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$\alpha\beta, \alpha'\beta'$ -Diepoxyketones are mechanism-based inhibitors of nucleophilic cysteine enzymes†

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Epoxides are an established class of electrophilic alkylating agents that react with nucleophilic protein residues. We report $\alpha\beta, \alpha'\beta'$ -diepoxyketones (DEKs) as a new type of mechanism-based inhibitors of nucleophilic cysteine enzymes. Studies with the L, D-transpeptidase Ldt_{MT2} from *Mycobacterium tuberculosis* and the main protease from SARS-CoV-2 (M^{Pro}) reveal that following epoxide ring opening by a nucleophilic cysteine, further reactions can occur, leading to irreversible alkylation.

Most covalently reacting enzyme inhibitors bear an electrophilic functional group that reacts with a nucleophile to enable covalent protein modification.¹ Although many such inhibitors work by apparently simple acylation, alkylation or conjugate addition reactions, some undergo further reaction after initial covalent modification. Such mechanism-based inhibitors can be found in drugs,^{2–4} with one such example being inhibitors of the nucleophilic serine- β -lactamases, such as clavulanic acid.^{5,6}

Despite the long-standing importance of covalently reacting drugs, concerns regarding potential toxicity have hindered their development. Covalently reacting drugs are, however, the subject of recent renewed interest,^{1,7} and are currently the basis for multiple drug development programs, including in oncology and antimicrobials.^{8–11} Covalent targeting of a prevalent oncogenic mutation in K-Ras (K-Ras^{G12C}) has led to development of sotorasib and adagrasib.¹² Various medicinal chemistry programs targeting the main protease (M^{Pro}) of SARS-CoV-2 have

focused on covalent reaction of the catalytic cysteine residue, with nirmatrelvir, a reversibly reacting nitrile-bearing inhibitor, being approved for COVID-19 treatment.^{13,14} The L,D-transpeptidase Ldt_{MT2} of *Mycobacterium tuberculosis*, which is a target for TB treatment,¹⁵ is amenable to covalent inhibition via reaction with its catalytic cysteine.^{16–18}

Epoxides are an established class of electrophilic alkylating agents, and are used to inhibit nucleophilic cysteine (and serine) proteases.^{1,19,20} Many epoxide inhibitors of cysteine or serine proteases contain peptide backbones, e.g. proteasome inhibitors,^{21–24} though the small molecule epoxide fosfomicin is a clinically important antibiotic (Fig. 1A).^{25,26}

We are interested in identifying new types of covalently reacting modulators of biological function. Recently, we

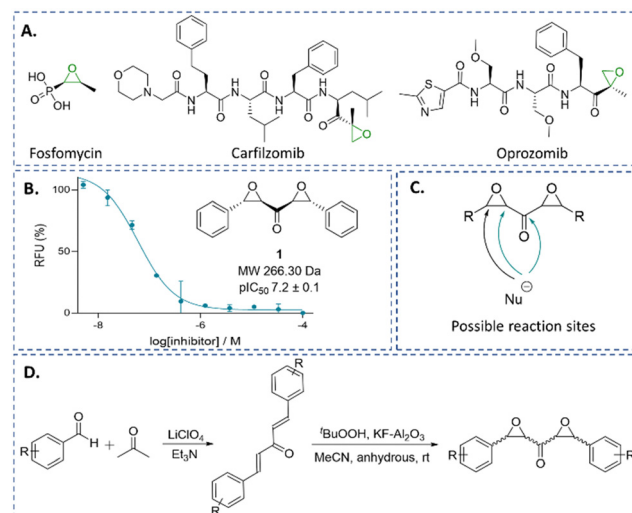


Fig. 1 $\alpha\beta, \alpha'\beta'$ -Diepoxyketones (DEKs) react with nucleophilic cysteine enzymes. (A) Examples of epoxide-bearing drugs. (B) DEK **1** was identified as a potent inhibitor of Ldt_{MT2}. (C) Symmetrical DEKs contain 3 potential sites for interactions with nucleophiles, as well as three oxygens that may react with electrophiles. Arrows in teal represent pathways consistent with mechanistic studies. (D) Synthesis of DEKs **1** and **4–11**.

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reported on a high-throughput screen aiming to identify new electrophilic inhibitors of Ldt_{Mt2} and other nucleophilic enzymes.²⁷ Here, we describe the identification of the small molecule *trans,trans* $\alpha,\beta,\alpha',\beta'$ -diepoxyketone (DEK) **1** (Fig. 1B), and the potency and mechanism of **1** and related DEKs **4–12** for Ldt_{Mt2} and SARS-CoV-2 M^{Pro} inhibition; the results reveal DEKs as a mechanistically interesting class of electrophile.

Symmetrical DEKs have 3 obvious positions that may react with nucleophiles and have potential to undergo further reactions (Fig. 1C). DEK **1** exhibited potent inhibition of Ldt_{Mt2} , with a pIC_{50} of 7.2 ± 0.1 , with 30 min pre-incubation (Fig. 1B). To investigate the mode of reaction of **1** with Ldt_{Mt2} , we carried out protein-observed mass spectrometry employing solid-phase extraction (SPE-MS). The results reveal that **1** covalently reacts with Ldt_{Mt2} , giving an initial adduct (**2**) with a +267 Da mass shift relative to unmodified Ldt_{Mt2} (Fig. 2B and Table S1, ESI[†]), corresponding to addition of one molecule of **1** to Ldt_{Mt2} , which has a single cysteine (Cys354). This adduct (**2**) was transient,

converting within 2 h into one with a mass shift of +160 Da relative to unmodified Ldt_{Mt2} , provisionally assigned as **3**. We proposed the reaction involves nucleophilic attack of Cys354 on the carbonyl-group adjacent carbon of one of the symmetrical epoxides, with ring opening to form **2**, followed by retro-aldol fragmentation, releasing benzaldehyde (Fig. 2C). Alternatively, the reaction may proceed through reaction at the carbonyl carbon to generate a hemithioacetal, after which rearrangement may occur (Fig. 2C).²⁸

The identity of **3** was validated by X-ray crystallography, using reported conditions,²⁷ wherein **1** was introduced through soaking; a structure of Ldt_{Mt2} reacted with **1** was obtained (2.15 Å resolution, $P12_11$ space group, PDB: 8BK3, Table S2, ESI[†]). As reported, Ldt_{Mt2} crystallised with two molecules (chains A and B) in the asymmetric unit. While this structure manifested clear additional electron density at the chain A active site, only partial density was observed at that of chain B, thus inhibitor modelling was only performed in chain A. The additional electron density in chain A supports the proposed structure of adduct **3** (Fig. 2). The carbonyl of **3** projects into the proposed oxyanion hole, formed by the backbone NH groups of His352, Gly353 and Cys354 (distances of 3.0 Å, 3.4 Å and 3.2 Å, respectively).²⁹ Extensive hydrophobic interactions of **3** with active site residues Tyr318, His352, Trp340, Thr320, and Met303 were observed.

In aqueous solution, **1** was found to be stable for at least 12 h (Fig. S3, ESI[†]). Cysteine reacted with **1**, apparently yielding a product analogous to adduct **3** (Fig. S4, ESI[†]). No evidence for reaction of **1** with serine, lysine, threonine, tyrosine, arginine, or histidine was observed by ¹H NMR or LCMS under the tested conditions (Fig. S5, ESI[†]).

To further analyse the inhibitory potency and mechanism of the DEKs, we prepared derivatives of **1**. Synthesis involved preparation of the diene ketones *via* solvent-free aldol condensation, mediated by lithium perchlorate and Et₃N,³⁰ followed by epoxidation using *t*-BuOOH and KF·Al₂O₃,^{31,32} to yield stereoisomeric mixtures of DEKs **1** and **4–11** (Fig. 1D and Table S3, ESI[†]).

No substantial difference in inhibition between diastereomerically pure **1** and enantiomerically pure **1** was observed. While we did not obtain the pure *cis,cis* diastereomer of **1**, a diastereomeric mixture of **1** (~1:3 ratio of *trans,trans*:*cis,cis* stereoisomers) manifested potent, but decreased, Ldt_{Mt2} inhibition compared to diastereomerically pure **1** (pIC_{50} 5.6 ± 0.04 compared to 6.2 ± 0.07 for diastereomerically pure **1**, with 15 min preincubation, Fig. S6, ESI[†]). The results imply the importance of the *trans,trans* stereochemistry for potent Ldt_{Mt2} inhibition by the DEKs. Recrystallisation of diastereomeric mixtures from ethanol afforded the corresponding pure *trans,trans* diastereomers, as supported by ¹H NMR analysis and small molecule X-ray diffraction (Table S4, ESI[†]), except for DEKs **5** and **8**, which were tested as diastereomeric mixtures (*trans,trans*:*cis,cis* ratio ~2:1 and ~1.2:1, respectively).

Dose–response assays of **4–11** with Ldt_{Mt2} showed decreased potency compared to **1** (Table S3 and Fig. S1, ESI[†]). Determination of the second-order rate constants for covalent target

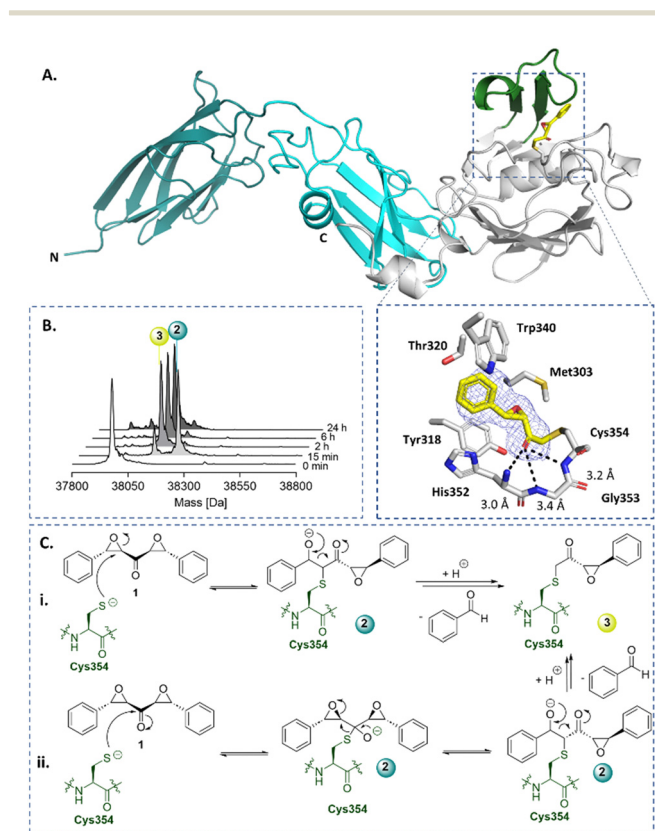


Fig. 2 X-ray crystallography and protein-observed SPE-MS studies inform on the mechanism of DEK inhibition. (A) Views from a crystal structure derived by reaction of Ldt_{Mt2} with DEK **1** (yellow, PDB: 8BK3). The immunoglobulin-like domains are in teal and cyan. The catalytic domain is grey, with the active site lid in green. The mF_o-DF_c polder OMIT map is contoured at 3.0 σ , carved around Cys354 bound **1** (refined as **3**) and shown in blue mesh. Polar interactions are shown in black dashes. (B) Protein-observed SPE-MS experiments inform on the mechanism of reaction of **1** (20 μ M) with Ldt_{Mt2} (1 μ M). (C) The proposed mechanisms for reaction of Cys354 of Ldt_{Mt2} (in green) with **1** *via* reaction with (i) the carbonyl adjacent carbon or (ii) the carbonyl carbon, followed by retro-aldol fragmentation.



inactivation ($k_{\text{inact}}/K_{\text{I}}$)³³ for Ldt_{Mt2} manifested the highest rate of inhibition for **1** ($k_{\text{inact}}/K_{\text{I}}$ of $484.3 \pm 28.4 \text{ M}^{-1} \text{ s}^{-1}$, Table S3 and Fig. S7, ESI[†]). DEKs **5–7** and **9** were observed to inhibit Ldt_{Mt2}, while no evidence for inhibition was observed with **4**, **8** and **10**. The kinetic rate constant for reactivity with GSH (k_{chem})^{27,34} was found to be below the assay limit for all DEKs (k_{chem} of $< 0.08 \text{ M}^{-1} \text{ s}^{-1}$ and half-life ($t_{1/2}$) $> 8.7 \text{ h}$), except **7** and **8** (k_{chem} of 1.71 ± 0.24 and $1.11 \pm 0.20 \text{ M}^{-1} \text{ s}^{-1}$, and $t_{1/2}$ of 24 min and 38 min, respectively; Table S3 and Fig. S8, ESI[†]). DEKs therefore apparently exhibit lower intrinsic reactivity compared to the common cysteine reactive acrylate, maleimide and isothiocyanate groups ($t_{1/2} < 1.0 \text{ min}$), and, with the exceptions of **7** and **8**, chloroacetamide (5.8 h).³⁵ MS studies of the reaction of GSH and **1** manifested an adduct analogous to **3** (Fig. S9, ESI[†]).

Protein-observed SPE-MS assays of **4–11** demonstrated covalent modification of Ldt_{Mt2} with **4–10**, which manifested adducts analogous to those with **1** (Fig. S2 and Table S1, ESI[†]) supporting the generality of the proposed mechanism. Additional peaks of +18 Da were observed with both unfragmented and fragmented adducts of **4–10**, likely due to ring opening of the second epoxide (Fig. S10, ESI[†]). With **1**, **5**, **6** and **7**, over 24 h, a second fragment adduct was observed with a +56 mass shift relative to the unmodified enzyme (Fig. S10, ESI[†]).

DEK **1** apparently displayed a low level of β -elimination of the reacted Cys354 residue, likely to form a dehydroalanine residue (Dha, $\sim 5\%$ in 24 h, as evidenced by a -34 Da mass shift relative to unmodified Ldt_{Mt2}, Fig. S2 and S10, ESI[†]).^{36–38} Interestingly, the *ortho*-trifluoromethoxy substituents on the phenyl groups of **5** promoted Dha formation ($\sim 30\%$ in 24 h). Dha formation was additionally observed following reaction with **4** ($\sim 2.5\%$ in 24 h) and **7** ($\sim 16\%$ in 24 h). In the cases of **6** and **8–10**, no evidence for Dha formation was observed.

While inhibition assays with the α,β -monoepoxyketone **12** did not manifest inhibition of Ldt_{Mt2}, protein-observed SPE-MS assays of Ldt_{Mt2} ($1 \mu\text{M}$) with **12** ($100 \mu\text{M}$) evidenced covalent reaction. As with DEKs **1** and **4–10**, initial measurements (2 h) showed the most abundant adduct to have a mass shift of +224 Da, corresponding to the addition of a single molecule of **12**. A +119 Da adduct was observed to become abundant after 6 h (Fig. S2, ESI[†]), indicating that the retro-aldol fragmentation is conserved between mono- and diepoxide derivatives.

While Ldt_{Mt2} contains only a single cysteine, in principle, the DEKs may alkylate other nucleophilic residues.^{39,40} To investigate whether the DEKs react selectively with Cys-354 of Ldt_{Mt2}, we performed protein-observed SPE-MS assays with Ldt_{Mt2} that had been preincubated with ebselen, which is known to selectively and irreversibly react with Cys354.¹⁶ When **1** and **4–10** were combined with the Ldt_{Mt2}-ebselen complex, no reaction was observed, evidencing that inhibition arises from at least partially, selective reaction with Cys354 (Fig. S11, ESI[†]).

To further investigate the reactivity of DEKs with nucleophilic cysteine enzymes, dose-response assays of **1** and **4–11** were performed with SARS-CoV-2 M^{Pro};^{41,42} note that the covalent reaction of SARS-CoV M^{Pro} with epoxides has been reported.⁴³ While DEKs **1** and **9** were inhibitors of M^{Pro} (pIC_{50} values of

4.6 ± 0.3 and 5.9 ± 0.2 , respectively), no inhibition was observed with **4–8** and **10–11** (Table S3 and Fig. S12, ESI[†]), providing further evidence for potential of the DEKs to react selectively.

Protein-observed SPE-MS experiments with M^{Pro} and the DEKs **1** and **9** (Fig. S13, ESI[†]) manifested a +266 Da adduct (analogous to species **2**, Fig. 2C), with a +160 Da adduct (analogous to species **3**) becoming apparent over time. A second molecule of **1** was observed to bind to M^{Pro} after 3 h (as evidenced by a mass shift of +266 Da relative to the +160 adduct), indicating reaction with a second residue, likely with one or more of the 12 cysteine residues of M^{Pro}. Notably, the second adduct did not fragment by retro-aldol reaction, implying that this pathway can be promoted by the active site, likely by binding of one of the DEK-derived oxygens in the oxyanion hole of M^{Pro}.⁴⁴ Incubation of M^{Pro} with **9** resulted in a single adduct of +186 Da, which can be assigned to a fragmented species analogous to species **3** (Fig. 2C).

As epoxide-bearing compounds may inhibit serine proteases, notably including proteasomes,^{45,46} we tested the ability of the DEKs to inhibit the nucleophilic serine enzyme BlaC, a class A β -lactamase of *M. tuberculosis*. None of compounds **1** and **4–12** exhibited inhibitory potency for BlaC (Fig. S14, ESI[†]).

The combined results of the reaction of DEKs with GSH, cysteine, Ldt_{Mt2} and SARS-CoV-2 M^{Pro}, imply a conserved reaction mechanism, involving epoxide opening followed by retro-aldol reaction. Importantly, the results reveal different reactivity of the 12 M^{Pro} cysteine residues with DEKs, indicating that selectivity for some proteins should be achievable; note that previous results showed that excess ebselen reacts covalently with all 12 cysteine residues.⁴⁷

The results identify DEKs as a new class of nucleophilic cysteine reacting covalent ligands. Variations on the DEK functionality can be readily envisaged *e.g.*, by substituting one or both epoxides for other covalently reacting electrophiles, such as aziridines or acylating agents. Notably, some natural products contain more than one epoxide, sometimes in a contiguous arrangement,⁴⁸ though to our knowledge the DEK functional group has not been identified in natural products. Interestingly, DEKs have 5 hypothetical sites for reaction with nucleophiles (Fig. 1C), and they hold potential for subsequent addition of a second nucleophile. This could be useful in enabling (i) formation of cross-linked enzyme-inhibitor complexes (as can occur with other mechanism based inhibitors, *e.g.*, certain β -lactamase inhibitors),⁴⁹ (ii) labelling of an inhibited protein for analytical or diagnostic purposes, (iii) the capture of enzyme substrates, and (iv) covalent gluing of protein-protein interactions; note that epoxides are used in commonly used polyepoxide glues.⁵⁰ The ability of DEKs to fragment after initial covalent reaction might be useful in releasing a functional fragment, *e.g.*, a cytotoxic agent (the cytotoxicity of benzaldehyde in tumour cells has been reported⁵¹).

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Conflicts of interest

There are no conflicts to declare.

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