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Research paper

Rapid preparation of (*3R,4S,5R*) polyhydroxylated pyrrolidine-based libraries to discover a pharmacological chaperone for treatment of Fabry disease

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ABSTRACT

The rapid discovery of a pharmacological chaperone toward human α-Gal A for the treatment of Fabry disease is described. Two polyhydroxylated pyrrolidines with the (*3R,4S,5R*) configuration pattern underwent rapid substituent diversity by conjugating the primary aminomethyl moiety of each with a variety of carboxylic acids to generate two libraries (2 × 60 members). Our bioevaluation results showed one member with the (*2R,3R,4S,5R*) configuration pattern and bearing a 5-cyclohexylpentanoyl group as a substituent moiety possessed sufficient chaperoning capability to rescue α-Gal A activity in the lymphocyte of the N215S Fabry patient-derived cell line and other α-Gal A mutants in COS7 cells.

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1. Introduction

Fabry disease (FD) is an inherited lysosomal metabolic disorder in which activity of lysosomal α-galactosidase A (α-Gal A) is deficient, resulting in the accumulation in cells of neutral glycosphingolipids bearing a terminal α-galactosyl residue, globotriaosylceramide (GL-3) [1]. More than 300 missense mutations in the *GLA* gene have been identified. FD is one of the most common lysosomal storage diseases; the incidence of FD was initially estimated to be between 1 in 47,600–117,000 live births, though this may be an underestimate [2] – a more recent study which screened newborn Taiwanese males found 1 in 1250 to carry the FD gene [3].

Enzyme replacement therapy (ERT) with recombinant human α-Gal A (rh-α-Gal A) is now available for the treatment of FD. Most patients being treated in this way experience clinical improvement or stabilization of the disease [4,5]. However, ERT is severely limited: treatments are expensive and inconvenient; the enzyme protein is unstable in blood (a short-circulating half-life of the properly folded active enzyme *in vivo*); and it cannot cross the blood-brain barrier (BBB) [6–9]. Novel approaches towards the treatment of FD or molecules to improve the efficacy of rh-α-Gal A are therefore urgently needed.

One approach is pharmacological chaperone therapy, in which small molecules are used to assist the folding of mutant enzymes to prevent their degradation by quality control in the endoplasmic reticulum (ER) prior to transfer to the lysosome [10]. 1-Deoxygalactonojirimycin (DGJ), a six-membered azasugar (iminosugar) and potent active site inhibitor of α-Gal A, has been proven as an effective pharmacological chaperone, able to restore mutant α-Gal A in Fabry cells and tissues [11–13]. DGJ was sent for phase III clinical trials, with encouraging results. Most previously reported pharmacological chaperones for FD patients with α-Gal A mutations are either DGJ-based derivatives, or piperidine-type iminosugars [2,12]. In contrast, five-membered iminosugars except 1,4-dideoxy- 1,4-imino-L-allitol (DIA) have not been extensively studied for this role [14].

It is known that though inhibition potency of small molecules does not highly correlate with their chaperoning activities [10,15], the primary inhibition screening of large compound libraries still is a practical way to quickly identify potential hits for further

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chaperoning studies. Unfortunately, a recent high throughput screening of a library of 230,000 compounds did not identify any promising inhibitors or activators of α-Gal A [16]. The scarcity of reported information regarding small molecule α-Gal A inhibitors has likely delayed the development of new pharmacological chaperones for the treatment of FD.

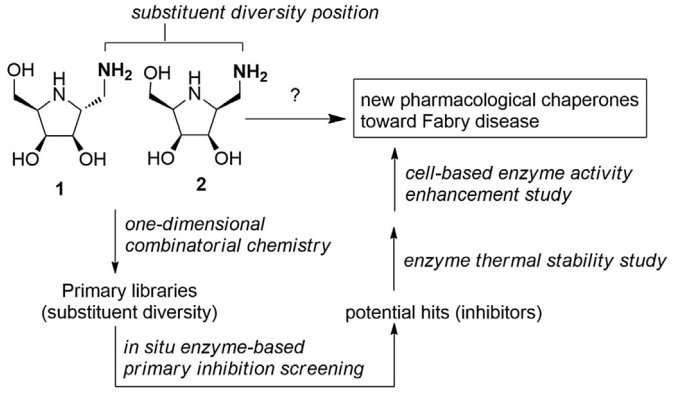
Our research interests include the preparation of various iminosugars as well as the use of these molecules for biological studies [17,18], and our recent preliminary study has found that two pyrrolidines **1** and **2** with the common (*3R,4S,5R*) configuration pattern displayed superior α-Gal A inhibitory activity (>60% inhibition at pH 4.6, 50 μM) than other isomers [19]. Structurally, these two molecules possess a pyrrolidine ring system, four chiral centers, polyhydroxylated groups, and one aminomethyl group. The latter functional group allows us to perform the substituent diversity. However, to the best of our knowledge, the use of pyrrolidine-based small molecules as templates to generate a small molecular library, followed by discovery of new pharmacological chaperones toward cell-based defect α-Gal A for the treatment of Fabry disease has not been extensively investigated.

Herein, we describe a straightforward approach to develop a new pharmacological chaperone for the treatment of Fabry disease. By using two pyrrolidines **1** and **2** with the common (*3R,4S,5R*) configuration pattern as initial scaffolds, rapid structural diversification via a combinatorial chemistry approach will be performed to generate pyrrolidine-based libraries.

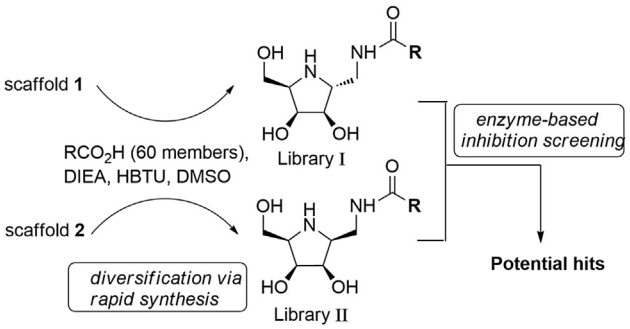
Subsequently, several biological studies including an enzyme inhibition study, enzyme thermal stability study, and cell-based enzyme activity enhancement study will also be investigated (Scheme 1).

2. Result and discussion

Following a method previously reported by us with using L-ribose instead of D-lyxose as starting material, pyrrolidines **1** and **2** were easily prepared from the key intermediate-chiral cyclic nitrone **3** (see Scheme S1) [20,21]. Subsequently, parallel synthesis of a library of amides was performed via an amide bond coupling with the exo amino methyl group of these two scaffolds and each constituent carboxylic acid of a randomly selected, 60-membered acid (Scheme 2), by prior activation of the carboxylic acids in the presence of HBTU (1.5 equiv) and DIEA (3 equiv) in DMSO. After 24 h, the reactions were analyzed and it was found that amine **1** or **2** had been completely consumed and significant amount of desired products formed (estimate conversion) [22–24]. The 60-membered primary Library I (from **1**) and Library II (from **2**) were directly



Scheme 1. General strategy for the development of new pharmacological chaperones toward Fabry disease.



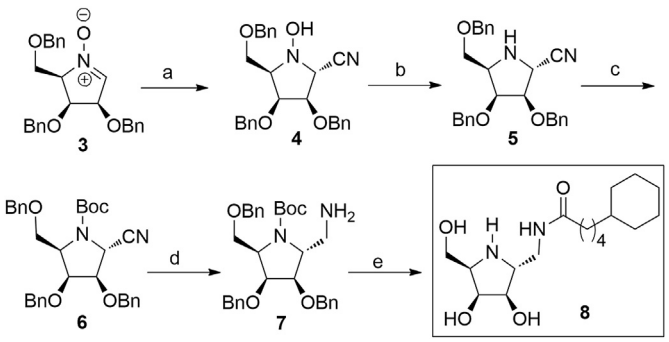
Scheme 2. Parallel synthesis of the primary library **I** and library **II**, followed by *in situ* enzyme-based inhibition study.

evaluated at 20 μM in an α-Gal A enzyme-based inhibition assay without further purification (Scheme 2).

Our primary screening result found five hits that exhibited >60% inhibition in library I (see Fig. S2). In contrast, no promising inhibitor was found in library **II** (see Fig. S2), suggesting that the configuration at the C2 positon plays an important role for inhibition. In order to more carefully characterize these inhibitors, these five hits, **8**–**12**, were re-synthesized and the synthetic routes were slightly modified for more rapid and effective purification.

Scheme 3 depicts our re-synthesis of **8**. The *N*–O bond in **4** was reduced with 0.1 M SmI_2, followed by N-protection with $(Boc)_2O$ to give **6**. The cyanide group was reduced to the primary amine by hydrogenation (Raney Ni/H_2) to give key intermediate **7**. Amide coupling was carried out with **7** and 5-cyclohexylpentanoic acid, followed by $Pd(OH)_2$/C-catalyzed hydrogenation to give a desired **8**, after purification. Likewise, others four inhibitors **9**–**12** were also prepared from amine **7** coupling with the corresponding acids (See in Fig. 1 and Fig. S1). Notably, **9** and **10** were prepared with using the same synthetic approach. For the preparation of **12**, debenzylation under acidic conditions (BBr_3, CH_2Cl_2, -78 °C) was conducted. The inhibition activity of these re-synthesized molecules, as well as reference compounds, DGJ and DIA, is shown in Table 1.

Two pH values were chosen for testing, because a pharmacological chaperone must tightly bind to the enzyme in the ER (neutral pH 7.0) but be released at the lysosome (acid pH 4.6) [10]. At pH 7.0, **8** and **10**–**12** showed *in vitro* rh-α-Gal A inhibition activity in the low-micromolar range, and expectedly all exhibited lower inhibition potency at pH 4.6. Although their inhibition activities were weaker than DGJ (IC_{50} = 0.013 μM) and DIA (IC_{50} = 0.75 μM) at pH 7.0 (Table 1), their potency still was qualified to do further *in vitro* enzyme thermal stability study, and these results might



Scheme 3. Re-synthesis of pyrrolidine **8**. Reagents and conditions: (a) TMSCN, MeOH, 50 °C, 10 h, 78%; (b) SmI_2, HOAc, THF, 0 °C to rt, 1 h, 70%; (c) Boc_2O, Et_3N, CH_2Cl_2, 12 h; (d) Raney Ni, H_2, MeOH, 3 h, 50%, over 2 steps; (e) 1) RCOOH, EDC, DMAP, CH_2Cl_2, 3 h, 2) $Pd(OH)_2$/C, H_2, HCl, 10 h, 48%, over 2 steps.

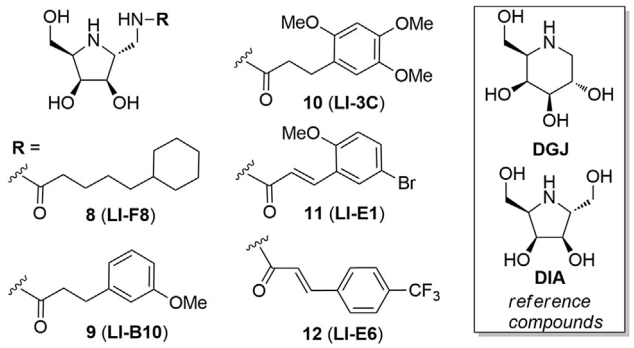


Fig. 1. Structures of five inhibitors **8**–**12**.

point out which ones are able to assist with the protein folding and protect enzymes from thermal denaturalization [25].

The results of this study are shown in Fig. 2. Without adding a compound, rh-α-Gal A activity was found to decrease to 10% of the initial enzyme activity after 30 min of incubation at 48 °C (pH 7.0). All our molecules could protect enzyme activity from thermal denaturation in a dose-dependent manner. Compounds **8** and **12** suppressed the activity reduction better than others at the concentration of 10 μM, and thus merit further study as chaperones. However, rh-α-Gal A activity dramatically decreased to below 40% when a lower compound concentration (1 μM) was treated after 30 min of incubation at 48 °C. Notably, our results showed that the ability of these molecules to stabilize rh-α-Gal A activity from thermal denaturation is moderately correlated with their corresponding inhibition activity.

Next, the chaperoning activity of these molecules in the lymphocytes of the N215S Fabry patient derived cell line was investigated. As shown in Fig. 3, **12** was a moderate pharmacological chaperone and its activity was better than its analogues **10** and **11**. Based on our results, **12** showed a preferable chaperoning performance than DIA but less potent than DGJ. Surprisingly, **8** exhibited the best chaperoning activity in our libraries to enhance the mutant α-Gal A activity in a wide range of concentrations (from 6.25 to 100 μM) and importantly, no significant drop of enzyme activity was observed even at high pulsed concentrations (up to 100 μM). Notably, treatment with **8** (25 μM) led to an approximately 18-fold α-Gal A activity increase compared with untreated cells (Fig. 3). The chaperoning effect of **8** was about 4-fold more potent than that of DGJ at the same concentration (25 μM) and about 13-fold more potent than DIA at the same concentration (100 μM). In addition, the chaperoning activity study of **8** towards the α-Gal A mutants R122H, S148 N, P205T, Q279E, R301Q, and R363C in COS7 cells was also investigated (Fig. 4). Compound **8** showed satisfactory

Table 1
Inhibitory of resynthesized compounds against rh-α-Gal A.

Entry	Compound	IC_{50} (μM)[a]	
		pH 4.6[c]	pH 7.0[c]
1	**9**	19.1 ± 2	13 ± 0.1
2	**10**	30.4 ± 1	7.7 ± 0.2
3	**11**	32.8 ± 4	3 ± 0.7
4	**12**	5.0 ± 0.7	1.1 ± 0.01
5	**8**	10.4 ± 2	2.1 ± 0.05
6	**DGJ**[b]	0.042 ± 0.0003	0.013 ± 0.0007
7	**DIA**[b]	1.0 ± 0.1	0.75 ± 0.09

[a] IC_{50} values were measured in triplicate experiments.
[b] Reference compounds were used for comparison purposes.
[c] 4MU-α-galactopyranoside as a substrate for this assay, K_M = 0.3 mM at pH 4.6 and 2.4 mM at pH 7.0.

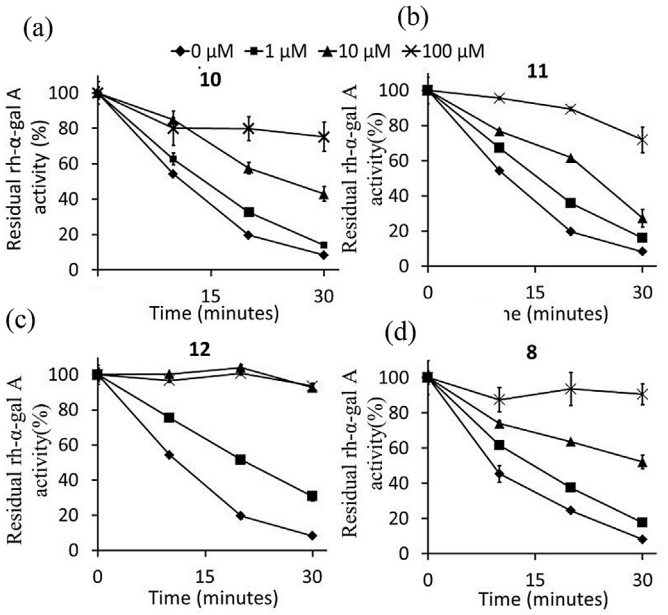


Fig. 2. The effects of compounds **8** (A), **10** (B), **11** (C) and **12** (D) on the thermal stability of rh-α-Gal A. The enzyme activity is compared to unheated cells (normalized to 100%). Each bar represents the mean ± SD of three determinations.

chaperoning behavior towards all human α-Gal A mutants except S148 N in COS7 cells, suggesting it is a versatile small molecule chaperone.

For the enzyme inhibition selectivity study (Table 2), **8** was tested against a panel of glycosidases. Compound **8** (100 μM) was found to be a poor inhibitor against all the glycosidases tested except α-galactosidase (from coffee beans) and rh-α-Gal A. Notably, no serious impairment of lysosomal β-galactosidase activity was observed, even the concentration of **8** was up to 100 μM. Interestingly, our enzyme kinetic studies (see Fig. S3) showed **8** acts as a non-competitive inhibitor with the 4MU artificial substrate, a different behavior with DGJ and DIA which play as active-site inhibitors against α-Gal A. Although we are unable to clearly point out the impacts between native or artificial substrates, our current findings suggest that **8** potentially is a new class of non-substrate competitive pharmacological chaperone toward mutant lysosomal α-Gal A with a high specificity and without affecting activities of other glycosidases including cellular lysosomal β-galactosidase. In addition, **8** didn't exhibit any toxic effects in cell culture at concentrations up to 200 μM (see Table S1).

The durability of chaperone behavior of **8** toward N215S

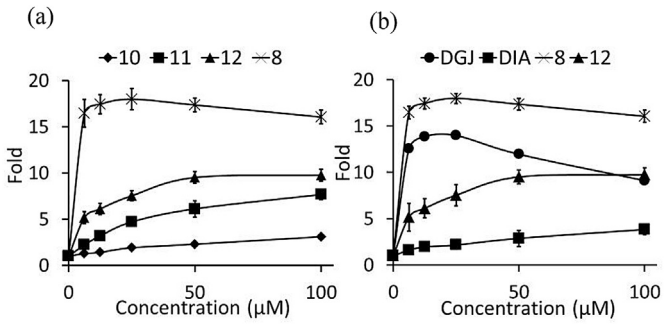


Fig. 3. In vivo study of chaperoning effects of **10**, **11**, **12**, **8**, **DGJ** and **DIA** on N215S lymphocytes. The fold change in enzyme activity is compared to untreated cells (normalized to 1). Each bar represents the mean ± SD of three determinations.

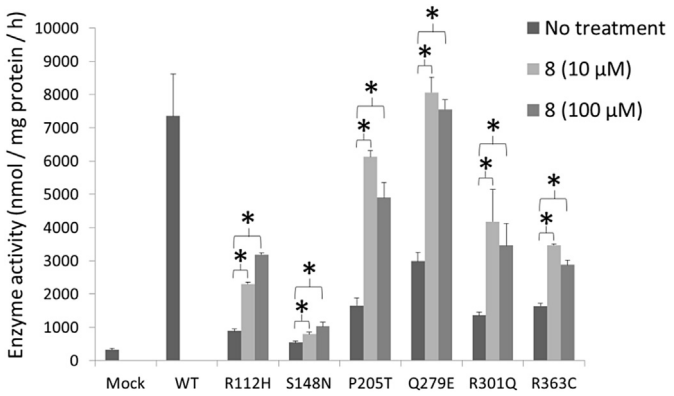


Fig. 4. In vivo study of chaperoning effects of **8** toward various human α-Gal A mutants in COS7 cells. Each bar represents the mean ± SD of three determinations. *$P < 0.05$.

lymphocytes was also investigated [26]. As shown in Fig. 5, N215S lymphocytes were pulsed with **8**, ranging in concentration from 0 to 50 μM, for four days and then washed away of it. Subsequently, cells were incubated in a fresh medium without **8**, and then the enzyme activity was measured on day 0, 2, and 4. To our delight, the shape of the activity curve only slightly shifted with time. All pulsed concentrations resulted in significant enhancement of enzyme activity during the chase period (from 0 to 4 days), indicating that the enzyme synthesized in the presence of **8** was stable in these cells for at least four days. Following the same conditions with the pharmacological chaperone DGJ, the enzyme activity in N215S lymphocytes was still elevated after removal of DGJ but the enhancement enzyme activity was dramatically decreased after 4 days (Fig. 5).

Through our rapid small molecule library synthesis with assistance of *in situ* screening, and following evaluations in this study, **8** was identified as a new pharmacological chaperone toward N215S Fabry patient lymphocyte cells and other human α-Gal A mutants in COS7 cells. Although its inhibition activity against rh-α-Gal A is not as potent as DGJ or DIA, its chaperoning activity toward N215S Fabry patient lymphocyte cells or other human α-Gal A mutants in COS7 cells is satisfactory. A small molecule with proper inhibition potency is sufficient to become a stabilizer or pharmacological chaperone toward mutant enzymes and these obsevations are consistent with previous research results reported by us and others [10,15]. It is thought that active site pharmacological chaperones might impair the activity of the target mutant proteins at higher concentrations, resulting in a narrow therapeutic window. Thus, developing a non-substrate competitive pharmacological chaperone is an attractive approach to potentially provide a wider therapeutic range. However, only limited strategies such as HTS or virtual screening provide information for the development of non-substrate competitive pharmacological chaperones [16,27]. In contrast, more structural designs currently are accessible for the development of competitive inhibitors converting into active site pharmacological chaperones, because their designs can be based on substrate mimics or transition state mimics. Most importantly, our unexpected finding of **8** might shed light on the development of a new series of Fabry non-substrate competitive pharmacological chaperones.

Table 2
Inhibition study of **8** toward various glycosidases.

Enzyme	Inhibition activity[a] (%)	Enzyme	Inhibition activity[a] (%)
α-glucosidase[b]	N.I.[i]	α-galactosidase[f]	94
β-glucosidase[c]	N.I.	rh-α-Gal A[g]	97
β-mannosidase[d]	N.I.	β-galactosidase[h]	N.I.
α-mannosidase[e]	36		

[a] Inhibitors were tested at 100 μM.
[b] From *Bacillus stearothermophilus*.
[c] From Almonds.
[d] From Helix.
[e] From Jack beans.
[f] From coffee beans.
[g] Recombinant human α-Gal A.
[h] From human lysate.
[i] Inhibition activity < 5%.

3. Conclusion

We have successfully discovered a new pharmacological chaperone toward human α-Gal A for the treatment of Fabry disease. Two polyhydroylated pyrrolidine-based scaffolds were applied to generate two libraries via a combinatorial chemistry approach. With these small molecule libraries in hand, several bio-evaluations including the primary enzyme inhibition screening, *in vitro* enzyme thermal stabilization study, and cell-based chaperoning activity study were performed. Compound **8** was found to be a promising pharmacological chaperone toward several α-Gal A mutants in COS7 cells and also in the lymphocytes of the N215S Fabry patient-derived cell line, and it does not affect seriously enzyme activity toward other glycosidases. More detailed examinations such as a binding site study of **8** toward α-Gal A are currently ongoing. The general strategy used in this work is currently being applied to the development of new pharmacological chaperone for other lysosomal storage diseases; the results of these studies will be reported in due course.

4. Experimental section

4.1. General information

Mass spectra were measured with a Bruker BioTOF III (ESIMS) NMR spectra (^{1}H at 600 MHz, ^{13}C at 150 MHz) were recorded in $CDCl_3$ or D_2O solvents. Flash column chromatography was carried out using Merck kieselgel Si60 (40–63 mm). All reagents and solvents were purchased from commercial suppliers, and used without further purification. CC refers to column chromatography (silica gel). Concentration refers to rotary evaporation.

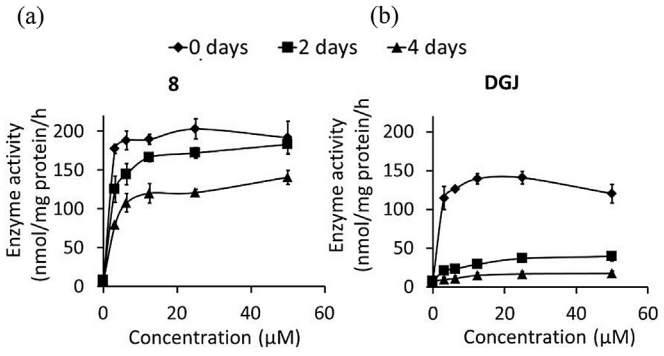


Fig. 5. Durability of chaperoning effects of **8** (a) and **DGJ** (b) toward N215S lymphocytes. Each bar represents the mean ± SD of three determinations.

4.2. Chemical synthesis

4.2.1. (2R,3R,4S,5R)-3,4-bis(benzyloxy)-5-((benzyloxy)methyl) pyrrolidine-2-carbonitrile **(5)**

A mixture of **4** (800.0 mg, 1.8 mmol), 75.0 ml samarium (II) iodide in THF (0.1 M) and HOAc (2.2 ml) were stirred in THF for 1 h at rt. After filtration through a Celite pad, the solvent was removed and the mixture of reaction was extracted with EtOAc and washed with $Na_2S_2O_{3(aq)}$. The organic layers were dried with $MgSO_4$, concentrated, and purified by CC (25% EtOAc in hexanes, silica gel) to give pure product as a colorless oil (540.0 mg, 0.9 mmol, 70%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.55–3.58 (m, 2H), 3.65 (dd, 1H, J = 9.2, 12.2 Hz), 4.07–4.09 (m, 2H), 4.26 (dd, 1H, J = 4.1, 6.6 Hz), 4.51–4.53 (m, 2H), 4.58 (d, 1H, J = 11.6 Hz), 4.65–4.68 (m, 2H), 4.77 (d, 1H, J = 11.6 Hz), 7.32–7.40 (m, 15H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 141.8, 141.7, 140.8, 132.5 × 2, 132.3 × 2, 132.2 × 2, 132.0, 131.8 × 2, 131.7 × 4, 131.6 × 2, 124.4, 87.5, 81.2, 81.0, 80.9, 80.8, 72.7, 62.9, 53.9. HRMS calcd for $[C_{27}H_{28}N_2O_3 + H]^+$429.2173, found 429.2174.

4.2.2. (2R,3R,4S,5R)-tert-butyl 2-(aminomethyl)-3,4-bis(benzyloxy)-5-((benzyloxy)methyl)pyrrolidine-1-carboxylate **(7)**

A mixture of **5** (540.0 mg, 1.3 mmol), Boc_2O (550.0 mg, 2.5 mmol) and triethylamine (0.17 ml, 1.2 mmol) in CH_2Cl_2 were stirred for 12 h at rt. The reaction solution was concentrated and followed by reduction of cyanide group with Raney Ni and $H_{2(g)}$ in MeOH for 3 h. The mixtures were concentrated, and purified by CC (6% MeOH in CH_2Cl_2, silica gel) to give pure product as a colorless oil (340.0 mg, 0.63 mmol, 50% over two steps). ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.43 (d, 9H, J = 16.8 Hz), 2.53 (dd, 1H, J = 8.5, 11.7 Hz), 2.87–2.97 (m, 1H), 3.59 (d, 1H, J = 8.9 Hz),3.74–3.80 (m, 1H), 3.93 (d, 1H, J = 3.8 Hz), 4.14–4.20 (m, 3H), 4.45–4.80 (m, 6H), 7.24–7.33 (m, 15H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 154.3, 138.5, 138.3, 138.2, 128.3 × 4, 128.2, 127.8, 127.6 × 3, 127.5 × 3, 127.3, 127.2, 127.1, 79.9, 79.5, 77.7, 73.1, 72.3, 71.9, 70.2, 64.3, 58.3, 42.6, 28.4 × 3. HRMS calcd for $[C_{32}H_{40}N_2O_5+ H]^+$533.3010, found 533.3015.

4.2.3. 5-cyclohexyl-N-(((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)pyrrolidin-2-yl)methyl)pentanamide **(8)**

A mixture of **7** (33.3 mg, 62.5 nmol), EDC●HCl (35.9 mg, 187.5 nmol), 4-dimethylaminopyridine (7.6 mg, 62.5 nmol) and cyclohexanepentanoic acid (17.3 mg, 93.7 nmol) in CH_2Cl_2 was stirred at rt for 3 h. The reaction was quenched with H_2O and extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and concentrated. The residue was purified by CC (33% EtOAc in hexanes, silica gel) to give an amide intermediate. A mixture of amide intermediate and 37% $HCl_{(aq)}$ in MeOH was refluxed at 50 °C. After 1 h, palladium hydroxide (50.0 mg) was added and the reaction mixture was stirred under a hydrogen atmosphere for anthor 10 h at rt. The reaction mixture was filtered through Celite, and the filtrate was concentrated, and purified by CC (4.5% H_2O and 28% MeOH in $CHCl_3$, silica gel) to give **8** as a yellowish oil (9.9 mg, 30 nmol, 48% over two steps). ^{1}H NMR (600 MHz, D_2O) δ 1.04–1.24 (m, 11 H), 1.49–1.63 (m, 6H), 2.18 (s, 2H), 3.09 (s, 1H), 3.22–3.24 (m, 2 H), 3.35 (d, 1 H, J = 11.6 Hz), 3.52-3–55 (m, 1H), 3.70–3.67 (m, 1H), 3.82 (s, 1H), 4.08 (s, 1H); ^{13}C NMR (150 MHz, D_2O) δ 176.4, 75.2, 71.9, 60.6, 59.9, 59.7, 41.7, 37.4, 37.1, 36.0, 33.2 × 2, 26.6, 26.4, 26.3 × 2, 25.9. HRMS calcd for $[C_{17}H_{32}N_2O_4+ H]^+$329.2435, found 329.2435.

4.2.4. N-(((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl) pyrrolidin-2-yl)methyl)-3-(3-methoxyphenyl)propanamide **(9)**

A mixture of **7** (53.9 mg, 0.1 mmol), EDC·HCl (57.5 mg, 0.3 mmol), 4-dimethylaminopyridine (12.2 mg, 0.1 mmol) and 3-(3-methoxyphenyl)-propionic acid (27.0 mg, 0.15 mmol) in CH_2Cl_2 was stirred at rt for 3 h. The reaction was quenched with H_2O and extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and concentrated. The residue was purified by CC (33% EtOAc in hexanes, silica gel) to give an amide intermediate. A mixture of amide intermediate and 37% $HCl_{(aq)}$ in MeOH was refluxed at 50 °C. After 1 h, palladium hydroxide (50.0 mg) was added, and the reaction mixture was stirred under a hydrogen atmosphere for another 10 h at rt. The reaction mixture was filtered through Celite and the filtrate was concentrated, and purified by CC (4.5% H_2O and 28% MeOH in $CHCl_3$, silica gel) to give **9** as a colorless oil (14.5 mg, 47.0 nmol, 47.1% over two steps). ^{1}H NMR (600 MHz, D_2O) δ 2.63–2.70 (m, 2H), 2.96 (t, 2H, J = 6.1 Hz), 3.03 (m, 1H), 3.19 (s, 1H), 3.32 (dd, 1H, J = 6.0, 14.6 Hz), 3.45 (dd, 1H, J = 3.3, 14.6 Hz), 3.58 (dd, 1H, J = 4.0, 8.7 Hz), 3.64 (dd, 1 H, J = 7.1, 11.2 Hz), 3.77 (dd, J = 6.0, 11.2 Hz), 3.86 (s, 3H), 4.03 (t, 1H, J = 3.3 Hz), 6.91–6.95 (m, 3H), 7.35 (t, 1H, J = 7.5 Hz); ^{13}C NMR (150 MHz, D_2O) δ 176.5, 158.9, 142.2, 129.9, 121.4, 121.2, 114.2, 111.9, 73.5, 71.3, 59.9, 59.7, 55.2, 40.0, 36.8, 31.1. HRMS calcd for $[C_{16}H_{24}N_2O_5+ H]^+$ 325.1763, found 325.1764.

4.2.5. N-(((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl) pyrrolidin-2-yl)methyl)-3-(2,4,5-trimethoxyphenyl)propanamide **(10)**

A mixture of **7** (40.1 mg, 0.075 mmol), EDC·HCl (43.0 mg, 0.225 mmol), 4-dimethylaminopyridine (9.2 mg, 0.075 mmol) and 3-(2, 4, 5-trimethoxy-phenyl)-propionic acid (27.0 mg, 0.1 mmol) in CH_2Cl_2 was stirred at rt for 3 h. The reaction was quenched with H_2O and extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and concentrated. The residue was purified by CC (33% EtOAc in hexanes, silica gel) to give amide intermediate. A mixture of amide intermediate and 37% $HCl_{(aq)}$ in MeOH was refluxed at 50 °C. After 1 h, palladium hydroxide (50.0 mg) was added and the reaction mixture was stirred under a hydrogen atmosphere for another 10 h at rt. The reaction mixture was filtered through Celite and the filtrate was concentrated, and purified by CC (4.5% H_2O and 28% MeOH in $CHCl_3$, silica gel) to give **10** as a yellowish solid (15.1 mg, 0.039 mmol, 52% over two steps). ^{1}H NMR (600 MHz, D_2O) δ 2.49–2.54 (m, 1H), 2.55–2.60 (m, 1H), 2.77–2.83 (m, 2H), 3.06 (t, 1H, J = 7.6 Hz), 3.22–3.37 (m, 2H), 3.47 (d, 1H, J = 12.9 Hz), 3.63 (dd, 1H, J = 3.5, 8.3 Hz), 3.68 (dd, 1H, J = 8.3 Hz, 11.6 Hz), 3.74 (s, 3H), 3.75 (d, 1H, J = 5.7 Hz), 3.77 (s, 3H), 3.79 (s, 3H), 3.97 (t, 1H, J = 3.5 Hz), 6.70 (s, 1H), 6.81 (s, 1H); ^{13}C NMR (150 MHz, D_2O) δ 177.6, 151.7, 147.5, 141.9, 120.2, 114.5, 98.8, 72.5, 70.4, 61.2, 60.2, 58.3, 56.7, 56.4, 55.9, 39.0, 35.5, 25.5. HRMS calcd for $[C_{18}H_{28}N_2O_7+ H]^+$ 385.1975, found 385.1978.

4.2.6. (E)-3-(5-bromo-2-methoxyphenyl)-N-(((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)pyrrolidin-2-yl)methyl)acrylamide **(11)**

A mixture of-*trans*5-bromo-2-methoxy-cinnamic acid(150.0 mg, 0.58 mmol, E1-acid, see Fig. S1), EDC·HCl (123.0 mg, 0.64 mmol) and *N*-hydroxysuccinimide (73.9 mg, 0.64 mmol) in CH2Cl2 was stirred at rt for 2 h. The reaction was quenched with H2O and extracted with CH2Cl2. The organic layer was dried over anhydrous MgSO4 and concentrated. The residue was purified by CC (33% EtOAc in hexanes, silica gel) to give E1-OSu (199.0 mg, 0.56 mmol, 96.6%). A mixture of 1 (19.0 mg, 0.12 mmol) and E1-OSu (45.7 mg, 0.13 mmol) in DMF was stirred at rt for 3 h. The reaction was quenched with NaOH(aq), concentrated and purified by CC (4.5% H2O and 28% MeOH in CHCl3, silica gel) to give 11 (8.0 mg, 0.02 mmol, 17% over two steps). 1H NMR (600 MHz, D2O) δ 3.40–3.43 (m, 1H), 3.49–3.52 (m, 1H) 3.55 (dd, 1H, J = 7.3, 14.3 Hz), 3.65–3.69 (m, 1H), 3.74 (dd, 1H, J = 7.3, 11.4 Hz), 3.86–3.88 (m, 1H), 3.90 (s, 3H), 4.09 (dd, 1H, J = 4.1, 8.6 Hz), 4.26 (t, 1H, J = 4.1 Hz), 6.67 (d, 1H, J = 16 Hz), 6.99–7.02 (m, 1H), 7.54 (dd, 1H, J = 1.7, 8.8 Hz), 7.67–7.72 (m, 2H). 13C NMR (150 MHz, D2O) δ 170.1,

157.0, 135.9, 134.0, 130.8, 124.9, 120.9, 113.9, 112.7, 73.3, 70.3, 61.5, 60.5, 58.2, 55.9, 39.8.HRMS calcd for $[C_{16}H_{21}BrN_2O_5+H]^+$401.0707, found 401.0712.

4.2.7. (E)-N-(((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl) pyrrolidin-2-yl)methyl)-3-(4-(trifluoromethyl)phenyl)acrylamide ***(12)***

A mixture of **7** (35.0 mg, 65.7 nmol), EDC·HCl (37.8 mg, 0.2 mmol), 4-dimethylaminopyridine (8.0 mg) and *trans*-4-(trifluoromethyl)cinnamic acid (21.3 mg, 0.13 mmol) in CH_2Cl_2 was stirred at rt for 3 h. The reaction was quenched with H_2O and extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and concentrated. The residue was purified by CC (33% EtOAc in hexanes, silica gel) to give amide intermediate. A mixture of amide intermediate and BBr_3 (32.0 μl, 0.33 mmol) in CH_2Cl_2 was stirred at −78 °C for 2 h. The reaction mixture was quenched by EtOH, concentrated, and purified by CC (4.5% H_2O and 28% MeOH in $CHCl_3$, silica gel) to give **12** as a yellowish solid (15.2 mg, 42.3 nmol, 64.4% over two steps). ^{1}H NMR (600 MHz, D_2O) δ 3.44 (d, 1H, J = 4.6 Hz), 3.51−3.58 (m, 2H), 3.70 (dd, 1H, J = , 4.6, 13.2 Hz), 3.75 (dd, 1H, J = 7.2, 11.3 Hz), 3.88 (dd, 1H, J = 6.2, 11.3 Hz), 4.10 (dd, 1H, J = 3.9, 8.4 Hz), 4.26 (t, 1H, J = 3.4), 6.75 (d, 1H, J = 15.8 Hz), 7.58 (d, 1H, J = 15.8 Hz), 7.76 (s, 4H); ^{13}C NMR (150 MHz, D_2O) δ 169.0, 140.1, 137.9, 130.6128.3 × 2, 125.8 × 2, 123.1, 122.1, 74.5, 71.3, 60.3, 59.9, 59.5, 41.2. HRMS calcd for $[C_{16}H_{19}F_3N_2O_4+H]^+$361.1380, found 361.1370.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.10.004.

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