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Systematic Exploration of the Structural Features of Yatakemycin Impacting DNA Alkylation and Biological Activity

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Abstract

A systematic examination of the impact of the yatakemycin left and right subunits and their substituents is detailed along with a study of its unique three subunit arrangement (sandwiched vs extended and reversed analogues). The examination of the ca. 50 analogues prepared illustrate that within the yatakemycin three subunit structure, the subunit substituents are relatively unimportant and that it is the unique sandwiched arrangement that substantially increases the rate and optimizes the efficiency of its DNA alkylation reaction. This potentiates the cytotoxic activity of yatakemycin and its analogues overcoming limitations typically observed with more traditional compounds in the series (CC-1065, duocarmycins). Moreover, a study of the placement of the alkylation subunit within the three subunit arrangement (sandwiched vs extended and reversed analogues) indicates that it has a profound impact not only on the rate and efficiency of DNA alkylation, but that it controls and establishes the DNA alkylation selectivity as well, where both enantiomers of such sandwiched agents alkylate the same adenine sites exhibiting the same DNA alkylation selectivity independent of their absolute configuration.

Introduction

Yatakemycin (**1**)¹ is the newest and most potent (IC_{50} , L1210 = 3–5 pM) member of a family of antitumor antibiotics that includes CC-1065 (**2**, IC_{50} = 20 pM),² duocarmycin A (**3**, IC_{50} = 200 pM),³ and duocarmycin SA (**4**, IC_{50} = 10 pM, Figure 1).⁴ Each derives its properties through a characteristic sequence-selective alkylation of duplex DNA.^{5–9} The yatakemycin structure is a remarkable hybrid of the preceding natural products, incorporating a central DNA alkylation subunit identical to that of duocarmycin SA, a left subunit identical to the DNA-binding subunits found in CC-1065, and a right subunit similar to that found in the duocarmycins. Distinct from the preceding agents, yatakemycin is the first naturally occurring member possessing a DNA binding subunit flanking each side of the alkylation subunit in a characteristic “sandwiched” arrangement. Recently, we disclosed first and second generation total syntheses^{10–13} of yatakemycin that served to reassign its structure as **1**¹⁰ and reported preliminary evaluations of (+)- and *ent*-(−)-yatakemycin and key partial structures.¹¹

Prior to the discovery of yatakemycin, we had utilized the C-terminus methyl ester of duocarmycin SA as a site for attachment of a second DNA binding subunit providing the first, albeit limited, series of such sandwiched analogues that were found to exhibit potent cytotoxic activity, an enhanced DNA alkylation rate, and a uniquely altered alkylation selectivity.¹⁴ The subsequent isolation of a naturally occurring member of this class has focused a more intense interest in such sandwiched compounds and now provides the incentive to more thoroughly

evaluate this unusual structural arrangement. Herein, we report efforts to systematically define the relationships between structure, reactivity, and biological activity of such sandwiched agents.

Extensive efforts have defined the structural features of the duocarmycins that contribute to their remarkable rate, selectivity, and efficiency of DNA alkylation.⁹ In these studies, the right-hand DNA binding subunit was identified as one that not only provides noncovalent DNA binding affinity and selectivity, but is also responsible for catalysis of the DNA alkylation reaction (Figure 2).^{14,15} The alkylation subunit cyclopropane is stabilized by the cross-conjugated delocalization of the N² lone pair via a vinylogous amide. Upon binding in the minor groove, the compound is forced to adopt a twisted conformation to accommodate the helical pitch of DNA, which disrupts the vinylogous amide conjugation and activates the cyclopropane for nucleophilic attack. Through use of this DNA binding-induced activation (shape-dependent catalysis), the compound avoids reaction with competitive nucleophiles until being selectively activated by and at its biological target.¹⁶

Results and Discussion

Synthesis of Analogues with Modifications in the Right Subunit

Studies conducted on duocarmycin SA have indicated that only the C5-methoxy substituent of the DNA binding subunit has a pronounced effect on the rate and efficiency of DNA alkylation, and the biological potency of the compounds.^{9,14} These effects are independent of the substituent electronic properties and could be attributed simply to its presence and the resulting increase in rigid length of the agent.¹⁷ This rigid extension increases the extent of the DNA binding-induced conformational change, increasing the degree of vinylogous amide disruption and enhancing the rate of DNA alkylation. Consequently, a series of key yatakemycin analogues were synthesized to establish the impact of its right subunit substituents in such sandwiched structures.

In route to these key analogues and for comparison purposes, the partial structures **9a–9f** were first prepared and lack the left subunit of yatakemycin (Scheme 1). As such, they represent not only key partial structures of yatakemycin, but also additional key analogues of duocarmycin SA itself. Boc deprotection of each enantiomer of **6**¹¹ (only natural enantiomer shown, 4 N HCl–EtOAc, 70 °C, 1 h), followed by coupling with the corresponding indole-2-carboxylic acid (**5a–f**, 4 equiv of EDCI, 3 equiv of NaHCO₃, DMF, 25 °C, 14 h, 62–78%) gave **7**. Benzyl ether deprotection of **7** (1 atm H₂, 10% Pd/C, 9:1 THF–MeOH, 2 h, 80–95%) provided **8**, and subsequent spirocyclization (sat. aq. NaHCO₃ in 2:1 DMF–H₂O, 25 °C, 1 h, 64–89%) provided (+)- and *ent*-(−)-**9a–f**.

The synthesis of sandwiched analogues **14b–f** was accomplished as shown in Scheme 2. Methyl ester hydrolysis of **7** (only natural enantiomer shown, 4 equiv of LiOH, 3:2:1 THF–MeOH–H₂O, 25 °C, 14 h, 78–94%) followed by benzyl ether deprotection of **11** (1 atm H₂, 10% Pd/C, 9:1 THF–MeOH, 2 h, 84–100%) gave **12**. Coupling of **12** with the hydrochloride salt of **10**¹¹ (4 equiv of EDCI, DMF, 25 °C, 16 h, 42–77%) furnished **13**, and subsequent spirocyclization (sat. aq. NaHCO₃ in 2:1 DMF–H₂O, 25 °C, 1 h, 63–98%) provided (+)- and *ent*-(−)-**14b–f**.

Synthesis of Analogues with Modifications in the Left Subunit

Yatakemycin is structurally unique among members of its class, possessing the additional left-hand DNA binding subunit. A series of left subunit analogues were prepared to establish its contribution to yatakemycin's properties. In previous studies, the CDPI¹⁸ and indole subunits (Scheme 3) were found to constitute unsubstituted and simplified replacements for the PDE

subunits found in CC-1065 that are readily accessible by chemical synthesis, maintain the full cytotoxic potency of the natural products and impart more efficacious *in vivo* activity.¹⁹ Thus, yatakemycin analogues incorporating these and related subunits were prepared for evaluation and constitute analogues in which the left-hand subunit substituents or key structural features are removed.

The synthesis of **16a–b** incorporating a pyrrolidine in place of the left subunit was accomplished as shown in Scheme 3. Coupling of carboxylic acids **12a** and **12c** with pyrrolidine hydrochloride (1.0 equiv, 4 equiv of EDCI, DMF, 25 °C, 16 h, 49–65%) gave **15a–b**, and subsequent spirocyclization (sat. NaHCO₃ in 2:1 DMF–H₂O, 25 °C, 1 h, 58–87%) provided (+)- and *ent*-(−)-**16a–b**.

The synthesis of yatakemycin analogues incorporating additional modifications in the left subunit was accomplished as shown in Schemes 4 and 5. Coupling of carboxylic acid **12a** with the CDPI-based amine hydrochlorides derived from **17**¹⁸ or **18** (1.0 equiv, 4 equiv of EDCI, DMF, 25 °C, 16 h, 37–41%) gave **19a–b**, and subsequent spirocyclization (sat. NaHCO₃ in 2:1 DMF–H₂O, 25 °C, 0.7 h, 66–91%) provided (+)- and *ent*-(−)-**20a–b**. Similarly, coupling of carboxylic acid **12f** with **21** (1.0 equiv, 4 equiv of EDCI, DMF, 25 °C, 24 h, 53%) gave **22**, and subsequent spirocyclization (sat. NaHCO₃ in 2:1 DMF–H₂O, 25 °C, 1 h, 93%) provided (+)- and *ent*-(−)-**23**.

Design and Synthesis of Extended, Sandwiched, and Reversed Analogues

Extended analogue **24**, sandwiched analogue **25**, and reversed analogue **26** were prepared to establish the importance of the agent structure and the location of the DNA binding subunits on the DNA alkylation selectivity (Figure 3). Each analogue in this series is composed of the alkylation subunit of duocarmycin SA and two PDE subunits attached in each of three possible arrangements. Extended analogue **24** contains the same DNA binding subunits organized in the same arrangement as found in CC-1065, but incorporates the more stable (4–5-fold) and more potent (4–5-fold) duocarmycin SA alkylation subunit.²⁰ As such, it would be expected to alkylate DNA with the same selectivity as CC-1065, but to be a more efficient DNA alkylating agent and a more potent cytotoxic compound.²¹ Moreover, the two enantiomers of **24**, like CC-1065, would be expected to exhibit characteristic and distinguishable alkylation selectivities constituting an adenine N3 alkylation at the terminus of a five base-pair AT-rich sequence albeit with opposite bound orientations and with that of the unnatural enantiomer offset by one base pair (i.e., nat. 5'-AAAAA vs unnat. 5'-AAAAA).^{6d} Sandwiched analogue **25** constitutes yatakemycin modified with incorporation of a PDE right-hand subunit. As such, the properties of **25** would be expected to be comparable to those of yatakemycin, both enantiomers of which exhibit identical alkylation selectivities (i.e., 5'-AAAAA)¹¹ and indistinguishable cytotoxic potencies. Reversed analogue **26** has two PDE subunits bound to the left of the alkylation subunit, and is capped on the right as its Boc derivative. Unlike the extended and sandwiched agents, the reversed agent lacks an extended heteroaromatic N² acyl substituent on the alkylation subunit to participate in a binding-induced activation of the alkylation reaction. Consequently, both its rate and efficiency of DNA alkylation would be expected to be diminished relative to **24** and **25**, resulting in a diminished cytotoxic potency. Just as significantly and because the DNA binding subunits extend in opposite directions relative to those found in the extended analogue **24**, they should exhibit a reversed enantiomeric alkylation selectivity if it is controlled by the noncovalent binding selectivity of the compounds. As such, the natural enantiomer of **26** would be expected to alkylate the same sites as the unnatural enantiomer of **24**, whereas its unnatural enantiomer should alkylate DNA with a selectivity indistinguishable from the natural enantiomer of **24**.¹⁴ Significantly, all three agents have comparable DNA-binding surfaces and the same inherent reactivity, thereby allowing a

direct comparison of the impact of the subunit arrangement on their DNA alkylation profile and biological properties.

The synthesis of extended analogue **24** was accomplished as shown in Scheme 6. Acid-catalyzed Boc deprotection of **27**¹¹ (4 N HCl–EtOAc, 25 °C, 3 h) followed by coupling with **28**²² (1.0 equiv, 4 equiv of EDCI, DMF, 25 °C, 22 h, 36%), and subsequent spirocyclization (sat. aq. NaHCO₃ in 2:1 DMF–H₂O, 25 °C, 0.5 h, 78%) provided (+)- and *ent*(−)-**24**. Sandwiched analogue **25** was prepared by the strategy outlined in Scheme 7. Boc deprotection of **6**¹¹ (4 N HCl–EtOAc, 25 °C, 3 h) followed by coupling with **30**^{11,22} (1.0 equiv, 4 equiv of EDCI, DMF, 25 °C, 14 h, 81%) provided **32**. Methyl ester hydrolysis (4 equiv of LiOH, 3:2:1 THF–MeOH–H₂O, 25 °C, 5 h, 96%) followed by benzyl ether deprotection of (1 atm H₂, 10% Pd/C, 9:1 THF–MeOH, 1 h, 93%) gave **34**. Coupling of **34** with the hydrochloride salt of **31**¹¹ (4 equiv of EDCI, DMF, 25 °C, 16 h, 69%) furnished **35**, and subsequent spirocyclization (sat. aq. NaHCO₃ in 2:1 DMF–H₂O, 25 °C, 1 h, 89%) provided (+)- and *ent*(−)-**25**. The reversed analogue **26** (Scheme 8) was prepared by Boc deprotection of **36**²² (4 N HCl–EtOAc, 25 °C, 0.5 h) followed by coupling with **37**^{14b} (1.0 equiv, 4 equiv of EDCI, DMF, 25 °C, 16 h, 67%), and subsequent spirocyclization (sat. aq. NaHCO₃ in 2:1 DMF–H₂O, 25 °C, 40 m, 68%) to provide (+)- and *ent*(−)-**26**. Partial structure **40** having the same two subunit arrangement as duocarmycin SA incorporating a single PDE subunit was also synthesized by benzyl ether deprotection of **32** (1 atm H₂, 10% Pd/C, 9:1 THF–MeOH, 2 h, 93%) followed by spirocyclization (sat. aq. NaHCO₃ in 2:1 DMF–H₂O, 25 °C, 1 h, 96%) to provide (+)- and *ent*(−)-**40** (Scheme 9).

Cytotoxic Activity

Summarized in Figure 4 is the cytotoxic activity of the series of right subunit analogues against a cell line (L1210 mouse leukemia) for which we have extensive comparisons for this class. Compound **14b** differs from yatakemycin only by switching the right subunit substituent locations, and exhibited cytotoxic potency that was indistinguishable from the natural product or its unnatural enantiomer. Analogue **14c** is a hybrid structure consisting of yatakemycin's three subunit sandwiched arrangement incorporating the 5,6,7-trimethoxyindole subunit found in the duocarmycins. Compound **14c** ($IC_{50} = 5$ pM) and *ent*(−)-**14c** ($IC_{50} = 5$ pM) were found to be potent cytotoxic agents, indistinguishable in the cellular assay and equipotent with (+)- and *ent*(−)-yatakemycin.¹¹ Both compounds, like yatakemycin, are 2-fold more potent than duocarmycin SA (**4**, $IC_{50} = 10$ pM) and 20-fold more potent than *ent*(−)-duocarmycin SA. The substantial increase in potency for the unnatural enantiomer relative to duocarmycin SA is a characteristic feature observed with the sandwiched agents. Presumably, the added left subunit contributes sufficient additional binding affinity²³ to compensate for the less effective DNA alkylation characteristic of the unnatural enantiomer of duocarmycins.

The contribution of each right subunit substituent was determined by evaluation of a series of key simplified analogues. Yatakemycin partial structure **9a** ($IC_{50} = 10$ pM) served as a valuable baseline comparison for identifying the contribution of the left subunit for each analogue. Both enantiomers of **14d** lacking only the C6-methoxy group were equipotent ($IC_{50} = 5$ pM) and indistinguishable from yatakemycin. In contrast, the corresponding partial structure **9d** ($IC_{50} = 30$ pM) was 6-fold less potent than yatakemycin and 3-fold less potent than **9a**, and its unnatural enantiomer ($IC_{50} = 600$ pM) was considerably less active. Similarly, **14e** lacking the C5-hydroxy group ($IC_{50} = 4$ pM; *ent*(−)-**14e**, $IC_{50} = 10$ pM) was essentially indistinguishable from yatakemycin, whereas the corresponding partial structure **9e** ($IC_{50} = 25$ pM) was 5-fold less potent than yatakemycin, 2–3 times less active than **9a**, and its unnatural enantiomer ($IC_{50} = 1300$ pm) was 260-fold less potent than the sandwiched structures.

A particularly interesting analogue was **14f**, incorporating a right subunit indole lacking additional substituents. The natural enantiomer was essentially equipotent with yatakemycin

($IC_{50} = 6$ pM) despite lacking a C5 substituent. Distinct from the preceding sandwiched analogues, the unnatural enantiomer of **14f** ($IC_{50} = 35$ pM) was 6-fold less potent than the natural enantiomer, and 7-fold less potent than the *ent*-(-)-yatakemycin. The corresponding partial structure **9f**^{14b} was found to be 10-fold less potent ($IC_{50} = 60$ pM) than **14f** and 6-fold less potent than **9a**, whereas the unnatural enantiomer *ent*-(-)-**9f** was nearly 50-fold less potent ($IC_{50} = 1700$ pM) than *ent*-(-)-**14f** and 4–5 fold less potent than *ent*-(-)-**9a**. Clear from these comparisons is the observation that the incorporation of a left subunit, which increases the DNA binding affinity of the sandwiched analogues, compensates for less effective right subunits, and substantially increases the cytotoxic potency of the unnatural enantiomers relative to the corresponding partial structures incorporating only the central and right-hand subunits. Only with the combination of a less effective right-hand subunit and the unnatural enantiomeric configuration in *ent*-(-)-**14f** was a detectable difference in cytotoxic activity observed between enantiomers. Even here, the difference in potency between the two enantiomers remains remarkably small (5-fold) relative to the typical partial structures incorporating only the central and right-hand subunits (10–40-fold). An immediate conclusion to these studies is that the right-hand subunit substituents significantly and predictably impact the cytotoxic activity of duocarmycin-like compounds (series **9**) especially improving the potency of the unnatural enantiomers,¹⁴ but they have a minor impact on the activity of yatakemycin.

Compound **40** is the most potent compound in the partial structure series ($IC_{50} = 5$ pM), being approximately 2-fold more potent than duocarmycin SA and equipotent with yatakemycin and **25** ($IC_{50} = 6$ pM). However, the unnatural enantiomer of **40** ($IC_{50} = 360$ pM) is still 60-fold less potent than the unnatural enantiomer of the corresponding sandwiched agent (*ent*-(-)-**25**, $IC_{50} = 6$ pM).

As shown in Figure 5, a separate series of compounds were evaluated to establish the impact of the left subunit. Its removal altogether providing **9a** afforded an analogue that was not distinguishable from duocarmycin SA and that was 2-fold (natural enantiomer) or 70-fold (unnatural enantiomer) less potent than yatakemycin and its unnatural enantiomer. The CDPI subunit lacking the methoxy and hydroxyl substituents was incorporated into analogue **20a** ($IC_{50} = 5$ pM, *ent*-(-)-**20a** $IC_{50} = 6$ pM) which was found to be equipotent with yatakemycin. The thiomethyl ester derivative of CDPI was similarly incorporated into analogue **20b** ($IC_{50} = 7$ pM, *ent*-(-)-**20b** $IC_{50} = 6$ pM) with no change in potency. Thus, like observations made with yatakemycin itself,¹¹ no unique effect was detected for the presence of the methyl thioester. To probe the impact of the pyrrolidine ring alone, **16a** was prepared which significantly reduces the size and length of the left subunit. The cytotoxic potency of the natural enantiomer ($IC_{50} = 7$ pM) was comparable to **1** and perhaps a bit more potent than duocarmycin SA and **9a**. The unnatural enantiomer was 10-fold less potent ($IC_{50} = 70$ pM), but notably 5-fold more potent than the corresponding methyl ester *ent*-(-)-**9a** ($IC_{50} = 400$ pM). Pyrrolidine analogue **16b** ($IC_{50} = 7$ pM; *ent*-(-)-**16b** $IC_{50} = 80$ pM) possessing a 5,6,7-trimethoxyindole right subunit was equipotent with **16a**. The small increase in agent length and rigidity provided by the pyrrolidine amide results in an increase in the potency of the unnatural enantiomer although a significant difference between each enantiomer's potency remains. The sandwiched analogue with two indole subunits **23** ($IC_{50} = 15$ pM) exhibited 3-fold reduced cytotoxic activity relative to yatakemycin or its indole analogue **14f**, but it was still nearly as potent as duocarmycin SA ($IC_{50} = 10$ pM). Nonetheless, the indole left subunit contributes sufficiently to exceed the potency of **9f** ($IC_{50} = 60$ pM). The unnatural enantiomer of **23** was approximately 20-fold less potent ($IC_{50} = 275$ pM) than the natural enantiomer, 50-fold less potent than yatakemycin and its enantiomer, and 8-fold less potent than the unnatural enantiomer of **14f**. Clearly, an optimal right subunit can compensate for substantial simplifications in the left subunit and this is especially true in the natural enantiomer series.

The cytotoxic activity of the extended, sandwiched, and reversed agents is summarized in Figure 6. The unnatural enantiomers of CC-1065²² and yatakemycin¹¹ exhibit cytotoxic potency that is indistinguishable from the natural enantiomers, which is distinct from the behavior of duocarmycin SA where the unnatural enantiomer is approximately 10-fold less active than the natural enantiomer.²⁰ The presence of two DNA binding subunits appears to compensate for the reduced potency that is characteristic of the unnatural enantiomer of duocarmycin SA. The extended agent **24** ($IC_{50} = 4$ pM) was essentially equivalent in potency with its unnatural enantiomer ($IC_{50} = 6$ pM), indistinguishable in potency with yatakemycin, but 4–5-fold more potent than CC-1065. The greater potency of **24** relative to CC-1065 can be attributed to the incorporation of the more stable and more potent duocarmycin SA alkylation subunit.²¹ The sandwiched analogue (+)-**25** ($IC_{50} = 6$ pM) was equipotent with its unnatural enantiomer *ent*-(−)-**25** ($IC_{50} = 6$ pM) and both enantiomers of yatakemycin ($IC_{50} = 5$ pM). In contrast, the reversed analogues were approximately 10-fold less potent than the preceding analogues ((+)-**26**, $IC_{50} = 40$ pM; *ent*-(−)-**26**, $IC_{50} = 60$ pM) but displayed similar potencies. The reversed agents lack an extended N²-acyl substituent that has been shown to be largely responsible for catalysis of the DNA alkylation reaction¹⁵ such that its removal results in less effective DNA alkylating agents as shown below.

Relative Rate and Efficiency of DNA Alkylation

To date, the biological properties of members of this class of natural products have typically mirrored their relative efficiencies of DNA alkylation. For the sandwiched analogues explored herein which contain three subunits, little or no differences in the final efficiencies of DNA alkylation were observed even though they often incorporated suboptimal DNA binding subunits or even possess the unnatural absolute configuration. These observations mirror the near equivalent cytotoxic activities observed within the **14** and **20** series and even between enantiomeric pairs of such analogues. The exceptions to these generalizations include only the unnatural enantiomers of the compounds bearing the simplest, unsubstituted indole right-hand subunit which proved to alkylate DNA with a marginally reduced efficiency, Figure 7. However, these differences are magnified if one examines the relative rates versus final efficiencies of DNA alkylation. Here, readily detectable differences in the rates of DNA alkylation are observed with each enantiomeric pair (natural > unnatural enantiomer) as well as between pairs of analogues. Illustrative of these differences and in a comparison that highlights the importance and role of the right-hand subunit, the relative rates of DNA alkylation in w836 DNA conducted at 5 °C for each enantiomer of yatakemycin were compared with those of **14f** bearing the unsubstituted indole as its right-hand subunit, Figure 7.

As observed with yatakemycin, the natural enantiomer of **14f** alkylates DNA much faster than its unnatural enantiomer (700-fold) and for each enantiomer, yatakemycin alkylates DNA faster than **14f** (1.5-fold for natural enantiomer and ca. 200-fold for unnatural enantiomer). Consistent with its role in catalysis, the right-hand subunit substituents (C5 substituent)^{14,17} increase the rate of DNA alkylation and this effect is most pronounced with the unnatural enantiomers.¹⁷

Notably, the natural enantiomer of **14f** incorporating a suboptimal right-hand subunit and even the unnatural enantiomer of yatakemycin alkylate DNA faster than the natural enantiomer of duocarmycin SA. In addition to highlighting the special characteristics of such sandwiched compounds, these studies illustrate that the incorporation of the yatakemycin left-hand subunit can compensate for suboptimal right-hand subunits (for catalysis) or structural features (unnatural enantiomer configuration) that would limit the behavior of typical two subunit members of this class including duocarmycin SA. So much so, that even the loss of 1,000-fold in the rate of DNA alkylation with the unnatural enantiomer of **14f** relative to yatakemycin

itself provides a very effective DNA alkylating agent whose DNA alkylation efficiency and resulting cytotoxic potency approach that of the natural product.

A second set of comparisons is summarized in Figure 6 entailing the PDE-based sandwiched, extended, and reversed analogues of yatakemycin which further illustrate the importance and role of the right-hand subunit to the catalysis of the DNA alkylation reaction. Both enantiomers of the sandwiched analogue **25** alkylated DNA at extraordinary rates comparable to yatakemycin itself, and the natural enantiomer of the extended analogue **24** also alkylated DNA at a rate that was intermediate of these two enantiomers. By contrast, the unnatural enantiomer of the extended analogue **24** was considerably slower (100-fold), but ultimately achieved the same efficiency of DNA alkylation. Although the reversed natural enantiomer was faster than its unnatural enantiomer, neither comes close to the rates exhibited by the sandwiched or extended analogues **24** and **25** and more closely approximate of the rates of DNA alkylation observed with N-Boc-DSA which lacks the DNA binding subunits altogether.⁸ Several important generalizations are illustrated with these comparisons including: 1) the observation of natural enantiomer > unnatural enantiomer rates within each series and this was especially pronounced for the extended analogue (100-fold) versus the sandwiched (2–4 fold) and reversed (3-fold) analogues, and 2) the relative DNA alkylation rates follow the order sandwiched > extended ≫ reversed analogues for each enantiomeric series. By far, the most striking observation is the extraordinarily slow rates (ca. 1,000-fold) and the reduced efficiency (10-fold) of DNA alkylation for the reversed analogues that may be attributed to their lack of an alkylation subunit N²-substituent that can participate in the binding-induced catalysis derived from disruption of the alkylation subunit vinylogous amide.¹⁴

DNA Alkylation Selectivity

The DNA alkylation selectivity of the yatakemycin analogues was examined within five 150 base-pair segments of DNA described previously.²⁴ The alkylation site identification and the assessment of the relative selectivity among the available sites were obtained by thermally-induced strand cleavage of the singly 5'-end-labeled duplex DNA after exposure to the compounds as detailed.^{5–8} Since the DNA alkylation properties of members of each class of these agents have been established in preceding studies including a description of the unaltered alkylation selectivity of (+)- and *ent*-(−)-yatakemycin within nucleosome packaged DNA^{5b} as well as a detailed comparison of the DNA alkylation properties of (+)- and *ent*-(−)-yatakemycin,¹¹ we focused our analysis on a select set of the new yatakemycin analogues. Most representative of this set are the PDE-based sandwiched, extended, and reversed analogues of yatakemycin (**24–26**). Here, the placement of the alkylation subunit within the trimer structure has a profound and controlling impact on the DNA alkylation selectivity, as well as exerts an influence on the efficiency of DNA alkylation, and exhibits a unique impact on the rate of DNA alkylation that was detailed in the preceding section.

Illustrated in Figure 8 is a representative comparison of the DNA alkylation selectivity of the PDE-based sandwiched, extended, and reversed analogues of yatakemycin within a six base-pair A-rich site found in w836 DNA which beautifully highlights the distinctions in the compounds. As might be anticipated, the PDE-based sandwiched analogue **25** exhibited a DNA alkylation selectivity essentially identical to the natural product yatakemycin itself. Most significant in this comparison is the observation that both enantiomers of PDE-DSA-PDE (**25**), like both enantiomers of yatakemycin, alkylate the same site(s) (adenines central to the six base-pair A-rich site, 5'-AAAAAA-3') with essentially the same relative efficiency.¹¹

In contrast, the two enantiomers of the extended analogue **24**, a close analogue of CC-1065 incorporating the yatakemycin alkylation subunit, exhibited very distinct alkylation selectivities which were both markedly different from yatakemycin and its enantiomer. The natural enantiomer (+)-DSA-PDE-PDE (**24**) alkylated the two adenines at the 3' end of six

base-pair A-rich site (5'-AAAAAA-3') consistent with alkylation only at the 3'-end of a five base-pair AT-rich site.⁶ In contrast, the unnatural enantiomer alkylated the three adenines at the opposite 5'-end of the site (5'-AAAAA-3'). These non overlapping alkylated adenines for the two enantiomers of **24** represent opposite bound orientations (3'→5' for nat. enantiomer and 5'→3' for unnat. enantiomer) of the compounds across a five base-pair AT-rich site starting with (nat. enantiomer)⁶ or preceding (unnat. enantiomer)^{6c} the alkylated adenine.

Most remarkable in this set of comparisons is the behavior of the two enantiomers of the reversed analogues PDE-PDE-DSA (**26**). Although much less efficient than preceding analogues even with extended reaction times (\geq 10-fold, requiring 10^{-4} vs 10^{-5} M concentrations), the two enantiomers of the reversed analogues exhibited distinct DNA alkylation selectivities that were not only different from that of the sandwiched compounds and their enantiomers, but were also opposite that of extended analogues **24**. That is, the natural enantiomer of the reversed analogue exhibited the identical selectivity as the unnatural enantiomer of the extended analogue **24** alkylating the adenines at the 5'-end of the site, whereas its unnatural enantiomer alkylated the same 3' adenines as the extended natural enantiomer **24**, Figure 8 and 9. This complete reversal of the enantiomeric selectivity^{14,25} between the extended versus reversed analogues indicates that it is not the alkylation subunit and its absolute configuration that dominates the alkylation selectivity, rather that it is the compound's AT-rich noncovalent binding selectivity²³ that delivers the modestly reactive electrophile to accessible nucleophiles within a noncovalent binding site.¹⁴

This distinct reversed enantiomer behavior of the extended versus reversed analogues is also and even more clearly illustrated in Figure 10. Within w794 DNA, the natural and unnatural enantiomers of the extended analogues **24** each alkylate a single distinct site within the 150 base-pair segment of DNA consistent with observations first made while examining CC-1065 and its unnatural enantiomer.²⁵ Examination of the unnatural enantiomer of the reversed analogue **26** alongside both enantiomers of the extended analogue **24** illustrate that it alkylates the natural enantiomer site (*ent*-(-)-PDE-PDE-DSA = (+)-DSA-PDE-PDE) beautifully highlighting this reversal of the enantiomer selectivity between the extended and reversed analogues.

Models of the Alkylation Products

Presented in Figure 11 are models of the sandwiched, extended, and reversed PDE-based analogues alkylation of their predominant w836 sites (see Figure 8) that visually illustrate the selectivity features discussed earlier. Most notable in the comparisons is the fact that all six compounds (two enantiomers of three compounds) bind across the identical five base-pair A-rich site with each enantiomer pair binding with opposite orientations and undergoing alkylation at the resulting adenine proximal to the reactive cyclopropane. The two enantiomers of the sandwiched analogues **25** alkylate the same adenine central to the site, the natural enantiomer of the extended analogue **24** and the unnatural enantiomer of the reversed analogue **26** alkylate the same adenine at the 3' end of the site, whereas the unnatural enantiomer of the extended analogue **24** and the natural enantiomer of the reversed analogue **26** alkylate an adenine at the opposite 5' end of the site (Figure 12).

Conclusions

A systematic examination of the yatakemycin left and right-hand subunits and their substituents is detailed establishing their impact on the cytotoxic potency and DNA alkylation properties of such sandwiched compounds. Within the context of this three subunit structural arrangement, the studies illustrate that the subunit substituents are now relatively unimportant and that the yatakemycin left-hand subunit, as well as those incorporating significant simplifications, can compensate for suboptimal right-hand subunits and substituents or

structural features (i.e. unnatural enantiomer configuration) that limit the more traditional compounds in the series. Significantly, this sandwiched subunit arrangement substantially increases the rate of DNA alkylation, maximizes the efficiency of DNA alkylation, and potentiates the cytotoxic potency of the compounds. Moreover, the placement of the alkylation subunit within the three subunit structure has a profound impact on not only the rate (PDE-DSA-PDE > DSA-PDE₂ ≫ PDE₂-DSA) and efficiency (PDE-DSA-PDE>DSA-PDE₂>PDE₂-DSA) of DNA alkylation, but controls the DNA alkylation selectivity as well. Most notable in this regard is the remarkable observation that both enantiomers of such sandwiched compounds alkylate the same sites exhibiting the same DNA alkylation selectivity independent of the enantiomeric configuration ((+)-PDE-DSA-PDE = *ent*-(−)-PDE-DSA-PDE). Contrasting this behavior is the distinct DNA alkylation selectivities of the enantiomeric pairs of extended or reversed analogues which are not only different from that of the sandwiched compounds, but which exhibit reversed enantiomeric selectivities (e.g., (+)-DSA-PDE₂ = *ent*-(−)-PDE₂-DSA). These remarkable observations, which are easily rationalized and visualized based on prevailing models of the DNA alkylation reaction,²⁶ are consistent with preferential noncovalent binding of the compounds across a five base-pair AT-rich site delivering the natural enantiomer of a modestly reactive electrophile to adenine positioned central (sandwiched) or at the 3' terminus (extended) and 5' terminus (reversed) of such sites, whereas the unnatural enantiomers bind within the same sites but with reversed orientations delivering the electrophile to adenine central (sandwiched) or at the opposite 5' terminus (extended) and 3' terminus (reversed) of the sites. Superimposed on this binding-derived alkylation selectivity is the impact of catalysis attributable to the alkylation subunit N² substituent. The subunit arrangements incorporating an extended heteroaromatic N² acyl substituent on the alkylation subunit (sandwiched, extended analogues) alkylate DNA with remarkable rates and efficiencies for such a modest electrophile, whereas those that lack this structural feature (reversed analogues) do so only at markedly reduced rates (\geq 1,000-fold). We have attributed this pronounced impact on the rate to a unique source of catalysis for the DNA alkylation reaction that is derived from a DNA binding-induced conformation change in the molecule that activates them for nucleophilic attack. Thus, the adoption of a DNA bound helical conformation disrupts the N² vinylogous amide conjugation stabilizing the alkylation subunit (sandwiched and extended, but not reversed) activating the cross-conjugated cyclopropane for nucleophilic attack.

Tucked into these studies, we have also prepared and evaluated a key analogue of CC-1065. The extended analogue **24**, (+)- and *ent*-(−)-DSA-PDE₂, constitutes the substitution of the duocarmycin SA/yatakemycin alkylation subunit into the CC-1065 structure replacing its CPI alkylation subunit. This substitution provided a hybrid analogue of CC-1065 and its unnatural enantiomer (L1210 IC₅₀ = 20 pM, both enantiomers) that exhibited an unaltered DNA alkylation selectivity relative to CC-1065, but which proved to be 5-fold more potent (IC₅₀ = 4 pM, Figure 6) than the natural product precisely in line with expectations²¹ based on its 5-fold enhanced stability.²⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

1. Igarashi Y, Futamata K, Fujita T, Sekine A, Senda H, Naoki H, Furumai T. *J Antibiot* 2003;56:107–113. [PubMed: 12715869]
2. Martin DG, Biles C, Gerpheide SA, Hanka LJ, Krueger WC, McGovren JP, Miszak SA, Neil GL, Stewart JC, Visser J. *J Antibiot* 1981;34:1119–1125. [PubMed: 7328053]
3. Takahashi I, Takahashi K, Ichimura M, Morimoto M, Asano K, Kawamoto I, Tomita F, Nakano H. *J Antibiot* 1988;41:1915–1917. [PubMed: 3209484]
4. Ichimura M, Ogawa T, Takahashi K, Kobayashi E, Kawamoto I, Yasuzawa T, Takahashi I, Nakano H. *J Antibiot* 1990;43:1037–1038. [PubMed: 2211354]
5. Yatakemycin: (a) Parrish JP, Kastrinsky DB, Wolkenberg SE, Igarashi Y, Boger DL. *J Am Chem Soc* 2003;125:10971–10976. [PubMed: 12952479] (b) Trzupek JD, Gottesfeld JM, Boger DL. *Nature Chem Biol* 2006;2:79–82. [PubMed: 16415862]
6. (a) CC-1065: Hurley LH, Lee C-S, McGovren JP, Warpehoski MA, Mitchell MA, Kelly RC, Aristoff PA. *Biochemistry* 1988;27:3886–3892. [PubMed: 3408734] (b) Hurley LH, Warpehoski MA, Lee CS, McGovren JP, Scahill TA, Kelly RC, Mitchell MA, Wicnienksi NA, Gebhard I, Johnson PD, Bradford VS. *J Am Chem Soc* 1990;112:4633–4649. (c) Boger DL, Johnson DS, Yun W, Tarby CM. *Bioorg Med Chem* 1994;2:115–135. [PubMed: 7922122] (d) Boger DL, Coleman RS, Invergo BJ, Sakya SM, Ishizaki T, Munk SA, Zarrinmayeh H, Kitos PA, Thompson SC. *J Am Chem Soc* 1990;112:4623–4632.
7. Duocarmycin A: (a) Boger DL, Ishizaki T, Zarrinmayeh H, Munk SA, Kitos PA, Sunturnwat O. *J Am Chem Soc* 1990;112:8961–8971. (b) Boger DL, Ishizaki T, Zarrinmayeh H. *J Am Chem Soc* 1991;113:6645–6649. (c) Boger DL, Yun W, Terashima S, Fukuda Y, Nakatani K, Kitos PA, Jin Q. *Bioorg Med Chem Lett* 1992;2:759–765.
8. Duocarmycin SA: Boger DL, Johnson DS, Yun W. *J Am Chem Soc* 1994;116:1635–1656.
9. Reviews: (a) Boger DL, Johnson DS. *Angew Chem Int Ed Engl* 1996;35:1438–1474. (b) Boger DL. *Acc Chem Res* 1995;28:20–29. (c) Boger DL, Johnson DS. *Proc Natl Acad Sci USA* 1995;92:3642–3649. [PubMed: 7731958] (d) Boger DL, Garbaccio RM. *Acc Chem Res* 1999;32:1043–1052.
10. Tichenor MS, Kastrinsky DB, Boger DL. *J Am Chem Soc* 2004;126:8396–8398. [PubMed: 15237994]
11. Tichenor MS, Trzupek JD, Kastrinsky DB, Futoshi S, Hwang I, Boger DL. *J Am Chem Soc* 2006;128:15683–15696. [PubMed: 17147378]
12. An independent route to the natural product has been reported: Okano K, Tokuyama H, Fukuyama T. *J Am Chem Soc* 2006;128:7136–7137. [PubMed: 16734447]
13. Review of related efforts: Boger DL, Boyce CW, Garbaccio RM, Goldberg JA. *Chem Rev* 1997;97:787–828. [PubMed: 11848889]
14. (a) Boger DL, Bollinger B, Hertzog DL, Johnson DS, Cai H, Mesini P, Garbaccio RM, Jin Q, Kitos PA. *J Am Chem Soc* 1997;119:4987–4998. (b) Boger DL, Hertzog DL, Bollinger B, Johnson DS, Cai H, Goldberg J, Turnbull P. *J Am Chem Soc* 1997;119:4977–4986.
15. Boger DL, Garbaccio RM. *Bioorg Med Chem* 1997;5:263–276. [PubMed: 9061191]
16. Reviews: (a) Wolkenberg SE, Boger DL. *Chem Rev* 2002;102:2477–2496. [PubMed: 12105933] (b) Tse WC, Boger DL. *Chem Biol* 2004;11:1607–1617. [PubMed: 15610844]
17. Parrish JP, Kastrinsky DB, Stauffer F, Hedrick MP, Hwang I, Boger DL. *Bioorg Med Chem* 2003;11:3815–3838. [PubMed: 12901927]
18. (a) Boger DL, Coleman RS. *J Org Chem* 1984;49:2240–2245. (b) Boger DL, Coleman RS, Invergo BJ. *J Org Chem* 1987;52:1521–1530. (c) Boger DL, Coleman RS. *J Org Chem* 1988;53:695–698.
19. (a) Warpehoski MA, Gebhard I, Kelly RC, Krueger WC, Li LH, McGovern JP, Praire MD, Wienienski N, Wierenga W. *J Med Chem* 1988;31:590–603. [PubMed: 3346875] (b) Boger DL, Ishizaki T, Sakya SM, Munk SA, Kitos PA, Jin Q, Besterman JM. *Bioorg Med Chem Lett* 1991;1:115–120. (c) Boger DL, Yun W, Han N. *Bioorg Med Chem* 1995;3:1429–1453. [PubMed: 8634824]
20. (a) Boger DL, Machiya K. *J Am Chem Soc* 1992;114:10056–10058. (b) Boger DL, Machiya K, Hertzog DL, Kitos PA, Holmes D. *J Am Chem Soc* 1993;115:9025–9036.
21. (a) Parrish JP, Hughes TV, Hwang I, Boger DL. *J Am Chem Soc* 2004;126:80–81. [PubMed: 14709069] (b) Boger DL, Munk SA, Ishizaki T. *J Am Chem Soc* 1991;113:2779–2780.

22. (a) Boger DL, Coleman RS. *J Am Chem Soc* 1987;109:2717–2727. (b) Coleman DLRS. *J Am Chem Soc* 1988;110:1321–1323.1988;110:4796–4807.
23. (a) Boger DL, Coleman RS, Invergo BJ, Zarrinmayeh H, Kitos PA, Thompson SC, Leong T, McLaughlin LW. *Chem-Biol Interact* 1990;73:29–52. [PubMed: 2406033] (b) Boger DL, Zarrinmayeh H, Munk SA, Kitos PA, Suntornwat O. *Proc Natl Acad Sci USA* 1991;88:1431–1435. [PubMed: 1847523]
24. Boger DL, Munk SA, Zarrinmayeh H, Ishizaki T, Haught J, Bina M. *Tetrahedron* 1991;47:2661–2682.
25. Boger DL, Johnson DS. *J Am Chem Soc* 1995;117:1443–1444.
26. Boger DL, Johnson DS, Yun W. *J Am Chem Soc* 1994;116:1635–1656.
27. This stability was measured by assessing solvolysis reactivity of the respective N-Boc derivatives at pH 3: N-Boc-CPI ($t_{1/2} = 37$ h, $k = 5.26 \times 10^{-6}\text{s}^{-1}$) versus N-Boc-DSA ($t_{1/2} = 177$ h, $k = 1.08 \times 10^{-6}\text{s}^{-1}$). See: Boger DL, Yun W. *J Am Chem Soc* 1994;116:5523–5524. Boger DL, Yun W. *J Am Chem Soc* 1994;116:7996–8006.

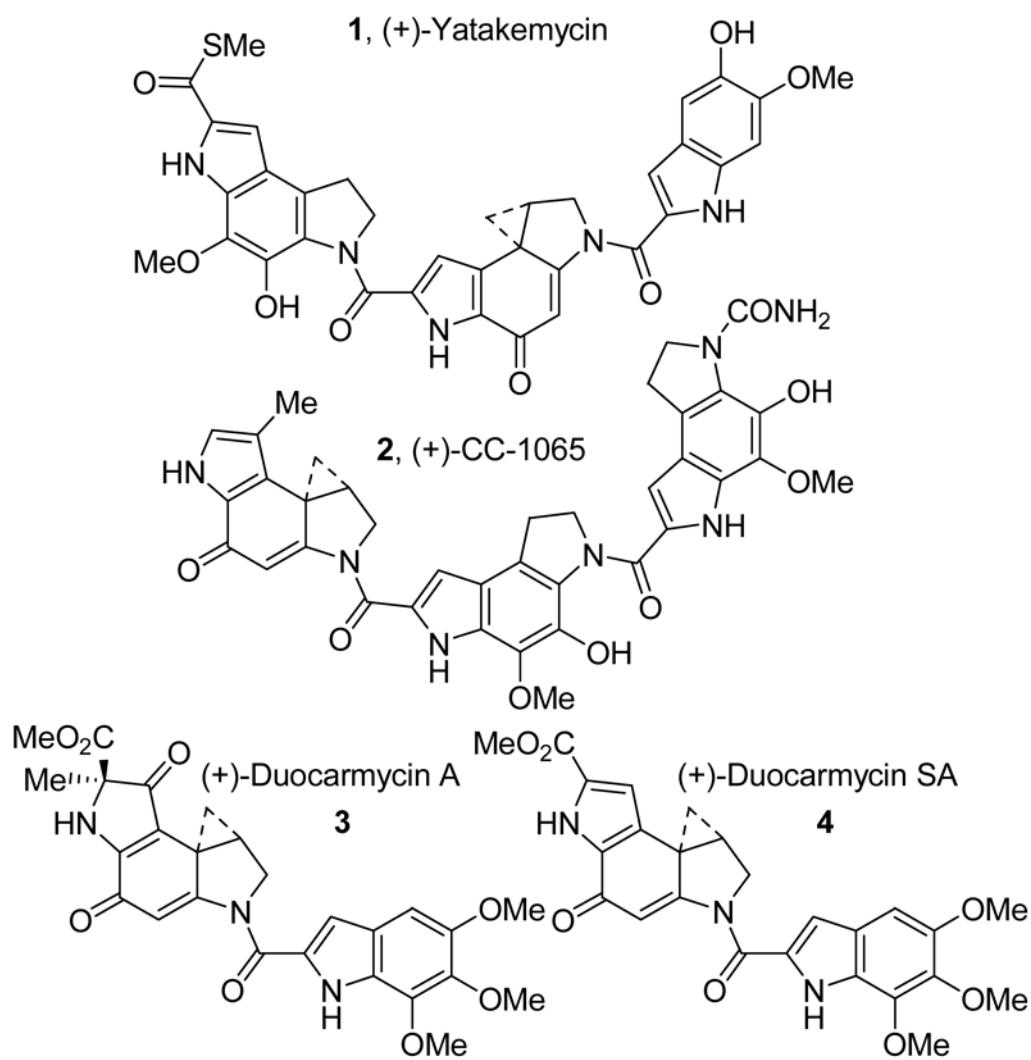
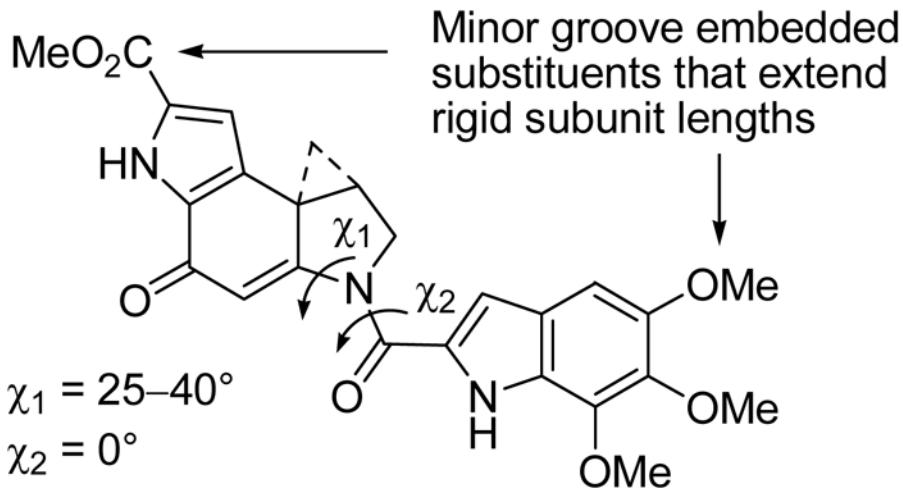


Figure 1.
Natural products.



- DNA bound agent adopts helical conformation with twist adjusted at linking amide (χ_1 : $0 \rightarrow 25\text{--}40^\circ$)
- DNA bound compound maintains full amide ($\chi_2 = 0^\circ$)
- Vinylogous amide conjugation diminished ($\chi_1 = 25\text{--}40^\circ$)
- Cyclohexadienone structure destabilized (activated)
- Shape-dependent catalysis: preferential activation in narrower, deeper AT-rich minor groove where binding induced twist greatest
- Target-based activation: binding-induced conformational change only at target activates compound for addition

Figure 2.

DNA alkylation catalysis and substituent effects on DNA alkylation efficiency, rate, and biological potency

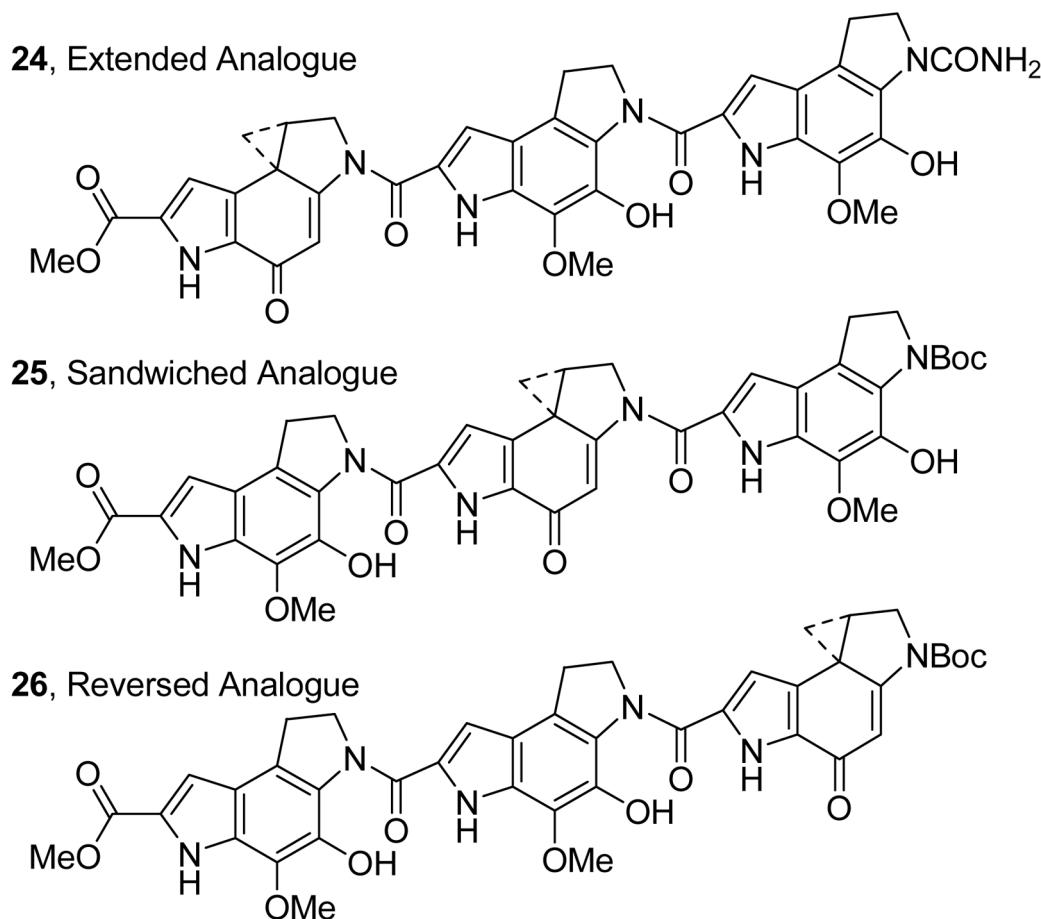


Figure 3.
Extended, reversed, and sandwiched analogues.

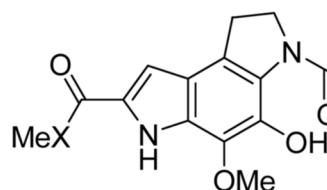
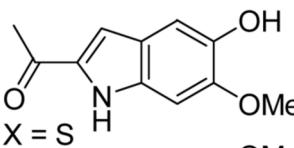
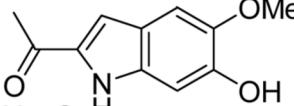
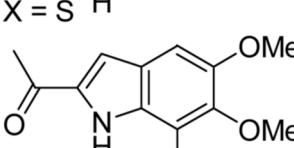
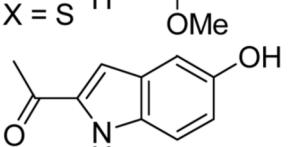
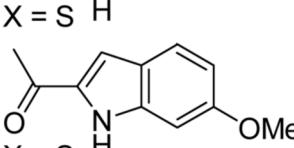
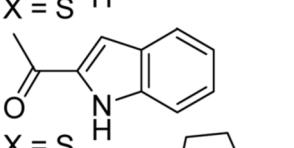
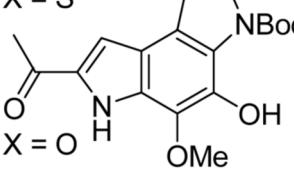
| |  | L-1210 IC ₅₀ (pM) | | L-1210 IC ₅₀ (pM) | |
|-------|---|------------------------------|---------|------------------------------|-----------|
| | | (+)- | ent(-)- | (+)- | ent(-)- |
| R = |  | 1 | 5 | 5 | 9a |
| X = S |  | 14b | 6 | 6 | 9b |
| X = S |  | 14c | 5 | 6 | 4 |
| X = S |  | 14d | 5 | 5 | 9d |
| X = S |  | 14e | 4 | 10 | 9e |
| X = S |  | 14f | 6 | 35 | 9f |
| X = O |  | 25 | 6 | 6 | 40 |

Figure 4.
L1210 Cytotoxic activity.

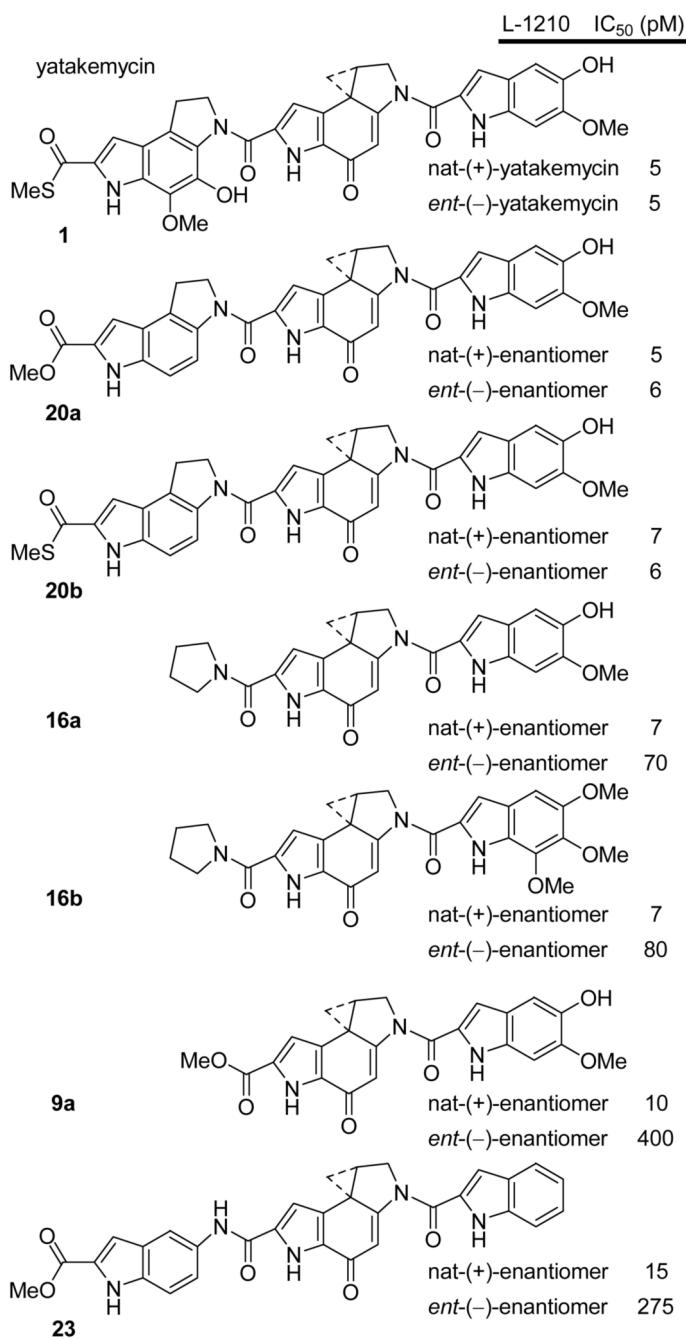


Figure 5.
L1210 Cytotoxic activity.

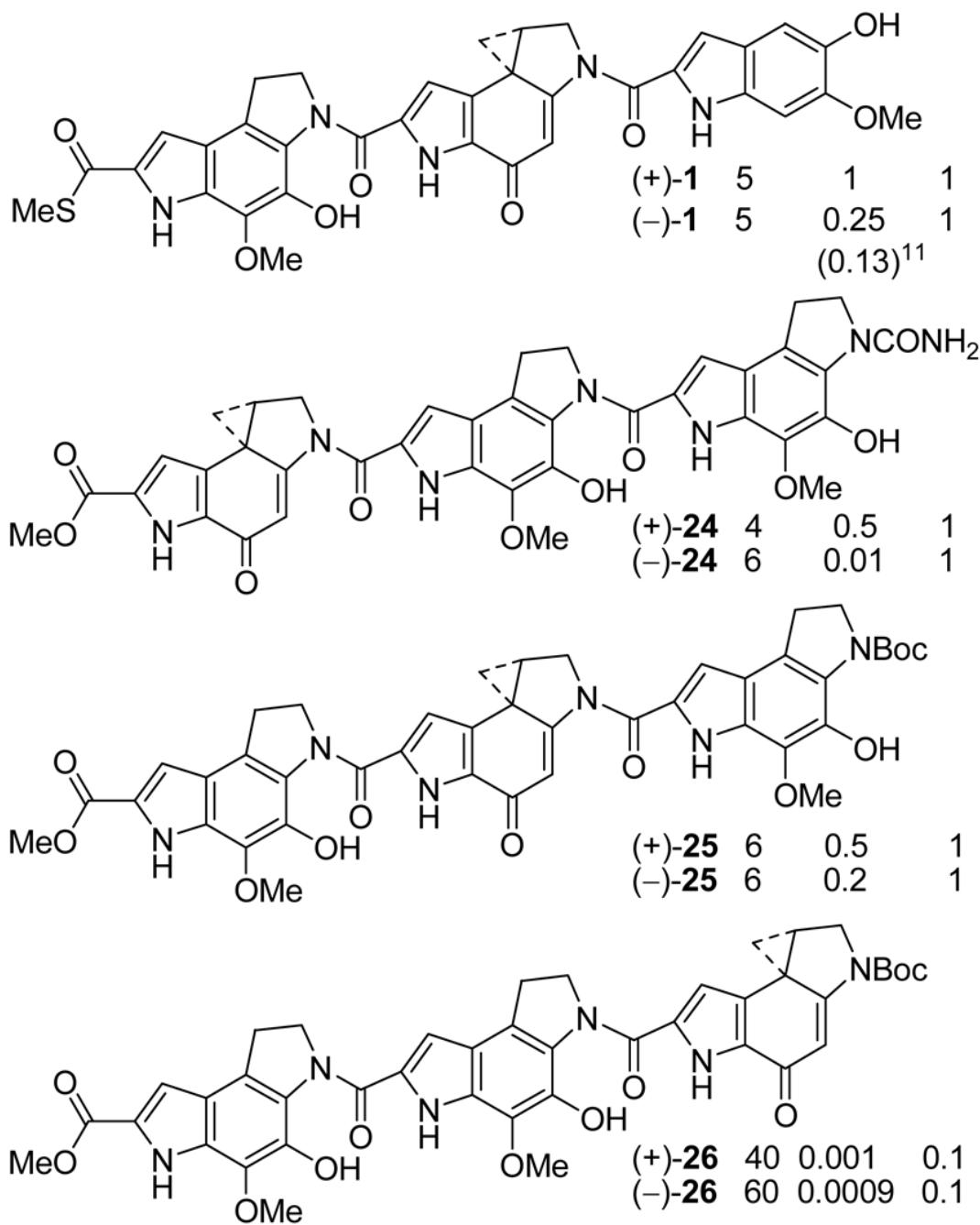


Figure 6.
Cytotoxic activity (L1210) and relative rates (k_{rel}) and efficiencies of DNA alkylation (w836).

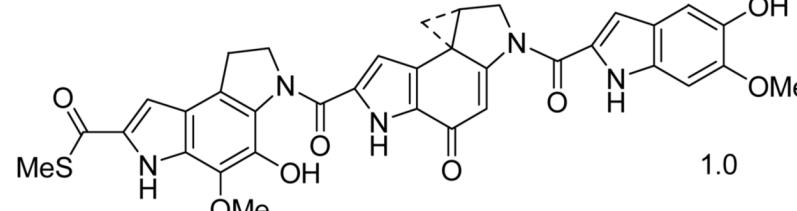
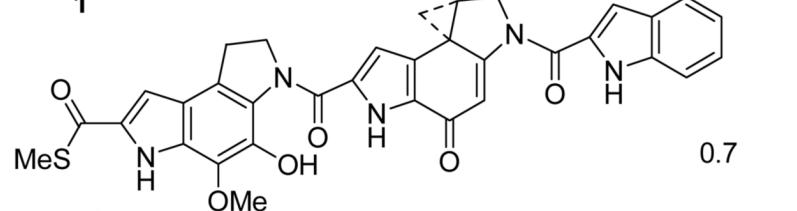
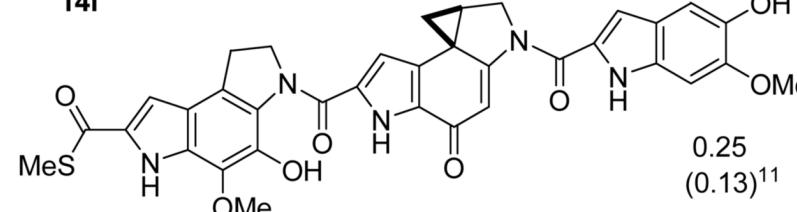
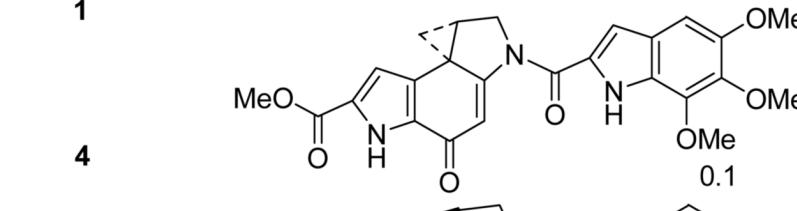
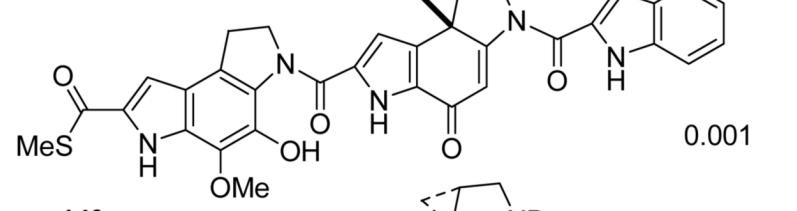
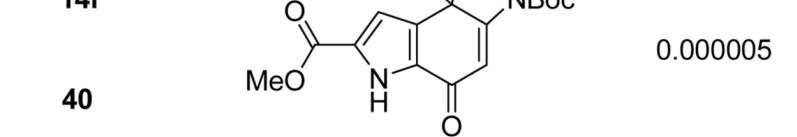
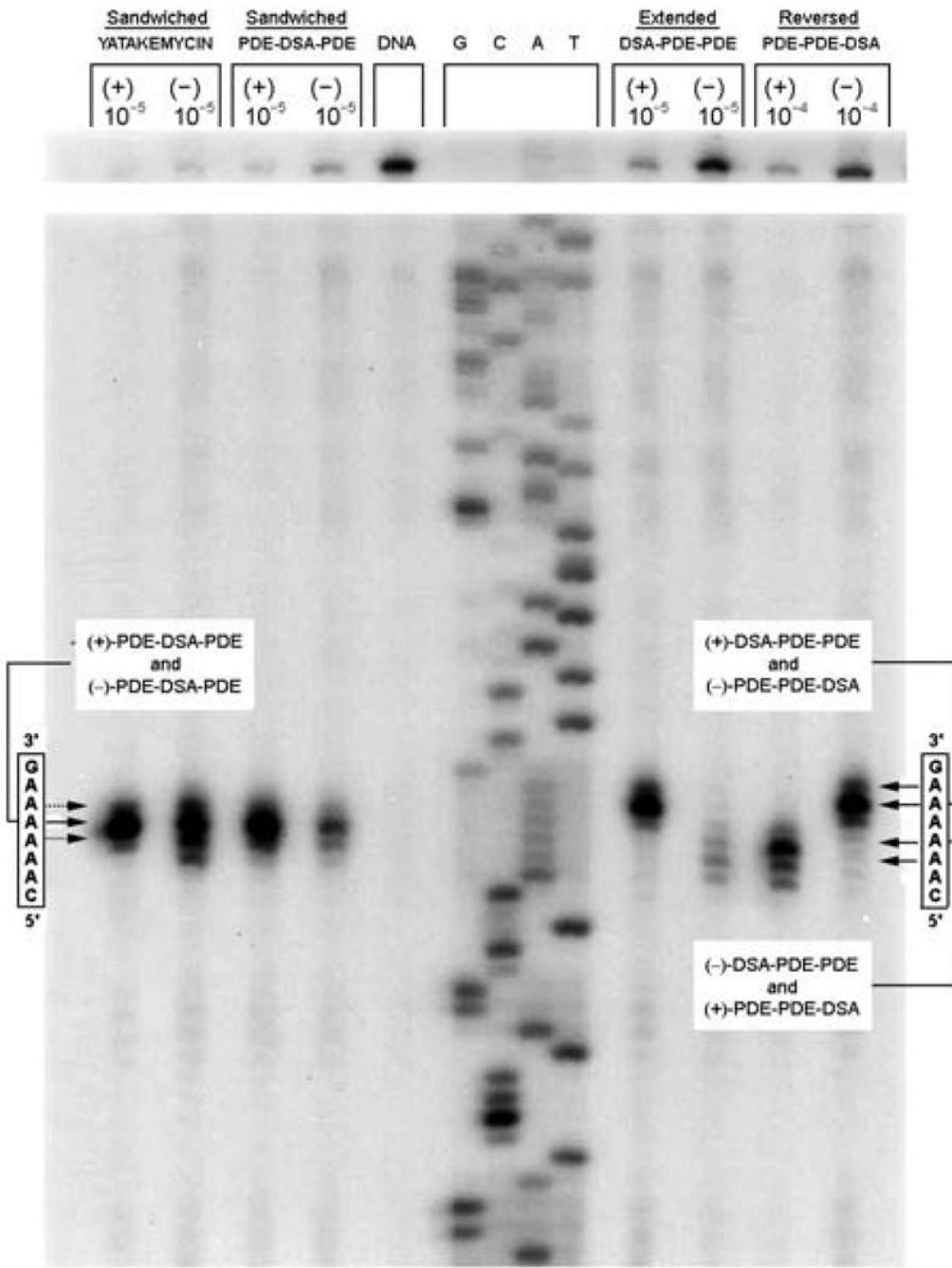
| Compound | k_{rel} | rel. efficiency |
|--|------------------------------|--------------------|
|  | 1.0 | 1.0 |
|  | 0.7 | 1.0 |
|  | 0.25 (0.13) ¹¹ | 1.0 |
|  | 0.1 | 1.0 |
|  | 0.001 | 0.75 |
|  | 0.000005 | 0.001 |

Figure 7.Relative rates (k_{rel}) and efficiencies of DNA alkylation (w836 DNA).

**Figure 8.**

Thermally-induced strand cleavage of w836 DNA (146 bp, nucleotide no. 5189-91) after DNA–agent incubation with DSA-PDE-PDE (**24**), PDE-DSA-PDE (**25**), and PDE-PDE-DSA (**26**, 23 h, 25 °C), removal of unbound agent by EtOH precipitation and 30 min thermolysis (100 °C), followed by denaturing 8% PAGE and autoradiography. Lanes 1 and 2, (+)-yatakemycin and *ent*(-)-yatakemycin (1×10^{-5} M); lanes 3 and 4, (+)-PDE-DSA-PDE (**25**) and *ent*(-)-PDE-DSA-PDE (**25**, 1×10^{-5} M); lane 5, control DNA; lanes 6–9, Sanger G, C, A, and T sequencing standards; lanes 10 and 11, (+)-DSA-PDE-PDE (**24**) and *ent*(-)-DSA-PDE-PDE (**24**, 1×10^{-5} M); lanes 12 and 13, (+)-PDE-PDE-DSA (**26**) and *ent*(-)-PDE-PDE-DSA (**26**, 1×10^{-4} M).

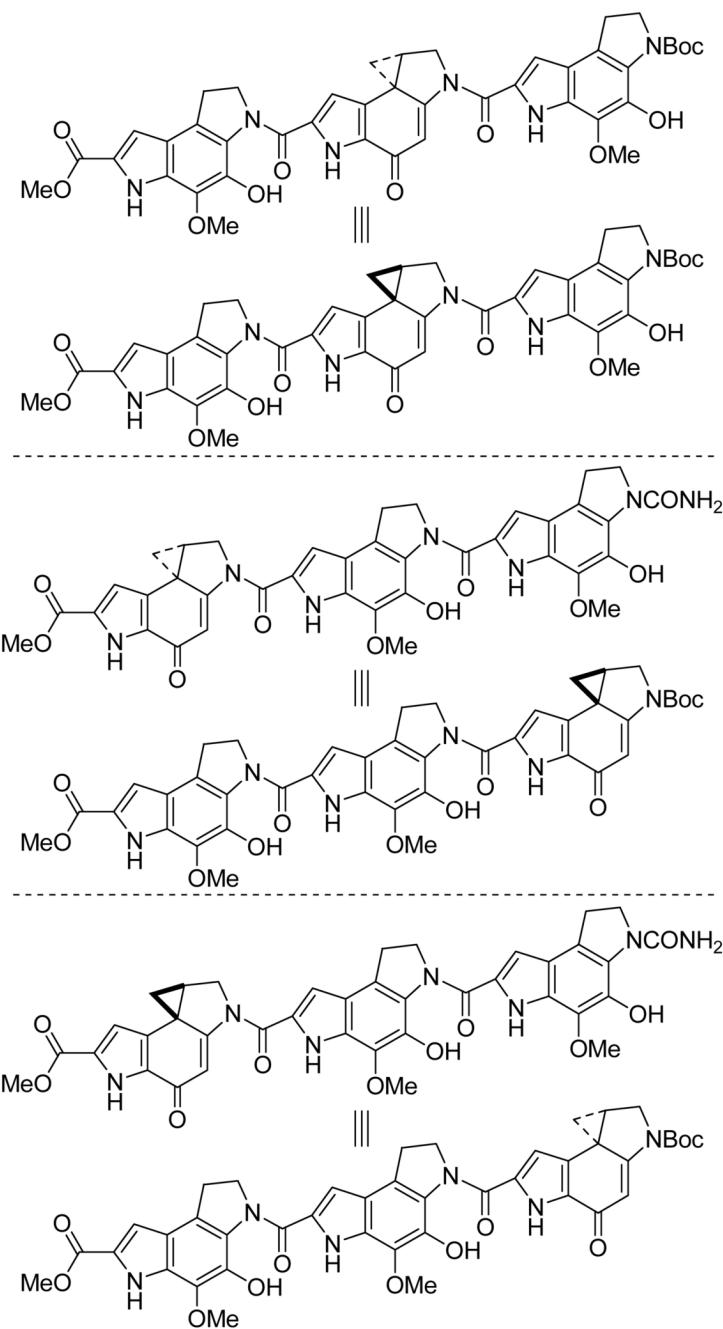
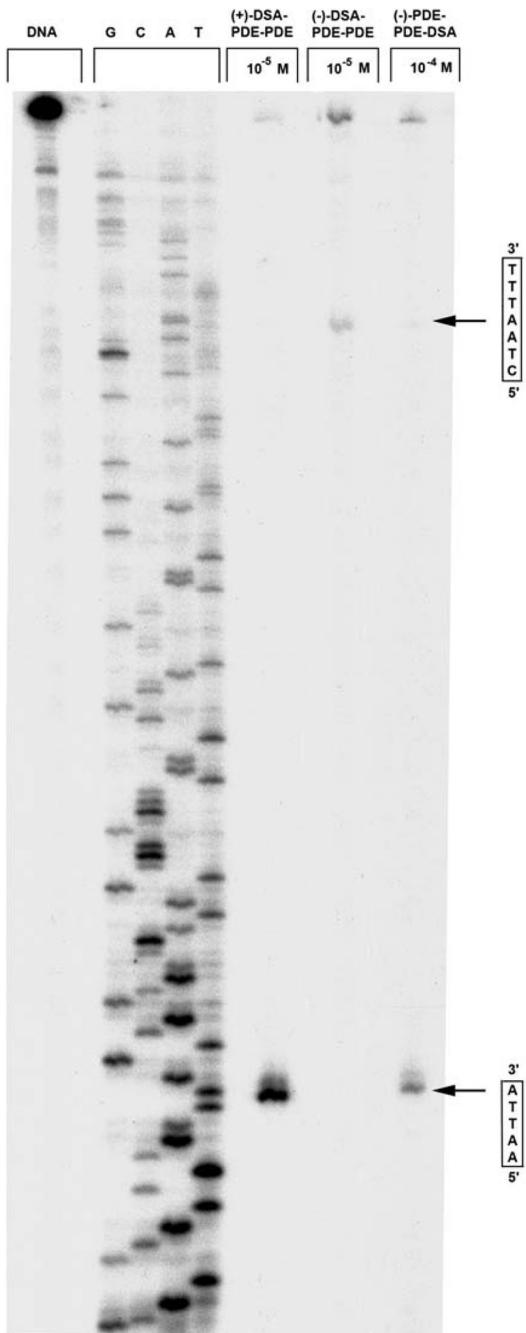
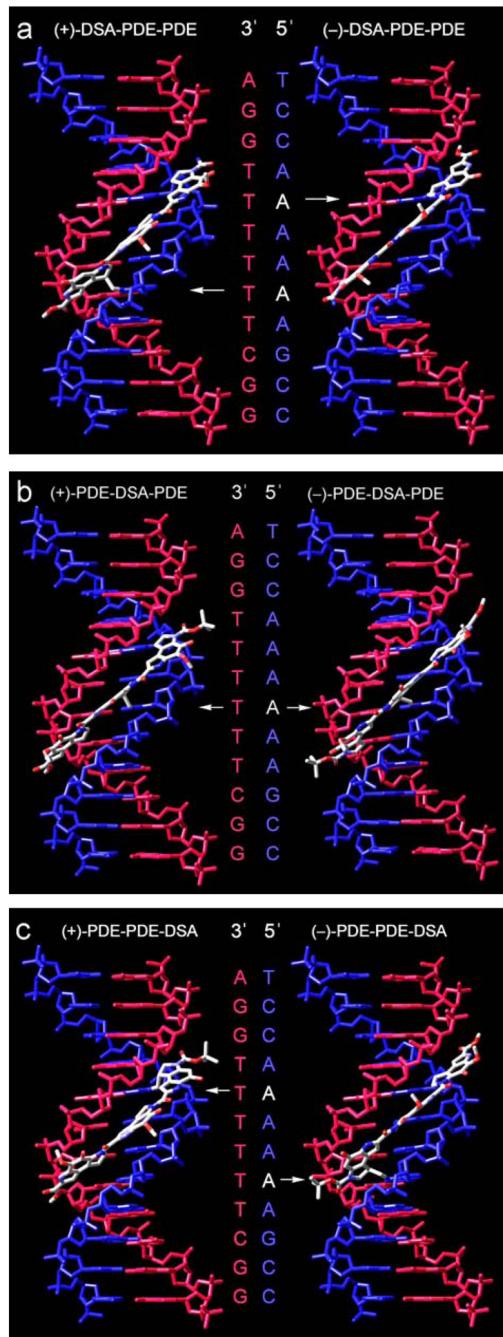


Figure 9.
Alkylation selectivities.

**Figure 10.**

Thermally-induced strand cleavage of w794 DNA (144 bp, nucleotide no. 5238-138) after DNA–agent incubation with DSA-PDE-PDE (**24**) (25 °C, 5 days) and PDE-PDE-DSA (**26**) (37 °C, 5 days), removal of unbound agent by EtOH precipitation and 30 min. thermolysis (100 °C), followed by denaturing 8% PAGE and autoradiography. Lane 1, control DNA; lanes 2-5, Sanger G, C, A, and T sequencing standards; lane 6, (+)-DSA-PDE-PDE (**24**, 25 °C, 1×10⁻⁵ M); lane 7, *ent*-(−)-DSA-PDE-PDE (**24**, 25 °C, 1×10⁻⁵ M); lane 8, *ent*-(−)-PDE-PDE-DSA (**26**, 37 °C, 1×10⁻⁴ M).

**Figure 11.**

a) Models of (+)-DSA-PDE₂ (left) and *ent*-(−)-DSA-PDE₂ (right). b) Models of (+)-PDE-DSA-PDE (left) and *ent*-(−)-PDE-DSA-PDE (right). c) Models of (+)-PDE₂-DSA (left) and *ent*-(−)-PDE₂-DSA (right). See Supporting Information for larger images of the models.

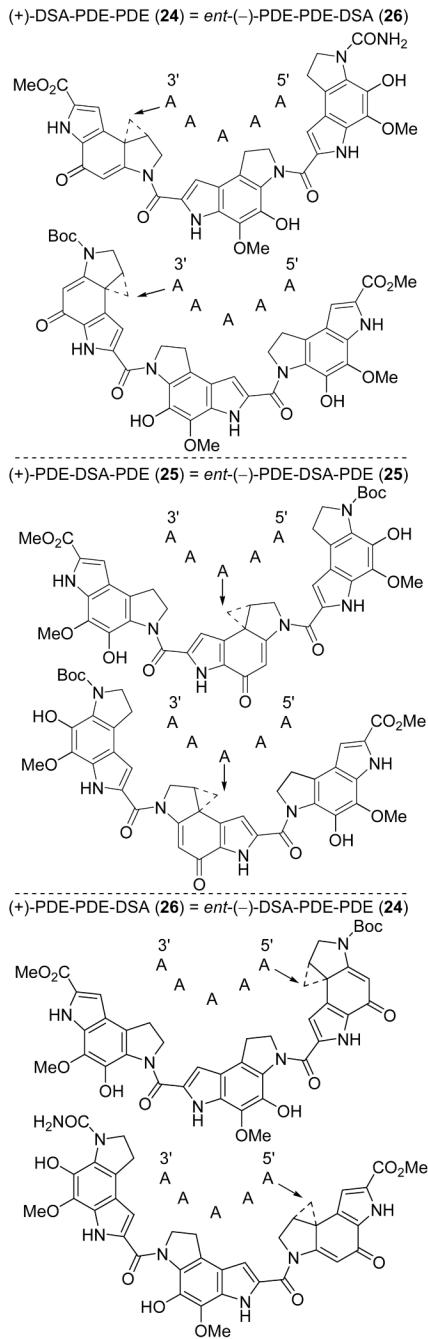
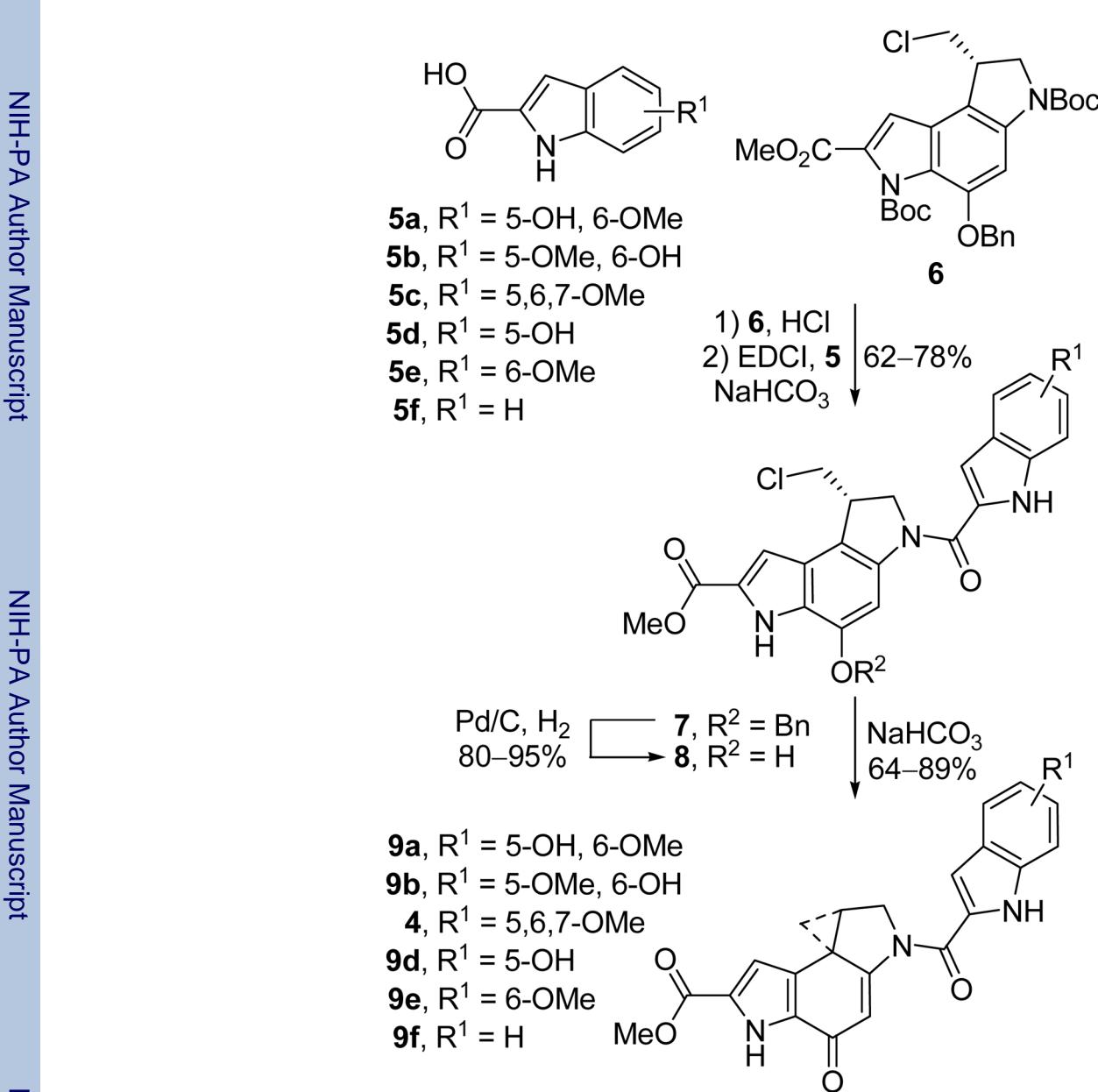
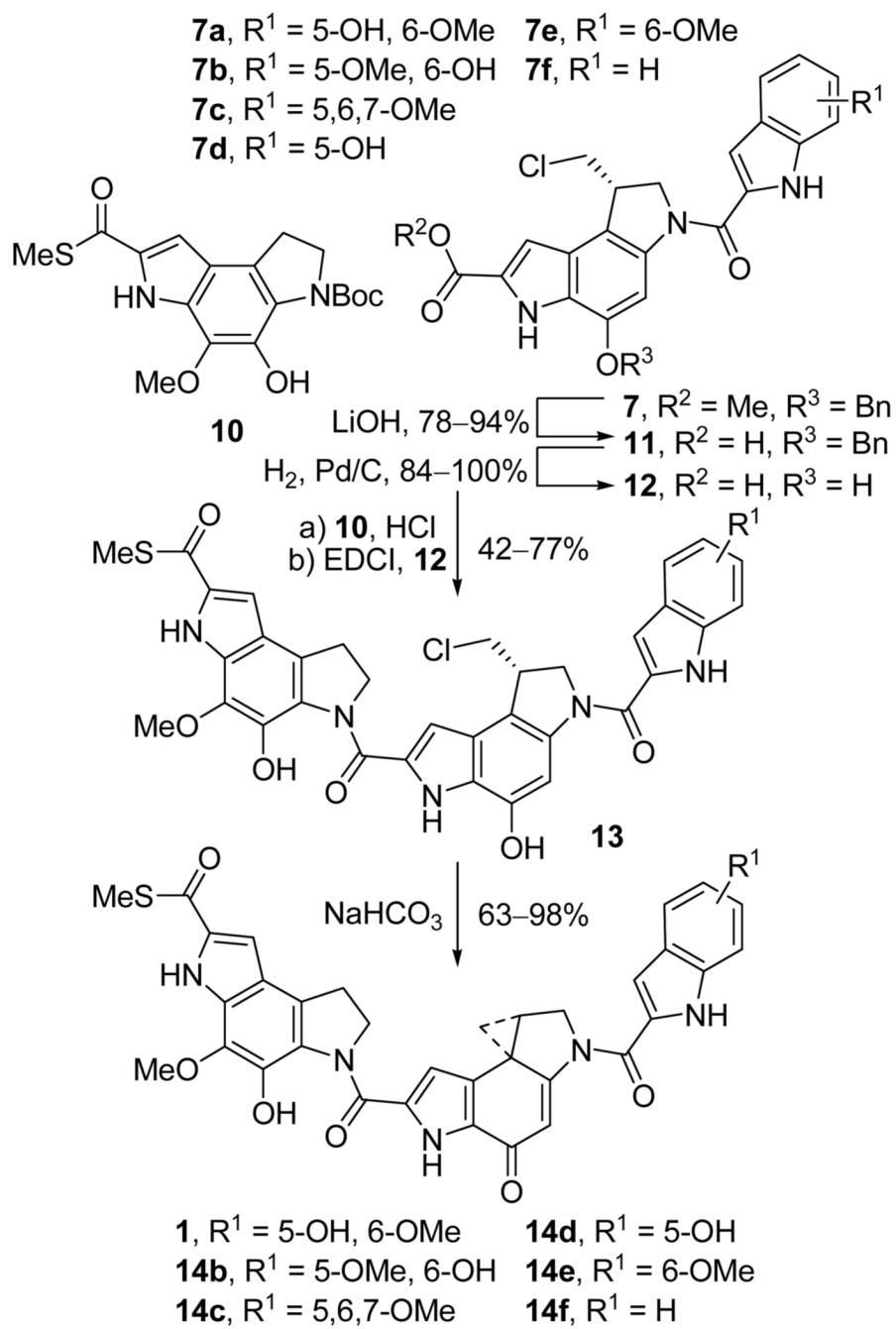


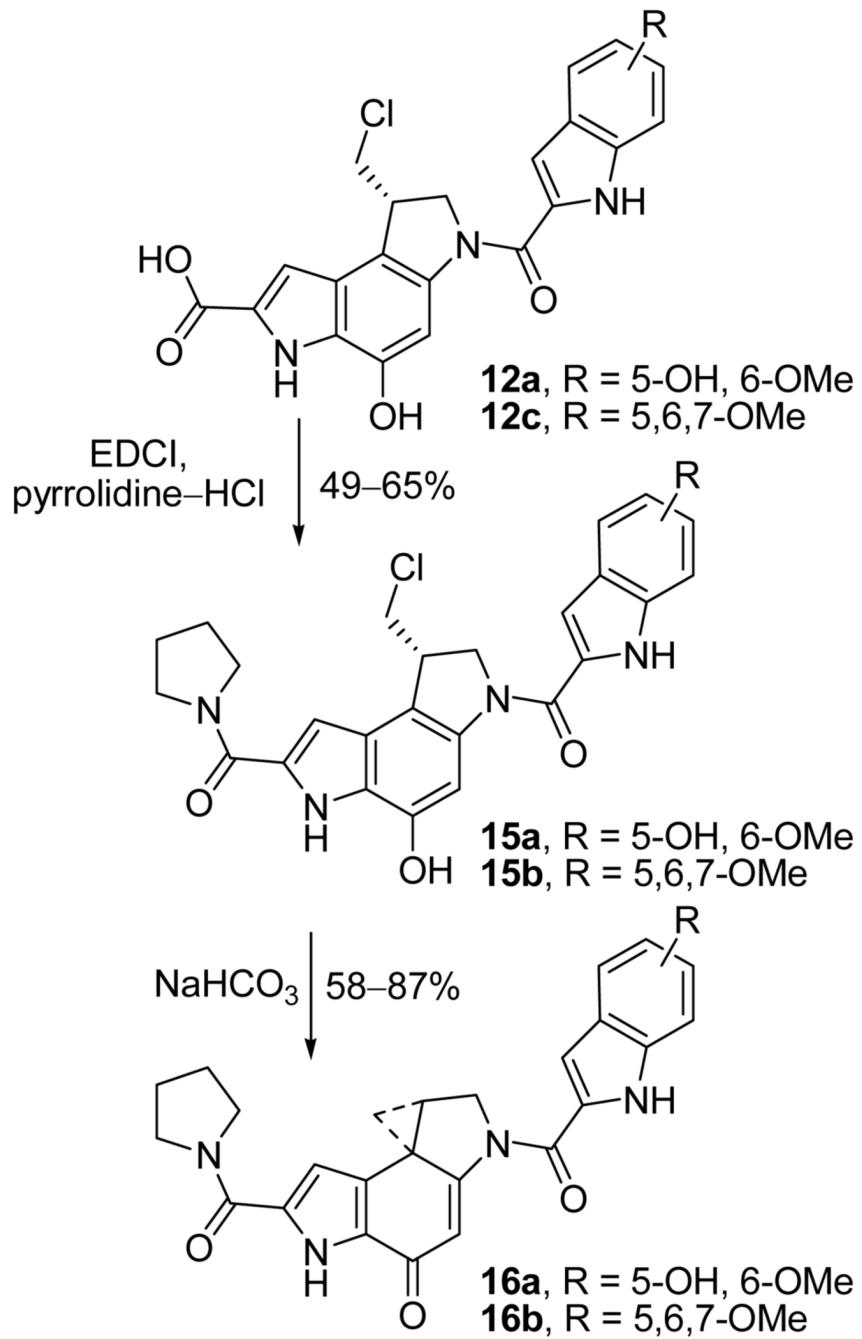
Figure 12.
Graphic representation of bound orientations and alkylation sites for the extended, sandwiched, and reversed analogues.



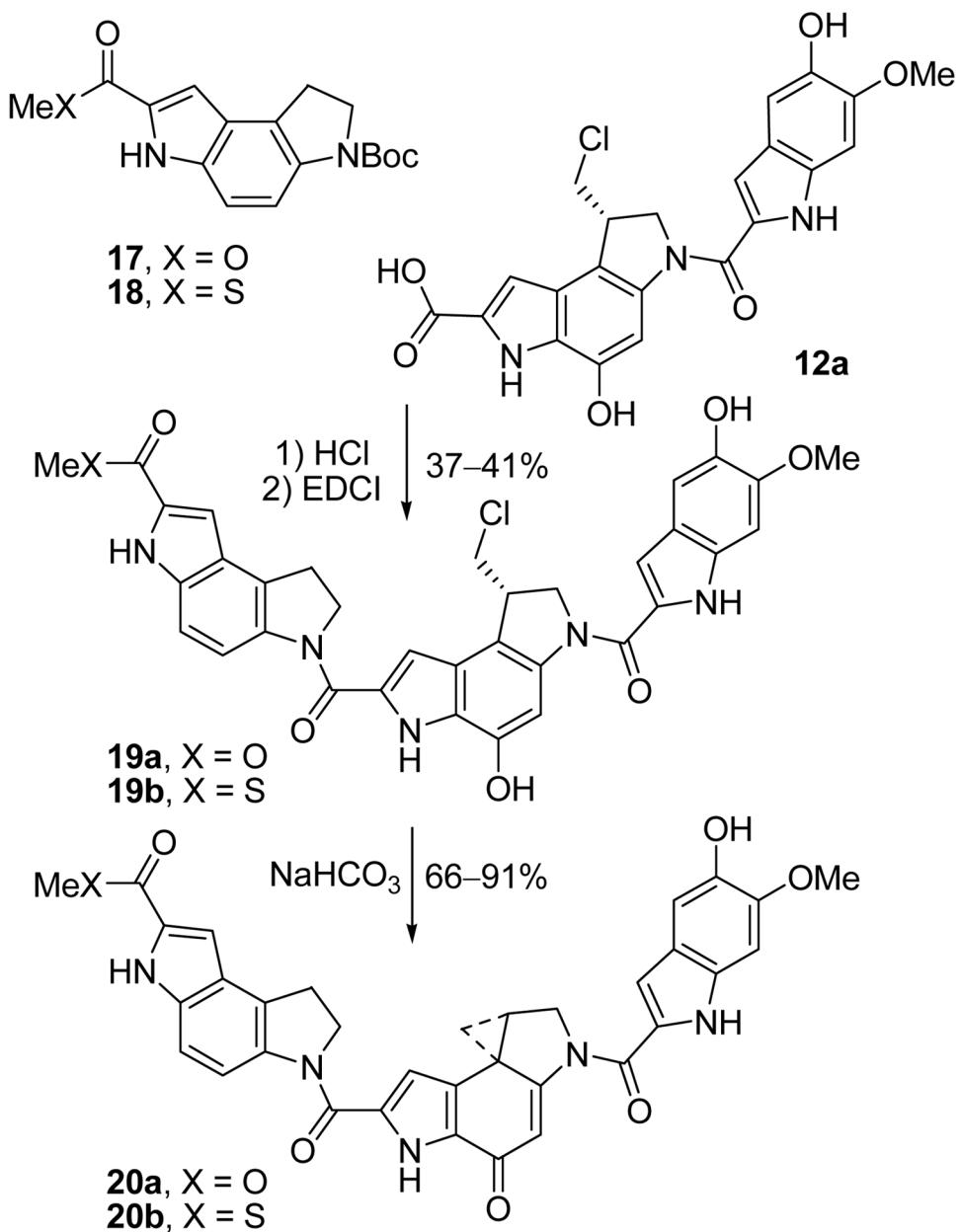
Scheme 1.



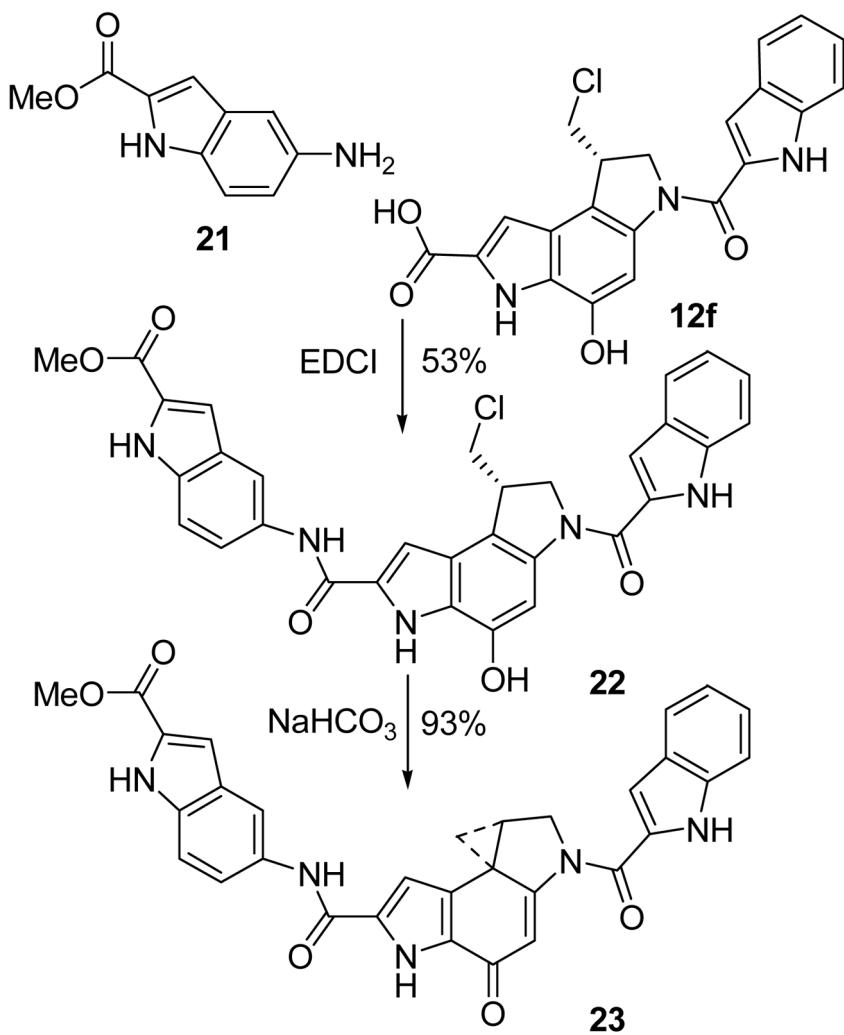
Scheme 2.



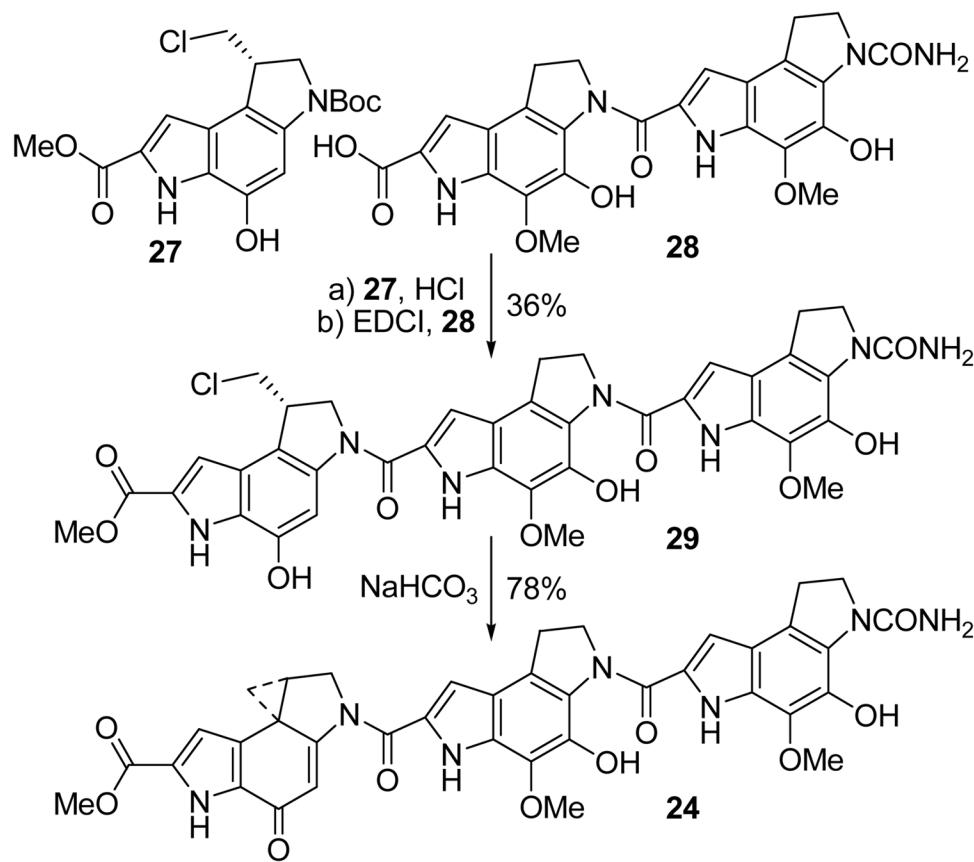
Scheme 3.



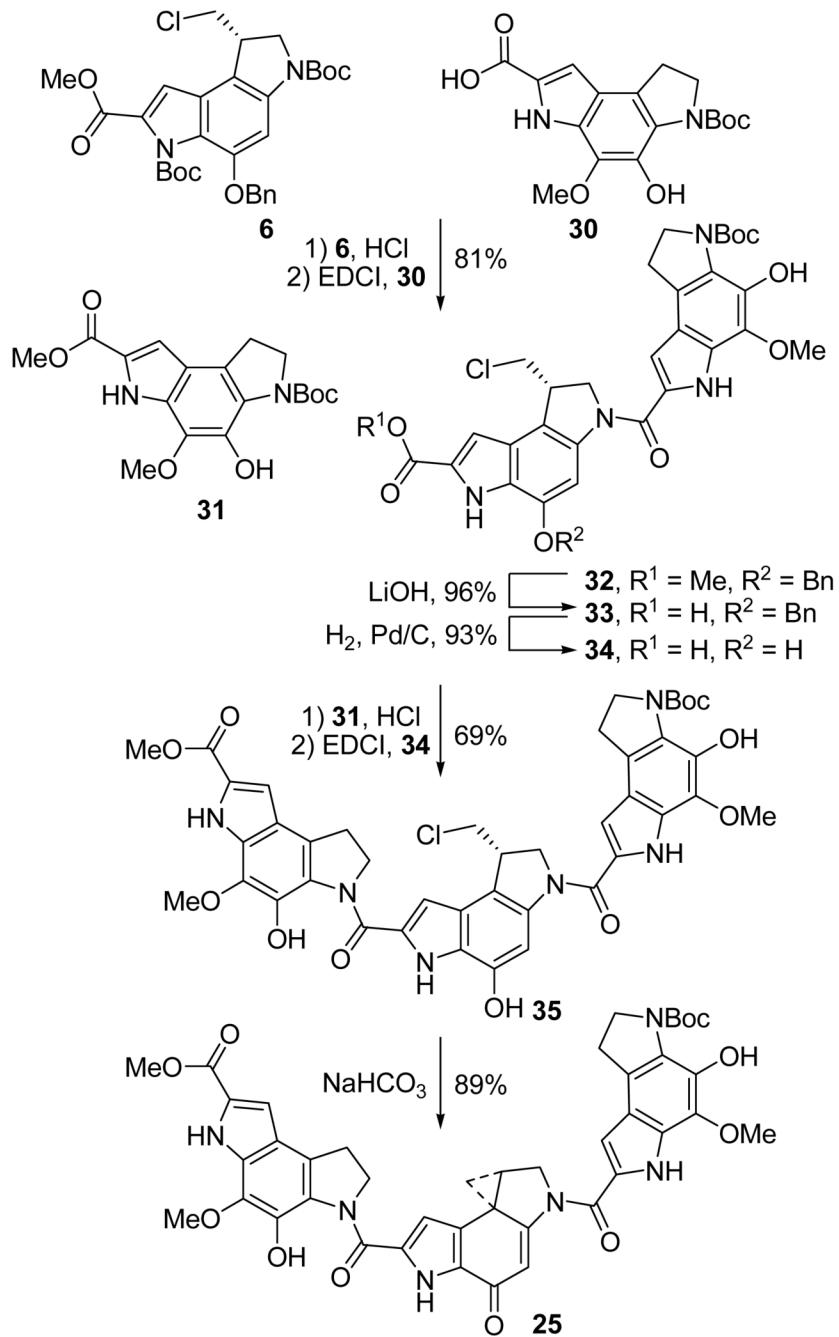
Scheme 4.



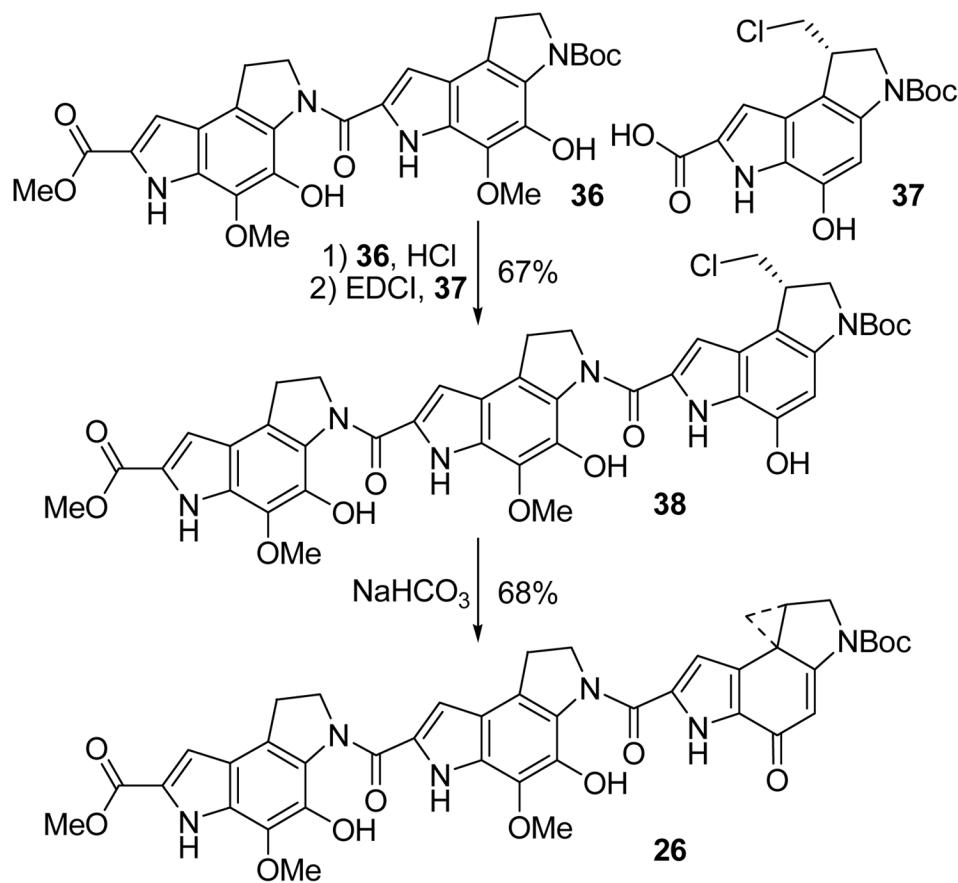
Scheme 5.



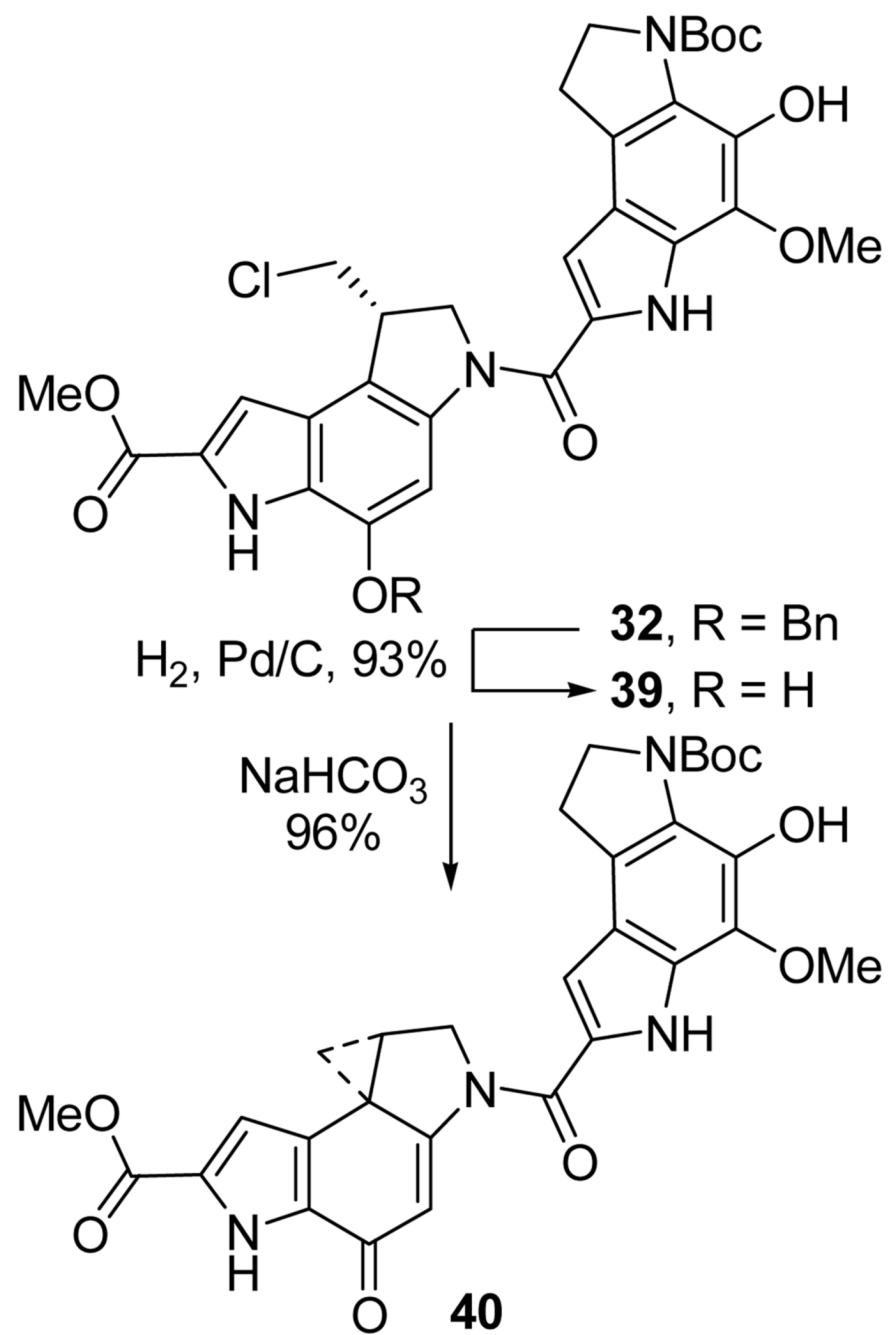
Scheme 6.



Scheme 7.



Scheme 8.



Scheme 9.