

Design, Synthesis, and Study of a Novel RXPA380–Proline Hybrid (RXPA380-P) as an Antihypertensive Agent

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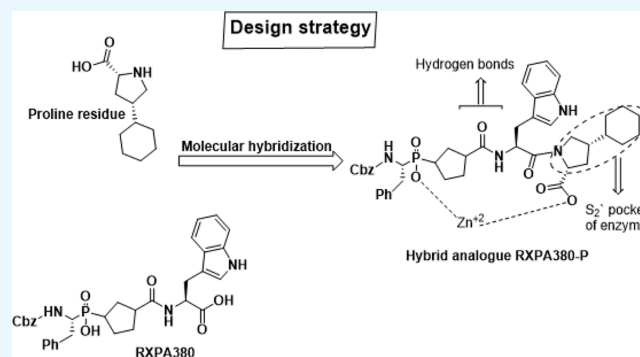
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ABSTRACT: In drug discovery, molecular modification over the lead molecule is often crucial for the development of a drug. Herein, we report the molecular hybridization design of a novel RXPA380–proline hybrid *via* linking the parent compound, phosphinic peptide RXPA380, with a proline analogue. The presented synthetic route is straightforward and produces the desired product RXPA380-P in moderate yield. The C- and N-domain constructs of the angiotensin-converting enzyme of RXPA380-P appeared to be poor inhibitors of ACE as compared to the parent compound RXPA380.



1. INTRODUCTION

Hypertension or high blood pressure has emerged as a major public health problem of the 21st century, affecting an estimated 26% of the world's adult population and is expected to increase to 29% by 2025.^{1–4} The upgrowth of hypertension as the most important risk factor in stroke and coronary heart disease has necessitated the development of new therapeutic agents that have centered upon the development of angiotensin-converting enzyme (ACE) inhibitors.^{5,6} Because of its activity profile, ACE is one of the major therapeutic targets for controlling hypertension and related cardiovascular diseases.^{7–10}

Recently, combination therapy, an effective medicinal tool used by clinicians to treat unresponsive patients¹¹ has attracted the attention of researchers globally toward molecular hybridization (multiple ligands) as a strategy for the rational design of new structures or prototypes.¹² Hybrid compounds based on linking pharmacophore subunits directly or with spacer agents, which through the adequate fusion of these subunits, lead to the design of new hybrid structures that maintain preselected characteristics of the original templates with improved therapeutic efficacy and bioavailability profile.¹³

One approach to protease inhibition that has proven to be very successful is the development of the phosphinic acid class of ACE inhibitors (Figure 1).^{14–19} The phosphinic peptide RXPA380 (Figure 3), bearing an unusual long side chain at the P1' position, was reported as the most potent and selective C-domain of ACE ($K_i = 10,000$ nmol/L for the N-domain; $K_i = 3$ nmol/L for the C-domain).^{20,21} On the other hand, an overview of the literature showed that most antihypertensive drugs contain a proline (or a proline derivative) residue within

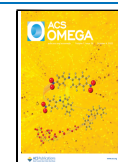
the basic framework (Figure 2).^{22–25} Another key feature of the proline ring is its ionophoric properties.^{26,27} The above arguments render the proline core a privileged structure, which is well worth studying.

This article describes a novel approach toward the synthesis of new phosphinic structures, a novel derivative combining the core of the phosphinic peptide RXPA380 linked covalently *via* an amide bond with a proline analogue at the C-terminus of the parent compound (Figure 3). This new derivative, RXPA380-P, might interact favorably with the ACE C-domain active site and prove to be more efficacious as an ACE inhibitor than RXPA380. Additionally, RXPA380-P meets the obligatory requirements that enable the inhibitor to fit into the constrained active site geometry of ACE:²¹ (i) the terminal –COOH group to satisfy the ionic interactions with the cationic site of ACE, (ii) the amide group to act as a H-bond acceptor, (iii) the ionizable function to coordinate with the Zn²⁺ ion (e.g., the –COOH group and –PO₂H₂ group), and (iv) the proline or proline-like ring at the C terminus fitting in the S₂ enzyme pocket offering optimal interactions for the amide bond hydrolysis.

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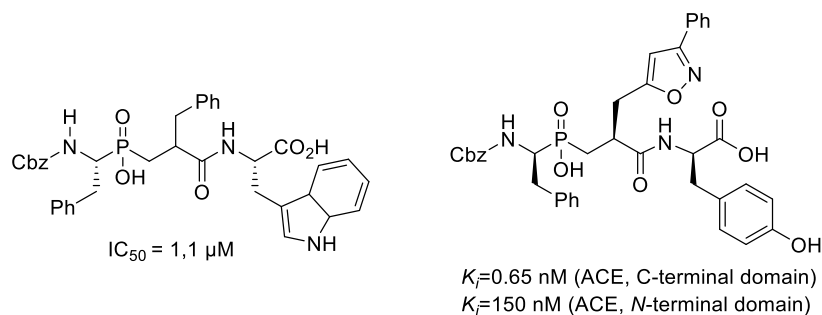


Figure 1. Representative examples of phosphinic peptide inhibitors of ACE.

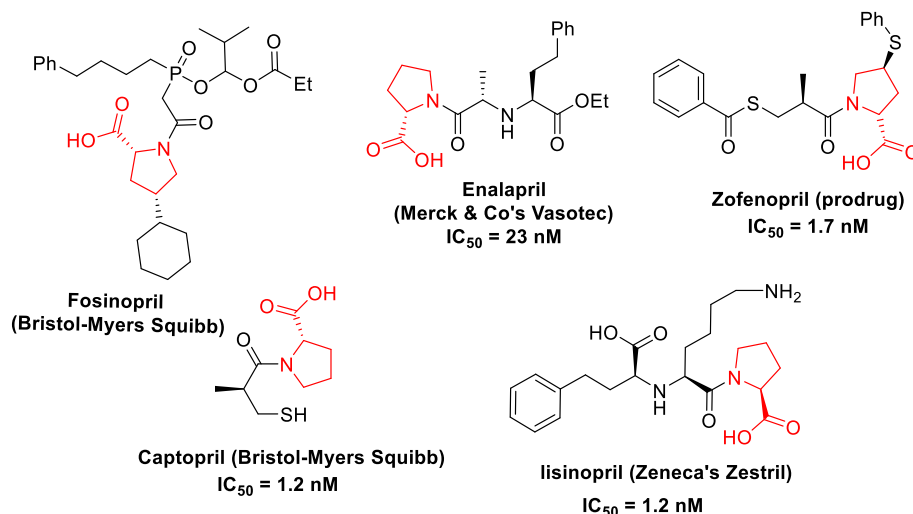


Figure 2. Examples of marketed antihypertensive drugs containing a proline residue.

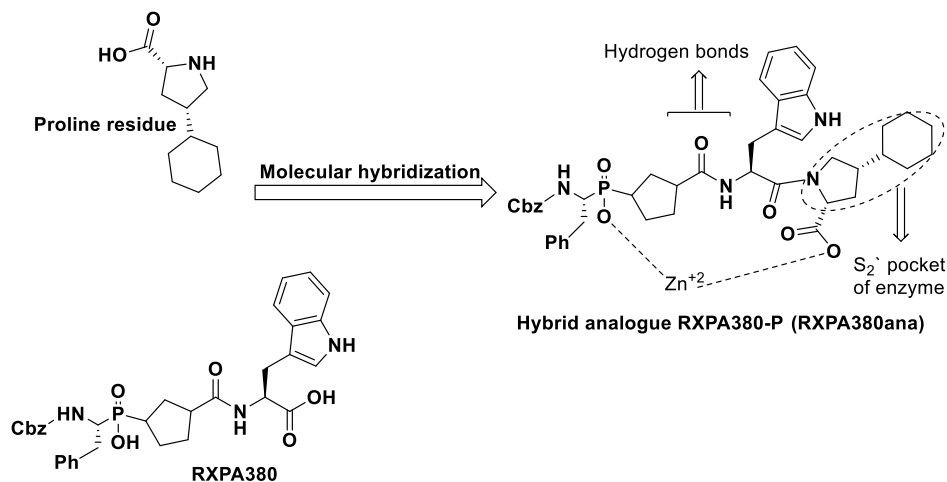


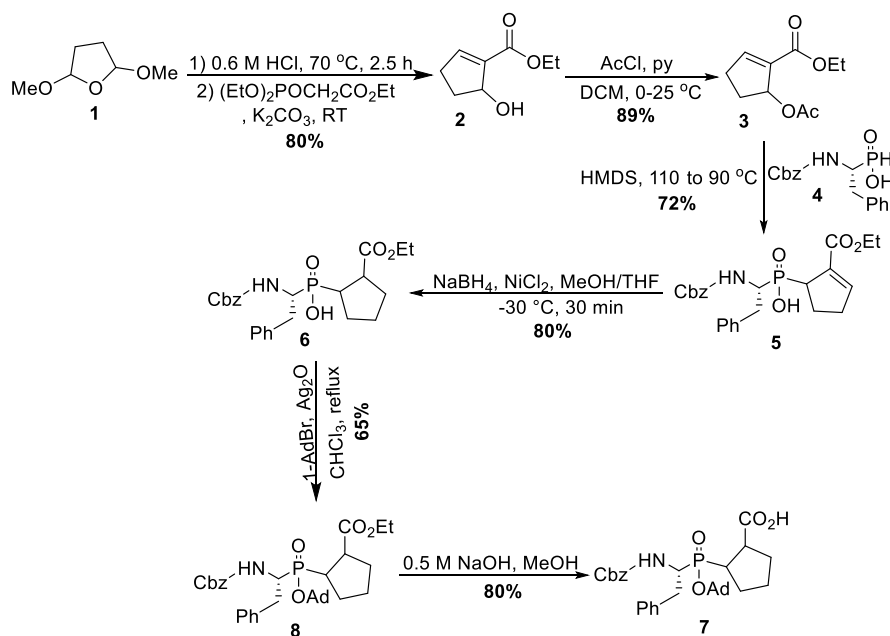
Figure 3. Design strategy of a new RXPA380–proline hybrid RXPA380-P (RXPA380ana).

2. RESULTS AND DISCUSSION

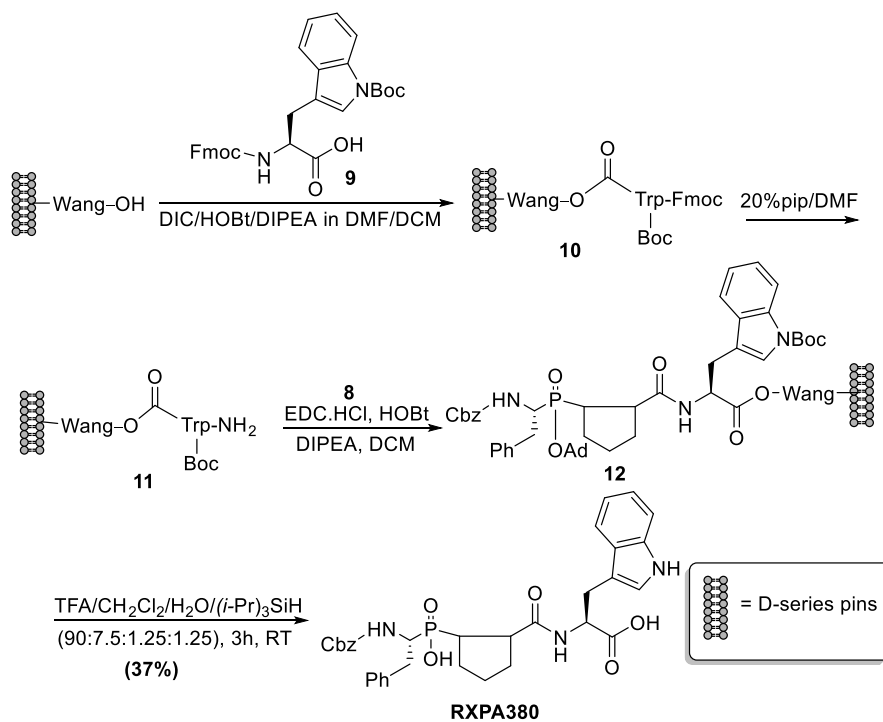
2.1. Chemistry. The key step in the strategy for the development of the new RXPA380–proline hybrid (RXPA380-P) is the construction of the tripeptidic unit RXPA380.²¹ The synthesis of starting material, ethyl 5-acetoxycyclopent-1-ene-1-carboxylate **3**, was accomplished in high yield through two steps beginning from a previously reported one-step reaction of 2,5-dimethoxytetrahydrofuran **1** and a phosphonate ester in an aqueous potassium carbonate solution to afford ethyl 5-hydroxycyclopent-1-enecarboxylate

2.^{28,29} Then, the hydroxyl group of **2** was esterified with acetyl chloride to give ethyl 5-acetoxycyclopent-1-ene-1-carboxylate **3**.^{28,29} The synthesis of phosphinic dipeptide **5** was performed via a Michael addition through the silylation of **4**, prepared according to the general procedure of Baylis et al.,³⁰ with HMDS followed by the addition of acrylate **3**. The reduction of alkene **5** was achieved by employing a sodium borohydride–nickel chloride–methanol catalytic system to access phosphinic acid **6**. Protection of the phosphinic group of **6** with an adamantyl group (Ad) in refluxing chloroform by portion-wise addition of silver(I) oxide afforded phosphinate **7**. Saponifica-

Scheme 1. Synthetic Pathway Used to Prepare Precursor 8 of the Phosphinic Inhibitor RXPA380



Scheme 2. Solid-Phase Synthesis of the Phosphinic Inhibitor RXPA380



tion of the ethyl ester **7** is completely selective under the reaction conditions applied and leads to the preparation of synthon **8** (Scheme 1).

A protocol for solid phase peptide synthesis (SPPS) elongation of the phosphinic block **8** has been established according to a methodology previously reported with minor modifications (Scheme 2).²¹ Phosphinic peptide **8**, which bears a free carboxylate group, is readily applicable for use in SPPS. The synthetic procedure commenced with the coupling of Fmoc-protected tryptophan **9** to pin lanterns bearing the Wang linker under standard conditions to afford the intermediate **10**. Piperidine-mediated removal of Fmoc

protection delivered **11**. Coupling of phosphinic peptide **8** with **11** gave **12**, which was then cleaved from the resin under acidic conditions, which also allowed the removal of the protecting groups to afford tripeptide block **RXPA380**.

The existing synthetic method does not seem suitable for large-scale synthesis, particularly considering the amount of expensive silver oxide required for the adamantylation step. Furthermore, this approach suffers a number of disadvantages such as the cost of the solid support and the limited number of “linker” groups on the surface of the beads.

To this effect, in order to overcome these drawbacks, we developed a new synthetic strategy using solution-phase

Scheme 3. Scaled-Up Synthesis of RXPA380-P

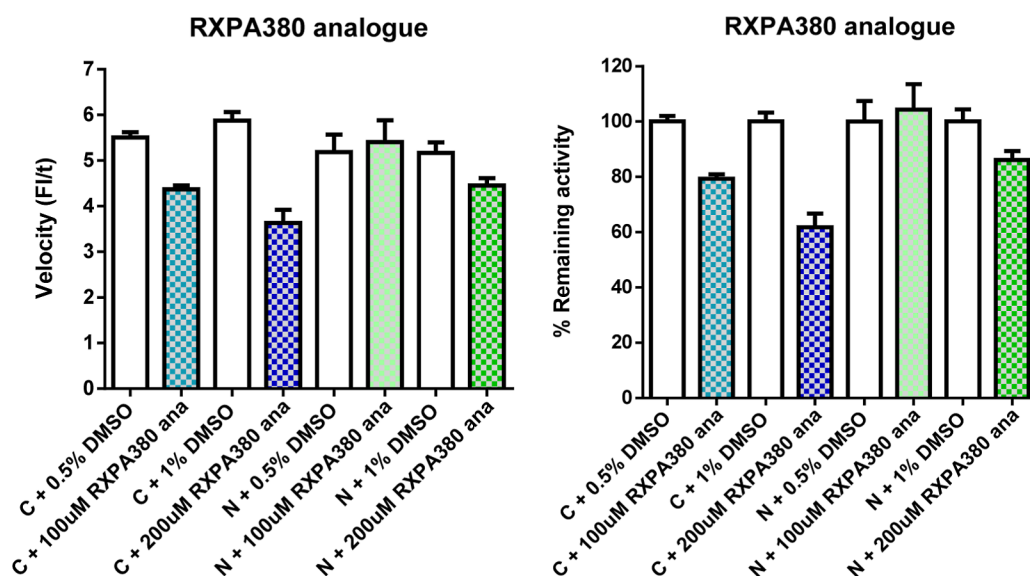
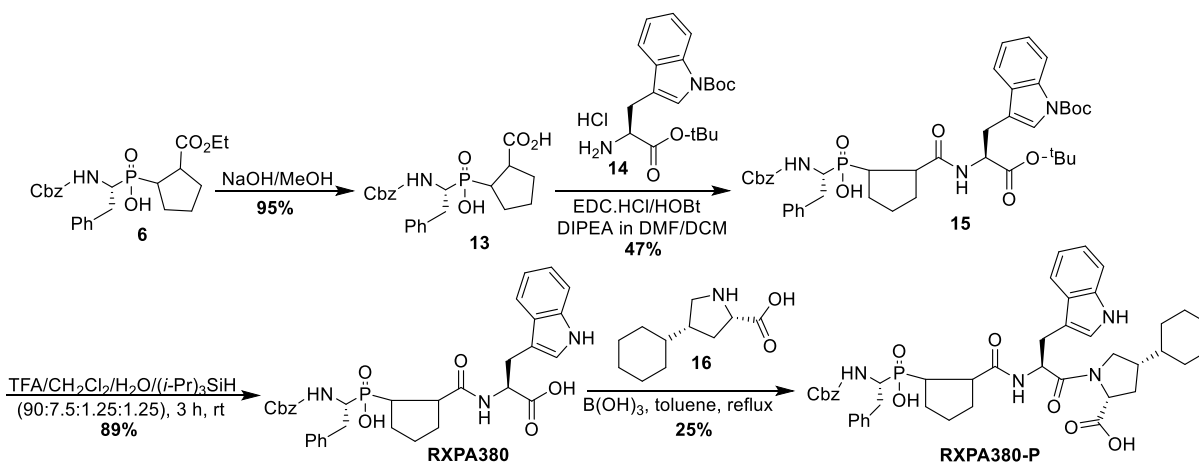


Figure 4. Inhibition screen of RXPA380-P (RXPA380ana) and its effect on N- and C-domain activity.

synthesis, avoiding the use of the Ad group, considering the fact that P–OH protection is not only unnecessary but also adds steps and complexity. This efficient and scalable synthetic approach of RXPA380-P is outlined in Scheme 3. This synthesis commenced with the saponification of the ethyl ester **6** with sodium hydroxide in methanol toward the preparation of carboxylic synthon **13**. This, in turn, was used in the coupling with H-Trp-OtBu hydrochloride **14** in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) and 1-hydroxybenzotriazole hydrate (HOBt) to afford **15**. The N-Boc and *tert*-butyl ester groups of the resulting dipeptide **15** were removed under acidic conditions to obtain the acid RXPA380. This analogue was further used to access the target compound RXPA380-P *via* boric acid-catalyzed amidation with *trans*-4-cyclohexyl-L-proline **16** in toluene under reflux. Owing to the difficulty in the separation of diastereoisomers of RXPA380-P, this compound was screened as a stereoisomeric mixture.

2.2. Biological Evaluation. **2.2.1. In Vitro Assay Results for RXPA380-P (RXPA380ana).** ACE activity in the presence or absence of the inhibitor was monitored as follows: A dilution of RXPA380-P at two concentrations was prepared in assay buffer (50 mM HEPES pH 6.8, 200 mM NaCl, and 10

μM ZnCl₂). The nonselective, quenched fluorogenic ACE substrate (Abz)-FRK-(Dnp)-P-OH was kindly donated by Dr. A.K. Carmona (Universidade Federal de São Paulo, Brazil). The peptide was dissolved in DMSO, and its concentration was determined by measuring the absorbance at 365 nm, and it had an extinction coefficient of 17,300 M⁻¹ cm⁻¹. Subsequent dilutions were made in assay buffer (50 mM HEPES, pH 6.8, 10 μM ZnCl₂, 200 mM NaCl).

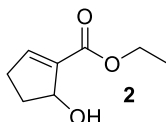
Truncated N-domain or C-domain ACE proteins were diluted to 80 nM in assay buffer. Equal volumes of enzyme and inhibitor or buffer containing DMSO (to serve as a negative control) were incubated for 15 min at RT in order to reach the inhibitor-binding equilibrium. Thereafter, 20 μL of aliquots of the enzyme–inhibitor/buffer reactions were added to triplicate wells of a 96-well plate on ice. The activity assays were commenced by addition of 280 μL of (Abz)-FRK-(Dnp)-P-OH to each well for a final substrate concentration of 4 μM. The hydrolysis of Abz-FRK(Dnp)P–OH was continuously monitored at RT with excitation and emission wavelengths of 320 and 420 nm, respectively, using a fluorescence spectrophotometer. Reaction velocities are represented as fluorescence intensity per unit time under initial reaction

conditions (first 3–5 min of collected data were used for analysis).

The presence of DMSO at these concentrations (given here as concentrations in the enzyme–inhibitor reaction prior to the addition of the substrate) did not appear to influence ACE activity. Inhibition of both the N- and C-domain was poor, with an estimated IC_{50} value for the C-domain of $250 \mu\text{M}$. The binding affinity is thus much lower than for the **RXPA380** parent molecule, which normally completely inhibits the C-domain at micromolar concentrations under identical assay conditions (Figure 4).

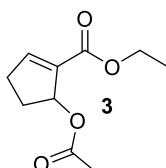
3. EXPERIMENTAL SECTION

3.1. Synthesis.



3.1.1. Ethyl 5-Hydroxycyclopent-1-enecarboxylate (**2**).^{31–33}

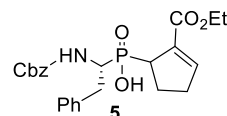
A solution of **1** (1.0 g, 8 mmol) in 0.6 M HCl (6.5 mL) was heated to $70 \text{ }^\circ\text{C}$ for 2.5 h under vigorous stirring. After cooling to $0 \text{ }^\circ\text{C}$, the mixture was neutralized with $\text{KHCO}_3(\text{aq})$ (10%, w/v, 4.5 mL), and triethyl phosphonoacetate (1.8 g, 8.1 mmol) and 6.4 M $\text{K}_2\text{CO}_3(\text{aq})$ (3.5 mL) were added. The reaction mixture was allowed to stir for 24 h at RT and then extracted with AcOEt. The combined organic extracts were washed with brine and then dried over MgSO_4 and evaporated *in vacuo*. The crude material was purified by column chromatography using (AcOEt/hexane, v/v, 3/10) as an eluent gave **2** (10 g, 80%) as a colorless oil with $R_f = 0.3$ (AcOEt/hexane, v/v, 3/10); IR (KBr) ν (cm^{-1}): 3427, 2983, 1715, 1290, 1088; ^1H NMR (400 MHz, CDCl_3): δ 1.33–1.28 (t, $J = 7.2$ Hz, 3H), 1.90–1.83 (m, 1H), 2.49–2.28 (m, 2H), 2.69–2.58 (m, 1H), 2.77 (s, 1H), 4.23 (q, $J = 7.2$ Hz, 2H), 5.08 (s, 1H), 6.91 (s, 1H); ^{13}C NMR (100 MHz, CDCl_3): δ 14.09, 30.82, 31.82, 60.47, 75.52, 138.21, 146.27, 165.15. HRMS (ESI/QTOF) m/z : $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_8\text{H}_{12}\text{O}_3\text{Na}$, 179.1708; found, 179.1705.



3.1.2. 2-Ethoxycarbonyl-1-acetyloxy-2-cyclopentene (**3**).^{34,35}

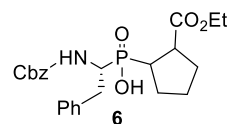
In a solution of **2** (1 g, 6.4 mmol) and pyridine (3.10 mL, 38.4 mmol) in DCM (3 mL), AcCl (2.7 mL, 38.4 mmol) was added dropwise at $0 \text{ }^\circ\text{C}$. The reaction mixture was allowed to stir overnight at RT. Then, the mixture was cooled at $0 \text{ }^\circ\text{C}$, and H_2O (5 mL) was slowly added. The resulting mixture was diluted with AcOEt (50 mL) and washed with 1 M HCl to pH ~ 3 , 5% $\text{NaHCO}_3(\text{aq})$ (30 mL), and brine (20 mL). The organic layer was dried with anhydrous Na_2SO_4 and evaporated *in vacuo*. Purification by column chromatography using DCM as an eluent afforded product **3** as a pale yellow liquid (1.13 g, 89%) with $R_f = 0.57$ (hexane/AcOEt, v/v, 3/1). IR (KBr) ν (cm^{-1}): 2882, 1723, 1638, 1295, 1240, 1102; ^1H NMR (400 MHz, CDCl_3): δ 1.26–1.22 (t, $J = 7.3$ Hz, 3H) 1.90–1.84 (m, 1H), 1.99 (s, 3H), 2.48–2.29 (m, 2H), 2.68–2.60 (m, 1H), 4.23–4.09 (m, 2H), 5.97 (d, $J = 7.5$ Hz, 1H), 7.06 (s, 1H); ^{13}C NMR (100 MHz, CDCl_3): δ 170.79, 163.73, 149.79, 135.34, 77.12, 60.28, 31.20, 21.11, 14.39. HRMS (ESI/

QTOF) m/z : $[\text{M}]^+$ calcd for $\text{C}_{10}\text{H}_{14}\text{O}_4$, 198.0892; found, 198.0889.



3.1.3. ((*R*)-1-(((Benzyloxy)carbonyl)amino)-2-phenylethyl(2-(ethoxy carbonyl)cyclopent-2-en-1-yl)phosphinic Acid (**5**).²¹

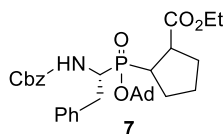
In a dry sealed flask under a flow of argon, a mixture of **4** (3.2 g, 10 mmol) and HMDS (10.5 mL, 50 mmol) was heated at $110 \text{ }^\circ\text{C}$ for 3 h. The reaction was accompanied by the liberation of NH_3 . Compound **3** (5.5 g, 12 mmol) was added dropwise to the opaque mixture within 35 min at this temperature, and this solution was then allowed to stir for 4 h at $90 \text{ }^\circ\text{C}$. This solution was cooled to $70 \text{ }^\circ\text{C}$, absolute EtOH (10 mL) was added dropwise thereto, and the mixture was allowed to stir at $70 \text{ }^\circ\text{C}$ for 30 min. After evaporating off the solvents, the residue was diluted with AcOEt (3×5 mL) and washed with H_2O (2×50 mL) and saturated brine (50 mL). The organic layer was dried with Na_2SO_4 , evaporated under reduced, and concentrated to afford a white solid. Purification on a column of silica using $\text{CHCl}_3/\text{MeOH}/\text{AcOH}$ (v/v, 7/0.3/0.3) as the mobile phase gave pure compound **5** in the form of a white solid (3.29 g, 72%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 1.27 (t, $J = 7.1$ Hz, 3H), 1.84–2.31 (m, 3H), 2.38–3.64 (m, 2H), 2.66–2.96 (m, 2H), 4.10–4.51 (m, 3H), 4.63–5.04 (m, 2H), 5.74–6.05 (m, 1H), 6.84–7.44 (m, 11H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 14.15, 21.21, 26.08/26.62, 31.12/31.28, 32.67/32.72, 34.35, 42.12/43.19, 50.48/52.26, 60.41/61.21, 66.66/67.03, 125.24, 126.53, 127.54, 127.68, 127.77, 127.85, 128.34, 129.05, 129.36, 135.14, 136.02, 136.58, 137.21/137.33, 148.10/148.92, 156.22, 163.67/165.52. ^{31}P NMR (162 MHz, $\text{DMSO}-d_6$): δ 49.23, 50.64. Data represents two diastereoisomers in the ratio 1:2.4. HRMS (ESI/QTOF) m/z : $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{28}\text{NO}_6\text{PH}$, 458.1732; found, 458.1696.



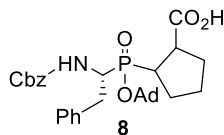
3.1.4. ((*R*)-1-(((Benzyloxy)carbonyl)amino)-2-phenylethyl(2-(ethoxy carbonyl)cyclopentyl)phosphinic Acid (**6**).²¹

To a magnetically stirred solution of compound **5** (1.4 g, 3.06 mmol) in a mixture of 20 mL of THF/MeOH (5/3, v/v), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (1.09 g, 9.2 mmol) was added gradually at RT. When the clear solution acquired a greenish color, the whole reaction mixture was brought to $-30 \text{ }^\circ\text{C}$, and NaBH_4 (0.58 g, 15.4 mmol) was added portionwise over 30 min. This mixture was allowed to stir for a further 10 min at $-30 \text{ }^\circ\text{C}$. The reaction mixture was filtered through Celite and was washed with AcOEt (25 mL). The filtrate was concentrated under reduced pressure, and the residue was purified by flash column chromatography ($\text{CHCl}_3/\text{MeOH}/\text{AcOH}$, v/v, 7/0.3/0.3) to provide the title compound **6** (1.12 g, 80%) as a colorless viscous gum. ^1H NMR (400 MHz, CDCl_3): δ 1.16–1.34 (m, 3H), 1.54–2.07 (m, 5H), 2.61–2.90 (m, 2H), 3.07–3.50 (m, 2H), 4.01–4.24 (m, 3H), 4.83–5.06 (m, 2H), 6.97–7.40 (m, 11H). ^{13}C NMR (100 MHz, CDCl_3): δ 14.13, 26.80, 27.04, 31.88, 32.11, 32.80, 34.31, 38.19, 43.96, 44.11, 44.62, 50.10, 50.55, 58.39, 60.43, 61.06 66.87, 67.27, 126.74, 127.70, 127.84, 127.97, 128.43, 129.22, 129.27, 129.32, 136.41, 155.99, 171.22; ^{31}P NMR (162 MHz, CDCl_3): δ 56.02, 55.29, 51.18,

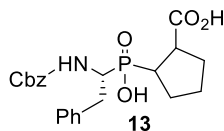
49.89. HRMS (ESI/QTOF) m/z : $[M + H]^+$ calcd for $C_{24}H_{30}NO_6PH$, 460.1889; found, 460.1862.



3.1.5. Ethyl-2-(((3R)-adamantan-1-yl)oxy)((R)-1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)phosphoryl)cyclopentane-1-carboxylate (7).²¹ Ag_2O (560 mg, 2.4 mmol) was added portionwise over 1 h to a refluxing solution of phosphinic acid **6** (920 mg, 2 mmol) and 1-adamantyl bromide (650 mg, 3 mmol) in $CHCl_3$ (15 mL). After the solution was allowed to reflux for 24 h, the reaction mixture was concentrated under reduced pressure, and the residue was treated with Et_2O (10 mL). The resulting mixture was filtered through Celite, the filtrates were evaporated, and the residue was purified by flash column chromatography using (DCM/*i*-PrOH, v/v, 9.8/0.2) to provide the title compound **7** (0.77 g, 65%) as a colorless gum. ^{31}P NMR (162 MHz, DMSO- d_6): δ 49.00, 49.35, 49.42, 49.72, 51.32, 51.45, 52.94, 53.54. HRMS (ESI/QTOF) m/z : $[M]^+$ calcd for $C_{34}H_{44}NO_6P$, 593.2906; found, 593.2921.

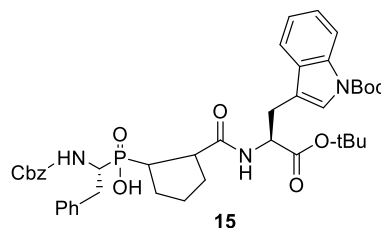


3.1.6. 2-(((1R)-1-(((Benzyloxy)carbonyl)amino)-2-phenylethyl)(adamantyl oxy)phosphinyl)cyclopentane-1-carboxylic acid (8).²¹ Phosphinate **7** (475 mg, 0.8 mmol) was dissolved in 2 mL MeOH and cooled to 0 °C. 4 M NaOH(aq) (0.7 mL) was added dropwise, and the reaction was allowed to warm to RT over 8 h. Then, the solvent was removed, and the residue was diluted with water and acidified with 3 M HCl in an ice-water bath. This aqueous solution was extracted with AcOEt, and the combined organic layers were dried over Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography using (DCM/MeOH, v/v, 15/1) as an eluent, affording **8** as a white solid (450 mg, 80%). ^{31}P NMR (162 MHz, DMSO- d_6): δ 51.07, 51.54, 51.77, 52.28, 53.02, 53.33. HRMS (ESI/QTOF) m/z : $[M]^+$ calcd for $C_{32}H_{40}NO_6P$, 565.2593; found, 565.2570.

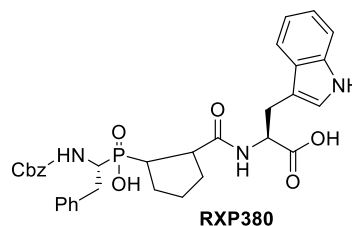


3.1.7. 2-(((R)-1-(((Benzyloxy)carbonyl)amino)-2-phenylethyl)(hydroxy)phosphoryl)cyclopentane-1-carboxylic acid (13). Pseudodipeptide **6** (368 mg, 0.8 mmol) was dissolved in EtOH (2 mL), and the solution was cooled to 0 °C. 4 M NaOH(aq) (0.7 mL) was added to the reaction mixture dropwise. The resulting opaque solution was allowed to stir for 5 h. Then, the solvent was removed *in vacuo*, and the residue was diluted with H_2O and acidified with 3 M HCl in an ice-water bath to adjust the pH to 2. This aqueous phase was extracted twice with AcOEt, and the combined organic layers were dried over anhydrous Na_2SO_4 and concentrated to dryness to afford a white solid. The residue was purified by silica gel chromatography using ($CHCl_3$ /MeOH/AcOH, v/v, 9.6/0.3/0.1) as an eluent. After purification, **13** was afforded as a white solid (330 g, 95%); 1H NMR (400 MHz, D_2O /

Na_2CO_3): δ 1.20–1.94 (m, 5H), 2.41 (s, 2H), 2.67–3.15 (m, 2H), 3.74 (d, 1H), 4.18–4.56 (m, 2H), 6.56–7.30 (m, 10H). ^{13}C NMR (100 MHz, D_2O/Na_2CO_3): δ 26.45, 27.49, 31.59, 33.00, 33.42, 34.42, 39.83, 47.59, 48.22, 52.17, 53.24, 66.64, 126.15, 127.07, 128.63, 129.28, 136.93, 138.50, 157.67, 184.75; ^{31}P NMR (162 MHz, D_2O/Na_2CO_3): δ 43.02, 42.64, 42.29, 41.25, 40.66. HRMS (ESI/QTOF) m/z : $[M + K]^+$ calcd for $C_{22}H_{26}NO_6PK$, 470.1135; found, 470.1118.



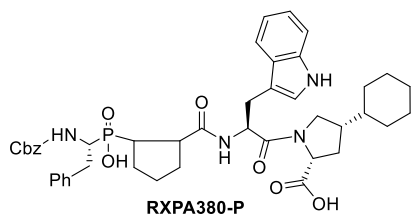
3.1.8. ((R)-1-(((Benzyloxy)carbonyl)amino)-2-phenylethyl)-2-(((S)-1-(tert-butoxy)-3-(1-(tert-butoxycarbonyl)-1H-indol-3-yl)-1-oxopropan-2-yl)carbamoyl)cyclopentyl)phosphinic Acid (15). Phosphinic pseudodipeptide **13** (430 mg, 1 mmol) was suspended in DCM (2 mL) and DIPEA (330 μ L, 1.94 mmol), and HCl-Trp(Boc)Ot-Bu **14** (400 mg, 1 mmol), HOBt (130 mg, 0.98 mmol), and EDC-HCl (770 mg, 4 mmol) were added. The mixture was allowed to stir for 1 h at RT and then diluted with DCM and washed with a solution of 1 M HCl. The organic layer was separated and washed with a sat. aq. NH_4HCO_3 (3 \times 1 mL), 1 M HCl to pH = 1, and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*, and the residue was purified by column chromatography using (DCM/MeOH/AcOH, v/v, 9/0.7/0.3) as an eluent, affording the target product **15** (380 mg, 47%) as a white solid. 1H NMR (400 MHz, D_2O/Na_2CO_3): δ 1.32 (s, 9H), 1.55–1.64 (m, 5H), 1.97 (s, 9H), 2.74 (s, 2H), 3.14–3.41 (m, 4H), 3.62 (d, 1H), 4.09–4.32 (m, 1H), 4.65–5.02 (m, 2H), 7.07–7.43 (m, 13H), 7.55–7.82 (m, 2H), 8.12 (d, 1H). ^{31}P NMR (162 MHz, D_2O/Na_2CO_3): δ 43.02, 42.64, 42.29, 41.25, 40.66. HRMS (ESI/QTOF) m/z : $[M + Na]^+$ calcd for $C_{42}H_{52}N_3O_9PNa$, 796.3339; found, 796.3299.



3.1.9. 2-(((R)-1-(((Benzyloxy)carbonyl)amino)-2-phenylethyl)(hydroxy)phosphoryl)cyclopentane-1-carboxylic acid-L-tryptophan (RXP380). **3.1.9.1. Method One.** A solution of **15** (232 mg, 0.30 mmol) in a mixture (3 mL) of (TFA/DCM/ H_2O /TIS, v/v, 90/7.5/1.25/1.25) was allowed to stir at RT for 3 h. After removal of the volatiles *in vacuo*, the residue was dried azeotropically using toluene, and then it was treated with dry Et_2O at 0 °C. The precipitate was collected by centrifugation, washed twice with dry Et_2O , and dried over P_2O_5 to afford **RXP380** (157 mg, 89%) as a light yellow solid.

3.1.9.2. Method Two: Procedure for RXP380 Synthesis on Wang Resin. The solid phase synthesis was carried out on a Wang-type linker (SynPhase Lanterns, D-series, 35 mmol/unit, Mimotopes, Inc., Australia). Wang resin was first swelled in DMF (0.5 mL/pin) for 30 min at RT then the solvent was drained off.

- **Fmoc-tryptophan coupling:** Fmoc-tryptophan **9** (3 equiv) was added to pins in DCM/DMF (6:1 *v/v*, 0.25 mL/pin) containing HOBt (5 equiv), and DIC (5 equiv) and DMAP (0.3 equiv) were added sequentially to the pins solution. The reaction mixture was shaken for 6 h. The resin was filtrated off and washed with DCM (2 × 5 min, 1 mL/pin), DMF (2 × 5 min, 1 mL/pin), and DCM (2 × 5 min, 1 mL/pin) and briefly air-dried.
- **Capping:** Ac₂O (5 equiv) and pyridine (1 equiv) in DMF were added to the resin and stirred for 30 min and then washed with DMF (2 × 5 min, 1 mL/pin).
- **Fmoc deprotection:** Resin **10** was treated with a solution of 20% piperidine/DMF solution (2:1 *v/v*, 1 mL/pin, 2 × 30 min, RT). The solvent was decanted, and the pins were washed with DMF (2 × 5 min, 1 mL/pin) and DCM (2 × 5 min, 1 mL/pin) and dried *in vacuo*.
- **Phosphinic block coupling:** Coupling of phosphinate **8** (1.5 equiv) was added to pins **11** in anhydrous DMF (0.25 mL/pin) containing DIPEA (1.5 equiv), HOBt (1.5 equiv), and EDC-HCl (4 equiv). The reaction mixture was stirred for 4 h. The pins were washed with DMF (4 × 5 min, 1 mL/pin).
- **Cleavage of the peptide from the resin: RXPA380** was obtained after treatment of resin **12** with a mixture of TFA/H₂O/TIS (95:2.5:2.5 *v/v*, 0.5 mL/pin) for 1 h at RT. After acidic cleavage and removal of the resin from the reaction mixture, the volatiles were concentrated *in vacuo*. The oily residue was decanted with cold dry Et₂O to afford **RXPA380** (37%) as a white solid. ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.42–1.68 (m, 2H), 1.73–1.86 (m, 4H), 2.46–2.62 (m, 1H), 2.71–2.93 (m, 1H), 2.99–3.24 (m, 4H), 3.92–4.14 (m, 1H), 4.38–4.50 (m, 1H), 4.91 (dd, 2H, *J* = 12.9, 21.7 Hz), 6.89–7.36 (m, 15H), 7.60 (s, 1H), 7.69 (d, 1H, *J* = 13.7 Hz), 8.41 (d, 1H, *J* = 9.7 Hz). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 25.93, 27.25, 29.49, 31.60, 34.06, 43.78, 44.54, 44.84, 51.30, 53.13, 60.86, 66.34, 106.55, 109.44, 111.03, 115.65, 118.49, 119.17, 121.13, 122.97, 124.03, 126.63, 127.84, 129.03, 128.7, 129.3, 129.4, 136.38, 136.74, 136.96, 137.86, 157.34, 157.76, 170.25, 174.70, 175.67; ³¹P NMR (162 MHz, DMSO-*d*₆): δ 47.46, 50.14. HRMS (ESI/QTOF) *m/z*: [M + H]⁺ calcd for C₃₃H₃₆N₃O₇PH, 618.2369; found, 618.2351.



3.1.10. RXPA380-P. The reaction vessel was charged with **RXPA380** (185 mg, 0.30 mmol), H₃BO₃ (18 mg, 0.30 mmol), and 2 mL of toluene. To the stirred reaction mixture was added *trans*-4-cyclohexyl-L-proline **16** (69 mg, 0.35 mmol) in one portion. The reaction mixture was heated at reflux for 18 h. The reaction mixture was concentrated, and the residue was purified by flash column chromatography to afford **RXPA380-P**, which had four diastereoisomers (by RP-HPLC). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 25.97, 26.03, 26.23, 28.48, 29.05, 29.69, 31.52, 31.89, 33.29, 35.83, 37.02, 43.21, 44.48, 44.88, 45.54, 49.20, 51.41, 52.85, 60.78, 65.78, 109.08, 111.19,

113.46, 115.57, 117.06, 118.82, 121.43, 121.99, 123.33, 126.01, 127.83, 129.05, 131.53, 136.94, 137.16, 137.43, 137.72, 153.03, 169.66, 171.92, 173.26; ³¹P NMR (162 MHz, DMSO-*d*₆): δ 41.17, 43.07, 44.17. HRMS (ESI/QTOF) *m/z*: [M + H]⁺ calcd for C₄₄H₅₃N₄O₈PH, 797.8953; found, 797.8618.

3.2. Biological Evaluation. The ACE inhibitory activity of **RXPA380-P** was determined by the fluorimetric method at two concentrations (200 and 100 μM).

3.2.1. Materials. All reagents were purchased from Merck Ltd. Structural analogues of **RXPA380-P** (**RXPA380ana**) were dissolved in DMSO and water, respectively, to yield a 50 mM stock solution. Aliquots of stock solutions were diluted to 10 mM working stocks with H₂O, followed by dilution in phosphate incubation buffer.

3.2.2. Data Analysis. Graphs of the percentage remaining activity of each individual ACE domain in the presence of the potential inhibitor were plotted and analyzed using GraphPad Prism 4.01 (GraphPad Software, La Jolla, CA) and/or Microsoft Excel (Figure 4).

4. CONCLUSIONS

A novel phosphinic derivative of **RXPA380**, the most potent and selective C domain of ACE incorporating a modified proline analogue designed *via* molecular hybridization, was synthesized with a very good yield through solution-phase synthesis. The sequence of reactions employed to access the target compound **RXPA380-P** is Michael addition of **4** to acrylate **3**, reduction of resulting phosphinic acid followed by saponification of ethyl ester, coupling reaction with H-Trp-OtBu hydrochloride followed by the acid hydrolysis of *tert*-butyl ester and N-Boc, and amidation with *trans*-4-cyclohexyl-L-proline. The current method is more convenient and operationally simple than the solid-phase protocol to synthesize **RXPA380** without the requirement of hydroxyphosphinyl protection, which further optimizes the previous work on this compound. This method has also been demonstrated to be a suitable method for the elongation of the phosphinic pseudopeptide at the C-terminus to access the title compound **RXPA380-P**. The title compound **RXPA380-P** has been employed successfully in the biological evaluation of the C- and N-domain constructs of ACE and appeared to be a poor inhibitor of ACE as compared to the parent compound **RXPA380**. Future work will focus on the resolution of the four diastereomeric forms of **RXPA380-P** and the studies of their pharmacokinetic properties toward ACE..

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Notes

The authors declare no competing financial interest.

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