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Total synthesis of grassystatin A, a probe for cathepsin E function

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ABSTRACT

The linear depsipeptide grassystatin A, a valuable probe for the study of cathepsin E function, has been synthesized by a [4+6] strategy. It exhibited specific inhibitory activity against cathepsin E with an IC_{50} value of 0.8 nM. Our studies indicated that inhibition of cathepsin E did not have an impact on ovalbumin antigen processing and peptide presentation, unique from studies of other aspartic protease inhibitors.

1. Introduction

Cathepsin E (CE) is an intracellular aspartic protease that is mainly present in immune cells and is highly homologous to the aspartic protease cathepsin D (CD). Like CD, CE has also been shown to have a role in antigen processing, but is localized in different cell compartments and tissues and has broader substrate specificity.[1,2] CE deficiency and over-expression have been shown to result in a variety of pathological conditions; however, its exact functional role remains to be elucidated.[3,4] Inhibitors developed to study the role of CE have not been forthcoming due to difficulties with purification or preparation in quantities sufficient for functional studies, and their non-specificity further complicates the progress in discriminating its unique role.

Grassystatins A–C were isolated from a cyanobacterium, identified as *Lyngbya* cf. *confervoides* by Luesch and co-workers.[5] During the biological evaluation of the three linear modified peptides, the major compound, grassystatin A (**1**, Fig 1) was 30-fold more selective for CE over CD with sub-nanomolar potency. It contains some notable structural features, including two *N*-methylamides and a (3*S*,4*S*)-statine unit. It also consists of several hydroxy acids linked through sterically hindered ester bonds which are very rare in the marine peptides. Due to the difficulties mentioned above and owing to the need of higher amounts of grassystatin A for biological assays, the total synthesis of grassystatin A (**1**) was undertaken and synthetic material subjected to further biological evaluation.

2. Results and discussion

The retrosynthetic analysis of **1** is outlined in Figure 2.

We envisioned that the molecule can be assembled via amidation between the Asn and Leu residues because of the versatility in the construction of the precursors (**4**, **5**), low hindrance and absence of racemization during fragment coupling.

First, we focused on the synthesis of fragment **4** as summarized in Scheme 1.

After the preparation of L- and D-Hiva-OBn through established protocol,[6] the synthesis was started with coupling of the commercially available Boc-L-Val and L-Hiva-OBn to afford **6** in 89% yield. Considering the possibility of racemization while using violent reaction conditions, we thought that **7** would easily be obtained by condensation between **6** and D-Hiva-OBn with a coupling reagent. However, the outcome did not appear as we expected. Various coupling reagents were tested, but the reactions failed. No product was observed and the reactants were not consumed. Since the large steric hinderance might have resulted in the low reactivity of the reactants, we made an attempt to find an alternative route for **7**. The preferred method was Yamaguchi esterification, which furnished **7** in 99% yield and was mild enough not to cause racemization. The intermediate was deprotected by 50% TFA/CH_2Cl_2 and underwent smooth reductive amination upon treatment with $Na(BH_3)CN$ to give **8** in 70% yield. Compound **8** was removed of the benzyl protecting group and then coupled with H-Leu-Obzl to afford fragment **4** in 75% yield.

The next effort was to synthesize fragment **5** as shown in Scheme 2. H-Pro-OMe and Boc-*N*-Me-D-Phe were prepared

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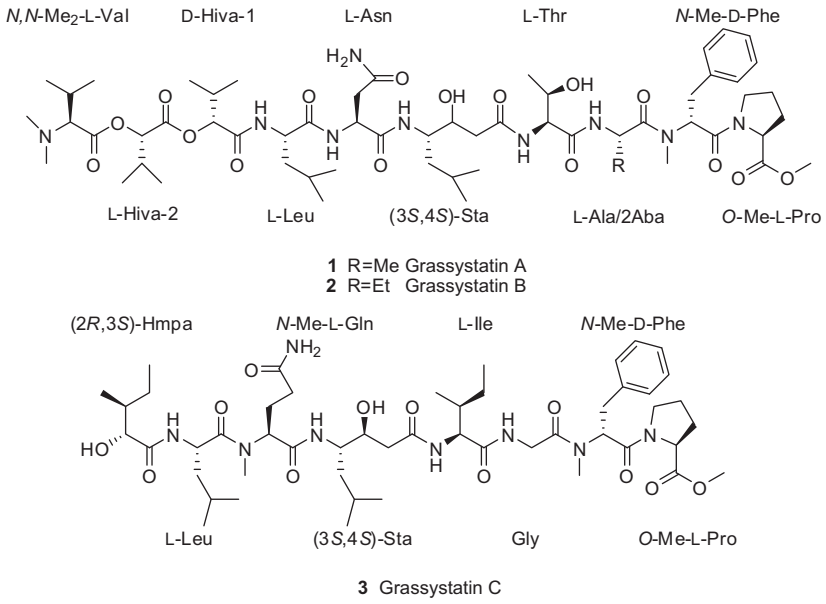


Figure 1. Grassystatins A-C.

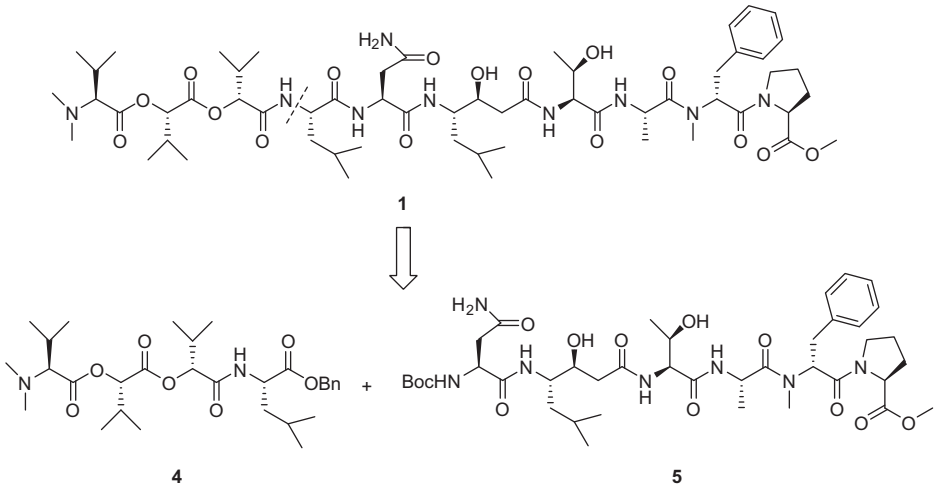


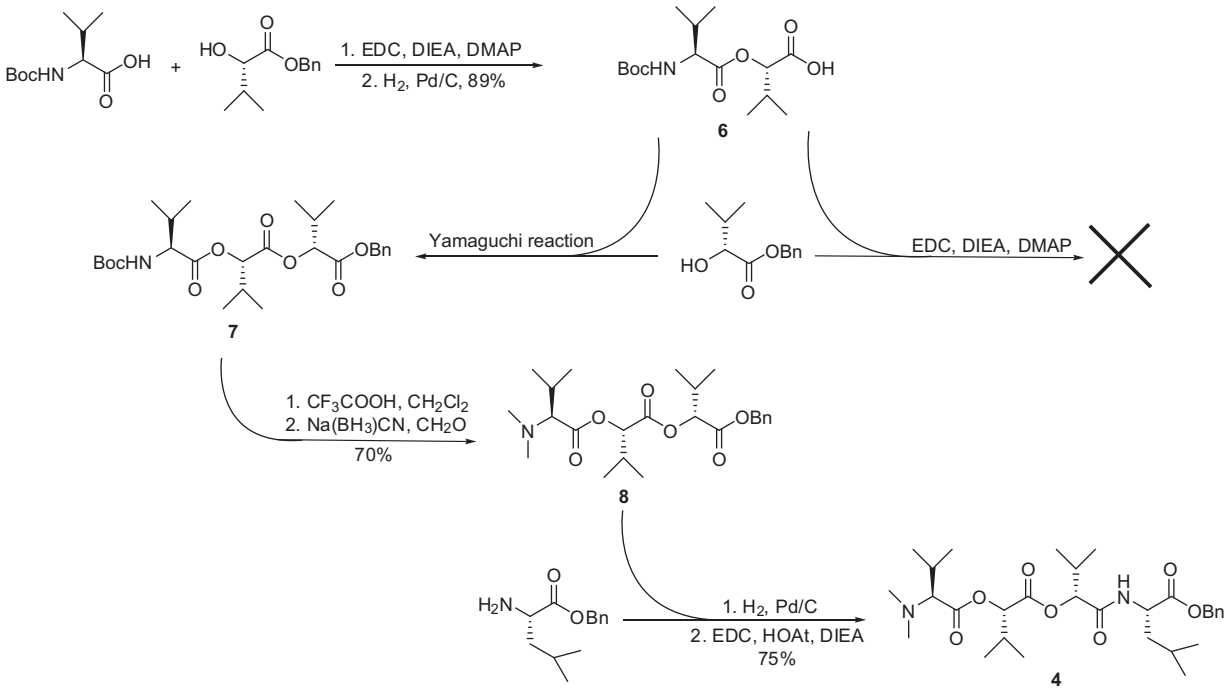
Figure 2. Retrosynthetic analysis of **1**.

according to the literature.[7,8] Their condensation was then carried out by using EDC/HOAt as coupling reagents to provide dipeptide **9** in 88% yield. Removal of the Boc group of 8 with 50% TFA/CH_2Cl_2 and incorporation with Boc-Ala (EDC, HOAt, DIEA) afforded the tripeptide **10** smoothly in 85% yield. With the same procedure, the condensation of **10** and Boc-Thr produced **11** in 75% yield.

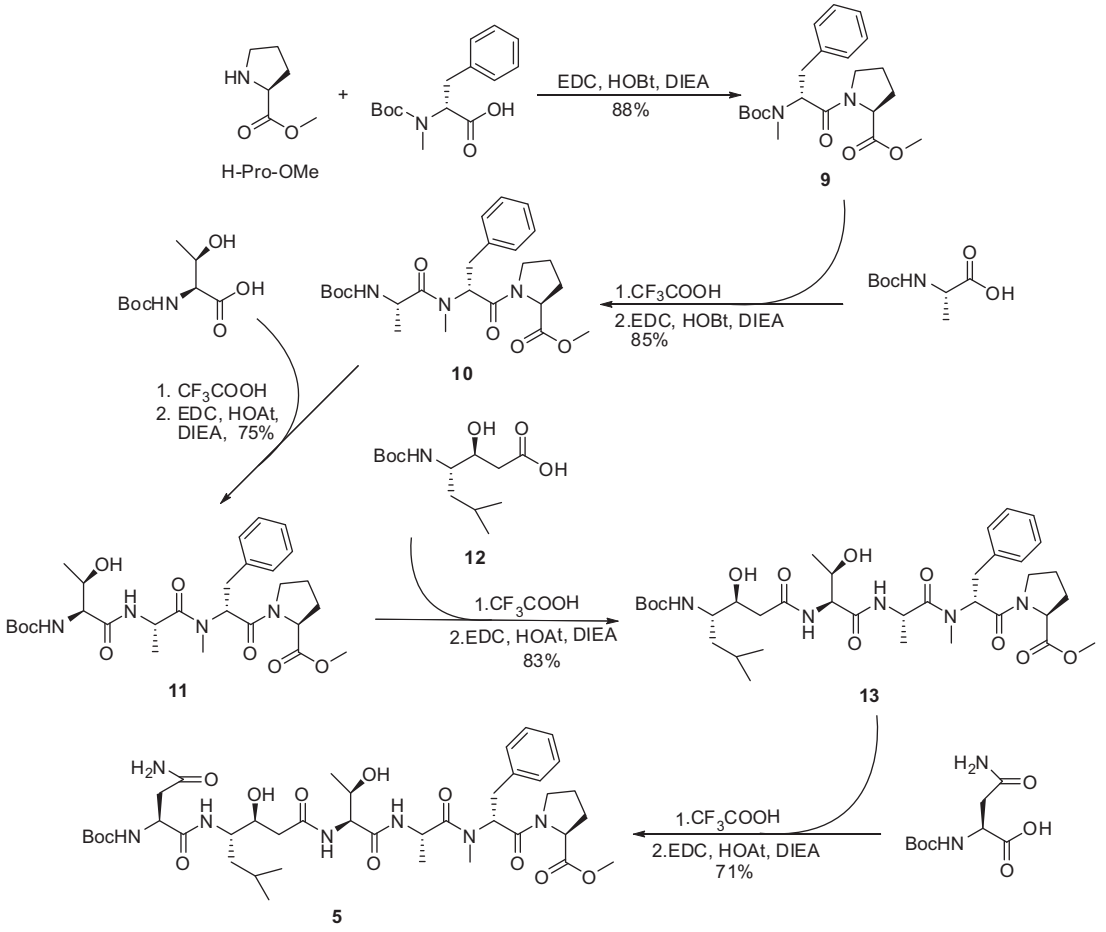
In order to construct fragment **13**, (*S*,*S*)-statine unit (**12**) had to be prepared first. Though several routes have been reported, most of them require specific reagents or too many steps to result in the product. Considering the situations above, we chose the method below.[9] The product of reduction and Dess–Martin oxidation of commercially available Boc-Leu-OH and then Claisen condensation with lithio ethyl acetate afforded Boc-Sta-OEt in 64% yield. After saponification of Boc-Sta-OEt, **12** was obtained. The method has a low stereoselectivity (*anti*:*syn* = 60: 40), but the fragment can be obtained in large amounts efficiently and economically.

With fragment **11** and **12** in hand, their coupling could then be carried out. Compound **12** was activated by HATU/HOAt and incorporated into the peptide chain **11** to obtain **13**. The last step was the condensation between **13** and Boc-Asn to afford the fragment **5** with a yield of 71% over two steps.

With fragment **4** and **5** in hand, we arrived at the final stage of the synthesis. First, the benzyl group and Boc of **4** and **5** were removed under catalytic hydrogenation and 50% TFA/CH_2Cl_2. The coupling was carried out with HATU/HOAt as coupling agents to provide grassystatin A (**1**, 45% for three steps). However, the purification did not work smoothly. After being subjected to column chromatography, the product decomposed into several fragments of larger polarity. Upon MS analysis, we presumed that the ester linkage between L-Hiva and D-Hiva was broken due to the acidity of the silica gel. Therefore, we changed the standard workup procedure and directly chromatographed and purified the target by HPLC afterwards to afford product **1** as a white solid (purity: 98.8%). Spectroscopic data of compound **1** were in full agreement with those reported for the natural product isolated from the cyanobacterium. Grassystatin A was found to be particularly prone to hydrolysis catalyzed by acid, and required storage under inert atmosphere at −20 °C. In fact, NMR spectra of samples stored at



Scheme 1. Synthesis of fragment **4**.



Scheme 2. Synthesis of fragment **5**.

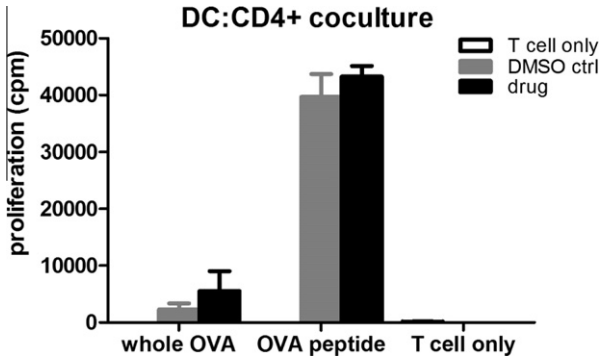


Figure 3. CD4+ T cell proliferation in response to antigen-loaded DC. Purified CD11c+ dendritic cells from spleen were treated with grassystatin A, then pulsed with whole OVA or OVAp 323–339 and cocultured with enriched CD4+ T cells. DMSO-treated DC were used as solvent controls.

room temperatures for a few days showed a gradual increase of the decomposed products.

We previously found that grassystatin A exhibited specific inhibitory activity against cathepsin E, an aspartic protease which has been shown to have an integral role in immune function. Consistent with our previous report,[5] synthetic grassystatin A also potently inhibited cathepsin E activity in vitro (IC_{50} 0.8 nM) and to a lesser extent cathepsin D activity (IC_{50} 40 nM). Our previous studies examining T cell response to exogenous antigen suggested that grassystatin A may alter antigen presentation,[5] and Yee et al. demonstrated that negative regulation of cathepsin E expression by CIITA (MHC class II transactivator) decreased antigen processing.[10] However, a clear mechanism in how cathepsin E inhibition may alter APC function remains unknown. We sought to determine whether grassystatin A may inhibit MHC class II-dependent antigen processing using the DO11.10 TCR transgenic mouse model, which possess MHC class II-restricted CD4+ T cells with a TCR that recognize the OVA peptide 323–339 (OVAp). OVAp-pulsed dendritic cells (DC) presenting this synthetic peptide to CD4+ T cells will result in T cell proliferation; alternatively DC that effectively process whole OVA antigen to present this OVA fragment can also elicit a T cell response. To assess the effect of grassystatin A on antigen processing, we measured the ability of drug-treated, whole antigen-pulsed DC to elicit a T cell response. We found that the T cell proliferative response to OVA-pulsed grassystatin-treated DC was comparable to that of OVA-pulsed DMSO control-treated DC, suggesting that grassystatin A does not have an impact on ovalbumin antigen processing (Fig. 3). Furthermore, grassystatin-treated DC that were loaded with OVAp were able to mount an equally robust response from T cells as controls, demonstrating that peptide presentation was unaffected (Fig. 3).

This finding is unique from studies of other aspartic protease inhibitors which implicated a role of cathepsin E in MHC class II antigen processing.[10–14] However, the MHCII antigen processing and presentation pathway involves a complex system of proteins and endocytic compartments, and the specific target within this pathway may vary between cathepsin E inhibitors. In dendritic cells, cathepsin E is present mainly in early endosomes and partially in lysosomes,[11,12,15] thus the effect of CE inhibitors may depend on its presence during the period of peptide–MHCII complex assembly within those compartments where both Ag processing and presentation activities occur. Our previous study demonstrated that T cell proliferation to peptide-APC stimulation was reduced in the presence of grassystatin A, suggesting impaired antigen presentation, but did not specifically address whether the upstream antigen processing machinery was also affected.[5] The routing of antigen through endocytic vesicles where whole antigen is cleaved into 9–22 mer peptide fragments is a critical step of processing before peptide presentation occurs.[16] However, the requirement for Ag processing may be bypassed to still allow T cell stimulation if APC are exposed to synthetic peptide fragments.[17] In this current study, we aimed to identify whether the observed grassystatin-associated Ag presentation was stunted due to inhibited Ag processing mechanisms using the OVA TCR transgenic MHCII-restricted T cell system. We pretreated DC with drug before incubation with whole OVA for co-culture with CD4+ T cells and found that both control and drug-treated DC were able to stimulate comparable T cell proliferation suggesting that antigen processing function remained intact. It is possible we did not see an effect because grassystatin A only induces a transient effect on cells, and the withdrawal of cells from drug exposure allowed the antigen processing and presentation machinery to recover function, an important observation for clinical considerations in determining drug half-life and dosage. Interestingly, a study using cathepsin E-deficient mice found that the antigen presentation ability of DC was enhanced, possibly due to increased phagocytic activity and higher expression of costimulatory molecules.[15] We also observed a minor enhancement in T cell response using drug-treated DC incubated with whole Ag or peptide though it is unknown whether this was due to enhanced processing or other heightened functions within the endocytic pathway. It has been suggested that inhibition of cathepsin E may not necessarily shut down antigen processing as compensatory functions by similar enzymes can occur depending on which compartment is affected. Furthermore, cathepsin E's role in Ag processing may be an indirect modulation of the cellular endosomal microenvironment rather than a direct influence on processing.[15] It remains to be determined where grassystatin exerts its effects along the endocytic pathway as our observations suggest its influence may require its presence within the cathepsin-E compartments during peptide–MHC complex assembly.

3. Conclusions

In summary, we have described the first total synthesis of grassystatin A, a promising linear depsipeptide which is a valuable probe for the study of cathepsin E function. We validated the potent in vitro cathepsin E inhibitory activity and have continued preliminary biological studies to study potential cellular effects on antigen processing and presentation. Further studies of the structure–activity relationship will be reported in due course. Specifically, it will be of interest to modulate the selectivity profile against various aspartic proteases, for example, to generate even more selective cathepsin E inhibitors or specifically enhance the inhibitory activity against cathepsin D based on the grassystatin scaffold. Our initial molecular docking studies with grassystatins suggested that the polar amino acid (Asn) in the P2 pocket adjacent to the statine unit (N-end) may contribute to the cathepsin E preference and that the *N*,*N*-dimethyl group is a likely contributor to the potency of these compounds against both cathepsins D and E.[5]

4. Experimental section

4.1. General information

Solvents were purified by standard methods. TLCs were carried out on Merck 60 F254 silica gel plates and visualized by UV irradiation or by staining with iodine absorbed on silica gel, ninhydrin solution or with aqueous acidic ammonium molybdate solution as appropriate. Flash column chromatography was performed on silica gel (200–300 mesh, Qingdao, China). Optical rotations were obtained using a JASCO P-1020 digital polarimeter. NMR spectra

were recorded on JEOLJNM-ECP 400 MHz spectrometers. Chemical shifts are reported in parts per million (ppm), relative to the signals due to the solvent. Data are described as followed: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), integration, and assignment. Mass spectra were obtained on a Q-Tof Ultima Global mass spectrometer.

4.2. Chemistry

4.2.1. *N*-Boc-Val-L-Hiva-OH (6)

A solution of Boc-L-Val (521.0 mg, 2.4 mmol) and L-Hiva (416.2 mg, 2.0 mmol) in anhydrous CH_2Cl_2 (10 ml) was treated sequentially with EDC (591.9 mg, 3.0 mmol), DIEA (1.01 mL, 6.0 mmol) and DMAP (24.4 mg, 0.2 mmol) at 0 °C. The mixture was warmed to room temperature and stirred overnight. After dilution with EtOAc (100 ml), the whole mixture was washed with 10% citric acid (2 × 30 ml), 5% $NaHCO_3$ (2 × 30 ml) and brine (2 × 30 ml), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash column chromatography with petroleum ether–EtOAc (9:1) to afford **6*** as a colorless oil (730.5 mg, 90%).

Hydrogenation of **6*** was carried out in CH_3OH–CH_2Cl_2 (1:1, 15 ml) in the presence of a catalytic amount of Pd-C (10%) under hydrogen at room temperature. Pd-C was removed by filtration and concentrated under reduced pressure to yield the acid **6**, which was used directly in the next step.

R_f 0.4 (10:1, $CHCl_3$–MeOH); $[\alpha]_D^{23}$ = +11.4° (*c* 0.04, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ: 5.06 (s, 1H), 4.93 (s, 1H), 4.32 (dd, 1H, *J* = 3.5, 8.6 Hz), 2.29 (d, 2H, *J* = 6.6 Hz), 1.44 (s, 9H), 0.93–1.05 (m, 9H), 0.89 (d, 3H, *J* = 6.5 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ: 174.00, 172.50, 155.91, 80.06, 76.89, 58.24, 31.06, 30.00, 28.29, 18.97, 18.80, 17.13, 16.98; ESIHRMS calcd for $C_{15}H_{27}N_1O_6$ $[M+Na]^+$ 340.1731; found 340.1729.

4.2.2. *N*-Boc-Val-L-Hiva-D-Hiva-OBn (7)

To a solution of acid **6** (380.6 mg, 1.2 mmol) and D-Hiva (208.1 mg, 1.0 mmol) in THF (10 mL) were added DIEA (1.01 mL, 6.0 mmol) and 2,4,6-trichlorobenzoyl chloride (1.20 mL, 1.8 mmol) at room temperature. The reaction mixture was allowed to stir at room temperature for 4 h before adding the tolulene (60 mL) to dilute the solution, then DMAP (5.10 g, 36 mmol) in toluene (30 mL) was added. After stirring at room temperature for 5 h, the solvent was removed under reduced pressure and the residue was chromatographed on silica gel with petroleum–EtOAc (9:1) to afford **7** (500.7 mg, 99%) as a yellow oil.

R_f 0.4 (5:1, petroleum–EtOAc); $[\alpha]_D^{23}$ = +7.1° (*c* 0.12, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ: 7.26–7.36 (m, 5H), 5.18 (dd, 2H, *J* = 5.2, 17.6 Hz), 5.05 (d, 1H, *J* = 4.4 Hz), 4.94 (d, 1H, *J* = 4.8 Hz), 4.84 (d, 1H, *J* = 4.0 Hz), 4.34 (dd, 1H, *J* = 4.4, 9.2 Hz), 2.23–2.30 (m, 3H,), 1.44 (s, 9H), 0.92–1.04 (m, 18H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ: 171.77, 168.95, 168.75, 155.66, 135.32, 128.54, 128.42, 128.39, 79.74, 77.52, 77.44, 77.23, 66.97, 31.34, 30.29, 30.11, 28.33, 19.05, 18.81, 18.66, 17.41, 17.34, 16.96; ESIHRMS calcd for $C_{27}H_{41}N_1O_8$ $[M+Na]^+$ 530.2724; found 530.2743.

4.2.3. *N*,*N*-Me-Val-L-Hiva-D-Hiva-OBn (8)

To a solution of **7** (507.3 mg, 1.0 mmol) in CH_2Cl_2 (10 mL) at 0 °C, TFA (10 mL) was added. The mixture was stirred for 30 min and then evaporated, the residual oil was dissolved twice in CH_2Cl_2 (15 mL) with evaporation each time to give TFA salt **7***, which was used directly in the next step.

To a solution of acid **7*** in MeCN was added DIEA (201.5 μL, 1.2 mmol) at 0 °C, then 37% aqueous formaldehyde solution followed by AcOH (0.1 mL). The reaction was stirred for 1 h before $Na(BH_3)CN$ (188.5 mg, 3.0 mmol) was added. AcOH was added periodically to maintain a pH of 5–7, and the reaction was stirred for 18 h at rt. The reaction was subsequently concentrated in vacuo and the residue redissolved in EtOAc. The organic fraction was then washed with saturated aqueous Na_2CO_3 solution and brine before drying over Na_2SO_4 and concentrating in vacuo. The residue was purified by column chromatography with petroleum–EtOAc (3:1) to obtain **8** as colorless oil (304.7 mg, 70%).

R_f 0.4 (5:1, petroleum–EtOAc); $[\alpha]_D^{23}$ = +9.8° (*c* 0.06, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ: 7.31–7.36 (m, 5H), 5.18 (q, 2H, *J* = 12.4, 14.4 Hz), 5.06 (d, 1H, *J* = 4.0 Hz), 4.96 (d, 1H, *J* = 4.8 Hz), 2.85 (d, 1H, *J* = 10.4 Hz), 2.35 (s, 6H), 2.23–2.34 (m, 2H), 2.01–2.06 (m, 1H), 0.92–1.07 (m, 18H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ: 171.12, 169.31, 168.80, 135.37, 128.52, 128.41, 128.33, 77.70, 76.23, 74.27, 66.93, 41.27, 30.20, 30.16, 27.62, 19.80, 19.40, 19.14, 18.58, 17.38, 17.14; ESIHRMS calcd for $C_{24}H_{38}N_1O_6$ $[M+H]^+$ 436.2694; found 436.2708.

4.2.4. *N*,*N*-Me-Val-L-Hiva-D-Hiva-Leu-OBn (4)

Hydrogenation of **8** (435.3 mg, 1.0 mmol) was carried out in CH_3OH–CH_2Cl_2 (1:1,15 ml) in the presence of a catalytic amount of Pd-C (10%) under hydrogen at room temperature. Pd-C was removed by filtration and concentrated under reduced pressure to yield the acid **8**, which was used directly in the next step.

To a solution of acid **8** and H-Leu-OBn·TosOH (471.8 mg, 1.2 mmol) in CH_2Cl_2 (10 mL) was added DIEA (1.00 mL, 6.0 mmol), HOAt (326.4 mg, 2.4 mmol) and EDC (473.5 mg, 2.4 mmol) at 0 °C. The mixture was warmed to room temperature and stirred overnight. After dilution with EtOAc (100 ml), the whole mixture was washed with 10% citric acid (2 × 30 ml), 5% $NaHCO_3$ (2 × 30 ml) and brine (2 × 30 ml), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash column chromatography with petroleum ether–EtOAc (4:1) to afford **4** as a colorless oil (411.2 mg, 75%).

R_f 0.3 (5:1, petroleum–EtOAc); $[\alpha]_D^{23}$ = +19.6° (*c* 0.07, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ: 7.33–7.36 (m, 5H), 6.90 (d, 1H, *J* = 8.4 Hz), 5.12–5.21 (m, 3H), 4.69–4.72 (m, 1H), 4.66 (d, 1H, *J* = 6.0 Hz), 2.85 (d, 1H, *J* = 10.4 Hz), 2.42–2.51 (m, 1H), 2.31 (s, 6H), 2.16–2.26 (m, 1H), 1.91–2.01 (m, 1H), 1.57–1.72 (m, 3H), 1.06 (q, 6H, *J* = 4.4, 6.8 Hz), 0.98 (t, 6H, *J* = 6.8 Hz), 0.93 (d, 3H, *J* = 6.8 Hz), 0.85–0.90 (m, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ: 172.51, 172.09, 169.37, 168.90, 135.57, 128.51, 128.27, 128.18, 78.36, 77.68, 73.80, 66.87, 50.64, 41.07, 40.80, 30.28, 29.84, 29.70, 27.62, 24.59, 22.76, 21.06, 19.57, 19.24, 18.85, 17.86, 16.19; ESIHRMS calcd for $C_{30}H_{49}N_2O_7$ $[M+H]^+$ 549.3534; found 549.3557.

4.2.5. *N*α-Boc-*N*α-Me-D-Phe-L-Pro-OMe (9)

A solution of L-proline methyl ester (1.30 g, 10 mmol) and *N*α-Boc-*N*α-Me-D-phenylalanine (2.80 g, 10 mmol) in anhydrous CH_2Cl_2 (50 ml) was treated sequentially with HOBt (1.63 g, 12 mmol), EDC (2.37 g, 12 mmol), and DIEA (4.45 mL, 26.4 mmol) at 0 °C. The mixture was warmed to room temperature and stirred overnight. After dilution with EtOAc (300 mL), the whole mixture was washed with 10% citric acid (100 mL), 5% $NaHCO_3$ (100 mL), and brine (100 mL), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash column chromatography with petroleum ether–EtOAc (5:1) to afford **9** as a white solid (3.43 g, 88%).

^1H NMR ($CDCl_3$, 400 MHz): δ: 7.12–7.26 (m, 10H), 5.16 (t, 1H, *J* = 7.4 Hz), 4.86 (dd, 1H, *J* = 5.1, 9.4 Hz), 4.42–4.48 (m, 2H), 3.73 (s, 6H), 3.39–3.54 (m, 4H), 3.20 (dd, 1H, *J* = 7.4, 14.1 Hz), 3.11 (dd, 1H, *J* = 5.1, 13.7 Hz), 2.86 (s, 4H), 2.81 (s, 4H), 2.16–2.22 (m, 2H), 1.83–2.00 (m, 6H), 1.61 (s, 9H), 1.20 (s, 9H)

4.2.6. *N*-Boc-Ala-*N*-Me-D-Phe-L-Pro-OMe (10)

To a solution of **9** (780.4 mg, 2.0 mmol) in CH_2Cl_2 (10 mL) at 0 °C, TFA (10 mL) was added. The mixture was stirred for 30 min

and then evaporated, the residual oil was dissolved twice in CH_2Cl_2 (15 mL) with evaporation each time to give TFA salt **9***, which was used directly in the next step.

A solution of **9*** and Boc-Ala-OH (378.2 mg, 2.0 mmol) in anhydrous CH_2Cl_2 (15 mL) was treated sequentially with HOAt (326.4 mg, 2.4 mmol), EDC (473.5 mg, 2.4 mmol), and DIEA (892.7 μL, 5.3 mmol) at 0 °C. The solution was stirred for 15 min, warmed to room temperature and stirred for another 12 h. The mixture was then treated as described for **8**. The residue was purified by flash column chromatography with petroleum ether–EtOAc (3:1) to afford **10** as a white solid (784.0 mg, 85%).

R_f 0.3 (2:1, petroleum–EtOAc); $[\alpha]_D^{23}$ = +56.4° (*c* 0.03, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): *δ*: 7.13–7.26 (m, 5H), 5.71 (dd, 1H, *J* = 6.4, 9.2 Hz), 5.17 (d, 1H, *J* = 8.4 Hz), 4.45 (t, 2H, *J* = 8.0 Hz), 3.74 (s, 3H), 3.47–3.49 (m, 1H), 3.35–3.40 (m, 1H), 3.22 (dd, 1H, *J* = 6.4, 14.4 Hz), 3.00 (s, 3H), 2.95 (dd, 1H, *J* = 9.6, 14.4 Hz), 2.20–2.25 (m, 1H), 1.82–1.97 (m, 3H), 1.39 (s, 9H), 0.82 (d, 3H, *J* = 7.2 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): *δ*: 173.05, 172.44, 168.35, 155.38, 136.90, 129.62, 128.20, 126.58, 79.50, 59.36, 55.63, 52.16, 30.22, 28.87, 28.32, 25.37, 17.54; ESIHRMS calcd for $C_{24}H_{35}N_3O_6$ $[M+Na]^+$ 484.2418; found 484.2410.

4.2.7. *N*-Boc-Thr-Ala-*N*α-Me-D-Phe-L-Pro-OMe (11)

To a solution of **10** (627.2 mg, 1.36 mmol) in CH_2Cl_2 (20 ml) at room temperature was added TFA (20 ml). The reaction mixture was stirred for 30 min and then reconcentrated from CH_2Cl_2 two times to remove excess TFA. A solution of the TFA salt **10*** and Boc-L-Thr-OH (298.0 mg, 1.36 mmol) in CH_2Cl_2 (10 ml) was treated sequentially with HOAt (222.2 mg, 1.63 mmol), EDC (313.1 mg, 1.63 mmol), and DIEA (570.9 μl, 3.4 mmol). The reaction mixture was stirred at 0 °C for 30 min and then stirred at room temperature for another 8 h. The reaction mixture was treated as described for **9**. The residue was purified by flash column chromatography with petroleum ether–EtOAc (20:1–3:1) to afford **11** as a white foam solid (573.6 mg, 75 %).

R_f 0.2 (2:1, petroleum–EtOAc); $[\alpha]_D^{23}$ = +34.5° (*c* 0.08, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): *δ*: 7.17–7.24 (m, 5H), 6.83–6.97 (br, 1H), 5.69 (q, 1H, *J* = 6.4, 9.2 Hz), 5.37 (d, 1H, *J* = 8.4 Hz), 4.71 (t, 1H, *J* = 8.0 Hz), 4.17–4.34 (m, 1H), 4.02–4.16 (m, 1H), 3.74 (s, 3H), 3.48–3.54 (m, 1H), 3.27–3.41 (m, 1H), 3.21 (dd, 1H, *J* = 6.8, 14.4 Hz), 3.02 (s, 3H), 2.97 (q, 1H, *J* = 9.6, 14.4 Hz), 2.21–2.24 (m, 1H), 1.80–1.97 (m, 3H), 1.44 (s, 9H), 1.11 (d, 3H, *J* = 6.4 Hz), 0.85 (d, 3H, *J* = 6.8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): *δ*: 172.51, 172.42, 170.56, 168.09, 155.98, 136.66, 129.58, 128.26, 126.66, 80.16, 67.27, 59.29, 57.93, 55.66, 52.24, 47.03, 45.76, 34.75, 30.32, 28.87, 28.28, 25.29, 17.96, 16.96; ESIHRMS calcd for $C_{28}H_{42}N_4O_8$ $[M+Na]^+$ 585.2895; found 585.2886.

4.2.8. *N*-Boc-Sta-Thr-Ala-*N*α-Me-D-Phe-L-Pro-OMe (13)

To a solution of **10** (562.3 mg, 1.0 mmol) in CH_2Cl_2 (10 mL) at room temperature was added TFA (10 mL). The reaction mixture was stirred for 30 min and then reconcentrated from CH_2Cl_2 twice to remove excess TFA. A solution of the TFA salt **11*** and **12** (275.2 mg, 1.0 mmol) in CH_2Cl_2 (10 ml) was treated sequentially with DIEA (646.8 μl, 3.8 mmol), HATU (456.0 mg, 1.2 mmol), and HOAt (163.2 mg, 1.2 mmol). The reaction mixture was stirred at 0 °C for 30 min and then stirred at room temperature for another 8 h. The reaction mixture was treated as described for **8**. The residue was purified by flash column chromatography with petroleum ether–EtOAc (2:1) to afford **13** as a white foam solid (597.1 mg, 83%).

R_f 0.15 (2:1, petroleum–EtOAc); $[\alpha]_D^{23}$ = +26.5° (*c* 0.02, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): *δ*: 7.12–7.24 (m, 5H), 6.94 (d, 1H, *J* = 7.6 Hz), 6.77 (d, 1H, *J* = 7.6 Hz), 5.67 (q, 1H, *J* = 6.8, 9.2 Hz), 4.78 (d, 1H, *J* = 9.6 Hz), 4.69 (t, 1H, *J* = 7.2 Hz), 4.40–4.47 (m, 2H), 4.20–4.24 (m, 1H), 3.97 (d, 1H, *J* = 10.4 Hz), 3.37 (s, 3H), 3.56–3.61 (m, 1H), 3.41–3.45 (m, 1H), 3.34–3.39 (m, 1H), 3.22 (dd, 1H, *J* = 6.4, 14.0 Hz), 3.02 (s, 3H), 2.96 (q, 1H, *J* = 9.2, 14.0 Hz), 2.36–2.51 (m, 2H), 2.21–2.24 (m, 1H), 1.77–2.00 (m, 3H), 1.63–1.70 (m, 1H), 1.50–1.57 (m, 1H), 1.44 (s, 9H), 1.31–1.36 (m, 1H), 1.10 (d, 3H, *J* = 6.4 Hz), 0.92 (d, 6H, *J* = 6.4 Hz), 0.86 (d, 3H, *J* = 5.2 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): *δ*: 173.10, 172.56, 172.46, 169.81, 168.08, 156.31, 136.68, 129.58, 128.29, 126.70, 79.37, 70.51, 66.98, 59.27, 56.97, 55.73, 52.40, 52.22, 47.02, 45.91, 41.62, 40.16, 34.82, 30.38, 28.89, 28.42, 25.26, 24.83, 23.14, 22.20, 18.14, 16.88; ESIHRMS calcd for $C_{36}H_{57}N_5O_{10}$ $[M+Na]^+$ 742.3998; found 742.3973.

4.2.9. *N*-Boc-Asn-Sta-Thr-Ala-*N*α-Me-D-Phe-L-Pro-OMe (5)

To a solution of **13** (719.4 mg, 1.0 mmol) in CH_2Cl_2 (10 mL) at room temperature was added TFA (10 mL). The reaction mixture was stirred for 30 min and then reconcentrated from CH_2Cl_2 twice to remove excess TFA. A solution of the TFA salt **13*** and Boc-L-Asn-OH (132.1 mg, 1.0 mmol) in CH_2Cl_2 (10 ml) was treated sequentially with DIEA (646.8 μl, 3.8 mmol), HATU (456.0 mg, 1.2 mmol), and HOAt (163.2 mg, 1.2 mmol). The reaction mixture was stirred at 0 °C for 30 min and then stirred at room temperature for another 8 h. The reaction mixture was treated as described for **9**. The residue was purified by flash column chromatography with petroleum ether–EtOAc (1:1) to afford **5** as a white foam solid (591.7 mg, 71%).

R_f 0.2 (15:1, $CHCl_3$–MeOH); $[\alpha]_D^{23}$ = −25.0° (*c* 0.02, $CHCl_3$); ^1H NMR (MeOD, 400 MHz): *δ*: 7.12–7.27 (m, 5H), 5.67 (dd, 1H, *J* = 3.6, 9.6 Hz), 4.68 (dd, 1H, *J* = 7.2, 14.0 Hz), 4.38–4.46 (m, 2H), 4.34 (d, 1H, *J* = 4.0 Hz), 4.08–4.18 (m, 1H), 3.88–4.02 (m, 2H), 3.72 (s, 3H), 3.37–3.52 (m, 2H), 3.13 (dd, 1H, *J* = 8.0, 14.4 Hz), 3.06 (s, 3H), 2.92 (dd, 1H, *J* = 4.4, 14.0 Hz), 2.73 (dd, 1H, *J* = 8.0, 15.2 Hz), 2.58 (dd, 1H, *J* = 8.8, 15.2 Hz), 2.40 (d, 2H, *J* = 6.0 Hz), 2.19–2.31 (m, 1H), 1.94–2.02 (m, 2H), 1.81–1.91 (m, 2H), 1.54–1.71 (m, 2H), 1.44 (s, 9H), 1.16 (d, 3H, *J* = 1.6 Hz), 0.85–0.94 (m, 9H). ^{13}C NMR (MeOD, 100 MHz): *δ*: 173.72, 173.02, 172.85, 172.60, 172.34, 170.75, 168.90, 156.21, 136.86, 129.34, 127.88, 126.22, 79.54, 70.03, 67.12, 59.50, 58.38, 58.38, 55.89, 53.40, 51.61, 51.30, 51.14, 45.58, 40.17, 39.99, 36.62, 34.21, 29.36, 28.60, 27.37, 24.84, 24.45, 22.48, 21.06, 18.57, 15.40; ESIHRMS calcd for $C_{40}H_{63}N_7O_{12}$ $[M+Na]^+$ 856.4427; found 856.4402.

4.2.10. Grassystatin A (1)

To a solution of **4** (83.3 mg, 0.1 mmol) in CH_2Cl_2 (10 mL) at room temperature was added TFA (10 mL). The reaction mixture was stirred for 30 min and then reconcentrated from CH_2Cl_2 twice to remove excess TFA. Hydrogenation of **5** (54.8 mg, 0.1 mmol) was carried out in CH_3OH–CH_2Cl_2 (1:1,15 ml) in the presence of a catalytic amount of Pd-C (10%) under hydrogen at room temperature. Pd-C was removed by filtration and concentrated under reduced pressure to yield the acid **5**, which was used directly in the next step. A solution of the TFA salt **4*** and acid **5** in CH_2Cl_2 (10 ml) was treated sequentially with DIEA (64.7 μl, 0.38 mmol), HATU (456.0 mg, 0.12 mmol), and HOAt (16.3 mg, 0.12 mmol). The reaction mixture was stirred at 0 °C for 30 min and then stirred at room temperature for another 8 h. The reaction mixture was treated as described for **8**. The residue was purified by HPLC to afford **1** as a white foam solid (53.8 mg, 45%).

R_f 0.3 (10:1, $CHCl_3$–MeOH); $[\alpha]_D^{23}$ = −5.3° (*c* 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): *δ*: 7.54 (d, 1H, *J* = 7.8 Hz), 7.09–7.24 (m, 5H), 7.03 (d, 1H, *J* = 5.8 Hz), 6.36 (br, 1H), 6.04 (br, 1H), 5.65 (dd, 1H, *J* = 6.6, 9.1 Hz), 5.12 (d, 1H, *J* = 3.3 Hz), 4.70–4.79 (m, 2H), 4.66 (d, 1H, *J* = 4.2 Hz), 4.44 (dd, 1H, *J* = 5.8, 8.0 Hz), 4.24–4.37 (m, 4H), 4.01–4.08 (m, 1H), 3.86–3.97 (m, 1H), 3.73 (s, 3H), 3.42–3.50 (m, 1H), 3.31–3.35 (m, 1H), 3.21 (dd, 1H, *J* = 6.6, 14.4 Hz), 3.02 (s, 3H), 2.95 (dd, 1H, *J* = 9.1, 14.2 Hz), 2.84–2.89 (m, 1H), 2.83 (d, 1H, *J* = 10.6 Hz), 2.65 (dd, 1H, *J* = 6.0, 15.2 Hz), 2.57 (dd, 1H, *J* = 8.9,

14.0 Hz), 2.37–2.44 (m, 2H), 2.31 (s, 6H), 2.18–2.24 (m, 2H), 1.95–2.01 (m, 2H), 1.81–1.91 (m, 2H), 1.56–1.72 (m, 5H), 1.05–1.11 (m, 6H), 0.85–1.01 (m, 27H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ: 173.17, 172.68, 172.45, 172.38, 171.80, 170.6, 170.33, 170.09, 169.53, 168.13, 136.68, 129.51, 128.24, 126.64, 78.08, 77.62, 73.78, 70.38, 67.44, 59.24, 58.05, 55.69, 52.78, 52.24, 51.91, 50.38, 47.00, 45.62, 41.12, 40.65, 40.05, 39.84, 37.33, 34.71, 30.18, 29.78, 28.87, 27.57, 25.26, 24.74, 24.47, 23.10, 22.61, 22.23, 22.01, 19.53, 19.17, 18.92, 18.82, 17.76, 16.96, 16.42; ESIHRMS calcd for $C_{58}H_{95}N_9O_{16}$ $[M+Na]^+$ 1196.6794; found 1196.6800.

4.3. Biology

4.3.1. Protease inhibition assays

In vitro enzymatic assays were carried out as described.[5]

4.3.2. Animals

Twelve-week old male and female DO11.10 mice were kindly provided as a gift by Lyle Moldawer, Department of Surgery, University of Florida College of Medicine.

4.3.3. Isolation of DC and T Cells from spleen

Spleens were processed using 100-micron nylon filters and depleted of red blood cells using ACK ammonium chloride lysis buffer. DC were first purified using CD11c+ magnetic beads separation (Miltenyi Biotec), then untouched CD4+ T cells were enriched from the flow-through negative fraction using a CD4+ T cell isolation kit that depletes positively-labeled CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, anti-MHC-class II, and Ter-119 cells (Miltenyi Biotec).

4.3.4. DC

4.3.4.1. T cell co-culture. Drug-treated DC were incubated in complete RPMI with grassystatin A at 37 °C for 1 h, and solvent control DC were incubated in cRPMI with 0.1% DMSO. DC were then washed 3× with PBS, pulsed with either whole ovalbumin (500μg/ml, Sigma) or ovalbumin peptide 323–339 (200 μg/ml, GenScript) for 1–2 h in cRPMI at 37 °C and washed 3×. DC were cocultured with 10^5 CD4+ T cells in 96-well round bottom plates for 72 h followed by 17 h 3H-thymidine (1 μCi/well, Perkin Elmer) incorporation. Cells were harvested and washed using an automated cell harvester and radioactivity was analyzed with a liquid scintillation counter. Uptake of ^{3}H-thymidine is reported as counts per minute (cpm).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.05.077.

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