Phosphinic Derivatives as New Dual Enkephalin-Degrading Enzyme Inhibitors: Synthesis, Biological Properties, and Antinociceptive Activities

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The development of dual inhibitors of the two zinc metallopeptidases, neprilysin (neutral endopeptidase) and aminopeptidase N involved in the inactivation of the opioid peptides, enkephalins, represents an attractive physiological approach in the search for new analgesics devoid of the major drawbacks of morphine. Phosphinic compounds, corresponding to the general formula H_3N^+-CH(R_1)-P(O)(OH)-CH_2-CH(R_2)-CONH-CH(R_3)-COO^-, able to act as transition-state analogues and to fit the S_1, S_1', and S_2' subsites of both enzymes were designed. Selection of the R_1, R_2, and R_3 residues for optimal recognition of these enzymes led to the first dual competitive inhibitors with K_i values in the nanomolar range for neprilysin and aminopeptidase N. These compounds induce potent analgesic responses after intracerebroventricular or intravenous administrations in mice (hot plate test), and several of them were shown to be, at least, 10 times more potent than the previously described dual inhibitors.

Introduction

The wide distribution of the opioid peptides enkephalins and dynorphin, and of their receptors (μ, δ, or κ), has suggested their involvement in a variety of physiological functions. Evidence for their implication in the control of respiratory, cardiovascular, gastrointestinal, or renal functions as well as in pituitary hormonal secretion or in immunological responses has been reported (reviewed in ref 1). However the most studied function of the opioid peptides Met- and Leu-enkephalins, in the central nervous system, concerns the control of pain, which is mainly associated with the activation of the μ opioid receptor.[2] This has been recently confirmed by disruption of the μ receptor gene leading to mice insensitive to morphine treatment.[3] Moreover, a clear demonstration of the antinociceptive properties of the endogenous enkephalins has been addressed by invalidation of the preproenkephalin gene giving mice with enhanced sensitivity to painful stimuli.[4]

These recent results reinforce our early physiological approach aimed at developing new analgesics by potentiating the antinociceptive properties of the endogenous enkephalins. This was achieved by inhibition of their two catabolizing enzymes, i.e., neutral endopeptidase (neprilysin, NEP, EC 3.4.24.11) and aminopeptidase N (APN, EC 3.4.11.2), both enzymes belonging to the family of zinc metallopeptidases (reviewed in ref 5). Selective inhibition of only one of these peptidases gives weak antinociceptive effects[6] due to the complementary role of NEP and APN in enkephalin inactivation, and strong analgesic responses could be obtained only when the two enzymes were jointly blocked.[7] This has been obtained with different classes of enzyme inhibitors,[8,9] which have been shown to induce antinociceptive responses in all animal models of pain where morphine is active and were demonstrated to be devoid of the severe drawbacks of opioids (respiratory depression, tolerance, reduction in intestinal transit, physical or psychic dependence, etc.) (reviewed in ref 5). However these compounds are poorly active after oral administration, limiting their possible use in human therapy. Consequently, a new strategy has been developed for obtaining orally active dual inhibitors.

This could be achieved by taking into account the particularities of both enzyme active sites, i.e., a carboxyl group facing the amino group of the anionic subsite in APN[10] and an arginine side chain interacting with the C-terminal carboxyl group of enkephalins[11] (Figure 1). The main difficulty, in the present case, was to develop a single molecule capable to efficiently block the active site of both an endopeptidase (NEP) and an exopeptidase (APN) with nanomolar affinities.

We have recently synthesized α-aminophosphinic derivatives of general formula H_3N^+-CH(R_1)-P(O)(OH)-CH_2-CH(R_2)-CONH-CH(R_3)-COO^-, interacting with the S_1, S_1', and S_2' subsites of the enzymes and fulfilling the requirements for either selective recognition of APN[12] or dual inhibition of both APN and NEP.[13] In these inhibitors, the phosphinic moiety binds the zinc atom in the catalytic site and mimics the transition-state intermediate formed during the cleavage of the substrate amide bond.[14]

It has been previously shown that a biphenyl group in the P_1' position increases significantly NEP inhibition[15] and that the presence of several aromatic or aliphatic bulky side chains in a pseudo-tripeptide impairs NEP recognition.[16] Therefore, a biphenyl group was introduced in the P_1' position and a methyl group as the P_2' component in the phosphinic inhibitors. These

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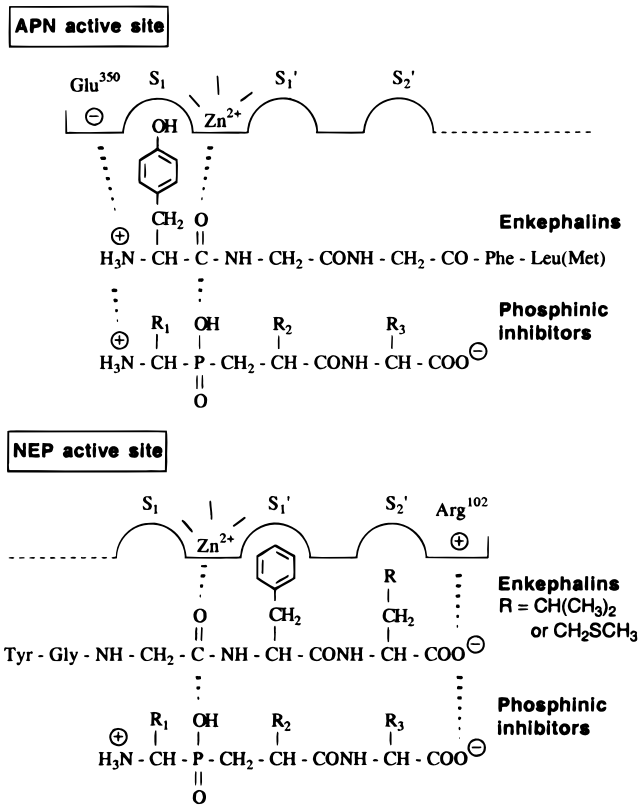


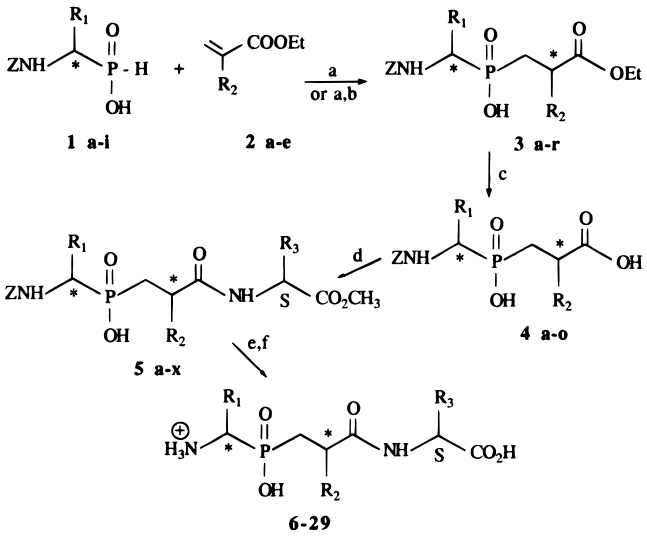
Figure 1. Schematic representation of Leu (or Met) enkephalin and phosphinic inhibitors within the active site of APN (above) and NEP (below).

compounds were the first reported dual competitive inhibitors with nanomolar affinities for both enzymes.[13]

On the basis of these preliminary results, structure−activity studies have been done in this study by using a large series of dual phosphinic inhibitors. Several of these compounds exhibit nanomolar affinities for APN and NEP. Among these compounds, those showing a poor recognition of related zinc metallopeptidases such as angiotensin-converting enzyme (ACE) and endothelin-converting enzyme (ECE) were selected for pharmacological studies. The synthesis, inhibitory potencies, selectivities, and antinociceptive properties, after icv or iv administration of these compounds, are reported in this paper.

Results

Synthesis. The synthetic pathway used for obtaining the various phosphinic inhibitors is depicted in Figure 2. The benzyloxycarbonyl-protected 1-aminoalkylphosphinic acids **1a**−**i** were prepared under racemic forms as previously described.[17] The Michael condensation of these phosphinic acids with various ethyl 2-substituted acrylates **2a**−**e** in the presence of *N*,*O*-bistrimethylsilylacetamide (BSA) led to the key intermediates **3a**−**o**. In the case of biphenyl-containing derivatives **3p**−**r**, a two-step reaction consisting in Michael condensation with the ethyl *p*-bromobenzylacrylate followed by Suzuki arylation using phenylboronic acid was carried out. After alkaline hydrolysis of esters, a coupling step with various amino acids was done followed by sequential cleavage of the protective groups yielding compounds **6**−**29**. These molecules were obtained as mixtures of



a, BSA ; b, $PhB(OH)_2/Pd(PPh_3)_4/2M\ Na_2CO_3$; c, 1N NaOH ; d, (S)-H_2N-CH(R_3)CO_2CH_3/BOP/DIEA ; e, 1N LiOH ; f, BBr_3/CH_2Cl_2.

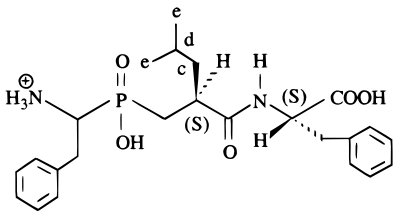
Figure 2. Synthetic pathway used to prepare the phosphinic inhibitors.

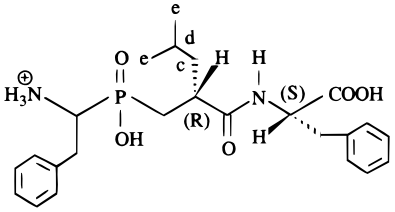
four stereoisomers, due to the presence of two unresolved asymmetric carbons. With the exception of compound **23**, it was not possible to completely separate by HPLC, the four diastereoisomers due to the closeness of their retention times. However, in all cases, two fractions were obtained from HPLC under semipreparative conditions. The first eluted fraction contained a mixture of two isomers (A+B) and the second fraction, the two other stereoisomers (C+D).

The optically pure diastereoisomers needed both for the determination of their absolute configurations and for studying the relationships between stereochemistry and inhibitory potencies were obtained by a slight modification of the synthetic pathway. First, the 1-(*N*-benzyloxycarbonyl)aminoalkylphosphinic acids **1a,e,h** were resolved by crystallization of their salts obtained by using the (+)- and (−)-α-methylbenzylamine as described.[17] The optical purity was checked by HPLC using a chiral column and found to be >99%. The *R* isomers, obtained from (+)-α-methylbenzylamine, and the *S* isomers, from (−)-α-methylbenzylamine, were then submitted to the Michael condensation. The new asymmetric carbon formed, during this step, led to mixtures of two diastereoisomers in different proportion (∼75/25). The (*R*)-α-aminophosphinic acids led to the isomers B (75%) and D (25%), whereas the (*S*)-α-aminophosphinic acids afforded the isomers A (25%) and C (75%), which were separated either by semipreparative HPLC or by chromatography on silica gel. Compounds **10**, **13**, **22**, **23**, **25**, and **28** were obtained by these procedures and correspond to pure B isomers. Compounds **30**−**33** were synthesized directly from the resolved aminophosphinic acids.

Absolute Configuration of the Asymmetric Carbons in the Designed Inhibitors Determined by ^{1}H NMR Spectroscopy. This study has been first performed on compound **23**, for which the four stereoisomers have been separated by semipreparative HPLC. The stereochemistry of the carbon belonging to the aminophosphinic moiety was deduced from the synthetic route (chiral amine separation) as reported above.

Table 1. Correlation between Chemical Shifts of the Leucyl Side Chain Protons and Absolute Configuration of This Residue in the Four Stereoisomers of the Phosphinic Inhibitor **23** and Stereochemical Dependence of the Inhibitory Protencies toward APN and NEP





		δ (ppm)			K_i (nM)[a]	
compd		H(c)	H(d)	H(e)	APN[b]	NEP[c]
23A	*(S,S,S)*	1.36/1.25	1.36	0.78/0.72	32 ± 6	140 ± 10
23B	*(R,S,S)*	1.36/1.18	1.37	0.79/0.72	2.3 ± 0.3	43 ± 4
23C	*(S,R,S)*	1.12/1.04	0.90	0.64/0.58	1700 ± 300	4000 ± 200
23D	*(R,R,S)*	1.17/1.1	0.88	0.64/0.58	1200 ± 100	10000 ± 500

[a] Values are the mean ± SEM from three independent experiments performed in triplicate. [b] APN activity was measured using Ala-pNA as substrate. [c] NEP activity was measured using DGNPA as substrate.

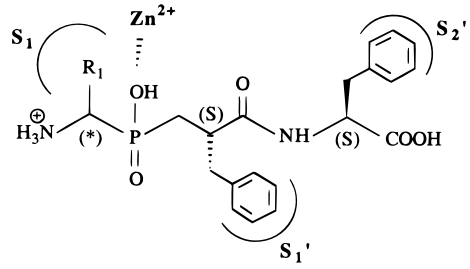
The absolute configuration of the second asymmetric carbon was determined by using a previously described ^{1}H NMR method.[18] Thus, it has been demonstrated that in a dipeptide unit containing one aromatic moiety, the relative absolute configuration of each amino acid could be deduced from the chemical shifts of the nonaromatic side chain protons: a shielding of these protons was observed when the two amino acids have opposite absolute configuration (*R,S* or *S,R*) as compared to those having identical configuration (*R,R* or *S,S*). Thus, as shown in Table 1, the isobutyl protons of **23** are more deshielded in fractions A and B than in fractions C and D. Accounting for the *S* configuration of the C-terminal phenylalanine, the isobutyl side chain has the *R* configuration in C and D and the *S* configuration in A and B. For compounds, such as **6**, which contain two benzyl moieties in positions R_1 and R_2, the same rule was shown to be valid and the chemical shift variations associated with changes in the absolute configuration of these groups were easily observed on the $CH_2\beta$ protons of both residues: a shielding of about 0.2 ppm was observed in the *RS* isomer as compared to the *SS* isomer (data not shown). Moreover, it is interesting to note that the absolute configuration of the α-aminophosphinic moiety has no significant influence on the proton chemical shifts of the other part of the molecule (data not shown). This is very likely related to the flexibility of the α-aminophosphinic moiety, which is linked to a more rigid dipeptide unit by a methylene group (Figure 1).

Similar chemical shift scattering was observed for all described inhibitors leading to the same relationship between the stereochemistry of each isomer and their HPLC elution order.

In Vitro Inhibitory Potency of Phosphinic Compounds on NEP, APN, and ACE. The inhibitory potencies of the four isomers of compound **23** were determined on both NEP and APN (Table 1) showing that the isomers A (*SSS*) and B (*RSS*) in which the P_1' residues have the *S* configuration, found in natural amino acids, are significantly more active than the C (*SRS*) and D (*RRS*) isomers on both enzymes.

In a preliminary screening, the inhibitory potencies of the phosphinic inhibitors were measured on NEP and APN, using the mixtures of stereoisomers (A+B and C+D). As illustrated for compound **23** (Table 1), the fraction A+B is more potent than the fraction C+D by at least 2 orders of magnitude on both enzymes, a result which was verified for all compounds (data not shown). Therefore, only the results corresponding to the A+B mixture are reported and discussed. Compounds reported in Table 2 contain benzyl moieties in the R_2 and R_3 positions for interaction with S_1' and S_2' subsites, respectively, and differ only by R_1 allowing the preference of the S_1 subsite of each enzyme to be investigated. All the inhibitors, except **12**, are very efficient against APN with inhibitory potencies in the nanomolar range, and four compounds, **6**, **7**, **13**, and **14**, which have small linear aliphatic, benzyl, or phenylethyl side chains in the P_1 position, respectively, exhibit K_i values lower than 3 nM. In contrast, the inhibitory potency of these compounds on NEP is only in the 10^{-7} M range (Table

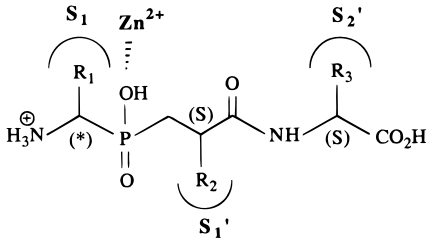
Table 2. Influence of R_1 Substituents of Various Phosphinic Compounds on Their Inhibitory Potencies toward APN and NEP



			K_i (nM)[a]	
compd		R_1	APN[b]	NEP[c]
6	(A+B)	CH_3	2.2 ± 0.2	146 ± 2
7	(A+B)	CH_2CH_3	2.3 ± 0.1	190 ± 10
8	(A+B)	$CH_2CH(CH_3)_2$	3.2 ± 0.5	790 ± 60
9	(A+B)	$CH_2CH_2SCH_3$	6.3 ± 0.3	180 ± 10
10	(A+B)	Ph	4.2 ± 0.5	104 ± 7
11	(A+B)	Ph(*p*-Me)	3.1 ± 0.3	160 ± 10
12	(A+B)	Ph(*o,p*-diMe)	130 ± 7	230 ± 8
13	(A+B)	CH_2Ph	2.9 ± 0.8	190 ± 50
14	(A+B)	CH_2CH_2Ph	2.3 ± 0.4	540 ± 90

[a] Values are the mean ± SEM from three independent experiments performed in triplicate. [b] APN activity was measured using Ala-pNA as substrate. [c] NEP activity was measured using DGNPA as substrate.

Table 3. Inhibitory Potencies of Various Phosphinic Compounds on APN and NEP Activities



compd		R_1	R_2	R_3	K_i (nM)[a] APN[b]	K_i (nM)[a] NEP[c]
13	(A+B)	CH_2Ph	CH_2Ph	CH_2Ph	2.9 ± 0.8	189 ± 70
15	(A+B)		CH_2Ph	CH_3	17.3 ± 0.3	185 ± 40
16	(A+B)		CH_2Ph	$CH_2CH(CH_3)_2$	6.4 ± 0.6	2200 ± 500
17	(A+B)		CH_2Ph	CH_2Ph(*p*-OH)	12.7 ± 1.3	560 ± 30
18	(A+B)		CH_2Ph	CH_2Ph(*p*-Ph)	11.7 ± 1.4	158 ± 10
19	(A+B)		CH_2Ph(*p*-Ph)	CH_3	15 ± 2	2.3 ± 0.1
20	(A+B)		CH_2Ph(*p*-Ph)	CH_2CH_3	33 ± 1	3.3 ± 0.2
21	(A+B)		CH_2Ph(*p*-Ph)	$CH_2CH(CH_3)_2$	6.3 ± 0.7	290 ± 40
22	(A+B)		CH_2Ph(*p*-Ph)	CH_2Ph	17 ± 0.3	150 ± 30
23	(A+B)		$CH_2CH(CH_3)_2$	CH_2Ph	4.6 ± 0.5	80 ± 4
10	(A+B)	Ph	CH_2Ph	CH_2Ph	3.8 ± 0.5	104 ± 7
24	(A+B)		CH_2Ph	Ph	10 ± 1	1100 ± 200
25	(A+B)		CH_2Ph(*p*-Ph)	CH_3	14 ± 1	2.9 ± 0.1
26	(A+B)		$CH_2CH(CH_3)_2$	CH_2Ph	7.6 ± 0.2	110 ± 5
6	(A+B)	CH_3	CH_2Ph	CH_2Ph	2.2 ± 0.2	146 ± 2
27	(A+B)		CH_2Ph(*p*-Ph)	CH_2Ph	3.7 ± 0.3	51 ± 8
28	(A+B)		CH_2Ph(*p*-Ph)	CH_3	5.2 ± 0.4	1.4 ± 0.2
29	(A+B)		CH_2Ph(*p*-iPr)	CH_3	11.4 ± 0.4	15.5 ± 1

[a] Values are the mean ± SEM from three independent experiments performed in triplicate. [b] APN activity was measured using Ala-pNA as substrate. [c] NEP activity was measured using DGNPA as substrate.

2), compounds **6** ($R_1 = CH_3$) and **10** (R_1 = Ph) showing the best affinities for this peptidase.

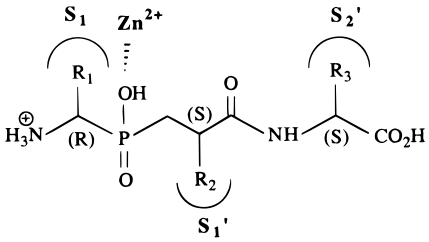
Accounting for these results, three R_1 side chains were selected (methyl, phenyl, and benzyl) for designing new inhibitors containing various R_2 and R_3 residues (Table 3). As compared to **13**, which contains three benzyl side chains in the R_1, R_2, and R_3 positions, all the modifications proposed for R_3 (methyl in **15**, isobutyl in **16**, *p*-hydroxybenzyl in **17,** and biphenylmethyl in **18**) led to less active compounds on APN with K_i values from 6 to 17 nM, without improvement in NEP recognition. The same remark could be made when **10** and **24** are compared. However, a significant increase in NEP inhibition was obtained by introducing both a biphenyl methyl moiety in the R_2 position and a small residue in the R_3 position (compounds **19**, **20**, **25,** and **28**) leading to K_i values in the nanomolar range. In contrast the association of the biphenylmethyl moiety and bulky residues in R_3 (compounds **21**, **22,** and **27**) reduced NEP inhibition.

Regarding APN recognition, the introduction of a biphenyl moiety in the P_1' position resulted in a decreased inhibition except for compounds **21**, **27,** and **28** which have inhibitory potencies lower than 10 nM. Furthermore, introduction in R_2 of an isobutyl side chain (compounds **23** and **26**) or a *p*-isopropylbenzyl group (**29**) was detrimental for both enzymes. From these data, the best arrangement for dual inhibition of both NEP and APN in the phosphinic series was to associate a biphenyl group in R_2 and a methyl group in R_3, with a methyl, phenyl, or benzyl group as the R_1 moiety (compounds **19**, **25,** and **28**). Finally, the most active inhibitors, which have been studied as a mixture of isomers (A+B), were tested under the optically pure B form (*RSS* isomer) (Table 4) resulting as expected in an increased affinity by a factor of around 2.

Some additional modifications in the structure of these very active compounds were investigated: compound **30B**, which contains a *p*-bromobenzyl moiety in the R_2 position, remains very efficient on both enzymes (4.9 and 1.9 nM on NEP and APN, respectively). Three others compounds **31B**−**33B** containing more hydrophilic side chains in the R_3 position were also tested (Table 4). The serine- and threonine-containing inhibitors **31B** and **32B** are less active on NEP with K_i values of 11 and 32 nM, respectively, while the glycine in **33B** improves the recognition of this enzyme (K_i = 0.9 nM). Regarding APN, the serine (**31B**) is well-accepted in the C-terminal position (K_i = 4.9 nM), but introduction of a threonine in **32B** (K_i = 10.2 nM) or a glycine in **33B** (K_i = 32.8 nM) reduces the inhibitory potencies. These compounds were also tested on ACE (Table 4). Compounds **10B**, **25B**, **31B**, and **32B**, which possess a phenyl group in position R_1, are very poor ACE inhibitors. Those containing a CH_3 in the R_1 position are slightly better with K_i values in the 10^{-6}−10^{-7} M range (compounds **28B**, **30B,** and **33B**). In contrast inhibitors bearing a benzyl moiety in R_1 are only 10 times less active on ACE than NEP and therefore poorly selective. Finally, compound **28B** was also tested on ECE inhibitor, and a K_i value of 3.5×10^{-4} M was measured (data not shown).

Analgesic Activity of the Dual Inhibitors. The antinociceptive properties of the most efficient inhibitors were investigated under their optically pure *RSS* isomers, on the mouse hot plate test, following icv administration 15 min before testing.

Table 4. Inhibitory Potencies of the Most Efficient Stereoisomers of α-Aminophosphinic Inhibitors on NEP and APN Activities



				K_i (nM)[a]		
compd	R_1	R_2	R_3	APN[b]	NEP[c]	ACE[d]
13B	CH_2Ph	CH_2Ph	CH_2Ph	1.5 ± 0.05	190 ± 10	660 ± 70
22B	CH_2Ph	CH_2Ph(*p*-Ph)	CH_3	5.3 ± 0.7	2.2 ± 0.3	39 ± 2
23B	CH_2Ph	$CH_2CH(CH_3)_2$	CH_2Ph	2.3 ± 0.3	43 ± 4	440 ± 20
10B	Ph	CH_2Ph	CH_2Ph	4.2 ± 0.1	70 ± 8	13000 ± 1000
25B	Ph	CH_2Ph(*p*-Ph)	CH_3	4.8 ± 0.7	2.0 ± 0.5	2500 ± 500
28B	CH_3	CH_2Ph(*p*-Ph)	CH_3	2.9 ± 0.3	1.2 ± 0.2	120 ± 8
30B	CH_3	CH_2Ph(*p*-Br)	CH_3	1.9 ± 0.1	4.9 ± 0.4	1400 ± 100
31B	Ph	CH_2Ph(*p*-Ph)	CH_2OH	4.9 ± 0.3	11.8 ± 1.2	>5000
32B	Ph	CH_2Ph(*p*-Ph)	$CH(CH_3)OH$	10.2 ± 1.4	32.5 ± 3.6	>20000
33B	Ph	CH_2Ph(*p*-Ph)	H	32.8 ± 2.0	0.94 ± 0.3	7800 ± 600

[a] Values are the mean ± SEM from three independent experiments performed in triplicate. [b] NEP activity was measured using DGNPA as substrate. [c] APN activity was measured using Ala-pNA as substrate. [d] ACE activity was measured using Cbz-Phe-His-Leu as substrate.

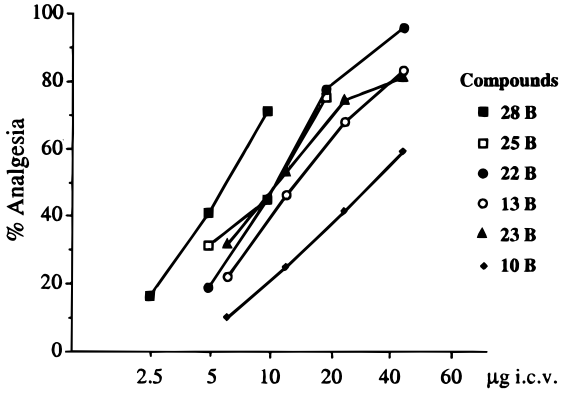


Figure 3. Antinociceptive responses induced by icv administration of various phosphinic inhibitors in the hot plate test in mice (jump response). ED_{50} values: ■ (**28B**) 6 μg; □ (**25B**) 10.5 μg; ● (**22B**) 11 μg; ○ (**13B**) 16 μg; ▲ (**23B**) 13.5 μg; ◆ (**10B**) 35 μg (see tables for formulas and inhibitory potencies of the various compounds).

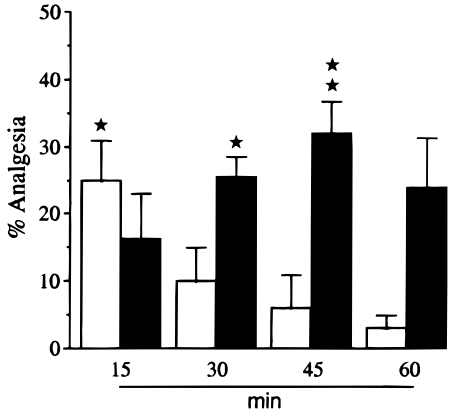


Figure 4. Time course of the antinociceptive responses induced by iv administration of 100 mg/kg **28B** (white columns) and its benzyl ester (black columns) in the hot plate test in mice (jump response).

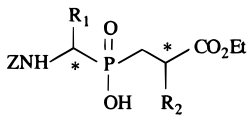
As observed in Figure 3, these compounds gave highly efficient antinociceptive responses on the jump latency time. The statistical analysis of the curves showed that they can be considered as parallel, allowing the relative efficiencies of the studied inhibitors to be compared using their ED_{50} values. The order of increasing efficiency was **10B** (35 μg) < **13B** (16 μg) < **23B** (13.5 μg) < **22B** (11 μg) < **25B** (10.5 μg) < **28B** (6 μg). The more efficient inhibitor compound **28B** gave 100% analgesia at 20 μg.

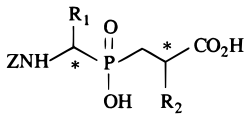
Compound **28B** was then studied on the same test after iv administration. At high doses, 100 mg/kg, **28B** induced significant antinociceptive responses (26% ± 3) in mice (Figure 4). With the same experimental conditions, the benzyl ester of **28B** was iv administered at the dose of 100 mg/kg in mice (Figure 4), and a delayed response and a longer duration of action were observed, with an analgesic peak at 45 min.

Discussion

The aim of this work was to develop a new series of potent dual inhibitors of NEP and APN able to induce in vivo antinociceptive responses by fully protecting the endogenous enkephalins from their degrading enzymes. These two peptidases belong to the thermolysin family of Zn^{2+} metallopeptidases (gluzincins) and have similar mechanisms of action[19] indicating that they can be inhibited by compounds containing a zinc ligand and residues selected for optimal recognition of their main binding subsites. In NEP, there is only two important binding sites, the hydrophobic S_1' and S_2' pockets, and inhibitors blocking these two domains have K_i values in the nanomolar range.[20] For APN, its exopeptidase nature requires essentially the occupancy of both the S_1 and the anionic subsites responsible for its N-terminus specificity,[21] but additional interactions with the S_1' and S_2' subsites have been shown to enhance the affinity of the inhibitors for this enzyme.[12] These requirements were fulfilled with α-aminophosphinic compounds giving highly potent APN inhibitors. Struc-

Table 5. Physical Data for 2-Alkyl-3-[hydroxy[1′-(*N*-benzyloxycarbonylamino)-2′-alkyl]phosphinyl]propanoate Derivatives **3** and **4**

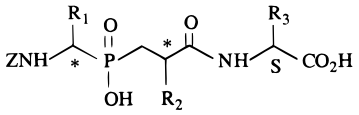




compd	R_1	R_2	yield (%)	mp (°C)	HPLC t_R, min (B%)	compd	yield (%)	mp (°C)	HPLC t_R, min (B%)	MS (ESI) ($M^+ + 1$)
3a	CH_3	CH_2Ph	68.9	154−155	9.1 (45)	**4a**	90.2	175−178	4.2 (65)	406.1
3b	CH_3	CH_2Ph(*p*-Br)	90	120−122	6.5 (60)	**4b**	98	176−177	4.2 (60)	484.1, 486.1
3c	CH_3	CH_2Ph(*p*-iPr)	77	110	9.4, 9.7 (60)	**4c**	96	158	5.4 (60)	448.2
3d	CH_2CH_3	CH_2Ph	64	104	7.1 (50)	**4d**	97	122	5.3 (50)	420.0
3e	$CH_2CH(CH_3)_2$	CH_2Ph	74.2	150−152	9.1, 9.4 (55)	**4e**	97	159−162	6.1 (55)	448.2
3f	$CH_2CH_2SCH_3$	CH_2Ph	52	120−122	8.0, 8.1 (50)	**4f**	65	134	5.8, 5.9 (48)	466.1
3g	Ph	CH_2Ph	55	152	6.0 (60)	**4g**	77	164	4.5 (60)	468.0
3h	Ph	CH_2Ph(*p*-Br)	88	191−193	7.5 (60)	**4h**	NP[a]			
3i	Ph	$CH_2CH(CH_3)_2$	51	147−149	5.8, 7.0 (60)	**4i**	96	160	7.2 (45)	434.1
3j	Ph(*p*-Me)	CH_2Ph	51.6	176−178	22.2 (45)	**4j**	90	182−184	11.7 (45)	482.0
3k	Ph(*o*,*p*-diMe)	CH_2Ph	51.2	170−172	7.7 (60)	**4k**	73	186−188	5.3 (60)	496.1
3l	CH_2Ph	CH_2Ph	67	161−163	23.2, 24.2 (45)	**4l**	100	178−179	12.6 (45)	482.0
3m	CH_2Ph	CH_2Ph(*p*-Br)	84.1	124−125	10.2, 10.7 (60)	**4m**	NP[a]			
3n	CH_2Ph	$CH_2CH(CH_3)_2$	81.3	143−144	7.7, 8.0 (60)	**4n**	99.8	170−172	4.7 (60)	448.21
3o	CH_2CH_2Ph	CH_2Ph	85.9	163−166	35.6 (45)	**40**	99.7	179−180	176, 18.1 (45)	496.1
3p	CH_3	CH_2Ph(*p*-Ph)	85	198	8.2 (60)	**4p**	88.2	216−218	4.2 (66)	482.0
3q	Ph	CH_2Ph(*p*-Ph)	82	163−164	15.2 (60)	**4q**	85	184	7.1 (60)	544.0
3r	CH_2Ph	CH_2Ph(*p*-Ph)	80	164−165	13.9, 14.6 (60)	**4r**	97.4	180−182	7.1, 8.0 (60)	558.2

[a] NP = not prepared.

Table 6. Physical Data for 2-Alkyl-3-[hydroxy[1′-(*N*-benzyloxycarbonylamino)-2′-alkyl]phosphinyl]propanoyl Amino Acids **5**



compd	R_1	R_2	R_3	yield (%)	mp (°C)	HPLC t_R, min (B%)
5a	CH_3	CH_2Ph	CH_2Ph	81.3	175−177	10.0, 14.3 (45)
5b	CH_3	CH_2Ph(*p*-Br)	CH3	74	178−180	4.3, 4.5 (60)
5c	CH_3	CH_2Ph(*p*-Ph)	CH_2Ph	56	186−188	6.1, 6.9 (60)
5d	CH_3	CH_2Ph(*p*-Ph)	CH_3	58.5	162−164	4.4, 5.1 (60)
5e	CH_3	CH_2Ph(*p*-iPr)	CH_3	43	230	7.2, 8.7 (50)
5f	CH_2CH_3	CH_2Ph	CH_2Ph	92	200	6.8, 8.4 (50)
5g	$CH_2CH(CH_3)_2$	CH_2Ph	CH_2Ph	69.9	192−193	6.2, 7.5 (60)
5h	$CH_2CH_2SCH_3$	CH_2Ph	CH_2Ph	47.5	152	5.5, 6.9 (55)
5i	Ph	CH_2Ph	CH_2Ph	31	152	4.6, 6.1 (60)
5j	Ph	CH_2Ph	Ph	81	220	7.5, 9.2 (60)
5k	Ph	CH_2Ph(*p*-Ph)	CH_3	80	156	9.8, 11.7 (50)
5l	Ph	$CH_2CH(CH_3)_2$	CH_2Ph	79	147−149	5.6, 7.1 (60)
5m	Ph(*p*-Me)	CH_2Ph	CH_2Ph	60	166−168	7.7, 9.6 (55)
5n	Ph(*o*,*p*-diMe)	CH_2Ph	CH_2Ph	61.5	184−185	7.0, 8.6 (60)
5o	CH_2Ph	CH_2Ph	CH_3	93	192−195	6.3, 7.4 (50)
5p	CH_2Ph	CH_2Ph	$CH_2CH(CH_3)_2$	77.6	196−199	5.4, 6.9 (60)
5q	CH_2Ph	CH_2Ph	CH_2Ph	91	203−205	8.6, 9.6 (60)
5r	CH_2Ph	CH_2Ph	CH_2Ph(*p*-OH)	68	207−209	13.9, 14.8, 16.1 (42)
5s	CH_2Ph	CH_2Ph	CH_2Ph(*p*-Ph)	56	210−212	10.5, 13.0, 13.6 (60)
5t	CH_2Ph	CH_2Ph(*p*-Ph)	CH_3	97.4	180−182	6.2, 7.2, 7.5 (60)
5u	CH_2Ph	CH_2Ph(*p*-Ph)	CH_2CH_3	97	181−182	6.6, 8.3, 9.1 (60)
5V	CH_2Ph	CH_2Ph(*p*-Ph)	$CH_2CH(CH_3)_2$	69	185−187	9.5, 11.9, 12.8 (60)
5w	CH_2Ph	CH_2Ph(*p*-Ph)	CH_2Ph	75	196−198	10.0, 12.0, 12.7 (60)
5x	CH_2Ph	$CH_2CH(CH_3)_2$	CH_2Ph	58.4	171−173	6.0, 7.4, 8.0 (60)
5y	CH_2CH_2Ph	CH_2Ph	CH_2Ph	78.9	181−182	7.1, 8.7 (60)

ture−activity relationships have shown that the presence of small hydrophobic residues in the P_1 position and hydrophobic aromatic or aliphatic side chains in the P_1' and P_2' positions are preferred by this peptidase.[12]

However, accounting for the specificity of NEP active site discussed above, it is somewhat surprising that α-aminophosphinic compounds such as those reported in Table 2 have modest inhibitory potencies on this enzyme with K_i values in the 10^{-7} M range. Three hypotheses could be proposed to explain these apparent discrepancies: (i) the presence of a free amino group would be detrimental for NEP inhibition; this argument can be discarded since we have shown that removing this function in **6** decreased its K_i value for NEP only by a factor of 2 (13); (ii) the phosphinic group, in these molecules, would not be well-oriented for an optimal interaction with the zinc ion; or (iii) the benzyl moiety may not fit perfectly the S_1' subsite. The two latter explanations are probably associated to account for the present results and for those previously reported in

other series of phosphorus-containing inhibitors. Indeed phosphonamidates such as phosphoramidon[22] and the shorter derivative phosphoryl-Leu-Phe[23] exhibit nanomolar affinities for NEP.

In contrast, other phosphonamidate peptide analogues[24] are relatively poor NEP inhibitors ($IC_{50} \geq 10^{-7}$ M). On the other hand, some *N*-phosphonomethyl dipeptides characterized by a free phosphonate group ($-CH_2-PO_3H_2$) exhibit nanomolar IC_{50} values,[25] but it can be noticed that in this series the best compounds contain a biphenylmethyl moiety in the P_1' position. It has been previously demonstrated that this large residue is well-recognized by NEP S_1' subsite.[15,26] Only one series of phosphinic acids ressembling the inhibitors reported in this study, but without a free amino group, have been tested on NEP.[27,28] None of the compounds contained a biphenylmethyl group, and the IC_{50} of the best inhibitor on NEP is 55 nM.[28]

In our series, the introduction of the biphenylmethyl group in the P_1' position, in association with a methyl side chain in the P_2' position, afforded inhibitors with nanomolar potencies on NEP (Table 3). Interestingly these two modifications did not perturb significantly APN recognition, and as shown in Table 4, three compounds which differ only by the nature of R_1 have nanomolar K_i values on both enzymes. A decrease in the size of the R_3 residue facilitates NEP recognition (compound **32B**), whereas an increase of R_3 enhances APN inhibition (compound **13B**). Another intriguing result is the fact that these molecules, which could be assumed to block the S_1, S_1', and S_2' subsites of NEP and act as transition-state analogues, are not better inhibitors than thiorphan and analogues. This suggests, as previously discussed,[16] that the occupancy of the assumed S_1 subsite of NEP is unable to significantly increase the affinity for this enzyme.

Since various phosphinic compounds, such as fosinopril, have been described as potent ACE inhibitors,[29] it was important to verify the selectivity of the compounds reported here. None of them behave as very efficient ACE inhibitors. However accounting for the specificity of the S_1 subsite of ACE,[30] compounds **18**−**21** which have a benzyl group in the R_1 position and hydrophobic residues in R_2 and R_3 have affinities for ACE around 10^{-8} M. Interestingly, the presence of a phenyl residue in R_1 precludes the recognition of ACE active site (compounds **10** and **24**−**26**) leading to the most selective NEP/APN inhibitors of the series.

Owing to their high efficiency on both NEP and APN in vitro, it was important to study the ability of these new inhibitors to protect the endogenous enkephalins by in vivo experiments. As expected (Figure 3) these molecules induced antinociceptive responses at low doses on the hot plate test in mice after icv administration. This test represents one of the most severe animal models of pain used for the selection of new analgesics.[31] The percentage of analgesia obtained at doses as low as 10 or 20 μg/kg reflects the high efficiency of these molecules and the validity of the concept of a true "dual inhibitor" for analgesia. It is worth noting that inhibition of only one enzyme, NEP or APN, was shown to produce slight antinociceptive responses as compared to dual inhibitors[32] even following icv administration.[7]

However, it is obvious that the development of new drugs for possible use in human therapy requires orally active compounds. It was interesting to observe that compound **28B**, administered iv at 100 mg/kg (Figure 4), induces 25% analgesia on the hot plate test. This indicates that aminophosphinic derivatives could be active by systemic route even under nonprotected forms. A significant increase in the bioavailability of these charged inhibitors is illustrated with the benzyl ester of **28B**. Its iv administration in mice (Figure 4) shows: (i) an increased antinociceptive response as compared to the free inhibitor and (ii) a delayed and prolonged effect reflecting an improvement in the pharmacokinetic properties. This relatively long duration of action is very different from that observed with RB 101, which is formed by the association through a disulfide bond, an inhibitor of NEP and a blocker of APN both exhibiting nanomolar affinity for their proper enzyme. Thus, the preliminary results obtained with the benzyl ester of **28B** appear very favorable since a more constant concentration−time profile as compared to RB 101 or analogues is observed. This interesting pharmacological result obtained with protection of only one ionizable group could be improved, for instance, by using protecting groups employed in ACE inhibitors such as fosinopril.[33] Indeed the ionization state of the inhibitor phosphinic group ($pK_a \sim 1.8-2.0$) restricts the bioavailability of this kind of compound.

Experimental Section

Chemistry. The natural amino acid derivatives were purchased from Bachem (Bubbendorf, Switzerland). Reagents were from Aldrich Chimie (Strasbourg, France). The solvents were from SDS (Peypin, France). TLC experiments were revealed with UV, iodine vapor, or ninhydrin. The purity of the compounds was checked by HPLC on a reverse-phase kromasil C_8 (5 μm, 100 Å) column (4.6 × 250 mm) with 0.05% TFA in H_2O (solvent A)/CH_3CN (solvent B), as mobile phase, using isocratic conditions at a flow rate of 1 mL/min on a Shimadzu apparatus (detector SPD-6AV, pumps LC-9A, recorder CR-6A). The eluted peaks were monitored at 210 nm.

The four stereoisomers of the final products were separated into two parts by semipreparative HPLC using a reverse-phase kromasil C_8 (10 μm, 100 Å) column (20 × 250 mm) with 0.05% TFA in H_2O (solvent A)/CH_3CN (solvent B), as mobile phase, in isocratic conditions at a flow rate of 15 mL/min on a Waters apparatus (detector Waters 486, pumps Waters 600, recorder Servogor 120). The eluted peaks were monitored at 210 nm.

The structure of all compounds was confirmed by ^{1}H NMR spectroscopy (Brüker AC 270 MHz) in DMSO-d_6 using HMDS as internal reference (values in δ, ppm). The signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Mass spectral analyses were performed by Quad Service (Poissy, France), using the electrospray ionization (ESI) technique. Melting points of the compounds were determined on an Electrothermal apparatus (Bûchi Melting Point B-450) and are reported uncorrected. Satisfactory analyses were obtained (C, H, N) for all final compounds.

pK_a determination of compound **28B** was obtained by classical titration methods (pH meter, Tacussel, Lyon, France).

Abbreviations: AcOH, acetic acid; BOP, 1*H*-benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; BSA, *N*,*O*-bistrimethylsilylacetamide; Chex, cyclohexane; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; Et_2O, ethyl ether; EtOH, ethanol; EtOAc, ethyl acetate; HMSD, hexamethyldisiloxane; Hex, hexane; Hep, heptane; MeOH, methanol; TFA, trifluoroacetic acid; Et_3N, triethylamine.

General Procedure for the Synthesis of 1-(Benzyloxycarbonyl)aminoalkylphosphonous Acids 1. They were

prepared, under racemic forms, in three steps as described by Baylis.[17] Compounds **1a**,**e**,**h** have been resolved by chiral amine as described.[17]

1a: $R_1 = CH_3$; white solid; mp 112−3 °C (110 °C lit.) (51%). Isomer *R*: $[\alpha]_D^{20}$ −48.3 °C (1% in AcOH).

1b: $R_1 = CH_2CH_3$; white solid; mp 106 °C (32%).

1c: $R_1 = CH_2CH(CH_3)_2$; white solid; mp 148−9 °C (150−2° lit.) (55%).

1d: $R_1 = CH_2CH_2SCH_3$; white solid; mp 105 °C (108−9 °C lit.) (21%).

1e: R_1 = Ph; white solid; mp 220−2 °C (47%). Isomer *R*: $[\alpha]_D^{20}$ +14.3 °C (1% in AcOH).

1f: R_1 = Ph(*p*-Me); white solid; mp 255 °C (52%).

1g: R_1 = Ph(*o*,*p*-diMe); white solid; mp 267 °C (49%).

1h: $R_1 = CH_2Ph$; white solid; mp 135−6 °C (137 °C lit.) (50%). Isomer *R*: $[\alpha]_D^{20}$ −72.6 °C (1% in AcOH).

1i: $R_1 = CH_2CH_2Ph$; white solid; mp 142−3 °C (44%).

General Procedure for the Synthesis of Acrylate Derivatives 2. Method A. To a solution of triethyl phosphoacetate in DMF, at −10 °C, were added sodium hydride (1.1 equiv) and, after 15 min, the alkyl or aryl bromide (1 equiv). The mixture was stirred at room temperature for 16 h and concentrated in vacuo. The residue was dissolved in EtOAc, washed with brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude product was purified by chromatography on silica gel using EtOAc/Hex, 1:1, as eluent.

The 2-substituted triethyl phosphoacetate (1 equiv), formaldeyde (5 equiv) and potassium carbonate (3 equiv) were treated at 100 °C under stirring for 3 h. Diethyl ether and water were added. The organic layer was separated, washed with brine, dried over Na_2SO_4 and evaporated to yield **2a**−**d**.

2a: $R_2 = CH_2Ph$; oil; R_f (Hep/EtOAc, 3:1) 0.59 (42%).

2b: $R_2 = CH_2Ph$(*p*-Br); oil; R_f (Hep/EtOAc, 9:1) 0.57 (33%).

2c: $R_2 = CH_2CH(CH_3)_2$; oil; R_f (Hep/EtOAc, 3.5:6.5) 0.32 (36.7%).

2d: $R_2 = CH_2Ph$(*p*-$CH(CH_3)_2$); oil; R_f (Chex/EtOAc, 10:1) 0.6 (34%).

Method B (for 2e). A solution of 4.8 g (16.1 mmol) of ethyl 4-phenylbenzylmalonic acid obtained from the corresponding diethyl ester[34] was treated by 1.68 mL (16.1 mmol) of diethylamine and 1.97 mL (24.2 mmol) of formaldehyde following the procedure of Mannich and Ritsert.[35] The crude product was purified by chromatography over silica gel using Hep/EtOAc/AcOH, 9:1:0.2, as eluents to yield 3.52 g (82.1%) of oil; R_f (Hep/EtOAc/AcOH, 9:1:0.2) 0.55.

General Procedure for the Synthesis of Ethyl 2-Alkyl-3-[hydroxy[1′-(*N*-benzyloxycarbonylamino)-2′-alkyl]phosphinyl]propanoate Derivatives 3 (Table 5). Method A (for 3a−o). The solution of various phosphonic acids **1** synthesized as described[15] (1 equiv) and the acrylate derivatives (1.2 equiv) in BSA (2−3 equiv) were stirred overnight at room temperature or at 70 °C. After cooling, the mixtures were taken off with water and extracted by EtOAc. The organic layers were washed with water and brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, the solid residues were triturated in hexane and purified by chromatography on silica gel using CH_2Cl_2/MeOH/AcOH, 9:1:0.3, as eluents.

Method B, Suzuki Condensation (for 3p−r). To a solution of the ethyl 2-*p*-bromobenzyl-3-[hydroxy[1′-(*N*-benzyloxycarbonylamino)alkyl]phosphinyl]propanoate (**3b,h,m**) (10 mmol) and $Ph(Ph_3)_4$ (0.3 mmol) in 20 mL of toluene were added under inert atmosphere 10 mL of 2 M aqueous solution of Na_2CO_3 and 12 mmol of the phenylboronic acid in 5 mL of MeOH. The vigorously stirred mixture was warmed to 80 °C for 10 h, then cooled, acidified at pH 3 and extracted with EtOAc. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation, the crude product was purified by chromatography on silica gel using CH_2Cl_2/MeOH/AcOH, 9:1:0.3, as eluents.

General Procedure for the Synthesis of 2-Alkyl-3-[hydroxy[1′-(*N*-benzyloxycarbonylamino)-2′-alkyl]phosphinyl]propanoic Acids 4a−r (Table 5). One equivalent of an ethyl 2-alkyl-3-[hydroxy[1′-(*N*-benzyloxycarbonylamino)-2′-alkyl]phosphinyl] propanoate **3a**−**r** was dissolved in ethanol, and 1 M NaOH (5 equiv) was added. The mixture was stirred for 6−8 h at room temperature. After acidification with 2 M HCl, ethanol was evaporated, diluted in water, and extracted with EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4 and evaporated in vacuo.

General Procedure for the Synthesis of 2-Alkyl-3-[hydroxy[1′-(*N*-benzyloxycarbonylamino)-2′-alkyl]phosphinyl]propanoyl Amino Acids 5a−y (Table 6). To a solution of 1 equiv of a 2-alkyl-3-[hydroxy[1′-(*N*-benzyloxycarbonylamino)-2′-alkyl]phosphinyl]propanoic acid **4a**−**r** in DMF were added the chlorhydrate of a given amino acid ester (1 equiv), Et_3N (1 equiv) and BOP (2.5 equiv). After stirring 30 min at room temperature, the mixture was treated with water and the formed product extracted using EtOAc. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by chromatography on silica gel using CH_2Cl_2/MeOH/AcOH, 9:1:0.5, as eluent.

The compound (1 equiv) was dissolved in ethanol and 1 M NaOH (5 equiv) or 1 M LiOH (5 equiv) was added. The mixture was stirred for 8 h at room temperature. After acidification with 2 M HCl, the solution was evaporated, diluted in water. The precipitate was filtered and dried.

General Procedure for the Synthesis of 2-Alkyl-3-[hydroxy(1′-amino-2′-alkyl)phosphinyl]propanoyl Amino Acid. The protected inhibitor (1 equiv) was dissolved in CH_2Cl_2 and 1 M BBr_3 in CH_2Cl_2 (5 equiv) was added at −10 °C. The mixture was stirred at this temperature for 1 h and at room temperature for 2 h. The organic layer was evaporated. The aqueous layer was washed with Et_2O and evaporated in vacuo.

Compound 6: white solid; mp 230 °C dec (91%); HPLC (30% B) 5.4 and 8.8 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.07−1.2 (dd, 3H, $CH_3\beta$); 1.45−2.2 (m, 2H, P−CH_2); 2.6−3.1 (m, 6H, 2xCH_2Ph, CHCO, NCHα); 4.5 (m, 1H, $CHCO_2$); 7.1−7.3 (m, 10H, Ar); 8.0 (s, br, 3H, NH_3); 8.38−8.62 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m*/*z* 419.0. Anal. ($C_{21}H_{28}BrN_2O_5P$) C, H, N.

Compound 7: white solid; mp 204 °C dec (57%); HPLC (28% B) 6.2 and 10.1 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.07−1.2 (dd, 3H, $CH_3\beta$); 1.45−2.2 (m, 4H, CH_2, P−CH_2); 2,6−3,1 (m, 6H, 2xCH_2Ph, CHCO, NCHα); 4,5 (m, 1H, $CHCO_2$); 7.1−7.3 (m, 10H, Ar); 8.0 (s, br, 3H, NH_3); 8.38−8.62 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m*/*z* 433.1. Anal. ($C_{22}H_{30}BrN_2O_5P$) C, H, N.

Compound 8: white solid; mp 195 °C dec (86%); HPLC (35% B) 5.3 and 7.2 min; ^{1}H NMR (DMSO-d_6 and TFA) 0.65−0.9 (m, 6H, 2xCH_3); 1.3−2.2 (m, 5H, $CH_2\beta$-CH, P−CH_2); 2.5−3.2 (m, 6H, 2xCH_2Ph, CHCO, NCHα); 4.4 (m, 1H, $CHCO_2$); 7.05−7.3 (m, 10H, Ar); 7.98 (s, br, 3H, NH_3); 8.3−8.55 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m*/*z* 461.0. Anal. ($C_{24}H_{34}BrN_2O_5P$) C, H, N.

Compound 9: white solid; mp 175 °C (85%); HPLC (30% B) 6.5 and 10.4 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.4−2.2 (m, 4H, $CH_2\beta$, P−CH_2); 1.96 (s, 3H, SCH_3); 2.35−3.08 (m, 7H, $CH_2\gamma$, 2xCH_2Ph, CHCO); 3.1−3.3 (m, 1H, NCHα); 4.45 (m, 1H, $CHCO_2$); 6.95−7.2 (m, 10H, Ar); 8.0 (s, br, 3H, NH_3); 8.35−8.6 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m*/*z* 479.1. Anal. ($C_{23}H_{32}BrN_2O_5PS$) C, H, N.

Compound 10: white solid; mp 173 °C dec (85%); HPLC (30% B) 8.25, 8.7 and 14.0 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.4−2.05 (m, 2H, P−CH_2); 2.55−3.05 (m, 5H, 2xCH_2Ph, CHCO); 4.35−4.5 (m, 2H, NCHα, $CHCO_2$); 6.9−7.4 (m, 15H, Ar); 8.2−8.55 (m, 1H, NH), 8.65 (s, br, 3H, NH_3); MS (ESI) $(M + 1)^+$ *m*/*z* 481.1. Anal. ($C_{26}H_{30}BrN_2O_5P$) C, H, N.

Compound 10B: HPLC (30% B) 8.25 min; $[\alpha]^{20}_D$ −6.5° (*c* 0.2 in CH_3CN/H_2O, 50/50).

Compound 11: white solid; mp 165 °C dec (90%); HPLC (35% B) 10.1, 10.5 and 12.7 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.4−2.02 (m, 2H, P−CH_2); 2.2 (s, 3H, CH_3Ar); 2.54−3.0 (m, 5H, 2xCH_2Ph, CHCO); 4.25−4.52 (m, 2H, NCHα, $CHCO_2$); 6.85−7.38 (m, 14H, Ar); 8.15−8.5 (m, 1H, NH), 8.6 (s, br, 3H, NH_3); MS (ESI) $(M + 1)^+$ *m*/*z* 495.0. Anal. ($C_{27}H_{32}BrN_2O_5P$) C, H, N.

Compound 12: white solid; mp 174 °C dec (91%); HPLC (30% B) 16.7, 28.8 and 31.9 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.4−2.0 (m, 2H, P−CH_2); 2.18 (d, 6H, 2xCH_2); 2.6−3.1 (m, 5H, 2xCH_2Ph, CHCO); 4.35−4.6 (m, 2H, NCHα, $CHCO_2$); 6.8−7.4 (m, 13H, Ar); 8.15−8.65 (m, 1H, NH), 8.5 (s, br, 3H, NH_3); MS (ESI) $(M + 1)^+$ *m/z* 509.0. Anal. ($C_{28}H_{34}BrN_2O_5P$) C, H, N.

Compound 13: white solid; mp 220 °C dec (89%); HPLC (30% B) 12.0, 12.8, 14.7 and 22.0 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.4−2.0 (m, 2H, P−CH_2); 2.6−3.1 (m, 7H, $CH_2\beta$, 2xCH_2-Ph, CHCO); 3.4 (m, 1H, NCHα); 4.4 (m, 1H, $CHCO_2$); 7.0−7.3 (m, 15H, Ar); 8.0 (s, br, 3H, NH_3); 8.4−8.6 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 495.0. Anal. ($C_{27}H_{32}BrN_2O_5P$) C, H, N.

Compound 13B: HPLC (30% B) 12.8 min; $[\alpha]^{20}_D$ −3.1° (*c* 0.1 in CH_3CN/H_2O, 50/50).

Compound 14: white solid; mp 205 °C dec (89%); HPLC (30% B) 5.1, 5.4 and 7.0 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.4−2.2 (m, 4H, $CH_2\beta$, P−CH_2); 2.5−3.2 (m, 8H, $CH_2\gamma$, 2xCH_2-Ph, CHCO, NCHα); 4.38 (m, 1H, $CHCO_2$); 6.9−7.3 (m, 15H, Ar); 8.1 (s, br, 3H, NH_3); 8.35−8.6 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 509.0. Anal. ($C_{28}H_{34}BrN_2O_5P$) C, H, N.

Compound 15: white solid; mp 210 °C dec (85%); HPLC (30% B) 4.4, 5.5 and 5.9 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.0−1.28 (m, 3H, CH_3); 1.4−2.25 (m, 2H, P−CH_2); 2.5−2.8 (m, 2H, $CH_2\beta$); 2.95 (m, 2H, CH_2Ph); 3.05 (m, 1H, CHCO); 3.3−3.52 (m, 1H, NCHα); 4.15 (m, 1H, $CHCO_2$); 7.1−7,3 (m, 10H, Ar); 8.0 (s, br, 3H, NH_3); 8.15−8.48 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 419.0. Anal. ($C_{21}H_{28}BrN_2O_5P$) C, H, N.

Compound 16: white solid; mp 230 °C dec (83%); HPLC (30% B) 9.2, 9.6, 15.6 and 17.2 min; ^{1}H NMR (DMSO-d_6 and TFA) 0.6−0.85 (m, 6H, 2xCH_3); 1.4−2.25 (m, 5H, P−CH_2, CH_2−CH); 2.5−2.8 (m, 2H, $CH_2\beta$); 2.9−3.15 (m, 3H, CH_2Ph, CHCO); 3.3−3.5 (m, 1H, NCHα); 4.15 (m, 1H, $CHCO_2$); 7.2 (m, 10H, Ar); 8.0 (s, br, 3H, NH_3); 8.12−8.42 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 461.0. Anal. ($C_{24}H_{34}BrN_2O_5P$) C, H, N.

Compound 17: white solid; mp 160 °C dec (44%); HPLC (25% B) 8.9, 12.0 and 14.3 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.3−2.2 (m, 2H, P−CH_2); 2.5−3.2 (m, 7H, C$H\beta$, 2xCH_2Ph, CHCO); 3.65 (m, 1H, NCHα); 4.6 (m, 1H, $CHCO_2$); 6.55−6.95 (dd, 4H, Ar); 7.0−7.3 (m, 10, Ar); 8.0 (s, br, 3H, NH_3); 8.3−8.5 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 511.0. Anal. ($C_{27}H_{32}BrN_2O_6P$) C, H, N.

Compound 18: white solid; mp 170 °C dec (50%); HPLC (43% B) 7.1, 8.8 and 9.2 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.45−2.2 (m, 2H, P−CH_2); 2.6−3.1 (m, 7H, $CH_2\beta$, 2xCH_2Ph, CHCO); 3.45 (m, 1H, NCHα); 4.45 (m, 1H, $CHCO_2$); 7.1−7.6 (m, 19H, Ar); 8.0 (s, br, 3H, NH_3); 8.4−8.6 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 571.1. Anal. ($C_{33}H_{36}BrN_2O_5P$) C, H, N.

Compound 19: white solid; mp 210 °C dec (86%); HPLC (30% B) 15.7, 21.3 and 23.2 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.04−1.3 (m, 3H, CH_3); 1.5−2.25 (m, 2H, P−CH_2); 2.55−3.1 (m, 5H, CH_2Ph, $CH_2\beta$, CHCO); 3.32−3.55 (m, 1H, NCHα); 4.15 (m, 1H, $CHCO_2$); 7.15−7.6 (m, 14H, Ar); 8.02 (s, br, 3H, NH_3); 8.2−8.5 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 495.0. Anal. ($C_{27}H_{32}BrN_2O_5P$) C, H, N.

Compound 20: white solid; mp 205 °C dec (82%); HPLC (30% B) 10.0, 14.4 and 15.5 min; ^{1}H NMR (DMSO-d_6 and TFA) 0.56−0.88 (2t, 2H, CH_3); 1.4−2.3 (m, 4H, CH_2, P−CH_2); 2.6−3.15 (m, 5H, CH_2Ph-Ph, $CH_2\beta$, CHCO); 3.35−3.55 (m, 1H, NCHα); 4.15 (m, 1H, $CHCO_2$); 7.15−7.6 (m, 14H, Ar); 8.02 (s, br, 3H, NH_3); 8.3−8.4 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 509.0. Anal. ($C_{28}H_{34}BrN_2O_5P$) C, H, N.

Compound 21: white solid; mp 221 °C dec (83%); HPLC (30% B) 11.4, 14.0 and 14.9 min; ^{1}H NMR (DMSO-d_6 and TFA) 0.7−0.9 (m, 6H, 2xCH_3); 1.4−2.3 (m, 5H, CH_2−CH, P−CH_2); 2.6−3.15 (m, 5H, CH_2Ph-Ph, $CH_2\beta$, CHCO); 3.3−3.55 (m, 1H, NCHα); 4.2 (m, 1H, $CHCO_2$); 7.15−7.6 (m, 14H, Ar); 8.0 (s, br, 3H, NH_3); 8.3−8.45 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 537.0. Anal. ($C_{30}H_{38}BrN_2O_5P$) C, H, N.

Compound 22: white solid; mp 225 °C dec (94%); HPLC (45% B) 8.7, 10.3 and 10.8 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.5−2.2 (m, 2H, P−CH_2); 2.6−3.15 (m, 7H, CH_2Ph, CH_2Ph-Ph, $CH_2\beta$, CHCO); 3.35−3.5 (m, 1H, NCHα); 4.36−4.48 (m, 1H, $CHCO_2$); 7.05−7.6 (m, 19H, Ar); 8.0 (s, br, 3H, NH_3); 8.38−8.5 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 571.1. Anal. ($C_{33}H_{36}BrN_2O_5P$) C, H, N.

Compound 22B: HPLC (45% B) 8.7 min; $[\alpha]^{20}_D$ +1.4° (*c* 0.1 in CH_3CN/H_2O, 50/50).

Compound 23: white solid; mp 230 °C dec (86%); HPLC (30% B) 8.5, 10.1, 14.3 and 15.1 min; ^{1}H NMR (DMSO-d_6 and TFA) 0.6−0.85 (4d, 6H, 2xCH_3); 1.15−1.5 (m, 3H, CH_2−CH); 1.55−2.0 (m, 2H, P−CH_2); 2.65 (m, 1H, CHCO); 2.7−3.1 (m, 4H, CH_2Ph, $CH_2\beta$); 3.45 (m, 1H, NCHα); 4.35 (m, 1H, $CHCO_2$); 7.1−7.3 (m, 10H, Ar); 7.05 (s, br, 3H, NH_3); 8.3−8.45 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 461.0. Anal. ($C_{24}H_{34}BrN_2O_5P$) C, H, N.

Compound 23B: HPLC (30% B) 10.1 min; $[\alpha]^{20}_D$ −4.9° (*c* 0.1 in CH_3CN/H_2O, 50/50).

Compound 24: white solid; mp 190 °C dec (46%); HPLC (30% B) 5.7 and 9.4 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.4−2.2 (m, 2H, P−CH_2); 2.6−3.2 (m, 3H, CH_2Ph, CHCO); 4.3−4.7 (m, 1H, NCHα); 5.3 (m, 1H, $CHCO_2$); 7.0−7.5 (m, 15H, Ar); 8.6 (s, br, 3H, NH_3); 8.5−8.8 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 467.0. Anal. ($C_{25}H_{28}BrN_2O_5P$) C, H, N.

Compound 25: white solid; mp 194 °C dec (45%); HPLC (33% B) 7.5 and 10.1 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.0−1.3 (dd, 3H, CH_3); 1.45−2.2 (m, 2H, P−CH_2); 2.55−3.05 (m, 3H, CH_2Ph, CHCO); 4.15 (m, 1H, $CHCO_2$); 4.4−4.7 (m, 1H, NCHα), 7.1−7.6 (m, 14H, Ar); 8.1−8.4 (m, 1H, NH); 8.65 (s, br, 3H, NH_3); MS (ESI) $(M + 1)^+$ *m/z* 481.1. Anal. ($C_{26}H_{30}BrN_2O_5P$) C, H, N.

Compound 25B: HPLC (45% B) 7.5 min; $[\alpha]^{20}_D$ −3.4° (*c* 0.1 in CH_3CN/H_2O, 50/50).

Compound 26: white solid; mp 150 °C dec (56%); HPLC (30% B) 8.0, 8.7 and 12.5 min; ^{1}H NMR (DMSO-d_6 and TFA) (DMSO-d_6 and TFA) 0.55−0.8 (dd, 6H, 2xCH_3); 0.8−1.4 (m, 3H, CH_2−CH); 1.5−2.0 (m, 2H, P−CH_2); 2.55−3.1 (m, 3H, CH_2-Ph, CHCO); 4.35−4.6 (m, 2H, NCHα, $CHCO_2$); 7.1−7.5 (m, 10H, Ar); 8.1−8.45 (m, 1H, NH); 8.65 (s, br, 3H, NH_3); MS (ESI) $(M + 1)^+$ *m/z* 447.0. Anal. ($C_{23}H_{32}BrN_2O_5P$) C, H, N.

Compound 27: white solid; mp 215 °C dec (91%); HPLC (35% B) 8.5 and 10.9 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.1 (4d, 3H, $CH_3\beta$); 1.45−2.2 (m, 2H, P−CH_2); 2.6−3.4 (m, 6H, 2xCH_2Ph, CHCO, NCHα); 4,4 (m, 2H, $CHCO_2$); 7.1−7.65 (m, 14H, Ar); 8.0 (s, br, 3H, NH_3); 8.3−8.6 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 495.0. Anal. ($C_{27}H_{32}BrN_2O_5P$) C, H, N.

Compound 28: white solid; mp 220°-222 °C (92%); HPLC (30% B) 7.2 and 10.3 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.15 (dd, 3H, $CH_3\beta$); 1.22 (m, 3H, CH_3); 1.5−2.2 (m, 2H, P−CH_2); 2.65 (m, 1H, CHCO); 2.95 (m, 2H, CH_2Ph); 3.2 (m, 1H, NCHα); 4.17 (m, 2H, $CHCO_2$); 7.24−7.6 (m, 9H, Ar); 8.0 (s, br, 3H, NH_3); 8.3−8.5 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 418.9. Anal. ($C_{21}H_{28}BrN_2O_5P$) C, H, N.

Compound 28B: HPLC (30% B) 7.2 min; $[\alpha]^{20}_D$ +1.6° (*c* 0.1 in CH_3CN/H_2O, 50/50).

Compound 29: white solid; mp 140 °C dec (38%); HPLC (27% B) 7.8, and 12.6 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.0−1.25 (m, 12H, 2xCH_3, $CH(CH_3)_2$); 1.45−2.2 (m, 2H, P−CH_2); 2.5−3.05 (m, 4H, $CH(CH_3)_2$, CH_2Ar, CHCO); 3.65 (m, 1H, NCHα); 4.1 (m, 1H, $CHCO_2$); 7.0−7.15 (dd, 4H, Ar); 8.0 (s, br, 3H, NH_3); 8.15−8.5 (m, 1H, NH); MS (ESI) $(M + 1)^+$ *m/z* 385.0. Anal. ($C_{18}H_{30}BrN_2O_5P$) C, H, N.

Compound 30B: white solid; mp 228 °C dec (57%); HPLC (20% B) 9.8 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.15 (dd, 3H, $CH_3\beta$); 1.2 (d, 3H, $CH_3\beta$); 1,55−2.1 (m, 2H, P−CH_2); 2.65−3.2 (m, 6H, 2xCH_2Ph, CHCO, NCHα); 4.15 (m, 2H, $CHCO_2$); 7.15−7.4 (dd, 4H, Ar); 8.0 (s, br, 3H, NH_3); 8.45 (d, 1H, NH), $[\alpha]^{20}_D$ −3.4° (*c* 0.1 in CH_3CN/H_2O, 50/50); MS (ESI) $(M + 1)^+$ *m/z* 497.1, 499.1. Anal. ($C_{21}H_{27}Br_2N_2O_5P$) C, H, N.

Compound 31B: white solid; mp 214 °C dec (52%); HPLC (35% B) 7.6 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.5−2.1 (m, 2H, P−CH_2); 2.6−3.05 (m, 3H, CHCO, CH_2Ph(p-Ph)); 4.2 (m, 2H, CH_2O); 4.5 (m, 2H, NCHα, $CHCO_2$); 7.1−7.6 (m, 14H, Ar); 8.38−8.58 (m, 1H, NH), 8.68 (s, br, 3H, NH_3); $[\alpha]^{20}_D$ −3.2° (*c* 0.1 in HAc); MS (ESI) $(M + 1)^+$ *m/z* 467.0. Anal. ($C_{25}H_{28}BrN_2O_6P$) C, H, N.

Compound 32B: white solid; mp 242 °C (55%); HPLC (35% B) 7.7 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.12 (dd, 3H, CH_3);

1.44−2.15 (m, 2H, P−CH_2); 2.55−3.2 (m, 3H, CHCO, CH_2Ph-(p-Ph)); 4.45 (m, 1H, NCHα); 4.58 (m, 1H, $CHCO_2$); 5.75 (m, 1H, CHO); 7.25−7.6 (m, 14H, Ar); 8.3−8.5 (m, 1H, NH), 8.65 (s, br, 3H, NH_3); $[\alpha]^{20}_D$ −3.4° (*c* 0.1 in HAc); MS (ESI) $(M + 1)^+$ *m/z* 511.6. Anal. ($C_{27}H_{32}BrN_2O_6P$) C, H, N.

Compound 33B: white solid; mp 230 °C dec (82%); HPLC (20% B) 7.9 min; ^{1}H NMR (DMSO-d_6 and TFA) 1.5−2.25 (m, 2H, P−CH_2); 2.6−3.1 (m, 3H, CH_2Ph, CHCO); 3.78 (d, 2H, CH_2-CO_2); 4.5 (m, 1H, NCHα); 7.15−7.2 (m, 14H, Ar); 8.5 (t, 1H, NH); 8.65 (s, br, 3H, NH_3), $[\alpha]^{20}_D$ +1.6° (*c* 0.1 in CH_3CN/H_2O, 50/50); MS (ESI) $(M + 1)^+$ *m/z* 467.0. Anal. ($C_{25}H_{28}BrN_2O_5P$) C, H, N.

Biological Tests. 1. Enzymes. APN from pig kidney was purchased from Boehringer Mannheim. NEP was purified to homogeneity from rabbit kidney.[36] ACE was purified from rat testis.[37]

2. Substrates. Inhibitory potencies were determined by using alanine-β-naphthylamide (Alaβ Na) (K_m = 50 μM) for APN, DGNPA[38] (DNS-Gly-(pNO_2)Phe-β-Ala) (K_m = 37 μM) for NEP, and N-CBz-Phe-His-Leu[39] (K_m = 50 mM) for ACE as substrates.

3. Assay for APN Activity. APN (final concentration 21 ng/mL) was preincubated for 10 min at 37 °C with or without increasing concentrations of a given inhibitor in a total volume of 200 μL in 50 mM Tris/HCl buffer, pH 7.4. Alaβ NA was added at a final concentration of 50 μM and the reaction was stopped after 30 min at 37 °C by adding 10 μM CH_3CO_2Na 1 M (pH 4.2). The fluorescence of the metabolite was measured at 400 nm (λ_{ex} = 340 nm) with a MPF44A Perkin-Elmer spectrofluorimeter. A calibration curve for β-naphthylamide was obtained by addition of increasing concentrations of βNA into 210 μL of 50 mM Tris/HCl buffer pH 7.4.

4. Assay for NEP Activity. NEP (final concentration 250 ng/mL) was preincubated for 15 min at 37 °C with or without increasing concentrations of a given inhibitor in a total volume of 225 μL of 50 mM Tris/HCl buffer, pH 7.4. DGNPA (25 μL) was added to a final concentration of 50 μM, and the reaction was stopped after 15 min at 37 °C by adding 250 μL of dioxane. The 500 μL mixture was then transferred into a quartz cell and the fluorescence measured (λ_{ex} = 342 nm, λ_{em} = 525 nm). Samples, corresponding to 0% hydrolysis, were obtained by adding the buffer and the substrate only, while samples corresponding to 100% of relative activity were prepared by adding all the reagents except the inhibitors. Both solutions were treated under the same conditions as above. The percentage of degradation was evaluated by reference to 100% of relative activity and the IC_{50} values of tested inhibitors were determined accordingly.

5. Assay for ACE Activity. ACE (final concentration 0.02 pmol/100 μL) was preincubated for 15 min at 37 °C with various concentrations of the inhibitors in 50 mM Tris/HCl buffer, pH 8.0. N-Cbz-Phe-His-Leu was added to a final concentration of 0.05 mM. The reaction was stopped after 15 min by adding 400 μL of 2 M NaOH. After dilution with 3 mL of water, the concentration of His-Leu was determined following the fluorimetric assay described by Cheung et al.[40] with a MPF44A Perkin-Elmer spectrofluorimeter (λ_{ex} = 365 nm, λ_{em} = 495 nm). The calibration curve for His-Leu was obtained by addition of increasing concentrations of His-Leu into 0.1 mL of 50 mM Tris/HCl buffer, pH 8.0, containing the denaturated enzyme.

K_i values were calculated from IC_{50} statistical values using the Cheng−Prussoff relationship.[41]

6. Pharmacological Assays. The inhibitors were dissolved in water and the pH of the solutions was adjusted to 7.0. Drugs and vehicle (controls) were administered to male Swiss mice (20−22 g; Depré, France). Mice were housed and used strictly in accordance with European Community guidelines for the care and use of laboratory animals and after approval of the proposed experiments by the ethical committee of the faculty.

7. Intracerebroventricular Injections. Inhibitors or vehicle were slowly (15 s) injected free hand into the left lateral ventricle of mice using a modified Hamilton microliter syringe in a volume of 10 μL/mouse according to the method of Haley and McCormick,[42] 15 min before the test.

8. Systemic Injections. Inhibitors or vehicle were slowly injected iv in a volume of 0.1 mL/10 mg in mice. The analgesic responses were measured at various times after the drug administration.

9. Hot Plate Test. The test was based on that described by Eddy and Leimbach.[31] A glass cylinder (16 cm high, 16 cm diameter) was used to keep the mouse on the heated surface of the plate (53 ± 0.5 °C) using a thermoregulated water-circulating pump. The latency period until the mouse jumped was registered by a means of a stopwatch (cutoff time 240 s). Dose−reponse curves were established by expressing the data as a percentage of analgesia calculated by the ratio: % analgesia = (test latency − control latency)/(cutoff time − control latency) × 100.

Statistical analysis of data was carried out by analysis of variance (ANOVA), followed by Dunnett's test or Newman−Keulss test. ED_{50} values and their 95% confidence limits were calculated by log−probit analysis according to the method of Litchfield and Wilcoxon.[43] The ED_{50} is defined as the dose required to elicit 50% analgesia.

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