Syntheses and Biological Activities of Rebeccamycin Analogues. Introduction of a Halogenoacetyl Substituent

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In the course of structure−activity relationships on rebeccamycin analogues, a series of compounds bearing a halogenoacetyl substituent were synthesized with the expectation of increasing the interaction with DNA, possibly via covalent reaction with the double helix. Two rebeccamycin analogues bearing an acetyl instead of a bromoacetyl substituent were prepared to gain an insight into the role of the halogen atom. The new compounds show very little effect on protein kinase C and no covalent reaction with DNA was detected. However, the drugs behave as typical topoisomerase I poisons, and they are significantly more toxic toward P388 leukemia cells than to P388/CPT5 cells resistant to camptothecin. The introduction of a bromo- or chloro-acetyl substituent does not affect the capacity of the drug to interfere with topoisomerase I either in vitro or in cells. One of the bromoacetyl derivatives, compound **8**, is the most cytotoxic rebeccamycin derivative among the hundred of derivatives we have synthesized to date. In addition, we determined the antimicrobial activities against two Gram-positive bacteria, *Bacillus cereus* and *Streptomyces chartreusis*, and against the Gram-negative bacterium *Escherichia coli*. The effect of the drugs on *Candida albicans* yeast growth and their anti-HIV-1 activities were also measured.

Introduction

DNA topoisomerase I participates in vital cellular processes such as replication and transcription.[1] The enzyme relaxes DNA by transient breaking of one strand of the double helix, passing the other strand through the break, and finally resealing the break.[2,3] Topoisomerase I has been identified as the target for several classes of anticancer agent,[4] the best known being camptothecin and its derivatives topotecan and irinotecan which are now approved as second-line chemotherapeutic agents against resistant cancers.[5,6]

Indolocarbazole compounds represent a novel class of topoisomerase inhibitors. The lead compound in the series is the synthetic derivative NB-506 which is currently undergoing clinical trials.[7−9] The antitumor antibiotic rebeccamycin (Chart 1) isolated from cultures of *Saccharotrix aerocolonigenes*[10,11] is structurally close to NB-506, and it is also similar to protein kinase C inhibitors such as bacterial metabolites staurosporine and K-252a. However, rebeccamycin has no effect on protein kinase C.[12] Its antitumor activity correlates with its ability to inhibit topoisomerase I.[13]

In previous structure−activity relationship studies with rebeccamycin analogues, we showed that the inhibition of topoisomerase I arises in part from the ability of the compounds to interact with DNA.[13−18] The sugar moiety is essential for tight binding to DNA since derivatives lacking the sugar moiety on the indolocarbazole chromophore have considerably lower affinities for DNA.[14,15] The two chlorine atoms in positions 1 and 11 on the indolocarbazole nucleus of rebeccamycin are detrimental to the interaction with DNA and the poisoning of topoisomerase I but generally not to the cytotoxicity.[13,14] Polar and nonpolar bulky groups (e.g., −NHCHO, −NH−CH(CH$_2$OH)$_2$, −CH$_3$) can be introduced on the imide nitrogen without affecting significantly the activity of the drugs. Given the potential relationships between DNA binding and antitumor activities we seek to introduce various substituents likely to reinforce the interaction with DNA. Among the variety of substituents available for covalent reaction with DNA, we chose chloro and bromoacetyl substituents which have been used in the past to trigger covalent binding of the minor groove binding antibiotic distamycin to DNA.[19,20] The approach has also been

Chart 1

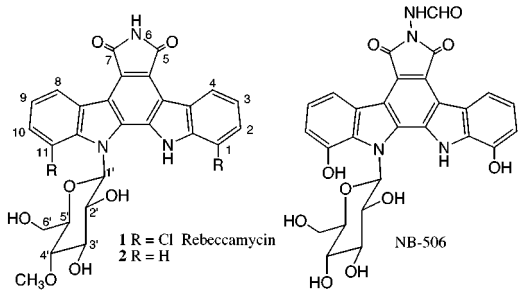

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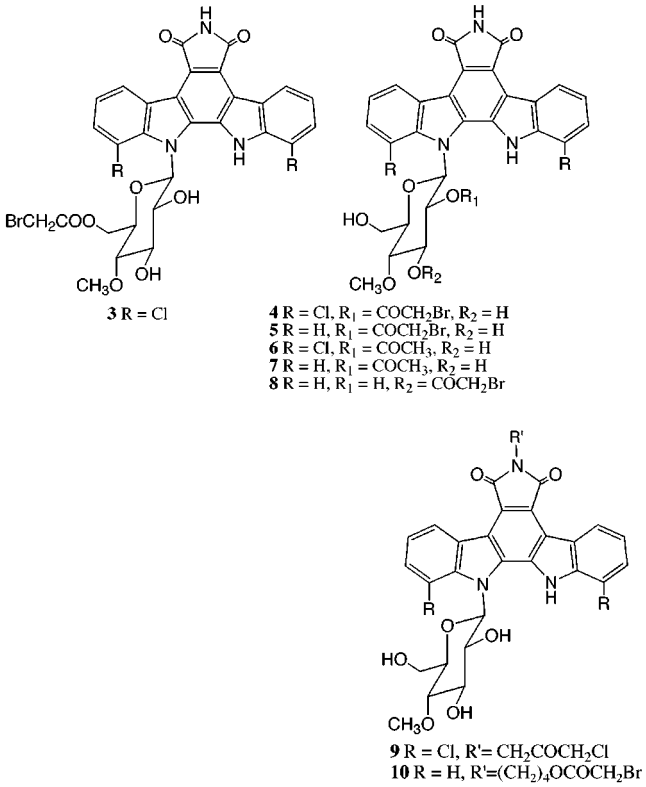
Chart 2



used for affinity labeling of the camptothecin binding site. The bromoacetyl-camptothecin derivative provided irreversible trapping of DNA−topoisomerase I covalent complex.[21] The reactivity of a halogenoacetyl group toward electronegative sites in DNA is generally weak, but we reasoned that it may be sufficient to promote stronger binding to DNA, possibly via a covalent bonding interaction. A series of rebeccamycin derivatives possessing a halogenoacetyl group at different positions on the sugar residue or attached directly to the imide nitrogen of the indolocarbazole ring were synthesized. Whenever possible, both the 1,11-bischlorinated and the dechlorinated compounds were prepared. Although the two chlorine atoms on the indole moieties do not favor interaction with DNA and inhibition of topoisomerase I, we observed in previous studies[13,17] that the chlorinated compounds were generally more active on cells than dechlorinated analogues. This is possibly due to their greater solubility and improved penetration in cells. We therefore prepared the chlorinated compounds **3**, **4**, **6**, and **9**. The chlorine atoms on the indole units could help membrane-crossing, and the halogen atom in an activated position could increase the interaction with DNA via a covalent bond. In addition, we examined the biochemical and biological activities of analogues **6** and **7** bearing an acetyl instead of a bromoacetyl substituent of compounds **4** and **5**, respectively, in order to evaluate the influence of the bromine atom (Chart 2). The biological activities of the new compounds were compared to those of rebeccamycin **1** and its dechlorinated analogue **2** which was previously found to be more active than the natural metabolite toward topoisomerase I but also more active against P388 leukemia cells. It is an exception among all the dechlorinated analogues we prepared. Compared to rebeccamycin, compound **2** exhibited a weaker antiproliferative activity against B16 melanoma cells and a weaker antibacterial activity toward the Gram-positive bacteria tested.[17] Compound **10**, generated by an unexpected reaction originally intented to occur at the 6′ position of the sugar moiety, led us to postulate the role of side chain length on the indole nitrogen consistent with a marked topoisomerase I inhibitory potency.

Chemistry

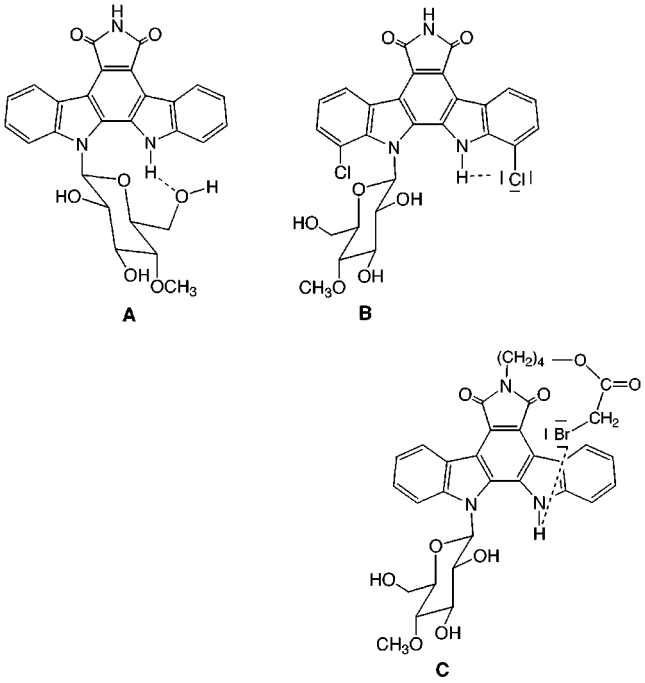
Rebeccamycin (**1**) was isolated from cultures of *S. aerocolonigenes* (ATCC 39243).[10] Covalent bonds to DNA at different sites of the drug were investigated using groups bearing a halogen atom in an activated position (α to carbonyl group) introduced either on the imide nitrogen or on the sugar moiety in the 2′, 3′, and 6′ positions.

Esterification by bromoacetyl bromide can occur on the sugar moiety either at the 2′ or 6′ position from rebeccamycin depending on the deprotonating base. Potassium carbonate (1 equiv) in THF led to the formation of the ester at the 6′ position (compound **3**) whereas sodium hydride (1 equiv) in DMF allowed esterification at the 2′ position (compound **4**). No substitution was observed on the imide nitrogen using sodium hydride (1 equiv) and bromoacetyl bromide (1 equiv) whereas Nettleton et al. obtained such a structure with the imide nitrogen linked to three carbonyl groups by peracetylation of rebeccamycin using acetic anhydride in pyridine.[10] Analogue **6** bearing an acetyl group at the 2′ position was prepared using NaH as a base in THF. Compound **7** was obtained from **6** by dechlorination with ammonium formate and Pd/C in methanol.[17] By treatment of dechlorinated rebeccamycin **2**[17] with bromoacetyl bromide, using ^{t}BuOK as the base in THF at room temperature, esterification occurred both at the 2′ and 3′ positions, leading to a mixture of esters **5** and **8**, respectively. Compound **5** was also prepared by treatment of dechlorinated rebeccamycin with NaH as the base in DMF. The orientations of the reactions were assessed from NMR ^{1}H−^{1}H COSY experiments.

To substitute the imide nitrogen of rebeccamycin with the 3-chloro-2-oxo-propyl group (compound **9**), the base used for deprotonation was sodium hydride (1 equiv) as described by Kaneko et al. for the synthesis of water soluble derivatives of rebeccamycin.[22] The reaction was carried out with commercial 1,3-dichloroacetone in dimethylformamide.

Ester **3** was obtained by reaction of rebeccamycin with bromoacetyl bromide using potassium carbonate as the base in THF. When the same reaction was carried out with dechlorinated rebeccamycin **2**, the reaction occurred on the imide nitrogen with a nucleophilic attack on THF yielding **10**. Two mechanisms are postulated for the reaction leading to compound **10**: THF ring opening after substitution of the intermediate with bromoacetyl bromide or the initial acylation of THF to give an acylonium ion and then attack by the imide anion. The first mechanism is more likely since if the acylonium ion was generated first, then in the reaction yielding compound **3**, the $-O-(CH_2)_4-O-COCH_2Br$ group at the 6′ position instead of the $-OCOCH_2Br$ substituent would be obtained. In most cases in the absence of chlorine atoms we assume sugar unit orien-

Chart 3



tation allows hydrogen bond formation between the hydroxyl oxygen in position 6′ and the indole NH (Chart 3, **A**). This intramolecular interaction would prevent substitution in position 6′, thus favoring substitution on the imide nitrogen. In the presence of chlorine atoms, a hydrogen bond between the indole NH and the neighboring chlorine frees the 6′ position for substitution (Chart 3, **B**). This hypothesis is consistent with the $N_{indole}-H$ chemical shift differences between chlorinated and dechlorinated analogues. In previous series[12,13,16] as well as for analogues **4** and **6** reported here, the presence of chlorine atoms always shielded the $N_{indole}-H$ in agreement with the hypothesis of a hydrogen bond with the chlorine. In dechlorinated compounds **2**, **5**, **7**, and **8**, the indole NH was exchangeable using D_2O at room temperature, whereas for compound **10**, the exchange was observed only by warming the solution up to 80 °C. For compound **10** we infer that the length of the chain at the imide nitrogen allows a hydrogen bond between the bromine atom and the indole NH (Chart 3, **C**). Replacing THF by DMF in identical experimental conditions did not yield the ester in position 6′, 90% of dechlorinated rebeccamycin was recovered unchanged, and 1% of compound **8** was obtained as well as small amounts of the aglycone.

Compounds **3**, **4**, **5**, **8**, **9**, and **10** were obtained in poor yields. Since a number of possible acylation sites are present, many compounds could be produced. However, in each case, they were the only reaction products. A large amount of the reactants was recovered unchanged and could be recycled.

Results and Discussion

Protein Kinase C Inhibition. Prior to determining the effect of the drugs on topoisomerase I, it is important to ascertain that the introduction of the halogenoacetyl moiety does not reactivate the anti-PKC activity. In the past we have observed occasionally that modification of the indolocarbazole chromophore not only affects topoisomerase I inhibition but, in some cases, can have a significant effect on the unwanted PKC activity.[12,13] The inhibitory properties toward PKC-α were tested using protamine sulfate as previously described.[13] The IC_{50} values are reported in Table 1. Only the bischlorinated derivative **4** exhibited a noticeable inhibitory effect on PKC. The other halogenoacetyl derivatives have less effect on PKC than rebeccamycin. The introduction of a chloro- or bromo-acetyl unit apparently leaves protein kinase activity unaffected. Compound **4**, which shows a moderate activity against PKC, is inactive against topoisomerase I. This drug displays a marked cytotoxicity toward P388 leukemia cells and, unlike the other drugs, it remains significantly toxic to P388/CPT5 cells resistant to campthotecin (Table 1). We can conclude that its cytotoxicity is not the result of an inhibition of topoisomerase I but may be due to a kinase inhibitory activity. Note that this compound also has the lowest MIC value against *Bacillus cereus* (Table 3). This example reinforces the idea that it is important to examine the effects of the drug on both PKC and topoisomerase I in order to understand their mechanism of action.

Table 1. Inhibitory Activities of Compounds **1**−**10** toward PKC and Topoisomerase I, Variation in Melting Temperature and Cytotoxic Activities

cpd	PKC IC_{50}[a]	ΔT_m (°C)	Topo I MIC[a]	P388[b] IC_{50}[a]	P388/CPT5[b] IC_{50}[a]	RI[c]
1	>175	0	1.75	1.22	10.5	8.6
2	>142	4.1	0.59	0.69	>20	>28
3	101	0.5	1.45	0.43	4.3	10
4	24	0.5	>14	0.26	0.94	2.5
5	>162	3.7	1.59	0.48	5.0	10.4
6	>163	0.5	>16	0.48	3.3	6.7
7	nd	nd	18	0.55	4.6	8.3
8	>161	3.2	1.61	0.16	3.2	20
9	>151	1.2	1.14	0.24	3.5	15
10	>144	0	1.44	1.94	>14.4	>7.4

[a] Micromolar concentration. [b] P388 and P388/CPT5 leukemia cells are sensitive and resistant to camptothecin, respectively. [c] The resistance index (RI) refers to the ratio $IC_{50}^{P388/CPT5}/IC_{50}^{P388}$.

DNA Binding. Attemps to evidence covalent reaction of the halogenoacetyl drugs with DNA were unsuccessful. A ^{32}P-labeled restriction fragment (four fragments were used: 90, 117, 160, or 265 bp) was incubated with the test drugs at various concentrations (up to 200 μM) for different times (up to 5 days), and the DNA samples were run on a native polyacrylamide gel. In every case, the migration of DNA was not retarded. Samples were boiled in 0.1 M piperidine prior to electrophoresis on sequencing gels but, here again, there was no sign of covalent interaction.

To measure the propensity of the drugs to bind to DNA, we determined their ability to alter the thermal denaturation profile of DNA. The change of the absorbance at 260 nm as a function of the temperature was measured using 20 μM calf thymus DNA with and without drugs (10 μM each). The variation of the T_m (ΔT_m) of helix-to-coil transition of DNA are collected in Table 1. The interaction of these compounds with DNA is weak since the maximum ΔT_m does not exceed 4 °C. The same conclusion was drawn when we examined the ability of the drugs to affect the fluorescence of ethidium bromide bound to DNA. In this series, dechlorinated rebeccamycin **2** is the only compound which competes significantly with ethidium for binding to DNA. Figure

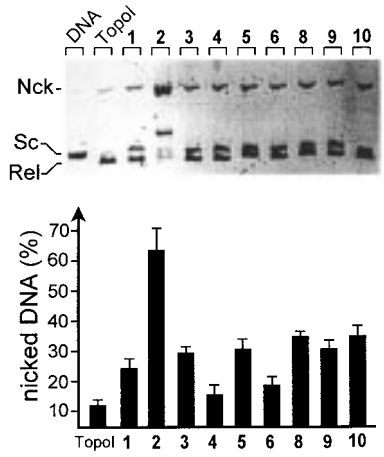


Figure 1. Inhibition of topoisomerase I-mediated relaxation of DNA by rebeccamycin analogues. Native supercoiled pAT DNA (0.5 μg) (lane DNA) was incubated for 30 min at 37 °C with 6 units human topoisomerase I without (lane Topo I) or with drug at 30 μM. Reactions were stopped with sodium dodecylsulfate 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. Densitometric analysis with a CCD camera gave the percentage of relaxed DNA form as well as mean standard errors (software GelAnalyst).

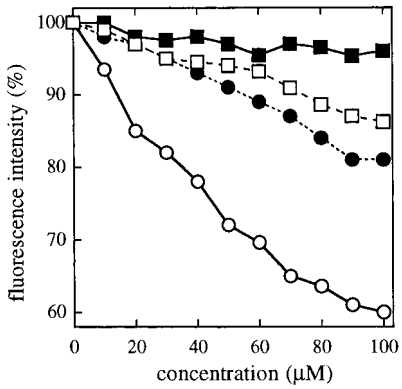


Figure 2. Quenching of fluorescence intensity of ethidium−DNA complexes by the compounds (E) **2**, (J) **5**, (G) **8**, and (B) **9**. Experimental conditions: 20 μM calf thymus DNA, 2 μM ethidium bromide (λ_{exc} = 546 nm, λ_{em} = 595 nm) in 0.01 M ionic strength buffer (9.3 mM NaCl, 2 mM Na acetate, 0.1 mM EDTA).[28]

2 shows typical fluorescence quenching curves obtained under high DNA−low fluorophore conditions for compounds **2**, **4**, **5**, and **9**. Compound **9** and the other bischlorinated drugs (**1**, **3**, **4**, and **7**) do not compete with ethidium. A small effect was observed with the compounds lacking chlorine atoms, such as **5** and **8**, but there was no major difference between compounds **4** and **6** or compounds **5** and **7**. We must conclude that the presence of a halogenoacetyl group does not confer tighter binding to DNA.

Topoisomerase I Inhibition. Two complementary assays were performed to investigate the effect of the drugs on topoisomerase I.

First, we resort to a relaxation assay using supercoiled plasmid as a substrate. The pAT plasmid was incubated with the test drug at a fixed concentration of 30 μM prior to the treatment with human topoisomerase I. The DNA samples were treated with SDS and proteinase K to remove any covalently bound protein and resolved on an agarose gel containing ethidium bromide. In the presence of ethidium, the electrophoretic mobility of relaxed and nicked DNA forms markedly differ because of DNA unwinding effects. In these conditions, the extent of drug-induced poisoning of topoisomerase I can be estimated by assaying for the presence of elevated levels of nicked DNA. The gel in Figure 1 shows that the level of nicked DNA is considerably increased in the presence of dechlorinated rebeccamycin **2**, i.e., it is the only drug that binds to DNA reasonably well. The densitometric analysis (of this and two other gels) reveals that the other compounds are significantly less effective; however, they promote the formation of nicked DNA species (histograms in Figure 1). The two 1,11-bis-chlorinated compounds **4** and **6** are significantly less effective than the corresponding dechlorinated analogues. The presence of the halogenoacetyl moiety does not affect the capacity of the drugs to inhibit topoisomerase I.

Second, we determined the minimum inhibitory concentration (MIC) using calf thymus topoisomerase I and the ^{32}P-labeled *Eco*RI-*Hin*dIII restriction fragment of pBR322 as a substrate. The labeled DNA fragment was incubated with topoisomerase I with and without drugs at concentrations ranging from 0.1 μM to 100 μM, and the resulting cleavage products were analyzed by agarose gel electrophoresis. The MIC values of the tested compounds are reported in Table 1. The drugs rank in the following order: **2** ≫ **1** > **9** > **10**, **3**, **5**, **8** ≫ **7**, **4**, **6**. Dechlorinated rebeccamycin is the most active compound. The effect has been attributed to the removal of the chlorine atoms of rebeccamycin which prevent the drug from intercalating into DNA.[14] The bromoacetyl derivative **4** and the two acetyl derivatives **6** and **7** lacking a halogen atom have very little effect on topoisomerase I. It is interesting to note that the bromoacetyl derivative **5** is 10 times more effective at inhibiting topoisomerase I than the counterpart **7** with no bromine atom. Although no covalent bonding to DNA was detected, the incorporation of the halogenoacetyl moiety may contribute positively to interaction with the topoisomerase I−DNA complex. However, little difference was detected when the halogenoacetyl group was present on the sugar residue or on the indolocarbazole ring. The MICs fall into the range of the values measured with other series of rebeccamycin derivatives.[13,16−18] The activity of the halogenated drugs toward topoisomerase I is certainly not reduced but it is not significantly improved compared with that of the reference compound **2**.

Cytotoxicity. The antiproliferative activities were tested in vitro against the P388 leukemia cell line. IC_{50} values are reported in Table 1. The cytotoxic activities of rebeccamycin analogues, including the tumor active drug NB-506, are correlated with their topoisomerase inhibition properties.[7,8,16−18,23,24] This prompted us to compare the toxicities of compounds **1**−**10** toward P388 cells sensitive or resistant to the topoisomerase I inhibitor camptothecin. The resistance of the P388/CPT5 cells has been ascribed to the expression of a deficient form of topoisomerase I as a result of a mutation in the *top1* gene of these cells.[25] The IC_{50} values measured with P388/CPT5 resistant cells are also given in Table 1. The

resistance index (RI) refers to the ratio of $IC_{50}^{P388/CPT5}/IC_{50}^{P388}$. This ratio provides information on the potential contribution of topoisomerase I inhibition to the drug antitumor activity. In terms of antiproliferative activity against P388 leukemia cells, the drugs rank in the following order: **8** > **9**, **4** > **3**, **5**, **6** > **7** > **2** > **1** > **10**. There is no direct correlation with the anti-topoisomerase I activity. Compounds **4**, **6**, and **7** have almost no effect on topoisomerase I whereas they exhibited a strong cytotoxicity against P388 leukemia cells. In contrast, the bromoacetyl derivatives **5** and **8**, which both behave as typical topoisomerase I poisons, are less active than compounds **6** and **7**. Surprisingly, compound **8** shows a potent cytotoxic activity; it is about 5 times more active than dechlorinated rebeccamycin **2**. Among the hundred indolocarbazole derivatives we have synthesized thus far, this compound **8** is the most cytotoxic compound. Interestingly, it is considerably less toxic to P388/CPT5 cells. It seems initially that topoisomerase I is a cellular target for this indolocarbazole derivative but the two complementary in vitro assays indicate that this drug is not a very potent topoisomerase I poison. It is incidentally worth noting that we repeated the topoisomerase I assay with compound **8** using a buffer lacking dithiothreitol (which may react with the alkylating functional group). However, even in this case no enhanced inhibition of topoisomerase I was detected. The origin of the high potency of **8** is at present unknown. A molecular target other than topoisomerase I or PKC may be involved.

Apparently, the presence of a bromine atom in the 2′ position reinforces the cytotoxicity because compounds **4** and **5** are slightly more active than the two analogues **6** and **7** lacking the bromine atom. But the effect can be attributed neither to an effect on PKC nor to an increase of the anti-topoisomerase I activity. As previously observed with other series of indolocarbazoles,[13−18] the 1,11-bis-chlorinated compounds are generally slightly more cytotoxic than the analogues lacking the Cl atoms (cf. compounds **4** and **5** or **6** and **7**).

Comparison of dechlorinated compounds **5** and **8** bearing a bromoacetyl group in positions 2′ and 3′, respectively, indicates that the position of the halogen atom had no influence on topoisomerase I inhibition. The antiproliferative activity against P388 cells was 3-fold stronger with **8** than **5**, and the resistance indices are quite different (RI = 20 for cpd **8** vs 10 for cpd **5**). Finally, when the halogenoacetyl group is attached to the imide nitrogen, the chain length may be crucial since compound **9** is 10 times more active than **10**.

Anti-HIV-1 Activity. The observation that topoisomerase I activates HIV-1 reverse transcriptase activity[26] and that the topoisomerase I-specific inhibitor topotecan inhibited this phenomenon[27] prompted us to investigate the activity of the test drugs against HIV-1. The anti-HIV-1 activity in CEM-SS cells was measured by quantification of the reverse transcriptase activity associated with virus particles released from HIV-1 infected cells in the culture medium. The cytotoxicity of the drugs (CC_{50}) was evaluated with the IC_{50} values, and the selectivity index (CC_{50}/IC_{50}) was calculated (Table 2). Although low IC_{50} values were measured for most of the tested compounds, the selectivity index was not sufficient enough to consider them as anti-HIV-1 drugs.

Table 2. Anti-HIV-1 Activities of Compounds **1**−**10** in HIV-1 Lai Infected CEM-SS Cells

cpd	IC_{50} (μM)	CC_{50} (μM)	SI[a]
1	0.52	1.05	2.0
2	0.16	0.90	5.6
3	0.33	1.45	4.4
4	0.98	2.3	2.3
5	0.12	0.46	3.8
6	1.4	1.2	
7	nd	nd	
8	0.15	0.44	2.9
9	0.45	1.2	2.7
10	0.50	0.57	1.1

[a] Selectivity index: SI = CC_{50}/IC_{50}.

Table 3. Antimicrobial Activities 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]

cpd	*B. cereus* ATCC 14579	*S. chartreusis* NRRL 11407	*E. coli* ATCC 11303	*C. albicans* IP 444	*B. cereus* MIC (μM)
1	++	++			10.9
2					>100
3	±				>72
4	+	++			1.81
5	++	++			5.02
6					>81
7	±	+++			46
8	++	++			5.02
9	+	+++			4.72
10	±				>72

[a] Growth inhibition zone size was 10−12 mm (+++), 8−9 mm (++), 7−8 mm (+), 6−7 mm (±).

Antimicrobial Properties. The antimicrobial activities against two Gram-positive bacteria (*B. cereus* and *Streptomyces chartreusis*), a Gram-negative bacterium (*Escherichia coli*), and a yeast (*Candida albicans*) were tested (Table 3). All the compounds were inactive against *C. albicans* and *E. coli*. With the exception of **2** and **6**, the others were active against *B. cereus*. The most active against *S. chartreusis* were **7** and **9**. However, as already observed with other series of rebeccamycin analogues,[13,16−18] the antimicrobial properties do not seem to be directly related to topoisomerase I inhibition. As mentioned above, the antimicrobial activity of compound **4** against *B. cereus* presumably accounts for its effect on PKC.

In conclusion, the introduction of a halogenoacetyl group is not sufficient to induce covalent reaction with DNA. The lack of covalent reaction with DNA may be connected with the enzymatic and/or hydrolytic lability of the ester and *N*-acylimide. But we are inclined to believe that it is the intrinsic reactivity of the alkylating moiety on the sugar residue or on the indolocarbazole chromophore which is too low. Other alkylating functionalities highly reactive toward DNA (such as nitrogen-mustards) are now being considered. However, this approach yielded the most active rebeccamycin analogue synthesized to date. PKC does not appear as a privileged target for this cytotoxic compound **8**, and topoisomerase I only contributes partially to the activity. Further study is required to elucidate the mechanism involved. We have shown in previous work[13,16] that different small substituents (−NH_2, −OH, −NHCHO, −CH_3) on the imide nitrogen could be introduced without impairing

the biological activity of the analogues. Other authors have obtained efficient topoisomerase I inhibitors and antitumor compounds involving bulkier groups ($-(CH_2)_n-NEt_2$ with $n = 2$ or 3),[22] but larger substituents such as those used in this study may be detrimental to the biological activity. Compared to dechlorinated rebeccamycin **2**, the weaker activity of compound **10** suggests that the length of the side chain on the imide nitrogen (longer than that for the substituents above) could be responsible for the difficulty of the drug to enter the active site of the enzyme. These results provide an insight regarding the size of the substituent on the imide nitrogen compatible with the biological activity.

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 (^1H, 400 MHz; ^{13}C, 100 MHz), the chemical shifts δ are reported in ppm, and the following abbreviations are used: singlet (s), doublet (d), doubled doublet (dd), triplet (t), pseudo-triplet (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. Purifications were performed by flash chromatography using silica gel Geduran SI 60 (Merck) 0.040−0.063 mm or Kieselgel 60 (Merck) 0.063−0.200 mm column. 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-(6-*O*-bromomethylcarbonyl-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 (400 mg, 0.70 mmol), THF (8 mL), and K_2CO_3 (97 mg, 0.70 mmol) was refluxed for 0.5 h. Bromoacetyl bromide (0.061 mL, 0.70 mmol) was then added, and the mixture was refluxed for 24 h. The solvent was removed and the residue purified by flash chromatography (eluent, EtOAc−toluene, 50:50) to give **3** (56 mg, 0.081 mmol, 12% yield) as a yellow solid. Mp: 70 °C. IR (KBr): $\nu_{C=O}$ 1710, 1750 cm^{-1}, $\nu_{NH,OH}$ 3200−3500 cm^{-1}. HRMS (FAB^+) (M^+): calcd for $C_{29}H_{22}N_3O_8Cl_2Br$, 688.9967; found, 688.9861. ^1H NMR (400 MHz, DMSO-d_6): 3.45 (1H, m), 3.55 (1H, pt, J = 8.7 Hz), 3.63 (3H, s, OCH_3), 3.65 (2H, m), 3.80 (1H, m), 4.24 (1H, dt, J_1 = 9.8 Hz, J_2 = 2.6 Hz), 4.72 (2H, m), 5.12 (1H, s), 5.57 (1H, s), 6.94 (1H, d, $H_{1'}$, J = 8.6 Hz), 7.43 (1H, t, J = 7.9 Hz), 7.44 (1H, t, J = 7.8 Hz), 7.67 (1H, d, J = 7.7 Hz), 7.74 (1H, d, J = 7.7 Hz), 8.99 (1H, d, J = 8.0 Hz), 9.19 (1H, d, J = 7.9 Hz), 10.45 (1H, s, $N_{imide}-H$), 11.35 (1H, s, $N_{indole}-H$). ^{13}C NMR (100 MHz, DMSO-d_6): 31.8 (CH_2Br), 60.3 (OCH_3), 63.3 ($C_{6'}$), 72.0, 77.0, 77.2, 80.2, 84.5 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 115.8, 116.4, 117.4, 119.6, 120.6, 123.1 (2C), 125.2, 129.5, 129.6, 136.8, 137.9 (C quat arom), 122.2, 123.7, 124.1, 125.3, 126.8, 130.0 (C tert arom), 170.2, 170.4, 170.5 (C=O).

1,11-Dichloro-12-(2-*O*-bromomethylcarbonyl-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 rebeccamycin (199 mg, 0.35 mmol) in DMF (5 mL) a suspension of NaH (60% dispersion in mineral oil, 14 mg) in DMF (1 mL) was added. The mixture was stirred at room temperature for 0.5 h, and then bromoacetyl bromide (0.03 mL, 70 mg, 0.35 mmol) was added. The mixture was stirred at room temperature for 30 h. Addition of water allowed the precipitation of a yellow solid which was filtered off. The filtrate was extracted with EtOAc, and the organic phase was dried over $MgSO_4$ and evaporated. The two fractions were purified by chromatography (eluent, EtOAc−toluene, 50:50) to give **4** (57 mg, 0.082 mmol, 23% yield). Mp: 190 °C. IR (KBr): $\nu_{C=O}$ 1710, 1720, 1760 cm^{-1}, $\nu_{NH,OH}$ 3200−3500 cm^{-1}. HRMS (FAB^+) (M^+): calcd for $C_{29}H_{22}N_3O_8Cl_2Br$, 688.9967; found, 688.9962. ^1H NMR (400 MHz, DMSO-d_6): 3.20 (1H, d, J = 12.4 Hz), 3.44 (1H, d, J = 12.4 Hz), 3.65 (3H, s, OCH_3), 3.85 (1H, t, $H_{4'}$, J = 9.1 Hz), 3.95 (1H, pt, $H_{3'}$, J = 9.1 Hz), 4.07 (3H, m, $H_{5'}$ and $2H_{6'}$), 5.22 (1H, t, $H_{2'}$, J = 9.1 Hz), 5.51 (1H, s, $OH_{6'}$), 5.81 (1H, s, $OH_{3'}$), 7.28 (1H, d, $H_{1'}$, J = 9.4 Hz), 7.45 (1H, t, J = 7.9 Hz), 7.48 (1H, t, J = 7.9 Hz), 7.69 (1H, dd, J_1 = 7.8 Hz, J_2 = 0.8 Hz), 7.76 (1H, d, J = 7.4 Hz), 9.09 (1H, d, J = 7.9 Hz), 9.22 (1H, dd, J_1 = 7.9 Hz, J_2 = 0.8 Hz), 10.63 (1H, s, $N_{imide}-H$), 11.42 (1H, s, $N_{indole}-H$). ^{13}C NMR (100 MHz, DMSO-d_6): 24.9 (CH_2Br), 59.4 ($C_{6'}$), 60.3 (OCH_3), 74.3 (2C), 78.7, 80.1, 81.6 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 116.1, 116.4, 118.0, 119.3, 120.5, 122.9, 123.4, 125.3, 129.6, 129.9, 136.5, 137.4 (C quat arom), 122.3, 123.1, 123.5, 124.3, 127.2, 130.1 (C tert arom), 165.3, 170.1, 170.2 (C=O).

12-(2-*O*-Bromomethylcarbonyl-4-*O*-methyl-*β*-D-glucopyranosyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]-carbazole-5,7-dione (5). The same procedure as described above for the preparation of compound **4** yielded, after purification by flash chromatography (eluent, EtOAc−CH_2Cl_2, 50:50), compound **5** in 13% yield from dechlorinated rebeccamycin **2**. Mp (decomposition): >200 °C. IR (KBr): $\nu_{C=O}$ 1700, 1750 cm^{-1}, $\nu_{NH,OH}$ 3300−3600 cm^{-1}. HRMS (FAB^+) (M^+): calcd for $C_{29}H_{24}N_3O_8Br$, 621.0747; found, 621.0756. ^1H NMR (400 MHz, DMSO-d_6): 2.80 (1H, d, J = 13.3 Hz), 3.41 (1H, d, J = 13.3 Hz), 3.71 (3H, s, OCH_3), 3.80−4.20 (5H, m), 5.01 (1H, pt, J = 9.3 Hz), 5.78 (1H, d, J = 6.4 Hz, OH), 6.44 (1H, t, J = 4.0 Hz, OH), 6.69 (1H, d, J = 8.9 Hz, $H_{1'}$), 7.43 (2H, t, J = 7.4 Hz), 7.65 (1H, t, J = 7.8 Hz), 7.67 (1H, t, J = 7.8 Hz), 7.77 (1H, d, J = 7.8 Hz), 8.01 (1H, d, J = 8.4 Hz), 9.12 (1H, d, J = 7.9 Hz), 9.15 (1H, d, J = 7.9 Hz),11.20 (1H, s, $N_{imide}-H$), 11.68 (1H, s, $N_{indole}-H$). ^{13}C NMR (100 MHz, DMSO-d_6): 25.2 (CH_2Br), 58.3 (C6′), 60.3 (OCH_3), 73.3, 75.4, 77.1, 77.3, 81.4 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 111.2, 112.3, 120.6, 121.3, 124.5, 124.8, 127.1 (2C) (C tert arom), 117.2, 118.6, 119.3, 121.5 (2C), 121.7, 128.5, 129.7, 140.8, 141.1 (C quat arom), 165.1, 170.9, 171.0 (C=O).

1,11-Dichloro-12(2-*O*-acetyl-4-*O*-methyl-*β*-D-glucopyranosyl)-6,7,12,13-tetrahydro(5*H*)-indolo[2,3-*a*]-pyrrolo[3,4-*c*]-carbazole-5,7-dione (6). NaH (60% in mineral oil, 77.2 mg, 1.93 mmol) was added to a solution of rebeccamycin (1 g, 1.75 mmol) in THF (150 mL), and the mixture was stirred at room temperature for 20 min. Acetyl chloride (137 μL, 1.93 mmol) was then added and the mixture stirred at room temperature for 2 h. Saturated aqueous $NaHCO_3$ was added. After extraction with EtOAc, the organic phase was washed with water and dried over $MgSO_4$. The solvent was removed and the residue purified by chromatography (first column: eluent CH_2Cl_2−EtOAc, 60:40; second column: eluent cyclohexane−EtOAc, 50−50) to yield compound **6** (511 mg, 0.83 mmol, 48% yield) as a yellow solid. Mp (decomposition) 285−295 °C. IR (KBr): $\nu_{C=O}$ 1710, 1715, 1745, 1750 cm^{-1}, $\nu_{NH,OH}$ 3200−3600 cm^{-1}. HRMS (FAB^+) (M^+): calcd for $C_{29}H_{22}N_3O_8Cl_2$, 611.0862; found, 611.0865. ^1H NMR (400 MHz, DMSO-d_6): 1.09 (3H, s), 3.65 (3H, s, OCH_3), 3.80 (1H, pt, J = 9.3 Hz), 3.92 (1H, dd, J_1 = J_2 = 8.9 Hz), 4.09 (3H, m), 5.10 (1H, pt, J = 9.1 Hz), 5.50 (1H, t, $OH_{6'}$, J = 4.5 Hz), 5.75 (1H, d, $OH_{3'}$, J = 6.2 Hz), 7.10 (1H, d, $H_{1'}$, J = 9.4 Hz), 7.48 (2H, t, J = 7.8 Hz), 7.75 (2H, t, J = 7.8 Hz), 9.11 (1H, d, J = 8.0 Hz), 9.29 (1H, d, J = 7.9 Hz), 10.77 (1H, s, $N_{imide}-H$), 11.45 (1H, s, $N_{indole}-H$). ^{13}C NMR (100 MHz, DMSO-d_6): 19.2 (CH_3), 59.6 ($C_{6'}$), 60.3 (OCH_3), 72.8, 74.3, 78.9, 80.3, 82.1 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 116.0, 116.4, 118.0, 119.2, 120.4, 123.0, 123.4, 125.1, 129.9, 130.0, 136.5, 137.3 (C quat arom), 122.3, 123.2, 123.6, 124.0, 127.2, 130.1 (C tert arom), 168.0, 170.2, 170.4 (C=O).

12(2-*O*-Acetyl-4-*O*-Methyl-*β*-D-glucopyranosyl)-6,7,12,13-tetrahydro(5*H*)-indolo[2,3-*a*]-pyrrolo[3,4-*c*]-carbazole-5,7-dione (7). A suspension of compound **6** (66 mg, 0.108 mmol) and 5% Pd/C (132 mg) in EtOAc (15 mL) was hydrogenated (1 atm) for 6 days. 5% Pd/C (132 mg) was added, and the mixture was further hydrogenated for 2 days. After filtration over Celite, the residue was washed with EtOAc and THF. The solvents were removed and the residue purified by chromatography (eluent cyclohexane−EtOAc, 50:50) to yield compound **7** (12.5 mg, 0.023 mmol, 21% yield). Mp (decomposition) > 175 °C. IR (KBr) $\nu_{C=O}$ 1720, 1730, 1740 cm^{-1}, $\nu_{NH,OH}$ 3200−3650 cm^{-1}. HRMS (FAB^+) (M^+): calcd for $C_{29}H_{25}N_3O_8$,

543.1641; found, 543.1642. ^{1}H NMR (400 MHz, DMSO-d_6): 1.02 (3H, s), 3.71 (3H, s, OCH_3), 3.90 (1H, pt, J = 9.5 Hz), 3.95 to 4.07 (3H, m), 4.16 (1H, d, J = 10.3 Hz), 4.95 (1H, pt, J = 9.5 Hz), 5.60 (1H, d, J = 6.4 Hz, OH), 6.35 (1H, t, J = 4.0 Hz, OH), 6.67 (1H, d, J = 9.5 Hz, $H_{1'}$), 7.43 (1H, t, J = 7.1 Hz), 7.45 (1H, t, J = 7.1 Hz), 7.64 (1H, t, J = 7.1 Hz), 7.69 (1H, t, J = 7.1 Hz), 7.79 (1H, d, J = 8.7 Hz), 8.04 (1H, d, J = 8.7 Hz), 9.14 (1H, d, J = 8.7 Hz), 9.20 (1H, d, J = 7.1 Hz), 11.22 (1H, s, N_{imide}−H), 11.75 (1H, s, N_{indole}−H). ^{13}C NMR (100 MHz, DMSO-d_6): 19.1 (CH_3), 58.3 ($C_{6'}$), 60.3 (OCH_3), 73.3, 73.6, 77.2, 77.3, 81.8 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 111.3, 112.2, 120.6, 121.1, 124.4, 124.7, 127.0, 127.1 (C tert arom), 117.1, 118.5, 119.3, 121.3, 121.4, 121.5, 128.8, 129.7, 140.7, 141.1 (C quat arom), 168.0, 170.9, 171.1 (C═O).

12-(3-*O*-Bromomethylcarbonyl-4-*O*-methyl-*β*-D-glucopyranosyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (8). To a solution of dechlorinated rebeccamycin (350 mg, 0.70 mmol) in THF (12 mL) was added ^{t}BuOK (79 mg). The mixture was stirred at room temperature for 0.5 h before bromoacetyl bromide (61 μL, 0.70 mmol) was added. The mixture was stirred at room temperature for 24 h prior to adding water. After extraction with EtOAc, the organic phase was dried over $MgSO_4$. The solvent was removed and the residue purified by flash chromatography (eluent, EtOAc−CH_2Cl_2, 30:70) to give **8** (30 mg, 0.05 mmol, 7% yield) as a yellow solid and **5** (58 mg, 0.09 mmol, 13% yield). Mp: 225 °C. IR (KBr): $\nu_{C=O}$ 1705, 1750 cm^{-1}, $\nu_{NH,OH}$ 3200−3600 cm^{-1}. HRMS (FAB$^+$) (M^+): calcd for $C_{29}H_{24}N_3O_8Br$, 621.0746; found, 621.0746. ^{1}H NMR (400 MHz, DMSO-d_6): 3.63 (3H, s, OCH_3), 3.80 (1H, m, $H_{2'}$), 3.99 (3H, m, $2H_{6'}$, $H_{4'}$), 4.09 (1H, d, J = 13.2 Hz), 4.21 (1H, d, J = 12.8 Hz), 4.28 (1H, d, J = 9.9 Hz, $H_{5'}$), 5.38 (1H, d, J = 5.9 Hz, $OH_{2'}$), 5.42 (1H, pt, J = 9.8 Hz, $H_{3'}$), 6.37 (1H, t, J = 4.0 Hz, $OH_{6'}$), 6.63 (1H, d, J = 8.9 Hz, $H_{1'}$), 7.43 (2H, t, J = 7.4 Hz), 7.64 (1H, dt, J_1 = 7.9 Hz, J_2 = 1.0 Hz), 7.66 (1H, dt, J_1 = 7.4 Hz, J_2 = 1.5 Hz), 7.77 (1H, d, J = 8.4 Hz), 8.03 (1H, d, J = 8.8 Hz), 9.15 (1H, d, J = 8.4 Hz), 9.20 (1H, d, J = 7.4 Hz), 11.22 (1H, s, N_{imide}−H), 11.58 (1H, s, N_{indole}−H). ^{13}C NMR (100 MHz, DMSO-d_6): 27.1 (CH_2Br), 58.3 ($C_{6'}$), 60.0 (OCH_3), 70.9, 75.3, 76.7, 77.0, 78.6, 83.5 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 111.7, 112.2, 120.6, 120.9, 124.6 (2C), 127.0, 127.2 (C tert arom), 117.2, 118.7, 119.6, 121.1, 121.3, 121.5, 128.1, 129.7, 140.8, 142.1 (C quat arom), 166.6, 170.9, 171.0 (C═O).

1,11-Dichloro-12-(4-*O*-methyl-*β*-D-glucopyranosyl)-6-*N*-(3-chloro-2-oxo-propyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (9). A suspension of NaH (60% w/w dispersion in mineral oil, 27 mg) in DMF (2 mL) was added to a solution of rebeccamycin (386 mg, 0.68 mmol) in DMF (4 mL). The mixture was stirred at room temperature for 0.5 h before the addition of 1,3-dichloroacetone (86 mg, 0.68 mmol) in DMF (1 mL). The mixture was then stirred at room temperature for 24 h prior to the addition of water. After extraction with EtOAc, the organic phase was dried over $MgSO_4$. The solvent was removed and the residue purified by flash chromatography (eluent, EtOAc−CH_2Cl_2, 35:65) to give compound **9** (22 mg, 0.033 mmol, 5% yield) as a yellow solid. Mp: 185 °C. IR (KBr) $\nu_{C=O}$ 1700, 1740, 1755 cm^{-1}, ν_{OH} 3300−3600 cm^{-1}. HRMS (FAB$^+$) $(M + H)^+$: calcd for $C_{30}H_{25}Cl_3N_3O_8$, 660.0707; found, 660.0699. ^{1}H NMR (400 MHz, DMSO-d_6): 3.65 (3H, s, OCH_3), 3.58−3.75 (3H, m), 3.9 (1H, m), 4.04 (2H, m), 4.89 (4H, s), 5.07 (1H, d, J = 5.4 Hz, OH), 5.41 (1H, broad s, OH), 5.48 (1H, broad s, OH), 6.96 (1H, d, J = 8.9 Hz, $H_{1'}$), 7.44 (1H, t, J = 7.8 Hz), 7.50 (1H, t, J = 7.8 Hz), 7.75 (2H, t, J = 7.9 Hz), 8.99 (1H, d, J = 7.9 Hz), 9.15 (1H, d, J = 7.9 Hz), 10.73 (1H, s, N_{indole}−H). ^{13}C NMR (100 MHz, DMSO-d_6): 44.7, 47.0 ($2CH_2$), 59.8 ($C_{6'}$), 60.1 (OCH_3), 72.1, 77.3, 79.1, 80.3 ($C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 84.5 (C1′), 116.3, 116.4, 117.8, 119.2, 119.6, 121.1, 123.0, 124.9, 129.7, 129.8, 137.2, 137.9 (C quat arom), 122.2, 122.7, 123.2, 123.9, 127.3, 130.2 (C tert arom), 168.0, 168.1, 196.7 (C═O).

12-(4-*O*-Methyl-*β*-D-glucopyranosyl)-6-*N*-butylbromoacetate-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (10). A mixture of dechlorinated rebeccamycin **2** (150 mg, 0.30 mmol), THF (5 mL), and K_2CO_3 (41 mg, 0.30 mmol) was refluxed for 0.5 h. Bromoacetyl bromide (26 μL, 0.3 mmol) was then added, and the mixture was refluxed for 48 h prior to adding water. After workup identical to that for **9** and purification by flash chromatography (eluent, EtOAc−CH_2Cl_2, 50:50), compound **10** (31 mg, 0.045 mmol, 15% yield) was obtained as a yellow solid. Mp: 155 °C. IR (KBr) $\nu_{C=O}$ 1710, 1760 cm^{-1}, $\nu_{NH,OH}$ 3300−3620 cm^{-1}. HRMS (FAB$^+$) $(M + H)^+$: calcd for $C_{33}H_{33}N_3O_9Br$, 694.1400; found, 694.1425. ^{1}H NMR (400 MHz, DMSO-d_6): 1.77 (2H, m), 1.93 (2H, m), 3.59 (2H, pt, J = 6.9 Hz), 3.71 (3H, s, OCH_3), 3.74−4.10 (6H, m), 4.24 (2H, pt, J = 6.4 Hz), 4.64 (2H, s), 5.05 (1H, d, J = 5.4 Hz, OH), 5.36 (1H, d, J = 5.4 Hz, OH), 6.24 (1H, t, J = 4.0 Hz, OH), 6.37 (1H, d, J = 8.9 Hz, $H_{1'}$), 7.43 (2H, t, J = 7.9 Hz), 7.65 (2H, t, J = 8.4 Hz), 7.78 (1H, d, J = 8.4 Hz), 8.03 (1H, d, J = 8.7 Hz), 9.10 (1H, d, J = 8.4 Hz), 9.17 (1H, d, J = 7.4 Hz), 11.73 (1H, s, N_{indole}−H). ^{13}C NMR (100 MHz, DMSO-d_6): 26.9, 28.8, 34.5, 38.9 (CH_2), 58.5 ($C_{6'}$), 60.1 (OCH_3), 64.5 (CH_2), 73.2, 76.3, 77.2, 77.3, 84.2 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 111.9, 112.3, 120.6, 120.9, 124.2, 124.3, 127.1, 127.3 (C tert arom), 117.3, 118.0, 118.8, 119.7, 120.9, 121.3, 128.4, 129.8, 140.9, 142.3 (C quat arom), 168.2, 168.7, 168.8 (C═O).

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 micromolar units.

DNA Melting Temperature Studies. Melting curves were measured using an Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat. For each series of measurements, 12 samples were placed in a thermostatically controlled cell holder, and the quartz cells (10 mm path length) were heated by circulating water. The measurements were performed in BPE buffer pH 7.1 (6mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA). The temperature inside the cell was measured with a platinum probe; it was increased over the range 20−100 °C with a heating rate of 1 °C/min. The "melting" temperature T_m was taken as the midpoint of the hyperchromic transition (from the first-derivative plot).

Fluorescence measurements were carried out on a Perkin-Elmer LS50B spectrofluorimeter. All measurements were made using a 10 mm light path cell in a 0.01 M ionic strength buffer (9.3 mM NaCl, 2 mM Na acetate, 0.1 mM EDTA) using 20 μM DNA and 2 μM ethidium bromide.[28] The DNA−ethidium complex was excited at 546 nm and the fluorescence measured at 595 nm.

Topoisomerase I Inhibition. (1) DNA Relaxation Experiments. Supercoiled pAT DNA (0.5 μg) was incubated with 6 units of topoisomerase I (TopoGen, Colombus, OH) at 37 °C for 1 h in the 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 test drug at 30 μM. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg/mL. DNA samples were then added to the electrophoresis dye mixture (3 μL) and electrophoresed in a 1% agarose gel containing ethidium bromide (1 μg/mL). After 2 h of electrophoresis at room temperature (about 180 V), the gel was washed with water and then photographed under UV light.

(2) Experiments with Linear Plasmid DNA on Agarose Gels.[29,30] 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 *Hind*III. 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 calf thymus topoisomerase I (40 units in 20 μL of 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, and 0.1% bromocresol green prior to loading on to a 1% agarose gel in TBE buffer containing 0.1% SDS. Electrophoresis was conducted at 2 V/cm for 18 h. The MIC values correspond to the lowest concentration (μM) producing a detectable DNA cleavage.

Growth Inhibition Assay. P388 and P388/CPT5 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 IC_{50} values (μM) refer to the minimum drug concentration requires to inhibit cell growth by 50%.

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 Institut Pasteur). 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* strains. Paper disks impregnated with solutions of **1**−**10** in DMSO (300 μg of drug per disk) were placed on Petri dishes. Growth inhibition was examined after 24 h incubation at 27 °C.

MIC of **1**−**10** were determined classically on *B. cereus* ATCC 14579 in Mueller−Hinton 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 μg/mL to 0.05 μg/mL.

Antiviral HIV-1 Activity. The cultures of CEM-SS cells were maintained at 37 °C in 5% CO_2 atmosphere in RPMI 1 640 medium supplemented with 10% decomplemented foetal 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 of adsorption, free virus particles were washed out, and cells were resuspended in RPMI 10% SVF at the final concentration of 10^5 cells/mL in the presence of different concentrations of test compounds. After 5 days, virus production was measured by the RT assay as already described.[31] The 50% inhibitory concentration (IC_{50}) was derived from the computer-generated median effect plot of the dose−effect data.[32] 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 (which quantity was given by the optical density at 540 nm).[33] 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.

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