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Efficient Solid-Phase Synthesis of Peptoid Analogs for the Development of RANK Receptor Inhibitors

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Osteoporosis is a disease of low bone mass which is associated with a destruction of trabecular architecture and loss of connectivity between plates of trabecular bone, resulting in an increased risk of fracture. Osteoporosis is most common in women, largely due to the marked loss in bone density associated with the withdrawal of estrogen that accompanies the loss of ovarian function after menopause.[1] The loss of bone density associated with estrogen withdrawal is a result of the marked increase in the activity of bone-resorbing osteoclasts.[2] Excessive bone resorption of osteoclasts is a major pathological factor in chronic inflammatory diseases such as osteoporosis and arthritis. The RANK/RANKL signaling pathway where Receptor Activator of Nuclear Factor κB (RANK) is expressed on the surface of osteoclasts and RANK ligand (RANKL) is found on the surface of osteoblasts, has been known to play a crucial role in the differentiation and activation of osteoclasts.[2a]

Recently, a cytoplasmic motif of RANK was identified to be essential in osteoclastogenesis.[3] In addition, Lee and coworkers developed a cell-permeable peptide inhibitor which targets this motif.[4] The RANK receptor inhibitor blocked the RANKL-induced formation and resorptive function of osteoclasts both *in vitro* and *in vivo* by regulating cytoskeleton integrity and survival of osteoclasts. Thus, the targeted inhibition of osteoclasts provides useful information for the development of new therapeutic agents for the treatment of osteoporosis and other osteoclasts-associated diseases. They designed cell-permeable peptide inhibitors spanning the RANK IVVY motif that were conjugated with cell-permeable sequences (YARVRRRGRRR) derived from the human transcription factor Hph-1.[5] The core peptide motif IIVVYV was identified as the minimal inhibitory sequence by using various peptides containing the IVVY motif. IIIIVV- and IIIIIV-containing peptides were also identified to possess the inhibitory activity on the formation of osteoclasts (unpublished results). Generally, peptides are interesting molecules which contain protein-binding properties as well as various biological functions. However, several undesirable problems including a low stability against proteolysis, a limited cell permeability and a poor bioavailability have limited the use of peptides as drugs. Thus, many peptidomimetics with improved pharmacological and pharmacokinetic characteristics have been developed to overcome such drawbacks.[6] In the previous study, the identified RANK receptor inhibitors were relatively long peptides which were fused with 11-mer cell-permeable peptide sequences in order to improve cell permeability. In this regard, the development of shorter peptidomimetics which have better cell permeability and stronger inhibitory activity on the RANK receptor could be valuable and necessary.

Peptoids, *N*-alkylated glycine oligomers whose side chains are attached to the amide backbone nitrogen atom instead of the α-carbon of peptides (Figure 1), are an attractive class of peptidomimetics because of their various advantages, compared to peptides. They can be easily synthesized on solid-phase with a tremendous diversity using submonomer strategy[7] and are proteolytically resistant.[8] They can also adopt stable secondary and even more complex structural features and have been identified to possess a variety of interesting bioactivities.[9] Moreover, peptoids proved to be much more cell-permeable than analogous peptides by a reporter gene-based assay, suggesting that peptoids might be useful drug candidates.[10] With this information in hand, we envisioned to develop short peptoid analogs without containing cell-permeable peptide sequences as new RANK receptor inhibitors for the treatment of osteoporosis. We also report herein the efficient solid-phase synthesis of cyclic peptoid analogs as well as linear peptoid analogs. Cyclic peptides and many naturally occurring cyclic molecules have received a great deal of attention because of their challenging chemical synthesis and numerous interesting bioactivities.[11] Generally, cyclic peptides can exhibit an enhanced cell permeability[12] and are much less sensitive to enzymatic degradation.[13] Additionally, cyclic molecules might also be presumed to bind more tightly

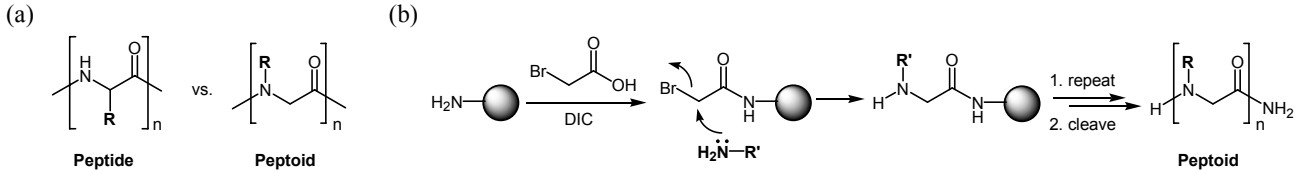

Figure 1. (a) Chemical structures of peptide and peptoid and (b) solid- phase synthesis of peptoid using submonomer strategy.

to their protein targets because of their more restricted conformational flexibility in some cases.[14] Thus, cyclic peptoid analogs could have a great potential as promising RANK receptor inhibitors.

For the purpose of this study, peptoid analogs (**L-PO1** – **L-PO3** and **C-PO1** – **C-PO3**) in both linear and cyclic forms were designed (Figure 2), based on three hexamer peptide sequences (IIVVYV, IIIIVV and IIIIIV) which possessed the inhibitory activity on RANK (unpublished results). The control peptoid (**Con-PO**) whose side chains are the same as those of the control peptide (ILAVYV) which did not have any inhibitory activity was also synthesized.

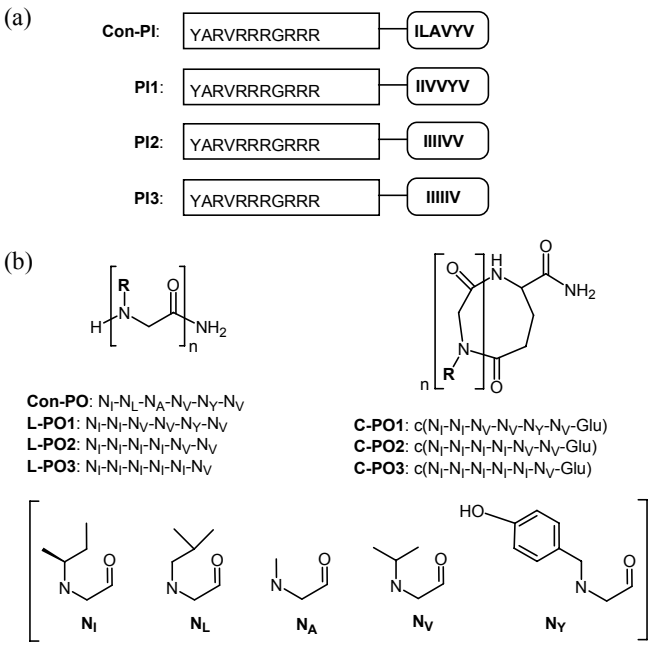

Figure 2. (a) Peptide sequences of RANK receptor inhibitors identified in the previous studies[4] and (b) peptoid analogs in both linear and cyclic forms employed in this study.

Peptoids were synthesized on solid-phase using a conventional submonomer strategy, which employed acylation with bromoacetic acid/DIC, followed by amination with primary amines under microwave conditions (Figure 3).[7] The desired side chains of peptoids could be obtained from 4 commercially available alkylamines such as (*S*)-(+)-2-butylamine, methylamine, isobutylamine and isopropylamine, and synthetically prepared 4-*tert*-butyloxybenzylamine. For the preparation of the side chain of tyrosine, 4-*tert*-butyloxybenzylamine was synthesized according to the literature.[15] Starting from 4-hydroxybezonitrile, the protection of the hydroxyl group as a *tert*-butyl ether using *tert*-butyl trichloroacetimidate which was prepared from *tert*-butanol, followed by the reduction of the nitrile group with $LiAlH_4$, furnished 4-*tert*-butyloxybenzylamine.[15] The *tert*-butyl protecting group could be removed at the cleavage step of peptoids from the resins using 92% trifluoroacetic acid (TFA). For the synthesis of linear peptoids, Fmoc of polystyrene AM RAM resin (0.6 mmol/g) was first removed with 20% piperidine, and then bromoacetic acid was attached to the resins using DIC under microwave conditions at a power of 100 W. The subsequent treatment of the beads with the primary amines (1 - 2 M in DMF) under microwave conditions completed the synthesis of each peptoid unit. The acylation and amidation steps were repeated to provide the desired peptoid sequences. At the final step, the resins were treated with a cleavage cocktail containing 92% TFA, 3% triisopropylsilane (TIS) and 5% H_2O to release the desired linear peptoids (**Con-PO** and **L-PO1** – **L-PO3**) which were purified through reverse-phase HPLC in high yields and lyophilized in a freeze-dryer.

Generally, it is very challenging to make cyclic compounds, especially on solid-phase. Cyclization of peptoids has been achieved through head to tail cyclization, ligation of side chains and polymerization.[16] Recently, we have also reported the efficient cyclization methods on solid-phase through an amide bond formation between the carboxyl group of Glu and the *N*-terminal amino function.[17] For the synthesis of cyclic peptoid analogs, we decided to employ the solid-phase macrocyclization strategy

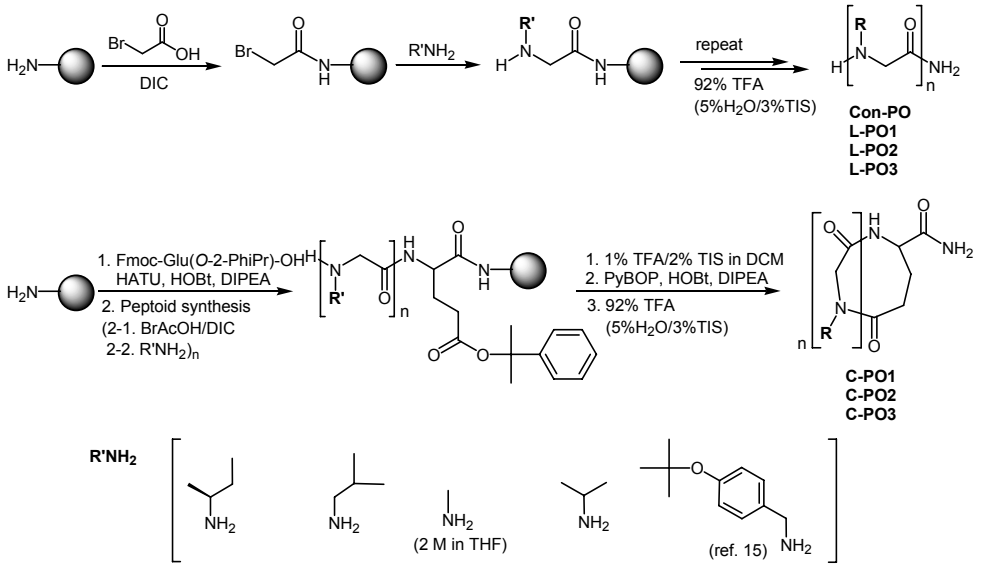

Figure 3. Solid-phase synthesis of peptoid analogs as RANK receptor inhibitors.

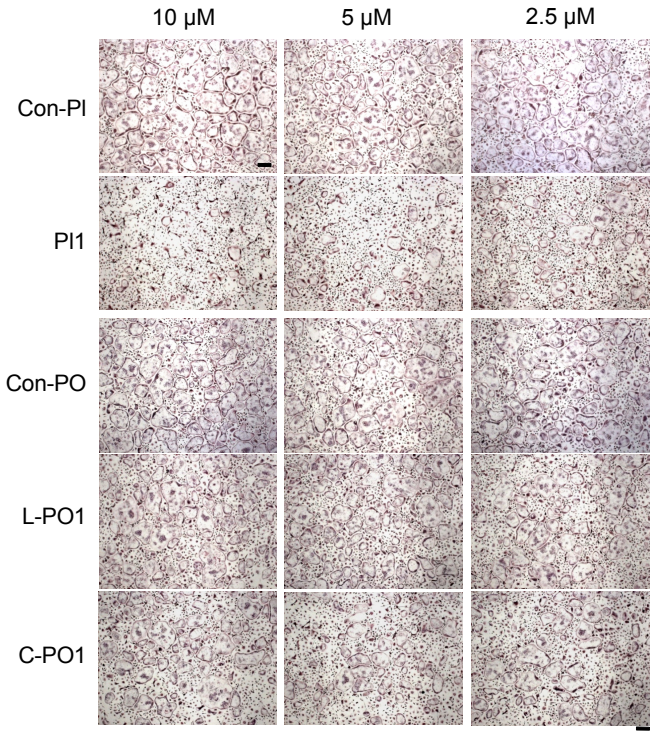

Figure 4. Inhibitory effect of peptoid analogs on osteoclasts formation: BMMs were cultured with M-CSF (30 ng/mL) and RANKL (200 ng/mL) for 4 days to generate osteoclasts. Peptoids or peptides were added at the time of RANKL addition. After osteoclasts formation, BMMs were stained for tartrate-resistant acid phosphatase (TRAP) activity; Scale bar: 200 μm.

which was already developed by our group (Figure 3). Generally, the low concentration is critical in most of solution-phase macrocyclization reactions. Similarly, the pseudodilution conditions that the resins have the low loading capacities should be helpful in the solid-phase macrocyclization. Thus, we used Rink amide AM resin LL with the capacity of 0.35 mmol/g, instead of polystyrene AM RAM resin (0.6 mmol/g). For the peptoid cyclization in the later stage, Fmoc-Glu(*O*-2-PhiPr)-OH, which contained two chemically orthogonal protecting groups (Fmoc and 2-PhiPr), was first attached to the resins using HATU and HOBt. After the selective removal of Fmoc with 20% piperidine, peptoid units were similarly introduced to the amino function of Glu using the submonomer protocol under microwave conditions. After the completion of peptoid sequences, cyclic peptoids were synthesized on the resins through the selective cleavage of the acid-labile 2-PhiPr group with 1% TFA and the subsequent cyclization with PyBOP and HOBt, which were identified to be the best cyclization conditions.[17] All of the peptoids were efficiently cyclized. The desired cyclic peptoids (**C-PO1** – **C-PO3**) were released from the resins with 92% TFA and purified through reverse-phase HPLC in high yields. The formation of the cyclic peptoids was fully confirmed through MALDI-TOF analysis.

In preliminary experiments, we simply tested whether the peptoid analogs (**L-PO1** and **C-PO1**) could inhibit RANKL-induced osteoclasts formation. **PI1** was also used as a positive control compound, and **Con-PI** and **Con-PO** as negative control compounds. Murine bone marrow-derived monocyte/macrophage precursor cells (BMMs) which were cultured with RANKL and M-CSF (macrophage colony stimulating factor) were incubated with either the peptides or the peptoids.[4] The RANKL-induced osteoclasts formation was inhibited by the wild type RANK receptor inhibitor (**PI1**) which was previously identified (Figure 4). However, the peptoid analogs (**L-PO1** and **C-PO1**) did not show the significant inhibition or showed relatively the very weak inhibition of osteoclasts formation at 10 μM even though the cellular images are not quite quantitative. Generally, the molecular conformations of compounds are closely related to their biological activities in many cases. Even though the peptoid analogs (**L-PO1** and **C-PO1**) contained the same side chains as the wild type peptide inhibitor (**PI1**), they might adopt the different conformations. Actually, it is also a significant issue to explore the relationships between peptoid structure and function.[9b,9c] Currently, peptoid model compounds are thoroughly tested for the inhibitory activities of osteoclasts formation and if necessary, new peptoid analogs will be designed from the biological data which will be obtained.

In summary, we have efficiently synthesized several peptoid analogs in both linear and cyclic forms as new RANK receptor inhibitors that we expect could have interesting cell permeability and inhibitory activity on the RANK receptor. The solid-phase synthesis of peptoids was synthetically straightforward. Especially, macrocyclization was well defined and very efficient on solid-phase, which can be further applied for the synthesis of many cyclic compounds. The detailed inhibition studies of various peptoid analogs against the RANK receptor are currently under way and the results will be reported elsewhere in due course though the preliminary results of some peptoids were not so good. These studies may provide significant insights into the relationships between peptoid structure and function as well as into the peptoid-based drug design for the treatment of osteoporosis.

Experimental Section

Materials and equipments. All of commercial reagents were used as obtained without further purification. The primary amines used in this study are as follows: (*S*)-(+)-2-butylamine, methylamine (2 M in THF), isobutylamine, isopropylamine and 4-*tert*-butyloxybenzylamine.[15] Polystyrene AM RAM macrobead (500 - 560 μm; 0.6 mmol/g) and Rink amide AM resin LL (100 - 200 mesh, capacity: 0.35 mmol/g) were purchased from Rapp Polymere and Novabiochem, respectively. Preparative HPLC was performed on a Shimadzu binary HPLC system with a C18 reverse-phase column using a gradient elution of water/acetonitrile with 0.1% TFA. MS (MALDI-TOF) was performed on a Voyager-DE STR biospectrometry workstation (Applied Biosystems) with α-hydroxy cinnamic acid as a matrix. Peptoids were synthesized in an incubator shaker (JEIO TECH, model: SI-600R) or under microwave conditions at a power of 100 W in a microwave oven (Daewoo, model: KR-B200R).

General procedure for the synthesis of linear peptoid analogs. The linear peptoids were synthesized on Polystyrene AM RAM macrobeads (20 - 30 μmol) under microwave conditions using a conventional submonomer strategy.[7b,17b] The desired products (**Con-PO** and **L-PO1** – **L-PO3**) were released from the resins

using 92% TFA containing 3% triisopropylsilane (TIS) and 5% water for 3 h, purified through reverse-phase HPLC using a gradient elution of water/acetonitrile with 0.1% TFA and lyophilized in a freeze dryer to give the isolated yields of 55 - 65%. The formation of the linear peptoid analogs was confirmed through MALDI-TOF analysis. MS (MALDI-TOF): *m/z*: **Con-PO:** calcd for $C_{34}H_{57}N_7NaO_7$ 698.4; found 698.7 $[M + Na]^+$, **L-PO1:** calcd for $C_{36}H_{61}N_7NaO_7$ 726.5; found 726.7 $[M + Na]^+$, **L-PO2:** calcd for $C_{34}H_{65}N_7NaO_6$ 690.5; found 690.6 $[M + Na]^+$, **L-PO3:** calcd for $C_{35}H_{67}N_7NaO_6$ 704.5; found 704.6 $[M + Na]^+$.

General procedure for the synthesis of cyclic peptoid analogs. Rink amide AM resins LL (20 - 30 μmol) swelled in DMF at room temperature for 1 h. Then DMF was drained, and the beads were incubated with 20% piperidine for 30 min. The beads were thoroughly washed with DMF (8 × 3 mL) and then treated with Fmoc-Glu(*O*-2-PhiPr)-OH (2.5 eq.) in the presence of HATU (2.5 eq.), HOBt (2.5 eq.) and *N*,*N*-diisopropylethylamine (DIPEA) (10 eq.) in DMF for 2 h. After the selective removal of Fmoc with 20% piperidine for 30 min, the peptoids were also synthesized under microwave conditions using a conventional submonomer strategy.[7b,17b] The 2-PhiPr group was selectively removed with the treatment of 1% TFA and 2% triisopropylsilane in DCM twice for 30 min. After the resins were thoroughly washed with 5% DIPEA in DCM and then DCM, cyclization was carried out using PyBOP (3 eq.), HOBt (3 eq.) and DIPEA (10 eq.) in DMF twice for 8 h. The desired cyclic peptoids (**C-PO1** – **C-PO3**) were cleaved from the resins using 92% TFA containing 3% TIS and 5% water for 3 h, purified through reverse-phase HPLC using a gradient elution of water/acetonitrile with 0.1% TFA and lyophilized in a freeze dryer to give the isolated yields of 38 - 45%. The formation of the cyclic peptoid analogs was confirmed through MALDI-TOF analysis. MS (MALDI-TOF): *m/z*: **C-PO1**: calcd for $C_{41}H_{66}N_8NaO_9$ 837.5; found 837.8 $[M + Na]^+$, **C-PO2**: calcd for $C_{39}H_{70}N_8NaO_8$ 801.5; found 801.6 $[M + Na]^+$, **C-PO3**: calcd for $C_{40}H_{72}N_8NaO_8$ 815.5; found 815.7 $[M + Na]^+$.

***In vitro* assays for osteoclasts formation.**[4] Bone marrow-derived macrophages (BMMs) were obtained from murine BM precursors of 6- to 8-week-old male C57BL/6 mice (The Jackson Laboratory). Bone marrow cells were suspended in α-minimal essential medium (α-MEM; Hyclone, Logan, UT) supplemented 10% fetal bovine serum (FBS; Hyclone, Logan, UT), penicillin (100 U/mL), streptomycin (100 μg/mL) and macrophage-colony stimulating factor (M-CSF; R&D Systems, 5 ng/mL) in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. The non-adherent cells were collected and cultured in α-MEM/10% FBS containing M-CSF (30 ng/mL) for 3 days. Floating cells were removed and adherent cells (BMMs) were used as osteoclast precursors of the monocyte/macrophage lineage. The precursor cells were seeded in a 48-well culture plate (3×10^4 cells/well) and cultured with M-CSF (30 ng/mL) and RANKL (200 ng/mL) for 4 days to generate osteoclasts. Peptoids or peptides were added at the time of RANKL addition. After osteoclasts formation, cells were fixed with 3.7% formaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) activity using a leukocyte acid phosphatase kit (Sigma, St Louis, MO). Cells were observed using a Zeiss Axiovert200 microscope (Carl Zeiss, Germany).

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