potency of AEC5 against *C. neoformans*, this compound was synthesized on Rink Amide resin, purified by HPLC, and evaluated by standard broth dilution assays to determine the MIC of AEC5 against *C. neoformans* H99S. The MIC against *C. neoformans* H99S was 6.3 μg/mL (Table 1), which is comparable to fluconazole and fluocytosine, two common clinical antifungal agents.[29] Having demonstrated the initial antifungal potency of AEC5, we sought to further evaluate this property by determining the MIC of AEC5 against other serotypes and strains of *C. neoformans*; namely, the serotype D lab strain (ATCC 24067), two serotype A clinical isolates (B18 and B24),[30] and two serotype D clinical isolates (AD 12−27 and AD 12−30).[31] The MIC of AEC5 against all these organisms ranged from 6.3 to 25 μg/mL, while the IC_{50} ranged from 0.68 to 12.2 μg/mL (Table 1 and Figure S3). Given that serotypes A and D are 15% different genetically[32] and the composition of the cell walls are likely different,[33,34] it is not surprising that this compound is not as potent against clinical isolates of serotype D.

We subsequently determined the fungicidal vs fungistatic properties of AEC5 at varying concentrations against *C. neoformans* H99S. The fungicidal concentration of a compound is the drug concentration needed to effectively kill a fungal microorganism, while the fungistatic concentration arrests fungal growth without killing the microorganism. This property was determined by preparing an MIC assay with varying AEC5 concentrations, pooling triplicate samples after the 72 h incubation, washing with PBS to remove AEC5, and spotting aliquots of the residual cells in PBS onto YPD agar plates. Spots of cells at concentrations of drug that are only fungistatic will

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Table 1. Potency Values for AEC5 against Several Serotypes and Strains of *C. neoformans* along with Toxicity Values for AEC5 against Several Mammalian Cell Types[a]

organism	assay	potency/toxicity (μg/mL)
C. neoformans H99S (serotype A lab strain)	MIC (IC_{50})	6.3 (0.68)
C. neoformans 24067 (serotype D lab strain)	MIC (IC_{50})	6.3 (3.9)
C. neoformans serotype A clinical isolate B18	MIC (IC_{50})	25 (12.2)
C. neoformans serotype A clinical isolate B24	MIC (IC_{50})	12.5 (10.7)
C. neoformans serotype D clinical isolate AD 12−27	MIC (IC_{50})	25 (4.0)
C. neoformans serotype D clinical isolate AD 12−30	MIC (IC_{50})	25 (6.9)
NIH/3T3 mouse fibroblasts	IC_{50}	48.6
HepG2 human hepatocellular carcinoma	IC_{50}	56.2
HPL1A human peripheral lung epithelial cells	IC_{50}	36.3
Human erythrocytes	HC_{10}	68.7

[a]MIC = minimum inhibitory concentration; IC_{50} = inhibitory concentration 50%; HC_{10} = hemolysis concentration 10%

lead to recovery of the fungal cells, resulting in colony growth on the YPD agar plate. Spots of cells at concentrations of drug that are fungicidal results in no colony growth, even after drug is removed (Figure S5). Using this assay, we determined that AEC5 is indeed fungicidal at the MIC of 6.3 μg/mL against *C. neoformans* H99S. No colony growth was observed at or above the MIC. Limited colony growth was observed at 3.1 μg/mL, the first concentration below the MIC, and robust growth was observed below this concentration and in the no-drug control.

Having established the potency of AEC5 as an antifungal agent against *C. neoformans*, we next set out to evaluate the unwanted cytotoxicity of this compound against mammalian cells. Specifically, concentrations of AEC5 ranging from 800 to 3.1 μg/mL were tested against NIH/3T3 mouse fibroblasts, HepG2 human hepatocellular carcinoma cells, HPL1A human peripheral lung epithelial cells,[35] and human erythrocytes. Although we were concerned about the potential cytotoxicity of AEC5 given the long 13 carbon alkyl tail, no cytotoxicity was observed in any of the mammalian lines tested at the *C. neoformans* H99S MIC of 6.3 μg/mL. The concentration of AEC5 resulting in a 50% inhibition of growth (IC_{50}) was determined against NIH/3T3, HepG2, and HPL1A cells. This concentration was found to be 50.3 μg/mL for NIH/3T3 cells, 43.6 μg/mL for HepG2 cells, and 36.3 μg/mL for HPL1A cells (Table 1 and Figure S6). These values are roughly 8, 9, and 6-fold higher than the MIC against *C. neoformans* H99S, respectively. Traditionally, hemolytic activity against human erythrocytes is defined as the drug concentration that results in lysis of 10% of the erythrocytes (HC_{10}), due to the sensitivity of an organism to red blood cell lysis. The HC_{10} of AEC5 against human erythrocytes was determined to be 68.7 μg/mL, nearly 11-fold higher than the MIC against *C. neoformans* H99S (Table 1 and Figure S6).

To gain a predictive understanding of the therapeutic potential of AEC5, several physicochemical properties were determined using ChemAxon's MarvinSketch Calculator Plugins.[36] The parameters for commonly administered antifungal agents (amphotericin B, fluconazole, and flucytosine) and commercial antimicrobial peptides (daptomycin, pexiganan, and colistin) were also determined (Table S1). The octanol−water partition coefficient (log*P*), distribution coefficient at pH 7.4 (log$D_{7.4}$), and water solubility coefficient (log*S*) for AEC5 were calculated to be 3.24, −1.18, and −4.13, respectively. Both log*P* and log*D* give a measure of a compound's lipophilicity, which plays a role in the bioavailability and absorptivity of a potential drug compound. The significant difference between log*P* and log*D* values shown here is because log*D* accounts for the ionization of a compound, while log*P* does not. Not surprisingly, with two ionizable amino groups, the predicted isoelectric point of AEC5 is 12.7, and at pH 7.4, the predominant species of AEC5 would be doubly protonated (Table S1), making log*D* the most accurate predictor of AEC5 lipophilicity. These data indicate that even though AEC5 contains a long hydrophobic tail, it still has excellent aqueous solubility. In fact, we have prepared stock concentrations of AEC5 in water up to 50 mg/mL. These physicochemical properties are comparable to the commercial antifungals and antimicrobial peptides analyzed here. Furthermore, the molecular weight, log$D_{7.4}$, and polar surface area are in line with a large number of natural products used to treat infections,[37] indicating that AEC5 may serve as a good lead for antifungal therapeutic development. Furthermore, the stability of AEC5 in human serum was determined following well established protocols.[38] No degradation of AEC5 was observed, even after incubation in human serum for 48 h (Figure S7), demonstrating the stability of antimicrobial peptoids compared to antimicrobial peptides.

In summary, the PLAD assay for high-throughput identification of peptoids with antimicrobial properties has been adapted and applied to screen two different libraries against the fungus *Cryptococcus neoformans*. This screening identified a relatively simple, biologically stable tripeptoid, AEC5, with excellent antifungal potency against various serotypes and strains of *C. neoformans*. Furthermore, the compound exhibited no observable cytotoxicity at the MIC and a significant therapeutic window between *C. neoformans* MIC values and IC_{50}/HC_{10} cytotoxicity values. The reported data regarding AEC5 are preliminary in scope, but with further *in vivo* characterization, AEC5 and other peptoids may prove to be valuable additions to a field of antifungal agents wrought with cytotoxicity and drug resistance issues. Work is currently underway to screen more tailored antifungal peptoid libraries against *C. neoformans* and other pathogenic fungi via the PLAD assay.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00338.

Materials, submonomer synthesis, peptoid synthesis, screening procedures, assay methods, and supplemental figures (PDF)

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Author Contributions

A.E.C., S.A.A., M.E.W., E.E.M., and K.L.B. designed experiments. A.E.C. performed assay development and identified AEC5. A.E.C. and S.A.A. characterized AEC5. M.E.W. performed NMR analysis of AEC5. A.E.C., S.A.A., E.E.M., and K.L.B. cowrote the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

PLAD, Peptoid Library Agar Diffusion; AMP, antimicrobial peptide; DTT, dithiothrietol; YPD, yeast extract peptone dextrose; MIC, minimum inhibitory concentration; IC_{50}, inhibitory concentration 50%; HC_{10}, hemolytic concentration 10%

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