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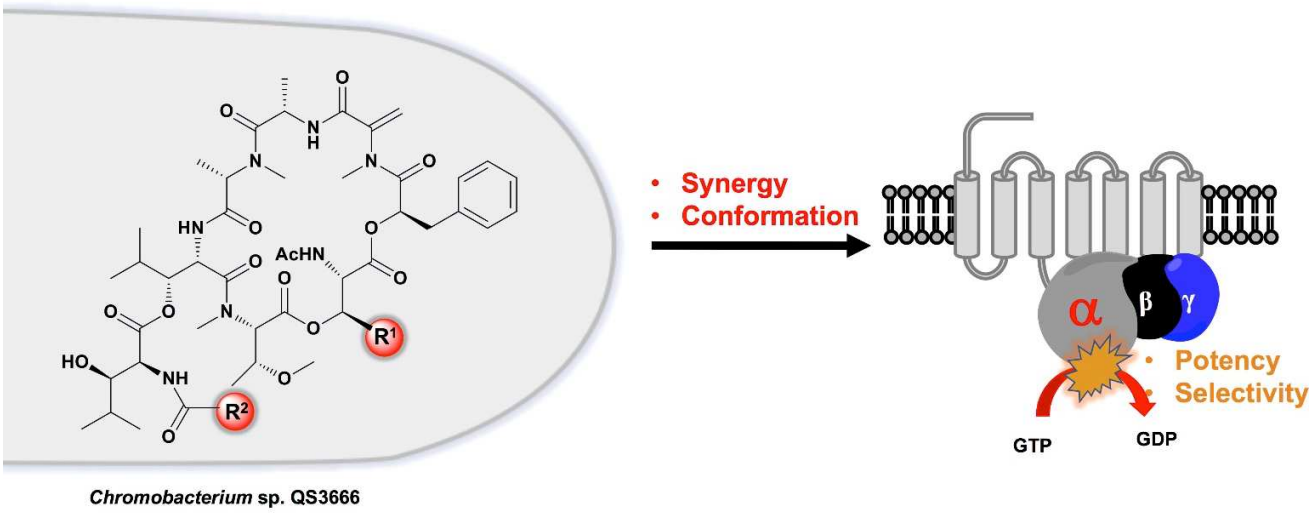

Graphical abstract

Structure–activity relationship and conformational studies of the natural product cyclic depsipeptides YM-254890 and FR900359

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ABSTRACT

G proteins are key mediators in the signaling of G protein-coupled receptors and involved in a plethora of important physiological processes. The natural product cyclic depsipeptides YM-254890 and FR900359 are the only known selective inhibitors of the G_q protein subfamily. So far, all reported YM-254890 and FR900359 analogs show no inhibition of other G protein subtypes except the G_q, G_{11} and G_{14} proteins. Here we report the rationalization of the high potency of FR900359 and efforts towards understanding the G protein subtype selectivity by synthesis of a collection of structurally and stereo-chemically diverse analogs of YM-254890 using an efficient synthetic protocol. We performed the first conformational study of YM-254890 in aqueous solution by NMR spectroscopy and replica exchange molecular dynamics, which suggested that the combined contribution of residues with appropriate size, stereochemistry and conformational stability are critical for inhibitory potency. Moreover, in addition to the fit of the binding pocket, more factors should be taken into consideration for the development of compounds targeting other G proteins.

INTRODUCTION

G protein-coupled receptors (GPCRs)[1] transduce extracellular stimuli into cellular responses, where the heterotrimeric guanine nucleotide binding proteins (G proteins)[2] are the key intracellular mediators and involved in a plethora of physiological process.[3, 4] The binding of an agonist to a GPCR introduces a conformational rearrangement in the receptor, which activates the intracellular G protein by exchanging the guanine nucleotide. This leads to dissociation of the heterotrimeric α, β and γ subunits of the G protein, which regulate downstream effectors and in turn trigger cellular responses through downstream signaling cascades.[5]

GPCR signaling plays a critical role in drug discovery and in fact approximately 34% of all marketed drugs target GPCRs,[6] which are encoded by more than 800 genes and comprise the largest family of membrane protein receptors in the human genome.[7] The role of G proteins in GPCR signaling pathways however is not well understood, primarily due to the lack of selective G protein modulators. The GPCR-G protein coupling is complex as there are 16 different G protein subtypes and either one of these can interact with a range of different GPCRs. The G proteins are divided into four subfamilies according to the sequence of their α subunits, named $G\alpha_{q/11}$, $G\alpha_{s}$, $G\alpha_{i/o}$, $G\alpha_{12/13.}$ Consequently, selective G protein modulators are in great demand to gain an improved understanding of G protein-mediated signaling.[8] However, only very few selective G proteins modulators have been reported, with pertussis toxin and cholera toxin[9, 10] as notable examples, being a selective $G_{i/o}$ inhibitor and G_{s} activator, respectively.

The two natural products YM-254890 and the structurally very similar FR900359, also known as UBO-QIC, were discovered during screening for platelet aggregation inhibitors. Specifically, YM-254890 (**1**, Figure 1a) was isolated from *Chromobacterium* sp. QS3666[11] and FR900359 (**2**, Figure 1a) was isolated from the plant *Ardisia crenata*.[12] Subsequently, YM-254890 and FR900359 were demonstrated as selec-

tive inhibitors of $G_{q/11}$-mediated signaling and used as unique pharmacological tools for the studies of $G_{q/11}$-mediated cell responses, and very recently also as templates in drug discovery efforts related to both cancer[13] and airway diseases.[14]

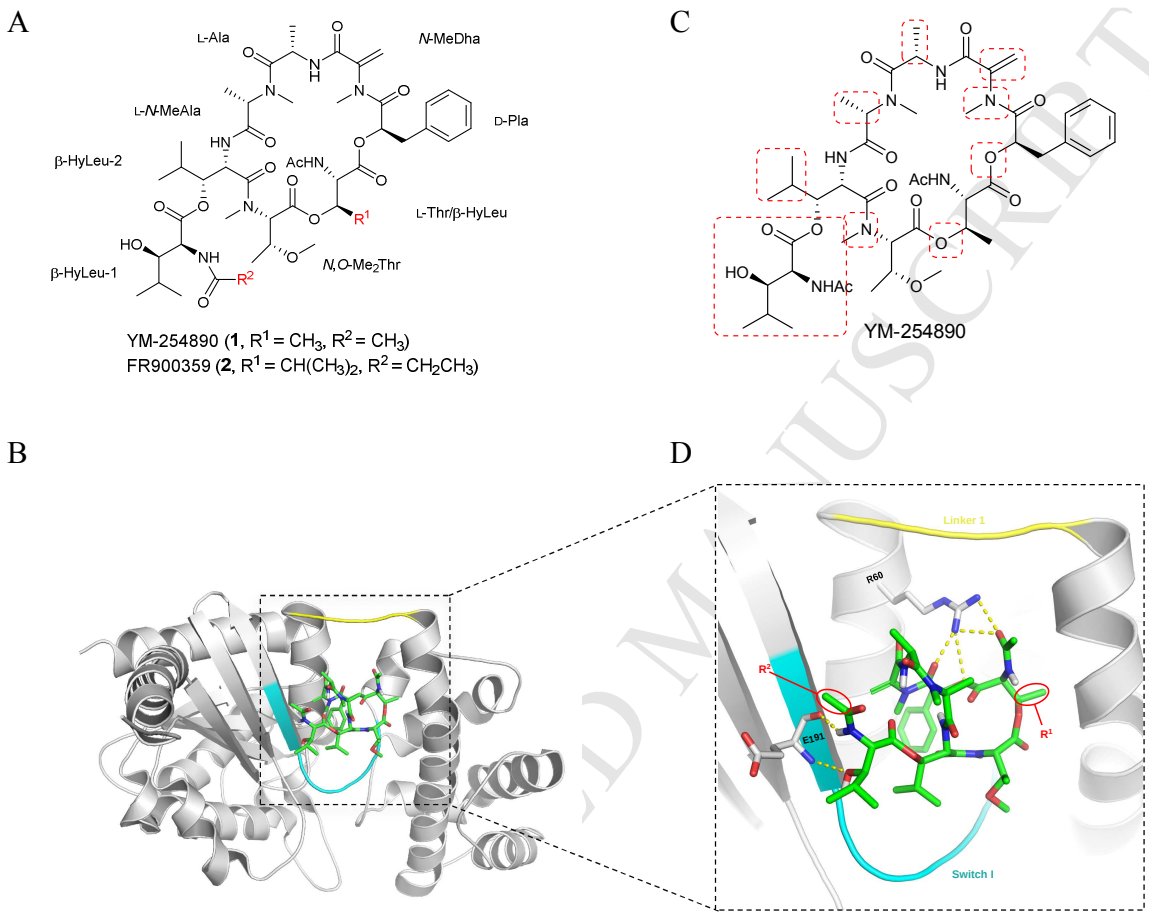


Figure 1. Structures and overview of chemical modifications of YM-254890 and FR900359: (A) Structures of YM-254890 (**1**) and FR900359 (**2**); (B) X-ray crystal structure of YM-254890 (PDB ID: 3AH8) (**1**, green carbons) bound to a chimeric $G_{q/i}$ protein (grey cartoon); (C) Overview of the modification sites of YM-254890. All the modification sites are highlighted (in red dash boxes); (D) Close up of the YM-254890 binding pocket (**1**, green carbons) of the $G_{q/i}$ protein (grey cartoon and surface) with highlighted important residues. The side chain of Thr (R^1, red solid box) and *N*-acyl group of β-HyLeu-1 (R^2, red solid box) of YM-254890, selected important residues (R60 and E191, white carbons) of the $G_{q/i}$ protein involved in the interaction between YM-284890 and linker I (yellow cartoon) and Switch I (cyan cartoon) are shown. Hydrogen bonds are presented as yellow dotted lines.

Structural elucidation revealed that YM-254890 is a macrocyclic depsipeptide comprising seven amino acids (alanine (Ala), threonine (Thr), *N*-methylalanine (*N*-MeAla), *N*-methyldehydroalanine (*N*-MeDha), two β-hydroxyleucines (β-HyLeu-1 and β-HyLeu-2), and *N*,*O*-dimethylthreonine (*N*,*O*-Me_2Thr)), as well as an α-hydroxy acid (D-3-phenyllactic acid (D-Pla)), three ester bonds and two acetyl groups (**1**, Figure 1a).[15] FR900359 differs from YM-254890 by only one amino acid (*N*,*O*-Me_2Thr to β-HyLeu) and one acetylation of β-HyLeu-1, where an *N*-acetyl group is replaced with a *N*-propionyl) (**2**, Figure 1a). Notably, the X-ray crystal structure of YM-254890 bound to a chimeric $G\alpha_{q/i}\beta\gamma$ protein (Figure 1b) revealed the molecular basis of the inhibition of $G_{q/11}$-mediated signaling.[16] It showed that YM-254890 bound to the Gα subunit and blocked the fundamental guanine nucleotide exchange from GDP to GTP by stabilization of the GDP-bound inactive state of the G_q protein.[16]

Although, YM-254890 and FR900359 attracted wide attention as tool compounds in $G_{q/11}$-mediated signaling studies, neither of the two compounds have, until very recently, been generally available.[17] The value and shortage of YM-254890 and FR900359 sparked attempts to achieve total synthesis,[18, 19] and the total synthesis of both YM-254890 and FR900359 was eventually achieved in 2016,[20] 28 years after the original isolation of FR900359.

With a general synthetic strategy in hand, we have subsequently performed systematic structure-activity relationship (SAR) studies of YM-254890 focusing on elucidating the molecular details of the interaction between YM-254890 and $G\alpha_{q/11}$. These and previous studies employing semi-synthetic and natural YM-254890 analogs[19-22] demonstrate that fairly diverse modifications of YM-254890 have been achieved, including changes in the backbone, modulation of various sub-regions and side-chains (Figure 1c, Supplementary Figure 1). Together, this shows that the structural integrity of YM-254890 is critical for biological activity, as changes particularly in the backbone and the *N*-MeDha residue are not well tolerated, while certain modifications in the side-chains are tolerated. So far, FR900359 is the only

compound that is slightly more potent (3-fold) than YM-254890.[20] Moreover, all analogs generated so far show exquisite selectivity for inhibition of $G_{q/11}$-mediated signaling.[20, 22]

Herein we report the design and synthesis of five new analogs focusing on the modification of the structural moieties that differ between YM-254890 and FR900359 to rationalize the difference in potency of $G_{q/11}$ signaling inhibition. These analogs were pharmacologically characterized by evaluating their inhibitory selectivity for $G_{q/11}$relative to G_s- and $G_{i/o}$-mediated signaling. Moreover, two additional new analogs were generated on the basis of sequence and structure diversity of the binding sites area between G_s and G_q proteins in an attempt to understand the subtype selective G protein inhibition.

RESULTS

Design of analogs. The X-ray crystal structure of the chimeric heterotrimer $G\alpha_{q/i}\beta\gamma$-YM-254890 complex showed that both the side chain of Thr (R^1, Figure 1a) and the *N*-acyl group of β-HyLeu-1 (R^2, Figure 1a) where YM-254890 and FR900359 are structurally different, are located at the exterior of the YM-254890 binding pocket in the $G\alpha_q$ subunit (Figure 1d) and has only limited direct interaction with $G\alpha_q$.[16] Thus, we designed three new analogs focusing on the modification of R^1 (Figure 1a) and two new analogs targeting R^2 (Figure 1a). Specifically, we examined the importance of the steric hindrance of R^1 by replacing Thr with the less bulky serine (Ser) (YM-26 (**3**), Figure 2a) or more bulky β-HyLeu (YM-27 (**4**), Figure 2a) and examined the influence of the stereochemistry of R^1 by changing Thr to the L-*allo*-Thr epimer (YM-28 (**5**), Figure 2a). We also examined the effect of introducing an electron-rich hexanoyl moiety at R^2 site (YM-29 (**6)**, Figure 2a) and the importance of the steric hindrance of R^2 by replacing the acetyl group with the bulkier benzoyl group (YM-30 (**7**), Figure 2a).

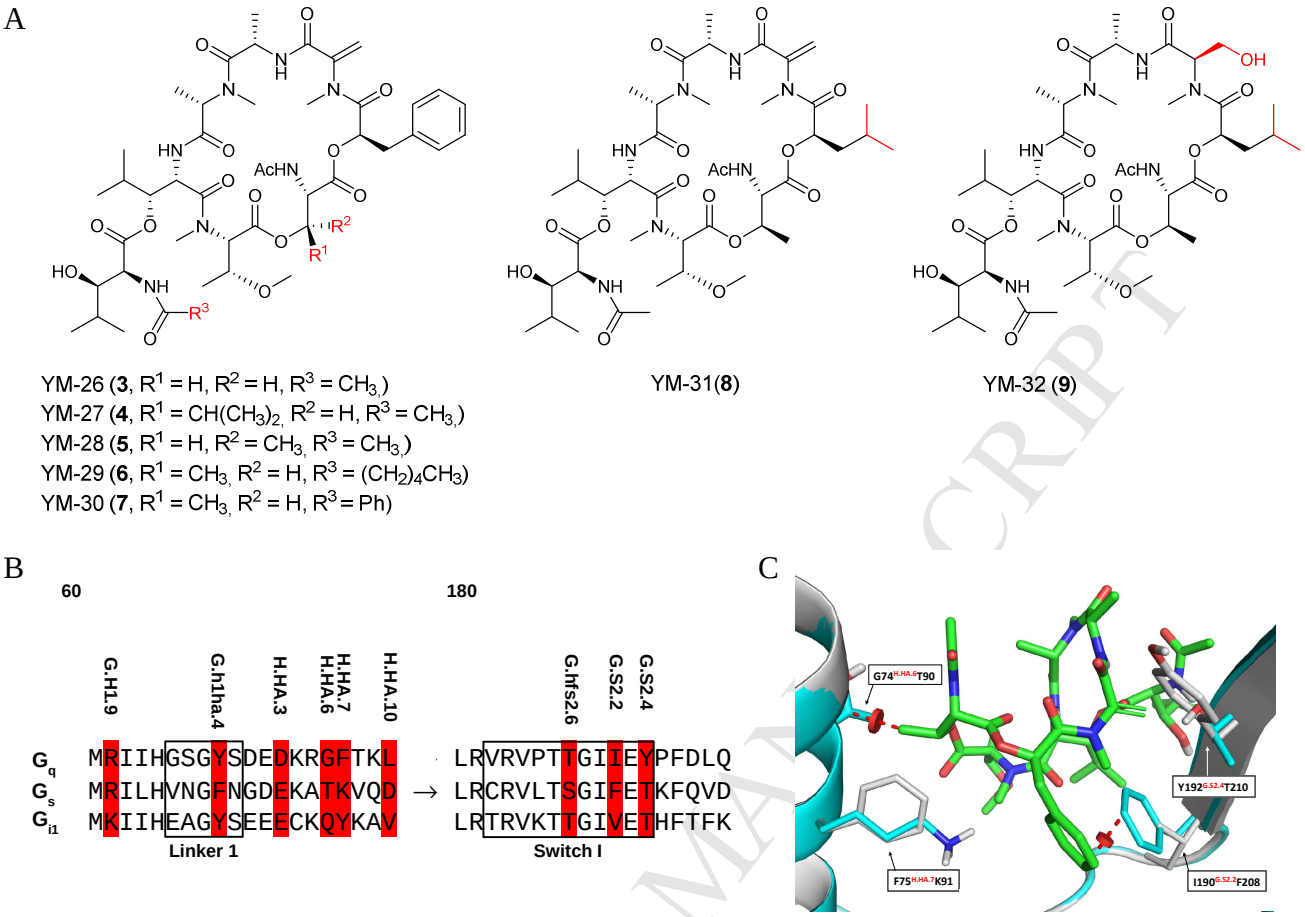


Figure 2. Design basis and structure of new analogs **3**-**9**:(A) structures of new analogs **3**-**9**;(B) sequence alignment of G protein α subunits around the YM-254890 binding site. Switch I and linker 1 regions are outlined in black. The residue numbering is based on $G\alpha_q$. The diverse residues between $G_q/G_s/G_i$ making up the YM-254890 binding site are highlighted with a red background; (C) illustration of the protein-protein interactions between YM-25489 (**1**, green carbons) and G_s/G_q (white cartoon), where important residues of G_s (T90, K91, T210 and F208, cyan carbons) were modeled into the corresponding positions of G_q (G74, F75, Y192 and I190, white carbons, PDB: 3AH8). The envisioned steric clashes and hydrogen bond are shown as red dotted lines and yellow arrow, respectively. Residue positions across G protein subtypes are used with the generic numbering system from the CGN server (www.mrclmb.cam.ac.uk/cgn/lookup_human_sequence.html).

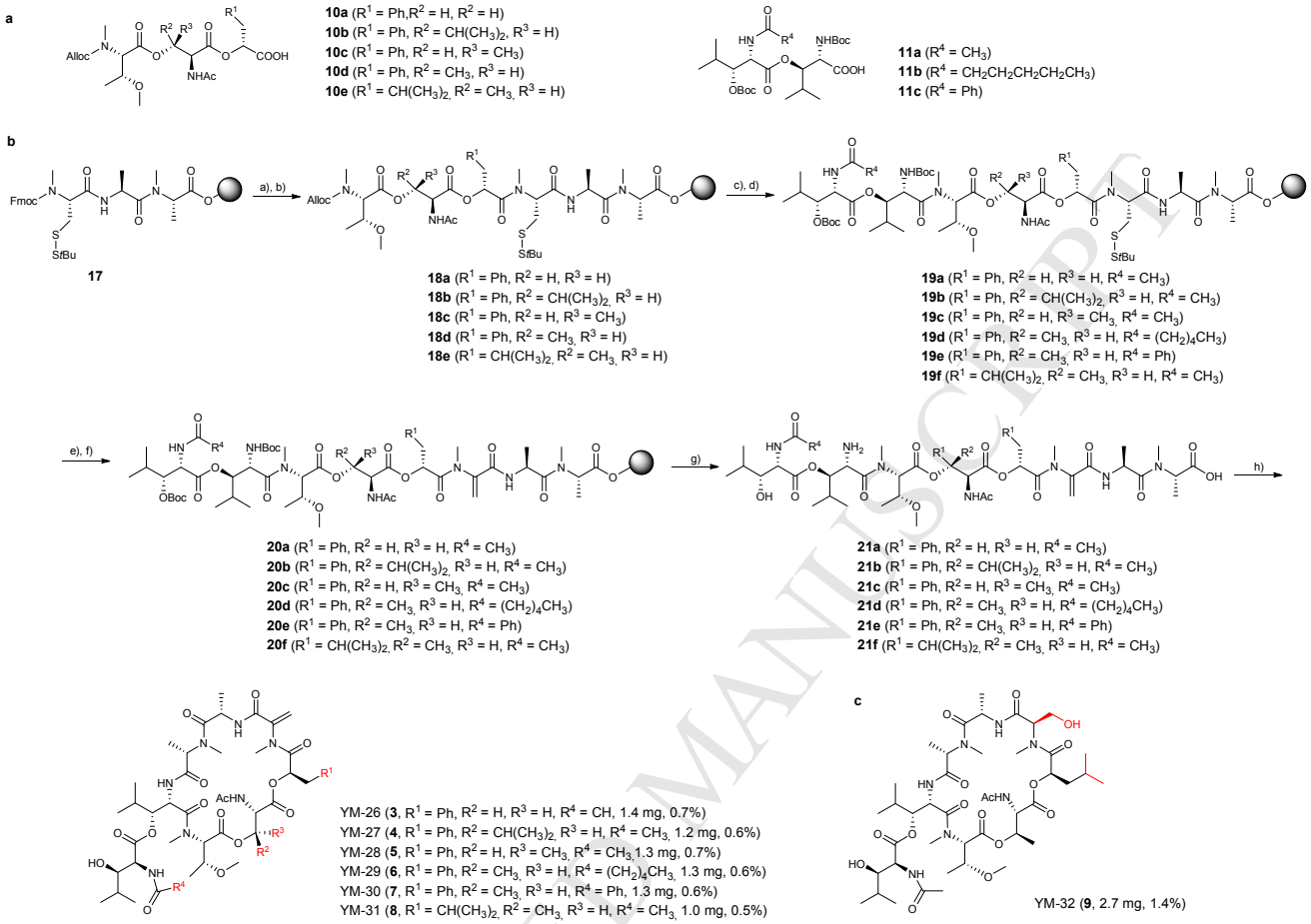
A sequence alignment of G proteins reveals that several of the residues forming the YM-254890 binding site are conserved between $G\alpha_q$, $G\alpha_s$, and $G\alpha_i$ proteins (Figure 2b), but also that residues in certain residues differ (highlighted in red, Figure 2b) and these residues may be responsible for the subtype selec-

tivity of YM-254890. Particularly two positions were considered in this study for the understanding of selectivity: Ile190 in $G\alpha_q$ is involved in defining the hydrophobic pocket accommodating the D-Pla side chain.[16] The corresponding Phe208 in $G\alpha_s$ show moderate steric clashes with the D-Pla aromatic side chain in YM-254890 and are in too close proximity to form aromatic (π-π) interactions, thus the Ile-to-Phe exchange may significantly decrease binding (Figure 2c). Thus, in order to relieve the steric clashes as well as maintain the hydrophobicity of YM-254890, the D-Pla in YM-254890 was replaced by D-leucic acid to potentially favor $G\alpha_s$ binding (**8**, Figure 2a). As multiple alterations are likely required for achieving any $G\alpha_s$ binding, the *N*-MeDha in YM-254890 was simultaneously modified with the replacement of *N*-Me-D-Ser (**9**, Figure 2a) to potentially add a hydrogen bond with Thr210 (Figure 2c). In addition, Gly74 in $G\alpha_q$ is replaced by Thr90 in $G\alpha_s$ predicted to clash with YM-254890 and the removal or inversion of the side chain of Thr (R^1, Figure 1a) in YM-254890 (**3** or **5**, respectively, Figure 2a) could relieve steric clash whilst analog **5** (Figure 2a) might also utilize the additional space around Lys91 in $G\alpha_s$ by favorable van der Waals interactions with parts of the hydrophobic carbon chain of the lysine (Figure 2c).

Synthesis of compounds 3-9. The building block based synthetic approach applied in the generation of YM-254890[20] was used here for the synthesis of the new analogs **3-9**. First, the ester building blocks (**10** and **11**, Scheme1a) were prepared from the synthesized or commercially available fragments D-Pla/D-leucic acid[23], Ser/Thr/L-*allo*-Thr, *N*,*O*-Me_2Thr[24] or β-HyLeu with the use of standard coupling conditions and fine-tuning of protecting groups (Supplementary Figure 2).[25, 26] With all building blocks in hand, we subsequently started the synthesis of compounds **3**-**9** on solid phase (Scheme 1b). The generation of analogs **3**-**8** commenced from the common resin-bound peptide **17**, which is synthesized by the standard solid-phase peptide synthesis (SPPS) procedure using the 2-chlorotrityl resin[27] and *N*-MeCys(S*t*Bu)-OH[28] as the *N*-MeDha precursor (Scheme 1b). Modifications were introduced via succes-

sive couplings of building blocks **10a-d** and **11a-c** to **17**, providing the full-length linear resin-bound depsipeptides **19a-e** (Scheme 1b). Next, after the generation of *N*-MeDha residue through a reduction-bisalkylation-elimination manipulation,[29, 30] cleavage of the resin afforded the linear precursors **21a-e** (Scheme 1b). Finally, the desired analogs **3-8** (Scheme 1b) were generated by macrolactamizations of **21a-e**, which were performed in solution under highly dilute conditions with HATU/collidine as coupling reagent/base pair. The synthesis route of analog **9** was similar with the replacement of *N*-MeCys(S*t*Bu)-OH, building blocks **10a-d** to *N*-Me-D-Ser(Bn)-OH, **10e**, respectively. In addition, the synthetic steps of *N*-MeDha were removed and an additional deprotection of the benzyl group at the last step was added (Supplementary Figure 3, Scheme 1c). The purity of analogs was determined by HPLC and the structure characterization was confirmed by ^{1}H- and ^{13}C-NMR spectroscopy, optical rotation, and HRMS. Analogs were generated in sufficient quantities (1.3-2.3 mg, 0.5-1.4% overall yields) with high purity (93-98%) to be used for pharmacological characterization.

Scheme 1. Total synthesis of new YM-254890 analogs.



[a]Structures of ester building blocks, **10a**-**10e** and **11a**-**11c**, used for the synthesis of analogs **3**-**9**; [b]Total synthesis of analogs **3**-**8**. a) piperidine/DMF (1:4), RT; b) **10a**-**10d**, HATU, collidine, DMF, RT; c) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2 ,N_2, RT; d) **11a**-**11c**, HATU, collidine, DMF, 35°C; e) DTT, DIEA, DMF, RT; f) 1,4-dibromobutane, K_2CO_3, DMF, RT; g) TFA/TIPS/CH_2Cl_2 (19:0.5:0.5, v/v), RT; h) HATU, collidine, DMF, RT. [c]Structures and yield of analog **9**.

Pharmacological characterization. We then examined the inhibitory effect of YM-254890, as well as the seven new analogs, on $G_{q/11}$-, G_s- and $G_{i/o}$-mediated signaling as previously described.[20] In general, all analogs showed decreased inhibitory activity of $G_{q/11}$-mediated signaling compared to YM-254890 (Table 1) and maintained $G_{q/11}$ selectivity (Supplementary Table 1).

Table 1. Inhibition of $G_{q/11}$-mediated signaling.[a]

Cmpd	IC_{50} (μM) [b]	95% CI (μM) [c]	pIC_{50} ± SEM [d]
YM-254890 (**1**)	0.15	0.10-0.23	6.81±0.08
YM-26 (**3**)	1.89	0.95-3.81	5.72±0.07
YM-27 (**4**)	0.31	0.21-0.46	6.51±0.04
YM-28 (**5**)	>50	-	-
YM-29 (**6**)	0.88	0.40-1.97	6.05±0.08
YM-30 (**7**)	2.34	0.27-20.7	5.63±0.22
YM-31 (**8**)	3.69	2.50-5.52	5.43±0.04
YM-32 (**9**)	>50	-	-

[a]The inhibition of carbachol-induced IP_1 production in CHO cells that stably express the M1 muscarinic receptor. [b]IC_{50} values represent the mean of three independent experiments performed 3-10 times. [c]The 95 % confidence intervals for the IC_{50} values. [d]The corresponding pIC_{50} values including the standard error of mean (SEM).

In the series of compounds **3**-**7** in which the two sites (R^1 and R^2, Figure 1a) distinguishing YM-254890 and FR900359 were modified, the modification of R^1 group (**3**-**5**, Table 1) led to dramatic changes of potency than the modification of R^2 group (**6**-**7**, Table 1). Specifically, the removal of the R^1 group by introducing a less bulky Ser residue instead of the Thr resulted in a 13-fold loss of potency (**3**, Table 1), whereas the replacement of Thr with more bulky β-HyLeu led to a 2-fold decrease in potency (**4**, Table 1). The most distinct effect was observed when Thr was replaced with the L-*allo*-Thr epimer, leading to no detectable $G_{q/11}$ inhibition (**5**, Table 1). Whereas, modification of the R^2 group seems to be better tolerated, both analogs **6** and **7** (Table 1) maintained inhibitory potency towards $G_{q/11}$-mediated signaling. Introduction of the electron-rich hexanoyl moiety led to a 6-fold loss of potency (**6**, Table 1) and the replacement of the acetyl group to the bulkier phenyl group resulted in a 16-fold decrease in potency (**7**, Table 1).

Conformational analysis. In general, YM-254890 and analogs exist as epimeric mixtures of typically two conformers in different ratios in NMR solvents. However, we had previously noted that one analog with an L-Ala to D-Ala modification in a position facing outside of the binding pocket, YM-17 (Figure 3b), existed as one conformer only as observed by NMR (in $CDCl_3$), but also was with low potency (IC_{50} = 36.6 μM, Figure 3b), with no obvious structural explanation for the great loss in potency.[22] Here we observed that the same was true for analog **4** (Figure 2a), with only one conformer, however in contrast to YM-17, **4** is almost equipotent YM-254890. We considered whether these two compounds might represent a biological active and inactive conformer, respectively and decided to perform a conformational analysis of YM-254890 in aqueous solution. This was done to examine the importance of conformation for selective inhibition of $G_{q/11}$-mediated signaling, where analog **4** (Figure 2a) and YM-17 (Figure 3b) were used as the positive and negative control, respectively.

We employed variable temperature ^{1}H NMR spectroscopy to probe the intramolecular hydrogen-bonding profile of YM-254890 and used replica exchange molecular dynamics[31, 32] to reveal the conformational landscape of YM-254890 (Figure 1a), analog **4** (Figure 2a) and YM-17 (Figure 3b) in solution. A ratio of conformers (3:1) was observed by NMR for YM-254890 in water, with notable conformational distinctions between the major and minor conformer. Temperature-chemical shift coefficients (T_{coeff}) revealed the Ala-NH as the single strong internal hydrogen bond donor in the major conformer, whereas Thr-NH interacted in intramolecular hydrogen bonding in the minor conformer (Supplementary Figure 4). Based on replica exchange simulations, the major conformer of YM-254890 and analog **4** seemed to adopt a strikingly similar conformation with Ala-NH hydrogen bonding to the carbonyl oxygen of D-Pla (Figure 3d) in agreement with the T_{coeff} (Supplementary Figure 4). Interestingly, YM-17 adopted a much different conformation with *cis-trans* inversion around the *N*-MeAla/D-Ala amide bond accommodating internal hydrogen bonding of Thr-NH to the Ala carbonyls. Lesser-occupied conforma-

tional clusters of YM-254890 seemed to adopt a similar conformation suggesting that YM-17 accommodates a conformation similar to the minor conformer of YM-254890 (Figure 3e). The Thr/Ac-2 in YM-254890 is involved in several hydrogen bonds with Arg60 of the G protein in the X-ray crystal structure.[16] This suggests that a large energetic penalty is required to break the intramolecular hydrogen bonds of YM-17 in solution to make the Thr/Ac-2 available for interactions with Arg60, thus allow the analog to adapt to a binding conformation of YM-254890, which is likely also accountable for the loss in potency in YM-254890 relative to FR900359. Overall, this suggests that conformational stability is crucially important for their inhibitory potency and, thus, design of YM-analogs.

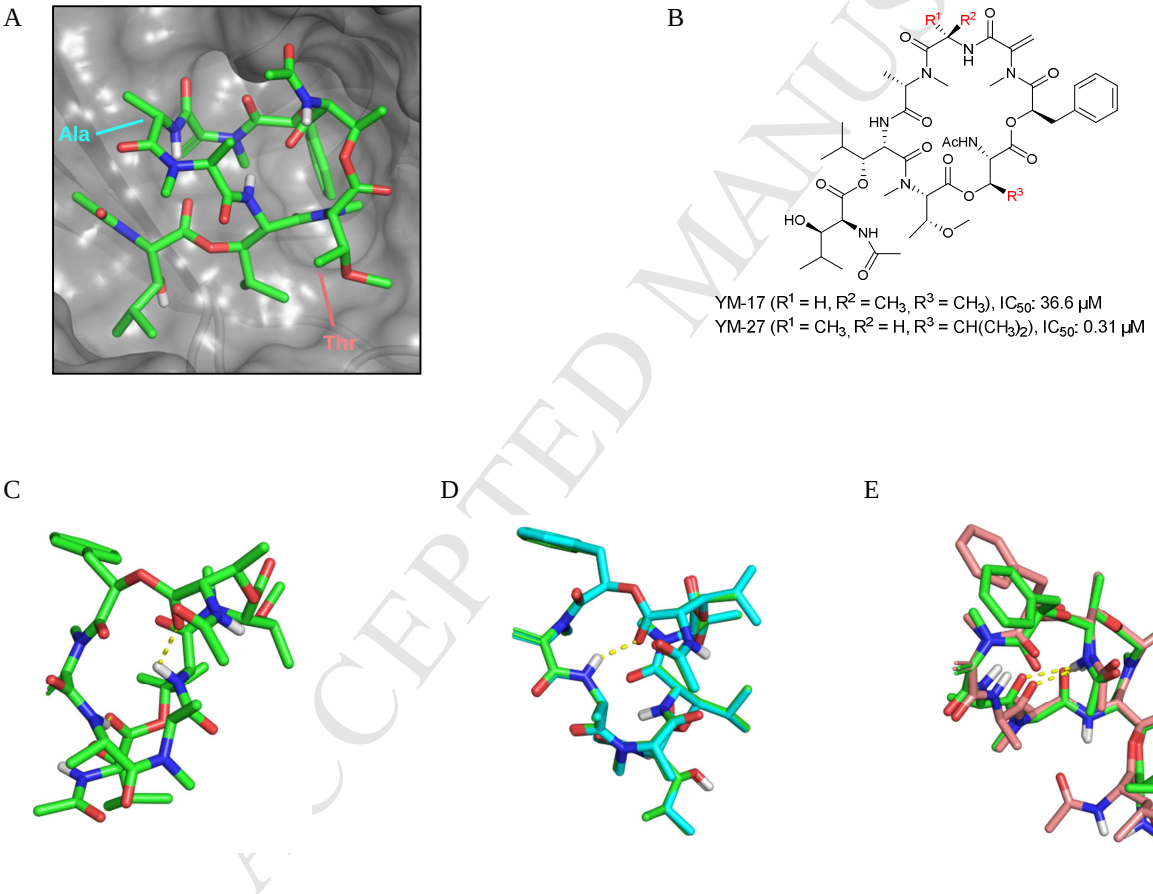


Figure 3. Replica exchange molecular dynamics of YM-254890 analogs: (A) X-ray crystal structure of YM -254890 (green carbon, PDB-ID 3AH8) in the $G\alpha_{q/i}$ binding pocket (grey surface). Residues where minor chemical changes led to single-conformer analogs are highlighted; (B) structure and IC_{50} values of the single-conformer analogs YM-17 and YM-27; (C)

binding-conformation of YM-254890 from the X-ray crystal structure (hydrogen bonds shown in yellow); (D) highest occupied structural cluster of YM-254890 (green carbon) and YM-27 (cyan carbon) 200 ns REMD simulations with Ala-NH as the primary internal hydrogen bond donor; (E) REMD cluster of YM-17 (salmon carbon) superimposed with the cluster of the proposed minor conformer of YM-254890 (green carbon) with Thr interacting as the major internal hydrogen bond donor.

DISCUSSION

Notably, analog **4** (Figure 2a) and the previously synthesized YM-10 (Supplementary Figure 1) are both structural hybrids of YM-254890 and FR900359, still both were 2-fold less potent than YM-254890.[20] However, FR900359 is 3-fold more potent than YM-254890,[20] which combines the modifications of analog **4** and YM-10. This indicates that the extra carbons of the R^1 and R^2 groups in FR900359 have synergistic effects to improve the potency. According to our previously published study,[20] it was proposed that the extra carbons in FR900359 had small favorable van der Waals interaction with the $G\alpha_q$ surface, which could explain the improvement of potency. In addition, from the X-ray crystal structure of the chimeric heterotrimer $G\alpha_{q/i}\beta\gamma$-YM-254890 complex, the free space around R^2 appeared to be larger than R^1 that is located close to the Gα helical domain (Figure 1d), which might be responsible for the less dramatic potency changes at the R^2 site. Interestingly, the strongly reduced potency of analogs with different stereochemistry, analog **5** (Table 1) and YM-17 (Supplementary Figure 1) in the present and previous[22] SAR studies, respectively, shows that a proper stereochemical arrangement of YM-254890 is required for its biological activity.

Compounds **8** and **9** were designed to examine the selectivity between $G_{q/11}$ and G_s (Supplementary Table 1) and potentially increase affinity towards G_s, however both compounds did not inhibit G_s and **9** did not inhibit $G_{q/11}$ either. The lack of inhibition at G_s may be due to several reasons, such as the conformational changes of new analogs, additional G_s binding pocket residues blocking the analogs or functional determinants such as efficacy or kinetics outside of the binding pocket.

CONCLUSION

To summarize, we have performed SAR and conformational studies of YM-254890 to rationalize the increased potency of FR900359 and an attempt to understand the exquisite selectivity of these compounds, the latter was achieved by the design and synthesis of a collection of structurally and stereochemically diverse analogs using a combination of solid- and solution-phase synthesis. The pharmacological characterization of the seven new analogs provided important SAR information. Specifically, the high potency of FR900359 could be a result of synergistic effects of the side chain of Thr (R^1, Figure 1a) and the *N*-acyl group of β-HyLeu-1 (R^2, Figure 1a) with appropriate size and stereochemistry. We also performed the first conformational study of YM-254890 in aqueous solution using variable temperature ^{1}H NMR spectroscopy to probe the intramolecular hydrogen-bonding profile and replica exchange molecular dynamics to reveal the conformational landscape. This analysis indicated that conformational stability is crucially important for potent inhibition of $G_{q/11}$-mediated signaling. Moreover, due to the lack of structural information of the inactive G_s protein and the poor understanding of the dynamic interaction between YM-254890 and the G_q protein, it seems to be challenging to rationally develop inhibitors of the G_s protein using YM-254890 as a template. Continuous investigations of selective G_s inhibition as well as more potent specific $G_{q/11}$ inhibitors are still ongoing.

EXPERIMENTAL SECTION

General Information. All starting materials were purchased from commercial sources and used without further purification, or purified according to the Purification of Laboratory Chemicals (Armarego, W. L. F., Chai, C. L. 5th edition). Solvents were dried under standard conditions. Reactions were monitored by thin-layer chromatography (TLC) using TLC silica gel coated aluminum plates 60F-254 (Merck). Silica gel chromatography was performed using Merck silica gel 60, 230-400 mech. NMR spectroscopy was performed on Bruker 400 and 600 MHz apparatus. NMR solvent was $CDCl_3$ unless otherwise stated. ^1H NMR chemical shifts are recorded in ppm using TMS as internal standard (0.00 ppm). The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad singlet. ^{13}C NMR shifts are recorded in ppm using the residual non deuterated solvent as internal standard ($CDCl_3$, 77.0 ppm). Analysis of all NMR spectra was performed with MestReNova version 8.1 by Mestrec Laboratories. Preparative HPLC was performed on an Agilent 1100 system using a C18 reverse phase column (Zorbax 300 SB-C18, 21.2 × 250 mm) with a linear gradient of the binary solvent system of H_2O/ACN/TFA (A: 95/5/0.1 and B: 5/95/0.1) with a flow rate of 20 mL/min, and gradient elution from 0-100% B in 60 min. Mass spectra were obtained with an Agilent 6410 Triple Quadrupole Mass Spectrometer instrument using electron spray coupled to an Agilent 1200 HPLC system (ESI-LC/MS) with a C18 reverse phase column (Zorbax Eclipse XBD-C18, 4.6 × 50 mm), auto sampler and diode-array detector using a linear gradient of the binary solvent system of H_2O/ACN/formic acid (A: 95/5/0.1 and B: 5/95/0.086) with a flow rate of 1 mL/min, and gradient elution from 0-100% B in 5 min. During ESI-LC/MS analysis evaporative light scattering (ELS) traces were obtained with a Sedere Sedex 85 Light Scattering Detector. Optical rotation data were examined in methanol solution at 20 °C. Purity was determined by Waters Acquity UPLC H-class system by using a C18 reverse phase column (ACQUITY UPLC BEH C18, 2.1 × 50 mm, 1.7 µm) or C8 reverse phase column (ACQUITY UPLCBEH C8, 2.1 ×

100 mm, 1.7 µm) with a linear gradient of the binary solvent system of H_2O/ACN/TFA (A: 95/5/0.1 and B: 5/95/0.1) or H_2O/ACN/ formic acid (A: 95/5/0.1 and B: 5/95/0.1) with a flow rate of 0.45 mL/min, and gradient elution from 5-95% B in 7 min. High-resolution mass spectra (HRMS) was obtained using a Micromass Q-Tof II instrument and were all within 5 ppm of theoretical values.

Molecular Modeling. Molecular modeling was performed with modules of the Schrödinger Small Molecule Drug Discovery Suite (Maestro), release 2016-3, using OPLS3 force field for parameterization.[33] The X-ray crystal structure of YM-254890 in complex with the chimeric $G_{q/i}$ protein[16] (2.9 Å; PDB ID: 3AH8) was imported from the Protein Data Bank (pdb.org),[34] and prepared using the Protein Preparation Wizard[35] with default setting skipping minimization steps. The depsipeptide analogs (YM-17 and YM-27) were built from YM-254890 in the G_q binding pocket. All computational results were visualized using PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.

Structure-based design. The sequences corresponding to the purchasable G proteins, G_s (P63094/NM_001077510.2) and G_{i1} were aligned with the sequence of G_q (human) and the $G_{q/i}$ (mouse) crystal structure (PDB ID: 3AH8) using Clustal X. Based on the similar conformations between G proteins, we assumed that all G proteins can adopt a conformation similar to that of the $G_{q/i}$-YM-254890 structure. Due to good sequence alignment of the binding site region we constructed reliable homology models of G_s and G_{i1} with YM-254890 in the binding site using Modeller using the $G_{q/i}$-YM-254890 structure template. Residue positions across G protein subtypes are used with the generic numbering system from the CGN server (www.mrclmb.cam.ac.uk/cgn/lookup_human_sequence.html).[36]

Replica Exchange Molecular Dynamics Simulations. Replica Exchange Molecular Dynamics (REMD) studies were conducted using Desmond (Desmond Molecular Dynamics System, version 4.4, D.E. Shaw Research, New York) and Maestro-Desmond Interoperability Tools version 4.4 (Schrödinger, New York). REMD was used rather than constant-temperature molecular dynamics to more thoroughly

sample the macrocyclic backbone as large conformational barriers might be required for sampling proposed *cis-trans* conversion around *N*-methyl amides. The constructed depsipeptides were imported into Desmond System Builder and solvated with the TIP3P solvent system in an orthorhombic 10-Å water box under periodic boundaries. Additional Na^+ and Cl^- ions were introduced for a physiological 0.15 M (salt concentration). The systems ($\approx$ 3500 atoms) were run for 200 ns (10 recorded frames/ns) using the NPT ensemble and Maestro's default relaxation protocol. A desired temperature span from 300-333 K was parameterized using the parallel tempering predictor algorithm[37] (http://folding.bmc.uu.se/remd/) with a desired acceptance probability of 15% (P_{Des} = 0.15) for 7 sequential replicas allowing for frequent switching between reference temperatures depending on the instantaneous potential energies and acceptance by the Metropolis criterion. Output trajectories were imported into the Maestro Suite and analyzed. RMSD Trajectory Clustering of the 20 highest occupied backbone conformations (*i.e.* the conformation that the depsipeptide spent most time in throughout the simulations) was performed with the Desmond Trajectory Clustering Script with 100% frequency. The most populated clusters were taken as the preferred structure and subjected to a restrained 0.3 Å minimization.

Variable temperature ^{1}H NMR. 1 mg of YM-254890 (Wako Chemicals) was dissolved in 90%H_2O/10% D_2O at an initial pH 5. ^{1}H-^{1}H TOCSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC were acquired at 290Kto 310K using a 600 MHz AVANCE III NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a cryogenically cooled probe. Spectra were recorded with an interscan delay of 1s. NOESY spectra were acquired with a mixing time of 200 ms, and TOCSY spectra were acquired with an isotropic mixing period of 80 ms.

NMR data of YM-254890 in 90% H_2O/ 10% D_2O (v/v)

Residue	Position	Major Conformer		Minor conformer		ROESY	HMBC
		^{1}H (mult, *J* in Hz)	^{13}C	^{1}H (mult, *J* in Hz)	^{13}C		
Ac-1	**1**	-	174.1	-	ND		
	2	2.11 (s)	22.1	2.12 (s)	22.0	NH (β-HyLeu-1)	1 (Ac-1)
β-HyLeu-1	**α**	5.18 (m)	ND	ND	ND	NH (β-HyLeu-1)	
	β	3.92 (m)	77.8	3.76 (m)	ND	α, γ, δ (β-HyLeu-1), 2 (Ac-1)	
	γ	1.66 (m)	30.7	1.73 (m)	30.8	β, δ (β-HyLeu-1)	
	δ	0.99 (m)	18.8	a. 0.92 (m)	18.7		
				b. 1.03 (m)	18.6	β, γ (β-HyLeu-1)	β, γ, δ (β-HyLeu-1)
	CO	-	ND	-	ND		
	NH	8.16 (d, 9.6)	-	8.08 (d, 8.6)	-	α, β (β-HyLeu-1), 2 (Ac-1)	
	β-OH	6.20 (m)	-	6.14 (br s)	-		
β-HyLeu-2	**α**	ND	ND	ND	ND		
	β	5.09	80.1	5.16 (m)	ND		
	γ	2.08 (m)	29.8	1.81 (m)	29.8	δ (β-HyLeu-2)	
	δ	a. 0.95 (d, 7.3)	19.9	a. 0.85 (d, 7.0)	17.4		
		b. 1.01 (m)	14.7	b. 1.11 (d, 6.9)	18.6	β, γ (β-HyLeu-2)	β, γ, δ (β-HyLeu-2)
	CO	-	ND	-	ND		
	NH	ND	-	ND	-		
***N,O*-Me$_2$Thr**	**α**	3.87 (m)	67.8	4.29 (d, 9.0)	64.4	α, γ (N,O-Me$_2$Thr), 2 (Ac-1)	
	β	4.00 (m)	74.2	4.01 (m)	73.4		
	γ	1.35 (d, 6.4)	17.7	1.24 (d, 6.3)	18.0	α & β (N,O-Me$_2$Thr)	α & β (N,O-Me$_2$Thr)
	CO	-	ND	-	ND		
	***N*-Me**	3.21 (s)	39.9	2.65 (s)	29.3	α (*N,O*-Me$_2$Thr)	
	***O*-Me**	3.32 (s)	56.4	3.41 (s)	57.5	γ (*N,O*-Me2Thr), δ (β-HyLeu-1)	β (N,O-Me2Thr)
Ac-2	**1**	-	174.9	-	174.7		
	2	2.13 (s)	22.1	2.20 (s)	22.1	NH (Thr), β (N-MeAla)	1 (Ac-2).
Thr	**α**	4.83 (m)	56.4	4.97 (m)	55.3		
	β	5.82 (m)	71.4	5.44 (m)	73.8	α, γ (Thr)	
	γ	1.27 (d, 6.7)	16.0	1.30 (d, 6.5)	17.8	α, β (Thr)	α, β (Thr)
	CO	-	ND	-	ND		
	NH	8.85 (d, 7.8)	-	7.85 (d, 9.2)	-	α, β, γ (Thr), 2 (Ac-2)	
D-Pla	1	-	170.3	-	170.8		
	2	5.69 (m)	70.8	5.69 (m)	70.8		
	3	3.20, 3.24 (m)	38.1	3.13 (m)	37.8		
	4	-	134.5	-	134.5		
	5-9	7.40-7.24 (m)	128.1-129.5	7.40-7.24 (m)	128.1-129.5		3, 4, 5-9 (D-Pla)
***N*-MeDha**	**α**	-	140.2	-	ND		
	β	4.27, 6.08 (m)	124.4	5.00, 6.26 (d, 2.0)	125.7	α, NH (Ala)	
	CO	-	ND	-	ND		
	***N*-Me**	2.97 (s)	37.3	3.03 (s)	37.3		α (*N*-MeDha), CO (D-Pla)
Ala	**α**	5.08 (m)	44.5	4.73 (m)	47.3		
	β	1.18 (d, 7.1)	16.3	1.33 (d, 6.6)	16.6		α, CO (Ala)
	CO	-	171.5	-	173.3		
	NH	7.53 (d, 7.9)	-	8.79 (d, 7.9)	-	β (*N*-MeDha)	
***N*-MeAla**	**α**	3.77 (m)	60.7	ND	ND	β (*N*-MeAla)	
	β	1.42 (d, 7.2)	12.1	1.47 (d, 7.0)	14.0	α (*N*-MeAla)	α, CO (*N*-MeAla)
	CO	-	172.6	-	173.1		
	***N*-Me**	3.17 (s)	37.2	2.85 (s)	31.3	β (*N*-MeAla), α (Ala), Ac-2	α (*N*-MeAla), CO (Ala)

ND: Not determined

Experimental procedures for building blocks. A. General procedure for ester bond formation: alcohol (5.0 mmol) and carboxylic acid (6.0 mmol) were dissolved in dichloromethane (DCM, 20 mL), followed by addition of dicyclohexylcarbodiimide (DCC, 6.0 mmol) and 4-dimethylaminopyridine (DMAP, 1.0 mmol) to the reaction mixture, which was stirred at room temperature overnight. The white precipitate was filtered off and the excess DCM was removed *in vacuo*. The resulting residue was purified by silica gel chromatography to afford the ester.

B. General procedure for removal of fluorenylmethyloxycarbonyl (Fmoc) protecting group by Et_2NH: Fmoc-protected compound (3.0 mmol) was dissolved in DCM (10 mL), followed by adding Et_2NH (3 mL) to the solution and the reaction was stirred at room temperature for 2 h. Another portion of Et_2NH (2 mL) was added to the reaction and the reaction mixture was allowed to stir for an additional 2 h until all the starting material was consumed. DCM and Et_2NH were removed *in vacuo*, and the remaining residue was purified by silica gel chromatography to afford the unprotected amine.

C. General procedure for protecting amine with acetyl group: amino compound (2.5 mmol) and triethylamine (TEA, 3.5 mmol) were dissolved in DCM (15 mL) and the mixture was stirred at 0^oC, followed by dropwise addition of acetyl chloride (3.0 mmol) to the reaction mixture. After that, the reaction was allowed to stir at 0 ^oC for 20 min and then at room temperature for an additional 40 min until all starting material was consumed. The reaction mixture was washed with brine (5 mL) and dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford the crude product, which was purified by silica gel column chromatography to afford pure protected amine.

Benzyl (*R*)-2-((*N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*O*-(*tert*-butyl)-L-seryl)oxy)-3-phenylpropanoate (**13a**). Compound **13a** was synthesized according to general procedure A. **12a** (2.56 g, 10.0 mmol) and Fmoc-Ser(*t*Bu)-OH (4.60 g, 12.0 mmol) were dissolved in dry DCM (40 mL), followed by adding DCC (2.47 g, 12.0 mmol) and DMAP (0.24 g, 2.0 mmol) to the reaction mixture,

which was stirred at room temperature overnight. After that, the white precipitate was filtered off and excess DCM was removed *in vacuo*. The resulting residue was directly purified by silica gel chromatography to afford **13a** (5.65 g, 91%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.62-7-60 (m, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.32-7.15 (m, 12H), 5.62 (d, *J* = 8.8 Hz, 1H), 5.33-5.29 (m, 1H), 5.14 (s, 2H), 4.60-4.57 (m, 1H), 4.37 (d, *J* = 8.0 Hz, 2H), 4.24 (t, *J* = 7.4 Hz, 1H), 3.68 (dd, *J* = 9.2, 2.8 Hz, 1H), 3.55 (dd, *J* = 9.2, 3.1 Hz, 1H), 3.21-3.09 (m, 2H), 1.02 (d, *J* = 1.7 Hz, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 168.9, 155.8, 143.9, 143.8, 141.2, 135.4, 135.1, 129.2, 128.5, 128.5, 128.4, 128.3, 127.6, 127.0, 125.1, 119.9, 73.9, 73.3, 67.1, 61.8, 54.5, 47.1, 37.3, 31.8, 27.2.

(*R*)-1-(benzyloxy)-1-oxo-3-phenylpropan-2-yl *N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*O*-(*tert*-butyl)-L-allothreoninate (**13b**). Compound **13b** was synthesized according to the same procedure as **13a**(2.57 g, 83%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.61-7.59 (m, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.34-7.17 (m, 12H), 5.40 (d, *J* = 8.4 Hz, 1H), 5.33 (dd, *J* = 8.9, 4.5 Hz, 1H), 5.20-5.12 (m, 2H), 4.51-4.49 (m, 1H), 4.43-4.30 (m, 2H), 4.25-4.21 (m, 1H), 3.87-3.85 (m, 1H), 3.25 (dd, *J* = 14.4, 4.5, 1H), 3.12 (dd, *J* = 14.4, 9.0, 1H), 1.14 (s, 9H), 0.89 (d, *J* = 6.1 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.8, 168.8, 158.8, 143.9, 141.3, 135.5, 135.0, 129.2, 128.6, 128.5, 128.4, 128.3, 127.7, 127.1, 127.0, 125.2, 119.9, 74.4, 73.8, 67.7, 67.2, 59.4, 47.1, 37.2, 28.6, 28.1, 18.5.

Benzyl (*R*)-2-((*N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*O*-(*tert*-butyl)-L-threonyl)oxy)-4-methylpentanoate (**13c**). Compound **13c** was synthesized according to the same procedure as **13a** (7.88 g, 96%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.64 (dd, *J* = 7.6, 3.8 Hz, 2H), 7.43-7.38 (m, 2H), 7.34-7.28 (m, 7H), 5.62 (d, *J* = 9.4 Hz, 1H), 5.21-5.14 (m, 2H), 5.10-5.05 (m, 1H), 4.43-4.35 (m, 3H), 4.28-4.22 (m, 2H), 1.84-1.72 (m, 2H), 1.67–1.63 (m, 1H), 1.23 (d, *J* = 6.2 Hz, 3H), 1.17 (s, 9H), 0.90 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 170.0, 156.4, 144.0,

143.9, 141.3, 135.3, 128.5, 128.3, 128.2, 127.7, 127.1, 127.1, 125.2, 125.2, 120.0, 77.2, 74.1, 72.1, 67.2, 67.0, 59.8, 47.2, 40.0, 28.5, 22.9, 22.7, 14.1.

Benzyl (*R*)-2-((*N*-acetyl-*O*-(*tert*-butyl)-L-seryl)oxy)-3-phenylpropanoate (**14a**). Compound **14a** was prepared according to the general procedures B and C. **13a** (4.35 g, 7.0 mmol) was dissolved in DCM (23 mL), followed by addition of Et_2NH (7.0 mL) to the mixture. The reaction was stirred at room temperature for 2 h and then another portion of Et_2NH (4.7 mL) was added to the reaction, which was stirred for another 2 h until all the starring material was consumed. The excess amount of Et_2NH and DCM were removed *in vacuo* and the residue was subjected to silica gel chromatography to afford the primary amine intermediate (1.68 g, 4.2 mmol). The primary amine was then dissolved in DCM (25 mL) and the mixture was cooled to 0^oC, followed by addition of TEA (0.58 g, 5.7 mmol) in one portion and then acetyl chloride (0.39 g, 4.9 mmol) dropwise. The reaction mixture was allowed to stir at 0^oC for 20 min then at room temperature and stirred for another 40 min until all the starting material was consumed. The reaction mixture was washed with brine (8 mL) and dried over $MgSO_4$, filtered and concentrated *in vacuo* to give the crude product, which was purified by silica gel chromatography to give **14a** (1.98g, 64% for two steps) as colorless oil. ^1H NMR (400 MHz, $CDCl_3$) δ 7.35-7.32 (m, 3H), 7.27-7.21 (m, 5H), 7.18-7.15 (m, 2H), 5.27 (dd, *J* = 7.9, 5.0 Hz, 1H), 5.14 (s, 2H), 4.83-4.80 (m, 1H), 3.66 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.51 (dd, *J* = 9.1, 3.2 Hz, 1H), 3.17-3.14 (m, 2H), 2.01 (s, 3H), 1.00 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 169.5, 168.9, 135.4, 135.1, 129.3, 128.5, 128.5, 128.4, 128.3, 127.1, 73.9, 73.3, 67.1, 61.7, 52.7, 37.3, 27.1, 23.1.

(*R*)-1-(benzyloxy)-1-oxo-3-phenylpropan-2-yl *N*-acetyl-*O*-(*tert*-butyl)-L-allothreoninate (**14b**). Compound **14b** was synthesized according to the same procedure as **14a** (1.10 g, 60% for two steps) as colorless oil. ^1H NMR (400 MHz, $CDCl_3$) δ 7.37-7.32 (m, 3H), 7.29-7.23 (m, 5H), 7.19-7.17 (m, 2H), 6.02 (d, *J* = 8.0 Hz, 1H), 5.32-5.29 (m, 1H), 5.15 (dd, *J* = 12.1, 6.6 Hz, 2H), 4.69 (dd, *J* = 8.0, 3.3 Hz,

1H), 3.84 (qd, *J* = 6.5, 3.3 Hz, 1H), 3.24 (dd, *J* = 14.4, 4.6 Hz, 1H), 3.12 (dd, *J* = 14.4, 8.7 Hz, 1H), 1.99 (s, 3H), 1.12 (s, 9H), 0.91 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.8, 169.5, 168.8, 135.5, 135.0, 129.2, 128.6, 128.5, 128.4, 128.3, 127.1, 74.2, 73.8, 67.7, 67.2, 57.6, 37.2, 28.1, 23.2,18.9.

Benzyl (*R*)-2-((*N*-acetyl-*O*-(*tert*-butyl)-L-threonyl)oxy)-4-methylpentanoate (14c). Compound **14c** was synthesized according to the same procedure as **14a** (3.15 g, 57% for two steps) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38-7.30 (m, 5H), 6.20 (d, *J* = 9.1 Hz, 1H), 5.17 (s, 2H), 5.02 (dd, *J* = 8.6, 4.7 Hz, 1H), 4.65 (dd, *J* = 9.1, 2.2 Hz, 1H), 4.22 (qd, *J* = 6.2, 2.2 Hz, 1H), 2.06 (s, 3H), 1.83-1.70 (m, 2H), 1.67-1.60 (m, 1H), 1.17 (d, *J* = 6.2 Hz, 3H), 1.14 (s, 9H), 0.93 (d, *J* = 6.3 Hz, 3H), 0.90 (d, *J* = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7, 170.1, 169.9, 135.3, 128.5, 128.3, 128.2, 74.1, 72.1, 67.2, 66.9, 57.6, 39.9, 28.4, 24.3, 22.9, 21.7, 20.6, 14.1.

(*S*)-2-acetamido-3-(((*R*)-1-(benzyloxy)-1-oxo-3-phenylpropan-2-yl)oxy)-3-oxopropyl *N*,*O*-dimethyl-L-threoninate (**15a**). **15a** (1.77 g, 4.0 mmol) was dissolved in DCM (10 mL), followed by addition of TFA (2.0 mL) to the mixture. The reaction was stirred at room temperature for 1 h, and then another portion of TFA (1.0 mL) was added to the reaction mixture and the reaction was kept at room temperature for an additional 1 h until all starting material was consumed. Excess TFA and DCM were removed *in vacuo* to give a crude product, which was purified by silica gel chromatography to generate the primary alcohol intermediate (1.16 g, 3.00 mmol). The secondary alcohol was coupled with Boc-*N*-Me-Thr(Me)-OH (0.89 g, 3.60 mmol) according to general procedure A to give the ester (1.35 g, 2.2 mmol), which was subsequently dissolved in DCM (5 mL) and TFA (1.0 mL) was added. The reaction was stirred at room temperature for 1 h and another portion of TFA (0.5 mL) was added to the reaction mixture. The reaction was stirred for another 1 h until all starting material was consumed. After that, excess TFA and DCM were removed *in vacuo*, the resulting residue was poured into sat. $NaHCO_3$ (15 mL) and the water phase was extracted with DCM (3 x 15 mL). The organic phase was collected, washed with

brine, dried over $MgSO_4$ and concentrated *in vacuo*to give crude product, which was subjected to silica gel chromatography to **15a** (0.86 g, 42% for three steps) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30-7.27 (m, 3H), 7.23-7.17 (m, 5H), 7.09-7.06 (m, 2H), 5.28 (dd, *J* = 8.1, 4.5 Hz, 1H), 5.23 (s, 1H), 5.09 (d, *J* = 5.8 Hz, 2H), 4.96-4.92 (m, 1H), 4.65 (dd, *J* = 11.2, 3.4 Hz, 1H), 4.19 (dd, *J* = 11.2, 2.3 Hz, 1H), 3.53-3.47 (m, 1H), 3.18 (s, 3H), 3.18-3.14 (m, 2H), 3.10-3.02 (m, 2H), 2.31 (s, 3H), 1.91 (s, 3H), 1.03 (d, *J* = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.9, 169.2, 168.7, 135.2, 134.9, 129.3, 128.7, 128.6, 128.5, 128.4, 127.2, 77.6, 73.9, 67.4, 65.1, 64.2, 56.6, 51.1, 37.2, 35.2, 22.8, 15.3.

(2*S*,3*S*)-3-acetamido-4-(((*R*)-1-(benzyloxy)-1-oxo-3-phenylpropan-2-yl)oxy)-4-oxobutan-2-yl *N*,*O*-dimethyl-L-threoninate(**15b**). Compound **15b** was synthesized according to the same procedure as **15a** (0.80 g, 62% for three steps) as colorless oil.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37-7.16 (m, 10H), 6.90(d, *J* = 9.0 Hz, 1H), 5.34 (dd, *J* = 9.0, 4.4 Hz, 1H), 5.22-5.12 (m, 3H), 4.85 (dd, *J* = 9.2, 3.4 Hz, 1H), 3.62 (qd, *J* = 6.3, 4.3, 1H), 3.36 (s, 3H), 3.28-3.23 (m, 2H), 3.15-3.09 (m, 1H), 2.35 (s, 3H), 1.91 (s, 3H), 1.10 (d, *J* = 2.7 Hz, 3H), 1.09 (d, *J* = 3.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 169.5, 168.6, 168.5, 135.4, 135.0, 129.2, 128.6, 128.5, 128.3, 127.2, 78.0, 74.1, 71.5, 67.3, 65.1, 56.7, 54.8, 37.2, 35.5, 22.8, 16.6, 15.2.

Benzyl (*R*)-2-((*N*-acetyl-*O*-(*N*,*O*-dimethyl- L-threonyl)- L-threonyl)oxy)-4-methylpentanoate (**15c**). Compound **15c** was synthesized according to the same procedure as **15a** (0.84 g, 52% for three steps) as colorless oil.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38-7.29 (m, 5H), 6.40 (d, *J* = 9.3 Hz, 1H), 5.52 (qd, *J* = 6.4, 2.7 Hz, 1H), 5.19-5.12 (m, 2H), 5.02 (dd, *J* = 9.2, 4.2 Hz, 1H), 4.94 (dd, *J* = 9.3, 2.7 Hz, 1H), 3.60-3.54 (m, 1H), 3.31 (s, 3H), 3.12 (d, *J* = 4.9 Hz, 1H), 2.35 (s, 3H), 2.08 (s, 3H), 1.85-1.62 (m, 3H), 1.31 (d, *J* = 6.4 Hz, 3H), 1.18 (d, *J* = 6.2 Hz, 3H), 0.93 (d, *J* = 6.4 Hz, 3H), 0.90 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.8, 170.1, 169.8, 169.4, 135.1, 128.6, 128.4, 128.1, 77.5, 72.4, 70.9, 67.7, 67.0, 56.7, 55.4, 39.6, 35.2, 24.5, 23.0, 23.0, 21.6, 17.1, 15.5.

(2*R*,5*S*,9*S*)-5-acetamido-2-benzyl-9-((*R*)-1-methoxyethyl)-10-methyl-4,8,11-trioxo-3,7,12-trioxa-10-azapentadec-14-enoic acid (10a).15a (0.86 g,1.68mmol) and 10% Pd/C (86 mg, 10% w/w) were mixed together in methanol (10 mL) under a hydrogen atmosphere. The reaction was stirred at room temperature for 2 h until all the starting material was consumed. The Pd/C was filtered off and the resulting organic phase was concentrated *in vacuo*, followed by purifying with reverse phase chromatography to afford the amino acid compound intermediate (0.79 g, 1.52 mmol). The intermediate (0.79 g, 1.52 mmol) was dissolved in THF (5 mL) and the mixture was kept at 0^{o}C, followed by addition of TEA (230 mg, 2.28mmol), and AllocCl (0.22 g, 1.82 mmol) slowly to the mixture. The reaction was stirred at 0 ^{o}C for another 1 h until all the starting material was consumed, then THF was removed *in vacuo* and the residue was poured into water (10 mL). To the water phase was added 0.5 M HCl to adjust the pH to 2-3, followed by extraction of the water phase with DCM (3 × 10 mL). The combined organic phases were washed with brine, dried over $MgSO_4$ and concentrated *in vacuo* to give the crude product, which subsequently purified by silica gel chromatography to afford **10a** (0.69 g, 81% for two steps) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31-7.29 (m, 5H), 6.60 (b r, 1H), 5.99-5.90 (m, 1H), 5.34-5.28 (m, 2H), 5.23 (dd, *J* = 10.5, 1.4 Hz, 1H), 4.96 (b r, 1H),4.75 (d, *J* = 4.8 Hz, 1H), 4.61 (dt, *J* = 5.5, 1.5 Hz, 2H), 4.55 (d, *J* = 11.7 Hz, 1H), 4.37-4.33 (m, 1H), 3.83-3.78 (m, 1H), 3.33-3.27 (m, 1H), 3.23 (s, 3H), 3.14 (dd, *J* = 14.4, 9.1 Hz, 1H), 2.93 (d, *J* = 8.6 Hz, 3H), 1.97 (d, *J* = 11.7 Hz, 3H), 1.12 (d, *J* = 6.3 Hz, 3H);^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 170.8, 169.2, 168.7, 157.6, 136.3, 132.6, 129.2, 128.4, 127.0, 117.3, 76.1, 66.5, 63.6, 62.8, 56.5, 53.4, 51.9, 37.1, 32.8, 22.6, 14.8.

(2*R*,5*S*,6*S*,9*S*)-5-acetamido-2-benzyl-9-((*R*)-1-methoxyethyl)-6,10-dimethyl-4,8,11-trioxo-3,7,12-trioxa-10-azapentadec-14-enoic acid (10c). Compound **10c** was synthesized according to the same procedure as **10a** (0.46 g, 59% for two steps) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$, 3:1 rotamer ratio) Major: δ 7.31-7.29 (m, 2H), 7.25-7.23 (m, 3H), 6.67 (d, *J* = 8.7 Hz, 1H), 5.98-5.92 (m, 1H), 5.35-

5.32 (m, 2H), 5.22-5.18 (m, 2H), 4.82 (dd, *J* = 8.7, 3.2 Hz, 1H), 4.74 (d, *J* = 4.8 Hz, 1H), 4.61 (dt, *J* = 5.5, 1.6, 2H), 4.03-3.99 (m, 1H), 3.36-3.32 (m, 1H), 3.30 (s, 3H), 3.14-3.10 (m, 1H), 2.98 (s, 3H), 1.99 (s, 3H), 1.17-1.15 (m, 3H), 1.00 (d, *J* = 6.8 Hz, 3H). Minor: δ 7.31-7.29 (m, 2H), 7.25-7.23 (m, 3H), 6.47 (d, *J* = 8.5 Hz, 1H), 5.90-5.88 (m, 1H), 5.31-5.30 (m, 2H), 5.24-5.23 (m, 2H), 4.89 (dd, *J* = 8.4, 2.8 Hz, 1H), 4.65 (d, *J* = 4.8 Hz, 1H), 4.62-4.55 (m, 2H), 3.96-3.94 (m, 1H), 3.36-3.32 (m, 1H), 3.30 (s, 3H), 3.14-3.10 (m, 1H), 2.95 (s, 3H), 1.98 (s, 3H), 1.17-1.15 (m, 3H), 0.96 (d, *J* = 6.6 Hz, 3H);^{13}C NMR (100 MHz, $CDCl_3$) Major: δ 170.9, 170.8, 169.0,168.2, 157.7, 135.7, 132.7, 129.2, 128.7, 127.3, 117.5, 76.1, 74.2, 71.2, 67.0, 63.4, 56.5, 55.5, 37.1, 33.3, 22.7, 16.2,15.1. Minor: δ 170.9, 170.7, 168.8, 168.3, 156.4, 135.8, 132.8, 129.3,128.7,127.2, 117.7, 76.0, 74.2, 71.0, 66.6, 63.1, 56.4, 55.3, 37.1, 33.3, 22.8, 15.8, 15.2.

(2*R*,5*S*,6*R*,9*S*)-5-acetamido-2-isobutyl-9-((*R*)-1-methoxyethyl)-6,10-dimethyl-4,8,11-trioxo-3,7,12-trioxa-10-azapentadec-14-enoic acid (10e). Compound **10e** was synthesized according to the same procedure as **10a** (0.38 g, 46% for two steps) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.28 (d, *J* = 9.3 Hz, 1H), 5.99-5.89 (m, 1H), 5.70 (qd, *J* = 6.4, 2.4 Hz, 1H), 5.34-5.30 (m, 1H), 5.25-5.22 (m, 1H), 5.03 (dd, *J* = 9.5, 3.6 Hz, 1H), 4.93 (dd, *J* = 9.4, 2.6 Hz, 1H), 4.77 (d, *J* = 4.9 Hz, 1H), 4.64-4.60 (m, 2H), 4.00-3.91 (m, 1H), 3.29 (s, 3H), 3.06 (s, 3H), 2.13 (s, 3H), 1.87-1.80 (m, 1H), 1.76-1.69 (m, 2H), 1.30 (d, *J* = 6.5 Hz, 3H), 1.18 (d, *J* = 6.3 Hz, 3H), 0.95 (d, *J* = 6.0 Hz, 3H), 0.91 (d, *J* = 6.0 Hz, 3H);^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0, 170.7, 169.0, 168.2, 158.4, 132.3, 117.8, 76.6, 72.7, 71.3, 67.1, 62.7, 57.1, 55.7, 39.3, 32.7, 24.7, 23.0, 23.0, 21.6, 17.0, 14.9.

(2*S*,3*R*)-2-(tert-butoxycarbonylamino)-3-((2*S*,3*R*)-3-(*tert*-butoxycarbonyloxy)-2-hexanamido-4-methylpentanoyloxy)-4-methylpentanoic acid (11b). Compound **11b** was synthesized was synthesized according to the general procedures B and C, followed by debenzylation of the benyl ester. **16** (1.97 g,

2.5 mmol) was dissolved in DCM (8.5 mL), followed by addition of Et_2NH (2.5 mL). The reaction was stirred at room temperature for 2 h, and then another portion of Et_2NH (1.7 mL) was added to the reaction and the reaction was stirred for an additional 1 h until all starting material was consumed. After that, excess Et_2NH and DCM were removed *in vacuo*, followed by purification by silica gel chromatography to afford the primary amine intermediate (0.99 g, 1.75 mmol), which was dissolved in DCM (11 mL) and TEA (248 mg, 2.45 mmol) was added to the solution. The mixture was kept in ice bath and hexanoyl chloride (283 mg, 2.1 mmol) was added to the mixture dropwise. The reaction was stirred at 0^oC for 20 min and then at room temperature for another 40 min until all starting material was consumed. After that, DCM (10 mL) was added to the reaction and the organic phase was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo* to give crude product (1.09 g). The crude benzyl ester (1.09 g) was dissolved in methanol (10 mL), followed by adding 10% Pd/C (109 mg, 10% w/w). The reaction was flushed with hydrogen and stirred at room temperature for 2 h until all starting material was consumed. The Pd/C was filtered off and MeOH was removed *in vacuo*, the remaining residue was purified by silica gel chromatography to afford**11b** (746 mg, 52% for three steps) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.27 (d, *J* = 9.3 Hz, 1H), 5.30 (d, *J* = 10.0 Hz, 1H), 5.15 (dd, *J* = 9.3, 2.5 Hz, 1H), 4.91 (d, *J* = 8.7 Hz, 2H), 4.65 (dd, *J* = 10.0, 2.6 Hz, 1H), 2.29-2.24 (m, 2H), 2.04-1.96 (m, 1H), 1.93-1.84 (m, 1H), 1.64 (p, *J* = 7.3 Hz, 2H), 1.46 (s, 9H), 1.46 (s, 9H), 1.34-1.30 (m, 4H), 1.01 (d, *J* = 6.7 Hz, 3H), 0.98 (d, *J* = 6.8 Hz, 3H), 0.95 (d, *J* = 6.7 Hz, 3H), 0.90 (d, *J* = 6.4 Hz, 6H);^{13}C NMR (100 MHz, $CDCl_3$) δ 174.4, 172.3, 169.4, 155.8, 152.9, 83.2, 81.1, 80.5, 80.2, 54.7, 53.0, 36.3, 31.3, 30.0, 29.3, 28.3, 27.6, 25.2, 22.3, 18.7, 18.3, 13.9.

(2*S*,3*R*)-3-((2*S*,3*R*)-2-benzamido-3-(*tert*-butoxycarbonyloxy)-4-methylpentanoyloxy)-2-(*tert*-butoxycarbonylamino)-4-methylpentanoic acid (11c). Compound **11c** (520 mg, 54% for three steps) was prepared according to the same strategy as **11b**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, *J* = 7.6 Hz,

2H), 7.51 (d, *J* = 7.3 Hz, 1H), 7.44 (t, *J* = 7.5 Hz, 2H), 7.00 (d, *J* = 9.4 Hz, 1H), 5.31 (d, *J* = 10.4 Hz, 1H), 5.15-5.11 (m, 2H), 5.04 (dd, *J* = 8.5, 3.3 Hz, 1H), 4.64-4.61 (m, 1H), 2.04-1.95 (m, 2H), 1.46 (s, 9H), 1.44 (s, 9H), 1.02-0.98 (m, 9H), 0.92 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0, 169.4, 168.1, 155.8, 153.1, 133.1, 132.1, 128.6, 127.4, 83.3, 81.1, 80.6, 80.2, 54.7, 53.9, 29.9, 29.2, 28.3, 27.6, 18.9, 18.7, 18.6, 18.1.

Building blocks **10b**, **10d** and **11a**was previously published and NMR spectra was in agreement with previous reports.[20]

Experimental procedures, NMR (^{1}H and ^{13}C) and HRMS data, optical rotation, purity for 3-9.

Synthesis of YM-26 (3): 2-chlorotrityl chloride resin (140 mg, 0.20 mmol) was pre-swelled in anhydrous DCM (4 mL) for 30 min, and then drained. A mixture of Fmoc-*N*-Me-Ala-OH (260 mg, 0.80 mmol) and DIEA (278 µL, 1.60 mmol) in anhydrous DCM (3 mL) was added to the resin, the reaction mixture was gently agitated at room temperature for 2 h. The resin was drained, washed with DCM/MeOH/DIEA (3 × 4 mL, 17:2:1, v/v), DCM (3 × 4 mL), DMF (3 × 4 mL). Then, a solution of 20% piperidine in DMF (4 mL) was added to the resin, the mixture was agitated for 10min, drained and washed with DMF (3 × 4 mL). This procedure was repeated once. After that, a mixture of Fmoc-Ala-OH (249 mg, 0.80 mmol), HATU (304 mg, 0.80 mmol) and collidine (210 µL, 1.60 mmol) in DMF (4 mL) was added to the resin. The reaction was agitated at room temperature for 2 h, after which the resin was drained and washed with DMF (3 × 4 mL). A solution of 20% piperidine (4 mL) in DMF was added to the resin and the reaction mixture was agitated for 10 min, the resin was drained and washed with DMF (3 × 4 mL). This procedure was repeated once. After that, a mixture of Fmoc-*N*-Me-Cys(S*t*Bu)-OH (356 mg, 0.80 mmol), HATU (304 mg, 0.80mmol) and collidine (210 µL, 1.60 mmol) in DMF (4 mL) was added to the resin and the reaction was agitated at room temperature overnight. The resin was drained, washed with DMF (3 × 4 mL), and 20% piperidine in DMF (4 mL) was added to the resin and

the mixture was agitated for 10 min, after which the resin was drained and washed with DMF (3 × 4 mL). This procedure was repeated once. A mixture of building block **10a** (406 mg, 0.80mmol), HATU (304 mg, 0.80 mmol) and collidine (210 μL, 1.60 mmol) in DMF (4 mL) was added to the resin, and the reaction was agitated at room temperature for overnight. The resin was drained, washed with DMF (3 × 4 mL). Then the reaction tube was bubbled with argon, followed by adding $Pd(PPh_3)_4$ (23 mg, 0.02 mmol) in anhydrous DCM (2 mL) and then $PhSiH_3$ (247 μL, 2.0 mmol). The reaction mixture was agitated at room temperature for 15 min under argon atmosphere, and washed with DCM (3 × 4 mL), DMF (3 × 4 mL) and DCM (3 × 4 mL), the same procedure was repeated once. A mixture of building block **11a** (207 mg, 0.40 mmol), HATU (152 mg, 0.40 mmol) and collidine (105 μL, 0.80 mmol) in DMF (2.0 mL) was added to the resin, and the mixture was agitated at 35 ^{o}C for 24 h. After that the resin was drained and washed with DMF (3 × 4 mL). This procedure was repeated once. A mixture of DTT (154 mg, 1.0mmol) and DIEA (348 μL, 2.0 mmol) in DMF (4 mL) was added to the resin, the reaction mixture was agitated at room temperature for 6 h. Then the resin was drained and washed with DMF (3 × 4 mL). After that, DMF (3 mL) was added to the resin, followed by addition of K_2CO_3 (138 mg, 1.0 mmol) and 1,4-dibromobutane (72 μL, 0.60 mmol), and the mixture was agitated at room temperature overnight. After that, the resin was drained, washed with DMF (3 × 4 mL), H_2O (3 × 4 mL), MeOH (3 × 4 mL) and DCM (3 × 4 mL). Then a mixture of TFA/TIPS/DCM (4 mL, 19/0.5/0.5, v/v) was added to the resin, and the reaction mixture was shaking at room temperature for 1 h. The solution was collected and the resin was washed with TFA (2 × 2 mL) and DCM (2 × 2 mL), the combined solution part was evaporated *in vacuo* to afford the crude depsipeptide, which was purified by preparative HPLC and monitored by LC-MS to provide the pure linear depsipeptide (11.0 mg). The linear depsipeptide (11.0 mg, 0.012 mmol) was dissolved in anhydrous DMF (12 mL), followed by addition of collidine (4.7 μL, 0.036 mmol) and HATU (4.6 mg, 0.012 mmol). The reaction was stirred at room temperature for 3 h until all the linear depsipeptide was consumed. Excess DMF was removed *in vacuo* and the residue sub-

jected to purification by preparative HPLC to afford YM-26 (**3**, 1.4 mg, 0.7%) as white powder.^{1}H NMR (600 MHz, $CDCl_3$) δ 8.52 (d, *J* = 9.1 Hz, 1H), 7.68 (d, *J* = 9.8 Hz, 1H), 7.41 (d, *J* = 8.1 Hz, 1H), 7.33 (d, *J* = 7.8 Hz, 1H), 7.30-7.29 (m, 3H), 7.25-7.23 (m, 2H), 6.68 (d, *J* = 9.8 Hz, 1H), 5.54 (d, *J* = 9.9 Hz, 1H), 5.38-5.36 (m, 2H), 5.23 (dd, *J* = 8.6, 4.0 Hz, 1H), 5.12-5.11 (m, 2H), 4.95-4.90 (m, 1H), 4.83 (q, *J* = 6.8 Hz, 1H), 4.69 (dd, *J* = 11.1, 2.3 Hz, 1H), 4.61 (dd, *J* = 8.0, 2.0 Hz, 1H), 4.26 (d, *J* = 9.5 Hz, 1H), 4.17 (dd, *J* = 11.1, 2.0 Hz, 1H), 3.76-3.74 (m, 1H), 3.64 (d, *J* = 1.9 Hz, 1H), 3.45 (s, 3H), 3.05 (s, 3H), 3.02-2.98 (m, 1H), 2.88 (s, 3H), 2.86 (s, 3H), 2.83-2.81 (m, 1H), 2.26 (s, 3H), 2.11 (s, 3H), 1.99-1.95 (m, 1H), 1.76-1.70 (m, 1H), 1.41 (d, *J* = 6.9 Hz, 3H), 1.32 (d, *J* = 6.4 Hz, 3H), 1.18 (d, *J* = 6.5 Hz, 3H), 1.11 (d, *J* = 6.7 Hz, 3H), 0.89 (d, *J* = 6.8 Hz, 6H), 0.84 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.8, 172.4, 171.4, 171.0, 170.0, 167.7, 166.7, 166.3, 164.0, 162.3, 145.4, 134.6, 129.4, 128.6, 127.0, 107.2, 78.7, 72.3, 71.0, 67.0, 64.3, 57.1, 56.0, 51.2, 46.6, 45.9, 37.5, 36.5, 31.9, 31.2, 29.9, 29.7, 29.3, 29.1, 27.2, 25.5, 22.3, 20.5, 19.4, 18.6, 18.3, 16.7, 14.1; $[\alpha]^{25}_{D}$ = +28.9 (*c* 0.069, MeOH); HRMS (MALDI): *m/z* $[M + Na]^+$ calculated for$C_{45}H_{66}N_7NaO_{15}$: 968.4587, found: 968.4536; Purity: 93%.

Synthesis of YM-27 (4): YM-27 (**4**, 1.2 mg, 0.6%) was synthesized as white powder by the same strategy as for **3**, using building block **10b** instead of **10a**. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.51 (d, *J* = 9.2 Hz, 1H), 7.63 (d, *J* = 9.9 Hz, 1H), 7.37 (d, *J* = 7.7 Hz, 1H), 7.31-7.28 (m, 3H), 7.24-7.22 (m, 2H), 6.88 (b s, 1H), 6.73 (d, *J* = 9.8 Hz, 1H), 5.36 (d, *J* = 9.8 Hz, 1H), 5.33-5.31 (m, 2H), 5.27 (dd, *J* = 10.0, 1.9 Hz, 1H), 5.22 (dd, *J* = 8.3, 4.0 Hz, 1H), 5.12 (dd, *J* = 10.0, 1.9 Hz, 1H), 5.08 (d, *J* = 2.3 Hz, 1H), 4.94-4.89 (m, 1H), 4.71 (q, *J* = 6.8 Hz, 1H), 4.55 (dd, *J* = 7.8, 2.0 Hz, 1H), 4.07 (d, *J* = 9.8 Hz, 1H), 3.78-3.73 (m, 2H), 3.41 (s, 3H), 3.17 (s, 3H), 3.12 (dd, *J* = 14.7, 3.9 Hz, 1H), 2.99 (dd, *J* = 14.7, 6.6 Hz, 1H), 2.88 (s, 3H), 2.70 (s, 3H), 2.28 (s, 3H), 2.26 (s, 3H), 2.02-1.97 (m, 2H), 1.92-1.86 (m, 1H), 1.39 (t, *J* = 6.4 Hz, 6H), 1.18 (d, *J* = 2.7 Hz, 3H), 1.17 (d, *J* = 3.3 Hz, 3H), 1.10 (d, *J* = 6.7 Hz, 3H), 1.01 (d, *J* = 6.7 Hz,

3H), 0.89 (d, *J* = 6.7 Hz, 3H), 0.86 (d, *J* = 6.9 Hz, 3H), 0.84 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ172.7, 172.1, 171.7, 171.2, 169.9, 169.0, 167.8, 166.4, 164.0, 145.3, 135.9, 129.6, 128.5, 127.0, 106.8, 78.1, 77.6, 72.6, 72.3, 64.5, 57.2, 57.1, 56.4, 50.9, 50.5, 46.7, 45.8, 36.7, 36.2, 31.4, 30.5, 30.0, 28.9, 28.8, 22.3, 22.2, 20.4, 19.4, 18.9, 18.9, 18.5, 18.2, 18.0, 16.3, 14.2; $[\alpha]^{25}_D$ = -43,3 (c 0.092, MeOH); HRMS (MALDI): *m/z* $[M + Na]^+$ calculated for$C_{48}H_{72}N_7NaO_{15}$: 1010.5062, found: 1010.5076; Purity: 96%.

Synthesis of YM-28 (5): YM-30 (**5**, 1.3 mg, 0.7%) was synthesized as white powder by the same strategy as for **3**, using building block **10c** instead of **10a**. ^{1}H NMR (600 MHz, $CDCl_3$) δ8.12 (d, *J* = 8.4 Hz, 1H), 7.64 (d, *J* = 9.7 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.32-7.29 (m, 3H), 7.21-7.20 (m, 2H), 6.70 (d, *J* = 9.7 Hz, 1H), 5.75 (d, *J* = 2.1 Hz, 1H), 5.42 (dd, *J* = 11.5, 4.4 Hz, 1H), 5.21-5.19 (m, 2H), 5.03-5.02 (m, 1H), 4.95-4.94 (m, 1H), 4.89-4.87 (m, 2H), 4.63 (dd, *J* = 8.0, 1.7 Hz, 1H), 4.29 (d, *J* = 6.2 Hz, 1H), 4.03-3.99 (m, 1H), 3.83 (dd, *J* = 9.3, 1.9 Hz, 1H), 3.40 (s, 3H), 3.12 (s, 3H), 3.04 (s, 3H), 2.92-2.90 (m, 2H), 2.86 (s, 3H), 2.26 (s, 3H), 2.08 (s, 3H), 1.97-1.94 (m, 1H), 1.86-1.83 (m, 1H), 1.63 (d, *J* = 6.6 Hz, 3H), 1.44 (d, *J* = 6.3 Hz, 3H), 1.30 (d, *J* = 6.5 Hz, 6H), 1.10 (d, *J* = 6.5 Hz, 3H), 1.05 (d, *J* = 6.7 Hz, 3H), 0.98 (d, *J* = 6.8 Hz, 3H), 0.85 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.0, 172.6, 171.8, 171.5, 171.3, 170.8, 167.5, 167.3, 166.5, 162.2, 141.3, 134.6, 130.0, 128.8, 127.8, 122.7, 78.7, 78.3, 77.8, 74.5, 70.5, 64.7, 57.6, 56.8, 56.1, 55.3, 46.6, 46.2, 38.7, 37.3, 32.2, 31.3, 30.80, 30.33, 30.0, 22.4, 22.3, 20.4, 19.6, 19.05, 18.8, 18.2, 16.5, 14.0; $[\alpha]^{25}_D$ = -20.0 (*c* 0.10, MeOH); HRMS (MALDI): *m/z* $[M + H]^+$ calculated for$C_{46}H_{70}N_7O_{15}$: 960.4930, found: 960.4935; Purity: 93%.

Synthesis of YM-29 (6): YM-29 (**6**, 1.3 mg, 0.6%) was synthesized as white powder by the same strategy as for **3**, using building block **10d** instead of **10a** and **11b** instead of **11a**. ^{1}H NMR (600 MHz, $CDCl_3$) δ8.53 (d, *J* = 9.1 Hz, 1H), 7.74 (d, *J* = 9.8 Hz, 1H), 7.37 (d, *J* = 8.2 Hz, 1H), 7.37-7.28 (m, 3H), 7.23 (d, *J* = 7.4 Hz, 2H), 6.85 (b s, 1H), 6.78 (d, *J* = 9.8 Hz, 1H), 5.50 (qd, *J* = 6.6, 1.8 Hz, 1H), 5.37 (d,

J = 9.5 Hz, 1H), 5.34 (d, *J* = 2.6 Hz, 1H), 5.32 (d, *J* = 9.9 Hz, 1H), 5.21 (dd, *J* = 8.7, 3.9 Hz, 1H), 5.09 (d, *J* = 2.0 Hz, 1H), 5.02 (dd, *J* = 9.9, 1.9 Hz, 1H), 4.90 (dd, *J* = 8.9, 6.6 Hz, 1H), 4.76 (q, *J* = 6.8 Hz, 1H), 4.57 (dd, *J* = 7.8, 2.1 Hz, 1H), 4.04 (d, *J* = 9.8 Hz, 1H), 3.78-3.73 (m, 2H), 3.41 (s, 3H), 3.20 (s, 3H), 3.14-3.10 (m, 2H), 3.00-2.95 (m, 1H), 2.89 (s, 3H), 2.68 (s, 3H), 2.29 (s, 3H), 2.02-1.94 (m, 2H), 1.40 (d, *J* = 2.6 Hz, 3H), 1.39 (d, *J* = 3.0 Hz, 3H), 1.35-1.30 (m, 6H), 1.27-1.25 (m, 3H), 1.19-1.17 (m, 3H), 1.09 (d, *J* = 6.7 Hz, 3H), 0.89-0.88 (m, 9H), 0.84 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ174.6, 172.5, 172.2, 171.3, 170.1, 170.0, 168.7, 167.9, 166.2, 164.1, 145.4, 135.9, 129.5, 128.5, 127.0, 107.0, 78.3, 72.7, 72.2, 69.5, 64.4, 57.1, 56.4, 52.9, 46.7, 45.9, 36.5, 36.3, 35.4, 31.6, 31.5, 30.5, 30.0, 28.9, 25.5, 25.5, 22.4, 22.4, 22.2, 20.5, 19.4, 18.5, 18.3, 18.0, 16.7, 16.4, 13.9, 13.9; $[\alpha]^{25}_{D}$ = -28.9 (c 0.069, MeOH); HRMS (MALDI): *m/z* $[M + Na]^+$ calculated for$C_{50}H_{76}N_7NaO_{15}$: 1038.5375, found: 1038.5389; Purity: 95%.

Synthesis of YM-30 (7): YM-30 (**7**, 1.3 mg, 0.6%) was synthesized as white powder by the same strategy as for **3**, using building block **10d** instead of **10a** and **11c** instead of **11a**.^{1}H NMR (600 MHz, $CDCl_3$) δ8.14-8.12 (m, 2H), 7.87 (d, *J* = 7.2 Hz, 1H), 7.77 (d, *J* = 9.8 Hz, 1H), 7.52-7.50 (m, 1H), 7.48-7.45 (m, 3H), 7.31-7.28 (m, 3H), 7.23-7.21 (m, 2H), 7.18-717 (m, 1H), 6.86 (d, *J* = 9.8 Hz, 1H), 5.53-5.49 (m, 1H), 5.37 (d, *J* = 9.5 Hz, 1H), 5.34 (d, *J* = 2.2 Hz, 1H), 5.32 (d, *J* = 9.9 Hz, 1H), 5.20 (dd, *J* = 8.7, 3.8 Hz, 1H), 5.09 (d, *J* = 2.4 Hz, 1H), 5.00 (dd, *J* = 9.8, 1.8 Hz, 1H), 4.81 (dd, *J* = 8.7, 6.4 Hz, 1H), 4.68-4.66 (m, 1H), 4.63 (dd, *J* = 7.2, 2.4 Hz, 1H), 4.03 (d, *J* = 9.9 Hz, 1H), 3.87-3.86 (m, 1H), 3.79-3.74 (m, 1H), 3.43 (s, 3H), 3.19 (s, 3H), 3.10 (dd, *J* = 9.6, 3.9 Hz, 1H), 2.96 (dd, *J* = 14.8, 8.9 Hz, 1H), 2.69 (s, 3H), 2.30 (s, 3H), 2.25 (s, 3H), 2.03-1.99 (m, 1H), 1.66-1.60 (m, 1H), 1.43 (d, *J* = 6.5 Hz, 3H), 1.29 (d, *J* = 6.9 Hz, 3H), 1.25 (d, *J* = 6.6 Hz, 3H), 1.20 (d, *J* = 4.4 Hz, 3H), 1.19 (d, *J* = 3.7 Hz, 3H), 1.06 (d, *J* = 6.7 Hz, 3H), 0.98 (d, *J* = 6.8 Hz, 3H), 0.85 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ172.1, 171.5, 170.1, 170.0, 169.2, 167.9, 167.7, 166.2, 164.1, 161.9, 145.5, 141.5, 136.0, 129.4, 128.5, 128.2,

128.2, 127.0, 122.1, 106.9, 77.9, 72.7, 72.2, 69.3, 64.3, 58.4, 57.1, 56.3, 52.8, 47.0, 46.0, 36.3, 31.0, 30.7, 30.5, 30.0, 28.8, 27.2, 22.1, 20.8, 19.5, 18.6, 18.0, 18.0, 16.7, 16.4, 14.1; $[\alpha]^{25}_{D}$ = -37.1 (c 0.054, MeOH); HRMS (MALDI): *m/z* $[M + Na]^+$ calculated for$C_{51}H_{70}N_7NaO_{15}$: 1044.4906, found: 1044.4920; Purity: 98%.

Synthesis of YM-31 (8): YM-31 (**8**, 1.0 mg, 0.5%) was synthesized as white powder by the same strategy as for **3**, using building block **10e** instead of **10a**. ^{1}H NMR (600 MHz, $CDCl_3$) δ8.51 (d, *J* = 8.0 Hz, 1H), 7.86 (d, *J* = 4.0 Hz, 1H), 7.50 (d, *J* = 8.0 Hz, 1H), 6.89 (br s, 1H), 6.74 (d, *J* = 8.0 Hz, 1H), 5.57 (dd, *J* = 8.0, 4.0 HZ, 1H), 5.37 (d, *J* = 8.0 Hz, 1H), 6.33-5.32 (m, 1H), 5.16 (d, *J* = 4.0 Hz, 1H), 5.10-5.07 (m, 1H), 4.92-4.88 (m, 1H), 4.71 (q, *J* = 4.0 Hz, 1H), 4.55 (d, *J* = 4.0 Hz, 1H), 4.05 (d, *J* = 8.0 Hz, 1H), 3.79-3.73 (m, 1H), 3.41 (s, 3H), 2.73 (s, 3H), 2.88 (s, 3H), 2.31 (s, 3H), 2.30 (s, 3H), 1.83-1.71 (m, 5H), 1.43 (d, *J* = 12.0 Hz, 3H), 1.38 (d, *J* = 4.0 Hz, 3H), 1.24 (d, *J* = 4.0 Hz, 1H), 1.20 (d, *J* = 4.0 Hz, 1H), 1.18 (d, *J* = 4.0 Hz, 1H), 1.10 (d, *J* = 4.0 Hz, 1H), 0.93 (d, *J* = 4.0 Hz, 1H), 0.90 (d, *J* = 4.0 Hz, 1H), 0.84 (d, *J* = 4.0 Hz, 1H); ^{13}C NMR: 172.8, 171.2, 170.0, 168.7, 166.2, 164.2, 145.3, 107.1, 78.1, 72.2, 70.8, 69.5, 65.3, 57.1, 56.5, 53.0, 46.7, 45.9, 38.2, 36.2, 31.5, 30.5, 30.1, 28.9, 24.7, 23.4, 22.1, 22.0, 21.2, 20.5, 19.5, 18.6, 18.1, 18.0, 16.8, 16.4, 14.1; $[\alpha]^{25}_{D}$ = +89.5 (c 0.067, MeOH); HRMS (MALDI): *m/z* $[M + Na]^+$ calculated for$C_{43}H_{71}N_7NaO_{15}$: 948.4906, found: 948.4872.

Synthesis of YM-32 (9): 2-chlorotrityl chloride resin (140 mg, 0.20 mmol) was pre-swelled in anhydrous DCM (4 mL) for 30 min, and then drained. A mixture of Fmoc-*N*-Me-Ala-OH (260 mg, 0.80 mmol) and DIEA (278 µL, 1.60 mmol) in anhydrous DCM (3 mL) was added to the resin, the reaction mixture was gently agitated at room temperature for 2 h. The resin was drained, washed with DCM/MeOH/DIEA (3 × 4 mL, 17:2:1, v/v), DCM (3 × 4 mL), DMF (3 × 4 mL). Then, a solution of 20% piperidine in DMF (4 mL) was added to the resin, the mixture was agitated for 10 min, drained and washed with DMF (3 × 4 mL). This procedure was repeated once. After that, a mixture of Fmoc-Ala-

OH (249 mg, 0.80 mmol), HATU (304 mg, 0.80 mmol) and collidine (210 µL, 1.60 mmol) in DMF (4 mL) was added to the resin. The reaction was agitated at room temperature for 2 h, after which the resin was drained and washed with DMF (3 × 4 mL). A solution of 20% piperidine (4 mL) in DMF was added to the resin and the reaction mixture was agitated for 10 min, the resin was drained and washed with DMF (3 × 4 mL). This procedure was repeated once. After that, a mixture of Fmoc-*N*-Me-D-Ser(*O*Bn)-OH[20] (345 mg, 0.80 mmol), HATU (304 mg, 0.80mmol) and collidine (210 µL, 1.60 mmol) in DMF (4 mL) was added to the resin and the reaction was agitated at room temperature overnight. The resin was drained, washed with DMF (3 × 4 mL), and 20% piperidine in DMF (4 mL) was added to the resin and the mixture was agitated for 10 min, after which the resin was drained and washed with DMF (3 × 4 mL). This procedure was repeated once. A mixture of building block **10e** (390 mg, 0.80mmol), HATU (304 mg, 0.80 mmol) and collidine (210 µL, 1.60 mmol) in DMF (4 mL) was added to the resin, and the reaction was agitated at room temperature for overnight. The resin was drained, washed with DMF (3 × 4 mL). Then the reaction tube was bubbled with argon, followed by adding $Pd(PPh_3)_4$ (23 mg, 0.02 mmol) in anhydrous DCM (2 mL)and then $PhSiH_3$ (247 µL, 2.0 mmol). The reaction mixture was agitated at room temperature for 15 min under argon atmosphere, and washed with DCM (3 × 4 mL), DMF (3 × 4 mL) and DCM (3 × 4 mL), the same procedure was repeated once. A mixture of building block **11a** (207 mg, 0.40 mmol), HATU (152 mg, 0.40 mmol) and collidine (105 µL, 0.80 mmol) in DMF (2.0 mL) was added to the resin, and the mixture was agitated at 35 ^{o}C for 24 h. After that, the resin was drained, washed with DMF (3 × 4 mL), MeOH (3 × 4 mL) and DCM (3 × 4 mL). Then a mixture of TFA/TIPS/DCM (4 mL, 19/0.5/0.5, v/v) was added to the resin, and the reaction mixture was shaking at room temperature for 1 h. The solution was collected and the resin was washed with TFA (2 × 2 mL) and DCM (2 × 2 mL), the combined solution part was evaporated *in vacuo* to afford the crude depsipeptide, which was purified by preparative HPLC and monitored by LC-MS to provide the pure linear depsipeptide (12.4 mg). The linear depsipeptide (12.4 mg, 0.012 mmol) was dissolved in anhydrous DMF

(12 mL), followed by addition of collidine (4.7µL, 0.036 mmol) and HATU (4.7 mg, 0.012 mmol). The reaction was stirred at room temperature for 6 h until all the linear depsipeptide was consumed. Excess DMF was removed *in vacuo* and the residue subjected to purification by preparative HPLC to afford cyclic precursor (**26**). The precursor was dissolved in MeOH (1 mL) together with Pd/C (1mg, 10%, w/w) and stirred for 3h under hydrogen atmosphere at room temperature. Pd/C was filtered off and the residue was concentrated, freeze dried without further purification to afford YM-32 (**9**, 2.7 mg, 1.4%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.36 (d, *J* = 8.9 Hz, 1H), 7.71 (d, *J* = 9.6 Hz, 1H), 7.28 (d, *J* = 7.7 Hz, 1H), 6.77 (d, *J* = 9.8 Hz, 1H), 5.6-5.58 (m, 1H), 5.45 (d, *J* = 9.9 Hz, 1H), 5.40 (d, *J* = 9.6 Hz, 1H), 5.15 (dd, *J* = 9.4, 2.5 Hz, 1H), 5.07-5.03 (m, 2H), 4.79-4.74 (m, 1H), 4.61 (dd, *J* = 7.8, 3.9 Hz, 1H), 4.59-4.57 (m, 1H), 4.10 (d, *J* = 9.8 Hz, 1H), 4.03 (dd, *J* = 12.4, 3.9 Hz, 1H), 3.95 (dd, *J* = 12.4, 7.8 Hz, 1H), 3.79-3.77 (m, 2H), 3.43 (s, 3H), 3.06 (s, 3H), 2.88 (s, 3H), 2.72 (s, 3H), 2.29 (s, 3H), 2.28 (s, 3H), 1.98-1.92 (m, 1H), 1.78-1.74 (m, 2H), 1.66-1.59 (m, 2H), 1.40 (d, *J* = 6.8 Hz, 3H), 1.34 (d, *J* = 6.5 Hz, 3H), 1.26-1.25 (m, 3H), 1.22 (d, *J* = 6.0 Hz, 3H), 1.13 (d, *J* = 6.5 Hz, 3H), 1.11 (d, *J* = 6.7 Hz, 3H), 0.92 (d, *J* = 6.6 Hz, 3H), 0.90-0.89 (m, 6H), 0.85 (d, *J* = 6.7 Hz, 3H; ^{13}C NMR (150 MHz, $CDCl_3$) δ172.7, 172.4, 172.3, 171.8, 170.1, 169.8, 169.8, 169.3, 168.9, 166.6, 166.0, 78.3, 72.3, 71.5, 70.0, 64.6, 60.9, 59.6, 57.1, 56.6, 53.3, 47.0, 46.1, 38.1, 31.5, 31.1, 30.7, 30.0, 29.4, 24.8, 23.5, 22.2, 22.1, 21.3, 20.1, 19.5, 18.4, 18.1, 17.9, 16.9, 16.4, 14.1; $[\alpha]^{25}_{D}$ = -19.3 (c 0.21, MeOH); HRMS (MALDI): *m/z* $[M + Na]^+$ calculated for$C_{43}H_{73}N_7NaO_{16}$: 966.5011, found: 966.5022; Purity: 93%.

Methods for Biological Evaluation. The cell lines CHO-k1, native HEK293 and GS-22A tested negative for mycoplasma infection in our lab, and the mGluR2-CHO cell lines were verified pharmacologically in our lab.

Cell Culturing. CHO-k1 cells that stably express the muscarinic M1 receptor were purchased from the cDNA Resource Center (www.cdna.org, catalogue No. CEM100TN00). The CHO cell line, which sta-

bly expresses the rat $mGlu_2$ receptor, was a generous gift from S. Nakanishi (Kyoto University). CHO-M1 cells were maintained in Ham's F12 media (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS) (Life Technologies), 1% (v/v) penicillin-streptomycin (10.000 units ml^{-1} (Life Technologies)) and 0.25 mg ml^{-1} G418 (Life Technologies). HEK293 cells that endogenously express the β_2 adrenergic receptor and CHO-$mGlu_2$ receptor cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) supplemented with 10% (v/v) FBS or dialysed FBS (Life Technologies), respectively, and 1% (v/v) penicillin-streptomycin (10.000 units ml^{-1} (Life Technologies)). CHO-$mGlu_2$ media was also supplemented with 1% (v/v) L-proline. GS-22A cells, derived from HEK293 purchased from ACTT, were cultured in DMEM supplemented with 10% (v/v) FBS. All the cells were kept at 37 °C in a humidified atmosphere (95% air and 5% CO_2).

Functional assays. The IP_1 HTRF assay (Cisbio) was used to quantify $G_{q/11}$ signalling activity, and the cAMP dynamic 2 assay (Cisbio) was used to quantify the G_s and G_i signalling. G protein inhibitors were dissolved in 20 mM DMSO stock solutions, diluted in assay buffer (Hanks' balanced salt solution buffer, 20 mM HEPES pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and added to a 384-well Optiplate (PerkinElmer) in triplicate unless stated otherwise. Subconfluent cells were detached from the culture dish with non-enzymatic cell-dissociation solution (Sigma).

IP_1 assay of the M1 receptor. CHO-M_1 cells were resuspended in assay buffer supplemented with 0.2% bovine serum albumin (BSA) to achieve a cell density of 2 million cells ml^{-1}. G protein inhibitors and cells (10.000 cells per well) were incubated at 37 °C for 1 h. After the incubation, carbamoylcholine chloride (carbachol (Sigma)) was dissolved in assay buffer supplemented with 200 mM LiCl (final concentration of LiCl in the assay was 20 mM). Carbachol was added to each well at a final concentration of 20 μM (~EC_{80} (drug concentration that induces 80% of the maximal response)) and the plate was incubated at 37 °C for 1 h followed by 15 min of incubation at room temperature. The detection solution

was prepared as follows: IP_1 conjugate and lysis buffer (Cisbio) + 2.5% anti-IP_1 cryptate Tb conjugate (Cisbio) + 2.5% D-myo-IP_1-d2 conjugate (Cisbio).

cAMP assay. HEK293 cells or CHO-$mGlu_2$ cells were resuspended in assay buffer supplemented with 0.2% BSA and 50 μM 3-isobutyl-1-methylxanthine (Sigma) to achieve a cell density of 500.000 cells ml^{-1}. G protein inhibitors and cells (2.500 cells per well) were incubated at 37 °C for 1 h. To stimulate G_s signalling, isoproterenol bitartrate salt (Sigma) was dissolved in the assay buffer and added to each well containing HEK293 cells at a final concentration of 20 nM (~EC_{80}). For G_i activity, L-glutamate (Sigma) was dissolved in assay buffer supplemented with forskolin (Sigma) (the final concentration of forskolin in the assay was 15 μM) and added to each well containing CHO-$mGlu_2$ cells at a final concentration of 5 μM (~EC_{80}). The plate was incubated at room temperature for 30 min on a plate shaker. The detection solution was prepared as follows: cAMP conjugate and lysis buffer (Cisbio) + 2.5% anti-cAMP cryptate conjugate (Cisbio) + 2.5% cAMP-d2 conjugate (Cisbio). For both assays, the detection solution was added to the plate (10 μl per well) and the plate was incubated in the dark for one hour at room temperature. The plate was read on an EnVision Multilabel Reader (PerkinElmer Life and Analytical Sciences), with excitation at 340 nm and measurements of emission at 615 and 665 nm. The fluorescence resonance energy transfer ratios (665/615 nm) were converted into IP_1 or cAMP concentrations, respectively, by interpolating values from an IP_1 or cAMP standard curve.

ASSOCIATED CONTENT

Supporting Information.

Additional figure displaying the chemical structures of YM-254890's analogs, chemical synthesis of building blocks and YM-32, variable-temperature ^{1}H-NMR of YM-254890, NMR spectra of building blocks and compound **3-9** in $CDCl_3$ and YM-254890 in H_2O/D_2O (v/v 9:1) and UPLC spectra of compound **3-9**. Inhibition of G_s- and $G_{i/o}$-mediated signaling.

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Notes

The authors declare no competing financial interest.

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- 5 analogues were designed and synthesized to rationalize the high potency of FR900359.
- 2 compounds were prepared to understand the subtype biased G protein inhibition.
- The first conformational study of YM-254890 was explored.
- The conformational stability of depesipeptides is crucial for potent G_q inhibition.
- The fit of binding pocket was not sufficient for G_s selective inhibitors discovery.