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► **To cite this version:**

Yves Janin. On the origins of SARS-CoV-2 main protease inhibitors. RSC Medicinal Chemistry, 2023, 10.1039/D3MD00493G . hal-04242040

HAL Id: hal-04242040

<https://hal.science/hal-04242040>

Submitted on 15 Oct 2023

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On the origins of SARS-CoV-2 main protease inhibitors

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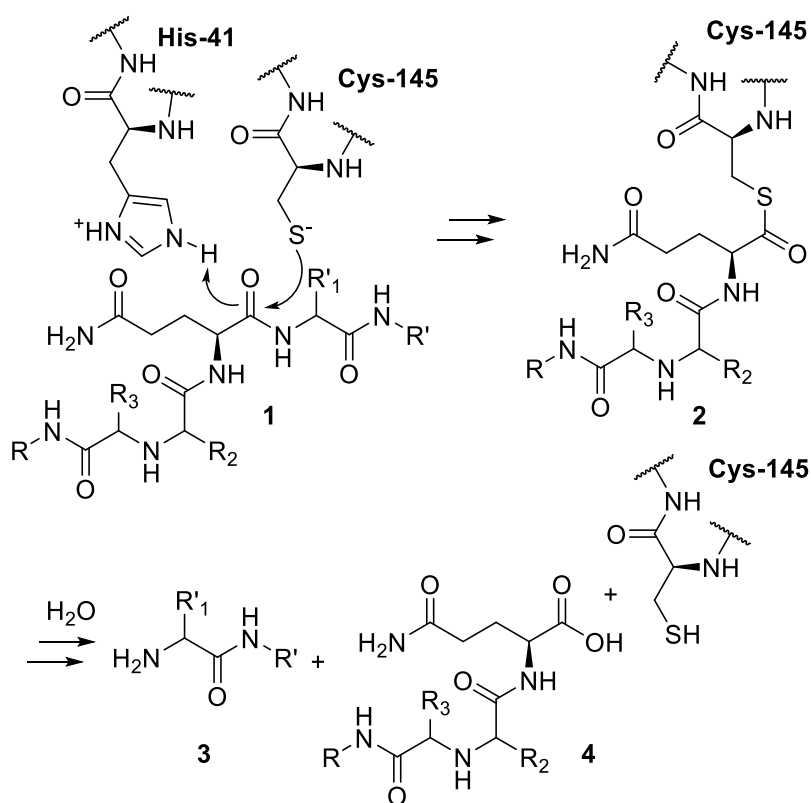
Abstract

In order to address the world-wide health challenge caused by the COVID-19 pandemic, the 3CL protease/SARS-CoV-2 main protease (SARS-CoV-2-M^{pro}) coded by its *nsp5* gene became one of the biochemical targets for the design of antiviral drugs. In less than 3 years of research, 4 inhibitors of SARS-CoV-2-M^{pro} have actually been authorized for COVID-19 treatment (Nirmatrelvir, Ensitrelvir, Leritrelvir and Simnotrelvir) and more such as EDP-235 and FB-2001 or six undisclosed compounds (CDI-988, ASC11, ALG-097558, STI-1558, QLS1128 and H-10517) are undergoing clinical trials. This review is an attempt to picture this quite unprecedented medicinal chemistry feat and provide insights on how these cysteine protease inhibitors were discovered. Since many series of covalent SARS-CoV-2-M^{pro} inhibitors owe some of their origins to previous work on other proteases, we first provided a description of various inhibitors of the cysteine-bearing human caspase-1 or cathepsin K, as well as inhibitors of serine proteases such as the human dipeptidyl peptidase-4 or the hepatitis C protein complex NS3/4A. This is then followed by a description of the results of the approaches adopted (repurposing, structure-based and high throughput screening) to discover corona viruses main protease inhibitors.

Introduction

The protein encoded by the *nsp5* gene of SARS-CoV-2 virus corresponds to the main protease of this coronavirus. This protein has also been named SARS-CoV-2 M^{pro}, 3C-like protease/3CLpro (CL for chymotrypsin-like) as well as C30 endopeptidase. Following an N and an O-terminal autocleavages, the mature enzyme is, at least,¹ able to hydrolyze 9 other conserved sites of the viral polyprotein produced by the host ribosome to generate the corresponding viral proteins. As well reviewed,²⁻⁴ the protease features a characteristic cysteine/histidine catalytic dyad. It is the cysteine-145 thiol anion, depicted in scheme 1, which is acting as a nucleophile and the histidine-41 imidazole as a general base in the course of the proteolysis. The peptide **1** cleavage to release **3** and **4** takes place via the occurrence of a transient S-acylcysteine (**2**) which is then hydrolyzed to regenerate the thiol function and release the protein **4**. Peptide cleavage-wise, the SARS-CoV-2 M^{pro} has the same hydrolysis selectivity as SARS-CoV-1 M^{pro} or other protease from coronaviruses.^{5,6} A recent report is actually describing remarkable structural insights in this sequence recognition process.⁷ As depicted, the residue R₁ of any substrate has to be a glutamine, whereas, upward of the cleavage site, R₂ is usually a leucine or another hydrophobic residue, and if R₃ can vary, R₄ (which is not depicted), remains small and usually aliphatic. Downward, only the R'₁ residue appears to be governing the cleavage selectivity as it can only be a serine, an alanine or an asparagine.⁷

Concerning host cell proteins, quite a few⁸⁻¹¹ have been reported are substrates of SARS-CoV-2 M^{pro} thus providing further insights in the many ways^{12,13} viruses play havoc on cellular biochemistry and innate immunity. A recent review on the proteins reported as substrates of this protease is also available.¹⁴



Scheme 1. Two stages of peptide **1** hydrolysis into **3** and **4** by SARS-CoV-2 M^{pro}

Of note is that comparatively less inhibitors have been reported for the other SARS-CoV-2 protease which is a papain-like cysteine-bearing enzyme. Two papers^{15,16} and few reviews¹⁷⁻²⁰ would probably be good starting points for further information on this different viral protease. Moreover, and as far better reviewed recently,^{21,22} the host cell lysosomal cysteine protease cathepsins B and L, the transmembrane protease serine 2 (TMPRSS2) or the subtilisin-like proprotein convertase furin, a calcium-dependent serine protease, are all capable of cleaving the viral spike protein coating the virus surface and this is an essential step for its cellular entry. Accordingly, in absence of *in vitro* selectivity controls, concerns have been raised on the true mechanism of observed antiviral effects for some series of unselective protease inhibitors reported,²³⁻²⁵ not to mention the recurrent frequent hitters.²⁶⁻²⁸ In fact, cathepsins B and L are actually the targets of a selective inhibitor which also displayed an antiviral effect *in cellulo*.²⁹ Moreover, as for past research against the MERS corona virus (MERS-CoV),^{30,31} the importance of these cellular proteases did suggest the use of Camostat (**5**), an approved TMPRSS2 inhibitor, for a treatment against COVID-19.³² However, even at high dose, the clinical trials with this anticancer agent pointed out a lack of any benefit.³³ Similar reasoning, originally based on MERS-CoV research,³⁴ have also led to trials (NCT04352400) with the nonspecific serine protease inhibitor Nafamostat (**6**). The conclusions of this 2021 human trials against COVID-19 have yet to be published although the short *in vivo* half-life of this iminoimide will remain of real concern.³⁵

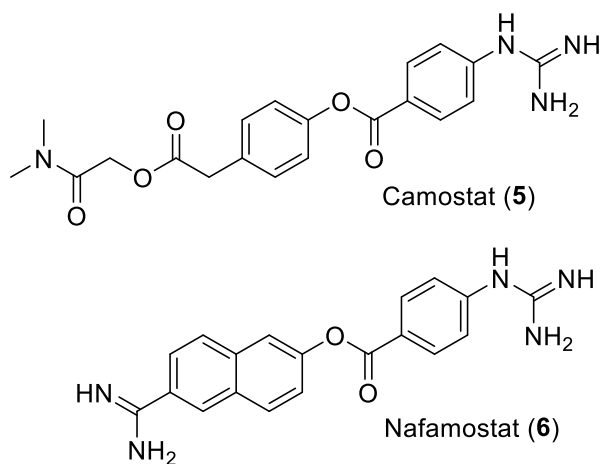


Figure 1: Structures of Camostat (5) and Nafamostat (6)

Two general approaches to discover inhibitors of serine or cysteine-bearing proteases have been used in the past. The main one is to design compounds which can arguably be described as covalent inhibitors. Such compounds will block the enzyme via the occurrence of a covalent bond with their catalytically essential OH or SH residues. Of note is that depending on the chemistry involved, the formation of covalent bond can sometime be reversed. The probably more difficult alternative is to discover inhibitors affecting the protease function because of a high and non-covalent affinity for either its catalytic site or another essential component of the enzyme. The latter approach can of course be useful for the former one since a well-placed incorporation of a reactive moiety into a high affinity compound will lead to a possibly more selective covalent inhibitor. Many reports have already listed all the, sometime related,³⁶ inhibitors reported for their effect on chymotrypsin-like proteases of human rhinovirus,³⁷⁻⁴⁰ enterovirus 71,⁴¹ SARS and MERS corona viruses⁴²⁻⁴⁶ and then SARS-CoV-2.^{2-4,6,17,18,47-70} In the present text, the many publications,²⁶ either solely based on *in silico* docking approaches^{71,72} and/or on traditional/ancestral medicine beliefs which only described frequent hitters/pan-assay interference compounds (PAINS)⁷³⁻⁷⁷ were ignored. This choice is a bid to discourage such all too obvious pollution of scientific literature,^{78,79} not to mention the issue of lack of reproducibility of some data from the academia.⁸⁰ Aside from these, quite a few virtual-based reports are describing modest inhibitions of SARS-CoV-2 M^{pro} by not too obvious frequent hitters which could lead to original series of inhibitors.⁸¹⁻⁹² However, as for the published results of high throughput or X-ray based screenings,^{84,93} we chose to wait for some reports focusing on the actual hit to lead progression before including them in this text.

Designing successful covalent inhibitors, few examples

The principle for designing a successful/selective covalent inhibitor in general is to first start with a substance with a degree of specific affinity for the active site of the targeted enzyme. Then, the inclusion of an electrophilic and thus reactive component to the structure of such compounds can lead to a far stronger and possibly more efficient *in vivo* inhibition effect. A counter example, which unfortunately keeps on attracting undue attention and funding,⁹⁴ would be the frequent hitter⁷⁴ Ebselen (7). This compound does feature a rather reactive nitrogen-

selenium bond but very little else in its structure is providing for any target selectivity. Indeed, a selective covalent inhibitor will rely on the principle that, when bound to its target, the reactive component of such compound is oriented toward a nucleophilic and essential part of the enzyme in order to favor a reaction selectivity. This requires a fine-tuning process not only to improve the affinity of the non-reactive part of the inhibitor for its target but also to secure the best orientation of its reactive component. In other words, Ebselen (**7**) does not comply with such criteria in contrast with, for instance, the recently authorized anticancer drug Sotorasib/AMG 510 (**8**). Indeed, the latter features a Michael accepting acrylamide moiety along with other structural components providing an affinity for its biochemical target. Accordingly, this anticancer substance does preferentially lead to the occurrence of a covalent bond with the oncogenic KRAS (glycine 12 cysteine) mutant.^{95,96} Of note is that more recent research, starting from BI-0474 (**9**), another covalent inhibitor of this KRAS mutant, actually led to the non-covalent pan-KRAS inhibitors BI-2865 (**10**).⁹⁷ Aside from providing a demonstration of the benefit of long-term research in medicinal chemistry, this illustrates the following facts: (i) in the course of designing inhibitors, it is indeed possible to add a reactive moiety and thus improve an inhibition effect and (ii) it is also sometime possible to remove such reactive moiety and, following some more structure-guided design of analogues, reach some very efficient non-covalent inhibitors.

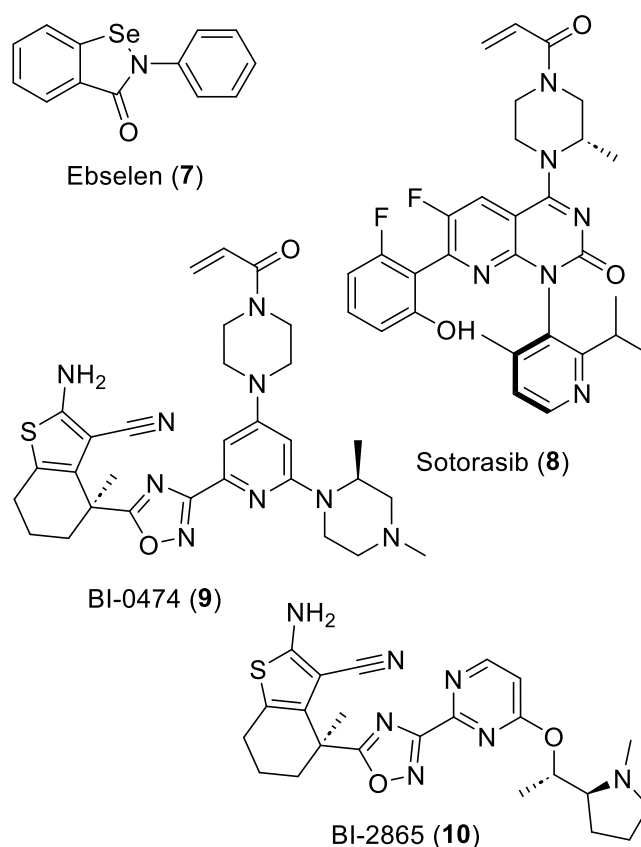


Figure 2: Structures of compounds **7-10**.

Concerning irreversible inhibitors of serine, cysteine or threonine proteases in general, few reviews⁹⁸⁻¹⁰¹ provides an extensive description of the compounds reported. The following are only illustrations, of some of the successes (and failures) of these classes of inhibitors.

- Inhibitors of caspase-1/interleukin-1 converting enzyme

The cysteine protease caspase-1/interleukin-1 converting enzyme (ICE)¹⁰² cleaves peptides after an aspartic acid residue, preferably endowed with the sequence Tyr–Val–Ala–Asp. As reviewed,¹⁰³ research for specific inhibitors started with the aldehyde-bearing peptide Ac-Tyr-Val-Ala-Asp-H and using extensive structure-based insights, proceeded in replacing/”deconstructing” most of these residues to reach more metabolically stable inhibitors. Belnacasan/VX-765 (**11**) is a hemiacetal prodrug, which first patent application was filed by Vertex in 2000.¹⁰⁴ This compound will, upon a hydrolysis, leads to the aldehyde-bearing VRT-043198 (**12**), a selective covalent inhibitor of ICE. Of note is the nitrile group of the analogue **13** which was introduced in 2010 as an alternative to the reactive aldehyde function of compound **11**.¹⁰⁵ Aside from these, few other series were also patented by Vertex. As depicted with the structures of the randomly selected N-substituted pyridinones **14** or **15**, both “drifted” from the ICE-favored peptidic sequence mentioned above and their central heterocycle provided another type of structural lock to favor a proper orientation of their reactive ketone.¹⁰⁶⁻¹⁰⁸ The possible extent of such peptide deconstruction approach is also well illustrated with the hemiacetal ICE inhibitor pralnacasan (**16**) which feature a notable bicyclic piperazic component.¹⁰⁹ More recent work on the design of ICE inhibitors led another research group to uracil-containing derivatives such as the most advanced compound **17**.^{110,111} Clinically, quite a few trials were conducted with Belnacasan (**11**) for treatments of conditions involving a possible dysfunction of ICE but this class of inhibitors has yet to reach an approved use in human health. Of note is the current trend in addressing the dreadful cytokine storm seen in some SARS-CoV-2 infected patients by inhibiting the many biochemical pathways involved in inflammation. In this regard, few 2022 patents are claiming ICE inhibitors such as Belnacasan (**11**) or the nitrile-bearing analogue **13** for their potential benefit against coronavirus infections.¹¹²⁻¹¹⁴ Similarly, a recent patent¹¹⁵ is claiming the use of inhibitors of the beta secretase 1 to suppress this storm however both cases are quite outside the scope of this review.

As well reviewed,¹²²⁻¹²⁷ the serine-containing dipeptidyl peptidase-4/CD26, which is a prolyl oligopeptidase, has many physiological roles. These are including the proteolysis of glucagon-like peptide 1 or the glucose-dependent insulinotropic polypeptide, which are both key factors in glucose homeostasis. From 1994, medicinal chemistry efforts focused on cyanopyrrolidine-containing inhibitors and culminated in the discoveries of Vildagliptin (**20**)¹²⁸ and Saxagliptin (**21**).¹²⁹ With their nitrile function, both compounds are covalent inhibitors of this serine protease, although the serine adducts formed¹³⁰ are slowly reversible.^{131,132} This class of covalent drugs have been prescribed for years to reduce hyperglycemia in patients with type 2 diabetes mellitus. Interestingly, many non-covalent inhibitors of this protease such as Sitagliptin (**22**)¹³³ or Alogliptin (**23**)¹³⁴ were also discovered. As demonstrated by X-ray based structures,^{130,135} these non-covalent inhibitors are also targeting the catalytic site of the protease as well and their wide structural diversity is a tribute to what can medicinal chemistry do.

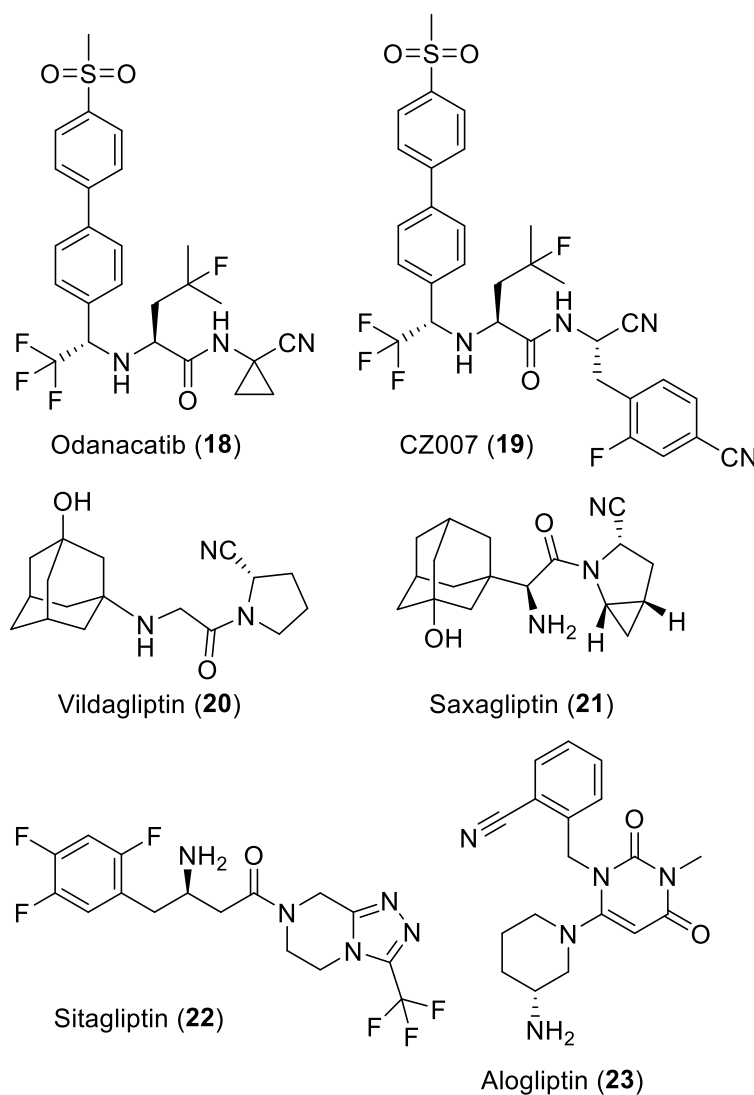


Figure 4: Structures of compound **18-23**.

- Inhibitors of hepatitis C serine protease NS3/4A

As described in an editorial on the 2020 Nobel prize award to Harvey J. Alter, Michael Houghton and Charles M. Rice, the quest to first characterize and then discover treatments against hepatitis C infection has been a 30 years-long story.¹³⁶ Indeed, medicinal chemistry research on the inhibition of a number of viral proteins of

hepatitis C viruses have today delivered treatment efficacies nearing 95% cure!¹³⁷ From the late 90's, many laboratories focused on the design of covalent or non-covalent inhibitors of the hepatitis C serine protease NS3/4A.^{138,139} The α -ketoamide-bearing covalent inhibitors boceprevir (**24**)¹⁴⁰⁻¹⁴² and telaprevir (**25**)¹⁴³ turned out to be the first clinically approved drugs. Interestingly, proof of concept was also achieved in patients with the remarkable macrocycle ciluprevir/BILN 2061 (**26**) which is a non-covalent inhibitor of NS3/4A.^{144,145} This discovery led to many series of macrocycle-bearing analogues which reached clinical approvals such as simeprevir (**27**)¹⁴⁶ or grazoprevir (**28**).¹⁴⁷ Finally, and as well accounted,¹⁴⁸ research on treatment of hepatitis C also focused on discovering inhibitors of its RNA-dependent RNA polymerase NS5B. This was concluded with the prodrug Sofosbuvir (**29**) which is instrumental for reaching the 95% clinical efficacy mentioned above. However, this last achievement also triggered the voluntary withdrawal, or project termination, of quite a few hepatitis C serine protease NS3/4A inhibitors.

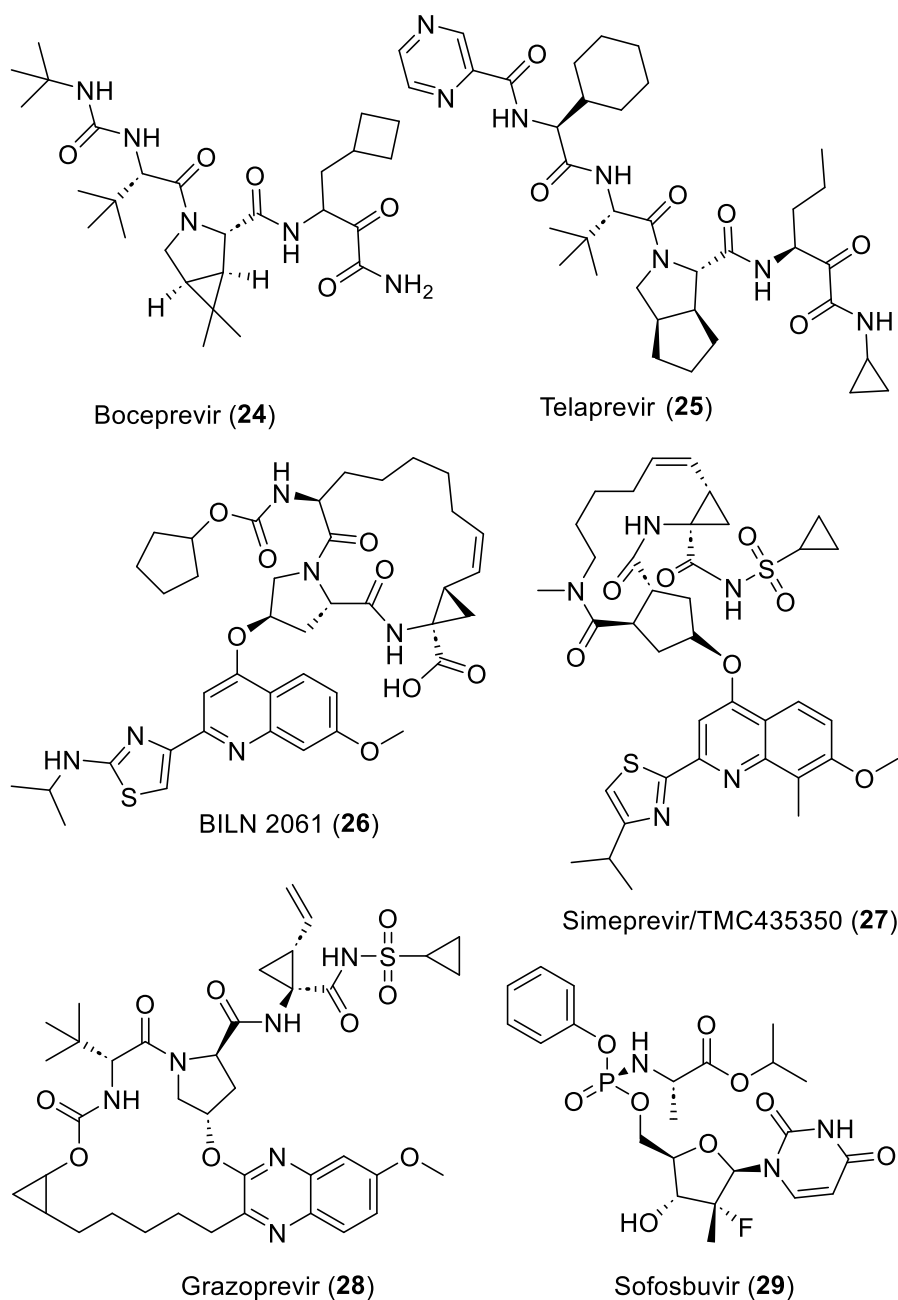


Figure 5: Structures of compound **24-29**.

Inhibitors of SARS-CoV-2 main protease

Since the main proteases of SARS-CoV-1 and SARS-CoV-2 share a 96% amino acid sequence identity (but only 50% with MERS-CoV main protease),² most if not all the research on their inhibition turned out to be useful for the renewed projects focusing on improving such inhibitors. It was also the case for drugs which had been “repurposed” as inhibitors of MERS or SARS-CoV-1 and these includes some potential inhibitors of their main protease.

- Concerning drug repurposing

Aside from the excessive number of reports describing *in vitro* inhibitions of SARS-CoV-1(or 2)-M^{Pro} by well-known frequent hitters/PAINS, another approach focused on assessing libraries of drugs already or previously used or tried in human. The SARS-CoV-2 pandemic thus saw the publication of many papers reporting *in vitro* inhibition of SARS-CoV-2-M^{Pro} by such compounds. The main result of this approach is an unfortunate illustration of a lack of medicinal chemistry culture in general. Indeed, most often only micromolar level of effects were observed *in vitro* for such drugs. Accordingly, it was more than unlikely that a patient would benefit from a treatment based on them, not to mention the issues of necessary dose increases which would be bound to lead to some side effects including some due to their main biological actions. In medicinal chemistry, compounds effective at the micromolar level on a given target can only be considered as hits (or early leads). Such compounds must undertake rounds of structure-activity relationship and selectivity studies to be further improved before preclinical and clinical trials can be envisaged. Attempts to cut this process short are oblivious to decades of experience in the domain and only slightly more rational than hoping for a miracle. Moreover, even in the rare event of finding a strong level of *in vitro* SARS-CoV-2-M^{Pro} inhibition for a prescribed drug, it is very likely that it will lack any *in vitro* or *in vivo* selectivity. Indeed, quite a few highly reactive compounds are found in present or past human pharmacopeia. In the following, we describe few drugs which were reported for their effect on SARS-CoV-2-M^{Pro}. Unfortunately, not all were the focus of some MedChem iterations to improve them before initiating wishful clinical trials which, predictably, led to disappointing results.^{27,149-152}

Out a screening which identified 14 known drugs, the antiepileptic drug perampanel (**30**) was found to be an inhibitor of the main protease of SARS-CoV-2.¹⁵³ Some MedChem helped by X-ray based structures (PDB 7L10 to 7L14) from the same research group provided 3-pyridyl-bearing analogues, such as compound **31-32** (**32** being much less cytotoxic than **31**).¹⁵⁴ And further work, helped by nine X-ray based structures in this case, gave improved inhibitors such as the pyrimidinediones **33-34a**,¹⁵⁵ as well as the even less cytotoxic N-methylated derivative **34b**.¹⁵⁶ A more recent report is describing analogues featuring a pyridone instead of the uracil moiety of compounds **33-34a-b**.¹⁵⁷ As described in more details below, the 3-pyridyl component of these improved analogues had actually been known since 2013 for its capability to interact with the histidine-163 of SARS-CoV-1-M^{Pro} catalytic site.¹⁵⁸ Also of note is that this class of inhibitors do have some structural similarities with alvelestat (**35**), a serine protease (elastase) inhibitor.¹⁵⁹ Independently from this work and as stated by its authors,

a remarkably lucky de novo-initiated search for inhibitors delivered the somehow related (but not computer-guided) N-substituted pyridinone derivative **36**. Further X-ray based crystallography studies are planned in order to improve its relatively modest inhibition level.¹⁶⁰

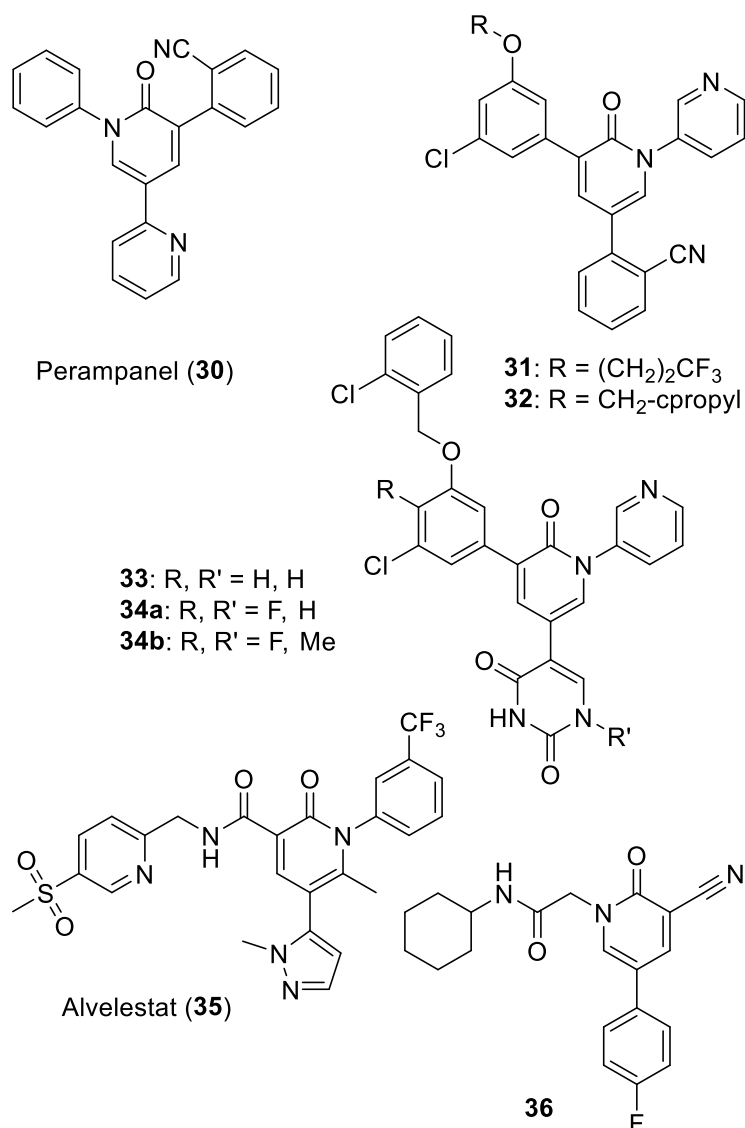


Figure 6: Structures of compound **30-36**.

A repurposed curiosity would be the veterinary anticancer drug masitinib (**37**), it is a tyrosine kinase inhibitor which has also been patented for its modest effect on the replication of SARS-CoV-2.¹⁶¹ Further research actually led to its co-crystallization with SARS-CoV-2-M^{pro} (PDB 7JU7).¹⁶² A note of caution would be that this rather sticky compound, as well as imatinib (**38**), have also managed to co-crystallize with human deoxycytidine kinase (PDB 5MQL).¹⁶³ In any case, undertaking clinical trials against COVID-19 (NCT05047783) with compound **37**, even in association with the polyphenolic frequent hitter isoquercetin (NCT04622865), is very likely to be disappointing. Indeed, as mentioned for the repurposing of imatinib (**38**) against COVID-19, which had also been found active *in vitro* on MERS-CoV,¹⁶⁴ clinically achievable doses in human will not be high enough to be effective on the virus replication.¹⁶⁵ One more examples of such “sticky” compounds would be dasatinib (**39**), another tyrosine kinase inhibitor, which was reported to have some effect on SARS-CoV-2-M^{pro}.¹⁶⁶ However,

the *in cellulo* antiviral effects observed for all these amine-bearing compounds are very likely due to a drug-induced phospholipidosis.^{167,168} This cellular-level effect (which impacts to some degree viral replications *in cellulo*) was unfortunately the cause of a considerable waste of money (as much as 6 billion dollars) when considering all the clinical trials against COVID-19 made with many amine-bearing compounds, especially chloroquine or hydroxychloroquine.¹⁵⁰ Concerning other cellular kinase, a recent report is describing far stronger *in cellulo* inhibition of SARS-CoV-2 replication by PI3K/mTOR inhibitors such as sapanisertib (**40**).¹⁶⁹ Future will tell if this translates into an *in vivo* effect although a precedent would be the inhibitors of cellular dihydroorotate dehydrogenase¹⁷⁰ which have yet to translate into effective RNA-based antivirals (including corona) in patients.¹⁷¹⁻¹⁷³ In 2004, niclosamide (**41**) was found to be endowed with a degree of antiviral effect on SARS-CoV-1 although it was not found to inhibit its main protease¹⁷⁴ and later on, this anthelmintic drug was also reported for its effect on many viruses which have been recently reviewed.¹⁷⁵ Finally, an inhibition of SARS CoV-2 replication was also reported¹⁷⁶ and, despite some undergoing structure-activity relationship studies,¹⁷⁷ clinical trials were undertaken (NCT04399356 and 04603924)¹⁷⁸ with compound **41** and have so far failed.¹⁷⁹ Even if mechanism of action-wise, niclosamide (**41**) should not be mentioned here,¹⁸⁰ some protease inhibitors such as **42** have a puzzling degree of structural similitude with this drug. Compound **42** actually resulted from attempts to prepare protease substrates which would release a fluorescent product. However, it turned out that that these were inhibiting SARS-CoV-2-M^{pro} and the ensuing structure activity relationship studies led to this compound.¹⁸¹ More recent work on closely related analogues of compound **42** have reported a very modest inhibition of SARS-CoV-2-M^{pro}.¹⁸² Another puzzling similitude is also seen with a patent¹⁸³ claiming the effect on SARS-CoV-2-M^{pro} of closantel (**43**), a rather toxic anthelmintic drug, which shares some degree of similitude with these two amides.

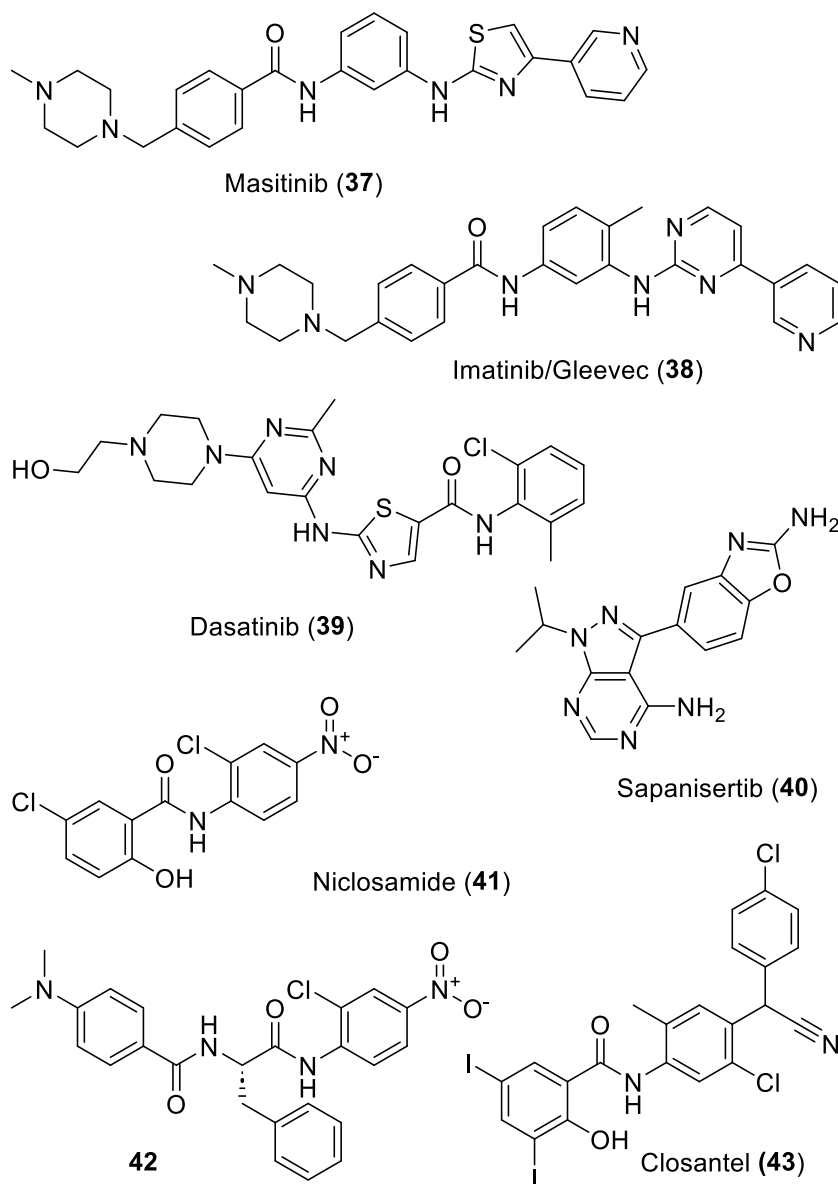


Figure 7: Structures of compound 37-43.

- Covalent or non-covalent inhibitors of SARS-CoV-2-M^{pro} from previous series

As for the series inhibiting other protease, one of the approaches to design inhibitors started from the structure of small peptides featuring sequences recognized by the protease (i.e.: Ser-Ala-Val-Leu-Gln) and the addition of various types of warheads next to the cleavage site: downward the glutamine (Gln) residue in the case of SARS-CoV-2-M^{pro}. It would be beyond the scope of this review to depict all the series of peptide-derived inhibitors made, especially since 2019 an even larger array of warheads was incorporated in such compounds.¹⁸⁴ Indeed, a non-exhaustive list used in the last decades includes: aldehyde (or the corresponding bisulfite adduct),^{41,185-202} ketone,²⁰³⁻²¹¹ α -ketoamide,^{153,185,212-214} Michael acceptors,^{194,215-218} 4-iminooxazolidin-2-one,²¹⁹ reactive halogens,²²⁰⁻²²⁴ β -lactam of some penicillins,^{225,226} phenylsulfide,²²⁷ thiocyanate,²²⁸ epoxide,²⁴ nitrile²²⁹⁻²³¹ and, last but not least, alkyne.^{232,233} Interestingly, aside from a 2005 patent²³⁴ targeting SARS-CoV-1-M^{pro}, the design of boron-containing inhibitors, which is fairly classic approach for proteases inhibition,^{235,236} was not reported in more recent time. Along with these warheads, intensive efforts were made to modify these peptides and/or

replaced them with non peptidic spacers. Moreover, compounds designed to block other viral cysteine proteases were of course assayed on the corona viruses. As an illustration of the multitude of approaches, the Michael-acceptor bearing Rupintrivir/AG7088 (**44**), initially designed in 1999 as a covalent inhibitor of human rhinovirus 3C cysteine protease,^{237,238} was suggested²³⁹ in 2003 to be a starting point to target the SARS-CoV-1-M^{pro}. Of further intensive to evaluate Rupintrivir (**44**) was the fact that it had been the subject of phase 1 and 2 clinical trials against rhinovirus.^{240,241} However, Rupintrivir (**44**) was “not able to significantly effect virus reduction or moderate disease severity and thus was terminated for clinical development”.²⁴² In any case, if compound **44** was reported inactive in 2005,²⁴³ the closely related analogue **45** turned out to modestly inhibit²⁴³ SARS-CoV-1-M^{pro} (or MERS-M^{pro})²⁴⁴ and the longer peptides N1 (**46**) and N3 (**47**) featuring the more adapted Ala-Val-Leu sequence were even better.²⁴⁵ Although Rupintrivir (**44**) was later found to only be a very modest inhibitor of SARS-CoV-2-M^{pro}, thanks to its reactive component, it still managed to co-crystallize with this protease (PDB 7L8I).²¹⁸ Also starting from the Ser-Ala-Val-Leu-Gln sequence, a research group reported in 2011 that the imidazole bearing aldehyde **48** was a modest inhibitor SARS-CoV-1-M^{pro}. The ensuing structure-guided iterations of synthesis and evaluation led to the much stronger inhibitor **49** which was also co-crystallized with SARS-CoV-1-M^{pro} (PDB 3ATW).²⁴⁶ More recent work actually reported related imidazole-bearing peptides.²⁴⁷ Of note is that a glutamine residue is prone to cyclize at the least with ketone or aldehyde warheads.²⁴⁸⁻²⁵⁰ Accordingly, effort to replace it, also stemmed from the search of human rhinovirus 3C cysteine protease inhibitors and provided the bioisosteric lactams depicted in the structures of compounds **44-45**. As well illustrated below, this bioisosteric replacement was repeatedly used in the structures of other virus proteases inhibitors.^{238,248,251} A recent review is actually describing in much more details this issue of glutamine replacement.²⁵² As for the results reported in 2000 focusing on the inhibition of Rhinovirus 3C protease,²⁰³ research on SARS-CoV-1-M^{pro}, dating from 2009, also explored the incorporation of large components downstream of the warhead. Similarly to peptide inhibitors of rhinovirus 3C protease,²⁰³ if the thiazole-2-ketone-bearing peptide **50** was only as a modest inhibitor of SARS-CoV-1-M^{pro},²⁵³ the benzothiazole analogue **51** which benzene filled a pocket of this protease provided a thousand-fold improvement.²⁵⁴ Also of interest is the nitrile-containing peptide **52** which was reported in 2013 to be a weak inhibitor of SARS-CoV-1 M^{pro} but established that this function could be used to covalently inhibit this protease.²⁵⁵

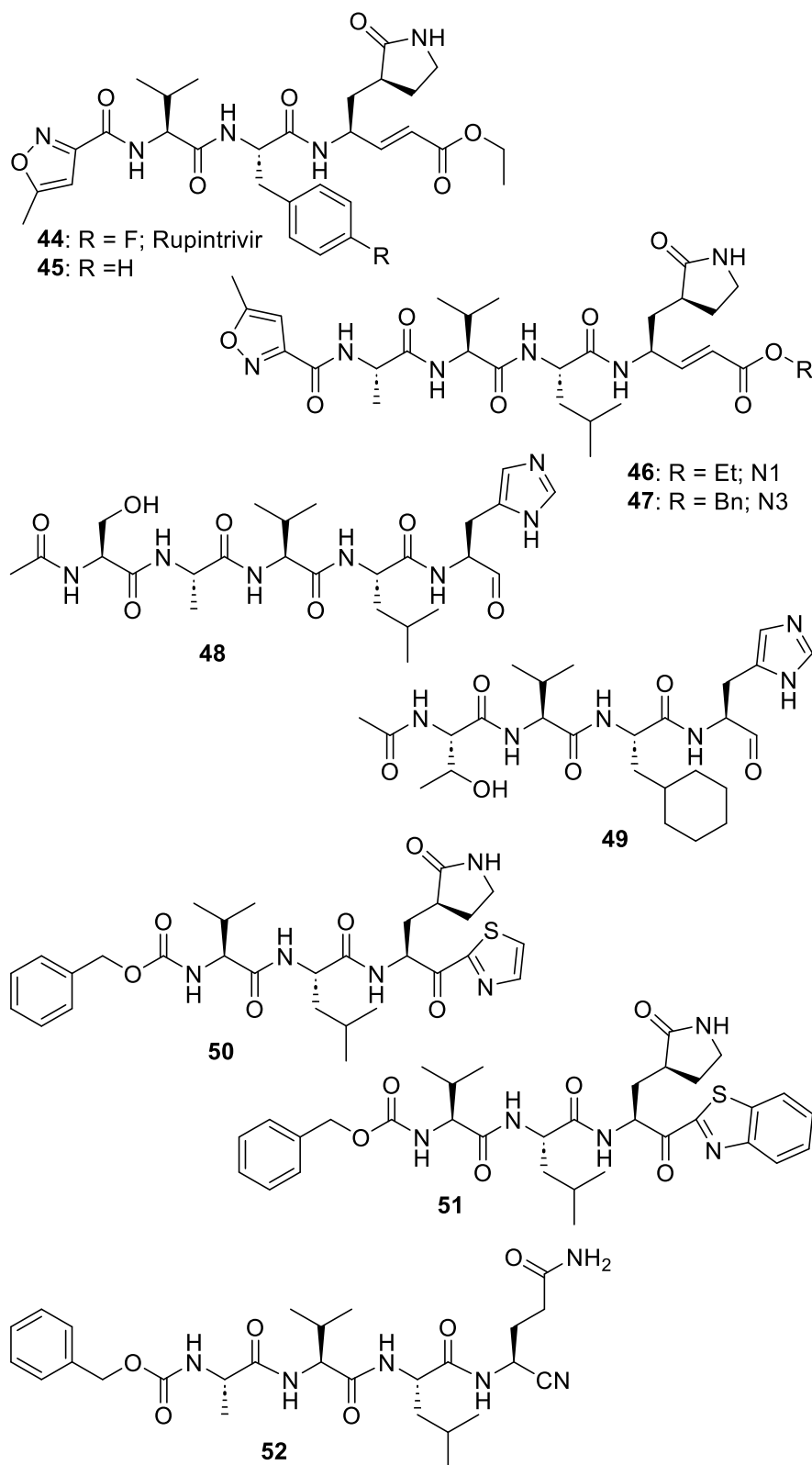


Figure 8: Structures of compound **44-52**.

The shorter bisulfite prodrug GC376 (**53**) was reported in 2012 as a norovirus 3CL protease inhibitor.²⁵⁶ As depicted, the bisulfite adduct **53** will release *in situ* the aldehyde function of the antiviral GC373 (**54**).²⁵¹ Interestingly, this compound has demonstrated a degree of *in vivo* effect against a Feline enteric coronavirus infection.^{257,258} Even it is only modestly effective *in vitro*²¹⁷ or *in vivo* on a mice model of SARS-CoV-2 infection,²⁵⁹ this pan 3CL virus protease inhibitor has often been used as a positive control in SARS-CoV-2-M^{PRO}

assays. Many analogues of GC376 (**53**) were reported^{185,260,261} such as compounds resulting from a “fluorine walk” on the benzyl moiety and/or its replacement by a substituted cyclohexyl, a bulkier adamantyl and even more elaborated substituents.^{195,199,201,262,263} As an illustration of the “leeway” on this position, the difluorocyclohexyl-bearing analogue **57** was found to be effective on a mice model MERS corona virus infection.¹⁸⁸ Moreover, a deuterated derivative of **50** was evaluated on a mice model of SARS-CoV-2 infection but this analogue showed no real advantage.¹⁹⁸ This result may not be too surprising, since a deuteration strategy usually addresses fast metabolic issues but GC376 (**53**) was reported to be reasonably stable in human plasma ($t_{1/2} > 240$ min) or in the presence of human liver microsomes ($t_{1/2} > 80$ min) as well as in mice ($t_{1/2}$, plasma > 240 min, $t_{1/2}$, microsome > 80).²¹¹ Further studies on the protease inhibition selectivity of GC376 (**53**) as well as analogues EB54 (**55**) and NK01-63/coronastat (**56**) pointed out the fact that these compounds are also very strong inhibitors of the host cell cathepsin L.²¹¹ Another investigation reported even more analogues of GC376 (**53**) but also described solubility issues, along with suggestions to administer such bisulfite adducts at higher concentration.²⁶⁴ The cyclohexyl group of compound **49** was also adopted in the design of the related compound **58** targeting the norovirus 3CL protease.²⁶⁵ However, even combined with other types of warheads, this cyclohexyl feature turned out to be associated with a cellular toxicity.^{209,266} Compound TG-0205221 (**59**) illustrates the structure-based improvements made in 2006 when focusing on the inhibition of SARS-CoV-1-M^{Pro} by this “Boc-derived” series.²⁶⁷ Much later, the O-tert-butyl-threonine component of the tripeptide TG-0205221 (**59**) was also found to be a key to improve the interactions of related analogues with the P3 site of SARS-CoV-2-M^{Pro} binding pocket and it also provided some cellular potency.²⁶⁸ Interestingly, research at Glaxo, part of it dating from 2018 and focusing on rhinovirus main protease,²⁶⁹ led to tripeptides such as **60** in which an α -ketoamide warhead was used instead of an aldehyde. However, a modest antiviral activity along with a degree of cellular cytotoxicity probably prevented further development of this series.²⁷⁰ The somehow larger “Boc” derivative **61** is amongst the compounds claimed in four patents by Cocrystal Pharma which has initiated a phase 1 study of the undisclosed coronavirus-norovirus proteases inhibitor CDI-988 (NCT05977140).²⁷¹⁻²⁷⁴

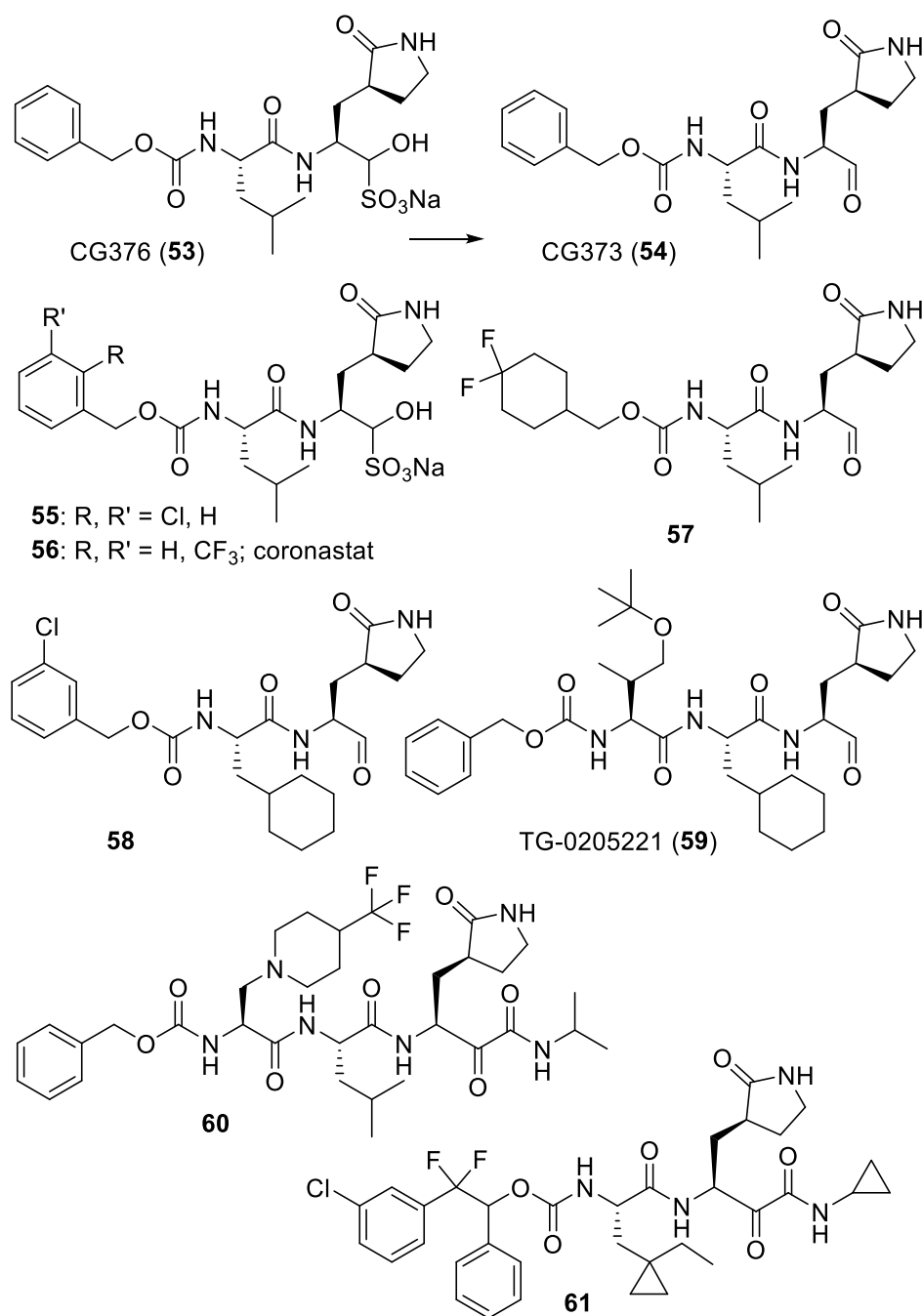


Figure 9: Structures of compound **53-61**.

Other series of short peptides were also designed when the world faced the first SARS epidemic. A 2005 patent claimed many isoleucine and lactam-containing inhibitors of SARS-CoV-1-M^{Pro}, including the noteworthy indole-bearing derivative PF00835231 (**62**).²⁷⁵ From 2020, the corresponding phosphate prodrug PF-07304814/Lufotrelvir (**63**) was then developed by Pfizer to fight COVID-19 infections,^{205,276} and this intravenous prodrug²⁰⁷ underwent successful phase 1 clinical trials.^{277,278} However, an additional trial (NCT05780541) was suspended by the FDA and Lufotrelvir (**63**) was then withdrawn. More recent work borrowed the cyclohexane and the indole elements of **62** and led to aldehyde **64** or to the benzyl-bearing analogues **65-66**.¹⁸⁹ Interestingly, FB-2001 (**64**) was further evaluated²⁷⁹ and has undergone some clinical trials (NCT04766931). The same research group reported two years later that the un-fluorinated analogue **65** was better suited as a broad-spectrum antiviral

since, aside from being active on SARS-CoV-2, it also displayed an effect on entero and rhinoviruses.⁴¹ Following extensive structure-activity relationship studies,²⁸⁰ Pardes Biosciences undertook clinical trials (NCT05011812 and NCT05543707) with the nitrile-bearing PBI-0451/Pomotrelvir (**67**) which had also been co-crystallized with SARS-CoV-2-M^{pro} (8TBE). However, a company statement reported that this compound “did not meet the primary endpoint measured by proportion of participants below the limit of detection for infectious SARS-CoV-2 on day 3 of treatment” and further development was thus suspended.²⁸¹ The 3-methoxyindole-bearing analogue **68**, also featuring the benzothiazole seen in compound **51**, was initially designed against SARS-CoV-1-M^{pro}.²⁸² It was then reported to be efficient against SARS-CoV-2-M^{pro} and the virus replication.^{206,210} An extensive study of various ester prodrugs such as compound **69** was reported and also demonstrated that no real difference in antiviral property was observed between compound featuring a 5 or a 6-membered lactams.²⁰⁹ In an approach which has been used in the past against rhinovirus 3C proteases²⁸³ and which incidentally addressed a racemization risk, the strong aza-bearing SARS-CoV-2-M^{pro} inhibitor YH-6 (**70**) featuring a chiral α,α -chlorofluoroacetamide warhead was reported.²²³ Two patents^{224,284} are also claiming related α,α -chlorofluoroacetamide-containing aza-peptides including the fairly elaborated compound **71** which is effective on a whole panel of coronaviruses.²²⁴

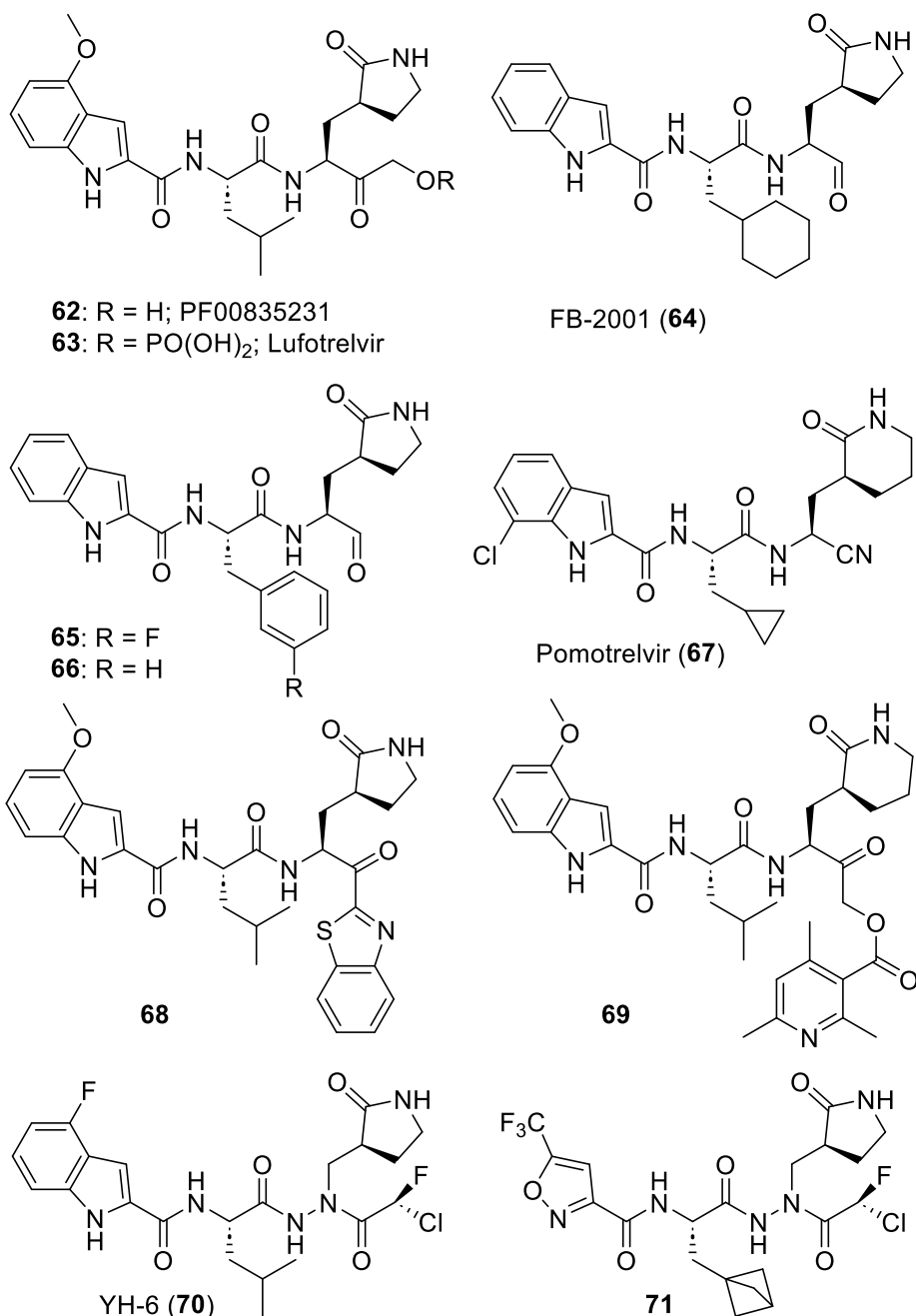


Figure 10: Structures of compound **62-71**.

By the end of 2019, the hepatitis C serine protease NS3/4A inhibitors boceprevir (**24**) and telaprevir (**25**) depicted above, or the related narlaprevir, had been repeatedly reported active in SARS-CoV-2-M^{pro} screening campaigns.^{153,186,187,285-287} This triggered X-ray based structural studies which pointed out that these reactive compounds did indeed bind to the catalytic site of SARS-CoV-2-M^{pro}.^{212,287,288} Of course the bicyclic 3-azabicyclo[3.1.0]hexane component of boceprevir (**24**), which not only enables the removal of an NH moiety but also probably acts as a conformational lock, provided a lot inspiration for the design of many series of SARS-CoV-2-M^{pro} inhibitors. In fact, research conducted at Pfizer led, for instance to the ketone-bearing inhibitor **72a** or the nitrile-bearing PF-07321332/Nirmatrelvir (**73**).^{63,231} A crucial point explaining that Nirmatrelvir (**73**) turned out to be orally active on a mice model of SARS-CoV-2 infection is the removal of this extra NH moiety with this bicycle which greatly improved its diffusion.²⁸⁹ The ensuing successful clinical trials of Nirmatrelvir

(**73**) then provided the first approved SARS-CoV-2-M^{pro} inhibitor which must be prescribed in association with ritonavir to alter a P4503A4-based metabolism and thus improve its pharmacology.^{290,291} Interestingly, compound **72a** was the focus of further optimization which led to the fluorinated derivative TKB245 (**72b**).^{292,293} Moreover, extensive search for an alternative to the trifluoroacetamide moiety of Nirmatrelvir (**73**) were conducted at Asclepis Bioscience.²⁹⁴ The undisclosed protease inhibitor ASC11 probably resulting from this approach is currently undergoing an Asclepis-sponsored phase 1 clinical trials, with the co-administration of ritonavir (NCT05718518). The 3-azabicyclo[3.1.0]hexane-bearing compounds MI-09 (**74**)¹⁹⁷ and UAWJ9-36-3 (**76**)²⁰⁰ or the cyclopenta[c]pyrrole-bearing inhibitors MI-30 (**75**)¹⁹⁷ and UAWJ-9-36-1 (**77**)²⁰⁰ also stemmed from this “structural lock” idea. Of note is that both **74** and **75** displayed *in vivo* antiviral effects as well.¹⁹⁷ The α -amido ketones MG-78 (**78a**) and MG-131 (**78b**) were designed according to similar lines and are very good inhibitors of SARS-CoV-2-M^{pro} as well.²⁹⁵ A recent patent is also claiming α -ketoamide-bearing derivatives in which this component is improving their cell permeability²⁹⁶ and SARS-CoV-2-M^{pro} structures binding such compounds have been released (PDB 7U92 and 7WQK). The notable cyclopenta[c]pyrrole-bearing SARS-CoV-2 M^{pro} inhibitor RAY1216/Leritrelvir (**79**) was developed by Raynovent^{297,298} and granted conditional market approval in China. Interestingly, this strong inhibitor also displayed an improved *in vivo* half-life in comparison with Nirmatrelvir (**73**) and appears to have been evaluated on COVID-19 patients with and without ritonavir or other P4503A4 inhibitors (NCT05620160).^{299,300} A structure of SARS-CoV-2 M^{pro} binding this compound has also been released (PDB 8IGN). The design and study of a related series of aldehyde-bearing compounds featuring various conformational locks actually reported that the cyclopenta[c]pyrrole was amongst the most efficient pan-corona proteases inhibitor *in cellulo*.³⁰¹ The dithia-7-azaspiro[4.4]nonane derivative (**80**) is a strong SARS-CoV-2 M^{pro} inhibitor which was developed by Sincere Pharmaceutical. In association with ritonavir,³⁰² it has also been granted conditional market approval in China under the name Xiannuoxin/Simnotrelvir^{303,304} and the results of a phase 1 clinical trials (NCT05339646) were reported recently.³⁰⁵ The undisclosed SARS-CoV-2 M^{pro} inhibitors ALG-097558 is developed by c, possibly without a co-administration of ritonavir and phase 1 clinical trial have been initiated (NCT05840952).²⁵ Three distinct patents,^{229,306,307} from this company and the catholic university of Leuven, are describing a large number of compounds featuring a variety of conformational lock. We (randomly) choose to depict the 5-azaspiro[bicyclo[2.2.1]heptane-2,1'-cyclopropane] derivative **81** since this strong SARS-CoV-2 M^{pro} inhibitor also illustrates what MedChem is about: innovation in chemistry leading to original and thus patentable compounds. The simpler azabicyclo[2.2.1]heptane ring system has also been introduced in the structure of such inhibitors.³⁰⁸ Further insights were provided in the course of an extensive study of boceprevir analogues which reports some key elements to achieve an antiviral effect in cellulo.³⁰⁹ Moreover, the same research group recently reported azaspiro[4.4]nonane derivative MPI-60 (**82**), which displays promising antiviral properties³¹⁰ and a structure of SARS-CoV-2 M^{pro} binding this compound was obtained (8STZ). Finally, the randomly chosen azaspiro[2,4]heptane derivative **83** described in a patent is again a tribute to the creativity required in MedChem³¹¹ and this ring system (and others) were also described in a more recent paper.³¹² Moreover, many strongly effective azapeptides, including some α,α -chlorofluoroacetamide-containing

derivatives²⁸⁴ related to **70** and **71** and featuring such conformational locks have been claimed.³¹³ Finally, an undisclosed prodrug (STI-1558/OVYDSO/Olgotrelviris) leading to AC1115, a covalent inhibitor of SARS-CoV-2-M^{pro} and cathepsin L³¹⁴ is currently undergoing phase 3 clinical trials, without the recourse to ritonavir. These trials are sponsored by Sorrento therapeutics/Zhejiang ACEA Pharmaceutical (NCT05716425 and NCT06044233) and the corresponding patent is claiming a lot SARS-CoV-2-M^{pro} inhibitors featuring structural elements seen in compounds **62-83** including some bisulfite-containing indole derivatives.³¹⁵

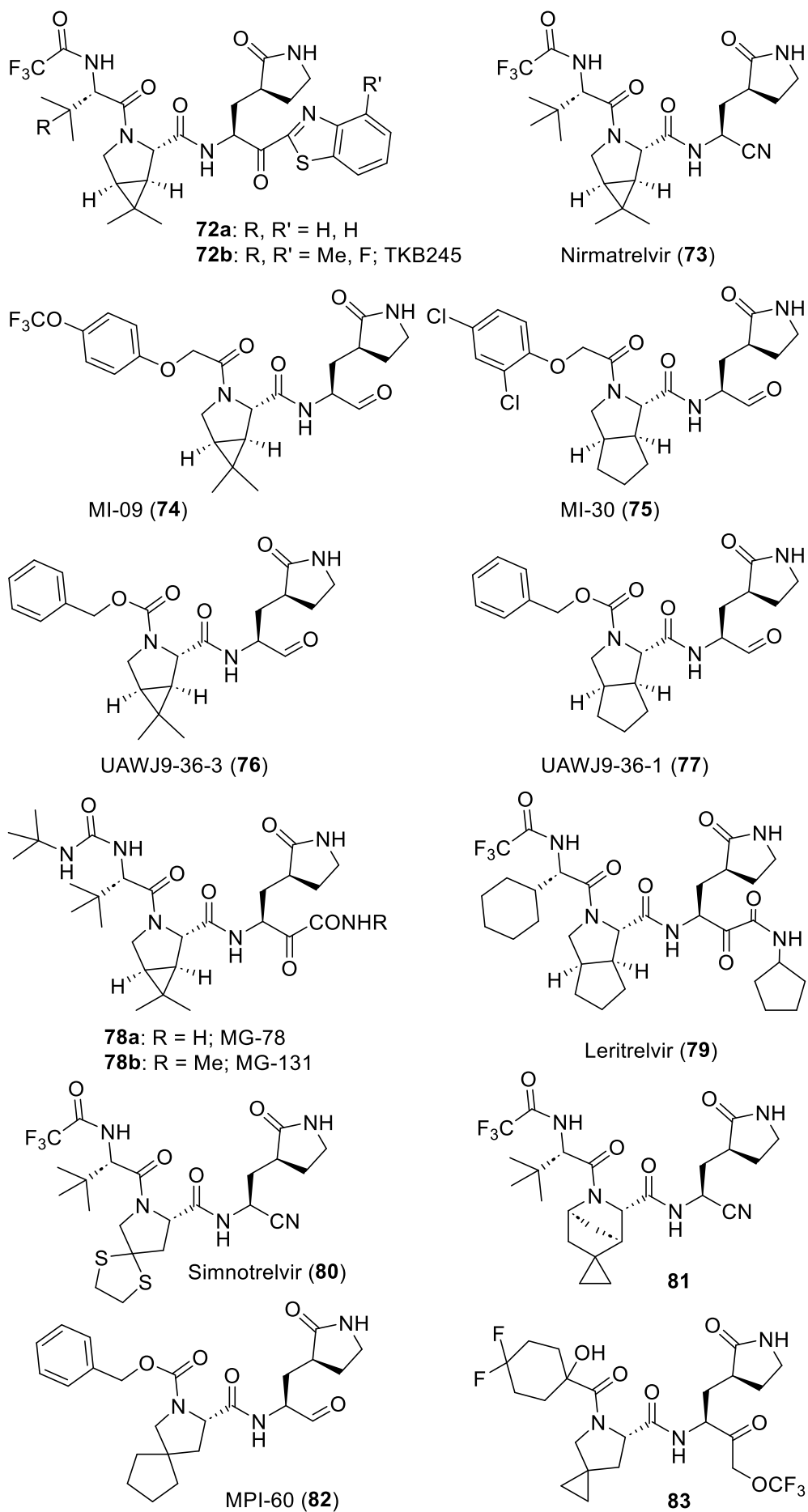


Figure 11: Structures of compound **72-83**.

Also of interest, the SARS-CoV-2 M^{pro} inhibitor EDP-235 (**85**) from Enanta Pharmaceuticals³¹⁶ recently met the primary end point of a phase 2 clinical trial without the coadministration of ritonavir (NCT05616728). In this case, 13 patents from this company are claiming a very large variety of peptides sometime featuring conformational locks related to the one depicted above. Amongst them, compound **84**³¹⁷ is not only featuring a cyclopenta[c]pyrrole in its structure but its warhead is a cyanopyrrolidine, reminiscent of Vildagliptin (**20**) or Saxagliptin (**21**). This cyanopyrrolidine is also rigidly connected to an indolinone which acts as the bioisoster replacement of the glutamine. Many more compounds featuring such unprecedented spiropyrrolidine along with a variety of warheads, were claimed. Amongst them EDP-235 (**85**),³¹⁸ which is also the subject of two process patents for its large-scale production,^{319,320} or compound **86**³²¹ both of which are noteworthy for their N-methyls. The NH homologs of compound **85** and **86** were also made but their antiviral effects were apparently not deemed of sufficient interest to be mentioned in the corresponding patents.^{318,321} Interestingly, the structurally very constrained macrocycle **87**³²² as well as the simplified compound **88**³²³ and **89**³²⁴ were also patented as effective inhibitors of SARS-CoV-2 M^{pro}.

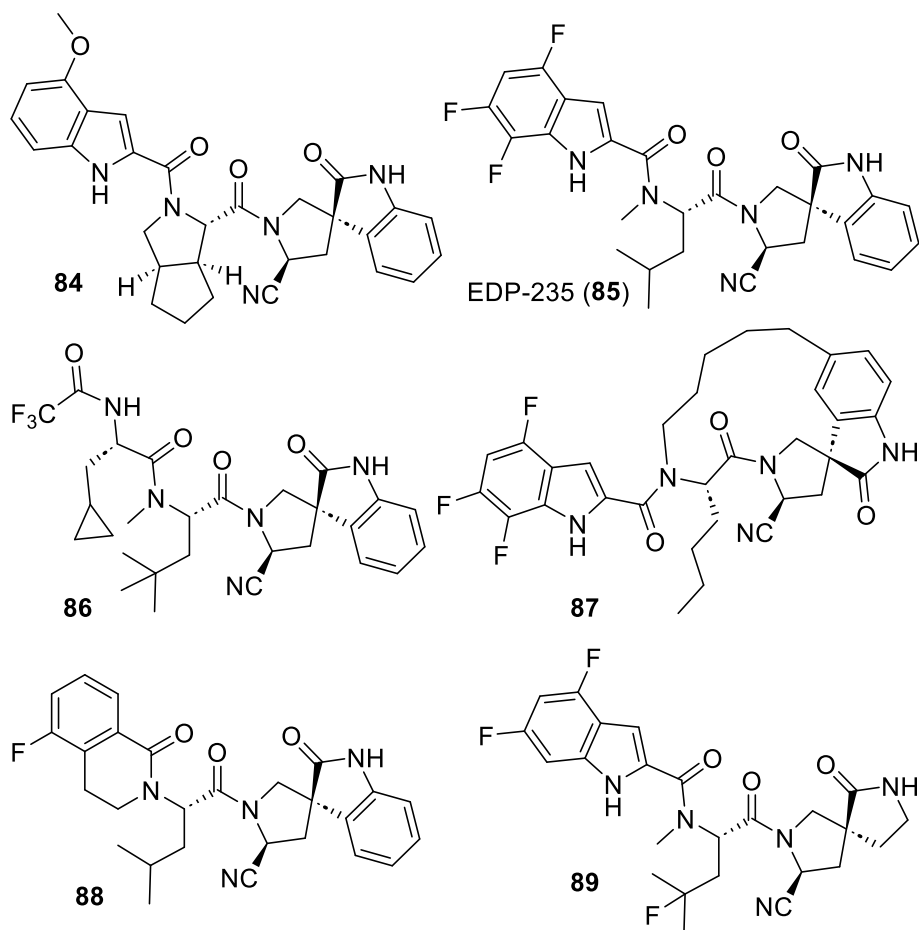


Figure 12: Structures of compound **84-89**.

The research of inhibitors of rhinovirus 3C protease conducted at Agouron/Pfizer at the turn of the century, which provided rupintrivir (**44**), also led to 2-pyridone-bearing analogues of this peptide.³²⁵⁻³²⁷ Amongst them, the orally available inhibitor AG7404 (**90**) underwent two phase 1 clinical trials,³²⁸ the latter, under the drug name V7404, was sponsored by ViroDefense and focused on enterovirus infections.³²⁹ As for rupintrivir (**44**), this alkyne-

bearing compound was reported in 2022 to modestly inhibit SARS-CoV-1 and SARS-CoV-2 main proteases and it was co-crystallized with both enzymes (PDB 7ZQW and 7ZQV).³³⁰ By following this approach, which consists in rigidifying the P2-P3 amide bond of the protease substrate, the pyridinone derivative (**91**) was designed and reported to inhibit SARS-CoV-2 M^{pro}. Moreover, this compound demonstrated an effect on cell-based virus replication assays.²¹² Interestingly, it was shown that the (*S*) enantiomer depicted here was the most active although the corresponding (*R*) enantiomer also had a degree of effect. In fact, even if the (*R*) isomer was 50 times less efficient as a protease inhibitor, both isomers co-crystallized within the SARS-CoV-2 M^{pro} catalytic site (PDB 8A4T and 8A4Q) with rather drastically different binding modes.²¹⁴ This probably illustrates the unselectivity bias induced by a reactive component in a given molecule. Indeed, if such compound can reach the nucleophilic part of the biochemical target, it is reasonable to assume that it will react with it, in spite of having a low overall affinity for the binding site. This known bias actually led in 2011 to the following comment: “the stringent substrate specificity of the SARS-CoV M^{pro} with respect to the P1 and P2 positions can be overruled by the highly electrophilic character of the aldehyde warhead. This constitutes a deviation from the dogma that peptidic protease inhibitors should comprise an amino-acid sequence corresponding to the cleavage specificity of the target enzyme.”³³¹ This comment is also likely valid for a number of aldehyde-bearing compounds which have been reported to be inhibitors of SARS-CoV-2 M^{pro}.^{91,196,332} In any case, a patent is describing further structure-activity relationship studies around such pyridone derivatives which led to the modest SARS-CoV-2 M^{pro} inhibitor **92**.³³³ In a 2013 patent, an array of inhibitors, such as the constrained azapeptide **93** featuring a rigidifying 2,6-dioxopiperidine component,³³⁴ or macrocyclic-bearing peptides such as **94**,³³⁵ were claimed for their effects on 3CL proteases of picornavirus, caliciviruses and coronaviruses. The related 3-amino-6-oxopiperidine spacer was also employed more recently and for instance, compound **95** turned out to be an effective SARS-CoV-2 M^{pro} inhibitor.³³⁶

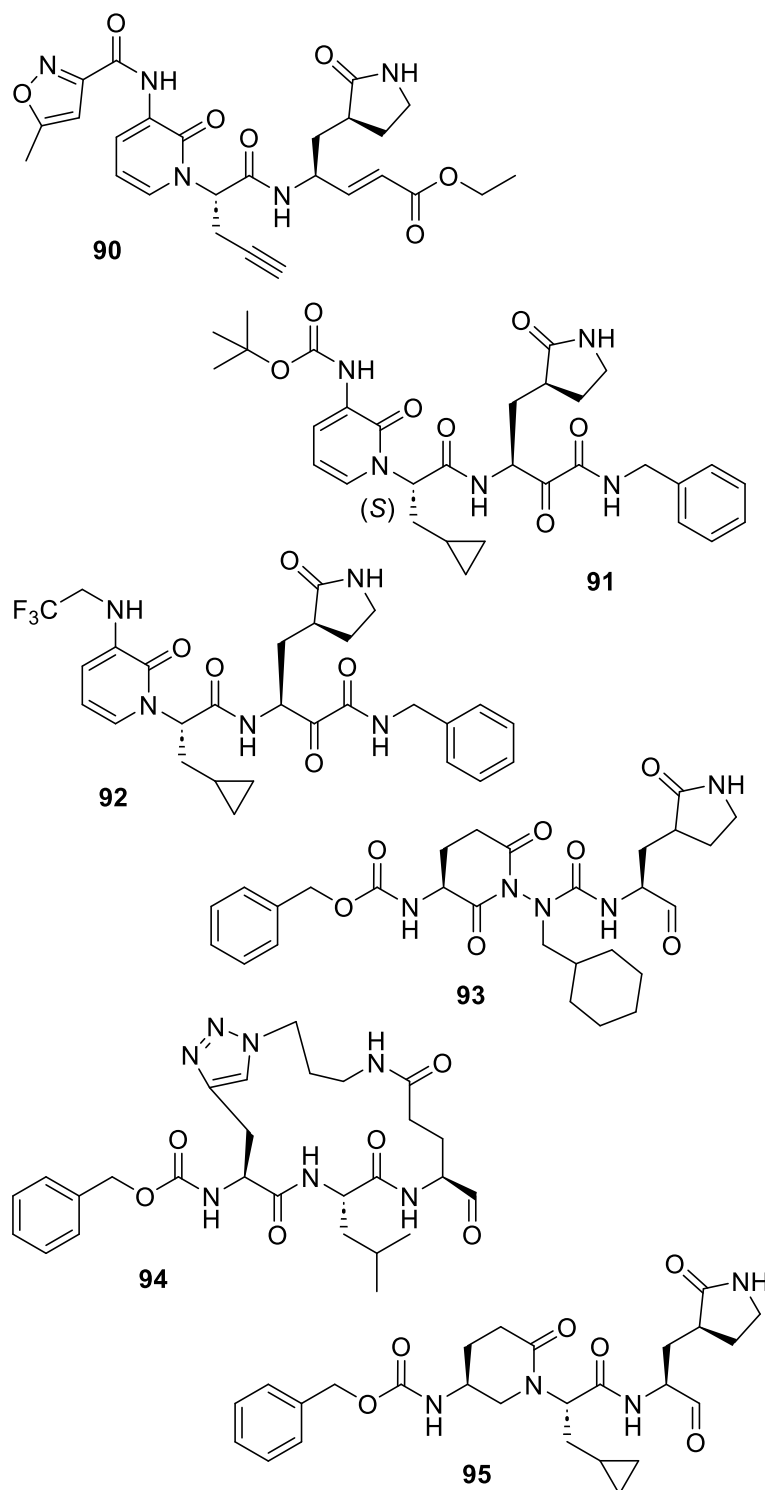


Figure 13: Structures of compound **90-95**.

Amongst SARS-CoV-1 M^{pro} covalent inhibitors of lesser interest, many 3-pyridyl esters with the general formula **96** were reported between 2004 and 2008.³³⁷⁻³⁴⁰ It was then demonstrated³³⁸ by mass spectroscopy that these aryl esters (unsurprisingly) react with the cysteine of the protease. Later on, some compounds were shown to also strongly inhibit SARS-CoV-2 M^{pro} but again these had a very modest antiviral effect.^{194,206,341} A more recent report provided an extensive demonstration of their chemical reactivity and further established that these compounds were probably the focus of too much attention in view of their lack of potential in medicinal chemistry.³⁴² Another recent report is actually describing covalent inhibitors in which the activated carboxyl was

replaced by a sulfone conjugated to a reactive oxadiazole ring system,³⁴³ and carmofur analogues also featuring an activated carboxamide side chain were reported.³⁴⁴ Moreover, somehow related thioesters have been reported to similarly react with the catalytic cysteine and displayed rather strong *in cellulo* antiviral effects.³⁴⁵ It remains to be seen if such compounds will retain some stability *in vivo*. To address this issue, an attempt was made in 2008 to replace the ester function of compounds **96** by a ketone, as seen in the structure of analogue **97**, but the results were disappointing.³⁴⁶ More interestingly, in 2013, a high throughput screening for inhibitors of SARS-CoV-1 M^{pro}, using the 293,000 compounds of the NIH molecular library, discovered more elaborated 3-pyridyl-bearing compounds such as ML188 (**98**) or benzotriazole derivatives such as ML300 (**99**).^{158,347} One of the reports also included a X-ray based structure of ML188 (**98**) bound to SARS-CoV-1 M^{pro} (PDB 3V3M),¹⁵⁸ and much later on, both inhibitors were co-crystallized with SARS-CoV-2 M^{pro} (PDB 7L0D³⁴⁸ PDB 7LME³⁴⁹). Of note is that these inhibitors are binding the catalytic site via key interactions between the imidazole ring of SARS-CoV-2 M^{pro} histidine-163 and their pyridine or benzotriazole nitrogen. Moreover, for these inhibitors the protease glutamine-166 NH is usually interacting with their recurrent carboxamide functions situated four “bonds away” from these nitrogens. As illustrated below this pattern is recurrent in most of the 3-pyridyl-bearing inhibitors of SARS-CoV main proteases.

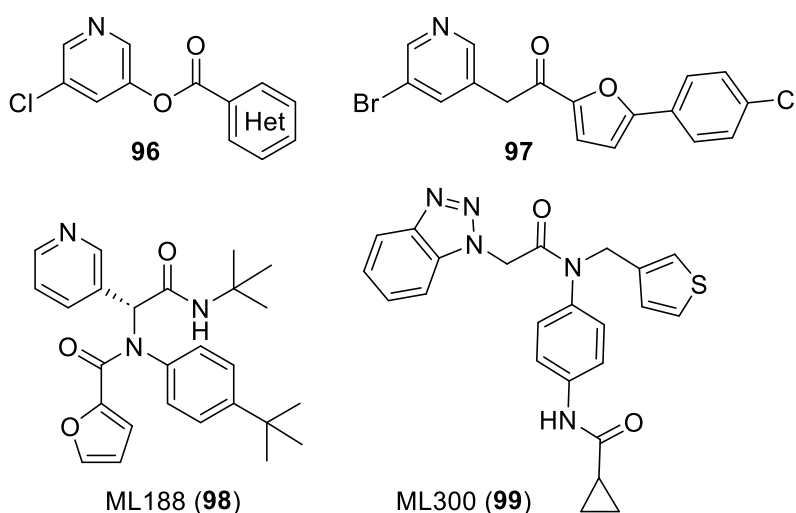


Figure 14: Structures of compound **96-99**.

By the end of 2019, these 2013 results became the starting point for research projects in many laboratories. In full collaboration with a large X-ray based facility, many structures of 3-pyridyl-bearing compounds bound to SARS-CoV-2 M^{pro} were thus made available.⁹³ Such structures were instrumental for the open science COVID Moonshot initiative which undertook a publicly available drug discovery project based on the iterative process required to improve 3-pyridyl-bearing compounds such as ML188 (**98**).³⁵⁰⁻³⁵⁴ As described,^{355,356} in one attempt, the COVID Moonshot initiative went from 3-pyridyl derivative TRY-UNI-714a760b-6 (**100**) to isoquinoline derivatives such as ADA-UCB-6c2cb422-1 (**101**), MAT-POS-b3e365b9-1 (**102**) or PET-UNK-29afea89-2 (**103**). In fact, these compounds became more or more elaborated not only to strengthen their affinity for SARS-CoV-2

M^{Pro} catalytic but also to start to address the myriad of problems associated with an eventual *in vivo* use. The next two inhibitors MAT-POS-4223bc15-23 (**104**) and VLA-UCB-29506327-1 (**105**), are illustrating some more possibilities for further iterations which required extensive profiling before choices were made. Meanwhile, another group also reported some related inhibitors using a docking-based virtual screen along with an X-ray based structure of the derivative X77 (**112**) bound to SARS-CoV-2 M^{Pro} (PDB 6W63). Interestingly, from the 3-pyridyl derivative **106** discovered with this strategy, the ensuing optimization led to the 300-fold more active inhibitor **107**.³⁵⁷ Moreover, out of a virtual screening using an X-ray based structure (PDB 6Y2G) of SARS-CoV-2 M^{Pro} covalently bound to the α -ketoamide inhibitor **91** depicted above, another research group also reported many (modest) hydantoin-bearing inhibitors related to compounds **105-107**.⁹¹

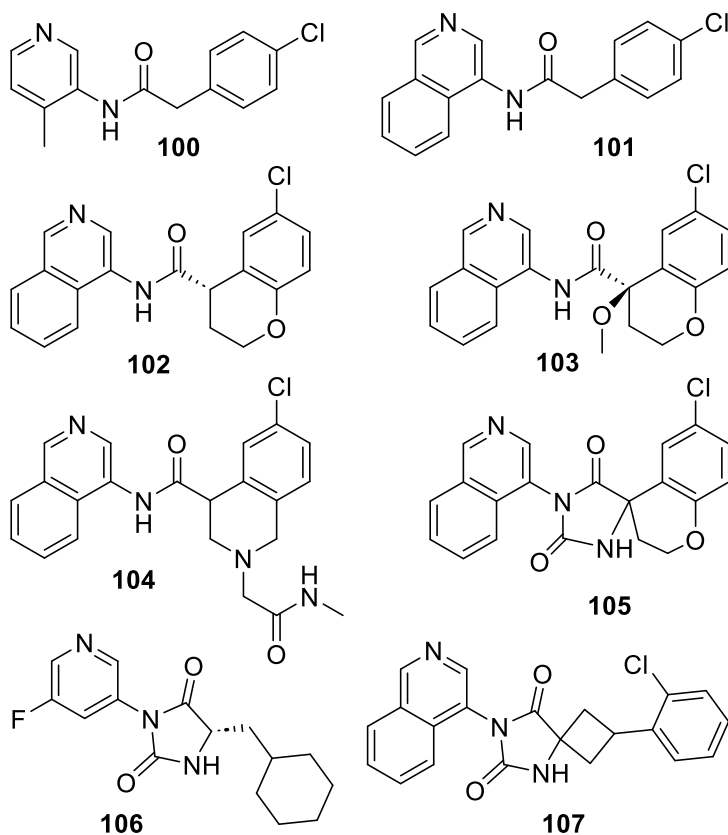


Figure 15: Structures of compound **100-107**.

Starting from the less deconstructed ML188 (**98**), the analogues **108** and **109**,³⁵⁸ or the related and MAT-POS-f2460aef-1 (**110**),³⁵³ explored some other possibilities and the more elaborated pyrazine-bearing analogue **111** was also reported.³⁵⁹ The design and synthesis of the analogue X77 (**112**) was never actually reported but this compound was co-crystallized with SARS-CoV-2 M^{Pro} in 2020 and the corresponding X-ray based structure (PDB 6W63) has been repeatedly used for virtual-based approaches. Finally, a search starting with DNA-encoded chemical libraries led to the very strong SARS-CoV-2 M^{Pro} inhibitor **113**. As demonstrated by X-ray (PDB 7UR9), the isoquinoline moiety of this compound do bind the protease catalytic site the same way the series depicted above are (via a hydrogen bond with His-163). However, in one instance in this series, replacing this isoquinoline by a pyridine led to a rather unexpected complete loss of activity.³⁶⁰

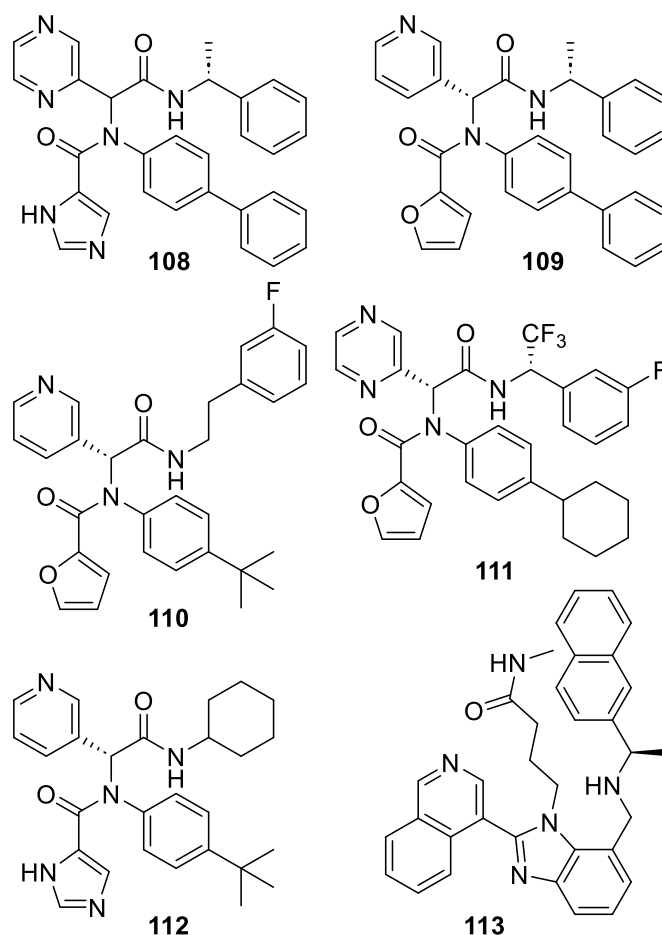


Figure 16: Structures of compound **108-113**.

Finally, many research groups resorted to the incorporation of a reactive component on these series.¹⁸⁴ As depicted, an acrylamide was used for compound **114**,³⁶¹ and an α,α -dichloroketone for compound **115**.²²¹ Of note, is the recourse the α,α -chlorofluoroacetamide moiety seen in the structures of compound **70** and **71** was claimed for these series as well³⁶² and an X-ray based structure of SARS-CoV-2 M^{PRO} covalently bound to the notable Jun10-90-3-C1 (**116**) has been released by another research group (PDB 8D4P). Out of two large patents from Pardes Biosciences, the randomly chosen α -aminonitrile **117**, or even more reactive 1-aminonitriles, are additional illustration of this approach.^{363,364} A report is describing a quite systematic introduction of an array of electrophilic moiety leading for instance to the chloroamide **118**.³⁶⁵ Even more reactive analogues are described in another publication¹⁸⁴ and in a patent.³⁶² Also of interest is the rather large α -ketoamide derivative **119** which turned out to be an orally available inhibitor of SARS-CoV-2 M^{PRO} endowed with a degree of antiviral effect *in vivo*.³⁶⁶ In another original approach, two patents from Novartis are claiming compounds such as the very strong inhibitors **120**³⁶⁷ or **121** both combining quite elegantly a reactive nitrile and structural features seen in this class. Concerning the series encompassing compound **121**, many analogues claimed in the patent³⁶⁸ do not feature a reactive moiety. Finally, the incorporation of a probably less reactive and hindered nitrile as seen in the structure of MAT-POS-e194df51 (**122**),^{353,355} or the more constrained MIK-ENA-5d9157e9 (**123**), provided some of the most advanced inhibitors of the remarkable COVID Moonshot initiative.³⁵⁶ In fact, compound **122** does not bind

covalently to SARS-CoV-2 M^{pro} as demonstrated by the X-ray based structure code P1788 accessible on the Fragalysis website.³⁶⁹

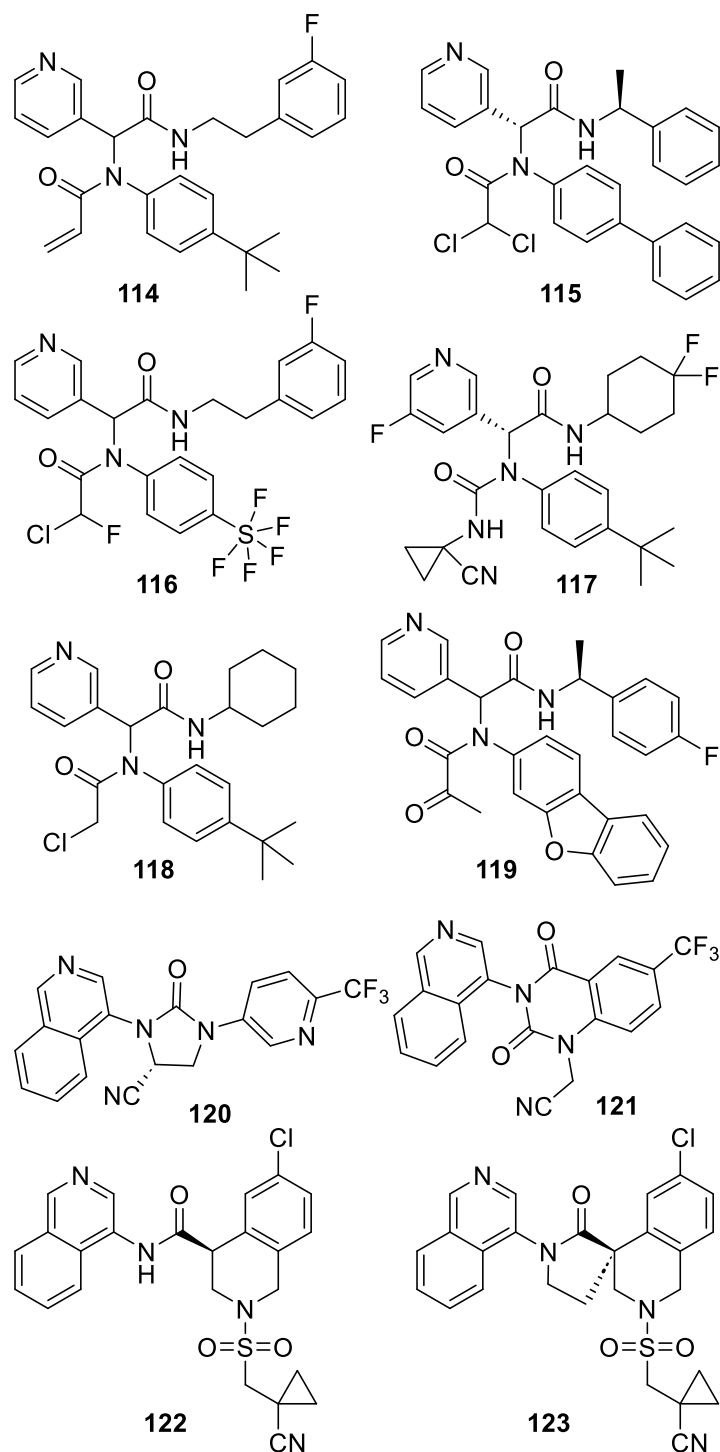


Figure 17: Structures of compound 114-123.

Interestingly, another class of isoquinoline-bearing inhibitors of SARS-CoV-2 M^{pro} (in which this heterocycle is also interacting with His-163, see PDB 7EN8) was found with the combined use of a DNA encoded chemical library and docking-based ranking. This resulted in WU-04 (**124**) which feature a so far required nitro group (for its notable interaction with the backbone CO of the protease Arg188) and is effective *in vivo* on a mice model of infection.³⁷⁰ Similar series of nitro-bearing compounds were the focus of extensive structure activity relationship

studies by another research group and are, so far, only illustrated by a recent patent from Qilu pharmaceuticals.³⁷¹ Moreover, a clinical trials of the undisclosed QLS1128 has been initiated by this company (NCT05458076). The benzotriazole-bearing inhibitor **99** reported³⁴⁷ in 2013 was also the focus of some more research. The MedChem efforts of the COVID Moonshot initiative led to improved analogues such as ALP-POS-6d04362c-2 (**125**).³⁵³ The imidazole-bearing analogue CCF981 (**126a**) was the fruit of extended SAR although this work also pointed out that a strong cytochrome P450 inhibition appeared to be a recurrent issue for these benzotriazole derivatives.³⁴⁹ Another report describe the triazole replacement by a 3-pyridyl moiety, as seen in the structure of compound **127**, which led to a less efficient inhibitor in comparison with **126b**. But then, the same research group used an isoquinoline and this provided the powerful SARS-CoV-2 M^{pro} inhibitor **128**³⁷² and many more examples, patented by the Cleveland Clinic Foundation, are claiming a range of inhibitors featuring alternatives to this benzotriazole.^{373,374} A recent reports is also describing additional analogues such as compound **129** which is combining structural elements of compound **125** as well as a 5-hydroxyisoquinoline. The X-ray structure of this inhibitor bound to SARS-CoV-2 M^{pro} was obtained (PDB 8SXR) and showed that the isoquinoline nitrogen is also interacting with the histidine residue 163 of the protease.³⁷⁵ Many strong inhibitors, in which the usual lactams were replaced by a whole array of other bioisosters, were patented by the Global Health Drug Discovery Institute.³⁷⁶ This patent is illustrated by compound **130**, which combine peptidic elements and a 3-pyridyl moiety. Moreover, patents from Exscientia or Pardes are claiming related compounds featuring even more varied heterocycles acting as lactam/3-pyridyl bioisosters.^{377,378} It is also very likely that an undisclosed derivative of **130** is undergoing clinical trials, sponsored by Jiangsu Hansoh Pharmaceutical, under the name H-10517 (NCT05779579). Finally, a recent patent from Merck³⁷⁹ is claiming a whole array of inhibitors such as compound **131** which feature a remarkable difluorinated side chain instead of the recurrent lactam. In a way, with these last two compounds are featuring most of the hard-won lessons of many structure-activity relationship studies and *la boucle est bouclée* (the circle is complete). From them it is actually tempting to suggest to alter the non-covalent 3-pyridyl bearing series of inhibitors by replacing their 3-pyridyl with all the other group described although it may be a real chemical challenge in some instances.

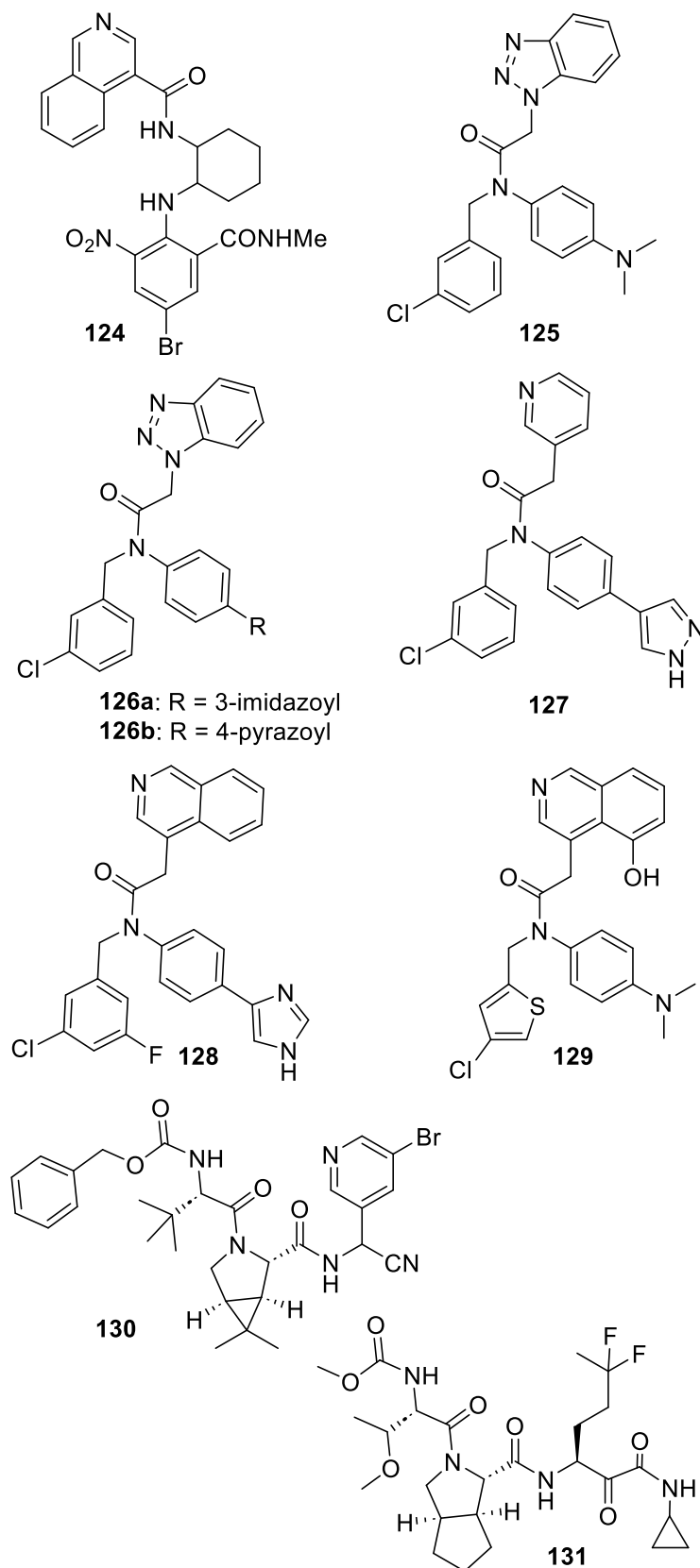


Figure 18: Structures of compound **124-131**.

- Original SARS-CoV-2-M^{pro} inhibitors

Aside from all the inhibitors described above, which are arguably continuations of the research made on the inhibition of various viral protease, including SARS-CoV-1 M^{pro}, one original approach involved the generation of a large number of random peptidic sequences which were then assayed. If a similar but more virtual-based

approach failed in an early instance,³⁸⁰ this also led to all D-peptides^{381,382} or to macrocyclic 11-14-mer peptides featuring modified amino acids³⁸³ endowed with affinities for SARS-CoV-2 M^{pro} catalytic site. In the latter case, the corresponding X-ray based structure (PDB 7Z4S) may be of future use to deconstruct such rather large peptides into more drug-like compounds. Concerning smaller compounds, a docking-based approach, using half a million bioactive compounds and the structure of SARS-CoV-2 M^{pro} binding the peptide derivative **47** (PDB 6LU7), had to be followed by more virtual-based selections, taking into account known inhibitors. This led to the very modest inhibitor **132** featuring a pyrimidinetrione component. A sulfamide derivative was also discovered by the same group but has yet to be developed.³⁸⁴ The related pyrimidinedione **133** was the result of another virtual screening of 6.5 million compounds commercially available using an array of X-ray based structure of SARS-CoV-2 M^{pro} binding various inhibitors (compounds **47**, X77 (**112**), telaprevir (**25**) and masitinib (**37**); see PDB 7BQY, 6LU7, 6W63, 7C7P, and 7JU7).³⁸⁵ Following this achievement, an X-ray based structure of pyrimidinedione **133** bound to SARS-CoV-2 M^{pro} was obtained and led to a medicinal chemistry program which has so far provided only modest results.³⁸⁶ Interestingly, another hit to lead project also started with compound **133** and led to the improved GC-14 (**134**) in which the pyrimidinedione was replaced by the recurrent 3-pyridyl component which interacts with the SARS-CoV-2 M^{pro} His-163 residue (PDB 8ACL). A replacement with an isoquinoline instead led to the same level of inhibition.³⁸⁷ Unexpectedly, adding instead an α -chloroketone warhead, as depicted for compound **135**, had a deleterious impact on the antiviral effect as compared with GC-14 (**134**).³⁸⁸ However, only a modest antiviral effect was observed *in cellulo*, for compound **134** possibly because of a low cell membrane permeability. From the non-covalent pyridyl-bearing inhibitors listed above, a computer-based “synthesis-directed de novo design model” provided the non-obvious quinolone **136** which has a degree of effect on the SARS-CoV-2 M^{pro} as well as on the seasonal OC43 coronavirus.³⁸⁹ Interestingly, the related inhibitor JZD-07 (**137**) which gather features found in **133** and **136** was reported later. In this case, it is the quinolone oxygen which is the bioisosteric replacement for the 3-pyridyl nitrogen (PDB 8GTV) and this inhibitor is endowed with an *in vivo* efficacy against a mice model of SARS-CoV-2 infection.³⁹⁰ A related analogue (JZD-26) has been co-crystallized with SARS-CoV-2 M^{pro} (PDB 8GTW) although a corresponding publication is still expected. The COVID Moonshot initiative also explored related quinolones such as the less rigid but rather modest inhibitor MAT-POS-3b536971-1 (**138**).³⁵³ Another series of 3-pyridyl-bearing inhibitor of SARS-CoV-2 M^{pro} such as the rather rigid compound **139** were also patented.³⁹¹

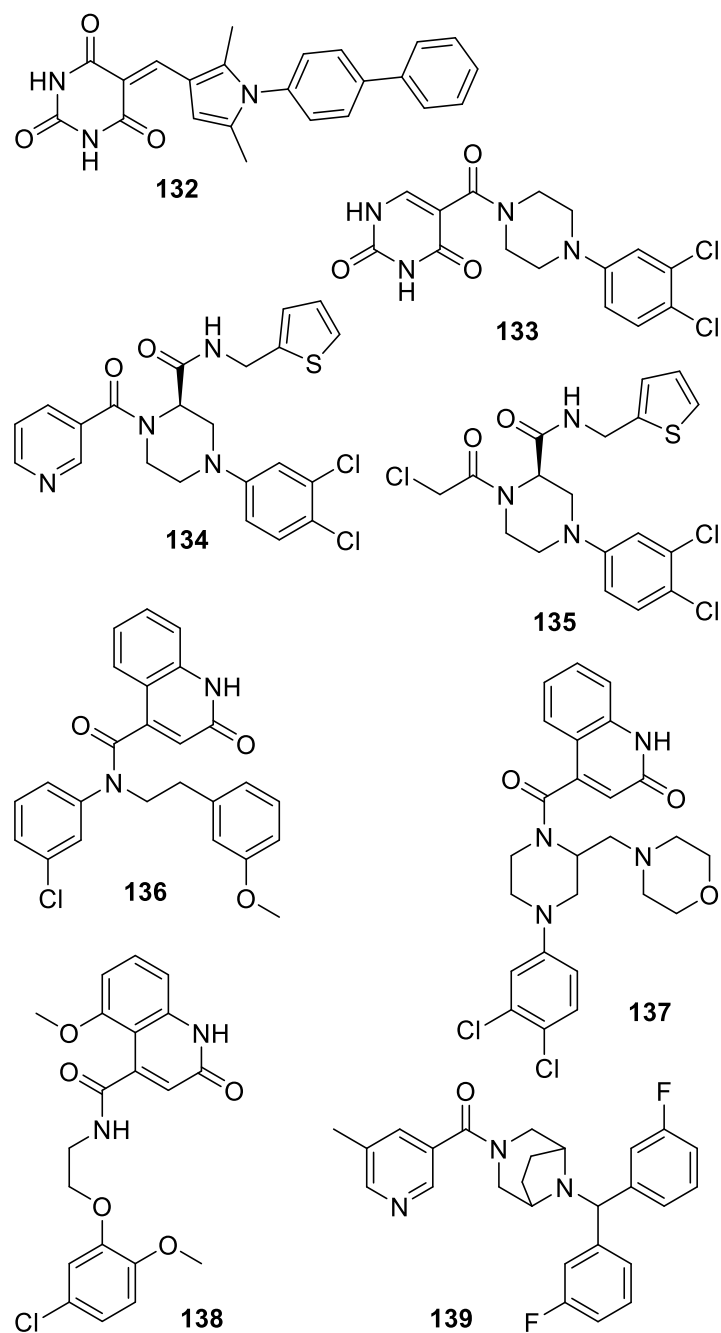


Figure 19: Structures of compound **132-139**.

Experimental screenings along with medicinal chemistry approaches were also used to search for SARS-CoV-2 M^{pro} binding inhibitors. The α -ketoamides **140**, its 2-pyridyl is actually in interaction with His-163 (PDB 7AEH),³⁹² and compound **141**³⁹³ were found in the course of a FRET-based screening of chemical library of 30.000 compounds. The ensuing structure-based (PDB 8HHT) hit to lead progression led to hundred-fold more active SARS-CoV-2 M^{pro} inhibitors such as compound SY110 (**142**).³⁹³ A remarkable feature of this original class of peptidic and covalent inhibitors is the absence of the lactam bioisoster of the glutamine residue seen in the many series depicted above. Earlier examples had actually been reported for a modest effect on SARS-CoV-1³⁹⁴ or SARS-CoV-2 multiplication although in the latter case these compounds were inhibiting other proteases and had anticancer properties as well.³⁹⁵ Since SY110 (**142**) displays a robust pan-coronavirus antiviral effects, an original binding mode to the catalytic site (PDB 8HHU), good preclinical characteristics and is effective in a

model animal of COVID-19 infection, further work on this class of inhibitors appears to be warranted. Another recent attempt focused on the variety of sequences found in the substates of SARS-CoV-2 M^{pro} including downstream of the cleavage site and the determination of X-ray based structures. This led to the recognition that the peptide Ala-Ile-PheOMe, derived from VKLQAIIFR a larger peptide which had been co-crystallized with SARS-CoV-2 M^{pro} (PDB 8GWS), retained a degree of affinity for the enzyme. Then, the addition of a warhead on this peptide and some structure-based improvements led to compounds such as the α -bromoacetamide **143** which is a modest inhibitor of SARS-CoV-2 M^{pro} and viral replication.³⁹⁶ It is possible that this original work along with the structure of **143** bound to the protease catalytic site reported (PDB 8JPQ) will pave the way for many research projects focusing on the discovery of non peptidic inhibitors targeting this previously unexplored pocket. The strong SARS-CoV-2 M^{pro} binding inhibitor and vinylsulfoxide-bearing PM-2-071 (**144**) was reported following a screen of 582 acrylamide or chloroacetamide-bearing compounds and some hit optimization.³⁹⁷ However, the issue of vinylsulfoxide-bearing compounds toxicity should be kept in mind for further development of this type of Michael acceptor.¹⁸⁴ The α -chloroamide-bearing benzodiazepine **145** was also found to be a modest inhibitor of SARS-CoV-2 M^{pro} following the screening of 5000 compounds. The ensuing hit progression has so far provided compound **146** with only a modest 10-fold improvement of activity, despite the obtention of an X-ray based structure (PDB 8JOP).³⁹⁸ Finally and to conclude this part, the truly original and authorized antiviral drug Ensitrelvir/S-217622 (**149**) was discovered in the course of a virtual-based selection using, again, the structure of SARS-CoV-2 M^{pro} binding X77 (**112**) (PDB 6W63).³⁹⁹ This selection from hundreds of thousand compounds of an in-house chemical library led to a smaller screening and the identification of compound **147** which had been made years before in the course of a research program focusing on the design of analgesics.⁴⁰⁰ An X-ray structure (PDB 7VTH) then revealed its binding mode and the ensuing hit to lead program proceeded, via compound **148**, to reach Ensitrelvir (**149**). Of note is that as seen by the X-ray based structure, it is the triazole ring which is in interaction with the His-163 residue for this class of inhibitors (PDB 8DZO).⁴⁰¹ In fact, closely related analogues featuring, amongst other heterocycles, the recurrent 3-pyridyl group instead were claimed later.⁴⁰² The clinical trials⁴⁰³⁻⁴⁰⁵ of Ensitrelvir (**149**), sponsored by Shionogi, turned out to be successful and this is so far the sole non-covalent SARS-CoV-2 M^{pro} inhibitor approved as an emergency treatment of COVID-19 in Japan. It is currently undergoing phase 3 clinical trials across the world (NCT05605093 and NCT05305547). Two more recent patents are focusing on analogues of Ensitrelvir (**149**), the first is claiming various deuterated analogs which may have improved pharmacological properties,⁴⁰⁶ the second undertook a rescaffolding of the core 1,3,5-triazine ring and is claiming analogs featuring a 1,2,4-triazine ring system instead.⁴⁰⁷

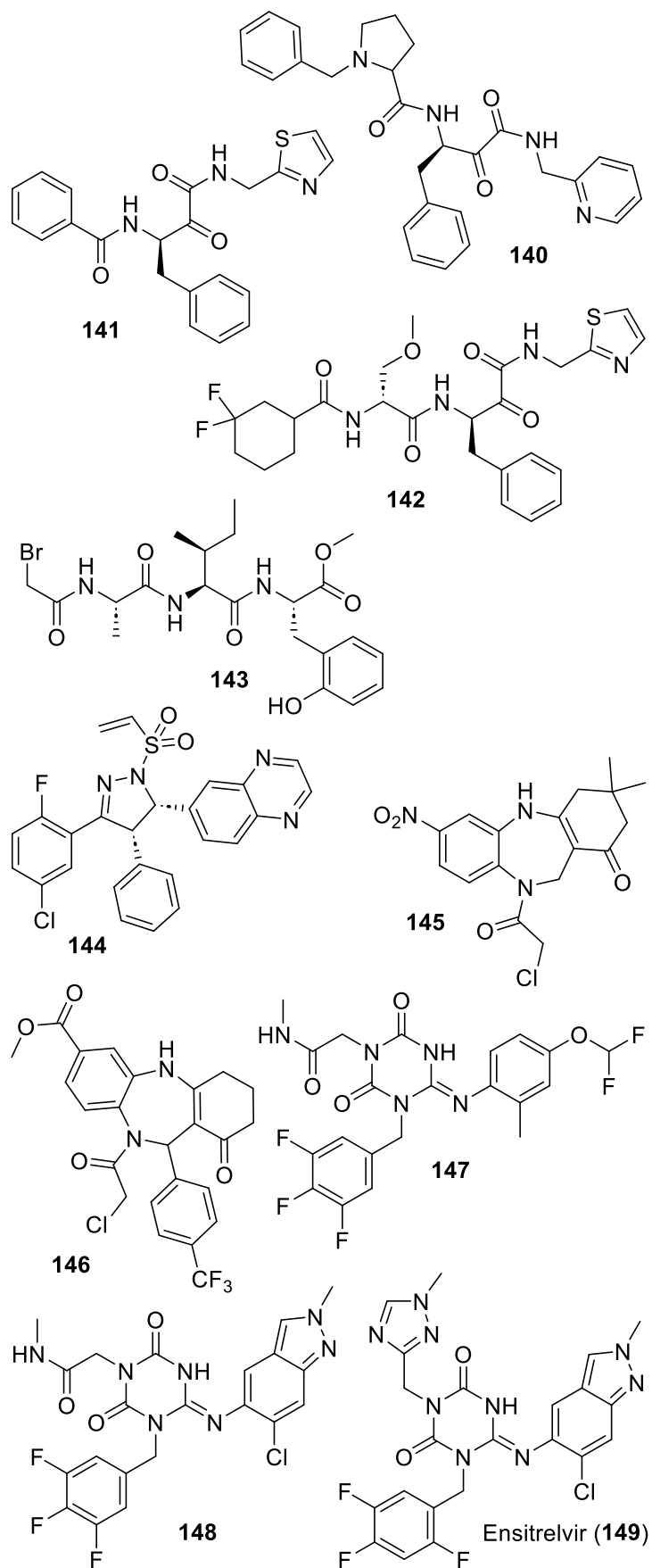
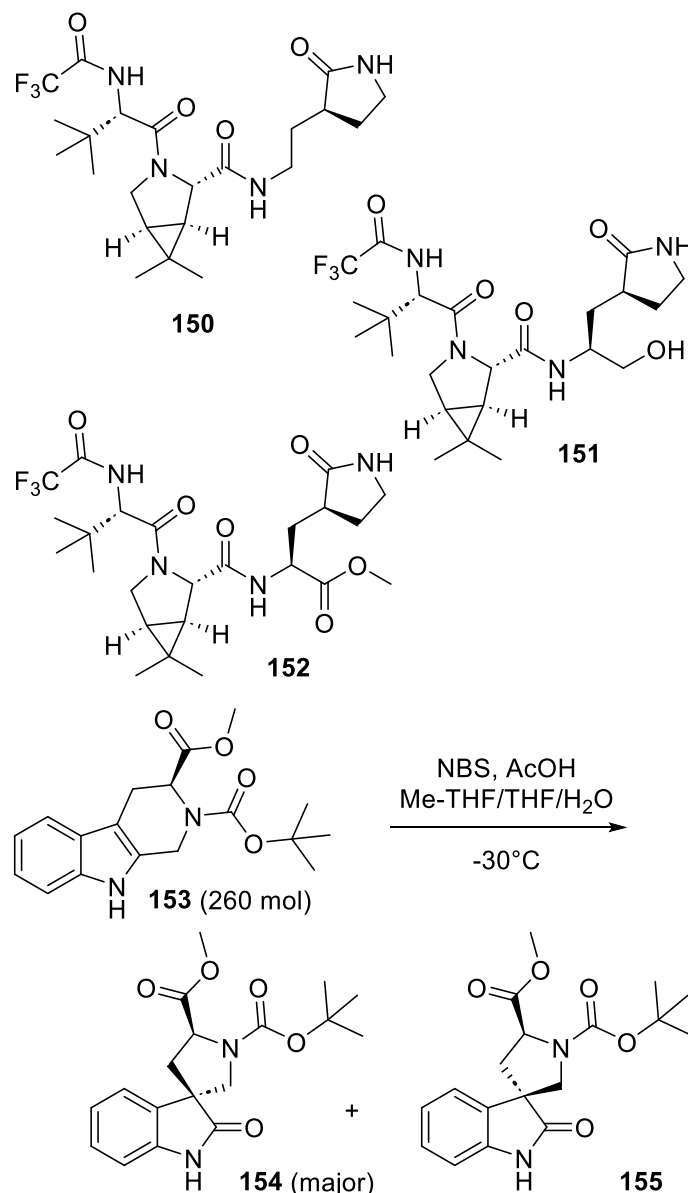


Figure 20: Structures of compound 140-149.

Conclusions

The list of chemicals described above is only representing a portion of all the compounds which have been evaluated around the world on many types of assays targeting SARS-CoV-2 M^{pro}. In spite of belief-based treatments (using natural or human-made compounds) which unfortunately mobilized a lot of money and claimed an unacceptable number of lives,¹⁵⁰ in spite of far too many unexploitable⁷¹ computer-involving reports (and patents) which also attracted much funds and raises acute questions on some authors, reviewers (if any), editors and publishers' integrity,⁷⁹ some approaches did deliver useful drugs. If drug repurposing was, as for the previous corona virus epidemics, an unmitigated and unfortunately predictable failure,^{151,152,408,409} past research results on various proteases as well as renewed screenings turned out to be successful starting points. Moreover, as probably not enough emphasized here, the staggering number of X-ray based structures solved greatly helped the resulting hit to lead progressions. A 2014 review on this subject does remain quite relevant.⁴² As described above, but for Ensitrelvir (**149**), all the most advanced or authorized inhibitors of SARS-CoV-2 M^{pro} owe their effect to the occurrence of a covalent bond with its catalytic cysteine. Interestingly, in a description of the invention of Nirmatrelvir (**73**),^{289,410} the choice of the chemically reactive component to be used (nitrile or the ketone function of analogue **72** in this case) remained a difficult one. This was the subject of some investigations^{193,411} especially in light of the metabolic and cytotoxicity concerns with aldehyde-bearing covalent inhibitors.²⁶⁸ In one instance,⁴¹¹ a systematic survey was made using Nirmatrelvir (**73**) structure as a template. From this work which provides precise biochemical and cell-based insights on warhead selection, another quite puzzling result emerged. In fact, if Nirmatrelvir (**73**) or the corresponding ketone-bearing analogue **72b** are a really effective inhibitor of SARS-CoV-2 M^{pro}, the peptides lacking these reactive moieties, such the decarboxylated derivative **150** or the alcohol **151** are devoid of inhibition effect. This experimental fact is in contrast with what is observed for the KRAS inhibitors **8** and **9** depicted in figure 2, since the analogues lacking their acrylamide moiety are still capable of a modest inhibition of this kinase. This suggest that, upon the chemical reaction between the warhead of covalent inhibitors and the cysteine of the SARS-CoV-2 M^{pro} catalytic site, a conformational shift occurs and the newly formed pocket can then bind some more elements of these inhibitors. Even the ester function of compound **152** which, once this compound settles in the catalytic site could react with the thiol function, does not. This suggests that **152** never binds to SARS-CoV-2 M^{pro} and this implies that before a reaction takes place with the cysteine thiol of SARS-CoV-2 M^{pro}, the catalytic site has a conformation devoid of affinity for the other components of these inhibitors. This could mean that ligands specific to this unknown site conformation have yet to be identified as such. At least one early report on the clustering of various inhibitors of SARS-CoV-2 M^{pro} according to the shape of the pocket binding them does point out a degree of flexibility.⁴¹² But to account for the complete lack of affinity of compounds **150-152** for SARS-CoV-2 M^{pro} catalytic site, one could suspect far larger conformational changes. Future will tell, but one way to detect and characterize such non-obvious conformations remains blind high throughput screenings for fully original and non-covalent inhibitors, followed by X-ray based structural studies of their interactions with the protease. On the chemistry point of view, many compounds described here are tributes to the creativity of organic chemists. Of note would the oxidative rearrangement of the tetrahydrocarboline **153** into the spiroprolidines **154** and **155**.³²⁰ As depicted in scheme 2, it is this synthetic

step which paved the way to the design and synthesis of EDP-235 (**85**) and led to 14 distinct patents claiming many spiropyrolidines series of SARS-CoV-2 M^{pro} inhibitors. Interestingly, this rearrangement⁴¹³ was previously used⁴¹⁴ in 1996 to prepare esters and nitrile derivatives somehow related to **154** and **155**. History will tell if it was their evaluations on SARS-CoV-2 M^{pro} which was at the source of these strong inhibitors.



Scheme 2: Structures of compound **150-152**, oxidative rearrangement of compound **153** into **154** and **155**.

Finally, in the following years some viral strains resistant to the currently available SARS-CoV-2 M^{pro} inhibitors will unfortunately emerge in the population⁴¹⁵⁻⁴²⁰ and these strains will likely compromise the corresponding drug efficacy. For that reason, as well as a price lowering effect, it is very important to have the widest possible range of efficient SARS-CoV-2 multiplication inhibitors so that a lack of cross-resistances between these drugs can be expected. In this regard, the possibility of a general/partial cross-resistance between all the covalent SARS-CoV-2 M^{pro} inhibitors currently used or developed today is a very relevant issue. In any case, any array of coronavirus-adapted antiviral drugs will be handy to address the next corona epidemic before specific vaccines are designed and mass-produced. Indeed, the recent major progresses made with RNA-based vaccines is challenging

MedChem and the much longer time usually required to provide a drug-based treatment. Past the next coronavirus pandemics, anticipating such challenges for other zoonotic diseases as well could be a good idea.

Conflicts of interest

There are no conflicts to declare

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