Syntheses and Biological Evaluation of Indolocarbazoles, Analogues of Rebeccamycin, Modified at the Imide Heterocycle

Pascale Moreau,† Fabrice Anizon,† Martine Sancelme,† Michelle Prudhomme,*,† Christian Bailly,‡ Carolina Carrasco,‡ Monique Ollier,§ Danièle Sevère,|| Jean-François Riou,|| Doriano Fabbro,⊥ Thomas Meyer,⊥ and Anne-Marie Aubertin▽

Synthèse et Etude de Systèmes à Intérêt Biologique, Université Blaise Pascal, UMR 6504 du CNRS, 63177 Aubière, France, INSERM U124 et Laboratoire de Pharmacologie Moléculaire Antitumorale du Centre Oscar Lambret, Place de Verdun, 59045 Lille, France, INSERM U71, Rue Montalembert, 63005 Clermont-Ferrand, France, Rhône-Poulenc Rorer, 13 Quai Jules Guesde, 93403 Vitry sur Seine, France, Département d'Oncologie, Novartis K-125-409, CH-4002 Bâle, Switzerland, and INSERM U74, Université Louis Pasteur, Strasbourg, France

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A series of 10 indolocarbazole derivatives, analogues to the antitumor antibiotic rebeccamycin, bearing modifications at the imide heterocycle were synthesized. They bear an *N*-methyl imide, *N*-methyl amide, or anhydride function instead of the original imide. Their inhibitory potencies toward topoisomerase I were examined using a DNA relaxation assay and by analyzing the drug-induced cleavage of ^{32}P-labeled DNA. Protein kinase C (PKC) inhibition and interaction with DNA were also studied together with the in vitro antiproliferative activities against B16 melanoma and P388 leukemia cells. The antimicrobial activities against two Gram-positive bacteria (*Bacillus cereus* and *Streptomyces chartreusis*), a Gram-negative bacterium (*Escherichia coli*), and a yeast (*Candida albicans*) were tested as well as their antiviral activities toward HIV-1. The efficiency of the anhydride compounds was compared to that of the parent compound rebeccamycin and its dechlorinated analogue. All the compounds studied were inactive against PKC. The structural requirements for PKC and topoisomerase I inhibition are markedly different. In sharp contrast with the structure−PKC inhibition relationships, we found that an anhydride function does not affect topoisomerase I inhibition, whereas a methyl group on the indole nitrogen prevents the poisoning of topoisomerase I. The compounds exhibiting a marked toxicity to P388 leukemia cells had little or no effect on the growth of P388CPT5 cells which are resistant to the topoisomerase I inhibitor camptothecin. This study reinforces the conclusion that the DNA−topoisomerase I cleavable complex is the primary cellular target of the indolocarbazoles and significantly contributes to their cytotoxicity and possibly to their weak but noticeable anti-HIV-1 activities. The structure−activity relationships are also discussed.

Introduction

DNA topoisomerase I catalyzes topological rearrangement of DNA essential for transcription, replication, and recombination processes.[1] This enzyme has been shown to be an important target in cancer therapy. Beside camptothecin and its derivatives,[2,3] indolocarbazole compounds including ED-110,[4] NB-506,[5] and rebeccamycin[6] exhibit antitumor properties very likely via topoisomerase I inhibition. These indolocarbazole compounds differ from indolocarbazole protein kinase C (PKC) inhibitors, such as staurosporine and UCN-01,[7,8] by a sugar moiety linked to only one indole nitrogen, while PKC inhibitors have their sugar unit attached to both indole nitrogens (Chart 1). This structural difference seems to be the major factor responsible for the selectivity in enzyme recognition.

In previous structure−activity relationship studies,[9,10] we have shown that the sugar moiety was a key element for both DNA-binding and topoisomerase I inhibition and that the rebeccamycin analogues were specific topoisomerase I inhibitors without any activity against topoisomerase II. The recognition of specific sequences in DNA was also investigated, and it was found that, unlike most DNA-binding small molecules, the rebeccamycin analogues appear to be highly sensitive to any modification of the exocyclic substituents on the bases in both the major and minor grooves of the double helix.[11] In this study, our purpose was to investigate the influence of functional modifications at the imide heterocycle and/or substitutions on the indole nitrogen on the biological activity as well as on topoisomerase I inhibition and interaction with DNA. Compounds **1**−**10** (Chart 2) were prepared by semisynthesis from rebeccamycin, an antibiotic isolated from cultures of *Saccharotrix aerocolonigenes*. To evaluate the role of the imide nitrogen in rebeccamycin, analogues were prepared with an anhydride or amide function instead of an imide function. Dibromoanhydride **7** was prepared to investigate the role of halogen atoms which in rebeccamycin probably increase membrane crossing.[9] The amide nitrogen was substituted with a methyl group in order to favor topoisomerase I poisoning compared to PKC

* Address correspondence to this author.
† Université Blaise Pascal.
‡ COL−INSERM U124.
§ INSERM U71.
|| Rhône-Poulenc Rorer.
⊥ Novartis.
▽ INSERM U74.

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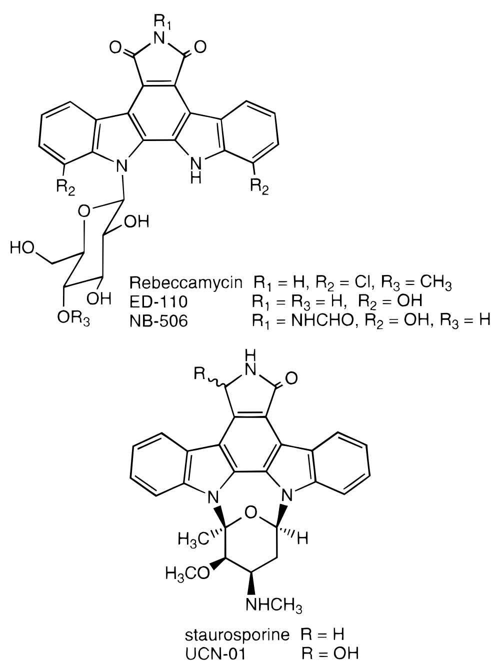
Chart 1



inhibition, as previously shown.[13] Since the substitution of the second indole nitrogen with a methyl group is consistent with PKC inhibition in indolocarbazole series, we investigated the effect of this substitution on topoisomerase I inhibition. The activity of the N-methylated and nonmethylated compounds was compared, thus providing useful information regarding the functional role of the indole nitrogen. The topoisomerase I inhibitory potencies of compounds **1**−**10** were examined using both DNA relaxation and cleavage assays. The PKC inhibition profiles were also determined, mainly to confirm the expected lack of effect toward this enzyme. In terms of biological activities, we investigated (i) the in vitro antiproliferative activities against murine B16 melanoma and P388 leukemia cell lines and (ii) their antimicrobial activities against two Gram-positive bacteria (*Bacillus cereus* and *Streptomyces chartreusis*), a Gram-negative bacterium (*Escherichia coli*), and a yeast (*Candida albicans*). In addition, the observation that topoisomerase I activity may be important for replication of human immunodeficiency virus type 1 (HIV-1),[14,15] prompted us to determine the activity of the drugs against HIV-1.

Chemistry

Compound **2** was prepared from the antibiotic rebeccamycin (**1**) by dimethylation with methyl iodide after deprotonation using sodium hydride. Fully dechlorinated rebeccamycin **3** was obtained by catalytic hydrogenolysis of **1** on palladium on activated carbon (Pd/C) in methanol in the presence of *tert*-butylamine. An alternative method for the dechlorination using ammonium formate and Pd/C proved equally efficient (76% and 77% yields, respectively). Reaction of **2** with ammonium formate and Pd/C in methanol yielded compound **4**.[16] The anhydride **5** was prepared by hydrogenolysis of rebeccamycin using Raney nickel in aqueous sodium hydroxide.[17] The same method was used for the preparation of the anhydride **6** from dimethylated rebeccamycin **2**. The dibromoanhydride **7** was synthesized by reaction of **5** with *N*-bromosuccinimide. From ^1H NMR data, the only possibilities for the bromo substituents on the indole moieties were at 3,9 or 2,10 positions. The structure of compound **7** was deduced from the literature data about electrophilic substitution on indolocarbazoles possessing electron-withdrawing groups in similar positions.[18−20] In an attempt to introduce a bromoacetyl group on its sugar moiety, a solution of anhydride **5** in dimethylformamide (DMF) was deprotonated with potassium *tert*-butylate (tBuOK; 1 equiv) at 120 °C before addition of bromoacetyl bromide. Compound **8** was obtained very likely via an addition−elimination reaction of the deprotonated hydroxyl at the 3′ position onto the carbonyl of DMF before the addition of bromoacetyl bromide. Effectively, the treatment of the anhydride **5** with tBuOK in DMF at the same temperature in the absence of bromoacetyl bromide furnished compound **8**. The position of the formyl group was deduced from $^1H-^1H$ COSY. *N*-Methylamides **9**,**9′** and **10**,**10′** were obtained from 6-*N*-methylrebeccamycin (**A**) and 6-*N*-methyl dechlorinated rebeccamycin **B**,[13] respectively, by reduction using zinc amalgam in acidic medium. The regioisomers could not be separated as already observed with amides **C**,**C′** and **D**,**D′** (Chart 3).[13,17] The regioisomeric ratios calculated from ^1H NMR spectra were 55:45 for **9**:**9′** and 62:38 for **10**:**10′**.

Results and Discussion

Self-Stacking of the Drugs in Solution. In preliminary experiments prior to investigating the interaction of the drugs with the complexes between DNA and topoisomerase I, we noticed that some of the drug solutions were highly sensitive to the temperature. As shown in Figure 1, the absorption spectrum of compound **8** changes drastically as the temperature varies from 30 to 60 °C. In contrast, little variation was observed with other drugs such as compound **9**. It turned out that the chlorinated drugs like rebeccamycin and compound **9** as well as the brominated derivative **7** were fairly insensitive to the variation of temperature, whereas the nonchlorinated drugs were very susceptible to the heating process. The nature of the other substituents on the drug chromophore and/or the sugar moiety can also affect the sensitivity of the drugs. For each drug solution we measured the variation of the absorbance at 280, 315, and 345 nm as a function of the temperature. The observed changes reflect the destacking of the drug chromophores in solution. As shown in Figure 2, the absorbance at 280 nm (open circles) only changes at temperatures of 60 °C for compound **6**, whereas at 60 °C compound **8** was already totally dissociated. The process is fully reversible. The changes are very weak with the chlorinated drug **9**. The different stacking properties of the drugs may account for their different capacity to interact with DNA and topoisomerase I. For instance, the greater efficiency of compound **8** compared to compound **6** in terms of topoisomerase I inhibition (see below) may be due, at least in part, to its higher propensity to dissociate in solution.

DNA Binding and Topoisomerase I Inhibition. The effects of the drugs on the activity of topoisomerase I were investigated by complementary assays using

Chart 2

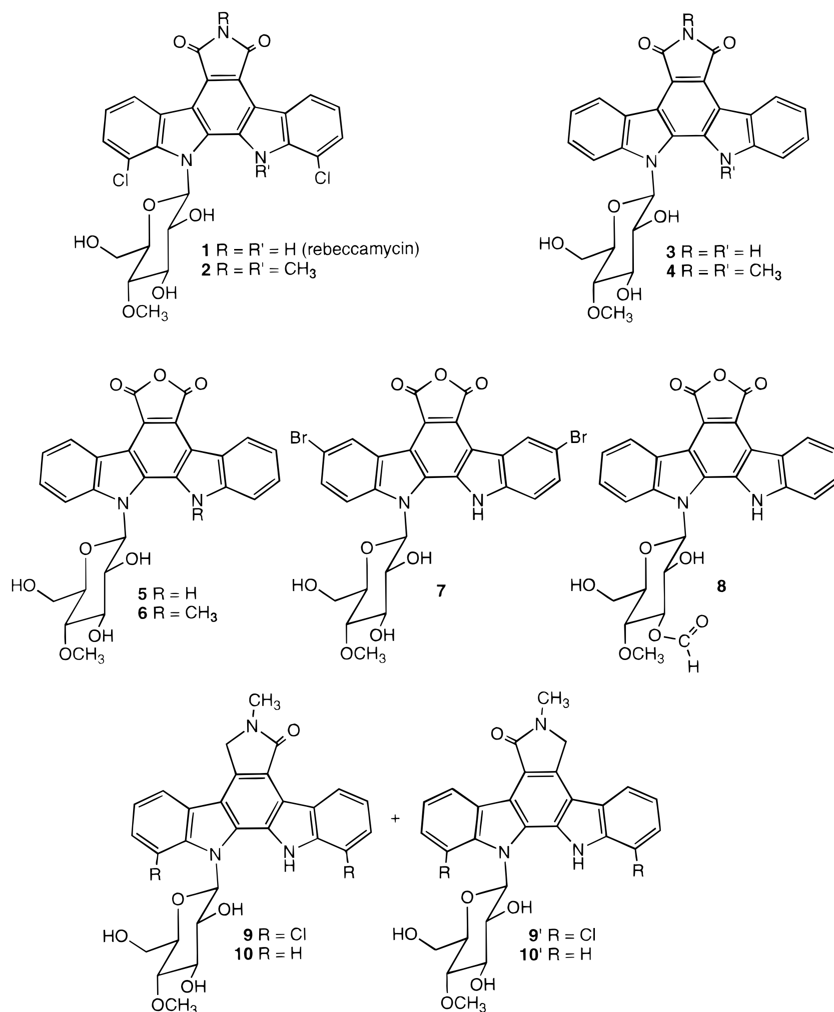
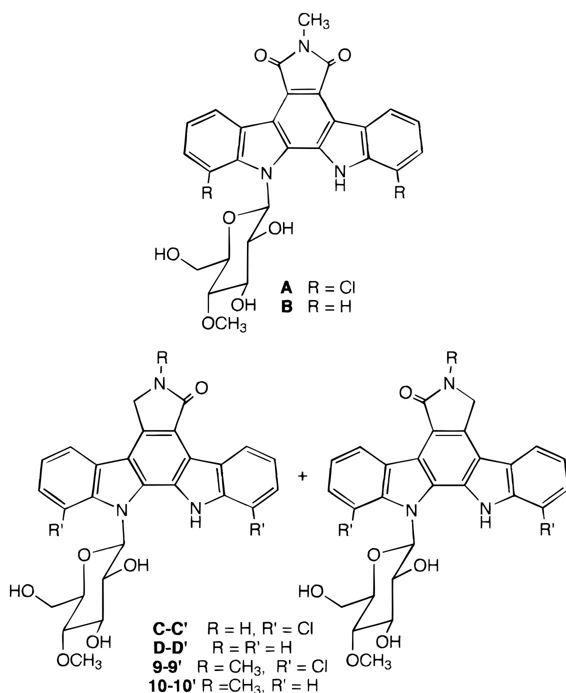


Chart 3



different DNA substrates. First, we resorted to a relaxation assay with supercoiled plasmid DNA. Closed circular DNA was treated with topoisomerase I in the absence and presence of the drug at 30 μM.

As shown in Figure 3, supercoiled DNA is fully relaxed by topoisomerase I in the absence of drug (compare lanes DNA and Topo I). The relaxed DNA migrates faster than the supercoiled plasmid on an agarose gel containing ethidium bromide. In the presence of the test drugs, topoisomerase I-mediated relaxation of DNA is either not affected (compound **2**) or partially (compound **6**) or completely (compound **10**) inhibited. In some cases the intensity of the band corresponding to the nicked form of DNA has increased significantly. This effect, observed with camptothecin, reflects the stabilization of topoisomerase I–DNA cleavable complexes. A substantial increase of the nicked DNA form is observed with different drugs, in particular compound **3** which seems to be the most active drug. In this assay, the drugs rank in the order **3** > **5** > **10** > **7** > **9** > **8** > **4** > **1** > **2** > **6**. A few topoisomerase I cleavage sites were sequenced. The cleavage sites detected using a 160-mer *Eco*RI-*Ava*I restriction fragment from plasmid pKM27 are shown in Figure 4 together with the positions of the drug binding sites inferred from DNaase I footprinting experiments. The results are fully compatible with those reported previously with another series of indolocarbazoles.[10] The two main cleavage sites detected with compound **5** correspond to a TpG step, and the footprint is located at a

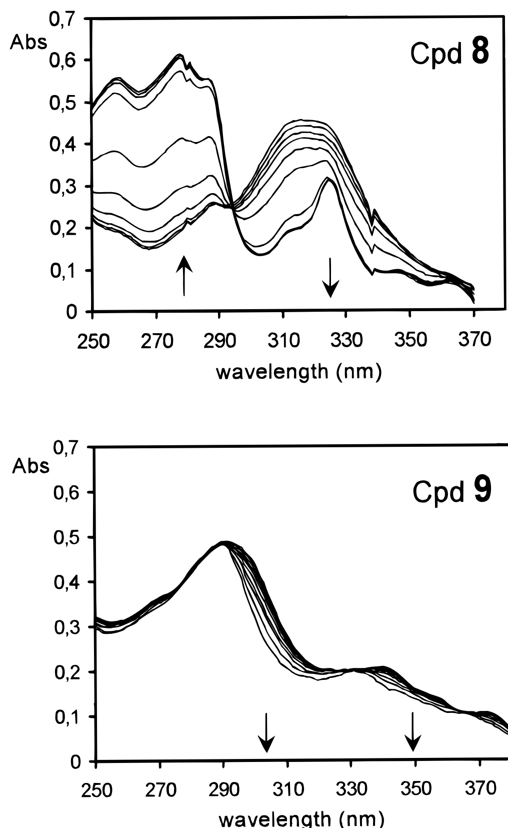


Figure 1. Absorption spectra of compounds **8** and **9** as a function of the temperature. A 3-mL drug solution at 10 μg/mL was heated from 20 to 90 °C with a heating rate of 1 °C/min, and the absorption spectra were recorded every 2 min. The temperature increased as follows (top to bottom curves at 310 nm): 21, 26, 32, 38, 44, 51, 56, 62, 67, and 75 °C. The measurements were performed in water. The concentration of DMSO (used to dissolve the drug) did not exceed 0.3% and could not interfere with the measurements.

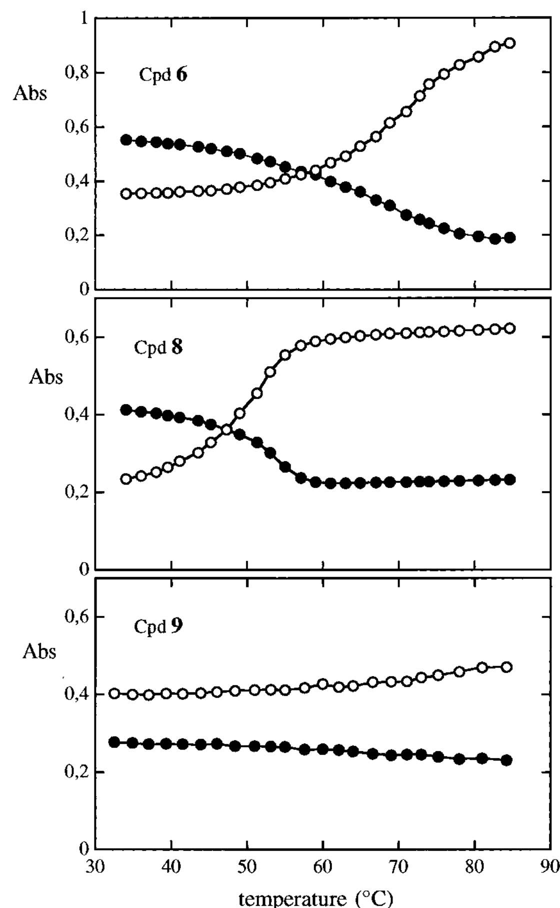


Figure 2. Plots of absorbance versus temperature. A 3-mL drug solution at 10 μg/mL was heated from 20 to 90 °C with a heating rate of 1 °C/min, and the absorbances at 280 nm (○) and at 315 or 345 nm (●) were measured simultaneously for compounds **6**, **8**, and **9**.

Figure 3. Inhibition of topoisomerase I-mediated relaxation of DNA by rebeccamycin analogues. Native supercoiled pKMp27 DNA (0.5 μg) (lane DNA) was incubated for 30 min at 37 °C with 6 units of human topoisomerase I in the absence (lane Topo I) or presence of the drug at 60 μg/mL. Reactions were stopped with sodium dodecyl sulfate and treatment with proteinase K. The DNA samples were run on an agarose gel containing ethidium bromide (1 mg/mL). Nck, nicked; Rel, relaxed; Sc, supercoiled. The gel was photographed under UV light.

CG site on the 3′ side of the cutting point. Compound **5** and the other analogues strongly discriminate against run of A and T. Binding occurs preferentially at sequences containing GC or GT sites. The footprinting data are also compatible with those reported very recently with related drugs.[11] From these data, we can conclude that the anhydride structure neither affects the capacity of the drugs to recognize specific sequences in DNA nor profoundly modifies the capacity of the drug to interfere with topoisomerase I.

In order to better compare the efficiency of the different drugs, the topoisomerase I inhibitory properties of compounds **1**−**10** were further examined using the ^{32}P-labeled *Eco*RI-*Hin*dIII restriction fragment of pBR322 as a substrate. The labeled DNA fragment was incubated with topoisomerase I in the presence and absence of the indolocarbazoles **1**−**10** at concentrations ranging from 0.01 to 10 μg/mL, and the resulting DNA cleavage products were analyzed by agarose gel electrophoresis under alkaline conditions. The inhibitory potency of the test compounds was assessed by comparing the cleavage of DNA by the enzyme in the absence and presence of the drug. The relative efficiency of the drugs to stimulate DNA cleavage varies markedly from one congener to another (MIC values in Table 1A).

The most potent topoisomerase I inhibitor in this series was the dechlorinated rebeccamycin derivative **3**. The introduction of a methyl substituent on the indole nitrogen of compounds **2**, **4**, and **6** led to inactivity toward topoisomerase I compared to the parent compounds **1**, **3**, and **5** which were efficient inhibitors. This key observation raises the question of the role of the indole NH which may be involved in a hydrogen bond with one oxygen of a hydroxyl group of the methoxyglucose residue. We believe that the 6′-OH group on

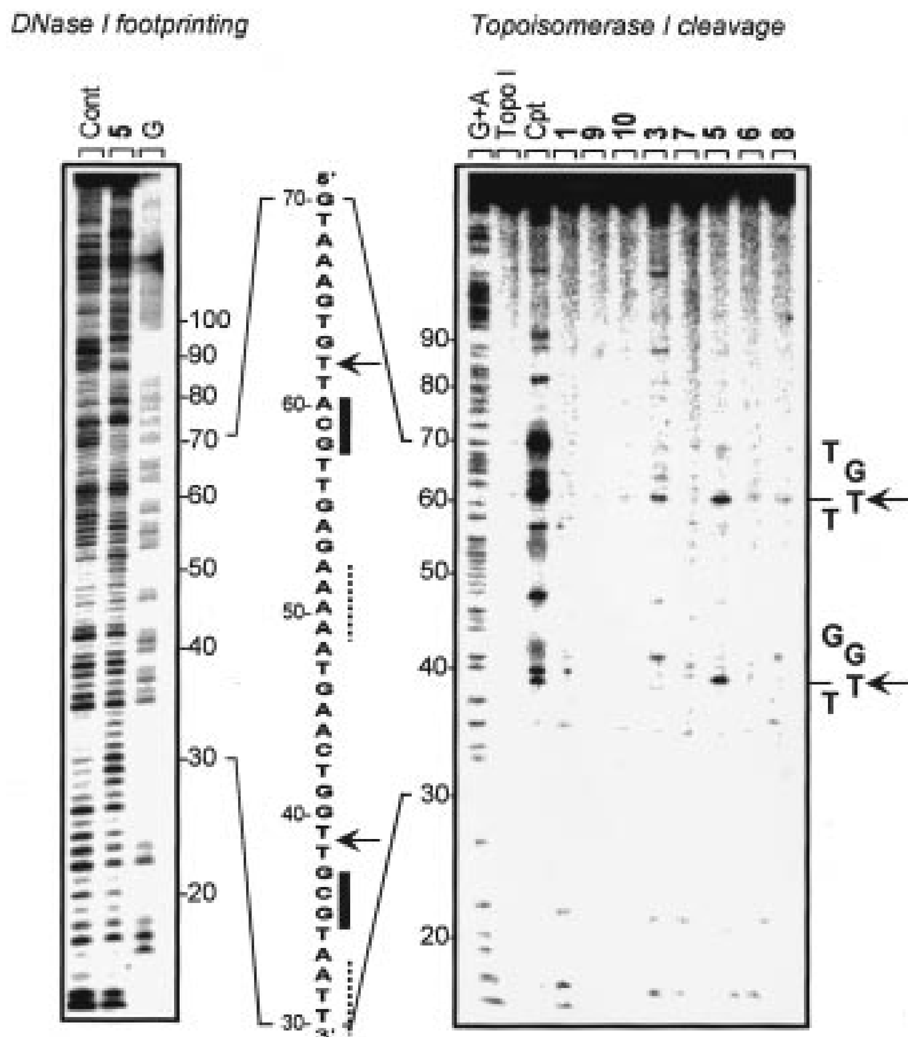


Figure 4. Sequencing of topoisomerase I cleavage sites (right panel) and sequence-selective binding (left panel). In each case, the 160-mer *Eco*RI-*Ava*I fragment from plasmid pKM27 was 3′-end-labeled at the *Eco*RI site with [α-^{32}P]dATP in the presence of AMV reverse transcriptase and then subjected to cleavage by DNaase I or topoisomerase I. The cleavage products were resolved on an 8% polyacrylamide gel containing 7 M urea. Guanine- and purine-specific sequence markers obtained by treatment of the DNA with dimethyl sulfate and formic acid, respectively, followed by piperidine were run in lanes marked G or G+A. The control track (Cont) contained no drug. The lane Topo I refers to the radiolabeled DNA substrate incubated with the enzyme but with no drug. Camptothecin was used at 0.3 μg/mL. Numbers on the side of the gels refer to the standard numbering scheme for the nucleotide sequence of the DNA fragment. The positions of the main topoisomerase I cleavage sites (arrows) and the binding sites (inferred from a quantitative analysis of the footprinting gel) are indicated by black boxes on the side of the sequence between the two panels. The sites where the cleavage by DNaase I is enhanced in the presence of the drug are indicated by a dashed line (at AT-rich sequences).

the carbohydrate is hydrogen bonding with the indole NH for three reasons. First, the molecular modeling analysis suggests that an internal hydrogen bond is formed between the 6′-OH and the indole NH.[21] Second, we have shown recently that the fucose derivatives of rebeccamycin are considerably less efficient than the corresponding glucose or galactose derivatives, suggesting also that the 6′-OH group (which is lacking on a fucose residue) is important for topoisomerase I inhibition and cytotoxicity.[13] Third, the presence of a hydrogen bond is known to induce deshielding. Actually, the chemical shifts observed for the triplet of 6′-OH are 5.40 ppm in rebeccamycin, 4.97 ppm in the analogue **2** methylated at the indole nitrogen, 6.22 ppm in **3**, 5.06 ppm in **4**, 6.22 ppm in anhydride **5**, and 5.06 ppm in the methylated analogue **6**. The H bond may serve to maintain the carbohydrate in a fixed conformation, optimal for interaction with the topoisomerase I–DNA complex.

Compound **5** with an anhydride function proved to be slightly less efficient at inhibiting topoisomerase I than compound **3** with an imide function at the upper heterocycle. The observation that compounds **5**, **7**, and **8** retained significant inhibitory potencies against the enzyme confirms that the anhydride function is not detrimental to the topoisomerase I inhibition process. Surprisingly, the introduction of bromo substituents at positions 3 and 9 on the indole moiety of compound **7** led to a better efficiency against topoisomerase I than the parent compound **5**. It is clear that the dechlorinated compounds are more active toward the enzyme than the corresponding 1,11-dichloro analogues (compare **1** and **3**, **9**, and **10**; also see our previous studies[9,13]). The introduction of a formyl group at the 3′ position reduces the capacity of the drug to inhibit topoisomerase I (compare **8** and **5**). On account of the strong inhibitory potencies of previously studied amides **C**,**C′** and **D**,**D′** (Chart 3) and given that a methyl group on the nitrogen

Table 1. Inhibitory Activities toward PKC and Topoisomerase I, Antiproliferative Activities in Vitro against Murine B16 Melanoma, P388 Leukemia Cells, and P388CPT5 Cells Resistant to Topoisomerase I Inhibitor Camptothecin ($R = IC_{50}$ P388CPT5/IC_{50} P388), Antimicrobial Activities against *B. cereus*, and Anti-HIV Activities in HIV-1 Lai-Infected CEM-SS Cells (Selectivity Index: SI = CC_{50}/IC_{50})

A. Compounds **1**–**10**

compd	IC_{50} (μM) B16	IC_{50} (μM) P388	IC_{50} (μM) P388CPT5 (*R*)	*B. cereus* MIC (μM)	HIV-1 Lai CEM-SS IC_{50} (μM), CC_{50} (μM), SI	topoisomerase I MIC (μM)
1	0.48	1.22	10.5 (8.5)	10.9	0.52, 1.05, 2.0	1.75
2	35	>16.7		>83	8.7, >16, >1.9	>16.7
3	0.90	0.69	>20 (>28)	>100	0.16, 0.90, 5.6	0.59
4	8	>19		94	>19, >19	>19
5	17.5	6.36	>20 (>3.1)	>99	>1.98, >1.98	1.99
6	31	>19		>97	>9.7, >9.7	>19
7	13	0.91	7.58 (8.3)	>75	4.85, 9.39, 1.9	1.52
8	nd	>19		>94	>9.4, >9.4	5.7
9,**9′**	0.48	0.52	4.03 (7.7)	>88	0.31, 1.72, 5.5	17.6
10,**10′**	5	3.39	>19.9 (>5.8)	24	0.60, 8.38, 14	19.9

B. Compounds **A**, **B**, **C**,**C′**, and **D**,**D′**

compd	IC_{50} (μM) PKC	IC_{50} (μM) B16	IC_{50} (μM) P388	*B. cereus* MIC (μM)	HIV-1 Lai CEM-SS IC_{50} (μM), CC_{50} (μM), SI	topoisomerase I MIC (μM)
A	>100	0.61	0.6	>85	5.06, 8.06, 1.6	>17
B	>100	1.06	0.7	1.55	4.17, 7.96, 1.9	0.6
C,**C′**	28.8	>125	3	5.6	0.36, 1.15, 3.2	1.8
D,**D′**	3.7	nd	3	25	4.1, 14.3, 3.5	0.02

of the upper heterocycle was consistent with topoisomerase I inhibition,[9,13] compounds **9**,**9′** and **10**,**10′** were prepared. Their biological activity could be compared to that of **C**,**C′**, **D**,**D′**, and N-methylated imides **A** and **B**.[13]

For this series (Table 1B) and as previously observed, we found once again that methyl substitution strongly decreases the activity against topoisomerase I and that chlorination leads to an important decrease of activity. In terms of topoisomerase I inhibition, the following sequence can be determined for the dechlorinated series: amide > imide ≈ *N*-methyl imide > *N*-methyl amide.

Protein Kinase C Inhibition. Indolocarbazole PKC inhibitors such as staurosporine are known antitumor agents which prevent the phosphorylation on serine/threonine residues of proteins involved in cell proliferation, differentiation, and gene expression.[7] But because PKC is able to phosphorylate a large number of substrates, this enzyme controls a wide range of cellular processes, and consequently, its inhibitors may induce serious side effects when used for therapies. Moreover, topoisomerase I activity is controlled by different kinases, and PKC is able to activate topoisomerase I by phosphorylating serine residues.[22] This prompted us to examine PKC inhibition by compounds **1**–**10** so as to establish whether or not PKC is involved in the antiproliferative activity of the studied indolocarbazoles. All compounds were inactive toward PKC-α, the most common PKC isoenzyme. The anticipated lack of effect on PKC is attributed either to the incorporation of the anhydride function or to the substitution of the upper nitrogen with a methyl group. In other series, such structural modifications have previously been correlated with the failure to inhibit PKC.[23,24] The intracellular target should be restricted to much fewer proteins, possibly to topoisomerase I only, and as a consequence the biological effect may be cell- and/or tissue-selective rather than directed to all cell populations as is generally the case with PKC inhibitors.

Table 2. In Vitro Growth Inhibitory Effect of Compounds **1**–**10** against Two Gram-Positive Bacteria (*B. cereus* and *S. chartreusis*), a Gram-Negative Bacterium (*E. coli*), and a Yeast (*C. albicans*)[a]

compd	*B. cereus* ATCC 14579	*S. chartreusis* NRRL 11407	*E. coli* ATCC 11303	*C. albicans* IP 444
1	++	++	−	−
2	++	±	−	−
3	±	−	−	−
4	+	+++	−	−
5	−	±	−	−
6	−	±	−	−
7	−	−	−	−
8	+	+++	−	−
9,**9′**	−	++	−	−
10,**10′**	++	++	−	−

[a] The size of zones of growth inhibition was 10–12 (+++), 8–9 (++), 7–8 (+), and 6–7 (±) mm.

Antimicrobial Activities. Antibiogram tests on two Gram-positive bacteria (*B. cereus* and *S. chartreusis*), a Gram-negative bacterium (*E. coli*), and a yeast (*C. albicans*) showed that **1**–**10** were inactive against *E. coli* and *C. albicans* (Table 2). Dimethylated compounds **2** and **4** that are neither topoisomerase I nor PKC inhibitors exhibited antimicrobial activities against *B. cereus* and *S. chartreusis* suggesting that other mechanisms may be involved.

Anti-HIV-1 Activity. The anti-HIV-1 activity of **1**–**10**,**10′** in CEM-SS cells was measured by quantification of the reverse transcriptase activity associated with virus particles released from HIV-1 Lai-infected cells in the culture medium. The cytotoxicity of the drugs (CC_{50}) was evaluated in parallel to the IC_{50} values, and the selectivity index (CC_{50}/IC_{50}) was calculated (Table 1A). Compounds **1**, **3**, **9**,**9′**, and **10**,**10′** were the most active compounds with IC_{50} values < 1 μM. The selectivity indexes are significant for dechlorinated rebeccamycin **3** and **9**,**9′** (about 5.5) and even more for the dechlorinated analogue **10**,**10′** (14). Their anti-HIV-1 activity could not be correlated to PKC inhibition. It could not be strictly correlated to the IC_{50} values

observed for topoisomerase I inhibition, but however, it is interesting to note that in the DNA relaxation experiments in the presence of topoisomerase I (Figure 3) compounds **3**, **9**,**9′**, and **10**,**10′** were among the most potent inhibitors. Therefore, it is plausible that topoisomerase I contributes, at least partially, to the anti-HIV-1 activity of the drugs. This hypothesis merits to be considered since it is known that topoisomerase I markedly enhanced HIV-1 reverse transcriptase activity in vitro and that the topoisomerase I-specific inhibitor topotecan inhibited this phenomenon.[25,26] Camptothecin also blocks the replication of the equine infectious anemia virus (EIAV is a retrovirus homologous to HIV) in chronically infected CF2Th cells.[27] The potential relationships between inhibition of topoisomerase I by indolocarbazoles and their antiviral properties open new synthetic strategies for the design of anti-AIDS drugs targeting topoisomerase I.

In vitro Antiproliferative Activity. The in vitro antiproliferative activity of **1**−**10** toward B16 melanoma and P388 leukemia cells was examined (Table 1). Amide **9**,**9′** had the strongest cytotoxicity against both cell lines but possessed 10-fold lower activity against topoisomerase I when compared to structural analogues of rebeccamycin on the upper heterocycle with similar cytotoxic activity (**1**, **3**, **7**). Consequently, the cytoxicity of this compound is likely not attributable, or to a very limited extent, to topoisomerase I inhibition. In contrast, for the most potent topoisomerase I inhibitor in this series, compound **3**, which also exhibits strong cytotoxicity, the inhibition of topoisomerase I is possibly involved in the antiproliferative process. To have an insight into the involvement of topoisomerase I inhibition in the cytotoxicity, we measured the toxicities of compounds **1**−**10** toward P388 and P388CPT5 leukemia cells sensitive and resistant to the topoisomerase I inhibitor camptothecin, respectively. The resistance of the P388CPT5 cell line has been attributed to the expression of a deficient form of topoisomerase I as a result of a mutation in the *top1* gene of these cells.[28] The results indicate that the drugs, when they exhibit any antiproliferative activity, are much more toxic against P388 cells than to the resistant cells suggesting that the toxicity is, at least partially, linked to topoisomerase I inhibition. The largest resistance index (i.e., the ratio between IC_{50} P388CPT5 and IC_{50} P388) was obtained for compound **3**. It is also noticeable that amide **9**,**9′** displays the higher cytotoxicity against resistant cells, suggesting a lower participation of topoisomerase I in its mechanism of action. Lack of direct relationship between topoisomerase I poisoning and antiproliferative activity could be due to different cellular uptake exhibited by drugs of various solubilities.

Summary

In conclusion, the collective picture which emerges from the variety of experiments reported in this paper is that (i) the newly introduced anhydride function does not prevent topoisomerase I inhibition, but however, it does not confer higher activities, in terms of DNA recognition, topoisomerase I inhibition, and cytotoxicity, compared to the amide function; (ii) the introduction of a methyl group on the indole nitrogen is not compatible with topoisomerase I inhibition, suggesting indirectly that the free NH is required for a proper positioning of the carbohydrate with respect to the topoisomerase I−DNA complex; (iii) topoisomerase I is the most likely cellular target for indolocarbazoles, even if no strict correlation emerges from the in vitro DNA cleavage assays and the biological activities; and (iv) for the first time, it is shown that some rebeccamycin analogues exhibit weak but noticeable anti-HIV-1 properties, thus providing new synthetic perspectives for the design of antiviral drugs. The challenge, revived by the increasing resistance of HIV to drugs such as Zidovudine (AZT), is to assist the development of efficient and nontoxic anti-AIDS agents.

Among the compounds tested in this study, the dechlorinated rebeccamycin analogue **3** proved to be the most efficient topoisomerase I inhibitor and is more efficient than the natural metabolite rebeccamycin in terms of both cytotoxicity against P388 cells and anti-HIV activity. The introduction of a water-solubilizing substituent on the phenyl moieties of **3** may help to improve its pharmacological profile. We are now investigating this field.

Experimental Section

Chemistry. IR spectra were recorded on a Perkin-Elmer 881 spectrometer (ν in cm^{-1}). NMR spectra were performed on a Bruker AC 400 spectrometer (^1H, 400 MHz; ^{13}C, 100 MHz) (chemical shifts in δ (ppm), with the following abbreviations: singlet (s), doublet (d), doubled doublet (dd), triplet (t), pseudotriplet (pt), doubled triplet (dt), multiplet (m), tertiary carbons (C tert), quaternary carbons (C quat)). The signals were assigned from $^1H-^1H$ COSY and $^{13}C-^1H$ correlations. Mass spectra (FAB+) were determined at CESAMO (Talence, France) on a high-resolution Fisons Autospec-Q spectrometer. Chromatographic purifications were performed by flash silica gel Geduran SI 60 (Merck) (0.040−0.063) mm or Kieselgel 60 (Merck) (0.063−0.200 mm) column chromatography. For purity tests, TLC was performed on fluorescent silica gel plates (60 F_{254} from Merck). Rebeccamycin was from our laboratory stock sample.

1,11-Dichloro-12-(4-*O*-methyl-*β*-D-glucopyranosyl)-6,13-dimethyl-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]-carbazole-5,7-dione (2). A suspension of NaH (102 mg, 60% dispersion in mineral oil, 2.55 mmol) in DMF (4 mL) was poured into a solution of rebeccamycin (500 mg, 0.88 mmol) in DMF (10 mL). The mixture was stirred for 1 h at room temperature before the addition of methyl iodide (0.128 mL, 2.05 mmol) and then stirred at room temperature for 20 h. After addition of water and extraction with EtOAc, the organic phase was dried over $MgSO_4$, the solvent was removed, and the residue was purified by chromatography (eluent, EtOAc−cyclohexane, 80:20) to give **2** (329 mg, 0.55 mmol, 63% yield) as a yellow solid. IR (KBr): $\nu_{C=O}$ 1690, 1750 cm^{-1}, ν_{OH} 3300−3500 cm^{-1}. Mp: 280 °C. HRMS (FAB+) $(M + H)^+$: calcd for $C_{29}H_{26}Cl_2N_3O_7$, 598.1148; found, 598.1129. ^1H NMR (400 MHz, DMSO-d_6): 3.05 (3H, s, N_{imide}-CH_3), 3.19 (1H, t, J = 9.3 Hz), 3.36 (1H, m), 3.53 (3H, s, OCH_3), 3.67 (2H, m), 3.80 (1H, m), 3.95 (1H, dd, J_1 = 10.3 Hz, J_2 = 5.4 Hz), 4.34 (1H, d, J = 6.9 Hz, OH), 4.38 (3H, s, N_{indole}-CH_3), 4.97 (1H, t, J = 5.0 Hz, $OH_{6'}$), 5.25 (1H, d, J = 4.4 Hz, OH), 5.48 (1H, d, J = 9.3 Hz, $H_{1'}$), 7.30 (1H, t, J = 7.9 Hz), 7.41 (1H, t, J = 7.9 Hz), 7.58 (2H, pt, J = 7.9 Hz), 8.96 (1H, d, J = 8.2 Hz), 9.12 (1H, d, J = 7.9 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): 23.6 (N_{imide}-CH_3), 36.9 (N_{indole}-CH_3), 59.8 (OCH_3), 60.9 ($C_{6'}$), 71.8, 77.3, 78.4, 78.9 ($C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 88.2 (C1′), 117.1, 119.0, 119.3, 119.4, 119.6, 121.0, 125.0, 128.4, 134.0, 134.6, 139.9, 140.3 (C quat arom), 122.4, 123.5, 123.7, 123.8, 129.1, 129.7 (C tert arom), 168.4, 168.5 (C=O).

12-(4-*O*-Methyl-*β*-D-glucopyranosyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (3). A mixture of rebeccamycin (50 mg, 0.088 mmol), methanol (30

mL), and catalytic amounts of 5% Pd/C and *tert*-butylamine (50 mg) was hydrogenated for 6 h (40 psi). After filtration over Celite and washing with THF, the filtrate was evaporated and the residue purified by flash chromatography (eluent, cyclohexane–EtOAc, 30:70) to give **3** (34 mg, 0.067 mmol, 76% yield) as a yellow solid.

Or, to a solution of rebeccamycin (1g, 1.75 mmol) in methanol (200 mL) were added 5% Pd/C (250 mg) and $HCOONH_4$ (884 mg). The mixture was stirred at room temperature for 72 h. After filtration, the catalyst was washed with THF, the solvents were removed, and the residue was purified by flash chromatography (77% yield). IR (KBr): $\nu_{C=O}$ 1705, 1750 cm^{-1}, $\nu_{NH,OH}$ 3200–3600 cm^{-1}. Mp: 239–241 °C. HRMS (FAB+) $(M + H)^+$: calcd for $C_{27}H_{24}N_3O_7$, 502.1614; found, 502.1625. ^1H NMR (400 MHz, DMSO-d_6): 3.63 (1H, m), 3.73 (3H, s, OCH_3), 3.74–4.10 (5H, m), 5.06 (1H, d, J = 5.4 Hz, OH), 5.37 (1H, d, J = 5.4 Hz, OH), 6.22 (1H, t, J = 4.0 Hz, OH), 6.36 (1H, d, J = 8.9 Hz, $H_{1'}$), 7.44 (2H, t, J = 7.4 Hz), 7.65 (2H, t, J = 7.9 Hz), 7.78 (1H, d, J = 7.9 Hz), 8.02 (1H, d, J = 8.4 Hz), 9.15 (1H, d, J = 7.9 Hz), 9.23 (1H, d, J = 7.9 Hz), 11.18 (1H, s, N_{imide}-H), 11.68 (1H, s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 58.5 ($C_{6'}$), 60.1 (OCH_3), 73.2, 76.3, 77.2, 77.3, 84.2 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 111.7, 112.2, 120.4, 120.7, 124.5 (2C), 126.8, 127.0 (C tert arom), 117.0, 118.5, 119.5, 121.1 (2C), 121.5, 128.3, 129.7, 140.8, 142.1 (C quat arom), 170.9, 171.0 (C=O).

6,13-Dimethyl-12-(4-*O*-methyl-*β*-D-glucopyranosyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (4). To a solution of compound **2** (119 mg, 0.20 mmol) in methanol (20 mL) were added ammonium formate (458 mg, 7.3 mmol) and 5% palladium on activated carbon (458 mg). The mixture was stirred at room temperature under argon atmosphere for 12 h. After filtration over Celite and washing with methanol and THF, the solvents were removed and the residue was purified by flash chromatography (eluent, EtOAc) to give **4** (47 mg, 0.09 mmol, 45% yield) as a yellow solid. IR (KBr): $\nu_{C=O}$ 1698, 1754 cm^{-1}, ν_{OH} 3200–3600 cm^{-1}. Mp: 286 °C. HRMS (FAB+) (M^+): calcd for $C_{29}H_{27}N_3O_7$, 529.1849; found, 529.1846. ^1H NMR (400 MHz, DMSO-d_6): 3.16 (3H, s, NCH_3), 3.33 (1H, t, J = 9.4 Hz), 3.51 (1H, m), 3.58 (3H, s, OCH_3), 3.76 (1H, m), 3.84 (2H, m), 3.95 (1H, m), 4.23 (3H, s, NCH_3), 4.53 (1H, d, J = 5.4 Hz, OH), 5.06 (1H, t, J = 5.4 Hz, OH), 5.20 (1H, d, J = 5.1 Hz, OH), 5.70 (1H, d, J = 8.9 Hz, $H_{1'}$), 7.41 (1H, t, J = 6.9 Hz), 7.44 (1H, t, J = 7.9 Hz), 7.58 (1H, t, J = 7.9 Hz), 7.67 (1H, t, J = 7.9 Hz), 7.72 (1H, d, J = 8.4 Hz), 7.97 (1H, d, J = 8.4 Hz), 9.02 (1H, d, J = 7.9 Hz), 9.08 (1H, d, J = 7.9 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): 23.6 (NCH_3), 34.2 (NCH_3), 59.8 (OCH_3), 60.6 ($C_{6'}$), 70.0, 76.9, 78.2, 79.0, 88.3 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 111.0, 115.5, 120.9, 122.0, 124.3, 124.8, 126.6, 127.6 (C tert arom), 118.7 (2C), 119.0, 120.1, 121.6, 124.4, 132.0, 132.1, 141.7, 144.4 (C quat arom), 169.2, 169.3 (C=O).

12-(4-*O*-Methyl-*β*-D-glucopyranosyl)-13-*N*-methyl-6,7,12,13-tetrahydro-5,7-dioxo(5*H*)indolo[2,3-*a*]furo[3,4-*c*]carbazole (6). A mixture of compound **2** (329 mg, 0.55 mmol) and NaOH (1 g) was suspended in water (80 mL) and refluxed before addition of Raney nickel (3 g) by portions. The mixture was refluxed for 3 h. After filtration over Celite and washing with hot water, the filtrate was acidified to pH 1 and extracted with EtOAc. The organic phases were dried over $MgSO_4$, the solvents were removed, and the residue was purified by flash chromatography (eluent, EtOAc–cyclohexane, 80:20) to give **6** (126 mg, 0.24 mmol, 45% yield) as a yellow solid. IR (KBr): $\nu_{C=O}$ 1754, 1825 cm^{-1}, ν_{OH} 3200–3600 cm^{-1}. Mp: >300 °C. HRMS (FAB+) $(M + H)^+$: calcd for $C_{28}H_{25}N_2O_8$, 517.1611; found, 517.1591. ^1H NMR (400 MHz, DMSO-d_6): 3.32 (1H, t, J = 9.4 Hz), 3.53 (1H, m), 3.57 (3H, s, OCH_3), 3.65–3.97 (4H, m), 4.31 (3H, s, NCH_3), 4.63 (1H, d, J = 5.9 Hz, OH), 5.06 (1H, m, OH), 5.23 (1H, d, J = 6.4 Hz, OH), 5.78 (1H, d, J = 8.9 Hz, $H_{1'}$), 7.54 (1H, t, J = 7.9 Hz), 7.55 (1H, t, J = 8.4 Hz), 7.69 (1H, t, J = 8.4 Hz), 7.79 (1H, t, J = 7.9 Hz), 7.87 (1H, d, J = 8.4 Hz), 8.05 (1H, d, J = 8.4 Hz), 8.95 (1H, d, J = 7.4 Hz), 8.98 (1H, d, J = 7.4 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): 34.4 (NCH_3), 59.8 ($C_{6'}$), 59.9 (OCH_3), 70.2, 76.8, 78.3, 78.9, 88.5 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 111.4, 115.7, 121.6, 122.5, 123.6, 124.0, 127.2, 128.2 (C tert arom), 117.2, 118.8, 119.0, 119.1, 121.1, 123.7, 132.9, 133.0, 141.5, 144.2 (C quat arom), 164.3 (2 C=O).

3,9-Dibromo-12-(4-*O*-methyl-*β*-D-glucopyranosyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (7). A solution of *N*-bromosuccinimide (NBS; 178 mg, 1 mmol) in THF (3 mL) was added dropwise into a solution of compound **5** (50 mg, 0.10 mmol) in THF (2 mL) at 0 °C. The mixture was stirred at room temperature for 1 week. After addition of water and extraction with EtOAc, the organic phase was dried over $MgSO_4$. The solvent was removed and the residue was purified by chromatography (eluent, EtOAc–cyclohexane, 70:30) to give **7** (47 mg, 0 07 mmol, 71% yield) as a yellow solid. IR (KBr): $\nu_{C=O}$ 1750, 1825 cm^{-1}, $\nu_{NH,OH}$ 3200–3600 cm^{-1}. Mp: >315 °C. HRMS (FAB+) $(M + H)^+$: calcd for $C_{27}H_{21}N_2O_8Br_2$, 660.9644; found, 660.9807. ^1H NMR (400 MHz, DMSO-d_6): 3.40 (1H, m), 3.68 (4H, s + m, 1H + OCH_3), 3.77 (2H, m), 4.01 (2H, m), 5.08 (1H, d, OH, J = 5.4 Hz), 5.38 (1H, d, OH, J = 5.9 Hz), 6.38 (1H, br s, OH), 6.40 (1H, d, $H_{1'}$, J = 8.9 Hz), 7.74 (1H, d, J = 8.3 Hz), 7.76 (1H, dd, J_1 = 8.8 Hz, J_2 = 1.9 Hz), 7.83 (1H, dd, J_1 = 8.8 Hz, J_2 = 2.0 Hz), 8.02 (1H, d, J = 9.3 Hz), 8.96 (1H, d, J = 2.0 Hz), 9.04 (1H, d, J = 2.0 Hz), 12.0 (1H, s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 58.5 ($C_{6'}$), 60.1 (OCH_3), 73.4, 76.0, 77.2, 77.4, 84.4 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 113.2, 113.4, 115.9, 117.5, 117.8, 119.5, 121.9, 122.4, 129.2, 130.4, 139.3, 140.8 (C quat arom), 114.4, 114.6, 125.3, 125.6, 129.7, 130.1 (C tert arom), 164.2, 164.3 (C=O).

12-(3-*O*-Formyl-4-*O*-methyl-*β*-D-glucopyranosyl)-6,7,12,13-tetrahydro-5,7-dioxo(5*H*)indolo[2,3-*a*]furo[3,4-*c*]carbazole (8). To a solution of anhydride **5** (263 mg, 0.53 mmol) in DMF (7 mL) was added tBuOK (59 mg, 0.53 mmol). The mixture was stirred at room temperature for 1 h. Bromoacetyl bromide (46 μL, 0.53 mmol) was then added, and the mixture was stirred at 120 °C for 24 h. After addition of water and extraction with EtOAc, the organic phase was dried over $MgSO_4$, the solvent was evaporated, and the residue was purified by chromatography (eluent, cyclohexane–EtOAc, 40:60) to give **8** (27 mg, 0.051 mmol, 10% yield). IR (KBr): $\nu_{C=O}$ 1750, 1825 cm^{-1}, $\nu_{NH,OH}$ 3100–3600 cm^{-1}. Mp: >250 °C. HRMS (FAB+) $(M + H)^+$: calcd for $C_{28}H_{23}N_2O_9$, 531.1403; found, 531.1399. ^1H NMR (400 MHz, DMSO-d_6): 3.63 (3H, s, OCH_3), 3.78 (1H, m), 3.96–4.10 (3H, m), 4.30 (1H, d, J = 9.8 Hz), 5.35 (1H, t, J = 9.8 Hz), 5.50 (1H, d, J = 5.9 Hz, OH), 6.44 (1H, br s, OH), 6.68 (1H, d, J = 9.3 Hz, $H_{1'}$), 7.50 (1H, t, J = 7.9 Hz), 7.51 (1H, t, J = 8.3 Hz), 7.70 (1H, dt, J_1 = 8.3 Hz, J_2 = 1.0 Hz), 7.72 (1H, dt, J_1 = 8.4 Hz, J_2 = 1.0 Hz), 7.84 (1H, d, J = 8.4 Hz), 8.10 (1H, d, J = 8.4 Hz), 8.40 (1H, s), 8.97 (1H, d, J = 8.4 Hz), 9.03 (1H, d, J = 7.9 Hz), 11.82 (1H, s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 58.2 ($C_{6'}$), 59.9 (OCH_3), 70.9, 75.2, 76.9, 77.2, 83.7 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 112.2, 112.7, 121.2, 121.5, 123.8 (2C), 127.5, 127.7 (C tert arom), 117.4, 117.5, 118.9, 119.1, 120.5, 121.0, 128.9, 130.4, 140.9, 142.0 (C quat arom), 162.0, 164.6 (2C) (C=O).

1,11-Dichloro-12-(4-*O*-methyl-*β*-D-glucopyranosyl)-6-*N*-methyl-6,7,12,13-tetrahydro-5-oxo(5*H*)indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (9) and 1,11-Dichloro-12-(4-*O*-methyl-*β*-D-glucopyranosyl)-6-*N*-methyl-6,7,12,13-tetrahydro-7-oxo(7*H*)indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (9′). A solution of 6 N HCl (3.5 mL) was added to a suspension of 6-methylrebeccamycin (140 mg, 0.24 mmol) in ethanol (20 mL) before addition of zinc amalgam (2.3 g). The mixture was refluxed for 24 h; then water was added. After extraction with EtOAc, the organic phase was washed with saturated aqueous $NaHCO_3$ and brine and dried over $MgSO_4$. The solvent was removed and the residue purified by chromatography (eluent, EtOAc) to give the isomeric mixture of **9**,**9′** (45 mg, 0.09 mmol, 33% yield) as an off-white powder. The isomeric ratio calculated from the ^1H NMR spectrum was 55% and 45%. IR (KBr): $\nu_{C=O}$ 1665 cm^{-1}, $\nu_{NH,OH}$ 3200–3600 cm^{-1}. Mp >300°C. HRMS (FAB+) $(M + H)^+$: calcd for $C_{28}H_{26}Cl_2N_3O_6$, 570.1198; found, 570.1183. ^1H NMR (400 MHz, DMSO-d_6): 3.14 and 3.20 (3H, 2s, NCH_3), 3.58–3.93 (4H, m), 3.65 (3H, s, OCH_3),

4.05 (2H, m), 4.78 (1H, m), 4.89–5.12 (2H, m), 5.44 (1H, t, J = 5.4 Hz), 5.50 (1H, t, J = 5.9 Hz), 6.98 and 7.02 (1H, 2d, $H_{1'}$, J = 9.3 Hz), 7.32 and 7.33 (1H, 2t, J = 7.9 Hz), 7.39 and 7.41 (1H, 2t, J = 7.4 Hz), 7.63 (2H, m), 7.89 and 7.93 (1H, 2d, J = 7.9 Hz), 9.29 and 9.58 (1H, 2d, J = 7.9 Hz), 10.34 and 10.46 (1H, 2s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 29.2, 29.3 (NCH_3), 51.1, 51.5 (NCH_2), 59.9, 60.0 ($C_{6'}$), 60.2 (OCH_3), 72.2, 72.3, 77.5 (2C), 79.2, 79.3, 79.9, 80.1, 84.2, 84.3 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 115.6 (2C), 115.9, 116.1, 116.2, 116.9, 117.6, 119.3, 119.5, 120.9, 123.9, 124.3, 125.7, 125.8, 125.9, 126.4, 127.7, 127.8, 130.3, 132.5, 136.0 (2C), 136.2, 136.4 (C quat arom), 119.8, 120.4, 120.7, 121.3, 121.7, 122.2, 124.1, 125.0, 125.3 (2C), 128.4 (2C) (C tert arom), 168.3 (C=O).

12-(4-*O*-Methyl-*β*-D-glucopyranosyl)-6-*N*-methyl-6,7,12,13-tetrahydro-5-oxo(5*H*)indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (10) and 12-(4-*O*-Methyl-*β*-D-glucopyranosyl)-6-*N*-methyl-6,7,12,13-tetrahydro-7-oxo(7*H*)indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (10′). The same procedure as described above afforded, from 6-methyl dechlorinated rebeccamycin (100 mg, 0.194 mmol) after purification by normal chromatography (eluent, EtOAc–cyclohexane, 90:10), the isomeric mixture of **10**,**10′** (76 mg, 0.152 mmol, 78% yield) as an off-white powder. The isomeric ratio calculated from the ^{1}H NMR spectrum was 62% and 38%. IR (KBr): $\nu_{C=O}$ 1660 cm^{-1}, $\nu_{NH,OH}$ 3200–3600 cm^{-1}. Mp: >230 °C. HRMS (FAB+) (M + H)$^+$: calcd for $C_{28}H_{28}N_3O_6$, 502.1978; found, 502.1978. ^{1}H NMR (400 MHz, DMSO-d_6): 3.30 and 3.31 (3H, 2s, NCH_3), 3.71 (3H, s, OCH_3), 3.58–4.10 (6H, m), 4.94 and 5.03 (1H, 2d, J = 5.4 Hz, OH), 5.12 (2H, s, NCH_2), 5.33 (1H, t, J = 5.9 Hz, OH), 6.13 (1H, m), 6.28 (1H, pt, J = 8.9 Hz), 7.28 (1H, t, J = 7.4 Hz), 7.38 (1H, t, J = 7.4 Hz), 7.49 (1H, t, J = 7.9 Hz), 7.55 (1H, t, J = 7.4 Hz), 7.68 and 7.74 (1H, 2d, J = 8.4 and 7.9 Hz), 7.89 and 7.96 (1H, 2d, J = 8.4 Hz), 8.05 and 8.10 (1H, 2d, J = 7.9 Hz), 9.37 and 9.47 (1H, 2d, J = 7.9 Hz), 11.31 and 11.47 (1H, 2s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 29.3 (NCH_3), 51.5, 51.7 (NCH_2), 58.7 ($C_{6'}$), 60.0 (OCH_3), 73.2, 73.3, 76.5, 77.1, 77.2, 77.4 ($C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 84.2 ($C_{1'}$), 111.1, 111.6, 111.7, 112.1, 119.1, 119.4, 120.1, 120.4, 120.8, 120.9, 125.1, 125.2, 125.3 (C tert arom), 115.1, 116.2, 116.9, 118.3, 118.6, 119.9, 122.0, 122.4, 122.5, 122.7, 124.7, 126.0, 126.8, 128.2, 129.6, 131.3, 139.8, 141.0, 141.1 (C quat arom), 169.2, 169.3 (C=O).

Biological Tests. 1. Temperature Studies. Absorption spectra were recorded on a Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat. For each series of measurements, 12 samples were placed in a thermostatically controlled cell holder (10-mm path length) and the quartz cuvettes were heated by circulating water. The temperature inside the cuvette was monitored by using a thermocouple in contact with the solution. The absorbances at 280, 315, and 345 nm were measured over the range 20–90 °C with a heating rate of 1 °C/min.

2. Topoisomerase I Inhibition. Relaxation of supercoiled DNA: Supercoiled pKMp27 DNA (0.5 mg) was incubated with 3 units of human topoisomerase I (TopoGen Inc., Columbus, OH) at 37 °C for 1 h in relaxation buffer (50 mM Tris, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA) in the presence of the drug under study at the indicated concentration. Reactions were terminated by addition of SDS and proteinase K. DNA samples were then added to the electrophoresis dye mixture (3 μL) and electrophoresed (35 V/cm) in a 1% agarose gel containing ethidium bromide (1 μg/mL) at room temperature for 15 h. Gels were washed and photographed under UV light.

Experiments with linear plasmid DNA on agarose gels: pBR322 DNA (Boehringer Mannheim, Germany) was linearized with *Eco*RI and labeled with [α-^{32}P]dATP in the presence of the Klenow fragment of DNA polymerase I. The labeled DNA was then digested to completion with *Hin*dIII. The cleavage reaction mixture contained 20 mM Tris HCl, pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 2 × 10^4 dpm of [α-^{32}P]pBR322 DNA, and the indicated drug concentrations. The reaction was initiated by the addition of topoisomerase I (40 units in 20-μL reaction volume) and allowed to proceed for 10 min at 37 °C. Reactions were stopped by adding SDS to a final concentration of 0.25% and proteinase K to 250 μg/mL, followed by incubation for 30 min at 50 °C. Samples were denatured by the addition of 10 μL of denaturing loading buffer consisting of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, 0.1% bromocresol green prior to loading onto a 1% agarose gel in TBE buffer containing 0.1% SDS. Electrophoresis was conducted at 2 V/cm for 18 h.

Sequencing of topoisomerase I-mediated DNA cleavage sites: Each reaction mixture contained 2 μL of 3′-end-^{32}P-labeled DNA (~1 μM), 5 μL of water, 2 μL of 10X topoisomerase I buffer, and 10 μL of drug solution at the desired concentration (50 μg/mL). After at least 30 min of incubation to ensure equilibration, the reaction was initiated by addition of 10 units of topoisomerase I. Samples were incubated for 40 min at 37 °C prior to adding SDS to 0.25% and proteinase K to 250 μg/mL to dissociate the drug–DNA–topoisomerase I cleavable complexes. The DNA was precipitated with ethanol and then resuspended in 5 mL of formamide–TBE loading buffer, denatured at 90 °C for 4 min, and then chilled in ice for 4 min prior to loading on the sequencing gel.

3. Footprinting Experiments. Cleavage reactions by DNaase I were performed essentially according to the previously detailed protocols.[29] Briefly, reactions were conducted in a total volume of 10 μL. Samples (3 μL) of the 160-bp ^{32}P-labeled *tyr*T DNA fragment were incubated with 5 μL of the buffer solution containing the desired drug concentration. After 30 min of incubation at 37 °C to ensure equilibration of the binding reaction, the digestion was initiated by the addition of 2 μL of DNaase I (0.01 unit/mL of enzyme in 20 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, pH 7.3). At the end of the reaction time (routinely 4 min at room temperature), the digestion was stopped by freeze-drying. After lyophilization each sample was resuspended in 4 μL of an 80% formamide solution containing tracking dyes prior to electrophoresis.

4. Growth Inhibition Assay. P388 murine leukemia cells: P388 murine leukemia cells were incubated at 37 °C for 96 h in the presence of various concentrations of drug and evaluated for viability by neutral red staining as previously described.[13] The concentrations of drugs giving 50% of growth inhibition (IC_{50}) were determined.

B16 cell cytotoxic assay: The antiproliferative activity is expressed as IC_{50} and was determined as previously described.[13]

Protein Kinase C Inhibition. Protamine sulfate was from Merck (Darmstadt, Germany). Unless specified, chemicals were from Sigma (St. Louis, MO). [γ-^{33}P]ATP (1000–3000 Ci/mmol) was obtained from Amersham. Recombinant baculoviruses from protein kinase C subtypes were supplied by Dr. Silvia Stabel, Köln, Germany. Expression and partial purification of PKCs together with measurements of activities were carried out as previously described.[13] Data show IC_{50} values expressed in μM.

Antibiogram Tests and MIC Determination. Four strains were tested, two Gram-positive bacteria (*B. cereus* ATCC 14579, *S. chartreusis* NRRL 11407), a Gram-negative bacterium (*E. coli* ATCC 11303), and a yeast (*C. albicans* 444 from the Pasteur Institute). Antimicrobial activity was determined by the conventional paper disk (Durieux No. 268; 6-mm in diameter) diffusion method using the following nutrient media: Mueller-Hinton (Difco) for *B. cereus* and *E. coli*, Sabouraud agar (Difco) for *C. albicans*, and Emerson agar (0.4% beef extract, 0.4% peptone, 1% dextrose, 0.25% NaCl, 2% agar, pH 7.0) for the *Streptomyces* strain. Paper disks impregnated with solutions of **1**–**10**,**10′** in DMSO (300 μg of drug/disk) were placed on Petri dishes. Growth inhibition was examined after 24 h incubation at 27 °C. MIC values of **1**–**10**,**10′** were determined classically on *B. cereus* ATCC 14579 in Mueller-Hilton broth, pH 7.4 (Difco), after 24-h incubation at 27 °C. The compounds diluted in DMSO were added to 12 tubes; the concentration range was from 100 to 0.05 μg/mL.

Anti-HIV-1 Activity. The cultures of CEM-SS cells were maintained at 37 °C in a 5% CO_2 atmosphere in RPMI 1640

medium supplemented with 10% decomplemented fetal bovine serum (FBS). The antiviral HIV-1 activity of a given compound in CEM-SS cells was measured by quantification of the reverse transcriptase activity (RT) associated with virus particles released from HIV-1 Lai-infected cells in the culture medium. CEM-SS cells were infected with 100 $TCID_{50}$ (the virus stock was titrated under the same experimental conditions); after 30-min adsorption, free virus particles were washed out and cells were resuspended in RPMI 10% SVF at a final concentration of 10^5 cells/mL in the presence of different concentrations of test compounds. After 5 days, virus production was measured by RT assay as already described.[30] The 50% inhibitory concentration (IC_{50}) was derived from the computer-generated median effect plot of the dose−effect data.[31] The cytotoxicity of the drugs was evaluated in parallel by incubating uninfected cells in the presence of different concentrations of antiviral products. The cell viability was determined by a measure of mitochondrial dehydrogenase activity, enzymes reducing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into formazan (quantity was given by the optical density at 540 nm).[32] The 50% cytotoxic concentration (CC_{50}) is the concentration of drug which reduces cell viability by 50% and was calculated with the program used in the determination of IC_{50}. The CEM-SS cells were obtained from P. Nara through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Bethesda, MD.

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