



Original article

Synthesis and biological evaluation of new N-alkylcarbazole derivatives as STAT3 inhibitors: Preliminary study

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ABSTRACT

The signalling pathway of Janus tyrosine Kinases-Signal Transducers and Activators of Transcription (JAK-STAT) is activated by a number of cytokines, hormones (GH, erythropoietin and prolactin), and growth factors. JAK-STAT signalling is involved in regulation of cell proliferation, differentiation and apoptosis. These activities are due to different members of JAK-STAT family consisting of: JAK1, JAK2, JAK3, Tyk2 and STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6. Recent studies suggest a key role for STAT family proteins, in particular for STAT3, in selectively inducing and maintaining a pro-carcinogenic inflammatory microenvironment, that promote tumour cells transformation. Moreover, a striking correlation between cancer development/progression and STAT3 persistent activation exists, probably due to STAT3 promoting of the pro-oncogenic inflammatory pathways, like NF- κ B, IL-6 and JAK family kinases. Recent study demonstrated that carbazoles can inhibit STAT3 mediated transcription. From these evidences, STAT3 represents a therapeutic target, so we have synthesized a new set of N-alkylcarbazole derivatives substituted in positions 2, 4 and 6, to evaluate their activity on STAT3. Some of these compounds showed an interesting activity as STAT3 selective inhibitors; in particular, compounds **9a**, **9b** and **9c** revealed to inhibit the STAT3 activation for the 50%, 90% and 95%, respectively.

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1. Introduction

Signal transducers and activators of transcription (STATs) are latent cytoplasmic transcription factors that once activated transduce signals from the cell membrane to the nucleus. To date, seven STATs have been identified in mammals (STATs 1, 2, 3, 4, 5A, 5B, 6). Among them, STAT3 is the most intimately linked to tumorigenesis. In fact, STAT3 is constitutively activated and overexpressed in various tumour types such as breast carcinoma [1,2], prostate cancer [3], melanoma [4], multiple myeloma [5], and leukemia [6].

In normal cells, where STAT3 is present in latent form, its activation can be induced by different cytokines, such as interleukins (IL2, IL5, IL6, IL7, IL9, IL10, IL11, IL15), by EGF (epidermal growth factor), PDGF (platelet-derived growth factor), GH (growth hormone), leptin, CT-1 (cardiotrophin-1), G-CSF (granulocyte colony-stimulating factor), and CSF-1 (colony-stimulating factor-1).

It is well established that the binding of a cytokine or growth factor to its receptor activates the receptor-associated tyrosine kinases, known as JAK1–3 and Tyk2. First, one or more members of the JAK family (JAK 1, 2, 3 and Tyk 2) of tyrosine kinases associated with a transmembrane receptor is phosphorylated and activated after ligand–receptor interaction. Activated JAKs recruit and phosphorylate one or more of the STAT proteins on tyrosine residues. Tyrosine phosphorylated STATs dimerize, translocate to the nucleus and bind specific promoter elements to regulate gene expression [7a]. A lot of papers state that STAT3 is implicated in promoting cell survival and it has been found to be persistently activated in many cancers. Therefore STAT3 is thought to confer protection against apoptosis in many of these transformed cells. Recent studies [7b] demonstrate that regulation of anti-apoptotic pathways by STAT3 is largely due to transcriptional control of genes that encode anti-apoptotic proteins (Bcl-xL, Bcl-2) and proliferation regulatory proteins (Cyclin D1, survivin).

Recent studies demonstrated the carbazole inhibition of: SVR (murine endothelial cells) cells proliferation; proinflammatory

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cytokine synthesis in particular of IL-15; α activation (vascular endothelial growth factor) VEGF-stimulated and STAT3-mediated transcription [8]. Moreover, in other studies carbazole derivatives showed a cytotoxic activity against a countless number of cancer cells: murine leukemia L1210 cell line [9a], SK-N-SH human neuroblastoma (NB) cells [9b], human solid cancer cells (PC3, DU145, and PA 1) [9c], human leukemic topoisomerase II sensitive (HL60N) and resistant (HL60 MX2) cell lines [9d].

From the aforementioned manuscripts, it seems that the carbazole moiety can be regarded as a privileged structure in the search of new antiproliferative chemotypes and several modifications of the carbazole scaffold led to different activity profiles against different tumor cell lines. Nevertheless, analysis of these studies reveals that the N9 position was never targeted and functionalized. Therefore, in the present paper a small series of new carbazoles modified by N-alkylation with C5, C6, C7 alkyl chains was obtained. Such a structural modification was also attempted to modulate the lipophilic properties of the carbazole ligands. Moreover, the alkyl chains were functionalized with dimethyl 5-hydroxyisophthalate or methyl salicylate as substituents. The characterization and biological evaluation of new N-alkylcarbazole derivatives as potential STAT3 inhibitors is also reported.

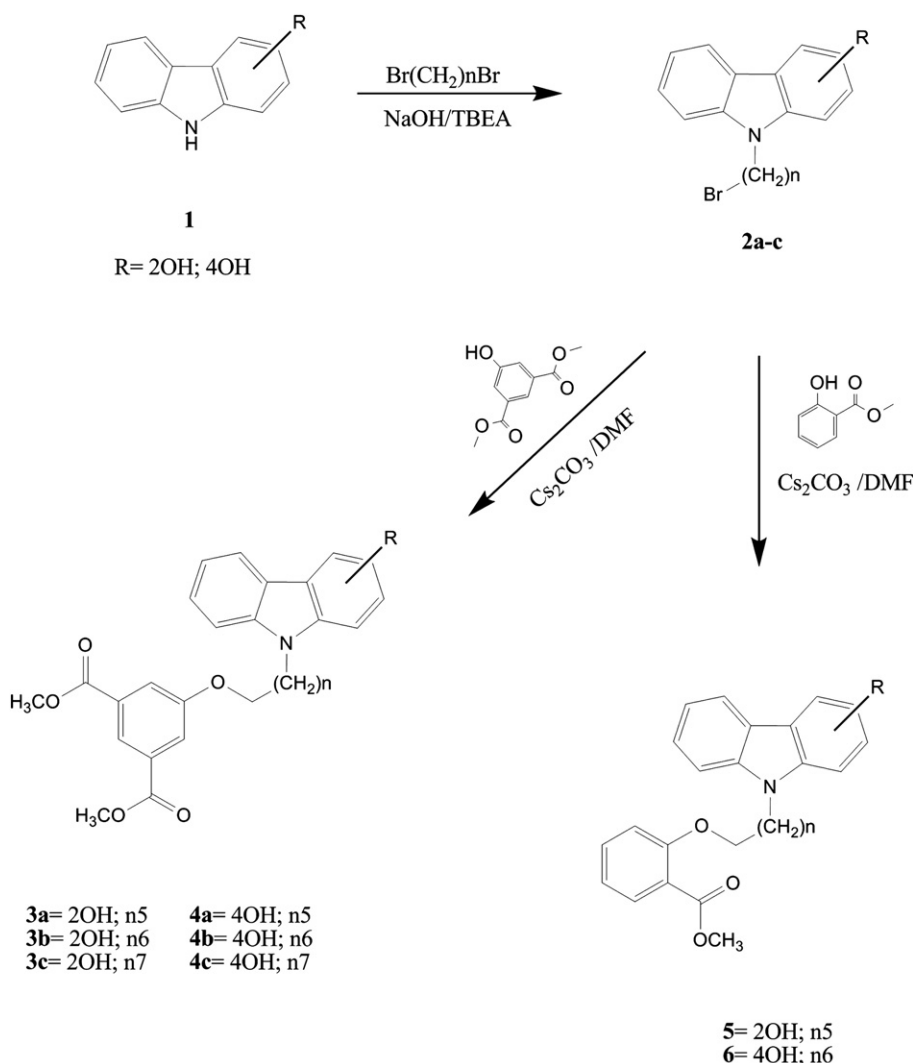
2. Results

2.1. Chemistry

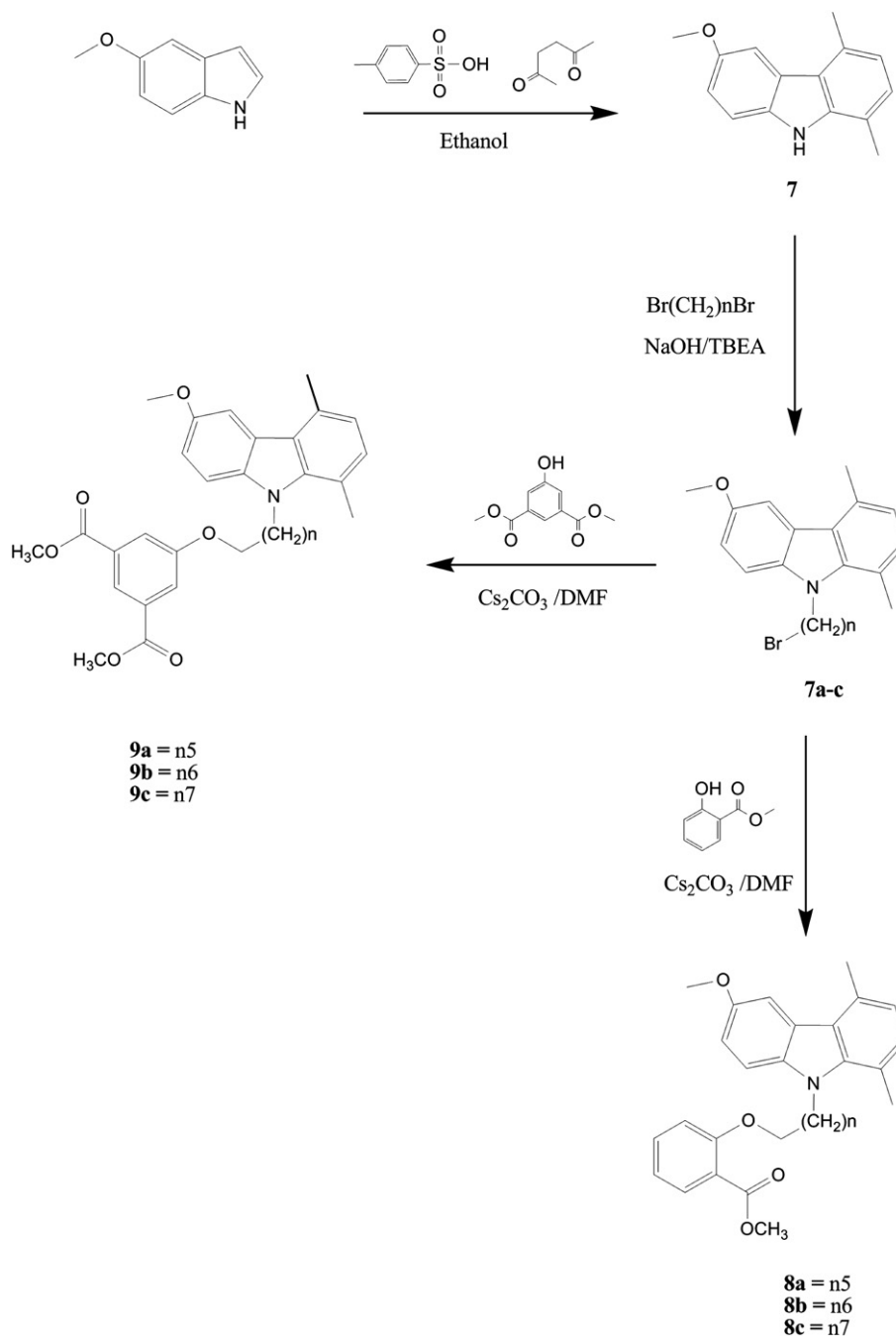
The synthesis of N-alkylcarbazole derivatives was accomplished using two different carbazole scaffolds. The compounds **3a–c**, **4a–c** and **5**, **6** were achieved from the 2 or 4 hydroxycarbazoles **1**, commercially available, in two steps of reaction as shown in Scheme 1. The first step was the N-alkylation of the hydroxycarbazoles with 1,5-dibromopentane; 1,6-dibromohexane or 1,7-dibromoheptane, to obtain the intermediates **2a–c**. These N-alkylated derivatives, in the second step were reacted to dimethyl 5-hydroxyisophthalate or methyl salicylate to give the final products. The compounds **8a–c** and **9a–c** were obtained from the same reactions, as shown in Scheme 2, from the 6-methoxy-1,4-dimethyl-9H-carbazole **7**. The compound **7** was synthesized from 5-methoxy-1H-indole, hexane-2,5-dione and 4-methylbenzenesulfonic acid dissolved in ethanol as shown in Scheme 2.

All synthesized compounds were characterized by NMR analysis and the elemental analysis carried out on them, are in agreement with the proposed formulations.

The compounds **8a–c** and **9a–c** were also characterized by mass spectrometry. The mass spectra of compounds **8a–c** and **9a–c**



Scheme 1. Synthetic route for the preparation of **3a**, **3b**, **3c**, **4a**, **4b**, **4c**, **5**, **6** compounds.



Scheme 2. Synthetic route for the preparation of **8a**, **8b**, **8c**, **9a**, **9b**, **9c** compounds.

show the molecular ion and the fragmentation of the aryl-ester group and sometimes of the OCH₃ group.

2.2. Pharmacology

In order to analyse the effect of a new set of N-alkylcarbazole derivatives on STAT3 signalling pathway, EMSA and Western blot analysis were performed in THP-1 cells treated with IL-6 (20 ng/ml) for 15 min. IL-6 increased predominantly the STAT3 DNA-binding activity as indicated by EMSA/supershift experiments with anti-STAT3 antibody (data not shown) in line with previous report [10]. Among different compounds examined only **9a**, **9b** and **9c** were able to inhibit, with different effectiveness, STAT3

DNA-binding activity at 50 μM (Fig. 1a) whereas all other compounds were ineffective also at higher doses (100 μM) (Table 1). In the [Supplementary materials](#) we also reported time- and dose-dependent effectiveness of **9a–c**. Moreover, the compounds **9a**, **9b** and **9c** were unable to inhibit IFN γ induced STAT1 nor TNF- α + LPS-induced NF- κB activation (Fig. 2a, b). One of the critical steps leading to the activation of STATs is their phosphorylation on specific tyrosine residues and successive translocation into the nucleus. In line with above described data, Western Blot analysis showed that the compounds **9a**, **9b** and **9c** decreased, in different grade, IL-6-induced tyrosine705 phosphorylation of cytosolic STAT3 without affecting the total amount of STAT3 protein (Fig. 1c).

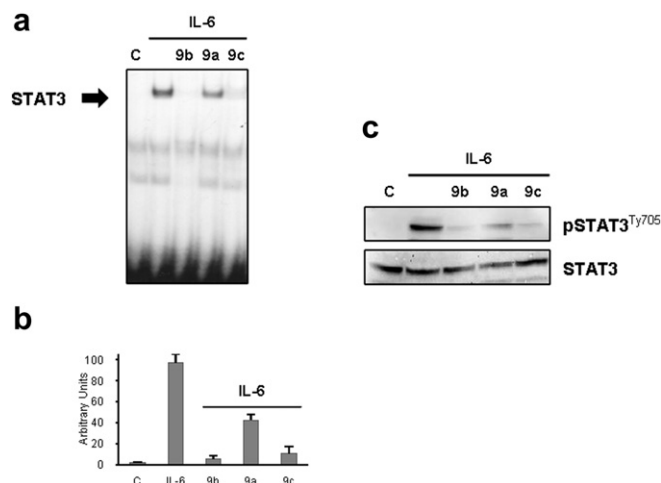


Fig. 1. Effect of compounds **9a–c** on STAT3 activation in THP-1 cell line. (a) THP-1 cells (1.5×10^6 /mL) were pretreated with 50 μ M of the compounds **9a**, **9b** or **9c** for 30 min and then stimulated with IL-6 (20 ng/mL) for 15 min. Nuclear extracts were then prepared and DNA-binding analysis was performed. EMSA shows that compounds **9b**, **9a** and **9c** decrease DNA-binding activity of STAT3 activated by 20 ng/mL IL-6. The gels are representative of five independent experiments. (b) Densitometric analysis of EMSA were performed as described in experimental protocols (Section 4.6) and are expressed as mean \pm S.D. of five experiments. (c) THP-1 cells (1.5×10^6 /mL) were pretreated with 50 μ M of the compounds **9a**, **9b** or **9c** for 30 min and then stimulated with IL-6 (20 ng/mL) for 15 min. Whole-cell extracts were prepared and p-STAT3 was detected by Western blot analysis. The same blot was stripped and reprobed with STAT3 antibody to verify equal protein loading.

3. Discussions and conclusions

Research within the past four years has provided convincing evidence for an oncogenic role of STAT3, a nuclear transcription factor belonging to the seven member STAT gene family of transcription factors, in different human cancers so that STAT3 is emerging as a critical transcription activator biomarker in antigenic therapy of tumour [11]. Therefore, inhibiting STAT3 pathway may be an effective approach to suppress tumour growth. The IL-6-induced activation of STAT3 is mediated by JAKs through a cytoplasmic domain of gp 130 of the IL-6 receptor [12]. The STAT3 phosphorylation at Tyr705 causes its homodimerization and

Table 1
Effect of examined compounds on STAT3 activation in THP-1 cell line.^a

Compound	Dose (μ M)	% of STAT 3 inhibition
3a	100	1 \pm 0.5
3b	100	1 \pm 0.5
3c	100	5 \pm 1.1
4a	100	20 \pm 2.2
4b	100	5 \pm 1.2
4c	100	6 \pm 1.3
5	100	1 \pm 0.3
6	100	3 \pm 0.4
8a	100	3 \pm 0.3
8b	100	1 \pm 0.3
8c	100	6 \pm 1.2
9a	50	50 \pm 5.2
9b	50	95 \pm 3.2
9c	50	90 \pm 6.5

^a THP-1 cells (1.5×10^6 /mL) were pretreated with 50 μ M or 100 μ M of all the synthesized compounds for 30 min and then stimulated with IL-6 (20 ng/mL) for 15 min. Nuclear extracts were subjected to EMSA analysis. Densitometric analysis of the gels were performed as described in experimental protocols (Section 4.6) and the inhibition of STAT3 DNA-binding is reported as mean percentage \pm SD respect to IL-6 induction alone.

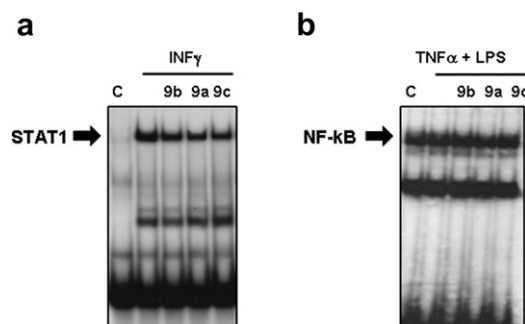


Fig. 2. Effect of compounds **9a–c** on STAT1 and NF- κ B activation in THP-1 cell line. THP-1 cells (1.5×10^6 /mL) were pretreated with 50 μ M of the compounds **9a**, **9b** or **9c** for 30 min and then stimulated with INF γ (a) or TNF α plus LPS (b) for 30 min. Nuclear extracts were then prepared and DNA-binding analysis was performed. EMSA shows that compounds **9b**, **9a** and **9c** are ineffective on DNA-binding activity of STAT1 nor NF- κ B activated with INF γ or TNF α plus LPS respectively. The gels are representative of five independent experiments.

subsequent translocation to the nucleus for binding to specific DNA sequences in the promoter of target genes [13,14] involved in apoptosis, cell survival, angiogenesis and metastasis. In the present study, we demonstrated that some of the tested compounds suppressed, with different effectiveness, the STAT3 phosphorylation and its nuclear translocation. In particular, compound **9a**, **9b** and **9c** revealed to inhibit the STAT3 activation for the 50%, 90% and 95%, respectively. These compounds represent a new class of carbazole leads endowed with antiproliferative activities. In fact, the addition of an N-alkyl chain provided an additional interaction point to the target protein as small modifications of its terminal phenyl ring cause dramatic changes in the activity profile (compare **8a**, **8b** and **8c** with **9a**, **9b** and **9c**). Also among the active ligand the length of the alkyl linker seems to be critical for the activity (compare **9a** with **9b** and **9c**). If some considerations of the structure-activity relationships (SARs) can be drawn, at present a deeper understanding of the chemical features responsible for the inhibition of the STAT3 activation would require a more systematic SAR study starting from the present preliminary work.

4. Experimental section

All reagents and solvents were purchased from Sigma–Aldrich s.r.l. (Milan, Italy), the 1,5-dibromopentane, 1,6-dibromohexane and 1,7-dibromoheptane were purified on Al₂O₃. The reactions were monitored by thin-layer chromatography (TLC), using silica gel aluminum plates Whatman K6F and Allumina (Merck), with fluorescence indicators and opportune solvents. A >95% purity could be inferred from the ¹H-NMR spectra. Melting points were taken on a Gallenkamp melting point apparatus and were uncorrected. The organic extracts were dried over sodium sulphate dry (Merck). The elemental analyses for C, H, N, were performed according to standard microanalytical procedures. Molecular weights were determined by ESI mass spectrometry. ESI-MS analysis in positive and negative ion mode, were made using a Finnigan LCQ ion trap instrument, manufactured by Thermo Finnigan (San Jose, CA, USA), equipped with the Excalibur software for processing the data acquired. The sample was dissolved in a mixture of acetonitrile and methanol (50/50) and injected directly into the electrospray source, using a syringe pump, which maintains constant flow at 5 μ L/min. The temperature of the capillary was set at 220 $^{\circ}$ C.

¹H NMR spectra were recorded at 298 K on a Bruker Avance 300 MHz spectrometer, using CDCl₃ and referred to internal tetramethylsilane. All chemicals used for pharmacological assays were

of the highest analytical grade, purchased from Sigma Chemical Company, Milan, Italy, unless otherwise specified. RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine (FBS) serum were obtained from Lonza, Verviers, BE.

4.1. Synthesis of 6-methoxy-1,4-dimethyl-9H-carbazole (**7**)

5-methoxy-1H-indole (6.8 mmol) was dissolved in ethanol (10 ml) and stirred at room temperature. To this solution were added hexane-2,5-dione (6.8 mmol) and 4-methylbenzenesulfonic acid (6.04 mmol). The mixture was stirred at reflux for 6 h. After the reaction, the solvent was evaporated, poured into water and extracted with chloroform (3 × 30 ml). The organic layer was dried over anhydrous MgSO₄, filtered and the solvent removed by evaporation. The resulting residue was purified on a silica gel column chromatography in petroleum ether/ethyl acetate (8/2) to give as white crystals (90% yield) (Scheme 2) [15–18].

4.2. Synthesis of N-alkyl carbazoles (**2a–c**; **7a–c**)

TBEA (benzyltriethylammonium bromide) (2.73 mmol), was dissolved in an aqueous solution of NaOH 50% p/p (15 ml) and toluene (20 ml) and stirred for 15 min. After was added a solution of a suitable hydroxycarbazole or 6-methoxy-1,4-dimethyl-9H-carbazole (5.45 mmol) in toluene (20 ml). To this mixture was added 1,5-dibromopentane or 1,6-dibromohexane or 1,7-dibromoheptane (16.3 mmol) and was stirred at reflux for 48 h. After the reaction, the solvent was evaporated, poured into water and extracted with chloroform (5 × 50 ml). The organic layer was dried over anhydrous MgSO₄, filtered and the solvent removed by evaporation. The resulting residue was purified on a silica gel column chromatography in petroleum ether/ethyl acetate (9/1) to give as white/yellow solid (60% yield) (**7a–c**). The resulting residue was extracted with hexane (3 × 20 ml), ethyl acetate, and diethyl ether. Final product was purified on a silica gel column chromatography in chloroform to give as yellow/orange solid (60% yield) (**2a–c**) (Schemes 1 and 2) [16–18].

4.3. General method for the synthesis of compounds **3a–c**, **4a–c**, **9a–c**

Dimethyl 5-hydroxyisophthalate (1.35 mmol) and Cs₂CO₃ (2.7 mmol) were dissolved in DMF (10 ml). A solution of N-bromoalkyl-substituted carbazole (1.35 mmol) in DMF was added at room temperature. The mixture was stirred for 20 h. After the reaction, the solvent was evaporated, poured into water and extracted with chloroform (5 × 50 ml). The organic layer was dried over anhydrous MgSO₄, filtered and the solvent removed by evaporation. Final product was an orange/brown solid (80% yield) (Schemes 1 and 2).

4.3.1. Dimethyl 5-(5-(2-hydroxy-9H-carbazol-9-yl)pentyl)oxy isophthalate (**3a**)

(δ ppm, CDCl₃ 300 MHz): 4.20 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 1H, s]; 4.10 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 4.03 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.94 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, s]; 1.27–2.02 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, m]; 6.80–8.22 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 10H, m].

Mass spectrum: 461.51 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂].

Elemental analysis of **3a**: Calcd. for C₂₇H₂₇NO₆ (%): C 70.27, H 5.90, N 3.03. Found (%): C 70.14, H 5.96, N 3.12.

4.3.2. Dimethyl 5-(6-(2-hydroxy-9H-carbazol-9-yl)hexyl)oxy isophthalate (**3b**)

(δ ppm, CDCl₃ 300 MHz): 4.17 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 1H, s]; 4.05 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.93 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.88 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, s]; 1.27–2.00 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 8H, m]; 6.70–8.30 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 10H, m].

Mass spectrum: 475.53 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂].

Elemental analysis of **3b**: calcd. for C₂₈H₂₉NO₆ (%): C 70.72, H 6.15, N 2.95. Found (%): C 70.54, H 6.23, N 2.86.

4.3.3. Dimethyl 5-(7-(2-hydroxy-9H-carbazol-9-yl)heptyloxy) isophthalate (**3c**)

(δ ppm, CDCl₃ 300 MHz): 4.20 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 1H, s]; 4.11 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.94 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.91 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, s]; 1.12–1.94 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 10H, m]; 6.80–8.30 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 10H, m].

Mass spectrum: 489.56 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂].

Elemental analysis of **3c**: Calcd. for C₂₉H₃₁NO₆ (%): C 71.15, H 6.38, N 2.86. Found (%): C 71.02, H 6.49, N 2.92.

4.3.4. Dimethyl 5-(5-(4-hydroxy-9H-carbazol-9-yl)pentyl)oxy isophthalate (**4a**)

(δ ppm, CDCl₃ 250 MHz): 4.22 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 1H, s]; 4.16 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.96 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.90 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, s]; 1.23–2.11 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, m]; 6.56–8.41 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 10H, m].

Mass spectrum: 461.51 [(OH–C₁₂H₇N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂].

Elemental analysis of **4a**: calcd. for C₂₇H₂₇NO₆ (%): C 70.27, H 5.90, N 3.03. Found (%): C 70.15, H 5.98, N 3.14.

4.3.5. Dimethyl 5-(6-(4-hydroxy-9H-carbazol-9-yl)hexyl)oxy isophthalate (**4b**)

(δ ppm, CDCl₃ 300 MHz): 4.10 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 1H, s]; 4.32 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.99 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.88 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, s]; 1.21–2.12 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 8H, m]; 6.57–8.50 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 10H, m].

Mass spectrum: 475.53 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂].

Elemental analysis of **4b**: calcd. for C₂₈H₂₉NO₆ (%): C 70.72, H 6.15, N 2.95. Found (%): C 70.59, H 6.27, N 2.84.

4.3.6. Dimethyl 5-(7-(4-hydroxy-9H-carbazol-9-yl)heptyloxy) isophthalate (**4c**)

(δ ppm, CDCl₃ 300 MHz): 4.23 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 1H, s]; 4.25 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.98 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.93 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, s]; 1.20–2.10

[(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 10H, m]; 6.50–8.40 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 10H, m]].

Mass spectrum: 489.56 [(OH–C₁₂H₇N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂].

Elemental analysis of 4c: calcd. for C₂₉H₃₁NO₆ (%): C 71.15, H 6.38, N 2.86. Found (%): C 71.02, H 6.49, N 2.97.

4.3.7. Dimethyl 5-(5-(6-methoxy-1,4-dimethyl-9H-carbazol-9-yl)pentyl)isophthalate (9a)

¹H NMR (δ ppm CDCl₃ 250 MHz): 4.06 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 3H, s]; 4.56 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 4.10 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 4.00 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, s]; 2.79 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 3H, s]; 2.74 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 3H, s]; 1.21–2.00 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, m]; 6.91–8.50 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂, 8H, m].

¹³C NMR (δ ppm CDCl₃ 300 MHz): 166.5 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂], 159, 153.5, 139.5, 136.2, 131.4, 128.9, 124.28, 123.6, 122.1, 120.4, 117.5, 113.4, 109.2, 106.6 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂]; 68.2 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂]; 56.3 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂]; 52.4 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂]; 44.8 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂]; 30.7, 30.5, 29.1 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂]; 20.3 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂]; 20.4 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂].

Mass spectrum: 503.59 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–(OCOCH₃)₂].

Elemental analysis of 9a: Calcd. for C₃₀H₃₃NO₆ (%): C 71.55, H 6.61, N 2.78. Found (%): C 71.44, H 6.81, N 2.91.

4.3.8. Dimethyl 5-(6-(6-methoxy-1,4-dimethyl-9H-carbazol-9-yl)hexyloxy)isophthalate (9b)

¹H NMR (δ ppm CDCl₃ 250 MHz): 3.95 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 3H, s]; 4.52 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 4.05 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.94 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, s]; 2.79 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 3H, s]; 2.83 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 3H, s]; 1.21–1.90 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 8H, m]; 6.80–8.30 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂, 8H, m].

¹³C NMR (δ ppm CDCl₃ 300 MHz): 166.5 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂], 159, 153.5, 139.5, 136.2, 131.4, 128.9, 124.28, 123.6, 122.1, 120.4, 117.5, 113.4, 109.2, 106.6 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂]; 68.2 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂]; 56.3 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂]; 52.4 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂]; 44.8 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂]; 30.8, 29.2, 26.9, 26.1 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂]; 20.3 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂]; 20.4 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂].

(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂]; 20.4 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂].

Mass spectrum: 517.61 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–(OCOCH₃)₂].

Elemental analysis of 9b: calcd. for C₃₁H₃₅NO₆ (%): C 71.93, H 6.82, N 2.71. Found (%): C 71.82, H 6.96, N 2.59.

4.3.9. Dimethyl 5-(7-(6-methoxy-1,4-dimethyl-9H-carbazol-9-yl)heptyloxy)isophthalate (9c)

¹H NMR (δ ppm CDCl₃ 300 MHz): 3.95 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 3H, s]; 4.48 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 4.00 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 2H, t]; 3.93 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 6H, s]; 2.77 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 3H, s]; 2.84 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 3H, s]; 1.23–1.87 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 10H, m]; 6.80–8.40 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂, 8H, m].

¹³C NMR (δ ppm CDCl₃ 300 MHz): 166.5 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂], 159, 153.5, 139.5, 136.2, 131.4, 128.9, 124.28, 123.6, 122.1, 120.4, 117.5, 113.4, 109.2, 106.6 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂]; 68.2 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂]; 56.3 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂]; 52.4 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂]; 44.8 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂]; 30.7, 29.2, 29.1, 27.2, 26.1 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂]; 20.3 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂]; 20.4 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂].

Mass spectrum: 531.64 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–(OCOCH₃)₂].

Elemental analysis of 9c: calcd. for C₃₂H₃₇NO₆ (%): C 72.29, H 7.01, N 2.63. Found (%): C 72.14, H 7.18, N 2.75.

4.4. General method for the synthesis of compounds 5, 6, 8a–c

Methyl salicylate (1.35 mmol) and Cs₂CO₃ (2.7 mmol) were dissolved in DMF (10 ml). A solution of N-bromoalkyl-substituted carbazole (1.35 mmol) in DMF was added at room temperature. The mixture was stirred for 20 h. After the reaction, the solvent was evaporated, poured into water and extracted with chloroform (5 × 50 ml). The organic layer was dried over anhydrous MgSO₄, filtered and the solvent removed by evaporation. Final product was an orange/brown solid (80% yield) (Schemes 1 and 2).

4.4.1. Methyl 2-(6-(2-hydroxy-6-methoxy-9H-carbazol-9-yl)hexyloxy)benzoate (5)

(δ ppm, CDCl₃ 300 MHz): 4.25 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 1H, s]; 4.18 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 2H, t]; 4.09 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 2H, t]; 3.89 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 1.21–1.97 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 8H, m]; 6.70–8.10 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 11H, m].

Mass spectrum: 417.5 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃].

Elemental analysis of 5: calcd. for C₂₆H₂₇NO₄ (%): C 74.80, H 6.52, N 3.35. Found (%): C 74.59, H 6.64, N 3.44.

4.4.2. Methyl 2-(6-(5-hydroxy-3-methoxy-9H-carbazol-9-yl)hexyloxy)benzoate (**6**)

(δ ppm, CDCl₃ 300 MHz): 4.32 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 1H, s]; 4.09 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 2H, t]; 3.99 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 2H, t]; 3.90 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 1.26–2.19 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 8H, m]; 6.54–8.50 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 11H, m].

Mass spectrum: 417.5 [(OH–C₁₂H₇N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃].

Elemental analysis of **6**: calcd. for C₂₆H₂₇NO₄ (%): C 74.80, H 6.52, N 3.35. Found (%): C 74.69, H 6.70, N 3.46.

4.4.3. Methyl 2-(5-(6-methoxy-1,4-dimethyl-9H-carbazol-9-yl)pentyl) benzoate (**8a**)

(δ ppm CDCl₃ 300 MHz): 3.89 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 4.52 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–OCOCH₃, 2H, t]; 4.00 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–OCOC H₃, 2H, t]; 3.86 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 2.80 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 2.83 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 1.10–2.00 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–OCOCH₃, 6H, m]; 6.60–8.40 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–OCOCH₃, 9H, m].

Mass spectrum: 445.55 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₃–CH₂O–C₆H₃–OCOCH₃].

Elemental analysis of **8a**: calcd. for C₂₈H₃₁NO₄ (%): C 75.48, H 7.01, N 3.14. Found (%): C 75.35, H 7.12, N 3.26.

4.4.4. Methyl 2-(6-(6-methoxy-1,4-dimethyl-9H-carbazol-9-yl)hexyloxy)benzoate (**8b**)

(δ ppm CDCl₃ 250 MHz): 3.99 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 4.53 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 2H, t]; 4.04 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOC H₃, 2H, t]; 3.94 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 2.82 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 2.84 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 1.30–1.90 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 8H, m]; 6.60–8.00 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃, 9H, m].

Mass spectrum: 459.58 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₄–CH₂O–C₆H₃–OCOCH₃].

Elemental analysis of **8b**: calcd. for C₂₉H₃₃NO₄ (%): C 75.79, H 7.24, N 3.05. Found (%): C 75.62, H 7.38, N 3.14.

4.4.5. Methyl 2-(7-(6-methoxy-1,4-dimethyl-9H-carbazol-9-yl)heptyloxy)benzoate (**8c**)

(δ ppm CDCl₃ 250 MHz): 3.95 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 4.50 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–OCOCH₃, 2H, t]; 3.95 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–OCOC H₃, 2H, t]; 3.85 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 2.87 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 2.95 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–OCOCH₃, 3H, s]; 1.20–1.90 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–OCOCH₃, 10H, m]; 6.76–8.12 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–OCOCH₃, 9H, m].

Mass spectrum: 473.60 [(CH₃O–C₆H₃–C₆H₃–(CH₃)₂–N)–CH₂–(CH₂)₅–CH₂O–C₆H₃–OCOCH₃].

Elemental analysis of **8c**: calcd. for C₃₀H₃₅NO₄ (%): C 76.08, H 7.45, N 2.96. Found (%): C 75.96, H 7.52, N 3.02.

4.5. Cell culture

Human monocytic leukemia THP-1 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 supplemented with 10% FBS, 100 UI/ml penicillin, 100 µg/ml streptomycin and 40 µg/ml gentamycin in a 5% CO₂ atmosphere at 37 °C.

4.6. Electrophoretic mobility shift assay – EMSA

Nuclear extracts of THP-1 cells were prepared according to Osborn et al. [19] in presence of 10 µg/ml leupeptin, 5 µg/ml anti-pain, 5 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Eight micrograms of nuclear extract were incubated with 2–5 × 10⁴ cpm of ³²P-labeled double-stranded oligonucleotides, the consensus STAT1/3 DNA binding site (sis-inducible factor-binding recognition element, SIE/m67) from the c-fos promoter (5'-gtcga-CATTTCGTAATCg-3'), in a 15 µl reaction mixture containing 20 mM Hepes, pH 7.9, 50 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 2 µg of poly(dI-dC), 1 µg of salmon sperm DNA, and 10% glycerol. Products were fractionated on a non-denaturing 5% polyacrylamide gel. The gels were dried and autoradiographed and the intensity of hybridization was quantified using the public domain NIH Image 1.61 program (developed at the U.S. National Institutes of Health and available on <http://rsb.info.nih.gov/nihi-image/>). Supershift assay was performed by incubating the nuclear extracts in a binding buffer for 1 h at 4 °C with 1 µl of antibody before addition of labelled oligonucleotide. Polyclonal antibodies against STAT3 and STAT1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

4.7. Western blot analysis

Cells were homogenized at 4 °C in 20 mM HEPES, pH 7.4, containing 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet-P40 (NP-40), 20% glycerol, protease cocktail inhibitors (GE Healthcare, Amersham Place, UK) and phosphatase cocktail inhibitors. Aliquots of the cell lysate (40 µg total protein/lane) were loaded on 7.5% SDS-polyacrylamide gels. Electrophoresis was performed at 100 V with a running buffer containing 0.25 M Tris HCl, pH 8.3, 1.92 M glycine, and 1% SDS. The resolved protein were electroblotted onto a PVDF membrane (Immobilon P, Millipore, Bedford MA) and incubated overnight at 4 °C with rabbit anti-phospho-Tyr⁷⁰⁵Stat3 (Cell Signaling Technology, Beverly, MA). After washing, membranes were developed using anti-rabbit IgG peroxidase-conjugated antibody (Cell Signaling Technology) and chemiluminescent detection system (Immun-Star™ WesternC™ Kit, Bio-Rad, Hercules, CA). Blotted proteins were detected and quantified using the ChemiDoc XRS Imaging System (Bio Rad). After stripping, membranes were re-hybridized with rabbit anti-STAT3 antibodies (Santa Cruz Biotechnology).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2012.11.004>.

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