N-Phosphonomethyl Dipeptides and Their Phosphonate Prodrugs, a New Generation of Neutral Endopeptidase (NEP, EC 3.4.24.11) Inhibitors[1]

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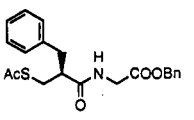
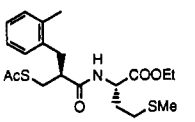
Inhibitors of the zinc protease neutral endopeptidase (NEP, EC 3.4.24.11) offer significant therapeutic interest as antihypertensives due to their ability to potentiate the biological action of the circulating natriuretic hormone ANF (atrial natriuretic factor). *N*-Phosphonomethyl dipeptides bearing a central (4-phenyl)phenylalanine residue have been designed to exert potent and selective NEP inhibition. In particular, (*S*)-3-[*N*-[2-[(phosphonomethyl)amino]-3-(4-biphenylyl)propionyl]amino]propionic acid (**10a**) (CGS 24592) displayed high inhibitory potency in vitro (IC_{50} = 1.9 ± 0.1 nM) and a long plasma half-life in rats but lacked oral bioavailability. This drawback was overcome by using esterase-sensitive (acyloxy)alkyl phosphonates. More remarkable, several diaryl phosphonate derivatives of **10a** also performed as effective prodrugs. Specifically, the structurally simple diphenyl phosphonate **18** (CGS 25462) induced potent inhibition of NEP ex vivo for at least 8 h after oral administration to rats (30 mg/kg). Its antihypertensive effect was demonstrated in DOCA-salt rats. At 30 mg/kg orally, **18** caused a significant reduction in mean arterial pressure measuring −35 ± 7 mmHg at 5-h postdosing. The α-aminomethyl phosphonate **18** represents a new generation of selective NEP inhibitors that combine high potency, long duration of action, and oral bioavailability. Therefore, it holds promise as a novel therapeutic agent for the treatment of human hypertension and congestive heart failure.

Introduction

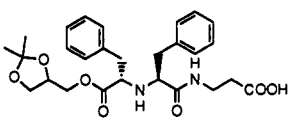
Atrial natriuretic factor (ANF) is a 28-amino-acid peptide secreted into the systemic circulation by heart muscle cells in response to atrial distension. By interacting with specific receptors, ANF elicits a number of responses including natriuresis, diuresis, and vasorelaxation.[2] Interestingly, the biological effects of ANF antagonize those of the renin angiotensin system (RAS).[3] ANF is believed to play an important role, separate from that of the RAS, in the regulation of blood pressure, fluid-volume, and electrolyte homeostasis.[4] The potential of ANF as an antihypertensive and diuretic agent has been recognized and demonstrated in clincical studies. Infusions of low doses of ANF into hypertensive patients have led to a gradual and long-lasting decrease in blood pressure and to an increase in urinary sodium excretion.[5] However, despite the significant efficacy and extreme potency of ANF, its clinical usefulness is limited by a lack of oral bioavailability and a short half-life.[6] One strategy to circumvent these limitations is to prevent ANF inactivation, thereby raising its endogeneous levels. ANF is cleared from the circulation by a receptor-mediated irreversible uptake and by enzymatic hydrolysis.[7,8] We, and others, have shown that the zinc metalloprotease neutral endopeptidase EC 3.4.24.11 (NEP) inactivates ANF in vitro[9-12] and in vivo[13-18] by cleavage of the Cys^{105}–Phe^{106} peptide bond. Although the relative importance of NEP in the metabolism of endogenous ANF remains to be clearly demonstrated,[19,20] NEP inhibitors have evoked, in animal models, pharmacological responses similar to those observed in low-dose ANF infusion studies.[21-23] In particular, lowering of blood pressure and increase of glomerular filtration rate and of urinary cyclic GMP excretion are among the indirect biological effects exerted by NEP inhibitors in DOCA-salt rats, a volume-dependent model of hypertension.[24-27] Despite these encouraging results in animals, recent human clinical studies with several NEP inhibitors such as the thioacetates sinorphan (prodrug of (*S*)-thiorphan) and SCH 42495 (prodrug of SCH 42354) or the carboxylic esters SCH 34826 (prodrug of SCH 32615) and UK 69578 (candoxatril) have shown, at best, only moderate antihypertensive activity.[28-36] Since these modest effects may



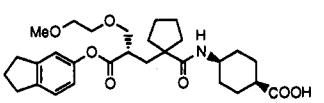
Sinorphan



SCH 42495



SCH 34826



Candoxatril

arise from poor pharmacokinetic properties or insufficient inhibitory potency, we have sought novel types of inhibitors possessing a more favorable pharmacological profile.

In our inhibitor design, we have taken into consideration the possible sites of ANF inactivation. While the kidneys contain most of the NEP activity,[37,38] there is evidence of other extrarenal localizations of NEP, in particular the vasculature, that also contribute to ANF metabolism.[39-41] This observation, in conjunction with the low volume distribution of ANF,[42] suggested that a compound confined to the extracellular volume would be particularly desirable. Such a compound should also be less prone to produce adverse side effects from the modulation of intracellular systems.[43,44] Since highly charged molecules do not readily distribute across cell membranes, phosphorus-

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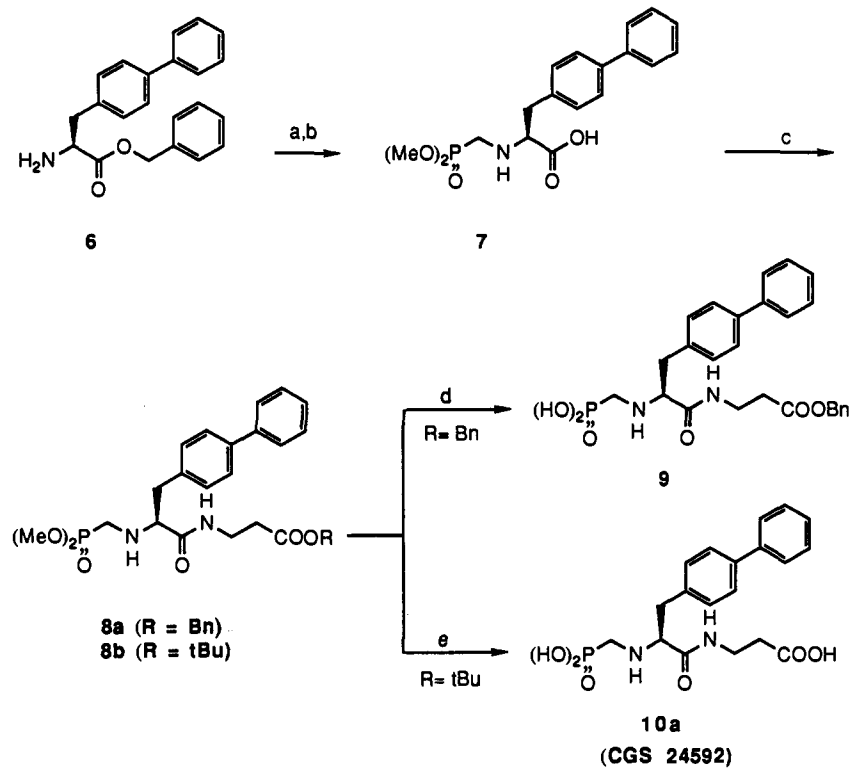
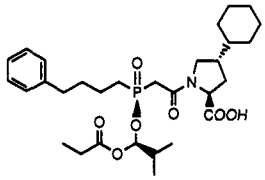
Scheme 1[a]

Method A

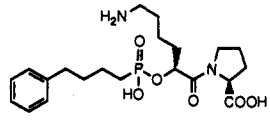


[a] (a) $(MeO)_2OPCH_2OSO_2CF_3$, $(iPr)_2NEt$ (92%); (b) H_2, Pd–C (88%); (c) $H_2N(CH_2)_2COOR$, *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt), Et_3N (**8a**, 100%; 8b, 90%); (d) Me_3SiBr (85%); (e) HBr, AcOH (51%).

containing compounds emerged as attractive candidates. Although few inhibitors of this type have been profiled in vivo, we noted that the angiotensin-converting enzyme (ACE) inhibitors fosinopril[45,46] and ceranapril[47-49] tend to be longer acting than other carboxylic acid- or thiol-containing analogues. However, charged compounds often present significant absorption problems, and a prodrug strategy was likely to be needed to achieve sufficient oral bioavailability.



Fosinopril



Ceranapril

A number of *N*-phosphorylated dipeptides, considered as transition-state analogues,[50] are potent inhibitors of zinc metalloproteases, including NEP.[51-54] The instability of the phosphorus–nitrogen bond under the acidic conditions encountered in the gastrointestinal tract severely limits the value of such compounds in oral formulation. Hence, we considered the replacement of this labile bond with a more stable phosphorus–oxygen or phosphorus–carbon linkage leading to phosphonic acids.[47,48,55,56]

Herein, we report the design of a series of *N*-phosphonomethyl dipeptides exhibiting potent NEP-inhibitory activity in vitro and long duration of action in vivo. We also present two types of orally bioavailable prodrugs that elicit antihypertensive effects in a rodent model.

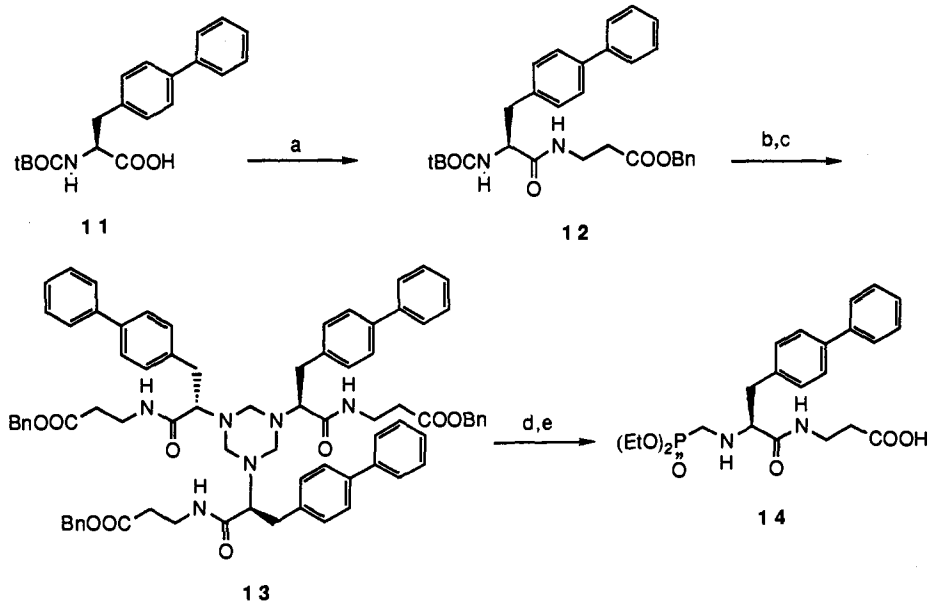
Chemistry

Phosphonates **1–5** (Table 1) were prepared by straightforward modifications of known methods.[48,55,57] The *N*-phosphonomethyl dipeptides were synthesized using one of the two following strategies: In method A (Scheme 1), the starting material, (*S*)-2-[[(dimethylphosphono)methyl]amino]-3-(4-biphenylyl)propionic acid (**7**), was prepared by alkylation of the free base of (*S*)-(4-phenyl)phenylalanine benzyl ester (**6**)[58] with (dimethylphosphono), (diethylphosphono), or (dibenzylphosphono)methyl trifluoromethanesulfonate[59] followed by deprotection of the carboxylic acid by hydrogenolysis. Condensation of **7** with an amino ester under standard peptide coupling conditions provided the protected *N*-phosphonomethyl dipeptide. Subsequent liberation of the phosphonic and carboxylic acids was carried out selectively, using trimethylsilyl bromide[60] or sodium hydroxide, respectively. The order of these two steps could be reversed. The use of a C-terminal *tert*-butyl ester allowed for the simultaneous deprotection of all the acidic functions using hydrobromic acid in acetic acid.[61] The preparation of **9** and **10a** by this method is representative.

Method A was particularly useful when highly nucleophilic and unhindered amines were used in the coupling step. With less activated amines, dimerization of **7** to the corresponding diketopiperazine was a competitive side reaction. Albeit slightly less convergent, method B (Scheme 2) offered an alternative and more generally applicable route to *N*-phosphonomethyl dipeptides. Treatment of an amino ester dipeptide with formaldehyde followed by reaction of the intermediate hexahydrotriazine **13**[62] with a dialkyl phosphite afforded the selectively protected *N*-phosphonomethyl dipeptides.[63,64] Final depro-

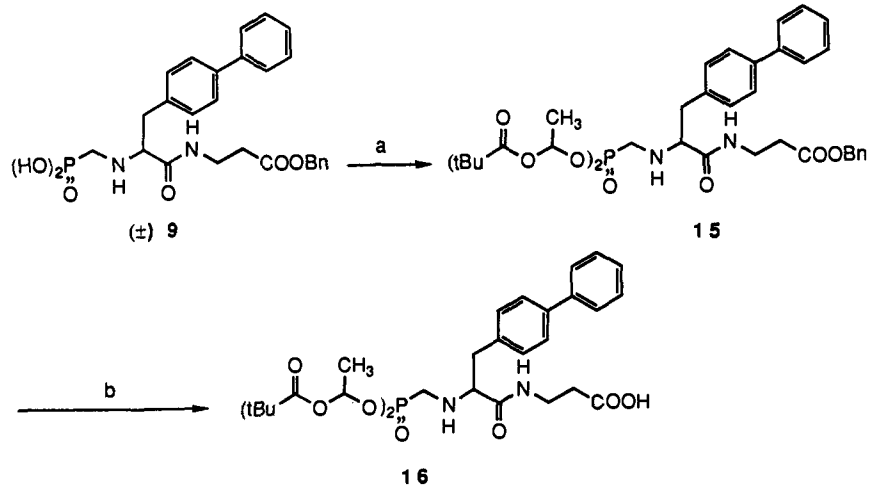
Scheme 2[a]

Method B



[a] (a) $H_2N(CH_2)_2COOBn$-*p*-TosOH, EDC, HOBt, Et_3N (86%); (b) HCl (93%); (c) aqueous HCHO, $NaHCO_3$ (84%); (d) $HPO(OEt)_2$ (56%); (e) H_2, Pd–C (76%).

Scheme 3[a]



[a] (a) $tBuCOOCH(CH_3)Cl$, DBU, NaI (40%); (b) H_2, Pd–C (27%).

tections of the acidic groups were carried out as described above. The preparation of **14** is illustrative.

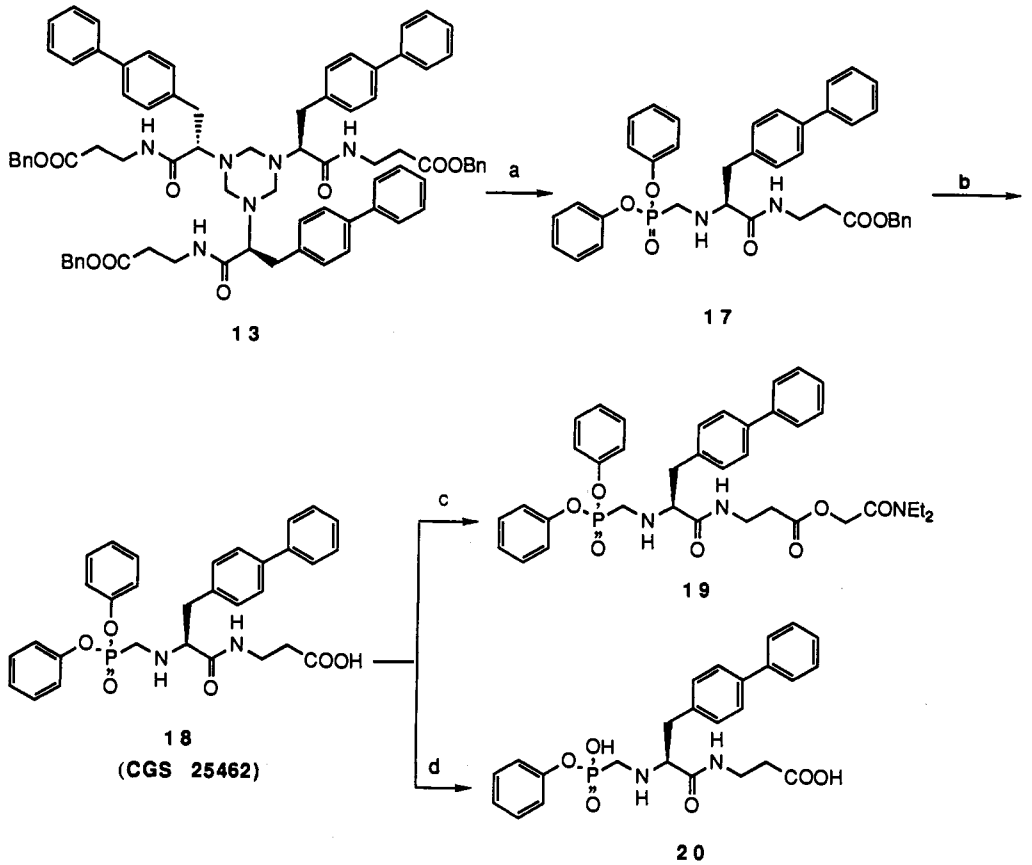
Monoalkyl phosphonates were obtained by selective dealkylation of dialkyl phosphonates with *tert*-butyl amine[65] or excess sodium hydroxide.[66] Bis-alkylation of phosphonic acids with (acyloxy)alkyl halides (Cl, Br, I)[67] in the presence of base afforded the corresponding phosphonates directly, albeit in low yield (Scheme 3). The use of (acyloxy)alkyl chlorides as alkylating agents required the presence of sodium iodide for proper activation. The preparation of **16** as a mixture of stereoisomers is illustrative.

Mono[(acyloxy)alkyl] phosphonates were synthesized by selective cleavage of the corresponding di[(acyloxy)-alkyl] phosphonates with 1 equiv of sodium hydroxide. Diaryl phosphonate derivatives of **10a** were prepared by reacting hexahydrotriazine **13** with a diaryl phosphite[68] (Scheme 4). This reaction occurred without erosion of enantiopurity (see below). Hydrogenolysis of the benzyl esters led to the desired carboxylic acids which could be further derivatized to other C-terminal ester prodrugs[69] by alkylation with an alkyl halide or condensation with an alcohol. Monophenyl phosphonates were obtained selectively by acidic hydrolysis of diphenyl phosphonates. The synthesis of **18** and some derivatives, **19** and **20**, is typical. The optical purity of the intermediate **17** was confirmed by ^1H NMR spectroscopy, using (*R*)-1,1′-binaphthyl-2,2′-diylphosphoric acid (BNPPA) as resolving agent.[70] Alternatively, **18** was analyzed by HPLC on a chiral column.[71] Both methods indicated the presence of a single enantiomer.

Results and Discussion

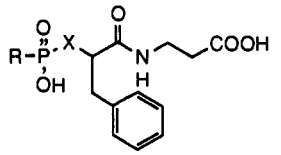
In Vitro NEP Inhibition. In the initial phase of our study, we needed a reliable and predictive method to rapidly assess the ability of the newly synthesized compounds to inhibit NEP.[72] We have shown previously that the synthetic substrate glutaryl-Ala-Ala-Phe-2-naphthylamide (GAAP)[73] constitutes a better predictor than the commonly used substrate [^3H]-Leu-enkephalin for eval-

Scheme 4[a]



[a] (a) $HPO(OPh)_2$ (91%); (b) H_2, Pd–C (86%); (c) $ClCH_2CONEt_2$, Et_3N, NaI (68%); (d) aqueous HCl, THF (95%).

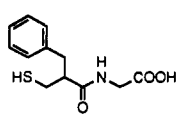
Table 1. Physicochemical Properties and in Vitro NEP-Inhibitory Activity of Phosphonic Acids



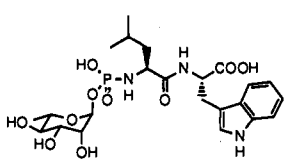
compd[a]	X	R	mp (°C)	formula[b]	analysis	IC_{50} (nM)
1	CH_2	OH	136	$C_{21}H_{26}NO_6P \cdot H_2O$	C, H, N	207
2	CH_2	$O(CH_2)_2Ph$	100–101	$C_{21}H_{26}NO_6P \cdot 3KCl$	C, N, H[c]	>1000
3	O	$(CH_2)_2Ph$	oil	$C_{20}H_{24}NO_6P$	C, H, N	>1000
4	O	$(CH_2)_4Ph$	75	$C_{22}H_{28}NO_6P$	C, H, N	>1000
5	O	CH_2NHCBz[d]	61–64	$C_{21}H_{25}N_2O_6P \cdot {}^1/_2H_2O$	C, H, N	>1000

[a] All compounds are racemic. [b] All compounds show IR and ^1H NMR data consistent with the assigned structures. Analytical results are within ±0.4% of the calculated value unless otherwise indicated. [c] Calcd for H: 4.07. Found: 4.49. [d] Abbreviations: CBz, $COOCH_2Ph$.

uating NEP inhibitors of ANF degradation.[74,75] This assay is amenable to an automated screening and has been used in this study. Both thiorphan (IC_{50} = 4.8 ± 0.3 nM) and phosphoramidon (IC_{50} = 27.1 ± 1.1 nM) served as controls in each round of IC_{50} determinations.



Thiorphan

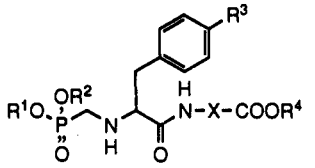


Phosphoramidon

Structure–Activity Relationship (SAR) Studies. In the GAAP assay, the known phosphonic acid 1[76] was a weaker NEP inhibitor (IC_{50} = 207 nM) than phosphoramidon (Table 1). SAR studies with other series of NEP inhibitors had shown that incorporation of lipophilic side chains, presumed to bind to the putative S_1 subsite,[77] usually resulted in an enhanced inhibitory activity.[76] However, phosphonates **2–5**, flanked by hydrophobic groups expected to interact similarly, displayed even lower inhibitory activity than 1 (IC_{50} > 1 μM).

The unfavorable steric hindrance around the zinc chelator, the improper orientation of the lipophilic chain and the absence of an α-amino group as a potential hydrogen-bond donor were considered as plausible causes for the poor binding affinity of these compounds. To overcome these limitations, new compounds were designed in which the N–H group was reintroduced and separated from the phosphonic acid moiety by an additional methylene bridge, leading to a series of α-aminomethyl phosphonic acids.[78] Although such modifications would directly affect the relative position of the zinc-chelating group in the target molecules, a certain degree of flexibility exists in this regard for NEP inhibitors.[79–81] The zinc-

Table 2. Physicochemical Properties and in Vitro NEP-Inhibitory Activity of α-Aminomethyl Phosphonic Acids and Esters[a]

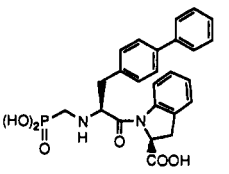


compd	R^1	R^2	R^3	X	R^4	conf[b]	mp (°C)	formula[c]	analysis	method	IC_{50} (nM)[d]
21	H	H	H	$(CH_2)_2$	H	S	244	$C_{13}H_{19}N_2O_6P$	C, H, N	A	1150
10a	H	H	Ph	$(CH_2)_2$	H	S	251–252	$C_{19}H_{23}N_2O_6P$	C, H, N	A	1.9[e]
10b	H	H	Ph	$(CH_2)_2$	H	R	250–251	$C_{19}H_{23}N_2O_6P \cdot H_2O$	C, H, N	B	1800
10c	H	H	Ph	$(CH_2)_2$	H	R,S	245–246	$C_{19}H_{23}N_2O_6P$	C, H, N	A	4
14	Et	Et	Ph	$(CH_2)_2$	H	S	112	$C_{23}H_{31}N_2O_6P$	C, H, N	B	>1000
22	Me	H	Ph	$(CH_2)_2$	H	R,S	270–280	$C_{20}H_{25}N_2O_6P \cdot HCl$	C, H, N[f]	A	301
23	Bn	H	Ph	$(CH_2)_2$	H	S	222–233	$C_{26}H_{29}N_2O_6P$	C, H, N	B	1000
24	H	H	Ph	$(CH_2)_2$	Et	R,S	251	$C_{21}H_{27}N_2O_6P$	C, H, N	A	120
25	H	H	Ph	$(CH_2)_3$	H	S	219–220	$C_{20}H_{25}N_2O_6P$	C, H, N	A	11
26	H	H	Ph	p-C_6H_4	H	S	270–274	$C_{23}H_{23}N_2O_6P \cdot H_2O$	C, H, N	B	10
27	H	H	Ph	CH_2	H	R,S	232	$C_{18}H_{21}N_2O_6P \cdot {}^1/_2H_2O$	C, H, N	A	5
28	H	H	Ph	(S)-CH(CH_3)	H	R,S	222–223	$C_{19}H_{23}N_2O_6P$	C, H, N	A	3
29	H	H	Ph	(S)-CH(Ph)	H	S	244–245	$C_{25}H_{27}N_2O_6P$	C, H, N	A	2
31	H	H	p-F-Ph	$(CH_2)_2$	H	S	230–232	$C_{19}H_{22}FN_2O_6P$	C, H, N	B	14
				thiorphan		R,S					4.8
				phosphoramidon							27

[a] Abbreviations: Ph, phenyl; Bn, benzyl; p-C_6H_4, *para*-phenylene; Py, pyridyl. [b] Configuration of the biphenylalanyl residue. [c] All compounds show IR and ^1H NMR data consistent with the assigned structures. Analytical results are within ±0.4% of the calculated value unless otherwise indicated. [d] NEP inhibition measured using GAAP as substrate ($n = 2$ except where stated otherwise). [e] ±0.1 nM (SEM, $n = 14$). [f] Contains ≤10% inorganic impurity. Calcd: C, 52.58; H, 5.74; N, 6.13. Found: C, 47.68; H, 5.21; N, 5.24.

binding group would then be placed as in the carboxyalkyl dipeptides[24] and the glutaric acid derivatives.[21,82] α-Aminomethyl phosphonic acids, as a class, were particularly attractive due to their stability in acidic medium and their affinity toward zinc.[83] Such compounds have been claimed to be potent inhibitors of the zinc matrix metalloprotease collagenase.[84] However, it had also been reported that replacing the carboxylic acid zinc ligand of the ACE inhibitor enalapril by a phosphonic acid group resulted in a markedly reduced ACE-inhibitory activity.[85,86] Probably for similar reasons, the simple *N*-phosphonomethyl dipeptide **21** displayed only weak NEP inhibition[87] (IC_{50} = 1150 nM) (Table 2).

In an attempt to maximize the interaction of α-aminomethyl phosphonic acids with the NEP active site, additional binding elements were introduced. Interestingly, it was known that in contrast to the glutaric acid and carboxyalkyl dipeptide series of inhibitors, α-aminoalkyl phosphonic acid analogues bearing a lipophilic benzyl group in the α-position did not achieve any significant inhibition of NEP.[66] This observation supports the interpretation that contrary to previous assumptions, a hydrophobic interaction with the S_1 subsite of NEP does not enhance the binding affinity of the inhibitor.[88,89] Instead, substituents at this location may affect the binding conformation of the group chelating to the zinc ion (i.e., mono- versus bidentate). Such substituents (sometimes as small as a methyl group) could exert a favorable effect in the case of carboxylic-acid-based inhibitors but be detrimental to the binding of phosphonic acid analogues. Therefore, we have investigated the structural optimization of the other substituents corresponding to P_1' and P_2'. Previous SAR studies have indicated that the NEP S_1' subsite delineates a large hydrophobic area able to accommodate substituents such as a (4-biphenyl)methyl group.[76] When **21** was modified with this substituent, the resulting compound, **10a**, exhibited a sharp increase in inhibitory potency, being a 600-fold more potent NEP inhibitor (IC_{50} = 1.9 ± 0.1 nM). As in the dicarboxylic acid series, NEP inhibition was associated with the (*S*)-enantiomer **10a**, the (*R*)-enantiomer **10b** being inactive. The racemic derivative **10c** (CGS 24128, IC_{50} = 4.3 nM) was originally prepared and used in our early biological studies. Using rat ANF (r-ANF) as the NEP substrate,[9] an IC_{50} value of 4.7 nM was determined for **10c**, correlating well with the result obtained in the GAAP assay. Esterification of the N-terminal phosphonic or C-terminal carboxylic acid caused a substantial loss of inhibitory potency, as illustrated with **14** and **22–24**. As in other series of NEP inhibitors,[21,82,90–93] some structural flexibility existed in the selection of the amino acid interacting with the S_2' subsite, provided that a free carboxylic acid terminus was present. Elongation (**25, 26**) or shortening (**27**) of the C-terminal residue had minimal influence on the inhibitory potency. Derivatives with C-terminal α-amino acids, such as alanine (**28**) or phenylalanine (**29**), maintained a high inhibitory potency. Incorporation of a cyclic amino acid in P_2', as in **30**, led to an inactive compound, as noticed in other series of NEP inhibitors.[81]



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Modification of the distal phenyl group on the biphenyl moiety with a *p*-fluoro substituent (**31**) resulted in a 7.5-fold decrease in inhibitory potency as compared to **10a**. This observation suggests that the binding of the biphenyl group to the S_1' pocket of NEP is highly sensitive to electronic effects.

Additional in vitro studies with (*S*)-3-[*N*-[2-[(phosphonomethyl)amino]-3-(4-biphenylyl)propionyl]amino]-propionic acid (**10a**) indicated an excellent selectivity for NEP versus other zinc metalloproteases. No inhibition of ACE (EC 3.1.15.1),[94] thermolysin (TLN, EC 3.4.24.4),[95] phosphoramidon-sensitive endothelin-converting enzyme

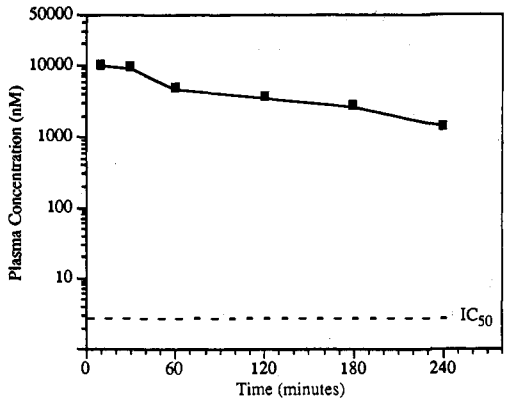


Figure 1. Plasma concentration of free inhibitor **10a** (10 mg/kg iv) measured in conscious DOCA-salt hypertensive rats by an ex vivo NEP-inhibition assay using GAAP as substrate. Values are the mean of four determinations ($p < 0.05$).

(ECE),[96] or stromelysin (SLN, EC 3.4.24.17)[97] was observed with **10a** at concentrations of compound below 20 μM.

Since **10a** displayed the desired in vitro inhibitory potency and selectivity, it was selected for further pharmacological studies.

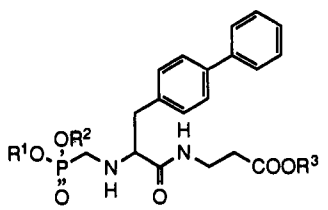
Pharmacokinetic Profile of 10a. The pharmacokinetic profile of **10a** was examined in conscious rats treated intravenously with a 10 mg/kg dose. An ex vivo method was devised in which blood samples from drug-treated animals were collected at intervals and tested in the GAAP assay for NEP inhibition (see Experimental Section). This method allowed the determination of the free (as opposed to protein-bound) fraction of active inhibitor present in the circulation. The results showed that **10a** maintained plasma concentration levels about 2–3 orders of magnitude higher than its IC_{50} for at least 4 h (Figure 1).

The plasma half-life of **10a** in rats was substantially longer than that of other potent NEP inhibitors such as thiorphan.[98–100] However, oral administration of **10a** resulted in extremely low plasma drug levels (<3% bioavailability). The highly charged nature of **10a** under physiological conditions clearly hindered its oral absorption. Addition of hydrolyzable lipophilic or basic chains at the C-terminus, as in **9** and **32–34** (Table 3), failed to promote oral absorption.[101] Subsequent efforts focused on decreasing the net charge of the inhibitor by temporarily masking the phosphonic acid moiety.

(Acyloxy)alkyl Prodrugs. The (acyloxy)alkyl derivatives of phosphates,[102] phosphinates,[103] and phosphonates[104–106] are sensitive to plasma esterases and were hence considered as potential prodrugs of α-aminomethyl phosphonic acids. After oral administration of several di[(acyloxy)methyl] phosphonates (**35–37**) to rats, high plasma concentration levels of active inhibitor **10c** were detected (300–800 times the IC_{50} of the active metabolite) (Table 3).

Other structurally related prodrugs that would not generate carcinogenic formaldehyde as byproduct were also investigated. Substitution of the acetal carbon, as in **16** and **38–41**, afforded compounds of generally lower oral bioavailability, possibly as a result of a reduced interaction with the esterase or a decreased chemical stability in the gastrointestinal tract. Yet, 5-h postdosing, phosphonate **16** produced levels of inhibitor 283-fold higher than the IC_{50} of the parent compound **10c**. Although the substituted proesters were converted in vivo to the single racemic compound **10c**, they were, nevertheless, obtained synthetically as a complex mixture of diastereomers. The mono[(acyloxy)alkyl] phosphonates, accessible as a mixture of two diastereomers, were considered as alternative prodrugs. However, as the phosphonic acid moiety was being only partially uncovered, such compounds were poorly absorbed relative to the corresponding di[(acyloxy)alkyl] phosphonates (data not shown). Derivatization with a very lipophilic acyl substituent, as in **42**, allowed for slightly improved absorption properties. Despite the usefulness of the di[(acyloxy)alkyl] phosphonates as orally bioavailable prodrugs, their physicochemical properties (mixtures of diastereomers, oils, or low-melting amorphous solids) were unsuitable for further development. Hence,

Table 3. Physicochemical Properties of α-Aminomethyl Phosphonic Acid Prodrugs and Plasma Concentration Levels of Active Inhibitor[a]



compd	R^1	R^2	R^3	conf[b]	mp (°C)	formula[c]	analysis	C/IC_{50}[d]
9	H	H	Bn	R,S	258–260	$C_{26}H_{29}N_2O_6P$	C, H, N	ND
32	H	H	$(CH_2)_5NH_2$	R,S	230–232	$C_{24}H_{34}N_3O_6P\cdot HBr$	H, N; C[e]	ND
33	H	H	$(CH_2)_9CH_3$	R,S	232–235	$C_{29}H_{43}N_2O_6P\cdot{}^1/_2H_2O$	C, H, N	ND
34	H	H	CH_2(3-Py)	R,S	218–220	$C_{21}H_{29}N_3O_6PCl\cdot{}^1/_2H_2O$	C, H, N	ND
35	$CH_2O(CO)tBu$	$CH_2O(CO)tBu$	Et	R,S	oil	$C_{33}H_{47}N_2O_{10}P$	C, H, N	816
36	$CH_2O(CO)iPr$	$CH_2O(CO)iPr$	Et	R,S	oil	$C_{31}H_{43}N_2O_{10}P$	C, H, N	535
37	$CH_2O(CO)tBu$	$CH_2O(CO)tBu$	H	R,S	oil	$C_{31}H_{43}N_2O_{10}P$	C, H, N	322
16	CH(Me)O(CO)Et	CH(Me)O(CO)Et	H	R,S	56–60	$C_{33}H_{47}N_2O_{10}P$	C, H, N	283
38	CH(iPr)O(CO)Et	CH(iPr)O(CO)Et	Et	R,S	oil	$C_{35}H_{51}N_2O_{10}P$	C, H, N	503
39	CH(cHex)O(CO)Et	CH(cHex)O(CO)Et	Et	R,S	oil	$C_{41}H_{59}N_2O_{10}P$	C, H, N	64
40	CH(iPr)O(CO)Et	CH(iPr)O(CO)Et	H	R,S	oil	$C_{33}H_{47}N_2O_{10}P$	C, H, N	380
41	CH(iPr)O(CO)iPr	CH(iPr)O(CO)iPr	Et	R,S	oil	$C_{37}H_{55}N_2O_{10}P$	C, H, N	97
42	CH(iPr)O(CO)nHep	H	Et	S	166–167	$C_{33}H_{49}N_2O_6P$	C, H, N	50

[a] Abbreviations: cHex, cyclohexyl (c-C_6H_{11}); nHep, *n*-heptyl (*n*-C_7H_{15}); ND, not detected. [b] Configuration of the biphenylalanine residue. [c] All compounds show IR and ^1H NMR data consistent with the assigned structures. Analytical results are within ±0.4% of the calculated values unless otherwise indicated. [d] Maximum plasma concentration (C, nM) measured ex vivo 4–8 h after oral administration of the compound in DOCA-salt rats at 30 mg/kg and normalized to the IC_{50} of the active metabolite **10a** (IC_{50} = 1.9 nM) or **10c** (IC_{50} = 4 nM). Values are the mean of two to four determinations. [e] Calcd for C: 50.36. Found: 49.91.

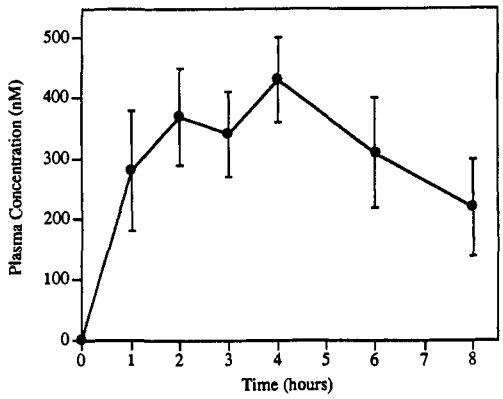


Figure 2. Plasma concentration of active inhibitor **10a** generated by prodrug **18** (30 mg/kg po) and measured in conscious DOCA-salt hypertensive rats by an ex vivo NEP-inhibition assay using GAAP as substrate. Values are the mean ±SEM (n = 3–5).

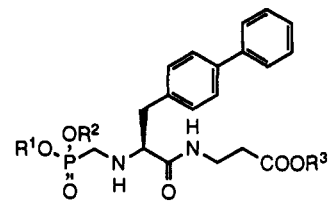
more practical prodrugs, devoid of unnecessary stereogenic centers, were sought.

Dialkyl and Diaryl α-Aminomethyl Phosphonates. Some diethyl esters of α-aminoalkyl phosphonic acids are selectively hydrolyzed enzymatically in vitro, either to the corresponding monoethyl phosphonates or to the free phosphonic acids, by the catalytic action of alkaline phosphatase (AP) or phosphodiesterase I (PDE I), respectively.[107-109] Since these enzymes are ubiquitous in mammals,[110,111] it was tempting to test if they could be invoked in the in vivo hydrolysis of the α-aminomethyl phosphonate **14**. Unfortunately, in this case, no ex vivo inhibitory activity was observed, indicating the resistance of the diethyl phosphonate to hydrolysis (chemical or enzymatic) in rat. In vitro experiments with PDE I and AP confirmed that **14** was not a suitable substrate of these enzymes either. Another bulkier analogue, **43**, also failed to produce NEP inhibition ex vivo.

In a first approximation, diaryl phosphonates can be viewed as mildly activated esters of phosphonic acids.[112] They should undergo more facile hydrolysis than the corresponding dialkyl phosphonates. The overall rate of hydrolysis in vivo would be critical for diaryl phosphonates to perform as efficient phosphonic acid prodrugs.[113,114] As expected,[115] the diphenyl phosphonate **18** was hydrolyzed rapidly with sodium hydroxide and slowly in strongly acidic medium not to **10a** but selectively to the corresponding monophenyl phosphonate **20**, a weak inhibitor in vitro (IC_{50} = 238 nM). The ability of **18** to generate a potent NEP inhibitor in plasma was assessed in rats (30 mg/kg po). The levels of ex vivo NEP inhibition from plasma samples were determined over an 8-h period and correlated with the concentration of the active inhibitor **10a**.[116] Remarkably, 4 h after oral administration of **18**, the concentration levels of **10a** in plasma reached 225 times its IC_{50} value. Even after 8 h, these values were still maintained at 115-fold the IC_{50} (Figure 2). The oral bioavailability of **18** was not enhanced by esterification of its C-terminal carboxylic acid group (see **17**, **19**, **44**, and **45** in Table 4).

To further evaluate the usefulness of diaryl phosphonates as prodrugs, a series of aryl-substituted derivatives of **18** were synthesized (**46–53**). Maximum plasma concentration levels of free inhibitor were generally attained 4–8 h after oral dosing. These values, normalized to the IC_{50} of the presumed bioactive metabolite **10a**, are reported in Table 4. Interestingly, **18** still remained among the most efficient prodrugs in this series. Para substitution of the phenol leaving group with alkyl- or electron-donating groups resulted in a significant loss of NEP inhibition.

Table 4. Physicochemical Properties of Dialkyl and Diaryl Phosphonate Prodrugs and Plasma Concentration Levels of Active Inhibitor[a]



compd	R^1	R^2	R^3	mp (°C)	formula[b]	analysis	^{31}P NMR (δ)	C/IC_{50}[c,d]
14	Et	Et	H	112	$C_{28}H_{31}N_2O_6P$	C, H, N	25.29[e]	ND
43	cHex	cHex	H	86–88	$C_{31}H_{43}N_2O_6P \cdot {}^1/_2H_2O$	C, H, N	16.51[f]	ND
18	Ph	Ph	H	109–110	$C_{31}H_{31}N_2O_6P$	C, H, N	18.46[g]	225[i]
20	Ph	H	H	222–223	$C_{25}H_{27}N_2O_6P$	C, H, N	4.74[g]	41
17	Ph	Ph	Bn	83–84	$C_{38}H_{37}N_2O_6P$	C, H, N	18.39[e]	75
19	Ph	Ph	CH_2CONEt_2	82–88	$C_{37}H_{42}N_3O_7P$	C, H, N	18.72[e]	135
44	Ph	Ph	Et	oil	$C_{33}H_{35}N_2O_6P$	C, H, N	18.41[e]	160
45	Ph	Ph	indanyl	89–91	$C_{40}H_{39}N_2O_6P$	C, H, N	18.39[e]	69
46	(4-Me)Ph	(4-Me)Ph	H	147–149	$C_{33}H_{35}N_2O_6P \cdot {}^1/_2H_2O$	C, H, N	23.89[e]	31
47	(4-nPr)Ph	(4-nPr)Ph	H	139–140	$C_{37}H_{43}N_2O_6P$	C, H, N	20.71[h]	18
48	(4-iPr)Ph	(4-iPr)Ph	H	145–147	$C_{37}H_{43}N_2O_6P$	C, H, N	12.51[f]	40
49	(3,4-diMe)Ph	(3,4-diMe)Ph	H	106–107	$C_{35}H_{39}N_2O_6P \cdot H_2O$	C, H, N	18.00[g]	40
50	(3,5-diMe)Ph	(3,5-diMe)Ph	H	56–58	$C_{25}H_{39}N_2O_6P$	C, H, N	20.35[e]	149
51	indanyl	indanyl	H	126–128	$C_{37}H_{39}N_2O_6P \cdot {}^1/_2H_2O$	C, H, N	12.26[f]	41
52	(4-MeO)Ph	(4-MeO)Ph	H	156–157	$C_{33}H_{35}N_2O_8P \cdot {}^1/_4H_2O$	C, H, N	21.19[g]	5
53	(3-MeO)Ph	(3-MeO)Ph	H	52–54	$C_{33}H_{35}N_2O_8P$	C, H, N	20.59[g]	240
54	Ph	H	Et	233–234	$C_{27}H_{31}N_2O_6P$	C, H, N	8.16[h]	ND
55	Ph	H	Bn	243–244	$C_{32}H_{33}N_2O_6P$	C, H, N	8.98[h]	16
56	Ph	H	CH_2CONEt_2	208–209	$C_{31}H_{39}N_3O_7P$	C, H, N	4.72[f]	9
57	Ph	H	$CH_2CONiPr_2$	208–209	$C_{33}H_{42}N_3O_7P$	C, H, N	4.56[f]	5

[a] All compounds have the (*S*) absolute configuration. [b] All compounds show IR and ^1H NMR data consistent with the assigned structures. Analytical results are within ±0.4% of the calculated value. [c] Plasma concentration (C, nM) measured ex vivo 4 h after oral administration of the compound in DOCA-salt rats at 30 mg/kg and normalized to the IC_{50} of the active metabolite **10a** (IC_{50} = 1.9 nM). Values are the mean of two to four determinations. [d] Abbreviations: ND, not detected. [e] In $CDCl_3$. [f] In DMSO/TFA. [g] In DMSO-d_6. [h] In CD_3OD. [i] ±44 nM (SEM, n = 5).

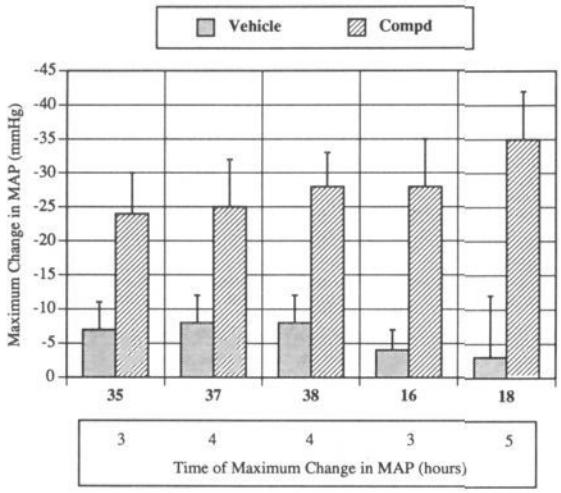


Figure 3. Maximum decrease in mean arterial pressure (MAP) relative to vehicle produced by prodrugs **35**, **37**, **38**, **16**, and **18** (30 mg/kg po) in conscious DOCA-salt hypertensive rats. Values are the mean ±SEM (n = 6–13).

Steric effects impairing the binding to a putative hydrolytic enzyme or the diminished leaving-group ability of the aryloxy moiety could prevent these derivatives from functioning as prodrugs. Such an adverse effect of a para substituent was not detected with similar meta substitution (compare **18** with **52** and **53**). Attempts to prepare diaryl phosphonate analogues bearing electron-withdrawing substituents (p-Cl, o- or p-COOEt) afforded chemically unstable compounds that hydrolyzed readily to the corresponding monoaryl phosphonates during purification. As anticipated, the monophenyl phosphonate **20** was not orally absorbed. Nevertheless, after intravenous administration, it produced potent and long-lasting inhibition of NEP, most likely via conversion to **10a**. Derivatization of the C-terminal carboxylic acid into enzymatically labile esters, such as ethyl, benzyl, or glycolamide esters,[69] did not improve the oral bioavailability of the monoaryl phosphonates (**54**–**57**).

The precise mechanism by which **18** is metabolized to **10a** remains to be elucidated. Since chemical hydrolysis of **18** generates selectivity the monophenyl phosphonate **20**,[117] subsequent conversion in vivo to the phosphonic acid **10a** likely requires a biological catalysis.

Although, upon oral administration, the diphenyl phosphonate **18** was not as efficient in rats as some di[(acyloxy)alkyl] phosphonate prodrugs, it still provided a very favorable pharmacological profile, considering its high inhibitory potency and long half-life in plasma. Among other advantages, **18** is crystalline, carries only one asymmetric center, and can be synthesized readily in optically pure form.

Antihypertensive Action of Diphenyl Phosphonate 18 and Selected Di[(acyloxy)alkyl] Phosphonate Prodrugs. The antihypertensive action of **18** and other selected prodrugs (**35**, **37**, **38**, **16**) was evaluated in the DOCA-salt rat, a model of volume-dependent hypertension, sensitive to NEP inhibitors (Figure 3).

The maximum changes in mean arterial pressure (MAP) generally occurred 3–5 h after oral administration of the prodrugs (30 mg/kg). Among the various compounds tested, **18** showed the most favorable and long-lasting antihypertensive response, albeit slow in onset. The maximum fall in blood pressure (–35 ± 7 mmHg) was reached 5-h postdosing.[118] Such a gradual and progressive hemodynamic effect is consistent with an initial potentiation of endogenous ANF.

Conclusions

We have found that an optimum hydrophobic interaction achieved with a (4-biphenylyl)methyl P_1' substituent allowed N-phosphonomethyl dipeptides such as **10a** (CGS 24592) to become NEP inhibitors rivaling in potency the thiol-containing series (e.g., thiorphan). However, unlike thiorphan, **10a** displayed not only a high specificity for NEP but, more importantly, a notably longer duration of action after intravenous administration in rats. Due to the poor oval bioavailabilty of the α-aminomethyl phosphonic acid **10a**, esterase-sensitive di[(acyloxy)alkyl] phosphonate derivatives were prepared and evaluated. After oral administration in rats, these prodrugs produced potent and long-lasting NEP inhibition. More remarkable, *the structurally simpler diphenyl α-aminomethyl phosphonate* **18** performed as an effective and long-acting orally active prodrug, generating **10a** in the circulation, likely by enzymatic hydrolysis. The two types of prodrugs lowered blood pressure significantly in DOCA-salt rats, probably due to potentiation of endogenous ANF levels. Due to its attractive chemical and biological properties and its unique pharmacological profile, **18** (CGS 25462) has emerged as a promising new therapeutic agent for the treatment of human hypertension and congestive heart failure.

Experimental Section

Melting points (mp) were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Nicolet 5SXB FTIR spectrometer. Proton NMR spectra were recorded on a Bruker AC-250, Varian XL-300, or Varian XL-400 spectrometer. A Bruker AC-300 spectrometer was used for ^{13}C NMR spectra (H-decoupled and DEPT). Proton noise decoupled ^{31}P spectra were recorded on a Varian XL-300 or Varian XL-400 spectrometer. Proton and carbon chemical shifts (δ) are reported in parts per million (ppm) using $CDCl_3$, CD_3OD, or DMSO-d_6 as internal standard. Trifluoroacetic acid (TFA, 1–5%) was occasionally needed to solubilize α-aminomethyl phosphonic acids in DMSO-d_6. Phosphorus chemical shifts are reported relative to 85% aqueous phosphoric acid as external standard (positive shifts are downfield). Optical rotations were measured with a Jasco DIP-370 instrument. Mass spectra were obtained on a Hewlett-Packard GC/MS 5985B spectrometer using chemical ionization (DCI) except for the α-aminomethyl phosphonic acids whose spectra were recorded by fast atom bombardment (FAB) on a Finnigan-Mat TSQ-70 spectrometer or after derivatization in situ to bis- and tris-methylated derivatives by treatment with a saturated ether solution of diazomethane. Microanalyses were carried out at Robertson Laboratory, Inc., Madison, NJ. All organic solvents used were of anhydrous grade. Chromatographic separations were performed under nitrogen pressure (flash chromatography) on silica gel 60 (0.04–0.06 mm)(Baker).

Method A. (*S*)-2-[*N*-[(Dimethylphosphono)methyl]-amino]-3-(4-biphenylyl)propionic Acid (7). To a mixture of (*S*)-4-phenylphenylalanine benzyl ester (8.6 g, 26 mmol) and diisopropylethylamine (6 mL, 34 mmol) in methylene chloride (60 mL) cooled at 0 °C was slowly added a solution of dimethyl [[[(trifluoromethyl)sulfonyl]oxy]methyl]phosphonate[59] (8.5 g, 31 mmol) in methylene chloride (20 mL). The reaction mixture was allowed to warm up to room temperature. After 18 h, the reaction mixture was extracted with saturated sodium bicarbonate and water. The organic layer was dried over anhydrous sodium sulfate, concentrated, and chromatographed, eluting with ethyl acetate, to yield benzyl (*S*)-2-[*N*-[(dimethylphosphono)methyl]-amino]-3-(4-biphenylyl)propionate as an oil (10.9 g, 92.5%). IR (CH_2Cl_2): *1733, 1600, 1240, 1175, 1059, 1035 cm^{-1}*. ^1H *NMR* ($CDCl_3$): δ 2.89 (t, 4H), 3.02 (d, 2H), 3.10 (dd, 1H), 3.69 (d, 3H),

3.71 (d, 3H), 5.11 (AB, 2H), 7.20–7.57 (m, 14H). ^{13}C NMR ($CDCl_3$): δ 14.12, 38.93, 41.85, 43.43, 52.78, 52.80, 52.85, 52.87, 60.29, 63.42, 63.58, 66.69, 126.88, 127.04, 127.13, 128.32, 128.36, 128.49, 128.67, 129.59, 135.25, 135.81, 139.61, 140.70, 173.38. ^{31}P NMR ($CDCl_3$): δ 27.64. $[\alpha]^{25}_D$ = −13.41° (c 1.53 CH_3OH). MS (DCI, CH_4): m/z 454 (MH^+). Anal. ($C_{25}H_{28}NO_5P \cdot H_2O$) C, H, N.

Benzyl (*S*)-2-[*N*-[(dimethylphosphono)methyl]amino]-3-(4-biphenylyl)propionate (5 g, 11 mmol) was dissolved in ethyl acetate (15 mL) and hydrogenated under 50 psi in the presence of 10% palladium on carbon (1 g) for 2 h. The catalyst was filtered and washed with methanol. The filtrate was concentrated under reduced pressure. The residue was triturated with ether to yield **7** as a crystalline solid (3.5 g, 88%): mp 133–134 °C. IR (KBr): 1725, 1618, 1247, 1059, 1029, 867, 826 cm^{-1}. ^{1}H NMR (CD_3OD): δ 2.90–3.01 (m, 2H), 3.09–3.21 (m, 2H), 3.62–3.70 (m, 1H), 3.70 (d, 6H), 7.20–7.45 (m, 5H), 7.53–7.61 (m, 4H). ^{13}C NMR (CD_3OD): δ 39.42, 42.00, 43.58, 48.26, 48.39, 48.48, 48.58, 48.69, 48.79, 48.90, 49.01, 49.22, 49.43, 49.65, 53.80, 53.87, 64.56, 64.69, 127.84, 127.86, 128.24, 129.84, 130.98, 137.89, 142.96, 142.18, 176.25. ^{31}P NMR (CD_3OD): δ 28.92. $[\alpha]^{25}_D$ = +77.24° (c 0.83, CH_3OH). MS (DCI, CH_4): m/z 364 (MH^+). Anal. ($C_{18}H_{22}NO_5P$) C, H, N.

Benzyl (*S*)-3-[*N*-[2-[[(Dimethylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionate (8a). To a stirred solution of (*S*)-2-[*N*-[(dimethylphosphono)methyl]amino]-3-(4-biphenylyl)propionic acid (**7**) (9 g, 24.77 mmol) in DMF (100 mL) were added successively β-alanine benzyl ester *p*-toluenesulfonic acid salt (10 g, 2.99 mmol), triethylamine (4.2 mL, 29.9 mmol), 1-hydroxybenzotriazole (HOBT, 3.35 g, 24.79 mmol), 4-(dimethylamino)pyridine (DMAP, 1.5 g, 12.2 mmol), and *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (EDC, 9.5 g, 49.54 mmol). The solution was stirred at room temperature for 10 h and then poured into ice/water (300 mL) and extracted with ethyl acetate (2 × 300 mL). The combined organic layers were washed successively with water (2 × 150 mL) and saturated sodium bicarbonate (2 × 100 mL) and then dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by chromatography, eluting with 5% methanol in methylene chloride, to yield **8a** as a pale yellow oil (12.9 g, 99.7%). IR ($CDCl_3$): 1732, 1670, 1601, 1521, 1239, 1178, 1060, 1037 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.56 (t, 2H), 2.78 (dd, 1H), 2.85–2.89 (m, 2H), 3.19 (dd, 1H), 3.45 (dd, 1H), 3.53–3.57 (m, 2H), 3.69 (dd, 6H), 5.08 (s, 2H), 7.26–7.58 (m, 14H). ^{13}C NMR ($CDCl_3$): δ 34.07, 34.65, 38.60, 41.80, 43.36, 52.86, 52.88, 64.65, 64.79, 66.41, 126.88, 127.24, 127.36, 128.16, 128.25, 128.30, 128.51, 128.72, 129.47, 135.56, 135.90, 139.88, 140.51, 171.78, 172.28. ^{31}P NMR ($CDCl_3$) δ 27.59. $[\alpha]^{25}_D$ = −23.66° (c 0.86, CH_2Cl_2). MS (DCI, CH_4): m/z 525 (MH^+). Anal. ($C_{28}H_{33}N_2O_6P$) C, H, N.

Benzyl (*S*)-3-[*N*-[2-[(Phosphonomethyl)amino]-3-(4-biphenylyl)propionyl]amino]propionate (9). To an ice-cold solution of (*S*)-3-[*N*-[2-[[(dimethylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionate **8a** (10 g, 19 mmol) in methylene chloride (5 mL) under nitrogen was added dropwise bromotrimethylsilane (12.4 mL, 95.8 mmol). The reaction mixture was allowed to warm up to room temperature. After 4 h, the solvent was evaporated under reduced pressure and ice/water was added to the residue. The suspension was stirred at room temperature for 2 h. The white precipitate was collected by filtration and identified as **9** (8 g, 85%): mp 246–247 °C. IR (KBr): 1736, 1668, 1558, 1173, 1078, 1008, 937, 760, 695 cm^{-1}. ^{1}H NMR (DMSO-d_6/TFA): δ 2.26–2.49 (m, 2H), 3.04–3.17 (m, 2H), 3.21–3.46 (m, 4H), 4.12 (dd, 1H), 4.97 (dd, 2H), 7.23–7.63 (m, 14H). ^{13}C NMR (DMSO-d_6/TFA): δ 33.05, 34.39, 38.37, 40.04, 40.59, 42.49, 61.50, 65.54, 126.45, 126.62, 127.31, 127.81, 127.91, 128.30, 128.82, 129.96, 133.85, 135.88, 139.00, 139.72, 166.17, 166.25, 170.87. ^{31}P NMR (DMSO-d_6/TFA): δ 12.50. $[\alpha]^{25}_D$ = −30.21° (c 0.65, TFA). MS (DCI, CH_4): m/z 525 (dimethylated, MH^+), 539 (trimethylated, MH^+). Anal. ($C_{26}H_{29}N_2O_6P \cdot {}^1/_2H_2O$) C, H, N.

***tert*-Butyl (*S*)-3-[*N*-[2-[[(Dimethylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionate (8b).** (*S*)-2-[*N*-[(dimethylphosphono)methyl]amino]-3-(4-biphenylyl)-propionic acid (**7**) (1.0 g, 2.75 mmol) was stirred with β-alanine *tert*-butyl ester hydrochloride (0.60 g, 3.31 mmol), triethylamine (1.07 mL, 7.63 mmol), HOBt (0.44 g, 2.89 mmol), and *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (EDC, 0.7 g, 3.64 mmol) in DMF (25 mL). After 3 h, the reaction mixture was diluted with ethyl acetate, washed with water, dried over anhydrous sodium sulfate, concentrated, and chromatographed, eluting with a mixture of methylene chloride/methanol/hexanes (76:4:20), to yield **8b** as a colorless oil (1.2 g, 90%). IR (CH_2Cl_2): 1722, 1674, 1519, 1239, 1156, 1059 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.44 (s, 9H), 2.42 (t, 2H), 2.76–3.00 (m, 3H), 3.21 (dd, 1H), 3.44–3.54 (m, 3H), 3.70 (d, 3H), 3.71 (d, 3H), 7.23–7.61 (m, 9H). MS (DCI, CH_4): m/z 491 (MH^+).

(*S*)-3-[*N*-[2-[(Phosphonomethyl)amino]-3-(4-biphenylyl)-propionyl]amino]propionic Acid (10a). A solution of *tert*-butyl (*S*)-3-[*N*-[2-[[[(dimethylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionate (**8b**) (0.4 g, 0.83 mmol) and 30% HBr in glacial acetic acid (9 mL) was stirred at room temperature. After 6.5 h, the solution was concentrated and treated with ether (80 mL). The resulting white solid was filtered, washed with water and ether, and dried under vacuum to yield **10a**, as a white solid (0.167 g, 51%): mp 251–252 °C dec. IR (KBr): 1715, 1658, 1561, 1170, 1067, 761 cm^{-1}. ^{1}H NMR (DMSO-d_6/TFA): δ 2.12–2.38 (m, 2H), 2.94–3.34 (m, 6H), 4.11 (dd, 1H), 7.20–7.70 (m, 9H), 8.41 (t, 1H). ^{13}C NMR (DMSO-d_6/TFA): δ 32.97, 34.44, 34.91, 40.72, 42.15, 61.45, 61.52, 126.42, 126.57, 127.18, 128.69, 129.86, 133.79, 138.99, 139.73, 166.05, 172.40. ^{31}P NMR (DMSO-d_6/TFA): δ 11.90. $[\alpha]^{25}_D$ = +21.73° (c 0.64, water containing 2 molar equiv of sodium hydroxide). MS (FAB, Xe): m/z 407 (MH^+). Anal. ($C_{19}H_{23}N_2O_6P$) C, H, N.

Method B. (*S*)-[*N*-(*tert*-Butoxycarbonyl)-4-phenylphenylalanyl]-β-alanine Benzyl Ester (12). *N*-(*tert*-Butoxycarbonyl)-(*S*)-4-phenylphenylalanine (**11**) (10 g, 29 mmol) was dissolved in DMF (200 mL) and treated sequentially with HOBT (4.7 g, 30.4 mmol), triethylamine (5.3 mL, 37.7 mmol), β-alanine benzyl ester *p*-toluenesulfonate (11.6 g, 34.8 mmol), triethylamine (5.3 mL, 37.7 mmol), and *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (EDC, 7.2 g, 37.7 mmol). The solution was stirred for 2 h at room temperature and then poured into ice/water (400 mL) and extracted with ether (2 × 300 mL). The combined organic layers were washed successively with water (2 × 150 mL), 1 N HCl (100 mL), water (100 mL), and saturated sodium bicarbonate (100 mL). The organic layer was dried over anhydrous sodium sulfate and decolorized with activated carbon. The filtrate was concentrated under reduced pressure to give **12** as a white solid (12.5 g, 86%): mp 103–105 °C. IR (Nujol): 1748, 1733, 1653, 1543, 1525, 1170 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.41 (s, 9H), 2.34–2.59 (m, 2H), 3.01–3.12 (m, 2H), 3.35–3.61 (m, 2H), 4.24–4.37 (m, 1H), 4.90–5.10 (m, 2H), 6.33 (t, 1H), 7.20–7.60 (m, 14H). $[\alpha]^{25}_D$ = +8.93° (c 0.87, CH_3OH). Anal. ($C_{30}H_{34}N_2O_5$) C, H, N.

1,3,5-Tris[(*S*)-1-(1-*O*-benzyl-β-alaninyl)-3-(4-biphenylyl)-1-oxo-2-propyl]hexahydro-1,3,5-triazine (13). A solution of [*N*-(*tert*-butoxycarbonyl)-(*S*)-4-phenylphenylalanyl]-β-alanine benzyl ester (**12**) (12.5 g, 24.9 mmol) in ether (200 mL) and methylene chloride (50 mL) at 0 °C was stirred with gaseous HCl for 5 min. Stirring was then continued at room temperature, and the disappearance of the starting material was monitored by TLC. After about 2 h, the solid was filtered and washed with ether before being dried in vacuo to give (*S*)-(4-phenylphenylalanyl)-β-alanine benzyl ester hydrochloride (10.18 g, 93%, mp 136 °C).

A solution of (*S*)-(4-phenylphenylalanyl)-β-alanine benzyl ester hydrochloride (10.1 g, 23 mmol) in a 1:1 mixture of ethyl acetate and water (400 mL) was cooled to 0 °C. Aqueous formaldehyde (37%, 1.73 mL, 23 mmol) was added followed by solid sodium bicarbonate (1.93 g, 23 mmol). The mixture was stirred at 0 °C for 1 h and then at room temperature for 18 h. The aqueous layer was extracted with ethyl acetate (100 mL). The combined organic layers were washed successively with water (50 mL) and brine (50 mL) and then dried over anhydrous sodium sulfate and evaporated in vacuo to give a white solid. Trituration with anhydrous ether (150 mL) followed by drying under vacuum at 45 °C afforded **13** as a white amorphous solid (7.98 g, 84%). This sensitive material was used as is in the next step. A small fraction was recrystallized from ethyl acetate/hexane: mp 117–118 °C. $[\alpha]^{25}_D$ = −45.93° (c 1.05, CH_2Cl_2). Anal. ($C_{78}H_{75}N_6O_9$) C, H, N.

(S)-3-[N-[2-[[(Diethylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionate (14). A solution of 1,3,5-tris[(S)-1-(1-O-benzyl-β-alaninyl)-3-(4-biphenylyl)-1-oxo-2-propyl]hexahydro-1,3,5-triazine (13) (0.37 g, 0.35 mmol) and diethyl phosphite (0.15 mL, 1.16 mmol) was refluxed for 2 h. The reaction mixture was cooled to room temperature and concentrated in vacuo. The residue was purified by chromatography, eluting with ethyl acetate, to yield the intermediate benzyl (S)-3-[N-[2-[[(diethylphosphono)methyl]amino]-3-(4-biphenylyl)-propionyl]amino]propionate as an oil (0.33 g, 56%). ^{1}H NMR ($CDCl_3$): δ 1.28 (t, 6H), 2.59 (t, 2H), 2.70–2.90 (m, 3H), 3.21 (dd, 1H), 3.40–3.49 (m, 1H), 3.50–3.62 (m, 2H), 3.98–4.16 (m, 4H), 5.10 (s, 2H), 7.23–7.60 (m, 14 H). ^{31}P NMR ($CDCl_3$): δ 25.38.

Benzyl (S)-3-[N-[2-[[(diethylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionate (0.33 g, 0.60 mmol) was dissolved in ethyl acetate (6 mL) and hydrogenated under 50 psi in the presence of 10% palladium on carbon (200 mg) for 2.5 h. The catalyst was filtered and washed with methanol. The filtrate was concentrated under reduced pressure. The residue was triturated with ether to yield 14 as a white amorphous solid (0.21 g, 76%): mp 112 °C. IR (film) 1723, 1648, 1519, 1025, 1224 cm^{-1}. ^{1}H NMR (CD_3OD): δ 1.21 (t, 6H), 2.42 (t, 2H), 2.74–3.10 (m, 4H), 3.39–3.50 (m, 3H), 3.94–4.10 (quintet, 4H), 7.22–7.60 (m, 9H). ^{13}C NMR (CD_3OD): δ 16.84, 16.92, 34.76, 36.33, 40.11, 42.91, 45.00, 63.97, 64.05, 64.14, 65.96, 66.15, 128.03, 128.29, 128.44, 130.00, 131.08, 138.01, 141.25, 142.27, 175.31, 175.65. ^{31}P NMR ($CDCl_3$): δ 25.29. $[\alpha]^{25}_D$ = −15.24° (c 0.96, CH_3CN); $[\alpha]^{25}_D$ = −12.01° (0.52, CH_3OH). Anal. ($C_{23}H_{31}N_2O_6P$) C, H, N.

Benzyl 3-[N-[2-[[[Bis[(α-pivaloyloxy)ethyl]phosphono]-methyl]amino]-3-(4-biphenylyl)-propionyl]amino]propionate (15). A stirred suspension of benzyl 3-[N-[2-[(phosphonomethyl)amino]-3-(4-biphenylyl)propionyl]-amino]propionate (9) (3 g, 6.07 mmol), 1,8-diazabicyclo[5.4.0]-undecene (DBU, 2.3 mL, 5.4 mmol), 1-chloroethyl pivaloate[67] (3 mL, 18.2 mmol), tetrabutylammonium hydrogensulfate (1.03 g, 3.03 mmol), and sodium iodide (0.45 g, 3.03 mmol) in anhydrous acetonitrile (30 mL) was heated to 75 °C under nitrogen for 2 h. The amber mixture was concentrated in vacuo. The residue was partitioned between ethyl acetate (150 mL) and brine (50 mL). The organic layer was dried over anhydrous sodium sulfate and filtered, and the solvent was evaporated under reduced pressure. The residue was purified by chromatography, eluting with ethyl acetate/hexane (45:55), to afford benzyl ester 15 as an oil (mixture of diastereomers, 1.83 g, 40%). IR (CH_2Cl_2): 1738, 1672, 1520, 1481, 1460, 1158, 948 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.06–1.30 (m, 18 H), 1.42–1.55 (m, 6H), 2.51–2.63 (m, 2H), 2.73–3.07 (m, 3H), 3.10–3.23 (m, 1H), 3.38–3.61 (m, 3H), 5.07 (s, 2H), 6.33–6.60 (m, 2H), 7.19–7.61 (m, 14H). MS (DCI, CH_4): m/z 753 (MH^+). Anal. ($C_{40}H_{53}N_2O_{10}P$) C, H, N.

3-[N-[2-[[[Bis[(α-pivaloyloxy)ethyl]phosphono]methyl]-amino]-3-(4-biphenylyl)propionyl]amino]propionic Acid (16). Benzyl 3-[N-[2-[[[bis[(α-pivaloyloxy)ethyl]phosphono]-methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionate (15) (0.55 g, 0.73 mmol) was dissolved in ethyl acetate (20 mL) and hydrogenated under 50 psi in the presence of 10% palladium on carbon (0.5 g) for 3 h. The catalyst was filtered off and washed with ethyl acetate. The filtrate was concentrated under reduced pressure. The residue was purified by chromatography, eluting with 5% methanol in methylene chloride, to yield 16 as a glassy solid (mixture of diastereomers, 0.132 g, 27%): mp 56–60 °C. IR (CH_2Cl_2): 1742, 1671, 1282, 1157, 851 cm^{-1}. ^{1}H NMR (CD_3OD): δ 1.07–1.33 (m, 9H), 1.35–1.57 (m, 6H), 2.30–2.52 (m, 2H), 2.78–2.96 (m, 2H), 2.98–3.12 (m, 3H), 3.38–3.58 (m, 2H), 6.37–6.60 (m, 2H), 7.22–7.66 (m, 9H). MS (DCI, CH_4): m/z 663 (MH^+). Anal. ($C_{33}H_{47}N_2O_{10}P$) C, H, N.

Benzyl (S)-3-[N-[2-[[(Diphenylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionate (17). 1,3,5-Tris[(S)-1-(1-O-benzyl-β-alaninyl)-3-(4-biphenylyl)-1-oxo-2-propyl]-hexahydro-1,3,5-triazine (13) (15 g, 36 mmol) was dissolved in toluene (175 mL) and treated with diphenyl phosphite (8.3 mL, 43 mmol) under a nitrogen atmosphere. The solution was heated at 70 °C for 1.25 h and then cooled to room temperature. Silica gel (80 g) was added, and the reaction mixture was stirred for 1 h. Filtration through silica gel (100 g), eluting with toluene (2 L) and then ethyl acetate (1 L), afforded a clear solution that was concentrated in vacuo. The residue was recrystallized from *tert*-butylmethyl ether (100 mL) to yield 17 (21.3 g, 90.7%): mp 83–84 °C. IR (CH_2Cl_2) 1733, 1674, 1591, 1489, 1087, 940 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.63 (t, 2H), 2.92 (dd, 1H), 3.21–3.41 (m, 3H), 3.55–3.72 (m, 3H), 5.13 (s, 2H), 7.11–7.72 (m, 24H). ^{13}C NMR ($CDCl_3$): δ 34.03, 34.86, 38.39, 42.09, 44.17, 64.49, 64.68, 66.47, 120.40, 120.46, 125.46, 126.99, 127.36, 127.55, 128.30, 128.57, 128.80, 129.64, 129.89, 135.63, 135.68, 140.08, 140.54, 149.89, 150.00, 171.67. ^{31}P NMR ($CDCl_3$): δ 18.39. $[\alpha]^{25}_D$ = −35.40° (c 1.04, $CHCl_3$). MS (DCI, CH_4): m/z 649 (MH^+). Anal. ($C_{38}H_{37}N_2O_6P$) C, H, N.

(S)-3-[N-[2-[[(Diphenylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionic Acid (18). A solution of benzyl (S)-3-[N-[2-[[(diphenylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionyl]amino]propionate (17) (15.6 g, 24.0 mmol) in ethyl acetate (250 mL) was hydrogenated in a Parr shaker in the presence of palladium on carbon (10%, 9 g) at 50 psi for 5.5 h. The catalyst was filtered off and washed with ethyl acetate (200 mL). After concentration of the filtrate in vacuo, the residue was crystallized from ethyl acetate/hexane (1:1) and the resulting crystalline solid dried under reduced pressure to give 18 (11.49 g, 86%). It was recrystallized to constant melting point from anhydrous acetonitrile (8.95 g, 67%): mp 108–109 °C. IR (CH_2Cl_2) 1717, 1672, 1643, 1591, 1488, 1188 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.63 (t, 2H), 2.92 (dd, 1H), 3.21–3.41 (m, 3H), 3.55–3.72 (m, 3H), 5.13 (s, 2H), 7.11–7.72 (m, 24H). ^{13}C NMR ($CDCl_3$): δ 33.85, 34.87, 38.82, 42.26, 44.37, 64.57, 64.75, 120.34, 120.40, 125.47, 126.95, 127.29, 127.43, 128.77, 129.59, 129.85, 136.01, 139.90, 140.55, 149.82, 149.94, 172.91, 174.76. ^{31}P NMR ($CDCl_3$): δ 18.46. $[\alpha]^{25}_D$ = −15.24° (c 0.96, CH_3CN); $[\alpha]^{25}_D$ = −44.24° (c 0.93, $CHCl_3$). Anal. ($C_{31}H_{31}N_2O_6P$) C, H, N.

(N,N-Diethylcarbamoyl)methyl (S)-3-[N-[2-[[(Diphenylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]-amino]propionate (19). To a stirred solution of (S)-3-[N-[2-[[(diphenylphosphono)methyl]amino]-3-(4-biphenylyl)-propionyl]amino]propionic acid (18) (0.6 g, 1.07 mmol) in ethyl acetate (9 mL) was added triethylamine (0.19 mL, 1.34 mmol) followed by 2-chloro-N,N-diethylacetamide (0.18 mL, 1.34 mmol) and sodium iodide (20 mg, 0.13 mmol). The solution was heated under nitrogen at 80 °C for 12 h. The precipitate was filtered off, and the filtrate was treated with water (10 mL) and 2 N HCl (0.5 mL). The organic layer was separated, dried over sodium sulfate, and filtered. After evaporation of the solvent under reduced pressure, the semisolid residue was purified by chromatography, eluting with ethyl acetate. The product, 19, was crystallized from ethyl acetate/hexane (0.89 g, 68%): mp 68–74 °C. IR (CH_2Cl_2) 1747, 1649, 1214, 1189, 938 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.10 (t, 3H), 1.16 (t, 3H), 2.48–2.63 (m, 2H), 2.86–2.97 (m, 1H), 3.06–3.22 (m, 4H), 3.30–3.41 (m, 3H), 3.55–3.67 (m, 3H), 4.68 (s, 2H), 7.02–7.62 (m, 19H), 7.49 (t, 1H). ^{13}C NMR ($CDCl_3$): δ 12.86, 13.94, 34.69, 35.10, 39.02, 40.59, 40.84, 42.17, 44.27, 61.03, 64.97, 65.17, 120.55, 120.56, 120.61, 120.62, 125.19, 126.94, 127.17, 128.73, 129.71, 129.76, 136.60, 139.54, 140.77, 150.04, 150.16, 165.62, 171.43, 172.62. ^{31}P NMR ($CDCl_3$): δ 18.72. $[\alpha]^{25}_D$ = −20.55° (c 0.84, $CHCl_3$). MS (DCI, CH_4): m/z 672 (MH^+). Anal. ($C_{37}H_{42}N_3O_7P$) C, H, N.

(S)-3-[N-[2-[[(Monophenylphosphono)methyl]amino]-3-(4-biphenylyl)propionyl]amino]propionic Acid (20). (S)-3-[N-[2-[[(Diphenylphosphono)methyl]amino]-3-(4-biphenylyl)-propionyl]amino]propionic acid (18) (300 mg, 0.54 mmol) was dissolved in a mixture of tetrahydrofuran (6 mL) and 2 N HCl (3 mL). The solution was stirred at room temperature for 10 h and then diluted with water (5 mL). The precipitate was filtered and washed with water and ether to yield 20 (0.247 g, 95.5%): mp 222–223 °C. IR (KBr): 1715, 1667, 1550, 1216, 1075, 920 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.11–2.35 (m, 2H), 2.75–2.97 (m, 2H), 3.05–3.35 (m, 4H), 4.08–4.19 (m, 1H), 6.95–7.67 (m, 14H), 8.51 (t, 1H). ^{13}C NMR (DMSO-d_6): δ 33.29, 34.77, 35.19, 41.17, 42.99, 61.68, 61.77, 120.66, 120.72, 122.59, 126.55, 126.59, 127.33, 128.89, 129.01, 129.97, 134.55, 128.76, 139.85, 152.77, 152.87, 166.72, 172.56. ^{31}P NMR (DMSO-d_6): δ 4.74. $[\alpha]^{25}_{435Hg}$ = +9.43° (c 1.08, DMSO). Anal. ($C_{25}H_{27}N_2O_6P$) C, H, N.

Enzyme Assay. The in vitro inhibition of neutral endopeptidase (NEP) 3.4.24.11 was determined by the hydrolysis of the substrate glutaryl-Ala-Ala-Phe-2-naphthylamide (GAAP) using a modification of the procedure of Orlowski and Wilk:[119] the incubation mixture (total volume 125 μL) contained washed rat

kidney cortex membrane homogenates[9] (4.2 μg of protein), 50 mM tris buffer (pH 7.4 at 25 °C), 500 μM GAAP (final concentration), and leucine aminopeptidase M (2.5 μg). The mixture was incubated for 10 min at 25 °C, and 100 μl of fast garnet (250 μg of fast garnet/mL of 10% Tween 20 in 1 M sodium acetate, pH 4.2) was added. Enzyme activity was measured spectrophotometrically at 540 nm. One unit of NEP 24.11 activity is defined as 1 nmol of 2-naphthylamine released/min at 25 °C at pH 7.4. To determine IC_{50} values of the inhibitors, increasing concentrations of each compound were preincubated with the membranes. Experiments were carried out in duplicate ($n = 2$), and inhibition curves were constructed on the basis of seven data points near the IC_{50}.

Neutral endopeptidase activity could also be determined using ANF as a substrate.[9] Atrial-natriuretic-factor-degrading activity was determined by measuring the disappearance of rat-ANF (r-ANF) using a 3-min reverse-phase HPLC separation. An aliquot of the enzyme in 50 mM tris–HCl buffer, pH 7.4, was preincubated at 37 °C for 2 min, and the reaction was initiated by the addition of 4 nmol of r-ANF in a total volume of 50 μL. The reaction was terminated after 4 min with the addition of 30 μL of 0.27% TFA. One unit of activity is defined as the hydrolysis of 1 nmol of r-ANF/min at 37 °C at pH 7.4. IC_{50} values were determined as described above.

Ex Vivo NEP Inhibition Assay. Pharmacokinetic studies were conducted in conscious unstrained rats which had been instrumented 24 h earlier with a catheter to obtain femoral arterial blood samples. Blood samples (0.25 mL) were taken, before and at various times after administration of the inhibitor or its prodrug, from the femoral artery and placed into a microfuge tube containing 25 μl of 27.5 mM EDTA. The blood was centrifuged immediately at full speed for 1.2 min at room temperature in a microfuge. The samples were kept at 0 °C. Plasma (150 μL) was pipetted into ultrafiltration units (Amicon product no. 4104, 30 000 MW cutoff) containing 5 μL of 0.1 N HCl in the collection cup. Ultrafiltration was accomplished by centrifuging for 20 min at 3000 rpm at 10 °C (Sorvall). The ultrafiltrates in the collection cups were capped and placed on ice or frozen for later analyses. The amount of unbound active NEP inhibitor present in the plasma samples was measured by the aforementioned GAAP in vitro assay.

Antihypertensive Effects in DOCA-Salt Rats. The antihypertensive effects of the inhibitors were determined in desoxycorticosterone acetate (DOCA)-salt hypertensive rats as follows: DOCA-salt hypertensive rats (280-380 g) with sustained hypertension were prepared by the standard method.[120] Two days before an experiment, the rats were anesthetized with methoxyflurane and instrumented with catheters in the femoral artery to measure arterial blood pressure. Forty-eight hours later, base-line arterial pressure and heart rate were recorded during a 1-h period. The test compound or vehicle was then administered at the indicated dose, and the same cardiovascular parameters were monitored for an additional 5 h.

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(71) HPLC conditions: Ultron ES-OV column; 20 mM KH_2PO_4/acetonitrile (75:25); 5 °C; pH 3.5; flowrate, 0.3 mL/min; limit of detection, 0.1%; retention times (R_t), (S)-enantiomer (CGS 25462) R_t = 39.84 min and (R)-enantiomer R_t = 34.01 min.
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