Syntheses and MTT Assays of Tetrathiafulvalene(TTF)-Carbohydrate Derivatives

Synthesen von Tetrathiafulvalen(TTF)-Kohlenhydratderivaten und deren Aktivitäten im MTT-Test

DISSERTATION

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List of Abbreviations

Ac	Acetone
Bn	Benzyl
d	Doublet
DCM	Dichloromethane
dd	Double doublet
DEE	Diethylether
DMF	Dimethylformamide
DMIT	1,3-Dithiole-2-thione-4,5-dithiolate
dt	Double triplet
ELISA	Enzyme-linked immunostaining assay
equiv	Equivalent
EtOH	Ethanol
FAB-MS	Fast atomic bombardment mass spectroscopy
FCS	Fetal calf serum
FD-MS	Field desorption mass spectroscopy
g	Relative centrifugal acceleration
GASPE	Gated spin echo
h	Hour
HVA	Homovanillic acid
IU	International unit
L	Liter
M	Molar
m	Multiplet
MeCN	Acetonitrile
MeOH	Methanol
mIBG	*meta*-Iodobenzylguanidine
min	Minute
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NBA	Nitrobenzyl alcohol
NLO	Non linear optics
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PE	Petroleum ether
ppm	Parts per million
q	Quadruplet
ROS	Reactive oxygen species
RPMI	Rosewell Park Memorial Institute
s	Singlet
SK-N-LO	Human neuroblastoma cell line
SK-N-SH	Human neuroblastoma cell line
t	Triplet
TLC	Thin-layer chromatography
Tol	Toluene
TTF	Tetrathiafulvalene, 2-(1,3-dithiol-2-ylidene)-1,3-dithiole
VMA	Vanillylmandelic acid
δ	Chemical shift unit

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A. Introduction

The synthesis and characterization of new heterocyclic systems based on chalcogens (oxygen, sulfur, selenium, tellurium) has been one of the central objectives in contemporary organic chemistry. Several interesting systems have been synthesized and characterized. Especially, sulfur-based heterocyclic systems have found widespread applications in modern material science and medicinal chemistry [1].

Tetrathiafulvalenes (TTF; Figure 1) and related heterocycles have received much interest due to their unique electron-donating capabilities [2]. A number of interesting properties of the TTF moiety includes its ability to form molecular metals and superconductors at low temperatures. It has also been incorporated in a number of macrocyclic systems for use as molecular sensors, enzyme biosensors, switches, wires and shuttles, exploiting the inherent electron donor properties [3].
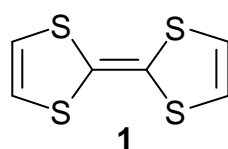
Figure 1. Structure of tetrathiafulvalene (TTF) **1**.

However, the biological importance of sulfur-containing heterocycles is still minor compared to the very wide applications of sulfur-based heterocycles in modern materials chemistry [1]. No studies have been made so far on the possible application of TTF to biological systems. Since biological assays are routinely done in aqueous media, the solubility of TTF in an aqueous medium

could pose a problem. Its solubility in water is extremely low [4]. In 1998, in a thesis by Alea, water-soluble TTF derivatives containing sugars were synthesized [5], which could be tested in biological systems.

This thesis deals with the synthesis of derivatives containing TTF and carbohydrate moieties. TTF in combination with carbohydrate molecules could possibly participate in a number of processes in the human body. Different metabolic pathways utilizing redox reactions can be influenced. Moreover, TTF derivatives are planar [2] and therefore might possibly intercalate with DNA or even influence membrane transport. These properties could be of use in the treatment of diseases or affect metabolic pathways. As such, part of this thesis deals with the performance of a MTT assay using SK-N-LO and SK-N-SH neuroblastoma cell lines to investigate the activity of the synthetic TTF-carbohydrate derivatives.

A.I. Review of Related Literature

A.I.1. Tetrathiafulvalene and related compounds

Only a very limited number of non-metals can be used by biological systems as a reversible redox switch, and sulfur is one of those [6]. It can be attached to different biomolecules. One of the examples, are iron/sulfur proteins. These function mainly as a part of electron transfer wires e.g.: in the energy-transducing respiratory chain of the mitochondria. Sulfur-based heterocycles are central building blocks of the chemistry of life [1] as is illustrated in Table 1.

Table 1. Some key functions of sulfur in biology [6].

Function	Example
Thiolate as a base	Thiol proteases
Thiol as a ligand	Metal-ion binding
Sulfide as a ligand	Metal-ion binding in clusters
Sulfide as a redox group	Lipoic acid
Thiolate in group transfer	Transfer of acetyl (CoA)
-S-S- as a cross link	Many extracellular enzymes
-S-S- in controls	Thioredoxine, glutathione
-S-CH$_3$ ether as a ligand and cross link	Cytochrome C
-S-CH$_3$ ethers (methyl transfers)	Coenzyme (methionyl CoA), coenzyme M
Coenzyme catalysis	Biotin, thiamin, lipoic acid
Inhibitors (drugs)	Penicillin

Among the sulfur-containing heterocycles, tetrathiafulvalene, 2-(1,3-dithiol-2-ylidene)-1,3-dithiole (TTF) derivatives have been the most intensively studied species during the past 25 years [7-12]. Its utility as building blocks in macromolecular and supramolecular structures [13-16], as ferromagnetic compounds [17] , as synthetic intermediates in organic chemistry [18-19], as a donor moiety in intramolecular donor-acceptor systems in nonlinear optic (NLO) materials , as well as in the preparation of liquid crystalline materials and Langmuir-Blodgett (LB) films [20-22], are only some applications, mentioned here.

The unique combination of properties which makes TTF a versatile component of materials is summarized as follows: (**1**) is a nonaromatic 14 π-electron system [23] in which oxidation can occur reversibly in two discrete steps with the formation of a radical cation (**2**) and a dication (**3**) (Scheme 1 [24]).
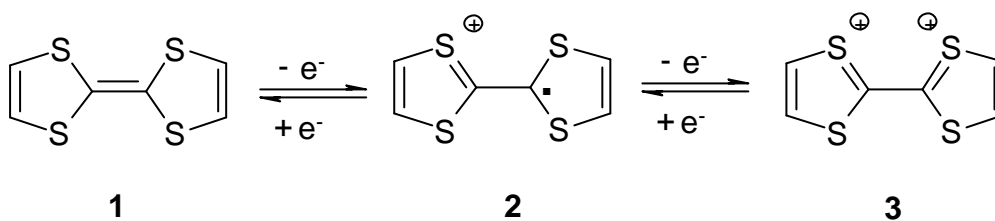
Scheme 1. Reversible oxidations of tetrathiafulvalene [24].

The redox potentials are relatively low with $E^{1/2}_1 = 0.34$ and $E^{1/2}_2 = 0.78$ V vs Ag/AgCl in MeCN [23]. The potential can be finely tuned by the attachment of the appropriate substituents on the ring system [25]. The TTF cation radical is thermodynamically very stable due to the contribution from a 6π electron heteroaromaticity of the 1,3-dithiolium ion [26]. It is stable to synthetic transformations, but it is important to avoid strongly acidic conditions and strong oxidizing agents [27]. A number of synthetic pathways have already been performed to synthesize the TTF core as shown in scheme 2 [28].

The use of 1,3-dithiole-2-thione-4,5-dithiolate or dimercaptoisotrithione (DMIT) (**4**)[29] is considered to be a key intermediate in the synthesis of TTF derivatives [28]. The name DMIT is a remnant from the nomenclature of the early days of heterocyclic sulfur chemistry [1].
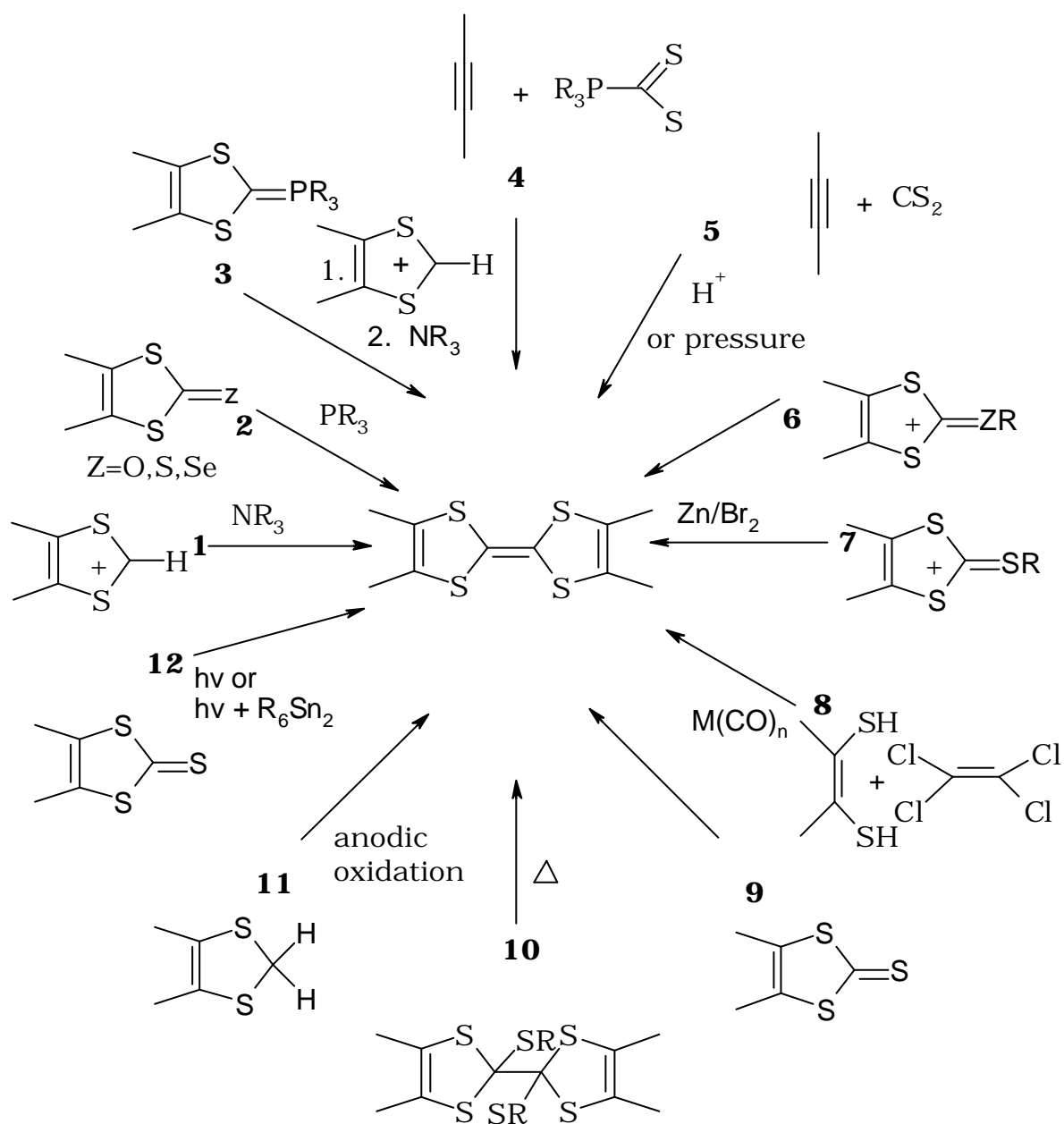
Scheme 2. Synthetic pathways for tetrathiafulvalene synthesis [28].

Scheme 3 surveys the various pathways in the synthesis of DMIT [28].
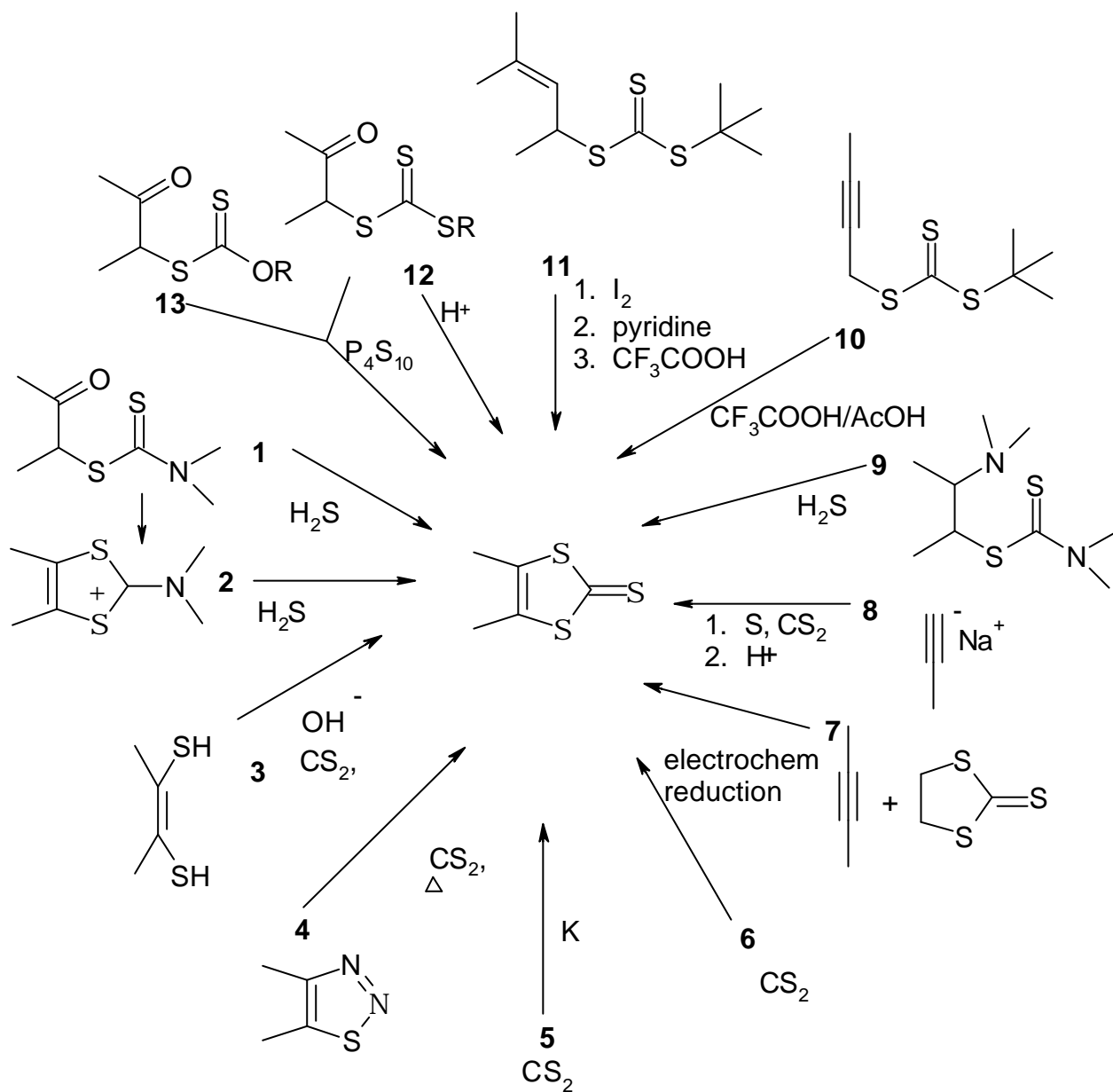
Scheme 3. Synthetic pathways for dimercaptoisotrithione synthesis [28].

The synthesis of choice for preparing DMIT is the DMF-mediated reduction of CS$_2$ by sodium metal followed by its isolation as zinc chelate, bis(tetraethylammonium)bis(1,3-dithiole-2-thione-4,5-dithiolato)zincate, was mainly developed in the laboratory of Hoyer [30].

A.I.2. MTT Assay

The non-radioactive, colorimetric assay system using MTT was first described by Mossman and co-workers [31]. It is used for the measurement of cell proliferation in response to growth factors, cytokines and nutrients as well as for the measurement of cytotoxocity. It can also be used to study cell activation [32]. MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (**5**), is a yellow tetrazolium salt which can be transformed into purple colored formazan crystals (**6**) by metabolic active cells. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH [33] (Scheme 4). The larger the number of mitochondria, the more MTT will be transformed into formazan. The formazans are purple crystals which are difficult to dissolve. Prior to measurement, it is first dissolved in isopropanol and the released, dissolved formazan reagent is measured spectrophotometrically. The photometric measurement of a 96-well plate is performed with an ELISA reader. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

MTT provides sensitive and reproducible indices of growth as well as drug sensitivity in individual cell lines over the course of multiple passages and several months of cultivation. It is suitable for initial stage *in vitro* drug testing [34].
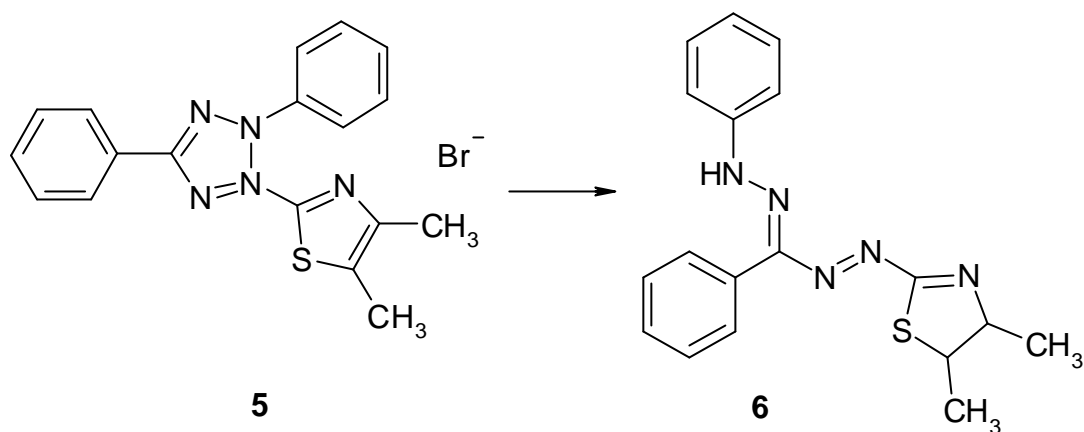
Scheme 4. Metabolization of MTT (**5**) to a formazan salt (**6**) by viable cells [33].

A.I.3. Doxorubicin

Doxorubicin hydrochloride (**7**), with the common name adriamycin, is named according to IUPAC nomenclature: (8S,10S)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-8-glycolyl-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride.
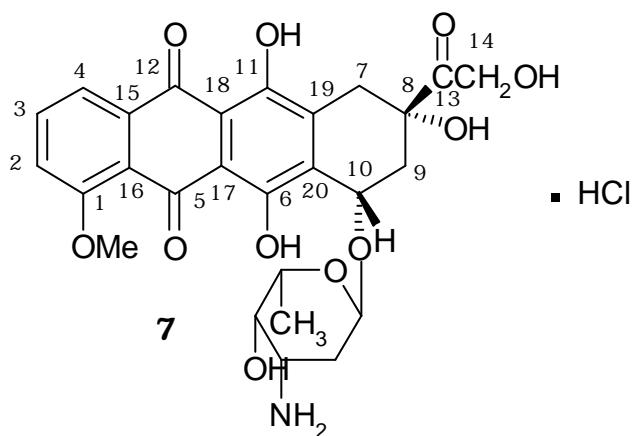
Figure 2. Structure of doxorubicin (**7**).

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Doxorubicin (**7**) is a cytotoxic anthracycline antibiotic, isolated from cultures of *Streptomyces peucetius* var*. caesius.* It consists of a naphthacenequinone nucleus linked through a glycosidic bond at ring atom 10 to an amino sugar, daunosamine. The cytotoxic effect of (**7**) on malignant cells and its toxic effects on various organs are among others thought to be related to its nucleotide base intercalation, presumably by specific intercalation of the planar anthracycline nucleus with the DNA double helix. Intercalation inhibits nucleotide replication and action of DNA and RNA polymerases. **7** also binds to cell membranes as well as plasma proteins. Its binding may effect a variety of cellular functions. Enzymatic electron reduction of **7** by a variety of oxidases, reductases and dehydrogenases generate highly reactive species including the hydroxyl free radical OH. Free radical formation has been implicated in its cardiotoxicity by means of Cu(II) and Fe(III) reduction at the cellular level [35].

A.I.4. Neuroblastoma

Cancer in children and adolescents is rare, but it is the leading cause of death from disease in children between the ages of 1 and 19. Neuroblastoma accounts for 97% of the cancers of the sympathetic nervous system which comprises one of the twelve major categories of the International Classification of Childhood Cancers (ICCC) [36].

It is predominantly a tumor of early childhood, with two-thirds of the cases presenting in children younger than five years of age [37]. It is a solid tumor cancer that originates in the adrenal medulla or the paraspinal sites where sympathetic nervous tissue is located.

The most common symptoms are due to a tumor mass or to bone pain from metastases. Occassionally, fever, anemia and hypertension are present. One of the rare symptoms is severe watery diarrhea due to secretion of vasoactive intestinal peptide by the tumor [37].

Diagnosis requires specialists who are expert in childhood tumors, because some neuroblastomas are hard to discriminate from other small round blue cells of childhood by conventional light microscopy. Evidence for sympathetic neuronal differentiation may be demonstrated by immunohistochemistry, electron microscopy, or by finding elevated levels of serum catecholamines (dopamine, norepinephrine) or urine catecholamine metabolites: (vanillamandelic acid (VMA) or homovanillic acid (HVA)). The minimum criterion for diagnosis established by international agreement is based on one of the following: 1) an unequivocal pathologic diagnosis made from tumor tissue by light microscopy; 2) the combination of bone marrow aspirate or trephine biopsy containing distinct tumor cells; 3) increased levels of serum or urinary catecholamines [38]. But nearly 70% of those children first diagnosed have a disease that has already metastasized to other parts of the body. The average age at diagnosis is two years old [36].

The treatment ranges from surgery, chemotherapy to radiation therapy alone or in some combination depending on the stage of the disease. Under evaluation are: monoclonal antibody therapy [39], myeloablative therapy and stem cell transplantation. The chemotherapeutic agents most commonly used include carboplatin, cyclophosphamide, doxorubicin, cisplatin, vincristine and teniposide or etoposide [38]. Furthermore, neuroblastoma can be detected by

scintigraphy, using the radiolabelled catecholamine-related substance meta-iodobenzylguanidine [^{123}I]mIBG.

Prognosis for neuroblastoma is dependent on age, stage of disease and the molecular biologic and cytogenetic characteristics of the tumor. There is very little known about why it occurs or about what factors increase the risk for occurrence [36].

Important research into neuroblastoma is under way right now all over the world. Scientists try to find out more about the disease, its prevention and improved treatment.

A.I.4.1. Adherently growing human neuroblastoma cell lines

The potential usefulness in cancer research of continous cell lines, established *in vitro* from human tumors is widely accepted [40]. Two human neuroblastoma cell lines were used in the experiment, namely: SK-N-SH and SK-N-LO.

SK-N-LO is a human cell line of neuroectodermal origin. It has no detectable DOPA-decarboxylase, the enzyme responsible for formation of dopamine [41]. SK-N-SH is a typical neuroblastoma cell line established and described by Biedler and her co-workers [40]. Aside from DOPA-decarboxylase, it also contains dopamine-β-hydroxylase, responsible for the production of norepinephrine [41].

B. Results and Discussion

B.I. Synthesis of Starting Materials

B.I.1. Synthesis of 2,3,6,7-tetrakis(2'-cyanoethylthio)tetrathiafulvalene (12)

The synthesis of a TTF derivative may proceed *via* numerous pathways. At present, the most common synthetic strategy for the preparation of structurally-modified TTFs is the direct coupling *via* desulfurization of modified 1,3-dithiole-2-thione-4,5-dithiolate or dimercaptoisotrithione (DMIT) **4**, by using trivalent phosphorus compounds (tri-alkyl/aryl/phenyl phosphites). It provides subs- tantial yield (40-90%) of the derivatized-TTF [29].

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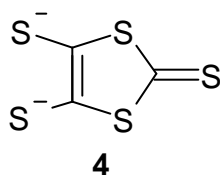
Figure 3. Structure of 1,3-dithiole-2-thione-4,5-dithiolate (dimercaptoisotri-thione, DMIT) (**4**).

However, the DMIT salts (Li, Na, and K) are all air- and moisture-sensitive. It is also unstable in acidic media and consequently cannot be isolated in its protonated neutral form [1]. The synthesis of choice for preparing DMIT is the DMF-mediated reduction of CS_2 by sodium metal followed by its isolation as zinc chelate, bis(tetraethylammonium)bis(1,3-dithiole-2-thione-4,5-dithiolato)-zincate (**8**), mainly developed in the laboratory of Hoyer [30]. The mechanism by which CS_2 is reduced is as follows:
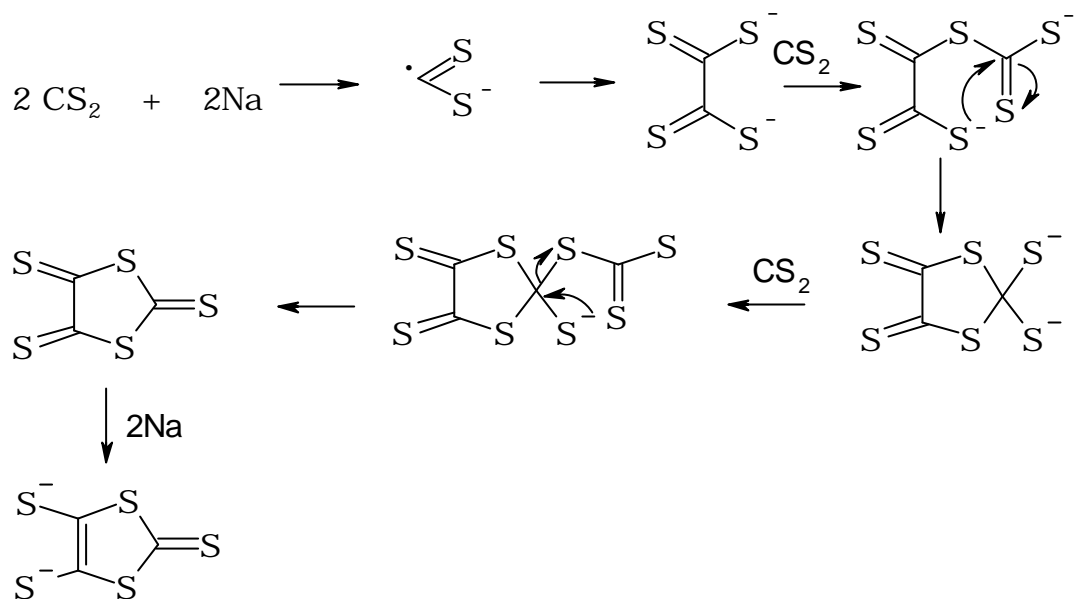
Scheme 5. Mechanism of carbon disulfide reduction by sodium.

The production of bis(tetraethylammonium)bis(1,3-dithiole-2-thione-4,5-dithio-lato)zincate (**8**) as shown in Scheme 6, gave a yield of 80%.

Sulfur nucleophiles are known to be among the most powerful heteroatom nucleophiles known. The zinc chelate and the DMIT salts are possible sources of nucleophilic synthons, although the zinc chelate is a less reactive substitute for the DMIT salts.

One can take advantage of the nucleophilicity by the use of protecting groups. Various studies have been done on possible protecting groups that can withstand the rigorous desulfurization coupling conditions of the thione/oxone precursors whose facile deprotection under basic/weakly acidic conditions generates the TTF mono-/di-/tetra- thiolates. However, a number of dithioles are unable to survive
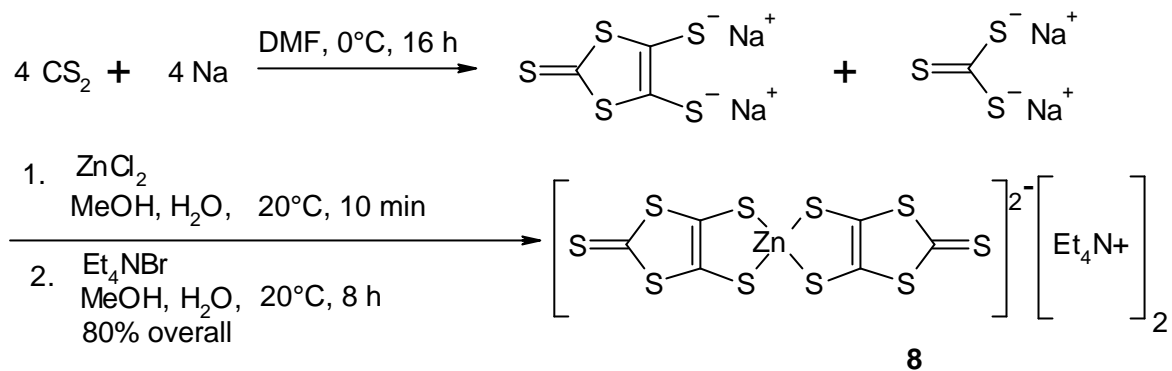
Scheme 6. Preparation of bis(tetraethylammonium)bis(1,3-dithiole-2-thione-4,5-dithiolato)zincate (**8**).

the reaction conditions of coupling, especially systems containing ester-, thioester/, urethane/, alcohol, or ketone-/aldehyde functionalities. That means, no coupling reaction would take place if a sugar moiety is attached to a DMIT structure as shown also in previous studies [46]. Scheme 7 shows the trivalent phosphorous coupling to produce TTF.

As such, an alternative route which makes use of a preformed TTF, tetrathiafulvalene-2,3,6,7-tetrathiolate (**9**), was done for the synthesis of TTF-carbohydrate derivatives.
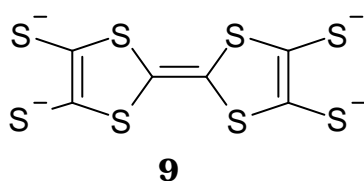
Figure 4. Structure of tetrathiafulvalene-2,3,6,7-tetrathiolate (**9**).
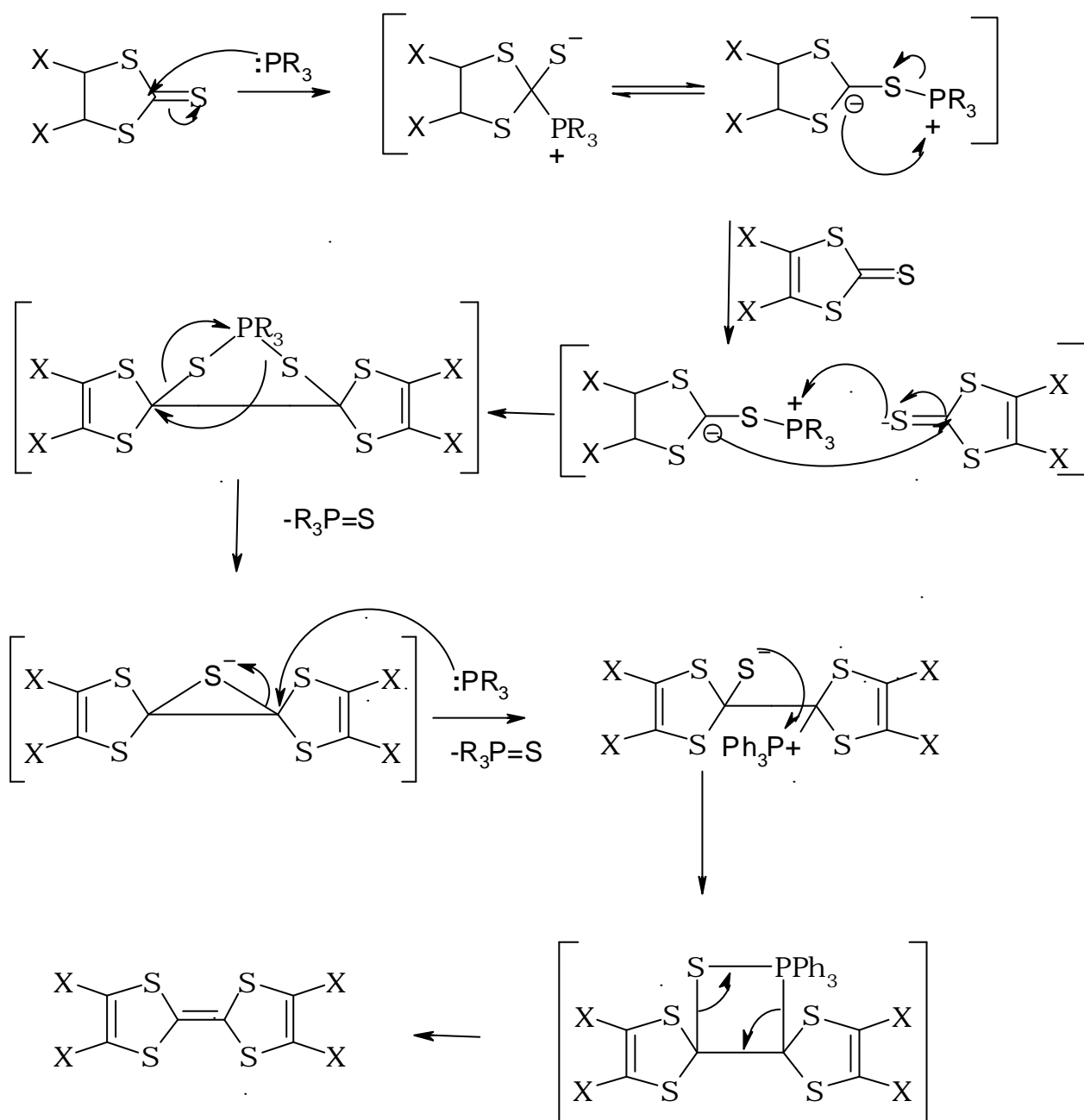
Scheme 7. Trivalent phosphorous coupling to TTF.

A number of precursors to **9** have been reported [42-47]. In this experiment, the use of 2,3,6,7-tetrakis(2-cyanoethylthio)tetrathiafulvalene (**12**) [47] as precursor to **9** was chosen.

12 is prepared in three steps from **8** by alkylation with 3-bromopropionitrile in refluxing acetonitrile giving 4,5-bis(2'-cyanoethylthio)-1,3-dithiole-2-thione (**10**) with 80 % yield. Mercuric acetate transchalcogenation of **10** gave the corresponding 4,5-bis(2'-cyanoethylthio)-1,3-dithiol-2-one (**11**) in near quantitative yield. Finally, self-coupling of **11** using triethylphosphite in refluxing toluene yielded **12** in 70% yield. (Scheme 8).

12 is an excellent precursor to **9**. Generation of **9** is achieved in quantitative yields at room temperature [47] by treating **12** with strong bases to afford cleavage of the cyanoethyl group. Either NaOMe/MeOH or CsOH•H_2O/MeOH was used for the cleavage reaction.

B.I.2. Synthesis of benzyl 2,3-anhydro-**b**-L-ribopyranoside (18) [48]

The synthesis of the compound **18** was successfully performed in 6 steps, starting from L-arabinose (**13**) (Scheme 9). Benzylation of L-arabinose in the presence of hydrogen chloride leads to the anomerically protected benzyl ß-L-arabinopyranoside (**14**) [49]. The *cis*-oriented hydroxyl groups at C-3 and C-4 were protected *via* 2,2-dimethoxypropane and *p*-toluene sulfonic acid (as a catalyst) in acetone, giving benzyl 3,4-di-O-isopropylidene-ß-L-arabinopyranoside (**15**). The free hydroxyl group at C-2 was directly tosylated with *p*-toluene sulfonyl chloride in pyridine, affording compound **16**. The isopropylidene protecting group was selectively removed from **16** with 90% acetic acid to yield **17** [50].
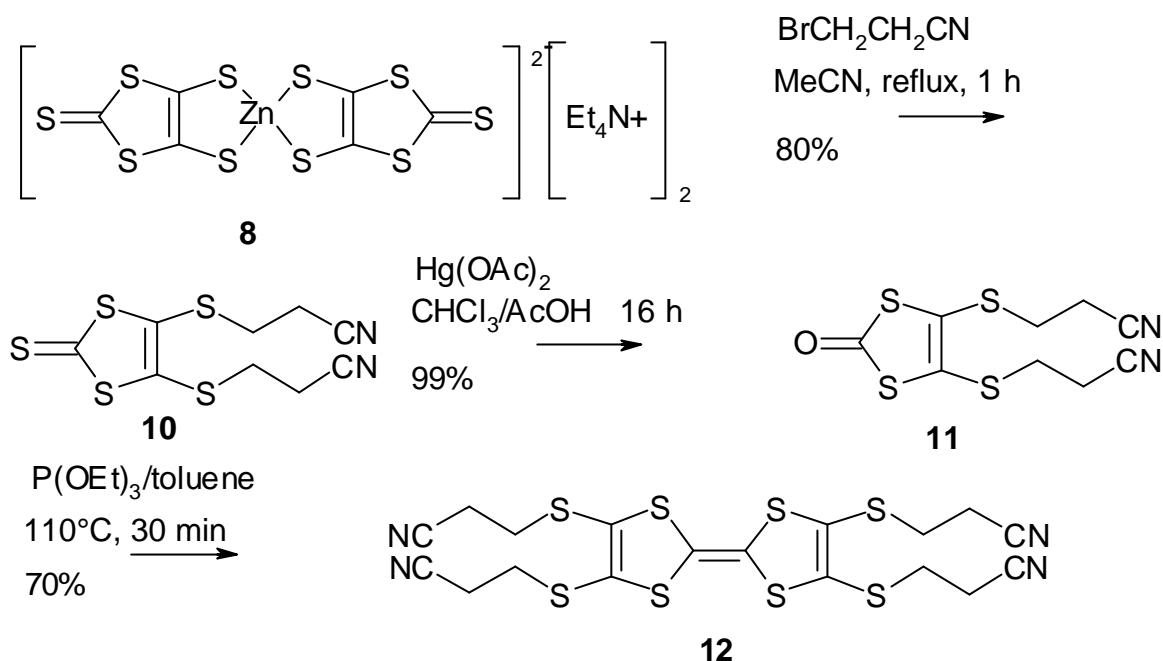
Scheme 8. Synthesis of 2,3,6,7-tetrakis(2'-cyanoethylthio)tetrathiafulvalene (**12**) from bis(tetraethylammonium)bis(1,3-dithiole-2-thione-4,5-dithiolato)zincate (**8**) *via* 4,5-bis(2'-cyanoethylthio)-1,3-dithiol-2-thione (**10**) and 4,5-bis(2'-cyano-ethylthio)-1,3-dithiol-2-one (**11**).
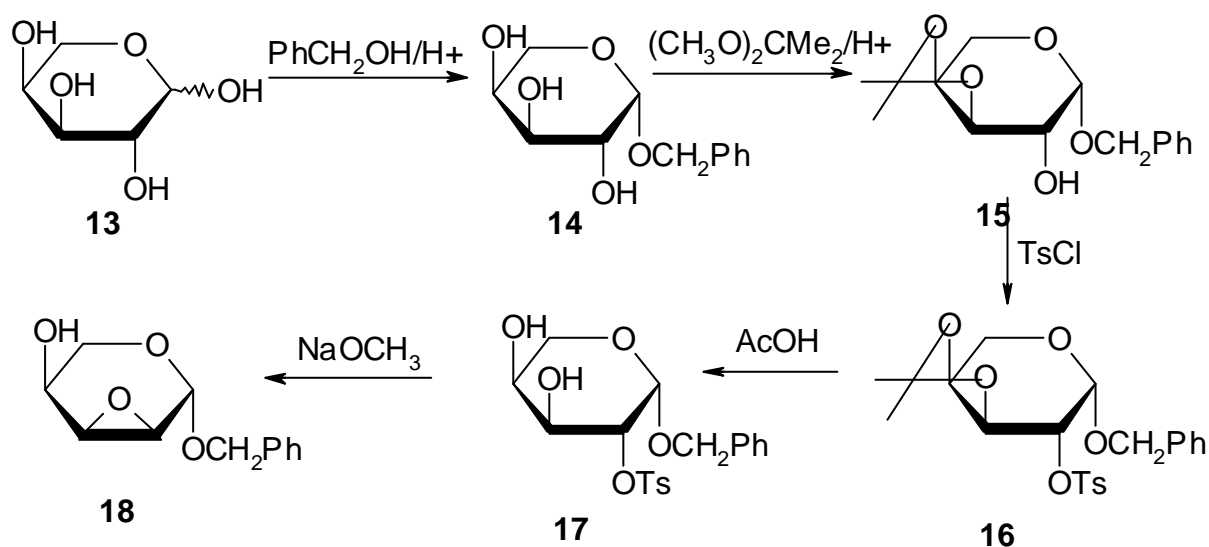
Scheme 9. Synthesis of benzyl 2,3-anhydro-β-L-ribopyranoside (**18**) from L-arabinose (**13**) *via* benzyl β-L-arabinopyranoside (**14**), benzyl 3,4-O-isopro-pylidene-ß-L-arabinopyranoside (**15**), benzyl 3,4-O-isopropylidene-2-O-p-tolylsul-fonyl-ß-L-arabinopyranoside (**16**) and benzyl 2-O-p-tolylsulfonyl-ß-L-arabinopyranoside (**17**).

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The target compound **18** was finally obtained by the action of sodium methoxide in methanol on compound **17**, followed by the neutralization with dilute hydrochloric acid.

B.I.3. Synthesis of benzyl 2,3-anhydro-4-O-triflyl-ribopyranoside (19) [48]

The triflation of the free hydroxyl group in **18** was achieved at low temperature (-20°C) by treatment with trifluoromethanesulfonic anhydride in dichloromethane. The triflate **19** was obtained in high yield upon work-up at 0°C (Scheme 10).
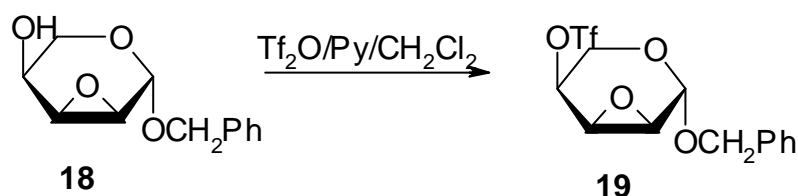
Scheme 10. Synthesis of benzyl 2,3-anhydro-4-O-triflyl-ß-L-ribopyranoside (**19**) from benzyl 2,3-anhydro-ß-L-ribopyranoside (**18**) [48].

B.I.4. Synthesis of 2,3,4,6-tetra-O-acetyl-a-D-glucopyranosylbromide (22) [51]

The synthesis of compound **22** was done in 2 steps, starting from the commercially available D-glucose (**20**). Acetylation of **20** in the presence of acetic anhydride/NaOAc leads to 1,2,3,4,6-penta-O-acetyl-β-D-glucopyranoside (**21**). Then, 45% HBr/HOAc was added to compound **21** to brominate the anomeric position, giving **22** with a yield of 89.5% (Scheme 11).
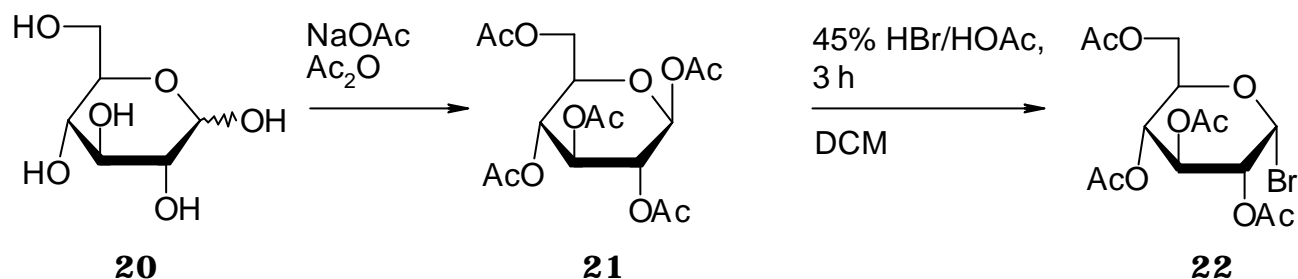
Scheme 11. Synthesis of 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (**22**), from D-glucose (**20**), *via* 1,2,3,4,6-penta-O-acetyl-β-D-glucopyranoside (**21**).

B.I.5. Synthesis of 1,2,3,4-tetra-O-acetyl-6-deoxy-6-iodo-**a**-D-glucopyrano-side (25) [52]

The primary hydroxyl group at C-6 in methyl α-D-glucopyranoside (**23**) was transformed into an iodo group upon treatment with triphenylphosphine, imidazole and iodine in toluene at 70°C.   Further treatment with acetic anhydride and pyridine produced methyl 2,3,4-tri-O-acetyl-6-deoxy-6-iodo-α-D-glucopyra-noside (**24**).   The anomeric position was then acetylated by the use of Ac$_2$O/HOAc giving **25** upon work-up (Scheme 12).
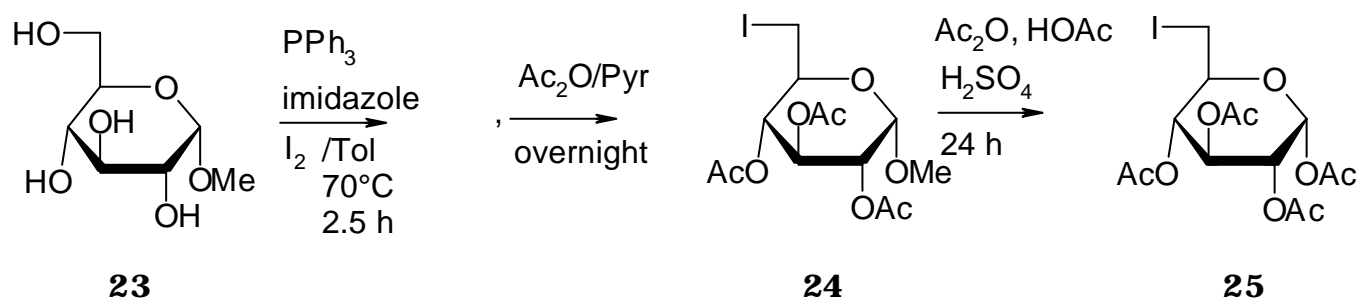
Scheme 12.   Synthesis of 1,2,3,4-tetra-O-acetyl-6-deoxy-6-iodo-α-D-gluco-pyranoside (**25**), from methyl α-D-glucopyranoside (**23**), *via* methyl 2,3,4-tri-O-acetyl-6-deoxy-6-iodo-α-D-glucopyranoside (**24**).

B.II. Synthesis of Tetrathiafulvalene-Carbohydrate Conjugates

Synthesis of the TTF derivatives made use of a tetrathiolate nucleophile first synthesized by Svenstrup [53] and his group in 1995. 2,3,6,7-tetra(2'-cyanoethylthio)tetrathiafulvalene (**12**), generates the tetrathiathiolate upon deprotection using basic conditions. The course of the reaction depends on the conditions, the nature of the electrophilic sites or the geometry of the electrophile [1]. The major problem encountered with the synthesis of the derivatives had to do with obtaining the products in moderate to high yield and the reproducibility of the results [54]. Different solvent systems and reaction conditions were used to optimize the results. Solvent plays a major role in most reactions [55]. Acetone, acetonitrile, methanol and DMF were tried for the reactions. Qualitative analysis of the products by TLC was performed to identify the solvent for optimal yield and little by-products. Carbohydrates of different stereochemistry and functionalization were used for the synthesis of the tetrathiafulvalene-carbohydrate conjugates.

The reaction of **12** with benzyl 2,3-anhydro-4-O-triflyl-ß-L-ribopyranoside (**19**), was performed first to get information about the feasibility of the reaction. Then, glucose (**20**) was protected by acetyl residues at different ring positions. Glucose was chosen because of it's wide availability and it's central role in biochemistry. Acetate as a protecting group was chosen, since the sugar can be deprotected under basic conditions whereby TTF is stable [1]. The syntheses made use of **22** with a bromo group at the anomeric position, **25** with an iodo group at position 6, and 3,4,6-tri-O-acetyl-D-glucal (**26**) with a double bond between positions 1 and 2.
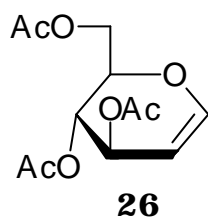
Figure 5. Structure of 3,4,6-tri-O-acetyl-D-glucal (**26**).

CsOH·H_2O in methanol and NaOMe in methanol were both used for the deprotection step. The reaction mixture proved to be very sensitive to the ratio of base used for deprotection. Moreover, a large excess of base is avoided because an excess could compete with the reaction of the thiolate with the carbohydrate moiety, in particular, deacetylation, producing a mixture of products. After preliminary experiments, the use of CsOH?H_2O is favoured, because of easier control in the number of equivalents used and yielding less side products compared to NaOMe/MeOH.

Deprotection of cyanoethyl groups proceeds *via* elimination: The base abstracts the alpha (α) proton neighbouring the cyano group, causing double bond formation generating the thiolate, acrylonitrile, and a protonated base. The acrylonitrile side product is volatile and can be conveniently removed *in vacuo*. The resonance stabilization of the tetrathiafulvalene tetrathiolate makes it a powerful nucleophile [56].

The reaction with the carbohydrate moieties proceeded *via* nucleophilic substitution with the tetrathiolate ion.
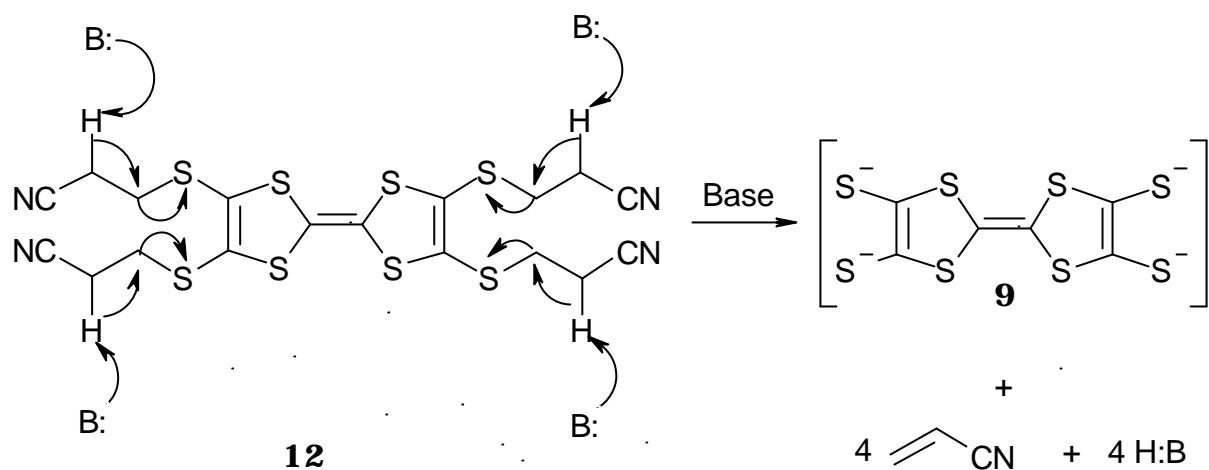
Scheme 13. Mechanism of tetrathiafulvalene-2,3,6,7-tetrathiolate (**9**), formation from 2,3,6,7-tetrakis(2'-cyanoethylthio)tetrathiafulvalene (**12**) under basic conditions.

The three sites identified for sugar attachment are: C-4 in **19**, the anomeric carbon (C-1) in **22** and **26**, and the primary carbon (C-6) in **25**. Although the anomeric carbon is the most reactive site in the sugar molecule followed by the least sterically hindered carbon 6, the structure of **19** made C-4, a more reactive site than the two because of the presence of the anhydro group in C-2 and C-3 and triflate in C-4 as the leaving group.

B.II.1. Synthesis of 2,3,6,7-bis(benzyl 3',4'-dithio-a-D-arabinopyranosyl)-tetrathiafulvalene (27)

The synthesis of 2,3,6,7-bis(benzyl 3',4'-dithio-α-D-arabinopyranosyl)tetrathia-fulvalene (**27**) was performed to know the feasibility of reacting a TTF molecule to a carbohydrate moiety. **12** was deprotected using 4 equivalents of CsOH·H$_2$O followed by the addition of **19**. Compound **19** contains the anhydro group functioning for both, as a protecting group and activator of position 4 of the sugar which in turn contains a very good leaving group, the triflate. It is

known that structures in which the anhydro group is between adjacent carbon atoms are generally too reactive. Ring opening could take place upon reaction with nucleophiles. The relief of ring strain is great enough that reaction could take place under relatively mild conditions [57]. From the results, the nucleophilic displacement of the triflyl group at C-4' by sulfur was followed by the simultaneous intramolecular ring opening of the epoxide to afford 2,3,6,7-bis(benzyl 3',4'-dithio-α-D-arabinopyranosyl)tetrathiafulvalene (**27**) in 80% yield. Flash column chromatography using 10% EtOAc in DCM afforded yellow orange powder with a melting point of 90.5°-92.0°C and $[a]_D^{25}$ = +37.66 (c = 0.10, DCM).

The structure of the product was established through IR, MS, ^{1}H and ^{13}C NMR spectroscopy and elemental analysis.

The infrared spectrum gives the following structural information: OH (3450 cm^{-1} associated OH stretching), phenyl ring, probably monosubstituted (2000 – 1600 cm^{-1} combination vibrations, 1500 cm^{-1}, 698 cm^{-1}, skeletal vibrations), olefinic carbon (3029 cm^{-1}, C-H stretching, 1605 and 1495 cm^{1-} , C=C stretching). The signal in the mass spectrum at m/z 740, is probably the molecular ion. The fragment m/z 329 corresponds to [M+ H$^+$- 2(C$_{12}$H$_{14}$O$_3$]] (C$_{12}$H$_{14}$O$_3$ = carbohydrate component), while m/z 207.1 corresponds to [M + H$^+$ - C$_6$S$_8$] (C$_6$S$_8$ = tetrathiafulvalene-tetrathiolate component). These, suggests the attachment of two carbohydrate moieties in the molecule and the absence of the ethylcyano leaving group.
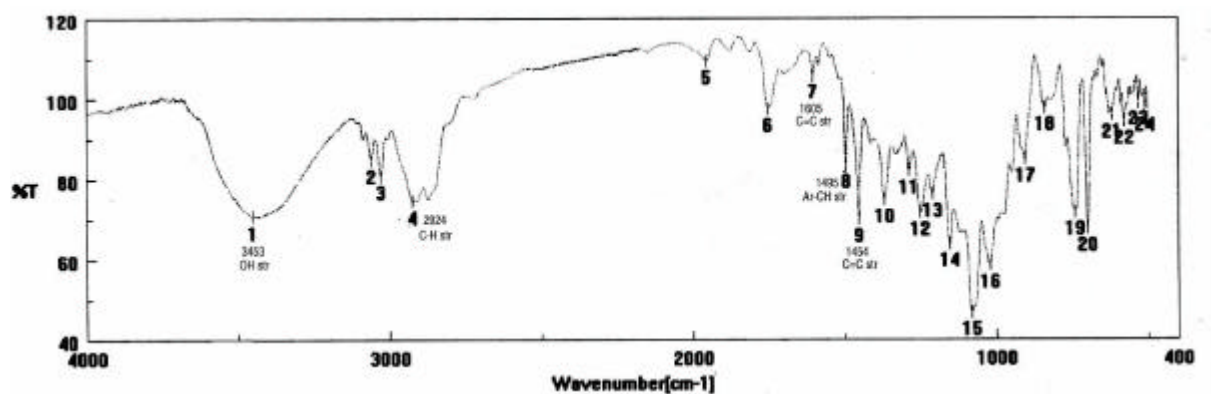
Figure 6. IR spectrum of 2,3,6,7-bis(benzyl 3',4'-dithio-α-D-arabinopyranosyl)-tetrathiafulvalene (**27**) recorded in KBr. Interpretation of the important bands (cm^{-1}): 3450, OH; 2000 –1600, arC-H; 1605, 1495 C=C.
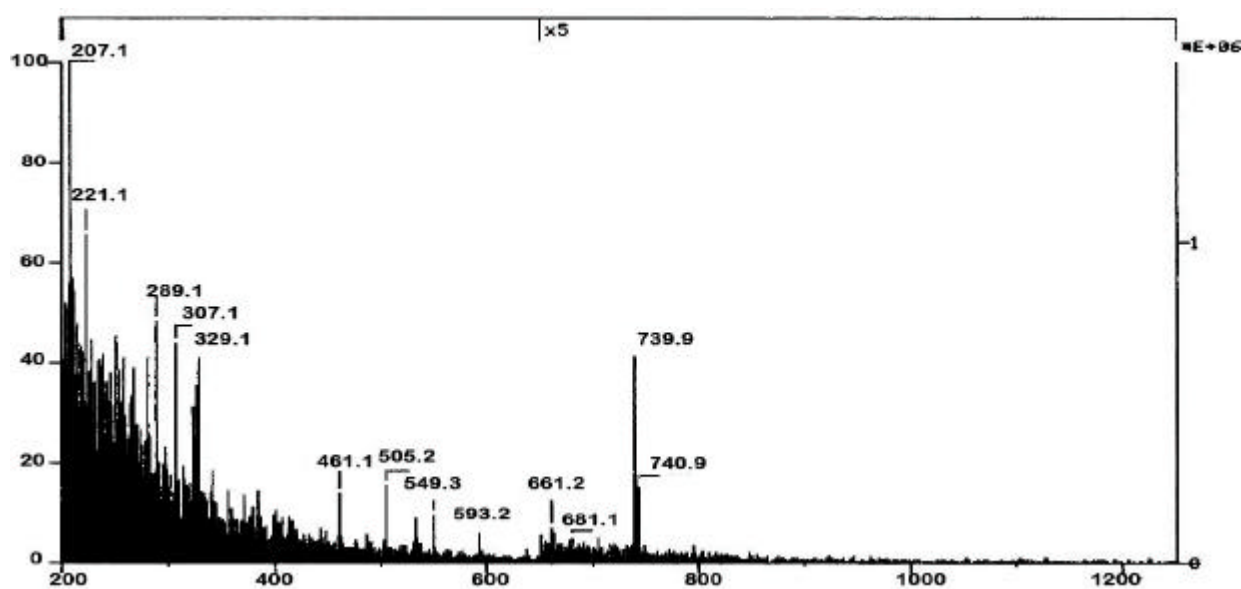
Figure 7. MS-FAB spectrum of 2,3,6,7-bis(benzyl 3',4'-dithio-α-D-arabinopy-ranosyl)tetrathiafulvalene (**27**), Interpretation of the important fragments: (matrix NBA at T=50°C) m/z: 740.9 [M + H$^+$], 739.9 [M], 329.1 [M+ H$^+$-2(C$_{12}$H$_{14}$O$_3$)] (C$_{12}$H$_{14}$O$_3$ = carbohydrate component), 307.1 [matrix], 207.1 [M + H$^+$ - C$_6$S$_8$] (C$_6$S$_8$ = tetrathiafulvalenetetrathiolate component).

The ^{13}C NMR spectrum of **27** shows a new signal at δ = 119.09 ppm besides the usual signals for the gluco moieties. This could infer the signal of the olefinic carbon due to presence of the TTF moiety in the molecule (Table 2). Indicative information about the structure of **27** can be received also from its ^1H NMR spectrum. The conformation adopted by the pyranoside ring in **27** was determined by the vicinal coupling constants and chemical shifts in the ^1H NMR spectrum. 5'-H and 5''-H were recognized from their large geminal coupling constant (~12 Hz) at δ = 4.61 and 4.18 ppm respectively. A symmetrical ddd pattern appeared for H-4' with $J_{4',5'}$ = 8.0, $J_{4',5''}$ = 3.0 and $J_{3',4'}$ = 10.0. This indicates an axial-axial relation between H-4' and H-5' and a quasi equatorial-axial relationship between H-4' and H-5''; conformation is, therefore predominantly $^1C_4$. This is further supported by large axial-axial coupling ($J_{1,2}$ = 7.5 Hz) for H-1' and H-2'.

Table 2. ^{13}C chemical shift assignment of 2,3,6,7-bis(benzyl 3',4'-dithio-α-D-arabinopyranosyl)tetrathiafulvalene (**27**).

Carbon chemical shift (d ppm)	Assignment
138.84	$\underline{C}_6H_5$
128.61, 128.52, 128.40	$\underline{C}_6H_5$
119.09	C=C
104.04	C-1'
95.20	C-2'
70.89	O$\underline{C}H_2$Ph, (B)
56.16	C-3'
52.23	C-5'
50.66	C-4'

Table 3. ^{1}H NMR chemical shifts of 2,3,6,7-bis(benzyl 3',4'-dithio-α-D-arabino-pyranosyl)tetrathiafulvalene (**27**).

Proton chemical shift (d ppm)	Integration	Multiplicity	Coupling constant, J (Hz)	Assignment
7.29-7.36	5H	m		C$_6$H$_5$
4.93	1H	d	12	OCHHPh
4.80	1H	d	12	OCHHPh
4.61	1H	dd	8, 12	H-5'
4.47	1H	d	7.5	H-1'
4.18	1H	dd	4, 12	H-5''
3.92	1H	ddd	4, 7, 10	H-4'
3.65	1H	dd	5, 7.5	H-2'
3.56	1H	dd	5, 10	H-3'
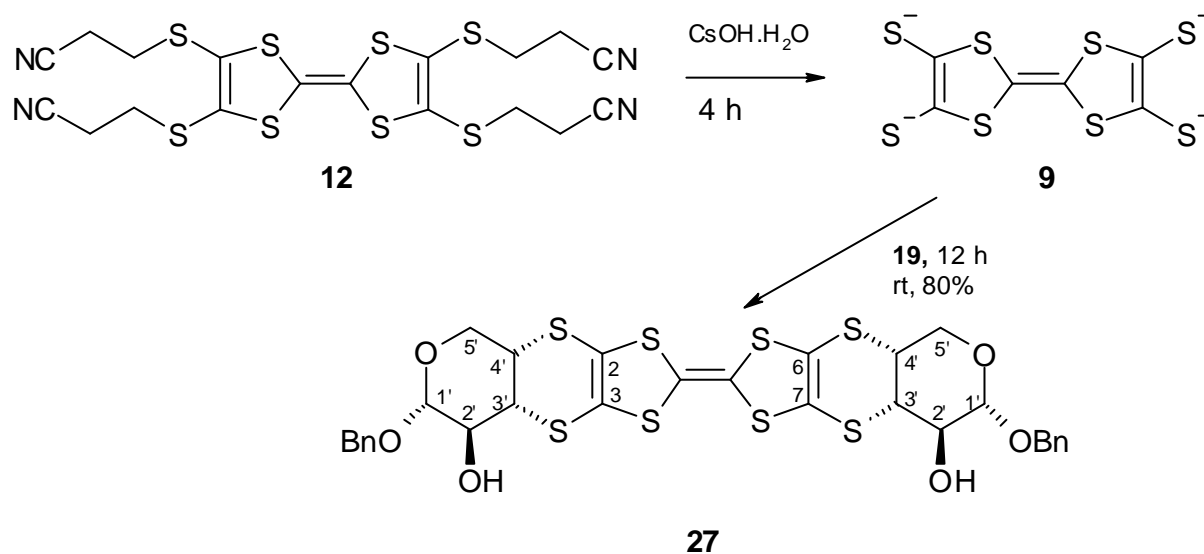
Scheme 14. Synthesis of 2,3,6,7-bis(benzyl 3',4'-dithio-α-D-arabinopyranosyl)-tetrathiafulvalene (**27**) from 2,3,6,7-tetrakis(2'-cyanoethylthio)tetrathiafulvalene (**12**) *via* tetrathiafulvalene-2,3,6,7-tetrathiolate, (**9**).

B.II.2. Synthesis of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-a-D-glucopyranosyl)tetrathiafulvalene (28) and 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-b-D-glucopyrano-syl)tetrathiafulvalene (29)

2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (**22**), and 1,2,3,4-tetra-O-acetyl-6-deoxy-6-iodo-α-D-glucopyranoside (**25**) were also used as starting materials to synthesize the TTF-carbohydrate derivatives. The anomeric carbon in **22** is the most reactive site in a sugar molecule followed by the least sterically hindered primary carbon of **25**.

The reaction involves the deprotection of **12**, followed by nucleophilic substitution of **25** to give the product 2,6-bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**28**) in 48.6% yield. Flash chromatography, using ethylacetate/DCM (1:9) as eluent yielded a yellow orange powder with a melting point of 186.7-187.0°C, $[\boldsymbol{a}]_{\mathrm{D}}^{25}$ = +101 (c = 0.1, DCM). Similarly, **22** yielded 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**29**) as a brown thick oily liquid in 59.8% yield with $[\boldsymbol{a}]_{\mathrm{D}}^{25}$ = -62.55 (c = 0.1, DCM).

The structures of the products were established through infrared, mass, ^{13}C and ^{1}H NMR spectroscopy. The infrared spectra of **28** and **29** give the following structural information: carbonyl group (1750 cm^{-1}, C=O stretching vibration, 1200-1000 cm^{-1}, C-O stretching vibration), nitrile group (2240 cm^{-1}, C≡N stretching vibration), olefinic carbon (2960 cm[1-] , CH stretching vibration, 1600 and 1400 cm[1-] C=C stretching vibration). The FAB-MS spectra of **28** and

29 show a peak at m/z 1098 which can be assumed as the molecular mass and there's the presence of fragment m/z 331 ($C_{14}H_{19}O_9$) that could correspond to the carbohydrate component. These suggest the presence of esters due to the carbohydrate moiety and the cyano group due to TTF in the molecule.
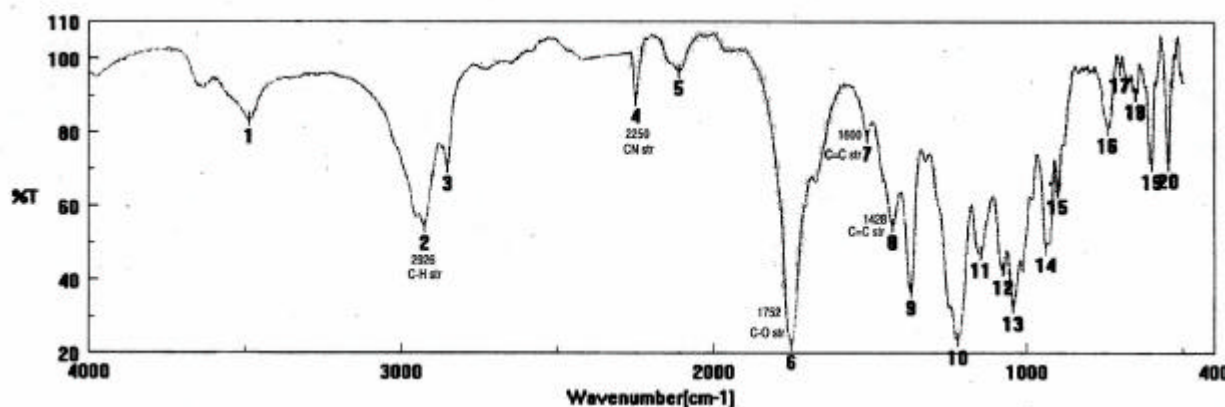
Figure 8. IR spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**28**) recorded in KBr. Interpretation of the important bands (cm^{-1}): 2200, C≡N; 1747, C=O; 1200-1000, C-O; 2926, C-H; 1600 and 1428, C=C.
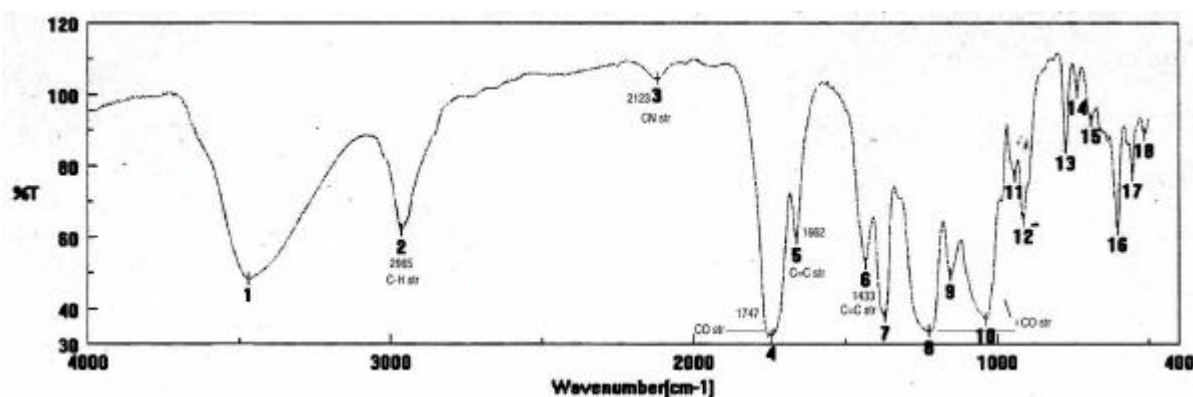
Figure 9. IR spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**29**) recorded in KBr. Inter-pretation of the important bands (cm^{-1}): 2200, C≡N; 1747, C=O; 1200-1000, C-O; 2965, C-H; 1662, 1433, C=C.
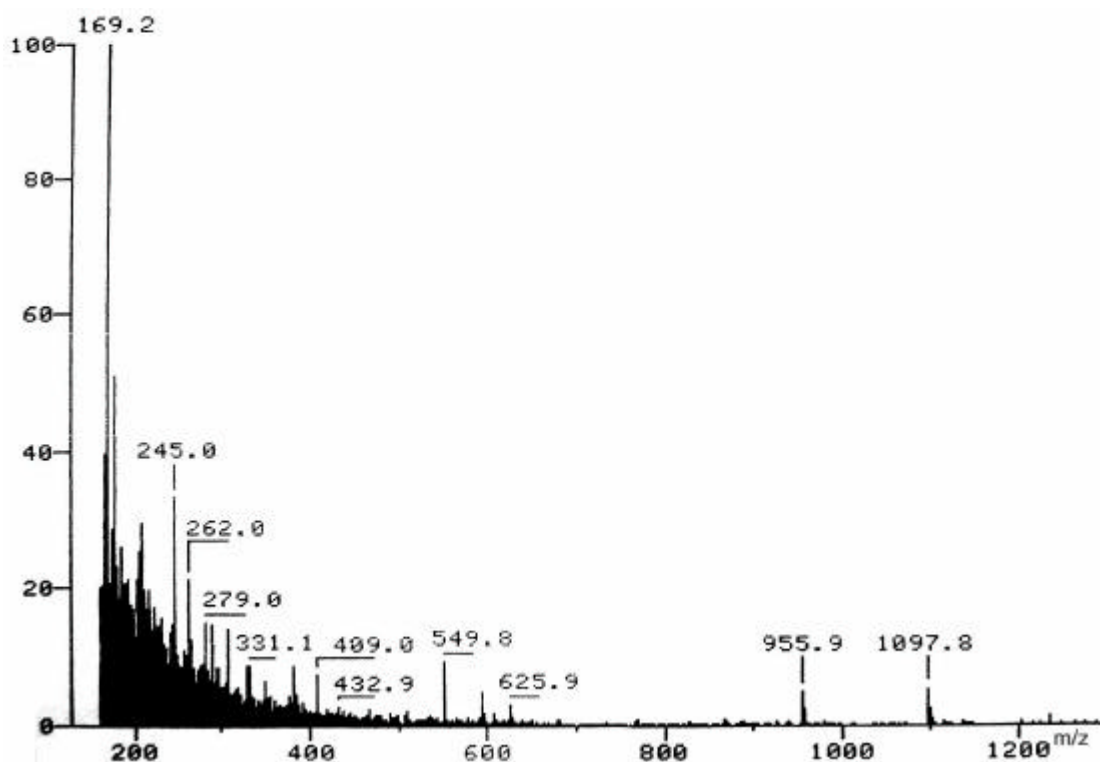
Figure 10. FAB-MS spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**28**). Interpretation of the important fragments: (matrix: NBA) m/z: 1098 [M$^+$], 331 = $C_{14}H_{19}O_9$ = carbohydrate component, 306.8 [matrix].

Figure 11. FAB-MS spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**29**) in NBA matrix at T=50°C. Interpretation of the important fragments: (matrix: NBA) m/z: 1120.8 [M$^+$ + 23], 1098 [M$^+$], 767.9 [M$^+$ - $C_{14}H_{19}O_9$] ($C_{14}H_{19}O_9$= carbohydrate component), 331 = $C_{14}H_{19}O_9$ =carbohydrate component, 307 [matrix].

Additional information can also be extracted from the ^{13}C NMR spectrum of **28** and **29**. Four carbonyl signals were found at δ =170.21 to 168.75 ppm while four acetyl methyl carbons lead to signals in the narrow range δ = 20.85 to δ = 20.44 ppm. This is in good agreement with the presence of esters as was suggested from the infrared spectrum. There is also the presence of the usual signals from the gluco moiety. The mass spectrum fragment at m/z 331 and the presence of resonances due to the acetylated pyranoside ring illustrate the presence of the carbohydrate moiety in the molecule. C-6' of **28** resonates significantly at higher field at δ = 37.21 ppm, and the anomeric carbon of **29** at δ = 84.35 ppm clearly indicate the attachment to a thiol functionality. The olefinic carbon signals at δ = 117.49 ppm and δ = 118.0 ppm for **28** and **29**, respectively, and the presence of resonances at δ = 31.40 ppm and δ = 18.81 for **28** and δ = 32.04 and δ = 18.79 for **29** are due to S$\underline{C}$H$_2$CH$_2$CN and SCH$_2$$\underline{C}H_2$CN, respectively and can be attributed to the TTF component. From the assumed molecular mass of 1098 one has to suggest the presence of two carbohydrate moieties attached to the TTF unit in the molecular structure.

Indicative information about the structure of **28** can be viewed also from its ^{1}H NMR spectrum. The ^{1}H NMR spectrum, integrated only half the number of hydrogen atoms expected from the proposed structure. The conformation adopted by the pyranoside ring in **28** is predominantly ^{1}C$_4$ due to equatorial-equatorial relationship between H-1' and H-2' (J = 4 Hz). Vicinal gauche protons with a ~60° dihedral angle have small J values between 1-4 Hz.

28

Figure 12. Structure of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**28**).

The glucose moiety in **29** adopts the $^4C_1$ as the predominant conformation due to diaxial relationship between H-1' and H-2' (J = 8 Hz). This high coupling constant is consistent with protons that have trans diaxial relationship with a dihedral angle of ~180º. The said conformation is favoured as can be illustrated by the mechanism in scheme 13. When bromide departs, influenced by the presence of cesium to give the oxocarbonium ion, it reacts with C-2' acetate to give a more stable dioxocarbonium ion. Nucleophilic attack of the thiolate salt results in the ring opening to give a bond trans to the participating acetate [5].

oxocarbonium ion   dioxocarbonium ion

Scheme 15. Mechanism for the favoured conformation of the glucopyranoside ring of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**29**).

Figure 13. Structure of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**29**).

Table 4. ^{13}C NMR chemical shifts of 2,6-bis(2''-cyanoethylthio)-3,7-bis (1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**28**).

Carbon chemical shift (đ ppm)	Assignment
170.23, 169.67, 169.59, 168.75	$\underline{C}OOCH_3$
117.50	C=C
88.88	C-1'
70.76 (2C), 69.69, 69.26	C-2'–C-5'
37.29	C-6'
31.40	S$\underline{C}H_2CH_2CN$
20.84, 20.73, 20.68, 20.46	$\underline{C}H_3COO$
18.82	$SCH_2\underline{C}H_2CN$

Table 5. ^1H NMR chemical shifts of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**28**).

| Proton chemical shift (đ ppm)	Integration	Multiplicity	Coupling constant, J (Hz)	Assignment
6.25	1H	d	4	H-1'
5.43	1H	t	10	H-3'
5.04	1H	dd	4, 10	H-2'
4.88	1H	t	10	H-4'
4.05	1H	m		H-5'
3.11	2H	dd	3, 7	S$\underline{C}H_2CH_2CN$
3.05	1H	dd	7, 12	H-6'
2.93	1H	dd	7, 14	H-6''
2.71	2H	dd	3, 7	$SCH_2\underline{C}H_2CN$
2.17, 2.07, 2.06, 2.01	12H	s		$C\underline{H}_3COO$

Table 6. ^{13}C NMR chemical shifts of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**29**).

| Carbon chemical shift (đ ppm)	Assignment
170.66-169.38	CH$_3$$\underline{C}$OO
118.86	C=C
82.98	C-1'
74.90	C-2'
72.66	C-3'
69.29	C-4'
67.69	C-2'
61.78	C-6'
31.75	S$\underline{C}$H$_2$CH$_2$CN
20.69-20.59	$\underline{C}$H$_3$COO
18.19	SCH$_2$$\underline{C}H_2$CN

Table 7. ^1H NMR chemical shifts of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**29**).

Proton chemical shift (đ ppm)	Integration	Multiplicity	Coupling constant, J (Hz)	Assignment
5.22	2H	t	9.5	H-3'
5.10	2H	t	9.5	H-4'
4.99	2H	dd	8, 9.5	H-2'
4.42	2H	d	8.0	H-1'
4.28	2H	dd	5, 12	H-6a'
4.15	2H	dd	3,12	H-6b'
3.72	2H	m		H-5'
3.27	2H	t	6.3	S$\underline{C}$H$_2$CH$_2$CN
2.76	2H	t	6.3	SCH$_2$$\underline{C}H_2$CN
2.08	4H	s		COC$\underline{H}$$_3$
2.05	4H	s		COC$\underline{H}$$_3$
2.04	4H	s		COC$\underline{H}$$_3$
1.98	4H	s		COC$\underline{H}$$_3$

B.II.3. Synthesis of 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-D-glucopy-ranosyl)tetrathiafulvalene (30) and 2,6-bis(2''-cyanoethylthio)-3,7-bis(1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (31)

The synthesis of 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-glucopyranosyl)-tetrathiafulvalene (**30**) and 2,6-bis(2''-cyanoethylthio)-3,7-bis(1'-thio-β-D-gluco-pyranosyl)tetrathiafulvalene (**31**) involved the simple hydrolysis of **28** and **29** using catalytic amounts of NaOMe in methanol. A quantitative amount of a brown oily product was produced. The products were also observed to slowly turn black upon exposure to the atmosphere. Not much information was obtained from the ^{1}H NMR spectrum of **30** and **31** in D_2O because of large water peaks at δ 4.60. However, the infrared spectrum showed the presence of a big broad band at ~3500 cm^{-1} due to OH group, and the absence of carbonyl functionality at 1750 cm^{-1}. The mass spectra for **30** and **31** end at m/z 482. This maybe assigned to [M- 2($C_6H_{11}O_5$) + 2 Na], where $C_6H_{11}O_5$ corresponds to the glucose moiety. The fragments at m/z 460 [M – 2($C_6H_{11}O_5$) + Na] and m/z 328 [M – 2($C_6H_{11}O_5$)] correspond to the TTF moiety. Therefore, the recovered water-soluble brown thick oily product was immediately used for the MTT assay.
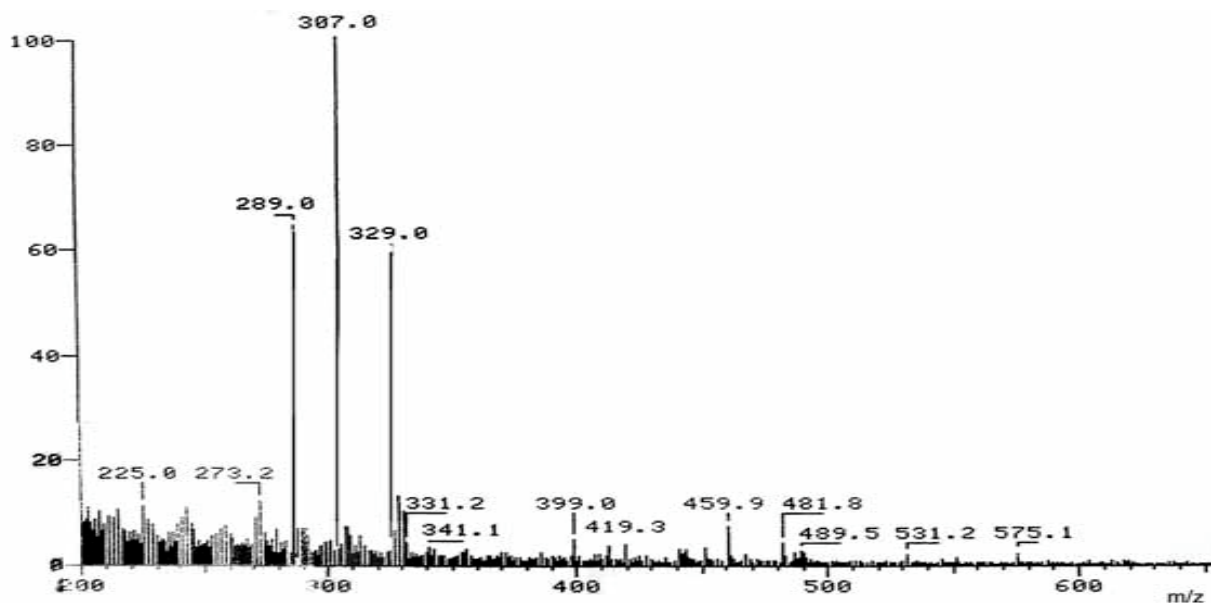
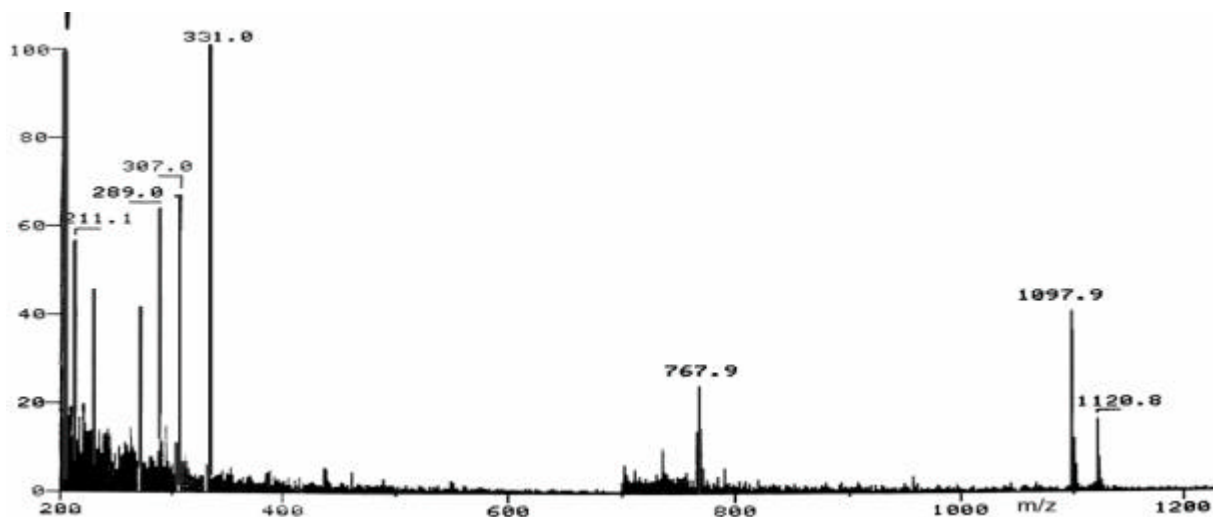**Figure 14**. FAB-MS spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-D-glucopyranosyl)tetrathiafulvalene (**30**) Interpretation of the important fragments: (matrix: NBA) m/z: 481.8 [M^+- 2($C_6H_{11}O_5$) + 2 Na], $C_6H_{11}O_5$ = glucose moiety, 460 [M^+ – 2($C_6H_{11}O_5$) + Na] 307, 289 [matrix].
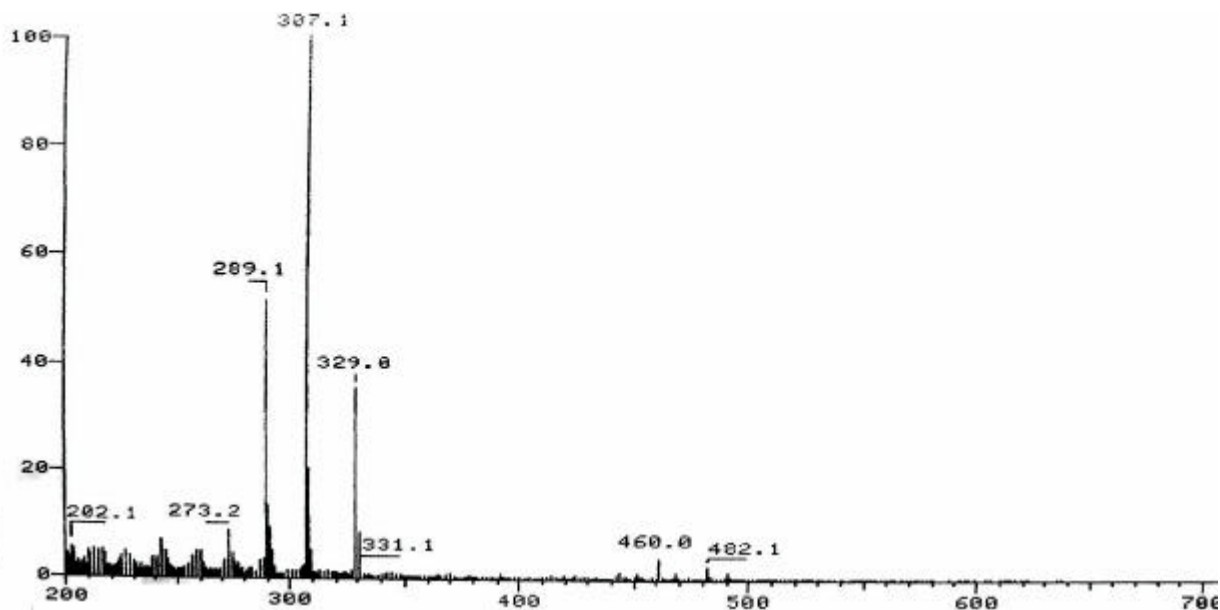
Figure 15. FAB-MS spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**31**). Interpretation of the important fragments: (matrix: NBA) m/z: 482 [M^+- 2($C_6H_{11}O_5$) + 2 Na], $C_6H_{11}O_5$ = glucose moiety, 460 [M^+ – 2($C_6H_{11}O_5$) + Na] 307, 289 [matrix].
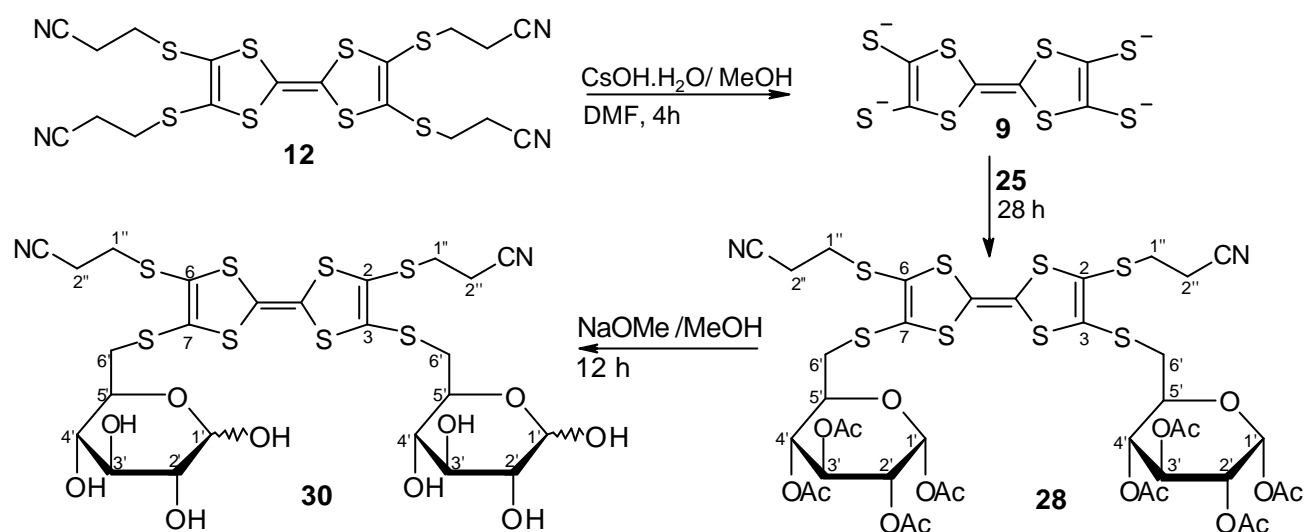
Scheme 16. Synthesis of 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-D-glucopyranosyl)tetrathiafulvalene (**30**) *via* tetrathiafulvalene-2,3,6,7-tetra-thiolate (**9**) an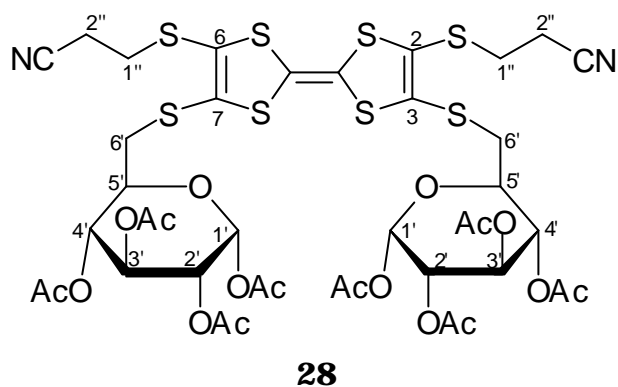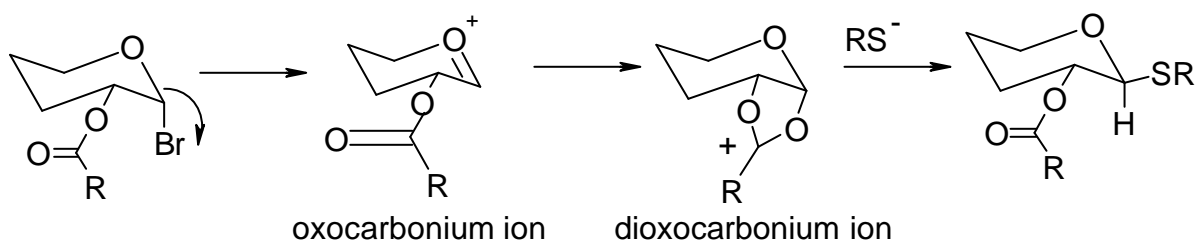d 2,6-bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**28**) from 2,3,6,7-tetrakis(2'-cyanoethylthio)tetrathiafulvalene (**12**) and 1,2,3,4-tetra-O-acetyl-6-deoxy-6-iodo-α-D-glucopyranoside (**25**).
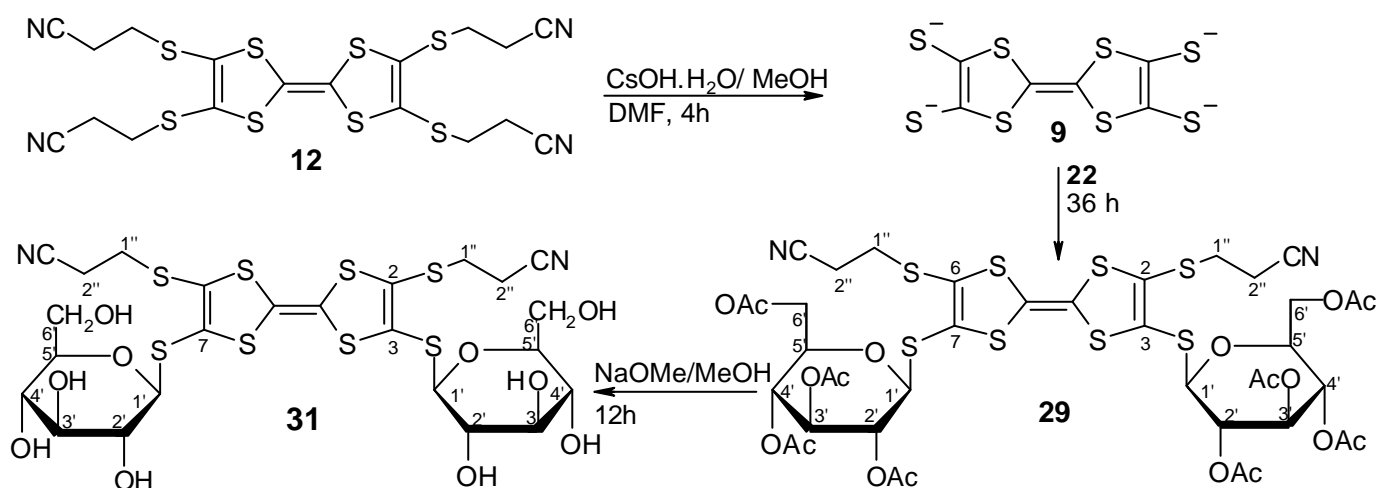
Scheme 17. Synthesis of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**31**) *via* tetrathiafulvalene-2,3,6,7-tetra-thiolate (**9**) and 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-a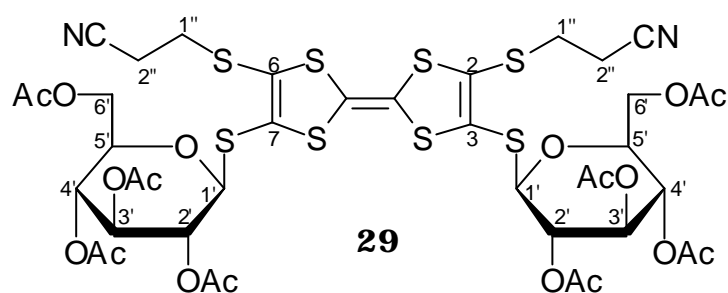cetyl-1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**29**) from 2,3,6,7-tetrakis(2'-cyano-ethylthio)tetrathiafulvalene (**12**) and 2,3,4,6-tetra-O-acetyl-α-D-glucopyrano-sylbromide (**22**).

B.II.4. **Reaction of 2,3,6,7-tetrakis(2'-cyanoethylthio)tetrathiafulvalene (12) with 3,4,6-tri-O-acetyl-D-glucal (26)**

An attempt was made to react **12** with 3,4,6-tri-O-acetyl-D-glucal (**26**). **26** contains a double bond between positions 1 and 2. The thiol group is capable of participation in reactions like addition to double bonds [58]. Two different mechanisms are possible, nucleophilic and free radical addition. The rate might be very slow unless there is a strong base catalyst. However, based on the results, a very polar product was obtained, which turned out to be just the deprotected **26**.
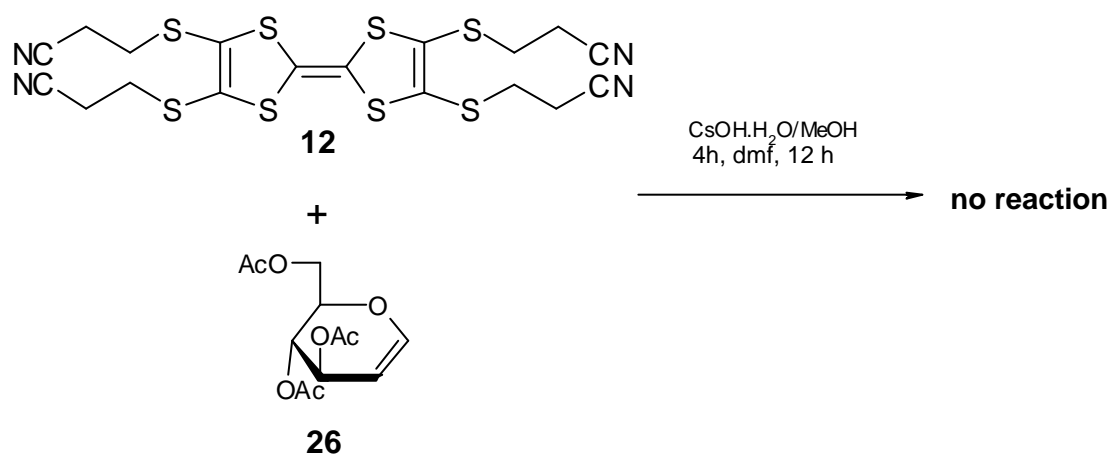
Scheme 18. Reaction of 2,3,6,7-tetrakis(2'-cyanoethylthio)tetrathiafulvalene (**12**) with 3,4,6-tri-O-acetyl-D-glucal (**26**).

B.II.5. Synthesis of tetrakis(2'-hydroxyethylthio)tetrathiafulvalene (32) and 2,6-bis(2''-cyanoethylthio)-3,7-bis(2'-carboxymethylthio)tetrathiaful-valene (33)

Tetrakis(2'-hydroxyethylthio)tetrathiafulvalene (**32**) and 2,6-bis(2''-cyanoethyl-thio)-3,7-bis(2'-carboxymethylthio)tetrathiafulvalene (**33**) were synthesized in an attempt to produce a water-soluble, carbohydrate-free, TTF derivative to be used for the biological assay. **32** was prepared by deprotecting **12** using freshly prepared NaOMe. 2-Chloroethanol was added, stirred over a period of 16 hours and this resulted in an orange oil which crystallized after the addition of hexane to give orange needles with a yield of 90% and a melting point of 142.9-143.6°C (Lit.[52] 139.0-140.0°C). ^{1}H NMR and ^{13}C NMR data are similar to the literature [52] (Scheme 19).
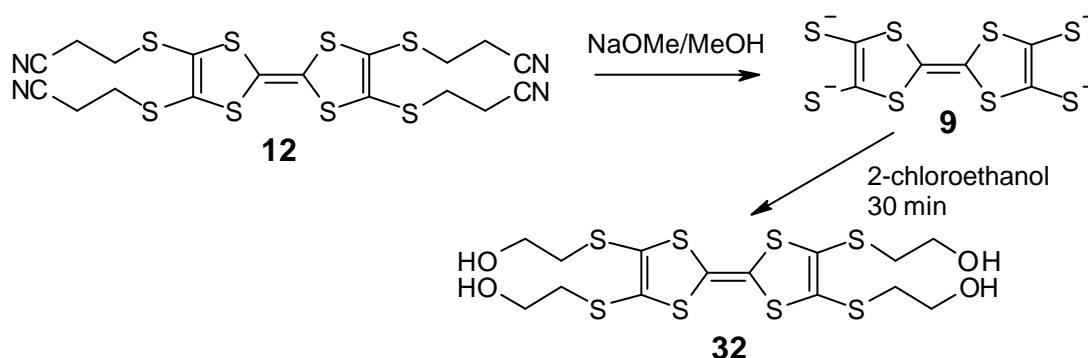
Scheme 19. Synthesis of tetrakis(2'-hydroxyethylthio)tetrathiafulvalene (**32**) *via* tetrathiafulvalene-2,3,6,7-tetrathiolate (**9**), from 2,3,6,7-tetrakis(2'-cyano-ethylthio)tetrathiafulvalene (**12**) and 2-chloroethanol.

Similarly, **33** was prepared by adding ICH2COOH to deprotected **12** and the mixture was stirred overnight at room temperature. This resulted in an orange

oil, methanol was then added and a red-orange precipitate formed. This was filtered to give 82% yield (mp 181-182°C). The FAB-MS spectrum ends at m/z 482 which did not correspond to the expected molecular mass of 544. However, the next fragment at m/z 460, corresponds to [M – 2(CH$_2$COOH) + Na], such that m/z 482 is [M – 2(CH$_2$COOH) + 2(Na)]. The ^{13}C NMR spectum of **33** showed the resonances at δ = 169.2 ppm, suggesting the presence of a carbonyl carbon, δ = 116.93 ppm, indicative for an olefinic carbon and δ = 31.00 and 18.15 ppm, that might be due to a SCH$_2$CH$_2$CN functionality. Additional information can be deduced from the ^{1}H NMR spectrum which showed an intense broad peak at δ = 3.70 ppm that can be attributed to the carboxylic acid functionality. Moreover, the presence of triplets at δ = 3.16 ppm (J = 7), and δ = 2.88 ppm (J= 7) confirmed the presence of the SCH$_2$CH$_2$CN group. Scheme 20 shows the synthesis for **33**.

Table 8. ^{13}C NMR chemical shift of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2'-carboxymethylthio)tetrathiafulvalene (**33**).

Carbon chemical shift (ð ppm)	Assignment
169.12	<u>C</u>OOH
118.93	<u>C=C</u>
31.00	S<u>C</u>H$_2$CH$_2$CN
18.15	SCH$_2$<u>C</u>H$_2$CN

Table 9. ^{1}H NMR chemical shift of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2'-carboxymethylthio)tetrathiafulvalene (**33**).

Proton chemical shift (ð ppm)	Integration	Multiplicity	Coupling constant, J (Hz)	Assignment
3.70		s		COO<u>H</u>
3.12	2H	t	7	SC<u>H</u>$_2$CH$_2$CN
2.88	2H	t	7	SCH$_2$C<u>H</u>$_2$CN
2.50	2H	s		C<u>H</u>$_2$COOH
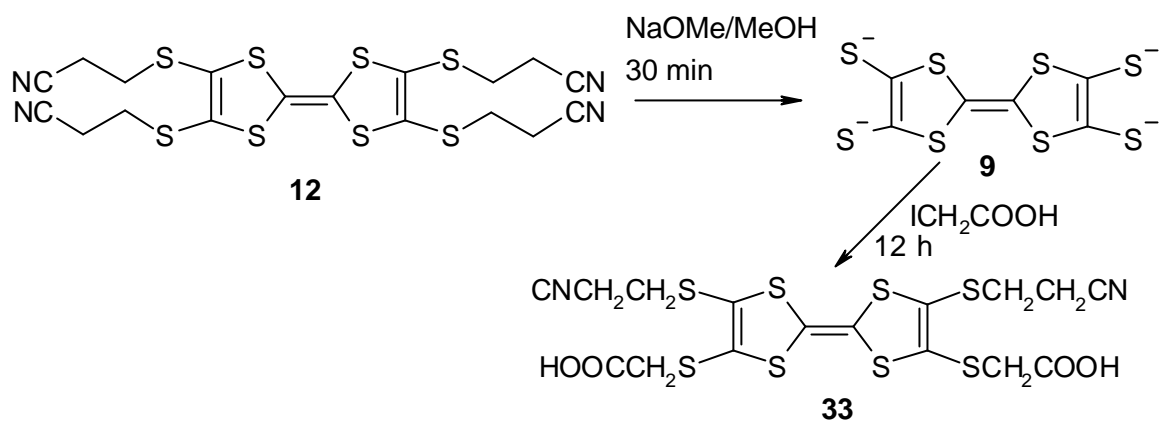
Scheme 20. Synthesis of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2'-carboxy-methylthio)tetrathiafulvalene (**33**) *via* tetrathiafulvalene-2,3,6,7-tetrathiolate (**9**), from 2,3,6,7-tetrakis(2'-cyanoethylthio)tetrathiafulvalene (**12**) and iodoacetic acid.

B.III. Synthesis of 1,3-Dithiole-2-thione-4,5-dithiolate (DMIT)-Carbohydrate Derivatives

The synthesis of different DMIT-carbohydrate derivatives (DMIT = 1,3-dithiole-2-thione-4,5-dithiolate or dimercaptoisotrithione) was performed to come to know the structure-related activity relationship between the DMIT- and TTF-carbohydrate derivatives using the MTT assay.

Two methods were employed for the formation of 1,3-dithiole-2-thione-4,5-dithiolate (DMIT) (**4**). Method A made use of the zinc complex **8,** suspended either in acetone, DMF or acetonitrile with the carbohydrate, and heated under reflux (120°C)[54]. Method B proceeded by the deprotection of **10** by a strong base [56] using either DMF or CH₃CN as solvent and adding finally the sugar (Scheme 21).
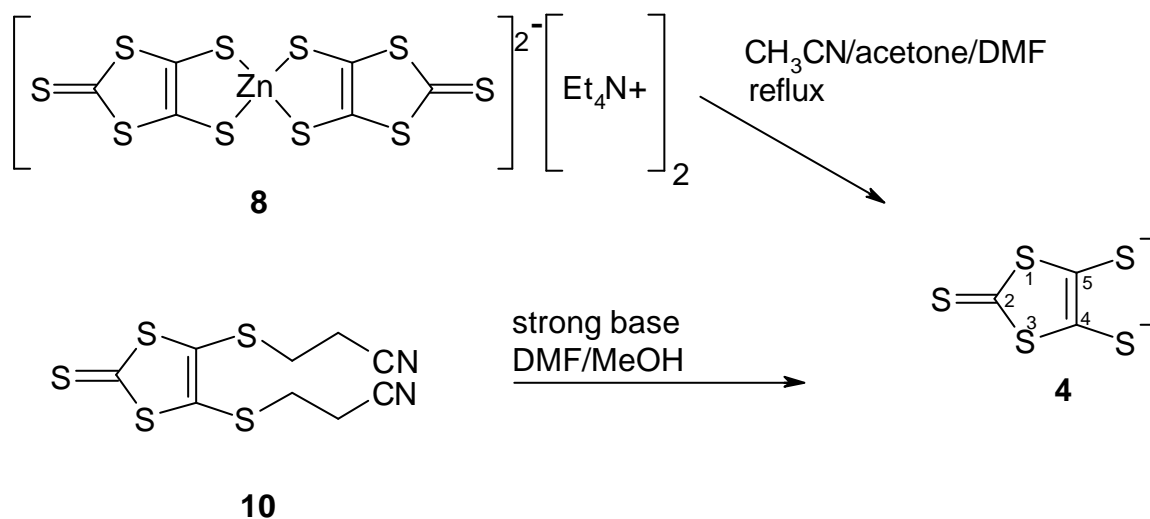
Scheme 21. Different pathways for the formation of 1,3-dithiole-2-thione-4,5-dithiolate (DMIT) (**4**) using bis(tetraethylammonium)bis(1,3-dithiole-2-thione-4,5-dithiolato)zincate (**8**) or 4,5-bis(2'-cyanoethylthio)-1,3-dithiole-2-thione (**10**).

Being a bidentate nucleophile, **4** can react with double and multiple electrophilic species in a number of ways. The course of the reaction depends on the conditions, the nature of the electrophilic sites or the geometry of the electrophile [1].

In the experiment, the DMIT-derivatives were synthesized by reacting **4** with the functionalized glucose **22**, **25** and **26,** ICH_2CH_2OH, and ICH_2COOH.

B.III.1. Synthesis of 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-**a**-D-gluco-pyranosyl)-1,3-dithiole-2-thione (34)

Reaction of **25** yielded *via* methods A and B the product 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)1,3-dithiole-2-thione (**34**) in 50-60% yield. (Scheme 22). **34** forms yellow needle with a melting point of 182.6° – 184 °C and $[a]_D^{25}$ = +147.6 (c = 0.05, DCM).

The structure of **34** was established through IR, MS, ^{1}H and ^{13}C NMR spectroscopy and elemental analysis.

The infrared spectrum gives the following structural information: carbonyl group (~1750 cm^{-1}, C=O stretching), and olefinic carbons (2928 cm^{1-}, C-H stretching, 1667 cm^{-1} and 1420 cm^{-1} C=C stretching). The signal in the mass spectrum at m/z 858 is probably due to the molecular ion. The fragment m/z 526.8 corresponds to [M$^+$ - (C$_{14}$H$_{19}$O$_9$]] (C$_{14}$H$_{19}$O$_9$ = carbohydrate component), while m/z 196 corresponds to [M$^+$ - 2(C$_{14}$H$_{19}$O$_9$)]. Therefore the attachment of two carbohydrate moieties in the molecule and the absence of the ethylcyano leaving group is suggested.
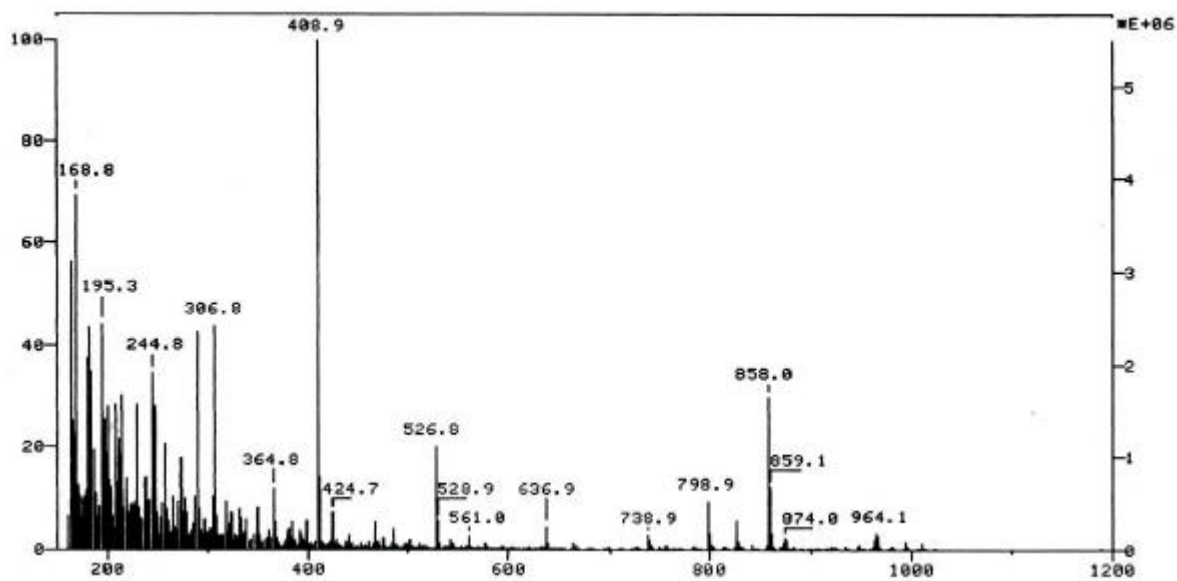
Figure 16. FAB-MS spectrum of 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)1,3-dithiole-2-thione (**34**). Interpretation of the important fragments: (matrix NBA at T=50°C) m/z: 859 [M+H$^+$], 858 [M], 798.9 [M-OAc], 738.9 [M-2(OAc)], 527 [M-C$_{14}$H$_{19}$O$_9$] (C$_{14}$H$_{19}$O$_9$ = carbohydrate component), 306.8 [matrix], 195.3 [M- C$_{14}$H$_{19}$O$_9$].
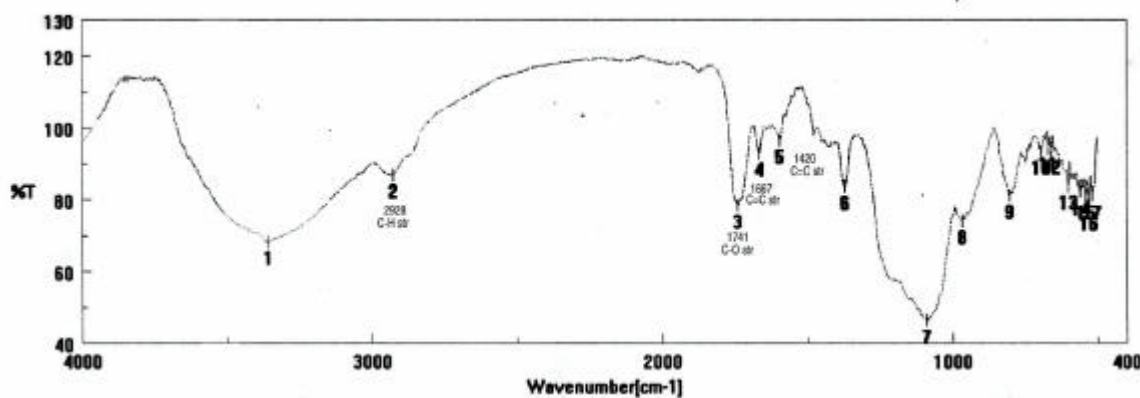
Figure 17. IR spectrum of 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)1,3-dithiole-2-thione (**34**) recorded in KBr. Interpretation of the important bands (cm^{-1}): 1747, C=O; 1200-1000, C-O; 2928, 1667, 1420, C=C.

The ^{13}C spectrum gave the usual resonance for the gluco moiety, but C-6' resonates at higher field δ = 36.93 ppm) indicating the attachment to a thiol group. The appearance of a new signal at δ =137.31 ppm illustrates the presence of an olefinic carbon due to the DMIT component of the molecule.

The ^{1}H NMR spectrum, integrated only half the number of hydrogen atoms which can be attributed to the compound being symmetrical. The conformation adopted by the pyranoside ring in **34** is predominantly $^1C_4$ due to equatorial-equatorial relationship between H-1' and H-2' (J = 3.6 Hz). Vicinal gauche protons with dihedral angles of ~60° have smaller J values between 1-4 Hz.

Table 10. ^{13}C chemical shifts of 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)-1,3-dithiole-2-thione (**34**).

Carbon chemical shift (δ ppm)	Assignment
169.65, 169.58, 169.37, 168.82	$\underline{C}OOCH_3$
137.31	C=C
88.03	C-1'
70.57	C-2'
69.86	C-3'
69.01	C-4'
68.70	C-5'
36.93	C-6'H_2S
20.58, 20.47, 20.32, 20.25	COO$\underline{C}H_3$

Table 11. ^{1}H NMR chemical shifts of 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)-1,3-dithiole-2-thione (**34**).

Proton chemical shift (δ ppm)	Integration	Multiplicity	Coupling constant, J (Hz)	Assignment
6.33	1H	d	3.6	H-1'
5.49	1H	t	10	H-3'
5.11	1H	dd	3.6, 10	H-2'
5.03	1H	t	10	H-4'
4.16-4.08	1H	m		H-5'
3.15	1H	dd	3, 14	H-6'
3.00	1H	dd	7, 14	H-6''
2.33	3H	s		C$\underline{H}_3$
2.27	3H	s		C$\underline{H}_3$
2.06	3H	s		C$\underline{H}_3$
2.03	3H	s		C$\underline{H}_3$
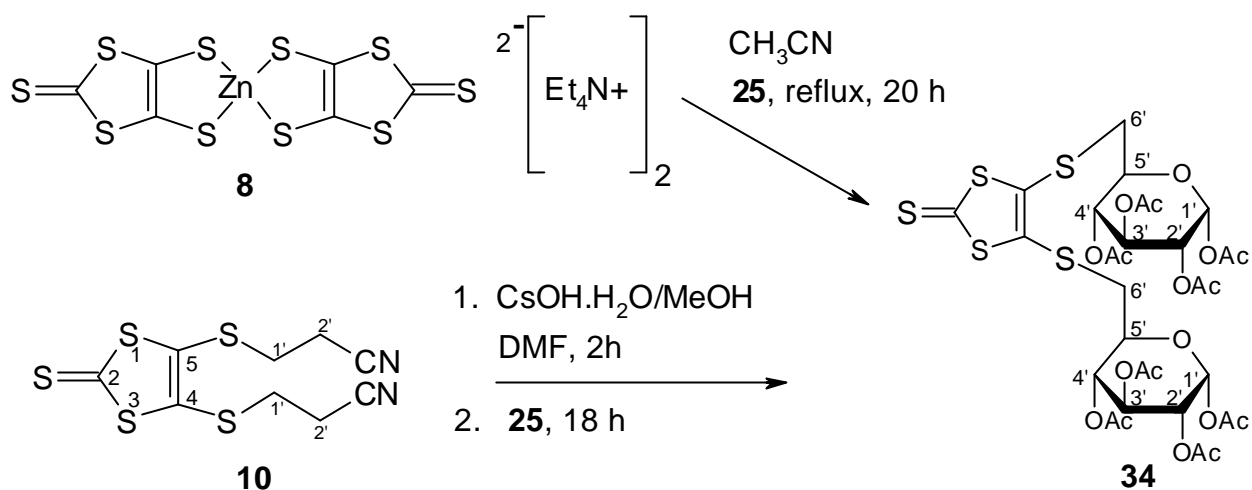
Scheme 22. Synthesis of 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-gluco-pyranosyl)-1,3-dithiole-2-thione (**34**) from bis(tetraethylammonium)-bis(1,3-dithiole-2-thione-4,5-dithiolato)zincate (**8**) or 4,5-bis(2'-cyanoethylthio)-1,3-dithiole-2-thione (**10**).

B.III.2. Synthesis of 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-b-D-glucopyranosyl)1,3-dithiole-2-thione (35)

Upon using method A for the synthesis of 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**35**), the 1,3-dithiole-2-thione-4,5-dithiolate (DMIT) (**4**) formed acted as a nucleophile when reacted with acetyl-protected glucosyl bromide **22** in a Koenig-Knorr type reaction. This resulted in the formation of a light yellow needles of **35** in 60 % yield melting point 178.1°-179.3ºC, and $[a]_D^{25}$ = +101.88 (c = 0.07, DCM). The infrared spectrum gave the following structural information: carbonyl group (1750 cm^{-1}, C=O stretching, 1200 cm^{-1} C-O stretching), nitrile group (2250 cm^{-1}, C≡N stretching), olefinic carbon (2951 cm^{1-} C-H stretching, 1638 cm^{1-} and 1433 cm^{1-}, C=C stretching). The signal in the mass spectrum at m/z 582 is probably caused by [M$^+$ + H]. The last signal in the MS-FAB spectrum at m/z 604 can be assigned to [M + 23], while the fragment at m/z 331 (C$_{14}$H$_{19}$O$_9$ = carbohydrate component) implies the presence of the carbohydrate moiety.

These data suggest the attachment of one carbohydrate residue and the ethylcyano group in the molecule.

The ^{13}C spectrum of the compound showed the usual glucose moiety resonances, but at higher field a resonance for the anomeric carbon at δ = 82.91 ppm is found. The new signals at δ = 31.68 and δ = 18.12 ppm are indicative for the presence of S$\underline{C}$H$_2$CH$_2$CN and SCH$_2$$\underline{C}H_2$CN residues, respectively, and at δ = 118.82 ppm for the olefinic carbon. These data confirm the attachment of the carbohydrate moiety to the DMIT unit. The conformation of the carbohydrate residue was defined by the ^{1}H NMR shift of the anomeric proton. The anomeric proton peak has moved significantly upfield and $J_{1,2}$ is also large (8 Hz) indicating a β configuration at the anomeric carbon. The glucose moiety in **35** adopts the $^{4}C_1$ as predominant conformation due to the diaxial relationship between H-1' and H-2' (J = 8 Hz). This high coupling constant is consistent with protons that are trans diaxial with a ~180º dihedral angle.
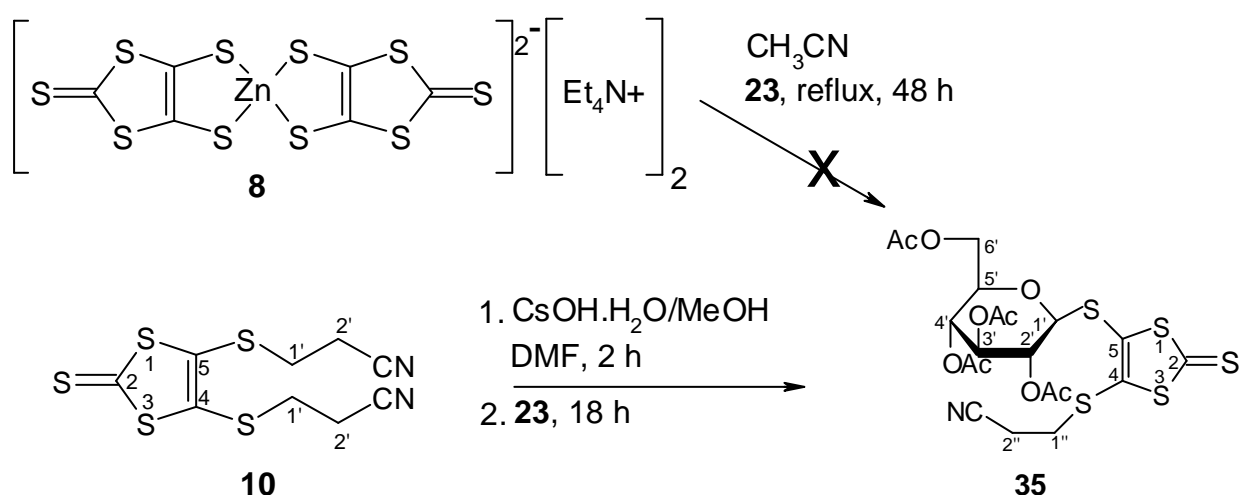
Scheme 23. Synthesis of 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**35**) from bis(tetraethyl-ammonium)bis(1,3-dithiole-2-thione-4,5-dithiolato)zincate (**8**) and 4,5-bis(2'-cyanoethylthio)-1,3-dithiole-2-thione (**10**).
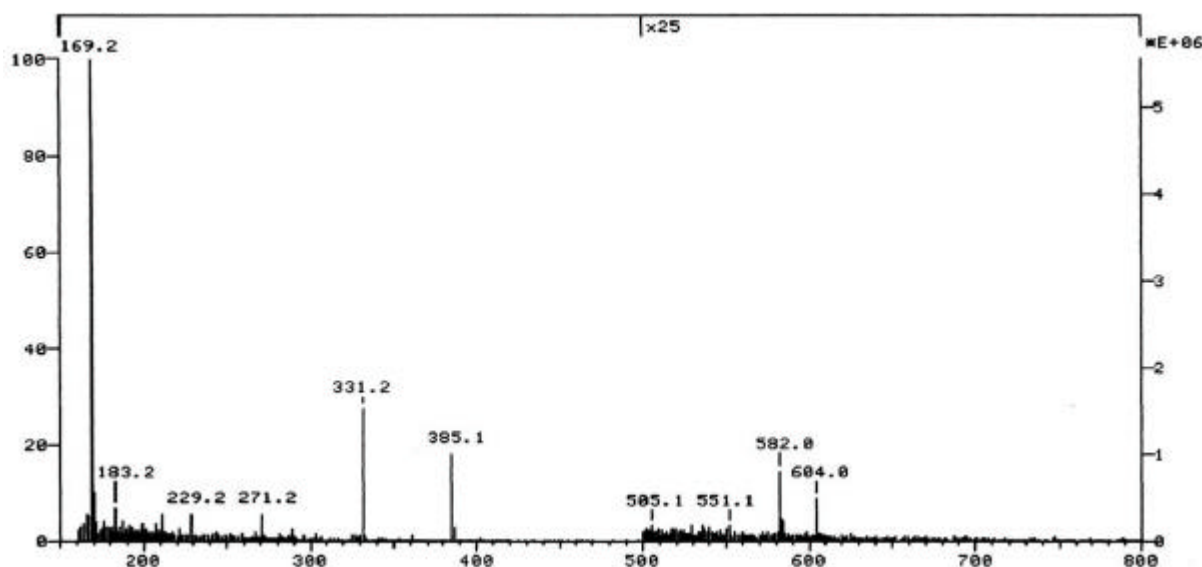
Figure 18. FAB-MS spectrum of 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**35**) . Interpretation of the important fragments: (matrix NBA at T=50°C.) m/z= 604 [M+Na], 582 [M + H$^+$], 385.1 [M+H$^+$-C$_3$S$_5$] (C$_3$S$_5$ = DMIT component), 331.2 [M+H$^+$-C$_{14}$H$_{19}$O$_9$], (C$_{14}$H$_{19}$O$_9$ = carbohydrate component).
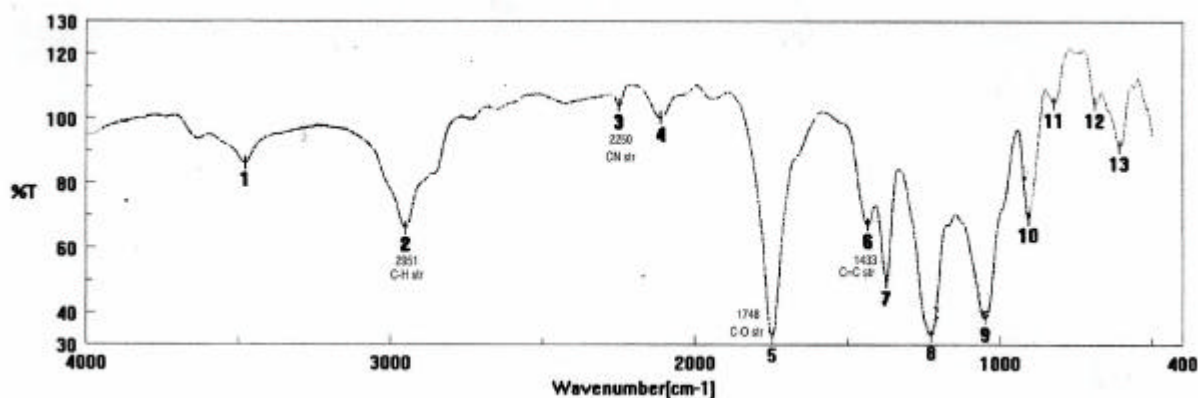
Figure 19. IR spectrum of 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**35**) recorded in KBr. Interpretation of the important bands (cm^{-1}): 1750, C=O; 1200, C-O; 2250, C≡N; 2951, C-H; 1638 and 1433, C=C.

Table 12. ^{13}C NMR chemical shifts of 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**35**).

Carbon chemical shift (d ppm)	Assignment
169.912, 169.503, 169.261	CH$_3$<u>C</u>OO
118.811	C=C
82.910	C-1'
74.824	C-2'
72.585	C-3'
69.227	C-4'
67.624	C-5'
61.713	C-6'
31.684	S<u>C</u>H$_2$CH$_2$CN
20.712, 20.425, 20.377, 20.247	<u>C</u>H$_3$COO
18.123	SCH$_2$<u>C</u>H$_2$CN

Table 13. ^{1}H NMR chemical shifts of 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**35**).

Proton chemical shift (d ppm)	Integration	Multiplicity	Coupling constant, J (Hz)	Assignment
5.23	1H	t	9.4	H-3'
5.11	1H	t	9.4	H-4'
5.00	1H	dd	7.9, 9.4	H-2'
4.43	1H	d	7.9	H-1'
4.29	1H	dd	4, 12	H-6'
4.21	1H	dd	4, 12	H-6''
3.72-3.65	1H	m		H-5'
3.25	2H	t	7	S<u>CH</u>$_2$CH$_2$CN
2.93	2H	t	7	SCH$_2$C<u>H</u>$_2$CN
2.18	3H	s		<u>C</u>H$_3$COO
2.15	3H	s		<u>C</u>H$_3$COO
2.13	3H	s		<u>C</u>H$_3$COO
2.09	3H	s		<u>C</u>H$_3$COO

B.III.3. Synthesis of 4,5-bis(6'-thio-D-glucopyranosyl)-1,3-dithiole-2-thione (36) and 4-(2''-cyanoethylthio)-5-(1'-thio-b-D-glucopyranosyl)-1,3-dithiole-2-thione (37)

The synthesis of of 4,5-bis(6'-thio-D-glucopyranosyl)-1,3-dithiole-2-thione (**36**) and 4-(2''-cyanoethylthio)-5-(1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**37**) involved the simple hydrolysis of **34** and **35** using catalytic amounts of NaOMe in methanol. Not much information was obtained from the ^{1}H spectrum of **36** and **37** in D$_2$O because of large water peaks at δ 4.60. However, the infrared spectrum showed the presence of a big broad band at ~3500 cm^{1-} due to OH group and the absence of carbonyl functionality at 1750 cm^{1-}. The m/z 413 peak in the mass spectrum corresponds to the expected molecular mass of **37**. However, the mass spectrum of **36** did not give the expected molecular mass. Instead a peak at m/z 530 may be caused by a completely protonated product and a fragment at m/z 219 may be assigned to [DMIT + 23]. Therefore, the recovered water-soluble brown thick oily product was immediately used for the MTT assay.

B.III.4 Reaction of 1,3-dithiole-2-thione-4,5-dithiolate (DMIT) with 3,4,6-tri-O-acetyl-D-glucal (26).

An attempt was made to react 1,3-dithiole-2-thione-4,5-dithiolate (DMIT) (**4**) with 3,4,6-tri-O-acetyl-D-glucal (**26**). **26** contains a double bond between positions 1 and 2. The thiol group is capable to add to double bonds [58]. However, based on the results, a very polar product was obtained from method A, which turned out to be just the deprotected **26**. The reaction using method B produced negative results.
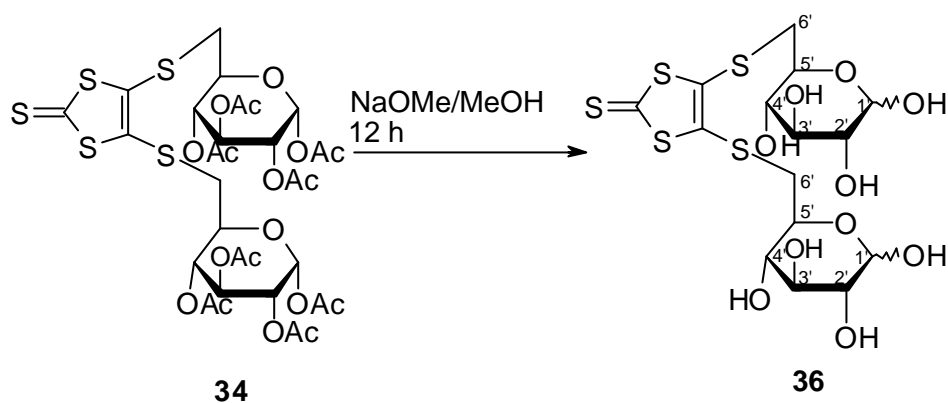
Scheme 24. Synthesis of of of 4,5-bis(6'-thio-D-glucopyranosyl)-1,3-dithiole-2-thione (**36**) from 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)-1,3-dithiole-2-thione (**34**).
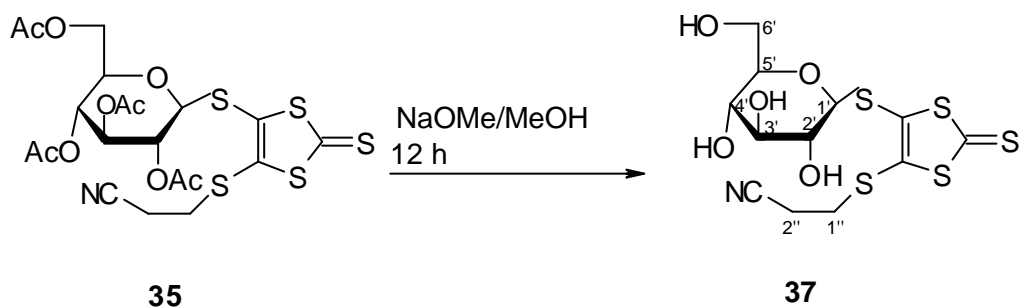
Scheme 25. Synthesis of 4-(2''-cyanoethylthio)-5-(1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**37**) from 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**35**).
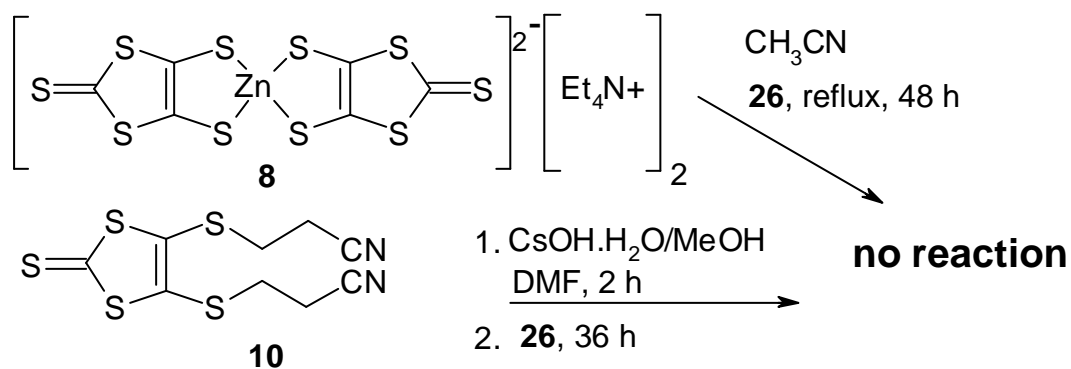
Scheme 26. Reaction of 3,4,6-tri-O-acetyl-D-glucal (**26**) with bis-(tetraethylammonium)-bis(1,3-dithiole-2-thione-4,5-dithiolato)zincate (**8**) or 4,5-bis(2'-cyanoethylthio)-1,3-dithiole-2-thione (**10**).

B.III.5. **Synthesis of 4,5-bis(2'-hydroxyethylthio)-1,3-dithiole-2-thione (38)**

The synthesis of the compounds 4,5-bis(2'-hydroxyethylthio)-1,3-dithiole-2-thione (**38**) and 4,5-bis(2'-carboxymethylthio)-1,3-dithiole-2-thione (**39**) was performed, to produce a non-carbohydrate-containing, water-soluble, DMIT-derivative to be used in the biological assay.

Only method A was used in the synthesis for 4,5-bis(hydroxyethylthio)-1,3-dithiole-2-thione (**38**), yielding 67% of yellow-green crystals with a melting point of 73.1°-75.7°C. The FAB-MS spectrum gave m/z 287 [M +1] which is the expected molecular mass of the compound. Additional information from the ^{13}C and ^{1}H NMR spectra confirmed the structure of the compound.

Table 14. ^{13}C NMR chemical shifts of 4,5-bis(2'-hydroxyethylthio)-1,3-dithiole-2-thione (**38**).

Carbon chemical shift (d ppm)	Assignment
206.62	C=S
136.11	C=C
60.07	SCH$_2$CH$_2$OH
38.64	SCH$_2$CH$_2$OH

Table 15. ^{1}H NMR chemical shifts of 4,5-bis(2'-hydroxyethylthio)-1,3-dithiole-2-thione (**38**).

Proton chemical shift (d ppm)	Integration	Multiplicity	Coupling constant, J (Hz)	Assignment
5.05	1H	t	5.5	CH$_2$OH
3.66	2H	dd	6.3, 12	SCH$_2$CH$_2$OH
3.08	2H	dd	6.3, 12	SCH$_2$CH$_2$OH
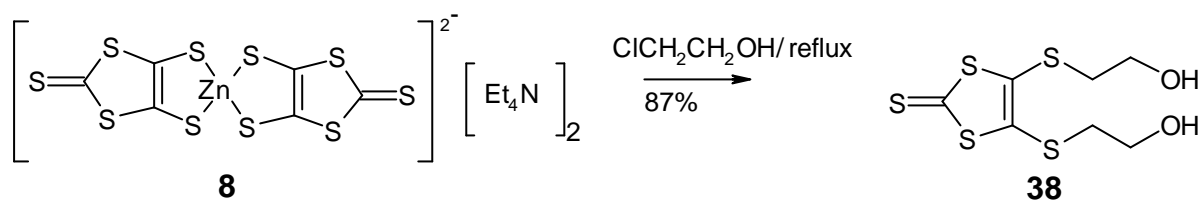
Scheme 27. Synthesis of 4,5-bis(2'-hydroxyethylthio)-1,3-dithiole-2-thione (**38**) from bis(tetraethylammonium)bis(1,3-dithiole-2-thione-4,5-dithiolato)-zincate (**8**).

B.III.6. Synthesis of 4,5-bis(2'-carboxymethylthio)-1,3-dithiole-2-thione (39)

4,5-bis(2'-carboxymethylthio)-1,3-dithiole-2-thione (**39**) was prepared following Hoyer et. al. [42]. Bis(tetraethylammonium)bis(1,3-dithiole-2-thione-4,5-dithiolato)zincate (**8**) in acetone is mixed with ICH_2COOH , NaOH and H_2O and heated under reflux until the disappearance of the red color of **8**. Acetone was removed *in vacuo*, HCl was added with cooling, forming yellow crystals. The product was recrystallized by dissolving the crystals in dilute NH_3 and slow addition of HCl (50%). Yellow crystals of **39**, with a melting point of 172º-174ºC (lit. [42] 171°-173°C) and m/z= 315 [M+1] and 337 [M+23] peaks in the FAB-MS were isolated. The ^{13}C and ^1H NMR spectral data obtained are similar to the literature [42].
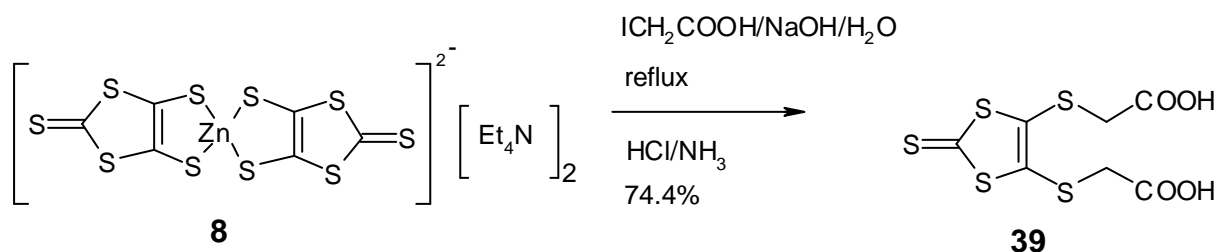
Scheme 28. Synthesis of 4,5-bis(2'-carboxymethylthio)-1,3-dithiole-2-thione (**39**) from bis(tetraethylammonium)-bis(1,3-dithiole-2-thione-4,5-dithiolato)zincate (**8**).

B.IV. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) Assay

The biological activity of the synthesized derivatives was tested on human neuroblastoma cell lines SK-N-LO and SK-N-SH using the MTT assay, with doxorubicin as a standard.

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (**5**), is a yellow tetrazolium salt which can be transformed into the purple coloured formazan (**6**) crystals, by metabolic active cells. The greater the number of vital mitochondria the more MTT will be transformed into formazan. The formazans are difficult to be dissolved. It is first dissolved prior to measurement in isopropanol and the released, dissolved formazan reagent is measured spectrophotometrically with an ELISA reader. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

The water-soluble derivatives were weighed and dissolved in an appropriate amount of sterile and deionized water to prepare the stock solution. The solution was then stored at –20ºC for further use.

There were four independent experiments, each was performed four fold. The first trial was performed using the freshly prepared solution of the synthesized derivatives. The second trial was done from a sample that was stored for 4 days at –20°C, the third one after 8 days of sample storage, and the fourth one after 12 days of sample storage. Figures 20-23 show the effect of different concentrations (0.030, 0.300, 1.50, 3.00 µM/mL) of the test samples against the neuroblastoma cell lines. The % mortality of the cells is ~ 95.0 - 99.0% at the highest concentration used which was 3.00 µM/mL. While at the lowest

concentration used, 0.030 μM/mL, there were trials wherein the number of the cells was large compared to the control. This can be explained by the resistance developed by the cells at a very small concentration of the sample, a well known phenomenon also observed in experiments with conventional cytotoxic drugs.

The results were compared to the doxorubicin (**7**) standard, which is normally employed in antitumor activity testing. This standard was chosen, based on its structure, and the possibility that the TTF could undergo a mechanism similar to **7,** namely intercalation and redox active mechanisms, upon its reaction with the tumor cells. At a concentration of 3.00 μM/mL the TTF-carbohydrate derivatives showed a tumor cell survival percentage between 2-10% which is comparable to **7** which at a concentration of 3.00 μM/mL showed a 17.5 % tumor cell survival . However, based on the experiment LD_{50} for TTF-carbohydrate conjugate has a range between ~ 0.070 – 2.00 μM/mL, while doxorubicin has an LD_{50} ~ 0.10 μM/mL (Table 16).

The cytotoxicity of 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-D-glucopyranosyl)-tetrathiafulvalene (**30**) and 2,6-bis(2''-cyanoethylthio)-3,7-bis(1'-thio-β-D-gluco-pyranosyl)tetrathiafulvalene (**31**), on both neuroblastoma cell lines tested, was dose dependent and was compared to doxorubicin. As shown in the figures, there is a significant difference in the sensitivity of SK-N-LO and SK-N-SH against **30** and **31**. At the moment it can only be speculated about the reason of this effect. It is known that SK-N-LO cells lack catalase activity, in contrast to SK-N-SH cells, and therefore, these cells are more reactive against reactive oxygen species (ROS). It may be that part of the effects of **30** and **31** is caused by the generation of ROS. However, other mechanisms explaining the

cytotoxicity of **30** and **31** are possible. The structural similarity to doxorubicin (both are planar molecules) may allow intercalation in the DNA-disturbing replication. Furthermore, it would be interesting to investigate the influence of these substances on the process of apoptosis. Unfortunately, not enough substances were available to study these processes in more detail. Nevertheless, the results obtained by the MTT test indicate that both substances have an effect on tumor cells in a concentration range not highly different from the well known and effective antitumor drug doxorubicin.

 The assay was also performed on the 1,3-dithiole-2-thione-4,5-dithiolate (DMIT)-carbohydrate derivatives 4,5-bis(6'-thio-D-glucopyranosyl)-1,3-dithiole-2-thione (**36**) and 4-(2''-cyanoethylthio)-5-(1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**37**). The samples did not exhibit significant cytotoxicity against SK-N-LO and SK-N-SH neuroblastoma cells. (Tables 17-20).

The MTT assay was not performed on compounds 4,5-bis(2'-hydroxyethylthio)-1,3-dithiole-2-thione (**38**), and 4,5-bis(2'-carboxymethylthio)-1,3-dithiole 2-thione (**39**), because these are only slightly soluble in water.

On the other hand, the following compounds are water-insoluble such that the MTT assay was not also performed: 2,3,6,7-bis(benzyl 3',4'-dithio-α-D-arabino-pyranosyl)tetrathiafulvalene (**27**), 2,6-bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra -O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**28**), 2,6-bis(2''-cyano-ethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-tetrathiafulvalene (**29**), 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopy-ranosyl)-1,3-dithiole-2-thione (**34**), and 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**35**).

Table 16. LD_{50} of 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-D-glucopyranosyl)-tetrathiafulvalene (**30**) and 2,6-bis(2''-cyanoethylthio)-3,7-bis(1'-thio-β-D-gluco-pyranosyl)tetrathiafulvalene (**31**) on SK-N-LO and SK-N-SH neuroblastoma cell lines.

Cell line	SK-	N-	SH		SK-	N-	LO	
Trial	1	2	3	4	1	2	3	4
LD 50 31 (μM/mL)	0.0773	0.0765	0.199	0.523	0.143	0.225	1.930	0.969
LD 50 30 (μM/mL)	0.152	0.300	0.119	0.469	0.0286	0.545	0.756	0.800

Table 17. Effect of different concentrations of 4,5-bis(6'-thio-D-glucopyranosyl)-1,3-dithiole-2-thione (**36**) on % of survival of SK-N-SH neuroblastoma cells. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 3 independent trials and the +/- standard deviation are given. First trial was performed using the freshly prepared solution. The second trial was performed in a sample that was stored for 4 days. Third trial was done after 8 days of sample storage.

Concentration μM/mL	Trial 1	Trial 2	Trial 3
3.00	2.34 ± 0.30	58.00 ± 5.1	58.51 ± 4.5
1.50	3.00 ± 0.40	93.00 ± 2.4	94.00 ± 1.5
0.30	97.00 ± 6.4	88.00 ± 0.7	104.00 ± 0.9
0.030	98.30 ± 2.7	100.00 ± 1.7	104.00 ± 1.9

Table 18. Effect of different concentrations of 4-(2''-cyanoethylthio)-5-(1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**37**) on % of survival of SK-N-SH neuro-blastoma cells. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 3 independent trials and the +/- standard deviation are given. First trial was performed using the freshly prepared solution. The second trial was performed in a sample that was stored for 4 days. Third trial was done after 8 days of sample storage.

Concentration μM/mL	Trial 1	Trial 2	Trial 3
3.00	24.50 ± 0.8	50.00 ± 0.7	69.70 ± 2.7
1.50	81.50 ± 2.3	49.50 ± 2.2	89.60 ± 2.6
0.30	98.50 ± 1.1	99.00 ± 1.0	93.00 ± 4.1
0.030	104.00 ± 0.9	117.00 ± 4.5	94.30 ± 4.4

Table 19. Effect of different concentrations of 4,5-bis(6'-thio-D-glucopyranosyl)-1,3-dithiole-2-thione (**36**) on % of survival of SK-N-LO neuroblastoma cells. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 3 independent trials and the +/- standard deviation are given. First trial was performed using the freshly prepared solution. The second trial was performed in a sample that was stored for 4 days. Third trial was done after 8 days of sample storage.

Concentration μM/mL	Trial 1	Trial 2	Trial 3
3.00	12.43 ± 0.6	25.60 ± 0.6	24.90 ± 1.6
1.50	71.56 ± 1.3	120.0 ± 1.5	191.0 ± 5.4
0.30	113.00 ± 1.3	120.0 ± 1.5	203.0 ± 3.3
0.030	114.00 ± 2.9	175.0 ± 5.4	179.4 ± 1.1

Table 20. Effect of different concentrations of 4-(2''-cyanoethylthio)-5-(1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**37**) on % of survival of SK-N-LO neuro-blastoma cells. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 3 independent trials and the +/- standard deviation are given. First trial was performed using the freshly prepared solution. The second trial was performed in a sample that was stored for 4 days. Third trial was done after 8 days of sample storage.

Concentration μM/mL	Trial 1	Trial 2	Trial 3
3.00	91.00 ± 6.2	93.50 ± 6.2	106.0 ± 3.1
1.50	192.0 ± 4.3	164.0 ± 4.3	107.0 ± 2.4
0.30	174.0 ± 6.8	152.0 ± 6.8	105.0 ± 6.8
0.030	132.6 ± 5.9	155.0 ± 3.9	186.3 ± 9.1

Effect of different concentrations of 31 on the % survival of SK-N-LO neuroblastoma cells

Figure 20. Activity of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1'-thio-β-D-glucopyrano-syl)tetrathiafulvalene (**31**) against SK-N-LO neuroblastoma cells. 200 μL of SK-N-LO cells were plated at 10,000 cells/well on a 96 well plate. After 24 h incubation at 37°C (5% CO_2 and 100 % humidity) 10 μL of compound **31** at concentrations of 0.030, 0.300, 1.50, 3.00 μM/mL was added to the wells. After 48 h in the incubator, the cell medium was removed and 100 μL of MTT solution was added to each well. After 3 h in the incubator, 100 μL of isopropyl alcohol was then added to each well . After overnight shaking, the absorbance of the colour in each well was measured by a microplate reader (MR 700) at wavelengths of 550 and 630 nm. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 4* independent trials and the +/- standard deviation was plotted.

*First trial was performed using the freshly prepared solution.
 Second trial was performed in a sample that was stored for 4 days.
 Third trial was done after 8 days of sample storage.
 Fourth trial was performed after 12 days of sample storage.

Effect of different concentrations of 30 on the % survival of SK-N-LO neuroblastoma cells

Figure 21. Activity of 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-D-glucopyrano-syl)tetrathiafulvalene (**30**) against SK-N-LO neuroblastoma cells. 200 μL of SK-N-LO cells were plated at 10,000 cells/well on a 96 well plate. After 24 h incubation at 37°C (5% CO_2 and 100 % humidity) 10 μL of compound **30** at concentrations of 0.030, 0.300, 1.50, 3.00 μM/mL was added to the wells. After 48 h in the incubator, the cell medium was removed and 100 μL of MTT solution was added to each well. After 3 h in the incubator, 100 μL of isopropyl alcohol was then added to each well . After overnight shaking, the absorbance of the colour in each well was measured by a microplate reader (MR 700) at wavelengths of 550 and 630 nm. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 4* independent trials and the +/- standard deviation was plotted.

*First trial was performed using the freshly prepared solution.
 Second trial was performed in a sample that was stored for 4 days.
 Third trial was done after 8 days of sample storage.
 Fourth trial was performed after 12 days of sample storage.

**Effect of different concentrations of 31
on the % survival of SK-N-SH
neuroblastoma cells**

Figure 22. Activity of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1'-thio-β-D-glucopyrano-syl)tetrathiafulvalene (**31**) against SK-N-SH neuroblastoma cells. 200 μL of SK-N-SH cells were plated at 10,000 cells/well on a 96 well plate. After 24 h incubation at 37°C (5% CO_2 and 100 % humidity) 10 μL of compound **31** at concentrations of 0.030, 0.300, 1.50, 3.00 μM/mL was added to the wells. After 48 h in the incubator, the cell medium was removed and 100 μL of MTT solution was added to each well. After 3 h in the incubator, 100 μL of isopropyl alcohol was then added to each well . After overnight shaking, the absorbance of the color in each well was measured by a Microplate Reader (MR 700) at wavelengths of 550 and 630 nm. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 4* independent trials and the +/- standard deviation was plotted.

*First trial was performed using the freshly prepared solution.
 Second trial was performed in a sample that was stored for 4 days.
 Third trial was done after 8 days of sample storage.
 Fourth trial was performed after 12 days of sample storage.

**Effect of different concentrations of 30
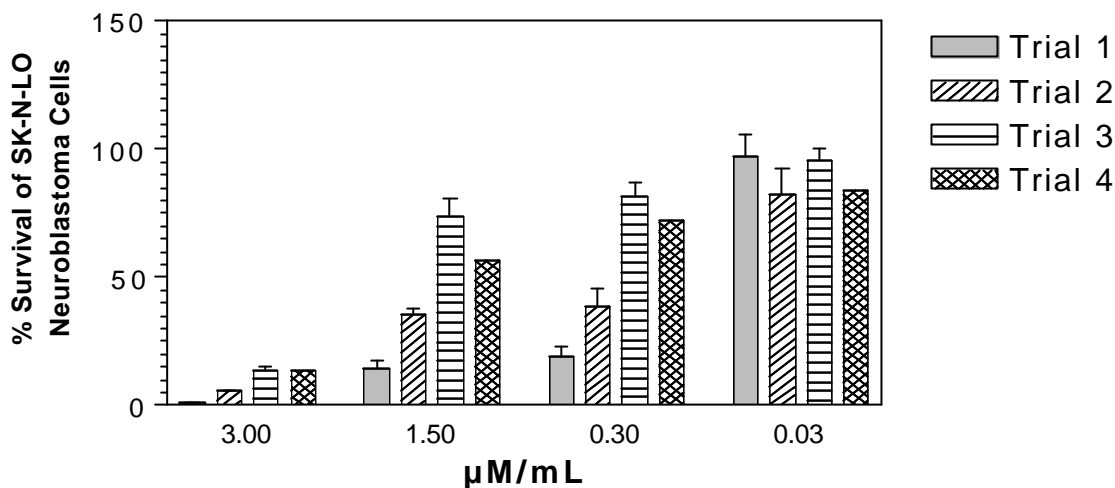on the % survival of SK-N-SH
neuroblastoma cells**

Figure 23. Activity of 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-D-glucopyrano-syl)tetrathiafulvalene (**30**) against SK-N-SH neuroblastoma cells. 200 µL of SK-N-SH cells were plated at 10,000 cells/well on a 96 well plate. After 24 h incubation at 37°C (5% CO_2 and 100 % humidity) 10 µL of compound **30** at concentrations of 0.030, 0.300, 1.50, 3.00 µM/mL was added to the wells. After 48 h in the incubator, the cell medium was removed and 100 µL of MTT solution was added to each well. After 3 h in the incubator, 100 µL of isopropyl alcohol was then added to each well . After overnight shaking, the absorbance of the colour in each well was measured by a microplate reader (MR 700) at wavelengths of 550 and 630 nm. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 4* independent trials and the +/- standard deviation was plotted.

*First trial was performed using the freshly prepared solution.
 Second trial was performed in a sample that was stored for 4 days.
 Third trial was done after 8 days of sample storage.
 Fourth trial was performed after 12 days of sample storage.

Experimental Part

Standard Experimental Procedures

All reactions were performed under an inert atmosphere (nitrogen) with dry reagents and oven-dried glasswares. The reactions were monitored by thin layer chromatography, carried out on 0.25 mm silica gel plates (60 F-254, Merck, Darmstadt, Germany). Plates were visualized by iodine, uv light(where appropriate) or sprayed with an orcinol/H_2SO_4/$FeCl_3$ solution and heated to develop coloration. Preparative thin layer chromatography was performed on 0.5x20x20 cm silica gel plates 860 F-254, Merck, Darmstadt, Germany).

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 250 (^{1}H NMR: 250 MHz, ^{13}C NMR: 63 MHz) or a Bruker WM 400-spectrometer (^{1}H NMR: 400 MHz, ^{13}C NMR: 100 MHz) either in $CDCl_3$, DMSO-d_6, acetone-d_6 or MeOH-d_4 with internal TMS. The chemical shifts are reported in parts per million (ppm) on the δ scale from TMS as an internal standard. Elemental analyses were performed on a Perkin-Elmer elemental analyser, model 240. The EI, FD and FAB mass spectra were recorded on a Finnigan MAT 312 mass spectrometer connected to a PDO 11/34 (DEC) computer system. Optical rotations were obtained with an LEP AZ polarimeter (Zeiss, Jena) at 546 nm.

C.I. Syntheses of Starting Materials

C.I.1. Synthesis of 2,3,6,7-tetrakis(2'-cyanoethylthio)tetrathiafulvalene (12)

C.I.1.1 Bis(tetraethylammonium)-bis(1,3-dithiole-2-thione-4,5-dithiolato)-zincate (8)

Finely shaved sodium metal (46 g, 2 mol) was added to anhydrous CS_2 (350 mL, 5.8 mol) and cooled in an ice bath (0°-5°C). Dry DMF (400 mL) was added slowly over a period of six hours. After stirring overnight, the solution was cooled in an ice bath, and methanol (100 mL) was added slowly to get rid of unreacted sodium. After diluting the solution with 1.5 $MeOH/H_2O$ (2.5 L), it was transferred into an Erlenmeyer flask. $ZnCl_2$ (40g) in ammonia solution (750 mL) and diluted with methanol (750 mL) were added to the flask. To the resulting solution, Et_4Br (66 g) in distilled H_2O (500 mL) was slowly added over a period of 4 hours and stirrred overnight. The solution was filtered using a large sintered glass funnel. The solid was washed with distilled H_2O, isopropanol until the filtrate was clear and lastly with diethylether (500 mL). After drying **8** was obtained as deep red precipitate (99.10 g, 55%); m.p. 202.4°-205.0° C, ref. [42]: 200-205°C. FAB–MS: m/z = 719.0 [M^+ + 1]. $C_{22}H_{40}N_2S_{10}Zn$ (718.6)

Calculated: C 36.77%   H 5.56%   N 3.89%   S 44.53%

Found: C 31.89%   H 4.23%   N 3.57%   S 44.10%

C.I.1.2. 4,5-Bis(2'-cyanoethylthio)-1,3-dithiole-2-thione (10)

To a solution of **8** (2.57 g, 3.57 mmole) in MeCN (50 mL) was added 3-bromopropionitrile (2.42 g, 18.0 mmol) and the mixture was refluxed for 2

hours. The resulting solution was cooled to room temperature and the precipitated salt was filtered. The brown-yellow filtrate was concentrated *in vacuo*, the resulting product was dissolved in CH_2Cl_2 (50 mL) , washed with water (4 x 100 mL) , dried (Na_2SO_4) and the solvent removed *in vacuo.* Recrystallization of the product from toluene/petroleum ether (bp 100-140°C) gave **10** as long yellow needles; yield 2.26 g (70%); mp 84.8-86.5°C, ref. [56]: 83°C.Rf 0.737 (DCM/toluene/hexane/acetone/; 1/1/1/1) FAB–MS: m/z = 305 [M^+ + 1]. $C_9H_8N_2S_5$ (304.5)

Calculated: C 35.53%   H 2.63%   N 9.21%   S 52.71%

Found: C 35.59%   H 2.30%   N 9.15%   S 54.00%

C.I.1.3. 4,5-Bis(2'-cyanoethylthio)-1,3-dithiol-2-one (11)

A mixture of **10** (1.5 g, 4.93 mmol) in DCM/AcOH (3:1, 50 mL) and $Hg(OAc)_2$ (4.11 g, 13.14 mmol) was stirred under N_2 at r.t. for 16 h. The resulting white precipitate was filtered using membrane filter and washed thoroughly with DCM. The combined organic phases were refluxed with activated charcoal, cooled to room temperature, washed with saturated $NaHCO_3$ solution (3 x 100 mL), water (100 mL), dried (Na_2SO_4) and concentrated *in vacuo* affording **11** as large pale yellow needles; yield :1.46 g, 99%, mp 64-65°C, ref. [56]: 64-65°C. FAB–MS: m/z = 289 [M^+ + 1]. $C_9H_8N_2S_4O$: (288.44)

Calculated: C 37.50%   H 2.77%   N 9.72%   S 44.44%

Found: C 37.62%   H 2.26%   N 9.79%   S 44.78%

C.I.1. 2,3,6,7-Tetrakis(2'-cyanoethylthio)tetrathiafulvalene (12)

11 (1.40 g, 4.85 mmol) was suspended in toluene (50 mL) and refluxed with stirring while freshly distilled triethylphosphite (5 mL) was added. After about 30 min a red-orange precipitate starts to form, the red solution was stirred for an additional 1 h at reflux and then allowed to cool at room temperature. MeOH (25 mL) was added, the product was filtered, washed with MeOH (3 x 25 mL) and dried *in vacuo*, to give **12** as intense red-orange crystals; yield 0.9 g (66%); mp 186.9-188.4°C, ref.[56]: 209-210°C. FAB–MS: m/z = 545 [M$^+$ + 1]

$C_{18}H_{16}N_4S_8$ (544.0)

Calculated: C 39.71%   H 2.93%   N 10.29%   S 47.06%

Found: C 39.71%   H 2.40%   N 10.33%   S 45.19%

C.I.2. Synthesis of benzyl 2,3-anhydro-ß-L-ribopyranoside (18)

C.I.2.1. Benzyl ß-L-arabinopyranoside (14)

14 was prepared from L-arabinose (**13**) 50 g (0.33 mol) and freshly distilled benzyl alcohol (250 mL) and absolute ether (1000 mL) as described in [48]. Yield 73 g (91%); m.p. 171-173°C (ethanol/water), ref. [48] : 168°-171° C (ethanol); $[a]_D^{25°C}$ = +210° (c = 1, H$_2$O) ref [48]: +206° (c = 3, H$_2$O).

$C_{12}H_{16}O_5$ (240.23)

Calculated: C 59.99%   H 6.70%

Found: C 59.55%   H 6.50%

C.I.2.2. Benzyl 3,4-O-isopropylidene-**b**-L-arabinopyranoside (15)

15 was prepared from **14** (72 g, 0.30 mol), 2,2-dimethoxypropane (500 mL) and p-toluenesulfonic acid (1 g) in acetone (500 mL). The resultant syrup **15** was used without further purification for the next step. Yield 53 g (89%).

C.I.2.3. Benzyl 3,4-O-isopropylidene-2-O-p-tolylsulfonyl-**b**-L-arabinopyranoside (16)

16 was prepared from **15** (75 g, 0.27 mol), dissolved in pyridine (500 mL) and p-toluenesulfonyl chloride (199 g) as described in [48]. Yield 107.3 g (92%); m.p. 93-95°C (ethanol/water), ref. [48]: 93°- 94° C (ethanol/water); $[a]_{D}^{25°C} =$ +183° (c = 1, CHCl$_3$), ref[48]: (c = 1, CHCl$_3$). C$_{22}$H$_{26}$O$_7$S: (434.47)

Calculated: C 60.81%   H 6.02%   S 7.37%

Found   C 60.61%   H 6.06%   S 7.15%

C.I.2.4. Benzyl 2-O-p-tolylsulfonyl-**b**-L-arabinopyranoside (17)

17 was prepared from **16** (107 g, 0.24 mol) and 90 % acetic acid (100 mL) as described in the ref [48]. Yield 88.7 g (91%); m.p. 119°-120°C (methanol/water), ref. [48]: 118°-120°C (methanol/water); $[a]_{D}^{25°C} =$ +128 (c= 1, CHCl$_3$), ref. [48]: +134 (c= 1, CHCl$_3$). C$_{19}$H$_{22}$O$_7$S: (394.41)

Calculated: C 57.81%   H 4.61%   S 8.12 %

Found   C 57.71%   H 5.50%   S 8.05%

C.I.2.5. Benzyl 2,3-anhydro-**b**-L-ribopyranoside (18)

18 was prepared from **17** (88 g, 0.22 mol), methanol (950 mL) and sodium (7.1 g, 0.30 mol). Yield 28.7 g (92.6%);   m.p. 76°-77° C (EE), ref. [48]: 76°-77° C (EE); $[a]_D^{25°C}$ = -3 (c= 1, EE); $[a]_D^{25°C}$ = -11 (c = 1, EE).

$C_{12}H_{14}O_4$ (222.22)

Calculated   C 64.86%   H 6.34%

Found   C 64.32%   H 6.32%

C.I.2. Benzyl 2,3-anhydro-4-O-triflyl-**b**-L-ribopyranoside (19)

19 was prepared from **18** (2.22 g, 10 mmol), CH_2Cl_2 (75 mL), pyridine (2 mL) and trifluromethanesulfonic anhydride (1.8 mL, 10.97 mmol), synthesized as described in ref. [48]. Yield 3.13 g (90%); m.p. 83°-84° C (ethanol), ref. [48]: 82°-83° C (ethanol); $[a]_D^{25°C}$ = +16° (c= 1, $CHCl_3$), ref. [48]: $[a]_D^{25°C}$ = +16° (c= 1, $CHCl_3$). $C_{13}H_{13}F_3O_6S$: (354.29)

Calculated   C 44.07%   H 3.69%   S 9.04%

Found   C 43.85%   H 3.60%   S 9.25%

C.I.3. Synthesis of 2,3,4,6-tetra-O-acetyl-**a**-D-glucopyranosylbromide (22) [51]

C.I.3.1. 1,2,3,4,6-Penta-O-acetyl-**a**-D-glycopyranoside (21)

Sodium acetate (5.5 g) in acetic anhydride (100 ml) were heated until boiling. D-(+)-glucose (**20**) (10 g, 55.5 mmole) were added slowly and remained heated until the solution became clear.   After cooling to room temperature, the solution was poured in crushed ice (100 g) and allowed to stand for 3 hours.

The resulting precipitate was filtered, washed with water and dried *in vacuo.* Recrystallization in ethanol (95%) gave **21** (18.2g, 83.9 %) mp 136-137°C, ref [51]: 128°-130°C. FAB–MS: m/z = 391. $C_{16}H_{22}O_{11:}$ (390.0)

Calculated: C 49.23% H 5.64%

Found: C 49.11% H 5.26%

C.I.3. 2,3,4,6-Tetra-O-acetyl-**a**-D-glucopyranosyl bromide (22)

To **21** (2.3 g, 5.8 mmole) was added 45% HBr/HOAc (15 ml) and stirred for 3 hours. Reaction was monitored by TLC. Solution was diluted with dichloromethane and added to crushed ice (~200 g) Organic phase was separated washed with $NaHCO_3$ (2 x 200 ml) , dried with Na_2SO_4, filtered and solvent evaporated *in vacuo.* The product was precipitated from the resulting liquid by adding diethyl ether (15 ml) and dropwise addition of petroleum ether with trituration. The product was obtained as white precipitate (2.176 g, 89.5%); mp 86°-87°C, ref [51] 87°-88°C Rf= 0.7 (acetone/DCM/ hexane/toluene). FAB–MS: m/z = 412. $C_{14}H_{19}O_9Br$: (411.0)

Calculated: C 40.88% H 4.62%

Found: C 40.44% H 3.55%

C.I.4. Synthesis of 1,2,3,4-tetra-O-acetyl-6-deoxy-6-iodo-**a**-D-glucopy-ranoside (25) [52]

C.I.4.1. Methyl 2,3,4-tri-O-acetyl-6-deoxy-6-iodo-**a**-D-glucopyranoside (24)

A mixture of methyl-α-D-glucopyranoside (**23**) (2.0 g, 10.3 mmol), triphenylphosphine (4.05 g, 15.5 mmol), imidazole (2.10 g, 30.9 mmol) and iodine (3.9 g, 15.5 mmol) in toluene (200 mL) was vigorously stirred at 70°C for

2.5 hours. Heating was stopped and resulting mixture was stirred for another 15 hours. After water (200 mL) was added, the mixture was vigorously stirred for 20 minutes and transferred to a separatory funnel. The organic phase was extracted with water until no product is left in the toluene layer. The combined aqueous phase was concentrated *in vacuo*. Acetic anhydride (40 mL) and pyridine (52 mL) were added and the solution was stirred overnight. After removing the solvent *in vacuo*, the residue is dissolved in toluene, washed with water, dried with Na_2SO_4, filtered, and concentrated *in vacuo*. Recrystallization in absolute ethanol gave **24** as a white solid (3.10 g, 88%), mp 151°-152°C, ref. [52]: 146°-147°C. FAB–MS: m/z = 431 [M$^+$ + 1]. $C_{13}H_{19}O_8I$: (430.0)

Calculated: C 36.28% H 4.42%

Found: C 36.31% H 4.13%

C.I.4. 1,2,3,4-Tetra-O-acetyl-6-deoxy-6-iodo-**a**-D-glucopyranoside (25)

To a solution of **24** (3.28 g, 7.6 mmol), in acetic anhydride (50 mL) and glacial acetic acid (20 mL) was slowly added conc. H_2SO_4 (1.6 mL). After stirring for 24 hours, solution was poured into crushed ice (100 g). The precipitate was filtered and thoroughly washed with saturated $NaHCO_3$ and distilled water. The precipitate was dried in a dessicator under high vacuum with silica and P_2O_5 for 2 hours. Recrystallization in absolute ethanol gave **25** as white crystals (3.0 g, 84.5%), mp 175°-176°C, ref [52]: 172°C. FAB–MS: m/z = 459 [M$^+$ + 1]. $C_{14}H_{19}O_9I$: (458.2)

Calculated: C 36.68% H 4.15%

Found: C 32.58% H 3.59%

C.II. Synthesis of Tetrathiafulvalene-Carbohydrate Conjugates

C.II.1. 2,3,6,7-Bis(benzyl 3',4'-dithio-*a*-D-arabinopyranosyl)tetrathiafulvalene (27)

Cesium hydroxide (CsOH.H$_2$O) (87.6 mg, 0.52 mmol) in MeOH (10 ml) was injected **12** (63.0 mg, 0.12 mmol) in dry and degassed DMF (10 ml) under nitrogen . Reaction was stirred for two hours, **19** (214 mg, 0.52 mmol) in DMF (5 ml) was added and solution stirred for 12 hours. After removing the solvent *in vacuo*, the residue was dissolved in DCM, washed with water, dried with Na$_2$SO$_4$, filtered and the solvent evaporated *in vacuo*. The residue was subjected to flash column chromatography using 10 % EtOAC/DCM as eluting solvents affording brown oil (76 mg, 80.0% yield); Rf 0.38 (10% EtOAC/DCM); $[a]_D^{25°C}$ = +62.55 (c = 0.1 DCM); FAB-MS m/z= 740 [M$^+$]

Calculated: C 48.65%   H 3.78%   S 34.59%

Found: C 48.99%   H 3.30%   S 32.26%

^{13}C (63 MHz, acetone d-6/TMS) δ = 138.84 (C$_6$H$_5$), 128.61, 128.52, 128.40 (C$_6$H$_5$), 119.09 (C=C), 104.04 (C-1'), 95.20 (C-2'), 70.80 (OCH$_2$Ph, (B), 56.16 (C-3'), 52.23 (C-5'), 50.66 (C-4').

^{1}H (250 MHz, CDCl$_3$ /TMS) δ = 7.29-7.36 (5H, m, C$_6$H$_5$), 4.93 (1H, d, J = 12, OCHHPh), 4.80 (1H, d, J = 12, OCHHPh), 4.61 (1H, dd, J = 8, 12, H-5'), 4.47 (1H, d, J = 7.5, H-1'), 4.18 (1H, dd, J = 4, 12, H-5''), 3.92 (1H, ddd, J = 4, 7, 10, H-4'), 3.65 (1H, dd, J = 5, 7.5, H-2'), 3.56 (1H, dd, J = 5, 10, H-3').
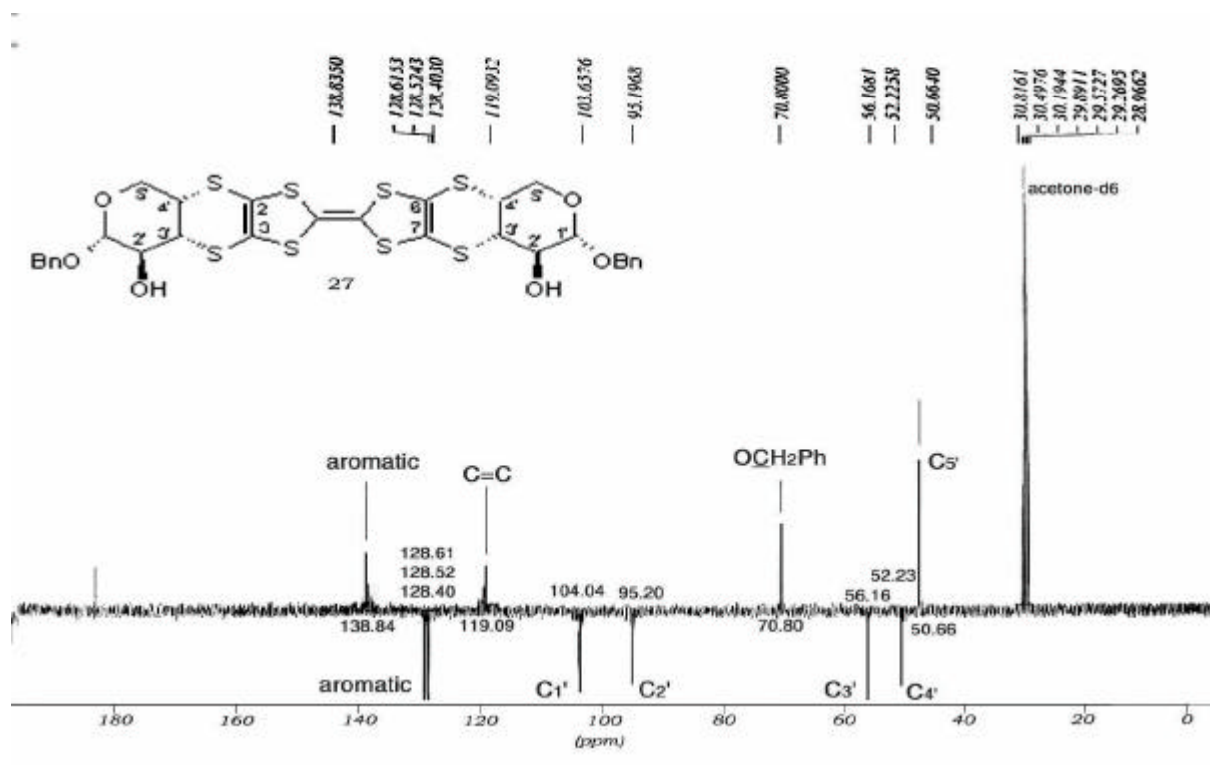
Figure 24. ^{13}C NMR spectrum of 2,3,6,7-bis(benzyl 3',4'-dithio-α-D-arabino-pyranosyl)tetrathiafulvalene (**27**). 63 MHz, acetone-d6. See also Table 2.
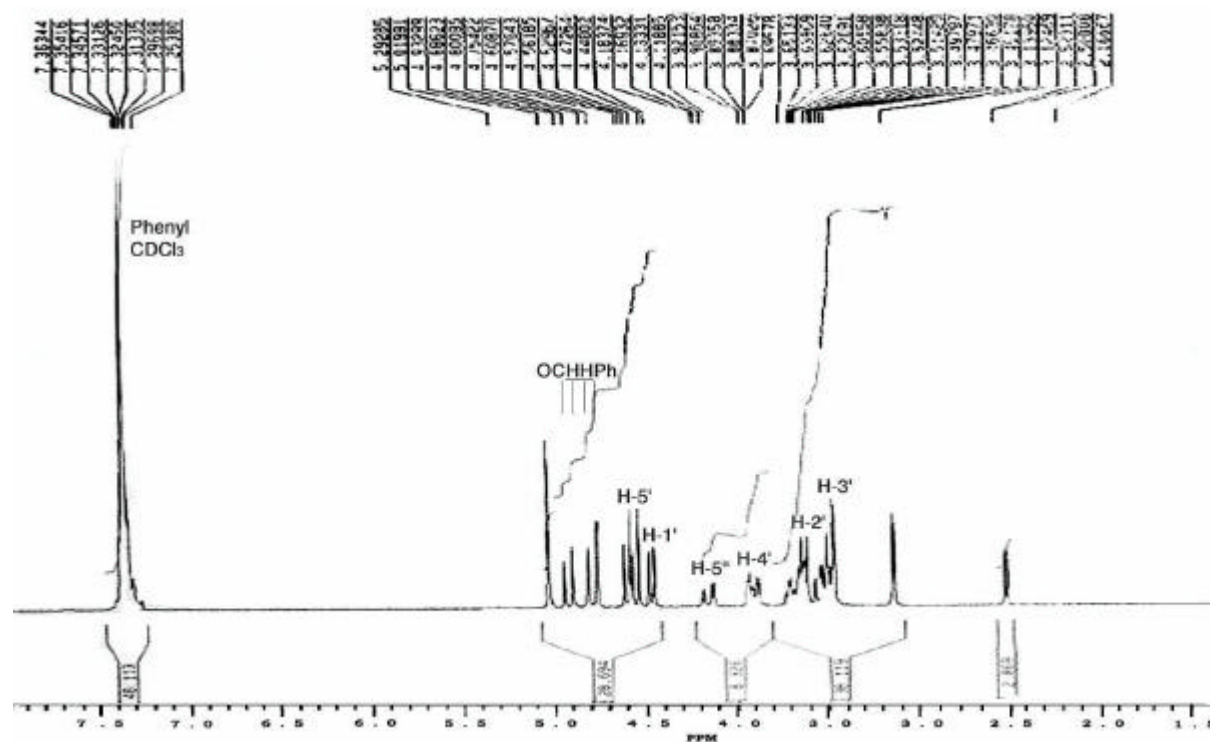
Figure 25. ^{1}H NMR spectrum of 2,3,6,7-bis(benzyl 3',4'-dithio-α-D-arabino-pyranosyl)tetrathiafulvalene (**27**). 250 MHz, CDCl₃. See also Table 3.

C.II.2. 2,6-Bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (28)

Cesium hydroxide (CsOH.H$_2$O) (64.9 mg, 0.39 mmol) in MeOH (10 ml) was injected to **12** (50.0 mg, 0.09 mmol) in dry and degassed DMF (3 ml) under nitrogen. Reaction was stirred for two hours, **25** (179 mg, 0.39 mmol) in DMF (3 ml) was added and solution stirred for 28 hours. After removing the solvent *in vacuo*, the residue was dissolved in DCM, washed with water, dried with Na$_2$SO$_4$, filtered and the solvent evaporated *in vacuo*. The residue was subjected to flash column chromatography using 10 % EtOAC/DCM as eluting solvents affording yellowish-brown oil (49 mg, 48.6% yield); Rf 0.40 (10% EtOAC/DCM); $[a]_D^{25°C}$ = +101 (c = 0.1 DCM). FAB-MS m/z = 1098 [M$^+$]

Calculated: C 44.08%   H 4.19%   S 23.32%   N 2.55%

Found: C 47.64%   H 2.74%   S 10.61%   N 3.57%

^{13}C (250 MHz, CDCl$_3$/TMS)

δ = 170.23, 169.67, 169.59, 168.75, (<u>C</u>OOCH$_3$), 117.50, (C=C), 88.88, (C-1'), 70.76 (2C), 69.69, 69.26, (C-2'–C-5'), 37.29, (C-6'), 31.40, (S<u>C</u>H$_2$CH$_2$CN), 20.84,20.73, 20.68, 20.46, (<u>C</u>H$_3$COO), 18.82, (SCH$_2$<u>C</u>H$_2$CN).

^{1}H (250 MHz, CDCl$_3$/TMS)

δ = 6.25, (1H, d, 4, H-1'), 5.43, (1H, t, J = 10, H-3'), 5.04, (1H, dd, J = 4, 10, H-2'), 4.88, (1H, t, J = 10, H-4'), 4.05, (1H, m, H-5'), 3.11, (2H, dd, J = 3, 7, SC<u>H</u>$_2$CH$_2$CN), 3.05, (1H, dd, J = 7, 12, H-6'), 2.93 (1H, dd, J = 7, 14, H-6''), 2.71 (2H, dd, J = 3, 7 , SCH$_2$C<u>H</u>$_2$CN), 2.17, 2.07, 2.06, 2.01, (12H, s, C<u>H</u>$_3$COO)

Figure 26. ^{13}C-NMR spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**28**).   63 MHz, CDCl$_3$. See also Table 4.

Figure 27. ^{1}H NMR spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**28**).   250 MHz, CDCl$_3$. See also Table 5.

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C.II.3. 2,6-Bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-b-D-glucopyranosyl)tetrathiafulvalene (29)

Cesium hydroxide (CsOH.H$_2$O) (87.6 mg, 0.52 mmol) in MeOH (10 ml) was injected to **12** (63.0 mg, 0.12 mmol) in dry and degassed DMF (10 ml) under nitrogen . Reaction was stirred for two hours, **23** (214 mg, 0.52 mmol) in DMF (5 ml) was added and solution stirred for 48 hours. After removing the solvent *in vacuo*, the residue was dissolved in DCM, washed with water, dried with Na$_2$SO$_4$, filtered and the solvent evaporated *in vacuo*. The residue was subjected to flash column chromatography using 10 % EtOAC/DCM as eluting solvents affording brown oil (76 mg, 59.8% yield); Rf 0.38 (10% EtOAC/DCM); $[a]_D^{25°C}$ = +62.55 (c = 0.1 DCM); FAB-MS: m/z =1098[M$^+$]. C$_{40}$H$_{46}$O$_{18}$N$_2$S$_8$

Calculated: C 44.08%   H 4.19%   S 23.22%   N 2.55%

Found: C 48.14%   H 5.87%   S 23.12%   N 3.44%

^{13}C (63 MHz, CDCl$_3$/TMS) δ = 170.66-169.38, (CH$_3$<u>C</u>OO), 118.86, (C=C), 82.98, (C-1'), 74.90, (C-2'), 72.66, (C-3'), 69.29, (C-4'), 67.69 (C-2'), 61.78 (C-6'), 31.75 (S<u>C</u>H$_2$CH$_2$CN), 20.69-20.59 (<u>C</u>H$_3$COO), 18.19 (SCH$_2$<u>C</u>H$_2$CN).

^{1}H (250 MHz, CDCl$_3$) δ = 5.22, (2H, t, J = 9.5, H-3'), 5.10, (2H, t, J = 9.5, H-4'), 4.99, (2H, dd, J = 8, 9.5, H-2'), 4.42 (2H, d, J = 8.0, H-1'), 4.28 (2H, dd, J = 5, 12, H-6a'), 4.15 (2H, dd, J = 3,12, H-6b'), 3.72 (2H, m, H-5'), 3.27 (2H, t, J = 6.3, SC<u>H$_2$</u>CH$_2$CN), 2.76(2H, t, J = 6.3, SCH$_2$C<u>H$_2$</u>CN), 2.08 (4H, s, COC<u>H$_3$</u>), 2.05, 4H, s, COC<u>H$_3$</u>), 2.04 (4H, s, COC<u>H$_3$</u>), 1.98 (4H, s, COC<u>H$_3$</u>)
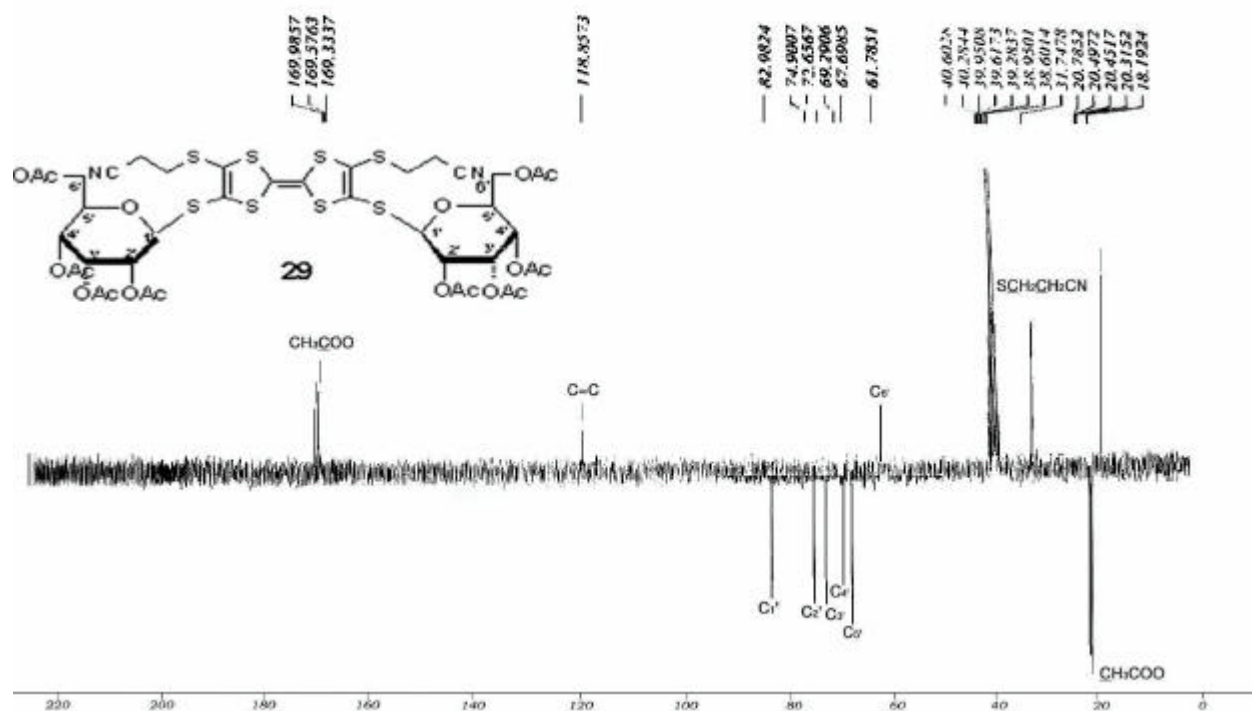
Figure 28. ^{13}C NMR of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**29**), 63 MHz, DMSO-d6. See also Table 6.
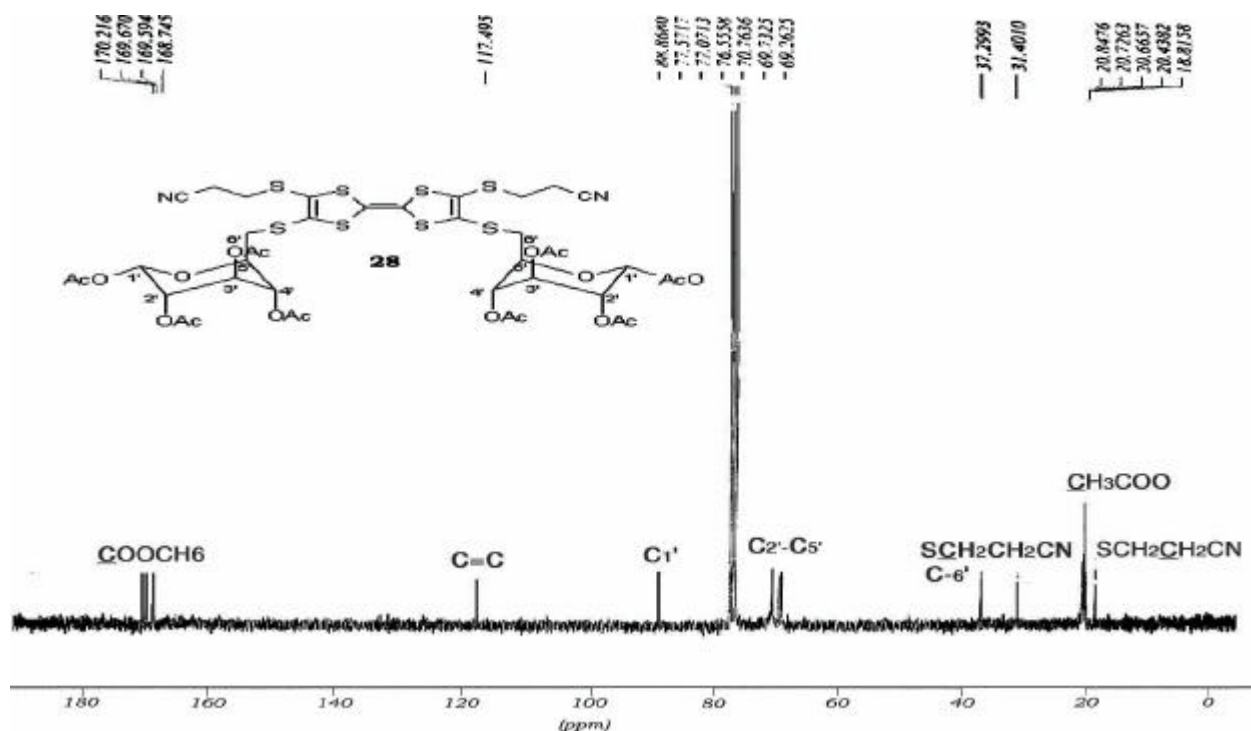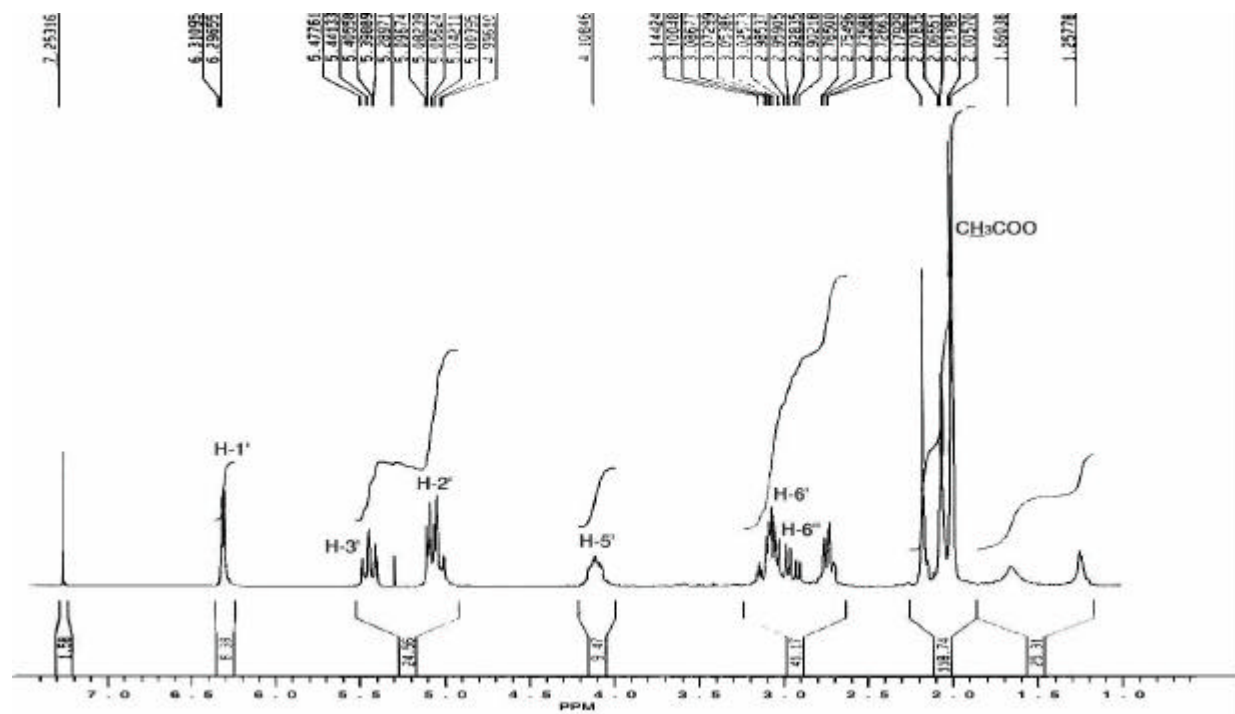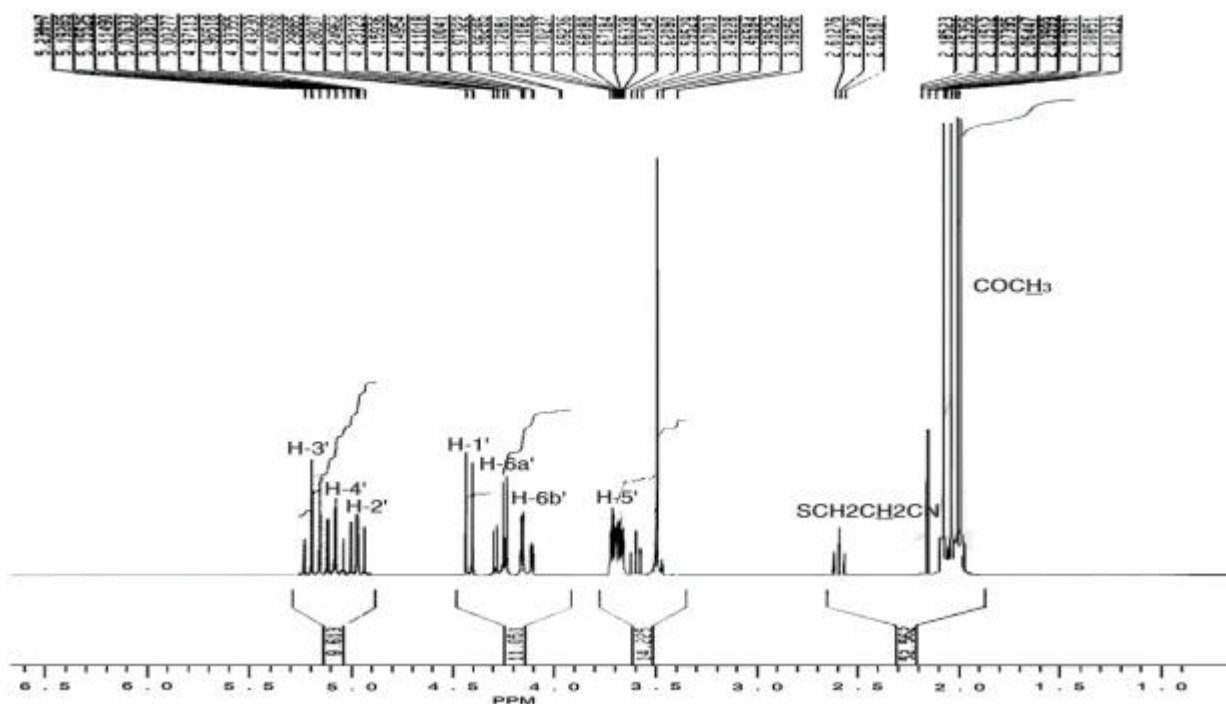

Figure 29. ^{1}H NMR spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**29**). 250 MHz, CDCl₃. See also Table 7.

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C.II.4. 2,6-Bis(2''-cyanoethylthio)-3,7-bis(6'-thio-D-glucopyranosyl)tetra-thiafulvalene (30)

Sodium (10 mg , 0.43 mmol) in MeOH (100 ml) was injected to **28** (50 mg, 0.045 mmol) in degassed MeOH (10 mL) and the solution stirred overnight. After removing the solvent *in vacuo*, a dark brown oily residue was left which turns black upon standing (33.2 mg, 96% yield), Rf = 0.59. (acetone/water: 1:1). FAB MS: m/z 482, [M- $2(C_6H_{11}O_5)$ + 2 Na], ($C_6H_{11}O_5$ = glucose moiety)

C.II.5. 2,6-Bis(2''-cyanoethylthio)-3,7-bis(1'-thio-**b**-D-glucopyranosyl)tetra-thiafulvalene (31)

Sodium (10 mg , 0.43 mmol) in MeOH (100 ml) was injected to **29** (50.0 mg, 0.045 mmol) in degassed MeOH (10 mL) and the solution stirred overnight. After removing the solvent *in vacuo*, a dark brown oily residue was left which turns black upon standing (32 mg, 94% yield), Rf = 0.59. (acetone/water: 1:1). FAB MS :m/z 482, [M- $2(C_6H_{11}O_5)$ + 2 Na], ($C_6H_{11}O_5$ = glucose moiety)

C.II.6. Tetrakis(2'-hydroxyethylthio)tetrathiafulvalene (32)

12 (100 mg, 0.185 mmol) was dissolved in anhydrous DMF (10 mL) in a 100 mL round bottomed flask under dry N_2. Freshly prepared NaOMe (7.4 mL, 0.10 M, 4.5 equiv) were added. After stirring for 30 minutes at room temperature. 2-chloroethanol (~0.06 mL, 10 equiv) were added through the septum and the mixture stirred for 14 h at r.t. H_2O (10 mL) were added and the stirring continued for another 30 min and the mixture was concentrated *in vacuo*. This resulted in an orange oil, which crystallized after the addition of hexane (4 mL) and Et_2O (10 mL). Isolation and recrystallization in methanol

gave orange needles; yield: mp 142.9-143.6°C (Lit.[56] 139°-140°C). MS (EI)
507.9

^{1}H NMR (250 MHz, DMSO-d$_6$/TMS) δ = 5.01 (t, 4H, J= 5.5 Hz), 3.60 (m, 8H),
2.96 (m, 8H). ^{13}C NMR (63 MHz, DMSO-d$_6$/TMS): δ = 126.94, 109.47 (C=C),
60.30 (SCH$_2$CH$_2$OH), 38.14 (SCH$_2$CH$_2$OH)

C.II.7. 2,6-Bis(2''-cyanoethylthio)-3,7-bis(2'-carboxymethylthio)tetrathia-fulvalene (33)

12 (100 mg, 0.185 mmol) was dissolved in anhy DMF (10 mL) in a 100 mL
round bottomed flask under dry N$_2$. Freshly prepared NaOMe (7.4 mL, 0.10 M,
4.5 equiv) were added. After stirring for 30 minutes at r.t ICH$_2$COOH (273.3
mg, 1.5 mmol, ~10 equiv) were added through the septum and the mixture
stirred overnight at room temperature (20 minutes after the addition, orange
precipitate starts to form). H$_2$O (15 mL) were added and the stirring continued
for another 30 min and the mixture was concentrated *in vacuo*. This resulted
in an orange oil, methanol was added and red-orange precipitate formed. This
was filtered to give a yield : 85.5 mg, (82%); mp 181-182°C

^{13}C NMR (DMSO-d$_6$/TMS): δ =169.12 (COOH), 118.93 (C=C), 31.00
(SCH$_2$CH$_2$CN), 18.15, (SCH$_2$CH$_2$CN). ^{1}H NMR (DMSO-d$_6$/TMS) δ = 3.70 (s,
COOH), 3.12 (2H, t, J = 7, SCH$_2$CH$_2$CN), 2.88 (2H, t, J = 7, CH$_2$CH$_2$CN), 2.50
(2H, s, CH$_2$COOH).
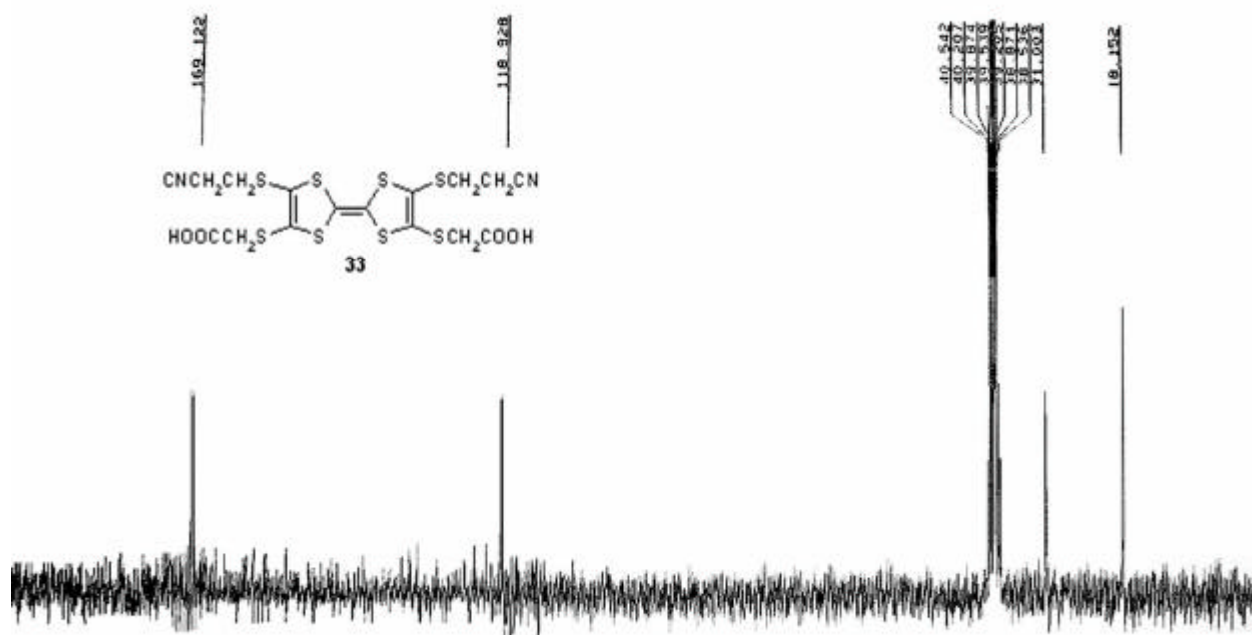
Figure 30. ^{13}C NMR spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2'-carboxy-methylthio)tetrathiafulvalene (**33**). 63 MHz, DMSO-d6. See also Table 8.
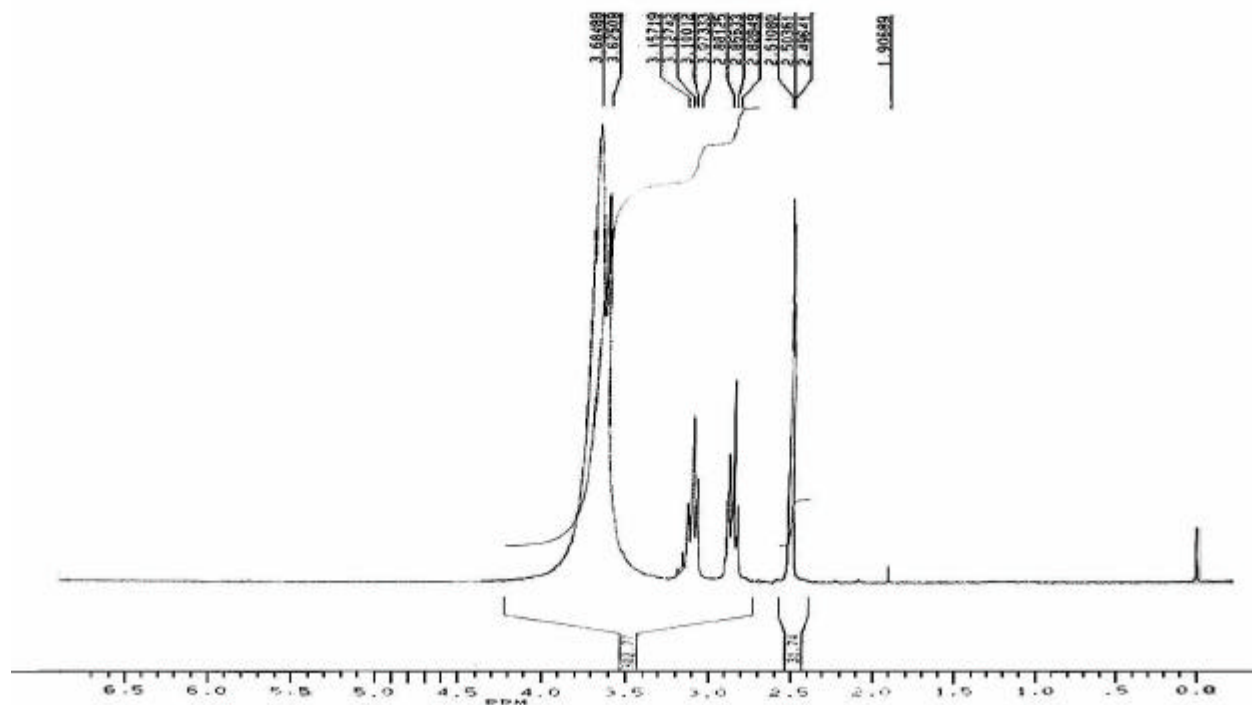
Figure 31. ^{1}H NMR spectrum of 2,6-bis(2''-cyanoethylthio)-3,7-bis(2'-carboxy-methylthio)tetrathiafulvalene (33). 250 MHz. DMSO-d6. See also Table 9.

C.III. Synthesis of 1,3-Dithiole-2-thione-4,5-dithiolate-Carbohydrate Derivatives

C.III.1. 4,5-Bis(1',2',3',4'-tetra-O-acetyl-6'-thio-**a**-D-glucopyranosyl)1,3-dithiole-2-thione (34)

8 (50 mg, 0.070 mmol) in DMF (5 mL) was degassed, **14** (150 mg, 0.33 mmol) were added and the mixture was refluxed (120-130°C) for 16 hours under N_2. After removing the solvent *in vacuo*, the residue was dissolved in DCM, washed with water, dried with Na_2SO_4 , filtered and the solvent evaporated *in vacuo*. The residue was subjected to flash column chromatography using 10% EtOAc/DCM . Yield: 90 mg, 74.3%, mp: 182.6-184°C. Rf: (solvent system: 1:1.1:1, hexane:toluene:DCM:acetone). $[\boldsymbol{a}]_D^{25°C}$ =+147.6 (c = 0.05M, DCM), FAB–MS: m/z = 858 [M+]. $C_{31}H_{38}O_{18}S_5$: (858.0).

Calculated	C 43.34%	H 4.42%	S 18.65%
Found	C 42.35%	H 4.15%	S 14.33%

[13] C NMR (63.5 MHz, CDCl$_3$) δ = 169.65, 169.58, 169.37, 168.82, (COOCH$_3$), 137.31 (C=C), 88.03 (C-1'), 70.57 (C-2'), 69.86 (C-3'), 69.01 (C-4'), 68.70, (C-5'), 36.93, (C6'H$_2$S), 20.58, 20.47, 20.32, 20.25, (COOCH$_3$).

^{1}H NMR (250 MHz, CDCl$_3$/TMS)6.33 (1H, d, J = 3.6, H-1'), 5.49 (1H, t, J = 10, H-3'), 5.11 (1H, dd, J = 3.6, 10, H-2'), 5.03 (1H, t, J = 10, H-4'), 4.16-4.08 (1H, m, H-5'), 3.15 (1H, dd, 3, 14, H-6'), 3.00 (1H, dd, J = 7, 14, H-6''), 2.33 (3H, s, CH$_3$), 2.27, 3H, s, CH$_3$), 2.06 (3H, s, CH$_3$), 2.03 (3H, s, CH$_3$).

C.III.2. 4-(2''-Cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-b-D-glucopyranosyl)-1,3-dithiole-2-thione (35)

Cesium hydroxide (CsOH.H$_2$O) (45.6 mg, 027 mmol) in MeOH (10 ml) was injected to **11** (33.0 mg, 0.109 mmol) in degassed MeCN (10 mL) after 30 minutes, **25** (111.38 mg, 0.27 mmol) in MeCN (10 ml) was added and solution stirred overnight. After removing the solvent *in vacuo*, the residue was dissolved in DCM, washed with water, dried with Na$_2$SO$_4$, filtered and the solvent evaporated *in vacuo*. The residue was subjected to flash column chromatography using 10 % EtOAC/DCM as eluting solvents affording yellow oil. Recrystallizing in EtOH afforded light yellow needle like crystals (57 mg, 90% yield); mp 178.1-179.3°C; Rf 0.47 (10% EtOAc/DCM) , $[a]_D^{25°C}$ = +101.88 (c = 0.07 M, DCM), FD-MS m/z= [M$^+$+1] 582, [M$^+$+23] 604. C$_{20}$H$_{23}$O$_9$NS$_5$: (581).

Calculated   C 41.30%   H 3.99%   N 2.41%   S 27.54%

Found   C 41.43%   H 4.54%   N 1.80%   S 26.00%

[13] C NMR (63.5 MHz, CDCl$_3$) δ= 169.91, 169.50, 169.26 (2C) (CH$_3$COO), 82.91 (C-1'), 118.81 (C=C), 74.82, 72.59, 69.23, 67.62 (C-2'–C-5'), 61.71 (C-6'), 31.68 (SCH$_2$CH$_2$CN), 20.71, 20.42, 20.38, 20.25 (4-CH$_3$COO), 18.12 (SCH$_2$CH$_2$CN)

[1] H (250 MHz, CDCl$_3$)δ = 5.23 (1H, t, J = 9.4, H-3'), 5.11 (1H, t, J = 9.4, H-4'), 5.00 (1H, dd, J = 7.9, 9.4, H-2'), 4.43 (1H, d, J = 7.9, H-1'), 4.29 (1H, dd, J = 4, 12, H-6'), 4.21 (1H, dd, J = 4, 12, H-6''), 3.72-3.65 (1H, m, H-5'), 3.25 (2H, t, J = 7, SCH$_2$CH$_2$CN), 2.93 (2H, t, J = 7, SCH$_2$CH$_2$CN), 2.18 (3H, s, CH$_3$COO), 2.15 (3H, s, CH$_3$COO), 2.13, (3H, s, CH$_3$COO), 2.09 (3H, s, CH$_3$COO).

C.III.3. 4,5-Bis(6'-thio-D-glucopyranosyl)-1,3-dithiole-2-thione (36)

Sodium (10 mg , 0.43 mmol) in MeOH (100 ml) was injected to **34** (50.0 mg, 0.058 mmol) in degassed MeOH (10 mL) and the solution stirred overnight. After removing the solvent *in vacuo*, a dark brown oily residue was left which turns black upon exposure to the atmosphere (30 mg, 99% yield), Rf = 0.57 (ethanol/acetone 1/1 v/v). FAB MS m/z 530 [M + 8H].

C.III.4. 4-(2''-Cyanoethylthio)-5-(1'-thio-b-D-glucopyranosyl)-1,3-dithiole-2-thione (37).

Sodium (10 mg , 0.43 mmol) in MeOH (100 ml) was injected to **35** (50.0 mg, 0.086 mmol) in degassed MeOH (10 mL) and the solution stirred overnight. After removing the solvent *in vacuo*, a dark brown oily residue was left which turns black upon standing (35 mg, 98 % yield), Rf = 0.70 (ethanol/acetone 1/1 v/v). FAB MS m/z 413 [M+].

C.III.5. 4,5-Bis(2'-hydroxyethylthio)-1,3-dithiole-2-thione (38)

8 (500 mg, 0.70 mmol) in acetonitrile (10 mL) were mixed with $ClCH_2CH_2OH$ (2 mL) and heated under reflux until the disappearance of the red color of **8**. After removing the solvent *in vacuo*, the residue was dissolved in DCM, washed with water, dried with Na_2SO_4 , filtered and the solvent evaporated *in vacuo*. The residue was recrystallized from ethanol forming yellow-green crystals. Yield: 280 mg, 70% m.p.: 73.1-75.7°C. Rf 0.32 (1:1:1:1, DCM/Tol/Acet/Hex), FAB–MS: m/z = 287 [M+ + 1]. $C_7H_{10}O_2S_5$: (286.0).

Calculated   C 29.37%   H 3.50%   S 55.94%

Found   C 28.57%   H 3.51%   S 55.20%

GASPE NMR (250 MHz, DMSO-d$_6$/TMS) δ= 206.62 (C=S), 136.11 (C=C), 60.07

(2C, SCH$_2$CH$_2$OH), 38.64, 38.50 (SCH$_2$CH$_2$OH)

^{1}H NMR (250 MHz, DMSO-d$_6$/TMS) δ= 5.05 (t, J = 5.5, 2H), 3.66 (dd, J = 6.3,

12 Hz, 4H), 3.08 (dd, J = 6.3, 12 Hz, 4H)

C.III.6. 4,5-Bis(2'-carboxymethylthio)-1,3-dithiole-2-thione (39)

8 (700 mg, 1.0 mmol) in acetonitrile (15 mL) were mixed with ICH$_2$COOH (1.1

g, 7 mmol), NaOH (0.3 g, 7.5 mmol) and H$_2$O (7.5 mL) and heated under reflux

until the disappearance of the red color of **8**. CH$_3$CN was removed *in vacuo*,

HCl (50%, 25 mL) were added with cooling, forming yellow crystals.   The

product was recrystallized by dissolving the crystals in dilute NH$_3$ and slow

addition of HCl (50%). Yield: 74.4%, mp 172-174°C (lit [42] 171-173°C , FAB–

MS: m/z = 315 [M$^+$+ 1], 337 [M$^+$+ 23]. C$_7$H$_6$O$_4$S$_5$ (314)

GASPE NMR (250 MHz, DMSO-d$_6$/TMS):   δ = 169.61(COOH) 136.50 (C=C),

37.58 (S-CH$_2$)

^{1}H NMR (250 MHz, DMSO-d$_6$/TMS) δ = 4.37 (broad, COOH), 3.84 (s, CH$_2$)
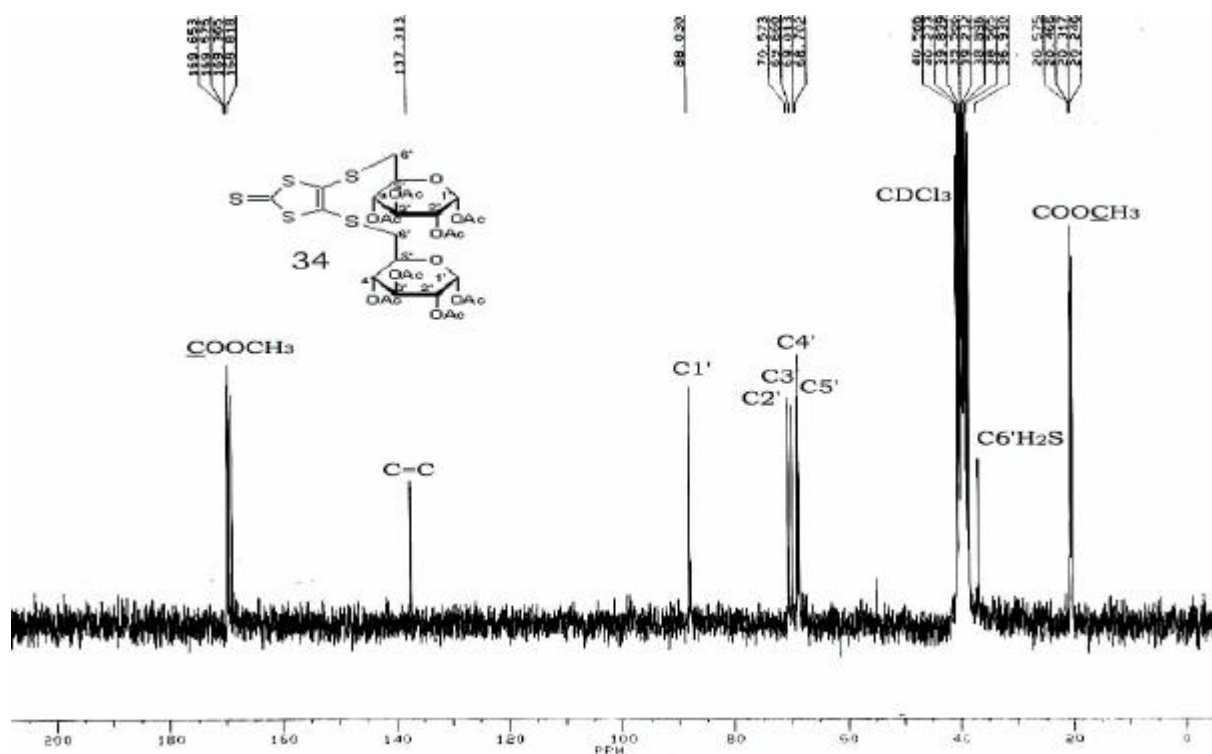
Figure 32. ¹C NMR spectrum of 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-α-D-glucopyranosyl)1,3-dithiole-2-thione (34). 63 MHz, DMSO-d6. See also Table 10.
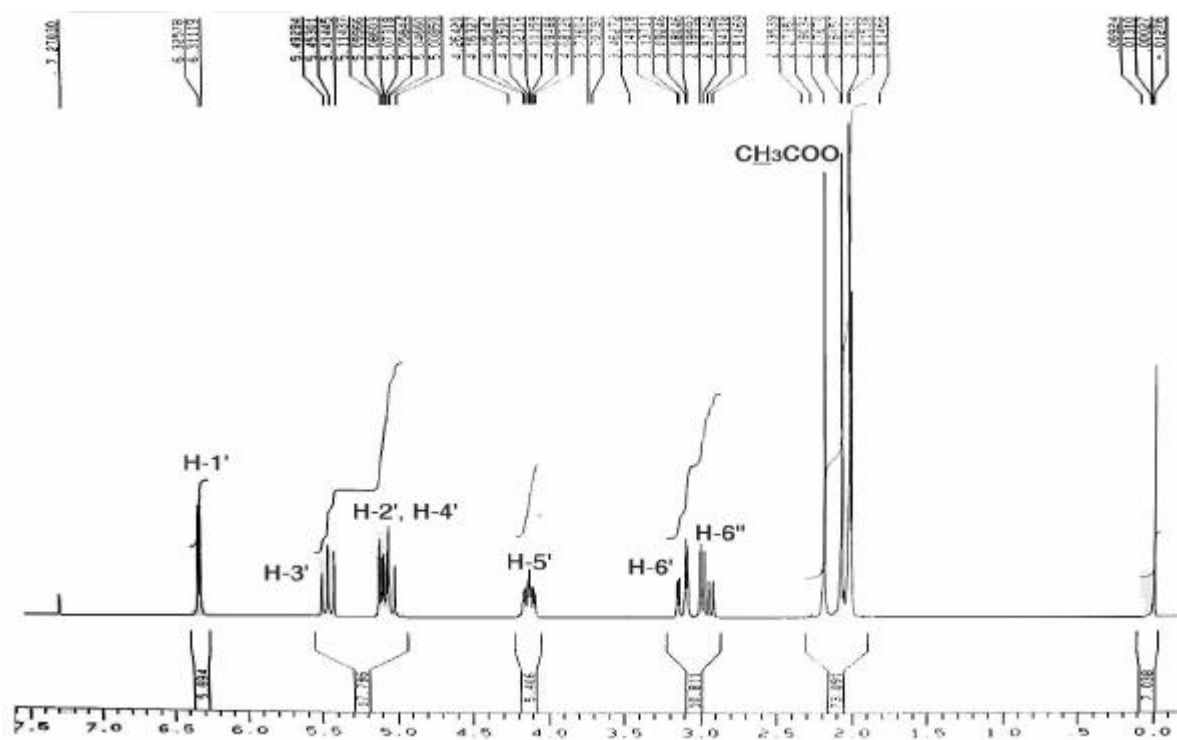
Figure 33. **¹H NMR spectrum of 4,5-bis(1',2',3',4'-tetra-O-acetyl-6'-thio-a-D-glucopyranosyl)1,3-dithiole-2-thione (**34**). 250 MHz, CDCl₃. See also Table 11.**
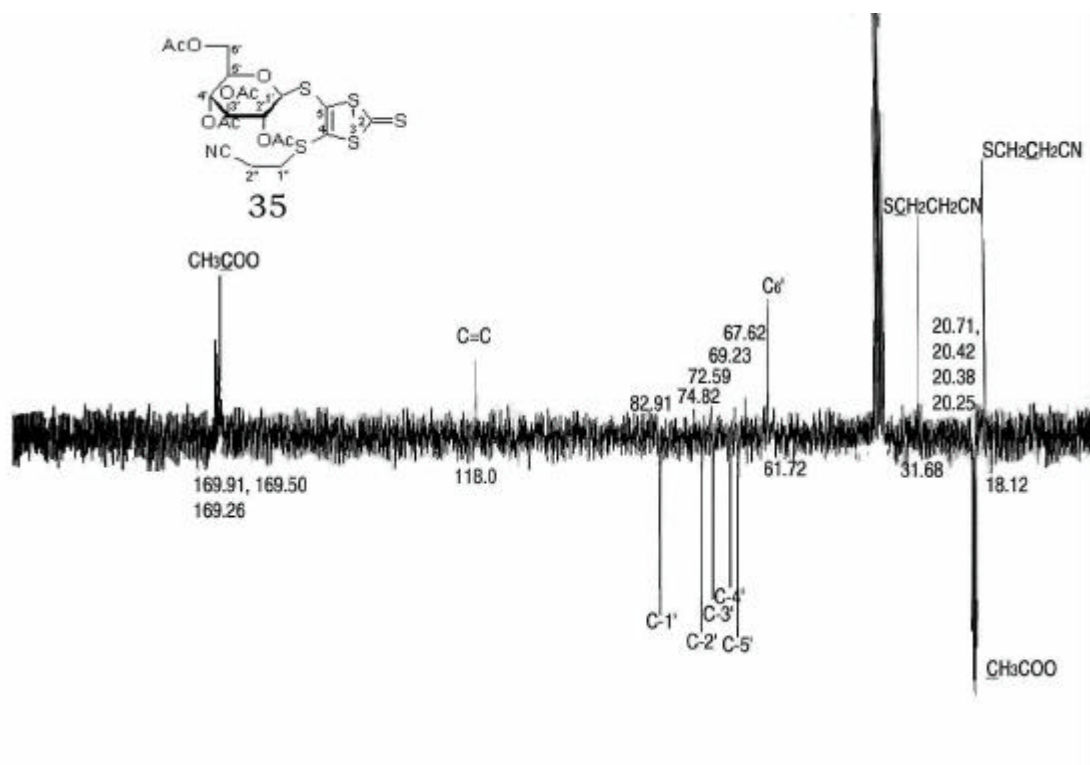
Figure 34. ¹³C NMR spectrum of 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (35). 63 MHz, DMSO-d6. See also Table 12.
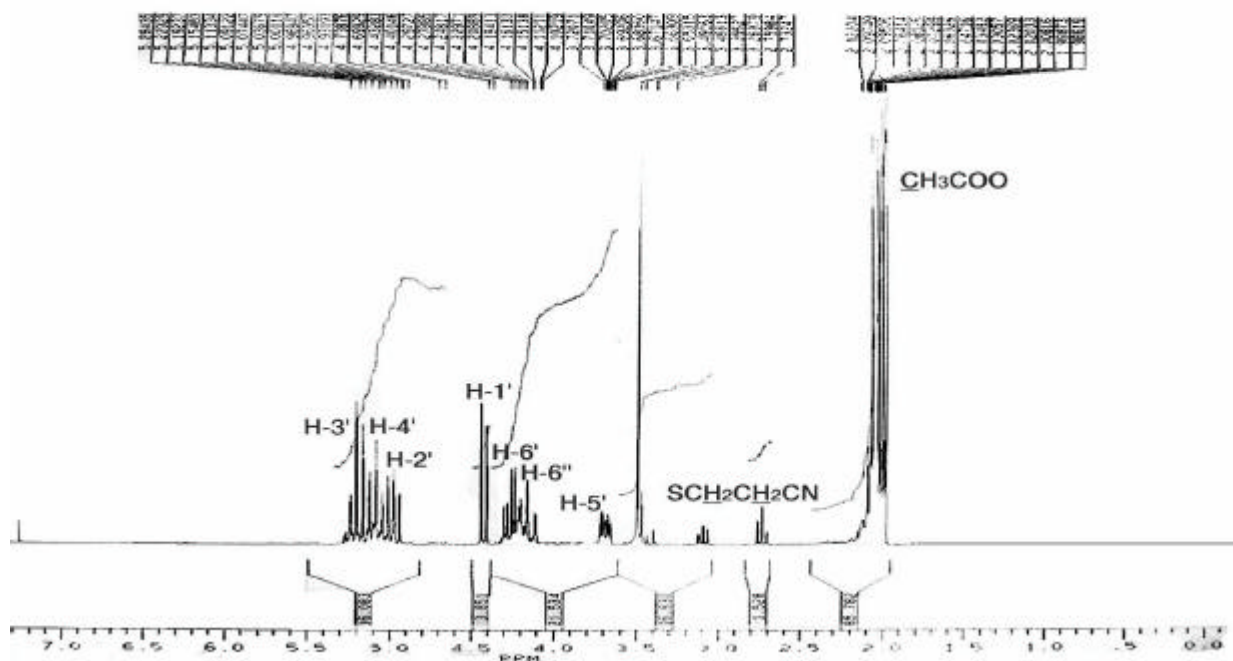
Figure 35. ¹H NMR spectrum of 4-(2''-cyanoethylthio)-5-(2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**35**). 250 MHz, CDCl₃. See also Table 13.

C.IV. MTT Assay

C.IV.1. Materials and reagents

The following materials were used for the biological assay:

Article	Company
Cell culture flasks (162 cm², standard cap (3150)	**Coster**
96 wells flat-bottomed microplate, catalog number 3596	**Coster**
Centrifuge tubes, 10 and 50 mL	Greiner
Securelock-Eppendorf tubes (1.5 mL)	Eppendorf
Bio-freeze vials 1.2 mL	**Coster**
Plastic pippettes, steripette 2, 5, 10, 25 mL	Coster
Pippette tips 10-1000 µL	Eppendorf
Combitips 0.5 and 5 mL	Eppendorf
Minisart NML filter, 0.2 µm porosity	Sartorius
Syringes 2-5 mL	B. Braun

Equipment	Company
ELISA Microplate Reader (MR 700)	Dynatech
Dispenser-Multipippette	Eppendorf
Varipette, 10-1000 µL, 10-100 µL	**Eppendorf**
Analytical balance	Sartorius
Centrifuge	Hettich
Sterile bank	Waldner
Incubator for cell culture	Heraus
Phase contrast microscope	**Olympus**
Waterbath	Kötterman
Shaker	Heidolph

Material	Company
Neubauer cell chamber	Blaubrand
Latex gloves	Safe Skin
Pasteur pippettes	Hirschmann

C.IV.2. Media:

C.IV.2.1. Cell culture medium:

RPMI 1640

Fetal calf serum (FCS) 10%

L-glutamine 2 mM

Penicillin 100 IU/mL

Streptomycin 100 IU/mL

C.IV.2.2. Medium for freezing the cells

Fetal calf serum (FCS) 90%

DMSO 10%

C.IV.3. Cell lines

The SK-N-SH cell line was obtained from the American Type Culture Collection, Virginia. Eagle's medium with 10-15 % FCS was used previously for its cultivation.

The SK-N-LO cell line was obtained from Dr. Helson and Dr. Fogh, Memorial Sloan Kettering Cancer Institute, New York.

C.IV.4. Methods

C.IV.4.1. Cell culture

Both cell lines grow adherent to the culture flask. Each were placed in 650 mL cell culture flask in RPMI medium with 10% FCS, glutamine100 IU/mL and penicillin/streptomycin 100 IU/mL. The flasks were laid down (area: 150 cm^2) for culture growth. The incubation of the cells was done at 37°C, with an atmosphere of 5% CO_2 and 100% humidity. The growth was monitored everyday under the microscope. The indicator phenol red is present in the medium to signal for pH changes. The change in color of the medium from red

to orange would require a change in the medium. An overview of the pH value with the corresponding color of the medium is given: 7.8: violet, 7.6: blue-red, 7.4: red, 7.0: orange, 6.5: yellow.

When the cells are fully grown, it is required to be split into another flask.

C.IV.4.2. Culture of neuroblastoma cells

Adherently Growing SK-N-SH and SK-N-LO were detached from the culture flasks as follows:

1. The cell culture medium was removed completely and approximately 2.5 mL of trypsin was added to each culture flask (150 cm²). The cells were incubated with trypsin at room temperature for 5 minutes.

2. The cells were detached from the bottom of the flask by shaking.

3. The effect of trypsin was blocked by the addition of 10 mL of cell culture medium.

4. The cell suspension was then centrifuged (10 minutes, 400g). The supernatant was removed and the cells were resuspended in 10 mL of cell culture medium. The cell count was determined with a Neubauer cell chamber in the presence of trypan blue. For this purpose, 20μL of cell suspension were mixed with 20 μL of trypan blue. The cells were counted in 4 large squares of the cell chamber, whereby only the non-stained cells were used to adjust the cell concentration. Since the volume of one large square is 0.1μL , the total cell count from 4 large squares was divided by 4 and multiplied by 10,000 in order to get the number of cells per 1 mL . This number was finally multiplied by 2 (dilution factor of trypan blue). A definite number of cells was used for further culture of the cells, whereas the rest of the cells was used for experiments.

C.IV.4.3. Freezing and defrosting of cells

The cells were removed from the flask as described in C.VII.3.1 and suspended in FCS containing 10% DMSO. The suspension is placed in 1 mL aliquot in "bio-freeze vials", wrapped in cell wool and placed in a deep-freezer (-70°C) for 24hours. Further freezing would require liquid nitrogen.

The cell samples are defrosted by quick immersion in a water bath (37°-40°C) and incubation for 40-60 seconds. The defrosted suspension is then placed in 10 mL centrifuge tubes and 10 mL of the medium is added. The cell suspension is centrifuged, the medium removed, the cell pelletized and fresh medium added. The new cell suspension can be placed in 2-3 culture flasks and cultivated in 35-50 mL medium.

C.IV.4.4. Preparation of MTT solution

5 mg of MTT are dissolved in 1 mL of PBS (phosphate buffered saline) . The solution should be filtered through a 0.2 µm filter, wrapped in aluminum foil (MTT is a light sensitive substance) and stored at 4°C.

C.IV.4.5. Preparation of isopropanol-triton X-100 solution

0.1 M HCl in isopropanol and 10% Triton X-100 are mixed. The solution should be stored at room temperature.

C.IV.5. Cytotoxicity test using the MTT assay

C.IV.5.1. MTT assay with neuroblastoma cells

200 µL of cell suspension (5.0×10^5 cells/mL of cell culture medium) are placed with a dispenser Multipipette (Eppendorf) into each well of the 96 well microtiter plate, 3 wells are filled with cell culture medium only and are used as blank. The plate is incubated overnight in an incubator at 37°C , 5% CO_2 and 100% humidity.

The next day, the substances to be tested are added in 10 µL volumes. In the control wells, the same volumes of PBS were added instead of the substances. The 96 well microtiter plate is further incubated for 48 hours at 37°C, 5% CO_2 and 100% humidity.

After 48 hours of incubation, the cell culture medium was removed completely by a Pasteur pipette connected a vacuum pump. Then, 100µL of MTT solution (MTT stock solution, diluted to 1:9 with RPMI-1640) is added into each well.

The microtiter plate was further incubated for 3 hours in the incubator. During this time, MTT is transformed into formazan, which microscopically show blue crystals between the cells at the bottom of the wells.

Then, 100 µL of isopropanol solution were added into each well and the plate was shaken horizontally for further 8-10 hours (usually overnight) at room temperature. During this time, formazan crystals are dissolved. After this step, microscopically no crystals should be present in any well. In all wells there is a homogenous colour to be observed.

Finally, the absorbance of the colour in each well was measured by a Microplate reader (MR 700) in a dual mode with a reference wavelength at 550 nm (filter number 5) and a test wavelength at 630 nm (filter number 4).

The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. There were four independent experiments, each was performed four fold. The first trial was performed using the freshly prepared solution of the synthesized derivatives. The samples were then stored at –20°C. The second trial was done from a sample that was stored for 4 days, the third one after 8 days of sample storage, and the fourth one after 12 days of sample storage. The average from

4 replicates in 4 independent trials and the +/- standard deviation was plotted.

C.IV.6. ELISA multiwell spectrophotometry

The light absorption of the sample in the 96 well microtiter plate was measured in a MR 700 Microplate Reader from Dyna. The absorption of the sample was measured at two wavelengths using 550 nm as the reference and 630 nm as the test wavelength.
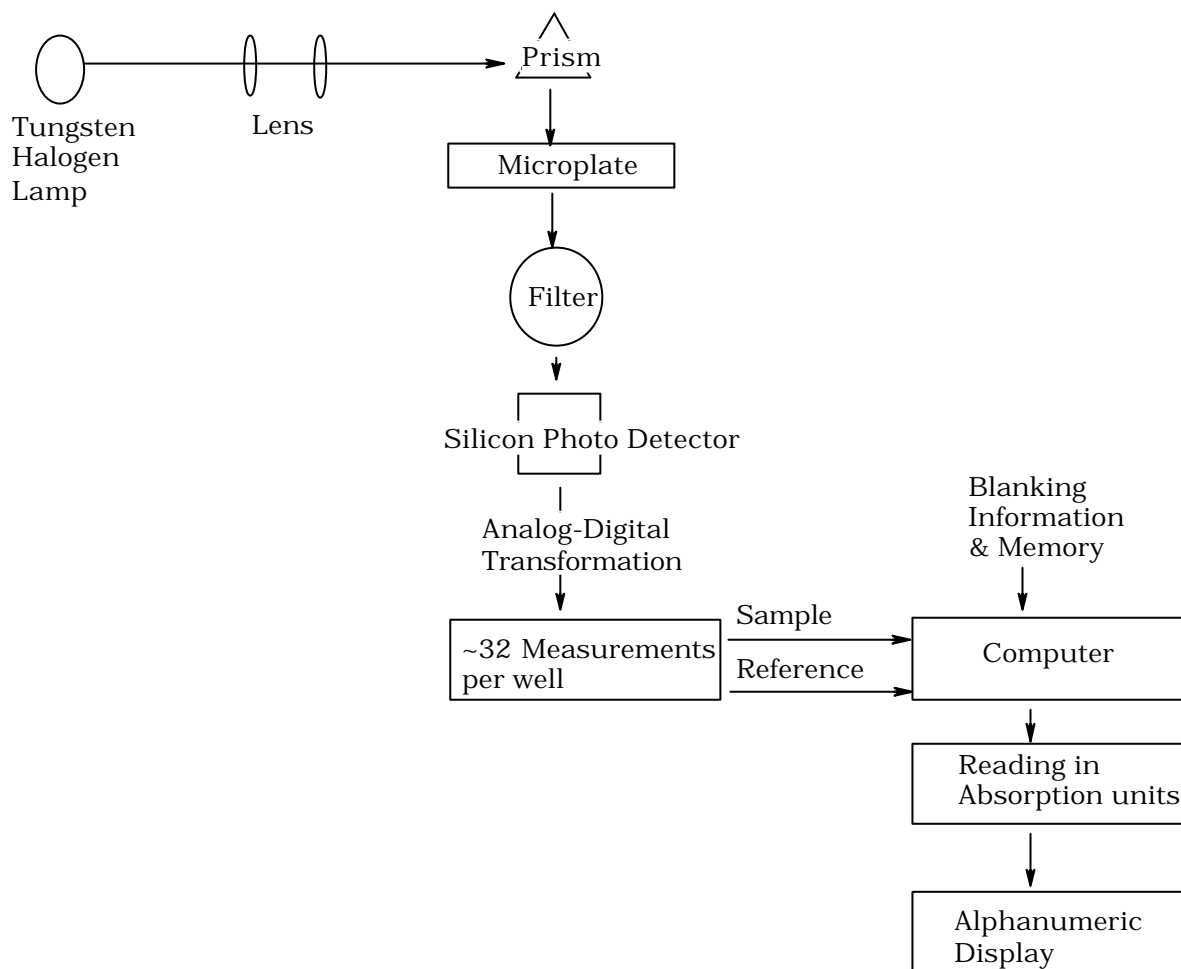

Figure 36. Schematic representation of the Microplate-Reader MR 700.

C.IV.7. Activity testing data

Table 21. Effect of different concentrations of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**31**) on % of survival of SK-N-LO neuroblastoma cells. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 4 independent trials and the +/- standard deviation are given. First trial was performed using the freshly prepared solution. The second trial was performed in a sample that was stored for 4 days. Third trial was done after 8 days of sample storage. Fourth trial was performed after 12 days of sample storage.

Concentration μM/mL	Trial 1	Trial 2	Trial 3	Trial 4
3.00	0.98 ± 0.10	5.49 ± 0.70	13.93 ± 1.40	13.54 ± 1.90
1.50	14.54 ± 3.30	35.71 ± 2.10	73.57 ± 7.00	56.60 ± 3.90
0.30	18.79 ± 4.00	38.46± 7.30	81.96 ± 5.10	71.88 ± 5.50
0.030	97.00 ± 9.10	82.41± 9.80	95.53 ± 4.70	84.02 ± 2.10

Table 22. Effect of different concentrations of 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**30**) on % of survival of SK-N-LO neuroblastoma cells. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 4 independent trials and the +/- standard deviation are given. First trial was performed using the freshly prepared solution. The second trial was performed in a sample that was stored for 4 days. Third trial was done after 8 days of sample storage. Fourth trial was performed after 12 days of sample storage.

Concentration μM/mL	Trial 1	Trial 2	Trial 3	Trial 4
3.00	5.89 ±0.20	9.89 ±0.60	24.82 ± 1.10	24.31 ± 1.20
1.50	10.78 ± 0.60	25.30 ± 0.40	27.14 ± 2.40	44.79 ± 0.70
0.30	37.25 ± 0.70	63.73 ± 5.00	78.75 ± 4.10	87.80 ± 7.40
0.030	45.58 ± 1.10	113.00 ± 6.60	81.25 ± 4.70	84.38 ± 5.20

Table 23. Effect of different concentrations of 2,6-bis(2''-cyanoethylthio)-3,7-bis(1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**31**) on % of survival of SK-N-SH neuroblastoma cells. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 4 independent trials and the +/- standard deviation are given. First trial was performed using the freshly prepared solution. The second trial was performed in a sample that was stored for 4 days. Third trial was done after 8 days of sample storage. Fourth trial was performed after 12 days of sample storage.

Concentration μM/mL	Trial 1	Trial 2	Trial 3	Trial 4
3.00	1.10 ± 0.40	1.65 ± 0.10	3.93 ± 0.50	4.32 ± 0.20
1.50	1.10 ± 0.10	3.30 ± 0.10	5.76 ± 2.30	7.40 ± 0.80
0.30	7.20 ± 0.40	4.79 ± 1.30	8.80 ± 0.90	78.39 ± 2.00
0.030	85.50 ± 6.40	88.30 ± 6.50	102.00 ± 10.50	113.00 ± 1.80

Table 24. Effect of different concentrations of 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-α-D-glucopyranosyl)tetrathiafulvalene (**30**) on % of survival of SK-N-SH neuroblastoma cells. The absorbance of the control was taken as 100% survival and the values given are the ratios between the absorbance of the test substance and the control. The average from 4 replicates in 4 independent trials and the +/- standard deviation are given. First trial was performed using the freshly prepared solution. The second trial was performed in a sample that was stored for 4 days. Third trial was done after 8 days of sample storage. Fourth trial was performed after 12 days of sample storage.

Concentration μM/mL	Trial 1	Trial 2	Trial 3	Trial 4
3.00	5.89 ± 0.30	6.44 ± 2.40	4.21 ± 1.0	8.33 ± 0.40
1.50	9.21 ± 1.20	6.61 ± 0.60	8.96 ± 1.2	12.04 ± 1.06
0.30	13.25±1.90	13.90±1.00	17.50 ± 2.9	66.67 ± 3.90
0.030	100.00±3.60	52.00 ± 16.40	90.77± 11. 2	119.00± 3.80

D. Abstract

Tetrathiafulvalenes (TTF) Figure 1. and related heterocycles have received much interest due to their unique electron-donating capabilities
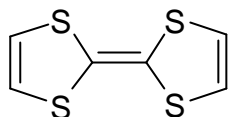

Figure 1. Structure of tetrathiafulvalene (TTF).

As such, they have a widespread application in modern material science chemistry: as a component of macrocycles, molecular metals and superconductors at low temperatures. Due to their planar structure (intercalation with DNA bases) and their redox properties they also might be potential interesting drugs in cancer therapy.

However, no studies have been made so far on the possible application of TTF to biological systems because of the insolubility of the known derivatives in water. The main task of this thesis was, therefore, to synthesize and characterise novel water-soluble TTF- and 1,3-dithiole-2-thione-4,5-dithiolate (DMIT) - carbohydrate derivatives. In an attempt to determine the biological activity of TTF-containing and related compounds. 2,6-bis(2''-cyanoethylthio)-3,7-bis(6'-thio-D-glucopyranosyl)tetrathiafulvalene (**30**) and 2,6-bis(2''-cyano-ethylthio)-3,7-bis(1'-thio-β-D-glucopyranosyl)tetrathiafulvalene (**31**) were found to be cytotoxic against SK-N-LO and SK-N-SH neuroblastoma cells using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide) assay. With this test system, proliferation and vitality of cells can be measured *in vitro*

due to the capability of vital mitochondria to reduce MTT to formazans. Using this test system LD_{50} values of substances **31** and **31** were found ~0.07 to 2.00 µM/mL, which is comparable to those of the well-known cytotoxic drug doxorubicin. On the other side, the DMIT-carbohydrate conjugates 4,5-bis(6'-thio-D-glucopyranosyl)-1,3-dithiole-2-thione (**36**) and 4-(2"-cyanoethylthio)-5-(1'-thio-β-D-glucopyranosyl)-1,3-dithiole-2-thione (**37**) did not exhibit any cytotoxic activity. This may be due to the different structural properties of DMIT compounds compared to compounds **30** and **31**.

In summary: 2,6-bis(2"-cyanoethylthio)-3,7-bis(6'-thio-D-glucopyranosyl)-tetrathiafulvalene (**30**) and 2,6-bis(2"-cyanoethylthio)-3,7-bis(1'-thio-β-D-gluco-pyranosyl)tetrathiafulvalene (**31**) are cytotoxic to both human neuroblastoma cell lines tested with the MTT assay. It may be that these effects can be further enhanced by combination with other redox active substances (Redox cycling). This question should be answered in further *in vitro-* and *in vivo-* experiments.

E. Zusammenfassung

Tetrathiafulvalen (Abb. 1) und verwandte Heterozyklen haben durch ihre spezielle Fähigkeit des Elektronentransfers grosses Interesse hervorgerufen.
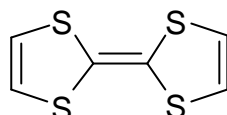

Abb. 1. Struktur von Tetrathiafulvalen (TTF).

Aus diesem Grund haben sie weitgefächerte Anwendungen in der modernen Materialstoffchemie als Komponenten von Makrozyklen, als "molekulare Metalle" und als Tieftemperatur-Supraleiter gefunden. Aufgrund ihrer planaren Struktur (mit der potentiellen Möglichkeit zur Intercalation zwischen DNS-Basen) und ihrer Redox-Eigenschaften könnten sie auch als cytotoxische Verbindungen in der Krebstherapie von Interesse sein. Allerdings sind bisher noch keine diesbezüglichen Applikationen von TTF beschrieben worden, u.a. wegen der Unlöslichkeit der existierenden Verbindungen in Wasser.

Das Hauptziel der vorliegenden Arbeit war es, neue wasserlösliche TTF und 1.3-dithiol-2-thion-4.5-dithiolat (DMIT)- Kohlenhydrate zu synthetisieren und zu charakterisieren, um die biologische Aktivität von TTF- Derivaten und verwandten Verbindungen zu bestimmen. Dabei zeigte sich, dass zwei TTF enthaltende Verbindungen 2.6-bis(2''-cyanoethylthio)-3.7-bis(6'-thio-D-glucopyranosyl)tetrathiafulvalen (**30**) und 2.6-bis(2''-cyanoethylthio)-3.7-bis(1'-thio-β-D-glucopyranosyl)tetrathiafulvalen (**31**) zytotoxisch auf die beiden humanen Neuroblastomzelllinien SK-N-LO und SK-N-SH reagierten. Unter

Verwendung des MTT-Testes, der die Vitalität und Proliferation durch Reduktion von MTT zu Formazanen durch Mitochondrien vitaler Zellen mißt, wurden durch Substanz **30** bzw **31** LD_{50} Werte von ~0.07 bis 2.00 µM/mL erhalten, was vergleichbar ist mit Werten, die bei Verwendung des etablierten Zytostatikums Doxorubicin erhalten wurden. Im Gegensatz dazu zeigten die synthetisierten DMIT-Kohlenhydrat-Derivate 4.5-bis(6'-thio-D-glucopyrano-syl)-1,3-dithiole-2-thione (**36**) and 4-(2''-cyanoethylthio)-5-(1'-thio-β-D-gluco-pyranosyl)-1,3-dithiole-2-thione (**37**) keine zytotoxische Aktivität. Möglicherweise sind die strukturellen Unterschiede zwischen beiden Substanzklassen für dieses unterschiedliche Verhalten verantwortlich.

Zusammenfassend läßt sich sagen, dass zumindest die Verbindungen 2.6-bis(2''-cyanoethylthio)-3.7-bis(6'-thio-D-glucopyranosyl)tetrathiafulvalen (**30**) and 2.6-bis(2''-cyanoethylthio)-3.7-bis(1'-thio-β-D-glucopyranosyl)tetrathia-fulvalen (**31**) eine ausgeprägte Zytotoxizität *in vitro* gegenüber den beiden untersuchten humanen Neuroblastomzellen zeigten. Möglicherweise lassen sich diese Effekte durch Kombination mit anderen Redox-aktiven Substanzen im Sinne eines Redox-Cycling weiter steigern. Die Beantwortung dieser Frage muß weiterführenden *in vitro-* und *in vivo-* Untersuchungen vorbehalten bleiben.

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