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Phosphinic Tripeptides as Dual Angiotensin-Converting Enzyme C-Domain and Endothelin-Converting Enzyme-1 Inhibitors

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A new series of phosphinic inhibitors able to interact with both angiotensin-converting enzyme (ACE) C-domain and endothelin-converting enzyme-1 (ECE-1), while sparing neprilysin (NEP), has been developed. The most potent and selective inhibitor in this series (compound $\mathbf{8_{F2}}$) displays K_i values of 0.65 nM, 150 nM, 14 nM and 6.7 μM toward somatic ACE C-domain, ACE N-domain, ECE-1, and NEP, respectively. Remarkably, in this series, the inhibitor's ability to discriminate between ECE-1 and NEP was observed to depend on the stereochemistry of the residue present in the inhibitor's P_1' position. After iv administration, compound $\mathbf{8_{F2}}$ (10 mg/kg) lowered mean arterial blood pressure by 24 ± 2 mmHg in spontaneously hypertensive rats, as compared with controls. Mixed ACE/ECE-1 inhibitor may lead to a new generation of vasopeptide inhibitors that should reduce the levels of angiotensin-II and endothelin-1, without interfering with bradykinin cleavage.

Introduction

The major involvement of membrane-bound zinc metallopeptidases like angiotensin-converting enzyme (ACE,[a] EC 3.4.15.1), neutral endopeptidase (neprilysin, NEP, EC 3.4.24.11), and endothelin-converting enzyme-1 (ECE-1, EC 3.4.24.71) in the control of peptide hormones exerting vasoconstrictive (angiotensin-II (Ang-II), endothelin-1 (ET-1)) and vasodilatory (natriuretic peptides, bradykinin (BK)) activities has made these enzymes the key targets for developing antihypertensive drugs.[1−4] Despite the success of ACE inhibitor therapy in treatment of hypertensive patients, blood pressure control remains suboptimal in a significant proportion of patients on this therapy or on other antihypertensive drug treatment.[5] This has justified the development of new strategies for improving treatment of patients with high blood pressure, a major risk factor for cardiovascular complications. To address this concern, dual inhibitors able to target potently ACE and NEP were first developed,[6] followed by dual NEP/ECE-1 inhibitors and ultimately triple inhibitors blocking ACE-NEP-ECE-1 simultaneously.[7] The dual ACE-NEP inhibitor omapatrilat (Chart 1) was reported in a clinical trial to be more effective than the use of a single ACE inhibitor. However, the higher incidence of angioedema observed in patients treated with omapatrilat versus patients treated with ACE inhibitors has halted the development of omapatrilat and raised concerns about the risk/benefit ratio of dual ACE-NEP inhibitors for therapeutic applications.[8] While a risk of angioedema associated with triple ACE-NEP-ECE-1 inhibitor treatment remains to be evaluated carefully, it has been suggested that NEP inhibition in a context of either dual or triple inhibitor treatment might be responsible for the occurrence of unwanted side effects.[7] Inhibiting NEP results in increased concentrations of BK and ET-1, two peptides associated with life-threatening side effects. The above concerns led us to consider the development of dual ACE/ECE-1 inhibitors, able to spare NEP. Targeting both ACE and ECE-1 should lower plasma concentrations of the two most potent vasoconstrictive peptides, Ang-II and ET-1. However, successful development of dual ACE/ECE-1 inhibitors relies on the possibility of identifying compounds able to differentiate ECE-1 from NEP while maintaining good potency toward ACE. Comparison between the crystallographic structures of NEP and ECE-1 reveals only slight differences in the topology of their active sites.[9,10] The most significant difference between these two peptidases, which could be exploited in the inhibitor design, is the replacement of Met^{579} in NEP by Val^{603} in ECE-1, which leads in the in ECE-1 crystal structure to a larger S_1' pocket, as compared with NEP. This consideration, together with the presence in ACE of an S_1' subsite forming a very large cavity,[11] led us to envisage the development of phosphinic derivatives containing bulky side chains in their P_1' position as

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[a] Abbreviations: ACE, angiotensin-converting enzyme; ECE-1, endothelin-converting enzyme 1; NEP, neprilysin; MMPs, matrix metalloproteinases; MCA, (7-methoxycoumarin-4-yl)acetyl; Dnp, 2,4-dinitrophenyl; DpaOH, N^3-(2,4-dinitrophenyl)-L-diaminopropionyl; Ac_2O, acetic anhydride; AcOH, acetic acid; AcOEt, ethyl acetate; Boc, *tert*-butoxycarbonyl; DCR, dipolar cycloaddition; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; HOBt, *N*-hydroxy benzotriazole; DIPEA, *N*,*N*-diisopropylethylamine; TIS, triisopropylsilane; Bu^t, *tert*-butyl; Cbz, benzyloxycarbonyl; DCM, dichloromethane; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; $DPM \cdot H_3PO_2$, aminodiphenylmethane hypophosphite; Et_2O, diethyl ether; EtOH, ethanol; HMDS, 1,1,1,3,3,3-hexamethyldisilazane; MeOH, methanol; NCS, *N*-chlorosuccinimide; Np, *para*-nitrophenyl; PE 40−60 °C, petroleum ether 40−60 °C; TMSCl, chlorotrimethylsilane; TFA, trifluoroacetic acid.

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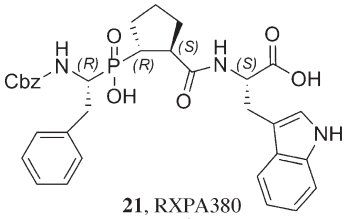
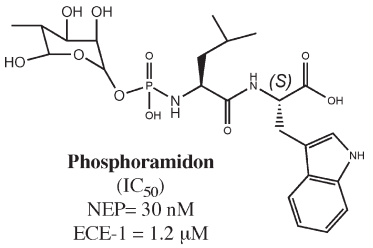
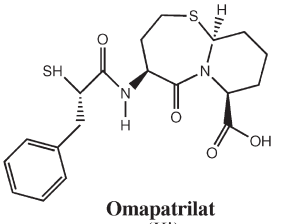
Chart 1. Chemical Structure of ACE, NEP, and ECE-1 Inhibitors



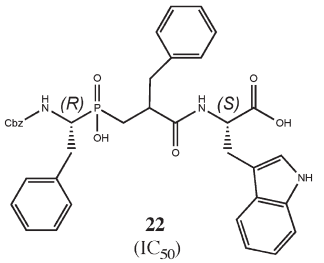
21, RXPA380
(Ki)
ACE-C = 3 nM
ACE-N= 2500 nM
NEP > 50 μM
ECE-1> 50 μM



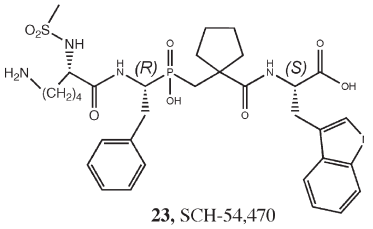
Phosphoramidon
(IC_{50})
NEP= 30 nM
ECE-1 = 1.2 μM



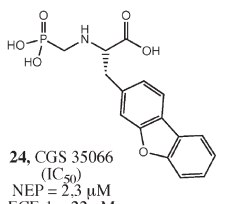
Omapatrilat
(Ki)
ACE = 6 nM
NEP = 9 nM



22
(IC_{50})
NEP= 1.4 μM
ECE-1 = 1.2 μM



23, SCH-54,470
(IC_{50})
ACE = 2.5 nM
NEP = 90 nM
ECE-1 = 80 nM



24, CGS 35066
(IC_{50})
NEP = 2.3 μM
ECE-1 = 22 nM

potential dual ACE/ECE-1 inhibitors. Among the phosphinic peptides we have developed in our laboratory as potent ACE inhibitors, compound **21** (RXPA380 in Chart 1) possesses both interesting structural features and selectivity profile.[12,13] Like phosphoramidon (Chart 1), one of the first phosphorus-containing peptide inhibitor of ECE-1, **21** also contains in its P_2' position a free Trp residue. Moreover, on the functional side, **21** is a potent and selective inhibitor of the ACE C-domain, the ACE active site responsible for the conversion of Ang-I into Ang-II and thus for the blood pressure control.[14] Somatic ACE contains two functional zinc-active sites, termed the N and C-domain, which displayed different physiological selectivity profiles and which can be differentiated by specific N- and C-domain selective inhibitors.[12,15] Comparison of the crystal structures of the N- and C-domain in interaction with their selective inhibitors has shown that only few mutations in the N- and C-domain active site were responsible for their different profiles of selectivity toward substrates and inhibitors.[13,16]

The above considerations have led us to start from the structure of **21** and see whether its modification at the P_1' position by bulky side chains may provide new inhibitors possessing the desired dual ACE C-domain and ECE-1 selectivity.

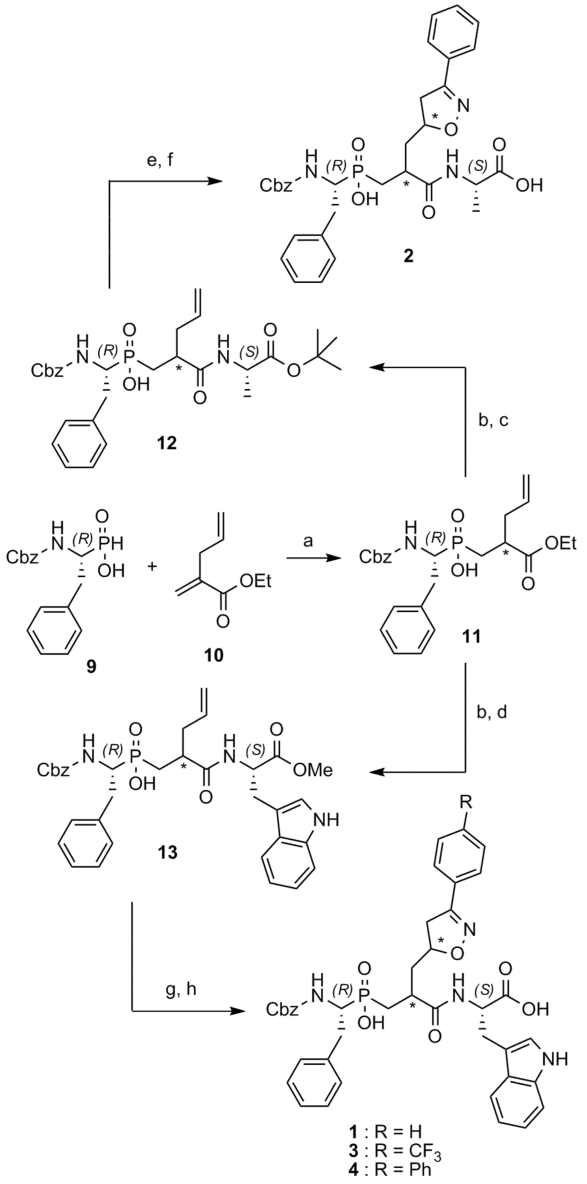
Results

Chemistry. A series of phosphinic tripeptides were synthesized, bearing a Cbz-protected pseudophenylalanine in the P_1 position, natural amino acid residues in the P_2' position, and isoxazoline- (**1**−**4**), biphenyl- (**6**), or isoxazole- (**5**, **7**, and **8**) containing side chains in the P_1' position.

The synthesis of isoxazoline-containing tripeptides **1**−**4** started from the phospha-Michael addition of the bis-(trimethylsilyl) phosphonite derived from the Cbz-protected aminophosphinic analogue of L-phenylalanine **9** to ethyl α-allyl acrylate **10** (Scheme 1).[17,18] From this reaction, phosphinic pseudodipeptide **11** was obtained as a mixture of two diastereoisomers in a 3:1 ratio. The isomer in excess possesses the *S*-stereochemistry in the P_1' position, as has been discussed in a previous report by our group.[18] Building block **11** was extended to tripeptides **12** and **13** after alkaline removal of the ethylester and carbodiimide-mediated coupling of H-Ala-OBu^t (for **12**) or H-Trp-OMe (for **13**) with the resulting diacid.

At the next synthetic step, compounds **12** and **13** served as ideal dipolarophilic substrates for the formation of isoxazoline rings in their P_1' position via a 1,3-dipolar cycloaddition (DCR) between the lateral terminal alkene and suitable nitrile oxides (Scheme 1).[19] These nitrile oxides are formed in situ by the corresponding aryl aldoximes after oxidative chlorination followed by base-induced dehydrochlorination of the intermediate hydroximinoyl chlorides. Correct choice of the chlorination conditions is essential in order to avoid possible oxidative decomposition of the substrate.[18] In particular, common bleach (aqueous NaOCl solution)[20] is compatible with tripeptide **12** but not with tripeptide **13** because the indole ring is prone to oxidation by this reagent. Thus, compound **2** was produced (as a mixture of 4 diastereoisomers) from the alkaline deprotection of the intermediate cycloaddition product between **12** and benzonitrile oxide. In the case of **13**, the terminal methyl ester was cleaved and the one-pot Huisgen protocol was applied to the product involving *N*-chlorosuccinimide as an oxidant.[21] Under these

Scheme 1. Synthesis of Isoxazoline Derivatives **1**−**4**[a]



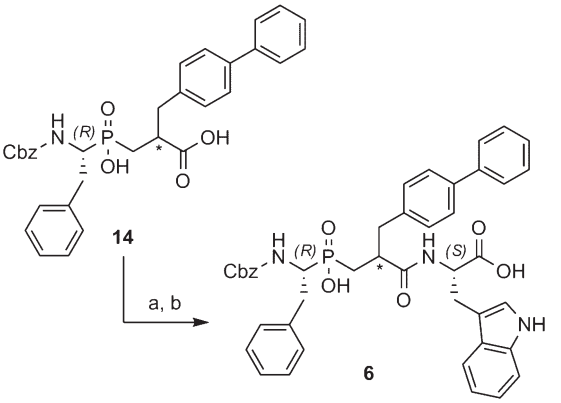
[a] Reagents and conditions: (a) HMDS, 110 °C, 1 h, then **10**, 100 °C, 3 h, then EtOH, 70 °C, 20 min, 84%; (b) NaOH, EtOH, H_2O, rt, 12 h; (c) HCl·H-Ala-O^{t}Bu, EDC·HCl, HOBt, DIPEA, CH_2Cl_2, rt, 1.5 h, 93%, two steps; (d) HCl·H-Trp-OMe, EDC·HCl, HOBt, DIPEA, CH_2Cl_2, rt, 1.5 h, 80%, two steps; (e) PhCH=NOH, common bleach, Et_3N, CH_2Cl_2, rt, 16 h; (f) NaOH, EtOH, H_2O, rt, 24 h, 88%, two steps; (g) NaOH, MeOH, H_2O, rt, 24 h; (h) *p*-R-PhCH=NOH, NCS, pyridine (cat.), $CHCl_3$, 45 °C, 3−4 h, then phosphinic substrate, Et_3N, 45 °C, 3 d, 92% (for **1**), 93% (for **3**), 90% (for **4**), two steps.

conditions, the indole ring remained intact and target compounds **1**, **3**, and **4** were obtained as mixtures of four diastereoisomers in high yields.

For the synthesis of biphenyl phosphinic tripeptide **6**, compound **14** was prepared according to a published procedure[22] and was subjected to carbodiimide-mediated coupling with H-Trp-OMe and subsequent alkaline deprotection. Compound **6** was obtained as an 1:1 mixture of (*R*,*R*,*S*) and (*R*,*S*,*S*) diastereoisomers (Scheme 2).

The synthesis of isoxazole-containing tripeptides **5**, **7**, and **8** started from the previously described propargylic diacid **15** (1:1 diastereoisomeric mixture).[18] For the synthesis of

Scheme 2. Synthesis of Biphenyl Derivative **6**[a]



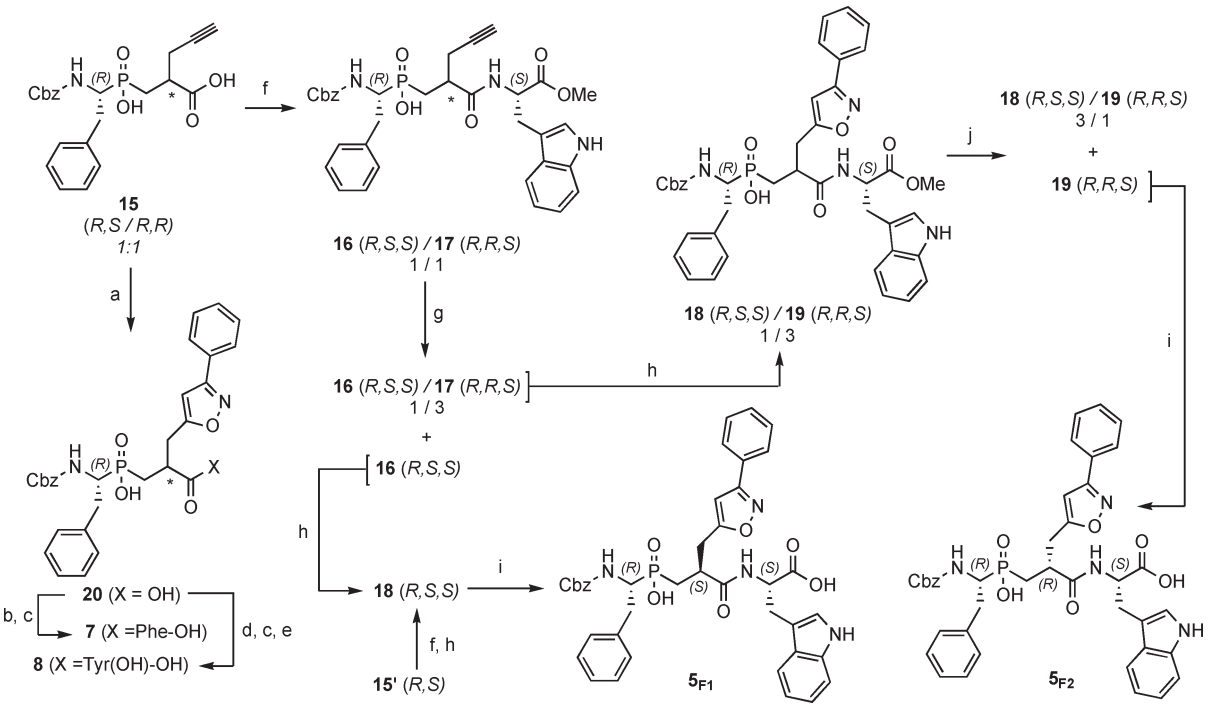
[a] Reagents and conditions: (a) HCl·H-Trp-OMe, EDC·HCl, HOBt, DIPEA, CH_2Cl_2, rt, 2 h; (b) NaOH, MeOH, H_2O, rt, 48 h, 81%, two steps.

stereochemically pure tripeptides $\mathbf{5_{F1}}$ (*R*,*S*,*S*) and $\mathbf{5_{F2}}$ (*R*,*R*,*S*) on a preparative scale, a sequence of crystallization resolving steps in carefully selected synthetic intermediates was devised (Scheme 3). Initially, diacid **15** was coupled with H-Trp-OMe to afford an 1:1 diastereoisomeric mixture of tripeptides **16** (*R*,*S*,*S*) and **17** (*R*,*R*,*S*). Attempts to separate the mixture by crystallization with CH_2Cl_2 furnished a white crystalline precipitate corresponding to **16** (*R*,*S*,*S*), while a 1:3 diastereoisomeric mixture of **16** (*R*,*S*,*S*) and **17** (*R*,*R*,*S*) was isolated from the filtrates [enriched to the more soluble **17** (*R*,*R*,*S*)]. Application of the one-pot Huisgen protocol to isomer **16** (*R*,*S*,*S*), for the introduction of the isoxazole ring, and alkaline deprotection of the resulting tripeptide **18** (*R*,*S*,*S*) efficiently led to pure diastereoisomer $\mathbf{5_{F1}}$ (*R*,*S*,*S*).[18] Transformation of the 1:3 diastereoisomeric mixture of **16** (*R*,*S*,*S*) and **17** (*R*,*R*,*S*) to the corresponding isoxazole derivatives by the one-pot Huisgen protocol afforded an 1:3 diastereoisomeric mixture of **18** (*R*,*S*,*S*) and **19** (*R*,*R*,*S*). Gratifyingly, from the latter mixture, isomer **19** (*R*,*R*,*S*) proved to be less soluble in $CHCl_3$ than **18** (*R*,*S*,*S*). On this basis, **19** (*R*,*R*,*S*) was isolated as a crystalline solid after crystallization by $CHCl_3$, while a 3:1 mixture of **18**/**19** was recovered from the filtrates. Compound $\mathbf{5_{F2}}$ was finally obtained after alkaline hydrolysis of methylester **19** (*R*,*S*,*S*). The stereochemical identity of $\mathbf{5_{F1}}$ (*R*,*S*,*S*) was confirmed by the conversion of the previously described pure *R*,*S*-diastereoisomer of **15** (noted as **15′** in Scheme 3) to tripeptide **18**.[18] This result unequivocally proves that derivative $\mathbf{5_{F1}}$ possesses the *S*-stereochemistry in the P_1' position and, consequently, derivative $\mathbf{5_{F2}}$ possesses the *R*-stereochemistry in the same position.

Finally, by following the one-pot Huisgen protocol, compound **15** was converted to isoxazole derivative **20** after 1,3-dipolar cycloaddition reaction with benzonitrile oxide (Scheme 3). Tripeptides **7** and **8** were obtained as mixtures of 2 diastereoisomers (1:1 ratio) after coupling of **20** with the appropriate amino acid and final deprotection. Isolation of fractions I (first eluting isomer, *R*,*S*,*S*) and II (second eluting isomer, *R*,*R*,*S*) from mixtures **7** and **8** was achieved by means of semipreparative RP-HPLC. [In each case, the stereochemical identity of the first eluting fraction was confirmed by a separate small-scale synthesis of $\mathbf{7_{F1}}$ and $\mathbf{8_{F1}}$ starting from the stereochemically pure diacid **15′**.]

In Vitro Potency and Selectivity Profile. Introduction of a phenyl substituted isoxazoline side chain in the P_1' position of **21** resulted in new compounds (**1**−**4**) displaying high potency toward ACE, a result in agreement with the presence

Scheme 3. Synthesis of Isoxazole Derivatives $\mathbf{5_{F1}}$, $\mathbf{5_{F2}}$, **7**, and **8**[a]

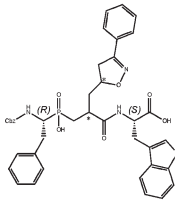
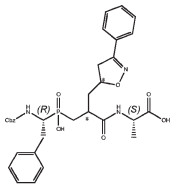
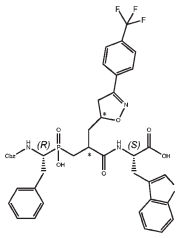
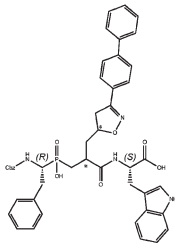
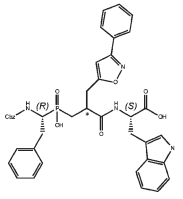
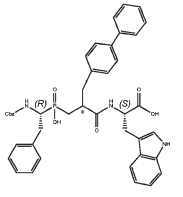


[a] Reagents and conditions: (a) PhCH=NOH, NCS, pyridine (cat.), $CHCl_3$, 45 °C, 3−4 h, then **15**, Et_3N, 45 °C, 3 d, 4 repetitions, 83%; (b) HCl·H-Phe-O^{t}Bu, EDC·HCl, HOBt, DIPEA, CH_2Cl_2, rt, 2 h; (c) NaOH, MeOH, H_2O, rt, 24 h, 87%, two steps; (d) HCl·H-Tyr(O^{t}Bu)-O^{t}Bu, EDC·HCl, HOBt, DIPEA, CH_2Cl_2, rt, 2 h; (e) HCO_2H, TIS, rt, 24 h, 90%, three steps; (f) HCl·H-Trp-OMe, EDC·HCl, HOBt, DIPEA, CH_2Cl_2, rt, 1.5 h; (g) crystallization by CH_2Cl_2, 33% of **16** + 43% of **16**/**17** (1:3), two steps; (h) PhCH=NOH, NCS, pyridine (cat.), $CHCl_3$, 45 °C, 3−4 h, then phosphinic substrate, Et_3N, 45 °C, 3 d, 70% (for **18**); (j) crystallization by $CHCl_3$, 59% for **19,** two steps; (i) NaOH, MeOH, H_2O, rt, 24 h, 86% (for $\mathbf{5_{F1}}$), 94% (for $\mathbf{5_{F2}}$).

of a very large and deep cavity S_1' cavity in this enzyme (Table 1). In contrast, the size of these side chains has more pronounced effects on the potency of these **21** analogues when tested with NEP and ECE-1. Interestingly, in this series only **1** and **2** displayed potency toward NEP and ECE-1, a result showing that the S_1' subsite in these two enzymes is of much reduced size compared with ACE. With an alanine instead of a tryptophan in its P_2' position, **2** exhibited a higher potency toward NEP, as compared with ECE-1, but the inverse situation was observed with **1**, which contains a Trp residue in its P_2' position. This result concurs with the ability of ECE-1 to accept a bulky residue in the inhibitor's P_2' position. It also suggests that there is a relationship between the size of the side chain present in the inhibitor's P_1' and P_2' positions, which differs in NEP and ECE-1. ECE-1 can tolerate the simultaneous presence of two bulky side chains in these positions, unlike NEP.

Further changes in the P_1' side chain structure were investigated in order to exploit subtle differences between the S_1' subsites of NEP and ECE-1. Instead of an isoxazoline side chain, two other "isosteric side chains" were assessed. The isoxazole side chain in **5** incorporates an additional double bond in the heterocycle, as compared with the isoxazoline ring. This difference results in a different orientation of these two side chains with respect to the inhibitor scaffold and thus may influence the potency of the inhibitor toward NEP and ECE-1. The same objective applied to **6**, containing a biphenyl side in the P_1' position. In this compound, the two aromatic rings are perpendicular to each other in their lower energy conformation, while in **5**, the two cycles are almost coplanar. Thus, here again subtle differences between the S_1' cavity of NEP and ECE-1 can be probed with these two additional compounds. As shown in Table 1, ACE can accommodate such P_1' substituents, but their effects on potency differ considerably between NEP and ECE-1. While a biphenyl provided a potent NEP inhibitor (K_i 52 nM), this side chain was not tolerated by ECE-1 ($K_i > 10\,\mu$M). In contrast, ECE-1 displayed a marked preference for the isoxazole side chain, the replacement of the isoxazoline with the isoxazole side chain resulting in a 65-fold increase in affinity (K_i 910 nM versus K_i 14 nM). The same substitution only provided a 10-fold increase in the inhibitor affinity for NEP. The above phosphinic peptides were evaluated as a mixture of four diastereoisomers, excepting **5** and **6**, which were synthesized as a mixture of two diastereoisomers (a single asymmetric center in their P_1' position). To assess the influence of the side chain stereochemistry in the inhibitor's P_1' position, the two diastereoisomers of compound **5** ($\mathbf{5_{F1}}$ and $\mathbf{5_{F2}}$) were prepared through a sequence of crystallization resolving steps and their respective K_i values for ACE, NEP, and ECE-1 were determined. As expected from previous studies, a better potency toward ACE was observed when the Cα carbon in the P_1' position had an S configuration. While the same trend held true for NEP, the most potent ECE-1 inhibitor displayed the R configuration. This difference in stereoselectivity provided a compound ($\mathbf{5_{F2}}$), which behaved as a dual ACE C-domain/ECE-1, with K_i values of 36 and 8 nM, respectively, for these two peptidases, but which displayed much lower potency with NEP (1850 nM). Toward the ACE N-domain, this compound exhibited a K_i value of 1250 nM, so $\mathbf{5_{F2}}$ is the first example of a dual ACE C-domain/ECE-1 inhibitor. However, preliminary in vivo evaluation of this inhibitor in lowering blood pressure in spontaneously hypertensive rats

Table 1. Potency of Phosphinic Peptide Inhibitors toward ACE C-Domain, NEP, and ECE-1

	ACE_C-dom Ki (nM)	NEP Ki (nM)	ECE-1 Ki (nM)
1 	13 ± 2	2500 ± 300	910 ± 150
2 	5 ± 0.5	85 ± 2	2100 ± 200
3 	20 ± 4	> 5 μM	> 5 μM
4 	25 ± 5	> 10 μM	> 10 μM
5 	16 ± 2	260 ± 18	14 ± 3
6 	12 ± 0.5	52 ± 8	> 10 μM

(SHR) indicated a short duration of action (few minutes, see below). Previous studies have suggested that high binding to plasma proteins might be responsible for such a short-lived effect[23] by limiting the inhibitor's access to the enzyme active. This led us to evaluate the extent of binding of compound $\mathbf{5_{F2}}$ to plasma proteins from this animal model.

Inhibitor Potency in the Presence of SHR Rat Plasma Proteins. To test the potential binding of compound $\mathbf{5_{F2}}$ to SHR rat plasma proteins, the potency of $\mathbf{5_{F2}}$ in blocking ACE activity in diluted rat plasma (1/50 and 1/100), complemented or not with serum albumin (5 μM final concentration) [in plasma 1/100, the concentration of serum albumin is still around 5 μM], was evaluated using an assay

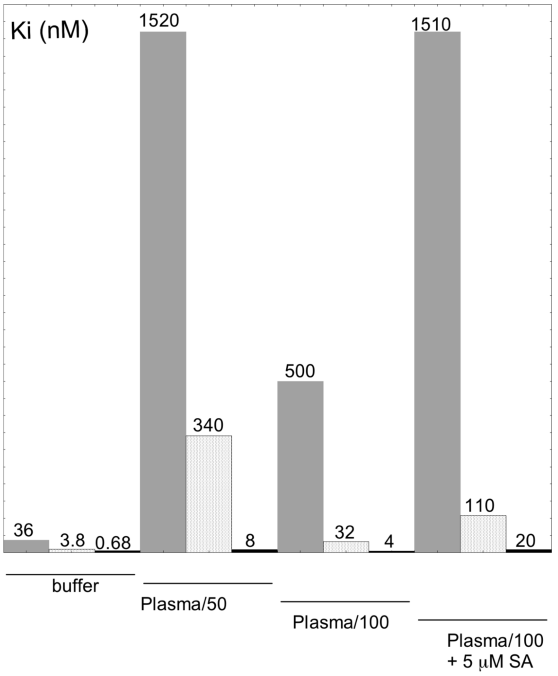


Figure 1. Apparent K_i values (numbers on the top of bars) displayed by compounds $\mathbf{5_{F2}}$ (dark gray), $\mathbf{7_{F2}}$ (dotted line), and $\mathbf{8_{F2}}$ (black) in different serum media.

based on the cleavage of a fluorogenic substrate.[24] The apparent K_i values of $\mathbf{5_{F2}}$, determined under these different conditions, are reported in Figure 1. The huge variation in the apparent K_i values, from 36 nM to 1520 in plasma 1/50, demonstrates that compound $\mathbf{5_{F2}}$ binds strongly to proteins present in rat plasma, probably to albumin as shown by the higher K_i value observed for this compound when the plasma 1/100 is complemented with this protein (500 versus 1510 nM). To alleviate this drawback, two additional compounds were synthesized by replacing the Trp residue by Phe and Tyr residues, as Trp is known to favor binding to albumin. Such substitution of the P_2' position by Phe and Tyr residues led to four additional compounds (resolved by reverse-HPLC), which confirm the above finding of the key role of the configuration of the P_1' position in this series of compounds, in affording dual ACE C-domain/ECE-1 inhibitors. Compounds $\mathbf{5_{F1}}$, $\mathbf{7_{F1}}$, and $\mathbf{8_{F1}}$ with the S configuration in the P_1' position behaved as triple ACE/NEP/ECE-1 inhibitors. Interestingly, compounds $\mathbf{7_{F2}}$ and $\mathbf{8_{F2}}$ exhibited higher potency toward the ACE C-domain, but more reduced potency toward NEP, while maintaining similar potency toward ECE-1. Toward the N-domain, $\mathbf{7_{F2}}$ and $\mathbf{8_{F2}}$ exhibited K_i values of 185 and 150 nM, respectively. Remarkably, the substitution of the Trp residue by Phe and Tyr resulted in a marked reduction in inhibitor binding to serum proteins. In diluted rat plasma (1/100), complemented with 5 μM of serum albumin, $\mathbf{8_{F2}}$ displayed an apparent K_i value of 20 nM toward ACE, while in the same media, $\mathbf{5_{F2}}$ was much less potent (K_i 1510 nM).

Potency toward Matrix-Metalloproteinases (MMPs). Finally, compounds **5**, **7**, and **8** were tested against MMP-13, a zinc metalloproteinase which is also characterized by the presence of a deep S_1' cavity in its active site. Compounds $\mathbf{5_{F1}}$, $\mathbf{7_{F1}}$, and $\mathbf{8_{F1}}$ behaved as rather potent inhibitors of MMP-13, confirming our previous findings which were based on phosphinic peptide containing in their P_1' position various substituted isozazoline side chains (Table 2).[18] But due to the sharp binding preference of MMPs for phosphinic peptide inhibitors containing in their P_1' position side chains with an S configuration, $\mathbf{5_{F2}}$, $\mathbf{7_{F2}}$, and $\mathbf{8_{F2}}$ were observed to be weak inhibitors of MMP-13 (Table 3). For the same reason, $\mathbf{5_{F2}}$, $\mathbf{7_{F2}}$, and $\mathbf{8_{F2}}$ are expected to display weak potency toward other MMPs.[25]

Effect on Blood Pressure. The in vivo efficacy of compounds $\mathbf{5_{F2}}$ and $\mathbf{8_{F2}}$ was evaluated by studying their ability to lower the blood pressure of SHR rats ($n = 6$). In the control group, SHR rats had elevated mean arterial pressure (MAP) of 156 ± 4 mmHg. Compound $\mathbf{5_{F2}}$ at a iv bolus dose of 10 and 30 mg/kg reduced the MAP to 148 ± 4 mm and 96 ± 7 mmHg, respectively. However, this potent effect was very short-lived, as after 5 min the blood pressure increased to baseline. In contrast, compound $\mathbf{8_{F2}}$ at 3 mg/kg and 10 mg/kg doses reduced the MAP to 147 ± 6 and 132 ± 5 mmHg, respectively, an effect that lasted 40 min.

Discussion and Conclusions

Several phosphorus-containing pseudopeptides have been reported previously as moderate or potent inhibitors of ECE-1. Phosphoramidon was probably the first example of such compounds (Chart 1). Lloyd et al. prepared more stable analogues of phosphoramidon by replacing the phosphonamide moiety (PO_2-NH) by a phosphinic function (PO_2CH_2), but these derivatives displayed similar potencies toward NEP and ECE-1 (compound **22**, Chart 1).[26] Better potency toward ECE-1 was observed with compound **23** (SCH-54,470, Chart 1),[27] but the major breakthrough in potency and selectivity was observed with compound compound **24** (CGS 35066, Chart 1), which has within the P_1' side chain a dibenzofuranyl.[23] Recently, highly selective ECE-1 inhibitors have been reported, but their chemical structures do not incorporate phosphoryl group.[28,29] Considering all these structures led us to suggest that further optimization of selective ECE-1 inhibitors can be achieved with a series of phosphinic compounds by keeping respectively in their structure a Z-Phe moiety in the P_1 position, a Trp-OH in the P_2' position, and various long side chains in their P_1' positions. Starting from the chemical structure of **21**, these simple ideas led us to rapidly identify a series of potent triple ACE/NEP/ECE-1 inhibitors ($\mathbf{5_{F1}}$, $\mathbf{7_{F1}}$, and $\mathbf{8_{F1}}$) or dual ACE/ECE-1 inhibitors ($\mathbf{5_{F2}}$, $\mathbf{7_{F2}}$, and $\mathbf{8_{F2}}$). These data demonstrate the key role played by the P_1' side chain configuration in discriminating between ECE-1 and NEP. It has been previously argued that NEP, because it has a larger S_1' cavity than ECE-1, can accommodate large P_1' side chains without stereochemical preference.[30] Here, the stereochemistry of compounds **5**, **7**, and **8** has more pronounced effect on their affinity toward NEP than ECE-1. A second factor contributing to ECE-1 selectivity was that the R configuration reduces the affinity of compounds **5**, **7**, and **8** toward NEP, as compared with the S configuration, but the reverse situation was observed for ECE-1. Recent efforts to develop inhibitors exhibiting similar selectivity profiles, based on a series of substituted urea congeners, failed to identify compounds with ACE-ECE-1 selectivity.[31]

Compounds $\mathbf{5_{F2}}$, $\mathbf{7_{F2}}$, and $\mathbf{8_{F2}}$ retain quite good selectivity for the C-domain of ACE, as compared with **21**. Determination of the 3D-structure crystal of **21** complexed with the ACE C-domain has not unveiled the molecular determinants of **21** responsible for its unique selectivity toward the ACE C-domain. The presence of a Trp in the P_2' position of the

Table 2. Potency of Phosphinic Peptide Inhibitors toward ACE C-Domain, NEP, and ECE-1

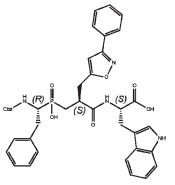
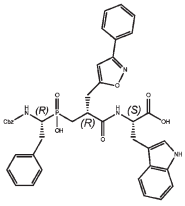
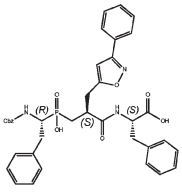
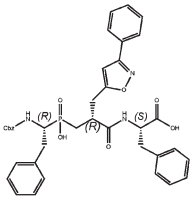
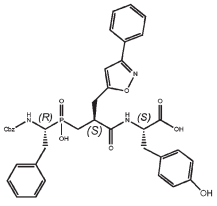
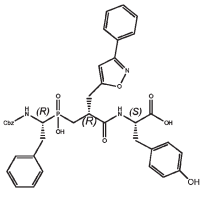
	ACE C-dom Ki (nM)	NEP Ki (nM)	ECE-1 Ki (nM)
 $\mathbf{5_{F1}}$	1.4 ± 0.2	130 ± 25	26 ± 6
 $\mathbf{5_{F2}}$	36 ± 5	1850 ± 120	8 ± 0.7
 $\mathbf{7_{F1}}$	0.4 ± 0.05	68 ± 4	240 ± 25
 $\mathbf{7_{F2}}$	3.8 ± 0.6	> 10 μM	7.7 ± 0.3
 $\mathbf{8_{F1}}$	0.41 ± 0.03	172 ± 24	275 ± 60
 $\mathbf{8_{F2}}$	0.65 ± 0.03	6700 ± 300	14 ± 2

Table 3. Inhibition of MMP-13

	$\mathbf{5_{F1}}$	$\mathbf{7_{F1}}$	$\mathbf{8_{F1}}$	$\mathbf{5_{F2}}$	$\mathbf{7_{F2}}$, μM	$\mathbf{8_{F2}}$
K_i nM	19 ± 2	30 ± 3	24 ± 2	1200 ± 20	> 10	4000 ± 35

inhibitors has been proposed to contribute to this selectivity, a suggestion that fits with the selectivity displayed by compounds $\mathbf{5_{F2}}$. The good potency exhibited by this compound fits also with the observation of a large S_1' cavity in the crystal structures of the ACE C-domain/**21** complex. However, it should be mentioned that assays to dock $\mathbf{8_{F2}}$ in the crystal structure of ACE C-domain in a "standard binding mode", the one adopted by **21** in this crystal structure, resulted in a model in which both the P_1' and P_2' inhibitor side chain atoms exhibit steric clashes with atoms of the S_1' and S_2' subsites of ACE C-domain. Thus, the exact mode of $\mathbf{8_{F2}}$ binding in the active sites of these enzymes remains to be experimentally determined. Such structures would certainly be of great value in revealing unexpected binding modes and thus be the

starting point for the development of nonpeptide inhibitors with a similar selectivity profile.

Blocking in vivo the C-domain of ACE by **21** was observed to prevent the conversion of Ang-I into Ang-II but not to abrogate the cleavage of BK by the N-domain, free of inhibitor.[12] On the basis of these observations, one may speculate that compounds $\mathbf{5_{F2}}$, $\mathbf{7_{F2}}$, and $\mathbf{8_{F2}}$ should behave in the same manner, blocking Ang-I conversion but not interfering in vivo with the BK degradation. Also, based on the weak potency displayed by these compounds toward NEP, the cleavage of BK by this peptidase should also occur in presence of these inhibitors. Thus, the increase in BK levels, which has been observed with the simultaneous blockade of ACE and NEP and seems to be responsible for angioedema, should not be observed with this novel series of vasopeptidase inhibitors.[7] Increase in ET-1 concentration has been also implicated in the development of side effects associated with dual ACE/NEP inhibitors through the blockade of NEP by these compounds. By targeting ECE-1, while sparing NEP, $\mathbf{8_{F2}}$ should result in vivo in a reduction of ET-1 levels. Compound $\mathbf{8_{F2}}$ was observed to reduce the MAP of SHR rats by 6 and 15% at iv doses of 3 and 10 mg/kg. These preliminary experiments are quite promising, but further work is required to determine the full dose−effect of compound $\mathbf{8_{F2}}$ on blood pressure. It will be mandatory also to examine the effect of compound $\mathbf{8_{F2}}$ on key vasoactive peptide mediators to explain its effect on blood pressure. This could be of fundamental importance, as to the best of our knowledge compound $\mathbf{8_{F2}}$ possesses a unique selective profile and so might be a good tool to improve understanding of the complex interplay between the different vasoactive peptide mediators. Finally, $\mathbf{8_{F2}}$ may also find important applications in diseases where both Ang-II and ET-1 overproduction is a main concern, like cardiovascular end-organ damage[32] or particular cancers.[33,34]

Experimental Section

General. Solvents were purchased as anhydrous grade and stored over 4 Å activated molecular sieves before use. Reagents were purchased from Aldrich, Acros, Fluka, Novabiochem, and Bachem and were used without further purification. Column chromatography was performed on silica gel (E. Merck, 70−230 mesh). TLC analyses were performed on silica gel plates (E. Merck silica gel $60F_{254}$), and components were visualized by the following methods: UV light absorbance and/or charring after spraying with a solution of NH_4HSO_4. A $CHCl_3$/MeOH/AcOH 7:2:1 solvent system was used for all R_f values reported in the Experimental Section unless otherwise noted. Melting points (measured on an electrothermal apparatus) are uncorrected. Optical rotation data were acquired on a Perkin-Elmer 343 polarimeter at 25 °C.

NMR spectra were recorded on either a Varian 200 MHz Mercury or a Bruker 500 MHz Avance III spectrometer at 26 °C. For NMR characterization, DMSO-d_6 containing 1% CF_3COOD was used as a solvent unless otherwise noted. ^1H and ^{13}C spectra are referenced according to the residual peak of the solvent based on literature data.[35] ^{31}P NMR chemical shifts are reported in ppm downfield from 85% H_3PO_4 (external standard). ^{13}C and ^{31}P NMR spectra are fully proton decoupled. For compounds **1**−**4**, **6**, **11**−**13**, **16**, and **18**−**20**, ^1H NMR peak assignments are based on 2D COSY experiments. For compounds $\mathbf{5_{F1}}$−$\mathbf{8_{F1}}$ and $\mathbf{5_{F2}}$−$\mathbf{8_{F2}}$, a full assignment of ^1H and ^{13}C NMR spectra, acquired on a 500 MHz instrument, is provided in Supporting Information. This assignment was performed on the basis of 1D DEPTQ and 2D COSY, TOCSY, HSQC, and HMBC spectra that were processed and analyzed with the Bruker TOPSPIN 2.0 program.

ESI mass spectral analysis was performed on a mass spectrometer MSQ Surveyor, Finnigan, using direct sample injection. Negative or positive ion ESI spectra were acquired by adjusting the needle and cone voltages accordingly. High-resolution mass spectroscopy (HRMS) data for compounds $\mathbf{5_{F1}}$−$\mathbf{8_{F1}}$ and $\mathbf{5_{F2}}$−$\mathbf{8_{F2}}$ were recorded using a 4800 MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA). Detection and resolution were optimized (delay time extraction and detector voltage multiplier) in positive reflectron mode in the m/z range of 200−1000 to obtain an accuracy of less than 5 ppm. All compounds were diluted in 50% acetonitrile/water with 0.1% TFA at a concentration of 100 μM or 0.1 mg/mL. Then 0.5 μL of each sample was spotted on a MALDI standard plate using the dried-droplet method with 0.5 μL of α-cyano-4-hydroxycinnamic acid matrix solution at 10 mg/mL in 50% acetonitrile/water with 0.1% TFA. Each spectrum was the result of 1000−2000 shots (20 different positions into each spot and 50 shots per subspectrum) and internal calibration was applied by using 4-HCCA matrix m/z.

RP-HPLC analyses were performed on a Hewlett-Packard 1100 model (C_{18}-Cromasil-RP, 5 μm, UV/vis detector, flow: 0.5 mL/min, 254 and/or 280 nm detection). The following gradients of buffers A and B (A buffer: 90% H_2O with 0.1% TFA, 10% CH_3CN; B buffer: 10% H_2O with 0.09% TFA, 90% CH_3CN) were used: method 1 (0 min, 30% B; 20 min, 60% B; 50 min, 100% B; 60 min, 40% B); method 2 (0 min, 0% B; 10 min, 25% B; 45 min, 75% B; 60 min, 100% B); method 3 (0 min, 0% B; 10 min, 35% B; 35 min, 100% B; 40 min, 35% B).

Purity of Compounds. Purity of compounds after preparative reverse-phase HPLC was assessed by analytical HPLC using both isochratic and gradient elution. On the basis of this criteria, all compounds possess purity at >95%.

Chemistry. (1*R*)-1-{[(Benzyloxy)carbonyl]amino}-2-phenylethyl[2-(ethoxycarbonyl)-pent-4-enyl] Phosphinic Acid (11). A mixture of aminophosphinic acid **9** (4.0 g, 12.5 mmol) and HMDS (13.1 mL, 62.5 mmol) was heated at 110 °C for 1 h under Ar, then ethyl α-allyl acrylate (**10**) (2.63 g, 18.8 mmol) was added dropwise. The resulting mixture was stirred at 100 °C for additional 3 h and slowly cooled to 70 °C. Then, dry EtOH (13 mL) was added portionwise, still under Ar. The cooled mixture was stirred at this temperature for 20 min. The solvent was removed under vacuum and the residue was purified by column chromatography ($CHCl_3$/MeOH/AcOH 7:0.3:0.3) to afford 4.8 g (84%) of compound **11** as a white solid; mp 120−122 °C; R_f = 0.65 ($CHCl_3$/MeOH/AcOH 7:0.5:0.5). ^1H NMR (200 MHz, CD_3OD) δ 1.22 (t, $^3J_{HH}$ = 6.8 Hz, 3H, CH_2CH_3), 1.63−1.89 (m, 1H, PCH*H*), 1.96−2.20 (m, 1H, PC*H*H), 2.24−2.44 (m, 2H, $CH_2CH{=}CH_2$), 2.58−3.02 (m, 2H, PCH_2CH, PCHCH*H*Ph), 3.18−3.34 (m, 1H, PCHC*H*HPh), 3.94−4.19 (m, 3H, CH_2CH_3, PC*H*), 4.93−5.16 (m, 4H, $CH{=}CH_2$, $PhCH_2O$), 5.48−5.81 (m, 1H, $CH{=}CH_2$), 7.12−7.29 (m, 10H, Ar). ^{13}C NMR (50 MHz, CD_3OD) δ 14.4, 28.8 (d, J_{PC} = 91.4 Hz), 34.6, 38.9 (d, J_{PC} = 9.5 Hz), 40.1, 53.2 (d, J_{PC} = 106.8 Hz), 61.9, 66.4, 119.1, 127.2, 127.8, 128.4, 128.7, 129.5, 129.7, 130.2, 130.5, 135.8, 139.3, 139.5, 157.5 (d, J_{PC} = 5.1 Hz), 175.9 (d, J_{PC} = 7.8 Hz). ^{31}P NMR (81 MHz, CD_3OD) δ 49.2, 49.7. ES-MS m/z: calcd for $[C_{24}H_{30}NO_6P - H]^-$ 458.2; found 458.1.

(1*R*)-1-{[(Benzyloxy)carbonyl]amino}-2-phenylethyl[2-({[(1*S*)-2-(*tert*-butyloxy)-1-methyl-2-oxoethyl]amino}carbonyl)-pent-4-enyl] Phosphinic Acid (12). Phosphinic pseudodipeptide **11** (750 mg, 1.63 mmol) were dissolved in EtOH (14 mL), and the solution was cooled to 0 °C. NaOH (1 M, 13 mL) was added portionwise, and the mixture was stirred at rt for 12 h. After acidification with 2 M HCl, ethanol was evaporated, and the residue was diluted with water and extracted with AcOEt (40 mL). The organic phase was washed with H_2O and brine, dried over Na_2SO_4, and evaporated under vacuum. The residue was dried over P_2O_5 for 24 h, and addition of CH_2Cl_2 (10 mL) followed. To the resulting suspension, DIPEA (0.81 mL,

4.9 mmol), hydrochloric L-alanine *tert*-butyl ester (311 mg, 1.71 mmol), HOBt (220 mg, 1.63 mmol), and EDC·HCl (1.88 g, 9.78 mmol) were added. The mixture was stirred for 1.5 h at rt. Then it was diluted with CH_2Cl_2 (40 mL) and the solution was washed with 1 M HCl (3 × 10 mL), H_2O (10 mL), 10% NH_4HCO_3 (2 × 1.5 mL), 1 M HCl (2 × 10 mL), and brine (10 mL). The organic phase was dried over Na_2SO_4 and evaporated under vacuum. Compound **12** (850 mg, 93%) was obtained as a white solid after addition of a Et_2O/light petroleum (1:3) mixture to the residue and filtration of the precipitate; mp 111−113 °C; R_f = 0.44/0.38 ($CHCl_3$/MeOH/AcOH 7:0.5:0.5). ^{1}H NMR (200 MHz) δ 1.23 (d, $^3J_{HH}$ = 6.7 Hz, 3H, CHCH_3), 1.34 [s, 9H, C(CH_3)$_3$], 1.51−2.05 (m, 2H, PCH_2), 2.11−3.17 (m, 5H, CH_2CH=CH_2, PCH$_2$C*H*, PCHCH_2Ph), 3.79−4.09 (m, 1H, PC*H*), 4.19−4.37 (m, 1H, NHC*H*CO), 4.64−5.17 (m, 4H, CH=CH_2, PhCH_2O), 5.46−5.82 (m, 1H, C*H* = CH$_2$), 7.04−7.44 (m, 10H, Ar), 7.63 (d, $^3J_{HH}$ = 9.6 Hz, OCON*H*), 8.39 (d, $^3J_{HH}$ = 9.6 Hz, N*H*CHCO). ^{13}C NMR (50 MHz) δ 17.1, 27.5 (d, J_{PC} = 88.6 Hz), 27.8, 33.1, 38.1, 47.9, 52.6 (d, J_{PC} = 95.9 Hz), 65.1, 81.1, 126.2, 127.0, 127.5, 128.1, 128.4, 128.9, 137.2, 138.5 (d, 1H, J_{PC} = 13.6 Hz), 156.1, 172.6 (d, 1H, J_{PC} = 7.9 Hz). ^{31}P NMR (81 MHz) δ 46.2−46.9. HPLC t_R(1) = 25.5, 26.6 min (2 isomers). ES-MS *m*/*z*: calcd for $[C_{29}H_{40}N_2O_7P + H]^+$ 559.3; found 559.1.

(1*R*)-1-{[(Benzyloxy)carbonyl]amino}-2-phenylethyl[2-({[(1*S*)-1-(1*H*-indol-3-ylmethyl)-2-methoxy-2-oxoethyl]amino}carbonyl)-pent-4-enyl] Phosphinic Acid (13). For the synthesis of **13**, phosphinic pseudodipeptide **11** (2.0 g, 4.4 mmol) was saponified and coupled to L-tryptophan methyl ester according to the synthetic protocol described for the preparation of compound **12**. Treatment of the residue obtained after the final workup with a mixture of Et_2O/light petroleum (2:1) and filtration of the precipitate furnished compound **13** (2.2 g, 80%) as a white solid; mp 184−187 °C; R_f = 0.44 ($CHCl_3$/MeOH/AcOH 7:0.5:0.5). ^{1}H NMR (200 MHz) δ 1.42−2.12 (m, 2H, PCH_2), 2.13−2.30 (m, 2H, CH_2CH=CH_2), 2.56−2.87 (m, 2H, PCH$_2$C*H*, CH*H*Ph), 2.90−3.42 (m, 3H, CH_2indolyl, C*H*HPh), 3.59 (s, 3H, OCH_3), 3.83−4.15 (m, 1H, PC*H*), 4.27−4.58 (m, 1H, NHC*H*CO), 4.62−5.02 (m, 4H, CH=CH_2, PhCH_2O), 5.05−5.28 (m, 0.25H, C*H*=CH$_2$, first diastereoisomer), 5.41−5.63 (m, 0.75H, C*H*=CH$_2$, second diastereoisomer), 6.83−8.41 (m, 18H, Ar, N*H*). ^{13}C NMR (50 MHz) δ 26.7 (d, J_{PC} = 89.7 Hz), 27.7 (d, J_{PC} = 89.7 Hz), 33.1, 33.6, 37.7 (d, J_{PC} = 10.1 Hz), 47.6, 51.5 (d, J_{PC} = 101.3 Hz), 51.8, 53.6, 65.4, 110.6, 111.1, 111.3, 116.8, 118.2, 120.8, 123.5, 126.2, 126.7, 127.1, 127.3, 127.4, 127.6, 128.2, 128.3, 129.0, 135.3, 135.7, 136.1, 136.2, 137.2, 138.3, 138.6, 156.3 (d, J_{PC} = 4.7 Hz), 173.2 (d, J_{PC} = 7.5 Hz). ^{31}P NMR (81 MHz) δ 46.7, 47.4. HPLC t_R(2) = 43.9 min (2 isomers). ES-MS *m*/*z*: calcd for $[C_{34}H_{38}N_3O_7P + H]^+$ 632.3; found 632.5.

(2*S*)-2-{[3-(4′,5′-Dihydro-3′-phenyl-5′-isoxazolyl)-2-{[hydroxyl-(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]amino}ethyl)phosphinyl]-methyl}-1-oxopropyl]amino} 1*H*-Indole-3-propanoic Acid (1). Phosphinic pseudotripeptide 13 (300 mg, 0.47 mmol) was dissolved in MeOH (5 mL), and the solution was cooled to 0 °C. NaOH (1 M, 5.0 mL) was added portionwise, and the mixture was stirred at rt for 24 h. Then, MeOH was evaporated and the residue was diluted with H_2O (10 mL) and acidified with 2 M HCl. The white precipitate was filtered off and dried over P_2O_5 for 24 h. In a separate flask, benzaldoxime (340 mg, 2.8 mmol) was dissolved in $CHCl_3$ (15 mL), and 3−4 drops of pyridine were added. Then NCS (374 mg, 2.8 mmol) was added at rt and after 10 min the resulting mixture was stirred at 45 °C for 3−4 h. In this solution, the dry tripeptide obtained from the saponification step was added, followed by slow addition of Et_3N (254 μL, 3.8 mmol) at the same temperature. The reaction mixture was stirred for 3 days at 45 °C. Then it was concentrated under vacuum and the residue was diluted with AcOEt (30 mL) and washed with 1 M HCl (2 × 10 mL), H_2O (10 mL), 10% NH_4HCO_3 (2 × 1 mL), 1 M HCl (2 × 10 mL), and brine (10 mL). The organic phase was dried over Na_2SO_4 and evaporated under vacuum. Compound **1** (319 mg, 92%) was obtained as a white solid after purification by column chromatography ($CHCl_3$/MeOH/AcOH 7:0.35:0.3) and precipitation by a Et_2O/light petroleum (1:9) mixture; mp 121−124 °C (dec); R_f = 0.49. ^{1}H NMR (200 MHz) δ 1.35−2.18 (m, 4H, CH_2CHO, PCH_2), 2.54−3.52 (m, 7H, CHCH_2Ph, C*H*CONH, CH_2indolyl, CH_2C=N), 3.65−4.22 (m, 1.75H, PC*H*, C*H*O of first diastereoisomer), 4.22−4.57 (m, 1.25H, C*H*O of second diastereoisomer, NHC*H*CO), 4.78−5.04 (m, 2H, PhCH_2O), 6.83−7.80 (m, 22H, Ar, indole-N*H*, OCON*H*), 7.98−8.19 and 8.36−8.52 (m, 1H, N*H*CHCO). ^{13}C NMR (50 MHz) δ 27.6 (d, J_{PC} = 88.2 Hz), 28.6 (d, J_{PC} = 88.2 Hz), 32.7 (br), 33.2, 36.9, 37.1, 42.3, 52.6 (d, J_{PC} = 97.8 Hz), 53.8, 65.1, 78.8/79.1, 110.6, 111.2, 112.1, 118.4, 118.6, 118.7, 120.7, 123.7, 126.2, 127.1, 127.2, 127.5, 128.2, 128.3, 128.9, 129.0, 131.3, 136.8, 137.3, 138.2, 138.6, 155.8 (d, J_{PC} = 3.2 Hz), 156.2, 172.1, 173.5, 173.9. ^{31}P NMR (81 MHz) δ 45.8, 45.9, 46.6, 47.1. HPLC t_R(2) = 38.9, 40.1(d) min, (4 isomers). HRMS *m*/*z*: calcd for $[C_{40}H_{41}N_4O_8P + H]^+$ 737.2740; found 737.2750.

(2*S*)-2-{[3-(4′,5′-Dihydro-3′-phenyl-5′-isoxazolyl)-2-{[hydroxyl-(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]amino}ethyl)phosphinyl]-methyl}-1-oxopropyl]amino} Propanoic Acid (2). Benzaldoxime (0.12 g, 1.0 mmol) was dissolved in CH_2Cl_2 (6 mL), and addition of phosphinic pseudodipeptide **12** (140 mg, 0.25 mmol) and Et_3N (35 μL, 0.25 mmol) followed. To this solution, common bleach (4.5% aqueous solution of NaOCl, 1.65 mL, 1.0 mmol) was slowly added, and the resulting biphasic mixture was vigorously stirred at rt for 16 h. Then, the mixture was diluted with CH_2Cl_2 (15 mL) and 1 M HCl was added for acidification of the aqueous layer. The aqueous phase was separated and the organic phase was washed with 1 M HCl (2 × 10 mL), H_2O (10 mL) and brine (10 mL). Then, the organic phase was dried over Na_2SO_4 and evaporated under vacuum. The residue was purified by column chromatography (eluent: $CHCl_3$/MeOH/AcOH = 70:3.5:3) and the pure cycloaddition product was dissolved in ethanol (2 mL). The resulting solution was cooled to 0 °C and 1 M NaOH (2 mL) was added portionwise. After stirring at rt for 24 h, EtOH was evaporated, the residue was diluted with H_2O, and the resulting solution was washed with Et_2O (2 × 5 mL). The aqueous phase was acidified with 2 M HCl, and extractions with AcOEt (3 × 10 mL) followed. The organic phase was washed with H_2O and brine, dried over Na_2SO_4, and evaporated under vacuum. Compound **2** (137 mg, 88%) was obtained as a white solid after addition of a Et_2O/light petroleum (2:1) mixture to the residue and filtration of the precipitate; mp 165−171 °C (dec); R_f = 0.39. ^{1}H NMR (200 MHz) δ 1.27 (appt, $^3J_{HH}$ = 6.9 Hz, 3H, CHCH_3), 1.64−2.40 (m, 4H, CH_2CHO, PCH_2), 2.59−3.23 (m, 4H, CHCH_2Ph, C*H*CONH, C*H*HC=N), 3.34−3.63 (m, 1H, CH*H*C=N), 3.76−4.08 (m, 1H, PC*H*), 4.10−4.33 (m, 1H, C*H*O), 4.49−4.77 (m, 1H, C*H*CH$_3$), 4.78−5.10 (m, 2H, PhCH_2O), 6.92−7.77 (m, 16H, Ar, OCON*H*), 8.15−8.55 (m, 1H, N*H*CHCO). ^{13}C NMR (50 MHz) δ 16.9, 17.0, 17.1, 17.3, 27.5 (d, J_{PC} = 88.4 Hz), 28.7 (d, J_{PC} = 88.4 Hz), 29.3 (d, J_{PC} = 88.4 Hz), 33.0 (br), 36.0, 36.4, 37.6, 47.8/47.9, 51.8 (d, J_{PC} = 103.7 Hz), 52.0 (d, J_{PC} = 103.7 Hz), 52.7 (d, J_{PC} = 103.7 Hz), 65.2, 79.1/79.3, 126.3, 126.6, 127.1, 127.6, 128.2, 128.4, 128.9, 129.1, 129.7, 130.1, 135.8, 137.3, 138.5 (d, J_{PC} = 14.1 Hz), 156.2 (d, J_{PC} = 3.4 Hz), 156.7 (br), 173.3, 173.5, 173.7, 174.1, 174.3. ^{31}P NMR (81 MHz) δ 46.2, 46.9, 47.0, 47.5. HPLC t_R(3) = 22.6−26.8 min, (4 isomers). HRMS *m*/*z*: calcd for $[C_{32}H_{36}N_3O_8P + H]^+$ 622.2318; found 622.2303.

(2*S*)-2-{[3-(4′,5′-Dihydro-3′-[4″-trifluoromethyl]phenyl-5′-isoxazolyl)-2-{[hydroxyl(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]-amino}ethyl)phosphinyl]methyl}-1-oxopropyl]amino} 1*H*-Indole-3-propanoic Acid (3). For the synthesis of **3**, phosphinic pseudodipeptide **13** (80 mg, 0.13 mmol) and 4-trifluoromethylbenzaldoxime were subjected to the same synthetic procedure as described for the preparation of compound **1**. Compound **3** (97 mg, 93%) was obtained as a white solid; mp 147−151 °C (dec); R_f = 0.60. ^{1}H NMR (200 MHz) δ 1.35−2.12 (m, 4H,

CH_2CHO, PCH_2), 2.58−3.49 (m, 7H, CHCH_2Ph, CHCONH, CH_2indolyl, CH_2C=N), 3.65−4.19 (m, 1.75H, PCH, CHO of first diastereoisomer), 4.23−4.52 (m, 1.25H, CHO of second diastereoisomer, NHCHCO), 4.77−5.02 (m, 2H, PhCH_2O), 6.96−7.85, 7.97−8.25 and 8.48−8.85 (m, 22H, Ar, NH). ^{13}C NMR (50 MHz) δ 27.6 (d, J_{PC} = 86.2 Hz), 28.3 (d, J_{PC} = 84.9 Hz), 32.6 (br), 33.6, 36.9, 37.1, 42.3, 52.6 (d, J_{PC} = 98.6 Hz), 53.5, 65.1, 78.7/79.1, 108.0, 110.6, 111.2, 112.1, 118.4, 118.6, 118.7, 120.7, 122.3, 123.7, 125.2, 126.2, 127.1, 127.2, 127.5, 128.2, 128.3, 128.9, 129.2, 129.4, 131.3, 136.8, 137.3, 138.2, 138.6, 155.7 (d, J_{PC} = 2.4 Hz), 156.2, 172.3, 173.9 (d, J_{PC} = 7.1 Hz). ^{31}P NMR (81 MHz) δ 45.7, 45.9, 46.6, 47.1. HPLC t_R(2) = 44.4, 46.0 min (4 isomers). HRMS m/z: calcd for $[C_{41}H_{40}F_3N_4O_8P + H]^+$ 805.2614; found 805.2604.

(2*S*)-2-{[3-(3′-[1,1′-Biphenyl]-4″-yl-4′,5′-dihydro-5′-isoxazolyl)-2-{[hydroxyl(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]amino}ethyl)-phosphinyl]methyl}-1-oxopropyl]amino} 1*H*-Indole-3-propanoic Acid (4). For the synthesis of **4**, phosphinic pseudodipeptide **13** (80 mg, 0.13 mmol) and (1,1′-biphenyl)-4-carbaldehyde oxime were subjected to the same synthetic procedure as described for the preparation of compound **1**. Compound **4** (95 mg, 90%) was obtained as a white solid; mp 174−177 °C (dec); R_f = 0.71. ^{1}H NMR (200 MHz) δ 1.34−2.17 (m, 4H, CH_2CHO, PCH_2), 2.55−3.52 (m, 7H, CHCH_2Ph, CHCONH, CH_2indolyl, CH_2C=N), 3.65−4.22 (m, 1.75H, PCH, CHO of first diastereoisomer), 4.26−4.57 (m, 1.25H, CHO of second diastereoisomer, NHCHCO), 4.78−5.02 (m, 2H, PhCH_2O), 6.83−8.19 (m, 26H, Ar, indole-NH, OCONH), 8.28−8.52 (m, 1H, NHCHCO). ^{13}C NMR (50 MHz) δ 27.4 (d, J_{PC} = 84.9 Hz), 28.5 (d, J_{PC} = 84.9 Hz), 32.7 (br), 33.4, 36.9, 37.3, 42.3, 52.3 (d, J_{PC} = 98.3 Hz), 53.6, 65.4, 78.8/79.1, 110.7, 111.2, 112.1, 118.4, 118.6, 118.7, 120.6, 123.7, 126.2, 127.1, 127.2, 127.5, 128.3, 128.9, 129.0, 131.4, 136.8, 137.3, 138.2, 138.6, 139.6, 155.8 (d, J_{PC} = 2.4 Hz), 156.1, 172.1, 173.8 (d, J_{PC} = 7.1 Hz). ^{31}P NMR (81 MHz) δ 45.9, 46.1, 46.8, 47.1. HPLC t_R(2) = 46.0, 47.4, 48.0 min, (4 isomers). HRMS m/z: calcd for $[C_{46}H_{45}N_4O_8P + H]^+$ 813.3053; found 813.3057.

(2*S*)-2-{[3-(1,1′-Biphenyl)-2-{[hydroxyl(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]amino}ethyl)phosphinyl]methyl}-1-oxopropyl]-amino} 1*H*-Indole-3-propanoic Acid (6). Phosphinic pseudodipeptide **14** (65 mg, 0.12 mmol) was suspended in CH_2Cl_2 (1.0 mL), and DIPEA (42 μL, 0.24 mmol), L-tryptophan methyl ester (28 mg, 0.13 mmol), HOBt (16 mg, 0.12 mmol), and EDC·HCl (138 mg, 0.72 mmol) were added to the resulting suspension. The mixture was stirred for 2 h at rt. Then the solvent was evaporated, AcOEt (15 mL) and 1 M HCl (5 mL) were added, and the organic layer was separated and washed with 1 M HCl (2 × 5 mL), H_2O (5 mL) and brine (5 mL). The organic phase was dried over Na_2SO_4, evaporated under vacuum, and the residue was purified by column chromatography ($CHCl_3$/MeOH/AcOH 70:1:1). The resulting tripeptide was suspended in MeOH (5 mL), and 1 M NaOH (2 mL) was added portionwise at 0 °C. After stirring at rt for 48 h, MeOH was evaporated, the residue was diluted with water and acidification followed with the addition of 2 M HCl. The mixture was extracted with AcOEt (3 × 15 mL) and the combined organic layers were washed with H_2O and brine, dried over Na_2SO_4, and evaporated under vacuum. Compound **6** (70 mg, 81%) was obtained as a white solid after addition of Et_2O to the residue and filtration of the precipitate; mp 196−204 °C (dec); R_f = 0.61. ^{1}H NMR (200 MHz) δ 1.57−2.09 (m, 2H, PCH_2), 2.56−3.24 (m, 7H, CHCH_2Ph, ArCH_2CHCONH, CH_2indolyl), 3.82−4.08 (m, 1H, PCH), 4.44−4.64 (m, 1H, NHCHCO), 4.74−5.02 (m, 2H, PhCH_2O), 6.86−7.77 (m, 26H, Ar, indole-NH, OCONH), 8.31 and 8.41 (d, $^3J_{HH}$ = 7.4 Hz, 1H, NHCHCO). ^{13}C NMR (50 MHz) δ 27.1 (d, J_{PC} = 88.6 Hz), 27.6 (d, J_{PC} = 88.6 Hz), 27.3, 27.4, 32.9 (br), 52.1 (d, J_{PC} = 103.2 Hz), 52.6 (d, J_{PC} = 103.2 Hz), 52.9, 53.1, 65.1/65.2, 109.9, 111.5, 118.3, 118.4, 120.1, 123.8, 126.2, 126.4, 126.6, 127.0, 127.1, 127.2, 127.3, 127.6, 128.2, 128.3, 128.9, 129.1, 129.8, 136.2, 136.3, 137.2, 137.3, 137.9, 138.2 (d, J_{PC} = 10.1 Hz), 138.6, 140.1, 140.2, 156.1 (appt, J_{PC} = 3.8 Hz), 173.2, 173.4, 173.5, 173.6. ^{31}P NMR (81 MHz) δ 46.4, 47.2. HPLC t_R(3) = 32.9, 34.2 min (2 isomers). HRMS m/z: calcd for $[C_{43}H_{42}N_3O_7P + H]^+$ 744.2760; found 744.2757.

(1*R*)-1-{[(Benzyloxy)carbonyl]amino}-2-phenylethyl[(2*S*)-2-({[(1*S*)-1-(1*H*-indol-3-ylmethyl)-2-methoxy-2-oxoethyl]amino}-carbonyl)-pent-4-ynyl] Phosphinic Acid (16). For the synthesis of **16**, phosphinic pseudodipeptide **15** (5.0 g, 11.6 mmol) was coupled to L-tryptophan methyl ester according to the synthetic protocol described for the preparation of compound **6**. After the end of the coupling reaction, the mixture was diluted with CH_2Cl_2 (200 mL) and the solution was washed with 1 M HCl (4 × 30 mL), H_2O (30 mL), and brine (30 mL). The organic phase was dried over Na_2SO_4, evaporated under vacuum, and a Et_2O/light petroleum (2:8) mixture was added to the residue. The precipitate was filtered, washed with Et_2O, and recrystallized by CH_2Cl_2 (~10 mL/g). After filtration, compound **16** (2.44 g, 33%) was isolated as a white solid; mp 161−171 °C; R_f = 0.59; $[\alpha]_D^{20}$ = −6.2 (c = 1.0, AcOH). ^{1}H NMR (200 MHz) δ 1.71−2.17 (m, 2H, PCH_2), 2.37−3.29 (m, 8H, CHCH_2Ph, CH_2indolyl, CHCH_2C≡CH), 3.54 (s, 3H, OCH_3), 3.82−4.09 (m, 1H, PCH), 4.45−4.67 (m, 1H, NHCHCO), 4.73−5.06 (m, 2H, PhCH_2O), 6.82−7.60 (m, 16H, Ar, indole-NH), 7.67 (d, $^3J_{HH}$ = 9.4 Hz, 1H, OCONH), 8.42 (d, $^3J_{HH}$ = 7.2 Hz, 1H, NHCHCO). ^{13}C NMR (50 MHz) δ 22.1 (d, J_{PC} = 7.5 Hz), 27.2, 27.4 (d, J_{PC} = 88.7 Hz), 32.9, 38.1, 52.3 (d, J_{PC} = 103.9 Hz), 51.6, 53.1, 65.2, 71.9, 81.7, 109.2, 111.3, 117.9, 118.3, 120.8, 123.7, 126.0, 126.9, 127.1, 127.4, 128.0, 128.1, 128.9, 136.1, 137.6, 138.2 (d, J_{PC} = 14.1 Hz), 156.0 (d, J_{PC} = 4.0 Hz), 172.0, 172.5 (d, J_{PC} = 9.5 Hz). ^{31}P NMR (81 MHz) δ 46.6. HPLC t_R(3) = 27.1 min. ES-MS m/z: calcd for $[C_{34}H_{36}N_3O_7P + H]^+$ 630.2; found 630.2. The filtrates were concentrated and recrystallized by CH_2Cl_2 (~10 mL/g). After filtration, the new filtrates were concentrated and triturated with a Et_2O/light petroleum (3:7) mixture. The mixture was filtrated and a white solid was obtained corresponding to a 1:3 mixture of **16**/**17** (3.21 g, 43%).

(2*S*)-2-({3-[Hydroxyl(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]-amino}ethyl)phosphinyl]-(2*S*)-2-[(3-phenylisoxazol-5-yl)methyl]-1-oxopropyl}amino) 1*H*-Indole-3-propanoic Acid, Methyl Ester (18). Benzaldoxime (1.35 g, 11.1 mmol) was dissolved in $CHCl_3$ (30 mL), and 5 drops of pyridine were added. Then, NCS (1.48 g, 11.1 mmol) was added at rt and after 10 min the resulting mixture was stirred at 45 °C for 3 h. In this solution, phosphinic tripeptide **16** (700 mg, 1.11 mmol) was added, followed by slow addition of Et_3N (1.8 mL, 12.9 mmol) at the same temperature. The reaction mixture was stirred for 4 days at 45 °C. Then, it was diluted with $CHCl_3$ (70 mL) and the resulting solution was washed with 1 M HCl (2 × 30 mL). The organic phase was concentrated (without prior drying) and the residue was recrystallized twice by AcOEt (~25 mL/g). Compound **18** (580 mg, 70%) was isolated as a white solid after filtration of the precipitate and washings with Et_2O; mp 193−195 °C; R_f = 0.61; $[\alpha]_D^{20}$ = −4.4 (c = 0.25, AcOH). ^{1}H NMR (200 MHz) δ 1.73−2.13 (m, 2H, PCH_2), 2.62−2.89 (m, 1H, CHCHHPh), 2.94−3.55 (m, 6H, CHCHHPh, CH_2indolyl, CHCH_2isoxazolyl), 3.40 (s, 3H, OCH_3), 3.83−4.10 (m, 1H, PCH), 4.40−4.60 (m, 1H, NHCHCO), 4.72−5.06 (m, 2H, PhCH_2O), 6.51 (s, 1H, H of isoxazole ring), 6.85−7.87 (m, 22H, Ar, OCONH, indole-NH), 8.73 (d, $^3J_{HH}$ = 7.2 Hz, 1H, NHCHCO). ^{13}C NMR (50 MHz) δ 27.2, 28.5 (d, J_{PC} = 93.0 Hz), 29.4, 32.8, 37.4, 51.7, 52.5 (d, J_{PC} = 103.8 Hz), 53.4, 65.2, 100.2, 109.3, 111.5, 118.0, 118.4, 121.0, 123.9, 126.3, 126.5, 127.0, 127.6, 128.2, 128.3, 128.9, 129.1, 130.1, 136.1, 137.2, 138.4 (d, J_{PC} = 14.1 Hz), 156.1 (d, J_{PC} = 3.4 Hz), 161.7, 171.6, 172.3, 172.8 (d, J_{PC} = 10.9 Hz). ^{31}P NMR (81 MHz) δ 45.4. HPLC t_R(3) = 31.1 min. ES-MS m/z: calcd for $[C_{41}H_{41}N_4O_8P + H]^+$ 749.3; found 749.2.

(2*S*)-2-({3-[Hydroxyl(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]-amino}ethyl)phosphinyl]-(2*R*)-2-[(3-phenylisoxazol-5-yl)methyl]-1-oxopropyl}amino) 1*H*-Indole-3-propanoic Acid, Methyl Ester (19). A 1:3 mixture of **16**/**17** (500 mg, 0.79 mmol), isolated during

the synthesis of **16**, was subjected to 1,3-DCR according to the synthetic protocol followed for the synthesis of **18**. After the end of the reaction, the mixture was diluted with $CHCl_3$ (100 mL) and the resulting solution was washed with 1 M HCl (3 × 20 mL), H_2O (20 mL), and brine (10 mL). The organic phase was dried over Na_2SO_4, evaporated under vacuum, and the residue was refluxed with $CHCl_3$ (50 mL). The hot mixture was filtrated, and the filtrates were concentrated and dissolved to a minimum quantity of $CHCl_3$. A white solid (corresponding to **19**) was precipitated after 24 h at room temperature which was isolated after filtration. A second crop of **19** was obtained after repeating the same crystallization procedure to the filtrates. The combined solids were washed with hot Et_2O to afford compound **19** (350 mg, 59%) as a white solid; mp 218−220 °C; R_f = 0.66; $[\alpha]_D^{20}$ = −28.6 (*c* = 0.50, DMSO). ^{1}H NMR (200 MHz) δ 1.64−1.89 (m, 1H, PC*H*H), 1.93−2.18 (m, 1H, PCH*H*), 2.61−2.82 (m, 1H, CHC*H*HPh), 2.89−3.34 (m, 6H, CHCH*H*Ph, CH_2indolyl, C*H*CH_2isoxazolyl), 3.51 (s, 3H, OCH_3), 3.80−4.08 (m, 1H, PC*H*), 4.40−4.60 (m, 1H, NHC*H*CO), 4.76−5.03 (m, 2H, PhCH_2O), 6.39 (s, 1H, H of isoxazole ring), 6.88−7.76 (m, 22H, Ar, OCON*H*, indole-N*H*), 8.71 (d, $^3J_{HH}$ = 7.0 Hz, 1H, N*H*CHCO). ^{13}C NMR (50 MHz) δ 27.3, 27.5 (d, J_{PC} = 88.5 Hz), 29.7, 32.9, 37.5, 51.3, 51.8, 53.3, 65.1, 100.2, 109.4, 111.5, 118.0, 118.5, 121.1, 123.8, 126.3, 126.4, 127.0, 127.6, 128.2, 128.3, 128.7, 129.1, 130.1, 136.1, 137.2, 138.4 (d, J_{PC} = 14.2 Hz), 156.0 (d, J_{PC} = 3.9 Hz), 161.6, 171.4, 172.2, 172.4 (d, J_{PC} = 9.7 Hz). ^{31}P NMR (81 MHz) δ 46.1. HPLC t_R(3) = 30.7 min. ES-MS *m*/*z*: calcd for $[C_{41}H_{41}N_4O_8P + H]^+$ 749.3; found 749.3.

(2*S*)-2-({3-[Hydroxyl(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]amino}ethyl)phosphinyl]-(2*S*)-2-[(3-phenylisoxazol-5-yl)methyl]-1-oxopropyl}amino) 1*H*-Indole-3-propanoic Acid (5_{F1}). Phosphinic pseudotripeptide **18** (211 mg, 0.28 mmol) was dissolved in MeOH (3 mL), and the solution was cooled to 0 °C. NaOH (1 M, 1.25 mL) was added portionwise, and the mixture was stirred at rt for 24 h. Then MeOH was evaporated, the aqueous residue was acidified with 2 M HCl, and the precipitate was filtrated. Compound $\mathbf{5_{F1}}$ (177 mg, 86%) was obtained as a white solid after washings of the solid product with hot Et_2O; mp 186−189 °C; R_f = 0.49; $[\alpha]_D^{20}$ = −7.8 (*c* = 0.25, AcOH). ^{1}H NMR (500.1 MHz, DMSO-d_6) δ 1.63−1.79 (m, 2H, PCH_2), 2.69 (dt, *J* = 6.0, 13.7 Hz, 1H, CHC*H*HPh), 3.02−3.13 (m, 5H, C*H*CH_2isoxazolyl, C*H*Hindolyl, CHCH*H*Ph), 3.24 (dd, *J* = 4.8, 14.6 Hz, 1H, CH*H*indolyl), 3.69−3.77 (m, 1H, PC*H*), 4.21−4.33 (m, 1H, NHC*H*CO), 4.72 (d, *J* = 13.0 Hz, 1H, PhC*H*HO), 4.91 (d, *J* = 13.0 Hz, 1H, PhCH*H*O), 6.53 (s, 1H, H of isoxazole ring), 6.94−7.72 (m, 21H, Ar, OCON*H*), 8.86 (br s, 1H, N*H*CHCO), 10.83 (d, *J* = 1.8 Hz, 1H, indole-N*H*). ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 26.8, 29.5, 30.7, 33.2, 38.7, 53.1 (d, J_{PC} = 106 Hz), 53.5, 53.9, 64.7, 99.7, 110.3, 111.3, 118.1, 118.2, 120.8, 125.7, 126.4, 126.8, 127.2, 127.3, 127.9, 128.1, 128.8, 128.9, 129.0, 129.8, 136.0, 137.4, 139.6, 155.9, 161.5, 172.2, 173.1, 173.3. HPLC t_R(3) = 29.4 min. HRMS *m*/*z*: calcd for $[C_{40}H_{39}N_4O_8P + H]^+$ 735.2583; found 735.2599.

(2*S*)-2-({3-[Hydroxyl(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]amino}ethyl)phosphinyl]-(2*R*)-2-[(3-phenylisoxazol-5-yl)methyl]-1-oxopropyl}amino) 1*H*-Indole-3-propanoic Acid (5_{F2}). For the synthesis of $\mathbf{5_{F2}}$, phosphinic pseudotripeptide **19** (112 mg, 0.15 mmol) was saponified and treated according to the synthetic protocol described for the preparation of compound $\mathbf{5_{F1}}$. Compound $\mathbf{5_{F2}}$ (103 mg, 94%) was obtained as a white solid; mp 183−185 °C; R_f = 0.52; $[\alpha]_D^{20}$ = −21.5 (*c* = 1.0, DMSO). ^{1}H NMR (500.1 MHz, DMSO-d_6) δ 1.62−1.76 (m, 2H, PCH_2), 2.62−2.70 (m, 1H, CHC*H*HPh), 2.72−2.80 (m, 1H, C*H*Hisoxazolyl), 2.95 (dd, *J* = 7.9, 15.3 Hz, 1H, CH*H*isoxazolyl), 2.98 (dd, *J* = 9.1, 14.8 Hz, 1H, C*H*Hindolyl), 3.06−3.12 (m, 2H, C*H*CH$_2$isoxazolyl, CHCH*H*Ph), 3.18 (dd, *J* = 4.7, 14.8 Hz, 1H, CH*H*indolyl), 3.66−3.81 (m, 1H, PC*H*), 4.34−4.42 (m, 1H, NHC*H*CO), 4.81 (d, *J* = 13.0 Hz, 1H, PhC*H*HO), 4.89 (d, *J* = 13.0 Hz, 1H, PhCH*H*O), 6.47 (s, 1H, H of isoxazole ring), 6.93−7.67 (m, 21H, Ar, OCON*H*), 8.67 (d, *J* = 7.4 Hz, 1H, N*H*CHCO), 10.77 (d, 1H, *J* = 2.2 Hz, indole-N*H*). ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 27.3, 29.8, 29.8, 30.7, 33.1, 38.1, 52.5 (d, J_{PC} = 106 Hz), 54.3, 64.7, 99.9, 110.3, 111.3, 118.1, 118.3, 120.8, 123.5, 125.7, 126.4, 126.8, 127.1, 127.3, 127.9, 128.1, 128.7, 128.9, 129.0, 129.9, 136.1, 137.5, 139.6, 155.8, 161.5, 171.9, 173.0, 173.5. HPLC t_R(3) = 30.7 min. HRMS *m*/*z*: calcd for $[C_{40}H_{39}N_4O_8P + H]^+$ 735.2583; found 735.2587.

3-[Hydroxyl(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]amino}-ethyl)phosphinyl]-2-[(3-phenylisoxazol-5-yl)methyl] Propanoic Acid (20). Benzaldoxime (340 mg, 2.8 mmol) was dissolved in $CHCl_3$ (15 mL), and 3 drops of pyridine were added. Then NCS (374 mg, 2.8 mmol) was added at rt and after 10 min the resulting mixture was stirred at 45 °C for 3−4 h. In this solution, phosphinic dipeptide **15** (300 mg, 0.7 mmol) was added, followed by slow addition of Et_3N (0.58 mL, 4.2 mmol) at the same temperature. The reaction mixture was stirred for 3 days at 45 °C. Then, it was concentrated under vacuum, and the residue was dissolved in 5% $NaHCO_3$ (10 mL). The resulting solution was washed with Et_2O (4 × 10 mL), and the aqueous phase was acidified with 1 M HCl and extracted with AcOEt (5 × 15 mL). The combined organic layers were washed with H_2O (10 mL) and brine (10 mL), dried over Na_2SO_4, and evaporated under vacuum. The reaction was repeated three more times in order to completely consume the unreacted starting material. Compound **20** (320 mg, 83%, 1:1 diastereoisomeric mixture) was obtained as a yellowish solid after addition of Et_2O to the final residue, boiling of the suspension, filtration of the hot mixture, and washings with Et_2O; mp 168−170 °C (dec); R_f = 0.42. ^{1}H NMR δ 1.67−1.93 (m, 1H, PC*H*H), 1.98−2.22 (m, 1H, PCH*H*), 2.59−2.86 (m, 1H, CHC*H*HPh), 2.92−3.29 (m, 4H, CHCH*H*Ph, C*H*CH_2isoxazolyl), 3.76−4.01 (m, 1H, PC*H*), 4.70−4.95 (m, 2H, PhCH_2O), 6.72 (s, 1H, H of isoxazole ring), 6.97−8.07 (m, 16H, Ar, N*H*). ^{13}C NMR δ 27.6 (d, J_{PC} = 88.5 Hz), 27.8 (d, J_{PC} = 88.5 Hz), 29.2, 29.3, 32.7 (br), 37.5, 37.8, 52.3 (d, J_{PC} = 105.0 Hz), 52.6 (d, J_{PC} = 105.0 Hz), 65.2, 100.6, 126.3, 126.6, 127.0, 127.1, 127.6, 128.2, 128.3, 128.8, 129.2, 130.2, 137.3, 138.4, (d, J_{PC} = 13.5 Hz), 156.1 (d, J_{PC} = 3.2 Hz), 161.8, 171.3, 174.5 (d, J_{PC} = 9.8 Hz). 179.5. ^{31}P NMR δ 45.4, 45.6. HPLC t_R(3) = 27.6 min (2 isomers). ES-MS *m*/*z*: calcd for $[C_{29}H_{29}N_2O_7P - H]^+$ 547.2; found 547.3.

(2*S*)-2-({3-[Hydroxyl(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]amino}ethyl)phosphinyl]-(2*S*)-2-[(3-phenylisoxazol-5-yl)methyl]-1-oxopropyl}amino)-3-phenyl Propanoic Acid (7_{F1}) and (2*S*)-2-({3-[Hydroxyl(2-phenyl-(1*R*)-1{[(benzyloxy)carbonyl]amino}ethyl) Phosphinyl]-(2*S*)-2-[(3-phenylisoxazol-5-yl)methyl]-1-oxopropyl}amino)-3-phenyl Propanoic Acid (7_{F2}). Phosphinic pseudodipeptide **20** (150 mg, 0.27 mmol) was suspended in CH_2Cl_2 (2.3 mL), and DIPEA (141 μL, 0.81 mmol), hydrochloric L-phenylalanine *tert*-butyl ester (75 mg, 0.29 mmol), HOBt (37 mg, 0.27 mmol), and EDC·HCl (310 mg, 1.62 mmol) were added to the resulting suspension. The mixture was stirred for 2 h at rt. Then the solvent was evaporated, AcOEt (30 mL) and 1 M HCl (15 mL) were added, and the organic layer was separated and washed with 1 M HCl (2 × 10 mL), H_2O (10 mL), and brine (10 mL). The organic phase was dried over Na_2SO_4, evaporated under vacuum, and the residue was purified by column chromatography ($CHCl_3$/MeOH/AcOH 70:1:1). The resulting tripeptide was suspended in MeOH (10 mL) and 1 M NaOH (4 mL) was added portionwise at 0 °C. After stirring at rt for 24 h, MeOH was evaporated, the residue was diluted with water, and acidification followed with the addition of 2 M HCl. The mixture was extracted with AcOEt (3 × 15 mL), and the combined organic layers were washed with H_2O and brine, dried over Na_2SO_4, and evaporated under vacuum. Compound **7** (165 mg, 87%) was obtained as a white solid after addition of Et_2O to the residue and filtration of the precipitate; mp 172−177 °C (dec); R_f = 0.41. HPLC t_R(3) = 30.1, 31.5 min (2 isomers). ES-MS *m*/*z*: calcd for $[C_{38}H_{38}N_3O_8P + H]^+$ 696.2; found 696.2. Diastereoisomers $\mathbf{7_{F1}}$ and $\mathbf{7_{F2}}$ were isolated after separation by preparative reverse-phase HPLC (AIT column 250 mm × 10 mm, kromasil C18, 10 μm, 3 mL/min) under isocratic elution

(43% CH_3CN in 0. 1% TFA) t_R ($\mathbf{7_{F1}}$) = 30.7 min; t_R ($\mathbf{7_{F2}}$) = 34.7 min.

$\mathbf{7_{F1}}$: ^{1}H NMR (500.1 MHz, DMSO-d_6) δ 1.56−1.60 (m, 2H, PCH_2), 2.59−2.66 (m, 1H, PHCHHPh), 2.83 (dd, J = 5.6, 15.4 Hz, 1H, CHHisoxazolyl), 2.89 (dd, J = 10.5, 13.5 Hz, 1H, CHHCHCOOH), 2.90−2.98 (m, 1H, CHCH_2isoxazolyl), 3.02 (dd, J = 8.5, 15.3 Hz, 1H, CHHisoxazolyl), 3.08 (d, J = 13.0, Hz, 1H, PCHCHHPh), 3.14 (dd, J = 3.9, 13.5 Hz, 1H, CHHCHCOOH), 3.56−3.69 (m, 1H, PCH), 3.98−4.10 (m, 1H, NHCHCO), 4.74 (d, J = 13.1 Hz, 1H, PhCHHO), 4.91 (d, J = 13.1 Hz, 1H, PhCHHO), 6.62 (s, 1H, H of isoxazole ring), 7.06−7.81 (m, 21H, Ar, OCONH), 8.84 (d, J = 7.0 Hz, 1H, NHCHCO). ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 29.9, 30.0, 31.9 (d, J_{PC} = 83.0 Hz), 33.6, 36.5, 39.0, 53.4 (d, J_{PC} = 105 Hz), 55.4, 64.6, 99.8, 125.6, 125.9, 126.5, 126.8, 126.9, 127.3, 127.8, 127.9, 128.1, 128.9, 129.0, 129.0, 129.2, 129.9, 137.5, 139.0, 140.0, 140.2, 155.8, 161.6, 172.4, 172.7, 173.1. HRMS, m/z: calcd for $[C_{38}H_{38}N_3O_8P + H]^+$ 696.2474; found 696.2507.

$\mathbf{7_{F2}}$: ^{1}H NMR (500.1 MHz, DMSO-d_6) δ 1.59−1.63 (m, 2H, PCH_2), 2.59−2.68 (m, 2H, CHHisoxazolyl, PCHCHHPh), 2.81 (dd, J = 10.0, 13.8 Hz, 1H, CHHCHCOOH), 2.90 (dd, J = 8.5, 15.5 Hz, 1H, CHHisoxazolyl), 3.00−3.10 (m, 3H, CHCH_2isoxazolyl, PCHCHHPh, CHHCHCOOH), 3.68−3.78 (m, 1H, PCH), 4.28−4.36 (m, 1H, NHCHCO), 4.82 (d, J = 13.0 Hz, 1H, PhCHHO), 4.89 (d, J = 13.0 Hz, 1H, PhCHHO), 6.57 (s, 1H, H of isoxazole ring), 7.00−7.78 (m, 21H, Ar, OCONH), 8.64 (d, J = 7.9 Hz, 1H, NHCHCO). ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 30.1, 30.2, 31.7 (d, J_{PC} = 88.0 Hz), 33.1, 37.0, 38.4, 52.4 (d, J_{PC} = 106 Hz), 54.9, 64.6, 99.9, 125.6, 126.0, 126.4, 126.8, 127.3, 127.8, 127.9, 128.1, 128.8, 128.9, 129.0, 129.0, 129.9, 137.6, 138.3, 139.9, 155.8, 161.5, 172.0, 173.2, 173.4. HRMS, m/z: calcd for $[C_{38}H_{38}N_3O_8P + H]^+$ 696.2474; found 696.2483.

(2*S*)-2-({3-[Hydroxyl(2-phenyl-(1*R*)-1-{[(benzyloxy)carbonyl]-amino}ethyl)phosphinyl]-2-[(3-phenylisoxazol-5-yl)methyl]-1-oxo-propyl}amino)-3-(4-hydroxy-phenyl) Propanoic Acid (8). For the synthesis of **8**, phosphinic pseudodipeptide **20** (175 mg, 0.32 mmol) was coupled to L-tyrosine(*tert*-butyl ether) *tert*-butyl ester and the product was subjected to saponification according to the synthetic protocol described for the preparation of compound **7**. The saponified product was dissolved in HCO_2H (5 mL), TIS (0.1 mL) was added, and the resulting solution was stirred for 24 h at rt. Then, HCO_2H was evaporated and Et_2O was added to the residue. Compound **8** (205 mg, 90%) was obtained as a white solid after filtration of the precipitate and washing with Et_2O; mp 189−192 °C (dec); R_f = 0.40. HPLC t_R(3) = 26.3, 26.8 min (2 isomers). ES-MS m/z: calcd for $[C_{38}H_{38}N_3O_9P + H]^+$ 712.2; found 712.2. Diastereoisomers $\mathbf{8_{F1}}$ and $\mathbf{8_{F2}}$ were isolated after separation with preparative reverse-phase HPLC (AIT column 250 mm × 10 mm, kromasil C18, 10 μm, 3 mL/min) under isocratic elution (52% CH_3CN in 0. 1% TFA) t_R ($\mathbf{8_{F1}}$) = 21.8 min; t_R ($\mathbf{8_{F2}}$) = 27.8 min.

$\mathbf{8_{F1}}$: ^{1}H NMR (500.1 MHz, DMSO-d_6) δ 1.53−1.64 (m, 2H, PCH_2), 2.64 (dt, J = 5.5, 13.2 Hz, 1H, PCHCHHPh), 2.75 (dd, J = 10.0, 14.0 Hz, 1H, CHHCHCOOH), 2.88 (dd, J = 5.2, 15.0 Hz, 1H, CHHisoxazolyl), 2.91−3.01 (m, 1H, CHCH_2isoxazolyl), 2.97 (dd, J = 5.5, 15.0 Hz, 1H, CHHCHCOOH), 3.04 (t, J = 7.7 Hz, 1H, CHHisoxazolyl), 3.10 (dt, J = 3.5, 14.5 Hz, 1H, PCHCHHPh), 3.59−3.67 (m, 1H, PCH), 3.92−4.01 (m, 1H, NHCHCO), 4.73 (d, J = 13.0 Hz, 1H, PhCHHO), 4.92 (d, J = 13.0 Hz, 1H, PhCHHO), 6.58−6.62 (m. 2H, Hε Tyr), 6.64 (s, 1H, H of isoxazole ring), 6.95−7.81 (m, 18H, Ar, OCONH), 8.86 (d, J = 7.4 Hz, 1H, NHCHCO). ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 29.8, 31.9 (d, J_{PC} = 83.0 Hz), 33.6, 36.2, 38.9, 53.4 (d, J_{PC} = 105 Hz), 55.9, 64.6, 99.7, 114.8, 125.5, 126.5, 126.8, 127.3, 127.8, 128.1, 128.9, 129.0, 129.1, 129.9, 130.1, 137.5, 140.1, 140.2, 155.5, 155.8, 161.6, 172.7, 172.8, 173.2. HRMS, m/z: calcd for $[C_{38}H_{38}N_3O_9P + H]^+$ 712.2424; found 712.2457.

$\mathbf{8_{F2}}$: ^{1}H NMR (500.1 MHz, DMSO-d_6) δ 1.51−1.66 (m, 2H, PCH_2), 2.60−2.67 (m, 2H, PCHCHHPh, CHHisoxazolyl), 2.71 (dd, J = 9.0, 13.7 Hz, 1H, CHHCHCOOH), 2.90−2.95 (m, 2H, CHHCHCOOH, CHHisoxazolyl), 2.99−3.07 (m, 1H, CHCH_2isoxazolyl), 3.09 (d, J = 14 Hz, 1H, PCHCHHPh), 3.63−3.69 (m, 1H, PCH), 4.16 (dt, J = 4.7, 8.4 Hz, 1H, NHCHCO), 4.80 (d, J = 13.0 Hz, 1H, PhCHHO), 4.89 (d, J = 13.0 Hz, 1H, PhCHHO), 6.53−6.58 (m. 2H, Hε Tyr), 6.61 (s, 1H, H of isoxazole ring), 6.93−7.80 (m, 18H, Ar, OCONH), 8.52 (br s, 1H, NHCHCO). ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 30.2, 30.3, 31.5 (d, J_{PC} = 87 Hz), 33.2, 36.4, 38.3, 52.5 (d, J_{PC} = 105 Hz), 55.8, 64.6, 99.9, 114.7, 125.5, 126.4, 126.8, 127.3, 127.8, 128.1, 128.7, 128.9, 129.0, 129.9, 137.6, 140.1, 155.5, 155.8, 161.5, 172.3, 173.1. 173.6. HRMS, m/z: calcd for $[C_{38}H_{38}N_3O_9P + H]^+$ 712.2424; found 712.2443

Inhibitor Potency. Enzymes and inhibitors were incubated for 45 min before the initiation of the reaction by substrate addition. Assays were carried out at 25 °C in 50 mM Hepes (pH 6.8), 200 mM NaCl, 10 μM $ZnCl_2$, and 0.02% Brij-35. Continuous assays were performed by recording the fluorescence increase at 405 nm (ε_{ex} = 320 nm) induced by the cleavage of fluorogenic substrates, using black, flat-bottomed, 96-well nonbinding surface plates (Corning-Costar, Schiphol-RijK, The Netherlands). Fluorescence signals were monitored using a Fluoroscan Ascent photon counter spectrophotometer (Thermo-Labsystems, Courtaboeuf, France) equipped with a temperature control device and a plate shaker. The substrate and enzyme concentrations for the experiments were chosen so as to remain well below 10% of substrate utilization and to observe initial rates. For each inhibitor, percentage inhibition was determined in triplicate experiments at five inhibitor concentrations, chosen to observe a 20−80% range of inhibition. K_i values were determined using the method proposed by Horovitz and Leviski.[36]

ACE. Inhibition assays were performed using Mca-Ala-Ser-Asp-Lys-Dpa-OH from Enzo Life Sciences, as substrate (8 μM) and human somatic ACE (0.5 nM) from R&D systems (929-ZN-010). The K_i values for the N- and C-domains were obtained as previously described.[37]

ECE-1. Inhibition assays were performed using Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)OH from Enzo Life Sciences, as substrate (8 μM, K_m = 4.6 μM) and human ECE-1 (0.1 nM) from R&D Systems (1784-ZN-010). The K_m value was estimated according to the direct linear plot.[38]

NEP. Inhibition assays were performed using Mca-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Dpa-OH from Enzo Life Sciences, as substrate (5 μM, K_m = 2 μM) and human NEP (0.5 nM) from R&D Systems (1182-ZN-010). The K_m value was estimated according to the direct linear plot.[38]

MMP-13. Inhibition assays were performed using Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, as substrate (13 μM, K_m = 8.5 μM) and human MMP-13 (3 nM) from R&D Systems (511-MM-010), as previously described.[39]

***K*$_i$ Values in the Presence of Serum Proteins.** Rat plasma was diluted (1/50 or 1/100) in 50 mM Hepes (pH 6.8), 200 mM NaCl, 10 μM $ZnCl_2$ and 0.02% Brij-35 at 25 °C. ACE (final concentration 1 nM) and inhibitors at various concentrations were incubated in the diluted plasma for 45 min. The inhibition assays were initiated by adding to these solutions Mca-Ala-Ser-Asp-Lys-Dpa-OH, as substrate (15 μM).[24]

Measurement of MAP. Male SHR rats (250 g, 12 weeks old, from Charles River, France) were anesthetized with Nesdonal (50 mg/kg, ip) and instrumented with an electronic transducer (SPR 407, Millar Instruments, Houston, TX) inserted into the femoral artery to measure mean arterial blood pressure (MAP) and with catheters in veins to administer inhibitors. A tracheotomy was performed and a cannula inserted in the trachea to ensure airway patency. The body temperature of the animals was maintained at 37 °C by means of a heating blanket. Following surgery, MAP was allowed to stabilize for 20 min before injection of inhibitors or vehicle (0.09% NaCl) and measured for 60 min (n = 6/dose).

Supporting Information Available: NMR characterization of compounds $\mathbf{5_{F1}}$, $\mathbf{5_{F2}}$, $\mathbf{7_{F1}}$, $\mathbf{7_{F2}}$, $\mathbf{8_{F1}}$, and $\mathbf{8_{F2}}$. This material is available free of charge via the Internet at http://pubs.acs.org.

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