A Short-Length Peptide YY Analogue with Anorectic Effect in Mice

Naoki Nishizawa, Ayumu Niida, Yasushi Masuda, Satoshi Kumano, Kotaro Yokoyama, Hideki Hirabayashi, Nobuyuki Amano, Tetsuya Ohtaki, and Taiji Asami

Pharmaceutical Research Division, Takeda Pharmaceutical Company, Ltd., Fujisawa 251-8555, Japan

Supporting Information

ABSTRACT: Peripheral administration of PYY$_{3-36}$, a fragment of peptide YY (PYY), has been reported to reduce food intake by activating the neuropeptide Y2 receptor (Y2R). An N-terminally truncated PYY analogue, benzoyl-[Ala$_{26}$,Ile$_{28},31$]PYY$_{25-36}$, showed a relatively potent agonist activity for Y2R but a weak anorectic activity by intraperitoneal administration (2000 nmol/kg) in lean mice because of its markedly poor biological stability in the mouse serum. Notably, two cyclohexylalanine (Cha) substitutions for Tyr residues at positions 27 and 36 (4) improved the stability in the mouse serum concomitant with enhanced anorectic activity. Further optimization at positions 27, 28, 30, and 31 revealed that 21, containing Cha$_{28}$ and Aib$_{31}$ residues, showed a more potent anorectic activity than PYY$_{3-36}$ at a low dose of 300 nmol/kg. The minimum effective dose by intraperitoneal administration of 21 was 30 nmol/kg (ca. 52 μg/kg) in mice, suggesting the biologic potential of short-length PYY$_{3-36}$ analogues with a potent anorectic effect.

INTRODUCTION

Obesity is considered to comprise an incredibly multifactorial chronic disease based on both genetic and behavioral factors.$^{1-3}$ Obesity is commonly associated with various diseases, such as diabetes mellitus, hypertension, stroke, cardiovascular disease, disability, gallbladder disease, osteoarthritis, sleep apnea, and certain types of cancer.$^1$ In addition, obesity, which is associated with an increased risk for cardiovascular and all-cause mortality, has been recognized as a worldwide epidemic.$^{2,4}$ When considering the treatment of obesity, it is required that the energy intake is decreased and/or the energy expenditure is increased to decrease the adipose tissue mass through the generation of a negative overall energy balance. For this, a restricted diet and exercise therapy represent the primary options; however, current nutritional optimization at positions 27, 28, 30, and 31 revealed that 21, containing Cha$_{28}$ and Aib$_{31}$ residues, showed a more potent anorectic activity than PYY$_{3-36}$ at a low dose of 300 nmol/kg. The minimum effective dose by intraperitoneal administration of 21 was 30 nmol/kg (ca. 52 μg/kg) in mice, suggesting the biologic potential of short-length PYY$_{3-36}$ analogues with a potent anorectic effect.

The homologies of PYY/NPY, PYY/PP, and NPY/PP are 70, 70, and approximately 50%, respectively.$^{9,10}$ NPY family receptors including six subtypes (Y1−Y5 and y6) are recognized in mammals. However, the functionality of Y3 remains controversial$^{11-13}$ and the gene for y6 is not expressed in primates.$^{14-16}$ Therefore, most pharmacological studies of the NPY family receptors have focused on Y1, Y2, Y4, and Y5, clarifying their different distributions and ligand affinities.$^{8,17-19}$ PYY is produced and secreted from L-cells in the intestinal epithelium of the ileum and colon. PYY has two endogenous isoforms: PYY$_{1-36}$ and its N-terminally truncated form, PYY$_{3-36}$ which constitutes the main isoform in circulation. The N-terminal dipeptide (Tyr-Pro) of PYY$_{1-36}$ is considered to be cleaved by a plasma protease, dipeptidyl peptidase IV.$^{20}$ PYY$_{1-36}$ binds to all Y family receptors with comparable affinity of a similar magnitude; conversely, PYY$_{3-36}$ displays increased Y2 receptor (Y2R) affinity and decreased Y1R and Y5R affinity.$^{21,22}$ Notably, PYY$_{3-36}$ has been reported to show anorectic activity by central and peripheral administration in mice.$^{23}$ The anorectic effect was not observed in Y2R KO mice, indicating that the effect was mediated by Y2R activation.$^{24}$ In initial clinical trials, approximately 30% of energy intake was suppressed after continuous intravenous infusion of PYY$_{3-36}$; in addition, an anorectic effect of PYY$_{3-36}$ was also found in subsequent clinical trials.$^{25}$

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| compound          | R        | AA26 | AA27 | AA28 | AA30 | AA31 | AA36 | IC_{50} nM (95% CI) | EC_{50} nM (95% CI) | agonist activity | bolus dosing (nmol/kg) | residual ratio (%) |
|-------------------|----------|------|------|------|------|------|------|----------------------|----------------------|------------------|-----------------------|------------------|
| PYY3–36           | benzoyl  | Ala   | Tyr  | Ile  | Leu  | Ile  | Tyr  | 0.30                 | 0.13                 | ND               | ND                    | 81               |
| 1                 | benzoyl  | Ala   | Tyr  | Ile  | Leu  | Ile  | Tyr  | 16 (9.6–27)          | 1.7 (1.3–2.2)        | 25 ± 7            | ND                    | ND               |
| 2                 | benzoyl  | Asn   | Asp  | Asn  | Asn  | Asn  | Asp  | 3.2 (2.0–5.0)        | 1.1 (0.68–1.7)       | ND               | ND                    | ND               |
| 3                 | benzoyl  | His   | Tyr  | Ile  | Leu  | Ile  | Tyr  | 15 (9.0–24)          | 1.9 (0.78–4.7)       | ND               | ND                    | ND               |
| 4                 | benzoyl  | His   | Cha  | Leu  | Ile  | Leu  | Tyr  | 7.2 (4.5–12)         | 3.6 (2.4–5.4)        | ND               | ND                    | ND               |
| 5                 | benzoyl  | His   | Cha  | Leu  | Thr  | Leu  | Tyr  | 9.8 (6.3–15)         | 2.3 (1.3–4.4)        | ND               | ND                    | ND               |
| 6                 | benzoyl  | His   | Cha  | Leu  | Thr  | Phe  | Tyr  | 11 (5.5–23)          | 2.9 (1.7–4.9)        | ND               | ND                    | ND               |
| 7                 | benzoyl  | His   | Cha  | Leu  | Thr  | Lys  | Tyr  | 13 (7.8–20)          | 2.4 (1.1–5.5)        | ND               | ND                    | ND               |
| 8                 | benzoyl  | His   | Cha  | Leu  | Nle  | Thr  | Tyr  | 25 (16–39)           | 7.3 (3.7–14)         | ND               | ND                    | ND               |
| 9                 | benzoyl  | His   | Cha  | Leu  | Lys  | Thr  | Tyr  | 1.6 (1.3–1.9)        | 0.62 (0.40–0.97)     | ND               | ND                    | ND               |
| 10                | benzoyl  | His   | Cha  | Leu  | Thr  | Thr  | Tyr  | 4.8 (3.4–6.9)        | 1.1 (0.68–1.7)       | 60 ± 7            | ND                    | ND               |
| 11                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 11 (7.1–18)          | 2.4 (1.4–4.1)        | 52 ± 5            | 36 ± 8                | 12 ± 3            |
| 12                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 2.4 (1.8–3.3)        | 0.84 (0.59–1.2)      | ND               | ND                    | 114              |
| 13                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 6.8 (5.3–8.6)        | 1.7 (0.79–3.9)       | ND               | ND                    | ND               |
| 14                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 15 (8.2–26)          | 11 (5.2–23)          | ND               | ND                    | ND               |
| 15                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 19 (13–28)           | 7.3 (4.2–13)         | ND               | ND                    | ND               |
| 16                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 49 (2.8–87)          | 1.6 (0.92–2.6)       | ND               | ND                    | ND               |
| 17                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 57 (3.5–92)          | 1.9 (1.3–2.8)        | ND               | ND                    | ND               |
| 18                | benzoyl  | His   | Thr  | Thr  | Thr  | Thr  | Tyr  | 64 (4.7–87)          | 1.6 (1.0–2.3)        | ND               | ND                    | ND               |
| 19                | benzoyl  | His   | Cha  | Val  | Thr  | Thr  | Tyr  | 9.1 (4.9–17)         | 2.7 (1.5–5.0)        | 1.9 (1.3–2.8)     | ND                    | ND               |
| 20                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 29 (17–48)           | 7.5 (4.3–13)         | ND               | ND                    | ND               |
| 21                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 7.4 (4.3–13)         | 2.0 (1.2–3.3)        | ND               | ND                    | ND               |
| 22                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 11 (6.2–19)          | 4.0 (2.0–8.0)        | ND               | ND                    | ND               |
| 23                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 3.1 (2.2–4.4)        | 1.2 (0.77–1.9)       | ND               | ND                    | ND               |
| 24                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 0.65 (0.50–0.79)     | 0.48 (0.36–0.65)     | ND               | ND                    | ND               |
| 25                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 18 (9.8–34)          | 5.8 (3.0–11)         | ND               | ND                    | ND               |
| 26                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 26 (16–41)           | 6.0 (2.9–12)         | ND               | ND                    | ND               |
| 27                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 8.9 (5.6–14)         | 2.2 (1.3–3.8)        | ND               | ND                    | ND               |
| 28                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 31 (19–49)           | 7.2 (4.8–11)         | ND               | ND                    | ND               |
| 29                | benzoyl  | His   | Cha  | Thr  | Thr  | Thr  | Tyr  | 18 (10–33)           | 14 (9.0–20)          | ND               | ND                    | ND               |

IC_{50} values and 95% confidence intervals (CI) of peptide analogues represent the concentrations required to displace the binding of the radiolabeled ligand by 50%. The IC_{50} value of PYY3–36 is calculated as the average value of 19 independent experiments. EC_{50} values and 95% CI of agonist activities were determined as the concentrations of peptide analogues that induced 50% of the maximum [35S]GTPγS binding. The EC_{50} value of PYY3–36 is calculated as the average value of 19 independent experiments. Percentage inhibition of food intake 3 h after administration of peptide analogues at doses of 250, 500, 1000, and 2000 nmol/kg, as compared to that after ip injection with saline as a vehicle in male C57BL/6J mice. Data are expressed as mean ± SD (n = 5–6 per group). ND: not determined. Residual ratio after 30 min of incubation in 20% mouse serum/phosphate-buffered saline (PBS) at 37 °C. ND: not determined.
Considering that PYY3−36 possesses a weak agonist activity for Y1R and Y4R, we were interested in determining the anorectic potential of PYY analogues with potent Y2R agonist activity and higher selectivity for Y2R over Y1R and Y4R. The selectivity of PYY analogues for Y2R has been shown to be increased by N-terminal truncation; in addition, amino acid substitutions at positions 32 and 34 in the NPY analogues affect the selectivity for Y1R and Y4R. Among the reported peptides, 12-amino acid peptides containing the N-terminal benzoyl group showed potent Y2R agonist activity and strict selectivity for Y2R over Y1R and YSR. Specifically, a series of N-terminally substituted benzoylated PYY analogues with 12 amino acids possessed high agonist activities with EC50 values in the nM range for Y2R and selectivity over Y1R and YSR, for example, benzoyl (6 nM) and 4-aminobenzoyl (3 nM). A variety of PYY analogues with amino acid substitutions, such as Ala26 or Ile28, were also disclosed to have Y2R agonism. Furthermore, PEGylation of the N-terminus of the short-length peptides maintained the selectivity for Y2R and elicited food intake and body weight reduction as well as improved glucose metabolism in rodents. However, few studies have used the in vivo activity of short-length peptides without the modification with PEG, albumin, or long-chain alkyl groups. We therefore designed and synthesized a new class of short-length PYY analogues with improved pharmacokinetic profiles as well as potent Y2R agonist activity, and demonstrated their anorectic activity in mice.

RESULTS AND DISCUSSION

Chemistry. All peptides were synthesized using standard Fmoc-based solid-phase synthetic methods. Subsequent preparative high-performance liquid chromatography (HPLC) purification of the obtained crude peptides exhibited ≥95% homogeneity. The purity of each peptide was verified by analytical reversed-phase HPLC (RP-HPLC), and the structure was assigned using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS).

Biological Activities. The binding affinity and agonist activity of all peptides synthesized were examined by receptor [35S]GTPγS binding assays using Chinese hamster ovary (CHO) cells expressing human cloned Y2R. Peptides with potent agonist activity were then screened for their anorectic activity in mice by intra/peritoneal (ip) administration (250, 500, 1000, or 2000 nmol/kg). Results of in vitro and in vivo screening are shown in Table 1.

Design and Syntheses of Dodecapeptide PYY Analogues Substituted at Positions 27 and 36. A short-length analogue of PYY3−36 with a simple benzoyl group at the N-terminus, benzoyl-[Ala26,Ile2831]PYY(25−36) (1), showed relatively high affinity and potent agonist activity for Y2R. Although 1 comprised the short-length peptide without any modification, for example, PEG or a long-chain alkyl, I showed moderate anorectic activity by ip administration at a high dose of 2000 nmol/kg in lean mice. Generally, short-length peptides tend to be quickly inactivated by proteases in the serum; therefore, we examined the serum stability of 1. Upon incubation in 20% mouse serum/PBS for 30 min, the residual ratio of the original peptide (1) was 5%. This result indicated the possibility that improvement of the serum stability might provide short-length peptides with a robust anorectic activity. To examine the effect of the substitution for a Tyr residue, which is a target of chymotrypsin-like proteases, on the serum stability, the unnatural amino acid, Cha, was introduced into positions 27 or 36. Compounds 2 (Cha36) and 3 (Cha30) showed good profiles of binding affinity and agonist activity for Y2R, as did 1. The serum stability of both 2 and 3 was increased with residual ratio values of 53 and 10%, respectively, indicating the increase in protease resistance effected by the substitution of Cha for Tyr. In addition, the combined Cha substitution at positions 27 and 36 (4) achieved further improvement of serum stability; in particular, 4 did not yield any apparent metabolites within 30 min in 20% mouse serum/PBS. Consistent with the enhanced stability afforded by the double-Cha substitution, 4 exerted an anorectic activity at a lower dose of 1000 nmol/kg but only minimally affected in vitro agonistic activities.

Design and Syntheses of Dodecapeptide PYY Analogues Substituted at Position 30 or 31. Further improvement of anorectic activity was investigated by generating amino acid substitutions at positions 30 and 31, which were found to be replaceable without loss of agonist activity for Y2R in our studies (data not shown). Specifically, aromatic amino acid substitutions at position 30, such as Trp (5) or Phe(4F) (6), induced comparable or slightly stronger food intake inhibition than 4, whereas the binding affinities and agonistic activities of 5 and 6 were moderate. The results imply that these hydrophobic residue substitutions improved the pharmacokinetic profiles of the peptides.

Substitutions of unnatural amino acids Nle31 (7) and Cha31 (8) decreased the binding affinity several-fold compared with 4; however, 8 showed slightly improved anorectic activity. In addition, a Lys analogue (9) displayed more than 4-fold increase in the agonist activity compared to that of 4 but no remarkable anorectic activity, which was likely caused by its decreased serum stability. The introduction of an α,α-disubstituted amino acid, 2-aminoisobutyric acid (Aib), into position 31 (10), which was expected to improve serum stability, increased both agonist activity and anorectic activity. Furthermore, the Aib31 residue, which is known to enhance peptide helicity, may have contributed to inducing a preferable conformation for Y2R recognition and to the resistance against serum proteases.

Comparison of the Biological Activities of Peptide Analogues with the Combination of Amino Acid Substitutions at Positions 30 and 31. Substitution of Trp30 (5), Lys31 (9), or Aib31 (10) contributed to the increase in in vitro or in vivo activity, which allowed us to synthesize analogues with combined substitutions at positions 30 and 31. The Trp30,Aib31 analogue (11) showed a moderate anorectic activity comparable to the parent compounds (5, 10). Compound 12 containing Trp30 and Lys31 substitutions, each of which individually showed increased in vivo or in vitro activity, exhibited potent in vitro activities; however, it was not taken into consideration for further studies of Lys31 derivatives owing to its weak anorectic activity. Among the analogues composed of amino acids at positions 30 (Leu or Trp) and 31 (Ile, Lys, or Aib), Leu30,Ile31 (4), Trp30,Ile31 (5), Leu30,Aib31 (10), and Trp30,Aib31 (11) showed similar anorectic activities at 1000 nmol/kg, which was more potent than that shown by the lead compound, 1, at 2000 nmol/kg, concomitant with their enhanced metabolic stability (4, 5, 10, 11) in 20% mouse serum/PBS compared to that in 1. Cha substitutions at positions 27 and 36 (4) were crucial for stability in the mouse serum, and additional substitutions, that is, Trp30 (5), Aib31 (10), and their combination, Trp30,Aib31 (11), allowed the maintenance of high stability. Compounds 4, 5, 10, and 11...
were comparable to or more stable than PYY3−36 in 20% mouse serum/PBS; 4, 5, 10, and 11 showed, however, lower anorectic activities than that shown by PYY3−36 at 1000 nmol/kg in lean mice; therefore, the subsequent optimization of 5, 10, and 11 was performed in terms of the anorectic activity in lean mice.

Design and Syntheses of Trp30-Ile31 Derivatives Substituted at the N-Terminus and at Positions 27 and 28. Further improvement of the anorectic activity of PYY analogues was examined by substitution at the N-terminus and at positions 27 and 28. For N-terminus substitutions, a cyclohexylcarbonyl analogue (13) of the Trp30-Ile31 derivatives showed similar in vitro activity and anorectic activity as the parent compound (5), indicating that an aromatic moiety of the N-terminus of dodocapeptide derivatives is not necessary for binding to Y2R. Although Cha introduction (14) at position 28 of 13 had some effect on the improvement of the anorectic activity, the binding affinity and agonist activity for Y2R were decreased. The 3-(2-naphthyl)alanine [Nal(2)] analogue (15) also possessed moderate anorectic activity but showed decreased in vitro activities.

Design and Syntheses of Leu30,Aib31 and Trp30,Aib31 Derivatives Substituted at the N-Terminus and at Positions 27, 28, and 30. The N-terminal substitution of the Leu30,Aib31 analogue (10) with a cyclohexylcarbonyl moiety (16) showed no increase in either in vitro or in vivo activities. The cyclohexylcarbonyl analogue (17) of 11 composed of Trp30,Aib31 possessed a moderately improved anorectic activity compared to that of 11. Among the Leu30,Aib31 derivatives substituted at position 28 (18−21), the Trp30,Aib31 analogue (18) possessed relatively potent in vitro activities comparable to those of 10 but did not reduce the food intake at a dose of 500 nmol/kg in mice. Conversely, the replacement of an unnatural amino acid, 3-(1-naphthyl)alanine [Nal(1)] (19), Nal(2) (20), or Cha (21), at position 28 improved the anorectic activity at the dose of 500 nmol/kg. In particular, 21 showed the most robust activity at 49% food intake reduction. Even at a dose of 250 nmol/kg, an apparent anorectic effect of 21 was observed with a more potent activity than that of PYY3−36. When comparing the effect of replacing the N-terminal moiety (21−26), a benzoyl group (21) was preferable in terms of in vitro and in vivo activities. Although the introduction of a basic moiety at the N-terminus (24) increased the binding affinity and agonist activity for Y2R, 24 did not show increased anorectic activity compared with 21. A bulky hydrophobic moiety, such as 1-naphthyl or 2-naphthoyl groups (25, 26), was not effective in increasing the binding affinity and agonist activity of the analogues for Y2R. Several preferable amino acid substitutions found in this study, for example, Trp30 (5), Phe(4F)30 (6), and Nal(2)27 (15), were also introduced into the basic structure of 22 containing a cyclohexanoyl moiety; however, these substitutions (27−29) did not lead to improved results in terms of food intake suppression.

Anorectic Activity of PYY3−36, 1, and 21 in Lean Mice. The anorectic activity of PYY3−36, 1, and 21 was evaluated by bolus ip administration in lean mice (Table 2, Figure 1). PYY3−36, and 21 significantly reduced food intake 3 and 6 h after dosing in a dose-dependent manner with a minimum effective dose of 30 nmol/kg (ca. 52 μg/kg) (Table 2, Figure 1A), whereas weak anorectic activity of the lead compound, 1, with low serum stability was observed at a higher dose of 2000 nmol/kg (Table 1, Figure 1B). Compound 21 thus represented a new short-length peptide that showed potent anorectic activity comparable to that of PYY3−36.

Pharmacokinetic Parameters of 21 in Mice. The pharmacokinetic parameters of 21 were examined in lean mice. Compound 21 was injected intravenously (iv) and ip at 1 mg/kg (Table 3). The total body clearance (Clb,tot) of 21 after iv administration was 162 mL/h/kg, indicating relatively low clearance compared to that of PYY3−36 (914 mL/h/kg) (Table S2). Compound 21 possessed lower tissue distribution than that of PYY3−36 (328 mL/kg) with a volume of distribution at steady state (Vdss) of 73 mL/kg. The pharmacokinetic properties of 21 were considered to be good in the blood circulation for such a short-length peptide and contributed the potent anorectic activity in mice, although the binding affinity and agonist activity of 21 were more than 10-fold lower than those of PYY3−36. The bioavailability (BA) value of 21 was 6.8 after ip dosing, indicating slow transfer into the blood vessels and the circulation. Thus, the possibility remains to obtain more potent analogues by increasing the absorption rate or improving the biological stability in peripheral tissues.

| Compound | Dose (nmol/kg) | % Food Intake Inhibition | Time after Administration (h) |
|----------|---------------|--------------------------|-------------------------------|
| 21       | 3             | 1 ± 6                    | 3                             |
| 21       | 30            | 20 ± 6                   | 6                             |
| 21       | 300           | 47 ± 3                   | 24                            |
| PYY3−36  | 3             | 18 ± 3                   | 5                             |
| PYY3−36  | 30            | 33 ± 6                   | 5                             |
| PYY3−36  | 300           | 33 ± 8                   | 2                             |

Table 2. Food Intake Inhibitory Activities by Bolus Ip Administration of PYY3−36 and 21

Pharmacokinetic Parameters of 21 in Mice.

CONCLUSIONS

We designed and synthesized short-length PYY3−36 analogues with 12 amino acid residues. A lead compound, benzoyl-[Ala26,Ile28,31]PYY(25−36) (1), showed a moderate anorectic activity at a dose of 2000 nmol/kg in lean mice by ip administration. However, 1 showed low biological stability in the mouse serum. The substitution of an unnatural amino acid residue, Cha, at position 27 (2), 36 (3), or at both positions (4) of 1, improved the serum stability; notably, 4 was completely resistant to serum proteases under the condition of 20% mouse serum/PBS for 30 min. Furthermore, a bolus ip administration of 4 showed marked anorectic activity at a dose of 1000 nmol/kg in mice. A subsequent investigation of the N-terminal modifications and amino acid substitutions at positions 27, 28, 30, and 31 led to the potent analogue (21) containing Cha28 and Aib31 residues. Compound 21, which exhibited a Y2R agonist activity comparable to that of 1, reduced food intake more potently than PYY3−36 at 300 nmol/kg in mice. Furthermore, ip-administered 21 inhibited food intake in a dose-dependent manner, with a minimum effective dose of 30 nmol/kg (ca. 52 μg/kg). Thus, the results of this study suggest that short-length PYY3−36 analogues without modification with PEG, albumin, or long-chain alkyl groups show potential as a new option of compounds with potent anorectic activity and an antiobesity effect.
**EXPERIMENTAL SECTION**

**Instruments and Materials.** Manual Fmoc solid-phase peptide syntheses were conducted, which consisted of Fmoc cleavage with 20% piperidine/N,N-dimethylformamide (DMF) (20 min) and an Fmoc amino acid condensation reaction using N,N′-disopropylcarbodiimide (DIPCDI)/1-hydroxy-7-azabenzotriazole (HOAt) (4 equiv), or by the ABI 433A automated peptide synthesizer (Applied Biosystems, Foster City, CA) as per the Fmoc/DCC/N-hydroxybenzotriazole (HOBT) 0.25 mmol protocol. All final compounds were purified to ≥95% homogeneity by the RP-HPLC analysis with a photodiode array detector across a wavelength range of 190–500 nm; the absence of co-eluting impurities (heterogeneous peaks) was confirmed by the liquid chromatography–mass spectrometry (LC/MS) analysis. The identity of the peptides was confirmed by the MALDI-TOF-MS analysis on a Bruker autoflex speed system (Billerica, MA). The purity, retention time, and molecular weight of each peptide are summarized in Table S1 of the Supporting Information. Commercially available amino acid derivatives and resins were purchased from Novabiochem (Billerica, MA), Watanabe Chemical Industries (Hiroshima, Japan), Peptide Institute (Osaka, Japan), Bachem (Bubendorf, Switzerland), AnaSpec (Fremont, CA), Chem-Impex International (Wood Dale, IL), and American Peptide Company (Sunnyvale, CA), whereas other reagents, such as coupling and deprotection reagents, were purchased from Wako Pure Chemical Industries (Osaka, Japan), Novabiochem, Watanabe Chemical Industries, and Nacalai Tesque (Kyoto, Japan).

**General Procedure for Synthesis of PYY Analogues.** All peptides were synthesized in the same manner as the following synthesis procedure for benzoyl-[Cha27,28,36,Aib31]-PYY(25–36) (21). Using commercially available Sieber Amide resin (347 mg, 0.25 mmol) as the starting material and an ABI433A peptide synthesizer (DCC/HOBt 0.25 mmol protocol), amino acids were successively condensed to give H-Arg(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, Pbf)-His(triphenylmethyl, Trt)-Cha-Asn(Trt)-Leu-Aib-Thr(3,30, and 300 nmol/kg) in lean mice. Data are expressed as mean ± SD (n = 5–6). *P < 0.05 vs the vehicle (saline) group by Student’s t test.

![Figure 1. Anorectic activity of PYY3–36, 1, and 21 in lean mice. (A) Dose-dependent anorectic activity of PYY3–36 and 21 by ip administration. Food intake was measured at 3, 6, and 24 h post injection of PYY3–36 and 21 (5, 30, and 300 nmol/kg) in lean mice. Data are expressed as mean ± SD (n = 5–6). * P < 0.025 vs the vehicle (saline) group by the Williams test. (B) Anorectic activity of PYY3–36 and 1 by ip administration. Food intake was measured at 3, 6, and 24 h post injection of PYY3–36 (250 nmol/kg) and 1 (2000 nmol/kg) in lean mice. Data are expressed as mean ± SD (n = 5–6). * P < 0.05 vs the vehicle (saline) group by Student’s t test.](image-url)
Establishment of Cloned Human Y2R-Expressing CHO Cells. The entire coding region of the human NPY2R cDNA was amplified by the polymerase chain reaction (PCR) from human brain cDNA (TaKaRa Bio, Shiga, Japan). The DNA sequence of the PCR product was confirmed to represent human Y2R and finally cloned into expression vector pAKKO-111H for the expression of Y2R in CHO cells. This expression vector was transfected into CHO (dhfr- ) cells, and CHO cells stably expressing human Y2R were established as described previously.36

Membrane Preparation from CHO Cells Expressing Human Y2R. The affinity of the synthesized peptides for human Y2R was determined by a competitive binding experiment with membranes of CHO cells expressing cloned human Y2R. The membrane was prepared as described previously.36 The CHO cells were detached from the culture dish with PBS-ethylenediaminetetraacetic acid (EDTA). The cells were recovered by centrifugation at 1000 rpm for 10 min. The cell pellets obtained were homogenized in ice-cold homogenizing buffer [10 mM NaHCO3, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL pepstatin A, 20 μg/mL leupeptin, 10 μg/mL E-64] with a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The homogenate was centrifuged at 700 g for 15 min at 4 °C, and then the supernatant obtained was ultracentrifuged at 100 000 g for 60 min. The resultant pellet was suspended in a suspending buffer (50 mM Tris, 5 mM MgCl2, 150 mM NaCl, 0.5 mM PMSF, 10 μg/mL pepstatin A, 20 μg/mL leupeptin, 10 μg/mL E-64, 0.03% NaN3, pH 7.4). The protein concentration was determined using the Coomassie Plus Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA).

Receptor Binding Assay for Human Y2R. First, 2 μL of the test compound was incubated in a 96-well plate with 100 μL of the membrane diluted with the assay buffer (50 mM Tris, 5 mM MgCl2, 150 mM NaCl, 0.03% NaN3, pH 7.4) to 0.5 μg protein/mL and 100 μL of 1 nM [35S]GTPγS (NEG030H, PerkinElmer). After incubation at room temperature for 120 min, the reaction mixture was filtered through a UniFilter-96 GF/C, washed, and dried, and the radioactivity was measured as done for the receptor binding assay. The data obtained were analyzed using Prism to calculate the EC50 value.

Food Intake Assay. Male C57BL/6J mice (seven-week-old) were purchased from Charles River (Kanagawa, Japan). The mice were housed in a room at 22 °C in a 12 h light/12 h dark cycle and maintained on a standard chow diet. Before the administration of peptides, the mice were acclimated by daily handling for 5 days. They were then acclimated daily to ip injection by sham injection with a 29-gauge needle for 3 days. They were fasted for 16 h with water available during the dark cycle and then were ip injected with peptides. Preweighed chow was provided, and the food intake was measured at 3, 6, and 24 h post injection.

Pharmacokinetics of PYY Analogues in Mice. Peptides were administered iv or ip to the C57BL/6J mice at a dose of 1 mg/kg in fed animals. After administration, blood samples were collected at 5, 10, 15, 30 min, 1, 3, 6, 8, and 24 h and centrifuged to obtain the plasma fraction. The plasma samples were deproteinized with acetonitrile, followed by centrifugation, and the supernatants were analyzed by LC/MC/MS to determine the plasma concentration of the peptides. The pharmacokinetics parameters were calculated by the moment analysis method. The plasma concentration 5 min after injection (C5 min), the area under the concentration–time curve from time zero to 24 h (AUC0–24 h), the mean residence time (MRT), Vd, and Cl were obtained. The plasma concentration (Cmax), time to maximum plasma concentration (Tmax), AUC0–24 h, MRT, and BA were obtained for each mouse after ip administration were also obtained.

Terminology. Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature.37 Amino acid symbols denote the l-configuration unless indicated otherwise.
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