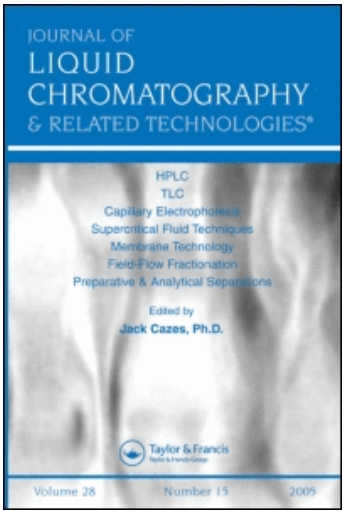

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Chromatographic Studies on the Racemization of Thiopeptides

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CHROMATOGRAPHIC STUDIES ON THE RACEMIZATION OF THIOPEPTIDES

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ABSTRACT

It was found by chromatographic, CD and NMR methods, that the thionation of piperazine-2,5-diones [*cyclo*(Aaa^1-Aaa^2) → *cyclo* (Aaa*t*^{1}-Aaa*t*^{2} (Aaa = -NH-CHR-CO-; Aaa*t* = -NH-CHR-CS-)] or piperazine-2,5-onthiones [*cyclo*(Aaa*t*^{1}-Aaa^2) → *cyclo* (Aaa*t*^{1}-Aaa*t*^{2})] and, occasionally, even the spontaneous cyclization of endo-thiodipeptide esters [H-Aaa*t*^{1}-Aaa^2-OR] result in enantiomeric (Aaa^1 or Aaa^2 = Gly) or diastereomeric mixtures of piperazine monothiones or dithiones. The diastereoisomers were separated by semipreparative HPLC and their quantitative product distribution was determined by an optimized HPLC method on Hypersil-silica column with CH_2Cl_2-EtOAc eluent mixtures. Isocratic RP-HPLC on ODS-Hypersil column and pre-column derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) were used to monitor the racemization of Ala and Pro residues and to de-

† Deceased 1991.

termine the ratio of enantiomers. Thionation of urethane protected dipeptide esters or dethionation of the corresponding endothiodipeptide derivatives were not found to result in significant racemization. However, during the thionation of cyclic dipeptides or thiopeptides or isolation of piperazine-2,5- mono- or dithiones a partial or complete racemization could always be detected. Moreover, the acidic hydrolysis of thiopeptides was also accompanied by racemization and resulted in partially racemized amino (oxo)acids.

INTRODUCTION

There is a growing interest in peptides containing one or more thioamide groups (1-10). Lawesson's reagent (LR) can be used for the conversion of amides into thioamides under mild conditions [heating in benzene or toluene at 30-80°C for 0.5-2 hrs] (9, 11-13). In an effort to study the chiroptical properties, and H-bond forming ability of thiopeptides, a great number of protected endothiopeptides and thionated acyl amino acid and dipeptide methylamides have been prepared in our laboratory (13-19). They were characterized chromatographically by TLC, LC and HPLC; by ^{1}H and ^{13}C NMR, UV, CD and IR spectroscopy, and also by MS (13-19). It has become generally accepted that LR converts the *trans* (Z) rotameric form of the amide group of urethane protected dipeptide esters to the corresponding thioamides selectively and without significant racemization (9, 13, 20).

Based on preliminary CD spectroscopic studies, the thionation of piperazine 2,5-diones (I), featuring *cis* (E) amide groups re-

sults, however, in partially or fully racemized piperazine-2,5-dithiones (14, 16).

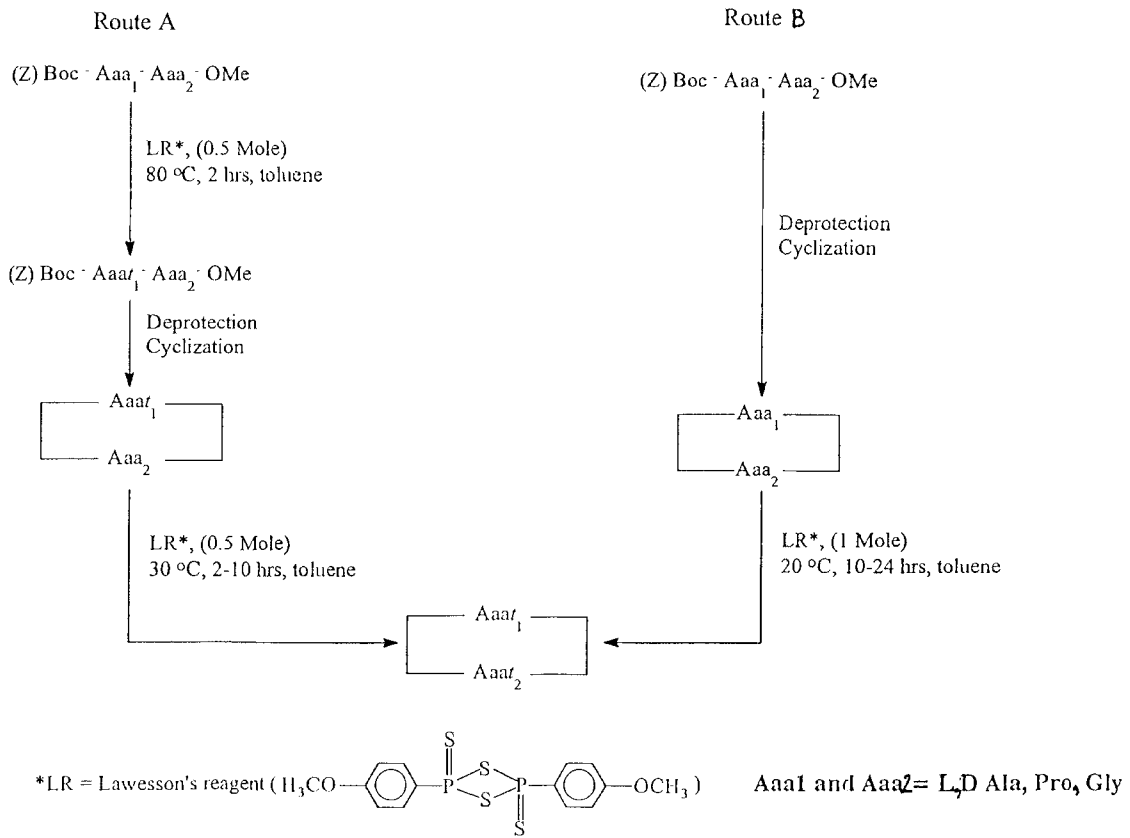
A violet-coloured crystal of *cyclo*(thioprolyl-thioprolyl), [*cyclo*(Pro*t*-Pro*t*), (III)]; prepared from optically pure L-proline diketopiperazine [*cyclo*(L-Pro-L-Pro)] has been reported to show crystallographic disorder (24) which was explained by the presence of co-crystallized enantiomorphous pairs in the sample (24). Comparative circular dichroism (CD), as well as ^{1}H and ^{13}C NMR studies have indicated that the sample, in addition to the L,L form (*ca.* 80%) contained *ca.* 20% enantiomeric (D,D) but no diastereomeric (L,D) form (14, 24).

Prompted by these findings, a number of single- and double-thionated diketopiperazines have been prepared (14, 16) to clarify the mechanism and conditions of their racemization. Herein we report results of comparative chromatographic studies on the racemization of linear and cyclic thiodipeptides and their non-thionated linear precursors.

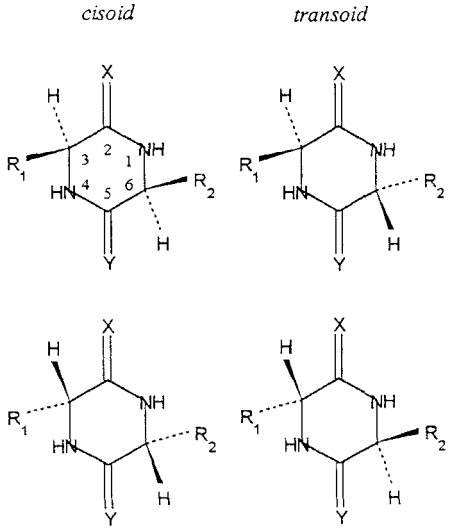
MATERIALS

Starting from Z- or Boc-protected linear endothiopeptide esters, (13, 14) a series (Table IV) of piperazine-2,5-onthiones and 2,5-dithiones has been synthesized according to Scheme I. (14) 400 MHz ^{1}H NMR and IR spectroscopy was used to characterize the cyclic thioamides. Details of the syntheses have been reported earlier (13, 14, 16).

SYNTHESIS



Scheme 1.



Possible stereoisomers

	configurations	
	C_3	C_6
cisoid	L	L
	D	D
transoid	L	D
(*mesoid* if	D	L
R_1=R_2 and		
X,Y=O or S)		

Figure 1. Possible stereoisomers of piperazine 2,5-onthiones (X = O, Y = S), 2,5-thion ones (X = S, Y = O) and 2,5-dithiones (X, Y = S).

The abbreviations used follow the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (25). A*aat* (e.g. Alat, Glyt, etc.) means a thioamino acid residue:

```
-NH-CH-C-
    |  ||
    R  S
```

METHODS

High Performance Liquid Chromatography

Separations were performed on a laboratory-assembled instrument consisting of a reciprocating piston pump (Model 1515,

Orlita, FRG), a variable wavelength UV monitor fitted to an 8 µl flow-cell (Model 212, Cecil, UK) and a sample injector (Rheodyne, USA), or on a Knauer HPLC-system consisting of two pumps Model 64 with analytical or preparative pumpheads, a gradient programmer Model 50 B, an injection valve with 20 µl sample loop and a spectral photometer with analytical and preparative flow cells (Knauer-GmbH, FRG).

Column effluents were monitored at 250, 254, 270, 279 or 281 nm (for thiopeptides) and at 340 nm (for Marfey's amino acid derivatives).

The packing materials were Hypersil-silica, Hypersil ODS-6 (Shandon Southern Products, UK), LiChroprep-silica (Merck, FRG) and Partisil M-9 silica (Whatman Ltd, UK). Peaks were recorded on a Model OH-314/1 chart recorder (Radelkis, Hungary) and the areas under them were calculated using programmed Simpson's rule. The chromatographs were operated isocratically with flow rates between 0.8 and 1.2 cm^3/min (analytical mode) and between 2.0 and 4 cm^3/min (preparative mode) at ambient temperature.

Hydrolysis

The linear protected and cyclic thiopeptides were subjected to acidic hydrolysis. The samples were treated at 105°C with 6 M hydrochloric acid for 48 hrs in sealed tubes. The acid was removed in vacuo, samples were neutralized and reacted with Marfey's reagent.

Derivatization

Derivatization was carried out according to Marfey (26) with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Pierce, USA). The hydrolysate prepared from 2-5 μmol starting thiopeptide was dissolved in 100 μl of 0.5 M $NaHCO_3$ solution and 200 μl of 1% solution of Marfey's reagent in acetone was added. The solution was incubated at 40°C for 90 min and cooled; then 25 μl of 2 M HCl was added at room temperature. After 20 fold dilution with methanol or eluant, 10-20 μl aliquots were used for HPLC injection on Hypersil ODS-6 column (27-28).

NMR studies

^1H NMR measurements were performed on a VARIAN 400 spectrometer at ambient temperature, c = 4-8 mg/mL. Solvents: DMSO-d_6 and $CDCl_3$. The assignment of the peaks is based on ^1H-^1H COSY experiments. Data are summarized in Table VIII.

CD measurements

CD spectra were recorded on Jobin-Yvon Dichrographs Mark III and V. D. Mark III is operated by an IBM AT computer. Spectograde solvents (Uvasol, E. Merck, Darmstadt) were used. Measurements were taken in 0.02-1.00 cm cells.

RESULTS AND DISCUSSION

Racemization is one of the major side reactions that may occur during peptide synthesis or in solution in the presence of bases (29). Piperazine-2,5-onthiones or -thionones and 2,5-dithiones, built up from two different chiral amino acid residues ($R_1 \neq R_2 \neq H$) have four stereoisomeric forms (Fig. 1). The number of stereoisomers is decreased by structural factors (incorporation of one glycine or two identical residues). However, steric factors may also lead to the decrease of stereoisomers. For example, the dithione *cyclo*(Prot-Prot), similarly to the parent dioxopiperazine *cyclo*(Pro-Pro), cannot exist in L, D *meso* form (24). Contrary to this, *cyclo*(Ala*t*-Ala*t*) was found to be present as a roughly 1:1 mixture of the enantiomeric (L, L + D, D) and mesoid (L, D) forms (Table II.). Preliminary CD spectroscopic and theoretical studies (30) have indicated that it is the enhanced tendency for thione → thiol tautomerisation of the piperazine-2,5-onthiones or 2,5-dithiones which explains the racemization of cyclic thioamides (30).

The strategy of the synthesis of piperazine-2,5-onthiones and 2,5-dithiones is shown in Scheme 1. The crude products were first pre-purified by chromatography on Kieselgel 60 columns using dichloromethane-ethylacetate mixtures (95:5, 98:2 v/v) as eluant. The diastereomeric mixtures were separated (see Table I) by semipreparative and preparative HPLC and their product distribution was determined (see Table II) by an optimized HPLC method

TABLE I
HPLC Separation Conditions for Diastereoisomeric Piperazine 2,5-Onthiones and Dithiones

	Analytical	Semipreparative	Preparative
Column	250 x 4 mm	500 x 8 mm	M 9 10/25 Whatman
Packing	Hypersil-silica 6 μm	LiChroprep silica 25-40 μm	Partisil-silica 10 μm
Detection	250, 254, 270 nm	250 nm	250 nm
Eluant:			
1. CH_2Cl_2-EtOAc	98:2	95:5 (v/v)	
2. EtOAc-MeOH-CH_2Cl_2		49.5:0.5:50	
3. Diisopropilaether-CH_2Cl_2-EtOAc		40:10:10	
4. Diisopropilaether-i-propanol-cyclohexane		5:1:60	5:1:80
Flow rate	0.8 mL min^{-1}	4 mL min^{-1}	2 mL min^{-1}
Instrument	Knauer-system	Knauer-system	Knauer-system
Sample	5 γ/μL	1 mg/200 μL	30 mg/500 μL
Solvent		Eluant + DMF	
Isolation		Fraction evaporation	
Purity checking	UV, CD, NMR	anal. HPLC	anal. HPLC

on Hypersil silica column with the same type of eluants (α = 1.2 - 1.6). UV detection at 250, 254, 270, 279 or 281 nm (at the absorption maxima of the cyclic thioamides) was used in the HPLC measurements. The semipreparative normal phase separation of diastereoisomers was performed on Lichroprep and Partisil M-9 silica columns with eluants 1 - 4: [dichloromethane - ethylacetate - methanol or diisopropyl ether and i-propanol - cyclohexane - diisopropyl ether eluant mixtures] (Table I). The purity (usually > 94-95%) of the crystalline endproducts was checked by the above analytical HPLC system.

The diastereoisomers (in the order of elution: I and II, see Table II) were identified by UV, ^{1}H and ^{13}C NMR, CD and IR spectroscopy (14). Chromatographic conditions were optimized to achieve high resolution and baseline separation. Best results were obtained on Hypersil-silica columns. Fig 2. shows the typical chromatographic pattern for the separation of a diastereoisomeric cyclic dithioamide mixture.

The scale up of analytical separations was performed on preparative silica columns, silica packings of 63-125 μm and 48-63 μm were not efficient enough. The separation was improved by applying silica with 25-40 μm size. The best resolution was achieved on 10 μm silica column.

Due to the low tendency of shift reagents to form complexes with linear or cyclic thioamides, NMR spectroscopy could not be used for determining the L/D or L,L/D,D ratio of enantiomeric mixtures. Thus, comparative racemization studies were performed on

TABLE II
Diastereomeric Distribution of Cyclic Thiopeptides

Compound	Preparation method (Route)	Diastereomers k' I	II	α	Distribution % from HPLC	^{1}H-NMR	Eluants
cyclo(Alat-Alat)	B	2.4	2.9	1.21	49.2:50.8	53:47	1
cyclo(Alat-Prot)*	B	1.0	1.5	1.50	49.1:50.9	41:59	3
cyclo(Alat-Prot)	A	2.2	2.5	1.14	49.0:51.0	45:35	4
crude product					39.1:60.9	40:60	
after preparative purification by HPLC (one-step)					5.1:94.9 (II)** 92.3:7.7 (I)	3:97 98:2	

*No separation in the eluent system 2.
**I = L, L + D, D
II = L, D + D, L
(I and II elution order of diastereoisomers; the compounds I and II were identified by CD and NMR)

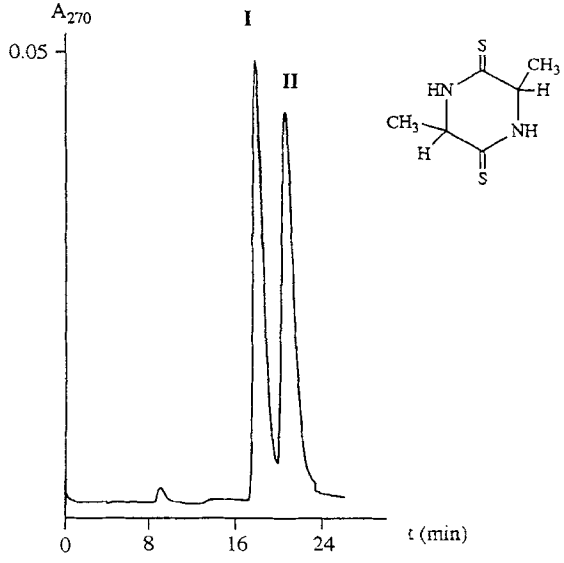


Figure 2. Analytical separation of diastereoisomeric cyclo-endothiopeptides. Col.: Hypersil-silica 250x4 mm; eluant: diisopropylaether-i-propanol-cyclohexane 5:1:60 v/v; flow rate: 0.80 ml min^{-1}; detection 270 nm; pressure 650 psi; sample: cyclo(L-Alat-L-Alat) PE 194 (I mixture of L,L and D,D enantiomers, II meso compound).

the amino acid mixtures obtained by acidic hydrolysis of linear and cyclic thiopeptides and on their (oxo)peptide precursors. The acidic hydrolysis using standard conditions (6M HCl, 48 hrs at 105°C in sealed glass tubes) was accompanied with complete dethionation. Isocratic reversed phase HPLC on ODS-Hypersil column with MeOH-CH_3CN or THF-NaOAc buffer (0.02M, pH 4) mobile phases and pre-column derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) were used to determine the ratio of D and L amino acids. Chromatographic data on Marfey's derivatives of amino acids contained in the models are summarized in Table III. The D/L amino acid ratio and rate of racemization of linear and cyclic thiopeptides and oxopeptides are given in Tables IV. and V.

We were able to monitor racemization for Ala and Pro residues and to determine the ratio of enantiomers by separation of the derivatized enantiomeric amino (oxo)acids (α = 1.5-4.2, R_s = 6.2-12.9) (26-28). For *cyclo*(L-Alat-L-Alat), Fig. 3 shows the separation of D and L-Ala derivatives. The hydrolysis is also a potential source of racemization (27) so that this step was also carefully monitored.

As expected (29), the parent Z- or Boc-protected dipeptide esters and piperazine-2,5-diones prepared from them (see Table IV and V) were not found to suffer significant racemization during peptide synthesis, cyclization and acidic hydrolysis.

As reported earlier, the CD spectra of urethane protected endothiodipeptide esters of types Z(Boc)-Aaa*t*^{1}-Aaa^2-OR and Z(Boc)-Aaa*t*-Gly-OR did not show dependence on the conditions (solvent,

TABLE III
Chromatographic Data of Amino Acid Marfey's Derivatives

Syst.	amino acid	k' L	k' D	α	R_s	Eluent system
1.	Ala	2.1	6.1	2.9	8.0	MeOH-CH_3CN-0.02M NaOAc buffer 20:10:70
	Marfey-OH*	15.1				
2.	Ala	4.7	7.3	1.5	9.1	THF-0.02M NaOAc buffer 15:85
	Gly	3.7				
	Marfey-OH*	34.0				
3.	Pro	5.1	11.0	2.1	6.2	MeOH-CH_3CN-0.02M NaOAc buffer 18:8:74
	Marfey-OH*	22.0				
	Gly	3.6				
	Ala	6.0	14.2	2.1	7.0	
4.	Val	3.0	12.5	4.2	12.9	MeOH-0.02M NaOAc buffer 40:60
	Marfey-OH*	5.1				
	Gly	0.8				

System 1 for cyclo(Alat-Alat)
2 for cyclo(Alat-Gly)
3 for the others, e.g. cyclo(Alat-Pro), cyclo)Pro-Gly), etc.
4 for Val peptides

(*Marfey-OH is the hydrolyzed reagent)

HPLC. Column: ODS-Hypersil-6 (125x4 mm)
Flow rate: 1.1 mol min^{-1}
Detection: at 340 nm

TABLE IV
Racemization Data of diketopiperazines

Diketopiperazines	D/L amino acid ratio		Rate of racemization %	
	without*	with correction	without*	with** correction
cyclo(Ala-Ala)	6.6:93.4	1.8:98.2	13.2	3.6
cyclo(Ala-Gly)	2.69:97.4	0.05:99.95	5.2	0.1
cyclo(Pro-Ala)	7.4:92.6 (Pro)	0.5:99.5	14.8	1.0
	5.5:94.5 (Ala)	0.7:99.3	11.0	1.4
cyclo(Pro-Gly)	7.6:92.4	0.7:99.3	15.2	1.4

*Without correction, the values are together with the background racemization of single amino acid components.
**These data are in good correlation with ones of Morinobu (31).

temperature, reaction time etc.) of thionation (Scheme 1) (13, 14). Similarly, ^{1}H and ^{13}C NMR studies did not reveal significant amounts (> 1-2%) of diasteroisomeric impurities in samples of Z(Boc)-Aaat^{1}-Aaa^{2}-OR thiopeptides (13, 15). Most importantly, the oxopeptide Z-Val-Gly-OEt obtained from Z-Valt-Gly-OEt by dethiation with Ag_2O in a dioxane/water mixture (13), gives 99.7% L- and only 0.3% D-valine after acidic hydrolysis, while its precursor, Z-Valt-Gly-OEt, results in 11.5% D-valine.

A comparison of the above data leads to the conclusion that, contrary to (oxo)peptides, linear protected thiopeptides suffer significant racemization during acidic hydrolysis. In the isomeric endothiopeptides Z-(Boc)-Prot-Gly-OMe and Z(Boc)-Glyt-Pro-OMe, the chiral center preceding the thioamide bond is less sensitive to

TABLE V
Racemization Data of Linear Thiopeptides*

Linear endothiopeptides	D/L amino acid ratio	Rate of racemization** %
Z-Ala-Ala-OEt	1.0:99	2.0
Z-Alat-Ala-OEt	24.5:75.5	49.0
Z-Val-Gly-OEt	0.2:99.8	0.4
Z-Valt-Gly-OEt	11.5:88.5	23.0
"Z-Valt-Gly-OEt"***	0.3:99.7	0.6
Z-Prot-Pro-OMe	20.0:80.0	40.0
Z-Alat-Gly-OEt	1.5:98.5	3.0
Z-Glyt-Pro-OMe	23.0:77.0	46.0
Z-Prot-Gly-OMe	6.0:94.0	12.0
Boc-Prot-Gly-OMe	2.0:98.0	4.0
Boc-Glyt-Pro-OMe	28.0:72.0	56.0
Boc-Pro-Gly-OMe	1.1:98.9	2.2
Boc-Pro-Ala-OMe	0.5:99.95 (Pro)	0.1
	0.05:99.55 (Ala)	0.1
Boc-Prot-Ala-OMe	33.0:67.0 (Pro)	66.0
	28.0:72.0 (Ala)	56.0
Boc-Ala-Pro-OMe	0.05:99.25 (Pro)	0.1
	1.4:98.6 (Ala)	2.8

*The data are corrected always with the background racemization of single amino acid components.

**Racemization rate (%) = {100 × [2D:(D+L)]}, where D and L are peak areas of isomers on the chromatograms.

***After dethiation. The physical properties (m.p., optical rotation) support this data, too.

racemization than the chiral center succeeding it (Table V). The high racemization rates of both chiral residues in Boc-Prot Ala-OMe is in contradiction with this finding. A thioalanyl (-Alat-) residue appears to be more resistant to racemization during hydrolysis than a thioprolyl (Prot-) one.

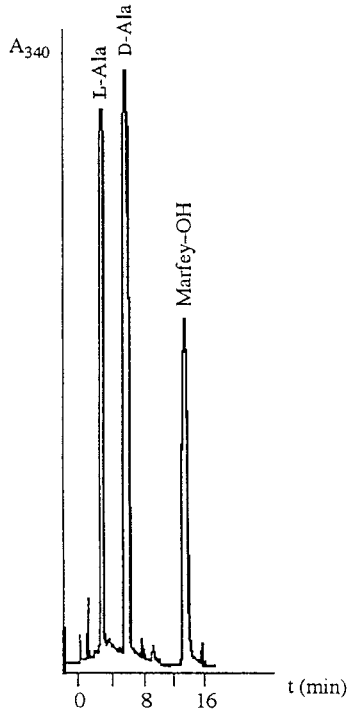


Figure 3. Separation of Marfey's derivatives. Col.: ODS-Hypersil-6, 125x4 mm; eluant: MeOH-CH_3CN-O,02M NaOAc buffer (pH 4); flow rate: 1.1 ml min^{-1}; detection: 340 nm; recorder speed: 15 cm/hr; pressure: 1350 psi; hydrolyzed sample: cyclo(L-Alat-L-Alat); derivatization: Marfey's reagent; sample volume: 10 µl.

The data listed in Tables II, IV-VII and the results of preliminary X-ray crystallographic, NMR and CD spectroscopic studes (14, 24) clearly indicate that piperazine-2,5-onthiones and 2,5-dithiones may undergo racemization not only during acidic hydrolysis but also in the course of their preparation, isolation or pu-

TABLE VI
Racemization Data of Cyclic Thiopeptides*

Cycloendothiopeptides	Preparation method (Route)	D/L amino acid ratio %	Rate of racemization**
cyclo(Alat-Ala)	A	23.0:77.0	46.0
cyclo(Alat-Alat)	B	49.5:50.5	99.0
cyclo(Prot-Prot)	B	29.8:70.2	59.6
cyclo(Alat-Gly)	A	44.0:56.0	88.0
cyclo(Alat-Glyt)*	A	0.0:100	0.0
cyclo(Prot-Pro)	A	18.0:82.0	36.0
cyclo(Prot-Gly)	A	46.7:53.3	93.4
cyclo(Glyt-Ala)	A	5.5:94.5	11.0
cyclo(Glyt-Pro)	A	30.0:70.0	60.0
cyclo(Prot-Ala)	A	46.0:54.0 (Pro)	92.0
		36.0:64.0 (Ala)	72.0
cyclo(Prot-Alat)	B	49.5:50.5 (Pro)	99.0

*Preparation at 20°C.

rification. The thionation of piperazine-2,5-diones and piperazine-2,5-onthiones is always accompanied with more or less extensive racemization, depending on the structure of the parent cyclic peptide or monothiopeptide and the conditions of thionation. It should be taken into account, that the formation of piperazine 2,5-diones (diketopiperazines) proceeds with some racemization (29). Their isomerization was studied theoretically and experimentally, too (21-23). The rate constant for racemization of *cyclo*-(L-Ala-Gly) (diketopiperazine) was only 2 times that of H-Gly-Ala-OH and 7 times the rate of H-Ala-Gly-OH; it racemized 20 times andH- Gly-Ala-OH

TABLE VII
Selected 400 MHz ^{1}H NMR Data on Piperazine Mono- and Dithiones[a]

	c(Alat-Glyt)[b]	c(Alat-Ala)[c]		c(Alat-Alat)[c]		c(Prot-Gly)[c]	c(Glyt)-Pro)[b]	c(Prot-Ala)[c]		c(Prot-Alat)[c]	
		LL(DD)	LD(DL)	LL(DD)	LD(DL)			LL(DD)	LD(DL	LL(DD)	LD(DL
Gly(t)α	4.28	-	-	-	-	3.72	4.07	-	-	-	-
						4.02	4.28				
Ala α	-	3.94	4.02	-		-	-	4.16	3.85	-	-
Ala β	-	1.34	1.32	-		-	-	1.32	1.33	-	-
Alat α	4.33	4.18	4.18	4.23	4.27	-	-	-	-	4.31	4.35
Alat β	1.58	1.47	1.44	1.53	1.51	-	-	-	-	1.54	1.43
Prot α	-	-	-	-	-	4.31	4.26	4.34	4.44	4.49	4.60
Prot β	-	-	-	-	-	2.12	2(3H)			2.35	2.10
						2.38		1.75-2.4	1.75-2.	2.42	2.50
Prot γ	-	-	-	-	-	1.8-1.9	2.29(1H)			1.9-2.0	1.9-2.0
Prot δ	-	-	-	-	-	3.44	3.63			3.60	3.65
							3.76	~3.4	~3.4	3.83	3.78
CONH	-	8.2	8.2	-	-	-	8.35	-	-		-
CSNH	10.24	10.48	10.46	10.64	10.61	n.a	-	10.52	10.60	10.76	10.81
$^3JCH_{\alpha,NH}$	n.a.	<1, or ~2~		5 or ~1		n.a.	n.a.	n.a	5.0+1.5	~2	4.1	<1	4.2

[a]δ(p.p.m) relative to internal TMS in DMSO-d_6 unless otherwise stated.
[b]Solvent DMSO d_6 + $CDCl_3$
[c]Solvent DMSO d_6

66 times faster than free alanine (23). Racemization may occur during treatment of peptides with LR in dry benzene, toluene or other solvents even at room temperature (Scheme 1). Considering the rate of racemization encountered during the acidic hydrolysis of linear thiopeptides, the data in Table V suggest that the preparation of piperazine-2,5-onthiones of type *cyclo*(Aaa*t*^{1}-Aaa^2) (Aaa^2 ≠ Gly) goes practically *without racemization* if the thionation takes place prior to ring closure (Route A in Scheme 1). Glycine appears to enhance the tendency for racemization, especially in Aaa*t*-Gly position. (Note, that during acidic hydrolysis of protected endothiopeptides, (Table V) glycine more promotes racemization in Gly*t*-Aaa position.) Apparently, the racemization may also take place following the thionation reaction. Chemically and optically pure Z- or Boc-Pro*t*-Gly-OMe can be N-deprotected without racemization. The dipeptide ester H-Pro*t*-Gly-OMe undergoes cyclization, in hot alcohol, in the presence of 0.05 - 0.1 equiv. of a tertiary amine base. Surprisingly, the monothione *cyclo*(Pro*t*-Gly) was found to show practically no optical activity. Optically active (or partially active) product cannot be prepared even at extremely mild thionation and isolation conditions. Acidic hydrolysis, derivatization with Marfey's reagent and HPLC separation (28) proved that this monothione suffers complete racemization during the cyclization in alcohols or the subsequent chromatographic purification in aqueous buffers (mono- and dithio derivatives of piperazine-2,5-diones are not soluble in nonpolar solvents). Though in lower extent, glycine also promotes racemization of alanine in Aaa*t*-Gly po-

sition. Contrary to these, the isomeric monothio derivatives *cyclo*(Gly*t*-Pro) and *cyclo*(Gly*t*-Ala) can be obtained from (Z- or Boc)-Gly*t*-Pro-OMe or Gly*t*-Ala through the same steps in practically pure L-enantiomeric form. Similarly to *cyclo*(Ala*t*-Ala) and *cyclo*(Pro*t*-Pro), the source of racemization here is the acidic hydrolysis rather than the cyclization or purification of the product (cf. Table IV and V).

The racemization studies based on HPLC chromatographic separation of diasteroisomeric cyclic thiodipeptides and the Marfey's derivatives of their amino acid components lead to the following conclusions:

1. In the case of dipeptides of type Z(Boc)-Aa*at*^{1}-Aaa^2-OR (R = CH_3, C_2H_5) thionation and subsequent nonhydrolytic dethionation do not cause considerable racemization under standard conditions. The spontaneous cyclization of dipeptide esters (H-Aa*at*^{1}-Aaa^2-OR) also goes without significant racemization.

2. Acidic hydrolysis of thiopeptides results in partially racemized amino acids. The rate of racemization is much higher (25-45%) at endothiodipeptides (Z[Boc)-Aa*at*^{1}-Aaa^2-OR] than in the corresponding (oxo)peptides (< 2%) [Table IV-V.].

3. Thionation of piperazine-2,5-diones or piperazine-2,5-onthiones is always accompanied by racemization. The ratio of diastereoisomeric and/or enantiostereoisomeric products depends on the structure of the parent cyclic peptide or thiopeptide and the conditions of thionation. Due to steric reasons, thionation of *cyclo*(Pro-Pro) proceeds with partial double racemization (see Table VI). In the case of *cyclo*(Ala*t*-Alat) the meso-compound is also formed to-

gether with the racemic pair.

4. Cyclization of optically pure endothiodipeptide esters (H-Aaa*t*^{1}-Aaa^2-OR; Aaa^2 ≠ Gly) gives rise to piperazine-2,5-onthiones of type *cyclo*(Aaa*t*^{1}-Aaa^2). The comparison of chromatographic and NMR-based racemization data suggests that the cyclization and isolation of the products are free from racemization. partial racemization of the chiral amino acid residues occurs during the acidic hydrolysis. Glycine has a special positional effect on racemization: *cyclo*(Pro*t*-Gly) and *cyclo*(Ala*t*-Gly) suffer total racemization during ring-closure and isolation while *cyclo*(Gly*t*-Pro) and *cyclo*(Gly*t*-Ala) can be prepared in optically pure form.

5. The acidic hydrolysis of thiopeptides yields amino (oxo)acids. Thus, the pre-column derivatization method with Marfey's reagent followed by RP-HPLC can be applied for measurement of racemization also in the case of thiopeptides. The diastereoisomeric cyclic thiodipeptides formed in consequence of racemization can be separated efficiently by HPLC on silica columns.

Starting from our observations the theoretical basis of stereochemical behaviour of cyclothiopeptides is discussed elsewhere (30).

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