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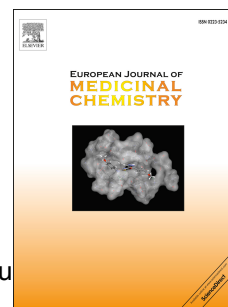
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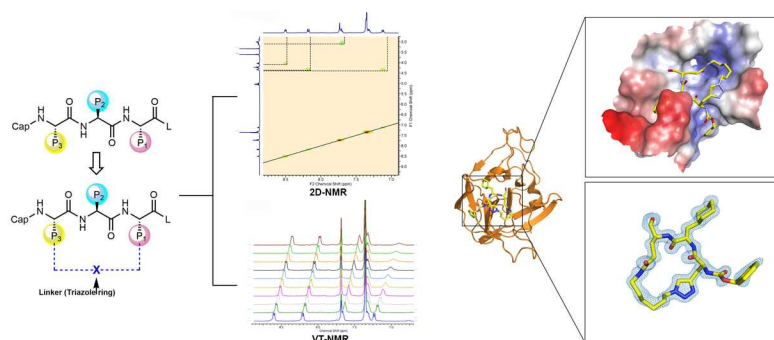
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Structure-Based Design and Synthesis of Triazole-based Macrocyclic Inhibitors of Norovirus Protease: Structural, Biochemical, Spectroscopic, and Antiviral Studies.

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Abstract

Outbreaks of acute gastroenteritis caused by noroviruses constitute a public health concern worldwide. To date, there are no approved drugs or vaccines for the management and prophylaxis of norovirus infections. A potentially effective strategy for the development of norovirus therapeutics entails the discovery of inhibitors of norovirus 3CL protease, an enzyme essential for noroviral replication. We describe herein the structure-based design of the first class of permeable, triazole-based macrocyclic inhibitors of norovirus 3C-like protease, as well as pertinent X-ray crystallographic, biochemical, spectroscopic, and antiviral studies.

1. Introduction

Noroviruses are the leading cause of foodborne illness in the U.S., accounting for >21 million infections per year and resulting in >70000 hospitalizations and nearly 800 deaths [1,3]. They are also the primary cause of sporadic and epidemic acute gastroenteritis worldwide [4,5]. The morbidity and mortality associated with norovirus infection impacts disproportionately the elderly, immunocompromised individuals [6] and, in developing countries, children [7]. Combating noroviruses presents a major challenge because noroviruses are highly contagious, genetically diverse, environmentally stable, display prolonged and profuse viral shedding, evolve rapidly, and are capable of spreading in many different ways [8,9]. There are currently no effective vaccines or norovirus-specific antivirals for norovirus infection. Consequently, there is a pressing need for the discovery and development of small molecule therapeutics for the treatment and prophylaxis of norovirus infection [10,13].

Norovirus is an RNA virus that belongs to the *Norovirus* genus of the *Caliciviridae* family. It carries a positive sense single stranded RNA genome (7.7 Kb) that consists of three open reading frames (ORF1-3). ORF1 encodes a 200 kDa precursor polyprotein which is processed by a virus-encoded cysteine endopeptidase to generate at least six mature non-structural proteins [14,16]. Polyprotein processing by norovirus 3C-like protease (3CLpro) is vital for the replication of the virus, consequently, norovirus 3CLpro has emerged as an attractive target for the discovery and development of antiviral agents against norovirus infections [10-11,17].

Norovirus 3CLpro is a chymotrypsin-like cysteine protease with an active site consisting of the catalytic triad of Cys139, His30 and Glu54 and located in a groove that is sandwiched between a β -sheet domain and a β -barrel domain [17,21]. The β -barrel domain accounts for most of the binding interactions involved between a peptide substrate and 3CLpro. A hallmark of norovirus 3CLpro, as well as other related 3C or 3CL viral proteases, is that its primary substrate specificity is dictated by the presence of a P₁ glutamine (and less frequently glutamic acid), at the S₁ subsite [22]. Furthermore, previous studies involving the mapping of the substrate specificity of 3CLpro have revealed that the protease prefers small hydrophobic amino acids such as a leucine residue at P2 and that the plasticity of its S₃ subsite allows the protease to accommodate His, Gln or Glu [16,18-19].

In continuing our studies in this area [12-13,17,23], we describe herein the structure-based design of cell-permeable macrocyclic transition state inhibitors (I) and (II) (Figure 1) of norovirus 3CLpro [23,24], as well as relevant structural, biochemical, spectroscopic, and cell-based studies.

2. Results and Discussion

2.1 Inhibitor Design. Inspection of crystal structures of norovirus 3CLpro with peptidyl inhibitors reveals that peptidyl inhibitors of norovirus 3CL^{pro} bind to the enzyme via a network of backbone hydrogen bonds that mimic an antiparallel β -sheet [25-26]. Among these hydrogen bonds, the backbone NH and carbonyl O of the Ala160 residue forms two hydrogen bonds with the backbone carbonyl O and NH of the P₃ residue of the inhibitor (Figure 2A). In addition, a third hydrogen bond exists between the backbone O of the Ala158 residue and the backbone NH of the P₁ residue of the inhibitor. This hydrogen

bonding arrangement resembles the hydrogen bonding pattern found in antiparallel β -sheets and serves to correctly orient and position the inhibitor (or substrate) backbone to the active site. The hydrogen bonding associated with an antiparallel β -sheet is more feasible when an inhibitor (or substrate) binds to the active site of a protease in a β -strand conformation, the extended “saw tooth” conformation of a peptide ($\phi = -120 \pm 20^\circ$ and $\psi = +120 \pm 20^\circ$) where the backbone NHs are exposed to the environment [27]. As a consequence, the aforementioned binding mode permits proteases to universally recognize their ligands in the β -strand conformation [25-26].

Based on the forgoing, we recently reported the results of preliminary studies related to the design of triazole-based macrocyclic inhibitors of 3C and 3C-like proteases of picornavirus, norovirus and coronavirus [24]. Macrocyclization of linear peptidyl inhibitors was accomplished by tethering the P₁ and P₃ residues of the linear inhibitor by a suitable linker, thereby pre-organizing the macrocycle in a β -strand conformation prior to binding to the enzyme active site (Figure 2B) [27-28]. This approach results in the formation of a pre-organized and semi-rigid entity that displays the amino acid side chains in a well-defined vector relationship for optimal binding and enhances the affinity of the inhibitor to the target enzyme by minimizing the entropy loss upon binding. Additional potential advantages of macrocyclic inhibitors include high stability to metabolizing enzymes, high selectivity and, frequently, increased cellular permeability [29-32]. We describe herein the structure-based design of macrocyclic inhibitors of norovirus 3CLpro where the side chain of the P1 Gln primary substrate specificity residue is tethered to the P3 residue via a triazole linker to yield macrocycles (I) (Figure 1) and explore the effect of ring size on conformation and its impact on pharmacological activity using high field NMR and X-ray crystallographic studies.

We also report the first examples of macrocyclic inhibitors of norovirus 3CLpro that are cellularly permeable.

2.2 Chemistry. The synthesis of macrocyclic compounds **1-11** (Table 1) was accomplished by first constructing key intermediates **15a-c**, **18a-e**, **19a-e**, and **20a-b** using well-established methodologies (Scheme 1). The commercially available N-Boc protected L-propargylglycine was reacted with L-leucine methyl ester, L-cyclohexylalanine methyl ester or L-phenylalanine methyl ester, in the presence of EDCI and HOBt to provide the corresponding dipeptide esters. Deblocking of the resulting esters was accomplished by treatment with 4M HCl in dioxane. The resulting amine hydrochlorides were reacted with benzylchloroformate followed by LiOH to yield the corresponding N-Cbz protected dipeptide acid intermediates **15a-c**. Preparation of the HCl salts of amino azides **18a-e** was effected by sequential reaction of commercially available N-Boc protected amino alcohols with methanesulfonyl chloride and sodium azide followed by deprotection with 4M HCl in dioxane. Coupling of N-Boc protected glutamic acid with the corresponding HCl salts of amino azides **18a-e** in the presence of EDCI and HOBt gave intermediates **19a-e**, in good yields.

The macrocyclization step was effected in two different ways. Specifically, the synthesis of macrocycles **2-3** entailed click chemistry-mediated macrocyclization using intermediates **15a-c** and **20a-b** (Scheme 2), while compounds **1** and **4-11** were obtained through macrolactamization starting with key intermediates **15a-c** and **19a-e** (Scheme 3). In general, the macrolactamization methodology was found to be superior, particularly in terms of ease of purification of the resultant cyclized products. Intermediates **15a** and **20a-b** were coupled in the presence of EDCI and HOBt to yield

the corresponding linear precursors **24a** and **24b** of the macrocyclic esters **25a** and **25b**. Intramolecular click reactions of the linear precursors **24a-b** were carried out using a CuI/DBU mixture in dichloromethane under a nitrogen atmosphere at room temperature for 24 h to provide macrocyclic esters **25a-b** in fairly good yields (45-50%). Intermediates **15a-c** and **19a-e** were coupled using intermolecular click reaction in the presence of CuSO₄ and sodium ascorbate in THF/H₂O solution to obtain the corresponding linear precursors **27a-i** of macrocyclic esters **28a-i**. Macrolactamization of compounds **27a-i** was carried out using EDCI and HOBt in DMF at room temperature for 24 h to yield macrocyclic esters **28a-i** in good yields (~60%). In both cyclization methods, the dilution factor of the reaction mixture was critical in controlling the intramolecular cyclization reaction over the intermolecular reaction. Reduction of macrocyclic esters using LiBH₄ followed by Dess-Martin periodinane oxidation provided the corresponding macrocyclic aldehydes **1-11** in fairly good yields (~50%).

The orientation of the triazole ring and its effect on pharmacological activity and conformation was probed by synthesizing macrocycle **12**, via intermediates **21** and **23**, as illustrated in Scheme 4. The acyclic variant of macrocycle **3** was synthesized according to Scheme 5 in order to determine the effect of macrocyclization on antiviral activity. As dictated by the primary substrate specificity of norovirus 3CLpro, the P₁ Gln residue remained invariant in all synthesized inhibitors. Furthermore, norovirus 3CLpro prefers a P₂ Leu or Cha [12,23a-c], consequently, the convergent nature of the synthetic plan allowed for variations in the P₂ residue via the judicious selection of appropriate natural or unnatural amino acid esters, thereby permitting the targeting of related viral proteases in the *Picornaviridae* and *Coronaviridae* families.

2.3 Biochemical Studies. The inhibitory activity of compounds **1-12** against norovirus 3CLpro *in vitro* and norovirus in a cell-based replicon system was evaluated as previously described [12-13] and the results are summarized in Table 1.

2.4 X-ray crystallography. In order to establish the precise mechanism of action of inhibitors (**1**) and gain insight and understanding into the structural determinants that impact potency and permeability that can be used to facilitate the structure-guided optimization of pharmacological activity, high-resolution X-ray crystal structures of NV 3CLPro with bound inhibitors **1**, **3**, and **10** were determined. Attempts to obtain crystal structures of permeable inhibitors **7** and **8** were unsuccessful, precluding a structural comparison between the permeable and non-permeable macrocyclic inhibitors that might perhaps illuminate the structural determinants responsible for the observed differences in potency and cellular permeability.

Examination of the active site revealed the presence of an antiparallel β -sheet-like hydrogen bonding network associated with the backbone of all three inhibitors, covalently bound to the catalytic Cys139 residue (Figure 3). The coordinates of the Ramachandran plot (see Figure S8 in Supporting information) of six ϕ and ψ angle pairs of the corresponding P₂ and P₃ residues, extracted from the X-ray crystal structures, lie in the upper left quadrant of the plot, indicating that bound inhibitors **1**, **3**, and **10** assume an extended β -strand conformation. The pseudo ϕ angles of the P₁ residue covalently bound to the Cys139 residue range from -129.5° to -137.8°. The electron density maps for the three bound inhibitors are shown in Figure 4A-C. As expected, the backbones of all three inhibitors are structurally more rigid compared to the atoms forming the macrocyclic linker, which is more flexible. This is evident from an analysis

of the *B*-factors for the inhibitors in each structure, which are slightly lower in the backbone region as shown in the right panels of Figure 4A-C. The electron density for the “cap” phenyl ring was not observed for **1** and **3** due to disorder and could not be modeled, while inhibitor **10** has somewhat weak electron density that permitted modelling of the phenyl ring. The triazole ring of inhibitor **3** has reasonable electron density, however, it is more disordered and its exact orientation is not clearly discernible (Figure 4).

Perusal of Table 1 indicates that, with the exception of compounds **8** and **11**, the rest of the compounds displayed low micromolar IC₅₀ values. The observed degree of inhibition prompted us to delineate the structural determinants associated with binding in order to gain insight and understanding into the relative potency of the inhibitors. Toward that end, superposition with GESAMT³³ of the high-resolution X-ray crystal structure of the NV 3CLpro:**10** complex (largest ring size) with the NV 3CLPro:**1** complex (smallest ring size) shows that the enzyme structures in both complexes are similar, with an RMSD between C α atoms of 0.76 Å (167 residues), except for the flexible loop spanning Lys121 to Glu140 (Figure 5A). The binding of inhibitor **10** (larger ring size) to the active site is accompanied by the movement of the flexible loop away from the inhibitor resulting in the loss of a hydrogen bond between the P₁ side chain (Gln C=O) of the inhibitor with Thr134 (backbone NH) which forms the S₁ pocket. His157, which is the other constituent of the S₁ pocket but is not a part of the flexible loop, maintains a hydrogen bond interaction with the P₁ Gln C=O (Figure 5B). It should be noted that the interaction of NV 3CLpro with acyclic dipeptidyl and tripeptidyl inhibitors involves the formation of two crucial hydrogen bonds between the carbonyl oxygen of the glutamine

surrogate C=O in the inhibitor and the Thr134 and His157 residues [12]. These two hydrogen bonding interactions, as well as the flexible backbone of the T123-G133 loop [34], appear to play a critical role in enzyme-substrate recognition and are conserved in all norovirus proteases. Thus, in the NV 3CLpro:**1** complex, the movement of the flexible loop and the shorter linker length of the inhibitor which positions the carbonyl oxygen of the P₁ Gln side chain away from His157 and Thr134, results in the loss of the Thr134 P₁ Gln C=O hydrogen bond (Figure 5C). Conversely, the binding of inhibitor **1** allows the flexible loop to move closer to the inhibitor such that a contact can be formed (3.3 Å) between Pro136 and the carbonyl oxygen of the warhead (Figure 5B).

The compact nature of the inhibitor positions the NH group of the P₁ Gln side chain within hydrogen bonding distance (3.2 Å) of the carbonyl oxygen of the Ala160 residue (see Figure **S1** in Supporting information for distance information related to the hydrogen bonds). The subtle structural shifts associated with the P₁ Gln side chain may account for the lower inhibitory activity observed with this series of macrocyclic triazole compounds, as compared to previously reported triazole-based macrocyclic calpain inhibitors [35] specifically and macrocyclic inhibitors generally [29,31]. In the former case, potency is solely dependent on the combined effect of hydrophobic interactions and the network of hydrogen bonding interactions between the inhibitor backbone and the enzyme active site which is facilitated by the β -strand conformation of the inhibitor.

The enhancement in the IC₅₀ values associated with the replacement of the P₂ isobutyl side chain with a cyclohexyl group observed with linear dipeptidyl inhibitors [12] is diminished in compound **10** (IC₅₀ 6.1 μ M) and is partly due to the loss of the key hydrogen bonding interaction with Thr134, as revealed by the X-ray crystal structure

and the larger flexible ring (see discussion of NMR studies). On the other hand, as mentioned above, the compact, rigid ring structure and the additional hydrogen bond with Ala160, discernible from the X-ray crystal structure, may account for the 4-fold decrease in the IC_{50} value ($1.6 \mu M$) of compound **1** as compared to compound **10**. The in vitro inhibitory activity of compounds **1** and **3** which form the same number of direct hydrogen bonds with the enzyme (7 hydrogen bonds) was found to be $1.6 \mu M$ and $2.4 \mu M$, respectively. Interestingly, compound **32**, the acyclic variant of compound **3**, was devoid of any inhibitory activity. Finally, the 18-membered macrocycle **12** (Figure 1) in which the triazole ring was constructed in a reverse fashion and presumably assumes a different orientation than inhibitors **1-11**, was effective against both 3CLpro and norovirus in replicon cells having IC_{50} and EC_{50} values $15.5 \mu M$ and $8.5 \mu M$, respectively.

In summary, the interaction of macrocyclic inhibitors (I) with NV 3CLpro induces conformational changes in the flexible loop and active site of the enzyme which are dependent on macrocycle ring size and structure. These changes result in sub-optimal angles and distances in forming the critical intermolecular hydrogen bonds between the P1 Gln side chain and the active site His157 and Thr134 residues, consequently, maximal binding potency and effective compensation for the energy penalty required to desolvate the molecule, are not attained.

2.5 NMR Studies. In order to gain further insight into the solution conformation and binding of the synthesized macrocyclic inhibitors, as well as confirm the absence of alternate structures, NMR studies were carried out using the precursor cyclic esters. Assignment of the proton resonances was done using 1H , COSY, ROESY and HMBC

spectral details. The ROESY spectra of the compounds show $H_{i\alpha}$ to NH ($i+1$) type correlation, where the Gln and Leu NH show correlation to the Leu H_α and carbamate H_α , respectively (see Figures S1-S5 in Supporting Information). This observation is indicative of a prearranged structure within the macrocycle [28]. The temperature dependence of amide NH chemical shifts experiments were performed using compounds **28a** (ring size: 17), **28e** (ring size: 20) and **28g** (ring size: 21), revealing important information about the solution structures of the macrocycles. Solvent shielded NH protons typically have $\Delta\delta/T$ values less than 4 ppb/K while solvent exposed NH protons have $\Delta\delta/T$ values greater than 4 ppb/K [28]. According to the results, the Gln, Leu, and propargylglycine NH protons have $\Delta\delta/T$ values greater than 4 ppb/K in all cases (Table 3). At larger ring sizes, the carbamate NH shows a higher temperature dependence (-7.0 and 6.4 ppb/K for ring sizes 20 and 21, respectively), indicating greater solvent exposure. However, for smaller ring size it shows comparatively low temperature dependence (4.2 ppb/K at 17 ring size), indicating less solvent exposure. This is further supported by the observed line broadening of the carbamate NH proton at elevated temperatures for compounds **28e** and **28g**, and the lower line broadening observed for **28a** (Figure S6 in Supporting Information). Previous studies have shown that amide signals exchanging with water impurity present in DMSO solvent show considerable broadening of the line width at elevated temperatures due to the enhanced exchange rate at higher temperatures [28]. At smaller ring size, all three NH protons show less temperature dependence, indicating solvent shielding which is partly due to the compact nature of the compound. The absence of very low temperature dependent NH protons (~ 2 ppb/K) would appear to rule out the presence of alternative turn

structures, such as γ -turns. Finally, the conformations of the peptide backbone of macrocycles **28a** and **28h** were determined based on 1D $^3J_{\text{NH-CH}\alpha}$ coupling constants (Table 4). The coupling constant $^3J_{\text{NH-CH}\alpha}$ correlates with the dihedral angle (ψ) according to the Karplus equation [28]. For unstructured random coil conformations the coupling constant is around 7 Hz while unusually high coupling constants (> 9 Hz) are observed for well-structured β -strands. According to the results shown in Table 4, the expected β -strand conformation was observed only for the 17-membered ring.

3. Conclusion

The studies described herein demonstrate the feasibility of generating cell-permeable triazole-based macrocyclic inhibitors of norovirus 3Cl protease. Valuable insights into the mechanism of action, conformation, and pharmacological activity were gained using an array of structural, spectroscopic, and antiviral techniques and methodologies. The interaction of inhibitor (I) with NV 3CLpro results in subtle structural shifts in the active site topography that impair hydrogen bonding interactions between the P1 Gln residue in the inhibitor and key amino acid residues and contribute to lower pharmacological activity. The results of ongoing studies are likely to illuminate further the interplay of ring size, conformation, and cellular permeability, and will be reported in due course.

4. Experimental Section

4.1 General. Reagents and dry solvents were purchased from various chemical suppliers (Aldrich, Acros Organics, ChemImpex, TCI America, and Bachem) and were used as obtained. Silica gel (230-450 mesh) used for flash chromatography was purchased from Sorbent Technologies (Atlanta, GA). Thin layer chromatography was performed using Analtech silica gel plates. Visualization was accomplished using UV light and/or iodine. The ^1H NMR were recorded in CDCl_3 or DMSO-d_6 using a Varian XL-400 spectrometer and are reported relative to TMS ($\delta_{\text{H}} = 0.00$ ppm). Chemical shifts are reported in ppm and spin multiplicities are represented by the following signals: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet) and m (multiplet). Melting points were recorded on a Mel-Temp apparatus and are uncorrected. High resolution mass spectrometry (HRMS) was performed at the University of Kansas Mass Spectrometry lab using an LCT Premier mass spectrometer (Waters, Milford, MA) equipped with a time of flight mass analyzer and an electrospray ion source. The purity of the compounds was established using HPLC and was >95%.

4.1.1. General Procedure A. Peptide coupling reaction. To a solution of N-protected amino acid derivative (1.0 eq) in dry DMF was added EDCI (1.22 eq), HOBt (eq) and the reaction mixture was stirred at room temperature for 40 minutes (Solution A). In the meantime, O-protected amino acid / amine (1.0 eq) in dry DMF kept at 0°C was added DIEA (4 eq) and stirred for 25 minutes (Solution B). After that Solution B was transferred to Solution A and stirred overnight at room temperature. The solvent was removed on the rotary evaporator and residue was taken up with ethyl acetate, washed sequentially with saturated sodium bicarbonate, 5% HCl and brine. The organic layer was dried over

anhydrous sodium sulfate, filtered and the solvent was removed on the rotary evaporator. See individual experiments for details.

4.1.2. General Procedure B. Hydrolysis of methyl esters. A solution of compound methyl ester (1.0 eq) in dry THF kept in an ice bath was treated with a solution of 1M lithium hydroxide (3.5 eq) and stirred for 4 hours at 0 °C until the starting material disappeared (as shown by TLC). The solvent was removed and the residue was diluted with water. The pH of the aqueous solution was adjusted to ~2 with 5% HCl and acidified solution was extracted with ethyl acetate and washed with brine. Organic extract was dried using anhydrous sodium sulfate, filtered and solvent was removed on the rotary evaporator. For details, see individual experiments.

4.1.3. General Procedure C. Removal of *N*-Boc protection. A solution of *N*-Boc protected compound (1.0 eq) in dry DCM was treated with 4M HCl in dioxane (10 eq) and stirred at room temperature for 3 hours while monitoring the disappearance of the starting material by TLC. Solvent was removed on the rotary evaporator, residue was treated with ether and concentrated. See individual experiments for details.

4.1.4. General Procedure D. Conversion of *N*-Boc protected alcohol to mesylate. To a solution of *N*-Boc protected alcohol (60 mmol) and TEA (60 mmol) in dry DCM (100 mL) kept in an ice bath (0 °C) was slowly added methane sulfonyl chloride (60 mmol) and reaction mixture was stirred for 30 minutes at 0 °C. The ice bath was removed and reaction mixture was further allowed to stir at room temperature for 5 hours (Completion of the reaction was monitored by TLC). Then water (50 mL) was added with stirring, the resulting solution was transferred to a reparatory funnel, organic

layer was separated and aqueous layer was extracted with DCM (2 x 75 mL). The combined organic layers were washed with brine (50 mL) dried over anhydrous sodium sulfate and concentrated on rotary evaporator. See individual experiments for more details.

4.1.5. General Procedure E. Conversion of *N*-Boc protected mesylate to azide.

To a solution of compound *N*-Boc protected mesylate (60 mmol) in dry DMF (150 mL) was added in small portions NaN₃ (180 mmol) with stirring over 30 minutes. Resulting solution was heated to 50 °C for 1 hour and it was further allowed to react overnight at room temperature. Then water (50 mL) was added with stirring, solvent was removed and residue was extracted with ethyl acetate (2 x 175 mL). The combined organic layers were washed sequentially with water (2 x 50 mL), brine (2 x 50 mL) and solvent was dried over anhydrous sodium sulfate and evaporated on rotary evaporator. See individual experiments for more details.

4.1.6. General Procedure F. Intermolecular click reactions. To a stirred solution of alkyne (5 mmol) in a mixture of water and THF (1:1, 25 mL) was added corresponding azide (5 mmol) followed by addition of sodium ascorbate (0.5 mmol) and CuSO₄·5H₂O (0.25 mmol). Resulting solution was further allowed to react 20 h at room temperature. See individual experiments for more details.

4.1.7. General Procedure G. Intramolecular click reaction. To a solution of macrocycle precursor (0.512 mmol) in dry DCM (1.2 M) under N₂ atmosphere, was added DBU (1.53 mmol) while solution was stirring vigorously at room temperature. After 15 minutes Cu(I)Br (0.512 mmol) was added and reaction mixture was stirred for

12 hours at room temperature. After that it was quenched by adding 3M HCl (100 mL), aqueous layer was separated and extracted with DCM (2 x 500 mL). The combined organic layers were washed sequentially with water (300 mL), brine (300 mL), dried over anhydrous sodium sulfate, filtered and concentrated on rotary evaporator. See individual experiments for more details.

4.1.8. General Procedure H. Macrolactamization reaction. To a solution of macrocycle precursor (1 mmol) in dry DMF (2 M) was added DIEA (4 mmol) and stirred for 30 minutes. After that, was added EDCI (1.25 mmol) followed by HOBt (1.25 mmol) and resulting solution was stirred for 20 hours at room temperature. The solvent was removed on the rotary evaporator and residue was taken up with DCM, washed sequentially with saturated sodium bicarbonate, 5% HCl and brine. The organic layer was dried over anhydrous sodium sulfate, filtered and the solvent was removed on the rotary evaporator. See individual experiments for details.

4.1.9. General Procedure I. Reduction of macrocycle esters. To a solution of compound macrocycle ester (1 mmol) in dry THF (6 mL) was added a solution of 2M LiBH_4 (3.0 mmol) drop wise followed by drop wise addition of absolute ethanol (11 mL) and was stirred at room temperature for 16 hours while monitoring the disappearance of the starting material by TLC. After that solvent was removed on the rotary evaporator and residue was partitioned between ethyl acetate and 1M KHSO_4 . The aqueous phase was extracted twice more chloroform and each organic extract was washed with brine separately, dried over anhydrous sodium sulfate and evaporated to give corresponding alcohol. See individual experiments for more details.

4.1.10. General Procedure J. Oxidation of macrocyclic alcohols. Corresponding macrocycle alcohol (0.36 mmol) was suspended in dry DCM (40 mL) under N₂ atmosphere and was added Dess-martin periodinane (1.08 mmol). Reaction mixture was stirred for 4 hours at room temperature and quenched with saturated sodium bicarbonate solution containing 10% Sodium thiosulfate (10 mL). Organic layer was separated and aqueous layer was extracted with DCM (2 x 60 mL), combined organic layers were washed with brine (40 mL), dried over anhydrous sodium sulfate and solvent was evaporated on rotary evaporator. See individual experiments for more details.

4.1.11. Methyl ((S)-2-((tert-butoxycarbonyl)amino)pent-4-ynoyl)-L-leucinate 13a. Boc-L-Propargylglycine (6.39 g; 30 mmol) in dry DMF (70 mL) was reacted with L-Leu-OMe(HCl) (30 mmol) according to general procedure **A** and crude product was purified using flash chromatography (silica gel; hexane/ethyl acetate 75:25) to give **13a** as a white solid (9.0 g ; 89% yield); M.p. 76-78 °C; ¹H NMR (400 MHz, DMSO-d₆): δ ppm 0.75 - 0.97 (m, 6 H), 1.38 (s, 9 H), 1.44 - 1.74 (m, 3 H), 2.43 (dd, J=8.70, 2.29 Hz, 1 H), 2.51 - 2.57 (m, 1 H), 2.82 (br s, 1 H), 3.52 - 3.71 (m, 3 H), 4.12 (d, J=5.19 Hz, 1 H), 4.29 (td, J=4.96, 2.90 Hz, 1 H), 6.97 (d, J=8.24 Hz, 1 H), 8.24 (d, J=7.63 Hz, 1 H); HRMS (ESI, *m/z*) calcd for C₁₇H₂₉N₂O₅ [M+H]⁺ 341.2076. found: 341.2069.

Compounds **13b** and **13c** were prepared using a similar procedure as the one described above.

4.1.12. Methyl (S)-2-((S)-2-((tert-butoxycarbonyl)amino)pent-4-ynamido)-3-cyclohexylpropanoate 13b. White solid (9.2 g ; 81% yield); M.p 85-87 °C; ¹H NMR

(400 MHz, DMSO- d_6): δ ppm 0.71 - 0.99 (m, 2 H), 1.02 - 1.25 (m, 3 H), 1.29 - 1.44 (m, 9 H), 1.45 - 1.76 (m, 8 H), 2.43 (dd, $J=8.79$, 2.15 Hz, 1 H), 2.48 (dd, $J=5.08$, 2.73 Hz, 1 H), 2.83 (br. s., 1 H), 3.60 (s, 3 H), 4.11 (d, $J=5.47$ Hz, 1 H), 4.32 (d, $J=5.08$ Hz, 1 H), 6.97 (d, $J=8.20$ Hz, 1 H), 8.24 (d, $J=7.42$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{20}H_{33}N_2O_5$ $[M+H]^+$ 381.2389. found: 381.2382.

4.1.13. Methyl ((S)-2-((tert-butoxycarbonyl)amino)pent-4-ynoyl)-L-phenylalaninate

13c. White solid (8.3 g ; 74% yield); M.p 81-83 $^{\circ}C$; 1H NMR (400 MHz, DMSO- d_6): δ ppm 1.38 (s, 9 H), 2.37 (dd, $J=8.85$, 2.14 Hz, 1 H), 2.45 (dd, $J=4.43$, 2.59 Hz, 1 H), 2.82 (br s, 1 H), 2.89 - 2.98 (m, 1 H), 2.99 - 3.12 (m, 1 H), 3.58 (s, 3 H), 4.06 - 4.21 (m, 1 H), 4.37 - 4.57 (m, 1 H), 6.90 (d, $J=8.85$ Hz, 1 H), 7.17 - 7.24 (m, 3 H), 7.25 - 7.33 (m, 2 H), 8.31 (d, $J=7.63$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{20}H_{27}N_2O_5$ $[M+H]^+$ 375.1920. found: 375.1909.

4.1.14. Methyl ((S)-2-(((benzyloxy)carbonyl)amino)pent-4-ynoyl)-L-leucinate 14a.

Compound **13a** (6.8 g; 20 mmol) in dry CH_2Cl_2 (30 mL) was treated with 1M HCl in dioxane to remove Boc protection according to general procedure **C** and solvent was removed after the reaction, under high vacuum to dryness. To a solution of resulting HCl salt of the amine in dry THF (70 mL) was added DIEA (12.9 g; 100 mmol) and stirred for 30 minutes. After that, benzyl chloroformate (3.75 g; 22 mmol) was added and the resulting solution was further reacted for 16 h at room temperature. The solvent was removed on the rotary evaporator and the residue was taken up in ethyl acetate (150 mL) and washed sequentially with saturated $NaHCO_3$ (50 mL), 5% HCl (2 x 50 mL) and brine (50 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and solvent was removed on the rotary evaporator to give **14a** as a white solid (6.7 g;

90% yield); M.p 79-80 °C. ¹H NMR (400 MHz, DMSO-d₆): δ ppm 0.79 - 0.93 (m, 6 H), 1.40 - 1.72 (m, 3 H), 2.36 - 2.47 (m, 1 H), 2.52 - 2.60 (m, 1 H), 2.84 (s, 1 H), 3.55 - 3.66 (m, 3 H), 4.15 - 4.32 (m, 2 H), 4.98 - 5.12 (m, 2 H), 7.24 - 7.42 (m, 5 H), 7.54 (d, *J*=8.54 Hz, 1 H), 8.36 (d, *J*=7.63 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₂₀H₂₇N₂O₅ [M+H]⁺ 375.1920. found: 375.1916.

Compounds **14b** and **14c** were prepared according to the same procedure as above.

4.1.15. Methyl ((S)-2-(((benzyloxy)carbonyl)amino)pent-4-ynamido)-3-cyclohexylpropanoate 14b. White solid (7.6 g: 92% yield); M.p 86-88 °C. ¹H NMR (400 MHz, DMSO-d₆): δ ppm 0.70 - 0.99 (m, 2 H), 1.02 - 1.24 (m, 3 H), 1.33 (br. s., 1 H), 1.43 - 1.56 (m, 2 H), 1.62 (d, *J*=12.21 Hz, 5 H), 2.37 - 2.48 (m, 1 H), 2.85 (s, 1 H), 3.60 (s, 3 H), 4.22 (td, *J*=8.85, 4.88 Hz, 1 H), 4.27 - 4.35 (m, 1 H), 5.04 (s, 2 H), 7.34 - 7.41 (m, 5 H), 7.54 (d, *J*=8.54 Hz, 1 H), 8.35 (d, *J*=7.63 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₂₃H₃₁N₂O₅ [M+H]⁺ 415.2233. found: 415.2228.

4.1.16. Methyl ((S)-2-(((benzyloxy)carbonyl)amino)pent-4-ynoyl)-L-phenylalaninate 14c.

White solid (6.9 g: 85% yield); M.p 106-108 °C. ¹H NMR (400 MHz, DMSO-d₆): δ ppm 2.31 - 2.44 (m, 1 H), 2.54 (dd, *J*=4.49, 2.54 Hz, 1 H), 2.84 (s, 1 H), 2.89 - 2.98 (m, 1 H), 2.99 - 3.08 (m, 1 H), 3.52 - 3.63 (m, 3 H), 4.23 (td, *J*=8.98, 4.69 Hz, 1 H), 4.41 - 4.54 (m, 1 H), 4.99 - 5.10 (m, 2 H), 7.15 - 7.23 (m, 3 H), 7.24 - 7.29 (m, 2 H), 7.30 - 7.42 (m, 5 H), 7.50 (d, *J*=8.98 Hz, 1 H), 8.44 (d, *J*=7.42 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₂₃H₂₅N₂O₅ [M+H]⁺ 409.1763. found: 409.1754.

4.1.17. ((S)-2-(((Benzyloxy)carbonyl)amino)pent-4-ynoyl)-L-leucine 15a. A solution of compound **14a** (5.6 g; 15 mmol) in in dry THF (60 mL) was hydrolyzed according to

general procedure B. The crude product was purified by Flash chromatography (Silica gel; Hexane/Ethyl acetate 50:50) to give **15a** as a colorless oil (4.5 g; 84% yield); ^1H NMR (400 MHz, DMSO- d_6): δ 0.80 - 0.95 (m, 6 H), 1.44 - 1.57 (m, 2 H), 1.59 - 1.70 (m, 1 H), 1.72 - 1.81 (m, 2 H), 2.35 - 2.48 (m, 1 H), 2.52 - 2.66 (m, 1 H), 2.79 - 2.86 (m, 1 H), 3.52 - 3.68 (m, 2 H), 4.11 - 4.32 (m, 2 H), 5.04 (s, 2 H), 7.25 - 7.43 (m, 6 H), 7.54 (d, $J=8.59$ Hz, 1 H), 8.18 (d, $J=8.20$ Hz, 1 H), 12.58 (br. s., 1 H). HRMS (ESI, m/z) calcd for $\text{C}_{19}\text{H}_{25}\text{N}_2\text{O}_5$ $[\text{M}+\text{H}]^+$ 361.1763. found: 361.1758.

Compounds **15b** and **15c** were prepared using the same procedure as described above.

4.1.18. (S)-2-(((S)-2-(((Benzyloxy)carbonyl)amino)pent-4-ynamido)-3-cyclohexylpropanoic acid 15b. Colorless oil (5.2 g; 88% yield); ^1H NMR (400 MHz, DMSO- d_6): δ 0.69 - 0.98 (m, 2 H), 1.05 - 1.21 (m, 3 H), 1.27 - 1.41 (m, 1 H), 1.53 (t, $J=7.17$ Hz, 2 H), 1.56 - 1.73 (m, 5 H), 2.36 - 2.48 (m, 1 H), 2.52 - 2.62 (m, 1 H), 2.82 (s, 1 H), 4.16 - 4.32 (m, 2 H), 5.05 (s, 2 H), 7.27 - 7.43 (m, 5 H), 7.55 (d, $J=8.85$ Hz, 1 H), 8.18 (d, $J=7.93$ Hz, 1 H), 12.50 (br. s., 1 H). HRMS (ESI, m/z) calcd for $\text{C}_{22}\text{H}_{29}\text{N}_2\text{O}_5$ $[\text{M}+\text{H}]^+$ 401.2076. found: 401.2068.

4.1.19. ((S)-2-(((benzyloxy)carbonyl)amino)pent-4-ynoyl)-L-phenylalanine 15c. Colorless oil (5.1 g; 86% yield); ^1H NMR (400 MHz, DMSO- d_6): δ 2.32 - 2.44 (m, 1 H), 2.51 - 2.59 (m, 1 H), 2.82 (s, 1 H), 2.86 - 2.97 (m, 1 H), 3.00 - 3.11 (m, 1 H), 4.23 (td, $J=9.18$, 4.30 Hz, 1 H), 4.42 (td, $J=8.10$, 5.27 Hz, 1 H), 5.01 - 5.07 (m, 2 H), 7.15 - 7.29 (m, 5 H), 7.30 - 7.42 (m, 5 H), 7.52 (d, $J=8.59$ Hz, 1 H), 8.21 (d, $J=7.81$ Hz, 1 H), 12.69

(br. s., 1 H). HRMS (ESI, m/z) calcd for $C_{22}H_{23}N_2O_5$ $[M+H]^+$ 395.1607. found: 395.1599.

4.1.20. Methyl N5-(3-azidoropyl)-N2-(tert-butyloxycarbonyl)-L-glutamate 19a. A solution of 2-(Boc-amino)-1-ethanol (9.6 g; 60 mmol) in dry DCM (100 mL) was converted in to mesylate according to general procedure **D** to yield **16b** as a colorless oil (13.4 g; 94% yield).

Compound **16a** (11.9 g; 50 mmol) was dissolve in dry DMF (120 mL) and converted in to azide according to general procedure **E** to yield **17a** as a light yellow oil (7.2 g; 78% yield).

A solution of compound **17a** (6.8 g; 37 mmol) in dry CH_2Cl_2 (30 mL) was treated with 1M HCl in dioxane to remove Boc protection according to general procedure **C** and solvent was removed after the reaction under high vacuum to dryness resulting compound **18a**.

Boc-Glu-OMe (9.67 g; 37 mmol) in dry DMF (100 mL) was reacted with compound **18a** (4.5 g; 37 mmol) according to general procedure **A** and the crude product was purified by flash chromatography (silica gel; hexane/ethyl acetate 70:30) to give **19a** as a colorless oil (9.5 g ; 78% yield); 1H NMR (400 MHz, DMSO- d_6): δ 1.27 - 1.47 (m, 9 H), 1.68 - 1.83 (m, 1 H), 1.86 - 1.97 (m, 1 H), 2.16 (t, $J=7.42$ Hz, 2 H), 3.23 (q, $J=5.60$ Hz, 2 H), 3.30 - 3.40 (m, 2 H), 3.62 (s, 3 H), 3.96 (d, $J=4.69$ Hz, 1 H), 7.24 (d, $J=7.42$ Hz, 1 H), 8.06 (br. s., 1 H). HRMS (ESI, m/z) calcd for $C_{13}H_{24}N_5O_5$ $[M+H]^+$ 330.1777. found: 330.1769.

Compounds **19b-e** were prepared using the same procedure as described above.

4.1.21. Methyl N⁵-(3-azidopropyl)-N²-(tert-butoxycarbonyl)-L-glutamate 19b.

Colorless oil (8.2 g; 72% yield); ¹H NMR (400 MHz, DMSO-d₆): δ 1.27 - 1.44 (m, 9 H), 1.63 (q, *J*=6.84 Hz, 2 H), 1.69 - 1.81 (m, 1 H), 1.85 - 1.98 (m, 1 H), 2.14 (t, *J*=7.42 Hz, 2 H), 3.01 - 3.18 (m, 2 H), 3.28 - 3.41 (m, 2 H), 3.62 (s, 3 H), 3.94 (dd, *J*=8.01, 3.32 Hz, 1 H), 7.23 (d, *J*=7.81 Hz, 1 H), 7.87 (d, *J*=4.69 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₁₄H₂₆N₅O₅ [M+H]⁺ 344.1934. found: 344.1929.

4.1.22. Methyl N⁵-(4-azidobutyl)-N²-(tert-butoxycarbonyl)-L-glutamate 19c.

Colorless oil (9.0 g; 85% yield); ¹H NMR (400 MHz, DMSO-d₆): δ 1.29 - 1.40 (m, 9 H), 1.40 - 1.58 (m, 4 H), 1.66 - 1.80 (m, 1 H), 1.84 - 1.97 (m, 1 H), 2.13 (t, *J*=7.42 Hz, 2 H), 3.04 (q, *J*=6.25 Hz, 2 H), 3.32 (t, *J*=6.84 Hz, 2 H), 3.62 (s, 3 H), 3.94 (dd, *J*=8.01, 3.71 Hz, 1 H), 7.23 (d, *J*=7.81 Hz, 1 H), 7.71 - 7.91 (m, 1 H). HRMS (ESI, *m/z*) calcd for C₁₅H₂₈N₅O₅ [M+H]⁺ 358.2090. found: 358.2083.

4.1.23. Methyl N⁵-(5-azidopentyl)-N²-(tert-butoxycarbonyl)-L-glutamate 19d.

Colorless oil (7.9 g; 69% yield); ¹H NMR (400 MHz, DMSO-d₆): δ 1.24 - 1.34 (m, 3 H), 1.35 - 1.44 (m, 10 H), 1.52 (q, *J*=7.13 Hz, 2 H), 1.66 - 1.81 (m, 1 H), 1.84 - 1.97 (m, 1 H), 2.13 (t, *J*=7.42 Hz, 2 H), 3.02 (q, *J*=6.12 Hz, 2 H), 3.26 - 3.37 (m, 2 H), 3.62 (s, 3 H), 3.94 (d, *J*=4.30 Hz, 1 H), 7.23 (d, *J*=7.81 Hz, 1 H), 7.78 (t, *J*=5.27 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₁₆H₃₀N₅O₅ [M+H]⁺ 372.2247. found: 372.2241.

4.1.24. Methyl N⁵-(6-azidohexyl)-N²-(tert-butoxycarbonyl)-L-glutamate 19e.

Yellow oil (6.5 g; 68% yield); ¹H NMR (400 MHz, DMSO-d₆): δ 1.24 - 1.30 (m, 3 H), 1.31 - 1.35 (m, 3 H), 1.36 - 1.41 (m, 9 H), 1.44 - 1.60 (m, 2 H), 1.73 (br. s., 1 H), 1.86 - 1.96 (m, 1 H), 2.12 (t, *J*=7.48 Hz, 2 H), 3.01 (q, *J*=6.21 Hz, 2 H), 3.22 - 3.36 (m, 2 H), 3.62 (s,

3 H), 3.94 (d, $J=4.27$ Hz, 1 H), 7.23 (d, $J=7.63$ Hz, 1 H), 7.76 (d, $J=5.19$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{17}H_{32}N_5O_5$ $[M+H]^+$ 386.2403. found: 386.2401.

4.1.25. Methyl N^5 -(3-azidopropyl)-L-glutamate hydrochloride 20a. A solution of compound **19a** (4.4 g; 15 mmol) in dry DCM (20 mL) was treated with 4M HCl in dioxane according to general procedure C to obtain compound **20a** as a yellow oil (4.0 g; 95% yield); 1H NMR (400 MHz, DMSO- d_6): δ 1.63 (q, $J=6.88$ Hz, 2 H), 1.67 - 1.80 (m, 1 H), 1.85 - 1.98 (m, 1 H), 2.14 (t, $J=7.42$ Hz, 2 H), 3.01 - 3.18 (m, 2 H), 3.28 - 3.41 (m, 2 H), 3.62 (s, 3 H), 3.94 (dd, $J=8.01, 3.32$ Hz, 1 H), 7.23 (d, $J=7.81$ Hz, 1 H), 7.87 (d, $J=4.69$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_9H_{19}ClN_5O_3$ $[M+H]^+$ 280.1176. found: 280.1172.

Compound **20b** was prepared using the same procedure as described above.

4.1.26. Methyl N^5 -(4-azidobutyl)-L-glutamate hydrochloride 20b. Yellow oil (4.8 g; 94% yield); 1H NMR (400 MHz, DMSO- d_6): δ 1.39 - 1.57 (m, 4 H), 1.66 - 1.80 (m, 1 H), 1.84 - 1.97 (m, 1 H), 2.13 (t, $J=7.42$ Hz, 2 H), 3.04 (q, $J=6.25$ Hz, 2 H), 3.33 (t, $J=6.84$ Hz, 2 H), 3.62 (s, 3 H), 3.94 (dd, $J=8.01, 3.71$ Hz, 1 H), 7.23 (d, $J=7.81$ Hz, 1 H), 7.71 - 7.91 (m, 1 H). HRMS (ESI, m/z) calcd for $C_{10}H_{21}ClN_5O_3$ $[M+H]^+$ 294.1333. found: 294.1330.

4.1.27. 5-azido-2-(((benzyloxy)carbonyl)amino)pentanoic acid 21.³⁴ To a solution of sodium azide (2.27 g; 35 mmol) suspended in anhydrous acetonitrile (35 mL) at 0 °C was added triflic anhydride (8.18 g; 29 mmol) dropwise over 10 minutes and the cooled solution was stirred for 2 hours. The mixture was filtered through cotton wool to give a crude triflic azide solution. To a vigorously stirred suspension of Z-L-ornithine (6.39 g;

24 mmol) in acetonitrile (100 mL) and water (40 mL) mixture was added trimethylamine (7.38 g; 73 mmol) and CuSO₄ (24 mmol). The resulting solution was cooled to 0°C and the triflic azide solution was added dropwise. After 30 minutes, cooling was removed and the reaction mixture was stirred at room temperature overnight. The solvent was removed on the rotary evaporator and the aqueous solution was washed with ethyl acetate (2 x 100 mL). The aqueous phase was then acidified with 2M HCl followed by concentrated HCl and extracted with ethyl acetate (3 x 100 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated to yield a crude product. After that chloroform was added and the solution was refrigerated and the precipitate was filtered off. The solvent was removed on the rotary evaporator and the crude product was purified using flash chromatography to obtain compound **21** as a light yellow oil (2.14 g; 30% yield); ¹H NMR (400 MHz, DMSO-d₆): δ 1.45 - 1.71 (m, 3 H), 1.72 - 1.87 (m, 1 H), 3.32 (t, J=6.41 Hz, 2 H), 3.96 - 4.02 (m, 1 H), 5.05 (s, 2 H), 7.23 - 7.46 (m, 5 H), 7.62 (d, J=7.93 Hz, 1 H), 12.10 (b. s., 1H). HRMS (ESI, *m/z*) calcd for C₁₃H₁₇N₄O₄ [M+H]⁺ 293.1250. found: 293.1248.

4.1.28. Methyl *N*²-(tert-butoxycarbonyl)-*N*⁵-(prop-2-yn-1-yl)-L-glutamate **22.** To a solution of Boc-Glu-OMe (5.2 g; 20 mmol) in dry DMF (60 mL) was added EDCI (4.67 g; 24.4 mmol), HOBt (3.7 g; 24.4 mmol) and the reaction mixture was stirred at room temperature for 30 minutes. Propargylamine (1.1 g; 20 mmol) was added to the mixture and the reaction mixture was stirred overnight at room temperature. The solvent was removed on the rotary evaporator and residue was taken up with DCM (400 mL) and washed sequentially with 10% aqueous citric acid (150 mL) and brine (150 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated to

yield a crude product which was purified by flash chromatography (silica gel; hexane/ethyl acetate 50:50) to give **22** as a colorless oil (3.8 g ; 64% yield); ^1H NMR (400 MHz, DMSO- d_6): δ ppm 1.35 (s, 9 H), 1.96 - 2.10 (m, 2 H), 2.19 - 2.30 (m, 1 H), 2.30 - 2.42 (m, 1 H), 3.12 (t, $J=2.29$ Hz, 1 H), 3.69 - 3.78 (m, 3 H), 3.85 (dd, $J=5.34$, 2.44 Hz, 2 H), 3.96 - 4.08 (m, 1 H), 7.81 (d, $J=6.81$ Hz, 1H), 8.48 (t, $J=5.19$ Hz, 1 H). HRMS (ESI, m/z) calcd for $\text{C}_{14}\text{H}_{23}\text{N}_2\text{O}_5$ $[\text{M}+\text{H}]^+$ 299.1607. found: 299.1602.

4.1.29. Methyl N^2 -((tert-butoxycarbonyl)leucyl)- N^5 -(prop-2-yn-1-yl)-L-glutamate

23. A solution of compound **22** (8.91 g; 30 mmol) in dry DCM (90 mL) was treated with 1M HCl in dioxane according to general procedure **C**. The resulting compound was coupled with Boc-Glu-OMe (7.74 g; 30 mmol) in dry DMF (70 mL) according to general procedure **A** and the crude product was purified by flash chromatography (silica gel; hexane/ethyl acetate 50:50) to give **19a** as a yellow oil (8.0 g ; 65% yield); ^1H NMR (400 MHz, CDCl_3): δ 0.95 (t, $J=6.56$ Hz, 6 H), 1.38 - 1.56 (m, 10 H), 1.65 (dd, $J=13.58$, 5.19 Hz, 2 H), 1.85 - 2.03 (m, 1 H), 2.18 - 2.39 (m, 4 H), 3.74 (s, 3 H), 4.04 (td, $J=5.04$, 2.59 Hz, 2 H), 4.08 - 4.20 (m, 1 H), 4.54 (br. s., 1 H), 5.02 (d, $J=7.63$ Hz, 1 H), 6.80 - 6.92 (m, 1 H), 6.94 - 7.07 (m, 1 H). HRMS (ESI, m/z) calcd for $\text{C}_{20}\text{H}_{34}\text{N}_3\text{O}_6$ $[\text{M}+\text{H}]^+$ 412.2448. found: 412.2445.

4.1.30. Methyl N^5 -(3-azidopropyl)- N^2 -(((S)-2-(((benzyloxy)carbonyl)amino)pent-4-ynoyl)-L-leucyl)-L-glutamate

24a. A solution of compound **15a** (11.7 g; 31 mmol) in dry DMF (100 mL) was coupled with compound **20a** according to general procedure **A** and the crude product was purified using flash chromatography (silica gel; DCM/methanol 95:5) to obtain compound **24a** as a white solid (8.3 g ; 46% yield); M.p 110-111 $^\circ\text{C}$. ^1H NMR (400 MHz, DMSO- d_6): δ 0.86 (dd, $J=15.43$, 6.44 Hz, 6 H), 1.45 (t,

$J=6.84$ Hz, 2 H), 1.63 (t, $J=6.64$ Hz, 3 H), 1.73 - 1.87 (m, 1 H), 1.89 - 2.02 (m, 2 H), 2.07 - 2.21 (m, 2 H), 2.35 - 2.48 (m, 2 H), 2.52 - 2.63 (m, 1 H), 2.82 (br. s., 1 H), 3.01 - 3.15 (m, 2 H), 3.60 (s, 3 H), 4.11 - 4.25 (m, 2 H), 4.31 (d, $J=7.03$ Hz, 1 H), 5.05 (s, 2 H), 7.23 - 7.41 (m, 5 H), 7.59 (d, $J=7.81$ Hz, 1 H), 7.82 (br. s., 1 H), 7.99 (d, $J=7.78$ Hz, 1 H), 8.29 (d, $J=7.42$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{28}H_{40}N_7O_7$ $[M+H]^+$ 586.2989. found: 586.2986.

Compound **24b** was prepared using the same procedure as described above.

4.1.31. Methyl N^5 -(4-azidobutyl)- N^2 -((((S)-2-(((benzyloxy)carbonyl)amino)pent-4-ynoyl)-L-leucyl)-L-glutamate 24b. White solid (9.6 g; 58% yield); M.p 106-108 °C. 1H NMR (400 MHz, DMSO- d_6): δ 0.75 - 0.94 (m, 6 H), 1.34 - 1.53 (m, 6 H), 1.54 - 1.69 (m, 2 H), 1.72 - 1.87 (m, 2 H), 1.94 (dd, $J=13.19, 5.49$ Hz, 2 H), 2.04 - 2.21 (m, 2 H), 2.77 - 2.90 (m, 1 H), 3.04 (q, $J=6.29$ Hz, 2 H), 3.54 - 3.67 (m, 3 H), 4.10 - 4.25 (m, 2 H), 4.26 - 4.39 (m, 2 H), 5.01 - 5.08 (m, 2 H), 7.28 - 7.44 (m, 5 H), 7.61 (d, $J=8.42$ Hz, 1 H), 7.81 (t, $J=5.40$ Hz, 1 H), 8.01 (d, $J=8.24$ Hz, 1 H), 8.31 (d, $J=7.14$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{29}H_{42}N_7O_7$ $[M+H]^+$ 600.3146. found: 600.3144.

4.1.32. Methyl (3S,6S,9S,Z)-3-(((benzyloxy)carbonyl)amino)-6-isobutyl-4,7,12-trioxo-1 1H -5,8,13-triaza-1(4,1)-triazolacyclohexadecaphane-9-carboxylate 25a. A solution of compound **24a** (0.3 g; 0.512 mmol) in dry DCM (420 mL) was cyclized according to general procedure **G** and the crude product was purified using flash chromatography (silica gel; DCM/methanol 95:5) to give compound **25a** as a white solid (0.14 g; 45% yield); M.p 210-211 °C. 1H NMR (DMSO- d_6): δ 0.73 - 0.99 (m, 6 H), 1.45 (q, $J=6.60$ Hz, 3 H), 1.62 - 1.80 (m, 2 H), 1.83 - 1.94 (m, 1 H), 2.00 - 2.30 (m, 4 H), 2.60

- 2.76 (m, 1 H), 3.04 (d, $J=4.30$ Hz, 2 H), 3.57 - 3.68 (m, 3 H), 4.08 - 4.20 (m, 1 H), 4.26 - 4.45 (m, 4 H), 5.04 (d, $J=9.76$ Hz, 2 H), 7.03 (d, $J=7.03$ Hz, 1 H), 7.24 - 7.42 (m, 5 H), 7.53 (s, 2 H), 8.16 (d, $J=7.42$ Hz, 1 H), 8.53 (d, $J=8.20$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{28}H_{40}N_7O_7$ $[M+H]^+$ 586.2989. found: 586.2984.

Compound **25b** was prepared using the same procedure as described above.

4.1.33. Methyl (3S,6S,9S,Z)-3-(((benzyloxy)carbonyl)amino)-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacycloheptadecaphane-9-carboxylate 25b.

White solid (0.15 g; 50% yield); M.p 213-215 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.87 (dd, $J=12.69, 6.44$ Hz, 6 H), 1.14 - 1.32 (m, 2 H), 1.35 - 1.56 (m, 3 H), 1.57 - 1.83 (m, 4 H), 2.03 - 2.32 (m, 3 H), 2.73 (t, $J=6.25$ Hz, 1 H), 2.98 - 3.14 (m, 2 H), 3.54 - 3.70 (m, 3 H), 4.11 - 4.32 (m, 3 H), 4.32 - 4.44 (m, 2 H), 4.92 - 5.16 (m, 2 H), 6.91 (d, $J=7.81$ Hz, 1 H), 7.26 - 7.41 (m, 5 H), 7.67 - 7.83 (m, 2 H), 8.22 (d, $J=7.42$ Hz, 1 H), 8.36 (d, $J=7.81$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{29}H_{42}N_7O_7$ $[M+H]^+$ 600.3146. found: 600.3142.

4.1.34. ((S)-2-(((benzyloxy)carbonyl)amino)-3-(1-(2-((S)-4-((tert-butoxycarbonyl)amino)-5-methoxy-5-oxopentanamido)ethyl)-1H-1,2,3-triazol-4-yl)propanoyl)-L-leucine 27a. Compound **15a** (2.04 g; 5 mmol) was reacted with

compound **19a** (1.92 g; 5 mmol) according to general procedure F and the crude product was purified using flash chromatography (silica gel; DCM/methanol 95:5) to give compound **27a** as a white solid (3.17 g; 80% yield); M.p 48-49 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.68 - 0.94 (m, 6 H), 1.25 - 1.41 (m, 9 H), 1.42 - 1.81 (m, 4 H), 1.84 - 2.01 (m, 1 H), 2.13 (t, $J=7.23$ Hz, 1 H), 2.76 - 3.12 (m, 2 H), 3.56 - 3.68 (m, 3 H), 3.94 (d, $J=5.08$ Hz, 1 H), 4.15 - 4.26 (m, 1 H), 4.27 - 4.42 (m, 3 H), 4.89 - 5.07 (m, 2 H), 7.24 (d,

$J=7.81$ Hz, 1 H), 7.28 - 7.40 (m, 7 H), 7.47 (d, $J=8.20$ Hz, 1 H), 7.69 - 7.80 (m, 1 H), 7.91 - 8.03 (m, 1 H), 8.24 (d, $J=7.81$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{32}H_{48}N_7O_{10}$ $[M+H]^+$ 690.3463. found: 690.3462.

Compound **27b-27i** was prepared using the same procedure as described above.

4.1.35. (S)-2-(((S)-2-(((benzyloxy)carbonyl)amino)-3-(1-(2-(((S)-4-((tert-butoxycarbonyl)amino)-5-methoxy-5-oxopentanamido)ethyl)-1H-1,2,3-triazol-4-yl)propanamido)-3-cyclohexylpropanoic acid 27b. White solid (3.4 g; 84%); M.p 51-53 °C. 1H NMR (400 MHz, DMSO- d_6): δ 0.67 - 1.00 (m, 2 H), 1.11 (d, $J=7.42$ Hz, 2 H), 1.28 - 1.45 (m, 10 H), 1.48 - 1.81 (m, 8 H), 1.83 - 1.95 (m, 1 H), 2.13 (t, $J=7.23$ Hz, 2 H), 2.87 (dd, $J=14.84$, 9.37 Hz, 1 H), 3.04 (dd, $J=14.84$, 4.69 Hz, 1 H), 3.40 - 3.52 (m, 3 H), 3.61 (s, 3 H), 3.87 - 3.98 (m, 1 H), 4.18 - 4.45 (m, 4 H), 4.86 - 5.10 (m, 2 H), 7.23 (d, $J=7.81$ Hz, 1 H), 7.27 - 7.39 (m, 5 H), 7.47 (d, $J=8.59$ Hz, 1 H), 7.75 (s, 1 H), 7.96 (t, $J=5.27$ Hz, 1 H), 8.22 (d, $J=7.81$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{35}H_{52}N_7O_{10}$ $[M+H]^+$ 730.3776. found: 730.3774.

4.1.36. (S)-2-(((S)-2-(((benzyloxy)carbonyl)amino)-3-(1-(3-(((S)-4-((tert-butoxycarbonyl)amino)-5-methoxy-5-oxopentanamido)propyl)-1H-1,2,3-triazol-4-yl)propanamido)-3-cyclohexylpropanoic acid 27c. White solid (3.2 g; 82% yield); M.p 52-53 °C. 1H NMR (400 MHz, DMSO- d_6): δ 0.68 - 0.99 (m, 2 H), 1.12 (d, $J=6.25$ Hz, 2 H), 1.24 - 1.44 (m, 10 H), 1.45 - 1.82 (m, 8 H), 1.83 - 1.97 (m, 3 H), 2.06 - 2.27 (m, 2 H), 2.77 - 2.94 (m, 1 H), 2.95 - 3.15 (m, 3 H), 3.62 (s, 3 H), 3.88 - 3.99 (m, 1 H), 4.16 - 4.40 (m, 4 H), 4.89 - 5.07 (m, 2 H), 7.25 (d, $J=7.42$ Hz, 1 H), 7.29 - 7.41 (m, 5 H), 7.49 (d,

$J=8.59$ Hz, 1 H), 7.78 (s, 1 H), 7.92 (d, $J=4.69$ Hz, 1 H), 8.24 (d, $J=7.42$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{36}H_{54}N_7O_{10}$ $[M+H]^+$ 744.3932. found: 744.3929.

4.1.37. (S)-2-(((S)-2-(((benzyloxy)carbonyl)amino)-3-(1-(4-(((S)-4-((tert-butoxycarbonyl)amino)-5-methoxy-5-oxopentanamido)butyl)-1*H*-1,2,3-triazol-4-yl)propanamido)-3-cyclohexylpropanoic acid 27d. White solid (3.3 g; 82% yield); M.p 58-60 °C. 1H NMR (400 MHz, DMSO- d_6): δ 0.64 - 0.10 (m, 2 H), 1.11 - 1.18 (m, 3 H), 1.22 - 1.43 (m, 10 H), 1.45 - 1.82 (m, 8 H), 1.83 - 1.99 (m, 3 H), 2.06 - 2.29 (m, 3 H), 2.78 - 2.94 (m, 1 H), 2.95 - 3.17 (m, 3 H), 3.64 (s, 3 H), 3.88 - 4.00 (m, 1 H), 4.15 - 4.40 (m, 4 H), 4.89 - 5.05 (m, 2 H), 7.28 (d, $J=7.47$ Hz, 1 H), 7.30 - 7.41 (m, 5 H), 7.50 (d, $J=8.78$ Hz, 1 H), 7.77 (s, 1 H), 7.92 (d, $J=4.46$ Hz, 1 H), 8.27 (d, $J=7.50$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{37}H_{56}N_7O_{10}$ $[M+H]^+$ 758.4089. found: 758.4087.

4.1.38. ((S)-2-(((benzyloxy)carbonyl)amino)-3-(1-(5-(((S)-4-((tert-butoxycarbonyl)amino)-5-methoxy-5-oxopentanamido)pentyl)-1*H*-1,2,3-triazol-4-yl)propanoyl)-L-leucine 27e. White solid (3.6 g; 87% yield); M.p 56-58 °C. 1H NMR (400 MHz, DMSO- d_6): δ 0.69 - 0.99 (m, 6 H), 1.10 - 1.25 (m, 3 H), 1.28 - 1.44 (m, 12 H), 1.47 - 1.81 (m, 5 H), 1.84 - 1.98 (m, 1 H), 2.12 (t, $J=7.23$ Hz, 2 H), 2.74 - 2.92 (m, 1 H), 2.99 (d, $J=5.86$ Hz, 3 H), 3.62 (s, 3 H), 3.94 (d, $J=4.69$ Hz, 1 H), 4.27 (br. s., 4 H), 4.99 (d, $J=4.30$ Hz, 2 H), 7.26 (d, $J=7.42$ Hz, 1 H), 7.30 - 7.41 (m, 5 H), 7.49 (d, $J=8.20$ Hz, 1 H), 7.68 - 7.87 (m, 2 H), 8.08 - 8.49 (m, 1 H). HRMS (ESI, m/z) calcd for $C_{35}H_{54}N_7O_{10}$ $[M+H]^+$ 732.3932. found: 732.3930.

4.1.39. (S)-2-(((S)-2-(((benzyloxy)carbonyl)amino)-3-(1-(5-(((S)-4-((tert-butoxycarbonyl)amino)-5-methoxy-5-oxopentanamido)pentyl)-1*H*-1,2,3-triazol-4-

yl)propanamido)-3-cyclohexylpropanoic acid 27f. White solid (3.5 g; 82% yield); M.p 55-57 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.71 - 0.99 (m, 2 H), 1.01 - 1.25 (m, 5 H), 1.26 - 1.45 (m, 12 H), 1.46 - 1.82 (m, 10 H), 1.82 - 1.97 (m, 1 H), 2.12 (t, *J*=7.23 Hz, 2 H), 2.84 (br. s., 1 H), 2.94 - 3.11 (m, 3 H), 3.62 (s, 3 H), 3.94 (d, *J*=4.69 Hz, 1 H), 4.27 (br. s., 4 H), 4.89 - 5.08 (m, 2 H), 7.20 - 7.41 (m, 6 H), 7.50 (d, *J*=8.20 Hz, 1 H), 7.77 (d, *J*=14.45 Hz, 2 H), 8.13 - 8.41 (m, 1 H). HRMS (ESI, *m/z*) calcd for C₃₈H₅₈N₇O₁₀ [M+H]⁺ 772.4245. found: 772.4243.

4.1.40. ((S)-2-(((benzyloxy)carbonyl)amino)-3-(1-(6-((S)-4-((tert-butoxycarbonyl)amino)-5-methoxy-5-oxopentanamido)hexyl)-1*H*-1,2,3-triazol-4-yl)propanoyl)-L-leucine 27g. White solid (3.9 g; 88% yield); M.p 59-61 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.72 - 0.94 (m, 6 H), 1.15 - 1.28 (m, 5 H), 1.29 - 1.43 (m, 11 H), 1.45 - 1.81 (m, 5 H), 1.83 - 1.96 (m, 1 H), 2.12 (t, *J*=7.23 Hz, 2 H), 2.78 - 2.92 (m, 1 H), 2.94 - 3.11 (m, 3 H), 3.61 (s, 3 H), 3.93 (d, *J*=4.69 Hz, 1 H), 4.27 (br. s., 4 H), 4.86 - 5.07 (m, 2 H), 7.26 (d, *J*=7.42 Hz, 1 H), 7.28 - 7.40 (m, 5 H), 7.48 (d, *J*=8.20 Hz, 1 H), 7.67 - 7.87 (m, 2 H), 8.13 - 8.40 (m, 1 H). HRMS (ESI, *m/z*) calcd for C₃₆H₅₆N₇O₁₀ [M+H]⁺ 746.4089. found: 746.4088.

4.1.41. (S)-2-((S)-2-(((benzyloxy)carbonyl)amino)-3-(1-(6-((S)-4-((tert-butoxycarbonyl)amino)-5-methoxy-5-oxopentanamido)hexyl)-1*H*-1,2,3-triazol-4-yl)propanamido)-3-cyclohexylpropanoic acid 27h. White solid (3.3 g; 84% yield); M.p 58-60 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.65 - 0.95 (m, 2 H), 1.08 (d, *J*=7.93 Hz, 2 H), 1.19 (br. s., 4 H), 1.26 - 1.39 (m, 13 H), 1.44 - 1.76 (m, 10 H), 1.78 - 1.93 (m, 1 H), 2.08 (t, *J*=7.17 Hz, 2 H), 2.73 - 2.88 (m, 1 H), 2.90 - 3.06 (m, 3 H), 3.58 (s, 3 H), 3.83 - 3.95 (m, 1 H), 4.23 (t, *J*=6.71 Hz, 4 H), 4.95 (d, *J*=3.97 Hz, 2 H), 7.20 (d, *J*=7.63 Hz, 1

H), 7.23 - 7.36 (m, 5 H), 7.40 - 7.48 (m, 1 H), 7.71 (br. s., 2 H), 8.10 - 8.32 (m, 1 H).

HRMS (ESI, m/z) calcd for $C_{39}H_{60}N_7O_{10}$ $[M+H]^+$ 786.4402. found: 786.4401.

4.1.42. ((S)-2-(((benzyloxy)carbonyl)amino)-3-(1-(6-((S)-4-((tert-butoxycarbonyl)amino)-5-methoxy-5-oxopentanamido)hexyl)-1*H*-1,2,3-triazol-4-yl)propanoyl)-L-phenylalanine 27i. White solid (3.41 g; 81% yield); M.p 112-116 °C. 1H NMR (400 MHz, DMSO- d_6): δ 1.12 - 1.44 (m, 14 H), 1.46 - 1.59 (m, 1 H), 1.64 - 1.81 (m, 2 H), 1.82 - 2.00 (m, 1 H), 2.12 (t, $J=6.44$ Hz, 2 H), 2.82 (br. s., 1 H), 2.89 - 3.12 (m, 5 H), 3.61 (d, $J=2.34$ Hz, 3 H), 3.94 (d, $J=4.69$ Hz, 1 H), 4.26 (t, $J=6.64$ Hz, 4 H), 4.39 - 4.53 (m, 1 H), 4.97 (s, 2 H), 7.10 - 7.40 (m, 11 H), 7.45 (d, $J=8.20$ Hz, 1 H), 7.69 (br. s., 1 H), 7.76 (d, $J=4.30$ Hz, 1 H), 8.08 - 8.41 (m, 1 H). HRMS (ESI, m/z) calcd for $C_{39}H_{54}N_7O_{10}$ $[M+H]^+$ 780.3932. found: 780.3929.

4.1.43. Methyl (8*S*,11*S*,14*S*,*Z*)-14-(((benzyloxy)carbonyl)amino)-11-isobutyl-5,10,13-trioxo-1*H*-4,9,12-triaza-1(1,4)-triazolacyclopentadecaphane-8-carboxylate 28a. A solution of compound **27a** (0.69 g; 1 mmol) in dry CH_2Cl_2 (5 mL) was treated with 1M HCl in dioxane according to general procedure **C**. The resulting compound was cyclized in dry DMF (500 mL) according to general procedure **H** and the crude product was purified by flash chromatography (silica gel; DCM/methanol 95:5) to give compound **28a** as a white solid (0.34 g; 60% yield); M.p 203-205 °C. 1H NMR (400 MHz, DMSO- d_6): δ 0.77 - 1.01 (m, 6 H), 1.34 - 1.59 (m, 3 H), 1.76 (dt, $J=13.28, 6.64$ Hz, 1 H), 2.12 (d, $J=16.80$ Hz, 1 H), 2.26 (t, $J=12.89$ Hz, 1 H), 2.37 - 2.48 (m, 1 H), 2.75 - 2.91 (m, 1 H), 3.01 - 3.11 (m, 1 H), 3.24 (d, $J=12.89$ Hz, 1 H), 3.59 - 3.69 (m, 3 H), 3.71 - 3.83 (m, 1 H), 4.01 - 4.20 (m, 2 H), 4.37 - 4.54 (m, 2 H), 4.61 - 4.74 (m, 1 H), 4.94 - 5.17 (m, 2 H), 6.60 (d, $J=8.20$ Hz, 1 H), 7.22 - 7.43 (m, 6 H), 7.68 (s, 1 H), 8.28 (d, $J=4.69$ Hz, 1

H), 8.45 (d, $J=9.76$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{27}H_{38}N_7O_7$ $[M+H]^+$ 572.2833. found: 572.2830.

Compound **28b-28i** was prepared using the same procedure as described above.

4.1.44. Methyl (8S,11S,14S,Z)-14-(((benzyloxy)carbonyl)amino)-11-(cyclohexylmethyl)-5,10,13-trioxo-1¹H-4,9,12-triaza-1(1,4)-

triazolacyclopentadecaphane-8-carboxylate 28b. White solid (0.37 g; 62% yield); M.p >230 °C. 1H NMR (400 MHz, DMSO- d_6): δ 0.79 - 0.96 (m, 2 H), 1.03 - 1.30 (m, 3 H), 1.46 (br. s., 4 H), 1.57 - 1.72 (m, 4 H), 1.75 - 1.89 (m, 1 H), 2.04 - 2.16 (m, 1 H), 2.18 - 2.33 (m, 1 H), 2.37 - 2.47 (m, 1 H), 2.74 - 2.91 (m, 1 H), 2.99 - 3.12 (m, 1 H), 3.15 - 3.26 (m, 1 H), 3.65 (s, 3 H), 3.71 - 3.82 (m, 1 H), 4.02 - 4.18 (m, 2 H), 4.37 - 4.52 (m, 2 H), 4.60 - 4.74 (m, 1 H), 5.04 (d, $J=7.03$ Hz, 2 H), 6.61 (d, $J=8.60$ Hz, 1 H), 7.22 - 7.44 (m, 6 H), 7.68 (s, 1 H), 8.25 (d, $J=4.41$ Hz, 1 H), 8.41 (d, $J=9.41$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{30}H_{42}N_7O_7$ $[M+H]^+$ 612.3146. found: 612.3144.

4.1.45. Methyl (3S,6S,9S,Z)-3-(((benzyloxy)carbonyl)amino)-6-(cyclohexylmethyl)-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclohexadecaphane-9-carboxylate

28c. White solid (0.4 g; 65% yield); M.p >230 °C. 1H NMR (400 MHz, DMSO- d_6): δ 0.73 - 0.95 (m, 2 H), 1.02 - 1.26 (m, 3 H), 1.30 - 1.53 (m, 3 H), 1.54 - 1.79 (m, 6 H), 1.83 - 1.93 (m, 1 H), 1.98 - 2.29 (m, 4 H), 2.66 (dd, $J=13.28, 6.25$ Hz, 1 H), 3.03 (d, $J=5.86$ Hz, 2 H), 3.19 - 3.30 (m, 1 H), 3.63 (s, 3 H), 4.13 (dt, $J=13.67, 6.44$ Hz, 1 H), 4.22 - 4.43 (m, 4 H), 4.94 - 5.11 (m, 2 H), 7.06 (d, $J=7.81$ Hz, 1 H), 7.25 - 7.41 (m, 5 H), 7.46 - 7.58 (m, 2 H), 8.14 (d, $J=7.42$ Hz, 1 H), 8.52 (d, $J=8.20$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{31}H_{44}N_7O_7$ $[M+H]^+$ 626.3302. found: 626.3329.

4.1.46. Methyl (3S,6S,9S,Z)-3-(((benzyloxy)carbonyl)amino)-6-(cyclohexylmethyl)-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacycloheptadecaphane-9-carboxylate

28d. White solid (0.39 g; 62% yield); M.p 206-207 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.75 - 0.99 (m, 2 H), 1.03 - 1.29 (m, 4 H), 1.44 (d, *J*=9.76 Hz, 3 H), 1.50 - 1.81 (m, 10 H), 2.04 - 2.31 (m, 3 H), 2.65 - 2.79 (m, 1 H), 2.95 - 3.16 (m, 2 H), 3.62 (s, 3 H), 4.10 - 4.20 (m, 1 H), 4.22 - 4.31 (m, 2 H), 4.32 - 4.49 (m, 2 H), 4.93 - 5.14 (m, 2 H), 7.00 (d, *J*=7.42 Hz, 1 H), 7.25 - 7.47 (m, 5 H), 7.76 (s, 2 H), 8.22 (d, *J*=7.42 Hz, 1 H), 8.35 (d, *J*=7.81 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₃₂H₄₆N₇O₇ [M+H]⁺ 640.3459. found: 640.3457.

4.1.47. Methyl (3S,6S,9S,Z)-3-(((benzyloxy)carbonyl)amino)-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclooctadecaphane-9-carboxylate **28e.**

White solid (0.36 g; 59% yield); M.p 209-211 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.86 (dd, *J*=19.53, 6.25 Hz, 6 H), 1.06 - 1.24 (m, 2 H), 1.28 - 1.56 (m, 4 H), 1.58 - 1.82 (m, 3 H), 1.91 (dt, *J*=14.06, 7.03 Hz, 1 H), 1.98 - 2.06 (m, 1 H), 2.07 - 2.22 (m, 2 H), 2.88 - 3.16 (m, 4 H), 3.62 (s, 3 H), 4.11 (q, *J*=6.77 Hz, 1 H), 4.16 - 4.25 (m, 1 H), 4.26 - 4.41 (m, 3 H), 4.92 - 5.15 (m, 2 H), 7.23 (d, *J*=7.42 Hz, 1 H), 7.28 - 7.43 (m, 5 H), 7.55 - 7.74 (m, 2 H), 8.21 (d, *J*=7.81 Hz, 1 H), 8.60 (d, *J*=6.25 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₃₀H₄₄N₇O₇ [M+H]⁺ 614.3302. found: 614.3301.

4.1.48. Methyl (3S,6S,9S,Z)-3-(((benzyloxy)carbonyl)amino)-6-(cyclohexylmethyl)-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclooctadecaphane-9-carboxylate

28f. White solid (0.42 g; 65% yield); M.p 215-216 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.70 - 0.98 (m, 2 H), 1.03 - 1.27 (m, 5 H), 1.30 - 1.47 (m, 4 H), 1.49 - 1.85 (m, 8 H), 1.90 (dt, *J*=14.06, 7.03 Hz, 1 H), 1.96 - 2.06 (m, 1 H), 2.11 (dq, *J*=14.84, 7.42 Hz, 2 H), 2.91 -

3.18 (m, 4 H), 3.54 - 3.77 (m, 3 H), 4.07 - 4.16 (m, 1 H), 4.16 - 4.24 (m, 1 H), 4.25 - 4.48 (m, 3 H), 4.93 - 5.18 (m, 2 H), 7.24 - 7.47 (m, 5 H), 7.59 - 7.79 (m, 2 H), 8.19 (d, $J=7.81$ Hz, 1 H), 8.57 (d, $J=6.64$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{33}H_{48}N_7O_7$ $[M+H]^+$ 654.3615. found: 654.3612.

4.1.49. Methyl (3S,6S,9S,Z)-3-(((benzyloxy)carbonyl)amino)-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclononadecaphane-9-carboxylate 28g.

White solid (0.38 g; 62% yield); M.p 218-219 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.85 (dd, $J=18.36, 6.25$ Hz, 6 H), 1.22 (d, $J=10.16$ Hz, 4 H), 1.35 (br. s., 2 H), 1.42 - 1.55 (m, 2 H), 1.59 - 1.71 (m, 1 H), 1.72 - 1.86 (m, 2 H), 1.95 (q, $J=6.51$ Hz, 2 H), 2.04 - 2.15 (m, 1 H), 2.16 - 2.27 (m, 1 H), 2.91 - 3.21 (m, 4 H), 3.62 (s, 3 H), 4.04 (q, $J=7.03$ Hz, 1 H), 4.16 - 4.28 (m, 2 H), 4.29 - 4.41 (m, 2 H), 4.89 - 5.16 (m, 2 H), 7.12 (d, $J=7.81$ Hz, 1 H), 7.27 - 7.44 (m, 5 H), 7.65 - 7.78 (m, 2 H), 8.18 (d, $J=8.20$ Hz, 1 H), 8.50 (d, $J=5.86$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{31}H_{46}N_7O_7$ $[M+H]^+$ 628.3459. found: 628.3463.

4.1.50. Methyl (3S,6S,9S,Z)-3-(((benzyloxy)carbonyl)amino)-6-(cyclohexylmethyl)-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclononadecaphane-9-carboxylate 28h.

White solid (0.4 g; 60% yield); M.p 207-209 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.72 - 0.97 (m, 2 H), 1.04 - 1.29 (m, 7 H), 1.30 - 1.46 (m, 4 H), 1.48 - 1.73 (m, 6 H), 1.74 - 1.83 (m, 2 H), 1.94 (q, $J=6.71$ Hz, 2 H), 2.09 (d, $J=7.02$ Hz, 1 H), 2.14 - 2.27 (m, 1 H), 2.90 - 3.20 (m, 4 H), 3.61 (s, 3 H), 4.03 (d, $J=6.71$ Hz, 1 H), 4.22 (t, $J=7.02$ Hz, 2 H), 4.28 - 4.45 (m, 2 H), 5.04 (s, 2 H), 7.18 (d, $J=7.63$ Hz, 1 H), 7.26 - 7.44 (m, 5 H), 7.63 - 7.80 (m, 2 H), 8.16 (d, $J=7.63$ Hz, 1 H), 8.46 (d, $J=6.10$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{34}H_{50}N_7O_7$ $[M+H]^+$ 668.3772. found: 668.3770.

4.1.51. Methyl (3S,6S,9S,Z)-6-benzyl-3-(((benzyloxy)carbonyl)amino)-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclononadecaphane-9-carboxylate 28i. White solid (0.4 g; 61% yield); M.p 209-211 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 1.12 - 1.30 (m, 5 H), 1.35 (d, J=5.08 Hz, 2 H), 1.51 (d, J=6.25 Hz, 1 H), 1.66 - 1.88 (m, 2 H), 1.96 (d, J=5.47 Hz, 1 H), 2.05 - 2.16 (m, 1 H), 2.17 - 2.27 (m, 1 H), 2.78 (d, J=13.28 Hz, 1 H), 2.93 - 3.17 (m, 4 H), 3.57 - 3.75 (m, 3 H), 4.08 (q, J=7.16 Hz, 1 H), 4.14 - 4.37 (m, 3 H), 4.53 (d, J=5.86 Hz, 1 H), 4.98 - 5.12 (m, 2 H), 6.97 (d, J=7.81 Hz, 1 H), 7.11 - 7.48 (m, 10 H), 7.68 (s, 1 H), 7.74 (br. s., 1 H), 8.26 (d, J=7.81 Hz, 1 H), 8.63 (d, J=5.86 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₃₄H₄₄N₇O₇ [M+H]⁺ 662.3302. found: 662.3329.

4.1.52. Methyl N²-(((S)-5-azido-2-(((benzyloxy)carbonyl)amino)pentanoyl)-L-leucyl)-N⁵-(prop-2-yn-1-yl)-L-glutamate 30. A solution of compound **23** (2.5 g; 6.075 mmol) in dry DCM (30 mL) was treated with 1M HCl in dioxane according to general procedure **C**. The resulting compound was coupled with compound **21** (1.77 g ; 6.075 mmol) in dry DMF (30 mL) according to general procedure **A** and the crude product was purified by flash chromatography (silica gel; hexane/ethyl acetate 75:25) to give **30** as a white solid (3.21 g ; 56% yield); M.p 106-107 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.87 (dd, J=17.09, 6.41 Hz, 6 H), 1.46 (d, J=6.71 Hz, 2 H), 1.50 - 1.72 (m, 5 H), 1.83 (br. s., 1 H), 1.94 (d, J=7.63 Hz, 1 H), 2.10 - 2.23 (m, 2 H), 3.07 (br. s., 1 H), 3.60 (s, 3 H), 3.78 - 3.89 (m, 2 H), 4.03 (d, J=5.80 Hz, 1 H), 4.19 (d, J=5.80 Hz, 1 H), 4.34 (d, J=7.32 Hz, 1 H), 5.03 (s, 2 H), 7.25 - 7.40 (m, 5 H), 7.46 (d, J=7.93 Hz, 1 H), 7.92 (d, J=7.93 Hz, 1 H), 8.23 (t, J=5.04 Hz, 1 H), 8.29 (d, J=6.71 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₂₈H₄₀N₇O₇ [M+H]⁺ 586.2989. found: 586.2985.

4.1.53. Methyl (7S,10S,Z)-13-(((benzyloxy)carbonyl)amino)-10-isobutyl-4,9,12-trioxo-1¹H-3,8,11-triaza-1(4,1)-triazolacyclohexadecaphane-7-carboxylate 31. A solution of compound **30** (0.3 g; 0.512 mmol) in dry DCM (420 mL) was cyclized according to general procedure **G** and the crude product was purified using flash chromatography (silica gel; DCM/methanol 95:5) to give compound **31** as a white solid (0.15 g; 48% yield); M.p 205-206 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.86 (dd, *J*=17.02, 6.40 Hz, 6 H), 1.45 (d, *J*=6.71 Hz, 2 H), 1.51 - 1.72 (m, 5 H), 1.84 (br. s., 1 H), 1.93 (d, *J*=7.65 Hz, 2 H), 2.10 - 2.23 (m, 2 H), 3.61 (s, 3 H), 3.78 - 3.88 (m, 2 H), 4.03 (d, *J*=5.81 Hz, 2 H), 4.17 (d, *J*=5.80 Hz, 1 H), 4.34 (d, *J*=7.33 Hz, 1 H), 5.03 (s, 2 H), 7.24 - 7.40 (m, 5 H), 7.45 (d, *J*=7.92 Hz, 1 H), 7.93 (d, *J*=7.92 Hz, 1 H), 8.20 - 8.24 (m, 2 H), 8.30 (d, *J*=7.10 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₂₈H₄₀N₇O₇ [M+H]⁺ 586.2989. found: 586.2992.

4.1.54. Benzyl ((3S,6S,9S,Z)-9-(hydroxymethyl)-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclohexadecaphane-3-yl)carbamate 26a. A solution of compound **25a** (0.57 g; 1 mmol) in dry THF (6 mL) was reduced using 2M LiBH₄ solution according to general procedure **I** and the crude product was purified using flash chromatography (silica gel; DCM/methanol 95:5) to give compound **26a** as a white solid (0.47; 84% yield); M.p 112-114 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.73 - 0.10 (m, 6 H), 1.44 - 1.46 (m, 3 H), 1.62 - 1.80 (m, 3 H), 1.84 - 1.94 (m, 2 H), 2.00 - 2.32 (m, 4 H), 2.61 - 2.76 (m, 2H), 3.02 - 3.06 (m, 2 H), 4.08 - 4.20 (m, 1 H), 4.26 - 4.45 (m, 4 H), 5.04 (d, *J*=9.76 Hz, 2 H), 7.01 (d, *J*=7.03 Hz, 1 H), 7.35 (br. s., 5 H), 7.45 (br. s., 1 H), 7.67 (br. s., 1 H), 7.90 (d, *J*=7.81 Hz, 1 H), 8.10 (d, *J*=6.25 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₂₇H₄₀N₇O₆ [M+H]⁺ 558.3040. found: 558.3038.

Compound **26b**, **29a-29i** and **32** were prepared using the same procedure as described above.

4.1.55. Benzyl ((3S,6S,9S,Z)-9-(hydroxymethyl)-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacycloheptadecaphane-3-yl)carbamate 26b. White solid (0.37 g; 70% yield); M.p 116-118 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.86 (dd, *J*=12.69, 6.44 Hz, 6 H), 1.15 - 1.32 (m, 2 H), 1.35 - 1.55 (m, 3 H), 1.57 - 1.84 (m, 4 H), 2.03 - 2.33 (m, 3 H), 2.71 - 2.74 (m, 1 H), 2.97 - 3.14 (m, 2 H), 4.10 - 4.32 (m, 3 H), 4.33 - 4.44 (m, 2 H), 4.93 - 5.16 (m, 2 H), 7.08 (d, *J*=7.42 Hz, 1 H), 7.26 - 7.42 (m, 5 H), 7.58 (br. s., 1 H), 7.77 (d, *J*=7.81 Hz, 1 H), 7.87 (s, 1 H), 8.13 (d, *J*=7.03 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₂₈H₄₂N₇O₆ [M+H]⁺ 572.3197. found: 572.3196.

4.1.56. Benzyl ((8S,11S,14S,Z)-8-(hydroxymethyl)-11-isobutyl-5,10,13-trioxo-1¹H-4,9,12-triaza-1(1,4)-triazolacyclopentadecaphane-14-yl)carbamate 29a. White solid (0.43 g; 78% yield); M.p 190-191 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.78 - 1.00 (m, 6 H), 1.35 - 1.59 (m, 3 H), 1.76 (dt, *J*=13.23, 6.64 Hz, 1 H), 2.12 (d, *J*=16.7 Hz, 1 H), 2.26 (t, *J*=12.89 Hz, 1 H), 2.37 - 2.48 (m, 1 H), 2.73 (s, 1H), 2.75 - 2.91 (m, 1 H), 3.01 - 3.11 (m, 1 H), 3.24 (d, *J*=12.89 Hz, 1 H), 3.70 - 3.82 (m, 1 H), 4.01 - 4.20 (m, 2 H), 4.37 - 4.55 (m, 2 H), 4.60 - 4.74 (m, 1 H), 4.94 - 5.17 (m, 2 H), 6.71 (d, *J*=8.20 Hz, 1 H), 7.21 (br. s., 1 H), 7.29 - 7.44 (m, 5 H), 7.78 (d, *J*=9.37 Hz, 1 H), 7.85 (s, 1 H), 8.19 (d, *J*=4.69 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₂₆H₃₈N₇O₆ [M+H]⁺ 544.2884. found: 544.2882.

4.1.57. Benzyl ((8S,11S,14S,Z)-11-(cyclohexylmethyl)-8-(hydroxymethyl)-5,10,13-trioxo-1¹H-4,9,12-triaza-1(1,4)-triazolacyclopentadecaphane-14-yl)carbamate 29b. White solid (0.45 g; 81% yield); M.p >230 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.78 -

0.96 (m, 2 H), 1.03 - 1.32 (m, 3 H), 1.46 (br. s., 4 H), 1.58 - 1.72 (m, 4 H), 1.75 - 1.89 (m, 1 H), 2.05 - 2.16 (m, 1 H), 2.17 - 2.31 (m, 1 H), 2.37 - 2.46 (m, 1 H), 2.74 - 2.91 (m, 1 H), 2.99 - 3.12 (m, 1 H), 3.15 - 3.26 (m, 1 H), 3.71 - 3.82 (m, 1 H), 4.02 - 4.18 (m, 2 H), 4.37 - 4.52 (m, 2 H), 4.60 - 4.74 (m, 1 H), 5.04 (d, $J=7.03$ Hz, 2 H), 6.71 (d, $J=8.59$ Hz, 1 H), 7.18 (br. s., 1 H), 7.26 - 7.43 (m, 5 H), 7.72 (d, $J=9.37$ Hz, 1 H), 7.95 (s, 1 H), 8.14 (d, $J=4.69$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{29}H_{42}N_7O_6$ $[M+H]^+$ 584.3197. found: 584.3197.

4.1.58. Benzyl ((3S,6S,9S,Z)-6-(cyclohexylmethyl)-9-(hydroxymethyl)-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclohexadecaphane-3-yl)carbamate 29c. White solid (0.46 g; 79% yield); M.p 200-202 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.73 - 0.94 (m, 2 H), 1.02 - 1.25 (m, 3 H), 1.30 - 1.52 (m, 3 H), 1.53 - 1.78 (m, 6 H), 1.83 - 1.94 (m, 1 H), 1.98 - 2.29 (m, 4 H), 2.66 (dd, $J=13.22$, 6.24 Hz, 1 H), 3.03 (d, $J=5.86$ Hz, 2 H), 3.19 - 3.30 (m, 1 H), 4.14 (dt, $J=13.65$, 6.44 Hz, 1 H), 4.22 - 4.43 (m, 4 H), 4.94 - 5.11 (m, 2 H), 7.04 (d, $J=7.42$ Hz, 1 H), 7.33 - 7.39 (m, 5 H), 7.42 (br. s., 1 H), 7.67 (s, 1 H), 7.88 (d, $J=8.20$ Hz, 1 H), 8.08 (d, $J=7.03$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{30}H_{44}N_7O_6$ $[M+H]^+$ 598.3353. found: 598.3351.

4.1.59. Benzyl ((3S,6S,9S,Z)-6-(cyclohexylmethyl)-9-(hydroxymethyl)-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacycloheptadecaphane-3-yl)carbamate 29d. White solid (0.43 g; 70% yield); M.p 201-203 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.75 - 0.99 (m, 2 H), 1.03 - 1.29 (m, 4 H), 1.44 (d, $J=9.76$ Hz, 3 H), 1.50 - 1.81 (m, 10 H), 2.04 - 2.31 (m, 3 H), 2.65 - 2.79 (m, 1 H), 2.95 - 3.16 (m, 2 H), 4.10 - 4.20 (m, 1 H), 4.22 - 4.31 (m, 2 H), 4.32 - 4.49 (m, 2 H), 4.93 - 5.14 (m, 2 H), 7.31 - 7.46 (m, 6 H), 7.67 (br. s., 1

H), 7.83 (d, $J=8.20$ Hz, 1 H), 7.96 (s, 1 H), 8.17 (d, $J=7.42$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{31}H_{46}N_7O_6$ $[M+H]^+$ 628.3823. found: 628.3822.

4.1.60. Benzyl ((3S,6S,9S,Z)-9-(hydroxymethyl)-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclooctadecaphane-3-yl)carbamate 29e. White solid (0.48 g; 86% yield); M.p 191-193 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.82 - 0.86 (m, 6 H), 1.09 - 1.23 (m, 2 H), 1.27 - 1.56 (m, 5 H), 1.59 - 1.84 (m, 3 H), 1.92 (dt, $J=14.02$, 6.99 Hz, 1 H), 1.98 - 2.05 (m, 1 H), 2.08 - 2.23 (m, 3 H), 2.89 - 3.17 (m, 4 H), 4.00 - 4.12 (m, 1 H), 4.14 - 4.23 (m, 1 H), 4.26 - 4.41 (m, 3 H), 4.93 - 5.15 (m, 2 H), 7.11 (d, $J=7.41$ Hz, 1 H), 7.28 - 7.45 (m, 5 H), 7.54 - 7.74 (m, 2 H), 8.11 (d, $J=7.86$ Hz, 1 H), 8.44 (d, $J=6.21$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{29}H_{44}N_7O_6$ $[M+H]^+$ 586.3353. found: 586.3350.

4.1.61. Benzyl ((3S,6S,9S,Z)-6-(cyclohexylmethyl)-9-(hydroxymethyl)-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclooctadecaphane-3-yl)carbamate 29f. White solid (0.44 g; 73% yield); M.p 193-195 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.70 - 0.98 (m, 2 H), 1.03 - 1.27 (m, 5 H), 1.30 - 1.47 (m, 4 H), 1.49 - 1.85 (m, 8 H), 1.89 - 1.90 (m, 1 H), 1.96 - 2.04 (m, 1 H), 2.11 (dq, $J=14.84$, 7.42 Hz, 2 H), 2.91 - 3.18 (m, 4 H), 4.07 - 4.16 (m, 1 H), 4.16 - 4.24 (m, 1 H), 4.25 - 4.48 (m, 3 H), 4.93 - 5.18 (m, 2 H), 7.25 - 7.43 (m, 5 H), 7.54 (br. s., 1 H), 7.74 - 7.87 (m, 1 H), 8.04 (d, $J=7.42$ Hz, 1 H) 8.55 (d, $J=6.64$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{32}H_{47}N_7O_6$ $[M+H]^+$ 626.3666. found: 626.3661.

4.1.62. Benzyl ((3S,6S,9S,Z)-9-(hydroxymethyl)-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclononadecaphane-3-yl)carbamate 29g. White solid (0.46 g; 84% yield); M.p 180-182 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.85 (dd, $J=18.36$, 6.25 Hz, 6 H), 1.22 (d, $J=10.16$ Hz, 4 H), 1.35 (br. s., 2 H), 1.42 - 1.55 (m, 2 H), 1.59 - 1.71

(m, 2 H), 1.72 - 1.86 (m, 2 H), 1.95 (q, $J=6.51$ Hz, 2 H), 2.04 - 2.15 (m, 2 H), 2.16 - 2.27 (m, 2 H), 2.91 - 3.21 (m, 4 H), 4.04 (q, $J=7.03$ Hz, 1 H), 4.16 - 4.28 (m, 2 H), 4.29 - 4.41 (m, 2 H), 4.89 - 5.16 (m, 2 H), 7.12 (d, $J=7.79$, 1 H), 7.21 - 7.40 (m, 5 H), 7.57 (t, $J=5.27$ Hz, 1 H), 7.81 (br. s., 1 H), 8.07 (d, $J=7.81$ Hz, 1 H), 8.49 (d, $J=5.79$, 1 H). HRMS (ESI, m/z) calcd for $C_{30}H_{46}N_7O_6$ $[M+H]^+$ 600.3510. found: 600.3508.

4.1.63. Benzyl ((3S,6S,9S,Z)-6-(cyclohexylmethyl)-9-(hydroxymethyl)-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclononadecaphane-3-yl)carbamate 29h. White solid (0.42 g; 73% yield); M.p 198-199 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.73 - 0.97 (m, 2 H), 1.04 - 1.29 (m, 7 H), 1.30 - 1.46 (m, 4 H), 1.48 - 1.73 (m, 6 H), 1.74 - 1.83 (m, 2 H), 1.92 - 1.94 (m, 2 H), 2.09 (d, $J=7.02$ Hz, 1 H), 2.13 - 2.27 (m, 1 H), 2.91 - 3.20 (m, 4 H), 4.02 (d, $J=6.72$ Hz, 1 H), 4.22 (t, $J=7.02$ Hz, 2 H), 4.28 - 4.45 (m, 2 H), 5.04 (s, 2 H), 7.26 - 7.44 (m, 6 H), 7.59 (br. s., 1 H), 7.78 (d, $J=8.24$ Hz, 1 H), 7.84 (s, 1 H), 8.08 (d, $J=7.63$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{33}H_{50}N_7O_6$ $[M+H]^+$ 640.3823. found: 640.3823.

4.1.64. Benzyl ((3S,6S,9S,Z)-6-benzyl-9-(hydroxymethyl)-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclononadecaphane-3-yl)carbamate 29i. White solid (0.45 g; 79% yield); M.p 219-221 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 1.10 - 1.29 (m, 5 H), 1.32 - 1.35 (m, 2 H), 1.53 (d, $J=6.24$ Hz, 1 H), 1.64 - 1.87 (m, 2 H), 1.94 - 1.98 (m, 1 H), 2.05 - 2.16 (m, 1 H), 2.18 - 2.29 (m, 2 H), 2.76 (d, $J=12.93$ Hz, 2 H), 2.93 - 3.15 (m, 4 H), 4.02 - 4.06 (m, 1 H), 4.14 - 4.36 (m, 3 H), 4.51 (d, $J=5.85$ Hz, 1 H), 4.97 - 5.12 (m, 2 H), 6.92 (d, $J=7.76$ Hz, 1 H), 7.12 - 7.47 (m, 10 H), 7.66 (s, 1 H), 7.72 (br. s., 1 H), 8.20 (d, $J=7.85$ Hz, 1 H), 8.68 (d, $J=5.88$ Hz, 1 H). HRMS (ESI, m/z) calcd for $C_{33}H_{44}N_7O_6$ $[M+H]^+$ 634.3353. found: 634.3352.

4.1.65. Benzyl ((7S,10S,Z)-7-(hydroxymethyl)-10-isobutyl-4,9,12-trioxo-1¹H-3,8,11-triaza-1(4,1)-triazolacyclohexadecaphane-13-yl)carbamate 32. White solid (0.43 g; 74% yield); M.p >230 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.88 (dd, *J*=17.08, 6.42 Hz, 6 H), 1.44 – 1.46 (m, 2 H), 1.53 - 1.73 (m, 5 H), 1.82 (br. s., 1 H), 1.91 – 1.94 (m, 1 H), 2.11 - 2.23 (m, 2 H), 3.79 - 3.89 (m, 2 H), 4.00 – 4.05 (m, 1 H), 4.16 – 4.21 (m, 1 H), 4.35 (d, *J*=7.34 Hz, 1 H), 5.04 (m, 2 H), 7.25 - 7.40 (m, 5 H), 7.48 (d, *J*=7.91 Hz, 1 H), 7.56 (br. S., 1H), 7.90 (d, *J*=7.91 Hz, 1 H), 8.23 (t, *J*=5.03 Hz, 1 H), 8.30 (d, *J*=6.75 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₂₇H₄₀N₇O₆ [M+H]⁺ 558.3040. found: 558.3038.

4.1.66. Benzyl ((S)-1-(((S)-1-(((S)-5-((3-azidopropyl)amino)-1-hydroxy-5-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-1-oxopent-4-yn-2-yl)carbamate 33. White solid (0.46 g; 82% yield); M.p 60-62 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.83-0.86 (m, 6 H), 1.46 (t, *J*=6.87 Hz, 2 H), 1.65 (t, *J*=6.62 Hz, 3 H), 1.71 - 1.87 (m, 2 H), 1.89 - 2.02 (m, 2 H), 2.07 - 2.21 (m, 2 H), 2.35 - 2.48 (m, 1 H), 2.52 - 2.63 (m, 1 H), 2.82 (br. s., 1 H), 2.98 - 3.20 (m, 4 H), 4.11 - 4.25 (m, 2 H), 4.31 (d, *J*=7.03 Hz, 1 H), 5.05 (s, 2 H), 7.23 - 7.41 (m, 5 H), 7.49 (d, *J*=7.76 Hz, 1 H), 7.81 (br. s., 1 H), 8.01 (d, *J*=7.76 Hz, 1 H), 8.21 (d, *J*=7.42 Hz, 1 H). HRMS (ESI, *m/z*) calcd for C₂₇H₄₀N₇O₆ [M+H]⁺ 558.3040. found: 558.3028.

4.1.67. Benzyl ((8S,11S,14S,Z)-8-formyl-11-isobutyl-5,10,13-trioxo-1¹H-4,9,12-triaza-1(1,4)-triazolacyclopentadecaphane-14-yl)carbamate 1. Macrocyclic alcohol **29a** (200 mg; 0.36 mmol) suspended in dry DCM (40 mL) was oxidized according to general procedure **J** to obtain compound **1** as a white solid (95 mg; 49% yield); M.p 127-128 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.77 - 1.01 (m, 6 H), 1.34 - 1.59 (m, 3 H), 1.76 (dt, *J*=13.28, 6.64 Hz, 1 H), 2.12 (d, *J*=16.80 Hz, 1 H), 2.26 (t, *J*=12.89 Hz, 1 H),

2.37 - 2.48 (m, 1 H), 2.75 - 2.91 (m, 1 H), 3.01 - 3.11 (m, 1 H), 3.24 (d, $J=12.89$ Hz, 1 H), 3.71 - 3.83 (m, 1 H), 4.01 - 4.20 (m, 2 H), 4.37 - 4.54 (m, 2 H), 4.61 - 4.74 (m, 1 H), 4.94 - 5.17 (m, 2 H), 6.62 (d, $J=8.21$ Hz, 1 H), 7.22 - 7.43 (m, 6 H), 7.68 (s, 1 H), 8.30 (d, $J=4.52$ Hz, 1 H), 8.42 (d, $J=9.69$ Hz, 1 H), 9.49 (s, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 21.7, 22.3, 22.9, 23.9, 28.0, 28.8, 30.6, 35.6, 48.6, 52.4, 52.8, 56.1, 65.5, 127.5, 127.7, 127.9, 128.2, 136.7, 140.8, 155.2, 162.2, 169.7, 171.1, 173.1, 201.1. HRMS-ESI: Calcd for $\text{C}_{26}\text{H}_{34}\text{N}_7\text{O}_6$ $[\text{M}-\text{H}]^+$, 540.2571. found: 540.2571.

Compound **2-12** and **34** were synthesized according to the same procedure as above.

4.1.68. Benzyl ((8S,11S,14S,Z)-11-(cyclohexylmethyl)-8-formyl-5,10,13-trioxo-1¹H-4,9,12-triaza-1(1,4)-triazolacyclopentadecaphane-14-yl)carbamate 2. White solid (102 mg; 51% yield); M.p 128-129 $^{\circ}\text{C}$. ^1H NMR (400 MHz, DMSO- d_6): δ 0.79 - 0.96 (m, 2 H), 1.03 - 1.30 (m, 3 H), 1.46 (br. s., 4 H), 1.57 - 1.72 (m, 4 H), 1.75 - 1.89 (m, 1 H), 2.04 - 2.16 (m, 1 H), 2.18 - 2.33 (m, 1 H), 2.37 - 2.47 (m, 1 H), 2.74 - 2.91 (m, 1 H), 2.99 - 3.12 (m, 1 H), 3.15 - 3.26 (m, 1 H), 3.71 - 3.82 (m, 1 H), 4.02 - 4.18 (m, 2 H), 4.37 - 4.52 (m, 2 H), 4.60 - 4.74 (m, 1 H), 5.04 (d, $J=7.03$ Hz, 2 H), 6.62 (d, $J=8.57$ Hz, 1 H), 7.22 - 7.44 (m, 6 H), 7.68 (s, 1 H), 8.26 (d, $J=4.43$ Hz, 1 H), 8.39 (d, $J=9.38$ Hz, 1 H), 9.48 (s, 1H). HRMS (ESI, m/z) calcd for $\text{C}_{29}\text{H}_{39}\text{N}_7\text{O}_6\text{Na}$ $[\text{M}+\text{Na}]^+$ 604.2860; found 604.2860.

4.1.69. Benzyl ((3S,6S,9S,Z)-9-formyl-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclohexadecaphane-3-yl)carbamate 3. White solid (91 mg; 48% yield); M.p 122-124 $^{\circ}\text{C}$. ^1H NMR (400 MHz, DMSO- d_6): δ 0.72 - 0.99 (m, 6 H), 1.42 - 1.44 (m, 3 H), 1.62 - 1.80 (m, 2 H), 1.82 - 1.93 (m, 1 H), 2.00 - 2.31 (m, 4 H), 2.61 - 2.76

(m, 1 H), 3.04 (d, $J=4.30$ Hz, 2 H), 4.08 - 4.22 (m, 1 H), 4.27 - 4.46 (m, 4 H), 5.03 (d, $J=9.75$ Hz, 2 H), 7.06 (d, $J=7.09$ Hz, 1 H), 7.23 - 7.42 (m, 5 H), 7.52 (s, 2 H), 8.19 (d, $J=7.47$ Hz, 1 H), 8.51 (d, $J=8.23$ Hz, 1 H), 9.44 (s, 1H). HRMS (ESI, m/z) calcd for $C_{27}H_{37}N_7O_6Na$ $[M+Na]^+$ 578.2703. found: 578.2711.

4.1.70. Benzyl ((3S,6S,9S,Z)-6-(cyclohexylmethyl)-9-formyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclohexadecaphane-3-yl)carbamate 4. White solid (96 mg; 49% yield); M.p 111-113 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.73 - 0.95 (m, 2 H), 1.02 - 1.26 (m, 3 H), 1.30 - 1.53 (m, 3 H), 1.54 - 1.79 (m, 6 H), 1.83 - 1.93 (m, 1 H), 1.98 - 2.29 (m, 4 H), 2.66 (m, 1 H), 3.06 (m, 2 H), 3.20 - 3.31 (m, 1 H), 4.13 (dt, $J=13.67$, 6.44 Hz, 1 H), 4.22 - 4.43 (m, 4 H), 4.94 - 5.11 (m, 2 H), 7.07 (d, $J=7.79$ Hz, 1 H), 7.25 - 7.41 (m, 5 H), 7.46 - 7.58 (m, 2 H), 8.13 (d, $J=7.41$ Hz, 1 H),, 8.51 (d, $J=8.22$ Hz, 1 H) 9.5 (s, 1H). HRMS (ESI, m/z) calcd $C_{30}H_{41}N_7O_6Na$ $[M+Na]^+$ 618.3016. found: 618.3016.

4.1.71. Benzyl ((3S,6S,9S,Z)-9-formyl-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacycloheptadecaphane-3-yl)carbamate 5. White solid (110 mg; 53% yield); M.p 110-112 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.89 (dd, $J=12.55$, 6.42 Hz, 6 H), 1.15 - 1.33 (m, 2 H), 1.36 - 1.56 (m, 3 H), 1.58 - 1.84 (m, 4 H), 2.03 - 2.33 (m, 3 H), 2.71 - 2.74 (m, 1 H), 2.98 - 3.16 (m, 2 H), 4.10 - 4.32 (m, 3 H), 4.32 - 4.45 (m, 2 H), 4.92 - 5.17 (m, 2 H), 6.93 (d, $J=7.88$ Hz, 1 H), 7.25 - 7.41 (m, 5 H), 7.67 - 7.82 (m, 2 H), 8.25 (d, $J=7.43$ Hz, 1 H), 8.35 (d, $J=7.80$ Hz, 1 H), 9.45 (s, 1H). HRMS (ESI, m/z) calcd for $C_{28}H_{39}N_7O_6Na$ $[M + Na]^+$ 570.3040. found: 570.3040.

4.1.72. Benzyl ((3S,6S,9S,Z)-6-(cyclohexylmethyl)-9-formyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacycloheptadecaphane-3-yl)carbamate 6. White solid (101

mg; 50% yield); M.p 119-122 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.75 - 0.99 (m, 2 H), 1.01 - 1.27 (m, 4 H), 1.42 (m, 3 H), 1.50 - 1.81 (m, 10 H), 2.04 - 2.31 (m, 3 H), 2.65 - 2.79 (m, 1 H), 2.95 - 3.16 (m, 2 H), 4.10 - 4.20 (m, 1 H), 4.22 - 4.31 (m, 2 H), 4.31 - 4.48 (m, 2 H), 4.93 - 5.14 (m, 2 H), 7.02 (d, *J*=7.41 Hz, 1 H), 7.25 - 7.47 (m, 5 H), 7.76 (s, 2 H), 8.24 (d, *J*=7.44 Hz, 1 H), 8.33 (d, *J*=7.78 Hz, 1 H), 9.51 (s, 1H). HRMS (ESI, *m/z*) calcd for C₃₁H₄₃N₇O₆Na [M+Na]⁺ 632.3173. found: 632.3182.

4.1.73. Benzyl ((3S,6S,9S,Z)-9-formyl-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclooctadecaphane-3-yl)carbamate 7. White solid (80 mg; 42% yield); M.p 126-127 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.75 - 0.95 (m, 6 H), 1.06 - 1.24 (m, 2 H), 1.28 - 1.56 (m, 4 H), 1.58 - 1.82 (m, 3 H), 1.85-1.93 (m, 1 H), 1.98 - 2.06 (m, 1 H), 2.07 - 2.22 (m, 2 H), 2.88 - 3.16 (m, 4 H), 4.13 (q, *J*=6.77 Hz, 1 H), 4.16 - 4.25 (m, 1 H), 4.26 - 4.41 (m, 3 H), 4.92 - 5.15 (m, 2 H), 7.22 (d, *J*=7.44 Hz, 1 H), 7.28 - 7.43 (m, 5 H), 7.55 - 7.74 (m, 2 H), 8.20 (d, *J*=7.82 Hz, 1 H), 8.60 (d, *J*=6.22 Hz, 1 H), 9.5 (s, 1H). HRMS (ESI, *m/z*) calcd for C₂₉H₄₁N₇O₆Na [M+Na]⁺ 606.3016. found: 606.2996.

4.1.74. Benzyl ((3S,6S,9S,Z)-6-(cyclohexylmethyl)-9-formyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclooctadecaphane-3-yl)carbamate 8. White solid (92 mg; 47% yield); M.p 119-121 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.71 - 0.96 (m, 2 H), 1.03 - 1.27 (m, 5 H), 1.32 - 1.48 (m, 4 H), 1.47 - 1.85 (m, 8 H), 1.86 - 1.92 (m, 1 H), 1.96 - 2.06 (m, 1 H), 2.10 - 2.18 (m, 2 H), 2.91 - 3.18 (m, 4 H), 4.07 - 4.16 (m, 1 H), 4.16 - 4.24 (m, 1 H), 4.24 - 4.47 (m, 3 H), 4.93 - 5.17 (m, 2 H), 7.25 - 7.47 (m, 5 H), 7.59 - 7.79 (m, 2 H), 8.19 (d, *J*=7.83 Hz, 1 H), 8.55 (d, *J*=6.62 Hz, 1 H), 9.5 (s, 1H). HRMS (ESI, *m/z*) calcd for C₃₂H₄₉N₇O₆ [M+H]⁺ 624.3510. found: 624.3508.

4.1.75 Benzyl ((3S,6S,9S,Z)-9-formyl-6-isobutyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclononadecaphane-3-yl)carbamate 9. White solid (105 mg; 51% yield); M.p 118-120 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.78 – 0.91 (m, 6 H), 1.23 (d, *J*=10.16 Hz, 4 H), 1.35 (br. s., 2 H), 1.42 - 1.54 (m, 2 H), 1.58 - 1.71 (m, 1 H), 1.71 - 1.85 (m, 2 H), 1.91 – 1.96 (m, 2 H), 2.04 - 2.16 (m, 1 H), 2.16 - 2.26 (m, 1 H), 2.92 - 3.21 (m, 4 H), 4.02 – 4.08 (m, 1 H), 4.16 - 4.28 (m, 2 H), 4.29 - 4.39 (m, 2 H), 4.90 - 5.16 (m, 2 H), 7.11 (d, *J*=7.76 Hz, 1 H), 7.27 - 7.43 (m, 5 H), 7.65 - 7.78 (m, 2 H), 8.18 (d, *J*=8.24 Hz, 1 H), 8.51 (d, *J*=5.85 Hz, 1 H), 9.5 (s, 1H). HRMS (ESI, *m/z*) calcd for C₃₀H₄₃N₇O₆Na [M+Na]⁺ 620.3173. found: 620.3164.

4.1.76. Benzyl ((3S,6S,9S,Z)-6-(cyclohexylmethyl)-9-formyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclononadecaphane-3-yl)carbamate 10. White solid (98 mg; 50% yield); M.p 120-121 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 0.71 - 0.95 (m, 2 H), 1.04 - 1.31 (m, 7 H), 1.31 - 1.46 (m, 4 H), 1.47 - 1.74 (m, 6 H), 1.74 - 1.82 (m, 2 H), 1.92 - 1.99 (m, 2 H), 2.01 – 2.09 (m, 1 H), 2.14 - 2.28 (m, 1 H), 2.91-3.10 (m, 4 H), 4.01 – 4.10 (m, 1 H), 4.22 (t, *J*=7.06 Hz, 2 H), 4.27 - 4.45 (m, 2 H), 5.02 (s, 2 H), 7.19 (d, *J*=7.66 Hz, 1 H), 7.25 - 7.44 (m, 5 H), 7.65 - 7.79 (m, 2 H), 8.17 (d, *J*=7.62 Hz, 1 H), 8.46 (d, *J*=6.14 Hz, 1 H), 9.5 (s, 1H); HRMS (ESI, *m/z*) calcd for C₃₃H₄₇N₇O₆Na [M+Na]⁺ 660.3486. found: 660.3475.

4.1.77. Benzyl ((3S,6S,9S,Z)-6-benzyl-9-formyl-4,7,12-trioxo-1¹H-5,8,13-triaza-1(4,1)-triazolacyclononadecaphane-3-yl)carbamate 11. White solid (91 mg; 48% yield); M.p 132-134 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 1.12 - 1.31 (m, 5 H), 1.31 – 1.39 (m, 2 H), 1.49 – 1.53 (m, 1 H), 1.65 - 1.87 (m, 2 H), 1.96 (d, *J*=5.46 Hz, 1 H), 2.07 - 2.18 (m, 1 H), 2.18 - 2.28 (m, 1 H), 2.78 - 2.83 (m, 1 H), 2.95 - 3.16 (m, 4 H), 4.02 –

4.09 (m, 1 H), 4.52 (d, $J=5.87$ Hz, 1 H), 4.96 - 5.11 (m, 2 H), 6.96 (d, $J=7.85$ Hz, 1 H), 7.12 - 7.48 (m, 10 H), 7.68 (s, 1 H), 7.76 (br. s., 1 H), 8.26 (d, $J=7.79$ Hz, 1 H), 8.67 (d, $J=5.85$ Hz, 1 H), 9.5 (s, 1H). HRMS (ESI, m/z) calcd for $C_{33}H_{40}N_7O_6$ $[M-H]^+$ 630.3040. found: 630.3040.

4.1.78. Benzyl ((7S,10S,Z)-7-formyl-10-isobutyl-4,9,12-trioxo-1¹H-3,8,11-triaza-1(4,1)-triazolacyclohexadecaphane-13-yl)carbamate 12. White solid (92 mg; 48% yield); M.p 118-121 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.84 - 0.88 (m, 6 H), 1.42 (d, $J=6.42$ Hz, 2 H), 1.49 - 1.72 (m, 5 H), 1.82 (br. s., 1 H), 1.91 - 1.93 (m, 2 H), 2.10 - 2.26 (m, 2 H) 3.76 - 3.88 (m, 2 H), 4.01-4.05 (m, 2 H), 4.16 (d, $J=5.84$ Hz, 1 H), 4.31 - 4.33 (m, 1 H), 5.04 (s, 2 H), 7.24 - 7.42 (m, 5 H), 7.40 (d, $J=7.66$ Hz, 1 H), 7.98 (d, $J=7.91$ Hz, 1 H), 8.21 - 8.24 (m, 2 H), 8.32 (d, $J=7.11$ Hz, 1 H) 9.49 (s, 1H). HRMS (ESI, m/z) calcd for $C_{27}H_{38}N_7O_6$ $[M+H]^+$ 556.2884. found: 556.2872.

4.1.79. Benzyl ((S)-1-(((S)-1-(((S)-5-((3-azidopropyl)amino)-1,5-dioxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-1-oxopent-4-yn-2-yl)carbamate 34. White solid (112 mg; 55% yield); M.p 60-62 °C. ¹H NMR (400 MHz, DMSO- d_6): δ 0.85 (dd, $J=14.98, 5.91$ Hz, 6 H), 1.41-1.49 (m, 2 H), 1.58-1.64 (m, 3 H), 1.76 - 1.86 (m, 2 H), 1.88 - 2.02 (m, 2 H), 2.07 - 2.24 (m, 2 H), 2.35 - 2.47 (m, 1 H), 2.51 - 2.64 (m, 1 H), 2.83 (br. s., 1 H), 3.01 - 3.12 (m, 2 H), 4.11 - 4.25 (m, 2 H), 4.32 (d, $J=7.05$ Hz, 1 H), 5.08 (s, 2 H), 7.21 - 7.45 (m, 5 H), 7.62 (d, $J=7.62$ Hz, 1 H), 7.82 (br. s., 1 H), 8.00 (d, $J=7.76$ Hz, 1 H), 8.28 (d, $J=7.44$ Hz, 1 H), 9.51 (s, 1H). HRMS (ESI, m/z) calcd for $C_{27}H_{38}N_7O_6$ $[M+H]^+$ 556.2884. found: 556.2886.

4.2. Enzyme assays and inhibition studies. The IC_{50} (50% inhibitory concentration in enzyme assay) values of protease inhibitors against 3CLpro were determined as described previously by our lab¹³. Each compound was evaluated for anti-3CLpro effects up to 100 μ M. For the cell based assay, one day old HG23 cells (NV replicon harboring cells) were treated with various concentrations (1.0 to 100 μ M) of each compound, and incubated for 48 h. Then total RNA was extracted from cells for qRT-PCR for norovirus and β -actin. The EC_{50} (50% inhibitory concentration in cell based assay) of each compound was calculated by the % reduction of RNA levels to Mock-treated cells after normalized with β -actin levels. Cell cytotoxicity using HG23 was measured by a CytoTox96® nonradioactive cytotoxicity assay kit (Promega, Madison, WI) with serial dilution of each compound up to 100 μ M following the manufacturer's instructions. The CC_{50} (50% cytotoxic concentration) was determined for each compound. The IC_{50} , EC_{50} and CC_{50} values were determined by at least two independent experiments.

4.3. X-ray crystallographic studies. Crystallization and data collection. Purified Norwalk virus 3CLpro (NV 3CLpro) in 100 mM NaCl, 50 mM PBS pH 7.2, 1 mM DTT at a concentration of 10 mg/mL was used for preparation of the NV 3CLpro-ligand complex. A stock solution of 100 mM compound **1**, **3**, or **10** was prepared in DMSO and each NV 3CLpro:inhibitor complex was prepared by mixing 12 μ L of **1**, **3** or **10** (3 mM) with 388 μ L (0.49 mM) of NV 3CLpro and incubating on ice for 1 hour. The buffer was exchanged to 100 mM NaCl, 20 mM Tris pH 8.0 using a Vivaspinn-20 concentrator (MWCO=5kDa, Vivaproducts, Inc.) and the samples were concentrated to 10, 8.4 and 12.0 mg/mL respectively for crystallization screening. All crystallization experiments

were conducted Compact Jr. (Rigaku Reagents) sitting drop vapor diffusion plates at 20 °C using equal volumes of protein and crystallization solution equilibrated against 75 µL of the latter. Crystals of NV 3CLpro-ligand **1** and NV 3CLpro-ligand **10** that displayed prismatic morphology were obtained in 1-2 days from the Index HT screen (Hampton Research) condition H12 (30% (w/v) PEG 2000 MME, 150 mM potassium bromide) (Table 2). Samples were transferred to a fresh drop composed of 80% crystallization solution and 20% glycerol and stored in liquid nitrogen. Crystals that displayed a plate morphology formed for NV 3CLpro-ligand **3** in 3 days from the Wizard 3 and 4 screen (Rigaku Reagents) A10 (20% (w/v) PEG3350, 200 mM sodium thiocyanate). The crystals tended to form clusters that needed to be separated in order to obtain a suitable sample for X-ray data collection. Samples were transferred to a fresh drop composed of 80% crystallization solution and 20% PEG 200 and stored in liquid nitrogen. X-ray diffraction data were collected at the Advanced Photon Source beamline 17-ID using a Dectris Pilatus 6M pixel array detector.

4.4. Structure Solution and Refinement. Intensities were integrated using XDS³⁶⁻³⁷ and the Laue class analysis and data scaling were performed with Aimless³⁸ which suggested that the highest probability Laue class was $2/m$ and space group $C2$ for NV CLpro:**1** and NV 3CLpro:**10**. For NV 3CLpro:**3**, the highest probability space group was $P1$. The Matthew's coefficient³⁹ suggested that there was a single molecule in the asymmetric unit ($V_m=1.8 \text{ \AA}^3/\text{Da}$, % solvent=32%) for NV 3CLPro:**1**, NV 3CLpro:**10** and two molecules for NV 3CLPro:3 ($V_m=2.2 \text{ \AA}^3/\text{Da}$, % solvent=45%). Structure solution was conducted by molecular replacement with Phaser⁴⁰ using a previously determined structure of inhibitor bound NV 3CLpro (PDB: 3UR9¹³) as the search model and for NV

3CLpro:**3**, a single solution was obtained for two independent molecules in the asymmetric unit. Structure refinement using and manual model building were conducted with Phenix⁴¹ and Coot⁴² respectively. Anisotropic atomic displacement parameters were refined for all atoms except solvent molecules for NV 3CLPro:**1** and NV 3CLpro:**10**. Disordered side chains were truncated to the point for which electron density could be observed. Structure validation was conducted with Molprobit⁴³ and figures were prepared using the CCP4MG package⁴⁴ and PyMOL⁴⁵.

4.5. NMR studies. The spectral details, 2D and variable temperature (VT) NMR spectra were taken by a Varian XL-400 MHz NMR spectrometer and sample was prepared by dissolving 30 mg of compound in 1 mL of DMSO-*d*₆. Assignment of NH and CH α resonances were done using 1D ¹H NMR, COSY, ROESY and HMBC spectral data recorded at 273 K. Temperature dependence of amide NH chemical shifts ($\Delta\delta/T$) were examined within the temperature range of 298-343 K in 5 degree increments.

4.6. Accession Codes

Coordinates and structure factors were deposited to the Worldwide Protein Databank (wwPDB) with the accession codes NV 3CLpro:1 (5E0G), NV 3CLpro:3 (5E0H) and NV 3CLpro:10 (5E0J).

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Supplementary Data

Supplementary data related to this article can be found at

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LEGENDS

Figure 1. General structures of macrocyclic inhibitor (I).

Figure 2. A) Schematic representation of antiparallel β -sheet like hydrogen bonding between NV 3CLpro and the tripeptidyl inhibitor. B) Macrocyclization of the tripeptidyl inhibitor by connecting the P₁ and P₃ residues.

Figure 3. Hydrogen bonding interactions derived from co-crystal structures of three inhibitors bound to NV 3CLpro. A) NV 3CLpro:**1** complex. B) NV 3CLpro:**3** complex. C) NV 3CLpro:**10** complex.

Figure 4. F_o-F_c electron density maps contoured at 3 σ for three inhibitors (left) and same inhibitors colored according to *B*-factors (right) (minimum=10, maximum=50). A) NV3CLpro:**10** complex. B) NV 3CLpro:**3** complex. C) NV 3CLpro:**1** complex.

Figure 5. Comparison of the high-resolution X-ray crystal structures of the NV 3CLpro:**10** (orange) and NV 3CLpro:**1** (magenta) complexes. Inhibitors **10** and **1** are colored yellow and cyan respectively. **A)** Superposition NV 3CLpro:**10** and NV 3CLpro:**1** highlighting the differences in the region spanning Thr 134 to His 157. The arrows indicate that conformation differences in Thr 134/Pro 136 in the flexible loop region. **B** and **C)** Differences in hydrogen bond/close contacts (dashed lines) in the flexible loop region for 3CLpro:**10** and NV 3CLpro:**1** respectively.

TABLE 1

Compound	R	m	n	Ring size	IC ₅₀ (μ M)	EC ₅₀ (μ M)	CC ₅₀ (μ M)
1	Isobutyl	1	2	17	1.6	53.2	> 100
2	Cyclohexyl	1	2	17	2.9	25.5	> 100
3	Isobutyl	1	3	18	2.4	81.5	> 100
4	Cyclohexyl	1	3	18	1.6	16.1	> 100
5	Isobutyl	1	4	19	6.4	46.3	> 100
6	Cyclohexyl	1	4	19	1.4	30.5	> 100
7	Isobutyl	1	5	20	3.7	6.2	> 100
8	Cyclohexyl	1	5	20	24.5	3.8	> 100
9	Isobutyl	1	6	21	4.1	42.1	> 100
10	Cyclohexyl	1	6	21	6.1	88.3	> 100
11	Phenyl	1	6	21	36.5	29.5	> 100

TABLE 2

	NV 3CLpro:1	NV 3CLpro:3	NV 3CLpro:10
Data Collection			
Unit-cell parameters (Å, °)	$a=65.84, b=39.28, c=61.32, \beta=118.7$	$a=38.10, b=48.77, c=53.08, \alpha=94.0, \beta=109.5, \gamma=101.3$	$a=66.67, b=37.16, c=61.86, \beta=110.0$
Space group	C2	P1	C2
Resolution (Å) ¹	33.24-1.20 (1.22-1.20)	37.01-1.95 (2.00-1.95)	32.63-1.20 (1.22-1.20)
Wavelength (Å)	1.0000	1.0000	1.0000
Temperature (K)	100	100	100
Observed reflections	147,835	43,724	264,169
Unique reflections	44,826	24,256	42,941
$\langle I/\sigma(I) \rangle$ ¹	14.1 (2.1)	8.0 (1.7)	16.3 (1.9)
Completeness (%) ¹	96.7 (94.2)	95.2 (94.0)	96.5 (93.7)
Multiplicity ¹	3.3 (3.2)	1.8 (1.8)	5.7 (3.6)
R_{merge} (%) ^{1, 2}	3.7 (58.0)	4.6 (44.2)	5.8 (78.7)
R_{meas} (%) ^{1, 4}	4.4 (69.4)	6.5 (62.5)	6.3 (92.4)
R_{pim} (%) ^{1, 4}	2.4 (37.7)	4.6 (44.2)	2.5 (47.6)
$CC_{1/2}$ ^{1, 5}	0.999 (0.758)	0.997 (0.755)	0.999 (0.688)
Refinement			
Resolution (Å) ¹	31.18-1.20	37.01-1.95	31.96-1.20
Reflections (working/test) ¹	42,608/2,205	22,998/1,244	40,906/2,020
$R_{\text{factor}} / R_{\text{free}}$ (%) ^{1, 3}	13.8/16.1	18.3/21.9	13.7/16.5
No. of atoms (Protein/Ligand/Water)	1,292/32/165	2,395/33/94	1,295/46/170
Model Quality			
R.m.s deviations			
Bond lengths (Å)	0.010	0.011	0.009
Bond angles (°)	1.109	1.136	1.087
Average B-factor (Å ²)			
All Atoms	20.1	38.6	19.0
Protein	18.5	38.6	17.4
Ligand	24.1	38.3	22.7
Water	32.3	38.9	30.5
Coordinate error(maximum likelihood) (Å)	0.12	0.21	0.10
Ramachandran Plot			
Most favored (%)	98.9	97.5	97.2
Additionally allowed (%)	1.1	2.5	2.8

1) Values in parenthesis are for the highest resolution shell.

2) $R_{\text{merge}} = \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity measured for the i th reflection and $\langle I(hkl) \rangle$ is the average intensity of all reflections with indices hkl

- 3) $R_{\text{factor}} = \sum_{hkl} ||F_{\text{obs}}(hkl) - |F_{\text{calc}}(hkl)|| / \sum_{hkl} |F_{\text{obs}}(hkl)|$; R_{free} is calculated in an identical manner using 5% of randomly selected reflections that were not included in the refinement.
- 4) R_{meas} = redundancy-independent (multiplicity-weighted) R_{merge} .⁴⁶ R_{pim} = precision-indicating (multiplicity-weighted) R_{merge} .⁴⁷⁻⁴⁸
- 5) $CC_{1/2}$ is the correlation coefficient of the mean intensities between two random half-sets of data.⁴⁹⁻⁵⁰

TABLE 3

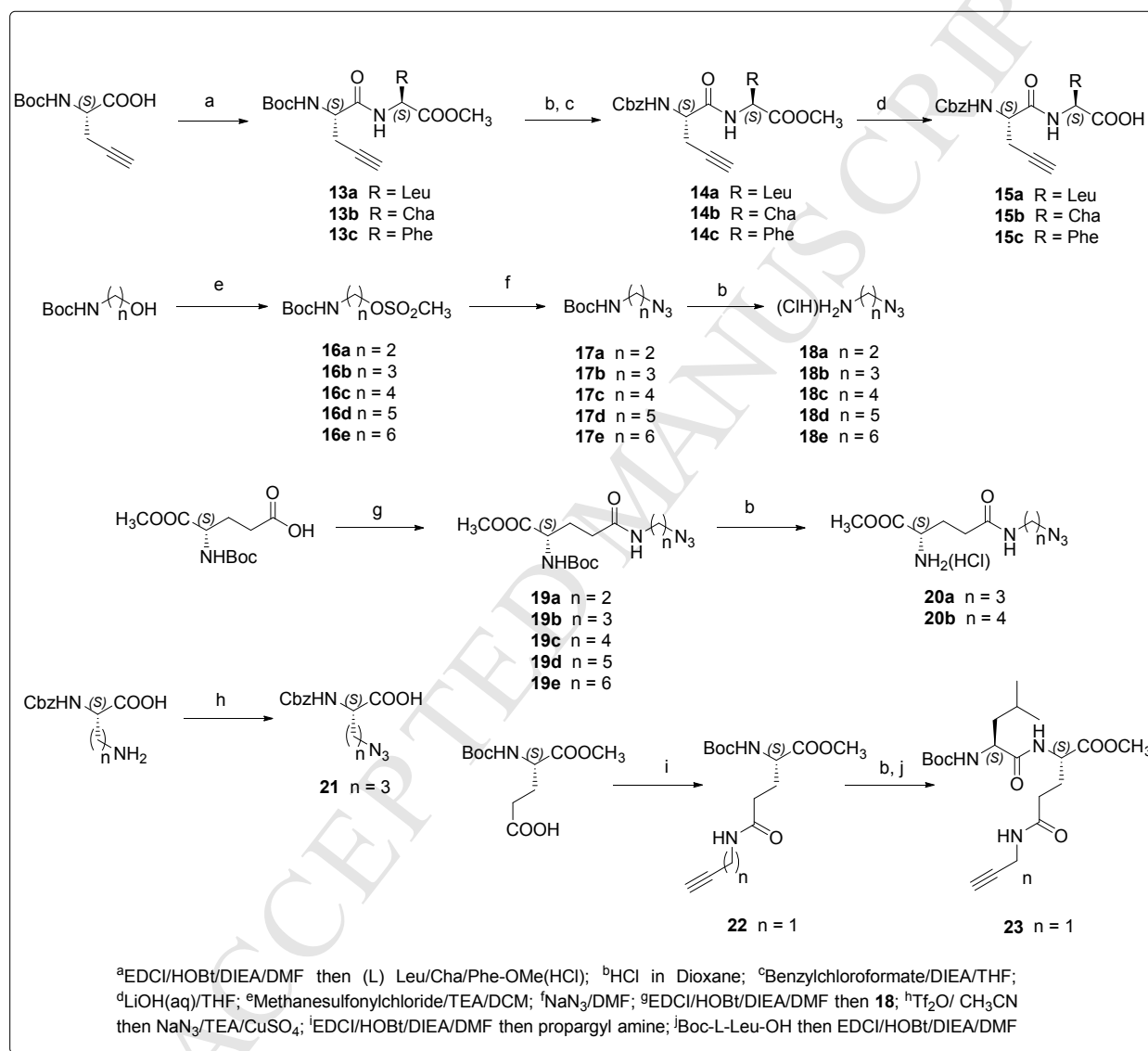
<i>Compound</i>	Ring Size	Temperature dependence of NH ($\Delta\delta/T$ ppb/K)		
		Glutamine (P ₁)	Leucine (P ₂)	Carbamate (P ₃)
28a	17	-4.5	-4.9	-4.2
28e	20	-5.5	-5.0	-7.0
28g	21	-4.5	-5.7	-6.4

TABLE 4

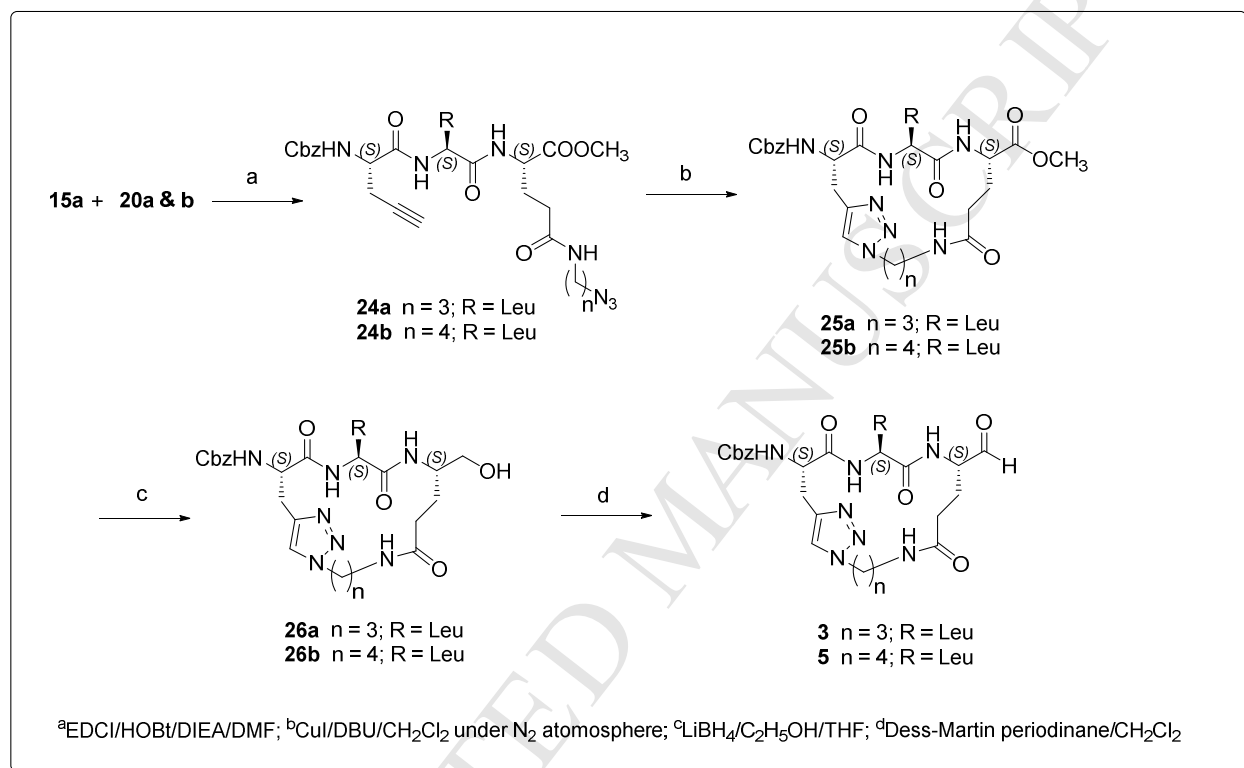
Compound	$^3J_{\text{NH-CH}\alpha}$ (Hz)				Ring size	β -strand ^a	IC ₅₀ (μM) ^b
	P ₁	P ₂		P ₃			
	Gln	Leu	Cha	Cab			
28a	9.76	4.69	-	8.20	17	Yes	1.2
28b	9.46	-	4.41	8.60	17	Yes	2.9
25a	8.20	7.42	-	7.03	18	No	2.4
28c	8.20	-	7.42	7.81	18	No	1.6
25b	7.81	7.42	-	7.81	19	No	6.4
28d	7.42	-	7.72	7.81	19	No	1.4
28e	7.42	7.81	-	8.6	20	No	3.7
28f	6.64	-	7.81	ND ^c	20	No	24.5
28g	7.81	8.2	-	5.86	21	No	4.1
28h	7.63	-	7.63	6.1	21	No	6.1

^a β -strand conformation with respect to P₁ and P₃ residues; ^bIC₅₀ values of corresponding aldehydes of the precursor esters; ^cNot detected due to overlapping resonances.

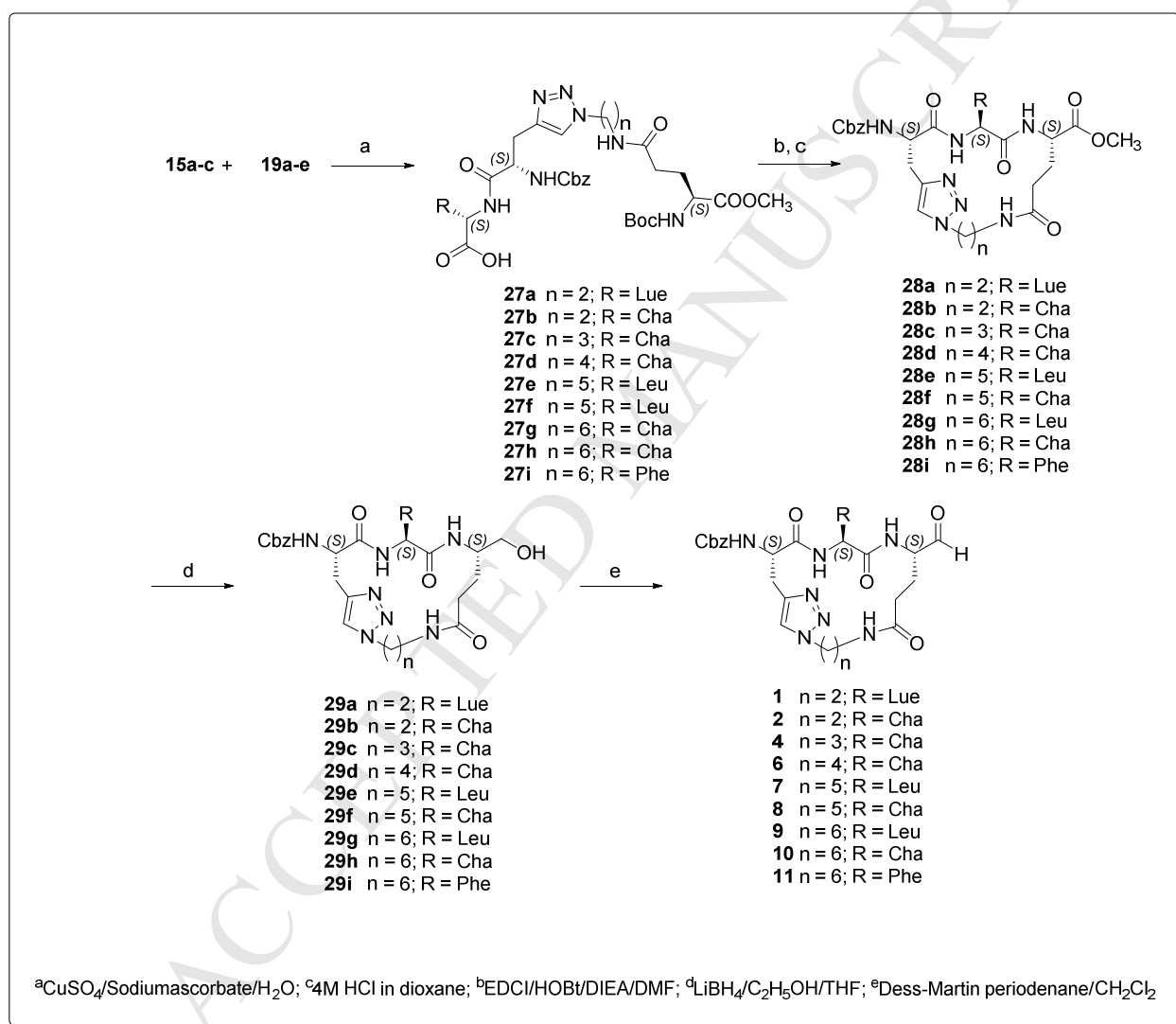
SCHEME 1



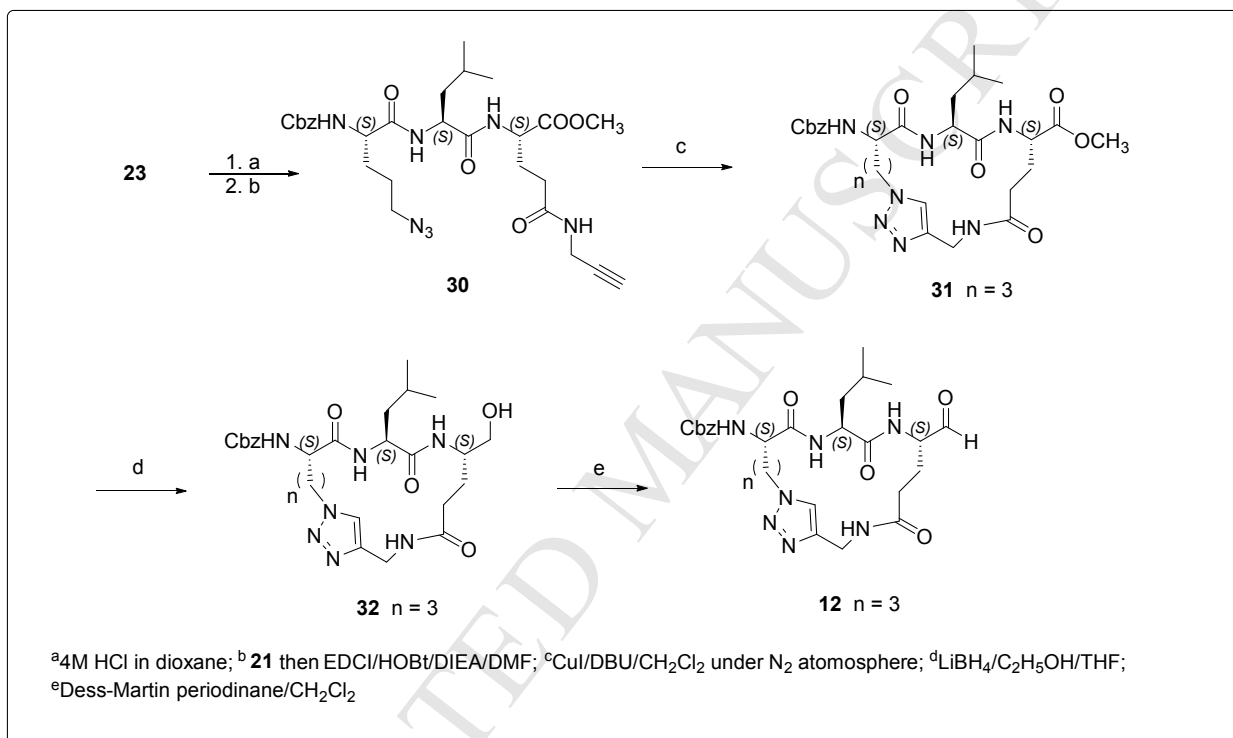
SCHEME 2



SCHEME 3



SCHEME 4



SCHEME 5

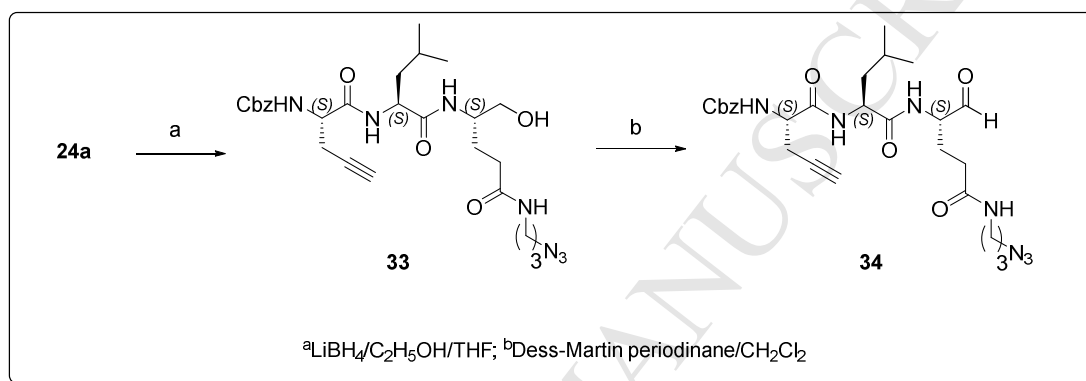
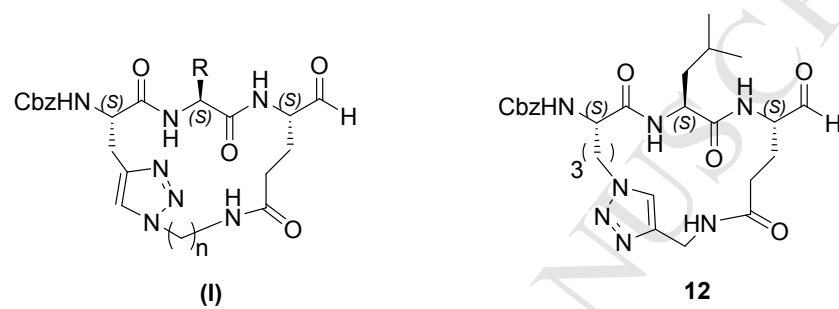


FIGURE 1



- 1** $n = 2$ $R = \text{Leu}$
2 $n = 2$ $R = \text{Cha}$
3 $n = 3$ $R = \text{Leu}$
4 $n = 3$ $R = \text{Cha}$
5 $n = 4$ $R = \text{Leu}$
6 $n = 4$ $R = \text{Cha}$
7 $n = 5$ $R = \text{Leu}$
8 $n = 5$ $R = \text{Cha}$
9 $n = 6$ $R = \text{Leu}$
10 $n = 6$ $R = \text{Cha}$
11 $n = 6$ $R = \text{Phe}$

FIGURE 2

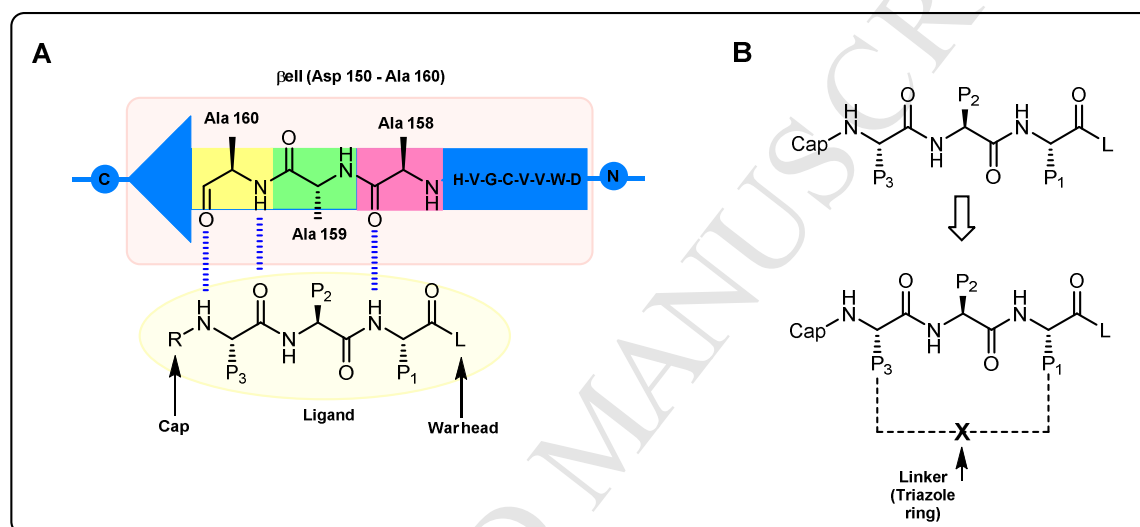
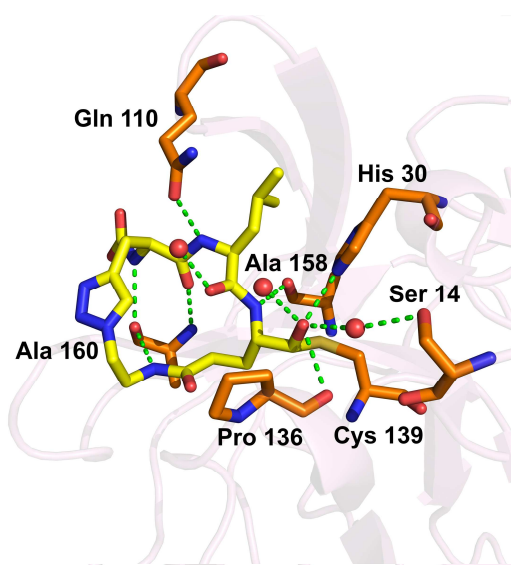
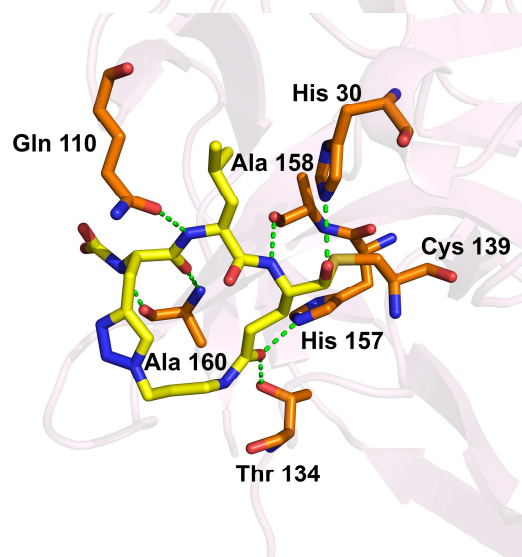


FIGURE 3

A



B



C

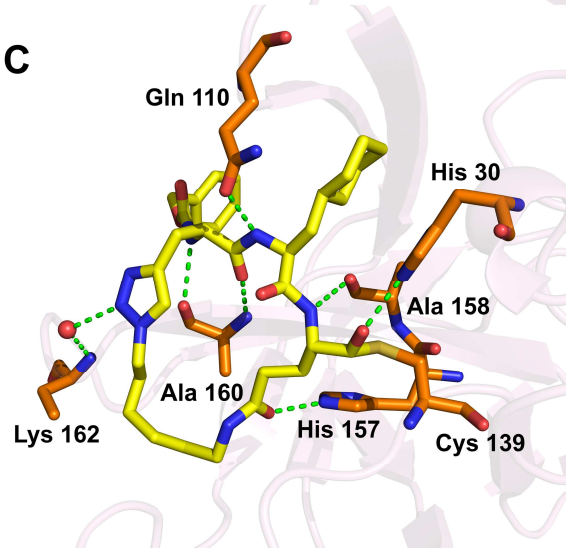


FIGURE 4

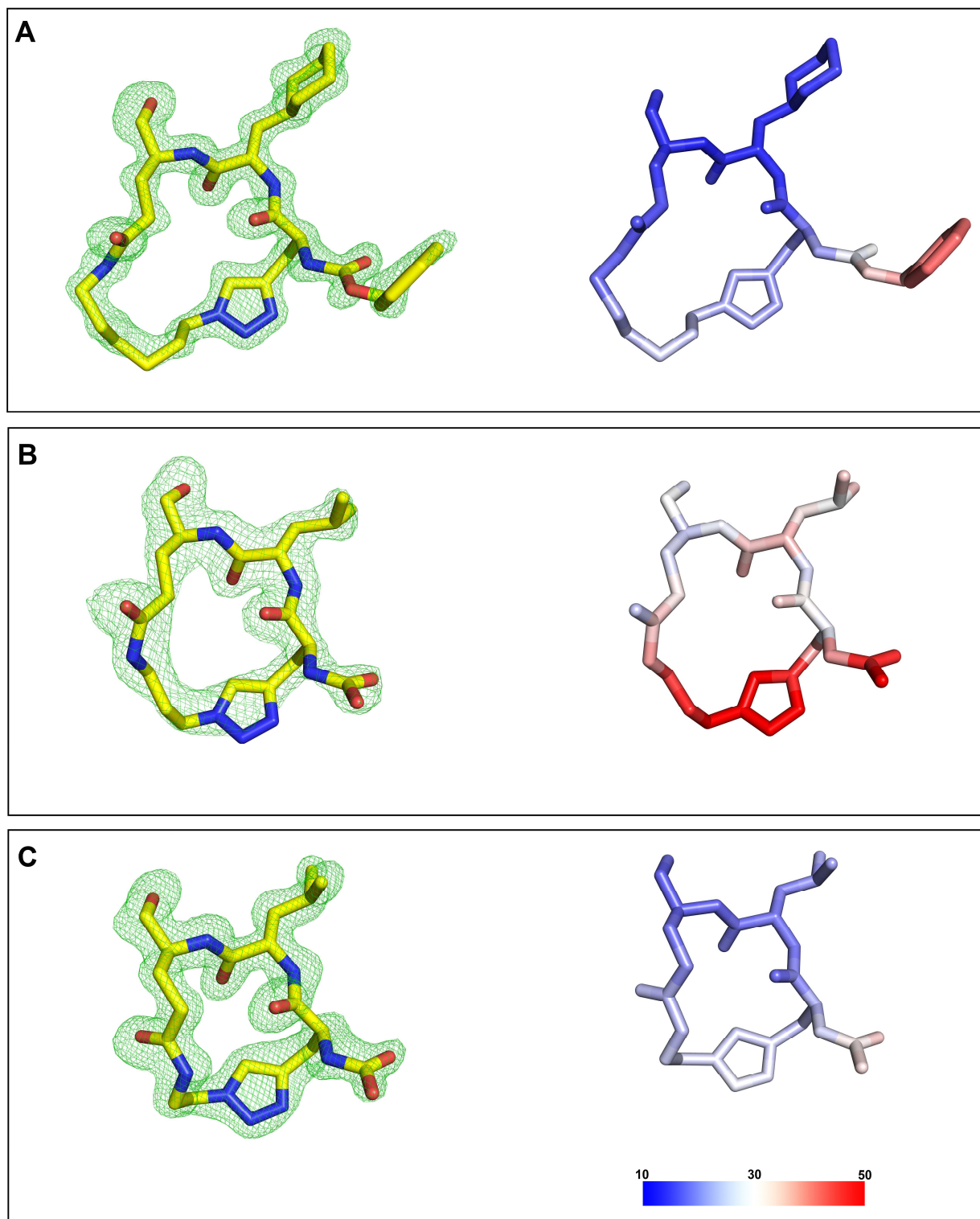
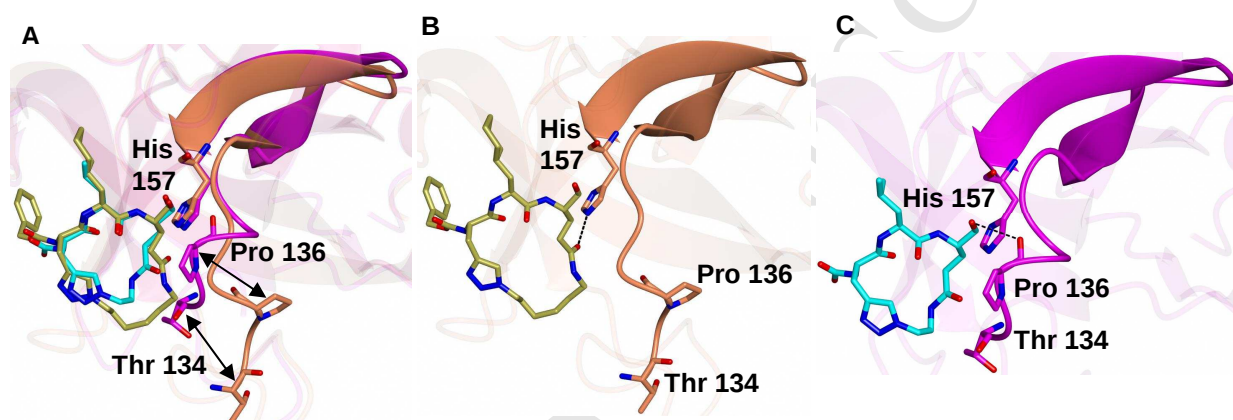


FIGURE 5



Highlights

- Novel triazole-based macrocyclic inhibitors of norovirus 3CL protease were synthesized.
- The interplay of conformation and activity was probed using NMR and X-ray crystallography.
- Bound inhibitors assume a β -strand conformation according to X-ray crystal structure
- Loss of critical hydrogen bonding interactions was revealed by X-ray crystallography.