Extracellular signals perceived by G protein-coupled receptors are transmitted via G proteins, and subsequent intracellular signaling cascades result in a plethora of physiological responses. The natural product cyclic depsipeptides YM-254890 and FR900359 are the only known compounds that specifically inhibit signaling mediated by the Gq subfamily. In this study we exploit a newly developed synthetic strategy for this compound class in the design, synthesis, and pharmacological evaluation of eight new analogues of YM-254890. These structure–activity relationship studies led to the discovery of three new analogues, YM-13, YM-14, and YM-18, which displayed potent and selective Gq inhibitory activity. This provides pertinent information for the understanding of the Gq inhibitory mechanism by this class of compounds and importantly provides a pathway for the development of labeled YM-254890 analogues.

Heterotrimeric G proteins are GTP/GDP-binding proteins made up of three subunits, α, β, and γ, which are key signal-transducing molecules activated by G protein-coupled receptors (GPCRs).[1,2] The propagation of G protein-mediated signals share a common mechanism: The ligand-activated GPCR engages with G proteins, which leads to the release of GDP from the G protein α subunit (Gα). Intracellular GTP is bound to the nucleotide-free G protein, allowing dissociation of the Gα subunit from the Gβγ dimer and GPCR, leading to the regulation of downstream effectors such as ion channels and phospholipase C to induce cellular responses.[3] Heterotrimeric G proteins are divided into four different classes, Gαi/o, Gq/11, and G12/13, which mediate various signaling pathways.

GPCRs are encoded by more than 800 genes, constitute the third-largest gene family in the human genome,[4] play important roles in detecting environmental stimuli, and are involved in a plethora of diseases.[5] It is estimated that approximately 40% of all current drugs target GPCRs.[6,7] In contrast to GPCRs, the precise function and regulation of G proteins is much less well understood, which, to a large degree, is due to lack of selective and potent inhibitors of G proteins. Only very few compounds have been shown to selectively modulate G protein function. Notably, pertussis toxin and cholera toxin[8–10] are known as selective modulators of Gq and Gi proteins, respectively.

The cyclic depsipeptides YM-254890 (1, Figure 1a) isolated from Chromobacterium sp. QS3666,[11] and FR900359 (2, Figure 1a) isolated from the plant Ardisia crenata,[12,13] are the only known compounds that specifically inhibit Gq signaling. They were originally discovered due to their inhibition of ADP-induced platelet aggregation,[14,15] but are now important pharmacological tools in studies of Gq-mediated cell responses.
Moreover, there is increasing interest in the potential development of such molecules as therapeutic agents, particularly for various forms of cancer.\cite{14, 17}

The molecular basis of how YM-254890 blocks G$_q$ signaling was revealed by an X-ray crystal structure of a complex between YM-254890 and a chimeric G$_{q/11}$ protein (Figure 1b).\cite{18}

This showed that G$_{q}$-mediated inhibition by YM-254890 is achieved by stabilizing an inactive GDP-bound form of G$_{q}$, which inhibits the fundamental GDP/GTP exchange of the G protein.

Although there is great interest and demand for studying G$_{q}$-mediated signaling using G$_{q}$ protein inhibitors, none of these inhibitors had been generally available.\cite{19}

This spurred great interest in generating them by total synthesis, as illustrated by a competition for generating 1 mg of YM-254890 (https://www.innocentive.com/ar/challenge/9933017), as well as recent efforts toward the total synthesis.\cite{20, 21}

Very recently, the first total synthesis of YM-254890 and FR900359 was achieved, which also provided sufficient material for verification of the proposed chemical structure, thorough chemical and pharmacological characterization.\cite{22}

Thus with a versatile synthetic approach in hand, we were interested in the design and synthesis of analogues to provide molecular insight into the structural elements required for biological activity, as well as to provide necessary structure–activity relationship (SAR) studies. Because YM-254890\cite{23} and FR900359 are nearly equipotent, and FR900359 is slightly more synthetically challenging,\cite{24} we used YM-254890 as a template. YM-254890 comprises seven amino acids (alanine (Ala), threonine (Thr), N-methylalanine (N-MeAla), N-methyldehydroalanine (N-MeDha), two β-hydroxybutyric acids (β-HyLeu-1 and β-HyLeu-2), and N,O-dimethylethionine (N,O-Me2Thr)), as well as an α-hydroxy acid (3-phenyllactic acid (3-Pla)), three ester bonds, and two acetyl groups (1, Figure 1a).\cite{25}

Not surprisingly, only very sparse SAR studies have been performed so far, using either structurally related natural products (YM-254891, YM-254892, and YM-280193),\cite{26} semisynthetic hydroxyanalogues (YM-385780 and YM-385781),\cite{27} a synthesized fragment WU-0704\cite{28} or analogues YM-1-1YM-10\cite{29} (Supporting Information Figure 1). In particular, the structural integrity of YM-254890 and the N-MeDha residue are critical for biological activity, and changes in the backbone and N-MeDha are generally not tolerated. We were therefore interested in examining the N-MeAla and Ala residues of YM-254890. Thus, herein we report the design and synthesis of eight new YM-254890 analogues generated by total synthesis and modification of the N-MeAla and Ala residues of YM-254890 (Figure 2).

The analogues were subsequently examined for their G protein inhibitory potency and selectivity by evaluation of pharmacological activity at G$_q^\gamma$, G$_s^\gamma$, and G$_{11}$-mediated signaling.

In our systematic evaluation of N-MeAla and Ala, we first designed four analogues replacing N-MeAla with residues in which steric hindrance was either increased, N-methylalanine (N-MeVal) or decreased, N-methylglycine (N-MeGly) (YM-11 (3) and YM-12 (4), respectively, Figure 2). Moreover, we examined the importance of stereochemistry by introducing N-methyl-α-alanine (N-Me-α-Ala) and effects of introducing an aromatic electron-rich moiety, N-methylphenyalanline (N-MePhe) (YM-13 (5) and YM-14 (6), respectively, Figure 2). For the other residue, Ala, we introduced principally similar substituents by incorporation of valine (Val), glycine (Gly), α-alanine (α-Ala), and phenylalanine (Phe) (YM-15-YM-18 (7–10), respectively, Figure 2).

In our retrosynthetic analysis (Scheme 1), which is inspired by our building-block approach used in the synthesis of YM-254890,\cite{30} the macrolactization site was chosen between the N-methylated residue of interest and the β-HyLeu residue. Thus, a linear precursor 11 should be generated with the N-MeDha residue to be accessed from the resin-bound depsipeptide 12 using an SbBu-protected cysteine derivative as a precursor (Scheme 1).\cite{31} A key feature is that 12 is generated from three building blocks: resin-bound 13 that would contain the residues where modifications would be introduced, as well as two ester-containing building blocks 14 and 15 that would be the same for all analogues.

The common building blocks 14 and 15 were prepared in good yield as previously reported.\cite{32} First, resin-bound peptides 13a–h were synthesized according to standard solid-phase peptide synthesis (SPPS) procedures using a 2-chlorotriyl resin and N-MeCys(SbBu)-OH as the N-MeDha precursor. The variable residues were the first two residues to be anchored to the solid support (Scheme 2). Subsequent coupling of 14 furnished the resin-bound depsipeptides 16a–h (Scheme 2). Next, 15 was introduced, providing the linear resin-bound depsipeptides 12a–h. After the assembly of all residues, the N-MeDha residue was generated through a reduction–bisalkylation–elimination process to give resin-bound compounds 17a–h. The linear precursors of the desired analogues, 11a–h, were then accessed by release from the resin and concomitant removal of the O- and N-Boc groups. Finally, the crude analogues 3–10 (Scheme 2) were generated by macrolactamization of 11a–h, carried out at highly diluted concentrations with HATU/collidine as coupling reagent/base pair, which was the “rate-limiting” synthetic step, with yields of 10–20%. All analogues were subsequently purified by semi-prepa-

Figure 2. Structures of designed analogues 3–10.
Reversed-phase HPLC providing the compounds in acceptable overall yields (0.7–1.4 %) and high purity (90–94 %). Furthermore, all analogues were characterized by 1H NMR spectroscopy, optical rotation, and HRMS.

The inhibitory effect of YM-254890 as well as the eight new analogues on Gq-mediated signaling (Table 1) was evaluated in Chinese hamster ovary (CHO) cells that stably express the human M1 muscarinic receptor. The M1 receptor is activated by carbachol, which leads to the Gq-mediated generation of inositol monophosphate (IP1), and Gq inhibition is determined as a decrease in IP1 accumulation. To address the selectivity of these analogues we also examined the compounds for their ability to inhibit Gs- and Gi-mediated signaling (Supporting Information Table 1). This was accomplished by examining the inhibition of isoproterenol-induced cyclic adenosine monophosphate (cAMP) production in human embryonic kidney cells.

Scheme 1. Retrosynthetic analysis of compounds 3–10.

Scheme 2. Total synthesis of compounds 3–10: a) piperidine/DMF (1:4), RT; b) 14, HATU, collidine, DMF, RT; c) Pd(PPh3)4, PhSiH3, CH2Cl2, N2, RT; d) 15, HATU, collidine, DMF, 35 °C; e) DTT, DIEA, DMF, RT; f) 1,4-dibromobutane, K2CO3, DMF, RT; g) TFA/TIPS/CH2Cl2 (19:0.5:0.5, v/v/v), RT; h) HATU, collidine, DMF, RT.

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(HEK) 293 cells that endogenously express the β2 adrenergic receptor (Gq signaling) and the inhibition of glutamic acid induced cAMP reduction in CHO cells that stably express the rat metabotropic glutamate receptor 2 (mGlu2) receptor (Gq signaling).

In general, we observed that all analogues selectively inhibited Gq-mediated signaling, as no inhibitory effect on Gs or Gi signaling was observed (Supporting Information Table 1). Moreover, changing the Ala residue next to N-MeDha (7–10) led to more dramatic changes than modifying the N-MeAla residue (3–6) (Table 1).

Interestingly, the most dramatic change in inhibitory activity was observed when changing the stereochemistry of L-Ala to D-Ala (9) leading to a striking 244-fold loss of potency, clearly underlining the requirement for an appropriate stereochemical arrangement at this position. Removing the side chain at the same position by introducing a Gly residue (8) led to a 17-fold decrease in potency, whereas introducing more bulk by a Val residue (7) decreased potency 6-fold relative to YM-254890. Notably, introducing Phe (10) led to a less than 2-fold change in potency, thereby being essentially equipotent to YM-254890. From the X-ray crystal structure of YM-254890, the Phe residue of 10 appears to reside outside the binding pocket and to not interact directly with the G protein (Figure 3a).

Table 1. Inhibition of Gq-mediated signaling by 3–10.[a]

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC50 [µM][b]</th>
<th>95 % CI [µM][c]</th>
<th>pIC50 ± SEM[d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>YM-254890 (1)</td>
<td>0.15</td>
<td>0.09–0.28</td>
<td>6.81 ± 0.08</td>
</tr>
<tr>
<td>YM-11 (3)</td>
<td>0.42</td>
<td>0.24–0.75</td>
<td>6.38 ± 0.08</td>
</tr>
<tr>
<td>YM-12 (4)</td>
<td>0.45</td>
<td>0.27–0.75</td>
<td>6.35 ± 0.07</td>
</tr>
<tr>
<td>YM-13 (5)</td>
<td>0.27</td>
<td>0.21–0.34</td>
<td>6.57 ± 0.03</td>
</tr>
<tr>
<td>YM-14 (6)</td>
<td>0.19</td>
<td>0.11–0.31</td>
<td>6.73 ± 0.07</td>
</tr>
<tr>
<td>YM-15 (7)</td>
<td>0.98</td>
<td>0.54–1.76</td>
<td>6.01 ± 0.08</td>
</tr>
<tr>
<td>YM-16 (8)</td>
<td>2.56</td>
<td>1.15–5.76</td>
<td>5.59 ± 0.11</td>
</tr>
<tr>
<td>YM-17 (9)</td>
<td>36.6</td>
<td>29.1–45.2</td>
<td>4.44 ± 0.03</td>
</tr>
<tr>
<td>YM-18 (10)</td>
<td>0.30</td>
<td>0.17–0.54</td>
<td>6.52 ± 0.08</td>
</tr>
</tbody>
</table>

[a] Inhibition of carbachol-induced IP3 production in CHO cells that stably express the M1 muscarinic receptor. [b] IC50 values are the mean of four independent experiments performed in triplicate. [c] 95% confidence intervals for the IC50 values. [d] Corresponding pIC50 values including standard errors of the mean (SEM).

Modifying the N-MeAla residue in general did not affect potency to the same degree as observed for Ala, and all analogues were within 3-fold of the potency of YM-254890. Most interestingly was the analogue in which N-MePhe was introduced (6), which gratifyingly was equipotent to YM-254890 and the most potent analogue of YM-254890 generated so far. Similar to the situation for 10, the N-MePhe residue in 6 is positioned at the exterior of the Gq protein with only minor or no contacts with the protein (Figure 3b). In addition, and in very stark contrast to the neighboring position, introducing N-Me-D-Ala (5) also led to less than 2-fold change in potency, showing that at this position stereoisomerism is not nearly as important.

Therefore, larger side chains can replace both Ala and N-MeAla residues. This agrees with the YM-254890/GqX-ray crystal structure, in which the side chains of these residues do not undergo direct interaction with the G protein[18] (Figure 1b). This suggests that it might be possible to introduce larger substituents in this region, which could eventually pave the way for a fluorescently labeled version of YM-254890, for example.

In summary, we have designed and prepared a collection of eight structurally and stereochemically diverse analogues of YM-254890 using an efficient synthetic protocol that uses a combination of solid- and solution-phase synthesis. The analogues were designed to probe the importance of changes in the N-MeAla and Ala residues of YM-254890 for selective inhibition of Gq-mediated signaling. The biological evaluation of the eight analogues, YM-11 to YM-18, provided important SAR information and notably resulted in the discovery of three new analogues, YM-13, YM-14, and YM-18, which are equipotent and selective Gq signaling inhibitors compared with YM-254890.

In general it has proven very difficult to improve or even maintain the potency of YM-254890 upon changing the structure, and thus it is particularly promising that YM-18, in which an aromatic moiety, Phe, has been introduced, is equipotent to YM-254890. This provides very important insight regarding where to introduce, for example, cross-linking moieties or fluorescent groups for further applications of the YM-254890 compounds.

Acknowledgements


Conflict of Interest

The authors declare no conflict of interest.

Keywords: biological activity · G protein-coupled receptors · peptide synthesis · structure–activity relationships · YM-254890
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