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Synthetic α-Hydroxytropolones as Inhibitors of HIV Reverse Transcriptase Ribonuclease H Activity†

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HIV Reverse Transcriptase-associated ribonuclease H activity is a promising enzymatic target for drug development that has not been successfully targeted in the clinic. While the α-hydroxytropolone-containing natural products β-thujaplicinol and manicol have emerged as some of the most potent leads described to date, structure-function studies have been limited to the natural products and semi-synthetic derivatives of manicol. Thus, a library of α-hydroxytropolones synthesized through a convenient oxidopyrylium cycloaddition/ring-opening sequence have been tested in *in vitro* and cell-based assays, and have been analyzed using computational support. These studies reveal new synthetic α-hydroxytropolones that, unlike the natural product leads they are derived from, demonstrate protective antiviral activity in cellular assays.

It is estimated that 37 million people worldwide are living with Human Immunodeficiency Virus (HIV), the causative agent of AIDS.[1] Resistant viral variants have been identified for virtually all HIV drugs in clinical use.[2] Combination therapies using drugs with different targets have significantly slowed this trend, but resistance development persists, even in patients with undetectable viral load. Thus, identification of new HIV antivirals, in particular those that work through new mechanisms of action, are of significant interest.

Inhibition of reverse transcriptase (RT), an enzyme critical for viral genome replication, has been a highly efficient method of HIV treatment over the last few decades.[3] Clinically, this is achieved using nucleoside and non-nucleoside drugs (NRTI and NNRTI, respectively) that inhibit DNA polymerase activity. Inhibitors of HIV RT polymerase activity account for over half of all HIV drugs currently on the market. HIV RT has a C-terminal ribonuclease H (RNase H) domain that is also necessary for viral replication.[4] Importantly, both the RNaseH and DNA polymerase-active sites can be engaged simultaneously,[5] raising the possibility for combination therapy employing both RNaseH and DNA polymerase inhibitors. HIV RT RNaseH is thus a promising enzymatic target for therapeutic development that remains unexploited clinically, and major efforts are underway to identify viable drug candidates that disrupt this function.[6]

In 2005, a high throughput screen of a National Cancer Institute library of purified natural products identified β-thujaplicinol (βTJ) and manicol as potent inhibitors of HIV RT RNaseH.[7] β-Thujaplicinol and manicol share a rare α-hydroxytropolone moiety that crystal-structure analysis revealed is key for the potent inhibition of the enzyme.[8] Unfortunately, both of these natural products displayed cytotoxicity in cell-based antiviral assays that precluded cellular antiviral activity. In order to address this limitation, a series of analogs of manicol were synthesized, several of which displayed significantly reduced cytotoxicity and moderate antiviral protective effects.[9]

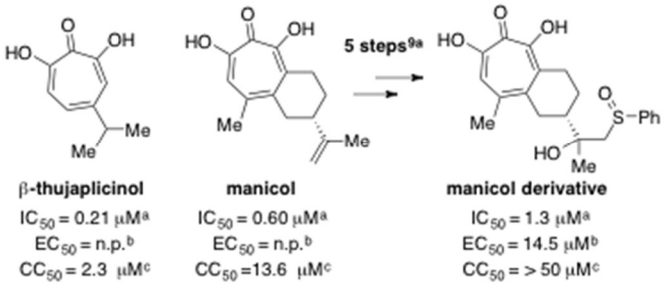


Figure 1. Natural Product α-Hydroxytropolones, and representative example of a derivative synthesized from manicol. [a]HIV RT RNaseH Inhibition Assay. [b]HIV-1_{RF} viral replication suppression. n.p. = non-protective. [b]Cytotoxicity of CEM-SS cells.

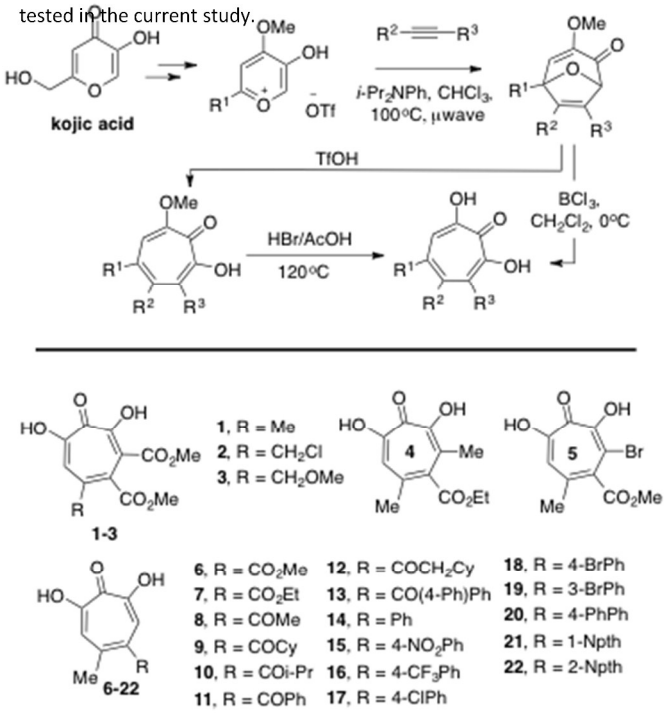
While these studies with manicol and its derivatives provide proof of principle that α-hydroxytropolones can elicit cell-based antiviral activity, there are two limitations to the approach.

†The authors declare no competing interests.

First, the source of manicol is the root bark of a rare Guyanan tree, *Dulacia guianeinsis*,[10] which limits the availability of the natural product. Second, the semisynthetic analogue strategy relies upon the modification of a single alkene appendage, which limits the structural diversity obtainable.

Driven by these limitations, over the last few years our lab has been studying an oxidopyrylium cycloaddition/ring-opening strategy for the synthesis of α-hydroxytropolones (Scheme 1).[11] The starting material is kojic acid, a common natural product used as an additive in food and cosmetics that can be purchased inexpensively (ie $0.80/g from Otava Chemicals). Kojic acid can be converted into pre-ylide oxidopyrylium salts in just 2-3 steps without any need for chromatography. The ready availability of kojic acid and ease of synthesis allows for large-scale generation of oxidopyrylium salts, which can be common intermediates for preparing α-hydroxytropolone libraries. To date, molecules made through this route have been used in preliminary structure-function studies for the aminoglycoside resistance enzyme ANT(2")-Ia,[12] Hepatitis B virus RNaseH,[13] Herpes simplex virus, and the terminase-associated nuclease of Herpes Simplex Virus.[14] In this report, we described the screening and structure-function analysis of a library of synthetic α-hydroxytropolones against HIV RT RNaseH. These studies reveal a series of easily accessible synthetic α-hydroxytropolones that demonstrate inhibition as good as βTJ in enzymatic assays, and superior protective effects in cell-based assays.

Scheme 1. General overview of the synthesis of α-hydroxytropolones from kojic acid, and compounds made through this route that were tested in the current study.



A library of 22 synthetic α-hydroxytropolones synthesized through the oxidopyrylium cycloaddition/ring-opening strategy, along with the natural product βTJ, was tested in two HIV assays. An enzymatic inhibition assay employed fluorescence resonance energy transfer (FRET), a high throughput assay used previously in the identification of α-hydroxytropolones as leads for HIV RT RNaseH (Figure 2A).[15] In parallel, the library was tested in a differential scanning fluorimetry (ThermoFluor) assay,[16] which assessed ligand-induced stabilization of the enzyme against thermal denaturation (Figure 2B).[17] One major difference between the two assays is the presence of oligonucleotide substrate in the former assay. A summary of the data is shown below, and compared to the natural product βTJ.

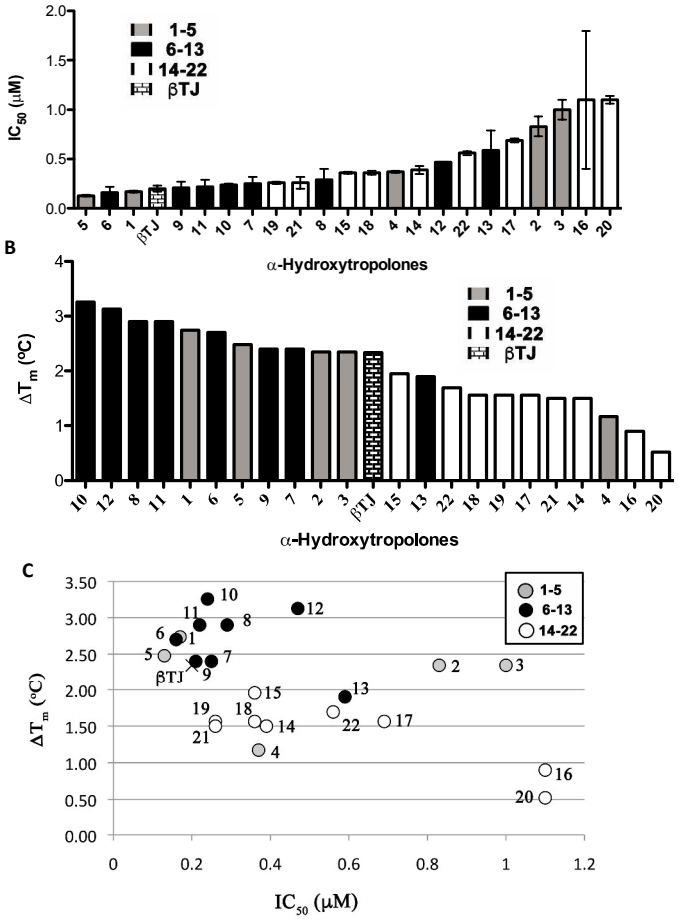


Figure 2. HIV RT RNaseH Enzyme-Associated Assays (A) Enzymatic Inhibition Assay employing FRET and serial dilution of tropolone inhibitors. (B) Thermal stabilization assay using Sypro® Orange dye at 20 μM of tropolones. (C) Plot of ΔT_m versus IC_{50} values. X = βTJ.

Of the two assays, the more robust structure-function trend was observed with the thermal stabilization assays. For example, molecules with aromatic appendages (**14-22**) almost universally gave rise to lower ΔT_m values then those with carbonyl appendages. The two exceptions to this trend were compound 4, which had a electron-donating methyl group at R^3, and a biphenyl ketone (**13**), which possessed a sterically bulky side chain.

One hypothesis that might explain these differences was the electronic effect on the tropolone ring by substituents. Indeed, among the monoaromatic series, nitroaryl 15 demonstrated the greatest ΔT_m, and among the monocarbonyl series, the top 4 stabilizers were ketones. Thus, to quantitatively measure this difference, Mulliken electronegativity values[18] were calculated *in silico* as the average of the absolute values of the HOMO and LUMO energies[19] of the 22 geometry optimized α-hydroxytropolones using the Gaussian program. These results were plotted against ΔT_m measurements, (Fig. 3A) and as expected, a modest correlation was observed (correlation coefficient r = 0.50). While it remains unclear what role, if any, electronegativity plays in increased stabilization, some possibilities for the advantages could be changes in pK_a, resulting in higher overall dianionic character, or enhancement in binding created through increased positive charge character of the tropolone ring.

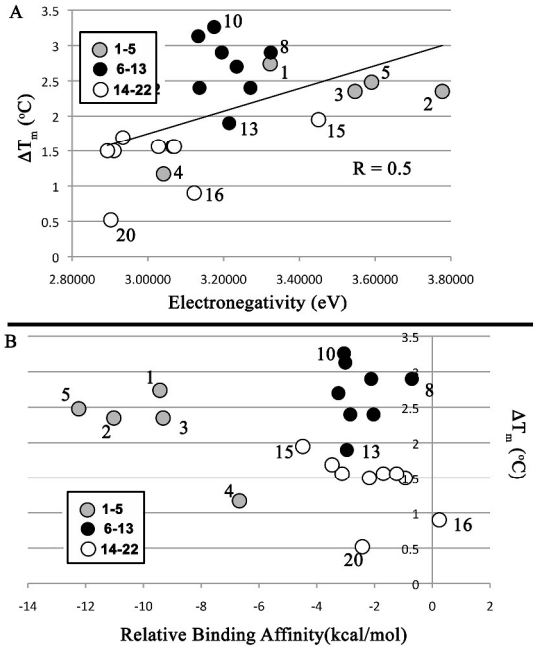


Figure 3. Thermal shift assay data of synthetic α-hydroxytropolones plotted against computationally predicted (A) electronegativity and (B) binding free energy relative to β-thujaplicinol.

It seemed equally possible, however, that the carbonyls present on compounds 1-13 at the R^2 position could have structural benefits either by providing increased flexibility of the side chain to adopt favourable conformations or by providing new favourable contacts between the carbonyl itself with the enzyme. Thus, complementary studies using molecular dynamics simulations were carried out. Structural models of the complexes were obtained by molecular docking[20] to the crystallographic structure of the RNaseH domain fragment of HIV-1 RT bound to manicol (PDB id 3K2P)[8] and alchemical binding free energy calculations were carried out to obtain relative binding free energy estimates (Figure 3B).[21] These give a free energy measure of the ratio of the dissociation constant of each compound relative to that of βTJ, the chosen reference compound (see Supplementary Information).

The binding free energy calculations do not shed light on the observed high ΔT_m values observed for the monocarbonyl-substituted compounds (**6-13**), suggesting that electronic effects may be at play. On the other hand, structural analysis reveals that substitutions at R^3 induce recruitment of Arg 557 that forms ionic interactions with one of the deproptonated hydroxyl groups of the α-hydroxytropolone ligand. The R^3 group itself is found sandwiched between Arg 557 and His 539 (Fig. 4). While no increases in potency are seen experimentally despite these predictions over compound **6**, which has a proton at R^3, the positioning of the group in proximity to this region suggests an important role in exploring this position more thoroughly in future optimization studies.

While these studies provided some promise for computer-guided optimization using thermal stabilization, the more biologically relevant assay was the enzymatic inhibition assay. Unfortunately, as had been seen in previous studies with manicol derivatives, only minimal differences were observed with the synthetic α-hydroxytropolones, with only an order of magnitude difference between the most potent and least potent inhibitors. Despite this small range of activity, however, some trends were observed. For example, while R^2 accommodated moderate sized groups without loss of inhibition, the largest side-chains were typically the least potent. More specifically, of the monocarbonyl series (**6-13**), molecules with the larger side chains (**12** and **13**) were the weakest inhibitors, and likewise **16** and **20** were among the weakest for the monoaromatic series (**14-22**). One of the more dramatic changes, on the other hand, was observed with only a slight increase to R^1 in series **1-3**. Among this series, the slight steric change from a methyl (**1**) to chloromethylene (**2**) to methoxymethylene (**3**) decreased inhibitory potency by close to a full order of magnitude.

Unfortunately, all observed changes seemed to be sterically driven, and any observed increases over the natural product βTJ were minimal. Furthermore, the free energy calculations provided almost no correlation, as may be expected given the higher complexity of the system. However, when comparing changes in inhibition versus T_m changes, some significant trends began to emerge. For example, while changes in inhibitory potency across compounds **1-3** reflected some of the larger changes in inhibition, compounds **2** and **3** remained among the more potent molecules in Thermofluor assays. Another discrepancy was compound **12**, which provided the second strongest ΔT_m, but was among the less potent group in inhibition assays. This suggested that greater steric limitations might apply in the inhibition assays. Since one of the major differences between the two assays was the presence or absence of substrate, and since βTJ has been shown to be a non-competitive inhibitor of RNaseH,[8] considerations for the entire

substrate-enzyme complex would be necessary for understanding changes in the enzymatic activity.

Thus, a homology model of the receptor bound to both the DNA/RNA substrate and α-hydroxytropolone inhibitors was constructed (Figure 4) by structural alignment of the RNaseH domain fragment used in the modeling studies to the RNaseH domain of the structure of full length HIV-1 RT bound to an DNA/RNA hybrid (PDB 4B3O).[22] In the model, the R^1 position overlapped closely with the substrate, suggesting that small changes to the size of the group could lead to destabilizing steric interactions, consistent with observed trends for R^1 in IC_{50} measurements. Furthermore, the R^2 position is in close proximity to the enzyme, but appears to be likely able to avoid some contact, but with larger changes could be impacted. Furthermore, Tyr 501, which is predicted computationally to be involved in interactions with the side chain, is blocked by the substrate. Collectively, it is possible that the differences between the enzymatic and thermal shift data could be explained by the presence of the substrate.

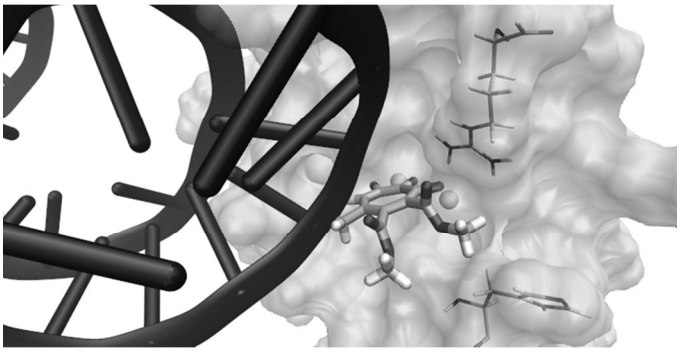


Figure 4. Homology model created between computationally-optimized RT RNaseH fragment bound to ligand **1** and HIV RT bound to RNA/DNA hybrid

This hypothesis is further supported by modeling results. The two metal coordination of α-hydroxytropolones presents a pseudo C2 symmetry axis through the carbonyl group and in the plane of the tropolone ring and the magnesium ions (Figure 4). Rotation by 180° around this axis produces an alternative bound conformation in which the interactions with the metal ions is preserved while the R^1 and R^2 positions exchange location. As it is evident from Fig 4, however, steric hindrance with the substrate likely limits binding to the orientation shown, with the smaller methyl group in closer proximity to the enzyme. When both orientations are considered, the calculations predict that a majority of compounds (17 out of 22) bind significantly more strongly (relative binding free energy -2 kcal/mol and lower) than β-thujaplicinol. This observation is in clear contrast with the IC_{50}'s measurements (Figure 2A) which show that only few compounds have inhibitory potency greater than βTJ. However, the number of compounds predicted to bind better than βTJ is reduced from 17 to 8 when the ligand orientation clashing with the substrate is excluded from the computational model. In addition, 5 of these 8 have a substitution at R^3, which as discussed above reflects receptor-ligand interactions achievable only in the substrate-allowed conformation. Hence computational predictions further support the hypothesis that the substrate has a determinant role in the mode of interaction between the inhibitors and the RNaseH active site.

The homology model further suggests an important role of R^3 in future inhibitor development. Unlike the R^2 position that is oriented mostly toward solvent, explaining the relatively minor variation in activity, R^3 is positioned toward a cavity in the enzyme rich with potential for favourable contacts. One of these residues is Arg557, which **1**-**3** and **5** are computationally predicted to contact. Another residue of interest is His539, which has been engaged in previous classes of RNaseH inhibitors,[6c] and should be possible to engage through modifications of R^3. Efforts are currently underway to adapt our oxidopyrylium cycloaddition/ring-opening method in order to explore this position of the tropolone in greater depth.

In addition to desired potency increases, a major obstacle in the development of α–hydroxytropolone inhibitors of RNaseH for use as antivirals to date has been their cytotoxicity. In addition to the obvious therapeutic disadvantages this creates, it also complicates SAR validation in cells by precluding antiviral activity testing. Thus, we next tested our library in a cellular HIV-1 antiviral assay, which assessed their ability to protect the human T4-lymphoblastoid cell line, CEM-SS, in the presence and absence of HIV-1.[23] Unlike the modest differences observed in HIV RT RNaseH enzymatic inhibition, cytotoxicity varied almost 2 orders of magnitude, with the most toxic analogs displaying cytotoxicity at concentrations of 290 nM, while the least toxic analogs had CC_{50} values of ~20 μM. These results represented approximately an order of magnitude increase and decrease in cytotoxicity over the natural product βTJ. Thus, changes in structure created through our synthetic method can have profound effects on cytotoxicity. It is expected that as greater structural diversity is achieved these differences will increase.

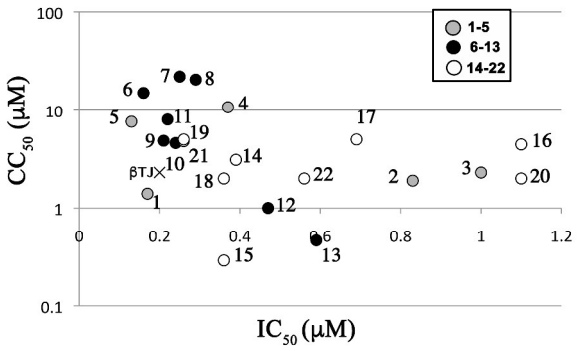
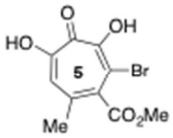
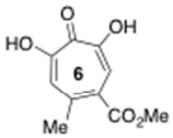
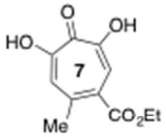
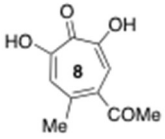


Figure 5. Cytotoxicity of synthetic α-hydroxytropolones and β-thujaplicinol against CEM-SS cells, plotted against HIV RT RNaseH inhibitory activity.

From these studies emerged four molecules, **5**-**8**,that demonstrated significant antiviral effects in cell-based assays (Table 1). As expected, these molecules were among the least cytotoxic of the α-hydroxytropolones tested, as well as among the most potent in enzymatic assays. Structurally, these molecules each shared a small carbonyl-containing moiety. Consistent with prior studies with manicol, the compounds show less potent cellular activity than the corresponding enzymatic activity, which can be attributed to several factors including cell permeability, high concentrations of the substrate, or differences in the native enzyme. Regardless of the reasons, the therapeutic index remains small and thus potency increases as well as decreases in cytotoxicity are clearly needed before these molecules can be studied in advanced models. In this regard, the oxidopyrylium cycloaddition/ring-opening method for the synthesis of α-hydroxytropolones will provide a means for optimization.

Table 1. Data summary of Synthetic α-Hydroxytropolones with Protective Effects in HIV-1/CEM-SS Assays.

	Enzymatic		Cytopathicity	
	IC_{50} (μM)	ΔTm (°C)	EC_{50} (μM)	CC_{50} (μM)
manicol	0.6	n.d.	n.p.	13.6
β-thujaplicinol	0.21	2.3	n.p.	2.3
	0.13	2.48	2.2	7.6
	0.16 (± 0.06)	2.7 (± 0.4)	7.5 (± 0.3)	15 (± 0.0)
	0.25 (± 0.07)	2.4 (± 0.1)	5 (± 0.2)	22 (± 0.0)
	0.29 (± 0.11)	2.9 (± 0.2)	6.9 (± 0.4)	20 (± 1.0)

Natural Product data reported previously in reference 9a. n.d. = not determined. n.p. = not protective. Compounds **6**-**8** are reported as the average of 3 runs ± standard deviation for enzymatic assays and 2 runs ± standard deviation for cytopathicity experiments. Compound **5** is reported from a single experiment.

In conclusion, synthetic α-hydroxytropolones synthesized through an oxidopyrylium cycloaddition/ring-opening have been evaluated against HIV RT RNaseH, and along with complementary computational methods, have provided new synthetic and medicinal chemistry directions for α-hydroxytropolones. The molecules also display a high level of variability in cytotoxicity experiments, demonstrating that the structural changes in the molecules can have strong effects on cytotoxic properties. From these studies, new analogs have emerged with protective effects against HIV in CEM-SS cell-based assays. The oxidopyrylium cycloaddition/ring-opening strategy should thus be valuable method for future studies aimed at further optimization and evaluation of the α-hydroxytropolone pharmacophore for HIV RT RNaseH inhibitor development.

Acknowledgments

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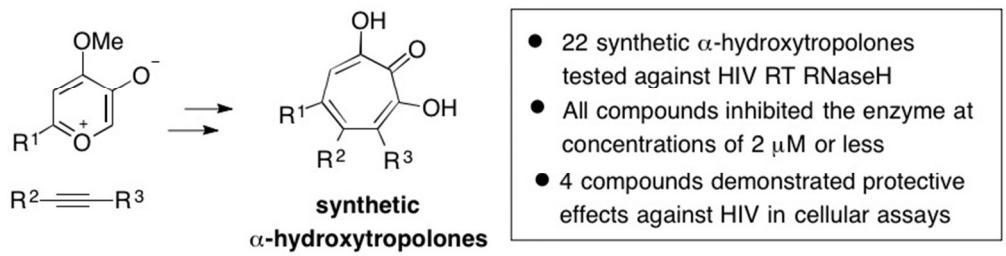

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