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DESIGN AND SYNTHESIS OF NATURAL COMPOUND ANALOGUES WITH CYTOTOXIC ACTIVITY

(CHIM/08)

SALVATORE CANANZI

Tutore

Prof.ssa Daniela Barlocco

Coordinatore

Prof. Carlo De Micheli

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To myself

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1

Introduction

Alkaloids are a diverse group of low molecular weight, nitrogen-containing molecules found in about 20% of plant species. Many of the 16,000 alkaloids for which structures have been described function in the defense of plants against herbivores, microbes, viruses and competing plants. Many alkaloids are infamous for their strong toxicity towards animals and humans. Most of the deadly alkaloids fall into the class of neurotoxins and the others have cytotoxic properties. A cytotoxic effect can be generated when cell membranes are made leaky (as by saponins or steroidal alkaloids), or when elements of the cytoskeleton are inhibited. The spindle poisons vinblastine, vincristine, colchicine, and taxol are particularly famous. Actin filament formation is blocked by fungal poisons such as phalloidin from *Amanita phalloides*. [1,2,3]

DNA can also be a target for alkaloids: planar and lipophilic alkaloids are intercalating compounds that assemble between the stacks of paired nucleotides in the DNA double helix. DNA intercalation can disturb replication, DNA repair, and DNA topoisomerases. Frameshift mutations are one of the adverse consequences of intercalating compounds. Some alkaloids, such as pyrrolizidine alkaloids, aristolochic acids, cycasin, and furoquinoline alkaloids, are known to form covalent adducts with DNA bases.[4] Mutations and tumor formation can be the result of such interactions. DNA alkylation occurs in some alkaloids only after activation by liver enzymes, such as cytochrome p450 oxidases (pyrrolizidine alkaloids, aristolochic acids).[5]

Ribosomal protein biosynthesis is often inhibited by alkaloids that interact with nucleic acids. There are also more specific inhibitors, such as emetine.

Disturbances of the cytoskeleton, DNA replication, and DNA topoisomerase, or DNA alkylation and intercalation usually lead to cell death by apoptosis. The cytotoxic properties are usually not specific for animals but also affect bacteria, fungi, other plants, and even viruses.

Alkaloids thus defend plants against a wide diversity of enemies. They have the disadvantage that a producing plant could theoretically kill itself by its own poison. Compartmentation, target-site insensitivity, and other mechanisms (which are largely unknown) must have evolved to overcome such problems.[6]

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. The estimated worldwide incidence of cancer is about 6 million new cases per year. In the United States, cancer is now the leading cause of death for people younger than 85 years, and it is only exceeded by heart disease in older individuals. The treatment of many diseases is highly dependent on natural products, and this is especially true for the treatment of cancer. Unique classes of natural products, such as alkaloids, have shown significant antitumor action.[7,8]

1.1

Antitumor alkaloids

1.1.1

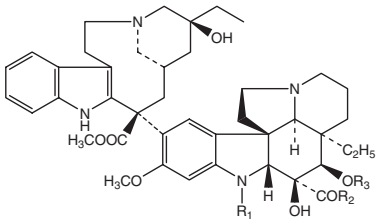
Antitumor alkaloids in clinical use

1.1.1.1

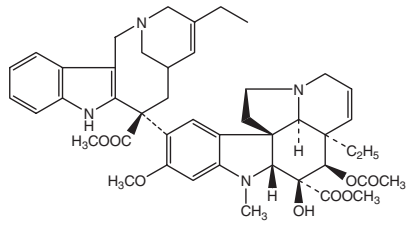
Vinca alkaloids

The vinca alkaloids, isolated from the Madagascar periwinkle, *Catharantus roseus* G. Don., comprise a group of about 130 terpenoid indole alkaloids. Their clinical value was clearly identified as early as 1965 and so this class of compounds has been used as anticancer agents for over 40 years and represents a true lead compound for drug development . Today, two natural compounds, *vinblastine* (VLB, 1) and *vincristine* (VCR, 2), and two semisynthetic derivatives, *vindesine* (VDS, 3) and *vinorelbine* (VRLB, 4), have been registered (Figure 1.1).[8]

Owing to the pharmaceutical importance and the low content of VLB and the related alkaloid VCR, *Catharanthus roseus* became one of the best-studied medicinal plants. Among the many biochemical effects observed after exposure of cells and tissues to the vinca alkaloids are disruption of microtubules, inhibition of synthesis of proteins and nucleic acids, elevation of oxidized glutathione, alteration of lipid metabolism and the lipid content of membranes, elevation of cyclic adenosine monophosphate (cAMP), and inhibition of calcium-calmodulin-regulated cAMP phosphodiesterase.[9]



Compound	R_1	R_2	R_3
1	CH_3	OCH_3	$COCH_3$
2	CHO	OCH_3	$COCH_3$
3	CH_3	NH_2	H



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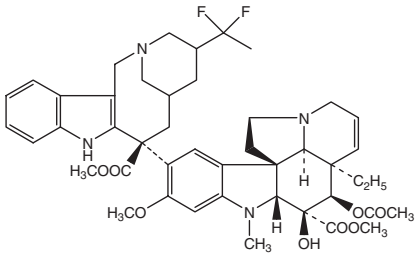


5

Fig 1.1

Despite these multiple biochemical actions, the antineoplastic activity of the vinca alkaloids is usually attributed to disruption of microtubules, resulting in dissolution of mitotic spindles and metaphase arrest in dividing cells. Microtubules are involved in many cellular processes in addition to mitosis, and exposure to vinca alkaloids gives rise to diverse biological effects, many of which could impair essential functions, both in dividing and in nondividing cells.[10]

Among anticancer drugs, the vinca alkaloids are classified as mitotic inhibitors, with their primary site of action being M phase of the cell cycle, but it is not certain that mitotic inhibition is the predominant in vivo

cytotoxic mechanism. Recent studies suggest that disruption of the cell cycle may lead to cell death through apoptosis. The many biological actions of the clinically active vinca alkaloids are seen over a wide range of drug concentrations, and there are selective effects in various normal and neoplastic tissues.[11]

1.1.1.2

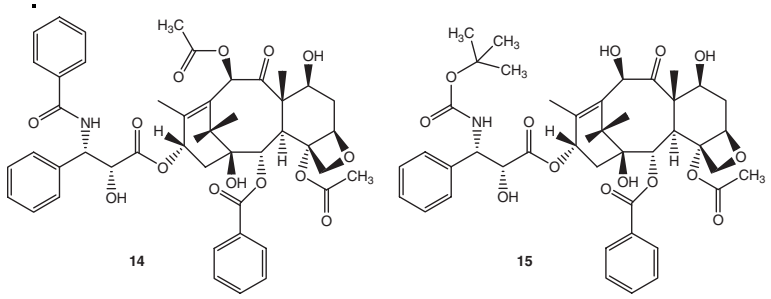
Camptothecin and Analogues

See chapter 3

1.1.1.3

Taxanes

Taxanes are a class of structurally complex yet homogenous diterpene alkaloids that occur in the genus *Taxus*, commonly known as the yew. This family of diterpenoids has long been known for its toxicity as well as for other biological activities. The first chemical study of the metabolites of the yew dates backs to the mid-nineteenth century, when a mixture of taxanes was obtained by the German pharmacist Lucas in 1856.[11] The structure characterization of these compounds, which were named *taxine* by Lucas was, however, extremely slow, owing to the complexity of the substance and the lack of modern spectroscopic techniques. In 1963, the constitution of the taxane nucleus was established for the first time by independent work of Lythgoe's group, Nakanishi's group, and Uyeo's group as tricyclic polyalcohols esterified with acids, such as *taxinine* , whose stereochemistry was established three years later.[12] More importantly, in 1971, Wani and Wall discovered the highly potent anticancer agent *paclitaxel* (14, Figure 1.2).[13, 14] This remarkable accomplishment not only shifted the attention of the scientific community to paclitaxel itself, but also attracted extensive studies on various species of yew tree that led to the isolation of many new taxane family members..



14 15

Fig 1.2

To date, over 100 taxanes have been isolated and structurally elucidated. Paclitaxel, and an analog, docetaxel, are currently regarded as very useful anticancer chemotherapeutic agents

Paclitaxel (14, Figure 1.3), a complex diterpenoid alkaloid, occurs widely in plants of Taxus species. Among various Taxus species and various parts of taxus trees, the bark of *T. brevifolia* Nutt. has the highest content of paclitaxel. However, even this content is relatively low – only about 0.01 % on a dry weight basis. Moreover, yew trees grow very slowly. Paclitaxel isolation is therefore restricted as a result of the relative scarcity of the Pacific yew tree and the low yield of its bark. However, other species and other parts of taxus trees also produce paclitaxel and related taxanes.[15] The major barrier to early clinical development of paclitaxel was its low abundance in the yew trees, since chemical synthesis had not been possible; there simply was not enough paclitaxel to perform the appropriate trials. This situation has changed because of the development of novel semisynthetic methods and the identification of new sources of taxanes. An important biosynthetic precursor of paclitaxel, 10-deacetylbaccatin III, was isolated in 1981 in reasonably good yield from the leaves of *Taxus baccata* L., as a renewable source, as well as from the bark of *Taxus brevifolia* in 1982.[16] It serves as the starting material for the production of paclitaxel through a coupling reaction with an appropriately protected side chain that can be prepared synthetically. The total synthesis of paclitaxel has also been a challenging target for a number of research groups. Both the Holton group and the Nicolaou group synthesized paclitaxel almost simultaneously in 1994, but both syntheses are too cumbersome to have commercial value.[17]

Among antineoplastic drugs that interfere with microtubules, paclitaxel exhibits a unique mechanism of action. Paclitaxel promotes assembly of microtubules by shifting the equilibrium between soluble tubulin and microtubules toward assembly, reducing the critical concentration of tubulin required for assembly. The result is stabilization of microtubules, even in the presence of conditions that normally promote disassembly of microtubules. The remarkable stability of microtubules induced by paclitaxel is damaging to cells because of the perturbation in the dynamics of various microtubule-dependent cytoplasmic structures that are required for such functions as mitosis, maintenance of cellular morphology, shape changes, neurite formation, locomotion, and secretion.[18-20] The binding site for paclitaxel is distinct from the binding sites for the vinca alkaloids, colchicine or podophyllotoxin. Cells treated with pharmacologic levels of paclitaxel are arrested in the G2 and M phases of the cell cycle and contain disorganized arrays of microtubules, often aligned in parallel bundles.

Docetaxel (15, Figure 1.2), a semisynthetic side-chain analog of paclitaxel, also has potent antitumor activities. It was first synthesized by Potier's group in 1984, from one of the yew tree taxanes, 10-deacetyl-baccatin III, found in the leaves of *Taxus baccata* L. This has the advantage of being a renewable source. Although the mechanisms of action of the taxanes docetaxel and paclitaxel are identical, docetaxel has almost a twofold higher binding affinity for the target site, b-tubulin.[16]

1.1.2

Antitumor alkaloids in clinical trials

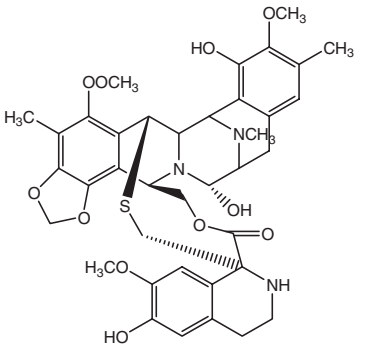
1.1.2.1

Ecteinascidin-743 (Yondelis, Trabectedin)

Ecteinascidin-743 (ET-743, 16, Figure 1.3) is a tetrahydroisoquinoline alkaloid isolated from *Ecteinascidia turbinata* Herdman, a tunicate that grows on mangrove roots throughout the Caribbean Sea. Most likely, the

compound is produced by the marine tunicate as a defense mechanism to survive in its natural environment.[21,22]

ET-743 binds to the N2 position of guanine in the minor groove of DNA with some degree of sequence specificity, altering the transcription regulation of induced genes. ET-743 was selected for clinical development



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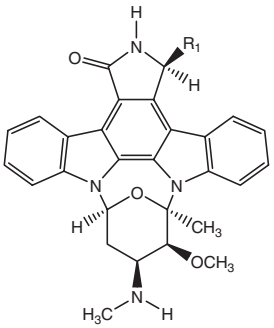


Compound	R_1
17	H
18	OH

Fig 1.3

because of its original mode of action involving DNA repair machinery and its cytotoxic activity against a variety of solid tumor cell lines, including sarcoma cell lines, even those resistant to many other cytotoxic agents.[23]

1.1.2.2

7-Hydroxystaurosporine (UCN-01)

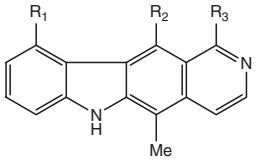
Staurosporine (17, Figure 1.3), an alkaloid isolated from *Streptomyces* spp., is a potent nonspecific protein and tyrosine kinase inhibitor. Thus, efforts to find analogs of staurosporine have identified compounds specific for protein kinases. UCN-01 (18, Figure 1.3) is a potent inhibitor of protein kinase C,[24] and has antiproliferative activity in several human tumor cell lines. Work from several groups supports the hypothesis that UCN-01 promotes its antitumor activity through induction of apoptosis by either modulation of cyclin-dependent kinases or inhibition of cell cycle checkpoints. UCN-01 may act by abrogating the G2 block often induced by cellular damage, thus causing inappropriate progression to G2 and subsequent apoptosis. Also, synergistic effects of UCN-01 have been

observed with many chemotherapeutic agents, including mitomycin C, 5-fluorouracil, and camptothecin.[25,26]

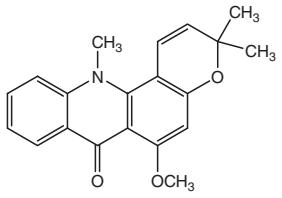
1.1.2.3

Ellipticine and analogs

Ellipticine (19) and its other two naturally occurring analogs, 9-methoxyellipticine (20) and olivacine (21), showed promising activity as potential anticancer drugs (Figure 1.4). Ellipticine was first isolated in 1959 from the leaves of the evergreen tree *Ochrosia elliptica* Labill., but its biological activities were only recognized in 1967.[27] Since then, the design, synthesis, and structure–activity relationships of this class of compounds have been studied by a number of laboratories. Studies on the mechanism of cytotoxicity and anticancer activity of the ellipticine analogs suggest a complex set of effects, including DNA intercalation, inhibition of topoisomerase, covalent alkylation of macromolecules, and generation of cytotoxic free radicals.[28,29]



Compound	R_1	R_2	R_3
19	H	CH_3	H
20	OCH_3	CH_3	H
21	H	H	CH_3



22

Fig 1.4

1.1.2.4

Acronycine and analogs

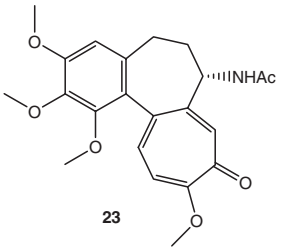
The pyranoacridone alkaloid, acronycine (22, Figure 1.4), isolated from *Acronychia baueri* Schott.,[30] was shown to exhibit promising activity against a broad spectrum of solid tumors. However, its moderate potency

and poor solubility in aqueous solvents severely hampered subsequent clinical trials, which were rapidly discontinued, owing to modest therapeutic effects and dose-limiting gastrointestinal toxicity after oral administration.[31] Consequently, the development of structural analogs with increased potency and/or better water solubility was highly desirable. Efforts to design more potent derivatives were guided by the hypothesis of in vivo bioactivation of the 1,2-double bond of acronycine into the corresponding epoxide. The high reactivity of acronycine 1,2-epoxide, which readily reacts with water to give the corresponding cis and trans diols, suggested that this compound could be the active metabolite of acronycine, able to alkylate some nucleophilic target within the tumor cell. Accordingly, significant improvements in terms of potency were obtained with derivatives modified in the pyran ring, which had a similar reactivity toward nucleophilic agents such as acronycine epoxide but improved chemical stability.[32]

1.1.2.5

Colchicine and analogs

Colchicine (23, Figure 1.5) is a three-ring amine alkaloid derived from the corms of *Colchicum autumnale* L. Like the anticancer indole alkaloids, vinblastine and vincristine, it depolymerizes microtubules at high concentrations and stabilizes microtubule dynamics at low concentrations. It was recognized as having damaging effects on tumor vasculature as long ago as the 1930s and 1940s, causing hemorrhage and extensive necrosis in both animal and human tumors.[33,34]



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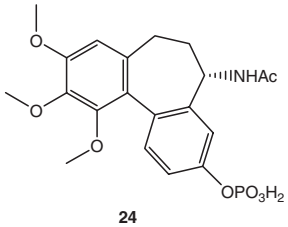

24

Fig 1.5

Although its toxicity precluded further clinical development, sporadic reports of tumor vascular damage induced by related compounds, such as podophyllotoxin, continued to emerge. Since the late 1990s, the combretastatins and N-acetylcolchinol-O-phosphate (ZD6126, 24, Figure 1.6), compounds that resemble colchicine and bind to the colchicine domain on tubulin, have undergone extensive development as antivascular agents. ZD6126 is a phosphate prodrug of the tubulin-binding agent N-acetylcolchinol. Profound disruption of the tumor blood vessel network has been noted in a wide variety of preclinical tumor models. The observed effects include vascular shutdown, and reduced tumor blood flow. Histologic assessment has revealed central tumor necrosis extending to within a few cell layers from the tumor margins. Following treatment, a ring of viable tumor cells is invariably found on the tumor periphery. Since the residual tumor tissue can serve as a foundation for tumor regrowth, the vascular disrupting agent could be combined with other treatment options so that the entire tumor cell population can be completely eradicated.[35]

1.1.3.6
Ukrain

Ukrain (NSC-631570) has been described by Novicky Pharma (Vienna, Austria) as a semisynthetic compound derived from *Chelidonium majus L*., which contains a range of alkaloids, most notably chelidonine. Ukrain consists of one molecule of thiophosphoric acid conjugated to three molecules of chelidonine. A mass-spectrometric analysis of Ukrain components failed to demonstrate the presence of the semisynthetic thiothepa derivative. Instead, analysis of the pharmaceutical preparation Ukrain revealed well-known alkaloids from *Chelidonium majus*, including chelidonine, chelerythrine, sanguinarine, protopine, and allocryptopine. Since 1990, preclinical investigations have pointed to the promising antineoplastic activity of Ukrain.[36-38] These studies suggested that Ukrain

exerts selective cytotoxic effects on tumor cells without adverse side effects on normal cells and tissues. However, observations on the selective cytotoxicity of Ukrain are still subject to controversy.[39]

1.1.3

Alkaloids used for MDR reversal

1.1.3.1

Cinchona alkaloids

Cinchona alkaloids isolated from the bark of several species of cinchona trees have been extensively studied for their antimalarial properties [197]. Later, quinine (25) and cinchonine (26) (Figure 1.6) were shown to have a potential use in reversing multidrug resistance in cancer patients, and are considered as first-generation blockers. In addition to their known role as inhibitors of Pgp, these chemomodulators have been suggested to have intracellular protein targets that may be involved in drug distribution.[40-42]

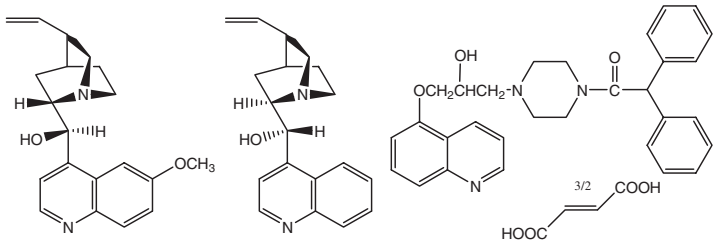

Fig 1.6

1.1.3.2

Dofequidar Fumarate (MS-209)

MS-209 (27, Figure 1.6), a quinoline derivative, was developed specifically as a selective Pgp inhibitor [208]. MS-209 alone has no antitumor activity and did not show serious toxicity at doses up to 2000 mg/kg. In preclinical models, MS-209 enhanced the antitumor effects of anticancer agents including adriamycin, vincristine, paclitaxel, and docetaxel in multidrug-

resistant tumor cell lines. It also enhanced the efficacy of anticancer agents against cancer cells sensitive to anticancer agents. MS-209 in combination with adriamycin was more effective than adriamycin alone against transplanted murine tumors, multidrug-resistant murine tumors, and human tumors transplanted to nude mice.[43]

1.1.4

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1.2

Topoisomerases and their inhibitors

DNA topoisomerases are marvelous molecular machines that manage the topological state of the DNA in the cell. These enzymes accomplish this feat by either passing one strand of the DNA through a break in the opposing strand (type I subfamily) or by passing a region of duplex from the same or a different molecule through a double-stranded gap generated in a DNA (type II subfamily). Besides altering the supercoiling of a closed DNA domain, the strand passing activities of topoisomerases can promote the catenation and decatenation of circular DNAs or the disentanglement of intertwined linear chromosomes. The fundamental need for topoisomerases derives from the double helical structure of DNA. For most processes that must access the information stored in the DNA, the two strands of the helix must separate either temporarily, as in transcription or recombination, or permanently, as in replication. The circular nature of bacterial chromosomes and the large size of eukaryotic DNA molecules preclude a simple winding solution to many of the topological problems that accompany the DNA transactions that occur in the cell.[1] Thus, during DNA replication, the two strands of the DNA must become completely unlinked by topoisomerases, and during transcription, the translocating RNA polymerase generates supercoiling tension in the DNA that must be relaxed.[1,2] The association of DNA with histones and other proteins introduces supercoiling that requires relaxation by topoisomerases. The immense interest in topoisomerases in recent years derives not only from the recognition of their crucial role in managing DNA topology, but also from two major advances in the field. First, a wide variety of topoisomerase-targeted drugs have been identified, many of which generate cytotoxic lesions by trapping the enzymes in covalent complexes on the DNA. These topoisomerase poisons include both antimicrobials and anticancer chemotherapeutics, some of which are currently in widespread clinical use.

DNA cleavage by all topoisomerases is accompanied by the formation of a transient phosphodiester bond between a tyrosine residue in the protein and one of the ends of the broken strand. DNA topology can be modified during the lifetime of the covalent intermediate, and the enzyme is released as the DNA is religated. Those enzymes that cleave only one strand of the DNA are defined as type I and are further classified as either type IA subfamily members if the protein link is to a 5′ phosphate (formerly called type I-5′) or type IB subfamily members if the protein is attached to a 3′ phosphate (formerly called type I-3′).

Topoisomerase[a]	Subfamily type	Subunit structure	Size(s) (aa)[b]
Eubacterial DNA topoisomerase I (*E. coli*)	IA	Monomer	865
Eubacterial DNA topoisomerase III (*E. coli*)	IA	Monomer	653
Yeast DNA topoisomerase III (*S. cerevisiae*)	IA	Monomer	656
Mammalian DNA topoisomerase IIIα (human)	IA	Monomer	1001
Mammalian DNA topoisomerase IIIβ (human)	IA	Monomer	862
Eubacterial and archaeal reverse DNA gyrase (*Sulfolobus acidocaldarius*)	IA	Monomer	1247
Eubacterial reverse gyrase (*Methanopyrus kandleri*)[c]	IA	Heterodimer	A, 358 B, 1221
Eukaryotic DNA topoisomerase I (human)	IB	Monomer	765
Poxvirus DNA topoisomerase (vaccinia)	IB	Monomer	314
Hyperthermophilic eubacterial DNA topoisomerase V (*Methanopyrus kandleri*)[d]	IB	Monomer	—[e]
Eubacterial DNA gyrase (*E. coli*)	IIA	A_2B_2 hetero-tetramer	GyrA, 875 GyrB, 804
Eubacterial DNA topoisomerase IV (*E. coli*)	IIA	C_2E_2 hetero-tetramer	ParC, 752 ParE, 630
Yeast DNA topoisomerase II (*S. cerevisiae*)	IIA	Homodimer	1428
Mammalian DNA topoisomerase IIα (human)	IIA	Homodimer	1531
Mammalian DNA topoisomerase IIβ (human)	IIA	Homodimer	1626
Archaeal DNA topoisomerase VI (*Sulfolobus shibatae*)	IIB	A_2B_2 hetero-tetramer	A, 389 B, 530

[a]The source of the most extensively studied family member is given in parentheses. The top portion of the table lists the type I topoisomerases; the bottom portion the type II enzymes.

[b]The subunit sizes are those corresponding to the most extensively studied family member.

[c]Included as the only known reverse gyrase with a heterodimeric structure.

[d]Only known representative at present. Probably present in other hyperthermophilic eubacteria.

[e]Gene not yet cloned; purified protein has a molecular size of 110 kDa.

Tab 2.1

Topoisomerases that cleave both strands to generate a staggered double-strand break are grouped together in the type II subfamily of topoisomerases. Further division of the subfamilies is based on structural considerations. Table 2.1 lists representatives of the various subfamilies of both prokaryotic and eukaryotic topoisomerases.[3]

Like the yeasts, all higher eukaryotes contain a single topoisomerase I enzyme that plays a major role in supporting fork movement during replication and in relaxing transcription related supercoils. Topoisomerase I is indispensable during development and probably also during cell division.[4,5] The realization that chromosome condensation during mitosis involves the generation of right-handed solenoidal supercoils by proteins belonging to the SMC family [6] means that topoisomerase I would also be required at this stage of the cell cycle to relax compensatory negative supercoils.

1.2.1

Human DNA Topoisomerase I

This section mainly focuses on the properties and structure of human DNA topoisomerase I, the best studied of the cellular type IB enzymes. Based on sequence comparisons, it is likely that most features described here for the human enzyme apply to the cellular topoisomerases I from other eukaryotic species as well.

Structure Based on conservation of sequence, sensitivity to limited proteolysis, hydrodynamic properties, and fragment reconstitution experiments, the 91-kDa human topoisomerase I protein has been subdivided into four distinct domains [7,8] (Figure 2.1).

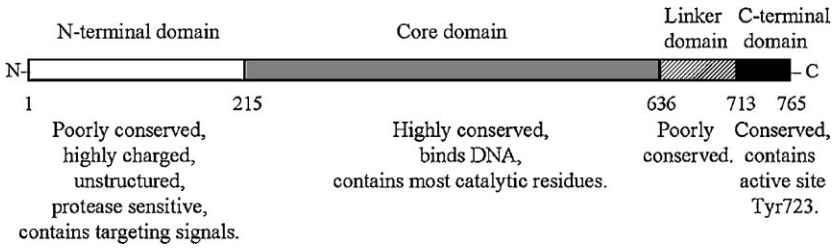


Fig 2.1

The N-terminal 214 aminoacids of the human enzyme are dispensable for relaxation activity in vitro and constitute a hydrophilic, unstructured, and highly protease-sensitive region of the protein. Contained with the N-terminal domain are four nuclear localization signals and sites for interaction with other cellular proteins, including such proteins as nucleolin, SV40 T-antigen, certain transcription factors, p53, and the WRN protein. The N-terminal domain is followed by a highly conserved, 421 amino acid core domain that contains all of the catalytic residues except the active site tyrosine. A protease-sensitive and poorly conserved linker domain comprising 77 amino acids connects the core domain to the 53 amino acid C-terminal domain. An active form of the enzyme can be reconstituted by mixing together fragments corresponding approximately to the core domain (residues 175 to 659) and the C-terminal domain (residues 713 to 765), and thus the linker is dispensable for relaxation activity in vitro. The active site Tyr723 is found within the C-terminal domain. The crystal structures of several forms of the human enzyme with both non- covalently and covalently bound DNA have been determined. These co-crystal structures represent the only examples to date of a topoisomerase containing bound DNA. Two views of the structure of human topoisomerase I non-covalently complexed with a 22 base pair DNA are shown in Figure 2.2. Although the crystals were grown with an N-terminal truncated active form of the protein missing the first 174 aminoacids, the X-ray density was only interpretable beginning at residue 215, and thus the entire N-terminal domain is missing from the structure (see Figure 2.1). Recently the crystal structure of another form of the protein extends the structure back to amino acid residue 203. The bi-lobed protein clamps tightly around the DNA with contacts between the protein and the DNA phosphate backbone that extend over 14 base pairs. Most of the contacts are clustered around the five base pairs upstream (−5 to −1) of the cleavage site, which is defined as occurring between DNA residues −1 and +1. The domain structure of the protein, including the subdivision of the core domain into core subdomains I, II, and III, is shown by the color

scheme in Figure 2.2. One of the lobes of the protein comprises core subdomains I and II (yellow and blue) and forms what has been referred to as the "cap" of the protein. The front end of the cap consists of a pair of long α-helices in a V-shaped configuration that had been seen earlier in the structure of a 26-kDa N-terminal fragment of yeast topoisomerase I. The other lobe forms a base that cradles the DNA and consists of core subdomain III (red) and the C-terminal domain (green).[9-13]

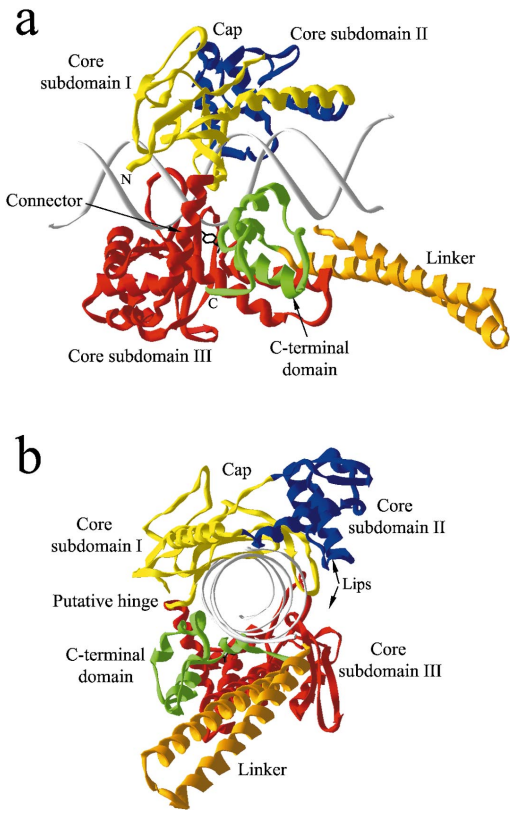


Fig 2.2

This second lobe is connected to the cap by a long α-helix labeled the "connector" in Figure 2.2*a*. On the side opposite to this α-helix are a pair of opposing loops called the "lips" that interact via six amino acids and one salt bridge to connect the cap to the base of the protein. Opening and closing of the protein clamp during DNA binding and release must involve the breaking of this interaction between the lips and the lifting of the cap away from the base as shown by the opposing arrows in Figure 2.2*b*. The hinge for this movement may be located at the top of the connector α-

helix as shown in Figure 2.2*b* (putative hinge).[13] The dispensable 77 amino acid linker domain (orange) is a coiled-coil structure that protrudes conspicuously from the base of the protein. Despite the clustering of basic amino acids on the DNA proximal sides of both the linker and the V-shaped α-helices of the cap, neither region of the protein is in direct contact with the DNA in the structure. Although it was necessary to inactivate the enzyme by replacing the active site Tyr723 with phenylalanine to obtain crystals, by modeling a tyrosine in place of the phenylalanine in the structure, it can be seen that the tyrosine side chain is buried between core subdomain III and the C-terminal domain and is close to the scissile phosphate (Figure 2.2*a*).

Despite very little sequence homology, most of core subdomain III of human topoisomerase I (residues 440–614) superimposes structurally on the catalytic core region of a family of tyrosine recombinases that includes bacteriophages HP1 and λ integrases, and *E. coli* XerD and bacteriophage P1 Cre recombinases.[12-13] In addition, a region near the active site of human topoisomerase I (residues 717–724) appears to correspond structurally to the active site region of the recombinases. In retrospect, this structural similarity is not surprising in view of the many biochemical properties shared by the two classes of enzymes, including a lack of Mg(II) dependence and the formation of a covalent intermediate involving attachment of the enzyme to the 3' phosphate of the cleaved strand. Most strikingly, the architecture of the active sites for the tyrosine recombinases are very similar to what is found for human topoisomerase I.[14] Thus, the tyrosine recombinases and type IB topoisomerases possess functional cores that use the same chemistry to carry out cleavage and religation. The two classes of enzymes are distinguishable by what occurs during the lifetime of the cleaved intermediate. The topoisomerases permit topological changes in the DNA and restore the original DNA linkage, whereas the recombinases promote strand exchange and join the DNA ends to new partners.[15]

Substrate Specificity The substrate specificity of eukaryotic topoisomerases I has been characterized at both the nucleotide sequence level and at the level of DNA tertiary structure. By mapping cleavage sites using detergent to trap the covalent complex,[15,16] the enzymes were found to nick the DNA with a preference for a combination of nucleotides that extends from positions −4 to −1. The preferred nucleotides in the scissile strand are 5′-(A/T)(G/C)(A/T)T-3′ with the enzyme covalently attached to the −1 T residue. Occasionally a C residue is found at the −1 position. A particularly strong cleavage site for all eukaryotic topoisomerases I was identified by Westergaard and his colleagues in the rDNA repeats found in *Tetrahymena pyriformis*,[17] and this sequence, which has a T at the −1 position, formed the basis for designing the 22 base pair oligonucleotide used in the crystallographic studies .[15]

The only base-specific contact between the protein and the DNA in the crystal structures involves a hydrogen-bond between Lys532 and the O-2 atom of the strongly preferred thymine base located at the −1 position. This base-specific contact would seem to provide a good explanation for the preference of a thymine at this location. The presence of a carbonyl oxygen at the same position in a cytosine base, which is also tolerated at this position, would be consistent with this conclusion. However, replacing Lys532 with an alanine drastically reduces the activity of the enzyme without detectably changing the cleavage specificity. Apparently other protein-DNA interactions in addition to the Lys532 contact with the −1 base play an important role in cleavage site selection. Thus, the structural basis for the weak nucleotide sequence preference of the enzyme remains elusive.

A number of studies have indicated that eukaryotic topoisomerase I prefers supercoiled over relaxed plasmid DNAs as substrates,[16,17] and the use of a mutant form of the protein with phenylalanine instead of tyrosine at the active site (Y723F) showed that the enzyme has a strong preference for binding to supercoiled DNA over relaxed DNA.[18] This binding property was localized to the core domain by showing that a fragment including

amino acid residues 175 to 659 exhibited the same preference for supercoils. Since the enzyme preferentially binds to supercoils of either sign, it seems likely that the structural feature recognized in the DNA is a node where two duplexes cross. Indeed, topoisomerase I has been observed to associate with nodes by electron microscopy.[18,19] The association of human topoisomerase I with a node would appear to require two DNA binding sites on the protein. One way to accommodate this requirement would be for the enzyme to dimerize upon binding DNA.[3] Although there is no evidence for dimerization, this possibility has not been ruled out. An alternative hypothesis is suggested by the crystal structure. The three-dimensional structure of core subdomain II was found to superimpose on the structure of the human Oct-1 POU homeodomain. Since Oct-1 belongs to a family of transcription factors that specifically bind DNA , this homeodomain-like region could represent a second DNA binding site on topoisomerase I. However the residues in Oct-1 that confer sequence specificity are not conserved in core subdomain II, so if this region of human topoisomerase I does bind DNA, it would seem to be a low- affinity, nonspecific interaction. Further experimentation will be required to test this hypothesis.

Mechanism of Catalysis Nucleophilic attack by the O-4 oxygen of Tyr723 on the scissile phosphate breaks the DNA strand to generate a phosphodiester link between the tyrosine and the 3' phosphate, releasing a 5' hydroxyl. Two different orientations of key active site residues in relation to the scissile phosphate were observed in the crystal structures, suggesting the interesting possibility that two distinct stages along the catalytic pathway have been observed. In noncovalent complexes in which the DNA contains a thymine at the −1 position (−1T structure) , Arg488 and Arg590 appear to hydrogen bond with one of the nonbridging oxygens of the scissile phosphate while His632 is hydrogen-bonded to the other nonbridging oxygen. In a structure where the −1 thymine has been replaced with cytosine (−1C structure), the phosphate group has rotated by 75° relative to its position in the −1T structure, leaving Arg488 and His632

hydrogen-bonded to the same two nonbridging oxygens, but now Lys532 rather than Arg590 is hydrogen-bonded to a nonbridging oxygen. This −1C configuration is shown in Figure 6 where it can be seen that Lys532 is also hydrogen-bonded to the carbonyl O-2 oxygen of the −1 cytosine base. In either orientation, it appears that Tyr723 is perfectly aligned for nucleophilic attack and that a triad of basic amino acids is positioned to stabilize the pentavalent transition state through interactions with the nonbridging oxygens of the scissile phosphate.

Although it has been hypothesized that the nucleophilic O-4 oxygen of Tyr723 could be activated by the proximity of a general base, none of the structures revealed an amino acid close enough to the tyrosine to play this role. However, a water molecule has been found hydrogen-bonded to Arg590 that is only 2.3 Å from the O-4 oxygen (not shown in Figure 2.3) and therefore is in a position to act as a specific base and accept the proton as catalysis proceeds. There are two reasons for suspecting that the −1C structure represents a more advanced stage in catalysis than the −1T structure. First, the orientation of the amino acid side chains in the structure of the covalent complex that is produced on cleavage is the same as that observed in the −1C structure. Second, after the 75° reorientation of the phosphate group on going from the −1T to the −1C structure, Arg590 is no longer within hydrogen-bonding distance of either non-bridging oxygen but is now brought into close proximity to the Tyr723 hydroxyl .Hence,analogous to the situation described above for Arg321 of *E. coli* topoisomerase I, Arg590 could facilitate nucleophilic attack by stabilizing the phenolate anion. Furthermore, a direct role for Lys532 in transition-state stabilization is consistent with the large effect on the rates of cleavage observed for the K532A mutation in the human enzyme (H Interthal, J Champoux, unpublished) and the corresponding K167A mutation in vaccinia topoisomerase .[20]

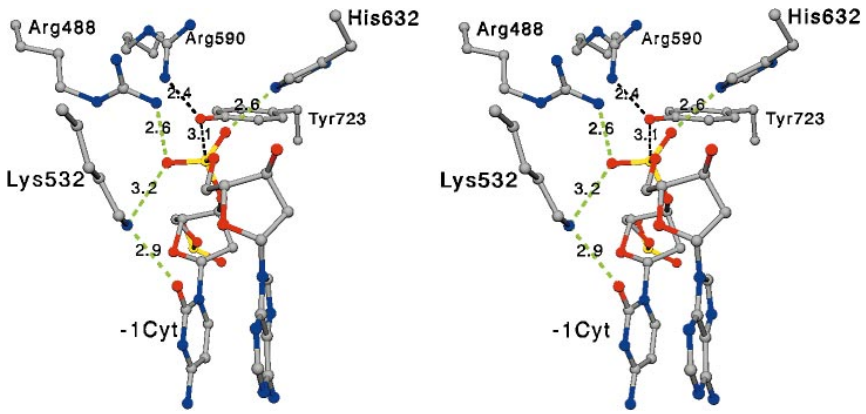


Fig 2.3

It was originally proposed that His632, in addition to stabilizing the transition state through its interaction with a nonbridging oxygen, might also act as a general acid to protonate the leaving 5 oxygen in the cleavage reaction. The findings that His632 can be replaced by glutamine with only a modest reduction in activity and that the H632Q mutation has the same pH profile as the wild-type enzyme rule out the possibility that His632 acts as a general acid.[20] A similar conclusion has been reached concerning the role of the corresponding His265 of vaccinia topoisomerase. Recent results indicate that Lys167 of vaccinia topoisomerase and the corresponding Lys532 in the human enzyme function instead as general acids to protonate the leaving 5' oxygen during cleavage. The distance of Lys532 from the 5'oxygen(~4 Å) in the human enzyme is compatible with this suggestion and confirms that Lys532 may very well play a key role in catalysis by human topoisomerase I. In principle, DNA religation could be mechanistically just the reverse of the cleavage reaction and the proximity of most of the same amino acids to the scissile phosphate in the crystal structure of the covalent complex is compatible with this possibility. However, the observation that the critical active site residue His632 is within a disordered region in the structure of the covalent complex suggests that there could be some mechanistic differences between the cleavage and religation reactions. Although the close proximity of the

attacking 5 oxygen nucleophile to the scissile phosphate likely contributes significantly to the rate of religation, other nucleophiles such as water, hydrogen peroxide, and certain alcohols can replace the 5 hydroxyl under some conditions.[20,21] Similar results have been reported for the vaccinia topoisomerase where there is also the suggestion that after mutating the active site tyrosine to histidine or glutamine, water may be able to participate in the cleavage reaction. These results indicate that the key to catalysis is the proper three-dimensional organization of the side chains of the active site residues around the scissile phosphate, leaving some flexibility with respect to the nature of the attacking nucleophiles.[21]

Mechanism of DNA Relaxation A schematic of one possible model for DNA relaxation is shown in Figure 2.4. the spatial arrangement of the DNA in the co-crystal structure of human topoisomerase I renders unlikely an enzyme-bridging model for DNA relaxation analogous to what has been proposed for *E. coli* topoisomerase I. Instead, it appears that once the DNA has been cleaved, the helical duplex downstream of the cleavage site rotates to relieve any torsional stress within the substrate DNA. Attempts to model this rotation within the confines of the hollow interior of the protein indicate that if the protein were to remain clamped around the DNA as shown in Figure 2.3, the rotating DNA would likely contact and perhaps shift the position of both the cap and the linker. Thus, the term "controlled rotation" was coined to indicate that these structural domains of the protein likely hinder or impede the rotation reaction. An alternative hypothesis is diagrammed in Figure 2.4, where it is proposed that once cleavage has occurred (Figure 2.4*d*), the enzyme opens up to allow rotation and perhaps assumes a conformation approximating that proposed for the DNA-free form of the enzyme (Figure 2.4*a*). In this model, there may be little or no hindrance to DNA rotation (Figure 2.4*e*). These two models differ mainly in the extent to which the DNA interacts with the cap and linker elements of the structure during rotation. Either model could accommodate multiple rotation events before religation of the DNA. Although the number of rotations per cleavage is not known for the human

enyzme, the vaccinia topoisomerase appears to allow, on the average, five rotations for each nicking-closing cycle.[22]

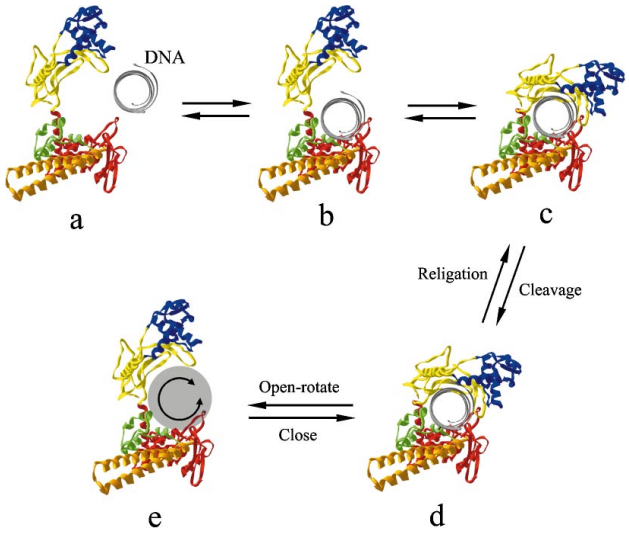


Fig 2.4

1.2.2

Human DNA Topoisomerase II

The following properties are shared by all type II topoisomerases[16]: (a) The dimeric enzymes bind duplex DNA and cleave the opposing strands with a four-base stagger (topoisomerase VI may generate a two-base stagger). (b) Cleavage involves covalent attachment of each subunit of the dimer to the 5′ end of the DNA through a phosphotyrosine bond. (c) A conformational change pulls the two ends of the cleaved duplex DNA apart to create an opening in what is referred to as the gated or G-segment DNA. A second region of duplex DNA from either the same molecule (relaxation, knotting or unknotting) or a different molecule (catenation or decatenation), referred to as the transported or T-segment, is passed through the open DNA gate. This feature of the reaction explains why the linking number is changed in steps of two when the supercoiling of a circular DNA is changed.[22] (d) The reactions require Mg(II), and ATP hydrolysis is required for enzyme turnover and rapid kinetics, although one cycle of relaxation or decatenation/catenation can occur in the presence of the nonhydrolyzable

analog of ATP, ADPNP (5'-adenylyl-β,γ-imidodiphosphate). (e) The crystal structures of several members, including the structurally distinct topoisomerase VI, reveal that the active site tyrosines are situated in a helix-turn-helix (HTH) motif found within a domain that strongly resembles the DNA binding region of the *E. coli* catabolite activator protein (CAP). In addition, acidic residues within a Rossmann fold on the opposing protomer appear to collaborate with the HTH region of the CAP-like domain to assemble the active site for catalysis and may be involved in metal ion binding in some cases.[23] This fold has also been referred to as a "toprim motif" because it is found in DNA primases as well as the topoisomerases and also in a number of other enzymes that catalyze phosphotransfers or hydrolyze phosphodiester bonds. Interestingly, both the CAP-like domain and the Rossmann/toprim fold of the type II enzymes are shared by the type IA, but not the type IB, subfamily of topoisomerases. (f) As with the type I enzymes, a highly conserved arginine residue is implicated in catalysis by its close proximity to the active site tyrosine.[24]

Several characteristics distinguish individual members of the type II family. All of the type II enzymes from both prokaryotic domains of life described to date contain two different subunits and are therefore heterotetrameric in structure, whereas the eukaryotic enzymes are homodimers (Table 2.1). Among all of the known type II enzymes, DNA gyrase stands alone as the only enzyme capable of using the energy from ATP hydrolysis to introduce negative supercoils into the DNA. Finally, different members of the type II family can be distinguished by their relative proficiency at DNA relaxation versus decatenation (or catenation), and this property likely reflects their specialized roles in the cell.[25]

1.2.3

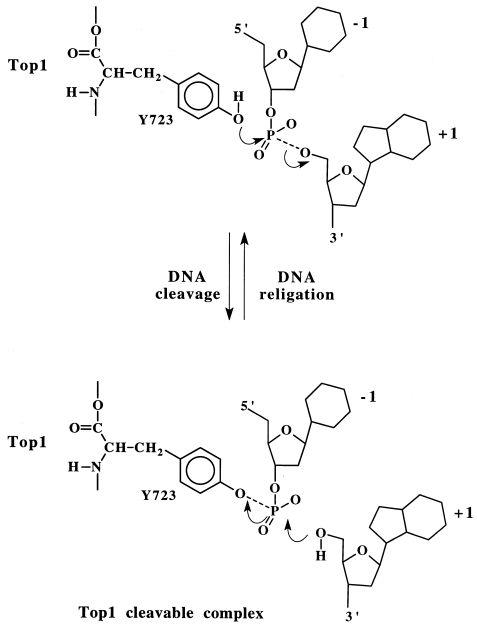
Topoisomerase – Targeted anticancer drugs

Topo I and II inhibitors are two promising new classes of anticancer agents[3] whose mechanism of action involves covalent binding to double-stranded DNA and subsequent interference with the DNA breakage-resealing process. Both inhibitor types form a cleavable complex by covalently binding to DNA-topo I or II; this binding still permits uncoiling of the double-stranded DNA but prevents its subsequent resealing. The advancing replication then is halted within the topoisomerase-DNA complex stage, resulting in unsealed DNA breakage and eventual cell death.

1.2.3.1

Topoisomerase I inhibitors

Topo I is a monomeric protein that breaks single strands of DNA by cleaving a phosphodiester bond, then covalently binds to the DNA 3'-phosphoryl end via a phosphotyrosine linkage. In general, topo I enzymes catalyze the removal of negative superhelical DNA twists, which is a thermodynamically favorable reaction.[26]



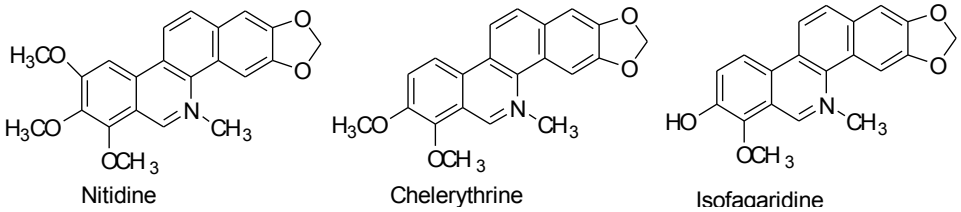
All cells appear to contain at least one type I and one type II topoisomerase. However, the levels of topo I in tumor specimens are higher than that in normal tissues, making inhibition of this enzyme an attractive target for anticancer agents. Surgical specimens from patients with colon cancer have a 14- to 16-fold increase in topo I levels in the malignant cells as compared with adjacent normal tissues.

Topo I inhibitors can be grouped into two main classes (a) topo I poisions, and (b) topo I suppressors. Both inhibit topo I catalytic activity (DNA relaxation). While topo I poisions trap cleavage complees, topo I suppressors do not. Topo I poisons kill cells by trapping cleavage complexes rather than inhibiting catalytic activity.[27, 28]

Camptotecin and analogues

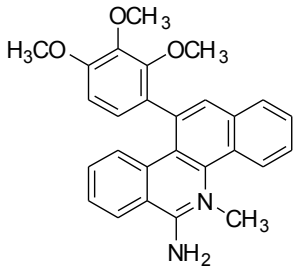
See chapter 3.

Benzophenanthridine Alkaloids



Three strongly topo I inhibitory principles, nitidine , chelerythrine and a new alkaloid isofagaridine were identified from a methanol extract of *Zanthoxylum nitidum* by Fang *et al.*[29] At 167 or 500 mM for 30 min. nitidine and isofagaridine, completely inhibited pSP64 DNA relaxation induced by 2.2 ng of calf thymus DNA topo I. Nitidine and isofagaridine were also effective at 50 mM, while chelerythrine, gave partial inhibition at this concentration. These results compared favorably with those obtained for

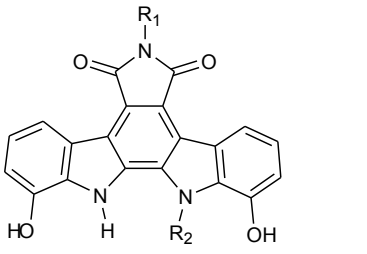
camptothecin, which inhibited[4] relaxation partially at 167 mM and completely at 500 mM.



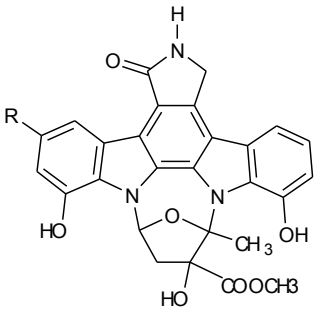
In 2005, Clement *et al*[30] reported 11-substituted 6-aminobenzo [*c*]phenanthridine derivatives, among them the above compound shows good activity *in vitro* as well as *in vivo*.

Indolocarbazole Derivatives

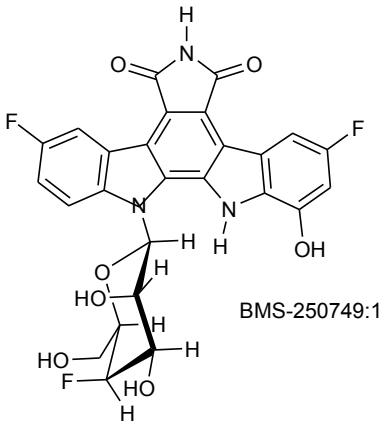
A indolocarbazole antitumor antibiotic, BE-13793C isolated [31] from the culture broth of the actinomycete strain *Streptoverticillium mobaraense* has topoisomerase inhibitory activity. BE-13793C strongly inhibited both topo I and topo II and inhibited the growth of doxorubicin-resistant or vincristine-resistant P388 murine leukemia cell lines, as well as their parent P388 cell line. Compound ED-110 is a new semisynthetic antitumor agent derived from BE-13793C by glucosylation.[32] Exposing P388 cells to ED-110 caused typical topoisomerase toxicity, i.e., formation of cleavable complexes, inhibition of nucleotide synthesis rather than protein synthesis, and cell cycle arrest in G2.



BE-13793C: R1=R2=H
ED:R1=H. R2=D-glucose
NB-506:R1==NHCHO, R2=D-glucose



K252A: R=H
KT6006:R=OH

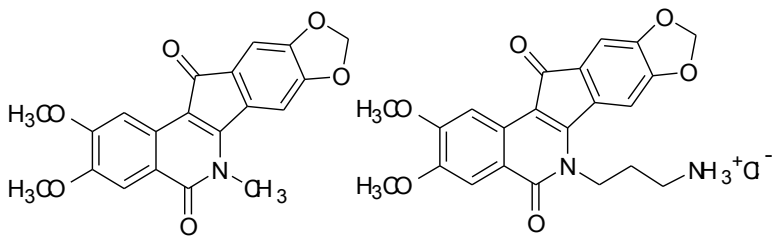


BMS-250749:1

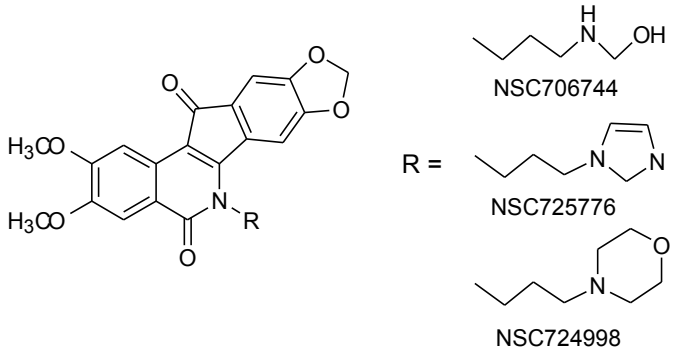
KT6006 and KT6528 , synthetic antitumor derivatives of the indolocarbazole antibiotic K252a, were potent inducers of a topo I cleavable complex.[33] A fluoroindolo[2,3-a]carbazole BMS-250749:1 shows a broad spectrum antitumour activity [34] comparable to that of the marketed CPT-11.

Indenoisoquinolines

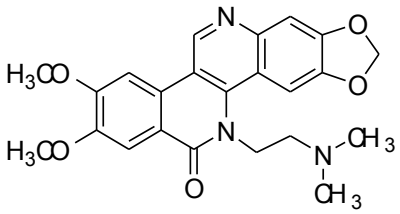
Indenoisoquinoline (NSC314622), an unexpected transformation product during the synthesis of nitidine chloride, was reported by Cushman *et a l*[35] to induce DNA cleavage in the presence of topI. Its cleavage site was not the same as that observed in camptothecin. In 2003, Cushman *et. al* reported that N-substitution by 3-aminopropyl substituents induced potent topoisomerase inhibitor activity. On the basis of these results, the substituents on the heterocyclic lactam as given below, showed greater activity than the parent indenoisoquinoline (NSC314622).[36,37]



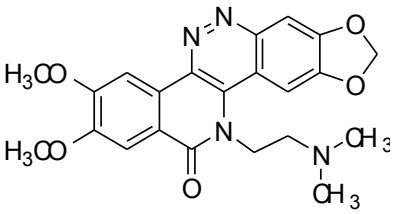
NSC 725776 3-aminopropyl substituents



Phenanthridines



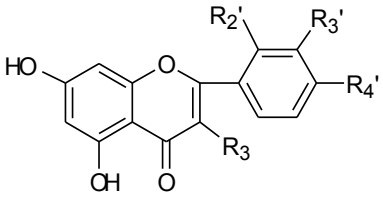
(A) ARC-111



(B)

Synthetic phenanthridine derivatives such as 5*H*-8,9-dimethoxy-5-(2-*N*,*N*-dimethylaminoethyl)-2,3-methylenedioxydibenzo[*c,h*][1,6]naphthyridin-6-one, **(A)**, and 11*H*- 2,3-dimethoxy-5-(2-*N*,*N*-dimethylaminoethyl)-8,9-methylenedioxy-5,6,11-triazachrysen-12-one (**B)** can exhibit potent TOP1-targeting activity and pronounced cytotoxicity.. Potent antitumor activity[38] was observed for **(A)** administered orally and parenterally to athymic nude mice bearing the human tumor xenograft, MDAMB-435.

Flavones



Quercetin: R_3= $R_{3'}$= $R_{4'}$= OH, R_2=H

Acacetin: R_3= $R_{2'}$= $R_{3'}$= OH, $R_{4'}$=OCH_3

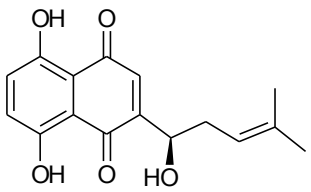
Apigenin: R_3= $R_{2'}$= $R_{3'}$= H, $R_{4'}$=OH

Kaempferol: $R_{2'}$= $R_{3'}$= H, R_3 = $R_{4'}$=OH

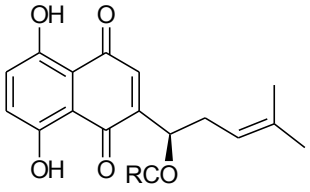
Morin: R_3= $R_{2'}$= $R_{4'}$= H, $R_{3'}$= H

Quercetin and the related natural flavones, acacetin, apigenin, kaempferol, and morin inhibit topo I-catalyzed DNA religation.[39]. In contrast to camptothecin these compounds do not act directly on the catalytic intermediate and also do not interfere with DNA cleavage. However, a ternary complex with topo I and DNA is formed during the cleavage reaction that inhibits the following DNA religation step.

Shikonin Analogs



Shikonin

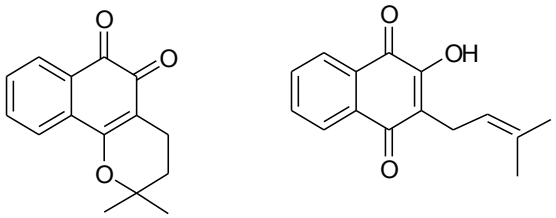


Acylshikonins, R = (C_2-C_{12} , C_{15}-C_{18})

The antineoplastic benzoquinone Shikonin isolated from the root of *Lithospermum erythrorhizon* (Boraginaceae), exhibited strong cytotoxicity against L1210 cells. Ahn *et al.* reported that shikonin and some synthetic 19-acylshikonins[40] are potent inhibitors of DNA topo I. In general, analogs with shorter acyl groups (C2–C6) were more potent than those with longer acyl chains (C7–C20). While halogen substitution at C-2 of the acetyl moiety failed to increase the inhibitory potency, placing double bonds in the acyl group (C5–C7) augmented the potency remarkably.

β-Lapachone

β-Lapachone is obtained by simple sulfuric acid oxidation of the naturally occurring lapachol[41] which is isolated from *Tabebuia avellanedae* growing mainly in Brazil. This compound inhibits topo I mediated DNA cleavage through direct interaction with topo I rather than DNA substrate.



β-Lapachone ***Lapachol***

The direct interaction of β-Lapachone with topo I may not affect the assembly of the enzyme-DNA complex but does inhibit the formation of cleavable complex.

1.2.3.3

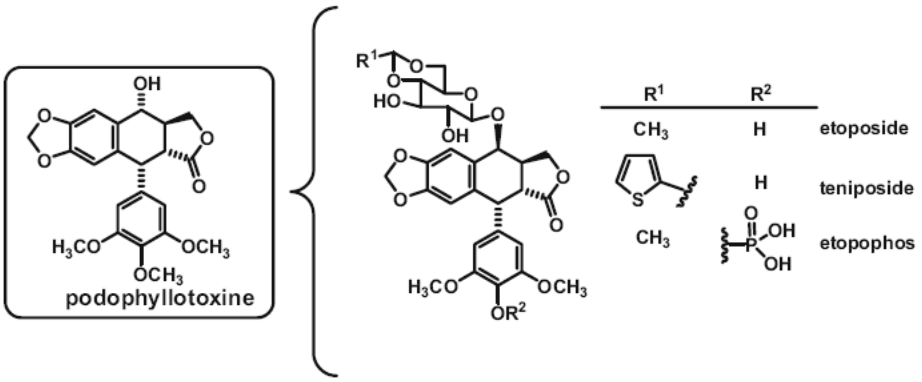
Topoisomerase II inhibitors

The level of topo II in normal cells is cell cycle-dependent and is controlled by the topo II gene, probably through a post-translation regulation mechanism.[42] Topo II activity is low in G_0 phase, is higher in cell division phase, and reaches a maximum during S phase.[43] Topo II activity also parallels the rate of cell proliferation; it is high in rapidly growing cells and lower in slowly growing cells. The half life of topo II activity in tumor cells (chicken hepatoma cells) is 4-fold longer than that in the corresponding normal cells (chicken embryo fibroblasts).

Topo II-targeted anticancer drugs achieve their selectivity for rapidly proliferating cancer cells over normal cells from this correlation between topo II activity and cell proliferation. Topo II inhibitors can be roughly classified into two groups: intercalators and nonintercalators. Nonintercalator topo II inhibitors include epipodophyllotoxins, such as etoposide, and some isoflavones, such as genistein. Intercalator topo II inhibitors are structurally diverse and usually have planar aromatic ring systems that can fill in (intercalate) between DNA base pairs and obstruct normal DNA functions.

Etoposide and its analogs

Etoposide (VP16,) and teniposide (VM26,) are two successful anticancer analogs developed from the anticancer lignan podophyllotoxin, which is isolated from *Podophyllum peltatum* or *P. emodii* (Berberidaceae).

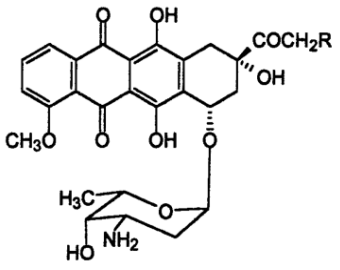


Etoposide–like compounds are potent irreversible inhibitors of DNA topoisomerase II and their action is based on the formation of nucleic acid drug enzyme complex, which induces single- and double- stranded DNA breaks as the intial step in a series of biochemical transformations that eventually lead to cell death.

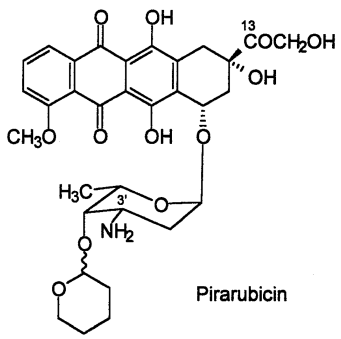
In order to overcome the limitations of these compounds with better anitumour activity, many structural modifications[44] have been perfomed on the cyclolignan skeleton.

Anthracyclines

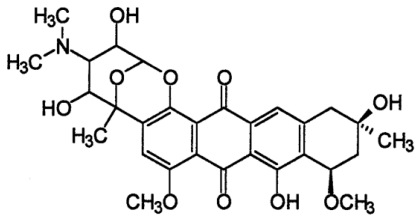
The anthracyclines are classical anticancer antibiotics and include doxorubicin (Adriamycin) and daunorubicin. These predominant topo II inhibitors have been used in the treatment of various tumors for many years. Doxorubicin, a standard anticancer drug, is widely used against leukemia, advanced urothelial, and other tumors. Antracyclines are intercalated in DNA due to their planar cyclic system.



Doxorubicin R= OH
Daunorubicin R = H



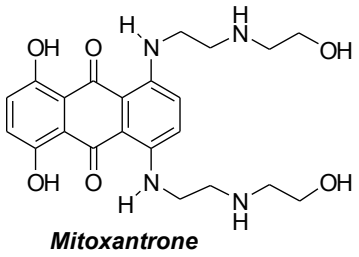
Pirarubicin



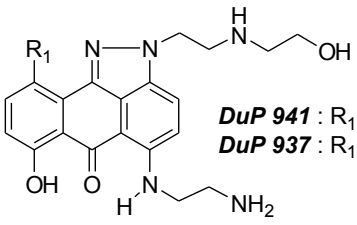
Menogaril

Menogaril, a new anthracycline agent, possesses weak double-helix unwinding activity. This anthracycline is for treatment of many tumors including leukemia[45] and malignant mesothelioma. Due to the side effects of doxorubicin , such as myelosuppression and irreversible cardiotoxicity, pirarubicin was recently developed[46]

Anthrapyrazoles (Mitoxantrone Analogs)



Mitoxantrone

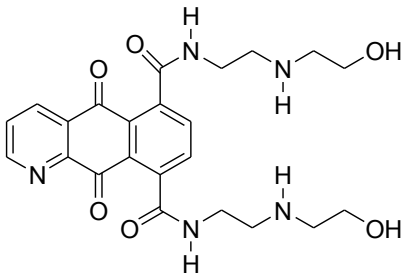


DuP 941 : R_1= H, R_2 =$(CH_2)_2OH$
DuP 937 : R_1= OH, R_2 = CH_3

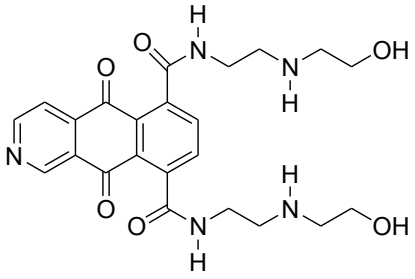
New DNA topo II inhibitory anthrapyrazoles structurally related to mitoxantrone were designed to suppress the redox reductions responsible for cardiac toxicity. e.g. DuP 941 (Losoxantrone) and DuP 937. These are potent topo II inhibitors closely related to mitoxantrone. Anthrapyrazoles are unlikely to produce free radicals and might be useful in the same

indications as mitoxantrone**,** especially for patients with cardiac risks and for pediatric patients.[47]

Azaanthracene-9,10-diones



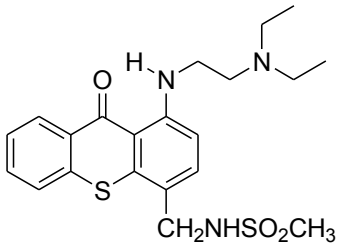
1-Azaanthracene-9-10-dione



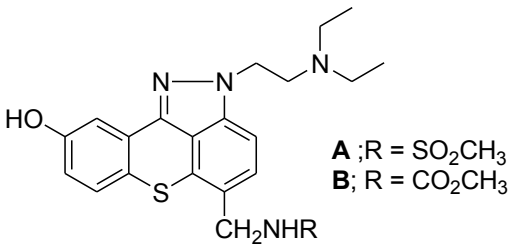
2-Azaanthracene-9-10-dione

Zwelling *et al.*[48] examined the activities of two novel aza-anthracene-9,10-diones, 1-aza and 2-aza-anthracene-9-10-diones in HL-60 human leukemia cell lines containing topo II with different sensitivities to other intercalating agents. Both aza compound induced, topo II-mediated DNA cross-linking in the cells revealed resistance and sensitivity patterns paralleling the cytotoxicity results.

Thioxanthones

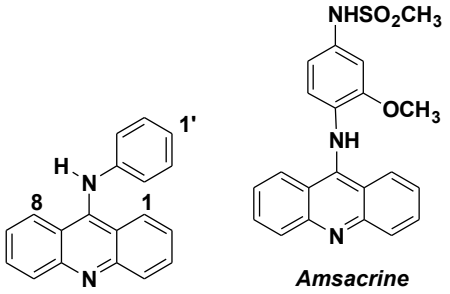


WIN 33377



Some new 4-aminomethylthioxanthones are active against[49] the murine tumor Panc 03. The methanesulfonamide analog SR 233377 (WIN 33377) is also a potent topo II inhibitor with a EC_{50} value of 3.0 µM. Derivatives **A, B** which incorporate a pyrazole ring at the 1- and 9-position of the thioxanthones[50] showed more topo II inhibition than WIN 33377.

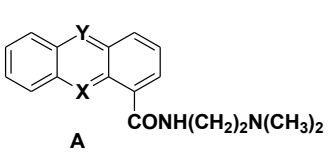
Acridone and Related Acridine Derivatives



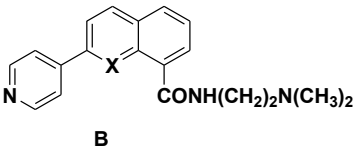
Amsacrine

(i), 1' -NHMe
(**ii)**, 1' -$NHSO_2Me$, 3' = OMe
(iii), 1' - $NHSO_2Me$, 3' = OMe, 3,6-diNH_2
(iv), 1' - SO_2NH_2, 3-NH_2
(v), 1' -SO_2NH_2, 3,6-diNH_2
(vi), 1' - NH_2 , 3,6-diNH_2
(vii), 1'-N(Me)Et
(viii), 1'-NHhexyl

Naturally occurring acridone alkaloids and related synthetic derivatives[51] possess many biological activities including antitumor, antiparasitic, and antiviral properties. 9-Anilinoacridines derivatives (**I – viii)** inhibit both the growth of Jurkat leukemia cells and activity of human DNA topo II *in vitro*[52].



A



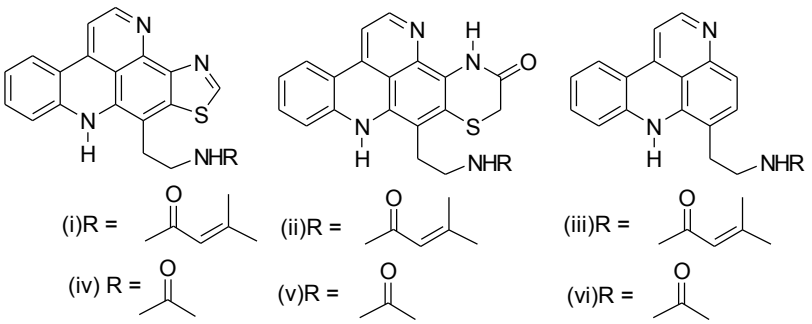
B

(ix): A : X = N, Y = $CONH_2$
(x) :A : X = N, Y = CN
(xi) : B : X = N
(xii) : B : X= Y=N
(xiii) : A : X= Y= CN

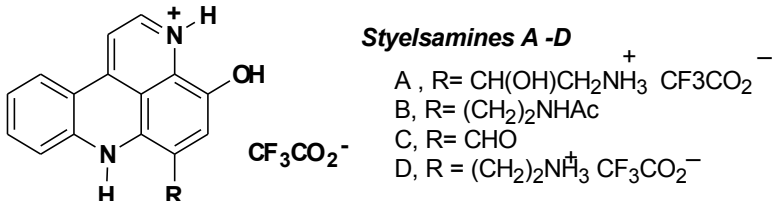
Antitumor intercalators acridine-4-carboxamide (**A**) and 2-(4-pyridyl)quinoline-8-carboxamide (**B**) are structurally related to the topo II-targeted drug amsacrine. These tricyclic carboxamides (**ix –xiii**) induce DNA lesions (which reflects stabilization of topo II cleavage complexes) in mouse fibrosarcoma cells (line 935.1).[53] The topo II inhibitory mechanisms by these tricyclic carboxamides differ from the classical trapping of topoisomerase in covalent cleavage complex.

Pyridoacridine Alkaloids

Several new pyridoacridine alkaloids, dehydrokuanoniamine B (**i**), shermilamine C (**ii**), and bystodytin J (**iii**), and the known compounds cystodytin A (**iv**), kuanoniamine D (**v**), shermilamine B (**vi**), were isolated from the Fijian *Cystodytes* sp.



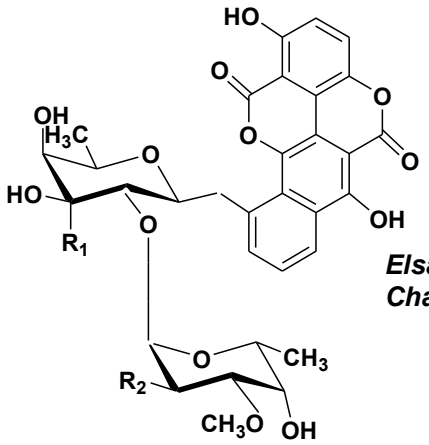
These pyridoacridines probably inhibit cellular proliferation by disrupting topo II function subsequent to intercalation. In incorporation studies, pyridoacridines disrupted DNA and RNA synthesis with little effect on protein synthesis. These results are consistent with those for known DNA intercalators.



A series of new alkaloids, styelsamines A-D **(a**-**d),** have been isolated from the Indonesian Ascidian, *Eusynstyela latericius*, which inhibit human colon HCT-116 cells in vitro[54] in the 1-100 µM range.

Elsamicin and Other Coumarin-Related Antitumor Agents

Elsamicin is structurally related to the anticancer compounds chartreusin



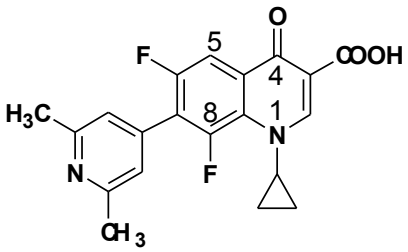
Elsamicin A : R_1= CH_3, R_2 = NH_2
Chartreusin : R_1= H, R_2 = OH

These compounds induced, to a different extent, DNA breakage in the human lung adenocarcinoma A549 cell line.[55] In addition all either bound to or intercalated into DNA.

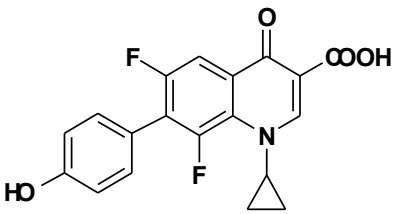
1,4-Dihydroquinoline Derivatives

The antibacterial properties of the quinolines have been known for over three decades. A 3-quinolinecarboxylic acid derivative (WIN 57294), a reported potent inhibitor of bacterial DNA gyrase, was found to be interactive with mammalian topo II by Wentland *et al*[56].

The 7-(4-hydroxyphenyl) analog (CP-115,953) was 6-fold more potent (EC50 = 1.2 mM) than *WIN 57294*



WIN 57294



CP-115,953

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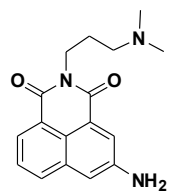
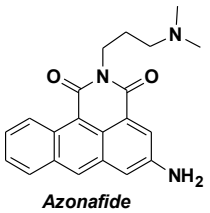
Mono- and Bis-naphthalimide Analogs

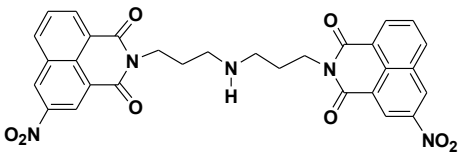
Amonafide and its analogs interfere with the breakage-rejoining reaction of purified mammalian DNA topo II by stabilizing a reversible enzyme-DNA

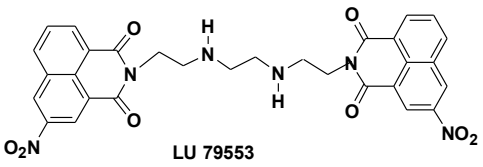
cleavable complex. In contrast to other topo II-active antitumor drugs, Amonafide induces specific DNA cleavage at a single major site on pBR322 DNA. This strong site specificity may allow detailed characterization of the intercalator-stabilized, topo II-DNA cleavable complex[57]

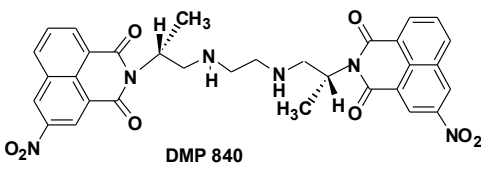
A dimethylaminoethyl group was the best N2 substitution, and the 4-, 5-, 7-, and 9-amino derivatives[58] showed significantly higher potency than the unsubstituted parent compound, Azonafide.





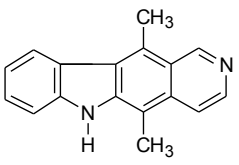


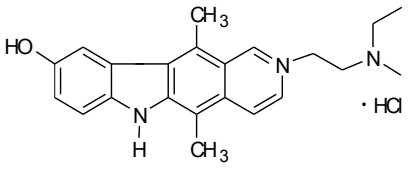


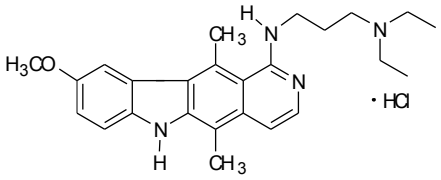


To increase binding capacity to DNA and improve the therapeutic properties of Aminofide and Azonafide two naphthalimides were joined by polyamine linkers.[59]

Ellipticine Analogs



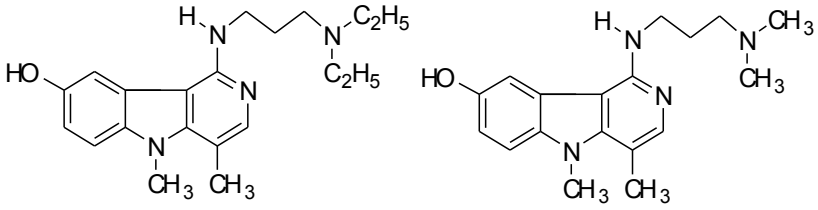




Ellipticine is an antitumor alkaloid isolated from *Ochrosia elliptica.*[60] It inhibits topo II and shows anticancer activity in several animal and human tumor systems.

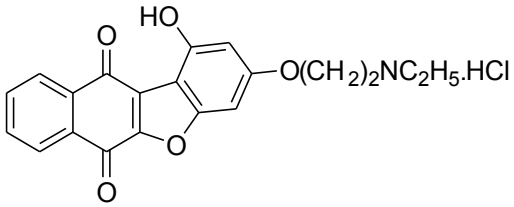
Compound (DHE) and Retelliptine are more cytotoxic than Ellipticine, this increased cytotoxicity was not accompanied by greater amount of DNA strand breakage or protein–DNA cross-linking. The single strand breaks caused by both compounds were protein associated and could result from double strand breaks. The DNA damage therefore was consistent with topo II inhibition.[61]

γ-Carbolines



γ-Carbolines are tricyclic aromatic compounds , they intercalate into DNA base pairs and exhibit significant cytotoxic and antitumor activities[62]. These two most cytotoxic γ-carbolines are also the most efficient stabilizers of the DNA-topo II covalent cleavable complex.

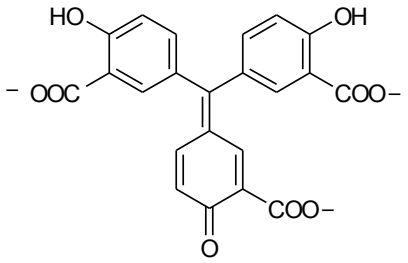
Benzonaphthofuran-6,11-diones



Based on the "2-phenylnaphthalene-type" structural pattern hypothesis, Liu *et al.* designed and synthesized a series of benzonaphthofuran-6,11-

diones. *In vitro* inhibitory action was evaluated against the growth of various human tumors and drug-stimulated topo II-mediated DNA cleavages.[63]

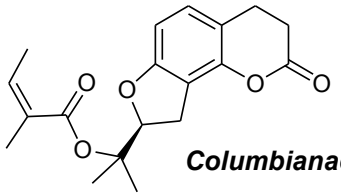
Aurintricarboxylic Acid



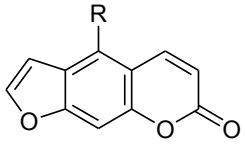
Aurintricarboxylic acid (ATA)

Aurintricarboxylic acid (ATA) is a polyanionic, polyaromatic compound derived from aurin, a triphenylmethane dye. Aurintricarboxylic acid inhibits apoptotic cell death induced by a variety of factors in various cell types. It is a potent inhibitor of the nuclear enzyme DNA topo II.[64] This compound effects on DNA-protein complex formation in living cells may result from a direct interaction with topo II. It prevents the binding of topo II to DNA and inhibits topo II-catalyzed ATP hydrolysis. [65]

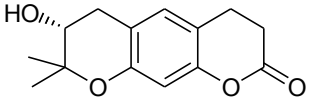
Coumarins and Derivatives



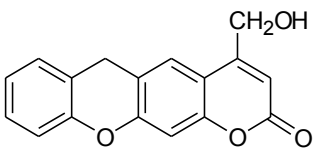
Columbianacin



5-Methoxypsoralen: R = OCH_3
Psoralen: R = H



Acetylumbelliferone

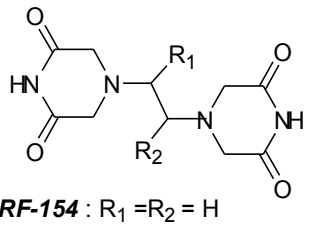


4-Hydroxymethyl-4',5'-benzopsoralen

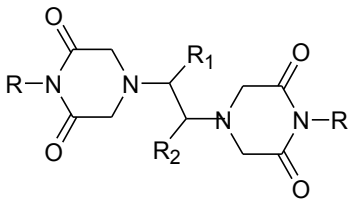
Columbianadin, 5-methoxylpsoralen, psoralen, and acetylumbelliferone were isolated from the roots of *Seseli mairei* Wolff [66] to significantly inhibit DNA topo II activity. 4-hydroxymethyl-4',5'-benzopsoraien appeared to inhibit mammalian topo II activity *in vitro,* in both the catenation and the deactivations assays.

Bis-dioxopiperazines (Dexrazoxane Analogs)

Bis-dioxopiperazines are recently developed novel antitumor agents. The parent bis(2,6-dioxopiperazine), ICRF-154, and its derivatives, ICRF-159, ICRF-193, and MST-16, all inhibit mammalian type II DNA topoisomerase[67].



ICRF-154 : $R_1 = R_2 = H$
ICRF-159 : $R_1 = H$, $R_2 = CH_3$(racemic)
ICRF-154 : $R_1 = R_2 = CH_3$(meso)

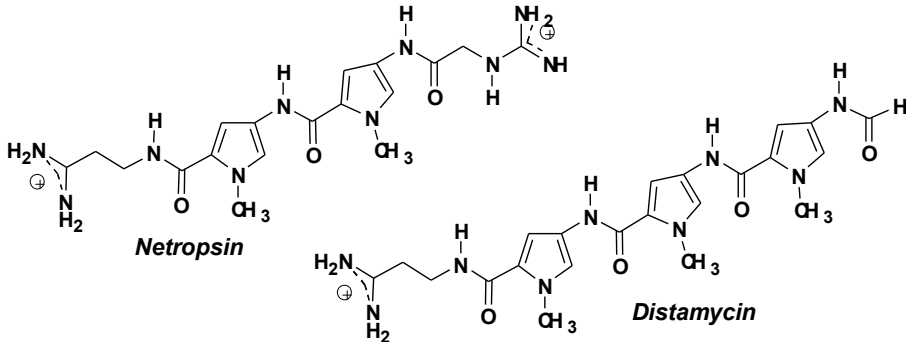


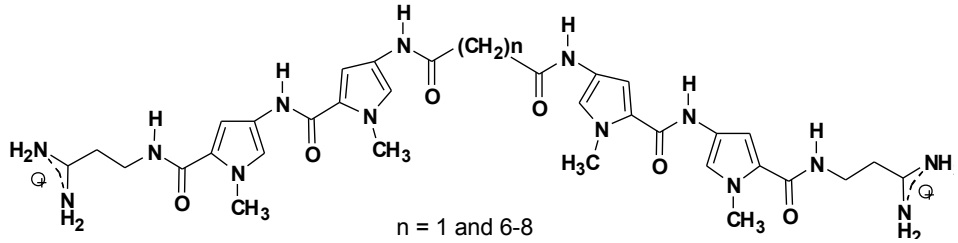
Sobuzoxane (MST-16):
$R = CH_2OCO_2CH_2CH(CH_3)_2$

ICRF -154 and related compounds are specific inhibitors of topo II with a unique mode of action: i.e., they interfere with some step(s) before formation of the intermediate cleavable complex in the catalytic cycle. Thus, bis-dioxopiperazines are quite distinct from previously known cleavable complex-forming type topo II-targeting antitumor agents such as acridines, anthracyclines, and epipodophyllotoxins.[68]

Netropsin and Its Dimers.

Lown *et al* [69] synthesized a group of oligopeptides that are structurally related to the natural antiviral antitumor antibiotics netropsin and distamycin. Polymethylene bridges [(CH2)*n, n* = 1 and 6–8] link two units of and markedly enhance both antitumor and antivaccinia virus activity, relative to the parent compounds **.**

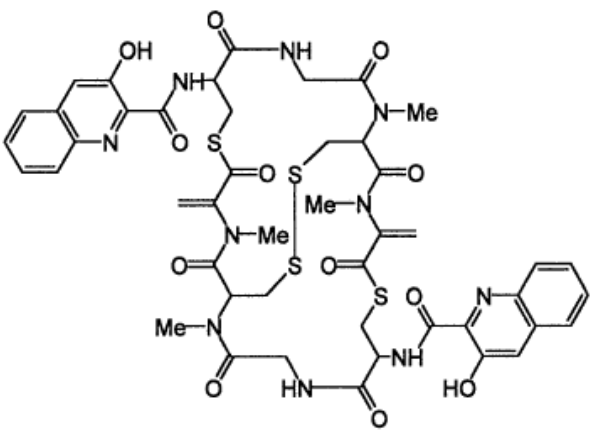




Both parent compounds and their dimers inhibit the catalytic activity of isolated topo II and interfere with stabilization of both topo II and topo I cleavable complexes in nuclei. [70]

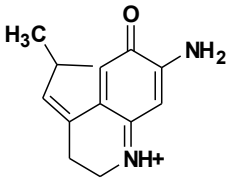
Cyclic Depsipeptides

BE-22179, a novel cyclic depsipeptide antibiotic, was isolated from the culture broth of *Streptomyces* sp. A22179 and showed potent antitumor activity against various tumor cell lines both *in vitro* and *in vivo* [71]

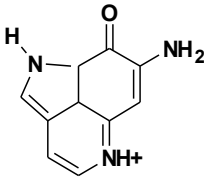


Compound BE-22179 inhibited the DNA-relaxing activity of L1210 topo II, also showed DNA-intercalating ability (DNA unwinding). The structure of BE-22179 contains two 3-hydroxyquinoline moieties and is quite novel for a topo II inhibitor.

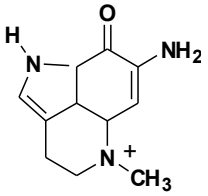
Makaluvamine



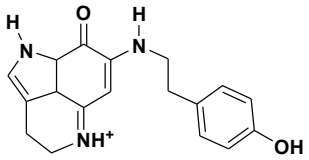
Makulavamine A



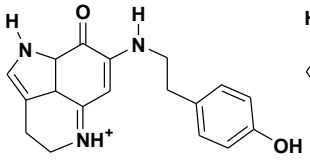
Makulavamine B



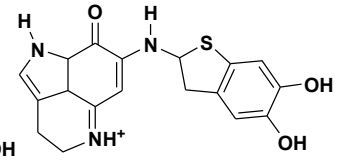
Makulavamine C



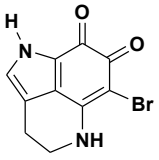
Makaluvamine D



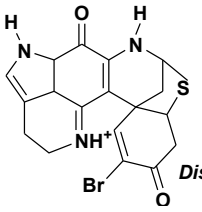
Makaluvamine E



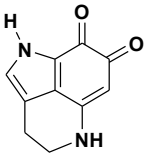
Makaluvamine F



Makaluvone



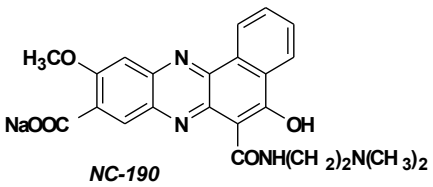
Discorhabdin A



Damirone B

The makaluvamines A–F, makaluvone, discorhabdin A, and damirone B were isolated from a sponge of the genus *Zyzzya* by following bioactivity against the human colon carcinoma cell line (HCT 116).[72] These compounds are considerably cytotoxic and may act through inhibition of DNA topo II. The compounds show enhanced toxicity toward a topo II-cleavable complex-sensitive cell line, and they inhibit topo II decatenation of kinetoplast DNA *in vitro.* Like amsacrine, makaluvamine C and makaluvamine A produced protein-linked DNA double-strand breaks in a dose-dependent fashion.

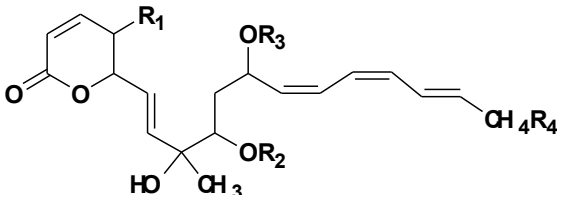
Benzophenazine Derivative



NC-190

NC-190, a novel benzophenazine derivative, is a potent antitumor compound. Compound NC-190 inhibited the DNA strand-passing and decatenation activity of DNA topo II,[73] but only weakly inhibited DNA topo I. In a topo II-dependent DNA cleavage assay, NC-190 inhibited the enzyme activity by stabilizing a topo II-DNA cleavable complex.

Fostriecin and Its Analogs



Fostriecin R_1 = H, R_2 = PO_3^-, R_3 = H, R_4 = OH
PD113271 R_1 = OH, R_2 = PO_3^-, R_3 = H, R_4 = OH
PD114631 R_1 = H, R_2 = H, R_3 = H, R_4 = OH
PD116425 R_1 = OH, R_2 = PO_3^-, R_3 = H, R_4 = OH

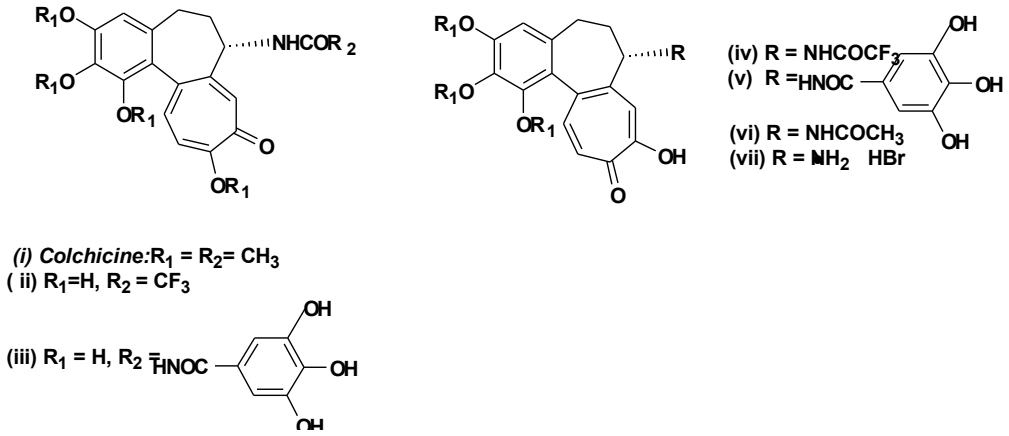
Fostriecin (phosphotrienin, FST) is a new antitumor antibiotic, derivatives being developed as anticancer agents based on their marked activity in murine leukemias.

Fostriecin and some of its analogs inhibit the catalytic activity of partially purified topo II [74]

Isocolchicine

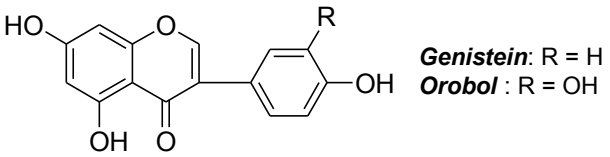
Colchicine (i) , a major alkaloid present in *Colchicum autumnale,* has been extensively investigated, and the principal biological action of this drug as a microtubule spindle toxin is well established. Recently, some

tetrademethylcolchicine and isocolchicine analogs were prepared and evaluated as inhibitors of mammalian DNA topoisomerase *in vitro.* [75]



Furthermore, **(ii)** and **(iii)** inhibited topo II activity and were 2-fold and 7-fold more potent than etoposide. Isocolchicine analogs **(iv)** to **(vii)** completely inhibited topo II-dependent DNA unknotting.

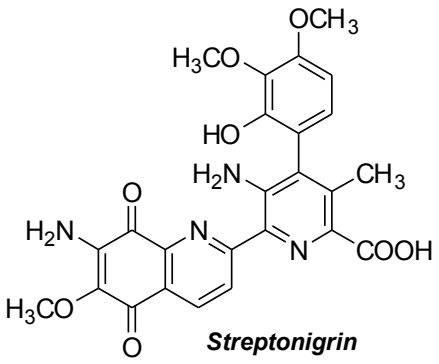
Isoflavones



Two isoflavones, genistein and orobol, induced mammalian topo II DNA cleavage *in vitro* with activities comparable to those of amsacrine and etoposide .

Streptonigrin

Streptonigrin is a nonintercalative antitumor antibiotic produced by *Streptomyces sp*. S-224 and S-502. Streptonigrin induces mammalian topo II dependent DNA cleavage *in vitro.* [76]



Streptonigrin

Streptonigrin[77] stimulated unique intensity patterns of topo II-mediated DNA that were not similar to those of doxorubicin, VM-26, amsacrine, genistein , and mitoxantrone. Streptonigrin may bind to DNA like a minor groove binder, and its pharmacophore, which is possibly different from other topo II inhibitors, may be an important determinant of its unique sequence position specificity.

1.2.4

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2

Aim of the work

This Ph.D. work was carried out as a part of a project focusing on the search of new naturally derived topoisomerase I inhibitors as potential antitumor compounds. Topoisomerase I is the target of anticancer drug campthotecin (CPT). First generation analogues of CPT are already approved as anticancer agents in human therapy, and several second and third generation derivatives are well advanced in clinical trials. Nevertheless, identification of new campthotecins or alternative molecular scaffolds endowed with similar properties is highly desirable because CPT derivatives were shown to be clinically problematic, above all for the instable α-hydroxy lactone moiety. Aim of this research is the synthesis of new CPT derivatives, in an effort to decrease the toxicity and improve the *E ring* stability of the natural alkaloid. At the same time, the search of new scaffolds showing topoisomerase I inhibitory activity was developed. Attention focuses on a new class of marine alkaloids called lamellarins that have been recently studied as Topo I inhibitors. In this Ph.D. work new lamellarin analogues are designed and synthesized. Molecular models of the ternary complex formed between the DNA-Topo I ensemble and new derivatives have been built to optimize the scaffold structure.

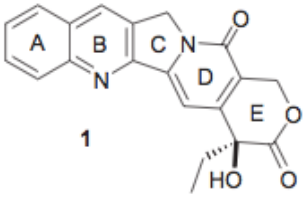
3

Synthesis of new cytotoxic E-ring modified camptothecins

3.1

Introduction

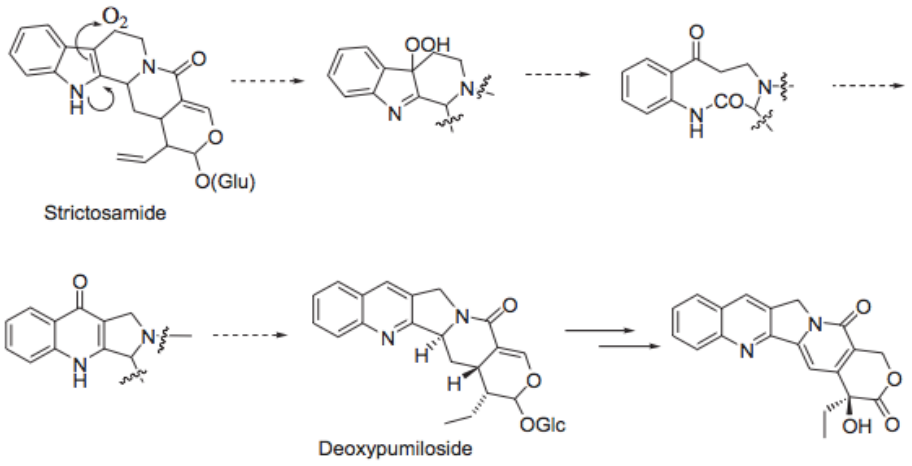
Camptothecin (CPT, 1) is a natural compound isolated for the first time [1] from the wood of *Camptotheca acuminata Decne* (*Nyssaceae*), a deciduous plant (xi shu, happy tree) of Southeastern China, but produced also by the *Indian icacinacea Nothapodytes foetida* (Wight) Sleumer (formerly Mappia foetida Miers) [2], and by some other plants [3], the two former being the major sources of the compound.



1

Although CPT is not basic, it certainly belongs to the alkaloid family, as its structure clearly shows the derivation from the main precursors of

monoterpenoid indole alkaloids, tryptamine and secologanin. The well-known intermediate of this pathway, strictosamide, has been shown to be a precursor of CPT, by incorporation of a radiolabeled sample.[4] The subsequent steps in the rearrangement of the indole to the quinoline nucleus most probably involve oxidation and recyclization of the C and D rings, oxidation of the D ring and removal of the C-21 glucose moiety, and oxidation of ring E. In agreement with this hypothesis is the isolation of 3-(*S*)- and 3-(*R*) deoxypumiloside and 3-(*S*)-pumiloside from *Ophiorrhiza* pumila, another plant producing CPT. (See ref. [3] for a detailed review of CPT biosynthesis.) (Scheme 3.1). ^{13}C NMR studies have established that the secologanin moiety is formed via the plastidic nonmevalonate (MEP) pathway,[5] but details of the last steps of the biosynthesis remain hypothetical.



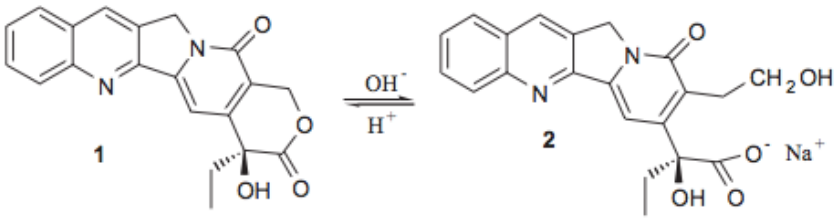
Scheme 3.1

Camptothecin was discovered during a program of screening plant extracts for antitumor activity, launched by NCI in 1955. The unusual activity of the extracts of *Camptotheca acuminata* against some leukemia cellular lines prompted a study of the components, which led to the bioguided fractionation, isolation, and structural elucidation of CPT in 1966. [1] As soon as sufficient material became available, further in vitro and in vivo assays were conducted, culminating in Phase I and II clinical trials in 1970–1972 .[6] Owing to its extremely low solubility in water, CPT had to be administered as the sodium salt of the hydroxycarboxylic acid **2** (Scheme 3.2). However,

shifting of the equilibrium toward the lactone form in tissutal compartments with acid pH caused precipitation of crystals of CPT, which caused severe hemorrhagic cystitis. This effect, together with other toxicities, led to the termination of clinical trials in 1972.

Interest in possible applications of CPT declined. However, renewed interest in CPT emerged when, as a result of a cooperative effort between Johns Hopkins University and SKB, it was found that DNA damage, which is the main reason for the antitumor activity, was due to inhibition of the ubiquitous nuclear enzyme topoisomerase I.[7] Elucidation of the mechanism of action of CPT and, therefore, of a definite biological target at which to aim new drugs gave rise to a fresh wave of research aimed at finding new more active and less toxic camptothecin derivatives. This is clearly shown by the sharp increase in the number of publications and patents that followed Liu's paper. To avoid the problems encountered with CPT itself, the introduction of functional groups able to make the compounds sufficiently water soluble to allow intravenous administration was a main issue.

The results of this effort were a detailed pattern of structure–activity relationships



Scheme 3.2

and the production of two compounds, topotecan **3** and irinotecan **4**,[9] which were approved for clinical use in 1996, the main indications being ovarian and small-cell lung cancer for the former and metastatic colorectal cancer for the latter. Irinotecan is a water-soluble prodrug of the active compound SN-38 [5] (Figure 3.2). Several reviews of this phase of research have been published. [10–12]

Together with the synthesis and screening of new derivatives and analogs, research continued unabated to unveil the details of the mechanism of action of CPT at the molecular level. The decade 1995–2005 brought new exciting results and some changes in the perspective of research in the CPT field.[13]

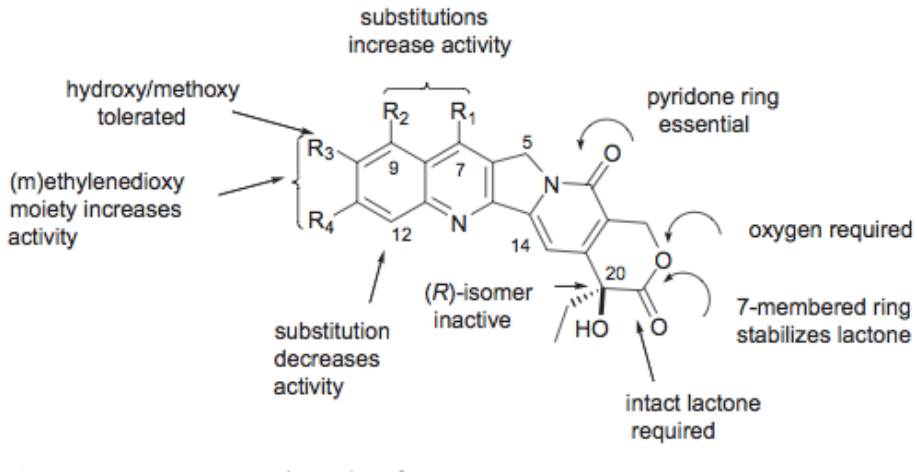


Fig 3.1

Camptothecin acts by forming a reversible ternary complex (‘‘cleavable complex’’) with DNA and topoisomerase I, preventing the religation of the DNA strand cut by topoisomerase to allow relaxation, and thus inducing apoptosis.[14] The X-ray structure of crystals of such a complex of a 22-base DNA fragment with topoisomerase I and topotecan has been reported,[15] and molecular models of the interaction have been proposed.[16–18] This kind of information should be of help in designing new active compounds, but so far no breakthrough substance seems to have been obtained on such a basis, and discussion on which feature of ring E of camptothecin is essential for activity is still lively.[19]

Over the years, the feature of interest for pharmacologists in camptothecins has progressively shifted, so that water solubility is no longer an essential requisite. Lipophilic compounds have the advantage of compartmentation in tissues, thus assuring the stabilization and enhanced persistence of the active lactone form, and allowing oral administration of the drug, with increased compliance by the patients.

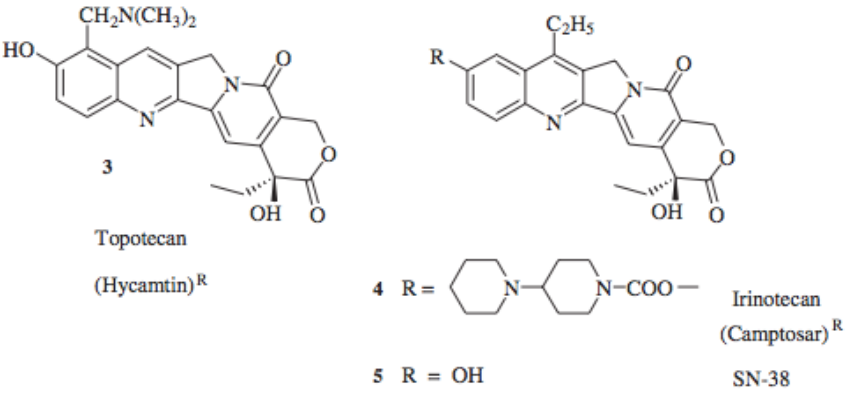


Fig 3.2

A seminal paper in this respect was published by Burke in 1993, [20] and now this trend is largely accepted.[21] These changes had important consequences in the design and synthesis of new analogs. In fact a series of lipophilic analogs of CPT are in preclinical development at the time of writing (2010) (Figure 3.3).

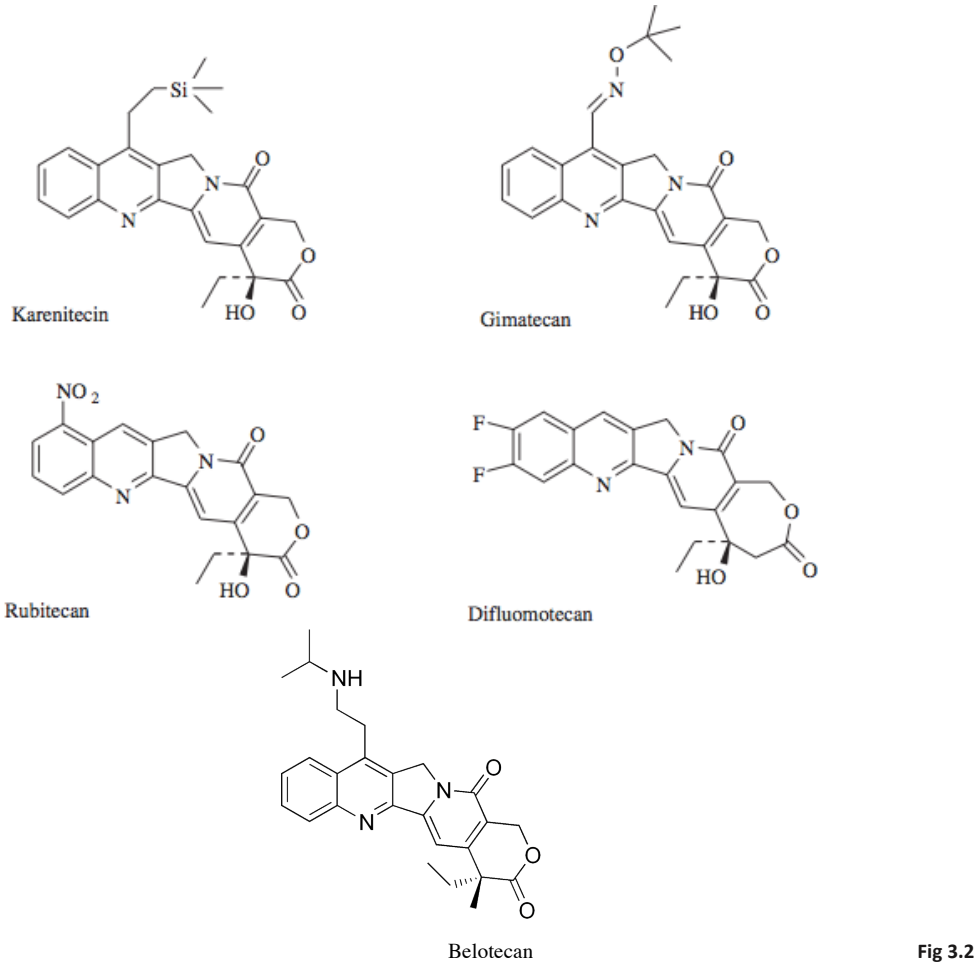
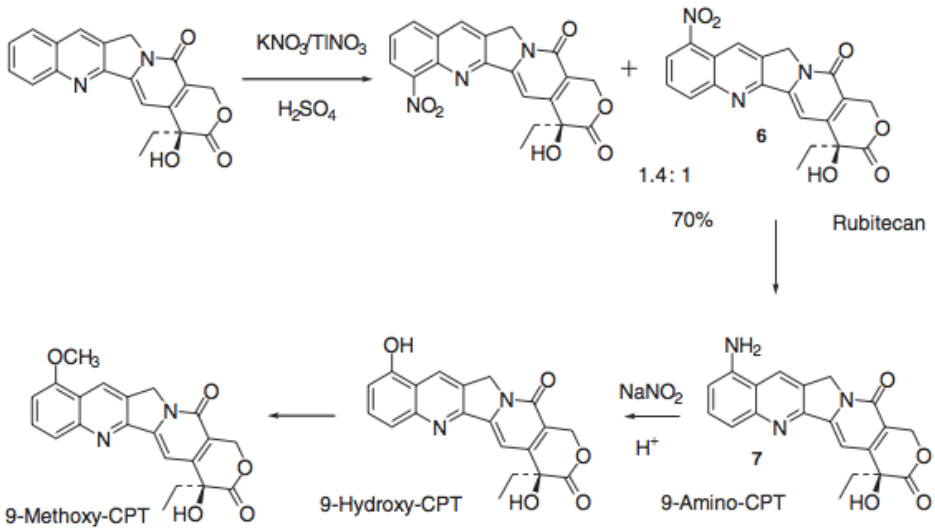


Fig 3.2

Another aspect of the progress toward the development of a camptothecin drug candidate concerns the study of proper formulations, such as liposomes,[22] and the finding of innovative drug delivery systems.[23]

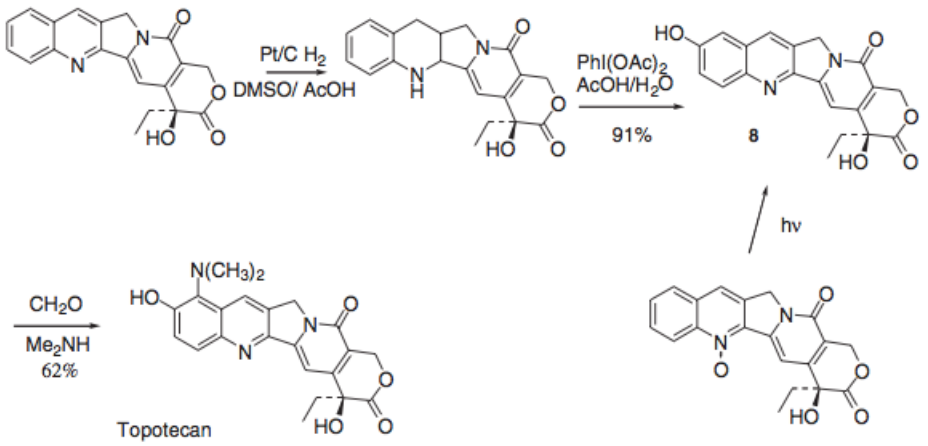
For the synthesis of a new camptothecin derivative, the first choice is between a semisynthesis starting from the natural compound CPT, or a total synthesis. Camptothecin is a chiral compound, with only one asymmetric center, carbon 20, the active compounds possessing the natural configuration (*S*). A semisynthesis has the advantages of starting from a compound that possesses all the necessary structural features, including the required 20-(*S*) configuration. The drawbacks of this approach can be the limited reactivity of the quinoline nucleus and the sensitivity of the lactone ring. For the development of a drug, difficulties could derive from the possible failure of an adequate and constant supply of the natural material, and from an unpredictable pattern of impurities in the different batches. Owing to the high potency of the drugs, doses are rather low, so that the amount of camptothecin required has so far been within the capacity of the Chinese and Indian producers, although some concern has been raised on the conservation of *Camptotheca acuminata*, which grows only in an area of China south of the Yangtze river. However, the plant has been shown to grow in other areas of the world, and considerable effort has already been spent toward the production of camptothecin by cell cultures.[3] On the other hand, a total synthesis offers the possibility of substitutions and structural modifications that depend only on the manageability of the synthetic scheme, so enlarging the diversity of the target compounds, and is free from the constraints indicated above. However, an asymmetric synthesis is required, with several steps, and so far most of the total syntheses appear too expensive. Actually, the two drugs currently in clinical practice and most of the candidates presently in an advanced stage of development are produced by semisynthesis. As Figure 3.2, 3.3 show, so far the most fruitful modifications of CPT to obtain an active antitumor compound have been the introduction of substituents

in positions 7,9, and 10.



Scheme 3.3

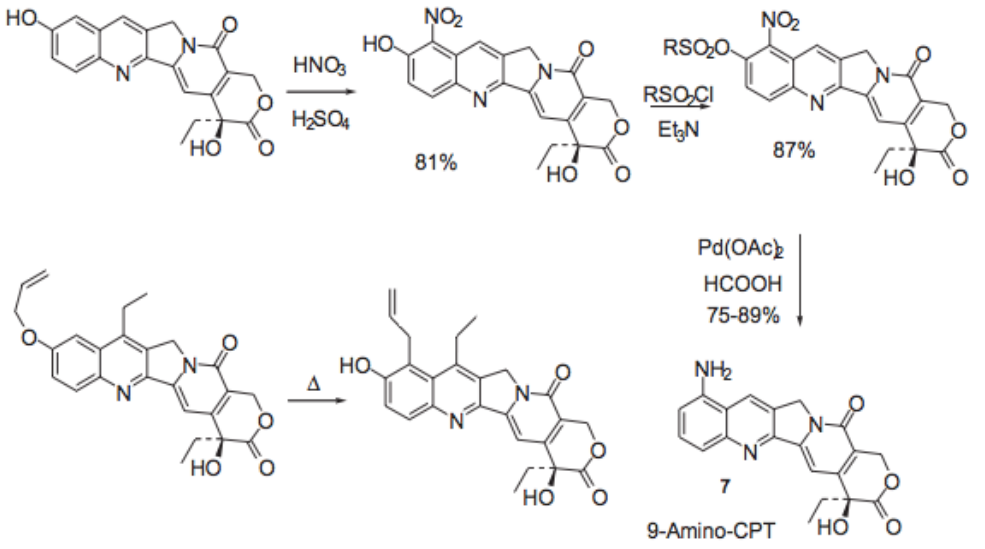
The electron-deficient ring of quinoline is not very reactive to electrophilic substitution, the preferred sites of attack being position 5 and 9.[24] Nitration of CPT (best yields 70 %) [25] gives in fact a mixture of 12- and 9-nitrocamptothecin **6**. The latter is itself a compound (Rubitecan) endowed with potent antitumor activity, [26] and is a precursor of many derivatives, as it can be easily reduced to 9-amino-CPT **7**, in turn convertible into 9-hydroxy- and 9-methoxycamptothecin, minor components of the plant extract (Scheme 3.3).



Scheme 3.4

The accessibility of position 9 becomes much higher when an activating

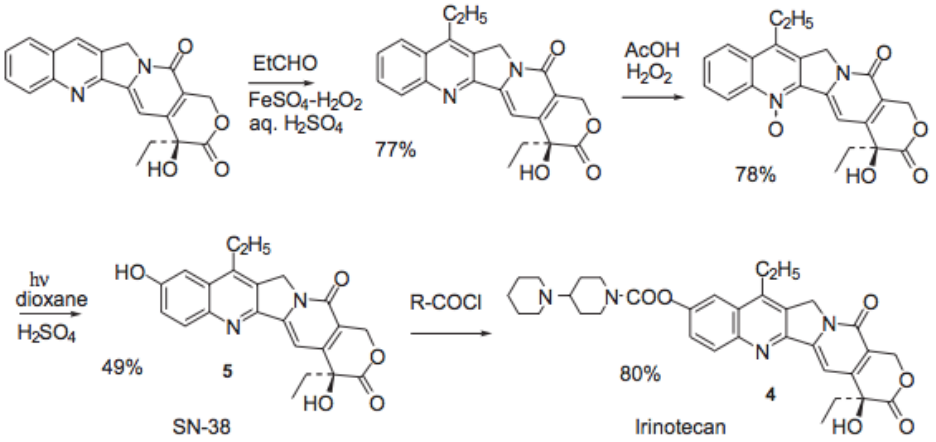
group, such as an OH, is present in position 10. Although 10-hydroxycamptothecin **8** is available in small amounts from the plant material, two efficient preparations of this compound were developed, via catalytic reduction of CPT in acid medium to a tetrahydroquinoline, followed by selective oxidation with lead tetraacetate,[8] or phenyliodonium diacetate,[27] or via a photochemical rearrangement of camptothecin N-oxide.[28] Thus activated, the nucleus smoothly undergoes the Mannich reaction to give topotecan **3** (Scheme 3.4).



Scheme 3.5

The 10-hydroxy group can facilitate the alkylation of C-9 via a Claisen rearrangement, as in the case of 7-ethyl-10-hydroxy-CPT,[29] or nitration in the same position, possibly followed by removal of the OH and reduction of the nitro group by palladium-catalyzed deoxygenation to give 9-aminoCPT **7**,[30] another drug candidate (Scheme 3.5). By contrast, substitution in position 7 is much easier thanks to the well-known Minisci reaction, which involves a nucleophilic radical attack on a protonated quinoline.[31] Moreover, due to the unavailability of position 2 of the quinoline nucleus, the reaction shows complete regioselectivity. Minisci alkylation with an ethyl radical produced in situ by decarbonylation of propionaldehyde is a crucial step in the process of preparation of irinotecan **4** (Scheme 16.6),[32] whereas the same kind of reaction led to the

semisynthesis (Scheme 16.7) of gimatecan **9**,[33] silatecan **10**,[34] and belotecan **11**.[35] This last compound entered clinical practice in Korea in 2005.



Scheme 3.6

As soon as the structure of camptothecin was published, the interest of many chemists, including some famous names, was directed toward this synthetic goal, encouraged by the relevance of the unusual antitumor activity. Later, when the compound had lost its novelty value, such studies were stimulated by the desire to achieve a process of production of the drugs derived from CPT and the preparation of new derivatives. Although at first sight the synthesis of CPT might not appear, by modern standards, a difficult task, the array of functional groups on ring E, not easily compatible with many synthetic procedures, has often required a number of steps and some detours to overcome the difficulties of a total synthesis. In some cases, the problem has been solved by the invention of new synthetic methods, so that the approaches have led to the addition of new tools to the arsenal of the synthetic organic chemist. The early syntheses have been reviewed by Schultz[38] and Hutchinson.[39] Other reviews, more medicinally oriented, have appeared.[40] One of the most recent and detailed, covering work from 1990 onward, is that of Du.[41]

3.2

Results and discussion

One of the major limitations of CPT and analogues is the severe toxicity, stemming in part from the instability of the α-hydroxylactone E-ring. Moreover, the CPT carboxylate binds tightly to serum albumin, which limits the fraction of drug in the active lactone form.

In early research, a number of E-ring modified CPT analogues were prepared. These studies showed that any change in the E-ring, such as deletion of the hydroxy group at C20 position, ring contraction to a five-membered lactone, replacement of the lactone by a lactam, a thiolactone or an imide, reduction of the lactone to lactol, synthesis of six-member ring or five-member ring ethers gave essentially inactive compounds. These results led to the conclusion that the intact six-membered ring was indispensable for antitumor activity.

However, further investigations demonstrated that other E-ring modified CPT analogues showed comparable or even better antitumor activity than the parent compound. The first successful approach was to expand the α-hydroxylactone ring by an additional methylene, thereby generating seven

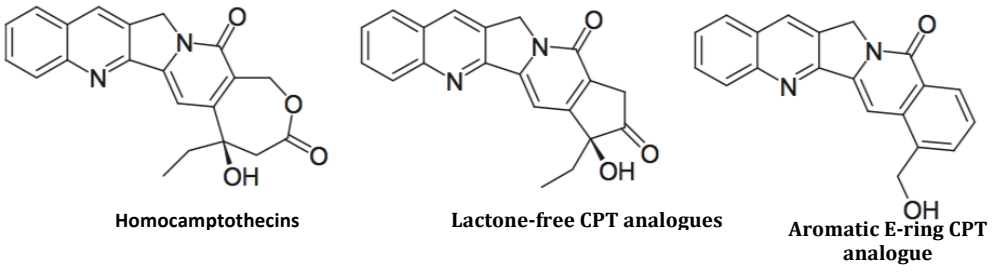


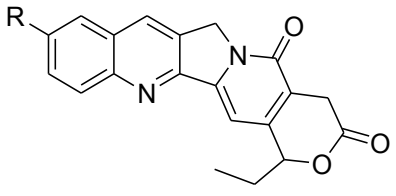
Figure 3.4

membered α-hydroxylactone E-ring analogues, which are referred to as homocamptothecins , expectedly less prone to ring opening. HomoCPTs were reported to exert potent inhibition of Topo I and elevated levels of cytotoxicity, while showing enhanced stability and decreased protein binding in human plasma. Their dehydrated conjugated ene-lactone

analogues were only modestly active in the TopoI cleavage test. Other successful approaches to the synthesis of stabile E-ring CPT derivatives were recently reported. Five-membered lactone-free CPT analogues, appeared to be potent inhibitors of TopoI and showed interesting cytotoxic activity. Aromatic E-ring CPT analogues and the natural pyrroloquinazolinoquinoline alkaloid luotonin A, were also found to have significant in vitro cytotoxic activity (Figure 3.4).

These results raise the possibility that novel E-ring modified analogues of CPT might retain or even possess enhanced antitumor activity.

The present study was undertaken to explore a new structural change at the E-ring, while keeping a lactone moiety in this portion of the molecule. Thus, we designed derivatives with an 'inverted' lactone ring (compounds **9** and **10**, Figure 3.5).



9 R=H

10 R=OCH_3

Figure 3.5

In these compounds the lactone ring is expected to be more stable, because the carbonyl group, lacking the activating α-hydroxy substituent typical of camptothecins, should be a weaker electrophile than the CPT α-hydroxylactone moiety (compare the HomoCPTs, see above).

Molecular modelling studies were performed to explore the binding mode for the planned compounds to the covalent TopoI–DNA complex. This study was based on the X-ray crystal structure of a ternary complex between a human TopoI construct covalently 100 attached to a DNA duplex with bound Topotecan. For comparison purposes, the same docking protocol used to study the complex containing Topotecan was applied in the present work. The intercalation binding site was created by conformational changes of the phosphodiester bond between the +1

(upstream) and -1 (downstream) base pairs of the uncleaved strand, which effectively 'open' the DNA duplex, with the evidence of only one direct hydrogen bond between the Asp533 residue of the enzyme and Topotecan in both the carboxylate and lactone models. The comparative analysis of the best poses obtained for compound **9** revealed that the molecule formed base-stacking interactions with both the and +1 base pairs, with a strong π-π stacking between the C-ring and guanosine at position +1 of the cleaved strand (+1G). The interactions between compound **9** and Topo I–DNA complex are given in Figure 3.6 (together with the experimental Topotecan conformation), where the interactions listed are hydrogen bonds.

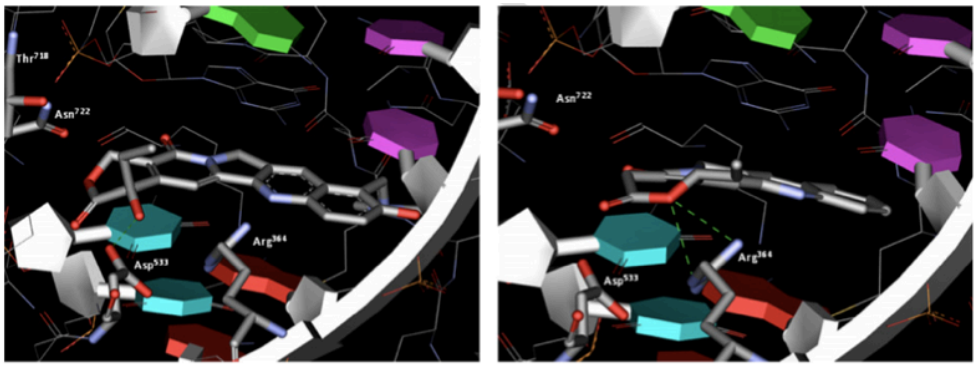


Figure 3.6

For compound **9**, the hydrogen bond between the Asp533 residue and Topotecan is replaced by two strong hydrogen bond interactions between the lactone ring oxygen atom and both the NH1 and NH2 hydrogen atoms of Arg364 (close to Asp533), equal to 2.85 Å and 3.00 Å, respectively. Thus, compound **9** appears to make use of distinct sets of interactions to stabilize the binding mode at the intercalation site in TopoI–DNA complex. The promising binding provides structural support to synthesis of **9** and analogues. Among them, compound **10**, bearing a methoxy group in position 10, showed a calculated free energy (9.12 kcal/mol) close to the value calculated for compound **9** (9.81 kcal/mol).

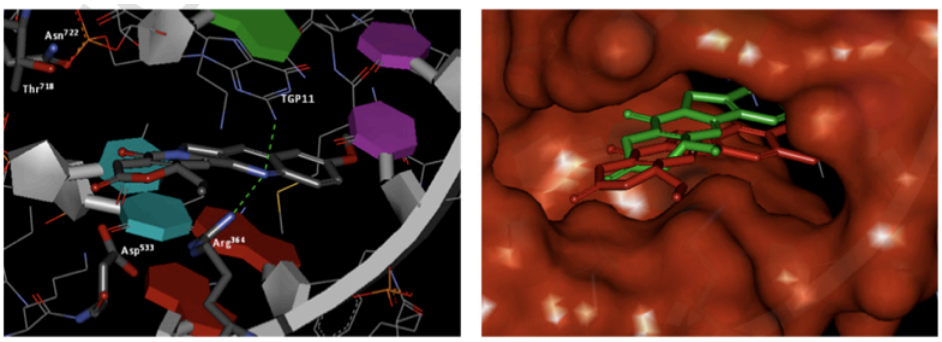
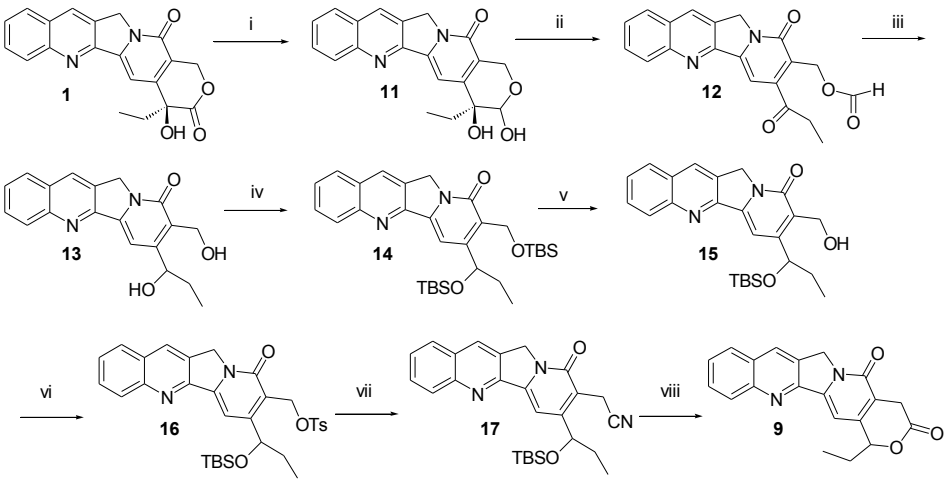


Figure 3.7

As shown in Figure 3.7, the ligand is placed in the TopoI–DNA complex in almost the same orientation as Topotecan and compound **9**, but the molecule is shifted towards the entrance of the binding site. Consequently, the strong π-π stacking interaction is now between the B-ring and +1G. Due to the new position of the ligand, compound 10 is connected to the Topo I–DNA complex through two new hydrogen bonds between the heterocyclic nitrogen of B-ring and NH1 Arg364 (2.93 Å) and N2 of guanosine +1 (2.78 Å).

The synthesis of compound **9** was performed using natural camptothecin as a starting material. CPT was readily reduced with sodium borohydride to the corresponding lactol **11**, whose 1,2-diol moiety was oxidatively cleaved with periodic acid to give compound **12**. Attempts to catalytically reduce the keto group failed, while treatment with sodium borohydride in DCM/methanol gave compound **13** in good yield. The diol was disilylated using TBDMSCl in DCM and selective primary desilylation was successfully achieved using catalytic PTSA in methanol. To install the carboxylic acid moiety in the right position for the subsequent lactonization, one-carbon homologation of the side chain at position 8 of compound **15** was required. Thus, compound **15** was converted into the corresponding tosylate that was immediately treated with sodium cyanide to furnish compound **17**. Finally, deprotection of the hydroxyl group and simultaneous hydrolysis and cyclization were achieved using HCl in ethanol (Scheme 3.7).

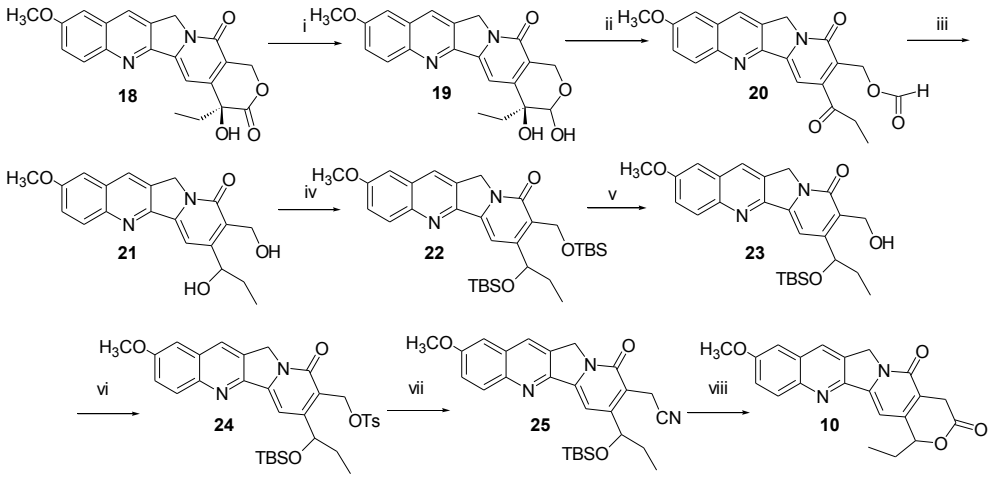


Scheme 3.7

Synthesis of compound **9** from natural camptothecin. Reagents and conditions: i NaBH4, MeOH, rt, overnight; ii NaIO4, AcOH, rt, overnight; iii NaBH4, DCM/MeOH 8:2, rt, 40 min; iv TBDMSCl, imidazole, DMF, rt, 24 h; v PTSA, DCM/MeOH 1:1, 0 °C then rt, 1 h; vi TsCl, DMAP, DCM, 0 °C, 24 h; vii NaCN, DMSO, 70 °C, 2 h; viii HCl, EtOH, 90 °C, 1.5 h.

The synthesis of the corresponding 10-methoxy derivative was achieved following the same pathway described for compound **9**, starting from natural 10-OH-CPT. It was previously converted in 10-methoxy derivative **18** according the procedure described in experimental methods. In this case the oxidative cleavage with sodium periodate appeared troublesome. Several attempts were performed but they all resulted unsuccessful, giving complex and unseparable mixtures of products. Finally, it was found that treatment of a DCM solution of compound **15** with silica-gel supported $NaIO_4$ afforded compound **16** in a satisfactory yield. The remaining steps to get compound **10** were the same as described previously for compound **9** (Scheme 3.8).

Compounds **9** and **10** were tested for their antiproliferative activity on human non-small lung cancer cells H-460 (1 h exposure), using Topotecan as a reference compound (IC_{50} = 1.38 ± 0.95 μM). Both analogues **9** and **10**, even if still in the racemic form, maintained a good cytotoxic activity, with IC_{50} less than 10 μM (IC_{50} 8.73 and 7.45 μM, respectively). The results



Scheme 3.8

Synthesis of compound **10** from 10-methoxycamptothecin. Reagents and conditions: i NaBH4, MeOH, rt, 2h; ii Silica gel-supported NaIO4, DCM, rt, overnight; iii NaBH4, DCM/MeOH 8:2, rt, 40 min; iv TBDMSCl, imidazole, DMF, rt, 24 h; v PTSA, DCM/MeOH 1:1, 0 °C then rt, 1 h; vi TsCl, DMAP, DCM, 0 °C, 24 h; vii NaCN, DMSO, 70 °C, 2 h; viii HCl, EtOH, 90 °C, 1.5 h.

suggest that the introduction of an 'inverted lactone' moiety in ring E gives compounds with retained antitumor activity, making them worthy of further investigation. Work is currently in progress to evaluate the effect of substituents and to study the Topoisomerase I inhibitory activity of these new camptothecin analogues.

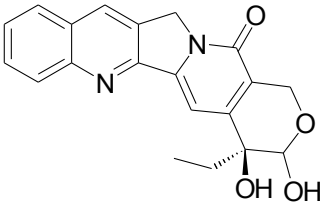
3.3

Eperimental methods

^{1}HNMR spectra were recorded in $CDCl_3$ solutions (where not otherwise stated) at room temperature on a Bruker AMX-300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts are reported as δ values in parts per million (ppm), and are indirectly referenced to tetramethylsilane (TMS) via the solvent signal (7.26 for ^{1}H, 77.0 for ^{13}C) in $CDCl_3$. Coupling constants (*J*) are given in Hz. Solvents were routinely distilled prior to use; anhydrous tetrahydrofuran (THF) and ether (Et_2O) were obtained by distillation from sodium-benzophenone ketyl; dry

methylene chloride (CH_2Cl_2) and toluene were obtained by distillation from $CaCl_2$. All reactions requiring anhydrous conditions were performed under a positive nitrogen flow, and glassware was oven-dried. Isolation and purification of the compounds were performed by flash column chromatography on silica gel 60 (230-400 mesh); when necessary deactivated silica gel was used. Analytical thin-layer chromatography (TLC) was conducted on Fluka TLC plates (silica gel 60 F_{254}, aluminium foil), and spots were visualized by UV light and/or by means of dyeing reagents. Melting points were determined on a Stuart Scientific SMP3 instrument and are uncorrected.

11. 4-Ethyl-3,4-dihydroxy-1,3,4,12-tetrahydro-2-oxa-6,12a-diaza-dibenzo[b,h]fluoren-13-one.

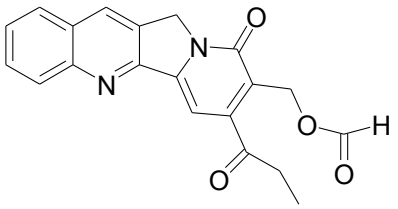


$C_{20}H_{18}N_2O_4$
Mol. Wt.: 350,37

Camptothecin **1** (1g, 2,87 mmol) was dissolved in methanol (75 mL) and $NaBH_4$ (0,163 g, 4,30 mmol) was added sequentially in 10' at r.t. After stirring overnight and evaporating the solvent, the mixture was poured in water. AcOH solution was dropped until pH 7 and the solution was cooled at 0°C to facilitate the precipitation. The white solid was filtered and dried (0, 900 g, 90 %).

^{1}H NMR (DMSO): 8.70 (1H, s), 8. 37 (1H, s), 8.10-8.20 (2H, m), 7.89 (1H, m), 7.75 (1H, m), 7.42 (1H, s), 5.20 (2H, s), 4.95 (2H, m), 4.50 (2H, m), 3.93 (3H, s), 1.75 (2H, m), 0.90 (3H, t, J=7.4).

12. Formic acid 9-oxo-7-propionyl-9,11-dihydro-indolizino[1,2-b]quinolin-8-ylmethyl ester

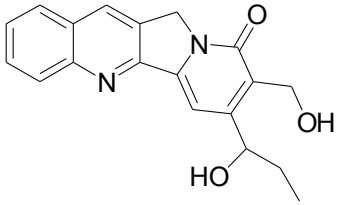


$C_{20}H_{16}N_2O_4$
Mol. Wt.: 348,35

Compound **11** (0.895 g, 2.57 mmol) was suspended in acetic acid (115 mL) at room temperature. To the suspension was added $NaIO_4$ (1.65 g, 7.71 mmol). The mixture reaction was stirred overnight and the solvent was evaporated under vacuo. Water was added to the and the mixture was extracted twice with dichloromethane. The combined organic layer was dried over $NaSO_4$ and evaporated. The residue was purified by flash chromatography (eluent dichloromethane: methanol, 95:5) to afford **12** (0.696 g, 78%).

^{1}H NMR (DMSO): 8.70 (1H, s), 8. 37 (1H, s), 8.10-8.20 (2H, m), 7.89 (1H, m), 7.75 (1H, m), 7.42 (1H, s) 5.30 (2H, s), 5.12 (2H, s), 2.90 (2H, q, J=7.04 Hz), 1.10 (3H, t, J=7.04 Hz).

13. 8-Hydroxymethyl-7-(1-hydroxy-propyl)-11H-indolizino[1,2-b]quinolin-9-one

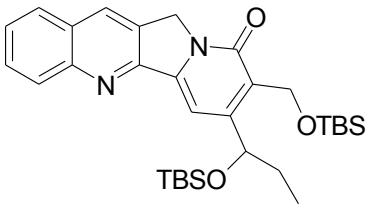


$C_{19}H_{18}N_2O_3$
Mol. Wt.: 322,36

Compound **2** (0.690 g, 2 mmol) was dissolved in dichloromethane-methanol (140 mL, 80:20). The solution was treated with $NaBH_4$ and stirred at room temperature. After 40' AcOH was dropped until pH 7, the solvent evaporated and the crude product purified by flash chromatography (dichloromethane: methanol, 9:1) to afford **13** (0.560 g, 87%)

^{1}H NMR (DMSO): 8.68 (1H, s), 8.10-8.20 (2H, m), 7.83 (1H, m), 7.70 (1H, m), 7.40 (1H, s), 5.42 (1H, m), 5.25 (2H, s), 4.80-4.95 (2H, m), 4.48-4.70 (2H, m), 1.75 (2H, m), 0.98 (3H, t, J=7.4 Hz).

14. 8-(tert-Butyl-dimethyl-silanyloxymethyl)-7-[1-(tert-butyl-dimethyl-silanyloxy)-propyl]-11H-indolizino[1,2-b]quinolin-9-one.

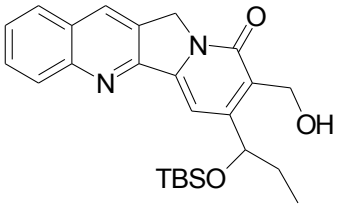


$C_{31}H_{46}N_2O_3Si_2$
Mol. Wt.: 550,88

A solution of compound **13** (0.555 g, 1.74 mmol) in dry DMF (28 mL) was treated sequentially with imidazole (1.89 g, 2784 mmol) and t-butyl chlorodimethylsilane (2.1 g, 13.92 mmol) under nitrogen atmosphere and stirred for 24 hours at room temperature. Solvent was evaporated and the residue was dissolved in dichloromethane and washed with NaCl solution. Organic layer was dried over $NaSO_4$ and evaporated under vacuo. The crude product was purified by flash chromatography (dichloromethane: methanol, 9:1) to afford compound **14** (0.574 g, 70%).

^{1}H NMR ($CDCl_3$): 8.42 (2H, m), 7.82-7.95 (3H, m), 7.62 (1H, m), 5.24 (2H, s), 5.00-5.10 (2H, m), 4.75 (2H, m), 1.82 (2H, m), 1.82 (2H, m), 1.10 (3H, t, J= 7.4 Hz), 0.98 (18H, s), 0.10 (12H, s)

15. 7-[1-(tert-Butyl-dimethyl-silanyloxy)-propyl]-8-hydroxymethyl-11H-indolizino[1,2-b]quinolin-9-one

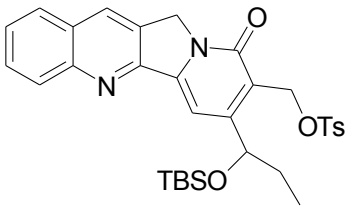


$C_{25}H_{32}N_2O_3Si$
Mol. Wt.: 436,62

A solution of **14** (0.570 g, 1.04 mmol) in dichloromethane: methanol (100 mL, 1:1) was cooled to 0°C and treated with PTSA (0.179 g, 0.52 mmol). The cooling bath was removed and the reaction mixture allowed to warme to room temperature. After 1h a $NaHCO_3$ solution was added, the mixture was concentrated under reduced pressure; dichloromethane was added and washed with water and NaCl. The organic layer was dried on Na_2SO_4 , evaporated and the crude product purified by flash chromatography (dichloromethane : methanol, 1:1) to afford product **15** (0.309 g, 68%).

^{1}H NMR ($CDCl_3$): 8.40 (1H, s), 8.30 (1H, m), 7.91 (1H, m), 7.85 (1H, m), 7.60-7.75 (2H, m), 5.27 (2H, s), 4.75-4.90 (3H, m), 1.80 (2H, m), 1.10 (3H, t, J= 7.4 Hz), 0.90 (9H, s), 0.15 (3H, s), 0.01 (3H, s).

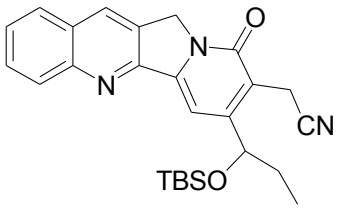
16. Toluene-4-sulfonic acid 7-[1-(tert-butyl-dimethyl-silanyloxy)-ethyl]-9-oxo-9,11-dihydro-indolizino[1,2-b]quinolin-8-ylmethyl ester



$C_{32}H_{38}N_2O_5SSi$
Mol. Wt.: 590,81

To an ice-cooled solution of compound **15** (0.303 g, 0.71 mmol) in dry dichloromethane (16 mL) was added DMAP (0.564 g, 4.61 mmol) and successively TsCl (0.675 g, 3.55 mmol) under nitrogen. After being stirred for 24h at 0°C, the mixture was added with water and washed with a $NaHCO_3$ solution. The organic layer was dried over Na_2SO_4 and evaporated affording crude product **16,** that was used for the next step without further purification (0.459 g).

17. {7-[1-(tert-Butyl-dimethyl-silanyloxy)-propyl]-9-oxo-9,11-dihydro-indolizino[1,2-b]quinolin-8-yl}-acetonitrile.

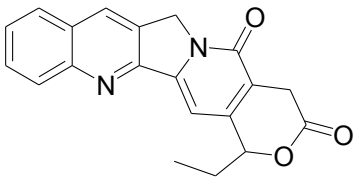


$C_{26}H_{31}N_3O_2Si$
Mol. Wt.: 445,63

To a solution of compound **16** (453 mg) in DMSO (7 mL) was added NaCN (0057 g, 1.2 mmol) and the mixture was heated at 70°C for 2 h. Dichloromethane was added and washed twice with NaCl solution. The organic layer was dried over Na_2SO_4, evaporated under reduced pressure and purified by flash chromatography (dichloromethane:methanol, 98:2) to afford product **17** (0.188 g,).

^{1}H NMR ($CDCl_3$): 8.50 (1H, s), 8.40 (1H, m), 7.90 (1H, m), 7.82 (1H, m), 7.60-7.75 (2H, m), 5.30 (2H, s), 4.80 (1H, m), 3.95 (2H, m), 1.82 (2H, m), 1.00 (3H, t, J= 7.4Hz), 0.90 (9H, s), 0.90 (9H, s), 0.10 (3H, s), -0.1 (3H, s).

9. 4-Ethyl-1,12-dihydro-4H-3-oxa-6,12a-diaza-dibenzo[b,h]fluorene-2,13-dione



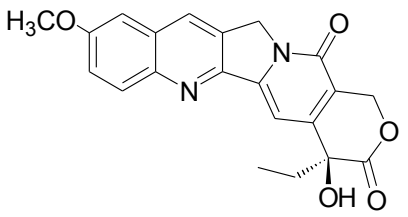
$C_{20}H_{16}N_2O_3$
Mol. Wt.: 332,35

Compound **17** (0.183 g, 0.42 mmol) was suspended in ethanol (2 mL), and added with ethanol saturated with HCl (2 mL) under stirring. The reaction mixture was heated at 90°C for 1.5 h, then AcOEt was added and washed with a NaCl solution. The organic layer was dried over Na_2SO_4, evaporated under reduced pressure, purified by flash chromatography (dichloromethane:acetone, 7:3) and crystallization from ethyl ether to afford product **8** (0.04 g, 29%). Mp 320 °C

^{1}H NMR (DMSO): 8. 70 (1H, s), 8.10-8.20 (2H, m), 7.86 (1H, m), 7.70 (1H, m), 7.30 (1H, s), 5.58 (1H, m), 5.31 (2H, s), 3.68 (2H, m), 2.10 9 (2H, m), 1.10 (3H, t, J= 7.4 Hz).

HRMS (ESI$^+$) calcd for $C_{20}H_{16}N_2O_3$ $[M+Na]^+$ 355.10531: found .355.10612

18. 10-methoxycamptothecin

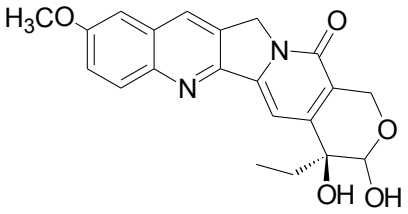


$C_{21}H_{18}N_2O_5$
Mol. Wt.: 378,38

To a suspension of 10-OH-camptothecin (1.70 g, 4.67mmol) in methanol-dioxane (350 mL, 1:1) was added dropwise a freshly distilled ethereal solution of diazomethane until the starting material was dissolved giving a yellow solution. The reaction mixture was stirred for 2 h. The solvent was evaporated under reduced pressure to afford 10-methoxycamptothecin **18** (1.77 g, 100%) as a yellow solid. Mp 287 ° C

^{1}H NMR (DMSO): 8.51 (1H, s), 8.00 (1H, m), 7.45-7.55 (2H, m), 7.25 (1H, s), 6.50 (1H, s), 5.50 (2H, s), 5.21 (2H, s), 4.00 (3H, s), 1.81 (2H, m), 0.80 (3H, t, J= 7.4 Hz).

19. 4-Ethyl-3,4-dihydroxy-9-methoxy-1,3,4,12-tetrahydro-2-oxa-6,12a-diaza-dibenzo[b,h]fluoren-13-one.

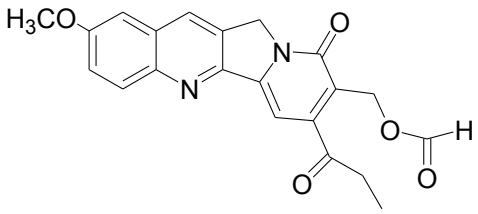


$C_{21}H_{20}N_2O_5$
Mol. Wt.: 380,39

10-methoxycamptothecin (1.75 g**,** 4.6 mmol) was suspended in methanol (180 mL). $NaBH_4$ (1.77g, 4.67 mmol) was added in portions in 10' at room temperature and the mixture was stirred for 2 h. Solvent was evaporated under reduced pressure and the crude mixture was filtered over a phosphate-buffered silica pad (dichloromethane:methanol, 7,5: 2,5) to affordi **2** (1.60 g, 90%). Mp 300 ° C

^{1}H NMR (DMSO): 8.50 (1H, s), 8.01 (1H, m), 7.49 (2H, m), 7.30 (1H, s), 6.70 (1H, d), 5.20 (2H, s), 4.95 (2H, m), 4.50 (2H, m), 3.93 (3H, s), 1.75 (2H, m), 0.90 (3H, t, J=7.4).

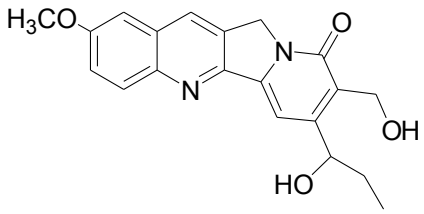
20. Formic acid 2-methoxy-9-oxo-7-propionyl-9,11-dihydro-indolizino[1,2-b]quinolin-8-ylmethyl ester.



$C_{21}H_{18}N_2O_5$
Mol. Wt.: 378,38

Compound **19** (1.55 g, 4.21 mmol) was suspended in dichloromethane (150 mL) and silica supported $NaIO_4$ (8.42 g, 1.72 g of $NaIO_4$) was added at room temperature. The reaction mixture was stirred for 24 h, filtered over a silica pad (dichloromethane:methanol, 8:2), affording a solution of compound **20**, which was used for the next step without further purification.

21. 7-(1-Hydroxy-ethyl)-8-hydroxymethyl-2-methoxy-11H-indolizino[1,2-b]quinolin-9-one.

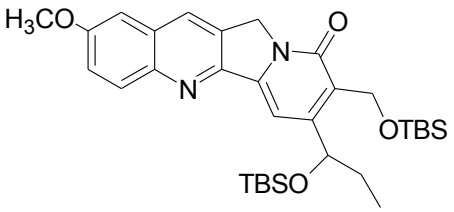


$C_{20}H_{20}N_2O_4$
Mol. Wt.: 352,38

To the above solution of **20** was added $NaBH_4$ (0.318 g, 8.40 mmol) in 10' at room temperature and the reaction mixture was stirred for 30'. THE Solvent was evaporated under reduced pressure and the crude product was purified by filtration of its solution in (dichloromethane:methanol, 8:2) over a silica pad to afford compound **21** (1g).

^{1}H NMR ($CDCl_3$): 8.20 (1H, s), 8.12 (1H, d, J=8.9 Hz), 7.60 (1H, s), 7.48 (1H, d, J=8.9 Hz), 7.12 (1H, s), 5.22 (2H, s), 5.95-5.02 (2H, m), 4.70 (1H, m), 4.00 (3H, s), 1.78 (2H, m), 0.98 (3H, m).

22. 8-(tert-Butyl-dimethyl-silanyloxymethyl)-7-[1-(tert-butyl-dimethyl-silanyloxy)-propyl]-2-methoxy-11H-indolizino[1,2-b]quinolin-9-one.

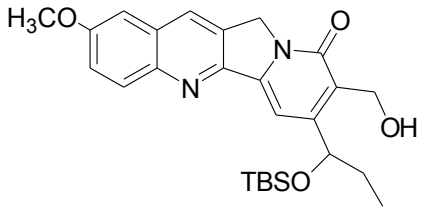


$C_{32}H_{48}N_2O_4Si_2$
Mol. Wt.: 580,91

An ice-cooled solution of compound **22** (0.995 g, 2.84 mmol) in dry DMF (100 mL) was treated sequentially with imidazole (3.09 g, 45 mmol) and t-butylchlorodimethylsilane (3.42 g, 23 mmol) under nitrogen atmosphere and stirred for 24 hours. The solid formed was isolated by filtration, dissolved in dichloromethane and washed with water and NaCl. The organic layer was dried over Na_2SO_4 and evaporated under vacuo to afford compound **22** (1.50 g, 96%).

^{1}H NMR ($CDCl_3$): 8.50 (1H, s), 8.00 (1H, d, J= 8.1 Hz), 7.41-7.50 (2H, m), 7.20 (1H, s), 5.21 (2H, s), 4.65-4.95 (3H, m), 3.93 (3H, s), 1.75 (2H, m), 0.80-1.05 (12H,s)

23. 7-[1-(tert-Butyl-dimethyl-silanyloxy)-propyl]-8-hydroxymethyl-2-methoxy-11H-indolizino[1,2-b]quinolin-9-one.

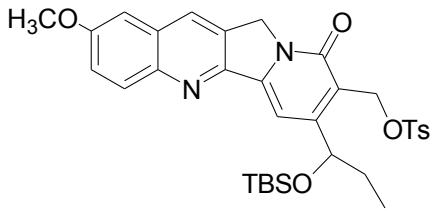


$C_{26}H_{34}N_2O_4Si$
Mol. Wt.: 466,64

A solution of **22** (1.45 g, 2.72 mmol) in dichloromethane:methanol (80 mL, 1:1) was cooled to 0°C and treated with PTSA (0.234 g, 1.36 mmol). The cooling bath was removed and the reaction mixture allowed to warm to room temperature. After 1h a $NaHCO_3$ solution was added, the mixture was concentrated under reduced pressure; dichloromethane was added and washed with water and NaCl. The organic layer was dried over Na_2SO_4 and evaporated to afford product **23** (0.570 g, 45%). Mp 200 ° C

^{1}H NMR ($CDCl_3$): 8.20 (1H, s), 8.11 (1H, d, J= 8.9 Hz), 7.10 (1H, s), 5.22 (2H, s), 4.70-4.90 (3H, m), 3.95 (3H, s), 1.80 (2H, m), 0.85-1.10 (12H, m), 0.10 (3H, s), 0.01 (3H, s).

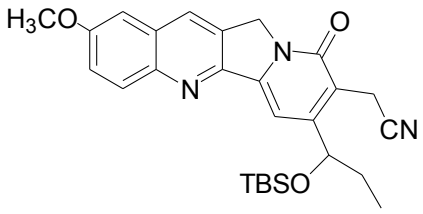
24. Toluene-4-sulfonic acid 7-[1-(tert-butyl-dimethyl-silanyloxy)-propyl]-2-methoxy-9-oxo-9,11-dihydro-indolizino[1,2-b]quinolin-8-ylmethyl ester.



$C_{33}H_{40}N_2O_6SSi$
Mol. Wt.: 620,83

To an ice-cooled solution of compound **23** (0.470 g, 0.99 mmol) in dry dichloromethane (20 mL) were added DMAP (0792 g, 6.49 mmol), then TsCl (0.949 g, 4.99 mmol) under nitrogen. After being stirred for 24h at 0°C the mixture was added w3ith water and washed with a $NaHCO_3$ solution. The organic layer was dried over Na_2SO_4 and evaporated affording crude product **24**, which was used for the next step without further purification (0.800 g).

25. {7-[1-(tert-Butyl-dimethyl-silanyloxy)-propyl]-2-methoxy-9-oxo-9,11-dihydro-indolizino[1,2-b]quinolin-8-yl}-acetonitrile.

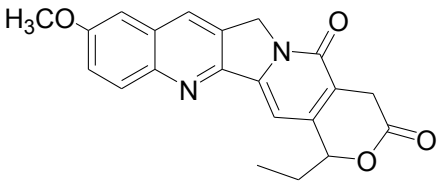


$C_{27}H_{33}N_3O_3Si$
Mol. Wt.: 475,65

To a solution of crude compound **24** (0.800 g) in DMSO (10 mL) was added NaCN (0.073 g, 1.49 mmol) and the mixture was heated at 50°C for 2 h. Dichloromethane was added and washed twice with NaCl solution. The organic layer was dried over Na_2SO_4, and evaporated under reduced pressure to afford compound **25** (0.350 g). Mp 210 °C.

^{1}H NMR ($CDCl_3$): 8.40 (1H, s), 8.12 (1H, d, J= 8.6 Hz), 7.50 (1H, d, J= 8.6 Hz), 7.40 (1H, s), 7.18 (1H, s), 5.29 (2H, s), 4.75 (1H, m), 3.95 (3H, s), 3.90 -3.95 (2H, m), 1.91 (2H, m), 1.10 (3H, t, J= 7.4 Hz), 0.90 (9H, m), 0.20 (3H, s), 0.01 (3H, s).

10. 4-Ethyl-9-methoxy-1,12-dihydro-4H-3-oxa-6,12a-diaza-dibenzo [b,h]fluorene-2,13-dione



$C_{21}H_{18}N_2O_4$
Mol. Wt.: 362,38

Compound **8** (0.100 g, 0.21mmol) was suspended in ethanol (1 mL), then ethanol saturated with HCl (1 mL) was added under stirring. The reaction mixture was heated at 50°C for 1.5 h, then dichloromethane was added and washed with a $NaHCO_3$ solution. The organic layer was dried over Na_2SO_4, evaporated under reduced pressure, and purified by flash chromatography (dichloromethane: acetone, 7: 3) to afford product **10** (0.01 g, 13%). Mp 278 ° C

^{1}H NMR ($CDCl_3$): 8.28 (1H, s), 8.10 (1H, d, J= 8.46 Hz), 7.50 (1H, dd, J=8.46-1.47), 7.19 (1H, d, J=1.47), 7.10 (1H, s), 5.41 (1H, m), 5.31 (2H, s), 4.00 (3H, s), 3.89 (1H, ab), 3.62 (1H, ab), 2.10 (2H, m), 1.10 (1H, d, J=1.47).
HRMS (ESI^+) calcd for $C_{21}H_{18}N_2O_4$ $[M+H]^+$ 363.13393; found 363.13426;
HRMS (ESI^+) calcd for $C_{21}H_{18}N_2O_4Na$ $[M+Na]^+$ 385.15500; found 385.11626

3.4

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4

Design and synthesis of new cytotoxic lamellarin D analogues.

4.1

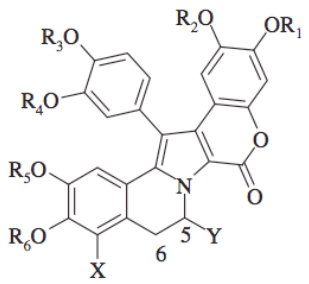
Introduction

Over 100 of the most important drugs in use today derive from terrestrial organisms, either bacteria, fungi, or plants.[1] By contrast, the chemical diversity of marine life, which is estimated to about two million species, remains largely underexplored.[2] This exceptional reservoir represents a vast chemical, structural, and biological diversity of molecules, often very distinct from those found in terrestrial natural compounds. The most frequent marine species used as sources of bioactive drugs are sponges, ascidians, mollusks, echinoderms, bryozoans, algae, and coelenterates (sea whips, sea fans, and coral).[3] One of the first key bioactive molecules of marine origin was spongothymidine, initially extracted from the sponge *Cryptotethia crypta* and identified by Werner Bergmann, an organic chemist in the 1950s.[4-6] This molecule is a nucleoside with a modified sugar, which interferes with DNA replication and inhibits the growth of some bacteria, viruses, and cancer cells. A few years later, Evans and coworkers synthesized other ‘‘false’’ nucleosides, including arabinosylcytosine (Cytarabine or ara-C) used mainly for the treatment of acute leukemia and non-Hodgkin’s lymphoma.[7] Cytarabine also displays antiviral activities, of potential use for the treatment of herpes virus infections. But during the following two decades, this field of research implicating molecules of marine origin evolved relatively slowly, at least compared to the huge efforts devoted to the identification of new molecules from plants and microorganisms. The reason is that the marine material was generally collected by hand, and in most cases only small

quantities of the living material could be obtained. It was (and remains) common to isolate less than one milligram of a bioactive substance from one kilogram (when available) of the marine organism. But today, extremely sensitive methods of NMR spectroscopy and mass spectrometry combined with liquid chromatography (HPLC, UPLC) are routinely used for the characterization of new drug structures.[8] Complex molecular structures can be solved with much less that 1 mg of compounds, without too much difficulty in most cases (although there are notorious exceptions). A few drugs derived from the sea are being developed for their biological activity in the field of inflammation (manoalide, pseudopterosins) and Alzheimer's disease (GTS-21), to cite only two examples.[9-12] The first totally marine-derived drug approved by the Food and Drug Administration was zicotinide (Prialt, developed by Elan Pharmaceuticals) in December 2004, for the treatment of chronic and severe pain.[13] The field of cancer chemotherapy has been also widely investigated using marine-derived molecules. Many natural products of marine origin are toxins and cytotoxic agents, potentially useful to inhibit cancer cell proliferation. A handful of marine compounds has reached phases II or III of clinical trials in oncology. This is the case for certain dolastatin derivatives (soblidotin, tasitodin) as mitotic inhibitors,[14-15] bryostatin 1, an activator of protein kinase C,[16] ecteinascidin-743, a powerful DNA alkylating drug,[17] neovastat, a pleiotropic antiangiogenic agent, which prevents the binding of the vascular endothelial growth factor (VEGF) to its receptors, inhibits gelatinolytic and elastinolytic activities of matrix metallo-proteinase MMP-2, MMP-9, and MMP-12, and promotes the activity of tissue-type plasminogen activator (t-PA),[18] squalamine, another antiangiogenic factor,[20] and kahalalide F, which alters the function of the lysosomal membrane. These are just a few examples, there are many more marine-based molecules of therapeutic interest in the field of cancer chemotherapy (see ref. 21 for a comprehensive survey). Ecteinascidin-743 (ET-743, Trabectidin, Yondelis1) is the most advanced marine natural product in clinical development, with a clinical efficacy established for the

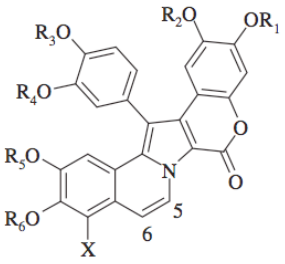
treatment of soft-tissue sarcoma.[22] This drug was originally developed by PharmaMar (Madrid, Spain), now in codevelopment with Johnson & Johnson (Ortho Biotech Products, NJ, USA). For more than 10 years, chemists and pharmacologists at PharmaMar have focused on the development of anticancer agents of marine origin (www.pharmamar.com). In addition to ET-743, the PharmaMar portfolio includes, at the clinical level, aplidin, an apoptotic inducer originally isolated from the tunicate Aplidium albicans, kahalalide F, a depsipeptide isolated from the sea slug Elysia rufescens ES-285, an aminoalcohol isolated from the clam Mactromeris polynyma, Zalypsis, an analog of ET-743 structurally related to the marine natural compound jorumycin obtained from mollusks and, at the preclinical level, thiocoraline, variolins, and the lamellarins, which represent original families of cytotoxic agents. These last molecules, the lamellarins, have attracted interest owing to their complex mechanism of action and structural originality (Figure 4.1).

Lamellarins were originally extracted from a marine prosobranch mollusk Lamellaria sp. and subsequently from primitive chordate ascidians (tunicates).[22-23] These ascidian

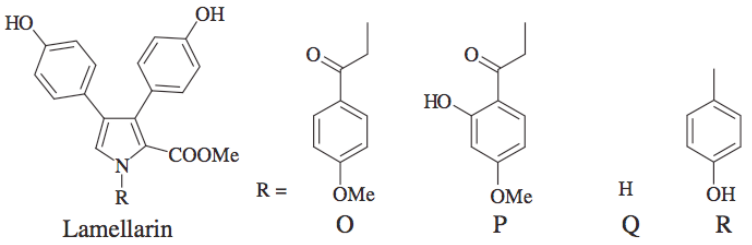


Lamellarin	R1	R2	R3	R4	R5	R6	X	Y
C	H	CH_3	H	CH_3	CH_3	CH_3	OCH_3	H
I	H	CH_3	CH_3	CH_3	CH_3	CH_3	OCH_3	H
K	H	CH_3	H	CH_3	CH_3	CH_3	OH	H
T	H	CH_3	CH_3	H	CH_3	CH_3	OCH_3	H
U	H	CH_3	CH_3	H	CH_3	CH_3	H	H
V	H	CH_3	CH_3	H	CH_3	CH_3	OCH_3	OH
γ	OH	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OH	H
χ-tri acetate	Ac	CH_3	Ac	CH_3	CH_3	Ac	H	H

Fig 4.1



Lamellarin	R1	R2	R3	R4	R5	R6	X
D	**H**	**CH_3**	**H**	**CH_3**	**CH_3**	**H**	**H**
H	H	H	H	H	H	H	H
M	H	CH_3	CH_3	CH_3	CH_3	CH_3	OH
N	H	CH_3	CH_3	H	CH_3	H	H
20 α sulfate	SO_3Na	CH_3	CH_3	H	CH_3	CH_3	H
α	H	CH_3	CH_3	H	CH_3	CH_3	H
ζ	H	CH_3	CH_3	CH_3	CH_3	CH_3	OCH_3
η	H	CH_3	CH_3	CH_3	CH_3	CH_3	H
ϕ tri-acetate	Ac	CH_3	CH_3	CH_3	Ac	CH_3	OCH_3



species, known to produce many bioactive metabolites, likely represent the original producer of lamellarins because these organisms are presumed to be the dietary source of the Lamellaria mollusks. Lamellarins have been isolated from different tunicates, including recently from the Indian ascidian *Didemnum obscurum*. Ascidians are rich sources ofcytotoxic compounds such as didemnin-B, eudistomin-C, lissoclinamides, asciddemnin, eilatin and segolins, lukianols, polycitrins, and ningalins. However, it may also be postulated that lamellarins are produced by microorganisms symbiotic to the ascidians, since numerous natural products from marine invertebrates have been demonstrated to originate from symbiotic organisms, cyanobacteria in particular. To date, 42 lamellarins (A–Z and a–z, including acetate and sulfate derivatives) have been isolated. Lamellarins z, h, f, and x are the most recent members of the family, having been isolated from the red colonial ascidian *Didemnum obscurum*.[24] They can be divided into three groups characterized by (i) the central ring pyrrole fused to adjacent rings having a single bond in the

isoquinoline moiety at position 5 and 6, or (ii) a double bond, or (iii) the central ring pyrrole unfused to adjacent rings. Each series includes derivatives in which the phenolic hydroxyl groups are substituted by methoxy, sulfate, or acetate groups. Although, the exact ‘‘natural’’ role of lamellar- ins in the ascidians is unknown, we can hypothesize that these molecules participate in some forms of chemical communication or defense mechanism (against predation or overgrowth by competing species), as is the case for many other secondary metabolites.[1,2] In the following sections, the different biological properties of lamellarins are briefly summarized, including MDR modulation activities, antioxidant properties, HIV integrase inhibition and cytotoxicity against tumor cells.

Multidrug resistance (MDR) is a term used to characterize the ability of tumors to exhibit simultaneous resistance to various chemotherapeutic agents. Different mechanisms can explain this behavior including alteration of the activity of target enzymes (such as glutathione-S transferase and topoisomerases), changes in apoptotic processes, and/or, more frequently, modification of drug efflux, implicating ABC transporters such as the multidrug resistance-associated protein (MRP) or P-glycoprotein (Pgp). Pgp proteins have been implicated in resistance to many chemotherapeutic drugs such as doxorubicin, vincristine, etoposide, or taxol. In fact, in tumor cells expressing Pgp, the efflux of a given anticancer drug increases across the plasma membrane, thereby reducing the intracellular drug concentration and hence its cytotoxicity. But Pgp activity can be reversed by a few specific molecules, referred to as MDR modulators . For example, the calcium channel blocker verapamil possesses this reversing capacity at a concentration range from 5 mM to 50 mM.[25] Lamellarin I is also an MDR modulator, directly inhibiting P-glycoprotein-mediated drug efflux at nonlethal doses. In P388/schabel cells, lamellarin I is able to reverse doxorubicin resistance at a concentration one-tenth of that necessary for verapamil. Other ‘‘sea world’’ molecules are endowed with this property to modulate MDR: discodermolide, pattelamide D, irciniasulphonic acid, agosterol A, and ningalin B.[26] This last compound bears a close structural

analogy with lamellarin T, which is also a modulator of multidrug resistance in addition to its cytotoxic and antibacterial properties. Ningalin B was recently incorporated with combretastatin A4-like structures to design new antimitotic agents. Combretastatin A-4 can easily isomerize to the thermodynamically more stable trans isomer, with reduced antimitotic activity. Its condensation with a lamellarin-type moiety provides an original approach to maintain the more potent cis configuration.[27,28]

Oxidant products are reactive species usually toxic to cells through lesions caused to lipids, proteins, and DNA in particular. In cells, mitochondria are the major source of oxidants. In fact, using oxygen to generate energy, mitochondria naturally produce reactive oxygen species (ROS). To avoid the toxic effects, cells have developed complex chemical and/or enzymatic processes to prevent damage, including thioredoxin, the glutathione system, superoxide dismutase, and catalase. When these protective systems are deficient, an abnormal ROS production arises and produces irreversible lesions potentially implicated in human pathologies such as cancer, artheriosclerosis, or neurodegeneration. Antioxidants reduce the rate of particular oxidation reactions and can help to protect against lethal damage induced by ROS. Certain lamellarins such as lamellarins G, K, U, I, and C-diacetate have revealed mild antioxidant properties. Their effects are fairly modest (with IC_{50} above 2 μM) compared to reference drugs such as Trolox and α-tocopherol (IC_{50} around 50 μM) but this property may be enhanced upon adequate substitutions of the molecule. However, this is certainly not the most advantageous property of the lamellarins, given their significant cytotoxicity.[29]

AIDS remains a devastating disease, responsible for the death of millions of humans, principally in central Africa. The prevalence of AIDS remains extremely high and, despite the efficacy of the highly active antiretroviral therapy (HAART) and the continuous development of chemotherapeutic agents targeting HIV-1 reverse transcriptase and protease, newer effective drugs are still eagerly awaited. HIV-1 integrase promotes the integration of proviral DNA into the host cell chromosomal DNA. It constitutes an

attractive target because no human cellular homolog for this enzyme exists. Two major classes of integrase inhibitors have been described: the catechol-containing hydroxylated aromatics (L-choric acid) and the diketo acid-containing aromatics (5CITEP, S-1360). A few members of the lamellarin family revealed integrase inhibition activities. Lamellarin A 20 sulfate in vitro showed potent inhibition of HIV-1 integrase. This compound, for which an efficient total synthesis has been reported, is able to act at two different steps of the catalytic cycle, terminal cleavage and strand transfer. Moreover, this compound inhibits viral replication in cultured cells at nontoxic doses. Its sulfate group plays a critical role because the analog lamellarin a is inactive against HIV-1 integrase. The nonsulfated analog lamellarin H may also be of interest as an HIV-1 integrase inhibitor (IC50 1⁄4 8 μM) but unfortunately this compound is markedly toxic to cells (LD50 of 5.7 μM measured with Hela cells). Nevertheless, the lamellarins have an intrinsic potential for the inhibition of HIV-1 integrase. But so far this pharmacological activity has not been thoroughly explored.[30-33]

The most common properties of the lamellarins are their cytotoxic activities and in particular their capacity to inhibit the proliferation of cancer cells. This activity is usually easy to measure and a large panel of tumor cells can be cultivated in vitro without much difficulty. Different levels of activity corresponding to a lethal or a growth inhibition activity (cytotoxic versus cytostatic) have been reported in the literature with different cell lines. A representative selection of these values is collated in Table 4.1. Although comparison cannot strictly be made, because of distinct experimental conditions from one study to another, it is nevertheless obvious that the majority of lamellarins are considerably cytotoxic, with IC_{50} (or LD_{50}) values in the nanomolar to micromolar range, depending on the experimental conditions and the nature of the compounds. A noticeable exception is that of the sulfated lamellarins (A, U, and V) which are not cytotoxic, presumably owing to reduced cell uptake.

Lamellarin D (Lam-D) is one of the most cytotoxic compounds of the family.

Its broad spectrum of toxicity to numerous tumor cell lines (Table 4.1) makes it a solid lead for the design of synthetic analogs. Different procedures have been reported for the total synthesis of Lam-D and related lamellarins, thus opening a route to the rational design of a variety of analogs. In the Lam-D series, precise structure–activity relationships have been delineated. Compounds possessing hydroxyl groups at both the C-8 and C-20 positions are more cytotoxic than other Lam-D analogs, whereas the OH at C-14 and methoxy groups at C-13 and C-21 seem to be less important to maintaining the cytotoxic potential.[34] These structure–activity relationships (Figure 4.2) have been extended to delineate the importance of the lactone of Lam-D in the cytotoxicity. Most of the Lam-D derivatives with an open lactone ring were found to be considerably less cytotoxic than Lam-D, except when a lactonization potential is preserved. In this case, a marked toxicity toward A-549 lung carcinoma, HT-29 colon carcinoma, and MDA-MD-231 breast adenocarcinoma cells was maintained .[35] The mode of action of these

Molecules	Cell lines	Time incubation	Test[a]	IC_{50}[b]	Reference
Lamellarin-ξ	COLO-205	16 h	MTT	5.6 nM	[24]
Lamellarin-η	COLO-205	16 h	MTT	178 nM	[24]
Lamellarin-ϕ triacetate	COLO-205	16 h	MTT	56 nM	[24]
Lamellarin-χ triacetate	COLO-205	16 h	MTT	0.2 nM	[24]
Lamellarin α	Hela	—	MTT	5 μM	[54]
Lamellarin α 20-sulfate	Hela	3 d	MTT	274 μM	[55]
LAM-D	MDCK	5–8 d	ICF	22 nM	[56]
	P388	3 d	MTS	136 nM	[57]
	CEM	3 d	MTS	14 nM	[57]
	Hela	5–8 d	ICF	10 nM	[56]
	XC	5–8 d	ICF	12 nM	[56]
	Vero	5–8 d	ICF	10 nM	[56]
Lamellarin F	COLO-205	1–6 h	MTT	9 nM	[24]
Lamellarin H	Hela	ND	MTT	5.7 μM	[54]
Lamellarin I	COLO-205	16 h	MTT	25 nM	[24]
Lamellarin J	COLO-205	16 h	MTT	50 nM	[24]
Lamellearin K triacetate	COLO-205	16 h	MTT	700 nM	[24]
Lamellarin L triacetate	COLO-205	16 h	MTT	0.25 nM	[24]
Lamellarin N	SK-MEL-5	—	—	187 nM	[55]
Lamellarin U 20 sulfate	Hela	3 d	MTT	145 μM	[55]
Lamellarin V 20 sulfate	Hela	3 d	MTT	130 μM	[55]
Lamellarin T	Hela	3 d	MTT	27 μM	[55]
Lamellarin T diacetate	COLO-205	16 h	MTT	180 nM	[24]
Lamellarin W	Hela	3 d	MTT	28 μM	[55]

[a]MTT and MTS are conventional tetrazolium dyes used to evaluate cell proliferation.
[b]IC_{50}, drug concentration to inhibit cell proliferation by 50 % (in some cases, the numbers refer to LD_{50}, dose to induce cell death, depending on the assay).

Tab 4.1

lactone-free derivatives of Lam-D is, however, unknown at present. It remains to be determined whether these cytotoxic derivatives can preserve an inhibitory activity against topoisomerase I, which is considered the privileged (but not unique) target of Lam-D. It is only since 2002 that

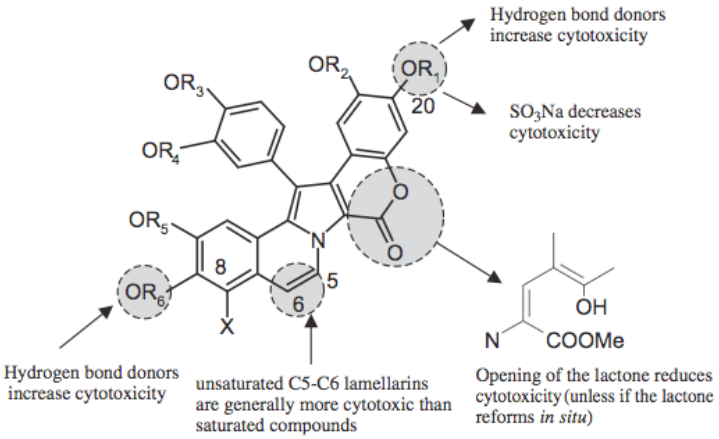


Fig 4.2

the molecular mechanism of action of cytotoxic lamellarins, in particular Lam-D, has been investigated, essentially in our laboratory.[36]

4.1.1

Topoisomerase I Inhibition

The non-CPT topoisomerase I poisons with potent activities include different indenoisoquinolines such as MJ-III-65, certain glycosyl indolocarbazoles such as NB-506 and edotecarin (formerly J-107088 and ED-749) and lamellarin D which was identified as a potent topoisomerase I poison in 2003.[35] Lam-D binds relatively weakly to DNA, presumably via the insertion of its planar pentacyclic chromophore between DNA base pairs. Intercalation of the flat chromophore between two adjacent base pairs exposes the perpendicular methoxyphenol moiety toward the major groove of DNA where the enzyme can be trapped.[37] This DNA interaction, although relatively weak, provides the necessary anchorage for stabilization of the enzyme–DNA complex. Inhibition of topoisomerase I by Lam-D has been demonstrated by a variety of approaches, both in vitro

using recombinant enzymes and model DNA substrates and in a cellular context using immunoblot assays to detect the drug-stabilized topoisomerase I–DNA complexes in cells. The key discovery that topoisomerase I was a major target for Lam-D has opened the door to the determination of structure–function relationships and the rational design of lamellarin analogs of pharmaceutical interest. The double bond between carbons 5 and 6 in the quinoline B-ring is a crucial element for topoisomerase I inhibition. This C-5′1/4C-6 double bond confers a planar structure on the molecule and when this double bond is lacking (as in Lam-K, for example) the planar conformation no longer exists and the drug loses its capacity to interfere with topoisomerase I. This was clearly demonstrated using the synthetic compound Lam-501, which only differs from Lam-D in the absence of the C-5′1/4C-6 double bond. This compound showed no inhibition of topoisomerase I, and its cytotoxicity was considerably reduced.[36] Additional structure–function relationships have been delineated, thanks to the synthesis of different series of Lam-D derivatives. In particular, using Lam-D analogs with distinct OMe/OH substitution on the core structure, it was demonstrated that the 8-OH and the 20-OH are crucial for topoisomerase I inhibition and in most cases a direct link could be established between enzyme inhibition and cytotoxicity .[37] The use of cancer cell lines with topoisomerase I mutated genes has also contributed to the characterization of topoisomerase I as the main target for Lam-D and related analogs. It is thus not surprising to understand why Lam-H, which is structurally close to Lam-D, has also been identified as a topoisomerase inhibitor. Lam-H was previously shown to inhibit topoisomerase I from the Molluscum contagiosum virus (MCV).

In terms of efficacy, Lam-D is less efficient than CPT in inhibiting topoisomerase I (IC50 of 0.42 versus 0.087 μM, respectively).[36] But the sequencing of DNA cleavage products revealed that the cleavage profiles obtained with Lam-D were distinct from those seen with CPT. Both molecules intercalate at the sites of DNA cleavage corresponding to T-G sites but in addition Lam-D can induce cleavage at certain C-G sites. Lam-D

and CPT share a common mechanism of action at the topoisomerase I–DNA complex level but the two drugs are positioned in a slightly different manner at the DNA–enzyme interface, thus providing distinct molecular contacts at the origin of the nonidentical cleavage profiles. Molecular modeling studies have greatly contributed to the explanation of how Lam-D fits into the topoisomerase I–DNA active site.[36,37]

But the story of Lam-D is not so simple. If topoisomerase I can be considered as the unique target of CPT, the situation is clearly more complicated for Lam-D because its cytotoxicity cannot be explained uniquely on the basis of the topoisomerase I targeting. The cytotoxicity of Lam-D has been evaluated using the mutant cell lines P388CPT5 and CEM-C2, which both express a mutant topoisomerase I conferring a high resistance to CPT and other CPT-derived topoisomerase I poisons.[38] The two cells lines are more resistant to CPT and Lam-D than the corresponding wild-type P388 and CEM cell lines. This observation confirmed the direct implication of topoisomerase I in the cytotoxicity of Lam-D. But the resistance indexes (i.e., the ratio of IC50 values for a pair of sensitive and resistant cell lines) was clearly different for Lam-D and CPT. For example, the top1 mutated gene in CEMC2 cells confers a huge resistance of these leukemia cells to CPT (RI > 2000) whereas the RI was about 30 times lower, around 70, with Lam-D. Moreover, it was discovered that Lam-D, but not CPT, was able to induce apoptosis of the P388CPT5-resistant cell line. These observation strongly suggested the existence of alternative target(s) for Lam-D in cancer cells. This hypothesis was rapidly validated with the discovery of a nuclear topoisomerase I-independent, direct effect of Lam-D on mitochondria.[29]

4.1.2

Targeting of mitochondria and proapoptotic activities

Mitochondria are the well-characterized intracellular organelles needed for the production of ATP for all cellular processes. They also play a major role in the regulation of calcium flux and redox state. Moreover, since the 1990,

mitochondria have been considered as central effectors for the regulation of cell apoptosis. Apoptosis is a cell death characterized by a number of distintive biochemical and morphological changes occurring in cells, such as DNA fragmentation, chromatin condensation, and loss of phospholipid asymmetry in the plasma membrane and membrane budding. During apoptosis, a family of proteases known as caspases is generally activated. These proteins cleave key cellular substrates required for normal cellular functions, including structural proteins of the cytoskeleton and nuclear proteins such as DNA repair enzymes. It is now well established that during apoptosis major changes occur at the level of mitochondria. A reduction of the mitochondrial membrane potential (DCm) is generally observed during the first steps of apoptosis, associated with the release into the cytosol of various proteins localized in the intermembrane space, such as cytochrome c, AIF, Smac/DIABLO and/or endoG. Initial studies on the apoptotic pathway induced by Lam-D revealed a very early mitochondrial dysfunction, including a reduction of DCm and the release of cytochrome c and AIF from the mitochondria to the cytosol. It was then observed that Lam-D could induce these alterations directly on isolated mitochondria. Both functional assays and direct observations by electron microscopy indicated that mitochondria represented targets for Lam-D, and similarly for Lam-M. Although the exact mechanism implicated in the targeting of mitochondria is not fully understood, we have accumulated evidence has been accumulated that an opening of the mitochondrial permeability transition pore (mPTP) is induced by Lam-D. The drug induces a swelling of isolated mitochondria and this effect can be prevented by a preincubation with the PTP inhibitor cyclosporin A. Each protein constituting the mPTP can be considered as a molecular target for Lam-D, including the adenine nucleotide translocator (ANT), voltage-dependent anion channel (VDAC), peripheral benzodiazepine receptor (PBR), hexokinase (HK), Bcl-2 family members (including Bax and Bcl-2), mitochondrial creatine kinase (CK), and cyclophilin D. These proteins and receptors represent potential drug targets to control tumor cell growth. But we also cannot exclude the

possibility that Lam-D induces an indirect opening of the mPTP, forming channels or pores through phospholipids from the outer or inner membranes or acting with specific complexes of the respiratory chain. The detailed mechanism is currently being investigated further.[38,39]

Based on our previous studies, we are confronted with an uncommon situation where Lam-D seems to reside in two preferred sites within cells: (i) the nucleus, where the drug forms tight and stable complexes with DNA and topoisomerase I; (ii) mitochondria, where Lam-D interacts with an as yet unknown molecular target, potentially associated with the mPTP. Both sites and their associated mechanisms can contribute to cell death induced by Lam-D but which is the major contributor, the nucleus or the mitochondrion? The question remains unresolved at this stage but recent data suggest that topoisomerase I targeting may be the main mechanism responsible for the antitumor effects of Lam-D. Recent results indicate that at low concentrations (<1 **μ**M), Lam-D affects preferentially the topoisomerase I pathway, without any direct interference with mitochondria. At higher concentrations (in particular for concentrations>5 **μ**M), Lam-D affects both the nucleus and mitochondria, with a dual action leading to massive and rapid cell death. We must also consider the hypothesis, as yet purely conjectural, that the drug can target simultaneously both the nuclear and the mitochondrial topoisomerase I. [40]The topology of the mitochondrial genome – a closed circular mtDNA – is regulated during replication by a protein encoded by the gene mtDNA topoisomerase I (top1mt) localized on human chromosome 8q24. This gene is highly homologous to the nuclear top1 gene and the mitochondrial topoisomerase I enzyme is fully inhibited by CPT.[29,41] The close functional homology between CPT and Lam-D suggests that the mitochondrial enzyme can also be targeted by Lam-D. It will be interesting to see if Lam-D does interfere with mitochondrial topoisomerase I and if this molecular effect is at the origin of the mitochondrial dysfunctions (reduction of DCm and mPTP opening) observed in cells upon treatment with Lam-D.

Lamellarin D is not the only marine compounds capable of altering

mitochondrial functions. This is also the case for stolonoxides, which are oxidized fatty acid metabolites isolated from the Mediterranean tunicate Stolonica socialis. These compounds are able to inhibit mitochondrial respiration; more precisely they are specific inhibitors of complexes II and III of the respiratory chain. Another example is that of cephalostatin 1, a bis-steroidal compound isolated from the marine worm Cephalodiscus gilchristi. Like Lam-D, cephalostatin 1 induces a reduction of the DCm, but interestingly this alteration is not associated with the release of cytochrome c or the apoptosis-inducing factor, but only with the release of the Smac/DIABLO protein. Drugs that induce apoptosis by directly disrupting mitochondrial functions or membrane integrity seem to have an attractive potential for preventing tumor growth and eliminating tumor cells [84]. But the next challenge will be to identify or to rationally design a mitochondriotoxic drug able to kill selectively cancer cells without affecting normal cells. At present this selectivity issue for cancer cells versus nontumor cells is a major obstacle to the development of anticancer drugs targeting mitochondria.[42]

4.2

Results and discussion

Although several lamellarin derivatives show potent anticancer potency in vitro, they suffer from several drawbacks that result from low water solubility and the relatively labile nature of the lactone ring under basic conditions. Therefore, a number of analogues of lamellarin have been synthesized with the aim of improving their inhibitory potencies as well as other desirable physicochemical properties. We decided to take Lamellarin D as lead compound to design new analogues One among the modifications that were investigated to achieve these goals was the replacement of the central core of the lead compound with a β-carbolin-1-one moiety and the replacement of lactone ring with other more stable rings, which would produced the desired analogues **2** and **3** (Fig 4.3)

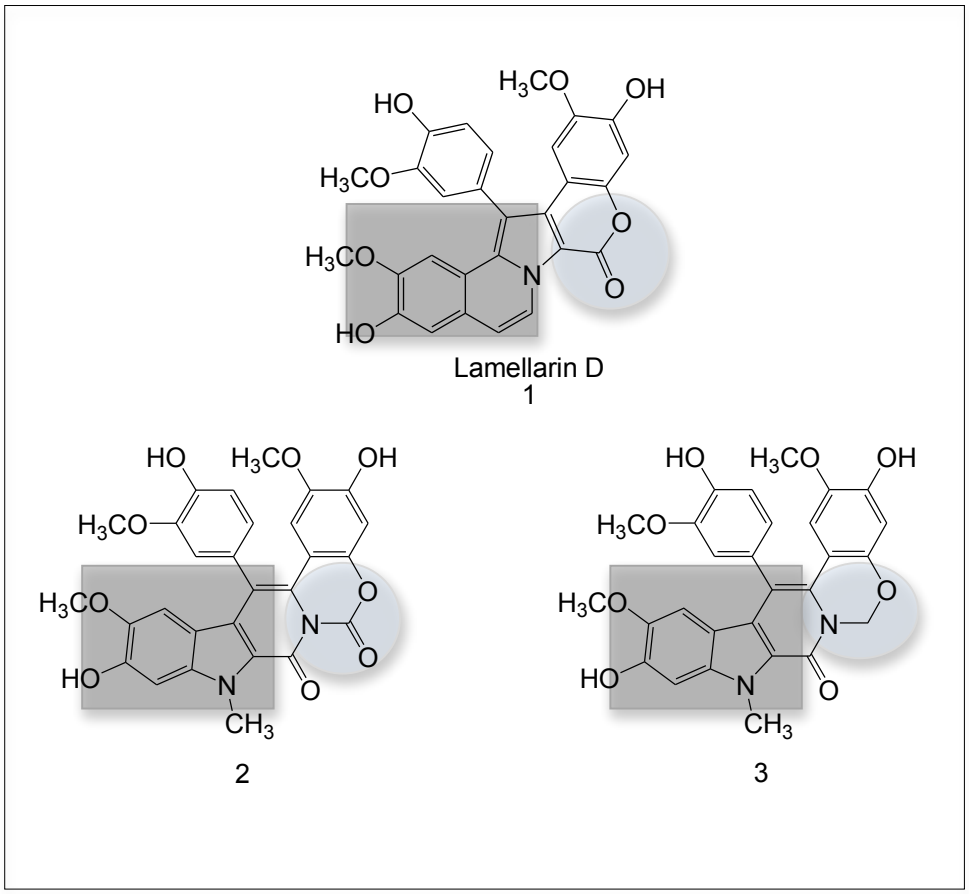
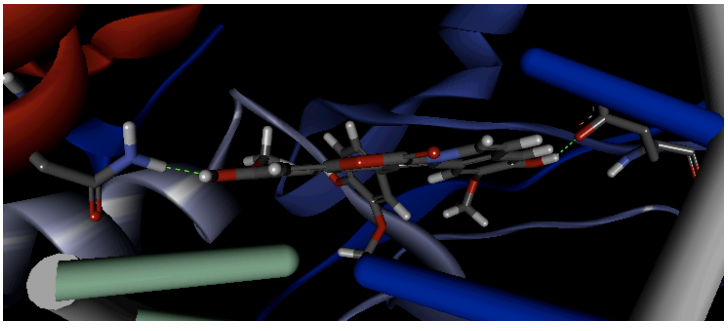
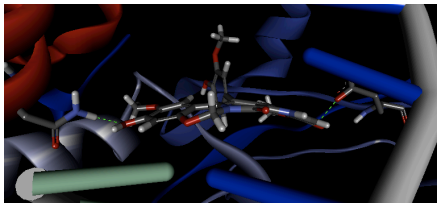


Fig 4.3

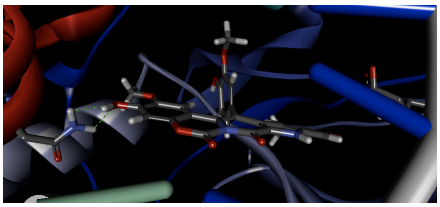
Molecular modelling studies were performed to explore the binding mode for the planned compounds to the covalent topo I-DNA complex. This study was based on the X-ray crystal structure of the well-know ternary complex between a human Topo-I construct covalently attached to a DNA duplex with bound topotecan.[7] For comparison purposes, the same docking protocol used to study the complex containing Lamellarin D was applied in the present work. As shown in figure 4.4 , the ligands is



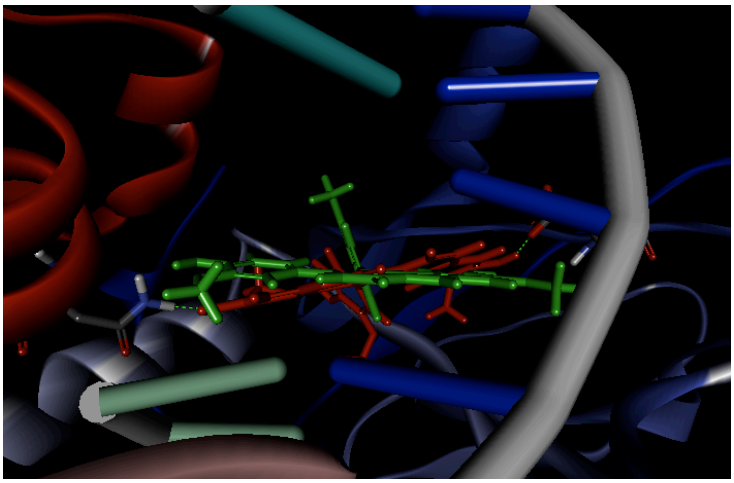
Lamellarin D



3



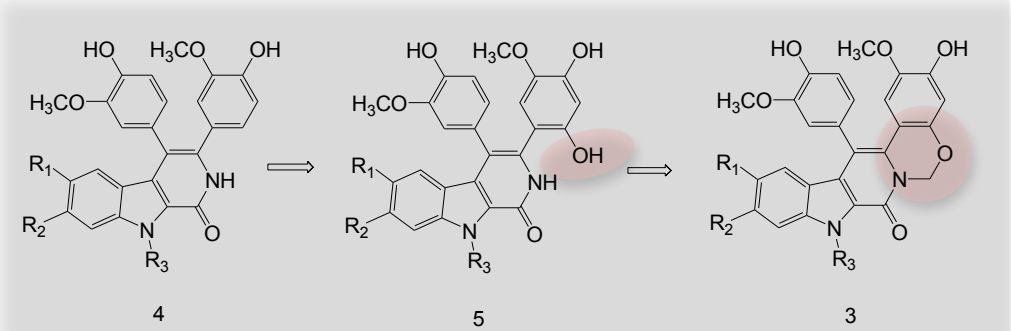
2



Overlap lamD (red) and 3 (green)

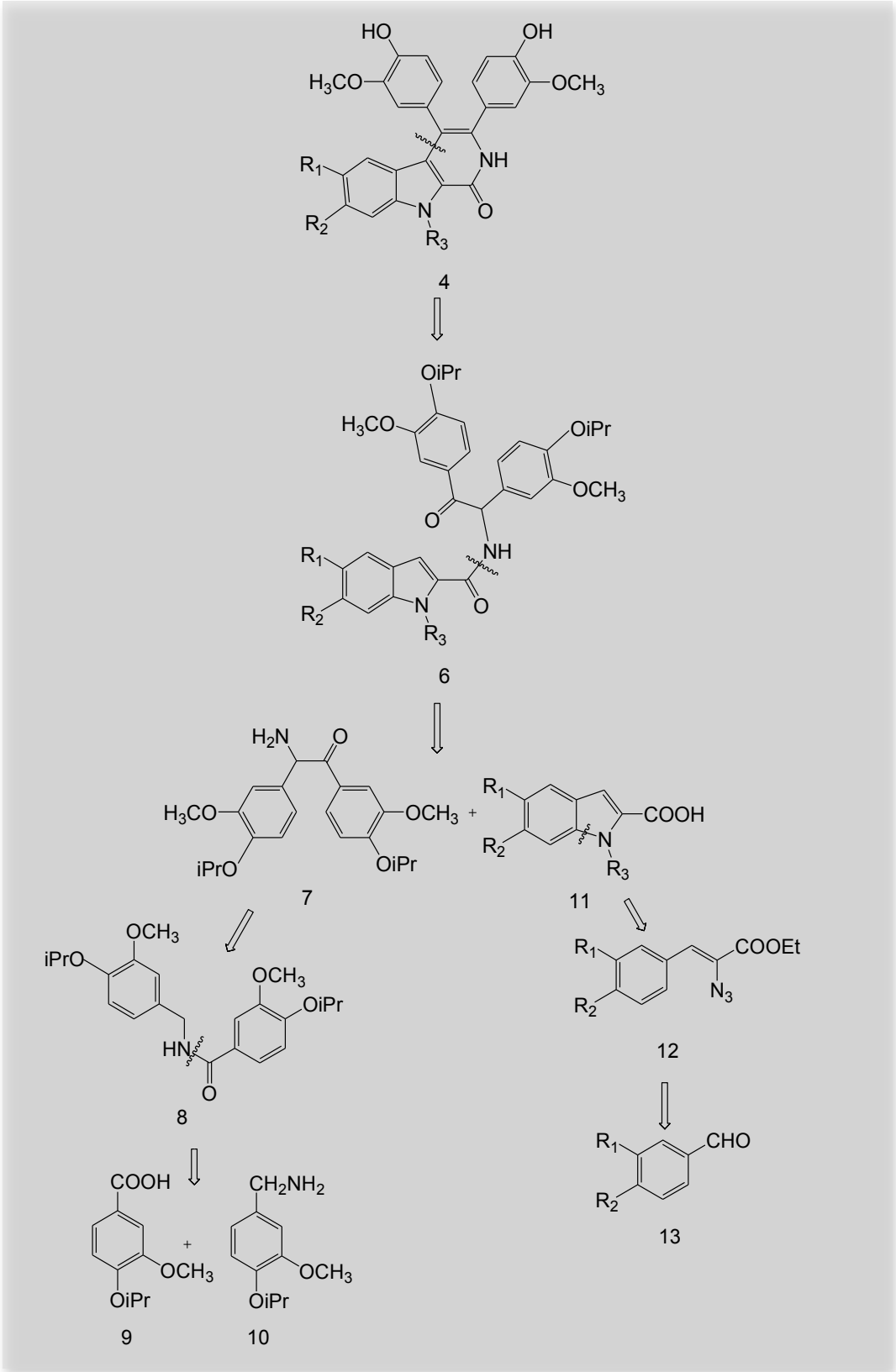
Fig 4.4

placed in the topo I-DNA complex in almost the same orientation as Lam-D. Compound **3** is connected better than compound **2** to the topo I-DNA complex through two new hydrogen bonds and it has been reasonable to assume that it might hold potent topoisomerase I inhibitory activity. To investigate this idea, we have developed a general synthetic route to this type of novel and promising Lamellarin D analogue starting from more simple analogues, which were synthesized to study reactions methods and structure-activity relationships (scheme 4.1)



Scheme 4.1

The simplified analogue **4** was obtained according a synthetic strategy in

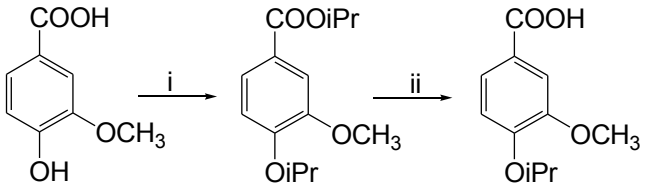


Scheme 4.2

which the key reaction is an intramolecular Friedel-Crafts cyclization of indole-2-carboxylic acid β-oxoamides, catalyzed by trifluoroacetic acid (TFA) or $InCl_3$. This method was developed io our laboratory [43] and represents a simple, straightforward synthesis of the β-carbolin-1-one nucleus. Due to the simplicity and efficiency of the synthesis, we decided to extend its scope to the preparation of 3,4-diaryl-β-carbolin-1-ones (**4**) characterized by the methoxy-hydroxy alternation typical of lamellarins.

The retrosynthetic pathway is reported in scheme 4.2. Following the above strategy, initial experiments were carried out to synthesize the necessary substrates **9** and **10.**

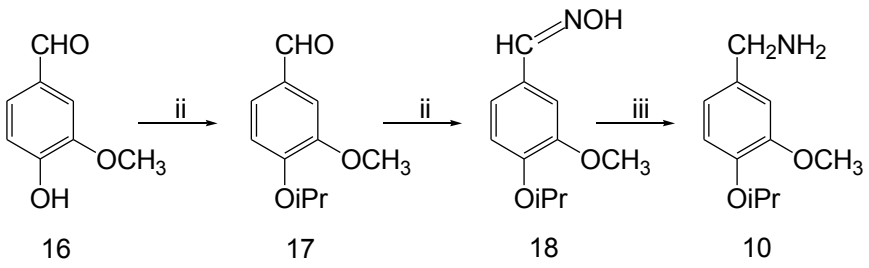
Compoud **9** was obtained from vanillic acid **14** after treatment with 2-bromopropane in basic condition and subsequentely ester hydrolysis of the ester **15** (scheme 4.3).



i: iPrBr, Ka_2CO_3, DMF, reflux; **ii**: KOH, ethanol, H_2O, reflux;

Scheme 4.3

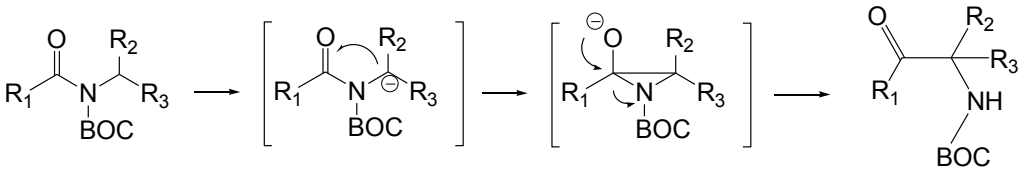
Isopropyl ether was used as a phenol protecting group in order to perform its selective cleavage at the end of synthetic route affording the desidered hydroxy-methoxy alternation.



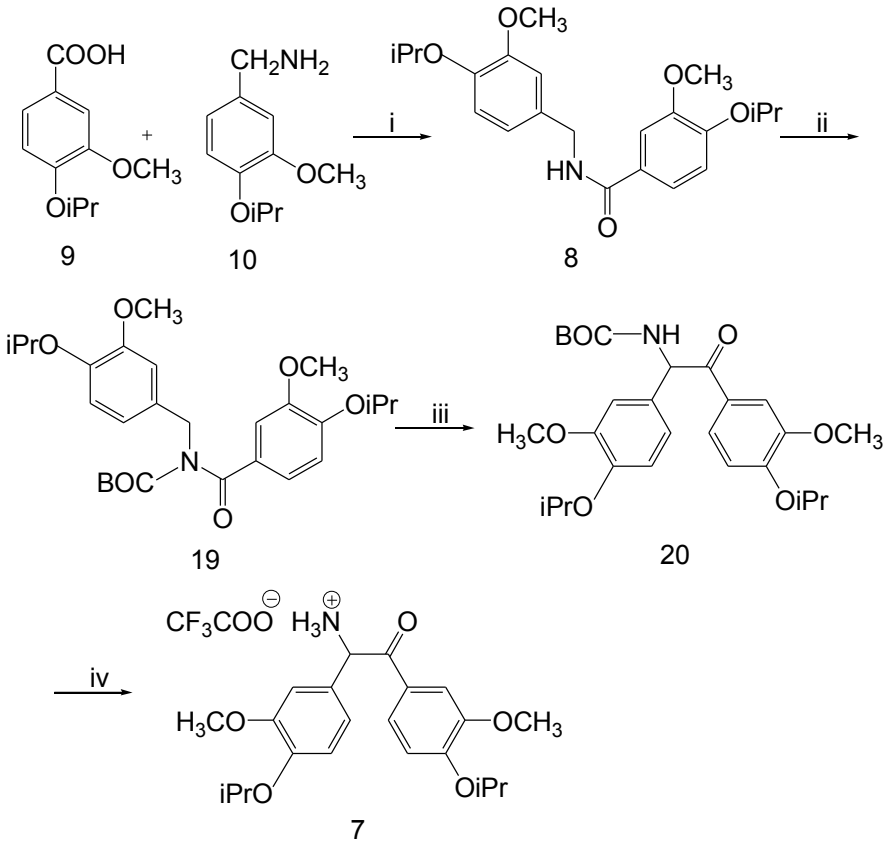
i: iPrBr, Na_2CO_3, DMF, reflux; **ii**: NH_3OHCl, pyridine, ethanol, RT; **iii**: Ni/Al alloy, NaOH 2N, ethanol,RT;

Scheme 4.4

Synthesis of compound **10** started from vanillin **16**. It was treated with 2-bromopropane under basic condition. After isopropyl protection, the resulting aldehyde **17** was trasformed using hydroxylamine chloride and pyridine in ethanol, into the oxime derivative **18,** which subsequently reduced with Raney nickel alloy and NaOH to afford the benzylamine derivative **10** (Scheme 4.4). The synthesis of the α-aminoketone derivative **7** was achieved by following the synthetic pathway outlined in scheme 4.5. The starting materials **9** and **10** were subjected to a coupling reaction using HOBt and WSC in THF at RT to afford compound **8.** This was subsequently treated with di-*tert*-butyldicarbonate in the presence of N,N'-dimethylaminopyridine in acetonitrile to protect the amide nitrogen affording BOC-derivative **19**. To obtain α-aminoketone **7** molecule a N-C migration reaction was performed [44]



followed by BOC cleavage under acid conditions (see mechanism above). In order to carry out the acyl migration, amide **19** was treated with an excess of lithium diisopropylamide (LDA). Although one equivalent of LDA is sufficient to form the carbanion, another equivalent was essential for completion of the reaction due to the proton abstraction from the α-position of the α-aminoketone. The optimized the yield of **20** was obtained using LDA (3 equiv.) in the presence of N,N'-dimethylpropyleneurea (DMPU, 3%) as an additive in THF at -78° C. Interestingly, other strong bases, such as NaH, KH, KHMDS, and s-BuLi with sparteine, did not give any acyl migrated product.

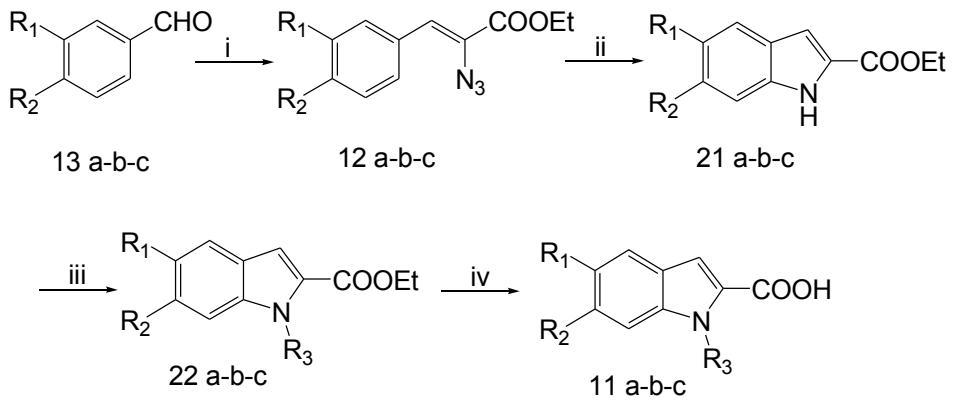


i: HOBt,WSC, THF, RT, N_2; **ii:** di-*tert*-butyldicarbonate, DMAP, acetonitrile, RT; **iii**: LDA, DMPU, THF, -78°C, N_2; **iv:** CF_3COOH, DCM,RT, N_2.

Scheme 4.5

Compound **20** was treated with TFA in DCM at RT to give α-aminoketone derivative **7** as a trifluoroacetate salt.

Indole-2-carboxylic acid derivatives **11 a-b-c** were achieved through the synthetic pathway reported in scheme (scheme 4.6) .



i: Ethy azidacetste: EtONa, EtOH, -15°C, N_2; **ii:** xylene, reflux;
iii: R_3Br, NaH, DMF, 0°C-RT;**III:** THF, MeOH, H_2O, LiOH, RT

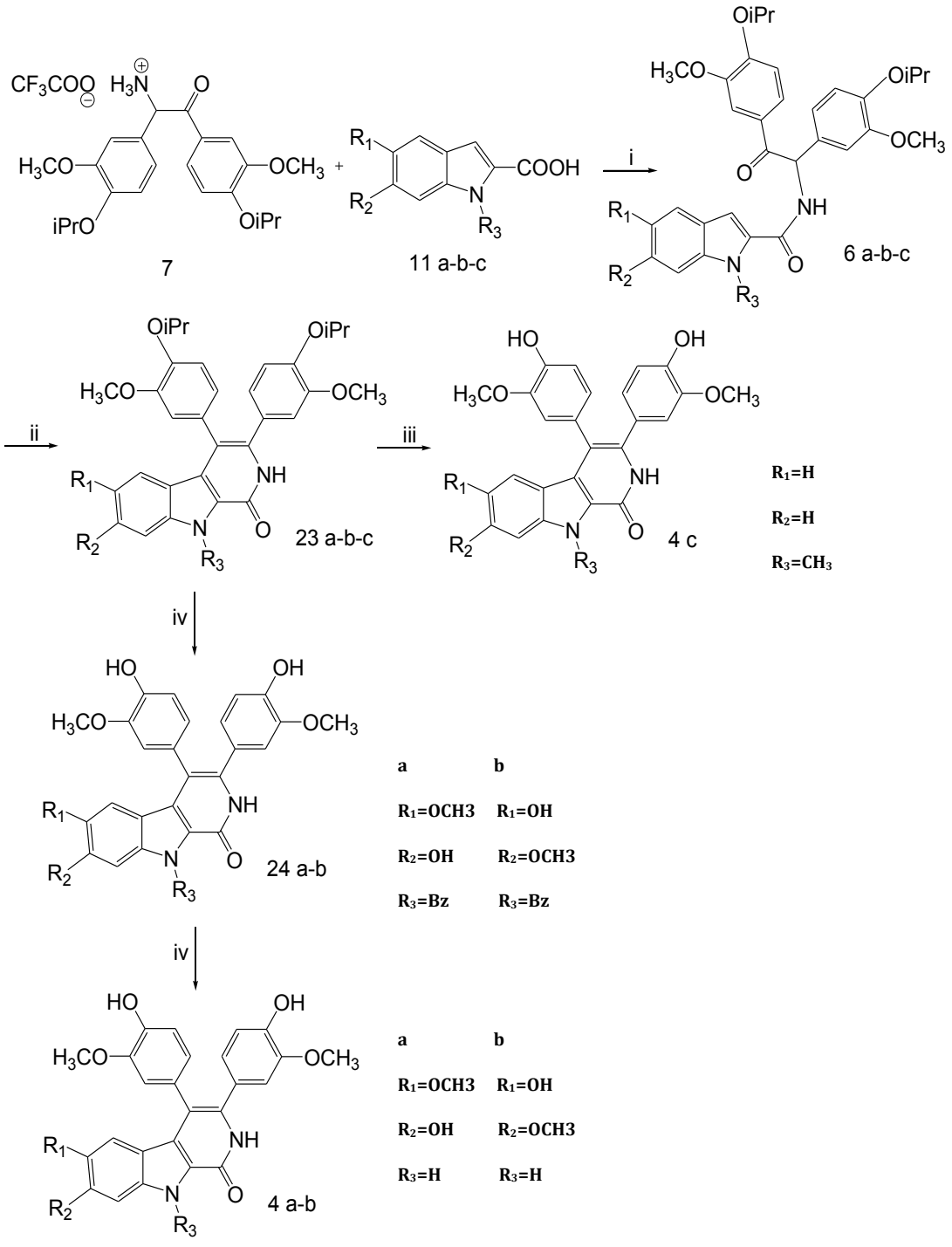
a	b	c
R_1=OCH_3	R_1=OBz	R_1=H
R_2=OBz	R_2=OCH_3	R_2=H
R_3=Bz	R_3=H	R_3=CH_3

Scheme 4.6

By allowing **13 a-c** to react with ethyl azidoacetate in the presence of sodium ethoxide at -15°C in ethanol, **12 a-c** were prepared in good yield. Subsequently the products were cyclized by heating to give indole derivates **21 a-c**. Than the indole was N-alkylated affording **22 a-c.** The ethyl ester moiety was hydrolyzed in basic conditions to obtain indolecarboxylic acids **11 a-c** .

The reaction sequence leading to compounds **4 a-c** is shown in scheme 4.7. It begins with a coupling reaction between the α-aminoketone **7** and the indolecarboxylic acid derivatives **11 a-c** in presence of HOBt and WSC in THF at RT giving amides **6 a-c** .

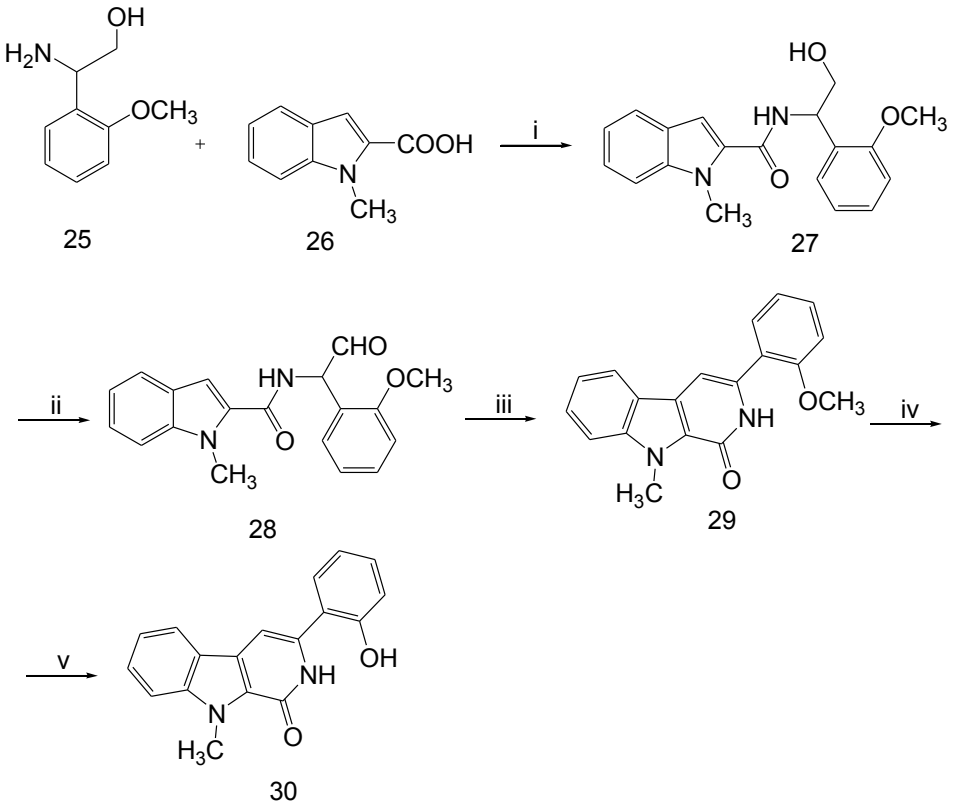
Compounds **6 a-c** were than treated with TFA in acetonitrile at 80°C to perform the crucial cyclization step by which the β-carbolin-1-one derivatives were obtained. At this point of the outlined synthetic route Intermediate **23 c** was subjected to isopropyl ether cleavage reaction carried out with $AlCl_3$ in DCM to afford expected compound **4c**. Intermediates **23 a-b** were treated in the same way, but this procedure gave **24 a-b**, without removing the N-benzyl group used as a protecting group in the indole moiety. For this reason compounds **2a-b** were subjected to react with $AlCl_3$ but in a different solvent (anisole) and under heating to remove the N.-benzyl protecting group, and to afford the expected products **4 a-b**.



i: HOBt,WSC, TEA, THF, RT, N_2; **ii:** acetonitrile, reflux; **iii, iv** and **v** : $AlCl_3$, DCM, N_2, 0°C- RT; **vi**: $AlCl_3$, anisole, reflux, N_2

Scheme 4.7

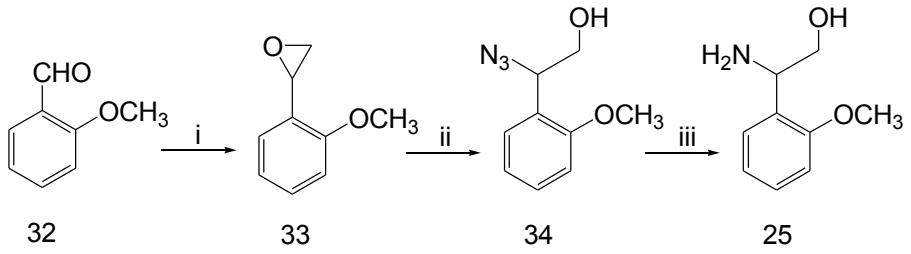
The synthetic strategy reported in scheme 4.2 was applied successfully to the synthesis of lamellarin analogue **3** but with further modifications to allow the introduction ofa further phenol group (see compound **5**) used subsequently to perform a cyclization reaction. This kind of reaction was first studied using as a simple model compound **30,** obtained via the following synthetic route (scheme 4.8), and then applied to the development of a lamellarin analogue.



i: HOBt, WSC, THF, N_2, RT; ii: IBX, DMSO, N_2, RT; iii: TFA, acetonitrile, riflux; iv: BBr_3, DCM, RT, N_2

Scheme 4.8

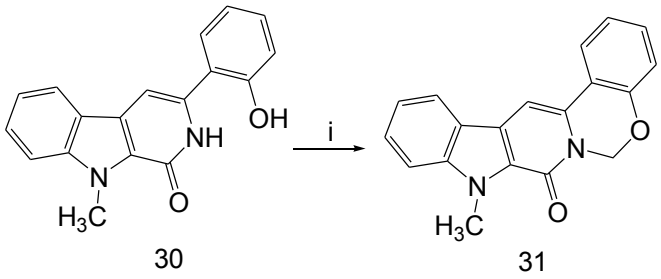
Following the above strategy, initial experiments were carried out to synthesize the necessary aminoalchol derivative **25** and the indolecarboxylic acid **26**. This latter was obtained by the synthetic route reported in scheme 4.6, the former one was achieved through the synthetic route reported in the following scheme (4.9).



i: $(CH_3)_2SOI$, NaH, DMSO, N_2, RT; ii: NaN_3, $LiClO_4$, acetonitrile, 60°C; iii: Pd(C), H_2, EtOH

Scheme 4.9

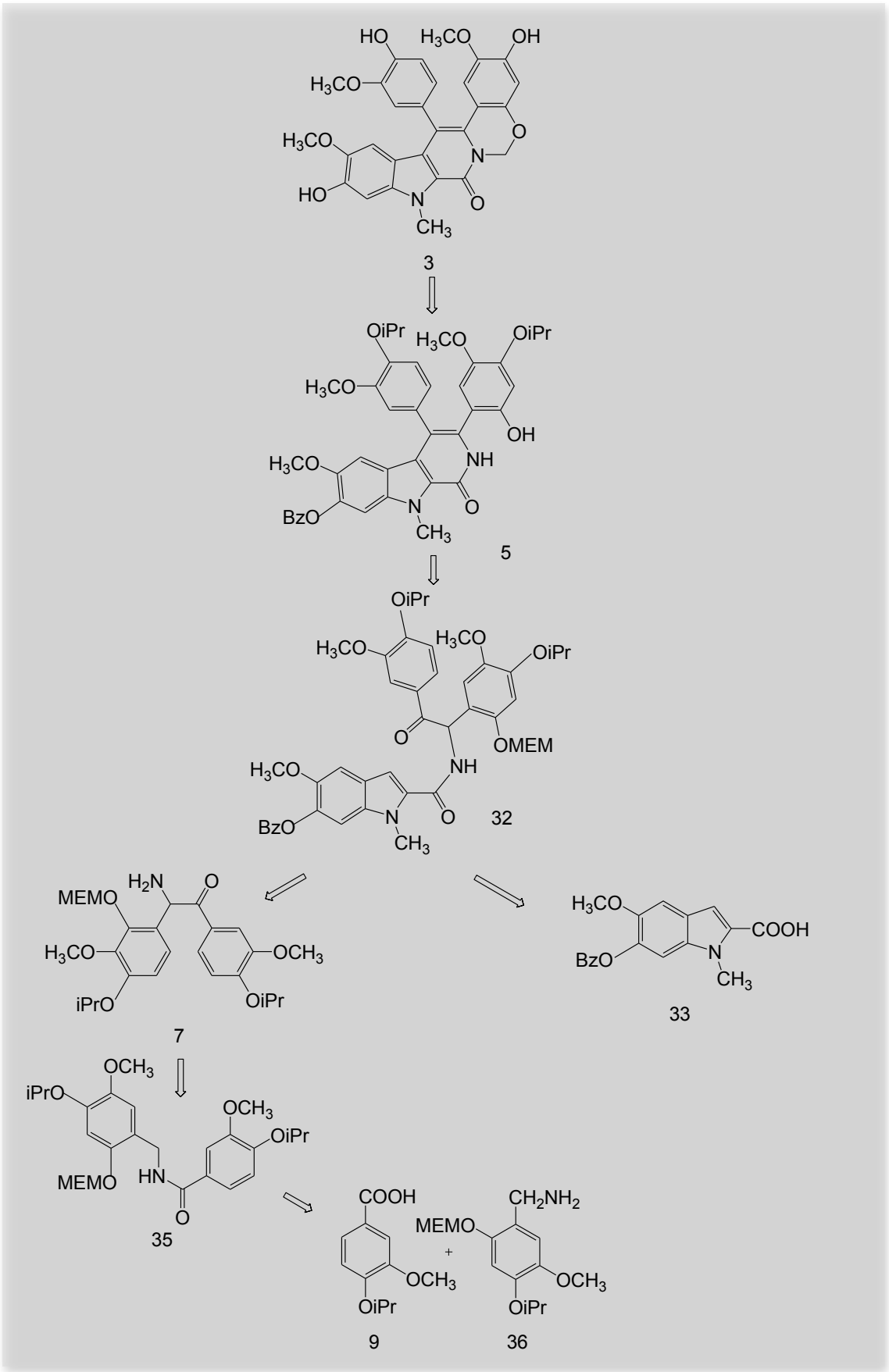
Compounds **25** and **26** were subjected to a coupling reaction using HOBt and WSC in THF at RT to afford the intermediate hydroxyamide **27.** This was subsequently oxidized to the corrisponding aldehyde derivative **28** using 2-iodoxybenzoic acid (IBX). The crucial cyclization step was obtained with TFA in acetonitrile at 80°C affording β-carbolin-1-one derivative **29**. The expected product **30**, obtained by cleavage of the methyl ether using BBr_3 in DCM, was successively used as a model compound to perform the cyclization reaction. The method developed is shown in the following



i: CH_2I_2, K_2CO_3, acetonitrile, N_2, reflux

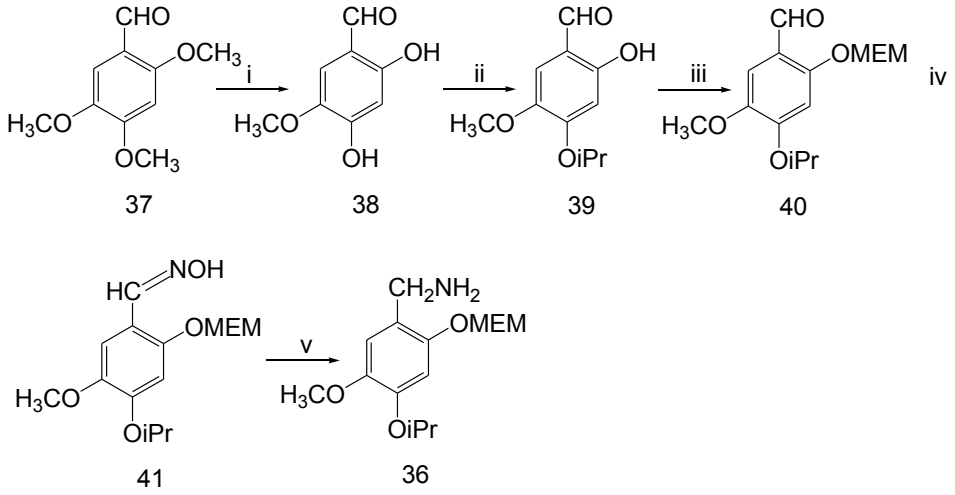
Scheme 4.10

The retrosynthetic pathway for this lamellarin analogue is reported in scheme 4.11


HO
H3CO
OH
H3CO
O
H3CO
N
HO
O
N
CH3
3
OiPr
H3CO
OiPr
H3CO
OH
H3CO
NH
N
O
BzO
CH3
5
OiPr
H3CO
H3CO
OiPr
O
OMEM
H3CO
NH
BzO
N
O
CH3
32
H2N
O
MEMO
H3CO
OCH3
iPrO
OiPr
7
H3CO
COOH
BzO
N
CH3
33
OCH3
iPrO
OCH3
OiPr
MEMO
HN
O
35
COOH
CH2NH2
MEMO
+
OCH3
OCH3
OiPr
OiPr
9
36

Scheme 4.11

Initial experiments were carried out to synthesize the necessary substrates **9** and **36.** The latter was achieved following scheme 4.13, by modification of the procedure already discussed



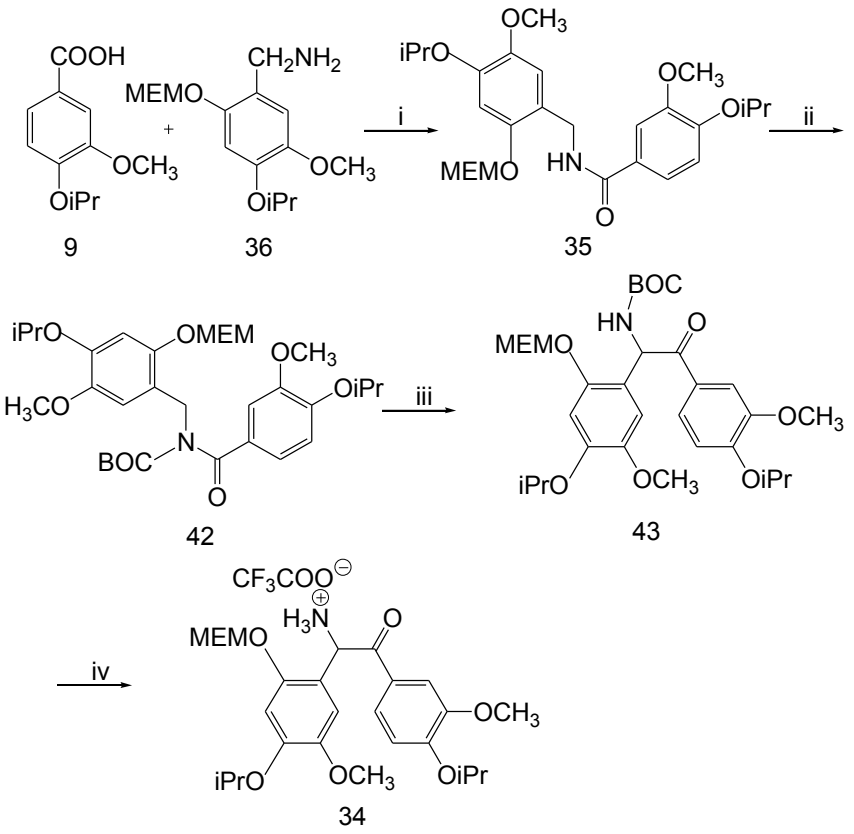
i: $AlCl_3$, DCM, N_2, RT; **ii**:IPrBr, $NaHCO_3$, DMF, 80°C, N_2; **iii**: NaH, THF, MEMCl, RT, N_2 ; **iv**: NH_3OHCl, pyridine, EtOH; **v**: Al/Ni alloy, EtOH.

Scheme 4.13

in order to have an additional phenol group in ortho posistion. Compound **9** was alkylated with a different protecting group because it must be removed in a different step with respect to the isopropyl ethers, which are maintained throughout the synthetic process. The synthesis of benzylamine derivative **36** was performed starting from 2,4,5-trymethoxybenzaldehyde **37**. It was subjected to a selective methyl ether cleavage using $AlCl_3$ in DCM, to afford compound **38** in good yield.. Here the possible different reactivity of the two phenol groups, one of them H-bonded to the ortho carbonyl, was taken into account and therefore a mild base was used to avoid a dialkylation reaction. Using 2-bromopropane and sodium bicarbonate in DMF allowed to protect selectively the p-OH group, to give compound **39**. . Now this compound was reacted with MEM chloride in the presence of the strong base NaH to alkylate the o-hydroxy group, obtaining compound **40,** which was thenconverted into oxime **41**

uby treatment with hydroxylamine chloride and pyridine in ethanol. Hydrogenation of **41** was carried out in ethanol using Ni/Al alloy and NaOH to give the benzyamine **36** in good yield.

The reaction sequence leading to compound **34** is shown in scheme 4.14. It begins with a coupling reaction between the benzylamine **36** and the vanillic acid derivative **9**. Reagents were allowed to react in the presence of HOBt and WSC in THF at RT giving amide **35,** that was subsequently treated with di-*tert*-butyldicarbonate in the presence of N,N'-dimethylaminopyridine in acetonitrile to protect the amide, affording BOC-derivative **42.** Synthesis of N-BOC- α-aminoketone **43** was achieved using

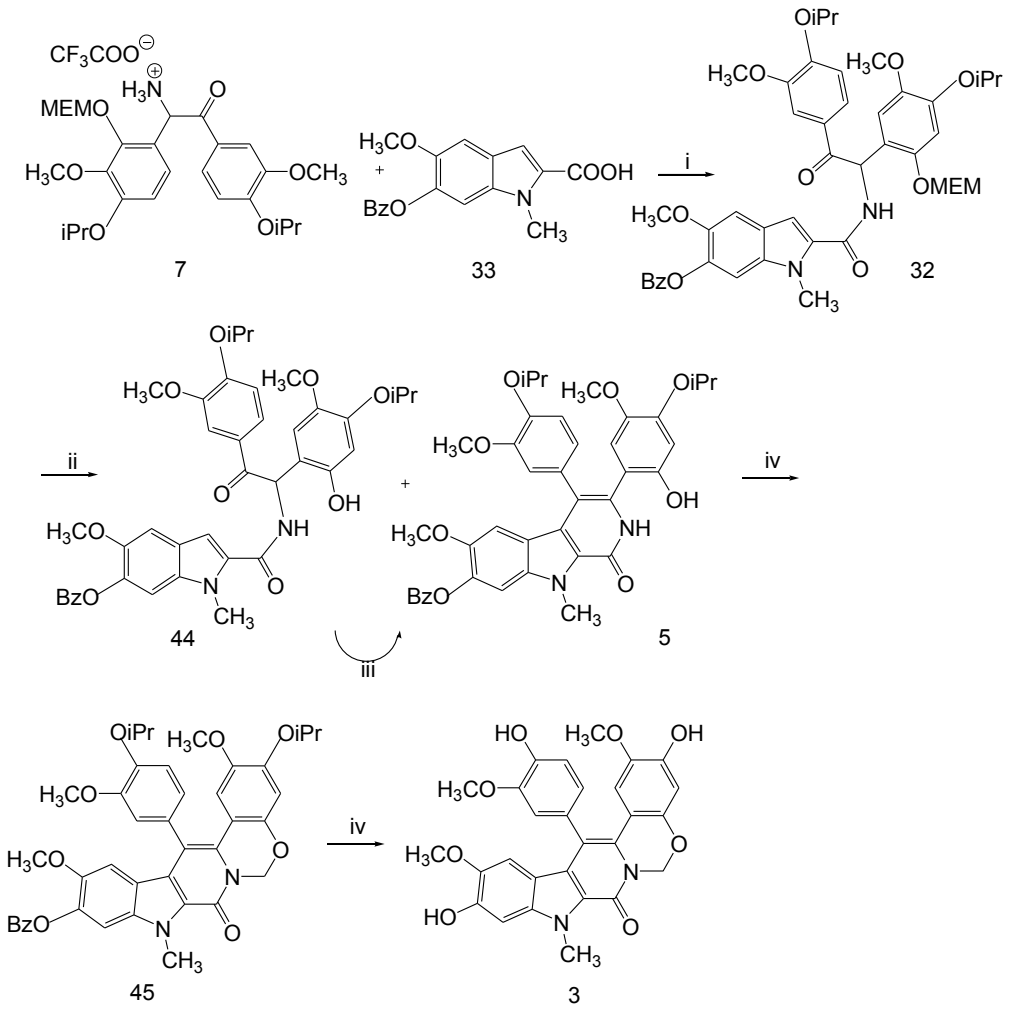


i: HOBt,WSC, THF, RT, N_2; **ii:** di-*tert*-butyldicarbonate, DMAP, acetonitrile, RT; **iii**: LDA, DMPU, THF, -78°C, N_2; **iv:** CF_3COOH, DCM,RT, N_2.

Scheme 4.14

the same procedure of *N-C migration reaction* discussed in the scheme 4.5. The so obtained compound **42** could not be reacted similarly to

compound **20** for N-BOC removal because of the presence of the MEM protecting group, which is cleavable in the same conditions. For this reason, alternative procedures were investigated, such as thermolytic treatment in solid phase under N_2, silica gel promoted cleavage and ceric ammonium nitrate treatment in acetonitrile. All these synthetic efforts failed to produce selective removal. Finally a different kinetic cleavage of the two protecting groups in acid conditions was attempted, finding that the N-BOC group could be removed faster than MEM. The reaction was carried out in DCM and TFA under N_2 at 0°C (its course being monitored by ^{1}H-NMR analysis) and afforded α-aminoketone **34** as the trifluoroacetate salt.



i: HOBt,WSC, THF, RT, N_2; **ii:** NaI, TMSCl**,** acetonitrile, -20°C ; **iii**: TFA, acetonitrile, reflux; **iv**: CH2I2, DCM, reflux; **v**: $AlCl_3$, DCM, N_2.

Scheme 4.15

According to the synthetic strategy reported in scheme 4.15, the building blocks previously obtained were subjected to a coupling reaction in the presence of HOBt and WSC in THF at RT, to give amide **32** in good yield. The key step to obtain β-carbolin-1-one scaffold **44** is the same discussed in scheme 4.8 but in this case it was not possible to follow the procedure described there, because in the acid cyclization conditions the MEM protecting group was not stable, liberating formaldehyde which attacked the indole moiety. To avoid this side reaction other conditions were investigated, and it was found that a convenient reagent was trimethylsilyl iodide (TMSI). Compound **32** was then reacted with 2 eq of TMSCl and 2 eq of NaI in dry acetonitrile at -20°C, affording a mixture of **44** and of the open-chain analogue **5**. and the two products were separated, and compound **5** was converted into compound **44** using the *intramolecular Friedel-Crafts cyclization method* as described in scheme …….. Compound **45** was obtained using the *ring closure method* reported in scheme 4.10, by reacting **44** with diiodomethane in DCM under basic conditions. In the final step of the synthetic route all the isopropyl and benzyl ether groups were removed using $AlCl_3$ in DCM at RT giving the valuable phenol derivative **3**.
Selected compounds were tested for their antiproliferative activity on human non-small lung cancer cells H-460 (1 h exposure), using LamD as a reference compound (IC_{50} = μM). The test results are reported in Table 4.2

Compound	**IC_{50}**
LamD	0,066 μM
3	0,2 μM
4a	>10 μM
4b	5 μM
4c	>10 μM

23a	>10 μM
23b	>10 μM
23c	>10 μM
24b	>10 μM
29	>10 μM
30	>10 μM
31	>10 μM

Tab. 4.2

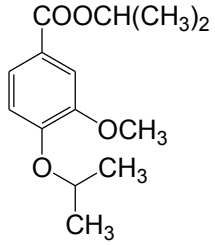
It was found that the planned lamellarin analogue **3** gave the highest citotoxicity profile of the series synthesized. This result means that a planar scaffold is important for enzyme binding mode. Moreover, coparing the activity of compoud **3** and compound **31** we can conclude that the presence of a methoxy-hydroxy alternation is fundamental for the cytotoxic activity. Results obtained are promising for a future development of new Lam D analogues. Further biological studies such as the evaluation of topoisomerase I-mediated DNA cleavage persistence in the presence of compound **3** are currently under evaluation.

4.3

Experimental methods

^{1}HNMR spectra were recorded in $CDCl_3$ solutions (where not otherwise stated) at room temperature on a Bruker AMX-300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts are reported as δ values in parts per million (ppm), and are indirectly referenced to tetramethylsilane (TMS) via the solvent signal (7.26 for ^{1}H, 77.0 for ^{13}C) in $CDCl_3$. Coupling constants (*J*) are given in Hz. Solvents were routinely distilled prior to use; anhydrous tetrahydrofuran (THF) and ether (Et_2O) were obtained by distillation from sodium-benzophenone ketyl; dry methylene chloride (CH_2Cl_2) and toluene were obtained by distillation from $CaCl_2$. All reactions requiring anhydrous conditions were performed under a positive nitrogen flow, and glassware was oven-dried. Isolation and purification of the compounds were performed by flash column chromatography on silica gel 60 (230-400 mesh); when necessary deactivated silica gel was used. Analytical thin-layer chromatography (TLC) was conducted on Fluka TLC plates (silica gel 60 F_{254}, aluminium foil), and spots were visualized by UV light and/or by means of dyeing reagents. Melting points were determined on a Stuart Scientific SMP3 instrument and are uncorrected.

15. 4-Isopropoxy-3-methoxy-benzoic acid isopropyl ester

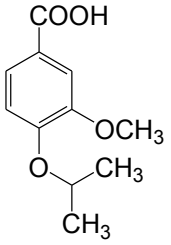


$C_{14}H_{20}O_4$
Mol. Wt.: 252,31

Vanillic acid **14** (12 g, 0.071 mol) was dissolved in DMF (70 mL) under nitrogen atmosphere and K_2CO_3 (24.53 g, 0.177 mol) was added. The suspension was stirred for 15 at room temperature and subsequently 2-bromopropane (35.11 g, 0.285 mol, 26 mL) was added dropwise. After refluxing for 4 h, the suspension was filtered and the solvent was evaporated under reduced pressure. The crude product was dissolved in ethyl acetate (80 mL) and the solution was washed with $NaHCO_3$ (3 x 20 mL) and brine (3 x 20 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure affording compound **15** as a yellow oil (17.65 g, 98%).

^{1}H NMR ($CDCl_3$): 7.62 (1H, dd, J= 1.7-8.4 Hz), 7.53 (1H, d, J= 1.7 Hz), 6.87 (1H, d, J= 8.4 Hz), 5.21 (1H, m), 4.62 (1H, m), 3.89 (3H, s), 1.38 (6H, d, J= 6.10 Hz), 1.34 (6H, d, J= 6. 10 Hz)

9. 4-Isopropoxy-3-methoxy-benzoic acid

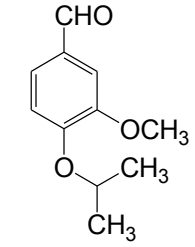


$C_{11}H_{14}O_4$
Mol. Wt.: 210,23

Compound **15** (17.60 g, 0.07 mol) was dissolved in 90% ethanol (100 mL) and KOH (5.89 g, 0.10 mol) was added. The solution was refluxed for 4 h. The solvent was evaporated under reduced pressure and the crude product was dissolved in water adding HCl solution until complete precipitation occurred (pH= 3). The suspension was extracted with ethyl acetate, the organic layer was dried over Na_2CO_3, filtered and evaporated under reduced pressure giving compound **9** (12.70 g, 87%) as a white solid. M.p.: 120°C

^{1}H NMR ($CDCl_3$): 7.73 (1H, dd, J= 2.1-8.9 Hz), 7.59 (1H, d, J= 2.1 Hz), 6.91 (1H, d, J= 8.9 Hz), 4.66 (1H, m), 3.91 (3H, s), 1.41 (6H, d, J= 6.10 Hz)

17. 4-isopropoxy-3-methoxybenzaldehyde

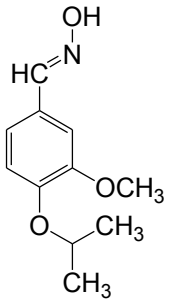


$C_{11}H_{14}O_3$
Mol. Wt.: 194,23

Vanillin **16** (14 g, 0.026 mol) was dissolved in DMF (20 mL) under nitrogen atmosphere and K_2CO_3 (4.13 g, 0.039 mol) was added. The suspension was stirred for 15 min.? at room temperature and subsequently 2-bromopropane (6.40 g, 0.052 mol, 4.88 mL) was added dropwise. After refluxing for 4 h, the suspension was filtered and the solvent was evaporated under reduced pressure. The crude product was dissolved in ethyl acetate (20 mL) and the solution was washed with brine (3 x 15 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure affording compound **15** as yellow solid (4.07 g, 81%), m.p.115°C.

^{1}H NMR ($CDCl_3$): 9.84 (1H, s), 7.36-7.45 (2H, m), 6.97 (1H, d, J= 8.2 Hz), 4.70 (1H, m), 3.92 (3H, s), 1.43 (6H, d, J= 5.9 Hz)

18. 4-isopropoxy-3-methoxybenzoxime

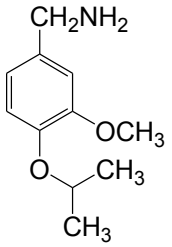


$C_{11}H_{15}NO_3$
Mol. Wt.: 209,24

A solution of **17** (4 g, 0.021 mol) in 95% ethanol (44 mL) was treated with a solution of hydroxylamine hydrochloride (1.82 g, 0.026 mol) in dry pyridine (8.13 mL) under stirring at room temperature for 90 min and at 0°C for 30 min to facilitate the oxime precipitation. The white solid was filtered, dried, (3.20 g, 71%) and used without further purification. 130 °C

^{1}H NMR ($CDCl_3$): 8.09 (1H, s), 7.23 (1H, d, J= 1.9 Hz), 7.03 (1H, dd, J= 1.9-8.2 Hz), 6.89 (1H, d, J=8.2 Hz), 4.60 (1H, m), 3.89 (3H, s,), 1.40 (6H, d, J= 6. 10 Hz)

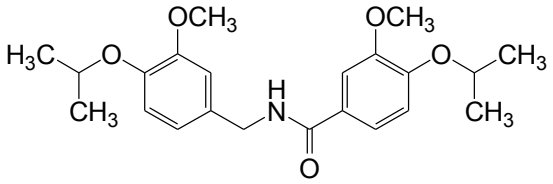
10. (4-isopropoxy-3-methoxyphenyl)methanamine



$C_{11}H_{17}NO_2$
Mol. Wt.: 195,26

A solution of **18** (3.15 g, 0.015 mol) in 95% ethanol (38.5 mL) was treated with an equal volume of 2M NaOH followed by Al/Ni alloy (4 g) under stirring at room temperature for 90 min. The Al/Ni alloy was removed by filtration and washed with fresh ethanol. Filtrate and washings were combined, acidified with 0.8 M HCl (pH=2) and extracted with DCM (3 X 15 mL). The aqueous phase was treated with solid KOH up to pH=12 and extracted with diethyl ether (3 X 10 mL). The extracts after drying over anhydrous Na_2SO_4, removal of the solvent afforded **10** (2.88 g, 98%) as a brown oil.

8. N-(4-isopropoxy-3-methoxybenzyl)-4-isopropoxy-3-methoxybenzamide

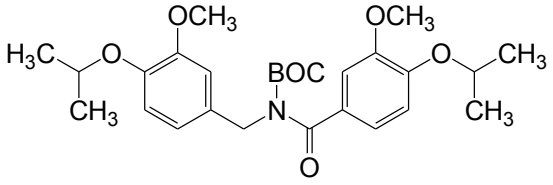


$C_{22}H_{29}NO_5$
Mol. Wt.: 387,47

To a suspension of HOBt (1.89 g, 0.014 mol) and WSC (2.68 g, 0.014 mol) in THF (110 mL) were added under nitrogen atmosphere **9** (1.46 g, 0.007 mol) and **10** (2.70 g, 0.014 mol). The reaction mixture was stirred at room temperature for 24 h and the solvent was evaporated under reduced pressure. The crude product was dissolved in ethyl acetate, the solution washed with $NaHCO_3$, HCl 2M and brine, dried over Na_2SO_4 and evaporated under reduced pressure to afford **8** as a yellow oil (2,60 g, 96%).

^{1}H NMR ($CDCl_3$): 7.47 (1H, s), 7.28 (1H, s), 6.81-6.93 (4H, m), 6.37 (1H, brs), 4.45-4.70 (4H, m), 3.91 (3H, s), 3.85 (3H, s), 1.40 (6H, d, J= 6.2 Hz), 1.37 (6H, d, J= 6.3 Hz).

19. N,N-BOC-(4-isopropoxy-3-methoxybenzyl)-4-isopropoxy-3-methoxybenzamide

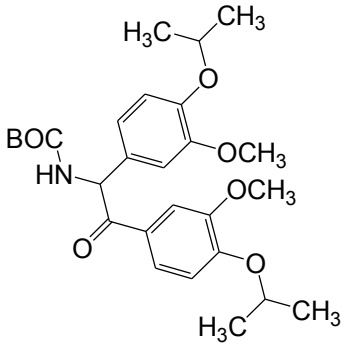


$C_{107}H_{146}N_4O_{28}$
Mol. Wt.: 1936,31

Compound **8** (2.20 g, 0.06 mol) was dissolved in acetonitrile (23 mL) under nitrogen flow and DMAP (0.073 g, 0.0006 mol) and di-terbutyldicarbonate (2.62 g, 0.012 mol) were added. After stirring the solution at RT for 16 h, the solvent was evaporated under reduced pressure and the crude product was dissolved in DCM. The solution was washed with saturated aqueous $NaHCO_3$ and the organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography (hexane: ethyl acetate, 6:4) affording **19** (2.60 g, 88%) as a yellow oil.

^{1}H NMR ($CDCl_3$):7.60 (1H, dd, 1.7-8.8 Hz), 7.53 (1H, d, 1.7 Hz), 6.77-6.94 (4H, m), 4.63 (1H, m), 4.48 (1H, m), 3.87 (3H, s), 3.83 (3H, s), 3.62 (1H, d, 9.9 Hz), 1.45 (9H, s), 1.35 (12H, d, 6.5 Hz).

20. tert-butyl 1,2-bis(4-isopropoxy-3-methoxyphenyl)-2-oxoethylcarbamate

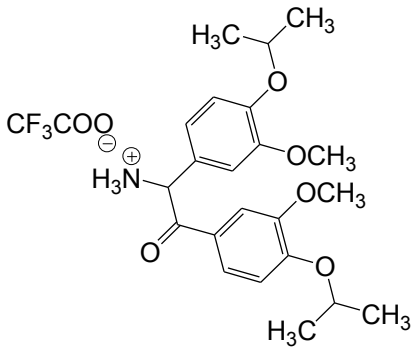


$C_{107}H_{146}N_4O_{28}$
Mol. Wt.: 1936,31

LDA was previously prepared by mixing diisopropylamine (1.52 g, 0.015 mol, 2.10 mL) and DMPU (1.05 mL) with n-BuLi (1.59 M in hexane, 5.55 mL, 0.015 mol) in THF (14 mL) at -78°C under nitrogen atmosphere. A solution of the imide **19** (2.40 g, 0.0049 mol) in THF freshly distilled (43 mL) was added to the LDA solution at -78°C and the mixture was stirred for ?? h. The mixture was quenched with aqueous NH_4Cl, diluted with ethyl acetate, washed with saturated aqueous NH_4Cl and brine, and dried over Na_2SO_4. Concentration in vacuo followed by flash chromatography (hexane: ethyl acetate, 7:3) gave product **20** (1.21 g, 55%) as a white solid. .Mp 120°C;

^{1}H NMR: 7.60(1H, dd, J= 1.7, 8.8 Hz), 7.53(1H, d, J= 1.7 Hz), 6.77-6.94(4H,m), 4.63(1H,m), 4.48(1H,m), 3.87(3H,s), 3.83(3H,s), 3.62(1H, d, J= 9.9 Hz), 1.45(9H, s), 1.35(12H, d, J= 6,5 Hz)

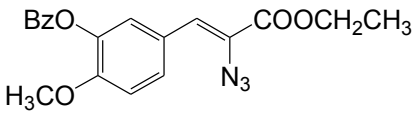
7. 2-amino-1,2-bis(4-isopropoxy-3-methoxyphenyl)ethanone (trifluoroacetate salt)



$C_{24}H_{30}F_3NO_7$
Mol. Wt.: 501,49

Imide **20** (1.21 g, 2.28 mmol) was dissolved in dry DCM (4mL) under nitrogen flow and TFA (2 mL) was added at 0°C. The solution was stirred at RT for 2h. The solvent was evaporated under vacuo and the crude product was used for the next step without purification.

12b. (Z)-ethyl 2-azido-3-(3-(benzyloxy)-4-methoxyphenyl) acrylate

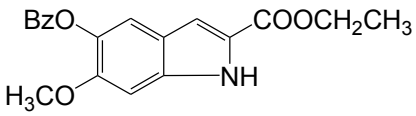


$C_{19}H_{19}N_3O_4$
Mol. Wt.: 353,37

Na (0.377 g, 0.016 mol) was dissolved in anhydrous EtOH (15mL), treated with 3-benzyloxy-4-methoxybenzaldheyde (1 g, 0.004 mol), and the mixture was cooled to -15°C. Ethyl azidoacetate (ethanol solution 25%, 0.016 mol, 1044 mL) was added dropwise to the reaction mixture. The mixture was stirred for 3 h at -15°C, then warmed to room temperature and treated with saturated aqueous NH_4Cl. The beige solid that formed was collected by filtration and washed with water to afford the title compound **13b** (1.20 g, 88%), m.p. 94-95 °C

^{1}H NMR ($CDCl_3$): 7.25-7.50 (7H, m), 6.85-6.95 (2H, m), 5.21 (2H, s), 4.38 (2H, q, 7.1 Hz), 3.95 (3H, s), 1.39 (3H, t, 7.1 Hz).

21b. ethyl 5-(benzyloxy)-6-methoxy-1H-indole-2-carboxylate

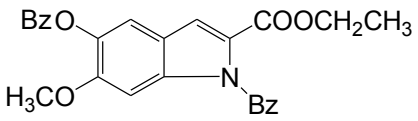


$C_{19}H_{19}NO_4$
Mol. Wt.: 325,36

Compound **12b** (1.15 g, 0.003 mol) was dissolved in xylenes (5 mL) and the solution was warmed at 130°C for 12 h. The solution was cooled to room temperature and hexane was added (5mL). The beige solid was collected by filtration. The filtrated was concentrated in vacuo and purified by flash chromatography (hexane:ethyl acetate, 8:2). The precipitate was combined with the product isolated by chromatography to afford **21b** (0.800 g, 76%), m.p. 177-178 °C.

^{1}H NMR ($CDCl_3$): 8.69(1H,s), 7.30-7.58 (6H, m), 7.12 (1H, m), 6.88 (1H, s), 5.25 (2H, s), 4.40 (2H,s), 4.40 (2H, q, 7.02 Hz), 3.95 (3H, s), 1.40 (3h, t, 7.07 Hz).

22b. ethyl 1-benzyl-5-(benzyloxy)-6-methoxy-1H-indole-2-carboxylate

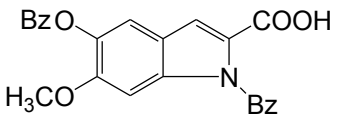


$C_{26}H_{25}NO_4$
Mol. Wt.: 415,48

Indole **21b** (0.740 g, 0.002 mol) was dissolved in dry DMF (7 mL) under nitrogen. The solution was cooled at 0°C and NaH (oil mineral susp. 60 %, 0.170 g, 0.003 mol) was added slowly. The reaction mixture was warmed to room temperature and stirred for 45 min. Then benzyl bromide (0.003 mol, 0.34 mL) was added and the mixture was reacted for 2 h. Reaction was completed and the solvent was removed under vacuo. The crude product was dissolved in ethyl acetate and washed with saturated aqueous brine. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure to afford **22b** (0.900 g, 97 %) as a white solid, m.p. 90-91 °C.

^{1}H NMR ($CDCl_3$): 7.20-7.48 (9H, m), 7.12 (1H, s), 7.02 (2H, m), 6.79 (1H, m), 6.79 (1H, s), 5.72 (2H, s), 5.18 (2H, s), 4.32 (2H, q, 7.1 Hz), 3.95 (3H, s), 1.36 (3H, t, 7.1 Hz).

11b. 1-benzyl-5-(benzyloxy)-6-methoxy-1H-indole-2-carboxylic acid

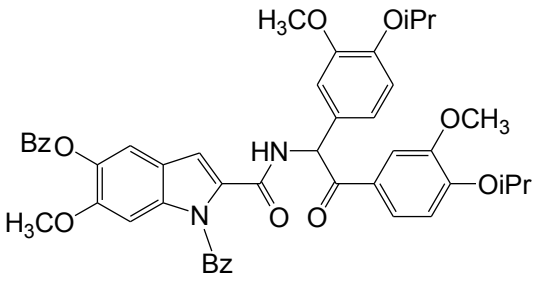


$C_{24}H_{21}NO_4$
Mol. Wt.: 387,43

Compound **22b** (0.890 g, 0.002 mol) was dissolved in 3:2:1 THF-MeOH-H_2O (5 mL), treated with LiOH-H_2O (0.359 g, 0.009 mol), and the mixture was stirred for 4 h at 25 °C. 1N aqueous HCl was added until pH less than 6, and the solution was extracted with ethyl acetate (3 x 10 mL). The combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo to afford **11b** (0.600 g, 72%). M.p: 130 °C

^{1}H NMR ($CDCl_3$): 7.17- 7.48 (9H, m), 7.10 (1H, s), 6.94-7.06 (2H, m), 6.75 (1H, s), 5.71 (2H, s), 5.15 (2H, s), 3.94 (3H, s)

6b. N-(1,2-bis(4-isopropoxy-3-methoxyphenyl)-2-oxoethyl)-1-benzyl-5-(benzyloxy)-6-methoxy-1H-indole-2-carboxamide

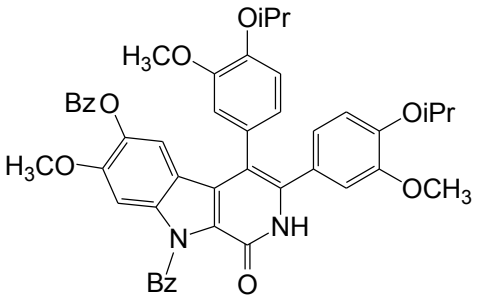


$C_{46}H_{48}N_2O_8$
Mol. Wt.: 756,88

To a suspension of HOBt (0.335 g, 0.003 mol) and WSC (0.474 g, 0.003 mol) in THF (20 mL) under nitrogen atmosphere **11b** (0.481 g, 0.001 mol) was added. The reaction mixture was stirred at room temperature for 12 h until the activated ester was formed. Then compound **7** dissolved in THF (5 mL) and TEA (0.002 mol, 0.346 mL) were added. After stirring the reaction mixture at room temperature for 14 h, the solvent was evaporated under reduced pressure. The crude product was dissolved in ethyl acetate, the solution washed with aqueous $NaHCO_3$, HCl 2M and brine, dried over Na_2SO_4, and evaporated under reduced pressure to afford **6b** as a yellow solid (0.520 g, 56%). Mp: 127 °C

^{1}H NMR ($CDCl_3$): 7.53-7.69 (3H, m), 7.23-7.44 (4H, m), 7.11-7.18 (3H, m), 7.07 (1H, s), 6.92-7.01 (5H, m), 6.77-6.87 (2H, m), 6.74 (1H,s), 6.61 (1H, d, J= 7.4 Hz), 5.74 (1H, ab, J= 16.2 Hz), 5.61 (1H, ab, J= 16.2 Hz), 5.12 (2H, s), 4.65 (1H, m), 4.49 (1H, m), 3.93 (3H, s), 3.88 (3H, s), 3.81 (3H, s), 1.40 (6H, d, J= 6.10 Hz), 1.34 (6H, d, J= 6.10 Hz).

23b 9-benzyl-6-(benzyloxy)-3,4-bis(4-isopropoxy-3-methoxyphenyl) -7-methoxy-2H-pyrido[3,4-b]indol-1(9H)-one

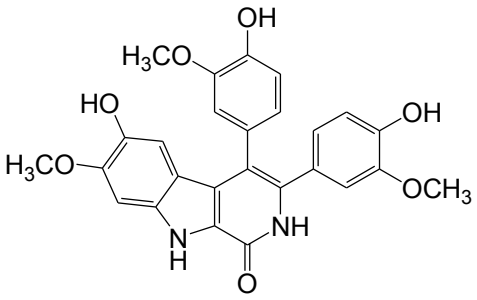


$C_{46}H_{46}N_2O_7$
Mol. Wt.: 738,87

To a solution of **6b** (0.500 g, 0.7 mmol) in acetonitrile (5 mL) was added TFA (0.9 mmol, 0,07 mL) and the reaction mixture was refluxed for 15'. The formed precipitate was filtered and washed with acetonitrile to afford **23b** as a white solid (0.340 g, 70%), Mp: 230 °C

^{1}H NMR ($CDCl_3$): 7.11-7.51 (11H, m), 6.65- 7.04 (7H, m), 6.43 (1H, s), 5.96 (2H, m), 5.17 (2H, s), 4.56 (1H, m), 4.44 (1H, m), 3.70 (3H, s), 3.57 (3H, s), 3.55 (3H, s), 1.40 (6H, d, J= 5.7 Hz), 1.35 (6H, d, J= 5.7 Hz)

4b. 6-hydroxy-3,4-bis(4-hydroxy-3-methoxyphenyl)-7-methoxy-2H-pyrido[3,4-b]indol-1(9H)-one

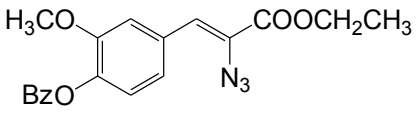


$C_{26}H_{22}N_2O_7$
Mol. Wt.: 474,46

Anhydrous $AlCl_3$ (0.094 g, 0.7 mmol) was suspended in dry DCM (4 mL) under nitrogen and compound **6b** (0.100g, 0.1 mmol) was added. After stirring the reaction mixture at room temperature for 24 h, saturated aqueous NH_4Cl was added and the mixture was extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The crude solid was crystallized from ethanol to give product **4b** (0.003 g, 48 %), m.p. > 350 °C.

^{1}H NMR ($CDCl_3$): 11.53 (1H, s), 11.15 (1H, s), 9.25 (1H, s), 9.08 (1H, s), 9.00 (1H, s), 6.60-6.95 (7H, m), 6.22 (1H, s), 3.65 (3H, s), 3.60 (3H, s), 2.45 (3H, s).

12a. (Z)-ethyl 2-azido-3-(4-(benzyloxy)-3-methoxyphenyl) acrylate

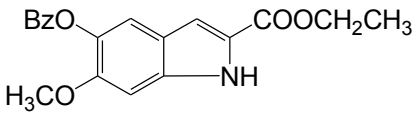


$C_{19}H_{19}N_3O_4$
Mol. Wt.: 353,37

Na (1.13 g, 0.049 mol) was dissolved in anhydrous EtOH (45mL), treated with 4-benzyloxy-3-methoxybenzaldheyde (3 g, 0.012 mol), and the mixture was cooled to -15°C. Ethyl azidoacetate (ethanol solution 25%, 0.049 mol, 25 mL) was added dropwise to the reaction mixture. The mixture was stirred for 3 h at -15°C, then warmed to room temperature and treated with saturated aqueous NH_4Cl. The beige solid that formed was collected by filtration and washed with water to afford the title ^1H compound **12b** (2.95 g, 73%), m.p. 102-103 °C .

^1H NMR ($CDCl_3$): 7.53 (1H, d, 1.9 Hz), 7.26-7.50 (6H, m), 6.85-6.93 (2H, m), 5.22 (2H, s), 4.38 (2H, q, 7.25 Hz), 3.95 (3H, s), 1.41 (3H, t, 7.25 Hz).

21a. ethyl 6-(benzyloxy)-5-methoxy-1H-indole-2-carboxylate

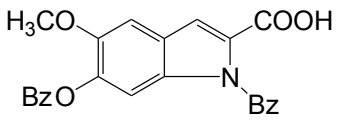


$C_{19}H_{19}NO_4$
Mol. Wt.: 325,36

Compound **12b** (2.90 g, 0.008 mol) was dissolved in xylenes (20 mL) and the solution was warmed at 130°C for 12 h. The solution was cooled to room temperature and hexane was added (5mL). The beige solid was collected by filtration. The filtrated was concentrated in vacuo and purified by flash chromatography (hexane:ethyl acetate, 8:2). The precipitate was combined with the product isolated by chromatography to afford **21a** (0.847 g, 32%), m.p. 220 °C.

^{1}H NMR ($CDCl_3$): 8.63 (1H, brs), 7.23-7.50 (5H, m), 7.01-7.12 (2H, m), 6.83 (1H, s), 5.20 (2H, s), 4.35 (2H, q, J= 7.1 Hz), 3.92 (3H, s), 1.38 (3H, t, J= 7.1 Hz)

22a. 1-benzyl-6-(benzyloxy)-5-methoxy-1H-indole-2-carboxylic acid

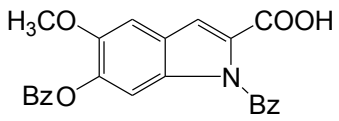


$C_{24}H_{21}NO_4$
Mol. Wt.: 387,43

Indole **21b** (0.840 g, 0.003 mol) was dissolved in dry DMF (8 mL) under nitrogen. The solution was cooled at 0°C and NaH (mineral oil susp. 60 %, 0.146 g, 0.004 mol) was added slowly. The reaction mixture was warmed to room temperature and stirred for 45 min. Then benzyl bromide (0.004 mol, 0.43 mL) was added and the mixture was reacted for 2 h. When the reaction was completed the solvent was removed under vacuo. The crude product was dissolved in ethyl acetate and washed with brine. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure to afford white solid **22a** (0.730 g, 59 %), m.p. 190-191 °C.

^{1}H NMR ($CDCl_3$): 7.11-7.47 (9H, m), 7.07 (1H, s), 6.91-7.00 (2H, m), 6.74 (1H, s), 5.69 (2H, s), 5.11 (2H, s), 4.29 (2H, q, J= 7.1 Hz), 3.91 (3H, s), 1.33 (3H, t, J= 7.1 Hz)

11a. 1-benzyl-6-(benzyloxy)-5-methoxy-1H-indole-2-carboxylic acid

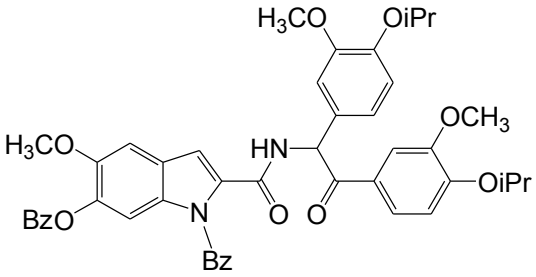


$C_{24}H_{21}NO_4$
Mol. Wt.: 387,43

Compound **22a** (0.725 g, 0.002 mol) was dissolved in 3:2:1 THF-MeOH- H_2O (6 mL), treated with LiOH-H_2O (0.292 g, 0.008 mol), and the mixture was stirred for 4 h at 25 °C. 1N aqueous HCl was added until pH was less than 6, and the solution was extracted with ethyl acetate (3 x 10 mL). The combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo to afford **11a** (0. 500 g, 64 %). M.p. 200 °C

^{1}H NMR ($CDCl_3$): 7.13-7.43 (9H, m), 7.07 (1H, s), 6.91-6.99 (2H, m), 6.72 (1H, s), 5.68 (2H, s), 5. 12 (2H, s), 3.91 (3H s)

6a. N-(1,2-bis(4-isopropoxy-3-methoxyphenyl)-2-oxoethyl)-1-benzyl-6-(benzyloxy)-5-methoxy-1H-indole-2-carboxamide

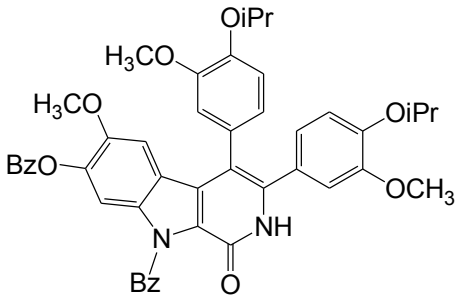


$C_{46}H_{48}N_2O_8$
Mol. Wt.: 756,88

To a suspension of HOBt (0.739 g, 0.005 mol) and WSC (1.04 g, 0.005 mol) in THF (44 mL) under nitrogen atmosphere **11a** (2.68 g, 0.005 mol) was added. The reaction mixture was stirred at room temperature for 12 h until the activated ester was formed. Then compound 7 dissolved in THF (10 mL) and TEA (0.005 mol, 0,76 mL) were added. After stirring the reaction mixture at room temperature for 14 h, the solvent was evaporated under reduced pressure. The crude product was dissolved in ethyl acetate, the solution washed with aqueous $NaHCO_3$, HCl 2M and brine, dried over Na_2SO_4 and evaporated under reduced pressure to afford **6a** as a yellow solid (1.13 g, 55%), m.p. 140-141 °C.

^{1}H NMR ($CDCl_3$): 7.50- 7.68 (3H, m), 7.21-7.43 (5H, m), 7.09-7.18 (3H, m), 7.06 (1H, s), 6.88-7.00 (5H, m), 6.76-6.87 (2H, m), 6.73 (1H, s), 6.59 (1H, d, J= 7.8 Hz), 5.72 (1H, ab, J= 16.1 Hz), 5.59 (1H, ab, J= 16.1 Hz), 5.10 (2H, s), 4.62 (1H, m), 4.49 (1H, m), 3.91 (3H, s), 3.86 (3H, s), 3.79 (3H, s), 1.38 (6H, d, J= 6.1 Hz), 1.32 (6H, d, J= 6.1 Hz)

23a. 9-benzyl-7-(benzyloxy)-3,4-bis(4-isopropoxy-3-methoxy phenyl)-6-methoxy-2H-pyrido[3,4-b]indol-1(9H)-one

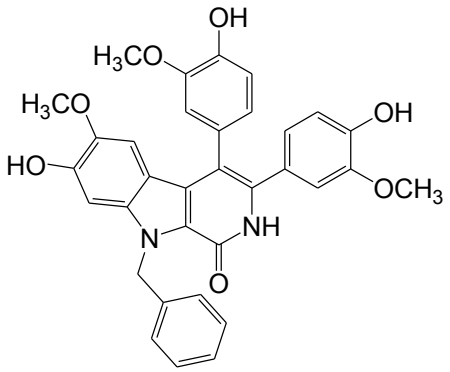


$C_{46}H_{46}N_2O_7$
Mol. Wt.: 738,87

To a solution of **6a** (1.10 g, 0.002 mol) in acetonitrile (8 mL) was added TFA (0.16 mL) and the reaction mixture was refluxed for 15'. The precipitate formed was filtered and washed with acetonitrile to afford **23a** as a white solid (0.764 g, 71%), Mp: 238 °C

^{1}H NMR (DMSO): 7.05-7.51 (12H, m), 6.63-7.03 (7H, m), 6.04 (2H, m), 5.16 (2H, s), 4.36-4.60 (2H, m), 3.60 (3H, s), 3.57 (3H, s), 3.37 (3H, s), 1.25 (6H, d, J= 5.5 Hz), 1.21 (6H, d, J= 5.6 Hz)

24a. 9-benzyl-7-hydroxy-3,4-bis(4-hydroxy-3-methoxyphenyl)-6-methoxy-2H-pyrido[3,4-b]indol-1(9H)-one

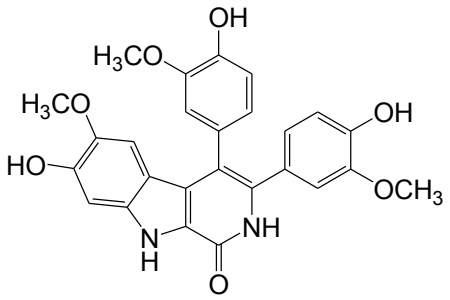


$C_{33}H_{28}N_2O_7$
Mol. Wt.: 564,58

Anhydrous $AlCl_3$ (0.281 g, 0.4 mmol) was suspended in dry DCM (5 mL) under nitrogen and compound **23a** was added. After stirring the reaction mixture at room temperature for 24 h, saturated aqueous NH_4Cl was added and the mixture was extracted with ethyl acetate. The organic layer was washed with brine , dried over Na_2SO_4 and evaporated under reduced pressure. The crude solid afforded was crystallized in ethanol to give product **24a** (0,026 g, 11%). M.p. 297 °C.

^{1}H NMR (DMSO): 11.25 (1H, s), 9.30 (1H, s), 9.07 (1H, s), 9.02 (1H, s), 7.15-7.35 (5H, m), 6.56-6.93 (7H, m), 6.13 (1H, s), 6.02 (2H, m), 3.64 (3H, s), 3.60 (3H, s), 3.39 (3H, s)

4a. 7-hydroxy-3,4-bis(4-hydroxy-3-methoxyphenyl)-6-methoxy-2H-pyrido[3,4-b]indol-1(9H)-one

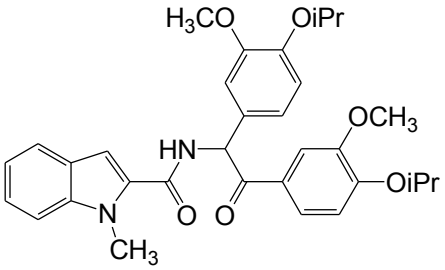


$C_{26}H_{22}N_2O_7$
Mol. Wt.: 474,46

Anhydrous $AlCl_3$ (0.188 g, 0.001 mol) was suspended in anisole (10 mL) under nitrogen and compound **23a** (0.200 g, 0.3 mmol) was added. After refluxing the reaction mixture at 100°C for 5 h, MeOH was added and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (DCM-MeOH, 9:1) affording product **4a** as a white solid (8 mg, 5%). M.p 315 °C.

^{1}H NMR (DMSO): 11.55 (1H, s), 11.13 (1H, s), 9.25 (1H, s), 9.05 (1H, s), 8.99 (1H, s), 6.58-6.91 (7H, m), 6.22 (1H, s), 3.62 (3H, s), 3.59 (3H, s), 3.42 (3H, s)

6c. N-(1,2-bis(4-isopropoxy-3-methoxyphenyl)-2-oxoethyl)-1-methyl-1H-indole-2-carboxamide

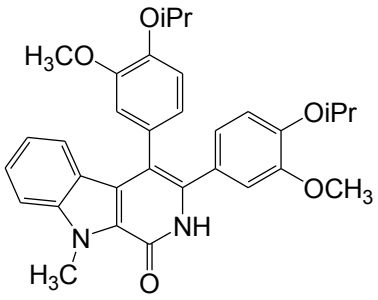


$C_{32}H_{36}N_2O_6$
Mol. Wt.: 544,64

To a suspension of HOBt (0.988 g, 0.007 mol) and WSC (1,39 g, 0.007 mol) in THF (60 mL) under nitrogen atmosphere **11c** (0.540 g, 0.004 mol) was added. The reaction mixture was stirred at room temperature for 12 h until the activated ester was formed. Then compound **7** dissolved in THF (10 mL) and TEA (0.007 mol, 1 mL) was added. After stirring the reaction mixture at room temperature for 14 h, the solvent was evaporated under reduced pressure. The crude product was dissolved in ethyl acetate, the solution washed with aqueous $NaHCO_3$, HCl 2M and brine, dried over Na_2SO_4 and evaporated under reduced pressure to afford **6c** as a white solid (0.978 g, 49 %), Mp 111 °C

^{1}H NMR (DMSO): 8.98 (1H, d, J= 7.07 Hz), 7.76 (1H, d, J= 8.2 Hz), 7.40-7.67 (3H,m), 7.23-7.33 (2H, m), 7.19 (1H, s), 6.80-7.15 (4H, m), 6.64 (1H, d, J= 7.07 Hz), 4.70 (1H, m), 4.50 (1H, m), 3.96 (3H, s), 3.79 (3H, s), 3.75 (3H, s), 1.27 (6H, d, J= 6.3 Hz), 1.22 (6H, d, J= 6.3 hz).

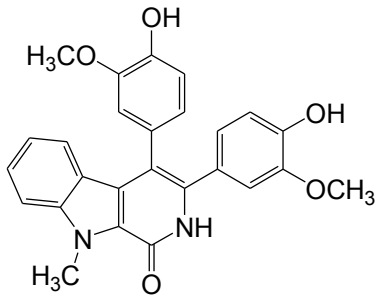
23c. 3,4-bis(4-isopropoxy-3-methoxyphenyl)-9-methyl-2H-pyrido[3,4-b]indol-1(9H)-one



$C_{32}H_{34}N_2O_5$
Mol. Wt.: 526,62

To a solution of **6c** (0.970 g, 0.002 mol) in acetonitrile (10 mL) was added TFA (0.2 mL) and the reaction mixture was refluxed for 15'. The precipitate formed was filtered and washed with acetonitrile to afford **23c** as a white solid (0.556 g, 58%), m.p. 287 °C.

4c. 3,4-bis(4-hydroxy-3-methoxyphenyl)-9-methyl-2H-pyrido[3,4-b]indol-1(9H)-one

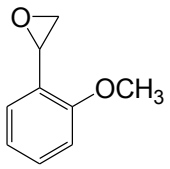


$C_{26}H_{22}N_2O_5$
Mol. Wt.: 442,46

Anhydrous $AlCl_3$ (0.197 g, 0.001 mol) was suspended in dry DCM (5 mL) under nitrogen and compound **23c** (0.300 g, 0.6 mmol) was added. After stirring the reaction mixture at room temperature for 6 h, saturated aqueous NH_4Cl was added and the mixture was extracted with ethyl acetate. The organic layer was washed with brine , dried over Na_2SO_4 and evaporated under reduced pressure. The crude solid afforded was crystallized from ethanol to give product **4c** (0.08 g, 18%), m.p. 315 °C.

^{1}H NMR (DMSO): 11.40 (1H, brs), 11.32 (1H, brs), 9.05 (1H, brs), 6.53-6.99 (10 H, m), 4.28 (3H, s), 3.60 (3H, s), 3.33 (3H, s).

32. 2-(2-methoxyphenyl) oxiarane

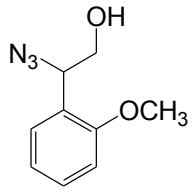


$C_9H_{10}O_2$
Mol. Wt.: 150,17

To an iced-cooled suspension of NaH (1.67 g, 70 mmol) in dry DMSO (30 mL) under nitrogen flow, trimethylsulfoxonium iodide (12.76 g, 58 mmol) was added gradually and the reaction mixture was stirred for 1 h. Then a solution of 2-methoxybenzaldehyde (8 g, 59 mmol) in dry DMSO (10 mL) was added dropwise. The cooling bath was removed and the reaction mixture allowed to warm to room temperature. After stirring for 3 h, water was added and the product extracted with ethyl acetate. the organic layer was washed several times with NaCl solution, dried over Na_2SO_4 , filtered over a silica pad and the solvent evaporated under reduced pressure to give **32** (8.50g, 97%) as a yellow oil.

^{1}H NMR (DMSO): 6.93 (1H, m), 6.36-6.65 (3H, m), 3.64 (1H, m), 3.37 (3H, s), 2.64 (1H, m), 2.25 (1H, dd, J= 2.8-5.5 Hz)

33. 2-azido-2-(2-methoxyphenyl) ethanol

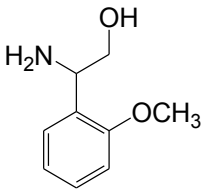


$C_9H_{11}N_3O_2$
Mol. Wt.: 193,2

To a solution of **32** (6 g, 40 mmol) in acetonitrile (20 mL) NaN_3 (5.20 g, 80 mmol) and $LiClO_4$ (8.51 g, 80mmol) were added. The reaction mixture was heated at 60°C . After being stirred for 2 h, the solvent was removed under reduced pressure and ethyl acetate was added. The solution was washed with water and NaCl, dried over Na_2SO_4, evaporated and purified by flash chromatography (hexane: ethyl acetate, 8:2) to afford **35** (2.96 g, 36%) as a yellow oil.

^{1}H NMR ($CDCl_3$): 7.39-7.56 (2H, m), 7.04-7.25 (2H, m), 5.32 (1H, m), 4.01 (3H, s), 3.78-4.01 (3H, m)

25. 2-amino-2-(2-methoxyphenyl) ethanol

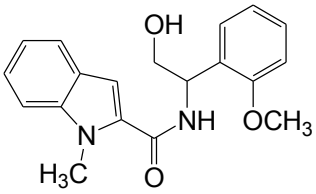


$C_9H_{13}NO_2$
Mol. Wt.: 167,21

To a solution of compound **33** (2.66 g, 16 mmol) in ethanol (30 mL) 10% Pd/C catalyst (0.327 g) was added at room temperature. The reaction mixture was stirred under hydrogen atmosphere for 24 h, the catalyst filtered and the solvent evaporated. Ethyl acetate was added to the crude product and the solution washed with water and NaCl, dried over Na_2SO_4 and evaporated under reduced pressure to afford compound **25** (1.98 g, 74%) as a colorless oil.

^{1}H NMR (DMSO): 7.42 (1H, d, J= 7.2 Hz), 7.19 (1H, m), 6.84-6.99 (2H, m), 4.70 (1H, brs), 4.20 (1H, dd, J= 3.4- 7.8 Hz), 3.77 (3H, s), 3.50 (1H, dd, J= 3.4-9.9 Hz), 3.14 (1H, m)

27. N-(2-hydroxy-1-(2-methoxyphenyl)ethyl)-1-methyl-1H-indole-2-carboxamide

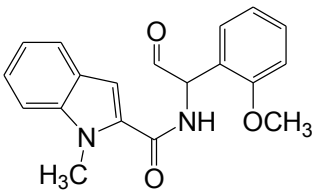


$C_{19}H_{20}N_2O_3$
Mol. Wt.: 324,37

To a suspension of HOBt (3.24 g, 24 mmol) and WSC (4.60 g, 24 mmol) in THF (40 mL) under nitrogen atmosphere, **25** (1.90 g, 0.012 mol) and **11c** (1.29 g, 0.012 mol) were added The reaction mixture was stirred at room temperature for 24 h and the solvent was evaporated under reduced pressure. The crude product was dissolved in ethyl acetate, the solution washed with $NaHCO_3$, HCl 2M and brine, dried over Na_2SO_4 and evaporated under reduced pressure to afford **7** as a white solid (2.33 g, 94%). M.p 95 °C.

^{1}H NMR ($CDCl_3$): 7.64 (1H, d, J= 8.3), 7.21- 7.43 (5H, m), 7.15 (1H, m), 6.29-7.03 (2H, m), 6.90 (1H, s), 5.51 (1H, m), 4.05 (3H, s), 3.93-4.03 (2H, m), 3.93 (3H, s)

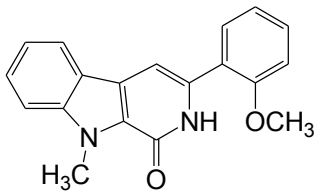
28. Preparation of N-(formyl(2-methoxyphenyl)ethyl)-1-methyl-1H-indole-2-carboxamide.



$C_{19}H_{18}N_2O_3$
Mol. Wt.: 322,36

To a solution of IBX (1.55 g, 5.6 mmol) in dry DMSO (10 mL) **7** (0.907 g, 2.8 mmol) was added under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 3 h, treated with ethyl acetate, washed with $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, and evaporated under reduced pressure to afford **28** as a yellow oil (0.900 g), used for the next step without further purification.

29. 3-(2-methoxyphenyl)-9-methyl-2H-pyrido[3,4,b]indol-1(9H)-one.

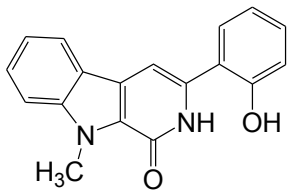


$C_{19}H_{16}N_2O_2$
Mol. Wt.: 304,34

To a solution of **28** (0.900 g) in acetonitrile (10 mL) was added TFA (1 mL) and the reaction mixture was refluxed for 15'. The precipitate formed was filtered and washed with acetonitrile to afford 2**9** as a white solid (0.540 g). Mp 250 °C

^{1}H NMR (DMSO): 7.88-8.12 (6H, m), 7.63-7.86 (4H, m), 3.90 (3H, s), 3.31 (3H, s), 2.52 (3H, s).

30. 3-(2-hydroxyphenyl)-9-methyl-2H-pyrido[3,4,b]indol-1(9H)-one.

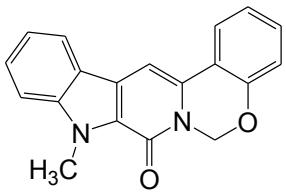


$C_{18}H_{14}N_2O_2$
Mol. Wt.: 290,32

To an ice-cooled suspension of **29** (0.540 g, 1.8 mmol) in dry dichloromethane (13 mL) 1 M BBr_3 in dichloromethane (1.33 g, 5.3 mmol, 5.3 mL) was added dropwise under nitrogen atmosphere. The reaction mixture was allowed to warm to room temperature, stirred overnight, quenched with aqueous HCl and extracted with dichloromethane. The extract was washed with brine, dried over Na_2SO_4, concentrated under reduced pressure and purified by flash chromatography (dichloromethane: methanol, 9.5:0.5) to afford **30** (0.200g, 38 %) as a yellow solid.Mp 270 °C

^{1}H NMR (DMSO): 7.88-8.12 (6H, m), 7.63-7.86 (4H, m), 3.31 (3H, s), 2.52 (3H, s).

31. 8-Methyl-8H-5-oxa-6a,8-diaza-indeno[2,1-b phen-anthren -7-one

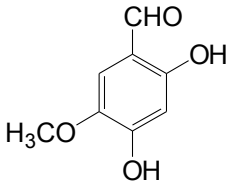


$C_{19}H_{14}N_2O_2$
Mol. Wt.: 302,33

A suspension of **30** (0.04 g, 0.14 mmol) and K_2CO_3 (0.05 g, 0.34 mmol) in DMF (4mL) was stirred for 30' at room temperature and treated with iodomethane (0.150 g, 0.56 mmol). After being refluxed for 1h, the solvent was evaporated and the crude product was dissolved in dichloromethane, washed with brine, dried over Na_2SO_4 and purified by flash chromatography (dichloromethane: methanol, 9,5:0,5) to afford **31** as a white solid (12 mg, 28%). M.p 280 °C.

^{1}H NMR (DMSO): 8.02- 8.19 (2H, m), 7.81 (1H, s), 7.65 (1H, d, J= 8.36 Hz), 7.52 (1H, m), 7.38 (1H, m), 7.18 -7.32 (2H, m), 7.13 (1H, d, J= 8.07 Hz), 5.92 (2H, S), 4.23 (3H, S).

38. 2,4-Dihydroxy-5-methoxy-benzaldehyde

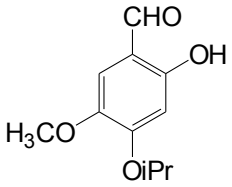


$C_8H_8O_4$
Mol. Wt.: 168,15

To a stirred suspension of $AlCl_3$ (68 g, 0.51 mol) in dry DCM (450 mL), a solution of 2,4,5-trimethoxybenzaldehyde **37** (25 g, 0.127 mol) in dry DCM (125 mL) was added dropwise. After stirring for 4 h at room temperature, another portion of $AlCl_3$ (68 g, 0.51 mol) was added. The suspension was further stirred for 19 h and the reaction mixture was poured into 1 Kg of ice to which 45 mL of concentrated hydrochloric acid weres added. The organic layer was separated and the aqueous phase was extracted twice with DCM (200 mL). The combined organic layers were filtered over silica gel, dried over Na_2SO_4, evaporated and the residue crystallized from ethyl acetate to give compound **38** (17.95 g, 84 %), m.p. 150 °C.

^{1}H NMR ($CDCl_3$): 11.95 (1H, s), 9.71 (1H, s), 7.29 (1H, s), 6.90 (1H, s), 6.55 (1H, s), 3.92 (3H, s).

39. 2-Hydroxy-4-isopropoxy-5-methoxy-benzaldehyde

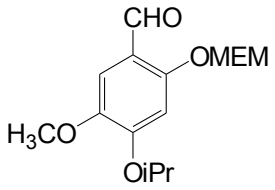


$C_{11}H_{14}O_4$
Mol. Wt.: 210,23

2,4-dihydroxy-5-methoxyxybenzaldehyde **38** (35.18 g, 0.209 mol) was dissolved in dry DMF (345 mL). $NaHCO_3$ (26.29 g, 0.313 mol) and 2-bromopropane (0.313 mol, 29.46 mL) were added under nitrogen flow. The reaction mixture was heated at 70 °C and stirred. After 4 h, the suspension was filtered and the organic layer was evaporated. To the crude product was added ethyl acetate and the suspension was filtered. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography (hexane:ethyl acetate, 1:1) affording a yellow oil (15 g, 34 %).

^{1}H NMR ($CDCl_3$): 11.36 (1H, s), 9.66 (1H, s), 6.89 (1H, s), 6.44 (1H, s), 4.63 (1H, s), 1.42 (6H, d, J= 5.9 Hz).

40. 4-Isopropoxy-5-methoxy-2-(2-methoxy-ethoxymethoxy)-benzaldehyde

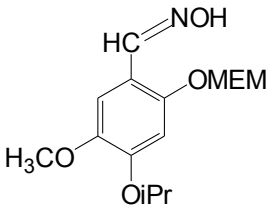


$C_{15}H_{22}O_6$
Mol. Wt.: 298,33

To a suspension of K_2CO_3 (29.16 g, 0.211 mol) in dry DMF (150 mL) was added compound **39** (14.80 g, 0.07 mol). After stirring for 15 min, MEMCl (26.63 g, 0.211 mol) was added slowly and reaction mixture was stirred for 12 h. The solvent was removed under reduced pressure and the crude product was dissolved in ethyl acetate, washed with brine, dried over Na_2SO_4, filtered and evaporated in vacuo, affording a yellow oil (19.70 g, 94 %).

^{1}H NMR ($CDCl_3$): 10.29 (1H, s), 7.29 (1H, s), 6.82 (1H, s), 5.31 (2H, s), 4.65 (1H, m), 3.80-3.90 (5H, m), 3.53-3.60 (2H, m), 3.36 (3H, s), 1.40 (6H, d, J= 6.1)

41. 4-Isopropoxy-5-methoxy-2-(2-methoxy-ethoxymethoxy)-benzaldehyde oxime

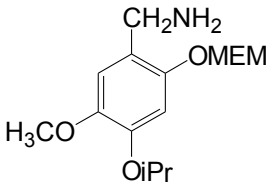


$C_{15}H_{23}NO_6$
Mol. Wt.: 313,35

A solution of **40** (17.70 g, 0.066 mol) in 95% ethanol (400 mL) was treated with a solution of hydroxylamine hydrochloride (5.73 g, 0,083 mol) in dry pyridine (40 mL) under stirring at room temperature for 90 min and at 0°C for 30 min to facilitate the oxime precipitation. The white solid was filtered, dried, and used (18.48 g, 89%) without further purification.

^{1}H NMR ($CDCl_3$): 8.94 (1H, brs), 8.40 (1H, s), 7.19 (1H, s), 6.80 (1H, s), 5.20 (2H, s), 4.55 (1H, m), 3.76-3.85 (2H, s), 3.35 (3H, s), 1.35 (6H, d, J= 6.10 Hz)

36. 4-Isopropoxy-5-methoxy-2-(2-methoxy-ethoxymethoxy)-benzylamine

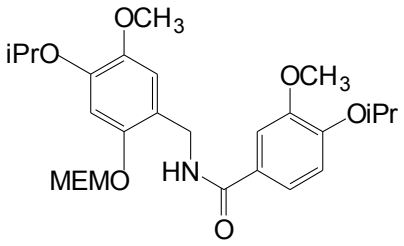


$C_{15}H_{25}NO_5$
Mol. Wt.: 299,36

A solution of **41** (18.40 g, 0.059 mol) in 95% ethanol (154 mL) was treated with an equal volume of 2M NaOH followed by Al/Ni alloy (15.97 g) under stirring at room temperature for 90 min. The Al/Ni alloy was removed by filtration and washed with fresh ethanol. Filtrate and washings were combined, acidified with 0,8 M HCl (pH=2) and extracted with DCM (3 X 50 mL). The aqueous phase was treated with solid KOH up to pH=14 and extracted with diethyl ether (3 X 30 mL). The extracts after drying over anhydrous Na_2SO_4 and removal of the solvent afforded **36** (17 g, 97%) as a brown oil.

^{1}H NMR ($CDCl_3$): 6.80 (1H, s), 6.77 (1H, s), 5.20 (2H, s), 4.46 (1H, m), 3.71-3.83 (7 H, m), 3.50- 3.58 (2H, m), 3.38 (3H, s), 1.31 (6H, d, J= 6.10 Hz)

35. 4-Isopropoxy-N-[4-isopropoxy-5-methoxy-2-(2-methoxy-ethoxymethoxy)-benzyl]-3-methoxy-benzamide

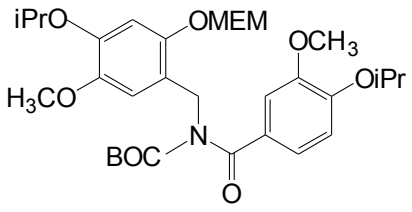


$C_{26}H_{37}NO_8$
Mol. Wt.: 491,57

To a suspension of HOBt (6.16 g, 0.046 mol) and WSC (8.79 g, 0.046 mol) in THF (250 mL) under nitrogen atmosphere, **9** (7.9 g, 0.04 mol) and **36** (16.90 g, 0.06 mol) were added. Reaction mixture was stirred at room temperature for 24 h and solvent was evaporated under reduced pressure. The crude product was dissolved in ethyl acetate, the solution washed with $NaHCO_3$, HCl 2M and brine, dried over Na_2SO_4 and evaporated under reduced pressure to afford **35** as a yellow oil (17.70 g, 94%).

^{1}H NMR ($CDCl_3$): 7.42 (1H, s), 7.21 (1H, dd, J= 1.7-8.4 Hz), 6.75-6.93 (3H, m), 6.65 (1H, t, J= 5.7), 5.24 (2H, s), 4.40-4.65 (4H, m), 3.88 (3H, s), 3.79-3.85 (2H, m), 3.80 (3H, s), 3.50-3.56 (2H, m), 3.30 (3H, s), 1.36 (6H, d, J= 6.5 Hz), 1.34 (6H, d, J= 6.5 Hz)

42. (4-Isopropoxy-3-methoxy-benzoyl)-[4-isopropoxy-5-methoxy-2-(2-methoxy-ethoxymethoxy)-benzyl]-carbamic acid tert-butyl ester

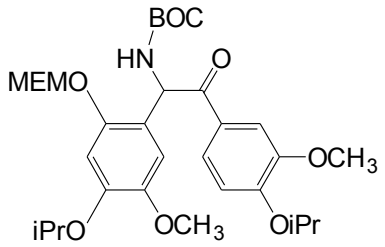


$C_{31}H_{45}NO_{10}$
Mol. Wt.: 591,69

Compound **35** (17.65 g, 0.04 mol) was dissolved in acetonitrile (150 mL) under nitrogen flow and DMAP (2.99 g, 0.024 mol) and di-terbutyldicarbonate (2.62 g, 0.02 mol) were added. After stirring the solution at RT for 16 h, the solvent was evaporated under reduced pressure and the crude product was dissolved in DCM. The solution was washed with saturated aqueous $NaHCO_3$ and the organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography (hexane:ethyl acetate, 6:4) affording **42** (13.95 g, 67 %) as a yellow oil.

^{1}H NMR ($CDCl_3$): 7.05-7.17 (2H, m), 6.88 (1H, s), 6.78-6.85 (2H, m), 5.10 (2H, s), 4.89 (2H, s), 4.57 (1H, m), 4.47 (1H, m), 3.81 (3H, s), 3.76 (3H, s), 3.70-3.74 (2H, m), 3.46-3.52 (2H, m), 3.33 (3H, s), 1.35 (6H,6H, d, J= 6.1 Hz), 1.31 (6H, d, J= 6.1 Hz), 1.15 (9H, s)

43 .1-[4-Isopropoxy-5-methoxy-2-(2-methoxy-etxy-methoxy)-phenyl]-2-(4-isopropoxy-3-methoxy-phenyl)-2-oxo-ethyl]-carbamic acid tert-butyl ester

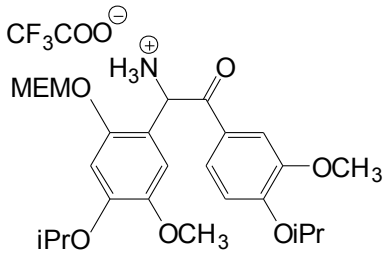


$C_{31}H_{45}NO_{10}$
Mol. Wt.: 591,69

LDA was previously prepared by mixing diisopropylamine (7.29 g, 0.07 mol, 2.10 mL) and DMPU (10.9 mL) with n-BuLi (2.7 M in heptane, 26.67 mL, 0.07 mol) in THF (70 mL) at -78°C under nitrogen atmosphere.A solution of the imide **42** (13.90 g, 0.024 mol) in freshly distilled THF (209 mL) was added to the LDA solution at -78°C and the mixture was stirred for ????? h. The mixture was quenched with aqueous NH_4Cl, diluted with ethyl acetate, washed with saturated aqueous NH_4Cl and brine, and dried over Na_2SO_4. Concentration in vacuo followed by flash chromatography (hexane:ethyl acetate, 7:3) gave product **43** (4.11 g, 29 %) as a yellow oil.

^{1}H NMR ($CDCl_3$):7.69 (1H, d, J= 8.2 Hz), 7.57 (1H, s), 6.68-6.85 (3H, m), 6.49 (1H, d, J= 7.8 Hz), 5.85 (1H, d, J= 7.8 Hz), 5.27 (2H, m), 4.62 (1H, m), 4.49 (1H, m), 3.74-3.95 (2H, m), 3.86 (3H, s), 3.77 (3H, s), 3.55-3.65 (2H, m), 3.38 (3H, s), 1.44 (9H, s), 1.28-1.40 (12 H, m).

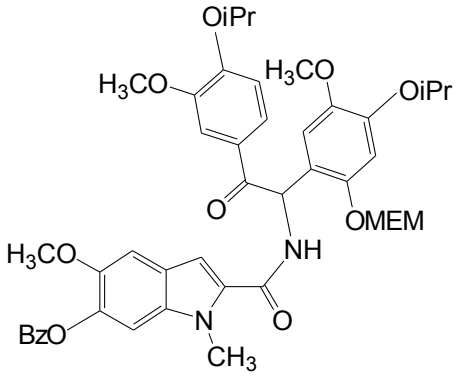
34. 1-[4-Isopropoxy-5-methoxy-2-(2-methoxy-ethoxy methoxy)-phenyl]-2-(4-isopropoxy-3-methoxy-phenyl)-2-oxo-ethyl-ammonium; trifluoro-acetate



$C_{28}H_{38}F_3NO_{10}$
Mol. Wt.: 605,60

Imide **43** (1.50 g, 2.5 mmol) was dissolved in dry DCM (30 mL) under nitrogen flow and TFA (3 mL) was added at 0°C. The solution was stirred at RT for 2h. Solvent was evaporated under vacuo and the crude product was used for the next step without purification.

32. 6-Benzyloxy-5-methoxy-1-methyl-1H-indole-2-carboxylicacid [1-[4-isopropoxy-5-methoxy-2-(2-methoxy ethoxymethoxy)-phenyl]-2-(4-isopropoxy-3-methoxyphenyl) -2-oxo-ethyl]-amide

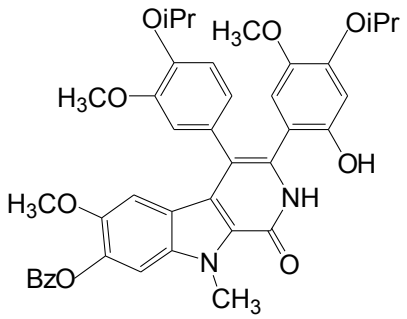


$C_{44}H_{52}N_2O_{11}$
Mol. Wt.: 784,89

To a suspension of HOBt (0.459 g, 0.003 mol) and WSC (0.650 g, 0.003 mol) in THF (30 mL) was added under nitrogen atmosphere **33** (0.519 g, 0.002 mol). The reaction mixture was stirred at room temperature for 12 h until the activated ester was formed. Then compound **34** dissolved in THF (10 mL) and TEA (0,004 mol, 0,64 mL) were added. After stirring the reaction mixture at room temperature for 14 h, the solvent was evaporated under reduced pressure. The crude product was dissolved in ethyl acetate, the solution washed with aqueous $NaHCO_3$, HCl 2M and brine, dried over Na_2SO_4 and evaporated under reduced pressure to afford **32** as a yellow solid (0.454 g, 55%), m.p. 134 °C.

^{1}H NMR (DMSO): 8.79 (1H, d, J= 7.5 Hz), 7.67 (1H, d, J= 8.6 Hz), 7.46-7.58 (3H, m), 7.32-7.46 (7.03-7.25 (4H, m), 6.83-6.98 (3H, m), 5.30 (2H, m), 5.30 (2H, m), 5.15 (2H, s), 4.69 (1H, s), 4.52 (1H, s), 3.90 (3H, s), 3.78 (6H, s), 3.67 (3H, s), 3.17 (3H, s), 1.20-1.29 (12H, m).

5. 7-Benzyloxy-3-(2-hydroxy-4-isopropoxy-5-methoxy-phenyl)-4-(4-isopropoxy-3-methoxy-phenyl)-6-methoxy-9-methyl-2,9-dihydro-b-carbolin-1-one

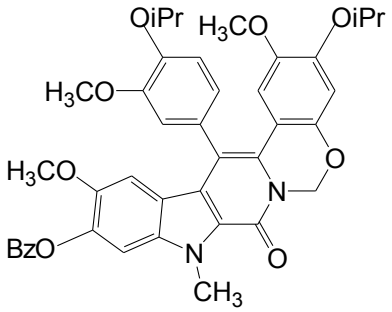


$C_{40}H_{42}N_2O_8$
Mol. Wt.: 678,77

TMSCl (0.67 mmol, 0.08 mL) and NaI (0.100 g, 0.67 mmol) were added to a solution of **32** (0.450 g, 0.67 mmol) in acetonitrile (40 mL) at –20 °C under nitrogen flow. After stirring at this temperature for 15 min, an additional equivalent of both NaI and TMSCl was added and the mixture stirred at –20 °C until no starting material remained (TLC analysis). The yellow-orange mixture was quenched with methanol and the solvent removed in vacuo. The residue was extracted with ethyl acetate and the organic layer washed with saturated sodium thiosulfate and brine. The crude product was purified by flash chromatography (hexane:ethyl acetate, 7:3) affording **5** (0.140 g, 31%) as a white solid, m.p.: 220 °C.

^{1}H NMR (DMSO): 11.00 (1H, s), 9.09 (1H, s), 7.19-7.56 (7H, m), 6.67-7.00 (3H,m), 6.54 (1H, s), 6.41 (1H, s), 6.27 (1H, s), 5.19 (2H, s), 4.50 (1H, s), 4.38 (1H, s), 4.24 (3H, s), 3.58 (3H, s), 3.43 (3H, s), 3.39 (3H, s), 1.22 (12 H, m)

45.10-Benzyloxy-3-isopropoxy-13-(4-isopropoxy-3-methoxy-phenyl)-2,11-dimethoxy-8-methyl-8H-5-oxa-6a,8-diaza-indeno[2,1-b]phenanthren-7-one

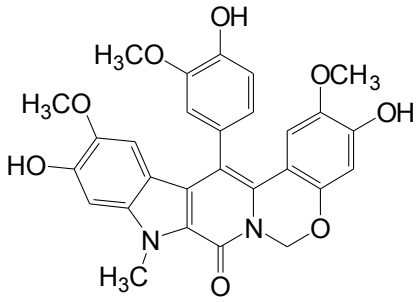


$C_{41}H_{42}N_2O_8$
Mol. Wt.: 690,78

A suspension of **5** (0.132 g, 0.2 mmol) and K_2CO_3 (0.072 g, 52 mmol) in DMF (6 mL) was stirred for 30' at room temperature and treated with iodomethane (0.7 mL, 82 mmol). After being heated at 100 °C for 1h, the solvent was evaporated under reduced pressure and the crude product was dissolved in dichloromethane, washed with brine, dried over Na_2SO_4 and purified by flash chromatography (petroleum ether-ethyl acetate, 1:1) to afford **45** as a white solid (14 mg, 12 %).

^{1}H NMR (DMSO): 7.17-7.58 (5H, m), 6.74-7.17 (4H, m), 6.46-6.72 (2H, m), 5.82-6.14 (3H, m), 5.23 (2H, s), 4.42-4.72 (2H, m), 4.24 (3H, s), 3.78 (3H, s), 3. 52 (3H, s), 3.26 (3H, s), 1.07-1.48 (12H, m)

3. 3,10-Dihydroxy-13-(4-hydroxy-3-methoxy-phenyl)-2,11-dimethoxy-8-methyl-8H-5-oxa-6a,8-diaza-indeno[2,1-b]phenanthren-7-one



$C_{28}H_{24}N_2O_8$
Mol. Wt.: 516,50

Anhydrous $AlCl_3$ (15.5 mg, 0.116 mmol) was suspended in dry DCM (5 mL) under nitrogen and compound **23c** (10 mg, 0.014 mmol) was added. After refluxing the reaction mixture for 6 h, saturated aqueous NH_4Cl was added and the mixture was extracted with ethyl acetate. The organic layer was washed with brine , dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by TLC preparative to give **3** (6 mg, 83 %), m.p. 290 °C.

^{1}H NMR (DMSO): 7.01 (1H, d, J= 8.2 Hz), 6.94 (1H, d, 1.7 Hz), 6.88 (1H, s), 6.76 (1H, dd, J=8.22-1.7 Hz), 6.63 (1H, s), 6.45 (1H, s), 5.87 (1H, ab, J=9.24 Hz), 4.11 (3H, s), 3.69 (3H, s), 3.16 93H, s)

4.4

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