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Design and Synthesis of Serine and Aspartic Protease Inhibitors

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Abstract

This thesis describes the design and synthesis of compounds that are intended to inhibit serine and aspartic proteases. The first part of the text deals with preparation of inhibitors of the hepatitis C virus (HCV) NS3 serine protease. Hepatitis C is predominantly a chronic disease that afflicts about 170 million people worldwide. The NS3 protease, encoded by HCV, is essential for replication of the virus and has become one of the main targets when developing drugs to fight HCV. The inhibitors discussed here constitute surrogates for the widely used *N*-acyl-hydroxyproline isostere designated 4-hydroxy-cyclopentene. The stereochemistry of the 4-hydroxy-cyclopentene scaffold was determined by nuclear overhauser effect spectroscopy (NOESY) and the regiochemistry by heteronuclear multiple bond correlation (HMBC). The scaffold was decorated with different substituents to obtain both linear and macrocyclic HCV NS3 protease inhibitors that display low nanomolar activity. The second part of the thesis describes the design and synthesis of potential aspartic protease inhibitors. The hydroxyethylene motif was used as a noncleavable transition state isostere. The synthetic route yielded a pivotal intermediate with excellent stereochemical control, which was corroborated by NOESY experiments. This intermediate can be diversified with different substituents to furnish novel aspartic protease inhibitors.

1 List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I.** Synthesis of Novel Potent Hepatitis C Virus NS3 Protease Inhibitors. Discovery of 4-Hydroxy-cyclopent-2-ene-1,2-dicarboxylic Acid as a *N*-Acyl-L-Hydroxyproline Bioisostere.

Fredrik Thorstensson, Fredrik Wångsell, Ingemar Kvarnström, Lotta Vrang, Elizabeth Hamelink, Anders Hallberg, Åsa Rosenquist, Bertil Samuelsson.

Submitted to *Bioorganic & Medicinal Chemistry*

- II.** Potent Macrocyclic Inhibitors of the Hepatitis C Virus NS3 Protease Incorporating Cyclopentane and Cyclopentene Derived Scaffolds.

Marcus Bäck, Per-Ola Johansson, Fredrik Wångsell, Fredrik Thorstensson, Ingemar Kvarnström, Lotta Vrang, Elizabeth Hamelink, Anders Hallberg, Åsa Rosenquist, Bertil Samuelsson.

In manuscript

2 Abbreviations

AcOH	acetic acid
AIBN	azobisisobutyronitrile
BACE	β -site-APP-cleaving enzyme
BF ₃ OEt ₂	boron trifluoride etherate
BnBr	benzyl bromide
Boc	<i>tert</i> -butyloxycarbonyl
Boc ₂ O	di- <i>tert</i> -butyl dicarbonate
BOP-Cl	bis-(2-oxo-3-oxazolidinyl)-phosphinic-chloride
Bu ₃ SnO	dibutyltin oxide
Cat D	cathepsin D
DCC	dicyclohexyl carbodiimide
DCM	dichloromethane
DIAD	diisopropyl azodicarboxylate
DIPEA	<i>N, N'</i> -diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	<i>N, N</i> -dimethylformamide
DPPA	diphenylphosphoryl azide
EC ₅₀	concentration of drug required to induce 50% of the maximum possible effect in a cell culture system
ER	endoplasmic reticulum
EtOAc	ethyl acetate
EtOH	ethanol
Fmoc	9-fluorenylmethyl carbamate
Fmoc-ONSu	<i>N</i> -(9-fluorenylmethoxycarbonyloxy)-succinimide
HATU	<i>O</i> -(7-azabenzotriazol-1-yl)- <i>N, N, N', N'</i> -tetramethyluronium hexafluorophosphate
HCV	hepatitis C virus
HE	hydroxyethylene
HEA	hydroxyethylamine
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple bond correlation
HOBt	1-hydroxy benzotriazole
HPLC	high performance liquid chromatography
IC ₅₀	concentration of an inhibitor required to inhibit an enzyme by 50%
Im	imidazole
K_i	inhibitory or affinity constant; $K_i = [E][I]/[EI]$
LC/MS	liquid chromatography/mass spectrometry
LDA	lithium diisopropylamide

LDL	low density lipoprotein
Maldi-TOF	matrix assisted laser desorption/ionization-time of flight
<i>m</i> CPBA	<i>m</i> -chloroperbenzoic acid
MeOH	methanol
MMLV asp	moloney murine leukemia virus
MS	mass spectroscopy
NaOAc	sodium acetate
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
NTPAse	nucleoside triphosphate
PhSeBr	phenylselenyl bromide
PPh ₃	triphenylphosphine
<i>p</i> -TsOH	<i>para</i> -toluenesulfonic acid
r.t.	room temperature
RCM	ring-closing metathesis
RNA	ribonucleic acid
ROESY	rotational nuclear overhauser effect spectroscopy
RSV asp	rous sarcoma virus
SAR	structure-activity relationship
TBAB	tetrabutylammonium bromide
TBTA	<i>tert</i> -butyl-2,2,2- <i>tri</i> -chloroacetimidate
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TOCSY	total correlation spectroscopy
<i>Z</i>	benzyloxycarbonyl

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3 Introduction

3.1 Proteases

Proteases are enzymes that selectively catalyze the hydrolysis of polypeptide bonds. These enzymes play important roles in the human body, because they are involved in protein synthesis and they regulate numerous physiological processes, such as digestion, fertilization, immunological defense, growth, and coagulation.[1] Many proteases are also essential for propagation of diseases, and hence inhibition of different proteases is emerging as a promising approach in the treatment of hepatitis,[2] herpes,[3] various forms of cancer,[4] malaria,[5] and Alzheimer's disease.[6, 7] Most proteases are sequence-specific in that they hydrolyze a specific amide bond after a certain amino acid sequence.[1] The standard nomenclature for the residues in a protease/substrate complex was first proposed by Schechter and Berger, and it is now widely used to describe the sites in such a complex that are recognized by the protease (Figure 1).[8] The residues in the *N*-terminal direction from the amide bond that are cleaved (scissile bond) by the protease are called the nonprime side and are designated P_n ($n = 1, 2, \text{etc.}$). The residues in the *C*-terminal direction are referred to as the prime side and are labeled P_n' , and the corresponding subsites in the enzyme are designated S_n and S_n' for the *N*- and *C*-terminal direction, respectively.

Proteases are divided into four major classes (aspartic, cysteine, metallo, and serine proteases) based on their catalytic mechanisms, and each class has several subclasses.[9] Proteases in different subclasses are often very similar, thus when designing a protease inhibitor it is important to consider possible cross inhibition of other proteases in the same subclass.[10] The research

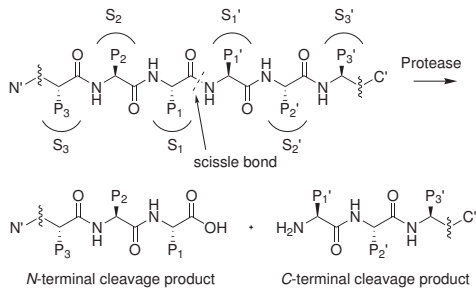


Figure 1: Schematic representation of the standard nomenclature of a protease/substrate complex and the products of proteolytic cleavage.

presented in this thesis focused on two classes of these enzymes: the serine proteases (Hepatitis C) and the aspartic proteases.

3.2 Serine Proteases

Three classes of serine proteases are known today, which are classified as trypsin-like, elastase-like, and chymotrypsin-like serine proteases according to the amino acid found in the P1 position of the substrate.[11] The active site of a serine protease consists of a catalytic triad that comprises closely situated Ser195, His57, and Asp102 (chymotrypsin numbering) and also an oxyanion hole (Gly193 and Ser195). The catalytic triad cleaves the amide bond, and the oxyanion hole stabilizes the tetrahedral intermediates that are formed during cleavage of the peptide bond in the substrate. The substrate binds to the active site to form a complex in which the scissile bond is in proximity to Ser195. The imidazole ring in His57 is activated by the deprotonated Asp102 and serves as a base catalyst by hydrogen bonding to the

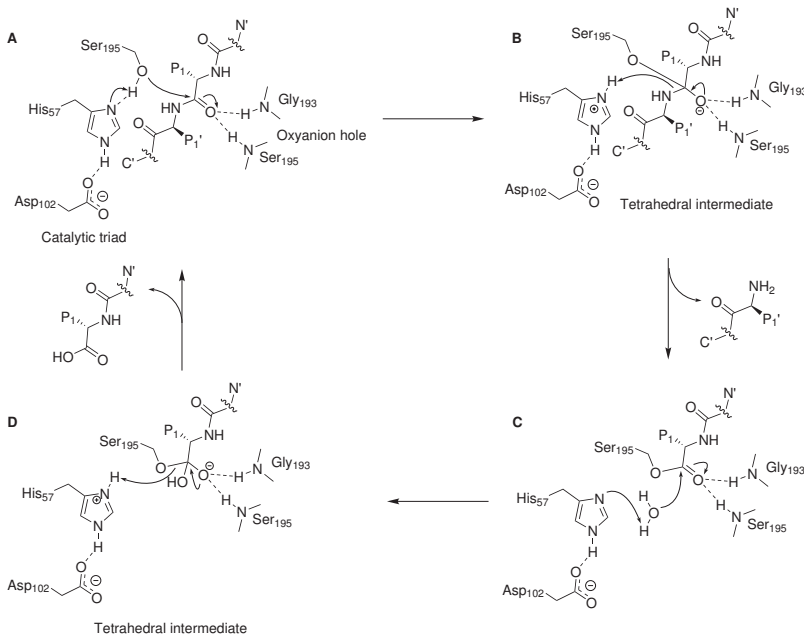


Figure 2: General catalytic mechanism of serine proteases.

hydroxyl group in Ser195. The carbonyl group of the scissile amide bond is more electrophilic due to the hydrogen bonding network in the oxyanion hole, and it undergoes a nucleophilic attack by the activated hydroxyl group in Ser195 (A, Figure 2). The resulting tetrahedral intermediate is stabilized by the hydrogen bonding network in the backbone of Ser195 and Gly193 in the oxyanion hole (B). Proton transfer from His57 to the amine in the tetrahedral intermediate facilitates expulsion of the amine fragment (*C*-terminal cleavage product) as leaving group, whereas the carboxylic part of the substrate remains covalently bound to the enzyme (C). A water molecule that is activated by the imidazole ring in His57 makes a nucleophilic attack on the acyl-enzyme complex, which results in a new tetrahedral intermediate (D). That intermediate subsequently collapses under the assistance of the protonated His57 as catalyst to release the carboxylic fragment of the substrate (*N*-terminal cleavage product). In doing so, the tetrahedral intermediate regenerates Ser195 (A), and the protease is then ready to hydrolyze another substrate.[1]

3.3 Aspartic Proteases

Aspartic proteases constitute a ubiquitous class of enzymes that use an Asp dyad to hydrolyze peptide bonds, and they have been studied and characterized extensively.[12, 13] Most of these proteases tend to bind to 6–10 peptide residues in the substrate, and this sequence can be used to design substrate-based inhibitors. Some aspartic proteases also have one or more flaps that close down and cover the inhibitor to give rise to further interactions in the protease-inhibitor complex.[1] Several mechanisms of action have been proposed for this protease family, but the most widely accepted is the acid-base

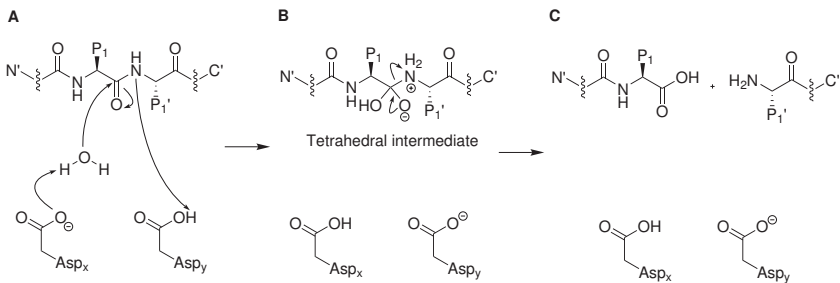


Figure 3: Catalytic mechanism of substrate hydrolysis by aspartic proteases.

system, depicted in Figure 3. It is generally believed that the water molecule is located between the aspartic residues in the active site, and when that molecule is partly activated by the deprotonated aspartic acid, it makes a nucleophilic attack at the carbonyl carbon in the substrate (**A**). The protonated aspartic acid donates a proton to the nitrogen and forms a zwitterionic tetrahedral intermediate (**B**) that collapses into two products: the acid (*N*-terminal cleavage product) and the amine (*C*-terminal cleavage product) (**C**, Figure 3).[1]

3.4 Designing Protease Inhibitors

A number of important properties should be taken into consideration when developing protease inhibitors as therapeutic agents. To be effective biological tools, the inhibitors must be very potent and highly selective for a specific protease. Furthermore, to be suitable potential drug candidates, protease inhibitors must have appropriate pharmacokinetic and pharmacodynamic characteristics. They should be minimally peptidic in nature, since peptides usually offer low bioavailability and poor pharmacological profiles. Protease inhibitors that are potential low molecular weight drugs should exhibit high efficacy, no toxicity, good oral bioavailability, a high therapeutic index, and a clearance rate that will make it possible to administer only one or two doses (*e.g.*, pills) a day.

To facilitate the search for potential drug candidates, certain properties have been suggested to improve the chances of a compound being well tolerated. A guideline with respect to permeation and solubility is the empirical Lipinski's "rule of five",[14] which postulates that drug candidates should fulfill as many as possible of the following requirements: molecular weight less than 500, no more than five hydrogen bond donors, no more than 10 hydrogen acceptors, and a calculated log *P* (octanol/water) less than 5. Other properties have also been discussed with regard to oral bioavailability, for instance, that the number of rotational bonds in the inhibitor should be equal to or less than 10, and the polar surface area should be equal to or less than 140 Å². [15] Protease inhibitors can be developed through natural product screening or by mimicking the natural peptide substrate and replacing the scissile amide bond with a noncleavable isostere. In recent years, development of potential inhibitors has been substantially improved by the implementation of computer-assisted substrate-based inhibitor design using three-dimensional structural information on a substrate-inhibitor complex determined by NMR spectroscopy and X-ray crystallography.

4 Discovery of a Novel *N*-Acyl-Hydroxyproline Isostere, and Design and Synthesis of Potential Hepatitis C Virus Inhibitors (Papers I and II)

4.1 Introduction

With the development of diagnostic tests for hepatitis A and B in the 1970s, it became evident that some unknown infectious agent was responsible for the majority of the cases of transfusion-associated hepatitis (initially called non-A, non-B hepatitis).[16, 17] The advent of recombinant DNA made it possible to clone the genome of the virus, which was done by Houghton and coworkers[18] in 1989, and the agent was named hepatitis C virus (HCV). It is assumed that HCV has been spread unknowingly since the 1960s, mainly through blood transfusions that were performed before reliable routine blood tests became available in 1992. Cases that occur today are usually associated with intravenous drug use or other unrecognized transmission factors.[19] It has been estimated that approximately 170 million people are infected with HCV, which represents about 3% of the world's population and nearly five times the number of individuals infected with human immunodeficiency virus (HIV).[20] HCV establishes a chronic infection in up to 85% of the cases, and it has become the major infectious agent giving rise to chronic liver disease in Western countries. In that part of the world, chronic HCV infection constitutes a very serious condition that is now the most common cause of hepatocellular carcinoma and the primary reason for liver transplantation among afflicted adults.[21] Approximately 20% of all patients who have had chronic HCV infection for 10–20 years can develop cirrhosis of the liver, and about 5% progress to liver cancer.[22] Most individuals who carry the virus are unaware of their condition, and in many cases the infection may not become apparent until liver failure or liver cancer develops several years after initial infection. Currently, the most effective therapy is to administer PEGylated interferon- α alone or in combination with ribavirin. However, such treatment has limited efficacy and is poorly tolerated in individuals with complications. Moreover, only about 40% of the patients with HCV genotype 1 show a sustained virological response to this therapy.[23, 24, 25] From the discussion above, it is clear that new and better therapeutic strategies are needed to battle HCV.

4.2 The HCV Genome

HCV has been classified as a small, enveloped, single-stranded positive RNA Hepacivirus of the Flaviviridae family based on a number of similarities with other viruses that cause diseases such as dengue fever and yellow fever in humans.[17] HCV has a narrow host range that includes only humans and chimpanzees.[26] The HCV genome and the proteins it encodes have been characterized in detail.[27] The positive RNA genome consists of a 5' non-translated region (NTR), a single open reading frame (ORF) of about 9,000 nucleotides that is translated into a single polyprotein, and a short 3'NTR (Figure 4).[28] The 5'NTR includes the internal ribosome entry site (IRES), which mediates the initiation of viral RNA translation. Both the 5'NTR and the 3'NTR are essential for efficient RNA replication. The polyprotein encoded in the ORF consists of approximately 3,000 amino acids, and it is cleaved at several sites by host and viral proteases to produce at least three structural proteins (C1, E1, and E2) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The core protein (C) is the first cleavage product, and it interacts with viral RNA to form the major constituent of the nucleocapsid. The envelope proteins (E1 and E2) are highly glycosylated type 1 membrane proteins that can interact with the plasma membranes of hepatocytes and other cells. Protein p7 is located at the carboxy terminus of E2; the exact function of this highly hydrophobic protein is not known, al-

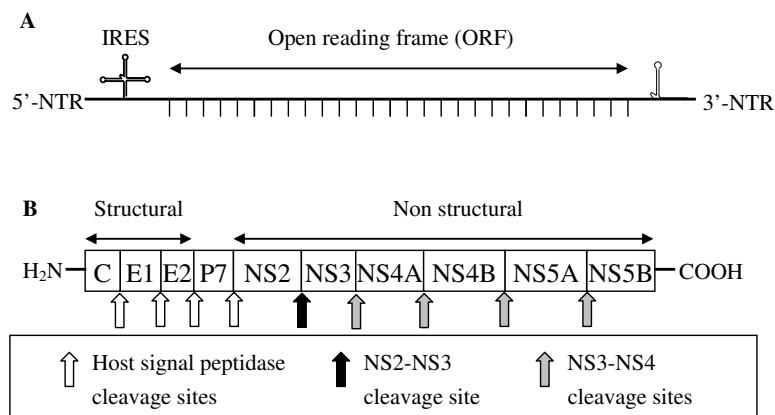


Figure 4: Schematic representation of the HCV genome (A) and the polyprotein it encodes (B). The figure is not drawn to scale.

though there is evidence that it may act as an ion channel.[29] Most of the NS proteins are enzymes or cofactors that are required to catalyze and regulate replication of the HCV RNA genome. Processing of the HCV NS polyprotein is catalyzed by the two virally encoded proteases NS2 and NS3. The zinc-dependent NS2-NS3 metalloproteinase undergoes autocatalytic cleavage to yield NS2 and NS3. The NS3 protease that is released is responsible for catalytic cleavage of the remaining NS proteins to produce NS4A, NS4B, NS5A, and NS5B. NS4A is a cofactor that is essential for the activity of NS3 protease, and it also forms a stable complex with the NS3 protein, which facilitates localization of the NS3-NS4A complex to the membrane of the endoplasmic reticulum (ER). The function of the relatively hydrophobic NS4B is not yet known. NS5A has been implicated in mediating resistance to interferon- α therapy, and it might also function to regulate viral replication by interacting with NS5B, which is an RNA polymerase that catalyzes the replication of HCV RNA.[17, 25, 30, 31]

4.3 The HCV Life Cycle

Our current understanding of the molecular mechanism of the HCV replicon is based primarily on analogies with closely related flavi- and pestiviruses, and on characterization of recombinant HCV proteins. A model of the HCV life cycle has been proposed[17, 25] and is illustrated in Figure 5.[28] In the first step, the viral particle becomes attached to the host cell (hepatocyte), and that event is mediated by specific interactions between the viral envelope proteins and receptors on the cell surface (**A**). The mechanism of virus entry has not yet been elucidated, and the receptors that are involved for this attachment are not fully understood. Nevertheless, it has been proposed that the glycoproteins E1 and E2 bind to the CD81 and LDL receptors on the hepatocyte, and the viral particle is subsequently transported into the cytoplasm of the cell by endocytosis (**B**). Once within the cytoplasm, the viral particle is uncoated (**C**) to release the positive single-stranded RNA genome, which is translated into the polyprotein (**D**). In the hepatocyte ER, host enzymes and the viral enzymes NS2 and NS3 process the polyprotein into at least ten active proteins (**E**). Three of these proteins (C, E1, and E2) form new viral envelopes (**F**), and the polymerase NS5B is responsible for replication of the positive single-stranded RNA to give double-stranded RNA (**G**), which is then unwound into negative and positive single-stranded RNA by the NS3 helicase (**H**). The negative-stranded RNA is subsequently used as a template for generation of new positive-strand RNAs, which are encapsulated to give new virions (**I**). Finally, it is presumed that infectious virions are released by transport through the Golgi compartment and are

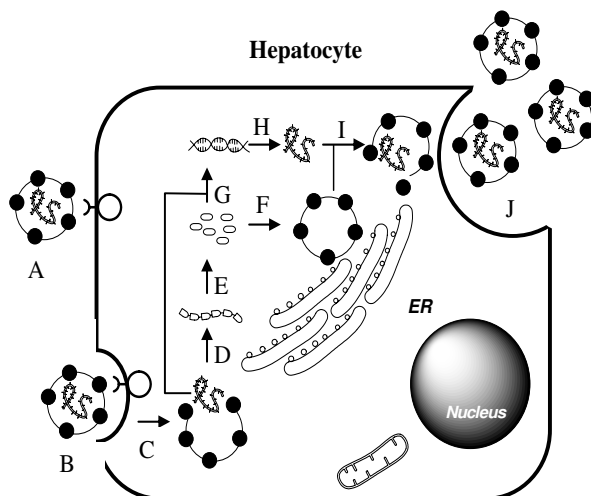


Figure 5: The life cycle of HCV.

ready to infect other hepatocytes (J). Practically all the steps in the HCV life cycle – from entry into the host cell to the replication and formation of new virion particles – constitute potential targets for therapeutic antiviral drugs, and one of the most promising of these is the NS3 protease.[17, 25, 30]

4.4 HCV NS3 Protease

The bifunctional NS3 protease is one of the most well-characterized of the HCV enzymes. This chymotrypsin-like serine protease comprises 180 amino acids, and it is located in the *N*-terminal domain of the NS3 protease.[32] The *C*-terminal domain of the NS3 protease also has NTPase/RNA helicase activity that is essential for translation and replication of the HCV genome. The structure of the full-length NS3 protease has been solved by X-ray crystallography.[33] Efficient processing by the NS3 protease requires a cofactor (NS4A) and a structural zinc molecule.[34] Direct comparison of the NS3 protease with and without NS4A has shown that NS4A is necessary to improve the anchoring and the orientation of the catalytic triad in the serine protease. The NS3 protease causes maturation of the viral polyprotein by catalyzing cleavages at four of five sites in the NS region (Figure 4): first a *cis*-cleavage at the NS3/NS4A junction and then hydrolysis of the polypep-

tide by intramolecular *trans*-cleavages at the NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B junctions. X-ray crystal structures of NS3 protease have shown that its active binding site is shallow and solvent-exposed, which makes the design of inhibitors of this enzyme a challenging task.[17, 25]

4.5 Inhibitors of HCV NS3 Protease

In substrate specificity studies of NS3 protease,[35] it was found that the enzyme is susceptible to feedback inhibition by several of the *N*-terminal cleavage products that are enzymatically released from the polyprotein. These cleavage products were identified and truncated to make novel inhibitors (*e.g.*, **A** in Figure 6), and that work was the starting point for developing smaller inhibitors of the HCV NS3 protease that take advantage of the feedback inhibition, which now constitute one of the most important classes of NS3 protease inhibitors. The distinctive feature of these product-based substances is the presence of a free carboxylic acid functionality in the *C*-terminal P1 residue, which subsite is hydrophobic and shallow.[36] The first generation of substrate-based analogs were modified from the natural amino acids of the hexapeptide *N*-terminal products and, to be highly potent, they had to have a dual anchor; a P1 anchor and an acidic anchor in the P5–P6 position. The preference displayed by NS3 protease for a cysteine residue as a P1 anchor was a problem for medicinal chemists, since the thiol group is a very reactive and unstable functionality and is not suitable in potential drug candidates.[37] Accordingly, the cited investigators focused on finding a more suitable and stable isostere that could replace the cysteine residue in the P1 position. By systematically modifying the natural amino acids of the truncated *N*-terminal cleavage product, several research groups have obtained potent hexapeptides.[37, 38, 39] Compound **B** ($IC_{50} = 51$ nM) is an example of the first generation of inhibitors based on the NS5A/NS5B product.[40]

Further truncation of the product-based hexapeptides resulted in a number of potent analogs that clearly demonstrated that the P5 and P6 residues are not required for enzymatic activity.[41, 42] In an additional attempt to reduce both the size and peptidic character of inhibitors, another series of tetrapeptides (P1–P4) was synthesized focusing on optimization of the P2 substituent on the P2 proline ring by introducing large lipophilic aromatic systems, which significantly enhanced the potency of the inhibitors.[43] Including aromatic systems in the P2 proline ring has been shown to stabilize the catalytic machinery in the correct geometry by shielding the catalytic triad of the protease from exposure to the solvent.[44, 45] Moreover, this P2 substituent moiety probably gives rise to favorable interactions with the heli-

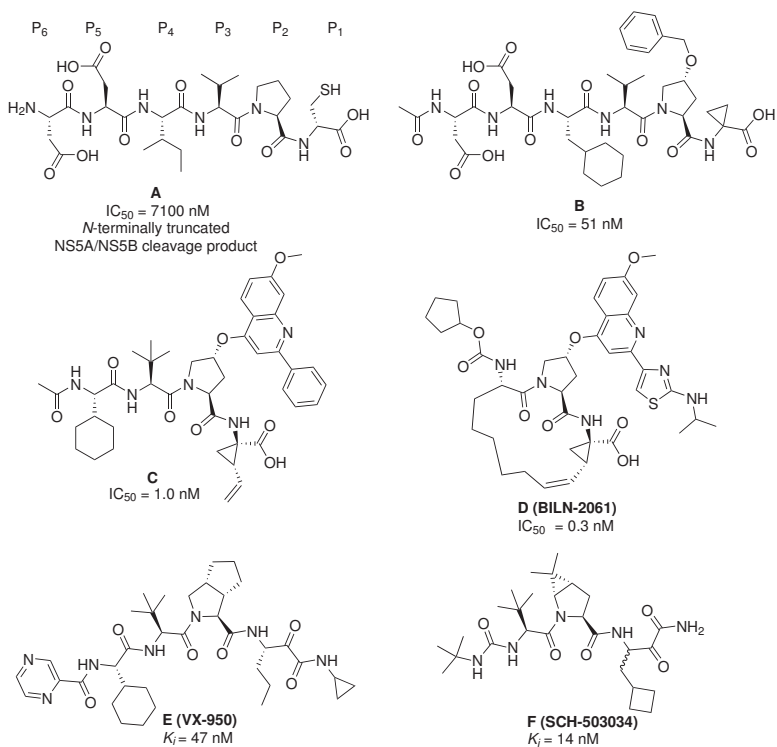


Figure 6: Examples of NS5A/NS5B product-based noncovalent inhibitors (**A–D**) and covalent inhibitors (**E–F**) of HCV NS3 protease.

case domain of the NS3 protease and thereby enhances the overall activity.[45] This strategy, in combination with optimization of the P1–P4 substituents, gave very potent tetrapeptides such as inhibitor **C** ($IC_{50} = 1.0 \text{ nM}$).[46] The compounds in this second generation of inhibitors were still not suitable as drugs due to poor membrane permeability, and thus it was obvious that some structural changes had to be made.

Earlier NMR analysis of the first generation of inhibitors showed that the P1 and P3 groups are in proximity to each other in the active conformation, which undergoes extensive rigidification upon binding.[47] Macrocyclization between P1 and P3 would further reduce the peptidic nature of an inhibitor and probably lead to a more desirable pharmacokinetic profile. Use of this strategy and further refinement resulted in exceptionally potent and less

peptidic inhibitors. The first member of this class to enter clinical trials (Phase II) is the extremely potent inhibitor **D** (BILN-2061), which has very good membrane permeability and an IC_{50} of 0.3 nM.[48] Unfortunately, the BILN-2061 trials are currently on hold due to toxicity observed in animals treated with high doses of the compound for four weeks. Notwithstanding, BILN-2061 can be looked upon as a milestone in the research aimed at finding effective inhibitors of HCV NS3 protease.

All the compounds discussed above are noncovalent inhibitors. Another class of structural inhibitors includes covalent peptides that have a reactive center at the cleavage site that traps the catalytic Ser139 in the active site of NS3 protease. Two of the most promising inhibitors in this group are the α -ketoamides **E** (VX-950, $K_i = 47$ nM)[49, 50, 51] and **F** (SCH-503034, $K_i = 14$ nM),[52, 53, 54] both of which are now in phase II clinical trials. Interestingly, in studies conducted *in vivo*,[55, 56] it was found that HCV clones that had developed resistance to BILN-2061 were still sensitive to VX-950 and vice versa. This finding suggests that, in analogy with treatment of HIV, combination therapy will probably be necessary for patients with HCV.

4.6 Design of Potential Inhibitors of HCV NS3 Protease

Earlier work in our laboratory that was focused on developing thrombin inhibitors revealed that *N*-acyl-proline (**G**, Figure 7) can be successfully replaced with five- and six-membered carboxylic ring isosteres, exemplified by structures **H** and **I**. [57, 58] Several research groups, with Boehringer Ingelheim in the foreground, have used the *N*-acyl-hydroxyproline moiety (**J**) as a central core in the P2 position to produce new and potent inhibitors of the HCV NS3 protease (*e.g.*, **C** and **D** in Figure 6).[59] In light of the results of our work on thrombin inhibitors, we wanted to ascertain whether the 4-hydroxy-cyclopentene moiety (**K**), which is based on molecular mod-

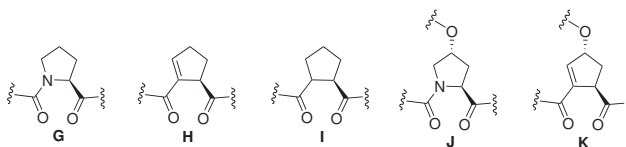


Figure 7: *N*-Acyl-proline (**G**), some previously reported *N*-acyl-proline isosteres (**H** and **I**), and *N*-acyl-hydroxyproline (**J**) shown in comparison with the cyclopentene isostere (**K**) used to develop novel HCV NS3 protease inhibitors.

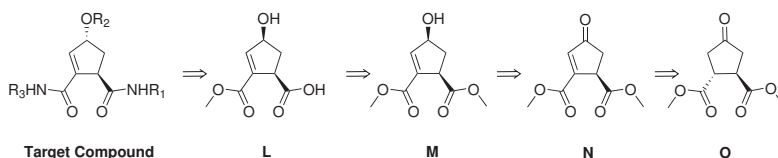


Figure 8: Retrosynthetic route to target compounds.

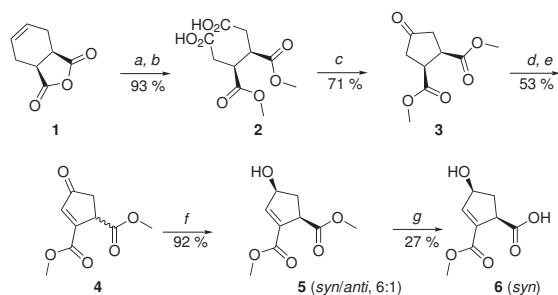
eling, could serve as a novel mimic of the *N*-acyl-hydroxyproline moiety in the previously reported inhibitors, without loss of potency.

The generic structure of our target compounds is depicted in Figure 8. The retrosynthetic analysis showed that they could be synthesized from compound **L** by use of two peptide coupling reactions and one Mitsunobu reaction. Compound **L** could be synthesized by regioselective monohydrolysis of the diester **M**. We reasoned that the alcohol **M** could be furnished by a stereoselective reduction of compound **N**. Synthesis of the chiral compound **O** had already been described in the literature,[60] thus we assumed that olefination of **O** could yield compound **N** with control of stereochemistry. We initially applied different olefination protocols to compound **O**, but, unfortunately, these attempts always yielded a racemate of **N**, so we instead chose the *syn*-derivative of **O**, compound **3**, as starting material.

4.7 Synthesis of the Novel Cyclopentene Scaffold

Synthesis of the scaffold **6** is depicted in Scheme 1, and synthesis of the ketone **4** from the anhydride **1** has been published.[60, 61, 62] The anhydride was opened with *p*-toluene sulfonic acid in methanol to give the corresponding diester, which was oxidatively cleaved with aqueous KMnO_4 to deliver the dicarboxylic acid **2** in 93% yield. The dicarboxylic acid was cyclized with acetic anhydride, and subsequent decarboxylation with NaOAc furnished the ketone **3** in 71% yield. Reacting ketone **3** with cupric bromide in refluxing THF gave the α -brominated ketone, which in the following step was β -eliminated with CaCO_3 in DMF (100 °C) to provide the $\alpha\beta$ -unsaturated ketone **4** in 53% yield.[61] Unfortunately this protocol resulted in racemization of compound **4**, probably due to the acidic α -hydrogen in the compound.

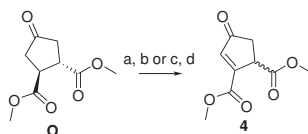
As discussed above, we tried to synthesize chiral ketone **4** from the enantiomerically pure ketone **O** (Scheme 2), which has been reported in the literature.[60] We used two different protocols: the above described olefination with cupric bromide and CaCO_3 ,[61] and a protocol with LDA/PhSeBr



Scheme 1: Synthesis of the scaffold. **Reagents and conditions:** (a) *p*-toluene sulfonic acid, methanol, reflux; (b) KMnO_4 (aq), 0 °C; (c) *i.* acetic anhydride, 130 °C; *ii.* NaOAc, 130 °C; (d) CuBr_2 , THF, reflux; (e) CaCO_3 , DMF, 100 °C. (f) NaBH_4 , methanol, -30 °C; (g) LiOH, 0 °C, water/dioxane (1:1).

followed by oxidation/elimination.[63] This strategy always resulted in the racemate of **4**. The racemization was corroborated by polarimetry and NMR analysis performed using the chiral shift reagent europium-tris[3-(heptafluoro-propyl-hydroxymethylene)-(+)-camphorate].[64]

Using sodium borohydride in cold methanol (-30 °C), the racemic ketone **4** was reduced to the corresponding alcohol **5** in 92% yield in a diastereomeric ratio of 6:1 (*syn/anti*). We supposed that the *5-syn* compound would be the major product and *5-anti* the minor product after the reduction, since we presumed that the $\beta\gamma$ -unsaturated ester in compound **4** would guide the hydride donation to the opposite face. This conclusion was substantiated by nuclear overhauser effect spectroscopy (NOESY) experiments on both the diastereomers. The diastereomeric mixture of **5** (*syn/anti* 6:1) could not be further purified, because the diastereomer of **5** that was formed in a smaller amount (*5-anti*) was always contaminated with the major diastereomer (*5-*



Scheme 2: Attempts to synthesize chiral **4**. **Reagents and conditions:** (a) CuBr_2 , THF, reflux; (b) CaCO_3 , THF, 100 °C; (c) *i.* LDA, -78 °C, THF, *ii.* PhSeBr; (d) H_2O_2 , DCM, 0 °C.

syn). The alcohol **5-syn** was converted to the **5-anti** by use of the Mitsunobu inversion reaction,[65] and NOESY experiments were performed on both **5-syn** and **5-anti**. Hydrogens that are close to each other in a compound exhibit nuclear overhauser effects (NOEs), which makes it possible to confirm that the compound has a specific stereochemistry (Figure 9). In the NOESY spectrum of **5-syn**, both H₁ and H₄ interact with H_{5β}, which is consistent with the *syn* configuration. The NOESY spectrum of the **5-anti** shows that H₁ and H₄ interact with different hydrogens, H₁ with H_{5β} and H₄ with H_{5α}, which indicates that our assumption is correct.

The diastereomeric mixture of **5** (*syn/anti* 6:1) was subjected to monohydrolysis with 1.0 equivalent of LiOH in dioxane/water (1:1) to provide the monomethylester **6** in 27% yield. We believed that selective monohydrolysis of the βγ-unsaturated ester would be possible due to two different effects. First, there may be a hydrogen bond between the hydroxyl group and the carbonyl carbon in the βγ-unsaturated ester, and such an NGP effect would make that carbonyl carbon more electropositive and more prone to hydrolysis.[66] Second, the αβ-unsaturated ester has greater electron density, and thus the αβ-unsaturated carbonyl carbon is less electropositive, which makes it less prone to hydrolysis. The regiochemistry of the monomethylester **6** was corroborated by an HMBC spectrum. An HMBC experiment reveals ¹³C-¹H couplings that are separated by two or three bonds between homo- and/or heteroatoms. The HMBC spectrum of compound **6** showed interactions between the alkene hydrogen and the αβ-unsaturated carbonyl carbon, and the hydrogens in the methyl ester interacted with the same carbonyl carbon. The results of this experiment prove that the βγ-unsaturated ester had been hydrolyzed.

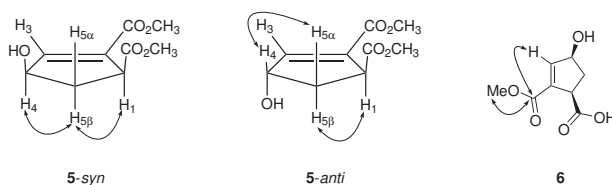
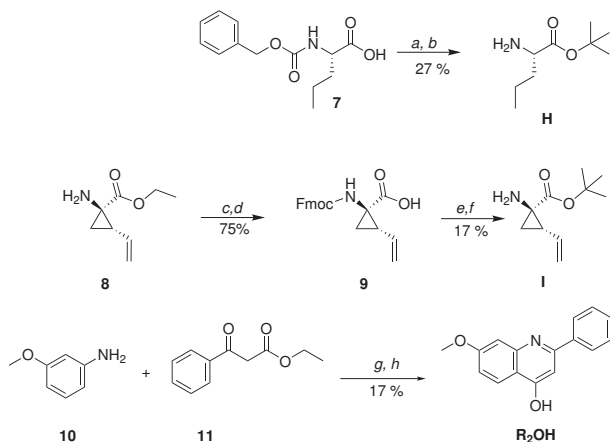


Figure 9: Significant NOE correlations in **5-syn** and **5-anti**, and the relevant HMBC correlations in compound **6**.

4.8 Synthesis of the Substituents Used in the Present Research

Three different P1 substituents (R_1NH_2), one P2 substituent (R_2OH), and six different P3/P4 substituents (R_3NH_2) were used to synthesize eighteen different inhibitors (Section 4.9). All the substituents were synthesized from commercially available amino acids, except the amines **F** and **G**, which could be purchased. The P1 substituent **H** was synthesized from (*S*)-2-benzyloxycarbonyl-amino-pentanoic acid (**7**), which was protected as a *tert*-butyl ester, and the *Z*-group was subsequently deprotected by catalytic hydrogenation to give the compound **H** in an overall yield of 27% (Scheme 3). The P1 substituent **I** was synthesized from (1*R*,2*S*)-1-amino-2-vinylcyclopropanecarboxylic acid ethyl ester (**8**) that had been produced as described in the literature.[67, 68, 69] The ethyl ester in compound **8** was hydrolyzed with LiOH, and the amine was protected as a carbamate with Fmoc-ONSu with LiOH, and the amine was protected as a carbamate with Fmoc-ONSu to afford the acid **9** in 75% yield over two steps.[70] Compound **9** was then protected as a *tert*-butyl ester using TBTA.[71] Last, the Fmoc group was deprotected with piperidine in DMF, which delivered compound **I** in only 17% yield. Unfortunately, the free amine **I** was not stable upon purification by flash column chromatography or after storage at room tem-

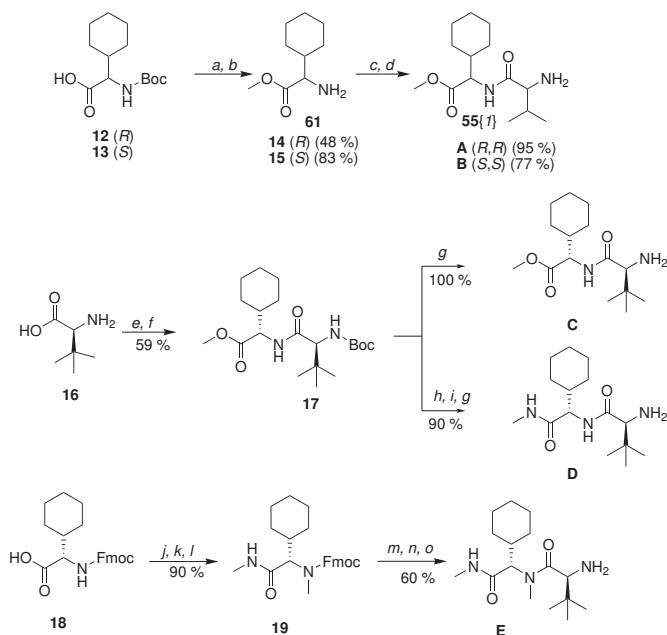


Scheme 3: Synthesis of **H** and **I** (R_1NH_2), and the R_2OH substituents. **Reagents and conditions:** (a) Boc_2O , DMAP, *tert*-butanol; (b) Pd-C, 95% ethanol, H_2 ; (c) LiOH, dioxane/water (1:1); (d) Fmoc-ONSu, $NaHCO_3$; (e) TBTA, 50 °C, DCM; (f) DMF, piperidine. (g) 4 M HCl, toluene, reflux; (h) diphenyl ether, 280 °C.

perature, which explains the low yield. The yield was improved (52%) when the crude amine **I** was coupled directly to scaffold **6** without purification.

Using a one-step protocol, the P2 substituent (**R₂OH**) was synthesized from *m*-anisidine (**10**) and ethyl benzoyl acetate (**11**) according to a procedure described in the literature.[46, 72] *m*-Anisidine makes a nucleophilic attack at the ketone carbonyl carbon in ethyl benzoyl acetate to form an imine. Next came a high temperature intramolecular condensation reaction, which furnished the desired 7-methoxy-2-phenyl-quinolin-4-ol (**R₂OH**) in 17% yield.

The same protocol was applied to synthesize amine **A** with (*R*)-amino



Scheme 4: Synthesis of the R_3NH_2 substituents (**A**, **B**, **C**, **D**, and **E**). **Reagents and conditions:** (a) Ag_2O , MeI, acetone; (b) TFA, triethylsilane, DCM; (c) *Z*-(*R*)-Val-OH or *Z*-(*S*)-Val-OH, HATU, DIPEA, DMF; (d) Pd-C, 95% ethanol, H_2 ; (e) Boc_2O , TEA, dioxane/water (1:1); (f) **15**-(*S*), HATU, DIPEA, DMF; (g) TFA, triethylsilane, DCM; (h) LiOH, dioxane/water (1:1); (i) methylamine, DIPEA, HATU, DMF. (j) paraformaldehyde, *p*-TsOH, toluene; (k) TFA, triethylsilane, DCM; (l) HATU, DIPEA, methylamine, DMF; (m) piperidine, DMF; (n) *Boc*-*tert*-leucine, DIPEA, HATU, DMF; (o) TFA, triethylsilane, DCM.

acids and amine **B** with (*S*)-amino acids (Scheme 4). Protection of Boc-(*R*)-cyclohexylglycine **12** and Boc-(*S*)-cyclohexylglycine **13** as methyl esters, followed by deprotection of the Boc group, gave the amines **14** and **15** in 48% and 83% yield, respectively. Those two amines were then coupled with *Z*-(*R*)-Val-OH and *Z*-(*S*)-Val-OH, respectively, using standard peptide coupling chemistry, and the *Z*-group was deprotected to deliver the amines **A** and **B** in 95% and 77% yield. Synthesis of the building blocks **C** and **D** started with Boc protection of the *tert*-leucine **16**, and the acid was subsequently coupled with amine **15** to obtain the amine **17** in 59% yield. This amine was used to synthesize both **C** and **D** by two different protocols. Deprotection of the amine **17** afforded the P3/P4 substituent **C** in quantitative yield. The methylamide derivative **D** was synthesized from compound **17**, which was hydrolyzed to the corresponding acid. Thereafter, the acid was linked to methylamine by standard peptide coupling chemistry and finally deprotected to provide the desired compound **D** in 90% yield. The *N*-methylated derivative **E** was synthesized from compound **18** as follows. The amide nitrogen in compound **18** was condensed with paraformaldehyde to give an imine that was attacked intramolecularly by the carboxylic acid to form an oxazolidinone intermediate.[73] The oxazolidinone ring was opened by hydrolysis to furnish the *N*-methylated acid, which was coupled with methyl amine by standard peptide coupling chemistry to afford compound **19** in an overall yield of 90%. The Fmoc group in compound **19** was removed to give the corresponding amine, which was coupled with Boc-*tert*-leucine. The Boc group was deprotected to deliver compound **E** in 60% yield.

4.9 Synthesis of the Final Products

The cyclopentene scaffold **6** was derivatized with three different amines in the P1 position (R_1NH_2 , **G–I**) and six different amines in the P3–P4 position (R_3NH_2 , **A–F**), and the P2 substituent position (R_2OH) was held constant. Figure 10 presents the generic structure of the cyclopentene scaffold and the substituents used in this work. During the entire process of synthesizing the desired final products, the intermediates consisted of a mixture of two diastereomers that in most cases could be separated by HPLC in the last step to give chiral final compounds, which were tested individually for their ability to inhibit the NS3 protease.

Scaffold **6** was coupled with the amines **G–I** (R_1NH_2) using HATU and DIPEA in DMF to give the amides **20–22** in 52–69% yield (Scheme 5). The alcohols **20–22** were subjected to a Mitsunobu reaction to attach the

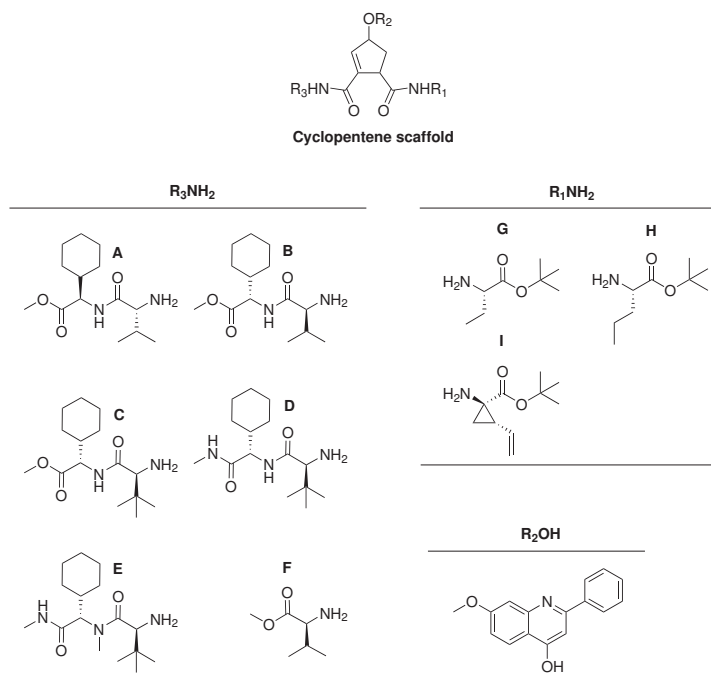
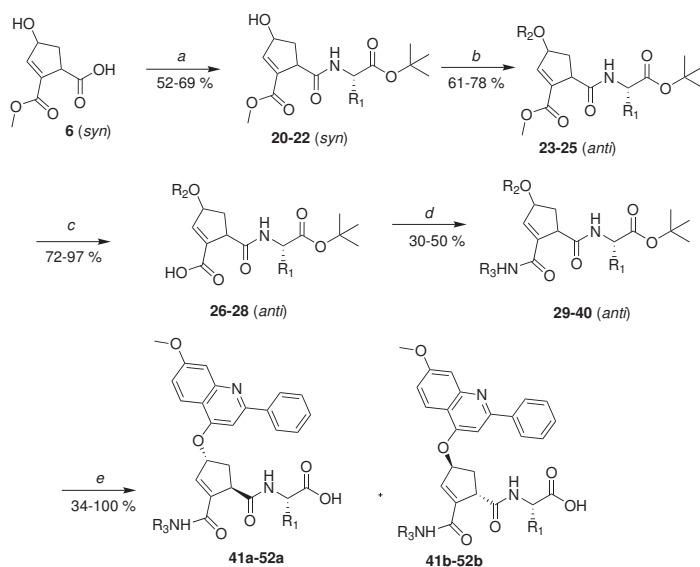


Figure 10: General structure of the central cyclopentene scaffold and the R_1 , R_2 , and R_3 substituents used in the present research.



Scheme 5: Synthesis of the final products. **Reagents and conditions:** (a) DIPEA, HATU, R_1NH_2 (**G-I**), DMF, 0 °C; (b) R_2OH , (methoxy-2-phenyl-quinolin-4-ol), PPh_3 , DIAD, THF; (c) LiOH, dioxane/water (1:1); (d) R_3NH_2 (**A-F**), DIPEA, coupling reagent (HATU and DMF or DCC/HOBt and THF); (e) triethylsilane, TFA, DCM.

7-methoxy-2-phenyl-quinolin-4-ol (R_2OH) moiety using PPh_3 and DIAD in THF, which provided the corresponding ethers **23–25** in 61–78% yield, with inversion of the configuration. The methyl esters were then hydrolyzed with LiOH in dioxane/water (1:1) to give the acids **26–28** in 72–97% yield. The amines **A–F** (R_3NH_2) were then coupled to the acids **26–28** using HATU, DIPEA in DMF or DCC/HOBt and DIPEA in THF to furnish the *tert*-butyl esters **29–40** in 30–50% yield. Thereafter, **29–40** were hydrolyzed using TFA and triethylsilane in DCM to deliver the final products **41–52** in 34–100% yield.

All the desired final products were purified by preparative HPLC, which successfully separated five out of 13 diastereomeric pairs into chiral compounds (designated **a** and **b** in Scheme 5 and Table 1). The chiral final compounds, the diastereomeric mixtures and two *tert*-butyl esters were tested regarding their inhibitory effects on the NS3 protease. The activity data along with the complete structures are presented in Table 1 (Section 4.10). The chiral final products in Table 1 have either the 1*R*,4*R* or the 1*S*,4*S*

stereochemistry in the cyclopentene ring, and the activity data on the chiral compounds (*e.g.*, **47a** and **47b**) reveal that the inhibitory properties differ substantially between the diastereomers.

Therefore, we assumed that the most active diastereomer (*e.g.*, **47a**) has the same stereochemistry (1*R*,4*R*) as the lead compound **C** (Figure 6), and the less active diastereomer (*e.g.*, **47b**) has the (1*S*,4*S*) stereochemistry. We conducted TOCSY, ROESY, and NOESY experiments to determine the stereochemistry of the final compounds, and the results showed that our assumption is probably correct (for more information see Paper I).

In concluding the discussion of this successful synthetic route, comments should be made about the stability of the final compounds. First, it is important to point out that, when performing the Mitsunobu reaction, the equivalents of the reagents (DIAD and PPh₃) must be kept under careful control. When we used PPh₃ and DIAD in excess (> 2 equiv.), the corresponding 1,4-DIAD adduct was the major product instead of the desired ether (Figure 11), and the identity of this byproduct was confirmed by NMR and Maldi-TOF. This problem was solved by using less than 2 equivalents of the reagents. Second, it is necessary to explain why compound **8**, with its ethyl ester protecting group, was not used to synthesize the final compounds. We did initially use **8** as a P1 substituent in the synthesis, but in the last hydrolysis step we observed a β -elimination of the P2 substituent (**R₂OH**) rather than hydrolysis of the ethyl ester. To avoid the problem, we tested several reaction protocols with different hydrolysis conditions (LiOH, KOH, HCl) and transesterification,[74] but all failed. Accordingly, we developed a

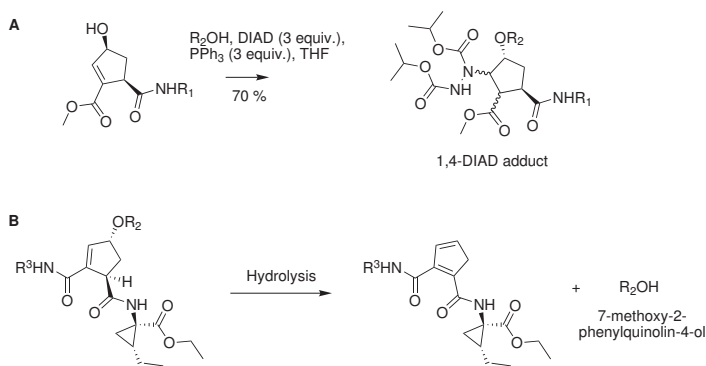


Figure 11: The problems encountered with Michael's addition (**A**) and β -elimination of the quinoline (**R₂OH**) (**B**).

synthetic route that would change the ethyl ester protecting group in compound **8** to a *tert*-butyl ester protecting group, compound **I** (Section 4.8). This strategy solved the problem. Summarizing the results, it can be said that these compounds tend to be unstable and show some preference for 1,4 Michael's addition and β -elimination of the quinoline (**R₂OH**) moiety.

4.10 SAR Analysis of Potential HCV NS3 Protease Inhibitors Incorporating the Cyclopentene Scaffold

All the final products that are discussed below (Table 1) contain the 7-methoxy-2-phenyl-quinolin-4-ol (**R₂OH**) group as P2 substituent, and they were evaluated in an *in vitro* assay that determines effects on the full-length NS3 protease. The activity data show that the final products with the (*R*, *R*) stereochemical configuration in the P2 hydroxycyclopentene moiety are more potent than the corresponding (*S*, *S*) diastereomers, for example, compare **46a** (*R*, *R*; K_i 102 nM) and **46b** (*S*, *S*; K_i 2,560 nM). This indicates that the *R*, *R* derivatives (designated **a** in Table 1) have the same stereochemistry as the *N*-acyl-hydroxyproline that has been incorporated in previously reported inhibitors.[46, 59, 75] We also had to determine which stereochemistry would be preferable at the P3/P4 position in our novel inhibitors. Our scaffold has a one-atom extension in the P3/P4 region compared to the substituted *N*-acyl-hydroxyproline scaffold incorporated in the lead compound **C** (Figure 6). All inhibitors that other investigators have synthesized using the *N*-acyl-hydroxyproline moiety as a building block appear to prefer *S*, *S* stereochemistry in the P3/P4 position.[46, 59, 75, 76] Consequently, our inhibitors have two carboxy termini and also a one-carbon extension in the P3/P4 amino acid chain. Comparison of the 2D structures of lead compound **C** and **51a** suggests that the *S*, *S* stereochemistry should be more favorable. This assumption was verified by comparing activity data on the P3/P4 *R*, *R* derivative and the corresponding *S*, *S* derivative, which made it evident that the *S*, *S* derivative **42** (K_i 523 nM) is more potent than the corresponding *R*, *R* derivative **41a** ($K_i > 2,000$ nM). From this result all inhibitors discussed below contain various amine derivatives with *S*, *S* configuration at the P3/P4 position.

The S3 pocket is considered to be relatively small. Despite that, we found that the enzyme-inhibiting capacity was enhanced when the P3 valine moiety in **46a** (K_i 102 nM) was replaced with the slightly more bulky substituent *tert*-butyl glycine in the P3 position to give compound **47a** (K_i 30 nM). Interactions with the S4 pocket in the enzyme are very important to achieve activity, which can be illustrated by comparing the P1–P3 inhibitor

45 ($K_i > 2,000$ nM) with the P1–P4 inhibitor **42** (K_i 523 nM). Furthermore, by comparing inhibitors containing the Abu residue **G** in the P1 position with inhibitors incorporating the homo-analog norvaline **H**, it can be seen that a one-carbon extension in the P1 position increases the inhibitory activity roughly fourfold, as exemplified by **44** and **48** (K_i values 280 and 67 nM, respectively). Llinàs-Brunet and coworkers[59, 77] have shown that the (1*R*,2*S*)-1-amino-2-vinylcyclopropane carboxylic acid **I** is an outstanding P1 substituent that fits well in the hydrophobic S1 pocket of the NS3 protease, and that compound has been incorporated into very potent inhibitors (Figure 6). The activity of our inhibitors was markedly improved by incorporation of the amine **I** instead of the norvaline residue **H**, as indicated by comparison of **47a** and **50a** (K_i values 30 and 1.1 nM, respectively). In the P1 position, the free carboxylic acid is essential for HCV NS3 protease inhibitor properties, which can be seen by comparing the acid **50a** (K_i 1.1 nM) with the corresponding *tert*-butyl ester **38** ($K_i > 2,000$ nM). Previous reports have indicated that the carboxylic acid in the P1 position probably forms important hydrogen bonds with Ser139 in the catalytic triad of NS3 protease.[77] The methyl ester compound **50a** (K_i 1.1 nM) has essentially the same potency as the corresponding methyl amide derivative **51a** (K_i 1.3 nM), which suggests that no hydrogen bonds need to be donated at this position. In contrast, when the amide nitrogen in the P4 position in compound **51a** is methylated to give compound **52**, the inhibitory properties are almost eradicated (K_i values 1.3 and $> 5,000$ nM, respectively), implying that the hydrogen in the P4 amide position is involved in important bonding interactions in the active site of the NS3 enzyme.

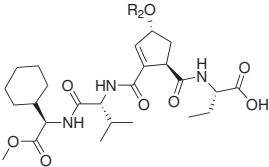
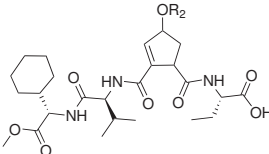
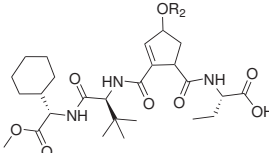
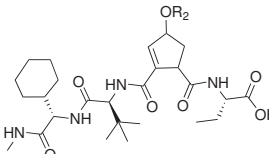
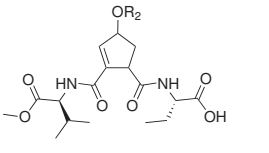
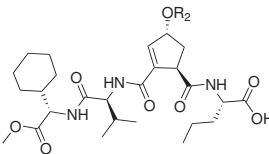
Compound	Structure	K_i (nM)
41a		>2000
42 (anti)		523
43 (anti)		503
44 (anti)		280
45 (anti)		>2000
46a		102

Table 1: Biological data of the final products

Compound	Structure	K_i (nM)
47a		30
48 (anti)		67
49 (anti)		24
50a		1.1
51a		1.3
52 (anti)		>5000

Table 1. (Continued)

Compound	Structure	K_i (nM)
46b		2560
47b		>2000
50b		108
51b (anti)		23
38 (anti)		>2000
39 (anti)		>2000

Table 1. (Continued)

5 Synthesis of Potent Macrocyclic HCV NS3 Protease Inhibitors Incorporating the Novel Cyclopentene as a Central Core (Paper II)

5.1 Design of Macrocyclic Inhibitors

Synthesis of the inhibitors discussed above revealed that the *N*-acyl-hydroxyproline moiety can be successfully replaced with our novel 4-hydroxy-cyclopentene moiety. That finding encouraged us to design a synthetic route to more rigid and less peptidic compounds in analogy with the development of BILN-2061 (Figure 6).[59, 78, 79, 80] It is believed that making inhibitors more rigid and locking them into the active bond conformation will reduce the entropy penalty paid upon binding and increase the overall binding energy of the inhibitor. The retrosynthetic analysis of the macrocyclic final compounds showed that they could be synthesized from the diolefin **A** by use of ring closing metathesis (RCM) (Figure 12).[81] It was possible to use peptide coupling chemistry to obtain **A** from our previously reported intermediate **28** and different Boc hydrazines **B** ($n = 1, 2$) in order to vary the size of the macrocycle ring. The R_1 substituent was held constant, 7-methoxy-2-phenyl-quinolin-4-ol (R_2OH), and the size of the macrocycle was varied by one carbon, which furnished 14- and 15-membered hydrazine-functionalized macrocyclic compounds as final products.

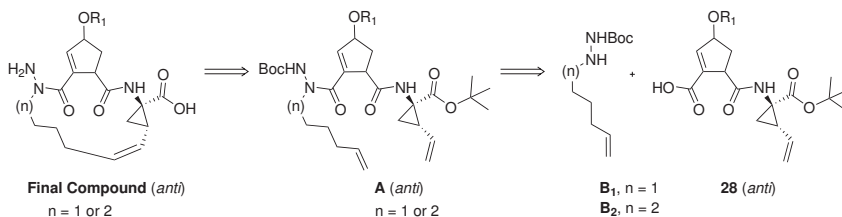
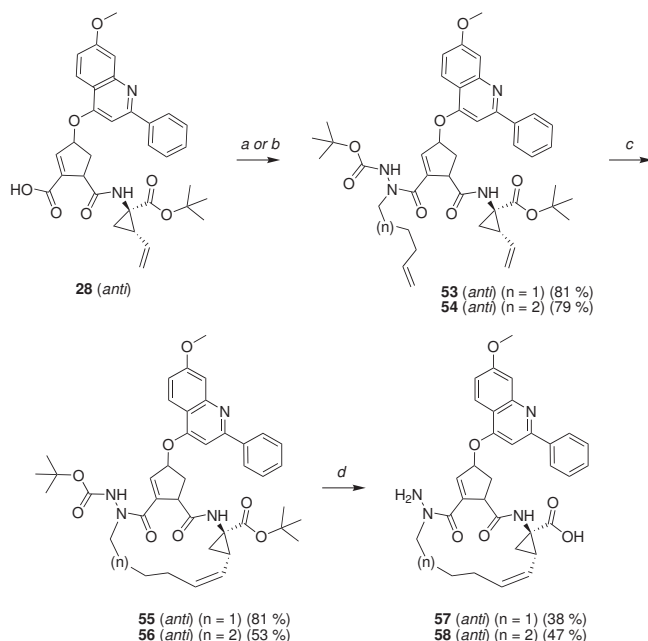


Figure 12: Retrosynthetic analysis of macrocyclic final compounds.

5.2 Use of the Cyclopentene Moiety to Synthesize Macrocyclic Inhibitors

Synthesis of the final compounds started from the previously described diastereomeric mixture (*anti*) of compound **28** (Paper I, Section 4). The Boc



Scheme 6: Reagents and conditions: (a) *N*'-Hex-5-enyl-hydrazinecarboxylic acid *tert*-butyl ester (**B**₁), DIPEA, HATU, DMF, 0 °C; (b) *N*'-hept-6-enyl-hydrazinecarboxylic acid *tert*-butyl ester (**B**₂), DIPEA, HATU, DMF, 0 °C; (c) Hoveyda-Grubbs catalyst 2nd generation (C₃₁H₃₈Cl₂N₂ORu), CH₂Cl₂, reflux; (d) TFA, triethylsilane, CH₂Cl₂, reflux.

hydrazines **B**₁ and **B**₂ were attached to compound **28** (Scheme 6) by peptide coupling chemistry (HATU, DIPEA in DMF) to give the dienes **53** and **54** in 81% and 79% yield, respectively (Paper II describes the synthesis of **B**₁ and **B**₂). Thereafter, **53** and **54** were subjected to RCM using Hoveyda-Grubbs Catalyst 2nd Generation as catalyst in refluxing methylene chloride to provide the macrocycles **55** and **56** in 81% and 53% yield, respectively.[79, 81] Lastly, deprotection of both the Boc group and the *tert*-butyl ester with TFA and triethylsilane in methylene chloride afforded the final compounds **57** (38%) and **58** (47%) as diastereomeric mixtures (*anti*) with regard to the stereochemistry of the cyclopentene ring. The diastereomeric mixtures could not be separated into chiral final compounds thus they were evaluated as diastereomeric mixtures with respect to their HCV NS3 inhibitory properties.

5.3 SAR Analysis of Potential Macrocyclic Inhibitors Incorporating the Cyclopentene Moiety as a Central Core

For the macrocyclic cyclopentane compounds, the biological data and structure-activity relationships from our laboratory revealed that, compared to the 13-, 15-, and 16-membered macrocyclic cyclopentane rings, the 14-membered ring gave better fit into the S1–S3 pocket of the NS3 enzyme. Furthermore, the hydrazine functionality gave rise to greater potency than the corresponding Boc hydrazine functionality did (for more information, see Paper II). Considering those observations, we focused on synthesizing 14- and 15-membered rings by introducing the cyclopentene moiety in the P2 position. The resulting compounds **57** and **58** (15 and 110 nM, respectively) display marginally lower potency against NS3 protease compared to the corresponding cyclopentane compounds **C** and **D** (6 nM and 120 nM, respectively) (Table 2). Unfortunately, as mentioned above, the diastereomeric mixture of the final products **57** and **58** could not be separated into chiral compounds, which decreases the inhibitory properties. Comparing the diastereomeric mixtures with the chiral linear compounds synthesized in the study reported in Paper I indicates that the (*R,R*) compound is superior to the diastereomer (*S,S*). The macrocyclic cyclopentene compounds also tend to be susceptible to Michael’s addition reactivity and β -elimination of the quinoline moiety, in analogy with our linear cyclopentene compounds (Section 4). These findings,

Compound	Structure	K_i (nM)	Compound	Structure	K_i (nM)
57 (<i>anti</i>)		15	C		6
58 (<i>anti</i>)		110	D		120

Table 2: Activity data on macrocyclic final compounds.

together with the inconvenient synthetic route and the zwitterionic nature of the final compounds, make it questionable whether inhibitors with favorable biopharmaceutical properties can be produced by this synthetic route.

6 Discovery of a Novel Hydroxyethylene Scaffold for Use in Designing Aspartic Protease Inhibitors (Appendix A)

6.1 Introduction

Some of the human aspartic proteases are illustrated in the evolutionary tree presented in Figure 13.[82] Therapeutic inhibition of aspartic proteases has been a challenging task, and inhibitors have been developed from truncated substrate analogs in which the scissile bond is replaced with a noncleavable isostere.[83, 84] Examples of noncleavable transition state isosteres that have been used in the discovery of aspartic protease inhibitors are depicted in Figure 14.[85, 86] The statine isostere is derived from the natural product pepstatin, which is a potent inhibitor of many aspartic proteases. The P1' group is deleted, which leads to a re-registration of the prime side amino acids relative to the peptide substrate. Hydroxymethylcarbonyl (norstatine) isosteres are chain contracted versions of the common statine transition state mimetic. The hydroxyethylene (HE) dipeptide isostere is one of the most direct dipeptide isosteres in which the scissile bond has been replaced with an all-carbon framework, and the hydroxyl group is positioned to engage the catalytic aspartates. In this isostere, registration of the amino acid side chain exactly mimics a peptide substrate.[87] This isostere and derivatives thereof had been used to develop inhibitors against *e.g.* renin[88] and HIV protease,[89] respectively. Hydroxyethylamine (HEA) isosteres normally become linked to the catalytic aspartic acid residues via a dual hydrogen bond from the secondary hydroxyl group, and this type of isostere was the starting point for the successful HIV protease inhibitor amprenavir.[90]

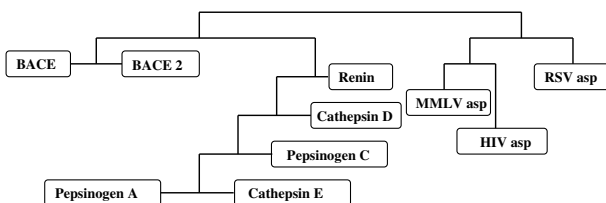


Figure 13: Evolutionary tree showing the relationships between the aspartic proteases.

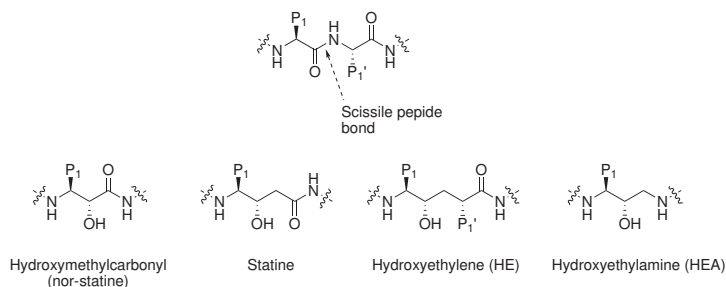


Figure 14: Natural peptide substrate together with, examples of transition state isosteres with a hydroxylgroup incorporated as stable peptide bond replacements

6.2 Design of the Novel HE Scaffold

As discussed above, we adopted the HE transition state analog as a central core, since this isostere is a good dipeptide mimetic and has been reported to be more potent than corresponding statine analogs.[91, 92] The (4*S*,5*S*) stereochemistry is generally preferred in hydroxyethylene peptide isostere inhibitors of aspartic proteases,[87, 93] and the chiral center at P₁' (C2) may have either the *R* (L-amino acids) or the *S* (D-amino acids) configuration. Therefore, we initiated a retrosynthetic analysis of the generic structure **A** (Figure 15), which could be varied at three positions using different substituents known from the literature to prepare novel aspartic inhibitors. The results of the analysis confirmed that **A** could be synthesized from the lactone **C**. Therefore it is possible that opening of **C** with different amines (R₂NH₂) would give intermediate **B**, and reduction of the azide to the corresponding

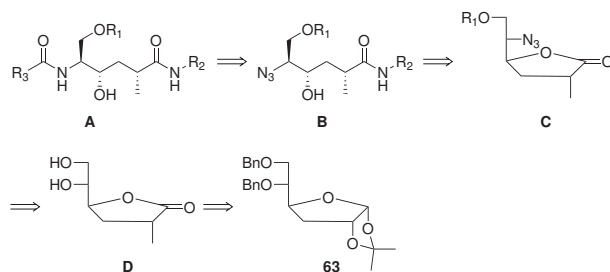
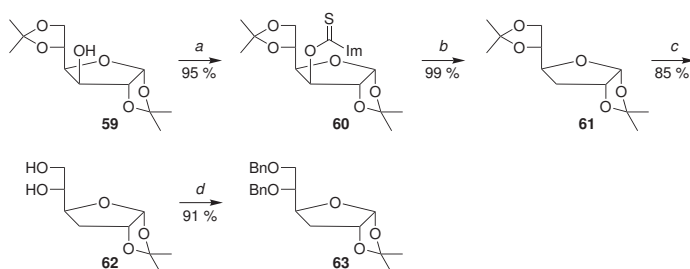


Figure 15: Retrosynthetic route to plausible compounds.

amine followed by peptide coupling chemistry with different acids (R_3COOH) might give final products in series **C** with the correct stereochemical assignment. Thereafter, a possible approach are to synthesize intermediate **C** from compound **D** by regioselective *O*-alkylation of the primary alcohol in **D** and then apply a Mitsunobu reaction at the secondary alcohol to yield compound **C** with inversion of configuration. We assumed that **D** could be prepared by a number of synthetic transformations of the acetal **63**, synthesis of which has previously been reported in the literature.[94, 95, 96, 97] In short, our approach involved hydrolysis of the acetal, deoxygenation of the formed secondary alcohol, followed by oxidation to the corresponding lactone. This lactone could then be alkylated with methyl iodide and deprotection of the benzyl ethers to give intermediate **D**.

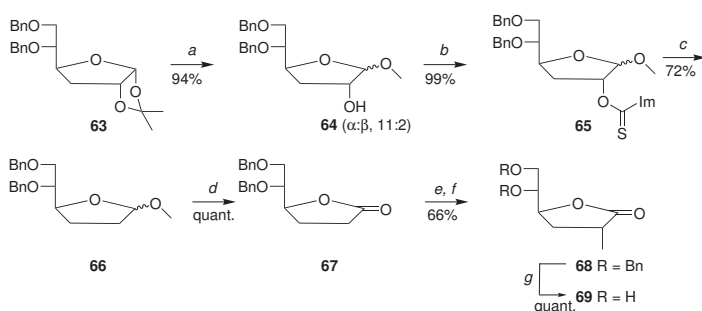
6.3 Synthesis of the Pivotal HE Scaffold

Compound **63** was generated from 1,2:5,6-di-*O*-isopropylidene- α -D-glucufuranose **59** in an over all yield of 73% over four steps according to a procedure described in the literature (Scheme 7).[94, 95, 96, 97] Treatment of compound **59** with thiocarbonyldiimidazole in THF delivered the thiocarbonyl derivative **60** in 95% yield. The thioester **60** was subjected to a radical reaction with tributyltin hydride and AIBN to give the monodeoxy sugar **61** in 99% yield. Selective deprotection of the terminal isopropylidene group in compound **61** under mild conditions by treatment with 70% AcOH (aqueous solution) furnished the diol **62** in 85% yield. Using NaH and benzyl bromide in DMF to protect the alcohols in compound **62** as benzyl ethers afforded compound **63** in 91% yield. The isopropylidene group in **63** was



Scheme 7: Synthesis of previously reported intermediates. **Reagents and conditions:** (a) Thiocarbonyldiimidazole, THF, reflux; (b) tributyltinhydride, AIBN, toluene, reflux; (c) 70% AcOH, 0 °C; (d) NaH, benzylbromide, DMF.

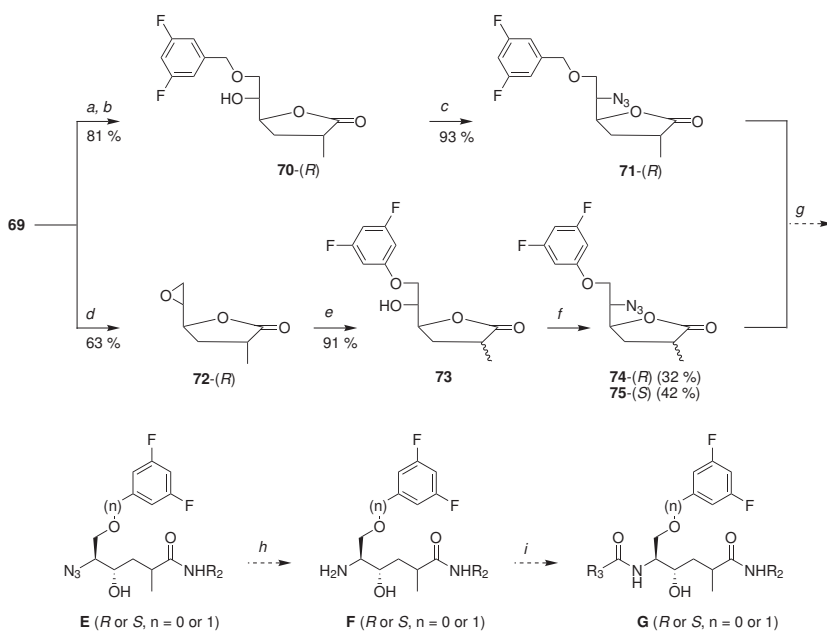
removed under acidic conditions (H_2SO_4 in methanol)[98] to give the α - and β -anomers of the methyl acetal **64** (Scheme 8) in a diastereomeric ratio of 11:2. Both the diastereomers were used in the subsequent synthesis, because this stereocenter would later be oxidized. Compound **64** was subjected to a deoxygenation described by Barton *et al.*[99, 100] by using thiocarbonyldiimidazole in refluxing THF to convert the alcohol to the thiocarbonyl derivative **65** (99% yield). This thioester was then subjected to a radical reaction with tributyltin hydride in refluxing toluene to give the methyl acetal **66** in 72% yield. Compound **66** was then oxidized to the corresponding γ -lactone **67** in quantitative yield by applying a procedure reported by Greico *et al.*,[101] which uses *m*-chloroperbenzoic acid in methylene chloride at 0 °C in the presence of boron trifluoride etherate. Introduction of the methyl group at C2 was accomplished by stereoselective alkylation of the γ -lactone **67** with methyl iodide.[102] The enolate of **67** was prepared by exposure to LDA for 30 min at -78 °C before addition of methyl iodide. Quenching with saturated ammonium chloride upon purification afforded the desired alkylated lactone **68** in 66% yield. Only one epimer of compound **68** was formed, probably due to steric hindrance from the benzyl groups at C5 and C6. We assumed that this isomer had the desired (*R*) configuration, since alkylation from that face was believed to be the least hindered. The diol **69** was obtained in quantitative yield by catalytic hydrogenolysis of compound **68**, and the stereochemical assignment and alkylation hypothesis considered above were confirmed by NOESY experiments on **69**, which are discussed in Section 6.5.



Scheme 8: Reagents and conditions: (a) H_2SO_4 , MeOH, 0 °C; (b) thiocarbonyldiimidazole, THF, reflux; (c) tributyltinhydride, toluene, reflux; (d) *m*-CPBA, BF_3OEt_2 , DCM; (e) LDA, THF, -78 °C; (f) MeI; (g) Pd/C, H_2 EtOH.

6.4 Synthesis of Potential Aspartic Protease Inhibitors

Synthesis of potential aspartic protease inhibitors was done according to two similar protocols in order to generate compounds with benzyl- and phenyl-substituted moieties in the P1 position (Scheme 9). Regioselective *O*-alkylation of the primary hydroxyl group in compound **69** was performed by reacting this diol with dibutyltin oxide in toluene to form the corresponding tin acetal, which was then allowed to react with 3,5-difluorobenzyl bromide in the presence of tetrabutylammonium bromide to afford the desired 6-*O*-benzylated compound **70** in 81% yield.[103] Compound **70** was then converted to the corresponding azide **71** in 93% yield with inversion of the configuration at C5; this was achieved using Mitsunobu conditions with DIAD, PPh₃, and DPPA in THF.[104]



Scheme 9: Reagents and conditions: (a) Bu₂SnO, toluene, reflux; (b) 3,5-difluorobenzyl bromide, toluene, 90 °C; (c) PPh₃, DIAD, DPPA, THF; (d) DIAD, PPh₃, CHCl₃, reflux; (e) 3,5-difluorophenol, K₂CO₃, DMF, 100 °C; (f) PPh₃, DIAD, DPPA, THF; (g) R₂NH₂, DIPEA, 2-hydroxypyridine, THF (dry), reflux; (h) PPh₃, MeOH, H₂O; (i) R₃COOH, DIPEA, HATU, DMF.

To obtain final products that were shortened by one carbon atom, the diol **69** was allowed to react with DIAD and PPh₃ in refluxing chloroform to provide the epoxide **72** in 63% yield.[105, 106] Epoxide **72** was regioselectively opened at the least hindered face by use of 3,5-difluorophenol in warm DMF (110 °C) in the presence of potassium carbonate, which gave the alcohol **73** in 91% yield.[104] Unfortunately, when using this reaction protocol, racemization occurred at the methyl group in compound **73**, and the racemic mixture could not be separated by flash column chromatography or HPLC. Protocols without a base catalyst were tested but resulted in lower yields and racemization. The racemic mixture of the alcohol **73** was converted to the corresponding azide with inversion of configuration at C5 under the Mitsunobu-like conditions described above to yield the separable diastereomers **74-(R)** and **75-(S)** in 32% and 42% yield, respectively.[104] The stereochemical assignment was confirmed by NOESY experiments (Section 6.5 and Appendix A).

The lactones **71**, **74-(R)**, and **75-(S)** could be further decorated with different substituents to provide novel aspartic protease inhibitors. It is possible that opening of these lactones with different amines (R₂NH₂) by use of DIPEA and 2-hydroxypyridine in refluxing THF[107], Weinreb amidation (Me₃Al)[108] or opening accomplished with LiOH followed by protection of the secondary alcohol[109] might give **E**. By applying reduction of the azide in compound **E**, compound **F** could be made. We assume that final products can be made by peptide coupling chemistry with different acids (R₃COOH) to give **G**.

6.5 Structure Determination by NOESY Experiments

Stereochemical assignment of the diol **69** was confirmed by ^1H - ^1H -NOESY experiments, which was possible because the compound has three significant NOE correlations. The NOESY spectrum of **69** shows that the hydrogen $\text{H}_{3\alpha}$ is in proximity to the hydrogen atoms in both the methyl group and H_4 (Figure 16). $\text{H}_{3\alpha}$ is spatially correlated with H_2 , which indicates that the methyl group at C2 in **69** has the desired (*R*) configuration. As mentioned above, opening of the epoxide **72** led to epimerization at the methyl group, resulting in an inseparable diastereomeric mixture of compound **73**. The alcohol in compound **73** was subjected to a Mitsunobu-like reaction to yield the corresponding azides as separable diastereomers, assumed to be **74**(*R*) and **75**(*S*). To corroborate the presupposed stereochemistry, ^1H - ^1H -NOESY experiments were performed on both **74**(*R*) and **75**(*S*). The NOESY spectrum of **74**(*R*) shows that H_4 is spatially correlated with the methyl hydrogen at C2, which is consistent with the (*R*) configuration in the lactone **74**(*R*) (Figure 16). In the NOESY spectrum of **75**(*S*), H_4 is correlated with H_2 , which indicates that the lactone **75**(*S*) has the presumed (*S*) configuration. NOESY spectra of compounds **69**, **74**(*R*), and **75**(*S*) are provided in Appendix A.

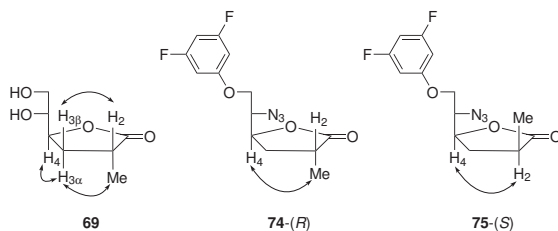


Figure 16: The significant NOE correlations observed in the NOESY spectra of compounds **69**, **74**(*R*) and **75**(*S*).

7 Summary

7.1 Conclusions (Paper I and II)

The compound 4-hydroxy-cyclopent-2-ene-1,2-dicarboxylic acid is a novel *N*-acyl-hydroxy-proline bioisostere, and it was used as scaffold **6** in the present research (Papers I and II). We synthesized this isostere from commercially available (*syn*)-tetrahydrophthalic anhydride, and its regio- and stereochemistry were confirmed by use of NOESY and HMBC spectra. Scaffold **6** was systematically and respectively derivatized with three different amines in the P1 position and six different amines in the P3/P4 position, and the P2 substituent (7-methoxy-2-phenyl-quinolin-4-ol) was held constant. Eighteen inhibitors were generated with this synthetic route, and their effects on HCV NS3 protease were tested. The most potent inhibitor has a K_i value of 1.1 nM. Moreover, we found that compound **51a**, which comprises our novel hydroxy-cyclopentene as a P2 *N*-acyl-hydroxyproline isostere, and the previously reported [46, 59, 75] *N*-acyl-hydroxy-proline analog compound **C** (Figure 6) are equipotent. This observation proves that our hydroxy-cyclopentene is a good isostere of the widely used *N*-acyl-hydroxyproline analog.

Encouraged by this result a synthetic route was developed, that successfully yielded two macrocyclic inhibitors, **57** and **58**, which have good inhibitory properties. Unfortunately, like the linear inhibitors, **57** and **58** are somewhat susceptible to Michael's addition and β -elimination of the quinoline moiety. Nevertheless, the compounds synthesized in the current research can provide useful information for future development of protease inhibitors.

7.2 Conclusions (Appendix A)

In our work focused on synthesizing aspartic protease inhibitors, we succeeded in developing a novel route from carbohydrates to interesting lactones. Notably, this approach yielded the key intermediate **69** with excellent stereochemical control that has been corroborated by NOESY experiments. Our synthesis protocol gave three different lactones that can be decorated with different substituents in the future to obtain novel aspartic protease inhibitors that differ with regard to linker size at the P₁ position and stereochemistry at the methyl group in the P₁' position.

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Appendices

A Experimental Section

General methods. Standard ^1H -NMR and ^{13}C -NMR spectra were recorded on a Varian 300 instrument using CDCl_3 or methanol- d_4 (CD_3OD) with TMS as an internal standard. NOESY spectra that were used to determine the structure of compound **69**, **74**-(*R*) and **75**-(*S*) were recorded on Varian 500 instrument using CDCl_3 or CD_3OD with TMS as an internal standard. In the recorded NMR spectra of the diastomeric mixture **73** the presumed diastomeric peaks were put into brackets with the general formula [nn.n & nn.n] for ^{13}C -NMR and [n.nn & n.nn, (x, yH)] for ^1H -NMR, respectively. TLC was carried out on Merck precoated 60 F₂₅₄ plates using UV-light and charring with ethanol/sulfuric acid/acetic acid/p-anisaldehyde (90:3:1:2) or a solution of 0.5% ninhydrine in ethanol (95%) for visualization. Flash column chromatography was performed using silica gel 60 (0.040–0.063 mm, Merck). Preparative HPLC was performed on a Gynkotek (pump: P580, detector: UVD 170S, software: Chromeleon) using a Kromasil 100-10-C18 (250 x 20 mm) column. Mixtures of deionized water and methanol with 0.1% TFA or 0.1% TEA were used as mobile phase. LC/MS was performed on a Gilson system (Column: Phenomenex C18 250x15 mm and Phenomenex C18 150x4.6 mm for preparative and analytical runs respectively; Pump: Gilson gradient pump 322; UV/VIS-detector: Gilson 155; MS detector: Thermo Finnigan Surveyor MSQ; Gilson Fraction Collector FC204, software: Gilson UniPoint ver. 4.0 and Xcalibur ver. 1.3) using methanol with 0.1% formic acid and deionized water with 0.1% formic acid as mobile phase. Concentrations were performed under diminished pressure (1-2 kPa) at a bath temperature of 40 °C. All low degree temperature reactions were accomplished by submerging the reaction vessels into an ethanol bath that had been cooled by additions of liquid nitrogen. All degassing of solvents were achieved using ultra sonification (Ultrasonik 104X) for at least two hours. Drying of solvents: THF was refluxed over sodium/benzophenone and distilled onto 4 Å MS, toluene and dichloromethane was refluxed over calcium hydride and distilled onto 4 Å MS. Organic extracts was dried over magnesium sulfate monohydrate and filtered. Filtrating was achieved using filter paper Munktell, OOH quality.

5,6-di-*O*-benzyl-3-deoxy-1,2-*O*-isopropylidene- α -D-glucofuranoside (63**).** Compound **63** was synthesized from commercially available 1,2:5,6-di-*O*-isopropylidene- α -D-glucofuranose in a four-step protocol according to literature procedure in an overall yield of 73%.^[94, 95, 96, 97]

Methyl 5,6-di-*O*-benzyl-3-deoxy- α -D-glucofuranoside (64 α**) and**

methyl 5,6-di-*O*-benzyl-3-deoxy- β -D-glucofuranoside (64 β). Compound **63** (6.21 g, 16.2 mmol) was dissolved in methanol and cooled in an ice bath (0 °C) and conc. H₂SO₄ (2.69 mL, 48.5 mmol) was slowly added to the solution. The reaction mixture was stirred overnight at room temperature and then neutralized with NaHCO₃ and concentrated. The residue was extracted with ethyl acetate (3 x 50 mL) and H₂O (50 mL). The combined organic layers were dried, filtered and concentrated. Purification by flash column chromatography (toluene/ethyl acetate 3:1) yielded the α - and β -anomers in an 11:2 ratio (**64 α** :**64 β**) as transparent oils (4.69 g, 80% and 0.84 g, 14% for the α - and β -anomers, respectively). **64 α** : ¹H-NMR (300 MHz, CDCl₃): δ 2.0 (dd, $J = 6.6, 13.7$ Hz, 1H), 2.14 (dq, $J = 4.7, 13.5$ Hz, 1H), 3.30 (s, 3H), 3.57-3.69 (m, 2H), 3.80 (dd, $J = 2.7, 10.2$ Hz, 1H), 4.23 (t, $J = 4.9$ Hz, 1H), 4.39-4.47 (m, 1H), 4.53-4.66 (m, 3H), 4.75-4.81 (m, 2H), 7.22-7.38 (m, 10H); ¹³C-NMR (75.5 MHz, CDCl₃): δ 35.5, 54.9, 71.3, 73.2, 73.7, 76.0, 79.0, 81.8, 109.8, 127.8, 127.9, 128.1, 128.6, 128.7, 138.7, 139.0. **64 β** : ¹H-NMR (300 MHz, CDCl₃): δ 1.76-1.88 (m, 1H), 2.20-2.30 (m, 1H), 2.40 (d, $J = 11.8$ Hz, 1H), 3.47 (s, 3H), 3.56 (d, $J = 5.5$ Hz, 1H), 3.68-3.75 (m, 1H), 4.20-4.30 (m, 1H), 4.31-4.40 (m, 1H), 4.54 (s, 2H), 4.57-4.64 (m, 1H), 4.70 (d, $J = 17.9$ Hz, 1H), 4.75-4.85 (m, 1H), 7.23-7.39 (m, 10H); ¹³C-NMR (75.5 MHz, CDCl₃): δ 32.8, 55.2, 70.4, 72.0, 73.3, 73.4, 76.0, 79.6, 102.5, 127.3, 127.5, 127.6, 127.7, 128.2, 128.3, 128.4, 129.0, 138.1, 138.7.

Methyl 5,6-di-*O*-benzyl-3-deoxy-2-*O*-(imidazolethiocarbonyl)-D-glucofuranoside (65). The alcohol **64** (α - and β -anomer mixture) (2.24 g, 6.25 mmol) and 1,1-thiocarbonyldiimidazole (1.67 g, 9.37 mmol) was dissolved in THF (31 mL) and the mixture was refluxed overnight. The crude residue was concentrated and purified by flash column chromatography (toluene/ethyl acetate 3:1) to yield compound **65** (2.92 g, 99%) as a pale yellow oil. ¹H-NMR (300 MHz, CDCl₃): δ 2.29-2.50 (m, 2H), 3.37 (s, 3H), 3.61-3.73 (m, 2H), 3.76-3.84 (m, 1H), 4.43-4.53 (m, 1H), 4.60 (dd $J = 7.4, 12.1$ Hz, 2H), 4.64 (d, $J = 11.5$ Hz, 1H), 4.80 (d, $J = 11.5$ Hz, 1H), 5.10 (s, 1H), 5.74 (d, $J = 4.1$ Hz, 1H), 7.05-7.07 (m, 1H), 7.27-7.39 (m, 10H), 7.60-7.62 (m, 1H), 8.33-8.34 (m, 1H); ¹³C-NMR (75.5 MHz, CDCl₃): δ 32.1, 54.9, 70.1, 72.7, 73.4, 79.2, 80.8, 86.1, 106.0, 117.7, 127.7, 127.5, 127.6, 128.2, 128.3, 130.9, 136.7, 138.0, 138.3, 183.0. MS m/z 469.3 [(M+H)⁺ calcd for C₂₅H₂₉N₂O₅S⁺ 469.18].

Methyl 5,6-di-*O*-benzyl-2,3-dideoxy-D-glucofuranoside (66). Tributyl tin hydride (5.18 g, 17.79 mmol) was dissolved in dry toluene (35 mL) under N₂-atmosphere and refluxed for 5 minutes. Compound **65** (5.56 g, 11.86 mmol) dissolved in dry toluene (35 mL) was added drop wise to the solution during 30 min. The combined solution was stirred at 110 °C and after 2 hours the mixture was concentrated. Purification by flash column

chromatography (toluene/ethyl acetate 18:1) yielded compound **66** (2.94 g, 72%) as colorless semi-crystals. $^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 1.87-1.98 (m, 4H), 3.25 (s, 3H), 3.55-3.66 (m, 2H), 3.81 (dd, $J = 2.2, 9.9$ Hz, 1H), 4.10 (dt, $J = 6.5, 8.2$ Hz, 1H), 4.45 (s, 1H), 4.61 (d, $J = 11.6$ Hz, 1H), 4.74 (d, $J = 11.6$ Hz, 1H), 4.80 (s, 1H), 4.92 (t, $J = 2.4$ Hz, 1H), 7.24-7.38 (m, 10H); $^{13}\text{C-NMR}$ (75.5 MHz, CD_3OD): δ 27.4, 33.5, 55.0, 71.8, 73.8, 74.4, 80.7, 82.7, 106.7, 128.6, 128.7, 128.8, 129.0, 129.2, 129.3, 139.8, 140.0. MS m/z 365.4 [(M+Na) $^+$ calcd for $\text{C}_{21}\text{H}_{26}\text{NaO}_4^+$ 365.17].

5,6-Di-O-benzyl-2,3-dideoxy-D-glucono-1,4-lactone (67). The methyl-acetal **66** (2.79 g, 8.16 mmol) was dissolved in dry CH_2Cl_2 (50 mL) and cooled to 0 °C in an ice bath. BF_3OEt (0.52 mL, 2.04 mmol) and *m*-chloroperbenzoic acid (2.20 g, 9.79 mmol) were added to the solution and the mixture was kept at 0 °C for 2 hours before it was allowed to reach room temperature. After 4 hours the mixture was concentrated and extracted with ethyl acetate (3 x 50 mL) and saturated NaHCO_3 (50 mL). The combined organic layers were dried, filtered and concentrated. The crude residue was purified by flash column chromatography (toluene/ethyl acetate 18:1) to yield the lactone **67** (2.66 g, quant.) as white crystals. $^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 2.14-2.25 (m, 2H), 2.44-2.50 (m, 2H), 3.60 (d, $J = 5.3$ Hz, 2H), 3.86 (dt, $J = 3.6, 5.3$ Hz, 1H), 4.49 (d, $J = 12.0$ Hz, 1H), 4.53 (d, $J = 12.0$ Hz, 1H), 4.56 (d, $J = 11.5$ Hz, 1H), 4.67 (d, $J = 11.5$ Hz, 1H), 4.68-4.75 (m, 1H), 7.25-7.34 (m, 10H); $^{13}\text{C-NMR}$ (75.5 MHz, CD_3OD): δ 23.2, 29.3, 69.9, 74.3, 74.5, 80.0, 81.9, 128.7, 128.8, 128.9, 129.0, 129.3, 129.4, 139.4, 139.6, 180.2. MS m/z 327.3 [(M+H) $^+$ calcd for $\text{C}_{20}\text{H}_{23}\text{O}_4^+$ 327.39].

5,6-Di-O-benzyl-2,3-dideoxy-2-methyl-D-glucono-1,4-lactone (68). The lactone **67** (1.31 g, 4.01 mmol) was dissolved in dry THF (40 mL) and cooled to -78 °C. After 15 min a solution of 2.0 M LDA (2.47 mL, 4.01 mmol) was added drop wise. After 30 min at -78 °C methyl iodine (2.5 mL, 40.1 mmol) dissolved in dry THF (5 mL) was slowly added. After further 2 hours at -78 °C the reaction was allowed to attain room temperature and quenched with saturated ammonium chloride (4 mL). The mixture was diluted with H_2O (50 mL) and extracted with ethyl acetate (3 x 50 mL). The combined organic layers were dried, filtered and concentrated. Purification by flash column chromatography (toluene/ethyl acetate 18:1) gave compound **68** (899 mg, 66%) as a pale yellow oil. $^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 1.23 (d, $J = 7.42$, 3H), 1.78-1.91 (m, 1H), 2.43-2.53 (m, 1H), 2.66-2.81 (m, 1H), 3.53-3.65 (m, 2H), 3.81-3.90 (m, 1H), 4.47-4.73 (m, 5H), 7.23-7.42 (m, 10H); $^{13}\text{C-NMR}$ (75.5 MHz, CDCl_3): δ 16.2, 30.4, 34.0, 68.9, 73.4, 73.5, 77.5, 78.5, 127.6, 127.7, 127.8, 127.9, 128.3, 128.4, 137.7, 137.8, 180.3. MS m/z 341.4 [(M+H) $^+$ calcd for $\text{C}_{21}\text{H}_{25}\text{O}_4^+$ 341.17].

2,3-Dideoxy-2-methyl-D-glucono-1,4-lactone (69). Compound **68**

(174 mg, 0.51 mmol) was dissolved in ethanol (2.5 mL, 95%) and Pd-C (~5 mg) was added. The reaction was performed under H₂-atmosphere, which was refilled until the reaction was complete. The mixture was filtered through a pad of celite and concentrated. This gave after co-concentration with methanol and toluene the diol **69** (83 mg, quant.) as white crystals. ¹H-NMR (300 MHz, CD₃OD): δ 1.23 (d, *J* = 7.4 Hz, 3H), 1.85-1.98 (m, 1H), 2.45-2.57 (m, 1H), 2.72-2.86 (m, 1H), 3.52-3.59 (m, 2H), 3.74-3.84 (m, 1H), 4.53-4.61 (m, 1H); ¹³C-NMR (75.5 MHz, CD₃OD): δ 15.3, 30.0, 34.3, 62.7, 72.4, 78.7, 181.8. MS *m/z* 182.9 [(M+Na)⁺ calcd for C₇H₁₂NaO₄⁺ 183.06].

2,3-Dideoxy-6-O-(3,5-difluorobenzyl)-2-methyl-D-glucono-1,4-lactone (70). The diol **69** (201 mg, 1.25 mmol) and dibutyltin oxide (405 mg, 1.63 mmol) was dissolved in toluene (7 mL). The mixture was refluxed for 5 hours before the temperature was lowered to 90 °C and tetrabutylammonium bromide (464 mg, 1.44 mmol) and 3,5-difluoro benzylbromide (0.186 ml, 1.44 mmol) were added. The mixture was allowed to stir at 90 °C overnight and thereafter concentrated. Purification by flash column chromatography (toluene/ethyl acetate 6:1) gave compound **70** (291 mg, 81%) as an colourless oil. ¹H-NMR (300 MHz, CD₃OD): δ 1.22 (d, *J* = 7.3 Hz, 3H), 1.92 (dt, *J* = 8.6, 13.0 Hz, 1H), 2.52 (ddd, *J* = 3.6, 9.5, 13.0 Hz, 1H), 2.79 (ddq, *J* = 7.3, 8.6, 9.5 Hz, 1H), 3.54 (dd, *J* = 5.7, 10.8 Hz, 1H), 3.58 (dd, *J* = 5.0, 10.8 Hz, 1H), 3.95 (dt, *J* = 4.7, 5.7 Hz, 1H), 4.55 (s, 2H), 4.59 (ddd, *J* = 3.6, 4.7, 8.6 Hz, 1H), 6.78-6.86 (m, 1H), 6.92-7.00 (m, 2H); ¹³C-NMR (75.5 MHz, CD₃OD): δ 16.4, 31.3, 35.3, 71.9, 72.5, 72.9, 79.7, 103.4 (t, *J*_{CF} = 25.8 Hz), 110.9 (d, *J*_{CF} = 25.5 Hz, 2C), 144.4 (t, *J*_{CF} = 8.9 Hz), 164.3 (d, *J*_{CF} = 247.5 Hz), 164.5 (d, *J*_{CF} = 247.5 Hz), 182.8. MS *m/z* 287.2 [(M+H)⁺ calcd for C₁₄H₁₇F₂O₄⁺ 287.11].

5-Azido-2,3,5-trideoxy-6-O-(3,5-difluorobenzyl)-2-methyl-L-ido-1,4-lactone (71). Compound **70** (150 mg, 0.53 mmol) was dissolved in dry THF (5 mL) and cooled to -15 °C (ice/acetone 1:1). Triphenylphosphine (207 mg, 0.79 mmol) and diisopropyl azodicarboxylate (0.156 mL, 0.79 mmol) were added. After 10 min the mixture was allowed to reach 0 °C and diphenylphosphoryl azide (217 mg, 0.79 mmol) was added. After 30 min of stirring at 0 °C the mixture was allowed to attain room temperature and was stirred overnight. The solvent was evaporated, and the crude mixture was purified by flash column chromatography (toluene/ethyl acetate 18:1) yielding the azide **71** (152 mg, 93%) as an colourless oil. ¹H-NMR (300 MHz, CD₃OD): δ 1.22 (d, *J* = 7.4 Hz, 3H), 2.05 (dt, *J* = 8.4, 13.2 Hz, 1H), 2.42 (ddd, *J* = 4.1, 9.6, 13.2 Hz, 1H), 2.83 (ddq, *J* = 7.4, 8.4, 9.6 Hz, 1H), 3.73 (dd, *J* = 8.0, 9.9 Hz, 1H), 3.79 (dd, *J* = 4.0, 9.9 Hz, 1H), 3.80-3.88 (m, 1H), 4.58 (s, 2H), 4.65 (dt, *J* = 4.1, 8.4 Hz, 1H), 6.77-6.86 (m, 1H), 6.91-6.99 (m, 2H); ¹³C-NMR (75.5 MHz, CD₃OD): 16.4, 33.5, 34.9, 65.4, 71.6, 72.8,

72.9, 103.6 (t, $J_{CF} = 25.8$ Hz), 110.9 (d, $J_{CF} = 25.2$ Hz, 2C), 143.9 (t, $J_{CF} = 8.9$ Hz), 164.4 (d, $J_{CF} = 247.2$ Hz), 164.6 (d, $J_{CF} = 247.2$ Hz), 182.0.

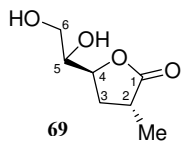
5,6-Anhydro-2,3-dideoxy-2-methyl-D-glucono-1,4-lactone (72).

The diol **69** (119 mg, 0.75 mmol) was dissolved in chloroform (37 mL), triphenylphosphine (293 mg, 1.12 mmol) and diisopropyl azodicarboxylate (220 μ L, 1.12 mmol) were added. The mixture was refluxed overnight before the solvent was evaporated. The crude mixture was purified by flash column chromatography (toluene/ethyl acetate 9:1) to give the epoxide **72** (66 mg, 63%) as an colorless oil. $^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 1.13 (d, $J = 6.32$ Hz, 3H), 1.76-1.87 (m, 1H), 2.06-2.17 (m, 1H), 2.50-2.56 (m, 1H), 2.57-2.68 (m, 1H), 2.74 (t, $J = 4.67$ Hz, 1H), 3.06-3.12 (m, 1H), 4.40-4.49 (m, 1H); $^{13}\text{C-NMR}$ (75.5 MHz, CDCl_3): δ 15.3, 30.2, 33.3, 44.2, 51.5, 76.5, 179.1.

2,3-Dideoxy-6-O-(3,5-difluorophenyl)-2-methyl-D-glucono-1,4-lactone and 2,3-dideoxy-6-O-(3,5-difluorophenyl)-2-methyl-D-manno-1,4-lactone (73). To the epoxide **72** (66 mg, 0.47 mmol) in DMF (2.4 mL) were 3,5-difluorophenol (92 mg, 0.71 mmol) and K_2CO_3 (37 mg, 0.24 mmol) added. The reaction mixture was heated to 110 $^\circ\text{C}$ for 4 hours. The DMF was removed by co-evaporation with toluene (3 x 15 mL). The crude residue was purified by flash column chromatography (toluene/ethyl acetate 9:1) to yield a diastereomeric mixture of compound **73** (116 mg, 91%) as an colourless oil. $^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 1.21-1.29 (m, 3H), 1.87-2.02 (m, 1H), 2.41-2.63 (m, 1H), 2.64-2.85 (m, 1H), [3.31 (d, $J = 4.94$ Hz, 0.5H) & 3.47 (d, $J = 5.22$ Hz, 0.5 Hz, 0.5H)], 3.98-4.04 (m, 2H), 4.10-4.20 (m, 1H), [4.46-4.53 (m, 0.5H), 4.55-4.63 (m, 0.5H)], 6.38-6.48 (m, 3H); $^{13}\text{C-NMR}$ (75.5 MHz, CD_3OD): δ [15.4 & 16.4], [31.5 & 32.8], [35.3 & 36.5], 70.8, [71.2 & 71.4], 79.3, 97.2 (t, $J_{CF} = 25.7$ Hz), 99.5 (d, $J_{CF} = 28.9$ Hz, 2C), 162.3 (t, $J_{CF} = 13.7$ Hz), 165.0 (d, $J_{CF} = 247.1$ Hz), 165.5 (d, $J_{CF} = 247.1$ Hz), [181.7 & 182.6]. MS m/z 272.8 [(M+H) $^+$ calcd for $\text{C}_{13}\text{H}_{15}\text{F}_2\text{O}_4^+$ 273.09].

5-Azido-2,3,5-trideoxy-6-O-(3,5-difluorobenzyl)-2-methyl-L-idono-1,4-lactone (74-(R)) and 5-azido-2,3,5-trideoxy-6-O-(3,5-difluorobenzyl)-2-methyl-L-gulono-1,4-lactone (75-(S)). A diastereomeric mixture of **73** (116 mg, 0.42 mmol) and triphenyl phosphine (168 mg, 0.64 mmol) was dissolved in dry THF (4.3 mL). The mixture was cooled to -15 $^\circ\text{C}$ (acetone/ice 1:1) and diisopropyl azodicarboxylate (126 μ L, 0.64 mmol) were added. The mixture was stirred for 30 min at -15 $^\circ\text{C}$ (acetone/ice 1:1) before the temperature was raised to 0 $^\circ\text{C}$ and diphenylphosphoryl azide (142 μ L, 0.64 mmol) was added. The reaction mixture was allowed to attain room temperature and was stirred overnight. The solvent was evaporated and the crude diastereomeric mixture was purified by flash column chromatography (toluene/ethyl acetate 18:1). The two diastereomers **74-(R)** (40 mg, 32%) and **75-(S)** (53 mg, 42%) were separated and collected as transparent oils.

74-(R): $^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 1.29 (d, $J = 7.1$ Hz, 3H), 2.02-2.16 (m, 1H), 2.40-2.53 (m, 1H), 2.82-2.98 (m, 1H), 3.86-3.95 (m, 1H), 4.21 (d, $J = 6.3$ Hz, 2H), 4.63-4.71 (m, 1H), 6.38-6.52 (m, 3H); $^{13}\text{C-NMR}$ (75.5 MHz, CDCl_3): δ 16.2, 32.6, 33.4, 63.3, 68.4, 75.2, 97.4 (t, $J_{CF} = 25.8$ Hz), 98.5 (d, $J_{CF} = 28.9$ Hz, 2C), 159.6 (t, $J_{CF} = 13.7$ Hz), 163.5 (d, $J_{CF} = 247.2$ Hz), 163.7 (d, $J_{CF} = 247.2$ Hz) 179.1. $[\alpha]_D^{22} = +63.5^\circ$ (chloroform). MS m/z 319.6 [(M+Na) $^+$ calcd for $\text{C}_{13}\text{H}_{13}\text{F}_2\text{N}_3\text{NaO}_3^+$ 320.08]. **75-(S)**: $^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 1.33 (d, $J = 4.1$ Hz, 3H), 1.87-2.01 (m, 1H), 2.43-2.56 (m, 1H), 2.66-2.80 (m, 1H), 3.80-3.87 (m, 1H), 4.17 (m, 2H), 4.52-4.62 (m, 1H), 6.41-6.52 (m, 3H); $^{13}\text{C-NMR}$ (75.5 MHz, CDCl_3): δ 15.1, 32.9, 35.0, 62.0, 68.2, 76.0, 97.4 (t, $J_{CF} = 26.1$ Hz), 98.5 (d, $J_{CF} = 29.2$ Hz, 2C), 163.5 (d, $J_{CF} = 247.4$ Hz), 163.8 (d, $J_{CF} = 247.4$ Hz), 177.9. $[\alpha]_D^{22} = +46.3^\circ$ (chloroform). MS m/z 319.6 [(M+Na) $^+$ calcd for $\text{C}_{13}\text{H}_{13}\text{F}_2\text{N}_3\text{NaO}_3^+$ 320.08].



Nomenclature of Protons:

Proton on C4 called H4

Proton on C2 called H2^α if on the same side as C4 otherwise H2^β

Proton on C3 called H3^α if on the same side as C4 otherwise H3^β

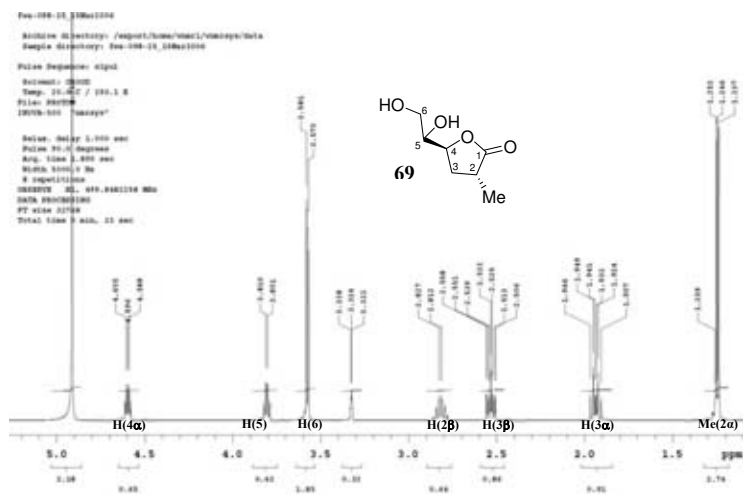


Figure 1. Proton spectrum of the compound **69** (CD₃OD)

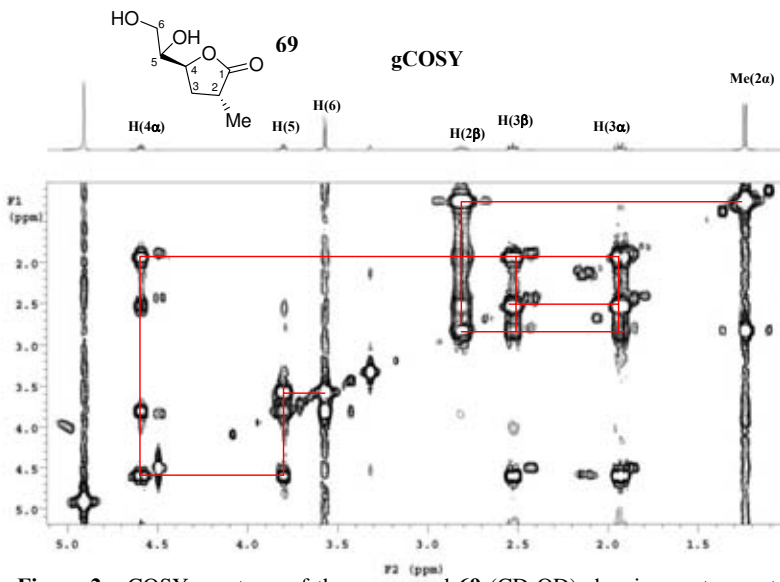


Figure 2. gCOSY spectrum of the compound **69** (CD_3OD) showing proton-proton correlation

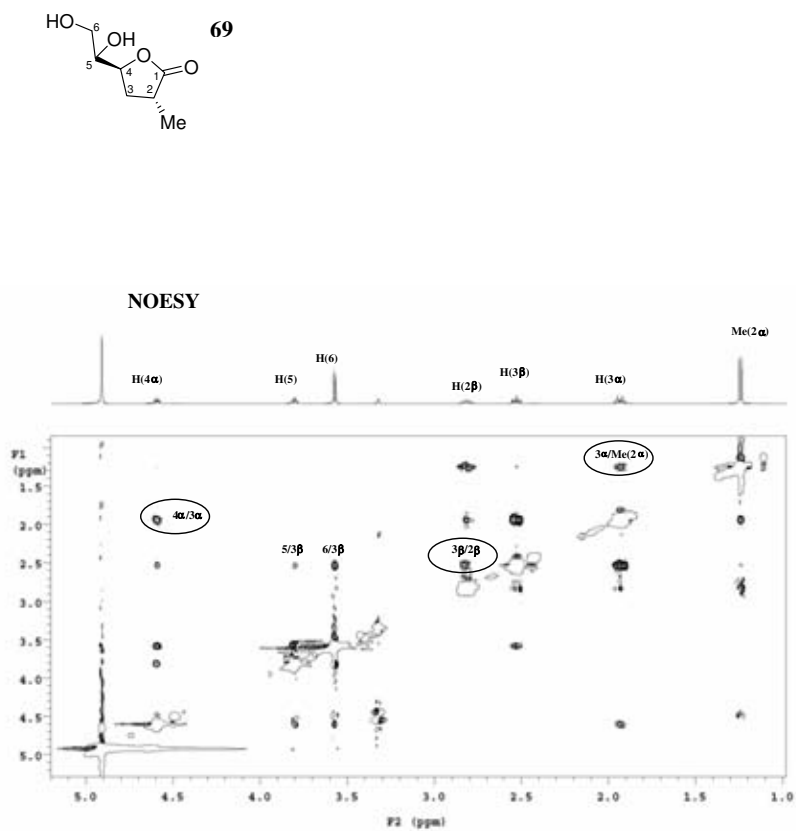
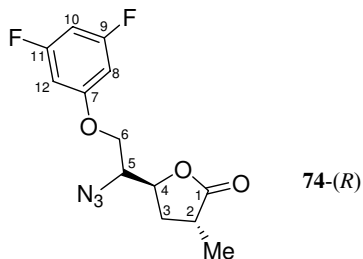


Figure 3. NOESY spectrum of the compound **69** (CD₃OD) showing proton-proton correlation through space.



Nomenclature of Protons:

Proton on C4 called H4^α

Proton on C2 called H2^β if on the same side as C4 otherwise H2^β

Proton on C3 called H3^α if on the same side as C4 otherwise H3^β

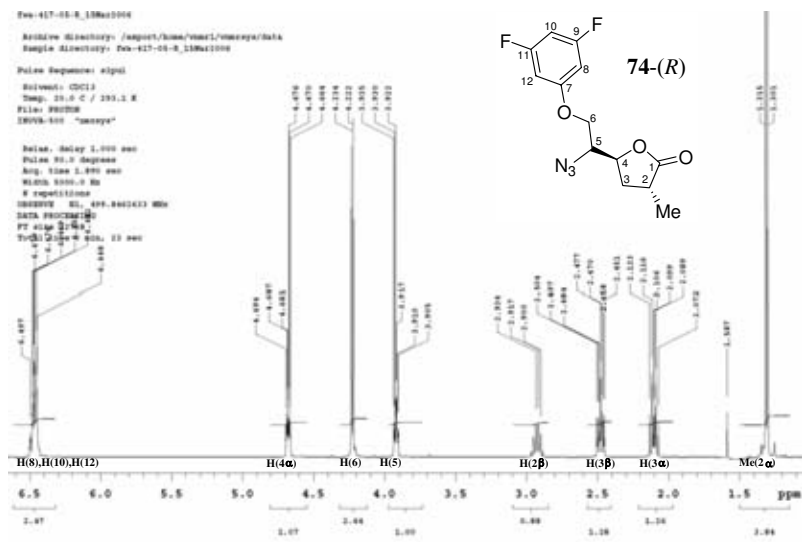


Figure 4. Proton spectrum of the compound **74-(R)** (CDCl_3)

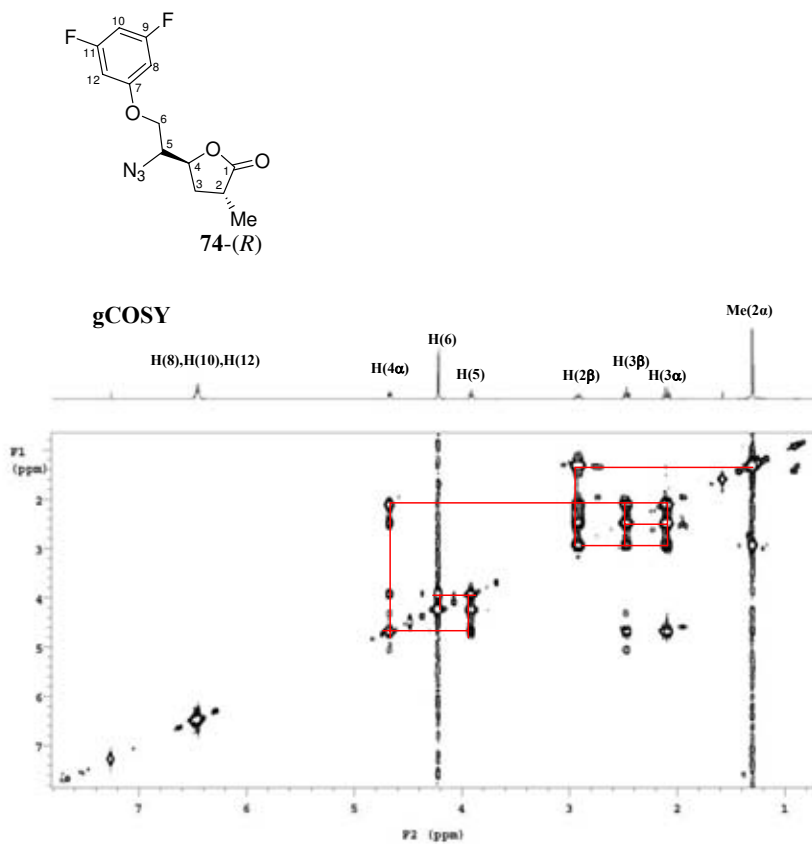


Figure 5. gCOSY spectrum of the compound 74-(R) (CDCl₃) showing proton-proton correlation

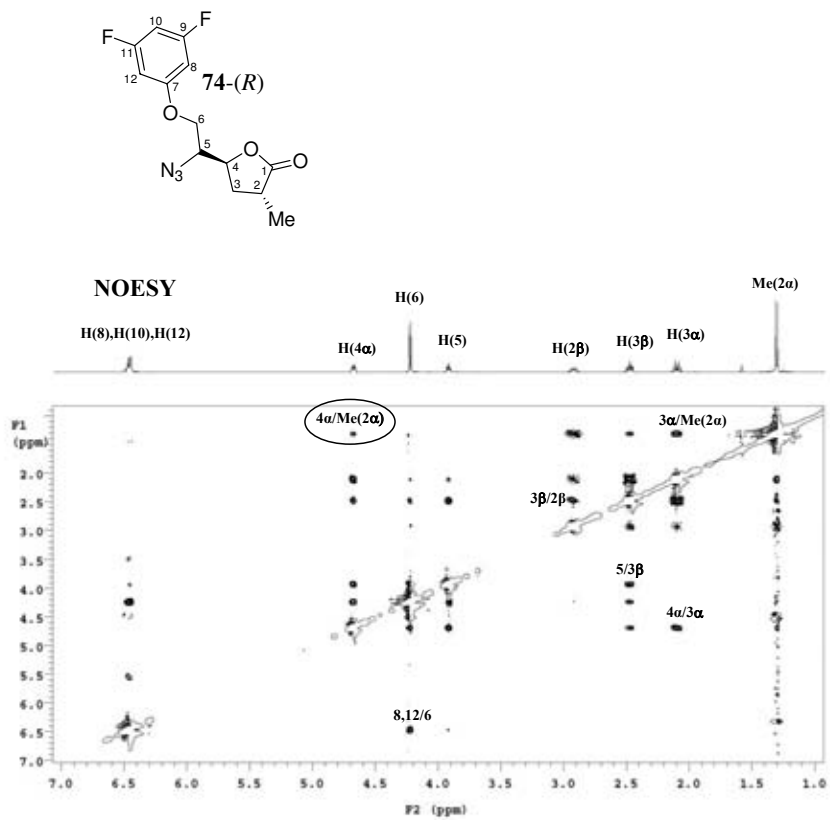
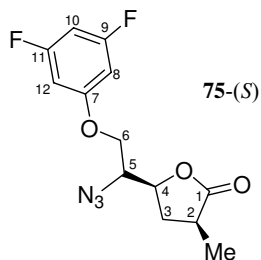


Figure 6. NOESY spectrum of the compound **74-(R)** (CDCl_3) showing proton-proton correlation through space.



Nomenclature of Protons:

Proton on C4 called H4^α

Proton on C2 called H2^α if on the same side as C4 otherwise H2^β

Proton on C3 called H3^α if on the same side as C4 otherwise H3^β

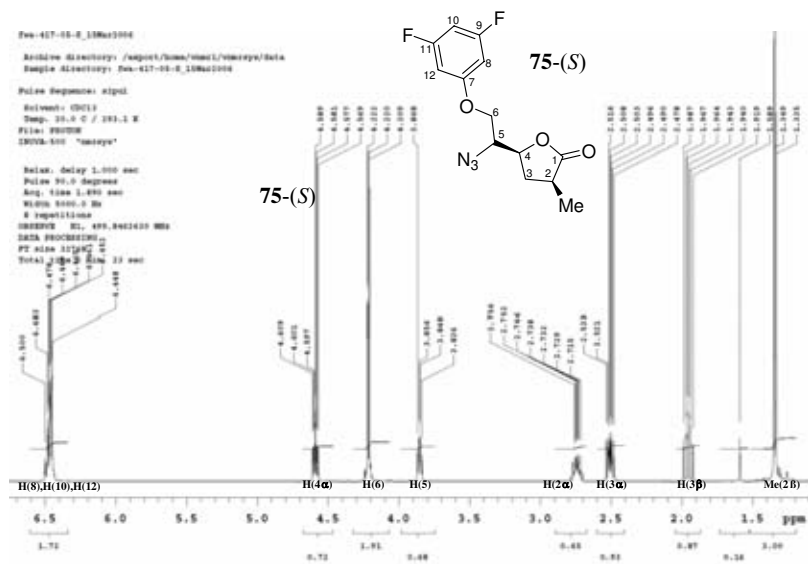


Figure 7. Proton spectrum of the compound **75-(S)** (CDCl_3)

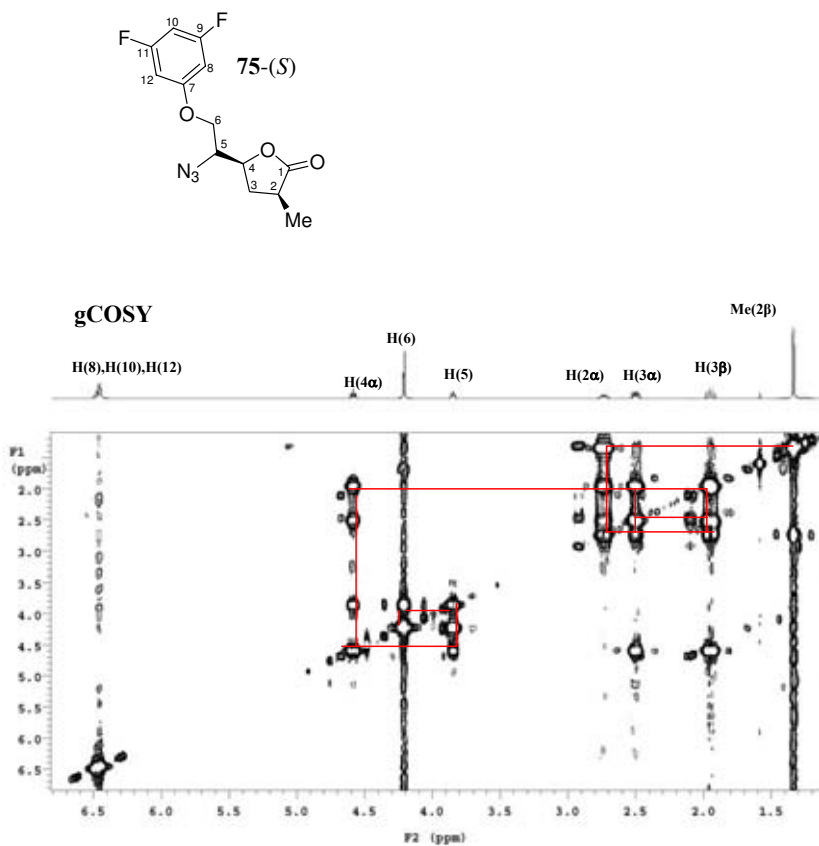


Figure 8. gCOSY spectrum of the compound **75-(S)** (CDCl_3) showing proton-proton correlation

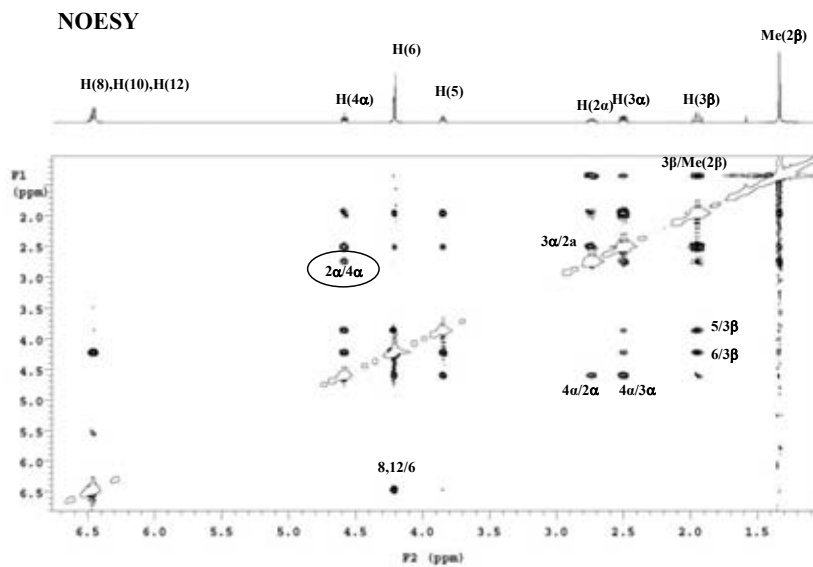
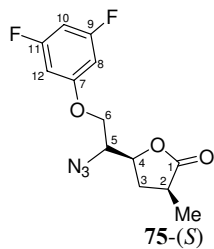


Figure 9. NOESY spectrum of the compound **75-(S)** (CDCl_3) showing proton-proton correlation through space.