



HAL
open science

Covalent-reversible peptide-based protease inhibitors. Design, synthesis, and clinical success stories

Anthony Feral, Anthony R. Martin, Alexandre Desfoux, Muriel Amblard,
Lubomir Vezenkov

► To cite this version:

Anthony Feral, Anthony R. Martin, Alexandre Desfoux, Muriel Amblard, Lubomir Vezenkov.
Covalent-reversible peptide-based protease inhibitors. Design, synthesis, and clinical success stories.
Amino Acids, In press, 10.1007/s00726-023-03286-1 . hal-04167336

HAL Id: hal-04167336

<https://hal.science/hal-04167336>

Submitted on 20 Jul 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Covalent-reversible peptide-based protease inhibitors. Design, synthesis, and clinical success stories.

Authors:

Anthony Feral¹ (0000-0001-8896-9710), Anthony R. Martin¹ (0000-0001-6187-6979), Alexandre Desfoux¹ (0009-0006-7058-4197), Muriel Amblard¹ (0000-0001-8922-1755), Lubomir L Vezenkov^{1*} (0000-0001-6348-7985)

Key words: Peptides, Covalent inhibitors, Covalent-reversible inhibitors, Drug discovery, Enzymes, Proteases

¹IBMM, University Montpellier, CNRS, ENSCM, Montpellier, France

* Correspondence: lubomir.vezenkov@enscm.fr

Abstract: Dysregulated human peptidases are implicated in a large variety of diseases such as cancer, hypertension, and neurodegeneration. Viral proteases for their part are crucial for the pathogens' maturation and assembly. Several decades of research were devoted to exploring these precious therapeutic targets, often addressing them with synthetic substrate-based inhibitors to elucidate their biological roles and develop medications. The rational design of peptide-based inhibitors offered a rapid pathway to obtain a variety of research tools and drug candidates. Non-covalent modifiers were historically the first choice for protease inhibition due to their reversible enzyme binding mode and thus presumably safer profile. However, in recent years, covalent-irreversible inhibitors are having a resurgence with dramatic increase of their related publications, preclinical and clinical trials, and FDA approved drugs. Depending on the context, covalent modifiers could provide more effective and selective drug candidates, hence requiring lower doses, thereby limiting off-target effects. Additionally, such molecules seem more suitable to tackle the crucial issue of cancer and viral drug resistances. At the frontier of reversible and irreversible based inhibitors, a new drug class, the covalent-reversible peptide-based inhibitors, has emerged with the FDA approval of Bortezomib in 2003, shortly followed by 4 other listings to date. The highlight in the field is the breathtakingly fast development of the first oral COVID-19 medication, Nirmatrelvir. Covalent-reversible inhibitors can theoretically provide the safety of the reversible modifiers combined with the high potency and specificity of their irreversible counterparts. Herein, we will present the main groups of covalent-reversible peptide-based inhibitors, focusing on their design, synthesis, and successful drug development programs.

1. Introduction

Proteases, also known as peptidases, represent one of the biggest classes of human and viral enzymes with over 600 human proteases discovered to date (compared to around 500 kinases for example) (Rawlings *et al.* 2018, <http://www.ebi.ac.uk/merops/>). Peptidases catalyze a very simple reaction, the hydrolysis of one or several peptide bonds, breaking down proteins to peptides fragments and amino acids. Behind this relative simplicity hides an extremely complex and diverse class of proteins (Bond 2019). This complexity stems first from their extreme difference in size, ranging from 20 kDa to 6 MDa for some proteolytic complexes, but also type of active site and catalytic residues, tissue, or cellular localizations and so on. This complexity translates in a preference to a variety of substrates with some proteases being promiscuous, while other being exquisitely specific to their substrates (Hedstrom 2010; Rawlings *et al.* 2018). The role of the proteases is far from limited to simple degradation, and a lot of the peptide bond cleavages represent highly biologically relevant post-translational modifications (Rogers and Overall 2013). Such modifications are implicated in cell cycle and morphology, homeostasis, immune response, hormone regulation and many other biological processes.

Dysregulation of peptidase's activity is implicated in various diseases, such as high blood pressure, cancers and neurodegenerative diseases to name a few (Bond 2019). Therefore, regulating their action has provided a great number of medications. Indeed, protease inhibitors represent about 3-4% of drugs approved by the FDA and are currently the 8th most explored target class just behind the kinases (Santos *et al.* 2017). On the other hand,

viral proteases, are crucial for the virus assembly and maturation; they are therefore highly explored as therapeutic targets. This is showcased by the fact that, out of the 26 HIV FDA approved drugs, 10 are protease inhibitors (Lv *et al.* 2015). This is even more striking in the case of Hepatitis C where the overwhelming majority (9 out of 13, ~70%) of approved drugs target viral proteases. Recently, the 2020 COVID-19 outbreak triggered a frenetic effort to discover SARS-CoV-2 main protease (M^{pro}) inhibitors (Ghosh *et al.* 2020; Ullrich and Nitsche 2020; Paul *et al.* 2021). In the end of the 2021, FDA issued an emergency approval of Nirmatrelvir in combination with Ritonavir™ for the treatment of mild-to-moderate coronavirus disease. Thus, Nirmatrelvir, a peptide-based inhibitor of SARS-CoV-2 M^{pro} , represents the first orally available FDA approved anti-COVID-19 drug (Joyce *et al.* 2022)

According to their hydrolytic mechanism, proteases could be divided into two main groups. The first group encompasses the aspartic (AspP), glutamic (GluP) and metalloproteases (MetalloP) that promote the one-step hydrolytic cleavage of the peptide bond through the activation of a water molecule (Fig 1A). The second one includes the serine (SerP), threonine (ThrP) and cysteine proteases (CysP) that perform the peptide bond cleavage in a two-step mechanism. In this case, the targeted amide bond undergoes a first nucleophilic attack by a Cys, Ser, or Thr residue, thereby forming a covalent conjugate with the substrate (Fig 1B). Next, an activated water molecule hydrolyses the newly formed enzyme-substrate conjugate.

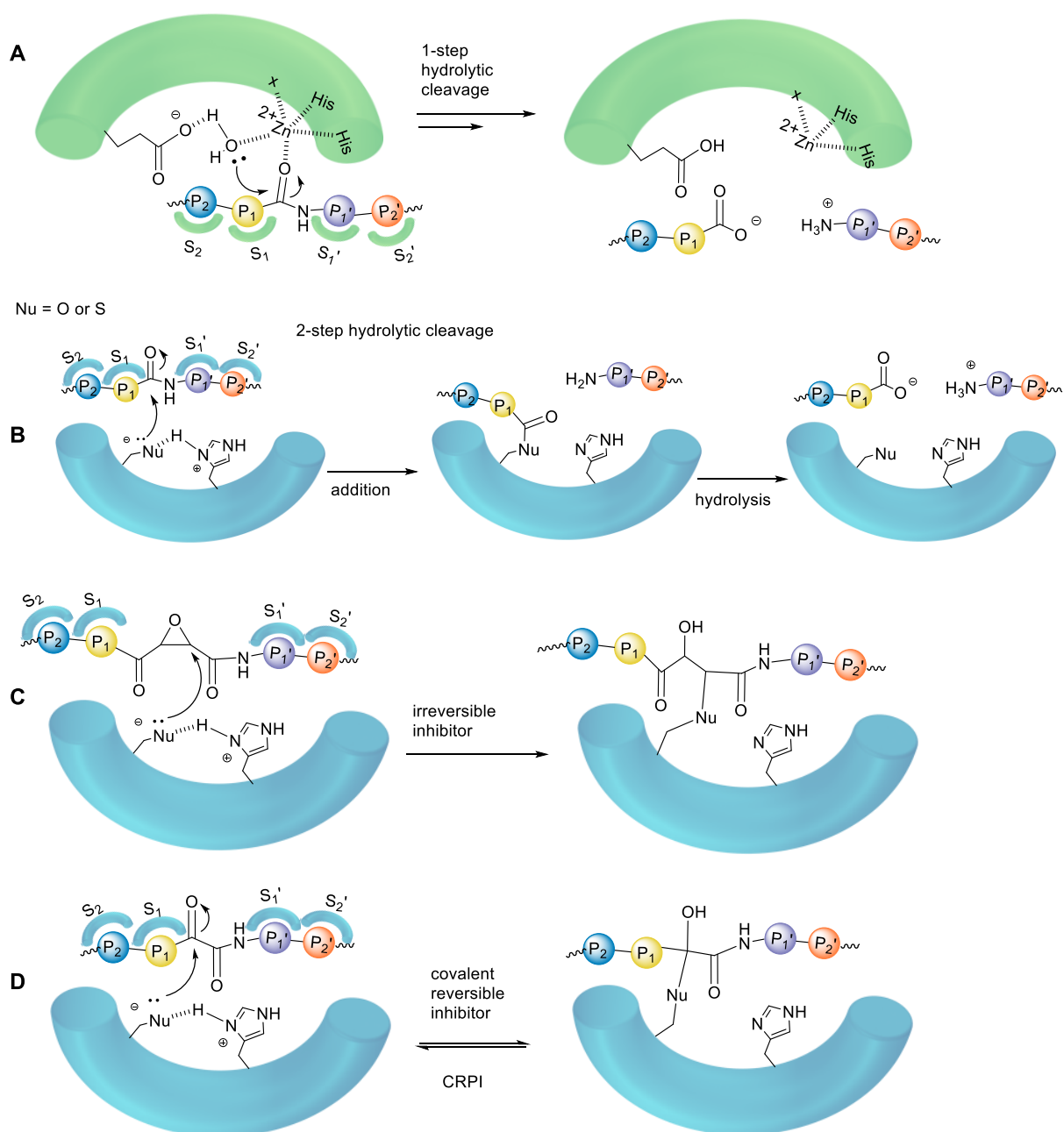


Fig 1: **A)** Schematic representation of one-step hydrolytic cleavage of a peptide substrate by an enzyme; **B)** Schematic representation of two-step hydrolytic cleavage of a peptide substrate by an enzyme; **C)** Schematic representation of an enzyme inhibition by a peptide-based covalent irreversible inhibitor; **D)** Schematic representation of an enzyme inhibition by a peptide-based covalent reversible inhibitor (CRPI).

Inhibitors are required for the different classes of enzymes as research tools and for drug development purposes. A possible method for discovering such compounds involves the screening of small molecules and/or natural products. Once identified, "hit" molecules are then further optimized into "lead" compounds using a QSAR study. Another approach is the rational design of peptide-based inhibitors considering the hydrolytic mechanism of the enzyme, the structure of the enzyme if available, and the structural features of its natural substrate. One way to achieve this is by substituting the substrates' scissile bond by a non-cleavable isostere such as a transition state mimic. Another approach is to introduce an electrophilic moiety such as an epoxide or a ketone that will form a covalent bond with the enzymes' catalytic residue faster than with the scissile amide bond (Fig 1C and 1D). This approach enables a rapid and straightforward rational design of lead inhibitors and can be applied even if the structure of the enzyme is still unknown. On the downside, extensive investment is necessary to enhance the pharmacokinetics (PK) of such compounds, as peptides are known to have low bioavailability, particularly when administered orally. Ironically, the first "blockbuster" protease inhibitor Captopril (*Capoten*®), targeting the angiotensin converting enzyme 1 (ACE1) was developed by combining both strategies. Indeed, the design of this antihypertensive drug was based on its natural substrate angiotensin 1 and a proline-rich alpha peptide issued from snake venom. Saquinavir (*Invirase*®), one of the first anti-AIDS drug, marketed in 1995, was a peptide-based inhibitor of HIV aspartyl protease containing a crucial statin residue which mimics the transition state formed during peptide bond hydrolysis. Both drugs are reversible competitive inhibitors of their respective target. Indeed, such non-covalent ligands were historically the first choice for the inhibition of both classes of proteases. This strategy was especially proficient in the case of 1-step catalytic enzymes. For example, all the FDA approved drugs targeting the HIV aspartyl protease are non-covalent inhibitors, most of them being peptide-based inhibitors. On the other hand, peptide-based covalent-irreversible modifiers, provided a fast strategy for targeting the 2-steps catalysis proteases. This approach consists in substituting the cleaved peptide bond of a preferred substrate by a highly electrophilic residue such as an epoxide, a Michael acceptor, or an alkyne group (Fig 1C). The advantages of the covalent inhibitors are their high potency and long duration of action. Additionally, in the case of viral and cancer treatments they are less prone to chemoresistance (Singh *et al.* 2011). However, concerns over the specificity and the safety profile of such compounds have greatly stalled their clinical applications. The first covalent inhibitor, none-other than *Aspirin*®, was approved in 1899, while the first peptide-based covalent-irreversible inhibitor, the proteasome targeting epoxide Carfilzomib (*Kyprolis*®), was approved more than a century later in 2012 (for an excellent review and a timeline, see (Boike *et al.* 2022)). Indeed, the somewhat dogmatic believe in the drug discovery field was that electrophilic warheads will react with a multitude of off-targets, thus providing unpredictable side effects and/or provoking hypersensitivity. The opposite argument is that covalent inhibition reduces the therapeutically active dose so significantly that it largely compensates for the potential side effects (Singh 2022; Boike *et al.* 2022). A recent study by the Klein group (Jöst *et al.* 2014) as well as our own experience with epoxide inhibitors (Rogowski *et al.* 2023, WO2023025861A1) indicate that in general the estimation of covalent inhibitors' reactivity and promiscuity is overstated. There is a recent trend towards the use of covalent inhibition strategy, highlighted by the success of two kinases (*Imbruvica*®, *Tagrisso*®) and one protease (*Opdivo*®) irreversible inhibitors for cancer treatment; both ranked comfortably among the top 20 best-selling drugs. However, it remains to be determined if this approach would yield similar success beyond the treatment of cancers.

An attractive compromise between the non-covalent and the covalent-irreversible inhibition is represented by the covalent-reversible peptide-based inhibitors (CRPIs). Indeed, electrophilic groups such as carbonyls, nitriles, boronic acids and cyanoacrylates can form covalent yet reversible bonds with nucleophilic residues located in the active sites of proteases (Fig 1D). Thus, CRPIs can retain much of the potency, specificity, and duration of action of covalent-irreversible inhibitors while alleviating the associated safety concerns thanks to the reversibility of their binding (Bandyopadhyay and Gao 2016; Faridoon *et al.* 2023). The clinical success of CRPIs is relatively recent, considering that Bortezomib was the first CRPI approved by the FDA in 2003 (see Fig 2 for a timeline of the clinically approved CRPIs). However, the amazingly quick development of Nirmatrelvir for COVID-19 treatment highlighted the high-potential of this novel drug class. Indeed, this nitrile SARS-CoV-2 M^{pro} oral inhibitor was developed and approved in less than a year.

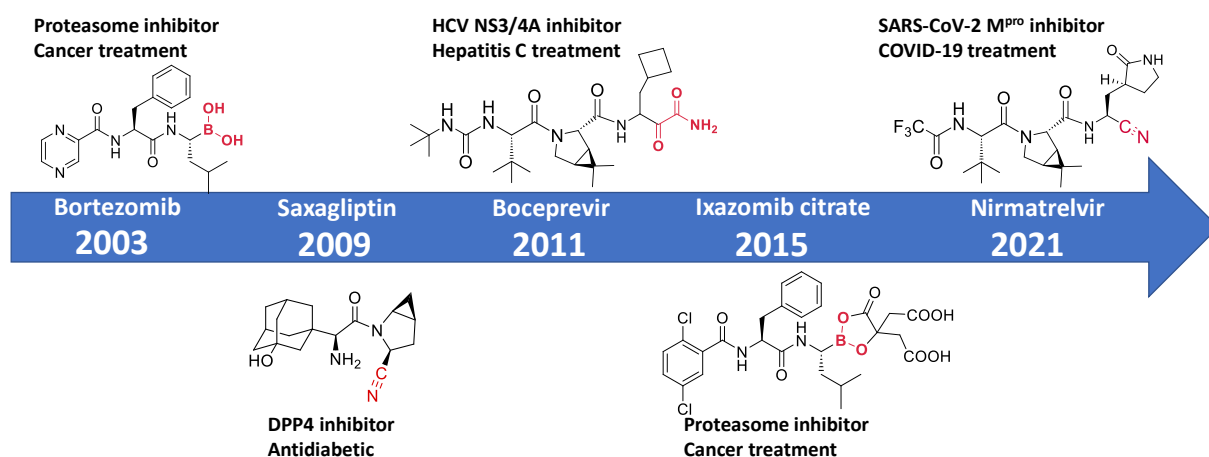


Fig 2: Timeline of the clinically approved CRPIs. Electrophilic groups are highlighted in red.

Could the CRPIs strategy really combine the best of both worlds and what could be its limitations? The current review will concentrate on the recent advances in the field, focusing on the most important classes of CRPIs, by exemplifying with clinically approved molecules. The goal of the review is not to provide an exhaustive list of inhibitors and targets but rather to outline general design strategies for the CRPIs and the most established synthetic pathways for obtaining them. One can view this article as a humble “getting started guide” in the field. Of note, an excellent review on the more general topic of covalent-reversible inhibitors was published during the revisions of this publication by Faridoon *et al.* (Faridoon *et al.* 2023).

2. CRPIs: design, choice of the electrophilic “warhead”

In a general manner, the design of CRPIs can be summarized as follows: 1) Identify a good substrate of the enzyme either based on the sequence of its natural substrate or *via* a screening. 2) Substitute the scissile bond in the chosen substrate by a suitable warhead that mimics the amide bond and allows a covalent reversible inhibition. 3) Improve the potency of the CRPI by QSAR. 4) Improve the PK and druglikeness of the CRPI by reducing its size. Enhance the CRPI enzymatic and metabolic stability by introducing unnatural amino acids and by modifying or suppressing some of its amide bonds.

In this oversimplified strategy the choice and positioning of the electrophile is crucial. Fig 3 summarizes the most widely used covalent-reversible electrophilic moieties and their localization within a CRPI sequence. In most clinically relevant CRPI cases, the warheads are positioned either in the middle of the sequence or at its C-terminus. It is important to note that, for all 4 FDA approved CRPIs the electrophile is positioned at the C-terminus (Fig 2). Theoretically, aminopeptidases could be targeted by installing the warhead at the N-terminus, however such examples are exceedingly scarce in the literature.

Importantly, the choice of reversible electrophiles provides a certain degree of specificity for the targeted protease. In fact, ketones are mainly used for the inhibition of CysP as they are not electrophilic enough to block the SerP/ThrP. Similarly, cyanoacrylates target exclusively CysP, through a reversible 1,4-Michael addition. Conversely, to the CysP specific electrophilic warheads, α -ketoamides and aldehydes allow the inhibition of both CysP and SerP/ThrP. It is noteworthy that the presence of α -proton on ketones, α -ketoamides or aldehyde can provoke an enolization-induced racemization of the α -carbon stereocenter and could erode the biological activity. Lastly, the boronates can selectively inhibit SerP/ThrP by the interaction of their empty *p* orbital with hydroxy lone pair of the Ser/Thr. Of note, the first FDA approved CRPI, Bortezomib, resorted a boronate warhead to target the proteasome’s $\beta 5$ and $\beta 1$ subunits (Fig 2 and Fig 8B).

Concerning the positioning of the above-mentioned warheads, α -ketoamides and ketones can be equally positioned at the C-terminus of the CRPI or embedded within its sequence. Heretofore, cyanoacrylates have only been reported within a CRPI. For structural reasons, α -ketoacids, boronates, aldehydes and nitriles are exclusively found at the C-terminus of the CRPI sequences. The nitrile warhead behaves as a less reactive analogue of aldehydes and in some cases can improve the safety profile of the CRPI. Additionally, nitriles are more metabolically stable compared to aldehydes that are often oxidized *in vivo* to their corresponding inactive carboxylic acids.

Herein, we will focus on each class of CRPIs according to the nature of their electrophilic warhead. For each group, we will provide several synthetic pathways followed by a case study of a clinically tested CRPI including its design and synthesis, except for aldehyde-CRPI since to date no clinical cases of aldehyde have been reported.

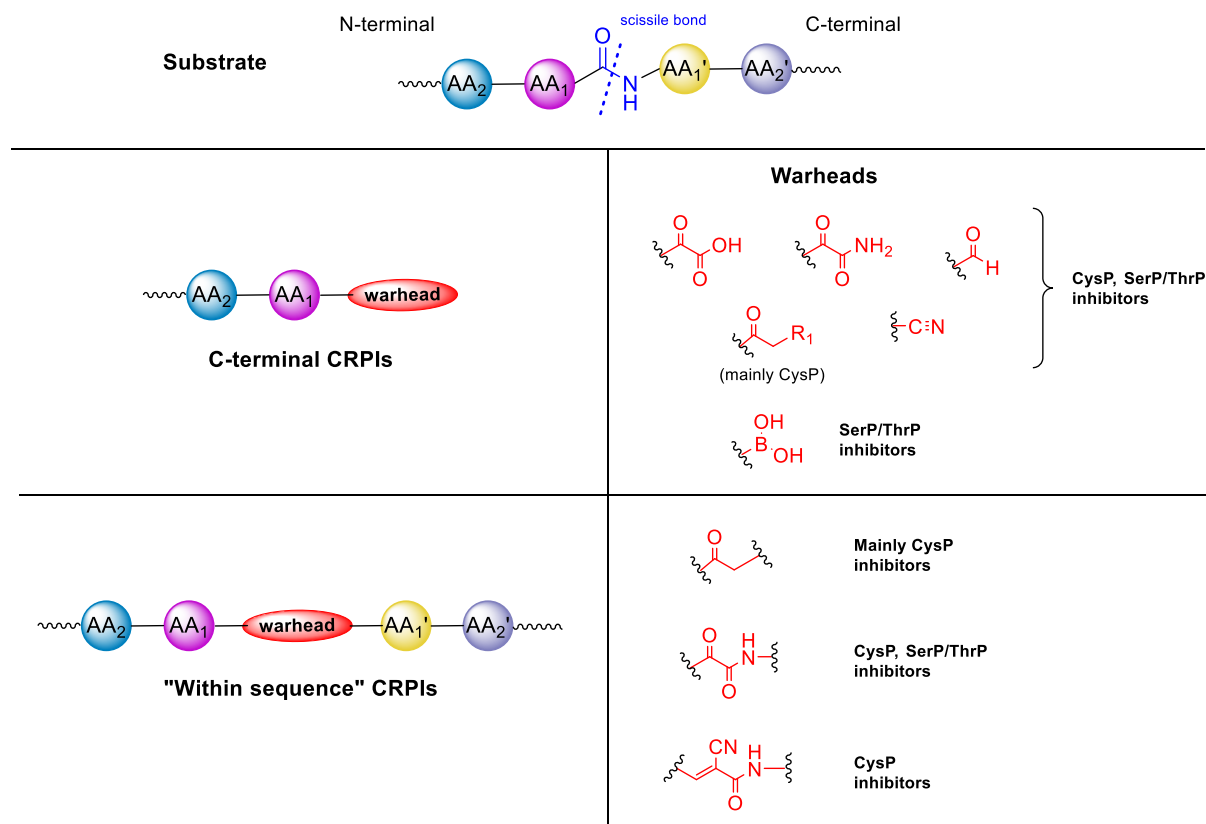


Fig 3: Summary of the most widely applied covalent-reversible electrophiles (warheads), their possible positioning within the CRPI sequence and their specificity for the different types of proteases.

3. Kinetic profiling of CRPIs

Kinetic profiling of CRPIs is a lot more complicated than evaluating fast-binding non-covalent small molecule ligands. Indeed, most CRPIs are slow binding inhibitors meaning that their inhibition is time dependent. Thus, estimating CRPIs' IC_{50} as a mean to study their potency could greatly underestimate their efficiency compared to fast-binding inhibitors. Properly determining CRPIs' kinetics, such as inhibitory constants and residence time, is critical for a successful drug discovery program. The complexity and importance of such study is supported by the fact that there is an independent scientific publication for the kinetic profile of each CRPI reported in this review. For Bortezomib and Ixazomib please see (Hasinoff 2018), for Saxagliptin (Wang *et al.* 2012), for Boceprevir (Zhang and Windsor 2013), and for Nirmatrelvir (Greasley *et al.* 2022). Additionally, excellent practical guides for kinetic profiling of slow binding inhibitors were reported (Mons *et al.* 2022), (McWhirter 2021), and (Zhang and Windsor 2013).

The most representative model for the kinetic evaluation of CRPI is the two-step slow binding reversible covalent inhibition (Fig 4). The CRPI and the enzyme will first form a non-covalent complex governed by weak interactions such as Van der Waals, hydrogen bonds, and π - π stacking. This first binding will be characterized by an initial inhibitory constant K_i ($K_i = k_4/k_3$, K_i^{app} will be calculated in the presence of a substrate, k_1 and k_2 are the rate constants for the substrate binding), and it will be crucial for the selectivity of the CRPI. Indeed, improving K_i via QSAR means increasing the non-covalent affinity of the CRPI for its target and thus its probability to be selective, non-toxic, and ultimately successful in clinical trials. Then in a second step, a covalent-reversible reaction takes place between a nucleophilic residue in the enzymes' active site and the CRPI electrophilic warhead. This reaction is characterized by the rate constants k_5 and k_6 which help to determine the overall inhibitory constant K_i^* ($K_i^* = K_i/[1 + k_5/k_6]$, K_i^{*app} will be calculated in the presence of a substrate). The difference between K_i^* and K_i is useful to estimate the contribution of the covalent bond formation in the inhibitor's efficiency. For example, in the case of Boceprevir, K_i^* was 10 times lower than K_i and the compound possessed a high residence time of 5.6 hours. Of

note, in some cases one of the two inhibition steps could be very fast on the scale of the assay. Such conditions simplify the kinetic study as the inhibition mechanism could be assimilated to a one step-slow binding. In this case, measuring K_i and the CRPI residence time are enough to assess the potency of the inhibitors.

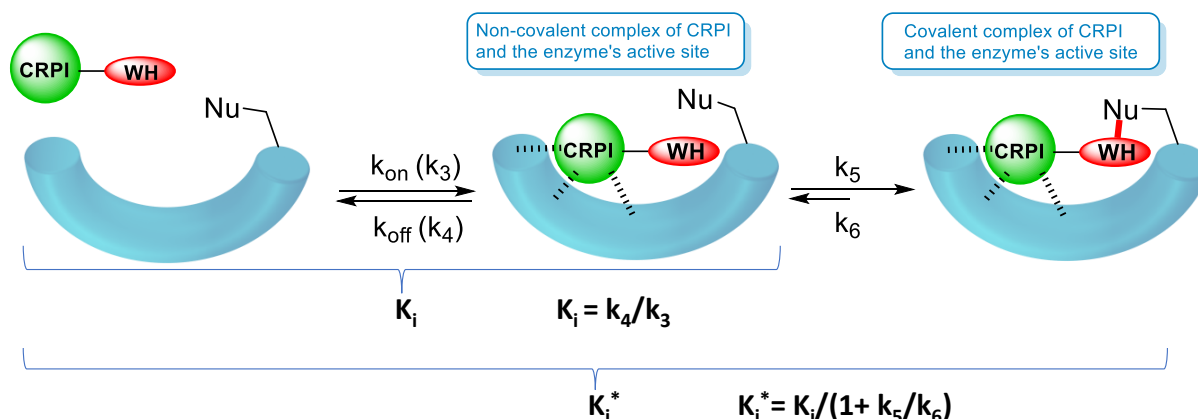


Fig 4: Kinetic profiling of CRPI. The figure does not show the reaction with the enzyme's substrate characterized by the corresponding association constant k_1 and dissociation constant k_2 . K_i and K_i^* are the initial and overall inhibition constants. k_{on} (or k_3) and k_{off} (or k_4) are, respectively, association and dissociation rate constant of the first step. k_5 and k_6 are the forward and backward modification rate constants of the second step. In the presence of a competitive substrate, which is the case for most CRPI, K_i^{app} and K_i^{*app} should be measured to take into account the competition between the substrate and the inhibitor. $K_i^{app} = K_i(1 + [S]/K_m)$ and $K_i^{*app} = K_i^*(1 + [S]/K_m)$.

On a practical level, developing a continuous enzyme assay for the targeted protease is crucial to determine the time dependent CRPIs' kinetics. Measuring the cleavage rate of a fluorogenic substrate by spectrophotometry is the most widely used technique for realizing such studies. Notably, this technique combined with the "progress curve analysis", was applied to characterize all the CRPIs reported in this review. Any time dependent inhibition detected in such a study will be indicative of slow-binding inhibitor. The reversible nature of the CRPI can be studied by preincubating the enzyme and the inhibitor at near stoichiometric binding, followed by a "jump dilution" (generally 100 times) with a substrate-containing buffer. Measuring the time dependent regain of enzymatic activity in these conditions confirms the reversibility and helps determine k_6 (or k_{off} in case of a one-step model) as well as the inhibitor's residence time.

4. Methyl ketone and Dicarboxyl CRPIs

4.1. Methyl ketone CRPIs

Methyl ketones are involved in numerous chemical reactions, as either electrophiles or nucleophiles, providing a wide range of relevant chemical products. In fact, they can be used to form C-C bond *via* aldol reactions, C-N bond *via* a reductive amination, C=C bond *via* Wittig-like reactions and so on (Murray 2020). Since ketones can be reactive in several experimental conditions, their carbonyl function is often masked during the synthesis, as di(thio)ketal analogs, to prevent side-products formation. This type of protection is commonly installed by treating the ketone under acidic conditions in the presence of a 1,2- or 1,3-diol or dithiol(2014). This reaction is in equilibrium and involves an unstable intermediate, a hemiketal or hemithioketal, which can give back the initial ketone function. Similarly, ketone and carbonyl containing CRPIs react within the catalytic sites of CysP to form covalent adducts (Fig 1D). This adduct is in equilibrium with its originating ketone and thereby provides a covalent yet reversible binding mode of the inhibitor. Ketones involved in CRPIs are mostly found at the C-terminus of CRPIs, but they can also be positioned within their sequence. These inhibitors preferentially bind Cys residues due the matching reactivity between the nucleophilic thiol of the CysP and the electrophilic carbonyl function of the inhibitor. Reported examples of SerP and ThrP inhibition by ketone CRPI are very seldom, possibly due to a nucleophilicity/electrophilicity mismatch.

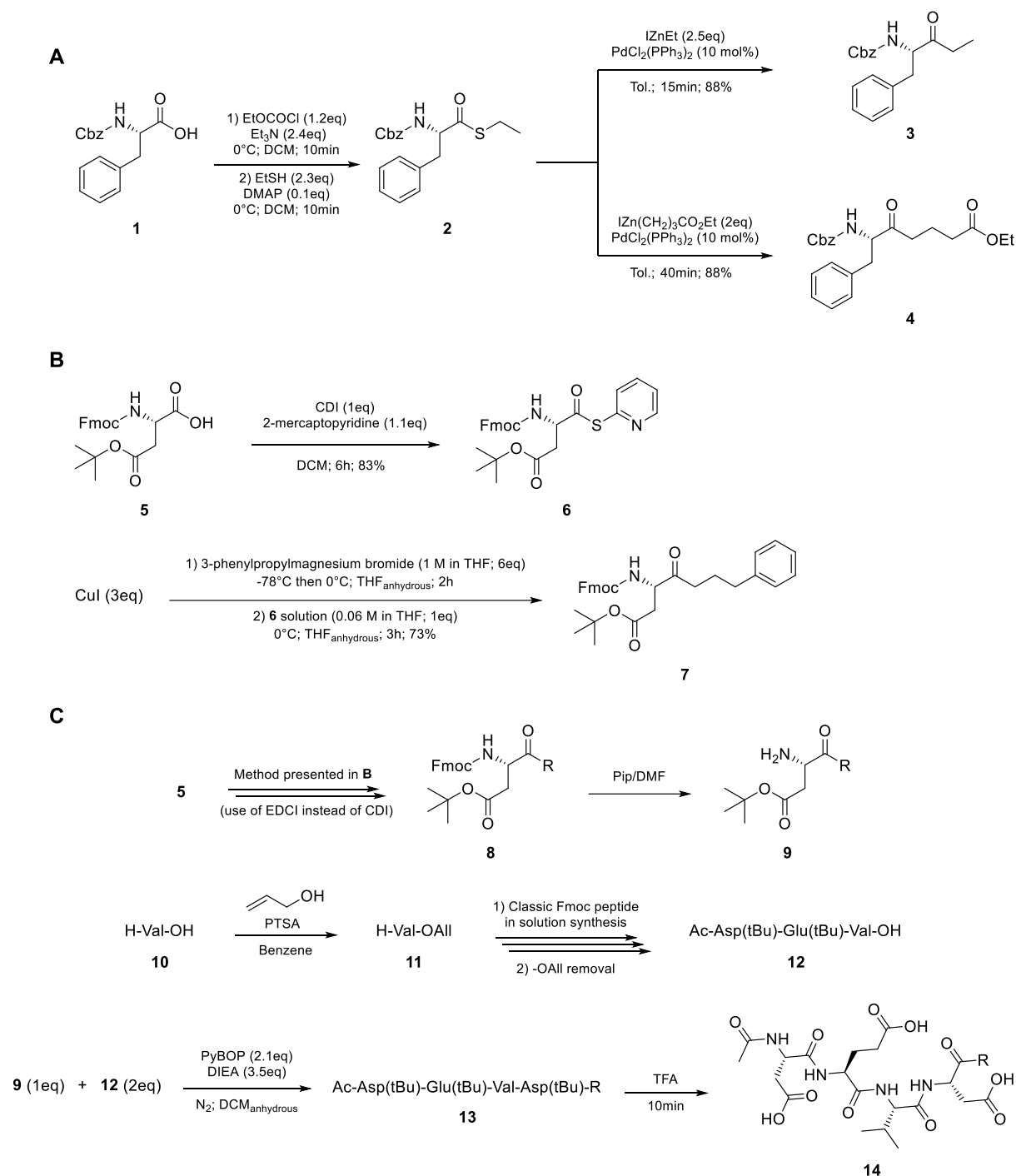
4.1.1. General synthesis

Many synthetic pathways to ketone based CRPIs have been reported over the years. These approaches are performed either on liquid (Scheme 1) or on solid phase (Scheme 2).

One of the most straightforward solution phase methods involves the reaction of a thioester intermediate with a soft organometallic nucleophile. This approach possesses the considerable advantage to avoid the use of hazardous reagents such as diazomethane or organolithiums. Tokuyama *et al.* applied this strategy by first converting Z-Phe-OH (**1**) to the corresponding ethyl thioester (**2**; Scheme **1A**) (Tokuyama *et al.* 1998).

The second step of the synthesis consists in the formation of the ketones **3** and **4** using respectively IZnEt and $\text{IZn}(\text{CH}_2)_3\text{CO}_2\text{Et}$ with a catalytic amount of $\text{PdCl}_2(\text{PPh}_3)_2$ in toluene (Tol). Compounds **3** and **4** were obtained with an 88% yield without racemization (**3**, 99% ee; **4**, 98% ee). This method was exemplified on several other compounds, including Glu and Pro derivatives, with good yields and no racemization. However, the use of ethanethiol can be an inconvenience because of its mephitic odor.

In 2002, Vázquez and Albericio described a Fmoc-protected amino ketone synthesis using 2-mercaptopyridine thioesters in presence of a magnesium organocuprate (Vázquez and Albericio 2002). As an example of their method, the authors reported the conversion of Fmoc-Asp(O^tBu)-OH (**5**) into Fmoc-Asp(O^tBu)-(CH₂)₃-Ph (**7**) (Scheme **1B**). This was performed in two steps. First, thioester **6** is prepared by reacting Fmoc-Asp(O^tBu)-OH (**5**) with carbonyldiimidazole (CDI) and 2-mercaptopyridine; thereafter, **6** was reacted with magnesium organocuprate in anhydrous THF to afford **7** with a 73% yield. Of note, 2-mercaptopyridine was chosen because it is odorless, inexpensive and its corresponding thioesters are highly reactive. In addition to compound **7**, seven other *N*-Fmoc-protected amino ketone compounds were obtained in their study with modest to good yields (46-77%). However, no data on racemization were reported. Concerning the synthesis of methyl ketone CRPIs in solution, Goode *et al.* presented in 2005 a method based on classic peptide synthesis (Goode *et al.* 2005). The authors used the method proposed by Vázquez and Albericio (Vázquez and Albericio 2002) to obtain the crucial C-terminal amino ketone compound (**9**) and proceeded to its condensation on the protected *N*-acylated peptide (**12**) (Scheme **1C**). They afforded several peptide ketones (**14**) in 7 steps and with 15% to 30% yields depending on the C-terminal amino ketone used. However, no data on epimerization/racemization were reported.



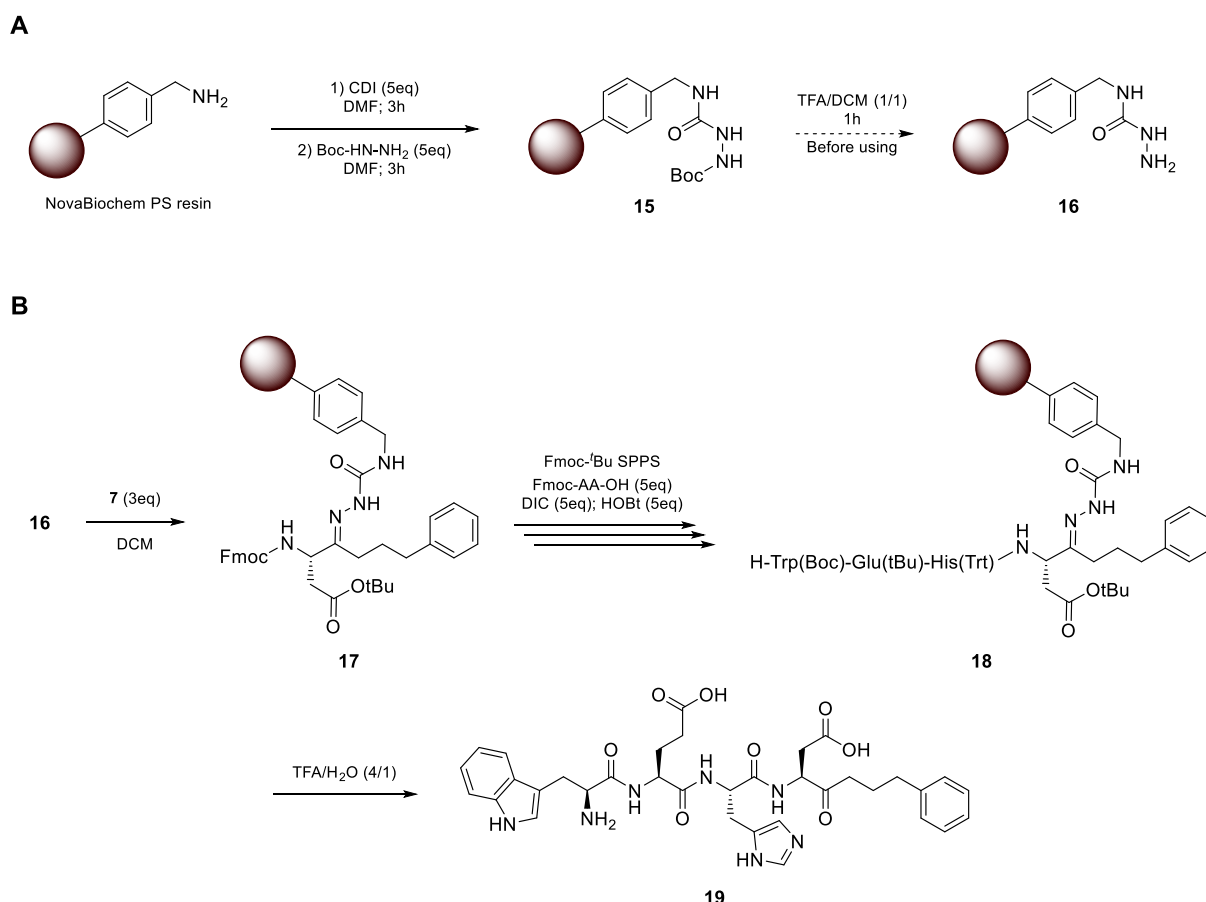
Scheme 1: Examples of aminoketones and peptide ketone synthesis in solution. **A)** Tokuyama *et al.* 1998; **B)** Vázquez and Albericio 2006; **C)** Goode *et al.* 2005.

In an alternative to solution phase synthesis, ketone CRPIs can be prepared using solid phase supported chemistry. Generally, after some initial optimizations, the latter strategy grants a more straightforward access to the target CRPIs while it eases purification processes. Moreover, SPPS is better suited when longer peptide sequences are prepared. However, one major drawback is that the ketone moiety must be generated in solution prior to its anchoring on the solid support.

One practical way to obtain ketone CRPIs on solid phase is by anchoring its C-terminal carbonyl moiety to a semicarbazone or semicarbazide resin. One such linker was developed by Lee *et al.* in 1999 and later modified by Vázquez and Albericio in 2006 (Lee *et al.* 1999; Vázquez and Albericio 2006). The principle is the same in both cases. First, the C-terminal amino-ketone residue is anchored *via* its carbonyl function, by chemoselective hydrazone bond formation with the resin. Then, peptide elongation takes place, and a final aqueous acidic cleavage

liberates the ketone CRPI from the resin with the concomitant unmasking of the peptide side chains. Importantly, in the two cases, the linker is not commercially available and needs to be synthesized following the procedure presented by the authors.

Vázquez and Albericio reported a resin and linker dependent study that highlights a general and convenient method to obtain ketone CRPIs *via* classical Fmoc-^tBu SPPS. Their optimized model consists in a semicarbazide polystyrene resin (**16**) obtained in two steps (Scheme 2A). In a first step, the activation of a benzylamine resin with CDI, followed by Boc-hydrazine treatment, yields the Boc semi-carbazide resin **15**. Before use, **15** must be deprotected using a TFA-DCM (1:1) mixture to provide resin **16**. The authors described a general method to incorporate *N*-Fmoc protected ketone moieties onto the resin using DCM as the solvent and 3 equiv. of the ketone. As a proof of concept, they synthesized the peptide H-WEHD-(CH₂)₃-Ph (**19**) *via* a standard Fmoc-^tBu protocol using DIC/HOBt as the activating mixture. After cleavage in TFA-H₂O (4:1), the keto-tetrapeptide **19** was obtained in 75% yield (Scheme 2B).



Scheme 2: Example of solid phase peptide-based methyl ketones synthesis. **A)** Preparation of the semicarbazide resin; **B)** Synthesis of a peptide-based methyl ketone (**16** → **19**) (Vázquez and Albericio 2006).

4.1.2. Example of Lufotrelvir, a SARS-CoV-1 and SARS-CoV-2 3CL^{pro} inhibitor

Lufotrelvir (PF-07304814; **27**) is the phosphate ester prodrug of PF-00835231 (**26**), a drug candidate against SARS-CoV-1 developed by Pfizer whose clinical advancement was suspended due to the pandemic end in 2003. However, due to the outbreak of the COVID-19 pandemic in 2020, PF-00835231 was repurposed as a potential drug candidate against SARS-CoV-2 (Hoffman *et al.* 2020). In both cases, **26** was able to inhibit 3CL^{pro} which is the M^{pro} of SARS-CoV viruses. These two enzymes share 96% sequence homology and 100% identity in their respective active sites (Zhang *et al.* 2020). In this context, Hoffman *et al.* (Hoffman *et al.* 2020) proposed PF-00835231 (**26**) as a potential COVID-19 treatment since it was highly efficient on the 3CL^{pro} of SARS-CoV-1. They described the rational design of **26**, using crystal structures of the two enzymes, and the biological results on 3CL^{pro} SARS-CoV-1 and SARS-CoV-2. Surprisingly, **26** displayed a K_i on the SARS-CoV-2 M^{pro} (0.27 ± 0.1 nM) that is about 10-fold lower compared with the SARS-CoV-1 M^{pro} (4 ± 0.3 nM) (Hoffman *et al.* 2020). Fig 5 summarizes the rational design of this CRPI. The preferred SARS-CoV-1 and SARS-CoV-2 3CL^{pro} substrates

included crucial Leu and especially Glu residues at positions P₂ and P₁ (Cannalire *et al.* 2022). Position P₁' , on the other hand, was less requiring and it allowed to substitute the scissile amide bond by a variety of electrophilic warheads. Bioisosteric electrophiles that closely mimic the amide bond were preferred. The authors choose the ketone warhead as it possessed the proper alignment with the oxyanion hole of the 3CL^{pro}, thus providing affinity and a strong covalent reversible bond with the enzyme. Position P₃ tolerated different aromatic lipophilic substituents that improved membrane permeation and metabolic stability as well as the potency of the inhibitor. Finally, a phosphate prodrug moiety was attached at position S₁' to improve solubility and bioavailability due to the increased serum proteins binding (Boras *et al.* 2021). Fig **5B** represents the cocrystal structure of the inhibitor **26** bound to SARS-CoV-2 3CL^{pro} and illustrates the different aforementioned covalent and non-covalent interactions. This rational substrate-based design provided highly potent, low nanomolar CRPI of the SARS-CoV-1 and SARS-CoV-2 M^{pro}.

Additionally, Lufotrelvir is highly selective, as it displayed an IC₅₀ superior to 10 μM for 6 human enzymes, including pepsin and caspase 2 and two viral proteases in HIV1 and HCV, compared to 0.3 nM for SARS-CoV-2 M^{pro}. Moreover, it only showed moderate inhibition of Cathepsin B and HRV with IC₅₀ of 1.3 μM and 1.7 μM, respectively, hence demonstrating its selectivity. Despite all these encouraging results, Lufotrelvir is not orally bioavailable and therefore had to be injected intravenously. This was likely the main reason why Lufotrelvir's Phase 2/3 clinical development was discontinued in February 2022 in favor of the development of another Pfizer CRPI: Nirmatrelvir.

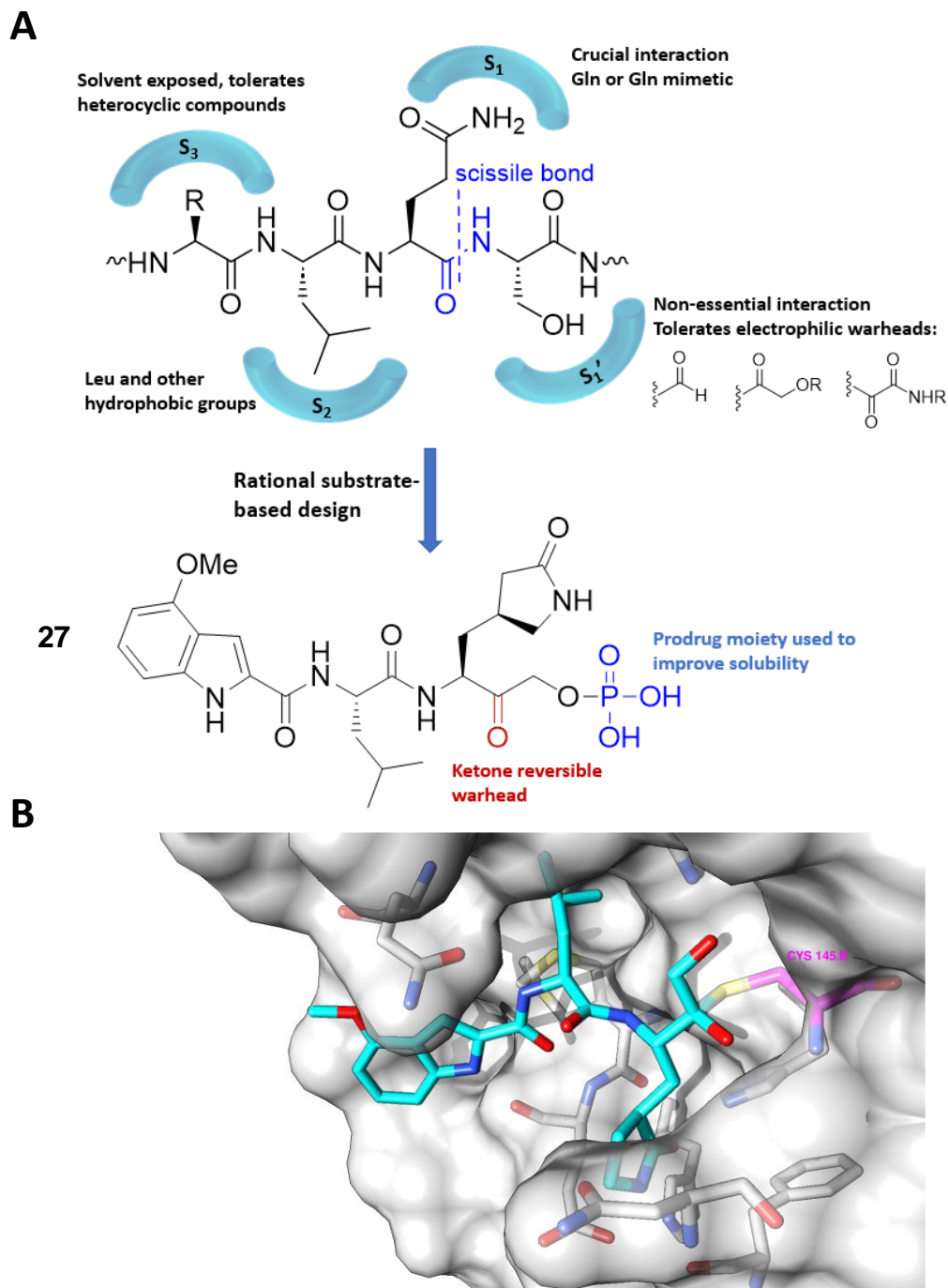
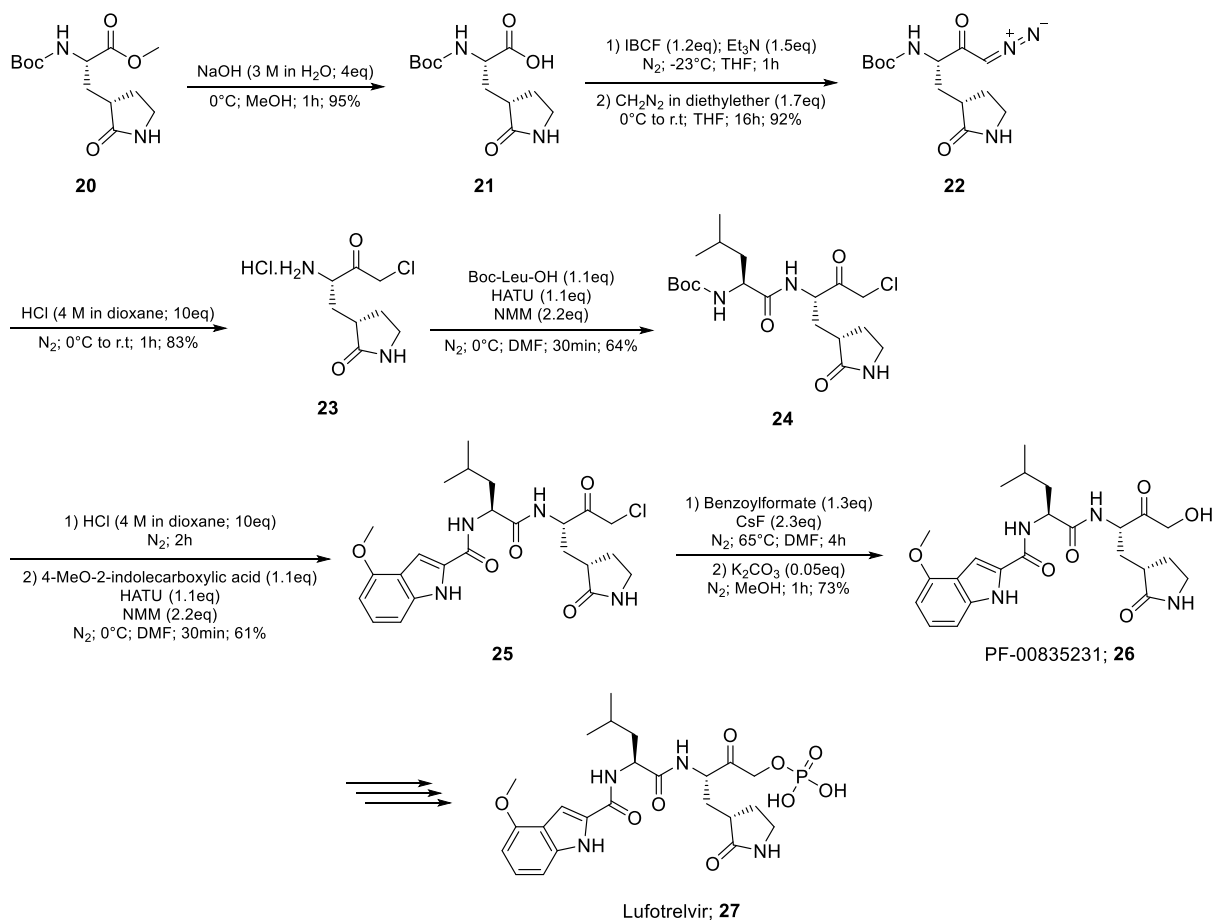


Fig 5: A) Substrate based design of Lufotrelvir (27); B) Cocystal structure of inhibitor 26 bound to SARS-CoV-2 3CL^{pro} (PDB: 6XHM). The nucleophile catalytic residue (here Cys145) is colored in magenta.

The synthesis of PF-00835231 (**26**) (Scheme 3) was described by Hoffman *et al.* following an eight steps route with an overall yield of nearly 21%. To obtain Lufotrelvir, 3 additional steps were required, reducing the overall yield to 10% (Boras *et al.* 2021). In this synthetic scheme, the ketone electrophile was introduced by activating the carboxylic acid function of **21** with isobutylchloroformate (IBCF) and Et₃N followed by a reaction with diazomethane to provide **22**. Compound **22** was subsequently treated with a HCl solution to afford the crucial α -chloroketone intermediate **23**. For more details on Lufotrelvir, please see (Chen *et al.* 2022).



Scheme 3: Synthesis of Lufotrelvir (PF-07304814; **27**).

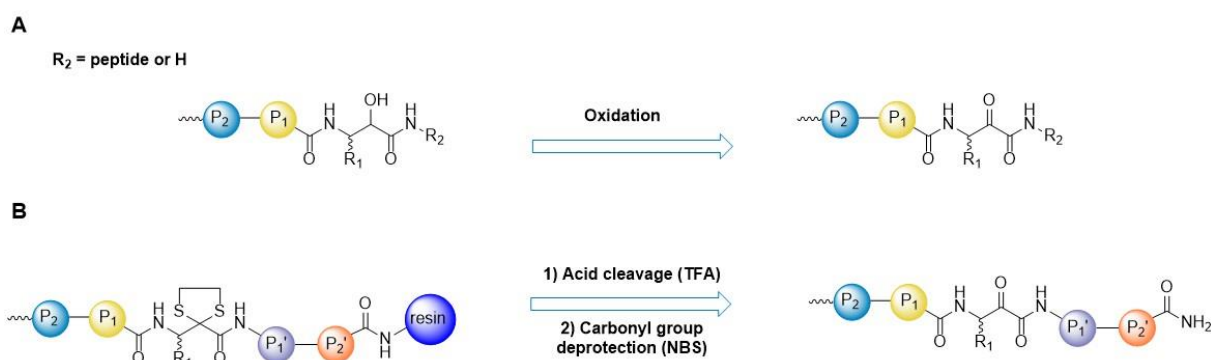
4.2. Dicarbonyl CRPIs

Dicarbonyl CRPIs refer to compounds containing an α -keto carbonyl group, including α -ketoacids, α -ketoketones (α -diketones), α -ketoesters and, one of the most commonly used, α -ketoamides (Fig 1D) (Robello *et al.* 2021). Their mechanism of action is very similar to the ketone inhibitors. The difference between ketone and dicarbonyl inhibitors is the electrophilicity of the carbon present in the ketone moiety. In fact, depending on the moieties present at the α -keto position, the reactivity of the inhibitor and the reversibility of the covalent adduct will be modulated. In the case of dicarbonyl inhibitors, the electron withdrawing group at the α -position increases the reactivity of the inhibitor and allows the electrophilic trapping of less reactive catalytic residues such as Ser or Thr (Michaud and Gour 1998). Similarly to the case of ketone CRPIs, α -chiral centers next to dicarbonyl moieties are prone to fast epimerization at physiological pH (Robello *et al.* 2021). Dicarbonyl moieties involved in CRPIs are mostly found on the C-terminus and more rarely within the CRPI sequence. Ketoamides generally possess higher metabolic stability and generally better PK profile compared to ketoesters and ketoacids (Robello *et al.* 2021).

4.2.1. General synthesis

This part will focus on the synthesis of α -ketoamides, which are the most represented type of dicarbonyl CRPIs in drug design due to their stability and cell-penetrant capacity (Robello *et al.* 2021). Numerous synthetic pathways to α -ketoamides have been reported (De Risi *et al.* 2016).

In particular, CPRI synthesis was reported in solution (Pacifico *et al.* 2019) and on solid phase (Papanikos and Meldal 2004; Rohrbacher *et al.* 2018; Van Kersavond *et al.* 2021). One important consideration when synthesizing α -ketoamide CRPIs is the CO group extrusion under acidic conditions that renders TFA usage undesirable (Papanikos and Meldal 2004). One way to circumvent this issue is to first introduce an α -hydroxy amide moiety within the peptide sequence. Then, at the end of the CRPI preparation the hydroxy group is oxidized to provide the C-terminal or internal peptide ketoamide (Scheme 4A). Similar strategy could be deployed on SPPS or alternatively the reactive carbonyl of the ketoamide could be protected as an acetal (Rohrbacher *et al.* 2018) or thioacetal (Papanikos and Meldal 2004). Then, after SPPS sequence elongation the CRPI is first cleaved from the resin and then the protection is removed in neutral conditions, thus avoiding the CO extrusion.



Scheme 4: **A)** General method to obtain peptide-based α -ketoamides: in solution. Ketoamide group is introduced either within the sequence or at the C-terminus; **B)** General method to obtain peptide-based α -ketoamides: synthesis on support and obtention in solution.

4.2.2. Design and synthesis of Boceprevir, an HCV NS3/4a SerP

Boceprevir (SCH 503034; **40**) is one of the first HCV NS3/4a SerP inhibitor approved by the FDA (May 2011) as a direct-acting antiviral drug to treat chronic hepatitis C in combination with pegylated interferon and ribavirin (Liverton 2019). Targeting NS3 represented a significant challenge as its active site, unlike most proteases, is shallow and solvent exposed. Thus, a relatively large minimal sequence of 10 amino acids was required for effective enzyme-substrate interactions. Additionally, the enzyme active site is modified upon interaction with its peptide substrates (Howe and Venkatraman 2013). Targeting such complicated large surface interaction with small non-covalent ligands was challenging and thus paved the way for the development of the second FDA approved CRPI, Boceprevir. This peptide-based α -ketoamide compound was discovered by Venkatraman *et al.* in 2006. The authors began their design with an undecapeptide α -ketoamide (**29**; Fig 6) based on the preferred consensus substrate of NS3 (**28**; Fig 6). This compound showed high *in vitro* inhibitory activity against HCV NS3/4a SerP ($K_i = 1.9$ nM) but suffered from poor PK, due to its numerous peptide bonds and large size. Moreover, it displayed poor selectivity against the closely structurally related Human Neutrophil Elastase (HNE). After several truncations and modifications, the authors identified the shorter and still active pentapeptide α -ketoamide **30** (Fig 6) ($K_i = 10$ nM; $EC_{90(\text{rep})} = 200$ nM). Nevertheless, it suffered from poor oral bioavailability in studied animal models. To address this issue, Venkatraman *et al.* truncated the C-terminal P_1' and P_2' of **30** and introduced a crucial methyl cyclobutyl moiety, that mimics the Leu residue at P_1 , to maintain potency. Interestingly, the authors showed that the configuration of the α -carbon was not essential for the biological activity of the compound. Some modifications at P_4 were additionally required to provide a selective (HNE/HCV = 2200), potent ($K_i = 14$ nM; $EC_{90(\text{rep})} = 350$ nM) and the orally bioavailable HCV NS3/4a inhibitor Boceprevir (**40**).

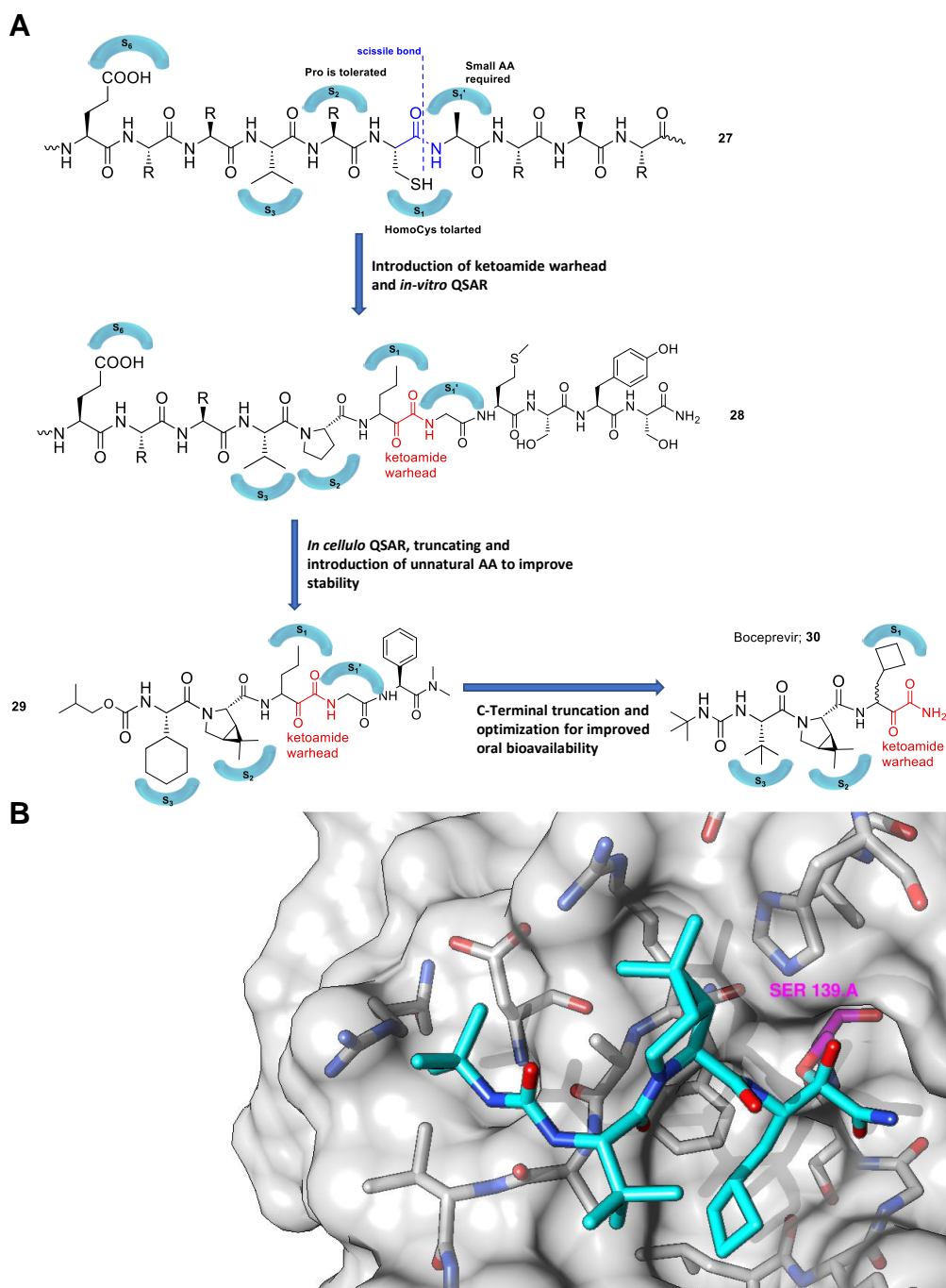
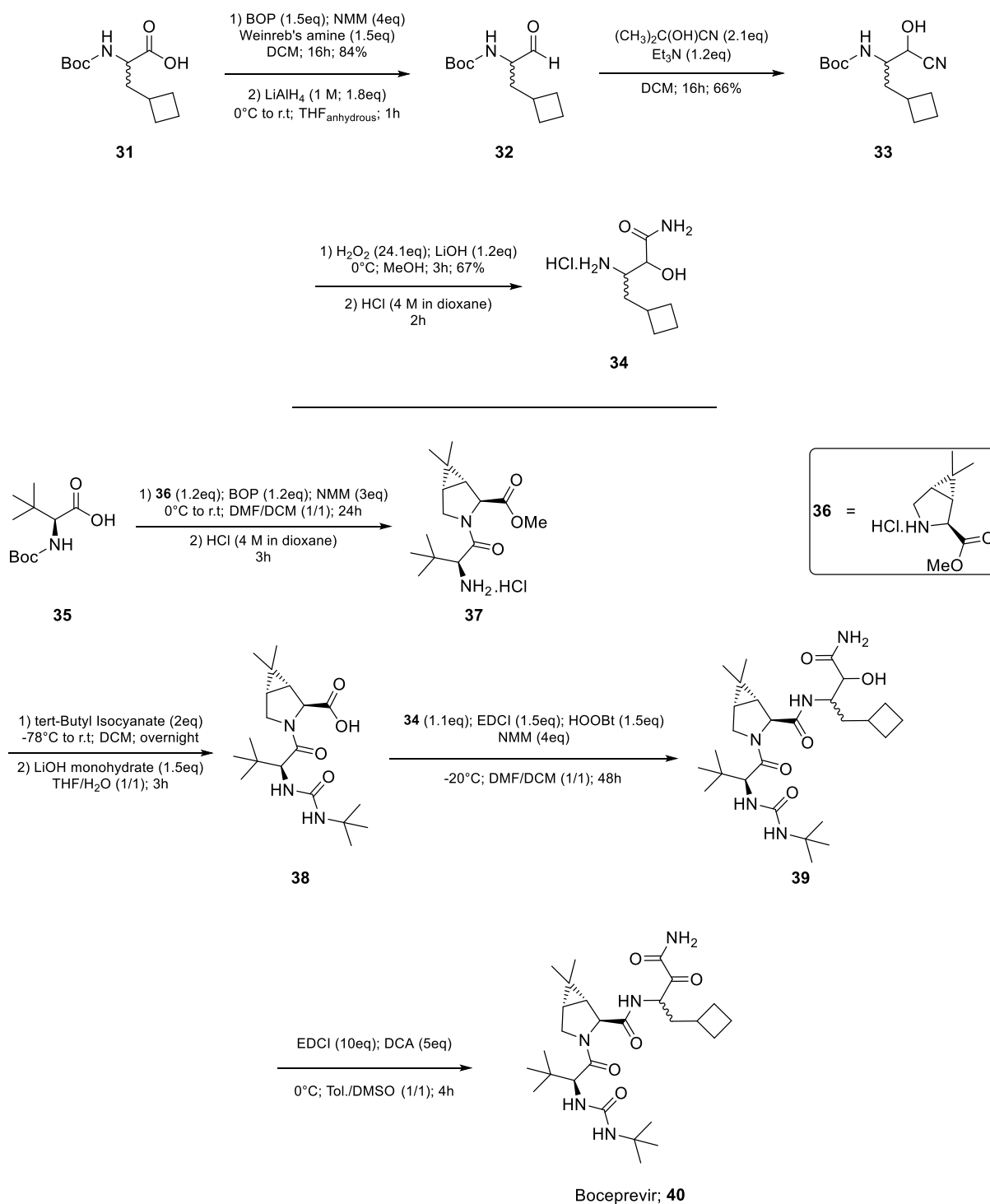


Fig 6: A) Rational design of Boceprevir (**40**); **B)** Cocrystal structure of **40** bound to HCV NS3/4a (PDB: 2OC8). The nucleophile catalytic residue (here Ser139) is colored in magenta.

Despite encouraging clinical results of Boceprevir, a novel treatment, named Sofosbuvir (*Sovaldi*TM), was FDA approved in December 2013. Sofosbuvir is a nucleotide prodrug of a potent HCV NS5B polymerase and present high potency, oral bioavailability, low side effects and low drug resistance induction (Bhatia *et al.* 2014). Regarding the better risk-benefit balance and more convenient posology of Sofosbuvir compared with Boceprevir, the latter was no longer recommended as a treatment against chronic hepatitis C and was discontinued in December 2015 (Liverton 2019). For further information on Boceprevir, please see (Chang *et al.* 2012).

A summarized synthesis of Boceprevir, as reported by Venkatraman *et al.* in 2006, is presented in Scheme 5. Starting from the racemic *N*-Boc-protected methyl cyclobutyl amino acid **31**, the authors proceeded to convert the acid function into the α -hydroxy amide compound **34**. The standard solution phase strategy then led to α -hydroxy amide peptide **39**, which was oxidized as a final step to give Boceprevir (**40**) as a diastereomeric mixture.



Scheme 5: Synthesis of Boceprevir (SCH 503034; **40**).

5. Aldehyde CRPIs

Aldehydes have a similar mechanism as ketone inhibitors, as they also form hemiacetals/hemithioacetals with their targeted enzymes. Peptide aldehydes are widely used as reversible inhibitors of AspP (Fehrentz *et al.* 1984; Sarubbi *et al.* 1993), SerP/ThrP (Kurinov and Harrison 1996), and CysP (Rosenthal 2004; Zhu *et al.* 2011). They were the first proteasome inhibitors to be identified, targeting a ThrP with three different catalytic subunits $\beta 5$ chymotrypsin-like, $\beta 2$ trypsin-like, and $\beta 1$ caspase-like activities (Thibaudeau and Smith 2019). One of the main

drawbacks of peptide aldehyde inhibitors is attributed to the sensitivity of the aldehyde function to metabolic oxidation reactions. This yields carboxylic acids in cell media, thereby inactivating the inhibitor. Moreover, alike ketone and dicarbonyl inhibitors, the keto-enol equilibrium progressively leads to the epimerization of the α -C-terminus amino acid. To counter this phenomenon, the aldehyde can be reversibly derivatized into Schiff bases (Adkison *et al.* 2006; Leban *et al.* 2008), bisulfite adducts (Galasiti Kankanamalage *et al.* 2017) or intramolecular acetals (Zhu *et al.* 2022).

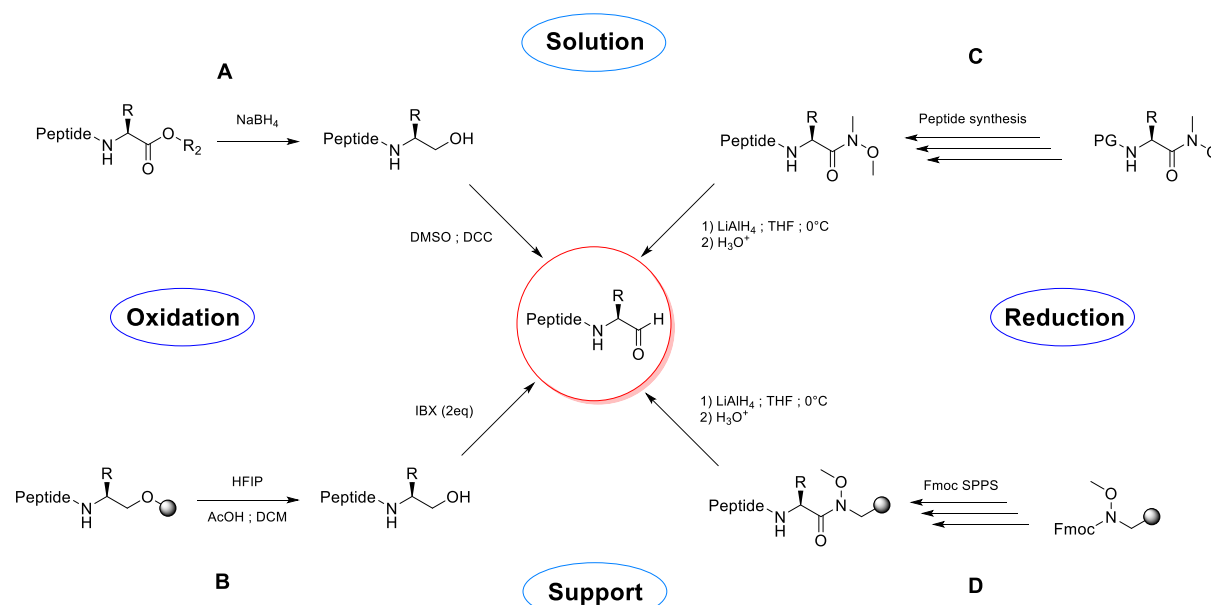
5.1. General synthesis

There are different strategies for the synthesis of peptide aldehydes (Scheme 6). They can be prepared either in solution or on solid phases, mainly by oxidation of an alcohol precursor or reduction of a Weinreb amide derivative.

The synthesis of peptide aldehydes from peptide alcohols in solution was described by Hamada and Shioiri in 1982 (Hamada and Shioiri 1982). The peptide alcohol is obtained by reduction of a peptide methyl ester using sodium borohydride (Scheme 6A). Next, it is directly engaged in a Pfitzner-Moffatt oxidation using DMSO and dicyclohexylcarbodiimine (DCC). Alternatively, the peptide alcohol can also be treated with sulfur trioxide and triethylamine (Hamada and Shioiri 1982). It is worth noting, this reaction is described as non-epimerizing. This methodology has been adapted to solid supports. The peptide alcohol, for which the C-terminal aminoalcohol was prepared prior to the anchoring on resin, is synthesized on trityl resin and then cleaved by treatment with a HFIP/AcOH/DCM mixture (Scheme 6B). Afterwards, it is oxidized into an aldehyde *via* a solid supported IBX reagent. This reaction is not quantitative and leads to an alcohol/aldehyde mixture which requires further purification by a catch and release threonine resin (Sorg *et al.* 2005).

Alternatively, peptide aldehydes can be synthesized through a reduction pathway. In solution, N-terminal protected peptide aldehydes can be obtained from C-terminal Weinreb amides (Scheme 6C). This strategy is one of the most widely used and is applicable for peptides protected by a variety of N-protecting groups such as Cbz, Fmoc or Boc. The first step is the preparation of a Weinreb amide of the C-terminal amino acid. Then, after peptide elongation, the amide is reduced by lithium aluminumhydride (LiAlH₄), followed by acidic treatment to provide the corresponding aldehyde CRPI (Fehrentz and Castro 1983; Guichard *et al.* 1993).

Interestingly, Fehrentz *et al.* transposed this strategy to solid support (Fehrentz *et al.* 1995) (Scheme 6D). For that purpose, they developed a linker that allowed the anchoring of the first amino acid to the solid support through a Weinreb amide bond. After SPPS elongation, the peptide is cleaved from the resin by LiAlH₄ treatment, following the previously described procedure to form the corresponding peptide aldehyde. Boc-Phe-Val-Ala-H was synthesized with a 40% yield after purification.



Scheme 6: General synthesis of peptide aldehydes. **A)** Oxidative in solution aldehydes formation; **B)** Supported preparation of peptide alcohols and oxidative in solution aldehydes formation; **C)** Reductive in solution aldehydes formation; **D)** Reductive on support aldehydes formation.

Other supported or in solution methodologies have been studied such as the prior preparation of amino aldehydes before their grafting onto cysteine-, semicarbazide-, or threonine-appended resin (Moulin *et al.* 2007).

5.2. Example of self-masked aldehyde CRPIs

To date, no peptide aldehyde drug is FDA approved albeit several aldehydes CRPIs are currently studied for the treatment of cancer (Quader *et al.* 2014), Dengue flaviviruses (Da Silva-Júnior and De Araújo-Júnior 2019) or SARS-CoV-1 (Akaji *et al.* 2011). The most likely explanation for this relative lack of clinical success is the metabolic instability and high reactivity of the aldehyde function.

One possible way to overcome this drawback is by masking the aldehyde as a prodrug moiety. Indeed, recent examples of SARS-CoV-2 M^{pro} inhibitors involving an aldehyde trap were described (Li *et al.* 2021; Zhu *et al.* 2022). The authors reported three potent self-masked aldehyde inhibitors (SMAIs; **46-48**; Scheme 7) as acetal prodrugs. Their mode of action is presented in Fig 7. The SMAIs are metabolically stable, as the sensitive aldehyde is protected in an acetal form. After cell penetration the ester is hydrolyzed by esterase activity releasing an unstable hemiacetal function that is in equilibrium with the active aldehyde CRPI.

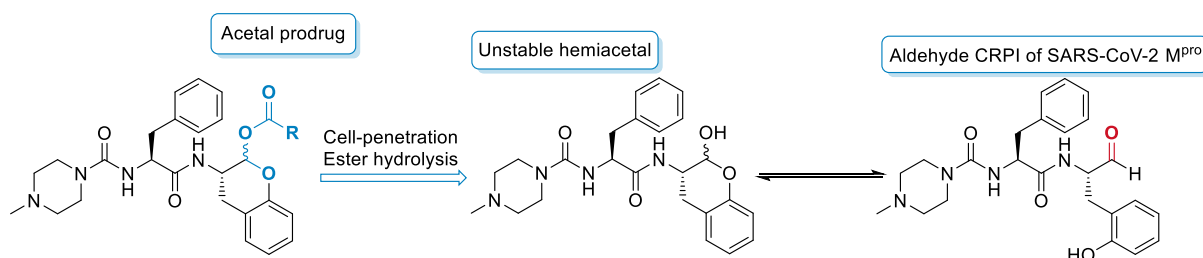
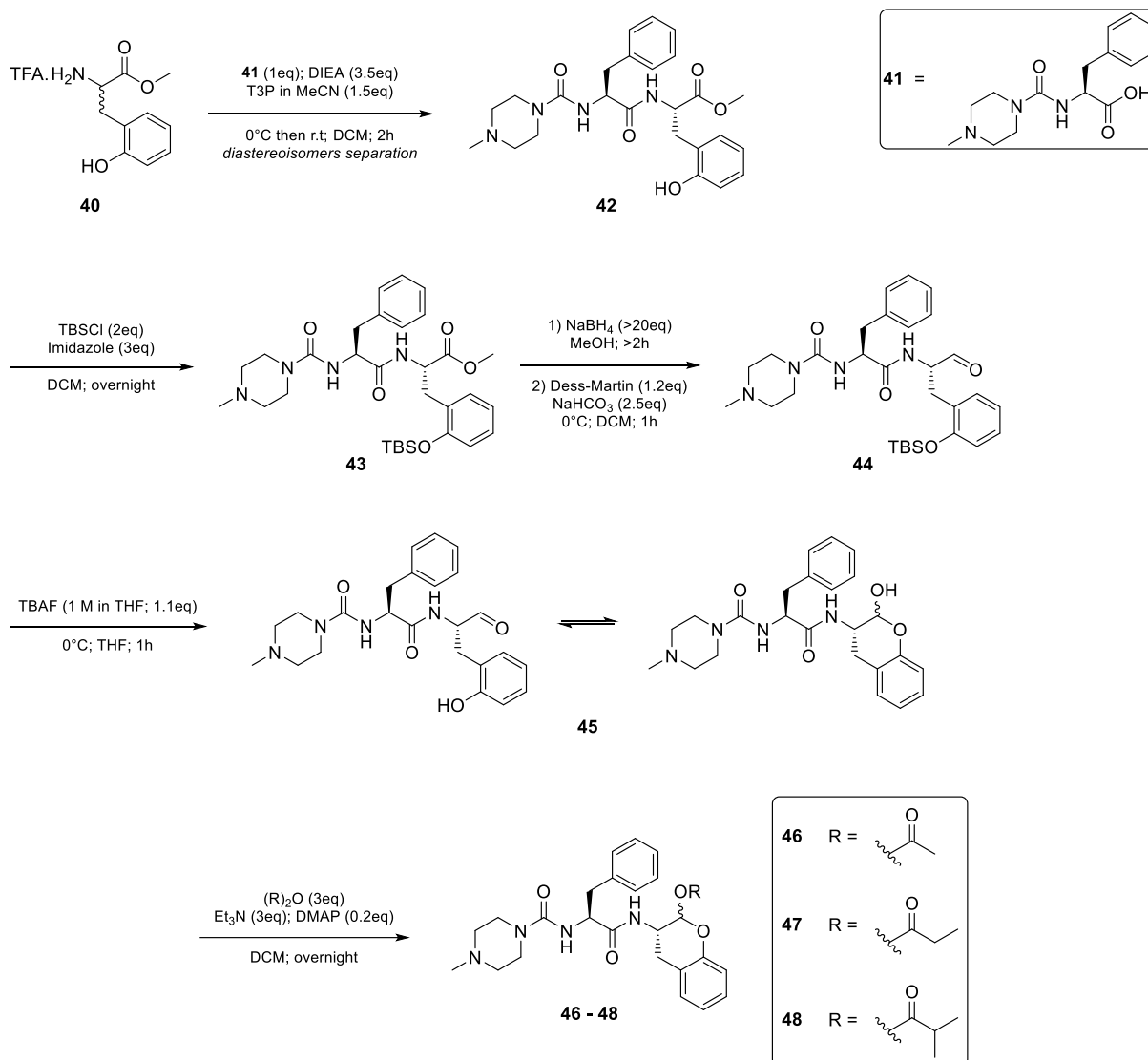


Fig 7: Prodrug strategy of self-masked aldehyde inhibitors. Prodrug moiety is highlighted in blue.

A synthesis outline of these compounds is presented in Scheme 7. Following the preparation of their building blocks **40** and **41** presented in their study (Li *et al.* 2021), the authors proceeded to a coupling step between these two molecules to afford the dipeptide ester **42** after diastereomeric separation. After protection of the phenol moiety, they converted the ester function into an aldehyde (**44**). The phenol was subsequently deprotected to allow the formation of the hemiacetal **45** which was acylated with different anhydrides to obtain the three ester SMAIs (**46-48**). The SMAI strategy permits better PK of the aldehyde CRPIs thereby avoiding the drawbacks mentioned earlier. Furthermore, the SMAIs presented in the study provide similar potencies as Nirmatrelvir (PF-07321232), a FDA-approved inhibitor of SARS-CoV-2 3CL^{pro} (Zhu *et al.* 2022). This interesting case should be followed closely as it presents an encouraging future for aldehyde CRPIs.



Scheme 7: Synthesis of three SMAIs as described by Li *et al.* 2021 and Zhu *et al.* 2022.

6. Boron containing CRPIs

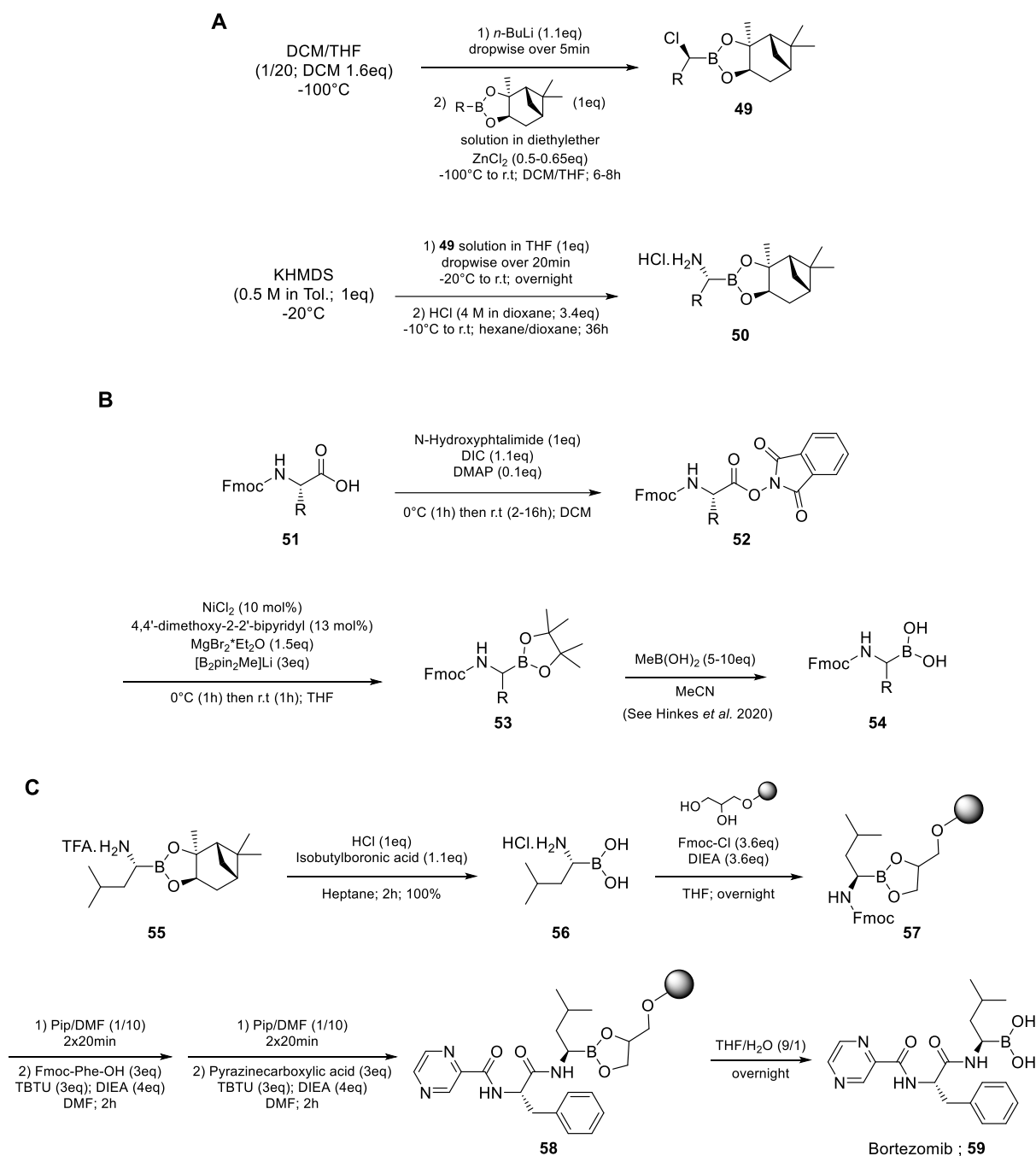
Boron containing molecules are a well-documented class of protease inhibitors (Smoum *et al.* 2012; Tan *et al.* 2021). Boronic acid compounds are widely used for the specific inhibition of SerP and ThrP (Philipp and Bender 1971). The mechanism of action of such compounds is due to the strong Lewis acidic nature of the boron atom. In fact, boron atoms feature an empty electronic orbital ($2p$) that permits strong coordination bonds with catalytic hydroxyl groups (Diaz and Yudin 2017). Two modes of inhibition of boronic acid compounds are accepted. The most common one, involves the binding of the boron atom with the nucleophilic residue of the target, thus mimicking the transition state of a peptide bond hydrolysis (Philipp and Bender 1971). The other mode, consists in the coordination of the boronic acid with the histidine residue of the catalytic triad of the SerP (Tsilikounas *et al.* 1993). To date, five boronic acid compounds were approved by the FDA for the treatment of various diseases, including fungal infection or pyelonephritis (M. António *et al.* 2019). Two of these drugs are CRPIs, Bortezomib (Velcade®; **59**) and Ixazomib citrate (Ninlaro®; **64**), and they both act by targeting the proteasome in the context of anti-cancer applications.

6.1. General synthesis

Several methods are available to afford α -amino-boronic and peptide-boronic acids (Andrés *et al.* 2016; Šterman *et al.* 2019).

The Matteson homologation is one of the most used method, in the solution phase, for producing enantiopure amino-boronic acids (Matteson and Majumdar 1983; Matteson and Sadhu 1983; Matteson 2013). However, this route, shown in Scheme **8A**, suffers from harsh experimental conditions, such as the use of organolithium, that limit the methodology to robust and insensitive side chains (*e.g.*, alkyl, aryl...). In addition to Matteson's approach, two solution phase methodologies were described by Baran and his team. They developed an elegant way to convert *N*-protected peptide acids into *N*-protected peptide boronic acids using nickel- (Li *et al.* 2017) or copper-catalysts (Wang *et al.* 2018) by direct decarboxylative borylation. For instance, Bortezomib could be produced starting from Boc-Phe-Leu-OH, in a five steps route, with an overall yield around 25% and a moderate epimerization of the C-terminal α -carbon (diastereomeric ratio of 5.1/1). Compared to Matteson homologation pathway, Baran's peptide boronic acid synthesis allows for broader functional group tolerance, at the expense of enantiopurity. A further illustration by Hinkes *et al.* emphasized the generality of the method by synthesizing the boronic acid derivatives of all proteinogenic amino acids (Scheme **8B**). However only racemates were obtained (Hinkes *et al.* 2020).

Concerning the solid-phase synthesis of peptide boronic acids, Daniels and Stivala described the use of a 1-glycerol polystyrene resin, where the C-terminal α -amino boronic acids is anchored, prior to peptide elongation (Daniels and Stivala 2018). As a proof-of-concept, they presented the synthesis of Bortezomib using the commercially available boroleucine pinanediol ester **55** (Scheme **8C**). The crucial step of this synthesis is the one pot anchoring of the boronic acid onto the glycerol linker through a boronate ester formation and the protection of its amino function with Fmoc-Cl. Importantly, the boronate tethering is stable enough to allow peptide elongation while it is conveniently cleaved by a TFA/H₂O mixture. Through their 7-steps method, Bortezomib and Ixazomib CRPIs could be obtained with respective overall yields of 54% and 49% and purity >95% in both cases.



Scheme 8: General synthesis of amino and peptide boronic acids. **A)** Matteson homologation and SN2 amine formation; **B)** Hinkes *et al.* general method to obtain racemic amino boronic esters (**53**) and acids (**54**); **C)** Supported synthesis of Bortezomib (**59**) as described by Daniels and Stivala in 2018.

6.2. Example of Ixazomib citrate, a proteasome inhibitor

Ixazomib citrate (MLN9708; **64**) is one of the two FDA approved boronic acid CRPI targeting the proteasome (November 2015) and used against multiple myeloma in combination with Lenalidomide. This prodrug of Ixazomib (MLN2238; **63**) (Muz *et al.* 2016; Raedler 2016), was the first orally administered proteasome inhibitor that has been approved by the FDA. Millennium Pharmaceuticals Inc. discovered this CRPI while conducting a screening assay to identify a structurally similar compound with improved PK properties relative to Bortezomib (Kupperman *et al.* 2010). Similarly, to Bortezomib, Ixazomib preferably inhibits the $\beta 5$ subunit of the proteasome. This subunit is a ThrP (Fig **8B**) that preferably cleaves substrates with lipophilic residues at P₁ and lipophilic/aromatic residues at P₃ (Huber *et al.* 2015). Fig **8A** shows the mode of action of Ixazomib citrate. A comparison of Bortezomib and Ixazomib-citrate showed that the latter provides improved PK and treatment comfort such as the administration route (**59**: IV/SC; **64**: Oral/IV).

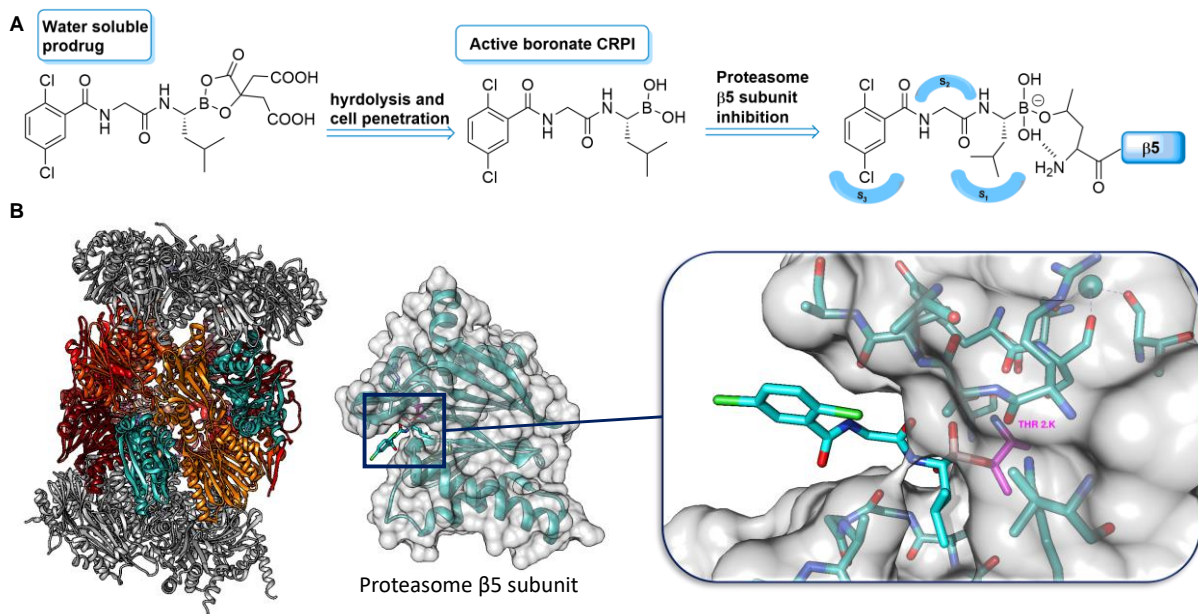
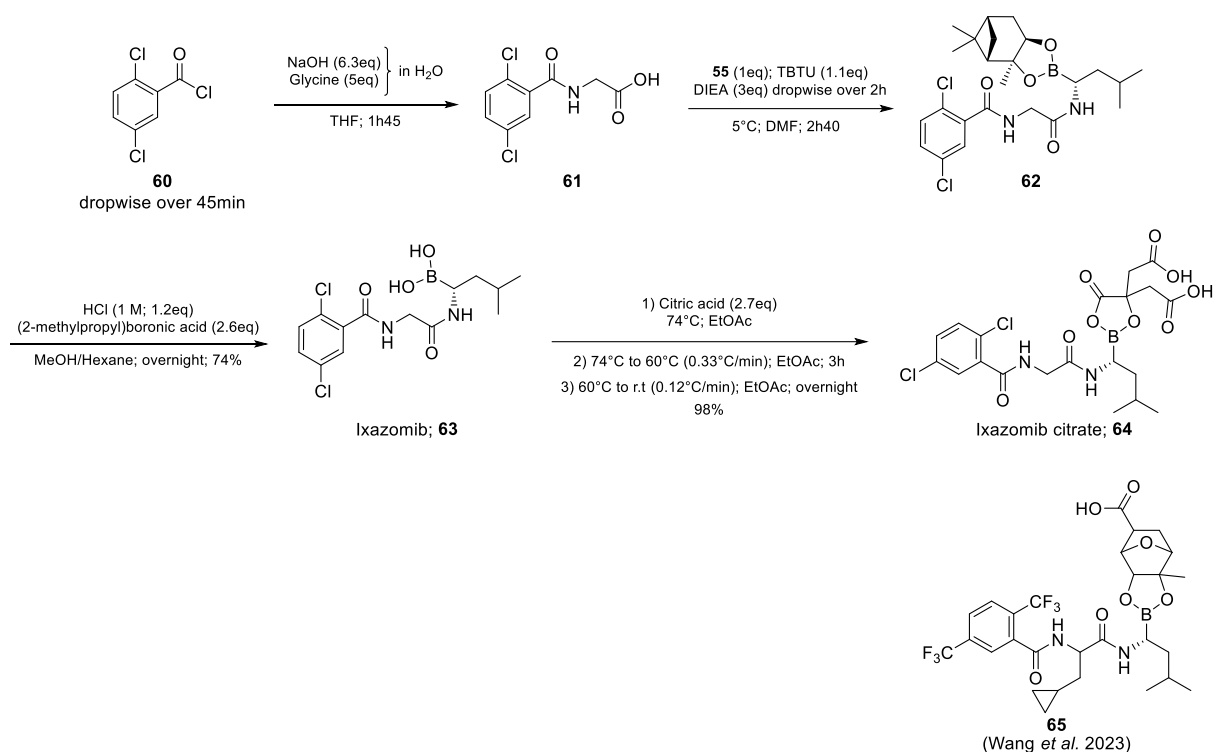


Fig 8: A) Mode of action of Ixazomib-citrate; **B)** Cocrystal structure of **63** bound to proteasome $\beta 5$ subunit (PDB: 5LF7). On the proteasome crystal structure (left side), the α -subunits are highlighted in grey, while the two $\beta 5$ subunits are colored in blue. The N-terminal Thr residue targeted by Ixazomib and bortezomib is highlighted in magenta.

While, a similar selectivity for the proteasome $\beta 5$ subunits was observed, Ixazomib was found to induce apoptosis of multiple myeloma cell lines resistant to conventional therapies such as Bortezomib (Offidani *et al.* 2014). The first synthesis of Ixazomib was reported in a patent in 2009 and is presented in Scheme 9 (Elliott *et al.* 2009, WO2009154737A1). This 4-steps synthesis affords Ixazomib citrate (**64**) in ~70% overall yield. Of note, this citrate prodrug is completely hydrolyzed within 2 min. of exposure to acid (gastric) or neutral (intestinal) aqueous media (Kupperman *et al.* 2010; Offidani *et al.* 2014; Wang *et al.* 2023).

A recent study, reported a moderately more potent *in cellulo* proteasome boronic acid CRPI (Wang *et al.* 2023). The authors described the obtention of an oral slowly-released prodrug (**65**) whereas toxicity and anticancer properties are similar to Ixazomib citrate. It remains to be determined whether this compound would be of any clinical significance. For further information on Ixazomib and Ixazomib citrate, please refer to (Gupta *et al.* 2019).



Scheme 9: Synthesis of Ixazomib (**63**) and Ixazomib citrate (**64**) as described in WO2009154737A1; Structure of the recent boronic acid CRPI **65** described by Wang *et al.* in 2023.

7. Nitrile CRPIs

Nitrile-containing molecules represent an important class of drugs in the field of medicinal chemistry (Brogi *et al.* 2022). In fact, these electrophilic warheads are commonly assumed as carbonyl bioisosteres, but with a milder electrophilic profile that allows for the development of safer drugs, reducing the risk of off-targets (Brogi *et al.* 2022). In the period 2010-2020, the FDA approved 24 nitrile-containing drugs, which corresponds to an average of approximately 2.2 nitrile drugs by year. These drugs are recommended for various illnesses such as cancer or cardiovascular disorders (Wang *et al.* 2021). Interestingly, the nitrile function allows for both non-covalent and covalent-reversible types of binding. This function possesses a *sp*-hybridized C atom linked to a N atom and can form H bond within the active site of a SerP and/or a CysP. Nitriles can also reversibly bind with these catalytic residues to form a (thio)imidate adducts (Fleming *et al.* 2010; De Cesco *et al.* 2017). Furthermore, in several examples, the incorporation of a nitrile moiety improved the PK properties of the inhibitor as this functional group appears to be metabolically stable and rarely toxic (Wang *et al.* 2021). Importantly, when embedded in peptide-based CRPI, the nitrile warhead induces much less racemization of the α -carbon stereocenter compared to carbonyl electrophiles, hence allowing for a more potent interaction at P₁. The first FDA approved nitrile CRPI was the antidiabetic Saxagliptin (Fig 2). Saxagliptin is a long-acting orally available Dipeptidyl Peptidase IV (DPP4) (Augeri *et al.* 2005). This molecule has a special place in the drug class, as it was the first CRPI to be used for a daily treatment of a chronic disease. Saxagliptin, taken daily as 2.5mg or 5mg tablet, regulates glycemia with very few side effects (Men *et al.* 2018). The other FDA approved CRPI, the beforementioned Nirmatrelvir, will be discussed in detail in section 7.2.

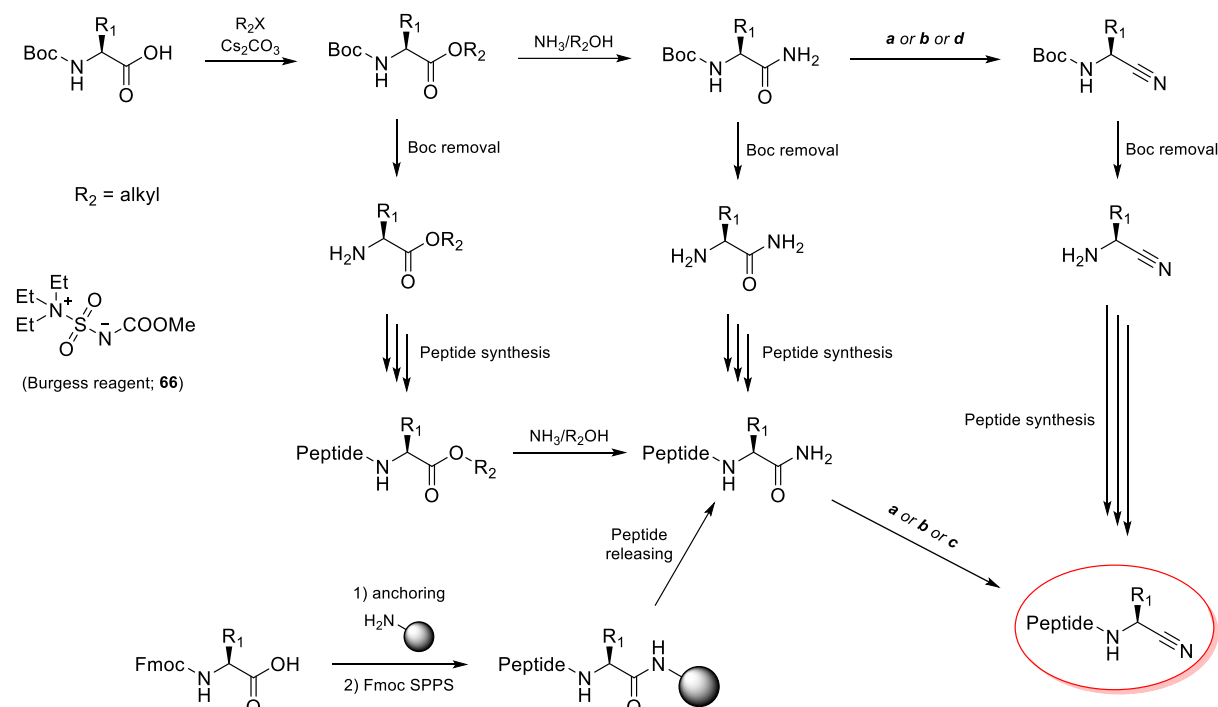
7.1. General synthesis

Similarly to the other CRPIs presented in this review, nitriles can be obtained by different synthetic routes (Mowry 1948; Madhusudana Reddy and Pasha 2010; Ganesan and Nagaraj 2020).

The most direct way to obtain a peptide nitrile is the dehydration of a C-terminal amide function. This step can be performed at the beginning or at the end of the CRPI synthesis. In the case of a solution-phase procedures, numerous pathways to obtain a peptide nitrile are possible such as 1) the peptide elongation with a C-terminus amide followed by the dehydration of the latter; 2) the peptide elongation with a C-terminus ester and formation of the amide at the end of the synthesis followed by a dehydration; 3) the peptide elongation with a C-terminus

nitrile function, since the nitrile moiety is compatible with peptide elongation conditions (Ward *et al.* 2002; Owen *et al.* 2021; Caravez *et al.* 2022). Selected examples are presented as general procedures in Scheme 10. Concerning the obtention of α -amino nitrile compounds, several methods were reported involving Burgess inner salt (**66**) (Burgess *et al.* 1973; Claremon and Phillips 1988), cyanuric chloride (Maetz and Rodriguez 1997) or trifluoroacetic anhydride (Caravez *et al.* 2022).

In the case of SPPS, the peptide is first synthesized on Rink-amide resin and after cleavage its C-terminal amide is transformed into a nitrile *via* Burgess inner salt method (Claremon and Phillips 1988; Owen *et al.* 2021), Cyanuric acid method (Maetz and Rodriguez 1997; Ward *et al.* 2002) or POCl₃ in presence of imidazole and pyridine (Owen *et al.* 2021). However, the trifluoroacetic anhydride method did not show good yields concerning the conversion of peptide amide into peptide nitrile (Stüber *et al.* 1988).



Scheme 10: General methods to afford peptide nitrile. **a**) Burgess reagent (**66**) method (Claremon and Phillips 1988; Owen *et al.* 2021); **b**) Cyanuric acid method (Maetz and Rodriguez 1997; Ward *et al.* 2002); **c**) POCl₃, imidazole, pyridine method (Owen *et al.* 2021); **d**) Trifluoroacetic anhydride method (Caravez *et al.* 2022).

7.2. Example of Nirmatrelvir, a SARS-CoV-2 3CL^{pro} inhibitor

Nirmatrelvir (PF-07321332; **73**) is the first oral drug approved by the FDA for emergency use, against COVID-19, in combination with ritonavir (*Paxlovid*®; December 2021) (Food and Drugs Administration 2021; U.S Department of Health & Human Services). This CRPI was discovered by Pfizer Inc. as a second generation of SARS-CoV-2 M^{pro} inhibitor (Owen *et al.* 2021). It is noteworthy that Nirmatrelvir combines the structure of both Lufotrelvir (also developed by Pfizer) (**20**) and Boceprevir (**30**). Indeed, Pfizer's medicinal chemistry program used compound PF-00835231 (**19**) as lead candidate for the development of potent oral anti-COVID-19 drugs. The main challenge was to decrease the number of hydrogen bond donors in **19**, that hampered intestinal epithelium permeation. To overcome this problem, the hydroxy ketoamide moiety was replaced by a nitrile warhead, which allowed better solubility and less racemization, while providing excellent potency (Fig 9A). Introduction of a Pro based bicyclic bioisoster of Leu removed another HBD while improving stability. Finally, the substitution of the C-terminal heterocycle by a trifluoroacetamide group removed another HBD and greatly improved the gut permeation by virtue of the high lipophilicity of the CF₃ group. The obtained CRPI showed good *in cellulo* antiviral activity (A549-ACE2 cells: EC₅₀ = 77.9 nM; EC₉₀ = 215 nM) with no cytotoxicity observed (up to 3 μ M). It also exhibited excellent selectivity with no off-target inhibition on several mammalian proteases (up to 100 mM) and a good oral bioavailability on mice model. Furthermore, the authors showed good antiviral activity, on mice, *via* oral administration (Owen *et al.* 2021). As a result, this CRPI development program yielded the first orally available COVID-19 drug in a breathtaking pace, from the initial idea to the first clinical trial in less than a year!

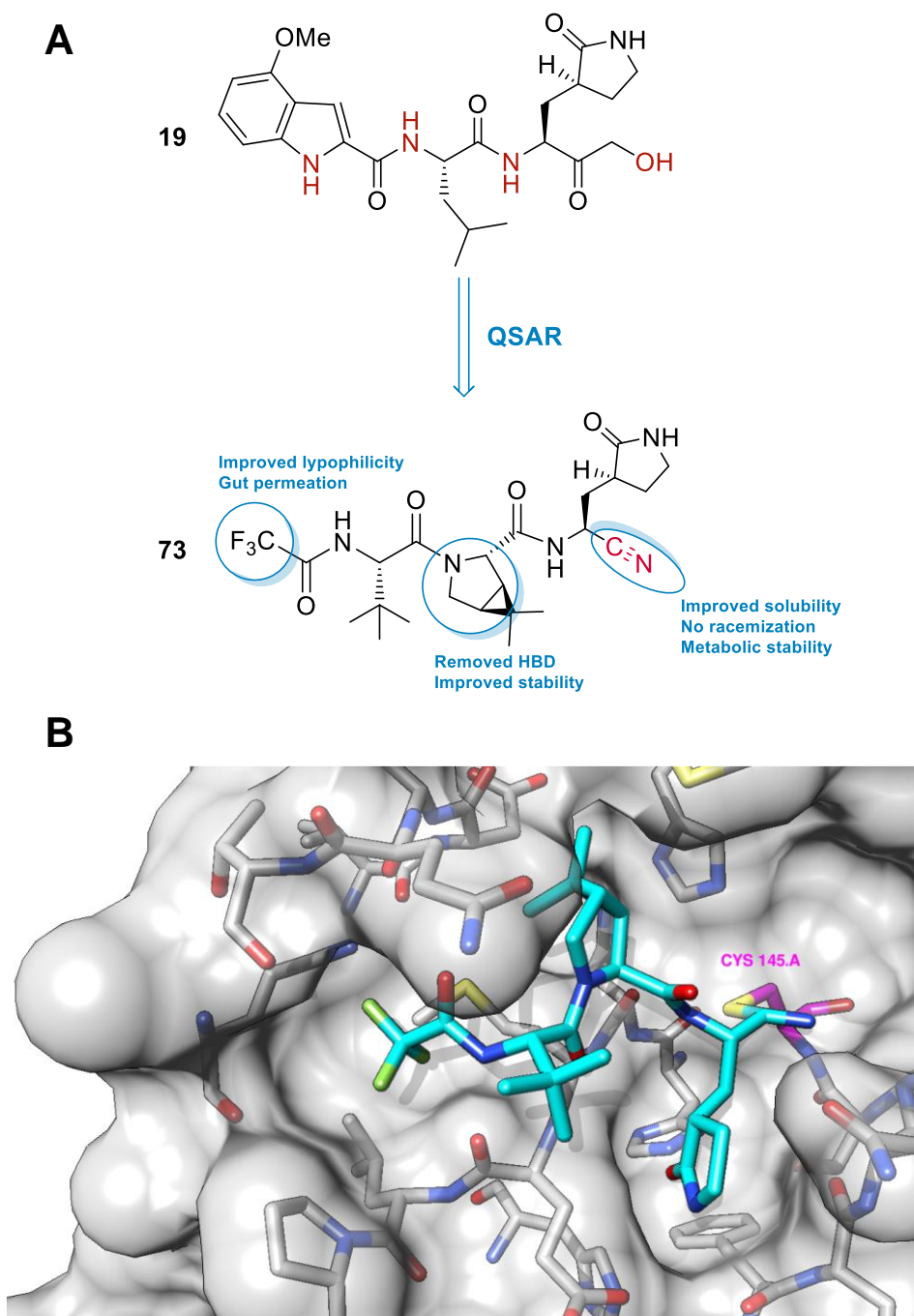


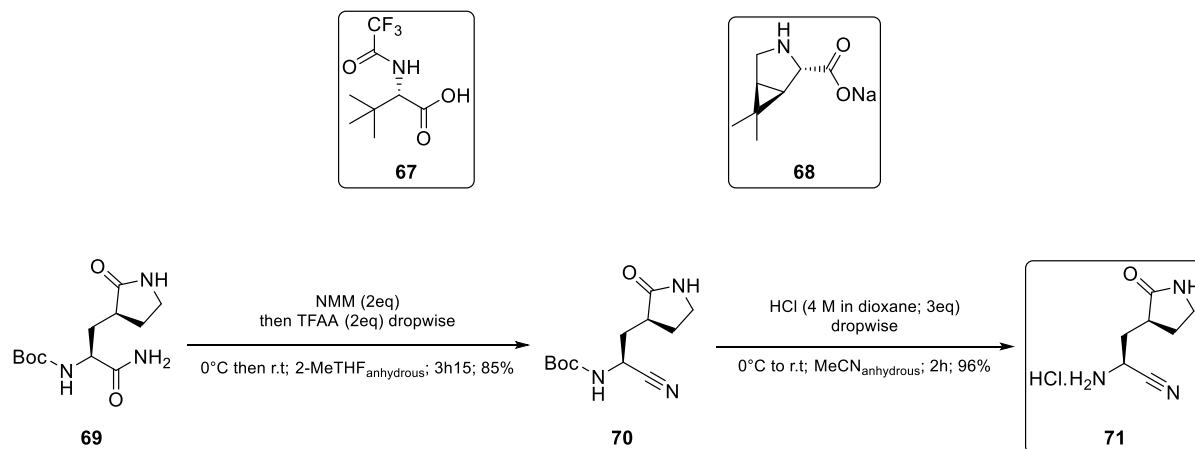
Fig 9: A) Development of Nirmatrelvir (**73**). H bond donors (HBD) are marked in red in the structure of PF-00835231 (**19**); **B)** Cocrystal structure of **73** bound to SARS-CoV-2 M^{pro} (PDB: 7TE0). The nucleophile catalytic residue (here Cys145) is colored in magenta.

Concerning the original synthesis of Nirmatrelvir, Owen *et al.* described a 4-steps pathway with an overall yield of 65% (Owen *et al.* 2021). The nitrile moiety was introduced in the final step, using Burgess reagent. Interestingly, this work inspired other medicinal chemists to develop other potent CRPIs against the SARS-CoV-2 M^{pro} (Göhl *et al.* 2022; Kneller *et al.* 2022).

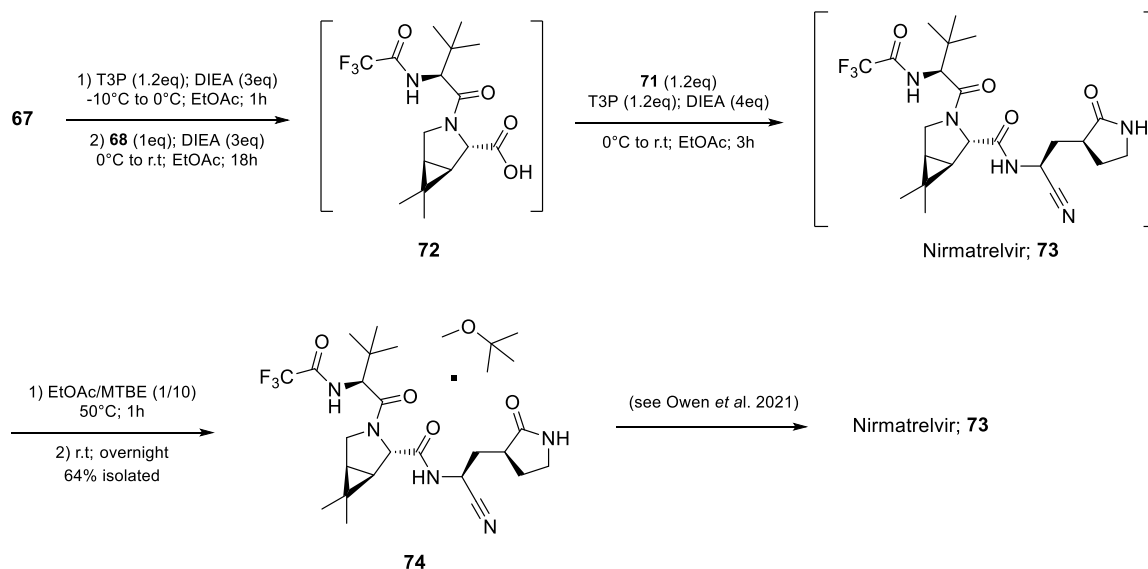
Recently, another synthetic route to obtain Nirmatrelvir was reported (Caravez *et al.* 2022). The authors described a one pot synthesis of the compound using three building blocks. However, none of them are not commercially available and need to be synthesized. This synthetic pathway (without considering the yields of the building blocks production) provides Nirmatrelvir in a 64% yield in an elegant and green way. The synthesis of the nitrile building block and the one pot synthesis are presented in Scheme 11. It is noteworthy that the product is isolated as a methyl tert-butyl ether (MTBE) solvate. Therefore, an additional step is needed to obtain Nirmatrelvir free of MTBE

traces. For more details on Nirmatrelvir, please refer to the publications (Naidu *et al.* 2022; Amani and Amani 2023).

A



B



Scheme 11: Synthesis of Nirmatrelvir (**73**) as described by Caravez *et al.* in 2022. **A)** Presentation of the building blocks (**67**, **68**, **71**) and synthesis of the α -amino nitrile (**71**); **B)** One pot synthesis of Nirmatrelvir (**73**).

8. Conclusion and perspectives

The field of peptide-base covalent-reversible protease inhibitors is still very young and dynamic. The success stories in drug discovery presented in this review suggest that CRPIs can combine the high specificity of peptides and the potency of the covalent modifiers. Moreover, the reversible nature of the inhibition alleviates some of the safety concerns related to the use of irreversible covalent warheads. The CRPI reported in this review, except for Saxagliptin, are aimed at intracellular targets, suggesting that this drug class provides cell penetrant molecules that are well adapted to the glutathione rich cytosolic environment. Remarkably, except for Bortezomib, all the FDA approved CRPIs are effectively administered orally even though this is a particularly challenging route for this class of therapeutics. The increase of inhibitor potency, provided by the covalent-reversible warhead, partially explains this surprising bioavailability as it allowed to significantly shorten the CRPI sequences while maintaining a strong target binding. Another advantage of the CRPI is their straightforward rational design. This was perfectly illustrated by the development of Nirmatrelvir. In less than a year of drug development, this nitrile CRPI entered

in clinical trials as oral COVID-19 medication. Nirmatrelvir had an important commercial success as *Paxlovid*®, becoming the fifth bestselling drug in 2022. Conversely, Boceprevir was discontinued only 4 years after its approval, in favor of the HCV polymerase inhibitor Sofosbuvir. Nevertheless, Boceprevir allowed the inhibition of a very challenging HCV protease with a relatively short and orally bioavailable CRPI sequence. These two examples suggest that CRPIs could have an important place in the field of antiviral therapeutics especially when drug development emergency meets rational design. Saxagliptin is the first CRPI used for the treatment of a chronic disease, diabetes. This nitrile SerP inhibitor, administered orally features an excellent safety profile as the adverse events reported during the clinical investigations are restricted to headaches in only 6% of the patients. This result is promising for considering the use of CRPIs beyond the fields of cancer and antivirals, where benefit-risk balance allows for some toxicity.

The clinically approved CRPI were initially developed by substrate-based design. Electrophile first-approach is an alternative technique, which consists in testing libraries of small electrophile containing fragments for the inhibition of the targeted enzyme. Such screenings could provide hit molecules not based on the natural substrate and/or not targeting the catalytic nucleophilic residues, thus greatly extending the structural space for CRPIs. In 2019, Resnick *et al.* used this strategy in combination with high-throughput crystallography to discover potent and selective ligands for two orphan enzymes (OTUB2 and NUDT7) (Resnick *et al.* 2019). Developing new electrophilic moieties is another way to enlarge the medicinal chemist's toolbox as highlighted by Gehringer and Laufer in 2019 (Gehringer and Laufer 2019). Additionally, warheads capable to target non-catalytic residues such as lysine could enlarge the field of CRPIs' applications. Pettinger *et al.* published a very interesting review on this subject and depicted a challenging but promising field of Lys-targeting covalent inhibitors (Pettinger *et al.* 2017). Targeting Asp and Glu residues with novel covalent-reversible electrophile is another exciting perspective as it could allow to develop CRPIs targeting the metalloproteases. In a recently published article, Thomas *et al.* reported the synthesis and screening of electrophilic fragment libraries targeting specifically the Asp and Glu residues (Thomas *et al.* 2023).

To date, CRPIs seem to indeed bring together the best of the covalent and non-covalent worlds of inhibition. In our opinion, the next couple of years, will be crucial to determine if this is a long-term trend that will eventually consolidate CRPIs as a bona fide class of drugs.

Acknowledgments

The authors acknowledge MT-act (Montpellier, France), Neoflow Therapeutics (Marseille, France) and the ANRT (Association Nationale de la Recherche et de la Technologie) for the financial support of A.F and A.D. The authors also thank the CNRS and the Université de Montpellier for literature access and financial support.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Adkison KK, Barrett DG, Deaton DN, et al (2006) Semicarbazone-based inhibitors of cathepsin K, are they prodrugs for aldehyde inhibitors? *Bioorganic Med Chem Lett* 16:978–983. <https://doi.org/10.1016/j.bmcl.2005.10.108>
- Akaji K, Konno H, Mitsui H, et al (2011) Structure-Based Design, Synthesis, and Evaluation of Peptide-Mimetic SARS 3CL Protease Inhibitors. *J Med Chem* 54:7962–7973. <https://doi.org/10.1021/jm200870n>
- Amani B, Amani B (2023) Efficacy and safety of nirmatrelvir/ritonavir (Paxlovid) for COVID-19: A rapid review and meta-analysis. *J Med Virol* 95:e28441. <https://doi.org/10.1002/jmv.28441>
- Andrés P, Ballano G, Calaza MI, Cativiela C (2016) Synthesis of α -aminoboronic acids. *Chem Soc Rev* 45:2291–2307. <https://doi.org/10.1039/C5CS00886G>

- Augeri DJ, Robl JA, Betebenner DA, et al (2005) Discovery and Preclinical Profile of Saxagliptin (BMS-477118): A Highly Potent, Long-Acting, Orally Active Dipeptidyl Peptidase IV Inhibitor for the Treatment of Type 2 Diabetes. *J Med Chem* 48:5025–5037. <https://doi.org/10.1021/jm050261p>
- Bandyopadhyay A, Gao J (2016) Targeting biomolecules with reversible covalent chemistry. *Current Opinion in Chemical Biology* 34:110–116. <https://doi.org/10.1016/j.cbpa.2016.08.011>
- Bhatia HK, Singh H, Grewal N, Natt NK (2014) Sofosbuvir: A novel treatment option for chronic hepatitis C infection. *J Pharmacol Pharmacother* 5:278–284. <https://doi.org/10.4103/0976-500X.142464>
- Boike L, Henning NJ, Nomura DK (2022) Advances in covalent drug discovery. *Nat Rev Drug Discov* 21:881–898. <https://doi.org/10.1038/s41573-022-00542-z>
- Bond JS (2019) Proteases: History, discovery, and roles in health and disease. *J Biol Chem* 294:1643–1651. <https://doi.org/10.1074/jbc.TM118.004156>
- Boras B, Jones RM, Anson BJ, et al (2021) Preclinical characterization of an intravenous coronavirus 3CL protease inhibitor for the potential treatment of COVID19. *Nat Commun* 12:6055. <https://doi.org/10.1038/s41467-021-26239-2>
- Brogi S, Ibba R, Rossi S, et al (2022) Covalent Reversible Inhibitors of Cysteine Proteases Containing the Nitrile Warhead: Recent Advancement in the Field of Viral and Parasitic Diseases. *Molecules* 27:2561. <https://doi.org/10.3390/molecules27082561>
- Burgess EM, Penton HRJr, Taylor EA (1973) Thermal reactions of alkyl N-carbomethoxysulfamate esters. *J Org Chem* 38:26–31. <https://doi.org/10.1021/jo00941a006>
- Cannalire R, Cerchia C, Beccari AR, et al (2022) Targeting SARS-CoV-2 Proteases and Polymerase for COVID-19 Treatment: State of the Art and Future Opportunities. *J Med Chem* 65:2716–2746. <https://doi.org/10.1021/acs.jmedchem.0c01140>
- Caravez JC, Iyer KS, Kavthe RD, et al (2022) A 1-Pot Synthesis of the SARS-CoV-2 Mpro Inhibitor Nirmatrelvir, the Key Ingredient in Paxlovid. *Org Lett* 24:9049–9053. <https://doi.org/10.1021/acs.orglett.2c03683>
- Chang MH, Gordon LA, Fung HB (2012) Boceprevir: A Protease Inhibitor for the Treatment of Hepatitis C. *Clin Ther* 34:2021–2038. <https://doi.org/10.1016/j.clinthera.2012.08.009>
- Chen W, Shao Y, Peng X, et al (2022) Review of preclinical data of PF-07304814 and its active metabolite derivatives against SARS-CoV-2 infection. *Front Pharmacol* 13:1035969. <https://doi.org/10.3389/fphar.2022.1035969>
- Claremon DA, Phillips BT (1988) An efficient chemoselective synthesis of nitriles from primary amides. *Tetrahedron Lett* 29:2155–2158. [https://doi.org/10.1016/S0040-4039\(00\)86697-6](https://doi.org/10.1016/S0040-4039(00)86697-6)

- Da Silva-Júnior EF, De Araújo-Júnior JX (2019) Peptide derivatives as inhibitors of NS2B-NS3 protease from Dengue, West Nile, and Zika flaviviruses. *Bioorg Med Chem* 27:3963–3978. <https://doi.org/10.1016/j.bmc.2019.07.038>
- Daniels BE, Stivala CE (2018) A solid-phase approach for the synthesis of α -aminoboronic acid peptides. *RSC Adv* 8:3343–3347. <https://doi.org/10.1039/C7RA13479G>
- De Cesco S, Kurian J, Dufresne C, et al (2017) Covalent inhibitors design and discovery. *Eur J Med Chem* 138:96–114. <https://doi.org/10.1016/j.ejmech.2017.06.019>
- De Risi C, Pollini GP, Zanirato V (2016) Recent Developments in General Methodologies for the Synthesis of α -Ketoamides. *Chem Rev* 116:3241–3305. <https://doi.org/10.1021/acs.chemrev.5b00443>
- Diaz DB, Yudin AK (2017) The versatility of boron in biological target engagement. *Nature Chem* 9:731–742. <https://doi.org/10.1038/nchem.2814>
- Elliott EL, Ferdous AJ, Kaufman MJ, et al (2009) Composés De Borates Esters Et Compositions Pharmaceutiques Contenant Des Composés
- Faridoon, Ng R, Zhang G, Li JJ (2023) An update on the discovery and development of reversible covalent inhibitors. *Med Chem Res*. <https://doi.org/10.1007/s00044-023-03065-3>
- Fehrentz J-A, Castro B (1983) An Efficient Synthesis of Optically Active α -(*t*-Butoxycarbonylamino)-aldehydes from α -Amino Acids. *Synthesis* 1983:676–678. <https://doi.org/10.1055/s-1983-30471>
- Fehrentz J-A, Heitz A, Bertrand C, et al (1984) Aldehydic peptides inhibiting renin. *FEBS Lett* 167:273–276. [https://doi.org/10.1016/0014-5793\(84\)80141-6](https://doi.org/10.1016/0014-5793(84)80141-6)
- Fehrentz J-A, Paris M, Heitz A, et al (1995) Improved solid phase synthesis of C-terminal peptide aldehydes. *Tetrahedron Lett* 36:7871–7874. [https://doi.org/10.1016/0040-4039\(95\)01646-Y](https://doi.org/10.1016/0040-4039(95)01646-Y)
- Fleming FF, Yao L, Ravikumar PC, et al (2010) Nitrile-Containing Pharmaceuticals: Efficacious Roles of the Nitrile Pharmacophore. *J Med Chem* 53:7902–7917. <https://doi.org/10.1021/jm100762r>
- Food and Drugs Administration (2021) Coronavirus (COVID-19) Update: FDA Authorizes First Oral Antiviral for Treatment of COVID-19. In: FDA. <https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-first-oral-antiviral-treatment-covid-19>. Accessed 3 Mar 2023
- Galasiti Kankanamalage AC, Kim Y, Rathnayake AD, et al (2017) Design, Synthesis, and Evaluation of Novel Prodrugs of Transition State Inhibitors of Norovirus 3CL Protease. *J Med Chem* 60:6239–6248. <https://doi.org/10.1021/acs.jmedchem.7b00497>
- Ganesan M, Nagaraaj P (2020) Recent developments in dehydration of primary amides to nitriles. *Org Chem Front* 7:3792–3814. <https://doi.org/10.1039/D0QO00843E>

- Gehringer M, Laufer SA (2019) Emerging and Re-Emerging Warheads for Targeted Covalent Inhibitors: Applications in Medicinal Chemistry and Chemical Biology. *J Med Chem* 62:5673–5724. <https://doi.org/10.1021/acs.jmedchem.8b01153>
- Ghosh AK, Brindisi M, Shahabi D, et al (2020) Drug Development and Medicinal Chemistry Efforts toward SARS-Coronavirus and Covid-19 Therapeutics. *ChemMedChem* 15:907–932. <https://doi.org/10.1002/cmdc.202000223>
- Göhl M, Zhang L, El Kilani H, et al (2022) From Repurposing to Redesign: Optimization of Boceprevir to Highly Potent Inhibitors of the SARS-CoV-2 Main Protease. *Molecules* 27:4292. <https://doi.org/10.3390/molecules27134292>
- Goode DR, Sharma AK, Hergenrother PJ (2005) Using Peptidic Inhibitors to Systematically Probe the S1' Site of Caspase-3 and Caspase-7. *Org Lett* 7:3529–3532. <https://doi.org/10.1021/ol051287d>
- Greasley SE, Noell S, Plotnikova O, et al (2022) Structural basis for the in vitro efficacy of nirmatrelvir against SARS-CoV-2 variants. *J Biol Chem* 298:101972. <https://doi.org/10.1016/j.jbc.2022.101972>
- Guichard G, Briand JP, Friede M (1993) Synthesis of arginine aldehydes for the preparation of pseudopeptides. *Pept Res* 6:121–124
- Gupta N, Hanley MJ, Xia C, et al (2019) Clinical Pharmacology of Ixazomib: The First Oral Proteasome Inhibitor. *Clin Pharmacokinet* 58:431–449. <https://doi.org/10.1007/s40262-018-0702-1>
- Hamada Y, Shioiri T (1982) New methods and reagents in Organic Synthesis. 29. A practical method for the preparation of optically active n-protected α -amino aldehydes and peptide aldehydes. *Chem Pharm Bull* 30:1921–1924. <https://doi.org/10.1248/cpb.30.1921>
- Hasinoff BB (2018) Progress curve analysis of the kinetics of slow-binding anticancer drug inhibitors of the 20S proteasome. *Arch Biochem Biophys* 639:52–58. <https://doi.org/10.1016/j.abb.2017.12.020>
- Hedstrom L (2010) Enzyme Specificity and Selectivity. In: *Encyclopedia of Life Sciences*. John Wiley & Sons, Ltd
- Hinkes SPA, Kämmerer S, Klein CDP (2020) Diversity-oriented synthesis of peptide-boronic acids by a versatile building-block approach. *Chem Sci* 11:9898–9903. <https://doi.org/10.1039/D0SC03999C>
- Hoffman RL, Kania RS, Brothers MA, et al (2020) Discovery of Ketone-Based Covalent Inhibitors of Coronavirus 3CL Proteases for the Potential Therapeutic Treatment of COVID-19. *J Med Chem* 63:12725–12747. <https://doi.org/10.1021/acs.jmedchem.0c01063>
- Howe AYM, Venkatraman S (2013) The Discovery and Development of Boceprevir : A Novel, First-generation Inhibitor of the Hepatitis C Virus NS3/4A Serine Protease. *J clin transl hepatol* 1:22–32. <https://doi.org/10.14218/JCTH.2013.002XX>

- Huber EM, de Bruin G, Heinemeyer W, et al (2015) Systematic Analyses of Substrate Preferences of 20S Proteasomes Using Peptidic Epoxyketone Inhibitors. *J Am Chem Soc* 137:7835–7842. <https://doi.org/10.1021/jacs.5b03688>
- Jöst C, Nitsche C, Scholz T, et al (2014) Promiscuity and Selectivity in Covalent Enzyme Inhibition: A Systematic Study of Electrophilic Fragments. *J Med Chem* 57:7590–7599. <https://doi.org/10.1021/jm5006918>
- Joyce RP, Hu VW, Wang J (2022) The history, mechanism, and perspectives of nirmatrelvir (PF-07321332): an orally bioavailable main protease inhibitor used in combination with ritonavir to reduce COVID-19-related hospitalizations. *Med Chem Res* 31:1637–1646. <https://doi.org/10.1007/s00044-022-02951-6>
- Kneller DW, Li H, Phillips G, et al (2022) Covalent narpilprevir- and boceprevir-derived hybrid inhibitors of SARS-CoV-2 main protease. *Nat Commun* 13:2268. <https://doi.org/10.1038/s41467-022-29915-z>
- Kupperman E, Lee EC, Cao Y, et al (2010) Evaluation of the Proteasome Inhibitor MLN9708 in Preclinical Models of Human Cancer. *Cancer Res* 70:1970–1980. <https://doi.org/10.1158/0008-5472.CAN-09-2766>
- Kurinov IV, Harrison RW (1996) Two crystal structures of the leupeptin-trypsin complex. *Protein Sci* 5:752–758. <https://doi.org/10.1002/pro.5560050420>
- Leban J, Blisse M, Krauss B, et al (2008) Proteasome inhibition by peptide-semicarbazones. *Bioorg Med Chem* 16:4579–4588. <https://doi.org/10.1016/j.bmc.2008.02.042>
- Lee A, Huang L, Ellman JA (1999) General Solid-Phase Method for the Preparation of Mechanism-Based Cysteine Protease Inhibitors. *J Am Chem Soc* 121:9907–9914. <https://doi.org/10.1021/ja992009a>
- Li C, Wang J, Barton LM, et al (2017) Decarboxylative borylation. *Science* 356:eaam7355. <https://doi.org/10.1126/science.aam7355>
- Li L, Chenna BC, Yang KS, et al (2021) Self-Masked Aldehyde Inhibitors: A Novel Strategy for Inhibiting Cysteine Proteases. *J Med Chem* 64:11267–11287. <https://doi.org/10.1021/acs.jmedchem.1c00628>
- Liverton NJ (2019) Evolution of HCV NS3/4a Protease Inhibitors. In: Sofia MJ (ed) *HCV: The Journey from Discovery to a Cure: Volume I*. Springer International Publishing, Cham, pp 231–259
- Lv Z, Chu Y, Wang Y (2015) HIV protease inhibitors: a review of molecular selectivity and toxicity. *HIV/AIDS - Res Palliat Care* 7:95–104. <https://doi.org/10.2147/HIV.S79956>
- Madhusudana Reddy MB, Pasha MA (2010) Efficient and High-Yielding Protocol for the Synthesis of Nitriles from Aldehydes. *Synth Commun* 40:3384–3389. <https://doi.org/10.1080/00397910903419894>
- Maetz P, Rodriguez M (1997) A simple preparation of N-protected chiral α -aminonitriles from N-protected α -amino acid amides. *Tetrahedron Lett* 38:4221–4222. [https://doi.org/10.1016/S0040-4039\(97\)00895-2](https://doi.org/10.1016/S0040-4039(97)00895-2)

- M. António JP, Russo R, Parente Carvalho C, et al (2019) Boronic acids as building blocks for the construction of therapeutically useful bioconjugates. *Chem Soc Rev* 48:3513–3536. <https://doi.org/10.1039/C9CS00184K>
- Matteson DS (2013) Boronic Esters in Asymmetric Synthesis. *J Org Chem* 78:10009–10023. <https://doi.org/10.1021/jo4013942>
- Matteson DS, Majumdar D (1983) Homologation of boronic esters to α -chloro boronic esters. *Organometallics* 2:1529–1535. <https://doi.org/10.1021/om50005a008>
- Matteson DS, Sadhu KM (1983) Boronic ester homologation with 99% chiral selectivity and its use in syntheses of the insect pheromones (3S,4S)-4-methyl-3-heptanol and exobrevicomin. *J Am Chem Soc* 105:2077–2078. <https://doi.org/10.1021/ja00345a074>
- McWhirter C (2021) Chapter One - Kinetic mechanisms of covalent inhibition. In: Ward RA, Grimster NP (eds) *Annual Reports in Medicinal Chemistry*. Academic Press, pp 1–31
- Men P, Li X, Tang H, Zhai S (2018) Efficacy and safety of saxagliptin in patients with type 2 diabetes: A systematic review and meta-analysis. *PLoS ONE* 13:e0197321. <https://doi.org/10.1371/journal.pone.0197321>
- Michaud S, Gour BJ (1998) Cathepsin B inhibitors as potential anti-metastatic agents. *Expert Opin Ther Pat* 8:645–672. <https://doi.org/10.1517/13543776.8.6.645>
- Mons E, Roet S, Kim RQ, Mulder MPC (2022) A Comprehensive Guide for Assessing Covalent Inhibition in Enzymatic Assays Illustrated with Kinetic Simulations. *Curr Protoc* 2:e419. <https://doi.org/10.1002/cpz1.419>
- Moulin A, Martinez J, Fehrentz J-A (2007) Synthesis of peptide aldehydes. *J Pept Sci* 13:1–15. <https://doi.org/10.1002/psc.787>
- Mowry DT (1948) The Preparation of Nitriles. *Chem Rev* 42:189–283. <https://doi.org/10.1021/cr60132a001>
- Murray BA (2020) Reactions of Aldehydes and Ketones and Their Derivatives. In: *Organic Reaction Mechanisms* · 2016. John Wiley & Sons, Ltd, pp 1–70
- Muz B, Ghazarian RN, Ou M, et al (2016) Spotlight on ixazomib: potential in the treatment of multiple myeloma. *Drug Des Devel Ther* 10:217–226. <https://doi.org/10.2147/DDDT.S93602>
- Naidu AH, Reddy KS, Swetha GS, et al (2022) Pfizer's inventive Nirmatrelvir/Ritonavir (Paxlovid™): Another arrow in the quiver against SARS-CoV-2. *NeuroQuantology* 3807–3812
- Offidani M, Corvatta L, Caraffa P, et al (2014) An evidence-based review of ixazomib citrate and its potential in the treatment of newly diagnosed multiple myeloma. *OncoTargets Ther* 7:1793–1800. <https://doi.org/10.2147/OTT.S49187>
- Owen DR, Allerton CMN, Anderson AS, et al (2021) An oral SARS-CoV-2 Mpro inhibitor clinical candidate for the treatment of COVID-19. *Science* 374:1586–1593. <https://doi.org/10.1126/science.abl4784>

- Pacifico S, Ferretti V, Albanese V, et al (2019) Synthesis and Biological Activity of Peptide α -Ketoamide Derivatives as Proteasome Inhibitors. *ACS Med Chem Lett* 10:1086–1092. <https://doi.org/10.1021/acsmchemlett.9b00233>
- Papanikos A, Meldal M (2004) Alpha-keto amide peptides: a synthetic strategy to resin-bound peptide isosteres for protease inhibitor screening on solid support. *J Comb Chem* 6:181–195. <https://doi.org/10.1021/cc030107t>
- Paul A, Sarkar A, Saha S, et al (2021) Synthetic and computational efforts towards the development of peptidomimetics and small-molecule SARS-CoV 3CLpro inhibitors. *Bioorg Med Chem* 46:116301. <https://doi.org/10.1016/j.bmc.2021.116301>
- Pettinger J, Jones K, Cheeseman MD (2017) Lysine-Targeting Covalent Inhibitors. *Angew Chem Int Ed* 56:15200–15209. <https://doi.org/10.1002/anie.201707630>
- Philipp M, Bender ML (1971) Inhibition of Serine Proteases by Arylboronic Acids. *Proc Natl Acad Sci USA* 68:478–480. <https://doi.org/10.1073/pnas.68.2.478>
- Quader S, Cabral H, Mochida Y, et al (2014) Selective intracellular delivery of proteasome inhibitors through pH-sensitive polymeric micelles directed to efficient antitumor therapy. *J Control Release* 188:67–77. <https://doi.org/10.1016/j.jconrel.2014.05.048>
- Raedler LA (2016) Ninlaro (Ixazomib): First Oral Proteasome Inhibitor Approved for the Treatment of Patients with Relapsed or Refractory Multiple Myeloma. *Am Health Drug Benefits* 9:102–105
- Rawlings ND, Barrett AJ, Thomas PD, et al (2018) The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. *Nucleic Acids Res* 46:D624–D632. <https://doi.org/10.1093/nar/gkx1134>
- Resnick E, Bradley A, Gan J, et al (2019) Rapid Covalent-Probe Discovery by Electrophile-Fragment Screening. *J Am Chem Soc* 141:8951–8968. <https://doi.org/10.1021/jacs.9b02822>
- Robello M, Barresi E, Baglini E, et al (2021) The Alpha Keto Amide Moiety as a Privileged Motif in Medicinal Chemistry: Current Insights and Emerging Opportunities. *J Med Chem* 64:3508–3545. <https://doi.org/10.1021/acs.jmedchem.0c01808>
- Rogers LD, Overall CM (2013) Proteolytic Post-translational Modification of Proteins: Proteomic Tools and Methodology. *Mol Cell Proteomics* 12:3532–3542. <https://doi.org/10.1074/mcp.M113.031310>
- Rogowski K, Amblard-Caussil M, Van DLS, et al (2023) New Vashs Inhibitors, Conjugates Thereof and Their Uses as Drugs or as Research Tools
- Rohrbacher F, Zwicky A, Bode JW (2018) Facile Synthesis of Internal and C-Terminal Peptide α -Ketoamides with Fmoc-Solid Phase Peptide Synthesis. *Helv Chim Acta* 101:e1800039. <https://doi.org/10.1002/hlca.201800039>
- Rosenthal PJ (2004) Cysteine proteases of malaria parasites. *Int J Parasitol* 34:1489–1499. <https://doi.org/10.1016/j.ijpara.2004.10.003>

- Santos R, Ursu O, Gaulton A, et al (2017) A comprehensive map of molecular drug targets. *Nat Rev Drug Discov* 16:19–34. <https://doi.org/10.1038/nrd.2016.230>
- Sarubbi E, Seneci PF, Angelastro MR, et al (1993) Peptide aldehydes as inhibitors of HIV protease. *FEBS Letters* 319:253–256. [https://doi.org/10.1016/0014-5793\(93\)80557-B](https://doi.org/10.1016/0014-5793(93)80557-B)
- Singh J (2022) The Ascension of Targeted Covalent Inhibitors. *J Med Chem* 65:5886–5901. <https://doi.org/10.1021/acs.jmedchem.1c02134>
- Singh J, Petter RC, Baillie TA, Whitty A (2011) The resurgence of covalent drugs. *Nat Rev Drug Discov* 10:307–317. <https://doi.org/10.1038/nrd3410>
- Smoum R, Rubinstein A, Dembitsky VM, Srebnik M (2012) Boron Containing Compounds as Protease Inhibitors. *Chem Rev* 112:4156–4220. <https://doi.org/10.1021/cr608202m>
- Sorg G, Thern B, Mader O, et al (2005) Progress in the preparation of peptide aldehydes via polymer supported IBX oxidation and scavenging by threonyl resin. *J Peptide Sci* 11:142–152. <https://doi.org/10.1002/psc.606>
- Šterman A, Sosič I, Gobec S, Časar Z (2019) Synthesis of aminoboronic acid derivatives: an update on recent advances. *Org Chem Front* 6:2991–2998. <https://doi.org/10.1039/C9QO00626E>
- Stüber W, Kosina H, Heimburger N (1988) Synthesis of a tripeptide with a C-terminal nitrile moiety and the inhibition of proteinases. *Int J Pept Protein Res* 31:63–70. <https://doi.org/10.1111/j.1399-3011.1988.tb00007.x>
- Tan Y, Wu J, Song L, et al (2021) Merging the Versatile Functionalities of Boronic Acid with Peptides. *Int J Mol Sci* 22:12958. <https://doi.org/10.3390/ijms222312958>
- Thibaudeau TA, Smith DM (2019) A Practical Review of Proteasome Pharmacology. *Pharmacol Rev* 71:170–197. <https://doi.org/10.1124/pr.117.015370>
- Thomas RP, Grant EK, Dickinson ER, et al (2023) Reactive fragments targeting carboxylate residues employing direct to biology, high-throughput chemistry. *RSC Med Chem* 14:671–679. <https://doi.org/10.1039/D2MD00453D>
- Tokuyama H, Yokoshima S, Yamashita T, Fukuyama T (1998) A novel ketone synthesis by a palladium-catalyzed reaction of thiol esters and organozinc reagents. *Tetrahedron Lett* 39:3189–3192. [https://doi.org/10.1016/S0040-4039\(98\)00456-0](https://doi.org/10.1016/S0040-4039(98)00456-0)
- Tsilikounas E, Kettner CA, Bachovchin WW (1993) ¹¹B NMR spectroscopy of peptide boronic acid inhibitor complexes of alpha-lytic protease. Direct evidence for tetrahedral boron in both boron-histidine and boron-serine adduct complexes. *Biochemistry* 32:12651–12655. <https://doi.org/10.1021/bi00210a013>
- Ullrich S, Nitsche C (2020) The SARS-CoV-2 main protease as drug target. *Bioorganic Med Chem Lett* 30:127377. <https://doi.org/10.1016/j.bmcl.2020.127377>
- U.S Department of Health & Human Services Paxlovid is an oral antiviral COVID-19 therapeutic. <https://aspr.hhs.gov/443/COVID-19/Therapeutics/Products/Paxlovid/Pages/default.aspx>. Accessed 3 Mar 2023

- Van Kersavond T, Konopatzki R, van der Plassche MAT, et al (2021) Rapid synthesis of internal peptidyl α -ketoamides by on resin oxidation for the construction of rhomboid protease inhibitors. *RSC Adv* 11:4196–4199. <https://doi.org/10.1039/D0RA10614C>
- Vázquez J, Albericio F (2002) Synthesis of Fmoc-protected amino ketones bearing tert-butyl based side-chain protecting groups. *Tetrahedron Lett* 43:7499–7502. [https://doi.org/10.1016/S0040-4039\(02\)01805-1](https://doi.org/10.1016/S0040-4039(02)01805-1)
- Vázquez J, Albericio F (2006) A convenient semicarbazide resin for the solid-phase synthesis of peptide ketones and aldehydes. *Tetrahedron Lett* 47:1657–1661. <https://doi.org/10.1016/j.tetlet.2005.12.101>
- Wang A, Dorso C, Kopcho L, et al (2012) Potency, selectivity and prolonged binding of saxagliptin to DPP4: maintenance of DPP4 inhibition by saxagliptin in vitro and ex vivo when compared to a rapidly-dissociating DPP4 inhibitor. *BMC Pharmacol* 12:2. <https://doi.org/10.1186/1471-2210-12-2>
- Wang J, Shang M, Lundberg H, et al (2018) Cu-Catalyzed Decarboxylative Borylation. *ACS Catal* 8:9537–9542. <https://doi.org/10.1021/acscatal.8b02928>
- Wang X, Wang Y, Li X, et al (2021) Nitrile-containing pharmaceuticals: target, mechanism of action, and their SAR studies. *RSC Med Chem* 12:1650–1671. <https://doi.org/10.1039/d1md00131k>
- Wang X, Zhang W, Wen T, et al (2023) Design and discovery of novel dipeptide boronic acid ester proteasome inhibitors, an oral slowly-released prodrug for the treatment of multiple myeloma. *Eur J Med Chem* 250:115187. <https://doi.org/10.1016/j.ejmech.2023.115187>
- Ward YD, Thomson DS, Frye LL, et al (2002) Design and Synthesis of Dipeptide Nitriles as Reversible and Potent Cathepsin S Inhibitors. *J Med Chem* 45:5471–5482. <https://doi.org/10.1021/jm020209i>
- Zhang L, Lin D, Sun X, et al (2020) Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α -ketoamide inhibitors. *Science* 368:409–412. <https://doi.org/10.1126/science.abb3405>
- Zhang R, Windsor WT (2013) In Vitro Kinetic Profiling of Hepatitis C Virus NS3 Protease Inhibitors by Progress Curve Analysis. In: Gong EY (ed) *Antiviral Methods and Protocols*. Humana Press, Totowa, NJ, pp 59–79
- Zhu J, Li L, Drelich A, et al (2022) Self-Masked Aldehyde Inhibitors of Human Cathepsin L Are Potent Anti-CoV-2 Agents. *Front Chem* 10:867928. <https://doi.org/10.3389/fchem.2022.867928>
- Zhu L, George S, Schmidt MF, et al (2011) Peptide aldehyde inhibitors challenge the substrate specificity of the SARS-coronavirus main protease. *Antiviral Res* 92:204–212. <https://doi.org/10.1016/j.antiviral.2011.08.001>
- (2014) Protection for the Carbonyl Group. In: *Greene's Protective Groups in Organic Synthesis*. pp 554–685

