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Cytotoxic and antitumoral properties in a series of new, ring D modified, olivacine analogues

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Abstract—The present study describes the synthesis and pharmacological profiles of new olivacine related compounds, possessing a modified D ring. The impact of this modification has been evaluated with respect to the cytotoxic and in vivo antitumoral effects of these molecules and in comparison with parent S 16020-2 previously prepared and investigated in our laboratory. The D ring size and number of nitrogen atoms as well as the position of the aminoalkyl substituent have a profound impact on the cytotoxic and antitumoral profiles. Thus out of the prepared pyrazinocarbazole compounds, **2** is devoid of any substantial cytotoxic and antitumoral activities while the pyrimidocarbazole **3** has a similar profile compared to **1** (S 16020-2). L1210 and P388 in vivo antitumoral effects are lost for both imidazocarbazoles **4** and **5**, but the former conserves an in vivo antitumoral effect on B16 melanoma, this effect being the largest in the series. Structural similarities and differences amongst the studied compounds could be evidenced by calculation of global properties such as molecular electrostatic potentials (MEP maps) and partition coefficients (log *P*), thus adding information on the impact of chemical changes on these two parameters known to influence biological behavior.

1. Introduction

Previous reports from our laboratory described new cytotoxic olivacine derivatives out of which compound **1** (Fig. 1, S 16020-2) was selected for clinical evaluation.

Pursuing our goals, we initiated a research project aimed to investigate the impact of ring D modifications on cytotoxic and antitumoral profiles of olivacine related compounds. Indeed, while the impact of substitution of the tetracycle has been studied,[1] no analogues containing modified D ring were known. Moreover, since literature reports stressed the role of D ring nitrogen in related tricyclics compounds,[2] it was decided to also evaluate such changes in **1**. Therefore, four types of structures namely pyrazinocarbazole, pyrimidocarbazole, imidazocarbazole and *N*-substituted imidazocarbazole (**2**, **3**, **4**, **5**, Fig. 1) were synthesized and pharmacologically evaluated. In these analogues, the number of nitrogens and the position of the basic side chain were varied. To begin with, we sought for practical synthetic approaches using simple reagents and no scaling up impediments.

2. Chemistry

Preparation of compound **2** (Scheme 1) started with carbazole **6** itself prepared in good yield by a literature described method.[3] Demethylation of the methoxy group as well as deacetylation of the acetamido group was achieved in good yield by treatment with 48% hydrobromic acid.[4] The amino and hydroxyl functions in compound **7** were then reacetylated with acetic anhydride. Compound **8** was then *N*-methylated using sodium hydride and iodomethane in DMF.[5] Selective nitration of **9** with nitric acid (fuming 100%) occurred at the *ortho*-position to the diacetylamino group to afford **10** in good yield.[6] The acetyl groups were removed in quantitative yield by treatment with sodium hydroxide in ethanol. Ni (catalytic) hydrogenation of the aromatic

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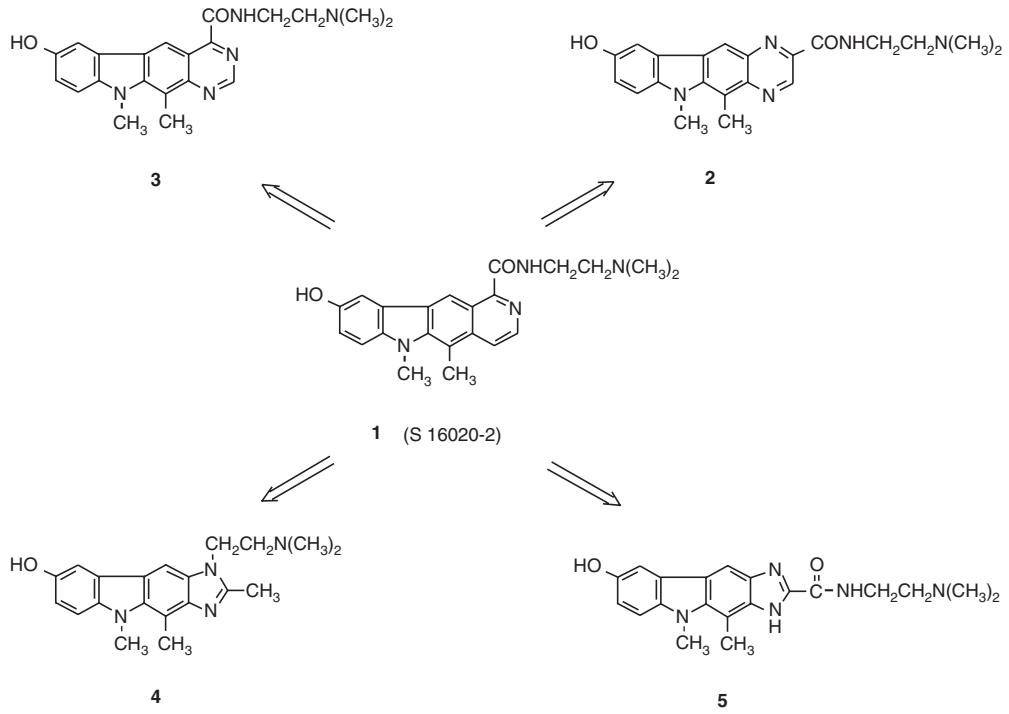
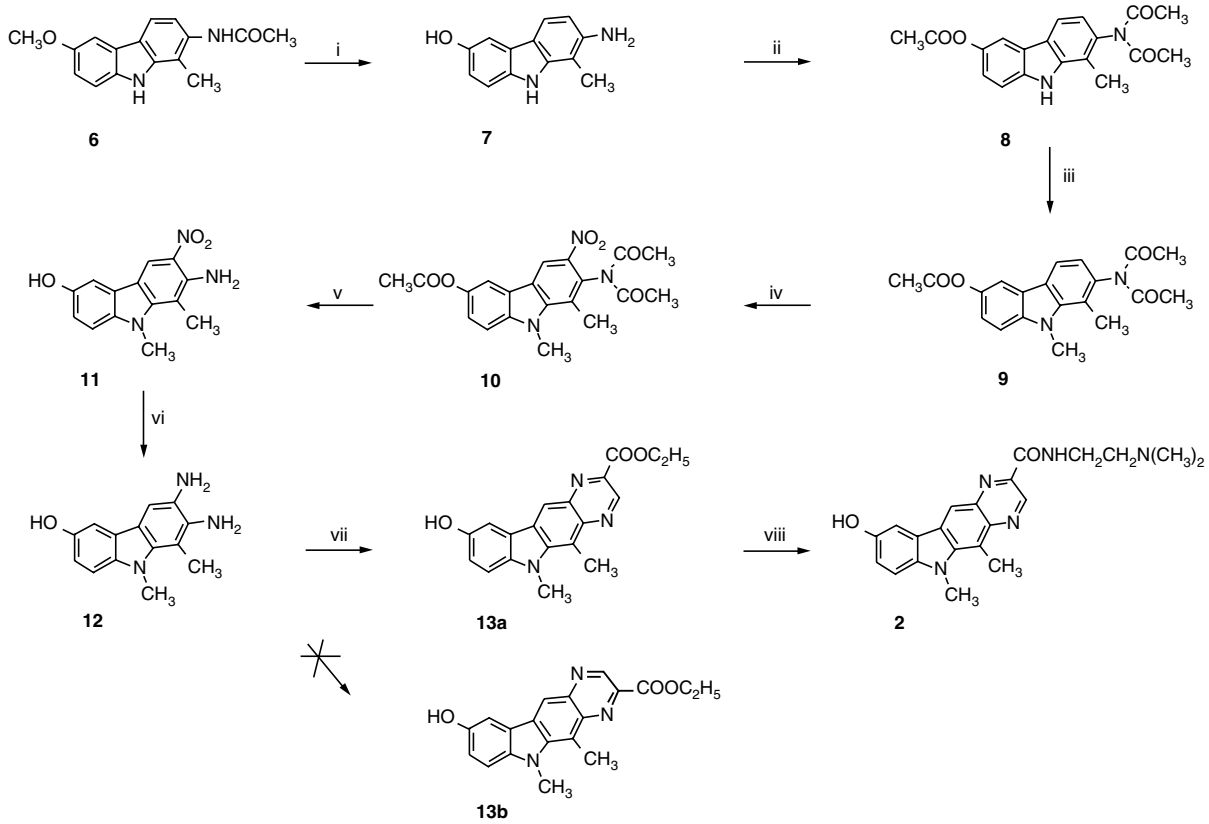


Figure 1. Ring D modified S 16020-2 analogues.



Scheme 1. Synthesis of pyrazocarbazole **2**: (i) 48% HBr (94%); (ii) Ac_2O (62%); (iii) NaH, CH_3I, DMF (64%); (iv) HNO_3 (53%); (v) NaOH, C_2H_5OH (100%); (vi) H_2–Ni, NMP (100%); (vii) $BrCH_2COCOOC_2H_5$, $N(C_2H_5)_3$, NMP (29%); (viii) $H_2N(CH_2)_2N(CH_3)_2$ (44%).

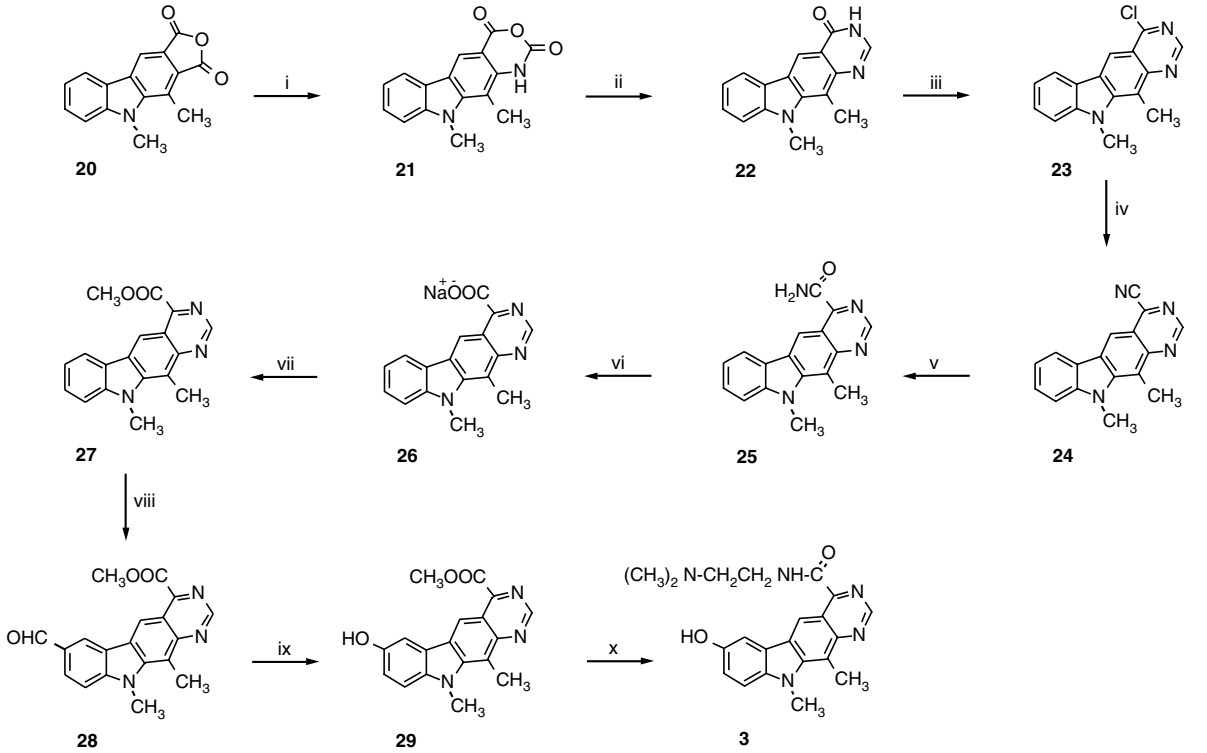
nitro group[6] in compound **11** afforded in good yield the diamino carbazole **12**. The pyrazo D cycle was constructed by condensation of diamino compound **12** with ethylbromopyruvate in the presence of triethylamine[7]

in NMP to afford compound **13a** in moderate yield. Although two different isomers **13a** and **13b** could be expected from this reaction, only isomer **13a** was obtained; its structure was proved at the next step by the NMR spectral data of compound **2**, itself obtained from compound **13a** on which the appropriate chain was introduced by condensation of the ethyl ester part with the suitable amine.[8] This procedure was easily performed on a multigram scale, thus allowing the preparation of **2** in fair quantities.

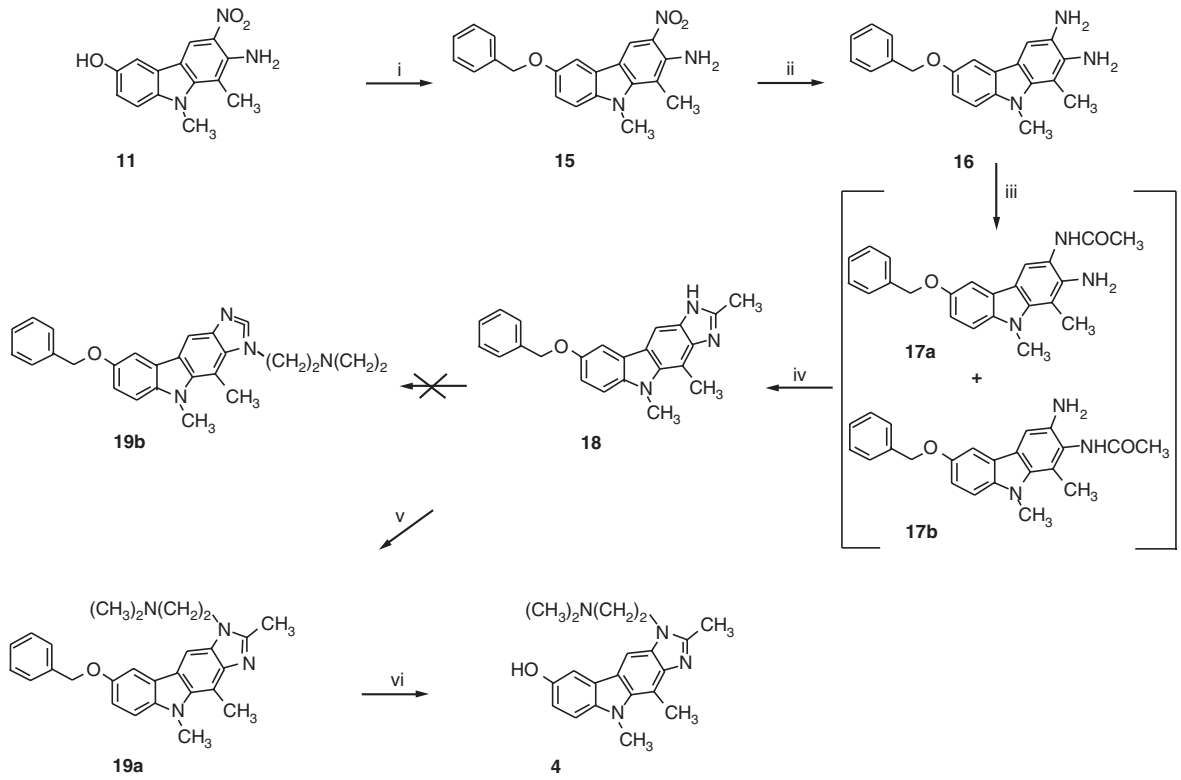
Preparation of compound **3** (Scheme 2) started with the known tetracyclic anhydride **20**.[9] Reaction with trimethylsilylazide in DMF afforded in good yield tetracycle **21**, which could be transformed into pyrimidocarbazole **22** according to a previously described procedure.[10] The cyano substituted pyrimidocarbazole **24** could be easily obtained by a two-step procedure involving quantitative reaction of **22** with phosphorous oxychloride affording the chloro derivative **23**[11] followed by chlorine displacement using potassium cyanide and a catalytic amount of sodium tosylate in DMSO.[12] Transformation of the nitrile **24** into the corresponding amide **25** was achieved by treatment with an excess of hydrogen chloride in a mixture of dichloromethane and methanol. The amide **25** was quantitatively transformed into carboxylic acid sodium salt **26** using sodium hydroxide in a mixture of ethanol and DMSO. The corresponding ester **27**, obtained in methanol in the presence of sulfuric acid, was formylated with hexamethylenetetramine in TFA[13] to give aldehyde **28** whose oxidation in methanol using hydrogen peroxide gave phenolic compound **29** in moderate yield.[13] The introduction of the amide chain was performed in good yield by reaction with *N*,*N*-dimethylethylenediamine to give **3**.

Preparation of compound **4** (Scheme 3) started with the benzylation of the phenolic function in compound **11** using sodium hydride and benzyl chloride in DMF to give carbazole **15** in good yield. Reduction of the nitro group with hydrazine hydrate in methanol and THF using Raney Nickel as catalyst[14] gave the diaminocarbazole **16** which was acetylated with acetyl chloride in THF in the presence of triethylamine to give a mixture of isomer **17a** and **17b** used without separation for the next step. Thermic cyclization[15] gave the expected imidazocarbazole **18** which reacted with 2-(dimethylamino)ethyl chloride hydrochloride in the presence of potassium carbonate[16] in DMF to give **19a**. Although two different isomers **19a** and **19b** could be expected from this reaction, only isomer **19a** was isolated; its structure was elucidated by NMR spectral data using NOE experiment. Debenzylation using cyclohexene in the presence of palladium on activated carbon[17] in *N*-methylpyrrolidone gave compound **4**.

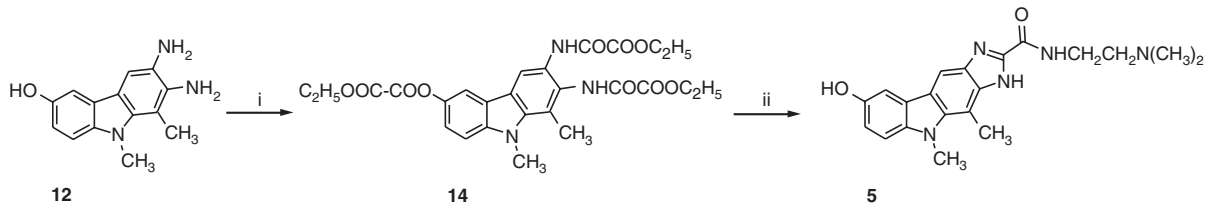
Preparation of compound **5** (Scheme 4) started with exhaustive acylation using ethylchlorooxoacetate of the previously described compound **12** in the presence of triethylamine in DMF. Transamidification of compound **14** using *N*,*N*-dimethylethylenediamine and concomitant cyclodehydration led to compound **5**.



Scheme 2. Synthesis of pyrimidocarbazole **3**: (i) $(CH_3)_3SiN_3$, DMF (52%); (ii) formamidine acetate, 1-methoxy-2-propanol (76%); (iii) $POCl_3$ (96%), (iv) KCN, NaOTos, DMSO (71%); (v) HCl, CH_2Cl_2, CH_3OH (76%); (vi) NaOH, C_2H_5OH, DMSO (100%); (vii) H_2SO_4, CH_3OH (68%); (viii) hexamethylenetetramine, TFA (83%); (ix) $KHSO_4$, H_2O_2, CH_3OH (68%); (x) $H_2N(CH_2)_2N(CH_3)_2$ (88%).



Scheme 3. Synthesis of imidazocarbazole **4**: (i) NaH, $C_6H_5CH_2Cl$, DMF (78%); (ii) $H_2N–NH_2$, H2O, Ni Raney, THF, CH_3OH (94%); (iii) CH_3COCl, $N(C_2H_5)_3$, THF (96%); (iv) Δ (68%); (v) $Cl(CH_2)_2N(CH_3)_2{\cdot}HCl$, K_2CO_3, DMF (79%); (vi) cyclohexene, Pd/C, NMP (74%).



Scheme 4. Synthesis of imidazocarbazole **5**: (i) $ClCOCOOC_2H_5$, $N(C_2H_5)_3$, DMF (83%); (ii) $H_2N(CH_2)_2N(CH_3)_2$ (59%).

3. Results and discussion

The cytotoxic effects of the newly synthesized compounds were evaluated on murine L1210 leukemia cells while the in vivo antitumoral effect was investigated on P388 leukemia and B16 melanoma (see experimental part for detailed protocols). These results were compared with those obtained for the parent compound **1** (Table 1). Thus compound **2** including a pyrazino D ring instead of the pyridine ring in **1** exhibits a much lower cytotoxicity (IC50 = 330 nM vs 13.1 nM). It might be assumed that the different lateral chain position plays a role in this diminished cytotoxicity. The same is true for the in vivo antitumoral activity since **2** reveals either a total lack of effect (P388; T/C = 105%) or a lower one (B16; T/C = 114% vs 157%, respectively).

Table 1. Cytotoxic and antitumoral profiles

Compound	Log *P*[a]	Cytotoxicity L1210 IC50 (nM)	Antitumoral activity			
			P388 Leukemia		B16 Melanoma	
			MTD[b]	T/C (%)	MTD[b]	T/C (%)
1	2.78	13.1	80	184–262	30	157
2	2.78	330	80	105	30	114
3	2.30	10	160	208	60	129
4	2.80	300	80	110	60	185
5	1.57	16900	80	102	20	138

[a] Log *P* values were calculated using Scilog P – Ultra version 1.5 software (MDL, San Leandro, CA, USA).
[b] MTD: maximal tolerated dose (mg/kg).

However if a pyrimidine ring replaces the pyridine D ring in **1** like in compound **3**, cytotoxic and antitumoral effects of the two olivacine derivatives are quite similar (cytotoxicity: L1210 IC50 = 10 nM vs 13.1 nM, respectively; antitumoral activity: P388: T/C = 208% vs 184–262%, respectively and B16: T/C = 129% vs 157%, respectively). In this case, unlike compound **2** the lateral chain position is identical for **1** and **3**.

Further insight on the factors influencing the pharmalogical profile was obtained by preparing and investigating analogues **4** and **5**. Thus cytotoxic effect as well as antitumoral effect on P 388 leukemia exhibited by compound **4** are much lower compared to reference compound **1** (IC50 = 300 nM vs 13.1 nM and T/C = 110% vs 184–262%, respectively). On the contrary, comparative to **1**, this imidazocarbazole tetracycle exhibited a more pronounced antitumoral effect on B16 melanoma T/C = 185% vs 157%, respectively). Unlike compound **4**, compound **5** was devoid of both cytotoxicity (L1210) and antitumoral effect on P388 leukemia. Antitumoral effect of B16 is lower for compound **5** than for reference **1** (T/C = 138% vs 157%, respectively).

Structural similarities and dissimilarities among structures cannot be simply based on superpositions that will evidence presence/absence of functional groups or atoms and interconnectivity patterns.

Indeed global properties that evidence molecular characteristics as a whole should be taken into consideration especially since it is well known that they largely impact on biological behavior.

Thus from simple atom by atom superposition of compounds **1**, **3** and **4**, one might hastily conclude on very large similarities between these molecules.

However when molecular electrostatic potentials (MEP maps) are calculated (Fig. 2, color coded and represented on Connolly surfaces), it is easily observed that while shape similarities are observed for all three compounds, distribution of electrostatic potential values are identical for **1** and **3**, they are very different for compound **4** (Fig. 2, **1**, **3**, **4** from right to left).

Lipophilicity, another global parameter may be appreciated from calculated log *P* (Table 1). Thus very close values are observed for compounds **1**, **2**, **3** and **4** while a less lipophilic value was calculated for **5**. This last compound exhibits no cytotoxicity under the experimental condition employed. It is clear that lipophilicity alone cannot be invoked to explain the difference in toxicity for the first four compounds.

Further studies[18] are needed in order to ascertain the precise impact of these two global parameters on biological behavior.

4. Conclusion

In this paper, the structure–activity relationship arising from the previously unknown ring D modification in compound **1** (S 16020-2) has been disclosed. It was shown that such structural variations strongly impact on the cytotoxic and in vivo properties of the studied molecules. Thus, while pyrazinocarbazole **2** is devoid of any substantial cytotoxic and antitumoral activity, pyrimidocarbazole **3** has a similar profile compared to **1**. Interestingly while L1210 cytotoxicity and P388 in vivo antitumoral effects are lost for imidazocarbazoles **4** and **5**, the compound **4** conserves an in vivo antitumoral effect against B16 melanoma, this effect being even the largest in the series.

5. Experimental

5.1. Chemistry

Melting points (cap) were determined on a Mel-temp capillary apparatus and are uncorrected. Melting points (K) were determined on a Kofler apparatus.

Elemental analysis were performed on a Carlo Erba analyser 1108. Column chromatography was performed using Merck silica gel 60 (0.040–0.063 mm) under a 1 bar nitrogen pressure (flash chromatography).

All reactions were carried out under a nitrogen atmosphere except where otherwise stated.

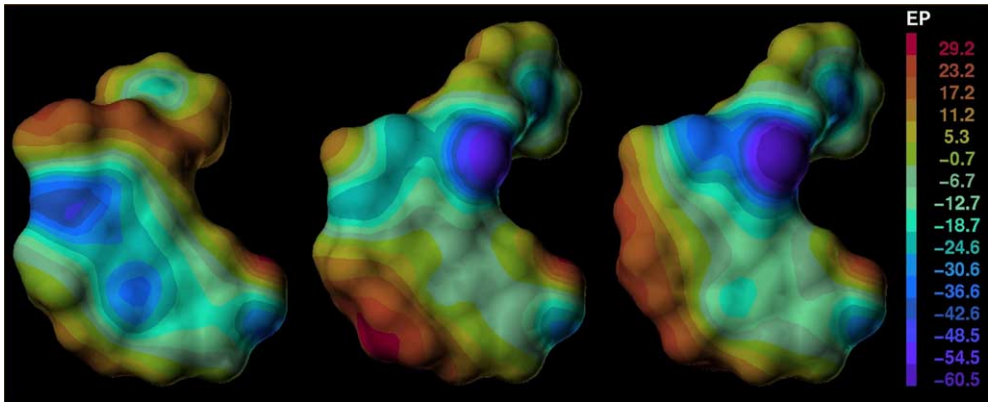


Figure 2. Color coded molecular electrostatic potentials for compounds **1**, **3** and **4**.

^{1}H NMR spectra were recorded at 200 MHz or 300 MHz (as indicated) on a Bruker AC 200 or a Bruker AM 300 spectrometer. Specific high field studies (NOESY experiment on compound **19a**) were performed at 500 MHz on a Bruker AMX 500 spectrometer. Solvent is indicated for each compound.

Significant ^{1}H NMR data are reported in the following order: chemical shifts expressed in ppm downfield from internal tetramethyl silane, number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet).

Electrospray (ESI) high resolution mass spectrometric analysis was performed using a QtoF2 instrument (Waters Micromass, Manchester, UK) operating in the positive mode. 10^{-6} M solutions of compounds x to y were introduced by flow injection in a 75/25 acetonitrile/water mixture. High measurements were precisely done by locking the mass calibration with an internal reference ion (resolving power ca 8500 FWHM at *m*/*z* 556). The low resolution mass spectra were recorded using a Nermag R 10-10 C simple quadrupole mass spectrometer fitted with a fast atom bombardment (FAB) source operating in the positive mode.

5.1.1. 7-Amino-8-methyl-9*H*-carbazol-3-ol (7). A mixture of **6** (88.7 g, 0.33 mol) and 48% hydrobromic acid (2.6 L) was heated under reflux for 2 h and then concentrated under vacuum. The residue was dissolved in water, filtered and the pH was brought to basic using sodium carbonate. The precipitate was filtered off, washed with water and dried at 40 °C under vacuum to yield compound **7** (66 g, 94%).

Mp (K): 239 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.25 (1H, s), 8.60 (1H, s), 7.45 (1H, d), 7.15 (1H, d), 7.1 (1H, s), 6.65 (1H, dd), 6.45 (1H, d), 4.8 (2H, bs), 2.2 (3H, s). Anal. Calcd for $C_{13}H_{12}N_2O$, O. 17 H_2O: C, 72.52; H, 5.78; N, 13.01. Found: C, 72.18; H, 5.75; N, 12.82.

HRMS calcd for $C_{13}H_{13}N_2O$ $(M + H)^+$: 213.1028; found: 213.1916.

5.1.2. 7-(Diacetylamino)-8-methyl-9*H*-carbazol-3-yl acetate (8). A mixture of **7** (66 g, 0.31 mol) and acetic anhydride (3 L) was heated under reflux for 2 h and then concentrated under vacuum. The residue was taken up in ligroin and the solid was collected by filtration and dried under vacuum. Chromatography of the residue eluting with a mixture of 4% THF in dichloromethane led to compound **8** (65.5 g, 62%).

Mp (K): 184 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.5 (1H, s, exchangeable for D_2O), 8.05 (1H, d), 7.9 (1H, d), 7.55 (1H, d), 7.2 (1H, dd), 7.0 (1H, d), 2.3 (6H, s), 2.2 (3H, s). Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.45; H, 5.36; N, 8.28. Found: C, 67.39; H, 5.52; N, 8.16.

5.1.3. 7-(Diacetylamino)-8,9-dimethyl-9*H*-carbazol-3yl acetate (9). Sodium hydride (60% dispersion in mineral oil, 7.6 g, 0.19 mol) was added at room temperature to a stirred solution of compound **8** (65.5 g, 0.19 mol) in DMF (1.1 L). After 30 min, methyl iodide (32.4 g, 0.228 mol) was added at room temperature for 2 h followed by concentration under vacuum. The residue was taken up with dichloromethane and water, the organic layer was washed with a 10% aqueous solution of lithium chloride, dried (Na_2SO_4) and concentrated under vacuum. Chromatography of the residue eluting with 3% acetone in dichloromethane afforded compound **9** (43.2 g, 64%).

Mp (K): 198 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.04 (1H, d), 7.91 (1H, d), 7.62 (1H, d), 7.22 (1H, dd), 7.02 (1H, d), 4.17 (3H, s), 2.57 (3H, s), 2.32 (3H, s), 2.21 (6H, s). Anal. Calcd for $C_{20}H_{20}N_2O_4$: C, 68.17; H, 5.72; N, 7.95. Found: C, 68.11; H, 5.80; N, 7.94.

5.1.4. 7-(Diacetylamino)-8,9-dimethyl-6-nitro-9*H*-carbazol-3-yl acetate (10). Fuming nitric acid ($d = 1.52$, 5.2 mL, 0.12 mol) was added at 15 °C to a stirred solution of **9** (43 g, 0.12 mol) in acetic acid (2 L). The mixture was stirred at room temperature for 3 h and concentrated under vacuum. The yellow residue was taken up with diethyl ether collected by filtration, washed with diethyl ether and dried under vacuum. Column chromatography of the residue eluting with 5% THF in toluene afforded compound **10** (25.3 g, 53%).

Mp (K): 266 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.07 (1H, s), 8.2 (1H, d), 7.8 (1H, d), 7.38 (1H, dd), 4.25 (1H, s), 2.72 (3H, s), 2.38 (3H, s), 2.28 (6H, s). Anal. Calcd for $C_{20}H_{19}N_3O_6$: C, 60.45; H, 4.82; N, 10.57. Found: C, 60.77; H, 4.97; N, 10.56.

5.1.5. 7-Amino-8,9-dimethyl-6-nitro-9*H*-carbazol-3-ol (11). Compound **10** (25.2 g, 0.063 mol) was dissolved in ethanol (350 mL) and 20% aqueous sodium hydroxide solution (350 mL). The mixture was stirred for 5 h at reflux temperature. Then, the cooled mixture was neutralized with 4 N hydrochloric acid and the ethanol was distilled off under vacuum. The red precipitate was collected by filtration, washed with water and diethyl ether and dried at 40 °C under vacuum to yield compound **11** (17.2 g, 100%).

Mp (K): 243 °C. ^{1}H NMR (200 MHz, DMSO-d_6) δ 9.15 (1H, br s, exchangeable for D_2O), 8.6 (1H, s), 7.37 (1H, d), 7.25 (1H, d), 7.04 (2H, br s, exchangeable for D_2O), 6.85 (1H, dd), 3.9 (3H, s), 2.52 (3H, s). Anal. Calcd for $C_{14}H_{13}N_3O_3$: C, 61.99; H, 4.83; N, 15.49. Found: C, 61.50; H, 4.93; N, 15.03.

5.1.6. 6,7-Diamino-8,9-dimethyl-9*H*-carbazol-3-ol (12). A solution of compound **11** (17 g, 0.062 mol) in NMP (680 mL) in the presence of activated nickel (4 mL) was hydrogenated under a 5 bar pressure of hydrogen during 16 h. the catalyst was removed by filtration and the filtrate was concentrated under vacuum. The residue was taken up with diethyl ether, collected by filtration, washed with diethyl ether and dried at 40 °C under vacuum to yield compound **12** (15.0 g, 100%).

Mp (cap): dec > 250 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.66 (1H, s, exchangeable for D_2O), 7.1 (1H, d), 7.02

(1H, d), 6.99 (1H, s), 4.35 (4H, br s, exchangeable for D_2O), 3.82 (3H, s), 2.48 (3H, s). Anal. Calcd for $C_{14}H_{15}N_3O$: C, 69.69; H, 6.27; N, 17.41. Found: C, 68.80; H, 6.25; N, 16.80. (Water dosage impossible).

5.1.7. Ethyl 9-hydroxy-5,6-dimethyl-6*H*-pyrazino[2,3-*b*]-carbazole-2-carboxylate (13). Ethyl bromopyruvate (1.63 mL, 0.013 mol) was added, dropwise, to a solution of **12** (3.0 g, 0.0124 mol) in NMP (120 mL). The mixture was stirred for 16 h at room temperature and concentrated under vacuum. Chromatography of the residue eluting with a mixture of 3% methanol in dichloromethane gave compound **13** (1.2 g, 29%).

Mp (K): 269 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.35 (1H, s, exchangeable for D_2O), 9.3 (1H, s), 8.75 (1H, s), 7.75 (1H, d), 7.50 (1H, d), 7.15 (1H, dd), 4.5 (2H, q), 4.2 (3H, s), 3.3 (3H, s), 1.45 (3H, t). This compound, which contained NMP, was used without further purification for the next step.

5.1.8. N-[2-(Dimethylamino)ethyl]-9-hydroxy-5,6-dimethyl-6*H*-pyrazino[2,3-*b*]carbazole-2-carboxamide (2). Compound **13** (1.2 g, 0.0036 mol) and *N*,*N*-dimethylethylenediamine (25 mL) was stirred in a autoclave at 120 °C for 20 h. The mixture was concentrated under vacuum. Chromatography of the residue eluting with a mixture of 1% ammoniac solution (28%) and 10% methanol in dichloromethane yielded compound **2** (0.6 g, 44%).

Mp (cap): 247–250 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.4 (1H, s), 9.3 (1H, s), 8.9 (1H, t), 8.7 (1H, s), 7.7 (1H, d), 7.55 (1H, d), 7.15 (1H, dd), 4.20 (3H, s), 3.55 (2H, q), 3.35 (3H, s), 2.6 (2H, t), 2.3 (6H, s). Anal. Calcd for $C_{21}H_{23}N_5O_2$: C, 66.83; H, 6.14; N, 18.55. Found: C, 66.25; H, 6.08; N, 18.17.

5.1.9. 6,7-Bis{[ethoxy(oxo)acetyl]amino}-8,9-dimethyl-9*H*-carbazol-3-yl ethyl oxalate (14). Ethyl chorooxoacetate (7.9 g, 0.0581 mol) was added to a stirred solution of compound **12** (4.0 g, 0.0166 mol) in DMF (100 mL) in the presence of triethylamine (5.9 g, 0.0581 mol) at a temperature below 10 °C. The mixture was stirred at 10 °C for 16 h and concentrated under vacuum. The residue was taken up with dichloromethane and water and the organic phase was dried (Na_2SO_4) and concentrated under vacuum. Chromatography of the residue eluting with 4% THF in dichloromethane yielded compound **14** (7.5 g, 83%).

Mp (K): 237 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.39 (1H, s, exchangeable for D_2O), 10.13 (1H, s, exchangeable for D_2O), 8.25 (1H, s), 8.04 (1H, d), 7.7 (1H, d), 7.38 (1H, dd), 4.44-4.27 (6H, m), 4.17 (3H, s), 2.64 (3H, s), 1.4-1.29 (9H, m). Anal. Calcd for $C_{26}H_{27}N_3O_{10}$: C, 57.67; H, 5.03; N, 7.76. Found: C, 57.53; H, 5.11; N, 7.56.

5.1.10. *N*-[2-(Dimethylamino)ethyl]-8-hydroxy-4,5-dimethyl-1,5-dihydroimidazo[4,5-*b*]carbazole-2-carboxamide (5). Compound **14** (3.5 g, 0.0065 mol) and *N*,*N*-dimethylethylenediamine (100 mL) are stirred in an autoclave at 120 °C for 20 h. The mixture was concentrated under vacuum. Chromatography of the residue eluting with 1% ammoniac solution (28%) and 10% methanol in dichloromethane yielded compound **5** (1.4 g, 59%) as a mixture of tautomers.

Mp (K): 273 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.02 (0.5H, s, exchangeable for D_2O), 12.94 (0.5H, s, exchangeable for D_2O), 8.93 (1H, s, exchangeable for D_2O), 8.6 (0.5H, t, exchangeable for D_2O), 8.51 (0.5H, t, exchangeable for D_2O), 8.13 (0.5H, s), 7.84 (0.5H, s), 7.5 (0.5H, d), 7.44 (0.5H, d), 7.3 (1H, d), 6.92 (1H, dd), 4.06 (1.5H, s), 4.05 (1.5H, s), 3.44 (2H, t), 3.1 (1.5H, s), 3.0 (1.5H, s), 2.5 (2H,t), 2.2 (6H, s). Anal. Calcd for $C_{20}H_{23}N_5O_2$, 1.6 H_2O: C, 60.93; H, 6.70; N, 17.76. Found: C, 60.40; H, 6.39; N, 17.29. MS calcd for $C_{20}H_{24}N_5O_2$ $(M + H)^+$: *m/z* 366.

5.1.11. 6-(Benzyloxy)-1,9-dimethyl-3-nitro-9*H*-carbazol-2-amine (15). Sodium hydride (60% dispersion in mineral oil, 0.86 g, 0.021 mol) was added at room temperature to a stirred solution of compound **11** (5.7 g, 0.021 mol) in DMF (120 mL). After 1 h at room temperature, benzyl chloride (3.59 g, 0.021 mol) was added, the mixture stirred 2 h and concentrated under vacuum. The residue was taken up with dichloromethane and water, the organic phase was dried (Na_2SO_4) and concentrated under vacuum. Chromatography of the residue eluting with dichloromethane yielded compound **15** (5.9 g, 78%).

Mp (K): 193 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.8 (1H, s), 7.9 (1H, s), 7.5 (2H, d), 7.4 (3H, m), 7.4 (1H, m), 7.1 (1H, dd), 7.10 (1H, s, exchangeable for D_2O), 5.2 (2H, s), 3.95 (3H, s), 2.55 (3H, s). Anal. Calcd for $C_{21}H_{19}N_3O_3$: C, 69.79; H, 5.30; N, 11.63. Found: C, 69.74; H, 5.35; N, 11.48.

5.1.12. 6-(Benzyloxy)-1,9-dimethyl-9*H*-carbazole-2,3-diamine (16). Compound **15** (6.5 g, 0.018 mol) was dissolved in THF (260 mL) and methanol (130 mL). Activated nickel was added and the mixture was heated at reflux temperature. Hydrazine hydrate was added dropwise and the mixture stirred for 15 min. The catalyst was removed by filtration and the filtrate concentrated and dried under vacuum to yield compound **16** (5.62 g, 94%).

Mp (K): 164 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.5(2H, d), 7.4 (3H, m), 7.35 (1H, d), 7.25 (1H, d), 7.1 (1H, s), 6.9 (1H, dd), 5.1 (2H, s), 4.4 (4H, m, exchangeable for D_2O), 3.9 (3H, s), 2.5 (3H, s). HRMS calcd for $C_{21}H_{22}N_3O$ $[M + H]^+$: 332.1763; found: 332.1765.

5.1.13. *N*-[2-Amino-6-(benzyloxy)-1,9-dimethyl-9*H*-carbazol-3-yl] acetamide (17). Acetyl chloride (1.2 g, 0.0143 mol) was added at 5 °C to a stirred solution of compound **16** (4.5 g, 0.0136 mol) in THF (225 mL) in the presence of triethylamine (1.45 g). The mixture was stirred 1 h at room temperature and concentrated under vacuum. The residue was taken up with dichloromethane and water, the organic phase was dried (Na_2SO_4), concentrated under vacuum and dried at 40 °C under vacuum to yield compound **17** (4.9 g, 96%).

Mp (cap): 198–204 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.1 (1H, s, exchangeable for D_2O), 7.65 (1H, s), 7.5 (1H, d), 7.5–7.35 (5H, m), 7.3 (1H, d), 6.95 (1H, dd), 5.2 (2H, s), 4.75 (2H, bs), 4.0 (3H, s), 2.55 (3H, s), 2.1 (3H, s). HRMS Calcd for $C_{23}H_{24}N_3O_2$ $[M + H]^+$: 374.1869; found: 374.1846.

5.1.14. 8-(Benzyloxy)-2,4,5-trimethyl-1,5-dihydroimidazo-[4,5-*b*]carbazole (18). Compound **17** (4.9 g, 0.013 mol) in DMF (260 mL) was heated under reflux for 4 h and then concentrated under vacuum. The residue was taken up with dichloromethane and water. The organic phase was dried (Na_2SO_4) and concentrated under vacuum. Chromatography of the residue eluting with a mixture of 4% methanol in dichloromethane afforded compound **18** (3.12 g, 68%) as a mixture of tautomers.

Mp (K): 251 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ (majority form) 12.00 (1H, m, exchangeable for D_2O), 7.9 5 (1H, s), 7.80 (1H, d), 7.50 (2H, d), 7.35 (4H, m), 7.10 (1H, dd), 5.20 (2H, s), 4.05 (3H, s), 3.90 (3H, s), 2.55 (3H, s). MS: *m*/*z* 356 $[M + H]^+$.

5.1.15. {2-[8-(Benzyloxy)-2,4,5-trimethylimidazo[4,5-*b*]carbazol-1(5*H*)-yl]ethyl}dimethylamine (19a). A mixture of compound **18** (0.3 g, 0.0008 mol), THF (20 mL), DMF (10 mL), potassium carbonate (0.22 g, 0.0016 mol), 2-dimethylamino ethyl chloride hydrochloride and adogene 464 (0.1 g) was stirred at reflux temperature for 16 h and concentrated under vacuum. The residue was taken up with water, collected by filtration, dissolved with a mixture of methanol and dichloromethane, dried (Na_2SO_4) and concentrated under vacuum. Chromatography of the residue eluting with a mixture of 5% methanol in dichloromethane afforded compound **19** (0.27 g, 79%).

Mp (K): 213 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.00 (1H, s), 7.85 (1H, d), 7.55 (2H, d), 7.45 (1H, d), 7.40 (3H, m), 7.10 (1H, dd), 5.20 (2H, s), 4.30 (2H, t), 4.05 (3H, s), 3.00 (3H, s), 2.65 (2H,t), 2.50 (3H, s), 2.25 (6H, s).

In a NOESY experiment, specific Overhauser were observed between, CH3 (δ = 2.50 ppm) and CH2 (δ = 4.30 ppm); between CH2 (δ = 4.30 ppm) and CH (δ = 8.00 ppm); between CH (δ = 8.00 ppm) and CH (δ = 5.85 ppm) and between CH (δ = 7.85 ppm) and CH2 (δ = 5.20 ppm). All these observations led to a non ambiguous determination of the position of substitution. MS: *m*/*z* 427 $[M + H]^+$.

5.1.16. 1-[2-(Dimethylamino)ethyl]-2,4,5-trimethyl-1,5-dihydroimidazo[4,5-*b*]carbazol-8-ol (4). A mixture of compound **19** (0.36 g, 0.0008 mol), cyclohexene (4 mL), NMP (12 mL) and 10% palladium on activated carbon (0.1 g) was stirred at 90 °C for 1 h. The catalyst was filtered off and the filtrate was concentrated under vacuum. The residue was washed with ether and dried under vacuum at 40 °C to yield compound **4** (0.2 g, 74%).

Mp (cap): 275–280 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.85 (1H, s, exchangeable for D_2O), 7.85 (1H, s), 7.45 (1H, d), 7.30 (1H, d), 6.90 (1H, dd), 4.30 (2H, t), 4.05 (3H, s), 3.00 (3H, s), 2.65 (2H, t), 2.60 (3H, s), 2.25 (6H, s). MS: *m*/*z* 337 $[M + H]^+$.

5.1.17. 10,11-Dimethyl-1,10-dihydro[1,3]oxazino[4,5-*b*]carbazole-2,4-dione (21). Trimethylsilylazide (5.1 mL, 0.0383 mol) was added to a stirred solution of compound **20** (1.8 g, 0.00678 mol) in DMF (90 mL) at 60 °C. The mixture was stirred at 80 °C for 2 h, at 100 °C for 15 min and at room temperature for 16 h. The solid was collected by filtration, washed with diethyl ether and dried at 40 °C under vacuum to yield compound **21** (1.0 g, 52%).

Mp (cap): 338–345 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.7 (1H, s), 8.25 (1H, d), 7.65 (1H, d), 7.5 (2H, t), 7.3 (2H, t), 4.1 (3H, s), 2.7 (3H, s). MS: *m*/*z* 281 $[M + H]^+$.

5.1.18. 10,11-Dimethyl-3,10-dihydro-4*H*-pyrimido[4,5-*b*]carbazol-4-one (22). Compound **21** (24.7 g, 0.088 mol) and formamidine acetate (36.6 g, 0.352 mol) were dissolved in 1-methoxy-2-propanol (2.47 L) and stirred for 1 h at reflux temperature. Silica gel (120 g) was added and the suspension was concentrated to dryness under vacuum. The residue was eluted with a mixture of 30% THF in dichloromethane to yield compound **22** (17.65 g, 76%).

Mp (cap): 307–315 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.9 (1H, s), 8.8 (1H, s), 8.3 (1H, d), 8.1 (1H, s), 7.65 (1H, d), 7.5 (1H, t), 7.25 (1H, t), 4.2 (3H, s), 3.15 (3H, s). MS: *m*/*z* 264 $[M + H]^+$.

5.1.19. 4-Chloro-10,11-dimethyl-10*H*-pyrimido[4,5-*b*]carbazole (23). Compound **22** (17.6 g, 0.0668 mol) was stirred at reflux temperature in phosphorus oxychloride (400 mL) for 3 h. The suspension was concentrated under vacuum. The residue was washed with diethyl ether, collected by filtration and stirred with an aqueous solution of sodium hydrogencarbonate. The yellow solid was collected by filtration, washed with water and dried at 40 °C under vacuum to yield compound **23** (18.0 g, 96%).

Mp (K): 258 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.1 (1H, s), 8.8 (1H, s), 8.25 (1H, d), 7.65 (1H, t), 7.4 (1H, d), 7.35 (1H, t), 4.25 (3H, s), 3.3 (3H, s). MS: *m*/*z* 282 $[M + H]^+$.

5.1.20. 10,11-Dimethyl-10*H*-pyrimido[4,5-*b*]carbazole-4-carbonitrile (24). Compound **23** (10.0 g, 0.035 mol), potassium cyanide (22.8 g, 0.35 mol) and sodium tosylate (2.3 g, 0.012 mol) were stirred at 80 °C for 4 h 30 min DMSO (500 mL). The suspension was poured in sodium chloride saturated aqueous solution (2 L) and extracted with dichloromethane. The organic phase was washed with water, dried (Na_2SO_4) and concentrated under vacuum. Chromatography of the residue eluting with dichloromethane yielded compound **24** (6.8 g, 71%).

Mp (K): 249 °C. ^{1}H NMR (300 MHz, CDCl3) δ 9.4 (1H, s), 8.8 (1H, s), 8.3 (1H, d), 7.7 (1H, t), 7.5 (1H, d), 7.4 (1H, t), 4.3 (3H, s), 3.3 (3H, s). MS: *m*/*z* 273 $[M + H]^+$.

5.1.21. 10,11-Dimethyl-10*H*-pyrimido[4,5-*b*]carbazole-4-carboxamide (25). Anhydrous hydrogen chloride was slowly bubbled for 5 h through a solution of compound **24** (3.2 g, 0.01175 mol) in methanol (500 mL) and dichloromethane (500 mL) at a temperature below 20 °C. The mixture was stirred for 16 h at room temperature and concentrated under vacuum to a volume of approximately 100 mL. The solid was collected by filtration, washed with water, ethanol and diethyl ether, dried at 40 °C under vacuum to yield compound **25** (2.6 g, 76%).

Mp (K): 226 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.3 (2H, 2s), 8.4 (1H, s, exchangeable for D_2O), 8.35 (1H, d), 8..0 (1H, s, exchangeable for D_2O), 7.7 (1H, d), 7.65 (1H, t), 7.35 (1H, t), 4.25 (3H, s), 3.3 (3H, s). MS: *m*/*z* 291 $[M + H]^+$.

5.1.22. 10,11-Dimethyl-10*H*-pyrimido[4,5-*b*]carbazole-4-carboxylic sodium salt (26). Compound **25** (4.3 g, 0.0154 mol) was dissolved in ethanol (200 mL) and DMSO (100 mL) in the presence of 1 N sodium hydroxide aqueous solution (46.2 mL). The mixture was stirred at reflux temperature for 20 h and concentrated under vacuum. The residue was taken up with ethanol and diethyl ether, collected by filtration, washed with diethyl ether and dried at 40 °C under vacuum to yield compound **26** (5.0 g) which was used without further purification for the next step.

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.0 (1H, s), 8.85 (1H, s), 8.20 (1H, d), 7.65 (1H, d), 7.60 (1H, t), 7.30 (1H, t), 4.25 (3H, s), 3.25 (3H, s). MS: *m*/*z* 314 $[M + H]^+$.

5.1.23. Methyl 10,11-dimethyl-10*H*-pyrimido[4,5-*b*]carbazole-4-carboxylate (27). Compound **26** (0.8 g, 0.00255 mol) was dissolved in methanol (500 mL) in the presence of concentrated sulfuric acid. The mixture was stirred at reflux temperature for 3 h. After cooling, the mixture was neutralized using sodium hydrogencarbonate, concentrated under vacuum, the residue taken up with water, collected by filtration, washed with water and dried at 40 °C under vacuum to yield compound **27** (0.53 g, 68%).

Mp (K): 222 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.30 (1H, s), 9.00 (1H, s), 8.40 (1H, d), 7.70 (2H, m), 7.35 (1H, m), 4.20 (6H, 2s), 3.35 (3H, s). HRMS calcd for $C_{18}H_{16}N_3O_2$ $[M + H]^+$: 306.12.43; found: 306.1236.

5.1.24. Methyl 7-formyl-10,11-dimethyl-10*H*-pyrimido-[4,5-*b*]carbazole-4-carboxylate (28). Compound **27** (3.5 g, 0.0115 mol) was dissolved in trifluoroacetic acid (175 mL). Hexamethylenetetramine (18.2 g) was added over a 30-min period. The mixture was stirred at reflux temperature for 40 min and concentrated under vacuum. The residue was taken up with a sodium hydrogencarbonate aqueous solution, the solid was collected by filtration, washed with water and dried at 40 °C under vacuum. Chromatography of this product eluting with 3% THF in dichloromethane gave compound **28** (3.15 g, 83%).

Mp (K): 268 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.10 (1H, s), 9.40 (1H, s), 9.15 (1H, s), 9.00 (1H, s), 7.85 (1H, d), 8.15 (1H, d), 4.30 (3H, s), 4.20 (3H, s), 3.35 (3H, s). Anal. Calcd for $C_{19}H_{15}N_3O_3$: C, 68.46; H, 4.54; N, 12.61. Found: C, 68.28; H, 4.53; N, 12.45.

5.1.25. Methyl 7-hydroxy-10,11-dimethyl-10*H*-pyrimido-[4,5-*b*]carbazole-4-carboxylate (29). Compound **28** (3.09 g, 0.0092 mol) and methanol (300 mL) were vigorously stirred at room temperature. Potassium hydrogensulfate (0.9 g) was added and 30% hydrogen peroxide (6 mL) was added over a 15 min period. The mixture was then heated at reflux temperature for 1 h. After cooling to 40 °C, the solid (mixture of starting material **28** and mineral products) was collected by filtration and reused, without further purification, in the conditions described above. This process was repeated several times until completion of the reaction. Each time, filtrates were poured in a mixture of dichloromethane (400 mL) and sodium hydrogencarbonate aqueous solution (400 mL). Combined organic phases were dried (Na_2SO_4) and concentrated under vacuum. Chromatography of the residue, eluting with a mixture of 5% THF in dichloromethane, afforded compound **29** (1.78 g, 68%).

Mp (K): 246 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.40 (1H, s), 9.35 (1H, s), 9.30 (1H, s, exchangeable for D_2O), 7.75 (1H, d), 7.55 (1H, d), 7.15 (1H, dd), 4.20(3H, s), 4.15 (3H, s), 3.25 (3H, s). HRMS calcd for $C_{18}H_{16}N_3O_3$ $[M + H]^+$: 322.1192; found: 322.1160.

5.1.26. *N*-[2-(Dimethylamino)ethyl]-7-hydroxy-10,11-dimethyl-10H-pyrimido[4,5-*b*]carbazole-4-carboxamide (3). Compound **29** (1.0 g, 0.0031 mol) and *N*,*N*-dimethylethylenediamine (40 mL) were stirred at 60 °C for 1 h and concentrated under vacuum. Chromatography of the residue eluting with a mixture of 0.5% ammonia solution (28%) and 5% methanol in dichloromethane yielded compound **3** (1.03 g, 88%).

Mp (cap): 235–240 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.35 (1H, s), 9.30 (1H, s, exchangeable for D_2O), 9.25 (1H, s), 8.25 (1H, t, exchangeable for D_2O), 7.60(1H, d), 7.50 (1H, d), 7.15 (1H, dd), 4.20 (3H, s), 3.55 (2H, q), 3.25 (3H, s), 2.55 (2H, t), 2.25 (6H, s). Anal. Calcd for $C_{21}H_{23}N_5O_2$: C, 66.83; H, 6.14; N, 18.55. Found: C, 66.72; H, 6.06; N, 18.57. HRMS calcd for $C_{21}H_{24}N_5O_2$ $[M + H]^+$: 378.1930; found: 378.1916.

5.2. Biology

5.2.1. Cell culture and cytotoxicity. The murine L1210 leukemia cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 10 mM HEPES buffer (pH = 7.4). Cytotoxicity was measured by the microculture tetrazolium assay as described.[19,20] Cells were exposed to graded concentrations of drug (nine serial dilutions in triplicate) for about four doubling times (48 h). Results were expressed as IC50, the concentration that reduced by

50% the optical density of treated cells with respect to untreated controls.

5.2.2. Antitumor activity. The antitumor activity of the compounds was evaluated on the P388 leukemia and the B16 melanoma, all provided by the NCI, Frederick, U.S.A. P388 cells were inoculated ip (10^6 cells/mouse) into B6D2F1 mice (Iffa credo) on day 0. The dichlorohydrate salts of the compounds were dissolved in water and injected iv on day 1. The results are expressed in terms of percent T/C survival (median survival time of treated animals/median survival time of control animals, $\times 100$). For the ip B16 melanoma, 0.5 mL of a tumor brei (1 g of tumor in 10 mL 0.9% NaCl) were injected ip on day 0, and compounds were administered ip on days 1, 5, 9. The optimal dose was the dose which gave the higher T/C without major toxicity (no toxic death, weight loss <20%). Results are expressed as % T/C survival.

5.2.3. Molecular modelling. Structures discussed in this study were modified using standard bond lengths as implemented in SYBYL version 6.9.1 from Tripos[21] running on an OCTANE R12000 Silicon Graphics workstation.

A comparison of electrostatic potentials was performed for molecules **1**, **3** and **4** (same side chain position relative to the tetracyclic skeleton. Thus the X-ray structure of **1** (S 16020-2) was used as a model to set the basic side chain position, followed by a short AM1 semiempirical optimization[22] to remove any unfavorable interactions.

Electrostatic potentials were then calculated using partial Gasteiger–Hückel charges, and were projected on the Connolly surfaces of the molecules, visualized using the MOLCAD software within SYBYL.

Energy levels (29.2 to −60.5 kcal/mol) are represented by a color scheme (Fig. 2).

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