

Efficient and Stereoselective Enzymatic Synthesis of Multisubstituted α,β -Diamino AcidRui Zhang,^{*,‡} Huijun Yang,[‡] Yu-Xin Hu,[‡] Weiqin Li, Ruo-Nan Ning,^{*} Min Jiang,^{*} and Cangsong Liao^{*}Cite This: <https://doi.org/10.1021/acscatal.6c03940>

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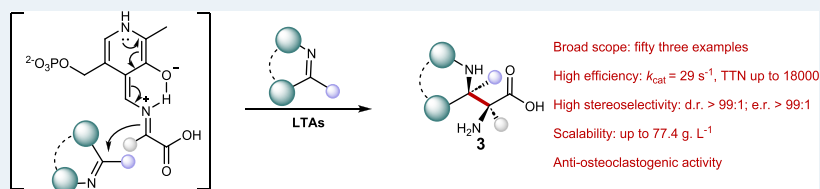
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ABSTRACT: α,β -Diamino acids are privileged motifs present in numerous bioactive natural products and synthetic molecules, yet their asymmetric synthesis remains challenging and limited in scope. Herein, we repurpose L-threonine aldolases to catalyze a stereoselective intermolecular Mannich-type reaction, enabling the direct and scalable synthesis of densely functionalized α,β -diamino acids. By exploiting enzyme sequence diversity, we accessed more than 50 derivatives from simple precursors with high efficiency (up to 97% yield) and selectivity (up to >99% d.r. and >99% e.r.). The reaction achieves total turnover numbers of up to 18,000 and titers reaching 77.4 g L⁻¹, representing the highest reported productivity for this class of biocatalytic transformation and meeting key benchmarks for industrial application. Mechanistic investigations suggest that the deprotonation of glycine contributes to the rate-determining step of the highly efficient catalytic cycle. Selected derivatives were shown to effectively suppress osteoclastogenesis, identifying a chemotype with potential for osteoporosis intervention.

KEYWORDS: biocatalysis, α,β -diamino acid, threonine aldolase, C–C bond formation, noncanonical amino acids

INTRODUCTION

Noncanonical amino acids (ncAAs) have become indispensable chemical building blocks in modern chemical biology and drug discovery. Their structural diversity and unique functional groups provide versatile motifs for diversifying the architectures of proteins, peptides, and synthetic small molecules.^{1–5} The inherent complexity of these nonproteinogenic scaffolds further highlights their untapped potential for the development of bioactive compounds.⁶ Indeed, nature itself produces a broad array of intricate amino acid frameworks that exhibit diverse biological activities, including antioxidant, antibiotic, and anticancer effects.⁷

Among these, α,β -diamino acids constitute a privileged structural class, serving as key pharmacophores in numerous bioactive natural products and synthetic molecules (Figure 1A). For example, the tyrosine analogue mimosine displays anticancer and anti-inflammatory activities,⁸ whereas β -N-oxalyl-L- α,β -diaminopropionic acid (β -ODAP) is a neurotoxin linked to irreversible paralytic disorders.⁹ Furthermore, α,β -diamino acids are essential subunits in many nonribosomal peptide natural products, such as antibiotic 593A.¹⁰ Given their pharmacological relevance, multiple catalytic strategies have been developed to access these motifs. However, although existing methods often deliver high stereoselectivity, they typically require high catalyst loadings and incur substantial costs.

Pyridoxal phosphate (PLP)-dependent enzymes represent efficient and sustainable biocatalysts for the synthesis of enantioenriched ncAAs through C–C bond formation from simple precursors.^{11–13} These enzymes mediate a wide range of transformations, including α -^{14–16} and β -aldol additions,^{17–21} β - and γ -substitutions,^{22–25} and radical alkylations.^{26–28} Their ability to install multiple stereocenters and functional groups in a single stereocontrolled step without protecting-group manipulations makes them particularly attractive for ncAA synthesis. Despite this versatility, the direct enzymatic Mannich reaction for the preparation of α,β -diamino acids remains largely unexplored. To date, only LolT from the loline alkaloid pathway has been shown to catalyze both intramolecular and intermolecular Mannich-type reactions toward such scaffolds (Figure 1B),^{29,30} while O-phospho-L-serine sulfhydrylase has been repurposed for their synthesis via β -substitution chemistry.³¹

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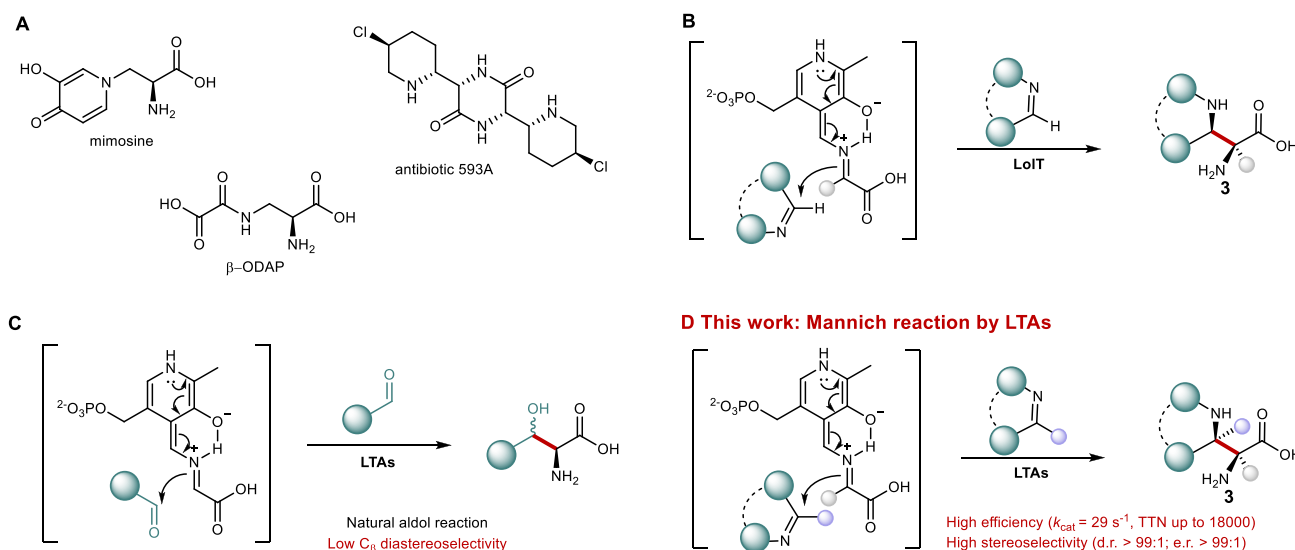


Figure 1. α,β -Diamino acids and PLP-dependent enzymes. (A) α,β -diamino acids found in bioactive molecules. (B) Natural enzymatic Mannich reaction by LolT to prepare α,β -diamino acids. (C) Natural intermediate and mechanism of threonine aldolases. (D) Repurposed Mannich reaction catalyzed by LTAs.

Within the PLP-dependent enzyme family, L-threonine aldolases (LTAs) constitute a broad group that catalyzes aldol reactions between glycine and aldehydes (Figure 1C). TAs are particularly valuable for synthesizing β -hydroxy- α -amino acids, key intermediates in the production of active pharmaceutical ingredients, antibiotics, and agrochemicals. Recent efforts have focused on improving their catalytic efficiency and diastereoselectivity through protein engineering, especially for industrial applications such as the synthesis of florfenicol.^{32–35} Recently, TAs have been repurposed in the development of photobiocatalytic alkylation reactions^{36–39} and nucleophilic substitution (S_N2) reaction⁴⁰ for synthesis of ncAAs. LTAs have also been engineered to catalyze α -functionalizing benzylamines for synthesis of 1,2-amino alcohols.^{41,42} We question whether TAs could be repurposed to accept imines as electrophiles, thereby providing access to enantiopure α,β -diamino acids and facilitating their broader application in medicinal chemistry and drug discovery.

Herein, we repurpose LTAs as efficient catalysts for a stereoselective intermolecular Mannich-type reaction (Figure 1D), providing direct access to >50 densely functionalized α,β -diamino acids from simple precursors.

Mechanistic analysis through steady-state kinetics and isotope-effect studies implicates glycine deprotonation in the rate-determining step. Moreover, phenotypic screening and mechanistic studies identified α,β -diamino acid derivatives as a novel chemotype targeting the TRAF6/p38/c-FOS/NFATc1 axis for potential osteoporosis intervention. The present system achieves a total turnover number and titer that are the highest reported to date for an intermolecular enzymatic Mannich reaction, in comparison with other biocatalytic platforms such as LolT,³⁰ lipases and artificial metalloenzymes,⁴³ and PLP-dependent decarboxylative aldolase.^{44,45}

MATERIALS AND METHODS

Materials

Chemicals, biochemicals, buffers, and solvents were purchased from Bidepharm, RHAUN, Macklin Biochemical Co., Ltd., J & K SCIENTIFIC Ltd., SINOPHARM, Merck KGaA, and CNPGC.

Protein Expression and Purification

E. coli BL21 (DE3) cells were transformed with pET-28a (+) expression plasmids via heat shock. The transformed cells were first cultivated on solid media (LB-AGAR plates with 50 $\mu\text{g}/\text{mL}$ kanamycin) and then on liquid media (LB with 50 $\mu\text{g}/\text{mL}$ kanamycin). After incubation at 37 $^{\circ}\text{C}$ overnight, 15 mL of preculture was used to inoculate fresh LB medium (1 L) with kanamycin (50 $\mu\text{g}/\text{mL}$). The cells were grown in a 3 L shaking flask at 37 $^{\circ}\text{C}$ (170 rpm) until the OD600 reached 1.0. The culture was cooled to 18 $^{\circ}\text{C}$ and supplemented with 100 μM isopropyl β -D-thiogalactopyranoside (IPTG). This culture was incubated at 18 $^{\circ}\text{C}$ for 20 h. The cells were harvested via centrifugation at 13,000 $\times$ g for 10 min and stored at -20°C .

The cell pellets (10 g) were suspended in 50 mL of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0). The cells were disrupted by a high-pressure cell disrupter 3 times. The lysates were subsequently centrifuged at 42,000 $\times$ g for 15 min at 4 $^{\circ}\text{C}$. The cleared lysate was loaded onto a Ni-NTA unionrose 6FF agarose column. To purify LTAs various mutants, the agarose beads were washed with 100 mL of lysis buffer containing 10 mM (50 mL) imidazole and 20 mM (50 mL) imidazole. The protein was eluted in a lysis buffer solution containing 250 mM imidazole. Protein-containing fractions were collected and dialyzed against dialysis buffer (50 mM sodium phosphate, 50 mM NaCl, 100 μM PLP, pH 7.4). Protein was used directly after dialysis. (If protein precipitated after dialysis, centrifugation was needed.)

General Procedure of Preparative Reactions

A 5–50 mL reaction containing LTA, Gly, substrate, 5% DMSO (v/v), 100 μM PLP, 50 mM sodium phosphate, and 50 mM NaCl at pH 7.4 was incubated at r.t. for 2–24 h and centrifuged. Products were loaded on a Biotage Isolera One with a SepaFlash column [$\text{CH}_3\text{OH}-\text{H}_2\text{O}$ (with 0.1% formic acid), 1%–50%, 9 column volume, 50 mL/min]. The purification was monitored with UPLC-MS. Fractions containing the products were collected, and the solvent was removed by a rotary evaporator to yield the products. Detailed substrate and enzyme concentrations were indicated in the Supporting Information.

RESULTS AND DISCUSSION

We began by evaluating benzoxathiazine-2,2-dioxide derivative (1a) as substrate for a panel of in-house L-threonine aldolases

(LTAs), aiming to explore the expansion of compatible electrophiles within this biocatalytic framework. We are particularly interested in functionalized benzosultam and sulfonamide derivatives, because they are widely regarded as highly privileged pharmacophores in medicinal chemistry,⁴⁶ and these moieties are integral to antiviral, anticancer, antibacterial, and antidiabetic agents, owing to their significant biological relevance.^{47,48}

All tested LTAs exhibited activity under standard reaction conditions (Figure S1). This preliminary screen revealed considerable potential for repurposing LTAs in Mannich-type reactions for the synthesis of α,β -diamino acids, as the selected variants represented significant diversity (Figure S2), sharing only 29%–80% sequence identity. Notably, four LTAs, PpLTA from *Pseudomonas putida*, AjLTA from *Aeromonas jandaei*, NmLTA from *Neptunomonas marina*, and LsLTA from *Leptomonas seymouri*, catalyzed the quantitative conversion of 5 mM substrate **1a** within five minutes, with excellent stereoselectivity (Figure S1).

Motivated by the high reactivity, we next optimized substrate loading with NmLTA. Systematic evaluation showed that NmLTA accepted 50 mM **1a** in full conversion with 400 mM glycine (Table S1). Further experiments confirmed the reaction reached completion within 30 min (Figure S3). While PpLTA demonstrated similar efficiency, LsLTA and AjLTA achieved only 83% and 52% conversion of 50 mM substrate **1a**, respectively, in the same timeframe. Notably, and in contrast to the typically low-selectivity aldol reactions of most LTAs, a single major diastereoisomer of the product was formed in this Mannich reaction.

After identifying the initial activity, we proceeded to assess the substrate tolerance of LTAs. Efficiency of NmLTA decreased significantly on substrates with substituents on the

aromatic ring. Therefore, we profiled the activity of the four LTAs with seventeen derivatives of **1a** with various substituents at different positions (Figure 2). To our delight, these LTAs showed complementary substrate scopes accepting substrates bearing halide, methyl, methoxy, and trifluoromethoxy groups. LsLTA showed highest efficiency on 3-methoxy, 4-methyl, 5-methoxy, 5-chloro, 5-bromo, and 5-trifluoromethoxyl substrates with 76% to >99% conversion. PpLTA showed highest efficiency on 3-chloro, 3-bromo, 4-fluoro, 4-chloro, 5-fluoro substrates with 56% to >99% conversion. AjLTA showed highest efficiency on 4-methoxy and 6-methyl substrates, while NmLTA was not efficient on most of the substrates with trace to 57% conversion.

The most activated 5-nitro-substituted substrate was not accepted by either enzyme, whereas electron-rich substrates bearing methyl or methoxy substituents exhibited moderate to high conversion. These results suggest that substrate binding and productive positioning within the enzyme active site play a more critical role than the intrinsic reactivity of the substrates in determining catalytic efficiency.

We then profiled the activity of the four LTAs with twelve derivatives of benzo[d]isothiazole-1,1-dioxide **1m** (Figure 2B). LsLTA showed highest efficiency on most of the substrates with over 97% conversion, while AjLTA showed lowest efficiency on the substrates. All these results underscore the influence of structural conformation, particularly the absence of an oxygen atom in the five-membered system, on reaction efficiency.

Building on these optimized conditions and activity profile, we next evaluated the efficiency and substrate scope of the Mannich reaction at a preparative scale. Guided by the reactivity observed in initial analytical assays, substrates showing conversions above 70% were selected for scale-up. The isolated yields

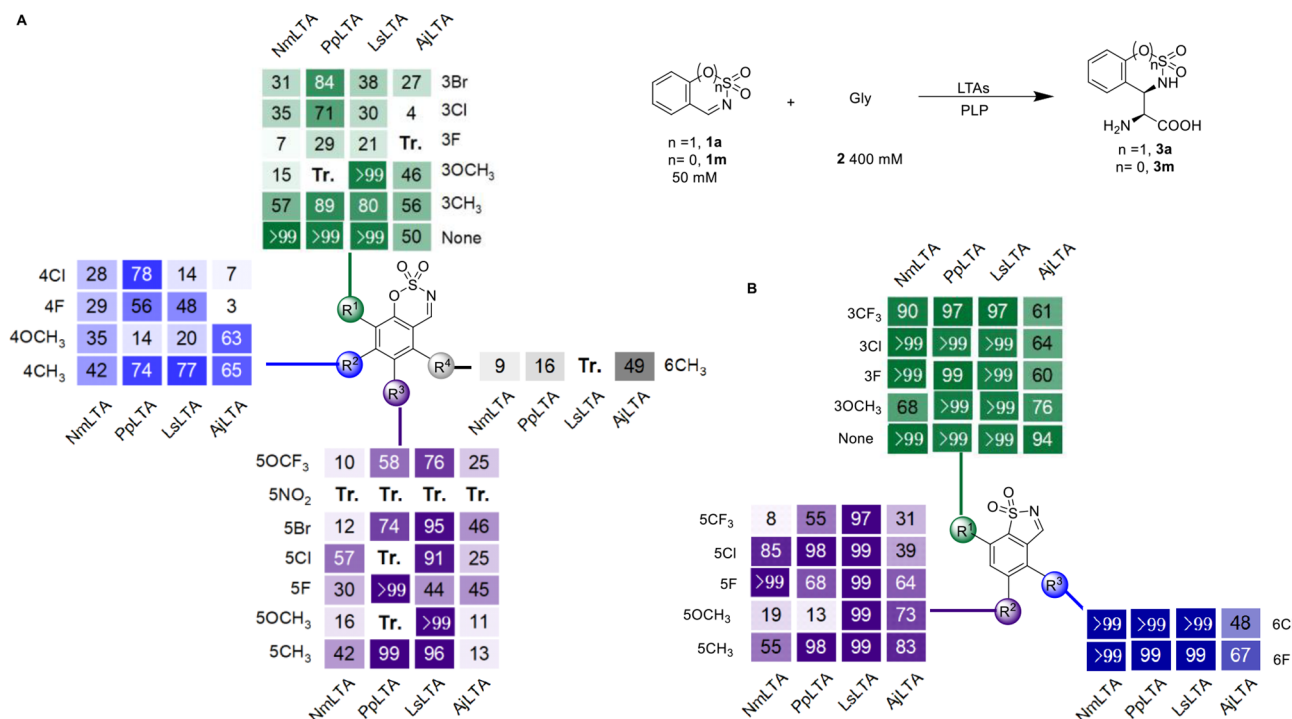


Figure 2. Screening of imine substrates across in-house LTAs. Reaction condition: a 1 mL reaction containing 2.5 μ M LTAs, 400 mM Gly, 50 mM **1**, 5% DMSO (v/v), 100 μ M PLP, 50 mM sodium phosphate, and 50 mM NaCl at pH 7.4 was incubated at r.t. for 2 h, and then diluted 20-fold with a 50% methanol/water solution, filtered, and analyzed by a Waters UPLC–MS, detection the consumption of **1**.

closely matched the conversions obtained from the analytical-scale experiments (Figure 3). Among these, product **3j** was obtained in the highest yield of 90% with a total turnover number (TTN) of 18,000, while **3m** was obtained in the highest yield of 86% with TTN of 17,200 among the products from benzo[*d*]isothiazole-1,1-dioxide derivatives.

Single-crystal X-ray diffraction analysis of representative compounds **3a** and **3p** established an *R* configuration at the C_β-position and an *S* configuration at the C_α-position. HPLC and ¹H NMR analysis confirmed excellent diastereoselectivity (at least 89:11) with all products, as no significant signals for diastereoisomers were detected. Furthermore, derivatization with Marfey's reagent verified that the enantioselectivity for all products exceeded 90%.

To further explore the scalability of this reaction for nCAs synthesis, we performed gram-scale preparative reactions using lyophilized *E. coli* cells expressing NmLTA as the biocatalyst, thereby circumventing protein purification. Using 20 mg/mL of whole-cell catalyst, the system effectively converted three sequential 100 mM additions of substrate **1a** and four sequential 100 mM additions of substrate **1m**, yielding product **3a** in

86% and **3m** in 80% yield. The final titers reached 66.6 g/L and 77.4 g/L, respectively, meeting the benchmark for industrially relevant biocatalytic processes (Figure 3).^{49–51}

Encouraged by the system's high efficiency, we sought to explore the scope of this biocatalytic platform for generating diverse α,β-diamino acids. Our investigation began with AjLTA and a related homologue, TmLTA from *Thermotoga maritima*, employing D-alanine and D-serine as nucleophiles to synthesize a series of enantiopure α,α-disubstituted variants (Figure 4).^{36,38,52} The reactions with D-alanine proceeded well, delivering fifteen diamino acid products (**4a1–4a15**) in moderate-to-good isolated yields and with high stereoselectivity. The total turn-over numbers were up close to 2000 after optimization (Figure S4), although the numbers decreased in general in comparison to that of reactions with glycine. However, the diastereoselectivity was somewhat lower than that observed with glycine. D-Serine also proved to be a good substrate, furnishing thirteen analogues (**4b1–4b13**) with similar yield and stereoselectivity profiles. Notably, a minor glycine-type byproduct was observed when D-serine was employed. Specifically, **3s** was detected at a concentration of 0.13 mM

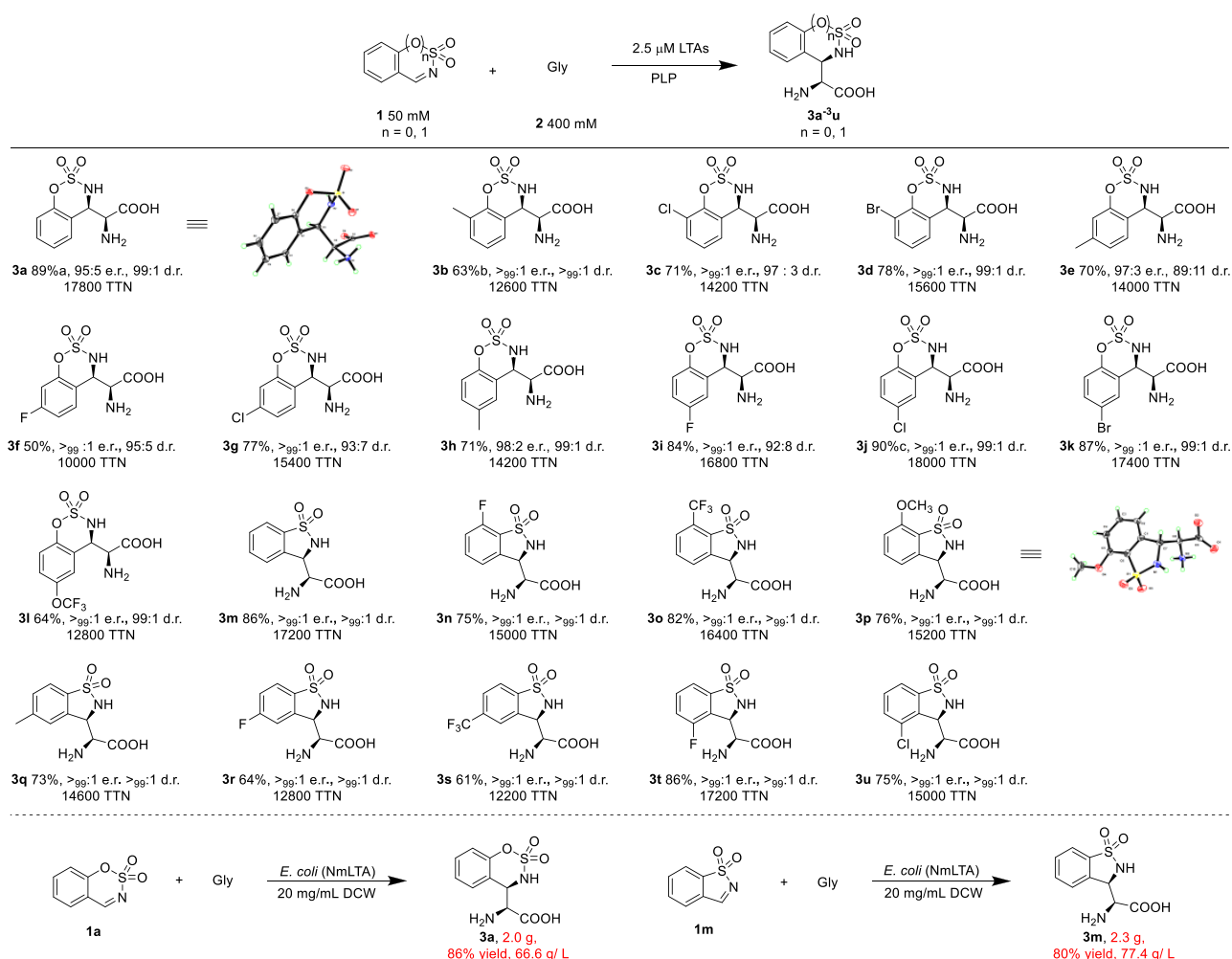


Figure 3. Substrate scope of LTAs. See the Supporting Information for reaction details. The isolated yield in a preparative reaction shown. e.r., enantiomeric ratio, d.r., diastereomeric ratio; TTN, total turnover number of catalyst based on the isolated yield and enzyme loading. ^aThe concentrations of cyclic imine substrate and NmLTA were 50 mM and 2.5 μM. ^bThe concentrations of cyclic imine substrate and PpLTA were 50 mM and 2.5 μM. ^cThe concentrations of cyclic imine substrate and LsLTA were 50 mM and 2.5 μM.

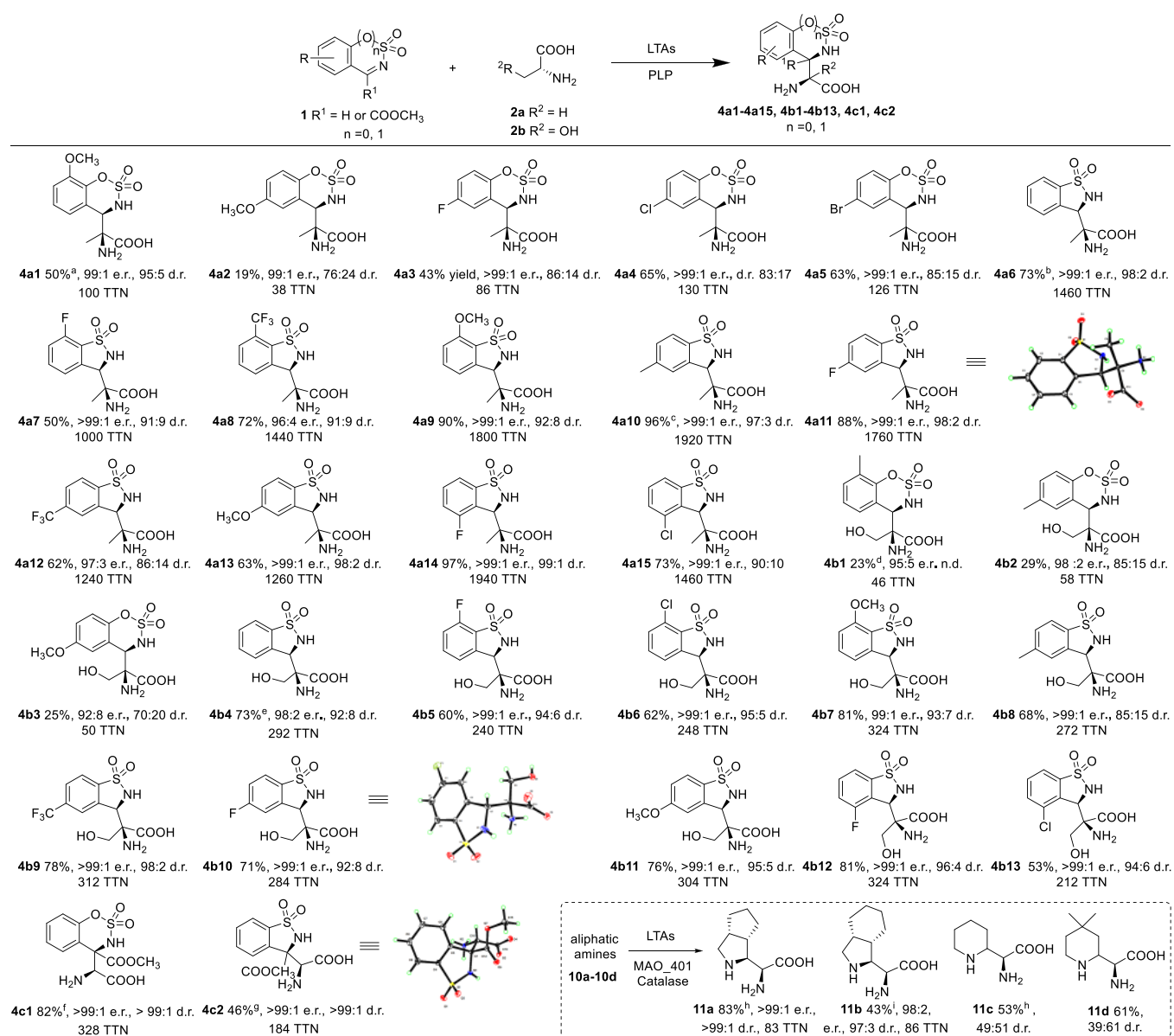


Figure 4. Substrate scope of LTAs. See the Supporting Information for reaction details. Isolated yield in a preparative reaction shown. E.r., enantiomeric ratio, d.r., diastereomeric ratio; TTN, total turnover number of catalyst. ^aThe concentrations of cyclic imine substrate and TmLTA were 5 mM and 25 μM . ^bThe concentrations of cyclic imine substrate and AjLTA were 50 mM and 25 μM . ^cThe concentrations of cyclic imine substrate and TmLTA were 50 mM and 25 μM . ^dThe concentrations of cyclic imine substrate and AjLTA were 5 mM and 25 μM . ^eThe concentrations of cyclic imine substrate and TmLTA were 10 mM and 25 μM . ^fThe concentrations of cyclic imine substrate and TmLTA were 10 mM and 25 μM . ^gThe concentrations of cyclic imine substrate and AjLTA were 10 mM and 25 μM . ^hThe concentrations of amine substrate, MAO_401, Catalase, and AjLTA were 10 mM, 100 mM, 1 mg/mL, 100 μM . ⁱThe concentrations of amine substrate, MAO_401, Catalase, and NmLTA were 10 mM, 50 mM, 1 mg/mL, 50 μM .

(Figure S5), representing a 1.3% yield relative to the 78% isolated yield of the major product **4b9**. This outcome is likely due to a TmTA-catalyzed retro-aldol process, which releases formaldehyde to generate the quinonoid intermediate that subsequently undergoes a Mannich reaction with the imine substrate.

Subsequently, we applied the platform to synthesize more challenging α,β -diamino acids containing a β -tertiary amine. These compounds (**4c1** and **4c2**) were produced in moderate yields with high stereoselectivity, although the overall turnover number was comparatively lower. The absolute configuration of these products were established by single-crystal X-ray

diffraction analysis of representative compounds **4a11**, **4b10**, and **4c2**.

Next, we investigated whether this biocatalytic platform could accept other cyclic imines. To this end, we coupled the LTAs with a monoamine oxidase variant, MAON401,⁵³ to generate reactive imines *in situ*. This strategy was employed in the study with LolT.³⁰ Two MAON401-generated bicyclic imines were well accepted by AjLTA and NmLTA respectively to give product **11a** and **11b** in moderate to good isolation yields and high stereoselectivity. *Anti*-diastereomers were obtained as the major products in opposite to the *syn*-diastereomers in previous cases. The opposite diastereoselectivity may be attributed to the

by the sterically hindrance of bicyclic imine intermediates, as same as the results observed in LolT study. Meanwhile, other tested cyclic imines were either accepted with low diastereoselectivity or low efficiency (Figure S6).

The catalytic efficiency and substrate scope of LTAs toward cyclic imines were limited in comparison to the activated sulfonylimines, likely due to differences in substrate structure and interactions within the enzyme active site. Cyclic imines often possess more flexible and sterically demanding frameworks, whereas benzoxathiazine-2,2-dioxide (**1a**) and benzo[*d*] isothiazole-1,1-dioxide (**1m**) derivatives feature more rigid aromatic scaffolds that may facilitate productive substrate positioning and binding. In addition, the distinct electronic properties of cyclic imines may influence their intrinsic electrophilicity and reactivity toward the LTA-bound nucleophilic intermediate. Nevertheless, the successful conversion of selected bicyclic imines highlights the potential of this platform, and further exploration and engineering of LTAs may enable access to a broader range of structurally diverse α,β -diamino acids.

Following the demonstration of its synthetic utility, we turned to elucidating the mechanistic details of this system. Steady-state kinetic analysis was conducted with substrates **1a** and **1m**, motivated by the known reversibility of enzymatic aldol reactions. This reversibility is often responsible for the low diastereoselectivity in LTA-catalyzed processes, as it permits equilibration toward thermodynamic byproducts. The measured kinetic parameters for **1a** revealed a k_{cat} of 29.4 s^{-1} and a K_M of 3.2 mM (Figure 5A), whereas **1m** showed a k_{cat} of 14.7 s^{-1} and a K_M of 8.5 mM (Figure 5B). Importantly, the reverse reactions (from **3a** and **3m**) proceeded with turnover numbers that were 65-fold and 26-fold slower, respectively (Figure 5C,D). Furthermore, a reaction conducted with high

NmLTA concentration resulted in the accumulation of 9 mM byproduct **3a'** in 82:18 diastereomeric ratio (Figure S7). The turnover frequency for this minor pathway was preliminary calculated to be 0.7 min^{-1} , which was more than 2500-fold lower than the k_{cat} for the main product **3a**. This kinetic preference for the major diastereoisomer was over 80 times greater than that reported for natural aldol reactions. These kinetic results collectively demonstrated that the major reaction is kinetically favored over the reverse reaction and the side-reaction, rationalizing the high stereoselectivity achieved in this reaction.

We next investigated the rate-limiting step of this reaction. A kinetic isotope effect (KIE) study was conducted by comparing the reaction rates of nondeuterated glycine and deuterated glycine in parallel reactions. The experiments yielded a KIE value of 5.9 with **1a** (Figure 5E) and a KIE value of 4.8 with **1m** (Figure 5F) respectively. According to the proposed native mechanism of LTAs,⁵⁴ these primary KIE values suggested that deprotonation of glycine in the external aldimine **2** (EA2) with PLP to form the quinonoid intermediate is involved the rate-determining step of the reaction (Figure 5G).

The sulfonamide motif represents a privileged scaffold in drug discovery, featured in numerous FDA-approved drugs across diverse therapeutic areas owing to its broad spectrum of bioactivities, such as carbonic anhydrase inhibition and antimicrobial, anticancer, anti-inflammatory, and central nervous system effects.⁵⁵ Notably, multiple studies have identified specific sulfonamide derivatives as effective inhibitors of osteoclast differentiation, providing a compelling chemical rationale for exploring this scaffold in anti-osteoporosis drug discovery.^{56–62} Postmenopausal osteoporosis, a prevalent and burdensome disorder caused by estrogen deficiency, is fundamentally characterized by excessive osteoclast-mediated bone resorption.^{63–65} Given that

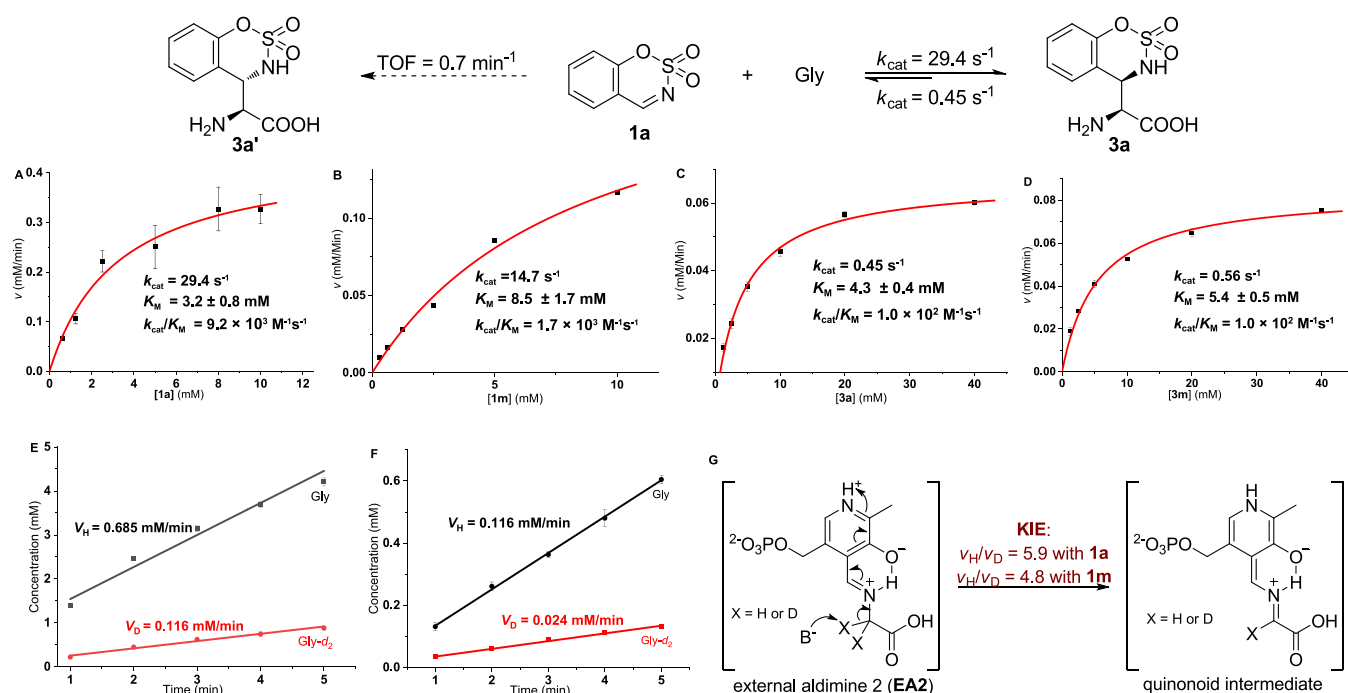


Figure 5. Enzyme kinetic and mechanism study. (A) Steady-state kinetic analysis of NmLTA with **1a** as substrate. (B) Steady-state kinetic analysis of NmLTA with **1m** as substrate. (C) Steady-state kinetic analysis of NmLTA-catalyzed retro-Mannich reaction from **3a**. (D) Steady-state kinetic analysis of NmLTA-catalyzed retro-Mannich reaction from **3m**. (E) Kinetic isotope effect (KIE) of glycine in reaction with **1a**. (F) Kinetic isotope effect (KIE) of glycine in reaction with **1m**. (G) Schematic representation of the key deprotonation step in the catalytic mechanism of TAs.

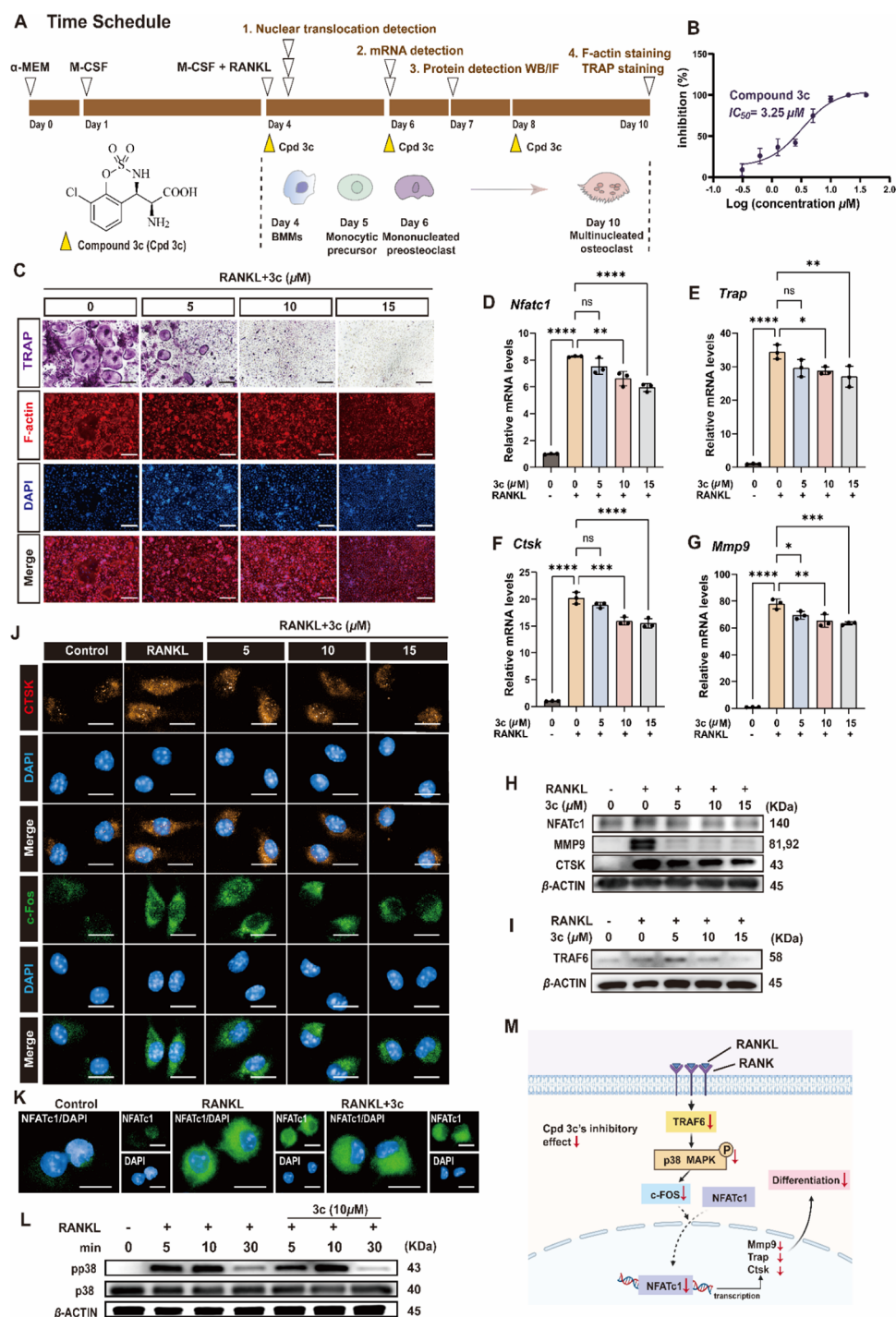


Figure 6. Compound 3c inhibits RANKL-induced osteoclast differentiation and function through suppressing key signaling pathways. (A) Schematic illustration of the experimental workflow for evaluating the effects of compound 3c on osteoclastogenesis *in vitro*. (B) Dose-response curve showing the inhibitory effect of compound 3c on osteoclast formation. The half-maximal inhibitory concentration (IC_{50}) is indicated. (C) Representative images of TRAP-stained and TRITC-phalloidin-stained osteoclasts derived from BMMs treated with the indicated concentrations of compound 3c (0, 5, 10, 15 μ M) for 7 days. Scale bar, 10 μ m. (D–G) Quantitative RT-PCR analysis of the mRNA expression in BMMs treated with compound 3c for 48 h during RANKL-induced differentiation. (H, I) Representative western blots showing the protein expression levels in BMMs treated with compound 3c for 72 h. (J) Immunofluorescence staining and representative images showing the expression and localization of CTSK and c-FOS in osteoclasts treated with compound 3c for 72 h. Scale bar, 10 μ m. (K) Representative immunofluorescence images demonstrating the inhibition of NFATc1 nuclear translocation by compound 3c after 1 h of treatment. Nuclei were counterstained with DAPI. Scale bar, 10 μ m. (L) Representative immunofluorescence images demonstrating the inhibition of NFATc1 nuclear translocation by compound 3c after 1 h of treatment. Nuclei were counterstained with DAPI. Scale bar, 10 μ m. (M) Proposed mechanism of compound 3c in inhibiting osteoclast differentiation and function via inhibiting the RANKL-induced TRAF6/p38/c-FOS/NFATc1 axis and downstream gene expression. Data in (D–G) are presented as mean $\pm$ SD from at least three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 vs the control (0 μ M) group.

the clinical utility of standard bisphosphonate therapies is compromised by adverse effects and poor patient adherence, there remains a pressing need for safer agents that specifically target osteoclastogenesis.⁶⁶ Motivated by the anti-osteoclastogenic potential for sulfonamides and the pressing clinical demand, we finally evaluated the anti-resorptive activity of our synthetic α,β -diamino acid library, which features conformationally constrained cyclic sulfonamides.

The anti-osteoclastogenic activity of the synthesized derivatives was first assessed using the classic tartrate-resistant acid phosphatase (TRAP) staining assay in RANKL-stimulated bone marrow-derived macrophages (BMMs), with alendronate as a positive control. Initial screening indicated that most compounds were active at 10 μM , and six derivatives maintained approximately 50% or greater inhibitory activity at 5 μM (Table S2). Among them, compound 3c demonstrated the most potent, dose-dependent inhibition. Parallel cell viability assays confirmed that this anti-osteoclastogenic efficacy was not an artifact of cytotoxicity at the tested concentrations (Table S2). Based on its superior potency and favorable safety profile, 3c was consequently selected as the lead candidate for further mechanistic investigation.

The classical RANKL/RANK signaling pathway drives osteoclast differentiation. We found that compound 3c potently inhibited this process with an IC_{50} of 3.25 μM (Figure 6B). Consistently, 3c dose-dependently diminished the number and size of multinucleated osteoclasts in TRAP staining and disrupted the bone-resorptive F-actin rings (Figure 6C). At the transcriptional level, 3c downregulated the mRNA expression of key players, including the master regulator *Nfatc1*, and its downstream effector genes, including *Acp5* (*Trap*), *Ctsk*, and *Mmp9* (Figure 6D–G). Corresponding suppression at the protein level of TRAF6, NFATc1, CTSK, and MMP9 was confirmed by western blot (Figure 6H,I and Figure S8). Immunofluorescence confirmed the attenuation of c-Fos and CTSK signals, and crucially, the blockade of RANKL-induced nuclear translocation of NFATc1 (Figure 6J,K). Further mechanistic tracing revealed that 3c pretreatment specifically inhibited RANKL-stimulated phosphorylation of p38 MAPK without affecting total p38 levels (Figure 6L and Figure S8). In summary, phenotypic screening identified 3c as a potent osteoclastogenesis inhibitor from an unnatural amino acid library. Its mechanism involves a multitiered suppression of the RANKL cascade: 3c downregulates the adaptor TRAF6, attenuates p38 MAPK activation, reduces the early transcription factor c-FOS expression, and thereby impairs the induction and nuclear translocation of the master regulator NFATc1. This leads to coordinated downregulation of critical effector genes (*TRAP*, *CTSK*, *MMP9*), disrupting osteoclast differentiation and function (Figure 6M). This work presents a novel chemotype for osteoporosis therapy that exerts its effects by interfering with the TRAF6/p38/c-FOS/NFATc1 signaling axis.

CONCLUSIONS

In summary, we have repurposed LTAs to catalyze a stereoselective intermolecular Mannich-type reaction, enabling the direct and scalable synthesis of densely functionalized α,β -diamino acids. By leveraging the broad sequence diversity of LTAs, a library of over fifty such derivatives were prepared from simple precursors. This transformation proceeds with outstanding efficiency (k_{cat} up to 29.4 s^{-1} , TTN up to 18,000), achieves a high

titer (up to 77.4 g/L), and exhibits excellent stereoselectivity, parameters that represent the highest reported to date for this class of enzymatic reaction and satisfy key benchmarks for industrial biocatalysis. Mechanistic studies suggest that glycine deprotonation contributes to the rate-determining step of the efficient catalytic cycle. We further identified the resulting α,β -diamino acid derivatives as novel osteoclastogenesis inhibitors that act via the TRAF6/p38/c-FOS/NFATc1 axis, thereby establishing a promising starting point for anti-osteoporosis therapy. Subsequent studies will focus on evaluating the *in vivo* efficacy and pharmacokinetic profile of the lead compound. Collectively, this work demonstrates the capacity of LTAs as versatile biocatalysts for the efficient synthesis of pharmaceutically relevant α,β -diamino acid derivatives.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscatal.6c03940>.

Supplementary tables and figures, general experimental information, experimental procedures and characterization data (PDF)

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Notes

The authors declare no competing financial interest.

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