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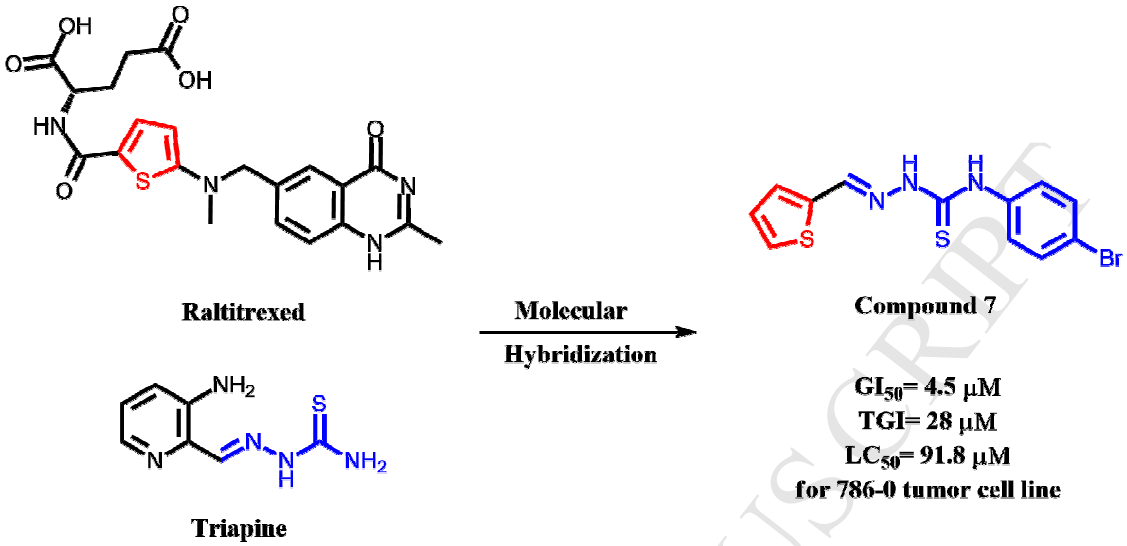
Graphical abstract



Synthesis of thiophene-thiosemicarbazone derivatives and evaluation of their *in vitro* and *in vivo* antitumor activities

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Abstract

A series of thiophene-2-thiosemicarbazones derivatives (**5-14**) was synthesized, characterized and evaluated for their antitumor activity. They were tested *in vitro* against human tumor cell lines through the colorimetric method. The results revealed that compounds **7** and **9** were the most effective in inhibiting 50% of the cell growth after 48 hours of treatment. As compound **7** showed a potent antiproliferative profile, it has been chosen for further studies in 786-0 cell line by flow cytometry. Treatments with compound **7** (50 µM) induced early phosphatidylserine exposure after 18 hours of exposure and this process progressed phosphatidylserine exposure with loss of cell membrane integrity after 24 hours of treatment, suggesting a time-dependent cell death process. Regarding the cell cycle profile, no changes were observed after treatment with compound **7** (25 µM), suggesting a mechanism of cell death independent on the cell cycle. The *in vivo* studies show that compound **7** possess low acute toxicity, being the doses of 30-300 $mgKg^{-1}$ chosen for studies in Ehrlich solid tumor model in mice. All doses were able to inhibit tumor development being the lowest one the most effective. Our findings highlight thiophene-2-thiosemicarbazones as a promising class of compounds for further studies concerning new anticancer therapies.

1. Introduction

Cancer is a leading cause of death worldwide, responsible for 8.2 million deaths in 2012. These numbers can be linked to population growth, the process of industrialization and changes in habit life [1-2]. In general, cancer treatment is based on radiotherapy, surgical removal, hormone therapy, immunotherapy and chemotherapy, alone or combined, aiming to increase the patients survival [3].

The design of new compounds prototypes based on medicinal chemistry followed by the biological approach has been a good strategy for the discovery of new therapies [4]. Molecular hybridization is a strategy for drug design widely used in medicinal chemistry and aims at the junction of two chemical moieties into a single compound, targeting an increase in biological activity [5]. The presence of heterocyclic systems prototype molecules is of great interest in design of the new drugs. Features as small molecular weight, presence of heteroatoms and aromatic structures can mimic endogenous substrates, which may lead to the development of a biological activity [6].

Raltitrexed (Tomudex®) (Figure 1) is a quinazoline folate analogue that has in its structure the thiophene heterocycle, which is also found in many promising antitumor compounds [6-9]. The mechanism of action of this drug is the selective inhibition of the enzyme thymidylate synthase, which leads to DNA fragmentation and subsequent cell death [10]. Raltitrexed is indicated for the treatment of colorectal cancer (CRC) been considered a promising alternative therapy when combined with 5-fluorouracil (5-FU) and capecitabine (Figure 1). This treatment schedule is better acceptance by patients since it results in lower clinical complications, such as thrombosis and cardiotoxicity, than the use of 5-FU and capecitabine alone [11-13].

PLEASE, INSERT FIGURE 1 HERE

Recent studies have been aimed to the discovery of new drugs for cancer treatment. The thiosemicarbazones comprise a class of molecules known for their diverse biological activities such as antimicrobial [14], antitumor [15-17], antileishmanial [18] and antiplasmodic [19] properties. Among the thiosemicarbazones, triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, 3-AP) is the most promising molecule undergoing clinical phase II studies [20]. The probable mechanism

of action of this drug is the inhibition of the enzyme ribonucleotide reductase [21], but there is also evidence of topoisomerase II inhibition [22].

Thus, this study aimed to describe the production of novel thiophene-2-thiosemicarbazones through molecular hybridization strategy between the thiophene core of raltitrexed and the thiosemicarbazone of triapine properly synthesized and characterized. The derivatives were evaluated for their antiproliferative activity in a panel of human tumor cell lines. The most promising molecule (compound **7**) was chosen for further studies concerning cell death and cell cycle profile by flow cytometry, focusing on the human renal adenocarcinoma cell line 786-0. Finally, we also describe the *in vivo* antitumor activity of **7** in the murine model of Ehrlich solid tumor in mice.

2. Results and Discussion

2.1 Chemistry

2-thiophene-thiosemicarbazone derivatives (**5-14**) were prepared in two steps. Thiosemicarbazide was obtained via nucleophilic addition reaction between hydrazine and substituted isothiocyanates. The synthesized thiosemicarbazides reacted with 2-thiophene-carboxaldehyde, in presence of a catalytic amount of AcOH, via condensation reaction, leading to the formation of compounds of interest [23]. Good reaction yields were observed using this methodology (40-87%) (Scheme 1). Some compounds evaluated in this study were previously synthesized (compounds **5**-**8**) [24]. However, such compounds have not been investigated against its antitumor potential. Thus, both compounds previously synthesized (**5**-**8**) as the novel compounds (**9**-**14**) were assessed for their pharmacological potential.

PLEASE, INSERT SCHEME1 HERE

The structures were determined by ^{1}H and ^{13}C NMR, DEPT, IR and high-resolution mass. Analyzes of these data were compatible with the proposed compounds. The IR spectra showed characteristic stretches of connections C=S, C=N and NH. Additionally, the absence of bands between 2700 cm^{-1} and 2500 cm^{-1} excluded the possibility of a thione-thiol tautomerism (e.g., H-N-C=S, -N=C-SH) [25].

As exemplified with the ^{1}H NMR analysis of *N*-(*p*-tolyl)-2-(thiophen-2-ylmethylene)-thiosemicarbazone (**9**), the singlet peak δ 2.50 corresponded to the *para* methyl group at phenyl ring. The phenyl protons occurred as doublets peak at 7.15 and 7.42 ppm. The protons of thiophene group occurred at δ 7.13, 7.52 and 7.69. Singlet peak at δ 8.33 confirms the success of the reaction between *N*-(*p*-tolyl)-thiosemicarbazone and 2-thiophene-carboxaldehyde, relative to iminic proton (H-C=N). In addition, signals related to N-H the presented chemical shifts δ 9.70 and 11.76 ppm. The IR spectrum showed stretches on the C=N and C=S at 1593 and 1557 cm^{-1}, respectively, and the absorption band of N-H at 3297 cm^{-1}. The predominance of the thione form of the synthesized thiosemicarbazones may be observed in the chemical shift of carbon of the thiocarbonyl ^{13}C NMR at δ 175.5. Quaternary carbon peaks were confirmed by DEPT experiments to appear at δ 134.3, 136.3, 138.3 and 175.5. In a

manner complementary, HRMS confirmed the identity of all the synthesized compounds.

All target compounds **5-14** could exist in either the *E* or *Z* isomeric form due to the imino bond (-CH=N-). However, in ^{1}H NMR spectra, the –N=CH- signal was seen as a single peak for each compound indicating that only one isomer was generated. The hydrogen attached to carbon of imine presented chemical shift in ^{1}H NMR spectra δ 8.24-8.40 for all target compounds. X-ray crystallography and NOESY studies confirm the preference of the *E* diastereoisomer [26,27].

Compound **10** was chosen for elucidation of isomerism of the derivatives obtained in this study. Many NOESY signals were observed for this compound. By on side, NOESY signals of the studied molecule were employed to assign and corroborate the obtained structure. By the other side, portion of the NOESY spectra reveals the interactions between H-C=N (at 8.33 ppm), N-H (at 9.70 ppm) and H-C=C (at 7.53 ppm) moieties. The close proximity between this group of protons suggests (*E*)-isomer as main product. Additionally, no signal relative to (*Z*)-isomer of the analyzed structure was detected in the NOESY spectrum (See supplementary material).

As reported in the literature, chemical shift values around 11 ppm (9-12 ppm) were correlated to the *E* isomers, whereas higher ppm values were reported for the *Z* forms (over 12 ppm) [28]. The most important signal in the process of describing the correct stereoisomer is that connected with NH group (CS)NH-proton [29]. The ^{1}H NMR of all synthesized compounds shows a singular peak around 11.50-11.97 ppm relative to the NH signal which indicates *E* isomer was generated.

2.2 Biological activities

2.2.1 *In vitro* studies

The antiproliferative effects of the thiophene-2-thiosemicarbazones derivatives on nine human tumor cell lines and one non-tumor cell line are summarized in Table 1, using doxorubicin as an positive control. The results were expressed as concentration required to inhibit in 50% the cell growth, named as GI_{50} in µM (Table 1). Moreover, the average activity was expressed as mean GI_{50} that was the arithmetical average of all GI_{50} values obtained for the human tumor cell lines. All ten derivatives evaluated have

the thiophene core in their structure, with modifications present in the distal portion of the compounds.

Considering the average activity (mean GI_{50}), three compounds (**6**, **7** and **9**) exhibited values lower than 50 µM, while six compounds (**5, 8, 10, 12**, **13** and **14**) showed mean GI_{50} between 50 - 100 µM, been **11** inactive (mean GI_{50} >100 µM).

Compound **5** represents the basic skeleton of this series of derivatives, with no replacements in the phenyl portion. This compound did not exert antiproliferative effect in most tumor cell lines evaluated been effective against K562 (leukemia cell line, GI_{50} = 16.7 µM) (Table 1).

The introduction of a halogen into phenyl group (**6** and **7**, mean GI_{50} < 50 µM) resulted in an important increase in cytostatic activity. The derivative with a *p*-bromo-phenyl moiety (**7**) stood out among the other compounds, being active against all cell lines excepting leukemia (K-562), with GI_{50} values ranging from 0.74 to 79.4 µM. Besides, the *p*-chlorophenyl analogue (**6**) was active against K-562, NCI-ADR/RES (multiresitant ovarian cells), 786-0 (renal cell adenocarcinoma) and U251 (glioma) with GI_{50} values of 2.0, 2.8, 7.4 and 8.3 µM, respectively (Table 1).

Moreover, the results observed for **9** and **10** seems to suggest that the size of the alkyl group introduced in the aromatic group affect greatly the cytostatic effective of a thiophene-2-thiosemicarbazones derivatives. This way, when a C2 chain (*p*-ethylphenyl, **10**) was attached to the base molecule, this resulted in lower inhibitory effect than that observed for **5** for almost cell lines, while a *p*-methylphenyl derivative (**9**) afforded an increment in cytostatic activity (Table 1). Also, it was interesting to notice that both **9** (*p*-methylphenyl) and **10** (*p*-ethylphenyl) showed a high selectivity to K-562 (leukemia, GI_{50} <0.9 and 1.6. µM, respectively), suggesting that the presence of an alkyl group introduced in the aromatic group affect increase the cytostatic effective against leukemia (Table 1).

Besides, when an ethyl group was introduced as a spacer group (compound **11,** 2-phenyl-ethyl derivative, a structural isomer of **10** (*p*-phenyl-ethyl) even the cytostatic activity against leukemia was not observed resulting in an inactive thiosemicarbazone derivative (mean GI_{50} >100 µM). These results suggest that large aliphatic carbon chains do not favor the antiproliferative activity. Also, the introduction of a hydroxyalkyl chain in the aromatic group (**8**, *p*-methoxyphenyl) might be considered unfavorable, since **8** was inactive for most of cell lines (mean GI_{50} < 89 µM) except for leukemia (GI_{50} = 1.7 µM, Table 1).

The substitution of phenyl ring for a 1-naphthyl ring (**12**) or for a 3-pyridyl group (**13**) resulted in an increased antiproliferative effect (mean GI_{50} > 59.5 and > 65.9 µM, respectively) compared to **6** (mean GI_{50} > 81.8 µM) mainly due the increment on activity against U251 (glioma), 786-0 (renal adenocarcinoma) for both **12** and **13** besides against NCI-ADR/RES (multidrug resistant ovarian adenocarcinoma) and K562 (leukemia) for **12** and NCI-H460 (no-small lung adenocarcinoma) for **13.** Finally, the structural modification presented by compound **14** (allyl), certainly did not favor an increase in the antiproliferative activity. Removing the phenyl ring and the insertion of an unsaturated aliphatic chain in this class of compounds significantly reduced their activity in the tumor cell lines used in this experiment (Table 1).

The non-tumor cell line HaCaT (immortalized human keratinocyte) was inserted into the panel in order to simulate normal human cells since immortalized cells share with tumor cells the characteristic of rapid mitosis of tumor cells without the presence of oncogenes. Considering the obtained results, the most potent derivatives 2-thiophene-thiosemicarbazones **6**, **7** and **9**, also showed cytostatic effect against non-tumor cell line (Table 1).

The thiosemicarbazones are known for their antiproliferative activity, and our results corroborate with previous studies that show a cytostatic profile for the cell line K-562 (leukemia) [30]. The *N*-acylhydrazones are analogous of thiosemicarbazone compounds. One of the common points between these two classes of compounds is the presence of the C=N bond. Recent studies show the importance of *N*- acylhydrazones for the antiproliferative activity against cell lines, as we can observe in our study for the compounds that possess the substitution *p*-methylphenyl and *p*-bromophenyl [31].

In summary, it can be suggested that the inclusion of aromatic rings in the structure of thiosemicarbazones improves the antiproliferative activity of this class of compounds. Furthermore, the presence of halogens directly connected to this core also has a large contribution to the increase in this activity. However, the presence of alkyl groups either directly connected to the thiosemicarbazone or attached to an aromatic ring skeleton does not appear to increase the effect on cell growth, unless the alkyl moiety is a short chain. With these preliminary results, it was possible to assume that these thiosemicarbazones derivatives show a remarkable antiproliferative activity.

PLEASE, INSERT TABLE 1 HERE

In view of the potency of **7** towards almost all cell lines (Table 1), this compound was selected for further studies against the human kidney tumor cell line 786-0, which is an epithelial adenocarcinoma that produces the parathyroid hormone (PTH) like peptide as breast and lung tumors [32]. First, the proliferation of 786-0 cells were determined after short time exposition (24 h) and a narrow concentration range (25, 50, 100 and 200 µM) of compound 8 in order to stablish better experimental condition for flow cytometric evaluations (phosphatidylserine exposition and cell cycle). This way, after 24h, the necessary concentration to promote total growth inhibition and 50% of cell death were 28 and 91.8 µM, respectively, thus an intermediary concentration (50 µM) was selected for further experiments.

One feature of some cell death subroutines, like apoptosis, parthantos and netosis [33], is the early exposure of phosphatidylserine (PS) on external cell membrane surface. This PS externalization signalizes for recognition and engulfment of dying cells, as viable cell membranes exhibits substantial phospholipid asymmetry, with most of the PS residing on the inner leaflet of the plasma membrane [34]. For assessment of PS exposure, we used the double staining with Annexin V and 7-amino-actinomycin D (7-AAD). Annexin V binds to PS translocated to the outer face of the cell membrane during the initial process of cell death while 7-AAD binds to the DNA only after loss of cell membrane integrity. This way, cells stained only with annexin-V-PE could be considered as in early stage of cell death subroutine.

After 24h of treatment, compound **7** (50 µM) increased 786-0 cell population with PS externalization (21.9 ± 2.4 %) and with PS externalization and loss of cell membrane integrity (35.7 ± 2.4%) in comparison to vehicle-treated cells (3.8 ± 0.5% and 3.6 ± 0.8%, respectively; $p<0.001$) (Figure 2). These results are in accordance with the antiproliferative activity assay, where after 24 hours of treatment, lethal concentration for 50% of the cells was 91.8 µM, thus cell death rate of around 35% of the cells was expected after a treatment with 50 µM of the compound. Moreover, the PS externalization induced by compound **7** (50 µM) seemed to be a dynamic, time-dependent process such as 18 hours of exposition resulted 15.5 ± 3.5% of 786-0 cells with PS externalization without damage to cell membrane in comparison to 7.2 ± 1.4% of vehicle-treated 786-0 cells with the same markup ($p<0.05$) (Figure 2).

PLEASE, INSERT FIGURE 2 HERE

For flow cytometer cell cycle evaluation, 786-0 cells were treated for 24 hours with a lower concentration of **7** (25 µM) in order to minimize a sub-G1 phase arrest. Colchicine (1.25 nM) was employed a positive control, as it promotes a cell cycle arrest on G2/M phase. The cell cycle distribution of treated 780-6 cells is presented in Table 2.

As expected, colchicine (1.25 nM) increased the population of 786-0 cells in G2/M phase (57.5 ± 3.3%) followed by a decreasing on G1 phase cell population (21.5 ± 2.2%) in comparison to vehicle-treated cells (Table 2). At these experimental conditions, no interference on cell cycle profile was observed for compound **7** at 25 µM (Table 2).

PLEASE, INSERT TABLE 2 HERE

Several studies have been conducted in order to understand the mechanism by which thiosemicarbazones act on tumor cells [35,36]. For example, a copper complex of thiosemicarbazone was evaluated for antitumor activity and it was able to induce PS exposition without loss of cell membrane integrity in a concentration-dependent way in the HepG2 (human hepatocellular carcinoma) cell line [37]. Another study showed that anthraquinone-thiosemicarbazone derivatives (AQ-thiosemicarbazone) were able to induce apoptosis in HeLa cell line (human cervix adenocarcinoma) in a caspase-dependent pathway, mainly through the mitochondrial pathway activation of caspase 8 [38]. These data are consistent with our findings, as compound **7** also led to PS exposition without loss of cell membrane integrity in a time-dependent way.

The complex network of cell death subroutines and cell cycle are closely linked, as many of cancer cells treatment with anticancer agents usually result in cell cycle arrest, which subsequently leads the cells to initiate a cell death subroutine [39]. The cell cycle analysis by flow cytometry enables to quantify the amount of cells in the different cell cycle phases and thereby infer a possible interference of the compounds evaluated on DNA replication [40]. Although compound **7** promotes cell death in 786-0 as evidenced by PS exposition, no interference on cell cycle was observed in our study.

There are several studies showing molecules that are also cytotoxic for non-tumor cells, by inducing cell death through a mechanism other then inhibition of DNA duplication, synthesis, cell cycle check points or inhibition of mitosis, as would be observed in the cell cycle experiment by flow cytometry. An example is Goniothalamin,

a styryl lactone with well-known antitumor activity *in vitro* and *in vivo* that also does not promote cell cycle arrest [41].

2.2.2 *In vivo* studies

Owing to the promising *in vitro* antiproliferative activity of **7**, we evaluated its *in vivo* antitumor activity in the Ehrlich solid tumor model in mice. In order to choose the properly doses to perform the antitumor studies in mice, the acute toxicity of compound **7** was evaluated.

2.2.2.1 Oral Acute Toxicity

Animals orally treated with compound **7** (200 and 400 $mgKg^{-1}$) showed no severe clinical side effects with no mortality (Table 3). Moreover, the single treatment with 600 $mgKg^{-1}$ of compound **7** promoted adhesion of organs. Based on these results, the *in vivo* anticancer assay was conducted with 30, 100 and 300 $mgKg^{-1}$of compound **7**.

PLEASE, INSERT TABLE 3 HERE

2.2.2.2 *In vivo* antitumor activity

In this experiment, Ehrlich tumor cells were injected in the paw subcutaneous of the mice and treatments began 3 days after the cells inoculation. The tumor development was accompanied by measures of paw volume using a pletismometer at time 0 (basal volume) and each three days until the end of the experiment (Table 4) [43], Treatment with compound **7**, at 30 $mgKg^{-1}$, was effective since the 6^{th} day of experiment, when inhibited tumor development in 30% in comparison to negative control group. At the same day, positive controls doxorubicin (3 $mgKg^{-1}$) and piroxicam (40 $mgKg^{-1}$) showed an inhibition of 35.8 and 34% respectively (Table 4). At the 9^{th} day, all treatments presented smaller tumor volumes than negative control group being compound **7** (30 $mgKg^{-1}$) and piroxicam (40 $mgKg^{-1}$) the most potent treatments. From the 12^{th} day until the 15^{th} day (end of experiment), all treatments presented statistical differences with negative control, being treatments with 30 $mgKg^{-1}$ of compound **7** the

most effective, inhibiting in 71.1%, in comparison to negative control group, in the 12th day, the tumor development (Table 4). However, the compound **7** at doses of 100 and 300 mg/Kg showed reductions of paw volume 39.6 and 49%, respectively, showing good activity of the compound in the model adopted. The measurement of variation in paw volume on Ehrlich tumor is a good indication of antitumor activity being well correlated with the antiinflammatory effect of compound **7**.

PLEASE, INSERT TABLE 4 HERE

The Ehrlich tumor is a murine breast adenocarcinoma, which presents an aggressive fast-growing behavior [44]. Ehrlich tumor cells generate an inflammatory response that leads to increase in vascular permeability, edema formation and cell migration [45]. In fact, the paw volume increased in the range of 25 to 40% (in comparison basal volume) in all experimental groups. Studies show that in the Ehrlich tumor occurs upregulation of cyclooxygenase-2 (COX-2) and activation of nuclear factor-kappa B (NF-κB), a transcription factor that regulates the expression of many pro-inflammatory and pro-proliferative genes [46]. Thus, the use of non-steroidal anti-inflammatory drugs (NSAID) as adjuvants on cancer therapy is a good approach, as they target COX-2 and downregulate NF-κB pathway, leading to inhibition of tumor proliferation [47]. Our results obtained for Piroxicam (NSAID) corroborate with these facts as this NSAID was inhibited effectively the Ehrlich tumor development (Table 4).

It is well-known that inflammation promotes all steps of carcinogenesis [48]. The inflammatory process establishes a microenvironment which is favorable to tumor growth and maintenance [49,50]. In fact, epidemiological data confirm the close relationship between cancer and inflammation, up to 20% of all cancer types are preceded by chronic inflammation [51].

Molecules presenting the thiophene moiety have antiinflammatory and antitumoral properties. 3-[4-(methylsulfonyl)phenyl]thiophene-2-carbaldehyde *O*-arylmethyl oximes derivatives are able to inhibit COX-2 activity [52] and as this moiety is present on compound **7**, it could be involved on the antiproliferative activity of this molecule, suggesting also an anti-inflammatory activity.

In our study, treatments with compound **7** were conducted by oral route and cell inoculation and tumor development were in the paw subcutaneous. The treatments conducted every two days (7 treatments in total) did not promote clinical side effects.

All these results highlight compound **7** as a promising molecule for further studies for cancer chemotherapy, as the toxicity promoted by most of the drugs used on chemotherapy is still a challenge to overcome.

3. Conclusions

The novel thiophene-2-thiosemicarbazone derivatives have been prepared and shown cytostatic activity against several cancer cell lines. It can be suggested that the inclusion of aromatic rings in the structure of thiosemicarbazones improves the antiproliferative activity of this class of compounds and that the presence of halogens directly connected to this core also has a large contribution to the increase in this activity.

Compound **7** promotes PS externalization as a cell death signaling mechanism on the human renal adenocarcinoma cell line 786-0 without changes on cell cycle profile. Moreover, compound **7** possesses low acute toxicity, being able to inhibit Ehrlich solid tumor development in low doses (30 $mgKg^{-1}$) through orally administration without evidence of toxic effects. Our findings highlight thiophene-2-thiosemicarbazones as a promising class of compounds for further studies concerning new anticancer therapies.

4. Experimental Section

4.1 Chemistry

All reagents used in this study are commercially available (Sigma-Aldrich, Acros Organics, Vetec). Progress of the reactions was followed by thin-layer chromatography (TLC) analysis (Merck, silica gel 60 F_{254} in aluminium foil). Melting points were determined on a Quimis 340 (Quimis, Brazil) capillary melting point apparatus and were uncorrected. IR spectra were recorded with a Bruker model IFS66 FT-IR spectrophotometer (Bruker, Germany) using KBr pellets. NMR spectra were measured on either a Varian UnityPlus 400 MHz (400 MHz for ^{1}H and 100 MHz for ^{13}C) or a Bruker AMX-300 MHz (300 MHz for ^{1}H and 75.5 MHz for ^{13}C) instruments. DMSO-d_6 was purchased from Sigma-Aldrich. Chemical shifts are reported in ppm and multiplicities are given as s (singlet), d (doublet), t (triplet), m (multiplet), dd (double

doublet), and coupling constants (*J*) in hertz. Mass spectrometry experiments were performed on a MALDI-TOF Autoflex III (Bruker Daltonics, Billerica, MA, USA).

4.1.1 General procedure for the synthesis of thiosemicarbazides (3)

80% hydrazine hydrate (**1**) (311 µL, 10 mmol) was added slowly to a solution of different isothiocyanates (**2**) (5 mmol) in dichloromethane (10 mL). The reaction was processed under magnetic stirring for 2 h at room temperature. The precipitate was filtered off, washed with dichloromethane and dried in desiccator under vacuum. Additional amount of desired compound could be recovered from the filtrate after cooling. After drying, the product was recrystallized from *n*-hexane.

4.1.2 General procedure for the synthesis of thiosemicarbazones (5-14)

Substituted thiosemicarbazide (**3**) (1 mmol) and few drops of acetic acid were added to a solution of 2-thiophene-carboxaldehyde (**4**) (93 µL, 1 mmol) in ethanol (10 mL). The reaction was processed under magnetic stirring for 2 h at room temperature. The precipitate was filtered off, washed with ethanol then dried in desiccator under vacuum. Additional amount of desired compound could be recovered from the filtrate after cooling. After drying, the product was recrystallized from toluene.

4.1.2.1 (*E*)-*N*-(*p*-tolyl)-2-(thiophen-2-ylmethylene)-thiosemicarbazone **(9)**

Compound **9** was obtained as white powder (119 mg, 40%): R_f= 0.45 (*n*-Hex/AcOEt 8:2); mp: 193-194°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ= 2.50 (s, 3H), 7.13 (t, *J*= 4.8 Hz, 1H), 7.15 (d, *J*= 8 Hz, 2H), 7.42 (d, *J*= 8.4 Hz, 2H), 7.52 (d, *J*= 3.6 Hz, 1H), 7.69 (d, *J*= 4.8 Hz, 1H), 8.33 (s, 1H), 9.70 (s, 1H), 11.76 ppm (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ= 20.5 (CH_3), 125.3 (CH Ar), 127.9 (CH Ar), 128.5 (CH Ar), 129.1 (CH Ar), 130.8 (CH Ar), 134.3 (Cq Ar), 136.3 (Cq Ar), 137.8 (C=N), 138.3 (Cq Ar), 175.5 (C=S) ppm; IR (KBr): $\tilde{\nu}$= 1557 (C=S), 1593 (C=N), 3297 (N-H) cm^{-1}; HRMS *m/z* $[M+H]^+$ calcd for $C_{13}H_{13}N_3S_2$: 276.058; found: 276.089.

4.1.2.2 (*E*)-*N*-(4-ethylphenyl)-2-(thiophen-2-ylmethylene)-thiosemicarbazone **(10)**

Compound **10** was obtained as white powder (162 mg, 55%): R_f= 0.46 (*n*-Hex/AcOEt 7:3); mp: 186-188°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ= 1.19 (t, *J*= 7.6 Hz, 3H), 2.60

(q, *J*= 7.6 Hz, 2H), 7.13 (t, *J*= 4.8 Hz, 1H), 7.18 (d, *J*= 8.4 Hz, 2H), 7.45 (d, *J*= 8 Hz, 2H), 7.53 (d, *J*= 4 Hz, 1H), 7.68 (d, *J*= 4.8 Hz, 1H), 8.33 (s, 1H), 9.70 (s, 1H), 11.76 ppm (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ= 15.6 (CH_3), 27.6 (CH_2), 125.4 (CH Ar), 127.3 (CH Ar), 127.9 (CH Ar), 129.1 (CH Ar), 130.8 (CH Ar), 136.5 (Cq Ar), 137.8 (C=N), 138.3 (Cq Ar), 140.7 (Cq Ar), 175.5 (C=S) ppm; IR (KBr): $\tilde{\nu}$= 1521 (C=S), 1547 (C=N), 3296 (N-H) cm^{-1}; HRMS *m/z* $[M+H]^+$ calcd for $C_{14}H_{15}N_3S_2$: 290.074; found: 290.098.

4.1.2.3 (*E*)-*N*-phenethyl-2-(thiophen-2-ylmethylene)-thiosemicarbazone **(11)**

Compound **11** was obtained as white powder (240 mg, 81%): R_f= 0.56 (*n*-Hex/AcOEt 7:3); mp: 169-170°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ= 2.90 (t, *J*= 8 Hz, 2H), 3.75 (q, *J*= 6 Hz, 2H), 7.11 (dd, J_1= 3.6 Hz, J_2= 4 Hz, 1H), 7.20-7.34 (m, 5H), 7.43 (d, *J*= 3.6 Hz, 1H), 7.66 (d, *J*= 5.2 Hz, 1H), 8.12 (t, *J*= 6 Hz, 1H), 8.24 (s, 1H), 11.50 ppm (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ= 34.7 (CH_2), 45.0 (CH_2), 126.2, 127.9 (CH Ar), 128.4 (CH Ar), 128.5 (CH Ar), 128.7 (CH Ar), 130.5 (CH Ar), 137.3 (C=N), 138.6 (Cq Ar), 139.2 (Cq Ar), 176.5 (C=S) ppm; IR (KBr): $\tilde{\nu}$= 1523 (C=S), 1546 (C=N), 3353 (N-H) cm^{-1}; HRMS *m/z* $[M+H]^+$ calcd for $C_{14}H_{15}N_3S_2$: 290.074; found: 290.083.

4.1.2.4 (*E*)-*N*-(naphthalen-1-yl)-2-(thiophen-2-ylmethylene)-thiosemicarbazone **(12)**

Compound **12** was obtained as white powder (411 mg, 80%): R_f= 0.59 (*n*-Hex/AcOEt 8:2); mp: 212-214°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ= 7.14 (dd, J_1= 5.4 Hz, J_2= 5.1 Hz, 1H), 7.51-7.55 (m, 4H), 7.57 (d, *J*= 5.4 Hz, 1H), 7.79 (d, *J*= 5.1 Hz, 1H), 7.83-7.99 (m, 3H), 8.40 (s, 1H), 10.10 (s, 1H), 11.92 ppm (s,1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ= 123.5 (CH Ar), 125.8 (CH Ar), 126.4 (CH Ar), 127.2 (CH Ar), 128.4, 129.6, 131.2, 132.5, 135.9, 138.4 (C=N), 151.5 (Cq Ar), 167.6 (Cq Ar), 177.7 (C=S) ppm; IR (KBr): $\tilde{\nu}$= 1519 (C=S), 1543 (C=N), 3328 (N-H) cm^{-1}; HRMS *m/z* $[M+H]^+$ calcd for $C_{16}H_{13}N_3S_2$: 312.058; found: 312.071.

4.1.2.5 (*E*)-*N*-(pyridin-3-yl)-2-(thiophen-2-ylmethylene)-thiosemicarbazone **(13)**

Compound **13** was obtained as white powder (233 mg, 79%): R_f= 0.40 (*n*-Hex/AcOEt 7:3); mp: 186-187°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ= 7.14 (t, *J*= 3.6 Hz, 1H), 7.39 (m, 1H), 7.54 (d, *J*= 3.6 Hz, 1H), 7.71 (d, *J*= 3.9 Hz, 1H), 7.97 (m, 1H), 8.36 (s, 1H), 8.38 (m, 1H), 8.66 (s, 1H), 9.97 (s, 1H), 11.97 ppm (s,1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ= 122.8 (CH Ar), 127.9 (CH Ar), 129.4 (CH Ar), 131.2 (CH Ar), 133.3 (CH Ar),

135.8 (CH Ar), 138.1 (CH Ar), 138.6 (C=N), 145.9 (Cq Ar), 147.1 Cq Ar), 176.1 (C=S) ppm; IR (KBr): $\tilde{\nu}$= 1519 (C=S), 1543 (C=N), 3295 (N-H) cm^{-1}; HRMS *m/z* $[M+H]^+$ calcd for $C_{11}H_{10}N_4S_2$: 263.038; found: 263.061.

4.1.2.6 (*E*)-*N*-allyl-2-(thiophen-2-ylmethylene)-thiosemicarbazone **(14)**

Compound **14** was obtained as white powder (223 mg, 65%): R_f= 0.50 (*n*-Hex/AcOEt 8:2); mp: 159-160°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ= 4.20 (t, *J*= 5.6 Hz, 2H), 5.09 (d, *J*= 10.4 Hz, 1H), 5.14 (d, *J*= 17.2 Hz, 1H), 5.95-5.86 (m, 1H), 7.11 (m, 1H), 7.44 (d, *J*= 3.6 Hz, 1H), 7.64 (d, *J*= 5.2 Hz, 1H), 8.23 (m, 1H), 8.26 (s, 1H), 11.51 ppm (s,1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ= 45.6 (CH_2), 115.5 (CH_2), 127.8 (CH allyl), 128.7 (CH Ar), 130.5 (CH Ar), 134.9 (CH Ar), 137.4 (C=N), 138.5 (Cq Ar), 176.8 (C=S) ppm; IR (KBr): $\tilde{\nu}$= 1533 (C=S), 1553 (C=N), 3358 (N-H) cm^{-1}; HRMS *m/z* $[M+H]^+$ calcd for $C_9H_{11}N_3S_2$: 226.043; found: 226.070.

4.2 Biological activities

4.2.1 *In vitro* assays

Considering that different cell lines display different sensitivities toward the same cytotoxic compound, the antiproliferative activity of all the 2-thiophene-thiosemicarbazone were evaluated *in vitro* against nine different human cancer cell lines [U251 (glioma), UACC-62 (melanoma), MCF-7 (breast), NCI-ADR/RES (multidrug resistant ovary carcinoma), 786-0 (renal), NCI-H460 (non-small cell lung cancer), PC-3 (prostate), HT-29 (colon), and K-562 (leukemia)]. The antiproliferative activity of each compound was also evaluated *in vitro* against spontaneously transformed keratinocytes from histologically normal skin (HaCat cells). Doxorubicin was employed as the positive control [53].

Cells in 96-well plates (100 µL cells/well) were exposed to 2-thiophene-thiosemicarbazonics derivates in concentrations 0.25, 2.5, 25 and 250 µg/mL in DMSO/RPMI at 37º C, 5% of CO_2 in air for 48 hours. Doxorubicin was used as standard (0.025, 0.25, 2.5 and 25 µg/mL). Final DMSO concentration did not affect cell viability (0.1%). Before (T_0 plate) and after (T_1 plates) sample addition, cells were fixed with 50% trichloroacetic acid and cell proliferation determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay. The

GI_{50} (concentration expressed in µM inhibiting 50% of cell growth or cytostatic effect) were determined through non-linear regression analysis using the concentration-response curve for each cell line in software ORIGIN 8.0® (OriginLab Corporation) [54-56]. To choose concentrations for flow cytometry analyses, 786-0 cells were treated for 24 hours with 25, 50, 100 and 200 µM of compound **7**. Cell growth was determined through sulforhodamine B assay and concentration that promote total growth inhibition (TGI) and lethal concentration 50 - LC_{50} (concentration that promote 50% of cell death) were calculated in software ORIGIN 8.0® (OriginLab Corporation).

4.2.2 Measurement of phosphatidylserine externalization

Phosphatidylserine externalization was analysed by flow cytometry using Guava® Nexin Assay Kit (Guava Technologies, Hayward, CA) in accordance with manufacturer's instructions. 786-0 cells were inoculated in 12 wells plate (3 x 10^4 cells/well) and incubated for 24 hours at 37 ºC, 5% of CO_2 in air. Then, cells were treated with compound **7** (50 µM) in DMSO/RPMI for 18 and 24 hours, harvested and resuspended at a density of 1 x 10^5 cells in 100 µL of supplemented medium. One hundred microliter of binding buffer containing annexin-V and 7-AAD were added on the cells and incubated in the dark for 20 minutes at room temperature. After, cells were analysed by flow cytometer (Guava Easycyte Mini - Guava Technologies, Hayward, CA). We collected 5.000 events, as suggested by the protocols of Guava Easycyte kits. To make it clear we added the following statement in the methods and figures legends: "A total of 5.000 events were collected". We performed three experiments in triplicate, as showed in the legends of figures and methodology. We performed statistical analyses for all experiments and all the significance was showed as asterisks, according with p value.

4.2.3 Cell cycle analyses

Cells cycle analyses were performed with the Guava®Cell Cycle reagent (Guava Technologies, Hayward, CA) in accordance with manufacturer's instructions. 780-0 cells were inoculated in 12 wells plate (3 x 10^4 cells/well) and incubated for 24 hours at 37 ºC, 5% of CO_2 in air. Afterwards, cells were deprived of serum for 24 hours for cell cycle synchronization and then treated with compound **7** (25 µM) and colchicine

(Sigma-Aldrich, 1.25 nM) in DMSO/RPMI, for 24 hours. After treatment, cells were harvested and resuspended at a density of 1 x 10^5 cells in 100 µL of PBS. The binding buffer containing propidium iodide (PI) was added to the cells (100 µL) and suspension was incubated in the dark for 20 minutes at room temperature. After, cells were analysed by flow cytometer (Guava Easycyte Mini - Guava Technologies, Hayward, CA). We collected 5.000 events, as suggested by the protocols of Guava Easycyte kits. To make it clear we added the following statement in the methods and figures legends: "A total of 5.000 events were collected". We performed three experiments in triplicate, as showed in the legends of figures and methodology. We performed statistical analyses for all experiments and all the significance was showed as asterisks, according with p value.

4.2.4 *In vivo* assays

4.2.4.1 Animals

Experiments were conducted with Balb/C mice (20 - 35 g) obtained from the Multidisciplinary Center for Biological Investigation on Laboratory Animals Sciences (CEMIB-UNICAMP). Animals were maintained at the Animal facilities of Pharmacology and Toxicology Division, CPQBA, University of Campinas (Campinas, Brazil), under controlled conditions (22 ± 3ºC for 12 h light/dark cycle, free access to food and water). All mice were fasted for 16 hours prior to each experiment because treatments were administered orally. Euthanasia was performed by cervical dislocation. Animal care, research and animal sacrifice protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Institute of Biology/ UNICAMP - Ethical Committee for Animal Research (number 3329-1).

4.2.4.2 Acute Oral Toxicity – Fixed Dose Procedure

A fixed dose procedure was adopted to evaluate the acute oral toxicity of compound **7** according to Organization for Economic Cooperation and Development (OECD) Guidelines for the Testing of Chemicals [42]. Groups of 5 mice were dosed in a stepwise procedure using the fixed doses of 600, 400 and 200 $mgKg^{-1}$. Clinical signs

and conditions associated with pain, suffering and impending death are described in detail in a separate OECD Guidance Document [57].

4.2.4.3 Ehrlich solid tumor in mice

The most active compound **7** was evaluated for its *in vivo* antiproliferative activity in the Ehrlich solid carcinoma model in mice. The procedures were developed according to Vendramini-Costa et al. (2010) [43] with few modifications on data analysis.

Cell maintenance and preparations: The Ehrlich ascitic tumor (EAT), derived from a spontaneous murine mammary adenocarcinoma, was maintained in the ascitic form by weekly intraperitoneal transplantations of 5 x 10^5 tumor cells/animal, in order to prepare cells for the following test. The ascitic fluid was removed by opening the belly and carefully collecting all the fluid using a sterile 3 mL syringe. Ascitic tumor cell counts were performed in a Neubauer hemocitometer, and the total number was determined by the Trypan blue dye exclusion method, with tumor cell viability always higher than 90%. The cells were then diluted in 0.9% phosphate buffer saline (PBS) into final inoculation density (2.5 x 10^6 cells/30µL).

Ehrlich solid tumor: After cells inoculation, basal volumes of the right hind paw were measured in a plethysmometer apparatus (7140 Ugo Basile). For the solid form implantation, 2.5 x 10^6 viable tumor cells in a volume of 30 µL were injected in subplantar site of the right hind paw of *Balb-C* mice (n= 8 per group) [58]. The compound **7** was emulsified with 1% Tween 80 (SIGMA®) and dissolved in phosphate buffered saline (PBS), pH 7,0. The test compound was administered on the 3th day after tumor inoculation, at doses of 30, 100 and 300 mgKg^{-1} orally. Doxorubicin (3 mgKg^{-1}, i.p.) and piroxicam (40 mgKg^{-1}, orally) were used as the standard drugs. After tumor cell inoculation, the paw volume was measured every three days using the plethysmometer apparatus till the 15th day, when the animals were sacrificed. The tumor growth was measured considering the following formula:

$$\textbf{Variation of the tumor growth (\%)} = \frac{\textbf{(Volume measured - Basal volume) x 100}}{\textbf{Basal volume}}$$

4.3 Statistical Analysis

The experimental results were expressed as the mean ± standard error (SE) and by analysis of variance (ANOVA), one-way and two-way, followed by Tukey or Bonferroni tests. P values lower than 0.05 ($p < 0.05$) were considered as indicative of significance and represented by: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. The calculations were performed using the statistical software GraphPad Prism version 5.0, San Diego California, USA.

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Conflict of Interest

The authors have declared that no competing interests exist.

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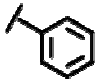
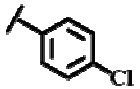
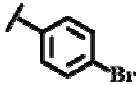
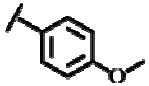
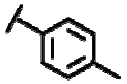
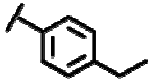
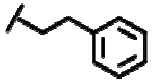
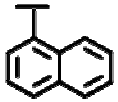
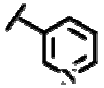
Table 1. *In vitro* antiproliferative activity (GI_{50}[a] in μM) of *N*-substituted-2-(thiophene-2-ylmethylene)-thiosemicarbazone derivatives **5-14**.

Compound		U251	UACC-62	MCF-7	NCI-ADR/RES	786-0	NCI-H460	PC-3	HT-29	K-562	Mean GI_{50}	HaCat
5		74.7	>100	94.6	>100	50.4	>100	>100	>100	16.7	>81.8	>100
6		8.3	15.8	>100	2.8	7.4	>100	>100	>100	2.0	>48.4	14.7
7		4.2	71.1	<0.74	1.7	4.5	4.7	79.4	17.7	>100	>31.5	0.8
8		>100	>100	>100	>100	>100	>100	>100	>100	1.7	>89.0	>100
9		12.6	16.8	38.6	4.8	19.5	80.6	29.7	>100	<0.9	>33.7	17.6
10		>100	>100	>100	>100	82.6	>100	>100	>100	1.6	>87.1	17.6
11		>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
12		12.7	>100	>100	6.6	15.2	>100	>100	>100	1.8	>59.5	82.4
13		26	>100	>100	>100	5.6	45.9	>100	>100	16.4	>65.9	45.4
14		>100	>100	>100	>100	21.2	>100	>100	>100	6.0	>80.8	>100
Dox	–	<0,046	0,05	<0,046	0,21	<0,046	<0,046	0,13	0,30	<0,046	<0.102	<0,046

[a]Concentration that promotes 50% of growth inhibition after 48 hours of treatment. GI_{50} was determined from nonlinear regression analyses by using ORIGIN 8.0 software (OriginLab Corporation). Experiments were conducted in triplicate. Dox: doxorubicine, positive control.

Table 2**.** Cell cycle profile of 786-0 cells treated with vehicle (DMSO), colchicine (1.25 nM) and compound **7** (25 µM) for 24 hours.

Treatments	Sub-G1	G1	S	G2/M
Vehicle	3.0 ± 0.7	46.7 ± 0.8	14.6 ± 1.3	35.7 ± 0.9
Colchicine	8.9 ± 0.8	21.5 ± 2.2***	12.6 ± 1.2	57.5 ± 3.3***
Compound 7	5.4 ± 0.9	44.9 ± 1.8	13.9 ± 0.7	35.9 ± 0.6

Cell population, expressed in percentage, for each phase of cell cycle, after 24h-treatment with vehicle, colchicine or compound **7**. Results represented by mean ± standard error, in percentage of cells, from three different experiments. A total of 5000 events were collected per experiment. ANOVA followed by Tukey's Multiple Comparison Test. ***p<0.001, statistically different from vehicle.

Table 3. Effects of compound **7** on acute oral toxicity assay–fixed dose procedure.

Dose ($mgKg^{-1}$)	Survival rate and side effects
200	100% survival rate, no severe toxic effect*
400	100% survival rate, no severe toxic effect*
600	100% survival rate, adhesions of organs

* Clinical signs and conditions associated with pain, suffering, and impending death, are described in detail in a separate OECD Guidance Document [42].

Table 4. Effect of 2-thiophene-thiosemicarbazone (compound **7**) on Ehrlich solid tumor development in mice.

Day	Paw volume variation (%) as compared to day-0 (mean ± SD)					
	Control	**Doxorubicin 3 $mgKg^{-1}$**	**Piroxicam 40 $mgKg^{-1}$**	**Compound 7 30 $mgKg^{-1}$**	**Compound 7 100 $mgKg^{-1}$**	**Compound 7 300 $mgKg^{-1}$**
3	40.2 ± 7.0	43.7 ± 4.5	42.2 ± 3.6	26.8 ± 1.8	33.4 ± 2.4	39.4 ± 3.0
6	86.1 ± 5.7	55.6 ± 4.3**	57.2 ± 5.0**	60.6 ± 4.6**	78.1 ± 3.1	75.1 ± 5.4
9	95.5 ± 5.2	69.8 ± 4.9*	52.3 ± 3.6***	57.1 ± 5.2***	73.7 ± 4.2	72.8 ± 5.5*
12	125.5 ± 7.5	39.6 ± 3.6***	34.9 ± 3.1***	36.3 ± 6.6***	75.8 ± 4.3***	64.0 ± 7.9***
15	166.5 ± 6.0	97.9 ± 12.5***	47.3 ± 3.0***	82.0 ± 11.6***	118.6 ± 9.4***	106.0 ± 17.5***

Data were expressed as the mean ± SE of 6 animals per group. Results were considered significant when *p <0.05; **p <0.01 and ***p <0.001 (in comparison to negative control group), determined by analysis of variance (ANOVA) followed by Bonferroni posttests.

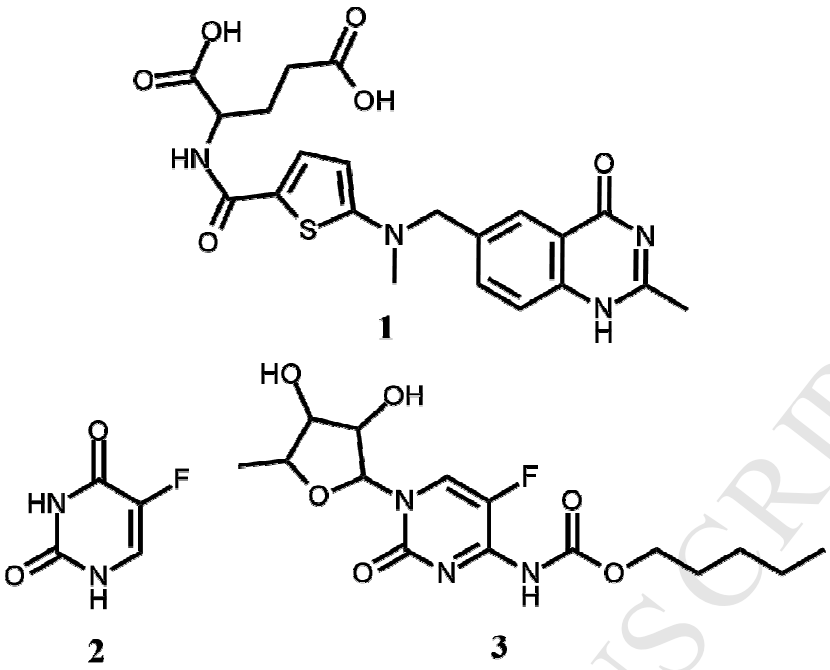


Figure 1. Structure of Raltitrexed (1); 5-FU (2) and Capecitabine (3)

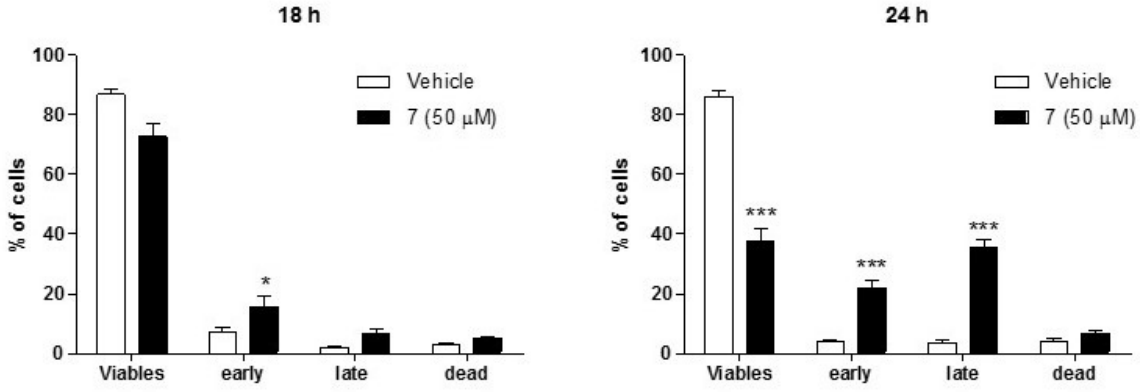
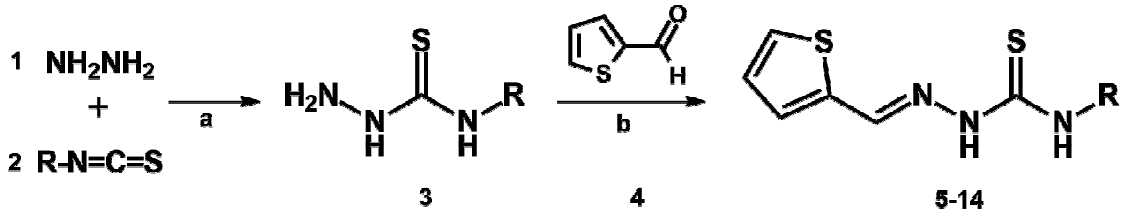


Figure 2. Percentage of 786-0 cells stained with annexin V-PE/7-AAD after 18 and 24h of treatment.

Viable (unstained cells - viable cells), early (annexin V-PE +/7-AAD - stain), dead (annexin V-PE -/7-AAD + stain) and late (annexin V-PE +/7-AAD + stain). Cells treated for 18 and 24 hours with vehicle (culture medium plus DMSO 0.25%) and compound 7 (50 µM). Results represented by mean ± standard error, in percentage of cells, from three different experiments. A total of 5000 events were collected per experiment. ANOVA followed by Tukey's Multiple Comparison Test. *$p<0.05$, ***$p<0.001$, * statistically different from vehicle.



Compound	R
5	phenyl
6	*p*-chlorophenyl
7	*p*-bromophenyl
8	*p*-methoxyphenyl
9	*p*-tolyl
10	*p*-ethylphenyl
11	2-phenylethyl
12	1-naphthyl
13	3-pyridyl
14	allyl

Scheme 1. Synthetic procedures for thiosemicarbazones (5-14).

Reagents and conditions: (a) hydrazine, substituted isothiocyanates, dichloromethane, r.t., 120 min; (b) substituted thiosemicarbazides, 2-thiophene-carboxaldehyde, ethanol, acetic acid (3 drops), r.t., 120 min.

Isomer *E*;

Cytotoxic *in vitro*, early apoptosis;

Promising antitumor activity *in vivo.*

Synthesis of thiophene-thiosemicarbazone derivatives and evaluation of their *in vitro* and *in vivo* antitumor activities

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[a]*Universidade Federal de Pernambuco (UFPE), Departamento de Antibióticos, 50670-901, Recife, PE (Brazil)*

[b]*Universidade Estadual de Campinas (Unicamp), Divisão de Farmacologia e Toxicologia - Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA) 13083-970, Campinas, SP (Brazil)*

[c]*Instituto Nacional de Tecnologia Nordeste/MCT, Centro de Tecnologias Estratégicas do Nordeste, 50740-540 - Recife, PE (Brazil)*

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[e]*Universidade Federal de Pernambuco (UFPE), Departamento de Química Fundamental, 50670-901, Recife, PE (Brazil)*

SUPPLEMENTARY INFORMATION

Figure S1. ^{1}H-NMR spectrum (DMSO) of compound 8

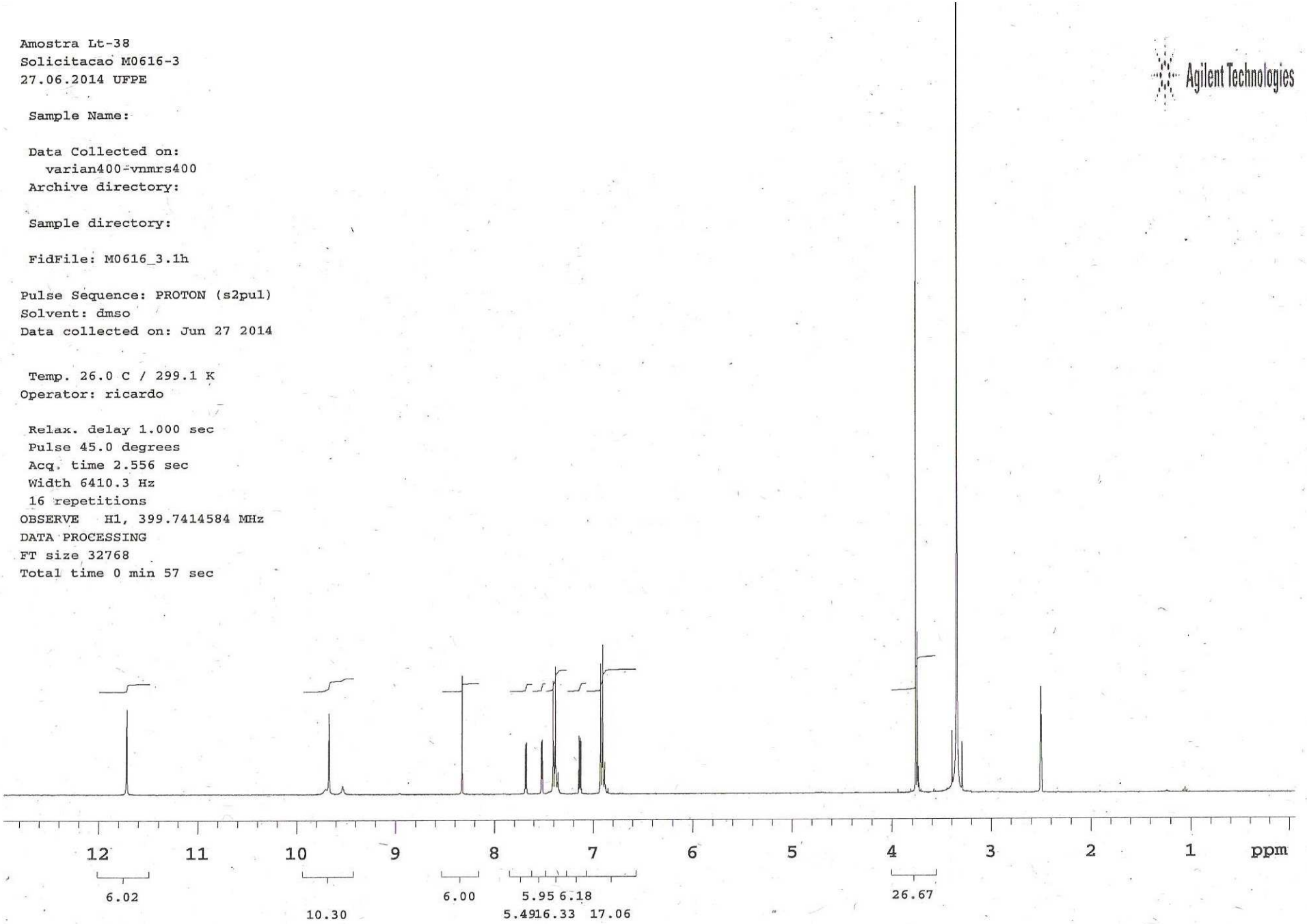
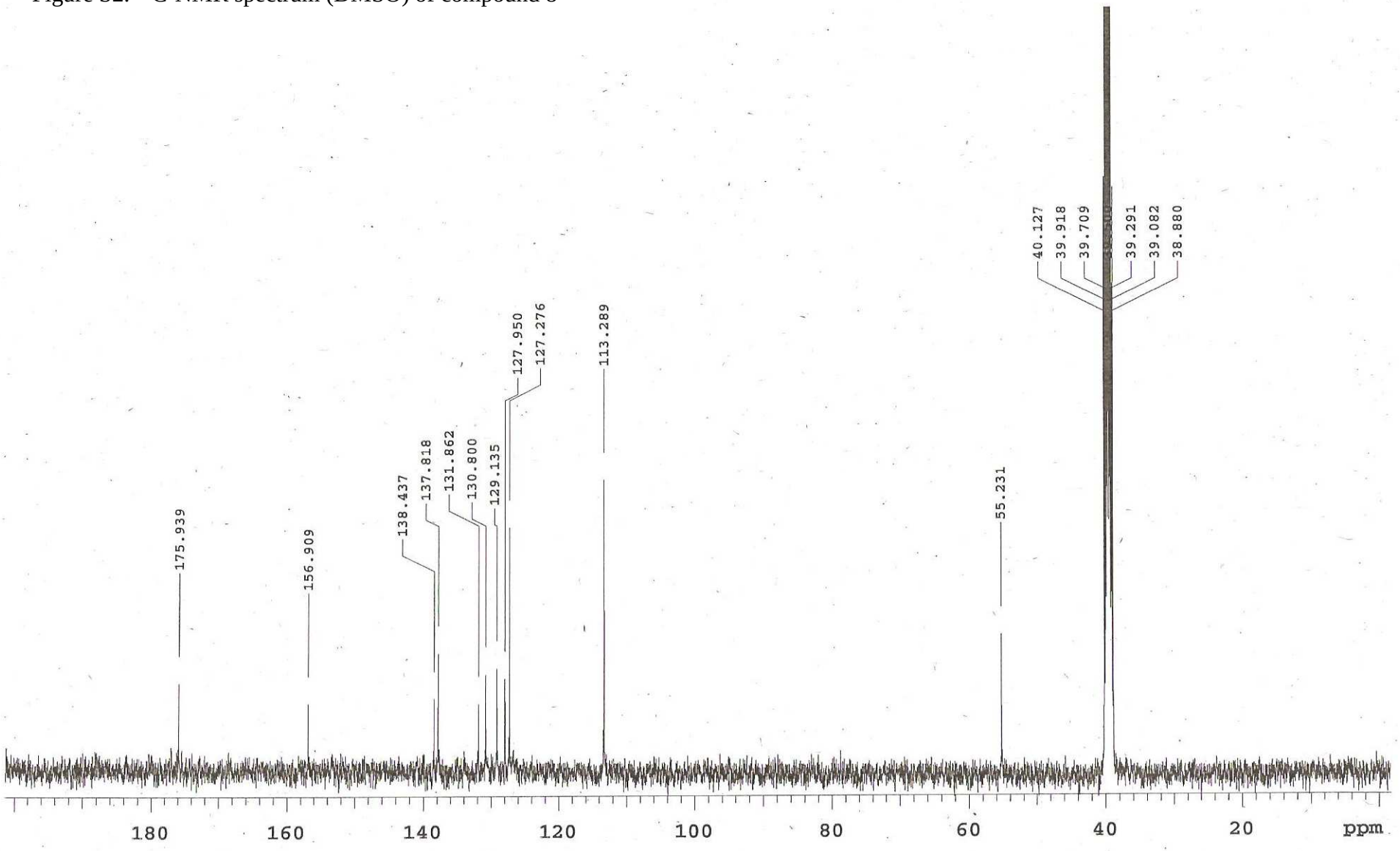


Figure S2. ^{13}C-NMR spectrum (DMSO) of compound 8



PULSE SEQUENCE Relax. delay 1.000 sec Pulse 45.0 degrees Acq. time 1.285 sec Width 25510.2 Hz 1024 repetitions	OBSERVE C13, 100.5151172 DECOUPLE H1, 399.7434654 Power 33 dB continuously on WALTZ-16 modulated	DATA PROCESSING Line broadening 2.0 Hz FT size 65536 Total time 38 minutes		Larissa Souza Amostra Lt-38 Solicitacao M0616-3 27.06.2014 UFPE Solvent: dmso Temp. 26.0 C / 299.1 K Operator: ricardo

Figure S3. ^{1}H-NMR spectrum (DMSO) of compound 9

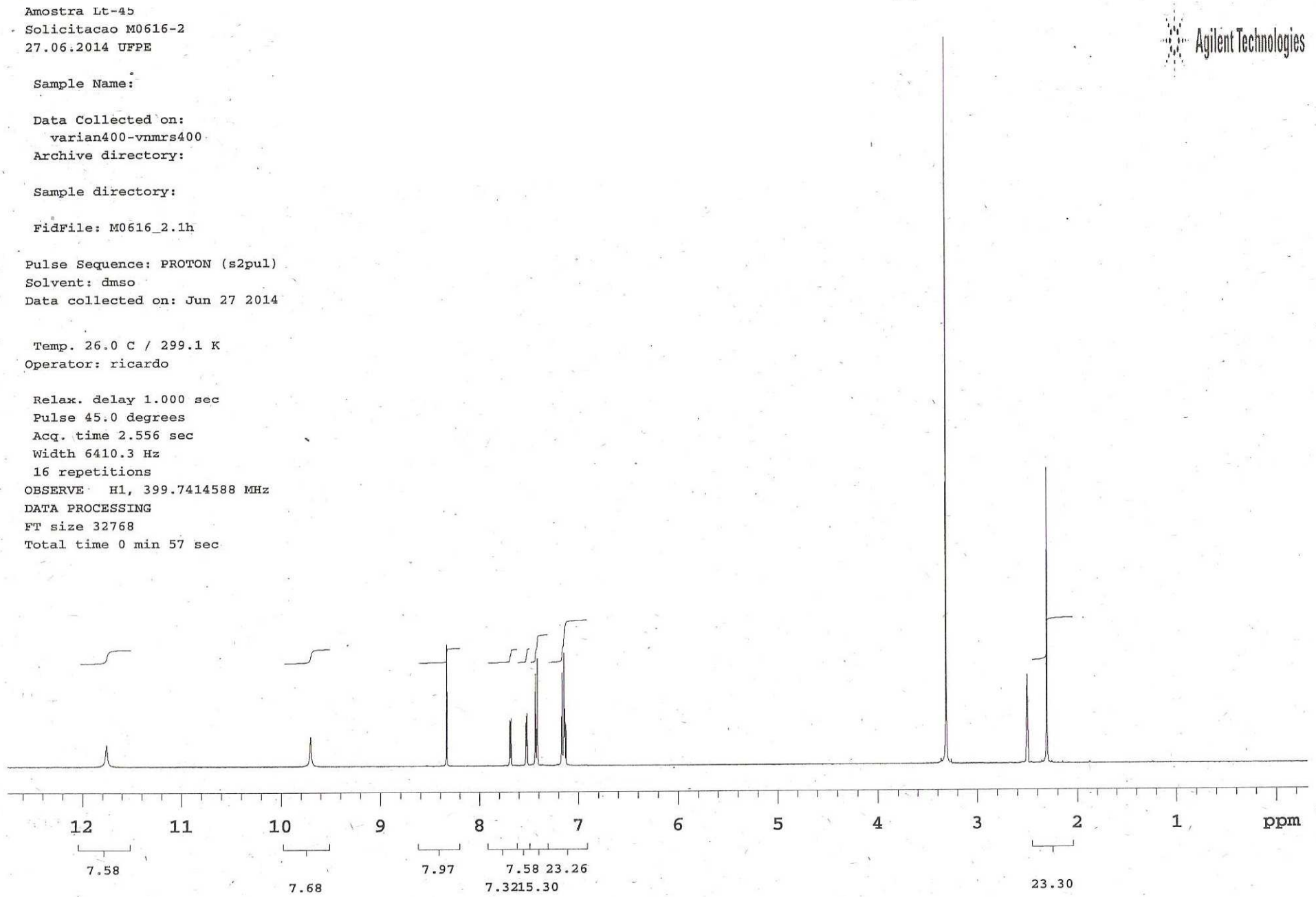
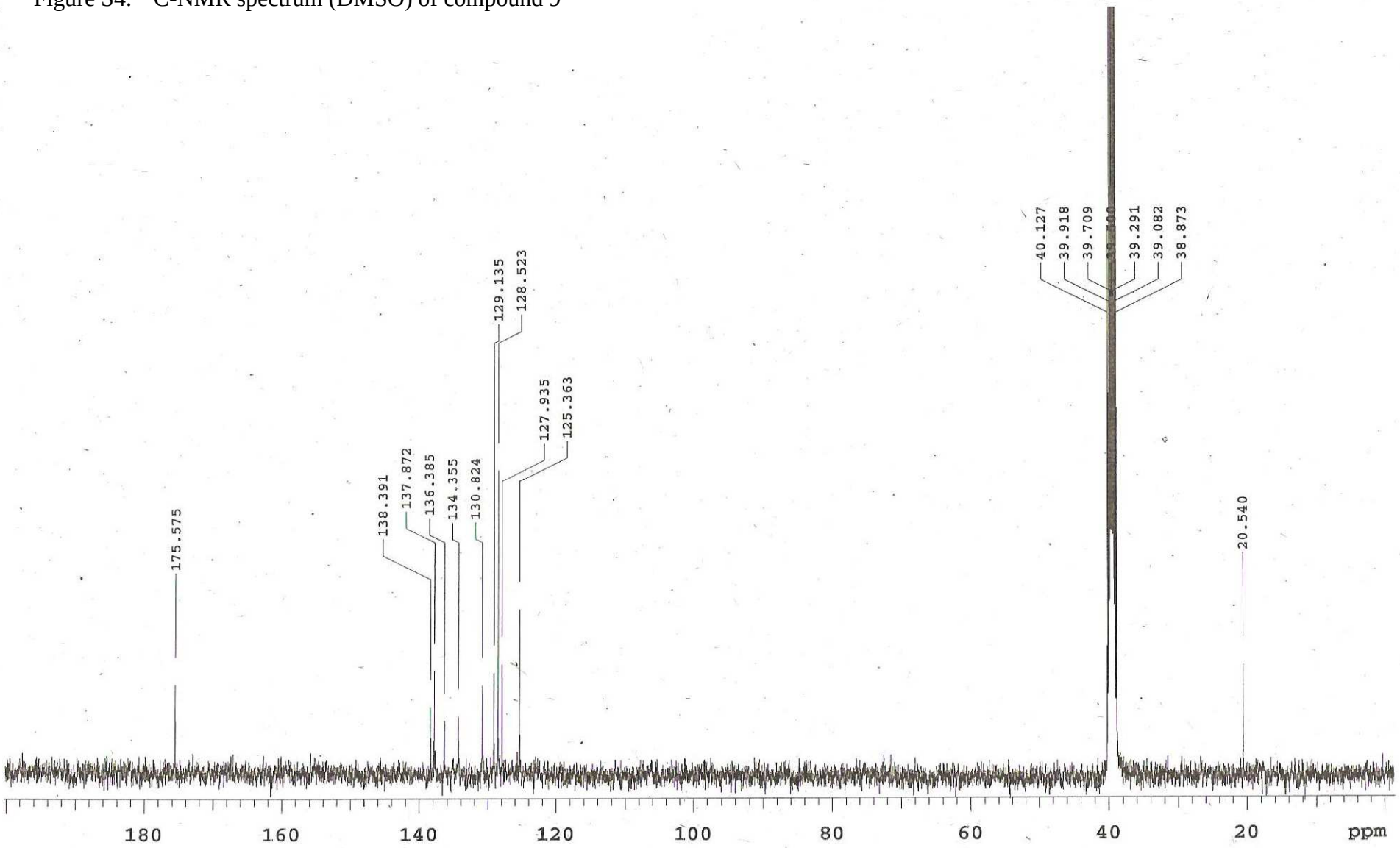


Figure S4. ^{13}C-NMR spectrum (DMSO) of compound 9



PULSE SEQUENCE Relax. delay 1.000 sec Pulse 45.0 degrees Acq. time 1.285 sec Width 25510.2 Hz 1024 repetitions	OBSERVE C13, 100.5151203 DECOUPLE H1, 399.7434654 Power 33 dB continuously on WALTZ-16 modulated	DATA PROCESSING Line broadening 2.0 Hz FT size 65536 Total time 38 minutes		Larissa Souza Amostra Lt-45 Solicitacao M0616-2 27.06.2014 UFPE Solvent: dmso Temp. 26.0 C / 299.1 K Operator: ricardo

Figure S5. ^{1}H-NMR spectrum (DMSO) of compound 10

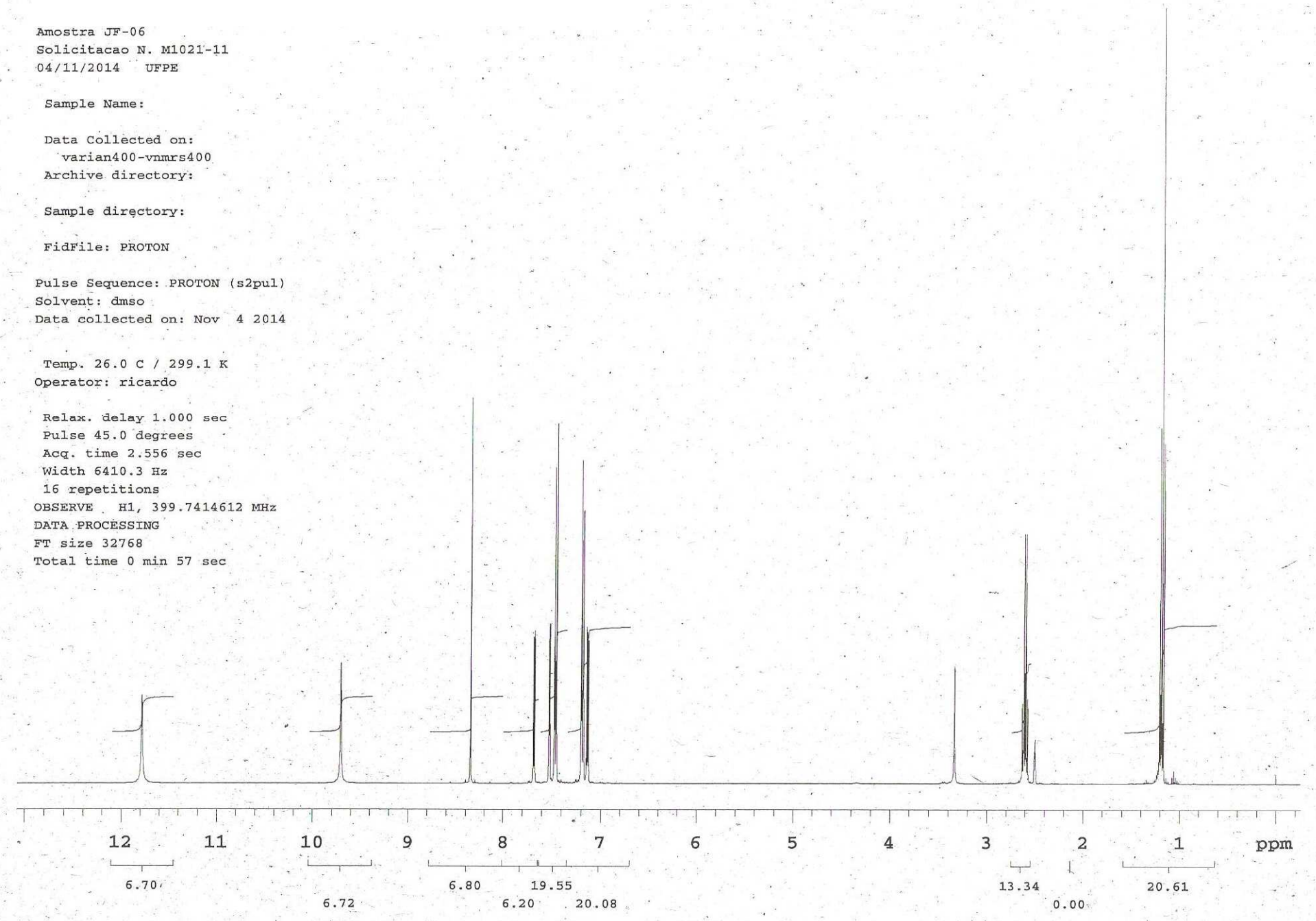
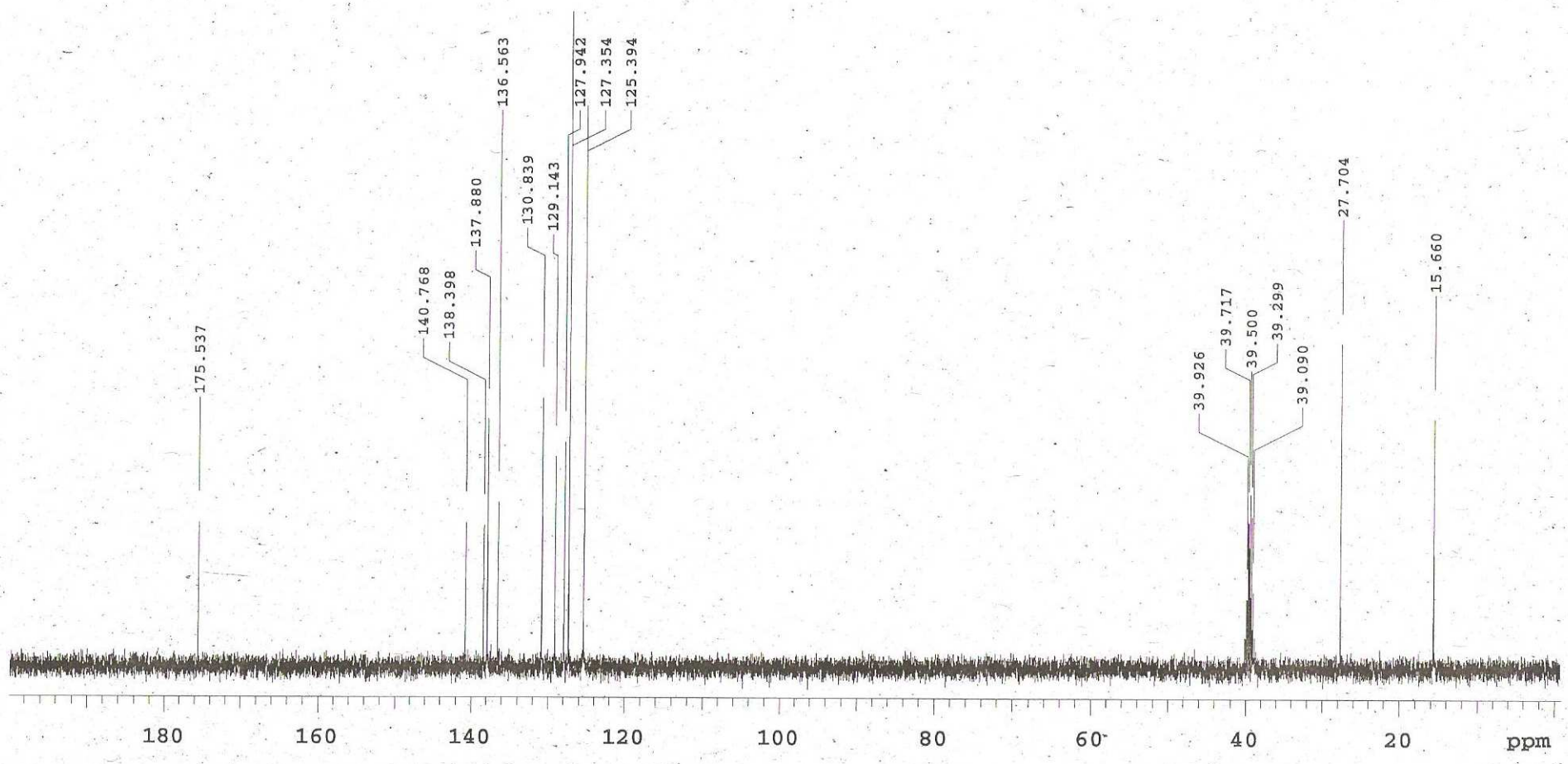


Figure S6. ^{13}C-NMR spectrum (DMSO) of compound 10



PULSE SEQUENCE: DEPT Relax. delay 1.000 sec Pulse 90.0 degrees Acq. time 1.285 sec Width 25510.2 Hz 32 repetitions	OBSERVE C13, 100.5151203 DECOUPLE H1, 399.7434654 Power 33 dB on during acquisition off during delay WALTZ-16 modulated	DATA PROCESSING Line broadening 0.5 Hz FT size 65536 Total time 10 minutes		Ana Daura Amostra JF-06 Solicitacao N. M1021-11 04/11/2014 UFPE Solvent: dmso Temp. 26.0 C / 299.1 K Operator: ricardo

Figure S7. ^{1}H-NMR spectrum (DMSO) of compound 11

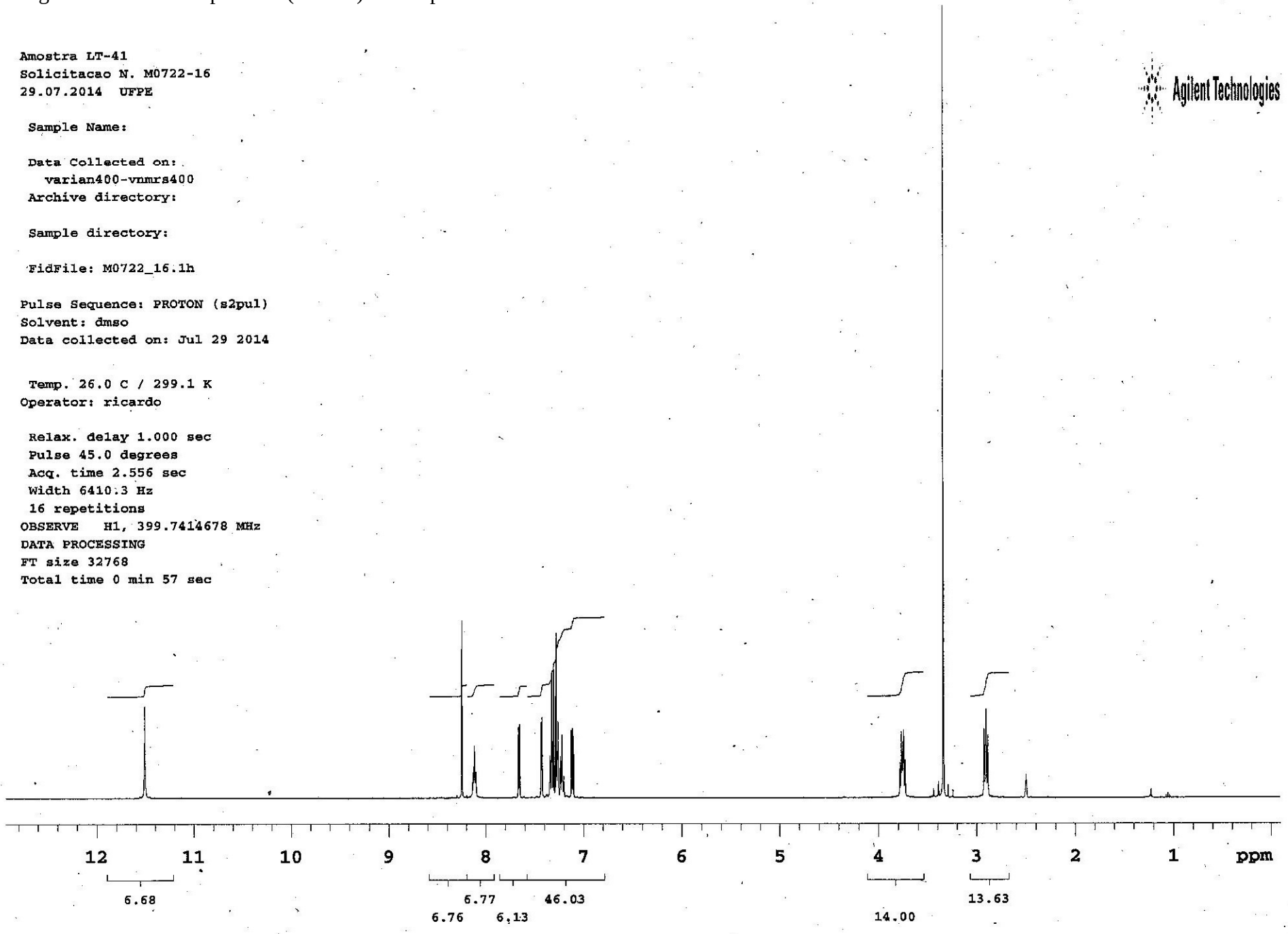
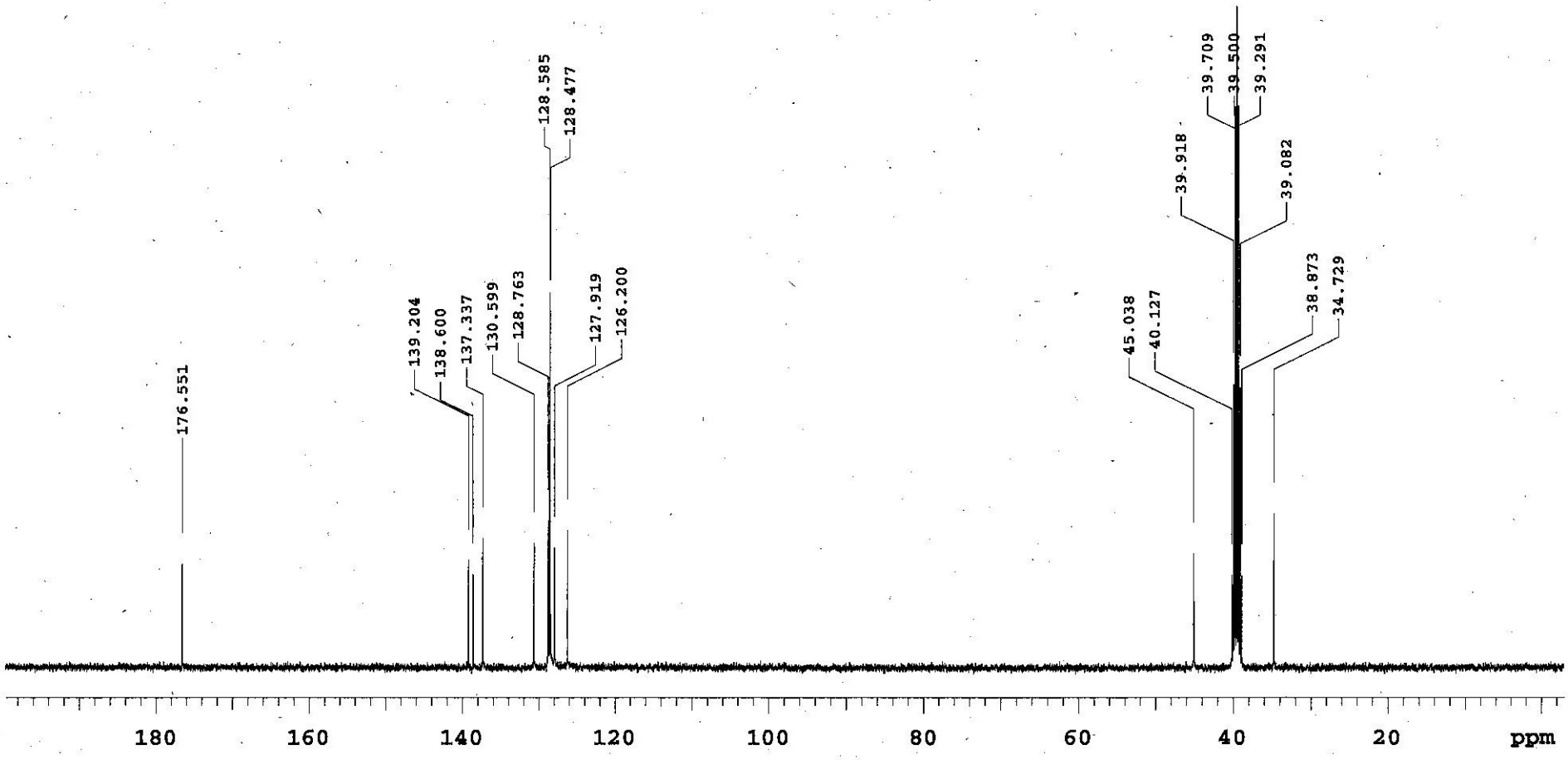


Figure S8. ^{13}C-NMR spectrum (DMSO) of compound 11



PULSE SEQUENCE Relax. delay 1.000 sec Pulse 45.0 degrees Acq. time 1.285 sec Width 25510.2 Hz 480 repetitions	OBSERVE C13, 100.5151211 DECOUPLE H1, 399.7434654 Power 33 dB continuously on WALTZ-16 modulated	DATA PROCESSING Line broadening 1.0 Hz FT size 65536 Total time 18 minutes		Jamerson/Antibioticos Amostra LT-41 Solicitacao N. M0722-16 29.07.2014 UFPE Solvent: dmso Temp. 26.0 C / 299.1 K Operator: ricardo

Figure S9. ^{1}H-NMR spectrum (DMSO) of compound 12

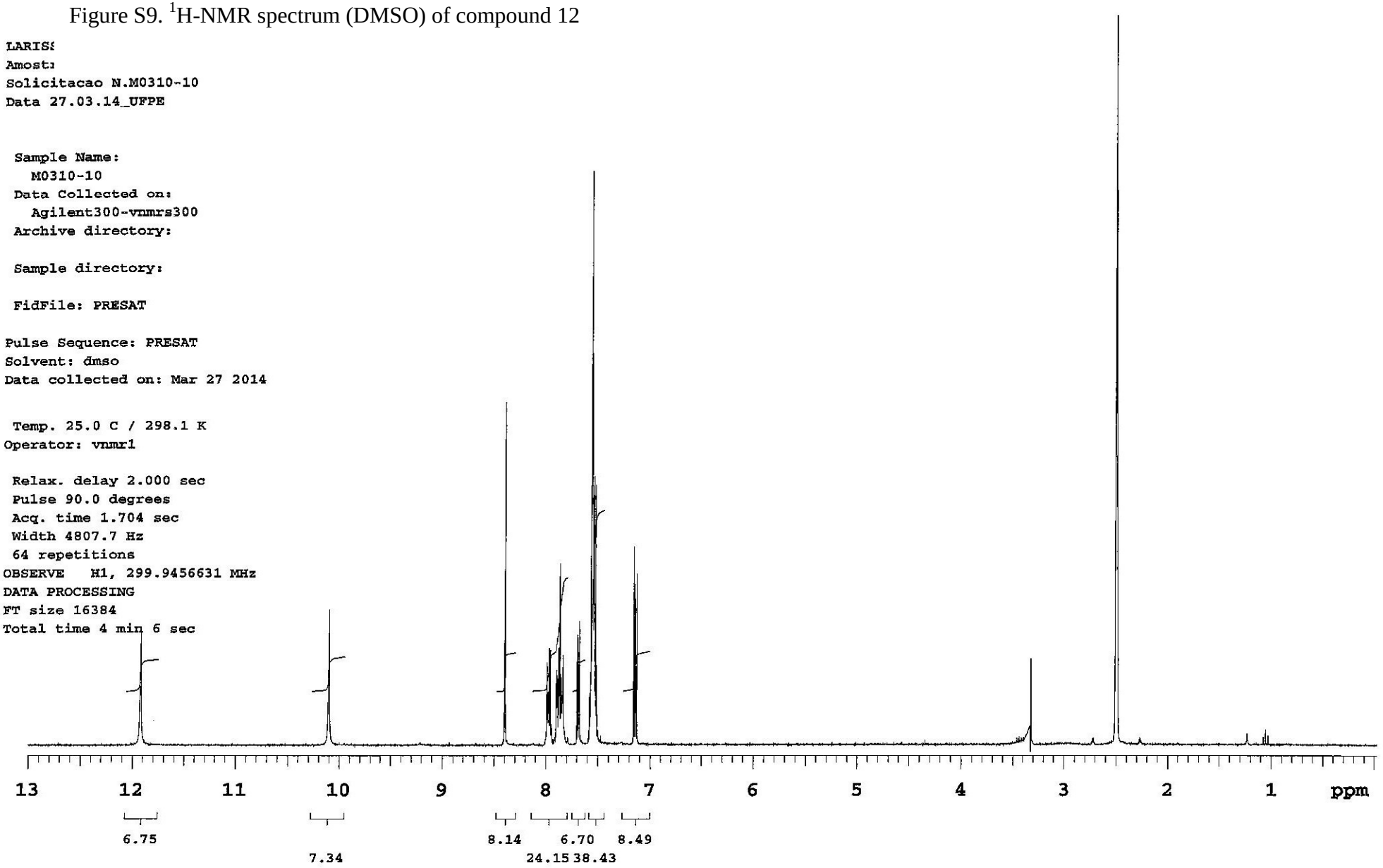


Figure S10. ^{13}C-NMR spectrum (DMSO) of compound 12

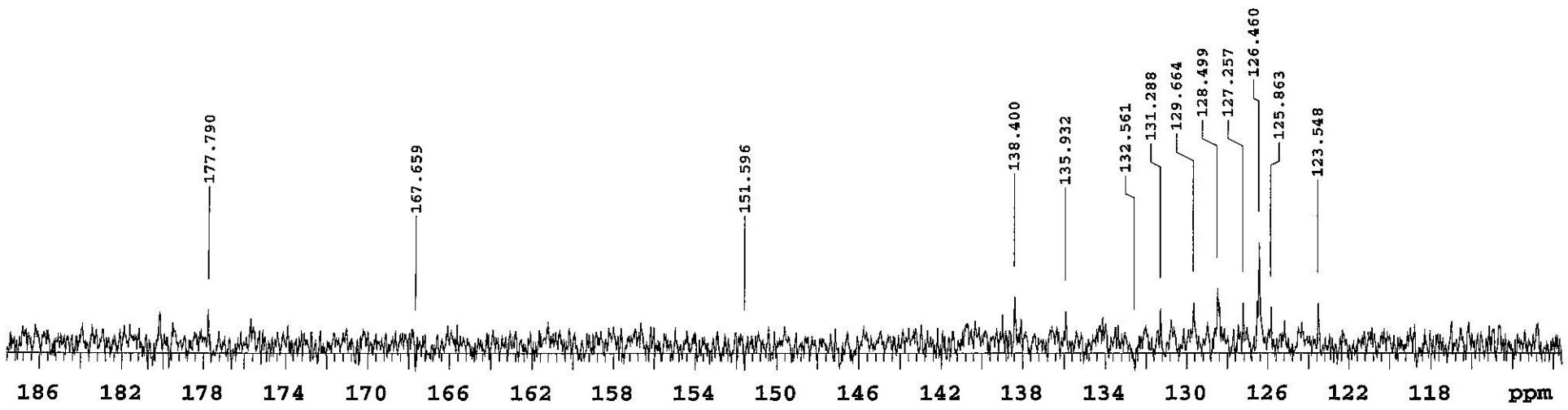


Figure S11. ^{1}H-NMR spectrum (DMSO) of compound 13

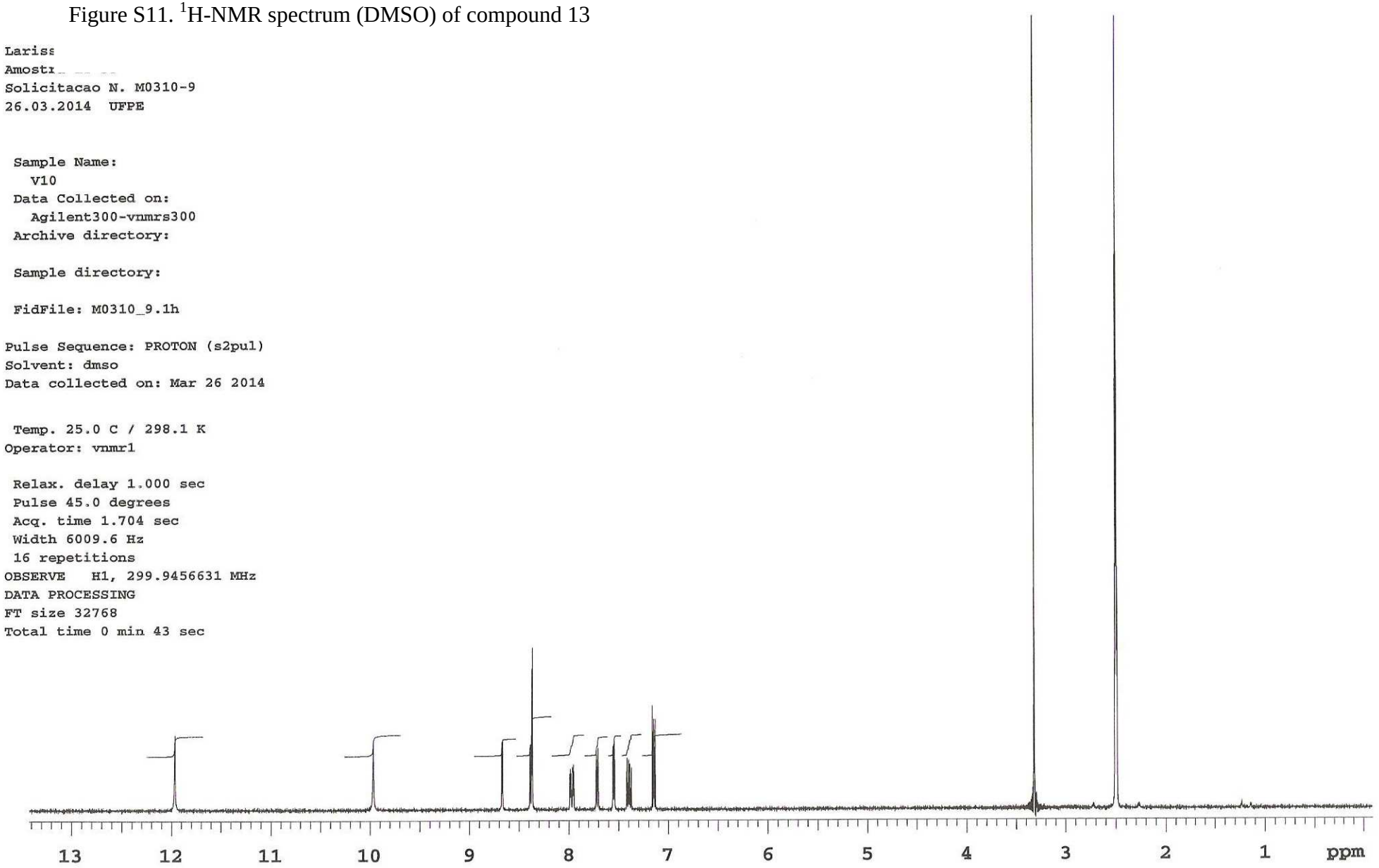
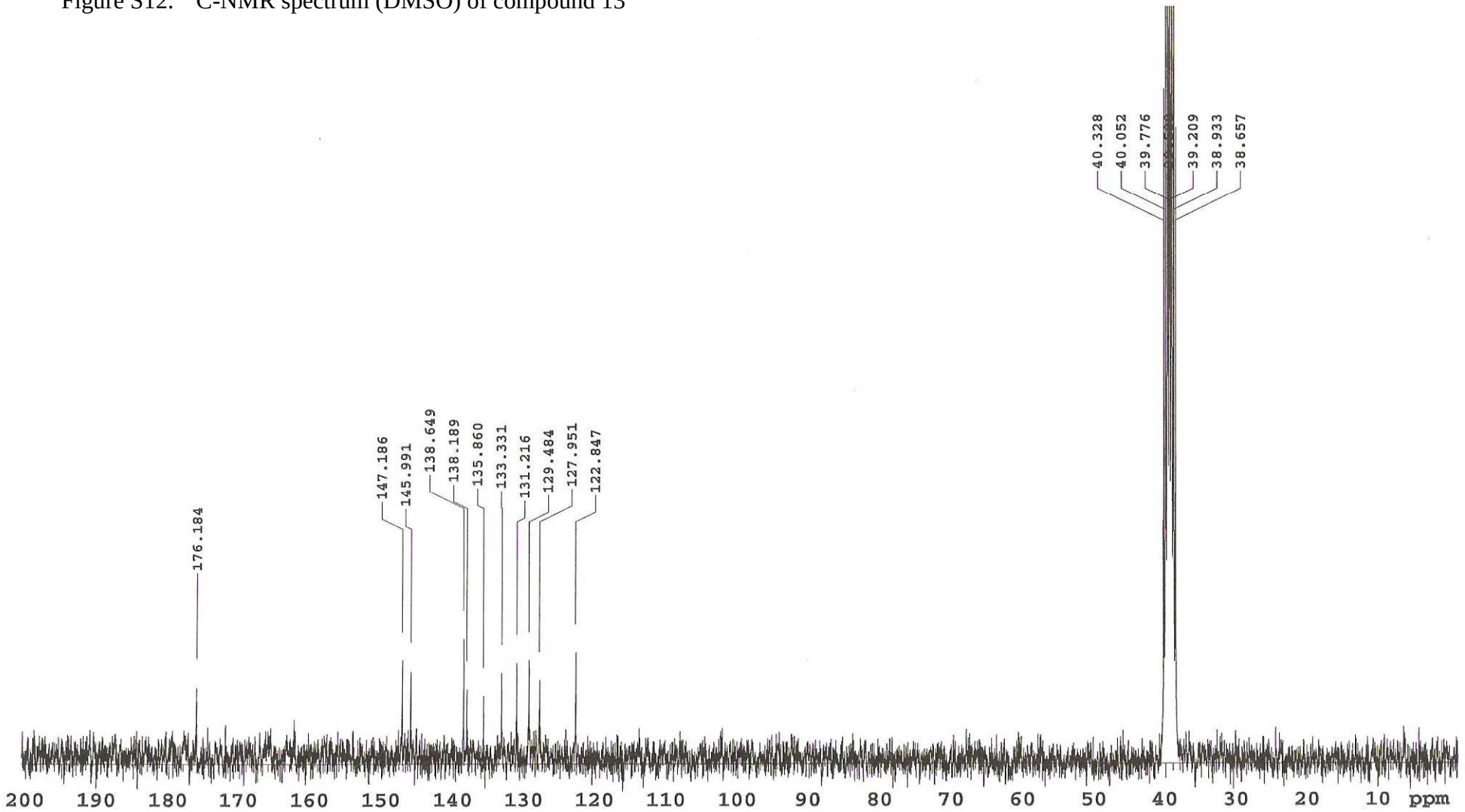


Figure S12. ^{13}C-NMR spectrum (DMSO) of compound 13



PULSE SEQUENCE Relax. delay 1.000 sec Pulse 45.0 degrees Acq. time 0.865 sec Width 18939.4 Hz 2048 repetitions	OBSERVE C13, 75.4214344 DECOUPLE H1, 299.9471704 Power 35 dB continuously on WALTZ-16 modulated	DATA PROCESSING Line broadening 2.0 Hz FT size 32768 Total time 63 minutes		Larissa Souza Amostra LT-35 Solicitacao N. M0310-9 26.03.2014 UFPE Solvent: dmso Temp. 25.0 C / 298.1 K

Figure S13. ^{1}H-NMR spectrum (DMSO) of compound 14

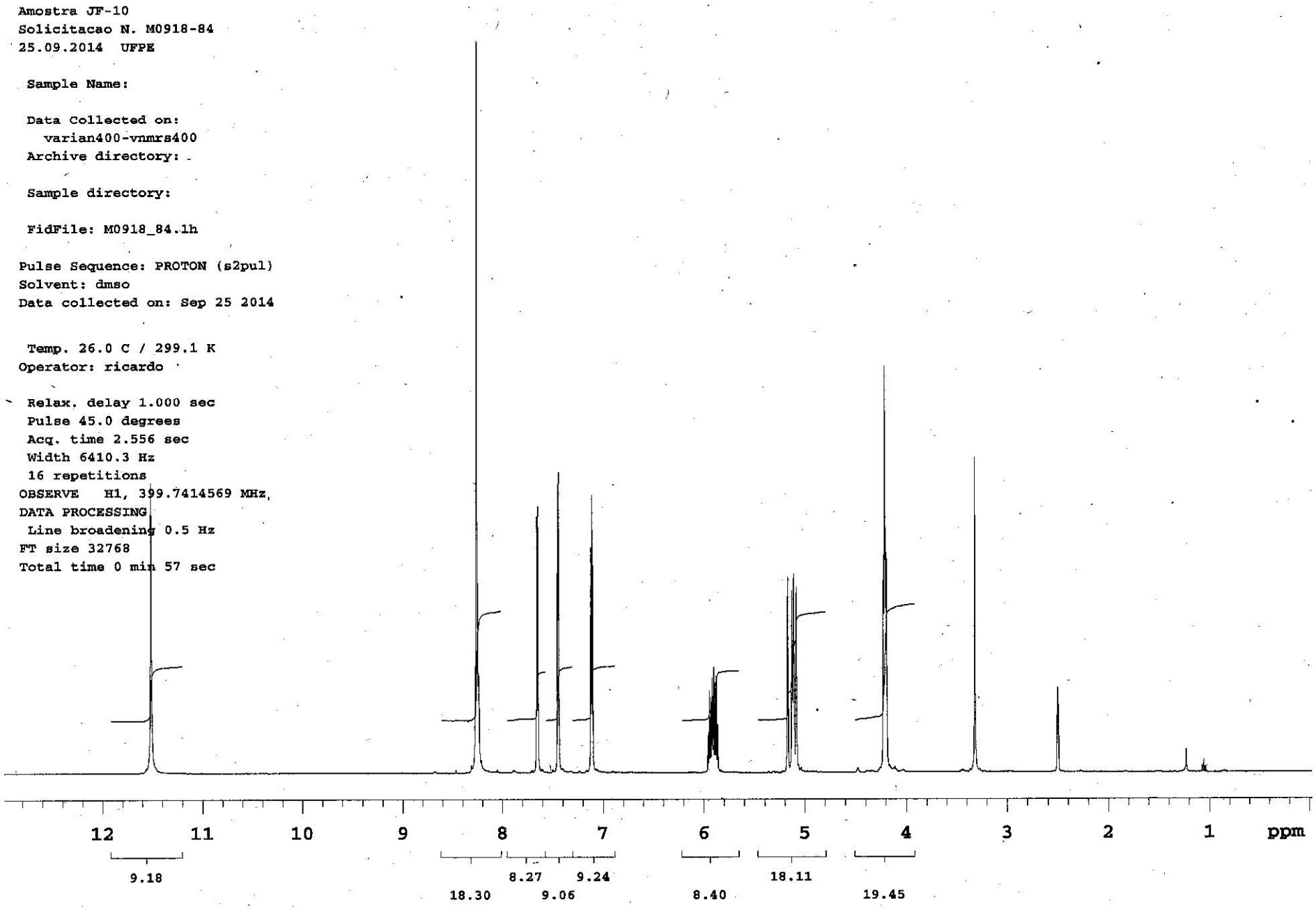
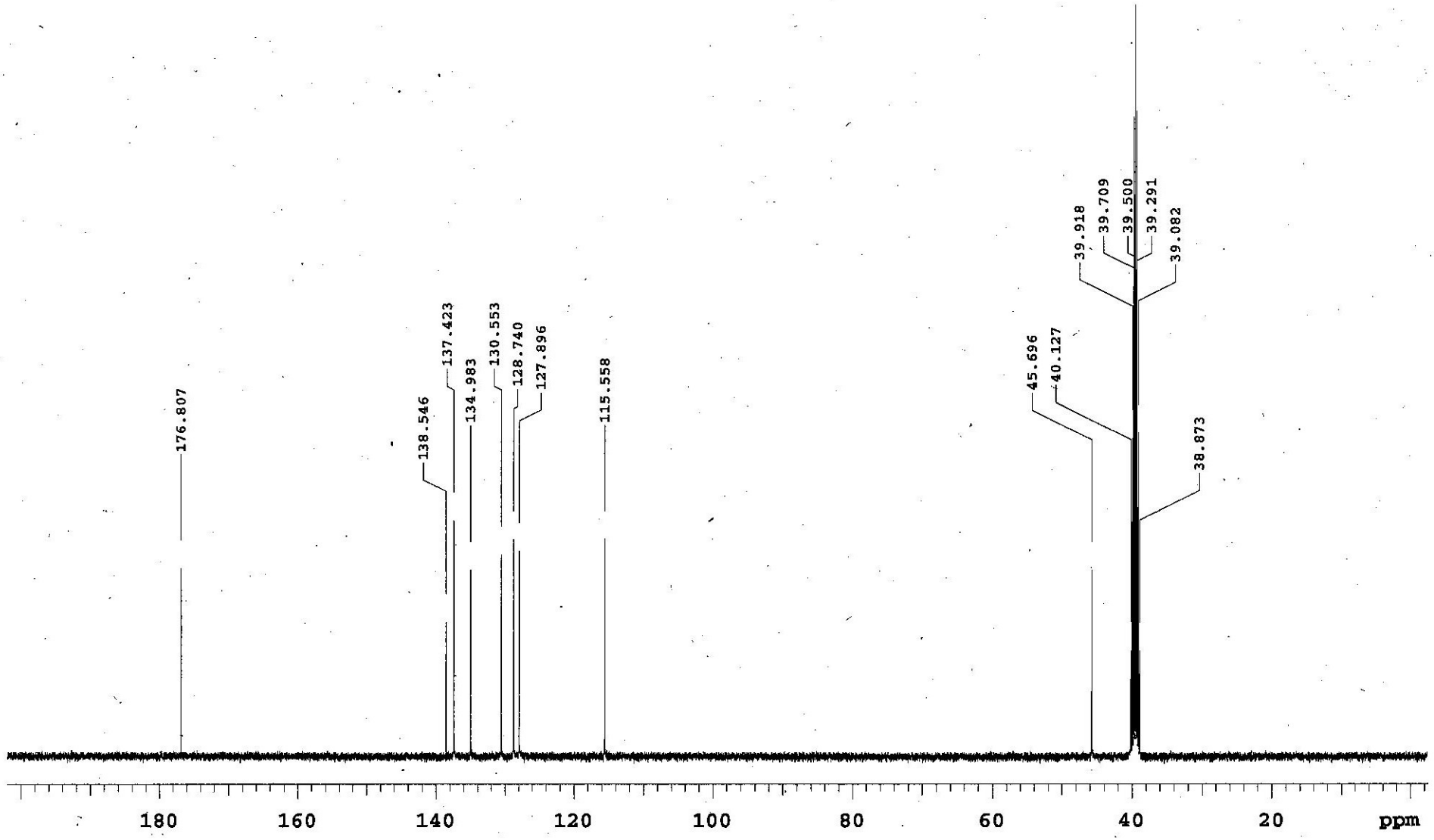


Figure S14. ^{13}C-NMR spectrum (DMSO) of compound 14



PULSE SEQUENCE Relax. delay 1.000 sec Pulse 45.0 degrees Acq. time 1.285 sec Width 25510.2 Hz 800 repetitions	OBSERVE C13, 100.5151203 DECOUPLE H1, 399.7434654 Power 33 dB continuously on WALTZ-16 modulated	DATA PROCESSING Line broadening 0.5 Hz FT size 65536 Total time 30 minutes		Edson Rubhens/Antibioticos Amostra JF-10 Solicitacao N. M0918-84 25.09.2014 UFPE Solvent: dmso Temp. 26.0 C / 299.1 K Operator: ricardo

Figure S15. NOESY spectrum (DMSO) of compound 10.