Dicarboxylic Acid Dipeptide Neutral Endopeptidase Inhibitors

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The synthesis of three series of dicarboxylic acid dipeptide neutral endopeptidase 24.11 (NEP) inhibitors is described. In particular, the amino butyramide **21a** exhibited potent NEP inhibitory activity (IC_{50} = 5.0 nM) *in vitro* and *in vivo*. Blood levels of **21a** were determined using an *ex vivo* method by measuring plasma inhibitory activity in conscious rats, mongrel dogs, and cynomolgus monkeys. Free drug concentrations were 10–1500 times greater than the inhibitory constant for NEP over the course of a 6 h experiment. A good correlation of free drug concentrations was obtained when comparing values determined by the *ex vivo* analysis to those calculated from direct HPLC measurements. Plasma atrial natriuretic factor (exogenous) levels were elevated in rats and dogs after oral administration of **19a**. Urinary volume and urinary sodium excretion were also potentiated in anesthetized dogs treated with **21a**.

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Atrial natriuretic factor (ANF)[1] is a potent diuretic, natriuretic, and vasorelaxant hormone. These properties have led many investigators to speculate that this peptide might be effective for treating hypertension, congestive heart failure, and renal diseases.[2] To circumvent the inherent problems with the development of a peptide as a therapeutic agent, several approaches can be taken including the identification of nonpeptidic agonists or agents that affect the clearance of the peptide. It is generally accepted that there are two mechanisms responsible for the clearance of ANF. These are receptor-mediated internalization and degradation by so-called C-receptors[3] and enzymatic hydrolysis.[4] Numerous groups have independently demonstrated that kidney membrane preparations degrade ANF enzymatically. Furthermore inactivation and the loss of biological activity *in vivo*,[5] at least in part, occur via cleavage of the Cys^7–Phe^8 peptide bond by neutral endopeptidase 24.11[6] (NEP; EC 3.4.24.11). Although the relative importance of NEP in the metabolism of endogenous ANF remains to be determined conclusively, NEP inhibitors have been shown to elicit ANF-like responses in animal models.[7a–g] Despite these encouraging experimental results, recent clinical trials have shown, at best, moderate pharmacologic activity. In these clinical studies[7h–p] several NEP inhibitors including sinorphan, SCH34826, and candoxatril have produced no or modest antihypertensive effects. Somewhat superior, albeit moderate, effects of these agents have been observed in patients with congestive heart failure. Since these poor clinical outcomes may arise from inferior pharmacokinetics or potency, we have sought to identify novel NEP inhibitors with superior pharmacologic properties.

Chemistry

The preparation of racemic unsymmetrical glutaric acids, Table 1, is outlined in Scheme 1. *p*-Phenylbenzaldehyde was converted in successive steps to the oxetane **4** by condensation with dimethyl malonate/NaOMe, hydrogenation, and LAH reduction to the diol followed by monotosylation and cyclization with *n*-butyllithium. The oxetane can be converted to a 2,4-alkyl-substituted γ-hydroxy ester (**5**) after boron trifluoride etherate-catalyzed condensation with various *tert*-butyl ester enolates at −78 to −90 °C. A variety of aliphatic, aryl-substituted, and heterosubstituted ester enolates[8] as well as phosphates readily react with 2-methylene biphenyl oxetane **4** to give diastereomeric mixtures separable by flash chromatography. However, separation at compound **7** was less tedious. The γ-hydroxy ester **5** was oxidized with pyridinium dichromate to give the 2,4-disubstituted mixed glutaric acid ester **6**. Activation of the carboxylic acid and coupling with a protected amino acid ester followed by sodium hydroxide and/or TFA hydrolysis gave the diacids **8**.

The symmetrical glutaramides **10**, Table 2, were prepared[9a] by heating β-alanine or *cis*-4-aminocyclohexanecarboxylic acid in a mixture of methylene chloride/pyridine with the appropriate *trans*-2,4-disubstituted glutaric acid anhydrides, Scheme 2.

Preparation of the chiral amino butyramides **20** (Table 3) is outlined in Scheme 3. *N*-Boc-D-tyrosine methyl ester is converted to the triflate **11** which undergoes Suzuki coupling reaction with phenylboric acid to give **12** in good yield. Conversion of the acid to the hydroxymate followed by lithium aluminum hydride reduction gave the aldehyde **15**. Wittig condensation of aldehyde **15** with (carbethoxyethylidene)triphenylphosphorane gave the olefin **16** as one geometric isomer. In the analogous series, aldehyde **15** was condensed with (carbethoxymethylene)triphenylphosphine leading to compound **21i**. Palladium-catalyzed hydrogenation of **16** gave a 6:1 diastereomeric mixture **17**. The Boc protecting group was removed with HCl and condensed with succinic anhydride affording the mixed acid ester **19**. The diastereomers are readily separated by flash chromatography after treatment of **19** with *N,N*-dimethylforamide di-*tert*-butyl acetal to give the mixed *tert*-butyl/ethyl ester **20**. Removal of the *tert*-butyl group with TFA gave the chiral prodrugs **19** which are readily converted to the chiral diacids **21** with

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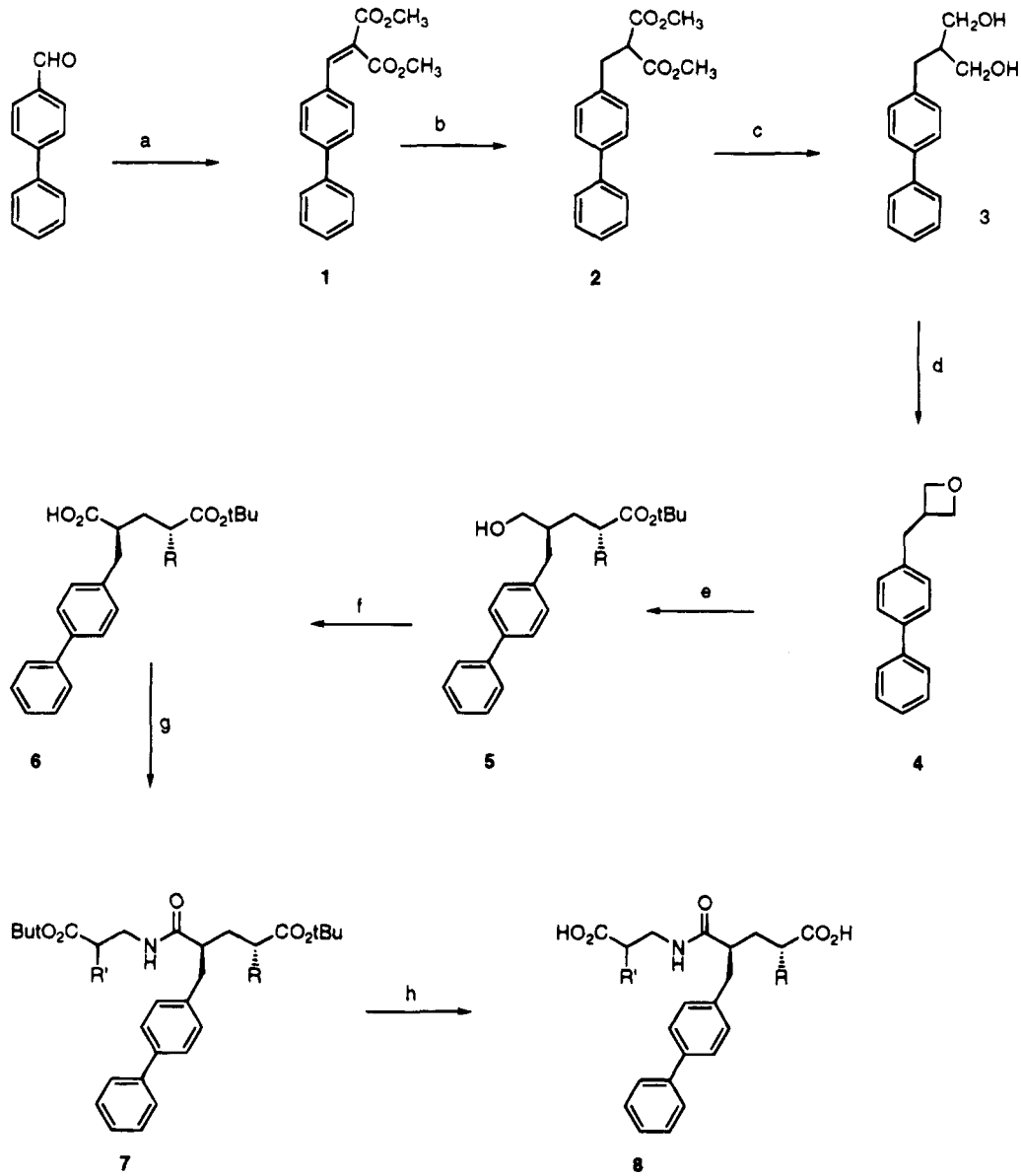
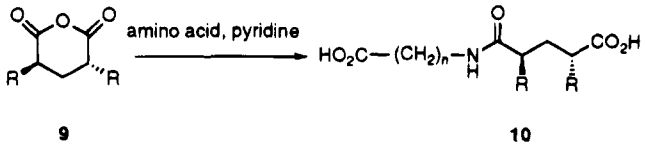
Scheme 1[a]



[a] (a) Dimethyl malonate, piperidine; (b) H_2, Pd/C; (c) $LiBH_4$; (d) TsCl, nBuLi; (e) ester enolate, $BF3OEt_2$; (f) pyridinium dichromate; (g) β-alanine *tert*-butyl ester, HOBT, EDCI; (h) TFA.

Scheme 2



lowing the same sequence of reactions except glutamic and adipic anhydride were substituted for succinic anhydride.

In Vitro Structure–Activity Discussion

A series of glutaramides[9a] were published several years ago describing very potent compounds as assessed by an *in vitro* assay using Leu enkephalin as substrate. Changing to our present assay using the synthetic substrate GAAP, we discovered that the previously potent compounds **10a** and **21j** were no longer active in the nanomolar concentrations, 8 and 2 nM, respec-

tions. The weak *in vitro* activity was consistent with the poor pharmacology observed with these compounds; however, many factors could lead to this observation. Therefore it was felt that this class of compounds should be reinvestigated.

Table 1 lists the series of racemic unsymmetrical glutaramides. The activity of other series of carboxyalkyl dipeptides/glutaric acids inhibitors[7d,9] has been reported previously. The P_1' substituent, biphenylmethyl, remained constant while the P_2' substituent (R') was interchanged between β-alanine and isoserine. The P_1 substituent was modified with alkyl, aralkyl, alkoxy, and arlyoxy groups. Potency improved by a modest 3–5-fold by changing β-alanine to isoserine (**8a–d**). When β-alanine and biphenylmethyl were kept constant, very little change in inhibitory activity was observed between alkyl, aralkyl, alkoxy, and aryloxy P_1 modifications. However, in the absence of a P_1 substituent (**8l**), the activity was decreased approximately 4-fold. The stereochemistry is predictably important

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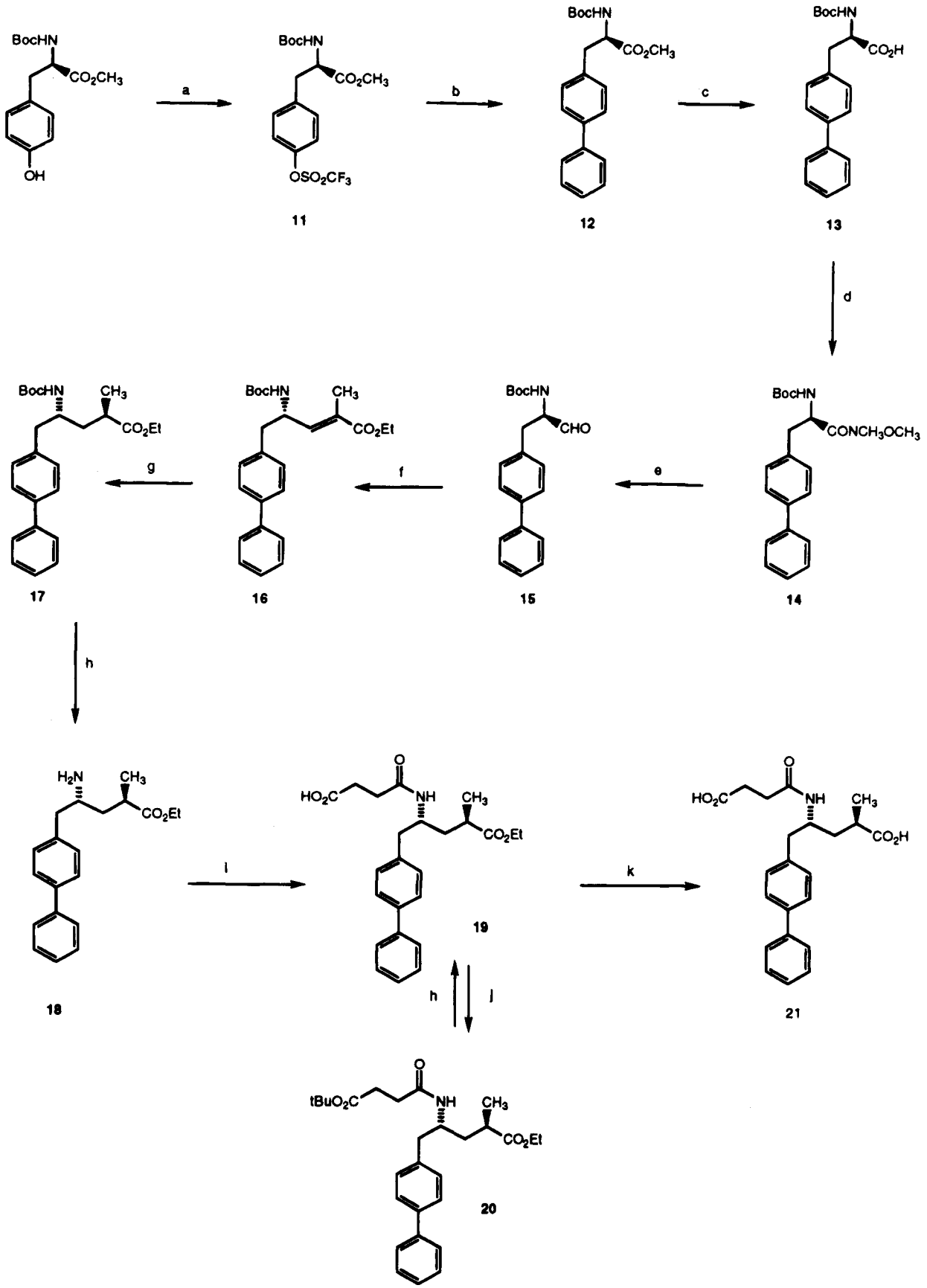
Scheme 3[a]



[a] (a) Triflic anhydride; (b) phenylboronic acid, tetrakis(triphenylphosphine)palladium(0); (c) NaOH; (d) $HNCH_3OCH_3$, EDCl, HOBT; (e) $LiAlH_4$; (f) (carbethoxyethylidene)triphenylphosphorane; (g) H_2/Pd/C; (h) HCl; (i) succinic anhydride; (j) *N,N*-dimethylformamide di-

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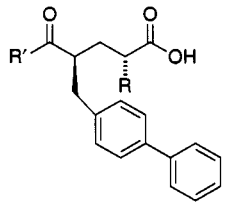
Table 1. *In Vitro* NEP Inhibition of Unsymmetrical Glutaramides



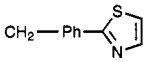
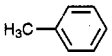
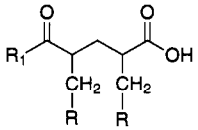
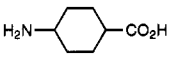
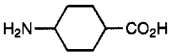
compd	R′	R	IC_{50} (nM)
8a	β-Ala	$CH_{2\text{-}Ph(3,4\text{-}OMe)}$	54
8b	isoserine	CH_2-Ph(3,4-OMe)	11
8c	β-Ala	nBu	66
8d	isoserine	nBu	21
8e	β-Ala	OPh	44
8f	β-Ala	OPh (erythreo)	>1000
8g	β-Ala	OCH_3	42
8h	β-Ala	OCH_3 (erythreo)	>1000
8i	β-Ala		19
8j	β-Ala	$CH_2CH_2OCH_3$	46
8k	β-Ala		54
8l	β-Ala	H	155
8m	β-Ala	CH_3	41

Table 2. *In Vitro* NEP Inhibition of Symmetrical Glutaramides

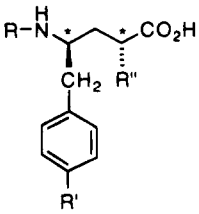


compd	R_1	R	IC_{50} (nM)
10a[9a]	β-Ala	Ph	1200
10b	β-Ala	Ph-Ph	49
10c	β-Ala		36
10d	β-Ala		>1000 (43% at 1μM)
10e	β-Ala		489
10f	β-Ala	Ph-O-Ph	203
10g			515
10h		Ph	52% at 10 μM

(**8g,h**) diastereomers, greater than a 100-fold difference in activity was observed.

Table 2 lists a series of racemic symmetrical glutaramides. The biaryl derivatives **10b,c** (IC_{50} = 49, 36 nM, respectively) were the most potent. This effect is governed entirely by the aryl−aryl P_1' substituent. The chiral β-alanine dibenzyl derivative **10a** is a relatively weak inhibitor in the GAAP assay with an IC_{50} of 1.2 μM. Substituting the monophenyl derivative in the para position with an isopropyl group (**10e**) or a phenoxy substituent (**10f**) improves activity as compared to the parent unsubstituted phenyl derivative **10a**. However, a 2−3-fold decrease in activity is observed when comparing these derivatives to the biphenylmethyl com-

Table 3. *In Vitro* NEP Inhibition of Amino Butyramides



compd	R	R′	R″	IC_{50} (nM)
21a (*R,S* isomer)	$CO(CH_2)_2CO_2H$	Ph	CH_3	5
21b (*S,R* isomer)	$CO(CH_2)_2CO_2H$	Ph	CH_3	190
21c (*R,R* isomer)	$CO(CH_2)_2CO_2H$	Ph	CH_3	700
21d (*S,S* isomer)	$CO(CH_2)_2CO_2H$	Ph	CH_3	27
21e	$CO(CH_2)_3CO_2H$	Ph	CH_3	90
21f	$CO(CH_2)_4CO_2H$	Ph	CH_3	324
21g	$COCH_2CO_2H$	Ph	CH_3	92
21h	$CO(CH_2)_3CO_2H$	Ph	OCH_3	49
21i	$CO(CH_2)_2CO_2H$	Ph	H	99
21j[9a]	$CO(CH_2)_2CO_2H$	H	CH_{2Ph}	4000

inhibitory activity of **10b,c**, was considerably weaker than other aryl−aryl derivatives. Comparison of **8i**, Table 1 (19 nM), with **10d** (>1 μM) implies that one of the heteroatoms, possibly the nitrogen, is adversely affecting the enzyme interaction in the P_1' pocket.

The inhibitory activities of the aminobutyramides are compiled in Table 3. On the basis of the data generated in the two previous series (Tables 1 and 2), we assumed aliphatic or aralkyl modifications at the P_1 site would not significantly alter the inhibitory activity. In addition, the P_1' biphenylmethyl substituent should be nearly optimum for this series. Therefore, a limited number of compounds were prepared (Table 3) with P_1 substituents being methyl and methoxy and P_1' substituents as biphenylmethyl, while the P_2' functionality was varied from malonyl to succinyl to butyryl to glutaryl to adipyl acids.

The most active compound was **21a**, IC_{50} = 5 nM. All four diastereomers, *R,S*-**21a**, *S,R*-**21b**, *R,R*-**21c**, and *S,S*-**21d**, were prepared, and IC_{50} values of 5, 190, 700, and 27 nM were determined, respectively. The succinic acid in the P_2' site appears to be optimal since extension of the carboxylic acid chain by one (**21e**) and two (**21f**) methylene units decreased activity 18- and 65-fold. In addition decreasing the chain length by one methylene (**21g**) also showed an 18-fold decrease in activity. Although in series 1 there was no difference in activity between the P_1 methyl and methoxy substituents, a 10-fold change in activity was observed in the aminobutyramide series (**21a,h**). As expected the P_1 desmethyl derivative **21i** was considerably less potent than **21a**.

Comparison of the P_1' benzyl substituent **21j** with other derivatives in Table 3 demonstrates the effects of a P_1' biphenylmethyl group. The biphenyl effect was also evident in the previous examples, shown in Table 2. This effect has been reported in the amino carboxylic acid[9b] and amino phosphonic acid[10] series. However, replacement of a benzyl group with the biphenylmethyl does not always result in a potency increase. In the thiol series,[11a] thiorphan (P_1' = benzyl, IC_{50} = 4 nM) is similar in potency to the biphenylmethyl compound (P_1' = biphenylmethyl, IC_{50} = 3 nM). In addition, a 50-fold

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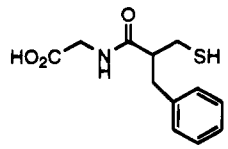
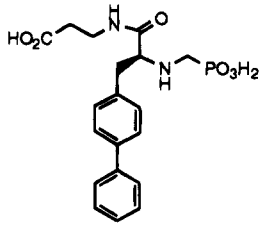
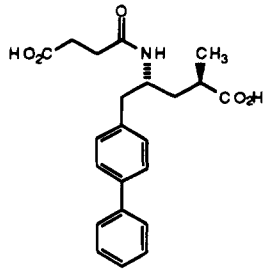
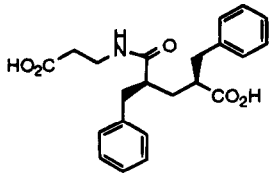
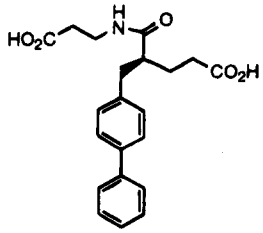
Table 4. Inhibitory Constants of NEP Inhibitors Determined from Different Substrates

Structures	IC_{50} (nM) GAAP	IC_{50} (nM) Leu ENK	IC_{50} (nM) ANF
	4.8	1.5	8.3
	1.6	0.81	2.0
	5.0	0.74	5.3
	1200	9	NT
	155	0.7	NT

Selectivity of these compounds for NEP over other Zn metalloproteases, e.g., stromelysin, endothelin converting enzyme, is not known; however, these compounds were inactive (<50% inhibition) in ACE at a concentration of 10 μM.

***In Vitro* Assays.** Leu enkephalin, glutaryl-Ala-Ala-Phe-β-naphthylamide, and ANF are used as substrates (K_{cat}/K_m = 56, 37, and 18, respectively) to identify inhibitors of NEP. We have compared the potencies of three different classes of NEP inhibitors, thiols, amino phosphonic acid, and carboxylic acid, in these three assays, Table 4. The IC_{50} values determined in these assays for thiorphan (a thiol), an amino phosphonate,[10] and **21a** (a dicarboxylic acid) were similar, although the Leu-ENK assay gave somewhat lower values, and predictive of functional potency *in vivo*. However, the correlation of inhibitory activity between assays was not always constant. Specifically within the dicarboxylic acid series of compounds, for example, **10a** and **8l** gave differences in potencies of greater than 2 orders of magnitude when tested against GAAP and Leu-ENK ences in the kinetics of individual compounds in each assay. Needless to say, the IC_{50} values for the dicarboxylic acid series of compounds vary significantly depending upon the assay used in determining these values and may not be predictive of *in vivo* functional responses.

Pharmacokinetic Profile

A pharmacokinetic profile was determined for each compound with adequate *in vitro* potency. Since pharmacokinetic measurements using HPLC techniques are labor intensive. The plasma concentrations of NEP inhibitors unbound to plasma proteins were determined by *ex vivo* analysis. This method measures the inhibitory activity in plasma and assumes that all activity is produced by the administered substance or from the active substance released via a suitable prodrug. This method does not measure total plasma levels of active substance but only the concentration of free inhibitor (i.e., not bound to plasma proteins). The total plasma concentration of compound can be calculated after the plasma protein binding is determined.

The pharmacokinetic profile of **19a,** the ethyl ester prodrug of **21a**, was determined using the *ex vivo* method by measuring plasma inhibitory activity of **21a** in conscious rats, anesthetized mongrel dogs, and conscious cynomolgus monkeys (Figure 1). In conscious rats, administration of **19a** at 30 mg/kg po produced free plasma levels of the active NEP inhibitor **21a** ranging from 0.64 to 0.05 μM over the course of the 6 h experiment. These values are 10–100 times greater than the inhibitory constant of **21a** (IC_{50} = 5 nM) for NEP. Intraduodenal administration of **19a** at 30 mg/kg in anesthetized mongrel dogs produced plasma levels of **21a** which ranged from 2.8 to 0.08 μM at 90 min and 6 h postdosing. In cynomolgus monkeys, dosing at 30 mg/kg po gave concentration values of 8.51 and 0.21 μM at 1 and 6 h, respectively. These concentrations of free inhibitor **21a** were 1500 and 38 times higher than its IC_{50} value for NEP. The apparent elimination half-life of **21a** was 4.6 ± 0.4 h in cynomolgus monkeys.

The accuracy of the *ex vivo* method was next examined using the monkey blood samples. Total drug levels of the active inhibitor **21a** were determined by HPLC.[15] The values obtained at 30, 60, 120, 180, 240, 300, 360, and 1440 min were 41, 98, 50, 21, 9.0, 4.3, 3.2, and 0.2 μM, respectively. The diacid **21a** was determined to be 94% plasma protein bound, and the free plasma concentrations of **21a** calculated from the HPLC data were in good agreement with those determined by *ex vivo* analysis (Figure 2).

ANF Potentiation Assay. Plasma ANF concentrations were determined in animals infused with exogenous ANF before and after administration of NEP inhibitors. Figure 3 shows the effects of **19a** and (±)-candoxatril[16] administered at 10 mg/kg po on plasma ANF levels in conscious rats. Plasma ANF levels are expressed as a percent of those measured in vehicle-treated animals which received the infusion of exogenous ANF. ANF levels were increased significantly at all time points (30–240 min) after the administration of **19a.** In contrast, the same dose of (±)-candoxatril produced a marked increase in plasma ANF levels initially, but this effect progressively diminished and

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