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Structure–Activity Relationship for DNA Topoisomerase II-Induced DNA Cleavage by Azatoxin Analogues

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Abstract—Eighteen analogues of the nonintercalative DNA topoisomerase II (topo II)-active epipodophyllotoxin-ellipticine hybrid, azatoxin, were synthesized and evaluated for their ability to induce topo II-mediated DNA strand breaks in vitro. In general, the SAR profile of the azatoxins showed more homology with that of the epipodophyllotoxins than with the ellipticines. Of the compounds studied, only fluoro substitution at the 8-, 9, and 10-positions of azatoxins enhanced activity, with 9-fluoroazatoxin being the most active compound in this series. © 1997 Elsevier Science Ltd.

Introduction

DNA topoisomerase II (topo II) has been identified as the target for a number of clinically important chemotherapeutic agents including adriamycin, mitoxanthrone, etoposide, and teniposide.[1–4] Several other drugs (amonafide, amsacrine (m-AMSA), menogaril) also target topo II.[1–4] These agents induce the formation of a ternary complex of enzyme, DNA and drug, termed the 'cleavable complex', in which enzyme-mediated DNA cleavage is observed. It is widely believed that the cleavable complex represents a reaction intermediate along the catalytic cycle that has been stabilized by the agent and that the drug-induced cleavable complex initiates an undefined cascade of intracellular signals that culminates in cytotoxicity.

We have hypothesized that the drug binds to DNA in the cleavable complex to induce a particular DNA deformation conducive to enzyme mediated DNA cleavage.[5] Regardless of whether the drug binds initially to DNA or to a DNA-enzyme complex, the specific site for binding remains DNA for all topo II-directed agents and the particular DNA deformations are similar for all agents. Based on this premise, we have described a composite pharmacophore (Fig. 1) that accounts for the apparent structural diversity among several classes of topo II inhibitors.[5,6] The model describes four domains: (1) a planar polyaromatic array responsible for intercalation or nonintercalative DNA association; (2) a pendant group with a hydrogen bond donor 5.5 Å below the plane of the intercalation domain responsible for minor groove binding; (3) a variable substituent domain also responsible for minor groove binding; and (4) the acyl functionality domain which dictates the conformational relationships available to the three aforementioned domains. As illustrated by the ellipticines and the 4′-demethyl-4-desoxypodophyllotoxin series, the variable substituent domain is not required for topo II inhibition, but can have a profound effect on the potency[7] and selectivity[8] of the drug.

As part of our program aimed at the design of topo II-targeted agents, we undertook the development of epipodophyllotoxin-ellipticine hybrids based on our model pharmacophore. As shown in Figure 2, the superimposition of the hypothetical domains of etoposide, m-AMSA, and ellipticine illustrates our rationale for the structural progression to our 'first generation' target hybrid: azatoxin **1**.[9] Our initial efforts were guided by two important observations from our laboratory: (1) the epipodophyllotoxin nucleus, as illustrated by etoposide and teniposide, is the most efficient structure yet identified at stabilizing cleavable complex per DNA association event;[6] and (2) 4′-demethyl-4-desoxypodophyllotoxin exhibits topo II-mediated activity comparable to etoposide and thus the glucose acetal moiety of the epipodophyllotoxins of etoposide is not critical for activity.

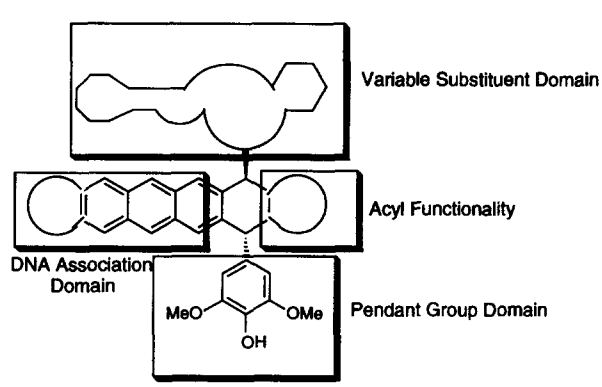


Figure 1. Composite pharmacophore for expression of DNA topoisomerase II activity.

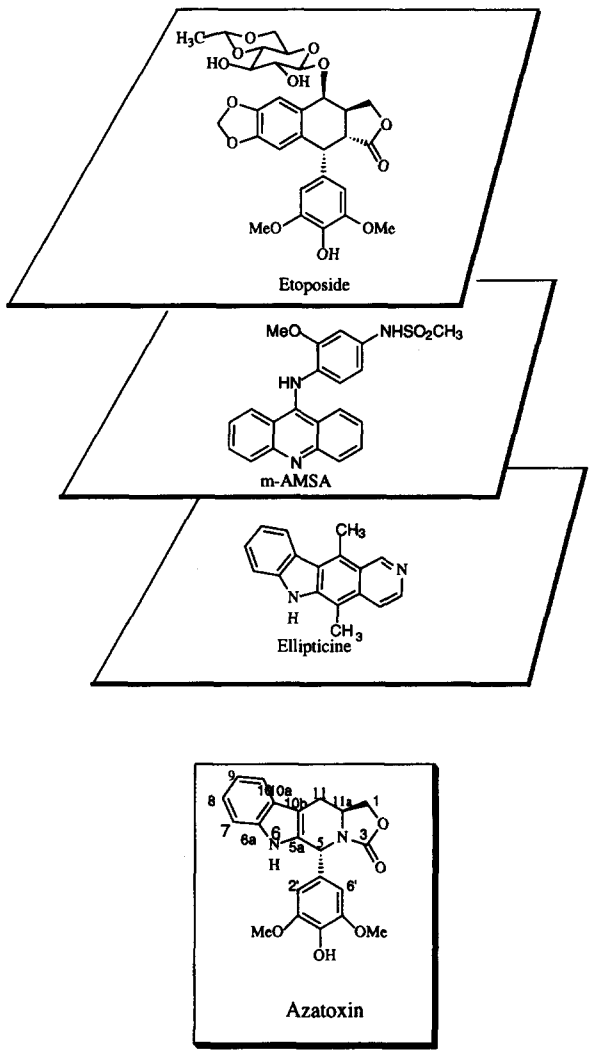


Figure 2. Development of model pharmacophore.

We have recently described the structure–activity relationship (SAR) of azatoxins substituted in the variable substituent domain and elucidated the effects of selectivity and potency of these substituted azatoxin analogues.[7,10] The studies described here detail the synthesis and biochemical evaluation of azatoxin analogues that have been modified in the domains embodied in the DNA associative, indole region and in the pendant dimethoxyphenol domains. These analogues help to further define the structural contributions to activity from each of these pharmacophore domains and to refine our model. In addition, our extensive structural analysis of azatoxin **1** by X-ray crystallography and molecular mechanics calculations revealed that significant overlap of the aromatic regions existed between **1** and its 5,11*a*-*cis*-diastereomer **5**. Therefore, our studies and analysis of this azatoxin diastereomer **5** are also detailed.

Chemistry

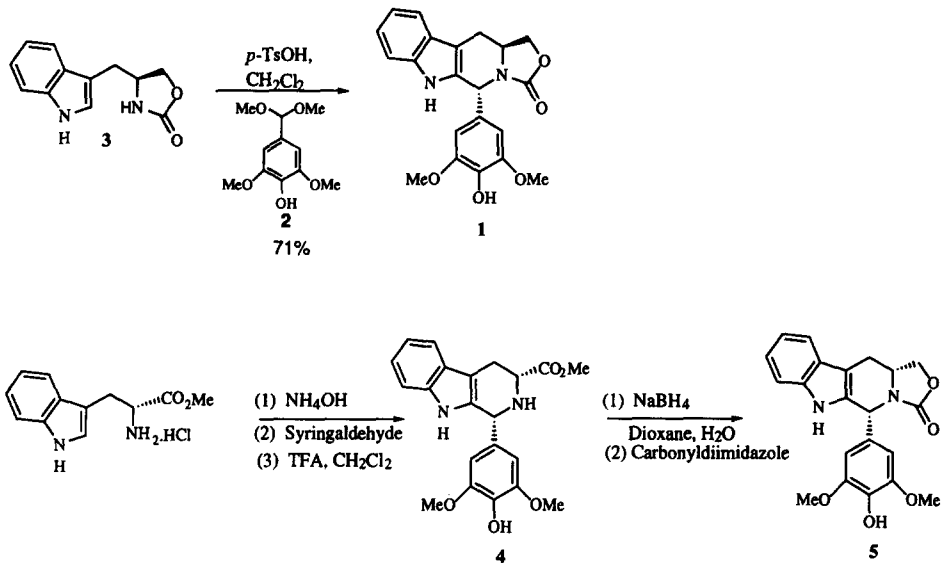
A mild method of effecting cyclization was developed by treating syringaldehyde dimethyl acetal **2** with catalytic *p*-TsOH (15 mol%) in CH_2Cl_2 followed by introduction of the oxazolidinone **3**.[7] Yields normally ranged 50–70% of a single diastereomer. The correct 1,3-*anti*/*trans*-stereochemistry was confirmed by a positive nuclear Overhauser effect (NOE) between the $C_{2'}$-hydrogens of the pendant aromatic ring and the C_{11a} ring junction hydrogen. This synthetic approach was applicable to the synthesis of the pendant group analogues by the condensation of the oxazolidinone with either the corresponding dimethyl acetal and a catalytic amount of *p*-TsOH or the corresponding aldehyde and 2 equiv of H_2SO_4.[11] Similarly, the indole substituted analogues could be synthesized from the appropriately substituted tryptophan methyl esters. All compounds in this series were synthesized by the aforementioned methodology, except **16** and **18**, which were synthesized with some minor modifications.

As illustrated in Scheme 1, Pictet–Spengler methodology[12] is also applicable to the synthesis of the 5,11*a*-*cis*-diastereomer. Condensation of tryptophan methyl ester with syringaldehyde in the presence of Na_2SO_4 followed by cyclization with TFA (2 equiv) at 0 °C yielded **4** as a single diastereomer in 93% yield.[13] Reduction of the ester **4** with $NaBH_4$ in 1:1 dioxane: water followed by cyclization with carbonyl diimidazole yielded the correct diastereomer **5** in 37% yield for both steps.

Results and Discussion

Compounds **1** and **5–22** were assayed for their ability to induce protein-associated DNA strand breaks in the presence of linearized pUC18 DNA and topo II isolated from human placenta,[14,15] which is a (1:1) mixture of the topo II a and b isozymes. The results are summarized in Tables 1 and 2. Azatoxin **1** was found to be as active as etoposide[9,16] in inducing the formation of cleavable complex and compound **6** exhibited significantly diminished activity; all other pendant group analogues were inactive (Table 1).

It was initially hypothesized that the aminoacridine substituent of amsacrine (m-AMSA) was located in the pendant group domain of our pharmacophore model. A comparison of the SAR for the aromatic pendant groups and the earlier reported variable substituent domain of azatoxin and m-AMSA analogues reveals that the aminoacridine substituent of m-AMSA is located in the variable substituent domain of azatoxin. The previously reported comparison of the SAR of the variable substituent domain of azatoxin and the aminoacridine substituent of m-AMSA (the 4′-aminobenzenesulfonamide portion of m-AMSA) shows that the SAR of the aminoacridine substituent of m-AMSA follows the SAR of the variable substituent domain of azatoxin.[5] In addition, there is also a key difference in the SAR profile of the aminoacridines and the azatoxins pendant group (Fig. 1) on the extent of steric crowding around the 4′-position and the resulting activity (numbers of the positions are illustrated in Fig. 2).



Scheme 1.

Azatoxins with increased steric crowding around the 4′-position (in the form of methoxy groups; i.e., **7** is inactive) exhibit increased activity, while the analogously substituted aminoacridines are generally less active.[5] However, if the different azatoxins impart a different DNA distortion on binding, molecular modeling may be required to discern the specific modes in which the different pendant groups affect .

The pendant group order of activity for the azatoxins follows the same order of activity found for etoposide: dimethoxyphenol **1** > methoxyphenol **6** > phenol **7** = trimethoxyphenyl **8**.[17] Of note, while in the azatoxin series the phenol **7** and trimethoxyphenyl **8** pendant group analogues are completely inactive, in the etoposide series, these pendant groups retain limited activity.

Further evidence for the homology in SAR profiles for the azatoxins and the epipodophyllotoxins is reflected in the activity of the azatoxin enantiomer which possesses the same absolute stereochemistry as etoposide: the other enantiomer **12** is inactive. In addition, although the diastereomer **5** possesses a similar relationship between the DNA intercalation/DNA association domain and the pendant group, **5** demonstrated no activity.

Substitution at the indole region of azatoxin showed that only fluoro substitution at the 8-, 9-, and 10-positions resulted in activity (numbers of the positions are illustrated in Fig. 2). The 9-fluoroazatoxin **18** was the most active in this series. Although the pendant group SAR profile exhibits homology with the epipodophyllotoxin SAR profile, a comparative evaluation of the ellipticine and the azatoxin SAR data reveals limited homology between the SAR profiles of these classes of agents. For example, while the 9-fluoro, 9-bromo, and 9-methoxy ellipticines are less active than ellipticine,[18] the analogous 9-fluoro azatoxin **18** is considerable more active than azatoxin **1** and the 9-bromo **20** (Fig. 3) and 9-methoxy **21** azatoxins are less

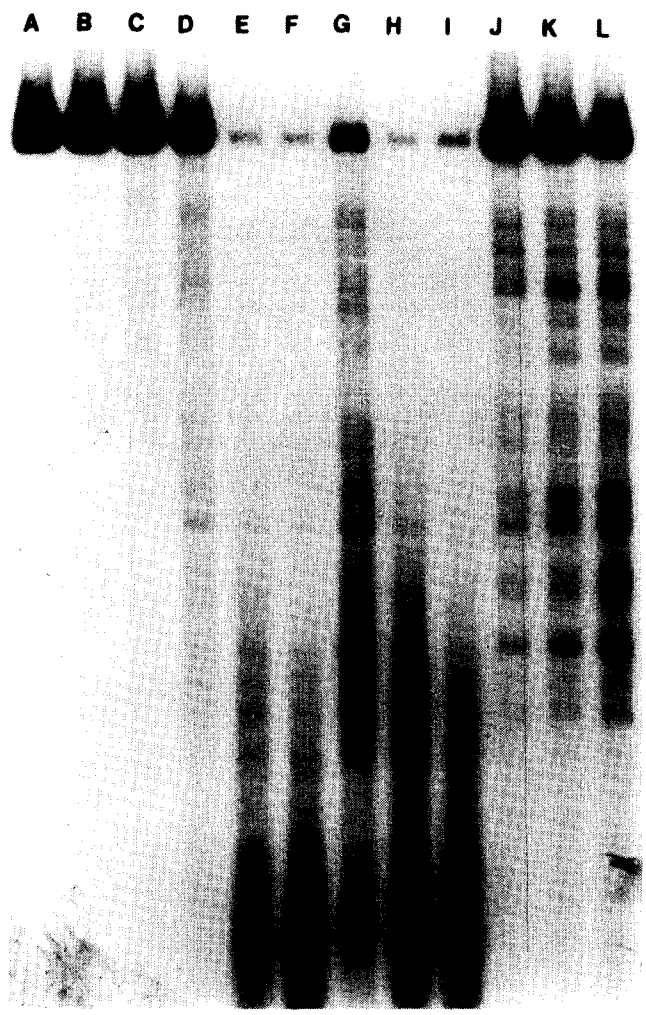
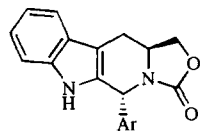
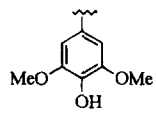
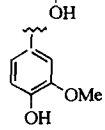
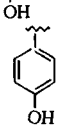
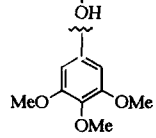
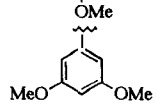
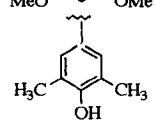
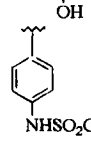


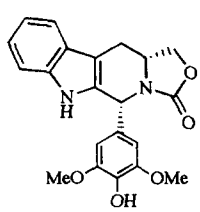
Figure 3. Lane A, DNA; lane B; DNA and topo II; lane C, DNA, topo II and 1% DMSO; lanes D–F, 10, 50, and 100 μM **1**; lanes G–I, 10, 50, and 100 μM **18**; lanes J–L, 10, 50, and 100 μM **17**; lanes M–O, 10, 50, and 100 μM **20**.

Table 1. Biological evaluation of azatoxin substituted in the pendant group domain

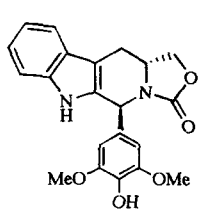


Compound	Ar	Activity
Etoposide		1[9,16]
Azatoxin, **1**		1[9,16]
6		0.3
7		Inactive
8		Inactive
9		Inactive
10		Inactive
11		Inactive

*Relative DNA cleavage was determined by densitometric analysis of the protein-associated DNA cleavage assays and quantified through comparison with the previously reported drug-induced cleavage of several of the azatoxin derivatives.[7-9] The cleavage activities are expressed relative to azatoxin and were reproducible within 30% of the reported activities.



5
inactive



12
inactive

active than **1**.[18-20] Furthermore, a discrepancy in the activity profiles is also reflected in the reduced activity of 9-hydroxy azatoxin **22** (vs **1**), while the corresponding 9-hydroxy ellipticine is the most active agent in that class of molecules.[21] In addition, while *N*-methylation of the 6-position of ellipticine generally increases activity, the analogous 6*N*-methyl azatoxin **16** is inactive.[21] An explanation for the discrepancy in activity between the most active analogues in each class of molecules is not readily apparent.

A general trend that is readily apparent in the azatoxin series is a relationship between the size of the substituents on the indole ring and activity. This is evidenced in the inactivity of all methylated and 9-methoxylated analogues; while the corresponding fluoro derivatives remain active. The reduced activity of the 9-bromo derivative **20** also supports this observation. The negative contribution to activity imparted by larger indole substituents is consistent with the assertion that the indole ring is strongly associated with the DNA base pairs. The bulky substituents would hinder this association or would influence the conformation of the drug–DNA complex. The activity difference between the 9-methyl **14** and 9-bromo **20** derivatives can thus be attributed to the rotational degrees of freedom available to **14** which would make this DNA association more difficult. Ascribing the azatoxin SAR for the F-, Br-, methyl-, methoxyl-, and hydroxyl- derivatives to electronic or hydrophobic effects is not consistent with observed relationships. In conclusion, we have illustrated that the SAR of the pendant group of azatoxin, as well as the DNA intercalation/association domain of azatoxin, as defiined in Figure 2, showed more homology with the epipodophyllotoxins series than with the ellipticines. Of the compounds studied, 9-fluoroazatoxin **18**, was the most potent topoisomerase II inhibitor, more potent than azatoxin and etoposide, which is currently the most widely prescribed antineoplastic agent.

Azatoxin **1** and its 9-fluoro derivative **18** do not exhibit DNA intercalative activity, as assessed by the abilities of these azatoxins to displace ethidium bromide[22] from calf thymus DNA at concentrations up to 10 mM (data not shown). Thus, these compounds represent a new class of potent, nonintercalative topo II active agents. In addition, the DNA cleavage pattern induced by the azatoxins is unique[9] when compared with known topo II agents, although more commonality exists between the patterns elicited by the azatoxins and the epipodophyllotoxins, than between the azatoxins and the ellipticines. In concert with the DNA associative activities of the azatoxins, epipodophyllotoxins and ellipticines and the patterns of DNA cleavage, the SAR profiles for the azatoxins and the epipodophyllotoxins share greater similarities than the profiles for the azatoxins and the ellipticines.[9] These studies have provided the foundations for the future development of additional classes of structurally unique, potent non-intercalative topo II-directed agents.

Experimental

General procedures

^{1}H NMR spectra were taken on a General Electric QE300 spectrometer at 300 MHz. Mass spectra were recorded on a Finnegan MAT4615 GC/MS/DS instrument using chemical or electron impact ionization techniques. Elemental analyses were determined by Atlantic Microlab Inc. (Norcross, GA). Melting points were determined on a Thomas–Hoover UNI-MELT apparatus and are uncorrected.

Thin-layer chromatography was performed using E. Merck glass plates precoated with silica-gel 60 F-254 and visualized with phosphomolybdic acid/ethanol solution. Woelm silica 32–63 was employed for column chromatography which was carried out using a modified short/flash column technique.

Tetrahydrofuran was distilled from sodium benzophenone immediately prior to use. Dichloromethane was distilled from CaH_2 immediately before use. All chemicals were purchased from the Aldrich Chemical Co. except for D- and L-tryptophan methyl ester–HCl which was purchased from the Sigma Chemical Co.

All reactions were carried out under an argon atmosphere.

4*R*-(1*H*-Indol-3-ylmethyl)-2-oxazolidinone. Sodium (1.56 g, 68.1 mmol) was dissolved in absolute ethanol (150 mL) and L-tryptophanol (12.94 g, 68.02 mmol) in ethanol (100 mL) and diethyl carbonate (8.83 g, 74.8 mmol) were added. The solution was heated at reflux for 5 h and after cooling was concentrated. Saturated NH_4Cl (100 mL) and CH_2Cl_2 (200 mL) were added and after mixing well, the layers were separated. The organic layer was washed with CH_2Cl_2 (2 × 100mL) and the organic fractions were combined, dried over Na_2SO_4 and concentrated. Recrystallization from MeOH/H_2O yielded 11.66 g (79%) of a white solid: mp 155 °C; $[\alpha]^{24}_D$ −8.3° (*c* 1.25, MeOH). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17 (sb, 1H), 7.57 (d, *J* = 7.94 Hz,1H), 7.40 (d, *J* = 8.06 Hz, 1H), 7.19 (m, 2H), 7.08 (d, *J* = 2.2 Hz, 1H), 5.22 (sb, 1H), 4.50 (m, 1H), 4.21 (m, 2H), 3.04 (m, 2H); Anal. ($C_{12}H_{12}N_2O_2$) C, H, N; theoretical: C, 66.65; H, 5.60; N, 12.96; found: C, 66.62; H, 5.82; N, 12.59.

General method for the preparation of dimethyl acetals

A catalytic amount of *p*-TsOH (40 mg) was added to a solution of the aromatic aldehydes (1 g) in trimethyl orthoformate (7 mL) and the reaction was followed to completion by TLC. The solvent was removed under reduced pressure and the remaining oil was dissolved in $CHCl_3$ and filtered through a plug of silica. The solvent was again removed under reduced pressure and the remaining oil was stored in a desiccator until use.

Preparation of 5,11*a*-*trans*-azatoxins

Method A. Concentrated H_2SO_4 (4 mmol) was added to a solution of **3** (2 mmol) and the corresponding aldehyde (2 mmol) in a CH_2Cl_2:MeOH (9:1) solution (6 mL). The reaction was followed to completion by TLC (20% acetone in $CHCl_3$). The reaction mixture was added to sat $NaHCO_3$, the layers were separated and the aqueous layer was washed with CH_2Cl_2 (3 ×). The combined organic fractions were dried over Na_2SO_4 filtered and concentrated. The product was purified by flash chromatography (acetone–$CHCl_3$).

Method B. *p*-Toluenesulfonic acid (2 mmol) in $CHCl_3$ (4 mL) was added to a solution of the corresponding dimethyl acetal (3 mmol) and the solution was allowed to stir for 5 min. The carbamate **3** (2 mmol) in $CHCl_3$ (4 mL) was then added and the reaction mixture was followed to completion by TLC. If the reaction proceeded too slowly, the reaction was heated at reflux. Saturated $NaHCO_3$ was added. The layers were separated, and the aqueous layer was washed with CH_2Cl_2 (2 × 20 mL). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated. The product was purified by flash chromatography (acetone–$CHCl_3$).

Method C. Anhydrous TFA (0.2 mmol) was added to a solution of the corresponding dimethyl acetal (3 mmol) in anhydrous THF (8 mL) followed by the carbamate **3** (2 mmol) and the solution was heated at reflux. The reaction was followed to completion by TLC. After cooling, the solution was added to saturated $NaHCO_3$, the layers were separated, and the aqueous layer was washed with CH_2Cl_2 (3 × 20 mL). The combined organic fractions were dried over Na_2SO_4, filtered and concentrated. The product was purified by flash chromatography (acetone–$CHCl_3$).

5*R*,11*aS*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-1*H*,6*H*-oxazolo[3′,4′:1,6]pyrido-[3,4*b*]indol-3-one (1). Azatoxin was prepared as described in method C. Purification by flash chromatography (12% acetone in $CHCl_3$, R_f = 0.28) produced a white solid in 91% yield: mp dec slow 175 °C; $[\alpha]^{22}_D$ −139.6° (*c* 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, CD_3CN) δ 7.94 (sb, 1H), 7.51 (d, *J* = 7.91 Hz, 1H), 7.30 (d, *J* = 7.57 Hz, 1H), 7.09 (m, 2H), 6.59 (s, 2H), 6.27 (s, 1H), 5.88 (d, *J* = 1.7 Hz, 1H), 4.54 (dd app t, *J* = 8.3 Hz, 1H), 4.33 (m, 1H), 4.21 (dd, *J* = 8.5, 4.7 Hz, 1H), 3.75 (s, 6H), 3.16 (dd, *J* = 15, 4.6 Hz, 1H), 2.76 (ddd, *J* = 15, 10.38, 1.73 Hz, 1H). Anal. ($C_{21}H_{20}N_2O_5$) C, H, N; theoretical: C, 66.31; H, 5.30; N, 7.36; found: C, 66.23; H, 5.52; N, 7.33.

5*S*,11*aR*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-1*H*,6*H*-oxazolo[3,4,:1,6]pyrido[3,4*b*]-indol-3-one (12). The same procedure as in **1** was applied using the *4R*-enantiomer. $[\alpha]^{22}_D$ + 139.4° (*c* 1.0, $CHCl_3$).

5*R*,11*aS*-5,4,11,11*a*-Tetrahydro-5-(3-methoxy-4-hydroxyphenyl)-1*H*,6*H*-oxazolo[3′,4′:1,6] pyrido[3,4*b*]-indol-3-one (6). Compound **6** was prepared as described in method A. Purification by flash chromatography (15% acetone in $CHCl_3$, R_f = 0.30) and recrystallization from CH_3CN gave a white solid in 46% yield: mp dec 190 °C: $[\alpha]^{22}_D$ −187° (*c* 0.86, DMSO); ^{1}H NMR (CD_3CN) δ 8.94 (s, 1H), 7.51 (d, *J* = 7.59 Hz, 1H), 7.29 (d, *J* = 7.95 Hz, 1H), 7.09 (m, 2H), 6.91 (d, *J* = 1.7 Hz, 1H), 6.79 (d, *J* = 8.08 Hz, 1H), 6.74 (dd, *J* = 8.08, 1.7 Hz, 1H), 6.55 (sb, 1H), 5.90 (d, *J* = 1.5 Hz, 1H), 4.52 (dd app t, *J* = 7.94 Hz, 1H), 5.27 (m, 1H), 4.20 (dd, *J* = 8.21, 4.83 Hz, 1H), 3.78 (s, 3H), 3.16 (dd, *J* = 14.93, 8.48 Hz, 1H), 3.77 (ddd, *J* = 14.91 Hz, 10.07, 1.59 Hz, 1H). Anal. ($C_{20}H_{18}N_2O_4$) C, H, N; theoretical: C, 68.56; H, 5.18; N, 7.99; found: C, 68.46; H, 5.20; N, 7.99.

5*R*,11*aS*-5,4,11,11*a*-Tetrahydro-5-(4-hydroxyphenyl)-1*H*,6*H*-oxazolo[3′,4′:1,6]pyrido[3,4-*b*]indol-3-one (7). Compound **7** was prepared as described in method C. Purification by flash chromatography (20% acetone in $CHCl_3$, R_f = 0.28) followed by recrystallization from CH_3CN gave a white solid in 89% yield: mp dec 278–280 °C; $[\alpha]^{22}_D$ −168° (*c* 0.8, DMSO); ^{1}H NMR (DMSO-d_6) δ 9.47 (s, 1H), 7.43 (d, *J* = 7.56 Hz, 1H), 7.24 (d, *J* = 7.91 Hz, 1H), 6.99 (m, 4H), 6.70 (d, *J* = 8.5 Hz, 2H), 5.81 (s, 1H), 4.49 (dd app t, *J* = 8.03 Hz, 1H), 4.14 (m, 2H), 3.10 (dd, *J* = 12.14 Hz, 4.69 Hz, 1H), 2.68 (dd, *J* = 14.3, 10.6 Hz, 1H). Anal. ($C_{19}H_{16}N_2O_3/CH_3CN$) C, H, N; theoretical: C, 69.79; H, 5.29; N, 11.62; found: C, 69.69; H, 5.39; N, 11.52.

5*R*,11*aS*-5,4,11,11*a*-Tetrahydro-5-(3,4,5-trimethoxyphenyl)-1*H*,6*H*-oxazolo[3′,4′:1,6]pyrido[3,4*b*]indol-3-one (8). Compound **8** was prepared as described in method C. Purification by flash chromatography (7% acetone in $CHCl_3$, R_f = 0.30) and recrystallization from CH_3CN/water yielded a white solid in 91% yield: mp 223 °C; $[\alpha]^{22}_D$ −117° (*c* 1.1, $CHCl_3$); ^{1} H NMR (CD_3CN) δ 8.95 (s,1H), 7.51 (d, *J* = 7.64 Hz, 1H), 7.31 (d, *J* = 7.93 Hz, 1H), 7.09 (m, 2H), 6.61 (s, 2H), 5.89 (s, 1H), 4.57 (dd app t, *J* = 8.24 Hz, 1H), 4.35 (m, 1H), 4.23 (dd, *J* = 8.42, 4.68 Hz, 1H), 3.74 (s, 6H), 3.70 (s, 3H), 3.17 (dd, *J* =15.02, 4.48, Hz, 1H), 2.77 (dd, *J* = 14.85, 10.41 Hz, 1H). Anal. ($C_{22}H_{22}N_2O_5$/H_2O) C, H (not tested for N); theoretical: C, 66.19; H, 5.78; found: C, 66.27; H, 5.66.

5*R*,11*aS*-5,4,11,11*a*-Tetrahydro-5-(4-methanesulfonilide)-1*H*,6*H*-oxazolo[3′4′:1,6]pyrido[3,4*b*]indol-3-one (11). Compound **11** was prepared as described in procedure A. Purification by flash chromatography eluting with 25% acetone in $CHCl_3$ (R_f = 0.35) produced a white solid in 23% yield: mp dec 183 °C; $[\alpha]^{22}_D$ −144° (*c* 1.0, $CDCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75 (sb, 1H), 7.55 (d, *J* = 7.51 Hz, 1H), 7.37–7.15 (m, 6H), 6.34 (sb, 1H), 6.09 (s, 1H), 4.57 (t, *J* = 8.28 Hz, 1H), 4.27 (dd, *J* = 8.52, 4.89 Hz, 1H), 4.23-4.15 (m, 1H), 3.20 (dd, *J* = 14.98, 4.68 Hz, 1H), 3.05 (s, 3H), 2.89 (ddd, *J* = 15.06 Hz, 10.52, 1.68 Hz, 1H). Not tested for elemental analysis.

5*R*,11*aS*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethoxyphenyl)-1*H*,6*H*-oxazolo[3′,4′:1,6]pyrido[3,4*b*]indol-3-one (9). Compound **9** was prepared as described in procedure B. Purification by flash chromatography eluting with 7% acetone in $CHCl_3$ (R_f = 0.31) and recrystallization from toluene gave a white solid in 66% yield: mp 215 °C; $[\alpha]^{22}_D$ −173° (*c* 0.67, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.81 (sb, 1H), 7.53 (d, *J* = 7.56 Hz, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.23–7.13 (m, 2H), 6.49 (d, *J* = 2.19 Hz, 2H), 6.43 (t, *J* = 2.19 Hz, 1H), 6.02 (s, 1H), 4.56 (t, *J* = 8.28 Hz, 1H), 4.27–4.18 (m, 2H), 3.76 (s, 6H), 3.16 (dd, *J* = 15.02, 4.63 Hz, 1H), 2.86 (ddd, *J* = 15.02, 10.22, 1.37 Hz, 1H). Anal. (($C_{21}H_{20}N_2O_4)_5$/toluene) C, H (not tested for N); theoretical: C, 69.21; H, 5.53; found: C, 70.69; H, 5.79.

5*R*,11*aS*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethyl-4-hydroxyphenyl)-1*H*,6*H*-oxazolo[3′4″:1,6]pyrido[3,4*b*]-indol-3-one (10). Compound **10** was prepared as described in procedure C. Purification by flash chromatography eluting with 15% acetone in $CHCl_3$ (R_f = 0.45) and recrystallization from ethyl acetate/cyclohexane yielded a white solid (62%): mp dec 194–196 °C; $[\alpha]^{22}_D$ −184° (*c* 0.82, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) 7.81 (sb, 1H), 7.54 (d, *J* = 7.45 Hz, 1H), 7.31 (d *J* = 7.48 Hz, 1H), 7.25–7.14 (m, 2H), 6.94 (s, 2H), 5.98 (s, 1H), 4.77 (sb, 1H), 4.52 (t, *J* = 9.53 Hz, 1H), 4.23–4.17 (m, 2H), 3.15 (dd, *J* = 14.87, 4.02 Hz, 1H), 2.85–2.77 (m, 1H), 2.19 (s, 6H). Anal. (($C_{21}H_{20}N_2O_3)_5$/cyclohexane) C, H (not tested for N); theoretical: C, 72.40 (73.00)*; H, 5.78 9 (6.18)*; found: C, 73.05; H, 6.49. (*May have crystallized solvent molecule.)

1*S*,3*S*-1-(3,5-Dimethoxy-4-hydroxyphenyl)-3-methoxycarbomyl-1,2,3,4-tetrahydro-*b*-carboline (4). To a solution of D-tryptophan methyl ester hydrochloride (9.52 g, 37.4 mmol) in $CHCl_3$ (150 mL) was added 14% ammonium hydroxide (30 mL) and the biphasic mixture was allowed to stir for 1 h. The layers were separated and the aqueous layer was extracted with $CHCl_3$ (2 × 100 mL). The organic layers were combined, dried over Na_2SO_4 and concentrated to yield a yellow oil. The oil was dissolved in benzene (200 mL). Syringaldehyde (6.81 g, 37.4 mmol, and Na_2SO_4 (10 g) were added, and the solution was allowed to stir for 60 h (white precipitate forms). The mixture was again concentrated, and anhydrous CH_2Cl_2 (150 mL) and anhydrous TFA (5.76 mL, 74.8 mmol) were added at 0 °C. The solution was allowed to stir at 0 °C for 12 h. The mixture was again concentrated, and the remaining solid was added to a biphasic mixture of saturated $NaHCO_3$ and ether. The mixture was allowed to stir for 1.5 h and the white solid that formed was collected in a scintered glass funnel. The solid was washed with water, dried in a vacuum oven and was recrystallized from CH_3CN/water to produce 13.34 g of a white solid in 93% yield: mp 174–175 °C; $[\alpha]^{22}_D$ +18.1° (*c* 0.8, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.27 (sb, 1H), 7.38 (d, *J* = 7.45 Hz, 1H), 7.18 (d, *J* = 7.51 Hz, 1H), 6.98–6.88

(m, 2H), 6.59 (s, 2H), 5.07 (sb, 1H), 3.84–3.77 (m, 1H), 3.68 (s, 9H), 2.98 (dd, J = 15.68, 3.22 Hz, 1H), 2.84–2.75 (m, 1H).

5*S*,11*aS*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-1*H*,6*H*-oxazolo[3′4′:1,6]pyrido[3,4-*b*]-indol-3-one (5). To a solution of **4** (2.01 g, 5.31 mmol) in 1:1 dioxane: water (20 mL) was added $NaBH_4$ (1.00 g, 26.6 mmol) and the solution was allowed to stir at rt for 3 h. The solvent was removed under reduced pressure and the remaining solid was redissolved. The product was precipitated by the addition of NaCl, and collected by filtration and drying in a vacuum desicator to yield 1.43 g (76%) of a white solid which was used without further purification.

To a suspension of the amino-alcohol (1.31 g 3.70 mmol) in THF (10 mL) was added carbonyl diimidazole (1.79 g, 11.1 mmol) and the suspension was allowed to stir for 5 h. The suspension was concentrated and 10% NaOH was added. After stirring for an additional 3 h, the solution was carefully acidified to pH 6 by the addition of concentrated HCl and the resulting mixture was extracted with EtOAc (3 × 50 mL), dried over Na_2SO_4 and concentrated. The product was recrystallization from CH_3CN: ^1H NMR ($CDCl_3$) δ 7.58 (s, 1H), 7.51 (d, J = 8.37 Hz, 1H), 7.19 (m, 3H), 6.57 (s, 2H), 5.52 (s, 1H), 5.24 (s, 1H), 4.63 (t, J = 6.65 Hz, 1H), 4.22 (m, 2H), 3.83 (s, 6H), 3.22 (m, 1H), 2.92 (ddd, J = 16.4, 10.1, 1.8 Hz, 1H). (Not tested for elemental analysis.)

General procedure for the synthesis of tryptophan ethyl esters

To a suspension of the substituted tryptophan (0.5 mmol) in absolute ethanol (2 mL) was added concentrated HCl (1 mmol) and the solution was allowed to heat at reflux overnight. The solution was allowed to cool and the product was precipitated as the hydrochloride salt by the addition of Et_2O. The product was collected in a scintered glass funnel and washed with additional ether and dried in a vacuum desicator.

Procedure for the synthesis of oxazolidinones

To a suspension of $NaBH_4$ (2 mmol) in 75% ethanol in H_2O (1 mL) was added the tryptophan ethyl ester hydrochloride (0.4 mmol) in 75% ethanol in H_2O and the solution was allowed to heat at reflux for 6 h. The ethanol was removed under reduced pressure and water (4 mL) was added. The resulting solution was washed with ethyl acetate (3 × 10 mL) and the combined organic extracts were dried over Na_2SO_4, filtered and concentrated. The tryptophanol was used without further purification. A solution of EtONa (0.4 mmol), tryptophanol (0.4 mmol) and diethyl carbonate (0.6 mmol) in absolute ethanol (2 mL) were heated at reflux for 5 h. The solvent was removed under reduced pressure and saturated NH_4Cl and CH_2Cl_2 were added. After thorough mixing, the layers were separated and the organic layer was dried over Na_2SO_4, filtered and concentrated. The product was purified by flash chromatography (acetone–chloroform).

General procedure for synthesis of indole substituted azatoxins

To a solution of syringaldehyde dimethylacetal (0.6 mmol) **2** in anhydrous THF (2 mL) was added first *p*-toluenesulfonic acid (0.15 mmol) and then the carbamate (0.4 mmol) in THF (2 mL). The solution was allowed to stir at room temperature for 3–4 h saturated $NaHCO_3$ was added followed by $CHCl_3$. The layers were separated and the organic layer was dried over Na_2SO_4, filtered and concentrated. The product was purified by flash chromatography (acetone–chloroform).

5,11*a-trans*-5,4,11,11*a*-tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-9-methoxy-1*H*,6*H*-oxazolo[3′,4′:1,6]-pyrido[3,4-*b*]indol-3-one (21). Compound **21** was prepared as described in procedure C (78% yield). Purified by flash chromatography eluting with 15% acetone in $CHCl_3$ (R_f = 0.35): mp slow dec 180 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 7.81 (sb, 1H), 7.22 (d, J = 8.76 Hz,1H), 6.98 (d, J = 2.3 Hz, 1H), 6.88 (dd, J = 8.78, 2.3 Hz, 1H), 6.55 (s, 2H), 6.03 (s, 1H), 5.55 (s, 1H), 4.56 (t, J = 8.2 Hz, 1H), 4.21 (m, 2H), 3.88 (s, 3H), 3.79 (s, 6H), 3.14 (dd, J = 14.99, 4.64 Hz, 1H), 2.85 (ddd, J = 14.96, 10.4, 1.4 Hz, 1H). Anal. ($C_{22}H_{22}N_2O_6/H_2O$) C, H, N; theoretical: C, 64.38; H, 5.40; N, 6.83; found: C, 62.36; H, 5.64; N, 6.58.

5,11*a-trans*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-9-bromo-1*H*,6*H*-oxazolo[3′,4′:1,6]-pyrido[3,4-*b*]indol-3-one (20). Compound **20** was prepared as described in procedure C (66%). Purified by flash chromatography eluting with 15% acetone in $CHCl_3$ (R_f = 0.28): mp > 300 °C; ^1H NMR (300 MHz, DMSO-d_6) 8.44 (s, 1H), 7.63 (d, J = 0.9 Hz, 1H), 7.23 (d, J = 8.59 Hz, 1H), 7.15 (dd, J = 8.6, 1.7 Hz, 1H), 6.47 (s, 2H), 5.8 (s, 1H), 4.53 (t, J = 7.77 Hz, 1H), 4.29–4.17 (m, 2H), 3.64 (s, 6H), 3.12 (dd, J = 15.04, 4.26 Hz, 1H), 2.66 (dd, J = 14.5, 10.05 Hz, 1H). Theoretical +FABMS 458.0477; found +FABMS 458.05.

5,11*a-trans*-5,4,11,11*a*-tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-6-methyl-1*H*,6*H*-oxazolo[3′,4′:1,6]-pyrido[3,4-*b*]indol-3-one (16). Compound **16** was prepared as described in procedure C (55%): mp 269–271 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 7.56 (d, J = 7.80 Hz, 1H), 7.32–7.24 (m, 2H), 7.17–7.14 (m, 1H), 6.52 (s, 2H), 6.08 (s, 1H), 4.55 (t, J = 8.31 Hz, 1H), 4.23 (dd, J = 4.69, 8.48 Hz, 1H), 4.16–4.11 (m, 1H), 3.77 (s, 6H), 3.38 (s, 3H), 3.22 (dd, J = 15.01, 4.92 Hz, 1H), 2.92-2.84 (m, 1H). (Not tested for elemental analysis.)

5,11*a-trans*-5,4,11,11*a*-tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-8-methyl-1*H*,6*H*-oxazolo[3′,4′:1,6]-pyrido[3,4-*b*]indol-3-one (15). Compound **15** was

prepared as described in procedure C (76%): mp dec 245 °C; ^{1}H NMR (300 MHz, CD_3CN) δ 8.95 (sb, 1H), 7.10 (d, J = 8.13 Hz, 1H), 6.96 (t, J = 8.09 Hz, 1H), 6.77 (d, J = 7.08 Hz, 1H), 7.64 (s, 2H), 5.84 (s, 1H), 4.52 (t, J = 8.25 Hz, 1H), 4.33–4.27 (m, 1H), 4.18 (dd, J = 8 .42, 6.07 Hz, 1H), 3.76 (s, 6H), 3.49 (dd, J = 15.1, 4.64 Hz, 1H), 3.00 (dd, J = 15.1, 12.1 Hz, 1H). Anal. ($C_{22}H_{22}N_2O_5$) C, H (not tested for N); theoretical: C, 66.99; H, 5.62; found: C, 66.86; H, 5.65.

5,11*a*-*trans*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-9-methyl-1*H*,6*H*-oxazolo[3′,4′:1,6]-pyrido[3,4-*b*]indol-3-one (14). Compound **14** was prepared as described in procedure C (70%). Purified by flash chromatography eluting with 10% acetone in $CHCl_3$ (R_f = 0.27): mp slow dec 250 °C; ^{1}H NMR (300 MHz, $CDCl_3$) 7.73 (sb, 1H), 7.33 (s, 1H), 7.21 (d, J = 8.29 Hz, 1H), 7.06 (d, J = 8.17 Hz, 1H), 6.56 (s, 2H), 6.07 (s, 1H), 5.54 (sb, 1H), 4.56 (t, J = 8.26 Hz, 1H), 4.27–4.17 (m, 2H), 3.80 (s, 6H), 3.15 (dd, J = 15.05, 4.67 Hz, 1H), 2.85 (ddd, J = 15.06, 10.42, 1.5 Hz, 1H). Anal. ($C_{22}H_{22}N_2O_5$) C, H (not tested for N); theoretical: C, 66.99; H, 5.62; found: C, 67.06; H, 5.63.

5,11*a*-*trans*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-10-methyl-1*H*,6*H*-oxazolo[3′,4′:1,6]-pyrido[3,4-*b*]indol-3-one (13). Compound **13** was prepared as described in procedure C (81%). Purified by flash chromatography eluting with 10% acetone in $CHCl_3$ (R_f = 0.31): mp dec 148 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 (sb, 1H), 7.42 (d, J = 8.01 Hz, 1H), 7.11 (s, 1H), 7.00 (d, J = 7.91 Hz, 1H), 6.56 (s, 2H), 6.01 (s, 1H), 5.54 (sb, 1H), 4.55 (t, J = 8.21 Hz, 1H), 4.27–4.15 (m, 2H), 3.80 (s, 6H), 3.16 (dd, J = 15.08, 4.65 Hz, 1H), 2.85 (ddd, J = 15.10, 11.89, 1.5 Hz, 1H), 2.46 (s, 3H). Anal. ($C_{22}H_{22}N_2O_5$) C, H (not tested for N); theoretical: C, 66.99; H, 5.62; found: C, 67.05; H, 5.65.

5,11*a*-*trans*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-8-fluoro-1*H*,6*H*-oxazolo[3′,4′:1,6]-pyrido[3,4-*b*]indol-3-one (19). Compound **19** was prepared as described in procedure C. Purification by flash chromatography eluting with 20% acetone in $CHCl_3$ (R_f = 0.31) yielded a white solid (68%). An analytical sample was obtained by recrystallization from CH_3CN: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87 (sb, 1H), 7.44 (dd, J = 8.59, 5.17 Hz, 1H), 7.01 (dd, J = 9.51, 2.14 Hz, 1H), 6.93 (td, J = 9.17, 2.16 Hz, 1H), 6.54 (s, 2H), 6.01 (s, 1H), 4.56 (t, J = 8.29 Hz, 1H), 4.27–4.16 (m, 2H), 3.81 (s, 6H), 3.15 (dd, J = 15.02, 4.59 Hz, 1H), 2.86 (ddd, J = 15.02, 10.29, 1.44 Hz, 1H). Theoretical: +FABMS, 398.1278; found: +FABMS 398.13.

5,11*a*-*trans*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-9-fluoro-1*H*,6*H*-oxazolo[3′,4′:1,6]-pyrido[3,4-*b*]indol-3-one (18). Compound **18** was prepared as described in procedure C. Purification by flash chromatography eluting with 20% acetone in $CHCl_3$ (R_f = 0.37) yielded the product (75%) as a white solid. An analytical sample was obtained by recrystallization from CH_3CN: mp 275–277 °C; ^{1}H NMR (300 MHz, DMSO-d_6) 8.43 (s, 1H), 7.26–7.19 (m, 2H), 6.88 (td, J = 9.49, 2.48 Hz, 1H), 6.48 (s, 2H), 5.80 (s, 1H), 4.53 (t, J = 7.9 Hz, 1H), 4.3–4.17 (m, 2H), 3.65 (s, 6H), 3.09 (dd, J = 14.98, 4.45 Hz, 1H), 2.65 (ddd, J = 15.00, 10.14, 1.44 Hz, 1H). Anal. ($C_{21}H_{19}N_2O_5F$) C, H (not tested for N). Theoretical: C, 63.31; H, 4.81; found: C, 62.89; H, 4.96.

5,11*a*-*trans*-5,4,11,11*a*-tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-10-fluoro-1*H*,6*H*-oxazolo[3′,4′:1,6]-pyrido[3,4-*b*]indol-3-one (17). Compound **17** was prepared as described in procedure C (69%) recrystallized from CH_3CN: mp 260 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.45 (s, 1H), 7.09 (d, J = 8.10 Hz, 1H), 7.03–6.95 (m, 1H), 6.74–6.68 (dd, J = 11.06, 7.63 Hz, 1H), 6.50 (s, 2H), 5.80 (s, 1H), 4.51 (t, J = 7.84 Hz, 1H), 4.31–4.20 (m, 2H), 3.66 (s, 6H), 3.24 (dd, J = 14.07, 4.36 Hz, 1H), 2.85 (dd, J = 10.07, 10.01 Hz). Theoretical: +FABMS, 398.1278; found: +FABMS, 398.13.

5*R*,11*aS*-5,4,11,11*a*-Tetrahydro-5-(3,5-dimethoxy-4-hydroxyphenyl)-9-hydroxy-1*H*,6*H*-oxazolo[3′4′:1,6]-pyrido[3,4-*b*]indol-3-one (22). Compound **22** was prepared as described in procedure C. Purification by flash chromatography eluting with 30% acetone in $CHCl_3$ (R_f = 0.34) yielded a white solid (11%) recrystallized from EtOAc/hexanes: mp dec slow >140 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.64 (s, 1H), 8.41 (s, 1H), 7.04 (d, J = 8.60 Hz, 1H), 6.73 (d, J = 1.95 Hz, 1H), 6.56 (dd, J = 8.62, 2.16 Hz, 1H), 6.48 (s, 2H), 5.75 (s, 1H), 4.52 (t, J = 7.71 Hz, 1H), 4.27–4.17 (m, 2H), 3.65 (s, 6H), 2.99 (dd, J = 14.65, 4.09 Hz, 1H), 2.61 (dd, J = 14.61, 10.55 Hz, 1H). Theoretical: +FABMS, 396.1321; found: +FABMS, 397.14.

Isolation of human DNA topoisomerase II

Human DNA topoisomerase II was isolated from human placenta by the same general method described by Spitzner and Muller,[14] with a few modifications. Briefly, the procedure involved nuclear extraction, followed by BioRex 70, MonoQ, and MonoS column chromatography. Activity was monitored by the kinetoplast DNA decatenation assay. MonoS fractions with activity were concentrated on the MonoQ column. SDS–PAGE of the concentrated sample revealed the presence of 170 and 180 kDa bands which correspond to the a and b isotypes of human topoisomerase II in an approximately 1:1 ratio, along with a few impurities of lower molecular weight.

DNA topoisomerase II cleavage assays

Assays for topoisomerase II-mediated DNA cleavage were performed according to the method of Rowe et al.,[23] with a few modifications. Reaction mixtures (25

mL) contained 50 ng of ^{32}P 3′-end-labeled pUC 18 DNA, 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 0.5 mM DDT, 0.5 mM EDTA, 30 mg/mL of bovine serum albumin, 2.5% glycerol, 1 mM ATP, and 120 ng protein from the concentrated MonoQ fractions containing topoisomerase II activity. The reactions were incubated for 30 min at 37 °C and were stopped with the addition of 2.5 mL of 10% SDS. EDTA (1 mL of a 0.5 M solution) and proteinase K (2 mL of a 1 mg/mL solution) were then added and the mixtures were incubated for 20 min at 37 °C. Loading solution [4 mL of a mixture containing 50% glycerol (v/v), 0.1 M EDTA, 1% SDS, and 0.1% bromophenol blue (w/v)] was added, and samples were electrophoresed on horizontal agarose gels (1.4%, 20 cm × 25 cm) containing 89 mM Tris–boric acid, pH 8.3, and 2 mM EDTA at 60 V for 14 h. Gels were dried on a Bio-Rad Model 583 gel drier, and autoradiography was performed at −70 °C using Kodak X-Omat AR film and a DuPont Lightning Plus intensifying screen.

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