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Endogenous alkaloids in man XXI.[☆] Analysis of glyoxylate-derived 1,3-thiazolidines and their precursors after trimethylsilylation by gas chromatography–mass spectrometry

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Abstract

A gas chromatographic procedure was developed for the simultaneous analysis of glyoxylate-derived 1,3-thiazolidines and their precursors, such as L-cysteine, cysteamine, and D-(−)-penicillamine as well as the toxic glyoxylic acid. The assay involves conversion of these highly polar compounds to volatile trimethylsilyl (TMS) derivatives, chromatography on a polar fused-silica capillary column, and determination using flame-ionization detection or electron-impact ionization mass spectrometry. On the base of this analytical device, the resolution of the diastereomeric pairs of 1,3-thiazolidine-2,4-dicarboxylic acids was achieved. Based upon the observation that no epimerization occurs during the silylation procedure, for the first time a reliable method was established for the determination of the diastereomeric ratio of such alkaloid-type heterocycles on a trace scale. Furthermore, studies concerning thiazolidine formation under derivatization conditions in the presence of the precursors glyoxylic acid and D-(−)-penicillamine are described.

1. Introduction

Primary hyperoxaluria (HOU) type I is a rare, but severe and hitherto incurable inherited disease [2,3]. Its toxic principle, an overproduction of oxalic acid, followed by an accumulation of calcium oxalate, is the result of an enzymatic block in the degradation pathway of glyoxylic acid (**1**). This highly reactive aldehyde is the most important metabolic precursor to oxalic acid, which itself cannot be detoxified further in humans [4]. Due to the low solubility of calcium oxalate, hyperoxaluria is characterized by serious pathological symptoms, such as urolithiasis, nephrocalcinosis, and, in severe cases, systemic as well as cerebral oxalosis. Most of the patients die from chronic renal failure in early adulthood [2,3]. But also exogenous factors, such as ethylene glycol intoxications by accident or suicide [5,6] as well as medicinally indicated therapies [7–9] cause an extreme increase in glyoxylate or oxalate concentration in the living organism. The consequences thereof are atrocious: severe central nervous system dysfunctions were observed upon treatment with glycine during transurethral surgery [7] or after post-operative administration of xylitol as an intravenous nutrient [8,9].

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☆ For part XX, see Ref. [1].

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Chemical deactivation of the toxin glyoxylic acid (**1**) by therapeutical administration of sulphur-containing binucleophilic amino acids or biogenic amines, leading to thiazolidine formation (see Fig. 1), is the key step of a therapeutical concept for the treatment of glyoxylate-induced oxalurias recently developed [10–14]. The realization of such a novel, still speculative approach requires the study of the metabolic "fate" of such chemical detoxication products, and thus the elaboration of an efficient analytical procedure.

This becomes evident from apparently contradictory results described in the literature for two possible glyoxylate scavengers. Thus, studies by Hamilton et al. [15–17] show that L-cysteine (**2**) and cysteamine (**3**) indeed spontaneously condense in vivo, leading to the thiazolidines **5a**, **5b** and **6**, respectively (see Fig. 1). These heterocycles, however, are subsequently metabolized by the organism, ultimately resulting in the undesired formation of oxalic acid as final product. By contrast, an Australian group [18,19] treated rats with daily doses of L-cysteine i.p., with a simultaneous addition of ethylene glycol to the drinking water, and found a distinct decrease in the excretion of oxalic acid. This reduction, which was explained by a possible formation of the thiazolidine **5**, was significant compared with control animals without a cysteine treatment.

Our concept [10–14] favours the non-natural amino acid D-(−)-penicillamine (**4**) as a scavenger for glyoxylic acid (**1**), since it does not undergo an extensive metabolism [20], and thus stays available for an in vivo cyclocondensation with **1** for a longer time. Meanwhile, we could demonstrate that the 1,3-thiazolidine-2,4-dicarboxylic acids **7a** and **7b** as resulting from this rapid and spontaneous condensation reaction (see Fig. 1), are extremely hydrophilic. First animals studies revealed their excellent physiological compatibility. Furthermore, the calcium thiazolidine dicarboxylate **Ca-7a** exhibits a high solubility, $1.8 \cdot 10^4$ times larger than that of calcium oxalate [12].

From the above-mentioned results it becomes evident that with respect to a possible therapeutic application of such scavenging reactions as presented in Fig. 1, a detailed knowledge of

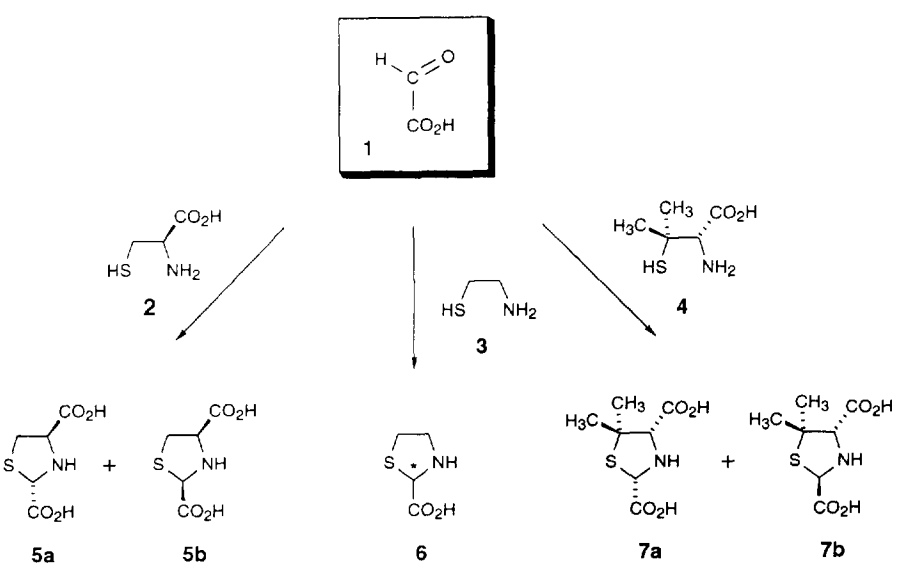


Fig. 1. Formation of the glyoxylate-derived 1,3-thiazolidines **5a**/**5b**, **6**, and **7a**/**7b** by condensation with L-cysteine (**2**), cysteamine (**3**), and D-(−)-penicillamine (**4**), respectively.

optimum condensation conditions, of the reaction course, and the epimerization behaviour of the thiazolidines **5a** and **5b** as well as **7a** and **7b** is indispensable. In this connection, especially investigations concerning the in vivo fate of these heterocycles are of great importance, too. This, however, requires the availability of appropriate analytical procedures that allow the unambiguous identification of these compounds even in low, biologically relevant concentrations.

TLC [21] and HPLC [22] analytical as well as spectroscopic [23] methods, as well as our own investigations [24] aiming at a direct detection of carboxy-substituted 1,3-thiazolidines show that, apparently because of their highly hydrophilic and their amphoteric character, these compounds are not easily accessible to a direct analysis. On the base of ion-pair assisted reversed-phase chromatography, we have developed an analytical device that allows, for the first time, the resolution of the highly functionalized diastereomeric 1,3-thiazolidine-2,4-dicarboxylic acids **5a** and **5b**, respectively **7a** and **7b**. Due to the absence of a characteristic UV chromophor and the low-intensity UV maximum, which is located almost exactly at the UV cut-off of the utilized solvents, application of this HPLC method is restricted to samples containing amounts of **5**, **6**, and **7** that are significantly above the trace range [24,25]. By contrast, derivatization of the thiazolidines with fluorescence reagents, e.g. dansyl chloride or 9-fluorenylmethyl chloroformate, leads to an enhanced sensitivity, allowing the separation and detection of the heterocycles **5**, **6**, and **7** by HPLC even from biological matrices. With respect to the required diastereomeric analysis, however, these reactions are most disappointing, because, probably due to steric reasons, only the *cis*-thiazolidines **5a** and **7a** react [25,26].

In this paper, we present a gas chromatographic analytic device, which, after trimethylsilylation, allows the separation of all the starting materials (**1**, **2**, **3**, and **4**) and products (**5a**, **5b**, **6**, and **7a**, **7b**) of the scavenging reactions as relevant for our therapeutical concept. Furthermore, it permits a direct physical identification on the base of mass spectrometry.

2. Experimental

2.1. Chemicals

Glyoxylic acid monohydrate was purchased from Merck (Darmstadt, Germany). Cysteamine hydrochloride and L-cysteine were obtained from Fluka (Buchs, Switzerland), whereas D-(−)-penicillamine was a generous gift from Degussa (Hanau, Germany). The derivatizing reagent *N*-methyl-trimethylsilyl-trifluoroacetamide (MSTFA) was supplied from Macherey and Nagel (Düren, Germany). All reagents and solvents used were of analytical grade quality. High-purity Milli-Q water (Millipore, Bedford, MA, USA) was used throughout.

2.2. Syntheses

The thiazolidines **5**, **6**, and **7** were synthesized according to procedures already published [27–30], and described in a previous paper [24]. The structures of the diastereomeric products **7a** and **7b** were unequivocally established by spectroscopic [31] and by X-ray crystallographic methods [31–33].

2.3. Gas chromatography

Gas chromatography was performed on a Model HRGC 5160 Mega gas chromatograph equipped with a split–splitless injector and a flame-ionization detector (FID) (Carlo Erba Instruments, Milan, Italy). The analyses were carried out on a DB 225 fused-silica capillary column (J&W Scientific, Folsom, CA, USA) with 0.25 μm film thickness (30 m × 0.32 mm I.D.). Helium was used as the carrier gas at a column head pressure of 80 kPa. The samples were introduced into the capillary column using the split injection mode at a split ratio of 1:60. The injection port and the detector were both maintained at 220°C. The column oven temperature was initially set at 100°C for 3 min, then programmed at 10°C/min to 220°C, then held for 5 min. The chromatograms were recorded and processed by computer-aided methods using Maxima evaluation software 820 (Millipore, Ven-

tura, CA, USA) in combination with a Maxima system Interface I-200.

2.4. Mass spectrometry

For mass spectral identification of TMS derivatives of the amino thiols, glyoxylic acid, and the glyoxylate-derived thiazolidines, a Varian Model 3700 gas chromatograph (Varian Instruments, Sunnyvale, CA, USA) coupled with a Finnigan MAT Model 8200 mass spectrometer (Finnigan MAT, San Jose, CA, USA) was used. All experimental work was done on a DB 17 fused-silica capillary column (J&W Scientific, Folsom, CA, USA) with 0.25 μm film thickness (30 m × 0.32 mm I.D.) using helium as the carrier gas. For the separation, the same temperature program as described above was applied. Electron-impact (EI) mass spectra were recorded at 70 eV and a helium carrier gas head pressure of 80 kPa. Operations in the chemical ionization (CI) mode were performed with isobutane as the reagent gas at a similar carrier gas head pressure, but the ion source pressure was increased by admitting isobutane via a separate capillary inlet (reagent gas pressure 0.3 mbar). The temperature of the transfer line was maintained at 250°C. The ion source temperature was set at 220°C in the EI mode (respectively 140°C in the CI mode). The emission current was 1 mA in the EI mode and 0.05 mA in the CI mode, respectively. The electron multiplier voltage was set at 3000 V. Full mass spectra (EI mode 35–550 amu, CI mode 60–600 amu) were recorded every 0.7 s over the entire elution profile.

2.5. Derivatization

Dimethylester derivatives

For esterification the thiazolidine **7** (**7a**/**7b** = 28:72) was dissolved in an ether solution of diazomethane [33] and stirred at room temperature for 12 h. After removal of the solvent in vacuo, the residue was chromatographed on silica gel with ether–petroleum ether (1:1) as an eluent to give the diastereomeric esters **8a** and **8b** as well as the *N*-methylated compounds **9a** and **9b**, respectively [31]. For GC analysis, the isolated pure dimethylester derivatives as well as aliquots of the reaction mixture were used. From a series of experiments with pure **7a** or mixtures **7a**/**7b** of different diastereomeric ratios, it could be deduced that **7a** gives rise to **8a** and **9a**, exclusively, whereas **7b** specifically delivers the esters **8b** and **9b**.

Trimethylsilyl (TMS) ester derivatives

The thiazolidines **5a**, **5b**, **6**, **7a** and **7b** as well as their precursors **1**, **2**, **3** and **4** can be derivatized by silylation reagents. Derivatives are formed by the exchange of the active hydrogens of amino or acid functions by trimethylsilyl groups. For the formation of TMS ester derivatives, the reaction procedure was as follows. An aliquot of a standard solution was placed in a screw-cap vial and evaporated to dryness. The dry residue was dissolved in 100 μl of MSTFA and the derivatization reaction was allowed to proceed at 80°C for 10 min. A 2-μl volume of this solution was directly injected onto the gas chromatograph.

2.6. Calibration graphs, reproducibility and linearity

For an examination of the reproducibility and linearity of the described silylation technique with MSTFA, as obtained by gas chromatographic analysis of the resulting TMS derivatives with flame-ionization detection, analysis functions $m = f(R)$, where m is the mass of the analyte and R is the detector response, were determined for the thiazolidines **5a**/**5b**, **6**, **7a**/**7b** and for the precursors **1** and **4**.

Stock solutions of the thiazolidines **5**, **6**, and **7** were prepared by dissolving 10.04 mg of **5** (**5a**/**5b** = 46:54) in 1 ml of 2 *M* HCl, 5.27 mg of **6** in 1 ml of methanol, and 20.34 mg of **7** (**7a**/**7b** = 37:63) in 2 ml of Milli-Q-water. Various aliquots of the undiluted (for **5** and **6**: 20, 40, 60 μl; for **7**: 30 μl) and of the ten-fold diluted stock solution (for **5** and **6**: 20, 40, 60, 80, 100 μl; for **7**: 20, 40, 70, 100 μl), respectively, were pipetted into Wheaton-V vials and evaporated to dryness. For the generation of data for calibration graphs, 2 μl of these solutions were injected onto the GC

system mentioned above, after derivatization with 100 μl of MSTFA.

For the aldehyde carboxylic acid **1** and the amino thiol **4**, respectively, six-point calibration graphs were generated by analyzing standard solutions silylated in the same way as described above. Stock solutions of **1** and **4** were prepared in Milli-Q water at concentrations of 5.3 mg/ml (solution of **1**) and 5.05 mg/ml (solution of **4**). For the calibration of the two substances, aliquots of the undiluted (30 and 50 μl) and of the ten-fold diluted stock solutions (20, 40, 70, and 100 μl) were used.

In each instance, calibration curves were constructed by plotting the integrated peak area against the corresponding standard concentration of the compounds **1**, **4**, **5a**, **5b**, **6**, **7a**, and **7b**. For all these substances, a linear correlation between the injected mass and the detector response was stated over the whole concentration range, with correlation coefficient $r > 0.98$.

2.7. Determination of the diastereomeric ratio of 7a/7b

In order to compare the diastereomeric ratio of **7a**/**7b**, as determined by ^{1}H NMR spectroscopy with those obtained by gas chromatography of the TMS derivatives **10a**/**10b**, samples containing only the *cis*-thiazolidine **7a** as well as mixtures of **7a** and **7b** were analyzed. For all measurements (^{1}H NMR, GC), pyridine was used as the solvent to guarantee epimerization-free conditions.

For the determination of the *cis*/*trans* ratio of mixtures of **7a** and **7b**, two series of tests were carried out. Stock solutions of the thiazolidine **7** were prepared by dissolving 10.04 mg (**7a**/**7b** = 50:50; solution A) and 6.43 mg (**7a**/**7b** = 29:71; solution B) in 1 ml of pyridine. Aliquots of the undiluted [60 μl (S 1), 40 μl (S 2), and 20 μl (S 3)] and of the ten-fold diluted stock solution A [100 μl (S 4), 60 μl (S 5), and 40 μl (S 6)] were evaporated to dryness. After derivatization with 100 μl of MSTFA, the diastereomeric ratio was determined by GC analysis. Volumes of 100 μl (S 7), 80 μl (S 8), and 60 μl (S 9) of the stock solution B were treated in the same way as described above.

Silylation of the pure *cis*-thiazolidine **7a** yielded only one peak for the corresponding TMS derivative **10a**. Samples containing an aliquot of 100 μl (S 10) and 50 μl (S 11) of a solution of 4.46 mg of **7a** in 1 ml of pyridine were used for GC analysis (see Table 3).

2.8. Condensation reaction of glyoxylic acid (1) and D-(−)-penicillamine (4) under silylation conditions

In order to determine the reliability of the derivatization procedure of **7** in the presence of the thiazolidine precursors **1** and **4**, we analyzed six samples concerning the formation of the heterocycles **7a** and **7b** under silylation conditions. The samples were prepared as follows. A volume of 20 μl of a solution of 10.12 mg of glyoxylic acid (**1**) in 2 ml of Milli-Q water was pipetted into a Wheaton-V vial and cooled down to −196°C. To this frozen sample an aliquot of 20 μl of a solution of 10.10 mg of D-(−)-penicillamine (**4**) in 2 ml of Milli-Q water was added and immediately frozen at −196°C. After lyophilization, the dry residue was dissolved in 100 μl of MSTFA. For derivatization, the sample solution was heated at 80°C for 10 min and the reaction mixture was subsequently analyzed by GC–FID and GC–MS, respectively.

In the chromatograms of these samples a hitherto not detected reaction product appeared, besides the well known bis-trimethylsilyl esters **10a**, **10b** as well as the additionally *N*-derivatized TMS compound **11**. Based upon GC–MS analysis, this new peak was interpreted as the silylated thiazolidine derivatives **20**/**21** (see Fig. 5) because of the following mass fragmentation pattern: GC–MS (70 ev): *m*/*z* (%) = 496 (3.9) $[M - CH_3]^{+\cdot}$, 468 (0.6) $[M - CO - CH_3]^{+\cdot}$, 421 (0.8) $[M - HOSi(CH_3)_3]^{+\cdot}$, 406 (2.5) $[M - HOSi(CH_3)_3 - Si(CH_3)_3]^{+\cdot}$, 394 (6.4) $[M - CO_2Si(CH_3)_3]^{+\cdot}$, 350 (2) $[M - CO_2Si(CH_3)_3 - CO_2]^{+\cdot}$, 292 (43) $[C_{11}H_{26}NO_2SSi_2]^{+\cdot}$, 219 (100) $[C_{11}H_{26}NO_2SSi - Si(CH_3)_3]^{+\cdot}$, 147 (32) (see also Section 3.7).

Quantitation of the amounts of starting material (**1** and **4**, respectively) and resulting products (**7a** and **7b**, respectively) was achieved by external calibration (for calibration graphs see Section 2.6). Estimation of the amount of the compounds **20/21** detected in these experiments in addition to the usually formed thiazolidine TMS derivatives **10a/10b** and **11** was done by use of the calibration graph of the *cis*-thiazolidine **7a** (see Fig. 6).

3. Results and discussion

For the investigation of each of the condensation reactions of glyoxylic acid (**1**) as presented in Fig. 1, the elaboration of a GC analytical device for the identification of all the starting materials and products was the first crucial task to be fulfilled. Thus, we aimed at the development of a derivatization procedure simultaneously suited for the aldehyde **1** and for the binucleophiles **2**, **3**, and **4** as well as for the thiazolidines **5a/5b**, **6**, and **7a/7b**. For this purpose, we primarily tested conventional methods like methylation with diazomethane [35,36], and silylation with commercially available reagents, such as *N*-methyl-trimethylsilyl-trifluoroacetamide (MSTFA) or bis-trimethylsilyl-trifluoroacetamide (BSTFA) [37–40].

3.1. Methylation

From the point of view of such a required uniform derivatization procedure for all the compounds to be analyzed, the methylation turned out to be unsatisfying: upon attempted esterification of the thiazolidines **7a** and **7b** with diazomethane, in each case two reaction products per thiazolidine were formed, namely the desired thiazolidine dicarboxylic esters **8a** and **8b**, giving yields of 60–70%, yet along with the *N*-methyl-thiazolidines **9a** and **9b** (ca. 30–40%) [31]. Although the methyl esters **8a**, **8b**, **9a**, and **9b** can efficiently be separated by gas chromatography on a polar DB 225 capillary column (see Fig. 2), a further use of this derivatization reaction did not seem rewarding, all the more as

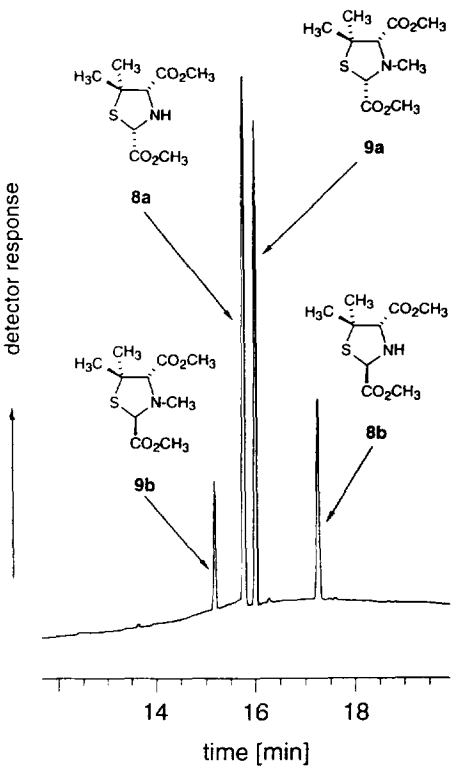


Fig. 2. Gas chromatographic analysis of the dimethylester derivatives **8a**, **8b**, **9a** and **9b** resulting from esterification of the penicillamine-derived thiazolidines **7a** and **7b** (*cis*/*trans* ratio 28:72) with diazomethane. GC separation was done on a DB 225 capillary column. For determination flame-ionization detection was used. Detailed synthetic procedures and chromatographic conditions are described in Section 2.

it is known from the literature [41] that also glyoxylic acid (**1**), on reaction with diazomethane, gives up to four reaction products.

3.2. Trimethylsilylation

By contrast, the goal of a generally applicable analysis should be the unambiguous derivatization to give only one single product, thus allowing a maximum of sensitivity. For this purpose, the transformation of the thiazolidines and their precursors into volatile trimethylsilyl (TMS) derivatives, using *N*-methyl-trimethylsilyl-trifluoroacetamide (MSTFA), seemed much more promising.

Of predominant interest was the analysis of the heterocycles **5a/5b**, **6**, and **7a/7b**. Thus, using a large excess of MSTFA, the dicarboxy-substituted thiazolidines **5** and **7** can each be reproducibly transformed at 80°C and a reaction time of 10 min, into a single volatile derivative. After significantly longer reaction times or in the case of retarded analysis, two additional peaks were observed in the chromatogram for the penicillamine-derived thiazolidines **7**, and one further product for the cysteine-derived heterocycles **5**, albeit to a small degree ($< 3\%$) in both cases.

For the cysteamine-derived thiazolidine **6**, which was treated with MSTFA under identical conditions, an immediate analysis frequently showed the presence of still two reaction products, one of which, however, was further converted in favour of the second one in course of the time.

3.3. Identification of the TMS derivatives by mass spectrometry

Mass spectroscopic investigations concerning the derivatization reaction of the penicillamine-derived heterocycles **7a/7b** as well as the cysteine-derived compounds **5a/b** with MSTFA revealed the main products of these two-fold carboxy-substituted thiazolidines to be the bis-trimethylsilyl esters **10a/10b** respectively **13a/13b**, whereas the side-products turned out to be the additionally *N*-derivatized trimethylsilyl esters **11** respectively **14** (see Fig. 3). In the case of the cysteamine-derived thiazolidine carboxylic acid **6**, the *N,O*-bis-derivatized compound **16** was the main product of the silylation reaction (see Fig. 3). The initially formed side-product (see Section 3.2) could not be elucidated by mass spectrometry.

Apparently, the high steric demand of the bulky trimethylsilyl group is responsible for the observed product selectivity in the trimethylsilylation of the 2,4-dicarboxy-substituted thiazolidines, compared with the introduction of the distinctly smaller methyl group using diazomethane.

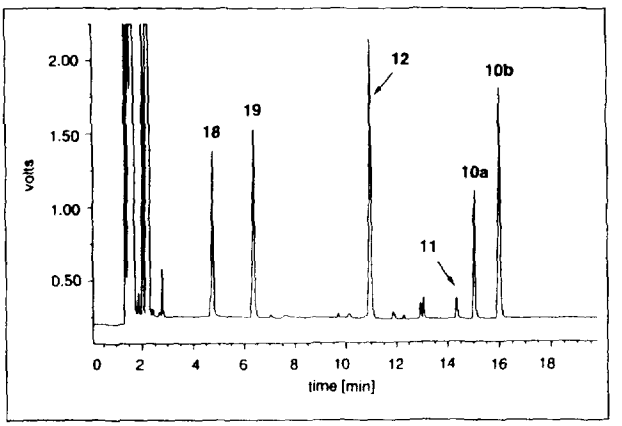
Fortunately, the mass spectrometric analysis using the electron-impact (EI) ionization mode showed a characteristic fragmentation pattern for the TMS derivatives **10a/10b**, **13a/13b**, and **16** (see Table 1). Thus, in all cases, the base peak (100%) of the obtained mass spectra was the $[M - COOSi(CH_3)_3]^{+\cdot}$ peak, which can be used as leading fragment for the compound, even in a complex environment. As an indicator for the entire molecular mass, the $[M - 15]^{+\cdot}$ and the $[M - 43]^{+\cdot}$ peaks have to be used, since the $[M]^{+\cdot}$ peak itself could be identified only in one single case. Characteristic for the thiazolidine system and thus independent from the silylation degree are those peaks that result from a loss of the whole functionality, i.e. signals at m/z 114 for the penicillamine-derived heterocycles **7a/7b**, and m/z 86 for the cysteine- as well as the cysteamine-derived thiazolidines **5a/5b** and **6**.

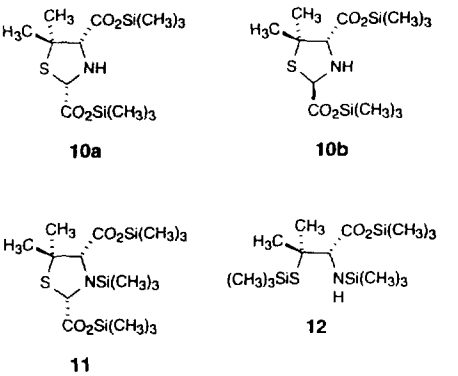
With the chemical ionization (CI) as a mild ionization technique, the $[MH]^+$ ion is obtained as the base peak, thus delivering unambiguous information on the molecular mass. As already described above for the EI spectra of the TMS derivatives **10a/10b**, **13a/13b**, and **16**, also in the CI mode mass fragments were observed hinting at the loss of a methyl group $[MH^+ - 16]$ respectively originating from a formal elimination of a trimethylsilylated carboxyl function $[MH^+ - 118]$ (see also Table 2).

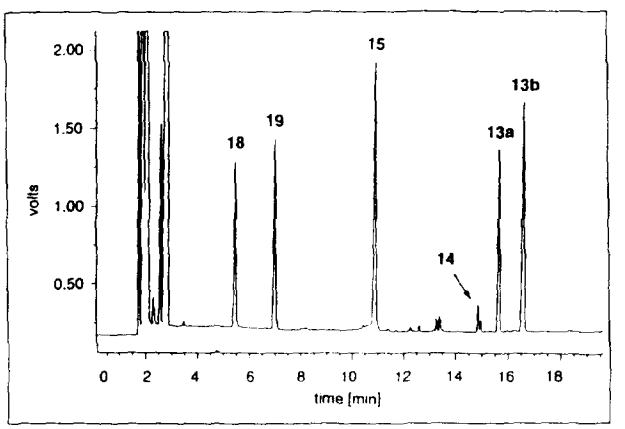
In Fig. 4 the EI and CI mass spectra, respectively, of the TMS derivative **10a** are given with the fragmentation pattern as typical for silylated thiazolidine ring systems. Table 1 (EI) and Table 2 (CI) show the characteristic mass spectroscopic data of all the glyoxylate-derived thiazolidines investigated here.

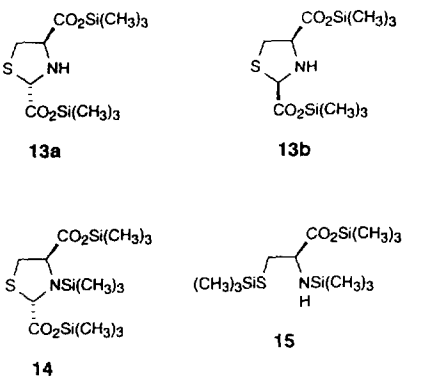
Similarly unambiguous and already described in the literature [42] is the trimethylsilylation of the amino acids L-cysteine (**2**) and D-(−)-penicillamine (**4**). This led to the formation of only one derivative each, which were identified to be the *N,O,S*-tris-trimethylsilylated compounds **15** and **12** (see Fig. 3) according to electron-impact mass spectrometrical analysis. Again, the characteristic $[M - COOSi(CH_3)_3]^{+\cdot}$ signals were used for the identification.

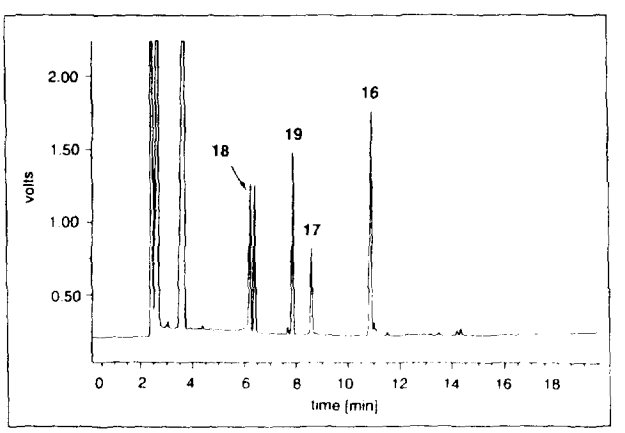
By contrast, the detection of the biogenic amine cysteamine (**3**) required further optimi-











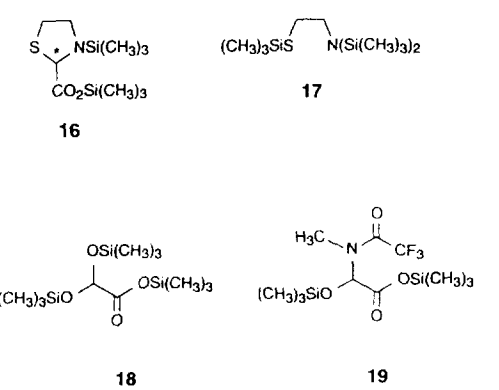


Fig. 3. Gas chromatographic analysis of the glyoxylate-derived thiazolidines **5a**/**5b**, **6** and **7a**/**7b** as well as their precursors **1**, **2**, **3** and **4**, after trimethylsilylation with MSTFA. GC separation of the resulting TMS derivatives (the inset formulas show their structures) was done on a DB 225 capillary column using flame-ionization detection for determination. For detailed derivatization and chromatographic conditions see Section 2.

Table 1
Electron-impact (70 eV) mass fragmentation pattern of the TMS derivatives **10a**, **10b**, **11**, **13a**, **13b**, **14** and **16** of glyoxylate-derived thiazolidines

Compound	$[M]^{+\bullet}$	$[M-15]^{+\bullet}$	$[M-43]^{+\bullet}$	$[M-117]^{+\bullet}$	$[M-234]^{+\bullet}$	$[M-307]^{+\bullet}$
10a	–	334 (10)	–	232 (100)	114 (25)	–
10b	–	334 (5)	–	232 (100)	114 (41)	–
11	421 (1)	406 (2)	378 (4)	304 (100)	–	114 (18)
13a	–	306 (4)	278 (1)	204 (100)	86 (52)	–
13b	–	306 (6)	278 (1)	204 (100)	86 (27)	–
14	–	378 (3)	350 (4)	276 (100)	–	86 (3)
16	–	262 (2)	234 (2)	160 (100)	–	–

Formal fragments: 117 = $COOSi(CH_3)_3$; 234 = $2COOSi(CH_3)_3$; 307 = $2COOSi(CH_3)_3 + Si(CH_3)_3$. Numbers in parenthesis represent relative intensities.

zation. As for the glyoxylate-derived compound **6**, also in this case initially two silylation products were found. In the presence of active silylation reagent, one of these products, probably an incompletely silylated compound, which could not fully be elucidated structurally, was transformed into the other derivative, identified as **17** by mass spectrometry (EI mode). The molecular mass of the TMS compound **17** was deduced from the mass peak *m/z* 278 $[M-15]^{+\bullet}$ resulting from the loss of a methyl group of a TMS unit, a decomposition process typical for TMS derivatives [43].

For the reaction of aldehydes with MSTFA the formation of addition products has been described [44]. Thus, for the trimethylsilylation of the aldehyde glyoxylic acid (**1**), the formation of two products had to be taken into account that should not be further convertable: **18**, the silylated hydrate of the aldehyde function, and **19**, the silylated product of an addition of the *N*-nucleophile *N*-methyl-trifluoroacetamide to the carbonyl group.

In the GC and MS analysis of silylation reactions of glyoxylic acid (**1**), indeed both the completely silylated product **18** and the substitution product **19** could be identified (see Fig. 3).

3.4. Gas chromatographic separation

Due to the formation of two different TMS derivatives, the detection sensitivity for glyoxylic acid (**1**) was diminished down to a factor of approximately 50%. With respect to the uniform derivatization procedure that is also easy to handle for all the precursors and products of the

Table 2
Chemical ionization (reagent gas: isobutane) mass fragmentation pattern of the TMS derivatives **10a**, **10b**, **11**, **13a**, **13b**, **14** and **16** of glyoxylate-derived thiazolidines

Compound	$[M+57]^+$	$[M+43]^+$	MH^+	$MH^+ - 16$	$MH^+ - 74$	$MH^+ - 118$	$MH^+ - 190$	$MH^+ - 235$
10a	406 (5)	392 (7)	350 (100)	334 (4)	276 (2)	232 (38)	–	114 (6)
10b	406 (3)	392 (5)	350 (100)	334 (3)	276 (2)	232 (36)	–	114 (5)
11	–	–	422 (100)	406 (5)	348 (39)	304 (58)	232 (22)	–
13a	378 (8)	364 (5)	322 (100)	306 (10)	248 (4)	204 (45)	–	87 (7)
13b	378 (8)	364 (6)	322 (100)	306 (9)	248 (3)	204 (40)	–	87 (5)
14	–	–	394 (100)	378 (6)	320 (46)	276 (63)	204 (18)	–
16	334 (9)	320 (6)	278 (100)	262 (5)	–	160 (49)	–	–

Formal fragments: 74 = $Si(CH_3)_3 + H$; 118 = $COOSi(CH_3)_3 + H$; 235 = $2COOSi(CH_3)_3 + H$; 190 = $COOSi(CH_3)_3 + Si(CH_3)_3$. Numbers in parenthesis represent relative intensities.

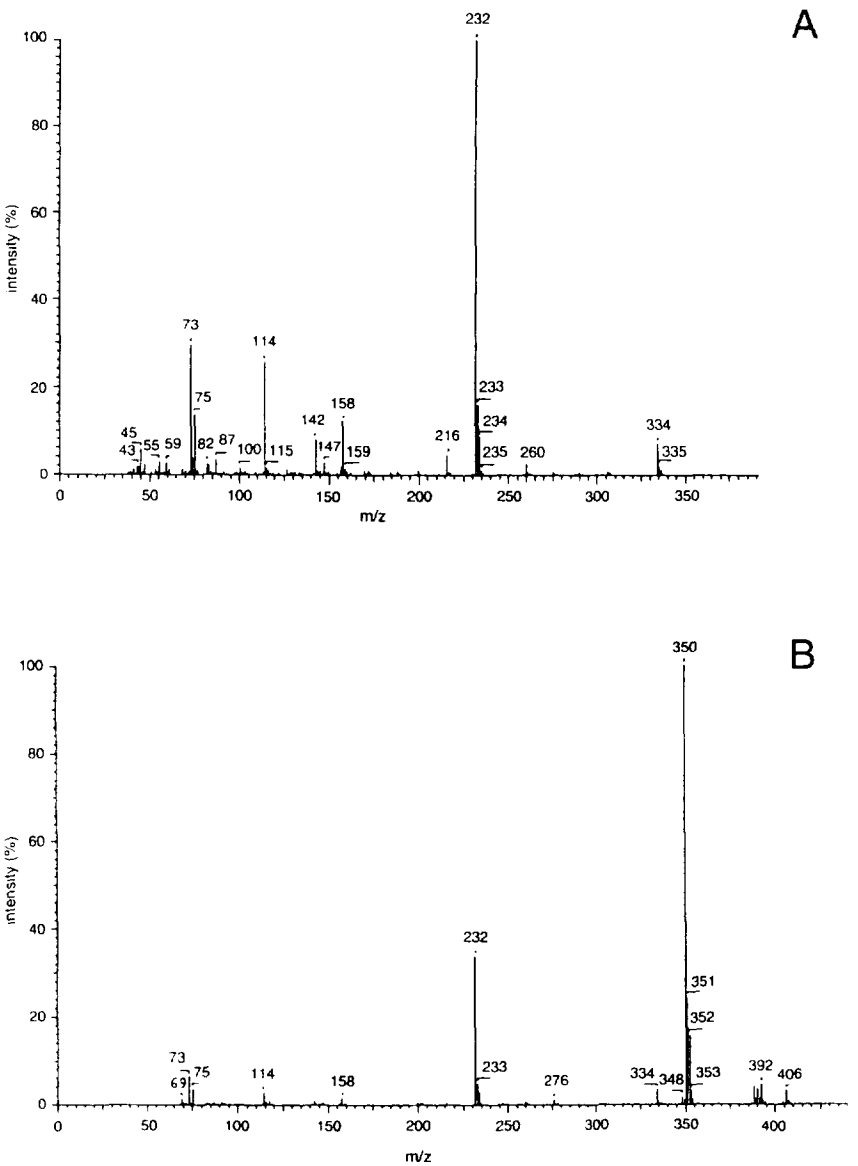


Fig. 4. GC–MS analysis of the trimethylsilylated derivative of the thiazolidine **7a**. The figure shows the EI (A) and CI (B) mass spectra of the TMS derivative **10a** yielded from silylation of **7a** with MSTFA. The identities of the characteristic ion fragments are described in Tables 1 and 2. For detailed GC–MS conditions see Section 2.

relevant condensation reactions, the application and further elaboration of this analytical concept was justified. Furthermore, separation of all the respective starting materials and thiazolidines on a fused-silica capillary column DB 255 or DB 17 within 20 min, using a special temperature program (see Section 2), proved to be greatly advantageous for a rapid and reliable identifica-

tion of these compounds by mass spectrometry in the same analytical run (see Fig. 3).

3.5. Investigations on the formation and epimerization of the thiazolidine 7 under silylation conditions

With respect to the planned in vivo experiments, exemplary for the penicillamine-derived compounds **7a** and **7b**, two important questions concerning the analytical concept had to be investigated based upon this derivatization procedure. Thus, it was necessary to study whether under silylation conditions, epimerization of the 2,4-disubstituted thiazolidine dicarboxylic acids takes place. Another important question was whether D-(−)-penicillamine (**4**) and glyoxylic acid (**1**) condense during the derivatization reaction with MSTFA.

3.6. Determination of the diastereomeric ratio

Especially the first of the two questions was relevant for an evaluation and interpretation of the results of the planned in vivo experiments concerning an enzymatic assistance in the endogenous condensation reaction. Furthermore, a chromatographic investigation on the epimerization behaviour was of most general interest since unambiguous information on diastereomeric ratios of thiazolidine mixtures could be obtained exclusively by NMR spectroscopy so far [12].

Thus, mixtures of **7a** and **7b** with stereoisomeric **7a**/**7b** ratios of 50:50, 25:75, and 100:0 as determined by NMR, were silylated under the conditions described above, and were then investigated by gas chromatography. In an impressive agreement, the given stereoisomeric ratios of **7a** vs. **7b** were reproduced by GC analysis of their silyl derivatives **10a** and **10b**, as illustrated in Table 3.

Thus, for the first time a chromatographic method was available that, like a snapshot, allowed a determination of the actual diastereomeric ratio and thus was clearly superior to the direct ion-pair assisted reversed-phase chromatography of **7a** and **7b** [24]. For the latter method, due to solubility problems, an epimerization had to be taken into account already while dissolving the compounds.

Table 3
Gas chromatographic studies on the stereochemically defined transformation of **7a** to **10a**, and **7b** to **10b** under silylation conditons

Sample[a]	*cis*/*trans* ratio of **7a**/**7b** determined by ^{1}H NMR	*cis*/*trans* ratio of **7a**/**7b** determined by GC as **10a**/**10b**	Calculated ratio *cis*/*trans* **7a**/**7b**
1	50:50	50:50	
2	50:50	51:49	
3	50:50	48:52	
4	50:50	47:53	51:49
5	50:50	51:49	
6	50:50	51:49	
7	25:75	24:76	
8	25:75	25:75	25:75
9	25:75	23:77	
10	100:0	100:0	
11	100:0	100:0	100:0

[a] For description of the preparation and treatment of sample solutions see Section 2.

3.7. Condensation of glyoxylate (1) and D-(−)-penicillamine (4) during silylation

The question whether silylating conditions are condensation conditions, is of crucial importance for all further investigations concerning the elaboration of a GC-suited workup procedure: if, during silylation of glyoxylic acid (**1**) and D-(−)-penicillamine (**4**), condensation to the heterocycles **7a** and **7b** takes place, the subsequent GC analysis can no longer distinguish between the substance genuinely present and the material that is formed de novo, during the silylation process in the sense of an artifact. In that case the analytical procedure would be invalid unless either a corresponding further workup procedure would prevent the non-desired condensation reaction or if this additional cyclocondensation reaction could be shown to convert reproducible percentages of the precursors into the thiazolidines. Thus, for these questions, qualitative as well as quantitative aspects have to be taken into consideration.

Consequently, the procedure was performed with a mixture of the condensation partners **1** and **4** in a realistic concentration range that also reflects the ratios of the silylating reagent and starting materials given in a normal analysis. Due to the impossibility of a direct weighing of the solid material in that order of magnitude, aqueous standard solutions of **1** and **4** were subsequently shock-frozen down to −196°C in the silylating vessels and then lyophilized. By this way, condensation of **1** and **4** upon mixing of the standard solutions was excluded. The solids of **1** and **4** as present after lyophilization were silylated without problems in the usual way. Subsequent GC–MS investigation of the reaction mixtures showed that the condensation between glyoxylic acid (**1**) and D-(−)-penicillamine (**4**) indeed occurred during the silylation reaction since both **7a** and **7b** were unambiguously identified as their silylation products **10a** respectively **10b** according to their retention times and mass spectra (see Fig. 5).

Interestingly, under these conditions, a third reaction product in the polarity range of the thiazolidines **10a** and **10b** was registered that had never been observed before. It seemed probable that a possible intermediate product resulting from the initial addition reaction of glyoxylic acid (**1**) and D-(−)-penicillamine (**4**) might have been trapped by the very active reagent MSTFA before the ring closure had been taken place to give a chromatographically stable product. Mass spectrometrical investigations revealed evidence for the formation of the silylated compounds **20** or **21** (see Fig. 5). Also in this case, as for the thiazolidines previously described, a characteristic $[M-15]^{+\cdot}$ peak, a valuable indicator for the molecular mass, was again registered besides several other signals from known decomposition processes (see Section 2). Yet, a definite structure elucidation, exclusively based upon the mass spectrum, did not succeed.

Because of this additional reaction product, it was not possible to quantitate the condensation reaction by a simple comparison of the calibration curves of the TMS derivatives **10a** and **10b** corresponding to the thiazolidines **7a** and **7b**, as initially assumed. For this reason, the amounts (in mol) of the reaction products **10a**, **10b**, and **20/21** originating in the different experiments were estimated by using the calibration curve of **10a** (derivative of **7a**) for **20/21**. For a further confirmation, this result was compared with the molar turnovers of the precursors **1** and **4** as likewise determined by calibration curves within the margins of error. A really good agreement between the quantities of the starting materials used and the products formed could be attained as seen in the bar graph (see Fig. 6), a hint that the applied procedure indeed allows a quantitative evaluation. Simultaneously, it becomes clear that, on the other hand, no reliable correlation between the experimental parameters and the degree of the condensation reaction can be stated.

4. Conclusions and outlook

Summarizing, the sulphur-containing compounds **2**, **3** and **4** as well as their condensation products **5a**, **5b**, **6**, **7a** and **7b** (see Fig. 1) can unambiguously be trimethylsilylated to give one

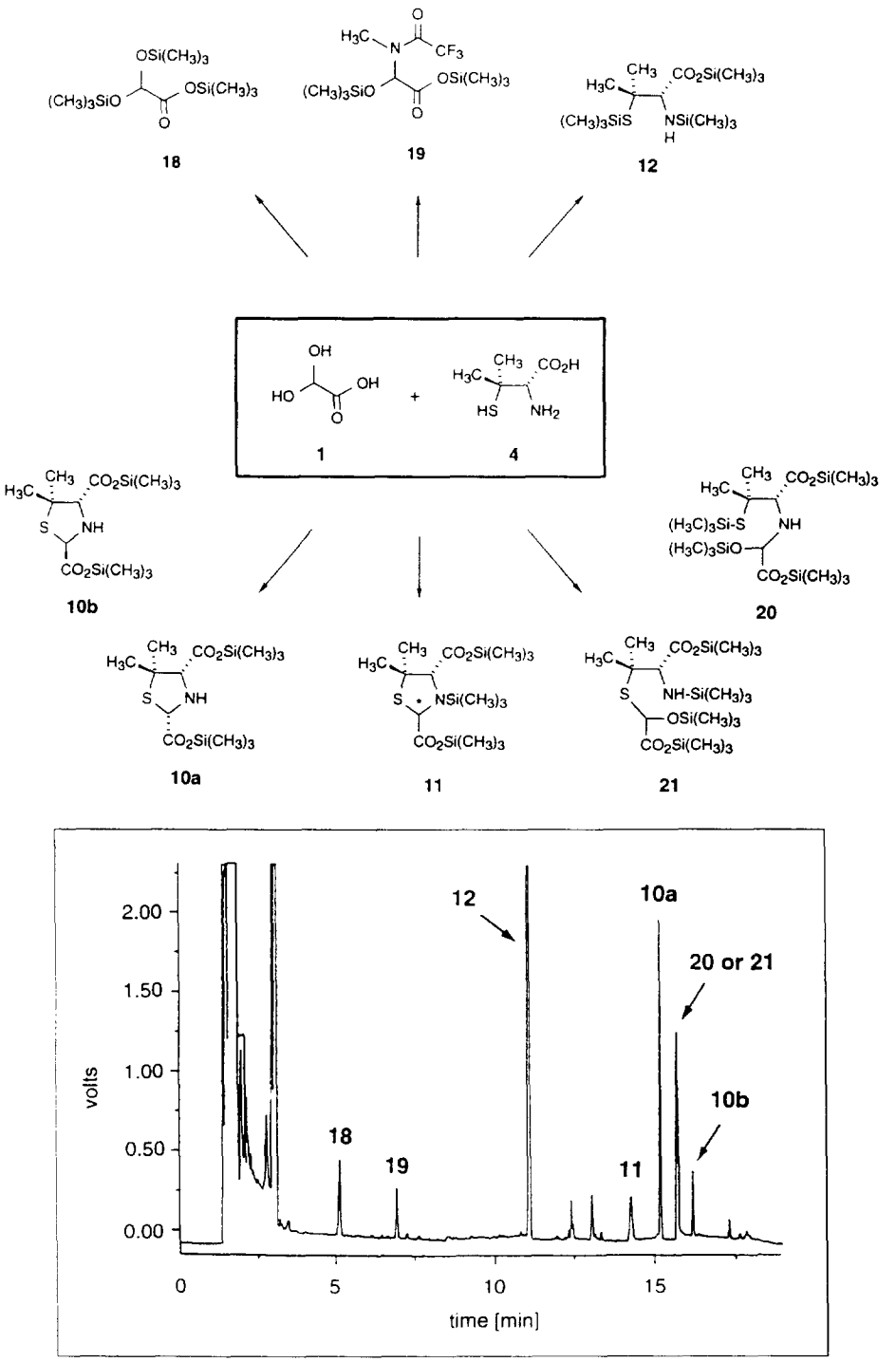


Fig. 5. Thiazolidine formation during trimethylsilylation. The figure shows a typical GC–FID chromatogram obtained from a mixture of glyoxylic acid (**1**) and D-(−)-penicillamine (**4**) after trimethylsilylation with MSTFA. The scheme above illustrates the formation of all the TMS derivatives detected and identified in the sample solution by GC–MS analysis. For detailed experimental conditions see Section 2.

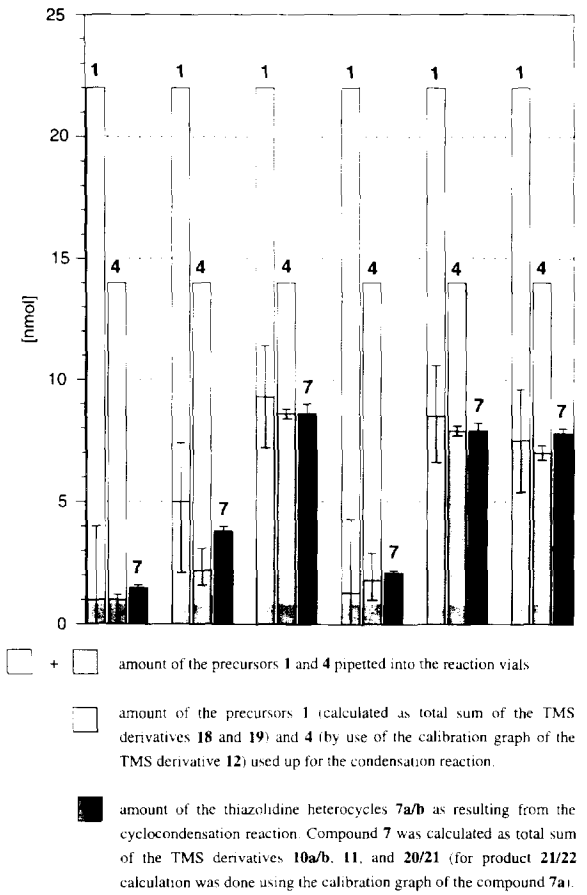


Fig. 6. Quantitation of the extent of thiazolidine formation caused by the condensation of glyoxylic acid (**1**) and D-(−)-penicillamine (**4**) under silylation conditions. For detailed experimental and chromatographic conditions see Section 2.

gas chromatographically analyzable main product each (see Fig. 3), although a time-dependence in the product ratio has to be taken into account. Thus, in combination with mass spectrometry, for the first time a method has become available that allows a sensitive and unequivocal identification of glyoxylate-derived thiazolidines. Still, the very rapid spontaneous condensation of glyoxylic acid (**1**) with binucleophilic amino acids to give alkaloid-type heterocycles, as essential for the presented therapeutic concept, constitutes a particular challenge to an analytical method for the quantitation of the thiazolidines in the presence of their precursors. Thus, the gas chromatographic procedure developed by us can be applied only if the non-desired condensation step during the silylating process can be excluded by a preceding workup step. It should be possible to prevent a spontaneous reaction of the starting material if at least one of the two reaction partners – glyoxylic acid or the amino acid – is immediately eliminated from the reaction mixture. Such a pre-separation to avoid artifact formation must be considered as the key step of the entire analytical device for the scheduled investigation program. Consequently, the present work aims at the elaboration of appropriate sample workup steps also suited for the analysis of these compounds from a complex biological matrix such as urine. Detailed investigations based upon ion chromatography are in progress.

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