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Insights into the impact of phenolic residue incorporation at each position along secretin for receptor binding and biological activity

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Abstract

Understanding of the structural importance of each position along a peptide ligand can provide important insights into the molecular basis for its receptor binding and biological activity. This has typically been evaluated using serial replacement of each natural residue with an alanine. In the current report, we have further complemented alanine scanning data with the serial replacement of each residue within secretin-27, the natural ligand for the prototypic class B G protein-coupled secretin receptor, using a photolabile phenolic residue. This not only provided the opportunity to probe spatial approximations between positions along a docked ligand with its receptor, but also provided structure-activity insights when compared with tolerance for alanine replacement of the same residues. The pattern of sensitivity to phenolic residue replacement was periodic within the carboxyl-terminal region of this peptide ligand, corresponding with alanine replacements in that region. This was supportive of the alpha-helical conformation of the peptide in that region and its docking within a groove in the receptor amino-terminal domain. In contrast, the pattern of sensitivity to phenolic residue replacement was almost continuous in the amino-terminal region of this peptide ligand, again similar to alanine replacements, however, there were key positions in which either the phenolic residue or alanine was differentially preferred. This provided insights into the receptor environment of the portion of this ligand most critical for its biological activity. As the structure of the intact receptor is elucidated, these data will provide a guide for ligand docking to the core domain and to help clarify the molecular basis of receptor activation.

Keywords

Secretin; structure-activity relationship study; benzoyl-phenylalanine; alanine scanning

1. Introduction

Replacement of each residue along a peptide ligand with an alanine residue represents a classical approach to determine the importance of each residue in hormone function, in making contributions to both receptor binding and biological activity [1–5]. Alanine has been chosen for this, based on its being the smallest of the uncharged non-polar amino acid

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residues that maintains the integrity of the peptide backbone, but that does not contribute to higher energy charge-charge interactions or strong hydrophobic interactions of more specialized residues. However, the usefulness of an alanine-scanning data set may be limited. Loss-of-function of a particular alanine-replacement mutant has been interpreted to suggest that the replaced residue is critical to function and might be difficult to replace in a peptide analogue. However, in preparing intrinsic photolabile probes for photoaffinity labeling of a hormone receptor, we have observed examples of successful incorporation of a bulky, hydrophobic photolabile moiety into a peptide position that poorly tolerates alanine replacement [6–8].

In the current work, we have focused on the natural peptide ligand for the secretin receptor, a prototypic class B peptide hormone-binding G protein-coupled receptor [9]. This receptor family typically binds and is activated by moderate length peptides having an extended pharmacophoric region, with the carboxyl-terminal region of the hormone contributing binding affinity to the structurally distinct, disulfide-bonded receptor amino terminus and the amino-terminal region of the hormone contributing biological activity and additional binding affinity, likely directed toward the core regions of the receptor [10]. Indeed, these features have been shown to be shared by secretin, a 27-residue peptide [11]. We previously reported the replacement of each secretin residue with alanines and demonstrated that the pattern of functional importance closely followed that of related peptide hormone receptors in this family [2]. We have also replaced sixteen natural residues along secretin with photolabile benzoyl-phenylalanine (Bpa) or benzyl-benzyl-lysine ((BzBz)Lys) for site-specific photoaffinity labeling studies of this receptor [6–8, 11–18]. In such studies, it is important for the photolabile hormone analogues to maintain adequate binding affinity and full biological activity to be certain that the probe occupy a site similar to that occupied by the natural hormone. In this experience, we have described three secretin analogues in which the photolabile moiety was successfully incorporated into a position in which alanine replacement has been poorly tolerated (positions 6, 22, and 26) [6–8].

We have, therefore, now prepared twelve additional analogues of secretin to complete the series of analogues with phenolic residue incorporations throughout the pharmacophore (every position from one through 26) and to compare this series with the alanine-replacement series, exploring how such data might provide insights into the molecular mechanism of peptide docking and receptor activation.

2. Methods

2.1. Reagents

Fmoc-protected amino acids for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY) and Pal resin was from Sigma-Aldrich (St. Louis, MO). ¹²⁵Iodine was from Perkin Elmer Life Sciences (Boston, MA). Dulbecco's modified Eagle's medium (DMEM) and soybean trypsin inhibitor were from Invitrogen (Carlsbad, CA). Fetal clone II culture medium supplement was from Hyclone laboratories (Logan, UT). Bovine serum albumin was from Serologicals Corp. (Norcross, GA). All other reagents were analytical grade.

2.2. Peptide synthesis

Eleven rat secretin peptide analogues were prepared for the current study, ten of which incorporated a Bpa into positions 2–4, 7–11, 17, and 19 and one of which incorporated a *p*-nitro-phenylalanine (NO₂-Phe) in position 15. Each probe (except for position 10 Bpa probe) incorporated a tyrosine residue in position 10 to replace the natural leucine residue in that position as a site for radioiodination that has previously been well tolerated [19, 20]. For

the probe incorporating a Bpa in position 10, a tyrosine for radioiodination was incorporated in position 26. All these peptides were synthesized using manual solid-phase synthesis techniques with Pal resin and Fmoc-protected amino acids and purified by reversed-phase HPLC using procedures described previously [21]. Expected molecular masses were verified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry.

2.3. Radioiodination

The radioligand was prepared as described previously by oxidative radioiodination of [Tyr¹⁰]rat secretin-27 using 1 mCi Na¹²⁵I and exposure for 15 s to the solid phase oxidant, Iodo-beads [21]. The radioiodinated peptide was purified by reversed-phase HPLC to yield specific radioactivity of ~2000 Ci/mmol [21].

2.4. Receptor source

A CHO cell line stably expressing the wild type rat secretin receptor (CHO-SecR) that had been previously established was used as source of receptor for the current study [22]. These cells were cultured in Ham's F-12 medium supplemented with 5% Fetal Clone II in a humidified atmosphere containing 5% CO₂ at 37 °C, and they were passaged approximately twice per week.

2.5. Receptor binding assay

Whole cell binding in 24-well tissue culture plates was performed to determine the binding affinity of each of the secretin analogues. In brief, 50,000 CHO-SecR cells were plated into each well and were allowed to grow to 80 percent confluence for approximately 72 h prior to the assay. After being washed twice with Krebs-Ringers/HEPES (KRH) medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄) containing 0.01% soybean trypsin inhibitor and 0.2% bovine serum albumin, cells were incubated with a constant amount of radioligand, [¹²⁵I-Tyr¹⁰]rat secretin-27 (3 to 5 pM, approximately 20,000 cpm) and increasing concentrations of secretin or each of the Bpa or NO₂-Phe replacement secretin analogues (ranging from 0 to 1 μM) for 60 min at room temperature. Cells were then washed twice with ice cold KRH medium to separate free from cell-bound radioligand, and bound cells were lysed with 0.5 M NaOH. Membrane-bound radioactivity was quantified with a γ-spectrometer. Nonspecific binding was determined in the presence of 1 μM secretin and represented less than 15% of total binding.

2.6. Biological activity assay

The biological activity of each of the secretin analogues was determined by examining its ability to stimulate cAMP responses in CHO-SecR cells grown in 96-well plates. In brief, ~8,000 cells per well were plated and allowed to grow for two days before being washed with PBS and stimulated with increasing concentrations of secretin or each secretin analogue (0 to 1 μM) in KRH medium containing 0.01% soybean trypsin inhibitor, 0.2% bovine serum albumin, 0.1% bacitracin, and 1 mM 3-isobutyl-1-methylxanthine for 30 min at 37 °C. After incubation, cells were lysed with 6% ice-cold perchloric acid for 15 min with vigorous shaking and the lysates were adjusted to pH 6 with 30% NaHCO₃. The cAMP levels in the lysates were determined in a 384-well white Optiplate using a LANCE kit from PerkinElmer (Boston, MA).

2.7. Statistical analysis

All assays were performed in duplicate in a minimum of three independent experiments and are expressed as the means ± S.E.M. Receptor competition binding and cAMP concentration-response curves were analyzed and plotted using the non-linear regression analysis program in the Prism software suite v3.0 (GraphPad Software, San Diego, CA).

Binding kinetics were determined by analysis with the LIGAND program of Munson and Rodbard [23]. Two-tailed *T*-tests were performed to evaluate the differences using InStat3 (GraphPad Software, San Diego, CA), with *P*-values less than 0.05 considered to be significant.

3. Results

3.1. Peptides

We have previously prepared and characterized secretin analogues incorporating phenolic Bpa or (BzBz)Lys residues into positions 1, 5, 6, 12–16, 18, 20–26 of the natural 27-residue peptide [6–8, 11–18]. In the current work, we have prepared eleven additional secretin analogue peptides, replacing each residue in positions 2–4, 7–11, 17 and 19 with a Bpa residue and replacing the residue in position 15 with NO₂-Phe, thereby completing the full series of replacements of each secretin residue with a phenolic residue. These peptides were synthesized by solid-phase techniques and purified to homogeneity by reversed-phase HPLC, and their identities were confirmed by mass spectrometry.

3.2. Binding affinity of phenolic-replacement analogues of secretin

A radioligand binding assay was used to determine the ability of each of the phenolic-replacement analogues of secretin to compete for binding to the secretin receptor expressed on CHO-SecR cells. These cells had $124,000 \pm 9,900$ secretin binding sites per cell ($B_{\max}$). Figure 1 shows the competition-binding curves for each of noted peptides. Of the ten Bpa-replacement analogues, only [Bpa¹⁰]sec, [Bpa¹¹]sec, and the [Bpa¹⁷]sec were able to fully displace all saturable radioligand binding (determined in the presence of 0.1 μM secretin competition), but the affinities of these peptides were lower than that of natural secretin (Fig. 1 and Table 1). All other noted Bpa-replacement analogues were only able to displace radioligand binding at high concentrations. The rank order of affinities of these analogues from highest to lowest was the following: Bpa¹⁰>Bpa¹⁷>Bpa¹¹>Bpa⁷>Bpa⁹>Bpa²>Bpa³>Bpa¹⁹>Bpa⁴>Bpa⁸.

Table 1 lists the calculated K_i values for the phenolic-replacement analogues of secretin characterized in the current study, as well as including the related secretin analogues that were previously reported. Figure 1 illustrates the data from the entire series, showing that multiple residues throughout the entire length of secretin had important contributions to the binding of this hormone to its receptor. The most pronounced decreases in binding affinity (>100-fold) were observed for phenolic substitution for residues Ser²Asp³Gly⁴Ser⁸Asp¹⁵ and Leu¹⁹. While phenolic substitution for residues His¹Thr⁷Glu⁹Ser¹¹Leu¹³Ala¹⁷Arg¹⁸ and Leu²³ also resulted in a substantial decrease in binding affinity (>10-fold, but <100-fold), substitution of residues Thr⁵Phe⁶Leu¹⁰Arg¹²Gln¹⁴Ser¹⁶Gln²⁰Arg²¹Leu²²Gln²⁴ and Gly²⁵ and Leu²⁶ resulted in only minimal decreases in affinity (<10-fold). It should be noted that replacing the acidic aspartate residue in position 15 with an acidic phenolic NO₂-Phe residue was four-fold better tolerated than replacing it with a Bpa (Table 1).

3.3. Biological activity of phenolic-replacement analogues of secretin

Intracellular cAMP responses in the CHO-SecR cells were used to assess the biological activity of each of the newly prepared secretin analogues. Figure 2 shows the cAMP concentration-response curves for each of these phenolic-replacement analogues of secretin. Although some of the Bpa-replacement analogues had very low binding affinity, all but one ([Bpa⁴]sec) were found to represent fully efficacious agonists, stimulating maximal cAMP responses in CHO-SecR cells that were similar to that stimulated by natural secretin. [Bpa¹⁰]sec had similar potency to that of natural secretin, while other analogues had

relatively lower affinities. The rank order of potencies from highest to lowest was the following: Bpa¹⁰>Bpa¹⁷>Bpa⁹>Bpa¹¹>Bpa²>Bpa⁷>Bpa¹⁹>Bpa⁸>Bpa³>Bpa⁴.

Table 1 lists the calculated EC₅₀ values for the phenolic-replacement analogues of secretin characterized in the current study, as well as for those previously reported. Figure 2 also illustrates the data from the entire series and shows that multiple residues spread throughout the length of this hormone were critical for its biological activity at the secretin receptor. The most pronounced decreases in stimulating cAMP responses in CHO-SecR cells (>100-fold) were observed for phenolic residue substitution for residues His¹Asp³Gly⁴Thr⁷Ser⁸Asp¹⁵Leu¹⁹ and Leu²³. While phenolic residue substitution for residues Ser²Thr⁵Phe⁶Glu⁹Ser¹¹Arg¹²Leu¹³Gln¹⁴Ala¹⁷Arg¹⁸ and Arg²¹ also resulted in a substantial decrease in biological activity (>10-fold, but <100-fold), substitution for residues Leu¹⁰Ser¹⁶Gln²⁰Leu²²Gln²⁴Gly²⁵ and Leu²⁶ only resulted in a slight decrease in potency (<10-fold). Consistent with the receptor binding data, replacing the acidic aspartate residue in position 15 with an acidic phenolic NO₂-Phe residue was four-fold better tolerated for biological activity than replacing it with a Bpa (Table 1).

3.4. Relationship between receptor binding and biological activity

Figure 3 illustrates the correlation between the effects of the phenolic residue substitution at each position along secretin on its binding affinity and on its potency. These were generally strongly correlated with each other ($R^2 = 0.73$), although a few notable positions diverged. Of interest, the most marked of these was the histidine in position one, having much more impact on biological activity than on binding affinity. Leucine in position 23 had similar disproportionate importance for biological activity. In contrast, secretin residues serine in position 2 and glutamic acid in position 9 had greater impact on binding affinity than on biological activity. These four residues are highlighted in this figure.

3.5. Effects of phenolic versus alanine substitutions

Figure 4 summarizes the impact of phenolic residue versus alanine substitution at each position along secretin on binding affinity and biological activity. The frequency and density of residues of importance are much greater in the amino terminus than in the carboxyl terminus of this peptide, where the major impact was periodic as expected for a helical structure with one face being most important. There are a few notable differences in accommodation of a phenolic residue or an alanine. The phenolic residue was much less well accommodated in positions 2 and 17 than an alanine. In contrast positions 6, 10, and 22 accommodated a phenolic residue much better than an alanine.

Figures 5 and 6 illustrate the relative impact of incorporation of a phenolic residue versus an alanine residue at each position within secretin on binding affinity (Fig 5) and biological activity (Fig 6). In each figure, the line of equivalence is shown, with the points above the line reflecting preference for a phenolic residue and those below the line reflecting preference for alanine. The residues having the most profound effects are highlighted in figures 5 and 6. Of note, the phenolic residue was preferred to alanine for both binding and biological activity when present in positions 10 and 22, while alanine was preferred to a phenolic residue in positions 2 and 17.

4. Discussion

Like the results of the common practice of serially replacing each residue along the length of a hormone with an alanine, the current series of replacing each residue with a photolabile phenolic residue was accommodated with different degrees of success at different positions. This approach of residue-replacement scanning yields insights into positions in which

receptor binding and stimulated biological activity are not affected or are affected in tandem, as well as those affecting each disproportionately. By taking this approach both with alanine and with a phenolic moiety, the insights turned out to be extremely interesting and substantially beyond those coming from only a single series of such replacements.

For secretin, alanine scanning results revealed that many, even most, residues within the amino-terminal region markedly interfered with binding affinity, while similar effects were observed in a periodic pattern, with every four or five residues in the carboxyl-terminal region interfering with binding [2]. A similar pattern was observed for biological activity in this series, although the most marked effects were observed only for a subset of the same residues [2]. Even if we did not know that the carboxyl-terminal region of secretin [13], and other natural peptide ligands for this family [24], were docked in an alpha helical conformation [13, 24], this pattern would have suggested the existence of such a conformation, along with definition of the face of the helix that lies within its binding cleft. Of interest, residues in positions 17 and 22 within the binding cleft of this helix have quite distinct characteristics, with position 17 tolerating alanine better than a phenolic residue and the opposite being true for position 22. Such insights may facilitate the development of a more active secretin receptor antagonist. While we know very little about the conformation of the amino terminus of these peptides as bound to their receptors, this pattern supports a non-helical conformation and suggests that many residues in that region might be contributing to important interactions involved in binding and biological activity. The amino-terminal histidine seems to hold a unique position in this series, having such a profound impact on biological activity, out of proportion to every other alanine-replacement mutant [2].

Phenolic residue scanning along secretin yielded a pattern similar to that of alanine scanning, with several amino-terminal residues markedly interfering with binding affinity and biological activity, and every four to five residues within the carboxyl-terminal region having similar effects. While the most profound effects of phenolic residue replacement involved the same residues as were most affected by alanine replacement within the carboxyl terminus, this was not true within the amino terminus. Similar levels of disruption of function by replacing the same periodic carboxyl-terminal residues with alanine and with the hydrophobic photolabile moiety supported the importance of the natural residues in those positions in contributing to specific interactions. When such residues are naturally charged, such as the aspartate in position 15, a charge-charge interaction or hydrogen bonding interaction would be predicted.

While alanine replacements for each of the amino-terminal ten residues within secretin had at least moderate negative effects, except for the residue in position two, the impact of hydrophobic replacement was more sporadic. Phenolic residue replacements had minimal effects in the same region in positions five, six, nine, and ten. Of note, hydrophobic photolabile moiety replacement for serine in position two did have a prominent effect on binding affinity, while alanine replacement for this residue had little effect. The residues in positions six, seven, and ten (phenylalanine, threonine, and leucine, respectively) have been implicated in forming an N-capping motif that has been implicated in stabilizing the carboxyl-terminal helix and also in biological activity [2, 25]. The phenolic residue replacements were accommodated well in these positions, suggesting that the hydrophobic nature of the residues was all that was necessary to reproduce the impact of this motif and that these residues do not have specific, selective interactions with receptor residues. This is different from the role predicted by Neumann et al. [25] when they pointed out the presence of this sequence in most members of this family. In contrast, the residues in positions one, three, and four (histidine, aspartate, and glycine, respectively) likely play specific and selective roles. Glycine may play such a role, even without a side chain, by allowing peptide

backbone mobility and/or by fitting into a relatively tight region in which steric hindrance of a side chain might interfere with function.

Looking at the correlation of impact on binding and biological activity of single residue replacements, the histidine in position one has disproportionate effects on biological activity. Of interest, this was true for both alanine replacement and for phenolic residue replacement. This residue likely has a very critical specific interaction with the receptor. Indeed, it is recognized that each of the natural ligands for this family can be stripped of its agonist activity by amino-terminal truncation [26–28].

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Abbreviations used are

Bpa	benzoyl-phenylalanine
(BzBz)Lys	benzyl-benzyl-lysine
CHO-SecR	Chinese hamster ovary cell line stably expressing the wild type rat secretin receptor
DMEM	Dulbecco's modified Eagle's medium
KRH	Krebs-Ringers/HEPES
NO₂-Phe	<i>p</i> -nitrophenylalanine

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Highlights

Each residue within secretin-27 was replaced with a phenolic residue.

The impact of structural modifications on binding and activity were quantified.

Results were compared with previous alanine scanning data.

Relative accommodation of two distinct types of residues provides key insights.

Fig. 1.

Binding activities of phenolic-replacement analogues of secretin. *Top* binding curves of increasing concentrations of secretin and phenolic-replacement analogues to compete for binding of secretin radioligand to CHO-SecR cells. Values illustrated represent saturable binding as percentages of maximal binding observed in the absence of the competing peptide and are expressed as the means $\pm$ S.E.M. of duplicate values from a minimum of three independent experiments. The rank order of affinities from highest to lowest is shown on the right of the graph (top to bottom). *Bottom* role of each residue in secretin on binding to its receptor. Shown are the K_i values of each of the secretin analogues incorporating a phenolic residue and the secretin sequence illustrating the role of each residue in binding to

the secretin receptor. *Open circles* represent residues whose replacement by phenolic residues resulted in less than 10-fold in binding affinity comparing to natural secretin. *Grey and black circles* represent residues whose replacement with phenolic residues resulted in more than 10-fold but less than 100-fold, and more than 100-fold increase in binding affinity (dashed lines), respectively, comparing to natural secretin.

Fig. 2.

Biological activities of the phenolic-replacement analogues of secretin. *Top* curves of intracellular cAMP responses in CHO-SecR cells stimulated by increasing concentrations of phenolic-replacement analogues. Data points represent the means $\pm$ S.E.M. of three independent experiments performed in duplicate, normalized relative to the maximal response to secretin. The rank order of potencies from highest to lowest is shown on the right of the graph (top to bottom). Basal and maximal cAMP levels by secretin were 3.7 ± 1.3 and 186 ± 40 pmol/million cells, respectively. *Bottom* role of each secretin residue on its biological activity. Shown are the EC₅₀ values of each of the secretin analogues incorporating a phenolic residue and the secretin sequence illustrating the role of each

residue in their biological activity. *Open circles* represent residues whose replacement by phenolic residues resulted in less than 10-fold in biological activity comparing to natural secretin. *Grey and black circles* represent residues whose replacement with a phenolic residue resulted in more than 10-fold but less than 100-fold, and more than 100-fold increase in biological activity (dashed lines), respectively, comparing to natural secretin.

Fig. 3. Relationship between binding affinities and biological activity potencies of phenolic-replacement analogues of secretin. Shown is the plot of the logarithmic transformations of K_i and EC_{50} values for these peptides, as well as their correlation coefficient. The points in the plot are numbered to indicate the positions of the residues in secretin. Data points representing residues having disproportionate impact on biological activity or on binding affinity are circled.

Binding affinity

Biological activity

Fig. 4.

Comparison of the roles of each secretin residue determined by phenolic replacement and by alanine replacement. *Top* role in binding affinity. *Bottom* role in biological activity. *Open circles* of little importance (K_i or EC_{50} values are less than 10-fold of that of natural ligand). *Grey circles* important (K_i or EC_{50} values are more than 10-fold but less than 100-fold of that of natural secretin). *Black circles* critical (K_i or EC_{50} values are more than 100-fold of that of natural secretin).

Fig. 5. Comparison of the binding affinities of each of the phenolic-replacement analogues of secretin relative to those of its Ala-replacement analogues. Shown is the plot of ratio of the logarithmic transformations of K_i values of Ala-replacement secretin peptides ($\log K_i^{\text{Ala}}$) and the natural secretin ($\log K_i^{\text{Sec}}$) vs. the ratio of the logarithmic transformations of K_i values for secretin peptides with phenolic substitutions ($\log K_i^{\text{Phenolic}}$) and the natural secretin ($\log K_i^{\text{Sec}}$). The points in the plot are numbered to indicate the positions of the residues in secretin. The line of equivalence is shown, and those data points above the line reflecting the greatest preference for a phenolic residue and those below the line reflecting the greatest preference for alanine are circled.

Fig. 6.

Comparison of the potencies in stimulating cAMP responses for each of the phenolic-replacement analogues of secretin relative to that of its Ala-replacement analogues. Shown is the plot of the ratio of the logarithmic transformations of EC_{50} values of Ala-replacement secretin peptides ($\log EC_{50}^{\text{Ala}}$) and natural secretin ($\log EC_{50}^{\text{Sec}}$) vs. the ratio of the logarithmic transformations of EC_{50} values for secretin peptides with phenolic substitutions ($\log EC_{50}^{\text{Phenolic}}$) and natural secretin ($\log EC_{50}^{\text{Sec}}$). The points in the plot are numbered to indicate the positions of the residues in secretin. The line of equivalence is shown, and those data points above the line reflecting the greatest preference for a phenolic residue and those below the line reflecting the greatest preference for alanine are circled.

Table 1

Binding and biological activities of phenolic-replacement analogues of secretin

Amino acid	Peptides		Receptor Residue labeled [ref.]	Binding		cAMP response		Relative affinity / Relative potency
	Position	Substitution		K _i (nM)	Relative affinity	EC ₅₀ (nM)	Relative potency	
His	1	Bpa	Phe ³⁸⁸ [11]	71 ± 4.0 ^{**}	20.9	27 ± 7 ^{**}	540	0.04
Ser	2	Bpa	N.D.	580 ± 81 ^{**}	170.6	2.0 ± 0.1 ^{**}	40	4.3
Ser	2	N ₃ -Phe	Phe ¹⁹⁹ [13]	231 ± 71 [*]	67.9	2.9 ± 0.8 [*]	58	1.2
Asp	3	Bpa	N.D.	990 ± 85 ^{**}	291.2	24 ± 3.2 ^{**}	480	0.6
Gly	4	Bpa	N.D.	>1,000 ^{**}	>300	>1,000 ^{**}	>20,000	
Thr	5	Bpa	Phe ³⁴⁹ [15]	7.7 ± 1.0 [*]	2.3	0.13 ± 0.01 ^{**}	2.6	0.9
Phe	6	Bpa	Val ⁴ [7]	26.3 ± 3.7 ^{**}	7.4	0.7 ± 0.2 [*]	14	0.6
Thr	7	Bpa	N.D.	202 ± 60 [*]	59.4	9.9 ± 1.4 ^{**}	198	0.3
Ser	8	Bpa	N.D.	>1,000 ^{**}	>300	13 ± 1.9 ^{**}	260	>1.0
Glu	9	Bpa	N.D.	299 ± 70 [*]	87.9	1.0 ± 0.18 ^{**}	20	4.4
Leu	10	OH-Bpa	N.D. [29]	25.3 ± 6.0 [*]	7.4	4.2 ± 0.7 [*]	84	0.1
Leu	10	Bpa	N.D.	15.9 ± 3.2 [*]	4.7	0.08 ± 0.01	0.6	7.8
Ser	11	Bpa	N.D.	72 ± 5.0 ^{**}	21.2	2.0 ± 0.1 ^{**}	40	0.5
Arg	12	(BzBz)Lys	Val ⁶ [16]	20.0 ± 3.3 ^{**}	5.9	0.1 ± 0.02	2	2.9
Leu	13	Bpa	Val ¹⁰³ [18]	56.0 ± 8.0 ^{**}	16.5	0.2 ± 0.02 [*]	4	4.1
Gln	14	(BzBz)Lys	Pro ³⁸ [16]	31.0 ± 4.1 ^{**}	9.1	0.1 ± 0.03 [*]	2	4.6
Asp	15	Bpa	Glu ¹⁹ [13]	370 ± 71 ^{**}	108.8	10.5 ± 2.3 [*]	210	0.5
Asp	15	NO ₂ -Phe	N.D.	86.3 ± 20.1 [*]	25.4	3.8 ± 0.7 ^{**}	76	0.3
Ser	16	Bpa	Leu ⁹⁹ [14]	10.2 ± 1.2 ^{**}	3	0.05 ± 0.01	1	3
Ala	17	Bpa	N.D.	53.5 ± 5.5 ^{**}	15.7	0.31 ± 0.11	6.2	2.5
Arg	18	(BzBz)Lys	Arg ¹⁴ [17]	68 ± 8 ^{**}	20	0.4 ± 0.1 [*]	8	2.5
Leu	19	Bpa	N.D.	>1,000 ^{**}	>300	10.7 ± 0.4 ^{**}	214	>1.4
Gln	20	Bpa	His ² [13]	5.0 ± 0.7	1.5	0.05 ± 0.01	1	1.5

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Amino acid	Position	Peptides		Receptor Residue labeled [ref.]	Binding		cAMP response		Relative affinity / Relative potency
		Substitution			K _i (nM)	Relative affinity	EC ₅₀ (nM)	Relative potency	
Arg	21	Bpa		Arg ¹⁵ [12]	17.9 ± 1.2 ^{**}	5.3	0.37 ± 0.02 ^{**}	7.4	0.7
Leu	22	Bpa		Leu ¹⁷ [8, 30]	5.0 ± 1.1	1.5	0.07 ± 0.02	1.4	1.1
Leu	23	Bpa		Arg ²¹ [12]	47.1 ± 14.2 [*]	13.9	5.2 ± 1.0 ^{**}	104	0.1
Gln	24	Bpa		Pro ⁹⁷ [13]	2.2 ± 0.3	0.6	0.02 ± 0.01	0.4	1.6
Gly	25	Bpa		His ²³ [13]	7.1 ± 1.5	2.1	0.08 ± 0.02	1.6	1.3
Leu	26	Bpa		Leu ²⁶ [30]	10.3 ± 2.4 [*]	3.0	0.07 ± 0.01	1.4	2.2
Val	27	-		N.D.	-	-	-	-	-

Shown are the K_i values of binding of secretin or each of its analogues to CHO-SecR cells and EC₅₀ values of the intracellular cAMP responses stimulated by these ligands. All values represent the means ± S.E.M. of data from three independent experiments performed in duplicate. Values that are significantly different from that of natural secretin are indicated by

* (*P*<0.05)
 ** (*P*<0.01).

Shown also are relative affinity (K_i^{Phenolic}/K_i^{Sec}), relative potency (EC₅₀^{Phenolic}/EC₅₀^{Sec}) and ratio of both for each analogue compared to data for natural secretin (K_i = 3.4 ± 0.4 nM and EC₅₀ = 0.05 ± 0.01 nM). N.D., not determined.