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Synthesis and activity of tetrapeptidic HTLV-I protease inhibitors possessing different P_3-cap moieties

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Abstract—The causative agent behind adult T-cell leukemia and tropical spastic paraparesis/HTLV-I-associated myelopathy is the human T-cell leukemia virus type 1 (HTLV-I). Tetrapeptidic HTLV-I protease inhibitors were designed on a previously reported potent inhibitor KNI-10516, with modifications at the P_3-cap moieties. All the inhibitors showed high HIV-1 protease inhibitory activity (over 98% inhibition at 50 nM) and most exhibited highly potent inhibition against HTLV-I protease (IC_{50} values were less than 100 nM).

1. Introduction

Human T-cell leukemia virus type 1 (HTLV-I) is the etiologic agent for adult T-cell leukemia (ATL) and other chronic inflammatory diseases, such as tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM) and HTLV-I-associated uveitis.[1] HTLV-I causes ATL, an invariably fatal expansion of virus-infected CD4+ T cells, in 2–6% of infected individuals after a long period of 20–50 years.[2] HTLV-I infections are endemic in high-risk groups in South Japan, the Caribbean Basin, South America, Central and West Africa, the Middle East, and the Pacific Region.[3] Currently, it is estimated that 20–30 million people worldwide are infected with HTLV-I.[4] All routes of HTLV-I virus transmission require close contact with infected T-lymphocytes. Three main routes of HTLV-I transmission have been recognized, including transmission from mother to child through breast-feeding, sexual, and blood–borne transmissions. In Japan, where 15–25% individuals are carriers, the infection is spread mainly via breast milk.[5,6] The prognosis of ATL patients is poor with a median survival time of 13 months in aggressive cases.[7] The poor prognosis for ATL patients is partly due to the innate resistance of HTLV-I-infected T-cells to apoptosis and thus to conventional chemotherapy regimens.[8] At the present time, there is no effective treatment for ATL and HTLV-I infection. Therefore, curative therapies against ATL are urgently needed.

HTLV-I was the first human retrovirus to be identified and isolated in the early 1980s from patients with ATL.[9,10] In common with other retroviruses, HTLV-I encodes a virus-specific aspartic protease responsible for the proliferation of the retrovirus. HTLV-I protease is a small homodimer composed of two identical subunits containing 125 amino acids each.[11–13] As in most human retroviruses, there are three large open reading frames in the genome of HTLV-I that encode for the Gag (48 kDa), Pol (99 kDa), and Env (54 kDa) polyproteins, and a number of spliced, open reading frames that encode for short regulatory proteins (Rex, Tax, etc.). HTLV-I proteolytic cleavage of the HTLV-I Gag precursor polyprotein yields matrix (MA), capsid (CA) and nucleocapsid (NC) proteins of the mature virion, while the cleavage of the Pol precursor polyprotein affords reverse transcriptase/ribonuclease H (RT–RH) and integrase (IN). Because of its crucial role in viral

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maturation, HTLV-I protease is an attractive target for the development of inhibitors to treat HTLV-I infection.

Although HTLV-I protease is in the same family of aspartic proteases as HIV-1 protease, HTLV-I protease has received little attention compared to its more famous cousin HIV-1 protease that has been extensively studied.[14] Due to 45% sequence similarity between HTLV-I and HIV-1 proteases at the active site, some HIV-1 protease inhibitors had been tested on HTLV-I protease.[15–17] However, differences in the amino acid sequences of HIV-1 and HTLV-I proteases produce differences in susceptibilities to these inhibitors. Consequently, we decided to design HTLV-I protease inhibitors on the sequence of a Gag substrate (**1**) that is processed at the matrix-capsid cleavage site,[18] and synthesized potent octapeptidic HTLV-I protease inhibitor KNI-10161 (**2**, IC_{50} = 159 nM) that possessed (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid (allophenylnorstatine, Apns) with a hydroxymethylcarbonyl isostere as a P_1–P_1' transition-state mimic (Fig. 1).[19] The replacement of the P_1' Pro residue by (*R*)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (Dmt) followed by truncation and natural amino acid substitution studies performed on compound **2** led to a more potent hexapeptidic HTLV-I protease inhibitor KNI-10166 (**3**, IC_{50} = 88 nM).[20] Compound **3** was further refined by non-natural amino acid isostere substitution with L-(+)-α-phenylglycine (Phg) and L-*tert*-leucine (Tle), and by a truncation study to uncover pentapeptidic inhibitor KNI-10247 (**4**, IC_{50} = 144 nM).[21] The replacement of the P_2' Ile residue by a P_1'-cap moiety increased HTLV-I protease inhibitory activity in tetrapeptidic inhibitor KNI-10516 (**5a**, IC_{50} = 107 nM). In our previous publications, we attempted to minimize the size of HTLV-I protease inhibitors to improve the chance for cellular penetration, and reduce the peptidic nature of the inhibitors so as to avoid premature digestion by other proteases. Thus far, the P_3-cap moiety has not been investigated to optimize the efficiency of inhibition. Our past study showed that the replacement of a proline P_4 moiety by an acetyl P_3-cap moiety significantly decreased HTLV-I protease inhibitory activity.[20] In the work described herein, using compound **5a** as a starting reference compound, we explored the significance of the capping moiety of the P_3 residue on HTLV-I protease inhibition.

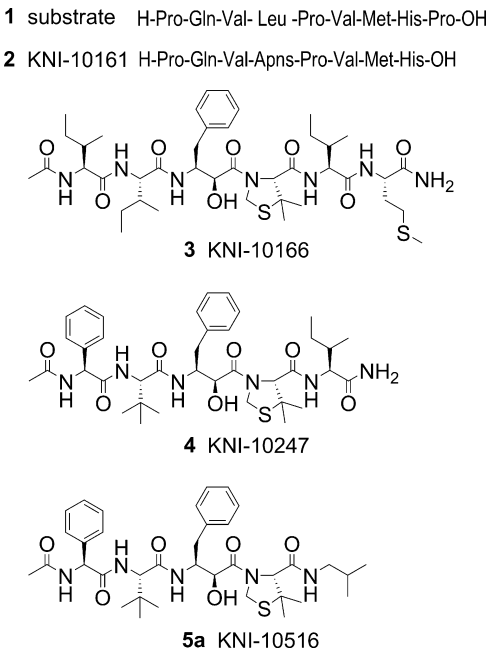


Figure 1. Processive development of potent HTLV-I protease inhibitors, KNI-10161 (**2**), KNI-10166 (**3**), KNI-10247 (**4**) and KNI-10516 (**5a**), from a MA/CA substrate (**1**).

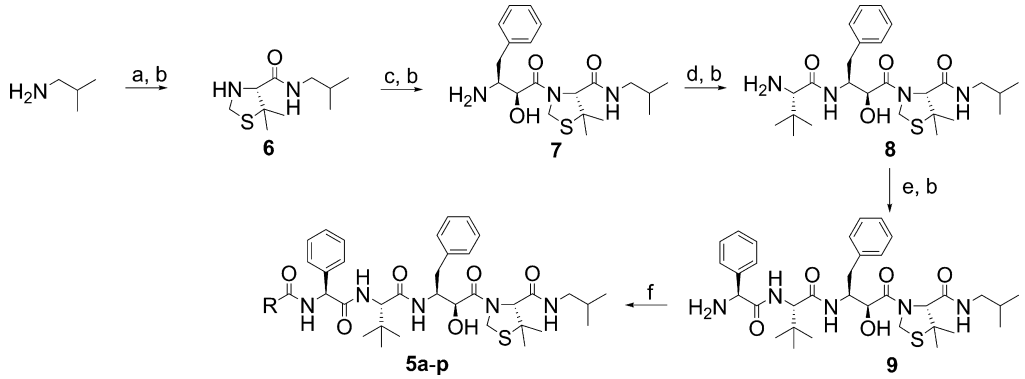
2. Chemistry

The synthesized HTLV-I protease inhibitors **9** and **5a**–**p** were prepared by standard solution phase peptide coupling chemistry (Scheme 1). Isobutylamine was coupled with N^{α}- *tert*-butyloxycarbonyl (*R*)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (Boc-Dmt-OH) using benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) in the presence of triethylamine (TEA), followed by Boc-deprotection with 4 N HCl in dioxane to obtain compound **6**. Compound **7** was prepared by coupling compound **6** and Boc-Apns-OH with BOP. The same deprotection/condensation procedure was repeated for the successive introduction of Boc-Tle-OH and Boc-Phg-OH. In the last step, inhibitors **5a**–**p** were obtained by coupling compound **9** with their respective carboxylic acids. Compounds **5k**–**l** are racemates.

3. Results and discussion

HTLV-I protease inhibition assay was performed using L40I HTLV-I protease mutant as previously reported.[21] Enzymatic assay data and computer assisted docking experiments from our previous studies suggested that the P_3-to-P_1' residues were strong determinants of inhibitory activity against HTLV-I protease.[20,21] In the current study, we described our efforts to further optimize KNI-10516-related compounds by exploring modifications at the P_3-cap position. We were particularly interested in studying the effect of attaching various moieties to the P_3 amide nitrogen atom. An X-ray crystal structure of a truncated, L40I mutant HTLV-I protease in complex with an inhibitor revealed that the S_4 subsite, where the P_3-cap moiety would presumably interact, would accommodate for hydrophobic residues.[22] Interestingly, similar associations had been made for peptidic substrates and the S_4 subsite.[12] In consideration of these facts, we opted to synthesize HTLV-I protease inhibitors that possess hydrophobic P_3-cap moieties. We believed that van der Waals interactions play a key role in the S_4 region of the active site, and that finding the perfect 'fit' would increase inhibitory activity against the enzyme.

Several interesting structure–activity relationships were observed with hydrogen bond interactions, chain length, branching, and bulkiness (Table 1). Most interestingly, the absence of a P_3-cap moiety in compound **9** greatly



Scheme 1. Synthesis of compounds **9** and **5a**–**p**. Reagents and conditions: (a) Boc-Dmt-OH, BOP, TEA, DMF, rt, 15 h; (b) 4 N HCl/dioxane, rt, 1 h; (c) Boc-Apns-OH, BOP, TEA, DMF, rt, 15 h; (d) Boc-Tle-OH, BOP, TEA, DMF, rt, 15 h; (e) Boc-Phg-OH, BOP, TEA, DMF, rt, 15 h; (f) RCOOH, BOP, TEA, DMF, rt, 15 h.

Table 1. Structures and activity of P_3-substituted HTLV-I Protease Inhibitors **9** and **5a**–**p**

Compound		R	HTLV-I protease inhibition, IC_{50} ± SEM (nM)
9	KNI-10673	Not applicable	331.6 ± 0.5
5a	KNI-10516	Me	107.1 ± 0.6
5b	KNI-10571	Et	104.5 ± 0.5
5c	KNI-10570	^{n}Pr	104.3 ± 0.5
5d	KNI-10616	^{n}Bu	92.0 ± 0.5
5e	KNI-10575	^{c}Pr	96.9 ± 0.6
5f	KNI-10574	^{i}Pr	93.5 ± 0.5
5g	KNI-10572	^{t}Bu	82.8 ± 0.6
5h	KNI-10573	^{i}Bu	101.7 ± 0.5
5i	KNI-10633	^{t}Pe	83.3 ± 0.6
5j	KNI-10614	C(Me_2)Et	80.4 ± 1.3
5k	KNI-10615	[a]CH(Me)^{n}Pr	84.2 ± 1.3
5l	KNI-10618	CH_2[a]CH(Me)Et	88.2 ± 0.5
5m	KNI-10617	^{i}Pe	96.5 ± 0.5
5n	KNI-10652	CH_2CH_2Ph	104.8 ± 0.6
5o	KNI-10651	^{c}Hx	98.2 ± 0.5
5p	KNI-10634	Ph	79.2 ± 0.6

[a] Racemate.

reduced the inhibitory activity against HTLV-I protease (cf. **5a**–**p**), because, according to computer-assisted docking experiments, the amide oxygen from the P_3-cap moiety might be involved in hydrogen bond interaction with Leu57A from the flap of the HTLV-I protease (Fig. 2B). We also examined the effect of P_3-cap chain elongation and observed that the inhibitory profiles of compounds **5a**–**d** suggested that HTLV-I protease inhibitory potency might improve proportionally with chain length. Intuitively, we explored branching on the P_3-cap moiety, and according to compounds **5b**,**e**–**g**, methyl branching at the α-carbon next to the amide carbonyl seemed to increase HTLV-I protease inhibitory activity, whereas methyl branching at the β-carbon also showed a trend with increased inhibitory potency as exemplified by compounds **5c**,**h**,**i**. To confirm the hypothesized structure–activity relationships on the P_3-cap moiety, compound **5j** showed a shared benefit of chain length (cf. **5c**) and α-carbon branching (cf. **5g**). From the same line of reasoning, compound **5k** was more potent than compounds **5d** (chain length) and **5f** (α-carbon branching), whereas compound **5l** exhibited higher inhibitory activity than compounds **5d** (chain length) and **5h** (β-carbon branching). Comparing compounds **5f** (α-carbon branching) to **5h** (β-carbon branching) suggested that α-carbon branching was a greater determinant of inhibitory activity than β-carbon branching, which was also confirmed by compounds **5j** (α-carbon branching) and **5i** (β-carbon branching), as well as compounds **5k** (α-carbon branching) and **5l** (β-carbon branching). However, increasing bulkiness at the γ-carbon reduced inhibitory activity against HTLV-I protease, as exemplified by compounds **5d**, **5m** and **5n**. Consequently, α-carbon branching effect was a greater determinant of HTLV-I protease inhibitory activity than β-branching, which in its turn, was greater than γ-branching. Hence, in agreement with the general trend observed with peptidic substrates,[12] we observed, by comparing α-, β- and γ-carbon branching effects, that the distance to the P_1–P_1' catalytic site was inversely proportional to the extent of change in HTLV-I protease inhibitory activity. Lastly, we examined the effect of introducing a six-member ring system at the α-carbon and observed that a compound with a P_3-cap cyclohexyl ring (**5o**) exhibited equipotency to that of a cyclopropyl ring (**5e**), but lower potency than compound **5k**. On the other hand, compound **5p** with a P_3-cap phenyl ring seemed to provide a better inhibitory profile than compounds **5e** and **5k**. This trend suggested that a P_3-cap phenyl ring moiety (**5p**) provided a more appropriate bulkiness than a cyclohexyl ring moiety (**5o**). Looking at the inhibitory profiles, although we hypothesized various structure–activity relationships from this group of compounds, the reliability of the trends is still in question considering that all of the amide P_3-cap moieties (**5a**–**p**) were extremely potent HTLV-I protease inhibitors with little variations in IC_{50} values, from 79 to 107 nM, although the standard-error-of-the-mean was only 1 nM. Nonetheless, this observation suggested that the nature of the amide P_3-cap moieties was a lower determinant of inhibitory activity. Computer-assisted docking experiments revealed that the pocket, where the P_3-cap moiety resides, was near the exposed surface of the enzyme (Fig. 2A), and thereby, other than a hydrogen bond interaction with the amide oxygen (cf. **9**), would have

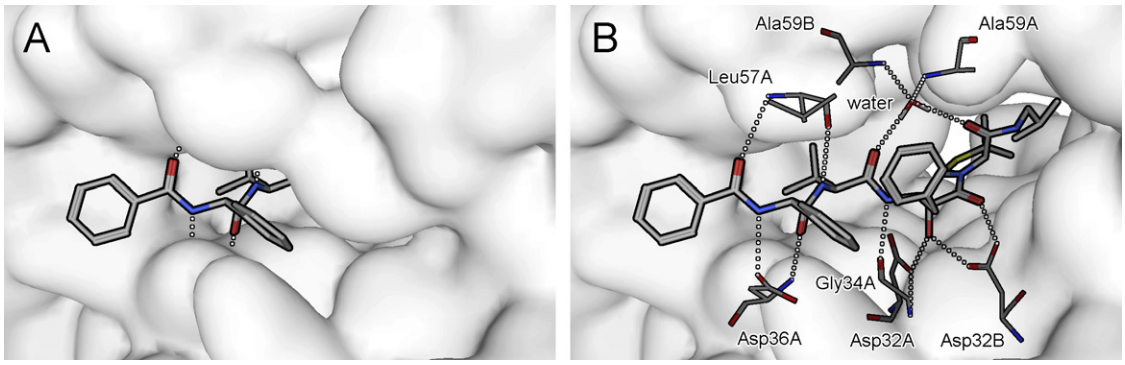


Figure 2. Computer model of KNI-10634 (**5p**) in the active site of HTLV-I protease. Dotted lines represent possible hydrogen bond interactions. (A) Portions of the P_3 moiety residue near the exposed surface of the HTLV-I protease. (B) The surface of some HTLV-I protease heavy atoms is hidden to clearly depict various hydrogen bond interactions.

a lesser influence on inhibitory activity. To confirm the speculative structure–activity relationships, various groups of compounds, where the P_1'-cap and P_3-cap moieties, respectively, differ from each other, are currently being evaluated so that structure–activity relationship trends can be statistically validated.

A computer-assisted docking model was generated for one of the more potent HTLV-I protease inhibitor, compound **5p**, described in the current study (Fig. 2). In the model, multiple possible hydrogen bond interactions existed throughout the backbone of compound **5p** and HTLV-I protease's Asp32A, Asp32B, Gly34A, Asp36A, Leu57A, Ala59A, and Ala59B of the dimer, and thereby anchoring the inhibitor in its docked position within the HTLV-I protease (Fig. 2B). Similar to HIV-1 and Plasmepsin II protease inhibitors, a water molecule mediated the hydrogen bond interactions between Ala59A and Ala59B in the hairpin regions of HTLV-I protease's flaps and the inhibitor. Moreover, compound **5p**'s transition-state mimic hydroxymethylcarbonyl isosteric moiety interacted with the protease's catalytic Asp32A and Asp32B. As a result, a hydrogen bond network was formed in the vicinity of the critical P_1–P_1' residues. As shown in Figure 2A, the P_3 residue's phenyl side-chain fitted snugly in the S_3 pocket, while the P_3-cap moiety resided near the edge of the active site in a region where various hydrophobic groups could be accommodated.

All synthesized inhibitors exhibited nearly complete anti-HIV-1 protease activity at 50 nM ($\geqslant$99% inhibition), and interestingly, our results reflected the observations made by Tözsér and co-workers that HTLV-I protease expresses higher specificity than its HIV-1 protease cousin.[15] Whereas the Tözsér research group reported narrower activation profiles from peptidic substrates, we observed narrower inhibition profile from our HTLV-I protease inhibitors. In simpler words, in agreement with our past reports,[20,21] in general, potent HTLV-I protease inhibitors exhibit extremely potent HIV-1 protease inhibition, whereas potent HIV-1 protease inhibitors do not necessarily exhibit potent HTLV-I protease inhibitory activity. Hence, differences in the amino acid sequences of HIV-1 and HTLV-I proteases do indeed produce differences in susceptibilities to inhibitors, in that HIV-1 protease is more susceptible to inhibitors than HTLV-I protease. On a side note, our past studies[23–27] have shown that HIV-1 protease is also less specific than Plasmepsin II, an aspartic protease associated with malaria. These observations also imply that should the need arise, HTLV-I protease inhibitors and Plasmepsin inhibitors could become lead compounds in the design of novel HIV-1 protease inhibitors.

4. Conclusions

In the present study, small peptide-based P_3-cap substituted HTLV-I protease inhibitors containing a hydroxymethylcarbonyl isostere were designed and synthesized on the basis of the substrate transition-state mimicry. The HTLV-I protease profiles of these new and extremely potent HTLV-I protease inhibitors showed chemical structure dependence at the P_3-cap moiety, especially the hydrogen bond interaction involving Leu57A. These inhibitors also exhibited high potency against HIV-1 protease. The overall most potent HTLV-I protease inhibitor within this study was KNI-10634 (**5p**, IC_{50} = 79 nM).

5. Experimental

5.1. Chemistry

5.1.1. General methods. Reagents and solvents were purchased from commercial suppliers and used without further purification. ^{1}H NMR spectra were measured on a JEOL JNM-AL300 FT (300 MHz) spectrometer with tetramethylsilane (TMS) as an internal standard at ambient temperature. Data are reported as follows: chemical shift (ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, bs = broad singlet, m = multiplet) and number of protons. LC MS was recorded on a Finnigan SSQ-7000 spectrometer. MALDI-TOF MS was performed on a

Voyager-DE RP spectrometer and all target compounds were less than 1 *m/z* from calculated values. Preparative HPLC was obtained from a C_{18} reverse phase column (20 × 250 mm; YMC Pack ODS SH-343-5AM). Assay HPLC was performed on a C_{18} reverse phase column (3.0 × 75 mm; YMC Pack ODS AS-3E7). Analytical HPLC was performed using a C_{18} reverse phase column (4.6 × 150 mm; YMC Pack ODS AM-302) with a binary solvent system: linear gradient of CH_3CN in 0.1% aqueous trifluoroacetic acid (TFA) at a flow rate of 0.9 mL/min, detected at UV 230 nm. The purity of the desired compounds and intermediates was confirmed by analytical HPLC and was greater than 99% in two different systems (gradient: system A, 0–100%/ 40 min; system B, 30–90%/ 40 min).

5.1.2. General method A: amide bond formation. To a solution of substituted amine (1.0 mmol) in *N*,*N*-dimethyl formamide (DMF, 5 mL) were added Boc-Xaa-OH or carboxylic acid (1.1 mmol), BOP (1.1 mmol), and Et_3N (adjusted to pH 8), and stirred for 15 h at rt. After removal of the solution in vacuo, the residue was dissolved in EtOAc (20 mL), washed with 10% citric acid aq (3 × 15 mL), 10% $NaHCO_3$ aq (3 × 15 mL) and brine (1 × 15 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo to give the crude product which was used directly to the next step without purification, or purified by preparative HPLC in the case of the target compounds. The purified target compounds were immediately lyophilized to afford their respective white amorphous powder.

5.1.3. General method B: Boc-deprotection. 4 N HCl in 1,4-dioxane (5 mL) was added to the afforded crude Boc-protected product (ca. 1.0 mmol) and stirred for 1 h at rt. The solution was evaporated under reduced pressure and used directly to the next step without purification. A small portion of the product was purified by preparative HPLC. The desired fractions were collected and immediately lyophilized to afford white amorphous powder, compound **6**, **7**, **8**, or **9**.

5.1.4. (*R*)-*N*-Isobutyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (H-Dmt-NH^{i}Bu, 6). Compound **6** was prepared from Boc-Dmt-OH and isobutylamine by general methods A and B. Yield: 98%; ^{1}H NMR ($CDCl_3$): δ = 0.90 (d, 2 × 3H), 1.42 (s, 3H), 1.64 (s, 3H), 1.77–1.86 (m, 1H), 3.12 (t, 2H), 4.35 (s, 1H), 4.53 (s, 2H), 6.4 (bs, 1H), 7.46 (t, 1H); MS (LC) *m/z* = 216.89 $[M+H]^+$; Analytical HPLC: R_t = 16.34 min (system A).

5.1.5. (*R*)-*N*-Isobutyl-3-[(2*S*,3*S*)-3-amino-2-hydroxy-4-phenyl]butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (H-Apns-Dmt-NH^{i}Bu, 7). Compound **7** was prepared from Boc-Apns-OH and compound **6** by general method A and B. Yield: 59%; ^{1}H NMR ($CDCl_3$): δ = 0.80–0.91 (m, 2 × 3H), 1.41–1.51 (m, 2 × 3H), 1.70–1.78 (m, 1H), 2.1 (bs, 1H), 2.94–3.02 (m, 2H), 3.03–3.19 (m, 2H), 3.75–3.88 (bs, 1H), 4.22–4.35 (m, 2 × 1H), 4.46 (d, 1H), 4.86 (d, 1H), 7.00–7.14 (bs, 2H), 7.24–7.35 (m, 5H), 7.9–8.2 (bs, 1H); MS (TOF) *m/z* = 394.615 $[M+H]^+$; Analytical HPLC: R_t = 21.66 min (system A).

5.1.6. (*R*)-*N*-Isobutyl-3-{(2*S*,3*S*)-3-[(2*S*)-2-amino-3,3-dimethyl]butanoylamino-2-hydroxy-4-phenyl}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (H-Tle-Apns-Dmt-NH^{i}Bu, 8). Compound **8** was prepared from Boc-Tle-OH and compound **7** by general method A and B. Yield: 81%; ^{1}H NMR (DMSO-d_6): δ = 0.67–0.99 (m, 5 × 3H), 1.37 (s, 3H), 1.51 (s, 3H), 1.70–1.77 (m, 1H), 2.65–2.80 (m, 2H), 2.82–3.10 (m, 2H), 3.46 (bs, 1H), 3.5 (bs, 1H), 4.11 (bs, 1H), 4.40–4.57 (m, 2 × 1H), 4.90–5.04 (m, 2H), 5.32 (bs, 2H), 6.90–7.40 (m, 5H), 8.17 (t, 1H), 8.52 (d, 1H); MS (TOF) *m/z* = 507.305 $[M+H]^+$; Analytical HPLC: R_t = 22.27 min (system A).

5.1.7. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-amino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenylbutanoyl}}-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (H-Phg-Tle-Apns-Dmt-NH^{i}Bu, 9). Compound **9** was prepared from Boc-Phg-OH and compound **8** by general method A and B. Yield: 77%; ^{1}H NMR (DMSO-d_6): δ = 0.51–0.98 (m, 5 × 3H), 1.31 (s, 3H), 1.46 (s, 3H), 1.65–1.76 (m, 1H), 2.50–2.78 (m, 2H), 2.80–2.95 (m, 2H), 3.5 (bs, 1H), 4.11 (bs, 1H), 4.20–4.33 (m, 2 × 1H), 4.38 (s, 1H), 4.86 (d, 1H), 4.98 (d, 1H), 5.07 (bs, 1H), 6.90–7.12 (m, 5H), 7.30–7.40 (m, 5H), 7.91 (t, 1H), 8.03 (d, 1H), 8.45 (d, 1H), NH_2 not observed; MS (TOF) *m/z* = 640.012 $[M+H]^+$, 663.239 $[M+Na]^+$; Analytical HPLC: R_t = 24.36 min (system A), 12.11 min (system B).

5.1.8. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-acetylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (Ac-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5a). Compound **5a** was prepared from acetic acid and compound **9** by general method A. Yield: 60%; ^{1}H NMR ($CDCl_3$)δ = 0.70–0.92 (m, 5 × 3H), 1.40–1.58 (m, 2 × 3H), 1.63–1.80 (m, 1H), 2.06 (s, 3H), 2.2–2.6 (bs, 1H), 2.58–2.85 (m, 2H), 2.88–3.22 (m, 2H), 4.04–4.96 (m, 4 × 1H + 2H), 5.58 (t, 1H), 6.32 (d, 1H), 6.35–6.40 (bs, 1H), 6.66 (t, 1H), 6.90–7.24 (m, 5H), 6.98 (d, 1H), 7.26–7.35 (m, 5H); MS (TOF) *m/z* = 704.762 $[M+Na]^+$, 720.763 $[M+K]^+$; Analytical HPLC: R_t = 28.35 min (system A), 17.94 min (system B).

5.1.9. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-propionylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (EtCO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5b). Compound **5b** was prepared from propionic acid and compound 9 by general method A. Yield: 65%; ^{1}H NMR ($CDCl_3$)δ = 0.78–0.92 (m, 5 × 3H), 1.16 (t, 3H), 1.40–1.57 (m, 2 × 3H), 1.62–1.80 (m, 1H), 2.2–2.7 (bs, 1H), 2.30 (q, 2H), 2.62–2.85 (m, 2H), 2.89–3.24 (m, 2H), 4.05–5.02 (m, 4 × 1H + 2H), 5.53–5.60 (m, 1H), 6.32 (d, 1H), 6.45–6.55 (bs, 1H), 6.70 (t, 1H), 6.90 (d, 1H), 6.92–7.21 (m, 5H), 7.26–7.35 (m, 5H); MS (TOF) *m/z* = 718.987 $[M+Na]^+$; Analytical HPLC: R_t = 29.64 min (system A), 20.26 min (system B).

5.1.10. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-butyrylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (^{n}PrCO-Phg-Tle-Apns-Dmt-NH^{i}Bu,

5c). Compound **5c** was prepared from *n*-butyric acid and compound **9** by general method A. Yield: 62%; ^{1}H NMR ($CDCl_3$)δ = 0.78–0.97 (m, 6 × 3H), 1.40–1.58 (m, 2 × 3H), 1.60–1.76 (m, 1H + 2H), 1.8–2.2 (bs, 1H), 2.24 (t, 2H), 2.66–2.84 (m, 2H), 2.86–3.23 (m, 2H), 4.04–4.99 (m, 4 × 1H + 2H), 5.51 (d, 1H), 6.15–6.25 (bs, 1H), 6.26–6.50 (bs, 1H), 6.66 (t, 1H), 6.73–6.82 (m, 1H), 6.91–7.25 (m, 5H), 7.26–7.36 (m, 5H); MS (TOF) *m*/*z* = 710.984 $[M+H]^+$, 732.931 $[M+Na]^+$, 748.858 $[M+K]^+$; Analytical HPLC: R_t = 30.56 min (system A), 22.19 min (system B).

5.1.11. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-pentanoylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (^{n}BuCO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5d). Compound **5d** was prepared from valeric acid and compound **9** by general method A. Yield: 59%; ^{1}H NMR ($CDCl_3$)δ = 0.71–0.94 (m, 6 × 3H), 1.30–1.37 (m, 2H), 1.47–1.64 (m, 2H + 2 × 3H), 1.6–2.0 (bs, 1H), 1.70–1.84 (m, 1H), 2.26 (t, 2H), 2.60–2.83 (m, 2H), 2.90–3.23 (m, 2H), 4.00–4.99 (m, 4 × 1H + 2H), 5.47–5.51 (m, 1H), 6.10–6.22 (m, 1H), 6.22–6.35 (bs, 1H), 6.65–6.71 (m, 1H), 6.72–6.80 (m, 1H), 6.91–7.25 (m, 5H), 7.26–7.36 (m, 5H); MS (TOF) *m*/*z* = 724.977 $[M+H]^+$, 746.951 $[M+Na]^+$, 762.969 $[M+K]^+$; Analytical HPLC: R_t = 32.30 min (system A), 24.36 min (system B).

5.1.12. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-cyclopropanecarbonylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (^{c}PrCO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5e). Compound **5e** was prepared from cyclopropanecarboxylic acid and compound **9** by general method A. Yield: 57%; ^{1}H NMR ($CDCl_3$)δ = 0.75–0.98 (m, 2H + 5 × 3H), 1.46–1.57 (m, 1H + 2 × 3H), 1.71–1.77 (m, 1H), 2.0–2.5 (bs, 1H), 2.68–2.82 (m, 2H), 2.83–3.25 (m, 2H), 4.07–5.04 (m, 4 × 1H + 2H), 5.53–5.58 (m, 1H), 6.35 (d, 1H), 6.54–6.68 (m, 2 × 1H), 6.72 (t, 1H), 6.92–7.25 (m, 5H), 7.26–7.35 (m, 5H); MS (TOF) *m*/*z* = 708.921 $[M+H]^+$, 730.972 $[M+Na]^+$, 746.905 $[M+K]^+$; Analytical HPLC: R_t = 30.46 min (system A), 21.40 min (system B).

5.1.13. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-isobutyrylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (^{i}PrCO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5f). Compound **5f** was prepared from isobutyric acid and compound **9** by general method A. Yield: 65%; ^{1}H NMR ($CDCl_3$)δ = 0.74–0.95 (m, 5 × 3H), 1.10–1.18 (m, 2 × 3H), 1.42–1.58 (m, 2 × 3H), 1.71–1.81 (m, 1H), 2.42–2.50 (m, 1H), 2.2–2.5 (bs, 1H), 2.67–2.84 (m, 2H), 2.87–3.25 (m, 2H), 4.05–5.04 (m, 4 × 1H + 2H), 5.48–5.53 (m, 1H), 6.29 (d, 1H), 6.45 (d, 1H), 6.68 (t, 1H), 6.82 (d, 1H), 6.92–7.25 (m, 5H), 7.26–7.35 (m, 5H); MS (TOF) *m*/*z* = 710.990 $[M+H]^+$, 732.939 $[M+Na]^+$, 748.938 $[M+K]^+$; Analytical HPLC: R_t = 30.99 min (system A), 22.34 min (system B).

5.1.14. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-pivaloylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (^{t}BuCO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5g). Compound **5g** was prepared from pivalic acid and compound **9** by general method A. Yield: 59%; ^{1}H NMR ($CDCl_3$)δ = 0.75–0.92 (m, 5 × 3H), 1.22 (s, 3 × 3H), 1.48–1.58 (m, 2 × 3H), 1.69–1.80 (m, 1H), 2.0–2.4 (bs, 1H), 2.62–2.81 (m, 2H), 2.85–3.26 (m, 2H), 3.99–5.06 (m, 4 × 1H + 2H), 5.42–5.46 (m, 1H), 6.21 (d, 1H), 6.29 (d, 1H), 6.72 (t, 1H), 6.86 (d, 1H), 6.92–7.25 (m, 5H), 7.26–7.37 (m, 5H); MS (TOF) *m*/*z* = 725.045 $[M+H]^+$, 747.044 $[M+Na]^+$, 763.027 $[M+K]^+$; Analytical HPLC: R_t = 32.27 min (system A), 25.20 min (system B).

5.1.15. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-(3-methyl)butanoylamino-2-phenyl]acetylamino-3,3-dimethyl}-butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (^{i}BuCO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5h). Compound **5h** was prepared from isovaleric acid and compound **9** by general method A. Yield: 56%; ^{1}H NMR ($CDCl_3$)δ = 0.77–0.96 (m, 7 × 3H), 1.47–1.58 (m, 2 × 3H), 1.61–1.78 (m, 1H), 2.0–2.4 (bs, 1H), 2.08–2.15 (m, 1H + 2H), 2.59–2.78 (m, 2H), 2.82–3.22 (m, 2H), 4.04–5.02 (m, 4 × 1H + 2H), 5.54–5.57 (m, 1H), 6.27 (d, 1H), 6.40 (d, 1H), 6.69 (t, 1H), 6.84 (d, 1H), 6.90–7.25 (m, 5H), 7.26–7.35 (m, 5H); MS (TOF) *m*/*z* = 724.933 $[M+H]^+$, 746.940 $[M+Na]^+$, 762.912 $[M+K]^+$; Analytical HPLC: R_t = 31.82 min (system A), 23.86 min (system B).

5.1.16. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-(3,3-dimethyl)butanoylamino-2-phenyl]acetylamino-3,3-dimethyl}-butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (^{t}PeCO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5i). Compound **5i** was prepared from *tert*-butylacetic acid and compound **9** by general method A. Yield: 58%; ^{1}H NMR ($CDCl_3$)δ = 0.70–0.93 (m, 5 × 3H), 1.01 (s, 3 × 3H), 1.47–1.58 (m, 2 × 3H), 1.67–1.80 (m, 1H), 2.0–2.4 (bs, 1H), 2.13 (d, 2H), 2.60–2.82 (m, 2H), 2.85–3.24 (m, 2H), 4.00–5.00 (m, 4 × 1H + 2H), 5.51 (d, 1H), 6.13 (d, 1H), 6.16–6.25 (bs, 1H), 6.67 (t, 1H), 6.73 (d, 1H), 6.90–7.26 (m, 5H), 7.27–7.36 (m, 5H); MS (TOF) *m*/*z* = 738.909 $[M+H]^+$, 760.904 $[M+Na]^+$, 776.911 $[M+K]^+$; Analytical HPLC: R_t = 33.15 min (system A), 25.55 min (system B).

5.1.17. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-(2,2-dimethyl)butanoylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide [EtC(Me$_2$)-CO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5j]. Compound **5j** was prepared from 2,2-dimethylbutyric acid and compound **9** by general method A. Yield: 61%; ^{1}H NMR ($CDCl_3$)δ = 0.78–0.92 (m, 6 × 3H), 1.18 (s, 2 × 3H), 1.48–1.59 (m, 2H + 2 × 3H), 1.7–2.2 (bs, 1H), 1.72–1.78 (m, 1H), 2.61–2.81 (m, 2H), 2.84–3.27 (m, 2H), 3.98–5.03 (m, 4 × 1H + 2H), 5.44 (d, 1H), 6.12 (d, 1H), 6.19 (d, 1H), 6.70 (t, 1H), 6.85 (d, 1H), 6.90–7.21 (m, 5H), 7.26–7.37 (m, 5H); MS (TOF) *m*/*z* = 739.093 $[M+H]^+$, 761.035 $[M+Na]^+$, 777.069 $[M+K]^+$; Analytical HPLC: R_t = 33.36 min (system A), 26.68 min (system B).

5.1.18. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-(2-methyl)pentanoylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-

dimethyl-1,3-thiazolidine-4-carboxamide [^{n}PrCH(Me)CO-Phg-Tle-Apns-Dmt-NH^{i}Bu, racemate, 5k]. Compound **5k** was prepared from 2-methyl-*n*-valeric acid and compound **9** by general method A. The compound was isolated by HPLC as a racemate. Yield: 55%; ^{1}H NMR ($CDCl_3$)δ = 0.80–0.91 (m, 6 × 3H), 1.13 (d, 3H), 1.20–1.78 (m, 1H + 2 × 2H + 2 × 3H), 2.30–2.35 (m, 1H), 2.5–2.9 (bs, 1H), 2.65–2.83 (m, 2H), 2.87–3.23 (m, 2H), 4.08–5.05 (m, 4 × 1H + 2H), 5.50–5.59 (m, 1H), 6.30–6.40 (bs, 1H), 6.50–6.56 (bs, 1H), 6.68 (t, 1H), 6.85 (d, 1H), 6.91–7.25 (m, 5H), 7.26–7.34 (m, 5H); MS (TOF) *m*/*z* = 760.894 $[M+Na]^+$, 776.931 $[M+K]^+$; Analytical HPLC: R_t = 32.94 min (system A), 26.63 min (system B).

5.1.19. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-(3-methyl)pentanoylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide [EtCH(Me)CH$_2$CO-Phg-Tle-Apns-Dmt-NH^{i}Bu, racemate, 5l]. Compound **5l** was prepared from 3-methyl-*n*-valeric acid and compound **9** by general method A. The compound was isolated by HPLC as a racemate. Yield: 54%; ^{1}H NMR ($CDCl_3$)δ = 0.78–0.92 (m, 7 × 3H), 1.11–1.29 (m, 1H), 1.30–1.43 (m, 1H), 1.45–1.58 (m, 2 × 3H), 1.60–1.82 (m, 1H), 1.83–1.95 (m, 1H), 1.97–2.08 (m, 1H), 2.20–2.30 (m, 1H), 2.60–2.83 (m, 2H), 2.88–3.26 (m, 2H), 4.02–5.00 (m, 4 × 1H + 2H), 5.53 (d, 1H), 6.18 (d, 1H), 6.22–6.36 (m, 1H), 6.60–6.70 (m, 1H), 6.75–6.84 (m, 1H), 6.90–7.25 (m, 5H), 7.26–7.36 (m, 5H), OH not observed; MS (TOF) *m*/*z* = 738.994 $[M+H]^+$, 761.034 $[M+Na]^+$, 777.036 $[M+K]^+$; Analytical HPLC: R_t = 33.31 min (system A), 25.71 min (system B).

5.1.20. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-(4-methyl)pentanoylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (^{i}PeCO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5m). Compound **5m** was prepared from 4-methyl-*n*-valeric acid and compound **9** by general method A. Yield: 59%; ^{1}H NMR ($CDCl_3$)δ = 0.76–0.92 (m, 7 × 3H), 1.47–1.58 (m, 1H + 2H + 2 × 3H), 1.6–2.0 (bs, 1H), 1.74–1.80 (m, 1H), 2.26 (t, 2H), 2.64–2.85 (m, 2H), 2.89–3.24 (m, 2H), 3.99–4.95 (m, 4 × 1H + 2H), 5.47–5.51 (m, 1H), 6.15 (d, 1H), 6.24 (d, 1H), 6.68 (t, 1H), 6.75 (d, 1H), 6.90–7.19 (m, 5H), 7.25–7.36 (m, 5H); MS (TOF) *m*/*z* = 738.962 $[M+H]^+$, 760.862 $[M+Na]^+$, 776.865 $[M+K]^+$; Analytical HPLC: R_t = 33.52 min (system A), 26.46 min (system B).

5.1.21. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-(3-phenyl)-propanoylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (PhCH$_2$CH$_2$CO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5n). Compound **5n** was prepared from 3-phenylpropanoic acid and compound **9** by general method A. Yield: 55%; ^{1}H NMR ($CDCl_3$)δ = 0.70–0.95 (m, 5 × 3H), 1.44–1.82 (m, 1H + 2 × 3H, partly covered by H_2O), 2.52–2.84 (m, 2 × 2H), 2.85–3.26 (m, 2 × 2H), 3.91–4.96 (m, 4 × 1H + 2H), 5.41 (t, 1H), 5.85–6.09 (m, 2 × 1H), 6.58–6.68 (m, 1H), 6.69–6.79 (m, 1H), 6.90–7.39 (m, 3 × 5H), OH not observed; MS (TOF) *m*/*z* = 772.855 $[M+H]^+$, 795.059 $[M+Na]^+$, 811.126 $[M+K]^+$; Analytical HPLC: R_t = 32.64 min (system A), 26.00 min (system B).

5.1.22. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-cyclohexanecarbonylamino-2-phenyl]acetylamino-3,3-dimethyl}-butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (^{c}HxCO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5o). Compound **5o** was prepared from cyclohexanecarboxylic acid and compound **9** by general method A. Yield: 55%; ^{1}H NMR ($CDCl_3$)δ = 0.78–0.91 (m, 5 × 3H), 1.22–1.58 (m, 3 × 2H + 2 × 3H), 1.67–1.90 (m, 1H + 2 × 2H), 2.15–2.24 (m, 1H), 2.6–2.9 (bs, 1H), 2.67–2.86 (m, 2H), 2.90–3.24 (m, 2H), 4.00–5.02 (m, 4 × 1H + 2H), 5.46–5.50 (m, 1H), 6.33 (d, 1H), 6.40 (d, 1H), 6.60–6.80 (m, 2 × 1H), 6.90–7.25 (m, 5H), 7.26–7.36 (m, 5H); MS (TOF) *m*/*z* = 750.277 $[M+H]^+$, 772.803 $[M+Na]^+$, 788.769 $[M+K]^+$; Analytical HPLC: R_t = 33.28 min (system A), 27.11 min (system B).

5.1.23. (*R*)-*N*-Isobutyl-3-{{(2*S*,3*S*)-3-{(2*S*)-2-[(2*S*)-2-benzoylamino-2-phenyl]acetylamino-3,3-dimethyl}butanoylamino-2-hydroxy-4-phenyl}}butanoyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (PhCO-Phg-Tle-Apns-Dmt-NH^{i}Bu, 5p). Compound **5p** was prepared from benzoic acid and compound **9** by general method A. Yield: 61%; ^{1}H NMR ($CDCl_3$)δ = 0.71–0.93 (m, 5 × 3H), 1.48–1.55 (m, 2 × 3H), 1.6–2.0 (bs, 1H), 1.65–1.80 (m, 1H), 2.62–2.85 (m, 2H), 2.88–3.20 (m, 2H), 4.03–4.91 (m, 4 × 1H + 2H), 5.68 (d, 1H), 6.19 (d, 1H), 6.22–6.35 (m, 2 × 1H), 6.67 (t, 1H), 6.90–7.26 (m, 5H), 7.30–7.86 (m, 2 × 5H); MS (TOF) *m*/*z* = 744.873 $[M+H]^+$, 766.838 $[M+Na]^+$, 782.867 $[M+K]^+$; Analytical HPLC: R_t = 32.19 min (system A), 25.16 min (system B).

5.2. HIV-1 and HTLV-I protease inhibition assays

Compounds **9** and **5a-p** were evaluated for HIV-1 protease inhibitory activity at 50 nM of the test compound, and HTLV-I protease inhibitory activity as IC_{50} values, using previously reported procedures.[21] The HTLV-I protease used in the assay was an L40I mutant of the wild-type protease to prevent autolysis and improve stability.[22]

5.3. Computer-assisted docking experiments

Attempts at ab initio computer-assisted docking of compound **5p**, which exhibited the more potent HTLV-I protease inhibitory activity, in a truncated mutant HTLV-I protease (AB dimer of PDB 2B7F; truncated from 125 to 116 residues; L40I mutation)[22] using all the different methods provided by the 2006.0804 release of Chemical Computing Group's Molecular Operating Environment software under MMFFX94 force-field, were unsuccessful in that a rational pose, that depicted the HMC transition-state mimic interactions usually observed under X-ray crystallography studies, could not be found. Consequently, we built a complex of compound **5p** and HTLV-I protease, based on the X-ray crystallography data of similar inhibitor-protease complexes, namely PDB 1MRX and 2B7F in the absence of water. The relative coordinates of the $P_1 - P_1'$ (Apns-Dmt) res-

idues of potent HIV-1 protease inhibitor KNI-577 (PDB 1MRX)[28] were merged into an HTLV-I protease inhibitor, Ac-Ala-Pro-Gln-Val-Statine-Val-Met-His-Pro (PDB 2B7F),[22] by triangulation using the carboxylic acid's carbons of the two proteases' two catalytic aspartic acids (AB dimers), and the two inhibitors' catalytic P_1 hydroxy group as points of alignment. After the HIV-1 protease was removed, all heavy atoms were fixed except for the side-chains of HTLV-I protease's Asp32A and Asp32B, the polarization state of the Asps was modified accordingly, and the model was energy minimized under MMFFX94 force-field. The merged inhibitor's positions of all heavy atoms that had hydrogen bond interactions in the respective original model were fixed and the inhibitor was energy minimized in the rigid HTLV-I protease. The model was meticulously modified accordingly to resemble compound **5p** while being energy minimized after each major modification. The generated model was "water-soaked" to identify the water molecule that mediated the interactions between the flaps of HTLV-I protease and the inhibitor. In the final step, the free inhibitor and water molecule were energy minimized inside the rigid HTLV-I protease inhibitor (Fig. 2).

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