material. A table of observed and calculated structure factors was submitted to the referees and may be obtained from J.V.S.

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Registry No. (±)-**2d**, 100448-10-0; **3**, 82359-62-4; **3**·oxalate, 100430-71-5; **4**, 82359-63-5; **4**·HCl, 100430-72-6; (±)-**5**, 100430-70-4; (±)-**6**, 100448-03-1; (±)-**6**·oxalate, 100448-11-1; (±)-**7**, 100448-04-2; (±)-**7**·HCl, 100448-05-3; **8**, 100448-06-4; **8**·HBr, 100448-07-5; **9**·HBr, 100448-08-6; **10**, 100448-09-7; **10**·HCl, 100448-12-2; veratrole, 91-16-7; 1-methyl-4-piperidone, 1445-73-4; allyl bromide, 106-95-6.

Supplementary Material Available: Tables of atomic parameters for all atoms, bond angles, and torsion angles (4 pages). Ordering information is given on any current masthead page.

^{1}H NMR Configurational Correlation for Retro-Inverso Dipeptides: Application to the Determination of the Absolute Configuration of "Enkephalinase" Inhibitors. Relationships between Stereochemistry and Enzyme Recognition

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A stereospecific synthesis of thiorphan [*N*-[2(*RS*)-(mercaptomethyl)-1-oxo-3-phenylpropyl]glycine] and *retro*-thiorphan [3-[[1(*RS*)-(mercaptomethyl)-2-phenylethyl]amino]-3-oxopropanoic acid], two highly potent inhibitors of enkephalinase, a neutral endopeptidase involved in enkephalin metabolism, is reported. Due to a rapid isomerization process, derivatives of *retro*-thiorphan, which contains a 2-substituted malonyl moiety, cannot be separated by classical methods. However, a separation of the diastereoisomeric mixtures of these *retro*-thiorphan derivatives was achieved by HPLC. The absolute configuration of each isomer was determined by using an NMR configurational correlation. The inhibitory potency of the various inhibitors indicates that, in the thiorphan series, the affinity for enkephalinase is independent of the stereochemistry of the 2-(mercaptomethyl)-1-oxo-3-phenylpropyl moiety. In contrast, in the *retro*-thiorphan series a 100-fold difference in the inhibitory activity of the two enantiomers is observed. This indicates that there are large differences in the conformational behavior of the two series of inhibitors at the active site of the enzyme.

The neutral endopeptidase (EC 3.4.24.11)[1] designated enkephalinase[2] is involved in the metabolism of the endogenous opioid peptides Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu). Inhibition of this enzyme has been shown to be a useful method for investigating the physiological functions of opioid peptides.[3-6] Moreover, inhibition of enkephalin metabolism represents a new approach in the search for analgesics.[3,7] The design of potent and specific inhibitors of a given peptidase requires the knowledge of both its mechanism of action and its substrate specificity. Crystallographic studies of various zinc metallopeptidases such as carboxypeptidase A[8] and thermolysin[9] have shown that they have a common mechanism of catalysis and that their specificity is related to well-defined interactions (ionic, hydrophobic, H bonds, etc.) between the side chains of the substrate and the enzyme subsites surrounding the catalytic site.

On the basis of these findings, highly potent inhibitors of metallopeptidases such as angiotensin converting enzyme, ACE (EC 3.4.15.1),[10] a metallopeptidase involved in blood pressure regulation, and enkephalinase[3,7,11,12] have been rationally designed after a careful characterization of their subsite specificity. The specificity of enkephalinase is essentially insured by the preferential interactions of the S_1' subsite with aromatic or large hydrophobic residues and of the S_2' subsite with small lipophilic moieties[13,14] (the nomenclature used for the individual subsites of the enzyme, S_1', S_2', ..., is that of Schechter and Berger[15]).

Taking these features into account, two highly potent enkephalinase inhibitors have been designed that are able to interact with the catalytic site by a thiol group and to recognize the S_1'-S_2' subsites. However, these two compounds, designated thiorphan [*N*-[2(*RS*)-(mercaptomethyl)-1-oxo-3-phenylpropyl]glycine][3] and *retro*-thiorphan [3-[[1(*RS*)-(mercaptomethyl)-2-phenylethyl]-amino]-3-oxopropanoic acid][7] were synthesized as racemic mixtures. The retro-inversion of the amide bond in thiorphan and derivatives was shown to induce a complete differentiation between enkephalinase and ACE inhibition.[7] Generally, enzymes bind preferentially to compounds that mimic the configuration of the natural substrate, and studies on enkephalinase inhibition, by various

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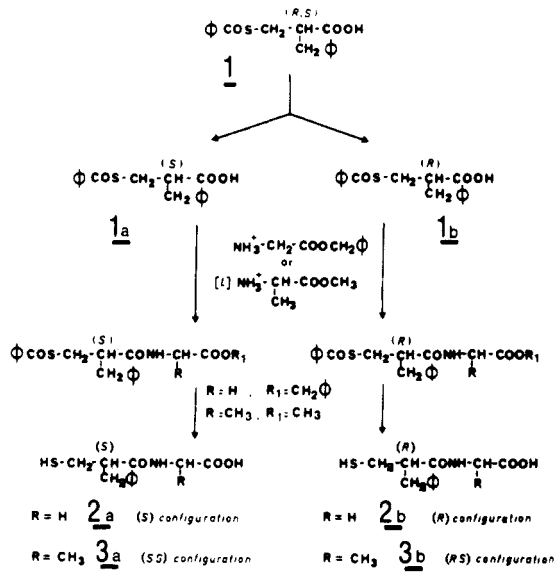

Figure 1. Scheme for the synthesis of pure stereoisomers of thiorphan **2a** and **2b** and "methylthiorphan" **3a** and **3b**.

peptides containing L or D residues, have confirmed the importance of this parameter.[13,14] It was, therefore, of interest to investigate the relationship between stereochemistry and activity in the thiorphan and *retro*-thiorphan series. Both compounds and a number of their analogues were synthesized either as optically pure forms or separated by analytical methods. The absolute configuration of the *retro*-thiorphan analogues was deduced from ^{1}H NMR measurements, and the respective inhibitory potency of each isomer on enkephalinase was determined.

Results and Discussion

Synthesis. (a) Synthesis of Pure Stereoisomers of Thiorphan and "Methylthiorphan". The synthesis of the two enantiomers of thiorphan is summarized in Figure 1. In the first step, racemic 2-[(benzoylthio)methyl]-3-phenylpropanoic acid (**1**) was prepared by the condensation of benzoyl chloride with (*RS*)-2-(mercaptomethyl)-3-phenylpropanoic acid.[16] The optical resolution of this compound was performed, as described,[17] with use of chiral α-methylbenzylamine. With the *d* (+) isomer of this amine, the salt containing the 2*S* isomer of 2-[(benzoylthio)methyl]-3-phenylpropanoic acid was crystallized, and the salt of the 2*R* isomer was obtained either from the mother liquors or after crystallization with *l*-(−)-α-methylbenzylamine. The two enantiomeric free acids **1a** and **1b** were then coupled with glycine benzyl ester. Alkaline deprotection of the thiol and carboxylate groups led to the formation of the two enantiomers of thiorphan, *N*-[2(*S*)-(mercaptomethyl)-1-oxo-3-phenylpropyl]glycine (**2a**) and *N*-[2(*R*)-(mercaptomethyl)-1-oxo-3-phenylpropyl]glycine (**2b**).

The two diastereoisomeric forms of "methylthiorphan" **3a** [*N*-[2(*S*)-(mercaptomethyl)-1-oxo-3-phenylpropyl]-L-alanine] and **3b** [*N*-[2(*R*)-(mercaptomethyl)-1-oxo-3-phenylpropyl]-L-alanine] were obtained by the same method, using L-alanine methyl ester in place of glycine benzyl ester (Figure 1).

(b) Stereospecific Synthesis of *retro*-Thiorphan and Analogues. The stereospecific synthesis of the two enantiomers of *retro*-thiorphan is schematized in Figure

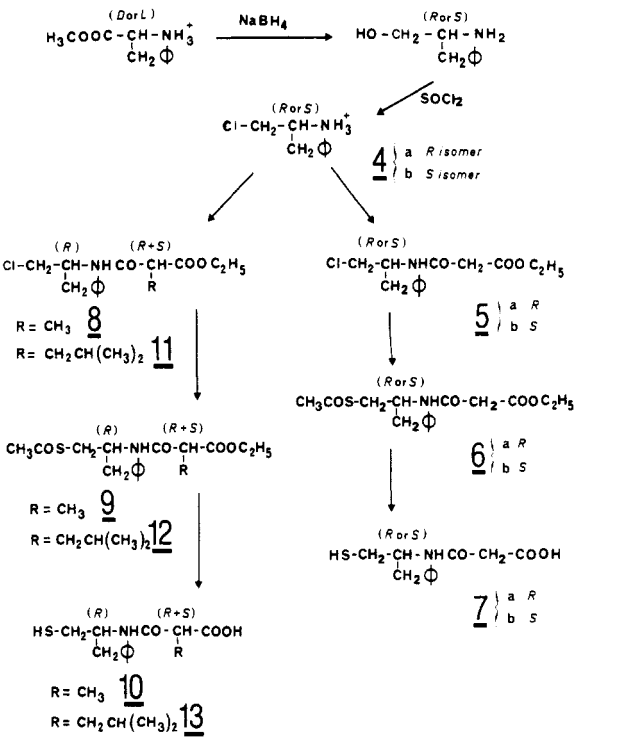

Figure 2. Scheme for the synthesis of pure stereoisomers of *retro*-thiorphan **7a** and **7b** and of its analogues **10** and **13**.

2. The transformation of phenylalanine methyl ester to 1-chloro-2-amino-3-phenylpropane was performed in two steps, which do not affect the asymmetric center of the molecule. The 2*S* isomer of the amine **4a** was obtained from L-phenylalanine methyl ester and the 2*R* isomer **4b** from D-phenylalanine methyl ester. The amines **4a** and **4b** were respectively coupled with monobenzyl malonate, leading to compounds **5a** and **5b**, and the nucleophilic displacement of chlorine by a thioacetate group yielded the expected acetylthio derivatives **6a** and **6b**. Finally, deprotection of the functional groups by alkaline hydrolysis gave the two separate isomers of *retro*-thiorphan **7a** [3-[[(1*R*)-1-(mercaptomethyl)-2-phenylethyl]amino]-3-oxopropanoic acid] and **7b** [3-[[(1*S*)-1-(mercaptomethyl)-2-phenylethyl]amino]-3-oxopropanoic acid].

The coupling of amine **4a** with methylmalonic or 2-methylpropyl malonic half-esters gave the analogues **10** and **13** of *retro*-thiorphan (Figure 2) as diastereoisomeric mixtures, since the half-esters of 2-substituted malonic acids exist only as racemic forms. The separation of the diastereoisomers was therefore performed by HPLC.

HPLC Separation of the Diastereoisomers of *retro*-Thiorphan Derivatives. It is well known that half-esters of 2-monosubstituted malonic acids are subject to a rapid racemization that prevents their resolution by classical methods.[18]

However, Pallai et al.[19] have recently shown that the diastereoisomeric mixture of a retro-inverso peptide H-Phe-g-Phe-(*R*,*S*)-*m*-Trp-OH, which contains a malonic moiety, can be separated by HPLC. Furthermore, the study of the epimerization rate of the optically pure isomers showed that this process is relatively slow (k = 1.06 × 10^{-2} h^{-1} at 37 °C).

The isomers of *retro*-thiorphan analogues **10** and **13** were therefore separated by HPLC, and the fractions were

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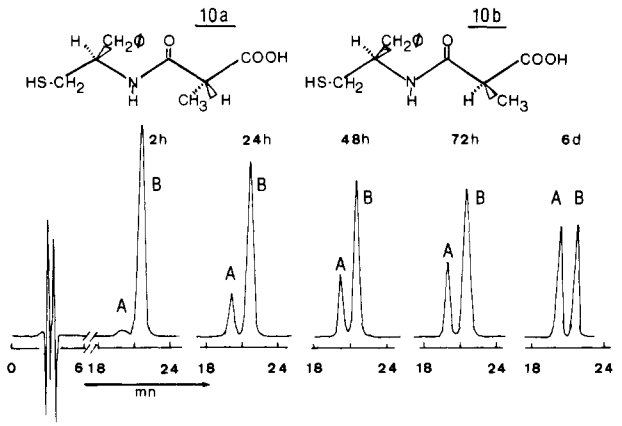

Figure 3. Isomerization process of the optically pure *retro*-thiorphan analogue **10b** in pH 7.4 buffer, 25 °C, as followed by HPLC, using a mixture of 2% acetic acid in water/CH_3CN (65/35) as eluent.

collected and cooled immediately at −20 °C, before lyophilization, in order to prevent the isomerization.

For all the separations performed by HPLC, the first eluted isomer was designated as peak A and the last eluted one as peak B.

For further studies on these isomers it was important to determine their epimerization rate under the experimental conditions employed. Isomer B of compound **10** was therefore incubated in 50 mM Tris-HCl, pH 7.5, at 25 °C, and the isomerization process was monitored by HPLC. As shown in Figure 3, isomer A was detectable only after a 2-h incubation, showing that both NMR and enzymatic studies could be performed on optically pure stereoisomers.

Determination of the Absolute Configuration of the Separate Isomers of *retro*-Thiorphan Derivatives by ^{1}H NMR Spectroscopy. The absolute configuration of the separated isomers of the *retro*-thiorphan analogues was determined by using the NMR configurational correlation observed for dipeptides and retro dipeptides. Indeed, ^{1}H NMR spectra of various phenylalanine-containing peptides shows that proton chemical shifts of the side chains of vicinal aminoacids are dependent on the absolute configuration of each residue.

As shown in Table I, the chemical shift of the methyl group of alanine in the natural dipeptides L-Phe-L-Ala or L-Ala-L-Phe is more deshielded [$\delta(CH_3)$ = 1.26 and 1.31 ppm, respectively] than in the D-Phe-L-Ala or D-Ala-L-Phe analogues [$\delta(CH_3)$ = 1.08 and 1.01 ppm, respectively]. The shielding observed for the methyl group of alanine in the D,L isomers may be related to a closer proximity between this side chain and the phenylalanine ring in the preferential conformation of these peptides.

This effect seems to be analogous to that observed by Mosher et al.,[20] with the chiral derivatizing agent α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) used for the NMR separation and configurational assignment of enantiomeric mixtures of chiral alcohols or amines. Thus, the NMR spectrum of the ester obtained by a reaction between (*R*)-MTPA and methyl-*tert*-butylcarbinol enriched in the *R* isomer showed that the *tert*-butyl group of the *R,S* isomer is significantly more shielded than that of the *R,R* isomer. As with the D,L dipeptides, the *R,S* isomer exists preferentially in the conformations containing the phenyl ring and the more hydrophobic substituent of the other asymmetric carbon in the "syn" position, with regard to the planar backbone.

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Table I. Stereochemical Dependency of Proton Chemical Shift of Methyl Group and Inhibitory Potency on Enkephalinase for Thiorphan, Retrothiorphan, and Analogues

compd	no.	absolute config	$\delta(CH_3)$[a]	inhibitory potency on enkephalinase: IC_{50},[b] nM
NH_3^+CHCONHCHCOO$^-$ (CH$_2$Ph, CH$_3$)		*S,S*	1.26	
		R,S	1.08	
NH_3^+CHCONHCHCOO$^-$ (CH$_3$, CH$_2$Ph)		*S,S*	1.31	
		R,S	1.01	
HSCH$_2$CHCONHCH$_2$COOH (CH$_2$Ph)	**2a**	*S*		1.90 ± 0.4
	2b	*R*		1.60 ± 0.4
HSCH$_2$CHCONHCHCOOH (CH$_2$Ph, CH$_3$)	**3a**	*S,S*	1.23	0.48 ± 0.08
	3b	*R,S*	1.05	0.41 ± 0.09
HSCH$_2$CHNHCOCH$_2$COOH (CH$_2$Ph)	**7a**	*R*		2.30 ± 0.70[c]
	7b	*S*		210 ± 10[c]
HSCH$_2$CHNHCOCHCOOH (CH$_2$Ph, CH$_3$)	**10a**	*R,R*	1.10	0.65 ± 0.03
	10b	*R,S*	0.95	0.54 ± 0.05
HSCH$_2$CHNHCOCHCOOH (CH$_2$Ph, CH$_2$-*i*-Pr)	**13a**	*R,R*	0.77	0.75 ± 0.10
	13b	*R,S*	0.67	1.50 ± 0.35

[a] The ^{1}H NMR spectra were performed in Me_2SO-d_6 at a concentration of 5 × 10^{-3} M. The chemical shifts are expressed in ppm (±0.02) from HMDS used as internal reference. [b] Concentration inhibiting 50% of the activity of enkephalinase in Tris-HCl (pH 7.4), 50 mM, 25 °C with 20 nM of [^{3}H]-D-Ala^2-Leu-enkephalin as substrate. Values are the means ± SEM from four independent experiments computed by log probit analysis of five inhibitor concentrations. [c] These values are the means ± SEM of 10 independent experiments.

This seems to be a general rule for inhibitors such as methylthiorphan **3a** and **3b**, which also contain a planar backbone due to the amide bond. In the *R,S* isomer **3b** the methyl group is more shielded (1.05 ppm) than in the *S,S* isomer **3a** (1.23 ppm) (Table I).

Before using this NMR parameter as a configurational probe for *retro*-thiorphan derivatives, it was important to verify its validity for retro dipeptides, as theoretical analyses on the conformational equilibrium of retro peptides indicate large differences with that of the corresponding peptides.[21,22]

For this purpose the strategy of Pallai et al.[23] for the configurational assignment of retro dipeptides was used, and the retro-dipeptide amide *N*-[2(*RS*)-(aminocarbonyl)-1-oxopropyl]-L-phenylalanine (**14**) was synthesized as reported in Figure 4. The two stereoisomers were separated by HPLC, and their ^{1}H NMR spectra were recorded. The two optically pure isomers **14a** and **14b** were converted into their corresponding dipeptides by reduction of the amide group by iodobenzene trifluoroacetate.[24] As it has already been observed,[23] HPLC analysis of the reaction mixture indicates that no racemization occurs during this step. The NMR spectra of the amides **14a** and **14b** and the resulting dipeptides indicate that isomer **14b** whose methyl group is the more shielded [$\delta(CH_3)$ = 1.00 ppm] was converted to D-Ala-L-Phe [$\delta(CH_3)$ = 1.01 ppm],

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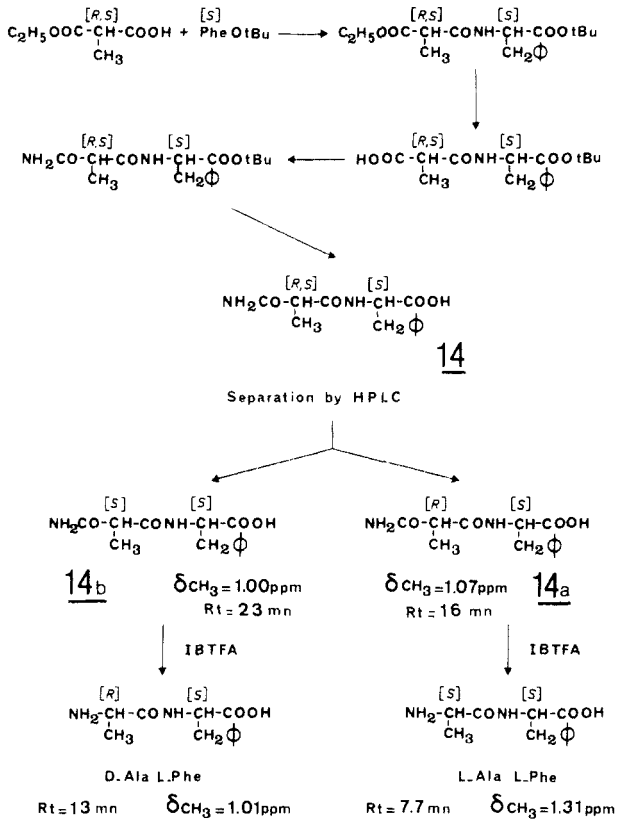

Figure 4. Scheme for the configurational correlation between retro dipeptides and their related dipeptides.

while the isomer **14a** [$\delta(CH_3)$ = 1.07 ppm] was converted to L-Ala-L-Phe [$\delta(CH_3)$ = 1.31 ppm].

This demonstrates that the correlation between the absolute configuration and the preferential conformations of phenylalanine-containing dipeptides is also valid for retro dipeptides and, consequently, could be used for the configurational assignment of retro-peptide analogues, such as substituted *retro*-thiorphan **10a**, **10b** and **13a**, **13b**.

Furthermore, it was interesting to observe that the HPLC retention times for the diastereoisomers **14a**, **14b**, and their related peptides L-Ala-L-Phe and D-Ala-L-Phe confirmed the proposed interpretation of the NMR data in terms of preferential conformations. Indeed chromatographic separation of diastereoisomeric forms of relatively planar molecules like amides, carbamates, or ureas is essentially related to the various interactions between the substituents, located on both sides of the planar backbone and the stationary phase.[25] The relative disposition of these substituents coupled with the extent and the sense of their interaction with the adsorbent (which are functions of their respective size, hydrophobicity, polarity, etc.) determines the elution order. Thus, it was shown that the isomer bearing in a "syn" position substituents exhibiting the same kind of interactions with the adsorbent is more retained than the isomer containing these substituents in an "anti" position. As shown in Figure 4, compound **14b** and the related dipeptide D-Ala-L-Phe which have the hydrophobic phenyl ring and methyl group in the "syn" position have the longest retention time (this additional parameter was also used for the configurational assignment of *retro*-thiorphan analogues).

The two diastereoisomers of compounds **10** and **13**, separated by HPLC, were analyzed by ^1H NMR spectroscopy. For compound **10**, the spectrum of the isomer eluted first (peak A = compound **10a**) shows a methyl group at 1.10 ppm and for the last eluted isomer (peak B = compound **10b**) the methyl group is at 0.95 ppm. Both NMR and HPLC data suggest a *R,R* configuration for **10a** and a *R,S* configuration for **10b**. Similarly with the two diastereoisomers of **13**, the *R,R* configuration was assigned to the first eluted isomer (peak A = compound **13a**) as the CH_3 protons of the isopropyl moiety are at 0.77 ppm and the *R,S* configuration was attributed to the second eluted isomer (compound **13b**) whose CH_3 is at 0.67 ppm.

Inhibitory Potency of the Various Stereoisomers of Thiorphan, *retro*-Thiorphan, and Their Analogues. The inhibitory potency of the various compounds was tested on purified rabbit kidney enkephalinase, using [^3H]-D-Ala^2-Leu-enkephalin as substrate (K_m = 30 ± 10 μM).[26]

As shown in Table I, the two isomers of thiorphan were equipotent in inhibiting enkephalinase, and the same result was obtained for the two isomers of methylthiorphan, which differ only by the configuration of the phenylpropyl moiety. By contrast, a large difference was found between the inhibitory potency of the two isomers of *retro*-thiorphan with the *R* isomer being 100 times more potent than the *S* isomer. On the other hand, the inhibitory potency of *retro*-thiorphan derivatives **10** and **13**, which differ only by the configuration of the malonyl moiety, was not dependent on their stereochemistry.

The high affinity (nanomolar range) of the two isomers of thiorphan and methylthiorphan indicates that the major interactions that stabilize the formation of the enzyme–inhibitor complex (thiol group, phenyl ring, and H bond involving the amide group) are satisfied whatever the stereochemistry of the 1-(mercaptomethyl)-2-oxo-3-phenylpropyl moiety.

The large difference in the inhibitory potencies of the two *retro*-thiorphan isomers indicates that inversion of the amide bond induces, in the complex, a conformation of the inhibitor such that only the *R* isomer is able to satisfy the three major interactions proposed. This very intriguing result was verified many times and was confirmed by binding experiments performed on rat kidney enkephalinase: the displacement curves of the tritiated enkephalinase inhibitor [^3H]-$HN(OH)C(O)CH_2CH(CH_2Ph)$-$CONHCH_2COOH$[27] by (*R*)- and (*S*)-*retro*-thiorphan gave the respective IC_{50} = 1.6 ± 0.3 × 10^{-9} and 3 ± 1 × 10^{-7} M, which are in good accordance with that obtained from their inhibitory potency (Waksman, G.; et al., unpublished results).

This result confirms the existence of differences in the conformational behavior of compounds bearing an amide bond and a retro amide bond, as demonstrated by theoretical analysis.[22]

Moreover, since only (*R*)-*retro*-thiorphan interacts strongly with enkephalinase, it may be assumed that the conformation of this inhibitor in the bound state resembles that taken up by thiorphan (either in the *R* or in the *S* configuration) and, conversely, that (*S*)-*retro*-thiorphan is not able to fit thoroughly one of these active conformations.

At the present time the difference in the inhibitory potency of the two isomers of *retro*-thiorphan as compared

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(27) Waksman, G.; Hamel, E.; Bouboutou, R.; Besselièvre, R.; Fournié-Zaluski, M. C.; Roques, B. P. *C. R. Acad. Sci., Ser. 3* **1984**, *299*, 613.

to the equipotency of the two isomers of thiorphan cannot be explained at the molecular level. A theoretical analysis of the conformational analogies between a dipeptide and its retro-inverso analogue, as a function of the relative configuration of both residues, may help to answer this question and facilitate a detailed analysis of the recognition process of inhibitors by enkephalinase. Furthermore, we have shown that enkephalinase resembles thermolysin,[28] a metallopeptidase that was extensively studied by crystallographic analysis.[29] Therefore, a computer graphics analysis of the conformations of the stereoisomers of thiorphan and *retro*-thiorphan at the active site of thermolysin could allow investigation of the differential interactions of these inhibitors with enkephalinase. This type of study recently performed with angiotensin converting enzyme inhibitors[30] is now in progress in our laboratory.

It should be noted that for compounds **10** or **13**, the stereochemistry of the malonyl part does not modify their inhibitory potency, indicating that this part of the molecule is not crucial for enzyme recognition.

Despite their equipotency to inhibit enkephalinase, the *R* and *S* isomers of thiorphan were recently reported to exhibit different antinociceptive properties.[31] However, this striking result was obtained by using only one concentration of both isomers, and the difference in analgesia was found to be hardly significant on the jump latency time in the mice hot plate test. Taking into account that proper analgesic effects of enkephalinase inhibitors are somewhat difficult to measure with accuracy, it would be preferable to investigate this interesting question by measuring the potentiating effect of exogenously administered enkephalinase sensitive Met-enkephalin.[32] Obviously, the various inhibitors whose stereochemistry was established in this paper will be of considerable interest to clarify this problem.

In conclusion, the NMR configurational correlation associated with the HPLC elution order seems to be a highly useful method for the determination of the absolute configuration of peptides and related structures. This method is applicable to peptides or derived compounds containing an aromatic residue, as recently shown in the determination of the absolute configuration of kelatorphan[32] [*N*-[(2*R*)-3-[(hydroxyamino)carboxy]-2-benzyl-1-oxopropyl]-L-alanine], the first complete inhibitor of all enkephalin degrading enzymes.[33] As expected, this compound displays greater analgesic properties[32] than selective enkephalinase inhibitors such as thiorphan and *retro*-thiorphan.

Experimental Section

Synthesis. The protected amino acids were from Bachem (Switzerland). Diethyl malonate, and diethyl methylmalonate were from Janssen-chimica (Belgium). All the other reagents and solvents (Normapur label) were from Prolabo (France). The diethyl ester of 2-(methylpropyl)malonate was prepared following Fedorynsky et al.[35] The monosaponification of the various diethyl malonates was performed by using the procedure of Curtius and Sieber.[36] L- and D-Phe-ol were prepared according to Seki et al.[37] The purity of all the synthesized compounds was checked by thin-layer chromatography on silica gel plates (E. Merck) with the following solvent systems (v/v): A, $CHCl_3$/MeOH (9:1), B, 1-BuOH/AcOH/H_2O (4:1:1), C, $CHCl_3$/MeOH (7:3), D = $CHCl_3$/Et_2O (5:5), E, CH_2Cl_2/Et_2O (9:1), F, CH_2Cl_2/MeOH (98:2).

1-Hydroxybenzotriazole was used in its hydrated form. The solution of this reagent in THF was therefore dried by shaking over a 5-Å molecular sieve for 10 min before use.

The structure of all the compounds was confirmed by ^1H NMR spectroscopy (Brüker WH 270 MHz) in Me_2SO-d_6 (5×10^{-3} M). Complete assignments of ^1H NMR signals were performed by classical double-resonance experiments. Chemical shifts (in parts per million ± 0.02) relative to HMDS as internal reference were reported only for the final compounds. Melting points of the crystallized compounds were determined on a Kofler apparatus (±2 °C) and are reported uncorrected.

The following abbreviations are used: MeOH, methanol; EtOH, ethanol; 1-BuOH, 1-butanol; AcOH, acetic acid; Et_2O, diethyl ether; THF, tetrahydrofuran; EtOAc, ethyl acetate; TFA, trifluoroacetic acid; Me_2SO-d_6, hexadeuterodimethyl sulfoxide; HMDS, hexamethyldisiloxane.

***N*-[2-[(Benzoylthio)methyl]-1-oxo-3-phenylpropyl]glycine Benzyl Ester.** To a solution of 2-[(benzoylthio)methyl]-3-phenylpropanoic acid (0.60 g, 2 mmol) in THF (30 mL), cooled at 0 °C, were successively added a solution of glycine benzyl ester *p*-tosylate (0.674 g, 2 mmol) and triethylamine (0.28 mL) in $CHCl_3$ (30 mL), a solution of 1-hydroxybenzotriazole (0.306 g, 2 mmol) in THF (20 mL), and finally a solution of *N*,*N*′-dicyclohexylcarbodiimide (0.494 g, 2.4 mmol) in $CHCl_3$ (25 mL). The mixture was stirred overnight at room temperature. After filtration of dicyclohexylurea and evaporation of solvents, the residue was dissolved in EtOAc and washed successively with water (2 × 30 mL), 10% citric acid (3 × 30 mL), water (30 mL), 10% $NaHCO_3$ (3 × 30 mL), water (30 mL), and finally with saturated NaCl (30 mL). The organic layer was dried over Na_2SO_4 and evaporated in vacuo. An oily product that crystallized slowly was obtained (0.79 g, 88%); R_f (F) = 0.58; mp 85 °C. Anal. ($C_{26}H_{25}NO_4S$) C, H, N, S.

***N*-[2-(Mercaptomethyl)-1-oxo-3-phenylpropyl]glycine (2a or 2b).** A solution of 0.40 g (0.89 mmol) of the preceding compound in MeOH (10 mL) was cooled at 0 °C, and 2 mL of 1 N NaOH was added dropwise during 1 h. The mixture was stirred for 2 h at 0 °C and for 2 h at room temperature. After evaporation of MeOH, the residue was diluted with 10 mL of water and washed with $CHCl_3$. The aqueous solution was acidified to pH 2 and a precipitate of disulfide derivative was obtained. The disulfide was dissolved in 5 mL of MeOH and the mixture was stirred for 1 h with 5 mL of 2 N HCl and 0.25 g of Zn powder. After filtration and evaporation of MeOH, the aqueous layer was extracted with degassed $CHCl_3$. The organic layer was dried over Na_2SO_4 and evaporated in vacuo. A white solid was obtained (0.212 g, 98%); R_f (B) = 0.85; mp 138 °C; $[\alpha]^{20}_{578}$ +43.5 for (*S*)-thiorphan **2a** in EtOH; $[\alpha]^{20}_{578}$ −42.5 for (*R*)-thiorphan **2b** in EtOH; ^1H NMR δ 2.19 (HS), 2.34 and 2.57 (CH_2SH), 2.64 (CH), 2.64 and 2.81 (CH_2Ph), 3.62 and 3.71 (Gly CH_2), 7.18 (Ph), 8.25 (NH), 12.41 (COOH). Anal. ($C_{12}H_{15}NO_3S$) C, H, N, S.

***N*-[2-[(Benzoylthio)methyl]-1-oxo-3-phenylpropyl]-L-alanine Methyl Ester.** These compounds were obtained with 0.086 g (0.29 mmol) of (*R*)- or (*S*)-2-[(benzoylthio)methyl]-3-phenylpropanoic acid and L-alanine methyl ester hydrochloride (0.04 g, 0.29 mmol) as the starting material following the procedure described for *N*-[2-[(benzoylthio)methyl]-1-oxo-3-phenylpropyl]glycine benzyl ester. A white solid (0.092 g, 84%) was obtained; R_f (A) = 0.86. Anal. ($C_{21}H_{23}NO_4S$) C, H, N, S.

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***N*-[2-(Mercaptomethyl)-1-oxo-3-phenylpropyl]-L-alanine (3a or 3b).** These compounds were obtained following the procedure described for thiorphan **2a** or **2b**. From 0.085 g (0.22 mmol) of the preceding compounds were obtained 0.055 g (93%) of the title compounds. R_f (B) = 0.88; mp 115 °C for compound **3a**; mp 157 °C for compound **3b**; ^{1}H NMR for compound **3a**, δ 1.22 (Ala CH_3), 2.08 (HS), 2.32 and 2.56 (CH_2SH), ~2.60 (CH), 2.60 and 2.83 (CH_2Ph), 4.17 (Ala H_α), 7.17 (Ph), 8.23 (NH), 12.40 (COOH); ^{1}H NMR for compound **3b**, δ 1.07 (Ala CH_3), 2.13 (HS), 2.35 and 2.67 (CH_2SH), ~2.76 (CH_2CH), 4.08 (Ala H_α), 7.17 (Ph), 8.13 (NH), 12.43 (COOH). Anal. ($C_{13}H_{17}NO_3S$) C, H, N, S.

1-Chloro-2-amino-3-phenylpropane Hydrochloride (4a or 4b). To a solution of L- or D-phenylalaninol (1.50 g, 10 mmol) in 25 mL of anhydrous DMF was added 0.8 mL of $SOCl_2$, and the mixture was stirred for 2 h at 120 °C. After evaporation of solvent in vacuo, the residue was triturated with Et_2O. A white solid (2.05 g, 99%) was obtained; R_f (C) = 0.70.

Ethyl 3-[[1-(Chloromethyl)-2-phenylethyl]amino]-3-oxopropanoate (5a or 5b). To a solution of 1.32 g (6.4 mmol) of compound **4a** and **4b** and 0.90 mL of triethylamine in $CHCl_3$ (20 mL), cooled at 0 °C, were added successively 0.85 g (6.4 mmol) of monoethyl malonate in THF (25 mL), 0.98 g (6.4 mmol) of 1-hydroxybenzotriazole in THF (25 mL), and 1.6 g (7.7 mmol) of *N*,*N*′-dicyclohexylcarbodiimide in $CHCl_3$ (15 mL). After 1 h at 0 °C, the mixture was stirred at room temperature overnight. The reaction was treated as described for *N*-[2-[(benzylthio)-methyl]-1-oxo-3-phenylpropyl]glycine benzyl ester. An oily product, which crystallized slowly, was obtained (1.50 g, 83%); mp 60 °C; R_f (A) 0.75. Anal. ($C_{14}H_{18}NO_3Cl$) C, H, N.

Ethyl 3-[[1-[(Acetylthio)methyl]-2-phenylethyl]amino]-3-oxopropanoate (6a or 6b). A solution of 0.80 g (7.0 mmol) of potassium thioacetate [prepared from 0.53 mL (7.0 mmol) of thioacetic acid and 285 mg (7.0 mmol) of KH] in CH_3CN (15 mL) was heated to 85 °C. A solution of 0.50 g (1.76 mmol) of compound **5a** or **5b** in CH_3CN (40 mL) was added dropwise during 2 h, and the mixture was stirred for 1 additional h at the same temperature. After filtration of the precipitate and evaporation of the solvents, the residue was dissolved in Et_2O. The organic layer was washed with water (2 × 10 mL) and saturated NaCl (10 mL) and dried over Na_2SO_4. After evaporation in vacuo, a white solid was obtained. Purification by flash chromatography on silica gel (CH_2Cl_2/Et_2O, 96:4) gave 0.46 g (80%) of compound **6**; mp 75 °C; R_f (D) 0.65. Anal. ($C_{16}H_{21}NO_4S$) C, H, N, S.

3-[[1-(Mercaptomethyl)-2-phenylethyl]amino]-3-oxopropanoic Acid (7a or 7b). A solution of compound **6a** and **6b** (0.20 g, 0.55 mmol) in EtOH (3 mL) was stirred under N_2 atmosphere at 0 °C, and 1.2 mL of 1 N NaOH was added. The mixture was stirred for 2 h at 0 °C and for 4 h at 20 °C. The reaction was treated as described for compound **2**. A white solid (0.12 g, 86%) was obtained; mp 137 °C; R_f (B) 0.80; $[\alpha]^{20}_{578}$ +5.16 for *R* isomer, $[\alpha]^{20}_{578}$ −5.25 for *S* isomer; ^{1}H NMR δ 2.27 (HS), ~2.48 (CH_2SH), 2.67 and 2.80 (CH_2Ph), 3.00 and 3.08 (CH_2CO), 3.90 (CH), 7.18 (Ph), 8.02 (NH), 12.45 (COOH). Anal. ($C_{12}H_{15}NO_3S$) C, H, N, S.

(2*RS*)-Ethyl 3-[[(1*R*)-1-(Chloromethyl)-2-phenylethyl]-amino]-2-methyl-3-oxopropanoate (8). This compound was obtained following the procedure used for the synthesis of **5**. From 0.70 g (3.4 mmol) of compound **4a** and 0.50 g (3.4 mmol) of half ethyl ester of methylmalonic acid were obtained 0.85 g (85%) of compound **8** after purification by chromatography on silica gel (CH_2Cl_2); R_f (A) 0.83; mp 109 °C. Anal. ($C_{15}H_{20}NO_3Cl$) C, H, N.

(2*RS*)-Ethyl 3-[[(1*R*)-1-[(Acetylthio)methyl]-2-phenylethyl]amino]-2-methyl-3-oxopropanoate (9). This compound was obtained following the procedure described for compound **6**. From 0.30 g (1 mmol) of compound **8** and 0.285 g (2.5 mmol) of potassium thioacetate was obtained 0.260 mg (78%) of compound **9**, which was recrystallized from Et_2O; R_f (D) 0.65; mp 79 °C. Anal. ($C_{17}H_{23}NO_4S$) C, H, N, S.

(2*RS*)-3-[[(1*R*)-1-(Mercaptomethyl)-2-phenylethyl]-amino]-2-methyl-3-oxopropanoic Acid (10). The title compound was obtained following the procedure used for compound **2**. From 0.26 g (0.77 mmol) of compound **9** was obtained 0.140 g (70%) of compound **10** as an oily product; R_f (B) 0.88; ^{1}H NMR for compound **10a**, δ 1.10 (CH_3 (malonate)), 2.22 (HS), ~2.75 (CH_2SH and CH_2Ph), 3.17 (CH (malonate)), 4.05 (CHNH), 7.17 (Ph), 8.00 (NH), 12.30 (COOH); ^{1}H NMR for compound **10b**, δ 0.95 (CH_3 (malonate)), 2.22 (HS), 2.53 and 2.62 (CH_2SH), 2.83 (CH_2Ph), 3.91 (CHNH), 7.17 (Ph), 7.98 (NH), 12.30 (COOH). Anal. ($C_{13}H_{17}NO_3S$) C, H, N, S.

(2*RS*)-Ethyl 3-[[(1*R*)-1-(Chloromethyl)-2-phenylethyl]-amino]-2-(2-methylpropyl)-3-oxopropanoate (11). This compound was obtained following the procedure used for the synthesis of **5**. From 0.66 g (3.2 mmol) of **4a** and 0.60 g (3.2 mmol) of 2-(methylpropyl)malonate half ethyl ester was obtained 0.93 g (85%) of compound **11**, which was purified by chromatography on silica gel (CH_2Cl_2 as eluent); R_f (A) 0.80; mp 81 °C. Anal. ($C_{18}H_{26}NO_3Cl$) C, H, N.

(2*RS*)-Ethyl 3-[[(1*R*)-1-(Mercaptomethyl)-2-phenylethyl]amino]-2-(2-methylpropyl)-3-oxopropanoate (12). This compound was obtained following the procedure used for compound **6**. From 0.28 g (0.82 mmol) of **11** and 0.235 g (2.06 mmol) of potassium thioacetate was obtained 0.26 g (84%) of an oily product, which crystallized slowly and was recrystallized from Et_2O; mp 69 °C; R_f (E) 0.60. Anal. ($C_{20}H_{29}NO_4S$) C, H, N, S.

(2*RS*)-3-[[(1*R*)-1-(Mercaptomethyl)-2-phenylethyl]-amino]-2-(2-methylpropyl)-3-oxopropanoic Acid (13). This compound was obtained as described for thiorphan **2**. From 0.15 g (0.40 mmol) of **12** was obtained 0.11 g (90%) of the titled compound as an oily product, which crystallized slowly; mp 95 °C; R_f (B) 0.85; ^{1}H NMR for compound **13a**, δ 0.77 (CH_3), 1.43 ($CH_2CH(CH_3)_2$), 2.16 (HS), 2.70 (CH_2SH and CH_2Ph), 3.12 (CH (malonate)), 4.06 (CH(NH)), 7.17 (Ph), 8.09 (NH), 12.10 (COOH); ^{1}H NMR for **13b**, δ 0.67 (CH_3), 1.35 ($CH_2CH(CH_3)_2$), 2.36 (HS), 2.58 and 2.76 (CH_2SH), 2.83 (CH_2Ph), 3.07 (CH (malonate)), 4.11 (CH(NH)), 7.17 (Ph), 8.14 (NH), 12.10 (COOH). Anal. ($C_{16}H_{23}NO_3S$) C, H, N, S.

***N*-[(*R*,*S*)-2-(Ethoxycarbonyl)-1-oxopropyl]-L-phenylalanine *tert*-Butyl Ester.** To a solution of methylmalonic acid half ethyl ester (1.7 g, 11.6 mmol) in THF (20 mL), cooled at 0 °C, were added successively a solution of L-phenylalanine *tert*-butyl ester hydrochloride (3.0 g, 11.6 mmol) and 1.6 mL of triethylamine in $CHCl_3$ (25 mL), a solution of 1-hydroxybenzotriazole (1.77 g, 11.6 mmol) in THF (30 mL), and a solution of *N*,*N*′-dicyclohexylcarbodiimide (2.6 g, 1.28 mmol) in $CHCl_3$ (15 mL). After 1 h at 0 °C, the mixture was stirred overnight at room temperature. The reaction was terminated as described for compound **5**. A white solid (3.9 g, 97%) was obtained; R_f (A) 0.80; mp 114 °C.

***N*-[(*R*,*S*)-2-Carboxy-1-oxopropyl]-L-phenylalanine *tert*-Butyl Ester.** A solution of the preceding compound (0.50 g, 1.44 mmol) in EtOH (15 mL) was cooled at 0 °C and 1.6 mL of 1 N NaOH was added. After 2 h at room temperature, the solvent was evaporated and the residue dissolved in water. The aqueous layer was washed with EtOAc (20 mL), acidified to pH 2 with 1 N HCl, and extracted with EtOAc (3 × 20 mL). The organic layer was washed with water (10 mL) and saturated NaCl (15 mL) and dried over Na_2SO_4. An oily product was obtained (0.73 g, 80%); R_f (B) 0.80.

***N*-[(*R*,*S*)-2-(Aminocarbonyl)-1-oxopropyl]-L-phenylalanine *tert*-Butyl Ester.** To a solution of the preceding compound (0.34 g, 1.07 mmol) in THF (2 mL), cooled at 0 °C, were successively added a solution of *N*-hydroxysuccinimide (0.123 g, 1.07 mmol) in THF (2 mL), a solution of *N*,*N*′-dicyclohexylcarbodiimide (0.242 g, 1.18 mmol) in THF (1 mL), and finally 10 mL of a saturated solution of NH_3 in THF. The reaction was terminated as described for compound **5**. A white solid (0.28 g, 82%) was obtained; mp 195 °C; R_f (A) 0.32.

***N*-[(*R*,*S*)-2-(Aminocarbonyl)-1-oxopropyl]-L-phenylalanine (14a and 14b).** The preceding compound (0.28 g, 0.87 mmol) was dissolved in CH_2Cl_2 (2 mL), cooled at 0 °C, and 2 mL of TFA was added. The mixture was stirred for 1 h at 0 °C and for 2 h at room temperature. The solvent and the excess of TFA were evaporated in vacuo, and the residue was triturated with Et_2O. A white solid (106 mg, 46%) was obtained; mp 128 °C; R_f (B) 0.53.

Reduction of Compound 14a or 14b to L-Ala-L-Phe or D-Ala-L-Phe. The separated amide **14a** or **14b** (0.050 g, 0.19 mmol) was dissolved in a mixture of CH_3CN/H_2O (60/40) (3 mL). A solution of [bis(trifluoroacetoxy)iodo]benzene (0.084 mg, 0.198 mmol) in CH_3CN (1 mL) was added, and the mixture was stirred for 3 h under N_2 atmosphere. The solvents were evaporated in

vacuo, and the oily residue was used for HPLC analysis.

HPLC Studies. HPLC studies were performed with use of a Waters apparatus, equipped with two M6000A pumps, a U6K injector, a M660 solvent programmer, and a M-450UV detector (Waters).

For analytical determinations, a 10-μm μ-Bondapak C_{18} column (Waters, 3.9 × 300 mm) was used. The separation of the diastereoisomers was obtained on a semipreparative 10-μm μ-Bondapak C_{18} column (Waters, 7.8 × 300 mm).

The eluted peaks were monitored at 220 or 260 nm when the eluent contained acetic acid.

The eluents used for the separation of the diastereoisomers **10a** and **10b** were 2% acetic acid in water/CH_3CN (65/35), for the mixture **13a** and **13b** 2% acetic acid in water/MeOH (55/45), and for the peptides **14a** and **14b** ammonium acetate buffer (pH 4.2)/CH_3CN (94/6).

Enzymatic Studies. Enkephalinase was purified to homogeneity from rabbit kidney by the method of Almenoff and Orlowski.[38] A single species was observed by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$.

The enkephalinase activity was checked following the procedure previously described.[26] The enzyme (at a final concentration of 0.9 ± 0.2 pmol/100 μL) was preincubated for 15 min at 25 °C with and without increasing concentrations of inhibitor in 100-μL total volume of 50 mM Tris-HCl buffer. [^{3}H]-D-Ala^2-Leu-enkephalin (k_m = 30 μM) was added to 20 nM final concentration, and the reaction was stopped after 30 min by adding 25 μL of 0.2 N HCl. The tritiated metabolites formed were separated on polystyrene beads.[39] Determination of IC_{50} values were performed as already described in detail.[13]

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Registry No. **1a**, 83024-49-1; **1b**, 100484-62-6; **2a**, 95909-00-5; **2b**, 95908-99-9; **3a**, 100484-63-7; **3b**, 100484-64-8; **4a**, 100431-27-4; **4b**, 100431-28-5; **5a**, 100431-29-6; **5b**, 100431-30-9; **6a**, 100431-31-0; **6b**, 100431-32-1; **7a**, 100484-65-9; **7b**, 100484-66-0; (R,R)-**8**, 100431-33-2; (R,S)-**8**, 100431-34-3; (R,R)-**9**, 100431-35-4; (R,S)-**9**, 100431-36-5; (R,R)-**10**, 100431-37-6; (R,S)-**10**, 100431-38-7; (*R*,*R*)-**11**, 100431-39-8; (*R*,*S*)-**11**, 100431-40-1; (*R*,*R*)-**12**, 100431-41-2; (*R*,*S*)-**12**, 100431-42-3; (*R*,*R*)-**13**, 100431-43-4; (*R*,*S*)-**13**, 100431-44-5; **14a**, 100431-45-6; **14b**, 100431-46-7; enkephalinase, 82707-54-8; (*S*)-*N*-[2-(benzoylthio)methyl]-1-oxo-3-phenylpropyl]glycine benzyl ester, 100431-47-8; (*R*)-*N*-[2-(benzoylthio)methyl]-1-oxo-3-phenylpropyl]glycine benzyl ester, 100431-48-9; glycine benzyl ester *p*-tosylate, 1738-76-7; (*S*)-*N*-[2-(benzoylthio)methyl]-1-oxo-3-phenylpropyl]-L-alanine methyl ester, 100431-49-0; (*R*)-*N*-[2-(benzoylthio)methyl]-1-oxo-3-phenylpropyl]L-alanine methyl ester, 100431-50-3; L-alanine methyl ester hydrochloride, 2491-20-5; L-phenylalaninol, 3182-95-4; D-phenylalaninol, 5267-64-1; monoethyl malonate, 1071-46-1; 1-hydroxybenzotriazole, 2592-95-2; potassium thioacetate, 10387-40-3; methylmalonic acid (half ethyl ester), 81110-31-8; 2-(methylpropyl)malonate (half ethyl ester), 78220-81-2; *N*-[(*R*,*S*)-2-(ethoxycarbonyl)-1-oxopropyl]-L-phenylalanine *tert*-butyl ester, 100431-51-4; L-phenylalanine *tert*-butyl ester hydrochloride, 15100-75-1; *N*-[(*R*,*S*)-2-carboxy-1-oxypropyl]-L-phenylalanine *tert*-butyl ester, 100431-52-4; *N*-[(*R*,*S*)-2-(aminocarbonyl)-1-oxopropyl]-L-phenylalanine *tert*-butyl ester, 100431-53-6; *N*-hydroxysuccinimide, 6066-82-6.

Preparation and Evaluation of Radioiodinated (Iodophenyl)cholines and Their Morpholinium and Piperidinium Analogues as Myocardial Perfusion Imaging Agents

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A series of nine radioiodinated quaternary ammonium salts related to phenylcholine were synthesized, characterized, and radiolabeled by exchange. These compounds were evaluated as myocardial perfusion imaging agents in mice, pigs, and humans. Mice biodistribution studies showed that five of the nine compounds were taken up in the heart to the same extent as $^{201}Tl^+$ at 5 min. At 60 min myocardial retention was significantly better than $^{201}Tl^+$ for six of the compounds. Several of the compounds showed more favorable heart/blood and heart/liver ratios when compared to $^{201}Tl^+$. Evaluation of three of the more promising compounds in pigs and humans however revealed no selective myocardial uptake.

Although $^{201}Tl^+$ is widely used for myocardial perfusion imaging, its properties are less than ideal.[1] Self-absorption of low-energy photons, redistribution during the imaging period, and interference of interpretation due to nearby hepatic or pulmonary activity are disadvantages of $^{201}Tl^+$ that have encouraged investigators to search for alternative myocardial perfusion imaging agents.

The favorable imaging qualities of ^{123}I and the in vivo stability of most aryliodo compounds[2,3] have stimulated research into radioiodinated aromatic quaternary ammonium compounds. A radioiodinated bretylium analogue (RIBA: (*o*-[^{125}I]iodobenzyl)trimethylammonium),[4] (*p*-[^{125}I]iodophenyl)trimethylammonium,[3] and a radioiodinated benzoylcholamine derivative[5] were all found to have initial high myocardial uptake in rats or mice. Unfortunately rapid myocardial washout or extensive hepatic uptake in higher species has not indicated promise for any of these agents to replace $^{201}Tl^+$ for myocardial perfusion imaging.

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