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Synthesis, DNA-binding ability and evaluation of antitumour activity of triazolo[1,2,4]benzothiadiazine linked pyrrolo[2,1-*c*][1,4]benzodiazepine conjugates

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ABSTRACT

A series of triazolobenzothiadiazine-pyrrolobenzodiazepine conjugates linked through different alkane spacers have been prepared. These compounds have exhibited significant cytotoxicity against most of the cell lines examined. Compound **5a** displays GI_{50} values from 1.83 to 2.38 μM against seven human tumour cell lines, and is identified as a promising lead compound from this series. Their DNA thermal denaturation studies have also been carried out, and one of the compounds **5c** elevates the DNA helix melting temperature of the CT-DNA by 2.6 °C after incubation for 36 h.

1. Introduction

Cancer has proven to be one of the most intractable diseases out of various human health problems due to uncontrolled cell proliferation, diminished apoptosis, invasion and metastasis. In spite of increasing number of inhibitory compounds against several molecular targets, there is an obvious need for better compounds. The identification of lead compounds remains an essential early step in the development of therapeutic agents that interact with the molecule of interest. Several analogues of the lead compounds are then designed and synthesized to define the key recognition elements for potential activity.[1,2]

Sulfonylurea derivatives are an important class of chemotherapeutic agents in medicinal chemistry.[3] Compound sulofenur (LY186641, **1**) (Fig. 1) has been clinically evaluated in lung, breast, colon, ovarian, pancreatic and gastric cancers,[4–6] and has reached clinical trials based on its impressive preclinical activity and apparent lack of toxicity to proliferating normal tissues.[7,8] These sulfonyl compounds exert their biological effect by inhibiting DNA, RNA or protein synthesis. Further, it is found to accumulate in the cell mitochondria, which may be target site for antitumour activity of these compounds.[9,10] Both the 1,2,4-benzothiadiazine 1,1-dioxide ring system and 2,10-dihydro-10-hydroxy-3*H*-imidazo[1,2-*b*][1,2,4]benzothiadiazine 6,6-dioxide (**2**) contain a built-in sulfonylhydroxyguanidine moiety and exhibit their activity against several tumour cell lines. This is probably due to the combination of their imino group of guanidine along with the hydroxylamino group of hydroxyurea. The potent antiviral and anticancer activities of this class of compounds are because of the inhibition of ribonucleotide reductase.[11,12] Moreover, triazole derivatives, for example, letrozole and anastrozole (arimidex) have shown considerable advances in the treatment of hormone-dependent breast cancer.[13] Further, 1,2,4-triazole-3,5-diamine analogues have also been reported as novel and potent anticancer cyclin-dependent kinase inhibitors.[14] A series of novel 1,4-bis-[4-amino-5-mercapto-1,2,4-triazol-3-yl-methoxy]phenylenes and their triazolothiadiazole derivatives are reported to exhibit anticancer activity against a panel of 60 human tumour cell lines.[15] Very recently, we have reported *N*-methyl/phenyl substituted triazolobenzothiadiazine–benzothiazole conjugates as potential antitumour agents.[16]

The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are a class of DNA-binding anticancer compounds that include the naturally occurring anthramycin and DC-81 (**3**).[17–19] The DNA-interactive ability and consequential biological effects are a result of the covalent bond formed between the N10/C11 carbinolamine/imine moiety in the central B-ring of the PBD moiety and N2 of the guanine residue in the minor groove of DNA.[20–22] 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.[2,23–27] Recently, we have been involved in the development of new synthetic strategies[28–30] for

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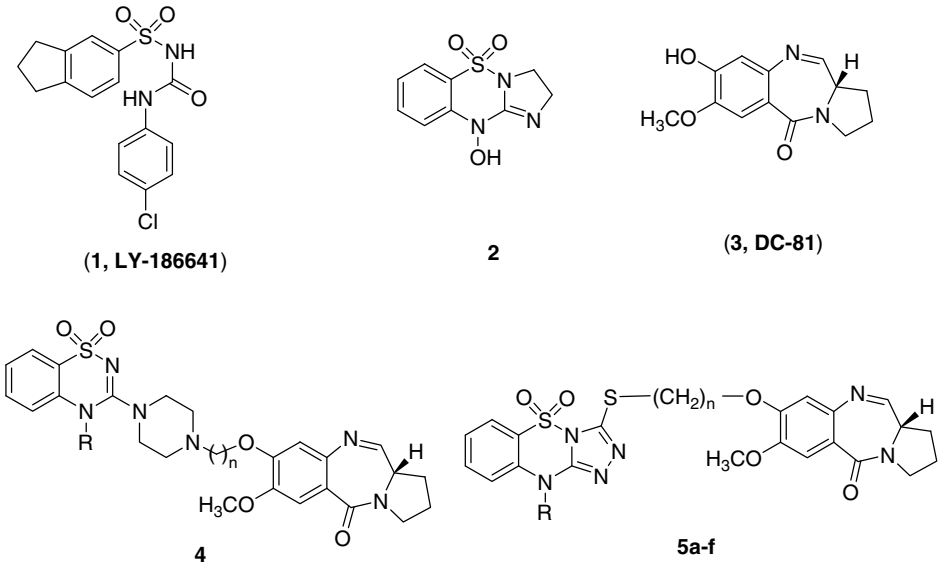


Figure 1. Chemical structures of sulfonylurea derivative (**1**, LY-186641), imidazobenzothiadiazine derivative (**2**), DC-81 (**3**), benzothiadiazine–PBD conjugates (**4**) and triazolobenzothiadiazine–PBD conjugates (**5a**–**f**).

the preparation of PBD ring system as well as in the design of structurally modified PBDs and their hybrids, in continuation of our efforts towards the search for more potent anticancer agents.[31–34]

In the previous studies, we had reported[35] new type of benzothiadiazine–pyrrolobenzodiazepine conjugates (**4**) linked through different 3-piperazinyl alkane spacers as significant cytotoxic and DNA-binding agents. Based on these preliminary investigations, we herein report the synthesis, DNA-binding ability and cytotoxicity of triazolo fused benzothiadiazine linked through various alkane spacers to the C8-position of the A ring of PBD ring system (**5**) to explore its potential as a new class of anticancer agents (Fig. 1 and Scheme 1).

2. Results and discussion

2.1. Chemistry

The preparation of mercaptotriazolobenzothiadiazine intermediates (**7a** and **b**) has been accomplished by the reaction of 3-hydrazino-4-methyl/phenyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxides (**6a** and **b**), which have been obtained by previously reported method[16] and carbon disulfide (CS_2) in ethanolic KOH under reflux conditions followed by the acidification with 1 M HCl to afford the mercapto substituted derivatives[36] (**7a** and **b**) as illustrated in Scheme 1.

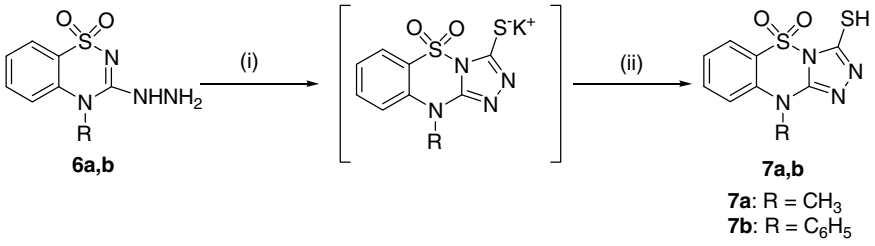
The synthesis of C8-linked triazolobenzothiadiazine–PBD conjugates (**5a–f**) has been carried out from the (2*S*)-*N*-{4-[3-bromoalkoxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehydediethylthioacetal (**8**), which has been prepared by the methods reported in our earlier studies[31–35] and mercapto precursors (**7a** and **b**) using K_2CO_3 in acetone to provide the nitrothioacetal intermediates **9a–f**. Finally, these upon reduction with $SnCl_2{\cdot}2H_2O$ in methanol followed by deprotection using $HgCl_2$/$CaCO_3$ afford the desired PBD conjugates **5a–f** (Scheme 2).

2.2. Biological activity

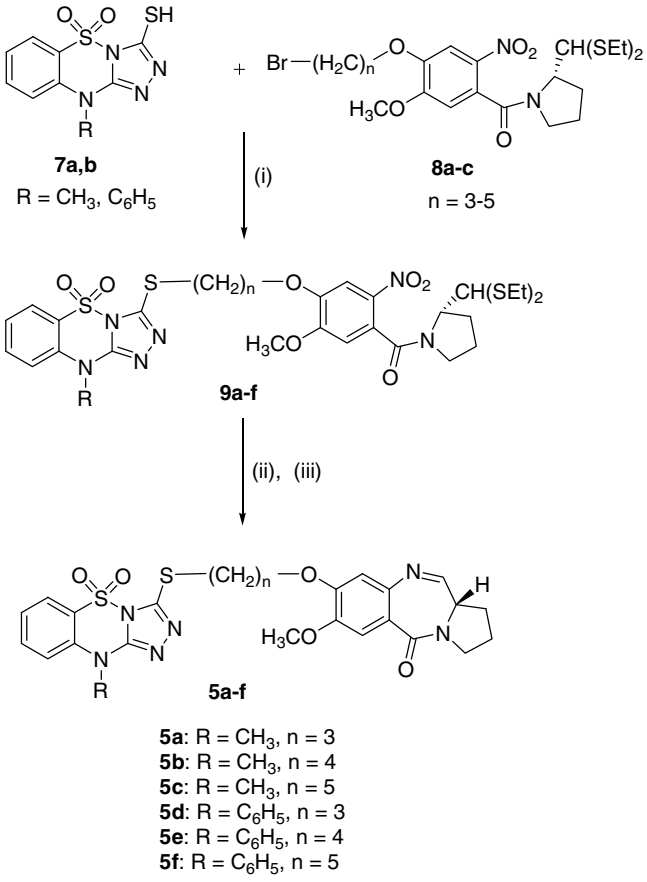
2.2.1. Cytotoxicity

Compounds **5a–f** have been evaluated for their in vitro cytotoxicity in selected human cancer cell lines of lung, breast, oral, colon, prostate and ovary by using sulforhodamine B (SRB) method.[37,38] The compounds exhibiting $GI_{50} \leqslant 10^{-5}$ M (10 μM) are considered to be active on the respective cell lines. The results expressed as GI_{50} are reported in Table 1. All the compounds **5a–f** have exhibited cytotoxicity with GI_{50} values ranging from 0.22 to 30.30 μM in comparison to adriamycin (GI_{50} 0.16–7.25 μM).

Compounds **5a** and **5c**, substituted by a methyl group at the 10 position of benzothiadiazine ring with odd number of alkyl spacers (n = 3 and 5) exhibit more activity than their counterpart with an even number of alkyl spacer (**5b**; n = 4). Replacement of 10-methyl group with a phenyl group in benzothiadiazine ring system (**5d–f**) has decreased the activity, and on increasing the number of carbons in the alkyl spacer results in some increase in activity. It is assumed that the alkyl spacer length and substituents at position 10 of the triazolobenzothiadiazine ring could play an important role in the increase or decrease of the binding ability based on the favourability of interaction with DNA. Compound **5c** has exhibited strong effect against DWD (AW13516),[39] Gurav (AW8507),[39]



Scheme 1. Reagents and conditions: (i) CS_2, KOH, EtOH, reflux, 12 h; (ii) dil HCl.



Scheme 2. Reagents and conditions: (i) K_2CO_3, acetone, reflux, 24 h; (ii) $SnCl_2{\cdot}2H_2O$, CH_3OH, 4 h, reflux; (iii) $HgCl_2$, $CaCO_3$, CH_3CN/H_2O, 12 h, rt.

A2780 (GI_{50} 1.95, 0.22 and 1.92 μM, respectively) and a GI_{50} of 2.45–28.50 μM against other cell lines, whereas compound **5a** exhibited significant activity in GI_{50} range of 1.83–2.29 μM against all the cell lines used in this study. Compound **5f** has shown selectivity against all the cell lines of oral cancer with GI_{50} value up to 1.78 μM. Therefore, from these data as illustrated in Table 1, it appears that there is potential in this new type of PBD conjugates for further exploitation with additional structural modifications.

2.2.2. DNA-binding affinity: thermal denaturation studies

The DNA-binding ability for these triazolobenzothiadiazine–PBD conjugates (**5a–f**) has been examined by thermal denaturation studies with duplex form of calf thymus (CT)-DNA by using reported procedures.[40,41] Thermal denaturation studies showed that these compounds stabilized the thermal helix → coil or melting stabilization (ΔT_m) for the CT-DNA duplex at pH 7.0, where PBD/DNA molar ratio is 1:5. Interestingly, all the benzothiadiazine–PBD conjugates (**5a–f**) elevate the helix melting temperature of CT-DNA in the range of 0.5–2.6 °C at 0 h and also examined after 18 and 36 h incubation at 37 °C (Table 2). Compound **5c** showed the highest ΔT_m of 2.1 °C at 0 h which increased up to 2.6 °C after 36 h incubation. The spacers in compounds **5a**, **5c**, **5d** and **5f** appear to impart the molecules a better fit in the minor groove of DNA, leading to enhanced binding affinity. In the same experiment, the naturally occurring DC-81 exhibits a ΔT_m of 0.7 °C upon incubation under similar conditions. This result shows the effect on DNA-binding affinity by linking the benzothiadiazine moiety to PBD through different alkyl spacers at C8-postion of the DC-81. It is expected that the anticancer activity of these compounds could be due to a similar mechanism to that of PBD, that is by DNA-binding and by the action of sulfonyl urea derivatives on the inhibition of DNA, RNA or protein synthesis.

3. Conclusion

The synthesis and evaluation of anticancer activity of a new series of benzothiadiazine–PBD conjugates has been investigated. Triazolobenzothiadiazine pharmacophore linked through an alkyl spacer at C8-position of the PBD ring system has emerged as potential antitumour agent. Compound **5c** has exhibited promising activity (GI_{50} 0.22 μM) against AW8507 cell line (oral cancer). Most

Table 2
Thermal denaturation data for triazolobenzothiadiazine–PBD conjugates (**5a–f**) with calf thymus (CT)-DNA

Compound	[PBD]:[DNA] molar ratio[b]	ΔT_m (°C)[a] after incubation at 37 °C		
		0 h	18 h	36 h
5a	1:5	1.2	1.5	1.7
5b	1:5	0.5	0.5	0.7
5c	1:5	2.1	2.4	2.6
5d	1:5	1.0	1.2	1.5
5e	1:5	0.5	0.6	0.7
5f	1:5	1.2	1.3	1.5
DC-81	1:5	0.3	0.7	0.7

[a] For CT-DNA alone at pH 7.00 ± 0.01, T_m = 69.2 °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].

Table 1
GI_{50} values[a] (in μM) for compounds **5a–f** in selected human cancer cell lines

Compound	A549[b]	HOP62[b]	Zr-75-1[c]	MCF7[c]	AW13516[d]	AW8507[d]	KB[d]	Colo205[e]	SiHa[f]	PC3[g]	A2780[h]
5a	2.29	2.13	2.19	2.06	2.24	2.24	2.38	1.93	2.12	2.15	1.83
5b	2.28	–	25.90	2.29	26.60	2.29	26.40	2.11	27.00	25.50	2.17
5c	2.45	–	28.50	–	1.95	0.22	25.60	2.19	27.90	25.51	1.92
5d	–	–	26.01	–	–	–	–	–	–	–	30.02
5e	29.71	–	–	–	1.98	25.80	–	–	–	28.80	2.27
5f	30.30	2.35	24.00	–	1.78	2.48	2.46	25.60	2.30	2.40	2.01
ADR	7.25	0.14	1.79	0.17	0.10	0.17	1.72	0.14	0.18	1.81	0.16

–, means GI_{50} values not attained at the concentrations used; ADR, adriamycin.
[a] 50% growth inhibition and the values are mean of three determinations.
[b] Lung cancer.
[c] Breast cancer.
[d] Oral cancer.
[e] Colon cancer.
[f] Cervix cancer.
[g] Prostate cancer.
[h] Ovarian cancer.

of the compounds have demonstrated appreciable cytotoxicity against selected cell lines. Moreover, these conjugates also exhibited DNA-binding ability and this investigation provides some useful lead towards the discovery of potential anticancer agents.

4. Experimental

4.1. General

All chemicals and reagents were obtained from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA) or Spectrochem Pvt. Ltd (Mumbai, India) and were used without further purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 F-254, and visualization on TLC was achieved by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H and ^{13}C NMR spectra were recorded on Gemini Varian-VXR-unity (200 MHz) or Bruker UXNMR/XWIN-NMR (300 M Hz) instruments. Chemical shifts (δ) are reported in ppm downfield from internal TMS standard. LC–MS was recorded on instrument LC-MSD-Trap-SL. ESI spectra were recorded on Micro mass, Quattro LC using ESI$^+$ software with capillary voltage 3.98 kV and ESI mode positive ion trap detector. High-resolution mass spectra (HRMS) were recorded on QSTAR XL Hybrid MS/MS mass spectrometer. Elemental analysis was within ±0.4% of the theoretical values. Elemental analyses were performed on a elemental analyzer, Model: VARIO EL, Elementar, Germany. Melting points were determined with an Electrothermal melting point apparatus, and are uncorrected.

4.2. General procedures

4.2.1. Synthesis of compound 10-methyl-3-sulfanyl-5,10-dihydro-5l^6-benzo[*e*][1,2,4] triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione (7a)

A mixture of 3-hydrazino-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**6a**, 1.5 g, 6.63 mmol) was treated with CS_2 (5 mL) and KOH (715 mg, 21 mmol) and refluxed in ethanol for 8 h to give potassium salt of compound **6a**. On further acidification of this with 1 M HCl, precipitate was obtained, which was filtered and washed with water and recrystallized from ethanol to give the pure compound **7a**.

Yield 1.33 g, 75%; mp 156–158 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.00 (d, J = 7.4 Hz, 1H), 7.76 (t, J = 7.4 Hz, 1H), 7.34 (t, J = 8.1 Hz, 2H), 3.64 (s, 3H); MS (LC) m/z 269 [M+H]$^+$.

4.2.2. 10-Phenyl-3-sulfanyl-5,10-dihydro-5l^6-benzo[*e*][1,2,4]-triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione (7b)

The compound **7b** was prepared according to the method described for compound **7a**, employing compound **6b** (1.5 g, 5.20 mmol).

Yield 1.40 g, 82%; mp 133–135 °C; ^{1}H NMR (200 MHz, $CDCl_3$): 8.03 (dd, J = 7.3, 1.4 Hz, 1H), 7.70–7.54 (m, 4H), 7.53–7.40 (m, 2H), 7.31 (t, J = 7.3 Hz, 1H), 6.62 (d, J = 8.1 Hz, 1H); MS (LC) m/z 331 [M+H]$^+$.

4.2.3. Synthesis of (2*S*)-*N*-{4-[3-[3-(10-Methyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]-thiadiazine-5,5-dione]propyl]oxy]-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehydediethylthioacetal (9a)

To a solution of (2*S*)-*N*-[4-(3-bromobutyl)oxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxarbaldehydediethylthioacetal **8a** (521 mg, 1 mmol) in acetone (10 mL) anhydrous K_2CO_3 (552 mg, 4 mmol) and the 3-mercaptotriazolobenzothiadiazine (**7a**, 268 mg, 1 mmol) were added. The reaction mixture was heated to reflux for 4–6 h. After completion of the reaction as indicated by TLC, potassium carbonate was removed by suction filtration and the solvent was removed under vacuum. The crude product thus obtained was purified by column chromatography using ethyl acetate/hexane (1:1) as eluant to afford the pure compound **9a**.

Yield 600 mg, 85%; mp 119–121 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02 (dd, J = 8.0, 1.4 Hz, 1H), 7.76 (dt, J = 7.3, 1.4 Hz, 1H), 7.66 (s, 1H), 7.40–7.25 (m, 2H), 6.80 (s, 1H), 4.86 (d, J = 3.6 Hz, 1H), 4.75–4.62 (m, 1H), 4.27 (t, J = 5.8 Hz, 2H), 3.94 (s, 3H), 3.80 (s, 3H), 3.54 (dt, J = 6.5, 1.4 Hz, 2H), 3.32–3.18 (m, 2H), 2.90–2.65 (m, 4H), 2.40 (t, J = 6.5 Hz, 1H), 2.30–1.80 (m, 2H), 1.42–1.24 (m, 7H), 0.99–0.85 (m, 2H); HRMS (ESI) m/z calcd for $C_{29}H_{37}N_6O_7S_4$ [M+H]$^+$, 709.1606; found, 709.1578; IR (KBr) (ν_{max}/cm^{-1}): 2921, 2872, 1626, 1596, 1526, 1422, 1339, 1278, 1216, 1184, 1032.

4.2.4. (2*S*)-*N*-{4-[4-[3-(10-Methyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione]butyl]-oxy]-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehydediethylthioacetal (9b)

This compound was prepared according to the method described for compound **9a**, employing compound **8b** (535 mg, 1 mmol) and compound **7a** (2268 mg, 1 mmol).

Yield 560 mg, 78%; mp 80–82 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.01 (dd, J = 8.1, 1.5 Hz, 1H), 7.80–7.72 (m, 1H), 7.64 (s, 1H), 7.36–7.27 (m, 2H), 6.80 (s, 1H), 4.86 (d, J = 3.7 Hz, 1H), 4.74–4.65 (m, 1H), 4.18–4.04 (m, 2H), 3.94 (s, 3H), 3.79 (s, 3H), 3.30–3.20 (m, 2H), 2.85–2.65 (m, 4H), 2.35–2.20 (m, 1H), 2.10–2.02 (m, 6H), 1.90–1.58 (m, 1H), 1.35 (q, J = 7.3 Hz, 4H), 1.28–1.24 (m, 2H), 0.95–0.90 (m, 2H); HRMS (ESI) m/z calcd for $C_{30}H_{39}N_6O_7S_4$ [M+H]$^+$, 723.1763; found, 723.1752, [M+H]$^+$; IR (KBr) (ν_{max}/cm^{-1}): 2925, 2870, 1672, 1637, 1582, 1520, 1423, 1335, 1275, 1220, 1177, 1017.

4.2.5. (2*S*)-*N*-{4-[5-[3-(10-Methyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione]pentyl]-oxy]-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehydediethylthioacetal (9c)

This compound was prepared according to the method described for compound **9a**, employing compound **8c** (549 mg, 1 mmol) and compound **7a** (268 mg, 1 mmol).

Yield 670 mg, 91%; mp 51–53 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.10 (d, J = 8.0 Hz, 1H), 7.77 (t, J = 8.0 Hz, 1H), 7.65 (s, 1H), 7.45–7.25 (m, 2H), 6.80 (s, 1H), 4.86 (d, J = 3.6 Hz, 1H), 4.75–4.64 (m, 1H), 4.18–4.05 (m, 2H), 3.95 (s, 3H), 3.18 (s, 3H), 3.25 (t, J = 7.3 Hz, 4H), 2.87–2.65 (m, 4H), 2.40–2.19 (m, 1H), 2.15–1.80 (m, 4H), 1.78–1.60 (m, 1H), 1.35 (p, J = 3.6 Hz, 4H), 1.30–1.20 (m, 4H), 0.98–0.80 (m, 2H); HRMS (ESI) m/z calcd for $C_{31}H_{40}N_6O_7S_4Na$ [M+Na]$^+$, 759.1739; found, 759.1752; IR (KBr) (ν_{max}/cm^{-1}): 2924, 2853, 1643, 1587, 1563, 1519, 1488, 1446, 1470, 1352, 1274, 1218, 1175, 723.

4.2.6. (2*S*)-*N*-{4-[3-[3-(10-Phenyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione]-propyl]oxy]-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehydediethylthioacetal (9d)

This compound was prepared according to the method described for compound **9a** by employing compound **8a** (521 mg, 1 mmol) and compound **7b** (330 mg, 1 mmol).

Yield 600 mg, 84%; mp 60–62 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.13 (dd, J = 8.1, 1.5 Hz, 1H), 7.70–7.62 (m, 4H), 7.60–7.52 (m, 1H), 7.48–7.35 (m, 3H), 6.82 (s, 1H), 6.77 (d, J = 8.1 Hz, 1H), 4.88 (d, J = 3.7 Hz, 1H), 4.76–4.64 (m, 1H), 4.24 (t, J = 6.0 Hz, 2H), 3.97 (s, 3H), 3.40 (t, J = 6.6, 1.5 Hz, 2H), 3.32–3.22 (m, 1H), 2.90–2.70 (m, 4H), 2.43–2.36 (m, 1H), 2.18–1.90 (m, 2H), 1.88–1.50 (m, 2H), 1.37 (q, J = 7.3 Hz, 4H), 1.30–1.24 (m, 2H), 0.98–0.92 (m, 2H); MS (LC)

m/z 793.2 [M+Na]$^+$; IR (KBr) (ν_{max}/cm^{-1}): 2923, 2866, 1639, 1579, 1553, 1519, 1485, 1420, 1351, 1218, 1185, 1040, 720.

4.2.7. (2*S*)-*N*-{4-[4-[3-(10-Phenyl-3-sulfanyl-5,10-dihydro-5-benzo-[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione]butyl]oxy]-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehydediethylthioacetal (9e)

This compound was prepared according to the method described for compound **9a**, employing compound **8b** (535 mg, 1 mmol) and compound **7b** (330 mg, 1 mmol).

Yield 675 mg, 85%; mp 64–66 °C; ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.13 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.68–7.60 (m, 4H), 7.58–7.50 (m, 1H), 7.45–7.34 (m, 3H), 6.81 (s 1H), 6.74 (d, *J* = 8.1 Hz, 1H), 4.87 (d, *J* = 3.7 Hz, 1H), 4.75–4.66 (m, 1H), 4.17–4.06 (m, 2H), 3.94 (s, 3H), 3.32–3.20 (m, 4H), 2.88–2.66 (m, 4H), 2.36–2.18 (m, 1H), 2.15–1.90 (m, 2H), 1.88–1.46 (m, 1H), 1.35 (q, *J* = 7.3 Hz, 4H), 1.30–1.20 (m, 2H), 0.96–0.84 (m, 4H); HRMS (ESI) *m/z* calcd for $C_{35}H_{40}N_6O_7S_4$-Na [M+Na]$^+$, 807.1739; found, 807.1727; IR (KBr) (ν_{max}/cm^{-1}): 2924, 2855, 1640, 1579, 1553, 1519, 1485, 1420, 1351, 1255, 1218, 1184, 1058, 744.

4.2.8. (2*S*)-*N*-{4-[5-[3-(10-Phenyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione]pentyl]-oxy]-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehydediethylthioacetal (9f)

This compound was prepared according to the method described for compound **9a**, employing compound **8c** (549 mg, 1 mmol) and compound **7b** (330 mg, 1 mmol).

Yield 640 mg, 86%; mp 74–76 °C; ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.13 (dd, *J* = 9.0, 1.5 Hz, 1H), 7.72–7.50 (m, 5H), 7.48–7.34 (m, 3H), 6.80 (s, 1H), 6.74 (d, *J* = 9.0 Hz, 1H), 4.87 (d, *J* = 3.7 Hz, 1H), 4.76–4.75 (m, 1H), 4.10 (dt, *J* = 6.0, 1.5 Hz, 1H), 3.95 (s, 3H), 3.30–3.15 (m, 3H), 2.88–2.65 (m, 4H), 2.35–2.20 (m, 1H), 2.15–2.05 (m, 1H), 1.90 (p, *J* = 7.5 Hz, 4H), 1.72–1.60 (m, 1H), 1.42–1.20 (m, 7H), 0.96–0.80 (m, 4H); HRMS (ESI) *m/z* calcd for $C_{36}H_{43}N_6O_7S_4$ [M+H]$^+$, 799.2076; found, 799.2039; IR (KBr) (ν_{max}/cm^{-1}): 2928, 2850, 1642, 1582, 1555, 1518, 1482, 1421, 1358, 1249, 1211, 1164, 1054, 741.

4.2.9. Synthesis of 7-methoxy-8-{3-(10-methyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*]-[1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione)]propyl}oxy-(11a*S*)-1,2,3,11a-5*H*-pyrrolo[2,1-*c*][1,4]-benzodiazepin-5-one (5a)

To a solution of compound **9a** (706 mg, 1 mmol) in methanol (20 mL), $SnCl_2{\cdot}2H_2O$ (5 mmol) was added and refluxed for 1–2 h. The methanol was evaporated in vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (20 × 30 mL). The combined organic phase was dried over anhydrous Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal, which due to potential stability problems[42] proceeded for the next step.

A solution of amino diethyl thioacetal (1 mmol), $HgCl_2$ (2.26 mmol) and $CaCO_3$ (2.46 mmol) in CH_3CN/water (4:1) was stirred slowly at room temperature until TLC indicated complete loss of starting material (12 h). The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through a Celite bed. The clear yellow organic supernatant was extracted with saturated 5% $NaHCO_3$ (20 mL) and brine (20 mL), and the combined organic phase was dried over anhydrous Na_2SO_4. The organic layer was evaporated in vacuum and purified by column chromatography (MeOH/EtOAc, 2:98) to give the final product **5a**.

Yield 280 mg, 51%; mp 86–88 °C; $[\alpha]_D^{25}$ +152.80 (*c* 0.5, CHCl$_3$); ^{1}H NMR (200 MHz, CDCl$_3$): δ 8.08 (dd, *J* = 7.9, 1.4 Hz, 1H), 7.80–7.64 (m, 2H), 7.52 (s, 1H), 7.46–7.24 (m, 2H), 6.84 (d, *J* = 2.2 Hz, 1H), 4.25 (t, *J* = 6.6 Hz, 1H), 4.00–3.88 (m, 4H), 3.80 (s, 3H), 3.72–3.35 (m, 2H), 2.48–2.24 (m, 1H), 2.16–1.95 (m, 2H), 1.78–1.52 (m, 2H), 1.25 (s, 2H), 0.87 (p, *J* = 5.1 Hz, 1H); MS (LC) *m/z* 555.1 [M+H]$^+$; IR (KBr) (ν_{max}/cm^{-1}): 2924, 2854, 1586, 1560, 1514, 1489, 1446, 1351, 1260, 1219, 1175, 1024. Anal. Calcd for $C_{25}H_{26}N_6O_5S_2$: C, 54.14; H, 4.72; N, 15.15. Found: C, 54.35; H, 4.81; N, 15.42.

4.2.10. 7-Methoxy-8-{4-(10-methyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione)] butyl}oxy-(11a*S*)-1,2,3,11a-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (5b)

The compound **5b** was prepared according to the method described for the compound **5a**, employing the compound **9b** (720 mg, 1 mmol) to afford the compound **5b**.

Yield 330 mg, 58%; mp 132–134 °C; $[\alpha]_D^{25}$ +174.64 (*c* 0.5, CHCl$_3$); ^{1}H NMR (200 MHz, CDCl3): δ ^{1}H NMR (200 MHz, CDCl$_3$): δ 8.11 (d, *J* = 7.5 Hz, 1H), 7.75 (t, *J* = 7.5 Hz, 1H), 7.66–7.50 (m, 2H), 7.53 (s, 1H), 7.42–7.20 (m, 2H), 6.81 (s, 1H), 4.22–3.96 (m, 4H), 3.82 (s, 3H), 3.74–3.40 (m, 4H), 3.21 (t, *J* = 6.5 Hz, 2H), 2.38–2.23 (m, 1H), 2.15–1.85 (m, 1H), 1.75–1.56 (m, 2H), 1.24 (s, 2H), 0.95–0.78 (m, 1H); MS (LC) *m/z* 569 [M+H]$^+$; IR (KBr) (ν_{max}/cm^{-1}): 2928, 2858, 1576, 1552, 1510, 1491, 1449, 1355, 1265, 1172. Anal. Calcd for $C_{26}H_{28}N_6O_5S_2$: C, 54.92; H, 4.96; N, 14.78. Found: C, 54.61; H, 4.86; N, 14.97.

4.2.11. 7-Methoxy-8-{5-(10-methyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione)]-pentyl}oxy-(11a*S*)-1,2,3,11a-5*H*-pyrrolo[2,1-*c*][1,4] benzodiazepin-5-one (5c)

The compound **5c** was prepared according to the method described for the compound **5a**, employing the compound **9c** (736 mg, 1 mmol) to afford the compound **5c**.

Yield 360 mg, 62%; mp 90–92 °C; $[\alpha]_D^{25}$ +181.00 (*c* 0.5, CHCl$_3$); ^{1}H NMR (200 MHz, CDCl$_3$): δ 8.10 (d, *J* = 7.3 Hz, 1H), 7.78 (t, *J* = 7.3 Hz, 1H), 7.65 (d, *J* = 4.4 Hz, 1H), 7.50 (s, 1H), 7.48–7.21 (m, 3H), 6.80 (s, 1H), 4.09 (d, *J* = 6.6 Hz, 1H), 3.95 (s, 3H), 3.80 (s, 3H), 3.76–3.48 (m, 4H), 3.26 (t, *J* = 6.6 Hz, 2H), 2.40–2.20 (m, 1H), 2.16–1.80 (m, 2H), 1.76–1.54 (m, 3H), 1.26 (s, 2H), 0.96–0.80 (m, 1H); HRMS (ESI) *m/z* calcd for $C_{27}H_{31}N_6O_5S_2$ [M+H]$^+$, 583.1763; found, 583.1781; IR (KBr) (ν_{max}/cm^{-1}): 2926, 2860, 1589, 1561, 1489, 1446, 1351, 1260, 1216, 1177, 1100, 1021, 755. Anal. Calcd for $C_{27}H_{30}N_6O_5S_2$: C, 55.66; H, 5.19; N, 14.42. Found: C, 55.93; H, 5.34; N, 14.70.

4.2.12. 7-Methoxy-8-{3-(10-phenyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione)] propyl}oxy-(11a*S*)-1,2,3,11a-5*H*-pyrrolo[2,1-*c*][1,4] benzodiazepin-5-one (5d)

The compound **5d** was prepared according to the method described for the compound **5a**, employing the compound **9d** (770 mg, 1 mmol) to afford the compound **5d**.

Yield 400 mg, 65%; mp 124–126 °C; $[\alpha]_D^{25}$ +144.62 (*c* 0.5, CHCl$_3$); ^{1}H NMR (200 MHz, CDCl$_3$): δ *8.20–8.08* (m, 1H), 7.74–7.50 (m, 6H), 7.48–7.35 (m, 4H), 6.80 (d, *J* = 5.4 Hz, 1H), 6.74 (s, 1H), 4.30–4.10 (m, 2H), 3.95 (s, 3H), 3.90–3.50 (m, 2H), 3.36 (t, *J* = 7.0 Hz, 2H), 2.45–4.25 (m, 1H), 2.16–1.96 (m, 1H), 1.33–1.23 (m, 2H), 0.98–0.82 (m, 2H); HRMS (ESI) *m/z* calcd for $C_{30}H_{29}N_6O_5S_2$ [M+H]$^+$, 617.1640; found, 617.1640; IR (KBr) (ν_{max}/cm^{-1}): 2929, 2852, 1597, 1552, 1484, 1437, 1353, 1256, 1221, 1186, 1024, 746. Anal. Calcd for $C_{30}H_{28}N_6O_5S_2$: C, 58.43; H, 4.58; N, 13.63. Found: C, 58.70; H, 4.42; N, 13.91.

4.2.13. 7-Methoxy-8-{4-(10-phenyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione)] butyl}oxy-(11a*S*)-1,2,3,11a-5*H*-pyrrolo[2,1-*c*][1,4] benzodiazepin-5-one (5e)

The compound **5e** was prepared according to the method described for the compound **5a**, employing the compound **9e** (784 mg, 1 mmol) to afford the compound **5e**.

Yield 410 mg, 66%; mp 76–78 °C; $[\alpha]_D^{25}$ +135.32 (*c* 0.5, $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$): δ 8.12 (d, *J* = 8.5 Hz, 1H), 7.70–7.48 (m, 7H), 7.46–7.30 (m, 3H), 6.77 (d, *J* = 4.6 Hz, 1H), 6.71 (s, 1H), 4.08 (d, *J* = 6.2 Hz, 1H), 3.92 (s, 3H), 3.86–3.42 (m, 2H), 3.23 (t, *J* = 6.2 Hz, 2H), 2.38–2.21 (m, 1H), 2.14–1.80 (m, 3H), 1.35–1.18 (m, 3H), 0.98–0.80 (m, 2H); HRMS (ESI) *m/z* calcd for $C_{31}H_{31}N_6O_5S_2$ $[M+H]^+$, 631.1797; found, 631.1785; IR (KBr) (ν_{max}/cm^{-1}): 2918, 1591, 1552, 1481, 1357, 1251, 1236, 1159, 1021, 754. Anal. Calcd for $C_{31}H_{30}N_6O_5S_2$: C, 59.03; H, 4.79; N, 13.32. Found: C, 59.35; H, 4.66; N, 13.58.

4.2.14. 7-Methoxy-8-{5-(10-phenyl-3-sulfanyl-5,10-dihydro-5-benzo[*e*][1,2,4]triazolo[4,3-*b*][1,2,4]thiadiazine-5,5-dione)] pentyl}oxy-(11a*S*)-1,2,3,11a-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (5f)

The compound **5f** was prepared according to the method described for the compound **5a**, employing the compound **9f** (798 mg, 1 mmol) to afford the compound **5f**.

Yield 640 mg, 68%; mp 84–86 °C; $[\alpha]_D^{25}$ +179.00 (*c* 0.5, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 8.13 (dd, *J* = 8.0, 1.46 Hz, 1H), 7.66 (d, *J* = 5.1 Hz, 1H), 7.65–7.48 (m, 6H), 7.46–7.32 (m, 3H), 6.78 (d, *J* = 5.1 Hz, 1H), 6.72 (s, 1H), 4.22 (dd, *J* = 5.8, 2.1 Hz, 1H), 4.07 (t, *J* = 6.5 Hz, 1H), 3.94 (s, 3H), 3.90–3.50 (m, 2H), 3.19 (t, *J* = 6.5 Hz, 2H), 2.40–2.25 (m, 1H), 2.06 (d, *J* = 7.3 Hz, 1H), 1.97–1.75 (m, 2H), 1.73–1.50 (m, 2H), 1.41–1.18 (m, 2H), 0.98–0.80 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): 165.2, 164.6, 162.3, 153.8, 150.8, 147.8, 140.8, 137.5, 136.0, 134.4, 130.8, 130.2, 128.9, 124.4, 123.8, 121.8, 120.0, 117.3, 111.5, 110.4, 68.7, 56.1, 53.7, 46.6, 31.0, 29.5, 28.7, 28.3, 25.0, 24.1; HRMS (ESI) *m/z* calcd for $C_{32}H_{31}N_6O_5S_2$ $[M+H]^+$, 645.1953; found, 645.1947; IR (KBr) (ν_{max}/cm^{-1}): 2922, 2860, 1602, 1556, 1475, 1438, 1361, 1248, 1230, 1162, 1026, 755. Anal. Calcd for $C_{32}H_{30}N_6O_5S_2$: C, 59.61; H, 5.00; N, 13.03. Found: C, 59.92; H, 5.17; N, 12.88.

4.3. In vitro evaluation of cytotoxic activity

The synthesized compounds (**5a–f**) have been evaluated for their in vitro cytotoxicity in selected human cancer cell lines of breast (Zr-75-1), lung (A-549), colon (Colo205), oral (DWD, Gurav), prostate (PC-3) and ovarian (A2780) origin. A protocol of 48 h continuous drug exposure has been used and a sulforhodamine B (SRB) protein assay has been used to estimate cell viability or growth.

The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine, and were inoculated into 96-well microtiter plates in 90 μL at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. Aliquots of 10 μL of the drug dilutions were added to the appropriate microtiter wells already containing 90 μL of cells, resulting in the required final drug concentrations. For each compound four concentrations (0.1, 1, 10 and 100 μM) were evaluated and each was done in triplicate wells.

Plates were incubated further for 48 h, and assay was terminated by the addition of 50 μL of cold trichloro acetic acid (TCA) (final concentration, 10% TCA) and plates were again incubated for 60 min at 4 °C. The plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (50 μL) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. The residual dye was removed by washing five times with 1% acetic acid. The plates were air-dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm with 690 nm reference wavelengths. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. The above determinations were repeated three times.

Percentage growth was expressed as the (ratio of average absorbance of the test well to the average absorbance of the control wells) multiplied by 100. Growth inhibition of 50% (GI_{50}) was calculated from $[(T_i - T_z)/(C - T_z)] \times 100 = 50$, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation.

Here, T_z is the optical density at time zero OD of control; and T_i, the OD of test growth in the presence of drug.

4.4. Thermal denaturation studies

Compounds were subjected to thermal denaturation studies with duplex-form CT-DNA using reported method.[40] Working solutions in aqueous buffer (10 mM NaH_2PO_4/Na_2HPO_4, 1 mM Na_2EDTA, pH 7.00 + 0.01) containing CT-DNA (100 μM in phosphate) and the PBD (20 μM) were prepared by addition of concentrated PBD solutions in DMSO to obtain a fixed [PBD]/[DNA] molar ratio of 1:5. The DNA–PBD solutions were incubated at 37 °C for 0, 18 and 36 h prior to analysis. Samples were monitored at 260 nm using a Beckman–Coulter DU 800 spectrophotometer fitted with high performance temperature controller, and heating was applied at 1 °C min^{-1} in the 40–90 °C range. DNA helix → coil transition temperatures (T_m) were obtained from the maxima in the $d(A_{260})/dT$ derivative plots. Results are given as means ± standard deviation from three determinations and are corrected for the effects of DMSO co-solvent using a linear correction term.[41] Drug-induced alterations in DNA melting behavior are given by $\Delta T_m = T_m(\text{DNA + PBD}) - T_m(\text{DNA alone})$, where the T_m value for the PBD-free CT-DNA is 69.2 ± 0.01 °C. The fixed [PBD]/[DNA] ratio used did not result in binding saturation of the host DNA duplex for any compound examined.

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