Two additional amino acids, other than proline, were also incorporated into **1** (region D). As shown in Scheme 6, 6-methoxytryptophan **61** was transformed into the cyclic dipeptides **65** and **66** with L-valine and L-phenylalanine, respectively, under conditions illustrated in Scheme 6. The 2-isoprenyl indole **20** was also transformed to analogues **67** and **68**, as shown in Scheme 7.

The synthesis of C-6 substituted analogues of tryprostatin A modified in region A is depicted in Scheme 7. The synthesis began with a highly regioselective process for nitration of **2**. Although several methods were attempted to incorporate the nitro group at the desired 6-position with only minimal success, this was successfully carried out when **2** was treated with $NaNO_2$ in the presence of TFA[49] at low temperature to afford **69**. To determine the regiochemistry, detailed NMR analysis of the 6-nitro analogue **69** was carried out. The coupling patterns of the aromatic ring protons could be employed to distinguish the 4- and 7-substituted indoles from the 5- and 6-substituted regioisomers, since one would not expect singlet protons in the spectrum of the 4- or 7-substituted indoles. The ^1H NMR spectrum of **69** clearly contained one singlet (8.29 ppm) corresponding to one proton in the aromatic region, consequently, the product of this mononitration was either the 5- or 6-substituted regioisomer. In case of the 5-nitrosubstituted indole this singlet would correspond to the proton at C(4), whereas in the case of 6-nitrosubstituted indole this singlet would correspond to the proton at C(7). The 6-nitro regioisomer would be expected to exhibit a much stronger NOE signal between the indole N(H) proton and the proton at C(7) than the one between the indole N(H) proton and the proton at C(4). A strong NOE signal was observed between this proton singlet and the indole N(H) and vice versa. This further ruled out the 5-nitro regioisomer. Reduction of the nitro group in **69** (Scheme 7) with hydrazine in the presence of $FeCl_3.H_2O$ and activated carbon in refluxing methanol[45] furnished analogue **70** which was purified by column chromatography and stored as the hydrochloride salt. Amine **70** was stirred with thiophosgene in dry chloroform to afford the 6-isothiocyanate analogue **71** in high yield. Treatment of amine **70** with triflyl azide (TfN_3) in the presence of copper sulfate afforded analogue **72** in 89% yield.[50,51]

3. Biological evaluation and discussion

3.1. Effects of analogues 1–8 on topoisomerase II

Tryprostatins **1–8** were evaluated as inhibitors of topoisomerase II in the topoisomerase II-mediated DNA relaxation assay.[52,53] This assay measures the ability of the compound to inhibit the ability of topoisomerase II to relax supercoiled DNA. The inhibitory activities against topoisomerase II of compounds **1–8** were evaluated by agarose gel electrophoresis experiments. The photopicture of **1–4**'s agarose gel electrophoresis experiment is presented in Figure 2. The agent, *m*-AMSA, a known inhibitor of topoisomerase II, was employed as the control (lane 3). The other controls employed were no-enzyme (lane 1), enzyme (lane 2), and 1% DMSO (lane 4). The gels were analyzed qualitatively by examination of the presence of DNA bands that migrate farther down on the gel than the negative controls. Topoisomerase II-mediated relaxation of the DNA prevents the band from migrating down the gel as far as one that is still in a supercoiled form. Therefore, DNA incubated with topoisomerse II inhibitors will migrate farther on the gel than the no-enzyme or DMSO controls. Lane 1 is DNA alone, existing in two forms- supercoiled DNA and loosened DNA; lane 2 is topoisomerase II together with DNA, and supercoiled DNA was relaxed by topoisomerase II completely. As illustrated in Figure 2, **1** (lane 5) and **2** (lane 7) are both weak inhibitors of topoisomerase II; however, the potency cannot be determined from this data. The laddering is evidence of inhibition of topoisomerase II. The enantiomers of tryprostatin A **3** (lane 6) and B **4** (lane 8) were both inactive. The four diastereomers **5–8** (data not shown) were also found to be inactive as topoisomerase II inhibitors in this assay. Tryprostatin A **1** and B **2** are, therefore, weak inhibitors of topoisomerase II but their enantiomers (**3** and **4**) and diastereomers (**5–8**) are not.

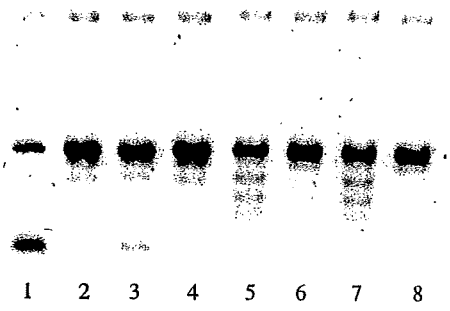


Figure 2. Representative agarose gel from the topoisomerase II-mediated DNA relaxation assay. Data for all compounds are not shown. Lane 1: DNA only; lane 2: DNA + topoisomerase II; lane 3: DNA + topoisomerase II + *m*-AMSA (100 μM); lane 4: DNA + topoisomerase II + 1% DMSO; lane 5: DNA + topoisomerase II + **1** (100 μM); lane 6: DNA + topoisomerase II + **3** (100 μM); lane 7: DNA + topoisomerase II + **2** (100 μM); lane 8: DNA + topoisomerase II + **4** (100 μM).

3.2. Effects of analogues 1–8 on tubulin polymerization

Tryprostatins **1–8** were also evaluated as inhibitors of tubulin polymerization.[13,54] Purified tubulin, containing MAPs and GTP, was incubated at 37 °C with either DMSO (as a solvent control), colchicine (standard), or analogues **1–8** and the change in absorbance was measured at 351 nM over 10 min. The concentration of the standard (colchicine) and analogues (**1–8**) was varied for different runs to obtain a delta absorbance versus concentration curve. Illustrated in Figures 3 and 4 are the results of the tubulin polymerization assay. Colchicine (the positive control) strongly suppressed tubulin

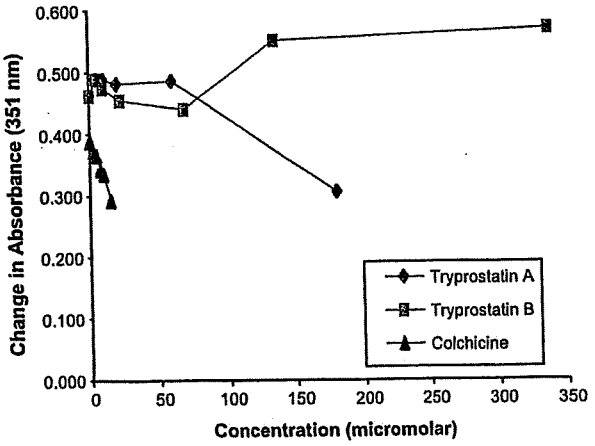


Figure 3. Inhibition of tubulin polymerization by tryprostatin A **1** and B **2**, colchicine, a known tubulin polymerization inhibitor, was used as a control.

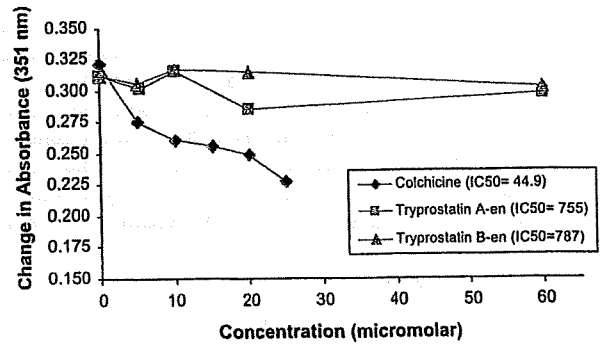


Figure 4. Inhibition of tubulin polymerization by analogues **3** and **4**. Colchicine was employed as a control.

assembly (IC_{50} = 44.9 μM), while **1** (Fig. 3) caused a moderate reduction in the rate of tubulin polymerization (IC_{50} = 250 μM). Tryprostatin B **2** (Fig. 3) as well as the enantiomers of tryprostatin A **3** and B **4** (Fig. 4) were inactive in this assay (IC_{50s} > 700 μM). Compounds **5–8** (data not shown) were also found to be inactive in this assay (less than 2% inhibition at 60 μM: IC_{50s} > 700 μM).

Osada et al.[13] recently reported that tryprostatin A **1** was a novel inhibitor of MAP-dependent microtubule assembly and through disruption of the microtubule spindle, specifically inhibited cell cycle progression at the M phase. Thus biological evaluation of analogues **1–8** illustrated above indicated that **1** was a weak inhibitor of both topoisomerase II and tubulin polymerization, whereas **2** was only a weak inhibitor of topoisomerase II. The enantiomers (**3** and **4**) and diastereomers (**5–8**) were inactive in both the tubulin polymerization assay and topoisomerase II-mediated DNA relaxation assay. In terms of the stereochemistry of the amino acids present in the diketopiperazine ring, biological evaluation indicated that ligands with the absolute configuration L-Tyr-L-Pro (natural stereochemistry as in **1** and **2**) were essential for inhibition of tubulin polymerization and/or topoisomerase II. Modification of the absolute configuration of the diketopiperazine ring from L-Tyr-L-Pro (**1** and **2**) to D-Tyr-D-Pro (**3** and **4**), D-Tyr-L-Pro (**5** and **6**), and L-Tyr-D-Pro (**7** and **8**) resulted in analogues that were very poor inhibitors of topoisomerase II and/or tubulin polymerization. Additionally, comparisons of analogues **1** and **2** indicated that presence of the 6-methoxy substituent in **1** resulted in analogues that are dual inhibitors of microtubule assembly and topoisomerase II.

In order to determine whether the absolute configuration of L-Tyr and/or L-Pro in the diketopiperazine ring was required to inhibit cell proliferation, analogues **1–8** were evaluated as inhibitors of three human lung (H520), breast (MCF-7), and prostrate (PC-3) cancer cell lines (Table 1).[12] Analogues **1–7** were not potent inhibitors (GI_{50} > 100 μM) of the growth of tumor cells in the three human cancer cell lines evaluated. However, the diastereomer **8** of tryprostatin B **2** exhibited potent cytotoxic activity at 100 μM against all three human cancer cell lines evaluated. It was found, in agreement with Danishefsky et al.,[55] that the inhibition of tryprostatin B **2** against the growth of the three human cancer cell lines evaluated occurred at higher concentrations (GI_{50} > 100 μM) than that reported earlier[6–8] for isolated tryprostatin B **2**. Danishefsky et al.[55] have shed some light on the apparent discrepancies in the cytotoxicity of isolated **2** versus synthetic **2**. Their studies[55]

Table 1. Cell growth inhibition of tryprostatins **1–8** (at 10, 100 μM) on human lung (H520), breast (MCF-7) and prostate (PC-3) cancer cell lines[a]

Compound	Percent cell survival[b]					
	H520		MCF-7		PC-3	
	10 μM	100 μM	10 μM	100 μM	10 μM	100 μM
1	80.1 ± 4.1	79.4 ± 4.2	>100	95.0 ± 4.7	99.2 ± 4.2	95.6 ± 5.0
2	77.6 ± 3.6	60.5 ± 3.5	88.2 ± 5.8	66.7 ± 5.3	95.5 ± 2.8	68.9 ± 6.6
3	81.7 ± 3.9	75.2 ± 3.5	>100	>100	>100	83.7 ± 4.2
4	>100	99.8 ± 1.6	>100	>100	95.8 ± 1.3	78.9 ± 2.1
5	>100	>100	>100	>100	>100	>100
6	>100	76.5 ± 11.2	>100	>100	97.3 ± 5.9	68.5 ± 3.4
7	99.3 ± 1.8	98.5 ± 3.1	>100	99.0 ± 4.6	>100	>100
8	88.3 ± 8.4	0.1 ± 0.1	73.6 ± 5.3	0.0 ± 0.0	59.3 ± 3.9	0.2 ± 0.0

[a] Table 1 published in its entirety in Ref. 12.

[b] CellTiter 96™ AQ_{ueous} non-radioactive cell proliferation assay (Promega) was used to determine growth inhibition. Percent inhibition values were calculated versus control wells and were done in quadruplicate. Control wells contained 0.2% DMSO and the positive control was either etoposide or *m*-AMSA (20 μM, 20 μM). Values are reported ± the standard deviation of the mean.

Table 2. Growth inhibition (GI_{50}) in μM of human cancer cell lines by 8 and etoposide

Compound	H520	MCF-7	PC-3
8[a]	15.8	15.9	11.9
8	11.9	17.0	12.3
Etoposide	8.7	55.6	11.1

[a] Data were obtained from NCI.

indicated that a DMSO solution of **2**, upon standing in air, undergoes slow transformation to a mixture of products. The solutions of **2** containing detectable byproducts were considerably more cytotoxic (ca. 50-fold) than those containing apparently homogenous tryprostatin. Growth inhibitory (GI_{50}) potency of **8** was also compared to that of etoposide against the growth of the three human lung (H520), breast (MCF-7), and prostrate (PC-3) cancer cell lines (Table 2).[12] Outlined in Table 2 are the results obtained from the National Cancer Institute (NCI)[56] screening of analogue **8** on the same three human cancer cell lines. The data obtained from the NCI for **8** were in complete agreement with the data obtained for **8** in the present study against all three human cancer cell lines evaluated. Analogue **8** was 3-fold more potent than etoposide in inhibition of the growth of the MCF-7 human cancer cell line. Also, analogue **8** was equipotent with etoposide against the growth of H520 and PC-3 human cancer cell lines.

If one examines the structures of tryprostatins (**1–7**) and compares them with that of the active analogue **8**, one can generate the following conclusion: the L-Tyr unit in the diketopiperazine ring was essential for potent tumor cell growth inhibition since none of the other tryprostatins (**3–6**), which contained the D-Tyr unit, exhibited activity. Biological evaluation of analogue **8** also indicated that the inhibition of the growth of human cancer cells by analogue **8** was not due to the inhibition of topoisomerase II or tubulin polymerization since analogue **8** was inactive against these two molecular targets. Further studies to identify the precise molecular targets are required. The presence of the 6-methoxy group on **7** compared to **8** nearly eliminated the potent tumor cell growth inhibitory activity against the three human cancer cell lines evaluated. The potent cytotoxic activity of analogue **8** against human cancer cells led to the evaluation of its activity against the growth of normal human cell lines. In preliminary studies, **8** was found to be cytotoxic to normal human cell lines; however, further studies are required in this regard.

Analogues **1–8** were selected by the NCI for evaluation in its in vitro preclinical antitumor screening program. The ability of compounds **1–8** to inhibit the growth of tumor cells was measured as GI_{50} values, the concentration required to inhibit the growth of tumor cells in culture by 50%, as compared to a control (Table 3). In two of the 60 tumor cell lines evaluated, tryprostatin A **1** showed GI_{50} values of $\leqslant 10^{-5}$ M. Again, tryprostatin B

Table 3. Cytotoxicity evaluation (GI_{50}, μM) of compounds **1–8** against selected tumor cell lines[56]

Cell line	1	2	3	4	5	6	7	8
Leukemia								
CCRF-CEM	>100	>100	>25	25.1	11.9	22.2	99.4	3.22
HL-60 (TB)	11.3	>100	>25	29.0	35.0	55.7	≥100	22.4
K-562	2.73	56.1	>25	25.8	17.7	31.8	>100	20.1
MOLT-4	ND	>100	>25	21.0	12.2	34.9	>100	5.96
RPMI-8226	37.1	>100	>25	12.9	7.76	18.6	92.0	5.54
SR	5.68	50.6	>25	12.1	11.3	25.4	76.9	9.46
Non-small cell lung cancer								
HOP-92	21.2	43.1	23.1	ND	ND	2.94	16.8	1.70
EKVX	>100	>100	>25	39.4	>50	20.7	>100	6.90
Colon cancer								
COLO 205	>100	>100	>25	21.2	12.4	39.3	>100	17.7
HT-29	ND	ND	ND	37.5	16.1	ND	40.3	5.25
Melanoma								
LOX IMVI	>100	>100	>25	>50	33.5	17.8	>100	9.23
Ovarian								
OVCAR-3	>100	>100	>25	38.2	>50	28.7	85.5	9.69
IGROV1	90.5	>100	>25	16.0	>50	32.3	50.0	11.5
Prostrate cancer								
PC-3	94.0	>100	>25	21.2	21.7	24.8	>100	11.9
DU-145	>100	>100	>25	>50	40.0	59.5	>100	14.0
Breast cancer								
MDA-MB-231/ATCC	>100	>100	>25	11.9	13.0	49.6	>100	14.9
BT-549	79.7	58.2	>25	7.21	9.77	26.9	>100	13.2
MCF-7	>100	>100	>25	40.0	25.5	25.9	>100	15.9
Renal cancer								
UO-31	>100	>100	>25	12.6	27.3	45.7	>100	14.7

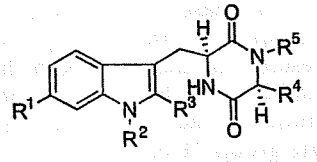
2 (GI_{50} > 40 μM) was considerably less active against the growth of the 60 tumor cell lines evaluated. In 9 of the 60 tumor cell lines evaluated, the most active analogue **8** showed GI_{50} values of $\leqslant 10^{-5}$ M. Analogue **5**, the diastereomer of tryprostatin A, **1** was more active than **1** in the inhibition of the growth of tumor cells in most of the tumor cell lines evaluated. Analogues **3** and **7** were both considerably less active than **1** in inhibition of the growth of the tumor cells in the NCI screening program. However, analogue **4**, the enantiomer of tryprostatin B **2**, as well as both of the diastereomers **6** and **8** were more active than **2** in inhibition of the growth of tumor cells in most of the tumor cell lines evaluated. It is noteworthy that compounds **4**, **5**, **6** and **8** were not general cell toxins but showed selectivity both within a type of tumor cell line and across different tumor cell lines, with inhibitory values, which in some instances, differed by 100-fold.

3.3. Structure–activity relationships of tryprostatin A analogues

Because **1** was an inhibitor of BCRP, the tryprostatin A-related analogues (**37–40**, **46**, **47**, **55**, **56**, **60**, and **62–72**) were evaluated in vitro for the ability to disrupt the cell cycle and to inhibit tsFT210 cell proliferation.[4,13,58] The inhibitory potency (IC_{50}) values are listed in Table 4 and compared with tryprostatin A **1**.

A 30 μM concentration of **1** arrested cell cycle progression in the M phase, as previously reported.[8,13] Many of these analogues were found to have similar activity as tryprostatin A against tsFT210 cell proliferation. Analogue **38**, which closely resembled tryprostatin A, was inactive. Substitution of the 2-isoprenyl moiety in **38** with a smaller methyl substituent (**37**) also resulted in an inactive analogue. Replacement of the N_a-isoprenyl group in analogue **38** with an allyl group (**40**) resulted in an analogue that was equipotent to **1** in the inhibition of cell proliferation. Similarly, replacement of the N_a-isoprenyl group in **38** with a N_a-benzyl group **39** also resulted in an analogue that was equipotent to **1** in inhibiting cell proliferation. However, analogues **39** and **40** inhibited cell cycle progression at the G1 phase. The biological data of analogues **39** and **40** indicated substitution of the indole N(H) with a benzyl moiety or allyl moiety was highly conducive for inhibition of cell proliferation and caused cell cycle arrest in the G1 phase. Tryprostatin A **1** analogues in which the indole NH was substituted with a benzyl moiety **46** or allyl moiety **47** also afforded active analogues that were equipotent with **1** in inhibition of the growth of tsFT210 cells. However, this inhibition was not cell cycle dependent. Removal of the 2-isoprenyl group in **1** afforded analogue **62** which was inactive. Similarly, removal of the 2-isoprenyl group in **1** and substitution of it with a bromine atom **63** or chlorine atom **64** also resulted in

Table 4. Effect of tryprostatin A-related analogues on cell cycle progression and tsFT210 cell proliferation



Compound	R^1	R^2	R^3	R^4	R^5	IC_{50} (μM)[a]	Effect on cell cycle arrest[b]
1	OMe	H	Isoprenyl	$-(CH_2)_3-$		68	M phase at 30 μM
37	OMe	Me	H	$-(CH_2)_3-$		>100	No effect[c]
38	OMe	Isoprenyl	H	$-(CH_2)_3-$		>100	No effect
39	OMe	Benzyl	H	$-(CH_2)_3-$		46	G1 phase at 100 μM
40	OMe	Allyl	H	$-(CH_2)_3-$		62	G1 phase at 100 μM
46	OMe	Benzyl	Isoprenyl	$-(CH_2)_3-$		55	No effect
47	OMe	Allyl	Isoprenyl	$-(CH_2)_3-$		75	No effect
55	OMe	H	Benzyl	$-(CH_2)_3-$		>100	No effect
56	OMe	H	Allyl	$-(CH_2)_3-$		60	G1, G2/M phase at 100 μM
60	OMe	H		$-(CH_2)_3-$		>100	No effect
62	OMe	H	H	$-(CH_2)_3-$		>100	No effect
63	OMe	H	Br	$-(CH_2)_3-$		96	No effect
64	OMe	H	Cl	$-(CH_2)_3-$		>100	No effect
65	OMe	H	H	Isopropyl	H	>100	No effect
66	OMe	H	H	Benzyl	H	100	M phase at 100 μM
67	OMe	H	Isoprenyl	Isopropyl	H	19	G1, G2/M phase at 100 μM
68	OMe	H	Isoprenyl	Benzyl	H	10	No effect
69	NO_2	H	Isoprenyl	$-(CH_2)_3-$		>100	No effect
70	NH_2	H	Isoprenyl	$-(CH_2)_3-$		>100	No effect
71	NCS	H	Isoprenyl	$-(CH_2)_3-$		50	G1, G2/M phase at 100 μM
72	N_3	H	Isoprenyl	$-(CH_2)_3-$		60	G1, G2/M phase at 100 μM

[a] Exponentially growing tsFT210 cells were treated with test compounds at 32 °C for 48 h. Cell viability was measured using the color reagent, WST-8™.

[b] Exponentially growing tsFT210 cells were treated with test compounds at 32 °C for 18 h. Then, flow cytometric analysis and nuclei staining were carried out, as described in Section 5.

[c] No effect even at 100 μM.

inactive analogues. Comparison of the analogues **62–64** with the activity of the active analogue **1** indicated the lipophilic 2-isoprenyl group in **1** played an important role in the inhibition of cell proliferation. The lipophilic 2-isoprenyl moiety may play an important role in the interaction with the molecular target and/or may increase the lipophilicity of the molecule thereby facilitating passive diffusion into the cells. Substitution of the 2-isoprenyl group of **1** with a 2-benzyl group **55** or 2-methyl acrylate moiety **60** afforded inactive analogues. However, a 2-allyl substituted analogue **56** of **1** was found to be equipotent to **1** in the inhibition of cell proliferation. Analogue **56** also arrested cell cycle progression at the G1, G2/M phase. Substitution of the L-proline residue in the diketopiperazine ring of **1** with an L-valine residue (**67**) afforded a 3.5-fold more potent inhibitor of the growth of tsFT210 cells than **1**. Similarly replacement of the L-proline residue in **1** with an L-phenyl alanine residue (**68**) resulted in an analogue that was 7-fold more potent than **1** in the inhibition of the growth of tsFT210 cells, but this inhibition was not cell cycle dependent. The biological data of analogues **67** and **68** indicated substitution of the L-proline residue in the diketopiperazine ring of **1** with other L-amino acids was highly conducive for inhibition of cell proliferation. Removal of the 2-isoprenyl group from analogue **67** afforded **65** which slightly inhibited (IC_{50} > 100 μM) cell proliferation. Again, removal of the 2-isoprenyl moiety from analogue **68** afforded analogue **66** which was 10-fold less potent than **68** in the cell proliferation assay again indicating the importance of the 2-isoprenyl moiety in the inhibition of cell proliferation. Analogue **66** also arrested cell cycle progression in the M phase at 100 μM. Replacement of the 6-methoxy group in **1** with a nitro group (**69**) or amino group (**70**) resulted in analogues that were poor (IC_{50} > 100 μM) inhibitors of the growth of tsFT210 cells. However, substitution of the 6-methoxy group in **1** with an isothiocyanate group **71** or azide group **72** resulted in analogues that were equipotent with **1** in the inhibition of the growth of tsFT210 cells. Both compounds **71** and **72** inhibited the cell cycle progression of tsFT210 cells at the G1, G2/M phase.

Turner and Sullivan et al.[57] have recently shown that tryprostatin A **1** is a specific and potent inhibitor of BCRP (breast cancer resistance protein), which further indicates the potential of analogues of tryprostatin A **1** synthesized in the present study in the potential inhibition of BCRP. Some of the analogues are currently being evaluated as inhibitors of BCRP and will be a topic of future communication. The isothiocyanate analogue **71** and the 6-azido analogue **72** may be excellent irreversible inhibitors in studies of BCRP.

4. Conclusion

In summary, the first structure–activity investigation into the cell cycle inhibitory effects of the tryprostatin A **1** analogues has been carried out. The SAR of tryprostatin A **1** suggests that the search for a potent and selective antitumor agent, in the tryprostatin series, still looks promising. Studies on elucidation of the mechanism of action of the tryprostatins indicate that tryprostatin A **1** is a weak inhibitor of topoisomerase II and tubulin polymerization, whereas tryprostatin B **2** is only a weak inhibitor of topoisomerase II. The absolute configuration of L-Tyr-L-pro in the diketopiperazine ring of the tryprostatins was shown to be essential for inhibition of tubulin polymerization and/or topoisomerase II. The 6-methoxy substituent in **1** was shown to promote inhibition of both topoisomerase II and tubulin polymerization in in vitro assays. Biological evaluation indicated that the presence of the 2-isoprenyl moiety on the indole scaffold in **1** was essential for inhibition of cell proliferation. Removal of the 2-isoprenyl group in **1** and substitution of the indole NH with a benzyl group or allyl group also afforded analogues that inhibited cell proliferation. The 6-methoxy substituent in **1** could be replaced with various groups to afford active analogues. Various L-amino acids other than L-proline could be incorporated into the diketopiperazine ring of **1** to afford active analogues. The nature of the substituent present on the indole NH or at the C-2 position influenced the mechanism of action of the analogue and highlights the versatility of the tryprostatin skeleton as a template for drug discovery. Analogue **8** was more potent than etoposide (a clinically used anticancer drug) against the three human cancer cell lines evaluated. However, preliminary biological evaluation against normal cells indicated that it was toxic which may limit its potential use in this regard. More work is required to define this. In the NCI preclinical screening program analogue **5**, the diastereomer of tryprostatin A **1**, was more active than **1** in the inhibition of growth of tumor cells in most of the tumor cell lines evaluated. Similarly, analogue **4**, the enantiomer of tryprostatin B **2** as well as both the diastereomers **6** and **8**, were more active than **2** in inhibition of the growth of tumor cells in most of the tumor cell lines evaluated.

5. Experimental

All melting points were determined using a Thomas-Hoover capillary melting point apparatus or an Electrothermal model IA8100 digital melting point apparatus and are uncorrected. Reagents and starting materials were obtained from commercial suppliers and used without further purification unless otherwise indicated. Unless specified otherwise, solvents were freshly distilled prior to use: tetrahydrofuran (THF), benzene, toluene, dioxane, and diethyl ether were distilled under nitrogen from sodium metal utilizing benzophenone as an indicator; MeOH and EtOH were distilled over Mg metal and I_2; dichloromethane was dried over $MgSO_4$ and then distilled over P_2O_5; triethylamine was dried over KOH and then distilled over KOH. Flash column chromatography was carried out on silica gel purchased from E. M. Laboratories (grade 60). HPLC grade solvents were used for all chromatography. Analytical thin-layer chromatography (TLC) was conducted on precoated plates: silica gel 60 F-254, 0.25 mm thickness, manufactured by E. Merck & Co., Germany. Indoles were visualized with Dragendorf's reagent or a saturated solution of ceric ammonium sulfate in 50% H_2SO_4. Ketones or aldehydes

were visualized with an aqueous solution of 2,4-dinitrophenylhydrazine in 30% H_2SO_4. The ^1H NMR spectra were recorded on a Bruker 300-MHz or 500-MHz multiple-probe instrument. Infrared spectra were recorded on a Nicolet Dx FTIR DX V5.07 spectrometer or a Perkin Elmer 1600 Series FT-IR spectrometer. Low resolution mass spectral data (EI/CI) were obtained on a Hewlett-Packard 5985B gas chromatography-mass spectrometer. High resolution mass spectral data were taken on a VG autospectrometer (Double Focusing High Resolution GC/Mass Spectrometer, UK). Optical rotations were measured on a JASCO DIP-370 polarimeter. Microanalyses were performed on a CE Elantech EA1110 elemental analyzer.

5.1. (3*S*,6*R*)-3-[(1-*tert*-Butyloxycarbonyl-6-methoxy)-3-indoyl]methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (12)

To a solution of **10** (3.41 g, 16.1 mmol) in dry THF (60 mL) under nitrogen, *n*-BuLi (2.5 M, 7.08 mL, 17.7 mmol) was added dropwise at −78 °C. The solution which resulted was stirred at −78 °C for 30 min and treated slowly with a solution of crude 3-bromo-methylindole **9a** (4.79 g, 14.1 mmol) in THF (30 mL). The mixture which resulted was stirred at −78 °C for 20 h, and then allowed to slowly warm to rt. The solution was concentrated under reduced pressure and diluted with a saturated aqueous solution of $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with brine (30 mL) and dried (K_2CO_3). After removal of solvent under reduced pressure, the residue was purified by flash chromatography (silica gel, hexane/EtOAc, 10:1) to afford **12** as an oil (6.04 g, 91%): $[\alpha]_D^{27}$ +24.7° (*c* 0.9, $CHCl_3$); IR ν_{max} (NaCl) 2970, 1730, 1690 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 0.63 (d, 3H, J = 6.8 Hz), 0.92 (d, 3H, J = 6.9 Hz), 1.21 (t, 3H, J = 7.1 Hz), 1.29 (t, 3H, J = 7.1 Hz), 1.62 (s, 9H), 2.15 (m, 1H), 3.13 (d, 2H, J = 4.8 Hz), 3.53 (t, 1H, J = 3.4 Hz), 3.84 (s, 3H), 3.94–4.16 (m, 4H), 4.25 (dd, 1H, J = 3.8 Hz), 6.80 (dd, 1H, J = 2.2 and 8.6 Hz), 7.21 (s, 1H), 7.42 (d, 1H, J = 8.6 Hz), 7.67 (br, s, 1H). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.43, 16.68, 19.04, 28.23, 29.37, 31.72, 55.58, 56.13, 60.42, 60.51, 60.67, 82.93, 99.03, 111.49, 116.74, 120.10, 122.83, 125.28, 136.10, 149.81, 157.67, 162.29, 163.49. EIMS *m/e* (relative intensity) 471 (M^+, 47), 261 (21), 212 (100), 169 (67), 141 (20), 57 (51). Anal. Calcd for ($C_{26}H_{37}N_3O_5$) C, H, N. This material was used directly in a later step.

5.2. (3*S*,6*R*)-3-(1-*tert*-Butyloxycarbonyl-3-indoyl)methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (13)

Indole **13** (6.7 g) was prepared in 92% yield from **9b** (5.1 g, 16.4 mmol) and **10** (3.7 g, 17.6 mmol) analogous to the procedure described for the synthesis of **12**. **13**: ^1H NMR (300 MHz, $CDCl_3$) δ 0.66 (d, 3H, J = 6.8, Hz), 0.95 (d, 3H, J = 6.9 Hz), 1.23 (t, 3H, J = 7.1 Hz), 1.32 (t, 3H, J = 7.1 Hz), 1.64 (s, 9H), 2.18 (m,1H), 3.19 (m, 2H), 3.56 (t, 1H, J = 3.4 Hz), 3.96–4.21 (m, 4H), 4.29 (dd, 1H, J = 4.3 Hz), 7.15–7.29 (m, 2H), 7.36 (s, 1H), 7.59 (d, 1H, J = 7.5 Hz), 8.08 (d, 1H, J = 7.6 Hz). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.40, 16.66, 19.01, 28.21, 29.26, 31.60, 31.73, 56.05, 60.56, 60.64, 83.11, 114.89, 116.73, 119.60, 121.97, 123.96, 124.19, 131.42, 135.13, 149.72, 162.25, 163.50. CIMS *m/e* (relative intensity) 442 (M^+, +1). HRMS Calcd for $C_{25}H_{35}N_3O_4$ *m/z* = 441.2628, found *m/z* = 441.2536. This material was used directly in a later step.

5.3. (3*R*,6*S*)-3-[(1-*tert*-Butyloxycarbonyl-6-methoxy)-3-indoyl]methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (14)

Indole **14** (5.5 g) was prepared in 92% yield from **9a** (4.4 g, 12.7 mmol) and **11** (3.06 g,14.5 mmol), analogous to the procedure described for the synthesis of **12**. **14**: $[\alpha]_D^{27}$ −25.1° (*c* 0.8, $CHCl_3$); IR ν_{max} (NaCl) 2970, 1730, 1690 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 0.63 (d, 3H, J = 6.8, Hz), 0.92 (d, 3H, J = 6.9 Hz), 1.21 (t, 3H, J = 7.1 Hz), 1.29 (t, 3H, J = 7.1 Hz), 1.62 (s, 9H), 2.15 (m, 1H), 3.13 (d, 2H, J = 4.8 Hz), 3.53 (t, 1H, J = 3.4 Hz), 3.83 (s, 3H), 3.94–4.16 (m, 4H), 4.25 (dd, 1H, J = 3.8 Hz), 6.80 (dd, 1H, J = 2.2 and 8.6 Hz), 7.21 (s, 1H), 7.42 (d, 1H, J = 8.6 Hz), 7.67 (br, s, 1H). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.43, 16.68, 19.04, 28.23, 29.37, 31.72, 55.58, 56.13, 60.42, 60.51, 60.67, 82.93, 99.03, 111.49, 116.74, 120.10, 122.83, 125.28, 136.10, 149.81, 157.67, 162.29, 163.49. CIMS *m/e* (relative intensity) 472 (M^+, +1). HRMS Calcd for $C_{26}H_{37}N_3O_5$ *m/z* = 471.2733, found *m/z* = 471.2739. This material was used directly in a later step.

5.4. (3*R*,6*S*)-3-(1-*tert*-Butyloxycarbonyl-3-indoyl)methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (15)

Indole **15** (6.6 g) was prepared in 91% yield from **9b** and **11**, analogous to the procedure described for the synthesis of **12**. **15**: ^1H NMR (300 MHz, $CDCl_3$) δ 0.66 (d, 3H, J = 6.8, Hz), 0.95 (d, 3H, J = 6.9 Hz), 1.23 (t, 3H, J = 7.1 Hz), 1.32 (t, 3H, J = 7.1 Hz), 1.64 (s, 9H), 2.18 (m, 1H), 3.19 (m, 2H), 3.56 (t, 1H, J = 3.4 Hz), 3.96–4.21 (m, 4H), 4.29 (dd, 1H, J = 4.3 Hz), 7.15–7.29 (m, 2H), 7.36 (s, 1H), 7.59 (d, 1H, J = 7.5 Hz), 8.08 (d, 1H, J = 7.6 Hz). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.40, 16.66, 19.01, 28.21, 29.26, 31.60, 31.73, 56.05, 60.56, 60.64, 83.11, 114.89, 116.73, 119.60, 121.97, 123.96, 124.19, 131.42, 135.13, 149.72, 162.25, 163.50. CIMS *m/e* (relative intensity) 442 (M^+, +1). HRMS Calcd for $C_{25}H_{35}N_3O_4$ *m/z* = 441.2628, found *m/z* = 441.2634. This material was used directly in a later step.

5.5. (3*S*,6*R*)-3-[(1-*tert*-Butyloxycarbonyl-2-isoprenyl-6-methoxy)-3-indoyl]-methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (16)

To a solution of pyrazine **12** (1.94 g, 4.11 mmol) in dry THF (30 mL) at −78 °C under nitrogen, a solution of lithium diisopropylamide (LDA, 1.5 M in THF, 4.2 mL, 6.17 mmol) was added dropwise. The mixture which resulted was stirred at −78 °C for 60 min. The dry (HBr free) 4-bromo-2-methylbutene (1.03 g, 6.91 mmol) was then added dropwise at −78 °C. The mixture was stirred at −78 °C for 1 h and allowed to

warm to rt overnight. The solvent was removed under reduced pressure. The residue was taken up in CH_2Cl_2 and washed with a 5% aqueous solution of $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were dried (K_2CO_3). After removal of the solvent under reduced pressure, the residue was separated by flash chromatography (silica gel, hexane/EtOAc, 15:1) to provide **16** (1.89 g, 85%) as an oil: IR ν_{max} (NaCl) 2970, 1730, 1690 cm^{-1}, ^1H NMR (250 MHz, $CDCl_3$) δ 0.61 (d, 3H, J = 6.8 Hz), 0.94 (d, 3H, J = 6.8 Hz), 1.18 (t, 3H, J = 7.1 Hz), 1.31 (t, 3H, J = 7.1 Hz), 1.61 (s, 3H), 1.63 (s, 9H), 1.70 (s, 3H), 2.19 (m, 1H), 2.88 (dd, 1H, J = 7.4 and 14.2 Hz), 3.23 (dd, 1H, J = 3.9 and 14.3 Hz), 3.56 (t, 1H, J = 3.4 Hz), 3.69 (d, 2H, J = 6.0 Hz), 3.83 (s, 3H), 3.94–4.22 (m, 5H), 5.16 (t, 1H, J = 5.8 Hz), 6.78 (dd, 1H, J = 2.3 and 8.5 Hz), 7.37 (d, 1H, J = 8.6 Hz), 7.65 (d, 1H, J = 2.3 Hz). EIMS *m/e* (relative intensity) 539 (M^+, 65), 439 (11), 328 (16), 272 (58), 228 (100), 212 (55), 169 (31), 141 (16), 57 (48); Anal. Calcd for ($C_{31}H_{45}N_3O_5$) C, H, N. This material was used directly in a later step.

5.6. (3*S*,6*R*)-3-[(1-*tert*-Butyloxycarbonyl-2-isoprenyl)-3-indoyl]-methyl-3,6-dihydro-6-isopropyl-2,5-diethoxypyrazine (17)

Indole **17** (3.4 g) was prepared in 82% yield from **13** under the conditions described above for the preparation of **16**. **17**: ^1H NMR (300 MHz, $CDCl_3$) δ 0.59 (d, 3 H, J = 6.8 Hz), 0.91 (d, 3 H, J = 6.9 Hz), 1.15 (t, 3 H, J = 7.1 Hz), 1.28 (t, 3 H, J = 7.1 Hz), 1.60 (s, 9 H), 1.62 (s, 3 H), 1.68 (s, 3 H), 2.09 (m, 1 H), 2.92 (m, 1H), 3.23 (m, 1 H), 3.56 (t, 1 H, J = 3.4 Hz), 3.71 (d, 2 H, J = 6.1 Hz), 3.98–4.20 (m, 5 H), 5.14 (t, 1 H, J = 6.0 Hz), 7.10–7.15 (m, 2 H), 7.49 (dd, 1H, J = 2.1 and 6.9 Hz), 7.99 (dd, 1H, J = 1.3 and 7.2 Hz). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.34, 14.36, 16.53, 18.13, 19.05, 25.60, 26.10, 28.09, 29.33, 31.41, 56.86, 60.36, 60.40, 60.69, 83.29, 114.98, 115.27, 119.04, 121.80, 122.40, 123.15, 130.54, 131.47, 135.94, 137.83, 150.47, 162.78, 163.23. CIMS *m/e* (relative intensity) 510 (M^+ +1). Anal. Calcd for ($C_{30}H_{43}N_3O_4 0.25H_2O$) C, H, N. This material was used directly in a later step.

5.7. (*S*)-1-*tert*-Butyloxycarbonyl-2-isoprenyl-6-methoxytryptophan ethyl ester (20)

To a solution of 2-prenylpyrazine **16** (1.27 g, 2.36 mmol) in THF (30 mL) at 0 °C was added an aqueous solution of 2 N HCl (10 mL). The reaction mixture was allowed to warm to rt and stirred for 1.5 h. A cold aqueous solution of 15% NH_4OH was added. The solution was concentrated under vacuum and diluted with CH_2Cl_2. The aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried (K_2CO_3) and the solvent was removed under vacuum. The residue was separated by flash chromatography (silica gel, EtOAc) to provide **20** (0.95 g, 94%) as an oil: $[\alpha]_D^{27}$ +15.2° (*c* 0.92, $CHCl_3$); IR ν_{max} (NaCl) 2975, 1730, 1615 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 1.22 (t, 3H, J = 7.1 Hz), 1.46–1.55 (m, 2H), 1.63 (s, 9H), 1.66 (s, 3H), 1.71 (s, 3H), 2.82 (dd, 1H, J = 8.8 and 14.2 Hz), 3.12 (dd, 1H, J = 5.0 and 14.2 Hz), 3.68 (d, 2H, J = 5.1 Hz), 3.70 (m, 1H), 3.83 (s, 3H), 4.13 (qd, 2H, J = 2.1 and 7.1 Hz), 5.16 (t, 1H, J = 5.1 Hz), 6.82 (dd, 1H, J = 2.3 and 8.5 Hz), 7.33 (d, 1H, J = 8.5 Hz), 7.69 (d, 1H, J = 2.3 Hz); ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.04, 18.02, 25.48, 26.03, 28.05, 30.35, 55.62, 60.85, 83.47, 100.31, 111.32, 113.98, 118.46, 122.28, 123.66, 131.70, 136.56, 136.95, 150.35, 157.41, 175.02; EIMS *m/e* (relative intensity) 430 (M^+, 3), 272 (36), 228 (100). HRMS Calcd for $C_{24}H_{34}N_2O_5$ *m/z* = 430.2468, found *m/z* = 430.2481. This material was used directly in a later step.

5.8. (*S*)-1-*tert*-Butyloxycarbonyl-2-isoprenyltryptophan ethyl ester (21)

Ester **21** (1.1 g) was prepared in 93% yield from **17** as described above for the preparation of **20**. **21**: $[\alpha]_D^{26}$ +19.7° (*c* 0.8, CH_3OH); ^1H NMR (300 MHz, $CDCl_3$) δ 1.23 (t, 3H, J = 7.1 Hz), 1.66 (s, 9H), 1.68 (s, 3H), 1.74 (s, 3H), 1.80 (br s, 2H), 2.89 (dd, 1H, J = 8.9 and 14.2 Hz), 3.19 (dd, 1H, J = 5.0 Hz and 14.3 Hz), 3.73–3.77 (m, 3H), 4.13–4.19 (m, 2H), 5.19 (t, 1H, J = 1.4 Hz), 7.20–7.25 (m, 2H), 7.49 (dd, 1H, J = 1.0 and 7.0 Hz), 8.08 (dd, 1H, J = 1.5 Hz and 8.8 Hz); ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.11, 18.15, 25.60, 25.99, 28.09, 30.27, 54.94, 61.02, 83.72, 114.04, 115.38, 118.09, 121.91, 122.43, 123.66, 129.68, 132.11, 136.00, 138.03, 150.33, 175.09. CIMS *m/e* (relative intensity) 401 (M^+ +1). This material was used directly in a later step.

5.9. (*R*)-1-*tert*-Butyloxycarbonyl-2-isoprenyl-6-methoxytryptophan ethyl ester (22)

Ester **22** (0.86 g) was prepared in 73% yield from **14** as described above for the preparation of **20**. **22**: $[\alpha]_D^{27}$ −15.9° (*c* 0.9, $CHCl_3$); All spectroscopic data were identical to that for **20** (the enantiomer of **22**) reported in the previous experiment except the optical rotation was opposite in sign. This material was used directly in a later step.

5.10. (*R*)-1-*tert*-Butyloxycarbonyl-2-isoprenyltryptophan ethyl ester (23)

Ester **23** (0.77 g) was prepared in 70% yield from **15** as described above for the preparation of **20**. **23**: $[\alpha]_D^{27}$ −19.9° (*c* 0.9, CH_3OH); All spectroscopic data were identical to that for **21** (the enantiomer of **23**) reported in the previous experiment except the optical rotation was opposite in sign. This material was used directly in a later step.

5.10.1. Tryprostation A (1). Fmoc-L-proline (126 mg, 0.374 mmol) was dissolved in thionyl chloride (1 mL). The solution which resulted was stirred overnight at rt. Excess thionyl chloride was removed under reduced pressure. The Fmoc-L-proline chloride **24** which resulted was dissolved in dry $CHCl_3$ (1 mL). This solution was added dropwise at 0 °C to a solution of **20** (107 mg, 0.249 mmol) and triethylamine (63.0 mg, 0.623 mmol) in dry $CHCl_3$ (6 mL). The mixture which resulted was stirred at 0 °C for 0.5 h and then at rt overnight. After

removal of solvent under reduced pressure, a solution of diethylamine (DEA, 2.5 mL) in acetonitrile (2.5 mL) was added in the same flask. The reaction mixture was stirred at rt for 2 h [monitored by TLC (silica gel) until the disappearance of starting material]. Acetonitrile and excess DEA were removed under reduced pressure. Xylene (25 mL) was added into the same reaction vessel and the solution degassed. The reaction mixture was stirred at reflux for 2 d at which time examination by TLC (silica gel) indicated the disappearance of starting material. After removal of xylene under reduced pressure, the residue was subjected to flash chromatography (silica gel, $CHCl_3/CH_3OH$, 95:5) to provide tryprostatin A **1** (78 mg, 82%) as a solid: $[\alpha]_D^{27}$ −65.9° (*c* 0.97, $CHCl_3$) [lit. [7] $[\alpha]_D^{27}$ −69.7° (*c* 0.70, $CHCl_3$)]; ^1H NMR (250 MHz, $CDCl_3$) δ 1.73 (s, 3H), 1.76 (s, 3H), 1.85–2.07 (m, 3H), 2.27–2.34 (m, 1H), 2.89 (dd, 1H, J = 11.4 and 15.0 Hz), 3.41 (d, 2H, J = 7.2 Hz), 3.53–3.72 (m, 3H), 3.81 (s, 3H), 4.05 (dd, 1H, J = 6.9 and 7.7 Hz), 4.32 (dd, 1H, J = 2.7 and 11.1 Hz), 5.28 (dd, 1H, J = 5.8 and 8.6 Hz), 5.61 (s, 1H), 6.74 (dd, 1H, J = 2.2 and 8.6 Hz), 6.81 (d, 1H, J = 2.1 Hz), 7.32 (d, 1H, J = 8.6 Hz), 7.80 (br s, 1H); ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 17.92, 22.63, 25.07, 25.69, 25.71, 28.32, 45.38, 54.56, 55.73, 59.23, 94.87, 104.38, 109.27, 118.33, 119.98, 122.27, 135.14, 135.27, 136.25, 156.31, 165.80, 169.37. EIMS *m/e* (relative intensity) 381 (M^+,4), 228 (100), 212 (14), 198 (9). HRMS Calcd for $C_{22}H_{27}N_3O_3$ *m/z* = 381.2052, found *m/z* = 381.2044. Anal. Calcd for $C_{22}H_{27}N_3O_3 \cdot 1/3H_2O$ (C, H, N). The spectral data for **1** were identical to that reported by Osada et al.[7] in the literature.

5.10.2. Tryprostatin B (2). Indole **2** (300 mg) was prepared in 81% yield under conditions described above for the preparation of tryprostatin A **1**. **2**: $[\alpha]_D^{26}$ −70.9° (*c* 0.80, $CHCl_3$) {lit.[7] $[\alpha]_D^{27}$ −71.1 (*c* 0.63, $CHCl_3$)}; IR ν_{max} (NaCl) 3303, 2971, 1678, 1661 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.76 (s, 3H), 1.79 (s, 3H), 1.75–2.03 (m, 3H), 2.32 (m, 1H), 2.96 (dd, 1H, J = 11.4 and 15.9 Hz), 3.46–3.49 (m, 2H), 3.59–3.72 (m, 3H), 4.06 (dd, 1H, J = 7.5, 8.0 Hz), 4.36 (dd, 1H, J = 3.5, 11.0 Hz), 5.31 (dd, 1H, J = 6.5, 7.0 Hz), 5.62 (br s, 1H), 7.09–7.18 (m, 2H), 7.31 (d, 1H, J = 7.7 Hz), 7.48 (d, 1H, J = 7.7 Hz), 8.00 (br s, 1H), ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 18.37, 23.03, 25.50, 25.98, 26.13, 28.74, 45.80, 54.95, 59.66, 105.03, 111.17, 118.13, 120.07, 120.30, 122.26, 128.37, 135.82, 135.91, 136.80, 166.183, 169.74. CIMS *m/e* (relative intensity) 352 (M^++1, 100), 198 (28). Anal. Calcd for $(C_{21}H_{25}N_3O_2 \cdot 1/4H_2O)$ C, H, N. The spectral data for **2** were identical to that reported by Osada et al.[7] in the literature.

5.10.3. Enantiomer of tryprostatin A (3). Enantiomer **3** (75 mg) was prepared in 78% yield from **22** as described above for the preparation of tryprostatin A **1**. The starting material used here was D-tryptophan derivative **22** and Fmoc-D-Pro-Cl **25**. **3**: $[\alpha]_D^{28}$ 70.3° (*c* 1.0, $CHCl_3$). Anal. Calcd for $(C_{22}H_{27}N_3O_3 \cdot 3/5H_2O)$ C, H, N. All spectroscopic data were identical to that reported for **1** (the enantiomer of **3**) in a previous experiment except the optical rotation was opposite in sign.

5.10.4. Enantiomer of tryprostatin B (4). Enantiomer **4** (80 mg) was prepared in 83% yield from **23** as described above for the preparation of tryprostatin A **1**. The starting material used here was D-tryptophan derivative **23** and Fmoc-D-Pro-Cl **25**. **4**: $[\alpha]_D^{26}$ 71.9° (*c* 1.1, $CHCl_3$). Anal. Calcd for $(C_{21}H_{25}N_3O_2 \cdot 3/4H_2O)$ C, H, N. All spectroscopic data were identical to that for **2** (the enantiomer of **4**) reported in the previous experiment except the optical rotation was opposite in sign. All spectroscopic data were identical to that reported for **2** (the enantiomer of **4**) in a previous experiment except the optical rotation was opposite in sign.

5.10.5. Diastereomer-1 of tryprostatin A (5). Indole **5** (105 mg) was prepared in 84% yield from **22** as described above for the preparation of tryprostatin A **1**. The starting material used here was D-tryptophan derivative **22** and Fmoc-L-Pro-Cl **24**. **5**: $[\alpha]_D^{26}$ −20.0° (*c* 0.12, $CHCl_3$), IR ν_{max} (NaCl) 3269, 2971, 1673, 1650 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.18–1.43 (m, 2H), 1.60–1.71 (m, 1H), 1.74 (s, 3H), 1.78 (s, 3H), 1.99–2.08 (m, 1H), 2.67 (dd, 1H, J = 6.3, 10.7 Hz), 3.08 (dd, 1H, J = 4.5, 14.7 Hz), 3.16 (dd, 1H, J = 2.5, 9.5 Hz), 3.35 (d, 1H, J = 5.0 Hz), 3.39 (d, 2H, J = 7.7 Hz), 3.47–3.57 (m, 1H), 3.81 (s, 3H), 4.23 (dd, 1H, J = 4.3, 8.5 Hz), 5.26 (tt, 1H, J = 1.3, 6.0 Hz), 6.26 (d, 1H, J = 3.6 Hz), 6.74 (dd, 1H, J = 2.3, 8.6 Hz), 6.78 (d, 1H, J = 2.1 Hz), 7.37 (d, 1H, J = 8.6 Hz), 7.92 (br s, 1H), ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 17.88, 21.51, 24.85, 25.74, 28.96, 29.33, 45.03, 55.62, 57.70, 58.62, 94.31, 104.20, 109.08, 118.95, 119.80, 122.70, 135.20, 135.34, 135.73, 155.97, 165.65, 169.23. EIMS *m/e* (relative intensity) 381 (M^+, 15), 228 (100). Anal. Calcd for $(C_{22}H_{27}N_3O_3 \cdot 3/4H_2O)$ C, H, N.

5.10.6. Diastereomer-1 of tryprostatin B (6). Indole **6** (77 mg) was prepared in 86% yield from **23** as described above for the preparation of tryprostatin A **1**. The starting material used here was D-tryptophan derivative **23** and Fmoc-L-Pro-Cl **24**. **6**: $[\alpha]_D^{26}$ −41.9° (*c* 0.45, $CHCl_3$). Anal. Calcd for $(C_{21}H_{25}N_3O_2 \cdot 1/5H_2O)$ C, H, N. All spectroscopic data were identical to that reported for **8** except the optical rotation was opposite in sign.

5.10.7. Diastereomer-2 of tryprostatin A (7). Indole **7** (48 mg) was prepared in 84% yield from **20** as described above for the preparation of tryprostatin A **1**. The starting material used here was L-tryptophan derivative **20** and Fmoc-D-Pro-Cl **25**. **7**: $[\alpha]_D^{27}$ 21.0° (*c* 0.32, $CHCl_3$). Anal. Calcd for $(C_{22}H_{27}N_3O_3 \cdot 3/8H_2O)$ C, H, N. All spectroscopic data were identical to that reported for **5** in a previous experiment except the optical rotation was opposite in sign.

5.10.8. Diastereomer-2 of tryprostatin B (8). Diastereomer **8** (104 mg) was prepared in 79% yield from **21** as described above for the preparation of tryprostatin A **1**. The starting material used here was L-tryptophan derivative **21** and Fmoc-D-Pro-Cl **25**. **8**: $[\alpha]_D^{26}$ 42.8° (*c* 0.65, $CHCl_3$). IR ν_{max} (NaCl) 3266, 2977, 1666 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 1.30–1.44 (m, 1H), 1.58–1.71 (m, 2H), 1.75 (s, 3H), 1.79 (s, 3H), 2.00–2.09 (m, 1H), 2.69 (dd, 1H, J = 6.3, 16.8 Hz), 3.08–3.19 (m, 2H), 3.37–3.43 (m, 1H), 3.44 (d, 2H, J = 6.3 Hz), 3.48–

3.58 (m, 1H), 4.24 (dd, 1H, J = 4.4, 8.6 Hz), 5.30 (tt, 1H, J = 1.4, 7.3 Hz), 6.09 (d, 1H, J = 3.4 Hz), 7.07–7.13 (m, 2H), 7.07–7.13 (m, 2H), 7.23–7.28 (m, 1H), 7.51 (dd, 1H, J = 2.1, 6.8 Hz), 8.00 (br s, 1H). ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 17.94, 21.53, 24.91, 25.76, 28.99, 29.27, 45.08, 57.74, 58.66, 104.46, 110.37, 118.27, 119.52, 119.67, 121.56, 128.33, 134.97, 135.55, 136.66, 165.59, 169.09. EIMS *m/e* (relative intensity) 351 (M^+, 13), 198 (100). Anal. Calcd for ($C_{21}H_{25}N_3O_2 \cdot 1/8H_2O$) C, H, N.

5.11. (5*R*,2*S*)-3,6-Diethoxy-5-[6-methoxy-2-(triethyl-silyl)-3-indolyl]methyl-2,5-dihydropyrazine (28)

To a three-neck flask (3 L) equipped with an overhead stir were added iodoaniline derivative **26** (150 g), Schöllkopf derivative **27** (265 g), LiCl (2.55 g), Na_2CO_3 (159 g), palladium (II) acetate (1.75 g) and anhydrous DMF (2 L). The mixture was then degassed with a vacuum pump three times at rt with Ar. The suspension which resulted was heated for 36 h at 100 °C under an atmosphere of Ar. At this point TLC (silica gel) indicated **26** had been consumed and the reaction mixture was cooled to rt and the DMF was removed under vacuum (aspirator). Methylene chloride (2 L) was added to the residue and the suspension which resulted was filtered to remove unwanted salts. After removal of the CH_2Cl_2, the crude product was purified by flash chromatography (silica gel, 2% EtOAc in hexane) to give 77% of the desired 6-methoxy substituted indole **28**. IR ν_{max} (NaCl) 3388, 2944, 1683 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.67 (d, 3H, J = 6.8 Hz), 0.85–1.05 (m, 18H), 1.20 (t, 3H, J = 7.1 Hz), 1.30 (t, 3H, J = 7.1 Hz), 2.25 (m, 1H), 2.80 (dd, 1H, J = 13.5 Hz and J = 10.6 Hz), 3.46 (dd, 1H, J = 14.1 Hz and J = 3.1 Hz), 3.84 (s, 3H), 3.88 (t, 1H, J = 3.9), 4.01–4.21 (m, 5H), 6.70 (dd, 1H, J = 8.7 Hz and J = 2.2 Hz), 6.82 (d, 1H, J = 2.1 Hz), 7.60 (d, 1H, J = 8.7 Hz), 7.77 (s, br, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 4.1, 7.9, 14.7, 14.8, 17.1, 19.5, 32.1, 32.5, 56.0, 59.3, 60.9, 61.0, 61.1, 93.9, 109.3, 121.8, 124.4, 124.7, 130.5, 139.5, 157.0, 163.1, 164.2. MS (CI, CH_4) *m/e* (relative intensity) 486 (M^++1, 100), 456 (13), 372 (51), 274 (27). HRMS Calcd for $C_{27}H_{43}N_3O_3Si$ *m/z* = 485.3074, found *m/z* = 485.3055. This material was used directly in a later step.

5.12. N_a-Methyl-(2*S*,5*R*)-3,6-diethoxy-5-[6-methoxy-2-(triethyl-silyl)-3-indolyl]methyl-2,5-dihydropyrazine (29)

Sodium hydride (60% in mineral oil, 0.2 g) in several portions was added to a mixture of **28** (1.5 g, 3.08 mmol), CH_3I (0.65 g, 4.55 mmol) and anhydrous DMF (20 mL) at 0 °C. After this mixture was stirred for 2 h, analysis by TLC (silica gel) indicated the absence of starting material. The reaction solution was quenched with water (1 mL) and then was neutralized with an aqueous solution of NH_4Cl after which it was extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine (2 × 30 mL) and dried (K_2CO_3). The solvent was removed under reduced pressure and the residue was subjected to a short wash column (silica gel, EtOAc/hexane, 1:4) to provide the pyrazine **29** (1.6 g, 99%). Mp 91–92 °C; IR ν_{max} (NaCl): 2945, 1688, 1613 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.63 (d, 3H, J = 6.8 Hz), 0.95 (m, 15H), 0.98 (d, 3H, J = 6.9 Hz), 1.14 (t, 3H, J = 7.1 Hz), 1.23 (t, 3H, J = 7.1 Hz), 2.23 (m, 1H), 2.80 (dd, 1H, J = 14.04 Hz and 4.53 Hz), 3.45 (dd, 1H, J = 14.02 and 3.51 Hz), 3.73 (s, 3H), 3.84 (s, 3H), 3.85 (m, 1H), 3.90–4.15 (m, 5H), 6.65 (m, 2H), 7.50 (d, 1H, J = 9.2 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 4.8, 7.6, 14.3, 14.4, 16.7, 19.1, 31.6, 31.9, 33.1, 55.7, 59.2, 60.4, 60.5, 60.7, 91.9, 108.2, 121.2, 124.2, 124.6, 132.3, 140.6, 156.7, 162.7, 163.9. MS (CI, CH4) *m/e* (relative intensity) 500 (M^++1, 100), 470 (16), 386 (14), 288(21). Anal. Calcd for ($C_{28}H_{45}N_3O_3Si$) C, H, N. This material was used directly in a later step.

5.13. N_a-Isoprenyl-(2*S*,3*R*)-3,6-diethoxy-5-[6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (30)

Indole **30** (2.1 g) was prepared in 75% yield from **28** and isoprenyl bromide under conditions described above for the preparation of **29**. **30**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.64 (d, 3H, J = 6.78 Hz), 0.92 (d, 3H, J = 6.87 Hz), 1.32 (m, 7H), 1.81 (d, 6H, J = 12.21 Hz), 2.06 (s, 2H), 3.24 (dd, 3H, J = 3.24 Hz and 2,34 Hz), 3.87 (s, 3H), 4.14 (m, 3H), 4.30 (s, 1H), 4.55 (d, 2H, J = 6.93 Hz), 5.34 (s, 1H), 6.74 (m, 2H), 7.50 (d, 1H, J = 2.64 Hz). This material was used directly in a later step.

5.14. N_a-Benzyl-(2*S*,5*R*)-3,6-diethoxy-5-[6-methoxy-2-(triethyl-silyl)-3-indolyl]methyl-2,5-dihydropyrazine (31)

Indole **31** (1.65 g) was prepared from **28** and benzyl bromide in 72% yield under conditions described above for the preparation of **29**. **31**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88–0.96 (m, 13H), 1.09 (d, 3H, J = 6.87 Hz), 1.21–1.36 (m, 9H), 2.09 (s, 1H), 2.30–2.40 (m, 1H), 3.02 (dd, 1H, J = 14.22 Hz and 9.21 Hz), 3.59 (dd, 1H, J = 16.61 Hz and 5.46 Hz), 3.88 (s, 3H), 3.93 (t, 1H, J = 3.33 Hz), 4.05–4.34 (m, 5H), 5.48 (br, 2H), 6.51 (d, 1H, J = 2.1 Hz), 6.74 (dd, 1H, J = 8.67 Hz and 2.19 Hz), 6.95 (d, 2H, J = 6.96 Hz), 7.20–7.30 (m, 2H), 7.63 (d, 1H, J = 8.67 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 4.57, 7.52, 14.17, 14.30, 16.62, 19.13, 20.94, 31.34, 31.40, 31.53, 34.62, 49.57, 55.46, 58.71, 60.38, 60.67, 92.98, 108.38, 121.01, 124.67, 125.01, 125.66, 126.80, 127.55, 127.70, 128.40, 128.94, 132.48, 138.49, 140.41, 156.67, 162.76, 163.95. This material was used directly in a later step.

5.15. N_a-Allyl-(2*S*,5*R*)-3,6-diethoxy-5-[6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (32)

Indole **32** (1.9 g) was prepared from **28** and allyl bromide in 80% yield under conditions described above for the preparation of **29**. **32:** ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.64 (d, 4H, J = 6.78 Hz), 0.92 (d, 3H, J = 6.87 Hz), 1.33 (m, 6H), 2.16 (t, 1H, J = 3.60 Hz), 3.32 (m, 3H), 3.86 (s, 3H), 4.16 (m, 3H), 4.60 (m, 2H), 5.02 (s, 1H), 5.18 (d, 1H, J = 1.35), 5.31 (s, 1H), 5.96 (m, 1H), 6.72 (m, 3H), 7.50 (d, 1H, J = 8.58). EIMS *m/e* (relative intensity) 411(M^+, 46). This material was used directly in a later step.

5.16. N_a-Methyl-6-methoxy-L-tryptophan ethyl ester (33)

Ester **33** (700 mg) was prepared in 84% yield from **29** (1.5 g, 3 mmol) as described above for the preparation of **20**. **33**: IR ν_{max} (NaCl) 3374, 3311, 2980, 1736, 1623 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.30 (t, 3H, J = 7.1 Hz), 1.62 (s, br, 2H), 2.99 (dd, 1H, J = 14.4 Hz and 7.7 Hz), 3.24 (dd, 1H, J = 14.3 Hz and 4.7 Hz), 3.65 (s, 3H), 3.79 (dt, 1H, J = 12.6 Hz and 7.4 Hz), 3.89 (s, 3H), 4.18 (q, 2H, J = 7.1 Hz), 6.75–6.83 (m, 3H), 7.48 (d, 1H, J = 8.6 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.1, 30.6, 32.5, 55.2, 55.8, 60.6, 93.1, 108.9, 109.8, 119.6, 122.6, 126.5, 137.8, 156.6, 175.0. EIMS *m/e* (relative intensity) 276 (M^+, 4), 174 (100), 159 (11). Anal. Calcd for ($C_{15}H_{20}N_2O_3$) C, H, N. This material was used directly in a later step.

5.17. N_a-Isoprenyl-6-methoxytryptophan ethyl ester (34)

Ester **34** (850 mg) was prepared from **30** in 85% yield under conditions described above for the preparation of **20**. **34**: ^1H NMR (300 MHz, $CDCl_3$) δ 1.24–1.30 (m, 4H), 1.83 (t, 3H, J = 18.21 Hz), 2.06 (s, 3H), 3.04 (dd, 1H, J = 14.37 Hz and 7.68 Hz), 3.22 (d, 1H, J = 4.8 Hz), 3.87 (s, 3H), 4.13–4.21 (m, 4H), 5.78 (br, 1H), 4.59 (d, 2H, J = 6.54 Hz), 5.37 (s, 1H), 6.82 (m, 3H), 7.48 (d, 1H, J = 8.04 Hz). This material was used directly in a later step.

5.18. N_a-Benzyl-6-methoxytryptophan ethyl ester (35)

Ester **35** (1.0 g) was prepared from **31** in 85% yield under conditions described above for the preparation of **20**. **35**: ^1H NMR (300 MHz, $CDCl_3$) δ 1.16–1.26 (m, 3H), 1.87 (s, 1H), 2.01 (s, 1H), 3.02 (dd, 1H, J = 14.28 Hz and 7.32 Hz), 3.18–3.25 (m, 1H), 3.87 (s, 3H), 4.06–4.18 (m, 3H), 5.17 (s, 1H), 6.71 (s, 1H), 6.77–6.80 (m, 1H), 6.87 (s, 1H), 7.08 (d, 1H, J = 7.38 Hz), 7.22–7.28 (m, 2H), 7.50 (d, 1H, J = 8.61 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.1, 30.6, 32.5, 55.2, 55.8, 60.6, 93.1, 108.9, 109.8, 119.6, 122.6, 126.5, 137.8, 156.6; ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.27, 20.06, 29.87, 48.89, 54.26, 54.66, 59.41, 59.92, 92.54, 108.13, 109.53, 118.81, 121.82, 124.99, 125.88, 126.64, 127.82, 136.55, 136.63, 155.62, 174.25. Anal. Calcd for ($C_{21}H_{24}N_2O_3H_2O$) C, H, N. This material was used directly in a later step.

5.19. N_a-Allyl-6-methoxytryptophan ethyl ester (36)

Ester **36** (640 mg) was prepared from **32** in 85% yield under conditions described above for the preparation of **20**. **36**: ^1H NMR (300 MHz, $CDCl_3$) δ 1.23–1.29 (m, 4H), 1.72 (s, 2H), 2.06 (s, 1H), 3.04 (d, 1H, J = 7.56 Hz), 3.87 (s, 3H), 4.12–4.18 (m, 2H), 4.63 (d, 1H, J = 1.5 Hz), 5.18 (dd, 2H, J = 17.2 Hz and 8.7 Hz), 5.98–6.00 (m, 1H), 6.75–6.87 (m, 3H), 7.50 (d, 1H, J = 8.43 Hz). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.09, 30.80, 48.55, 55.02, 55.60, 60.45, 93.24, 108.85, 110.06, 117.09, 119.56, 122.53, 125.39, 133.27, 137.10, 156.32, 175.19. This material was used directly in a later step.

5.20. 3-(6-Methoxy-1-methyl-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (37)

Indole **37** (280 mg) was prepared as described above for the preparation of **1** in 82% yield. The starting material used here was L-tryptophan derivative **33** (360 mg, 1.3 mmol) and Fmoc-L-pro-Cl **24** (690 mg, 2.05 mmol). **37**: IR ν_{max} (NaCl) 3430, 1610, 1550, 1390 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 2.02–2.05 (m, 4H), 2.89 (dd, 1H, J = 15.03 Hz and 10.92 Hz), 3.59–3.74 (m, 5H), 3.88 (s, 3H), 4.08 (dd, 1H, J = 14.8 Hz and 7.14 Hz), 4.32 (d, 2H, J = 10.92 Hz), 5.78 (br, 1H), 6.77–6.91 (m, 3H), 7.48 (d, 1H, J = 14.0 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 22.54, 26.66, 28.20, 32.66, 45.29, 54.44, 55.65, 59.13, 93.01, 108.25, 109.36, 119.20, 121.37, 126.71, 138.18, 156.77, 165.49, 169.21. EIMS *m/e* (relative intensity) 327 (M^+, 14), 174 (100). Anal. Calcd for ($C_{18}H_{21}N_3O_3$) C, H, N.

5.21. 3-(6-Methoxy-1-(3-methyl-but-2-enyl)-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (38)

Indole **38** (190 mg) was prepared from **34** in 80% yield under conditions described above for the preparation of **1**. **38**: ^1H NMR (300 MHz, $CDCl_3$) δ 1.76–1.85 (m, 3H), 2.00–2.03 (s, 3H), 2.05–2.14 (s, 3H), 2.32 (d, 1H, J = 6.84 Hz), 2.87 (dd, 1H, J = 15.09 Hz and 11.04 Hz), 3.58–3.70 (m, 3H), 3.88 (s, 3H), 4.08 (dd, 1H, J = 14.80 Hz and 7.14 Hz), 4.32 (d, 2H, J = 10.92 Hz), 4.61 (d, 1H, J = 6.87 Hz), 5.38 (t, 1H, J = 1.38 Hz), 5.78 (br, 1H), 6.80–6.82 (m, 2H), 6.90 (s, 1H), 7.45 (d, 1H, J = 6.33 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 17.99, 22.53, 25.59, 26.82, 28.22, 44.00, 45.29, 53.34, 54.52, 55.63, 59.13, 93.47, 109.22, 119.20, 119.46, 121.66, 125.27, 136.50, 137.47, 156.55, 165.50, 169.19. EIMS *m/e* (relative intensity) 381 (M^+, 16), 160 (100), 228 55. Anal. Calcd for ($C_{22}H_{27}N_3O_3$) C, H, N.

5.22. N_a-Benzyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (39)

Indole **39** (130 mg) was prepared from **35** in 74% yield under conditions described above for the preparation of **1**. **39**: mp 124–126 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 1.70–1.81 (m, 4H), 2.03–2.06 (m, 2H), 2.82 (dd, 1H, J = 10.74 Hz and 6.69 Hz), 3.11–3.18 (m, 2H), 3.40 (dd, 1H, J = 14.55 Hz and 5.67 Hz), 3.51–3.61 (m, 1H), 3.82 (s, 3H), 4.22–4.27 (m, 1H), 5.21 (s, 1H), 6.01 (d, 1H, J = 3.09 Hz), 6.72 (s, 1H), 6.80 (d, 1H, J = 8.70 Hz), 6.91 (s, 1H), 7.13–7.16 (m, 2H), 7.28–7.33 (m, 2H), 7.49 (d, 1H, J = 8.70Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 12.41, 28.74, 30.68, 44.97, 49.98, 55.54, 57.77, 58.44, 93.29, 108.48, 109.34, 119.82, 122.03, 126.77, 126.89, 127.69, 128.77, 137.24, 156.53, 165.31, 169.14. Anal. Calcd for ($C_{24}H_{25}N_3O_30.5H_2O$) C, H, N.

5.23. N_a-Allyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (40)

Indole **40** (220 mg) was prepared from **36** in 80% yield under conditions described above for the preparation of **1**. **40**: mp 84–86 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 1.20–1.25 (m, 3H), 2.07 (d, 1H, J = 7.56 Hz), 2.25 (d,

1H, J = 22 Hz), 3.24 (d, 2H, J = 5.70 Hz), 3.70 (dd, 1H, J = 13.92 Hz and 7.01 Hz), 3.83 (s, 3H), 4.06–4.13 (m, 3H), 4.60 (m, 3H), 5.18 (d, 1H, J = 3.10 Hz), 5.89–5.94 (m, 1H), 6.70–6.83 (m, 3H), 7.42 (d, 1H, J = 5.61 Hz). Anal. Calcd for ($C_{20}H_{23}N_3O_3$) C, H, N.

5.24. (2*S*,5*R*)-3,6-Diethoxy-2-isopropyl-5-[6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (41)

A solution of **16** (500 mg, 0.93 mmol) in xylene was stirred at reflux for 3 d at which time examination by TLC (silica gel) indicated the disappearance of starting material. After removal of xylenes under reduced pressure, the residue was subjected to flash chromatography (silica gel, hexane/EtOAc, 10:1) to provide **41** (330 mg, 80%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.61 (d, 3H, J = 6.75 Hz), 0.91 (d, 3H, J = 6.87 Hz), 1.22 (t, 3H, J = 7.08 Hz), 1.35 (t, 4H, J = 7.14 Hz), 1.77 (d, 5H, J = 7.92), 2.15 (s, 1H), 3.24 (m, 3H), 3.42 (d, 2H, J = 7.20 Hz), 3.83 (s, 3H), 3.98–4.30 (m, 4H), 5.29 (s, 1H), 6.73 (m, 2H), 7.42 (t, 1H, J = 8.61 Hz), 7.65 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.29, 16.32, 17.71, 18.99, 25.00, 25.67, 28.88, 30.82, 55.58, 57.23, 59.92, 60.24, 60.45, 93.97, 106.77, 108.10, 119.53, 120.94, 123.99, 125.28, 127.67, 134.16, 135.57, 155.45, 162.74, 163.41. EIMS *m/e* (relative intensity) 439 (M^+, 13), 212 (54), 169 (100). Anal. Calcd for ($C_{26}H_{37}N_3O_3$) C, H,N. This material was used directly in a later step.

5.25. N_a-Benzyl-(2*S*,5*R*)-3,6-diethoxy-2-isopropyl-5-[6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (42)

Indole **42** (550 mg) was prepared in 91% yield from **41** and benzyl bromide as described above for the preparation of **29**. **42**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.62 (d, 3H, J = 6.69 Hz), 0.93 (d, 3H, J = 6.84 Hz), 1.17–1.22 (m, 7H), 1.62 (t, 7H, J = 9.4 Hz), 2.10 (m, 1H), 3.21–3.39 (m, 4H), 3.77 (s, 3H), 4.10 (m, 4H), 5.04 (s, 1H), 5.22 (s, 1H), 6.60 (s, 1H), 6.70 (d, 1H, J = 8.55 Hz), 6.88 (d, 2H, J = 6.87 Hz), 7.27 (m, 3H), 7.48 (d, 1H, J = 8.55 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.28, 16.33, 17.80, 19.08, 24.02, 25.57, 28.07, 29.14, 30.77, 46.43, 55.59, 57.35, 59.96, 60.17, 60.44, 92.95, 107.46, 107.91, 119.77, 122.23, 123.16, 125.67, 126.82, 128.47, 131.87, 136.55, 137.08, 138.30, 155.61, 162.67, 163.3. This material was used directly in a later step.

5.26. N_a-Allyl-(2*S*,5*R*)-3,6-diethoxy-2-isopropyl-5-[6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (43)

Indole **43** (135 mg) was prepared in 82% yield from **41** (150 mg, 0.34 mmol) and allyl bromide (50 mg, 0.40 mmol) as described above for the preparation of **29**. **43**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.60 (t, 3H, J = 6.75 Hz), 0.91 (t, 3H, J = 6.84 Hz), 1.31 (m, 7H), 1.69 (t, 7H, J = 19.7 Hz), 2.10 (m, 1H), 3.14–3.27 (m, 3H), 3.42 (d, 1H, J = 6.45 Hz), 3.85 (s, 3H), 4.12 (m, 4H), 4.58 (s, 2H), 4.78 (d, 1H, J = 8.67 Hz), 5.09 (d, 2H, J = 9.12 Hz), 5.85 (m, 1H), 6.70 (m, 2H), 7.45 (d, 1H, J = 8.58 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.29, 16.32, 17.83, 18.98, 19.56, 23.87, 25.45, 29.17, 30.79, 45.31, 55.65, 57.41, 59.94, 60.18, 60.43, 92.96, 107.16, 107.67, 115.57, 119.70, 122.45, 123.18, 131.67, 133.62, 136.12, 136.71, 155.46, 162.78, 163.26. EIMS *m/e* (relative intensity) 479 (M^+, 13), 268 (100). This material was used directly in a later step.

5.27. N_a-Benzyl-2-isoprenyl-6-methoxytryptophan ethyl ester (44)

Ester **44** (330 mg) was prepared in 87% yield from **42**, as described above for the preparation of **20**. **44**: IR ν_{max} (KBr) 3054, 2305, 1733, 1422, 1265 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.18–1.32 (m, 3H), 1.60 (d, 8H, J =15.30 Hz), 3.01 (t, 1H, J = 9.30), 3.40 (d, 2H, J = 6.30 Hz), 3.80 (s, 3H), 4.17 (m, 2H), 5.04 (s, 1H), 5.26 (s, 1H), 6.66 (s, 1H), 6.80 (d, 1H, J = 3.96 Hz), 6.92 (d, 2H, J = 7.17 Hz), 7.25 (d, 3H, J = 7.50 Hz), 7.48 (d, 1H, J = 8.61 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.06, 17.86, 24.01, 25.40, 30.64, 46.54, 55.52, 55.67, 60.78, 93.51, 106.85, 108.49, 118.89, 121.57, 122.43, 125.72, 126.98, 128.53, 132.68, 136.34, 137.40, 137.92, 155.99, 175.33. EIMS *m/e* (relative intensity) 420 (M^+, 13), 318 (100). This material was used directly in a later step.

5.28. N_a-Allyl-2-isoprenyl-6-methoxytryptophan ethyl ester (45)

Ester **45** (88 mg) was prepared in 86% yield from **43** (130 mg, 0.27 mmol) as described above for the preparation of **20**. **45**: IR ν_{max} (NaCl) 3365, 1730, 1625 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26 (m, 3H), 1.78 (t, 10H, J = 10.44), 2.94 (dd, 1H, J = 8.46 and J = 5.82), 3.20 (d, 1H, J = 5.04), 3.44 (d, 1H, J = 6.03), 3.86 (s, 3H), 4.16 (m, 2H), 4.61 (t, 2H, J = 2.43), 4.84 (d, 1H, J = 15.93), 5.12 (m, 2H), 5.90 (s, 1H), 6.75 (t, 2H, J = 6.33), 7.44 (d, 1H, J = 8.55); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.05, 17.91, 23.86, 25.49, 30.54, 45.44, 55.47, 55.71, 60.78, 93.50, 106.40, 108.28, 115.91, 118.81, 121.72, 122.40, 132.54, 133.40, 136.18, 136.95, 155.84, 175.30. EIMS *m/e* (relative intensity) 370 (M^++1, 12), 268 (100). Anal. Calcd for ($C_{22}H_{30}N_2O_3$) C, H, N. This material was used directly in a later step.

5.29. N_a-Benzyl-2-isoprenyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (46)

Indole **46** (125 mg) was prepared in 87% yield from **44** as described above for the preparation of **1**. **46**: IR ν_{max} (KBr) 3010, 2408, 1735 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.75 (dd, 3H, J = 7.14 Hz and J = 7.41 Hz), 1.55 (s, 2H), 1.64 (d, 4H, J = 11.82 Hz), 2.06 (d, 2H, J = 2.52), 2.28 (d, 2H, J = 2.70 Hz), 2.75 (m, 1H), 3.00 (m, 1H), 3.38 (m, 2H), 3.82 (s, 3H), 4.14 (m, 3H), 5.15 (d, 1H, J = 7.47 Hz), 5.27 (t, 1H, J = 8.88 Hz), 6.60 (s, 1H), 6.86 (m, 2H), 6.91 (d, 2H, J = 6.18 Hz), 7.05 (d, 2H, J = 2.70 Hz), 7.30 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.93, 17.83, 21.31, 23.87, 24.32, 25.38, 28.28, 46.44, 55.69, 59.19, 61.22, 64.48, 84.64, 93.94, 108.52, 118.68, 121.60, 126.09, 126.93, 127.21, 127.88, 128.46, 128.68, 129.40, 137.99, 155.72, 169.02. EIMS *m/e* (relative intensity) 472 (M^+, 5), 318 (48) 91 (100). Anal. Calcd for ($C_{29}H_{33}N_3O_3H_2O$) C, H, N.

5.30. N_a-Allyl-2-isoprenyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (47)

Indole **47** (80 mg) was prepared as described above for the preparation of **1**. The starting material used here was L-tryptophan derivative **45** (85 mg, 0.23 mmol) and Fmoc-L-pro-Cl **24** (126 mg, 0.37 mmol) in 82% yield. **47**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.27 (s, 1H), 1.51–1.76 (m, 9H), 2.37 (t, 1H, J = 3.60 Hz), 3.00 (s, 1H), 3.29 (t, 1H, J = 4.20 Hz), 3.40 (m, 1H), 3.60 (d, 1H, J = 5.70 Hz), 3.92(s, 3H), 4.23 (d, 1H, J = 9.60 Hz), 4.70 (d, 1H, J = 10.2 Hz), 5.14 (s, 1H), 5.54 (s, 1H), 6.95 (d, 1H, J = 7.71 Hz), 7.18 (s, 1H), 7.24 (d, 1H, J = 5.70 Hz). EIMS *m/e* (relative intensity) 421 (M^+, 14), 268 (100). +TOF MS HRMS Calcd for $(C_{25}H_{31}N_3O_3 + Li)^+$ *m/z* = 428.2525, found *m/z* = 428.2519.

5.31. (5*S*,2*R*)-3,6-Diethoxy-2-bromo-5-[1-*tert*-butyloxycarbonyl-6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (48)

A solution of NBS (183 mg, 1.03 mmol) which had been dissolved in acetonitrile (10 mL) was syringed into a solution of **28** (500 mg, 1.03 mmol) in acetonitrile (40 mL) at 0 °C. The reaction mixture was allowed to stir at 0 °C for 30 min at which time analysis by TLC (silica gel) indicated the absence of starting material. To this solution was then added 4-dimethylaminopyridine (DMAP, 7 mg, 0.057 mmol) and di-*tert*-butyl-dicarbonate (450 mg, 2.06 mmol) at rt. After the reaction solution was stirred for another 1 h, analysis by TLC (silica gel) indicated the disappearance of the intermediate. The solvent was removed under reduced pressure, and the residue was partitioned between CH_2Cl_2 (100 mL) and H_2O (100 mL). The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 80 mL). The combined organic layers were dried (Na_2SO_4) and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (silica gel, EtOAc/hexanes, 4:96) to afford **48** (492 mg) as an oil in 87% yield. **48**: IR ν_{max} (NaCl) 2970, 1735, 1690 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.62 (d, 3H, J = 6.8 Hz), 0.97 (d, 3H, J = 6.8 Hz), 1.17 (t, 3H, J = 7.1 Hz), 1.28 (t, 3H, J = 7.1 Hz), 1.67 (s, 9H), 2.20 (m, 1H), 2.92 (dd, 1H, J = 8.1 and 13.9 Hz), 3.28 (dd, 1H, J = 4.6 and 13.9 Hz), 3.68 (t, 1H, J = 3.3 Hz), 3.84 (s, 3H), 3.95–4.25 (m, 5H), 6.80 (dd, 1H, J = 2.2 and 8.6 Hz), 7.38 (d, 1H, J = 8.6 Hz), 7.63 (d, 1H, J = 2.1 Hz); ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.31, 14.35, 16.59, 19.09, 28.24, 31.04, 31.46, 55.62, 55.78, 60.49, 60.74, 84.46, 99.59, 107.81,111.61, 119.69, 120.59, 123.66, 137.34, 149.35, 157.65, 162.77, 163.37. EIMS *m/e* (relative intensity) 551 (M^+, 17), 549 (M^+, 17), 470 (64), 414 (61), 370 (58), 341 (32), 240 (49), 238 (49), 212 (72), 169 (100), 141 (39). Anal. Calcd for ($C_{26}H_{36}N_3O_5Br$) C, H, N. This material was used directly in a later step.

5.32. (2*S*,5*R*)-3,6-Diethoxy-2-5-[1-*tert*-butyloxycarbonyl-2-benzyl-6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (49)

Indole **49** (800 mg) was prepared in 85% yield from **29** and benzyl bromide, as described below for the preparation of **50**. **49**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.65 (d, 3H, J = 3.75 Hz), 0.98 (d, 3H, J = 6.87 Hz), 1.20 (t, 3H, J = 7.11 Hz), 1.29 (m, 6H), 1.41 (s, 10H), 2.87 (s, 1H), 3.64 (d, 1H, J = 3.36), 3.88 (s, 3H), 4.00 (m, 1H), 4.56 (s, 2H), 6.89 (d, 1H, J = 8.58 Hz), 7.01 (d, 2H, J = 7.08 Hz), 7.20 (m, 3H), 7.52 (d, 1H, J = 8.58 Hz), 7.75 (s, 1H). EIMS *m/e* (relative intensity) 561 (M^+, 14), 461 (11), 212 (100). Anal. Calcd for ($C_{33}H_{43}N_3O_5$) C, H, N. This material was used directly in a later step.

5.33. (2*S*,5*R*)-3,6-Diethoxy-2-allyl-5-[1-*tert*-butyloxycarbonyl-6-methoxy-3-indolyl]methyl-2,5-dihydropyrazine (50)

A solution of *n*-BuLi (2.5 M in hexane, 0.22 mL, 0.54 mmol) was added dropwise to a solution of 2-bromopyrazine **29** (250 mg, 0.46 mmol) in dry THF (8 mL) at −78 °C under nitrogen. The mixture which resulted was stirred at −78 °C for 30 min and then warmed to 0 °C for 10 min. Then allyl bromide (82.8 mg, 0.69 mmol) was added quickly at 0 °C. The mixture was stirred at 0 °C for 1 h and allowed to warm to rt overnight. The solvent was removed under reduced pressure. The residue was taken up in CH_2Cl_2 and washed with a 5% aqueous solution of $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried (Na_2SO_4). After removal of the solvent under reduced pressure, the residue was separated by flash chromatography (silica gel, hexane/EtOAc, 15/1) to provide **50** (198 mg, 85%) as an oil. **50**: IR ν_{max} (KBr) 3054, 2306, 1733, 1422 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.65 (d, 3H, J = 6.78 Hz), 0.97 (d, 3H, J = 6.90 Hz), 1.23 (t, 10H, J = 6.54 Hz), 1.66 (d, 8H, J = 7.17 Hz), 2.20 (m, 1H), 3.31 (d, 1H, J = 3.30 Hz), 3.64 (s, 1H), 3.88 (s, 3H), 4.07 (m, 3H), 4.21 (m, 2H), 4.94 (d, 2H, J = 6.36 Hz), 5.98 (m, 1H), 6.84 (d, 1H, J = 2.34 Hz), 7.70 (d, 1H, J = 2.25 Hz), 7.98 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.23, 16.46, 18.97, 28.08, 29.19, 30.59, 31.33, 55.54, 60.32, 83.25, 98.73, 99.77, 110.94, 114.75, 119.42, 136.23, 136.70, 157.07. EIMS *m/e* (relative intensity) 511 (M^+, 10), 244 (50), 200 (100). +TOF MS HRMS Calcd for ($C_{29}H_{41}N_3O_5$+ H)$^+$ *m/z* = 512.3124, found *m/z* = 512.3126. This material was used directly in a later step.

5.34. (5*R*,2*S*)-3,6-Diethoxy-5-[6-methoxy-2-phenyl-3-indolyl]methyl-2,5-dihydropyrazine (51)

A solution of *n*-BuLi (2.5 M in hexane, 0.27 mL, 0.68 mmol) was added dropwise to a solution of 2-bromopyrazine **29** (250 mg, 0.46 mmol) in dry THF (10 mL) at −78 °C under nitrogen. The mixture which resulted was stirred at −78 °C for 30 min and then warmed to 0 °C for 10 min. Then dry anhydrous pure zinc chloride (0.68 mL, 0.69 mmol) was added quickly at 0 °C. The mixture which resulted was stirred at 0 °C for 1 h and iodobenzene was added and this was followed by addition of tri-2-furyl phosphine (21 mg, 0.1 mmol) and $Pd(OAc)_2$ (12 mg, 0.05 mmol). The mixture which resulted was then stirred overnight. The solvent was removed under reduced pressure. The residue was taken up in CH_2Cl_2 and washed with a 5% aqueous solution of $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried (Na_2SO_4). After removal of the solvent under reduced pressure, the residue was separated by flash chromatography (silica gel, hexane/EtOAc, 15/1) to pro-

vide **51** (170 mg, 65%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.95 (d, 3H, J = 3.75 Hz), 1.22 (m, 3H), 1.33 (m, 6H), 1.41 (s, 10H), 1.65 (m, 3H), 2.81 (s, 1H), 3.34 (m, 3H), 3.90 (s, 3H), 6.88 (m, 2H), 7.40 (m, 3H), 7.56 (d, 2H, J = 8.58 Hz), 7.71 (s, 1H), 7.52 (d, 1H, J = 2.10 Hz), 7.75 (s, 1H). EIMS *m/e* (relative intensity) 547 (M^+, 56), 236 (100). Anal. Calcd for ($C_{32}H_{41}N_3O_5H_2O$) C, H, N. This material was used directly in a later step.

5.35. (*S*)-1-*tert*-Butyloxycarbonyl-2-benzyl-6-methoxy-tryptophan ethyl ester (52)

Ester **52** (185 mg) was prepared in 87% yield from **49** as described above for the preparation of **20**. **52**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (m, 3H), 1.39 (s, 9H), 1.67 (s, 3H), 2.96 (d, 1H, J = 8.28 Hz), 3.18 (d, 1H, J = 5.46 Hz), 3.89 (s, 3H), 4.10 (m, 2H), 4.49 (s, 2H), 6.90 (d, 1H, J = 2.34 Hz), 7.02 (d, 2H, J = 6.93 Hz), 7.23 (m, 3H), 7.44 (d, 1H, J = 8.58 Hz), 7.80 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.10, 27.66, 28.09, 30.13, 32.20, 54.86, 55.58, 64.16, 83.61, 100.01, 111.64, 115.22, 119.38, 122.52, 125.81, 127.44, 128.03, 130.13, 136.79, 139.84, 150.13, 157.60, 174.92. This material was used directly in a later step.

5.36. 2-Benzyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (55)

Indole **55** (95 mg) was prepared in 81% yield from **52**, as described above for the preparation of **1**. **55**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26 (t, 2H, J = 5.16 Hz), 1.64 (s, 1H), 2.01 (m, 2H), 2.33 (m, 1H), 3.03 (t, 1H, J = 11.25 Hz), 3.58–3.73 (m, 2H), 3.83 (s, 3H), 4.09 (m, 2H), 4.32 (d, 1H, J = 10.80 Hz), 5.65 (s, 1H), 6.78 (s, 2H), 7.18 (d, 2H, J = 6.90 Hz), 7.28 (t, 2H, J = 5.70 Hz), 7.40 (d, 2H, J = 9.60 Hz), 7.80 (s, 1H); ^{13}C NMR (75.7 MHz, $CDCl_3$) δ 22.53, 25.65, 28.24, 32.20, 45.32, 54.54, 55.64, 59.15, 94.79, 105.76, 109.56, 118.46, 122.03, 126.48, 128.43, 128.70, 128.89, 136.49, 138.03, 156.53, 165.60, 169.24. EIMS *m/e* (relative intensity) 403 (M^+, 25), 335 (8), 250 (100), 218 (6). Anal. Calcd for ($C_{24}H_{25}N_3O_3$) C, H, N.

5.37. 2-Allyl-3-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (56)

Indole **56** (88 mg) was prepared in 81% yield from **53**, as described above for the preparation of **1**. **56**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (m, 2H), 1.76 (s, 1H), 2.02 (m, 2H), 2.34 (s, 1H), 3.01 (t, 1H, J = 5.73 Hz), 3.48 (t, 1H, J = 6.30 Hz), 3.67 (s, 2H), 4.05–4.11 (m, 3H), 4.32 (d, 1H, J = 9.60 Hz), 5.15 (m, 1H), 5.88 (m, 1H), 6.78 (t, 2H, J = 3.30 Hz), 7.38 (m, 1H), 8.08 (s, 1H). EIMS *m/e* (relative intensity) 353 (M^+, 8), 200 (100). +TOF MS HRMS Calcd for $(C_{20}H_{23}N_3O_3 + Li)^+ m/z$ = 360.1899, found m/z = 360.1896.

5.38. (2*S*,5*R*)-3,6-Diethoxy-2-isopropyl-5-[1-*tert*-butyloxycarbonyl-2-methyl-acrylate-6-methoxy-3-indolyl] methyl-2,5-dihydropyrazine (58)

Indole **29** (200 mg, 0.36 mmol) and $Pd(PPh_3)_4$ (21 mg, 0.018 mmol) were placed in a round-bottomed flask (50 mL) and purged with argon. Toluene (10 mL) was added and this was followed by methyl acrylate (0.16 mL) and dicyclohexylmethylamine (0.09 mL, 0.43 mmol). The reaction was heated to 95 °C for 48 h, cooled and then filtered through celite. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography to afford **58** (190 mg, 94%). **58**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.77 (d, 3H, J = 6.78 Hz), 0.89 (t, 3H, J = 3.48 Hz), 1.07 (d, 3H, J = 6.87 Hz), 1.25 (m, 6H), 1.67 (s, 9H), 2.18 (m, 1H), 2.89 (t, 1H, J = 9.27 Hz), 3.46 (d, 1H, J = 10.74 Hz), 3.81 (s, 3H), 3.90 (s, 3H), 4.14 (m, 3H), 6.50 (d, 1H, J = 11.46 Hz), 6.86 (d, 1H, J = 2.64 Hz), 7.54 (d, 1H, J = 8.67 Hz), 7.73 (s, 1H), 8.02 (d, 1H, J = 13.53 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.00, 14.15, 14.28, 16.58, 18.97, 22.54, 28.06, 30.30, 31.48, 31.67, 51.34, 55.48, 56.57, 60.53, 60.70, 60.85, 84.17, 99.08, 112.27, 118.04, 119.50, 122.68, 124.06, 131.18, 137.94, 150.24, 158.86, 162.31, 163.29, 167.39. EIMS *m/e* (relative intensity) 555 (M^+, 64), 455 (37), 412 (22), 288 (18), 244 (100), 212 (92). Anal. Calcd for ($C_{30}H_{41}N_3O_7$) C, H, N. This material was used directly in a later step.

5.39. 2-Methyl acrylyl-6-methoxytryptophan ethyl ester (59)

Indole **59** (180 mg) was prepared in 87% yield from **58** as described above for the preparation of **20**. **59**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23 (m, 3H), 1.58 (s, 2H), 1.67 (m, 10H), 3.04 (d, 1H, J = 8.67 Hz), 3.23 (d, 1H, J = 4.83 Hz), 3.82 (s, 3H), 3.90 (s, 3H), 4.17 (d, 2H), 6.38 (d, 1H, J = 15.0 Hz), 6.91 (d, 1H, J = 2.16 Hz), 7.48 (d, 1H, J = 8.67 Hz), 7.75 (s, 1H), 8.01 (d, 1H, J = 15.09 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.96, 22.09, 28.06, 30.68, 51.55, 55.04, 55.52, 61,13, 84.54, 99.34, 112.66, 118.63, 120.19, 120.88, 123.40, 131.37, 136.29, 137.77, 150.06, 158.99, 164.46, 167.17, 174.88. EIMS *m/e* (relative intensity) 446 (M^+, 35), 244 (100). This material was employed directly in the next step.

5.40. 2-Methyl acrylyl-3-(6-methoxy-1*H*-indol-3-yl-methyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (60)

Compound **60** (110 mg) was prepared in 87% yield from **59** as described above for the preparation of **1**. **60**: mp 135–137 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.24 (m, 4H), 3.54 (m, 2H), 3.80 (s, 3H), 3.91 (s, 3H), 4.14 (m, 2H), 6.02 (s, 1H), 6.70 (d, 1H, J = 2.82 Hz), 7.08 (d, 1H, J = 2.79 Hz), 7.38 (s, 1H), 7.68 (d, 1H, J = 12.76 Hz), 8.25 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.12, 21.24, 24.35, 28.36, 44.27, 51.64, 55.36, 61.41, 64.01, 93.64, 102.72, 111.40, 119.39, 121.00, 127.26, 128.53, 138.36, 138.61, 159.00, 167.32, 175.08. EIMS *m/e* (relative intensity) 397 (M^+, 34), 324 (77), 293 (100). HRMS Calcd for $C_{21}H_{23}N_3O_5 m/z$ = 397.1638, found m/z = 397.1657.

5.41. 6-Methoxy-L-tryptophan ethyl ester (61)

Ester **61** (700 mg) was prepared in 86% yield from **28** (1.5 g, 31 mmol) as described above for the preparation of **20**. **61**: IR ν_{max} (NaCl) 3365, 1730, 1625 cm^{-1}; ^{1}H

NMR (300 MHz, $CDCl_3$) δ 1.23 (t, 3H, J = 7.1 Hz), 1.60 (s, br, 2H), 3.0 (dd, 1H, J = 14.3 Hz and 7.7 Hz), 3.22 (dd, 1H, J = 14.4 Hz and 4.8 Hz), 3.78 (m, 1H), 3.81 (s, 3H), 4.15 (q, 2H, J = 7.2 Hz), 6.80 (m, 2H), 6.90 (d, 1H, J = 2.0 Hz), 7.45 (d, 1H, J = 8.5 Hz), 8.15 (s, br, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.0, 30.7, 54.5, 54.9, 60.8, 94.7, 109.2, 111.0, 119.1, 121.8, 121.9, 136.5, 156.1, 175.2. MS (CI) *m/e* 263 (M^++1, 100). Anal. Calcd for ($C_{14}H_{18}N_2O_3$) C, H, N. This material was used directly in a later step.

5.42. 3-(6-Methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (62)

Indole **62** (280 mg) was prepared as described above for the preparation of **1** in 82% yield. The starting material used here was L-tryptophan derivative **61** (360 mg, 1.37 mmol) and Fmoc-L-pro-Cl **24** (690 mg, 2.05 mmol). mp 133–135 °C; IR ν_{max} (KBr) 3100, 1700, 1400, 1050 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.98–2.06 (m, 3H), 2.29–2.36 (m, 1H), 2.94 (dd, 1H, J = 15.01 and 10.93 Hz), 3.55–3.77 (m, 3H), 3.87 (s, 3H), 4.10–4.15 (m, 1H), 4.36 (d, 1H, J = 11.8 Hz), 5.76 (br, 1H), 6.83 (dd, 1H, J = 8.6 and 2.2 Hz), 6.90 (d, 1H, J = 2.0 Hz), 6.98 (s, 1H), 7.47 (d, 1H, J = 8.6 Hz), 8.03 (br, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 21.41, 28.80, 30.54, 44.94, 55.52, 57.76, 58.19, 94.58, 109.01, 109.58, 119.48, 121.40, 123.12, 136.93, 156.42, 165.66, 169.80. EIMS *m/e* (relative intensity) 313 (M^+, 14), 160 (100). Anal. Calcd for ($C_{17}H_{19}N_3O_3$) C, H, N. This material was used directly in a later step.

5.43. 3-(2-Bromo-6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (63)

A solution of NBS (28.5 mg) which had been dissolved in THF (1 mL) was syringed into a solution of **62** (50 mg, 0.16 mmol) in THF (10 mL) at −78 °C. The reaction mixture which resulted was allowed to stir at rt overnight at which time analysis by TLC (silica gel) indicated the absence of starting material. The solvent was removed under reduced pressure. The residue was purified by preparative TLC (silica gel, 5% EtOH/CH_2Cl_2) to afford **63** as a powder in 80% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.76–1.89 (m, 2H), 2.11 (dd, 2H, J = 16.2 Hz and 8.8 Hz), 2.97 (dd, 1H, J = 10.44 and 6.6 Hz), 3.12–3.37 (m, 3H), 3.52–3.60 (m, 1H), 3.83 (s, 3H), 4.27 (d, 1H, J = 4.2 Hz), 6.37 (br, 1H), 6.75–6.84 (m, 2H), 7.39 (d, 1H, J = 8.6 Hz), 8.92 (br, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 21.52, 28.95, 29.77, 45.18, 55.54, 57.78, 58.06, 94.32, 108.61, 108.70, 110.00, 118.88, 121.30, 136.71, 156.59, 165.33, 168.96. EIMS *m/e* (relative intensity) 391 (M^+, 18), 393 (M^+, 18), 154 (100), 240 (78).

5.44. 3-(2-Chloro-6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (64)

Indole **64** (55 mg) was prepared in 85% yield from **62** under conditions described above for the preparation of **63**. **64**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.81–1.90 (m, 2H), 2.15 (dd, 2H, J = 16.2 Hz and 8.8 Hz), 2.97 (dd, 1H, J = 10.44 Hz and 6.6 Hz), 3.17–3.32 (m, 3H), 3.52–3.60 (m, 1H), 3.82 (s, 3H), 4.26 (t, 1H, J = 4.2 Hz), 6.43 (br, 1H), 6.74–6.84 (m, 2H), 7.35 (d, 1H, J = 9.4 Hz), 9.08 (s, br, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 18.30, 21.49, 28.96, 45.13, 55.54, 58.00, 58.32, 94.44, 105.26, 109.94, 118.96, 121.12, 121.42, 135.13, 156.57, 165.40, 169.11. EIMS *m/e* (relative intensity) 347 (M^+, 14), 194 (100), 154 (91). +TOF MS HRMS Calcd for ($C_{17}H_{18}N_3O_3$ + Na)$^+$*m/z* = 370.0934, found *m/z* = 370.0935.

5.45. 3-Benzyl-6-(6-methoxy-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (66)

Indole **66** (78 mg) was prepared from **61** in 73% yield under conditions described above for the preparation of **1**. **66**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.99–2.05 (m, 2H), 2.60–2.63 (m, 2H), 3.23–3.29 (m, 3H), 3.50 (d, 1H, J = 6.80 Hz), 3.82 (s, 3H), 4.14–4.16 (m, 2H), 4.92 (s, 1H), 6.74–6.83 (m, 3H), 7.22–7.29 (m, 2H), 7.76 (s, 1H), 8.42 (br, 1H). MS (EI) *m/e* (relative intensity) 363 (M^+, 100), 287(62.5). Anal. Calcd for ($C_{21}H_{21}N_3O_30.5$-H_2O) C, H, N.

5.46. (3*S*,6*S*)-3-Isopropyl-6-[6-methoxy-2-(3-methylbut-2-enyl)-1*H*-indol-3-ylmethyl]piperazine-2,5-dione (67)

Indole **67** (63 mg) was prepared from **20** in 85% yield under conditions described above for the preparation of **1**. **67**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.95–0.97 (d, 3H, J = 6.8 Hz), 1.06–1.08 (d, 3H, J = 7.1 Hz), 1.77–1.80 (d, 6H, J = 9.8 Hz), 2.42 (m, 1H), 2.91–2.99 (dd, 1H, J = 11.0 and 14.4 Hz), 3.43–3.46 (d, 2H, J = 7.2 Hz), 3.58–3.64 (dd, 1H, J = 3.3 and 14.5 Hz), 3.85 (s, 3H), 3.92 (s, 1H), 4.25–4.29 (d, 1H, J = 9.4 Hz), 5.29–5.32 (t, 1H, J = 8.2 Hz), 5.79 (s, 1H), 6.00 (s, 1H), 6.77–6.81 (dd, 1H, J = 8.6 and 2.2 Hz), 6.84 (d, 1H, J = 2.1 Hz), 7.38–7.41 (d, 1H, J = 8.6 Hz), 7.83 (br, 1H). MS (EI) *m/e* (relative intensity) 383 (M^+). +TOF MS HRMS Calcd for ($C_{22}H_{29}N_3O_3$ + H)$^+$*m/z* = 384.2287, found *m/z* = 384.2282.

5.47. (3*S*,6*S*)-3-Benzyl-6-[6-methoxy-2-(3-methylbut-2-enyl)-1*H*-indol-3-ylmethyl]piperazine-2,5-dione (68)

Indole **68** (45 mg) was prepared from **20** in 75% yield under conditions described above for the preparation of **1.68**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.81 (d, 6H, J = 9.8 Hz), 1.90–1.98 (m, 1H), 3.12–3.26 (m, 2H), 3.41–3.43 (d, 2H, J = 7.5 Hz), 3.85 (s, 3H), 4.05–4.08 (d, 1H, J = 10.1 Hz), 4.25 (m, 2H), 5.32–5.35 (t, 1H, J = 8.4 Hz), 5.53 (s, 1H), 5.75 (s, 1H), 6.78–6.85 (m, 4H), 7.25–7.28 (m, 3H), 7.43–7.46 (d, 1H, J = 9.3 Hz), 7.83 (br, 1H). MS (EI) *m/e* (relative intensity) 431 (M^+, 100). +TOF MS HRMS Calcd for ($C_{26}H_{29}N_3O_3$+ H)$^+$*m/z* = 432.2287, found *m/z* = 432.2292.

5.48. 2-Isoprenyl-3-(6-nitro-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (69)

Tryprostatin B **2** (20 mg, 0.056 mmol) was dissolved in anhydrous THF (5 mL) and trifluoroacetic acid (2 mL) was added, after which the mixture was cooled to −78 °C. A solution of $NaNO_2$ (20 mg, 0.28 mmol) in

TFA (2 mL) was slowly added to the cooled solution via a syringe over a 10 min period. The reaction mixture was stirred for an additional 30 min and then allowed to warm to −20 °C for 30 min, after which CH_2Cl_2 (10 mL) and cold aq NH_4OH (14%) were added until pH 8. The layers were separated and the aqueous layer was washed with CH_2Cl_2 (3 × 10 mL). The combined organic layers were washed with brine (10 mL) and dried (Na_2SO_4). After removal of the solvent under reduced pressure, the residue was purified by flash chromatography (silica gel, CH_2Cl_2/ethanol 20:1) to provide **69** (17.5 mg, 76%) as a yellow powder. **69**: IR ν_{max} (KBr) 3249, 2923, 1650, 1540, 1451, 1304.5 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.60 (s, 3H), 1.76 (s, 3H), 1.84–2.06 (m, 3H), 2.34 (s, 2H), 3.04 (dd, 1H, J = 10.86 Hz and J = 4.32 Hz), 4.12 (m, 2H), 4.35 (d, 1H, 8.74 Hz), 5.34 (s, 1H), 5.53 (s, 1H), 7.53 (d, 1H, J = 8.82 Hz), 8.03 (d, 1H, J = 6.00 Hz), 8.29 (s, 1H), 8.53 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.08, 17.96, 22.47, 24.30, 25.66, 28.28, 45.38, 54.57, 59.15, 95.47, 106.08, 107.60, 115.52, 117.38, 118.34, 128.05, 129.42, 132.82, 143.15, 165.78, 169.19. EIMS *m/e* (relative intensity) 396 (M^+, 22), 355 (10), 381 (23), 243 (100). Anal. Calcd for ($C_{21}H_{24}N_4O_4$) C, H, N.

5.49. 2-Isoprenyl-3-(6-amino-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (70)

Iron (III) chloride hexahydrate (25 mg, 0.063 mmol) and active carbon (2 mg, 0.16 mmol) were added to a solution of **69** in MeOH (4 mL). After the reaction mixture was heated to reflux, hydrazine monohydrate (0.1 mL) was added dropwise. The reaction mixture was allowed to stir at reflux until analysis by TLC (silica gel) indicated the disappearance of starting material (3 h). The reaction was then cooled to rt and the catalysts were removed by filtration through a pad of celite. The CH_3OH was removed under vacuum and the residue was dissolved in CH_2Cl_2. The organic layer was then washed with water, brine, and dried (Na_2SO_4). After the $CH_2Cl_2$was removed under reduced pressure, the residue was purified by a short wash column (silica gel, CH_2Cl_2/ethanol, 20:1) to provide **70** as an oil (20.6 mg, 88%). **70**: ^1H NMR (300 MHz, $CDCl_3$) δ 1.77 (m, 4H), 1.99–2.06 (m, 5H), 2.33 (s, 1H), 2.89 (t, 1H, J = 14.04 Hz), 3.41 (m, 3H), 3.59–3.67 (m, 4H), 4.12 (s, 1H), 4.34 (d, 1H, J = 9.03 Hz), 5.29 (dd, 1H, J = 4.80 Hz and J = 7.20 Hz), 5.67 (s, 1H), 6.55 (d, 1H, J = 6.60 Hz), 6.64 (s, 1H), 7.28 (d, 1H, J = 3.60 Hz), 7.69 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 17.85, 22.55, 24.94, 25.63, 27.47, 28.26, 45.29, 54.46, 59.17, 96.49, 104.42, 110.30, 118.34, 120.04, 121.27, 134.02, 134.90, 136.74, 142.04, 165.78, 169.20. EIMS *m/e* (relative intensity) 366 (M^+, 10), 198 (62), 165 (100). Anal. Calcd for ($C_{21}H_{26}N_4O_2H_2O$) C, H, N. This material was converted into the hydrochloride salt for storage purposes.

5.50. 2-Isoprenyl-3-(6-isothiocyanato-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (71)

6-Aminotryprostatin B **70** (20 mg, 0.055 mmol) was dissolved in $CHCl_3$ (4 mL) and thiophosgene (0.2 mL, 0.003 mmol) was added dropwise at rt. The reaction mixture was stirred for 4 h and then the solution was treated with triethylamine (2 mL). After the solvent was removed under reduced pressure, the residue was purified by a short wash column (silica gel, CH_2Cl_2/ethanol, 20:1) to provide **71** as an oil (20.0 mg, 91%). **71**: IR ν_{max} (KBr) 2961, 2120, 1615 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.59 (s, 3H), 1.75 (s, 3H), 1.82 (s, 3H), 2.34 (m, 1H), 2.97 (dd, 1H, J = 10.86 Hz and J = 11.16 Hz), 3.50 (s, 2H), 3.63 (m, 3H), 4.14 (s, 1H), 4.32 (d, 1H, J = 6.96 Hz), 5.31 (s, 1H), 5.61 (s, 1H), 7.03 (d, 1H, J = 6.72 Hz), 7.28 (s, 1H), 7.42 (d, 1H, J = 8.52 Hz), 8.07 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 17.95, 22.53, 25.13, 25.49, 25.68, 28.31, 29.62, 45.36, 54.49, 59.19, 105.33, 108.07, 118.41, 118.50, 118.94, 124.68, 127.27, 134.81, 136.20, 138.83, 165.37, 169.26. EIMS *m/e* (relative intensity) 408 (M^+, 18), 255 (100). Anal. Calcd for ($C_{22}H_{24}N_4O_2S0.3H_2O$) C, H, N.

5.51. 2-Isoprenyl-3-(6-azido-1*H*-indol-3-ylmethyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione (72)

6-Aminotryprostatin B **70** (15 mg, 0.041 mmol) was dissolved in CH_2Cl_2 (2 mL). Triethylamine (0.1 mL, 0.72 mmol) and a solution of $CuSO_4$ (2.0 mg, 0.014 mmol) in H_2O (0.05 mL) were added to the reaction mixture. A freshly prepared solution of TfN_3 (21 mg, 0.12 mmol) in CH_2Cl_2 (1 mL) was then added, and the solution which resulted was brought to homogeneity by adding MeOH (1 mL). The solution which resulted was stirred at rt for 2 h. The reaction solution was then poured into a saturated solution of aq $NaHCO_3$ (5 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were washed with brine (10 mL) and dried (Na_2SO_4). After the solvent was removed under reduced pressure, the residue was purified by a short wash column (silica gel, CH_2Cl_2/EtOH, 20:1) to provide **72** as an oil (14.5 mg, 90%). **72**: IR ν_{max} (KBr) 2960, 2112.2, 1615 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.77 (m, 6H), 2.06 (s, 3H), 2.34 (s, 1H), 3.00 (t, 1H, J = 8.67), 3.47 (s, 2H), 3.60 (m, 3H), 4.12 (t, 1H, J = 7.20), 4.33 (d, 1H, J = 9.63), 5.32 (s, 1H), 5.61 (s, 1H), 6.83 (d, 1H, J = 6.60), 6.99 (s, 1H), 7.38 (d, 1H, J = 12.00), 7.96 (s, 1H). EIMS *m/e* (relative intensity) 392 (M^+, 28). HRMS Calcd for $C_{21}H_{24}N_6O_2$ *m/z* = 392.1961, found *m/z* = 392.1957.

5.52. Topoisomerase II-mediated DNA relaxation assay[52,53]

The DNA relaxation assay tests the ability of a drug to inhibit the Topo II-mediated relaxation of supercoiled DNA. The assay was performed in a total volume of 10 μL and contained 62.5 μg of plasmid pUC18 DNA (from *Escherichia coli*) and 100 μM drug in an incubation assay buffer (0.05 M Tris–HCl, pH 8.0, 0.12 M KCl, 0.01 M $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT, 30 mg/mL BSA). Stock solutions of drug were made up in either DMSO or EtOH and the total percentage of these in the assay mixture was kept to less than 1%. The mixture was allowed to warm to 37 °C and the reaction was initiated by the addition of 2.0 U of Topo II (TopoGEN, Inc.). The reaction was allowed to proceed for 30–45 minutes before being stopped by addition of

2.5 μL decatenation buffer (5% sarkosyl, 25% glycerol, and 0.0025% bromophenol blue). The drugs were then extracted from the incubation with 10 μL of 24:1 $CHCl_3$: isoamyl alcohol and the samples loaded onto a 1% agarose gel to run for 90 min at 90 V. The gel was stained with ethidium bromide and destained with H_2O. The DNA bands were detected on a UV light box and photographed with Polaroid 525 film. Controls were no-enzyme, enzyme, 100 μM *m*-AMSA, and 1% DMSO.

The gels were analyzed qualitatively by looking for the presence of DNA bands that migrate farther down on the gel than the negative controls. Topo II-mediated relaxation of the DNA prevents the band from migrating down the gel as far as one that is still in a supercoiled form. Therefore, DNA incubated with Topo II inhibitors will migrate farther on the gel than the no-enzyme or DMSO controls.

5.53. Microtubule assembly assay[13,54]

Tubulin, containing MAPs (microtubule-associated proteins), was prepared as described in the literature.[59] The tubulin polymerization assay was run at 37 °C by adding to 240 μL of PME buffer (100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$), 8 μL of GTP (50 mM), 32 μL of drug in DMSO, and 120 μL of tubulin (added last). The change in absorbance was measured at 351 nm over 10 min. The sample cuvette was zeroed against a reference cuvette containing 360 μL TBE buffer, 8 μL GTP (50 mM), and 32 μL DMSO. The concentration of the drug solution was varied for different runs to obtain a delta absorbance versus concentration curve. Standard curves were prepared on each batch of separately prepared tubulin using colchicine as the standard. Polymerization assays were conducted on the tryprostatins (**1–8**) and similar derivative (colchicine).

5.54. Cytotoxicity assay

Three human cell lines were purchased from American Type Culture Collection (ATCC) and used in all cytotoxicity assays. The MCF-7 breast adenocarcinoma cells, NCI-H520 lung squamous cell carcinoma cells and PC-3 prostate adenocarcinoma cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were subcultured twice a week in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 0.1 mM non-essential amino acids.

Normally growing cells were plated at 1×10^4 cells/well into 96-well plates and incubated for 24 h at 37 °C. After 24 h, the cells were drugged for initial screening with 100 μM, 50 μM, and 10 μM drug dissolved in a DMSO or EtOH vehicle (less than 1% in culture medium). Any drug showing <50% cell survival at 100 μM was further tested using appropriate drug concentrations to determine its growth inhibition-50% (GI_{50}). Drugs were run in quadruplicate or greater and control wells contained an appropriate percentage of DMSO or EtOH, usually 0.2%. Positive controls were either etoposide (ETOP) or amsacrine (*m*-AMSA).

After incubation with drug for 72 h, the CellTiter 96™ AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) was used to evaluate cell survival. Cells were treated with a solution of MTS [3-(4,5-dimethylthiozol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and an electron coupling reagent, PMS (phenazine methosulfate) diluted in RPMI-1640 medium. MTS (Owen's reagent)[60] was bioreduced by viable cells into formazan, and the amount of formazan present can be measured by reading the absorbance at 490 nm.[61] The amount of formazan present was proportional to the number of living cells in culture. Vehicle control lanes were assumed to have 100% cell survival and the percentage of cells remaining in the drug-treated wells was calculated as a percentage of these control wells. The absorbance of wells containing only the MTS reagent (the plate blank) was subtracted from all wells.

5.55. Cell culture

A temperature-sensitive *cdc2* mutant cell line, tsFT210, which was isolated from the mouse mammary carcinoma cell line FM3A, was a kind gift from Dr. F. Hanaoka (RIKEN).[4] The tsFT210 cells were maintained in RPMI 1640 with 10% fetal calf serum at the permissive temperature of 32 °C.

5.56. Cell cycle analysis

In a synchronous-culture assay, cells were seeded at a density of 1×10^5 cells/mL in 0.5 mL into a 24-well plate and were preincubated at 32 °C for 1 h. Then, 5 μL of each sample solution was added, and the cells were incubated at 32 °C for 18 h. After incubation, morphological characteristics of the cells were examined by microscopic observation. The cells were subjected to flow cytometric analysis as described below to confirm the DNA contents in cells.

Flow cytometric analysis was performed essentially as described by previous reports.[4,58] The harvested cells were stained with solution containing 50 μg/mL propidium iodide, 0.1% sodium citrate, and 0.2% NP-40 and analyzed for DNA contents using a flow cytometer (Coulter Co., Hialeah, FL).

5.57. Cell staining

Carnoy fixation and staining were performed with slight modification. Cells were treated with 0.55% of KCl for 20 min, fixed in Carnoy's solution and dropped onto a wet glass slide. The chromosomes and intact nuclei were stained with 1 mg/mL of Hoechst 33258, and examined by using fluorescent microscopy (Olympus, Tokyo, Japan).

5.58. Proliferation assay

Exponentially growing tsFT210 cells were treated with test compounds at 32 °C for 48 h. The cell number was evaluated by the subsequent color reaction. The 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-

disulfophenyl)-2*H* tetrazolium, mono-sodium salt, WST-8™ (Nakalai Tesque, Kyoto, Japan) was added, and the cells were further incubated for 4 h at 37 °C. The absorbance (A_{450}) of each well was measured by a Wallac 1420 multilabel counter (Amersham Biosciences, Piscataway, NJ).

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Appendix A

Elemental analyses

Compound	Formula	Calculated (%)			Found (%)		
		C	H	N	C	H	N
1	$C_{22}H_{27}N_3O_3 \cdot 1/3H_2O$	68.19	7.20	10.84	68.21	7.16	10.85
2	$C_{21}H_{25}N_3O_2 \cdot 1/4H_2O$	70.86	7.22	11.81	70.88	7.25	11.85
3	$C_{22}H_{27}N_3O_3 \cdot 3/5H_2O$	67.36	7.25	10.71	67.33	7.26	10.75
4	$C_{21}H_{25}N_3O_2 \cdot 3/4H_2O$	69.11	7.32	11.51	69.11	7.28	11.55
5	$C_{22}H_{27}N_3O_3 \cdot 3/4H_2O$	66.90	7.27	10.64	66.92	7.26	10.67
6	$C_{21}H_{25}N_3O_2 \cdot 1/5H_2O$	71.04	7.21	11.84	71.06	7.24	11.79
7	$C_{22}H_{27}N_3O_3 \cdot 3/8H_2O$	70.41	7.25	11.73	70.44	7.26	11.75
8	$C_{21}H_{25}N_3O_2 \cdot 1/8H_2O$	71.31	7.20	11.88	71.34	7.21	11.85
12	$C_{26}H_{37}N_3O_5$	66.22	7.91	8.91	66.23	7.90	8.55
16	$C_{31}H_{45}N_3O_5$	68.99	8.40	7.79	68.69	8.66	7.40
17	$C_{30}H_{43}N_3O_4 \cdot 1/4H_2O$	70.08	8.53	8.17	70.10	8.56	8.15
29	$C_{28}H_{45}N_3O_3Si$	67.29	9.08	8.41	67.49	9.16	8.34
33	$C_{15}H_{20}N_2O_3$	65.18	7.30	10.14	64.96	7.36	10.24
35	$C_{21}H_{24}N_2O_3 \cdot H_2O$	68.11	7.03	7.58	67.71	6.68	7.81
37	$C_{18}H_{21}N_3O_3$	66.04	6.47	12.84	65.80	6.75	13.06
38	$C_{22}H_{27}N_3O_3$	69.27	7.13	11.02	69.03	7.28	11.29
39	$C_{24}H_{25}N_3O_3 \cdot 0.5H_2O$	69.90	6.31	10.19	69.54	5.89	9.87
40	$C_{20}H_{23}N_3O_3$	67.97	6.56	11.89	68.20	6.28	11.63
41	$C_{26}H_{37}N_3O_3$	71.04	8.48	9.56	71.39	8.01	9.82
45	$C_{22}H_{30}N_2O_3$	71.32	8.16	7.56	70.90	7.81	7.89
46	$C_{29}H_{33}N_3O_3 \cdot H_2O$	71.02	7.14	8.57	70.61	6.75	8.28
48	$C_{26}H_{36}N_3O_5Br$	56.73	6.59	7.63	56.97	6.47	7.48
49	$C_{33}H_{43}N_3O_5$	70.56	7.72	7.48	70.23	7.98	7.14
51	$C_{32}H_{41}N_3O_5 \cdot H_2O$	67.96	7.61	7.43	67.51	7.24	7.94
55	$C_{24}H_{25}N_3O_3$	71.44	6.25	10.41	71.09	6.56	10.05
58	$C_{30}H_{41}N_3O_7$	64.85	7.44	7.56	65.22	7.10	7.98
61	$C_{14}H_{18}N_2O_3$	64.12	6.87	10.69	63.96	6.98	10.54
62	$C_{17}H_{19}N_3O_3$	65.16	6.11	13.41	64.93	6.36	13.68
66	$C_{21}H_{21}N_3O_3 \cdot 0.5H_2O$	67.74	5.91	11.29	67.36	5.49	11.73
69	$C_{21}H_{24}N_4O_4$	63.62	6.10	14.13	63.96	5.73	14.47
70	$C_{21}H_{26}N_4O_2 \cdot H_2O$	65.62	7.29	14.58	65.20	14.26	15.02
71	$C_{22}H_{24}N_4O_2S \cdot 0.3H_2O$	63.86	5.95	13.55	63.52	5.54	13.13

High resolution mass spectra (HRMS) (EI)

Compound	Formula	Calculated mass	Found Mass
1	$C_{22}H_{27}N_3O_3$	381.2052	381.2044
13	$C_{25}H_{35}N_3O_4$	441.2628	441.2536
14	$C_{26}H_{37}N_3O_5$	471.2733	471.2739
15	$C_{25}H_{35}N_3O_4$	441.2628	441.2634
20	$C_{24}H_{34}N_2O_5$	430.2468	430.2481
47	$C_{25}H_{31}N_3O_3 + Li^+$	428.2525	428.2519

(*continued on next page*)

Appendix A (*continued*)

Compound	Formula	Calculated mass	Found Mass
50	$C_{29}H_{41}N_3O_5 + H^+$	512.3124	512.3126
56	$C_{20}H_{23}N_3O_3 + Li^+$	360.1899	360.1896
60	$C_{21}H_{23}N_3O_5$	397.1638	397.1657
64	$C_{21}H_{23}N_3O_5 + Na^+$	370.0934	370.0935
67	$C_{22}H_{29}N_3O_3 + H^+$	384.2287	384.2282
68	$C_{26}H_{29}N_3O_3 + H^+$	432.2287	432.2292
72	$C_{21}H_{24}N_6O_2$	392.1961	392.1957

References and notes

1. Nasmyth, K. *Science* **1996**, *274*, 1643.
2. Paulovich, A. G.; Toczyski, D. P.; Hartwell, L. H. *Cell* **1997**, *88*, 315.
3. Pardee, A. B. *PNAS* **1974**, *71*, 1286.
4. Osada, H.; Cui, C.-B.; Onose, R.; Hanaoka, F. *Bioorg. Med. Chem.* **1997**, *5*, 193.
5. Osada, H. *J. Antibiotics* **1998**, *51*, 973.
6. Cui, C.-B.; Kakeya, H.; Okada, G.; Onose, R.; Ubukata, M.; Takahashi, I.; Isono, K.; Osada, H. *J. Antibiotics* **1995**, *48*, 1382.
7. Cui, C.-B.; Kakeya, H.; Osada, H. *J. Antibiotics* **1996**, *49*, 534.
8. Cui, C.-B.; Kakeya, H.; Okada, G.; Onose, R.; Osada, H. *J. Antibiotics* **1996**, *49*, 527.
9. Gan, T.; Cook, J. M. *Tetrahedron Lett.* **1997**, *38*, 1301.
10. Ma, C.; Liu, X.; Li, X.; Flippen-Anderson, J.; Yu, S.; Cook, J. M. *J. Org. Chem.* **2001**, *66*, 4525.
11. Zhao, S.; Gan, T.; Yu, P.; Cook, J. M. *Tetrahedron Lett.* **1998**, *39*, 7009.
12. Zhao, S.; Smith, K. S.; Deveau, A. M.; Dieckhaus, C. M.; Johnson, M. A.; Macdonald, T. L.; Cook, J. M. *J. Med. Chem.* **2002**, *45*, 1559.
13. Usui, T.; Kondoh, M.; Cui, C.-B.; Mayumi, T.; Osada, H. *Biochem. J.* **1998**, *333*, 543.
14. Amos, L. A. *Curr. Opin. Struct. Biol.* **2000**, *10*, 236.
15. Maiato, H.; Sampaio, P.; Sunkel, C. E. *Int. Rev. Cytol.* **2005**, *241*, 53.
16. Hamel, E.; Campo, A. A. d.; Lowe, M. C.; Waxman, P. G.; Lin, C. M. *Biochemistry* **1982**, *21*, 503.
17. Zhou, J.; Giannakakou, P. *Anti-Cancer Agents Med. Chem.* **2005**, *5*, 65.
18. Shi, Q.; Chen, K.; Morris-Natschke, S. L.; Lee, K.-H. *Curr. Pharm. Des.* **1998**, *4*, 219.
19. Mani, S.; Macapinlac, M.; Goel, S.; Verdier-Pinard, D.; Fojo, T.; Rothenberg, M.; Colevas, D. *Anti-Cancer Drugs* **2004**, *15*, 553.
20. Beckers, T.; Mahboobi, S. *Drugs Future* **2003**, *28*, 767.
21. Rowinsky, E.; Donehower, R. C. In *Cancer Principles and Practice of Oncology*; DeVita, V. T., Hellman, S., Rosenburg, S. A., Eds.; Lippincott-Raven: Philadelphia, 2001; pp. 431–452.
22. Pettit, G. R.; Singh, S. B.; Hamel, E.; Lin, C. M.; Alberts, D. S.; Garcia-Kendall, D. *Experientia* **1989**, *45*, 209.
23. Blokhin, A. V.; Yoo, H.-D.; Geralds, R. S.; Nagle, D. G.; Gerwick, W. H.; Hamel, E. *Mol. Pharmacol.* **1995**, *48*, 523.
24. Damayanthi, Y.; Lown, J. W. *Curr. Med. Chem.* **1998**, *5*, 205.
25. Flörsheimer, A.; Altmann, K.-H. *Expert Opin. Ther. Patents* **2001**, *11*, 951.
26. Poncet, J. *Curr. Pharm. Des.* **1999**, *5*, 139.
27. Simpson, D.; Wagstaff, A. J. *Am. J. Cancer* **2003**, *2*, 373.
28. Mazumdar, M.; Parrack, P. K.; Mukhopadhyay, K.; Bhattacharyya, B. *Biochemistry* **1992**, *31*, 6470.
29. Lage, H.; Dietel, M. *Lancet Oncol.* **2000**, *1*, 169.
30. Doyle, L. A.; Yang, W.; Abruzzo, L. V.; Krogmann, T.; Gao, Y.; Rishi, A. K.; Ross, D. D. *PNAS* **1998**, *95*, 15665.
31. Abbott, B. L. *Hematol. Oncol.* **2003**, *21*, 115.
32. Allen, J. D.; Schinkel, A. H. *Mol. Cancer Ther.* **2002**, *1*, 427.
33. Doyle, L. A.; Ross, D. D. *Oncogene* **2003**, *22*, 7340.
34. Benderra, Z.; Faussat, A.-M.; Sayada, L.; Perrot, J.-Y.; Chaoui, D.; Marie, J.-P.; Legrand, O. *Clin. Cancer Res.* **2004**, *10*, 7896.
35. Uggla, B.; Stahl, E.; Wagsaeter, D.; Paul, C.; Karlsson, M. G.; Sirsjoe, A.; Tidefelt, U. *Leukemia Res.* **2005**, *29*, 141.
36. Candeil, L.; Gourdier, I.; Peyron, D.; Vezzio, N.; Copois, V.; Bibeau, F.; Orsetti, B.; Scheffer, G. L.; Ychou, M.; Khan, Q. A.; Pommier, Y.; Pau, B.; Martineau, P.; Del Rio, M. *Int. J. Cancer* **2004**, *109*, 848.
37. Ross, D. D. *Leukemia* **2000**, *14*, 467.
38. Sauerbrey, A.; Sell, W.; Steinbach, D.; Voigt, A.; Zintl, F. *Br. J. Haematol.* **2002**, *118*, 147.
39. Ross, D. D.; Yang, W.; Abruzzo, L. V.; Dalton, W. S.; Schneider, E.; Lage, H.; Dietel, M.; Greenberger, L.; Cole, S. P.; Doyle, L. A. *J. Natl. Cancer Inst.* **1999**, *91*, 429.
40. Robey, R. W.; Medina-Perez, W. Y.; Nishiyama, K.; Lahusen, T.; Miyake, K.; Litman, T.; Senderowicz, A. M.; Ross, D. D.; Bates, S. E. *Clin. Cancer Res.* **2001**, *7*, 145.
41. Houghton, P. J.; Germain, G. S.; Harwood, F. C.; Schuetz, J. D.; Stewart, C. F.; Buchdunger, E.; Traxler, P. *Cancer Res.* **2004**, *64*, 2333.
42. Rabindran, S. K.; Ross, D. D.; Doyle, L. A.; Yang, W.; Greenberger, L. M. *Cancer Res.* **2000**, *60*, 47.
43. Woehlecke, H.; Osada, H.; Herrmann, A.; Lage, H. *Int. J. Cancer* **2003**, *107*, 721.
44. Maliepaard, M.; van Gastelen, M. A.; Tohgo, A.; Hausheer, F. H.; van Waardenburg, R. C. A. M.; de Jong, L. A.; Pluim, D.; Beijnen, J. H.; Schellens, J. H. M. *Clin. Cancer Res.* **2001**, *7*, 935.
45. Larock, R. C.; Yum, E. K.; Refvik, M. D. *J. Org. Chem.* **1998**, *63*, 7652.
46. Larock, R. C.; Yum, E. K. *J. Am. Chem. Soc.* **1991**, *113*, 6689.
47. Feldman, K. S.; Eastman, K. J.; Lessene, G. *Org. Lett.* **2002**, *4*, 3525.
48. Dieck, H. A.; Heck, F. R. *J. Organomet. Chem.* **1975**, *93*, 259.
49. Lifer, S. Master thesis, University of Wisconsin—Milwaukee, Milwaukee, WI, 1987.
50. Nyffeler, P. T.; Liang, C.-H.; Koeller, K. M.; Wong, C.-H. *J. Am. Chem. Soc.* **2002**, *124*, 10773.
51. Liu, Q.; Tor, Y. *Org. Lett.* **2003**, *5*, 2571.
52. Spitzner, J. R.; Muller, M. T. *Nucleic Acids Res.* **1988**, *16*, 5533.

53. Muller, M. T.; Spitzner, J. R.; DiDonato, J. A.; Mehta, V. B.; Tsutsui, K.; Tsutsui, K. *Biochemistry* **1988**, *27*, 8369.
54. Kondoh, M.; Usui, T.; Mayumi, T.; Osada, H. *J. Antibiotics* **1998**, *51*, 801.
55. Depew, K. M.; Danishefsky, S. J.; Rosen, N.; Sepp-Lorenzino, L. *J. Am. Chem. Soc.* **1996**, *118*, 12463.
56. We thank the Developmental Therapeutics Program of the National Cancer Institute for performing the in vitro anticancer evaluation.
57. Turner, J. G.; Gump, J. L.; Zhang, C.; Cook, J. M.; Marchion, D.; Hazlehurst, L.; Munster, P.; Schell, M. J.; Dalton, W. S.; Sullivan, D. M. *Blood* **2006**, *108*, 3881.
58. Kakeya, H.; Onose, R.; Liu, P. C.; Onozawa, C.; Matsumura, F.; Osada, H. *Cancer Res.* **1998**, *58*, 704.
59. Bulman, A. L. Kinases and Drugs Targeting the microtubule System. Ph.D. Thesis, University of Virginia, Charlottesville, VA, 1998.
60. Barltrop, J. A.; Owen, T. C.; Cory, A. H.; Cory, J. G. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 611.
61. Cory, A. H.; Owen, T. C.; Barltrop, J. A.; Cory, J. G. *Cancer Commun.* **1991**, *3*, 207.

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NOTE

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Fungal Metabolite, Epoxyquinol B, Crosslinks Proteins by Epoxy-thiol Conjugation

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Abstract Epoxyquinol B (EPQB) is a fungal metabolite, containing two α,β-epoxy ketones. We previously showed that EPQB inhibited the signal transduction involved in angiogenesis through the binding to cysteine residues of receptor kinases. However, the inhibitory mechanism was undefined. In this report, we found that one EPQB molecule is covalently bound to two *L*-cysteine molecules through two epoxide residues on EPQB. Furthermore, EPQB crosslinked binding proteins through the cysteine residues. These results suggest that EPQB inhibits receptor kinases by crosslinking with other protein or by intramolecular crosslinking.

Keywords epoxyquinol B, crosslink, epoxide, cysteine, epoxyquinoid, fungal metabolite

It is well known that epoxides can be opened by direct attack of the nucleophile on the epoxide ring. Epoxide containing compounds are able to directly form stable adducts with proteins containing thiol or phenolic group, under mild experimental conditions [1]. We previously showed that epoxyquinol B (EPQB), a fungal metabolite and the epoxyquinoid containing two α,β-epoxy ketones, inhibited angiogenesis through the covalent binding to cysteine residues of VEGFR2, EGFR, FGFR, and PDGFRβ [2~4]. In this paper, we report the reaction mechanism between EPQB and cysteine residues of binding proteins, resulted in crosslinking of the binding proteins.

EPQB possesses the highly reactive functional group; two α,β-epoxy ketones which could form covalent bonds with nucleophiles such as a thiol (Fig. 1A). Therefore, we anticipated that EPQB has several reaction sites with cysteine residue of the binding protein. To investigate this possibility, we analyzed the reaction products of EPQB and *L*-cysteine by LC/MS analysis. EPQB and *L*-cysteine were incubated together in water for 10, 30, 60, and 120 minutes. Then, the products were analyzed by QTRAP LC/MS/MS system. As shown in Fig. 1B, four major peaks were detected in a time dependent manner. *L*-cysteine and EPQB were detected with *m/z* 120 and 387, ($[M-H]^-$) with retention times (Rt) of 1.74 and 29.2 minutes, respectively. Two *L*-cysteine adducts of EPQB were detected, and

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