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Kilogram-Scale Synthesis of Osteogenic Growth Peptide (10–14) Using a Fragment Coupling Approach

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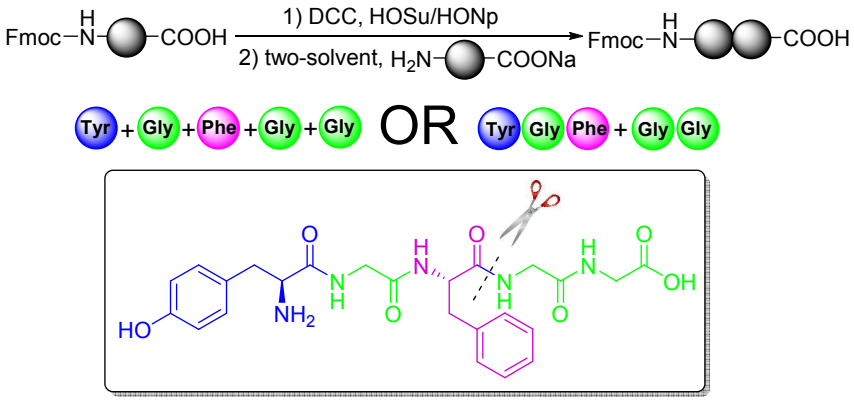


Abstract: Kilogram-scale synthesis of a bioactive pentapeptide in solution by "3+2" fragment coupling strategy has been successively accomplished in the development of OGP (10-14), a minimal OGP-derived sequence that retains the full proliferative activity of the osteogenic growth peptide. The synthetic scheme, coupling conditions and scaling up of the process are systematically studied, the epimerization of the tripeptide fragment and pentapeptide are also evaluated.

Keywords: Osteogenic growth peptide; OGP(10-14); Liquid-phase synthesis; Fragment coupling

Introduction

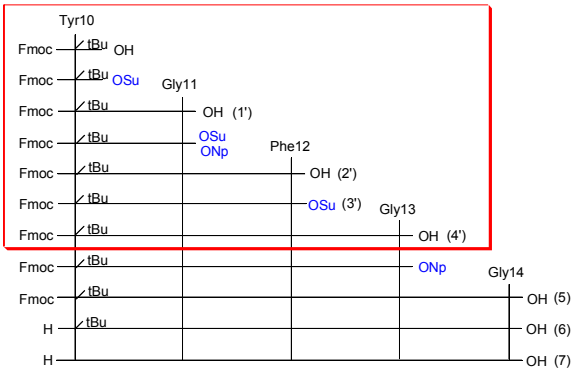
The osteogenic growth peptide (OGP) is an endogenous tetradecapeptide physiologically present in the blood circulation at micromolar concentrations.[1] It is identical to the C-terminal sequence of histone H4 spanning residues 89-102 (ALKRQGRTLYGFGG). Native and synthetic OGP are mitogenic to both osteoblastic and fibroblastic cells and can enhance osteogenesis and hematopoiesis in vivo. The structure-activity relationship revealed that C-terminal pentapeptide OGP (10-14), with the sequence of Tyr-Gly-Phe-Gly-Gly, is the minimal fragment that retains the full OGP-like activity.[2] The analogues of OGP (10-14) are used as bone anabolic agents and stimulators of hematopoiesis and will be carried out clinical trials soon. The promising activity makes it also applicable for a wide range of uses, including pharmaceuticals, intermediates, food additives and cosmeceuticals. Therefore, the demand for this product has increased significantly in recent years.

Though there are considerable number of research on the medicinal activity of OGP-peptides, few studies have investigated the synthetic method of these oligopeptide mimetics. Most of the described synthesis have been based on the solid-phase chemistry.[3] However, it is not the most cost-effective method to produce kilograms or even tens of kilograms of peptides due to limitation of resin carriers.[4] In some cases, the synthesis of OGP (10-14) was performed by enzyme catalysis or photochemical method,[5] but both of them only produce a small batch of products and cannot meet practical demands. An excellent example of enfuvirtide,[6] which is the first peptide-drug substance that is produced in

multi-tons, gave us incentive to explore a facile and cost-effective approach for large scale synthesis of OGP (10-14). Alternatively, a solution process could provide considerable freedom regarding the choice of synthetic schemes, protecting groups, solvent systems, monitoring measures and scaling up,[7] these properties suggest that it is possible to get substantial amounts of OGP (10-14) to meet the commercial demands. Therefore, we set the goal of synthesizing OGP (10-14) in solution and investigating the effect of different coupling conditions on the yield.

Results and Discussion

Initially, we had planned to prepare the OGP(10-14) by the stepwise construction of the peptide chain starting from the protected tyrosine moiety. Except for protected tyrosine, free glycine and phenylalanine are used directly in this approach, showed lower costs and is suitable for industrial-scale production. In our published patent,[8] this scheme has satisfactorily performed on tens of grams synthesis of the pentapeptide (Scheme 1). However, when it was applied to hundreds of gram-scale preparation, some impurities gradually accumulated and the solubility of peptide chain decreased substantially, especially after four links of amino acids were completed (**4'**).



Scheme 1. Step-wise construction of OGP (10-14).

Further analyses show that O-acylisourea tends to be the primary source of impurities (Figure 1), it is one of the most reactive active species and could be rearranged into the N-acylurea, which is irreversible and consumes starting amino acid without generating product.[9] In addition, due to excess carbodiimide is used, traces of DCU are accumulated gradually and are difficult to be removed. Although some of impurities can be removed by using column chromatography, this process is undesirable, time consuming and limiting amplification. What's more, the purity of crude pentapeptide obtained after all side-chain deprotection was about 40–60%, which led to the overall yield of product at 25–35% seemed too low to achieve commercial objectives. Therefore, it is necessary to separate the peptide chain into different fragments to decrease interference from impurities.

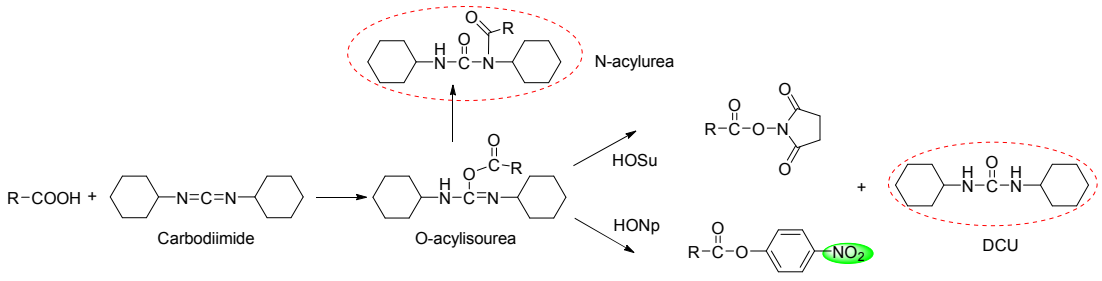
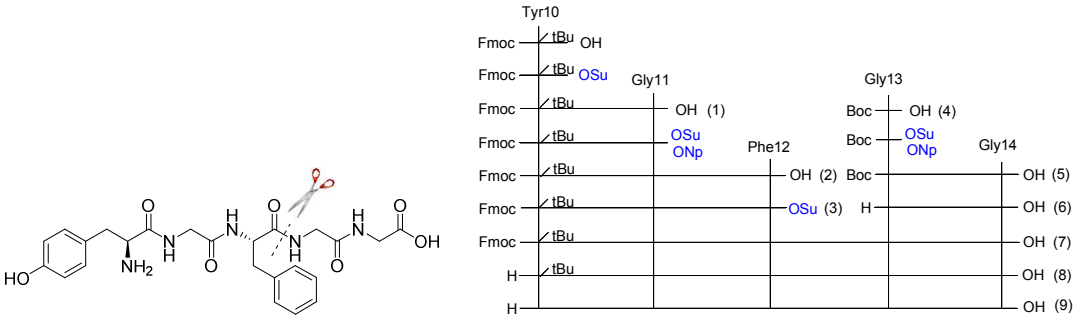


Figure 1. Mechanism of active ester formation from an O-acylisourea intermediate.

In selecting the fragments, both “3+2” and “2+3” convergence would avoid the need to purify tetrapeptide (**4'**) and shorten the synthetic cycle. But based on the consideration of atom economy, the protected phenylalanine, which had to used as the starting amino acid in “2+3” strategy, is comparatively expensive, while Boc_2O is much less expensive in “3+2” strategy, the synthesis of

Boc-Gly-OH could be carried out under moderate conditions and facilitate industrial production. Therefore, we decided to perform the synthesis by "3+2" fragment coupling strategy (Scheme 2).



Scheme 2. "3+2" fragment condensation of OGP (10-14).

Due to the aforementioned difficulties with impurity and material costs, the peptide chain was finally divided into two different sequences: Fmoc-Tyr(tBu)-Gly-Phe-OH (**2**) and H-Gly-Gly-OH (**6**). The intermediates were obtained as follows. The N-terminal amines of tyrosine and glycine13 were protected by the Fmoc- and Boc- groups, respectively. The phenolic hydroxyl group of tyrosine was protected by formation of its t-butyl ether. The peptide bonds were facilitated by the use of "activated ester", which was obtained in a nearly stoichiometric yield and used in the next step without further purification. The carboxyl groups of glycine11, phenylalanine and Gly14 were converted into their salt forms before reacting with the relevant activated ester. The Boc- protecting group was easily removed with 50% trifluoroacetic acid in dichloromethane (v/v). After condensation of the phenylalanine and glycine13 residues of two fragments, the protected pentapeptide (**7**) was obtained with a satisfactory yield. Finally, the Fmoc- group was deprotected with 10% piperidine in dioxane (v/v) and the tBu-

Group was removed by 50% trifluoroacetic acid in dichloromethane (v/v). The crude product was purified on cation exchange chromatography to yield the pure pentapeptide (**9**).

"3+2" condensation is a crucial step in the amplifying synthesis of the protected pentapeptide (**7**). Because the tripeptide ester (**3**) is freely soluble in organic solvent, while glycylglycine (**6**) was hydrophilic, undissolving in the organic layer, the fragment condensation must be carried out in two miscible liquids. Consequently, the proportion of mixed solvents directly affects the yield of the product (**7**). From figure 2, the expected yield was not obtained when a lower percentage of water was added to the reaction system, because water was essential for dissolving glycylglycine to ensure the homogeneity of two-solvent system. The yield was satisfactory when the proportion of water was between 23% and 38% (v/v), but it decreased when the water content was greater 40% due to the increase of hydrolysis of the tripeptide ester (**3**). Therefore, the optimal water proportion for the synthesis of protected pentapeptide (**7**) was approximately 30% (v/v).

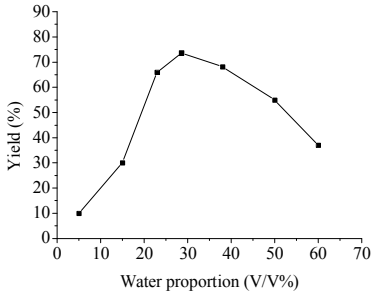


Figure 2. The effects of water proportion on the yield of protected pentapeptide (**7**) in "3+2" condensation.[a]

[a]Mole ratio of tripeptide ester (**3**), glycylglycine, and sodium bicarbonate was about 1:1.2:1.2. Solvent system is a mixture of water and tetrahydrofuran. All reactions were performed at 25°C for 10 hours and analyzed by HPLC using a Kromasil C18 column (4.6 mm diameter × 250 mm) with a linear

gradient from 10% to 90% aqueous acetonitrile (0.1% trifluoroacetic acid) over 30 min at a flow rate of 1.0 mL/min and detected at 215 nm.

The aminolysis of peptide ester has been demonstrated to be a general base-catalyzed reaction, however, the hydrolysis of active esters is performed under the same condition.[10] Therefore, the type and the amount of base in the solvent system of "3+2" condensation were also necessary to be investigated. Figure 3 shows that the amount of base had substantial effects on the yield of protected pentapeptide (**7**). Compared with the 10% yield of the free base, the synthetic yields promoted by the general base were measurably increased. When the amount of base was added to 1.0-1.5 molar quantity, $NaHCO_3$, TEA and DIEA resulted in a high yield, whereas Na_2CO_3 generated the lowest yield (30%) followed by DEA (40%). This result was due to the strong basicity that makes the hydrolysis of the ester faster than the coupling reaction, especially with a large excess of base. To be environmentally friendly, sodium bicarbonate was often used as a additional base with 1.2 equivalents in our research.

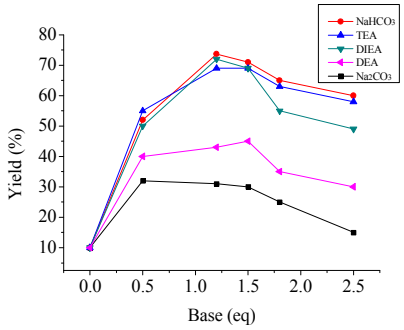


Figure 3. The effect of the type and amount of base on the yield of protected pentapeptide (**7**).[a]
[a]Mole ratio of tripeptide ester (**3**) and glycylglycine was about 1:1.2; Volume ratio of water and tetrahydrofuran was about 2:5; The determination method was the same as described in Fig. 2.

A major problem in the repetitive process is the separation of the peptide products from liquid mixtures. We have envisioned that this problem could be easily solved by reacting the full-protected peptide ester with excess free amino acid to produce hydrophobic peptide acids products (Figure 4), which could be precipitated by addition of 10% aqueous solution of citric acid (pH 3) after remove the organic phase by reduced pressure distillation. While excess amino acids, base and other hydrophilic byproducts remain soluble in the aqueous solution and can be easily removed by filtration. Using this protocol, a series of peptide intermediates were quickly prepared in high yields with excellent purity. Additionally, a low coupling yield would be obtained if 1,4-dioxane was used as a solvent during the kilograms preparation of Fmoc-Tyr(tBu)-Gly-Phe-OH (**2**). It is because 1,4-dioxane can easily form azeotropes with water which makes it hard to be distilled, result in the uncomplete precipitation of the tripeptide and ultimately lead to the loss of product during the post-treatment.

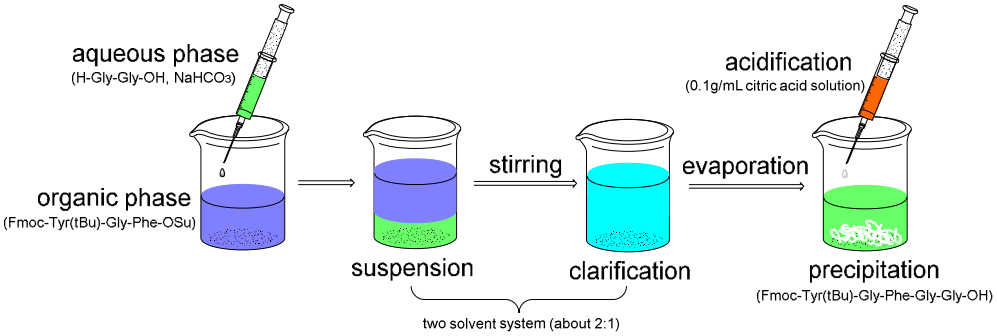


Figure 4. Schematic representation of the separation of the hydrophobic products from two solvents.

Under above-mentioned optimal conditions, the production time for each fragment is less than five days. At present, pilot scale of operation provides each fragment in 2 to 5kg batches. The cycle time from building the fragments to completion of the analytical and preparative scale purification on the pentapeptide (**9**) is approximately half a month. The efficiency of fragment coupling is approximately 70-80% and the overall yield of pure product is about 45%. Therefore, "3+2" fragment coupling approach could satisfy kilogram-scale needs of OGP(10-14) and support late-stage sustained development.

A potential problem in solution phase fragment condensation could be the epimerization leading to diastereomers. In our research, several positive parameters were applied to minimize racemization during peptide coupling reactions. A key issue is the use of an appropriate additive and condensing reagent. HOSu is a relatively stable additive to HONp, while HONp is more prone to peptide-coupling than other additives. Both of them are less reactive than the O-acylisourea toward peptide bond formation (Figure 1). Therefore, except the optically inactive glycine residues, which can be activated as -ONp or -OSu at room temperature, tyrosine and phenylalanine must be activated as -OSu in ice bath. Although such procedures could possibly reduce racemization during the activation reaction, it is necessary to further ensure the absolute configuration of the peptide chain by HPLC chiral analysis. Therefore after replace L-amino acid with D-amino acid, we successfully obtained various epimers of the tripeptide and pentapeptide. By comparing the chromatogram of desired peptide with the

resynthesized epimers for "3+2" fragment coupling, both tripeptide (**2**, 17.418min) and pentapeptide (**9**, 40.00min) show a single peak with the different retention time of their isomers in the chromatograms (Figure 5). These results show that the epimerization at the carboxyl terminus of tripeptide during the coupling reaction is not observed and the configuration of pentapeptide is maintained under the optimal synthetic process, which also indicated that "3+2" condensation is an excellent method for successfully obtaining OGP(10-14) with high optical purity.

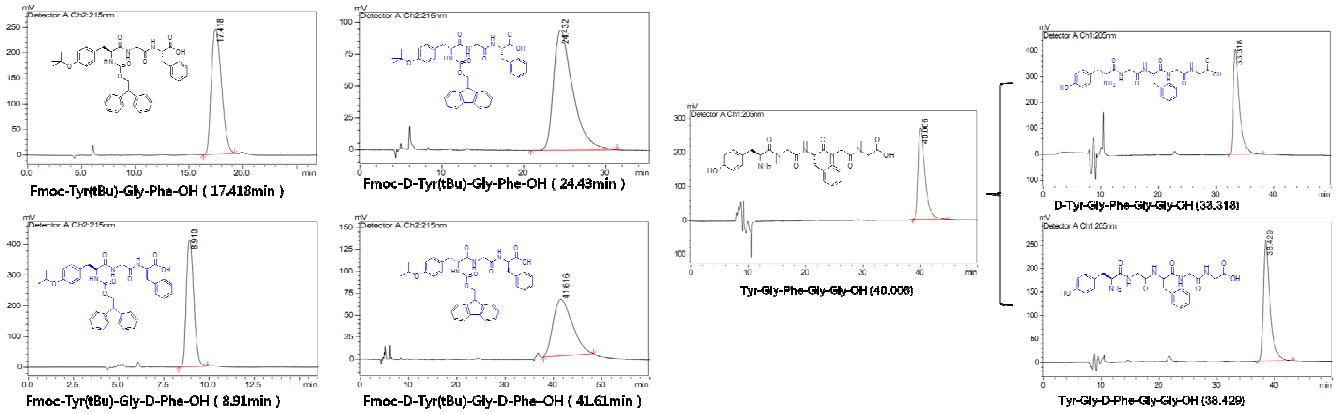


Figure 5. Chiral analysis of epimerization for "3+2" fragment coupling step.

Conclusions

An efficient process for kilogram-scale synthesis of OGP(10-14) has been developed in solution by "3+2" fragment coupling approach using free amino acids for each coupling cycle. Compared with our original stepwise protocol, the yield obtained by fragment condensation was significantly improved (from 25–35% to 45%) and the post-processing was more convenient. Furthermore, some important factors such as route selection, aqueous proportion, base quantity, post-treatment and epimerism

evaluation were optimized for "3+2" fragment coupling step. All results indicate that "3+2" route is a reasonably appropriate way for the synthesis OGP(10-14) on kilogram-scale with good yield and high optical purity. Our study not only provides a rapid, economic and environmental friendly method for the synthesis of OGP (10-14) on a large scale but also gives some useful information for the liquid phase synthesis of peptides, especially for oligopeptides.

Experimental Section

Thin-layer chromatograms are run on GF254 plates in the following systems:

a_1, ethyl acetate-n-hexane-acetic acid (10:10:1);

a_2, ethyl acetate-n-hexane-acetic acid (10:5:1)

a_3, ethyl acetate-n-hexane-acetic acid (15:5:1);

b, ethyl acetate-n-hexane-methanol-acetic acid (10:10:1:1);

c, n-butyl alcohol-acetic acid-water-0.05 g/ml Nin (8:3:1:1).

Analysis conditions of high performance liquid chromatography are as follows:

d: A(0.1% TFA/ACN), B(0.1% TFA/H_2O), 30 min 10-90%;

e: A(0.1% TFA/ACN), B(0.1% TFA/H_2O), 30 min 50-90%;

f: A(0.1% TFA/ACN), B(0.1% TFA/H_2O), 30 min 55-90%.

TLC measurements were performed on silica gel GF254 plates (Qingdao Haiyang Chemical Co., Ltd, China). Melting points were determined using a XT-4 melting point apparatus with temperature uncorrected (Beijing Tech Instrument Co., LTD, China). Compounds were visualized by irradiation with UV light or by treatment with 0.05 g/ml ninhydrin in ethanol or potassium iodide reagent. Optical rotations were determined using an Autopol II automatic polarimeter for solution in methanol. Elemental analysis was performed by Vario EL III CHNOS analyzer. ^{1}H-NMR and FT-IR spectra were recorded on a Bruker Avance-400 MHz and Perkin Elmer-spectrum instrument, respectively. MS were obtained with a LTQ-XL apparatus. HPLC was performed by Hitachi L2000 instrument equipped with a diode array detector (L-2455). Chiral analysis of tripeptide and pentapeptide were carried out respectively on CHIRALPAK AD-H (0.46cm×25cm× 5μm) and CHIRALPAK ZWIX(+) (0.40cm×15cm×3μm) columns using Shimadzu LC 20 with UV detector SPD-20A. The crude pentapeptide was purified by cation exchange chromatography using a SP sepharose fast flow column (GE Healthcare), and desalted by reversed phase liquid chromatography on a SBC MCI GEI column (Sci-Bio-Chem Co.Ltd.), finally lyophilized with a Biocool lyophilizer. Saline refers to a saturated sodium chloride solution. The L-amino acids (protected or free) were obtained from GL Biochem Ltd. (Shanghai, China). Other reagents were provided by Sigma-Aldrich. The organic solvents were commercially available product (Sinopharm Chemical Reagent Co., Let, China) and were dried with anhydrous sodium sulfate before application. During the coupling reaction, material ratio was strictly

controlled and evaporation was performed in vacuo below 37°C. Most of the solvents after distillation can be reused.

Abbreviations: acetonitrile (ACN), tert-butyloxycarbonyl (Boc-), di-*tert*-butylpyrocarbonate (Boc_2O), N, N'-dicyclohexy- carbodiimide (DCC), dichloromethane (DCM), N, N'-dicyclohexylurea (DCU), diethylamine (DEA), diisopropylethylamine (DIEA), dimethylformamide (DMF), Ethyl acetate (EtOAc), 9-Fluorenylmethoxycarbonyl (Fmoc), n-hexane (HEX), p-nitrophenol (HONp), N-hydroxysuccinimide (HOSu), tert-Butyl (tBu), triethylamine (TEA), trifluoroacetic acid (TFA), Tetrahydrofuran (THF), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), ninhydrin (Nin), N-methylmorpholine (NMM).

Fmoc-Tyr(tBu)-Gly-OH (1)

Fmoc-Tyr(tBu)-OH (2.3 kg, 5mol) and HOSu (0.69 kg, 6mol, 1.2eq) were dissolved in dry 1,4-dioxane (20 L) and stirred in an ice-bath. A solution of DCC (1.24 kg, 6mol, 1.2eq) in dry 1,4-dioxane (5 L) was slowly added. The mixture stirred in ice bath for 4h, and then at ambient temperature for further 8h. After cooling in refrigerator for 1h, the precipitate of DCU was removed to leave the Fmoc-Tyr(tBu)-OSu in 1,4-dioxane as a colorless transparent liquid, TLC: a_1, R_f=0.4, which was used without further purification.

Glycine (0.45 kg, 6mol, 1.2eq) and $NaHCO_3$ (0.5 kg, 6mol, 1.2eq) was dissolved in H_2O (10 L), The aqueous solution was added to the solution of Fmoc-Tyr(tBu)-OSu (get by above step) in 1,4-dioxane (25 L) in 2h. The mixture was stirred at about 25°C until the active ester disappeared by TLC (about 8h). The solution was concentrated to a small volume (6 L) and treated with ice ether (8 L). At the same time, the pH value was adjusted close to 2-3 by addition of 10% aqueous solution of citric acid. The white solid was precipitated, filtered and washed with cold water. After dried under vacuum, Fmoc-Tyr(tBu)- Gly-OH (2.42 kg, 93.7%) was yield as white pulverous solid, m.p. 108.9～110.9°C, TLC: a_1, R_f=0.2, HPLC: *d*, 25.63min, purity 89.38% , Anal. Calc. for $C_{30}H_{32}N_2O_6$: C, 69.75; H, 6.24; N, 5.42. Found: C, 69.55; H, 6.31; N, 5.38. ESI-MS for [M] calcd: 516.58, found: 540.87 ($[M+Na]^+$, 100%); 521.81 (70%). $[\alpha]_D^{20.5}$-35.51 (c=1.0, CH_3OH). IR (KBr): 3679.9, 3331.0, 2973.0, 1676.0, 1525.6, 1237.5, 1032.6, 738.1, 616.4 cm^{-1}. ^1H NMR (400 MHz, MeOD) δ 7.81 (d, *J* = 7.5 Hz, 2H), 7.62 (t, *J* = 7.7 Hz, 2H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.33 (t, *J* = 7.1 Hz, 2H), 7.19 (d, *J* = 8.3 Hz, 2H), 6.86 (t, *J* = 9.8 Hz, 2H), 4.42 (dd, *J* = 10.1, 4.3 Hz, 1H), 4.30 (dd, *J* = 10.1, 7.0 Hz, 1H), 4.14 (dt, *J* = 13.7, 7.1 Hz, 2H), 3.95 – 3.81 (m, 2H), 3.49 (dd, *J* = 12.8, 5.8 Hz, 1H), 3.22 (dd, *J* = 13.7, 4.4 Hz, 1H), 2.88 – 2.73 (m, 2H), 1.40 – 1.09 (m, 9H).

Fmoc-Tyr(tBu)-Gly-Phe-OH (2)

Fmoc-Tyr(tBu)-Gly-OH (2.42 kg, 4.68mol) was dissolved in dry THF (25 L). HOSu (0.65kg, 5.62mol, 1.2eq) or HONp (0.78 kg, 5.62mol, 1.2eq) were added and stirred at ambient temperature. A solution of DCC (1.25 kg, 6.05mol, 1.3eq) in dry THF (5 L) was added and the mixture stirred for 10h. After cooling for 1h, the precipitate of DCU was filtered off and the solution was used to the next step directly, TLC: *a1*, R_f=0.3/0.4.

A suspension of L-phenylalanine (1 kg, 6.08mol, 1.3eq) in H_2O (15 L) was mixed with $NaHCO_3$ (0.51 kg, 6.08mol, 1.3eq), the mixture was drop-wise added to the solution of Fmoc-Tyr(tBu)-Gly-OSu in THF (30 L). The reaction mixture was stirred at about 25°C until the solution become clear and then for further 5h. The solvent was evaporated, the residue was distributed by H_2O (30 L), EtOAc (10L) and light petroleum (10 L). After organic layer removed, the aqueous phase was acidified to a pH 2-3 with 10% citric acid aqueous solution and further extracted with EtOAc (3×15 L). The combined organic extracts were washed with saline (2×10 L), dried with magnesium sulfate and evaporated. After ultrasonic extractions with ether (10 L), Fmoc-Tyr(tBu)-Gly-Phe-OH (2.76 kg, 89.12%) was obtained as a white crystal, m.p. 136.7～140.1°C, TLC: *a2*, R_f=0.2, HPLC: *e*, 22.14min, purity 87.52%, Calc. for $C_{39}H_{41}N_3O_7$: C, 70.57; H, 6.23; N, 6.33. Found: C, 70.33; H, 6.31; N, 6.27. ESI-MS for [M] calcd: 663.75, found: 686.48 ($[M+Na]^+$, 40%); 686.41 ($[M+Na]^+$, 40%); 413.53 (100%). $[\alpha]_D^{20.5}$-20.46 (c=1.0, CH_3OH). IR (KBr): 3292.6, 3193.3, 2956.4, 1660.0, 1541.6, 1400.7, 1301.5, 616.43 cm^{-1}. ^1H NMR (400 MHz, DMSO) δ 8.52 (t, *J* = 5.7 Hz, 1H), 8.17 (dd, *J* = 17.4, 7.2 Hz, 2H), 7.87 (d, *J* = 7.5 Hz, 2H),

7.65 (t, J = 7.6 Hz, 3H), 7.40 (t, J = 7.4 Hz, 3H), 7.34 – 7.27 (m, 3H), 7.25 (d, J = 4.1 Hz, 3H), 7.17 (d, J = 8.5 Hz, 2H), 4.56 (s, 1H), 4.19 (d, J = 11.2 Hz, 1H), 4.16 – 4.06 (m, 3H), 3.89 – 3.76 (m, 3H), 3.05 (dd, J = 13.9, 4.2 Hz, 1H), 2.95 (d, J = 9.8 Hz, 1H), 2.79 – 2.68 (m, 2H), 1.41 – 0.85 (m, 9H).

Fmoc-Tyr(tBu)-Gly-Phe-OSu (3)

To a stirred solution of Fmoc-Tyr(tBu)-Gly-Phe-OH (2.76 kg, 4.16mol) and HOSu (0.67 kg, 5.83mol, 1.4eq) in dry THF (25 L), a solution of DCC (1.21 kg, 5.83mol, 1.4eq) in dry THF (5 L) was added slowly under ice-cooling within 3h, then the solution was stirred overnight at ambient temperature. After cooling for 1 h, the resulting precipitate was filtered and filtrate was used further reaction without purification. TLC: a_2, R_f=0.3.

Boc-Gly-OH (4)

The mixture of glycine (1 kg, 13.32mol) and $NaHCO_3$ (1.12 kg, 13.32mmol) in H_2O (15 L) was added slowly to the solution of Boc_2O (3.06 L, 13.32mol) in THF (10 L) and stirred at ambient temperature for 5h. When TLC indicated the absence of starting material, the residue was evaporated and distributed by H_2O (20 L), EtOAc (7 L) and light petroleum (7 L). The organic layer was removed and the aqueous phase adjusted to pH 2-3 with 10% citric acid solution. After extracted with EtOAc (3×10 L), the aqueous layer was separated and the combined organic extracts were washed with saline (2×10 L), dried with magnesium sulfate and evaporated to give a white crystal (1.98 kg, 84.85%). m.p.85.7～86.5°C,

TLC: *a1*, R_f=0.2, HPLC: *d*, 10.40min, purity 94.7%, Calc. for $C_7H_{13}NO_4$: C, 47.99; H, 7.48; N, 8.00. Found: C, 47.40; H, 7.02; N, 8.31. ESI-MS for [M] calcd: 175.18, found: 198.78 ($[M+Na]^+$, 100%); 339.24 (100%). IR (KBr): 3340.6, 3116.5, 2982.0, 2732.4, 2534.0, 1746.4, 1672.8, 1538.4, 1413.5, 1202.67, 1058.2, 958.97, 856.54, 587.65cm^{-1}.

Boc-Gly-Gly-OH (5)

Boc-Gly-OH (1.98 kg, 11.3mol) and HONp (1.73 kg, 12.43mol, 1.1eq) or HOSu (1.43 kg, 12.43mol, 1.1eq) were dissolved in dry 1,4-dioxane (25 L), a solution of DCC (2.8 kg, 13.56mol, 1.2eq) in dry 1,4-dioxane (10 L) was added and the mixture stirred for 8-10h. After cooling for 2h, the precipitate was removed, the filtrate was used directly for next reaction without purification, TLC: *a1*, R_f=0.4, 0.3. The mixture of glycine (0.94 kg, 12.43mol, 1.1eq) and $NaHCO_3$ (1.05 kg, 12.43mol, 1.1eq) in H_2O (10 L) was slowly added to the solution of Boc-Gly-ONp (get by above step) in 1,4-dioxane (25 L). The solution was stirred at about 25°C for 7 h, then evaporated under reduced pressure. The residue was acidified to a pH 2-3 with 10% citric acid and extracted with EtOAc (3×20 L). The organic phase was successively washed with saline (20 L), dried, and evaporated to give Boc-Gly-Gly-OH (2.37 kg, 90.3%) as a white schistose crystals. m.p.123.5～125.5°C, TLC: *a3*, R_f=0.2, HPLC: *e*, 6.53min, purity 93.5%, Calc. for $C_7H_{13}NO_4$: C, 46.55; H, 6.94; N, 12.06. Found: C, 45.40; H, 6.02; N, 14.13. ESI-MS for [M] calcd: 232.23, found: 255.03 ($[M+Na]^+$, 100%); 233.06 ([M+H], 20%).

Glycylglycine (6)

TFA (15 L) was added to a solution of Boc-Gly-Gly-OH (2.37 kg, 10.2mol) in DCM (15 L) with stirring. After starting material run out indicated by TLC (*a*$_3$), the solution was evaporated to a small volume (3 L) and treated with ice ether (15 L). The resulting solid was collected to give H-Gly-Gly-OH (1.15 kg, 84.9%) as a white solid. m.p.220.7～222.1°C, TLC: *c*, R_f=0.2, HPLC: *d*, 4.35min, purity 95.7%, Calc. for $C_4H_8N_2O_3$: C, 36.36; H, 6.01; N, 21.2. Found: C, 36.01; H, 5.92; N, 21.31. ESI-MS for [M] calcd: 132.05, found: 132.76(100%); 132.69 (85%). IR (KBr): 3267.0, 3199.7, 2937.2, 1455.1, 1099.2, 1023.0, 930.16, 718.9, 622.86 cm^{-1}. ^1H NMR (400 MHz, D_2O) δ 3.77 (d, *J* = 2.6 Hz, 1H), 3.74 (d, *J* = 2.5 Hz, 1H), 3.69 (d, *J* = 8.6 Hz, 2H), 3.67 – 3.62 (m, 2H), 3.56 (dd, *J* = 11.6, 5.9 Hz, 2H).

"3+2" condensation (7)

The mixture of glycylglycine (0.66 kg, 4.99mol, 1.2eq) and $NaHCO_3$ (0.42 kg, 4.99mol, 1.2eq) in H_2O (13 L) was added to a solution of Fmoc-Tyr(tBu)-Gly-Phe-OSu (4.16mol) in THF (30 L) and stirred for 8h at ambient temperature. After the tripeptide ester could not be detected by TLC, the solution was evaporated and the residue was adjusted to pH 2-3 with 10% citric acid. The precipitate was filtered and washed with cold water. The crude product was triturated with ice ether to give the protected pentapeptide (2.43 kg, 75.1%) as a white solid. m.p.105.0～107.8°C. TLC: *b*, R_f=0.2, HPLC: *e*, 15.46min, purity 95.3%, Calc. for $C_{43}H_{47}N_5O_9$: C, 66.39; H, 6.09; N, 9.00. Found: C, 66.34; H, 6.11; N, 9.02. ESI-MS for [M] calcd: 777.58, found: 777.92 (100%); 800.32 ($[M+Na]^+$, 85%); 876.42 (80%).

$[\alpha]_D^{20.5}$-27.13 (c=1.0, CH_3OH). IR (KBr): 3705.5, 3286.2, 3084.5, 1672.8, 1644.0, 1551.2, 1202.3, 1135.0, 699.68 cm^{-1}. ^1H NMR (400 MHz, DMSO) δ 8.41 (s, 1H), 8.19 (d, *J* = 19.8 Hz, 2H), 7.87 (d, *J* = 7.1 Hz, 2H), 7.64 (d, *J* = 7.7 Hz, 3H), 7.50 – 7.34 (m, 3H), 7.35 – 7.26 (m, 3H), 7.17 (d, *J* = 8.3 Hz, 3H), 6.79 (d, *J* = 8.3 Hz, 2H), 4.53 (s, 1H), 4.21 (s, 1H), 4.17 – 4.01 (m, 3H), 3.94 – 3.78 (m, 2H), 3.75 (dd, *J* = 13.6, 5.5 Hz, 2H), 3.62 (s, 1H), 3.17 (d, *J* = 5.1 Hz, 2H), 3.05 (d, *J* = 9.1 Hz, 2H), 2.95 (d, *J* = 10.2 Hz, 2H), 2.82 – 2.65 (m, 2H), 1.27 (dd, *J* = 39.1, 28.2 Hz, 9H).

H-Tyr(tBu)-Gly-Phe-Gly-Gly-OH (8)

Fmoc-Tyr(tBu)-Gly-Phe-Gly-Gly-OH (2.43 kg, 3.13mol) was added to a solution of piperidine in 1,4-dioxan (10% v/v, 30L) and stirred for 1h. After TLC (*b*) indicated the absence of starting material, the solution was evaporated to dryness. The residue was distributed by H_2O (30 L) and DCM (3×10 L). The organic layer was removed and the aqueous phase evaporated to give H-Tyr(tBu)-Gly-Phe-Gly-Gly -OH (1.68kg, 96.47%) as a gummy solid. TLC: *c*, R_f=0.3, HPLC: *e*, 14.32min, purity 87.5%, ESI-MS for [M] calcd: 555.62, found: 555.72 (20%); 555.00 (10%); 496.23 ([M-59], 100%).

H-Tyr-Gly-Phe-Gly-Gly-OH (9)

H-Tyr(tBu)-Gly-Phe-Gly-Gly-OH (1.68 kg, 3.02mol) was dissolved in a solution of TFA in H_2O (95% v/v, 30L) and stirred for 1h. The solution was concentrated to a small volume (2 L) and ice ether was added (9 L), then the solvent decanted and the residue triturated with more ether (5 L). The resulting

solid was collected and dried under vacuum to yield the crude pentapeptide with white solid (1.43 kg, 94.79%, purity 80-85%). The crude product was dissolved in the minimum volume of 0.02mol/L acetic acid-sodium acetate aqueous buffer (pH 4, K=0.36) and purified by a column (10×50cm) of SP sepharose which had been equilibrated previously with the same buffer. The column was eluted with 0.02mol/L acetic acid-sodium acetate with 1mol/L sodium chloride aqueous buffer (pH 4) from 40% to 60% for 60min at a flow rate of 100mL/min and the effluent monitored by U.V. absorption at 254nm. Fractions were pooled and evaporated. Desalting is done on a SBC MCI GEI column (10×50cm) with buffer A (0.25% TFA/ACN) and B (0.25% TFA/H_2O). The column was eluted with buffer A from 15%-25% for 30min at a flow rate of 50mL/min and monitored as in the case of cation exchange chromatography. The fractions containing the major component were pooled and evaporated, which was further lyophylized at -50°C for 3 days to obtain chromatographical homogeneous Try-Gly-Phe-Gly-Gly (1.14 kg) as a white crystalline solid. The purity is more than 97%, the overall yield is 45.64%. m.p.176.8～180.0°C. TLC: *c*, R_f=0.2. HPLC: *d*, 11.34min. Calc. for $C_{22}H_{29}N_5O_7$: C, 57.71; H, 5.85; N, 14.02. Found: C, 57.73; H, 5.80; N,14.01. ESI-MS for [M] calcd: 449.51, found: 500.51 ([M+H], 100%); 500.45 (90%). IR (KBr): 3293.4, 3080.8, 2933.3, 1666.3, 1638.8, 1519.1, 1448.1, 1241, 1202, 1139.8, 839.38, 723.63, 699.77 cm^{-1}. $[\alpha]_D^{20.5}$+24.13 (c =0.65, MOH). ^1H NMR (400 MHz, CD_3OD) δ 6.55 – 6.38 (m, 5H), 6.28 (d, *J* = 8.4 Hz, 2H), 5.97 (d, *J* = 8.4 Hz, 2H), 3.79 (dd, *J* = 8.9, 5.7 Hz, 1H), 3.19 (dd, *J* = 12.1, 7.5 Hz, 2H), 3.14 (d, *J* = 10.2 Hz, 3H), 3.02 (d, *J* = 16.8 Hz, 1H), 2.90 (d, *J* = 16.4

Hz, 1H), 2.42 (dd, *J* = 14.0, 5.7 Hz, 2H), 2.31 (dd, *J* = 14.2, 6.3 Hz, 1H), 2.13 (ddd, *J* = 14.2, 8.5, 5.6 Hz, 2H).

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Supporting Information Available

Experimental details and spectroscopic data for all peptides described here.

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