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# A disulfide bond replacement strategy enables the efficient design of artificial therapeutic peptides

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ABSTRACT

We demonstrate that disulfide bond replacement is an efficient strategy for engineering therapeutic peptides. In previous work, short peptide fragments, known as WP9QY, with sequence homology with the predicted ligand contact surface of the receptor activator of NF-κB (RANK) were crosslinked through intramolecular disulfide bonds to inhibit RANK ligand (RANKL)-induced signaling, osteoclastogenesis, bone resorption in vitro, and bone loss in vivo. We report that replacement of the disulfide bond of WP9QY with an amine cross-linkage results in a significant improvement in enzymatic stability, with only a slight loss of bone resorption-blocking activity in vitro. Furthermore, the WP9QY derivative inhibits bone loss significantly in vivo, whereas the native form of WP9QY was not effective under the same conditions.

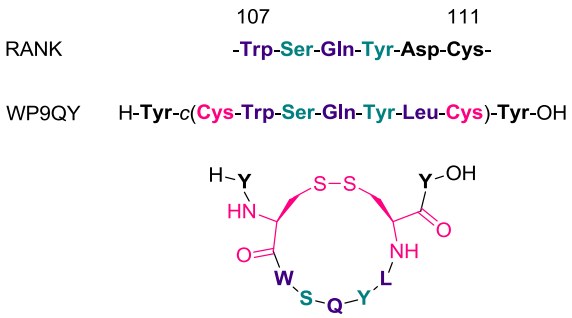


## 1. Introduction

Protein–protein interactions are central to all aspects of cellular function and widely recognized as potential targets for human therapeutics. The recent discovery of a variety of small molecules that can disrupt and/or modulate protein–protein interactions has resulted in the possibility of viable routes for a large number of new medicinal candidates. However, the identification of such low-molecular weight compounds remains challenging, partly because of the shallow nature of many protein–protein interfaces, lacking deep pockets as possible binding sites for small molecules. Although short peptide fragments of interfaces offer promising lead compound design, their flexible nature creates an additional challenge. When considering their potential as therapeutics, the limited stability of short peptides in biological fluids, mainly due to proteolytic enzymes, should also be addressed.

Cyclic peptides are of particular interest as scaffolds whose amino acid sequence is pre-organized into a rigid conformation, thereby imparting and/or enhancing their biological activity and stabilizing their structure.[1] Naturally occurring peptides utilize disulfide bonds between cysteine residues to form intramolecular cross-links and achieve their particular physiological functions.[2] Such disulfide bond-containing peptides also provide an opportunity for the development of novel artificial therapeutic agents, as their analogs may have greater activity than the native form.

It was previously found that a short peptide fragment, known as WP9QY, which has sequence homology with the predicted ligand contact surface of the receptor activator of NF-κB (RANK), could be cross-linked through an intramolecular disulfide bond to inhibit RANK ligand (RANKL)-induced signaling, osteoclastogenesis, and bone resorption in vitro (Scheme 1).[3] It also acted in vivo to protect mice against experimentally induced bone loss, suggesting that the




**Scheme 1.** Comparison of the sequence of RANK and peptide WP9QY.

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development of engineered WP9QY may prove to be useful for the treatment of several diseases in which bone resorption is increased.

While the disulfide bond is an effective structural motif for control of biological activities, it can be reduced under physiological conditions, potentially resulting in loss of the desired function.[4] Many elegant alternative cross-linkage chemistries have been adopted in synthetic peptides to improve stability, and some of these peptides exhibit the desired activities.[5] For this purpose, amine cross-linkages are intriguing because they supply an additional modifiable site that has a minimal impact on the active sequence. We have been developing hydrophobic tag-assisted liquid-phase techniques that are applicable to the versatile large-scale production of peptides, enabling not only effective deprotection and coupling, but also reliable modification based on various existing synthetic chemistries.[6] In light of previous studies, we sought to focus on the synthesis and characterization of WP9QY derivatives incorporating amine cross-linkages instead of disulfide bonds with the aim of proposing an engineering strategy for therapeutic peptides.

## 2. Results and discussion

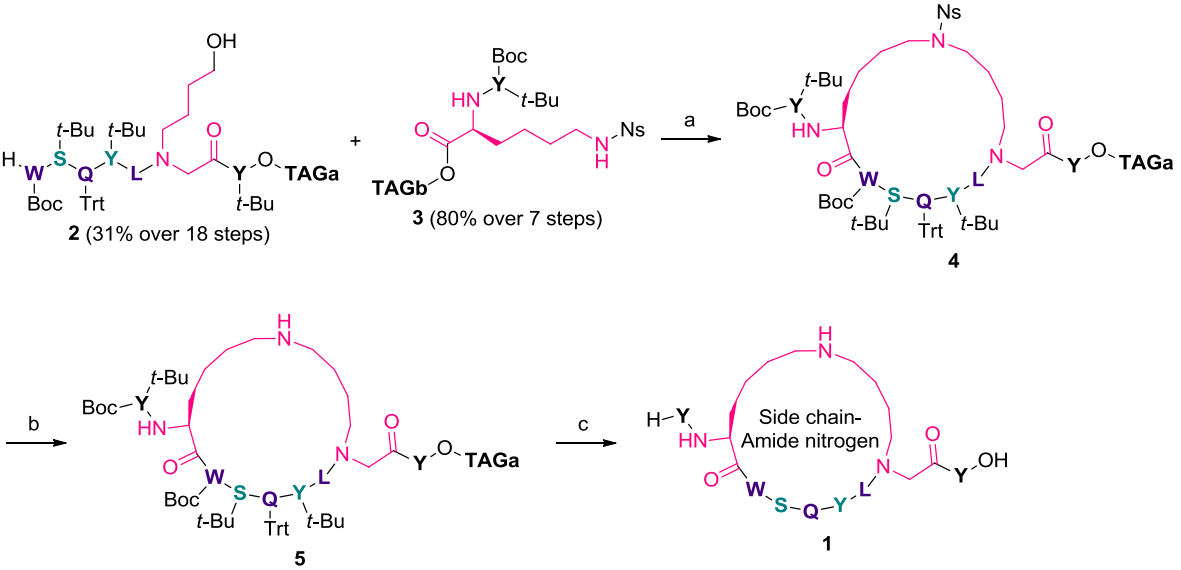
The present work began with the synthesis of the WP9QY derivative (**1**), incorporating an artificial amine cross-linkage between the lysine side chain and the glycine amide nitrogen in place of the disulfide bond (Scheme 2). To this end, we employed Mitsunobu reactions between alcohols and *N*-nosyl amines to make secondary amines that act as cross-linkages (Scheme S1, Supplementary data).[7] We also utilized two hydrophobic tags, which could be cleaved selectively under different acidic conditions, to elaborate the desired cyclic peptide (Scheme S2, Supplementary data). The alcohol fragment (**2**) and the *N*-nosyl amine fragment (**3**) were prepared in 31% yield over 18 steps (Scheme S3, Supplementary data) and 80% yield over 7 steps (Scheme S4, Supplementary data), respectively. These fragments were connected via a Mitsunobu reaction, followed by intramolecular cyclization to afford the cyclic peptide (**4**), which was deprotected in a stepwise fashion to give the WP9QY derivative (**1**) in 21% yield over five steps.

Enzymatic stability was tested in the presence of carboxypeptidase A and chymotrypsin (Table S1, Supplementary data). The WP9QY derivative (**1**) exhibited greatly improved stability relative to its native form—a 5.6-fold and a 3.3-fold improvement in half-life against carboxypeptidase A and chymotrypsin, respectively. However, (**1**) did not show any bone resorption-blocking activity in murine bone marrow cells (data not shown), suggesting that a large conformational change might have been induced by the altered structure of the cross-linkage.

On the other hand, the acetylated version (**6**) of WP9QY derivative (**1**) did demonstrate bone resorption-blocking activity: at 3 μM concentration, its inhibition of osteoclastogenesis was comparable to that of the native form (also at 3 μM), but with improved enzymatic stability (Scheme 3 and Fig. 1). Cytotoxicity assays showed clearly that the inhibition of osteoclastogenesis by (**6**) was not induced by a toxic effect (Fig. S1, Supplementary data). The differences in basicity and/or solubility between secondary amines and amides were also expected to affect the activity.

We then turned our attention to the synthesis of the WP9QY derivatives (**7**–**9**), in which the acetylated forms of the bridgehead positions were varied (Scheme 4 and Schemes S5–S7, Supplementary data). In a manner analogous to the WP9QY derivative (**1**), alcohol fragments (**2**, **10**, and **11**) and *N*-nosyl amine fragments (**12** and **13**) were linked through Mitsunobu reactions, followed by intramolecular cyclization to construct the cyclic peptides. The *N*-nosyl groups were replaced with *N*-acetyl groups, which were deprotected to give the WP9QY derivatives (**7**–**9**). While improved enzymatic stabilities were obtained, neither WP9QY derivative (**8**), in which the bridgehead positions were opposite to those of (**6**), nor WP9QY derivative (**9**), in which the bridgehead positions were both on the amide nitrogen, showed bone resorption-blocking activity in murine bone marrow cells (Fig. 1). In contrast, the WP9QY derivative (**7**), in which the bridgehead positions were both on the side chain, achieved the desired activity with improved enzymatic stability (Fig. 1). These results suggest that the installation of amine cross-linkages offers opportunities for fine-tuning the properties of cyclic bioactive peptides.

To confirm the therapeutic potential of the WP9QY derivatives, their bone resorption-blocking activities were re-evaluated using murine low-dietary calcium models (Fig. 2). Remarkably, reduction of trabecular bone mineral density in tibiae was inhibited




Reagents and conditions: (a) (i) DEAD, $Ph_3P$, THF, 75%; (ii) TFA, TFE, $CH_2Cl_2$; (iii) HATU, HOAt, DIPEA, THF, 56% over 2 steps. (b) PhSH, DBU, THF, 96%. (c) TFA, TIS, $H_2O$, 84%.

**Scheme 2.** Hydrophobic tag-assisted liquid-phase synthesis of the WP9QY derivative (**1**).