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Design, synthesis and biological evaluation of Pontin ATPase inhibitors through a molecular docking approach

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ABSTRACT

A virtual screening strategy, through molecular docking, for the elaboration of an electronic library of Pontin inhibitors has resulted in the identification of two original scaffolds. The chemical synthesis of four candidates allowed extensive biological evaluations for their anticancer activity. Two compounds displayed an effect on Pontin ATPase activity, and one of them also exhibited a noticeable effect on cell growth. Further biological studies revealed that the most active compound induced apoptotic cell death together with necrosis, this latter effect being likely related to the cellular balance of ATP regulation.

Pontin and Reptin are ATPases that belong to the AAA+ (ATPases associated with diverse cellular activities) family.[1] As such, these proteins are implicated in multiple and very different cellular functions,[2] as reflected by the variety of names they are called, such as RuvBL1 (RuvB-like),[3] Rvb1,[4] TAP54a (TIP60 associated protein)[5] or TIP49 (TATA-box binding protein)[6–8] for Pontin, and RuvBL2, Rvb2, TAP54b or TIP48[9] for Reptin.[10] They contain characteristic Walker A and B domains, essential respectively for the binding and the hydrolysis of ATP and structural studies have highlighted their ability to form ring-shaped oligomeric complexes.[1,11,12] The crystallographic structure of Pontin alone was resolved as an hexamer in 2006,[13] and in 2011, a dodecameric assembly composed of two heterohexamers of alternating units of Pontin and Reptin has been published by the same team.[14] The structures display bound ADP for Pontin alone, or a mixture of bound ATP and ADP for the dodecamer of Pontin and Reptin. A similar heterododecameric structure has been reported by another group using cryo-electron microscopy.[15] The ATP/ADP binding site, which is located at the interface of two subunits, seems to involve residues from both chains.[13,14]

Recently, several reports have hinted at a link between Pontin and Reptin and various types of cancer.[16] We notably found that both proteins were overexpressed in human hepatocellular carcinoma where they are required for cell viability and proliferation.[17,18] They also interact with oncogenic transcription factors such as beta-catenin or c-Myc.[19,9] Finally, they are also required for the assembly and function of telomerase[20] and for the stability and function of mTOR.[21,22] As suggested by the use of mutants devoid of ATPase activity, most functions of these proteins require an ATPase activity, including those implied in cancer,[9,20–25] indicating that the inhibition of the ATPase activity of Pontin and/or Reptin could be of special interest for cancer therapy. In a previous Letter,[26] we have disclosed the discovery of the first four small molecules able to inhibit the ATPase activity of Pontin. This was achieved through the combination of molecular docking of compounds from commercially available libraries into the ATP binding site and in vitro ATPase assays. Further testing showed that one of these four molecules was competitive with ATP. Encouraged by these promising results we decided to look for Pontin inhibitors among original molecules from our laboratories. At this time, 4-hydroxy-2-pyridone and 4-hydroxy-2-quinolone moieties were of special interest to us because of their apparent similitude with quinones and napthoquinones. Starting from these scaffolds, simple chemical transformations were envisioned to introduce chemical diversity in an easy manner and prepare a set of innovative

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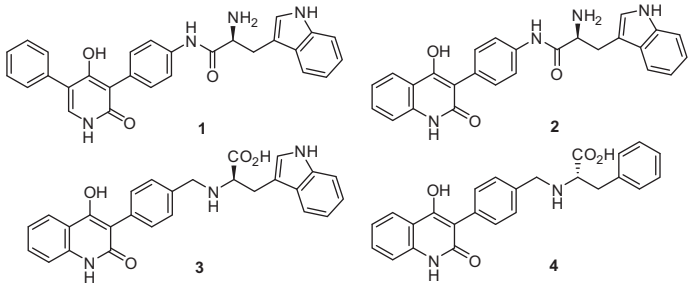
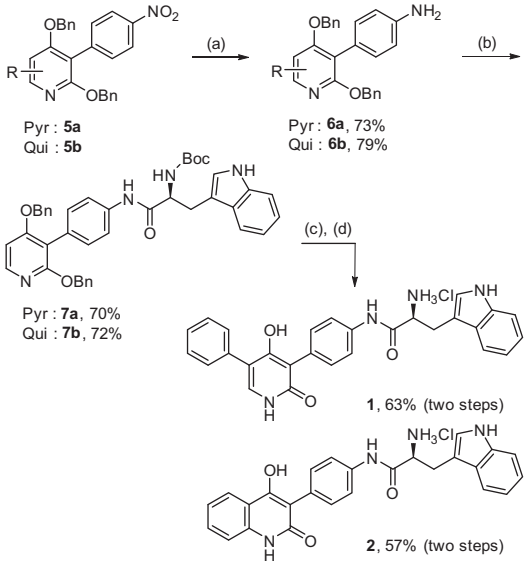
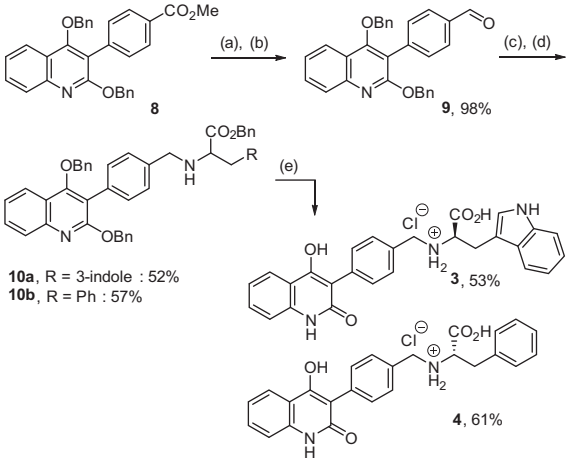


Figure 1. Selected structures from in silico studies to be prepared by chemical synthesis.



Scheme 1. Preparation of compounds **1**–**2**. Reagents and conditions: (a) Fe, acetic acid, reflux, 0.5 h; (b) Boc-(L)-Trp-OH, EDC, HOBt, di-isopropyl ethyl amine, DMF, 25 °C, 12 h; (c) TFA, CH_2Cl_2, 0–25 °C, 0.5 h; (d) H_2, Pd/C, MeOH, 25 °C, 12 h, then HCl.



Scheme 2. Preparation of compounds **3**–**4**. Reagents and conditions: (a) $LiAlH_4$, THF, 0–25 °C, 0.5 h; (b) stabilized 2-iodoxy benzoic acid, THF, 60 °C, 0.5 h; (c) L-phenylalanine benzyl ester or D-tryptophan benzyl ester, MS 4 Å, CH_2Cl_2, MeOH, 25 °C, 2 h; (d) $NaBH_4$, −5 °C, 3 h; (e) H_2, Pd/C, MeOH, 25 °C, 12 h, then HCl.

small compounds. Parallel to the organic chemistry effort, an in-house virtual chemical database based on the chosen scaffolds was simultaneously prepared, then extended along with the syntheses progress. This database contains all the compounds believed to be accessible through synthetic chemistry.

The already published[26] procedure was repeated in this study. Briefly, the crystal structure of the hexamer of Pontin alone with bound ADP was obtained from the Protein Data Bank, code 2C9O.[13] Two adjacent subunits, forming a complete ATP/ADP binding site, were extracted from the ring-shaped hexamer. The resulting homodimer was used as the receptor for docking. It was formed of one subunit as the main docking area, while the second sub-unit was positioned on top of the cavity, as if to forbid access to the exterior, as observed in the published crystal structure. Two missing loops in the crystal structure (residues 142–155 and 248–276) were not completed since they were far enough from the nucleotide binding site and did not impact the docking results. Water molecules were removed from the receptor, then missing hydrogen atoms were added while applying Charmm Forcefield in Discovery Studio 3.1 (Accelrys). The structure with bound ADP was minimized while the backbone was fixed, using a Steepest Descent algorithm (2000 step, gradient 0.01).

The virtual chemical database was prepared with Discovery Studio 3.1. Two original moieties of special interest within our group were chosen: pyridone[27] and quinolone.[28] Several modifications and implementations were proposed and introduced on the original scaffolds. The changes were oriented either by the experience of the organic chemists in the synthesis of this family of compounds, or by the potential activity increase brought by some functional groups, as suggested by previous virtual screening. If the envisioned chemical modifications were not judged feasible, they were not incorporated. The final database contained more than 800 original molecules designed for their easy access. All docking calculations were conducted using Autodock Vina 1.0.2.[29] The docking grid was a box with the following dimensions: $20 \times 22 \times 20$ Å, in order to incorporate all residues of the cavity with a margin of 3 Å in all directions. As already stated, 74 amino acids from the first sub-unit, plus 6 from the second sub-unit were included, at least partially, in the docking box. The whole receptor was kept rigid during the docking, while all the ligands were fully flexible. The poses obtained were rescored with DrugScore[30,31] and XScore[32] then consensus scoring determined the most probable ligands as already published.[26] A short list of 15 compounds was proposed to the chemists and 4 were immediately chosen to be prepared, because of their synthetic accessibility.

Molecular docking of molecules from the virtual database led us to the selection of several compounds, among which molecules **1**–**4**, depicted in Figure 1, were the most easily accessible for the campaign of chemical synthesis.

We recently described the preparation of 3-aryl-2,4-oxypyridines and 3-aryl-2,4-oxyquinolines through a practical Pd/C-catalyzed Suzuki–Miyaura reaction.[33] Thereby, with the aid of this efficient methodology, we prepared the 4-hydroxy-2-pyridone (Pyr) and 4-hydroxy-2-quinolone (Qui) cores of our targets **1**–**4**.

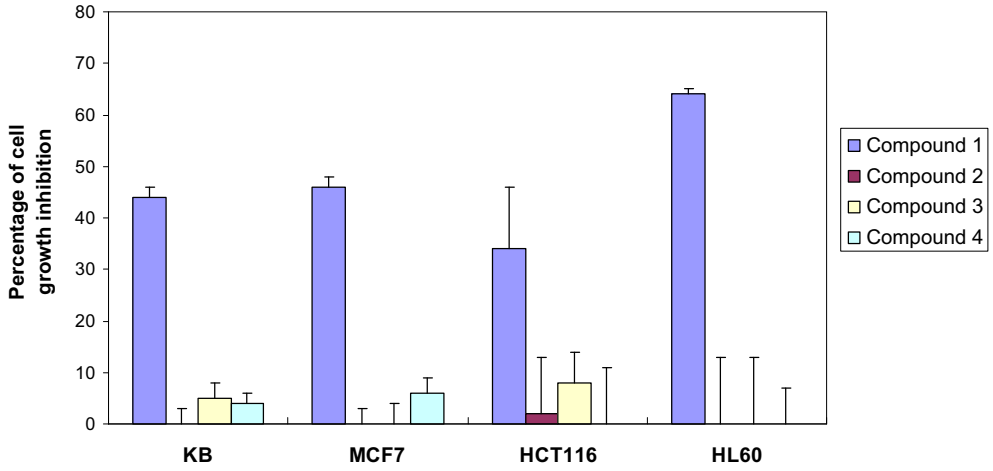


Figure 2. Growth inhibition in human cancer cell lines.

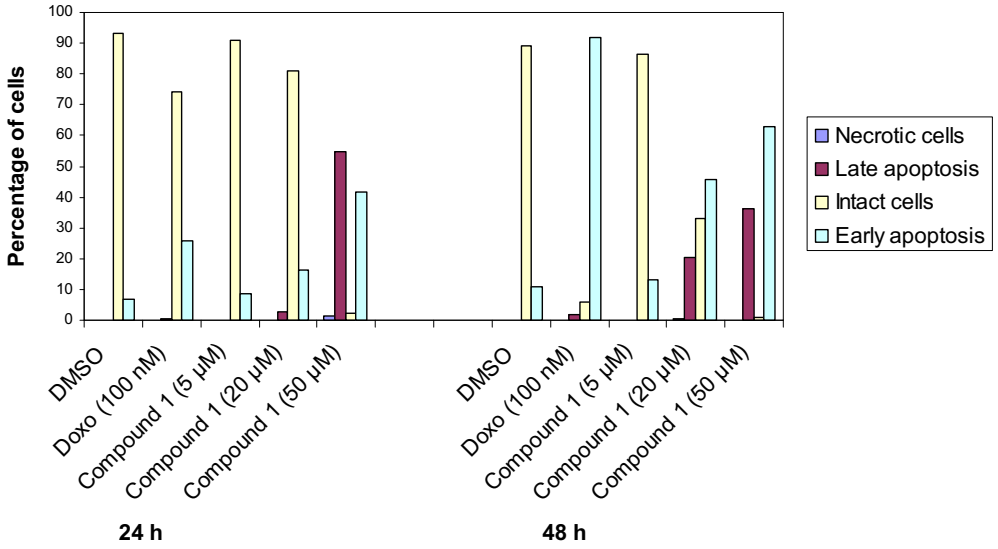


Figure 3. Apoptosis in HL60 cells treated with compound **1** (percentage of cells).

Table 1
Activation of caspase 3 in HL60 cells by compound **1**

	Caspase 3 activity	
	24 h	48 h
HL60	100 ± 7	100 ± 16
Doxo 1 µM	178 ± 6	1251 ± 18
1 5 µM	116 ± 5	119 ± 6
1 10 µM	111 ± 7	131 ± 12
1 20 µM	134 ± 15	247 ± 87
1 50 µM	306 ± 22	169 ± 29

The cleavage of DEVD was measured and expressed as a percentage of activity in control cells.

For instance, compounds **1**–**2** were synthesized from the nitro-derivatives **5a**–**b** following the sequence depicted in Scheme 1. Iron-mediated reduction of the nitro function led to the corresponding anilines **6a**–**b** in 73% and 79% yields respectively. Then, ligation of the latter, with *N*-Boc-L-tryptophan followed by protecting groups cleavage furnished the targeted compounds **1**–**2** with good overall yields.[34]

On the other hand, the preparation of compounds **3**–**4** started from the 3-(4-methylbenzoate)-2,4-benzyloxyquinoline **8** (Scheme 2). Conversion of the ester function into aldehyde was realized through an efficient two step sequence giving a quantitative yield of the expected compound **9**. The reductive amination of **9** with D-tryptophan benzyl ester and L-phenylalanine benzyl ester provided **10a** and **10b** in correct yields. Last, a Pd/C-catalyzed hydrogenolysis of the benzyl protecting groups furnished the expected targets **3** and **4**.

As mentioned, our previous study revealed that 4 commercial compounds were able to inhibit the ATPase activity of His-tagged purified Pontin in the micromolar range concentration. Three among these also inhibited cell tumor growth in vitro, indicating that the enzymatic assay could be helpful for the identification of novel inhibitors. The Malachite Green assays were conducted following the already published protocol.[26] The ATPase activity of the four synthesized molecules **1**–**4** were measured at a 100 µM concentration. From this study, we noticed that compounds **1** and **2** reduced the inorganic phosphate (Pi) release in a dose dependent manner. ATPase inhibitors **1** and **2** displayed an IC_{50} at 9 and 18 µM, respectively, and reduced the enzymatic activity

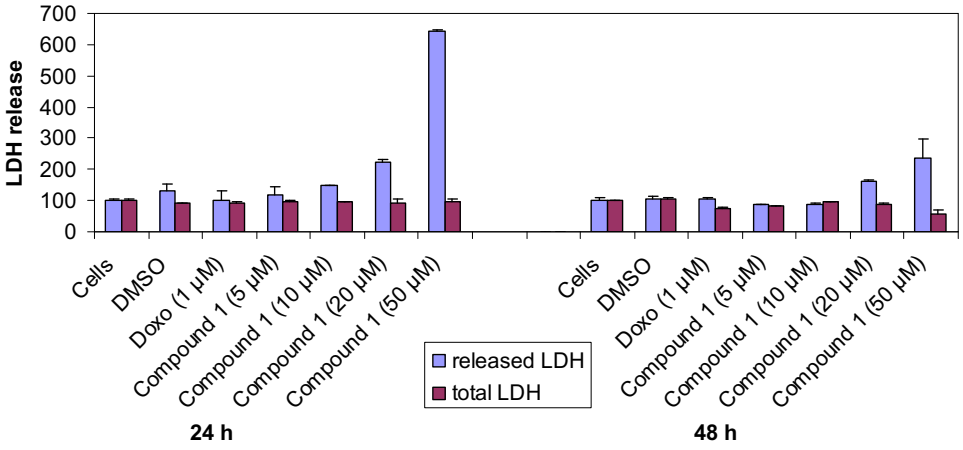


Figure 4. Total LDH and LDH release in the culture medium after treatment of HL60 cells with compound **1**.

Table 2
FACS analysis of HL60 cells treated with compound **1** for 24 h

	SubG1	G0/G1	S phase	G2/M
HL60 24 h	1.2	47.6	48.7	2.5
Doxo 50 nM	1.8	14.5	34.4	49.3
1 5 µM	2.7	48.3	44.7	4.3
1 20 µM	10.9	46.8	34.4	8.0
1 50 µM	69.5	14.7	14.6	1.3

DNA was tagged with propidium iodide and results expressed as the percentage of cells in the different phases of cell cycle.

by competing with ATP, in agreement with the in silico experiments and chemical design work.

By contrast, compounds **3** and **4** did not show significant inhibition, while being structurally related. From these very informative results, we tentatively established a preliminary structure–activity relationship. The calculated K_i of 4.5 and 9 µM for compounds **1** and **2**, respectively, suggested that the 3-hydroxy-5-phenyl-2-pyridone moiety was more suited for the inhibitory activity than the 3-hydroxy-2-quinolone core. Moreover, the absence of inhibition observed for compounds **3** and **4** in the enzymatic assay highlighted the crucial role of the α-aminoamide function (R^1–NH–CO–CH(NH_2)–R^2), characteristic of compounds **1** and **2**, for the interaction with the catalytic center.

Cell culture, cell proliferation assay, cell cycle analysis, flow cytometric detection of apoptosis, caspase activity assay and necrosis assay were performed as previously detailed.[35] Only compound **1** demonstrated an antiproliferative activity in cancer cell lines at 10^{-5} M (Fig. 2). IC_{50} of 9 and 15 µM were measured respectively for KB and HL60 cells after 72 h of exposure. The other synthesized molecules displayed no cytotoxic activity, including compound **2** despite its ability to partially inhibit Pontin ATPase activity in vitro. Compound **1** also reduced cell numbers with an IC_{50} of 25 µM in the non-dividing EPC cells, suggesting a cytotoxic effect.

The mode of action of compound **1** in HL60 cells was then investigated in more detail. First, apoptosis was evaluated with annexin and 7-AAD labeling using FACS analysis. A dose-dependent cell apoptosis was evidenced in HL60, demonstrating an early cell death after 24 h of exposure at a concentration of 20 µM, increasing at 48 h. At 50 µM, compound **1** induced 95% of cell death at 24 h (Fig. 3).

As it is generally assumed that caspase 3 is the terminal effector in the apoptotic cascade pathway, the activation of caspase 3 after treatment of HL60 cells with compound **1** was measured, using doxorubicin as positive control (Table 1).

After 24 h, caspase 3 activity was dose-dependently activated by treatment with compound **1**, with a 3-fold increase at 50 µM. Thus compound **1** is able to stimulate apoptosis through the activation of caspase 3.

However the moderate activation of the caspase 3 cannot account for the whole cytotoxic effect of compound **1**. Therefore, the release of cytosolic LDH into the culture medium as a marker for cell necrosis was also measured (Fig. 4).

After 24 h, the total LDH activity was not noticeably modified indicating that the cell number was unaffected at that time. However, compound **1** induced a clear release of LDH into the culture medium at concentrations above 20 µM indicating that cells entered the necrotic process. After 48 h, a dose-dependent decrease of total LDH paralleled the cytotoxic effect of compound **1** while the release of LDH remained observable. In the same conditions, 1 µM doxorubicin elicited a reduction in the total LDH content without any discernible leakage of the cytosolic LDH. Therefore we can conclude that necrosis is involved in the cell death mode of compound **1**. Finally, a possible effect of compound **1** on cell division was investigated. As shown in Table 2, doxorubicin promoted an early blockade of the cell cycle in phase G2/M. Compound **1** did not block the cell cycle but promoted a direct and dose-dependent accumulation of dead cells (SubG1 phase). This is in line with the cytotoxic effect of compound **1** in the non-dividing cell line EPC demonstrating that compound **1** could promote cell death irrespective of the cell cycle.

Guided by the information obtained from the initial virtual screening, a series of original molecules was synthesized and evaluated for their effect on Pontin and potential anti-cancer activity. Among the four selected candidates from the docking process, only compounds **1** and **2** displayed a comparable effect on Pontin ATPase activity. However, only compound **1** displayed a cytotoxic effect, while compound **2** had no noticeable effect on cell growth. This lack of activity of compound **2** could result from cell permeability constraint, or instability. Further measures of the biological activity of compound **1** emphasized the induction of apoptotic cell death together with necrosis. This latter effect could be directly related to the cellular balance of ATP regulation. With this new lead in hand, we are working on the establishment of a structure activity-relationship in order to find agents with lower IC_{50}. Parallel to this study, is the search for a deeper understanding of the mechanism of action on the biological target.

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34. *34(S)-1-(4-(4-Hydroxy-2-oxo-5-phenyl-1,2-dihydropyridin-3-yl)phenylamino)-3-(1H-indol-3-yl)-1-oxopropan-2-aminium chloride* **1**: Trifluoroacetic acid (TFA, 500 μL) was added dropwise to a stirred solution of **7a** (100 mg, 0.13 mmol, 1 equiv) in CH_2Cl_2 (500 μL) at 0 °C. The solution was stirred at 0 °C for 30 min then concentrated under vacuum to provide the amine as a TFA salt. The product was then diluted with EtOH and 1 M HCl and concentrated to provide the chloride salt. The residue was diluted with EtOH (5 mL) then Pd/C (10 mol %, 14 mg, 0.1 equiv) was added and the suspension was placed under H_2 atmosphere (balloon) and stirred at room temperature for 12 h. The solution was diluted with EtOH (20 mL) and heated to 60 °C then warm filtered over a celite pad. The cake was washed twice with warm EtOH. Combined organic fractions were concentrated to provide the crude compound. The product was purified by octadecyl-functionalized silica gel column chromatography (100% H_2O to 100% MeOH) to afford the chloride salt in 63% yield as an amorphous white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 8.04 (1H, d, J = 8.1 Hz), 7.69 (1H, d, J = 7.8 Hz), 7.60–7.00 (13H, m), 4.26 (1H, t, J = 7.0 Hz), 3.50 (1H, dd, J = 6.8 14.5 Hz), 3.37 (1H, dd, J = 6.8 14.5 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 167.6, 164.5, 160.3, 137.7, 136.87, 136.80, 131.6, 130.7, 129.2, 128.1, 126.9, 124.2, 123.3, 121.9, 120.4, 118.9, 117.8, 116.4, 115.0, 111.26, 111.20, 106.6, 54.3, 27.7; IR (ZnSe) ν 2967, 2902, 1706, 1614, 1575, 1471, 1410, 1401, 1375, 1336, 1211, 1057, 751, 701; HRMS (ESI): m/z calcd for $[(M+H)^+]$ = 465.1927, found = 465.1920.
(S)-1-(4-(4-Hydroxy-2-oxo-1,2-dihydroquinolin-3-yl)phenylamino)-3-(1H-indol-3-yl)-1-oxopropan-2-aminium chloride **2**: Following the procedure described for the synthesis of **1**, the compound was obtained from **7b** in 57% yield as an amorphous solid after purification by octadecyl-functionalized silica gel column chromatography (100% H_2O to 100% MeOH). ^{1}H NMR (300 MHz, CD_3OD) δ 7.69 (1H, d, J = 7.5 Hz), 7.57 (1H, d, J = 8.4 Hz), 7.51–7.30 (7H, m), 7.29–7.00 (3H, m), 4.26 (1H, t, J = 6.9 Hz), 3.50 (1H, dd, J = 6.6, 14.7 Hz), 3.37 (1H, dd, J = 7.5, 14.4 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 167.2, 162.5, 159.5, 137.0, 136.9, 134.1, 132.9, 131.4, 129.1, 128.8, 128.1, 127.3, 126.9, 124.3, 121.5, 120.4, 118.9, 117.8, 116.6, 111.3, 111.2, 106.5, 54.3, 27.5; IR (ZnSe) ν 3250, 3028, 2966, 2921, 1674, 1634, 1600, 1517, 1378, 1202, 1142, 1045, 834, 758, 675 cm^{-1}; HRMS (ESI): m/z calcd for $[(M+H)^+]$: 439.1765, found: 439.1801.
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