

**Synthesis of N-substituted 3,5-bis(arylidene)-4-piperidones  
with high antitumor and antioxidant activity**

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## Synthesis of *N*-substituted 3,5-bis(arylidene)-4-piperidones with high antitumor and antioxidant activity

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4 A series of 3,5-bis(arylidene)-4-piperidones (DAP compounds) are considered as synthetic  
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6 analogs of curcumin for anticancer and anti-inflammatory properties. We performed structure-  
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8 activity relationship studies by synthesizing a number of 3,5-bis(arylidene)-4-piperidones *N*-  
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10 alkylated or acylated with nitroxides or their amine precursors as potent antioxidant moieties.  
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12 Both substituents on arylidene rings and on piperidone nitrogen (five- or six-membered, 2- or  
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14 3- or 3,4-disubstituted, isoindoline nitroxides) were varied. The anticancer efficacy of the new  
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16 DAP compounds was tested by measuring their cytotoxicity to cancer cell lines A2780  
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18 (human epithelial ovarian cancer cell line) and MCF-7 (human breast cancer cell line) and to  
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20 H9c2, a noncancerous (healthy) cardiac cell line. The results showed that all DAP compounds  
21  
22 induced a significant loss of cell viability in both the human cancer cell lines tested, however  
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24 only pyrroline appended nitroxides (**5c**,<sup>1</sup> **5e**, **7**, **9**) showed limited toxicity toward  
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26 noncancerous cell lines. Computer docking simulations support the biological activity tested.  
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28 These results suggest that antioxidant-conjugated DAPs will be useful as a safe and effective  
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30 anticancer agent for cancer therapy.  
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## Introduction

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)1,6-heptadien-3,5-dion), a natural component of the rhizome of *curcuma longa*, proved to be a powerful chemopreventive and anticancer agent<sup>2-5</sup> having also anti-inflammatory<sup>6</sup>, antibacterial<sup>7</sup> and antioxidant properties.<sup>8</sup> However, the clinical use of curcumin has been limited due to its low anticancer activity and poor bioabsorption. In the last decade, a novel class of curcumin analogs, diarylidene piperidones (DAPs) has been developed by incorporating a piperidone link to the beta-diketone structure and fluoro-, methoxy-, hydroxyl-, chloro-, nitro-, dimethylamino substituents on the phenyl group.<sup>9,10</sup> These curcumin analogs exhibited multi-drug-resistance reverting<sup>11,12</sup> and antimycobacterial<sup>13</sup> properties as well. The idea of evaluation of these compounds as antineoplastic agents is based on the assumption that these compounds may be considered as Mannich base of dienone and  $\alpha,\beta$ -unsaturated ketones display anticancer properties via a mechanism of action comprising interactions with cellular thiols with little or no affinity for hydroxyl and amino group in nucleic acids. The 1,5-diaryl-3-oxo-1,4-pentadienyl groups considered to react at a primary binding site, however the bioactivity will be influenced by others structural units: such as acylating piperidone nitrogen increased cytotoxic potencies and increasing the electron-withdrawing properties of substituents on aromatic ring has advantageous effect on cytotoxicity.<sup>14-18</sup> However, dimethylenbridge between C2-C6 atoms in piperidone was accompanied by reduction of cytotoxic properties exerting a steric impedence to alignment at one or more binding sites as well as variation of hydrophobicity and hence membrane transportation properties.<sup>16</sup>

In general the DAP compounds were more effective than curcumin in inhibiting the proliferation of a variety of cancer cell lines. EF24, with ortho-fluorinated phenyl group exhibited anticancer activity *in vitro* when tested using breast cancer, colon cancer and ovarian epithelial cancer.<sup>19-21</sup> Its para-fluorinated derivative H-4073 was more potent than

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2  
3 EF24 in inducing cytotoxicity to ovarian cancer cells.<sup>21-23</sup> DAP compounds have also been  
4 shown to be more readily bioavailable than the parent compound, curcumin.[Dayton A, et al,  
5  
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8 2010]

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10 A nonspecific cytotoxic compound may have side effects caused by damage to normal cells.  
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12 Many chemotherapeutic agents act by producing free radicals, causing oxidative stress in  
13 normal cells.<sup>24</sup> It is well known that nitroxides or their precursors (hydroxylamines and  
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3 The new N-acyl-3,5 bis(4- fluorobenzilydene)piperidin-4-ones were prepared by treatment of  
4 compound **1** with freshly prepared paramagnetic acyl chloride **2a**<sup>29</sup> in the presence of Et<sub>3</sub>N in  
5 CH<sub>2</sub>Cl<sub>2</sub> or treatment with isocyanate **2b**<sup>30</sup> generated in situ by Curtius-rearrangement of acyl  
6 azide in THF to yield compound **3a** and **3b**. The N-alkyl 3,5-bis(4-  
7 fluorobenzilydene)piperidin-4-ones were achieved by alkylation of compound **1** with  
8 equivalent amount paramagnetic alkyl halides **4a**,<sup>31</sup> **4b**,<sup>31</sup> **4c**,<sup>32</sup> **4d**,<sup>33</sup> **4e**,<sup>34</sup> **4f**,<sup>35</sup> **4g**<sup>36</sup> in  
9 acetonitrile in the presence of K<sub>2</sub>CO<sub>3</sub> to give compounds **5a**, **5b**, **5c**, **5d**, , **5f**, **5g**, **5h**.  
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Compound **5e** was synthesized by reduction of nitroxide function of compound **5c** with iron powder in glacial acetic acid<sup>37</sup> (Scheme 1).

Alkylation of 3,5-bis(2-fluorobenzylidene)piperidin-4-one **6**<sup>19</sup>, 3,5-bis(4-trifluoromethylbenzylidene)piperidin-4-one **8** and with 3,5-bis(2,3,4-trimethoxybenzylidene)piperidin-4-one **10** with compound **4c** as above yielded compounds **7**, **9**, **11** respectively (Scheme 2). Compounds **8** and **10** were synthesized by condensation of piperidine-4-one HCl (**12**) salt with 4-trifluoromethyl benzaldehyde or 2,3,4-trimethoxybenzaldehyde in AcOH saturated previously with HCl gas (Scheme 3).

## Results and discussion

The anticancer efficacy of the DAP compounds with various substituents on aromatic rings and on piperidone nitrogen was evaluated by measuring the cytotoxicity of the compounds to well-established cancer cell lines, namely A2780 (human epithelial ovarian cancer cell line) and MCF-7 (human breast cancer cell line) using MTT assay. The measurements were performed by exposing the cells to 10- $\mu$ M concentration of the compound for 24 h. The results, in the form of percent cell viability as compared to respective control, are summarized in Tables 1-3. The results showed that all DAP compounds induced a significant loss of cell viability in both the human cancer cell lines tested. In particular 3,5-bis(arylidene)-4-

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3 piperidone compounds without nitroxide tag (**1**, **6**, **8**, **10**) demonstrated a substantial cytotoxic  
4 effect against A2780 and MCF-7 cells. The electron-withdrawing substituents (F, CF<sub>3</sub>)  
5 containig derivatives (**1**, **6**, **8**) exhibited greater cytotoxicity than **10** trimethoxy derivative  
6 (**10**) in accordance with previous findings.<sup>14,15</sup> In all caseses the toxicity can be increased by  
7 modifying the 3,5-bis(arylidene)-4-piperidone compounds with nitroxides by acylation (**3a**,  
8 **3b**) and by alkylation (**5a-h**, **7**, **9**, **11**). In particular **3a**, **3b** demonstrated a substantial  
9 cytotoxic effect against A2780 and MCF-7 cells. Comparable cytotoxic efficacies were  
10 observed with **5c**, **5e**, **5f** and **9** derivatives. The results further indicated that the DAPs were  
11 more cytotoxic to ovarian (A2780) cancer cell when compared to breast cancer cell.  
12  
13 Compounds containing 2-substituted pyrrolidine nitroxide (**5a**, **5b**), 3,4-disubstituted  
14 nitroxide (**5d**), isoindoline-type nitroxides (**5g**, **5h**) exhibited limited toxicity toward breast  
15 cancer cell lines.  
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19 We also compared the cytotoxicity of DAPs to a noncancerous (healthy) cardiac cell line,  
20 namely H9c2, an undifferentiated neonatal rat cardiomyoblast. Most of the compounds  
21 induced a significant loss of cell viability, although to different extents (Table 1-3), the  
22 pyrroline-appended DAPs, **5c**, **5e** and **7** were significantly less toxic to the healthy cell.  
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24 Particularly, the results of **5c** seem to suggest a strikingly differential effect on cancer *versus*  
25 noncancerous cells. The compound **6**, which was toxic to breast cancer cell, was toxic to  
26 healthy cells to the same extent. In addition, this differential effect could stem from the *N*-  
27 hydroxypyrroline function. Overall the viability results seem to implicate the  
28 diarylidenylpiperidone group in inducing cytotoxicity and *N*-hydroxypyrroline group in  
29 protecting noncancerous cells.  
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33 We recently reported the anticancer efficacy of four DAPs, namely **1** and **8** without NOH  
34 function and **5c** and **9** with NOH function against a number of cancerous (breast, colon, head  
35 and neck, liver, lung, ovarian, and prostate cancer) and noncancerous (smooth muscle, aortic  
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3 endothelial, and ovarian surface epithelial cells) human cell lines.<sup>22</sup> We observed that all four  
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5 compounds induced significant loss of cell viability in cancer cells, while **5c** and **9** showed  
6  
7 significantly less cytotoxicity in noncancerous (healthy) cells. Electron paramagnetic  
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9 resonance (EPR) measurements showed a metabolic conversion of the *N*-hydroxylamine  
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11 function to nitroxide with significantly higher levels of the metabolite and superoxide radical-  
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13 scavenging (antioxidant) activity in noncancerous cells when compared to cancer cells.<sup>22</sup>  
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15 Among the new compound synthesized compound **5c** (HO-3867) exhibited the best selective  
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17 toxicity against cancerous cell. Hence this compound was chosen as a new lead compound for  
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19 further evaluations. Western-blot analysis showed that the DAP-induced growth arrest and  
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21 apoptosis in cancer cells were mediated by inhibition of STAT3 phosphorylation at Tyr705  
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23 and Ser727 residues and induction of apoptotic markers of cleaved caspase-3 and PARP  
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25 suggesting that the antioxidant-conjugated DAPs will be useful as a safe and effective  
26  
27 anticancer agent for cancer therapy.<sup>22</sup> In a subsequent study, we further established the  
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29 anticancer efficacy of **5c** in a number of established human ovarian cancer cell lines (A2870,  
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31 A2780cDDP, OV-4, SKOV3, PA-1 and OVCAR3), as well as in a murine xenograft tumor  
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33 (A2780) model.<sup>1</sup> Compound **5c** (HO-3867) demonstrated a preferential toxicity towards  
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35 ovarian cancer cells, while sparing healthy cells. It induced G2/M cell-cycle arrest in A2780  
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37 cells by modulating cell-cycle regulatory molecules p53, p21, p27, cdk2 and cyclin, and  
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39 promoted apoptosis by caspase-8 and caspase-3 activation. It also caused an increase in the  
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41 expression of functional Fas/CD95 and decreases in STAT3 (Tyr705) and JAK1  
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43 phosphorylation. There was a significant reduction in STAT3 downstream target protein  
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45 levels including Bcl-xL, Bcl-2, survivin, and vascular endothelial growth factor (VEGF),  
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47 suggesting that **5c** exposure disrupted the JAK/STAT3-signaling pathway. In addition,  
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49 compound **5c** significantly inhibited the growth of the ovarian xenografted tumors in a  
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51 dosage-dependent manner without any apparent toxicity. Western-blot analysis of the  
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3 xenograft tumor tissues showed that compound **5c** inhibited pSTAT3 (Tyr705 and Ser727)  
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5 and JAK1 and increased apoptotic markers cleaved caspase-3 and PARP. Overall, compound  
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8 **5c** exhibited significant cytotoxicity towards ovarian cancer cells by inhibition of the  
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10 JAK/STAT3-signaling pathway.<sup>1</sup>  
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### 13 14 15 **Molecular docking**

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17 We performed theoretical modeling calculations using AutoDock (version 4.2) to elucidate  
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19 the mode of STAT3-inhibition by these compounds using *in silico* docking simulations. The  
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21 simulation results have provided additional support for the hypothesis that the mechanism of  
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23 action of the DAP compounds is via targeting of the STAT3 pathway. The computations  
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25 demonstrate that the DAP compounds have high docking affinity for the STAT3 dimer (PDB  
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27 ID: 3CWG) at the DNA binding domain of the molecule (Table 4, Figure 1). This, in turn,  
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29 would prevent the activated STAT3 molecule from binding with DNA, thereby inhibiting  
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31 transcription of downstream signaling. The *N*-hydroxypyrroline-bearing DAP molecules were  
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33 found to have substantially higher binding affinity than their non-antioxidant promoting  
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35 counterparts. Table 4 lists the tested compounds, and the 5 lowest calculated binding energies  
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37 and the corresponding constants of inhibition ( $K_i$ , nmol). The DAP compound binding affinity  
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39 to the STAT3 dimer determined is to be ranked as follows: HO-3867 > HO-4200 >> H-4073  
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41 > HO-4138. The rankings also correlate well with the biological activity. Many small  
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43 molecules identified as potential drug candidates have reported binding energies in the -7 to -  
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45 9 kcal/mol range, but few of these potential drug candidates have  $K_i$  values <50 nM. In  
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47 addition, many STAT3 inhibitors target the SH2 binding-domain of the molecule, whereas the  
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49 DAP compounds favor the DNA-binding region. The identification of a previously-  
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51 unreported docking site for molecular inhibition of STAT3 activity would be of significant  
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53 benefit for future drug design.  
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## Conclusions

The present study demonstrated that the earlier known 3,5-bis(arylidene)-4-piperidones (DAP compounds) can be *N*-alkylated or acylated with nitroxides or their amine precursors as potent antioxidant moieties. Measurement of the cytotoxicity of the new compounds to cancer cell lines A2780 (human epithelial cancer cell line) and MCF-7 (human breast cancer cell line) and to H9c2 noncancerous (healthy) cardiac cell line has shown that the modified compounds are more effective as anticancer compounds, but at the same time was less toxic to noncancerous (healthy) cells. Computer docking simulations support the empirical data collected. Among the compounds tested HO-3867 (**5c**) was chosen as lead compound for further studies. These results support the earlier findings that nitroxides and their precursors do not compromise the anticancer effect of the modified molecules, but they have beneficial effect on the original activity.

## Experimental Section

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on Fisons EA 1110 CHNS elemental analyzer. Mass spectra were recorded on a Thermoquest Automass Multi and VG TRIO-2 instruments and in the EI mode. <sup>1</sup>H NMR spectra were recorded with Varian UNITYINOVA 400 WB spectrometer. Chemical shifts are referenced to Me<sub>4</sub>Si. Measurements were run at 298K probe temperature in CDCl<sub>3</sub> solution. ESR spectra were taken on Miniscope MS 200 in 10<sup>-4</sup> M CHCl<sub>3</sub> solution and monoradicals gave triplett line.

Flash column chromatography was performed on Merck Kieselgel 60 (0.040-0.063 mm). Qualitative TLC was carried out on commercially prepared plates (20 x 20 x 0.02 cm) coated with Merck Kieselgel GF<sub>254</sub>. All chemicals were purchased from Aldrich, compounds **1**,<sup>10</sup> **4a**,<sup>31</sup> **4b**,<sup>31</sup> **4c**,<sup>32</sup> **4d**,<sup>33</sup> **4e**,<sup>34</sup> **4f**,<sup>35</sup> **4g**,<sup>36</sup> **5c**,<sup>1</sup> **6**<sup>19</sup> was prepared as described earlier.

**3,5-Bis(4-fluorobenzylidene)-1-[(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)carbonyl]piperidin-4-one Radical (3a):**

To a solution of compound **1** HCl salt (1.73g, 5.0 mmol) and Et<sub>3</sub>N (1.0 g, 10.0 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (35 mL) freshly prepared **2a** (1.01 g, 5.0 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was added dropwise at 0 °C and the mixture was allowed to warm to rt. and stirred for 1h. The organic phase was washed with brine (20 mL), the organic phase was separated, dried (MgSO<sub>4</sub>), filtered and evaporated. The residue was purified by flash column chromatography (CHCl<sub>3</sub>/Et<sub>2</sub>O) to yield the title compound as yellow solid 1.26 g (49%), mp 168-170 °C, R<sub>f</sub> 0.55 (CHCl<sub>3</sub>/Et<sub>2</sub>O, 2:1). MS (EI) m/z (%): 477 (M<sup>+</sup>, 6), 463 (15), 447 (10), 310 (30), 133 (100). Anal calcd. for C<sub>28</sub>H<sub>27</sub>F<sub>2</sub>N<sub>2</sub>O<sub>3</sub> C 70.43, H 5.70, N 5.87, found: C 70.24, H 5.88, N 6.01.

**3,5-Bis(4-fluorobenzylidene)-4-oxo-N-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)piperidine-1-carboxamide Radical(3b):**

To a solution of compound **1** (933 mg, 3.0 mmol) in anhydr. THF (40 mL) compound **2b** (627 mg, 3.0 mmol) was added and the mixture was heated under reflux for 4h. After colling the THF was evaporated off under reduced pressure, the residue was partitioned between CH<sub>2</sub>Cl<sub>2</sub> (40 mL) and brine (10 mL), the organic phase was washed with 10% aq. K<sub>2</sub>CO<sub>3</sub> (20 mL), water (10 mL), the organic phase was separated, dried (MgSO<sub>4</sub>), filtered and evaporated. The residue was purified by flash column chromatography (CHCl<sub>3</sub>/MeOH) to yield the title compound as yellow solid 915 mg (62%), mp 175-177 °C, R<sub>f</sub> 0.43 (CHCl<sub>3</sub>/Et<sub>2</sub>O, 2:1). MS

(EI) m/z (%): 492 (M<sup>+</sup>, 1), 462 (1), 460 (8), 310 (22), 133 (100). Anal calcd. for C<sub>28</sub>H<sub>28</sub>F<sub>2</sub>N<sub>3</sub>O<sub>3</sub> C 68.28, H 5.73, N 8.53, found: C 68.12, H 5.90, N 8.68.

**General procedure for *N*-alkylation of 3,5-Bis(arylidene)piperidin-4-one (5a, 5b, 5c, 5d, 5f, 5g, 5h, 7, 9, 11):**

A mixture of **1** or **6** or **8** or **10** HCl salt (5.0 mmol), K<sub>2</sub>CO<sub>3</sub> (1.38g, 10.0 mmol) in acetonitrile (20 mL) was stirred at rt. for 30 min., then alkyl bromide **4a-h** (5.0 mmol) was added dissolved in acetonitrile (5 mL) and the mixture was stirred and refluxed till the consumption of the starting materials (~3 h). After cooling the inorganic salts were filtered off on sintered glass filter, washed with CHCl<sub>3</sub> (10 mL), the filtrate was evaporated and the residue was partitioned between CHCl<sub>3</sub> (20 mL) and water (10 mL). The organic phase was separated, the aqueous phase was washed with CHCl<sub>3</sub> (20 mL), the combined organic phase was dried (MgSO<sub>4</sub>), filtered and evaporated. The residue was purified by flash column chromatography (Hexane/EtOAc) to give the title compounds in 35-71%.

**3,5-Bis(4-fluorobenzylidene)-1-[(2*E*)-3-(1-oxyl-2,5,5-trimethylpyrrolidin-2-yl)prop-2-enyl]piperidin-4-one Radical (5a)** mp 168-170 °C (2HCl salt), R<sub>f</sub> 0.62 (hexane/EtOAc, 2:1). MS (EI) m/z (%): 477 (M<sup>+</sup>, 2), 311 (14), 282 (17), 133 (100). Anal calcd. for C<sub>29</sub>H<sub>34</sub>F<sub>2</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub> C 63.16, H 6.21, N 5.08, found: C 62.99, H 6.08, N 6.15.

**3,5-Bis(4-fluorobenzylidene)-1-[3-(1-oxyl-2,5,5-trimethylpyrrolidin-2-yl)prop-2-ynyl]piperidin-4-one Radical (5b)** mp 132-134 °C (2HCl salt), R<sub>f</sub> 0.62 (hexane/EtOAc, 2:1). MS (EI) m/z (%): 475 (M<sup>+</sup>, 1), 461 (1), 311 (12), 148 (100). Anal calcd. for C<sub>29</sub>H<sub>32</sub>F<sub>2</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub> C 63.39, H 5.87, N 5.10, found: C 63.28, H 6.00, N 5.05.

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**3,5-Bis(4-fluorobenzylidene)-1-[(1-oxyl-2,2,3,5,5-petramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl]piperidin-4-one Radical (5d):** mp 178-180 °C  $R_f$  0.50 (hexane/EtOAc, 2:1). MS (EI)  $m/z$  (%): 477 ( $M^+$ , 13), 447 (6), 404 (10), 303 (29), 133 (100). Anal calcd. for  $C_{29}H_{31}F_2N_2O_2$  C 72.94, H 6.54, N 5.87, found: C 72.92, H 6.65, N 5.82.

**3,5-Bis(4-fluorobenzylidene)-1-[4-(1-oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydro-1H-pyridin-4yl)methyl]piperidin-4-one Radical (5f):** mp 157-159 °C,  $R_f$  0.43 (hexan/EtOAc, 2:1). MS (EI)  $m/z$  (%): 477 ( $M^+$ , 5), 447 (7), 324 (93), 133 (70), 121 (100). Anal calcd. for  $C_{29}H_{31}F_2N_2O_2$  C 72.94, H 6.54, N 5.87, found: C 73.00, H 6.52, N 5.83.

**3,5-Bis(4-fluorobenzylidene)-1-[5-(2-oxyl-1,1,3,3-tetramethyl-1,3-dihydro-2H-isoindol)methyl]piperidin-4-one Radical (5g):** mp 169-172 °C,  $R_f$  0.46 (hexan/EtOAc, 2:1). MS (EI)  $m/z$  (%): 513 ( $M^+$ , 24), 499 (30), 483 (11), 310 (25), 133 (100). Anal calcd. for  $C_{32}H_{31}F_2N_2O_2$  C 74.83, H 6.08, N 5.45, found: C 74.85, H 6.10, N 5.53.

**3,5-Bis(4-fluorobenzylidene)-1-[6-(2-oxyl-1,1,3,3-tetramethyl-1,3-dihydro-2H-pyrrolo[3,4-c]pyridine)methyl]piperidin-4-one Radical (5h):** mp 155-157 °C,  $R_f$  0.11 (hexan/EtOAc, 2:1). MS (EI)  $m/z$  (%): 514 ( $M^+$ , < 1), 411 (15), 311 (12), 133 (100). Anal calcd. for  $C_{31}H_{30}F_2N_3O_2$  C 62.69, H 5.82, N 5.22, found: C 62.80, H 5.90, N 8.13.

**3,5-Bis(2-fluorobenzylidene)-1-[(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl]piperidin-4-one Radical (7):** mp 195-197 °C (2 HCl salt),  $R_f$  0.75 ( $CHCl_3/Et_2O$ , 2:1). MS (EI)  $m/z$  (%): 463 ( $M^+$ , 6), 433 (8), 327 (37), 310 (40), 133 (100). Anal calcd. for  $C_{28}H_{31}Cl_2F_2N_2O_2$  C 72.36, H 5.82, N 5.22, found: C 72.20, H 6.00, N 5.13.

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6 **3,5-Bis(4-trifluoromethylbenzylidene)-1-[(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-**  
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8 **pyrrol-3-yl)methyl]piperidin-4-one Radical (9):** mp 188-190 °C, R<sub>f</sub> 0.36 (CHCl<sub>3</sub>/Et<sub>2</sub>O,  
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10 2:1). MS (EI) m/z (%): 563 (M<sup>+</sup>, 6), 533 (5), 490 (16), 424 (100), 410 (60), 107 (83). Anal  
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12 calcd. for C<sub>30</sub>H<sub>29</sub>F<sub>6</sub>N<sub>2</sub>O<sub>2</sub> C 63.94, H 5.19, N 4.97, found: C 63.80, H 5.11, N 4.88.  
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20 **3,5-Bis(2,3,4-trimethoxybenzylidene)-1-[(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-**  
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22 **pyrrol-3-yl)methyl]piperidin-4-one Radical (11):** mp 186-188 °C, R<sub>f</sub> 0.42 (CHCl<sub>3</sub>/Et<sub>2</sub>O,  
23  
24 2:1). MS (EI) m/z (%): 607 (M<sup>+</sup>, 1), 577 (5), 424 (21), 191 (100). Anal calcd. for C<sub>34</sub>H<sub>43</sub>N<sub>2</sub>O<sub>8</sub>  
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26 C 67.20, H 7.13, N 4.61, found: C 67.25, H 7.02, N 4.56.  
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32 **3,5-Bis(4-fluorobenzylidene)-1-[(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-**  
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34 **yl)methyl]piperidin-4-one (5e):** To a solution of nitroxide **5c** (2.31 g, 5.0 mmol) in AcOH  
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36 (25 mL) iron powder (2.8g, 50.0 mmol) was added and the mixture was stirred at 60 °C for 30  
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38 min. After cooling, the reaction mixture was diluted with water (40 mL) and filtered. The  
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40 filtrate was basified with solid K<sub>2</sub>CO<sub>3</sub> to pH=8 (intensive foaming). The aq. phase was  
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42 extracted with CHCl<sub>3</sub> containing 10% MeOH (2 x 30 mL) and the combined organic phase  
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44 was dried (MgSO<sub>4</sub>), filtered and evaporated. The residue was purified by flash column  
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46 chromatography (CHCl<sub>3</sub> / MeOH) to give the title compound as a yellow solid 1.25g (56%),  
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48 mp 158-160 °C, R<sub>f</sub> 0.31 (CHCl<sub>3</sub>/MeOH, 9:1). MS (EI) m/z (%): 448 (M<sup>+</sup> <1), 433 (12), 324  
49  
50 (13), 133 (56), 124 (100). Anal calcd. for C<sub>28</sub>H<sub>30</sub>F<sub>2</sub>N<sub>2</sub>O C 74.98, H 6.54, N 5.87, found: C  
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52 75.01, H 6.68, N 5.70. <sup>1</sup>H NMR (CD<sub>3</sub>OD) : 7.73 (s, 2H); 7.46 (q, J=15.4 Hz, ArH, 4H); 7.17  
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54 (t, J = 8.7 Hz, ArH, 4H) 5.38 (s, CH, 1H); 3.82, (s, N(CH<sub>2</sub>)<sub>2</sub>, 4H); 3.18 (s, CH<sub>2</sub>, 2H); 1.12 (s,  
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56 CH<sub>3</sub>, 6H); 0.99 (s, CH<sub>3</sub>, 6H).  
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**3,5-Bis[4-(trifluoromethyl)benzylidene]piperidin-4-one (8):**

A solution of 4-(trifluoromethyl)benzaldehyde (3.48 g, 20.0 mmol) and 4-piperidone hydrate hydrochloride (1.53 g, 10.0 mmol) was allowed to stay in glacial acetic acid (saturated with HCl gas previously) for 2 days. The precipitated yellow solid was filtered, washed with Et<sub>2</sub>O (30 mL) and the yellow hydrochloride salt 3.30g (74%) was air-dried and used in the next step without further purification. For analytical characterization 300 mg of the salt was dissolved in water (10 mL) and basified by addition of 250 mg K<sub>2</sub>CO<sub>3</sub> and extracted with CHCl<sub>3</sub> (3 x 10 mL). The combined extracts were dried (MgSO<sub>4</sub>), filtered and evaporated to give yellow solid mp 195-197 °C, R<sub>f</sub> 0.40 (CHCl<sub>3</sub>/Et<sub>2</sub>O, 2:1). MS (EI) m/z (%): 411 (M<sup>+</sup>, 23), 382 (16), 284 (69), 115 (100). Anal calcd. for C<sub>21</sub>H<sub>15</sub>F<sub>6</sub>NO C 61.32, H 3.68, N 3.41, found: C 61.22, H 3.63, N 3.31. <sup>1</sup>H NMR (CD<sub>3</sub>OD) : 7.79 (s, 2H); 7.74 (d, J= 8 Hz, ArH, 4H); 7.62 (d, J= 8 Hz, ArH, 4H), 4.12 (s, N(CH<sub>2</sub>)<sub>2</sub>, 4H).

**3,5-Bis(2,3,4-trimethoxybenzylidene)piperidin-4-one (10):**

A solution of 2,3,4-trimethoxybenzaldehyde (3.92 g, 20.0 mmol) and 4-piperidone hydrate hydrochloride (1.53 g, 10.0 mmol) was allowed to stay in glacial acetic acid (saturated with HCl gas previously) for 2 days. The precipitated yellow solid was filtered, washed with Et<sub>2</sub>O (30 mL) and the yellow hydrochloride salt 3.04g (62%), mp 211-214 °C was air-dried and used in the next step without further purification. For analytical characterization 300 mg of the salt was dissolved in water (10 mL) and basified by addition of 250 mg K<sub>2</sub>CO<sub>3</sub> and extracted with CHCl<sub>3</sub> (3 x 10 mL). The combined extracts were dried (MgSO<sub>4</sub>), filtered and evaporated to give yellow solid R<sub>f</sub> 0.41 (CHCl<sub>3</sub>/MeOH, 9:1). MS (EI) m/z (%): 455 (M<sup>+</sup>, 13), 424 (100), 191(63). <sup>1</sup>H NMR (CD<sub>3</sub>OD) : 8.17 (s, 2H); 7.05 (d, J=8.7 Hz, ArH, 2H); 6.90 (d,

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3  $J=8.7$  Hz, ArH, 2H) 4.47, (s, N(CH<sub>2</sub>)<sub>2</sub>, 4H); 3.91 (s, OCH<sub>3</sub>, 6H); 3.89 (s, OCH<sub>3</sub>, 6H), 3.84 (s,  
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6 OCH<sub>3</sub>, 6H).

### 10 **General procedure for synthesis of hydroxylamine salts:**

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12 To achieve the *N*-hydroxy compound HCl salt, nitroxide (**3a-b**, **5a-d**, **5f-h**, **7**, **9**, **11**) was  
13 dissolved in EtOH (20 mL, saturated with HCl gas previously) and refluxed for 30 min., then  
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15 solvent was evaporated off and the procedure was repeated till the disappearance of the EPR  
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17 triplet line (taken from a sample dissolved in MeOH, 10<sup>-4</sup> M).  
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### 25 **Cell lines and cultures**

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27 The cytotoxicity assays were performed using the following cell lines: A2780 (human  
28 epithelial ovarian cancer cell line), MCF-7 (human breast cancer cell line), and H9c2  
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30 (undifferentiated neonatal rat cardiomyoblasts). The A2780 and MCF-7 cells were grown in  
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32 RPMI 1640 medium and H9c2 cells were grown in Dulbecco's modified Eagle's medium. The  
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34 medium was supplemented with 10% FBS, 2% sodium pyruvate, 1% penicillin and 1%  
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36 streptomycin. Cells were grown in a 75-mm flask to 70% confluence at 37°C in an  
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38 atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were routinely trypsinized (0.05% trypsin/EDTA)  
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40 and counted using an automated counter (NucleoCounter, New Brunswick Scientific, Edison,  
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42 NJ).  
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### 49 **Cell viability by MTT assay**

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51 Cell viability was determined by a colorimetric assay using MTT. In the mitochondria of  
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53 living cells, yellow MTT undergoes a reductive conversion to formazan, giving a purple  
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55 color. Cells, grown to ~80% confluence in 75-mm flasks, were trypsinized, counted, seeded in  
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57 96-well plates with an average population of 7,000 cells/well, incubated overnight, and then  
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59 treated with the compounds (10 μM) for 24 hours. The dose and time of incubation were  
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3 determined from a set of preliminary experiments. All experiments were done using 8  
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5 replicates and repeated at least three times. Cell viability was expressed as a percent MTT  
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7 viability of untreated cells.  
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### 10 11 12 ***In Silico* Docking Simulations** 13

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15 Previously published work by our group<sup>1,22</sup> has demonstrated that these compounds act upon  
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17 the signal transducer and activator of transcription 3 (STAT3) pathway. This phenomenon  
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19 was investigated in more detail through *in silico* molecular docking simulations using the  
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21 freely-available program AutoDock (version 4.2).<sup>38,39</sup> The target macromolecule used in these  
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23 studies was a non-transformed murine STAT3 dimer downloaded from the RCSB Protein  
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25 Data Bank (PDB ID: 3CWG).<sup>40</sup> Energy-minimized 3D molecular topographies of the DAP  
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27 compounds were obtained using the Dundee PRODRG2 server.<sup>41</sup>  
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31 To identify the site on the STAT3 dimer with the highest binding affinity for the DAP  
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33 compounds, blind docking was accomplished using 0.625 Å grid spacing with 128 points in  
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35 each of the X, Y, and Z directions. This grid covered the majority of the previously-defined  
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37 3CWG molecular structure, including the entire SH2, linker, and DNA-binding domains, with  
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39 partial coverage of the coil-coil domain.<sup>40</sup> Specific docking at the preferential site identified  
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41 by blind docking was accomplished using 0.375 Å grid spacing with 100 points in each of the  
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43 X, Y, and Z directions. Dockings were automatically ranked by AutoDock according to the  
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45 lowest calculated binding energies (kcal/mol).  
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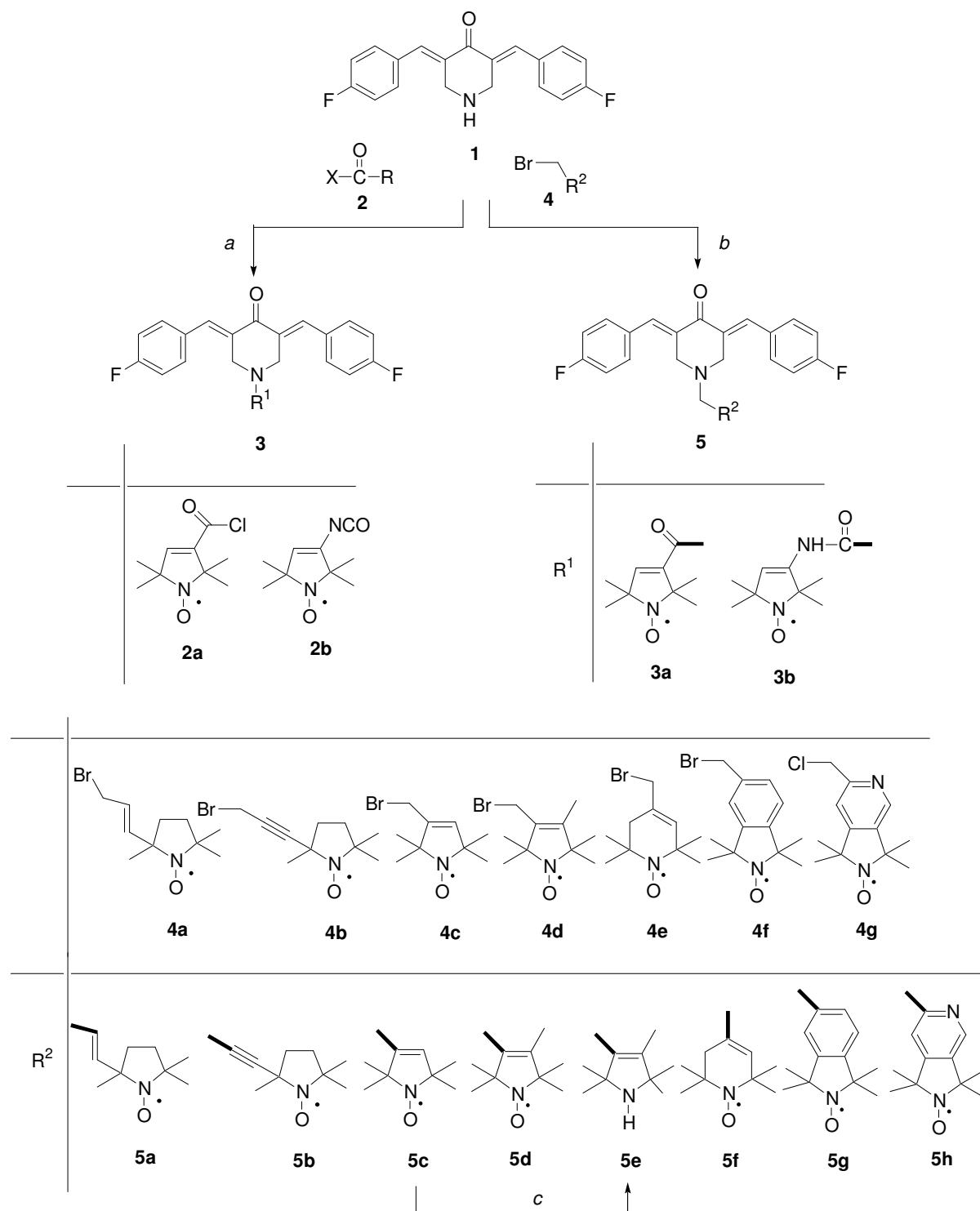
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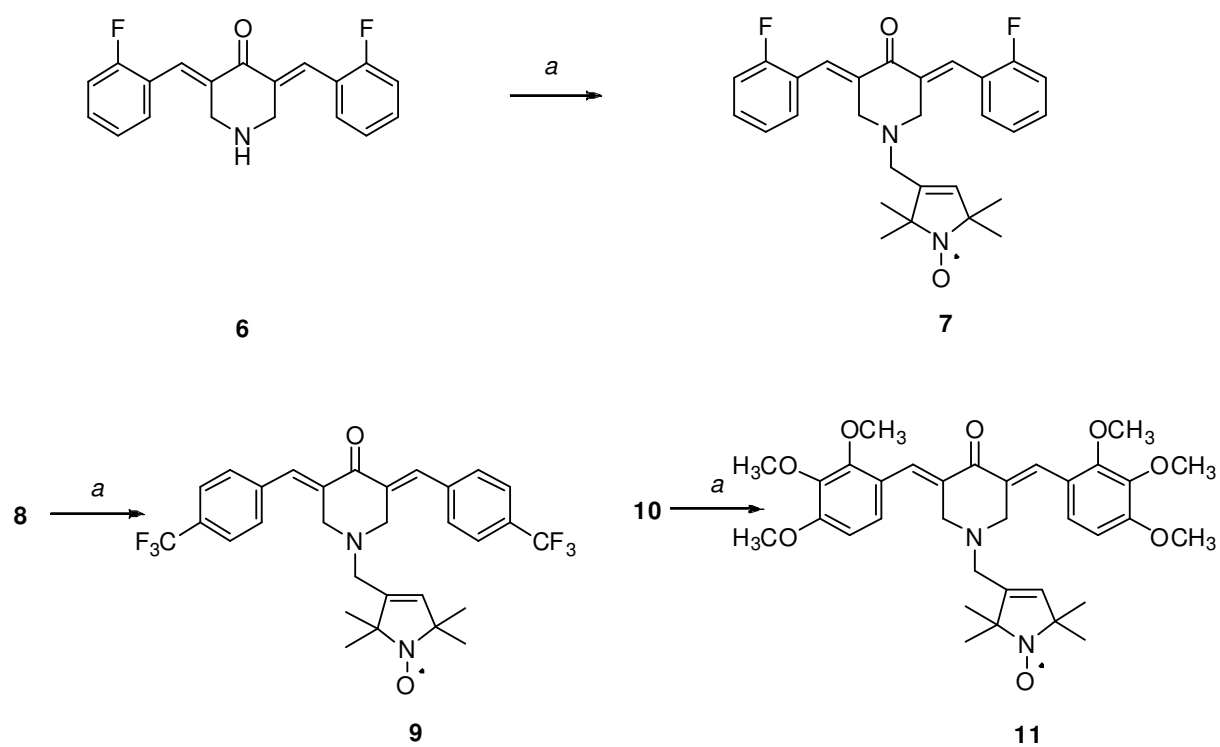
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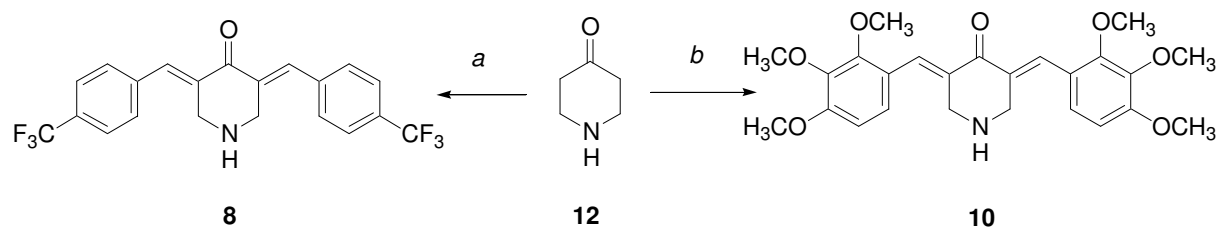
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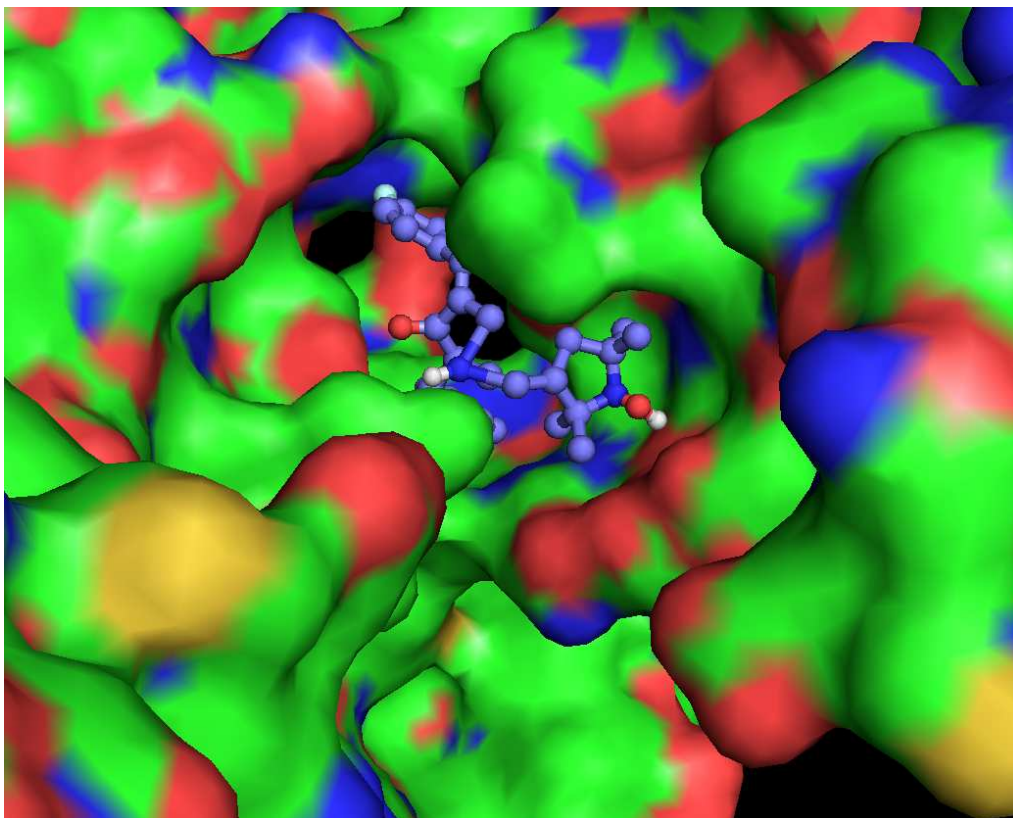


**Scheme 2.** Reagents and conditions: (a) Compound **4c** (1.0 equiv.),  $K_2CO_3$ , (1.0 equiv.), reflux, 3h, 55-71%.

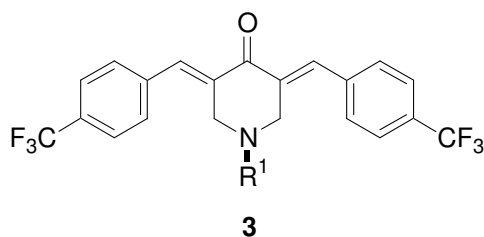


**Scheme 3.** Reagents and conditions: (a) 4-trifluoromethylbenzaldehyde (2.0 equiv.), AcOH sat. with HCl gas, 48h, rt., 74%; (b) 2,3,4-trimethoxybenzaldehyde (2.0 equiv.), AcOH sat. with HCl gas, 48h, rt., 62%.



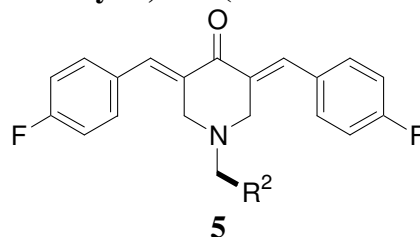


**Figure 1:** Compound **5c** docked to STAT3 dimer. This image generated with the free version of PyMol using computational data from the AutoDock simulations.

Table 1. Biological activity of new *N*-Acyl -3,5-bis(4-fluorobenzylidene)piperidin-4-ones.

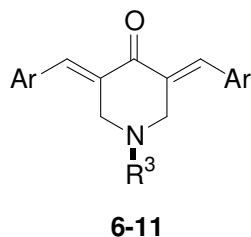
Compound	R <sup>1</sup>	A2780 Viability (%)*	MCF-7 Viability (%)	H9c2 Viability (%)
<b>1</b> H-4073	H	12.40± 2.62	17.14±1.98	61.40±13.74
<b>3a</b> HO-4049		4.54±0.66	12.42± 2.70	33.29± 5.58
<b>3b</b> HO-4060		5.90±1.57	12.64±1.41	36.70± 6.73

\*Control is 100 %.

Table 2. Biological activity of *N*-alkyl-3,5-bis(4-fluorobenzylidene)piperidin-4-ones.

Compound	R <sup>2</sup>	A2780 Viability (%)	MCF-7 Viability (%)	H9c2 Viability (%)
<b>5a</b> HO-4151		5.81±1.25	43.15±6.30	51.90±10.08
<b>5b</b> HO-4147		5.89±1.36	38.34±3.81	50.66±8.72
<b>5c</b> HO-3867		20.48±4.60	16.56±3.69	88.50±13.25
<b>5d</b> HO-4146		6.75±0.64	41.98±4.59	56.79±6.74
<b>5e</b> HO-3868		4.79±0.55	28.28±0.90	78.83±10.47
<b>5f</b> HO-4059		6.86±1.05	20.12±4.83	51.36±4.81
<b>5g</b> HO-4104		9.50±3.71	46.21±5.63	26.35±4.81
<b>5h</b> HO-4180		5.26±0.86	42.71±5.60	46.57±7.40

**Table 3. Biological activity of *N*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-ylmethyl)-3,5-bis(4-arylidene)piperidin-4-ones.**



Compound	Ar	R <sup>3</sup>	A2780 Viability (%)	MCF-7 Viability (%)	H9c2 Viability (%)
<b>6</b> L-2359	<i>o</i> -F-Ph	H	12.79±2.65	34.55±2.26	32.88±6.22
<b>7</b> HO-3865	<i>o</i> -F-Ph		12.48±1.14	53.50±8.36	86.35±7.82
<b>8</b> H-4138	<i>p</i> -CF <sub>3</sub> -Ph	H	13.10±0.46	20.19±2.49	51.74±12.10
<b>9</b> HO-4200	<i>p</i> -CF <sub>3</sub> -Ph		5.85±0.63	14.23±1.71	17.06±0.86
<b>10</b> H-4139	2,3,4-MeO-Ph	H	10.99±1.10	56.56±6.18	32.26±5.43
<b>11</b> HO-4196	2,3,4-MeO-Ph		26.07±4.43	27.96±1.94	53.13±7.14

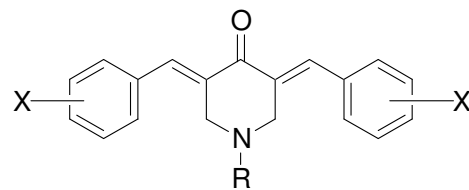
**Table 4. Summary of DAP compound docking simulations with the murine STAT3 dimer (PDB ID: 3CWG)**

<i>Simulation Test Compound</i>	<i>Lowest 5 Binding Energies (kcal/mol)</i>	<i>Corresponding Constant of Inhibition (<math>K_i</math>, nmol)</i>
<b>1</b> (H-4073)	-8.76*	380.28
	-8.76*	379.04
	-8.76*	378.52
	-8.76*	379.50
	-8.75	386.19
<b>8</b> (H-4138)	-7.89*	1660
	-7.89*	1660
	-7.88	1670
	-7.87*	1710
	-7.87*	1700
<b>5c</b> (HO-3867)	-11.15*	6.75
	-11.15*	6.76
	-11.10	7.24
	-11.00	8.67
	-10.99	8.80
<b>9</b> (HO-4200)	-10.66	15.33
	-10.62	16.32
	-10.61	16.68
	-10.59*	17.22
	-10.59*	17.34

\* The similarity of docked structures is measured by computing the root-mean-square deviation (rmsd) between the coordinates of the atoms and creating a *clustering* of the conformations based on these rmsd values. In this run, multiple binding conformation *clusters* were found, some with equivalent binding energies.

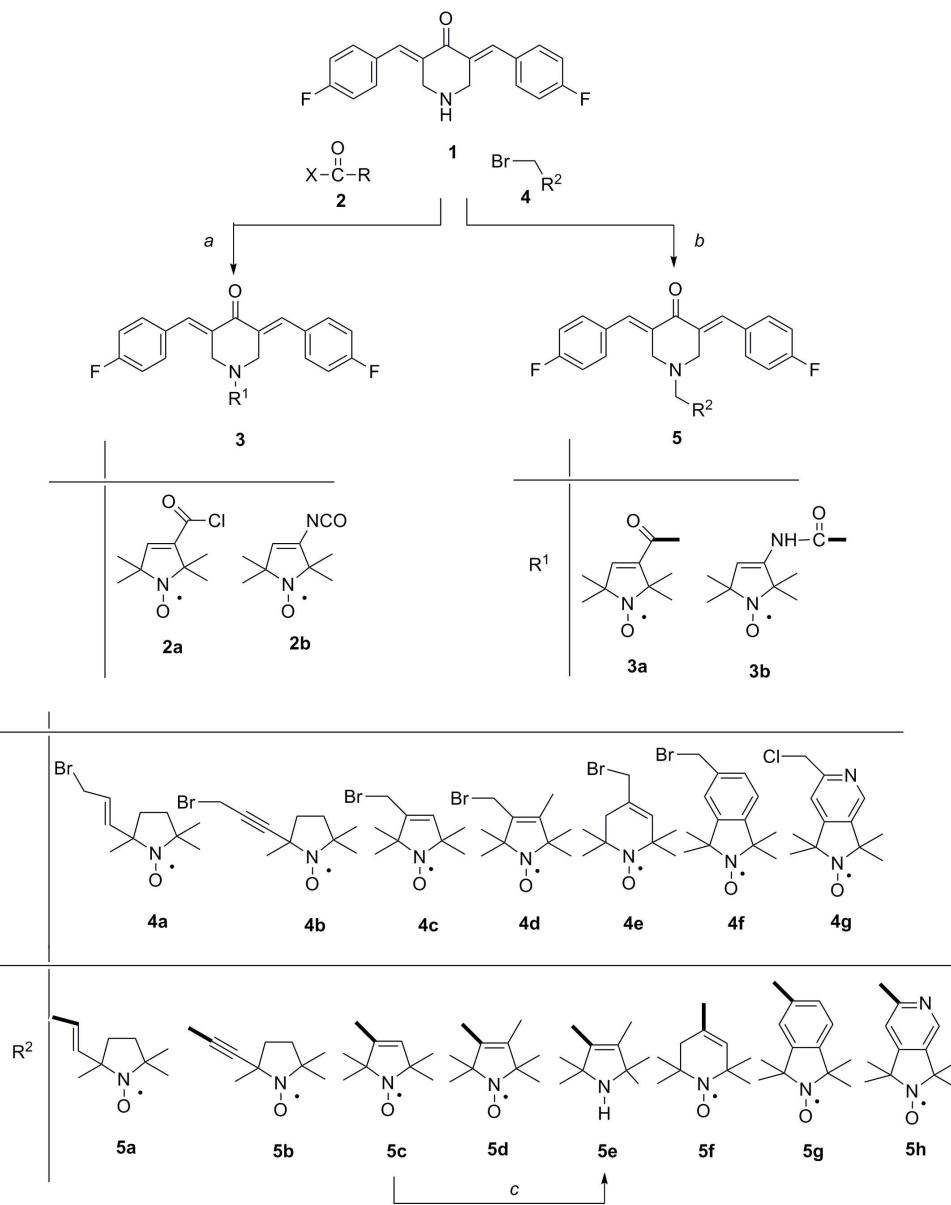
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A series of 3,5-bis(arylidene)-4-piperidones (DAP) were synthesized and *N*-alkylated or acylated with nitroxides or their precursors (amine and hydroxylamine). It was found that DAP part have cytotoxic (anti-cancer) activity, while the NOH moiety functioned as a tissue-specific modulator (anti-oxidant) of cytotoxicity.

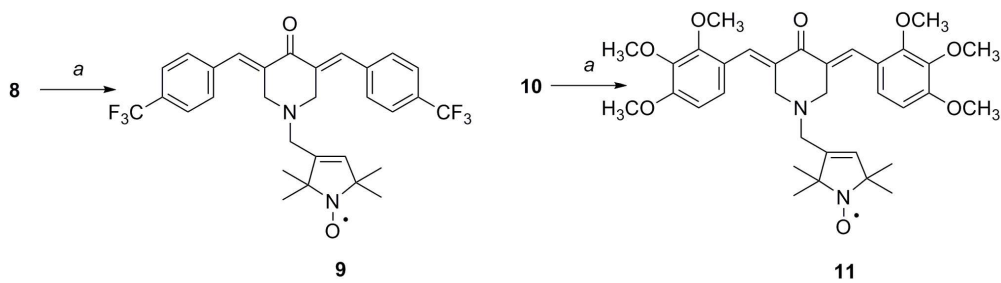
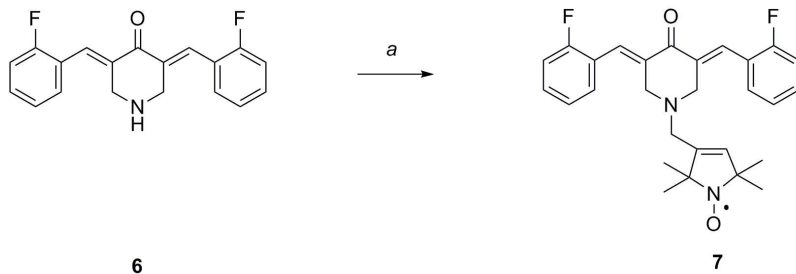


X: F, CF<sub>3</sub>, OCH<sub>3</sub>

R: nitroxides and their precursors

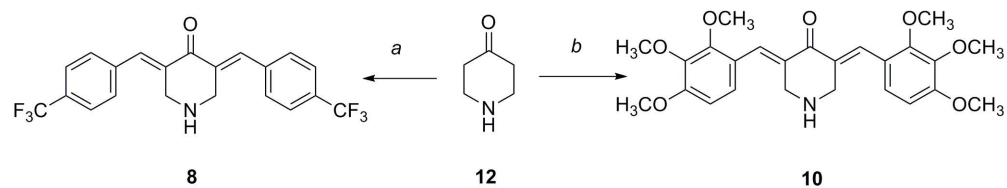


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