N_2 to lyse the organelles. Protein content was measured by the method of Bradford.[32] Acid phosphatase activity was determined with β-glycerophosphate as substrate by the method of Barrett.[35] Homogenates and lysosomal lysates were flash frozen in liquid N_2 and stored at -80 °C.

In Vitro Lysosomal Degradation Experiments. Conjugates or derivatives were incubated with homogenates or lysosomal lysates at different pH's. Conjugates and derivatives were dissolved in appropriate solutes. Incubations contained 250 μL of the homogenate or 100 μL of the lysate and were diluted with a 0.1 M phosphate buffer (pH 5.0 or 7.4) to a final volume of 1.0 mL. The final concentrations of the conjugates and derivatives based on parent compound present in the incubation media were as follows (μmol·L^{-1}): 1a–t, 10; **2a–g**, 250; **3a–h**, 600; **4a**, **5a**, **6a**, 2000; **7a–b**, 75; **8a,b**, 4; **9a-I-III**, 4; **10a–b**, 70. The buffers contained a suitable internal standard for HPLC analysis (Chromatography Section). Samples (100 μL) were taken at various times and were diluted with either 2 volumes of ACN, 3 volumes of MeOH, or 1 volume of 2 N HCl to precipitate proteins. Samples were placed on ice for 15 min and then centrifuged at 3000*g* for 2 min. The supernatants were injected into the HPLC (Tables I and II). Peak ratios of derivative and parent compound were measured. The percentage parent compound generated, was calculated as

$$R(\text{PC, IS}) \times 100\% / (R(\text{Der, IS}) + R(\text{PC, IS}))$$

where R(PC, IS) and R(Der, IS) refer to the ratio (R) of the peak areas of the parent compound (PC) and the internal standard (IS) and that of the peak areas of the derivative (Der) and IS, respectively. In the case of protein conjugates, release was calculated as the percentage of drug released from the drug–protein conjugate (the initial total amount of drug present in the incubation media was set 100%).

Chromatography Section. TLC Analysis. TLC plates (silica gel 60F254, 10 × 5 or 20 × 20 cm) were from Merck (Darmstadt, FRG). Eluents were v/v(/v) MeOH/dichloromethane (50/50); BuOH/water/HAc (35/10/5); 1,4-dioxane/water/HAc (35/10/5), and ethyl acetate/hexane/HAc (33/66/1).

HPLC Analysis. The column was μ-Bondapack C-18. The following eluent systems and flow rates were used: I (a/b), water/ACN/concentrated HAc (60/40/1) at 1 (a) and 2 (b) mL/min; II, water/ACN/concentrated HAc (70/30/1) at 1.5 mL/min; III, phosphate buffer (67 mM, pH 7.0)/ACN (70/30) at 1.5 mL/min; IV, phosphate buffer (67 mM, pH 7.0)/ACN (60/40) at 1 mL/min; V, water/ACN/concentrated HAc (25/75/1) at 1.5 mL/min; VI, phosphate buffer (67 mM, pH 7.0)/MeOH (90/10) at 1 mL/min; VII (a/b), phosphate buffer (15 mM, pH 5.0)/ACN (80/20) at 1 (a) and 1.5 (b) mL/min; VIII, water/ACN/concentrated H_3PO_4 (85/15/0.2) at 2 mL/min; IX, water/ACN/concentrated H_3PO_4 (90/10/0.2) at 2 mL/min; X, water/ACN (60/40) at 2 mL/min; XI, phosphoric acid (10 mM, pH 3.2)/ACN (70/30) at 1.5 mL/min.

For the individual compounds the internal standards were (compound/internal standard) naproxen/sulfamethoxazole or 2-aminobenzoic acid, indomethacin/flurbiprofen, benzoic acid/salicylic acid and sulfamethoxazole/6-desmethylnaproxen.

Detection was performed by UV at 254 nm (indomethacin, benzoic acid, triamterene), 206 nm (Z-Ala-Pro, Z-Phe-Ala) or 260 nm (sulfamethoxazole) or by fluorescence (λ_{ex}, λ_{em}): naproxen (330, 360 nm), adriamycin (470, 560 nm), or triamterene (365, 440 nm).

FPLC. Pure soluble naproxen–lysozyme (1:1) was obtained by chromatography on the FPLC (Pharmacia). Mono-S HR 5/5 (Pharmacia) was used as cation exchanger. Proteins were separated by gradient elution: eluent A, 10 mM Tris HCl (pH 7.4); eluent B, 10 mM Tris HCl (pH 7.4) and 1 M NaCl. The flow was 1.0 mL/min and the eluent program was as follows: 0–5 min, 20% B, (sample application at 3 min); 5–15 min, 20% → 22.5% B; 15–19 min, 22.5% B; 19–20 min, 22.5% → 20% B.

On-line UV detection was performed at 280 nm for protein, and on-line selective fluorescence was done at excitation wavelength 330 nm and emission wavelength 360 nm for naproxen (Figure 2). Naproxen–lysozyme (0.5:1), stored at -20 °C, was dissolved in FPLC buffer (20% B). Undissolved protein was filtered off and the filtrate was passed through a Millex-GV (0.2 μm) filter, prior to injection into the FPLC. During each run, 2.5 mg of protein was applied to the column and 1.2 mg of pure soluble naproxen–lysozyme (1:1) was obtained (0.2 mg/mL).

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Potent and Systemically Active Aminopeptidase N Inhibitors Designed from Active-Site Investigation

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Derivatives of amino acids bearing various zinc-coordinating moieties (SH, COOH, CONHOH, and PO_3H_2) were synthesized and tested for their ability to inhibit aminopeptidase N (APN). Among them, β-amino thiols were found to be the most efficient with IC_{50}'s in the 11–50 nM range. These results suggest that the S_1 subsite of APN is a deep but not very large hydrophobic pocket, optimally fitting side chains of moderate bulk endowed with some degree of freedom. The iv administration of the inhibitors, alone, did not induce antinociceptive responses on the hot plate test in mice. However, in presence of 10 mg/kg acetorphan, a prodrug of the neutral endopeptidase inhibitor thiorphan, these compounds gave a large increase in the jump latency time with ED_{50}'s of 2 and 2.4 mg/kg for the disulfides of methioninethiol $[H_2NCH(CH_2CH_2SCH_3)CH_2S]_2$ and *S*-oxomethioninethiol $[H_2NCH(CH_2CH_2S(O)CH_3)CH_2S]_2$, respectively. These results show that the disulfide forms of β-amino thiols are efficient prodrugs of aminopeptidase N inhibitors capable of crossing the blood–brain barrier.

Introduction

The opioid peptides Met-enkephalin and Leu-enkephalin are degraded in the central nervous system by two zinc-metallopeptidases, neutral endopeptidase, NEP (EC 3.4.24.11)[1] and aminopeptidase N, APN (EC 3.4.11.2).[2,3] It has been proposed that inhibitors of both peptidases

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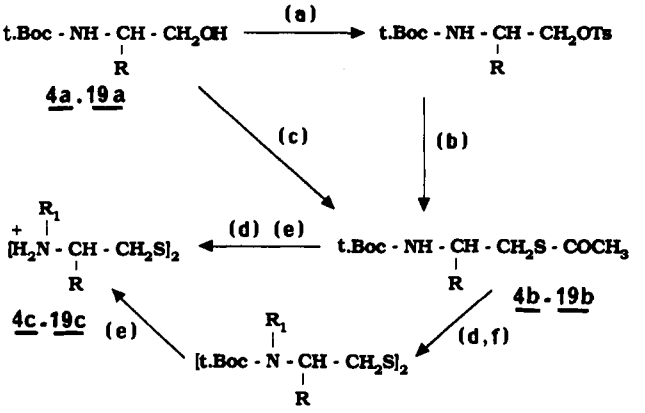
would be expected to provide new "physiological" analgesics devoid of the major side effects elicited by morphine and surrogates. Accordingly, using our concept of "mixed inhibitors", based on the occurrence of structural similarities in the active sites of zinc-metallopeptidases, compounds inhibiting both NEP and APN have been developed[4-6] and were shown to induce potent antinociceptive responses,[4-6] almost devoid of the severe drawbacks of opiates.[7]

The critical point in designing mixed NEP/APN inhibitors was the efficiency of these compounds toward APN. A large number of membrane-bound aminopeptidases, most of them belonging to the group of zinc-metallopeptidases, are present in brain and in peripheral tissues.[8] Due to the similarity in the mechanism of action of these enzymes, the commonly used aminopeptidase inhibitors (bestatin and amastatin) interact with the pool of aminopeptidases with inhibitory potencies in the micromolar range.

APN was purified from brush border membranes several years ago,[9] but until recently, little was known about its structure and mechanism of action, although the enzyme was shown to remove preferentially N-terminal hydrophobic amino acids of substrates[8] and to bind, with a higher affinity, compounds possessing aromatic or highly hydrophobic residues interacting with the S'_1 and S'_2 subsites.[10] Large sequence similarities have been found between recently cloned aminopeptidase N from various species.[11-14] In contrast, a very low sequence homology



(a) TsCl. DMF (b) $CH_3COS^-K^+$. DMF

(c) $P\Phi_3$. iPrOOC - N = N - COOiPr. CH_3COSH

(d) OH^-. I_2 (e) CF_3COOH. CH_2Cl_2 (f) NaH. R_1X

Figure 1. Scheme for the synthesis of the β-amino thiols.

was observed with the sequence of other zinc-metallopeptidases, except for the consensus sequence V-x-x-H-E-x-x-H which contains the two histidines coordinating the zinc atom and the glutamate involved in catalysis.[15] This highly conserved sequence has been previously found in zinc-endopeptidases such as NEP,[16,17] angiotensin-converting enzyme,[18] and thermolysin, whose structure is known at the atomic level,[19] but not in zinc-carboxypeptidases.[15] Furthermore, chemical modifications by selective reagents have shown the presence of four essential residues in the active site of APN;[20] one arginine and a carboxylic amino acid (glutamic or aspartic) in addition to the histidine and the tyrosine residues previously hy-

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pothesized in the pioneering work of Femfert and Pfleiderer.[21,22] Taken together, these data suggest that the essential amino acids of the active site of APN and the mechanism of action of this enzyme are similar to those proposed for the group of the zinc-metalloendopeptidases, except for the presence in APN of a negatively charged amino acid capable of interacting with the N-terminal amino group of the substrate and thus ensuring the exopeptidase specificity of this enzyme.[20] Based on these results, the structural properties of the S_1 subsite and the catalytic site of APN have been explored in this study using a large series of amino alkyl derivatives bearing various zinc-coordinating moieties. These molecules have P_1 side chains of different size and hydrophobicity which are directed toward the S_1 subsite of APN by a positively charged free amino group, thus allowing the specificity of the subsite to be characterized.

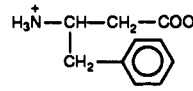
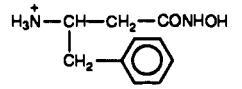
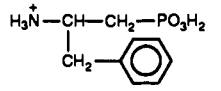
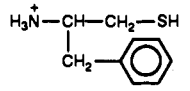
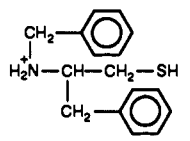
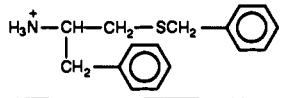
The potencies of these compounds in inhibiting APN activity have been determined with [^{3}H]Leu-enkephalin as substrate and their pharmacological properties were tested after iv administration, using the classical nociceptive mouse hot plate test, in order to study the relationship between enzyme affinity and in vivo analgesic potency. The most efficient APN inhibitor, both in terms of inhibitory potency and ability to cross the blood–brain barrier, could thus be selected.

Results

Chemistry. The various β-amino thiols described in this paper were synthesized from the corresponding β-amino alcohols, some of which such as L-phenylalaninol, L-leucinol, and L-methioninol, are commercially available. The amino function of these latter compounds was protected by a *tert*-butyloxycarbonyl group (Boc) for subsequent steps in the synthesis. The other *N*-Boc-amino alcohols were prepared by reduction of the corresponding *N*-Boc-amino esters by sodium borohydride treatment. Two different methods were used to transform the hydroxyl group in mercaptan (Figure 1). The first method was similar to that described by Chan[23] in the synthesis of leucinethiol, i.e. activation of the OH group using a tosylate intermediate, which was then substituted by a thioacetate group. An alternative route was the direct thioacetylation of the hydroxyl function by the Mitsunobu reaction[24] using diisopropyl azodicarboxylate, triphenylphosphine, and thioacetic acid as reagents. The thioesters were hydrolyzed by saponification and the disulfide derivatives isolated after iodine oxidation. Finally the *t*-Boc group was eliminated by TFA treatment (Figure 1).

The L-3-(*N*-Boc-amino)-4-phenylbutanoic acid (*N*-Boc-β-phenylalanine) was prepared by Arndt–Eistert homologation of *N*-Boc-L-phenylalanine as previously described,[25] with retention of configuration. Deprotection of the amino group by TFA, yielded the corresponding L-β-amino acid **1**.

Table I. Inhibitory Potency of L-Phenylalanine Derivatives on Aminopeptidase N

no.	compounds	IC_{50}, μM
1		800 ± 100
2		75 ± 5
3		500 ± 80
4c		0.030 ± 0.004
5c		160 ± 25
6c		80 ± 12

The reaction of *O*-benzylhydroxylamine on 3-(*N*-Boc-amino)-4-phenylbutanoic acid in the presence of DCC/HOBt, led, after successive deprotections of the two functional groups, to the L-β-phenylalanine N′-hydroxyamide (**2**).

The phosphonic derivative **3** was obtained, as previously described[26] from the tosylamino tosylate of L-phenylalaninol.

Inhibition of Aminopeptidase N. The inhibitory potency of the various compounds was tested on APN purified from hog kidney, using [^{3}H]Leu-enkephalin as substrate. The thiol-containing inhibitors were used as disulfide derivatives and were incubated in situ with 100 equiv of DTT to reduce the disulfide bridge. The cleavage followed by HPLC was complete in less than 5 min. At this concentration DTT was without effect on APN activity. In the absence of DTT, the IC_{50}'s of all disulfide inhibitors were around 10^{-5} M (data not shown).

The inhibitory potencies of compounds derived from L-phenylalanine are reported in Table I. In all these molecules, the functional group able to interact with the zinc ion of the APN catalytic site was in the β position, with regard to the essential amino group. Very large differences in the inhibitory potencies of these molecules were observed, with the lowest activities being found for the carboxylate **1** and the phosphonate **3** which inhibit the enzyme with IC_{50}'s in the 10^{-4} M range. An increase of 1 order of magnitude was observed with the hydroxamate **2** (IC_{50} in the 10^{-5} M range) but the most efficient compound was the thiol **4c** with an IC_{50} of 30 nM.

The substitution of the amino or the thiol function by a benzyl group in **5c** and **6c** led to a large decrease in inhibitory potency. However it was interesting to observe that the formation of a thiol ether in **6c**, induced a loss of affinity which was less drastic than benzylation of the free amino group in **5c**, despite the fact that this substitution preserves the protonation of the nitrogen atom shown to be essential for the interaction with APN.[27]

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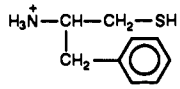
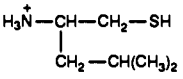
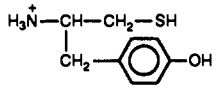
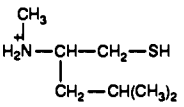
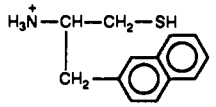
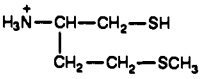
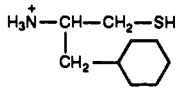
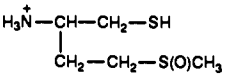
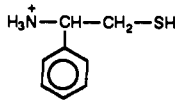
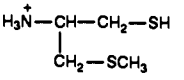
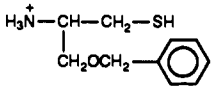
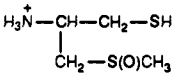
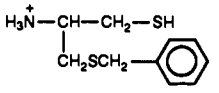
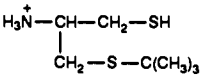
Table II. Inhibitory Potency of Thiol-Containing Inhibitors on Aminopeptidase N Activity

no.	compounds	IC_{50}, nM	no.	compounds	IC_{50}, nM
4c		30 ± 5	13c		22 ± 2
7c		45 ± 5	14c		4000 ± 100
8c		130 ± 12	15c		11 ± 1
9c		45 ± 3	16c		20 ± 2
10c		25 ± 2	17c		20 ± 5
11c		56 ± 6	18c		21 ± 3
12c		90 ± 7	19c		40 ± 5

The inhibitory potencies of various β-amino thiols differing only by the size and the hydrophobicity of the lateral chain are reported in Table II. All these compounds inhibit APN with IC_{50}'s in the 10^{-8} M range (except compound **14c** which contains a secondary amino group), indicating that all the selected side chains fit efficiently the S_1 subsite of APN. Nevertheless, compounds bearing aliphatic side chains (compounds **13c** and **15c–18c**) were slightly more potent than those containing aromatic or cyclic moieties (compounds **4** and **7–12**). Furthermore inhibitors **15c** and **16c**, derived from methionine, were slightly more active than those containing branched chains such as **13c** and **19c**. Interestingly, the thiol inhibitor **10c** derived from the nonnatural amino acid phenylglycine, also exhibited a good affinity for APN.

HPLC Studies of the Biologically Dependent Activation of the Inhibitor 15c. After incubation, under its disulfide form at a final concentration of 10^{-4} M with rat plasma serum (2.8 mg of protein/mL) at 37 °C for 1 h, inhibitor **15c** was found to be unchanged. Contrastingly, when it was incubated with a homogenate of rat brain membranes (3.0 mg/mL) under the same conditions of time and temperature the disulfide form disappeared and the free thiol analogue was found. This bioactivation process was prevented by a prior incubation of the membranes with perchloric acid.

Analgesic Properties. The antinociceptive properties of the inhibitors were tested on the hot plate test in mice, 15 min after their iv administration under disulfide forms and compared with carbaphethiol, a S-protected derivative of **4c**, previously reported to be slightly active after systemic administration.[28] Under our conditions, iv administration of carbaphethiol or of the disulfide of **4c** did not induce significant antinociceptive responses even at the highest dose used (40 mg/kg).

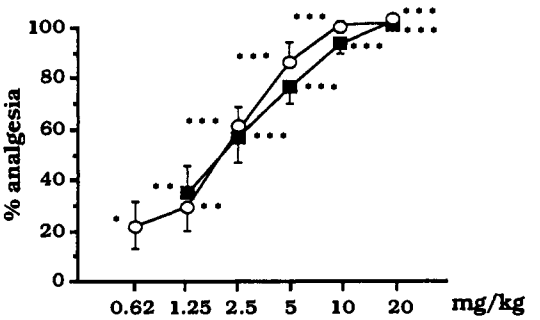


Figure 2. Antinociceptive activity induced on the jump latency in the hot plate test in mice by methionine thiol **15c** (○) and methionine thiol sulfoxide **16c** (■) in presence of 10 mg/kg acetorphan.

Therefore, in order to evaluate their in vivo activity, the thiol-containing APN inhibitors were coadministered, following a previously described procedure[4] with a fixed, subactive dose of acetorphan (10 mg/kg), which has previously been shown to inhibit endopeptidase activity in vivo. As shown in Table III, this test was extremely sensitive, since significant differences in the analgesic properties of the inhibitors studied were observed despite their similar in vitro inhibitory potencies.

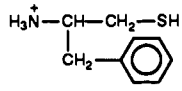
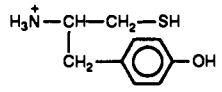
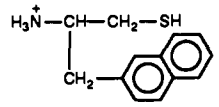
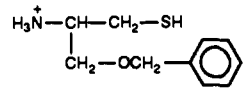
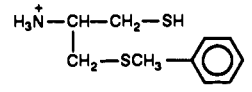
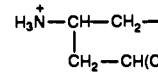
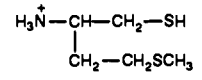
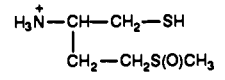
The most efficient compounds were **15c** and **16c** which contain a linear chain with a thioether, oxidized in **16c**. Thus at 10 mg/kg, iv, compound **16c** (0.7×10^{-6} mol per mouse) led to 97% analgesia while carbaphethiol at the same concentration gave 56% analgesia. Compounds containing the tyrosine side chain (**7c**), the β-naphtylalanyl moiety (**8c**), or the *O*-benzyl (**11c**) or the *S*-benzyl ether (**12c**) were significantly less active, in agreement with their lower affinity for APN.

As the two methioninethiol derivatives **15c** and **16c**, were highly efficient, complete dose–response curves could be measured (Figure 2). The ED_{50}'s were 2.2 (1.58–3.06) and

(27) Thorsett, E. D.; Wyvratt, M. J. Inhibition of zinc peptidases that hydrolyse neuropeptides. In *Neuropeptides and their peptidases*; Turner, A. J., Ed.; Ellis Horwood series in biomedecine: Sussex, 1987; pp 229–292.

(28) Gros, C.; Giros, B.; Schwartz, J. C.; Vlaiculescu, A.; Costentin, J.; Lecomte, J. M. Potent inhibition of cerebral aminopeptidases by carbaphetiol, a parenterally active compound. *Neuropeptides* 1988, *12*, 111–118.

Table III. Antinociceptive Responses Induced in the Hot Plate Test in Mice (Jump Latency) after Intravenous Coadministration of Acetorphan (10 mg/kg) and Various Doses of APN Inhibitors (under Disulfide Forms)

			doses,[a] mg/kg				
no.	compounds		control	5	10	20	40
4c		jump latency	69.6 ± 5.2	90 ± 12	147.9 ± 8.8	204.2 ± 6.8	
		% analgesia		12 ± 7[NS]	46 ± 5***	79 ± 4***	(-)
7c		jump latency	82 ± 6.4		112.9 ± 11.3	119 ± 12.6	166.4 ± 10.8
		% analgesia			19.5 ± 7*	23.7 ± 8**	53.4 ± 6.8***
8c		jump latency	91.1 ± 8.8		109.4 ± 13.7	106.6 ± 8.4	132.8 ± 6.6
		% analgesia			12.2 ± 9.2[NS]	10.4 ± 5.6[NS]	28.0 ± 4.4**
11c		jump latency	74.4 ± 4.8		127.1 ± 12.6	166.5 ± 16	229.4 ± 7.1
		% analgesia			31.8 ± 7.6***	55.6 ± 9.6***	93.6 ± 4.2***
12c		jump latency	112.8 ± 9.9		200.4 ± 19.5	232.2 ± 7.3	233.6 ± 4.3
		% analgesia			68.9 ± 15.3***	93.9 ± 5.7***	95 ± 3.4***
13c		jump latency	67.9 ± 5.7		184.5 ± 15.3	225.6 ± 102	240 ± 0
		% analgesia			67.8 ± 8.9***	91.6 ± 6***	100***
15c		jump latency	73.3 ± 6.0	200.8 ± 13.9	225.4 ± 7	240 ± 0	
		% analgesia		76.5 ± 10.8***	91.2 ± 4.2***	100***	
16c		jump latency	94.7 ± 6.8	216.9 ± 10.8	236.0 ± 2.7	240 ± 0	
		% analgesia		84.1 ± 7.4***	97.3 ± 1.9***	100***	

[a] *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS = nonsignificant vs control group.

2.4 (1.51–3.82) mg/kg for **15c** and **16c**, respectively.

Discussion

The results obtained in this study provide interesting information on the structure of the active site of APN and on the relationship between the in vitro inhibitory potencies of the most potent inhibitors synthesized and their in vivo antinociceptive action. As shown in Table I, there are large differences between the thiol inhibitor **4c** and the other phenylalanine derivatives **5c** and **6c**. The increase of the IC_{50} of **6c**, by a factor 2500, reflects the expected loss of interaction of the free thiol group of **4c** with the zinc ion. The 5000-fold lower affinity of **5c**, as compared to **4c**, seems more surprising, since **5c** contains a thiol group and an amino group expected to be positively charged at physiological pH. The benzyl chain being relatively large, a less bulky group was tested and a methyl group was introduced on the amino function of leucinethiol (**13c**). The compound obtained, **14c**, was 200 times less active than its precursor. Using the same approach, Pickering et al.[29] have shown that (*N*-butylamino)-ethanethiol and (dimethylamino)ethanethiol are more than 1000-fold less active than aminoethanethiol. The drastic decrease in the inhibitory potencies of these N-substituted amino thiols is very likely due to a severe steric hindrance between the constituting amino acids of the active site of APN and of the substituted amino group, suggesting the presence of a weakly accessible glutamate (or aspartate) in the active site of the enzyme.[20]

A comparison of the IC_{50}'s of compounds **1** to **3** and **4c** to **6c**, which are all endowed with a primary protonatable amino group, suggests that the functional groups, carboxylate, hydroxamate, or phosphonate, interact very weakly with the catalytic site of APN. This hypothesis was confirmed by comparing the inhibitory potencies of the β-substituted derivatives **1–3** and those of their α-analogues. Thus, L-phenylalanine or L-leucine[30,31] inhibit APN with IC_{50}'s around 10^{-3} M. Likewise, α-amino acid hydroxamates, such as Leu-NHOH,[32] Tyr-NHOH or Phe-NHOH[32,33] inhibit APN in the (2–5) $\times 10^{-5}$ M range, which is not very different from that of β-Phe-NHOH **2** ($IC_{50} = 7.5 \times 10^{-5}$ M). The α-amino phosphonate NH_2-$CH(CH_2Ph)PO_3H_2$ which is an efficient leucine aminopeptidase inhibitor[34,35] was found to be slightly more active on APN ($K_i = 27.5 \times 10^{-6}$ M)[35] than its β analogue **3** ($IC_{50} = 5 \times 10^{-4}$ M).

These results seem to indicate that the interaction of these inhibitors within the catalytic site of APN was not optimized, whatever the position (α or β) of the zinc chelating group, related to the P_1 residue.

Interestingly, it has generally been observed that, for

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(30) DiGregorio, M.; Pickering, D. S.; Chan, W. W. C. Multiple sites and synergism in the binding of inhibitors to microsomal aminopeptidase. *Biochemistry* **1988**, *27*, 3613–3617.

(31) Chan, W. W. C.; Dennis, P.; Demmer, W.; Brand, K. Inhibition of leucine aminopeptidase by amino acid hydroxamates. *J. Biol. Chem.* **1982**, *257*, 7955–7957.

(32) Coletti-Previero, M. A.; Crastes de Paulet, A.; Mattras, M.; Previero, A. Amino acid hydroxamates as inhibitors of the human enkephalin degrading aminopeptidase. *Biochem. Biophys. Res. Commun.* **1982**, *107*, 465–469.

(33) Baker, J. O.; Wilkes, S. H.; Bayliss, M. E.; Prescott, J. M. Hydroxamates and aliphatic boronic acids: marker inhibitors for aminopeptidase. *Biochemistry* **1983**, *22*, 2098–2103.

(34) Giannousis, P. P.; Bartlett, P. A. Phosphono amino acid analogues as inhibitors of leucine aminopeptidase. *J. Med. Chem.* **1987**, *30*, 1603–1609.

(35) Lejczak, B.; Kafarski, P.; Zygment, J. Inhibition of aminopeptidases by aminophosphonates. *Biochemistry* **1989**, *28*, 3549–3555.

endopeptidases such as NEP or ACE, inhibitors interacting with the Zn^{2+} ion and the S'_1 and S'_2 subsites are more efficient when they possess a methylene spacer between the zinc chelating group and the P'_1 residue. For instance, the inhibitory potencies of the two analogues HS-CH-(CH_2Ph)CO-L-Leu and HS-CH_2-CH(CH_2Ph)CO-L-Leu[36] for NEP are 50 and 4.5 nM, respectively. This has also been observed for the hydroxamate NEP inhibitors HONHCO-CH(CH_2Ph)CO-Gly (IC_{50} = 1.5 × 10^{-8} M) and HONHCO-CH_2-CH(CH_2Ph)CO-Gly (IC_{50} = 1.4 × 10^{-9} M).[37] Taken together these results, and those of this study, may be interpreted by a nonsymmetrical disposition of the coordinating moiety toward the zinc atom, when the inhibitors interact with subsites located on each side of the catalytic side, i.e. S_1 subsite in one case and S'_1 and S'_2 subsites in the other case. In agreement with this hypothesis the phenylalaninethiol **4c** is 200 times more potent as an APN inhibitor than the β-phenylalanine hydroxamate **2**, both compounds fitting the S_1 subsite of the peptidase. In contrast, the hydroxamate HONH-$COCH_2$-CH(CH_2Ph)CO-L-Phe[38] (IC_{50} = 130 nM on APN) is 800-fold more potent than the thiol inhibitor HS-CH_2-CH(CH_2Ph)CO-L-Phe (IC_{50} = 10^{-4} M), both compounds interacting with the S'_1 and S'_2 subsites of APN (to be published).

In contrast to compounds **1–3**, the inhibitory potencies of the various β-amino thiols reported in Table II indicate that for this type of molecule, which interacts with the S_1 subsite and the zinc ion of APN, the positions of the two functional groups are optimized. The IC_{50}'s reported in this study are in the 10^{-8} M range and confirm the results previously published on leucinethiol[23,29,39] or phenylalaninethiol.[40] Furthermore, it seems that this type of structure is well adapted for this family of zinc-metallopeptidases, since using the same strategy, Ocain and Rich[41] have synthesized lysinethiol which behaves as the most efficient known inhibitor of aminopeptidase B (IC_{50} = 0.9 nM). Since these β-amino thiols are very small molecules, it was tempting to increase inhibition of aminopeptidase N which prefers large substrates[42] with catalytic site-directed compounds able to interact not only with the S_1 but also with the S'_1 or S'_1 and S'_2 subsites. Such inhibitors containing a thiol group as zinc chelating agent have been previously synthesized. Surprisingly, thiol bestatin analogues[39] or dipeptidyl diamino thiols,[43] assumed to interact with the catalytic site and to fit the S_1 and S'_1 subsites, are 2 orders of magnitude less active than the β-amino thiols. However, the tripeptidyl amino thiols[42] which bind the S_1, S'_1, and S'_2 subsites gave IC_{50}'s in the nanomolar range on the pool of soluble brain aminopeptidases.

Various studies have shown that the S_1 subsite of APN is hydrophobic, and we have introduced lipophilic side chains in this position which differ only by length or bulk. In the series containing a cyclic moiety (compounds **4c** and **7c–12c**), the best affinity was obtained for inhibitors **4c** and **10c** derived from phenylglycine and phenylalanine which contain the less-hindering and less-bulky side chains. An increase in the length of the chain in *O*-benzylserine **11c** and *S*-benzylcysteine **12c** led to decreased activity by factors of 2 and 3, respectively. Similarly, the introduction of more bulky groups, such as cyclohexylalanyl in **9c** and 2-naphtylalanyl in **8c** led to a loss of activity by a factor of 2 and 5, respectively. For compounds containing an aliphatic side chain, the linear chains were slightly preferred to branched chains. *S*-Methylcysteine and methionine side chains in **17c** and **15c**, respectively, led to lower IC_{50}'s. The isopropyl group of leucine in **13c** and the *S*-*tert*-butyl group of compound **19c** were less efficient for S_1 subsite recognition. The sulfoxide **16c** and **18c** have about the same IC_{50}'s as their precursors **15c** and **17c**. However, it is possible that under the incubation conditions used to determine IC_{50}'s (100 equiv of DTT) the sulfoxide group was reduced. Taken together these results seem to indicate that the S_1, subsite of APN is a deep, but not very large hydrophobic pocket fitting optimally side chains of moderate bulk endowed with some degree of freedom.

The analgesic properties of the various thiol inhibitors were tested on the hot plate test in mice after intravenous administration. None of the aminopeptidase inhibitors tested were able to induce significant antinociceptive responses on the jump or the paw lick tests when tested alone. This is in agreement with previous studies showing that potent analgesic responses require in vivo inhibition of both NEP and APN, for example by mixed inhibitors such as kelatorphan or derivatives.[4,6] Therefore, in order to study their in vivo properties, APN inhibitors were iv coinjected under their disulfide forms, which are poor inhibitors of APN (IC_{50} > 10^{-5} M), with a constant subanalgesic dose of 10 mg/kg of acetorphan, the prodrug of the NEP inhibitor thiorphan.[1] By taking into account both the lack of activity of iv administered free thiol analogues (not shown here) and the in vitro bioactivation of the disulfide inhibitors by brain membranes, but not by serum, the naloxone reversible antinociceptive responses observed (Table III) suggest that the disulfide forms of these inhibitors were able to cross the blood–brain barrier and that the active mercaptans were released in the brain, probably through a reductive enzymatic pathway.

Another interesting result concerns the high efficiency of inhibitors **15c** and **16c**, since their ED_{50}'s on the hot plate test were 2 and 2.4 mg/kg, respectively (Figure 2). At 10 mg/kg of each inhibitor the cutoff time of the experiment was reached, without any sign of secondary effects. In agreement with the low doses used to obtain strong antinociceptive responses, the inhibitors appear to exhibit a rather good selectivity for the aminopeptidase N physiologically involved in enkephalin metabolism.

(36) Fournié-Zaluski, M. C.; Lucas, E.; Waksman, G.; Roques, B. P. Differences in the structural requirements for selective interaction with neutral metalloendopeptidase (enkephalinase) or angiotensin converting enzyme. *Eur. J. Biochem.* 1984, *139*, 267–274.

(37) Bouboutou, R.; Waksman, G.; Denin, J.; Fournié-Zaluski, M. C.; Roques, B. P. Bidentate peptides: Highly potent new inhibitors of enkephalin degrading enzymes. *Life Sci.* 1984, *35*, 1023–1030.

(38) Xie, J.; Soleilhac, J. M.; Schmidt, C.; Peyroux, J.; Roques, B. P.; Fournié-Zaluski, M. C. New kelatorphan related inhibitors of enkephalin metabolism: Improved antinociceptive properties. *J. Med. Chem.* 1989, *32*, 1497–1503.

(39) Ocain, T. D.; Rich, D. H. Synthesis of sulfur containing analogues of bestatin inhibition of aminopeptidase by α-thiol bestatin analogues. *J. Med. Chem.* 1988, *31*, 2193–2199.

(40) Weiss, B.; Hui, K. S.; Hui, M.; Lajtha, A. Aminothiols: Synthesis and effect on chicken brain aminopeptidases. *Res. Commun. Chem. Pathol. Pharmacol.* 1988, *62*, 113–123.

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(42) Kim, Y. S.; Kim, Y. W.; Sleisenger, M. H. Studies of the properties of peptide hydrolases in the brush-border and soluble fractions of small intestinal mucosa of rat and man. *Biochim. Biophys. Acta* 1974, *370*, 283–296.

(43) Gordon, E. M.; Godfrey, J. D.; Delaney, N. G.; Asaad, M. M.; Von Langen, D.; Cushman, D. W. Design of novel inhibitors of aminopeptidases. Synthesis of peptide derived diaminothiols and sulfur replacement analogues of bestatin. *J. Med. Chem.* 1988, *31*, 2199–2211.

In conclusion, highly efficient inhibitors of APN have been obtained by introduction in the same molecule of a thiol group as a zinc chelator and hydrophobic linear side chains able to interact with the S_1 subsite. The other classical metal coordinating groups such as carboxylate, phosphonate, or hydroxamate are much less potent in this series of compounds.

All the aminoalkyl thiols synthesized under their disulfide forms, are easily purified and characterized by physicochemical methods. The antinociceptive properties obtained with these compounds suggest that these oxidized and lipophilic forms were able to cross the blood–brain barrier before releasing the active mercaptans, leading therefore to a very simple prodrug methodology. This opens a new promising approach for designing parentally active, mixed NEP/APN inhibitors.

Experimental Section

Inhibitory Potency. Aminopeptidase from hog kidney was purchased from Boehringer Mannheim (Meylan, France) as a suspension in 3.2 M, ammonium sulfate, 50 mM Tris buffer, pH 7.4. [^{3}H]Tyr^1-Leu^5-enkephalin (30 Ci/mmol) was from Amersham. Determination of IC_{50}'s: the solutions of thiol inhibitors were prepared in Tris buffer pH 7.4 containing DTT (100 equiv per equivalent of inhibitor). APN was preincubated for 15 min at 25 °C with or without increasing concentrations of inhibitors, in a total volume of 100 μL in 50 mM Tris-HCl buffer pH 7.4. [^{3}H]Tyr^1-Leu^5-enkephalin (K_m = 50 μM) was added at a final concentration of 10 nM and the reaction was stopped after 15 min by adding 10 μL of 0.5 M HCl. The tritiated metabolite [^{3}H]Tyr was separated on polystyrene beads.

Antinociceptive Properties. Analgesic activities of the various aminopeptidase N inhibitors coinjected with acetorphan (10 mg/kg) were assessed by using the mouse hot plate test at 55 ± 0.5 °C. All the inhibitors were dissolved in the following vehicle: ethanol (10%)/cremophor EL (10%)/distilled water (80%). Drugs or vehicle (controls) were administered intravenously to male Swiss mice (20–22 g, Depré, France) in a volume of 0.1 mL per 10 g, 15 min before the hot plate test. The latency of jumping (cut-off time 240 s) was measured. A percentage of analgesia was calculated by the ratio: % analgesia = (test latency − control latency)/(cut-off time − control latency) × 100. Statistical analysis was carried out by analysis of variance (ANOVA), followed by Dunnett's t test. The ED_{50} was defined as the dose of APN inhibitors required to elicit 50% analgesia when they were iv coadministered with 10 mg/kg of acetorphan.

ED_{50} values and their 95% confidence limits were calculated by log-probit analysis according to the method of Litchfield and Wilcoxon.[44]

HPLC Studies of Prodrug Inhibitor 15c Bioactivation. The in vitro formation of the active component of the prodrug inhibitor **15c** was monitored by HPLC. Compound **15c** (10^{-4} M final concentration) was incubated for 60 min at 37 °C in the presence of rat brain membranes (3.0 mg of protein/mL) or rat serum (2.8 mg of protein/mL) in 450 μL of 50 mM Tris-HCl buffer, pH 7.4. The reaction was stopped by addition of 50 μL of 4 M $HClO_4$, and kept at 0 °C for 10 min. Acetonitrile (200 μL) was added to the suspension to extract products adsorbed to proteins and the mixture was vigorously agitated and centrifuged for 5 min at 100000g. Controls were performed under the same conditions in the absence of protein or with protein inactivated by prior addition of 4 M $HClO_4$.

The products formed were analyzed by HPLC on a nucleosil 5-μm C_{18} column (4.6 × 250 mm). The mobile phase consisted of (A) trifluoroacetic acid 0.05% and (B) acetonitrile/H_2O, 7:3 with 0.05% trifluoroacetic acid. The elution was carried out under isocratic conditions (14% of B) at a flow rate of 1 mL/min.

The products were detected at 214 nm and identified by comparison with synthetic markers (solubilized in Tris-HCl buffer/EtOH, 90:10). The elution times were 24.2 min for compound **15c** and 7.4 min for the free thiol analogue $NH_2CH(CH_2CH_2S-CH_3)CH_2SH$.

Chemistry. Amino acids and amino esters were from Bachem (Bubendorf, Switzerland). Amino alcohols and other reagents were from Aldrich Chemie (Steinheim, West Germany). The solvents were from SDS (Peypin, France).

Carbaphethiol was synthesized in our laboratory following the reported procedure. β-Phenylalanine phosphate[26] was prepared as described. The purity of the synthesized compounds was checked by thin-layer chromatography on silica gel plates (60F254, Merck). The following solvent systems (v/v) were used: (A) CH_2Cl_2/MeOH, 9:1; (B) CH_2Cl_2/MeOH, 9:0.5; (C) cyclohexane/EtOAc/acetic acid, 7:3:0.5; (D) CH_2Cl_2/MeOH, 12:0.5; (E) $CHCl_3$/MeOH, 9:7; (F) cyclohexane/EtOAc, 7:3; (G) cyclohexane/EtOAc, 8:2; (H) n-hexane/EtOAc, 9:1; (I) cyclohexane/EtOAc, 9:1; (J) n-hexane/EtOAc, 7:3; (K) cyclohexane/EtOAc/acetic acid, 8:2:0.5; (L) cyclohexane/EtOAc/acetic acid, 6:4:0.5; (M) cyclohexane/EtOAc/acetic acid, 9:1:0.5. The purity of the final compounds was also checked by HPLC using a reverse-phase nucleosil C_8 column (SFCC, France) with CH_3CN/TFA 0.07% buffer (pH = 4.0) as solvent. The eluted peaks were monitored at 210 nm. The structure of all the compounds synthesized was confirmed by ^{1}H NMR spectroscopy (Brüker WH 270 MHz) in DMSO-d_6 solutions (5×10^{-3} M). Melting points of the crystallized compounds were determined on an electrothermal apparatus and are reported uncorrected. The structure of the final compounds was verified by mass spectroscopy (Nermag R 10 C, DIC NH_3 mode) and satisfactory analyses (<±0.4%) were obtained (C, H, N) for all compounds. The following abbreviations are used: EtOH, ethanol; Et_2O diethyl ether; DMF, dimethylformamide; Et_3N, triethylamine; $(Boc)_2O$, di-*tert*-butyl dicarbonate; TsCl, tosyl chloride; EtOAc, ethyl acetate; THF, tetrahydrofuran; TFA, trifluoroacetic acid; BuOH, butanol; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole.

Synthesis of β-Phenylalanine *N'*-Hydroxyamide 2. The *N*-Boc-β-phenylalanine *N'*-(benzyloxy)amide (0.23 g, 0.6 mM) prepared as previously described[5] was dissolved in 2 mL of TFA at 0 °C and 1.77 mL of a 1 M solution of boron tris(trifluoroacetate) in TFA was added. The mixture was stirred 1.5 h at 0 °C and evaporated in vacuo. The crude residue was taken off in Et_2O and the white precipitate was filtered. Purification by flash chromatography in silica gel using EtOAc/pyridine/acetic acid/water (50:20:6:11) as eluent: R_f 0.29 in this system (52%); ^{1}H NMR δ CH_2(CO) 2.2, $CH_2\beta$ 2.7 and 2.88, $CH\alpha$ 3.60, Ar-Phe 7.25, NH_3^+ 7.85, OH 8.83, NH 10.61. Anal. ($C_{10}H_{14}N_2O_2$) C, H, N.

General Procedure for the Synthesis of *N*-Boc-Amino Alcohols a. Method A. The Boc-amino ester (10 mM) was dissolved in 30 mL of EtOH/H_2O (50:50). $NaBH_4$ (4 equiv) in EtOH/H_2O (50:50) was added at −10 °C. The mixture was stirred for 30 min at 0 °C and for 2 h at room temperature. The mixture was evaporated in vacuo and the residue dissolved in Et_2O. The organic layer was successively washed with H_2O, CH_3COOH, H_2O, and saturated NaCl solution and dried over Na_2SO_4. After evaporation in vacuo the Boc-amino alcohols were obtained. Yield 80–95%: **7a**, oily product R_f (A) 0.52; **8a**, white solid, mp 138 °C, R_f (B) 0.60; **11a**, oily product, R_f (C) 0.40; **12a**, oily product, R_f (D) 0.38; **17a**, oily product, R_f (C) 0.30; **19c**, oily product, R_f (C) 0.42.

Method B. The amino alcohols (10 mM) were dissolved in DMF (10 mL) and at 0 °C Et_3N (1 equiv) and $(Boc)_2O$ (1.1 equiv) were added. The mixture was stirred for 30 min at 0 °C and for 2 h at room temperature. After evaporation in vacuo, the residue was dissolved in Et_2O. The organic layer was successively washed with H_2O, 10% citric acid solution, H_2O, and saturated NaCl solution and dried over Na_2SO_4. After evaporation in vacuo, the Boc-amino alcohols were isolated. Yield 90–95%: **4a**, white solid, mp 97 °C, R_f (E) 0.86; **13a**, oily product, R_f (B), 0.53; **15a**, oily product, R_f (A) 0.55; **9a**, oily product, R_f (C) 0.48; **10a**, oily product, R_f (B) 0.34.

Preparation of Acetylthio Derivatives b. Method A. The Boc-amino alcohols were dissolved in a mixture pyridine/KOH and 1.1 equiv of TsCl in pyridine/KOH was added at 0 °C. The mixture was stirred overnight at 0 °C. The precipitate was filtered and the solution evaporated in vacuo. The residue was dissolved in EtOAc and washed with H_2O, acetic acid, H_2O, and saturated

(44) Litchfield, J. I.; Wilcoxon, F. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* **1949**, *96*, 99–113.

NaCl solution, and dried over Na_2SO_4 and evaporated in vacuo.

A white solid was obtained; yield 70–80%. The *N*-Boc-amino tosylate was dissolved in DMF and a solution of $CH_3COS^-K^+$ in DMF (prepared from CH_3COSH and KOH) was added at 0 °C. The mixture was stirred overnight at the same temperature. The mixture was poured in iced water, and the white precipitate was filtered off washed with iced water and dried. Yield 90–98%: **4b**, R_f (F) 0.53; **13b**, R_f (G) 0.38.

Method B. In a mixture of diisopropyl azodicarboxylate (2 equiv) and triphenylphosphine (2 equiv) in THF were added successively at 0 °C 2 equiv of CH_3COSH and the Boc-amino alcohols (1 equiv). The mixture was stirred overnight at room temperature. After evaporation in vacuo the residue was dissolved in EtOAc, washed successively with a 10% $NaHCO_3$ solution, H_2O, and a saturated NaCl solution, and then dried over Na_2SO_4. After evaporation the residue was poured in EtOAc/*n*-hexane and the precipitate was eliminated. The filtrate was evaporated and residue purified by flash chromatography on silica gel column, using cyclohexane/EtOAc (9:1) as eluent: **7b**, oily product R_f (H) 0.30; **8b** R_f (I) 0.20; **9b**, oily product R_f (C) 0.64; **10b**, white solid, mp 64 °C, R_f (J) 0.44; **11b**, oily product, R_f (L) 0.67; **12b**, white solid, mp 72 °C, R_f (K) 0.43; **15b**, white solid, mp 67 °C, R_f (L) 0.48; **17b**, oil, R_f (K) 0.42; **19b**, white solid, mp 75 °C, R_f (M) 0.32.

Compounds 16b and 18b Were Obtained by Oxidation of Their Precursors 15b and 17b. The Boc-amino thioester was dissolved in EtOH. An aqueous solution of $NaIO_4$ (0.2 M, 2.2 equiv) was added at 0 °C, and the mixture was stirred overnight at the same temperature. The precipitate was filtered, and the solution was evaporated in vacuo. The residue was dissolved in EtOAc; the organic layer was filtered, washed with H_2O and NaCl (saturated solution), dried over Na_2SO_4, and evaporated in vacuo: **16b**, R_f (A) 0.28; **18b**, R_f (A) 0.34.

General Procedure for the Preparation of Bis(amino sulfides) c. The *N*-Boc-amino acetylthio derivatives were dissolved in EtOH and 1 M NaOH solution (2 equiv) was added at 0 °C. The mixture was stirred for 1 h at 0 °C and for 3 h at room temperature. A solution of I_2 in EtOH was added until a persistent yellow color was obtained. The excess of iodine was reduced by $Na_2S_2O_3$ and the solution was evaporated in vacuo. The residue was dissolved in EtOAc, washed with H_2O and saturated NaCl solution, dried over Na_2SO_4, and evaporated, yield 80–90%. The residue was dissolved in CH_2Cl_2 and TFA (10 equiv) was added at 0 °C. The mixture was stirred for 30 min at 0 °C and 2 h at room temperature and then was evaporated in vacuo. The residue was dissolved in Et_2O. The white precipitate obtained was extensively washed with Et_2O. The inhibitors were isolated as trifluoroacetate salts.

The mass spectra and the elemental analyses were performed on the deprotonated forms of the inhibitors. **4c**: white solid (72%), mp 148 °C; ^1H NMR δ CH_2S 2.6, CH_2(Ph) 2.75 and 2.90, $CH\alpha$ 3.5, Ph 7.25, NH_3^+ 7.95; MS m/z 333 (M + 1). Anal. ($C_{18}H_{24}N_2S_2$) C, H, N.

5c: white solid (63%), mp 177 °C; ^1H NMR δ CH_2S 2.70, CH_2(Ph) 2.70 and 3.20, $CH\alpha$ 3.2, NCH_2(Ph) 4.22, Ph centered on 7.25 and 7.40, NH_2^+ 8.95; MS m/z 515 (M + 1). Anal. ($C_{32}H_{36}N_2S_2$) C, H, N.

7c: white solid (83%), mp 112 °C; ^1H NMR δ CH_2S 2.70, $CH_2\beta$ 2.75, $CH\alpha$ 3.45, Ph 6.75 and 6.95, NH_3^+ 7.90, OH 9.35; MS m/z 365 (M + 1). Anal. ($C_{18}H_{24}N_2O_2S_2$) C, H, N.

8c: oily compound (58%); ^1H NMR δ CH_2S 2.58, $CH_2\beta$ 2.90, $CH\alpha$ 3.18, naphtyl 7.3, 7.4, 7.6, and 7.75, NH_3^+ 7.92; MS m/z 365 (M + 1). Anal. ($C_{26}H_{28}N_2S_2$) C, H, N.

9c: white solid (60%); mp 172 °C; ^1H NMR δ CH_2S 2.70, $CH_2\beta$ 0.8, cyclohexyl 1.1, 1.4, and 1.8, CH_2S 2.90, $CH\alpha$ 3.35, NH_3^+ 7.9; MS m/z = 345 (M + 1). Anal. ($C_{18}H_{24}N_2S_2$) C, H, N.

10c: oily product (76%); ^1H NMR δ CH_2S 3.2, $CH\alpha$ 4.4, Ph 7.35, NH_3^+ 8.42; MS m/z 305 (M + 1). Anal. ($C_{16}H_{20}N_2S_2$) C, H, N.

11c: white solid (75%); mp 98 °C; ^1H NMR δ CH_2S 2.95, $CH_2\beta$ 3.55, $CH\alpha$ 3.65, OCH_2 4.5, Ph 7.30, NH_3^+ 8.1; MS m/z 393 (M + 1). Anal. ($C_{20}H_{28}N_2O_2S_2$) C, H, N; C: calcd, 61.21; found, 60.80.

12c: oily product (78%); ^1H NMR δ CH_2S 2.70, $CH_2\beta$ 2.94, $CH\alpha$ 3.2, CH_2(Ph) 3.70, Ph 7.20, NH_3^+ 8.00; MS m/z 425 (M + 1). Anal. ($C_{20}H_{28}N_2S_4$) C, H, N.

13c: white solid (56%); mp 112 °C; ^1H NMR δ CH_3 0.85, $CH_2\beta$ 1.45, $CH\gamma$ 1.70, CH_2S 2.85 and 2.95, $CH\alpha$ 3.35, NH_3^+ 7.2; MS m/z 265 (M + 1). Anal. ($C_{12}H_{28}N_2S_2$) C, H, N.

14c: oily product (66%); ^1H NMR δ CH_3 0.8, $CH_2\beta$ 1.28, $CH\gamma$ 1.65, NCH_3 2.30, CH_2S 2.95, $CH\alpha$ 3.30, NH_2^+ 8.00; MS m/z 293 (M + 1). Anal. ($C_{14}H_{32}N_2S_2$) C, H, N.

15c: oily compound (66%); ^1H NMR δ $CH_2\beta$ 1.87, SCH_3 2, $CH\gamma$ 2.55, CH_2S 2.9 and 3.05, $CH\alpha$ 3.47, NH_3^+ 8.00; MS m/z 301 (M + 1). Anal. ($C_{10}H_{24}N_2S_4$) C, H, N.

16c: oily product (76%); ^1H NMR δ $CH_2\gamma$ 2.05, SCH_3 2.4, $CH\beta$ 2.80 and 2.90, CH_2S 2.92 and 3.05, $CH\alpha$ 3.50, NH_3^+ 8.01; MS m/z 333 (M + 1). Anal. ($C_{10}H_{24}N_2O_2S_4$) C, H, N.

17c: oily compound (66%); ^1H NMR δ CH_2S 2.60 and 2.70, SCH_3 2.65, $CH_2\beta$ 2.85 and 3.00, $CH\alpha$ 3.30, NH_3^+ 7.98; MS m/z 273 (M + 1). Anal. ($C_8H_{20}N_2S_4$) C, H, N.

18c: white solid (68%); mp 72 °C; ^1H NMR δ SCH_3 2.65, $CH_2\beta$ 3.10, $CH\alpha$ 3.9, NH_3^+ 8.2; MS m/z 305 (M + 1). Anal. ($C_8H_{20}N_2O_2S_4$) C, H, N.

19c: white solid (76%); mp 126 °C; ^1H NMR δ $C(CH_3)$ 1.25, $CH\beta$ 2.85, CH_2S 2.90 and 3.05, $CH\alpha$ 3.50, NH_3^+ 8.10; MS m/z 357 (M + 1). Anal. ($C_{14}H_{32}O_8S_2$) C, H, N.

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