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New Orally Active Enkephalinase Inhibitors: their Synthesis, Biological Activity, and Analgesic Properties

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Abstract—A series of (4*S*)-4-[(2*S*)-benzyl-3-mercaptopropionylamino]-4-(*N*-phenylcarbamoyl)-butyric acids has been identified as potent systemically active enkephalinase inhibitors. Structure–activity relationships (SAR) are discussed. Further chemical modification of the inhibitors was carried out in order to identify the inhibitors which are orally active in an animal model. Compounds of particular interest are the prodrug-like analogues, including **5b** (ONO-9902). Their analgesic effects after oral administration were evaluated.

Introduction

The discovery of endogenous opioid peptides in the brain[1], enkephalins, which are associated with both a heterogeneity of binding sites[2–4] and a well-defined metabolic pathway might offer new approaches to the prevention or treatment of addiction following analgesic use. In vitro studies performed with rat brain slices showed that three metalloenzymes are involved in enkephalin degradation: (1) a neutral metalloendopeptidase, EC 3. 4. 24. 11,[5] designated enkephalinase,[6] cleaving the Gly-Phe (positions 3 and 4) bond, (2) an aminopeptidase releasing the *N*-terminal tyrosine, and a dipeptidylaminopeptidase[7] cleaving the Gly-Gly (positions 2 and 3) amide bond.[8] The importance of enkephalinase is supported by the analgesic effects induced by the administration of potent enkephalinase inhibitors such as carboxyalkyl dipeptides[9,10], thiorphan **1**,[11,12] retrothiorphan **2** and kelatorphan **4** (Chart 1)[13]. The oral activity of the compounds **3a**–**c** has also been reported[14].

The analgesic activity induced by the intracerebroventricular (icv) administration of the aminopeptidase inhibitor *bestatin* suggested that this enzyme could also be physiologically involved in enkephalin degradation.[15] Therefore, it is of interest to design compounds which are able to inhibit the three enkephalin-degrading enzymes belonging to the group of metallopeptidases.

Despite the considerable interest in this field during the last few decades, therapeutic applications of an enkephalinase inhibitor have failed to materialize. This failure has been due largely to oral activity problems encountered when the inhibitors are used as a drug.

During the course of a screening of our compound library, an angiotensin-converting enzyme (ACE) inhibitor **6**[16] was discovered to show moderate inhibitory activity against enkephalinase ($IC_{50} = 0.7\,\mu M$). The structural similarity of this compound to thiorphan **1** prompted us to prepare compounds **7a**,**b** and evaluate their inhibitory activity (Chart 2). Compounds **7a**,**b** showed potent in vitro inhibitory activity (**7a**: $IC_{50} = 4.25\,nM$, **7b**: $IC_{50} = 10.5\,nM$), although they were not effective in systemic administration (Table 1). According to the reported modes of binding of a substrate and various inhibitors with the active site of enkephalinase,[17] the R residue of general formula I should be lipophilic, while it was hydrophilic in **7a**,**b**. In light of this, we were surprised that the glutamic acid residue was accepted as a surrogate instead of the glycine residue of thiorphan **1**. In the present report, we

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describe the successful modification of compound **6** to obtain new orally active enkephalinase inhibitors. The full details of the synthesis, structure–activity relationships, and some pharmacological evaluations are discussed.

Chemistry

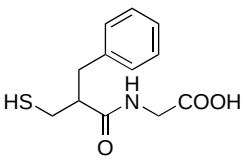
The syntheses of **8b** and **9c–17c** are described in Scheme 1. The preparation of anilide **28** from **27** was carried out by an acid anhydride method using pivaloyl chloride followed by acidic deprotection, respectively. The *N*-acylation of **28** with an acid chloride **29** followed by alkaline hydrolysis afforded **8b**. Compounds **9c–17c** were prepared from **27** by the following sequential reactions: The preparation of acid anhydride with pivaloyl chloride, the addition of an appropriate amine, and acidic deprotection afforded **9a–17a**. Compounds **9a–17a** were converted to **9c–17c** by the same procedure as described in the preparation of **8b** from **28**.

Scheme 2 demonstrates the syntheses of **18a** and **18b**. Compound **18a** was prepared from **31**. The preparation of an anilide **32** from **31** was carried out by the acid anhydride method using pivaloyl chloride followed by deprotection with trifluoroacetic acid to afford **33**, which was acylated with **29** to give **34**. The aminolysis of the acetylthio moiety with mercaptoethylamine followed

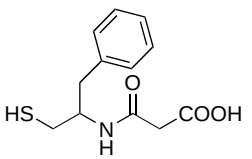
Table 1. Enkephalinase inhibition and/or the effect on the bradykinin-induced biting-like response of **1**, **2**, **3a–c**, **6** and **7a–b** in male SD rats

Compound	IC_{50} (nM)	BK-biting[b] (mg/kg ip)	N[e]
1	3.0	NE[c] (30 > mg/kg ip)	7
2	700	NT[d]	—
3a[a]	2.5	33.3% (5 mg/kg ip)	6
3b	33	NT[b]	—
3c	26	NT[b]	—
6	700	NT[b]	4
7a	4.25	NE[c] (30 mg/kg ip)	4
7b	10.5	NE[c] (10 mg/kg ip)	4

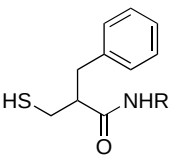
[a]Oral activity was reported.[14]
[b]Bradykinin-induced biting-like response.
[c]NE: Not effective.
[d]NT: Not tested.



1 (Thiorphan)



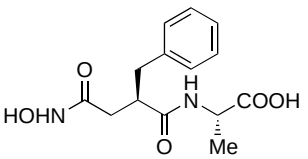
2 (Retrothiorphan)



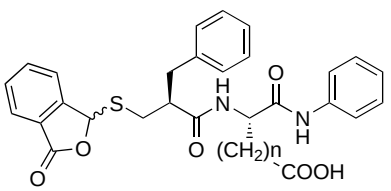
3a (Ru 42827) : R = N • HCl

3b : R =

3c : R = OMe



4 (Kelatorphan)



5a : n = 1
5b : n = 2 (ONO-9902)

Chart 1. Enkephalinase inhibitors.

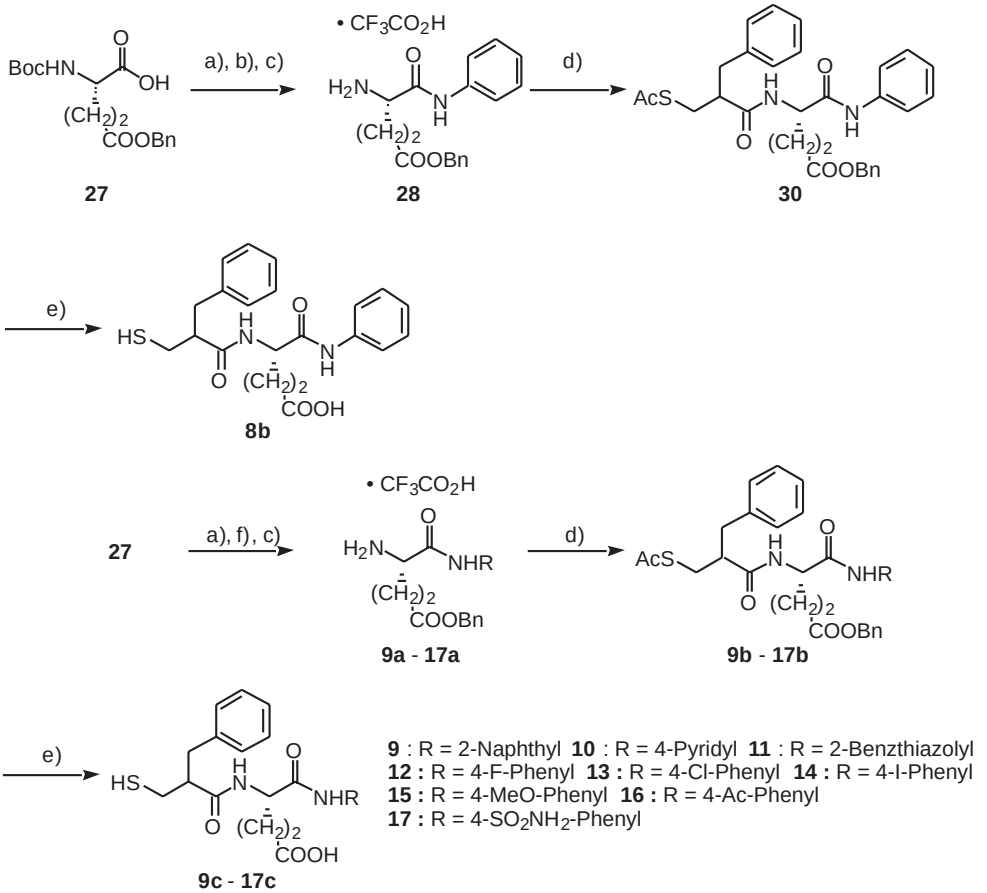
by alkaline hydrolysis provided **18a**. An anilide **37** was prepared from **36** by the same procedure as described in the preparation of **32** from **31**. Compound **37** was converted to **18b** by these sequential reactions: hydrogenation; acylation with **29**; acidic deprotection; aminolysis of thioacetate; hydrolysis with sodium hydroxide.

The preparation of **19a–c** was carried out as shown in Scheme 3. The alkaline hydrolysis of **41a–c** followed by the Michael addition of thiobenzoic acid or thioacetic acid provided **42a–c**, respectively. The conversion of **42a–c** to the corresponding acid chloride with oxalyl chloride followed by the addition of **28** afforded **43a–c**, respectively. The alkaline hydrolysis of **43a–c** gave **19a–c**, respectively.

The preparation of optically active diastereomers **8a**(*S*), **8a**(*R*), **8b**(*S*), **8b**(*R*), **11**(*S*) and **11**(*R*) are described in Scheme 4.

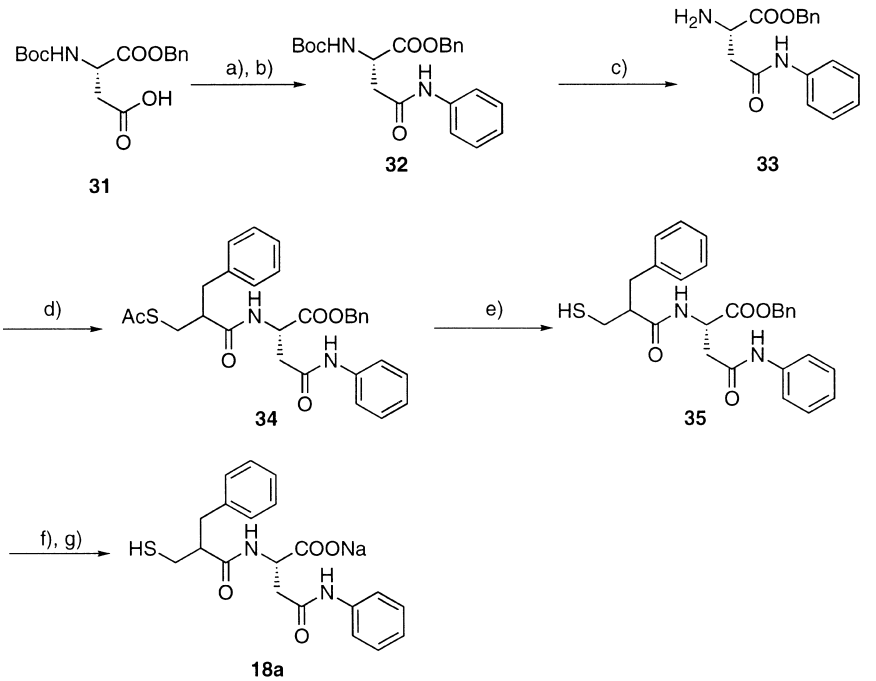
The treatment of **44**(*S*) and **44**(*R*) with oxalyl chloride followed by the addition of **45a** provided **46a**(*S*) and **46a**(*R*), respectively, which were converted to **8a**(*S*) and **8a**(*R*) by methanolysis followed by deprotection with trifluoroacetic acid (TFA), respectively. The *N*-acylation of **45b**,**c** with **44**(*S*) and **44**(*R*) provided **46b**(*S*), **46c**(*S*) and **46b**(*R*), **46c**(*R*), respectively, which were converted to **8b**(*S*), **11**(*S*) and **8b**(*R*) and **11**(*R*) by alkaline hydrolysis, respectively.

Compounds **20–24** were prepared as shown in Scheme 5. The condensation of **47** with aniline provided **48**. The hydrogenation of **48** followed by the addition of 1 equivalent of hydrogen chloride afforded **49**, which was acylated with an acid chloride prepared from **44**(*S*), affording **50a**. The methanolysis of **50a** provided **50b**, which was acylated with an appropriate acyl chloride and then deprotected to give **20** and **24**, respectively. The following sequential reactions: The protection of

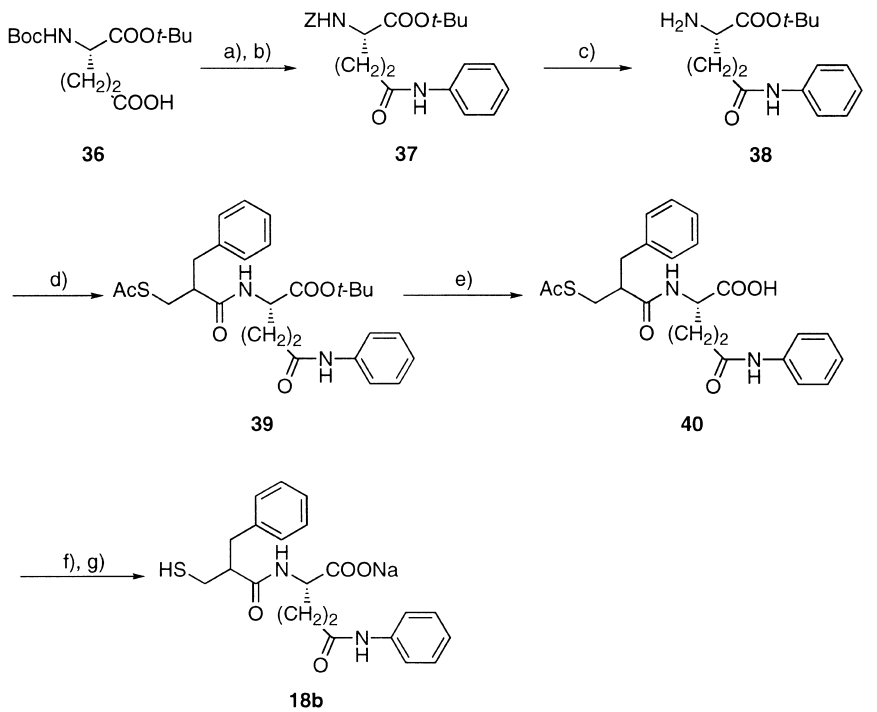


Reagents: a) *t*-BuCOCl, Et_3N, CH_2Cl_2, 5 °C, 0.5 h; b) $PhNH_2$, 5 °C, 1 h; c) CF_3CO_2H, CH_2Cl_2, 25 °C, 2.5 h; d) 2-benzyl-3-acetylthiopropionyl chloride (**29**), Py, CH_2Cl_2, 5 °C then 25 °C, 15 min; e) LiOH, DME - THF - H_2O, 25 °C, 0.5 h; f) RNH_2, 5 °C, 1 h.

Scheme 1. Synthesis of **8b** and **9c–17c**.



Reagents: a) *t*-BuCOCl, Et_3N, CH_2Cl_2, 25 °C, 1 h; b) $PhNH_2$, Et_3N, 5 °C then 25 °C, 1 h; c) CF_3CO_2H, 25 °C, 15 h; d) **29**, Py, CH_2Cl_2, 5 °C, 0.5 h then 25 °C, 1 h; e) $HS(CH_2)_2NH_2$, CH_3CN, 45 °C, 2 h; f) KOH, MeOH - THF, 25 °C, 4 h; g) NaOH (1eq.), H_2O, 25 °C.



Reagents: a) *t*-BuCOCl, Et_3N, CH_2Cl_2, 25 °C, 10 min; b) $PhNH_2$, Et_3N, 5 °C then 25 °C, 15 h; c) H_2, 10% Pd - C, MeOH, 25 °C, 50 min; d) **29**, Py, CH_2Cl_2, 5 °C, 0.5 h then 25 °C, 0.5 h; e) CF_3CO_2H, 25 °C, 2 h; f) $HS(CH_2)_2NH_2$, CH_3CN, 50 °C, 0.5 h; g) NaOH (1eq.), H_2O, 25 °C.

Scheme 2. Synthesis of **18a** and **18b**.

the carboxylic acid moiety of **8b**(*S*) with *t*-butylchlorodiphenylsilane; acylation with an appropriate acyl chloride or with an appropriate carboxylic acid in the presence of DPPA; deprotection with *n*-butylammoniun fluoride, afforded **21**–**23**, respectively.

Compounds **5a**,**b** were prepared from **46a** and **8b**(*S*) by *S*-alkylation with 3-chlorophthalide followed by deprotection with TFA and simple *S*-alkylation with 3-chlorophthalide, respectively. The preparation of **7b**, **25** and **26** is illustrated in Scheme 6. After protection of the carboxylic acid function of **11c**(*S*) as a silyl ester, the *S*-alkylation with 3-chlorophthalide followed by acidic deprotection afforded **25**. The alkaline hydrolysis of **51** afforded **7b**(*S*). The methanolysis of **44**(*S*) provided **26**.

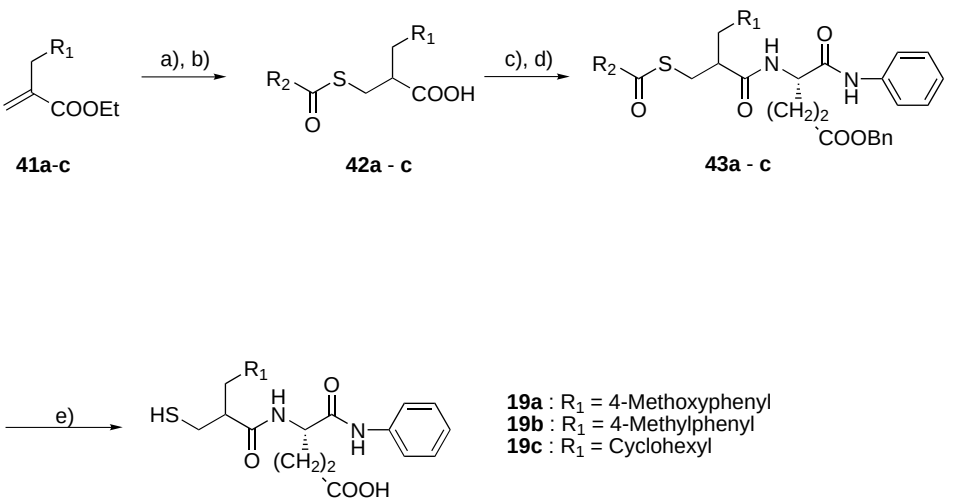
Results and discussion

The enkephalinase inhibitory activity of the synthetic inhibitors was studied using an in vitro approach.[18]

Their antinociceptive effect was evaluated with the bradykinin (BK)-induced biting-like response,[19] the formalin-induced licking method, the acetic acid (AcOH)-induced writhing method, and potentiation effects on stress-induced analgesia (SIA) in a hot-plate test.

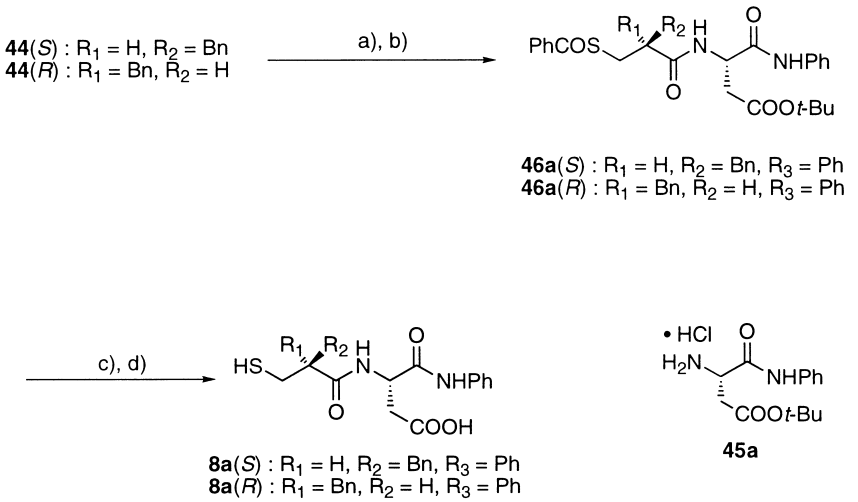
Although the selected compound **5b** showed low in vitro enkephalinase inhibition ($IC_{50} = 0.3\,\mu M$ as compared to 21 nM of **8b** or 3 nM of thiorphan **1**), this orally active compound suppressed all of the above experimental pain responses in a dose-dependent manner. The potentiation of the SIA-induced antinociceptive action of rodents in the hot-plate test was also observed. An evaluation of **5b** using the BK-induced nociceptive biting-like and AcOH-induced writhing responses indicated a 30 mg/kg ED_{50} and 10 mg/kg as the minimum effective dose (MED) values in oral administration. Though this enkephalinase inhibitor was effective in the above tests, it did not elicit significant suppressive effects on naive rodents examined with the hot-plate test, a property intrinsic to the opioid agonist–antagonist pentazocine, as well.

Enkephalinase is a zinc metallopeptidase homologous to ACE, and previous work provided some precedents for the design of enkephalinase inhibitors.[10] The design of enkephalinase inhibitors of the general formula II was based on the subsite preferences. According to the reported modes of binding of a substrate and various inhibitors with the active site of enkephalinase, enkephalinase inhibitors such as SCH-32615[20], which was reported during our work, were estimated to possess a strong interaction with the two subsites S1′ and S2′ of enkephalinase using the two aromatic moieties, besides the regular interaction using zinc ligand and acidic moiety, as illustrated in Chart 3(a) and (b). Based on this information, the conversion of the α-carboxylic acid moiety of **7b** to an anilide provided **8b**, whose modes of interaction with the enzyme were expected to be similar to those of SCH-32615 as shown in Chart 3(c), with a

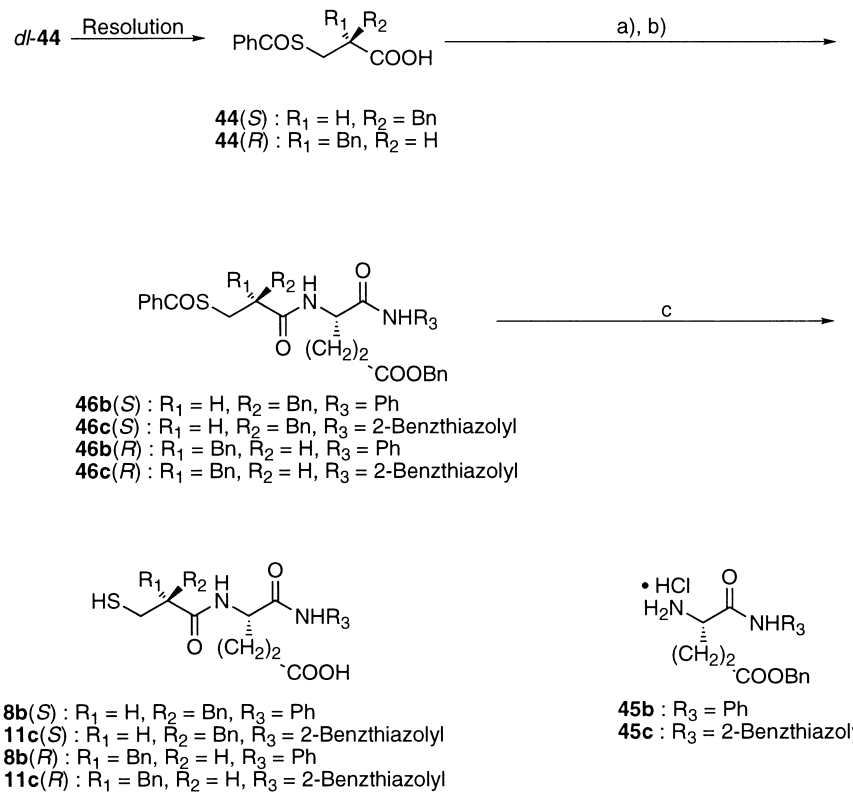


Reagents: a) KOH, EtOH, reflux, 1.5 h; b) PhSH, CH_2Cl_2, reflux or AcSH, Ph-H, reflux, 2 h; c) $(COCl)_2$, CH_2Cl_2, 5 - 25 °C, 0.5 h; d) L-GluNHPh(OBn) • CF_3CO_2H (**28**), Py, CH_2Cl_2, 5 °C, 1 h; e) LiOH, DME - THF, 25 °C, 1 h.

Scheme 3. Synthesis of **19a**–**19c**.

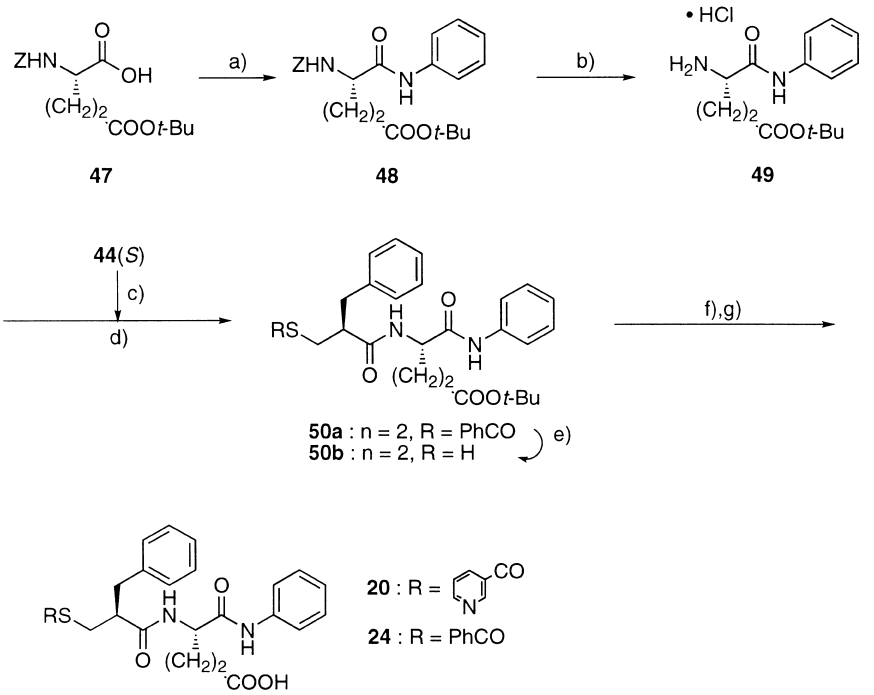


Reagents; a) $(COCl)_2$, CH_2Cl_2, 25 °C, 1 h; b) **45a**, Py, CH_2Cl_2, 5 °C then 25 °C, 15 min; c)K_2CO_3, MeOH, 25 °C, 0.5 h; d) CF_3CO_2H, CH_2Cl_2, 25 °C, 2 h.

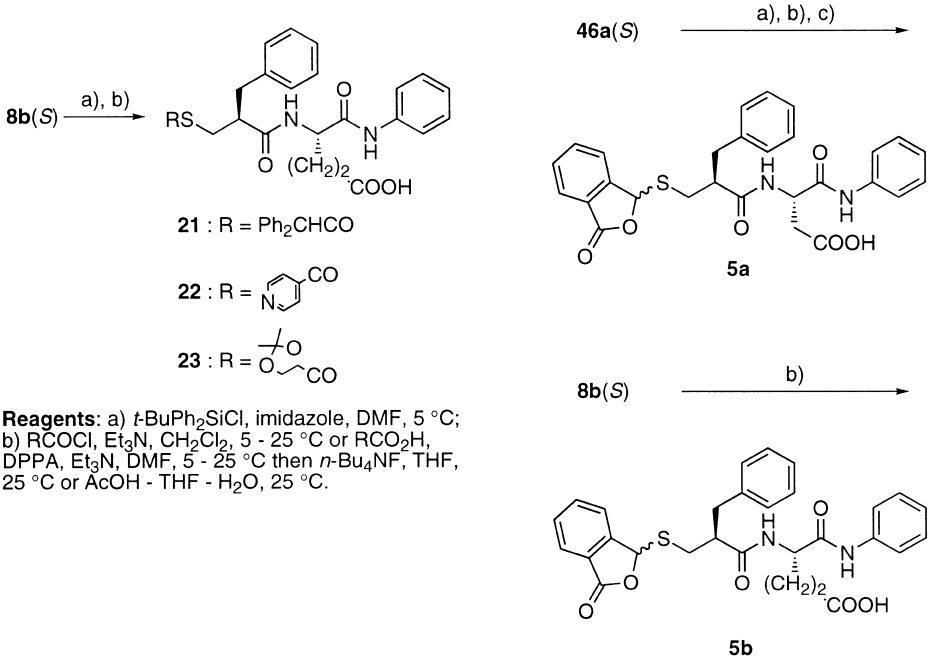


Reagents; a) $(COCl)_2$, CH_2Cl_2, 5 - 25 °C, 1 h; b) **45b** or **45c**, Py, CH_2Cl_2, 5 °C then 25 °C, 15 min; c) LiOH, THF, 25 °C, 1 h.

Scheme 4. Synthesis of **8a**(*S*), **8a**(*R*), **8b**(*S*), **8b**(*R*), **11c**(*S*) and **11c**(*R*).



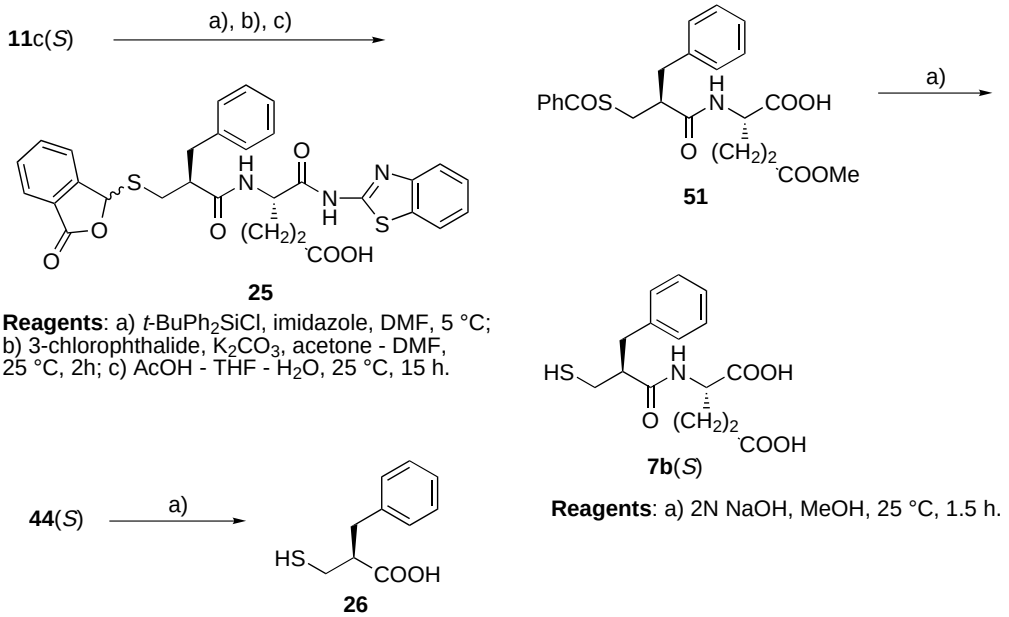
Reangents: a) DCC, $PhNH_2$, CH_2Cl_2, 5 °C, 1 h; b) H_2, 10% Pd - C, AcOEt, 25 °C, 1 h, then HCl (1eq), AcOEt; c) $(COCl)_2$, CH_2Cl_2, 25 °C, 1 h; d) Py, CH_2Cl_2, 5 °C, 1 h;e) K_2CO_3, MeOH, 25 °C, 1h; f) RCOCl, Et_3N, CH_2Cl_2, 25 °C; g) 4N HCl - AcOEt, AcOEt, 25 °C.



Reagents: a) *t*-$BuPh_2SiCl$, imidazole, DMF, 5 °C; b) RCOCl, Et_3N, CH_2Cl_2, 5 - 25 °C or RCO_2H, DPPA, Et_3N, DMF, 5 - 25 °C then *n*-Bu_4NF, THF, 25 °C or AcOH - THF - H_2O, 25 °C.

Reagents: a) K_2CO_3, MeOH - CH_2Cl_2, 25 °C, 1.5 h; b) 3-chlorophthalide, K_2CO_3, acetone - DMF, 25 °C; c) CF_3CO_2H, CH_2Cl_2, 25 °C, 1 h.

Scheme 5. Synthesis of **20**–**24** and **5a** and **5b**.



Scheme 6.

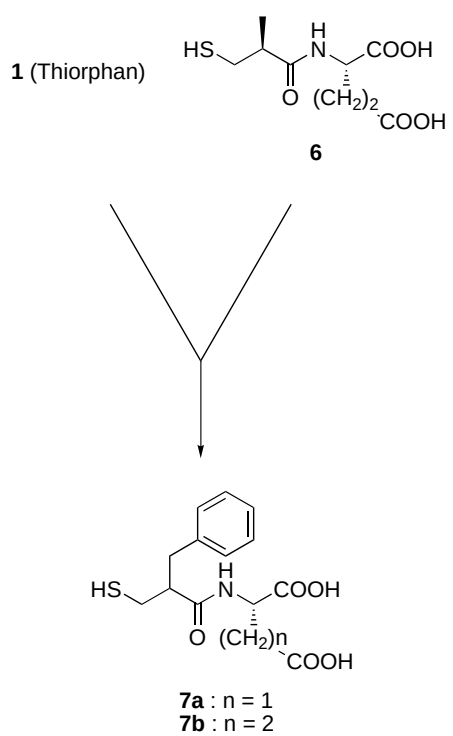


Chart 2. Hybridization of **1** and **6**.

slightly less in vitro potency. However, compound **8b** showed systemic activity after intraperitoneal (ip) administration (Table 3), while **7b** at 10 mg/kg ip did not affect the BK-induced biting-like response of rats (Table 1). The compounds shown in Table 2 were made in order to explore the subsite generalization and to test our hypothesis described in Chart 3(c). The naphthylamide moiety of **9c** was estimated to be the one which is difficult to fit into the S2′ pocket of enkephalinase, based on its lesser in vitro activity compared to the other compounds listed in Table 2. Modifications of the anilide moiety provided **12c**–**17c** with retained in vitro activity (Table 3). The compounds **8**–**17** demonstrated antinociceptive effects on the BK-induced biting-like response after ip administration regardless of their in vitro potency.

Regioisomers **18a**,**b** (Table 4) exhibited less antinociceptive effects in the BK-induced biting-like model described above regardless of their in vitro potency. As a result, the conversion of one carboxylic acid group of **7a**,**b** to an anilide was found to improve the pharmacodynamics of the inhibitors. We had to revise our hypothesis at this stage.

In order to obtain more effective compounds for systemic administration, a further chemical modification of the anilide moiety was made. As shown in Table 3, a

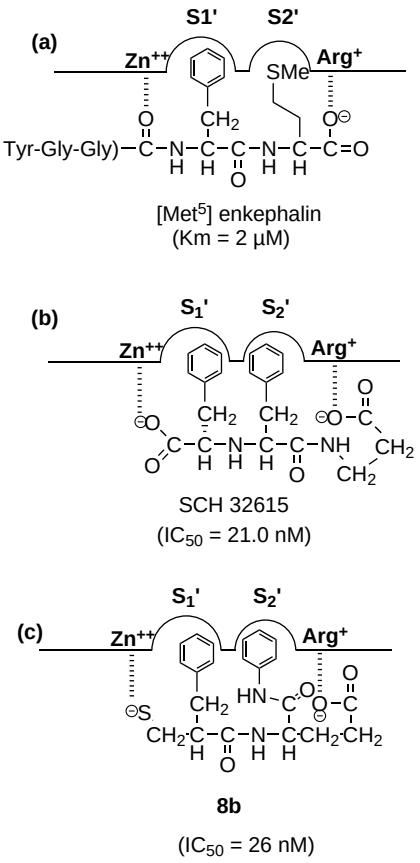


Chart 3. Model of active site of enkephalinase with proposed models of binding of [Met^5]-enkephalin, and the synthetic inhibitors.

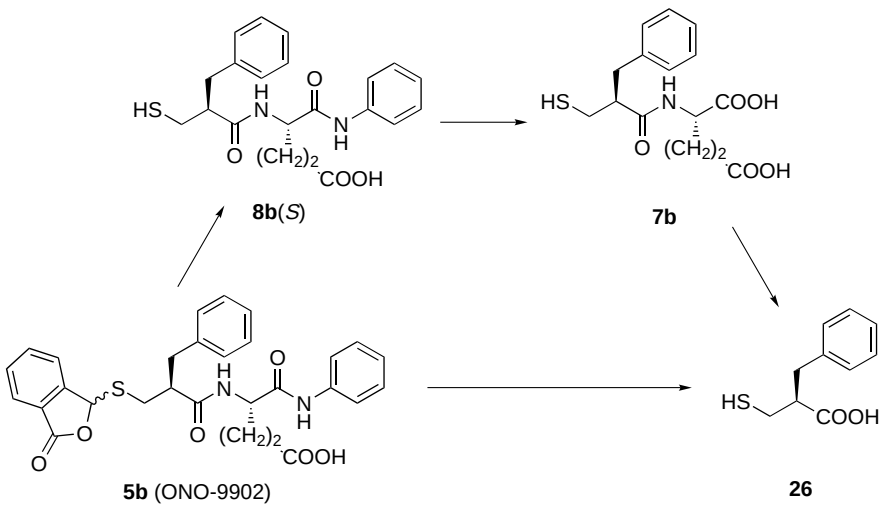
significant increase in the in vitro activity was not obtained with this modification.

Additionally, the in vivo effects of **8**–**17** did not show a close relationship to their in vitro potency. These results strongly suggested that the in vivo efficacy of these compounds is largely dependent upon their metabolic pathway and/or rate (Scheme 7). In addition, the percentage of the parent compound and the active metabolites, and their pharmacodynamic parameters such as the permeability through the blood–brain barrier (BBB) were estimated to give a reasonable explanation of the in vivo efficacy of compounds **8**–**17**.

Among compounds **8**–**17**, **8b** exhibited the most potent antinociceptive effects on the BK-induced biting-like response by ip administration.

In order to further optimize the benzyl moiety, a modification of R of the general formula 4 was carried out. As shown in Table 5, the aromatic moiety such as *p*-methoxyphenyl of **19a** or *p*-methylphenyl group of **19b** was more preferable to the aliphatic moiety such as the cyclohexyl group of **19c**. A phenyl group was found to be the best among the synthesized compounds.

As illustrated in Table 6, a series of the synthetic inhibitors possessing an *S* configuration of the benzyl moiety showed more potent inhibitory activity than their corresponding *R*-isomers. The BK-induced biting-like response was also more potently suppressed by the *S*-isomer **8b**(*S*) than its *R*-isomer **8b**(*R*), based on the comparison of the effects of **8b**(*S*) and **8b**(*R*). None of the synthesized inhibitors were orally active, although they showed an in vivo effect by ip administration.



Scheme 7. Plausible metabolic pathway and metabolites of **5b** (ONO-9902) in rats and dogs after oral administration.

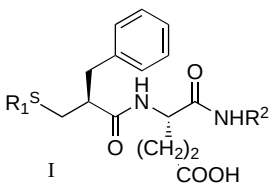
The purpose of this investigation was to identify an orally active enkephalinase inhibitor as a drug candidate. The metabolic instability of the naked thiol group was considered to be one of the most plausible reasons of the oral inactivity encountered. The protection of the thiol group as a prodrug was regarded as one reasonable way to solve this problem.

As described in Tables 7 and 8, several kinds of presumed prodrugs, **5a**,**b** and **20**–**25**, were prepared as the candidates and evaluated in the BK-induced biting test and formalin- induced licking test following oral administration. Compounds **5a**,**b**, **20**, **21**, **23** and **25** exhibited analgesic effects on at least the BK-induced biting-like response. Among the synthesized compounds, **5b** afforded the best result in all the test pain models and was least toxic in the acute toxicity test. A more detailed comparison was then performed to differentiate **5a** and **5b**, because of their structural similarity.

As shown in Table 8, a simultaneous comparison of the synthetic inhibitors **5a** and **5b** by an in vivo evaluation was carried out. In this evaluation, **5b** gave better results than **5a**, especially in acute toxicity. As a result, **5b** (ONO-9902) was selected for further evaluation. It was of interest to investigate the metabolism of **5b** because it possesses metabolically unstable functions such as one ester and two amides in its molecule. The metabolic pathway of **5b** in rat was presumed as described in Scheme 7 based on its metabolites. The metabolism of **5b** was estimated to proceed differently in the rate and/or the pathway dependent upon the species, judging from the different percentage of its metabolites as shown in Table 9.

A main metabolite of **5b** in rat plasma and dog plasma was **26**. The parent compound was not detected in rat plasma, while it was detected in dog plasma as a minor metabolite. Compound **7b** was detected as one of the metabolites in rat plasma. It was also detected in dog

Table 2. Enkephalinase inhibition and the effect on the bradykinin-induced biting-like response of **8a**(*S*),**b** and **9c**–**11c** in male SD rats



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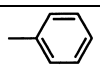
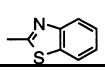
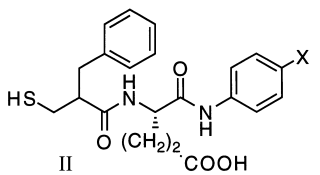
Compound	*n*	R	IC_{50} (nm)	BK-biting[a] (mg/kg ip) (EN/N)[b]
8a(*S*)	1		30	10 (4/8)
8b	2		21	3 (4/8) 10 (7/8)
9c	2		349	10 (2/4)
10c	2		38	10 (1/4)
11c	2		43.8	10 (3/4)

[a]Bradykinin-induced biting-like response.
[b]EN/N: Number of affected rats/number of tested rats.

Table 3. Enkephalinase inhibition and the effect on the bsdykinin-induced biting-like response of **12c**–**17c** in male SD rats

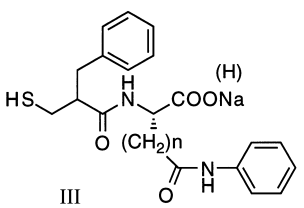


II

Compound	X	IC_{50} (nm)	BK-biting[a] (mg/kg ip) (EN/N)[b]
12c	F	94	10(2/4)
13c	CI	49	10 (3/4)
14c	I	48	10 (5/8)
15c	OMe	31.9	10 (2/4)
16c	COMe	20.8	10 (3/4)
17c	SO_2NH_2	23.3	10 (1/4)

[a]Bradykinin-induced biting-like response.
[b]EN/N: Number of affected rats/number of tested rats.

Table 4. Enkephalinase inhibition and the effect on the bradykinin-induced biting-like response of **18a** and **18b** in male SD rats

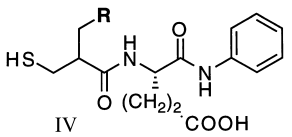


III

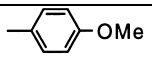
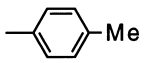
Compound	*n*	IC_{50} (nM)	BK-biting[a] (mg/kg ip) (EN/N)[b]
18a	1	40	10 (1/4)
18b	2	68	10 (3/6)

[a]Bradykinin-induced biting-like response.
[b]EN/N: Number of affected rats/number of tested rats.

Table 5. Enkephalinase inhibition and the effect on the bradykinin-induced biting-like response of **19a–c** in male SD rats



IV

Compound	R	IC_{50} (nM)	BK-biting[a] (mg/kg ip) (EN/N)[b]
19a		51	10 (2/4)
19b		71	10 (2/4)
19c		400	NTC[c]

[a]Bradykinin-induced biting-like response.
[b]EN/N: Number of affected rats/number of tested rats.
[c]NT: Not tested.

plasma. The bioavailability of **5b** after oral administration was 40% in rats with a half-life of about 7 h.

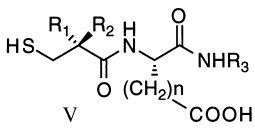
In summary, structurally new enkephalinase inhibitors were discovered based on the chemical modification of an ACE inhibitor, compound **6**. Systemically effective enkephalinase inhibitors were designed based on the reported modes of interaction between the enzyme and the inhibitor, although they showed less in vitro activity than the lead compound **7b**. Thus, these newly found synthetic inhibitors possessing a naked thiol group were not orally active because of their presumed instability (mainly against oxidative metabolism) and/or an oral absorption problem, although they exhibited systemic effects by ip administration. The protection of the thiol group with the known prodrug-like moiety illustrated in Table 7 provided **5b**, **20**, **21** and **23**, which were orally active. Some of these orally active enkephalinase inhibitors elicited potent analgesic effects when tested with experimental nociceptive methods, and could be of significant benefits in clinical application.

Experimental

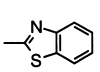
Chemistry

General directions. All ^{1}H-NMR spectra were taken on a JEOL FX- 90Q, Varian VXR-200s, or 500s spectrometer. MS spectra were obtained on a JEOL JMS-DX-303HF. IR spectra were measured on a Perkin Elmer FT-IR 1760X. Melting points were uncorrected. Column chromatography was carried out on silica gel (particle size 0.063–0.02 mm; E. Merck, Darmstadt,

Table 6. Enkephalinase inhibition and the effect on the bradykinin-induced biting-like response of **8a**(*S*), **8a**(*R*), **8b**(*S*), **8b**(*R*), **11**(*S*) and **11**(*R*) in male SD rats



V

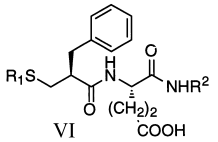
Compound	*n*	R_1	R_2	R_3	IC_{50} (nM)	BK-biting[a] (mg/kg ip) (nM) (EN/N)[b]
8a(*S*)	1	H	Bn		30	10 (4/8)
8a(*R*)	1	Bn	H		47	NT[c]
8b(*S*)	2	H	Bn		23	10(7/8)
8b(*R*)	2	Bn	H		175	10(0/8)
11(*S*)	2	H	Bn		16.8	10 (5/8)
11(*R*)	2	Bn	H		240	NT[c]

[a]Bradykinin-induced biting-like response.
[b]EN/N: Number of affected rats/number of tested rats.
[c]NT: Not tested.

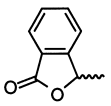
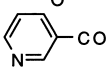
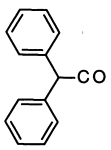
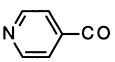
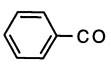
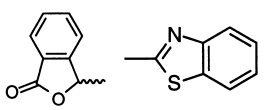
Germany). Thin layer chromatography was performed on silica gel (Merck Art. no. 5715). All solvents were distilled before use. Thiorphan (**1**), retrothiorphan (**2**), **3a–c** and kelatorphan (**4**) were prepared according to the reported methods.[12–14,21]

Preparation of 2-substituted-3-acylthiopropionic acids (42a-c): 2-(4-methoxybenzyl)-3-benzoylthiopropionic acid (42a). To a stirred solution of **41a** (6.49 g, 29.5 mmol), which was prepared from diethyl malonate (10 mL, 0.065 mol), *p*-anisaldehyde (9.8 mL, 0.081 mol) and paraformaldehyde[22] was added potassium hydroxide (3.89 g, 59.0 mmol), and the mixture was refluxed for 1.5 h. After evaporation, the reaction mixture was dissolved in water (30 mL), acidified with concentrated hydrogen chloride (6 mL) and extracted with dichloromethane. The organic layer was washed with distilled water and dried (Na_2SO_4). The resulting product was purified by chromatography on silica-gel (*n*-hexane: ethyl acetate = 5:2) to yield a corresponding carboxylic acid (3.38 g, 59.6%) as a white powder: R_f = 0.42 (*n*-hexane:ethyl acetate = 2:1); ^{1}H-NMR ($CDC1_3$) δ 3.67 (2H, s), 3.80 (3H, s, -OMe), 5.45–5.56 (1H, m, vinylic), 6.36 (1H, brd, J = 1.0 Hz), 6.85 (2H, brd, J = 8.5 Hz), 7.14 (2H, brd, J = 8.5 Hz).

Table 7. Enkephalinase inhibition, analgesic properties and acute toxicity of **5b** and **20–25**



VI

Compound	R^1	R^2	IC_{50} (nM)	Analgesic test (po)		Acute toxicity[f] ip N = 5, 500 mg/kg
				BK test[b] N = 8, % mg/kg	Licking[c] N = 6, 300 mg/kg	
5b		Ph	340	50 (30) 75 (100)	++	(−)
20		Ph	(1600)[a]	37.5 (30) 50 (100)	NE[e]	(+)
21		Ph	1900	37.5 (30) 81.5 (100)	+	(+)
22		Ph	160	0 (100)	NT[d]	NT[d]
23		Ph	135	62.5 (100)	NE[e]	(−)
24		Ph	3300	0 (100)	NT[d]	NT[d]
25			310	0 (30) 62.5 (100)	NE[e]	(−)

[a]These compounds were studied as diastereomeric mixtures of *R*, *S* and *S*, *S* isomers.
[b]BK test: Bradykinin-induced biting-like response. Values are inhibition %.
[c]Licking: Formalin-induced licking test. Significant difference from the control group: ++, $p < 0.01$ at 0–60 min; +, $p < 0.01$ at 0–15 min.
[d]NT: Not tested.
[e]NE: Not effective.
[f]Acute toxicity: (−), no effect; (+), slight loss of body weight.

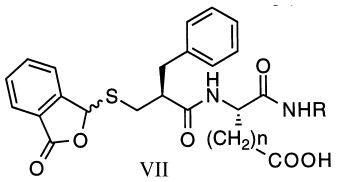
To a stirred solution of the resulting carboxylic.acid (3.37 g, 17.6 mmol) in dichloromethane (30 mL) was added thiobenzoic acid (2.18 mL, 17.6 mmol), and the mixture was refluxed for 15 h. After evaporation, the residue was purified by chromatography on silica-gel (*n*-hexane:ethyl acetate = 4:1 followed by 2:1) to yield **42a** (3.29 g, 56.6%): $R_f = 0.25$ (*n*-hexane:ethyl acetate = 2:1); ^{1}H-NMR ($CDC1_3$) δ 2.92–3.40 (5H, m), 3.79 (3H, s, -OMe), 6.85 (2H, brd, $J = 8.5$ Hz), 7.17 (2H, brd, $J = 8.5$ Hz), 7.32–7.62 (3H, m), 7.96 (2H, brd, $J = 8.5$ Hz).

Compound **42b** was prepared according to the same method as described above (43.0% in 2 steps).

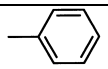
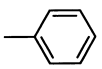
2-(4-Methylbenzyl)-3-benzoylthiopropionic acid (42b). $R_f = 0.28$ (*n*-hexane:ethyl acetate = 2:1); ^{1}H-NMR ($CDC1_3$) δ 2.33 (3H, s, Me), 2.80–3.40 (SH, m), 7.12 (4H, brs), 7.30–7.61 (3H, m), 7.97 (2H, brd, $J = 8.5$ Hz).

2-Cyclohexylmethyl-3-acetylthiopropionic acid (42c). To a stirred solution of carboxylic acid (3.38 g, 20.1 mmol) which was prepared from diethyl malonate,

Table 8. Enkephalinase inhibition, analgesic properties and acute toxicity of **5a**,**b** and **26**



VII

Compound	*n*	R	IC_{50} (nM)	Analgesic test (po)				Acute toxicity[e] (ip) N = 5 500 mg/kg
				BK test[a] N = 8, % (mg/kg)	Licking[b] N = 6, 300 mg/kg	Acetic acid[c] N = 10, % (mg/kg)	SIA[d] N = 5 (mg/kg)	
5a	1		135	50 (30) 75 (100)	+ +	20.1(10) 37.6 (30)	+ + + (30) + + + (100)	(±)
5b	2		340	50 (30) 75 (100)	+ + +	40.7 (10) 49.6 (30)	+ + (30) + + + (100)	(-)

[a]BK test: Bradykinin-induced biting-like response. Values are inhibition %.
[b]Licking: Formalin-induced licking test. Significant difference from the control group: + + +, $p < 0.01$ at 0–60 min; + +, $p > 0.01$ at 0–15 min.
[c]Acetic acid: Acetic acid-induced writhing test. Values are inhibition %.
[d]SIA: Stress-induced analgesia in the hot-plate test. Significant difference from the control group: + + +, $p < 0.001$; + +, $p < 0.01$.
[e]Acute toxicity: (−), no effect; (±), slight loss of body weight.

cyclohexylaldehyde and paraformaldehyde according to the method described above, was added thioacetic acid (2.5 mL, 35.0 mmol), and the mixture was refluxed for 2 h. After evaporation, the residue was purified by chromatography on silica-gel (*n*-hexane:ethyl acetate) to yield **42c** (3.76 g, 76.0%): ^{1}H-NMR ($CDC1_3$) δ 0.64–1.20 (6H, m), 1.30–1.65 (6H, m), 2.08 (lH, brd, $J = 7.5$ Hz), 2.32 (3H, s, -SCOMe), 2.50–2.71 (1H, m), 3.02 (2H, brt, $J = 7.0$ Hz); Mass *m*/*e* 244 (M^+), 201 (M^+–Ac), 168 (M^+–AcSH).

Preparation of optically active carboxylic acid (44): (2*S*)-Benzyl-3- benzoylthiopropionic acid [44(*S*)]. To a stirred solution of *dl*-**44** (2292 g, 7.64 mol) in acetonitrile (27 L) was added (1*R*, 2*S*)–(–)–2-amino-1,2-diphenylethanol (814 g, 3.82 mol), and the mixture was stirred at 25 °C for 2 h. The resulting salt was recrystallized from acetonitrile three times to afford optically active salt (1080 g, 27.6%) as a white solid.

A suspension of the salt (1080 g) in ethyl acetate (25 L) was treated with 0.5 N hydrochloric acid (24 L). The organic layer was washed with water and dried ($MgSO_4$), followed by evaporation of the solvent to give a residue which was crystallized from dichloromethane–*n*-hexane to yield **44**(*S*) (505 g, 79.9%) as a white powder: $R_f = 0.30$ (*n*-hexane:ethyl acetate = 1:1); ^{1}H-NMR ($CDCl_3$) δ 2.90-3.20 (3H, m), 3.20–3.40 (2H, m), 7.15–7.38 (5H, m), 7.38–7.52 (2H, m), 7.52–7.62 (1H, m), 7.90–8.00 (2H, m); Mass *m*/*e* 300 (M^+), 282, 254, 196 (M^+ -AcSH); IR (KBr, cm^{-1}) 3030, 1696, 1597, 1581, 1496, 1448, 1423; $[\alpha]_D$ −40.8° ($c = 1.035$, chloroform).

Table 9. Percentage of the identified metabolites of **5b** in rats and dogs after its oral administration

Compound[a]	Rat (%)	Dog (%)
5b	ND[b]	0.53
8b(*S*)	2.79	11.38
7b	24.00	15.91
26	73.21	72.17

[a]The identified metabolites were **8b**(*S*), **7b** and **26**. The percentage of the each metabolite and **5b** was obtained based on the total AUC of these four compounds as 100%.
[b]ND: Not detected.

(2*R*)-Benzyl-3-benzoylthiopropionic acid [44(*R*)]. 44(*R*) was prepared according to the method described in ref 23: $[\alpha]_D$ + 42.48° ($c = 1.025$, chloroform).

Preparation of (4*S*)-4-(2-substituted-3-mercaptopropionyl-amino)-4-(*N*-arylcarbamoyl)-butyric acid and (3*S*)-3-(2-substituted-3-mercapto-propionylamino)-3-(*N*-arylcarbamoyl)-propionic acid derivatives

(1) General procedure for (4*S*)-4-(2-substituted-3-mercaptopropionyl-amino)-4-(*N*-arylcarbamoyl)-butyric acid derivatives: (4*S*)-4-(2-benzyl-3-mercaptopropionylamino)-4-(*N*-phenylcarbamoyl)-butyric acid (8b). Step 1: To a stirred solution of *t*-butoxycarbonyl-L-glutamic acid

γ-benzyl ester (3.37 g, 10 mmol) in dichloromethane (30 mL) were added pivaloyl chloride (1.35 mL, 11 mmol) and triethylamine (1.4 mL, 10 mmol) at 5 °C, followed by stirring at 25 °C for 30 min. The reaction mixture was treated with aniline (1 mL, 11 mmol) at 5 °C, then stirred at 25 °C for 1 h. After dilution with ethyl acetate, the reaction mixture was washed with 1N hydrochloric acid, 1N sodium hydroxide, brine and dried ($MgSO_4$). After evaporation, the residue was crystallized from *n*-hexane to afford *t*-butoxycarbonyl-L-glutamylanilide γ-benzyl ester (2.9 g, 70.4%) as a white powder: R_f = 0.74 (*n*-hexane:ethyl acetate = 1:1); ^{1}H-NMR ($CDCl_3$) δ 1.42 (9H, s), 1.70–2.40 (2H, m), 2.40–2.80 (2H, m), 4.10–4.40 (1H, m), 5.10(2H, s), 5.35 (1H, brd, J = 7.5 Hz, -NH-), 6.90–7.55 (10H, m), 8.40 (1H, brs, -NHPh); Mass *m*/*e* 412 (M^+), 356, 339, 292.

Step 2: To a stirred solution of *t*-butoxycarbonyl-L-glutamylanilide γ-benzyl ester (2.9 g, 7.04 mmol) in dichloromethane (2 mL) was added trifluoroacetic acid (5.4 mL, 70 mmol) at 5 °C, followed by stirring at 25 °C for 2.5 h. The solvent was evaporated to yield **31** (3.0 g, quantitative yield): R_f = 0.23 (ethyl acetate); ^{1}H-NMR ($CDCl_3$) δ 1.90–2.50 (2H, m), 2.50–2.70 (2H, m), 4.30–4.60 (1H, m), 5.00 (2H, s), 6.80–7.60 (10H, m), 9.50 (1H, brs, -NHPh); Mass *m*/*e* 312 (M^+), 204, 193, 192.

Step 3: 2-benzyl-3-acetylthiopropionic acid (833 mg, 3.5 mmol) was treated with excess oxalyl chloride in dichloromethane (1 mL) at 25 °C for 30 min. After evaporation, the resulting **29** was used for the next reaction without purification. To a stirred mixture of **28** (1.64 g, 3.83 mmol) and pyridine (2.83 mL, 35 mmol) in dichloromethane (16 mL) was added a solution of **29** in dichloromethane (2 mL) at 5 °C, followed by stirring at 25 °C for 15 min. The reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with 1N hydrochloric acid, 1N sodium hydroxide, brine and dried ($MgSO_4$), and then evaporated. The residue was purified by chromatography on silica-gel (eluted with *n*-hexane:dichloromethane = 1:2 followed by methanol:dichloromethane = 1:20) followed by recrystallization from ethyl acetate and *n*-hexane to yield **30** (743 mg, 40.0%): R_f = 0.48 (*n*-hexane:ethyl acetate = 1:1); ^{1}H-NMR ($CDCl_3$) δ 1.82–2.20 (2H, m), 2.22, 2.28 (3H, s), 2.34–2.76 (3H, m), 2.82–3.00, 3.00–3.24 (4H, m), 4.30–4.60 (1H, m), 5.10, 5.12 (2H, s), 6.24–6.44 (1H, m), 7.00–7.34 (15H, m), 8.38, 8.52 (1H, brs, -NHPh); Mass *m*/*e* 532 (M^+), 489, 440, 370.

Step 4: To a stirred solution of **29** (743 mg, 1.4 mmol) in tetrahydrofuran (9 mL)-1,2-dimethoxyethane (1.4 mL) was added lithium hydroxide monohydrate (283 mg, 6.74 mmol) in water (5 mL) under argon at 25 °C, followed by stirring for 30 min. The reaction mixture was treated with 1N hydrochloric acid at 5 °C and extracted with ethyl acetate. The organic layer was washed with brine and dried ($MgSO_4$), and evaporated. The residue was purified by chromatography on silica-gel (chloroform:acetic acid = 99:1) to yield **8b** (387 mg, 69.1%): mp. 158.5–160.0 °C; R_f = 0.48 (chloroform:acetic acid = 95:5); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.54 (1H, t, J = 7.5 Hz), 1.68–2.40 (4H, m), 2.40–3.02 (6H, m), 4.46–4.72 (1H, m), 6.92–7.36 (9H, m), 7.40–7.56 (2H, m), 8.86–9.04 (1H, m); Mass *m*/*e* 400 (M^+), 382, 366, 353, 335; IR (KBr, cm^{-1}) 3275, 1700, 1640, 1600, 1540, 1440, 1300, 1250, 750, 695.

Compounds **9c**–**17c** were prepared from **27b** according to the same method as described above for the preparation of **8b** from **27**. Compounds **19a**–**c** were prepared by the acylation of **28** with **42a**–**c**, respectively. Compounds **8b**(*S*) and **8b**(*R*) were prepared by the acylation of **45b** with **44**(*S*) and **44**(*R*), respectively. Compounds **11c**(*S*) and **11c**(*R*) were prepared by the acylation of **45c** with **44**(*S*) and **44**(*R*), respectively.

(4*S*)-4-(2-Benzyl-3-mercaptopropionylamino)-4-[*N*-(2-naphthyl)-carbamoyl]-butyric acid (9c). R_f = 0.25 (5% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.50–3.60 (10H, m), 4.50–4.88 (1H, m), 6.80–8.30 (13H, m), 9.35, 9.57 (1H, s); Mass *m*/*e* 450 (M^+), 432, 416, 389, 385; IR (KBr, cm^{-1}) 3260, 3025, 1695, 1630, 1525, 1500, 1430, 1395, 1265.

(4*S*)-4-(2-Benzyl-3-mercaptopropionylamino)-4-[*N*-(4-pyridyl)-carbamoyl]-bubric acid (10c). R_f = 0.18 (chloroform:methanol:acetic acid = 30:5:1) ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ 1.64–2.22 (3H, m), 2.35–2.45 (1H, m), 2.50–3.00 (5H, m), 4.35–4.50 (1H, m), 7.00–7.35 (5H, m), 7.55 (2H, brt, J = 6.0 Hz), 8.38 (2H, brt, J = 6.0 Hz); Mass *m*/*e* 401 (M^+), 383, 368, 349, 336, 310; IR (KBr, cm^{-1}) 3250, 1720, 1700, 1630, 1590, 1500.

(4*S*)-4-(2-Benzyl-3-mercaptopropionylamino)-4-[*N*-(2-benzothiazoly)-carbamoyl]-butyric acid (11c). R_f = 0.52 (chloroform:methanol:acetic acid = 30:3:1); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.52–3.20 (10H, m), 4.56–4.85 (1H, m), 6.95–7.50 (8H, m), 7.67–8.06 (3H, m); Mass *m*/*e* 439 (M^+), 405, 392, 367; IR (KBr, cm^{-1}) 3275, 1700, 1640, 1530, 1440, 1305, 1285.

(4*S*)-4-(2-Benzyl-3-mercaptopropionylamino)-4-[*N*-(4-fluorophenyl)-carbamoyl]-bubric acid (12c). R_f = 0.19 (8% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.56, 1.60 (1H, t, J = 8.0 Hz), 1.70–2.30 (3H, m), 2.30–3.04 (6H, m), 4.40–4.64 (1H, m), 6.86–7.02 (2H, m), 7.04–7.32 (5H, m), 7.40–7.64 (3H, m), 9.18, 9.20 (1H, brs); Mass *m*/*e* 418 (M^+), 400, 368, 353, 308; IR (KBr, cm^{-1}) 3275, 1705, 1640, 1540, 1505, 1440, 1405.

(4*S*)-4-(2-Benzyl-3-mercaptopropionylamino)-4-[*N*-(4-chlorophenyl)-carbamoyl]-butyric acid (13c). R_f = 0.28 (8% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.77, 1.82 (1H, t, J = 8.0 Hz), 1.70–2.30 (3H, m), 2.30–3.06 (6H, m), 4.40–4.66 (1H, m), 7.00–7.34 (7H, m), 7.35–7.70 (3H, m), 9.34 (1H, brs); Mass m/e 434 (M^+), 416, 368, 308; IR (KBr, cm^{-1}) 3275, 1705, 1640, 1600, 1530, 1495.

(4*S*)-4-(2-Benzyl-3-mercaptopropionylamino)-4-[*N*-(4-iodophenyl)-carbamoyl]-bubric acid (14c). R_f = 0.34 (8% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.55, 1.60 (1H, t, J = 8.0 Hz), 1.80–2.25 (2H, m), 2.30–3.10 (7H, m), 4.40–4.65 (1H, m), 7.00–7.38 (6H, m), 7.50–7.70 (3H, m), 9.30, 9.32 (1H, s); Mass m/e 526 (M^+), 508, 474, 461, 417, 331; IR (KBr, cm^{-1}) 3300, 1700, 1650, 1601, 1580, 1520, 1480, 1390.

(4*S*)-4-(2-Benzyl-3-mercaptopropionylamino)-4-[*N*-(4-methoxyphenyl)-carbamoyl]-butyric acid (15c). R_f = 0.25 (20% acetic acid in ethyl acetate); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.55, 1.63 (1H, t, J = 8.0 Hz), 1.70–2.30 (3H, m), 2.40–3.10 (6H, m), 3.78, 3.81 (3H, s), 4.40–4.70 (1H, m), 6.80, 6.83 (2H, d, J = 8.0 Hz), 7.00–7.30 (4H, m), 7.30–7.60 (3H, m), 8.95, 9.05 (1H, brs); Mass m/e 430 (M^+), 412,398, 368, 334, 321, 307; IR (KBr, cm^{-1}) 3275, 1700, 1640, 1510, 1440, 1420, 1300, 1240.

(4*S*)-4-(2-Benzyl-3-mercaptopropionylamino)-4-[*N*-(4-acetylphenyl)-carbamoyl]-butyric acid (16c). R_f = 0.34 (10% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.52–3.40 (13H, m), 4.42–4.66 (1H, m), 6.98–7.36 (5H, m), 7.59–7.76 (2H, m), 7.80–8.00 (3H, m), 9.76, 9.82 (1H s); Mass m/e 442 (M^+), 424, 390, 377, 333; IR (KBr, cm^{-1}) 3350, 1710, 1640, 1595, 1525, 1405, 1360, 1320, 1270, 1180, 840, 700.

(4*S*)-4-(2-Benzyl-3-mercaptopropionylamino)-4-[*N*-(4-sulfamoylphenyl)-carbamoyl]-butyric acid (17c). R_f = 0.52 (5% acetic acid in ethyl acetate); ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ 1.11, 1.20 (1H, t, J = 8.0 Hz), 1.60–2.30 (3H, m), 2.30–2.52 (1H, m), 2.54–3.10 (5H, m), 4.40–4.60 (1H, m), 6.96–7.36 (5H, m), 7.58–7.75 (2H, m), 7.75–7.90 (2H, m); Mass m/e 427 (M^+ -H_2O - H_2S), 414, 383, 368, 320, 284; IR (KBr, cm^{-1}) 3300, 1700, 1645, 1595, 1520, 1400, 1330, 1255, 1160.

(4*S*)-4-[2-(4-Methoxybenzyl)-3-mercaptopropionylamino]-4-(*N*-phenyl-carbamoyl]-bubric acid (19a). R_f = 0.40 (10% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$) δ 1.40–2.96 (10H, m), 3.50, 3.77 (3H, s), 4.76–5.00 (1H, m), 6.54–7.15 (10H, m), 9.13, 9.16 (1H, s); Mass m/e 430 (M^+), 412, 337, 310; IR (KBr, cm^{-1}) 3270, 3040, 2920, 1630, 1505, 1440, 1295, 1240, 1 175.

(4*S*)-4-[2-(4-Methylbenzyl)-3-mercaptopropionylamino]-4-(*N*-phenyl-carbamoyl]-bubric acid (19b). R_f = 0.47 (10% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$) δ 1.42–2.98 (13H, m), 4.75–5.01 (1H, m), 6.62–7.56 (10H, m), 9.12, 9.16 (1H, s); Mass m/e 414 (M^+), 467, 349, 321, 294; IR (KBr, cm^{-1}) 3270, 3040, 2925, 1705, 1640, 1600.

(4*S*)-4-(2-Cyclohexylmethyl-3-mercaptopropionylamino)-4-(*N*-phenyl-carbamoyl]-butyric acid (19c). R_f = 0.33 (10% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$) δ 0.70–1.10 (3H, m), 1.10–1.40 (6H, m), 1.40–1.90 (5H, m), 2.00–2.20 (1H, m), 2.20–2.40 (1H, m), 2.40–2.82 (5H, m), 4.80–5.10 (1H, m), 6.90–7.30 (2H, m), 7.30–7.60 (3H, m), 7.55 (2H, brd, J = 8.0 Hz), 9.27 (1H, brs); Mass m/e 406 (M^+), 388, 373, 355, 326, 277, 259, 184; IR (KBr, cm^{-1}) 3600–2400, 1710, 1640, 1540, 1440, 1245.

(4*S*)-4-[(2*S*)-Benzyl-3-mercaptopropionylamino]-4-(*N*-phenylcarbamoyl)-butyric acid [8b(*S*)]. mp 153–155 °C; R_f = 0.39 (chloroform:tetrahydrofuran:acetic acid = 15:4:1); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.60 (1H, t, J = 8.0 Hz), 1.82–2.27 (2H, m), 2.42 (2H, t, J = 8.0 Hz), 2.50–3.54 (6H, m), 4.58 (1H, ddd, J = 4.0, 8.0 Hz), 6.99–7.20 (6H, m), 7.25 (2H, t, J = 8.0 Hz), 7.50 (2H, d, J = 8.0 Hz), 7.76 (1H, d, J = 8.0 Hz), 9.30 (1H, s); Mass m/e 400 (M^+), 382, 353, 335, 307, 291, 280, 274,262;IR (KBr,cm^{-1}) 3275,3050, 1700, 1640, 1530, 1500, 1440; $[\alpha]_D$ −16.11° (c 1.00, methanol); Anal. Calcd. $C_{21}H_{24}N_2O_4S$: C, 62.98; N, 6.04; S, 8.01. Found: C, 62.93; N, 7.02; S, 8.10.

(4*S*)-4-[(2*R*)-Benzyl-3-mercaptopropionylamino]-4-(*N*-phenylcarbamoyl)-bubric acid [8b(*R*)]. mp 164–168 °C; R_f = 0.30 (5% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.58 (1H, t, J = 8.0 Hz), 1.70–1.90 (1H, m), 1.90–2.34 (2H, m), 2.54–3.04 (5H, m), 4.42–4.60 (1H, m), 7.04 (1H, brt, J = 8.0 Hz), 7.10–7.34 (8H, m), 7.40 (1H, t, J = 8.0 Hz), 7.50 (2H, dd, J = 1.0, 8.0 Hz), 9.09 (1H, s); Mass m/e 400 (M^+), 382, 353, 335, 307; IR (KBr, cm^{-1}) 3275, 1700, 1640, 1600, 1530; $[\alpha]_D$ −91.72° (c 1.01, ethanol); Anal. Calcd. $C_{21}H_{24}N_2O_4S$: C, 62.98; N, 6.04; S, 8.01. Found: C, 63.12; N, 7.06; S, 8.17.

(4*S*)-4-[(2*S*)-Benzyl-3-mercaptopropionylamino]-4-[*N*-(2-benzothiazolyl)-carbamoyl]-bubric acid [11(*S*)]. R_f = 0.26 (5% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$) δ 1.56 (1H, t, J = 8.0 Hz), 1.85–3.00 (9H, m), 4.99 (1H, ddd, J = 4.0, 8.0 Hz), 6.64 (1H, d, J = 8.0 Hz), 6.83–7.20 (6H, m), 7.29–7.55 (2H, m), 7.70–7.85 (2H, m); Mass m/e 457 (M^+), 439, 392; IR (KBr, cm^{-1}) 3280, 2910, 2525, 1640, 1600, 1530, 1440, 1300, 1270; $[\alpha]_D$ −5.27° (c 1.12, chloroform).

(4*S*)-4-[(2*R*)-Benzyl-3-mercaptopropionylamino]-4-[*N*-(2-benzothiazolyl)-carbamoyl]-bubric acid [11(*R*)]. R_f = 0.29 (chloroform:tetrahydrofuran:acetic acid = 30:8:1);

^{1}H-NMR ($CDCl_3 + CD_3OD$) δ 1.60–1.90 (1H, m), 2.00–2.30 (4H, m), 2.55–3.00 (5H, m), 4.60–4.70 (1H, m), 7.05–7.50 (7H, m), 7.60–795 (2H, m); Mass *m/e* 457 (M^+), 439, 392, 368, 278; IR (KBr, cm^{-1}) 3250, 1710–1666, 1640, 1600, 1530, 1440; $[\alpha]_D$ −35.6° (*c* 1.00, chloroform)

General procedure for (3*S*)-3-(2-substituted-3-mercapto-propionyl-amino)-3-(*N*-arylcarbamoyl)-propionic acid derivatives

(3*S*)-3-[(2*S*)-Benzyl-3-mercaptopropionylamino]-3-(*N*-phenylcarbamoyl)-propionic acid[8a(*S*)]. Step 1: To a stirred mixture of **45a** (832 mg, 3.2 mmol) and pyridine (0.5 mL, 6.2 mmol) in dichloromethane (10 mL) was added a solution of an acyl chloride which was prepared from **44**(*S*) by the usual method. After being stirred for 30 min, the reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with 1N hydrochloric acid, 1N sodium hydroxide, brine and dried ($MgSO_4$). Removal of the solvent by evaporation afforded a residue which was purified by chromatography on silica-gel (ethyl acetate:dichloromethane = 1:4) to yield **46a**(*S*) (1.45 g, 88.4%) as a white powder: R_f = 0.40 (*n*-hexane:ethyl acetate = 2:1); ^{1}H-NMR ($CDCl_3$) δ 1.40 (9H, s), 2.35–3.20 (4H, m), 3.35 (2H, brd, *J* = 8.0 Hz), 4.70–5.00 (1H, m), 6.83 (1H, brd, *J* = 8.0 Hz), 7.00–7.70 (13H, m), 8.00 (2H, brdd, *J* = 2.0, 8.0 Hz), 8.25 (1H, brs); Mass *m/e* 546 (M^+), 473 (M^+ -*t*-BuO), 454, 398.

Step 2: **46a**(*S*) (1.41 g, 2.58 mmol) was dissolved in methanol (20 mL). To this solution was added potassium carbonate (714 mg, 5.16 mmol), followed by stirring under argon at 25 °C for 30 min. The reaction mixture was acidified with 1N hydrochloric acid and extracted with diethyl ether. The organic layer was washed with brine, dried ($MgSO_4$), and then evaporated to give a residue which was purified by chromatography on silica-gel (ethyl acetate:*n*-hexane = 1:2) to afford thiol (1.04 g, 91.2%) as a white amorphous powder: R_f = 0.50 (*n*-hexane:ethyl acetate = 1:1) ^{1}H-NMR ($CDCl_3$) δ 1.43 (9H, s), 1.50 (1H, t, *J* = 8.0 Hz), 2.50–3.04 (7H, m), 4.78–4.91 (1H, m), 6.86 (1H, brd, *J* = 8.0 Hz), 7.02–7.22 (5H, m), 7.25–7.50 (5H, m), 8.27 (1H, brs); Mass *m/e* 442 (M^+), 386, 369.

Step 3: The resulting thiol (913 mg, 2.07 mmol) was treated with trifluoroacetic acid (3.2 mL, 41.54 mmol) in dichloromethane (5 mL) at 25 °C for 2 h. The reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with distilled water, brine, dried (Na_2SO_4) and evaporated to give a residue which was purified by chromatography on silica-gel (eluted with dichloromethane followed by ethyl acetate:dichloromethane = 1:4) to yield **8a**(*S*) (284 mg, 35.6%) as a white powder: R_f = 0.43 (chloroform:tetrahydrofuran:acetic acid = 15:4:1); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.56 (1H, t, *J* = 7.5 Hz), 2.50–3.00 (7H, m), 3.00–4.00 (1H, m), 4.91 (1H, q, *J* = 7.5 Hz), 7.00–7.20 (6H, m), 7.29 (2H, t, *J* = 7.5 Hz), 7.43 (2H, d, *J* = 7.5 Hz), 7.58 (1H, d, *J* = 7.5 Hz), 8.69 (1H, s); Mass *m/e* 386 (M^+), 368, 335, 321; IR (KBr, cm^{-1}) 3250, 1675, 1640, 1601, 1520, 1435; $[\alpha]_D$ −28.6° (*c* 1.00, methanol).

Compound **8a**(*R*) was prepared from **44**(*R*) according to the same method as described above (19.4% in three steps).

(3*S*)-4-[(2*R*)-Benzyl-3-mercaptopropionylamino]-3-(*N*-phenylcarbamoyl)-propionic acid [8a(*R*)]. R_f = 0.46 (5% acetic acid in chloroform×2); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.75 (1H, brt, *J* = 7.5 Hz), 2.40–3.00 (7H, m), 4.80–5.00 (1H, m), 7.05 (1H, t, *J* = 7.5 Hz), 7.09–7.38 (6H, m), 7.53 (2H, d *J* = 7.5 Hz), 7.86 (1H, d, *J* = 7.5 Hz), 9.05 (1H, s); Mass *m/e* 386 (M^+), 368, 335, 321; IR (KBr, cm^{-1}) 3300, 1700, 1680, 1670, 1645, 1600, 1530, 1440; $[\alpha]_D$ −86.0° (*c* 0.82, methanol).

Preparation of (2*S*)-2-(2-benzyl-3-mercapto-propionyl-amino)-2-(*N*-phenylcarbamoylmethyl)-acetic acid and (2*S*)-2-(2-benzyl-3-mercapto-propionylamino)-2-(*N*-phenylcarbamoylethyl)-acetic acid derivatives

(1)(2*S*)-2-(2-Benzyl-3-mercapto-propionylamino)-2-(*N*-phenylcarbamoyl-methyl)-acetic acid (18a). Step 1: To a stirred solution of **31**(4.00 g, 12.4 mmol) and triethylamine (1.90 mL, 13.6 mmol) in dichloromethane (40 mL) was added pivaloyl chloride (1.68 mL, 13.6 mmol) at 5 °C and stirred at 25 °C for 1 h. The resulting solution was treated with aniline (1.23 mL, 13.6 mmol) and triethylamine (1.90 mL, 13.6 mmol) at 5 °C and then stirred at 25 °C for 1 h. The reaction mixture was diluted with ethyl acetate and washed with 1N hydrochloric acid, brine, dried (Na_2SO_4) and evaporated to give a residue. The residue was triturated with ethyl acetate:*n*-hexane to afford **32** (3.80 g, 77.0%) as a white powder: R_f = 0.43 (*n*-hexane:ethyl acetate = 1:1); ^{1}H-NMR ($CDC1_3$) δ 1.63 (9H, s), 2.64–3.20 (2H, m), 4.40–4.70 (1H, m), 5.16 (2H, s), 5.68 (1H, brd, *J* = 8.0 Hz), 6.90–7.60 (11H, m); Mass *m/e* 398 (M^+), 342, 250; IR (KBr, cm^{-1}) 3290, 1725, 1670, 1595, 1535, 1295.

Step 2: The solution of **32** (2.5 g, 6.27 mmol) in dichloromethane (5 mL) was treated with trifluoroacetic acid (4.83 mL, 62.7 mmol) at 25 °C for 15 h. The reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with 1N sodium hydroxide, brine, dried ($MgSO_4$) and evaporated to give a residue. The residue was purified by chromatography on silica-gel (ethyl acetate followed by 10% methanol in ethyl acetate) to yield **33** (1.74 g, 93.0%): R_f = 0.46 (10% methanol in ethyl acetate);

^{1}H-NMR ($CDCl_3$) δ 2.48–2.95 (2H, m), 3.00 (2H, s), 3.88 (1H, dd, J = 7.5 Hz), 5.02 (2H, s), 6.90–7.60 (10H, m), 9.36 (1H, brs).

Step 3: To a stirred solution of **33** (1.3 g, 4.35 mmol) and pyridine (0.53 mL, 6.53 mmol) in dichloromethane (20 mL) was added a solution of **29** which was prepared from the corresponding acid (1.04 g, 4.35 mmol) and excess oxalyl chloride in dichloromethane (10 mL) at 5 °C, and then stirred at 25 °C for 1 h. The reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with 1N hydrochloric acid, brine, dried ($MgSO_4$). Removal of the solvent by evaporation afforded a residue which was purified by chromatography on silica-gel (dichloromethane:ethyl acetate = 10:1 to 6:1) to yield **34** (1.37 g, 60.7%). R_f = 0.29 (*n*-hexane:ethyl acetate = 1:1); ^{1}H-NMR ($CDCl_3$) δ 2.05, 2.23 (3H, s), 2.28–3.20 (7H, m), 4.62–4.84 (1H, m), 5.12, 5.13 (2H, s), 6.55–6.70 (1H, m), 6.95–7.78 (16H, m); Mass *m*/*e* 518 (M^+), 429, 410; IR (KBr, cm^{-1}) 3280, 1720, 1680, 1650, 1535, 690.

Step 4: **34** (1.31 g, 2.52 mmol) was treated with 2-aminoethanethiol (292 mg, 3.8 mmol) in acetonitrile (20 mL)–dichloromethane (4 mL) at 45 °C for 2 h under argon. The reaction mixture was poured into 1N hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with 1N hydrochloric aid, brine, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica-gel (dichloromethane:ethyl acetate = 6:1) to yield **35** (860 mg, 72.0%): mp. 148–154.5 °C; R_f = 0.24 (ethyl acetate); ^{1}H-NMR ($CDCl_3$) δ 1.36–1.60 (1H, m), 2.37–3.34 (7H, m), 4.74–4.92 (1H, m), 5.10–5.20 (2H, m), 6.65–6.90 (1H, m), 6.96–7.60 (16H, m); Mass *m*/*e* 476 (M^+), 429, 368, 335, 321; IR (KBr, cm^{-1}) 3275, 2950, 1760, 1650, 1595, 1540, 1440, 690.

Step 5: To a stirred solution of **35** (667 mg, 1.40 mmol) in methanol (10 mL)–tetrahydrofuran (3 mL) was added a solution of potassium hydroxide (184 mg, 2.80 mmol) in distilled water (2 mL) at 25 °C. The mixture was stirred for 4 h, and then diluted with ethyl acetate. The organic layer was washed with 1N hydrochloric acid, 1N sodium hydroxide, brine, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica-gel (dichloromethane:tetrahydrofuran:methanol = 60:2:1 followed by 40:2:1) to afford thiol (330 mg, 0.90 mmol). The resulting thiol (330 mg, 0.90 mmol) was treated with 1N sodium hydroxide (0.9 mL) in methanol (2 mL) at 25 °C followed by evaporation to afford **18a** (367 mg, 64.1% in 2 steps): R_f = 0.35 (chloroform:tetrahydrofuran:acetic acid = 15:4:1); ^{1}H-NMR (CD_3OD + $CDCl_3$) δ 2.25–3.30 (7H, m), 4.34–4.58 (1H, m), 6.85–7.56 (10H, m).; Mass *m*/*e* 386 (M^+), 368, 335, 321, 293. (as a carboxylic acid); IR (KBr, cm^{-1}) 3640–2400, 1650, 1630, 1595, 1530, 1380, 1300.

(2)(2*S*)-2-(2-benzyl-3-mercapto-propionylamino)-2-(*N*-phenylcarbamoyl-ethyl)-acetic acid (18b). Step 1: To a stirred solution of **36** (2.00 g, 6.0 mmol) and triethylamine (0.92 mL, 6.6 mmol) in dichloromethane (30 mL) was added pivaloyl chloride (0.74 mL, 6.0 mmol) at 5 °C, followed by stirring at 25 °C for 10 min. The resulting solution was treated with aniline (0.66 mL, 7.2 mmol) and triethylamine (0.92 mL, 6.6 mmol) at 5 °C, followed by stirring at 25 °C for 15 h. The reaction mixture was diluted with ethyl acetate. The organic layer was washed with 1N hydrochloric acid, 1N sodium hydroxide, brine, dried ($MgSO_4$) and evaporated. The residue was triturated with ethyl acetate:*n*-hexane (1:9) to yield **37** (2.07 g, 83.9%) as a white powder: R_f = 0.48 (*n*-hexane:ethyl acetate = 1:1); ^{1}H-NMR ($CDCl_3$) δ 1.42 (9H, s), 1.70–2.60 (4H, m), 4.05–4.40 (1H, m), 5.08 (2H, s), 5.40–5.70 (1H, m), 6.80–7.60 (10H, m), 8.00–8.30 (1H, m); Mass *m*/*e* 356 (M^+-56), 311 (M^+-COOt-Bu).

Step 2: To a solution of **37** (2.14 g, 5.20 mmol) in methanol (20 mL) was added 10% Pd on carbon (214 mg). The mixture was stirred under hydrogen at 25 °C for 50 min. The removal of the catalyst by filtration followed by evaporation afforded **38** (1.37 g, 94.2%) as a colorless oil: R_f = 0.42 (10% methanol in chloroform); ^{1}H-NMR ($CDCl_3$) δ 1.43 (9H, s), 2.40–1.80 (2H, m), 2.40–2.60 (2H, m), 3.40 (1H, dd, J = 4.0, 8.0 Hz), 6.90–7.60 (5H, m), 8.30–8.62 (1H, m).

Step 3: To a cold solution of **38** (285 mg, 1.03 mmol) and pyridine (0.5 mL, 6.18 mmol) in dichloromethane (5 mL) was added a solution of **29**, which was prepared from the corresponding acid (268.4 mg, 1.13 mmol) and excess oxalyl chloride, at 5 °C. The mixture was stirred at 25 °C for 2 h, and then poured into water and extracted with ethyl acetate. The organic layer was washed with 1N hydrochloric acid, 1N sodium hydroxide, brine, dried (Na_2SO_4) and evaporated. The residue was purified by chromatography on silica-gel (ethyl acetate:*n*-hexane = 1:2 followed by 1:1) to yield **39** (302 mg, 59.1%): R_f = 0.42 (*n*-hexane:ethyl acetate = 1:1); ^{1}H-NMR ($CDCl_3$) δ 1.39, 1.42 (9H, s), 1.60–2.48 (4H, m), 2.32, 2.36 (3H, s), 2.58–2.84 (1H, m), 2.84–3.00 (2H, m), 3.04–3.28 (2H, m), 4.22–2.50 (1H, m), 6.12 (1H, brt, J = 8.0 Hz), 6.98–7.40 (8H, m), 7.48–7.80 (2H, m), 8.56, 9.08 (1H, brs); Mass *m*/*e* 498 (M^+), 422, 425, 409, 397.

Step 4: A solution of **39** (301 mg, 0.60 mmol) in dichloromethane (2 mL) was treated with trifluoroacetic acid (1.15 mL, 15 mmol) at 25 °C for 2 h. The reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with distilled water, brine, dried (Na_2SO_4) and evaporated. The residue was triturated with ethyl acetate:*n*-hexane (1:2) to yield **40** (150 mg, 56.6%) as a white powder: R_f = 0.10 (ethyl acetate); Mass *m*/*e* 442 (M^+), 424.

Step 5: Compound **40** (442 mg, 1.0 mmol) was treated with 2-aminoethanethiol (154 mg, 2.0 mmol) in acetonitrile (12 mL) at 50 °C for 30 min under argon. The reaction mixture was poured into 1N hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with 1N hydrochloric acid, brine, dried (Na_2SO_4) and evaporated. The residue was purified by chromatography on silica-gel (ethyl acetate:*n*-hexane = 1:1) to afford thiol (242 mg, 0.60 mmol) which was treated with 1N sodium hydroxide (0.6 mL) in methanol (2 mL) at 25 °C followed by evaporation to give **18b** (235 mg, 55.6% in 2 steps): mp 131–135 °C; R_f = 0.23 (chloroform:tetrahydrofuran:acetic acid = 16:3:1); ^{1}H-NMR (CD_3OD) δ 1.09–1.42 (1H, m), 1.68–2.09 (2H, m), 2.10–2.57 (2H, m), 2.58–3.12 (5H, m), 4.12–4.36 (1H, m), 6.96–7.36 (8H, m), 7.52 (2H, brd, J = 8.0 Hz); Mass *m*/*e* 309 (M^+-NHPh), 237, 213, 160, 145, 117; IR(KBr, cm^{-1}) 3300, 1660, 1640, 1600, 1540, 1500, 1440.

Preparation of 5a, b and 20–25

(a) General Procedure for (4*S*)-4-amino-4-(*N*-arylcarbamoyl)-butyric acid derivatives: Preparation of (4*S*)-4-[(2*S*)-benzyl-3-nicotinoylthio-propionylamino]-4-(*N*-phenylcarbamoyl)-butyric acid (20)

(4*S*)-4-(Benzyloxycarbamoyl)-4-(*N*-phenylcarbamoyl)-butyric acid *t*-butyl ester (48). To a stirred solution of **47** (8.00 g, 23.7 mmol) and aniline (2.27 mL, 24.9 mmol) in dichloromethane (60 mL) was added *N*, *N*-dicyclohexylcarbodiimide (DCC, 5.13 g, 24.9 mmol) at 5 °C. After being stirred for 1 h, the resulting urea was removed by filtration and the filtrate was evaporated. The residue was triturated with ethyl acetate:*n*-hexane(1:9) to yield **48** (8.5 g, 86.9%) as a white powder: R_f = 0.80 (ethyl acetate:*n*-hexane = 1:1); ^{1}H-NMR ($CDCl_3$) δ 1.50 (9H, s), 1.70–2.70 (4H, m), 4.37 (1H, dt, J = 7.5 Hz), 5.16 (2H, s), 5.84 (1H, d, J = 7.5 Hz), 7.04–7.64 (10H, m), 8.53 (1H, brs).; Mass *m*/*e* 412 (M^+), 336, 295.; IR (KBr, cm^{-1}) 3300, 1740, 1700, 1660, 1600, 1520, 1450.

(4*S*)-4-Amino-4-(*N*-phenylcarbamoy)-butyric acid *t*-butyl ester (49). To a solution of **48** (8.50 g, 20.6 mmol) in ethyl acetate–acetic acid (160 mL–0.1 mL) was added 10% Pd on carbon (850 mg) followed by stirring under a hydrogen atmosphere for 1 h. Removal of the catalyst by filtration followed by treatment with 1 equivalent of hydrogen chloride in ethyl acetate afforded **49** as a hydrochloride salt, which was used without further purification in the next step: R_f = 0.20 (ethyl acetate:*n*-hexane = 1:1)

(4*S*)-4-[(2*S*)-Benzyl-3-benzoylthiopropionylamino]-4-(*N*-phenyl-carbamoyl)-bubric acid *t*-butyl ester (50). To a stirred solution of **49** (6.48 g, 20.6 mmol) and pyridine (12 mL, 0.15 mol) in dichloromethane (110 mL) was added a solution of an acid chloride, which was prepared from **44**(*S*) by the usual method, in dichloromethane (8 mL) at 5 °C followed by stirring for 1 h. The reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with 1N hydrochloric acid, 1N sodium hydroxide, brine, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica-gel (ethyl acetate:dichloromethane = 1:10 followed by 1:6) to yield **50** (8.46 g, 73.2% in 2 steps): R_f = 0.80 (*n*-hexane:ethyl acetate = 1:1) ^{1}H-NMR ($CDCl_3$) δ 1.48 (9H, s), 1.73–3.20 (6H, m), 3.35 (2H, d, J = 7.0 Hz), 4.28–4.64 (1H, m), 6.36–6.60 (2H, m), 6.85–8.07 (15H, m), 8.53 (1H, brs); Mass *m*/*e* 560 (M^+), 504, 487, 469, 432, 412, 399, 384; IR (KBr, cm^{-1}) 3300, 1730, 1640, 1600, 1540, 1450.

(4*S*)-4-[(2*S*)-Benzyl-3-mercaptopropionylamino]-4-(*N*-phenylcarbamoyl)-bubric acid *t*-butyl ester (50b). 50a (8.46 g, 15.1 mmole) was dissolved in methanol (180 mL) degassed with argon. To the stirred solution was added potassium carbonate (4.38 g, 31.7 mmole) under argon at 25 °C for 1 h. The reaction mixture was acidified with 1N hydrochloric acid and extracted with diethyl ether. The organic layer was washed with brine, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica-gel (ethyl acetate:dichloromethane = 1:9) to yield **50b** (5.6 g, 81.2%) as a white amorphous powder: R_f = 0.43 (ethyl acetate:dichloromethane = 1:6); ^{1}H-NMR ($CDC1_3$) δ 1.49 (9H, s), 1.78–3.17 (9H, m), 4.51 (1H, dt, J = 7.0 Hz), 6.79 (1H, d, J = 7.0 Hz), 6.89–7.69 (10H, m), 8.62 (1H, brs); Mass *m*/*e* 456 (M^+), 400, 383, 364.

(4*S*)-4-[(2*S*)-Benzyl-3-nicotinoylthiopropionylamino]-4-(*N*-phenyl-carbamoyl)-butyric acid (20). To a stirred solution of **50b** (2.20 g, 4.82 mmol) and triethylamine (2.69 mL, 19.3 mmol) in dichloromethane (20 mL) was added nicotinoyl chloride hydrochloride (0.994 g, 5.30 mmol) at 5 °C followed by stirring at 25 °C for 3 h. The reaction mixture was diluted with ethyl acetate and washed with 1N sodium hydroxide, and then brine. The organic layer was dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica-gel (ethyl acetate:dichloromethane = 1:6 followed by 1:4) to afford 2.20 g (3.92 mmol, 81.3%) of (4*S*)-4-[(2*S*)-benzyl-3-nicotinoylthiopropionylamino]-4-(*N*-phenyl-carbamoyl)-butyric acid *t*-butyl ester as a white powder: R_f = 0.24 (ethyl acetate:*n*-hexane = 1:1); ^{1}H-NMR ($CDC1_3$) δ 1.49 (9H, s), 1.80–3.10 (7H, m), 3.26–3.42 (2H, m), 4.46 (1H, dt, J = 7.5 Hz), 6.80 (1H, d, J = 7.5 Hz), 7.00–7.20 (5H, m), 7.11–7.50 (5H, m), 8.19 (1H, ddd, J = 1.5, 5.0, 8.0 Hz), 8.61 (1H, brs), 8.77 (1H, dd, J = 2.0, 5.0 Hz), 9.15 (1H, d, J = 2.0 Hz).

The resulting solution of *t*-butyl ester (2.20 g, 3.92 mmol) in ethyl acetate–dichloromethane (20 mL–10 mL) was

treated with 4N hydrogen chloride in ethyl acetate (20 mL) at 25 °C and stirred for 2 h. The resulting precipitate was collected by filtration and washed with ethyl acetate to yield **20** (1.96 g, 75.0% in 2 steps) as a white powder: R_f = 0.35 (10% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.82–3.45 (9H, m), 4.58 (1H, dt, J = 7.5 Hz), 7.00–7.36 (8H, m), 7.53 (2H, d, J = 7.0 Hz), 7.98 (1H, dd, J = 5.0, 8.0 Hz), 8.19 (1H, d, J = 9.0 Hz), 8.65 (1H, d, J = 8.0 Hz), 8.97 (1H, dd, J = 2.0, 5.0 Hz), 9.19 (1H, d, J = 2.0 Hz), 9.50 (1H, s); Mass m/e 505 (M^+), 487; IR (KBr, cm^{-1}) 3270, 3040, 1720, 1665, 1640, 1595, 1520, 1440, 1210, 930, 810, 750, 695, 680; $[\alpha]_D$ −50.6° (c = 1.00, methanol).

Compounds **24** was prepared from **50b** according to the same method as described above.

(4*S*)-4-[(2*S*)-Benzyl-3-benzoylthiopropionylamino]-4-(*N*-phenyl-carbamoyl)-butyric acid (24). R_f = 0.29 (5% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.80 (4H, m), 2.79–3.38 (5H, m), 4.47–4.63 (1H, m), 6.99–7.33 (8H, m), 7.40–7.65 (5H, m), 7.85–8.00 (3H, m), 9.28 (1H, s); Mass m/e 504 (M^+), 486, 412, 395, 384; IR (KBr, cm^{-1}) 3270, 3050, 1705, 1655, 1640, 1600, 1530, 1490, 1445, 1205, 910, 750, 685; $[\alpha]_D$ −77.9° (c = 0.96, methanol).

Preparation of 21–23 from 8b(*S*)

Direct preparation of (4*S*)-4-[(2*S*)-benzyl-3-[(1*RS*)-1,3-dihydro-3-isobenzofuran-1-ylthio]-propionylamino]-4-(*N*-phenylcarbamoyl)-butyric acid (5b) from 8b(*S*). To a stirred solution of **8b**(*S*) (221.4 g, 0.55 mol) and 3-chlorophthalide (140 g, 0.83 mol) in acetone (1.2 L)-*N*, *N*-dimethylformamide (DMF, 600 mL) was added potassium carbonate (152.8 g, 1.11 mol) at 5 °C followed by stirring at 25 °C for 1.5 h. The reaction mixture was poured into ice-water and extracted with ethyl acetate. The organic layer was washed with diluted water, brine, dried ($MgSO_4$) and evaporated. The residue was recrystallized from acetone-*n*-hexane (1:4) to yield **5b** (234 g, 79.3%) as a white amorphous powder: mp 180–183 °C; R_f = 0.45 (5% methanol in ethyl acetate); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) 1.85–2.30 (2H, m), 2.35–2.58 (2H, m), 2.65–3.11 (5H, m), 4.50–4.70 (1H, m), 6.68, 6.78 (1H, s, 1:1.6), 7.00–7.38 (8H, m), 7.48–7.65 (4H, m), 7.65–7.80 (2H, m), 9.22–9.37 (1H, m); Mass m/e 532 (M^+), 514, 496, 440, 412.; IR (KBr, cm^{-1}) 3250, 1750, 1640, 1600, 1525, 1440; Anal. Calcd. $C_{29}H_{28}N_2O_6S$: C, 65.40; N, 5.26; S, 6.02. Found: C, 64.92; N, 5.32; S, 6.07.

General procedure for the preparation of 21–23 via silyl ester of 8b(*S*)(4*S*)-4-[(2*S*)-benzyl-3-diphenylacetylthio-propionylamino]-4-(*N*-phenyl-carbamoyl)-butyric acid (21). A solution of **8b**(*S*) (4.30 g, 10.7 mmol) and imidazole (1.61 g, 23.6 mmol) in DMF (30 mL) was treated with *t*-butylchlorodiphenylsilane (3.13 mL, 11.8 mmol) at 5 °C followed by stirring at 25 °C for 4 h. The reaction mixture was diluted with ethyl acetate, washed with 1N sodium hydroxide and then brine. The organic layer was dried ($MgSO_4$) and evaporated. To a stirred solution of the residue in dichloromethane (30 mL) was added triethylamine (3.9 mL, 28 mmol) and then diphenylacetyl chloride, which was prepared from the corresponding acid (2.97 g, 14.0 mmol) and an excess amount of oxalyl chloride (10 mL). The reaction mixture was diluted with diethyl ether and washed with water and then brine. The organic layer was dried ($MgSO_4$) and evaporated. The resulting silyl ester was treated with acetic acid:tetrahydrofuran: water (3:1:1) at 25 °C for 15 h and evaporated. The residue was triturated with *n*-hexane-diethyl ether to yield **21** (3.20 g, 50.3%) as a white powder: R_f = 0.40 (10% methanol in chloroform); ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ 1.60–2.35 (4H, m), 2.65–3.25 (5H, m) 4.45–4.70 (1H, m), 5.19 (1H, s), 6.92–7.51 (20H, m); Mass m/e 594 (M^+), 557, 548, 502; IR (KBr, cm^{-1}) 3270, 1690, 1640, 1600, 1535, 1490, 1440; $[\alpha]_D$ −52.9° (c = 1.035, chloroform).

(4*S*)-4-[(2*S*)-Benzyl-3-(2,2-dimethyl- 1,3-dioxolane-4*S*-carbonyl)thio-propionylamino]-4-(*N*-phenyl-carbamoyl)-butyric acid (23). To a solution of diphenylphosphoryl azide (DPPA, 1.80 mL, 8.00 mmol) and silyl ester of **8b**(*S*) (5.117 g, 8.00 mmol) ,which was prepared according to the method described above, in DMF (8 mL) was added triethylamine (1.12 mL, 8.00 mmol) at 5 °C followed by stirring at 25 °C for 24 h. The reaction mixture was diluted with ethyl acetate–diethyl ether (2:1), washed with distilled water and then brine. The organic layer was dried ($MgSO_4$) and evaporated. The residue was dissolved in tetrahydrofuran (10 mL) and treated with tetra-*n*-butylammonium fluoride (1 M in tetrahydrofuran, 2.32 mL, 2.32 mmol) at 5 °C for 5 min. The reaction mixture was diluted with ethyl acetate, washed with water and then brine. The organic layer was dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica-gel (eluted with ethyl acetate followed by 10% methanol in ethyl acetate) to yield **23** (1.50 g, 35.0%) as a white powder: R_f = 0.25 (ethylacetate); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) 1.35 (3H, s), 1.50 (3H, s), 1.80–3.50 (9H, m), 4.01 (1H, dd, J = 5.0, 9.0 Hz), 4.18 (1H, dd, J = 7.0, 9.0 Hz), 4.50–4.70 (2H, m), 6.95–7.30 (8H, m), 7.58 (2H, d, J = 7.0 Hz), 8.18 (1H, d, J = 8.0 Hz), 9.78 (1H, brs); Mass m/e 510 ($M^+ - H_2O$), 495, 424; IR (KBr, cm^{-1}) 3280, 2990, 1640, 1590, 1535, 1440, 1380, 1310, 1250, 1220; $[\alpha]_D$ −58.8° (c = 1.085, chloroform).

Compound **22** was prepared from **8b**(*S*) according to the same procedure as described in the preparation of **21** or **23**.

(4*S*)-4-[(2*S*)-Benzyl-3-isonicotinoylthio-propionylamino]-4-(*N*-phenyl-carbamoyl)-bubric acid (22). $R_f = 0.36$ (chloroform:tetrahydrofuran:acetic acid = 15:4:1); ^{1}H-NMR ($CDCl_3 + CD_3OD$) 1.75–2.00, 2.00–2.25 (2H, m), 2.38 (2H, brt, $J = 7.5$ Hz), 2.75–3.13 (3H, m), 3.20–3.50 (2H, m), 4.43–4.60 (1H, m), 7.00–7.21 (6H, m), 7.31 (2H, brt, $J = 8.0$ Hz), 7.40–7.50 (2H, m), 7.80 (2H. dd, $J = 2.5$, 5.0 Hz), 8.71 (2H, dd, $J = 2.5$, 5.0 Hz); Mass *m*/*e* 505 (M^+), 487, 413, 385; IR (KBr, cm^{-1}) 3275, 1700, 1670, 1640, 1600, 1530, 1500, 1440, 1410; $[\alpha]_D$ −77.5° (*c* = 1.00, methanol).

(4*S*)-4-[(2*S*)-Benzyl-3-[(1*RS*)-1,3-dihydro-3-isobenzofuran-1-ylthio]-propionylamino]-4-[*N*-(2-benzothiazoly)carbamoyl] -butyric acid (25). Compound **26** was prepared from **11**(*S*) according to the same procedure as described in the preparation of **21** or **23**. $R_f = 0.25$ (2% acetic acid in chloroform); ^{1}H-NMR ($CDCl_3 + CD_3OD$) δ 1.90–2.30 (2H, m), 2.30–2.60 (2H, m), 2.65–3.05 (5H, m), 4.60–4.72 (1H, m), 6.75 (1H, s), 6.95–7.18 (5H, m), 7.27–7.68 (4H. m), 7.75–7.90 (4H, m); Mass *m*/*e* 571 ($M^+ - H_2O$), 454, 438, 421, 405; IR (KBr, cm^{-1}) 3300, 1760, 1640, 1600, 1540, 1440.

(b) General procedure for (3*S*)-3-amino-3-(*N*-arylcarbamoyl)-propionic acid derivative: Preparation of (3*S*)-3-[(2*S*)-benzyl-3-[(1*RS*)-1,3-dihydro-3-isobenzofuran-1-ylthio]propionylamino]-3-(*N*-phenylcarbamoyl)-propionic acid (5a)

(3*S*)-3-[(2*S*)-Benzyl-3-[(1*RS*)-1,3-dihydro-3-isobenzofuran-1-ylthio]-propionylamino]-3-(*N*-phenylcarbamoyl)-propionic acid (5a). 46a(*S*) (8.95 g, 16.8 mmol), which was prepared according to the general procedure for the preparation of (3*S*)-3-(2-substituted-3-mercaptopropionyl-amino)-3-(*N*-arylcarbamoyl)-propionic acid derivatives, was dissolved in methanol (80 mL)–dichloromethane (20 mL). To the solution was added potassium carbonate (4.52 g, 32.7 mmol) under argon at 25 °C for 1.5 h. The reaction mixture was acidified with 1N hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with brine, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica-gel (ethyl acetate:dichloromethane = 1:15) to afford thiol (8.95 g, 12.0 mmol). To a solution of the resulting thiol (5.20 g, 11.8 mmol) and 3-chlorophthalide (3.28 g, 17.6 mmol) in acetone (40 mL) was added potassium carbonate (2.65 g, 19.4 mmol) at 25 °C followed by stirring for 5 h. The reaction mixture was diluted with ethyl acetate–diethyl ether (1:1) and washed with water, then brine. The organic layer was dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica-gel (ethyl acetate:*n*-hexane = 1:2 followed by 2:3) to afford 6.36 g (11.1 mmol) of *S*-alkylated product as a white amorphous powder: $R_f = 0.17$ (ethyl acetate:*n*-hexane = 1:2); ^{1}H-NMR ($CDCl_3$) δ 1.40, 1 .45 (9H, s), 2.55–3.15 (7H, m), 4.77–4.91 (1H, m), 6.55 (1H, s), 6.92–7.44 (11H, m), 7.51–7.78 (3H, m), 7.88, 7.90 (1H, d, $J = 8.0$ Hz), 8.30, 8.35 (1H, s); Mass *m*/*e* 574 (M^+), 518, 501, 482.

A solution of *S*-alkylated product (6.35 g, 11 mmol) in dichloromethane (20 mL) was treated with trifluoroacetic acid (25 mL, 0.32 mol) at 25 °C for 1 h. The reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with 1N sodium hydroxide, brine, dried (Na_2SO_4) and evaporated. The residue was triturated with diethyl ether:*n*-hexane to yield **5a** (4.89 g, 58.8% in 3 steps) as a white powder: $R_f = 0.51$ (chloroform:tetrahydrofuran:acetic acid = 15:4:1); ^{1}H-NMR ($CDCl_3$) δ 2.50–3.10 (7H, m), 4.85–5.06 (1H, m), 6.50, 6.53 (1H, s), 6.95–7.88 (15H, m), 9.46, 9.60 (1H, s); Mass *m*/*e* 500 ($M^+ - H_2O$), 482, 407, 367; IR (KBr, cm^{-1}) 3280, 3030, 1760, 1640, 1600, 1525, 1440, 1285, 1170, 940, 760, 725, 695.

Preparation of metabolites 7b(*S*) and 26(4*S*)-4-[(2*S*)-benzyl-3-mercaptopropionyl]-4-carboxybutyric acid (7b). To a stirred solution of cyclohexylamine salt of **51**[24] (5.42 g, 10 mmol) in methanol (25 mL) was added 2N sodium hydroxide (16.5 mL, 33 mmol). After being stirred at 25 °C for 1.5 h under argon, the reaction mixture was poured into cold 0.5N hydrochloric acid (100 mL, 50 mmol) and extracted with ethyl acetate. The organic layer was washed with water, brine, dried ($MgSO_4$) and evaporated. The residue was recrystallized from ethyl acetate–*n*-hexane to yield **7b**(*S*) (2.6 g, 80.0%) as a white powder: $R_f = 0.50$ (chloroform:tetrahydrofuran:acetic acid = 20:2:1); ^{1}H-NMR ($CDCl_3$ + DMSO-d_6) δ 1.60–1.75 (1H, m), 1.78–2.23 (2H, m), 2.25–2.45 (4H, m), 2.63–2.85 (2H, m), 2.80–4.00(2H, m, OH), 2.90–3.15 (1H, m), 4.37–4.50 (1H, m), 7.12–7.32 (5H, m), 7.88 (1H, d, $J = 8.5$ Hz); Mass *m*/*e* 325 (M^+), 292, 278, 260, 234; $[\alpha]_D$ −31.5° (*c* = 0.93, methanol).

(2*S*)-Benzyl-3-mercaptopropionic acid (26). To a stirred solution of **44**(*S*) (6.0 g 20 mmol) in methanol (50 mL) was added potassium carbonate (5.52 g, 40 mmol). After being stirred under argon at 25 °C for 2 h, the reaction mixture was acidified with 1N hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with brine, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica-gel (*n*-hexane:dichloromethane = 1:1 followed by dichloromethane) to yield **26** (3.6 g, 92.0%): $R_f = 0.68$ (chloroform:tetrahydrofuran:acetic acid = 20:2:1); ^{1}H-NMR ($CDCl_3$) δ 1.55 (1H, t, $J = 10$ Hz), 2.58–3.23 (5H, m), 7.03–7.21 (5H, m); Mass *m*/*e* 196 (M^+), 162, 117.

Biological evaluation. Assay for enkephalinase inhibitory activity. Mouse striatal membranes were used as the enzyme source. Male ddY mice (20–25 g) were sacrificed by decapitation; striata were rapidly dissected out on ice

and homogenized by sonication in 10 mL of ice-cold 50 mM Tris-HCl buffer, pH 7.4. The homogenate was centrifuged immediately for 1 min at 200,000 *g*. The resulting pellet was sonicated in the same volume of cold buffer and used as an enzyme source. Aliquots of the suspension (50 mL) were preincubated for 5 min at 37 °C with 30 μL of 100 μM bestatin, 100 μM puromycin and 1 μM captopril with or without the inhibitor. Incubation for 180 min at 37 °C was started by adding 300 μM Leu^5-enkephalin and 10 nM ^{3}[H]Leu^5-enkephalin (1472.6 Gbq/mmol) and terminated by the addition of 0.2 M HCl. Aliquots of the incubation medium were spotted on TLC silica gel sheets, and the metabolites Tyr (aminopeptidase metabolite), Tyr-Gly (dipeptidyl aminopeptidase metabolite), and Tyr-Gly-Gly (enkephalinase metabolite) were separated according to their R_f values.[18] Their radioactivity was estimated by a liquid scintillation counter.

Pharmacological evaluation. Antinociceptive test

1. **Bradykinin (BK)-induced biting-like response**: Male Sprague–Dawley rats weighing 200–250 g were used after overnight starvation. Implantation of the bradykinin-fed cannula onto the tooth pulp and the fixation of it on the lower incisor surfaces were carried out under diethyl ether anesthesia according to the previous report[19]. At least 2 h were allowed for recovery from anesthesia. Microapplications of BK (0.5–1 μL, 10^{-7} M) onto the tooth pulp of the right mandibular incisor of the animals produced biting-like responses, and these responses were taken as the measure for assaying the analgesic potencies of compounds. Only rats that showed two serial biting-like responses with a latency less than 1 min and a duration of more than 20 min were used for the analgesic assays. BK was applied at 30 min intervals. When the biting duration was shortened to 4 min or less after drug administration, the effect of the compound was considered analgesic.
2. **Formalin-induced licking test**: Male Sprague–Dawley rats weighing 200–300 g were used after overnight starvation. A rat was placed in a metal cage and left there for 10 min or more. A group of three animals was used for each treatment. For noxious stimulation, the rat was injected with 5 μL of diluted formalin (5% formaldehyde solution) into the plantar region of the left hindpaw, and the duration of the licking response was measured by stopwatch from 10 min to 60 min after the injection. Compounds were administered per os (po) 30 min prior to the injection of formalin. Saline (1 mL/kg) was given as a vehicle. A mirror was placed on the opposite side of the enclosure for the unhindered observation of the paw of the rat treated with formalin. No restraint was applied to the rat during the behavioral observations.
3. **Acetic acid-induced writhing test**: Male ddY mice weighing 20–23 g were used after overnight starvation. Compounds and vehicle were administered po at a 20 mL/kg dose 30 min prior to the ip injection of 0.6% acetic acid (AcOH) at 10 mL/kg to conscious animals. Mice were then placed into perspex observation cages, and the number of writhings was counted from 5 min to 20 min after the ip injection of AcOH.
4. **Stress-induced analgesia (SIA) in the hot-plate test**: Male ICR mice weighing 20–23 g were used after overnight starvation. As inescapable stress (a 2 mA electric footshock) was delivered in 60×16 msec pulses once every 2 s for 3 min to mice. After 1, 5, and 10 min of the stress exposure, jump latencies on a 55 °C hot-plate test were measured. The vehicle or compound was administered 60 min prior to the stress exposure. The potentiation effects of the compound/vehicle on SIA in the mice were judged by determining the extension seen in the jump latencies before and 10 min after the stress. Student's *t*-test was used for statistical analysis.

Pharmacokinetic study

The bioavailability of **5b** in rats was obtained from the area under the plasma concentration–time curves ($AUC_{0-\infty}$), which were determined by high-performance liquid chromatography (HPLC), after oral (10 mg/kg) and intravenous (iv) (1 mg/kg) administrations of **5b**. The half-life of **5b** in rats was obtained from the plasma concentration-time curve after the oral (10 mg/kg) administration of **5b**.

The percentages of metabolites of **5b** were obtained from their AUC_{0-48} values after the oral (100 mg/kg) administration of **5b** to fasted male rats and beagle dogs.

The metabolites of **5b** were detected as their derivatives by reduction with tri-*n*-butylphosphine followed by treatment with 4-fluoro-7-sulfamoylbenzofurazan (ABD-F).

(1) Evaluation of the metabolism of 5b in rats. Four male Sprague–Dawley rats weighing 210–260 g (8 weeks old, Charles River Japan, Atsugi, Japan) were fasted for 20 h prior to and for 12 h after the drug administration, but were allowed free access to water. **5b** suspended in 0.5% carboxymethyl cellulose (CMC) in water was administered orally at a dose of 100 mg/5 mL/kg. Blood samples were taken from the jugular vein with heparinized syringes. Then, 0.1 M potassium borate buffer

containing 2M EDTA (0.5 mL, pH8.2), **11c**(*S*) as an internal standard (1 μg/mL, 0.05 mL) and 2% tri-*n*-butylphosphine in DMF (10 μL) were added to the plasma (0.2 mL) prepared from the above-mentioned blood. After incubation at 37 °C for 10 min, 0.1 M citric acid (0.1 mL) was added to the samples in an ice bath. The resulting samples were charged to Bond Elut C18 and eluted with CH_3CN (1 mL). After treatment with 0.1 M potassium borate buffer containing 2M EDTA (0.5 mL, pH8.2) and ABD-F (0.1 mL, 1 mg/mL in 0.1 M pH8.2 potassium borate buffer) at 37 °C for 45 min, 0.1 M citric acid (0.5 mL) was added in the ice bath. Samples were extracted with ethyl acetate (2 mL) twice and evaporated. The residue was dissolved in the mobile phase (0.24 mL), and 80 μL of this solution was loaded on an HPLC column.

(2) Evaluation of the metabolism of 5b in beagle dogs. Three male beagle dogs weighing 13 to 15 kg were fasted for 20 h prior to and for 12 h after the drug administration, but were allowed free access to water. **5b** charged in 1/2 oz capsule was administered orally at a dose of 100 mg/kg. Blood samples were taken from the cephalic vein with heparinized syringes. Potassium borate buffer (0.1 M) containing 2M EDTA (1 mL, pH8.2), **11c**(*S*) as an internal standard (1 μg/mL, 0.5 mL) and 2% tri-*n*-butylphosphine in DMF (10 μL) were added to the plasma (0.2 mL) prepared from the aforementioned blood. After incubation at 37 °C for 10 min, 0.1 M citric acid (0.1 mL) was added to the samples cooling with an ice bath. The resulting samples were charged to Bond Elut C18 and eluted with CH_3CN (1 mL). After treatment with 0.1 M potassium borate buffer containing 2M EDTA (0.5 mL, pH8.2) and ABD-F (0.1 mL, 1 mg/mL in 0.1 M pH8.2 potassium borate buffer) at 37 °C for 45 min, 0.1 M citric acid (0.5 mL) was added cooling with the ice bath. Samples were extracted with ethyl acetate (2 mL) twice and evaporated. The residue was dissolved in the mobile phase (0.24 mL), and 80 μL of this solution was loaded on an HPLC column.

The HPLC was carried out using a YMC A-302 ODS (150×4.6 mm i.d.) column, and the HPLC analysis of metabolites **8b**(*S*), **7b**, **26** and **5b** was carried out under the following conditions:

Metabolites **8b**(*S*), **7b** and **26**:
Eluent A: 0.02 M KH_2PO_4 (adjusted to pH2.1 with H_3PO_4)/CH_3CN = 70/30 (v/v)
Eluent B: 0.02 M KH_2PO_4 (adjusted to pH2.1 with H_3PO_4)/CH_3CN = 30/60 (v/v)
Linear gradient (Eluent A/Eluent B = 100/0 at 0 min followed by Eluent A/Eluent B = 34/66 at 30 min)
Flow rate: 0.8 mL/min. Detection: Ex = 380 nm, Em = 510 nm.

5b:
Eluent A: 0.02 M KH_2PO_4 (adjusted to pH2.1 with H_3PO_4)/CH_3CN = 70/30 (v/v)
Eluent B: 0.02 M KH_2PO_4 (adjusted to pH2.1 with H_3PO_4)/CH_3CN = 30/60 (v/v)
Linear gradient (Eluent A/Eluent B = 100/0 at 0 min followed by Eluent A/Eluent B = 34/66 at 30 min)
Flow rate: 0.8 mL/min. Detection: 236 nm.

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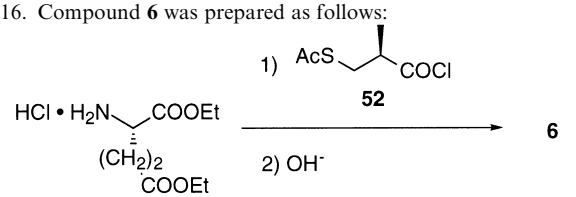
16. Compound **6** was prepared as follows:



6: R_f = 0.13 (chloroform:tetrahydrofuran:acetic acid = 10:2:1); ^{1}H-NMR ($CDCl_3$ + acetone-d_6) δ 1.21 (3H, d, J = 7.5 Hz), 1.63 (1H, t, J = 7.5 Hz), 1.80–3.00 (7H, m), 4.48–4.80 (1H, m), 7.10–

7.70 (3H, m); Mass *m/e* 249 (M^+), 216, 205; IR (KBr, cm^{-1}) 3600–3200, 3200–2300, 1700, 1640, 1520, 1240; $[\alpha]_D$ −39.2° (*c* 1.01, methanol); mp. 102–107 °C. Preparation of **52**: (a) Imaki, K.; Sakuyama, S.; Okada, T.; Toda, M.; Hayashi, M.; Miyamoto, T.; Kawasaki, A.; Okegawa, T. *Chem. Pharm. Bull.* **1981**, *29*, 2210. b) Suh, J. T.; Skiles, J. W.; Williams, B. E.; Youssefyeh, R. D.; Jones, H.; Loev, B.; Neiss, E. S.; Schwab, A.; Mann, W. S.; Khandwala, A.; Wolf, P. S.; Weinryb, I. *J. Med. Chem.* **1985**, *28*, 57.

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