D,L-N-*Phthalyl-o-carboranylalanine t-butyl ester.* D,L-*N*-Phthalyl-propargylglycine *t*-butyl ester [6] was condensed with bis (acetonitril)-decaborane as described for **2**. The product crystallized from ethanol/H_2O: m.p. 179–180° (rhombohedral crystals), Rf 0.8 (E). – IR.: 2550 (B—H), 1770 and 1705 (C=O, phthalyl), 1730 (C=O, ester).

$C_{17}H_{27}B_{10}NO_4$	Calc.	C 49.12	H 6.55	B 25.57	N 3.37%
(415.72)	Found	,, 49.08	,, 6.39	,, 25.37	,, 3.50%

L-N-(o-*Carboxybenzoyl*)-o-*carboranylalanine.* 68 mg (0.18 mmol) of **2** were dissolved in 3 ml of EtOH, treated with 3.6 ml of 0.1 N KOH and kept for 18 h at 20°. The product was extracted by the usual procedure (ethyl acetate). Recrystallization from chloroform: 65 mg (95%), $[\alpha]_D^{30} = -32.1°$ ($c = 1$, EtOH), Rf 0.65 (A), 0.1 (F), ninhydrin negative, *Reindel-Hoppe* positive.

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226. Hormone-Receptor Interactions. Synthesis and Conformational Study of *cyclo*-L-Cystathionine[1])

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(15. VI. 76)

Summary. The purpose of this work was to see whether the replacement of a sulfur atom in a cystine disulfide bridge by a methylene group is an only superficial 'isosteric' substitution, *i.e.* with regard to size, hydrophobia, bond angles, *etc.*, or whether it would also encompass such parameters as preferred conformations in solution (*M*- or *P*-helicity of the bridge). The methods involved the synthesis of a model compound, *cyclo*-L-cystathionine (*cyclo*-L-carbacystine), and its investigation by ^{1}H- and ^{13}C-NMR. It is concluded that the conformations of the $CH_2(\beta)$—$CH_2(\gamma)$—S—$CH_2(\beta')$ bridge, and of the diketopiperazine ring are closely similar to the analogous elements in *cyclo*-L-cystine (DMSO as solvent). This knowledge might help to explain the fact that carba analogs of heterodetic-cyclic polypeptide hormones are often biologically very active.

1. Introduction. – It has been established that neither of the two conspicuous heteroatom groupings of amino-acid and peptide hormones – namely the intrachenar

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[1]) Presented in the poster session of the 14th European Peptide Symposium, Wépion, Belgium, April 1976. Abbreviations and nomenclature according to *Wünsch* [1]. This work was supported by the *Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung.*

disulfide bridges of oxytocin, vasopressin, and insulin, and the iodine atoms of the thyroxins – participate functionally in the chemical mechanisms of hormonal action. This was proved by the synthesis of analogs containing so-called isosteric replacements. Various groups of similar size and hydrophobia can be substituted for iodine without abolishing the biological activity of thyroid hormone [2]. *Rudinger* replaced one or both of the intrachenar sulfur atoms of the neurohypophyseal hormones [3] (see also *Sakakibara* [4]), and of insulin [5] by methylene groups, and demonstrated the biological activity of the resulting carba analogs.

The question arises as to whether the similarity between the methylene group and the sulfur atom extends only to such parameters as size, hydrophobia, electronegativity, bond angles, *etc.*, or also encompasses the preferred conformation in solution (*M*- or *P*-helicity of the bridges). The latter property is, of course, essential for the detailed understanding of the hormone-receptor interaction [6].

It has been shown in this laboratory that relatively simple intrachenar cystine compounds with additional stabilizing features, *e.g.* [2,7-cystine]-gramicidin S [7] and *cyclo*-L-cystine [8], contain the $CH_2(\beta)-S-S-CH_2(\beta')$ bridge in a preferred solution conformation[2]).

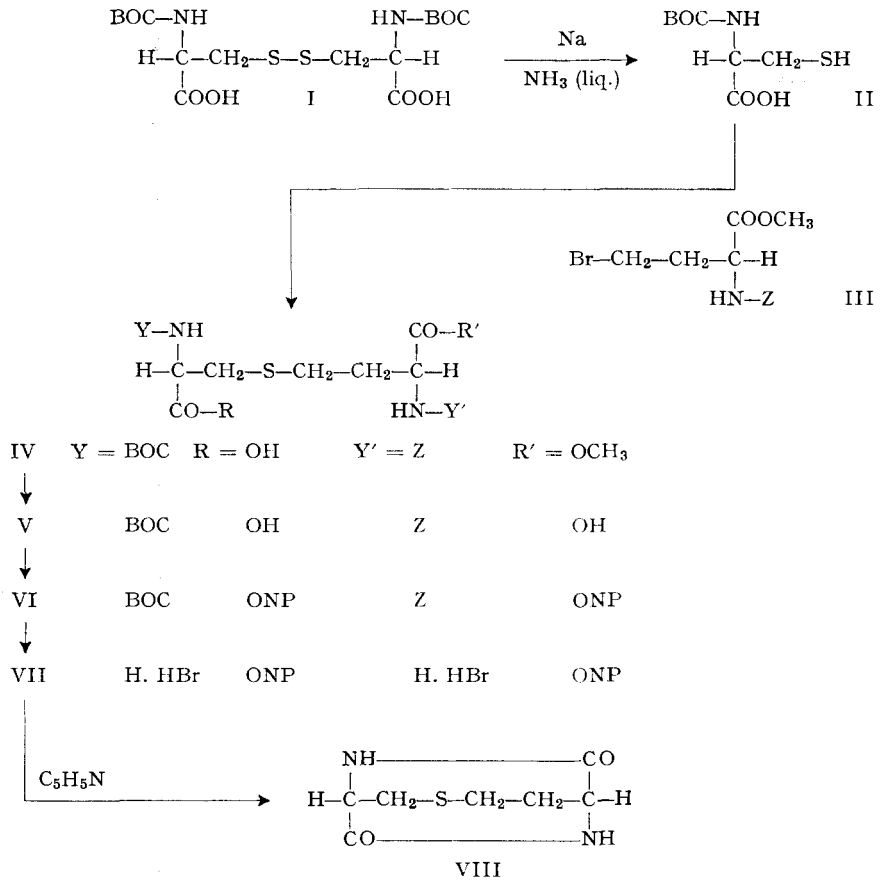
We therefore decided to prepare the carba-analog of *cyclo*-L-cystine, *cyclo*-L-cystathionine (VIII), and to study its conformation in solution by NMR. methods.

2. Chemical synthesis. – The general course of the synthesis of *cyclo*-L-cystathionine is shown in the *Scheme*. Starting from *N,N'*-bis(*t*-butoxycarbonyl)-L-cystine (I), *t*-butoxycarbonyl-L-cysteine (II) was prepared by reduction with sodium in liquid ammonia, and condensed with methyl L-α-(benzyloxycarbonylamino)-γ-bromobutyrate (III) according to the procedure of *Rudinger* [11]. The resulting dicyclohexylammonium salt of *O'*-methyl *N*-(*t*-butoxycarbonyl)-*N'*-benzyloxycarbonyl-L,L-cystathioninate (IV) was hydrolysed to the *N,N'*-asymmetrically substituted cystathionine V, a homogeneous compound that was used without further purification for the next step, the preparation of the active ester, *N*-(*t*-butoxycarbonyl)-*N'*-benzyloxycarbonyl-L,L-cystathionine di(*p*-nitrophenyl)ester (VI). Both amino-protecting groups were removed selectively by HBr in acetic acid without affecting the active ester groups [12], thus producing the dihydrobromide of L-cystathionine di(*p*-nitrophenyl)ester (VII). This key compound was cyclized in pyridine to VIII by the usual procedure for preparing homodetic-cyclic peptides [13].

3. Chemical and spectroscopic characterization of VIII. – The purity of crystalline VIII was checked by TLC., elemental analysis, and ^{1}H-NMR. The latter indicated traces of water as the only impurity. The mass spectrum was also in full agreement with the expected structure, displaying in particular the characteristic (M^+–CH_3S)-peak (19% of the M^+-peak intensity). No detectable absorption was present in the UV. region above 200 nm. The CD. spectrum exhibited only peptide

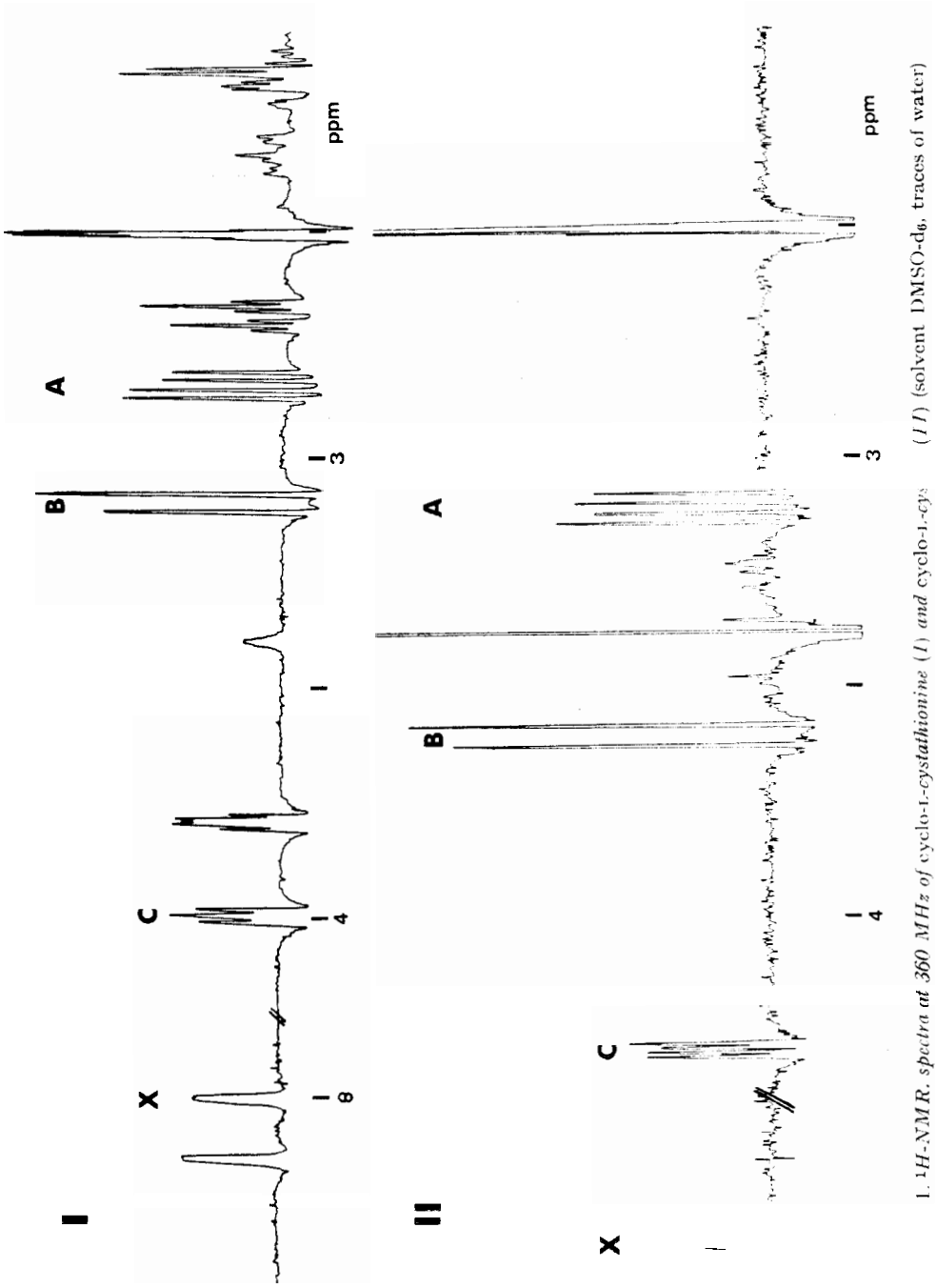
[2]) *Cyclo*-L-cystine shows evidence for the presence at room temperature of small amounts of the other diastereomer. At elevated temperatures, this conformer becomes more important [9]. Whereas *P*-helicity was tentatively assigned to the low-temperature conformer [8], crystals are composed of the *M*-helical diastereomer [10]. The question of preferred helicity in solution remains unsolved.

Scheme



contributions at 190 nm ($[\theta] = +230{,}000$) and at 220 nm ($[\theta] = -65{,}000$ deg · cm³ · decimol^{-1}). Compound VIII was – rather unexpectedly – soluble in water ($> 2 \cdot 10^{-1}$ M), whereas *cyclo*-L-cystine [8] was very insoluble.

4. Conformational properties of VIII as revealed by NMR. – *The ^1H-NMR. spectrum of VIII* obtained at 360 MHz using convolution techniques [14] is shown in Fig. 1. Unequivocal assignment of the signals was made by double resonance experiments. The only peaks off scale are those of the solvents DMSO (2.5 ppm) and water (3.4 ppm). VIII contains 10 protons in two independent spin systems separated in the molecular structure by the two carbonyl groups and by the sulfur atom (Fig. 2): (i) The four-spin-system gives rise to an approximately first order *ABCX* pattern. The amide proton appears as a broad singulet at 7.99 ppm (*X*), the H–C(α) as a quartet with two median peaks, not well resolved, at 4.03 (*C*), and the two H–C(β) as 6 resolved lines: a doublet with some fine structure centered at 3.12 (*B*) and a well resolved quartet centered at 2.85 ppm (*A*). (ii) The 6 protons of



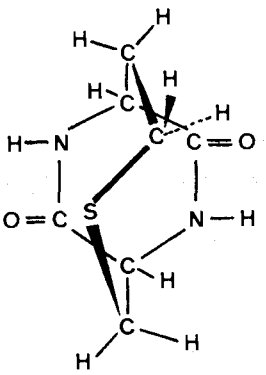
1. *1H-NMR. spectra at 360 MHz of* cyclo-L-*cystathionine* (*I*) *and* cyclo-L-cys (*II*) (solvent DMSO-d_6, traces of water)

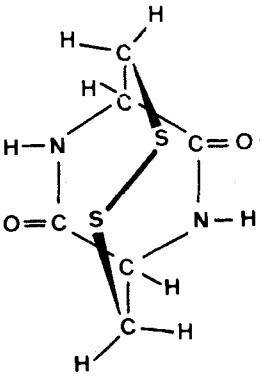
the $A'B'C'D'E'X'$ system give rise to a more complex pattern, although none of its resonances overlap with those of the $ABCX$ system. Its signals appear at 8.1 (X', broad singlet), 3.8 (E', quintet), 2.7 (D', double triplet), 2.33 (C', triplet with fine structure), and 2.23 to 2.08 ppm (A', B', multiplet with complex fine structure).

Evaluation of the ^{1}H-NMR. spectra immediately reveals the quite inflexible conformation of VIII: the geminal protons of all three methylene groups are unequivalent and display different chemical shifts and coupling constants.

The $ABCX$ and $A'B'C'D'E'X'$ spectra were computer-simulated separately as well as simultaneously, giving the same result: the two spin systems are indeed independent. The spectral parameters were approximated by the NMR. 8P program and then refined by the iterative LAOCOON II procedure [15] to give the chemical shifts and coupling constants of Fig. 2.

For the purpose of comparison, the ^{1}H-NMR. spectrum of *cyclo*-L-cystine [8] was again recorded under the same conditions as for VIII. Its spectral parameters were recalculated and assembled in Fig. 2.





spin systems

$ABCX$ ($\approx$ 1. order), and	$ABCX$ ($\approx$ 1. order, symmetrical)
chemical shifts δ (ppm)	
H_A: 2.85	3.04
H_B: 3.12	3.53
H_C: 4.03	4.12
H_X: 7.99	8.03
coupling constants (Hz)	
$J(A,B)$ = – 14.46	– 14.46
$J(A,C)$ = 6.25	6.43
$J(A,X)$ = 0	0
$J(B,C)$ = 0.44	1.06
$J(B,X)$ = 0	0
$J(C,X)$ = 3.64	3.17

Fig. 2. *Formulae and ^{1}H-NMR. spectral parameters of* cyclo-L-*cystathionine and* cyclo-L-*cystine* (molecules tentatively displayed as the *P*-helical diastereomers)

The preferred conformation of VIII. Space-filling molecular models of VIII can be built without strain from commercially available sets (*e.g. Ealing CPK*). The two possible diastereomeric forms, L configuration at the two C(α) atoms and either *M*- or *P*-helical conformation of the $CH_2(\beta)-CH_2(\gamma)-S-CH_2(\beta')$ bridge (Fig. 2), are obtained with equal ease.

From our NMR. results we can conclude that VIII, synthesized as described, exists in only one conformation at 26° in 0.03M DMSO solution. A single set of chemical shifts and coupling constants accounts for the observed spectrum, thus excluding the presence in the sample of both the *M*- and *P*-conformers (for a detailed argumentation, see [8]). However, it is impossible to decide on the screw sense of the bridge in VIII from NMR. parameters alone.

A useful conclusion can be drawn from the comparison of the spectra of *cyclo*-L-cystine and VIII. There is a striking agreement between the general pattern and between the coupling constants of the *ABCX*-systems of both compounds (Fig. 1 and 2). Examination of the molecular models shows that interconversion from one helical form to the other would result, to a good approximation, in an exchange of the *AC* and *BC vicinal* couplings in the *ABCX* system without changing the chemical shifts. Introducing this exchange into the simulation process, one obtains the theoretical spectrum of the other diastereomer of *cyclo*-L-cystine, which is very different from the experimental *ABCX* pattern of both *cyclo*-L-cystine and VIII. This is a strong piece of evidence that *cyclo*-L-cystine and its carba analog VIII have the same preferred conformation under the conditions used here.

The value of about 45° determined according to *Karplus* [16] and *Bystrov* [17] for the dihedral H–N–C(α)–H angle of the *ABCX*-system agrees with a boat conformation of VIII (and of *cyclo*-L-cystine [8]). A chair conformation is incompatible with the bridged structures.

The configurational stability of the prevalent form of VIII as a function of temperature was tested by recording the ^{13}C-NMR. spectra at room temperature and at 60° (Fig. 3). *Cyclo*-L-cystine has been reported [9] to partly interconvert (*P* to *M*) at higher temperatures, and to contain even at room temperature about 15% of the minor conformer. In our spectra of VIII, the signals of the seven carbon atoms of the molecule were readily identified, and no other signals, except those of the solvent DMSO, were detectable, even at 60°. Neither the observed chemical shifts nor the coupling constants were affected by the temperature change.

5. Conclusions. – The carba analog of *cyclo*-L-cystine, *cyclo*-L-cystathionine (VIII), is readily prepared from an active ester of L-cystathionine. That the crystalline compound VIII is more soluble in water than the disulfide is hard to explain at the present stage.

VIII is present in DMSO as *one* diastereomer with the same conformation and configuration as *cyclo*-L-cystine (*M*- or *P*-helical arrangement of the $CH_2(\beta)-CH_2(\gamma)-S-CH_2(\beta')$ bridge in conjunction with L-configuration at C(α)). This shows that an isosteric modification in the refined sense, preserving dimensions as well as conformational preferences, has been achieved in this example.

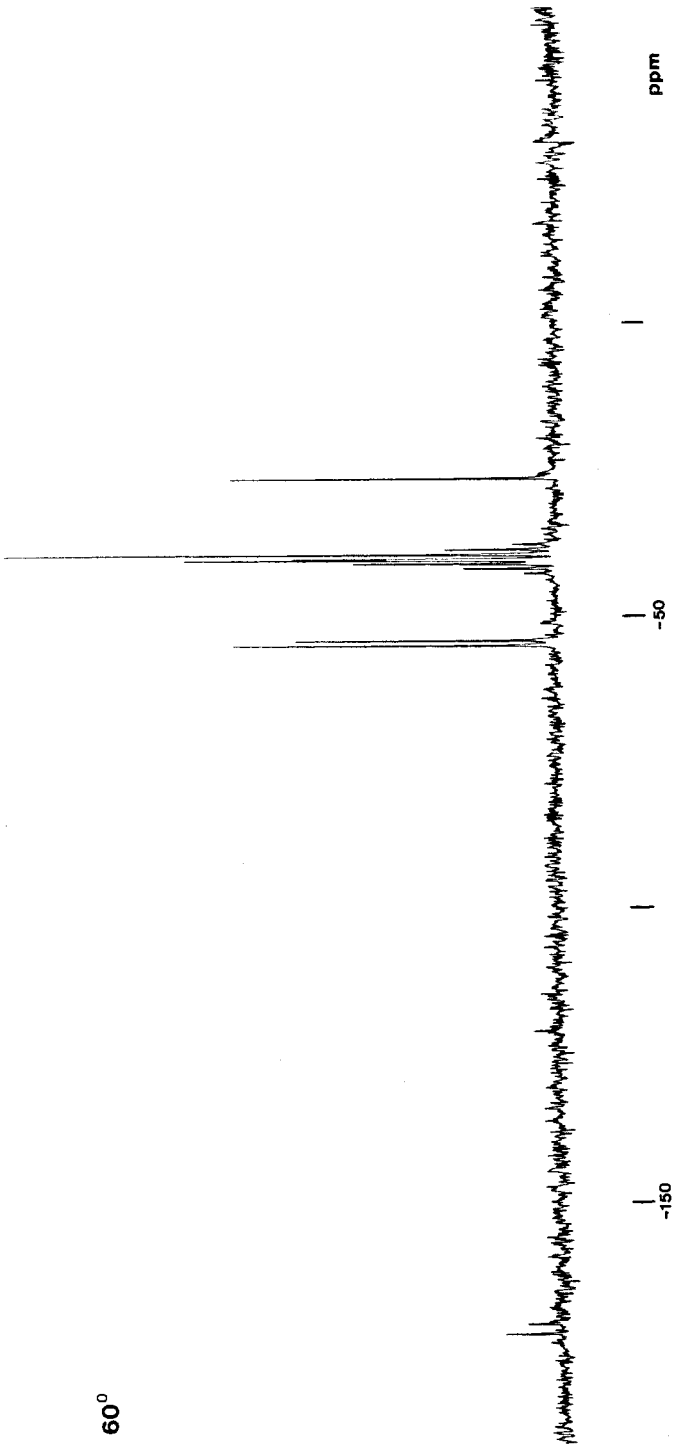


Fig. 3. ^{13}C-*NMR. spectrum of* cyclo-L-*cystathionine at 60°*. The spectrum at 26° is practically identical (chemical shifts δ (ppm): 171.3, 170.1 (2CO); 54.6, 53.6 (2C(α)); 40.0, 38.9, 26.0 (2C(β), C(γ)); the septet centered at 39.8 is from DMSO)

The existence of the preferred conformer of VIII is much less temperature-dependent than in the case of *cyclo*-L-cystine, as determined by ^{13}C-NMR. A qualitative explanation for that would invoke a more restricted rotation about the $CH_2(\beta)-CH_2(\gamma)$ bond of VIII compared to the equivalent $CH_2(\beta)-S$ bond of *cyclo*-L-cystine, and other factors including bond length and bond polarizability.

A possible general tendency of carba analogs to be present in the same preferred conformations as the parent compounds containing disulfide bridges might offer additional, refined explanations for the strong biological activities observed for many carba hormone analogs. It should be kept in mind that similar studies of complete hormonal carba analogs are difficult because of the complexity of their spectra (see *e.g.* [18]).

Experimental Part

^1H-NMR. spectra were determined in hexadeuteriodimethylsulfoxide, DMSO-d_6, at 360 MHz on a *Bruker* HXS spectrometer. Tetramethylsilane (TMS) was used as internal reference. The concentration of VIII was 5.6 mg/ml (0.03 M) and the temperature 26°. ^{13}C-NMR. spectra at 25.14 MHz, with proton noise decoupling, were obtained on the *Varian* XL100 spectrometer using the *Fourier* Transform technique. The sample contained 57 mg of VIII dissolved in 1.5 ml of DMSO-d_6. The chemical shifts are relative to TMS. 2000 pulses were accumulated: pulse with 10 μsec, acquisition time 0.66 sec, pulse delay 0.8 sec.

Purity of all compounds was checked by thin-layer chromatography (TLC.) on commercially available (*Merck*) precoated silica gel plates with fluorescence indicator. M.p.'s (uncorrected) were determined with an automatic apparatus of *Mettler Co.*, using a very slow rate of heating. Optical rotation: *Perkin-Elmer* polarimeter 141. UV. spectra: *Beckman* Acta V spectrophotometer, solvent: water. Mass spectrum: *Hitachi-Perkin-Elmer* RMU 6D. Circular dichroism (CD.): *Jouan* Roussel dichrograph, solvent: water.

Additional abbreviations: DMF = dimethylformamide, DCCI = dicyclohexyl-carbodiimide.

N-(t-*Butoxycarbonyl*)-N'-*benzyloxycarbonyl-cystathionine* (V). 652 mg (1 mmol) of the dicyclohexylammonium salt of IV was dissolved in 10 ml of 2N NaOH and 5 ml of methanol, and the solution stirred at room temp. during 2 h. After removing the methanol the aqueous solution was completely evaporated at pH 7. The solid residue was then dissolved in a small amount of water, and extracted into ethyl acetate at pH 2 after cooling. Washing with saturated sodium chloride solution, and removing of the solvent afforded a solid compound, which proved to be homogeneous by TLC. (1-butanol/AcOH/H_2O 4:1:1), Rf 0.66. It was used in the next step without further purification. Yield 400 mg (87%).

N-(t-*Butoxycarbonyl*)-N'-*benzyloxycarbonyl-cystathionine di*(p-*nitrophenyl*) *ester* (VI). 456 mg (1 mmol) of V and 305 mg (2.2 mmol) of *p*-nitrophenol were dissolved in 5 ml of DMF. The solution was cooled to 0°, and 453 mg (2.2 mmol) of DCCI were added. The reaction was allowed to proceed for 1 h at 0°, and 16 h at room temp. The mixture was filtered, and the filtrate evaporated at *ca.* 1–3 Torr. Repeated crystallization from ether afforded the active ester. The product was homogeneous on TLC. ($CHCl_3$/MeOH 9:1, Rf 0.75), whereas elemental analysis showed a contamination of about 5 weight-% of dicyclohexylurea. M.p. 91°, yield 617 mg (88%), $[\alpha]_D^{20} = +44.3°$ (c = 1, ethanol).

$C_{32}H_{34}O_{12}N_4S$	Calc.	C 55.0	H 4.91	N 8.02	S 4.59%
(698.58)	Found	,, 55.7	,, 5.20	,, 8.24	,, 4.44%

Cystathionine di(p-*nitrophenyl*) *ester dihydrobromide* (VII). One-step deprotection of both amino groups of VI was accomplished with 33% HBr in acetic acid (5 ml for 1.4 g of VI) in the presence of a few drops of anisole as scavenger. After 1 h at room temp. the product was precipitated adding dry ether, and, after filtration, kept in a dessicator over NaOH pellets. Yied 1.1 g (67%).

The hygroscopic compound was ninhydrin positive, and homogeneous on TLC. (1-butanol/AcOH/H_2O 4:1:1, Rf 0.35).

Cyclo-L-*cystathionine* (VIII). 830 mg (1.3 mmol) of VII were dissolved in 10 ml of DMF containing a few drops of acetic acid. This solution was slowly added to 600 ml of pyridine at 65° over a period of 10 h. Then the reaction was allowed to run overnight. The solvents were evaporated, and the gummy residue triturated with dry ether, then with chloroform, and finally extracted into water. The aqueous layer was washed with chloroform, then stirred with 1.5 ml of an acidic, then with 1.5 ml of an alcaline Dowex 50 resin. After filtration the aqueous solution was evaporated. The solid residue was triturated in ethanol, and centrifuged. The pellet was crystallized from ethanol/chloroform: 65 mg (35%). Purity was checked by TLC. ($CHCl_3$/MeOH 1:1, Rf 0.6; 2-BuOH/AcOH/H_2O 10:1:3, Rf 0.44). M.p. 304°, $[\alpha]_D^{20} = +181.1$ ($c = 1$, H_2O). – MS.: 186 (100, M^+), 171 (2, $M^+ - CH_3$); 158 (4.5, $M^+ - CO$); 143 (7, $M^+ - CH_3 - CO$, $M^+ - NH - CO$); 139 (19, $M^+ - CH_3S$).

$C_7H_{10}O_2N_2S$	Calc.	C 45.16	H 5.41	N 15.05	S 17.22%
(186.1)	Found	,, 44.98	,, 5.50	,, 14.82	,, 17.25%

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