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Synthesis and biological evaluation of anilino substituted pyrimidine linked pyrrolobenzodiazepines as potential anticancer agents

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ABSTRACT

A series of new anilino substituted pyrimidine linked pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) conjugates were prepared and evaluated for their anticancer activity. The effects of four promising PBD conjugates on cell cycle of cancerous cell line A375 were investigated. These compounds showed the characteristic features of apoptosis like enhancement in the levels of p53, release of cytochrome *c*, and cleavage of PARP.

The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are a group of naturally occurring DNA-interactive antitumour antibiotics, examples of which include anthramycin, tomamycin, sibiromycin, chicamycin, prothracarin, DC-81 (**3**) and dextochrysin.[1,2] The covalent bond formation in the minor groove of DNA by nucleophilic attack of 2-amino group of guanine base to form an amino linkage to C-11 is mainly responsible for the biological activities of PBDs.[3,4] Recently a series of C2-fluorinated PBDs have been synthesized and evaluated for anticancer activity against a number of cancer cell lines,[5] in which fluorine substitution plays an important role for their biological activity. In the past few years, several hybrid compounds, in which known antitumour agents tethered to PBD moiety, have been designed, synthesized and evaluated for their biological activity.[6,7] Moreover, we have been involved in the development of new synthetic strategies[8–10] for the preparation of PBD ring system and also in the design of structurally modified PBDs and their hybrids for the development of more potent anticancer agents.[11–14]

An anilino substituted pyrimidine moiety present in the structures of agents like STI571 (Imatinib mesylate, gleevec (**1**)), nicotinib (**2**) (Fig. 1) is considered responsible for the potent antitumor properties.[15,16]

Many chemotherapeutics are known to induce apoptosis, which is a promising strategy for cancer drug discovery. Among the different approaches available to promote apoptosis, the development of conjugates is one of the main strategies. From the previous studies it is known that anilino primidines are reported to possess apoptosis inducing ability,[17,18] similarly some of the PBD conjugates are also known to induce apoptosis.[19,20] Therefore, in continuation of our efforts in the development of apoptosis inducers as potential anticancer agents, it has been considered of interest to synthesize these PBD conjugates with a view to increase the efficacy of the compounds, that is, additive or synergistic effect in comparison to both the precursors. Therefore in continuation of these efforts, we herein report the synthesis and biological evaluation of some new hybrids wherein anilino substituted pyrimidine moieties were linked to the PBD ring system.

Synthesis of pyrimidine intermediates **13a**,**b** and **15** was carried out from the compounds **11a,b** which were prepared by the literature method.[21] Compounds **11a**,**b** were coupled to 5-bromo valeryl chloride (**12**) to provide compounds **13a,b** whereas this **11a** was treated with 4-(bromomethyl)benzoyl chloride (**14**) to obtain compound **15** as shown in Scheme 1.

Synthesis of C8-linked anilino substituted pyrimidine—PBD conjugates (**4a–c** and **5a,b**) was carried out by employing the hydroxy nitrothioacetal compounds **16a,b** as the starting material, which was prepared by previously reported methods.[11–14] These upon etherification with the intermediates **13a,b**, and **15** using K_2CO_3 in acetone provided the corresponding nitro thioacetals

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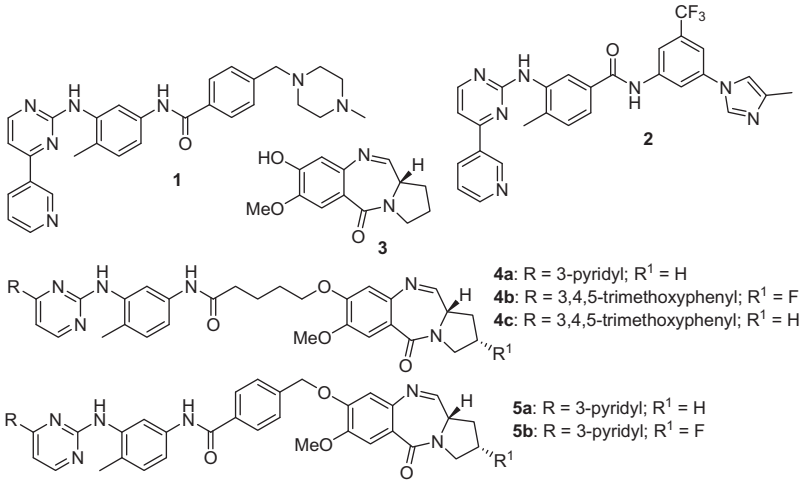
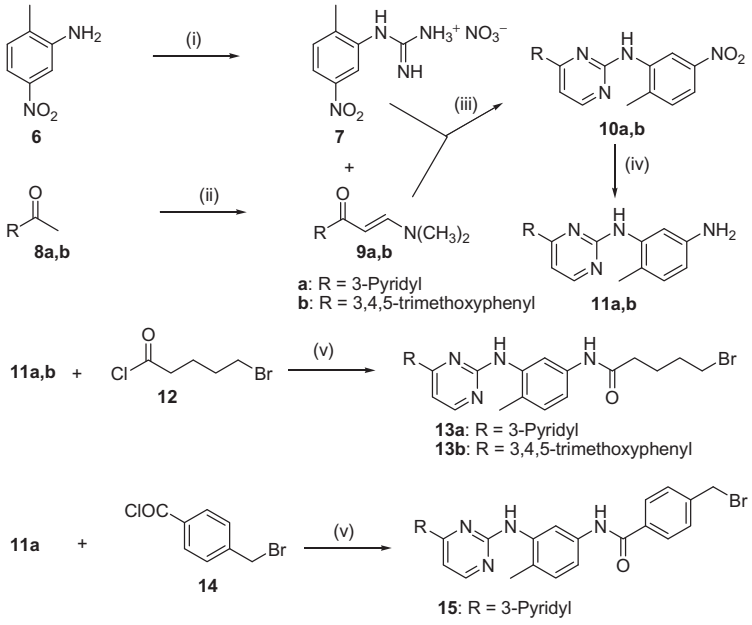


Figure 1. Chemical structures of biologically important imatinib mesylate or gleevec (**1**), nilotinib (**2**), DC-81 (**3**), and PBD conjugates (**4a–c** and **5a**,**b**).

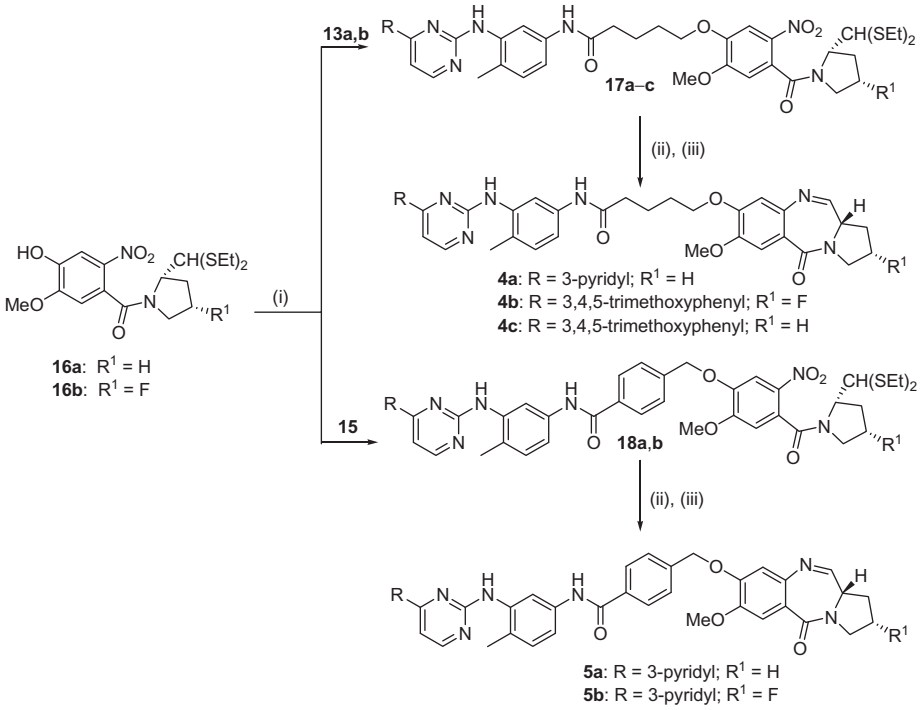


Scheme 1. Reagents and conditions: (i) H_2NCN, HNO_3, EtOH, reflux, 24 h, 58%; (ii) DMF–DMA, toluene, reflux, 24 h, 75%; (iii) NaOH, *n*-butanol, reflux, 48 h, 60%; (iv) $SnCl_2{\cdot}2H_2O$, HCl, rt, 2 h, 80%; (v) K_2CO_3, dry DMF, rt, 6 h, 85%.

(**17a–c** and **18a**,**b**). Further, reduction of these nitro compounds by using $SnCl_2{\cdot}2H_2O$ in MeOH followed by deprotective cyclization employing $HgCl_2/CaCO_3$ afford the desired PBD conjugates (**4a–c** and **5a**,**b**) as shown in Scheme 2.[22]

DNA-binding ability of these PBD conjugates (**4a–c** and **5a**,**b**) was investigated by thermal denaturation studies using calf thymus (CT) DNA. The increase in the helix melting temperature (ΔT_m) for each compound was examined at 0 and 18 h incubation at 37 °C. Interestingly, all the compounds **4a–c** and **5a**,**b** elevated the helix melting temperature of CT-DNA in the range of 1.2−7.1 °C. Compounds **5a**,**b** have shown highest ΔT_m ranges from 5.0 to 5.8 °C at 0 h and increased up to 6.8−7.1 °C after 18 h incubation. Moreover, the naturally occurring PBD, that is, DC-81 (**3**) exhibits a ΔT_m of 0.7 °C under similar experimental conditions as illustrated in Table 1.

A quantitative restriction enzyme digestion (RED100) assay was previously developed in which the inhibition of DNA cleavage by *BamH*I is used to probe the DNA binding capability of PBD compounds. Further, from the previous studies it was reported that, PBD prefers G-rich sequences (5′-G′GATCC-3′) in DNA binding which could be due to the affinity of PBDs in covalent interaction with the free amino group attached to the N2 of guanine in the DNA.[23] Therefore, in order to investigate the binding capability of these compounds to DNA at G-rich regions, restriction endonuclease *BamH*I assay was performed. The pBR322 vector DNA was incubated with these compounds **4a**,**b** and **5a**,**b** at 4 and 8 μM concentrations and digested with *BamH*I enzyme. The results showed that PBD conjugates could not inhibit DNA digestion at 4 μM concentration. However, compounds **5a**,**b** inhibited DNA digestion at 8 μM concentration as shown in Supplementary Figure 1. Further, it was observed from Figure 2 that one of the promising compounds **5b** was found to inhibit the restriction digestion at 8 μM concentration, whereas DC-81 showed similar effects at a higher concentration of 16 μM. These results showed an enhanced DNA binding ability for the compound **5b** compared to the naturally occurring DC-81(**3**).



Scheme 2. Reagents and conditions: (i) K_2CO_3, dry CH_3COCH_3, 48 h, 80–89%; (ii) $SnCl_2.2H_2O$, CH_3OH, reflux, 2 h; (iii) $HgCl_2$, $CaCO_3$, CH_3CN–H_2O, (4:1), 8 h, 52–59%.

Table 1
Thermal denaturation data for PBD conjugates **4a–c** and **5a**,**b** with calf thymus (CT)-DNA

Compound	[PBD]/[DNA] molar ratio[b]	ΔT_m (°C)[a] after incubation at 37 °C for	
		0 h	18 h
4a	1:5	2.0	2.1
4b	1:5	1.5	2.5
4c	1:5	1.2	1.4
5a	1:5	5.0	6.8
5b	1:5	5.8	7.1
DC-81 (3)	1:5	0.3	0.7

[a] For CT-DNA alone at pH 7.00 ± 0.01, ΔT_m = 68.5 °C ± 0.01 (mean value from 10 separate determinations), all ΔT_m values are ± 0.1−0.2 °C.

[b] For a 1:5 molar ratio of [PBD]/[DNA], where CT-DNA concentration = 100 μM and ligand concentration = 20 μM in aqueous sodium phosphate buffer [10 mM sodium phosphate + 1 mM EDTA, pH 7.00 ± 0.01].

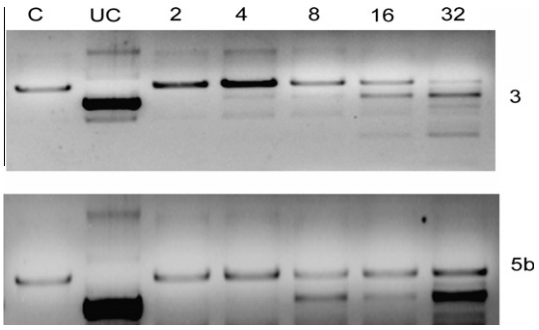


Figure 2. RED 100 assay: The figure in the top panel depicts the Red assay using DC-81 (**3**) and figure in the bottom panel depicts the compound **5b**. C, the Cut vector DNA with *BamH*I enzyme in the absence of compound and UC, the uncut vector DNA. The numbers 2, 4, 8, 16, and 32 depicts the concentration in micro molar of the compound used in the RED assay.

Compounds **4a–c** and **5a**,**b** were also evaluated for their cytotoxicity in selected human cancer cell lines of breast, oral, ovary, colon, lung, prostate, and cervix by using sulforhodamine B (SRB) method. The compounds exhibiting $GI_{50} \leqslant 10^{-5}$ M (10 μM) are considered to be active on the respective cell lines. Table 2 reveals that these compounds exhibited broad-spectrum activity in all the cell lines employed and the activity of the compounds ranged from GI_{50} <0.1–2.7 μM. Adriamycin was employed as the positive control that demonstrated GI_{50} values in the range of <0.1 to 2.1 μM, whereas DC-81 showed GI_{50} values in the range of 0.11 to 2.37 μM. Overall, significant cytotoxicity was observed for these new PBD conjugates.

MTT assay was also carried out to identify the cytotoxic effect of the four promising PBD conjugates (**4a**,**b** and **5a**,**b**) on A375 cell lines. IC_{50} values were found to be 5.5, 5.6, 1.1, and 0.16 μM for **4a**,**b** and **5a**,**b**, respectively. Further, the cell viability of A375 cells was determined and the values found to be 72% (**4a**), 64.9% (**4b**), 48% (**5a**), and 33.7% (**5b**), respectively, at 4 μM concentration as shown in Supplementary Figures 2 and 3. The data clearly indicated that compound **5b** to be the most effective among all the compounds studied. Further, to confirm the synergistic effect of the promising conjugates (**5a**,**b**), they were compared to both the precursors **3** (DC-81) and **15** (leftside conjugate partner). As shown in Supplementary Figure 4, both the conjugates (**5a**,**b**) exhibited an increase in the cytotoxicity in relation to **3** (DC-81) and **15** in A375 cells.

In order to study the effect of these conjugates on cell cycle progression, human melanoma cell lines (A375) were treated with compounds **4a**,**b**, and **5a**,**b** and analyzed using fluorescence activated cell sorter (FACS shown in Supplementary Fig. 5). Treatment of cells with these compounds at 4 μM concentration for 24 h induced apoptotic effects up to 41.59%, 36.15%, 49.70%, and 83.86% compared to 5.61% of control cells. Increased cells in sub G1 phase, decrease of G1 and G2/M phase cells clearly showed that all the compounds are effective in causing apoptosis as shown in Figure 3. Further, to evaluate the additive effect in the apoptosis inducing ability, conjugate (**5b**) was compared to both the precursors **3**

Table 2
GI_{50} values[a] (in μM) for compounds **4a–c** and **5a**,**b** in selected human cancer cell lines

Cell lines	4a	4b	4c	5a	5b	DC-81(3)	ADR[i]
MCF7[b]	0.17 ± 0.010	0.16 ± 0.007	2.0 ± 0.159	0.14 ± 0.007	<0.1 ± 0.008	0.17 ± 0.016	<0.1 ± 0.012
A2780[c]	2.4 ± 0.250	0.18 ± 0.011	0.18 ± 0.141	0.13 ± 0.009	0.13 ± 0.003	0.14 ± 0.006	<0.1 ± 0.005
KB[d]	0.18 ± 0.012	0.17 ± 0.002	0.17 ± 0.005	0.14 ± 0.003	0.12 ± 0.006	0.17 ± 0.006	0.11 ± 0.01
PC3[e]	2.03 ± 0.067	0.17 ± 0.012	1.8 ± 0.028	0.15 ± 0.005	0.14 ± 0.004	0.20 ± 0.014	0.17 ± 0.005
SiHa[f]	2.2 ± 0.116	1.8 ± 0.069	1.8 ± 0.050	0.17 ± 0.006	0.12 ± 0.005	0.17 ± 0.008	2.1 ± 0.118
GURAV[d]	<0.1 ± 0.007	0.15 ± 0.003	2.0 ± 0.073	<0.1 ± 0.012	0.13 ± 0.008	0.16 ± 0.003	0.12 ± 0.007
DWD[d]	2.5 ± 0.089	0.18 ± 0.008	1.8 ± 0.026	0.13 ± 0.003	0.14 ± 0.003	1.49 ± 0.038	<0.1 ± 0.006
Colo205[g]	2.3 ± 0.086	2.1 ± 0.038	2.3 ± 0.108	0.12 ± 0.003	<0.1 ± 0.001	0.11 ± 0.001	<0.1 ± 0.004
HOP62[h]	0.16 ± 0.008	0.12 ± 0.001	2.4 ± 0.141	0.17 ± 0.022	0.15 ± 0.008	0.15 ± 0.029	0.14 ± 0.008
Zr-75-1[b]	0.17 ± 0.007	2.7 ± 0.186	2.6 ± 0.205	0.16 ± 0.016	0.15 ± 0.008	2.37 ± 0.122	0.13 ± 0.005
A549[h]	2.3 ± 0.016	2.5 ± 0.073	2.3 ± 0.053	0.14 ± 0.001	0.16 ± 0.002	0.16 ± 0.001	0.15 ± 0.001

[a] 50% growth inhibition and the values are mean of three determinations and are reported as mean ± standard error of the mean.
[b] Breast cancer.
[c] Ovarian cancer.
[d] Oral cancer.
[e] Prostate cancer.
[f] Cervix cancer.
[g] Colon cancer.
[h] Lung cancer.
[i] ADR (adriamycin).

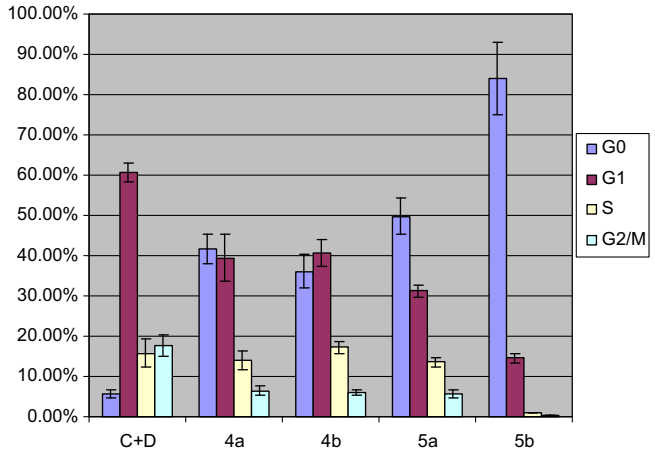


Figure 3. FACS analysis of cell cycle distribution of A375 cells after treatment with PBD conjugates (**4ab** and **5ab**) at 4 μM for 24 h.

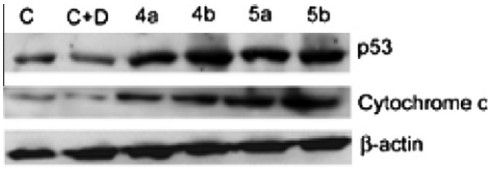


Figure 4. Activation of tumor suppressor gene p53 and release of cytochrome *c* from mitochondria. A375 cells were treated with 4 μM concentration of PBD conjugates for 24 h. C: control (untreated) C + D: control (untreated) + DMSO. β-Actin was used as internal loading control.

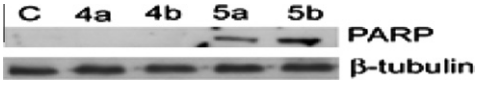


Figure 5. PBD conjugates induced levels of cleaved PARP. A375 cells were treated with 4 μM concentration of PBD conjugates for 24 h. β-Tubulin was used as loading control. C: control (untreated) cells.

(DC-81) and **15** (leftside conjugate partner). As shown in Supplementary Figure **6a** and **b**, conjugate (**5b**) exhibited 99.58% of apoptosis in G0/G1 phase in comparison to **3** (86.73%) and **15** (68%) in A375 cells.

Increased levels of p53 are found to be important in drug-induced apoptosis.[24] To investigate whether these PBD conjugates are acting in p53 dependent apoptotic pathway, the expression levels of p53 protein were checked by treating the cells with these compounds and western blot analysis was carried out using p53 antibody. It is observed that p53 levels were up regulated in all the compounds tested and it was more prominent in case of **4b** and **5b** indicating a p53 dependent pathway as shown in Figure 4.

An important consequence of intrinsic apoptotic pathway is the mitochondrial dysfunction and cytochrome *c* release. Cytosolic cytochrome *c* is known to cause activation of caspase-3.[25] To investigate the effect on cytochrome *c* A375 cells were treated with PBD conjugates at 4 μM concentration for 24 h and we observed that there is an increase in the cytochrome *c* protein levels. However, the enhancement in cytochrome *c* level was more pronounced in case of compound **5b** as shown in Figure 4. The data clearly shows that PBD conjugates act through the involvement of mitochondria in apoptotic pathway.

During apoptotic event, Procaspase-3 is degraded to active caspase-3 and cleaves PARP.[26] As we observed an increase in the levels of cytochrome-*c* there could be an increase in the levels of cleaved PARP. As expected the levels of cleaved PARP were more prominent in the case of **5b** as shown in Figure 5.

In the present study, a new series of anilino substituted pyrimidine linked pyrrolobenzodiazepine conjugates were prepared. The thermal denaturation studies showed that these conjugates have better DNA binding ability compared to DC-81. Moreover, these binding studies were further validated by restriction endonuclease *BamH*I digestion. Further, these PBD conjugates (**4a–c** and **5a**,**b**) showed good cytotoxicity against 11 human cancer cell lines. The MTT proliferation assay for the four promising compounds in A375 cells showed potent cytotoxicity at 4 μM. The FACS analysis showed more population in sub-G1 phase indicating that all these PBD conjugates have apoptosis inducing ability. It was observed from the results that the p53 levels were enhanced. Pronounced increase in the levels of cytosolic cytochrome *c* and cleaved PARP was observed particularly, it was more pronounced in case of **5b**. Hence, these PBD conjugates induce apoptosis by acting through the mitochondrial mediated pathway. Further, these results indicated that the PBD conjugate (**5b**) is an effective cytotoxic agent compared to DC-81 (**3**), with increased accumulation of cells in

sub G1 phase of A375 cells. Further, it can be concluded that this conjugate shows synergistic effect in comparison to the conjugate partners. Finally, it suggests that one of the PBD conjugate like **5b** would be a potential lead for its development as a new anticancer agent.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.147.

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22. *Spectral data for compound* (**4a**): Yield 59%; mp 114–116 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.85 (s, 1H, NH), 9.27 (d, 1H J = 1.6 Hz), 8.93 (s, 1H, NH), 8.59 (d, 1H, J = 6.6 Hz), 8.48 (d, 1H, J = 5.2 Hz), 8.45–8.41 (m, 1H), 7.69 (d, 1H, J = 3.6 Hz), 7.51–7.43 (m, 2H), 7.39 (s, 1H), 7.23 (d, 1H, J = 8.2 Hz), 7.17 (d, 1H, J = 4.4 Hz), 7.14 (d, 1H, J = 8.1 Hz,), 6.80 (s, 1H), 4.15 (t, 2H, J = 5.8 Hz), 3.90 (s, 3H), 3.94–3.54 (m, 3H), 2.59–2.49 (m, 2H), 2.32 (s, 3H), 2.04–1.90 (m, 4H), 1.31–1.24 (m, 4H); MS (ESI): m/z 606 $(M+1)^+$; HRMS (ESI m/z) for $C_{34}H_{36}N_7O_4$, calcd 606.2828, found 606.2819 $(M+1)^+$. Compound (**5a**): Yield 54%; mp 108–110 °C; ^{1}H NMR (DMSO, 300 MHz): δ 10.18 (s, 1H, NH), 9.24 (d, 1H, J = 1.8 Hz), 8.63 (dd, 1H, J = 3.6, 2.2 Hz), 8.51 (d, 1H, J = 5.2 Hz), 8.48 (d, 1H, J = 8.1 Hz), 8.12 (s, 1H, NH), 7.85 (d, 2H, J = 7.4 Hz), 7.64 (d, 1H, J = 3.6 Hz), 7.56–7.36 (m, 6H), 7.19 (d, 1H, J = 8.2 Hz), 7.14 (d, 1H, J = 4.4 Hz), 6.80 (s, 1H), 5.25 (s, 2H), 3.96 (s, 3H), 3.89–3.47 (m, 3H), 2.34 (s, 3H), 2.13–1.97 (m, 2H), 1.82–1.56 (m, 2H); MS (ESI): m/z 640 $(M+1)^+$; HRMS (ESI m/z) for $C_{37}H_{34}N_7O_4$, calcd 640.2672, found 640.2668 $(M+1)^+$. The detail spectral data of other compounds are available in Supplementary data.
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