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Synthesis and DNA binding affinity of novel *A*-C8/*C*-C2-*exo* unsaturated alkoxyamido-linked pyrrolo[2,1-*c*][1,4]benzodiazepine dimers

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Abstract—The synthesis of novel *A*-**C8**/*C*-**C2**-*exo* unsaturated alkoxyamido-linked pyrrolo[2,1-*c*][1,4]benzodiazepine dimers is reported and these dimers show significant DNA binding affinity and they also exhibit moderate anticancer activity.

1. Introduction

In the last decade there has been increasing interest towards the synthesis of DNA sequence selective agents, particularly low molecular weight antitumour antibiotics. Pyrrolo[2,1-*c*][1,4]benzodiazepines are naturally occurring antitumour antibiotics that interact within the minor groove of DNA forming monocovalent adducts.[1] Recently, there has been tremendous interest in the design and synthesis of DNA interstrand cross-linking agents that are likely to enhance the sequence selectivity and in turn could increase selectivity for tumour cells.[2] In this context a large number of PBD dimers have been designed and synthesized with a view to explore their DNA cross-linking ability and their sequence selectivity.[3] These PBD dimers have been joined through different positions such as *A*-**C7**/*A*-**C7′**, *A*-**C8**/*A*-**C8′** (**3**) and *C*-**C2**/*C*-**C2′** (**2**), amongst these *A*-**C8**/*A*-**C8′** linked PBD dimers have shown promising cytotoxicity and efficient cross-linking property.[4] Further it is observed from the literature that **C2**-*exo* unsaturation as in case of tomaymycin significantly enhances the anticancer potential and DNA binding ability[5] in comparison to non-**C2**-*exo* unsaturation (DC-81) and this feature has been extensively investigated for many synthetic analogues.[6]

Thurston and co-workers[7] have developed new pyrrolobenzodiazepine dimers with remarkable DNA cross-linking ability. Moreover, the same group has reported the first example of *A*-**C8**/*C*-**C2** amido-linked PBD dimer[8] (**4**) and recently we have reported *A*-**C8**/*C*-**C2** alkoxyamido-linked tail to head type PBD dimer (**5**) with significant DNA binding ability and low anticancer activity.[9] Therefore, introduction of **C2**-*exo* unsaturation is an important component that probably is causing the flattening of the C-ring to produce an improved isohelical fit within the DNA minor groove.[10] In view of these findings and in conjunction with our pursuit towards the design and synthesis of PBD hybrids,[11] we have considered the synthesis of *A*-**C8**/*C*-**C2**-*exo* unsaturated alkoxyamido linked PBD dimers (**6a**–**b** and **7a**–**b**) as a significant and important aspect in the development of novel PBD dimers with potential anticancer activity and DNA binding ability. Further, we have also prepared such PBD dimers (**8a**–**b**) with amide functionality at N10–C11position of *A*-**C8** component to investigate the effect of the absence of one of the imine functionality on the DNA binding profile.

2. Results and discussion

2.1. Synthesis

Synthesis of *A*-**C8**/*C*-**C2**-*exo* unsaturated alkoxyamido-linked PBD dimers **6a**–**b** has been carried out by

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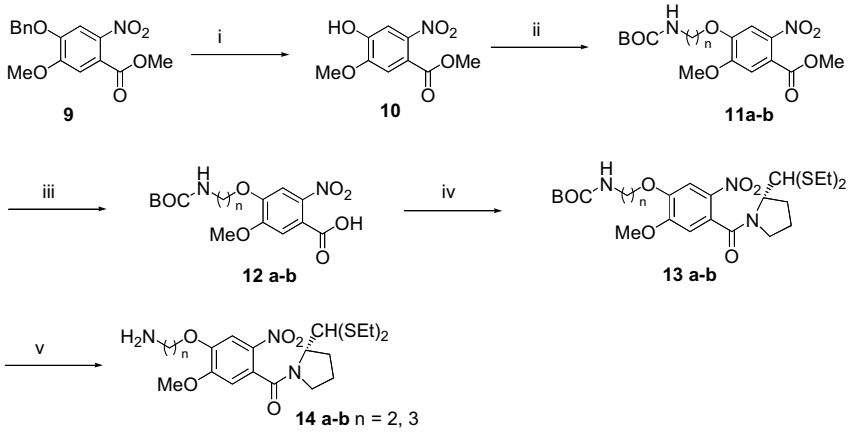
amidation of the *A*-**C8** alkoxyamine components (**14a**–**b**) and the *C*-**C2**-*exo* unsaturated acid component (**22a**). The **C8** alkoxyamine has been synthesized in the following manner; the precursor methyl 4-benzyloxy-5-methoxy-2-nitrobenzoate **9** has been prepared by employing the literature method,[12] which upon debenzylation with $BF_3{\cdot}OEt_2$–EtSH gives compound **10** and etherification with Boc-protected bromoalkylamines provide **11a**–**b**. These compounds have been hydrolyzed and coupled with (2-*S*)-pyrrolidinecarboxaldehyde diethyl thioacetal to give **13a**–**b**, which upon deprotection provide the desired intermediate amines **14a**–**b** (Scheme 1). The other precursor **C2**-*exo* unsaturated acid (**22a**) has been prepared by employing 4-benzyloxy-5-methoxy-2-nitrobenzoic acid **15a** as the starting material, which has been obtained by the procedure described in the literature.[12] *trans*-4-Hydroxy-L-proline methyl ester hydrochloride has been coupled to compound **15a** to give the nitro ester **16a**. The hydroxy group is protected with TBDMS-Cl followed by reduction with DIBAL-H to produce the corresponding aldehyde, which is protected with EtSH/TMS-Cl. Interestingly, in this reaction protection of aldehyde to diethyl thioacetal and deprotection of TBDMS takes place in the same step to afford compound **19a**. Then **C2**-hydroxy group is oxidized with TPAP/NMO to give compound **20a**, which upon Horner–Emmons olefination with methyl diethylphosphonoacetate affords compound **21a**. In this reaction, (*E*) ester[3d] (**21a**) has been obtained exclusively, which upon hydrolysis affords the corresponding acid **22a**. The key intermediates **23a**–**b** have been prepared by amidation of compound **22a** with **14a**–**b**. The compounds **23a**–**b** have been reduced with $SnCl_2{\cdot}2H_2O$ to afford the corresponding amino diethyl thioacetal (**25a**–**b**). Deprotection of amino diethyl thioacetal with $HgCl_2$/$CaCO_3$ provides the target molecules **6a**–**b** (Scheme 2). The compounds **7a**–**b** have been prepared in the same manner by employing with commercially available 4,5-dimethoxy-2-nitrobenzoic acid (Scheme 2). The compounds **30a**–**b** have been prepared by amidation of compounds **22a** and **29a**–**b**, then subsequent reduction followed by deprotection of diethyl thioacetal group affords the desired compounds **8a**–**b** (Scheme 3).

2.2. DNA interactions: thermal denaturation studies

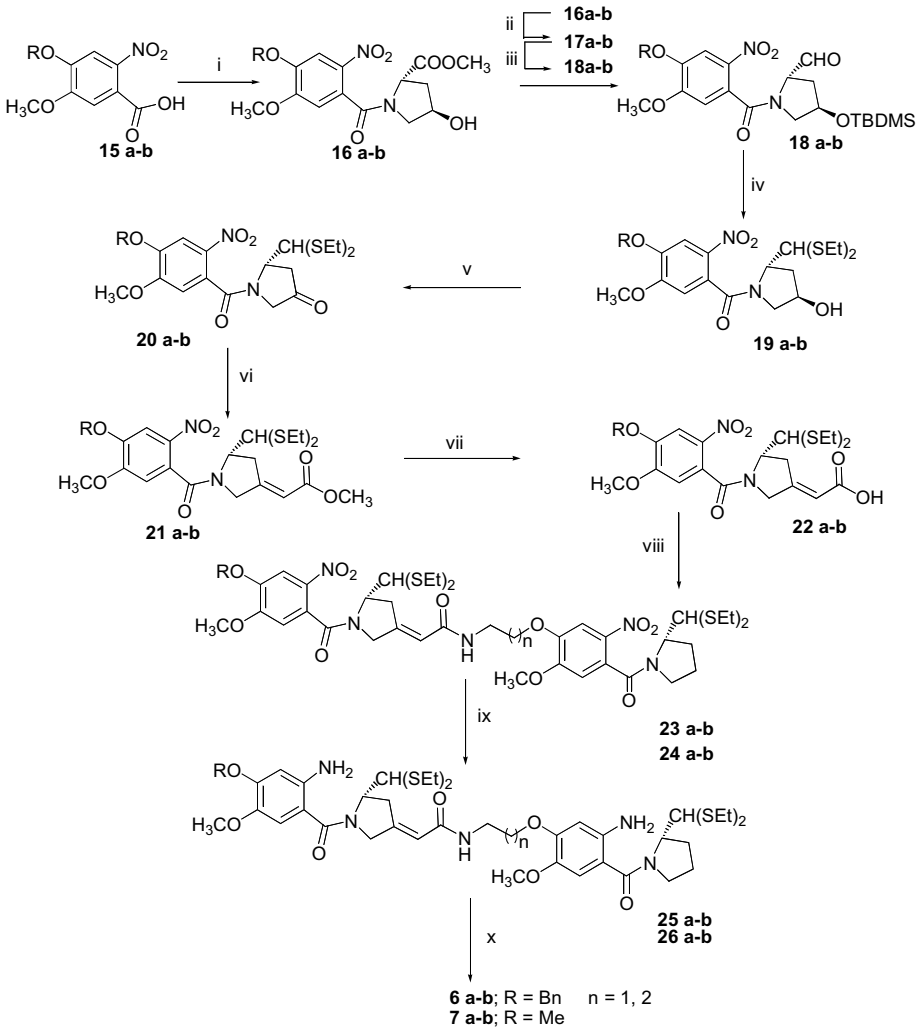
The DNA binding ability for these *A*-**C8**/*C*-**C2**-*exo* unsaturated alkoxyamido-linked PBD dimers has been determined by thermal denaturation studies using calf thymus (CT) DNA. Interestingly, in this assay four of these PBD dimers that is, **6a**,**b**, **7a** and **7b** elevate the helix melting temperature of CT-DNA by a 5.7, 8.3, 5.6 and 6.4 °C, respectively, after incubation for 18 h at 37 °C. On the other hand, the *A*-**C8**/*A*-**C8**′ PBD dimer, DSB-120 exhibits a ΔT_m of 15.3 °C after 18 h of incubation. Further, the values of T_m enhances with the increase of incubation time. This demonstrates that these PBD dimers show significant DNA binding affinity but not as high as DSB-120. On the contrary, the imine–amide dimers (**8a** and **8b**) do not exhibit significant DNA binding ability (Table 1).

2.3. Cytotoxicity

Compounds **6a** and **8b** have been evaluated for their in vitro cytotoxicity against 60 human tumour cell lines derived from nine cancer types (leukaemia, nonsmall cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer). According to the in vitro screening data from the National Cancer Institute (NCI) initial primary test, the compounds **6a** and **8b** have cytotoxic potency against many cell lines. Compound **6a** exhibits a wide spectrum activity against 32 cell lines in nine cell panels, with GI_{50} values of $<10\,\mu M$, whereas compound **8b** exhibits activity against 24 cell lines in seven cancer cell panels, with GI_{50} values of $<50\,\mu M$. A reference compound of the same family DRG-16 ranges from 0.001 to 7.94 nM (mean 0.12 nM).[7] The GI_{50} values of compounds **6a** and **8b** for different cell lines have been illustrated in Table 2.



Scheme 1. Reagents and conditions: (i) EtSH–BF_3OEt_2, CH_2Cl_2, 12 h, rt, 75%; (ii) Boc-protected bromo alkylamine, K_2CO_3, DMF, rt, 24 h, 85–88%; (iii) 1 N LiOH, THF–H_2O–MeOH, 2 h, rt, 80–83%; (iv) 2(*S*)-pyrrolidinecarboxaldehyde diethyl thioacetal, EDCl–HOBt, CH_2Cl_2–H_2O, 24 h, rt, 60–65%; (v) TFA, CH_2Cl_2, 8 h, rt.



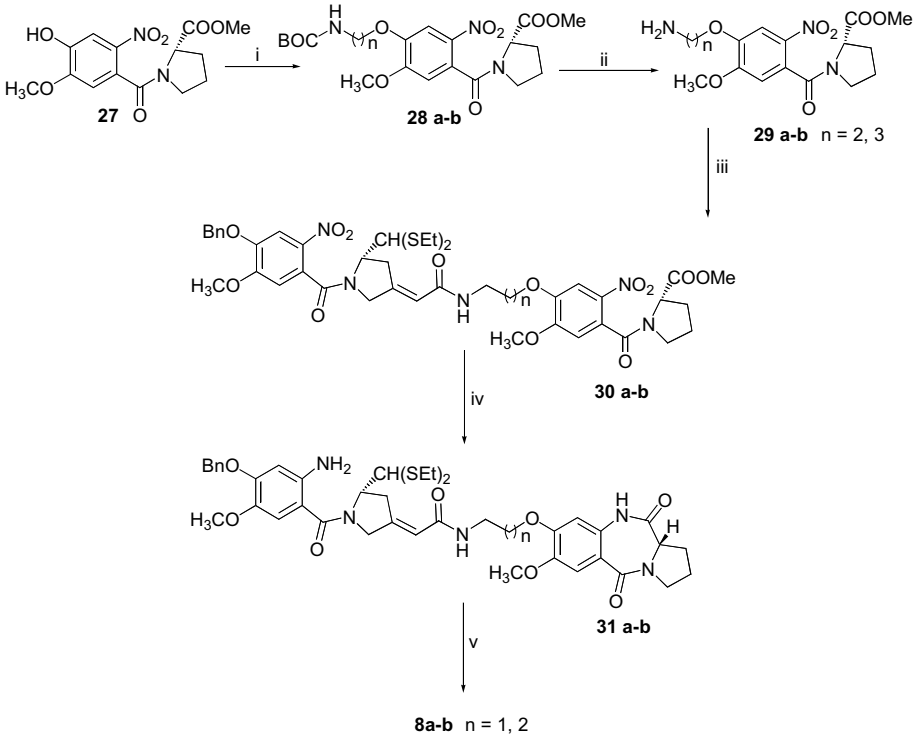
Scheme 2. Reagents and conditions: (i) $SOCl_2$, C_6H_6, *trans*-4-hdroxy-L-proline methylester hydrochloride, Et_3N, H_2O, THF, 0 °C, 1 h, 75–80%; (ii) TBDMS-Cl, CH_2Cl_2, 12 h, rt, 90–92%; (iii) DIBAL-H, CH_2Cl_2, −78 °C, 45 min, 65–68%; (iv) EtSH, TMS-Cl, $CHCl_3$, 18 h, rt, 72–75%; (v) TPAP–NMO, CH_2Cl_2–MeCN, 2.5 h, rt, 76–78%; (vi) methyl diethylphosphonoacetate, NaH, THF, 0 °C, 2 h, 80–84%; (vii) 1 N LiOH, THF–H_2O–MeOH, 2 h, rt, 83–87%; (viii) EDCl–HOBt, compound **14a**–**b**, CH_2Cl_2–H_2O, 24 h, rt, 54–60%; (ix) $SnCl_2$–$2H_2O$, MeOH, reflux, 3 h, 70–75%; (x) $HgCl_2$–$CaCO_3$, CH_3CN–H_2O, 12 h, rt, 48–55%.

2.4. RED_{100}-restriction endonuclease digestion assay

Many studies have employed restriction endonuclease inhibition to confirm the relative binding affinity of DNA-interactive small molecule ligands.[13–16] A quantitative restriction enzyme digest (RED_{100}) assay was developed in which the inhibition of DNA cleavage by *Bam*H1 was used to probe the DNA binding capability of PBD monomers.[17] We have earlier investigated this assay for preferences of base pair selectivity of the imine–amide PBD dimers.[18] Recently this technique has also been used to study the covalent DNA interaction of PBD dimers and it is capable to distinguish between the monomeric and dimeric families.[7] The *Bam*H1 cleavage sequence G$^{\downarrow}$GATCC overlaps with several favoured PBD binding sites suggesting ligand binding has the potential to inhibit the *Bam*H1 cleavage activity. The study has been carried out to determine the ability of *A*-**C8**/*C*-**C2**-*exo* unsaturated alkoxyamido-linked PBD dimers, which inhibit the DNA linearization by *Bam*H1. The results of this experiment for compounds **6a**,**b**, **7a** and **7b** shown in Figure 2 suggest that the PBD dimers inhibit *Bam*H1. There are differences in the inhibitory activity exhibited by PBDs evaluated in this assay. It is observed that the ranking order is **6b** > **7b** > **6a** > **7a** for inhibition of *Bam*H1 cleavage is in agreement with the DNA binding affinity as determined by thermal denaturation. These results clearly demonstrate that as the linker chain increases from two to three carbon spacer as in case of **6b** there is an enhancement in the inhibitory activity (Fig. 1).

2.5. Conclusions

The increase of alkane spacer from two to three carbons in the newly designed *A*-**C8**/*C*-**C2**-*exo* unsaturated



Scheme 3. Reagents and conditions: (i) Boc-protected bromo alkylamine, K_2CO_3, DMF, rt, 24 h, 80–82%; (ii) TFA, CH_2Cl_2, 8 h, rt; (iii) EDCl–HOBt, compound **22a**, CH_2Cl_2–H_2O, 24 h, rt, 65–67%; (iv) $SnCl_2$–$2H_2O$, MeOH, reflux, 3 h, 76–78%; (v) $HgCl_2$–$CaCO_3$, CH_3CN–H_2O, 12 h, rt, 51–53%.

Table 1. Thermal denaturation data for *A*-**C8**/*C*-**C2**-*exo* unsaturated alkoxyamido-linked PBD dimers with CT-DNA

PBD dimers	[PBD]/[DNA] molar ratio[b]	ΔT_m (°C)[a] After incubation at 37 °C			
		0 h	18 h	36 h	48 h
6a	1:5	1.2	5.7	7.1	8.4
6b	1:5	2.3	8.3	10.0	12.6
7a	1:5	1.5	5.6	6.8	7.7
7b	1:5	2.0	6.4	7.8	9.0
8a	1:5	0.3	0.6	0.7	1.0
8b	1:5	0.5	0.9	1.1	1.4
DC-81	1:5	0.3	0.7		
DSB-120	1:5	10.1	15.3		

[a] For CT-DNA alone at pH 7.00 ± 0.01, T_m = 69.0 °C ± 0.01 (mean value from 30 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].

alkoxyamido-linked PBD dimers considerably enhances the DNA melting temperatures. The restriction endonuclease study also demonstrates this aspect and suggests these molecules selectively interact with G sequences in DNA and low affinity with AT-rich sequences. However, the DNA binding potential of these PBD dimers is comparatively less than the *A*-**C8**/*A*-**C8**′ PBD dimers. The in vitro anticancer activities for the representative compounds **6a** and **8b** are not that significant.

3. Experimental

3.1. Synthetic chemistry

Reaction progress was monitored by thin-layer chromatography (TLC) using GF_{254} silica gel with fluorescent indicator on glass plates. Visualization was achieved with UV light and iodine vapour unless otherwise stated. Chromatography was performed using Acme silica gel (100–200 and 60–120 mesh). The majority of reaction solvents were purified by distillation under nitrogen from the indicated drying agent and used fresh: dichloromethane (calcium hydride), tetrahydrofuran (sodium benzophenone ketyl), methanol (magnesium methoxide) and acetonitrile (calcium hydride).

^{1}H NMR spectra were recorded on Varian Gemini 200 MHz spectrometer using tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Spin multiplicities are described as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Coupling constants are reported in Hertz (Hz). Low resolution mass spectra (LRMS) were recorded on VG-7070H Micromass mass spectrometer at 200 °C, 70 eV with trap current of 200 μA and 4 kV acceleration voltage. Optical rotations are measured on Horiba, high sensitive polarimeter, SEPA-300.

3.2. Methyl-4-hydroxy-5-methoxy-2-nitrobenzoate (10)

To a stirred solution of EtSH (1.20 g, 19 mmol) and $BF_3{\cdot}OEt_2$ (1.419 g, 10 mmol) was added dropwise to the

Table 2. In vitro cytotoxicity of compound **6a** and **8b** in selected human cancer cell lines

Cancer panel/cell line	GI_{50} (μM)	
	6a	**8b**
Leukaemia		
CCRF-CEM	1.1	40.8
K-562	2.7	—
MOLT-4	1.1	29.6
SR	1.4	17.5
RPMI-8226	1.2	43.6
Nonsmall cell lung		
HOP-62	4.2	41.3
HOP-92	—	47.2
NCI-H23	9.2	—
NCI-H460	—	38.9
NCI-H522	1.6	31.3
Colon		
HCT-116	2.9	—
HT-29		45.2
KM-12	9.3	47.5
SW-620	5.3	33.3
CNS		
SF-268	2.1	39.6
SF-539	—	32.4
SNB-19	8.6	43.3
SNB-75	—	32.7
U251	2.8	33.3
Melanoma		
LOX IMVI	2.7	43.3
SK-MEL-5	1.3	22.5
MALME-3M	2.2	—
SK-MEL-2	1.5	—
UACC-62	2.6	—
Ovarian		
IGROV1	1.4	—
OVCAR-3	2.1	—
OVCAR-5	4.0	—
OVCAR-8	3.8	—
Prostate		
PC-3	3.5	—
Renal		
RXF-393	1.9	33.6
SN12C	2.6	18.4
786-0	3.6	—
TK-10	7.0	—
Breast		
MCF-7	2.6	32.0
HS-578T	5.6	34.4
MDA-MB-435	2.9	44.2
BT-549	—	23.9
T-47D	2.7	—

compound **9** (317 mg, 1 mmol) in dichloromethane (15 mL) at room temperature. Stirring was continued until TLC completed the reaction and then the solvent was evaporated in vacuum. The residue was quenched with 5% $NaHCO_3$ solution (20 mL) and then extracted with dichloromethane (2×20 mL). The combined organic phases were washed with saturated brine (10 mL), dried over Na_2SO_4 and the solvent removed by vacuum and purified by column chromatography (30% EtOAc–hexane) to afford compound **10** (170 mg, 75%). ^{1}H NMR ($CDCl_3$): δ 3.89 (s, 3H), 3.95 (s, 3H), 6.25 (br s, 1H), 7.05 (s, 1H), 7.45 (s, 1H). MS (EI) m/z 227 $[M]^{+\cdot}$.

3.3. Methyl-4-(*N*-*t*-butoxycarbonyl)aminoethoxy-5-methoxy-2-nitrobenzoate (11a)

To a solution of compound **10** (227 mg, 1 mmol) in dimethylformamide (10 mL) was added, anhydrous K_2CO_3 (553 mg, 4 mmol) and Boc-protected bromo ethylamine (269 mg, 1.2 mmol) and the mixture was stirred for 24 h at room temperature. The reaction was monitored by TLC using EtOAc–hexane (1:1) and K_2CO_3 was removed by filtration and the solvent was evaporated under the vacuum. The residue was dissolved in ethyl acetate (2×20 mL) and washed with water. The combined organic phases were dried over Na_2SO_4 and the solvent was removed by vacuum and purified by column chromatography (25% EtOAc–hexane) to afford compound **11a** (315 mg, 85%). ^{1}H NMR ($CDCl_3$): δ 1.45 (s, 9H), 3.89 (s, 3H), 3.60 (t, 2H, $J = 6.22$ Hz), 3.95 (s, 3H), 4.10 (t, 2H, $J = 5.98$ Hz), 5.0 (br s, 1H), 7.05 (s, 1H), 7.45 (s, 1H). MS (EI) m/z 370 $[M]^{+\cdot}$.

3.4. Methyl-4-(*N*-*t*-butoxycarbonyl)aminopropoxy-5-methoxy-2-nitrobenzoate (11b)

The compound **11b** was prepared according to the method described for the compound **11a** employing compound **10** (227 mg, 1 mmol) and Boc-protected bromopropylamine (286 mg, 1.2 mmol) to afford crude compound, which was purified by column chromatography (30% EtOAC–hexane) to give compound **11b** (338 mg, 88%). ^{1}H NMR ($CDCl_3$): δ 1.45 (s, 9H), 1.90–2.05 (m, 2H), 3.89 (s, 3H), 3.60 (t, 2H, $J = 6.22$ Hz), 3.95 (s, 3H), 4.10 (t, 2H, $J = 5.98$ Hz), 5.0 (br s, 1H), 7.05 (s, 1H), 7.45 (s, 1H). MS (EI) m/z 384 $[M]^{+\cdot}$.

3.5. 4-(*N*-*t*-Butoxycarbonyl)aminoethoxy-5-methoxy-2-nitrobenzoicacid (12a)

Lithium hydroxide monohydrate (2 N, 1.22 mL) was added to a solution of methyl-4-(*N*-*t*-butoxycarbonyl)aminoethoxy-5-methoxy-2-nitrobenzoate **11a** (370 mg, 1 mmol) in THF–H_2O–MeOH (4:1:1) and the mixture stirred at room temperature for 2 h. After most of the THF and methanol were evaporated, the aqueous phase was acidified with 12 N HCl to pH 7 and re-extracted with EtOAc to give a 4-(*N*-*t*-butoxycarbonyl)-aminoethoxy-5-methoxy-2-nitrobenzoic acid **12a** (285 mg, 80%). ^{1}H NMR ($CDCl_3$): δ 1.45 (s, 9H), 3.60 (t, 2H, $J = 6.4$ Hz), 3.95 (s, 3H), 4.20 (t, 2H, $J = 6.2$ Hz), 5.0 (br s, 1H), 7.20 (s, 1H), 7.40 (s, 1H). MS (EI) m/z 356 $[M]^{+\cdot}$.

3.6. 4-(*N*-*t*-Butoxycarbonyl)aminopropoxy-5-methoxy-2-nitrobenzoicacid (12b)

The compound **12b** was prepared according to the method described for the compound **12a**

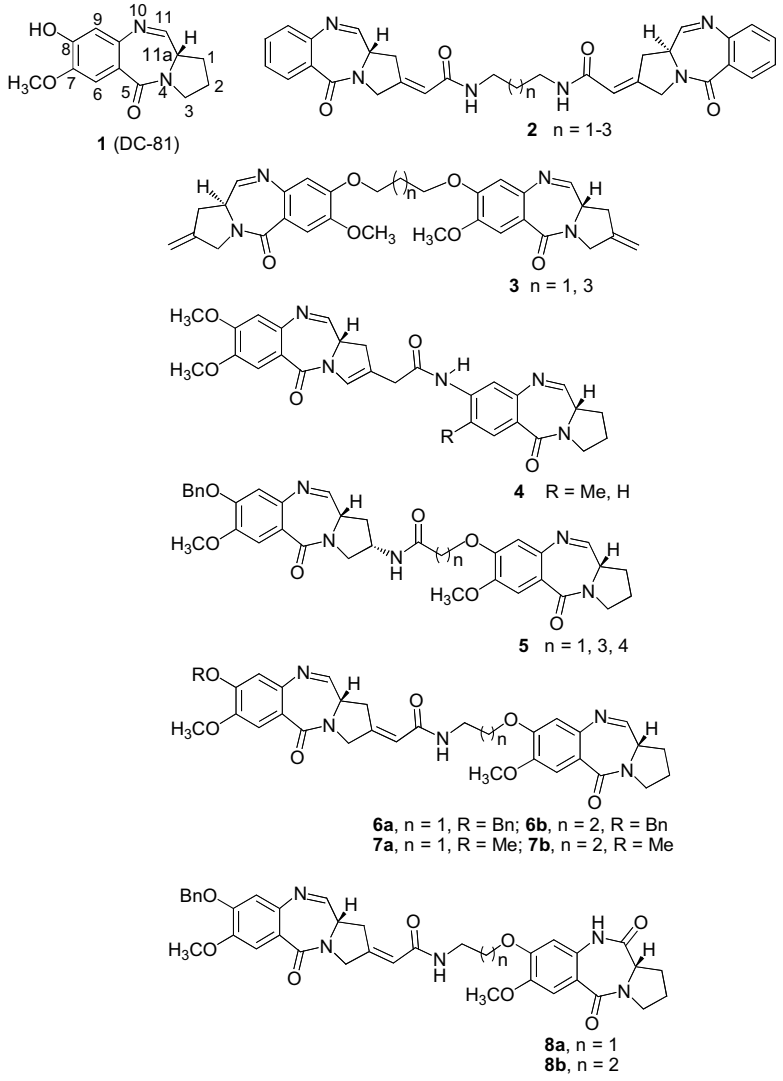


Figure 1. Structures of pyrrolobenzodiazepines and their dimers.

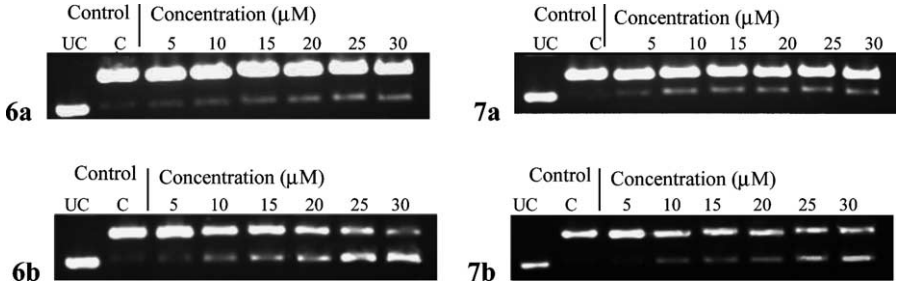


Figure 2. RED_{100}-restriction endonuclease digestion assay for *A*-**C8**/*C*-**C2**-*exo* unsaturated alkoxyamido-linked PBD dimers with CT-DNA inhibitory activity of **6a**,**b**, **7a** and **7b** on the cleavage of plasmid pBR322 by restriction endonuclease *Bam*H1 (20 units in 2 μL) for 1 h at 37 °C. The cut (C) and uncut (UC) products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining under UV illumination. Lane 1: control pBR322; lane 2: complete digest of pBR322 by *Bam*H1.

employing compound **11b** (384 mg, 1 mmol) to afford compound **12b** (409 mg, 87%). ^{1}H NMR ($CDCl_3$): δ 1.45 (s, 9H), 1.95–2.05 (m, 2H), 3.60 (t, 2H, $J = 6.4$ Hz), 3.98 (s, 3H), 4.25 (t, 2H, $J = 6.2$ Hz), 5.0 (br s, 1H), 7.20 (s, 1H), 7.45 (s, 1H). MS (EI) m/z 370 $[M]^{+\cdot}$.

3.7. (2*S*)-*N*-[4-(*N*-*t*-Butoxycarbonyl)aminoethoxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (13a)

To a solution of **12a** (356 mg, 1 mmol) in dichloromethane–water (20:20 mL), were added EDCI (384 mg,

2 mmol), HOBt (270 mg, 2 mmol) and (2*S*)-pyrrolidine-2-carboxaldehyde diethyl thioacetal (205 mg, 1 mmol) and the mixture was stirred for 24 h at room temperature. The dichloromethane was evaporated in vacuum, the residue was dissolved in ethyl acetate and washed with saturated sodium bicarbonate solution and then with brine solution. The combined organic phases were dried over Na_2SO_4, the solvent was removed under vacuum and purified by column chromatography (50% EtOAc–hexane) to afford compound **13a** (524 mg, 60%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.40 (m, 6H), 1.45 (s, 9H), 1.80–2.10 (m, 4H), 2.60–2.90 (m, 4H), 3.20–3.35 (m, 2H), 3.60 (t, 2H, $J = 6.22$ Hz), 3.95 (s, 3H), 4.10 (t, 2H, $J = 5.98$ Hz), 4.63–4.73 (m, 1H), 4.80 (d, 1H, $J = 4.3$ Hz), 5.0 (br s, 1H), 6.75 (s, 1H), 7.60 (s,1H). MS (FAB) 544 $[M+H]^{+\cdot}$.

3.8. (2*S*)-*N*-[4-(*N*-*t*-Butoxycarbonyl)aminopropoxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (13b)

The compound **13b** was prepared according to the method described for the compound **13a** by employing **12b** (370 mg, 1 mmol) to afford crude compound, which was purified by column chromatography (55% EtOAC–hexane) to give compound **13b** (362 mg, 65%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.40 (m, 6H), 1.45 (s, 9H), 1.70–2.35 (m, 6H), 2.65–2.90 (m, 4H), 3.18–3.30 (m, 2H), 3.40 (t, 2H, $J = 6.20$ Hz), 3.95 (s, 3H), 4.18 (t, 2H, $J = 6.10$ Hz), 4.63-4.75 (m, 1H), 4.80 (d, 1H, $J = 4.29$ Hz), 5.20 (br s, 1H), 6.80 (s, 1H), 7.62 (s, 1H). MS (FAB) 558 $[M+H]^{+\cdot}$.

3.9. (2*S*)-*N*-[4-(2-Aminoethoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (14a)

To a solution of Boc-compound **13a** (543 mg, 1 mmol) in dry dichloromethane was added trifluoroacetic acid (1 mL) at 0 °C and stirred under nitrogen for 8 h, the reaction mixture was then concentrated in vacuum and then it was used directly in the next step.

3.10. (2*S*)-*N*-[4-(3-Aminopropoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (14b)

The compound **14b** was prepared according to the method described for the compound **14a** by employing **13b** (557 mg, 1 mmol) and TFA.

3.11. Methyl-(2*S*,4*R*)-*N*-[4-benzyloxy-5-methoxy-2-nitrobenzoyl]-4-hydroxypyrrolidine-2-carboxylate (16a)

To a stirred suspension of compound **15a** (303 mg, 1 mmol) and thionyl chloride (476 mg, 4.0 mmol) in dry benzene (15 mL) was added DMF (four–five drops) and the stirring was continued for 6 h. The benzene was evaporated in vacuum and the resultant oil dissolved in dry THF (20 mL) and added drop wise over a period of 30 min to a stirred suspension of *trans*-4-hydroxy-L-proline methylester hydrochloride (270 mg 1.5 mmol), triethylamine (303 mg, 3 mmol) and ice water (20 mL) cooled in an ice bath. After the completion of addition, the reaction mixture was brought to ambient temperature and stirred for an additional 1 h. The THF was evaporated in vacuum and the aqueous layer was washed with ethyl acetate (10 mL). The aqueous phase was then adjusted to pH 3 using 6 N HCl and extracted with ethyl acetate and washed with brine, dried over Na_2SO_4 and evaporated in vacuum and was purified by column chromatography (70% EtOAc–hexane) to afford compound **16a** (323 mg, 75%). ^{1}H NMR ($CDCl_3$): δ 2.10–2.22 (m, 1H), 2.30–2.45 (m, 1H), 2.95–3.20 (m, 2H), 3.42–3.50 (m, 1H), 3.80 (s, 3H), 4.0 (s, 3H), 4.40–4.50 (m, 1H), 4.75–4.85 (m, 1H), 5.20 (s, 2H), 6.82 (s, 1H), 7.3–7.50 (m, 5H) 7.70 (s, 1H). MS (FAB) 431 $[M+H]^{+\cdot}$.

3.12. Methyl-(2*S*,4*R*)-*N*-[4,5-dimethoxy-2-nitrobenzoyl]-4-hydroxypyrrolidine-2-carboxylate (16b)

The compound **16b** was prepared according to the method described for the compound **16a** employing compound 4,5-dimethoxy nitro benzoic acid (227 mg, 1 mmol) to give crude product, which was purified by column chromatography (80% EtOAc–hexane) to afford compound **16b** (283 mg, 80%). ^{1}H NMR ($CDCl_3$): δ 2.0–2.20 (m, 1H), 2.25–2.45 (m, 1H), 2.80–3.20 (m, 2H), 3.50–3.60 (m, 1H), 3.80 (s, 3H), 3.98 (s, 3H), 4.0 (s, 3H), 4.40–4.50 (m, 1H), 4.75–4.85 (m, 1H), 6.82 (s, 1H), 7.70 (s, 1H); MS (EI) 354 $[M]^{+\cdot}$.

3.13. Methyl-(2*S*,4*R*)-*N*-[4-benzyloxy-5-methoxy-2-nitrobenzoyl]-4-(*t*-butyldimethylsilyl)-oxypyrrolidine-2-carboxylate (17a)

Solid TBDMS-chloride (226 mg, 1.5 mmol) was added in one portion to a solution of compound **16a** (430 mg, 1 mmol) and imidazole (136 mg, 2 mmol) in anhydrous dichloromethane (20 mL) and allowed to stir at room temperature for 12 h under a nitrogen atmosphere. The reaction mixture was poured into water and then extracted with dichloromethane (2×20 mL). The combined organic phases were dried over Na_2SO_4 and the solvent was removed by vacuum and purified by column chromatography (30% EtOAc–hexane) to afford compound **17a** (490 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 0.18 (s, 6H), 0.85 (s, 9H), 2.08–2.20 (m, 1H), 2.25–2.40 (m, 1H), 3.0–3.10 (m, 1H), 3.40–3.55 (m, 1H), 3.80 (s, 3H), 4.00 (s, 3H), 4.40–4.50 (m, 1H), 4.75–4.85 (m, 1H), 5.20 (s, 2H), 6.80 (s, 1H), 7.30–7.50 (m, 5H) 7.70 (s, 1H). MS (FAB) 545 $[M+H]^{+\cdot}$.

3.14. Methyl-(2*S*,4*R*)-*N*-[4,5-dimethoxy-2-nitrobenzoyl]-4-(*t*-butyldimethylsilyl)oxypyrrolidine-2-carboxylate (17b)

The compound **17b** was prepared according to the method described for the compound **17a** employing compound **16b** (354 mg, 1 mmol) to give the crude product, which was purified by column chromatography (40% EtOAc–hexane) to afford the compound **17b** (430 mg, 92%). ^{1}H NMR ($CDCl_3$): δ 0.18 (s, 6H), 0.85 (s, 9H), 2.10–2.20 (m, 1H), 2.25–2.40 (m, 1H), 3.0–3.10 (m,

1H), 3.40–3.50 (m, 1H), 3.80 (s, 3H), 3.98 (s, 3H), 4.0 (s, 3H), 4.40–4.50 (m, 1H), 4.70–4.80 (m, 1H), 6.80 (s, 1H), 7.65 (s, 1H). MS (FAB) 469 $[M+H]^{+\cdot}$.

3.15. (2*S*,4*R*)-*N*-[4-Benzyloxy-5-methoxy-2-nitrobenzoyl]-4-(*t*-butyldimethylsilyl)oxypyrrolidine-2-carboxaldehyde (18a)

Diisobutyl alminium hydride solution (2.5 mL of 1.0 M solution in hexane) was added drop wise to a vigorously stirred solution of the compound **17a** (544 mg, 1 mmol) in anhydrous dichloromethane (10 mL) under dry nitrogen at −78 °C (dry ice–acetone). After the mixture was stirred for an additional 45 min, excess of reagent was decomposed by careful addition of methanol (2 mL) followed by 5% HCl (2 mL). The resulting mixture was allowed to warm room temperature and the organic layer was removed, the aqueous layer was extracted with ethylacetate (4 × 10 mL). The organic layers were combined, dried over Na_2SO_4 and the solvent was evaporated in vacuum to afford the crude aldehyde **18a** (334 mg, 65%). ^{1}H NMR ($CDCl_3$): δ 0.16 (s, 6H), 0.80 (s, 9H) 2.10–2.30 (m, 1H), 2.42–2.56 (m, 1H), 3.20–3.30 (m, 1H), 3.40–3.52 (m, 1H), 4.0 (s, 3H), 4.40–4.50 (m, 1H), 4.75–4.90 (m, 1H), 5.20 (s, 2H), 6.75 (s, 1H), 7.30–7.50 (m, 5H), 7.75 (s, 1H), 9.78–9.80 (d, 1H, $J = 2.0$ Hz). MS (FAB) 515 $[M+H]^{+\cdot}$.

3.16. (2*S*,4*R*)-*N*-[4,5-Dimethoxy-2-nitrobenzoyl]-4-(*t*-butyldimethylsilyl)oxypyrrolidine-2-carboxaldehyde (18b)

The compound **18b** was prepared according to the method described for the compound **18a** employing compound **17b** (468 mg, 1 mmol) to afford compound **18b** (298 mg, 68%). ^{1}H NMR ($CDCl_3$): δ 0.18 (s, 6H), 0.85 (s, 9H) 2.10–2.20 (m, 1H), 2.25–2.40 (m, 1H), 3.0–3.10 (m, 1H), 3.40–3.50 (m, 1H), 3.98 (s, 3H), 4.0 (s, 3H), 4.40–4.50 (m, 1H), 4.70–4.80 (m, 1H), 6.80 (s, 1H), 7.65 (s, 1H), 9.78–9.80 (d, 1H, $J = 2.2$ Hz). MS (FAB) 439 $[M+H]^{+\cdot}$.

3.17. (2*S*,4*R*)-*N*-[4-Benzyloxy-5-methoxy-2-nitrobenzoyl]-4-hydroxypyrrolidine-2-carboxaldehyde diethyl thioacetal (19a)

Ethanethiol (278 mg, 4.4 mmol) was added to a stirred solution of nitroaldehyde **18a** (514 mg, 1 mmol) in dry chloroform (15 mL) under nitrogen atmosphere. The mixture was stirred for a further 30 min followed by the addition of trimethylsilyl chloride (540 mg, 5 mmol). After a further 18 h of stirring of the mixture, when TLC indicated that reaction was completed, the reaction mixture was carefully neutralized with sodium bicarbonate solution and extracted with chloroform (2 × 10 mL). The combined organic phase was dried over Na_2SO_4 and evaporated in vacuum to give the crude diethyl thioacetal, which was purified by column chromatography (60% EtOAc–hexane) to afford compound **19a** (364 mg, 72%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.40 (m, 6H), 2.20–2.35 (m, 2H), 2.62–2.90 (m, 4H), 3.10–3.20 (d 1H, $J = 11.15$ Hz), 3.41–3.49 (dd, 1H, $J = 3.7$, 3.7 Hz), 3.95 (s, 3H), 4.25–4.38 (m, 1H), 4.80–4.90 (m, 2H), 5.20 (s, 2H), 6.80 (s, 1H), 7.30–7.50 (m, 5H), 7.70 (s, 1H). MS (FAB) 507 $[M+H]^{+\cdot}$.

3.18. (2*S*,4*R*)-*N*-[4,5-Dimethoxy-2-nitrobenzoyl]-4-hydroxypyrrolidine-2-carboxaldehyde diethyl thioacetal (19b)

The compound **19b** was prepared according to the method described for the compound **19a** employing compound **18b** (438 mg, 1 mmol) to afford crude compound, which was purified by column chromatography (70% EtOAc–hexane) to give compound **19b** (323 mg, 75%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.40 (m, 6H), 1.80–2.35 (m, 3H), 2.62–2.90 (m, 4H), 3.20 (d, 1H, $J = 10$ Hz), 3.40–3.50 (dd, 1H, $J = 8.5$, 3.5 Hz), 3.95 (s, 3H), 3.98 (s, 3H), 4.25–4.38 (m, 1H), 4.80–4.90 (m, 2H), 6.80 (s, 1H), 7.65 (s, 1H). MS (FAB) 431 $[M+H]^{+\cdot}$.

3.19. (2*S*)-*N*-[4-Benzyloxy-5-methoxy-2-nitrobenzoyl]-4-oxo-pyrrolidine-2-carboxaldeyde diethyl thioacetal (20a)

A solution of **19a** (506 mg, 1 mmol) in dichloromethane (15 mL) was treated with acetonitrile (2 mL), molecular sieves (4 Å, 500 mg) and NMO (176 mg, 1.5 mmol). After the mixture was stirred for 15 min at room temperature, TPAP (18 mg, 0.05 mmol) was added to the reaction mixture and it was stirred for further 2.5 h at room temperature until TLC (50% EtOAc–hexane) indicated complete consumption of starting material. Concentration of the reaction mixture in vacuum followed by column chromatography (40% EtOAc–hexane) gave the pure ketone **20a** (393 mg, 78%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.40 (m, 6H), 2.600–2.90 (m, 6H), 3.55 (d, 1H, $J = 17.0$ Hz), 3.78–3.95 (d, 1H, $J = 17.0$ Hz), 4.0 (s, 3H), 4.60 (d, 1H, $J = 4.26$ Hz), 5.15–5.25 (m, 3H), 6.75 (s, 1H), 7.30–7.50 (m, 5H), 7.75 (s, 1H). MS (FAB) 505 $[M+H]^{+\cdot}$.

3.20. (2*S*)-*N*-[4,5-Dimethoxy-2-nitrobenzoyl]-4-oxo-pyrrolidine-2-carboxaldeyde diethyl thioacetal (20b)

The compound **20b** was prepared according to the method described for the compound **20a** employing compound **19b** (430 mg, 1 mmol) to give crude compound, which was purified by column chromatography (50% EtOAc–hexane) to afford compound **20b** (325 mg, 76%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.40 (m, 6H), 2.70–3.0 (m, 6H), 3.55 (d, 1H, $J = 15.0$ Hz), 3.85 (d, 1H, $J = 15.0$ Hz), 3.95 (s, 3H), 4.0 (s, 3H), 4.65 (d, 1H, $J = 4.40$ Hz), 5.15–5.25 (m, 1H), 6.75 (s, 1H), 7.75 (s, 1H). MS (FAB) 429 $[M+H]^{+\cdot}$.

3.21. (2*S*)-*N*-[4-Benzyloxy-5-methoxy-2-nitrobenzoyl]-4-(methoxycarbonylmethylidene)-pyrrolidine-2-carboxaldehyde diethyl thioacetal (21a)

Dry THF (10 mL) was added to the sodium hydride (80 mg of 60% dispersion in oil, 2 mmol) under a nitrogen

atmosphere and the mixture was cooled to 0 °C. The cool solution was treated drop wise with a solution of methyl diethylphosphonoacetate (420 mg, 2 mmol) in THF (5 mL) under nitrogen and allowed to stir for 1 h at room temperature. The reaction mixture was cooled 0 °C and treated drop wise with a solution of the ketone **20a** (504 mg, 1 mmol) in THF (2 × 5 mL) under nitrogen. The reaction mixture was stirred for a further 2 h until TLC (50% EtOAc–hexane) indicated complete consumption of starting material. The THF was evaporated in vacuum and the mixture was washed with sodium bicarbonate solution and then extracted with ethyl acetate (2 × 20 mL). The combined organic phases were washed with saturated brine and dried over Na_2SO_4. The solvent was removed under vacuum and purified by column chromatography (30% EtOAc–hexane) to afford compound **21a** (448 mg, 80%). ^{1}H NMR ($CDCl_3$): δ 1.25–1.45 (m, 6H), 2.65–2.90 (m, 4H), 3.05–3.30 (m, 2H), 3.65 (s, 3H), 3.95 (s, 3H), 4.20–4.30 (m, 1H), 4.45–4.60 (m, 1H), 4.80 (d, 1H, $J = 4.2$ Hz), 4.90–5.0 (m, 1H), 5.20 (s, 2H), 5.35–5.40 (m, 1H), 5.90 (s, 1H), 6.80 (s, 1H), 7.30–7.50 (m, 5H), 7.75 (s, 1H). MS (FAB) 561 $[M+H]^{+\cdot}$.

3.22. (2*S*)-*N*-[4,5-Dimethoxy-2-nitrobenzoyl]-4-(methoxycarbonylmethylidene)pyrrolidine-2-carboxaldehyde diethyl thioacetal (21b)

The compound **21b** was prepared according to the method described for the compound **21a** employing compound **20b** (428 mg, 1 mmol) to give crude product, which was purified by column chromatography (40% EtOAc–hexane) to afford compound **21b** (402 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 1.25–1.45 (m, 6H), 2.65–2.90 (m, 4H), 3.05–3.30 (m, 2H), 3.60–3.75 (m, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 4.20–4.30 (m, 1H), 4.45–4.60 (m, 1H), 4.80 (d, 1H, $J = 4.2$ Hz), 5.35–5.40 (m, 1H), 5.90 (s, 1H), 6.80 (s, 1H), 7.68 (s, 1H). MS (FAB) 485 $[M+H]^{+\cdot}$.

3.23. (2*S*)-*N*-[4-Benzyloxy-5-methoxy-2-nitrobenzoyl]-4-(carboxymethylidene)pyrrolidine-2-carboxaldehyde diethyl thioacetal (22a)

Lithium hydroxide monohydrate (2 N, 1.22 mL) was added to a solution of **21a** (560 mg, 1 mmol) in THF–H_2O–MeOH (4:1:1) and the mixture stirred at room temperature for 2 h. After most of the THF and methanol were evaporated, the aqueous phase was acidified with 12 N HCl to pH 7 and extracted with EtOAc to give **22a** (452 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 1.25–1.45 (m, 6H), 2.65–2.90 (m, 4H), 3.10–3.25 (m, 2H), 3.90–4.10 (m, 5H), 4.78 (d, 1H $J = 4.28$ Hz), 5.20 (s, 2H), 5.35–5.40 (m, 1H), 5.95 (s, 1H), 6.80 (s, 1H), 7.35–7.55 (m, 5H), 7.75 (s, 1H). MS (FAB) 545 $[M+H]^{+\cdot}$.

3.24. (2*S*)-*N*-[4,5-Dimethoxy-2-nitrobenzoyl]-4-(carboxymethylidene)pyrrolidine-2-carboxaldehyde diethyl thioacetal (22b)

The compound **22b** was prepared according to the method described for the compound **22a** employing compound **21b** (484 mg, 1 mmol) to afford compound **22b** (409 mg, 87%). ^{1}H NMR ($CDCl_3$): δ 1.25–1.45 (m, 6H), 2.65–2.90 (m, 4H), 3.10–3.25 (m, 2H), 3.90–5.10 (m, 8H), 4.78 (d, 1H, $J = 4.28$ Hz), 5.35–5.40 (m, 1H), 5.92 (s, 1H), 6.80 (s, 1H), 7.68 (s, 1H). MS (FAB) 471 $[M+H]^{+\cdot}$.

3.25. (2*S*)-*N*-{4-[(2*S*)-*N*-(4-Benzyloxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-*N*-ethyl]oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (23a)

To a solution of **22a** (544 mg, 1 mmol) in dichloromethane–water (20:20 mL), were added EDCI (384 mg, 2 mmol), HOBt (270 mg, 2 mmol) and compound **14a** (443 mg, 1 mmol) and the mixture was stirred for 24 h at room temperature. The dichloromethane was evaporated in vacuum, the residue was dissolved in ethyl acetate and washed with saturated sodium bicarbonate solution and then with brine solution. The combined organic phases were dried over Na_2SO_4, the solvent was removed under vacuum and purified by column chromatography (80% EtOAc–hexane) to afford compound **23a** (524 mg, 54%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.45 (m, 12H), 1.75–2.35 (m, 4H), 2.60–2.90 (m, 8H), 3.0–3.30 (m, 4H), 3.55–3.65 (m, 2H), 3.90–4.10 (m, 8H), 4.15 (t, 2H, $J = 5.98$ Hz), 4.63–4.72 (m, 1H), 4.80 (m, 2H), 5.20 (s, 2H), 5.30–5.35 (m, 1H), 5.90 (s, 1H), 6.50–6.55 (m, 1H), 6.75 (s, 1H), 6.82 (s, 1H), 7.30–7.50 (m, 5H), 7.65 (s, 1H), 7.75 (s, 1H). MS (FAB) 971 $[M+H]^{+\cdot}$.

3.26. (2*S*)-*N*-{4-[(2*S*)-*N*-(4-Benzyloxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-*N*-propyl]oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (23b)

The compound **23b** was prepared according to the method described for the compound **23a** by employing **22a** and **14b** (457 mg, 1 mmol) to afford crude compound, which was purified by column chromatography (85% EtOAC–hexane) to give compound **23b** (562 mg, 57%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.45 (m, 12H), 1.75–2.35 (m, 6H), 2.65–2.90 (m, 8H), 2.95–3.30 (m, 4H), 3.40–3.50 (m, 2H), 3.80–4.05 (m, 8H), 4.15 (t, 2H, $J = 5.80$ Hz), 4.60–4.70 (m, 1H), 4.75–4.82 (m, 2H), 5.20 (s, 2H), 5.35–5.40 (m, 1H), 5.92 (s, 1H), 6.55 (m, 1H), 6.75 (s, 1H), 6.80 (s, 1H), 7.30–7.50 (m, 5H), 7.60 (s, 1H), 7.70 (s, 1H). MS (FAB) 986 $[M+H]^{+\cdot}$.

3.27. (2*S*)-*N*-{4-[(2*S*)-*N*-(4,5-Dimethoxy-2-nitrobenzoyl)-pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-*N*-ethyl]oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (24a)

The compound **24a** was prepared according to the method described for the compound **23a** employing compound **22b** (470 mg, 1 mmol) and **14a** (443 mg,

1 mmol) to afford crude product, which was purified by column chromatography (90% EtOAc–hexane) to give compound **24a** (501 mg, 56%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.45 (m, 12H), 1.75–2.35 (m, 4H), 2.60–2.90 (m, 8H), 2.95–3.30 (m, 4H), 3.55–3.70 (m, 2H), 3.90–4.10 (m, 11H), 4.05–4.15 (m, 2H), 4.60–4.70 (m, 1H), 4.75–4.85 (m, 2H), 5.30–5.40 (m, 1H), 5.90 (s, 1H), 6.50 (br s, 1H), 6.75 (s, 1H), 6.82 (s, 1H) 7.60 (s, 1H), 7.65 (s, 1H). MS (FAB) 896 $[M+H]^{+\cdot}$.

3.28. (2*S*)-*N*-{4-[(2*S*)-*N*-(4,5-Dimethoxy-2-nitrobenzoyl)-pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-*N*-propyl]oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (24b)

The compound **24b** was prepared according to the method described for the compound **24a** employing compound **22b** (470 mg, 1 mmol) and **14b** (457 mg, 1 mmol) to afford crude product, which was purified by column chromatography (95% EtOAc–hexane) to give compound **24b** (545 mg, 60%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.45 (m, 12H), 1.72–2.35 (m, 6H), 2.65–2.90 (m, 8H), 2.95–3.30 (m, 4H), 3.55–3.70 (m, 2H), 3.90–4.05 (m, 11H), 4.02–4.15 (m, 2H), 4.60–4.70 (m, 1H), 4.75–4.82 (m, 2H), 5.30–5.40 (m, 1H), 5.92 (s, 1H), 6.55 (br s, 1H), 6.75 (s, 1H), 6.80 (s, 1H) 7.60 (s, 1H), 7.68 (s, 1H). MS (FAB) 910 $[M+H]^{+\cdot}$.

3.29. (2*S*)-*N*-{4-[(2*S*)-*N*-(4-Benzyloxy-5-methoxy-2-aminobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-*N*-ethyl]oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (25a)

The compound **25a** (970 mg, 1 mmol) was dissolved in methanol (20 mL) and added $SnCl_2{\cdot}2H_2O$ (2.256 g, 10 mmol) was refluxed for 3 h or until the TLC indicated that reaction was complete. The methanol was evaporated under vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (2×30 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal, **25a**, which due to potential stability problems, was briefly characterized by ^{1}H NMR and then used directly in the next step (655 mg, 72%). ^{1}H NMR ($CDCl_3$): δ 1.15–1.45 (m, 12H), 1.70–2.35 (m, 4H), 2.55–2.85 (m, 8H), 2.95–3.25 (m, 4H), 3.45–3.85 (m, 10H), 3.98–4.05 (t, 2H, $J = 6.0$ Hz), 4.45–4.50 (m, 1H), 4.62–4.70 (m, 2H), 5.10 (s, 2H), 5.25–5.35 (m, 1H), 5.85 (s, 1H), 6.15–6.25 (m, 2H), 6.65-6.85 (m, 3H), 7.35–7.50 (m, 5H).

3.30. (2*S*)-*N*-{4-[(2*S*)-*N*-(4-Benzyloxy-5-methoxy-2-aminobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-*N*-propyl]oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (25b)

The compound **25b** was prepared according to the method described for the compound **25a** employing the compound **23b** (984 mg, 1 mmol), to afford the amino diethyl thioacetal **25b** (693 mg, 75%). ^{1}H NMR ($CDCl_3$): δ 1.15–1.45 (m, 12H), 1.60–2.35 (m, 6H), 2.55–2.85 (m, 8H), 2.90–3.20 (m, 2H), 3.35–3.65 (m, 6H), 3.75 (s, 3H), 3.77 (s, 3H), 4.05 (t, 2H, $J = 6.0$ Hz), 4.52–4.70 (m, 3H), 5.10 (s, 2H), 5.35–5.45 (m, 1H), 5.85 (s, 1H), 6.15–6.25 (m, 2H), 6.60–6.80 (m, 3H) 7.30–7.50 (m, 5H).

3.31. (2*S*)-*N*-{4-[(2*S*)-*N*-(4,5-dimethoxy-2-aminobenzoyl)-pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-*N*-ethyl]oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (26a)

The compound **26a** was prepared according to the method described for the compound **25a** employing compound **24a** (895 mg, 1 mmol) to afford compound **26a** (593 mg, 71%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.45 (m, 12H), 1.70–2.40 (m, 4H), 2.60–2.90 (m, 8H), 2.95–3.25 (m, 2H), 3.45–3.70 (m, 4H), 3.80 (s, 6H), 3.85 (s, 3H), 4.0–4.20 (m, 3H), 4.55–4.75 (m, 4H), 5.40–5.50 (m, 1H), 5.90 (s, 1H), 6.18 (s, 1H), 6.22 (s, 1H), 6.70–6.80 (m, 3H).

3.32. (2*S*)-*N*-{4-[(2*S*)-*N*-(4,5-Dimethoxy-2-aminobenzoyl)-pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-*N*-propyl]oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (26b)

The compound **26b** was prepared according to the method described for the compound **25a** employing compound **24b** (909 mg, 1 mmol) to afford compound **26b** (620 mg, 73%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.45 (m, 12H), 1.70–2.45 (m, 6H), 2.60–2.90 (m, 8H), 2.95–3.25 (m, 2H), 3.45–3.70 (m, 4H), 3.80 (s, 6H), 3.85 (s, 3H), 4.0–4.20 (m, 3H), 4.55–4.75 (m, 4H), 5.40–5.50 (m, 1H), 5.90 (s, 1H), 6.18 (s, 1H), 6.22 (s, 1H), 6.70–6.80 (m, 3H).

3.33. 7-Methoxy-8-{2-[8-benzyloxy-7-methoxy-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo-[2,1-*c*][1,4]benzodiazepine-5-one-2-(carboxamidomethylidene)ethyl]oxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (6a)

A solution of **25a** (910 mg, 1 mmol), $HgCl_2$ (1.226 g, 4.52 mmol) and $CaCO_3$ (492 mg, 4.92 mmol) in acetonitrile–water (4:1) was stirred slowly at room temperature until TLC indicates complete loss of starting material. The reaction mixture was diluted with EtOAc (30 mL) and filtered through a Celite bed. The clear yellow organic supernatant was washed with saturated 5% $NaHCO_3$ (20 mL), brine (20 mL) and the combined organic phase is dried over Na_2SO_4. The organic solvent was evaporated in vacuum and purified by column chromatography (92% EtOAc–MeOH) to give compound **6a** (318 mg, 48%). This compound was repeatedly evaporated from chloroform in vacuum to generate the imine form. $[\alpha]_D^{25}$ +290.333 (*c* 0.5, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 2.03–2.16 (m, 3H), 2.25–2.35 (m, 3H), 3.15–3.82 (m, 6H), 3.85 (s, 3H), 3.90 (s, 3H), 4.20 (t, 2H,

$J = 4.8$ Hz), 4.40–4.50 (m, 2H), 5.20 (s, 2H), 5.88 (s, 1H), 6.60–6.65 (m, 1H), 6.80 (s, 1H), 6.85 (s, 1H), 7.25–7.75 (m, 7H), 7.65 (d, 2H, $J = 4.46$ Hz). MS (FAB) 664 $[M+H]^{+\cdot}$. Anal. Calcd for ($C_{37}H_{37}N_5O_7$): C, 66.96, H, 5.62, N, 10.55. Found: C, 66.85, H, 5.69, N, 10.32.

3.34. 7-Methoxy-8-{3-[8-benzyloxy-7-methoxy-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo-[2,1-*c*][1,4]benzodiazepine-5-one-2-(carboxamidomethylidene)propyl]oxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (6b)

The compound **6b** was prepared according to the method described for the compound **6a** employing **25b** (924 mg, 1 mmol) to give crude product, which was purified by column chromatography (90% EtOAc–MeOH) to afford compound **6b** (338 mg, 52%). $[\alpha]_D^{25}$ +454.0 (*c* 0.5, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 2.0–2.20 (m, 4H), 2.25–2.40 (m, 2H), 3.20 (d, 1H, $J = 4.8$ Hz), 3.50–3.90 (m, 7H), 3.95 (s, 6H), 4.10–4.30 (m, 2H), 4.40–4.50 (m, 2H), 5.20 (s, 2H), 5.89 (s, 1H), 6.80 (s, 1H), 6.85 (s, 1H), 6.88–6.92 (m, 1H), 7.30–7.50 (m, 5H), 7.52 (s, 1H), 7.55 (s, 1H), 7.66 (d, 2H, $J = 4.46$ Hz). MS (FAB) 678 $[M+H]^{+\cdot}$. Anal. Calcd for ($C_{38}H_{39}N_5O_7$): C, 67.34, H, 5.80, N, 10.33. Found: C, 67.42, H, 5.77, N, 10.21.

3.35. 7-Methoxy-8-{2-[7,8-dimethoxy-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]-benzodiazepine-5-one-2-(carboxamidomethylidene)-ethyl]oxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (7a)

The compound **7a** was prepared according to the method described for the compound **6a** employing **26a** (835 mg, 1 mmol) to give crude product, which was purified by column chromatography (87% EtOAc–MeOH) to afford compound **7a** (294 mg, 50%). $[\alpha]_D^{25}$ +430.417 (*c* 0.4, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 2.0–2.15 (m, 2H), 2.25–2.40 (m, 2H), 3.20–3.25 (m, 1H), 3.45–3.60 (m, 2H), 3.65–3.85 (s, 4H), 3.90–4.0 (m, 9H), 4.05–4.20 (m, 3H), 4.40–4.55 (m, 2H), 5.90 (s, 1H), 6.40–6.55 (m, 1H), 6.80 (s, 1H), 6.83 (s, 1H), 7.50 (s, 1H), 7.53 (s, 1H), 7.66 (d, 1H, $J = 4.46$ Hz), 7.70 (d, 1H, $J = 4.46$ Hz). MS (FAB) 588 $[M+H]^{+\cdot}$. Anal. Calcd for ($C_{31}H_{33}N_5O_7$): C, 63.36, H, 5.66, N, 11.92. Found: C, 63.45, H, 5.74, N, 11.83.

3.36. 7-Methoxy-8-{3-[7,8-dimethoxy-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]-benzodiazepine-5-one-2-(carboxamidomethylidene)-propyl]oxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (7b)

The compound **7b** was prepared according to the method described for the compound **6a** employing **26b** (849 mg, 1 mmol) to give crude product, which was purified by column chromatography (85% EtOAc–MeOH) to afford compound **7b** (331 mg, 55%). $[\alpha]_D^{25}$ +480.333 (*c* 0.4, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 2.0–2.15 (m, 4H), 2.25–2.40 (m, 2H), 3.10–3.25 (m, 1H), 3.50–3.85 (m, 6H), 3.90–4.0 (m, 9H), 4.05–4.30 (m, 3H), 4.35–4.55 (m, 2H), 5.90 (s, 1H), 6.80 (s, 1H), 6.83 (s, 1H), 6.91–6.93 (m, 1H), 7.50 (s, 1H), 7.54 (s, 1H), 7.66 (d, 1H, $J = 4.30$ Hz), 7.70 (d, 1H, $J = 3.80$ Hz). MS (FAB) 602 $[M+H]^{+\cdot}$. Anal. Calcd for ($C_{32}H_{35}N_5O_7$): C, 63.88, H, 5.86, N, 11.64. Found: C, 63.92, H, 5.94, N, 11.56.

3.37. Methyl-(2*S*)-*N*-[4-(*N*-*t*-butoxycarbonyl)aminoethoxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxylate (28a)

The compound **28a** was prepared according to the method described for the compound **11a** employing compound **27** (324 mg, 1 mmol) and Boc-protected bromo ethyl amine (269 mg, 1.2 mmol) to give crude product, which was purified by column chromatography (60% EtOAc–hexane) to afford compound **28a** (373 mg, 80%). ^{1}H NMR ($CDCl_3$): δ 1.40 (m, 9H), 1.85–2.15 (m, 4H), 2.20–2.40 (m, 1H), 3.15–3.22 (m, 1H), 3.23–3.38 (m, 1H), 3.60 (t, 2H, $J = 6.20$ Hz), 3.80 (s, 3H), 4.0 (s, 3H), 4.15 (t, 2H, $J = 5.98$ Hz), 4.65–4.78 (m, 1H), 5.0 (br s, 1H), 6.82 (s, 1H), 7.62 (s, 1H). MS (FAB) 468 $[M+H]^{+\cdot}$.

3.38. Methyl-(2*S*)-*N*-[4-(*N*-*t*-butoxycarbonyl)aminopropoxy-5-methoxy-2-nitrobenzoyl]-pyrrolidine-2-carboxylate (28b)

The compound **28b** was prepared according to the method described for the compound **11a** employing compound **27** (324 mg, 1 mmol) and Boc-protected bromo propyl amine (286 mg, 1.2 mmol) to give crude product, which was purified by column chromatography (65% EtOAc–hexane) to afford compound **28b** (395 mg, 82%). ^{1}H NMR ($CDCl_3$): δ 1.40 (m, 9H), 1.85–2.18 (m, 5H), 2.20–2.40 (m, 1H), 3.15–3.40 (m, 4H), 3.80 (s, 3H), 3.95 (s, 3H), 4.16 (t, 2H, $J = 5.96$ Hz), 4.65–4.72 (m, 1H), 5.22 (br s, 1H), 6.82 (s, 1H), 7.62 (s, 1H). MS (FAB) 482 $[M+H]^{+\cdot}$.

3.39. Methyl-(2*S*)-*N*-[4-(2-aminoethoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxylate (29a)

The compound **29a** was prepared according to the method described for the compound **14a** by employing **28a** (467 mg, 1 mmol) and TFA.

3.40. Methyl-(2*S*)-*N*-[4-(3-aminopropoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxylate (29b)

The compound **29b** was prepared according to the method described for the compound **14a** by employing **28b** (481 mg, 1 mmol) and TFA.

3.41. Methyl-(2*S*)-*N*-{4-[(2*S*)-*N*-(4-benzyloxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-*N*-ethyl]oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxylate (30a)

The compound **30a** was prepared according to the method described for the compound **23a** by employing

22a and **29a** (367 mg, 1 mmol) to give crude product, which was purified by column chromatography (90% EtOAc–hexane) to afford compound **30a** (582 mg, 65%). ^{1}H NMR ($CDCl_3$): δ 1.22–1.42 (m, 6H), 1.55–2.45 (m, 6H), 2.65–2.90 (m, 4H), 3.0–3.40 (m, 4H), 3.60 (t, 2H, $J = 6.2$ Hz), 3.80 (s, 3H), 4.0 (s, 6H), 4.15 (t, 2H, $J = 5.98$ Hz), 4.65–4.75 (m, 1H), 4.80 (d, 1H, $J = 4.4$ Hz), 5.20 (s, 2H), 5.35–5.40 (m, 1H), 5.92 (s, 1H), 6.55–6.60 (m, 1H), 6.75 (s, 1H), 6.85 (s, 1H), 7.30–7.50 (m, 5H), 7.60 (s, 1H), 7.75 (s, 1H). MS (FAB) 896 $[M+H]^{+\cdot}$.

3.42. Methyl-(2*S*)-*N*-{4-[(2*S*)-*N*-(4-benzyloxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-*N*-propyl]oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxylate (30b)

The compound **30b** was prepared according to the method described for the compound **23a** by employing **22a** and **29b** (381 mg, 1 mmol) to give crude product, which was purified by column chromatography (87% EtOAc–MeOH) to afford compound **30b** (609 mg, 67%). ^{1}H NMR ($CDCl_3$): δ 1.22–1.42 (m, 6H), 1.85–2.20 (m, 5H), 2.35–2.40 (m, 1H), 2.65–2.85 (m, 4H), 2.90–3.40 (m, 5H), 3.50–3.60 (m, 2H), 3.80 (s, 3H), 4.0 (s, 6H), 4.05–4.20 (m, 3H), 4.65–4.75 (m, 1H), 4.85 (d, 1H, $J = 4.38$ Hz), 5.20 (s, 2H), 5.30–5.40 (m, 1H), 5.92 (s, 1H), 6.55–6.60 (m, 1H), 6.75 (s, 1H), 6.85 (s, 1H), 7.30–7.50 (m, 5H), 7.60 (s, 1H), 7.75 (s, 1H). MS (FAB) 910 $[M+H]^{+\cdot}$.

3.43. 7-Methoxy-8-{2-[(2*S*)-*N*-(4-benzyloxy-5-methoxy-2-aminobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)-ethyl]oxy}-(11a*S*)-1,2,3,10,11,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]-benzodiazepin-5,11-dione (31a)

The compound **31a** was prepared according to the method described for the compound **25a** employing the compound **30a** (895 mg, 1 mmol) to afford the amino diethyl thioacetal **31a** (610 mg, 76%). ^{1}H NMR ($CDCl_3$): δ 1.15–1.40 (m, 6H), 1.60–2.10 (m, 6H), 2.50–2.80 (m, 4H), 2.95–3.20 (m, 2H), 3.35–3.60 (m, 4H), 3.75 (s, 3H), 3.82 (s, 3H), 4.0 (t, 2H, $J = 5.98$ Hz), 4.50–4.60 (m, 2H), 5.0 (s, 2H), 5.35–5.40 (m, 1H), 5.82 (s, 1H), 6.20 (s, 1H), 6.45–6.55 (m, 1H), 6.80 (s, 1H), 6.90 (s, 1H), 7.25–7.42 (m, 6H), 9.10 (br s, NH exchangeable).

3.44. 7-Methoxy-8-{3-[(2*S*)-*N*-(4-benzyloxy-5-methoxy-2-aminobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal-4-(carboxamidomethylidene)propyl]oxy}-(11a*S*)-1,2,3,10,11,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]-benzodiazepin-5,11-dione (31b)

The compound **31b** was prepared according to the method described for the compound **25a** employing the compound **30b** (895 mg, 1 mmol) to afford the amino diethyl thioacetal **31b** (629 mg, 78%). ^{1}H NMR ($CDCl_3$): δ 1.15–1.40 (m, 6H), 1.85–2.10 (m, 6H), 2.50–2.80 (m, 4H), 2.95–3.20 (m, 3H), 3.35–3.60 (m, 4H), 3.75 (s, 3H), 3.85 (s, 3H), 3.90–4.0 (m, 3H), 4.45–4.60 (m, 2H), 5.0 (s, 2H), 5.30–5.40 (m, 1H), 5.82 (s, 1H), 6.20 (s, 1H), 6.45 (s, 1H), 6.55–6.65 (m, 1H), 6.80 (s, 1H), 7.25–7.40 (m, 6H), 8.60 (br s, NH exchangeable).

3.45. 7-Methoxy-8-{2-[8-benzyloxy-7-methoxy-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo-[2,1-*c*][1,4]benzodiazepine-5-one-2-(carboxamidomethylidene)ethyl]oxy}-(11a*S*)-1,2,3,10,11,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5,11-dione (8a)

The compound **8a** was prepared according to the method described for the compound **6a** employing **31a** (803 mg, 1 mmol), $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) to give crude product, which was purified by column chromatography (87% EtOAc–MeOH) to afford compound **8a** (346 mg, 51%). $[\alpha]_D^{25}$ +352.667 (*c* 0.5, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.90–2.20 (m, 4H), 2.75–3.80 (m, 8H), 3.85 (s, 3H), 3.95 (s, 3H), 4.0 (d, 2H, $J = 4.8$ Hz), 4.35–4.55 (m, 2H), 5.20 (s, 2H), 5.90 (s, 1H), 6.55 (s, 1H), 6.80 (s, 1H), 6.95–7.0 (m, 1H), 7.20–7.50 (m, 6H),7.55 (s, 1H), 7.66 (d, 1H, $J = 4.40$ Hz), 8.7 (br s, NH exchangeable). MS (FAB) 680 $[M+H]^{+\cdot}$. Anal. Calcd for ($C_{37}H_{37}N_5O_8$): C, 65.38, H, 5.49, N, 10.30. Found: C, 65.23, H, 5.53, N, 10.35.

3.46. 7-Methoxy-8-{3-[8-benzyloxy-7-methoxy-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo-[2,1-*c*][1,4]benzodiazepine-5-one-2-(carboxamidomethylidene)propyl]oxy}-(11a*S*)-1,2,3,10,11,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]-benzodiazepin-5,11-dione (8b)

The compound **8b** was prepared according to the method described for the compound **8a** by employing **31b** (817 mg, 1 mmol) to give crude product, which was purified by column chromatography (87% EtOAc–MeOH) to afford compound **8b** (368 mg, 53%). $[\alpha]_D^{25}$ +410.256 (*c* 0.325, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.90–2.20 (m, 6H), 2.75–3.80 (m, 8H), 3.85 (s, 3H), 3.95 (s, 3H), 4.0 (d, 2H, $J = 4.6$ Hz), 4.35–4.50 (m, 2H), 5.20 (s, 2H), 5.90 (s, 1H), 6.60 (s, 1H), 6.65–6.70 (m, 1H), 6.85 (s, 1H), 7.20–7.50 (m, 6H), 7.55 (s, 1H), 7.65 (d, 1H, $J = 4.40$ Hz), 8.82 (br s, NH exchangeable). MS (FAB) 694 $[M+H]^{+\cdot}$. Anal. Calcd for ($C_{38}H_{39}N_5O_8$): C, 65.79, H, 5.67, N, 10.09. Found: C, 65.84, H, 5.60, N, 10.03.

3.47. Thermal denaturation studies

Compounds were subjected to thermal denaturation studies with duplex-form calf thymus DNA (CT-DNA) using an adaptation of a reported procedure.[19] 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 MeOH to obtain a fixed [PBD]/[DNA] molar ratio of 1:5. The DNA–PBD solutions were incubated at 37 °C for 0, 18, 36 and 48 h prior to analysis. Samples were monitored at 260 nm using a Beckman DU-7400 spectrophotometer fitted with high performance tem-

perature 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 $(dA_{260})/dT$ derivative plots. Results are given as the mean ± standard deviation from three determinations and are corrected for the effects of MeOH co-solvent using a linear correction term.[20] Drug-induced alterations in DNA melting behaviour are given by: $\Delta T_m = T_m(\text{DNA} + \text{PBD}) - T_m$ (DNA alone), where the T_m value for the PBD-free CT-DNA is 69.0 ± 0.01. The fixed [PBD]/[DNA] ratio used did not result in binding saturation of the host DNA duplex for any compound examined.

3.48. In vitro evaluation of cytotoxic activity

In routine screening, compounds have been tested against 60 human tumour cell lines derived from nine cancer types (leukaemia, nonsmall cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer). For each compound, dose response curves for each cell line were measured at a minimum of five concentrations at 10-fold dilutions. A protocol of 48 h continuous drug exposure was used, and a sulforhodamine B (SRB) protein assay was used to estimate cell viability or growth. The concentration causing 50% cell growth inhibition (GI_{50}), total cell growth inhibition (TGI, 0% growth) and 50% cell death (LC_{50}, −50% growth) compared with the control was calculated.

3.49. Restriction endonuclease inhibition

Stock solutions of each PBD (100 μM) were prepared by dissolving each compound in DMSO (Sigma). These were stored at −20 °C. Plasmid (pBR322) containing single *Bam*H1 site was used in this assay. Restriction endonuclease and the relevant buffer were obtained from NEB. The DNA fragment (500 ng) was incubated with each PBD (see Fig. 2 for PBD concentrations) in a final volume of 16 μL for 16 h at 37 °C. Next 10 × *Bam*H1 buffer (2 μL) was added, and the reaction mixture was made up to 20 μL with *Bam*H1 (20 units) and then incubated for 1 h at 37 °C. Then loaded on to a 1% agarose gel electrophoresis in Tris–acetate EDTA buffer at 80 V for 2 h. The gels were stained with ethidium bromide and photographed.

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