Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 1448–1474
Bioorganic & Medicinal Chemistry

Phenylglycine as a novel P2 scaffold in hepatitis C virus NS3 protease inhibitors

Pernilla Örtqvist,[a] Shane D. Peterson,[a] Eva Åkerblom,[a] Thomas Gossas,[b] Yogesh A. Sabnis,[a] Rebecca Fransson,[a] Gunnar Lindeberg,[a] U. Helena Danielson,[b] Anders Karlén[a] and Anja Sandström[a,*]

[a]*Department of Medicinal Chemistry, Uppsala University, BMC, Box 574, SE-751 23 Uppsala, Sweden*
[b]*Department of Biochemistry, Uppsala University, BMC, Box 576, SE-751 23 Uppsala, Sweden*

Received 2 June 2006; revised 30 October 2006; accepted 2 November 2006
Available online 6 November 2006

Abstract—Molecular modeling and inhibitory potencies of tetrapeptide protease inhibitors of HCV NS3 proposed phenylglycine as a new promising P2 residue. The results suggest that phenylglycine might be capable of interacting with the NS3 (protease-helicase/NTPase) in ways not possible for the common P2 proline-based inhibitors. Thus, a series of tripeptides, both linear and macrocyclic, based on *p*-hydroxy-phenylglycine in the P2 position were prepared and their inhibitory effect determined. When the *p*-hydroxy group was replaced by methoxy, isoquinolin-, or quinolinyloxy functions, inhibitors with improved potencies were obtained. The P2 phenylglycine-based inhibitors were further optimized by C-terminal extension to acyl sulfonamides and by P1–P3 cyclization, which gave products with inhibition constants in the nanomolar range (~75 nM).

1. Introduction

Hepatitis C virus (HCV) is the major cause of chronic liver disease worldwide as well as the primary indication for liver transplantation.[1–3] With a prevalence of 123 million people globally, according to the most recent estimate from the World Health Organization, HCV infection constitutes a serious threat to global health.[1] The current standard therapy, comprised of pegylated α-interferon and the broad-spectrum antiviral agent ribavirin, is effective in roughly 50% of patients.[4] Consequently, development of new therapies is of utmost importance, especially since the number of people diagnosed with chronic HCV infection is expected to increase dramatically over the next decade.[3]

Hepatitis C is an encapsulated (+)-RNA virus targeting hepatocytes. Translation of the RNA gives a single polyprotein of about 3000 amino acids, which is proteolytically cleaved into 10 mature proteins: C, E1, E2, p7, and the non-structural (NS) proteins (2, 3, 4A, 4B, 5A, and 5B). NS3 is a bifunctional enzyme with serine protease and helicase/NTPase activities. The NS3 protease is responsible for *cis*-cleavage at the NS3/4A junction and *trans*-cleavages at the NS4A/4B, NS4B/5A, and NS5A/5B junctions. The catalytic efficiency of the NS3 protease has been shown to be improved by interaction with the NS4A cofactor.[5]

The NS3 protease has been one of the primary targets for anti-HCV drug development since the discovery of HCV in 1989.[6–10] Proof-of-concept was recently established by BILN 2061 (ciluprevir), the first HCV NS3 inhibitor to reach clinical trials.[11] While further development of ciluprevir has been halted due to cardiotoxicity in animals,[9] two other NS3 protease inhibitors, VX-950 (telaprevir) and SCH 503034, have demonstrated potent anti-HCV activity and have recently advanced into phase II trials.[12–16] The development of resistance mutations in the NS3 protease has been observed in in vitro tests against the three candidate drugs, indicating that a combination therapy may be necessary in the future, as has been effective in the treatment of HIV/AIDS.[17–19]

A substantial research effort has been devoted to P2 proline-based HCV NS3 protease inhibitors, including

Keywords: HCV; Phenylglycine; Protease inhibitor; Acyl sulfonamide.
* Corresponding author. Tel.: +46 18 417 4957; fax +46 18 471 4474; e-mail: Anja.Sandstrom@orgfarm.uu.se

0968-0896/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2006.11.003

development of the three clinical candidates mentioned above. To yield reasonable inhibitory activity, decoration of the proline ring has been necessary, indicating that P2–S2 interactions are important for potency.[9]

Previous studies by us have shown that 2-naphthylalanine is a good P2 building block in hexa- and tetrapeptides as protease inhibitors of the full-length NS3.[20,21] 2-Naphthylalanine was found to be better than both cyclohexylalanine and leucine in the P2 position,[20,21] which are two commonly used P2 residues.[22,23] These results were contradictory to results achieved from a peptide library by Ingallinella et al. where an assay comprising truncated NS3 (i.e., the protease domain alone) was used for evaluation. In that case P2 cyclohexylalanine was found to be superior to a diverse set of amino acids including 2-naphthylalanine and leucine.[24] Consequently, we considered the involvement of the helicase domain in protease inhibitor binding as a possible explanation for the differences in measured activity.[20,21]

In a subsequent study of tetrapeptides, we found that inhibitors with phenylglycine in P2 were of similar potency as those with P2 2-naphthylalanine (unpublished results) in the full-length NS3 assay. It should be noted that the potential of phenylglycine was also revealed in the Ingallinella library, where it was shown to be the best of the aromatic amino acids and similar in potency to both 2-naphthylalanine and leucine.[24] While cyclohexylalanine, leucine, and proline have been thoroughly examined as P2 residues in HCV NS3 protease inhibitors, no one has yet, to our knowledge, explored substituted phenylglycine analogues.[9,22,23]

We became interested in using phenylglycine in the P2 position of HCV NS3 protease inhibitors due to the possibility of forming interactions not possible with proline-based inhibitors. Another advantageous property of phenylglycine is its aromatic nature that allows synthetic decorations, including preparation of proprietary structures. The project was begun with the hope of producing inhibitors with potencies superior to analogous proline-based structures.

Herein, we present the synthesis, biochemical data and structure–activity relationships for a series of tripeptide P2 phenylglycine-based HCV NS3 protease inhibitors. Both linear and macrocyclic inhibitors with substituted *p*-hydroxyphenylglycine have been evaluated. Furthermore, the new phenylglycine series allows the comparison of carboxylic acids and acylsulfonamides as C-terminal functionalities and demonstrates the impact of various P1 residues. The combination of quinolinyloxy-type substitution of the P2 phenylglycine, C-terminal acylsulfonamides, and macrocyclization was successful in producing inhibitors with potencies in the nanomolar range. In addition, molecular modeling suggests differences in the relative positions of the P1 and P2 side chains, as compared to proline-based inhibitors, and a possible pi-stacking interaction between phenylglycine and the imidazole side chain of the catalytic histidine residue.

2. Results

2.1. Chemistry

The tetrapeptide HCV NS3 protease inhibitors **1** and **2** (Fig. 1) were synthesized by standard Fmoc/*t*-Bu solid-phase peptide synthesis.[25,26] Preparations of the linear and macrocyclic tripeptide inhibitors included in this study are outlined in Schemes 1–5.

The synthesis of the tripeptide inhibitors started with the assembly of the phenylglycine-based P2 building blocks presented in Scheme 1. The alkylated or heteroarylated 4-hydroxy-phenylglycine compounds **5**–**10** were synthesized from Boc-protected 4-hydroxy-L-phenylglycine, which was prepared from the commercially available 4-hydroxy-L-phenylglycine according to a published procedure.[27] The methyl ether **4** in Scheme 1 was prepared using an excess of methyl iodide and cesium carbonate in dry DMF to achieve methylation of both the hydroxy and the carboxylic group. The carboxylic acid **5** was produced by alkaline hydrolysis of the corresponding ester. Under the basic conditions employed in the hydrolysis, racemization occurred at the phenylglycine α-carbon, which was detected by the formation of epimers in the subsequent coupling step.

As depicted in Scheme 1, the heteroaryl ethers **6**–**9** were synthesized from **3** in nucleophilic aromatic substitution reactions using 4-chloro-7-methoxy-2-phenylquinoline[28] or commercially available 2,6-dichloropyridine, 2-fluoropyridine, and 1,3-dichloroisoquinoline, respectively, and potassium-*tert*-butoxide in DMSO. The nucleophilic substitutions with 4-hydroxy-phenylglycine (as nucleophile) were, as expected, slow at 40 °C. Attempts to increase the reaction speed by raising the temperature resulted in partial loss of the Boc group. In addition to their temperature sensitivity, the reactions were also very sensitive to moisture and minor impurities present in the reaction mixture. In the synthesis of **6**, this sometimes resulted in the transformation of the 4-chloro-7-methoxy-2-phenyl-quinoline to its 4-hydroxy, 4-alkoxy, or dechlorinated analogue. The substitution reactions were followed by LC–MS for several days, until the lim-

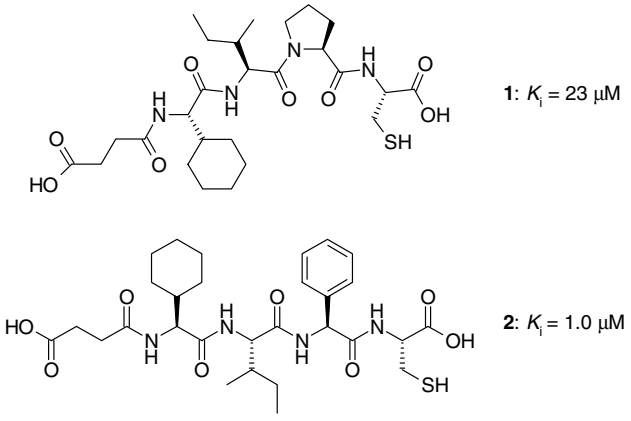
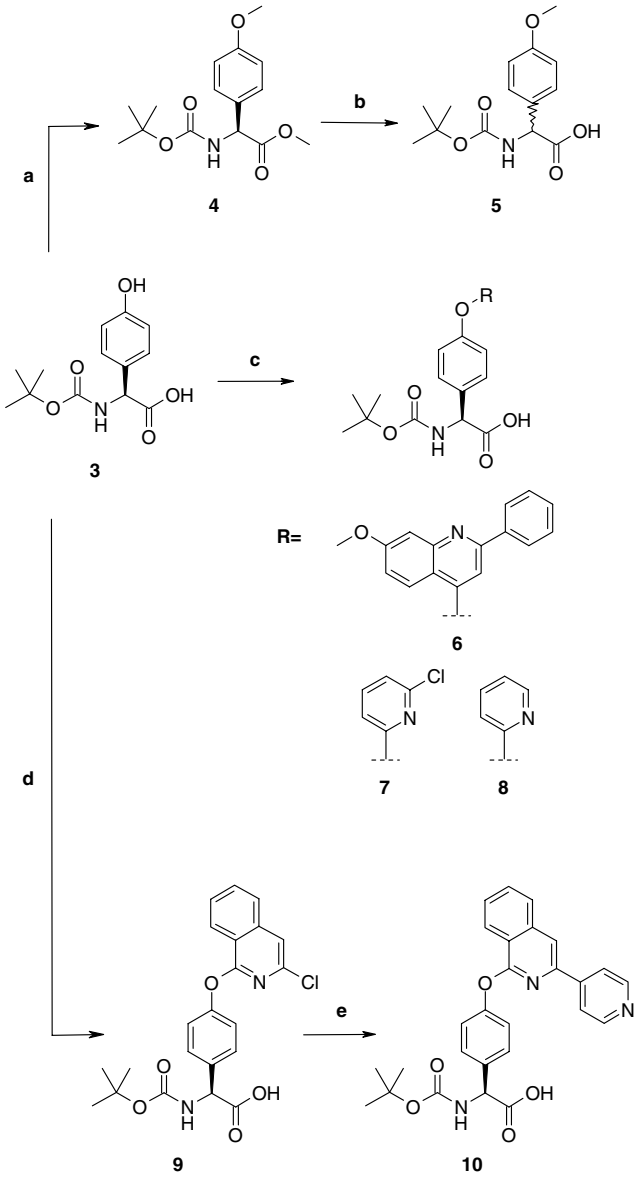


Figure 1. K_i values of two tetrapeptide protease inhibitors of full-length NS3 having proline (**1**) or phenylglycine (**2**) in P2 position.



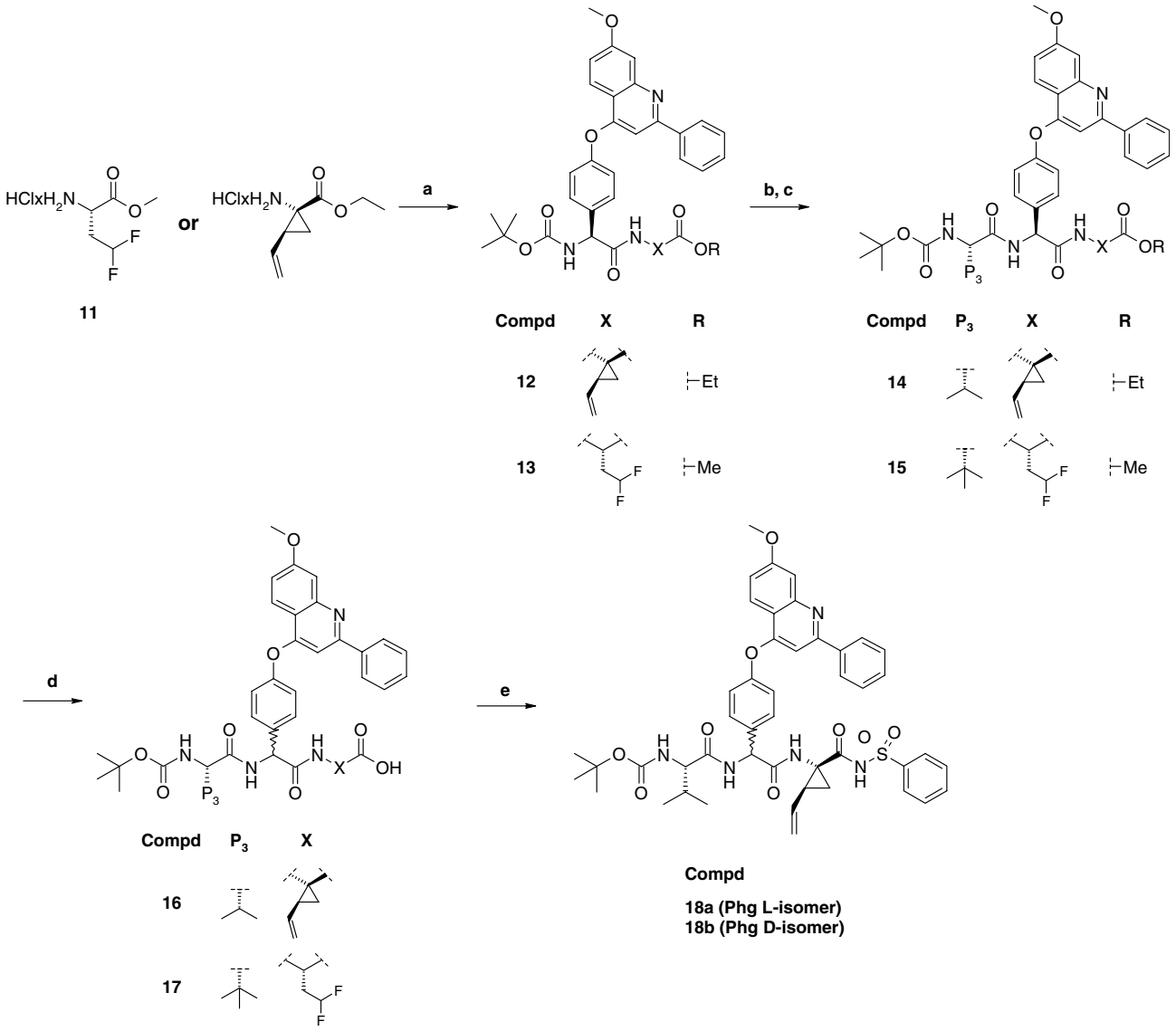
Scheme 1. Reagents: (a) MeI, Cs_2CO_3, DMF; (b) LiOH, THF/H_2O/MeOH; (c) 4-chloro-7-methoxy-2-phenylquinoline or 2,6-dichloropyridine or 2-fluoropyridine, KO-*t*-Bu, DMSO; (d) 1,3-dichloroisoquinoline, KO-*t*-Bu, DMSO; (e) 4-pyridine boronic acid, $Pd_2(dba)_3$, [(*t*-Bu)$_3$PH]BF_4, KF, THF.

iting starting material was fully consumed, which resulted in a 71–75% yield of **6**, **7**, and **8**, and a quantitative yield of **9**. The products were precipitated as their zwitterions by acidification of an alkaline aqueous solution to pH 5.2 for **6** and pH 3.6 for **7**–**9**. The P2 building block **10** was synthesized from its chlorine-substituted analogue **9** and 4-pyridineboronic acid in a microwave-heated Suzuki reaction.[29–31] After purification by preparative LC–MS, the desired product was isolated in 31% yield. The main side-product detected during the Suzuki reaction was the dechlorinated **9**.

All tripeptide inhibitors were prepared in solution by standard peptide coupling methods using *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) or *N*-[(1*H*-benzotriazole-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), and diisopropylethylamine in DMF, but using different synthetic routes as presented in Schemes 2 and 3. The synthetic route in Scheme 2 was designed to produce the C-terminal carboxylic acids, for comparison with the corresponding acyl sulfonamide bioisosteres. The main drawback of this approach was the necessity of alkaline ester hydrolysis leading to epimerization at the phenylglycine α-carbon.

The starting material **11** in Scheme 2 was prepared from the commercially available (*S*)-2-[(benzyloxycarbonyl)amino]-4,4-difluorobutanoic acid methyl ester (Cbz-difluoro-Abu-OMe) by catalytic hydrogenation. Since the corresponding free amine is volatile, the product was converted to its hydrochloride salt. The hydrochloride salt of (1*R*,2*S*)-1-amino-2-vinyl-cyclopropanecarboxylic acid ethyl ester and **11** were used in the coupling to **6**, producing dipeptides **12** and **13** in 54% and 55% yield, respectively. After removal of the Boc group in HCl/dioxane, Boc-L-valine was coupled to **12** and Boc-L-*tert*-leucine to **13** to give the ester-protected tripeptides **14** (77%) and **15** (52%). The esters were hydrolyzed using lithium hydroxide, to give the tripeptides **16** and **17** in 80% and 39% yield, respectively. The diastereomers could not be separated at this stage. The tripeptide **16** was transformed into the corresponding acyl sulfonamide using benzenesulfonamide, 1,1-carbonyldiimidazole (CDI), and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dry THF.[32] As sulfonamides, the P2 L-isomer **18a** and the corresponding D-isomer **18b** could be separated and isolated in 18% and 17% yield, respectively. When **17** was submitted to the same coupling conditions, it also racemized at the P1 (difluoro-Abu) α-carbon, which resulted in a mixture of diastereomers too complex to separate.

To minimize the problem of epimerization at the phenylglycine α-carbon, it was most convenient to couple the P2 building block to a preformed P1-acyl sulfonamide, as depicted in Scheme 3. The P1-acyl sulfonamide building blocks were prepared according to the first step in Scheme 3. Thus, the starting material (1*R*,2*S*)-1-[(*tert*-butoxycarbonyl)amino]-2-vinyl-cyclopropancarboxylic acid[33] was synthesized in two steps from the hydrochloride salt of (1*R*,2*S*)-1-amino-2-vinyl-cyclopropanecarboxylic acid ethyl ester by Boc-protection followed by ester hydrolysis using lithium hydroxide. (*S*)-2-[(Benzyloxycarbonyl)amino]-4,4-difluorobutanoic acid[34] was prepared from commercially available (*S*)-2-[(benzyloxycarbonyl)amino]-4,4-difluorobutanoic acid methyl ester by alkaline hydrolysis as described above. (1*R*,2*S*)-1-[(*tert*-butoxycarbonyl)amino]-2-vinyl-cyclopropancarboxylic acid, (*S*)-2-[(benzyloxycarbonyl)amino]-4,4-difluorobutanoic acid, and the commercially available amino acids Boc-L-norvaline and Boc-1-aminocyclopropane-1-carboxylic acid (Boc-ACCA) were transformed into the corresponding acyl sulfonamides **19**,[35,36] **20**,[36] **21**,[37] **22** (62%), and **23** (62%) using cyclopropylsulfonamide[28] or commercially available benzenesulfonamide, CDI, and DBU in dry THF. After acidic removal of the Boc groups and Cbz-removal

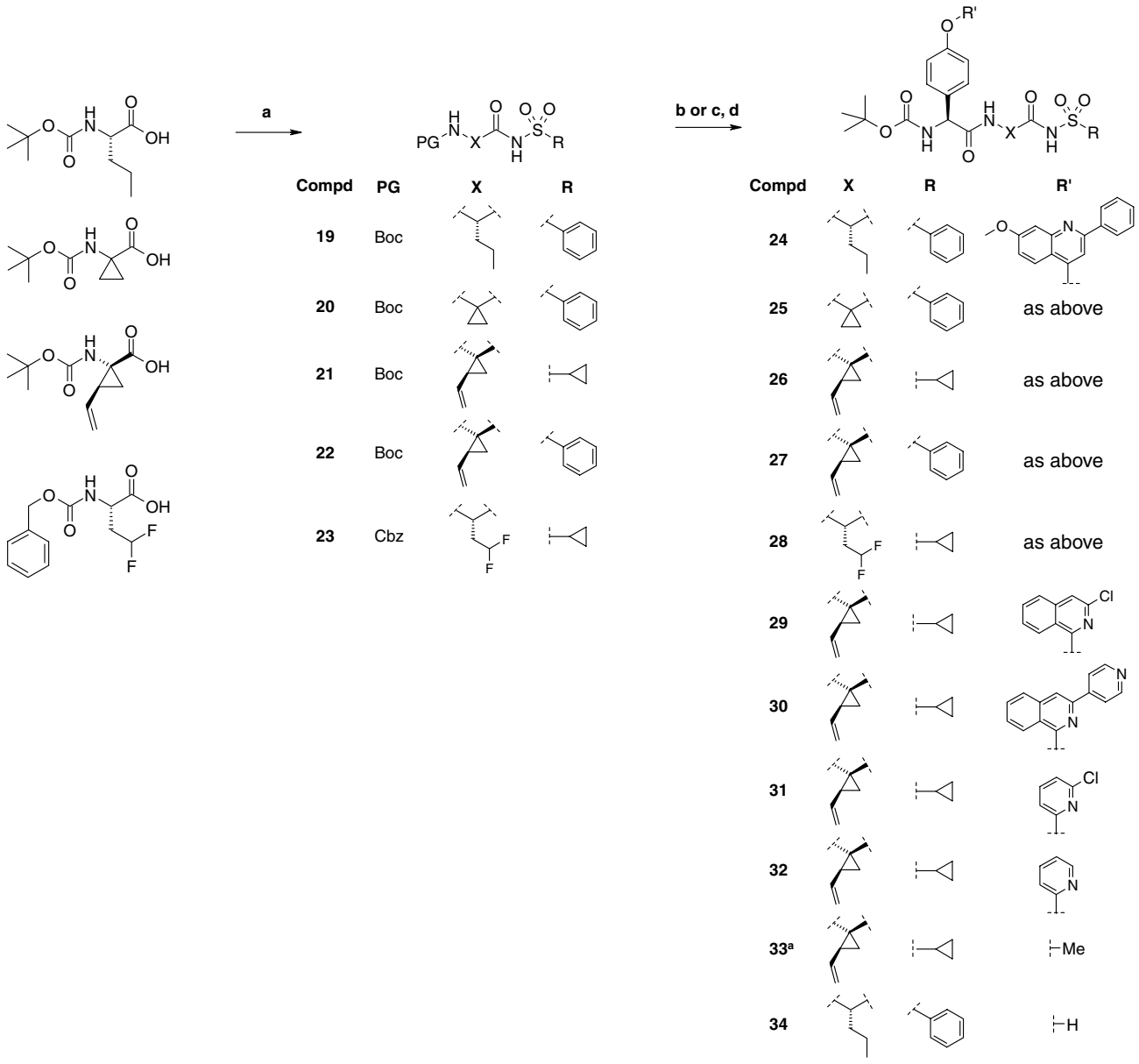


Scheme 2. Reagents: (a) compound **6**, HATU or HBTU, DIEA, DMF; (b) HCl/1,4-dioxane; (c) Boc-Val-OH or Boc-Tle-OH, HBTU or HATU, DIEA, DMF; (d) LiOH, THF/H_2O/MeOH; (e) benzenesulfonamide, CDI, DBU, THF.

using catalytic hydrogenation, the building blocks were used in the following peptide couplings as their hydrochloride salts or as the corresponding zwitterions, producing dipeptides **24**–**34**. The dipeptides were either purified chromatographically (38–87% yield), or used in the following step as crude products after extractions (**25**, **28**, and **34**).

In the final step, Boc-L-valine was used in the synthesis of tripeptide inhibitors **45**–**47** and **18a** and Boc-L-*tert*-leucine for the preparation of inhibitors **35**–**44a** and **b** in 8–64% yield (Scheme 4). To remove the Boc groups from the dipeptides, HCl in 1,4-dioxane or HCl in ethyl acetate was used. Due to the risk of hydrolysis of ethyl acetate to acetic acid it was preferable to use HCl in 1,4-dioxane. In cases where the coupling step is slow, the acetic acid may acetylate the P2–P1′ sulfonamide building block. This occurred in the synthesis of **41**, where it caused an unusually low yield (8%) of the desired product, whereas the acetylated form was recovered in 29% yield. Moreover, it should be noted that the phenylglycine α-carbon is also isomerized to some extent (less than 15%) under standard coupling conditions. The unwanted diastereomers were removed from the target compounds by chromatography. In the case of compound **40**, both diastereomers **40a** (P2 L-isomer) and **40b** (P2 D-isomer) were isolated and used in the biochemical evaluation.

The macrocyclic inhibitors presented in Scheme 5 were synthesized from the P2 building blocks **6**, **9**, and **10**. Synthesis began with the coupling of these to the P1 residue (1*R*,2*S*)-1-amino-2-vinyl-cyclopropanecarboxylic acid ethyl ester resulting in dipeptides **12**, **48**, and **49**. After hydrolysis of the Boc groups, (2*S*)-*N*-Boc-2-amino-8-nonenoic acid was coupled to the deprotected **12**, **48**, and **49**, affording tripeptides **50**–**52** in 56–84% yield. In the cases of **51** and **52**, HCl in ethyl acetate was used in the deprotection step, which led to the problem of acetylated dipeptides as mentioned above. The cyclization was

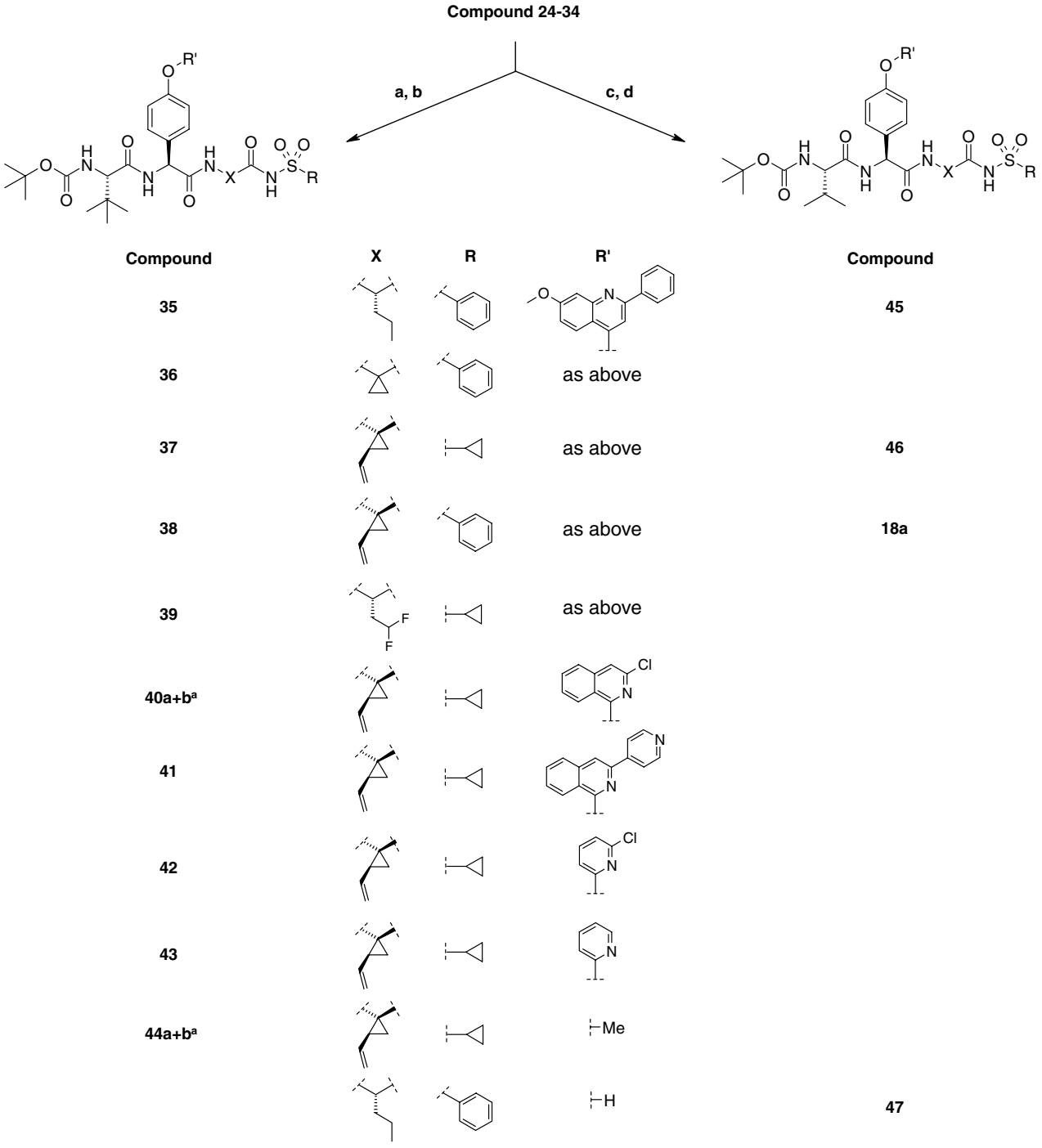


Scheme 3. Reagents: (a) benzenesulfonamide or cyclopropylsulfonamide, CDI, DBU, THF; (b) HCl in 1,4-dioxane or EtOAc; (c) H_2, Pd/C, MeOH; (d) compounds **3**, **5**–**10**, HATU or HBTU, DIEA, DMF. [a]Epimeric mixture of L- and D-phenylglycine.

successfully accomplished in a ring-closing olefin metathesis reaction (RCM) in dry toluene at 80 °C for 1–1.5 h and catalyzed by the second generation Grubb's catalyst, to yield **53** (46%) and **54** (72%).[38,39] The pyridine analogue **52** was less reactive and required heating overnight and addition of more catalyst to give a comparable yield of **55** (64%). Thus, 15-membered rings could be effectively formed using RCM even in the cases of phenylglycine-based tripeptides as previously demonstrated for proline compounds, despite the greater flexibility of phenylglycine.[40] Following alkaline hydrolysis of the ethyl esters to the carboxylic acids **56**–**58**, the synthesis of inhibitors **59**–**62** was accomplished by coupling of benzenesulfonamide or cyclopropylsulfonamide using CDI and DBU in dry THF.[32] The inhibitors were isolated in 11–33% yield. Interestingly, the cyclized compounds did not epimerize at the phenylglycine α-carbon when submitted to the alkaline conditions of the ester hydrolysis that readily epimerized the linear tripeptides.

2.2. Biochemical evaluation

The inhibitors **1**, **2**, **16**–**18b**, **35**–**47**, and **56**–**62** were evaluated in an in vitro assay using the full-length NS3 protein, the central part of the NS4A as cofactor, and an internally quenched fluorescent peptide substrate.[21,41] K_i values of the inhibitors are presented in Tables 1–3. Inhibitors **16**–**18a** and **b**, **35**–**40a**, **41**, **56**, and **59**–**61** were also evaluated for cell activity in the HCV replicon assay.[42] Compounds **16** and **60** were the most active with ED_{50} values of 8.5 and 4.6 μM, respectively. For all remaining compounds, the ED_{50} values exceeded 10 μM.

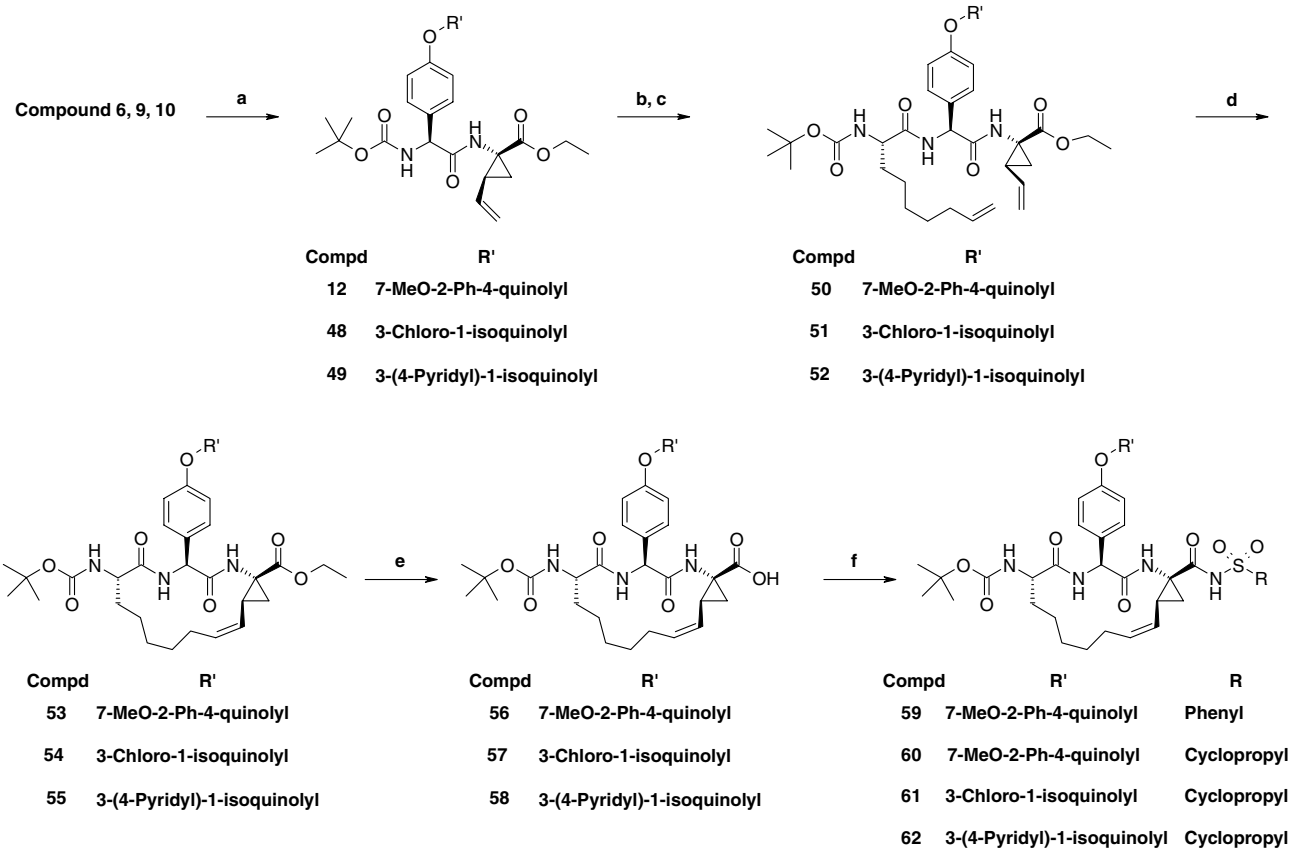


Scheme 4. Reagents: (a) HCl/1,4-dioxane or HCl/EtOAc; (b) Boc-Tle-OH, HATU, DIEA, DMF; (c) HCl/1,4-dioxane; (d) Boc-Val-OH, HATU, DIEA, DMF. [a]D-phenylglycine.

2.3. Molecular modeling

To be consistent with biochemical testing methodology, the full-length NS3 (protease-helicase/NTPase) (PDB code 1CU1) was used for modeling studies.[43] We have used the full-length NS3 protein previously due to the fact that the full-length NS3 is the native form of the protein and due to discrepancies between structure–activity relationships observed using the truncated and full-length NS3 proteins.[20,21] Furthermore, previously published modeling studies on proline-based inhibitors (**A** and **B**, Fig. 2) suggested the importance of the helicase domain, showing a close fit between quinoline-substituted P2 side chains and the helicase domain.[35] To date, there is no published crystal structure of an HCV protease inhibitor bound to the full-length NS3 protein. Docking has been used to suggest likely binding poses of ligands and to help provide a qualitative assessment of protein–ligand interactions.

The active site has been described as a flat and relatively featureless area on the surface of the protease



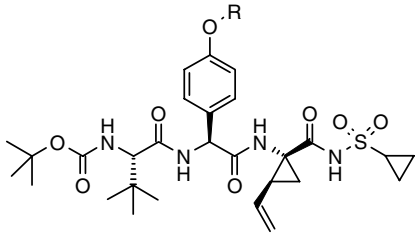
Scheme 5. Reagents: (a) HCl × vinyl-ACCA-OEt, HATU, DIEA, DMF; (b) HCl/1,4-dioxane or HCl/EtOAc; (c) (2*S*)-*N*-Boc-2-amino-8-nonenoic acid, HATU, NMM, DMF; (d) Grubb's catalyst (2nd generation), dry toluene; (e) LiOH, THF/H_2O/MeOH; (f) benzenesulfonamide or cyclopropylsulfonamide, CDI, DBU, THF.

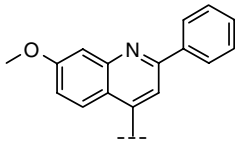
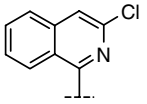
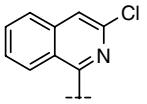
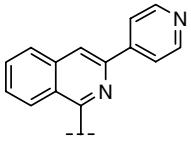
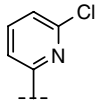
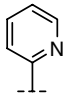
domain.[44,45] However, this only applies to the truncated NS3 where the helicase is not present. Inclusion of the helicase domain results in a more feature-rich binding site that exists at the interface of the protease and helicase domains.[43] Since compounds designed in this study have been optimized to form interactions with the S1 and S2 pockets, understanding of the chemical features in those regions is important. The S1 pocket forms a shallow hole in the protease domain in which the P1 side chain resides. The P1 side chain is encircled by K136, G137, S138, and S139, while the bottom of the S1 pocket is formed by the aromatic side chain of F154. While the S1 pocket is comprised of residues in the protease domain, S2 is formed by residues in both the helicase and protease domains. According to the orientation shown in Figures 3 and 4, the right and back sides of the S2 pocket are formed by the H57 and R155 side chains (protease domain), respectively, while the left side and ceiling of the pocket are formed by Q526 and M485 (both residues in the helicase domain), respectively. Before ligand binding, the R155 side chain lies in close proximity to Q526, but must move backward to accommodate the large quinoline-substituted P2 phenylglycines employed in this study. After accommodation of the large P2 substituent, the R155 side chain is in the position to form the previously described hydrogen bond with D168, as suggested in the crystal structure of an analogue to BILN 2061 and the NS3 protease.[46]

Docking poses of the two tetrapeptides comprising a P2 proline or a P2 phenylglycine in Figure 1 were established and compared (Fig. 3). Some notable features include: First, the phenylglycine side chain may be able to participate in a pi-pi stacking interaction with H57. Here, the distance between the phenyl and imidazole centroids is approximately 3.5 Å. Second, docking studies suggest that the αNH of the phenylglycine may be able to participate in a hydrogen bonding interaction with Q526 of the NS3 helicase domain. As seen in Figure 3 the distance between the αNH and the carbonyl of Q526 is 2.2 Å, indicating that hydrogen bonding is likely. Further differences in the binding poses of the phenylglycine- and proline-based inhibitors can be seen in the P1 position. In Figure 3 where compounds **1** and **2** are overlaid it can be seen that the cyclic nature of the proline ring induces a conformational change in this position so that the P1 may be positioned deeper in the S1 pocket, lying closer to F154.

A similar phenylglycine-proline comparison with the quinoline-substituted inhibitors **18a** (Table 2) and compound **B** (Fig. 2) was performed, and the overlayed poses can be seen in Figure 4. Binding poses of the phenylglycine-based tripeptides were similar to those observed in the tetrapeptide series mentioned above. The H57-phenylglycine pi-pi stacking interaction and the hydrogen bond with Q526 were observed in both

Table 1. Inhibition of NS3 protease activity of the full-length NS3 (protease-helicase/NTPase)



Compound	R	$K_i \pm SD^a$ (μM)
37		0.18 ± 0.04
40a		0.41 ± 0.09
40b[b] D-Phg		1.1 ± 0.2
41		0.29 ± 0.08
42		2.6 ± 0.1
43		7.0 ± 0.8
44a		5.4 ± 0.3
44b[b] D-Phg		34 ± 2
47[c]		130 ± 20

[a] SD = standard deviation.
[b] D-Phenylglycine instead of L-phenylglycine in P2.
[c] Valine in P3 and norvaline phenyl acyl sulfonamide in P1.

cases. Also, the difference in the relative position of the P1 side chain as compared to proline-based inhibitors was consistent for both phenylglycine-based inhibitors.

In agreement with published NMR studies,[47] docking studies indicate that the phenylglycine side chain may adopt a conformation perpendicular to the inhibitor backbone. This conformation is favored due to the repulsive interaction between phenylglycine hydrogens and those of the backbone. However, for the bulky quinoline or isoquinoline substituent to fit into the active site, some strain is introduced in the ether linker (C–O–C approximately 108°). A second plausible phenylglycine conformation may exist where the phenyl ring lies parallel to the backbone (not shown), allowing a more comfortable fit of the quinoline in the S2 pocket and a reduction in the strain in the ether linker (C–O–C approximately 115°). However based on the documented NMR findings and the fact that the possible pi-pi stacking interaction with H57 is possible, one may consider the perpendicular phenylglycine conformation to be the more likely conformation.

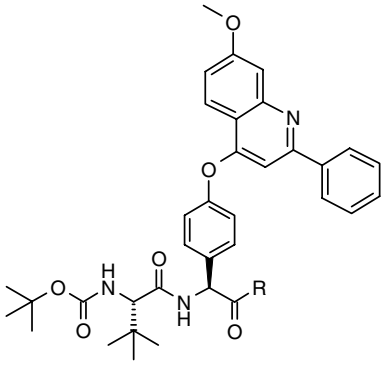
Docking studies of the phenylglycine-based D-isomers (not shown) suggest that they adopt binding conformations similar to the L-isomers. Although the phenylglycine was found to be perpendicular to the inhibitor backbone, the positions of the large P2 quinoline substituents differed. The phenylglycine of the D-isomer is closer to the R155 side chain, which reduces the available volume in the S2 pocket for the quinoline substituent so that it could not fit in the pocket as it did in the case of the corresponding L-isomer. Rather than being found between H57 and Q526, the quinoline moiety was instead located between Q526 and R155.

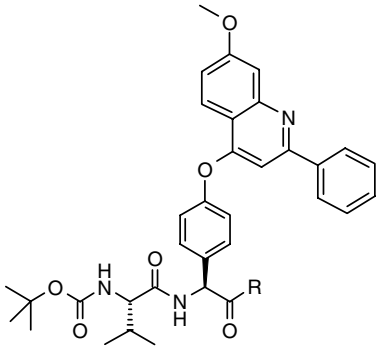
3. Discussion

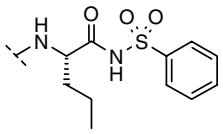
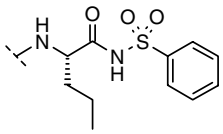
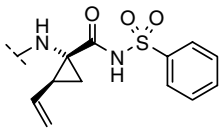
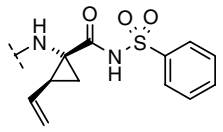
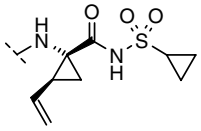
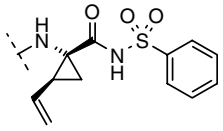
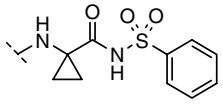
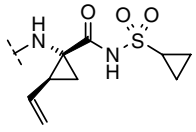
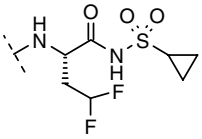
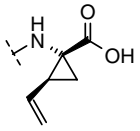
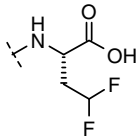
There are numerous examples of potent peptidomimetic tripeptide HCV NS3 inhibitors based on P2 proline in the literature today.[9] Such inhibitors include BILN 2061 and derivatives thereof, designed at Boehringer Ingelheim, and acyl sulfonamides on a similar scaffold previously published by us and others (Fig. 2).[11,28,35] As mentioned in the introduction, preliminary studies by us indicated that phenylglycine might be a useful alternative to proline as a P2 building block in HCV NS3 protease inhibitors. This idea was further strengthened by docking studies that suggested a possible pi-pi stacking interaction with the catalytic H57 and phenylglycine side chain, as discussed above (Fig. 3). Moreover, the NH of the phenylglycine may be able to participate in a hydrogen bonding interaction with Q526 of the NS3 helicase domain, although this is more speculative. In agreement with this hypothesis, we found that a tetrapeptide inhibitor (**2**) with phenylglycine in P2 was more than 20 times more potent than the corresponding inhibitor (**1**) with proline in the P2 position (Fig. 1).

The effectiveness of phenylglycine as compared to proline in simple tetrapeptides (Fig. 1) prompted us to explore the potential of phenylglycine-based inhibitors using decorations proven to be effective in proline-based inhibitors. We anticipated that these tripeptide phenylglycine compounds would be of greater potency than

Table 2. Inhibition of NS3 protease activity of the full-length NS3 (protease-helicase/NTPase)



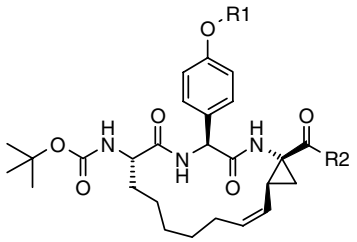


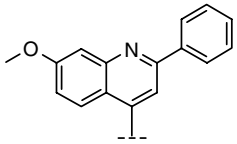
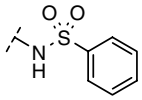
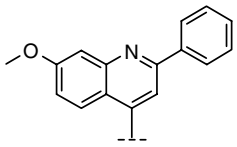
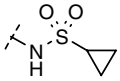
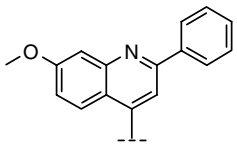
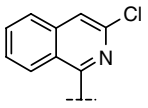
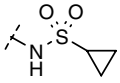
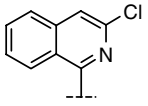
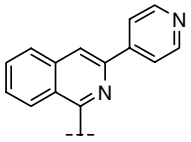
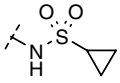
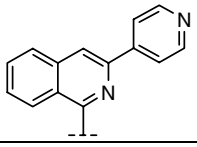
Compound	R	$K_i \pm SD$[a] (μM)	Compound	R	$K_i \pm SD$[a] (μM)
35		0.23 ± 0.04	**45**		0.28 ± 0.02
38		0.23 ± 0.05	**18a**		0.27 ± 0.06
37		0.18 ± 0.04	**18b**[c] D-Phg		0.40 ± 0.03
36		1.2 ± 0.2	**46**		0.20 ± 0.04
39		0.78 ± 0.1	**16**[d]		2.4 ± 0.5
17[b]		2.8 ± 0.6			

[a] SD = standard deviation.
[b] 68:32 ratio of L:D phenylglycine epimers.
[c] D-Phenylglycine instead of L-phenylglycine in P2.
[d] 60:40 ratio of L:D phenylglycine epimers.

the similar proline-based inhibitors. Therefore, alkyl- and heteroaryl-substituted compounds were synthesized via an ether linkage of P2 phenylglycine (Table 1). This range of P2 substitutions was also tested in combination with a variety of common P1 and P3 residues (Table 2). Furthermore, cyclization was performed to understand

Table 3. Inhibition of NS3 protease activity of the full-length NS3 (protease-helicase/NTPase)



Compound	R1	R2	$K_i \pm SD$[a] (μM)
59			0.095 ± 0.01
60			0.076 ± 0.02
56			0.79 ± 0.2
61			0.074 ± 0.01
57			1.1 ± 0.2
62			0.24 ± 0.04
58			1.3 ± 0.5

[a] SD = standard deviation.

the effect of a reduction in conformational flexibility (Table 3). While most inhibitors employed acyl sulfonamide C-terminals, the corresponding carboxylic acid derivatives were synthesized to allow comparisons (Tables 2 and 3).

p-Methoxyphenylglycine incorporated in a tripeptide sequence with (1*R*,2*S*)-1-amino-2-vinyl-cyclopropanecarboxylic acid (vinyl-ACCA) in P1 and *tert*-leucine in P3 produced a promising inhibitor of low molecular weight, with a K_i of 5.4 μM (**44a**, Table 1). A similar

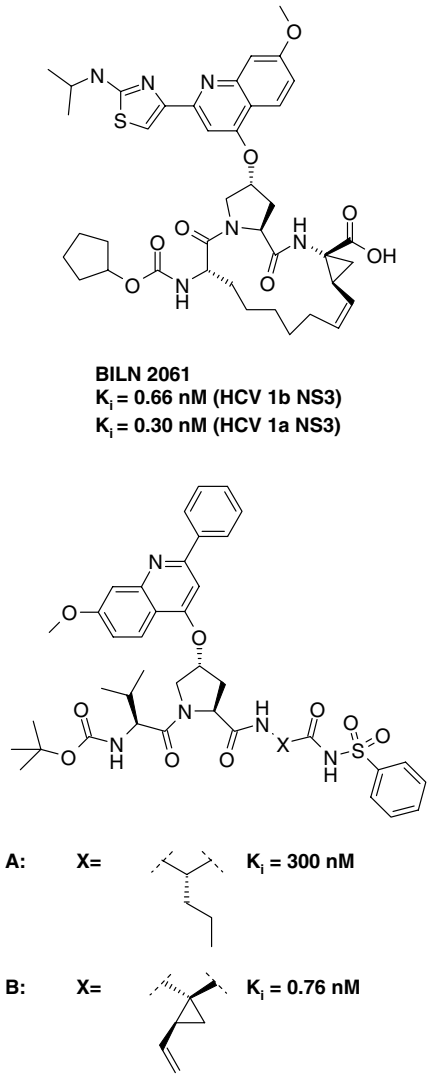


Figure 2. K_i values of three HCV NS3 protease inhibitors based on P2 proline: BILN 2061, the first protease inhibitor to enter clinical trials,[11,28,35] and two linear acyl sulfonamide inhibitors evaluated in our assay system with norvaline (A) or vinyl-ACCA (B) in P1.[11,28,35]

inhibitor but with a P2 proline moiety and C-terminal carboxylic acid had a reported K_i of >600 μM.[48] Interestingly, the pyridinyloxy- and the chloropyridinyloxy-based inhibitors (**42** and **43**) were roughly equipotent with the methoxy-based inhibitor **44a**. As was found for the proline-based inhibitors, the introduction of bulky quinoline ethers yielded much more potent inhibitors of NS3 protease.[48] Thus, replacement of methoxy with a 7-methoxy-2-phenylquinolin-4-yloxy resulted in a 30-fold improvement in potency (**37**, K_i = 0.18 μM). The 3-chloro- and 3-pyridine isoquinolines (**40a** and **41**) were approximately 2-fold less potent than the quinoline **37**.

In order to investigate whether phenylglycine is compatible with P1 residues previously used in different types of HCV NS3 inhibitors,[36,49–56] the compounds in Table 2 were synthesized. In that series, various P1 residues and C-terminals were evaluated on *p*-(7-methoxy-2-phenylquinolin-4-yloxy)phenylglycine-based inhibitors, using either Boc-protected valine or *tert*-leucine as the P3 residue. P3 *tert*-leucine has been proved to produce not only slightly improved HCV NS3 protease inhibitors as compared to P3 valine, but also inhibitors with higher solubility and better pharmacokinetics.[51,57–59] In the present phenylglycine series, P3 *tert*-leucine and P3 valine generate equipotent inhibitors (**35** compared with **45**, **38** compared with **18a**, and **37** compared with **46**, Table 2).

Molecular modeling suggests that the relative position of P1 residues in the present phenylglycine series is different from that of the P1 of the analogous proline compounds. Hence, in the P2 proline series of phenyl acylsulfonamides, the replacement of norvaline in P1 with vinyl-ACCA resulted in a 400-fold improvement in potency (Fig. 2).[35] This same replacement in the P2 phenylglycine series resulted in no significant change in activity (**35** compared to **38**, and **45** compared to **18a**, Table 2). The difference in activity of these two analogous series may accordingly be attributed to differences in conformation in the P1 position as discussed above (see Fig. 3), which may affect protein–ligand interactions

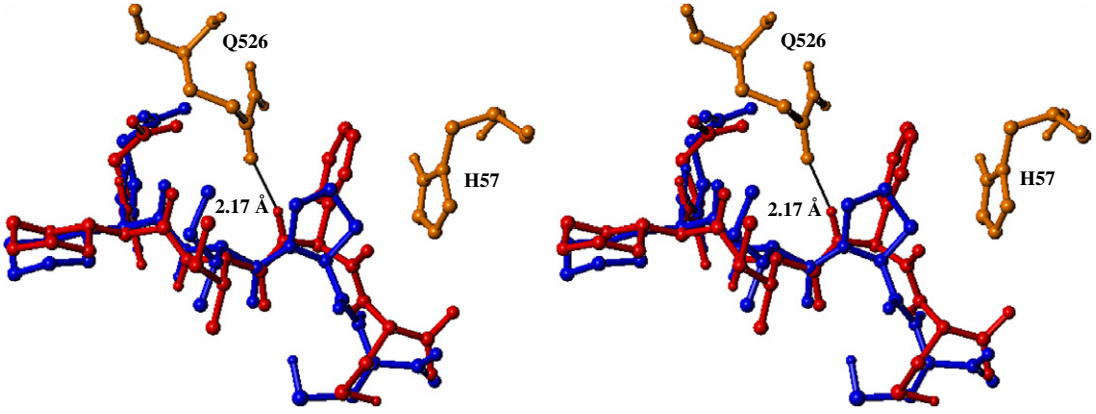


Figure 3. Stereographic image of **1** (red) and **2** (blue) shown with the residues H57 and Q526 (orange). A pi–pi stacking interaction is possible based on the distance (3.5 Å) between the centroids of the H57 imidazole side chain and the phenylglycine P2 side chain. Structures of both the inhibitor and amino acid residues were obtained from docking studies and the positions of the protein atoms were obtained from the docking results of **1**.

in the S1 pocket. However, one should note that the phenylglycine- and proline-based inhibitors with norvaline in the P1 position were of very similar potency (**45**, K_i = 280 nM, Table 2, and **A** in Fig. 2, K_i = 300 nM). The phenylglycine-based inhibitors are more flexible than the corresponding proline compounds and modeling suggests at least two distinct binding modes (in the P2 position) for the phenylglycine compound as compared to one for the proline compound. Conceivably, the phenylglycine-based compound can benefit from one, but perhaps not two fragments that have been optimized for the proline compounds. For example, in the proline series, the vinyl-ACCA and 7-methoxy-2-phenylquinoline-4-yloxy were found to be optimal in the P1 and P2 positions,[58] respectively, but the same combination in the phenylglycine series does not work equally well. However, the vinyl group of the P1 vinyl-ACCA continued to have a large effect on potency as inhibitors with only ACCA in the P1 position were lower in inhibitory potency than those with either norvaline or vinyl-ACCA (compare **36** with **35** and **38** in Table 2).

2-Amino-4,4-difluorobutanoic acid (difluoro-Abu) was elegantly designed by Narjes et al. as a replacement of the native P1 cysteine in NS3 protease inhibitors, and has worked well in inhibitors with C-terminal carboxylates, phenethylamides, as well as in electrophilic α-ketoacids and α-ketoamides.[34,51,53,56] Prior to this study, the effect of this amino acid in acyl sulfonamide-based inhibitors had not been evaluated. Difluoro-Abu was therefore used as an additional P1 residue in phenylglycine-based inhibitors in an attempt to improve potency. Unfortunately, difluoro-Abu did not prove to be a good P1 residue in a phenylglycine-based inhibitor with a C-terminal acylsulfonamide. A 4-fold decrease in potency was observed, as compared to the corresponding vinyl-ACCA compound (**37** and **39**, Table 2). On the other hand, the carboxylates of difluoro-Abu and vinyl-ACCA were equal in activity in the phenylglycine series (**16** and **17**, Table 2), indicating that it is the difluoro-Abu and acyl sulfonamide combination that is not optimal.

Phenylglycine-based compounds are prone to racemization under basic conditions, due to the risk of conjugation resulting from the phenyl group being adjacent to the α-carbon.[60] Partial epimerization was noted for several of the phenylglycine-based inhibitors during synthesis. Epimerization was less than 15% under normal peptide coupling conditions, and more than 32% during treatment with lithium hydroxide (see Scheme 2). Interestingly, the macrocyclic analogues were free of epimerization under the same conditions. After epimeric separation of some of the linear inhibitors, biochemical evaluation of both the D- and L-epimers was possible. The D-epimers were of lower potency than the corresponding L-epimers but retained a reasonable inhibition constant (only a 2.5-, 6-, and 1.5-fold loss in potency, respectively, for the pairs **40a**,**40b** and **44a**,**44b** in Table 1, and **18a**,**18b** in Table 2). As discussed above, docking studies show that the D- and L-isomers bind in a similar way but still with some difference in the relative positions of the quinoline substituents, which might explain the relatively small difference in potencies between the D- and L-epimers of P2 phenylglycine.

Formation of macrocyclic compounds has been achieved by connecting the P1 and P3 side chains of proline-based inhibitors, such as BILN 2061 (Fig. 2).[46,48] Similar 15-membered ring macrocyclic phenylglycine-based inhibitors were synthesized in this study, producing compounds with nanomolar potencies (Table 3). A 2- to 6-fold gain in potency was achieved by inhibitor cyclization, with the exception of the pyridine-based inhibitor **62**, which was not affected. Inhibitors **60** and **61** with 7-methoxy-2-phenylquinolin-4-yloxy and 3-chloroisoquinolin-1-yloxy substituted phenylglycines have K_i values of 76 and 74 nM, respectively, and are

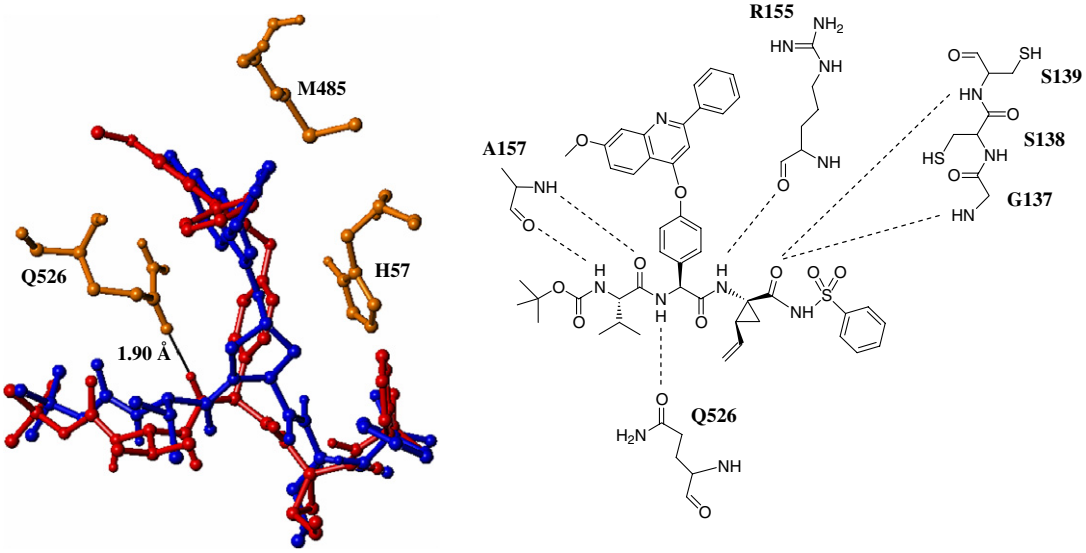


Figure 4. On left, comparison of the suggested binding poses for **18a** (red, Table 2) and compound **B** (blue, Fig. 2) shown with residues H-57, Q-526, and M-485 (orange). The distance between the centroids of the imidazole group of H-57 and the phenylglycine side chain is 3.8 Å, indicating a possible pi-pi interaction. Structures of both the inhibitor and amino acid residues were obtained from docking studies and the positions of the NS3 residues were obtained from the docking results of **18a**. Shown on right is a schematic representation of the H-bonding network for **18a**.

the most potent inhibitors presented herein. Additionally, the macrocyclic inhibitor **60** had the best cellular potency in the series, although in the micromolar range (EC_{50} = 4.6 μM). The structure-activity relationship of the inhibitor series presented herein indicates that the phenylglycine-based inhibitors require their own optimization studies in order to improve both enzymatic and cellular potencies.

We previously discovered that the C-terminal carboxylates of product-based inhibitors of HCV NS3 protease could be successfully replaced with classical carboxylic acid bioisosteres, of which the acyl sulfonamides seemed most promising.[35,36] Subsequent studies showed the acyl sulfonamides to be more selective for the HCV NS3 protease as compared with other serine proteases.[61] The same substitution in the present phenylglycine series of inhibitors was also associated with a 10-fold gain in potency (Tables 2 and 3). In previous work, several acyl sulfonamide substituents were tested and phenyl was shown to be a favorable substituent.[35] The cyclopropyl acyl sulfonamide substituent was originally explored by Campbell et al.[28] and used in this study of phenylglycine-based inhibitors. Biochemical testing data indicate that it is as good, and in some cases, slightly better than the phenyl substituent (**38**, **37**, **18a**, and **46**, Table 2, and **59**, **60**, Table 3).

4. Conclusion

In summary, phenylglycine is shown to be a useful P2 residue in protease inhibitors of the HCV NS3. It is thought to interact with the protease-helicase/NTPase in ways not possible for the proline-based inhibitors, as suggested by modeling and inhibitory potencies. However, the structure–activity relationship for the phenylglycine-based inhibitors is not identical to the well-known proline-based compounds. The phenylglycine compounds do not benefit from a combination of fragments optimized for the proline-based compounds, as for example the vinyl-ACCA in P1 and 7-methoxy-2-phenylquinolin-4-yloxy as a P2 phenylglycine substituent. These results indicate that phenylglycine-based inhibitors may adopt a slightly different binding pose relative to the previously synthesized proline series. Nevertheless, the combination of C-terminal acylsulfonamides, isoquinolin- or quinolinyloxy substituted phenylglycine in P2, and P1–P3 cyclization yielded effective inhibitors with potencies in the nanomolar range (~75 nM). Further optimization studies are underway to fully exploit the promising P2 phenylglycine scaffold.

5. Experimental

5.1. Chemistry

Reagents and solvents were obtained commercially and used without further purification. The microwave reactions were preformed in a Smith-Synthesizer single mode cavity with controlled irradiation at 2450 MHz. The reactions were executed in Smith vials. Thin-layer chromatography (TLC) was performed using aluminum sheets precoated with silica gel 60 F_{254} (0.2 mm, E. Merck) or RP-TLC RP-18 F_{254S}, (E. Merck). Chromatographic spots were visualized using UV-detection or by spraying with a 2% ethanolic ninhydrin solution followed by heating, or both. Column chromatography was performed using Merck silica gel 60 (40–63 μm) (or Merck Silica gel 60 RP-18 (40–63 μm) for compounds **21** and **22**). Analytical RP-HPLC–MS was preformed on a Gilson–Finnigan ThermoQuest AQA system (Chromolith Performance RP-C18 column, 100 × 4.6 mm, MeCN/H_2O gradient with 0.05% HCOOH) in ESI mode, using UV (214 and 254 nm) and MS detection. Preparative RP-HPLC–MS was performed on a Gilson–Finnigan ThermoQuest AQA system equipped with a C8 column (Zorbax SB-C8, 5 μm, 150 × 21.2 mm) using a MeCN/H_2O (0.05% HCOOH) gradient with UV (214 and 254 nm) and MS (ESI, 10 eV) detection. Preparative RP-HPLC was performed on a system equipped with a phenyl column (ACE 5 Phenyl, 150 × 21.2 mm) or a C18 column (Vydac 218TP, 10 μm, 250 × 22 mm), in both cases with UV detection at 230 nm. The purity of each of the inhibitors was determined by RP-HPLC in two of the following systems (UV detection at 220 nm): column 1 (ACE 5 C18, 50 × 4.6 mm, H_2O/MeCN gradient with 25 mM NH_4OAc, pH 6.3), column 2 (Thermo Hypersil C4, 50 × 4.6 mm, 5 μM, H_2O/MeCN gradient with 0.1% TFA) or column 3 (ACE 5 Phenyl, 50 × 4.6 mm, H_2O/MeCN gradient with 0.1% TFA). ^1H and ^{13}C NMR spectra, except for compound **41**, were recorded on a Varian Mercury-400 or on a JEOL JNM-EX400 spectrometer at 399.8 MHz or 100 MHz, respectively, at ambient temperature. The ^1H NMR for compound **41** was recorded on a Varian UNITY INOVA spectrometer at 499.9 MHz. Chemical shifts are reported as δ values (ppm) indirectly referenced to TMS via the solvent signal (^1H: CHD_2OD δ 3.31, δ $CHCl_3$ 7.26, CHD_2COCD_3 δ 2.05; ^{13}C: CD_3OD δ 49.0, δ $CDCl_3$ 77.16, $(CD_3)_2CO$ δ 49.0). Exact molecular masses were determined on Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source.

5.2. General procedure for the synthesis of tetrapeptides (1 and 2)

The peptides were prepared manually on a 120 μmol scale by standard Fmoc/*t*-Bu solid-phase peptide synthesis in 5 mL disposable syringes equipped with porous polyethylene frits. The starting polymer was H-Cys(Trt)-2-chloro-trityl resin (0.48 mmol/g). Fmoc-amino acids (3 equiv) were coupled in DMF (1.5 mL) for 2 h using HBTU (3 equiv) in the presence of DIEA (6 equiv). The Fmoc group was removed by repeated treatment with 20% piperidine in DMF (5+10 min). Succinylation was accomplished by reaction with succinic anhydride (10 equiv) and DIEA (10 equiv) in DMF for 2 h. The resins were then washed with DMF, DCM, and MeOH, and dried in vacuo. Part of the products (60 μmol) were deprotected and cleaved from the resin by treatment with triethylsilane (175 μL) and 95% aqueous TFA (2 mL) for 1.5 h. The resins were filtered off and washed with 2 × 0.3 mL TFA. The filtrates

were evaporated in a stream of nitrogen to less than 0.5 mL, the products precipitated by addition of ether (13 mL), collected by centrifugation, and washed with ether (2 × 6 mL). The dried products **1** and **2** weighed 11.2 mg and 25.9 mg, respectively. LC–MS analysis showed the occurrence of two components of the expected m/z ratio in the crude phenylglycine-containing peptide and, in addition, a substantial amount of the peptide lacking the phenylglycine residue. The peptides were purified by RP-HPLC on a Zorbax SB-C8 column using a buffer system comprised of (A) 25 mM aqueous NH_4OAc, pH 6.3, and (B) 25 mM NH_4OAc, pH 6.3, in 60% acetonitrile at a flow rate of 5 mL/min and with detection by UV absorption at 220 nm.

5.3. Suc-Chg-Ile-Pro-Cys-OH (1)

Amino acid analysis Chg, 0.99; Ile, 0.99; Pro, 0.99; Cys, 1.03. HRMS calcd for $C_{26}H_{41}N_4O_8S$ ($M-H^+$) 569.2645, found: 569.2634. RP-HPLC purity (column 1: 99.4%, column 2: 100%).

5.4. Suc-Chg-Ile-L-Phg-Cys-OH (2)

Amino acid analysis Chg, 0.99; Ile, 0.99; Phg, 1.00; Cys, 1.01. HRMS calcd for $C_{29}H_{41}N_4O_8S$ ($M-H^+$) 605.2645, found: 605.2667. RP-HPLC purity (column 1: 94.4%, column 2: 94.8%). Data for the corresponding D-isomer: Suc-Chg-Ile-D-Phg-Cys-OH amino acid analysis Chg, 0.99; Ile, 0.99; Phg, 1.01. HRMS calcd for $C_{29}H_{43}N_4O_8S$ ($M+H^+$) 607.2802, found: 607.2810.

5.5. (*S*)-*tert*-Butoxycarbonylamino(4-hydroxyphenyl)acetic acid (3)[27]

Prepared as described in the literature.[27]

5.6. (*S*)-*tert*-Butoxycarbonylamino(4-methoxyphenyl)acetic acid methyl ester (4)

Cs_2CO_3 (0.525 g, 1.6 mmol) was dissolved in dry DMF (10.0 mL) under N_2 flow. Compound **3**[27] (0.171 g, 0.64 mmol) dissolved in dry DMF (5.0 mL) and methyl iodide (0.12 mL, 1.9 mmol) were added. The reaction mixture was stirred at room temperature for 21 h under N_2 atmosphere. Water (50 mL) was added and the aqueous phase extracted with EtOAc (3 × 30 mL). The combined organic phases were washed with brine (1 × 30 mL), dried with $MgSO_4$, filtered, and evaporated to yield **4** (0.168 g, 89%) as a yellow oil. ^{1}H NMR (CD_3OD) δ 7.28 (m, 2H), 6.90 (m, 2H), 5.14 (s, 1H), 3.78 (s, 3H), 3.68 (s, 3H), 1.44 (s, 9H). ^{13}C NMR (CD_3OD) δ173.5, 161.2, 157.6, 129.9, 129.8, 115.2, 80.8, 58.8, 55.7, 52.8, 28.7. MS ($M+H^+$) 296.2. HRMS calcd for $C_{15}H_{22}NO_5$ ($M+H^+$) 296.1498, found: 296.1504.

5.7. *tert*-Butoxycarbonylamino(4-methoxyphenyl)acetic acid (5)

Compound **4** (0.120 g, 0.406 mmol) was dissolved in THF (15 mL) and MeOH (7 mL). LiOH (0.145 g, 6.09 mmol) dissolved in water (2 mL) was added and the reaction stirred at room temperature for 24 h. pH was adjusted to 5 using 1 M HCl, and the organic solvents removed in vacuo. pH was adjusted to 3 with 1 M HCl whereafter the aqueous phase was extracted twice with EtOAc (2 × 10 mL). The combined organic phases were washed with brine, dried with $MgSO_4$, filtered, and the solvents removed in vacuo, to give **5** (0.080 g, 70%) racemized at the Phg α-carbon (as determined by epimer formation in subsequent peptide couplings). ^{1}H NMR (CD_3OD) δ 7.31 (m, 2H), 6.90 (m, 2H), 5.1 (s, 1H), 3.78 (s, 3H), 1.44 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.6, 161.1, 157.5, 130.5, 129.8, 115.1, 80.7, 58.6, 55.7, 28.7. HRMS calcd for $C_{14}H_{20}NO_5$ ($M+H^+$) 282.1341, found: 282.1335.

5.8. (*S*)-*tert*-Butoxycarbonylamino[4-(7-methoxy-2-phenylquinolin-4- yloxy)phenyl]acetic acid (6)

4-Chloro-7-methoxy-2-phenylquinoline (0.328 g, 1.22 mmol), **3** (0.651 g, 2.44 mmol) and KO-*t*-Bu (0.548 g, 4.88 mmol) were dried over P_2O_5 in vacuo before use. Dry DMSO (3.6 mL) was added to KO-*t*-Bu under N_2 atmosphere in a sealed reaction vessel tube. Compound **3** was dissolved in dry DMSO (3.6 mL) and added dropwise to the KO-*t*-Bu solution. The solution was stirred for 30 min, whereafter a solution of 4-chloro-7-methoxy-2-phenyl-quinoline in dry DMSO (3.6 mL) was added dropwise. The reaction mixture was stirred at 40 °C in a heating block for 8 days and was monitored by LC–MS. When only traces of 4-chloro-7-methoxy-2-phenyl-quinoline could be detected, the reaction mixture was filtered and poured into H_2O (60 mL). The aqueous phase was washed with ether (2 × 30 mL) to remove the side-products (see Section 5.1). The pH was adjusted to 5.15 using 1 M HCl (~2.4 mL). The precipitated product was collected by centrifuge filtration through Whatman PVDF-filters (0.45 μm), since it was too fine-grained for ordinary filtration, to yield **6** (0.434 g, 71%) as a white solid. ^{1}H NMR (CD_3OD) δ 8.25 (d, J = 9.2 Hz, 1H), 7.81 (m, 2H), 7.58 (m, 2H), 7.47 (m, 4H), 7.27 (m, 3H), 6.89 (s, 1H), 4.0 (s, 3H), 1.46 (s, 9H). ^{13}C NMR (CD_3OD) δ 175.4, 167.7, 166.2, 160.0, 158.2, 155.1, 148.4, 139.1, 136.9, 131.8, 131.1, 129.9, 125.9, 122.9, 122.2, 116.7, 104.4, 81.7, 60.0, 57.4, 29.5. MS ($M+H^+$) 501.1. HRMS calcd for $C_{29}H_{29}N_2O_6$ ($M+H^+$) 501.2026, found: 501.2024.

5.9. (*S*)-*tert*-Butoxycarbonylamino[4-(6-chloro-pyridin-2-yloxy)-phenyl]acetic acid (7)

Compound **7** was prepared as described for **6** using 2,6-dichloropyridine (0.115 g, 0.778 mmol), **3** (0.104 g, 0.389 mmol), KO-*t*-Bu (0.087 g, 0.778 mmol), and DMSO (2 mL). The reaction mixture was stirred for 4 days. The pH of the water solution was adjusted to 3.5 and the precipitated product was filtered to give **7** (0.110 g, 75%) as a white solid. ^{1}H NMR (CD_3OD) δ 7.78 (dd, J = 7.6, 8.2 Hz, 1H), 7.47 (m, 2H), 7.13 (m, 3H), 6.87 (m, 1H), 5.23 (br s, 1H), 1.45 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.1, 164.8, 157.5, 155.0, 149.9, 143.6, 135.5, 130.2, 122.3, 119.9, 110.8, 80.9, 58.7, 28.7. HRMS calcd for $C_{18}H_{18}N_2O_5Cl$ ($M-H^+$) 377.0904, found: 377.0901.

5.10. (*S*)-*tert*-Butoxycarbonylamino[4-(pyridin-2-yloxy)-phenyl]acetic acid (8)

Compound **8** was prepared as described for **6** using 2-fluoropyridine (0.096 g, 0.988 mmol), **3** (0.132 g, 0.494 mmol), KO-*t*-Bu (0.111 g, 0.988 mmol), and DMSO (2 mL). The reaction mixture was stirred for 4 days. The aqueous phase was acidified to 3.6 and cooled in a refrigerator. The precipitated product was filtered to give **8** (0.056 g, 33%) as a white solid. The water phase was extracted with ethyl acetate (2 × 10 mL). The combined organic phases were dried with $MgSO_4$, filtered, and the solvent removed in vacuo to give another 0.032 g, 19% of **8**. ^{1}H NMR (CD_3OD) δ 8.12 (dd, J = 2.2, 5.0 Hz, 1H), 7.82 (ddd, J = 2.1, 7.3, 8.3 Hz, 1H), 7.45 (m, 2H), 7.11 (m, 3H), 6.95 (m, 1H), 5.21 (s, 1H), 1.46 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.1, 164.8, 157.5, 155.6, 148.4, 141.7, 135.1, 130.2, 122.0, 120.3, 113.0, 80.8, 58.7, 28.7. HRMS calcd for $C_{18}H_{21}N_2O_5$ ($M+H^+$) 345.1450, found: 345.1454.

5.11. (*S*)-*tert*-Butoxycarbonylamino[4-(3-chloroisoquinolin-1-yloxy)phenyl]acetic acid (9)

Compound **9** was prepared as described for **6** using 1,3-dichloroisoquinoline (0.396 g, 2.0 mmol), **3** (1.069 g, 4.0 mmol), KO-*t*-Bu (0.898 g, 8.0 mmol) and DMSO (12 mL). The reaction was stirred for 14 days. The aqueous phase was acidified to 3.6 and cooled in a refrigerator. The precipitated product was filtered to give **9** (0.849 g, 99%) as a gray solid. ^{1}H NMR (CD_3OD) δ 8.37 (dd, J = 1.0, 8.3, Hz, 1H), 7.84–7.79 (m, 2H), 7.66 (ddd, J = 1,6, 6.6, 8.3 Hz, 1H), 7.53–7.48 (m, 3H), 7.26 (m, 2H), 5.26 (s, 1H), 1.47 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.1, 161.0, 157.5, 154.6, 143.0, 141.4, 135.6, 133.2, 130.0, 128.7, 126.9, 125.1, 123.0, 119.3, 116.1, 80.8, 58.7, 28.7. HRMS calcd for $C_{22}H_{20}N_2O_5Cl$ ($M-H^+$) 427.1061, found: 427.1064.

5.12. (*S*)-*tert*-Butoxycarbonylamino[4-(3-pyridin-4-yl-isoquinolin-1- yloxy)phenyl]acetic acid (10)

A microwave vial was charged with $Pd_2(dba)_3$ (0.016 g, 0.018 mmol), [(*t*-Bu)$_3$PH]BF_4 (0.010 g, 0.035 mmol), and KF (0.067 g, 1.16 mmol). The vial was sealed and flushed with N_2 for 30 min. Dry THF (0.5 mL) was added and the reaction was stirred for 1 h before addition of **9** (0.150 g, 0.350 mmol), 4-pyridineboronic acid (0.172 g, 1.4 mmol), and THF (1.5 mL). The reaction mixture was exposed to microwave heating for 15 min at 170 °C. The solution was centrifuged through a Whatman polypropylene filter (0.45 μm) and the THF was removed under reduced pressure. The residue was purified by preparative RP-HPLC–MS using an acetonitrile/H_2O gradient with 0.05% HCOOH to yield **10** (0.0517 g, 31%) as white solid after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.75 (m, 2H), 8.56–8.44 (m, 4H), 8.10 (m, 1H), 7.92 (ddd, J = 1.4, 6.9, 8.3 Hz, 1H), 7.84 (ddd, J = 1.4, 6.9, 8.3 Hz, 1H), 7.57 (m, 2H), 7.36 (m, 2H), 5.32 (s, 1H), 1.48 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.2, 161.7, 156.7, 154.7, 142.7, 141.9, 140.0, 135.8, 133.5, 131.4, 129.9, 129.4, 125.3, 124.4, 123.3, 122.0, 119.0,115.6, 81.0, 58.8, 28.7. HRMS calcd for $C_{27}H_{25}N_3O_5$ ($M-H^+$) 470.1716, found: 470.1701.

5.13. (2*S*)-2-amino-4,4-difluorobutanoic acid methyl ester hydrochloride (11)

(*S*)-2-[(Benzyloxycarbonyl)amino]-4,4-difluorobutanoic acid methyl ester (0.150 g, 0.52 mmol) was dissolved in MeOH (9.0 mL) and 10% Pd/C (0.045 g) was added. H_2 was led into the flask for 2 h. The reaction mixture was centrifuge filtered through a Whatman PP filter (0.45 μm) and acidified to pH 4–5 using 4 M HCl/1,4-dioxane before the solvents were removed in vacuo to give **11**[53,62] (0.118 g, toluene still present). The crude product was used in the following synthesis without further purification.

5.14. Compound 12

Compound **6** (0.150 g, 0.30 mmol), the hydrochloride salt of (1*R*,2*S*)-1-amino-2-vinyl-cyclopropane-carboxylic acid ethyl ester (0.067 g, 0.43 mmol), and HATU (0.137 g, 0.36 mmol) were dissolved in DMF (2.0 mL). DIEA (0.27 mL, 1.6 mmol) was added and the pH of the solution was controlled to be >10. The reaction mixture was stirred at room temperature for 23 h. EtOAc (25 mL) was added to the reaction mixture and the organic phase was washed with 0.035 M aqueous $NaHSO_4$ (5 × 5 mL), saturated aqueous $NaHCO_3$ (2 × 5 mL), and brine (1 × 5 mL). The organic phase was dried with $MgSO_4$, filtered, and the solvent removed in vacuo. The crude product was purified by column chromatography (*i*-hexane/EtOAc 7:3) to give **12** (0.141 g, 74%). ^{1}H NMR (CD_3OD) δ 8.17 (d, J = 9.1 Hz, 1H), 7.78 (m, 2H), 7.59 (m, 2H), 7.42 (m, 4H), 7.25 (m, 2H), 7.20 (dd, J = 2.5, 9.1 Hz, 1H), 6.80 (s, 1H), 5.79–5.70 (ddd, J = 8.9, 10.4, 17.2 Hz, 1H), 5.31–5.26 (ddd, J = 0.8, 1.9, 17.2 Hz, 1H), 5.20 (br s, 1H), 5.10 (ddd, J = 0.6, 1.8, 10.4 Hz, 1H), 4.04 (m, 2H), 3.96 (s, 3H), 2.14 (m, 1H), 1.74 (m, 1H), 1.45 (s, 9H), 1.32 (m, 1H), 1.11 (m, 3H). ^{13}C NMR (CD_3OD) δ 173.8, 171.4, 163.9, 163.4, 160.9, 157.3, 155.6, 152.7, 140.9, 136.7, 135.0, 130.9, 130.6, 129.8, 128.6, 123.9, 122.2, 120.0, 118.2, 116.2, 107.6, 102.4, 81.0, 62.4, 59.4, 56.1, 41.0, 34.7, 28.7, 23.3, 14.6. MS ($M+H^+$) 638.1.

5.15. Compound 13

Compound **6** (0.150 g, 0.30 mmol), HBTU (0.137 g, 0.36 mmol), and **11** (0.52 mmol) were dissolved in DMF (2.0 mL). DIEA (0.287 mL, 1.68 mmol) was added after which pH was controlled and found to be <7. Another portion of DIEA (0.050 mL, 0.29 mmol) was added resulting in pH >10. Stirring was continued at room temperature overnight. EtOAc (25 mL) was added and the organic phase was washed with 0.035 M aqueous $NaHSO_4$ (4 × 5 mL), saturated aqueous $NaHCO_3$ (2 × 5 mL), and brine (1 × 5 mL). The organic phase was dried with $MgSO_4$, filtered, and the solvent removed in vacuo. The crude product was purified by column chromatography (hexane/EtOAc 6:4) to give **13** (0.105 g, 55%). ^{1}H NMR (CD_3OD) δ 8.08 (d, J = 9.2 Hz, 1H), 7.75 (m, 2H), 7.56 (m, 2H), 7.39 (m, 4H), 7.20 (m, 2H), 7.11 (dd, J = 2.5, 9.2 Hz, 1H), 6.77 (s, 1H), 6.01 (tm, 1H), 5.28 (s, 1H), 4.64 (dd, J = 4.9, 9.2 Hz, 1H), 3.91 (s, 3H), 3.61 (s, 3H), 2.51–2.20 (m,

2H), 1.45 (s, 9H). ^{13}C NMR (CD_3OD) δ 172.9, 172.1, 163.8, 163.3, 160.8, 157.4, 155.6, 152.6, 140.8, 136.1, 131.0, 130.5, 129.7, 128.6, 123.8, 122.2, 119.9, 116.9 (t, J = 237 Hz), 116.2, 107.6, 102.2, 81.0, 61.5, 59.3, 56.0, 53.0, 36.6 (t, J = 23 Hz), 28.7. MS ($M+H^+$) 636.1.

5.16. Compound 14

Compound **12** (0.122 g, 0.191 mmol) was dissolved in 4 M HCl/1,4-dioxane (3.0 mL) and stirred at room temperature for 1 h 45 min. The solvent was removed in vacuo. The resulting Boc-deprotected product was dissolved in DMF (2.0 mL) and Boc-Val-OH (0.083 g, 0.383 mmol) was added followed by HBTU (0.174 g, 0.460 mmol) and DIEA (0.26 mL, 1.5 mmol). The reaction was stirred overnight. EtOAc (30 mL) was added and the organic phase was washed with 0.035 M aqueous $NaHSO_4$ (3 × 5 mL), saturated aqueous $NaHCO_3$ (2 × 5 mL), and brine (1 × 5 mL). The organic phase was dried with $MgSO_4$, filtered, and the solvent removed in vacuo. The crude product was purified by column chromatography (pentane/EtOAc 2:1) to give **14** (0.108 g, 77%). ^{1}H NMR ($CDCl_3$) δ 8.10 (d, J = 9.1 Hz, 1H), 7.89 (m, 2H), 7.64–7.31 (m, 6H), 7.21–7.05 (m, 3H), 6.91 (s, 1H), 5.72 (ddd, J = 8.7, 10.3, 17.1 Hz, 1H), 5.63 (d, J = 7.3 Hz, 1H), 5.31 (d, J = 7.9 Hz, 1H), 5.23 (dd, J = 1.8, 17.1 Hz, 1H), 5.14 (d, J = 7.1 Hz, 1H), 5.09 (dd, J = 1.9, 10.3 Hz, 1H), 4.12–3.94 (m, 3H), 3.97 (s, 3H), 2.23–2.06 (m, 2H), 1.84 (m, 1H), 1.41 (s, 9H), 1.47–1.32 (m, 1H), 1.09 (m, 3H), 0.99–0.83 (m, 6H). ^{13}C NMR ($CDCl_3$) δ 171.2, 170.2, 169.6, 161.9, 158.8, 156.2, 155.9, 154.6, 151.0, 138.8, 134.6, 133.4, 133.2, 129.6, 128.7, 127.5, 122.8, 120.8, 119.2, 118.0, 115.1, 106.8, 101.6, 80.5, 61.3, 60.4, 56.4, 55.6, 39.9, 33.7, 30.5, 28.2, 23.0, 19.3, 17.7, 14.1. MS ($M+H^+$) 737.2.

5.17. Compound 15

Compound **15** was prepared as **14** using the Boc-deprotected **13** (0.154 mmol), Boc-Tle-OH (0.071 g, 0.308 mmol), HATU (0.141 g, 0.370 mmol), and DIEA (0.205 mL, 1.2 mmol) in DMF (1.0 mL). The crude product was purified by column chromatography using gradient elution (DCM/MeOH 100:0–98:2) to give **15** (0.060 g, 52%). ^{1}H NMR ($CDCl_3$) δ (mixture of rotamers, major rotamer reported) 8.15 (d, J = 9.2 Hz, 1H), 7.84 (m, 2H), 7.53 (m, 2H), 7.49–7.30 (m, 4H), 7.20 (dd, J = 2.5, 9.2 Hz, 1H), 7.14 (m, 2H), 6.82 (s, 1H), 5.93 (tt, J = 4.6, 56 Hz, 1H), 5.74 (d, J = 7.1 Hz, 1H), 5.51 (d, J = 9.0 Hz, 1H), 4.71 (dt, J = 5.0, 7.7 Hz, 1H), 4.07 (m, 1H), 4.0 (s, 3H), 3.66 (s, 3H), 2.52–2.25 (m, 2H), 1.34 (s, 9H), 1.02 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 171.1, 170.6, 169.7, 163.8, 163.1, 158.2, 156.0, 152.9, 142.6, 134.5, 130.5, 129.6, 128.8, 128.1, 123.1, 121.1, 120.2, 119.8, 115.1 (t, J = 234 Hz), 114.8, 104.6, 101.5, 79.9, 66.6, 62.4, 56.4, 56.0, 52.8, 35.9 (t, J = 23 Hz), 35.0, 28.2, 26.6. MS ($M+H^+$) 749.2.

5.18. Compound 16

Compound **16** was prepared as described for **5**, using **14** (0.108 g, 0.147 mmol), LiOH (0.053 g, 2.21 mmol), THF (6.0 mL), MeOH (3.2 mL), and water (0.8 mL) to give **16** partly epimerized (L:D 68:32) at the Phg α-carbon (0.083 g, 80%) as a solid. ^{1}H NMR (CD_3OD, mixture of diastereomers) δ [8.18 (d, J = 9.2 Hz), 8.17 (d, J = 9.2 Hz), 1H], 7.77 (m, 2H), 7.59 (m, 2H), 7.47–7.42 (m, 4H), 7.25–7.16 (m, 3H), 6.79 (m, 1H), 5.80 (m, 1H), [5.58 (br s), 5.53 (br s), 1H], [5.29 (dd, J = 1.9, 17.1 Hz), 5.27 (dd, J = 1.9, 17.1 Hz), 1H], [5.10 (dd, J = 1.9, 10.3 Hz), 5.09 (dd, J = 1.9, 10.3 Hz), 1H], 3.96 (s, 3H), [3.88 (d, J = 7.3 Hz), 3.88 (d, J = 7.3 Hz), 1H], 2.24–1.97 (m, 2H), 1.77 (m, 1H), [1.43 (s), 1.42 (s), 9H], 1.30 (m, 1H), 0.98–0.85 (m, 6H). HRMS calcd for $C_{40}H_{43}N_4O_8$ ($M-H^+$) 707.3081, found: 707.3057. RP-HPLC purity (column 2: 95.2%, column 3: 90.2%).

5.19. Compound 17

Compound **17** was prepared as described for **5**, using **15** (0.059 g, 0.079 mmol), LiOH (0.028 g, 1.18 mmol), THF (3.0 mL), MeOH (1.5 mL) and water (0.4 mL). The crude product was purified using RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) to give **17** partly epimerized (L:D 60:40) at the Phg α-carbon (0.023 g, 39%) as a solid after freeze-drying. ^{1}H NMR ($CDCl_3$, mixture of diastereomers) δ 8.30 (m, 1H), 7.62 (m, 4H), 7.57 (m, 2H), 7.39 (m, 2H), 7.22–6.97 (m, 3H), 6.55 (m, 1H), 5.66 (tm, 1H), 5.37 (m, 1H), 4.81–4.57 (m, 1H), 4.11 (m, 1H), [4.04 (s), 4.03 (s), 3H], 2.55–2.21 (m, 2H), [1.40 (s), 1.38 (s), 9H], [1.02 (s), 0.93 (s), 9H]. HRMS calcd for $C_{39}H_{43}N_4O_8F_2$ ($M-H^+$) 733.3049, found: 733.3074. RP-HPLC purity (column 2: 99.8%, column 3: 99.1%).

5.20. Compounds 18a and 18b

All solid chemicals used in the synthesis were dried in vacuo over P_2O_5 before use. CDI (0.029 g, 0.181 mmol) was dissolved in dry THF (0.2 mL). Compound **16** (0.064 g, 0.090 mmol) was dissolved in dry THF (1.45 mL) and added dropwise to the CDI solution. The reaction mixture was stirred at room temperature for 1 h under N_2 atmosphere. Benzenesulfonamide (0.057 g, 0.36 mmol) dissolved in dry THF (0.3 mL) and DBU (0.027 mL, 0.180 mmol) were added. The reaction mixture was stirred at room temperature for 22 h. The mixture was diluted with EtOAc and washed with 0.1 M NaOAc buffer, pH 4.0 (3 × 3 mL), and brine (1 × 3 mL). The organic phase was dried with Na_2SO_4, filtered, and the solvent removed in vacuo. The crude product was purified by column chromatography using gradient elution (DCM/MeOH 100:0–98:2), to give the pure P2 L-isomer **18a** (0.014 g, 18%). The fractions containing the corresponding D-isomer (0.013 g, 17%) were purified using RP-HPLC (Vydac C18 (6), MeCN/H_2O gradient with 0.1% TFA), to give **18b** (0.013 g, 17%).

5.20.1. Phg L-isomer (18a). Compound **18a** was synthesized in two ways. For characterization, see under General method for synthesis of tripeptides **18a** and **35**–**47** (method B). HRMS calcd for $C_{46}H_{50}N_5O_9S$ ($M+H^+$) 848.3329, found: 848.3315.

5.20.2. Phg D-isomer (18b). ^{1}H NMR (CD_3OD) δ 8.46 (d, J = 9.2 Hz, 1H), 7.95 (m, 2H), 7.83 (m, 2H), 7.72–7.52

(m, 9H), 7.48 (dd, J = 2.5, 9.2 Hz, 1H), 7.43 (m, 2H), 6.99 (s, 1H), 5.44 (s, 1H), 5.37 (m, 1H), 5.16 (dm, 1H), 4.92 (m, 1H, partly overlapped by the water signal), 4.07 (s, 3H), 3.89 (d, J = 8.4 Hz, 1H), 2.18 (m, 1H), 2.03 (m, 2H), 1.72 (dd, J = 5.5, 8.1 Hz, 1H), 1.43 (s, 9H), 1.00–0.90 (m, 7H). HRMS calcd for $C_{46}H_{48}N_5O_9S$ ($M-H^+$) 846.3173, found: 846.3186. RP-HPLC purity (column 2: 98.2, column 3: 98.6%).

5.21. *tert*-Butyl (*S*)-1-{[(phenylsulfonyl)amino]carbonyl}-butylcarbamate (19)

Prepared as described by us previously.[35,36]

5.22. *tert*-Butyl 1-{[(phenylsulfonyl)amino]carbonyl}-cyclopropylcarbamate (20)

Prepared as described by us previously.[36]

5.23. *tert*-Butyl (1*R*,2*S*)-1-{[(cyclopropylsulfonyl)amino]-carbonyl}-2-vinylcyclopropylcarbamate (21)

Compound **21** has been previously described in the literature.[37] Prepared as described for compound **22**.

5.24. *tert*-Butyl (1*R*,2*S*)-1-{[(phenylsulfonyl)amino]carbonyl}-2-vinylcyclopropylcarbamate (22)

(1*R*,2*S*)-1-*tert*-Butoxycarbonylamino-2-vinylcyclopropancarboxylic acid (0.282 g, 1.2 mmol), benzene sulfonamide (0.382 g, 2.4 mmol), CDI (0.389 g, 2.4 mmol), and glassware were dried in vacuo over P_2O_5 before use. CDI and the (1*R*,2*S*)-1-*tert*-butoxycarbonylamino-2-vinylcyclopropancarboxylic acid were dissolved in dry THF (8.0 mL) and stirred at room temperature for 1 h under N_2 atmosphere. Benzenesulfonamide dissolved in dry THF (3 mL) followed by DBU (0.9 mL, 6.0 mmol) was added. The reaction mixture was stirred at room temperature overnight. pH was adjusted to 1 using 1 M HCl whereafter the THF was evaporated and the aqueous phase extracted with EtOAc (3×40 mL). The combined organic phases were dried with $MgSO_4$, filtered, and the solvent removed in vacuo. The crude product was purified by column chromatography using RP-silica (MeCN/H_2O 1:1) to give **22** (0.274 g, 62%) after freeze-drying. ^{1}H NMR (CD_3OD) δ 7.99 (m, 2H), 7.67 (m, 1H), 7.55 (m, 2H), 5.14 (m, 2H), 2.1 (m, 1H), 1.69 (dd, J = 7.9, 5.4 Hz, 1H), 1.46 (s, 9H), 1.2 (J = 9.4, 7.9 Hz, 1H). ^{13}C NMR (CD_3OD) δ 170.6, 158.1, 140.9, 134.7, 133.9, 129.8, 129.3, 118.1, 81.6, 43.7, 34.7, 28.6, 21.8. MS ($M+H^+$) 367.1.

5.25. Benzyl (*S*)-1-{[(cyclopropylsulfonyl)amino]carbonyl}-3-difluoropropylcarbamate (23)

Compound **23** was prepared as described for **22** using (*S*)-2-[(benzyloxycarbonyl)amino]-4,4-difluorobutanoic acid (0.186 g, 0.68 mmol), cyclopropylsulfonamide (0.169 g, 1.4 mmol), CDI (0.227 g, 1.4 mmol), and DBU (0.21 mL, 1.4 mmol) in dry THF (4+2 mL) and stirred for 4 h. The crude product was purified by column chromatography using gradient elution (DCM/MeOH 98:2–95:5), to give **23** (0.162 g, 62%). ^{1}H NMR (CD_3OD) δ 7.36–7.31 (m, 5H), 6.00 (ddt, J = 3.9, 5.5, 56.2 Hz, 1H), 5.10 (s, 2H), 4.31 (dd, J = 4.9, 9.2 Hz, 1H), 2.85 (m, 1H), 2.42–2.13 (m, 2H), 1.16 (m, 2H), 1.02 (m, 2H). ^{13}C NMR (CD_3OD) δ 174.0, 158.2, 138.0, 129.5, 129.1, 128.9, 116.9 (t, J = 239 Hz), 67.9, 52.4, 37.3 (t, J = 22.3 Hz), 31.7, 6.09, 6.05. MS ($M+H^+$) 377.0.

5.26. General method for preparation of dipeptides 24–34 (method A)

The P1-sulfonamide building blocks **19**–**22** respectively, were dissolved in 4 M HCl/1,4-dioxane (approximately 10 mL/mmol starting material) and stirred at room temperature for 2–4 h, until the starting material could no longer be detected by LC–MS. The solvent was removed in vacuo and the hydrochloride salt of Boc-deprotected product was used in subsequent couplings without further purification. The P2 building block (**3**, **5**–**10**), the N-deprotected **19**–**22** (and **23**, N-deprotected by catalytic hydrogenation), and HATU or HBTU were dissolved in DMF. DIEA was added and the pH was controlled. If the pH of the solution was less than 10, more DIEA was added until pH 10 was reached. The reaction mixture was stirred at room temperature overnight. EtOAc (approximately 10 mL/mL DMF) was added to the mixture and the organic phase was washed first with 0.1 or 0.2 M aqueous $NaHSO_4$-solution, then repeatedly with 0.035 M aqueous $NaHSO_4$ until the pH of the aqueous phase was $\leqslant$3, and finally with brine. The organic phase was dried with $MgSO_4$, filtered, and the solvent removed in vacuo. The crude product was purified chromatographically if necessary, either on a silica gel column or by preparative RP-HPLC.

5.27. Compound 24

Compound **24** was prepared according to method A using **6** (0.132 g, 0.26 mmol), Boc-deprotected **19** (0.31 mmol), HATU (0.117 g, 0.31 mmol), and DIEA (0.30 mL, 1.76 mmol) in DMF (2.5 mL). Purification by column chromatography (DCM/MeOH 95:5) gave **24** (0.160 g, 83%). ^{1}H NMR (CD_3OD) δ 8.20 (d, J = 9.2 Hz, 1H), 7.89 (m, 2H), 7.81 (m, 2H), 7.55–7.39 (m, 10H), 7.23 (dd, J = 2.5, 9.2 Hz, 1H), 7.15 (m, 2H), 6.83 (s, 1H), 5.28 (br s, 1H), 4.30 (dd, J = 5.5, 8.4 Hz, 1H), 3.98 (s, 3H), 1.75–1.56 (m, 2H), 1.44 (s, 9H), 1.36–1.26 (m, 2H), 0.87 (dd, J = 7.3, 7.4 Hz, 3H). ^{13}C NMR (CD_3OD) δ 174.4, 172.7, 164.3, 163.7, 160.8, 157.5, 155.3, 152.1, 141.8, 140.3, 136.5, 134.1, 130.8, 129.9, 128.75, 128.72, 124.1, 122.0, 120.2, 116.3, 107.2, 102.5, 81.0, 58.9, 56.1, 55.5, 34.8, 28.7, 19.8, 14.0. HRMS calcd for $C_{40}H_{43}N_4O_8S$ ($M+H^+$) 739.2802, found: 739.2799.

5.28. Compound 25

Compound **25** was prepared according to method A using **6** (0.100 g, 0.20 mmol), Boc-deprotected **20** (0.096 g, 0.4 mmol) (Note: the hydrochloride salt of deprotected **20** had been removed by treatment with propeneoxide in EtOH), HBTU (0.091 g, 0.24 mmol), DIEA (0.14 mL, 0.8 mmol), and DMF (0.8 mL).

Amount **25**: 0.106 g (73%). ^{1}H NMR (CD_3OD) δ 8.20 (d, J = 9.3 Hz, 1H), 7.95 (m, 2H), 7.82–7.34 (m, 11H), 7.32–7.18 (m, 3H), 6.82 (s, 1H), 5.08 (s, 1H), 3.97 (s, 3H), 1.84–1.23 (m, 2H), 1.53 (s, 9H), 0.94 (m, 2H). ^{13}C NMR (CD_3OD) δ 174.8, 173.0, 166.2, 164.9, 159.5, 158.5, 155.2, 148.6, 140.6, 137.0, 135.6, 134.7, 132.0, 131.3, 130.2, 129.9, 129.1, 129.0, 124.9, 122.4, 121.2, 116.0, 104.4, 102.9, 81.8, 60.8, 56.5, 39.0, 28.7, 19.0. MS ($M+H^+$) 723.2.

5.29. Compound 26

Compound **26** was prepared according to method A using **6** (0.135 g, 0.27 mmol), Boc-deprotected **21** (0.33 mmol), HATU (0.125 g, 0.33 mmol), and DIEA (0.32 mL, 1.86 mmol) in DMF (2.5 mL). Purification by column chromatography (DCM/MeOH 97:3) gave **26** (0.137 g, 70%) as a solid. ^{1}H NMR (CD_3OD) δ 8.17 (d, J = 9.2 Hz, 1H), 7.79 (m, 2H), 7.57 (m, 2H), 7.43 (m, 4H), 7.26 (m, 2H), 7.21 (dd, J = 2.5, 9.2 Hz, 1H), 6.8 (s, 1H), 5.70 (ddd, J = 8.9, 10.2, 17.3 Hz, 1H), 5.27 (dd, J = 1.9, 17.1 Hz, 1H), 5.12 (dd, J = 1.9, 10.1 Hz, 1H), 5.01 (s, 1H), 3.96 (s, 3H), 2.9 (m, 1H), 2.12 (m, 1H), 1.84 (dd, J = 5.5, 8.1 Hz, 1H), 1.48 (s, 9H), 1.18 (m, 3H), 1.05 (m, 2H). ^{13}C NMR (CD_3OD) δ 174.6, 170.8, 164.0, 163.5, 160.9, 158.1, 155.9, 152.5, 140.6, 135.2, 134.2, 131.0, 130.7, 129.8, 128.6, 124.0, 122.4, 120.1, 118.6, 116.3, 107.5, 102.5, 81.5, 60.2, 56.1, 42.8, 35.5, 31.9, 28.7, 23.0, 6.6, 6.5. MS ($M+H^+$) 713.2.

5.30. Compound 27

Compound **27** was prepared according to method A using **6** (0.215 g, 0.43 mmol), Boc-deprotected **22** (0.572 mmol), HATU (0.194 g, 0.51 mmol), DIEA (0.5 mL, 2.9 mmol), and DMF (4.5 mL). Purification by column chromatography (DCM/MeOH 98:2) gave **27** (0.186 g, 58%). ^{1}H NMR (CD_3OD) δ 8.18 (d, J = 9.2 Hz, 1H), 7.93 (m, 2H), 7.85 (m, 2H), 7.58 (m, 3H) 7.51 (m, 2H) 7.44 (d, J = 2.5 Hz, 1H), 7.42 (m, 3H), 7.26 (m, 2H), 7.22 (dd, J = 2.5, 9.2 Hz, 1H), 6.82 (s, 1H), 5.44 (m, 1H), 5.15 (dd, J = 1.8, 17.1 Hz, 1H), 5.10 (s, 1H), 4.93 (m, 1H), 3.96 (s, 3H), 2.04 (m, 1H), 1.69 (m, 1H), 1.50 (s, 9H), 1.10 (m, 1H). ^{13}C NMR (CD_3OD) δ 174.6, 170.6, 164.2, 163.7, 160.7, 158.1, 155.8, 152.1, 141.2, 140.3, 135.4, 134.4, 134.0, 131.1, 130.8, 129.9, 129.8, 129.1, 128.7, 124.1, 122.4, 120.2, 118.3, 116.2, 107.2, 102.6, 81.5, 60.2, 56.1, 42.9, 35.4, 28.8, 22.8. MS ($M+H^+$) 749.2.

5.31. Compound 28

Compound **23** (0.133 g, 0.354 mmol) was dissolved in MeOH (6 mL) and 10% Pd/C (0.031 g) was added. The flask was sealed with a rubber septum and flushed with H_2 for 3 h at which time no starting material could be detected on LC–MS. The mixture was centrifuge filtered through a Whatman PP filter (0.45 μm) and the solvent was evaporated. Compound **28** was then prepared as described in method A by addition of **6** (0.133 g, 0.265 mmol), HATU (0.120 mg, 0.318 mmol), DMF (2 mL) and DIEA (0.212 mL, 1.24 mmol). Amount **28**: 0.173 g (90%). ^{1}H NMR (CD_3OD) δ 8.16 (d, J = 9.3 Hz, 1H), 7.77 (m, 2H), 7.60 (m, 2H), 7.54–7.37 (m, 4H), 7.32–7.17 (m, 3H), 6.82 (s, 1H), 6.01 (ddt, J = 3.8, 5.4, 56.1 Hz, 1H), 5.3 (s, 1H), 4.59 (m, 1H), 3.94 (s, 3H), 2.93 (m, 1H), 2.48–2.19 (m, 2H), 1.46 (s, 9H), 1.26–1.08 (m, 2H), 1.06–0.93 (m, 2H). ^{13}C NMR (CD_3OD) δ 172.9, 171.8, 164.9, 164.1, 160.1, 157.4, 155.2, 150.5, 138.8, 136.3, 131.3, 131.1, 130.0, 128.8, 124.3, 122.2, 120.5, 116.8 (t, J = 240 Hz), 116.1, 106.0, 102.5, 81.2, 59.3, 56.3, 50.5, 36.6 (t, J = 23.1 Hz), 31.7, 28.7, 6.3, 6.2. MS ($M+H^+$) 725.1.

5.32. Compound 29

Compound **29** was prepared according to method A using **9** (0.094 g, 0.22 mmol), Boc-deprotected **21** (0.18 mmol), HATU (0.100 g, 0.26 mmol), DIEA (0.188 mL, 1.10 mmol), and DMF (1 mL) and stirred for 45 h. Purification by column chromatography using gradient elution (DCM: MeOH 99:1–98:2) gave **29** (0.044 g, 38%). ^{1}H NMR (CD_3OD) δ 8.36 (dd, J = 1.0, 8.4 Hz, 1H), 7.86–7.76 (m, 2H), 7.66 (ddd, J = 1.6, 6.6, 8.3, 1H), 7.52 (m, 2H), 7.48 (s, 1H), 7.27 (m, 2H), 5.72 (ddd, J = 8.9, 10.3, 17.2 Hz, 1H), 5.28 (dd, J = 1.9, 17.2 Hz, 1H), 5.11 (dd, J = 1.9, 10.3 Hz, 1H), 2.95 (m, 1H), 2.15 (m, 1H), 1.84 (dd, J = 5.4, 8.1 Hz, 1H), 1.49 (s, 9H), 1.35–1.17 (m, 3H), 1.07 (m, 2H). ^{13}C NMR (CD_3OD) δ 174.8, 171.2, 161.0, 158.2, 155.1, 142.9, 141.5, 134.4, 133.3, 130.3, 128.8, 127.0, 125.1, 123.4, 119.4, 118.4, 116.2, 81.4, 60.3, 42.8, 35.5, 31.9, 28.7, 23.1, 6.6, 6.4. MS ($M+H^+$) 641.1, 643.1

5.33. Compound 30

Compound **30** was prepared according to method A using **10** (0.104 g, 0.22 mmol), Boc-deprotected **21** (0.28 mmol), HATU (0.100 g, 0.26 mmol), DIEA (0.21 mL, 1.25 mmol), and DMF (1.5 mL). Purification by preparative RP-HPLC–MS, using an MeCN/H_2O gradient with 0.05% HCOOH, gave **30** (0.060 g, 40%) after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.42 (m, 2H), 8.32 (d, J = 8.2 Hz, 1H), 7.91 (m, 1H), 7.84–7.73 (m, 3H), 7.66 (m, 1H), 7.55 (m, 2H), 7.31 (m, 2H), 5.74 (m, 1H), 5.26 (dd, J = 1.7, 17.1 Hz, 1H), 5.12–5.07 (m, 2H), 2.97 (m, 1H), 2.13 (m, 1H), 1.86 (dd, J = 5.5, 8.1 Hz, 1H), 1.49 (s, 9H), 1.21 (m, 3H), 1.04 (m, 2H). ^{13}C NMR (CD_3OD) δ 174.9, 170.9, 165.8, 161.1, 158.1, 148.3, 145.0, 140.3, 134.1, 132.8, 129.9, 129.6, 128.6, 124.9, 123.5, 122.1, 120.9, 118.7, 115.2, 81.4, 60.3, 42.8, 35.5, 31.9, 28.7, 23.1, 6.6, 6.5. MS ($M+H^+$) 684.2.

5.34. Compound 31

Compound **31** was prepared according to method A using **7** (0.072 g, 0.19 mmol), Boc-deprotected **21** (0.22 mmol), HATU (0.091 g, 0.24 mmol), DIEA (0.191 mL, 1.12 mmol), and DMF (1.0 mL), and stirred for 19 h. Purification by column chromatography (DCM/MeOH 95:5) gave**31** (0.091 g, 81%) in the presence of another diastereomer (<10%) as determined by NMR. ^{1}H NMR (CD_3OD, major isomer (**31**) reported) δ 7.98 (s, 1H), 7.78 (dd, J = 7.6, 8.1 Hz, 1H), 7.47 (m,

2H), 7.14 (3H), 6.90 (m, 1H), 5.70 (m, 1H), 5.28 (ddd, $J = 0.8$, 1.9, 17.1 Hz, 1H), 5.11 (m, 1H), 5.03 (s, 1H), 2.94 (m, 1H), 2.13 (m, 1H), 1.83 (dd, $J = 5.4$, 8.1 Hz, 1H), 1.47 (s, 9H), 1.22 (m, 3H), 1.07 (m, 2H). ^{13}C NMR (CD_3OD) 174.7, 170.9, 164.3, 158.1, 155.3, 149.8, 143.6, 134.2, 130.4, 122.6, 122.5, 119.9, 118.5, 110.9, 81.4, 60.3, 42.8, 35.5, 31.9, 28.7, 23.1, 6.6, 6.5. MS ($M+H^+$) 591.1.

5.35. Compound 32

Compound **32** was prepared according to method A using **8** (0.067 g, 0.19 mmol), Boc-deprotected **21** (0.22 mmol), HATU (0.091 g, 0.24 mmol), DIEA (0.191 mL, 1.12 mmol), and DMF (1.0 mL) and stirred for 19 h. Purification by column chromatography (DCM/MeOH 95:5) gave **32** (0.095 g, 87%) in the presence of another diastereomer (<10%) as determined by NMR. ^{1}H NMR (CD_3OD, major isomer (**32**) reported) δ 8.12 (ddd, $J = 0.9$, 2.1, 5.0 Hz, 1H), 7.83 (ddd, $J = 2.1$, 7.3, 8.3 Hz, 1H), 7.46 (m, 2H), 7.11 (m, 3H), 6.98 (m, 1H), 5.70 (m, 1H), 5.27 (dd, $J = 2.0$, 17.1 Hz, 1H), 5.11 (dd, $J = 2.0$, 10.3 Hz, 1H), 5.01 (s, 1H), 2.93 (m, 1H), 2.12 (m, 1H), 1.82 (dd, $J = 5.2$, 8.0 Hz, 1H), 1.47 (s, 9H), 1.22 (m, 3H), 1.06 (m, 2H). ^{13}C NMR (CD_3OD) δ 174.7, 170.9, 164.8, 158.1, 155.9, 148.3, 141.7, 134.3, 133.8, 130.4, 122.2, 120.4, 118.5, 113.2, 81.4, 60.3, 42.8, 35.5, 31.7, 28.7, 23.1, 6.6, 6.4. MS ($M+H^+$) 557.2.

5.36. Compound 33

Compound **33** was prepared according to method A using **5** (0.056 g, 0.20 mmol), Boc-deprotected **21** (0.22 mmol), HATU (0.091 g, 0.24 mmol), DIEA (0.191 mL, 1.12 mmol), and DMF (1.1 mL), and stirred for 24 h. Purification by column chromatography using gradient elution (pentane/EtOAc 70:30–50:50) gave **33** (0.079 g, 80%) as a 50:50 mixture of diastereomers. ^{1}H NMR (CD_3OD) δ 7.32 (m, 2H), 6.92 (m, 2H), 5.70 (m, 1H), [5.28 (dd, $J = 1.9$, 17.3 Hz), 5.27 (dd, $J = 2.0$, 17.1 Hz),1H], [5.12 (dd, $J = 1.9$, 10.4 Hz), 5.10 (dd, $J = 2.0$, 10.3 Hz), 1H], [4.91 (s), 4.88 (s), 1H], [3.78 (s), 3.78 (s), 3H], 2.92 (m, 1H), 2.08 (m, 1H), [1.81 (dd, $J = 5.4$, 8.2 Hz), 1.80 (dd, $J = 5.3$, 8.2 Hz), 1H], [1.47 (s), 1.45 (s), 9H], 1.21 (m, 3H), 1.06 (m, 2H). ^{13}C NMR (CD_3OD) δ 175.4, 175.2, 171.0, 161.4 (two peaks), 158.5, 158.1, 134.2, 134.1, 130.0, 129.2, 128.8, 118.6, 118.5, 115.4, 115.3, 81.6, 81.4, 61.0, 60.3, 55.8 (two peaks), 42.7 (two peaks), 35.5, 35.3, 31.9 (two peaks), 28.7 (two peaks), 23.1, 6.6, 6.5, 6.4. MS ($M+H^+$) 494.2.

5.37. Compound 34

Compound **34** was prepared according to method A using **3** (0.027 g, 0.10 mmol), Boc-deprotected **19** (0.052 g, 0.20 mmol) (Note: the hydrochloride salt of deprotected **19** had been removed by treatment with propeneoxide in EtOH), HBTU (0.049 g, 0.13 mmol), DIEA (0.070 mL, 0.40 mmol), and DMF (1.0 mL). The crude product **34** (0.066 g) was used in the following coupling without further purification.

5.38. General method for synthesis of tripeptides 18a and 35–47 (method B)

The *N*-Boc-protected dipeptides **24**–**34**, respectively, were dissolved in 4 M HCl/1,4-dioxane (~10 mL/mmol) and stirred at room temperature for 4–6 h or as long as needed for the starting material to be consumed. The progress of the reaction was followed by LC–MS, TLC or both. The solvent was removed in vacuo to give the deprotected hydrochloride of dipeptide **24**–**34**. To the hydrochloride salt, Boc-Tle-OH or Boc-Val-OH, HATU, and DMF were added. DIEA was added to the reaction mixture until pH >10. The mixture was stirred at room temperature overnight. EtOAc (approximately 10 mL/mL DMF) was added to the mixture and the organic phase was washed first with 0.1 or 0.2 M aqueous $NaHSO_4$-solution, then repeatedly with 0.035 M aqueous $NaHSO_4$ until the pH of the aqueous phase was $\leqslant 3$, and finally with brine. After drying the organic phase with $MgSO_4$ and filtration, the solvent was removed in vacuo. The product was purified by RP-HPLC (MeCN/H_2O with 0.1% TFA) and the pure fractions were pooled and freeze-dried. The yields were affected by the fact that the Phg-residue always isomerized to some extent during the couplings.

5.39. Compound 18a

Compound **18a** was prepared according to method B using the hydrochloride salt of deprotected **27** (0.098 mmol), Boc-Val-OH (0.031 g, 0.14 mmol), HATU (0.061 g, 0.016 mmol), DIEA (0.22 mL, 1.3 mmol), and DMF (1.5 mL). Purification by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) gave **18a** (0.033 g, 43%) as a white solid, after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.50 (d, $J = 9.3$ Hz, 1H), 7.94 (m, 2H), 7.80 (m, 2H), 7.71 (m, 2H), 7.66 (m, 3H), 7.60 (m, 3H), 7.44 (m, 2H), 6.9 (s, 1H), 5.42–5.33 (m, 2H), 5.16 (dd, $J = 1.9$, 17.1 Hz, 1H), 4.93 (dd, $J = 1.9$, 10.1 Hz, 1H), 4.08 (s, 3H), 3.96 (d, $J = 7.2$ Hz, 1H), 2.10 (m, 2H), 1.68 (dd, $J = 5.5$, 8.1 Hz, 1H), 1.45 (s, 9H), 1.07 (dd, $J = 5.5$, 9.5 Hz, 1H), 1.00 (d, $J = 6.8$, 3H), 0.94 (d, $J = 6.8$, 3H). ^{13}C NMR (CD_3OD) δ 174.9, 173.6, 169.7, 168.6, 166.7, 158.5 158.2, 154.4, 145.1, 140.7, 136.5, 134.8, 133.7, 133.6, 131.9, 130.7, 129.9, 129.5, 129.2, 125.9, 122.7, 122.4, 118.6, 115.8, 103.2, 101.3, 80.7, 61.3, 58.9, 57.0, 42.8, 35.3, 32.3, 28.8, 22.8, 19.9, 18.7. HRMS calcd for $C_{46}H_{50}N_5O_9S$ ($M+H^+$) 848.3329, found: 848.3347. RP-HPLC purity (column 2: 98.7%, column 2: 99.1%).

5.40. Compound 35

Compound **35** was prepared according to method B using the hydrochloride salt of deprotected **24** (0.17 mmol), Boc-Tle-OH (0.062 g, 0.27 mmol), HATU (0.127 g, 0.33 mmol), DIEA (0.24 mL, 1.4 mmol), and DMF (1.8 mL). Purification by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) gave **35** (0.0138 g, 10%) as a white solid, after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.53 (m, 2H), 7.91 (m, 2H), 7.84 (m, 2H), 7.7–7.5 (m, 9H), 7.32

(m, 2H), 6.92 (s, 1H), 5.58 (m, 1H), 4.29 (dd, J = 5.7, 8.7, 1H), 4.10 (s, 3H), 3.97 (m, 1H), 1.68–1.51 (m, 2H), 1.42 (s, 9H), 1.38–1.24 (m, 2H), 0.99 (s, 9H), 0.89 (dd, J = 7.3, 7.3 Hz). ^{13}C NMR (CD_3OD) δ 173.1, 172.4, 171.6, 168.8, 166.7, 158.5, 157.9, 153.9, 145.2, 140.8, 137.7, 134.7, 133.9, 133.6, 131.6, 130.8, 129.9, 129.6, 129.1, 125.9, 122.3, 121.9, 115.9, 111.2, 103.2, 101.4, 80.7, 63.7, 57.2, 57.0, 54.9, 35.3, 34.2, 28.7, 27.2, 19.9, 13.9. HRMS calcd for $C_{46}H_{54}N_5O_9S$ ($M+H^+$) 852.3642, found: 852.3625. RP-HPLC purity (column 1: 98.1%, column 2: 99.7%).

5.41. Compound 36

Compound **36** was prepared according to method B using the hydrochloride salt of deprotected **25** (0.147 mmol), Boc-Tle-OH (0.068 g, 0.294 mmol), HBTU (0.134 g, 0.352 mmol), DIEA (0.22 mL, 1.29 mmol), and DMF (1.2 mL). Purification by RP-HPLC (Sorbax SB-C8 column, MeCN/H_2O gradient with 0.1% TFA) gave **36** (0.053 g, 43%) as a white solid, after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.55 (d, J = 9.2 Hz, 1H), 7.99 (m, 2H), 7.82 (m, 2H), 7.74–7.55 (m, 10H), 7.44 (m, 2H), 6.95 (s, 1H), 5.22 (s, 1H), 4.10 (s, 3H), 3.98 (s, 1H), 1.43 (s, 9H), 1.38 (m, 2H), 1.05 (s, 9H), 0.90 (m, 2H). HRMS calcd for $C_{45}H_{48}N_5O_9S$ ($M-H^+$) 834.3173, found: 834.3160. RP-HPLC purity (column 1: 98.8%, column 2: 99.8%).

5.42. Compound 37

Compound **37** was prepared according to method B using the hydrochloride salt of deprotected **26** (0.076 mmol), Boc-Tle-OH (0.035 g, 0.15 mmol), HATU (0.069 g, 0.18 mmol), DIEA (0.11 mL, 0.67 mmol), and DMF (1.0 mL). Purification by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) gave **37** (0.009 g, 15%) as a white solid, after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.55 (d, J = 9.3 Hz, 1H), 7.82 (m, 2H), 7.71 (m, 3H), 7.66–7.61 (m, 3H), 7.57 (dd, J = 2.4, 9.3 Hz, 1H), 7.46 (m, 2H), 6.93 (s, 1H), 5.70 (ddd, J = 8.7, 10.4, 17.1 Hz, 1H), 5.29 (s, 1H), 5.28 (dd, J = 2.0, 17.2), 5.14 (dd, J = 1.9, 10.3 1H), 4.10 (s, 3H), 3.98 (s, 1H), 2.94 (m, 1H), 2.17 (m, 1H), 1.85 (dd, J = 5.4, 8.1 Hz, 1H), 1.44 (s, 9H), 1.23–1.17 (m, 3H), 1.08–0.97 (m, 2H), 1.02 (s, 9H). ^{13}C NMR (CD_3OD) δ 173.7, 173.5, 170.5, 169.1, 167.0, 158.3, 158.0, 154.3, 144.5, 136.7, 134.1, 133.8, 133.4, 132.0, 130.9, 129.6, 126.1, 122.7, 122.6, 118.7, 115.8, 103.3, 100.8, 80.7, 63.3, 59.1, 57.1, 42.9, 35.6, 35.4, 28.8, 28.7, 27.2, 22.9, 6.64, 6.57. HRMS calcd for $C_{44}H_{50}N_5O_9S$ ($M-H^+$) 824.3329, found: 824.3322. RP-HPLC purity (column 1: 95.1%, column 2: 98.8%).

5.43. Compound 38

Compound **38** was prepared according to method B using the hydrochloride salt of deprotected **27** (0.098 mmol), Boc-Tle-OH (0.031 g, 0.14 mmol), HATU (0.062 g, 0.016 mmol), DIEA (0.23 mL, 1.3 mmol), and DMF (1.5 mL). Purification by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) gave **38** (0.032 g, 41%) as a white solid after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.53 (d, J = 9.3 Hz, 1H), 7.94 (m, 2H), 7.81 (m, 2H), 7.71 (m, 2H), 7.67 (m, 2H), 7.64–7.59 (m, 4H), 7.57–7.51 (m, 4H), 7.46 (m, 2H), 6.92 (s, 1H), 5.44–5.31 (m, 2H), 5.14 (dd, J = 2.0, 17.2 Hz, 1H), 4.92 (dd, J = 2.0, 10.3 Hz, 1H), 4.10 (s, 3H), 4.01 (s, 1H), 2.10 (m, 1H), 1.68 (dd, J = 5.5, 8.1 Hz, 1H), 1.44 (s, 9H), 1.09 (dd, J = 5.5, 9.5 Hz, 1H), 1.04 (s, 9H). ^{13}C NMR (CD_3OD) δ 173.8, 173.5, 169.7, 169.0, 166.9, 158.3, 158.0, 154.3, 144.5, 140.6, 136.7, 134.8, 133.8, 133.7, 133.3, 132.1, 130.8, 129.6, 129.3, 126.0, 122.7, 122.6, 118.6, 115.8, 103.3, 100.8, 80.7, 63.4, 59.1, 57.0, 42.8, 35.6, 35.4, 28.8, 27.3, 22.7. HRMS calcd for $C_{47}H_{52}N_5O_9S$ ($M+H^+$) 862.3486, found: 862.3491. RP-HPLC purity (column 2: 98.9%, column 3: 98.9%).

5.44. Compound 39

Compound **39** was prepared according to method B using the hydrochloride salt of deprotected **28** (0.239 mmol), Boc-Tle-OH (0.119 g, 0.516 mmol), HATU (0.235 g, 0.619 mmol), DIEA (0.37 mL, 2.19 mmol), and DMF (2.0 mL). Purification by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) gave **39** (0.057 g, 28%) as a white solid, after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.50 (d, J = 9.3 Hz, 1H), 7.83 (m, 2H), 7.71–7.61 (m, 5H), 7.58 (d, J = 2.4 Hz, 1H), 7.52 (dd, J = 2.5, 9.3 Hz, 1H), 7.40 (m, 2H), 6.95 (s, 1H), 6.02 (ddt, J = 3.6, 5.5, 56 Hz, 1H), 5.57 (s, 1H), 4.60 (dd, J = 5.2, 9.0 Hz, 1H), 4.09 (s, 3H), 4.00 (s, 1H), 2.83 (m, 1H), 2.46–2.18 (m, 2H), 1.43 (s, 9H), 1.6 (m, 2H), 1.04–0.98 (m, 2H), 1.01 (s, 9H). ^{13}C NMR (CD_3OD) δ 173.2, 171.7, 171.1, 168.5, 166.5, 158.7, 157.9, 154.2, 145.5, 137.3, 134.2, 133.4, 131.8, 130.7, 129.5, 125.8, 122.5, 122.2, 116.6 (t, J = 237 Hz), 115.9, 103.2, 101.6, 80.7, 63.6, 57.9, 56.9, 50.2, 36.4 (t, J = 23 Hz), 35.4, 31.8, 28.7, 27.2, 6.4, 6.3. HRMS calcd for $C_{42}H_{48}N_5O_9SF_2$ ($M-H^+$) 836.3141, found: 836.3149. RP-HPLC purity (column 1: 97.8%, column 2: 99.4%).

5.45. Compounds 40a and 40b

Compound **40** was prepared according to method B using the hydrochloride salt of deprotected **29** (0.07 mmol), Boc-Tle-OH (0.037 g, 0.16 mmol), HATU (0.073 mg, 0.19 mmol), DIEA (0.164 mL, 0.96 mmol), and DMF (0.6 mL). Purification by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) gave the Phg L-isomer **40a** (0.017 g, 32%) as a white solid, after freeze-drying. The mixed fractions were purified using RP-HPLC (Zorbax SB-C8 column, (21.2 × 150 mm), MeCN/H_2O gradient with 0.1% TFA), to give the Phg D-isomer **40b** (0.0018 g, 3%) as a solid after freeze-drying. Phg L-isomer **40a**: ^{1}H NMR (CD_3OD) δ 9.18 (s, 1H), 8.83 (d, J = Hz, 1H), 8.38 (d, J =8.4, 1.1 Hz, 1H), 7.85 (m, 1H), 7.81 (ddd, J = 1.4, 6.8, 8.2 Hz, 1H), 7.68 (ddd, J = 1.5, 6.7, 8.3 Hz, 1H), 7.57 (m, 2H), 7.50 (s, 1H), 7.29 (m, 2H), 5.72 (m, 1H), 5.29 (dd, J = 1.9, 17.1 Hz, 1H), 5.21 (d, J = 4.4 Hz, 1H), 5.12 (dd, J = 1.9, 10.3 Hz, 1H), 4.00 (s, 1H), 2.96 (m, 1H), 2.17 (m, 1H), 1.86 (dd, J = 5.4, 8.2 Hz, 1H), 1.45 (s, 9H), 1.23 (m, 3H), 1.10–1.01 (m, 2H), 1.04 (s,

9H). ^{13}C NMR (CD_3OD) δ 173.8, 173.6, 170.8, 161.1, 157.9, 155.3, 143.0, 141.6, 134.2, 133.8, 133.3, 130.7, 128.8, 127.0, 125.1, 123.5, 119.4, 118.6, 116.2, 80.6, 63.1, 59.5, 42.8, 35.8, 35.6, 32.1, 28.8, 27.2, 23.4, 6.7, 6.5. HRMS calcd for $C_{37}H_{43}N_5O_8ClS$ ($M-H^+$) 752.2521, found: 752.2505. RP-HPLC purity (column 1: 98.1%, column 2: 98.7%). Phg D-isomer **40b**: ^{1}H NMR (CD_3OD) δ 8.39 (ddd, J = 0.96, 2.1, 8.4 Hz, 1H), 7.87–7.79 (m, 2H), 7.6 (m, 1H), 7.56 (m, 2H), 7.51 (s, 1H), 7.32 (m, 2H), 5.75 (ddd, J = 8.7, 10.3, 17.1 Hz, 1H), 5.30 (dd, J = 2.0, 17.1 Hz, 1H), 5.24 (s, 1H), 5.13 (dd, J = 2.0, 10.5 Hz, 1H), 3.98 (s, 1H), 2.98 (m, 1H), 2.22 (m, 1H), 1.85 (m, 1H), 1.45 (s, 9H), 1.33–1.18 (m, 3H), 1.15–1.04 (m, 2H), 1.00 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.7, 174.4, 170.8, 161.1, 158.1, 155.3, 143.0, 141.6, 134.2, 133.3, 130.8, 128.8, 127.0, 125.1, 123.5, 119.4, 118.6, 116.2, 106.2, 80.9, 63.5, 60.0, 42.6, 36.1, 35.5, 32.0, 28.9, 27.0, 23.5, 6.7, 6.6. HRMS calcd for $C_{37}H_{45}N_5O_8$ ClS ($M+H^+$) 754.2677, found: 754.2689. RP-HPLC purity (column 1: 98.6%, column 2: 99.0%).

5.46. Compound 41

Compound **30** (0.060 g, 0.09 mmol) was dissolved in 4 M HCl in EtOAc (2 mL) and stirred for 3 h at room temperature, after which the solvent was removed in vacuo. With the exception of this deviation from protocol, **41** was prepared according to method B using the hydrochloride of the deprotected **30**, Boc-Tle-OH (0.042 g, 0.18 mmol), HATU (0.082 g, 0.22 mmol), DIEA (0.15 mL, 0.88 mmol), and DMF (0.8 mL). Purification by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) gave **41** (0.006 g, 8%) as a white solid, after freeze-drying. ^{1}H NMR (CD_3OD, 500 MHz) δ 8.67 (m, 2H), 8.51 (dm, 1H), 8.45 (m, 1H), 8.35 (m, 2H), 8.12 (d, J = 8.2 Hz, 1H), 7.93 (m, 1H), 7.85 (m, 1H), 7.62 (m, 2H), 7.41 (m, 2H), 5.71 (m, 1H), 5.29 (dd, J = 1.9, 17.1 Hz, 1H), 5.27 (s, 1H), 5.15 (dd, J = 1.9, 10.3 Hz, 1H), 4.02 (s, 1H), 2.95 (m, 1H), 2.2 (m, 1H), 1.88 (dd, J = 5.5, 8.2 Hz, 1H), 1.45 (s, 9H), 1.27–1.17 (m, 3H), 1.11–1.01 (m, 2H), 1.05 (s, 9H). ^{13}C NMR (CD_3OD, 100 MHz) δ 174.1, 173.6, 170.6, 161.6, 158.0, 155.4, 153.0, 146.4, 143.6, 140.3, 134.1, 133.9, 133.2, 130.7, 130.5, 129.1, 125.2, 123.6, 123.4, 121.6, 118.8, 117.4, 80.7, 63.3, 59.4, 43.0, 35.7, 35.5, 32.1, 28.8, 27.2, 22.3, 6.7, 6.6. HRMS calcd for $C_{42}H_{47}N_6O_8S$ ($M-H^+$) 795.3176, found: 795.3193. RP-HPLC purity (column 1: 96.4%, column 2: 98.0%).

5.47. Compound 42

Compound **42** was prepared according to method B using the hydrochloride salt of deprotected **31** (0.139 mmol), Boc-Tle-OH (0.064 g, 0.278 mmol), HATU (0.127 g, 0.334 mmol), DIEA (0.209 mL, 1.224 mmol), and DMF (1.5 mL). Purification by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) gave **42** (0.063 g, 61%) as a white solid, after freeze-drying. ^{1}H NMR (CD_3OD) δ 9.1 (s, 1H), 7.79 (dd, J = 7.6, 8.2 Hz, 1H), 7.51 (m, 2H), 7.14 (m, 3H), 6.91 (m, 1H), 5.71 (m, 1H), 5.28 (dd, J = 2.0, 17.1 Hz, 1H), 5.18 (s, 1H), 5.12 (dd, J = 2.0, 10.3 Hz, 1H), 3.99 (s, 1H), 2.94 (m, 1H), 2.16 (m, 1H), 1.85 (dd, J = 5.4, 8.2 Hz, 1H), 1.44 (s, 9H), 1.23 (m, 3H), 1.08 (m, 2H), 1.03 (s, 9H). ^{13}C NMR (CD_3OD) δ 173.8, 173.5, 170.7, 164.3, 157.9, 155.5, 149.8, 143.6, 134.2, 133.4, 130.8, 122.7, 119.9, 118.6, 111.0, 80.6, 63.1, 59.4, 42.8, 35.8, 35.5, 32.1, 28.7, 27.1, 23.3, 6.7, 6.5. HRMS calcd for $C_{33}H_{41}N_5O_8SCl$ ($M-H^+$) 702.2364, found: 702.2360. RP-HPLC purity (column 1: 96.1%, column 2: 97.7%).

5.48. Compound 43

Compound **43** was prepared according to method B using the hydrochloride salt of deprotected **32** (0.162 mmol), Boc-Tle-OH (0.075 g, 0.324 mmol), HATU (0.148g, 0.389 mmol), DIEA (0.244 mL, 1.426 mmol), and DMF (2.0 mL). Purification by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) gave **43** (0.070 g, 64%) as a white solid, after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.14 (ddd, J = 0.88, 2.0, 5.1 Hz, 1H), 7.86 (ddd, J = 2.0, 7.3, 8.3 Hz, 1H), 7.50 (m, 2H), 7.15 (m, 3H), 6.99 (m, 1H), 5.70 (ddd, J = 8.8, 10.4, 17.1 Hz, 1H), 5.28 (dd, J = 2.1, 17.1 Hz, 1H), 5.17 (s, 1H), 5.12 (dd, J = 2.1, 10.3 Hz, 1H), 3.99 (s, 1H), 2.94 (m, 1H), 2.15 (m, 1H), 1.83 (dd, J = 5.4, 8.1 Hz, 1H), 1.44 (s, 9H), 1.21 (m, 3H), 1.07 (m, 2H), 1.02 (s, 9H). ^{13}C NMR (CD_3OD) δ 173.8, 173.5, 170.7, 164.6, 157.8, 156.0, 148.0, 142.1, 134.2, 133.2, 130.9, 122.3, 120.5, 118.6, 113.2, 80.6, 63.1, 59.4, 42.8, 35.8, 35.6, 32.1, 28.7, 27.2, 23.2, 6.7, 6.5. HRMS calcd for $C_{33}H_{44}N_5O_8S$ ($M+H^+$) 670.2911, found: 670.2914. RP-HPLC purity (column 2: 99.1%, column 3: 99.2%).

5.49. Compounds 44a and 44b

Compound **44** was prepared according to method B using the hydrochloride salt of deprotected **33** (0.154 mmol), Boc-Tle-OH (0.071 g, 0.308 mmol), HATU (0.141 mg, 0.370 mmol), DIEA (0.205 mL, 1.202 mmol), and DMF (1.5 mL). Purification by RP-HPLC (Zorbax SB-C8 column, MeCN/H_2O gradient with 0.1% TFA) gave the Phg L-isomer **44a** (0.017 g, 19%) and Phg D-isomer **44b** (0.017g, 19%) as white solids after freeze-drying. Phg L-isomer **44a** ^{1}H NMR (CD_3OD) δ 7.27 (m, 2H), 6.84 (m, 2H), 5.60 (m, 1H), 5.19 (dd, J = 2.0, 17.2 Hz, 1H), 5.02 (dd, J = 2.0, 10.4 Hz, 1H), 4.98 (s, 1H), 3.88 (s, 1H), 3.70 (s, 3H), 2.84 (m, 1H), 2.02 (m, 1H), 1.74 (dd, J = 5.4, 8.1 Hz, 1H), 1.35 (s, 9H), 1.15 (m, 3H), 1.06 (m, 2H), 0.93 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.2, 173.4, 170.8, 161.6, 157.9, 134.2, 130.5, 128.6, 118.5, 115.3, 80.6, 63.1, 59.4, 55.8, 42.7, 35.8, 35.6, 32.0, 28.7, 27.2, 23.3, 6.7, 6.5. HRMS calcd for $C_{29}H_{43}N_4O_8S$ ($M+H^+$) 607.2802, found: 607.2789. RP-HPLC purity (column 1: 95.0%, column 2: 97.9%). Phg D-isomer **44b** ^{1}H NMR (CD_3OD) δ 7.34 (m, 2H), 6.94 (m, 2H), 5.75 (ddd, J = 17.2, 10.4, 8.7 Hz, 1H), 5.28 (dd, J = 17.2, 2.0 Hz, 1H), 5.11 (dd, J = 10.4, 2.0 Hz, 1H), 5.06 (s, 1H), 3.95 (s, 1H), 3.80 (s, 3H), 2.96 (m, 1H), 2.15 (m, 1H), 1.80 (dd, J = 8.3, 5.2 Hz, 1H), 1.44 (s, 9H), 1.26 (m, 2H), 1.08 (m, 2H), 0.96 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.7, 174.6,

170.8, 161.6, 158.0, 134.3, 130.5, 128.1, 118.6, 115.4, 80.8, 63.3, 59.9, 55.8, 42.5, 36.2, 35.5, 32.0, 28.9, 27.0, 23.4, 6.64, 6.58. HRMS calcd for $C_{29}H_{41}N_4O_8S$ ($M-H^+$) 605.2645, found: 605.2657. RP-HPLC purity (column 2: 98.1%, column 3: 97.2%).

5.50. Compound 45

Compound **45** was prepared according to method B using the hydrochloride salt of deprotected **24** (0.079 mmol), Boc-Val-OH (0.034 g, 0.16 mmol), HBTU (0.072 g, 0.19 mmol), DIEA (0.12 mL, 0.70 mmol), and DMF (0.7 mL). Purification by RP-HPLC (Vydac C18 (L), MeCN/H_2O gradient with 0.1% TFA) gave **45** as a solid. ^{1}H NMR (CD_3OD) δ 8.55 (d, J = 9.3 Hz, 1H), 7.91 (m, 2H), 7.84 (m, 2H), 7.71–7.47 (m, 10H), 7.53 (m, 2H), 6.93 (s, 1H), 5.59 (s, 1H), 4.30 (m, 1H), 4.10 (s, 3H), 3.93 (d, J = 6.8 Hz, 1H), 2.03 (m, 1H), 1.66–1.51 (m, 2H), 1.43 (s, 9H) 1.43–1.23 (m, 2H), 0.97–0.86 (m, 9H). ^{13}C NMR (CD_3OD) δ 174.1, 172.3, 171.6, 169.2, 166.9, 158.3, 158.1, 153.8, 144.6, 140.8, 138.0, 134.7, 133.8, 133.3, 131.5, 130.9, 129.9, 129.6, 129.0, 126.0, 122.5, 122.3, 115.8, 103.2, 100.9, 80.7, 69.9, 61.5, 57.0, 55.0, 34.2, 31.9, 28.7, 19.9, 19.7, 18.5, 13.9. HRMS calcd for $C_{45}H_{52}N_5O_9S$ ($M+H^+$) 838.3486, found: 838.3499. RP-HPLC purity (column 1: 98.5%, column 2: 100%).

5.51. Compound 46

Compound **46** was prepared according to method B using the hydrochloride salt of deprotected **26** (0.11 mmol), Boc-Val-OH (0.037 g, 0.17 mmol), HATU (0.076 g, 0.20 mmol), DIEA (0.21 mL, 1.2 mmol), and DMF (1.4 mL). Purification by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) gave **46** (0.039 g, 43%) as a white solid, after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.54 (d, J = 9.3 Hz, 1H), 7.83 (m, 2H), 7.73–7.69 (m, 3H), 7.65–7.61 (m, 3H), 7.57 (dd, J = 2.4, 9.3 Hz, 1H), 7.46 (m, 2H), 6.93 (s, 1H), 5.73–5.63 (m, 1H), 5.33 (s, 1H), 5.29 (dd, J = 1.9, 17.1 Hz, 1H), 5.14 (dd, J = 1.9, 10.3 Hz, 1H), 4.10 (s, 1H), 3.94 (d, J = 7.1 Hz, 1H), 2.93 (m, 1H), 2.18 (m, 1H), 2.06 (m, 1H), 1.85 (dd, J = 5.5, 8.1 Hz, 1H), 1.44 (s, 9H), 1.21–1.15 (m, 3H), 1.09–1.05 (m, 2H), 0.97 (d, J = 6.9 Hz, 3H), 0.92 (d, J = 6.9 Hz, 3H). ^{13}C NMR (CD_3OD) δ 174.18, 173.5, 170.4, 169.1, 167.0, 158.3, 158.2, 154.2, 144.4, 136.8, 134.0, 133.8, 133.3, 131.9, 130. 8, 129.6, 126.0, 122.7, 122.6, 118.7, 115.8, 103.3, 100.8, 80.7, 61.2, 58.9, 57.1, 42.9, 35.2, 32.2, 32.0, 28.8, 22.9, 19.8, 18.7, 6.6, 6.5. HRMS calcd for $C_{43}H_{50}N_5O_9S$ ($M+H^+$) 812.3329, found: 812.3353. RP-HPLC purity (column 1: 100%, column 2: 100%).

5.52. Compound 47

Compound **47** was prepared according to method B using the hydrochloride salt of deprotected **34** (0.13 mmol), Boc-Val-OH (0.062 g, 0.29 mmol), HBTU (0.084 g, 0.22 mmol), DIEA (0.10 mL, 0.57 mmol), and DMF (1.0 mL). Purification by RP-HPLC (Vydac C18 (L) MeCN/H_2O gradient with 0.1% TFA) gave **47** (0.021 g, 27%) as a white solid after freeze-drying. ^{1}H NMR (CD_3OD) δ 7.94 (m, 2H), 7.66 (m, 1H), 7.54 (m, 2H), 7.11 (m, 2H), 6.69 (m, 2H), 5.35 (s, 1H), 4.26 (dd, J = 5.6, 8.4 Hz, 1H), 3.89 (d, J = 6.8 Hz, 1H), 2.02 (m, 1H), 1.63 (m, 2H), 1.27 (m, 2H), 0.89 (m, 9H). ^{13}C NMR (CD_3OD) ^{13}C NMR (CD_3OD) δ 174.0, 172.4, 158.6, 158.1, 140.7, 134.8, 130.0 (two overlapping signals), 129.1, 128.8, 116.4, 80.7, 61.4, 57.6, 54.8, 34.4, 31.9, 28.7, 19.8, 18.4, 13.9. HRMS calcd for $C_{29}H_{39}N_4O_8S$ ($M-H^+$) 603.2489, found: 603.2491. RP-HPLC purity (column 1: 99.4%, column 2: 98.7%).

5.53. Compound 48

Compound **48** was in all essentials prepared as described for **12**, using **9** (0.257 g, 0.60 mmol), the hydrochloride salt of (1*R*,2*S*)-1-amino-2-vinyl-cyclopropanecarboxylic acid ethyl ester (0.159 g, 0.83 mmol), HATU (0.274 g, 0.72 mmol), and DIEA (0.53 mL, 5.2 mmol) in DMF (2.5 mL). The crude product was purified by column chromatography (*i*-hexane/EtOAc 7:3) to give **48** (0.224 g, 66%). ^{1}H NMR ($CDCl_3$) δ 8.34 (d, J = 8.3 Hz, 1H), 7.70 (m, 2H), 7.58 (ddd, J = 1.5, 6.6, 8.4 Hz, 1H), 7.46 (m, 2H), 7.27 (m, 3H), 6.83 (br s, 1H), 5.90 (br s, 1H), 5.77 (ddd, J = 8.7, 10.3, 17.1 Hz, 1H), 5.30 (dd, J = 1.8, 17.1, Hz, 1H), 5.13 (dd, J = 1.8, 10.3 Hz, 1H), 4.11 (m, 2H), 2.14 (m, 2H), 1.81 (dd, J = 5.4, 8.1 Hz, 1H), 1.44 (s, 9H), 1.18 (dd, J = 7.2, 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 171.1, 170.0, 159.6, 155.4, 153.5, 142.1, 140.1, 135.1, 133.5, 132.0,128.9, 127.4, 125.8, 124.6, 122.2, 118.5, 118.3, 115.2, 80.5, 61.8, 58.0, 40.2, 34.5, 28.6, 23.2, 14.5. MS ($M+H^+$) 566.3.

5.54. Compound 49

Compound **49** was in all essentials prepared as described for **12**, using **10** (0.170 g, 0.36 mmol), the hydrochloride salt of (1*R*,2*S*)-1-amino-2-vinyl-cyclopropanecarboxylic acid ethyl ester (0.096 g, 0.50 mmol), HATU (0.163 g, 0.43 mmol), and DIEA (0.36 mL, 2.1 mmol) in DMF (2.0 mL). The product **49** (0.218 g) obtained after extraction was used in the subsequent synthesis without further purification. ^{1}H NMR ($CDCl_3$) δ 8.66 (m, 2H), 8.42 (m, 1H), 8.03 (m, 3H), 7.91 (m, 1H), 7.79 (m, 1H), 7.70 (ddd, J = 1.3, 7.0, 8.3 Hz, 1H), 7.57 (m, 2H), 7.30 (m, 2H), 5.96 (d, J = 6.9 Hz, 1H), 5.73 (m, 1H), 5.34 (s, 1H), 5.28 (dd, J = 2.0, 17.1 Hz, 1H), 5.11 (dd, J = 2.0, 10.3 Hz, 1H), 4.09 (m, 2H), 2.15 (m, 1H), 1.84 (m, 1H), 1.43 (s, 9H), 1.23 (m, 1H), 1.15 (dd, J = 6.8, 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 170.9, 169.8, 160.0, 155.1, 153.2, 150.1, 145.7, 142.6, 138.5, 135.0, 133.3, 131.8, 129.0, 128.7, 128.6, 127.5, 124.3, 122.1, 121.7, 120.3, 118.2, 80.2, 61.4, 57.9, 39.9, 34.1, 28.3, 22.8, 14.2. MS ($M+H^+$) 609.1.

5.55. Compound 50

Compound **12** (0.280 g, 0.44 mmol) was dissolved in 4 M HCl/1,4-dioxane (5 mL) and stirred at room temperature for 3 h. The solvent was removed in vacuo and the product dried in vacuo. *N*-Boc-amino-8-nonenoic acid (0.183 g, 0.67 mmol) was dissolved in DMF (2.0 mL) and added to the hydrochloride salt of N-deprotected **12**. HATU (0.453 g, 1.19 mmol), and DMF

(7 mL) were added followed by NMM (0.73 mL, 7.1 mmol). The reaction was stirred at room temperature for 24 h. EtOAc (20 mL) was added and the organic phase was washed with 0.1 M $NaHSO_4$ (2 × 30 mL), saturated aqueous $NaHCO_3$ (1 × 30 mL), and brine (1 × 15 mL). The organic phase was dried with $MgSO_4$, filtered, and the solvent removed in vacuo. The crude product was purified by column chromatography (*i*-hexane/EtOAc 6:4), to give **50** (0.292 g, 84%) as a solid. ^{1}H NMR (CD_3OD) δ 8.20 (d, J = 9.2 Hz, 1H), 7.79 (m, 2H), 7.61 (m, 2H), 7.46 (d, J = 2.5 Hz, 1H), 7.44 (m, 3H), 7.27 (m, 2H), 7.23 (dd, J = 2.5, 9.2 Hz, 1H), 6.83 (s, 1H), 5.85–5.68 (m, 2H), 5.44 (s, 1H), 5.29 (m, 1H), 5.10 (m, 1H), 4.96 (m, 1H), 4.89 (m, 1H, partly overlapped with the water signal), 4.11–4.01 (m, 2H), 3.98 (s, 3H), 3.65 (m, 1H), 2.22 (m, 1H), 2.02 (m, 2H), 1.75 (m, 2H), 1.60 (m, 1H), δ 1.43 (s, 9H), δ 1.38 (m, 7H), δ 1.12 (dd, J = 7.1, 7.1, 3H). ^{13}C NMR (CD_3OD) δ 175.0, 172.9, 171.3, 163.9, 163.5, 161.1, 158.3, 155.8, 152.8, 141.0, 140.0, 136.2, 135.0, 131.2, 130.6, 129.8, 128.6, 123.9, 122.2, 120.1, 118.3, 116.3, 114.9, 107.7, 102.5, 80.9, 67.8, 62.4, 58.0, 56.5, 56.1, 41.0, 34.8, 29.9, 29.8, 28.8, 28.7, 26.7, 23.3, 14.7. MS (M+H$^+$) 791.3.

5.56. Compound 51

Compound **51** was in all essentials prepared as described for **50**, except that **48** (0.186 g, 0.33 mmol) was deprotected at room temperature for 2.5 h in 4 M HCl/EtOAc (4.5 mL) before the solvent was removed in vacuo. In the coupling reaction, *N*-Boc-amino-8-nonenoic acid (0.138 g, 0.50 mmol), HATU (0.194 g, 0.50 mmol), NMM (0.274 mL, 2.65 mmol), and DMF (2.5 mL) were used. The product **51** (0.232 g, 97%) obtained after extraction was used in subsequent reaction without further purification. ^{1}H NMR ($CDCl_3$) 8.33 (dd, J = 1.1, 8.4 Hz, 1H), 7.72 (ddd, J = 1.3, 6.7, 8.3 Hz, 1H), 7.66 (m, 2H), 7.58 (ddd, J = 1.4, 6.7, 8.4 Hz, 1H), 7.46 (m, 2H), 7.25 (m, 2H), 7.17 (d, J = 6.8 Hz, 1H), 5.80–5.70 (m, 2H), 5.53 (d, J = 7.0 Hz, 1H), 5.32 (dd, J = 2.1, 17.0 Hz, 1H), 5.12 (dd, J = 2.0, 10.3 Hz, 1H), 5.04 (d, J = 5.2 Hz, 1H), 5.00–4.89 (m, 2H), 4.08 (m, 3H), 2.29 (m, 2H), 2.01 (m, 2H), 1.82 (m, 2H), 1.61 (m, 1H), 1.45 (s, 9H), 1.45–1.28 (m, 7H), 1.17 (dd, J = 7.2, 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 171.7, 170.3, 169.8, 159.4, 156.18, 153.3, 141.8, 139.9, 138.7, 134.0, 133.6, 131.8, 129.1, 127.3, 125.5, 124.4, 122.0, 118.3, 117.9, 115.0, 114.4, 104.8, 80.9, 61.4, 56.9, 55.6, 40.0, 33.6, 31.9, 28.7, 28.6, 28.2, 25.5, 23.0, 14.3. MS (M+H$^+$) 719.4, 721.4.

5.57. Compound 52

Compound **52** was in all essentials prepared as described for **51**, using **49** (0.186 g, 0.31 mmol) and 4 M HCl/EtOAc (4.0 mL) for the deprotection. In the coupling reaction, *N*-Boc-amino-8-nonenoic acid (0.126 g, 0.47 mmol), HATU (0.177 g, 0.47 mmol), NMM (0.371 mL, 3.37 mmol), and DMF (2.5 mL) were used. The crude product was purified by column chromatography (DCM/MeOH 96:4) to give **52** (0.133 g, 56%). ^{1}H NMR ($CDCl_3$) δ 8.64 (m, 2H), 8.38 (dd, J = 3.7, 8.3 Hz, 1H), 7.93–7.87 (m, 4H), 7.76 (ddd, J = 1.4, 6.9, 8.2 Hz, 1H), 7.66 (ddd, J = 1.3, 7.0, 8.3 Hz, 1H), 7.54 (m, 2H), 7.30 (m, 2H), 5.79–5.66 (m, 2H), 5.58 (d, 7.0 Hz, 1H), 5.27 (ddd, J = 2.1, 8.3, 17.1 Hz, 1H), 5.22 (s, 1H), 5.10 (dd, J = 2.2, 10.5 Hz, 1H), 4.96–4.86 (m, 2H), 4.15–3.98 (m, 3H), 2.23 (m, 1H), 2.20–1.78 (m, 5H), 1.52 (m, 1H), 1.51–1.20 (m, 6H), 1.41 (s, 9H), 1.15 (dd, J = 7.2, 7.4 Hz). ^{13}C NMR ($CDCl_3$) δ 171.7, 170.4, 169.8, 159.8, 156.1, 153.4, 148.2, 147.6, 147.1, 143.4, 138.7, 134.1, 133.5, 133.3, 131.6, 128.9, 128.7, 127.3, 124.3, 122.0, 121.2, 120.0, 117.9, 114.4, 80.6, 61.3, 56.8, 55.5, 40.0, 33.5, 32.0, 28.64, 28.56, 28.3, 28.2, 25.5, 22.9, 14.2. MS (M+H$^+$) 762.3.

5.58. Compound 53

Compound **50** (0.263 g, 0.33 mmol) was dried in vacuo over P_2O_5 before use. Compound **50** was dissolved in dry toluene (600 mL, dried over sodium metal) under N_2 atmosphere. Grubb's catalyst, second generation (0.032 g, 0.038 mmol), was added to the solution. The reaction mixture was stirred at room temperature for 45 min, after which it was heated to 80 °C for 1.5 h. After cooling to room temperature, the solvent was removed in vacuo. The crude product was purified by column chromatography using gradient elution (DCM/MeOH 99:1–97:3), to give **53** (0.117 g, 46%) as a solid. ^{1}H NMR ($(CD_3)_2CO$) δ 8.19 (d, J = 9.1 Hz, 1H), 8.04 (m, 2H), 7.71 (m, 2H), 7.49–7.46 (m, 4H), 7.34 (m, 2H), 7.24 (dd, J = 2.5, 9.1 Hz, 1H), 7.07 (s, 1H), 5.62–5.50 (m, 3H), 4.23 (m, 1H), 4.05 (m, 2H), 4.02 (s, 3H), 2.28–2.20 (m, 2H), 2.02 (m, 1H), 1.97–1.88 (m, 2H), 1.80–1.72 (m, 1H), 1.68 (dd, J = 5.0, 9.6 Hz, 1H), 1.56 (m, 2H), 1.45–1.33 (m, 3H), 1.40 (s, 9H), 1.30 (m, 2H), 1.12 (m, 3H). ^{13}C NMR ($(CD_3)_2CO$) δ 173.0, 170.8, 170.2, 162.2, 161.9, 159.0, 155.4, 154.6, 151.2, 133.8, 132.7, 129.9, 129.6, 128.8, 127.6, 125.3, 122.8, 121.0, 119.2, 115.2, 107.0, 101.4, 79.9, 61.4, 56.2, 55.7, 53.9, 53.5, 52.2, 40.4, 40.3, 33.3, 31.7, 28.4, 27.2, 26.0, 23.5, 23.4, 14.3. MS (M+H$^+$) 763.2.

5.59. Compound 54

Compound **54** was prepared as described for **53** using **51** (0.200g, 0.22 mmol), dry toluene (600 mL), and Grubb's catalyst, second generation (0.030 g, 0.035 mmol). The crude product was purified by column chromatography (DCM/MeOH 98:2) to give **54** (0.140 g, 72%). ^{1}H NMR ($CDCl_3$) δ 8.4 (m, 1H), 7.72 (m, 2H), 7.60 (m, 1H), 7.53 (m, 2H), 7.46 (m, 1H), 7.37 (m, 2H), 7.29 (m, 1H), 5.85–5.29 (m, 3H), 5.01 (m, 1H), 4.41–4.09 (m, 3H), 2.25 (m, 1H), 2.14–1.70 (m, 6H), 1.53–1.30 (m, 6H), 1.44 (s, 9H), 1.24 (m, 3H). HRMS calcd for $C_{37}H_{44}N_4O_7Cl$ (M+H$^+$) 691.2899, found: 691.2892.

5.60. Compound 55

Compound **55** was prepared as described for **53** using **52** (0.066g, 0.087 mmol), dry toluene (180 mL), and Grubb's catalyst, second generation (0.0095 g, 0.011 mmol). The reaction mixture was stirred at 85° for 6 h thereafter another portion of catalyst (0.0095 g, 0.011 mmol) was added and heating was continued overnight. The toluene was removed in vacuo and the resi-

due was purified by column chromatography (DCM/MeOH 97:3) to give **55** (0.040 g, 64%). ^{1}H NMR (CD_3OD) δ 8.56–8.30 (m, 3H), 8.19–7.62 (m, 8H), 7.31 (m, 2H), 5.82 (s, 1H), 5.59 (m, 1H), 5.03 (m, 1H), 4.38 (m, 1H), 4.08 (m, 2H), 2.58 (m, 1H), 2.15–1.80 (m, 4H), 1.76 (m, 1H), 1.61 (dd, J = 5.1, 9.3 Hz, 1H), 1.45–1.14 (m, 6H), 1.43 (s, 9H), 1.09 (dd, J = 7.1, 7.1 Hz, 3H). HRMS calcd for $C_{42}H_{48}N_5O_7$ ($M+H^+$) 734.3554, found: 734.3555.

5.61. Compound 56

Compound **53** (0.060 g, 0.082 mmol) was dissolved in THF (3.0 mL) and MeOH (1.5 mL). LiOH (0.027 g, 1.12 mmol) dissolved in water (0.5 mL) was added and the solution stirred for 22 h at room temperature. The pH was adjusted to ~7 using 1 M HCl and the organic solvents were removed in vacuo. The pH was adjusted to 3 using more 1M HCl followed by extraction with EtOAc (2× 15 mL). The organic phase was washed with brine, dried with $MgSO_4$, and removed in vacuo to give **56** (0.056 g). 0.009 g of crude **56** was purified for biological testing using RP-HPLC (ACE 5 Phenyl S/N-A15161 column (150 × 21.2 mm), MeCN/H_2O gradient with 0.1% TFA) to give **56** (0.0016 g) as a white solid after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.36 (dd, J = 1.0, 9.3 Hz, 1H), 7.80 (m, 4H), 7.59–7.53 (m, 4H), 7.36 (m, 3H), 6.9 (s, 1H), 5.8 (br s, 1H), 5.64–5.57 (m, 2H), 5.51–5.46 (m, 1H), 4.24 (dd, J = 4.2, 7.8 Hz, 1H), 4.03 (s, 3H), 2.28–2.18 (m, 2H), 1.82 (m, 1H), 1.67–1.61 (m, 2H), 1.54–1.28 (m, 8H), 1.44 (s, 9H). ^{13}C NMR (CD_3OD) δ 175.3, 173.8, 173.5 167.2, 165.6, 159.3, 157.5, 154.5, 151.9, 147.6, 136.1, 134.1, 132.6, 132.0, 130.4, 129.3, 129.2, 127.0, 125.2, 122.3, 121.6, 116.0, 103.5, 102.9, 80.5, 57.7, 56.7, 55.1, 41.1, 33.8, 32.6, 29.5, 28.7, 28.6, 27.3, 24.2, 23.9. HRMS calcd for $C_{42}H_{45}N_4O_8$ ($M-H^+$) 733.3237, found: 733.3224. RP-HPLC purity (column 2: 97.4%, column 3: 98.2%).

5.62. Compound 57

Compound **57** was prepared as described for **56** using **54** (0.095 g, 0.14 mmol), THF (6 mL), MeOH (3.2), and LiOH (0.049 g, 2.1 mmol) dissolved in water (0.8 mL). The crude product was purified by column chromatography (DCM/MeOH 95:5) to give **57** (0.0357 g, 39%). ^{1}H NMR (CD_3OD) δ 8.34 (dd, J = 1.0, 8.4 Hz, 1H), 7.75 (m, 2H), 7.60 (m, 3H), 7.39 (s, 1H), 7.23 (m, 2H), 5.62–5.44 (m, 3H), 4.26 (m, 1H), 2.24–2.11 (m, 3H), 1.77–1.65 (m, 4H), 1.46–1.27 (m, 6H), 1.42 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.2, 173.4, 172.9, 160.5, 156.6, 154.2, 142.5, 140.8, 133.8, 133.4, 132.6, 130.2, 128.1, 126.4, 126.3, 124.8, 122.6, 118.9, 115.7, 80.4, 57.4, 55.6, 54.2, 40.8, 33.6, 32.2, 29.0, 28.6, 27.8, 26.7, 23.5. HRMS calcd for $C_{35}H_{38}N_4O_7Cl$ ($M-H^+$) 661.2429, found: 661.2422. RP-HPLC purity (column 1: 95.4%, column 2: 97.0%).

5.63. Compound 58

Compound **55** (0.040 g, 0.055 mmol) was dissolved in THF (2.0 mL) and MeOH (1.0 mL). LiOH (0.020 g, 0.83 mmol) was dissolved in water (0.3 mL) and added to the reaction. After stirring at room temperature overnight, the pH was adjusted to ~6 by addition of 1 M aqueous HCl after which the organic solvents were removed in vacuo. The aqueous phase was diluted with water and acidified to pH ~3. A precipitate was formed that was sonicated and cooled in the refrigerator before filtration. After vacuum-drying, the crude **58** was purified by RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA), giving **58** (0.0043 g, 11%) as a solid after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.77 (m, 2H), 8.55 (m, 4H), 8.15 (m, 1H), 7.95 (ddd, J = 1.4, 7.0, 8.2 Hz, 1H), 7.89 (ddd, J = 1.3, 7.0, 8.3 Hz, 1H), 7.78 (m, 2H), 7.37 (m, 2H), 5.79 (s, 1H), 5.61 (m, 1H), 5.04 (m, 1H), 4.32 (m, 1H), 2.60 (m, 1H), 2.26–2.01 (m, 3H), 1.91–1.64 (m, 3H), 1.58–1.14 (m, 6H), 1.42 (s, 9H). ^{13}C NMR (CD_3OD) δ 174.6, 174.0, 173.3, 162.0, 157.0, 154.7, 142.8, 141.9, 140.0, 135.7, 133.9, 133.5, 131.5, 130.6, 129.5, 128.6, 125.3, 124.5, 123.3, 122.1, 119.0, 106.2, 80.6, 56.8, 54.9, 54.4, 39.9, 33.4, 31.5, 31.5, 28.7, 28.3, 27.4, 23.2, 22.4. HRMS Calcd for $C_{40}H_{44}N_5O_7$ ($M+H^+$) 706.3241, found: 706.3254. RP-HPLC purity (column 1: 95.8%, column 2: 97.2%).

5.64. Compound 59

The starting material, reagents, and glassware were dried in vacuo over P_2O_5. CDI (0.042 g, 0.26 mmol) and **56** (0.095 g, 0.129 mmol) were dissolved in dry THF (3.3 mL) under N_2 atmosphere. The reaction mixture was stirred at 60 °C for 2 h. When the solution had cooled to room temperature, benzenesulfonamide (0.041 g, 0.26 mmol) dissolved in dry THF (1.2 mL) and DBU (0.036 mL, 0.26 mmol) were added. The reaction mixture was stirred at room temperature for 23 h. EtOAc (10 mL) was added and the organic phase washed with 1 M NaOAc buffer (pH 4). The organic phase was dried with $MgSO_4$, filtered, and the solvent removed in vacuo. Purification was performed using RP-HPLC, (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA) to give **59** (0.013 g, 11%) as a white solid after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.50 (d, J = 9.5 Hz, 1H), 7.90–7.83 (m, 6H), 7.65–7.58 (m, 5H), 7.54–7.50 (m, 5H), 7.00 (s, 1H), 5.49 (s, 1H), 5.35 (m, 1H), 4.55 (m, 1H), 4.21 (m, 1H), 4.09 (s, 3H), 2.23–2.05 (m, 3H), 1.79 (m, 2H), 1.58 (m, 1H), 1.50–1.30 (m, 7H), 1.44 (s, 9H). ^{13}C NMR (CD_3OD) δ 177.2, 175.8, 169.4, 167.8, 166.1, 162.7, 159.0, 157.5, 154.9, 146.5, 140.5, 136.0, 135.1, 134.9, 133.1, 132.0, 130.7, 129.9, 129.4, 129.2, 125.6, 125.1, 122.7, 122.0, 116.0, 103.1, 102.4, 80.6, 58.2, 56.8, 55.0, 44.5, 33.7, 31.5, 29.3, 28.7, 28.5, 27.5, 23.8, 20.3. HRMS calcd for $C_{48}H_{52}N_5O_9S$ ($M+H^+$) 874.3486, found: 874.3481. RP-HPLC purity (column 1: 99.0%, column 2: 97.0%).

5.65. Compound 60

Compound **60** was in all essentials prepared as described for **59**, using **56** (0.040 g, 0.054 mmol), CDI (0.018 g, 0.108 mmol), and dry THF (0.5 mL) but stirred at room temperature for 60 min before addition of cyclopropanesulfonamide (0.0261 g, 0.216 mmol), dry THF (0.5 mL), and DBU (0.016 mL, 0.108 mmol). The crude

product was purified using RP-HPLC (Vydac C18, MeCN/H_2O gradient with 0.1% TFA), to give **60** (0.0122 g, 27%) as a solid after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.43 (d, J = 9.3 Hz, 1H), 7.88–7.72 (m, 4H), 7.64–7.51 (m, 4H), 7.48–7.31 (m, 3H), 6.94 (s, 1H), 5.70 (m, 1H), 5.48 (s, 1H), 5.05 (m, 1H), 4.18 (m, 1H), 4.06 (s, 3H), 2.89 (m, 1H), 2.38–2.15 (m, 3H), 1.84–1.69 (m, 3H), 1.61–1.18 (m, 7H), 1.43 (s, 9H), 1.09–0.96 (m, 3H). ^{13}C NMR (CD_3OD) δ 177.1, 175.8, 170.1, 167.4, 165.9, 159.2, 157.5, 154.9, 147.1, 145.6, 136.3, 135.8, 134.9, 132.8, 131.9, 130.6, 129.3, 125.6, 125.4, 122.6, 121.8, 116.0, 103.0, 80.5, 58.2, 56.7, 55.0, 44.6, 33.7, 31.8, 31.6, 30.1, 29.3, 28.7, 27.6, 23.8, 20.7, 6.8, 6.6. HRMS calcd for $C_{45}H_{50}N_5O_9S$ ($M-H^+$) 836.3329, found: 836.3310. RP-HPLC purity (column 1: 96.6%, column 2: 98.2%).

5.66. Compound 61

Compound **61** was prepared as described for **59**, using **57** (0.029 g, 0.044 mmol), CDI (0.014 g, 0.088 mmol), dry THF (1.1 mL + 0.4 mL), cyclopropanesulfonamide (0.021 g, 0.18 mmol), and DBU (0.013 mL, 0.088 mmol). The crude product was purified using RP-HPLC (ACE 5 Phenyl S/N-A15161 column, MeCN/H_2O gradient with 0.1% TFA), to give **61** (0.0113 g, 33%) as a solid after freeze-drying. ^{1}H NMR (($CD_3)_2CO$) δ 10.6 (s, 1H), 8.79 (s, 1H), 8.42 (dd, J = 1.0, 8.4 Hz, 1H), 8.27 (m, 1H), 7.95 (m, 1H), 7.88 (m, 1H), 7.74 (m, 1H), 7.62 (m, 3H), 7.38 (m, 2H), 5.81 (d, J = 7.6 Hz, 1H), 5.69 (m, 1H), 5.54 (d, J = 6.8 Hz, 1H), 4.99 (dd, J = 9.5, 10.8 Hz, 1H), 4.37 (m, 1H), 2.37–2.18 (m, 4H), 1.89–1.83 (m, 2H), 1.73 (dd, J = 5.8, 13.9 Hz, 1H), 1.58–1.40 (m, 5H), 1.40 (s, 9H), 1.34–1.29 (m, 2H), 1.20 (m, 1H), 1.13–1.00 (m, 3H). HRMS calcd for $C_{38}H_{43}N_5O_8S$ ($M-H^+$) 764.2521, found: 764.2535. RP-HPLC purity (column 1: 98.9%, column 2: 99.0%).

5.67. Compound 62

Compound **62** was prepared as described for **59**, using **58** (0.021 g, 0.030 mmol), CDI (0.0097 g, 0.060 mmol), dry THF (1.5 mL + 0.4 mL), and cyclopropanesulfonamide (0.014 g, 0.12 mmol). The reaction mixture was stirred at room temperature for 48 h. The solvent was evaporated and the residue was treated with 0.1 M $NaHSO_4$ (0.6 mL), which gave a precipitate. After sonication, 0.1 M $NaHSO_4$ was added until the pH was 5–6. After centrifuge filtration through a Whatman PP 0.45 μm filter the solid residue was vacuum-dried. The crude product was purified by RP-HPLC (Sorbax SB-C8 column, MeCN/H_2O gradient with 0.1% TFA), to give **62** (0.0041 g, 17%) as a solid after freeze-drying. ^{1}H NMR (CD_3OD) δ 8.72 (m, 2H), 4.49–4.40 (m, 4H), 8.11 (m, 1H), 7.93 (ddd, J = 1.2, 6.9, 8.3 Hz, 1H), 7.84 (ddd, J = 1.2, 7.1, 8.3 Hz, 1H), 7.69 (m, 2H), 7.40 (m, 2H), 5.81 (s, 1H), 5.66 (m, 1H), 5.02 (m, 1H), 4.33 (m, 1H), 2.90 (m, 1H), 2.63 (dd, J = 9.0, 17.2 Hz, 1H), 2.17 (m, 1H), 2.10–2.0 (m, 2H), 1.99–1.82 (m, 2H), 1.79 (m, 1H), 1.69 (m, 1H), 1.56–1.14 (m, 7H), 1.42 (s, 9H), 1.31–0.98 (m, 2H). HRMS calcd for $C_{43}H_{47}N_6O_8S$ ($M-H^+$) 807.3176, found: 807.3148. RP-HPLC purity (column 2: 95.5%, column 3: 96.6%).

5.68. Enzyme inhibition

The protease activity of the full-length HCV NS3 protein (protease-helicase/NTPase) was measured using a FRET-assay as previously described.[21,41] In short, 1 nM enzyme was incubated for 10 min at 30 °C in 50 mM HEPES, pH 7.5, 10 mM DTT, 40% glycerol, 0.1% *n*-octyl-β-D-glucoside, 3.3% DMSO with 25 μM of the peptide cofactor 2K-NS4A (KKGSVVIVGRIVLSGK), and inhibitor. The reaction was started by the addition of 0.5 μM substrate (Ac-DED(Edans)EEAbuψ[COO]ASK(Dabcyl)-NH_2) obtained from AnaSpec Inc. (San Jose, USA). Non-linear regression analysis of the data was made using Grafit 5.0.8 (Erithacus software limited).

5.69. Computational methodology

The FLO+ docking suite was used to dock all compounds into the active site of the HCV NS3 protein.[63] This docking program was chosen partly due to its treatment of protein flexibility, which is thought to more realistically simulate protein–ligand interactions.[64] The NS3 protease/helicase crystal structure (protein access code 1CU1)[43] was refined and used to build the active site for docking. The crystal structure was subjected to constrained minimization followed by extraction of the residues within 9 Å of the last eleven residues of the 1CU1 c-terminal. This active site has been previously used and further details regarding its derivation may be found in the work of Rönn et al.[35] Ligand structures were prepared using LZM and were minimized prior to docking using the modified version of the AMBER force field included in the FLO+ docking package.

Ligand docking included both conformational analysis and simulated annealing. Conformational analysis was performed using 2000 steps of limited Monte Carlo (mcldock) perturbation. Residues R155 and K136 as well as the ligand were allowed full conformational freedom without energy penalty, while movement of all other critical active site residues in excess of 0.2 Å was penalized by 20 kJ/(mol$Å^2$). Due to the shallow and relatively featureless nature of the HCV NS3 active site, several zero-order bonds were added to hold the ligand in the active site but still allow conformational freedom. The 10 lowest-energy, unique conformations from the Monte Carlo perturbation were retained and further optimized using 50 steps of simulated annealing. To preserve the conformation obtained in the Monte Carlo step, an energy penalty of 20 kJ/(mol$Å^2$) was applied when the similarity distance between two consecutive conformations differed by more than 0.2 Å.

Acknowledgments

We gratefully acknowledge support from the Swedish Foundation for Strategic Research and the Swedish Research Council. We also thank Dr Aleh Yahorau, Department of Pharmaceutical Biosciences, Uppsala University, for help with HRMS analyses.

References and notes

1. Shepard, C. W.; Finelli, L.; Alter, M. J. *Lancet Infect. Dis.* **2005**, *5*, 558–567.
2. Willems, M.; Metselaar, H. J.; Tilanus, H. W.; Schalm, S. W.; de Man, R. A. *Transplant. Int.* **2002**, *15*, 61–72.
3. National Institutes of Health Consensus Development Conference Statement. Management of Hepatitis C: 2002, June 10–12, 2002. http://consensus.nih.gov/2002/2002HepatitisC2002116PDF.pdf (accessed May 2006).
4. Moreno-Otero, R. *J. Viral. Hepat.* **2005**, *12*, 10–19.
5. Bartenschlager, R.; Lohmann, V. *J. Gen. Virol.* **2000**, *81*, 1631–1648.
6. De Francesco, R.; Migliaccio, G. *Nature* **2005**, *436*, 953–960.
7. Tan, S. L.; Pause, A.; Shi, Y.; Sonenberg, N. *Nat. Rev. Drug. Disc.* **2002**, *1*, 867–881.
8. Choo, Q. L.; Kuo, G.; Weiner, A. J.; Overby, L. R.; Bradley, D. W.; Houghton, M. *Science* **1989**, *244*, 359–362.
9. Goudreau, N.; Llinàs-Brunet, M. *Expert Opin. Investig. Drugs* **2005**, *14*, 1129–1144.
10. Chen, S.-H.; Tan, S.-L. *Curr. Med. Chem.* **2005**, *12*, 2317–2342.
11. Lamarre, D.; Anderson, P. C.; Bailey, M.; Beaulieu, P.; Bolger, G.; Bonneau, P.; Bos, M.; Cameron, D. R.; Cartier, M.; Cordingley, M. G.; Faucher, A. M.; Goudreau, N.; Kawai, S. H.; Kukolj, G.; Lagace, L.; LaPlante, S. R.; Narjes, H.; Poupart, M. A.; Rancourt, J.; Sentjens, R. E.; St George, R.; Simoneau, B.; Steinmann, G.; Thibeault, D.; Tsantrizos, Y. S.; Weldon, S. M.; Yong, C. L.; Llinàs-Brunet, M. *Nature* **2003**, *426*, 186–189.
12. Malcolm, B. A.; Liu, R.; Lahser, F.; Agrawal, S.; Belanger, B.; Butkiewicz, N.; Chase, R.; Gheyas, F.; Hart, A.; Hesk, D.; Ingravallo, P.; Jiang, C.; Kong, R.; Lu, J.; Pichardo, J.; Prongay, A.; Skelton, A.; Tong, X.; Venkatraman, S.; Xia, E.; Girijavallabhan, V.; Njoroge, F. G. *Antimicrob. Agents Chemother.* **2006**, *50*, 1013–1020.
13. Lin, K.; Perni, R. B.; Kwong, A. D.; Lin, C. *Antimicrob. Agents Chemother.* **2006**, *50*, 1813–1822.
14. Perni, R. B.; Almquist, S. J.; Byrn, R. A.; Chandorkar, G.; Chaturvedi, P. R.; Courtney, L. F.; Decker, C. J.; Dinehart, K.; Gates, C. A.; Harbeson, S. L.; Heiser, A.; Kalkeri, G.; Kolaczkowski, E.; Lin, K.; Luong, Y. P.; Rao, B. G.; Taylor, W. P.; Thomson, J. A.; Tung, R. D.; Wei, Y.; Kwong, A. D.; Lin, C. *Antimicrob. Agents Chemother.* **2006**, *50*, 899–909.
15. Press releases at the Schering-Plough homepage: http://www.schering-plough.com/schering_plough/news/release.jsp?releaseID=809986 and http://www.schering-plough.com/schering_plough/news/release.jsp?releaseID=845117 (accessed Sep 2006).
16. Press releases at the Vertex homepage: http://www.vrtx.com/Pressreleases2006/pr052306.html http://www.vrtx.com/Pressreleases2006/pr052106.html (accessed Sep 2006).
17. Lin, C.; Lin, K.; Luong, Y. P.; Rao, B. G.; Wei, Y. Y.; Brennan, D. L.; Fulghum, J. R.; Hsiao, H. M.; Ma, S.; Maxwell, J. P.; Cottrell, K. M.; Perni, R. B.; Gates, C. A.; Kwong, A. D. *J. Biol. Chem.* **2004**, *279*, 17508–17514.
18. Lin, C.; Gates, C. A.; Rao, B. G.; Brennan, D. L.; Fulghum, J. F.; Luong, Y. P.; Frantz, J. D.; Lin, K.; Ma, S.; Wei, Y. Y.; Perni, R. B.; Kwong, A. D. *J. Biol. Chem.* **2005**, *280*, 36784–36791.
19. Tong, X.; Chase, R.; Skelton, A.; Chen, T.; Wright-Minogue, J.; Malcolm, B. A. *Antiviral Res.* **2006**, *70*, 28–38.
20. Johansson, A.; Hubatsch, I.; Åkerblom, E.; Lindeberg, G.; Winiwarter, S.; Danielson, U. H.; Hallberg, A. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 203–206.
21. Johansson, A.; Poliakov, A.; Åkerblom, E.; Lindeberg, G.; Winiwarter, S.; Samuelsson, B.; Danielson, U. H.; Hallberg, A. *Bioorg. Med. Chem.* **2002**, *10*, 3915–3922.
22. Lahm, A.; Yagnik, A.; Tramontano, A.; Koch, U. *Curr. Drug. Targets* **2002**, *3*, 281–296.
23. Steinkühler, C.; Koch, U.; Narjes, F.; Matassa, V. G. *Curr. Med. Chem.* **2001**, *8*, 919–932.
24. Ingallinella, P.; Altamura, S.; Bianchi, E.; Taliani, M.; Ingenito, R.; Cortese, R.; DeFrancesco, R.; Steinkühler, C.; Pessi, A. *Biochemistry* **1998**, *37*, 8906–8914.
25. Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.
26. Sewald, N.; Jakubke, H.-D.. *Peptides: Chemistry and Biology*; Wiley-VCH: Weinheim, 2002.
27. Salituro, G. M.; Townsend, C. A. *J. Am. Chem. Soc.* **1990**, *112*, 760–770.
28. Campbell, J. A.; Good, A. WO 02/060926, 8 August 2002.
29. Netherton, M. R.; Fu, G. C. *Org. Lett.* **2001**, *3*, 4295–4298.
30. Littke, A. F.; Dai, C.; Fu, G. C. *J. Am. Chem. Soc.* **2000**, *122*, 4020–4028.
31. Larhed, M.; Moberg, C.; Hallberg, A. *Acc. Chem. Res.* **2002**, *35*, 717–727.
32. Dhanoa, D. S.; Bagley, S. W.; Chang, R. S.; Lotti, V. J.; Chen, T. B.; Kivlighn, S. D.; Zingaro, G. J.; Siegl, P. K.; Patchett, A. A.; Greenlee, W. J. *J. Med. Chem.* **1993**, *36*, 4239–4249.
33. Park, K.-H.; Olmstead, M. M.; Kurth, M. J. *J. Org. Chem.* **1998**, *63*, 6579–6585.
34. Colarusso, S.; Koch, U.; Gerlach, B.; Steinkühler, C.; De Francesco, R.; Altamura, S.; Matassa, V. G.; Narjes, F. *J. Med. Chem.* **2003**, *46*, 345–348.
35. Rönn, R.; Sabnis, Y. A.; Gossas, T.; Åkerblom, E.; Danielson, U. H.; Hallberg, A.; Johansson, A. *Bioorg. Med. Chem.* **2005**, *14*, 544–559.
36. Johansson, A.; Poliakov, A.; Åkerblom, E.; Wiklund, K.; Lindeberg, G.; Winiwarter, S.; Danielson, U. H.; Samuelsson, B.; Hallberg, A. *Bioorg. Med. Chem.* **2003**, *11*, 2551–2568.
37. Wang, X. A.; Sun, L.-Q.; Sit, S.-Y.; Sin, N.; Scola, P. M.; Hewawasam, P.; Good, A. C.; Chen, Y.; Campbell, J. A. WO 03/099274, 2003.
38. Trnka, T. M.; Grubbs, R. H. *Acc. Chem. Res.* **2001**, *34*, 18–29.
39. Astruc, D. *New J. Chem.* **2005**, *29*, 42–56.
40. Faucher, A.-M.; Bailey, M. D.; Beaulieu, P. L.; Brochu, C.; Duceppe, J.-S.; Ferland, J.-M.; Ghiro, E.; Gorys, V.; Halmos, T.; Kawai, S. H.; Poirier, M.; Simoneau, B.; Tsantrizos, Y. S.; Llinàs-Brunet, M. *Org. Lett.* **2004**, *6*, 2901–2904.
41. Poliakov, A.; Hubatsch, I.; Shuman, C. F.; Stenberg, G.; Danielson, U. H. *Protein Expr. Purif.* **2002**, *25*, 363–371.
42. Lohmann, V.; Korner, F.; Koch, J. O.; Herian, U.; Theilmann, L.; Bartenschlager, R. *Science* **1999**, *285*, 110–113.
43. Yao, N.; Reichert, P.; Taremi, S. S.; Prosise, W. W.; Weber, P. C. *Structure* **1999**, *7*, 1353–1363.
44. Kim, J. L.; Morgenstern, K. A.; Lin, C.; Fox, T.; Dwyer, M. D.; Landro, J. A.; Chambers, S. P.; Markland, W.; Lepre, C. A.; O'Malley, E. T.; Harbeson, S. L.; Rice, C. M.; Murcko, M. A.; Caron, P. R.; Thomson, J. A. *Cell* **1996**, *87*, 343–355.
45. Love, R. A.; Parge, H. E.; Wickersham, J. A.; Hostomsky, Z.; Habuka, N.; Moomaw, E. W.; Adachi, T.; Hostomska, Z. *Cell* **1996**, *87*, 331–342.
46. Tsantrizos, Y. S.; Bolger, G.; Bonneau, P.; Cameron, D. R.; Goudreau, N.; Kukolj, G.; LaPlante, S. R.; Llinàs-Brunet, M.; Nar, H.; Lamarre, D. *Angew. Chem. Int. Ed.* **2003**, *42*, 1355–1360.

47. Seco, J. M.; Quinoa, E.; Riguera, R. *J. Org. Chem.* **1999**, *64*, 4669–4675.
48. LaPlante, S. R.; Llinàs-Brunet, M. *Curr. Med. Chem. — Anti-Infect. Agents* **2005**, *4*, 111–132.
49. Han, W.; Jiang, X.; Hu, Z.; Wasserman, Z. R.; Decicco, C. P. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3487–3490.
50. Han, W.; Hu, Z.; Jiang, X.; Wasserman, Z. R.; Decicco, C. P. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1111–1114.
51. Victor, F.; Lamar, J.; Snyder, N.; Yip, Y.; Guo, D.; Yumibe, N.; Johnson, R. B.; Wang, Q. M.; Glass, J. I.; Chen, S. H. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 257–261.
52. Perni, R. B.; Britt, S. D.; Court, J. C.; Courtney, L. F.; Deininger, D. D.; Farmer, L. J.; Gates, C. A.; Harbeson, S. L.; Kim, J. L.; Landro, J. A.; Levin, R. B.; Luong, Y. P.; O'Malley, E. T.; Pitlik, J.; Rao, B. G.; Schairer, W. C.; Thomson, J. A.; Tung, R. D.; Van Drie, J. H.; Wei, Y. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4059–4063.
53. Orvieto, F.; Koch, U.; Matassa, V. G.; Muraglia, E. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2745–2748.
54. Rancourt, J.; Cameron, D. R.; Gorys, V.; Lamarre, D.; Poirier, M.; Thibeault, D.; Llinàs-Brunet, M. *J. Med. Chem.* **2004**, *47*, 2511–2522.
55. Llinàs-Brunet, M.; Bailey, M.; Fazal, G.; Ghiro, E.; Gorys, V.; Goulet, S.; Halmos, T.; Maurice, R.; Poirier, M.; Poupart, M. A.; Rancourt, J.; Thibeault, D.; Wernic, D.; Lamarre, D. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2267–2270.
56. Narjes, F.; Koehler, K. F.; Koch, U.; Gerlach, B.; Colarusso, S.; Steinkühler, C.; Brunetti, M.; Altamura, S.; De Francesco, R.; Matassa, V. G. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 701–704.
57. Bold, G.; Fassler, A.; Capraro, H. G.; Cozens, R.; Klimkait, T.; Lazdins, J.; Mestan, J.; Poncioni, B.; Rosel, J.; Stover, D.; Tintelnot-Blomley, M.; Acemoglu, F.; Beck, W.; Boss, E.; Eschbach, M.; Hurlimann, T.; Masso, E.; Roussel, S.; Ucci-Stoll, K.; Wyss, D.; Lang, M. *J. Med. Chem.* **1998**, *41*, 3387–3401.
58. Llinàs-Brunet, M.; Bailey, M. D.; Ghiro, E.; Gorys, V.; Halmos, T.; Poirier, M.; Rancourt, J.; Goudreau, N. *J. Med. Chem.* **2004**, *47*, 6584–6594.
59. Attwood, M. R.; Bennett, J. M.; Campbell, A. D.; Canning, G. G. M.; Carr, M. G.; Conway, E.; Dunsdon, R. M.; Greening, J. R.; Jones, P. S.; Kay, P. B.; Handa, B. K.; Hurst, D. N.; Jennings, N. S.; Jordan, S.; Keech, E.; O'Brien, M. A.; Overton, H. A.; King-Underwood, J.; Raynham, T. M.; Stenson, K. P.; Wilkinson, C. S.; Wilkinson, T. C. I.; Wilson, F. X. *Antiviral Chem. Chemother.* **1999**, *10*, 259–273.
60. Bodanszky, M.; Bodanszky, A. *Chem. Commun.* **1967**, 591–593.
61. Poliakov, A.; Johansson, A.; Åkerblom, E.; Oscarsson, K.; Samuelsson, B.; Hallberg, A.; Danielson, U. H. *Biochim. Biophys. Acta* **2004**, *1672*, 51–59.
62. Chen, K. X.; Njoroge, F. G.; Yang, W.; Vibulbhan, B.; Girijavallabhan, V. M.; Venkatraman, S.; Velazquez, F. WO 2005/021584, March 10, 2005.
63. McMartin, C.; Bohacek, R. S. *J. Comput. Aided. Mol. Des.* **1997**, *11*, 333–344.
64. Teague, S. J. *Nat. Rev. Drug Disc.* **2003**, *2*, 527–541.