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Rebeccamycin Analogues from Indolo[2,3-*c*]carbazole

Aline Voldoire,[a] Martine Sancelme,[a] Michelle Prudhomme,[a,*] Pierre Colson,[b] Claude Houssier,[b] Christian Bailly,[c] Stéphane Léonce[d] and Stéphanie Lambel[d]

[a]*Université Blaise Pascal, Synthèse, Electrosynthèse et Etude de Systèmes à Intérêt Biologique, UMR 6504, 63177 Aubière, France*

[b]*Laboratoire de Chimie Macromoléculaire et Chimie Physique, Université de Liège, Liège 4000, Belgium*

[c]*Centre Oscar Lambret and INSERM U-524, IRCL, Place de Verdun, 59045 Lille, France*

[d]*Institut de Recherches SERVIER, 11 Rue des Moulineaux, 92150 Suresnes, France*

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Abstract—Glycosylated indolocarbazoles related to the antibiotic rebeccamycin represent an important series of antitumor drugs. In the course of structure–activity relationship studies, we report the synthesis of two new derivatives containing an indolo[2,3-*c*]carbazole chromophore instead of the conventional indolo[2,3-*a*]carbazole unit found in the natural metabolites. The *N*-methylated compound **8** containing one glucose residue behaves as a typical DNA intercalating agent, as judged from circular and electric linear dichroism measurements with purified DNA. In contrast, the bis-glycosylated derivative **7** containing a glucose residue on each indole nitrogen has lost its capacity to form stable complexes with DNA. DNA relaxation experiments reveal that the two drugs **7** and **8** have weak effects on human DNA topoisomerase I. The modified conformation of the indolocarbazole chromophore is detrimental to the stabilization of topoisomerase I–DNA complexes. The lack of potent topoisomerase I inhibition leads to decreased cytotoxicity but, however, we observed that the DNA-intercalating mono-glycosyl derivative **8** is about 5 times more cytotoxic than the bis-glycosyl analogue **7**. The study suggests that the naturally-occurring indolo[2,3-*a*]carbazole skeleton should be preserved to maintain the topoisomerase I inhibitory and cytotoxic activities.

Introduction

Indolocarbazoles represent an important class of antitumor agents.[1] Antibiotics such as staurosporine and K-252a for which the sugar unit is linked to both indole nitrogens are potent protein kinase C (PKC) inhibitors.[2,3] In contrast, the antibiotics rebeccamycin,[4] AT-2433-A1/B1,[5] and the semisynthetic derivatives, ED-110[6] and NB-506,[7] for which the carbohydrate residue is attached to only one indole nitrogen, mainly act as topoisomerase I inhibitors (Scheme 1). Like the camptothecins, these drugs stabilize topoisomerase I–DNA covalent complexes.[8] Their antitumor activity is attributed to their capacity to induce topoisomerase I-dependent DNA strand breaks. NB-506 is currently undergoing clinical trials.

In previous works, we showed that the sugar residue on the indolocarbazole ring system is a key element for both DNA binding and topoisomerase I inhibition.[9,10] Moreover, the interaction is stereospecific. Indolocarbazoles linked to the carbohydrate via a β-*N*-glycosidic linkage are potent DNA intercalators and topoisomerase I inhibitors, whereas an α-*N*-glycosidic linkage results in a complete loss of both DNA intercalation and topoisomerase I inhibition.[11,12]

As part of structure–activity relationship studies, we investigated the synthesis of rebeccamycin analogues **7** and **8** possessing an indolo[2,3-*c*]carbazole framework instead of the conventional indolo[2,3-*a*]carbazole found in indolocarbazoles from bacterial sources. The orientation of the indole rings is reversed and, consequently, the sugar residue is now pointing toward the imide heterocycle. The altered orientation of the carbohydrate moiety is expected to modify the capacity of the drug to interact with DNA and/or DNA–topoisomerase I complexes.

*Corresponding author. Tel.: 33-473-40-71-24; fax: 33-473-40-77-17; e-mail: mprud@chimtp.univ-bpclermont.fr

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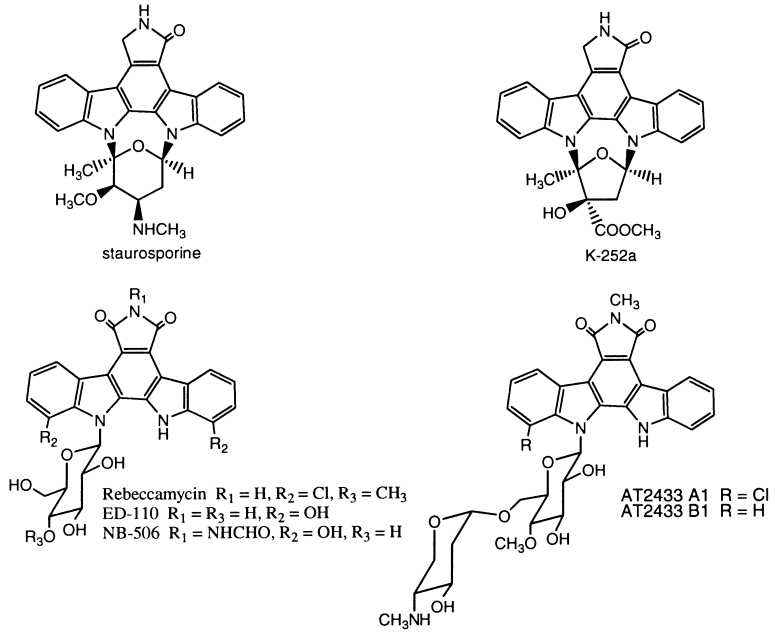
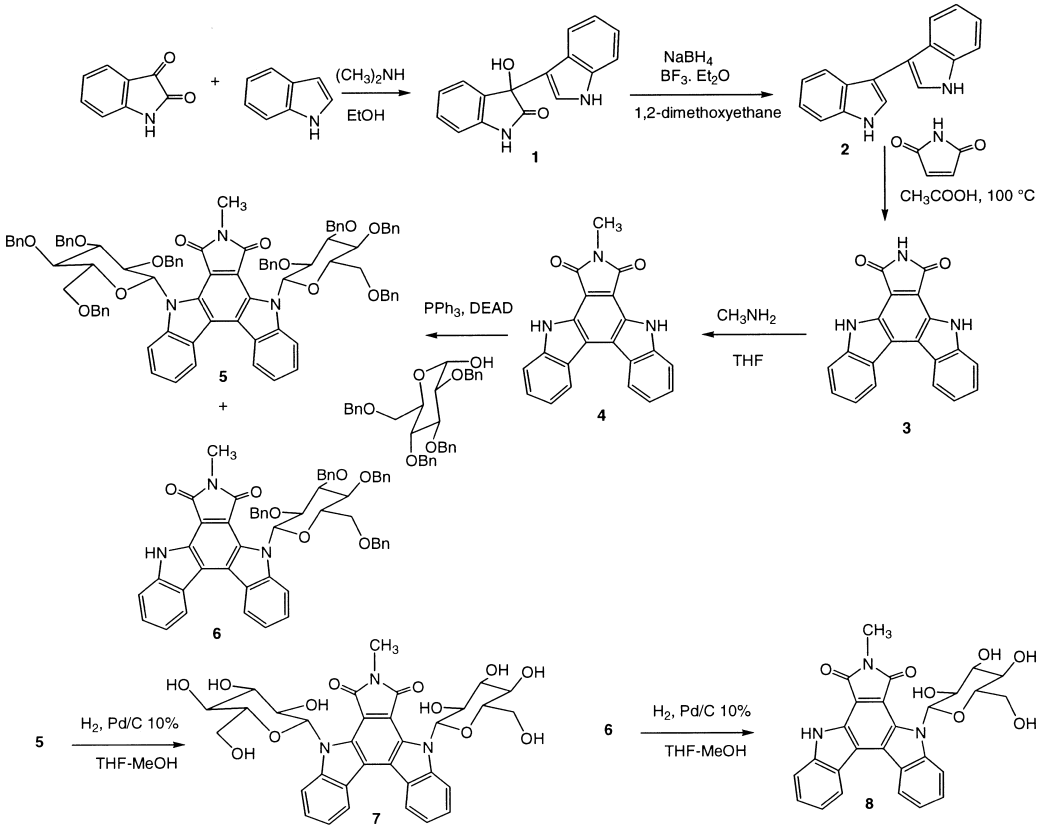
We have shown previously that the introduction of a methyl group on the imide nitrogen led to stronger topoisomerase I inhibition.[11] For this reason, the newly designed compounds **7** and **8** were both synthesized with a methyl group on the imide nitrogen. The original synthesis, DNA binding and topoisomerase I inhibitory properties of the mono- and bis-glycosylated indolo[2,3-*c*]carbazole derivatives **7** and **8** are reported here. These compounds were examined for their in vitro antiproliferative activities against L1210 leukemia cells, and their antimicrobial activities against the Gram-positive bacterium *Bacillus cereus*.



Scheme 1.



Scheme 2.

Results and Discussion

Chemistry

3-Hydroxy-2-oxo-1,3-dihydro-1′*H*-3,3′-bisindolyl **1** (Scheme 2) was prepared by reaction of commercial isatin with indole in the presence of dimethylamine in ethanol. Intermediate **1** was then reduced to **2** by generating B_2H_6 in situ using $NaBH_4$ and BF_3/Et_2O according to the procedure described by Berens et al.[13] Indolocarbazole **3** was obtained from **2** via a Diels–Alder cycloaddition in acetic acid at 100 °C.[14] *N*-Methylation of indolocarbazole **3** with 2 N solution of methylamine in THF in a sealed tube at 70 °C gave **4** with an overall yield of 97.8%. This procedure proved much more efficient than the conventional method[14] based on a Diels–Alder reaction with *N*-methylmaleimide. Several methods have been investigated for coupling a carbohydrate unit onto an indolocarbazole aglycone.[11,15–17] The method from 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide and silver oxide led mainly to α-coupling products. But, as mentioned in the Introduction, only β-coupling products were desired due to their superior activity profiles.[11,12] The glycosylation of the indole ring was achieved using the Mitsunobu reaction with 2,3,4,6-tetra-*O*-benzyl-D-glucose (α-dominant, Sigma)[17] in the presence of both diethylazodicarboxylate (DEAD) and triphenylphosphine. Di- and mono-β-*N*-glycosylated compounds **5** and **6** were the major products of the reaction and were isolated in 14.7 and 28.9% yields, respectively. The di- and mono-α-*N*-glycosylated compounds were also detected as minor products of the reaction. Debenzylation of **5** and **6** performed by hydrogenolysis with 10% Pd/C in THF:methanol (1:1 v/v) afforded analogues **7** and **8** in 46.6 and 43.6% yields, respectively. The β-*N*-glycosidic bonds were identified from the ^1H NMR coupling constants between protons $H_{1'}$ and $H_{2'}$ of the sugar moiety. The coupling constant of about 9 Hz corresponds to an axial–axial coupling.

DNA binding

The mono-glycosylated compound **8** has significant interaction with DNA. The melting temperature (T_m) of the polynucleotide poly(dA-dT)•(dA-dT) is markedly increased in the presence of **8** whereas **7** has absolutely no effect, even at high drug/DNA ratios (Fig. 1). The T_m value reaches 6.5 °C with **8** whereas the T_m remains unchanged at 41 °C with **7**. The difference between the two compounds was also evident from the absorption measurements (not shown). The interaction of **8** with DNA caused a hypochromism in the absorption band centered at 450 nm whereas the spectrum of **7** was not affected by the addition of DNA. The incorporation of a second carbohydrate residue seems to abolish the capacity of the drug to stabilize the double stranded DNA structure. Complementary biophysical analyses fully support this view.

Figure 2 shows the circular dichroism (CD) spectra of **7** and **8** either free in solution or bound to calf thymus DNA. One can immediately observe that the CD spectrum of **7** is little modified upon addition of DNA whereas the spectrum of **8** shows marked changes. In this case the peak at 458 nm is shifted to 515 nm and the molar dichroism intensity becomes less negative. The CD

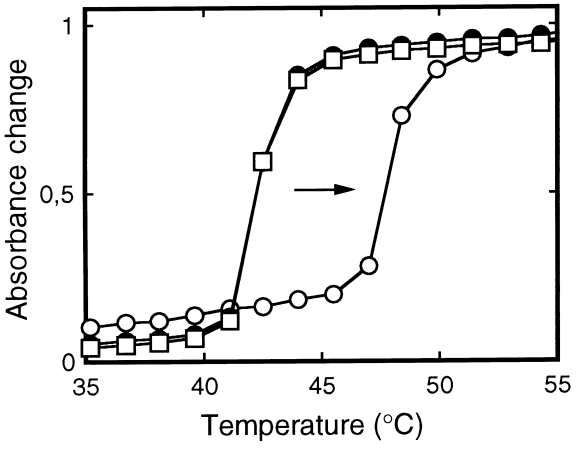


Figure 1. Thermal denaturation curves for (□) poly(dA-dT)•(dA-dT) alone or in interaction with (●) **7** or (○) **8** at a drug/DNA ratio of 0.25. The T_m values were determined from the corresponding first-derivative plots.

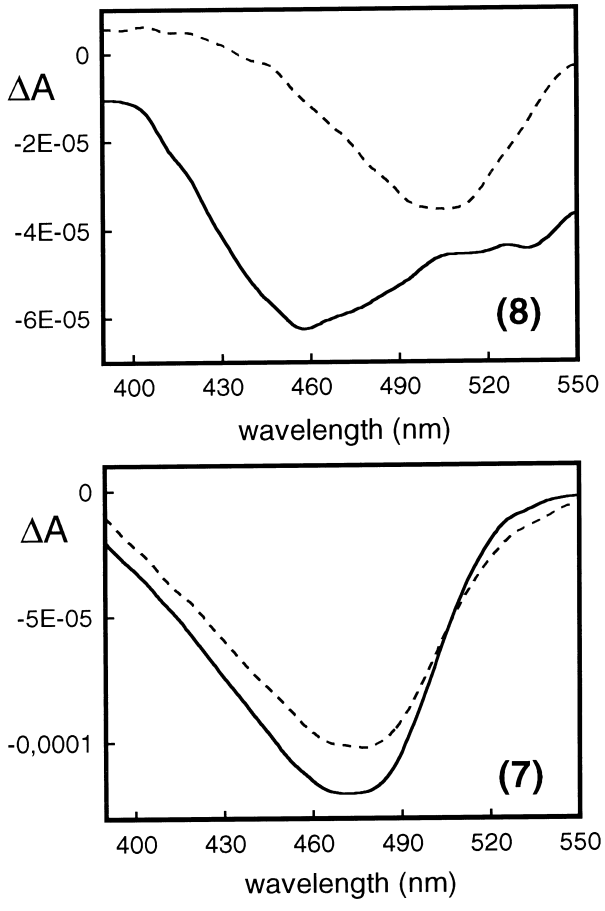


Figure 2. Circular dichroism spectra of (solid lines) **7** and **8** free in solution or (dashed lines) bound to calf thymus DNA. The circular dichroism amplitude ΔA was measured in 3 mL quartz cuvettes using 20 μM drug±500 μM DNA.

data corroborate the T_m and UV measurements. To define more precisely the mode of binding of the two drugs to DNA, we recorded the electric linear dichroism spectra of the drug–DNA complexes.

Figure 3 shows the dependence of the reduced dichroism $\Delta A/A$ on (A) the wavelength and (B) the electric field strength. The drug/DNA ratio was fixed at 20. Very weak signals were measured with **7**. Clearly this bis-glycosyl compound does not form stable complexes with calf thymus DNA. No particular binding mode could be determined for this compound, even using synthetic polynucleotides. In contrast, the ELD spectrum of the complexes between **8** and calf thymus DNA reveals that the reduced dichroism is always negative in sign in the 400–500 nm region where the indolocarbazole chromophore absorbs the light (Fig. 3A). The intensity of the ELD signal is a function of the degree of alignment of the DNA molecules in the electric field. With **8**, there is a clear parallelism between the electric field dependence of the reduced dichroism measured at 260 nm for the DNA bases and at 500 nm for the drug (Fig. 3B). This indicates that the indolo[2,3-*c*]carbazole ring is tilted close to the plane of the DNA bases, consistent with an intercalative mode of binding. Similar data were previously reported with indolo[2,3-*a*]carbazole such as dechlorinated rebeccamycin or synthetic derivatives including the antitumor drug NB-506.[10,18]

Further ELD studies were undertaken to compare the binding of the drugs to the synthetic polynucleotides poly(dA-dT)•poly(dA-dT) and poly(dG-dC)•poly(dG-dC). Measurements were carried out at a fixed P/D ratio of 20 and under the same buffer and temperature conditions as with calf thymus DNA. Here again, **7** failed to bind to the polynucleotides (Fig. 4). The spectrum of the complex between **8** and the AT polymer is reminiscent of that obtained with calf thymus DNA. The reduced dichroism is negative and the amplitude of the signal is the same as with the polynucleotide alone at 260 nm (Fig. 4B). This is the expected behavior for a drug parallel to the DNA base pair plane. But, interestingly, very weak ELD signals were obtained with the GC polymer with which compound **8** interacts only poorly. In this case the reduced dichroism is much weaker than that of the GC polymer alone (Fig. 4B). From the ELD data, we therefore concluded that **8** can intercalate into AT sequences but not into GC repeats. However, no particular sequence selectivity with **8** could be discerned using footprinting methods. It may be the peculiar conformation of poly(dG-dC)•poly(dG-dC) rather than the primary sequence per se which is recognized by **8**.

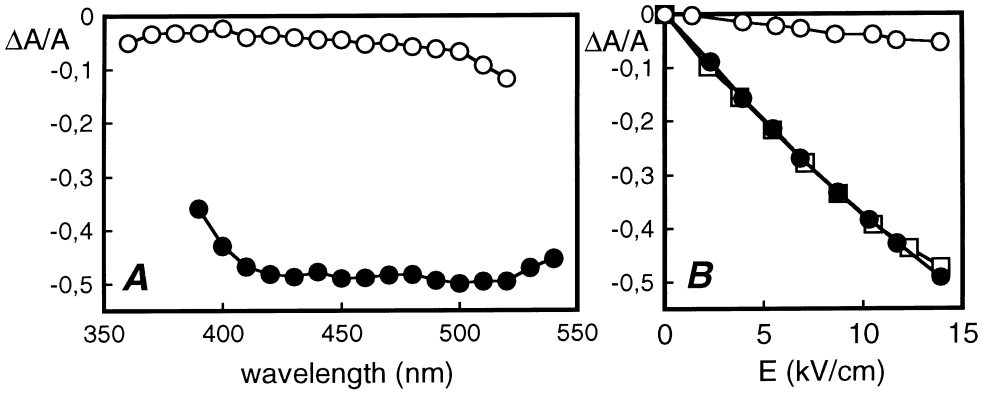


Figure 3. Dependence of the reduced dichroism $\Delta A/A$ on (A) the wavelength and (B) the electric field strength. (E) **7**, (J) **8**, (G) calf thymus DNA alone. Conditions: (A) P/D = 20, 13 kV/cm, (B) 460 nm for **7**, 500 nm for **8**, P/D = 20 in 1 mM sodium cacodylate buffer, pH 7.0.

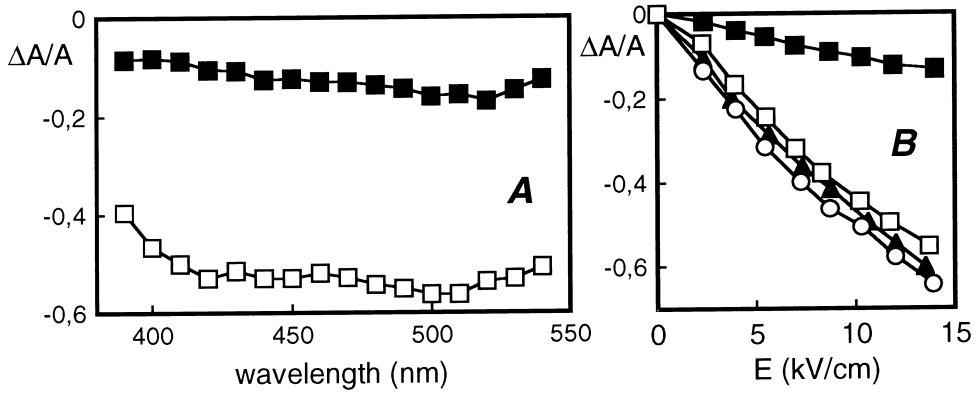


Figure 4. (A) Electric linear dichroism spectra of **8** bound to (G) poly(dA-dT)•poly(dA-dT) and (B) poly(dG-dC)•poly(dG-dC). Panel B shows the field strength dependence for (E) drug-free poly(dA-dT)•poly(dA-dT), (H) drug-free poly(dG-dC)•poly(dG-dC) and the complexes: (G) **8**-poly(dA-dT)•poly(dA-dT) and (B) **8**-poly(dG-dC)•poly(dG-dC). In each case the drug was present at 20 μM and the polymer at 400 μM. Conditions: (A) P/D = 20, 13 kV/cm, (B) 500 nm, P/D = 20 in 1 mM sodium cacodylate buffer, pH 7.0.

Topoisomerase I inhibition

We studied the effects of **7** and **8** on the relaxation of plasmid DNA by topoisomerase I. Negatively supercoiled plasmid pKMp27 was incubated with human topoisomerase I and increasing concentration of the test drug, ranging from 1 to 100 μM. DNA samples were treated with SDS and proteinase K to remove any covalently bound protein and resolved in a 1% agarose gel. The gel shown in Figure 5A indicates that **8**, but not **7**, interferes with the relaxation of DNA mediated by topoisomerase I. The mono-glycosyl compound shifts the distribution of the population of the topoisomers. The experiments presented in Figure 5A were performed without ethidium bromide in the gel during the electrophoresis. The effect of **8** may be attributed to a direct effect on the catalytic activity of the enzyme (stabilization of enzyme–DNA complexes) but it may also result from a nonspecific inhibition. Indeed, **8** is an intercalating agent and as such it can reduce the binding of the enzyme to the DNA template, especially at high drug concentrations. Similar effects have been recently observed with an indolo[2,3-*a*]carbazole derivative.[8]

To distinguish the specific and nonspecific effects, we repeated the DNA relaxation experiments using gels pre-stained with ethidium bromide (Fig. 5B). With ethidium the electrophoretic mobility of relaxed DNA, but not that of nicked DNA, is markedly changed because of DNA unwinding effects, whereas under the

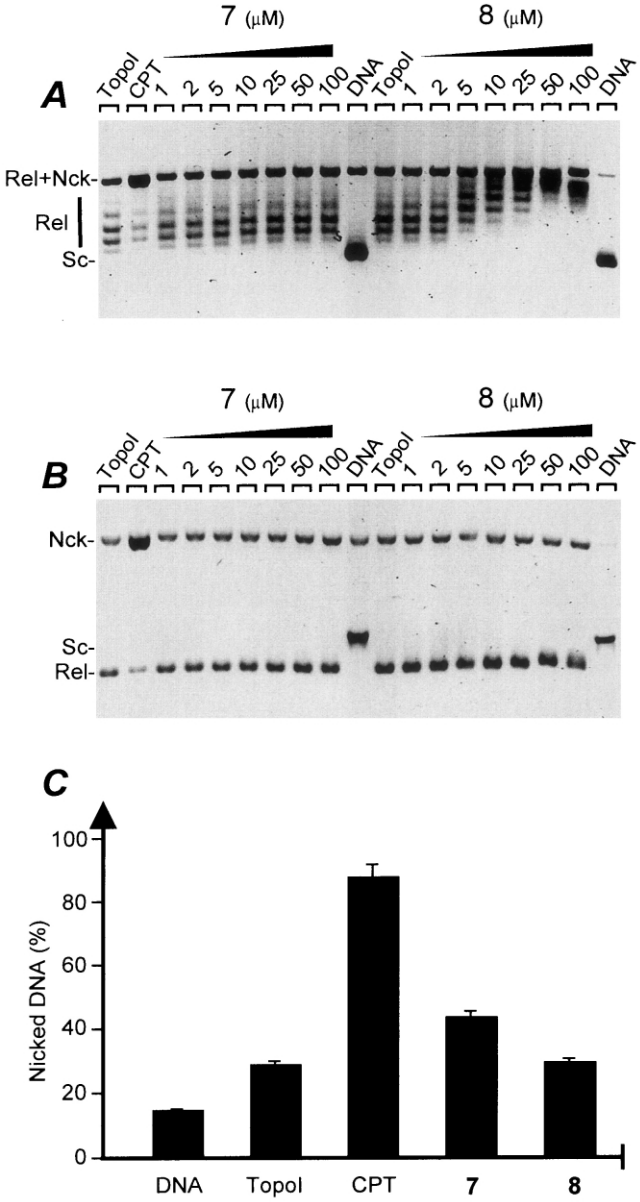


Figure 5. Effect of increasing concentrations of **7** and **8** on the relaxation of plasmid DNA by topoisomerase I. Native supercoiled pKMp27 DNA (0.5 μg) (lane DNA) was incubated for 30 min at 37 °C with 6 units topoisomerase I (lane TopoI) in the absence or presence of drug at the indicated concentration (μM). The reactions were stopped with sodium dodecylsulfate and treatment with proteinase K. DNA samples were separated by electrophoresis on an agarose gel (A) without or (B) with ethidium bromide. Nck, nicked; Rel, relaxed; Sc, supercoiled. The histograms in panel C compare the topoisomerase I-mediated cleavage efficiency of the different drugs. Data were compiled from quantitative analysis of three gels as the one shown in (B) and must be considered as a set of averaged values.

standard conditions used in Figure 5A, nicked DNA and relaxed (closed circular) DNA comigrate at the same level in the agarose gel matrix. The gel shown in Figure 5B and the quantification in Figure 5C reveal that in fact the two drugs have minor effects on topoisomerase I. In the presence of **8**, the amount of nicked DNA produced by topoisomerase I is about the same as that formed without drug. With **7**, the percentage of nicked DNA is slightly higher indicating that this compound weakly stabilizes DNA–topoisomerase I covalent complexes. However, the effect is poor compared to what can be achieved with the reference inhibitor camptothecin. Therefore we must conclude that the inhibition of topoisomerase I observed with **8** under standard (ethidium-free) conditions (Fig. 5A) is nonspecific and arises solely from DNA binding rather than from interaction between the drug and covalent enzyme–DNA species. It is worth mentioning here that we also studied the effect of the drugs on the cleavage activity of the enzyme using radiolabeled DNA substrates but the profiles of DNA cleavage were not affected by the two compounds, even using concentrations as high as 100 μM. The indolo[2,3-*c*]carbazole derivatives **7** and **8** cannot be considered as good topoisomerase I poisons, in contrast to the corresponding indolo[2,3-*a*]carbazole.

Table 1. In vitro antiproliferative activities against murine leukemia L1210 cells: percentage of L1210 cells in the G2 + M phase (for a drug concentration of 1 μM) and antimicrobial activities against *Bacillus cereus*

Compd	L1210 IC_{50} μM	% of L1210 cells in the G2 + M phase[a]	*B. cereus* MIC μM
Rebeccamycin	0.14	69 (1 μM)	10.9
Dechlorinated rebeccamycin	0.11	71 (1 μM)	> 97
7	48.9	n.e.	19
8	10.4	n.e.	20

[a]24% of untreated control cells were in the G2 + M phase of the cell cycle; n.e.: not evaluated.

Biological activities

The antiproliferative activities were tested in vitro against L1210 leukemia cells. The IC_{50} values reported in Table 1 reveal that the two new compounds are much less cytotoxic than the parent drugs rebeccamycin and dechlorinated rebeccamycin. However, the mono-glycosyl molecule is about 5 times more toxic to leukemia cells than the corresponding bis-glycosyl analogue. The differences between the two compounds may be attributed to the higher propensity of **8** to interact with DNA as compared to **7**. The effect on the cell cycle of rebeccamycin and dechlorinated rebeccamycin, that exhibited the strongest antiproliferative activities, were studied and it was observed that, at a concentration of 1 μM, about 70% of L1210 cells were accumulated in the G2 + M phase. The antimicrobial assay shows that the two compounds are equally active at inhibiting the growth of the Gram-positive bacterium *B. cereus* (Table 1).

Discussion

All rebeccamycin derivatives synthesized thus far contained a carbohydrate moiety on one of the two indole nitrogens. Substitution of both indoles was not possible due to steric hindrance. In the present case, the change of the indolocarbazole structure allows for the introduction of two sugar units on each indole nitrogen. The reversal of the indole rings markedly modifies the shape of the molecules. The indolo[2,3-*a*]carbazole structure is extended whereas the corresponding indolo[2,3-*c*]carbazole chromophore is essentially curved (Fig. 6). The geometrical changes may modify significantly stacking interactions with DNA bases or amino acid residues of topoisomerase I (e.g., with the catalytic tyrosine 723 residue).

The results indicate that the mono-glycosyl analogue **8** retains the capacity to intercalate into DNA. Therefore, the curvature introduced into the chromophore is apparently not an obstacle for intercalation. In contrast, the indolocarbazole structural change is clearly detrimental to the inhibition of topoisomerase I. The carbohydrate pointing toward the imide nitrogen may not be recognized by the enzyme or the overall configuration of the chromophore may not permit optimal stacking interactions with certain amino acid residues of topoisomerase I (e.g., with the tyrosine 723 residue which is believed to stack with camptothecin).[19]

Figure 6. Conformation of the (A) indolo[2,3-*a*]carbazole and (B) indolo[2,3-*c*]carbazole chromophores. The energy minimized structures were built using the softwares HyperChem™ 5.01 and Alchemy 2000®.

The presence of a sugar residue on each indole ring abolishes the capacity of the drug to intercalate into DNA. The fact that the bis-glycosyl derivative **7** is considerably less cytotoxic than the mono-glycosyl analogue **8** suggests indirectly that DNA-binding contributes to the cytotoxicity. This bis-glycosyl compound is reminiscent of the molecule ED-954 (also called RBM-1) identified as an 11-*O*-glucuronide biliary metabolite of the antitumor drug NB-506.[20] At this stage, substitution of the indolocarbazole structure with two sugar residues does not seem to represent a profitable route for the design of tumor active compounds.

In recent years, we have elaborated over 100 rebeccamycin derivatives with various side chains on the different rings of the indolocarbazole unit. We have elucidated important structure–activity relationships and also a few compounds endowed with marked cytotoxic activities were obtained. Here, for the first time, we modified the chromophore structure. This work opens a new area to define novel SARs in the rebeccamycin series.

Experimental

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) (chemical shifts δ in ppm, the following abbreviations are used: singlet (s), doublet (d), triplet (t), multiplet (m), tertiary carbons (C tert), quaternary carbons (C quat)). The signals were assigned from exhange with D_2O and inverse gate decoupling. 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.

7-Methyl-6,7,8,9-tetrahydro-5*H*-indolo[2,3-*c*]pyrrolo[3,4-*a*]carbazole-6,8-dione 4. Indolocarbazole **3** (300 mg, 0.92 mmol) and a 2 M solution of dimethylamine in THF was heated at 70 °C in a sealed tube for 4 days. After cooling, the mixture was poured into water. The precipitate was filtered off, washed with water and with Et_2O to give **4** (305 mg, 0.90 mmol, 97.8%) as an orange solid. Mp, IR and NMR spectra were identical with the descriptions in the literature.[11]

7-Methyl-8,9-di(2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranosyl)-6,7,8,9-tetrahydro-5*H*-indolo[2,3-*c*]pyrrolo[3,4-*a*]carbazole-6,8-dione 5 and 7-methyl-8-(2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranosyl)-6,7,8,9-tetrahydro-5*H*-indolo[2,3-*c*]pyrrolo[3,4-*a*]carbazole-6,8-dione 6. To a mixture of **4** (100 mg, 0.31 mmol), PPh_3 (244 mg, 0.93 mmol), tetra-*O*-benzyl-D-glucopyranose and THF (20 mL) at −55 °C was added dropwise DEAD (162 mg, 146 μL). The reaction mixture was stirred at −55 to 0 °C until TLC indicated complete consumption of the indole (2 h). The mixture was poured into 0.2 M aq HCl, then extracted with EtOAc. The organic phase was washed with $NaHCO_3$ and brine, and dried over $MgSO_4$. The solvent was removed and the residue purified by flash chromatography (eluent cyclohexane:EtOAc, 7:3, then EtOAc:methanol, 95:5) to give **5** (63 mg, 0.045 mmol, 14.7% yield) and **6** (77 mg, 0.090 mmol, 29% yield) as red powders. The ^1H NMR spectra of **5** and **6** showed a mixture of conformers.

7-Methyl-8,9-di(β-D-glucopyranosyl)-6,7,8,9-tetrahydro-5*H*-indolo[2,3-*c*]pyrrolo[3,4-*a*]carbazole-6,8-dione 7. Hydrogenolysis of compound **5** (53 mg, 0.038 mmol) in THF:methanol (1:1 v/v, 10 mL) using 10% Pd/C (15 mg) with hydrogen at atmospheric pressure for 24 h. After filtration over Celite, the solvent was removed and the residue purified by flash chromatography (eluent CH_2Cl_2:methanol, 85:15) to give **7** (12 mg, 0.023 mmol, 47% yield) as a red solid. Mp 225–227 °C. IR (KBr) ν_{CO} 1738 cm^{-1}, ν_{OH} 3200–3600 cm^{-1}. HRMS (FAB+) calcd for $C_{33}H_{33}N_3O_{12}$ (M^+) 633.2059, found 633.2069. ^1H NMR (400 MHz, DMSO-d_6) δ 3.24 (3H, s, NCH_3), 3.42–3.55 (4H, m), 3.66–3.78 (4H, m), 3.90 (2H, m), 4.00 (2H, m), 4.75 (2H, br s, 2 OH), 4.85 (2H, br s, 2 OH), 5.19 (4H, br s, 4 OH), 7.51 (2H, d, J = 9.1 Hz, 2 $H_{1'}$), 7.53 (2H, t, J = 7.7 Hz), 7.68 (2H, t, J = 7.7 Hz), 8.12 (2H, d, J = 8.4 Hz), 8.97 (2H, d, J = 8.2 Hz); ^{13}C NMR (100 MHz, CH_3OD) δ 16.5 (NCH_3), 63.2 ($C_{6'}$), 72.1, 72.3, 79.7, 81.3, 89.0 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 114.4, 125.3, 126.2, 135.4, 143.4 (C quat arom), 116.7, 122.3, 125.2, 128.9 (C tert arom), 170.3 (C=O).

7-Methyl-8-(β-D-glucopyranosyl)-6,7,8,9-tetrahydro-5*H*-indolo[2,3-*c*]pyrrolo[3,4-*a*]carbazole-6,8-dione 8. Hydrogenolysis of **6** (67 mg, 0.078 mmol) in the same conditions as described above gave, after 4 days, compound **8** (17 mg, 0.034 mmol, 44% yield) as a red solid. Mp > 300 °C. IR (KBr) ν_{CO} 1681, 1697 cm^{-1}, $\nu_{NH,OH}$ 3200–3600 cm^{-1}. HRMS (FAB+) calcd for $C_{27}H_{23}N_3O_7$ (M^+) 501.1531, found 501.1521. ^1H NMR (400 MHz, DMSO-d_6) δ 3.22 (3H, s, NCH_3), 3.50 (2H, m), 3.71 (2H, m), 3.90 (1H, m), 4.06 (1H, m), 4.74 (1H, t, J = 4.9 Hz, $OH_{6'}$), 4.92 (1H, d, J = 5.3 Hz, OH), 5.16 (1H, s, OH), 5.21 (1H, s, OH), 7.46 (1H, t, J = 7.3 Hz), 7.53 (1H, t, J = 7.3 Hz), 7.57 (1H, d, J = 9.5 Hz, $H_{1'}$), 7.64 (1H, t, J = 7.7 Hz), 7.66 (1H, t, J = 7.4 Hz), 7.88 (1H, d, J = 8.2 Hz), 8.08 (1H, d, J = 8.3 Hz), 8.90 (1H, d, J = 8.2 Hz), 8.95 (1H, d, J = 8.1 Hz), 12.01 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 24.0 (NCH_3), 61.2 ($C_{6'}$), 70.1, 70.2, 77.7, 80.1, 87.1 ($C_{1'}$,$C_{2'}$,$C_{3'}$,$C_{4'}$,$C_{5'}$), 110.7, 111.9, 120.3, 122.6, 123.0, 123.1, 129.4, 132.4, 140.2, 143.2 (C quat arom), 112.7, 114.9, 119.9, 120.7, 123.5, 123.8, 126.9, 127.6 (C tert arom), 168.2, 168.4 (C=O).

Biochemicals. Human topoisomerase I was purchased from TopoGen Inc. (Columbus, OH). Calf thymus DNA and the double-stranded polymers poly(dA-dT)•poly(dA-dT) and poly(dG-dC)•poly(dG-dC) were from Pharmacia (Uppsala, Sweden). Calf thymus DNA was deproteinized with sodium dodecylsulfate

(protein content $<0.2\%$) and all nucleic acids were dialyzed against 1 mM sodium cacodylate buffer, pH 7.0. All solutions were prepared using doubly deionized, Millipore filtered water.

Absorption spectra and 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 cuvettes (10 mm pathlength) were heated by circulating water. Measurements were performed in BPE buffer, pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA). The temperature inside the cuvette 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.

Circular dichroism (CD). CD spectra were recorded on a Jobin-Yvon CD 6 dichrograph interfaced to a microcomputer. Solutions of drugs, nucleic acids and their complexes in 1 mM sodium cacodylate buffer (pH 7.0) were scanned in 2 cm quartz cuvettes. Measurements were made by progressive dilution of drug–DNA complex at a high P/D (phosphate/drug) ratio with a pure ligand solution to yield the desired drug/DNA ratio. Three scans were accumulated and automatically averaged.

Electric linear dichroism (ELD). ELD measurements were performed according to the procedures outlined previously.[21] All experiments were conducted at 20 °C with a 10 mm pathlength Kerr cell having 1.5 mm electrode separation, in 1 mM sodium cacodylate buffer, pH 7.0. The DNA samples were oriented under an electric field strength of 13 kV/cm, and the drug under test was present at 10 μM together with the DNA or polynucleotide at 100 μM unless otherwise stated. This electro-optical method has proved most useful as a means of determining the orientation of drugs bound to DNA, and has the additional advantage that it senses only the orientation of the polymer-bound ligand: free ligand is isotropic and does not contribute to the signal.[22]

DNA relaxation experiments. Supercoiled pKMp27 DNA (0.5 μg) was incubated with 6 units human topoisomerase I 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 varying concentrations of the drug under study. 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 with or without ethidium bromide (1 mg/mL), at room temperature for 4 h. Gels were washed and photographed under UV light.

Growth inhibition assay. L1210 cells (Murine Leukemia) provided by the NCI, Frederik, USA were cultivated in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES buffer (pH 7.4). Cytotoxicity was measured by the microculture tetrazolium assay as described.[23] Cells were exposed to graded concentrations of the compounds for 48 h and results expressed as IC_{50} (concentration which reduced by 50% the optical density of treated cells with respect to untreated controls).

Cell cycle analysis. For the cell cycle analysis, L1210 cells (2.5×10^5 cells/mL) were incubated for 21 h with various concentrations of the compounds, then fixed by 70% ethanol (v/v), washed and incubated in PBS containing 100 μg/mL RNase and 25 μg/mL propidium iodide for 30 min at 20 °C. For each sample, 10^4 cells were analyzed on an XL/MCL flow cytometer (Beckman Coulter). The fluorescence of propidium iodide was collected through a 615 nm long-pass filter. Data are displayed as linear histograms and results are expressed as the percentage of cells found in the G2 + M phase of the cell cycle.

Antibiogram tests and MIC determination. MICs of **7** and **8** were determined 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 μg/mL to 0.05 μg/mL.

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