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Prodrugs

Unlocking Amides: A General Method for the Self-Immolative Release of Amide-Containing Molecules

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Abstract: The controlled liberation of molecules from a constructed framework is a subject of profound interest across various chemical fields. It allows for the masking of a molecule's properties and precise deployment upon a single controllable release event. While numerous methodologies have been developed for amines, alcohols, and thiols, approaches for utilising amides as payload-release handles are still in their early stages of development, despite the prevalence of amides in therapeutic compounds and materials. Herein, is presented a comprehensive strategy for the controlled and selective release of a diverse range of amides with stable linkers. The versatility of this approach is demonstrated by its successful application in the targeted release of various amide-containing drugs in their natural form via the use of commonly used trigger motifs, such as dipeptides or glycosides. As a proof of concept, the FDA-approved antibiotic linezolid has been successfully converted into a prodrug form and released selectively only in the presence of the trigger event. Significantly, in its prodrug state, no activity against *Mycobacterium tuberculosis* was exhibited. Linezolid's full potential was achieved only upon controlled release, where an equipotent efficacy to the free linezolid control was demonstrated, thus emphasising the immense potential of this method.

Introduction

Strategies that enable the selective release of a molecule in its native form from a larger construct are very attractive in many fields of chemical and biological sciences. One effective approach utilises so called 'self-immolative' or 'traceless' linker systems which trigger construct fragmentation in response to specific events, thereby releasing the target molecule.[1,2] Typically, the fragmentation is initiated by the removal or conversion of functional groups, leading to spontaneous 1,4- or 1,6-elimination reactions, cyclisation, and/or the irreversible loss of gases like CO_2 and SO_2.[3–6] These systems temporarily mask specific properties of the molecule while integrated into the construct, with these properties (such as biological activity) being restored upon release. To this end, such systems are used for a range of applications such as prodrugs and chemical probes,[4,5,7–13] antibody–drug conjugates (ADCs),[14–20] solid supported chemical synthesis,[21] and materials science.[22–26]

To achieve controlled release, specific chemically or enzymatically labile triggers are chosen; examples include cathepsin-cleavable valine–citrulline (Val–Cit) and valine–alanine dipeptides,[16,17] reducible disulphides[27,28] and nitroaryls,[7,9,29] glycosidase,[5,20,30,31] peroxide,[10,32] and sulphatase[33] cleavable groups. Whilst many of these approaches rely on biologically present stimuli, such as enzymes, others employ external stimuli like tetrazines[34] and light responsive[35] triggers. For prodrugs and ADCs, a controlled release enables the targeting of the therapeutic only near the site of action, thus reducing off-target effects and increasing efficacy. Evidently, the ability to release a molecule of interest in its native form is highly attractive, not only to preserve its known properties but also to allow

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new constructs to be synthesised using existing molecules of interest to expedite research programs and allow the exploitation of said molecules.

Self-immolative linker systems often employ common and readily modifiable nucleophilic groups like amines,[36,37] alcohols,[13,16,19,20] or thiols[18] (Figure 1a and Figure 1b, *Previous strategies*) as the payload linkage points, with numerous release methods and variations described.[2] Indeed, all FDA-approved ADCs with cleavable linkers utilise one of these three key functional group handles.[14] However, a controlled release of other functional groups, such as amides, appears to be a challenging task. Amides are heavily underrepresented as payload-release handles, despite their ubiquity in FDA-approved drugs, polymers, and immense biological importance in proteins and peptides. A few approaches have been developed to release amides,[38–41] with a desire to enhance physicochemical properties, safety, and efficacy, however, with limited success.

To the best of our knowledge, a comprehensive method for an amide-based release system, accommodating diverse amide and trigger components, remains unavailable. Additionally, existing approaches rely on specific or unstable trigger motifs, limiting controlled fragmentation.[38–40] Many amide-containing drugs lack other modifiable release handles, potentially constraining their use as prodrugs. One of the best examples of this is linezolid, a synthetic oxazolidinone antibiotic used mainly for the treatment of Gram-positive and mycobacteria infections, where it prevents protein synthesis initiation by binding to ribosomal subunits.[42–47] Whilst libraries of analogues have been synthesised to try improve the properties of linezolid,[48–50] the number of strategies that release the native molecule remain scarce due to the lack of a conventional free amine, alcohol or thiol function group.[39,41]

Herein, a general and modular synthesis of an aminomethyl carbamate linker system that allows for complete choice over the amide and trigger components is described. It enables the creation of stable constructs that undergo selective and controlled self-immolative release using a range of trigger systems. The use of the Curtius rearrangement, often utilised in the synthesis of carbamates, ureas, and primary amines in natural product synthesis,[51,52] allowed for one-pot attachment of nitrobenzyl, dipeptide and glycoside-based triggers. The method was showcased with the selective and efficient release of various aliphatic and aryl amides such as linezolid, lidocaine, and sulfamethi-

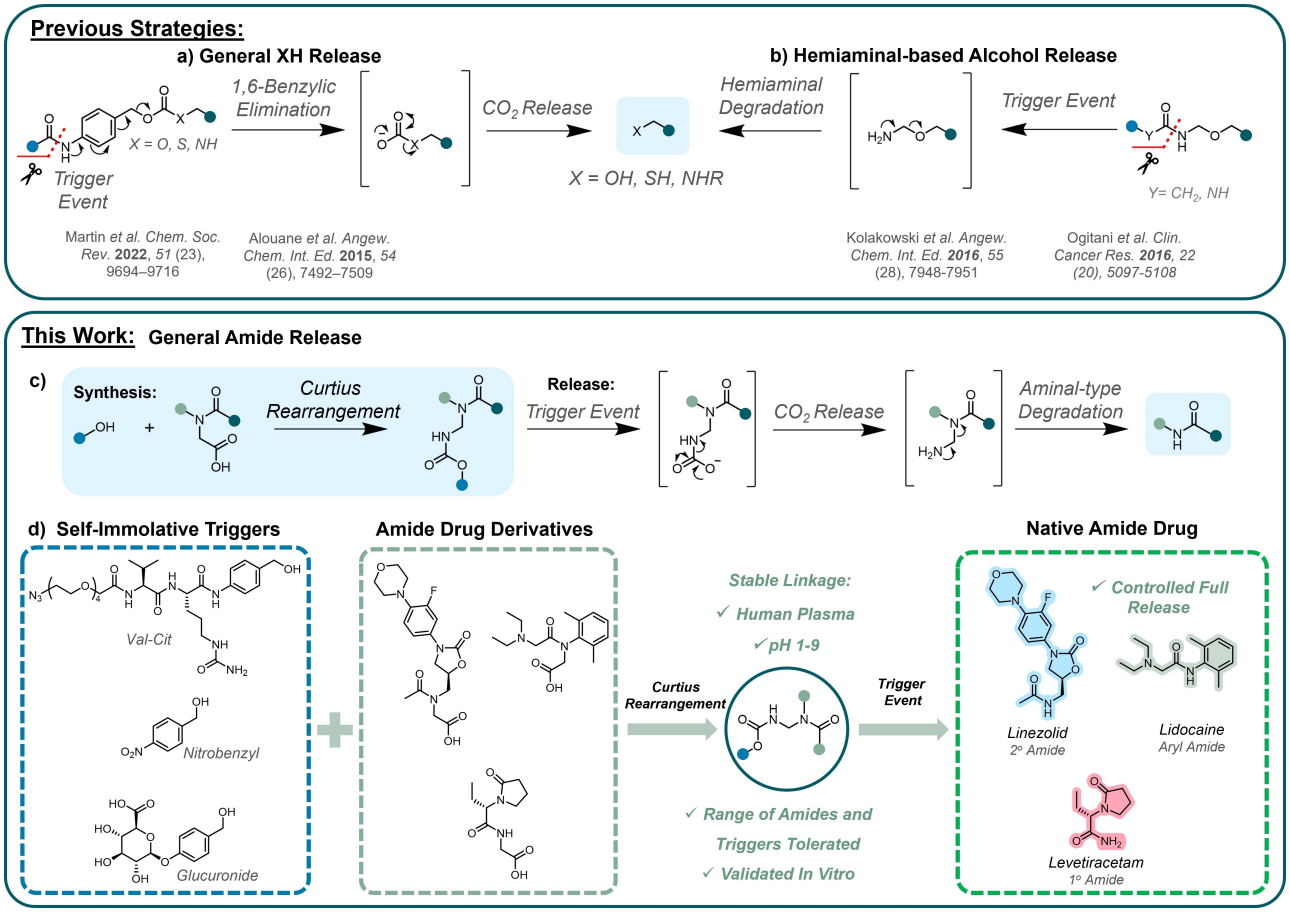


Figure 1. Overview of self-immolative release strategies. *Previous work:* a) general methods to release alcohols, thiols, and amines, b) hemiaminal-based alcohol release strategies. *This work:* c) Synthetic route and release mechanism of amide based self-immolative linkers, d) scope of the method showing the variety of diverse triggers and amides employed in this work. All enabled the generation of stable constructs with selective and efficient release rates.

zole. Further biological testing showed that a ValCit–linezolid prodrug exhibited equipotency to a free linezolid control upon a selective enzymic trigger event, validating this amide release approach for therapeutic use.

Results and Discussion

With previous positive experience using the versatile carbamate-based amine release, it was envisaged that a stable linkage and controllable amide release could be enabled by utilising an aminomethyl carbamate linkage between the desired amide and the trigger component (Figure 1c). Carbamates are widely used in self-immolative linker systems as they have drug-like properties, stability when incorporated into a linker, and crucially can be readily eliminated following a trigger event.[53] In this case, the carbamate is eliminated by an appendaged trigger to reveal an unstable terminal carbamic acid that spontaneously releases CO_2, affording an *N*-aminomethyl amide. This can then degrade via an aminal-type mechanism to release the native parent amide (Figure 1c),[38] similar to methods previously used to release alcohols.[19,20]

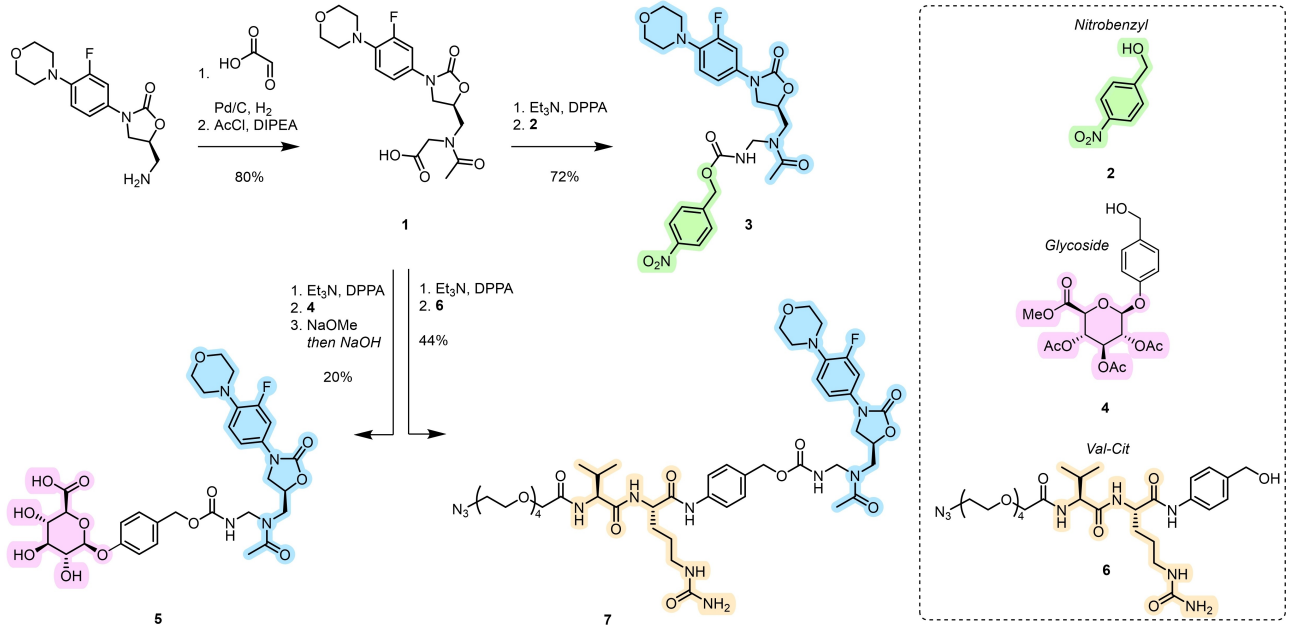
As a proof-of-concept, a suitable drug and trigger combination had to be chosen. Crystallography of linezolid binding to ribosomal subunits has shown that the area around the acyl amide motif fits deep into the active site[54] and so while modification is tolerated, activity can be easily lost by the inclusion of bulky groups in this area.[50] Linezolid was, therefore, chosen as a model amide drug for our proof-of-concept studies due to the lack of available release strategies and the importance of the drug clinically. Linking the drug via the amide could greatly reduce activity, therefore ensuring the model construct would only show a biological effect upon fragmentation. Added to this, a prodrug strategy could help to reduce off-target toxicity associated with linezolid, such as nerve damage.[47]

Firstly, a simple nitrobenzyl trigger was chosen and incorporated into a linezolid prodrug, **3** (Scheme 1). Nitrobenzyl-based triggers have been utilised in anticancer therapy[7,55] and antimicrobial probes[56] where their reduction is facilitated by the hypoxic tumour environment or nitroreductase enzymes,[9,57] respectively. Under reducing conditions, the electron withdrawing nitro group is rapidly converted to an electron donating amine which can then undergo spontaneous 1,6-benzylic elimination to release the carbamate. Decarboxylation followed by aminal-type degradation should then reveal the parent amide.

The hypothesis was that prodrug **3** could be synthesised via the Curtius rearrangement of *β*-carboxylic acid linezolid derivative **1**, trapping the isocyanate intermediate with nitrobenzyl alcohol (Scheme 1).[51,52] Starting from a commercially available amino-linezolid precursor, a reductive amination with glyoxylic acid followed by acetylation afforded the *β*-carboxylic acid **1** in good yield. Multiple approaches for the direct alkylation of linezolid itself were attempted, but with limited success, presumably due to a lower nucleophilicity of the amide. Moreover, it was observed that the use of strong bases led to the opening of the oxazolidinone ring and decomposition of linezolid.

A diphenylphosphoryl azide (DPPA)[58] mediated Curtius rearrangement of **1** in DMF afforded an isocyanate intermediate which was trapped in situ with nitrobenzyl alcohol **2** to produce the nitrobenzyl-linezolid **3** (Scheme 1).[63]

With nitrobenzyl-linezolid **3** in hand, attention shifted towards investigating the method's versatility, specifically examining whether further diverse triggers could be accom-



Scheme 1. Synthesis of model linezolid prodrugs **3**, **5**, and **7**, respectively, via Curtius rearrangement of linezolid acid **1**. *Abbreviations:* DPPA = Diphenylphosphoryl azide.

modated. Glycosidase and cathepsin protease cleavable moieties (such as glycosides and the dipeptide Val–Cit, respectively) are commonly used in cancer targeting ADCs and prodrug strategies due to the prevalence of these enzymes in tumours.[16,17,30] Therefore, glycoside-linezolid **5** and ValCit–linezolid **7** were synthesised analogously to nitrobenzyl-linezolid **3** via Curtius rearrangement (Scheme 1). Consequently, the inclusion of a glycoside meant that the drug release mechanism would go via a phenolic rather than aniline intermediate; further showing the robustness of this approach.

To test if the aminomethyl carbamate linkage could successfully release linezolid, nitrobenzyl-linezolid **3** was subjected to mild reduction using aqueous sodium dithionite at 37 °C in phosphate buffered saline (PBS) buffer (pH 7.4). Pleasingly, the construct was shown to fully fragment within 24 hours, as observed via UV-LCMS (Figure 2) and showed no signs of fragmentation in the absence of the reducing agent. UV-LCMS analysis allowed the intermediates to be tracked during the fragmentation. The initial reduction of the nitrobenzyl to the aniline is extremely fast, occurring within 5 minutes from treatment with sodium dithionite and was therefore not observed via either UV or mass detection

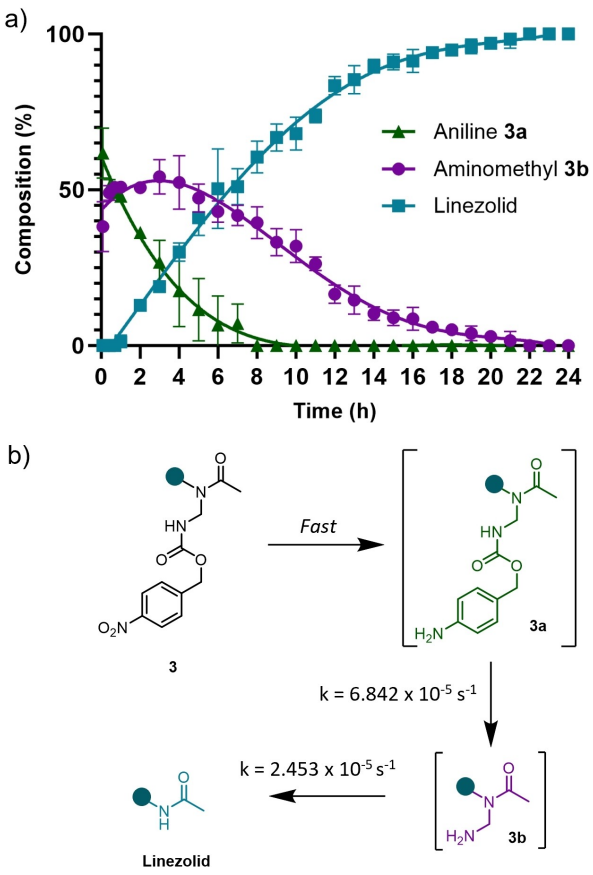


Figure 2. a) Graph showing the fragmentation of nitrobenzyl-linezolid **3**; b) Proposed mechanism of fragmentation. **3** is fully reduced within 5 minutes, revealing aniline **3a** which quickly undergoes a 1,6-benzylic elimination and loss of CO_2 to afford aminomethyl **3b. 3b** degrades more slowly to release linezolid fully within 24 hours.

(Figure S5). The aniline **3a** is observed but, as hypothesised, degrades to reveal the longer-lived aminomethyl **3b** species via a 1,6-benzylic elimination to the carbamic acid which rapidly decarboxylates. Finally, the aminomethyl **3b** fragments to release the native linezolid within 24 hours.

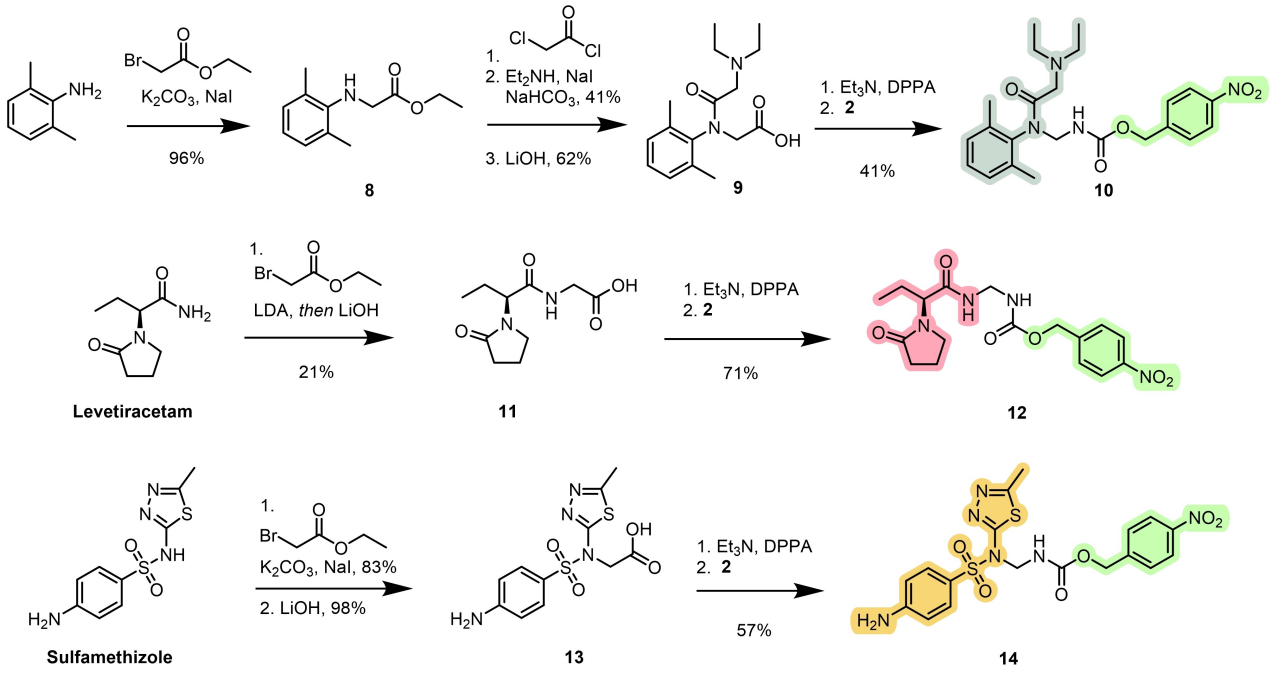
Importantly, degradation of excess sodium dithionite led to a decrease in pH which hindered the release kinetics of **3a** to linezolid, probably due to amine protonation (Figure S4). Therefore, to mitigate this shift, the pH was maintained between 7–8 throughout the study which enabled physiologically relevant conditions to be maintained and a steady drug release. When sodium dithionite was not used to cause the fragmentation trigger event, it was found that the pH did not shift away from that of the buffer (see below), suggesting that the byproducts produced during the fragmentation do not adversely impact the pH and that the acidity observed during the fragmentation of **3** was due wholly to the sodium dithionite.

To gain a better understanding of the release, the rate constants for the two intermediate fragmentation steps were calculated: assuming irreversible fragmentation and first-order kinetics. Both rates were found to be in the order of 10^{-5} s^{-1} (Figure 2b), which is favourably comparable to other self-immolative linker systems,[36] and corresponds to previously reported findings for aminomethyl amide fragmentation.[38]

To demonstrate that the release was a result of nitro reduction rather than mere molecular instability, investigations were conducted on nitrobenzyl-linezolid **3** to assess the applicability of these constructs in biologically relevant scenarios. Nitrobenzyl-linezolid **3** underwent incubation at 37 °C in human plasma and PBS buffer (pH 7.4) for a duration of 10 days. Encouragingly, no evidence of degradation was observed, as confirmed by UV-HPLC and LCMS (see Supporting Information).

Furthermore, pH studies were conducted across a range of aqueous buffers, spanning from pH 1 to 14 (see Supporting Information). Notably, following a 10-day incubation at 37 °C, **3** exhibited complete stability within the pH range of 1 to 9.2, with degradation only occurring at pH 14 over a 48-hour period. These findings provide robust evidence that the aminomethyl carbamate linker remains stable under physiologically relevant conditions and is solely cleaved upon activation of the trigger motif.

With the construct stability and selective fragmentation proven, attention turned to expanding the scope of amide types that could be released with this method. The mechanism hinges on the ability of the final aminomethyl amide to collapse down to the parent amide, therefore a range of drugs containing different types of amide functionality were chosen to see if their release was possible. Linezolid showcased the possibility of secondary amide release, and so molecules to release an aryl amide (lidocaine), primary amide (levetiracetam), and sulphonamide (sulfamethizole) were chosen. These drugs were also chosen since they contain few classical linker attachment points and therefore release via the amide could be highly beneficial.



Scheme 2. Synthesis of nitrobenzyl prodrugs of lidocaine **10**, levetiracetam **12**, and sulfamethizole **14**. All syntheses utilise a Curtius rearrangement of a carboxylic acid derivative of the parent drug and the desired trigger alcohol. *Abbreviations:* LDA = lithium diisopropylamide.

Consequently, model prodrugs **10**, **12**, and **14** were synthesised utilising a nitrobenzyl trigger (Scheme 2). Compounds **12** and **14** where successfully synthesised via direct alkylation of the native drug, showcasing the opportunity of late-stage functionalisation using this approach.

The stability of these compounds was then tested against human plasma and in pH 4 and 9.2, analogously to compound **3**.

Pleasingly, the true amides **10** and **12** showed complete stability in all tests. However, although sulphonamide **14** showed stability in acidic media (pH 4) it showed instability in basic media (pH 9.2) and in human plasma with near complete loss of **14** over 24 hours (Figure S1). Neutral conditions (pH 7.2) slowed the release rate greatly (c. 33 % released over 3 days), though clearly this instability would hinder the use of **14** in a biological setting.

The release rate of prodrugs **5**, **10**, **12**, and **14** was then tested in the same way as nitrobenzyl linezolid **3** (see above). Pleasingly, release studies of **10** and **12** with sodium dithionite in PBS buffer (pH 7.4) and **5** with glucuronidase showed similar fragmentation rates to nitrobenzyl linezolid **3**, all fully releasing within 24 hours (Figure 3, Figure S11).

Interestingly, sulphonamide **14** was shown to fully release in only 4 hours. An aminomethyl intermediate was not observed, suggesting that the final aminal-type degradation is much faster for the sulphonamide than for the true amides, probably due to the more acidic nature of the sulphonamide NH and therefore it being a better leaving group. The acidity of the leaving amide speeding up release would also explain the increased rate for the fragmentation of **10**, as the aryl amide will have a lower pKa than the alkyl amides in **3** and **12**.

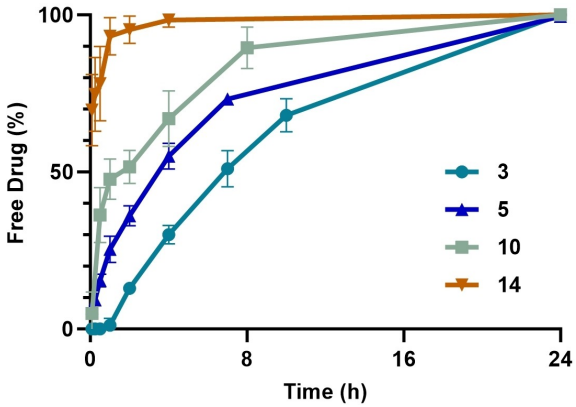


Figure 3. Drug release studies from nitrobenzyl triggered prodrugs of linezolid **3**, lidocaine **10**, and sulfamethizole **14**, and glycosidase triggered linezolid prodrug **5**. Graph shows percentage of released drug over time as measured by UV-LCMS or HPLC peak integration. Error bars show standard deviation of three replicates.

This propensity for cleavage is probably also the cause of the instability of **14**, though it is of note that a triggered release is far faster than spontaneous degradation. Therefore, whilst the instability of **14** in aqueous media is disappointing, a successful triggered release has been shown and the release of sulphonamides may be of interest in other areas of chemistry where aqueous stability may be less of a concern such as in solid-support synthesis or materials science.

Release studies on glycoside-linezolid **5** using *E. coli* β-glucuronidase showed efficient drug release, with the

enzymic cleavage complete within 10 minutes (50 μg/ml enzyme).

Interestingly, unlike the aniline intermediates in the release of prodrugs **3** and **10**, the analogous phenol intermediate from the fragmentation of **5** was not observed by UV-LCMS and so must be extremely short lived, quickly breaking down to the aminomethyl intermediate; this leads to an overall faster release than **3** which is slowed by the fragmentation of aniline intermediate **3a**. The pH during the release study of **5** never dropped below 7, showing that it was the sodium dithionite that was causing the decreased pH in the initial release studies. Because levetiracetam exhibits poor UV absorbance, the full release from **12** could not be followed by UV-LCMS and therefore a full drug release was instead proven by NMR analysis after 24 hours (Figure S11). This data shows that a range of amide and amide-like motifs can be released using the aminomethyl carbamate linkage.

Having successfully shown that the linker system could be used to produce stable constructs able to selectively fragment, the next aim was to assess whether the prodrug strategy would enable effective therapeutics. Therefore, the effect of the ValCit–linezolid prodrug **7** was tested in vitro. Since linezolid is an active agent against *Mycobacterium tuberculosis (Mtb)*,[43,44] ValCit–linezolid **7** was tested against luminescent *Mtb* to assess the effect on planktonic growth (Figure 4). Pleasingly, **7** showed equipotency to native linezolid, when incubated with cathepsin B protease, at all concentrations (Figure 4b).

This indicates that the presence of cathepsin B was able to trigger the release of linezolid from **7** efficiently and to completion, allowing it to exhibit the same potency. In contrast, no significant effect on *Mtb* growth was observed both in the absence of cathepsin B or in the presence of a cathepsin B inhibitor (Figure 4). This strongly indicates the stability of the linker system to resist spontaneous linezolid release and, gratifyingly, a complete lack of efficacy of the prodrug itself.

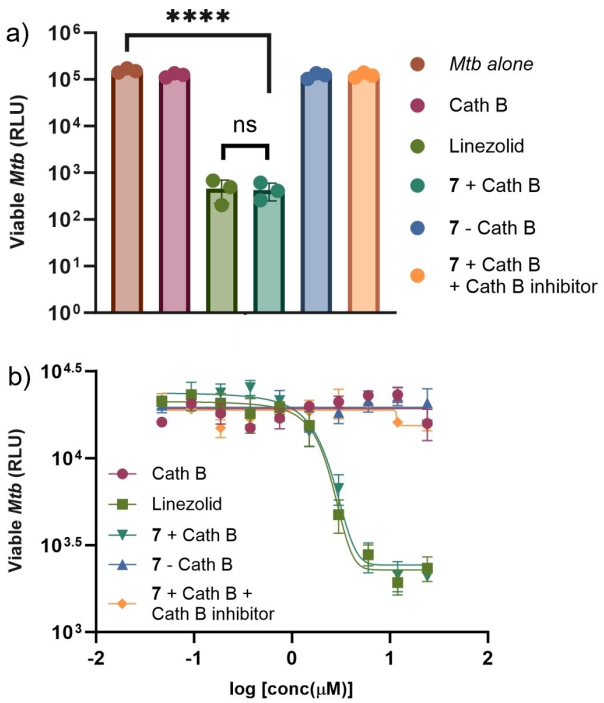


Figure 4. a) Number of viable luminescent *M. tuberculosis* (*Mtb*) cells after incubation with 6 μM solutions of linezolid, ValCit–linezolid **7**, and various controls after 5 days; detected by luminescence. ValCit–linezolid **7** showed significant reduction in viable cells and equipotency to free linezolid when cathepsin B enzyme was present. Studies of **7** without cathepsin B or while inhibiting the enzyme showed complete lack of efficacy. b) Number of viable luminescent *Mtb* cells at various concentrations of linezolid, and ValCit–linezolid **7** with/without cathepsin B enzyme. Results suggest that linezolid is being fully released from **7** selectively on the addition of cathepsin B, leading to an equipotency with free linezolid. Error bars show standard deviation of 3 biological replicates. Sigmoidal line of best fit. T-test: $p = < 0.0001$ between *Mtb* alone and **7** + Cath B. *Abbreviations:* Cath B = Cathepsin B, ns = not significant, RLU = relative light units.

Conclusion

A facile and stable linker system capable of the efficient and controlled release of amides has been developed using aminomethyl carbamate linkages. A range of amide functionalities have been successfully released, demonstrating that this method could be further utilised for many primary or secondary amides and sulphonamides. Furthermore, the application of a range of different trigger systems has been described, and it is proposed that any trigger system capable of allowing self-immolative elimination could therefore be used. This allows the choice of amide to be supplemented with a suitable trigger of choice, enabling considered combinations to be prepared based on the purpose of the construct. In many cases, late-stage functionalisation of the amide motif allows for a facile synthesis of the key aminomethyl carbamate linkage without the need for elaborate synthetic routes. The method was proven in a biological setting, with a prodrug releasing linezolid selectively and showing equipotency to the free drug. Moreover, this method adds to the arsenal of self-immolative linker options, enabling a greater flexibility of linker attachment to a molecule and therefore allowing structure–activity relationship and molecular properties to be considered when choosing how to release a molecule of interest. Ultimately, this opens up new possibilities for amide containing molecules to be released for the fields of prodrugs, chemical probes, ADCs, and in materials science.

Supporting Information

The authors have cited additional references within the Supporting Information.[59–62]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Amides · Linezolid · Prodrugs · Release · Self-immolative

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[63] The Curtius rearrangements were initially carried out in toluene, however, poor solubility of many of the carboxylic acids led to long reaction times and poor yields. To improve this, a partial optimisation was carried out and it was found that the use of DMF and 10 mol % dibutyltin dilaurate greatly improved the yields and shortened the reaction time to 2 hours overall.

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Research Articles

Prodrugs

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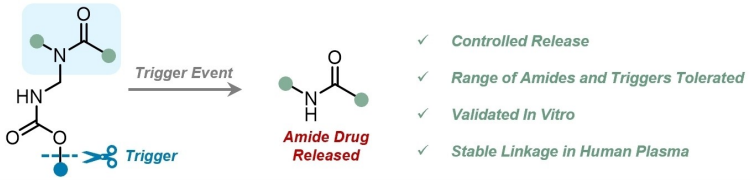
Unlocking Amides: A General Method for the Self-Immolative Release of Amide-Containing Molecules



Methodologies for the self-immolative release of amide motifs remain scarce despite the prevalence of amides in molecules. Herein, is presented a general and comprehensive strategy for the selective release of a diverse range of amides using a range of common triggers. A stable prodrug of the FDA approved antibiotic linezolid showcased the method and exhibited selective release and equipotency to free linezolid against *Mycobacterium tuberculosis*.