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Note

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Discovery and optimization of two Eis inhibitor families as kanamycin adjuvants against drug-resistant *M. tuberculosis*

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KEYWORDS *Aminoglycoside acetyltransferase; Bacterial resistance; Enzyme inactivation; Drug combination; Structure-activity-relationship analysis.*

ABSTRACT: Drug-resistant tuberculosis (TB) is a global threat, and innovative approaches such as using adjuvants of anti-TB therapeutics are required to combat it. High-throughput screening yielded two lead scaffolds of inhibitors of *Mycobacterium tuberculosis* (*Mtb*) acetyltransferase Eis, whose upregulation causes resistance to the anti-TB drug kanamycin (KAN). Chemical optimization on these scaffolds resulted in several potent Eis inhibitors. One compound restored the activity of KAN in a KAN-resistant *Mtb* strain. Model structures of Eis-inhibitor complexes explain the structure-activity-relationship. In the future, these Eis inhibitors may potentially be developed into KAN adjuvant therapies against KAN-resistant *Mtb*.

Despite extensive efforts to discover new antitubercular agents in recent years, tuberculosis (TB) remains the top bacterial cause of mortality worldwide. The proportion of new multidrug-resistant (MDR)-TB cases has not changed in recent years. MDR strains of *Mycobacterium tuberculosis* (*Mtb*) are resistant to the first-line antituberculars isoniazid and rifampicin. Extensively drug-resistant (XDR) strains of *Mtb* are additionally resistant to fluoroquinolones and at least one of the second-line injectable anti-TB drugs, capreomycin, kanamycin (KAN), or amikacin. XDR-TB has very poor therapeutic outcomes. Therefore, the discovery and development of new therapeutics is needed.

KAN is currently used to treat MDR- and XDR-*Mtb* infections. Resistance to KAN observed in one-third of KAN-resistant infections is due to the upregulation of the enhanced intracellular survival (Eis) protein in *Mtb*.[1] We previously established that Eis modifies aminoglycosides (AGs),[2] capreomycin,[3] and other lysine-containing biological molecules[3] by a unique multiacetylating mechanism.[4-6] We also reported crystal structures of Eis-CoA complexes and that of an Eis_*Mtb*-CoA-tobramycin complex.[7-10]

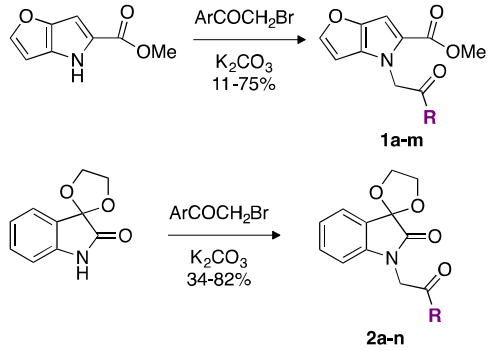
New Eis inhibitors that can be used in combination with AGs such as KAN is a potential way to overcome the resistance due to Eis upregulation. We reported some structural scaffolds with inhibitory activity against the purified Eis enzyme *in vitro*.[11] Here, we report two new scaffolds: the methyl 4*H*-furo[3,2-*b*]pyrrole-5-carboxylate (scaffold **1**) and the 3-(1,3-dioxolano)-2-indolinone (scaffold **2**) identified by high-throughput screening HTS, the synthesis of 13 and 14 analogues of these scaffolds, respectively, and their biochemical and biological testing.

First, we screened ~123,000 small molecules for inhibition of KAN acetylation by Eis_*Mtb*. The HTS yielded two promising scaffolds **1** and **2** (Fig. S1A). Sixty compounds containing scaffold **1** (Fig. S2) were present in the HTS library. In these sixty compounds, the pyrrole ring was decorated with different groups including alkyl chains, aryl and oxazole rings, as well as amides. Fifty-nine of these scaffold **1** molecules did not inhibit Eis_*Mtb*, however compound **1a** (Fig. S1B and Scheme 1, also labeled as **4kk** in Fig. S2) displayed some inhibition in the HTS. Because **1a** contained an aryl ketone group, we opted to synthesize **1a** along with 12 additional analogues (**1b-1m**) comprised of different aryl groups (Scheme 1). For scaffold **2**, ten compounds with different groups attached to the indolinone ring were in the HTS library (Fig. S3). A hydrogen (**8a**), an alkyl (**8b**), an alkyl ketone (**8c**), a carboxylic acid (**9a**), or an amide (**9b**) substituent resulted in no Eis_*Mtb* inhibitory activity. Similarly to the scaffold **1** analogues, aryl ketones of scaffold **2** (**8f-8h**) displayed Eis_*Mtb* inhibition, with the exception of the trifluoromethyl substituted aryl ketones (**8d** and **8e**). Three compounds synthesized from scaffold **2** (**8f-8h**, Fig. S3) were found to be active. These and 11 of their analogues containing different aryl substituents for further study (*Note*: **8f-8h** are numbered **2g-2i** in scheme 1 and Fig. S1 to represent the scaffold **2** series).

Compound **1a** and 12 analogues (**1b-1m**) as well as 14 analogues for scaffold **2** (**2a-2n**) with different R substituents were synthesized for structure-activity-relationship (SAR) analysis of Eis_*Mtb* inhibition *in vitro* and *in cellulo* (Scheme 1). All new compounds were characterized by ^{1}H, ^{13}C NMR (Figs. S5-S56), mass spectrometry, and were established to be ≥95% pure by HPLC prior to further testing.

We evaluated biochemical (inhibition (IC_{50}) of purified Eis_*Mtb* enzyme) and biological (effect on the KAN MIC values for KAN-sensitive *Mtb* H37Rv and KAN-resistant *Mtb* K204 cells) properties of these compounds, in parallel (Table 1; Fig. S57). The freshly synthesized **1a**, which displayed some inhibition of Eis_*Mtb* in the HTS campaign, was confirmed to be a good Eis_*Mtb* inhibitor *in vitro* (IC_{50} = 3 ± 1 μM). In the presence of **1a**, KAN displayed an MIC of 5-10 μg/mL against *Mtb* K204. Having confirmed the weak inhibitory activity of **1a**, we explored the effect of substitution on the phenyl ring on the aryl ketone part of scaffold **1**. *Ortho* substitution, as in **1b** with a *o*-fluoro substituent, resulted in almost the same Eis_*Mtb* inhibitory activity (IC_{50} = 2.9 ± 0.9 μM) as that for the parent **1a**. KAN MIC against *Mtb* K204 was unaffected by **1b** (MIC_{KAN} = 10 μg/mL). To establish if *meta* or *para* substitution would be more favorable than *ortho* substitution, we generated compounds **1c-1j**. For both *meta* and *para* substitutions, bulkier substituents led to weaker Eis_*Mtb* inhibition (IC_{50} > 200 μM for *m*-methoxy (**1f**) and *p*-bromo (**1i**) compared to IC_{50} = 0.16 ± 0.07 and 0.3 ± 0.1 μM for *m*-fluoro (**1c**) and *p*-fluoro (**1g**), respectively). Most of these derivatives (**1c-1i**) did not improve KAN activity against *Mtb* K204. The *p*-methyl derivative **1j** displayed almost the same Eis_*Mtb* inhibitory activity (IC_{50} = 5.8 ± 1.8 μM) and MIC value against *Mtb* K204 as that of **1a**. We generated **1k**

(naphthyl substituted) with the hope of strengthening any possible π–π interaction between the inhibitor and the AG-binding site of the *Mtb* Eis. This compound was found to be completely inactive (IC_{50} > 200 μM and MIC_{KAN} = 10 μg/mL against *Mtb* K204). Finally, replacing the phenyl ring with alkyl chains (ethyl (**1l**) and *t*-butyl (**1m**)) did not improve the Eis inhibition or MIC_{KAN} for K204 *Mtb*.



R: **a** = Ph; **b** = *o*-F-Ph; **c** = *m*-F-Ph; **d** = *m*-Cl-Ph; **e** = *m*-Br-Ph; **f** = *m*-OMe-Ph; **g** = *p*-F-Ph; **h** = *p*-Cl-Ph; **i** = *p*-Br-Ph; **j** = *p*-Me-Ph; **k** = naphthyl; **l** = Et; **m** = *t*-Bu; **n** = *m*-NO_2-Ph

Scheme 1. Preparation of potential Eis inhibitors scaffold **1** scaffold **2** core structures generated in this study.

For scaffold **2**, compound **2g-2i**, which displayed Eis_*Mtb* inhibition in the HTS, were freshly synthesized. The *p*-fluoro substituted **2g** displayed good Eis inhibitory activity (IC_{50} = 0.09 ± 0.03 μM) and when used in combination with KAN resulted in MIC_{KAN} of 5 μg/mL against K204 *Mtb*. The *p*-chloro substituted **2h** was less active (IC_{50} = 2.2 ± 0.7 μM) than the *p*-fluoro substituted **2g** and did not sensitize *Mtb* K204 to KAN (MIC_{KAN} = 5-10 g/mL). The *p*-bromo substituted **2i** was found to be completely inactive (IC_{50} > 200 μM and MIC_{KAN} ≥10 μg/mL against *Mtb* K204), while it displayed limited Eis inhibition in the HTS, which could indicate that the compound in the HTS library was not completely pure. We also found that the *p*-methyl derivative **2j** displayed a 100-fold decrease in Eis inhibitory activity (IC_{50} = 8.7 ± 2.2 μM) from **2g** and in combination with KAN resulted in almost the same KAN MIC (5-10 μg/mL) against KAN-resistant *Mtb* as **2g** did. The non-substituted counterpart of parent **2g**, derivative **2a**, displayed weaker Eis inhibitory activity (IC_{50} = 0.33 ± 0.16 μM) and improved the activity of KAN against *Mtb* K204 (MIC_{KAN} = 5 μg/mL). We also synthesized the *m*-fluoro, *m*-chloro, and *m*-bromo derivatives **2c**, **2d**, and **2e**. The *m*-chloro substituted **2d** showed the same inhibitory activity as that of **2g**, but did not sensitize *Mtb* K204 to KAN (MIC_{KAN} ≥10 μg/mL). The *m*-fluoro and *m*-bromo substituted **2c** and **2e** resulted in a 3- and 5-fold worse Eis inhibitory activity (IC_{50} = 0.30 ± 0.08 and 0.54 ± 0.25 μM), respectively. When used with the *m*-bromo-substituted **2e**, KAN had an MIC of 5-10 μg/mL against *Mtb* K204. However, when used with the *m*-fluoro substituted **2c**, KAN displayed a better MIC value of 2.5-5 μg/mL. As observed with scaffold **1**, the presence of *m*-methoxy-phenyl, naphthyl, ethyl, and *t*-butyl groups in scaffold **2** resulted in molecules that were completely inactive (IC_{50} > 200 μM and MIC_{KAN} ≥10 μg/mL against *Mtb* K204). For scaffold **2**, we also synthesized a *m*-nitro substituted compound (**2n**) to investigate the potential effect of a strong electron-withdrawing group on Eis inhibitory activity. Compound **2n** was less active (IC_{50} = 1.2 ± 0.4 μM) than **2g**, but it sensitized *Mtb* K204 to KAN (MIC_{KAN} = 5-10 μg/mL). The absence of antibacterial activity of these compounds when used alone along with a general correlation between IC_{50} and MIC values indicated that inhibition of Eis by these compounds is the main mechanism of sensitization to KAN.

Table 1. IC_{50} values against purified Eis_*Mtb* and MIC values against *Mtb* H37Rv and *Mtb* K204 with the compounds at the concentrations specified.

Cpd	IC_{50} (μM)[a]	Concentration tested (μM)[b]	H37Rv MIC_{KAN} (μg/mL)[c]	K204 MIC_{KAN} (μg/mL)[d]
-	-		≤1.25	≥10, 10
1a	3 ± 1	100	≤ 1.25	5, 10
1b	2.9 ± 0.9	100	≤ 1.25	10, 10
1c	0.16 ± 0.07	15.5	≤ 1.25	10, 10
1d	0.25 ± 0.08	25.1	≤ 1.25	≥10, 10
1e	0.5± 0.2	52.6	≤ 1.25	≥10, 10
1f	>200	100	≤ 1.25	≥10, 10
1g	0.3 ± 0.1	33.9	≤ 1.25	10, 10
1h	0.6 ± 0.3	64.5	≤ 1.25	10, ≥10
1i	>200	100	≤ 1.25	≥10, ≥10
1j	5.8 ± 1.8	100	≤ 1.25	5, 10
1k	>200	100	≤ 1.25	10, 10
1l	>200	100	≤ 1.25	≥10, 10
1m	>200	100	≤ 1.25	≥10, 10
2a	0.33 ± 0.16	32.6	≤ 1.25	5, 5
2b	23 ± 10	100	≤ 1.25	10, 10
2c	0.30 ± 0.08	29.7	≤ 1.25	5, 2.5
2d	0.015 ± 0.005	1.5	≤ 1.25	≥10, 10
2e	0.54 ± 0.25	54.2	≤ 1.25	10, 5
2f	>200	100	≤ 1.25	≥10, 10
2g	0.09 ± 0.03	8.9	≤ 1.25	5, 5
2h	2.2 ± 0.7	100	≤ 1.25	10, 5
2i	>200	100	≤ 1.25	≥10, 10
2j	8.7 ± 2.2	100	≤ 1.25	5, 10
2k	>200	100	≤ 1.25	10, 10
2l	>200	100	≤ 1.25	≥10, 10
2m	>200	100	≤ 1.25	≥10, 10
2n	1.2 ± 0.4	100	≤ 1.25	10, 5

[a]IC_{50} against purified Eis_*Mtb* enzyme, [b]Concentrations of Eis inhibitor in the MIC assays. At these concentrations, these compounds did not inhibit the growth of *Mtb* H37Rv or that of *Mtb* K204 when tested in the absence of KAN. Concentrations of Eis inhibitors were 100x their IC_{50} when IC_{50} <1 μM, or 100 μM for IC_{50} >1 μM. [c]Activity of KAN against *Mtb* H37Rv. [d]Activity of KAN against *Mtb* K204. For [c] and [d], results are from two experiments.

To investigate the selectivity of our inhibitors towards Eis_*Mtb*, we tested two of our derivatives, one from each series, **1c** and **2c**, against three other AAC enzymes with different acetylation regiospecificities: AAC(2')-Ic from *Mtb*,[5] AAC(3)-IV from *E. coli*,[12] and AAC(6')-Ie/APH(2")-Ia from *Staphylococcus aureus*.[13] Similarly to other known non-Eis AACs, these three enzymes were previously shown to be strictly regiospecific, but, like Eis, each enzyme was capable of acetylating structurally distinct AGs. Neither **1c** nor **2c** inhibited KAN acetylation by these three AACs at concentrations as high as 200 μM, which indicated that our compounds were highly selective against Eis_*Mtb*.

To explain the results of our SAR study, we used previously published crystal structures of ternary Eis_*Mtb*-CoA-compound **A** (**13g** in ref [14]; PDB ID 5EC4) and **B** (**11c** in ref [14]; PDB ID 5EBV) complexes to model our inhibitors **1a** and **2g** in a position similar to that of inhibitors **A** and **B** (Fig. S4). Without the crystal structures, *de novo* computational modeling of and screening for Eis inhibitors, including pharmacophore-based computer modeling, are invalidated by significant conformational changes in the Eis active site upon inhibitor binding.[13] The inhibitors occupy the site overlapping with the AG-binding site of Eis. The models show that the cores of inhibitors **1a** and **2g** are surrounded by the side chains of hydrophobic amino acid residues (Trp36, the aliphatic part of

Glu401, Ile28, Phe24, and Val400). The cores stack with the indole of Trp36, and in the orthogonal direction they are sandwiched between Glu401, the Eis C-terminus on one side and Ile28 on the other. The acetophenone rings of both series **1** and **2** with different substituents stack with Phe84, explaining why replacing these aromatic rings with alkyl chains resulted in a loss of activity for **1l**, **1m**, **2l**, and **2m**. The acetophenone rings are also surrounded by several hydrophobic amino acid residues (Phe84, Trp36, Met65, Ala33). Therefore putting a polar methoxy group in this hydrophobic environment would likely destabilize Eis binding, explaining the IC_{50} values of >200 μM for **1f** and **2f**. The *para* position of the acetophenone rings is flanked by Phe84 and Trp36, and it is ~5 Å away from Trp13 and Met65, explaining why the bulkier bromo substituents of **1i** and **2i** resulted in lower Eis inhibitory activity, whereas the small fluoro substituents of **1g** and **2g** improved Eis inhibitory activity. The *ortho* position of the acetophenone rings is flanked by Phe402, explaining why an *ortho* substituent, as in **1b** and **2b**, resulted in a loss of Eis inhibition. The models shows that there is space for small substitution in the *meta* position of the acetophenone rings (a ~5 Å-gap). Small substituents such as the fluoro and chloro of **1c**, **1d**, **2c** and **2d** fit well in the cavity, explaining why these compounds displayed good Eis inhibition. However, bulkier substituents such as the bromo of **1e** and **2e** or the nitro of **2n** are too big to be accommodated at this site and would clash with Eis residues, accounting for the poor Eis inhibition by these compounds. We also determined that the calculated LogP values of all compounds are in the desirable range (0.98-3.75; Table S1).

In sum, we have discovered two scaffolds with Eis inhibitory activity. From 27 synthesized analogues of these scaffolds with the variable acetophenone appendage, we identified potent inhibitors of Eis. Growth inhibition studies of our inhibitors in combination with KAN in KAN-susceptible *Mtb* H37Rv (MIC_{KAN} ≤1.25 μg/mL) and KAN-resistant *Mtb* K204 (MIC_{KAN} ≥ 10 μg/mL) showed that some of our inhibitors were able to sensitize *Mtb* K204 to KAN. Smaller substituents, like hydrogen and fluorine, yielded the best compounds. In contrast, larger substituents, such as bromo or methoxy dramatically decreased the potency of the compounds. The best compound identified was **2c** with the 3-(1,3-dioxolano)-2-indolinone core and a *m*-fluoro-phenyl substituent. This compound when used in combination with KAN reduced the MIC_{KAN} for KAN-resistant *Mtb* to 2.5-5 μg/mL. While CLSI recommends MIC_{KAN} of 5 μg/mL on Middlebrook 7H10 agar, it has no recommendation for susceptibility testing by Alamar Blue, the method used here. One study suggests a critical MIC_{KAN} of 2.5 μg/mL for Alamar Blue testing. Since our inhibitors are able to return KAN-resistant isolates to an MIC_{KAN} below the critical concentration, essentially making resistant *Mtb* isolate KAN-susceptible, such inhibitors could play a crucial role in recovering KAN as a treatment option. However, clinical studies to support this hypothesis are yet to be undertaken. We are actively pursuing these avenues.

ASSOCIATED CONTENT

Supporting Information. The supporting information includes the structures of scaffolds **1** and **2** (Figs. S1-S3) and models of Eis inhibitors bound to Eis (Fig. S4), experimental procedures, and the characterization data of all new compounds synthesized and their ^{1}H and ^{13}C NMR spectra (Figs. S5-S56). Representative IC_{50} curves are also provided (Fig. S57). This material is available free of charge *via* the Internet at http://pubs.acs.org."

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version.

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ABBREVIATIONS

AG, aminoglycoside; Eis, enhanced intracellular survival; HTS, high-throughput screening; KAN, kanamycin; MDR, multidrug-resistant; *Mtb*, *Mycobacterium tuberculosis*; SAR, structure-activity-relationship; TB, tuberculosis; XDR, extensively drug-resistant.

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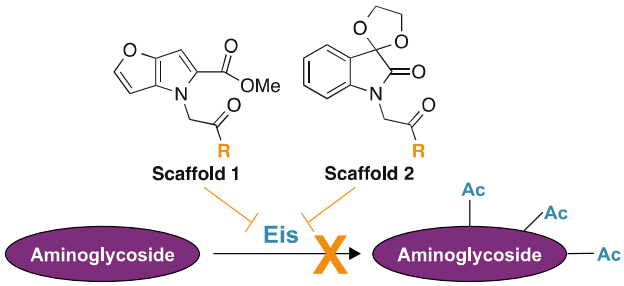
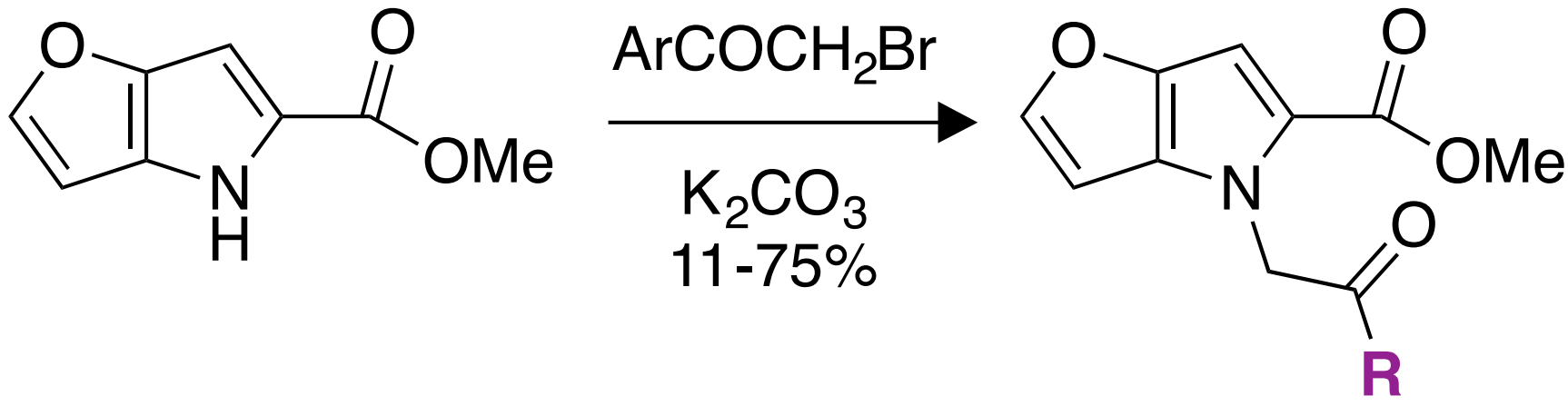
Table of Contents Graphic and Synopsis.



Aminoglycoside acetylation no more: Inhibitors of the aminoglycoside multiacetylating enzyme Eis from *Mycobacterium tuberculosis* (*Mtb*) were discovered and developed and were shown to restore kanamycin sensitivity of kanamycin-resistant *Mtb* bacteria.



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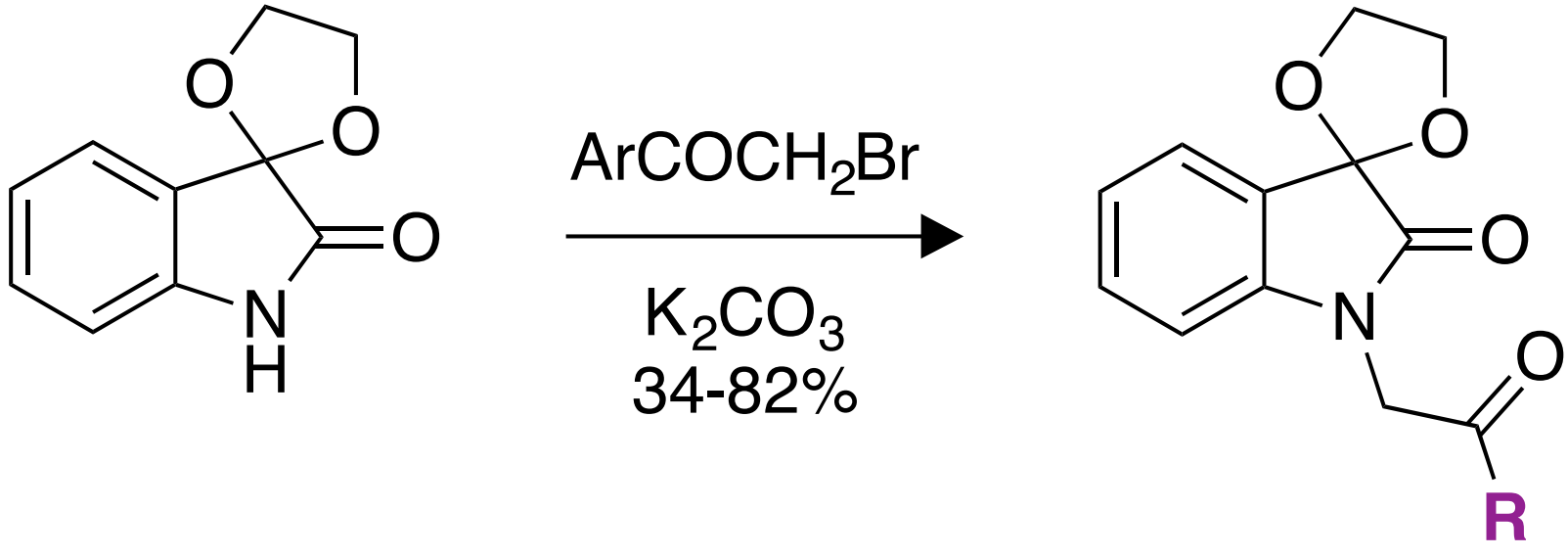
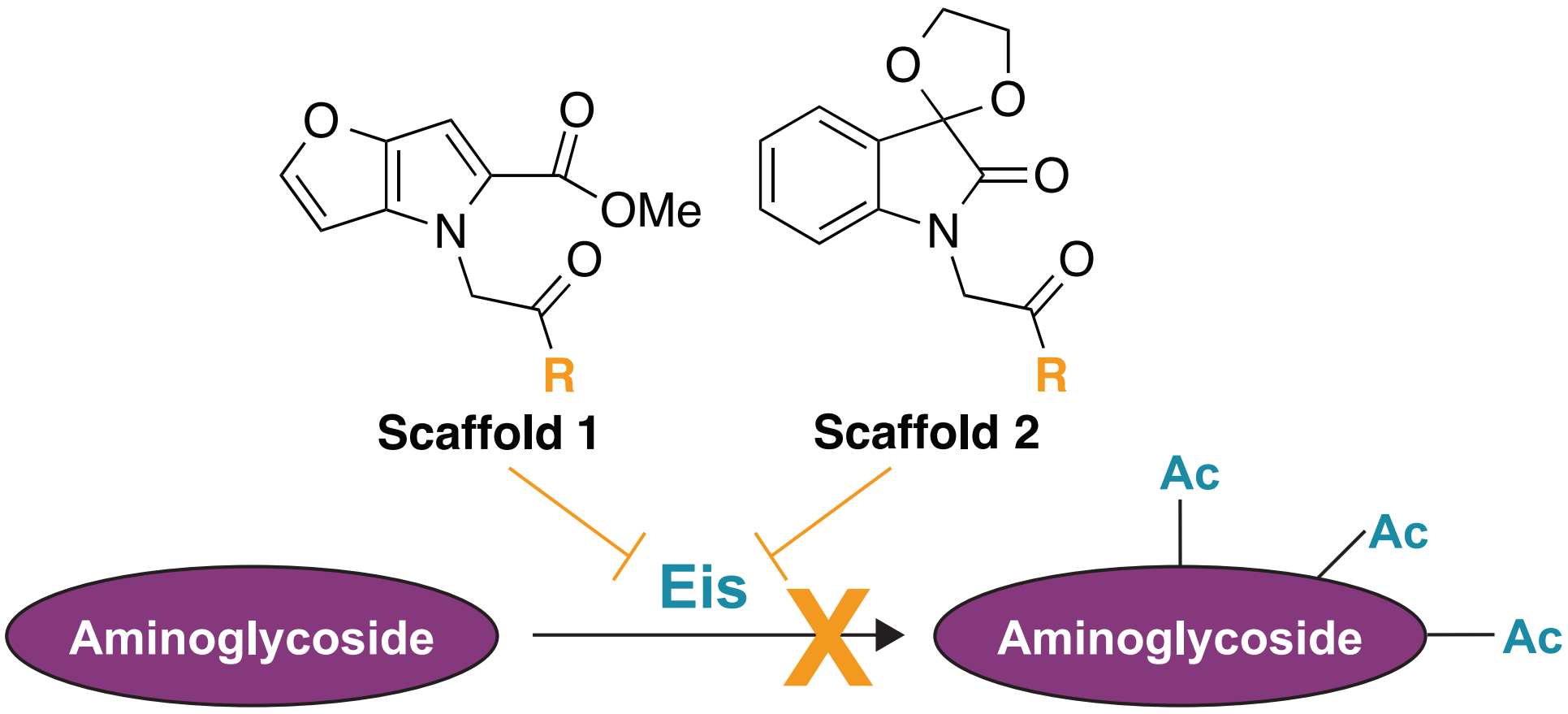


2a-n

R: **a** = Ph; **b** = *o*-F-Ph; **c** = *m*-F-Ph; **d** = *m*-Cl-Ph; **e** = *m*-Br-Ph; **f** = *m*-OMe-Ph; **g** = *p*-F-Ph; **h** = *p*-Cl-Ph; **i** = *p*-Br-Ph; **j** = *p*-Me-Ph; **k** = naphthyl; **l** = Et; **m** = *t*-Bu; **n** = *m*-NO_2-Ph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