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# Synthesis and Biological Evaluation of Cyclopenta[*c*]thiophene Related Compounds as New Antitumor Agents

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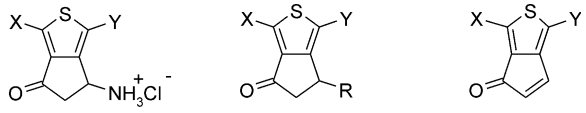
**Abstract**—A series of 22 cyclopenta[*c*]thiophene related compounds was obtained by the pharmacomodulation of 6-amino-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-ones **1a–g**. All compounds were evaluated for potential anticancer activity in the NCI's in vitro human disease-oriented tumor cell line screening panel that consisted of 60 human tumor cell lines arranged in nine subpanels, representing diverse histologies. Among these tested compounds, seven were found to be cytotoxic, especially against leukemia cell lines, allowing us to point out some structure–activity relationships. These derivatives were further evaluated for potential in vivo anticancer activity in the hollow fiber assay developed at the NCI, which selected two compounds, **1f** and **3a** for standard xenograft testing. 

## Introduction

A few years ago, within the context of our work aiming at researching new compounds derived from thiophene and showing a biological interest, we reported the synthesis of the first aminocyclopenta[*c*]thiophenones **1** (Fig. 1).[1] The cytotoxic properties of those substituted on thiophene by two halogen atoms had been brought to the fore in vitro towards leukemia L1210.[2] In order to go more closely into the potential antitumor interest of this original chemical series not yet represented in any active anti-neoplastic compound, and to make a first approach of its structure–activity relationships, we set out a pharmacomodulation of the parent compounds **1**, whose products were submitted first to the NCI in vitro human disease-oriented tumor cell line screening panel.[3] This pharmacomodulation particularly concerned the primary amine function which was either conserved and involved in different amino groups or replaced by hydroxyl group or halogen atoms (**2–8**). The amine function was also eliminated to give access to the cyclopentenones **9**. Finally, with regard to some of the synthesized compounds, the pharmacomodulation also concerned the thiophene substituents X,Y such as hydrogen or halogen atoms (Br, Cl, I) or methyl groups.




**1** **2-8** **9**

**Figure 1.** Structures of the 6-substituted cyclopenta[*c*]thiophenones **1–8** and cyclopentenones **9**.

## Chemistry

We recently described the access to the cyclopenta[*c*]thiophene system.[1] The *N*-protected 6-amino-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-ones **10a–f** were obtained starting from thiophene-3-carboxaldehyde **11d** by a multistep reaction sequence (Fig. 2). The latter was also used to give the new dimethyl derivative **10g** obtained in a similar manner from 2,5-dimethylthiophene-3-carboxaldehyde **11g**, itself produced by the Vilsmeier–Haack formylation of 2,5-dimethylthiophene **12** (Scheme 1).

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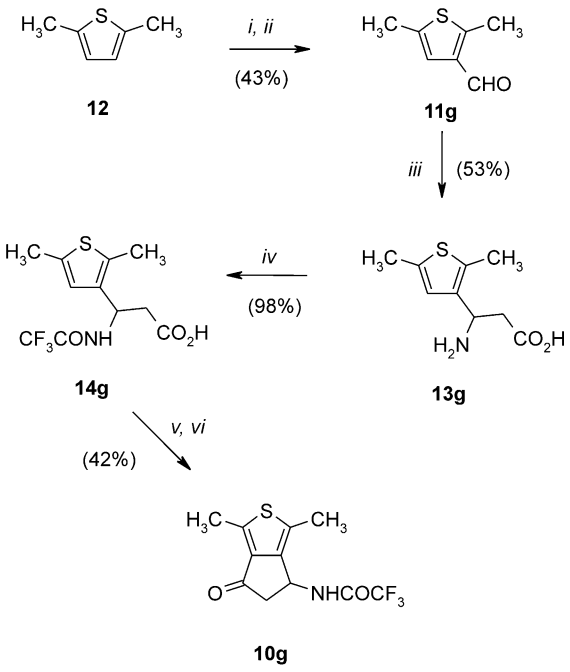
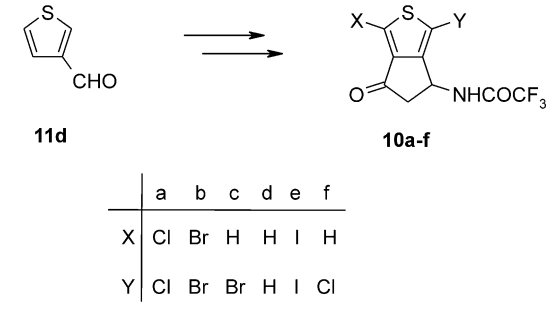



**Scheme 1.** Reagents: (i) $POCl_3$, DMF (rt/30 min, 80 °C/3 h); (ii) NaOH, $H_2O$ (10 °C); (iii) $CH_2(CO_2H)_2$, $AcONH_4$, EtOH (rflx/24 h); (iv) $TFA_2O$, $Et_2O$ (rt/30 min); (v) $SOCl_2$ (rflx/30 min); (vi) $AlCl_3$, $CH_2Cl_2$ (rflx/2 h).



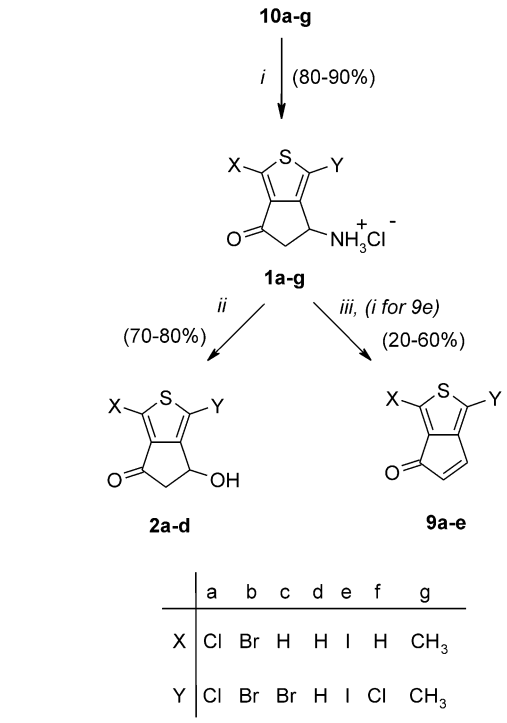

| | a | b | c | d | e | f |
|---|---|---|---|---|---|---|
| X | Cl | Br | H | H | I | H |
| Y | Cl | Br | Br | H | I | Cl |

**Figure 2.** Synthetic route to 6-trifluoroacetylamino-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-ones **10a**–**f**.

Thus, **11g** was reacted with malonic acid in the presence of ammonium acetate in refluxing ethanol, according to the Rodionow–Johnson method,[4,5] to give 3-amino-3-(2,5-dimethylthien-3-yl)propanoic acid **13g**. The amino group of **13g** was then protected with $TFA_2O$ prior to the Friedel–Crafts intramolecular cyclization of **14g**, leading to **10g** in a global yield of 9% from **12**.

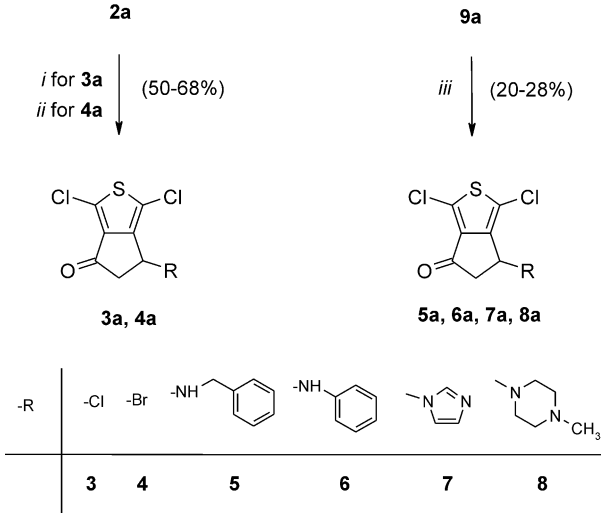
The amino group of **10a**–**g** was deprotected by acidic hydrolysis to give the ammonium chlorides **1a**–**g** (Scheme 2). Diazotization of the latter by sodium nitrite in various conditions allowed the synthesis of the hydroxycyclopentanones **2a**–**d** (20% aqueous AcOH solution) or cyclopentenones **9a**–**e** (50% aqueous TFA solution).[6] A more drastic acidic hydrolysis of **1e** led directly to **9e**.

The replacement of the hydroxyl group of **2a** by a chlorine or a bromine atom, using either thionyl chloride or bromine in acetic acid, led to the trihalo derivatives **3a** and **4a** respectively (Scheme 3). On the other hand, the Michaël adducts **5a, 6a, 7a** and **8a** were obtained by the treatment of **9a** in refluxing chloroform with benzylamine, aniline, imidazole sodium salt or *N*-methyl-piperazine, respectively.

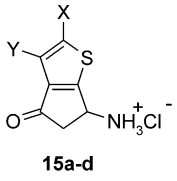



| | a | b | c | d | e | f | g |
|---|---|---|---|---|---|---|---|
| X | Cl | Br | H | H | I | H | $CH_3$ |
| Y | Cl | Br | Br | H | I | Cl | $CH_3$ |

**Scheme 2.** Reagents: (i) HCl, $H_2O$ (rflx, 12 h); (ii) $NaNO_2$, AcOH, $H_2O$ (rt/4 h); (iii) $NaNO_2$, TFA, $H_2O$ (rt/5 h).




**Scheme 3.** Reagents: (i) $SOCl_2$ (rflx/15 min); (ii) $Br_2$, AcOH (rt/5 h); (iii) RH, $CHCl_3$ (rflx/3 h).




| | a | b | c | d |
|---|---|---|---|---|
| X | Cl | H | Br | Cl |
| Y | Cl | Br | H | H |

**Scheme 4.**

## Results and Discussion

### Evaluation of the cytotoxicity of the synthesized compounds

Twenty-two cyclopenta[*c*]thiophene related compounds were evaluated in the in vitro human disease-oriented tumor cell line screening panel developed at the NCI. The log $GI_{50}$ values ($GI_{50}$ being the molar drug concentration required for half growth inhibition) obtained with selected cell lines, along with the mean graph midpoint (MGM) values, are summarized in Table 1. The MGM is based on a calculation of the average log $GI_{50}$ for all of the cell lines tested (approximately 60) in which $GI_{50}$ values below and above the test range ($10^{-4}$–$10^{-8}$ M) are taken as the minimum ($10^{-8}$ M) and maximum ($10^{-4}$ M) drug concentrations used in the screening test. These results indicated an indisputable cytotoxicity linked to the series since nine derivatives (**1a**, **1b**, **1f**, **2a**, **2b**, **3a**, **4a**, **6a** and **9a**) showed a MGM log $GI_{50}$ below to −5, corresponding to MGM $GI_{50}$ values ranging from 1.2 to 9.5 μM (Table 2). Qualitatively, the complete set of data obtained in this screening panel showed that the leukemia cell lines were relatively more sensitive to some tested compounds than were other cell lines. This selectivity is expressed through the ratio calculated on plotting the average $GI_{50}$ values corresponding to the leukemia cell lines against the MGM $GI_{50}$ values (Table 3). The most significative ratio ranging from 3.2 to 10 and for example **6a** was cytotoxic against leukemia cell lines with an average $GI_{50}$ value of 0.12 μM ($GI_{50} < 10$ nM for RPMI-8226 leukemia cell line).

The analysis of the structure–activity relationships seems first to link the cytotoxicity of the tested compounds to the position of the sulfur atom in the thiophene ring, since any cyclopenta[*b*]thiophene isomeric compounds **15a**–**d**, we recently described,[7] exhibited the least cytotoxicity (MGM log $GI_{50} > -4$) (Scheme 4).

On the other hand, the results of the evaluation of compounds **1a**–**g** showed a relationship between the presence and the nature of the thiophene substituents and the activity (Table 1). Thus, the unsubstituted derivative **1d** and the dimethyl one **1g** were inactive or poorly cytotoxic respectively, contrary to the mono and dihalo compounds. Amongst those, the most active were the dichloro **1a** and dibromo **1b** compounds (MGM log $GI_{50} = -5.54$ and −5.46, respectively), while the monochloro **1f**, monobromo **1c** and diiodo **1e** compounds exhibited weaker values (MGM log $GI_{50}$ between −5.13 and −4.78).

**Table 1.** Cytotoxicity of cyclopenta[*c*]thiophene related compounds

| Compd | Log molar drug concentration required for 50% growth inhibition (log $GI_{50}$) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Leukemia RPMI 8226 | Lung NCI-H522 | Colon SW-620 | CNS SF-539 | Melanoma UACC-257 | Ovarian ovcar-4 | Renal CAKI-1 | Prostate PC-3 | Breast MDA-231 | MGM[a] |
| **1a** | −5.63 | −5.87 | −5.82 | −5.77 | −5.89 | −5.72 | −6.26 | −5.48 | −6.00 | *−5.54* |
| **1b** | −6.53 | −5.81 | −5.50 | −5.61 | −7.23 | −5.71 | −5.76 | −5.13 | −5.68 | *−5.46* |
| **1c** | −5.52 | −5.69 | −5.35 | −5.70 | −4.75 | −4.78 | −4.85 | −4.97 | −4.81 | *−4.88* |
| **1d** | −5.35 | NT[b] | −4.75 | −4.43 | −4.03 | > −4.00 | −4.80 | −4.28 | > −4.00 | *−4.36* |
| **1e** | −5.30 | −5.11 | −4.84 | −5.30 | −4.72 | −4.70 | −4.74 | −4.71 | −4.70 | *−4.78* |
| **1f** | −6.85 | −5.71 | −5.79 | −6.36 | −4.79 | −4.63 | −5.75 | −5.15 | −5.33 | *−5.13* |
| **1g** | −5.57 | −5.38 | −5.36 | −5.55 | −4.76 | −4.45 | −4.83 | −4.76 | −4.72 | *−4.63* |
| **2a** | −5.14 | NT | −5.67 | −5.04 | −5.15 | −4.70 | −5.47 | −4.83 | −4.71 | *−5.02* |
| **2b** | −5.61 | −5.08 | −5.71 | −5.19 | −5.41 | NT | −5.68 | −4.89 | −4.82 | *−5.06* |
| **2c** | −4.84 | −4.81 | −4.82 | NT | −4.97 | −4.50 | −4.42 | −4.49 | −4.62 | *−4.59* |
| **2d** | −4.45 | −4.38 | −4.42 | > −4.00 | > −4.00 | > −4.00 | > −4.00 | > −4.00 | > −4.00 | *−4.09* |
| **3a** | −6.81 | −5.82 | −5.77 | −5.67 | −5.33 | −5.05 | −5.72 | −5.66 | −5.58 | *−5.54* |
| **4a** | −5.85 | −4.77 | −5.74 | −5.08 | −5.40 | NT | −5.51 | −4.97 | −5.00 | *−5.14* |
| **5a** | −5.65 | −4.89 | −4.61 | NT | −4.59 | −4.50 | −4.65 | −4.76 | −4.61 | *−4.62* |
| **6a** | < −8.00 | −6.90 | −6.50 | −6.82 | −5.69 | −5.73 | −6.58 | −5.83 | −5.74 | *−5.92* |
| **7a** | −5.64 | −5.02 | −4.41 | −4.76 | −4.61 | −4.63 | −4.69 | −4.78 | −4.68 | *−4.74* |
| **8a** | −5.36 | −4.66 | −4.72 | −4.80 | −4.39 | −4.45 | −4.68 | −4.53 | −4.44 | *−4.54* |
| **9a** | −6.17 | NT | −6.01 | −5.83 | −5.74 | −5.61 | −5.64 | −5.74 | −5.69 | *−5.77* |
| **9b** | −4.99 | −4.59 | −4.74 | −4.23 | −4.58 | NT | −4.71 | −4.71 | −4.66 | *−4.56* |
| **9c** | NT | −4.73 | −4.65 | NT | −4.17 | −4.15 | > −4.00 | −4.19 | −4.04 | *−4.31* |
| **9d** | −4.69 | −4.71 | −4.48 | > −4.00 | > −4.00 | −4.09 | −4.56 | −4.38 | −4.50 | *−4.38* |
| **9e** | NT | −5.70 | −4.87 | −4.59 | −4.70 | −4.70 | −4.73 | NT | −4.52 | *−4.69* |

[a]Mean Graph Midpoint for all human cancer cell lines tested.
[b]NT, not tested.

**Table 2.** Cytotoxicity of cyclopenta[*c*]thiophene related compounds

| MGM ($GI_{50}$ in μM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **6a** | **9a** | **1a** | **3a** | **1b** | **4a** | **1f** | **2b** | **2a** |
| 1.2 | 1.7 | 2.9 | 2.9 | 3.5 | 7.2 | 7.4 | 8.7 | 9.5 |

**Table 3.** Selectivity of some cyclopenta[*c*]thiophene related compounds towards leukemia cell lines

| Ratio leukemia cell lines average $GI_{50}$/MGM $GI_{50}$ | | | | |
|---|---|---|---|---|
| **6a** | **1f** | **3a** | **9a** | **2b** |
| 10 | 5.6 | 4.6 | 4.6 | 3.2 |

Concerning the cyclopentane substituents, the replacement of the amino group of **1a** by a methylpiperazine, a benzylamine or an imidazole moiety (compounds **8a**, **5a**, **7a**), dramatically decreased the activity (MGM log $GI_{50}$ between −4.54 and −4.74). The decrease was weaker with an hydroxyl group or a bromine atom (**2a**, **4a**) which maintained the MGM log $GI_{50}$ values around −5, while the substitution of the amino group by a chlorine atom (**3a**) did not affect the cytotoxicity. However the latter was strongly increased by the presence in C-6 of an aniline moiety since **6a** exhibited the higher cytotoxicity level with a MGM log $GI_{50}$ value = −5.92. The analysis of the results of the compounds **2a**–**d** confirms moreover the greater activity of the dichloro and dibromo thiophene derivatives comparatively to their monohalo or unsubstituted analogues. The evaluation of the cytotoxicity of the cyclopentenones **9a**–**e**, issued from the elimination of the amino group of **1a**–**e**, showed inconsistent results since the activities of **9a** and **9e** are slightly increased relatively to **1a** and **1e** (MGM log $GI_{50}$ = −5.77 and −4.69 respectively), whereas those of **9c** and especially **9b** were dramatically decreased relatively to **1c** and **1b**.

The complete set of data obtained in this screening panel has been used through the COMPARE algorithm[8] with a view to approaching the mechanism of action of the designed cyclopenta[*c*]thiophene derivatives. This program compares a complete set of cell sensitivity exerted towards a test compound to those of other compounds and especially of standard agents present in the NCI database. The results of this comparison (Table 4) showed significant correlations (correlation coefficients ≥0.6) between, on the one hand, many of the tested compounds and, on the other hand, rifamycin SV **16** (NSC 133100) and L-cystein ethyl ester methyl carbamate **17** (NSC 303861) which further correlated together, respectively. However, the mechanism of the cytotoxic activity of these standard agents has not yet been fully determined.

### Hollow fiber assay for preliminary in vivo testing

On the basis of these results, seven derivatives were selected for a preliminary in vivo testing (**1a**, **1b**, **1f**, **2a**, **3a**, **6a** and **9a**). The hollow fiber assay, developed at the NCI, is a screening tool for assessing the potential anticancer activity of compounds against human tumor cells cultivated in hollow fibers and implanted intraperitoneally and subcutaneously in mice.[9] After treatment, fiber cultures were collected and the viable cell mass was determined. A scoring system was developed to simplify evaluation of the results (Table 5). For this, a value of 2 was assigned for each compound dose which results in a 50% or greater reduction (%T/C ≤50) in viable cell mass. The intraperitoneal and subcutaneous samples were scored separately. Compounds with a combined IP + SC score ≥20, a SC score ≥8 or a net cell kill of one or more cell lines are considered as active in this testing. Two derivatives met these criteria: compounds **1f** and **3a** on account of a net cell kill. Accordingly, these derivatives have been selected for further in vivo testing in standard subcutaneous xenograft models.

**Table 4.** Correlation coefficients calculated by the COMPARE algorithm using the $GI_{50}$ values

| | 6a | 9a | 1a | 3a | 1b | 4a | 1f | 2b | 2a | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **6a** | 1.00 | 0.74 | 0.61 | 0.81 | 0.68 | 0.62 | 0.86 | 0.61 | 0.61 | *0.65* | *0.64* |
| **9a** | | 1.00 | 0.69 | 0.74 | 0.66 | 0.67 | 0.72 | 0.59 | 0.79 | *0.66* | *0.69* |
| **1a** | | | 1.00 | 0.66 | 0.73 | 0.52 | 0.66 | 0.50 | 0.68 | *0.66* | *0.60* |
| **3a** | | | | 1.00 | 0.66 | 0.60 | 0.70 | 0.56 | 0.69 | *0.68* | *0.59* |
| **1b** | | | | | 1.00 | 0.68 | 0.72 | 0.62 | 0.61 | *0.63* | *0.59* |
| **4a** | | | | | | 1.00 | 0.64 | 0.90 | 0.68 | *0.46* | *0.59* |
| **1f** | | | | | | | 1.00 | 0.64 | 0.68 | *0.49* | *0.61* |
| **2b** | | | | | | | | 1.00 | 0.58 | *0.40* | *0.42* |
| **2a** | | | | | | | | | 1.00 | *0.54* | *0.59* |
| **16** | | | | | | | | | | 1.00 | *0.69* |
| **17** | | | | | | | | | | | 1.00 |

**Table 5.** Results of the hollow fiber assay for preliminary in vivo testing

| Compd | ip score | sc score | ip + sc score | Cell kill | MTD[a] (mg/kg) |
|---|---|---|---|---|---|
| **6a** | 0 | 0 | 0 | No | 6.3 |
| **9a** | 0 | 0 | 0 | No | 12.5 |
| **1a** | 0 | 2 | 2 | No | 200 |
| **3a** | 4 | 0 | 4 | Yes | 200 |
| **1b** | 0 | 2 | 2 | No | 200 |
| **1f** | 8 | 2 | 10 | Yes | 400 |
| **2a** | 2 | 0 | 2 | No | 100 |

[a]Maximum Tolerated Dose.

## Conclusion

The pharmacomodulation of the cyclopenta[*c*]thiophene system afforded several new derivatives whose cytotoxicity was assessed in vitro in a screening panel consisting of 60 human tumor cell lines. Among 22 test compounds, seven were found to be active (MGM log $GI_{50}$ values below to-5) especially against leukemia cell lines. The RSA study established that activity was related to the presence of some halogen atoms on the thiophene ring and of an adequate substituent on the C-6 position of the cyclopentanone moiety. Among these compounds, two furthermore exerted an in vivo activity, assessed in the NCI's hollow fiber assay, and thus were currently selected for standard xenograft testing. Although these active compounds showed correlations with some of the standard agents, the mechanism of action of this new group of antitumor agents has not yet been clearly established. The elucidation of the latter will constitute the prolongation of this work.

## Experimental

### General experimental procedures

Melting points were determined on a Kofler block and are uncorrected. $^1H$ and $^{13}C$ NMR spectra were measured on a Jeol JNM-LA 400 spectrometer. Chemical shifts are reported in δ (ppm). Column chromatography was performed on Merck silica gel 60, 0.063–0.200 mm, 70–230 mesh. Precoated silica gel plates (Polygram SIL G/UV254, 0.25 mm) were used for TLC analysis. All products and reagents were purchased from Acros, Belgium.

**1,3-Dimethyl-6-oxo-5,6-dihydro-4*H*-cyclopenta[*c*]thien-4-ylammonium chloride (1g).** This was prepared by refluxing for 12 h a solution of 2 g (0.006 mol) of **10g** in 100 mL of a 10 N aqueous HCl solution. After evaporation of the reaction mixture, the resulting crystals (81%) were recrystallized from isopropanol: White crystals, mp >260 °C; $^1$H NMR (DMSO-$d_6$) δ 8.7 (bs, 3H, $NH_3$), 4.8 (bs, 1H, H-4), 3.44 (dd, $J$=19, 8 Hz, 1H, H-5a), 2.90 (dd, $J$=19, 4 Hz, 1H, H-5b), 2.61 (s, 3H, $CH_3$), 2.56 (s, 3H, $CH_3$); $^{13}$C NMR (DMSO-$d_6$) δ 193.9 (C-6), 144.3 (C-6a), 139.9 (C-1), 139.1 (C-3), 132.9 (C-3a), 49.2 (C-4), 42.6 (C-5), 12.8 ($CH_3$), 12.7 ($CH_3$). Anal. calcd for $C_9H_{12}NOClS$: C, 49.65; H, 5.56; N, 6.43. Found: C, 49.45; H, 5.58; N, 6.52.

**1,3-Dichloro-6-hydroxy-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-one (2a).** This was prepared by adding at 0 °C, 7 g (0.102 mol) of $NaNO_2$ to a solution of 4.5 g (0.017 mol) of **1a** in 100 mL of a 20% aqueous AcOH solution. The reaction mixture was then stirred at room temperature for 4 h. Crystals (80%) were formed, filtered, washed with water, dried and recrystallized from $Et_2O$: Beige crystals, mp 73 °C; $^1$H NMR (DMSO-$d_6$) δ 5.8 (bs, 1H, OH), 5.06 (dd, $J$=7, 1 Hz, 1H, H-6), 3.31 (dd, $J$=19, 7 Hz, 1H, H-5a), 2.68 (dd, $J$=19, 1 Hz, 1H, H-5b); $^{13}$C NMR (DMSO-$d_6$) δ 192.7 (C-4), 149.9 (C-3a), 138.2 (C-6a), 126.9 (C-3), 121.8 (C-1), 63.4 (C-5), 53.6 (C-6). Anal. calcd for $C_7H_4O_2Cl_2S$: C, 37.69; H, 1.81; S, 13.76. Found: C, 37.46; H, 2.21; S, 13.69.

**1,3-Dibromo-6-hydroxy-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-one (2b).** This was prepared from **1b** using the same method and concentrations as for the synthesis of **2a**. Crystals (76%) were formed, filtered, washed with water, dried and recrystallized from $Et_2O$: Beige crystals, mp 75 °C; $^1$H NMR (DMSO-$d_6$) δ 5.7 (bs, 1H, OH), 5.00 (dd, $J$=7, 2 Hz, 1H, H-6), 3.31 (dd, $J$=19, 7 Hz, 1H, H-5a), 2.67 (dd, $J$=19, 2 Hz, 1H, H-5b); $^{13}$C NMR (DMSO-$d_6$) δ 192.5 (C-4), 158.1 (C-3a), 154.1 (C-6a), 115.0 (C-3), 111.8 (C-1), 64.4 (C-5), 63.2 (C-6). Anal. calcd for $C_7H_4O_2Br_2S$: C, 26.95; H, 1.29; S, 10.28. Found: C, 26.74; H, 1.37; S, 10.35.

**1-Bromo-6-hydroxy-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-one (2c).** This was prepared by adding at 0 °C, 5.5 g (0.08 mol) of $NaNO_2$ to a solution of 2.7 g (0.01 mol) of **1c** in 50 mL of a 20% aqueous AcOH solution. The reaction mixture was stirred at room temperature for 6 h, neutralized with $NaHCO_3$ and extracted with $CHCl_3$. The organic layer was separated, dried over $CaCl_2$ and evaporated. The resulting brown oil was purified by column chromatography (3.5×30 cm, silica gel 60, 0.063–0.200 mm) with petroleum ether–EtOAc (80:20) as the eluent, resulting in a colorless oil (70%): $^1$H NMR (DMSO-$d_6$) δ 8.12 (s, 1H, H-3), 5.7 (bs, 1H, OH), 5.04 (dd, $J$=7, 2 Hz, 1H, H-6), 3.35 (dd, $J$=19, 7 Hz, 1H, H-5a), 2.67 (dd, $J$=19, 2 Hz, 1H, H-5b). Anal. calcd for $C_7H_5O_2BrS$: C, 36.07; H, 2.16; S, 13.76. Found: C, 36.02; H, 2.04; S, 13.58.

**6-Hydroxy-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-one (2d).** This was prepared from **1d** using the same method and concentrations as for the synthesis of **2c**. The crude oil was purified by column chromatography (3.50×30 cm, silica gel 60, 0.063–0.200 mm) with petroleum ether-EtOAc (80:20) as the eluent, resulting in a colorless oil (70%): $^1$H NMR (DMSO-$d_6$) δ 8.07 (d, $J$=2 Hz, 1H, H-3), 7.55 (d, $J$=2 Hz, 1H, H-1), 5.65 (dd, $J$=7, 2 Hz, 1H, H-6), 5.1 (bs, 1H, OH), 3.25 (dd, $J$=19, 7 Hz, 1H, H-5a), 2.67 (dd, $J$=19, 2 Hz, 1H, H-5b). Anal. calcd for $C_7H_6O_2S$: C, 54.53; H, 3.92; S, 20.80. Found: C, 54.69; H, 4.08; S, 21.02.

**1,3,6-Trichloro-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-one (3a).** This was prepared by refluxing for 15 min, 0.7 g (0.003 mol) of **2a** in 10 mL of thionyl chloride. After evaporation, the resulting oil was dissolved in 30 mL of $Et_2O$ and the solution was washed with water. The organic layer was separated, dried over $MgSO_4$ and the solvent was removed to afford crystals (68%) which were recristallized from ether–petroleum ether (50:50): Beige crystals, mp 75 °C; $^1$H NMR ($CDCl_3$) δ 5.27 (dd, $J$=8, 2 Hz, 1H, H-6), 3.61 (dd, $J$=20, 8 Hz, 1H, H-5a), 3.20 (dd, $J$=20, 2 Hz, 1H, H-5b); $^{13}$C NMR ($CDCl_3$) δ 192.0 (C-4), 147.21 (C-3a), 138.0 (C-6a), 132.0 (C-3), 121.0 (C-1), 54.6 (C-5), 46.1 (C-6). Anal. calcd for $C_7H_3OCl_3S$: C, 34.81; H, 1.25; S, 13.28. Found: C, 34.66, H, 1.32; S, 13.05.

**6-Bromo-1,3-dichloro-5,6-dihydro-4*H*-cyclopenta[*c*] thiophen-4-one (4a).** This was prepared by adding at 0 °C, 0.25 mL of bromine (0.005 mol) to a suspension of 1.2 g (0.005 mol) of **2a** in 30 mL of AcOH. The reaction mixture was stirred at room temperature for 5 h and then poured into 100 mL of cold water. Crystals (50%) were formed, filtered, washed with water, dried and recrystallized from $Et_2O$: Yellow crystals, mp 82 °C; $^1$H NMR (DMSO-$d_6$) δ 5.50 (dd, $J$=8, 2 Hz, 1H, H-6), 3.74 (dd, $J$=20, 8 Hz, 1H, H-5a), 3.10 (dd, $J$=20, 2 Hz, 1H, H-5b). Anal. calcd for $C_7H_3OBrCl_2S$: C, 29.40; H, 1.06; S, 11.21. Found: C, 29.35; H, 1.30; S, 11.49.

**6-Benzylamino-1,3-dichloro-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-one (5a).** This was prepared by refluxing for 3 h a solution of 0.6 g (0.003 mol) of **9a** and 0.4 g (0.0037 mol) of benzylamine in 20 mL of chloroform. After evaporation of the reaction mixture, the resulting oil was dissolved in 30 mL of $Et_2O$ and the solution was washed with water. The organic layer was separated and dried over $MgSO_4$. After filtration and evaporation, the crude oil was purified by column chromatography (3.5×30 cm, silica gel 60, 0.063–0.200 mm) with petroleum ether–EtOAc (90:10) as the eluent, resulting in a yellow oil (20%): $^1$H NMR ($CDCl_3$) δ 7.30 (m, 5H, phenyl), 4.40 (m, 1H, H-6), 4.2 (bs, 1H, NH), 3.80 (m, 2H, $CH_2$), 3.21 (dd, $J$=19, 7 Hz, 1H, H-5a), 2.88 (dd, $J$=19, 3 Hz, 1H, H-5b); $^{13}$C NMR ($CDCl_3$) δ 193.4 (C-4), 149.7 (C-3a), 139.3 (C-6a), 137.5 (C-3), 128.6 (C-2α and C-6α), 128.5 (C-1′), 128.2 (C-3′ and C-5′), 127.4 (C-4′), 119.0 (C-1), 51.6 ($CH_2$), 51.2 (C-5), 51.1 (C-6). Anal. calcd for $C_{14}H_{11}NOCl_2S$: C, 53.86; H, 3.55; N, 4.49. Found: C, 53.97; H, 3.52; N, 4.42.

**6-Anilino-1,3-dichloro-5,6-dihydro-4*H*-cyclopenta[*c*] thiophen-4-one (6a).** This was prepared from **9a** and aniline using the same method as for the synthesis of **5a**.

Workup and purification were performed identically, resulting in beige crystals (20%), mp 130 °C: $^1$H NMR ($CDCl_3$) δ 7.18 (m, 2H, H-2′ and H-6′), 6.81 (m, 1H, H-4′), 6.64 (m, 2H, H-3′ and H-5′), 4.98 (m, 1H, H-6), 4.5 (bs, 1H, NH), 3.45 (dd, $J$ = 19, 7 Hz, 1H, H-5a), 2.92 (dd, $J$ = 19, 3 Hz, 1H, H-5b); $^{13}$C NMR ($CDCl_3$) δ 192.8 (C-4), 149.9 (C-3a), 138.2 (C-6a), 129.4 (C-2′ and C-6′), 129.2 (C-1′), 129.0 (C-3′ and C-5′), 128.9 (C-4′), 118.6 (C-3), 113.6 (C-1), 52.4 (C-5), 47.8 (C-6). Anal. calcd for $C_{13}H_9NOCl_2S$: C, 52.36; H, 3.04; N, 4.70. Found: C, 52.09; H, 2.98; N, 4.89.

**1,3-Dichloro-6-(1*H*-imidazol-1-yl)-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-one (7a).** This was prepared from **9a** and imidazole sodium salt, using the same method as for the synthesis of **5a**. Workup and purification were performed identically, resulting in beige crystals (25%), mp 150 °C: $^1$H NMR (DMSO-$d_6$) δ 9.35 (m, 1H, H-2′), 7.98 (m, 1H, H-4′), 7.72 (m, 1H, H-5′), 6.23 (dd, $J$ = 8, 4 Hz, 1H, H-6), 3.63 (dd, $J$ = 19, 8 Hz, 1H, H-5a), 3.27 (dd, $J$ = 19, 4 Hz, 1H, H-5b). Anal. calcd for $C_{10}H_6N_2OCl_2S$: C, 43.97; H, 2.21; N, 10.23. Found: C, 43.75; H, 2.70; N, 10.03.

**1,3-Dichloro-6-(4-methylpiperazin-1-yl)-5,6-dihydro-4*H*-cyclopenta[*c*]thiophen-4-one (8a).** This was prepared from **9a** and 4-methylpiperazine, using the same method as for the synthesis of **5a**. Workup and purification were performed identically, resulting in brown oil (28%): $^1$H NMR ($CDCl_3$) δ 4.37 (dd, $J$ = 7, 3 Hz, 1H, H-6), 3.01 (dd, $J$ = 18, 7 Hz, 1H, H-5a), 2.97 (dd, $J$ = 18, 3 Hz, 1H, H-5b), 2.64 (s, 3H, $CH_3$), 2.3 (m, 8H, 4 $CH_2$). Anal. calcd for $C_{12}H_{14}N_2OCl_2S$: C, 52.76; H, 5.17; H, 10.26. Found: C, 52.59; H, 5.08; N, 10.33.

**1,3-Dichloro-4*H*-cyclopenta[*c*]thiophen-4-one (9a).** This was prepared by adding at 0 °C, 5.8 g (0.084 mol) of $NaNO_2$ to a solution of 2.6 g (0.01 mol) of **1a** in 100 mL of a 50% aqueous TFA solution. The reaction mixture was stirred at room temperature for 5 h and extracted with $Et_2O$. The organic layer was washed twice with a saturated aqueous solution of $NaHCO_3$, separated, dried over $MgSO_4$ and evaporated. The crystals were left to stand at the ambient air for 24 h and then purified by column chromatography (3.5×30 cm, silica gel 60, 0.063–0.200 mm) with petroleum ether–EtOAc (80:20) as the eluent, resulting in yellow crystals (40%): $^1$H NMR ($CDCl_3$) δ 7.33 (d, $J$ = 6 Hz, 1H, H-6), 6.13 (d, $J$ = 6 Hz, 1H, H-5); $^{13}$C NMR ($CDCl_3$) δ 186.4 (C-4), 142.1 (C-3a), 141.4 (C-6a), 136.5 (C-3), 133.1 (C-1), 129.0 (C-5), 120.1 (C-6). Anal. calcd for $C_7H_2OCl_2S$: C, 41.00; H, 0.98; S, 15.64. Found: C, 39.82; H, 1.18; S, 15.79.

**1,3-Dibromo-4*H*-cyclopenta[*c*]thiophen-4-one (9b).** This was prepared from **1b** and purified using the same method and concentrations as for the synthesis of **9a**. Workup was performed identically, resulting in a yellow powder (40%): $^1$H NMR ($CDCl_3$) δ 7.29 (d, $J$ = 6 Hz, 1H, H-6), 6.23 (d, $J$ = 6 Hz, 1H, H-5); $^{13}$C NMR ($CDCl_3$) δ 206.9 (C-4), 141.7 (C-3a), 136.6 (C-6a), 124.9 (C-3), 113.9 (C-1), 109.6 (C-5), 106.0 (C-6). Anal. calcd for $C_7H_2OBr_2S$: C, 28.60; H, 0.69; S, 10.91. Found: C, 28.32; H, 0.78; S, 11.12.

**1-Bromo-4*H*-cyclopenta[*c*]thiophen-4-one (9c).** This was prepared from **1c** and purified using the same method and concentrations as for the synthesis of **9a**. Workup was performed identically, resulting in a yellow oil (20%): $^1$H NMR ($CDCl_3$) δ 7.96 (s, 1H, H-3), 7.69 (d, $J$ = 6 Hz, 1H, H-6), 6.29 (d, $J$ = 6 Hz, 1H, H-5). Anal. calcd for $C_7H_3OBrS$: C, 39.09; H, 1.41; S, 14.91. Found: C, 39.21; H, 1.52; S, 15.09.

**4*H*-Cyclopenta[*c*]thiophen-4-one (9d).** This was prepared from **1d** and purified using the same method and concentrations as for the synthesis of **9a**. Workup was performed identically, resulting in an yellow oil (20%): $^1$H NMR ($CDCl_3$) δ 7.72 (d, $J$ = 6 Hz, 1H, H-6); 7.71 (d, $J$ = 2 Hz, 1H, H-3), 7.52 (d, $J$ = 2 Hz, 1H, H-1), 7.51 (d, $J$ = 6 Hz, 1H, H-5). Anal. calcd for $C_7H_4OS$: C, 61.74; H, 2.96; S, 23.55. Found: C, 62.01; H, 3.12; S, 23.19.

**1,3-Diiodo-4*H*-cyclopenta[*c*]thiophen-4-one (9e).** This was prepared by refluxing for 1 h a solution of 0.5 g (0.001 mol) of **1e** in 50 mL of an 6 N aqueous HCl solution. After evaporation, the residual solid (60%) was washed with acetone, filtered, dried and recrystallized from isopropanol: Orange crystals, mp 260 °C; $^1$H NMR ($CDCl_3$) δ 7.30 (d, $J$ = 6 Hz, 1H, H-6); 6.30 (d, $J$ = 6 Hz, 1H, H-5). Anal. calcd for: $C_7H_2OI_2S$: C, 21.67; H, 0.52; S, 8.26. Found: C, 21.77; H, 0.81; S, 8.52.

***N*-(1,3-Dimethyl-6-oxo-5,6-dihydro-4H-cyclopenta[*c*] thien-4-yl)trifluoroacetamide (10g).** This was prepared by refluxing for 30 min a solution of 3 g (0.01 mol) of **14g** in 50 mL of $SOCl_2$. After evaporation of the solvent, the resulting green oil was triturated in petroleum ether. The precipitate was filtered, washed with petroleum ether and immediately dissolved in 50 mL of dichloromethane. To the solution was added 3.4 g (0.025 mol) of $AlCl_3$ and the reaction mixture was refluxed for 2 h. After cooling, the solution was poured into an iced 6 N aqueous HCl solution and then extracted twice with $Et_2O$. The organic layers were collected and the solvent was removed under reduced pressure. The resulting crystals (42%) were recrystallized from ether: White crystals, mp > 260 °C; $^1$H NMR (DMSO-$d_6$) δ 9.94 (d, $J$ = 8 Hz, 1H, NH), 5.29 (ddd, $J$ = 9, 8, 3 Hz, 1H, H-4), 3.29 (dd, $J$ = 19, 9 Hz, 1H, H-5a), 2.95 (dd, $J$ = 19, 3 Hz, 1H, H-5b), 2.49 (s, 3H, $CH_3$), 2.19 (s, 3H, $CH_3$). Anal. calcd for $C_{11}H_{10}NO_2F_3S$: C, 47.65; H, 3.64; N, 5.05. Found: C, 47.54; H, 3.69; N, 5.12.

**2,5-Dimethylthiophene-3-carboxaldehyde (11g).** This was prepared by adding dropwise at 0 °C a solution of 40 g (0.37 mol) of dimethylthiophene **12** in 100 mL of DMF to a Vilsmeier's complex, prepared starting from 133 mL (1.7 mol) of DMF and 290.4 g (1.9 mol) of $POCl_3$. The reaction mixture was stirred at room temperature for 30 min and then heated up to 80 °C for 3 h. After cooling to 10 °C, an alkaline hydrolysis was performed with a 2 N sodium hydroxide solution until pH = 10. Extraction of the solution with 200 mL of dichloromethane and then evaporation of the organic layer yielded a brown oil (43%) which was used without further purification: $^1$H NMR (DMSO-$d_6$) δ 9.88 (s, 1H, CHO), 6.97 (s, 1H, H-4), 2.35 (s, 3H, $CH_3$), 2.33 (s, 3H, $CH_3$);

$^{13}$C NMR (DMSO-$d_6$) δ 184.9 (CO), 150.7 (C-2), 137.1 (C-3), 136.7 (C-5), 123.7 (C-4), 14.5 ($CH_3$), 12.7 ($CH_3$).

**3-Amino-3-(2,5-dimethylthien-3-yl)propanoic acid (13g).** This was prepared by refluxing for 24 h a solution of 20 g (0.142 mol) of **11g**, 33 g (0.426 mol) of ammonium acetate and 14.9 g (0.142 mol) of malonic acid in 250 mL of ethanol. Crystals (53%) were formed, filtered and washed with hot ethanol: White crystals, mp $>260$ °C; $^1$H NMR (DMSO-$d_6$) δ 12.5 (bs, 1H, OH), 6.72 (s, 1H, H-4′), 4.38 (d, $J=7$ Hz, 1H, H-3), 3.7 (bs, 2H, $NH_2$), 2.61 (d, $J=7$ Hz, 2H, H-2), 2.36 (s, 3H, $CH_3$), 2.29 (s, 3H, $CH_3$); $^{13}$C NMR (DMSO-$d_6$) δ 172.1 (C-1), 138.2 (C-5′), 136.1 (C-3′), 132.6 (C-2′), 123.1 (C-4′), 47.6 (C-3), 39.8 (C-3), 14.8 ($CH_3$), 12.4 ($CH_3$). Anal. calcd for $C_9H_{13}NO_2S$: C, 54.25; H, 6.58; N, 7.03. Found: C, 54.40; H, 6.55, N, 7.09.

**3-(2,5-Dimethylthien-3-yl)-3-trifluoroacetylamino-propanoic acid (14g).** This was prepared by adding dropwise at 0 °C, 14.2 mL (0.1 mol) of trifluoroacetic anhydride to a stirred suspension of 13 g (0.05 mol) of **13g** in 100 mL of $Et_2O$. Stirring was maintained at room temperature for 30 min. After evaporation of the reaction mixture, the resulting colorless oil was dissolved in water. The solution was left to stand overnight. Crystals (98%) were formed, filtered, washed with water and dried: White crystals, mp $>260$ °C; $^1$H NMR (DMSO-$d_6$) δ 12.5 (bs, 1H, OH), 9.80 (d, $J=8$ Hz, 1H, NH), 6.68 (s, 1H, H-4′), 5.22 (ddd, $J=9$, 8, 6 Hz, 1H, H-3), 2.87 (dd, $J=16$, 9 Hz, 1H, H-2a), 2.66 (dd, $J=16$, 6 Hz, 1H, H-2b), 2.32 (s, 3H, $CH_3$), 2.31 (s, 3H, $CH_3$). Anal. calcd for $C_{11}H_{12}NO_3F_3S$: C, 44.74; H, 4.10; N, 4.75. Found: C, 44.81; H, 4.05; N, 4.88.

## Cytotoxic assays

The cytotoxic activity of tested compounds was evaluated in the NCI's in vitro human disease-oriented antitumor screen. This screening panel consists of 60 human tumor cell lines. Nine subpanels represent diverse histologies, that is nonsmall cell lung, renal, breast cancers, central nervous system, colon, melanoma, prostate, ovarian, and leukemia. Compounds were tested at a minimum of five concentrations at 10-fold dilutions. Results are evaluated in terms of specificity and potency. The cytotoxic effects of each compounds are expressed as the molar drug concentration required for 50% growth inhibition ($GI_{50}$).

## Hollow fiber assay

Human tumor cells were cultivated in polyvinylidene fluoride hollow fibers, and a sample of each cell line was implanted into each of two physiological compartments (intraperitoneal and subcutaneous) in mice. After treatment with tested compounds at each of two test doses using a QD×4 schedule, fiber cultures were collected and the viable cell mass was determined using a formazan dye conversion assay. A scoring system was developed to simplify evaluation of the results. The cell lines used were: MDA-MB-231 and MDA-MB-435 (breast cancer), NCI-H23 and NCI-H522 (lung cancer), OVCAR-3 and OVCAR-5 (ovarian cancer), SF-295 and U-251 (CNS cancer), LOX IMVI and UACC-62 (melanoma), COLO 205 and SW-620 (colon cancer).

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