# Structure−Activity Studies Including a Ψ($CH_2$-NH) Scan of Peptide YY (PYY) Active Site, PYY(22−36), for Interaction with Rat Intestinal PYY Receptors: Development of Analogues with Potent in Vivo Activity in the Intestine[#]

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*Received February 11, 2000*

Peptide YY (PYY) is a gut hormone that inhibits secretion and promotes absorption and growth in the intestinal epithelium. We have performed structure−activity studies with the active site, N-α-Ac-PYY(22−36)-$NH_2$, for interaction with intestinal PYY receptors. Investigation of aromatic substitutions at position 27 resulted in analogues that exhibited potent in vitro antisecretory potencies with N-α-Ac-[$Trp^{27}$]PYY(22−36)-$NH_2$ exhibiting even greater potency than intact PYY. In vivo studies in dogs revealed that this analogue also promoted intestinal absorption of water and electrolytes during continuous intravenous and intraluminal infusion. Investigations carried out to identify features that would enhance stability revealed that incorporation of $Trp^{30}$ increased affinity for PYY receptors. A "$CH_2$-NH" scan revealed that incorporation of reduced bonds at position 28−29 or 35−36 imparted greater receptor affinity. In general, disubstituted analogues designed based on the results of single substitutions exhibited good receptor affinity with N-α-Ac-[$Trp^{27}$,$CH_2$-$NH^{35-36}$]PYY(22−36)-$NH_2$ having the greatest affinity ($IC_{50}$ = 0.28 nM). Conservative multiple substitutions with Nle→Leu and Nva→Val also imparted good affinity. An analogue designed to encompass most of the favored substitutions, N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$,$CH_2$-$NH^{35-36}$]PYY(22−36)-$NH_2$, exhibited a proabsorptive effect in dogs comparable to, but longer lasting than, that of intact hormone. Selected analogues also exhibited good antisecretory potencies in rats with N-α-Ac-[$Trp^{30}$]PYY(22−36)-$NH_2$ being even more potent than PYY. However, the potencies did not correlate well with the PYY receptor affinity or the proabsorptive potencies in dogs. These differences could be due to species effects and/or the involvement of multiple receptors and neuronal elements in controlling the in vivo activity of PYY compounds. PYY(22−36) analogues exhibited good affinity for neuronal Y2 receptors but poor affinity for Y1 receptors. Also, crucial analogues in this series hardly bound to Y4 and Y5 receptors. In summary, we have developed PYY(22−36) analogues which, via interacting with intestinal PYY receptors, promoted potent and long-lasting proabsorptive and antisecretory effects in in vivo models. These compounds or analogues based on them may have useful clinical application in treating malabsorptive disorders observed under a variety of conditions.

## Introduction

Peptide YY (PYY) is a 36-residue peptide amide first isolated from porcine intestine.[1] PYY is essentially a gut hormone mainly localized in the endocrine cells of colon, rectum, and ileum.[2,3] Nevertheless, small quantities of PYY have also been found in the enteric nerves surrounding the stomach and in rat brain.[4] PYY, which is released into circulation in response to feeding,[5,6] exhibits a variety of effects on the mammalian gastrointestinal tract. These effects include inhibition of gastric acid and exocrine pancreatic secretions,[1] delaying of gastric emptying,[7] slowing of intestinal transit,[8−9] enhancement of basal and postprandial absorption,[10−13] and inhibition of basal and secretagogue-induced intestinal secretions.[14−19] The PYY homologue, neuropeptide Y (NPY), mimics these activities in the intestine but is 5−10-fold less potent than PYY.[14,15,20] In addition, PYY, but not NPY, promotes intestinal growth in normal rats[21] as well as those maintained on parenteral nutrition.[22] Moreover, plasma PYY levels and its mucosal mRNA levels are elevated in certain malabsorptive disorders.[23,24] These findings suggest that PYY may play a significant role in the physiological regulation of the mammalian gastrointestinal tract.

A PYY receptor system coupled negatively to adenylyl

[#] Supported by grants from NIH GM47122 (A.B.) and DK53548 (S.S.) and Biomeasure, Inc. (A.B.). PYY(22−36) analogues are covered by U.S. Patents #5,604,203 and #6,046,167.

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10.1021/jm000052z CCC: $19.00 
Published on Web 08/04/2000

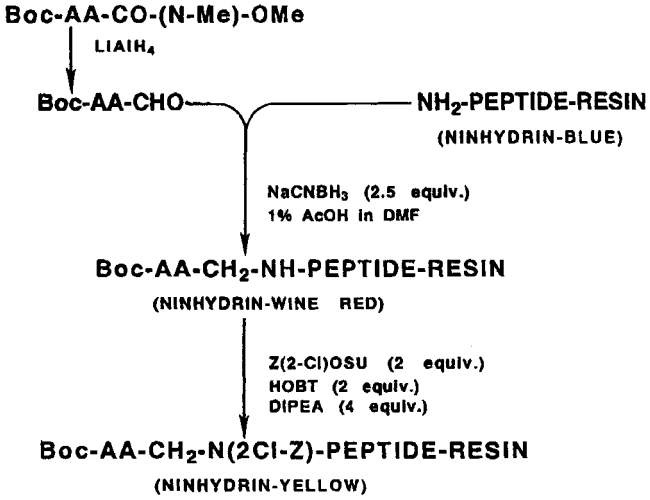
cyclase has been characterized in rat intestinal crypt epithelial cells.[20,25,26] It appears that this receptor may mediate the effects of PYY on intestinal absorption, secretion, and growth.[20] PYY receptors exhibit high affinity for PYY and NPY but bind poorly to pancreatic polypeptide (PP). These same receptors also exhibit high affinity to the C-terminal fragments of NPY and PYY. The latter finding suggests that PYY receptors have characteristics of classical neuronal Y2 receptors of the NPY−PYY−PP family, which also bind well to NPY, PYY, and their C-terminal fragments. On the other hand, typical Y1 receptors bind well only to intact PYY and NPY (see ref 27 for a review). However, elegant investigations by Cox and co-workers have shown that several potent Y2 agonists, including cyclic truncated analogues of NPY[28] and PYY[29] as well as several Ala-substituted NPY analogues,[30] exhibit only weak to moderate antisecretory activity in rat intestinal preparations. Thus, it appears that intestinal PYY receptors, which remain to be cloned, may represent a novel subclass of Y2 receptors.

Malabsorption, which often results following a loss of a critical mass of gut mucosa, may produce chronic diarrhea, malnourishment, and dehydration. Such conditions affect millions of people worldwide per year. Moreover, no effective therapy exists at present for treating this condition. These observations, and the findings that PYY inhibits intestinal secretion and promotes absorption and growth through specific receptors, suggest that it is possible to dissociate various effects of PYY and develop PYY receptor-selective compounds for treating malabsorptive disorders. Our previous efforts toward this goal resulted in the identification of PYY(22−36), A S L R H Y L N L V T R Q R Y, as the active site for interaction with intestinal PYY receptors[26,31] and demonstrated that N-α-Ac-PYY-(22−36)-$NH_2$ analogues could mimic the antisecretory effects of PYY in rat jejunal membrane preparations.[32] In continuation of these investigations, we have now performed further studies with N-α-Ac-PYY(22−36)-$NH_2$ to improve the affinity, selectivity, and in vivo activity. These investigations that are described in this manuscript have resulted in the development of analogues exhibiting long-lasting and potent in vivo effects on intestinal absorption and secretion.

## Results and Discussion

The PYY(22−36) analogues with standard substitutions were synthesized by t-Boc solid-phase method and purified by reversed-phase chromatography in good yields following our previously published procedures.[33,34] To synthesize PYY(22−36) analogues with a $CH_2$−NH bond, we made use of the elegant methods developed by Fehrentz and Castro[35] and by Saski and Coy[36] to obtain optically pure Boc-AA-CHO and couple it directly to the α-amino group of the peptide-resin, respectively. However, the possibility of branching at the IIry amino group has prevented general applicability of this method, especially for the synthesis of long peptides containing a $CH_2$−NH bond in the C-terminal region as in PYY-(22−36). Moreover, this method is not compatible with solid-phase synthesis involving capping procedures because the unprotected IIry amine of $CH_2$-NH is also prone to acetylation. In fact, during our initial attempts

**Scheme 1**




to synthesize N-α-Ac-[$CH_2$-$NH^{35-36}$]PYY(22−36)-$NH_2$ the major product had a mass corresponding to a compound with two acetyl groups, possibly due to the formation of N-α-Ac-[$CH_2$-N-$Ac^{35-36}$]PYY(22−36)-$NH_2$. To ensure no such problems were encountered, we investigated the possibility of temporarily protecting the IIry amine in $CH_2$-NH with Tos, Z, or (2-Cl)Z which could be simultaneously removed during the final HF cleavage to obtain the free peptide. Initial investigations revealed that the treatment of peptide-resin containing $CH_2$-NH with Tos-Cl or Z-Cl in the presence of DIPEA resulted in the complete capping of the IIry amine within 30 min. The red wine color of ninhydrin with IIry amine turned yellow at the end of the capping reaction. However, the known lability of the Z group during repeated acidolysis to remove N-α-Boc groups, and the apparent stability of the Tos group attached to the IIry amine to HF, led us to choose the (2-Cl)Z group for the temporary protection of $CH_2$-NH. This protection was introduced according to Scheme 1 by reacting the N-α-Boc-AA-$CH_2$-NH-peptide-resin with (2-Cl)Z-OSu in the presence of HOBT and DIPEA and monitoring the reaction with ninhydrin. As judged by analytical HPLC, the crude peptides obtained using this strategy contained 70−90% of the target peptides. This procedure has additional advantages including: (1) it could be adapted for automated procedures and (2) it precluded the formation of deletion peptides as the excess of (2-Cl)Z used will also cap the α-amino group, which has not undergone reductive alkylation. All the peptides used in this study had the expected amino acid composition and mass (see Supporting Information) and were >95% homogeneous by analytical HPLC.

Previous investigations have shown that Phe→$Tyr^{27}$ substitution in PYY(22−36) increased intestinal antisecretory potency.[32] Therefore, we investigated the effect of substituting a series of aromatic hydrophobic residues at this position. These substitutions in general imparted good PYY receptor affinity and exhibited the following order of potency: Thi > Phe > Bip > Trp ∼ Tic ∼ Bth > Nal > Pcp > Dip (Table 1: analogues **5**−**13**). As is evident, there was no straightforward correlation between the affinities and the hydrophobicity of the analogues. Probably, other factors such as steric hindrance and changes in the conformation might have

**Table 1.** Affinities of PYY(22−36) Analogues for Intestinal PYY (rat jejunum), Y-1 (SK-N-MC), and Y-2 (SK-N-BE2) Receptors

| | | $IC_{50}$ (nM) | | |
|---|---|---|---|---|
| no. | compound | intestine (SCC[a]) | SK-N-BE2 | SK-N-MC |
| **1** | PYY | 0.14 ± 0.04 (1.0) | 0.07 ± 0.006 | 0.28 ± 0.05 |
| **2** | NPY | 2.00 ± 0.70 (9.0) | 0.32 ± 0.11 | 3.07 ± 0.52 |
| **3** | [Leu$^{31}$,Pro$^{34}$]PYY | 54 ± 9 | 261 ± 89 | 2.69 ± 0.60 |
| **4** | N-α-Ac-PYY(22−36)-$NH_2$ | 0.91 ± 0.11 (35.7) | 1.30 ± 0.50 | >1000 |
| **5** | N-α-Ac-[Phe$^{27}$]PYY(22−36)-$NH_2$ | 2.3 ± 0.6 (15.1) | 1.83 ± 0.82 | 764 ± 398 |
| **6** | N-α-Ac-[Pcp$^{27}$]PYY(22−36)-$NH_2$ | 7.49 ± 5.00 | 8.09 ± 3.35 | >10000 |
| **7** | N-α-Ac-[Thi$^{27}$]PYY(22−36)-$NH_2$ | 1.00 ± 0.10 (100) | 2.63 ± 0.83 | 257 ± 8.5 |
| **8** | N-α-Ac-[Tic$^{27}$]PYY(22−36)-$NH_2$ | 3.40 ± 0.50 | 186 | ND |
| **9** | N-α-Ac-[Bip$^{27}$]PYY(22−36)-$NH_2$ | 2.42 ± 0.90 | 14.2 ± 6.2 | 279 ± 9 |
| **10** | N-α-Ac-[Dip$^{27}$]PYY(22−36)-$NH_2$ | 16.5 ± 8.2 | 10.00 ± 4.22 | 447 ± 281 |
| **11** | N-α-Ac-[Nal$^{27}$]PYY(22−36)-$NH_2$ | 6.60 ± 5.30 (0.1) | 7.08 ± 4.65 | 88.3 ± 17 |
| **12** | N-α-Ac-[Bth$^{27}$]PYY(22−36)-$NH_2$ | 3.99 ± 0.53 (1.0) | 5.4 ± 2.53 | 105 ± 57 |
| **13** | N-α-Ac-[Trp$^{27}$]PYY(22−36)-$NH_2$ | 3.20 ± 1.23 (0.005) | 2.09 ± 1.1 | 76.95 ± 17 |
| **14** | N-α-Ac-[Trp$^{27}$]PYY(22−36)NH-$CH_3$ | >100 | >100 | >1000 |
| **15** | N-α-Ac-[Trp$^{24}$]PYY(22−36)-$NH_2$ | 2.10 ± 0.10 | ND | ND |
| **16** | N-α-Ac-[Trp$^{28}$]PYY(22−36)-$NH_2$ | 0.60 ± 0.30 (5.00) | 2.08 ± 0.79 | 398 ± 116 |
| **17** | N-α-Ac-[Trp$^{29}$]PYY(22−36)-$NH_2$ | 100 | 158 ± 83 | 762 ± 537 |
| **18** | N-α-Ac-[Trp$^{30}$]PYY(22−36)-$NH_2$ | 0.60 ± 0.30 (2.00) | 0.98 ± 0.26 | 250 ± 207 |
| **19** | N-α-Ac-[D-Trp$^{30}$]PYY(22−36)-$NH_2$ | >100 | 56.5 ± 9.8 | 47.9 ± 10.619. |
| **20** | N-α-Ac-[Nal$^{30}$]PYY(22−36)-$NH_2$ | 3.54 ± 0.72 | 1.09 ± 0.17 | 159 ± 56 |
| **21** | N-α-Ac-[Bth$^{30}$]PYY(22−36)-$NH_2$ | 1.12 ± 0.24 | 3.3 ± 1.79 | 103 ± 31 |
| **22** | N-α-Ac-[Dip$^{36}$]PYY(22−36)-$NH_2$ | >100 | 1672 ± 430 | >10000 |
| **23** | N-α-Ac-[$CH_2$-NH$^{35-36}$]PYY(22−36)-$NH_2$ | 0.50 ± 0.08 | 1.32 ± 0.80 | >10000 |
| **24** | N-α-Ac-[$CH_2$-NH$^{33-34}$]PYY(22−36)-$NH_2$ | >100 | >10000 | 1248 ± 495 |
| **25** | N-α-Ac-[$CH_2$-NH$^{32-33}$]PYY(22−36)-$NH_2$ | 5.03 ± 1.18 | 52 ± 11 | 1091 ± 987 |
| **26** | N-α-Ac-[$CH_2$-NH$^{31-32}$]PYY(22−36)-$NH_2$ | 11.89 ± 3.28 | 105 ± 20 | 1597 ± 906 |
| **27** | N-α-Ac-[$CH_2$-NH$^{30-31}$]PYY(22−36)-$NH_2$ | >100 | >3000 | 769 ± 125 |
| **28** | N-α-Ac-[$CH_2$-NH$^{28-29}$]PYY(22−36)-$NH_2$ | 0.36 ± 0.10 | 55 ± 14 | 1390 |
| **29** | N-α-Ac-[$CH_2$-NH$^{27-28}$]PYY(22−36)-$NH_2$ | >100 | 864 | 1270 |
| **30** | N-α-Ac-[$CH_2$-NH$^{25-26}$]PYY(22−36)-$NH_2$ | 4.22 ± 1.23 | 41 ± 14 | >1000 |
| **31** | N-α-Ac-[$CH_2$-NH$^{24-25}$]PYY(22−36)-$NH_2$ | 2.76 ± 0.98 | 59 ± 19 | 735 ± 147 |
| **32** | N-α-Ac-[Phe$^{27}$,Thi$^{36}$]PYY(22−36)-$NH_2$ | 4.50 ± 1.50 | ND | ND |
| **33** | N-α-Ac-[Phe$^{27,36}$]PYY(22−36)-$NH_2$ | 14.10 ± 5.30 | 1.16 ± 0.51 | >10000 |
| **34** | N-α-Ac-[Phe$^{22,27}$]PYY(22−36)-$NH_2$ | 11.20 ± 3.18 | 5.61 ± 3.02 | 275 ± 76 |
| **35** | N-α-Ac-[Tyr$^{22}$,Phe$^{27}$]PYY(22−36)-$NH_2$ | 3.40 ± 2.6 | 2.68 ± 1.38 | 48.9 ± 7.2 |
| **36** | N-α-Ac-[Ala$^{26}$,Phe$^{27}$]PYY(22−36)-$NH_2$ | 5.30 ± 2.7 | 3.84 ± 2.04 | 674 ± 616 |
| **37** | N-α-Ac-[Trp$^{28,30}$]PYY(22−36)-$NH_2$ | 1.07 ± 0.13 | 3.17 ± 0.51 | 233 ± 68 |
| **38** | N-α-Ac-[Trp$^{30}$,$CH_2$-NH$^{35-36}$]PYY(22−36)-$NH_2$ | 0.28 ± 0.07 | 1.39 ± 0.53 | 1514 ± 514 |
| **39** | N-α-Ac-[Trp$^{27}$,$CH_2$-NH$^{35-36}$]PYY(22−36)-$NH_2$ | 1.12 ± 0.34 | 1.05 ± 0.26 | >1000 |
| **40** | N-α-Ac-[Nle$^{24,28,30}$,Nva$^{31}$]PYY(22−36)-$NH_2$ | 0.59 ± 0.02 | 57.0 ± 10.1 | 1586 ± 13 |
| **41** | N-α-Ac-[Nle$^{24,28}$,Trp$^{30}$,Nva$^{31}$]PYY(22−36)-$NH_2$ | 0.56 ± 0.20 | 3.73 ± 0.89 | 183 ± 25 |
| **42** | N-α-Ac-[Nle$^{24,28}$,Trp$^{30}$,Nva$^{31}$,$CH_2$-NH$^{35-36}$]PYY(22−36)-$NH_2$ | 0.31 ± 0.11 | 1.75 ± 0.44 | 1567 ± 304 |
| **43** | bis(24/24′){N-α-Ac-[Cys $^{24}$]PYY(22−36)-$NH_2$} | 0.63 ± 0.27 | 0.39 ± 0.17 | 110 ± 22 |

[a] SCC, short circuit current; antisecretory potency of peptides was determined by measuring short circuit current in rat jejunal membrane preparation clamped in an Ussing chamber (from refs 17 and 32). ND, not determined.

contributed to the net receptor affinity of the analogues. Alkylation of the C-terminal amide group is known to prolong the half-life of peptide hormones.[37] However, in this instance, methylation, as in **14**, substantially decreased the affinity possibly due to steric hindrance at the binding site.

Further SAR studies revealed that substitution of Leu residues in N-α-Ac-PYY(22−36)-$NH_2$ with Trp at 28 or 30 increased the affinity, while substitution at 29 decreased it (analogues **15**−**18**). The increase in affinity with Trp$^{28}$ or Trp$^{30}$ substitutions may be due to the fact that hydrophobic residues are favored at this position, they increase proteolytic stability, or both. In fact, it has been reported that the Asn$^{29}$−Leu$^{30}$ bond is susceptible to cleavage by endopeptidase-24.11.[38] This observation is also in agreement with that of Fauchere and Thurieau,[39] who have suggested that the location of modifications used to stabilize the active sites of a number of polypeptide hormones have been or could have been guided by prior identification of the cleavage sites. Although Trp-like residues, Bth and Nal, were also tolerated at position 30, D-Trp$^{30}$ substitution substantially decreased affinity (analogues **19**−**21**).

The ability of these analogues to inhibit intestinal secretion was then compared by measuring the short circuit current (SCC) in rat jejunal mucosa according to our previously published procedures.[17] Most of these analogues modified at position 27 exhibited greater antisecretory potency than even intact PYY, with N-α-Ac-[Trp$^{27}$]PYY(22−36)-$NH_2$ (**13**) exhibiting the most potent effect (Table 1). However, the antisecretory potencies did not correlate well with the PYY receptor affinities of the analogues. The reasons for the discrepancy remain unclear at present.

Previous studies have shown that repeated treatment of intestinal mucosa with PYY causes desensitization of the receptor system in this tissue as evidenced by reduced SCC response.[17,18] Therefore, we investigated this phenomenon with N-α-Ac-[Trp$^{27}$]PYY(22−36)-$NH_2$ (**13**). In contrast to PYY,[17] this analogue was more effective in inhibiting the SCC response in a cumulative dose−response study than in experiments where each dose was tested in a fresh tissue (Figure 1). Moreover,

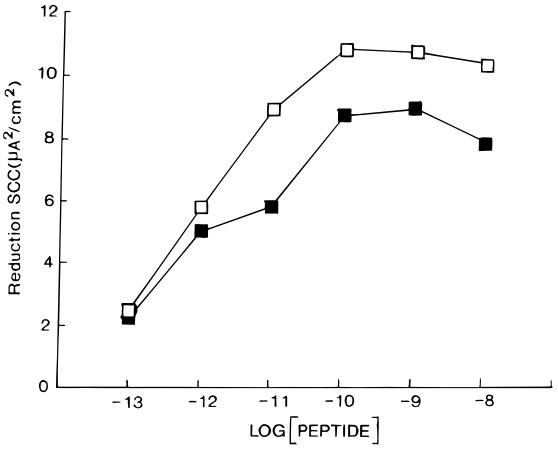



**Figure 1.** Effects of increasing concentrations of N-α-Ac-[Trp[27]]PYY(22−36)-$NH_2$ (**13**) on short circuit current (SCC) in rat jejunal mucosal membranes mounted on Ussing chamber. Dose−response curve obtained using a single tissue which received all the doses cumulatively (□) or a fresh tissue for each dose (■). Each point represents the mean of 8−10 tissues. SE was less than 10% and is not included in the figure for clarity. This is in contrast to PYY where the SCC dose−response curve obtained in cumulative experiment was shifted to the right relative to that obtained with fresh tissue for each dose.[17]

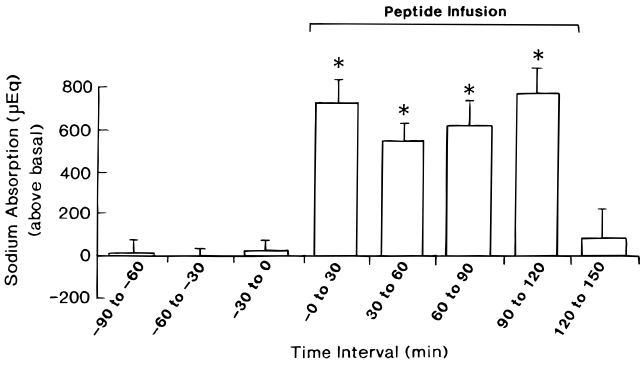



**Figure 2.** Ileal sodium fluxes above basal in six awake dogs with exteriorized jejunal loops infused (iv) with N-α-Ac-[Trp[27]]-PYY(22−36)-$NH_2$ (**13**) (200 pmol/kg/h) for 2 h starting at time zero. Water and chloride absorption paralleled that of sodium; $p < 0.05$ vs basal by ANOVA. Peak effect of **13** is comparable to that of PYY (see Figure 3).

it was determined that the pretreatment of the mucosal preparations with N-α-Ac-[Trp[27]]PYY(22−36)-$NH_2$ (**13**) did not significantly attenuate the SCC response of PYY (100 nM) (not shown). These observations suggest that PYY(22−36) analogues may not cause desensitization observed with the intact hormone. It appears therefore that the N-terminal segment of PYY may also be required to trigger desensitization of PYY receptors.

N-α-Ac-[Trp[27]]PYY(22−36)-$NH_2$ (**13**), being the most potent analogue in this series, was also selected to investigate whether PYY(22−36) analogues could promote intestinal absorption similar to intact PYY during intravenous or intraluminal administration in dogs.[10−12] These experiments were performed in awake dogs with isolated neurovascularly intact ileal loops (Thiry-Villa Fistulas). Intravenous infusion of 200 pmol/kg/h for 2 h significantly increased the ileal absorption of water and electrolytes (e.g., Figure 2). The proabsorptive effects of intraluminal administration of N-α-Ac-[Trp[27]]-PYY(22−36)-$NH_2$ (**13**) in the ileum (200 pmol/kg/h) or colon (300 pmol/kg/h) were also investigated using the same dog model (Table 2). Ileal administration of the analogue significantly enhanced the absorption of water and electrolytes in the ileum and had no effects in colon. Similarly colonic administration only enhanced the absorption in the colon. The confined effects of the analogue within the isolated segment suggest the involvement of PYY receptors on the luminal side. The significance of this observation is that the synthetic analogue could also be adapted for oral delivery using a stable formulation that resists degradation in the gut. However, the proabsorptive effects of both the analogue (Figure 2) and intact PYY[10−12] reached statistically insignificant levels soon after the iv or intraluminal infusions were stopped. This decrease in effect is not surprising because both PYY and the analogue are known to be susceptible to proteolysis.[38]

**Table 2.** Peak Proabsorptive Effects of Intraluminal Infusion of N-α-Ac-[Trp[27]]PYY(22−36)-$NH_2$ (**13**) in the Ileum and Colon of Awake Dogs Bearing Ileal and/or Colonic Thiry-Vella Fistulas[a]

| | ileum | | colon | |
|---|---|---|---|---|
| | basal | peak[b] | basal | peak[b] |
| $H_2O$ (μL/min) | 437 ± 92 | 725 ± 98 | 204 ± 25 | 503 ± 49 |
| $Na^+$ (μequiv/min) | 68 ± 13 | 120 ± 14 | 41 ± 5 | 78 ± 9 |
| $Cl^-$ (μequiv/min) | 63 ± 9 | 101 ± 13 | 45 ± 5 | 77 ± 8 |

[a] Peptides were infused for 1 h starting at time zero: in ileum at 200 pmol/kg/h and in colon at 300 pmol/kg/h. [b] $p < 0.05$ vs basal by ANOVA. Peak absorptions were observed 30 and 90 min after starting intraluminal infusion in the ileum and colon, respectively.

Further SAR studies were therefore performed to identify features which would enhance the half-life of PYY(22−36) analogues without jeopardizing PYY receptor affinity. Incorporation of a $CH_2$−NH bond has been reported to increase the stability and impart dramatic changes in the property and/or potency of the active sites of a number of polypeptide hormones.[39,40] Therefore, we investigated the effects of incorporating $CH_2$−NH bonds in PYY(22−36) except those involving His, Asn, and Gln (analogues **23**−**31**).While Asn and Gln are not compatible with this procedure, we had difficulty in preparing Boc-His(Bom)-CHO in good yields. The $CH_2$−NH bonds at 27−28 (**29**) and 33−34 (**24**) substantially reduced the receptor affinity, while those analogues with reduced bonds at 35−36 (**23**) and 28−29 (**28**) exhibited the highest affinity in this series (Table 1). This increase in affinity may be due to increased resistance to proteolysis, easy adaptation of the bioactive conformation due to increased flexibility of the bond, or both. The $CH_2$−NH bond in **28** may also increase the stability of its penultimate bond at 29−30, which is prone to endopeptidase-24.11.[37,39] Analogues with $CH_2$−NH bonds at either 31−32 (**26**) or 32−33 (**25**) also exhibited 10 times greater selectivity for PYY receptors relative to Y2 receptors. Incorporation of $CH_2$−NH bonds at other positions resulted in analogues with moderate affinity (Table 1).

On the basis of the information gathered from analogues **4**−**31**, we then synthesized analogues incorporating modifications at two sites. Most of these modifications, analogues **32**−**39**, were tolerated and also resulted in the development of one of the most potent analogues: N-α-Ac-[Trp[30],$CH_2$-NH[35−36]]PYY(22−36)-$NH_2$

**Table 3.** Potencies of PYY(22−36) Analogues for Inhibiting VIP-Induced Secretion in Isolated Jejunum in Anesthetized Rats[a]

| no. | compound | $ID_{50}$ (pmol/kg) | 95% conf limit |
|---|---|---|---|
| **1** | PYY | 6.0 | 3.9−9.3 |
| **13** | N-α-Ac-[$Trp^{27}$]PYY(22−36)-$NH_2$ | 33.3 | 17.1−64.8 |
| **18** | N-α-Ac-[$Trp^{30}$]PYY(22−36)-$NH_2$ | 2.1 | 0.47−9.44 |
| **38** | N-α-Ac-[$Trp^{30}$,$CH_2$-$NH^{35-36}$]PYY(22−36)-$NH_2$ | 190 | 95−370 |
| **40** | N-α-Ac-[$Nle^{24,28,30}$,$Nva^{31}$]PYY(22−36)-$NH_2$ | 29.5 | 16.6−52.3 |
| **41** | N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$]PYY(22−36)-$NH_2$ | 13 | 8.7−19.4 |
| **42** | N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$,$CH_2$-$NH^{35-36}$]-PYY(22−36)-$NH_2$ | 59 | 31−116 |
| **43** | bis(24/24′){N-α-Ac-[$Cys^{24}$]PYY(22−36)-$NH_2$} | 365 | 95−1400 |

[a] VIP was administered at 30 μg/kg/h. Each point was investigated in 6−8 animals.

(**38**). We also investigated the effects of conservative multiple substitutions with Nle→Leu and Nva→Val because these modifications, if tolerated, would further enhance the proteolytic stability. This analogue, N-α-Ac-[$Nle^{24,28,30}$,$Nva^{31}$]PYY(22−36)-$NH_2$ (**40**), exhibited high affinity to PYY receptors, but $Trp^{30}$ substitution as in **41** was also essential for potent in vivo activity (Table 3). We then synthesized **42**, N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,-$Nva^{31}$,$CH_2$-$NH^{35-36}$]PYY(22−36)-$NH_2$ encompassing all the modifications in **38**, **40**, and **41**. This analogue exhibited an affinity comparable to the most potent analogue, #**38**, and in preliminary studies exhibited greater proteolytic stability than **38**, **40**, and **41**. Therefore, we tested the in vivo proabsorptive effects of **42** in dogs in collaboration with Townsend and co-workers.[41] During iv infusions, this analogue promoted intestinal absorption to a level comparable to that of PYY, and as expected its peak effects were longer lasting than those of PYY (Figure 3).

Since dimerization of the active sites has been reported to increase potency and/or stability,[42] we synthesized the PYY(22−36) dimer via $Cys^{24}$ (**43**). This dimerization did not improve the receptor affinity very much, compared to its monomer (Table 1). It is possible that this may not be the optimal dimerization site. Further investigations are therefore necessary to determine whether this approach will yield potent PYY-(22−36) analogues.

PYY has been shown to inhibit basal and secretagogue-induced intestinal secretion in a number of animal models including humans.[14−19] Therefore, we selected those analogues exhibiting high affinity to PYY receptors and compared their ability to inhibit intestinal secretion with that of PYY in anesthetized rats, according to our previously published procedures.[15] In this study, 30-min inhibitory effects of bolus doses (iv) of PYY(22−36) analogues on VIP (30 μg/kg/h)-induced intestinal secretion were investigated. All these analogues dose-dependently inhibited the intestinal secretion with varying degree of potencies (Table 3). N-α-Ac-[$Trp^{30}$] PYY(22−36)-$NH_2$ (**18**), which is stable to endopeptidase-24.11, exhibited potent antisecretory activity, greater than even that of intact PYY. On the other hand, although both N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$]PYY(22−36)-$NH_2$ (**41**) and N-α-Ac-[$Nle^{24,28,30}$,$Nva^{31}$]PYY(22−36)-$NH_2$ (**40**) are stable to endopeptidase-24.11 and exhibit comparable affinity to PYY receptors, **41** exhibited greater antisecretory activity. This observation suggests that $Trp^{30}$ plays a greater role than just imparting proteolytic stability. N-α-Ac-[$Trp^{27}$]PYY(22−36)-$NH_2$

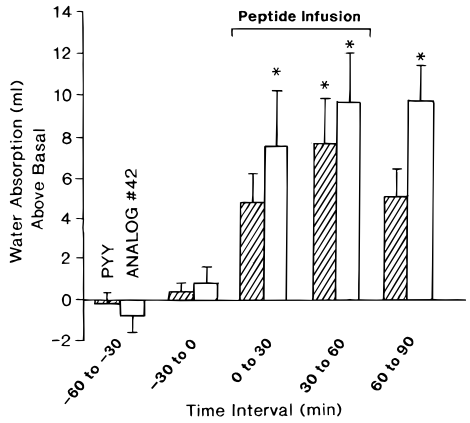



**Figure 3.** Comparison of the ileal proabsorptive effects of N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$,$CH_2$-$NH^{35-36}$]PYY(22−36)-$NH_2$ (**42**) with that of PYY in six awake dogs with exteriorized jejunal loops. Each compound was infused (iv) at the rate of 200 pmol/kg/h for 60 min starting at time zero; $p < 0.05$ vs basal by ANOVA. Peak effect of **42** was sustained for a longer period than that of PYY.

(**13**) and N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$,$CH_2$-$NH^{35-36}$]PYY-(22−36)-$NH_2$ (**42**), which exhibited potent and/or long-lasting proabsorptive effects in dogs, were only moderately potent as antisecretory agents. Moreover, N-α-Ac-[$Trp^{30}$,$CH_2$-$NH^{35-36}$]PYY(22−36)-$NH_2$ (**38**), which exhibited the greatest PYY receptor affinity, was one of the weakest in this series. So was the dimer, **43**. As is always the case with in vivo experiments, the antisecretory data cannot be interpreted based solely on PYY receptor affinity. This disparity could be due to many factors including differences in in vivo stability, accessibility to receptors, species differences, and involvement of multiple receptors. Moreover, one cannot exclude the involvement of neuronal factors, at least in part, in controlling PYY effects on intestinal secretion.[16] These results, together with future time course experiments, may prove useful in identifying potent and long-acting antisecretory analogues.

While our studies were in progress, cloning of additional receptor subtypes, Y4 and Y5, of the NPY−PYY−PP family of hormones was reported (see ref 27 for a review). Moreover, one of us (M.L.) has recently determined that Y2, Y4, and Y5 receptors are also present in colonic or intestinal mucosa.[43] Although these receptors may also be involved in mediating the effects of PYY peptides in the intestine, our recent investigation with crucial analogues in this series, N-α-Ac-[$Trp^{27}$]-PYY(22−36)-$NH_2$ (**13**), N-α-Ac-[$Trp^{30}$]PYY(22−36)-$NH_2$ (**18**), and N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$,$CH_2$-$NH^{35-36}$]PYY-(22−36)-$NH_2$ (**42**), revealed that they exhibit lower affinity ($IC_{50}$ > 1000 nM) to Y4 and Y5 receptors (not shown). Moreover, these analogues bound poorly to Y1 receptors (Table 1). These observations are consistent with our previous results that PYY(22−36) analogues do not cause hypertension,[31] an effect predominantly mediated by Y1 receptors. On the other hand, PYY(22−36) analogues, with few exceptions, also bound well to Y2 receptors (Table 1). It appears therefore that Y2 receptors, in addition to PYY receptors, may also be involved in the in vivo effects of PYY(22−36) analogues. Alternatively, since PYY receptors have not been cloned yet, we cannot strictly rule out the possibility that they represent a peripheral form of Y2 receptors in intestine.

Although one could not rule out these possibilities, the following observations suggest otherwise: (1) PYY exhibited a more pronounced effect than NPY on actin reorganization and induction of CD63 and clustering transcripts in an intestinal epithelial cell line;[44] (2) PYY, and not NPY, enhanced intestinal growth;[21] (3) intestinal distributions of Y2 receptor mRNAs are different from those of functional PYY receptors;[43] (4) potent Y2 agonists exhibited poor antisecretory potencies.[28−30] Experiments with receptor knock-out models and receptor-antisense and receptor-selective ligands may shed light on whether different receptors are involved in mediating PYY effects in the intestine.

The observation that some of the potent Y2 agonists elicit weak effects on PYY receptor-mediated antisecretory functions and the finding that analogues **25**, **26**, and **40** were 10−100 times selective to rat intestinal PYY receptors relative to human neuroblastoma Y2 receptors (Table 1) suggest that it is possible to dissociate Y2 activity from PYY(22−36) analogues and develop ligands highly selective for intestinal PYY receptors. However, apparently no adverse effects were observed with the PYY(22−36) analogues in the animal models used in this study. Therefore, one questions whether there is a need to dissociate Y2 activity. Moreover, the peripheral adverse effects, if any, could be avoided via oral delivery, because analogues delivered luminally do not appear to enter into circulation in the active form.

In summary, we have developed analogues that bind to intestinal PYY receptors and promote potent and long-lasting in vivo effects on intestinal absorption and secretion. Although it remains to be proven, these compounds may also promote repletion of intestine similar to intact PYY. Therefore, it appears that these compounds or analogues based on them may prove useful in treating malabsorptive disorders observed under a variety of conditions including chronic diarrhea, short bowel syndrome, and intestinal bowel diseases.

## Experimental Section

**Materials.** N-α-Boc-amino acids with benzyl-based protecting groups (Midwest Biotech, Indianapolis, IN) and peptide synthesis reagents (Applied Biosystem, Inc., Foster City, CA) and solvents (Tedia Inc., Cincinnati, OH) were obtained commercially and used without further purification. All protease inhibitors and buffer reagents were purchased from Sigma Chemical Co., St. Louis, MO, Biowhitaker, Walkersville, MA, and/or Life Technologies, Grand Island, NY. Neuroblastoma cells, SK-N-MC and SK-N-BE2 cells, were gifts from Dr. June Biedler, Sloan−Kettering Memorial Institute, NY. Sources of rats and other reagents have been reported previously.[33,34]

**Peptide Synthesis.** Peptides were synthesized according to our previously published procedures for the synthesis of NPY.[33] Briefly, the protected amino acids were assembled sequentially on *p*-methylbenzhydrylamine resin (0.45 mmol amino group) using an automated Applied Biosystem instrument employing a program supplied by the manufacturers for single coupling procedures. All amino acids were coupled using 4.4 equiv of pre-formed 1-hydroxybenzotriazole esters.

**Synthesis of Peptides Containing $CH_2$−NH Bonds.** Coupling of t-Boc-amino acid aldehydes was performed manually as described by Saski and Coy.[36] Briefly, t-Boc-amino acid aldehyde (4 equiv), obtained by $LiAlH_4$ reduction of the corresponding *N*-methoxy-*N*-methylamide derivatives,[35,45] was reacted immediately with the free α-amino group resin containing the appropriate peptide sequence with protected side chains and in DMF containing 1.0% HOAC in the presence of an equivalent quantity of $NaBH_3CN$. At the end of the reaction (2−3 h), the formation of the secondary amino group was confirmed with ninhydrin (wine-red color). To prevent acylation of the $CH_2$−NH bond during subsequent coupling or capping steps, the peptide-resin with the secondary amine was reacted with 2 equiv of Z(2-Cl)OSU, 2 equiv of HOBT, and 4 equiv of diisopropylethyamine until the ninhydrin test gave a yellow color. Automated synthesis was then resumed by reintroducing the peptide-resin into the recation vessel of the synthesizer.

**Synthesis of Dimers.** Peptides were dimerized on the resin by coupling 0.5 equiv of Boc-Cys in the presence of equivalent quantities of HBTU, HOBT and DIEA. This coupling which was also performed manually was generally complete within 10−18 h. In some instances, the uncoupled amino group was capped by acetylation. Stepwise synthesis was then continued to obtain the desired sequences. If required, the α-amino group was acetylated at the end of the synthesis.

**HF Cleavage.** At the end of the synthesis, the N-α-Boc group was removed as programmed, and if required the α-amino group was acetylated automatically with $Ac_2O$. $N^{in}$-CHO, if present, was then removed with 20% piperidine−DMF, and the free peptides were obtained by treating the peptide-resins (∼0.25 mmol) with HF (∼10 mL) containing 5% *p*-cresol for about 1 h at −2 to −4 °C. In the case of peptides with Trp or Cys, HF reaction mixture also contained ∼2.5% dimethyl sulfide. The residue after HF cleavage was washed repeatedly with diethyl ether, and then extracted with 30% acetic acid (2 × 15 mL), diluted to 10% and lyophilized.

**Purification.** Peptides were purified according to our previously published procedures for NPY,[33] using a Waters Instrument with model 600 multisolvent delivery system, U6K injector, model 481 spectrphotometer and Baseline 810 data collection. Analytical HPLC was performed on a Vydac C18 column (250 × 4.6 mm, 5-μm particle size, 300-Å pore size) at a flow rate of 1 mL/min. Semipreparative HPLC was carried out on a Vydac column (250 × 10 mm, 10-μm particle size, 300-Å pore size) at a flow rate of 4.7 mL/min. Detection was at 214 nm. A combination of either 0.1% TFA−$H_2O$ (A) and 60% MeCN in A (B) or 0.1% triethylyammonium phosphate buffer, pH 2.25 (C) and 60% MeCN in C (D) were used to purify the peptides. If sovent system C and D were used, peptides were desalted using A and B, before lyophilization. All the purified peptides were characterized by analytical reversed-phase chromatography using an isocratic gradient and amino acid and mass spectral analyses.

**Cell Culture.** The neuroblastoma cells were grown in Eagle's essential medium (EMEM), supplemented with 10% fetal calf serum, penicillin (100 IU/mL) and streptomycin (50 μg/mL) in a 95% air−5% $CO_2$ humidified atmosphere at 37 °C. When the cells became 90% confluent (4−5 days), the growth medium was aspirated, cells washed with Dulbecco's phosphate-buffered saline (DPBS) with $Ca^{2+}$ and $Mg^{2+}$, then dispersed by incubating with 0.1% trypsin in DPBS, pH 7.4, without $Ca^{2+}$ and $Mg^{2+}$ for 5 min at 37 °C.

**Receptor Binding.** The SK-N-MC and SK-N-BE2 cell lines were cultured in EMEM media containing 10% fetal calf serum and 5% chicken embryo extract in a humidified atmosphere (37 °C) of 95% air and 5% $CO_2$. The appropriate cells were harvested, homogenized (Polytron, setting 6, 15 s) in ice-cold 50 mM Tris-HCl (buffer A), and centrifuged twice at 39000*g* (10 min), with an intermediate resuspension in fresh buffer. The final pellets were resuspended in 50 mM Tris-HCl, containing 0.1 mg/mL bacitracin, and 0.1% BSA (buffer B), and held on ice for the receptor binding assay. Aliquots (0.4 mL) were incubated with 0.05 mL of [$^{125}$I]PYY (SK-N-MC cells) or [$^{125}$I]PYY(3−36) (SK-N-BE2) (∼2200 Ci/mmol, New England Nuclear) and buffer B, with and without 0.05 mL of unlabeled competing peptides. After a 120-min incubation (25 °C), the bound [$^{125}$I]PYY or [$^{125}$I]PYY(3−36) was separated from the free by rapid filtration through GF/C filters previously soaked in 0.3% poly(ethylenimine). The filters were then washed three times with 5-mL aliquots of ice-cold buffer A. Specific binding was defined as the total [$^{125}$I]PYY bound minus that bound in the presence of intact PYY.

**Binding Studies with Rat Intestinal Crypt Epithelial membranes.** This was performed according to our previously published procedures.[25,26] Jejunal segments were removed and flushed free of content, filled with 0.34 M NaCl. Crypt cells were separated from villus cells by shaking the everted jejunum for successive periods in a dispersing solution containing 2.5 mM EDTA and 0.24 M NaCl, pH 7.5. Villus cells were released first and crypt cells after a prolonged shaking. Crypt cells were then sedimented at 2000*g* for 2 min and washed 4 times with Krebs-Ringer phosphate buffer, pH 7.5. Crude membranes were prepared from isolated crypt cells as described.[22,26] Displacement studies were performed in a total volume of 0.25 mL of 20 mM HEPES assay buffer, pH 7.4, containing 2% bovine serum albumin and 0.1% bacitracin. In a standard assay 200 $\mu$g of membrane protein/tube were incubated for 2 h at 15 °C in a shaking water bath with [$^{125}$I]-PYY (50 pM) and increasing concentrations of peptides. At the end of incubation tubes were vortexed and 150-$\mu$L aliquots transferred into polypropylene tubes containing 250 $\mu$L of ice-cold assay buffer. Unbound [$^{125}$I]PYY were separated by centrifugation at 20000*g* for 10 min followed by aspiration of the supernatant. The tubes containing the pellet were counted for bound radioactivity in a Micromedic gamma counter. Each point in an experiment was carried out in triplicate and the experiment repeated at least three times. Displacement curves were plotted using LIGAND program and the $IC_{50}$ values determined.

**In Vivo Experiments in Rats To Determine the Effects of PYY Analogues on VIP-Induced Jejunal Secretions.** This was carried out according to the procedures developed by Roze and co-workers.[15] Briefly, rats were anesthetized, and a saphenous vein was cannulated to infuse peptides. The abdomen was opened, and a jejunal loop (~20 cm long) was delimited by two ligatures. At time zero, 2 mL of 0.9% saline prewarmed to 37 °C was instilled into the jejunal loop. The loop was then returned into the abdomen and closed. VIP (30 $\mu$g/kg/h) was infused through the saphenous vein at the rate of 2.5 mL/h for 30 min starting at time zero. PYY analogues (3−900 pmol/kg) or saline (controls) was injected as bolus doses 15 min before starting VIP infusions. At the end of the experiment rats were sacrificed, and the jejunal loop excised. The loop was then measured and weighed before and after removing the instilled fluid. The net water flux expressed as $\mu$L/cm/30 min was calculated using the formula $(F - E) - 2000/L$, where $F$ and $E$ are the weights of the loop before and after emptying the remaining fluid, $L$ is the length of the loop in cm, and 2000 is the volume in $\mu$L of the fluid instilled initially. Net absorption is indicated by a negative value and net secretion by a positive value. Each dose was investigated in 6−8 animals.

**In Vivo Experiments in Dogs To Determine the Effects of PYY Analogues on Intestinal Absorption.** In vivo effects of peptides on water and electrolyte absorption by the jejunum, ileum and colon under basal conditions were investigated in awake dogs with jejunal, ileal and/or colonal Thiry-Vella loops according to our previously published procedures.[10−12] Each experiment consisted of a 90-min basal period followed by a 150-min experimental period. The small bowel segment was perfused through the proximal cannula at 2 mL/min using a roller pump. The perfusate, pH 7.4 maintained at 37 °C, contained (in nmol/L) 140 $Na^+$, 5.2 $K^+$, 119.8 $Cl^-$, 25 $HCO_3^-$, 1.2 $Ca^{2+}$, 1.2 $Mg^{2+}$, 2.4 $HPO_4^{4-}$, 0.4 $H_2PO_4^-$, 10 glucose and 10 $\mu$C [$^{14}$C]poly(ethylene glycol) in 5 g/L poly(ethylene glycol). After a 20-min washout period, perfusate was collected every 15 min for determination of $Na^+$, $Cl^-$ and water contents.

**Supporting Information Available:** HPLC, MS, and amino acid analysis data of PYY(22−36) analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM000052Z