



The novel economical synthesis and antimicrobial activity of a trithiocarbonate derivative

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ABSTRACT

The compound diethyl 2,2'-(thiocarbonyl-bis(sulfaneyl))-diacetate **4** belongs to the trithiocarbonate class containing a trithiocarbonate function group flanked by ethyl acetate. In this procedure, a novel economic synthesis route to obtain compound **4** is described. This compound proved to possess broad-spectrum antimicrobial activity both *in vitro* and *in vivo*, and could be used as a lead compound. It is worth mentioning that this compound has been patented [No. US 9,988,348 B1; date of patent: June 5, 2018].

1. Introduction

The trithiocarbonates have been investigated for years, with various synthetic routes having been employed for their synthesis [1]. They are simply and efficiently incorporated into many organic compounds and pharmaceutical therapeutic agents. In organic synthesis, for instance, they have been used in surface and colloidal nanotechnology [2], as reagents for platinum group metals flotation [3], as an electrolyte additive in lithium batteries [1], for monodisperse surface modification [4] and for polymer formation [5]. As pharmaceutical therapeutic agents, they represent a promising class. As leads for novel biologically active compounds, they act as biological toxicants [6], anti-radiation agents in mice [7], antitumor agents [8,9], inhibitors for carbonic anhydrase, and anti-glaucoma effects *in vivo* [10]. Their activity as an inhibitor for carbonic anhydrase (CA) is seen through the formation of a mono-coordination bond of its sulfur atoms with the Zn(II) ion from the CA enzyme active site, as proved by X-ray crystal structure [11–13].

The development of antimicrobial agents never stops, but there is no

doubt that the major microbial pathogens are increasingly developing resistance to the current antimicrobial drugs. Broad-spectrum antimicrobial agents must kill or inhibit specific targets in pathogenic microorganisms. These targets exist in most human microbial pathogens as part of an essential pathway. Absent from animal cells, not inhibited by broadly applied antimicrobial drugs, easy to evaluate *in vitro* and *in vivo*, highly specific for the pathogenic microorganisms, non-toxic for animals or humans, not result in the quick gaining of resistance [14,15].

Broad-spectrum antimicrobials have several advantages in initial empirical therapy, particularly against serious microbial infections. In a severe microbial disease, a therapy must be administered before the identification of the pathogenic microbe, because the time of diagnosis is a powerful predictor of mortality [16,17]. The patents of novel antifungal drugs include azole compounds (new imidazole, fuconazole analogs, azole with 2,6-di-*tert*-butyl-4-methylphenol), enzyme inhibitors (chitin inhibitors, β -1,3-D-glucan synthases inhibitors), peptides (fusion peptides), phenylalkane nitriles (esters of 2-phenylalkane nitriles), boron compounds, pyridine derivatives, cyclic guanidines and

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Table 2
Selected geometric parameters (Å, °).

S1—C4	1.788 (5)	O1—C3	1.308 (9)
S1—C5	1.729 (5)	O2—C3	1.198 (8)
S2—C5	1.617 (4)	O3—C7	1.213 (8)
S3—C5	1.740 (5)	O4—C7	1.313 (9)
S3—C6	1.784 (5)	O4—C8	1.486 (11)
O1—C2	1.450 (12)		
C4—S1—C5	103.6 (2)	S1—C5—S2	126.5 (3)
C5—S3—C6	103.1 (2)	S1—C5—S3	108.1 (2)
C2—O1—C3	116.6 (7)	S2—C5—S3	125.5 (3)
C7—O4—C8	118.5 (6)	S3—C6—C7	114.4 (4)
O1—C2—C1	117.2 (10)	O3—C7—O4	124.8 (6)
O1—C3—O2	124.4 (6)	O3—C7—C6	124.6 (6)
O1—C3—C4	108.9 (5)	O4—C7—C6	110.6 (5)
O2—C3—C4	126.6 (6)	O4—C8—C9	111.7 (9)
S1—C4—C3	113.2 (4)		

The x-ray single diffraction analysis clearly elucidated the molecular structure of compound **4**. The crystallographic data and refinement information for this compound are summarized in Table 1. The selected bond lengths and bond angles are listed in Table 2. The asymmetric unit contains one independent molecule, as shown in Fig. 1. All the bond lengths and angles are within normal ranges [21]. Regarding the crystal packing, shown in Fig. 1, the molecules are linked via three non-classical intermolecular hydrogen bonds (Table 3).

2.2. Biological activity tests

Antimicrobial agents are used in the treatment and prevention of microbial infections. These agents (drugs) have been produced by both biological and chemical methods. Many antimicrobial agents, such as sulfonamids, oxazolidinones and quinolones, are produced via chemical synthesis [22]. The production of a broad-spectrum antimicrobial agent is extremely significant when countering the threat of pathogenic microorganisms, particularly antibiotic resistant microorganisms. The production of broad-spectrum antibacterial and antifungal agents through chemical synthesis was the principle objective of this work.

The biological activity of compound **4** as an antimicrobial and anticancer agent was first evaluated. The results indicated that the compound showed no biological activity as an anticancer against the MCF-7 human breast cancer cell line. The data in Table 4 did confirm that compound **4** showed biological activity as an antibacterial and antifungal agent against all pathogenic microorganisms that were used in this research. Both the water- and DMSO-soluble forms indicated efficacy against the microorganisms.

All tested fungi (*C. albicans*, *Cr. neoformans*, *A. niger*, *P. chrysogenum*, *Fusarium* sp.) except *C. parapsilosus* were significantly ($P < 0.05$) susceptible to the compound. Regarding the *A. fumigatus* mold, the inhibition shown by the compound was significantly ($P < 0.05$) stronger than that of the standard antifungal drugs Cycloheximide, Canesten and

Table 3
Hydrogen-bond geometry (Å, °).

D—H...A	D—H	H...A	D...A	D—H...A
C4—H4A...O2i	0.9900	2.4600	3.319(7)	145.00
C6—H6A...O3iii	0.9900	2.5800	3.353(7)	135.00
C6—H6A...O3iii	0.9900	2.6000	3.260(8)	124.00

Symmetry codes: (i) $x, y - 1, z$; (ii) $x, y + 1, z$; (iii) $-x + 1, y + 1/2, -z + 3/2$.

Caspofungin. The *A. fumigatus* used in this work is one of the strains isolated from humans who suffered from aspergillosis disease in the city of Riyadh, KSA, as seen in our previous study [23]. The *A. fumigatus* mold, which lives both outdoors and indoors, is considered a real risk for individuals with immunodeficiency or lung illnesses [24]. Compound **4** also showed high biological activity as an antifungal agent against *Cr. neoformans*, with the inhibition zone and minimal inhibitory concentration reaching 30 mm and 0.03125 mg/ml, respectively. The *Cr. neoformans* strain used in this study confirmed that Caspofungin has limited activity against this fungi [25]. This pathogenic encapsulated yeast causes several diseases called cryptococcosis [26]. The results in Table 4 refer to the efficiency of compound **4** to inhibit both pathogenic yeasts and molds, particularly its effectiveness in inhibiting Cycloheximide, Canesten and Caspofungin resistant *C. parapsilosus*. Both the gram-positive and -negative bacteria which were used in this research were inhibited by compound **4** (Table 4). Additionally, the biological activity against *S. mutans* (plays a major role in tooth decay) and *N. meningitides* (can cause meningitis) was also very good.

Regarding in vitro uses, the present work concluded that compound **4** exhibits biological activity as a novel broad-spectrum antimicrobial agent.

To confirm the results obtained from the *in vitro* study, an animal experiment was also implemented. In this stage, the role of the water-soluble compound **4** was evaluated to treat mice infected with *C. albicans* (candidemia). The animal experiments add abundant evidence to support the use of Compound **4**. In the following results, Co1, Co2, FluCal, ComCal and Comp are given to several groups of mice, treated with sterile normal saline, infected with *C. albicans*, treated with Diflucan (fluconazole) (Pfizer Inc. New York, N.Y, U.S.A), treated with compound **4** or injected only with the chemical, respectively. In Table 5, a percentage change refers to the change in weight of the treated groups compared to the control group (Co1). At the end of the 15th day, the data showed that the percentage change of groups treated with fluconazole and compound **4** were convergent changes, whereas the percentage change of the group infected with *C. albicans* (Co2) was the highest. Food consumption (g/day/mice) and water consumption (ml/day/mice) were arranged as noted in Tables 6 and 7. Both fluconazole and compound **4** had nearly identical effects on food and water consumption. The investigated compound **4** had no negative side effects

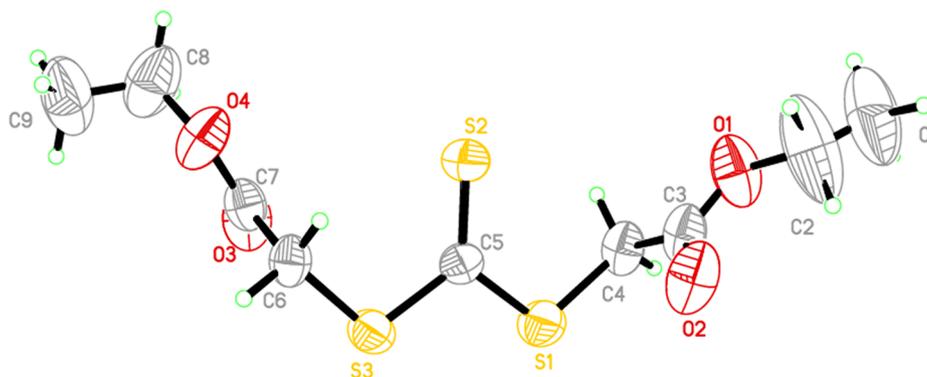


Fig. 1. Molecular packing of compound **4** viewed as hydrogen bonds drawn as dashed lines along the *b* axis.

Table 4
Biological activity of soluble water compound 4 as an antimicrobial agent against some medical microorganisms.

Microorganisms	Compound 4 (4 mg/disk)		Inhibition zone diameter (mm) of standard antimicrobial agents						
	Inhibition zone diameter (mm)	Minimal inhibitory concentration (mg/ml)	Standard antibacterial agent			Standard antifungal agent			
			Tobramycin (10 µg/desk)	Chloramphenicol (30 µg/disk)	Fusidic acid (10 µg/disk)	Cycloheximide (30 µg/disk)	Canesten (10 µg/disk)	Casopfungin (10 µg/disk)	
Gram positive bacteria	<i>S. aureus</i>	10.6 ± 0.5F*	2	14	30	23	N.T	N.T	N.T
	<i>S. mutans</i>	20 ± 1 D	0.0625	35	20	10	N.T	N.T	N.T
Gram negative bacteria	<i>E. coli</i>	11.6 ± 0.5 E	1	20	40	0	N.T	N.T	N.T
	<i>N. meningitides</i>	22.6 ± 0.5C	0.0625	21	34	20	N.T	N.T	N.T
	<i>Sa. typhimurium</i>	8.6 ± 0.5F	2	14	30	0	N.T	N.T	N.T
Yeasts	<i>C. albicans</i>	20 ± 1 D	0.0625	N.T	N.T	N.T	0	26	20
	<i>C. neoformans</i>	30 ± 1B	0.03125	N.T	N.T	N.T	32	25	0
	<i>C. parasilosus</i>	13 ± 1 E	0.05	N.T	N.T	N.T	0	0	0
Molds	<i>P. chrysoginum</i>	30.3 ± .5B	0.03125	N.T	N.T	N.T	23	30	0
	<i>A. fumigatus</i>	34.6 ± 0.5 A	0.03125	N.T	N.T	N.T	22	23	20
	<i>Fusarium sp.</i>	30 ± 1B	0.03125	N.T	N.T	N.T	30	15	0

* The mean of inhibition zone diameter (mm) ± Standard division. In the same column, the means with different letters are significant at P < 0.05.

on food and water consumption compared to the control group (Tables 6 and 7). Table 8 shows the daily mortality rate (%) in albino male mice infected with *C. albicans* and treated with fluconazole or compound 4. The *C. albicans* caused dramatic increases in daily mortality compared to the control group. The results demonstrated that compound 4, at a loading dose of 800 mg, followed by 400 mg/d for 2 weeks, reduced the mortality (%) caused by *C. albicans*. The results confirmed that compound 4 has very similar effects to fluconazole (Table 8). The mortality in the group treated only with compound 4 was also very similar to the

control group.

The effectiveness of the previous treatments on the serum marker enzymes of the mice is shown in Table 9. The activity of the serum marker enzymes (SGOT, SGPT, alkaline phosphatase, GGT and bilirubin) significantly ($p < 0.001$) increased in the group infected with *C. albicans* (negative control) when compared with the control group. The activity of serum marker enzymes also significantly ($p < 0.001$) increased in the group infected with *C. albicans* and treated with fluconazole, more so than the group only infected with *C. albicans*. The

Table 5
Daily body weight (gm) of albino male mice infected with *C. albicans* and treated with fluconazole and compound 4 for 15 days.

Days↓	Co1*	Co2	FluCal	Comp.	CompCal.
0	19.20 ± 0.35**	19.9 ± 0.43	19.70 ± 0.47	20.20 ± 0.38	21.00 ± 0.36
1	20.10 ± 0.31 4.68†***	19.9 ± 0.37 0.00	19.80 ± 0.35 0.50	20.30 ± 0.36 0.49	21.50 ± 0.26 2.38
2	21.1 ± 0.31 9.89	20.8 ± 0.38 4.52	21.80 ± 0.24 10.65	22.10 ± 0.23 9.40	22.00 ± 0.25 4.76
3	21.7 ± 0.21 13.02	21.7 ± 0.3 9.04	22.30 ± 0.21 13.19	23.60 ± 0.33 16.83	22.3 ± 0.21 6.19
4	22.2 ± 0.20 15.62	22.22 ± 0.27 11.66	22.40 ± 0.22 13.70	24.20 ± 0.20 19.80	22.90 ± 0.27 9.04
5	22.3 ± 0.21 16.14	24.00 ± 0.26 20.60	23.90 ± 0.31 21.31	24.90 ± 0.23 23.26	23.55 ± 0.37 12.16
6	23.2 ± 0.24 20.83	25.37 ± 0.26 27.51	24.00 ± 0.29 21.82	25.70 ± 0.21 27.22	24.22 ± 0.32 15.34
7	23.9 ± 0.23 24.47	25.12 ± 0.29 26.25	24.22 ± 0.22 22.95	26.20 ± 0.24 29.70	24.66 ± 0.23 17.46
8	24.2 ± 0.24 26.04	26.12 ± 0.29 31.28	24.44 ± 0.24 24.08	26.70 ± 0.26 32.17	24.87 ± 0.29 18.45
9	24.9 ± 0.23 29.68	27.00 ± 0.48 35.67	24.50 ± 0.18 24.36	26.80 ± 0.29 32.67	25.25 ± 0.25 20.23
10	25.00 ± 0.22 30.20	27.42 ± 0.48 37.83	24.87 ± 0.29 26.29	27.00 ± 0.27 33.66	25.37 ± 0.26 20.83
11	25.70 ± 0.22 33.85	27.85 ± 0.40 39.98	25.25 ± 0.25 28.17	27.40 ± 0.35 35.64	26.14 ± 0.26 24.48
12	26.3 ± 0.27 36.97	27.83 ± 0.40 39.86	25.37 ± 0.26 -28.80	27.66 ± 0.28 36.96	26.28 ± 0.28 25.17
13	26.7 ± 0.38 39.06	29.50 ± 0.42 48.24	26.00 ± 0.30 31.97	27.77 ± 0.27 35.51	27.28 ± 0.28 29.93
14	27.55 ± 0.29 43.51	29.16 ± 0.47 46.56	26.28 ± 0.28 33.43	28.00 ± 0.37 38.61	28.33 ± 0.49 34.92
15	28.00 ± 0.40 45.83	30.20 ± 0.73 51.78	26.71 ± 0.28 35.60	28.62 ± 0.41 41.70	28.66 ± 0.33 36.50

* Co1 = control group treated with sterile normal saline, Co2 = negative control infected with *C. albicans*, Flucal = group infected with *C. albicans* and treated with fluconazole, Comp = group treated with compound 4 and CompCal = group infected with *C. albicans* and treated with compound 4.

** Mean ± SE, ***% change in daily body weight (gm) compared with control group.

Table 6
Food consumption (g/day/mouse) of albino male mice infected with *C. albicans* and treated with fluconazole and compound 4.

	Days														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Co1*	4.5**	4.6	4.6	4.7	4.8	5.0	5.2	5.5	5.7	5.7	5.9	5.9	6.0	6.1	6.2
Co2	4.1	4.1	4.3	4.2	4.5	4.6	4.8	4.8	4.5	4.8	5.5	5.6	5.5	5.8	6.0
FluCal	4.3	4.3	4.5	4.6	4.6	4.7	5.0	5.1	4.7	5.00	5.1	5.62	5.2	5.2	5.5
Comp.	4.2	4.5	4.4	4.5	4.6	4.6	4.8	4.9	4.9	5.1	5.2	6.0	6.0	6.1	6.1
CompCal.	4.5	4.6	4.7	4.9	4.00	4.2	4.2	4.3	4.5	4.5	4.7	5.0	5.14	5.3	5.5

* Co1 = control group treated with sterile normal saline, Co2 = negative control infected with *C. albicans*, Flucal = group infected with *C. albicans* and treated with fluconazole, Comp = group treated with compound 4 and CompCal = group infected with *C. albicans* and treated with compound 4.

** Food consumption (g/day/mice).

results obtained from the group treated with compound 4 showed significantly ($p < 0.001$) enhancement in activity, with the exception of SGOT activity. In the group injected only with compound 4, there was significantly ($p < 0.01$) less SGOT activity than in the control group. Table 10 includes the concentration of serum lipoproteins (mg/dl) in the tested mice. The data confirmed that the presence of *C. albicans* led to a significant ($p < 0.001$) rise in cholesterol, triglycerides, VLDL and LDL level, whereas it caused a significant ($p < 0.001$) decrease in the HDL level. These effects of *C. albicans* significantly ($p < 0.001$) decreased in the groups treated with compound 4 and fluconazole. The compound 4 group showed a significant ($p < 0.001$) decrease in cholesterol, triglycerides, VLDL and LDL levels, along with a non-significant increase in HDL, compared to the control group. The data in Table 11 demonstrates that *C. albicans* caused a significant ($p < 0.001$) increase in glucose and LDH, whereas it caused a significant ($p < 0.001$) decrease in the total protein level in the serum blood of treated mice. The effects of *C. albicans* (Table 11) significantly decreased in the groups treated with compound 4 and fluconazole ($p < 0.01, p < 0.001$ respectively). The glucose and total protein level in serum blood of mice injected only with the compound 4 significantly decreased ($p < 0.01$) and increased ($p < 0.05$) respectively, while no significant changes were seen in LDH level.

The MDA, total protein and NP-SH in the liver tissues of the mice were estimated in the previous treatments (Table 12). Significant ($P < 0.001$) differences were found between the control group and the negative control group infected with *C. albicans*. Compound 4 and fluconazole demonstrated similar results in reducing the biological effects of *C. albicans* (Table 12). Table 13 illustrates that all hematologic parameters in the mice decreased in the group infected with *C. albicans*, when compared with those injected with sterile normal saline. The lymphocytes excluded from the previous results in Table 13 showed significant ($p < 0.001$) increase. The compound 4 and fluconazole groups noted a decrease in the hematologic changes resulting from *C. albicans*, with the exception of monocytes (%).

The data obtained from the *in vivo* test confirmed that compound 4 significantly reduces the activity of aspartate aminotransferase (AST), cholesterol, triglycerides, VLDL, LDL, and glucose, MDA, WBC and

Table 7
Water consumption (ml/day/mouse) of albino male mice infected with *C. albicans* and treated with fluconazole and compound 4.

	Days														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Co1*	5.1**	5.1	5.2	5.3	5.3	5.4	5.4	5.6	5.8	5.7	5.8	5.8	5.9	6.1	6.2
Co2	4.4	4.5	4.5	4.4	4.6	4.8	5.0	5.1	5.0	5.1	5.1	5.0	5.3	5.5	6.0
FluCal	4.8	4.8	4.9	5.0	5.2	5.2	5.1	5.2	5.2	5.3	5.3	5.5	5.4	5.4	5.7
Comp.	5.2	5.2	5.4	5.5	5.5	5.7	5.8	5.8	5.9	5.9	6.0	6.11	6.2	6.2	6.3
CompCal.	4.5	4.5	4.7	4.9	4.2	4.6	5.7	4.6	4.6	4.8	5.1	5.4	5.5	5.5	5.8

* Co1 = control group treated with sterile normal saline, Co2 = negative control infected with *C. albicans*, Flucal = group infected with *C. albicans* and treated with fluconazole, Comp = group treated with compound 4 and CompCal = group infected with *C. albicans* and treated with compound 4.

** Water consumption (g/day/mice).

lymphocytes in the serum of mice. Additionally, it significantly increases other hematologic parameters, the total protein of serum and the total protein of liver tissue.

Antimicrobial agents can be grouped based on their chemical structures into aminoglycosides, anasamcolides, beta-lactams, chloramphenicol and analogues, lincosamides, macrolides, nucleosides, puromycin, peptides, phenazines, polyenes, polyethers and tetracyclines [27]. Recent scientific research has focused on the function and inhibition of fungal β -carbonic anhydrases and bacterial carbonic anhydrases (α -, β -, and/or γ - carbonic anhydrases familie) as novel anti-fungal and antibacterial targets [28,29]. Based on its chemical structure, compound 4 belongs to the trithiocarbonates class. This class is considered a strong inhibitor of carbonic anhydrases [10,30], which may explain the effects of compound 4 on the tested microorganisms.

The present results obtained from *in vitro* and *in vivo* experiments suggest that compound 4 may be a novel antibacterial and antifungal drug.

3. Material and methods

3.1. Chemistry

All chemicals were obtained from commercial sources, including Sigma-Aldrich (St. Louis, MO, USA), and were used as received without further purification, unless otherwise stated. Melting points were measured on a Gallenkamp melting point apparatus (Thermo Fisher Scientific, Paisley, UK) in open glass capillaries and are uncorrected. Infrared spectra (IR) were recorded using the KBr disc technique on a Perkin Elmer FT-IR spectrophotometer 1000 (PerkinElmer, Waltham, MA, USA). ^1H - and ^{13}C -NMR spectra were obtained with either a JEOL ECP 600 NMR spectrometer (Tokyo, Japan) operating at 600 MHz z in deuterated chloroform (CDCl_3) or dimethyl sulfoxide ($\text{DMSO}-d_6$). Chemical shifts are expressed in δ units and J -coupling constants are given in Hz. Mass spectra were acquired with the aid of a Shimadzu GCMS-QP 1000 EXmass spectrometer (Tokyo, Japan) at 70 eV. Elemental analysis was carried out on a Perkin Elmer 2400Elemental Analyzer; CHN mode.

Table 8Daily mortality (%) in albino male mice infected with *C. albicans* and treated with fluconazole and compound 4.

	Days														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Co1*	0**	0	0	0	0	0	0	0	0	0	0	0	0	10	10
Co2	0	0	0	10	20	20	20	20	30	30	30	40	40	40	50
FluCal	0	0	0	0	0	0	10	10	20	20	20	30	30	30	30
Comp.	0	0	0	0	0	0	0	0	0	0	0	0	10	10	10
CompCal.	0	0	0	0	10	10	10	20	20	20	30	30	30	30	30

* Co1 = control group treated with sterile normal saline, Co2 = negative control infected with *C. albicans*, FluCal = group infected with *C. albicans* and treated with fluconazole, Comp = group treated with compound 4 and CompCal = group infected with *C. albicans* and treated with compound 4.

** Mortality (%).

3.1.1. Synthesis of diethyl 2,2'-(thiocarbonylbis(sulfanediyl))diacetate 4

A mixture of ethyl cyanoacetate 1 (0.1 mol) and anhydrous K_2CO_3 (30 g) in DMF (35 ml) was stirred vigorously at 25 °C for 10 min, and then CS_2 2 (0.2 mol) was added with stirring for 50 min. The mixture reaction was cooled in an ice bath. Then, ethyl 2-chloroacetate 3 (0.2 mol) was added with continued stirring for 15 min. The cooling bath was subsequently removed, and the mixture was stirred for 3 h. The solid product was precipitated by the addition of H_2O collected by filtration, washed with H_2O , and dried. The compounds were recrystallized from EtOH to afford two crystalline materials: ethyl 3-amino-4-cyano-5-((2-ethoxy-2-oxoethyl)thio)thiophene-2-carboxylate, Yield: 60%; m.p. 93 ° and diethyl 2,2'-(thiocarbonylbis(sulfanediyl))diacetate 4. These compounds had the following characteristics: Yield: 20%; m. p. 40 °C; IR (KBr, cm^{-1}) δ = 2978, 2930, 1732, 1475, 1367, 1300, 1202, 1155, 1095, 1069; 1H NMR (500 MHz, $CDCl_3$) δ = 1.23 (t, J = 7 Hz, 3H, $-CH_3$), 4.14 (s, 2H, $S-CH_2-CO$), 4.16 (q, 2H, J = 7 Hz, $-O-CH_2-$); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 14.0, 38.9, 62.1, 167.0, 220.2; MS m/z : 283.0 $[M+H]^+$, 267.1, 239.1, 207.0; Elemental Analysis: C, 38.28; H, 5.00; S, 34.06, Found: C, 38.31; H, 5.12; S, 33.97.

3.1.2. Single-crystal X-ray diffraction of compound 4

Compound 4 was obtained as single crystals by slow evaporation from an ethanol solution of the pure compound at room temperature. Data were collected on a Bruker APEX-II D8 Venture area diffractometer, equipped with graphite monochromatic Cu K α radiation, λ = 1.54178 Å at 293 (2) K. Cell refinement and data reduction were carried out by a Bruker SAINT. SHELXT [31,32] was used to reveal the structure. The final refinement was carried out using the full-matrix least-squares technique with anisotropic thermal data for non-hydrogen atoms on ?? . CCDC 1522608 contains the supplementary crystallographic data for this compound and can be obtained free of charge

Table 9Activity of serum marker enzymes of albino male mice infected with *C. albicans* and treated with fluconazole and compound 4.

	SGOT(AST)U/L		SGPT(ALT) U/L		Alkaline phosphatase E(U/L)		GGT (U/L)		Bilirubin (mg/dl)	
	Mean \pm SE	% Change	Mean \pm SE	% Change	Mean \pm SE	% Change	Mean \pm SE	% Change	Mean \pm SE	% Change
Co1*	107.45 \pm 2.71		27.07 \pm 0.53		296.88 \pm 4.35		4.94 \pm 0.14		0.54 \pm 0.01	
Co2	204.8 \pm 5.86*** a	90.59 \uparrow	125.20 \pm 7.45*** a	362.37 \uparrow	414.80 \pm 6.75*** a	39.71 \uparrow	9.70 \pm 0.29*** a	9617 \uparrow	1.67 \pm 0.04*** a	209.46 \uparrow
FluCal	143.28 \pm 2.88*** b	30.03 \downarrow	50.48 \pm 3.17*** b	59.67 \downarrow	321.28 \pm 6.91*** b	22.54 \downarrow	5.60 \pm 0.17*** b	42.26 \downarrow	0.75 \pm 0.03*** b	55.13 \downarrow
Comp.	89.07 \pm 3.82*** a	17.10 \downarrow	28.67 \pm 1.15 a	5.89 \downarrow	286.62 \pm 4.67 a	3.45 \downarrow	4.40 \pm 0.13 ^a	11.10 \downarrow	0.57 \pm 0.017 a	6.04 \downarrow
CompCal.	169.33 \pm 2.69*** b	17.31 \downarrow	83.50 \pm 2.35*** b	33.30 \downarrow	354.66 \pm 5.57*** b	14.49 \downarrow	7.18 \pm 0.20*** b	25.94 \downarrow	1.15 \pm 0.03*** b	31.16 \downarrow

* Co1 = control group treated with sterile normal saline, Co2 = negative control infected with *C. albicans*, FluCal = group infected with *C. albicans* and treated with fluconazole, Comp = group treated with compound 4 and CompCal = group infected with *C. albicans* and treated with compound 4. All values represent mean \pm SEM.

*** $p < 0.001$; Student's t -test.

^a As compared with control group.

^b As compared with *C. albicans* group.

from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

3.2. Biological activity tests

3.2.1. In vitro, antimicrobial activity

To evaluate the antimicrobial activity of compound 4 as a novel anti-bacterial and anti-fungal agent, the inhibition zone diameter (mm) and the minimal inhibitory concentration were determined according to literature reports [33,34]. The tests were carried out on several clinical microorganisms, including gram positive bacteria (*Staphylococcus aureus* ATCC and *Streptococcus mutans* ATCC 35668), gram negative bacteria (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028 and *Neisseria meningitidis* ATCC 1302), yeasts (*Candida albicans* ATCC 60193, *Cryptococcus neoformans* Wild strain and *Candida parapsilosis* ATCC 22019), and molds (*Aspergillus fumigatus* AUMC 8794, *Penicillium chrysogenum* AUMC 9476 and *Fusarium* sp.).

The clinical microorganisms were cultivated at least three times with suitable culture mediums and incubating conditions before the susceptibility tests. The concentrations of microbial inoculation suspensions in sterile sodium chloride solution (0.89%) were adjusted to 0.65 at 620 nm by spectrophotometer. In the first stage, 150 mg of compound 4 was dissolved in 2000 μ L of DMSO, then 40 μ L of solution was added on a sterile filter disk (6 mm) to obtain 3 mg per disk. To prepare a water soluble form of compound 4, 150 mg of compound 5 was treated with sodium ethoxide and dissolved with 2000 μ L of sterile distilled water; then, 40 μ L of solution was added to the filter disk (3 mg/disk). DMSO- and water-soluble forms of compound 4 were investigated using disk diffusion assay to determine the inhibition zone (mm) resulting from 3 mg of compound per disk.

The disks were then dried at 25 °C under sterile conditions using a safety biological cabinet. Mueller-Hinton agar (MHA) (Scharlau Microbiology, Spain) and potato dextrose agar (PDA) (Scharlau, Spain) were used to perform antibacterial and antifungal tests, respectively. The media were prepared according to the manufacturer's guidelines. To prepare one liter of MHA and one liter of PDA, 21 g of MHA and 39 g of PDA were dissolved in one liter of sterile distilled water, then sterilized at 121 °C for 15 min by autoclave (HL-321, Taiwan). The sterilized media were poured into sterile plastic petri dishes at 50 °C. The media were allowed to solidify at room temperature. Next, 0.1 ml of microbial inoculation suspension was spread on the dried surface of the medium and kept at 25 °C for 5–10 min. Compound 4 and the standard antimicrobial drug discs (Table 1) were dispensed on the surface of the inoculated media. The bacteria were incubated at 37 \pm 1 °C for 19–22 h and the fungi at 25 \pm 1 °C for 48–72 h. After incubation, the diameters of the inhibition zones (millimetre) resulting from the biological activity of the compound were recorded.

The minimal inhibitory concentration (MIC) of compound 4 (water-

Table 10
Serum lipoproteins of albino male mice infected with *C. albicans* and treated with fluconazole and compound 4.

Treatments	Cholesterol (mg/dl)		Triglycerides (mg/dl)		HDL(mg/dl)		VLDL(mg/dl)		LDL(mg/dl)	
	Mean ± SE	% Change	Mean ± SE	% Change	Mean ± SE	% Change	Mean ± SE	% Change	Mean ± SE	% Change
Co1*	98.17 ± 2.1		85.3 ± 2.7		39.0 ± 0.7		17.0 ± 0.5		42.0 ± 2.2	
Co2	212.8 ± 6.0 ^{*** a}	116.7↑	186.8 ± 6.0 ^{*** a}	118.8↑	21.1 ± 0.9 ^{*** a}	45.8↓	37.3 ± 1.21 ^{*** a}	118.8↑	154.2 ± 5.7 ^{*** a}	267.1↑
FluCal	134.8 ± 3.6 ^{*** b}	36.6↓	105.1 ± 2.2 ^{*** b}	43.7↓	29.8 ± 0.9 ^{*** b}	41.1↑	21.0 ± 0.4 ^{*** b}	43.7↓	83.9 ± 3.3 ^{*** b}	45.5↓
Comp.	78.68 ± 3.2 ^{*** a}	19.85↓	68.5 ± 1.6 ^{*** a}	19.6↓	41.6 ± 2.1 ^{*** a}	6.42↑	13.7 ± 0.3 ^{*** a}	19.6↓	23.3 ± 3.0 ^{*** a}	44.35↓
CompCal.	159.8 ± 3.4 ^{*** b}	24.8↓	134.8 ± 3.8 ^{*** b}	27.8↓	26.9 ± 1.1 ^{*** b}	27.2↑	26.9 ± 0.7 ^{*** b}	27.8↓	105.9 ± 4.6 ^{*** b}	31.3↓

* Co1 = control group treated with sterile normal saline, Co2 = negative control infected with *C. albicans*, Flucal = group infected with *C. albicans* and treated with fluconazole, Comp = group treated with compound 4 and CompCal = group infected with *C. albicans* and treated with compound 4. All values represent mean ± SEM.

** p < 0.01.

*** p < 0.001; Student's- *t*-test.

^a As compared with control group.

^b As compared with *C. albicans* group.

Table 11
Serum glucose, total protein and LDH of albino male mice infected with *C. albicans* and treated with fluconazole and compound 4.

Treatments	Glucose level (mg/dl)		Total protein (mg/dl)		LDH	
	Mean ± SE	% Change	Mean ± SE	% Change	Mean ± SE	% Change
Co1*	113.95 ± 2.96		7.11 ± 0.15		158.84 ± 3.00	
Co2	204.2 ± 7.58 ^{*** a}	79.19↑	3.55 ± 0.08 ^{*** a}	50.05↓	245.18 ± 5.12 ^{*** a}	54.35↑
FluCal	143.85 ± 2.47 ^{*** b}	29.55↓	5.64 ± 0.11 ^{*** b}	58.79↑	184.92 ± 5.08 ^{*** b}	24.57↓
Comp.	96.38 ± 3.62 ^{*** a}	15.41↓	7.66 ± 0.18 ^{*** a}	7.69↑	162.50 ± 1.84 ^{*** a}	2.29↑
CompCal.	166.83 ± 4.32 ^{*** b}	18.29↓	4.91 ± 0.09 ^{*** b}	38.14↑	220.98 ± 3.96 ^{*** b}	9.86↓

* Co1 = control group treated with sterile normal saline, Co2 = negative control infected with *C. albicans*, Flucal = group infected with *C. albicans* and treated with fluconazole, Comp = group treated with compound 4 and CompCal = group infected with *C. albicans* and treated with compound 4. All values represent mean ± SEM.

** p < 0.01.

*** p < 0.001; Student's- *t*-test.

^a As compared with control group.

^b As compared with *C. albicans* group.

Table 12
MDA, Total Protein and NP-SH in liver tissue of albino male mice infected with *C. albicans* and treated with fluconazole and compound 4.

Treatments	MDA (nmol/g)	Total protein (g/l)	NP-SH (nmol/g)
Co1*	1.24 ± 0.02	115.23 ± 1.80	5.70 ± 0.14
Co2	6.36 ± 0.25 ^{*** a}	65.14 ± 2.06 ^{*** a}	1.61 ± 0.14 ^{*** a}
FluCal	2.33 ± 0.07 ^{*** b}	92.72 ± 1.54 ^{*** b}	4.21 ± 0.18 ^{*** b}
Comp.	0.94 ± 0.03 ^{*** a}	129.04 ± 2.80 ^{*** a}	6.88 ± 0.22 ^{*** a}
CompCal.	4.57 ± 0.25 ^{*** b}	77.84 ± 2.37 ^{*** b}	3.56 ± 0.16 ^{*** b}

* Co1 = control group treated with sterile normal saline, Co2 = negative control infected with *C. albicans*, Flucal = group infected with *C. albicans* and treated with fluconazole, Comp = group treated with compound 4 and CompCal = group infected with *C. albicans* and treated with compound 4. All values represent mean ± SEM.

** p < 0.01.

*** p < 0.001; Student's- *t*-test.

^a As compared with control group.

^b As compared with *C. albicans* group.

soluble form) was determined by microdilution assay, using a Mueller-Hinton broth for bacteria and a potato dextrose broth for fungi. Compound 4 was dissolved in a sterile culture broth (2 mg/mL). Two-fold serial dilutions were carried out to obtain concentrations ranging from 2 to 0.008 mg/mL. Each dilution of 95 µL was added to 96-well plates, then each well was inoculated with 5 µL of inoculation microbial suspension (5 × 10⁸ colony form unit (CFU)/ml). The plates were incubated at conditions suitable for the tested microorganism. After incubation, microbial growth was detected in each well by a *p*-iodonitrotetrazolium violet reagent (Sigma, USA). Each well received 20 µL of

the reagent (0.5 mg/ml), and was then incubated at 37 ± 1 °C for 30 min. A violet color indicated microbial growth in the well. The minimal inhibitory concentration (mg/ml) of compound 4 was estimated as the lowest concentration that inhibited microbial growth.

3.2.2. Anticancer test

The biological activity of compound 4 (water soluble form) as an anticancer agent for human breast cancer was evaluated according to a previous study [35]. Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma, USA) was prepared according to the manufacturer's guidelines and supplemented with fetal bovine serum (10%). To avoid microbial contamination, streptomycin and penicillin were added to RPMI-1640. Several concentrations of compound 5, ranging from 4 to 0.008 mg/mL, were tested.

3.2.3. In vivo, antifungal activity

To confirm that the water-soluble form of compound 4 has biological activity as a novel antimicrobial agent, mice were treated with candidemia and the effectiveness of compound 4 was investigated. The experiment was performed in the Experimental Animal Care Center (EACC), College of Pharmacy, King Saud University, Riyadh. Fifty albino male mice weighing nearly 30 ± 2 g were used. The mice were arranged in five groups, ten mice in each cage, and given water and feed ad libitum. The negative control group was infected intravenously with 0.2 ml of *C. albicans* suspension (10⁶ CFU/mouse), while the control groups received 0.2 ml of sterile normal saline (0.89% NaCl). The group treated with the standard drug (fluconazole) was infected intravenously with 0.2 ml of *C. albicans* suspension (10⁶ CFU/mouse) and treated (orally) by 12 mg/kg of fluconazole (loading dose) on the first day, followed by 6 mg/ml for 2 weeks. The group treated with the investigated

Table 13

Hematological studies of albino male mice infected with *C. albicans* and treated with fluconazole and compound 4.

Treatments	Co1*	Co2		FluCal		Comp.		CompCal.	
	Mean ± SE	Mean ± SE	% Change	Mean ± SE	% Change	Mean ± SE	% Change	Mean ± SE	% Change
WBC(Cell/mm ³)	8582.2 ± 17.7	6513.6 ± 24.0 ^{*** a}	24.1↓	8233.2 ± 83.7 ^{*** b}	26.4↑	8795 ± 45.9 ^{** a}	2.48↑	7540.8 ± 87.3 ^{*** b}	15.9↑
RBC(10 ⁶ /mm ³)	8.14 ± 0.17	4.46 ± 0.19 ^{*** a}	45.23↓	7.50 ± 0.23 ^{*** b}	68.16↑	9.26 ± 0.1 ^{** a}	13.72↑	6.16 ± 0.1 ^{*** b}	38.2↑
HGB(g/dL)	11.9 ± 0.18	7.62 ± 0.16 ^{*** a}	17.9↓	11.1 ± 0.18 ^{*** b}	46.7↑	12.4 ± 0.18 ^a	3.4↑	9.8 ± 0.14 ^{*** b}	28.6↑
HCT	42.48 ± 1.47	23.3 ± 1.54 ^{*** a}	44.9↓	35.05 ± 1.5 ^{*** b}	49.9↑	45.9 ± 1.51 ^a	8.2↑	31.3 ± 0.42 ^{*** b}	33.8↑
PLT(10 ³ /mm ³)	618.0 ± 15.5	441.2 ± 24.7 ^{*** a}	28.6↓	563.8 ± 17.4 ^{*** b}	27.8↑	670.7 ± 15.3 ^{** a}	8.5↑	522.3 ± 10.1 ^{* b}	18.3↑
MCV(fL)	75.84 ± 3.72	43.5 ± 2.5 ^{*** a}	42.5↓	72.04 ± 2.6 ^{*** b}	65.4↑	87.31 ± 1.74 ^{** a}	15.1↑	53.7 ± 2.0 ^{* b}	23.4↑
MCH(pg)	29.9 ± 0.97	21.2 ± 0.5 ^{*** a}	28.9↓	28.2 ± 0.7 ^{*** b}	32.6↑	39.0 ± 1.0 ^{*** a}	30.4↑	26.3 ± 0.5 ^{*** b}	23.6↑
MCHC(g/dL)	27.87 ± 0.94	19.3 ± 0.7 ^{*** a}	30.6↓	24.3 ± 0.5 ^{*** b}	25.9↑	32.6 ± 0.9 ^{** a}	17.0↑	23.4 ± 1.0 ^{* b}	21.1↑
Neutrophils(%)	28.88 ± 0.71	21.0 ± 0.4 ^{*** a}	27.3↓	25.9 ± 0.5 ^{*** b}	23.3↑	33.3 ± 1.1 ^{** a}	15.3↑	24.1 ± 0.2 ^{*** b}	15.0↑
Lymphocytes(%)	5.49 ± 0.10	8.8 ± 0.2 ^{*** a}	60.7↑	6.2 ± 0.1 ^{*** b}	29.2↑	4.8 ± 0.09 ^{** a}	11.3↓	7.10 ± 0.07 ^{*** b}	19.4↓
Eosinophils(%)	1.74 ± 0.04	0.96 ± 0.05 ^{*** a}	44.8↓	1.61 ± 0.10 ^{*** b}	68.3↑	2.38 ± 0.09 ^{*** a}	36.7↑	1.30 ± 0.02 ^{*** b}	36.1↑
Monocytes (%)	1.04 ± 0.02	0.96 ± 0.02 ^{** a}	7.98↓	1.06 ± 0.02 ^{* b}	11.16↑	1.15 ± 0.01 ^{** a}	10.7↑	0.88 ± 0.07 ^b	7.9↓

* Co1 = control group treated with sterile normal saline, Co2 = negative control infected with *C. albicans*, Flucal = group infected with *C. albicans* and treated with fluconazole, Comp = group treated with compound 4 and CompCal = group infected with *C. albicans* and treated with compound 4. All values represent mean ± SEM.

** p < 0.01.

*** p < 0.001; Student's- *t*-test.

^a As compared with control group.

^b As compared with *C. albicans* group.

compound 4 was infected intravenously with *C. albicans* at 10⁶ CFU/mouse (0.2 ml) and treated (orally) by 12 mg/kg of compound 4 (loading dose) on the first day, followed by 6 mg/ml for 2 weeks. To study the biological effects of compound 4, a fifth group was designated. In this group, the mice were treated intravenously (I.V) with 12 mg/kg of compound 4 on the first day, followed by 6 mg/ml for 2 weeks. All doses of the standard drug and compound 4 were given twice daily.

The effectiveness of the treatments on some biological aspects was evaluated. The biological parameters included daily body weight (gm), mortality (%), food consumption (g/day/mouse), water consumption (ml/day/mouse), serum glutamic oxaloacetic transaminase [SGOT(AST) (U/L)], serum glutamic-pyruvic transaminase [SGPT(ALT) (U/L)], alkaline phosphatase (U/L), gamma-glutamyl transferase (GGT) (U/L), bilirubin (mg/dl), cholesterol (mg/dl), triglycerides (mg/dl), high-density lipoprotein (HDL) (mg/dl), very-low-density lipoprotein (VLDL) (mg/dl), low-density lipoprotein (LDL) (mg/dl), lactate dehydrogenase (LDH) (U/L), glucose (mg/dl), total protein (mg/dl), malondialdehyde (MDA) (nmol/g), nonprotein sulfhydryl (NP-SH) (nmol/g). Additional hematologic constituents, including white blood cells (WBC) (cell/mm³), red blood cells (RBC) (10⁶/mm³), hemoglobin (HGB) (g/dL), HCT, platelets (PLT) (10³/mm³), mean corpuscular volume (MCV) (fL), mean corpuscular hemoglobin (MCH) (pg), mean corpuscular hemoglobin concentration (MCHC) (g/dL), neutrophils (%), lymphocytes (%), eosinophils (%) and monocytes (%) were also evaluated [36–38]. SGOT (AST), SGPT (ALT), ALP, GGT, bilirubin LDH and total protein were estimated using a Reflotron Plus Analyzer and Roche kits (Roche Diagnostics GmbH, Mannheim Germany) and United diagnostic kits assay. Hematological tests were performed by Mindray BC-2800VET Auto Hematology Analyzer.

3.2.4. Experimental design and statistical analysis

The present experiment was designed as a completed random design (CRD). The data are arranged as mean ± standard deviation (SD) or standard error of mean (SEM). The Statistical Package for the Social Sciences (SPSS 13.0) was used to analyze the mean of the biological parameters by using the Tukey post-hoc and Student's- *t*-test.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2019.103157>.

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