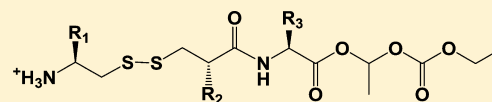


New Orally Active Dual Enkephalinase Inhibitors (DENKIs) for Central and Peripheral Pain Treatment

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Supporting Information

ABSTRACT: Protecting enkephalins, endogenous opioid peptides released in response to nociceptive stimuli, is an innovative approach for acute and neuropathic pain alleviation. This is achieved by inhibition of their enzymatic degradation by two membrane-bound Zn-metallopeptidases, neprilysin (NEP, EC 3.4.24.11) and aminopeptidase N (APN, EC 3.4.11.2). Selective and efficient inhibitors of both enzymes, designated enkephalinases, have been designed that markedly increase extracellular concentrations and half-lives of enkephalins, inducing potent antinociceptive effects. Several chemical families of Dual ENKephalinase Inhibitors (DENKIs) have previously been developed but devoid of oral activity. We report here the design and synthesis of new pro-drugs, derived from co-drugs combining a NEP and an APN inhibitor through a disulfide bond with side chains improving oral bioavailability. Their pharmacological properties were assessed in various animal models of pain targeting central and/or peripheral opioid systems. Considering its efficacy in acute and neuropathic pain, one of these new DENKIs, **19-IIIa**, was selected for clinical development.

**19-IIIa:** R₁ = CH₂CH₂SCH₃; R₂ = CH₂Ph; R₃ = H

INTRODUCTION

In spite of the constant quest for new analgesics devoid of the severe side effects of morphine (tolerance, dependence, respiratory depression, sedation, constipation, nausea), few new painkillers have been developed during the last decades.¹ This is particularly true for neuropathic and inflammatory pain, which are not efficiently treated by morphine or synthetic opiates.² However, in the 1970s, the discovery that morphine and its surrogates bind to specific targets designated opioid receptors³ and physiologically activated by two peptides, Met- and Leu-enkephalins (Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu),⁴ has led to a new approach for pain alleviation. These opioid peptides, expressed as preproenkephalin, are processed within specific neurons and released through a Ca²⁺-dependent mechanism, to interact with two G protein-coupled receptors, the μ and δ -opioid receptors, to act as physiological regulators of pain.⁵ The affinity of enkephalins (ENKs) for μ receptors is similar to that of morphine (MO) and is 10-fold higher for δ receptors.⁶

Consistently, the intracerebroventricular administration of enkephalins in rodents in animal models of acute pain induced antinociceptive responses⁷ similar to morphine. However, this effect was transient due to the rapid enzymatic degradation of ENKs.^{7,8} Consequently, enzyme-resistant ENKs, such as (D-Ala²-N-Me-Phe⁴-Met(O)⁵-OL)enkephalin (FK33-824⁹), have been synthesized and were shown to induce potent antinociceptive responses in clinical trials while keeping all unwanted effects of morphine.¹⁰ This was a consequence of the exogenous administration of these compounds (MO or modified ENKs) which interact ubiquitously with all opioid receptors in the body,

including those which are not physiologically involved in pain control but are accountable for MO side effects.^{11,12}

We have therefore proposed a novel physiological approach for pain alleviation based on endogenous ENKs protection from their enzymatic degradation.¹³ In vivo, enkephalins are inactivated by two membrane-bound Zn-metallopeptidases, neprilysin (NEP, EC 3.4.24.11), which cleaves the Gly³-Phe⁴ bond, and aminopeptidase N (APN, EC 3.4.11.2), which releases the N-terminal Tyr¹ of these pentapeptides.¹³

The inhibition of neprilysin by thiorphan,¹⁴ or of APN by bestatin,¹⁵ was insufficient for completely protect ENKs^{16,17} and therefore unable to show antihyperalgesic or analgesic efficient effects in rodents¹⁸ and in humans,¹⁹ respectively. The combination of both inhibitors was more effective²⁰ in humans, but dual inhibitors (i.e., one molecule inhibiting both enzymes) were found more appropriate as shown with the first described Dual ENKephalinase Inhibitor (DENKI)¹⁷ essentially for pharmacokinetic reasons.^{13,17,18,21}

Accordingly, selective and potent inhibition of both ENK-metabolizing peptidases largely increases extracellular concentrations and half-life of ENKs released in response to a noxious stimulus.¹⁶ This occurs at the three levels of pain control (peripheral, spinal, and central) without any modification in ENK secretion, as demonstrated by brain microdialysis and spinal superfusion experiments in rats^{16,22,23} without any saturation of opioid receptors, taken as one of the responsible effects of exogenous opioids such as MO, as shown by binding experiments.²⁴

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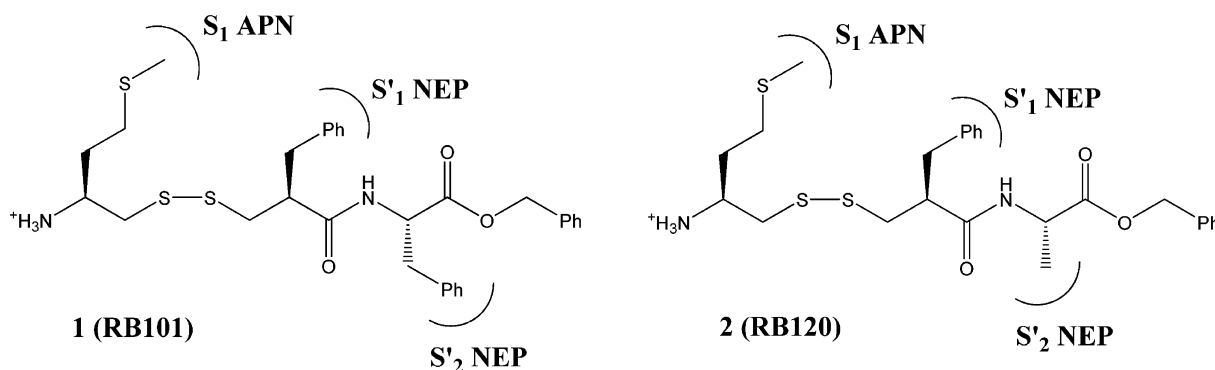
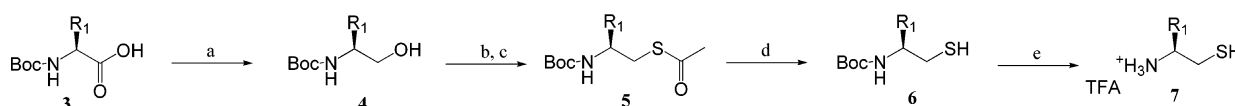


Figure 1. Representation of **1** (RB101) and **2** (RB120) in the subsites of neprilysin (NEP) and aminopeptidase N (APN) (from the nomenclature of Schechter and Berger⁴³).

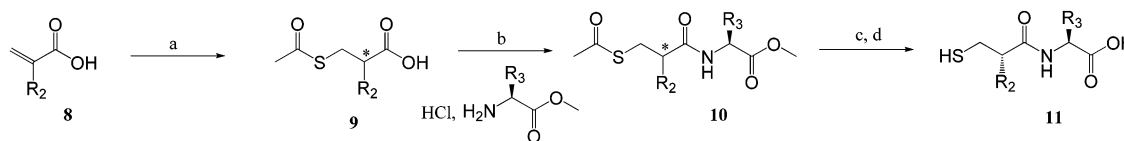
Scheme 1. Synthesis of APN Inhibitor^a



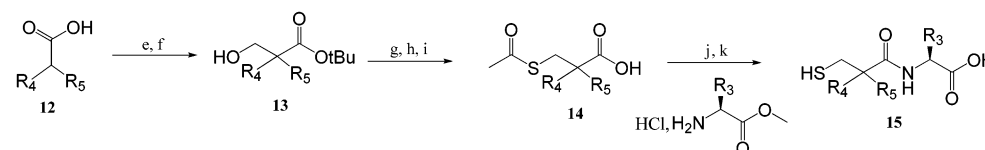
^aReagents and conditions: (a) (i) NMM, *i*BuOCOCl, DME, (ii) NaBH₄, THF; (b) CHCl₃, Et₃N, CH₃SO₂Cl; (c) CH₃COSK, DMF; (d) NaOH 1N, MeOH; (e) TFA, CH₂Cl₂.

Scheme 2. Synthesis of NEP Inhibitor^a

i) R₂ = benzyl or 3-thienyl methyl



ii) R₄, R₅ = cyclopentyl or cyclohexyl



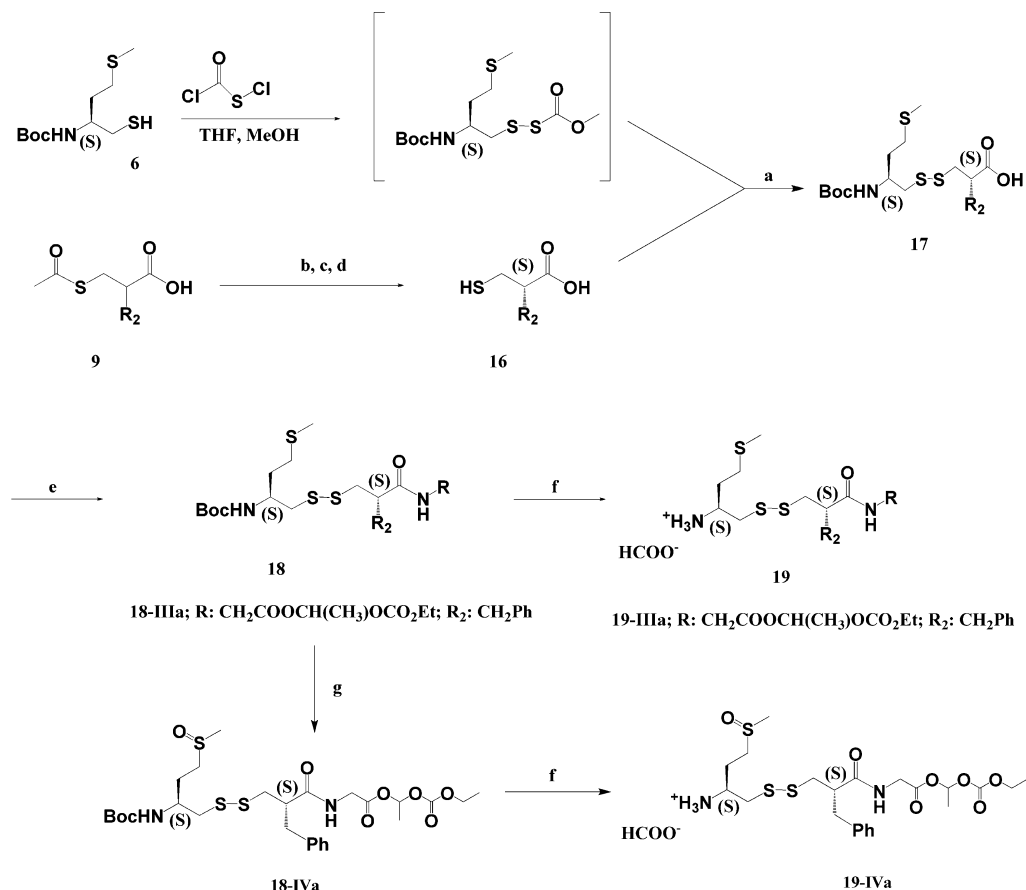
^aReagents and conditions: (a) CH₃COSH, CH₂Cl₂, overnight reflux; (b) EDCI, HOBT, DIEA, CH₂Cl₂; (c) semipreparative HPLC; (d) NaOH 1N, MeOH; (e) isobutylene, H₂SO₄ cat., *t*BuOH or (*t*Bu)₂CHNMe₂, toluene, 80 °C; (f) LDA, -78 °C, paraformaldehyde; (g) MsCl, Et₂O, Et₃N; (h) CH₃COSK, DMF, 50 °C; (i) TFA, CH₂Cl₂, RT; (j) EDCI, HOBT, DIEA, CH₂Cl₂; (k) NaOH 3N, MeOH.

Different chemical families of NEP/APN inhibitors (abbreviated in DENKIs)^{17,18,25–27} were developed in order to improve their efficacy and druggability. Their efficacy in reducing nociception¹⁷ was unambiguously demonstrated in various rodent pain models.^{18,25} It was also shown *in vivo* that enkephalins, when fully protected from catabolism by DENKIs, have an intrinsic efficacy higher than that of morphine, which is actually a partial agonist.²⁸ Among DENKIs, benzyl *N*-(3-[(2*S*)-2-amino-4-(methylthio)butyl]dithio}-2-benzylpropanoyl)-*L*-phenylalaninate **1**¹⁸ and *N*-((*S*)-2-benzyl-3[(*S*)-2-amino-4-methylthio]butylthio)-*L*-alanine benzyl ester **2**²⁶ (Figure 1), two benzyl ester pro-drugs derived from co-drugs formed by the combination, through a disulfide bridge, of an APN and a NEP inhibitor, have been extensively investigated.^{18,25,26} The disulfide bond was shown to be reduced *in vivo*, after administration of the pro-drug, releasing the two Zn-metallopeptidase inhibitors endowed with nanomolar inhibitory potency for their target-peptidase.²⁵

Accounting for its high hydrophobicity, **1** was solubilized in lipophilic solvent and most of the pharmacological tests were performed by *iv* route, where it exhibited high antinociceptive effects, but this vehicle was not suitable for human use.^{18,25} The lack of oral pharmacological activity of **1**, solubilized in lipophilic solvent, is probably related to its precipitation in gut after oral administration.¹⁸ **2** had an improved bioavailability and was slightly active orally but only at very high doses.²⁶

The endogenous enkephalinergic system (made up of μ and δ receptors, enkephalins, and their degrading enzymes) is not only present in the medulla and in the brain but also at the peripheral level²⁹ where noxious stimuli are generated.³⁰ It was therefore relevant to develop compounds able to target pain through the peripheral components of the endogenous enkephalinergic system to avoid central side effects.^{31,32}

The present paper shows how these new disulfide co-drugs were designed, aiming at improving their oral bioavailability, and reports the pharmacokinetic properties, making them efficacious and safe painkillers suitable for oral administration in humans.

Scheme 3. Synthesis of Co-drugs and Pro-drugs 19^a

^aReagents and conditions: (a) CHCl_3 , Et_3N , RT; (b) EDCl, HOBt, MeOH, DIEA, CH_2Cl_2 ; (c) (1*R*,2*S*)-ephedrine, Et_2O or α -chymotrypsin, pH 7.8, DMSO; (d) (i) NaOH 1*N*, MeOH, (ii) Zn, 3*N* HCl, THF; (e) HX, NH_2R (25 or 27), BOP, DIEA, DMF, RT; (f) HCOOH; (g) NaIO_4 (0.2 *M* water solution), EtOH, 4 °C.

The first step was to design less hydrophobic inhibitors by keeping their high affinity for their target enzymes. In a second step, pro-drugs endowed with good solubility in aqueous solvents were tested for the ability to either cross the gastrointestinal or the blood–brain barrier in relation with a recruitment of the peripheral or the central enkephalinergic system. In this paper, we describe the synthesis of various pro-drugs and their pharmacological properties in various rodent models of pain. From these results, **19-IIIa** (designated PL37),³³ a new highly efficient DENKI pro-drug, was selected for clinical development. A complete set of **19-IIIa** pharmacological and clinical studies results will be published elsewhere.

RESULTS

Chemistry. Synthesis of APN Inhibitor Moieties. The synthesis of the β -aminothiols was performed as previously described from their corresponding Boc α -amino acids³⁴ leading to optically pure APN inhibitors (Scheme 1).

Synthesis of NEP Inhibitor Moieties. For industrial development, it could be interesting to remove the chiral center in P_1' position by inhibitors with a cyclopentyl or a cyclohexyl moiety in P_1' position which were reported to be NEP inhibitors.³⁵

The preparation of the pseudodipeptides NEP inhibitors included two main steps: (i) the synthesis of the different acetylthioalkanoic acids **9** or **14** and (ii) the coupling of several α -amino acids leading to compounds **11** and **15**, respectively (Schemes 2 and 3).

The acetylthioalkanoic acids **9** were obtained as racemic mixtures by addition of thioacetic acid on various acrylic acids **8**, which were synthesized by alkylation of triethylphosphonoacetate³⁶ or by Knoevenagel condensation with diethylmalonate.³⁷

Condensation of these different acids **9** with various α -amino acids methyl esters using the classical EDCl/HOBt coupling method led to the corresponding *N*-(mercaptoacyl)amino methyl esters **10** (Scheme 2), and the diastereoisomers were separated by HPLC. The ester and the thioacetate groups were hydrolyzed, leading to the corresponding inhibitors **11** as optically pure compounds for the determination of their NEP inhibitory potency.

For compounds containing a cyclopentyl or a cyclohexyl moiety in P_1' position, the acetylthioalkanoic acids **14** were obtained by nucleophilic substitution of the corresponding alcohols **13**, prepared from the commercially available acids **12**, as described by Neustadt et al.³⁸ Condensation of **14** with various α -amino acid methyl esters using the classical EDCl/HOBt coupling method followed by saponification led to the NEP inhibitors **15** (Scheme 2).

Synthesis of the Co-drugs: Formation of the Disulfide Bond. The dissymmetric disulfides **17** were obtained by activation of the Boc- β -aminothiols **6** with methoxycarbonylsulfonyl chloride, prepared in situ from chlorocarbonylsulfonyl chloride and MeOH,³⁹ then this intermediate was coupled with racemic or optically pure thioalkanoic acids **16**, issued from **9** by resolution

Table 1. Inhibitory Potency (K_i) of Thiols 11 and 15 on NEP

A)			B)			C)			
Compound	R ₃	K _i NEP (nM)	Compound	R ₃	K _i NEP (nM)	Compound	R ₃	n	K _i NEP (nM)
11a ^a	CH ₃	0.7 ± 0.2	11f	CH ₃	2.0 ± 0.5	15a	H	0	44.1 ± 0.4
11b ^a	H	1.5 ± 0.2				15b	CH ₃	0	59 ± 1
11c	CH ₂ OH	2.9 ± 0.5	11g	CH ₂ OH	24 ± 2	15c	CH ₂ COOH	0	3.45 ± 0.3
11d	CH(CH ₃)OH	2.5 ± 0.4				15d	CH ₂ COOH	1	17.9 ± 0.3
11e	CH ₂ COOH	1.5 ± 0.3	11h	CH(CH ₃)OH	8.2 ± 2.5				

^aSee ref 14 for literature.

with chiral amines^{25,40} such as (1*R*,2*S*) ephedrine or by enzymatic process (α -chymotrypsin)⁴¹ (Scheme 3).

Synthesis of the Pro-drugs. Several esters pro-drugs **19** were synthesized by coupling dissymmetric disulfide **17** with several hydrophilic α -amino acid alkyl esters **25** or **27**, including 1-(ethoxycarbonyloxy) groups obtained from a procedure described in the literature⁴² (see Supporting Information for experimental procedure for the synthesis of the α -amino acid alkyl esters (**25** or **27**) and the table with the corresponding pro-drugs **19** starting from dissymmetric disulfide racemic or optically active **17** (Supporting Information Scheme SI-12)).

The introduction of β -methionine sulfoxide as APN moiety in the compound **18-IVa** was achieved by oxidation of β -methionine. Therefore, compound **19-IVa** was obtained by action of NaIO₄ on **18-IIIa** in EtOH,³⁴ followed by final deprotection in HCOOH (Scheme 3).

Inhibitory Potencies of the New NEP and APN Inhibitors. As these co-drugs are a combination of a NEP and an APN inhibitor, through a disulfide bond, each moiety was tested under its free form for its NEP- or APN-inhibiting activity.

For the final selection of the NEP inhibitor moiety, two parameters were taken into account: (a) the inhibitory potency versus NEP, and (b) the easiest synthesis including the resolution step to obtain the inhibitor with a (S) configuration, corresponding to the most active form.¹⁸

NEP Inhibitory Potency. Accounting for the hydrophobic character of the S₁' subsite (as defined by Schechter and Berger⁴³) of NEP,^{44,45} various aromatic and cyclic structures were introduced in this position and the inhibitory potency of the obtained molecules was measured on NEP (Table 1). Thiophene being an usual isostere of benzene,⁴⁶ 3-thienyl methyl was tested instead of benzyl group, found in the P₁' position of the NEP inhibitor moiety of **1** or **2**.

The S₂' subsite of NEP, which is widely open toward the external medium,^{44,45} did not present a clear specificity: small and bulky chains, hydrophilic and hydrophobic residues were well-accepted. Consequently, compounds **11a–11e** with a benzyl group (Table 1A) or **11f–11h** with a 3-thienyl methyl

group (Table 1B) in P₁' and various residues in P₂' position were synthesized.

Compounds **11a–11e** (Table 1A) inhibited NEP with K_i values in the nanomolar range. For compounds containing a 3-thienyl methyl group in P₁' position, the best K_i value (2.0 ± 0.5 nM) was obtained for **11f** (Table 1B).

The introduction of a symmetric cyclic moiety without chiral center in P₁' position led to inhibitors **15** (Scheme 2) and their inhibitory potencies were evaluated toward NEP (Table 1C). Compounds **15a–15c**, with a cyclopentyl moiety in P₁' position, had K_i values in the 10⁻⁸ M range except when the P₂' residue was the hydrophilic aspartic acid, compound **15c** (K_i = 3.4 ± 0.3 nM). Compound **15d**, with a more flexible cyclohexyl moiety, in P₁' position, was found less efficient than its cyclopentyl analogue **15c**.

However, the most potent inhibitor **15c**, bearing two equivalent carboxylic functions, requested the esterification of both groups leading to a too high molecular weight molecule for a good bioavailability. Therefore, among the inhibitors reported in Table 1, compounds **11b**, **11d**, and **11f** were selected for the synthesis of the co-drugs due to their favorable inhibitory potency.

APN Inhibitory Potency. In a previous report,³⁴ β -aminothiols containing various aliphatic or aromatic side chains were tested as potential APN inhibitors such as **7a** and **7b**; compounds with aliphatic side chains have IC₅₀ values around 10–20 nM, and those with aromatic or bulky side chains display greater IC₅₀ values (30–130 nM). Methioninethiol **7a** was the most efficient, with a K_i value of 11 ± 2 nM. New β -aminothiols, derived from natural or synthetic α -amino acids, with linear aliphatic side chains, were prepared and tested toward APN (Table 2). Interestingly, compound **7c**, without a heteroatom in its side chain, has an inhibitory potency (13 ± 2 nM) close to **7a**. The three compounds derived from homoserine (**7d–7e**) and serine (**7f**) were significantly less active.

Moreover, **7a** (Table 2) could be easily transformed in a more soluble sulfoxide **7b**, which could be reduced enzymatically in vivo.⁴⁷

Table 2. Inhibitory Potency (K_i) of Amino Thiols 7a–7f on APN

Compound	R ₁	K _i APN (nM)
7a ^b	CH ₂ CH ₂ SCH ₃	11 ± 2
7b ^b	CH ₂ CH ₂ SOCH ₃	11 ± 2 ^a
7c	CH ₂ CH ₂ CH ₂ CH ₃	13 ± 2
7d	CH ₂ CH ₂ OCH ₃	35 ± 2
7e	CH ₂ CH ₂ OCH ₂ CH ₃	47 ± 8
7f	CH ₂ OCH ₂ CH ₃	55 ± 10

^adiS homodimer reduction was performed with DTT present in the APN buffer. ^bSee ref 34 for literature.

According to these *in vitro* results, new DENKIs pro-drugs were selected among compounds containing, for APN inhibition, a methioninethiol or its sulfoxide and for NEP inhibition, a pseudodipeptide, made of either benzyl or 3-thienyl methyl as P₁' components and α -amino acid (Gly, Ala, or Thr) for S₂' recognition.

On the basis of these criteria, four co-drugs (**19-I** to **19-IV**, Figure 2) and their pro-drugs, obtained after introduction of hydrophilic esters, were tested for their antinociceptive properties (Tables 3–5).

Pharmacological Studies. The Hot Plate Test (HPT) in Mice, A Model of Acute Central Pain.⁴⁸ Consistent with their charged forms well-known to inhibit membrane crossing, the selected co-drugs **19-I**–**19-IV** (Figure 2) were found inactive in the hot plate test at a single dose after either *iv* (10 mg/kg) or oral administration (150 mg/kg) (data not shown), highlighting the necessity of the ester pro-drugs to cross the gastrointestinal or the BBB barrier.

To evaluate the interest of relatively hydrophilic esters in improving bioavailability of dual inhibitors, a preliminary study was performed with a series of pro-drugs derived from the co-drug **19-I**, used as a diastereoisomeric mixture (Table 3). These compounds (**19-Ia**–**19-Ig**) were tested in the HPT after *iv*

administration, at 20 mg/kg in water/mannitol (50 mg/mL) as vehicle. The percentage of antinociception was reported in parallel with the calculated average Log *P*^{49,50} (Table 3).

These esters (except **19-Ig**) elicited significant antinociceptive effects 10 min after injection.

Two compounds emerged with an efficacy of 40% analgesia, **19-Ic** and **19-Ie**, and their average Log *P* (1.72 and 0.96, respectively) correspond to a good balance between solubility and permeability.^{49,50} The most hydrophilic and consequently more soluble in aqueous solvent, compounds **19-Ib**, **19-Ie**, and **19-Ig** (average Log *P* < 0.4), were significantly less active. However, due to the complex synthesis of the triglyceride **19-Ie**, the sole acyloxyethyl ester of **19-Ic** was selected for the synthesis of pro-drugs in the benzyl series.

19-IIa and **19-IIIa** were tested at different doses after *iv* administration (Table 4). At 40 mg/kg, threonine-containing derivative **19-IIa** induced significant responses (61% analgesia), but glycine-containing derivative **19-IIIa**, which produced 35% analgesia at 10 mg/kg and 100% analgesia at 40 mg/kg, was the most interesting pro-drug in this series.

The responses to **19-IIIa** after *iv* administration were then evaluated in various vehicles. In water/mannitol, this compound induced dose-dependent antinociceptive effects (ED₅₀ = 16 mg/kg) (Figure 3A) with a relatively short time-course (less than 40 min), measured at the dose of 40 mg/kg (Figure 3B). In EtOH/Tween80/water (1/1/8), strong dose-dependent antinociceptive effects were obtained with low doses of **19-IIIa** (ED₅₀ = 4.5 mg/kg) (Figure 3C). The full reversion of **19-IIIa**-induced antinociception by prior subcutaneous injection of naloxone (0.5 mg/kg) (Nlx) supports the selective involvement of opioid receptors (Figure 3D).

Then, antinociceptive responses of **19-IIIa** and its sulfoxide analogue **19-IVa** were compared at 10 mg/kg in EtOH/Tween80/water (1/1/8) after *iv* administration (Figure 4A). A significant lower efficacy of **19-IVa** (30% analgesia) compared to **19-IIIa** (78% analgesia) was observed 10 min after administration.

Accounting for the marked efficacy of **19-IIIa** by *iv* route, this compound and other acyloxylalkyl ester pro-drugs (**19-IIIb**–**19-IIIe**) were studied after oral administration (150 mg/kg in EtOH/PEG400/H₂O 10/40/50 as vehicle), in HPT in mice

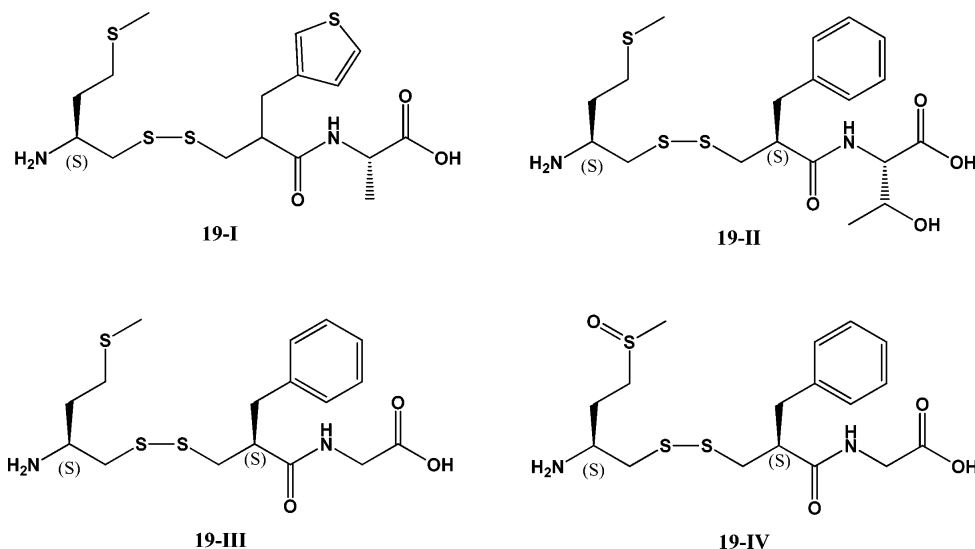
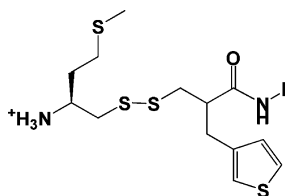
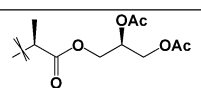
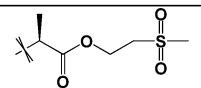
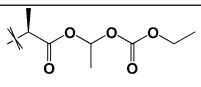
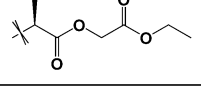
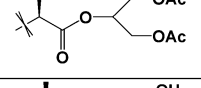
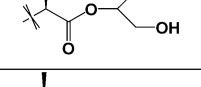
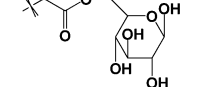
**Figure 2.** **19-I**–**19-IV** co-drug structures.

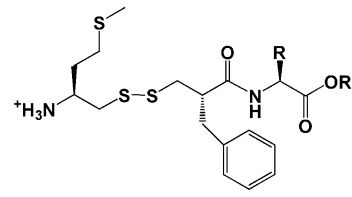
Table 3. Percentage of Analgesia after Intravenous Injection of 19-Ia–19-Ig Pro-drugs Measured on Hot Plate Test in Mice

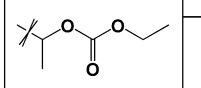
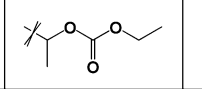


Compound	R	% Analgesia ^a	Average LogP ^b
19-Ia		29 ± 8 ^{**}	0.96
19-Ib		22 ± 7 ^{**}	0.39
19-Ic		44 ± 7 ^{***}	1.72
19-Id		32 ± 5 ^{***}	1.14
19-Ie		40 ± 9 ^{***}	0.96
19-Ig		22 ± 8 ^{**}	-0.17
19-Ig		10 ± 7 ^{ns}	-1.12

^aHot plate test, male mice. Dose: 20 mg/kg, iv administration, 10 min postinjection. Vehicle: water/mannitol (50 mg/mL). ANOVA + Newman Keuls: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. ^bALOGPs 2.1 Program, Virtual Computational Chemistry Laboratory.

Table 4. Percentage of Analgesia after Intravenous Injection of 19-IIa or 19-IIIa Pro-drugs Measured on Hot Plate Test in Mice



Compound	R	R'	% Analgesia ^a				Average LogP ^b
			10 mg/kg	20 mg/kg	40 mg/kg	60 mg/kg	
19-IIa	CH(CH ₃)OH		---	29 ± 5 ^{**}	61 ± 8 ^{**}	84 ± 8 ^{**}	1.49
19-IIIa	H		35 ± 8 ^{**}	58 ± 6 ^{***}	97 ± 2 ^{***}	-	1.73

^aHot plate test, male mice. Dose: iv administration, 10 min postinjection. Vehicle: water/mannitol (50 mg/mL). ANOVA + Newman Keuls: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. ^bALOGPs 2.1 Program, Virtual Computational Chemistry Laboratory.

(Table 5). The percentage of antinociception, measured 20, 40, and 60 min after gavage, showed that **19-IIIa** remained the most active pro-drug of this series. The dose–response curve (Figure 4B), established 20 min after administration, gave an ED₅₀ of 133 mg/kg. The duration of action of all these compounds remained relatively short (less than 1 h).

*Tail-Flick Test in Rat (TFT), A Model of Acute Centropinal Nociception.*⁵¹ As shown in Figure 3E, **19-IIIa** produced a significant dose-dependent antinociceptive effects 10 and 20 min after iv administration at doses of 2.5, 5, 10, and 20 mg/kg in EtOH/Tween80/H₂O (1/1/8). The maximum effect (40% analgesia) was obtained 10 min after injection of 20 mg/kg. At

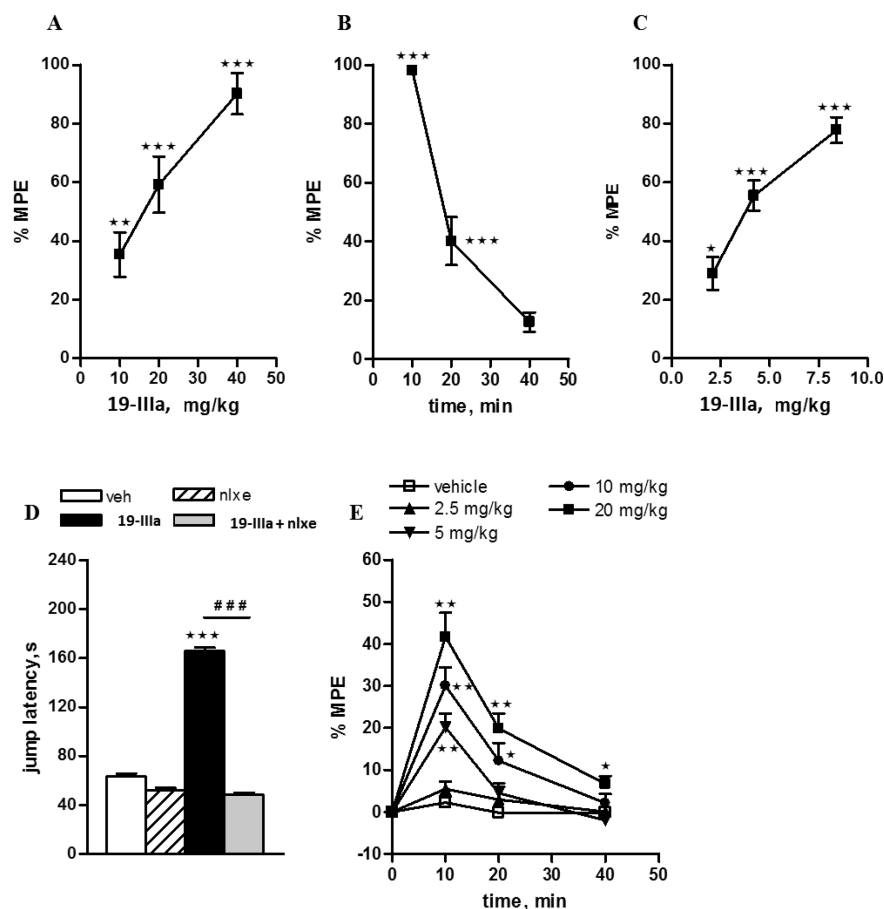


Figure 3. Antinociceptive effects of compound **19-IIIa** by intravenous route. (A) Dose–responses of **19-IIIa** obtained in a vehicle (mannitol, 50 mg/mL in water) 10 min after iv injection in the hot plate test (jump latency) in mice, $n = 10$ per group. (B) Duration of action of **19-IIIa** injected by iv route, 40 mg/kg, in a vehicle (mannitol, 50 mg/mL in water), in the hot plate test (jump latency) in mice, $n = 9–10$ per group. (C) Dose–responses of **19-IIIa** were obtained in ethanol/Tween80/water (1/1/8), 10 min after iv injection in the hot plate test (jump latency) in mice, $n = 18–21$ per group. (D) Inhibition of antinociceptive responses induced by **19-IIIa** (10 mg/kg, iv) by prior injection of naloxone (0.5 mg/kg, sc) in the hot plate test in mice, $n = 7–10$ per group. Results are expressed as mean % MPE \pm SEM, or mean jump latency (s) \pm SEM. $\star p < 0.05$, $\star\star p < 0.01$, $\star\star\star p < 0.001$ versus vehicle, $\#\#\# p < 0.001$ versus drug, ANOVA followed by Bonferroni test. (E) Tail flick in rat. Antinociceptive effects of **19-IIIa** injected by iv route at different doses in the tail-flick test in rat, $n = 8–9$ per group. Results are expressed as mean % MPE \pm sem. $\star p < 0.05$, $\star\star p < 0.01$, $\star\star\star p < 0.001$ versus vehicle, $\#\#\# p < 0.001$ versus drug, ANOVA followed by Bonferroni test.

this dose, the antinociceptive effects remained significant until 40 min after administration.

At the oral dose of 100 mg/kg, **19-IIIa** was poorly active (10% analgesia 20 min after administration, data not shown).

Antinociceptive Responses in the Formalin Test, A Model of Persistent Pain.⁵² Formalin solution injected subcutaneously into the plantar hindpaw of mice induces a tissue injury associated with persistent pain and inducing licking of the affected paw. Compound **19-IIIa** and its sulfoxide analogue, **19-IVa**, were compared in the early phase (0–5 min after formalin injection) of the test at 50 mg/kg po in EtOH/methyl cellulose 0.5% in water (1.5/98.5) as vehicle (Figure 5). At 30 min after drug administration, **19-IVa** was slightly, but not significantly, more active than **19-IIIa** (54% vs 41% analgesia) in reducing licking. Interestingly, both compounds remained equipotent at 90 min (41% analgesia).

Model of Neuropathic Pain: Partial Sciatic Nerve Ligation (PSNL) in Mice. The partial sciatic nerve ligation in mice induces characteristic symptoms as profound and prolonged mechanical allodynia and thermal hyperalgesia, characterized respectively by a lowered paw withdrawal threshold to pressure (von Frey test⁵³) and thermal stimuli (plantar test⁵²), related to neuropathic

pain.^{54–56} As shown in Figure 6A (left panel), PSNL induced a dramatic decrease in the threshold for paw withdrawal during von Frey filament stimulation, significant when compared to the noninjured contralateral side (Figure 6A, right panel). Oral administration of **19-IIIa**, 25 mg/kg, significantly increased mechanical threshold values by 51% at 20 min. Likewise, **19-IIIa** (25 mg/kg, po) induced a moderate antihyperalgesic effect (increase of thermal threshold values to half of those obtained on contralateral side) (Figure 6B, left panel). These results support the antiallodynic and antihyperalgesic effects of **19-IIIa** at low oral doses in animal model of neuropathic pain.

Kinetics of Pro-drug Bioactivation. In Vitro Bioactivation. Compound **19-IIIa** was incubated at a final concentration of 800 μ M in rat plasma (66 mg protein/mL) at 37 °C and the proportion of intact pro-drug and major metabolites, measured in function of time, was quantified by LC/MS analysis (Figure 7).

After 5 min, all the pro-drug **19-IIIa** had disappeared, transformed into the co-drug **19-III** (100%), which decreased slowly (80% after 1 h) without appearance of new metabolite. This latter effect was likely due to plasma protein binding.

In Vivo Bioactivation. Compound **19-IIIa** (200 mg) was administered orally to male rats. Iterative blood samples (0.3

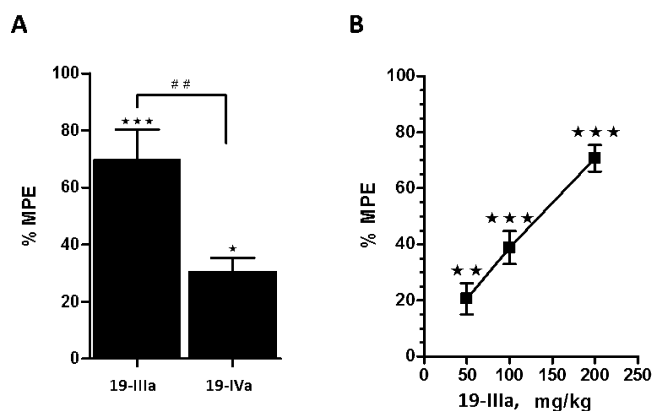


Figure 4. Antinociceptive effects of 19-IIIa and 19-IVa. (A) Antinociceptive effects of 19-IIIa and 19-IVa (10 mg/kg) by intravenous injection in ethanol/Tween80/water (1/1/8) in the hot plate test in mice, 10 min after injection. Results are expressed as mean % MPE \pm SEM, $n = 8$ per group. $\star p < 0.05$, $\star\star\star p < 0.001$ versus vehicle; $\#\#\# p < 0.01$ versus drug, ANOVA followed by Bonferroni test. (B) Antinociceptive effects of 19-IIIa after p.o. administration in EtOH/PEG400/H₂O (10/40/50), at different doses in the hot plate test in OF1 mice, $n = 8-9$ per group mice. Results are expressed as mean % MPE \pm sem. $\star\star p < 0.01$, $\star\star\star p < 0.001$ versus vehicle, $\#\#\# p < 0.001$ versus drug, ANOVA followed by Bonferroni test.

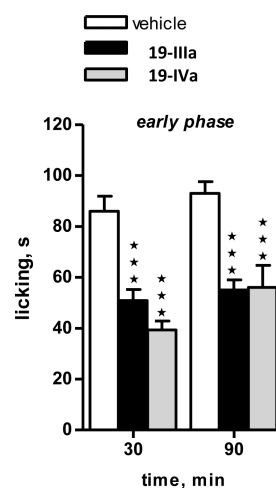
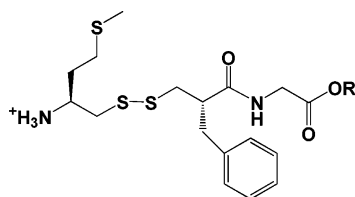


Figure 5. Antinociceptive effects of oral administration of compounds 19-IIIa and 19-IVa (50 mg/kg) in the formalin test in mice. Formalin solution was injected sc into the plantar surface of the hindpaw, and licking behavior (seconds) was recorded during the following 5 min (0–5 min post injection), corresponding to the early phase of the test. Compounds 19-IIIa or 19-IVa or vehicle (ethanol/methylcellulose 0.5% in water, 1.5/98.5) were given orally 30 or 90 min before formalin injection. Results are expressed as mean licking time (s) \pm SEM, $n = 5-7$ per group. $\star\star\star p < 0.001$ versus vehicle, ANOVA followed by Bonferroni test.

Table 5. Percentage of Analgesia after Oral Administration of 19-IIIa–19-IIIe Pro-drugs Measured on Hot Plate Test in Mice



Compound	R	Dose	% Analgesia ^a			Average LogP ^b
			20 min	40 min	60 min	
19-IIIa		150 mg/kg	58 \pm 5 ^{***}	28 \pm 5 ^{**}	7 \pm 2 ^{ns}	1.73
19-IIIb		150 mg/kg	32 \pm 7 ^{***}	24 \pm 5 ^{***}	8 \pm 4 ^{ns}	2.38
19-IIIc		150 mg/kg	46 \pm 6 ^{***}	18 \pm 5 ^{ns}	0 ^{ns}	2.48
19-IIId		150 mg/kg	8 \pm 4 ^{ns}	10 \pm 5 ^{ns}	12 \pm 6 ^{ns}	3.22
19-IIIE		150 mg/kg	17 \pm 4 ^{ns}	3 \pm 2 ^{ns}	8 \pm 4 ^{ns}	3.32

^aHot plate test, male mice. Dose: oral administration 150 mg/kg. Vehicle: EtOH/PEG400/H₂O (10/40/50). ANOVA + Newman Keuls: ^{***} $p < 0.001$; ^{**} $p < 0.01$; ^{*} $p < 0.05$. ^bALOGPs 2.1 Program, Virtual Computational Chemistry Laboratory.

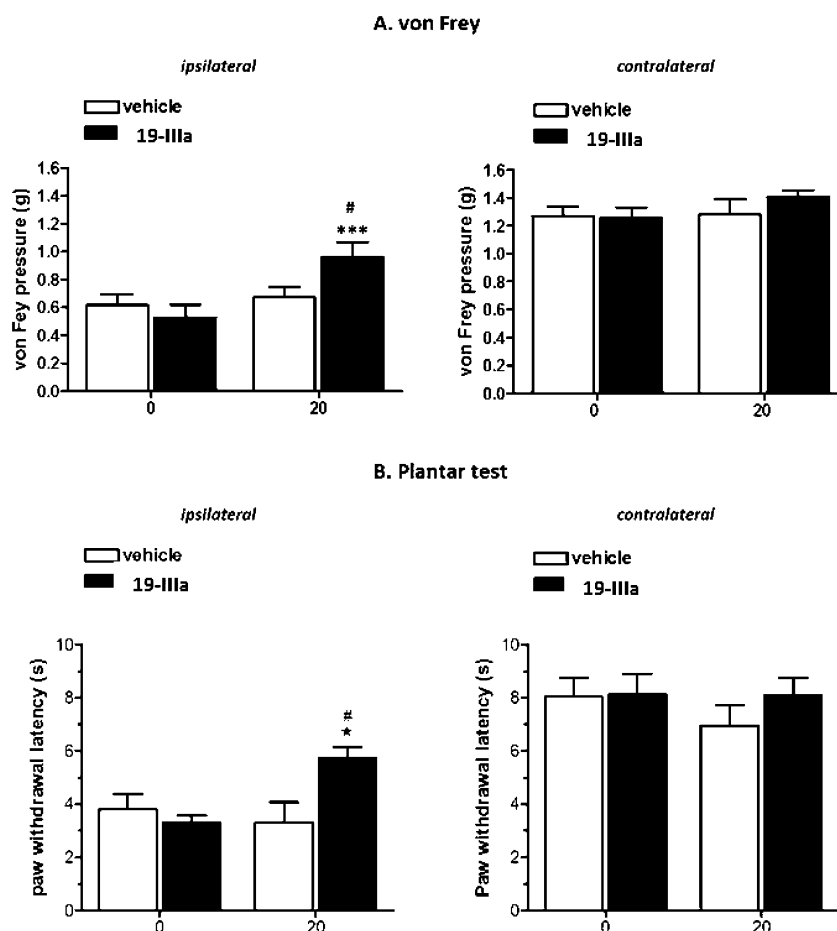


Figure 6. Antiallodynic and antihyperalgesic effects of **19-IIIa** on neuropathic pain in the partial sciatic nerve ligation model in mice. (A) Mechanical allodynia (von Frey filament) and (B) thermal hyperalgesia (plantar test) induced by partial sciatic nerve ligation in mice were assessed between day 10 and day 20 postsurgery, before (t0) and 20 min after po administration of **19-IIIa** (25 mg/kg) or vehicle (ethanol/methylcellulose 0.5% in water, 1.5/98.5), on both ipsilateral (left panel) and contralateral (right panel) sides and on sham-operated mice. Results are expressed as mean von Frey pressure (g) \pm sem (allodynia) and mean paw withdrawal latency (s) \pm sem (hyperalgesia), $n = 6-9$ per group. ★★ $p < 0.01$, ★★★ $p < 0.001$, versus vehicle, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus t0, ANOVA + Bonferroni.

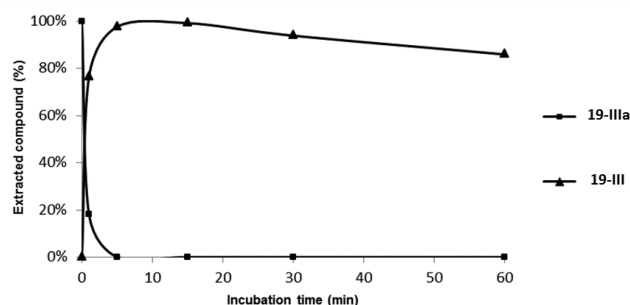


Figure 7. Incubation in vitro of **19-IIIa** (800 μ M) in male rat plasma (66 mg protein/mL) at 37 $^{\circ}$ C.

mL) were collected during 8 h after administration. Plasma samples were centrifuged at 8 $^{\circ}$ C for analysis by LC/MS/MS. After 30 min gavage, the pro-drug **19-IIIa** disappeared as well as 98% of the co-drug **19-III** in favor of the different metabolites from the two moieties formed by disulfide bridge cleavage (methioninethiol **7a** (20%) and thiorphan **11b** (28%), which were mainly transformed into their S-Me forms (c (20%) and d (48%), respectively) and various oxidized derivatives such as sulfoxide from the S-Me derivatives of the latter two (Figure 8).

For the NEP moiety, at 30 min in rat plasma, the main metabolite corresponds to the S-methylation of thiorphan (d).

DISCUSSION

The aim of this study was to develop new druggable analgesics based on the now well-accepted concept of dual enkephalinase inhibition.^{12,57,58} However, the poor solubility of early DENKIs (Figure 1) in aqueous medium and their mediocre bioavailability have precluded the possibility to develop them.

To overcome this problem, improvements in the chemical structures of the co-drug and pro-drug moieties were performed. The oral bioavailability of each compound was estimated by calculating their average Log P , which is optimal between 1 and 2.^{49,50}

As a first step, we attempted to decrease the lipophilicity of the NEP thiol inhibitor HS-CH₂CH(CH₂Ph)CONHCH(CH₃)-CO₂H (**11a**) present in **2**. The substitution of the benzyl group by its more hydrophilic isostere 3-thienyl methyl group in **11f** (Table 1) yielded an interesting inhibitor. However, the difficulty to obtain an optically pure intermediate (S)-**9a** in large amounts precluded its use for further development and forced us to test pro-drugs from **19-I** series as a diastereoisomeric mixture (for details see Supporting Information). Another possibility to decrease the hydrophobicity of the molecule without loss of

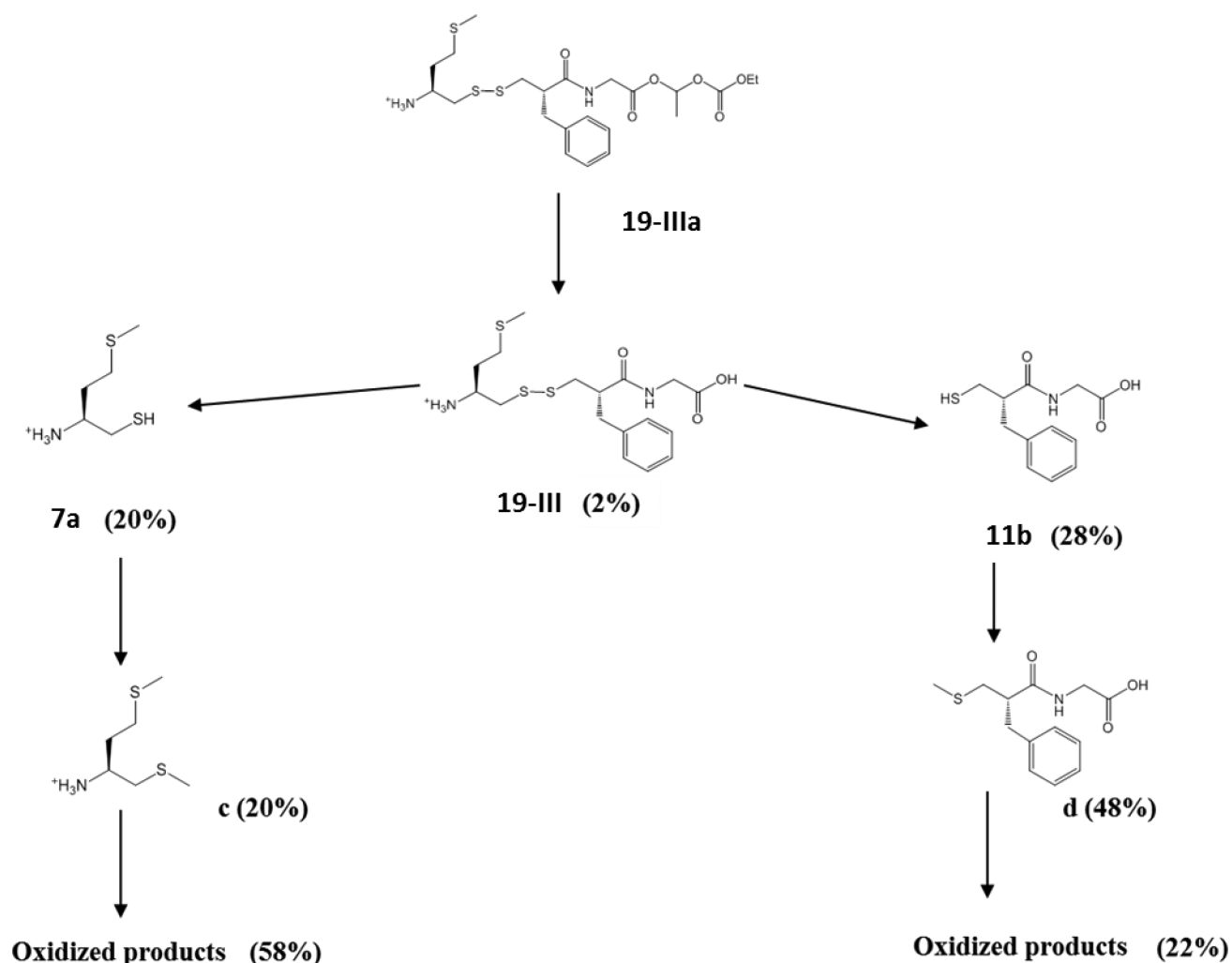


Figure 8. Metabolism of **19-IIIa**: percentage of metabolites from each moiety, measured in male rat plasma, 30 min after po administration of **19-IIIa**.

potency was to change the C-terminal amino acid by substituting the alanine by a glycyl residue, like in thiorphan.¹⁴

As a second step, with the aim of removing any chiral center present in the NEP inhibiting moiety, mercaptomethyl-cyclopentanoyl and -cyclohexanoyl synthons were tested, instead of the mercaptomethylbenzoyl group. Because of its planar structure, the cyclopentyl group was relatively well accepted^{13,5,38} (see Table 1C) as compared to the cyclohexyl moiety. Nevertheless, a nanomolar inhibitory potency on NEP was obtained only with a cyclopentyl group combined with an aspartic acid as P₂' component (compound **15c**) optimizing the recognition of Arg-102 and Arg-104, present in human NEP active site S₂' subsite.^{13,44,45} However, the presence of two carboxylate groups requires a dual esterification with cascade ester, of which synthesis is difficult and would lead to a molecule with a too high molecular weight.

Hydrophobic and linear β -aminothiol side-chains have been shown to give the best APN inhibition.³⁴ None of the tested modifications, such as substitution of the sulfur atom by an oxygen or a CH₂ group, increased the inhibitory potency as compared to the methionine side chain (Table 2).

Four series of co-drugs (Figure 2) were selected and tested for their antinociceptive properties. As expected, they did not induce any significant response, showing that, without ester moiety, they

were unable to cross the blood–brain and/or the intestinal barrier.

Therefore, various other pro-drugs were synthesized. Accounting for the bis-ionic form of the co-drugs, transient protection of the C-terminal carboxylic or N-terminal groups could be considered. In this study, the carboxylate protection was selected. Therefore, esterification of co-drugs with alcohols, known for their capacity to improve drugs bioavailability, such as diacetoxy-propyl alcohol, carbethoxyalkyl alcohol, or ethoxycarbonyloxyalkyl alcohol,^{59–61} was performed.

Co-drug **19-I** esters were prepared and evaluated for their efficacy in the HPT. Compounds **19-Ic** and **19-Ie** were found the most effective (Table 3). However, due to the intrinsic chemical instability⁶² of the triglyceride found in **19-Ie**, this compound was not developed.

The ethoxycarbonyloxyethyl alcohol, present in **19-Ic**, was therefore selected and introduced in co-drugs **19-II**, **19-III**, and **19-IV**, leading to **19-IIa**, **19-IIIa** (Table 4), and **19-IVa** (Figure 4A), respectively. Tested, by iv, in water/mannitol, on the HPT, **19-IIIa** was significantly more active than **19-IIa** (Table 4) and **19-IVa** (Figure 4A), likely in relation with a better brain penetration due to a higher hydrophobicity.

For iv administration of **19-IIIa**, another vehicle (ethanol/Tween 80/H₂O: 1/1/8) well-known to facilitate the blood–brain barrier crossing,⁶³ was used (Figure 3C). As expected, the

lowest value ($ED_{50} = 4.5$ mg/kg) in the HPT was obtained using this vehicle. The complete reversal of the antinociceptive effects by naloxone, an antagonist of opioid receptors, demonstrated that the responses were mediated by the opioidergic system at the brain and/or spinal levels (Figure 3D).

Considering the results obtained with **19-IIIa** on intravenous administration, various other ethoxycarbonyloxyalkyl esters (**19-IIIc** and **19-IIIe**) and carbethoxyalkyl esters (**19-IIId** and **19-IIId**) were evaluated in the HPT (Table 5) by oral route. The level of analgesia measured under these conditions reflected the ability of the molecules to cross both gastrointestinal and blood–brain barriers. Once more, **19-IIIa**, endowed with an average Log *P* in the proper range, was found to be the more efficient inhibitor of the series. In a more severe antinociceptive model, the tail flick test in rats, **19-IIIa**, induced significant effect at relatively low doses (40% analgesia at 20 mg/kg iv, Figure 3E).

Since the efficacy of **2** has been previously measured in these two tests,²⁶ it was interesting to evaluate the increased bioavailability induced by the ethoxycarbonyloxyethyl ester present in **19-IIIa** in place of the benzyl ester in **2**. Thus, administered by iv route in a lipophilic vehicle containing 10% EtOH known to facilitate brain crossing, the two pro-drugs gave similar responses in HPT: **2** ($ED_{50} = 3$ mg/kg) in EtOH/cremophor/H₂O and **19-IIIa** ($ED_{50} = 4.5$ mg/kg) in EtOH/Tween80/H₂O (Figure 3C). In the tail-flick test, **2** induced 35% analgesia at 25 mg/kg and **19-III** 40% analgesia at 20 mg/kg (Figure 3E). By contrast, antinociceptive responses observed in HPT after oral administration, are 4-fold better for **19-IIIa** ($ED_{50} = 133$ mg/kg) than for **2** ($ED_{50} = 410$ mg/kg).

Two other nociceptive tests, which are known to involve the peripheral opioid system,⁶⁴ were achieved: the formalin test which produces persistent pain by tissue injury⁶⁵ and the partial ligation of sciatic nerve, which is a currently used predictive model of neuropathic pain.⁶⁶

The efficiency of **19-IIIa** and its sulfoxide analogue **19-IVa** at the same dose (50 mg/kg, per os) was compared in the formalin test (Figure 5). No difference, neither in the intensity of the response nor in the duration of action, was observed between these two compounds. This observation was different from that obtained in the hot plate test after iv administration, where **19-IVa** was about two times less efficient than **19-IIIa** (Figure 4A). It could be assumed that **19-IVa** was rapidly reduced in **19-IIIa** after oral administration, most probably at the level of the gastrointestinal barrier.^{59,60}

The chronic pain associated with neuropathic syndromes, such as allodynia and hyperalgesia, results from a wide variety of pathophysiological mechanisms and the multiplicity of factors involved in this pathology.^{67,68} In this study, we used a model of neuropathic pain based of the lesion of peripheral nerves,^{53,56} in which opiates have been demonstrated to be active,^{69–71} although associated with severe side effects due to their repetitive administration. In our experiments with **19-IIIa**, antiallodynic and antihyperalgesic responses were obtained at relatively low doses: **19-IIIa**, given orally at 25 mg/kg, reduced both thermal hyperalgesia and mechanical allodynia by about 50% (Figure 6).

Accounting for the significant increase in the pharmacological properties observed by modifying the co-drug **19-III** into the pro-drug **19-IIIa**, it was interesting to study in details the metabolism of this latter in vitro and in vivo to understand its action in both the central nervous system and the periphery. After in vitro incubation, of **19-IIIa**, in plasma, the release of the ester was very rapid, but the disulfide bridge was not cleaved (Figure 7). However, the pro-drug ester was essential for an iv or

po activity in a central test such as the hot plate assay. Even if the cleavage of the cascade ester is very rapid, its short lifetime is sufficient to allow the brain crossing followed by formation of the two active NEP and APN moieties after cleavage of the disulfide bridge inside the CNS³⁶ or more likely during the BBB crossing.

By oral route, the duration of action was longer than by iv administration and the metabolism in vivo was very similar to that obtain in vitro. The important difference was the rapid transformation of the two active inhibitor entities, thiorphan and methionine thiol, by *S*-methylation. In the case of thiorphan, the metabolite possesses a non-negligible NEP inhibitory potency ($K_i = 46.3 \pm 0.3$ nM, **d** in Figure 8), while the *S*-methylation of APN blocker methionine thiol leads to an inactive compound (**c** in Figure 8), explaining, at least partly, the relatively short duration of inhibitory potency of the pro-drug **19-IIIa**.

CONCLUSION

Taken together, these results indicate that the objective of obtaining a DENKI, active by oral route on various types of pain was reached. The DENKI **19-IIIa**, is active either on acute centrally controlled pain or on neuropathic pain, in this latter case essentially by acting at the peripheral level, as shown by reversion of the analgesia following administration of methyl-naloxonium, an opioid antagonist unable to enter the brain,⁷² which has been previously demonstrated by experiments using DENKIs and methyl-naloxonium.^{73,74} Furthermore, as expected from the mechanism of action of DENKIs and previous extensive pharmacological studies with **1**,^{25–28} none of the unwanted effects of morphine was observed in any of the animal models of pain tested (not shown), which was confirmed in phase I human clinical trials with **19-IIIa** (to be published). This demonstrates that DENKIs have the potential to be novel potent analgesics, effective on various types of pain when administered via appropriate routes and vehicles. In neuropathic and inflammatory pain, two severe long-lasting painful diseases,⁷⁵ these DENKIs, acting at the source of the nocifensive stimulation by increasing locally the levels and lifetime of endogenous enkephalins, are devoid of morphine side effects and represent a novel promising approach of painkillers.

EXPERIMENTAL SECTION

Chemistry. All reagents were obtained from Sigma-Aldrich and solvents from Carlo Erba-SDS (France). TLC plates were Merck TLC aluminum sheets coated with silica gel 60 F254. Compounds were purified by flash chromatography (silica gel Si 60, 40–63 μ m) or by semipreparative HPLC purification. ¹H NMR spectra were recorded on 200 MHz Bruker instrument. Chemical shift were reported in ppm with the solvent as internal standard (CDCl₃, 7.26 ppm; DMSO-*d*₆, 2.54 ppm). Data are reported as followed: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet). Electron spray mass spectroscopy (ESI) was performed with a LCMS Quad Electrospray Agilent 6120. RP-HPLC separations were respectively performed on Shimadzu Prominence HPLC at a flow rate of 1 mL/min for analytical column (Kromasil C18, 100 Å, 5 μ m, 250 mm \times 4.6 mm, ACE C18, 100 Å, 5 μ m, 250 mm \times 4.6 mm, or Atlantis T3, 100 Å, 3 μ m, 100 mm \times 4.6 mm) and on Waters prep 600 HPLC at a flow rate of 10 mL/min on the corresponding semipreparative column (Kromasil C18, 100 Å, 5 μ m, 250 mm \times 21.2 mm, ACE C18, 100 Å, 5 μ m, 250 mm \times 21.2 mm, or Atlantis T3, 100 Å, 3 μ m, 100 mm \times 21.2 mm). Purity (%) was determined from a surface integral of detected peaks by reversed-phase HPLC using UV detection (210 nm) and all compounds showed purities greater than 95%. All commercial reagents and solvents were used without further purification.

General Procedure for the Synthesis of Dissymmetric Disulfide 2-Alkyl-3-((S)-2-(tert-butoxycarbonylamino)-4-

(methylthio)butyl)disulfanyl)propanoic Acid (17). A mixture of MeOH (23 mL) and THF (23 mL) was cooled to 4 °C, under inert atmosphere. Then chlorosulfonyl chloride (1.3 mL, 15.25 mmol, 1.09 equiv) was added dropwise. The reaction mixture was stirred for 15 min at 4 °C to give the methoxycarbonylsulfonyl chloride. Compound 6 (14.86 mmol, 1.06 equiv) in 16 mL of THF/MeOH was added once. The reaction mixture was warmed to room temperature and stirred for 30 min. The previous solution was added dropwise to a solution of compound 16 (14.02 mmol, 1 equiv) in 100 mL of degassed CH₂Cl₂ in the presence of Et₃N (1 equiv). The reaction mixture was stirred 1 h at room temperature. The solvent was removed under reduced pressure, and the product was portioned in CH₂Cl₂ and 10% citric acid solution. The organic layer was washed with brine and dried over Na₂SO₄ to give a crude product, which was purified on silica gel with CHex/AcOEt 8/2 and then CHex/AcOEt 6/4 as eluent to give compound 17.

Because of the poor resolution of 9a (see Supporting Information), compound 17a was used as a diastereoisomeric mixture.

No loss of optical resolution was observed during the following steps of the synthesis.

(S)-3-(((S)-2-(tert-Butoxycarbonylamino)-4-(methylthio)butyl)disulfanyl)-2-(thiophen-3-ylmethyl)propanoic Acid (17a) (R₂ = CH₂-3-Thiophene). White solid (yield 77.0%). ¹H NMR (CDCl₃): δ tBu 1.46 (9H, s), CH₂β 1.72 (1H, m) and 1.98 (1H, m), SCH₃ 2.12 (3H, s), CH₂γ 2.56 (2H, m), SCH₂ + CH + CH₂Ar + SCH₂ 2.70–3.20 (7H, m), CHα 3.95 (1H, m), NH 4.73 (1H, m), Ar 6.93 (1H, m), Ar 7.08 (1H, m), Ar 7.30 (1H, m). HPLC Kromasil C18, 100 Å, 5 μm, 250 mm × 4.6 mm, CH₃CN/H₂O (0.1% TFA) 70–30: 7.36 min.

(S)-2-Benzyl-3-(((S)-2-(tert-Butoxycarbonylamino)-4-(methylthio)butyl)disulfanyl)propanoic Acid (17b) (R₂ = CH₂Ph). White solid (yield 65.9%); ee 88%. ¹H NMR (CDCl₃): δ tBu 1.48 (9H, s), CH₂β 1.68 (1H, m) and 1.97 (1H, m), SCH₃ 2.11 (3H, s), CH₂γ 2.56 (2H, m), SCH₂ + CH + CH₂Ar + SCH₂ 2.70–3.20 (7H, m), CHα 3.95 (1H, m), NH 4.72 (1H, d), Ar 7.20–7.40 (5H, m). HPLC Kromasil C18, 100 Å, 5 μm, 250 mm × 4.6 mm, CH₃CN/H₂O (0.1% TFA) 70–30: 8.20 min.

General Synthesis of 2-(3-(((S)-2-Amino-4-(methylthio)butyl)disulfanyl)-2-alkylpropanamido)-2-alkylacetic Ester (19). The amino acid ester was coupled using BOP (1.2 equiv) and DIEA (1.2 equiv) as reagents, and final deprotection gave the crude compound 19.

The disulfide 17 (0.54 mmol) was solubilized in DMF (4 mL) (Scheme 3). BOP (1.2 equiv; 1.0 g) was added, followed by DIEA (284 μL) and the corresponding amino acid ester 25 or 27 (1.3 equiv) (see Supporting Information for the experimental procedure for synthesis of the appropriate 25 or 27 leading to the corresponding pro-drug 19, Supporting Information Scheme SI-12). The reaction mixture was stirred for 20 min at room temperature, and then DMF was removed under reduced pressure. The crude mixture was portioned in AcOEt, and the organic layer was washed with 10% citric acid solution, 10% NaHCO₃ solution, and brine and dried over Na₂SO₄ to give a crude product, which was purified on silica gel with CHex/AcOEt 8/2 and then CHex/AcOEt 6/4 as eluent to give corresponding compound 18.

Compound 18 (0.38 mmol) was solubilized in HCOOH (1 mL). The reaction mixture was stirred for 1 h at room temperature. The excess HCOOH was removed under reduced pressure, and the reaction mixture was coeluted with cyclohexane to give crude which was purified by reverse-phase semipreparative HPLC to give the appropriate pro-drug 19.

Because of the poor resolution of 9a, 19-I series compounds (Supporting Information Scheme SI-11) were synthesized starting from a diastereoisomeric mixture of 17a and tested as a mixture (Table 3).

1-(2-(1-(2,3-Diacetoxypropoxy)carbonyl)-ethylcarbamoyl)-3-thiophen-3-ylpropyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (19-1a) (R₂ = CH₂-3-Thiophene, R = CH-(S)-CH₂-COOCH₂CH(OAc)CH₂OAc). Solid (yield 48.4%). ¹H NMR (DMSO-*d*₆ + TFA): δ CH₃ 1.12 and 1.28 (3H, d), CH₂β 1.72–1.92 (2H, m), COCH₃ 1.92 (6H, s), SCH₃ 1.98 (3H, s), CH₂γ 2.52 (2H, m), SCH₂ + CH + CH₂Ar + SCH₂ 2.60–2.95 (7H, m), CHα 3.41 (1H, m), CHα + CH₂OCO 4.05–4.30 (5H, m), CHOAc 5.12 (1H, m), Ar 6.93 (1H, m), Ar 7.12 (1H, m), Ar 7.38 (1H, m), NH₃⁺ 7.88 (3H, br), NH 8.45 (1H, br). HPLC Kromasil C18, 100 Å, 5 μm, 250 mm × 4.6 mm,

CH₃CN/H₂O (0.1% TFA) 40–60: 9.07 and 10.18 min. ESI(+): [M + H]⁺ = 581.2.

1-(2-(1-(2-Methanesulfonylethoxycarbonyl)-ethylcarbamoyl)-3-thiophen-3-ylpropyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (19-1b) (R₂ = CH₂-3-Thiophene, R = CH-(S)-CH₂-COOCH₂CH₂SO₂CH₃). Solid (yield 54.7%). ¹H NMR (DMSO-*d*₆ + TFA): δ CH₃ 1.12 and 1.28 (3H, d), CH₂β 1.72–1.92 (2H, m), SCH₃ 1.98 (3H, s), CH₂γ 2.52 (2H, m), SCH₂ + CH + CH₂Ar + SCH₂ 2.60–2.95 (7H, m), SO₂CH₃ 3.0 (3H, s), CHα + CH₂SO₂ 3.35–3.50 (3H, m), CHα 4.17 (1H, m), CH₂O 4.20–4.40 (2H, m), Ar 6.92 (1H, m), Ar 7.09 (1H, m), Ar 7.38 (1H, m), NH₃⁺ 7.87 (3H, br), NH 8.40 (1H, br). HPLC Kromasil C18, 100 Å, 5 μm, 250 mm × 4.6 mm, CH₃CN/H₂O (0.1% TFA) 40–60: 5.0 and 5.35 min. ESI(+): [M + H]⁺ = 529.2.

1-(2-(1-(1-Ethoxycarbonyloxyethoxycarbonyl)-ethylcarbamoyl)-3-thiophen-3-yl-propyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (19-1c) (R₂ = CH₂-3-Thiophene, R = CH-(S)-CH₂-COOCH₂CH₂OCO₂CH₂CH₃). Solid (yield 51.4%). ¹H NMR (DMSO-*d*₆ + TFA): δ CH₃CH 1.12 (3H, m), CH₃CH₂ 1.22 (3H, m), CH₃CH 1.35 (3H, m), CH₂β 1.72–1.92 (2H, m), SCH₃ 1.98 (3H, s), CH₂γ 2.52 (2H, m), SCH₂ + CH + CH₂Ar + SCH₂ 2.60–2.95 (7H, m), CHα 3.42 (1H, m), CH₃CH₂ 4.08 (2H, q), CH₃CH 4.28 (1H, m), CHCH₃ 6.58 (1H, m), Ar 6.95 (1H, m), Ar 7.12 (1H, m), Ar 7.39 (1H, m), NH₃⁺ 7.92 (3H, br), NH 8.57 (1H, br). HPLC Kromasil C18, 100 Å, 5 μm, 250 mm × 4.6 mm, CH₃CN/H₂O (0.1% TFA) 50–50: 3.84 and 4.03 min. ESI(+): [M + H]⁺ = 539.2.

1-(2-(1-(1-Ethoxycarbonylmethyl)ethoxycarbonyl)-ethylcarbamoyl)-3-thiophen-3-yl-propyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (19-1d) (R₂ = CH₂-3-Thiophene, R = CH-(S)-CH₂-COOCH₂CO₂CH₂CH₃). Solid (yield 59.8%). ¹H NMR (DMSO-*d*₆ + TFA): δ CH₃ 1.10–1.30 (6H, m), CH₂β 1.80–2.0 (2H, m), SCH₃ 1.98 (3H, s), SCH₂ + CH + CH₂Ar + SCH₂ + CH₂γ 2.60–3.05 (9H, m), CHα 3.46 (1H, m), CH₃CH₂ 4.08 (2H, m), CH₃CH 4.38 (1H, m), OCH₂CO₂ 4.52 (2H, m), Ar 6.98 (1H, m), Ar 7.12 (1H, m), Ar 7.38 (1H, m), NH₃⁺ 7.92 (3H, br), NH 8.52 (1H, br). HPLC Kromasil C18, 100 Å, 5 μm, 250 mm × 4.6 mm, CH₃CN/H₂O (0.1% TFA) 50–50: 4.04 and 4.23 min. ESI(+): [M + H]⁺ = 509.2.

1-(2-(1-(2-Acetoxy-1-acetoxymethyl)ethoxycarbonyl)-ethylcarbamoyl)-3-thiophen-3-ylpropyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (19-1e) (R₂ = CH₂-3-Thiophene, R = CH-(S)-CH₂-COOCH(CH₂OAc)₂). Solid (yield 27.1%). ¹H NMR (DMSO-*d*₆ + TFA): δ CH₃CH 1.15 (3H, m), CH₂β 1.70–2.0 (2H, m), CH₃CO 2.0 (6H, s), SCH₃ 1.90 (3H, s), CH₂γ + SCH₂ + CH + CH₂Ar + SCH₂ 2.60–2.95 (9H, m), CHα 3.42 (1H, m), CH₃CH + CH₂O 4.0–4.30 (5H, m), CHO 5.12 (1H, m), Ar 6.95 (1H, m), Ar 7.05 (1H, m), Ar 7.25 (1H, m), NH₃⁺ 7.85 (3H, br), NH 8.40 (1H, br). HPLC Kromasil C18, 100 Å, 5 μm, 250 mm × 4.6 mm, CH₃CN/H₂O (0.1% TFA) 40–60: 7.35 and 8.09 min. ESI(+): [M + H]⁺ = 581.1.

1-(2-(1-(2-Hydroxy-1-hydroxymethyl)ethoxycarbonyl)-ethylcarbamoyl)-3-thiophen-3-ylpropyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (19-1f) (R₂ = CH₂-3-Thiophene, R = CH-(S)-CH₂-COOCH(CH₂OH)₂). Solid (yield 18.7%). ¹H NMR (DMSO-*d*₆ + TFA): δ CH₃CH 1.10–1.30 (3H, m), CH₂β 1.70–2.0 (2H, m), SCH₃ 1.95 (3H, s), CH₂γ + SCH₂ + CH + CH₂Ar + SCH₂ 2.60–3.0 (9H, m), CHα + CH₂O (2x) 3.20–3.60 (5H, m), CH₃CH 3.90 (1H, m), CHCH₂OH 4.28 (1H, m), Ar 6.90 (1H, m), Ar 7.08 (1H, m), Ar 7.35 (1H, m), NH₃⁺ 7.85 (3H, br), NH 8.40 (1H, br). HPLC Kromasil C18, 100 Å, 5 μm, 250 mm × 4.6 mm, CH₃CN/H₂O (0.1% TFA) 30–70: 5.22 and 6.90 min. ESI(+): [M + H]⁺ = 497.1.

3-Methylsulfanyl-1-(2-(1-(3,4,5,6-tetrahydroxytetrahydropyran-2-yl)methoxycarbonyl)-ethylcarbamoyl)-3-thiophen-3-yl-propyl)disulfanylmethyl)-propyl-ammonium Trifluoroacetate (19-1g) (R₂ = CH₂-3-Thiophene, R = CH-(S)-CH₂-COGlucose). Solid (yield 12.9%). ¹H NMR (DMSO-*d*₆ + TFA): δ CH₃ 1.10–1.30 (3H, m), CH₂β 1.75–1.95 (2H, m), SCH₃ 1.98 (3H, s), SCH₂ + CH + CH₂Ar + SCH₂ + CH₂γ 2.40–3.05 (9H, m), CHα + CHO 3.10–3.70 (4H, m), CH₃CH + OCH₂CHO 3.95–4.40 (5H, m), OCHOH 4.85 (1H, m), Ar 6.94 (1H, m), Ar 7.12 (1H, m), Ar 7.38 (1H, m), NH₃⁺ 7.90 (3H, br), NH 8.42 (1H, br). HPLC Kromasil C18, 100 Å, 5 μm, 250 mm × 4.6 mm, CH₃CN/H₂O (0.1% TFA) 50–50: 2.34 min. ESI(+): [M + H]⁺ = 585.2.

1-(2-((1-(1-Ethoxycarbonyloxy-ethoxycarbonyl)-2-hydroxypropylcarbamoyl)-3-phenylpropyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (**19-IIa**) ($R_2 = (S)\text{-CH}_2\text{-Phenyl}$, $R = \text{CH}(\text{S})\text{-CH}(\text{CH}_3)\text{OH-COCH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$). Solid (yield 65.2%). $^1\text{H NMR}$ (DMSO- d_6 + TFA): δ CH_3CHOH 1.20 (3H, d), CH_3CH_2 1.35 (3H, t), CH_3CH 1.60 (3H, d), $\text{CH}_2\beta$ 2.01–2.18 (2H, m), SCH_3 2.19 (3H, s), $\text{CH}_2\gamma$ 2.74 (2H, t), $\text{SCH}_2 + \text{CH} + \text{CH}_2\text{Ar} + \text{SCH}_2$ 2.90–3.20 (7H, m), $\text{CH}\alpha$ 3.76 (1H, m), $\text{CH}_3\text{CH}_2 + \text{NHCH}\alpha + \text{OHCH}\alpha$ 4.10–4.40 (4H, m), CHCH_3 6.84 (1H, m), Ar 7.25–7.40 (5H, m), NH_3^+ 7.90 (3H, br). HPLC Kromasil C18, 100 Å, 5 μm , 250 mm \times 4.6 mm, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (0.1% TFA) 45–55: 7.0 min. ESI(+): $[\text{M} + \text{H}]^+ = 563.2$.

1-(2-((1-Ethoxycarbonyloxy-ethoxycarbonylmethyl)-carbamoyl)-3-phenyl-propyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (**19-IIIa**) ($R_2 = (S)\text{-CH}_2\text{-Phenyl}$, $R = \text{CH}_2\text{-COOCHCH}_2\text{CO}_2\text{CH}_2\text{CH}_3$). Solid (yield 72.8%); $F = 114^\circ\text{C}$. IR (KBr): 3298, 2918, 1765, 1651, 1538, 1445, 1375, 1266, 1183, 1078, 983, 932, 851, 790, 743, 702, 651, 559, 500 cm^{-1} ; $[\alpha]_D^{20} = +0.7^\circ$ (5% p/v $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1/1, v/v)). $^1\text{H NMR}$ (DMSO- d_6 + TFA): δ CH_3CH_2 1.37 (3H, t), CH_3CH 1.58 (3H, d), $\text{CH}_2\beta$ 2.03–2.16 (2H, m), SCH_3 2.19 (3H, s), $\text{CH}_2\gamma$ 2.72 (2H, t), $\text{SCH}_2 + \text{CH} + \text{CH}_2\text{Ar} + \text{SCH}_2$ 2.89–3.17 (7H, m), $\text{CH}\alpha$ 3.74 (1H, m), NHCH_2 3.90–4.10 (2H, m), CHCH_3 6.84 (1H, m), Ar 7.26–7.40 (5H, m), NH_3^+ 7.90 (3H, br). $^{13}\text{C NMR}$ (DMSO- d_6): δ 14.3, 14.7, 19.6, 19.7, 29.3, 32.2, 37.8, 40.5, 41.9, 47.0, 49.3, 64.5, 91.7, 126.6, 128.6, 129.3, 139.1, 152.7, 168.4, 173.4. HPLC Kromasil C18, 100 Å, 5 μm , 250 mm \times 4.6 mm, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (0.1% TFA) 50–50: 5.33 min. ESI(+): $[\text{M} + \text{H}]^+ = 519.1$.

1-(2-((1-Ethylcarbonyloxy-isobutoxycarbonylmethyl)-carbamoyl)-3-phenyl-propyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (**19-IIIB**) ($R_2 = (S)\text{-CH}_2\text{-Phenyl}$, $R = \text{CH}_2\text{-COOCHCH}(\text{CH}_3)_2\text{OCOCH}_2\text{CH}_3$). Solid (yield 34.0%). $^1\text{H NMR}$ (DMSO- d_6 + TFA): δ $(\text{CH}_3)_2\text{CH}$ 1.12 (6H, m), CH_3CH_2 1.20 (3H, m), $\text{CHCH}(\text{CH}_3)_2$ 1.90 (1H, m), $\text{CH}_2\beta$ 2.00–2.20 (2H, m), SCH_3 2.20 (3H, s), CH_3CH_2 2.35 (2H, m), $\text{CH}_2\gamma$ 2.70 (2H, t), $\text{SCH}_2 + \text{CH} + \text{CH}_2\text{Ar} + \text{SCH}_2$ 2.90–3.20 (7H, m), $\text{CH}\alpha$ 3.75 (1H, m), NHCH_2 3.90–4.10 (2H, m), $\text{CHCH}(\text{CH}_3)_2$ 6.62 (1H, m), Ar 7.26–7.40 (5H, m), NH_3^+ 7.90 (3H, br). HPLC Kromasil C18, 100 Å, 5 μm , 250 mm \times 4.6 mm, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (0.1% TFA) 50–50: 8.82 min. ESI(+): $[\text{M} + \text{H}]^+ = 531.1$.

1-(2-((1-Ethoxycarbonyloxy-isobutoxycarbonylmethyl)-carbamoyl)-3-phenyl-propyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (**19-IIIC**) ($R_2 = (S)\text{-CH}_2\text{-Phenyl}$, $R = \text{CH}_2\text{-COOCHCH}(\text{CH}_3)_2\text{OCO}_2\text{CH}_2\text{CH}_3$). Solid (yield 38.5%). $^1\text{H NMR}$ (DMSO- d_6 + TFA): δ $(\text{CH}_3)_2\text{CH}$ 1.12 (6H, m), CH_3CH_2 1.30 (3H, t), $\text{CHCH}(\text{CH}_3)_2$ 1.90 (1H, m), $\text{CH}_2\beta$ 2.00–2.20 (2H, m), SCH_3 2.20 (3H, s), $\text{CH}_2\gamma$ 2.70 (2H, t), $\text{SCH}_2 + \text{CH} + \text{CH}_2\text{Ar} + \text{SCH}_2$ 2.90–3.20 (7H, m), $\text{CH}\alpha$ 3.75 (1H, m), NHCH_2 3.90–4.10 (2H, m), CH_3CH_2 4.30 (2H, q), $\text{CHCH}(\text{CH}_3)_2$ 6.62 (1H, m), Ar 7.26–7.40 (5H, m), NH_3^+ 7.90 (3H, br). HPLC Kromasil C18, 100 Å, 5 μm , 250 mm \times 4.6 mm, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (0.1% TFA) 50–50: 9.33 min. ESI(+): $[\text{M} + \text{H}]^+ = 547.1$.

1-(2-((1-Ethylcarbonyloxy-cyclohexylmethoxycarbonylmethyl)-carbamoyl)-3-phenyl-propyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (**19-IIID**) ($R_2 = (S)\text{-CH}_2\text{-Phenyl}$, $R = \text{CH}_2\text{-COOCH}(\text{C}_6\text{H}_{11})\text{OCOCH}_2\text{CH}_3$). Solid (yield 44.0%). $^1\text{H NMR}$ (DMSO- d_6 + TFA): δ CH_3CH_2 1.20 (3H, m), cyclohexyl 1.10–1.80 (11H, m), $\text{CH}_2\beta$ 2.00–2.20 (2H, m), SCH_3 2.20 (3H, s), CH_3CH_2 2.35 (2H, m), $\text{CH}_2\gamma$ 2.70 (2H, t), $\text{SCH}_2 + \text{CH} + \text{CH}_2\text{Ar} + \text{SCH}_2$ 2.90–3.20 (7H, m), $\text{CH}\alpha$ 3.75 (1H, m), NHCH_2 3.90–4.10 (2H, m), $\text{CHC}_6\text{H}_{12}$ 6.63 (1H, m), Ar 7.26–7.40 (5H, m), NH_3^+ 7.90 (3H, br). HPLC Kromasil C18, 100 Å, 5 μm , 250 mm \times 4.6 mm, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (0.1% TFA) 50–50: 17.50 min. ESI(+): $[\text{M} + \text{H}]^+ = 571.2$.

1-(2-((1-Ethoxycarbonyloxy-cyclohexylmethoxycarbonylmethyl)-carbamoyl)-3-phenyl-propyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (**19-IIIE**) ($R_2 = (S)\text{-CH}_2\text{-Phenyl}$, $R = \text{CH}_2\text{-COOCH}(\text{C}_6\text{H}_{11})\text{OCO}_2\text{CH}_2\text{CH}_3$). Solid (yield 50.0%). $^1\text{H NMR}$ (DMSO- d_6 + TFA): δ cyclohexyl 1.10–1.80 (11H, m), CH_3CH_2 1.30 (3H, t), $\text{CH}_2\beta$ 2.10 (2H, m), SCH_3 2.20 (3H, s), $\text{CH}_2\gamma$ 2.70 (2H, t), $\text{SCH}_2 + \text{CH} + \text{CH}_2\text{Ar} + \text{SCH}_2$ 2.90–3.20 (7H, m), $\text{CH}\alpha$ 3.75 (1H, m), NHCH_2 3.90–4.10 (2H, m), CH_3CH_2 4.30 (2H, q), $\text{CHC}_6\text{H}_{11}$ 6.65 (1H, m), Ar 7.20–7.50 (5H, m), NH_3^+ 7.90 (3H, br). HPLC Kromasil

C18, 100 Å, 5 μm , 250 mm \times 4.6 mm, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (0.1% TFA) 60–40: 7.72 min. ESI(+): $[\text{M} + \text{H}]^+ = 587.2$.

Synthesis of 1-(2-((1-Ethoxycarbonyloxy-ethoxycarbonylmethyl)-carbamoyl)-3-phenyl-propyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate **19-IVa.** Compound **18a** was treated by NaIO_4 in EtOH^{34} as described for the synthesis of **7b**. Final deprotection by HCOOH as previously described led to compound **19-IVa**.

1-(2-((1-Ethoxycarbonyloxy-ethoxycarbonylmethyl)-carbamoyl)-3-phenyl-propyl)disulfanylmethyl)-3-methylsulfanylpropyl-ammonium Trifluoroacetate (**19-IVa**). Solid (yield 64.0%). $^1\text{H NMR}$ (DMSO- d_6 + TFA): δ CH_3CH_2 1.37 (3H, t), CH_3CH 1.58 (3H, d), $\text{CH}_2\beta$ 2.07 (2H, m), SOCH_3 2.52 (3H, d), $\text{CH}_2\gamma$ 2.72 (2H, t), $\text{SCH}_2 + \text{CH} + \text{CH}_2\text{Ar} + \text{SCH}_2$ 2.89–3.17 (7H, m), $\text{CH}\alpha$ 3.74 (1H, m), NHCH_2 3.90–4.10 (2H, m), CH_3CH_2 4.27 (2H, q), CHCH_3 6.84 (1H, m), Ar 7.26–7.40 (5H, m), NH_3^+ 7.90 (3H, br). HPLC Kromasil C18, 100 Å, 5 μm , 250 mm \times 4.6 mm, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (0.1% TFA) 30–70: 14.57 min. ESI(+): $[\text{M} + \text{H}]^+ = 535.1$.

Biochemistry. Nephrilysin (NEP) enzymatic activity was assessed using Suc-Ala-Ala↓Phe-AMC⁷⁶ (20 μM , Bachem) and aminopeptidase N (APN) activity using L-Ala↓β-NA⁷⁷ (50 μM , Sigma-Aldrich). Fluorescence was read on a Berthold fluorimeter ($\lambda_{\text{ex}} = 340$ nm and $\lambda_{\text{em}} = 360$ nm for NEP, and $\lambda_{\text{em}} = 405$ nm for APN, lamp energy = 10000). Inhibitory potencies of active inhibitors were determined using recombinant human (rh) enzymes (50 ng/mL; R&D Systems). After 30 min of preincubation at 37 °C with increasing concentrations of inhibitor in Tris 50 mM pH 7.4, their corresponding substrates were added, and the reaction was left to proceed for 30 min at 37 °C. Each experiment was performed in duplicate, three times independently.

Animals. Experiments were performed in male OF1 mice, 20–32 g (Charles River Laboratories, France), and male Sprague–Dawley rats, 200–280 g (Janvier, France). Animals were housed for at least 2 days before experiments in a room with controlled temperature ($21 \pm 2^\circ\text{C}$) and a 12 h alternating light–dark cycle. Food and water were available ad libitum. Great care was taken to avoid or minimize discomfort of animals. Animal experiments were carried out with the European Communities Council directive (89/609/CEE) and in accordance with the ethical guidelines of the International Association of Pain. The maximal volume administered was 0.1 mL/100g for intravenous injection and 0.2 mL/100g orally.

Pharmacological Assays. Hot Plate Test. A glass cylinder (16 cm \times 16 cm) was used to keep the mouse on the heated surface of the plate, which was maintained at a temperature of $52 \pm 1^\circ\text{C}$ using a thermoregulated water-circulating pump. Jump latency was registered by a stop-watch, and cutoff time was set to 240 s. Results are expressed as percentage of maximum possible effect: % MPE = (drug latency – control latency)/(cutoff time – control latency) \times 100.

Tail-Flick Test. Tail-flick latency was measured automatically using a tail-flick analgesimeter (Bioseb, France). For each rat, three to four determinations were performed once a day until response values were stable. Then at least two determinations were carried out before (t0) and after drug injection at each time point. A cutoff time of 15 s was selected to minimize tail damage. Responses are expressed as percentage of maximal possible effect (% MPE).

Formalin Test. Mice received subcutaneous (plantar surface of the hindpaw) injection of 5% formalin solution (20 μL , in saline) and were placed back for nociceptive behavior observation (licking) for the initial early phase (0–5 min). Vehicle (ethanol/PEG 400/water, 1/4/5) or compounds were administered by gavage 30 or 90 min before formalin injection. Results are expressed as licking time (in seconds).

Mechanical Allodynia: von Frey Test. Mechanical allodynia was quantified by measuring the hindpaw withdrawal in response to von Frey filament stimulation (bending force ranging from 0.02 to 2 g). First, the filament of 0.4 g was used. Then the weight of the next filament was decreased if the mouse responded or increased if the mouse did not respond. This up–down procedure was stopped four measures after the first change in animal responding. The threshold of response was calculated by using the up–down Excel program kindly provided by the A. Basbaum's laboratory (UCSF, San Francisco, USA). Clear paw

withdrawal, shaking, or licking was considered as nociceptive-like response. Both ipsilateral and contralateral side were tested.

Hyperalgesia to Noxious Thermal Stimuli: Plantar Test. Briefly, paw withdrawal latency in response to radiant heat stimulus was measured using a plantar test apparatus (Bioseb, France). The intensity of the noxious stimulus was calibrated to obtain paw withdrawal latencies in a range of 8–10 s in mice. A cutoff time of 20 s was used to prevent tissue damage. Mean paw withdrawal latencies for ipsilateral and contralateral side were determined from the average of three separate trials, taken at 5 min intervals to prevent thermal sensitization and behavioral disturbances. Both ipsilateral and contralateral side were tested.

Seltzer Model of Neuropathic Pain. A partial ligation of the sciatic nerve at midhigh level was used to induce neuropathic pain. Mice were anaesthetized by isoflurane (TEM, France; induction 4%, surgery, 1.5%), and the common sciatic nerve was exposed at the level of the midhigh of the right hindpaw. At about 1 cm proximally to the nerve trifurcation, a tight ligation was created around 33–50% of the sciatic nerve using 8–0 18 in. nonabsorbable virgin silk suture (Alcon France), leaving the rest of the nerve uninjured. The muscle was then stitched, and the incision was closed with wound clips. Control animals (sham-operated mice) underwent the same surgical procedure except that the sciatic nerve was not ligated.

Habituation periods were conducted for two consecutive days on mice. Then baseline thresholds to pressure (von Frey test) and heat (plantar test) stimulation were assessed during two consecutive days. Sciatic nerve surgery was performed the day after. Hypersensitivity to pain was assessed on mice using von Frey test (mechanical allodynia) followed by the plantar test (thermal hyperalgesia) between day 3 and day 20 after surgery (baseline). Compound was tested between day 10 and day 20 after surgery. Mechanical and thermal hypersensitivity were assessed before (t₀) and 20 min after vehicle (ethanol/PEG400/water, 1/4/5) or **19-IIIa** (50 mg/kg) oral administration.

■ ASSOCIATED CONTENT

● Supporting Information

Supplemental figures and synthesis and analytical data of all intermediates not described in the Experimental Section. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AMC, 7-amino-4-methylcoumarin; APN, aminopeptidase N; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DENKI, dual enkephalinase inhibitor; DIEA, diisopropylethylamine; DMSO, dimethylsulfoxide; EDCl, 1-[3-(dimethylamino)propyl]-3-ethyl-carbodiimide methiodide; ED₅₀, median effective dose; ee, enantiomeric excess; ENK, enkephalin; ESL, electrospray ionization; HCOOH, formic acid; HOBt, hydroxybenzotriazole; HPLC, high performance liquid chromatography; HPT, hot plate test; iv, intravenous; LC/MS, liquid chromatography mass spectroscopy; MO, morphine; MPE, maximal possible effect; β -NA, β -naphthylamine; NEP,

nepilysin; Nlx, naloxone; OR, opioid receptor; PEG, poly(ethylene glycol); po, per os; PSLN, partial sciatic nerve ligation; TFA, trifluoroacetic acid; TFT, tail flick test

■ REFERENCES

- (1) Melzack, R. The future of pain. *Nature Rev. Drug Discovery* **2008**, *7*, 629.
- (2) Dellemijn, P. Are opioids effective in relieving neuropathic pain? *Pain* **1999**, *80*, 453–462.
- (3) (a) Pert, C. B.; Snyder, S. A. Opiate receptor: demonstration in nervous tissue. *Science* **1973**, *179*, 1011–1014. (b) Simon, E. J.; Hiller, J. M.; Edelman, I. Stereospecific binding of potent narcotic analgesic 3H-etorphine to rat brain homogenate. *Proc. Natl. Acad. Sci. U. S. A.* **1973**, *70*, 1947–1949. (c) Terenius, L. Characteristics of the “receptor” for narcotic analgesics in synaptic plasmas membrane fraction from rat brain. *Acta Pharmacol. Toxicol.* **1973**, *33*, 372–384.
- (4) Hugues, J.; Smith, T. W.; Kosterlitz, H. W.; Fothergill, L. A.; Morgan, B. A.; Morris, H. R. Identification of the two related pentapeptides from the brain with potent opiate agonist activity. *Nature* **1975**, *258*, 577–579.
- (5) North, R. A.; Williams, J. T.; Surprenant, A.; Christie, M. J. Mu and delta receptors belong to a family of receptors that are coupled to potassium channel. *Proc. Natl. Acad. Sci. U. S. A.* **1987**, *84*, 5487–5491.
- (6) Dhawan, B. N.; Cesselin, F.; Raghurir, R.; Reisine, T.; Bradley, P. B.; Portoghese, P. S.; Hamon, M. International union of Pharmacology. XII. Classification of opioid receptors. *Pharmacol. Rev.* **1996**, *48*, 567–592.
- (7) Belluzzi, J. D.; Grant, N.; Garsky, V.; Sarantakis, D.; Wise, C. D.; Stein, L. Analgesia induced in vivo by central administration of enkephalin in rat. *Nature* **1976**, *260*, 625–626.
- (8) Mosmaim, A. D.; Nguyen, T. D.; Tse, R.; Puente, J.; Couceyro, P.; Wolf, M. E. In vitro methionine5-enkephalin degradation kinetics by human brain preparations. *Neurochem. Res.* **2008**, *33*, 81–86.
- (9) Römer, D.; Buscher, H. H.; Hill, R. C.; Pless, J.; Bauer, W.; Cardinaux, F.; Closse, A.; Hauser, D.; Huguenin, R. A. A synthetic enkephalin analogue with prolonged parenteral and oral analgesic activity. *Nature* **1977**, *268*, 547–549.
- (10) Stacher, G.; Bauer, P.; Steininger, H.; Schreiber, E.; Schmierer, G. Effects of the synthetic enkephalin analogue FK33-824 on pain threshold and pain tolerance in man. *Pain* **1979**, *7*, 159–172.
- (11) Williams, J. T.; Christie, M. J.; North, R. A.; Roques, B. P. Potentiation of enkephalin action by peptidase inhibitors in rat locus ceruleus in vitro. *J. Pharmacol. Exp. Ther.* **1987**, *243*, 397–401.
- (12) Roques, B. P.; Fournie-Zaluski, M. C.; Wurm, M. Inhibiting the breakdown of endogenous opioids and cannabinoids to alleviate pain. *Nature Rev. Drug Discovery* **2012**, *11*, 292–310.
- (13) Roques, B. P.; Noble, F.; Daugé, V.; Fournie-Zaluski, M. C.; Beaumont, A. Neutral endopeptidase 24.11: structure, inhibition, and experimental and clinical pharmacology. *Pharmacol. Rev.* **1993**, *45*, 87–146.
- (14) Roques, B. P.; Fournie-Zaluski, M. C.; Soroca, E.; Lecomte, J. M.; Malfroy, B.; Llorens, C.; Schwartz, J. C. The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. *Nature* **1980**, *288*, 286–288.
- (15) Umezawa, H.; Aoyagi, T.; Suda, H.; Hamada, M.; Takeuchi, T. Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *J. Antibiot.* **1976**, *29*, 97–99.
- (16) Bourgoin, S.; Le Bars, D.; Artaud, F.; Clot, A. M.; Bouboutou, R.; Fournie-Zaluski, M. C.; Roques, B. P.; Hamon, M.; Cesselin, F. Effects of kelatorphan and other peptidase inhibitors on the in vitro and in vivo release of methionine-enkephalin-like material from the rat spinal cord. *J. Pharmacol. Exp. Ther.* **1986**, *238*, 360–366.
- (17) Fournie-Zaluski, M. C.; Chaillet, P.; Bouboutou, R.; Coulaud, A.; Cherot, P.; Waksman, G.; Costentin, J.; Roques, B. P. Analgesic effects of kelatorphan, a new highly potent inhibitor of multiple enkephalin degrading enzymes. *Eur. J. Pharmacol.* **1984**, *102*, 525–528.
- (18) Noble, F.; Soleilhac, J. M.; Soroca-Lucas, E.; Turcaud, S.; Fournie-Zaluski, M. C.; Roques, B. P. Inhibition of the enkephalin metabolizing

enzymes by the first systemically active mixed inhibitor pro-drug RB101 induces potent analgesic responses in mice and rats. *J. Pharm. Exp. Ther.* **1992**, *261*, 181–190.

(19) Willer, J. C.; Roby, A.; Ernst, M. The enkephalinase inhibitor, GB 52, does not affect nociceptive flexion reflexes nor pain sensation in humans. *Neuropharmacology* **1986**, *25*, 819–822.

(20) Meynadier, J.; Dalmás, S.; Lecomte, J. M.; Gros, C. I.; Schwartz, J. C. Potent analgesic effects of inhibitors of enkephalin metabolism administered intrathecally to cancer patients. *Pain Clinic* **1988**, *2*, 201–205.

(21) Wisner, A.; Dufour, E.; Messaoudi, M.; Nejd, A.; Marcel, A.; Ungeheuer, M.-N.; Rougeot, C. Human opiorphin, a natural antinociceptive modulator of opioid-dependent pathways. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 17979–17984.

(22) Le Guen, S.; Mas Nieto, M.; Canestrelli, C.; Chen, H.; Fournie-Zaluski, M. C.; Cupo, A.; Maldonado, R.; Roques, B. P.; Noble, F. Pain management by a new series of dual inhibitors of enkephalin degrading enzymes: long lasting antinociceptive properties and potentiation by CCK2 antagonist or methadone. *Pain* **2003**, *104*, 139–148.

(23) Dauge, V.; Mauborgne, A.; Cesselin, F.; Fournie-Zaluski, M. C.; Roques, B. P. The dual peptidase inhibitor RB101 induces a long-lasting increase in the extracellular level of Met-enkephalin-like material in the nucleus accumbens of freely moving rats. *J. Neurochem.* **1996**, *67*, 1301–1308.

(24) Ruiz-Gayo, M.; Baamonde, A.; Turcaud, S.; Fournie-Zaluski, M. C.; Roques, B. P. In vivo occupation of mouse brain opioid receptors by endogenous enkephalins: blockade of enkephalin degrading enzymes by RB 101 inhibits [³H]diprenorphine binding. *Brain Res.* **1992**, *571*, 306–312.

(25) Fournie-Zaluski, M. C.; Coric, P.; Turcaud, S.; Lucas, E.; Noble, F.; Maldonado, R.; Roques, B. P. Mixed inhibitor-pro-drug as a new approach toward systemically active inhibitors of enkephalin-degrading enzyme. *J. Med. Chem.* **1992**, *35*, 2473–2481.

(26) Noble, F.; Smadja, C.; Valverde, O.; Maldonado, R.; Coric, P.; Turcaud, S.; Fournie-Zaluski, M. C.; Roques, B. P. Pain-suppressive effects on various nociceptive stimuli (thermal, chemical, electrical and inflammatory) of the first orally active enkephalin-metabolizing enzyme inhibitor RB120. *Pain* **1997**, *73*, 383–391.

(27) Chen, H.; Noble, F.; Coric, P.; Fournie-Zaluski, M. C.; Roques, B. P. Aminophosphinic inhibitors as transition state analogues of enkephalin-degrading enzymes: a class of central analgesics. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 12028–12033.

(28) Noble, F.; Roques, B. P. Assessment of endogenous enkephalins efficacy in the hot plate test in mice: comparative study with morphine. *Neurosci. Lett.* **1995**, *185*, 75–78.

(29) Obara, I.; Przewlocki, R.; Przewlocka, B. Local peripheral effects of mu-opioid receptor agonists in neuropathic pain in rats. *Neurosci. Lett.* **2004**, *360*, 85–89.

(30) Basbaum, A. I.; Bautista, D. M.; Scherrer, G.; Julius, D. Cellular and molecular mechanisms of pain. *Cell* **2009**, *139*, 267–284.

(31) Stein, C.; Schafer, M.; Macheltska, H. Attacking pain at its source: new perspectives on opioids. *Nature Med.* **2003**, *9*, 1003–1008.

(32) Wu, H. E.; Hung, K. C.; Mizoguchi, H.; Nagase, H.; Tseng, L. F. Roles of endogenous opioid peptides in modulation of nocifensive response to formalin. *J. Pharmacol. Exp. Ther.* **2002**, *300*, 647–654.

(33) Fournie-Zaluski, M. C.; Roques, B. P. New amino acid derivatives, useful to treat depression and different types of pains e.g. acute pain, inflammatory pain and neurogenic pain, are neprilysine and N-amino peptidase inhibitors. Patent WO EP67711, 2006.

(34) Fournie-Zaluski, M. C.; Coric, P.; Turcaud, S.; Bruetschy, L.; Lucas, E.; Noble, F.; Roques, B. P. Potent and systemically active aminopeptidase N inhibitors designed from active-site investigation. *J. Med. Chem.* **1992**, *35*, 1259–1266.

(35) James, K.; Palmer, M. J. gem-Cycloalkyl substituted thiol inhibitors of neutral endopeptidase 24.11. Synthesis via nucleophilic opening of 2,2-spiro-β-lactones. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 825–830.

(36) Fournie-Zaluski, M. C.; Coric, P.; Turcaud, S.; Rousselet, N.; Gonzalez, W.; Barbe, B.; Pham, I.; Jullian, N.; Michel, J. B.; Roques, B. P.

New dual inhibitors of neutral endopeptidase and angiotensin-converting enzyme: rational design, bioavailability, and pharmacological responses in experimental hypertension. *J. Med. Chem.* **1994**, *37*, 1070–1083.

(37) Allen, C. F. C.; Spangler, F. W. Ethyl benzalmalonate. *Org. Synth.* **1945**, *25*, 42–44.

(38) Neustadt, B. R.; Smith, E. S.; Nechuta, T. L.; Bronnenkant, A. A.; Haslanger, M. F.; Watkins, R. W.; Foster, C. J.; Sybertz, E. J. Mercaptoacyl amino acid inhibitors of atriopeptidase. I. Structure-activity relationship studies of methionine and S-alkylcysteine derivatives. *J. Med. Chem.* **1994**, *37*, 2461–2476.

(39) Sanchez, J. P. A ring opening reaction of benzisothiazolones. A new route to unsymmetrical disulfides. *J. Heterocycl. Chem.* **1997**, *34*, 1463–1467.

(40) Bindra, J. S. Enkephalinase enzyme inhibiting compounds. U.S. Patent 4,329,495, 1982.

(41) Kim, D. H.; Kim, Y. J. Synthesis and inhibitory activity of optically active 2-benzyl-3-mercaptopropanoic acid against carboxypeptidase A. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2681–2684.

(42) Barcelo, G.; Senet, J. P.; Sennyey, G.; Bensoam, J.; Loffet, A. Alkyl 1-chloroalkylcarbonates: reagents for the synthesis of carbamates and protection of amino groups. *Synthesis* **1986**, *8*, 627–632.

(43) Schechter, I.; Burger, A. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157–162.

(44) Oefner, C.; Roques, B. P.; Fournie-Zaluski, M. C.; Dale, G. Structural analysis of neprilysin with various specific and potent inhibitors. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2004**, *60*, 392–396.

(45) Oefner, C.; D'Arcy, A.; Hennig, M.; Winkler, F. K.; Dale, G. Structure of human neutral endopeptidase (neprilysin) complexed with Phosphoramidon. *Mol. Biol.* **2000**, *296*, 341–349.

(46) Kilbourn, M. R. Thiophenes as phenyl bio-isosteres: application in radiopharmaceutical design-I. Dopamine uptake antagonists. *Nucl. Med. Biol.* **1989**, *16*, 681–686.

(47) Kim, H. Y.; Gladyshev, V. N. Methionine sulfoxide reduction in mammals: characterization of methionine-R-sulfoxide reductase. *Mol. Biol. Cell* **2004**, *15*, 1055–1064.

(48) Eddy, N. B.; Leimbach, D. Synthetic analgesics: dithienylbutenyl and dithienylbutylamines. *J. Pharm. Exp. Ther.* **1953**, *107*, 385–389.

(49) Kerns, E. H.; Di, L. *Drug-Like Properties: Concepts, Structure Design and Methods*; Academic Press: New York, 2008; pp 43–47.

(50) ALOGPs 2.1 Program; Virtual Computational Chemistry Laboratory, 2005; <http://www.vcclab.org/lab/alogps/>.

(51) D'Amour, F. E.; Smith, D. L. A method for determining loss of pain sensation. *J. Pharm. Exp. Ther.* **1941**, *72*, 74–79.

(52) Hunskaar, S.; Fasmer, O. B.; Hole, K. Formalin test in mice, a useful technique for evaluating mild analgesics. *J. Neurosci. Methods* **1985**, *14*, 69–76.

(53) Chaplan, S. R.; Bach, F. W.; Pogrel, J. W.; Chung, J. M.; Yaksh, T. L. Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods* **1994**, *53*, 55–63.

(54) Hargreaves, K.; Dubner, R.; Brown, F.; Flores, C.; Joris, J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **1988**, *32*, 77–88.

(55) Bennett, G. J.; Xie, Y. K. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **1988**, *33*, 87–107.

(56) Seltzer, Z.; Dubner, R.; Shir, Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* **1990**, *43*, 205–218.

(57) Thanawala, V.; Kadam, V. J.; Ghosh, R. Enkephalinase inhibitors: potential agents for the management of pain. *Curr. Drug Targets* **2008**, *9*, 887–894.

(58) Yaksh, T. L.; Sabbe, M. B.; Lucas, D.; Mjanger, E.; Chipkin, R. E. Effects of [n-(1-(1-carboxy-2-phenyl)ethyl)-1-phenylalanyl-beta-alanine (SCH32615), a neutral endopeptidase (enkephalinase) inhibitor, on levels of enkephalin, encrypted enkephalins and substance P in cerebrospinal fluid and plasma of primates. *J. Pharmacol. Exp. Ther.* **1991**, *256*, 1033–41.

(59) Stella, V. J.; Borchards, R. T.; Hageman, M. J.; Oliyai, R.; Maag, H.; Tilley, J. W. *Prodrugs: Challenges and Rewards, Part 2*; AAPS Press: New York, 2007; pp 3–29.

(60) Daehne, W. V.; Frederiksen, E.; Gundersen, E.; Lund, F.; Morch, P.; Petersen, H. J.; Roholt, K.; Tybring, L.; Godtfredsen, W. O. Acyloxymethyl esters of ampicillin. *J. Med. Chem.* **1970**, *13*, 607–612.

(61) Bodin, N. O.; Ekström, B.; Forsgren, U.; Jalar, L. P.; Magni, L. Bacampicillin: a new orally well-absorbed derivative of ampicillin. *Antimicrob. Agents Chemother.* **1975**, *8*, 518–525.

(62) Bentley, P. H.; McCrae, W. An efficient synthesis of symmetrical 1,3-diglycerides. *J. Org. Chem.* **1970**, *35*, 2082–2083.

(63) Pardridge, W. M. The blood–brain barrier: bottleneck in brain drug development. *NeuroRx* **2005**, *2*, 3–14.

(64) Stein, C. Targeting pain and inflammation by peripherally acting opioids. *Front. Pharmacol.* **2013**, *4*, 1–3.

(65) Hunskaar, S.; Hole, K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* **1987**, *30*, 103–114.

(66) Malmberg, A. B.; Basbaum, A. I. Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. *Pain* **1998**, *76*, 215–222.

(67) Porreca, F.; Ossipov, M. H.; Gebhart, F. G. Chronic pain and medullary descending facilitation. *Trends Neurosci.* **2002**, *25*, 319–325.

(68) Review in *Emerging Strategies for the Treatment of Neuropathic Pain*; Campbell, J. N., Basbaum, A. I., Dray, A., Dubner, R., Dworkin, R. H., Sang, C. N., Eds; IASP Press: Seattle, WA, 2006.

(69) Attal, N.; Chen, Y. L.; Kayser, V.; Guilbaud, G. Behavioural evidence that systemic morphine may modulate a phasic pain-related behavior in a rat model of peripheral mononeuropathy. *Pain* **1991**, *47*, 65–70.

(70) Desmeules, J. A.; Kayser, V.; Guilbaud, G. Selective opioid receptor agonists modulate mechanical allodynia in an animal model of neuropathic pain. *Pain* **1993**, *53*, 277–285.

(71) Martin, T. J.; Hairston, C. T.; Lutz, P. O.; Harris, L. S.; Porreca, F. Anti-allodynic actions of intravenous opioids in the nerve injured rat: potential utility of heroin and dihydroetorphine against neuropathic pain. *Eur. J. Pharmacol.* **1998**, *357*, 25–32.

(72) Bianchi, G.; Fiocchi, R.; Tavani, A.; Manara, L. Quaternary narcotic antagonist relative ability to prevent antinociception and gastrointestinal transit inhibition in morphine-treated rats as an index of peripheral selectivity. *Life Sci.* **1982**, *30*, 1875–1883.

(73) Maldonado, R.; Valverde, O.; Turcaud, S.; Fournie-Zaluski, M.-C.; Roques, B. P. Antinociceptive response induced by mixed inhibitors of enkephalin catabolism in peripheral inflammation. *Pain* **1994**, *58*, 77–83.

(74) Menendez, L.; Hidalgo, A.; Meana, A.; Poras, H.; Fournie-Zaluski, M.-C.; Roques, B. P. Inhibition of osteosarcoma-induced thermal hyperalgesia in mice by the orally active dual enkephalinase inhibitor PL37. Potentialisation by gabapentin. *Eur. J. Pharmacol.* **2008**, *596*, 50–55.

(75) Stein, C.; Schafer, M.; Hassan, A. H. Peripheral opioid receptors. *Ann. Med.* **1995**, *27*, 219–221.

(76) Spillantini, M. G.; Panconesi, A.; Del Bianco, P. L.; Sicuteri, F. Enkephalinase and angiotensin converting enzyme activities in human venous and arterial plasma. *Neuropeptides* **1986**, *8*, 111–117.

(77) Greenberg, L. J. Fluorimetric measurement of alkaline phosphatase and aminopeptidase activities in the order of 10^{-14} moles. *Biochem. Biophys. Res. Commun.* **1962**, *9*, 430–435.