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Communication

Tyrosinase-Catalyzed Peptide Macrocyclization for mRNA Display

Matthew C. Fleming, Matthew M. Bowler, Rodney Park, Konstantin I. Popov, and Albert A. Bowers*

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ABSTRACT: mRNA display of macrocyclic peptides has proven itself to be a powerful technique to discover high-affinity ligands for a protein target. However, only a limited number of cyclization chemistries are known to be compatible with mRNA display. Tyrosinase is a copper-dependent oxidase that oxidizes tyrosine phenol to an electrophilic *o*-quinone, which is readily attacked by cysteine thiol. Here we show that peptides containing tyrosine and cysteine are rapidly cyclized upon tyrosinase treatment. Characterization of the cyclization reveals it to be widely applicable to multiple macrocycle sizes and scaffolds. We combine tyrosinase-mediated cyclization with mRNA display to discover new macrocyclic ligands targeting melanoma-associated antigen A4 (MAGE-A4). These macrocycles potently inhibit the MAGE-A4 binding axis with nanomolar IC_{50} values. Importantly, macrocyclic ligands show clear advantage over noncyclized analogues with ∼40-fold or greater decrease in IC_{50} values.

mRNA display is a powerful tool for discovery of macrocyclic peptide ligands against challenging protein targets.[1−4] Macrocyclization typically rigidifies and preorganizes peptide hits into a high-affinity binding conformation. Additionally, macrocyclization tends to contribute to improved stability and cell permeability for potential therapeutic leads or probes.[5−7] Key examples include the peptide macrocycles MK-0616[8−10] from Merck and RA101495[11] from UCB Pharma, both of which are derived from mRNA display hits and have advanced in clinical trials. Despite the success of this technology, the list of peptide macrocyclization chemistries available to mRNA display is limited. Current strategies include cysteine alkylation with exogenous dihaloalkanes,[10,12] copper-catalyzed click reactions,[13] cysteine attack at dehydroalanines,[14] and spontaneous cysteine cyclization onto an N-terminal N^{α}-chloroacetyl group.[15,16] While extant methods are powerful, new cyclization methods will benefit mRNA display by expanding the diversity of libraries through generation of novel macrocyclic scaffolds[17,18] and allowing derivatization of linkages themselves.[19]

Enzymes can offer new routes for peptide cyclization in mRNA display.[20,21] Many enzymes function under mild, aqueous conditions. Additionally, many peptide-modifying enzymes exhibit high levels of substrate promiscuity,[22−24] which make them ideal candidates for application to mRNA display. One such enzyme is tyrosinase, an industrially utilized copper-dependent oxidase, which converts tyrosine phenols to electrophilic *o*-quinones.[25] In biological context, the reactivity of *o*-quinones toward cysteine nucleophiles is essential to formation of melanin and pheomelanin submonomers.[26−28] Recent work has exploited this chemistry for intermolecular bioconjugation, where the reaction has been found to be selective for ligation at C5 rather than C2 (Figure 1A).[24,28,29] Given the speed and biocompatibility of this conjugation strategy, we wondered whether a suitable tyrosinase might be capable of effecting cyclization of peptides containing tyrosine/cysteine pairs.

Herein we describe tyrosinase-catalyzed peptide macrocyclization and its application to mRNA display (Figure 1A). We explore the substrate scope and regiochemistry of peptide cyclization with megTYR, from the bacterium *Bacillus megaterium*,[30] and demonstrate compatibility with mRNA display. We further deploy this enzymatic chemistry in the effective selection of new macrocyclic peptide inhibitors of the cancer-testes antigen MAGE-A4.[2,31] This work provides first evidence of the ability to use tyrosinase in peptide cyclization and uncovers key knowledge for the use of tyrosinase as well as other peptide-modifying enzymes in selection campaigns.

We began our efforts by testing whether tyrosinase could activate and effect *in situ* cyclization of tyrosine-containing peptides. Previous work suggested that abTYR, from mushroom *Agaricus bisporus*, might be incompatible with nucleic acid-fused substrates but that megTYR does not share this sensitivity.[24] Thus, we chose to proceed with megTYR. A model peptide (gene G1; Figure 1B and Supporting Information) was initially employed to test cyclization in an *in vitro* context. G1 contains a C-terminal Cys and N-terminal Tyr, the latter of which we replaced with 3,5-dideuterotyrosine under conditions of translation using the PURExpress *in vitro* translation (IVT) kit. We anticipated that use of 3,5-dideuterotyrosine could simultaneously allow confirmation of cyclization and quantitation of potential C2- and C5-cyclized products by relative integration of the A (monoisotopic) and A +1 peaks of the mass spectrum (MS) (Figure 1B). In the event, treatment of the dideutero G1 peptide with 1 μM megTYR cleanly afforded the Cys-cyclized product. The mass envelope for this intramolecular cyclization product is consistent with a

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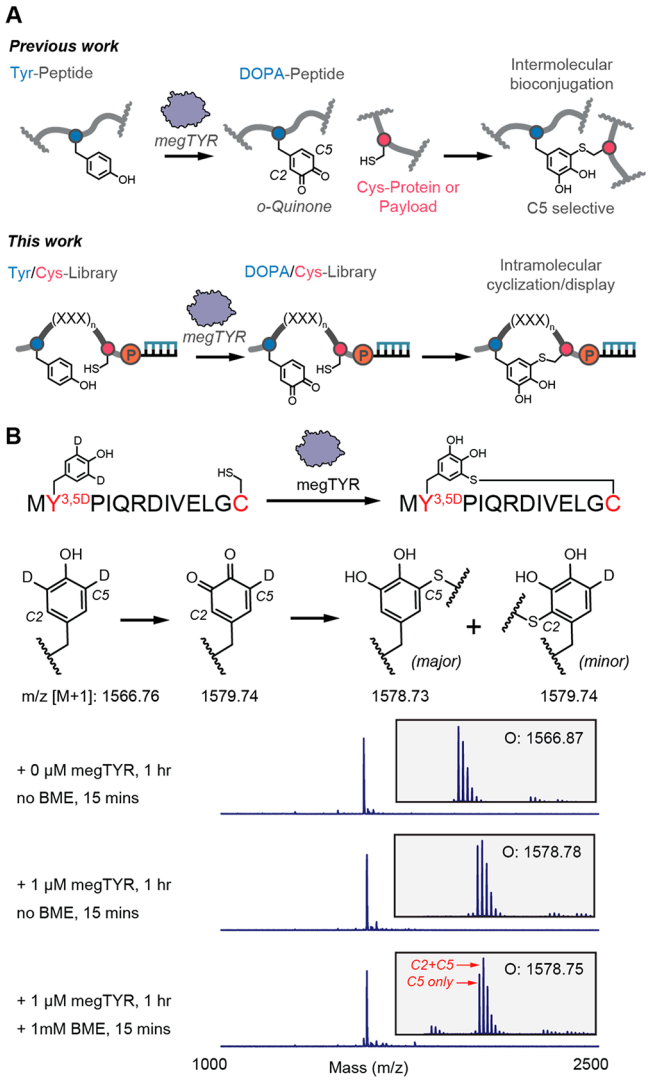

Figure 1. Tyrosinase-catalyzed peptide macrocyclization validation and regioselectivity. (A) Comparison of tyrosinase-catalyzed bioconjugation of macromolecules to proposed mRNA display cyclization showing oxidation of tyrosine to *o*-quinone and subsequent thiol attack. (B) Substrate for proof-of-principle experiments including site of incorporation of 3,5-dideuterotyrosine ($Y^{3,5D}$) and MALDI traces. Treatment with 1 μM megTYR yields predominant cyclization product at C5 (C5:C2 ratio calculated from peak intensity). Product identity can be further validated by BME chase.

2.8:1 mixture of the C5- and C2-modified products (Figure 1B and Supporting Information). Exclusive formation of the macrocycle was further confirmed by BME chase experiments to ensure that no *o*-quinone was left. We additionally tested two analogues of this model peptide, each with a different nucleophilic amino acid at the C-terminus: a Ser hydroxyl or Lys amine. Consistent with literature precedent from the intermolecular reaction, neither Ser- nor Lys-containing substrates show cyclization at 1 μM megTyr (Figures S1 and S2).

We further sought to probe the substrate scope of this cyclization. Employing the same kind of isotope MS assay, we interrogated whether relative positioning of the Cys nucleophile impacted cyclization ability or regiochemistry. The position of the Cys residue was varied within our model sequence to allow formation of three-, six-, or nine-residue macrocycles (Figure 2A, entries 2−4). We additionally examined whether internal and C-terminal Tyr residues could also be activated and cyclized (Figure 2A, entries 5 and 6). After 1 h incubation with 1 μM megTYR treatment, all tested peptides exhibited complete conversion to the cyclic products, with varying C5:C2 product ratios, although C5 remained the major (Figure 2A and Supporting Information). Collectively, these results suggest that regiochemistry may be context-dependent but that cyclization should be broadly applicable to a variety of macrocycle architectures.

Given the robustness of the tyrosinase cyclization with tyrosine itself, we also investigated compatibility with tyrosine and phenylalanine analogues. Such analogues might allow for additional scaffold diversity in future display libraries. Thus, we selected a focused panel of nine analogues, most of which have previously been incorporated via the tyrosyl-tRNA synthetase (Figure 2A, entries 7−14).[32] Of the amino acid analogues tested, L-Dopa, 3-aminotyrosine, and 3-fluorotyrosine exhibit quantitative expression (Figure 2A, entries 7, 8, and 10). In contrast, 3-methyltyrosine, 3-chlorotyrosine, 3-bromotyrosine, 4-nitrophenylalanine, and 4-aminophenylalanine all suffer from background incorporation of trace tyrosine from the translation mixtures (Figure 2A, entries 9 and 11−14). Interestingly, 3-aminotyrosine undergoes facile oxidation/cyclization (Figure 2A, entry 8). Although 3-aminotyrosine has previously been characterized as an inhibitor of abTyr, apparently it can be a substrate for megTyr when embedded in a peptide sequence.[33] Peptides containing 3-methyltyrosine, 3-fluorotyrosine, and 3-chlorotyrosine do not show significant cyclization. These data demonstrate that tyrosine analogues can be used to replace tyrosine in PURExpress IVT to incorporate non-canonical phenolic residues with varied substrate propensities for megTYR.

We next developed a substrate display assay to test compatibility of tyrosinase with mRNA display and assess potential substrate bias. As envisioned, the assay would exploit the *inter*molecular capture of an mRNA-displayed tyrosine with a biotin thiol to report on permissible substrates (Figure 2B). We designed a small RNA library encoding an N-terminal tyrosine next to a randomized four-residue sequence. This library was amplified, P-linked, and displayed, and in the presence of tyrosinase, in a dose-dependent manner, the library could be efficiently captured with biotin thiol (Figure 2C,D). We chose to sequence replicates of the library treated with 125 nM tyrosinase, where incomplete modification might reveal patterns in substrate bias. Three key motifs can be gleaned from the heat map of this display data (Figure 2E): (1) *intra*molecular cyclization is efficient and outcompetes the *inter*molecular capture, as indicated by deprecation of Cys residues; (2) proline directly adjacent to the Tyr hinders activation; and (3) some bias toward negative charge, previously noted by Francis et al.[24] is also present here. Nonetheless, there was high sequence diversity recovered and low sequence convergence, suggesting overall broad substrate promiscuity.

We finally sought to integrate tyrosinase-based peptide cyclization into an mRNA display selection against melanoma-associated antigen A4 (MAGE-A4). MAGE-A4 is an exploratory cancer target that has proven tractable to mRNA display selection as a well-behaved, soluble target with robust assays in hand.[2,31] We next designed a peptide library with 10−12 NNK randomized positions bookended by an N-terminal

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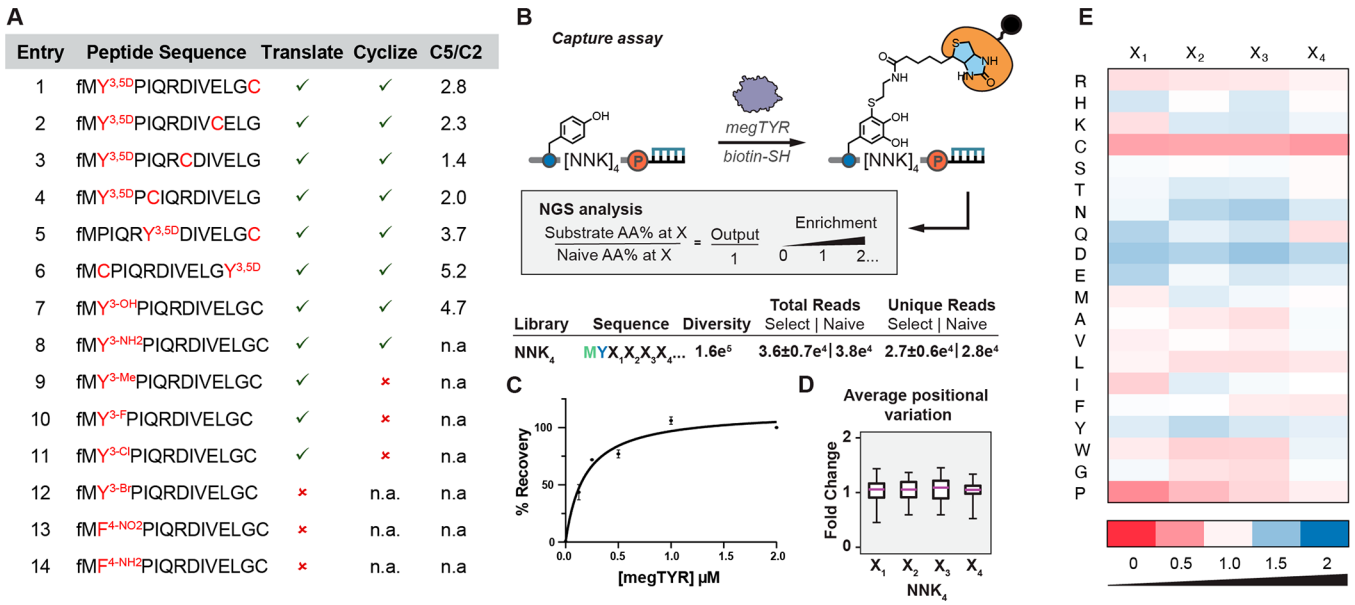

Figure 2. (A) Table of substrate scope for peptide macrocyclization. A green check indicates complete conversion to macrocyclic mass after 1 h in the presence of 1 μM megTYR. (B) Schematic of biotin capture assay developed to assess substrate promiscuity of tyrosinase activation, including table detailing library used and NGS statistics for the assay. (C) qPCR results of streptavidin-captured material (n = 3). Samples treated with 125 nM tyrosinase were sent for NGS sequencing. (D) NGS results for average amino acid positional variation at each randomized position of sequences isolated from capture assay. (E) Heat map of positional enrichment of canonical amino acids in single-round capture assay.

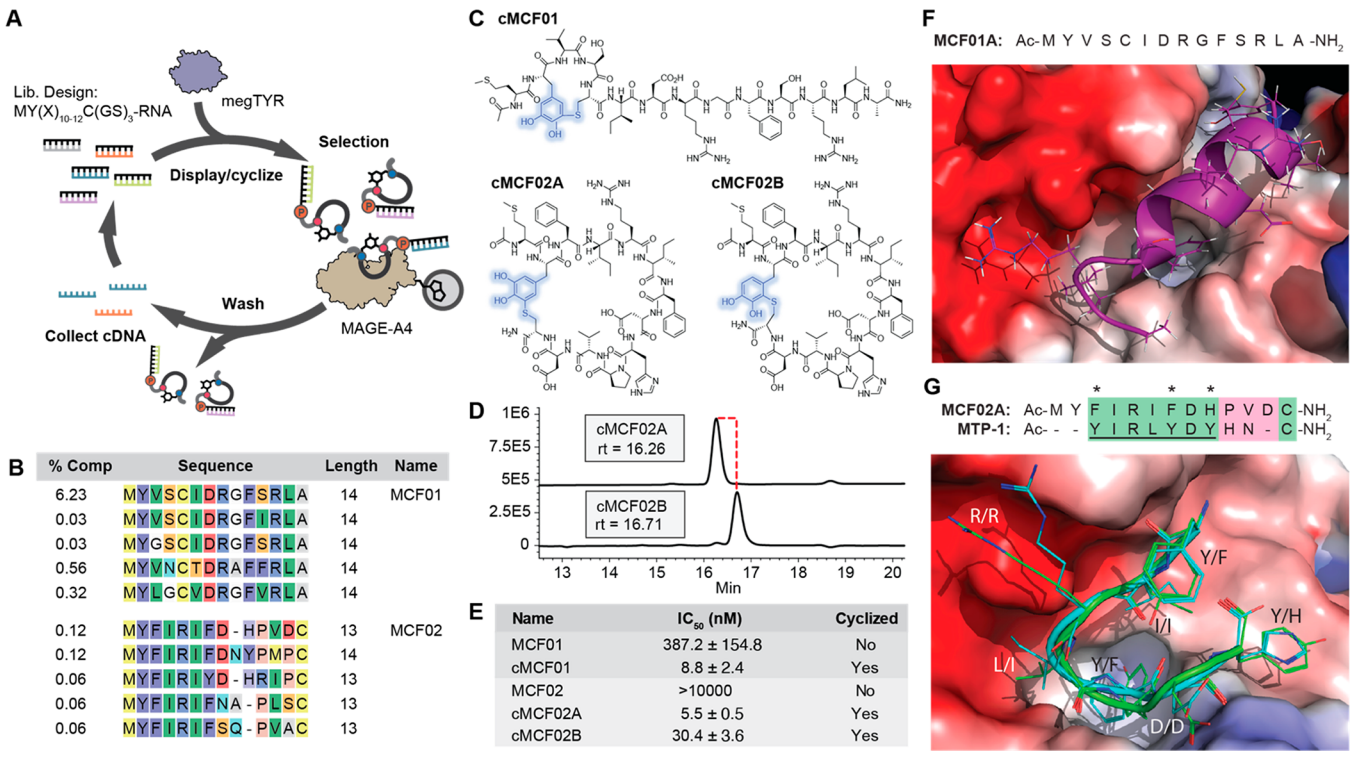

Figure 3. mRNA display of tyrosinase-cyclized peptides against MAGE-A4. (A) Scheme depicting incorporation of tyrosinase into mRNA display. (B) MSA of top two peptide families based on percent NGS composition after four rounds of selection. Names refer to linear peptides (c indicates cyclic variant). (C) Chemical structures of the cyclized peptide hits chosen for synthesis. (D) LCMS traces depicting the difference in retention time between cMCF02A (C5-cyclized) and cMCF02B (C2-cyclized). (E) IC_{50} values for inhibition of FRET between MAGE-A4 and MTP-4 (titration curves in Figure S8). (F, G) Docked poses of energy-minimized MCF01 (F) and MCF02 (G) to depict predicted binding modes. MCF02 is overlaid on the crystal structure of MAGE-A4-MHD bound to homologous MTP-1 binding epitope [PDB entry 7UOA].

tyrosine and a C-terminal cysteine (Figure 3A). The library was translated and treated with megTYR for 1 h, and resulting macrocycle−RNA fusions were selected against immobilized MAGE-A4. After four rounds of selection, robust enrichment was indicated by qPCR, and the cDNA was sent for sequencing (Figure S4). Sequence analysis resulted in two promising families, representing two distinct macrocycle sizes (Figure 3B). The top hit represents a family of Cys-to-Ala

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mutants of the original library with smaller, four-residue macrocycles at their N-termini, while the second family sustain the larger macrocycles of the initial library. The latter sequences also bear a strong similarity to cyclic MAGE-A4 inhibitors identified in a prior Flexizyme-based selection (Figure 3G).[2]

To validate the selection, one representative peptide from each family was chosen for synthesis and testing. Small-scale assays confirm that both peptides are good substrates for megTYR under selection conditions (Figure S7) but that MCF02 forms two potential regioisomers in a ratio of 70:30 (Figure S6). On scale, the enzymatic cyclization products of MCF02 again eluted as two separate peaks of identical mass. Comparison of the retention times to those of products from small-scale reactions with a substrate containing 3,5-dideuterotyrosine confirmed that the major product, cMCF02A, is the C5 cyclization product and the minor product, cMCF02B, is the C2 cyclization product (Figures 3D and S7).[27,28] Both regioisomers as well as cMCF01 and the respective linear precursors were tested in a previously developed TR-FRET assay to assess disruption of the MAGE-A4 binding interaction (Figures 3E and S8).[2] All three macrocycles exhibited potent inhibitory activity with IC_{50} values in the low nanomolar range, while linear versions were significantly less active. The major C5 regioisomer of MCF02 proved more potent than the minor C2 isomer by almost 5-fold, and the IC_{50} of the linear precursor shows little or no inhibition up to 10 μM. Linear MCF01 exhibits an IC_{50} value of 387 nM, while cyclization of the N-terminal region provides an almost 50-fold improvement. Energy-minimized and docked poses of the two peptides suggest that they adopt very different binding modes (Figure 3F,G). MCF01, while not helical in solution, is predicted to bind in a helical conformation, while MCF02 aligns nicely with the binding pose of MTP-1.

Cumulatively, this work adds a new macrocyclization strategy to the mRNA display toolbox. megTYR can activate RNA-conjugated tyrosine residues toward intramolecular Cys-thiol conjugate addition and cyclization. Cyclization is amenable to various macrocycle sizes and accommodates N-terminal, internal, and C-terminal tyrosine residues. This knowledge allowed us to integrate this chemistry into an mRNA display selection that uncovered three new macrocyclic peptides, cMCF01, cMCF02A, and cMCF02B, all potent nanomolar inhibitors of the MAGE-A4 binding axis. cMCF01, the most enriched sequence from this campaign, is a novel sequence, not seen in the prior campaign, suggesting that this cyclization strategy can elicit new structures. Although this work establishes efficacy, the full scope and capability of the methodology remain to be elucidated. Importantly, multiple tyrosines may not be well-tolerated, but supplementing future libraries with tyrosine mimetics through amber codon suppression or other means could potentially surmount this challenge.[32−36] Tyrosine analogues could be particularly helpful in preventing side reactions while promoting library diversity; tyrosine itself could be replaced at internal positions with one of the unreactive analogues, 3-methyltyrosine or 3-chlorotyrosine. Additionally, a better understanding of the substrate promiscuity and/or bias toward this cyclization reaction could be garnered through the more extensive investigation of our substrate display assay, as has recently been employed with several other chemistries adapted for display platforms.[37−39] Lastly, although we have focused on megTYR in this work, additional tyrosinase homologues or engineered variants may prove more adept in future applications.[24,40]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c12629.

Detailed information regarding experimental methods, primers, genes, MALDI-TOF traces, and LCMS characterization (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Albert A. Bowers − *Division of Chemical Biology and Medicinal Chemistry, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States; Center for Integrative Chemical Biology and Drug Discovery, Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, United States; Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States;* orcid.org/0000-0001-8214-7484; Email: abower2@email.unc.edu

Authors

Matthew C. Fleming − *Division of Chemical Biology and Medicinal Chemistry, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States; Center for Integrative Chemical Biology and Drug Discovery, Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, United States;* orcid.org/0000-0003-0258-5025

Matthew M. Bowler − *Division of Chemical Biology and Medicinal Chemistry, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States; Center for Integrative Chemical Biology and Drug Discovery, Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, United States*

Rodney Park − *Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599, United States*

Konstantin I. Popov − *Division of Chemical Biology and Medicinal Chemistry, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States; Center for Integrative Chemical Biology and Drug Discovery, Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy and Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.2c12629

Notes

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