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The Synthesis of Amino-Acid Functionalized β-Carbolines as Topoisomerase II Inhibitors

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Abstract—The synthesis and biological activity of amino acid functionalized β-carboline derivatives, which are structurally related to azatoxin and the tryprostatins, are reported. These compounds were assayed for their growth inhibition properties in H520 and PC3 cell lines and were examined for their abilities to inhibit topoisomerase II-mediated DNA relaxation.

Topoisomerase II (topo II) is a ubiquitous nuclear enzyme that modulates topological relationships within the genome of eukaryotic cells; topo II adjusts and stabilizes DNA during replication, transcription, and recombination, and also functions in chromosomal condensation and segregation reactions.[1–7] Unlike other members of the topoisomerase superfamily, topo II is required for proper cell function because it possesses the ability to catenate and decatenate DNA.[2,4] The essential nature of topo II coupled with its elevated expression in tumor cells make it an exceptional target for chemotherapy. In fact, two of the most widely utilized anticancer drugs, etoposide and adriamycin (Fig. 1), inhibit topo II.[2,4,5,8]

Azatoxin (Fig. 1), a compound designed as a hybrid of etoposide and ellipticine (Fig. 1), inhibits topo II action and also halts tubulin polymerization at variable concentrations.[9–11] The intriguing dual activity of azatoxin coupled with the disclosure of a model pharmacophore[9] for topo II inhibitors (Fig. 2) has facilitated the design and synthesis of novel derivatives that possess heightened efficacy and specificity for topo II.[12,13] Despite the enhanced topo II activity of azatoxin derivatives synthesized in recent years,[13–15] undesirable physiochemical and solubility problems preclude the full realization of their clinical utility.

Recently, a novel class of amino acid (AA)-based azatoxin congeners was engineered in an effort to improve bioavailabilty and enhance potency. The design concept for these compounds originates from recognition of the similarities between azatoxin and the tryprostatins[16–18] (Fig. 1). Although both azatoxin and the tryprostatins possess good in vitro growth inhibition properties and a characteristic L-tryptophan backbone, the tryprostatins contain a terminal diketopiperazine moiety in place of azatoxin's carbamate region. Functionalizing azatoxin's carboline core with amino acids and subsequent cyclization to form a terminal diketopiperazine emerged as a viable synthetic initiative (Figs. 1 and 3). Amino acids are attractive substrates not only because they are the fundamental building blocks of biological systems and many natural products, but also because they are commercially available and possess structurally diverse side chains. Therefore, by utilizing both D- and L-AAs containing either nonpolar, acidic, basic, or aromatic side chains, varied functionalities with absolute stereochemistry are introduced to azatoxin's tetrahydrobetacarboline core. The chemodivergent methodology developed provides access to a wide range of AA-substituted compounds: the AA-functionalized β-carbolines **8a**–**w** and diketopiperazines **8x**–**z** (Fig. 3). As a result of this work, SARs detailing stereochemical and substituent preferences for AA-substituted topo II-directed agents have been elucidated. The 'acyl region' domain of the topo II-pharmacophore has also been further defined.

Synthesis of Target Compounds

Amino acid functionalized β-carboline derivatives **8a**–**z** were prepared in accord with known procedures as

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depicted in Figure 3. The 1,3-*trans*-1,2,3,4-tetrahydrobetacarboline intermediate **4** was obtained via a Pictet–Spengler reaction utilizing L-tryptophan methyl ester hydrochloride **2** and benzyl protected syringaldehyde **3**; a chromatographically separable 65:35/*trans*/*cis* mixture was obtained after 20 h of vigorous reflux.[19] The requisite 1,3-*trans* relationship of **4** was verified by ^{13}C spectroscopy as previously described by Cook et al.[20] β-Carboline **4** was then saponified with LiOH in refluxing THF to the acid **5**. After a survey of coupling processes, **5** was subsequently coupled to the methyl ester-protected AA of choice **6a**–**w** using benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate (PyBop) in CH_2Cl_2 with either diisopropylethylamine (DIEA) or triethylamine (TEA) as the base. The selective protection of AA side chains in compounds **6o**–**p**, while the phenolic and alcohol bearing moieties of **6k**–**l** and **q**–**r** were masked as *tert*-butyldimethylsilyl (TBS) ethers. The resulting coupled species **7a**–**w** were refluxed in *N,N*-dimethylformamide (DMF) or with catalytic *p*-toluenesulfonic acid (*p*-TsOH) in toluene to promote diketopiperazine formation. Cyclization was only successful for **7b**, **d**, and **w**. These diketopiperazines and the aforementioned AA-substituted β-carboline derivatives **7a**–**w** were appropriately deprotected to afford final compounds **8a**–**z**. TBS-ether cleavage of compounds of **7k**–**l** and **q**–**r** with tetrabutylammonium fluoride (TBAF) was required prior to hydrogenolysis, which was conveniently utilized as the final deprotection protocol for all compounds.

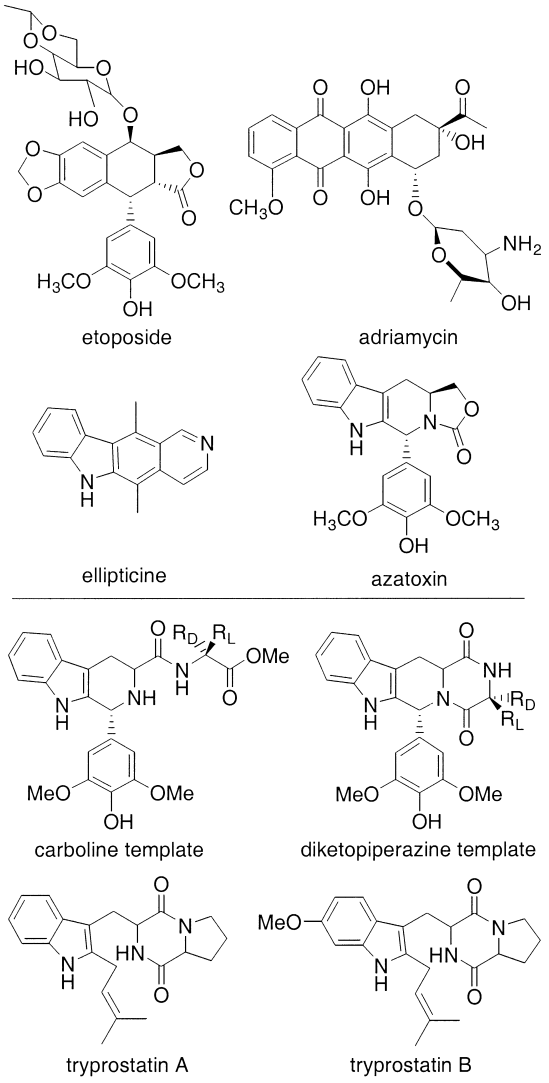

Figure 1. Structures of known topo II inhibitors (top), and structures of the tryprostatins and AA-functionalized synthetic targets (bottom).

Discussion

While AA-substituted β-carbolines **8a**–**w** were readily realized, diketopiperazines **8x**–**z** proved to be challenging synthetic targets. We postulate that this difficulty may be attributed to the inaccessibility of the sterically hindered secondary amine in the β-carboline ring or to the bulkiness and/or stereochemistry of the respective AA side chain. Existence of an unfavorable amide bond conformation in **7** may also impede diketopiperazine formation. Despite the synthetic problems encountered, it is important to note that Fischer cyclization conditions (NH_3/MeOH), and a peptide deblocking strategy using piperidine[21] were also effective for the synthesis of **8x**–**z** (Fig. 3). We continue to seek conditions that will promote the cyclization of the remaining AA derivatives.

Growth inhibition data as an indicator of cytotoxicity for AA-functionalized β-carbolines **8a**–**z** in H520 and PC3 cell lines are summarized in Figure 4. Of the 26 compounds screened, only those containing the AAs Trp or Phe possessed significant activity. Interestingly, there does not appear to be a trend correlating AA stereochemistry (D or L) to growth inhibition; H520 cells exhibit approximately a 7% cell survival rate when treated with compounds **8e**, **f**, **i**, or **j**. Additionally, we found the GI_{50} for **8e** in H520 cells to be 22.4 μM. Since the growth inhibition profiles of **8e** and **i** are similar, the extrapolated GI_{50} for **8i** would also be expected to be in this range.

The topo II assay data for **8a**–**z** are presented in Figures 4 and 5. Compounds **8g**, **h**, and **i** significantly inhibit topo II's ability to relax supercoiled DNA. Although **8h** is not cytotoxic in the cell lines examined, the data for topo II-mediated DNA relaxation suggests that we are on track with the rational design of our molecules; **8a**, **c**,

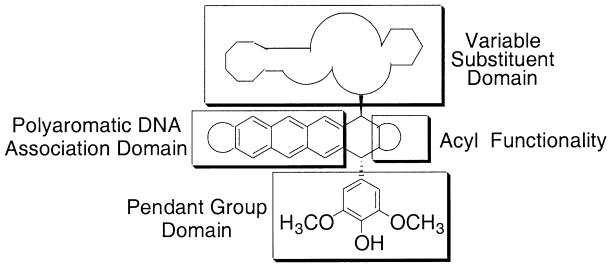

Figure 2. Model pharmacophore for topo II inhibitors.

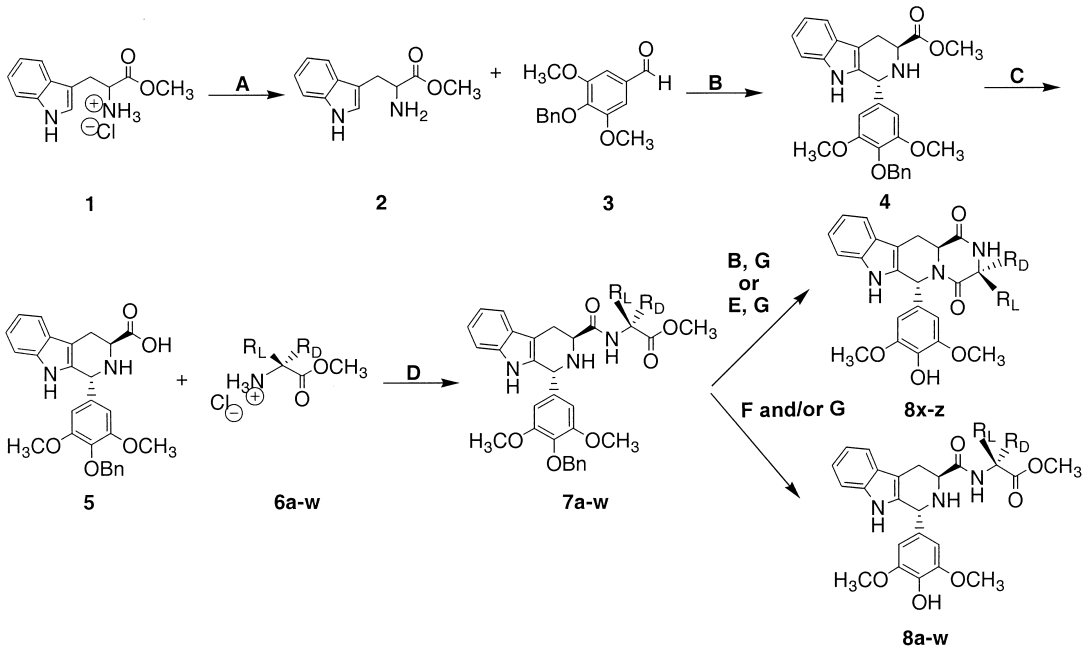

Figure 3. Synthesis of AA-functionalized β-carboline derivatives. Reagents: (A) TEA, MeOH; (B) *p*-TsOH (cat), toluene, reflux; (C) LiOH, THF, reflux; (D) ByBop, TEA, CH_2Cl_2; (E) DMF, reflux; (F) TBAF, THF; (G) H_2, Pd/C, THF/MeOH.

e, **f**, **j**, **k**, **m**, **o**, **s**, and **u** also interact with topo II but to a lesser extent. Most significantly, compounds **8a**, **c**, **g**, **h**, **k**, **m**, **o**, and **u** exhibit measurable topo II activity, but do not inhibit cancer cell growth. It is conceivable that growth inhibition may not be observed despite the documented inhibition of topo II in the DNA relaxation assay; these polar derivatives may be unable to cross the plasma membrane. Additionally, it is possible that these compounds may be degraded by peptides or acyl transferases under cellular assay conditions.

The compounds possessing the greatest topo II activity incorporate either nonpolar or aromatic AAs into their structural framework. When comparing **8h** to **8f**, the addition or removal of one carbon in the AA side chain causes a marked difference in the respective compound's growth and topo II-inhibition abilities (Fig. 4). This observation is consistent with a hydrophobic and/or aromatic binding region on the DNA/topo II complex that enhances activity against topo II when occupied by a compound. Previous work in our laboratory has found that azatoxin derivatives substituted with aniline functionalities in the variable substituent domain are more potent topo II toxins than azatoxin itself.[13] Thus, the terminal AA region of **8a**–**w** may indeed mimic the variable substituent domain of our model pharmacophore (Fig. 2). Data from compounds **8a**, **c**, **e**–**j**, **s**, and **u** further support this hypothesis. The remaining topo II-active compounds (**8k**, **m**, and **o**) also follow this trend because a segment of their side chain is either nonpolar or aromatic.

In contrast to the growth inhibition data, a stereochemical preference within the topo II data for D- over L-amino acids appears to exist. This trend is evident upon examination of the topo II data for **8a**, **c**, **k**, **m**, **o**, **s**, and **u** (Fig. 4). While compounds **8e**–**j** do not follow this pattern, the hydrophobic and aromatic nature of their side chains may alone satisfy the binding requirements to generate topo II-active compounds.

The diketopiperazine analogues **8x**–**z** do not inhibit H520/PC3 cell growth or topo II-mediated DNA relaxation (Fig. 4). While these results are negative, they help us to further define the acyl region of the proposed topo II model pharmacophore (Fig. 2). There appears to be a preference for the five-membered carbamate moiety of azatoxin[9,15] over the six-membered diketopiperazine ring present in compounds **8x**–**z**. It is possible that the ketone functionalities of the diketopiperazine may force a conformation that ultimately restricts rotation of the pendant group (Fig. 2). The spatial orientation and mobility of the pendant group is known to be important in drug recognition and DNA binding.[14]

Conclusion

We have realized the synthesis of AA-functionalized β-carbolines **8a**–**w** and diketopiperazines **8x**–**z**. These compounds were analyzed for in vitro growth inhibition of H520 and PC3 cell lines, and for their abilities to inhibit topo II-mediated DNA relaxation. Of the compounds synthesized, **8e**, **f**, **i**, and **j** inhibit both cell growth and topo II action. **8h** does not inhibit cell growth but was found to be active in our topo II-mediated DNA relaxation assay. Data from these compounds suggest that a hydrophobic and/or aromatic binding region may exist and promote further refinement of our model pharmacophore for topo II/drug

Compd Number	Amino Acid[c]	Percent Cell Survival[a]						Topo II Assay Data[b]
		H520			PC3			DNA Relaxation
		10 μM	*50μM*	*100 μM*	*10 μM*	*50μM*	*100 μM*	(+)=positive (−)=negative
8a	D-Pro	94.3	86.1	61.0	98.0	99.4	66.3	+
8b	L-Pro	80.5	83.4	86.6	96.8	98.3	>100	–
8c	D-Ala	>100	>100	>100	96.9	>100	>100	++
8d	L-Ala	>100	>100	>100	>100	96.6	95.8	–
8e	D-Phe	77.6	33.0	6.70	>100	>100	63.6	+
8f	L-Phe	59.4	97.4	14.8	97.8	90.6	18.0	+
8g	D-Phg	>100	>100	99.2	>100	>100	>100	+++
8h	L-Phg	82.7	89.9	86.8	88.5	>100	64.5	++++
8i	D-Trp	>100	35.2	7.20	>100	84.9	62.4	+++
8j	L-Trp	67.9	61.1	2.80	95.7	77.0	6.70	+
8k	D-Tyr	>100	79.0	83.2	88.5	>100	89.8	+
8l	L-Tyr	>100	>100	98.6	>100	96.4	94.9	–
8m	D-Met	>100	>100	89.6	>100	>100	>100	+
8n	L-Met	99.2	91.2	79.9	96.7	88.2	76.8	–
8o	D-Lys	78.3	76.9	87.7	>100	>100	>100	++
8p	L-Lys	99.3	>100	>100	95.2	91.2	90.6	–
8q	D-Ser	75.7	80.4	85.0	72.8	81.8	79.1	–
8r	L-Ser	87.0	90.0	79.6	99.7	98.5	>100	–
8s	D-Val	90.0	>100	98.3	>100	>100	>100	+
8t	L-Val	87.6	85.4	93.6	91.9	93.1	95.3	–
8u	D-Leu	85.2	84.3	80.6	>100	>100	99.4	++
8v	L-Leu	84.0	89.2	83.2	>100	98.7	87.6	–
8w	Gly	>100	>100	>100	99.7	>100	88.8	–
8x	Gly	91.6	69.2	51.9	>100	>100	86.6	–
8y	D-Ala	93.8	99.7	91.5	82.6	99.5	95.3	–
8z	L-Pro	>100	>100	>100	>100	>100	>100	–

Figure 4. Summary of in vitro cell growth and topo II[22] assays for **8a–z**. Compounds that inhibit *both* cell growth and topo II-mediated DNA relaxation are highlighted in red. Compounds that *only* inhibit topo II-mediated DNA relaxation are highlighted in blue. [a]CellTiter 96^{TM} Aq$_{ueous}$ Non-Radioactive Cell Proliferation Assay (Promega) was used to determine growth inhibition. All data points run in quadruplicate. Controls were DMSO (0.20%) and m-AMSA[23] (20, 40 μM). Standard error is ±5 μM. [b] Densitometer qualitation of topo II-mediated DNA relaxation assay. Gel was run in triplicate. Scale: (+), drug interacts with topo II; (−), drug does not interact with topo II. A representative gel is shown in Figure 5.[22] [c] Amino acid noted was used in synthesis as described in Figure 3.

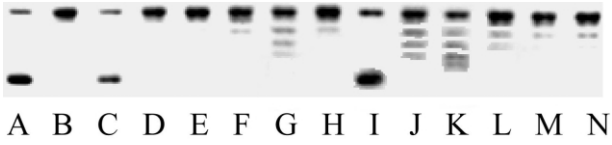

Figure 5. [22] Representative agarose gel from the topo II-mediated DNA relaxation assay. Data for all compounds are not shown. Lane A: DNA only; lane B: DNA + topo II; lane C: DNA, topo II, and m-AMSA; lane D: DNA, topo II, and DMSO; lane E: **8y**; lane F: **8a**; lane G: **8c**; lane H: **8e**; lane I: **8h**; lane J: **8g**; lane K: **8i**; lane L: **8k**; lane M: **8m**; lane N: **8o**.

interactions. Because the AA-functionalized agents synthesized are structurally similar to azatoxin and the podophyllotoxins, it is important to note that our compounds could also inhibit either topo II-mediated cleavable complex formation or tubulin polymerization.

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22. The DNA relaxation assay tests the ability of topo II to relax supercoiled DNA in the presence of drug. Each incubation contained 1 μL 10× incubation assay buffer (0.5 M Tris–Cl, pH 8.0, 1.2 M KCl, 0.1 M $MgCl_2$, 5 mM ATP, 5 mM DTT, 300 mg/mL BSA), 0.5 μL of 2.0 units topo II (Note: 1 unit of topo II can decatenate 0.2 microgram KDNA in 30 min at 37 °C. Topo II was obtained from TopoGEN, Inc., Columbus, OH, USA), 0.5 μL of 0.125 μg/mL plasmid pUC18 DNA from *Escherichia coli* (obtained from Boehringer Mannheim GmbH, Germany), 1 μL of 1 mM drug in 10% DMSO and H_2O to 10 μL. The samples were prepared with enzyme added last and incubated at 37 °C for 30–45 min. The reaction was stopped with 2.5 μL of decatenation buffer (5% sarkosyl, 25% glycerol, and 0.0025% bromophenol blue). The drugs were extracted from the incubation with 10 μL of 24:1 chloroform/isoamyl alcohol. The aqueous DNA containing samples were loaded on a 1% agarose gel and 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, m-AMSA (100 μM), and DMSO (1%).
23. m-AMSA, the topo II-inhibitor utilized as a control in our DNA relaxation assay, is also a known DNA intercalator