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Preparation and Properties of Glutathion Conjugates of 2,4,5,6-Tetrachloro-1,3-dicyanobenzene

Zdenko Hameršak[a], Miklos Hollósi[b]*, Darko Kontrec[a] Branko Ladešić[a], Zsuzsa Majer[b], Vitomir Šunjić[a]*

[a]"Ruđer Bošković" Institute, P. O. B. 1016, 41001 Zagreb, Croatia
[b]Deparment of Organic Chemistry, Eötvös University Budapest, P.O.B. 32, H-1518 Budapest, 112 Hungary

Abstract.-Preparation and spectroscopic properties of 2,5,6-trichloro-4-(S-γ-L-glutamyl-L-cysteinyl-glycine)-1.3-dicyanobenzene (**2**), 2,5-dichloro-4,6-di-(S-γ-L-glutamyl-L-cysteinyl-glycine)-1,3-dicyanobenzene (**3**), and 5-chloro-2,4,6-tri(S-γ-L-glutamyl-L-cysteinyl-glycine)-1,3-dicyanobenzene (**4**), gluthation conjugates of the general fungicide chlorothalonil (2,4,5,6-tetrachloro-1,3-dicyanobenzene, **1**) are reported. Optimal conditions for preparation and chromatographic separation of the single conjugates are described. NMR-spectroscopic data allowed unambiguous determination of regioselectivity of conjugation reaction of chlorothalonil with glutathione. UV Spectra of **2-4** revealed significant long-wavelength band shift and enhancement of intensity with increasing number of thioalkyl groups attached to the aromatic ring. The UV band assignement is based on the UV-VIS spectra of the model compounds **5-7**. The far-UV CD spectra are dominated by the chiral contribution of the amide chromophores. In a trifluoroethanol/water (70:30) mixture the peptide backbone of conjugate **2** likely adopts an ordered (folded) conformation, whereas peptides **3** and **4** show no significant tendency for forming ordered structures.

INTRODUCTION

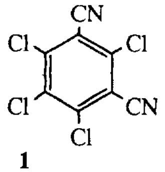
Chlorothalonil (2,4,5,6-tetrachloro-1,3-dicyanobenzene, **1**) represents one of the most effective fungicides used in the agriculture[1]. It is selectively toxic to funghi by effecting primarily the activity of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAP)[2], but also by its ability to deplete cellular glutathione (GSH) reserves[3]. In the higher animals, indeed, conjugation of the xenobiotica with GSH has generally been considered a depletion pathway[4]. Previous *in vivo* and *in vitro* studies have indicated that chlorothalonil is first transformed into mono-, di-, and tri-conjugates with GSH, and then converted to various thio-metabolites[5,6].

In view of the importance of glutathionyl conjugates of chlorothalonil **2-4**, it is surprising that no specific protocol for their preparation, separation and identification is available in the literature. Vincent and Sisler[7] were the first to suggest that glutathione reacts with chlorothalonil by nucleophilic replacement of chlorine in positiones 4, 6 and 2, respectively, leading to the formation of mono-, di- and tri-conjugated products. The evidence for this reaction sequence was somewhat circumstantial, however. In the paper of Davies[8], some spectroscopic data and Rf values for thin layer and paper chromatography are cited for the monoglutathion conjugate. For diglutathion conjugate only Rf value is reported, while triglutathion derivative is not mentioned. Mono-, di- and tri-conjugates are not registered in Chem Abstract data bank, and do not possess CAS number. Prompted by this situation, in order to get more insight into relation between structure and toxic behaviour of these xenobiotics, and in view of the application of such structures as "chiral selectors" in the peptide-like chiral columns for enantioselection, we report herewith the preparation, spectroscopic characterization and chiroptical properites of three glutathionyl conjugates of 2,4,5,6-tetrachloro-1,3-dicyanobenzene.

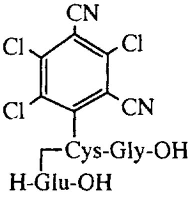
RESULTS AND DISCUSSION

Preparation. Alkyl thiolates are known to react through the halogen displacement in reactions with unactivated or slightly activated mono- and polyhalobenzenes under different conditions. Various metal ions promote this reaction, in particular copper (I)[9], lead, zinc, and mercury[10], and also nickel (II)[11]. In some instances, simple heating of an aryl halide with sodium or potassium salt of the alkyl thiol in an aprotic solvent is sufficient to effect substitution reactions[12,13].

The use of the promoting metal ions in preparation of **2-4** is *a priori* excluded because of the strong complexing properties of the small peptides like GSH. Therefore we focussed our attention on the selection of reaction conditions wherein the accumulation of one particular conjugate occurs. With less than equimolar amounts of glutathione, the reaction mixture contained at best *ca* 70% of monoglutathionyl **2**, *ca.* 15% of diglutathionyl **3**, and 1% of triglutathionyl conjugate **4**. With 2:1 molar ratio of glutathione the optimal distribution of the products was *ca.* 30% of **2**, *ca.* 60% of **3**, and *ca* 2% of **4**. With 4:1 molar ratio of glutathione, over 80% of triglutahione conjugate **4** was formed. All three conjugates contained various amounts of the isomeric products; the 4-monosubstituted derivative **2** contained 10-20% of the 2-isomer, bis-derivative **3** 15-20% of 2,4-isomer, while the isomeric purity of **4** was usually over 99% . Obviously, the *ortho*-positions to cyano groups in **1** are activated more than 5-position for the *in vitro* nucleophilic displacement of chlorine.



1



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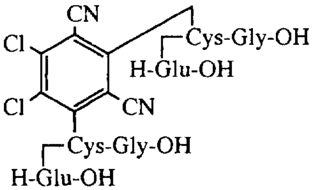

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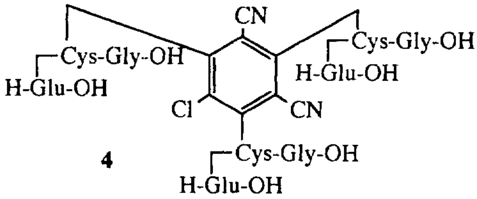

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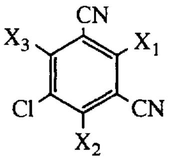

	X_1	X_2	X_3
5	Cl	SCH_3	Cl
6	SCH_3	SCH_3	Cl
7	SCH_3	SCH_3	SCH_3

All reactions were monitored by HPLC, preparative separations were performed by gel permeation/adsorption chromatography on Sephadex G-25 column, and ulterior purification on Amberlite XAD-4 column. Fractions containing over 96% pure **2-4**, according to HPLC, were collected, lyophylized and analyzed; to this aim compound **3** was isolated as bis-ammonium salt and compound **4** as tris-ammonium salt. Analytical HPLC reveales that mono- and diglutatione conjugates are 92:8 and 85:15 mixtures of two isomers, respectively, whereas triglutathione conjugate is present as over 99.5% pure 2,4,6-isomer; complete separation of the positional isomers on the preparative scale cannot be achieved, however.

With this experience at hand, we also optimezed the conditions for preparation and isolation of the thiomethyl model compounds **5-7**, as descibed in the Experimental.

Spectroscopic Properties. In the ^{13}C-NMR spectra of the conjugates **3** and **4** , showing more constitutional symmetry, four signals for the aromatic carbons appear. The non-symmetrically substituted derivative **2** gives rise to six signals in the aromatic region, indicating that the main isomer is the 4-substituted conjugate. Two non-equivalent cyano groups are present in **2** (113.72 and 114.67 ppm), and two equivalent in **3** and **4**, at 114.61 ppm, and 115.74 ppm, *resp.* The relative position of ^{13}C signals reveals that the third thio-group enters in the position C-2, since the signals for C-2 and C-4,6 atoms in **4** are found at 144.58 ppm, and 141.81 ppm, *resp.*

UV spectra of the conjugates **2-4** exhibit several bands, the main maximum above 200 nm is found around 230 nm (ε 35000). This absorbtion is likely due to the ^{1}B band of the highly substituted benzene ring. In order to assign these aromatic bands with confidence mono-, di- and tri-methylthio derivatives **5-7** are prepared. Due to their low solubility in water, the UV spectra of the methylthio derivatives were measured in trifluoroethanol (TFE, see Experimental).

Chlorothalonil exhibits the first maximum at 234 nm (ε *ca* 48000), a shoulder at 255 nm, which is atributable to the ^1L_a band, and two distinct maxima of low intensity at 313 (ε *ca.* 1000) and 326 nm (ε *ca.* 1500). The latter two bands belong to ^1L_b-band, and change significantly their position and intensity with number of thioalkyl groups, Fig. 1.

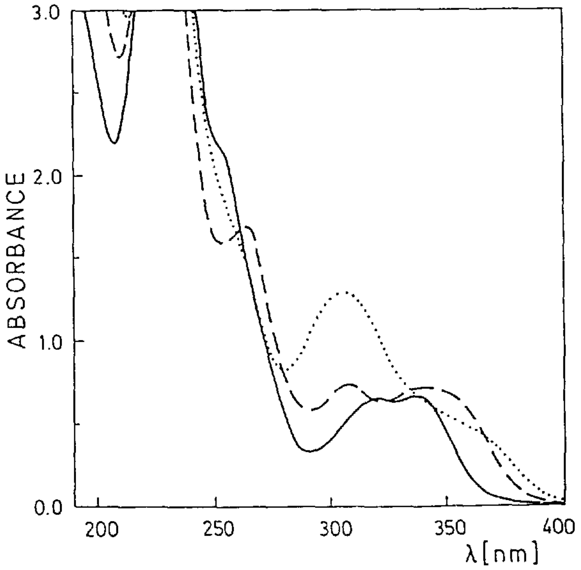

Figure 1. UV Spectra of **2** (——), **3** (- - -), and **4** (····); c 1x10^{-4} M in water.

The ε value of the first band above 300 nm rises from 4500 in **2** to 8300 in **4**, whereas for the second one it drops from *ca* 4500 in **2** to a shoulder at 360 nm, ε *ca.* 300, in **4**. Thus, the strongly red-shifted ^1L_b band region in UV spectra of glutathionyl conjugates of chlorothalonil is highly convenient for identification purposes of small quanities of metabolites.

Chiroptical properties of glutathionyl conjugates. The CD spectra of the glutathionyl derivatives **2-4** show a series of low-intensity positive bands above 230 nm. In the spectrum of **2** in 70% TFE two groups of bands are separated, Fig. 2 (insert). The maxima between 235 and *ca* 290 nm may be asssigned to the ^1L_a band whereas those above 300 nm to the ^1L_b band of the fully substituted benzene ring. In the CD spectrum of **3** and **4** the two regions are not well separated. There are several overlapping maxima with Δε *ca* 1 values (not shown).

In the far-UV CD spectrum of the conjugates **2-4** the aromatic bands below 230 nm overlap with those of the amide groups. The 190-235 nm region of the spectra shows strong negative bands, Fig. 2. The higher intensity negative band at 223 nm of the monoglutathionyl derivative **2** is contributed by the amide n-π* and the aromatic ^1B transition (see the UV data in TFE of the compounds **5-7** in Experimental). However, taking into account the three bonds distance of the closest perturbing chiral center of cystein, it is reasonable to suppose that this negative band is mainly due to the n - π* transitions of the amide groups. Thus, the solvent-dependence of the negative band near 220 nm of conjugate **2** suggests that at higher TFE concentrations the peptide chain adopts a significant population of the ordered conformation. In water, at low TFE concentrations or pH ≥ 9,values the intensity of this band is decreased and the spectra are dominated by the strong negative band near 200 nm which is accompanied by a shoulder at about 220 nm, Fig. 3.

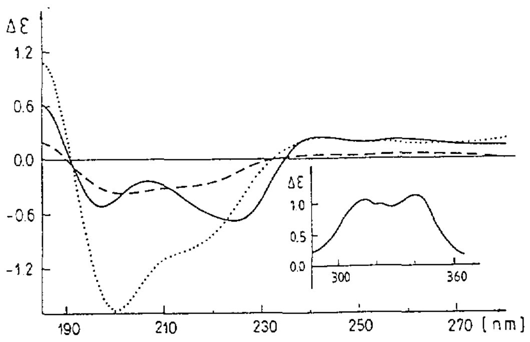

Figure 2. CD Spectra of **2** (———), **3** (-----), and **4** (·········) in 70% TFE.

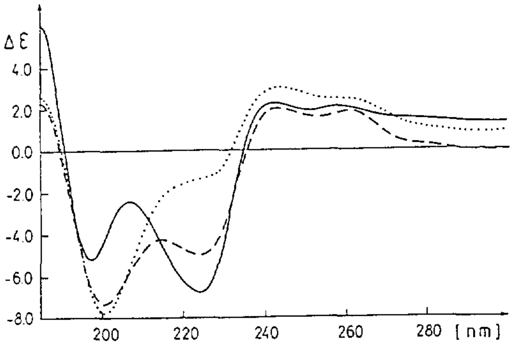

Figure 3. CD Spectra of **2** in the media of different basicity; 70% TFE (———), water (-----), and water, pH ≥ 9 (·········)

The comparison of the relative band intensities at pH≥9 and pH 6-7 reflects the importance of a salt bridge possibly stabilizing the ordered (folded) conformation of the peptide backbone of **2** in 70% TFE.

The CD spectrum of **4** shows in 70% TFE a positive band below 185 nm, a strong negative band at 200 nm and, a negative shoulder near 215 nm, Fig. 3. The blue-shift, and the increase of intensity of the *ca* 200 nm band in water (not shown) may be indicative of some ordered conformation of the peptide chains.

The low intensity of the CD spectrum of **3** in 70% TFE is surprising, Fig. 2. Moreover, the CD spectrum of **3** depends not only on the solvent but also on the concentration and the time passed after dissolving the sample (not shown) may be indicative of some ordered conformation. This behaviour is likely due to slow association.

In conclusion we can state that gluthationyl conjugates of chlorothalonil represent an interesting group of the peptide-derived xenobiotica, in which both the chromophoric properties and the abilitiies to adopt ordered conformation depend on the number of the bound peptide chains. Their structural properties indicate that simple aromatic structures like **1**, are potential targets for the template assembled synthetic proteins[14], in particular those which are expected to serve as "chiral selectors" bound on the solid support, for GLC and HPLC of the enantiomeric mixtures[15,16].

EXPERIMENTAL

Melting points are determined on Electrothermal apparatus. 1H- and 13C-NMR spectra were recorded on Varian Gemini 300 FT spectrometer; shifts are given in ppm downfield from TMS (for 13C-NMR spectra dioxane was used as internal standard, 67.4 ppm downfild from TMS). IR spectra were recorded on Perkin-Elmer 297 spectrometer. UV Spectra were run on Pay Unicam 8700 Series spectrophotometer. Rotations were determined on Optical Activity AA-10 Polarimeter. CD spectra were recorded on a Jobin-Yvon VI dichrograph calibrated with epiandrosterone. Measurements were performed at room temperature in 0.02 and 1.0 cm cells. TFE (Aldrich, NMR grade) and double-distilled water were used as solvents.

Materials. Chlorothlonil (**1**, 99% purity)) was obtained from the production of Caffaro S.p.A., and was used without ulterior purification. Gluthathion (puriss.), Sephadex G-15 and Amberlite XAD-4 were purchased from Fluka.

HPLC Analysis. HPLC system consisted of Hewlet Packard HP 1050 quaternary pump, HP 1050 UV detector fixed at 240 nm, and HP 3396 integrator. Analysis was performed on 5 µm C-18 column (Waters, 3.9x130 mm) using gradient technique. As eluent A 0.05M NaH_2PO_4 in water with 5% acetonitrile, as eluent B 50% acetonitrile in water was used. Gradient from 100% A to 60% A was used over 25 min, hold 10 min, and returned to 100% A. With a flow rate of 0.7 mL/min retention times were; for **2** between 25-30 min, for **3** between 16-22 min, for **4** between 4-6 min.

HPLC technique was used for the preparative separations with a flow rate 2.5 mL/min on 10 µm Nucleosil C-18 column (8x250 mm).

2,5,6-Trichloro-4-(S-γ-glutamyl-L-cysteinylglycine)-1,3-dicyanobenzene (2)

Chlorothalonil (**1**, 532 mg, 2.0 mmol) was dissolved in MeOH (120 ml) on brief heating and stirring under nitrogen, heated on oil bath at 90°C. To the resulting solution glutathione (522 mg, 1.7 mmol), and K_2CO_3 (234 mg, 1.7 mmol), dissolved in water (15 ml) were added. The resulting solution was heated under gentle reflux, and progress of reaction was followed by HPLC. After 90 min the reaction mixture consists of over 70% of of monogluthationyl derivative **2**, *ca* 15% of diglutathionyl conjugate **3**, *ca* 1% of **4**, and some unreacted material. The reaction mixture was evaporated to *ca* 15 ml, to precipitate crude **2**. It was separated by centrifugation, washed with cold water and repeatedly centrifuged. This treatment permitted separation of the main quantity of dissolved **3** formed as the side-product, whereas 335 mg of **2** with 97% purity remained.

Second crop was obtained on collection of the supernatant, evaporation to *ca* 10 ml, and dissolution of solid material on addition of $(NH_4)_2HPO_4$. The resulting solution was applied on a Sephadex G-15 column (2.5x95 cm), pretreated with 0.02 M AcOH. The same diluted acid was used for elution; after elution of some bis-conjugate **3**, fractions (ca 8ml/10 min) with over 96% of **2** were collected and evaporated *in vacuo* affording additional 276 mg. Total yield on **2** was 611 mg (67%), m.p >180°C (decc). $[\alpha]_D$.+18° (c=1, in 0,1M $(NH_4)_2HPO_4$) buffer). IR (KBr): 3260, 3050, 2915, 2240, 1650, 1540, 1355, 1240, cm^{-1} ^{1}H NMR (D_2O): 1.95-2.10 (m, 2H), 2.30-2.50 (m, 2H), 3.33 (dd, J=14,6 Hz, J=9.1 Hz, 1H), 3.50-3.75 (m, 4H), 4.33 (dd, J=9.1 Hz, J=4.7 Hz, 1H) ppm. ^{13}C NMR (D_2O, 0,1 M $(NH_4)_2HPO_4$): 27.13, 32.48, 37.39, 44.38, 54.32, 55.14, 113.72, 114.67, 117.68, 120.77, 139.98, 140.20, 142.90, 145.68, 171.65, 175.05, 175.91, 176.92 ppm. UV (H_2O), λ in nm (ε): 230.1 (42300), 320.8 (4500), 338,4 (4500) nm. Anal. Calcd. for $C_{18}H_{16}N_5O_6SCl_3$ (536.78): C 40.27, H 3.00 N 13.05. Found: C 40.34, H 3.31, N 13.14.

2,5-Dichloro-4,6-di(S-γ-glutamyl-L-cysteinylglycine)-1,3-dicyanobenzene diammonium salt (3)

Chlorothalonil (266 mg, 1.0 mmol) was dissolved in MeOH (80 ml) as described for **2**, then 615 mg (2.0 mmol) of glutathione and K_2CO_3 (276 mg, 2.0 mmol) dissolved in 20 ml of water were added. After 120 min HPLC control revealed *ca* 60% of **3**, *ca* 30% of **2**, and *ca* 2% of **4**. On evaporation to a small volume a part of **2** precipitated, and was removed by centrifugation. The remaining solution was applied on a Sephadex G-15 column, prepared and eluted as described for **2**. Fractions containing over 85% of **3** were collected, and evaporated affording 320 mg (40%) of yellowish product. It was dissolved in a small quantity of water, acidified to pH 3, and purified by chromatography on Amberlite XAD-4 (2.0x32 cm). On elution with 0.01 M NH_4OH fractions that contained over 98% **3** were collected and lyophylized affording 270 mg (33%) of pure product, m.p. >180°C (decc), $[\alpha]_D$ +15°(c=1 in H_2O). IR(KBr): 3230, 2930, 2230, 1645, 1530, 1400, 1350, 1230 cm^{-1}.^{1}H NMR (D_2O): 1.90-2.15 (m, 4H), 2.30-2.50 (m, 4H), 3.25-3.40 (m, 2H), 3.45-3.70 (m, 8H), 4.25-4.40 (m, 2H) ppm. ^{13}C NMR (D_2O): 26.86, 32.28, 37.26, 43.95, 54.06, 54.91, 114.61, 122.19, 139.73, 144.98, 145.54, 171.63, 174.68, 175.65, 176.37 ppm. UV (H_2O), λ in nm (ε): 228.3 (35000), 264.2 (11800), 305.8 (5200), 340.3 (5000). Anal. calcd. for $C_{28}H_{38}N_{10}O_{12}S_2Cl_2$ (841.70): C 39.96, H 4.55, N 16.64. Found: C 39.78, H 4.26, N 16.84.

5-Chloro-2,4,6-tri(S-γ-glutamyl-L-cysteinylglycine)-1,3-dicyanobenzene triammonium salt (4)

Chlorothalonil (212 mg, 0.8 mmol) dissolved in 80 ml of MeOH as described for **2**, and to the solution were added 982 mg (3.2 mmol) of glutathion and 441 mg (3.2 mmol) of K_2CO_3 dissolved in water (22 ml). HPLC monitoring revealed after after 90 min 83% of **4**, 4% of **3**, and 2% of **2**. Solution was evaporated to ca 5 ml, and crude **4** isolated on chromatography on Sephadex G-15 column, prepared as described for **2**. Fractions containing over 90% of **4** were collected, evaporated and repeatedly chromatographed on AmberliteXAD-4 column as described for **3**. On elution with 0.01 M. NH_4OH 355 mg (40%) of **4** were obtained; over 99% purity was determined by HPLC, m.p.>180°C (decc.), $[\alpha]_D$ +10° (c=1 in H_2O). IR (KBr): 3230, 2930, 2230, 1650, 1590, 1400 cm^{-1}. ^{1}H NMR (D_2O): 1.95-2.15 (m, 6H), 22.30-2.55 (m, 6H), 3.25-3.40 (m, 3H), 3.50-3.75 (m, 12H), 4.30-4.45 (m, 3H) ppm. ^{13}C NMR (D_2O): 26.88, 32.28, 37.34, 38.11, 43.93, 53.87, 54.00, 54.90, 115.74, 127.77, 141.87, 144.58, 147.72, 171.67, 171.70, 174.72, 175.59, 175.66, 176.35 ppm. UV (H_2O), λ in nm (ε): 192.0 (44000), 231.3 (30000), 305.4 (8300). Anal. Calcd. for $C_{38}H_{57}N_{14}O_{18}S_3Cl$ (1129.60): C 40.40, H 5.09, N 17.36. Found: C 40.16, H 5.34, N17.52.

2,5,6-Trichloro-4-thiomethyl-1,3-dicyanobenzene (5)

To the solution of chlorothalonil (1.0 g, 3.76 mmol) in DMF (40 ml), cooled to -12°C, a solution of sodium methylthiolate (263 mg, 3.76 mmol, Fluka 95%) in methanol (80 ml) was added dropwise during 1 hr. The resulting solution was stirred for another hour at ambient temperature, then the solvents were evaporated *in vacuo* to a final volume of *ca* 10 ml. On the addition of water crude **5** precipitated; it was filtered off, washed, and dried (920 mg). GLC control revealed the presence of *ca* 50% of the unreacted **1**, *ca* 40% of **5** and *ca* 10% of **6**. Chromatography on silicagel (120 g) with carbontetrachloride/dichloromethane (4:1) as eluant afforded 230 mg of 94.1% pure **5**, containing 2.4% of **1** and 2.9% of **6**, m.p. 160-162°C. UV (CF_3CH_2OH; nm (ε)): 347 (3070), 310 (3300), 275, sh (7307), 248, sh (10560), 228 (34600). IR (KBR): 2210, 1540, 1510, 1430, 1370, 1320, 1250, 1140, 970, 720 cm^{-1}. ^{1}H-NMR ($CDCl_3$); 2.7 (s, 3H), ^{13}C-NMR ($CDCl_3$): 111.81, 112.65, 115.26, 117.34, 136.76, 139.57, 140.90, 149.14.

2,5-Dichloro-4,6-dithiomethyl-1,3-dicyanobenzene (6)

To the suspension of **1** (2.0 g, 7.5 mmol) in methanol (150 ml) sodium thiomethylate (2.28 g, 32.5 mmol) was added in small portions at room temperature with stirring. After 1 hr. water (1200 ml) was added, the precipitate was separated by filtraton, washed with water, and dried (1.93 g). GLC control revealed the presence of 13% of **6** and 87% of **7**.

Using the same chromatographic system as for **5**, **6** was the first eluated, and on crystallization afforded 190 mg of 96.2% isomericaly pure product, mp. 126-127°C. UV (CF_3CH_2OH; nm (ε)): 358 (4770), 336 (5440), 302 (8020), 261 (11700), 225 (43100). IR (KBR): 2210, 1520, 1490, 1460, 1420, 1380, 1340, 1310, 1230, 1130, 1040, 970, 740, 720, 680 cm-1. ^{1}H-NMR ($CDCl_3$): 7.27 (s, 6H). ^{13}C-NMR ($CDCl_3$): 113.05, 117.63, 139.57, 140.75, 147.45.

5-Chloro-2,4,6-trithiomethyl-1,3-dicyanobenzene (7)

The next fractions contained chromatographically pure **7**, which on repeated crystallization from chloroform afforded 420 mg of 99.8% pure product. UV (CF_3CH_2OH; nm (ε)): 360 (3570), 302 (11340), 247 (11600), 226 (35260). IR (KBR): 2210, 1610, 1450, 1410, 1380, 1340, 1310, 1220, 970, 890, 710, 690 cm-1. ^{1}H-NMR ($CDCl_3$): 2.69 (s, 3H), 2.72 (s, 6H). ^{13}C-NMR ($CDCl_3$): 19.26 (2C, 4,6-CH_3), 19.76, 114.49, 122,82, 142.72, 145.38, 146.89, 154.73.

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