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Inhibition of the ANT(2″)-Ia Resistance Enzyme and Rescue of Aminoglycoside Antibiotic Activity by Synthetic α-Hydroxytropolones

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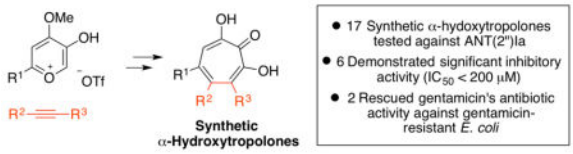
Abstract

Aminoglycoside-2″-O-nucleotidyltransferase ANT(2″)-Ia is an aminoglycoside resistance enzyme prevalent among Gram-negative bacteria, and is one of the most common determinants of enzyme-dependant aminoglycoside-resistance. The following report outlines the use of our recently described oxidopyrylium cycloaddition/ring-opening strategy in the synthesis and profiling of a library of synthetic α-hydroxytropolones against ANT(2″)-Ia. In addition, we show that two of these synthetic constructs are capable of rescuing gentamicin activity against ANT-(2″)-Ia-expressing bacteria.

Graphical Abstract



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‡**Author Contributions**

These authors contributed equally.

Supplementary Material

Supplementary material, including ^{1}H and ^{13}C data of all new compounds, can be accessed online free of charge.

Keywords

α-Hydroxytropolones; Aminoglycoside-2″-O-nucleotidyltransferase; Bacterial Resistance; Aminoglycosides; Gentamicin

Antibiotic resistance is a global health threat that jeopardizes not only our ability to treat bacteria infections, but healthcare as we know it.[1] One strategy to overcome resistance development is to inhibit or disrupt resistance elements.[2] Clavulanic acid, for example, is a β-lactamase inhibitor commonly used in combination with β-lactam antibiotics to prolong their effectiveness.[3] Much like β-lactam antibiotics, aminoglycoside antibiotics are also prone to enzymatic deactivation.[4] One of these enzymes is the aminoglycoside-2″-O-nucleotidyltransferase [ANT(2″)-Ia], which catalyzes the adenylation of several clinically used aminoglycoside antibiotics, such as gentamicin and tobramycin, rendering them inactive (Scheme 1).[5] ANT(2″)-Ia is highly prevalent among pathogenic Gram-negative bacteria, and has been classified along with N-acetyltransferase-6′ [AAC-(6′)] as the most common determinant of enzyme-dependant aminoglycoside resistance in *Pseudomonas aeruginosa*.[6] Thus, therapeutically viable inhibitors of ANT(2″)I-a could find use in combination with ANT(2″)-Ia susceptible aminoglycoside antibiotics.

In 1982, researchers at Eli Lilly reported that α-hydroxytropolone and the α-hydroxytropolone natural product β-thujaplicinol are capable of inhibiting ANT(2″)-Ia and thus restoring aminoglycoside activity against ANT(2″)-Ia-expressing bacteria (Figure 1).[7] Tropolone, meanwhile, showed no activity against the enzyme, illustrating the importance of the 3 contiguous oxygen atoms. While several compounds have emerged for targeting aminoglycoside-modifying enzymes,[8] in particular aminoglycoside mimics,[9] ANT(2″)-Ia inhibitors have been elusive,[10] and α-hydroxytropolones remain one of the only leads.

Our lab has been developing an oxidopyrylium cycloaddition/ring-opening strategy to access α-hydroxytropolones that we believe has the potential to be a general strategy to access these molecules (Scheme 2).[11] Driving these studies is the large number of therapeutically relevant targets that α-hydroxytropolones have been identified as lead compounds for,[12] and the relative scarcity of accompanying synthetic chemistry-driven structure-function studies.[13] We believe that a general, reliable, and practical *de novo* method to access these compounds would be instrumental in fully assessing their therapeutic potential.

Herein, we report the use of this synthetic strategy in a structure-function study of ANT(2″)-Ia. These studies have revealed important structural information related to both the inhibition of ANT(2″)-Ia and the effectiveness at suppressing the enzymatic function in bacteria. As the first report on the use of our synthetic strategy in a medicinal chemistry-based structure-function study, we also highlight the advantages and limitations of the method in its current form.

ANT(2″)-Ia was overexpressed in *E. coli* BL21 (λDE3), and the purified enzyme's activity was monitored in 96 well format through the detection of pyrophosphate (EnzCheck pyrophosphate assay), a by-product of the adenylation of kanamycin B (cf. Scheme 1).

Previously described synthetic α-hydroxytropolones (**3a–3h**)[11] and natural product β-thujaplicinol were tested for their inhibitory activity through an *in vitro* screen with duplicate serial dilutions, and these data are represented by IC_{50} values (Table 1). K_i experiments were obtained on active compounds (IC_{50} < 200 μM) through more rigorous dose curves with carefully maintained concentrations of substrates ATP and kanamycin B. Where K_i values were determined, all compounds demonstrated competitive inhibition with ATP and mixed inhibition with the aminoglycoside antibiotic substrate, suggesting that α-hydroxytroplones bind at or near the ATP binding site.

Among the compounds tested, β-thujaplicinol was capable of inhibiting the enzyme with the greatest potency, with a K_i value of 6.4 μM. While the majority of the synthetic constructs were unable to inhibit the enzyme, there was some significant inhibitory potency of methyl ketone **3e** and nitroaryl **3g**. Notably, these compounds were among the least sterically demanding of the synthetic constructs and were roughly 5–10 fold less potent then β-thujaplicinol, which has the least substitution. This trend may suggest that the compounds bind to an enzymatic pocket that does not as readily accommodate added substitution, and that monosubstituted α-hydroxytropolones may be desired in future optimization studies. Unfortunately, the inability to access monosubstituted derivatives (ie R^1=H, Scheme 2) represent a shortcoming of our synthetic method as it currently stands, and efforts are currently underway to overcome these limitations. Alternatively, other methods are available to target monosubstituted α-hydroxytropolones that could be used.[14]

α-Hydroxytropolones appear to have privilege for dinuclear metalloenzymatic inhibition, with established activity against several enzymes of this class including ribonuclease,[15] integrase,[16] phosphatase[17] and phospholipase[18] enzymes. In each of these cases, it has been proposed (and against certain RT RNase H confirmed crystallographically)[19] that the binding mode leverages all three contiguous oxygen atoms to bind to the two metals (ie Scheme 3). This is made possible by the highly charged character at physiological pH,[18] and Lewis basicity of the carbonyl oxygen due to the stabilized tropylium. Consistent with studies on ANT(2″)-Ia, tropolone is generally inactive versus α-hydroxytropolone-inhibiting dinuclear metalloenzymes.

While not definitive, previously reported kinetic data suggests that the adenylation of ANT(2″)-Ia may work through a mechanism involving two magnesium ions in the enzyme's active site.[20] Meanwhile, prior studies on α-hydroxytropolone inhibition of ANT(2″)-Ia showed that inhibitory activity is influenced much more strongly by ATP then the aminoglycoside substrate,[7a] and we confirmed this trend in our own studies. While direct binding of α-hydroxytropolones to ATP cannot be ruled out, this seems unlikely due to the observed IC_{50} values relative to the concentration of the ATP (25 μM) in the inhibition assay (as low as 6 μM). The more likely explanation is that the α-hydroxytropolones are competing for an ATP binding site of ANT(2″)-Ia. Further supporting this hypothesis is that the distance between metal ions found in α-hydroxytropolone-bound HIV RT RNase H crystal structures demonstrate a metal-metal bond distance comparable to that found in a recent two metal ATP-enzyme complex (3.76Å vs. 3.91Å, Figure 2).[21]

One advantage of our synthetic method is that it allowed us to access methoxytropolone congeners of the ANT(2″)-Ia-active compounds **3e** and **3g** (**4e** and **4g**, Figure 3),[11b] which we found inactive against ANT(2″)-Ia. While certainly not confirmatory, and other explanations could exist such as steric increases due to the methyl group, this inactivity of these compounds is at least consistent with binding to 2 metals analogous to the aforementioned α-hydroxytropolone-inhibiting enzymes. Furthermore, we would like to raise the possibility that other small-molecule ligands that target dinuclear metalloenzymes through similar binding mechanisms, such as napthiridinones[22] and pyrimidinols,[23] may also inhibit the enzyme and could also be useful in future ANT(2″)-Ia-inhibitor development.

We next set out to synthesize and test close variants of the active compounds **3e** and **3g**. Thus, a series of new α hydroxytropolones were synthesized through our oxidopyrylium cycloaddition/ring-opening method (Table 2). The overall processes were in general high yielding, and details of these procedures can be seen in the supporting information. Among the aryl series, phenylacetylene-derived **3i** showed comparable potency to nitroaryl-containing **3g**, but napthyl derivatives **3j** and **3k** showed no activity at all, supporting the theory of a sterically congested binding pocket. Analogs with electron-withdrawing substituents were also synthesized. Excitingly, **3l** and **3m** both showed almost 4 fold increase in activity over the other synthetic α-hydroxytropolones. **3n**, on the other hand, had no activity at all. This was surprising given the similar electronics of trifluomethylarenes and haloarenes, but might be explained due to greater sterics in lieu of the sp3-hybridized nature of the CF_3 group. Among the ketone series, only **3o** showed appreciable inhibitory activity, which was comparable to **3e**. Given that **3p** and **3q** have larger side-chains, their inactivity could again be indicative of an enzymatic pocket that does not readily accommodate higher levels of substitution.

With a series of ANT(2″)-Ia-inhibiting α-hydroxytropolones, we wanted to gauge whether or not the molecules could potentiate the activity of ANT(2″)-Ia-susceptible aminoglycoside antibiotics against ANT(2″)-Ia-expressing bacteria. A hindrance often associated with the identification and development of inhibitors against a specific target involves the lack of antibacterial activity.[24] Often, the molecule exhibits potent inhibition of the enzyme *in vitro*, however, the lack of cellular activity is largely attributed to the inability of the molecule to enter the bacterial cell or is actively effluxed from the cell. Thus, a hyper-permeable, ANT(2″)-Ia-expressing strain of *E. coli* BW25113 was constructed by cloning the *aadB* gene into the pGDP4 vector,[25] which led to gentamicin resistance (MIC of 64 μg/mL vs. 0.25 μg/mL of wild type). These resistant bacteria were treated to serial dilutions of gentamicin and the ANT(2″)-Ia inhibiting α-hydroxytropolones in a checkerboard fashion in order to test the tropolone's ability to rescue antibiotic activity in resistant cells (Figure 4). Quantitative assessment of synergy, or the ability of the molecules to work better in concert than the sum of their parts, is determined by calculating the FIC index (FICI)[26] according to CLSI guidelines.[27] FICI values less then 0.5 are considered synergistic, while values between 0.5-4 are classified as no interaction and FICI > 4 are antagonistic.[28] More qualitatively, one can observe the shapes of the shaded parts of the 2D graphs, and notice that those deemed synergistic (FICI < 0.5) are triangular in shape as a result of the effects of

the compounds in combination with one another. For a negative control, we used an isogenic *E. coli* mutant with the gene encoding aminoglycoside phosphotransferase (APH[2″]-Id), an enzyme conferring resistance to gentamicin that is not inhibited by α-hydroxytropolones.

Consistent with the previous reports by researchers at Eli Lilly, β-thujaplicinol showed synergy with gentamicin in our experiments, with an FICI of 0.375 (Figure 4). Meanwhile, the molecule showed no synergy with gentamicin against the ANT(2″)-Ia-negative, APH(2″)-Id-positive bacteria, which supports that this synergy is likely due to inhibition of the ANT(2″)-Ia enzyme. This also shows that these molecules are not simply promiscuous for ATP-binding proteins and may be selective for ANT(2″)-Ia. Synergy is also seen with ketone-containing **3e** and **3n**. Biaryl compounds **3g**, **3h**, **3l**, and **3m**, all showed no synergistic activity with ANT(2″)-Ia. It is at present unclear why these molecules are inactive in the cellular experiments, however a few possibilities involve low cell permeability of this class, alternative changes to the cells by the molecules, or other targets within the cell that may reduce this group's effectiveness.

Among the active compound, the relative inhibitory potency revealed itself during the checkerboard analysis. At 12 μM of β-thujaplicinol and **3e**, gentamicin has an MIC of 8 μg/mL and 32 μg/mL respectively (See Figure 4), a trend that can be explained based on the relative potency determined in the ANT(2″)-Ia inhibition assay. On the other hand, while all of the bacterial cells have died at 50 μM of β-thujaplicinol, a healthy amount of cells (~80%) remain at 50 μM of **3e**. At these elevated concentrations of **3e**, no growth is seen at the lowest concentration of gentamicin tested (2 μg/mL). Thus, the highest concentrations of **3e** non-toxic to *E. coli* are more effective at inhibiting ANT(2″)-Ia and restoring aminoglycoside activity in cells then the highest non-toxic concentrations of β-thujaplicinol are. While this doesn't have therapeutic advantages, it suggests that **3e** could have advantages over the natural product in studying ANT(2″)-Ia in cellular contexts where background antibacterial activity could be problematic.

Herein, we have described several synthetic α-hydroxytropolones that are capable of inhibiting ANT(2″)-Ia, and two that have specific synergistic activity with gentamicin against gentamicin-resistant, ANT(2′)-Ia-expressing *E. coli*. One of these compounds has significantly less antibacterial activity with respect to *E. coli* than the natural product lead, β-thujaplicinol, and could have advantages in the study of the enzyme in cellular contexts. These results validate the oxidopyrylium cycloaddition/ring-opening method as a viable approach to generating new ANT(2″)-Ia-inhibitors, and provide some preliminary insight into the structural changes required for inhibitory activity and cellular efficacy

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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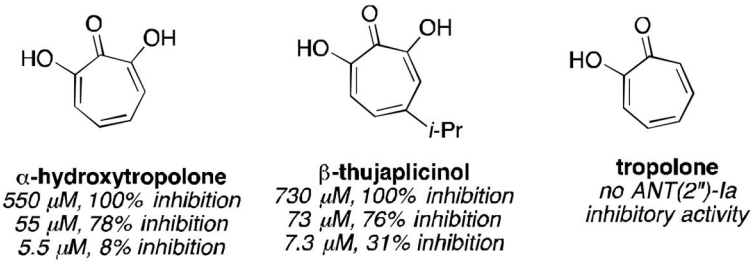


Figure 1.
ANT(2″)Ia inhibition data of troponoids (Eli Lilly, 1982)[7a]

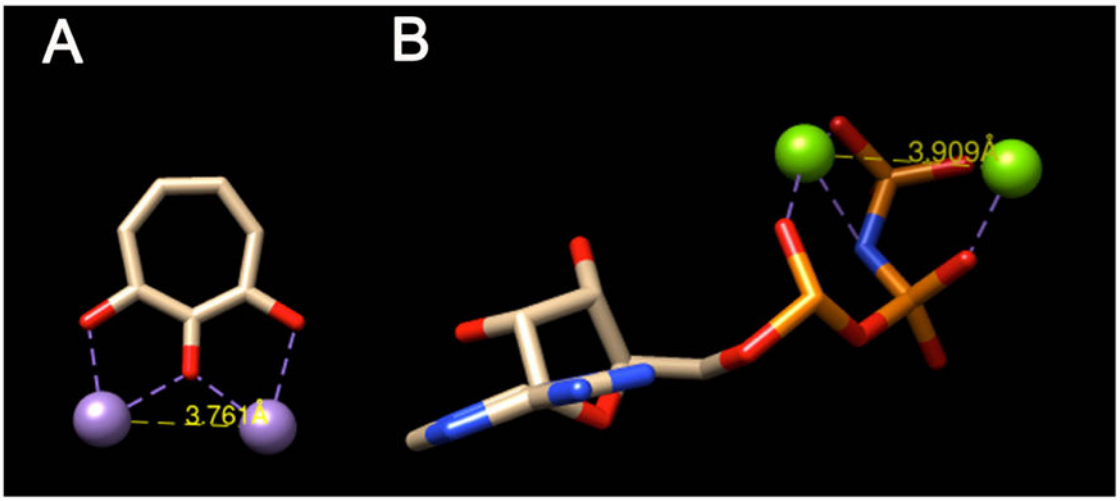


Figure 2.

(A) α-Hydropolone bound to two Mn ions, adapted for clarity from crystal structure data of β-thujaplicinol bound HIV RT RNase H domain (pdb3K2B). (B) ATP mimic bound to two Mg ions, adapted for clarity from crystal structure data of the compound bound to Protein Kinase A (pdb4HPU). Structures were modified using UCSF Chimera to include only ligand and metals, and distances were also calculated from these pdb files.

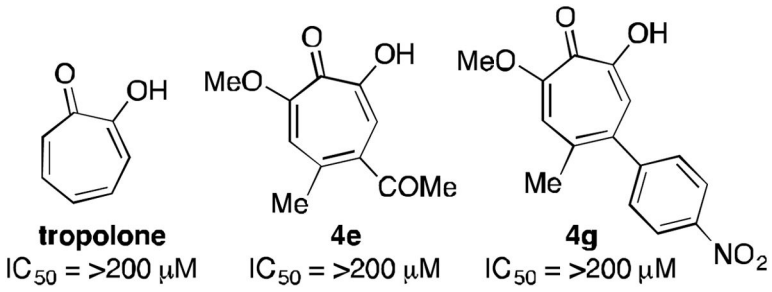


Figure 3.

ANT(2″)-Ia-inactive α-hydroxytropolone congeners that are consistent with a bimetallic binding mechanism

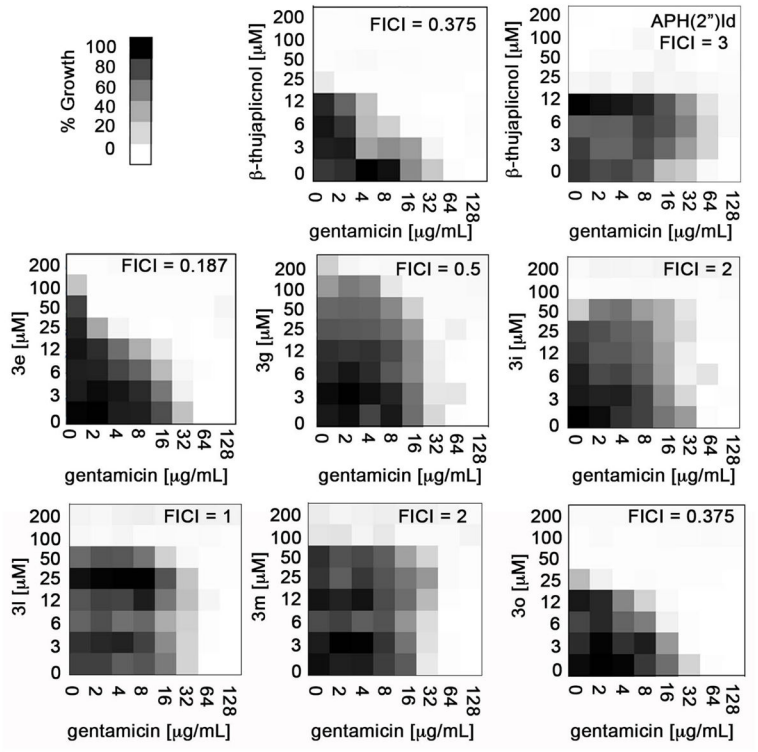
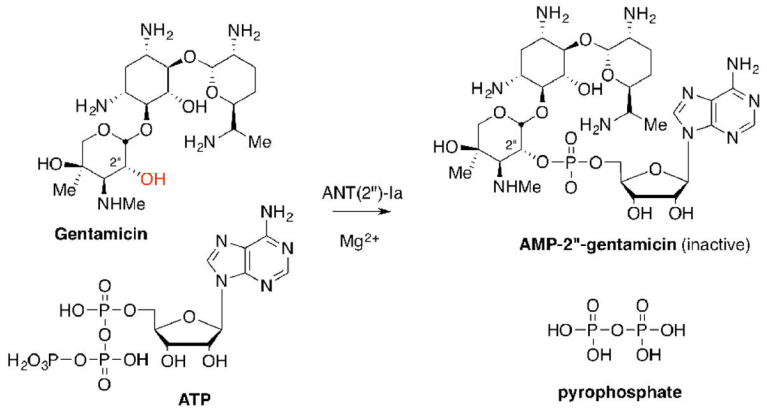


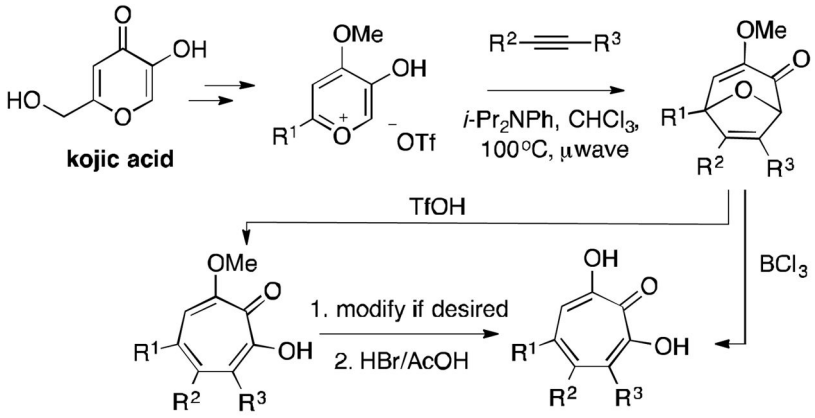
Figure 4.

Checkerboard analysis of ANT(2″)-Ia inhibitors with gentamicin against an ANT(2″)-Ia-expressing *E. coli* along with FICI values. Also shown is data with APH(2″)-Id-expressing bacteria with β-thujaplicinol



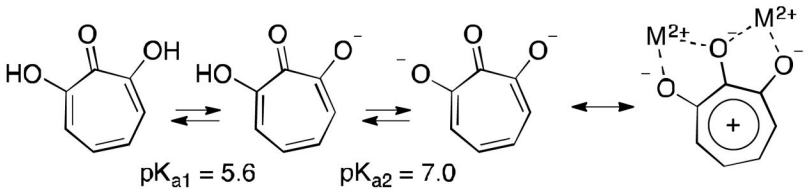
Scheme 1.

Deactivation of gentamicin by the aminoglycoside resistance enzyme, ANT(2″)-Ia, through magnesium-dependent adenylation



Scheme 2.

Synthetic method for the synthesis of α-hydroxytropolones



Scheme 3.

Tridentate array of negatively charged oxygens and proposed binding mode for several dinuclear metalloenzymes. It is possible that ANT(2″)-Ia may also be bound in a similar manner.

Table 1

ANT(2″)-Ia inhibition by known α-hydroxytropolones

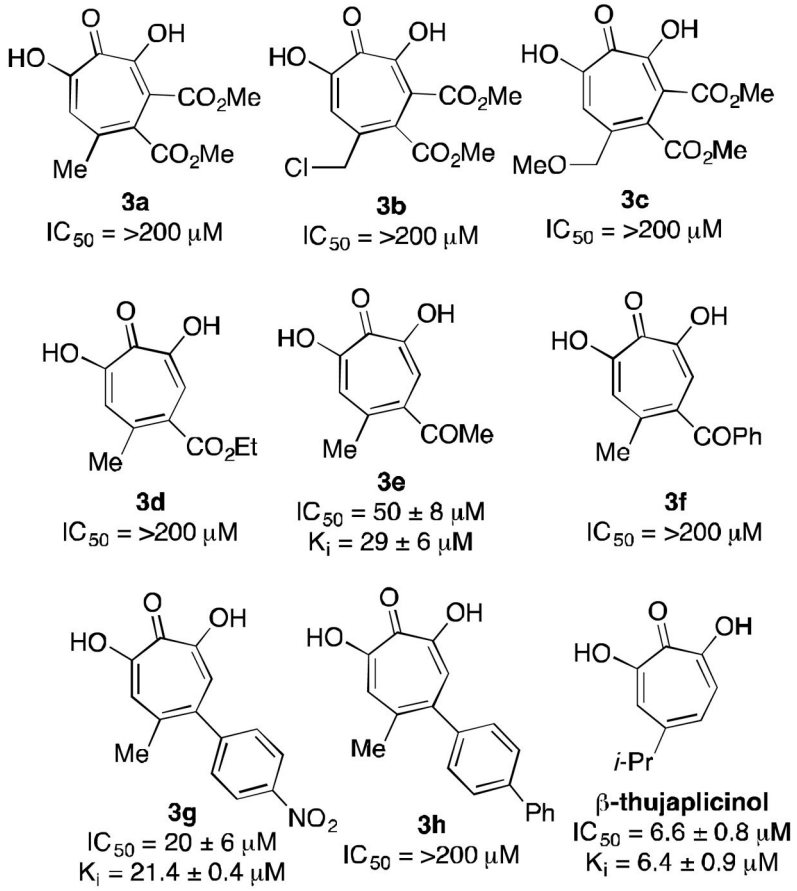
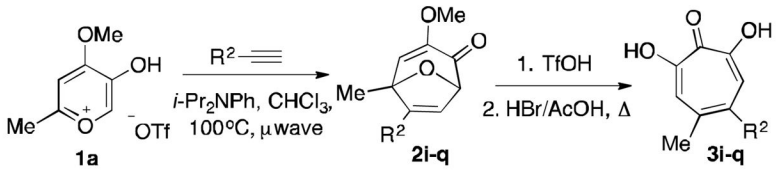
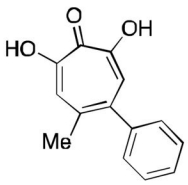


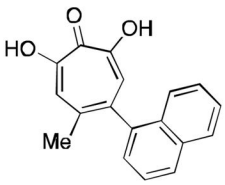
Table 2

Synthesis of α-hydroxytropolones 3i–q and ANT(2″)-Ia inhibition data

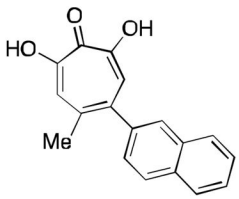




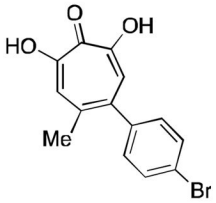
3i
% yield **2i** = 57%
% yield **3i** = 95%
IC_{50} = 117 ± 68 μM
K_i = 25 ± 3 μM



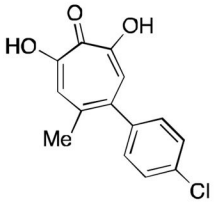
3j
% yield **2j** = 79%
% yield **3j** = 77%
IC_{50} = >200 μM



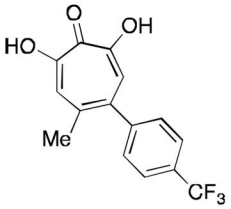
3k
% yield **2k** = 84%
% yield **3k** = 85%
IC_{50} = >200 μM



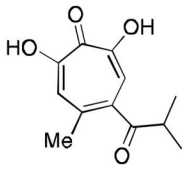
3l
% yield **2l** = 61%
% yield **3l** = 86%
IC_{50} = 8 ± 1 μM
K_i = 5.7 ± 0.8 μM



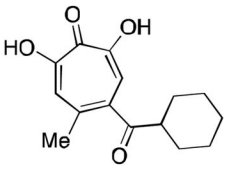
3m
% yield **2m** = 40%
% yield **3m** = 90%
IC_{50} = 9.9 ± 0.6 μM
K_i = 6.9 ± 0.2 μM



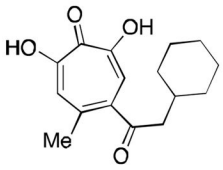
3n
% yield **2n** = 72%
% yield **3n** = 63%
IC_{50} = >200 μM



3o
% yield **2o** = 70%
% yield **3o** = 77%
IC_{50} = 73 ± 31 μM
K_i = 19 ± 0.3 μM



3p
% yield **2p** = 87%
% yield **3p** = 84%
IC_{50} = >200 μM



3q
% yield **2q** = 64%
% yield **3q** = 82%
IC_{50} = >200 μM