Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 1597–1604

BIOORGANIC & MEDICINAL CHEMISTRY

Syntheses, Biochemical and Biological Evaluation of Staurosporine Analogues from the Microbial Metabolite Rebeccamycin

Fabrice Anizon,[a] Pascale Moreau,[a] Martine Sancelme,[a] Aline Voldoire,[a] Michelle Prudhomme,[a,*] Monique Ollier,[b] Danièle Sevère,[c] Jean-François Riou,[c] Christian Bailly,[d] Doriano Fabbro,[e] Thomas Meyer[e] and A. M. Aubertin[f]

[a]*Université Blaise Pascal, Synthèse, Electrosynthèse et Etude de Systèmes à Intérêt Biologique, UMR 6504, 63177 Aubière, France*
[b]*INSERM U71, Rue Montalembert, 63005 Clermont-Ferrand, France*
[c]*Rhône-Poulenc Rorer, 13, Quai Jules Guesde, 93403 Vitry sur Seine, France*
[d]*Centre Oscar Lambret et INSERM U124, Place de Verdun, 59045 Lille, France*
[e]*Novartis, Department of Oncology, K-125-409, CH-4002, Basle, Switzerland*
[f]*INSERM U74, Université Louis Pasteur, Strasbourg, France*

Received 5 February 1998; accepted 8 April 1998

Abstract—The indolocarbazole antibiotics staurosporine and rebeccamycin (**1**) are potent antitumor drugs targeting protein kinase C and topoisomerase I, respectively. To obtain staurosporine analogues from rebeccamycin, different structural modifications were performed: coupling of the sugar moiety to the second indole nitrogen, dechlorination and then reduction of the imide function to amide. The newly synthesized compounds (**3**–**6**) were tested for their abilities to bind to DNA and to inhibit topoisomerase I and protein kinase C. Their antiproliferative effects in vitro against B16 melanoma and P388 leukemia (including the related P388CPT cell line resistant to camptothecin) as well as their anti-HIV-1 and antimicrobial activities against various strains of microorganisms were determined. The cytotoxicity of the dechlorinated imide analogue **5** correlates well with its DNA binding and anti-topoisomerase I activities. These findings provide guidance for the development of new topoisomerase I-targeted antitumor indolocarbazoles equipped with a carbohydrate attached to the two indole nitrogens.

Introduction

Rebeccamycin is an antitumor antibiotic isolated from cultures of *Saccharotrix aerocolonigenes*.[1,2] Its structure contains an indolocarbazole framework onto which a sugar unit is attached via a β-*N*-glycosyl bond with one of the indole nitrogens. Its antitumor activity, as well as those of related compounds ED-110, NB-506, and derivatives of the antibiotic BE-13793C, is attributed to topoisomerase I inhibition.[3–5] Unlike the well known protein kinase C (PKC) inhibitor staurosporine, rebeccamycin does not inhibit this enzyme.[6,7] The two main differences between the structures of rebeccamycin and staurosporine are the functionality of the upper heterocycle (imide function in rebeccamycin, amide function in staurosporine) and, most importantly, the sugar moiety which is attached to both indole nitrogens in staurosporine but to only one indole nitrogen in rebeccamycin, ED-110 and NB-506 (Fig. 1). These factors could explain the discrepancy in the enzyme inhibition.

In the course of structure–activity relationship studies on rebeccamycin analogues, we investigated the possibilities to obtain, from rebeccamycin, compounds for which the sugar moiety would be linked to both indole nitrogens. In this paper, we report the synthesis,

Key words: Topoisomerase I; protein kinase C; indolocarbazoles; rebeccamycin; staurosporine.
*Corresponding author. Tel.: 33 4 73 40 71 24; Fax: 33 4 73 40 77 17.

0968-0896/98/$—see front matter
PII: S0968-0896(98)00096-0

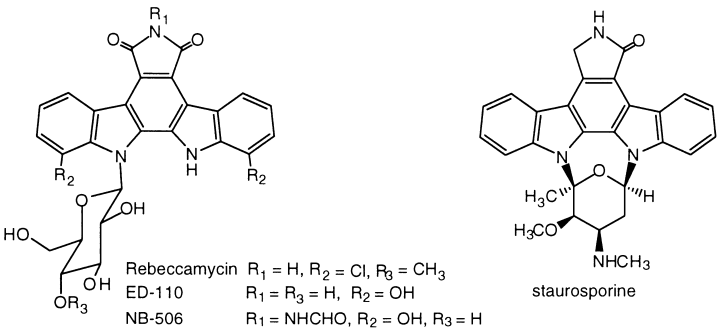

Figure 1.

biochemical (DNA binding and PKC and topoisomerase I inhibition) and biological (antimicrobial, antitumor and anti-HIV-1 activities) evaluation of compounds **3**–**6** prepared by semi-synthesis from rebeccamycin (Fig. 2).

Results and Discussion

Chemistry

Compound **2** was prepared from rebeccamycin by *O*-tosylation at the 2′ position using *p*-toluenesulfonylchloride and potassium carbonate as a base. The 2′ position of the ester was assigned from ^{1}H-^{1}H COSY and exchange with D_2O. Reaction of tosylate **2** with sodium azide led to a mixture of azide **3** and compound **4**. Azide **3** is probably formed via 2′,3′-epoxide resulting from the elimination of *p*-toluenesulfonic acid before the nucleophilic attack by the azide anion. A nucleophilic attack by the indole nitrogen leads to **4**. Compound **4** could be formed either by a direct nucleophilic substitution on tosylate **2** leading to inversion of the configuration at C2′ or by nucleophilic attack at the 2′ position of the transient epoxide leading to retention of configuration at C2′. In compound **4**, $C_{2'}$ and $C_{3'}$, at 56.6 and 68.7 ppm, respectively, are shielded compared to $C_{2'}$ and $C_{3'}$ of rebeccamycin (72.0 and 77.5 ppm); $H_{3'}$ is coupled with $OH_{3'}$. The weak coupling constant (6 Hz) between protons at the 1′ and 2′ positions observed in cyclized compound **4** is in the favor of the nucleophilic attack of the epoxide. However the weak coupling constants between protons at the 2′ and 3′ positions in compounds **4** and **5** (3.2 and about 0 Hz, respectively) are in the favor of an axial-equatorial coupling resulting from a direct nucleophilic substitution. Molecular modeling experiments (Program MOPAC version 6.0, QCPE no 455) were carried out on compounds **4** and **5** with both configurations *R* and *S* at the C2′. The coupling constants $J_{1',2'}$ and $J_{2',3'}$ were 2.0 and 3.2 Hz for **4***S*, 9.0 and 10.2 Hz for **4***R*, 2.4 and 3.6 Hz for **5***S*, 8.7.and 10.8 for **5***R*, respectively. These values fit

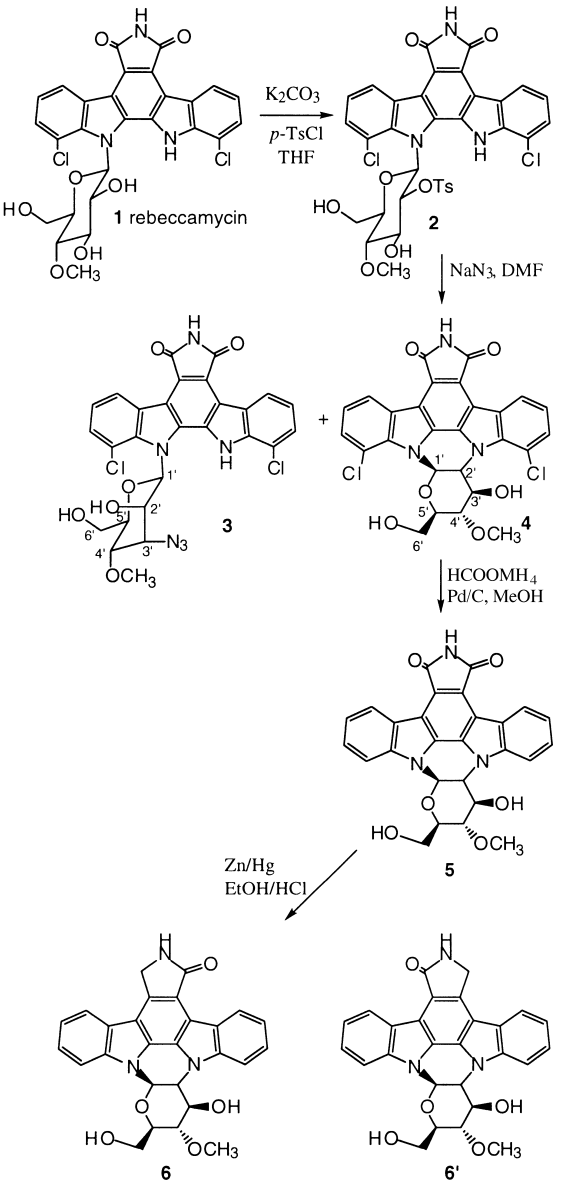

Figure 2.

better with a S configuration except for $J_{1',2'}$. The correct assignment of the structures could be obtained from cristallographic data (under investigations). Compound **4** was the major product (53% yield) whereas azide **3** was obtained in 36% yield. The position of the azide group in compound **3** and the stereochemistry on the sugar moiety were determined from ^{1}H-^{1}H COSY, exchange with D_2O and ^{13}C-^{1}H correlations. The position of N_3 group was assigned from the chemical shift of $C_{3'}$ (59.9 ppm) compared to the shielded $C_{3'}$ in rebeccamycin (77.5 ppm) whereas the chemical shift of $C_{2'}$ (69.3 ppm) remains almost unchanged (72.0 in rebeccamycin). The coupling constants $H_{2'}$-$H_{3'}$ (4.0 Hz) and $H_{3'}$-$H_{4'}$ (3.5 Hz) are consistent with axial-equatorial and equatorial-equatorial coupling. Dechlorination of **4** was achieved using ammonium formate and palladium on activated carbon in methanol. Reduction of **5** was performed with zinc-amalgam in ethanol/HCl,[8,9] a mixture of regioisomers **6**–**6′** which could not be separated by chromatography was obtained. The isomeric ratio calculated from ^{1}H NMR spectrum was 1.2:1.

Protein kinase C inhibition

The inhibitory properties toward PKC-α were tested using protamine sulfate as a substrate. The IC_{50} values are reported in Table 1. Unexpectedly, all the compounds in this series are inactive toward PKC. A very weak effect was noted with compound **4** at a high concentration but the effect is extremely weak compared to what can be achieved with staurosporine (IC_{50} PKC-α:28 nM).[10] This suggests that the rigid tetrahydropyrazine ring of compounds **4**–**6** in which are located both indole nitrogens is not consistent with PKC inhibitory effect. In staurosporine, this heterocycle contains seven atoms, instead of six, including an oxygen atom and therefore is more flexible.

DNA binding

The capacity of the test compounds to interact with DNA was investigated by means of absorption and fluorescence spectroscopy. As shown in Figure 3, addition of purified DNA has practically no effect on the absorption band of compounds **3** or **4** centered 326 nm. Similarly, the main absorption band of compound **6** at 288 nm remains unmodified in the presence of DNA, even in the presence of a large excess of DNA. In sharp contrast, compound **5** has significant interaction with DNA as judged from the 5 nm bathochromic shift and the 39% hypochromism observed upon addition of DNA. Rebeccamycin can also interact with DNA but its absorption spectrum shows no red shift and the hypochromism is weak (8%). We have shown previously that the presence of bulky chlorine atoms on the indolocarbazole chromophore (as with rebeccamycin) markedly hinders the capacity of the drug to intercalate into DNA and to recognise specific sequences.[11,12] The fluorescence data in Figure 4 provide complementary information. The fluorescence spectrum of compound **3** or **4** shows very little variation in the presence of DNA. Only a slight decrease of the fluorescence peak at 550 nm is detected. Conversely, under identical conditions the spectrum of compound **5** is drastically changed. Addition of DNA causes a significant increase in the fluorescence peak at 540 nm. The absorption and fluorescence spectral changes with compound **5** reflect the perturbation of the complexed indolocarbazole chromophore system upon binding to DNA. This dechlorinated imide analogue **5** is apparently the only drug in the series capable of detectable complex formation with DNA.

Topoisomerase I inhibition

The topoisomerase I inhibitory properties were tested on purified calf thymus topoisomerase I using the ^{32}P-labelled *Eco*RI-*Hind*III restriction fragment of pBR322 as a substrate. The labelled DNA fragment was incubated with topoisomerase I in the presence and absence of the drugs at concentrations ranging from 0.01 μg/mL to 10 μg/mL and the resulting cleavage products were analysed by agarose gel electrophoresis. The relative efficacy of the drugs to stimulate DNA cleavage varies considerably from one congener to one another. For each drug, we determined the MIC which corresponds

Table 1. Inhibitory activities toward PKC, topoisomerase I, in vitro antiproliferative activities against murine B16 melanoma and P388 leukemia cells, antimicrobial activities against *B. cereus*, and anti HIV-1 activities in HIV-1 Lai infected CEM-SS cells (Selectivity index $SI = CC_{50}/IC_{50}$)

Compd	PKC IC_{50} μM	B16 IC_{50} μM	P388 IC_{50} μM	*B. cereus* MIC μM	Topoisomerase I MIC μM	HIV-1 Lai CEM-SS IC_{50}-CC_{50} (μM)-SI
1	> 175	0.48	1.22	10.9	1.75	0.52–1.05–2.0
3	nd	nd	3.95	> 84	17	nd
4	118	2.9	5.44	> 90	18.1	0.58–4.2–7.2
5	> 207	2	0.70	> 93	2.07	3.7–6.6–1.8
6–**6′**	> 213	8.9	6.82	> 106	21.3	5.7–6.3-1.1

to the minimum drug concentration at which topoisomerase I-mediated DNA cleavage was detected (Table 1). As expected from previously results,[7,13] dechlorinated imide **5** exhibited the strongest topoisomerase I inhibitory effect. This compound is 10 times more active than the corresponding reduced analogues **6**–**6′**. There is no doubt that the carbonyl group that distinguishes these compounds must be important for both DNA binding and topoisomerase I inhibition.

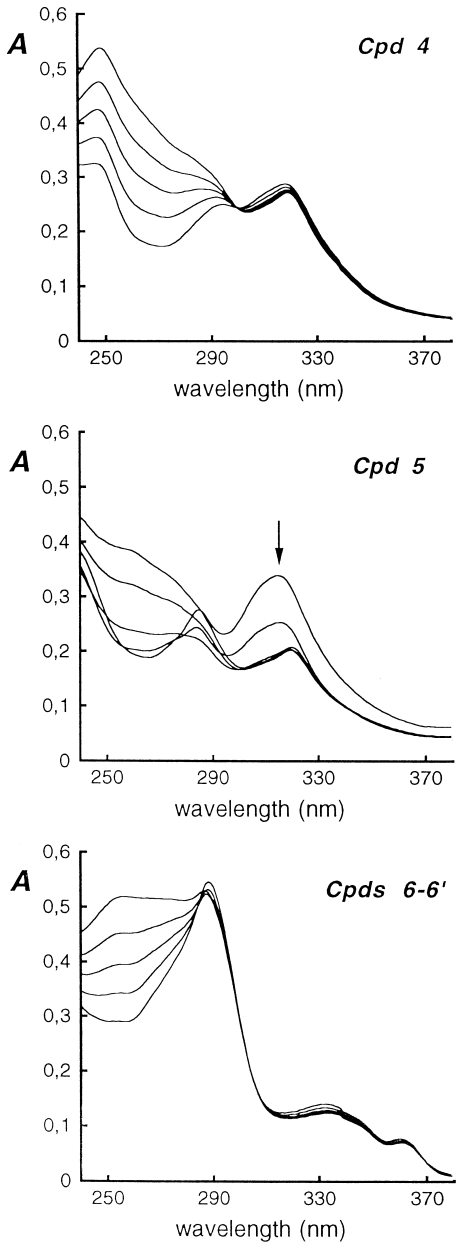

Figure 3. Absorption spectra of compounds **4**, **5**, and **6**–**6′** in the absence and presence of calf thymus DNA. To 3 mL of drug solution (10 μM in 10 mM Tris, 10 mM NaCl buffer, pH 7.1) were added successive amounts of DNA. The DNA/drug ratio increased as follows (bottom to top curves at 260 nm): 0, 1, 2, 3, and 4. With compound **5**, at higher P/D (phosphate–DNA/drug) ratios no further qualitative changes occurred and the hypochromism at 320 nm plateaued at about 40%.

In vitro antiproliferative activity

The antiproliferative activities were tested in vitro against two murine cell lines, B16 melanoma and P388 leukemia. IC_{50} values are reported in Table 1. The most efficient compound was dechlorinated imide **5**. In order to have an insight into the involvement of topoisomerase I inhibition in the cytotoxicity, the effects of compounds **1**–**6** on the growth of P388 CPT cells resistant to the topoisomerase I inhibitor camptothecin were evaluated (Table 2). From the resistance index measured

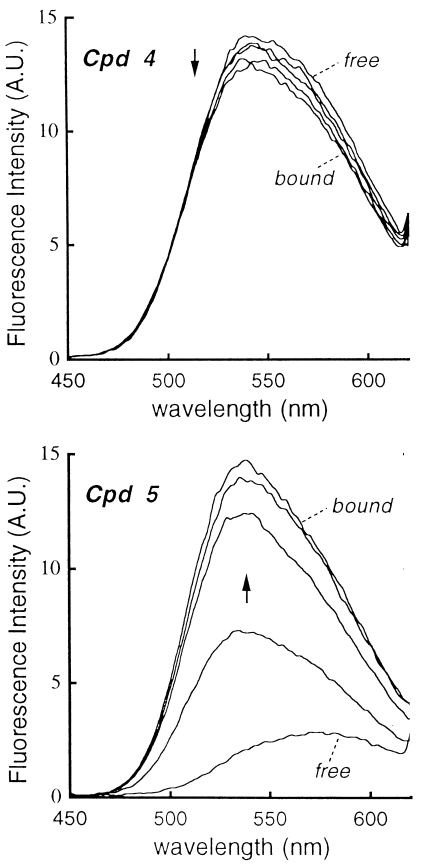

Figure 4. Fluorescence emission spectra of compounds **4** and **5** in the absence and presence of increasing amounts of calf thymus DNA. To 3 mL of a drug solution (10 μM drug, 80 μM CT-DNA, in 10 mM Tris, 10 mM NaCl buffer, pH 7), successive amounts of DNA were added from a stock solution. Spectra were recorded after 10 min of equilibration with an excitation wavelength of 320 nm. The DNA/drug ratio increased from 1 to 4 (the first curve corresponds to free DNA).

Table 2. Toxicities of compounds **1**–**6**–**6′** towards P388 cells and P388 CPT cells, resistant to the topoisomerase I inhibitor camptothecin. Resistance index, $R = IC_{50}$ P388 CPT/IC_{50} P388

Compd	IC_{50} P388 μM	IC_{50} P388 CPT μM	*R*
1	1.22	10.5	8.5
3	3.95	17	4.2
4	5.44	5.08	0.93
5	0.70	> 20	> 29
6–**6′**	6.82	> 21	> 3.1

with compound **4**, it seems obvious that topoisomerase I plays little or no role in the cytotoxicity of this compound. Its cytotoxic effect may be attributed to its action at a protein kinase level because it is the only drug in the series exhibiting a weak inhibitory effect toward PKC. The high resistance index determined for compound **5** suggests a major contribution of topoisomerase I inhibition to the cytotoxicity of this compound.

Anti-HIV-1 activity

Since topoisomerase I is known to activate HIV-1 reverse transcriptase activity,[14,15] the anti-HIV-1 activity of compounds **2**–**6** was tested. For that purpose, we quantitated the reverse transcriptase activity associated with virus particles released from HIV-1 infected CEM-SS 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 1). The best selectivity index was obtained for the chlorinated imide **4**. Compound **5** presents no particular effect and therefore there seems to be no straightforward correlation between topoisomerase I inhibition and anti-HIV-1 activity in this series.

Antimicrobial properties

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 (Table 3). All the compounds were inactive against *C. albicans*. Compound **5** was weakly active against the two Gram-positive strains tested and weakly active against *E. coli*. The activity of chlorinated imide **4** against *S. chartreusis* was similar to that observed with rebeccamycin.

Conclusion

A direct correlation can be established between DNA binding, topoisomerase I inhibition and the cytotoxic effects. Indeed, compound **5** is the only drug in the series which produces detectable complexes with DNA and stabilizes DNA-topoisomerase I cleavable complexes and, as a result, exhibits marked cytotoxic activities. As a corollary, the other compounds which fail to form complexes with DNA, do not inhibit topoisomerase I, or very weakly, and are not cytotoxic. It is therefore tempting to conclude that DNA binding is a prerequisite for topoisomerase I inhibition and antiproliferative activity. In contrast, no obvious correlation can be established between topoisomerase I poisoning and the anti-HIV-1 or antimicrobial effects.

Compound **5** represents a useful starting point for the future design of potent antitumor indolocarbazoles acting specifically on topoisomerase I without unwanted effects on PKC. We are now introducing polar groups to increase its solubility.

Table 3. Antimicrobial activities of compounds **1**–**6**–**6′** against two Gram-positive bacteria, *B. cereus* and *S. chartreusis*, a Gram-negative bacterium *E. coli* and a yeast *C. albicans*

Compd	*B. cereus* ATCC 14579	*S. chartreusis* NRRL 11407	*E. coli* ATCC 11303	*C. albicans* IP 444
1	++	++	—	—
3	—	—	—	—
4	—	++	—	—
5	±	±	±	±
6–**6′**	—	—	—	—

The size of zones of growth inhibition was 8–9 mm (++), 7–8 mm (+),6–7 mm (±).

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), doubled doublet (dd), triplet (t), multiplet (m), tertiary carbons (C tert.), quaternary carbons (C quat.)). The signals were assigned from ^1H-^1H COSY, ^{13}C-^1H correlations, exchange 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 silicagel Geduran SI 60 (Merck) 0.040–0.063 mm or Kieselgel 60 (Merck) 0.063–0.200 mm column chromatography. For purity tests, TLC were 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-(2′-*O*-tosyl)-glucopyranosyl)-6,7,12,13-tetrahydro(5*H*)-indolo[2,3-*a*]-pyrrolo-[3,4-*c*]-carbazole-5,7-dione (2). Potassium carbonate (235 mg, 1 equiv) and *p*-toluenesulfonylchloride (323 mg, 1.7 mmol) were added to a solution of rebeccamycin (1 g, 1.7 mmol) in THF (200 mL). The mixture was refluxed for 48 h. After removal of the solvent, the residue was purified by chromatography (eluent, cyclohexane:EtOAc, 70:30) to give **2** (579 mg, 0.80 mmol, 47% yield) as a yellow powder. Melting point 168–170 °C; IR(KBr): $\nu_{C=O}$ 1720, 1770 cm^{-1}, $\nu_{NH,OH}$ 3200–3600 cm^{-1}; HRMS (FAB+) M^+ calcd for $C_{34}H_{27}N_3O_3SCl_2$ 723.0845 found 723.0787; ^1H NMR (400 MHz, acetone-d_6) δ 2.20 (3H, s), 3.78 (3H, s, OCH_3), 4.08 (1H, $H_{4'}$, t, J = 9.0 Hz), 4.17 (2H, $H_{3'}$, $H_{5'}$, m), 4.27 (2H, $H_{6'}$, m), 4.86 (1H, t, J = 5.4 Hz, OH), 5.10 (1H, $H_{2'}$, dd, J_1 = 9.3 Hz, J_2 = 8.4 Hz), 5.12 (1H, d, J = 5.9 Hz, OH), 6.58 (2H, d, J = 8.5 Hz), 6.68 (2H, d, J = 7.9 Hz), 7.17 (1H, t, J = 7.9 Hz), 7.28 (1H, t, J = 7.9 Hz), 7.37 (1H, dd, J_1 = 7.9 Hz, J_2 = 1.0 Hz), 7.56 (1H, d, $H_{1'}$, J = 9.3 Hz), 7.58 (1H, dd, J_1 = 7.9 Hz, J_2 = 1.0 Hz), 8.86 (1H, d, J = 7.9 Hz), 8.89 (1H, dd, J_1 = 7.8 Hz, J_2 = 1.4 Hz), 10.06 (1H, s, NH), 10.40 (1H, s, NH); ^{13}C NMR (100 MHz, acetone-d_6) δ 20.5 (CH_3 of the tosyl group); 60.2 (OCH_3); 60.3 (CH_2); 75.5, 79.4, 80.4, 81.1, 81.4 (CH); 115.2, 116.6, 118.4, 119.2, 120.1; 122.2, 123.6, 124.9, 128.2, 129.1, 132.8, 135.4, 137.7, 144.1 (C quat. arom.); 121.7, 121.8, 123.5, 124.6, 125.6 (2C), 126.9, 128.9 (2C), 129.8 (C tert. arom.); 170.0, 170.1 (C=O).

1,11-Dichloro-12(4-*O*-methyl-β-D-(3′-azido)-altropyranosyl)-6,7,12,13-tetrahydro(5*H*)-indolo[2,3-*a*]-pyrrolo[3,4-*c*]-carbazole-5,7-dione (3) and 1,11-dichloro-12,13-[1,2-(4-*O*-methyl-D-mannopyranosyl)]-6,7,12,13-tetrahydro(5*H*)-indolo[2,3-*a*]-pyrrolo[3,4-*c*]-carbazole-5,7-dione (4). To a solution of compound **2** (449 mg, 0.620 mmol) in DMF (16 mL) was added sodium azide (403 mg, 6.2 mmol, 10 equiv). The mixture was stirred at 70 °C for 6 days, then cooled, poured into water and extracted with EtOAc. The organic phase was washed with saturated aqueous $NaHCO_3$ and brine, then dried over $MgSO_4$. The solvent was removed and the residue purified by chromatography (eluent, EtOAc:CH_2Cl_2, 10:90) affording **3** (69.4 mg, 0.117 mmol, 19% yield) as a yellow–orange solid and **4** (226.1 mg, 0.401 mmol, 65% yield) as a yellow solid.

Compound **3**: mp >300 °C; IR(KBr): $\nu_{C=O}$ 1710, 1750 cm^{-1}, $\nu_{N=N}$ 2110 cm^{-1}, $\nu_{NH,OH}$ 3200–3600 cm^{-1}; HRMS (FAB+) $(M+H)^+$ calcd for $C_{27}H_{21}N_6O_6Cl_2$ 595.0899 found 595.0937; ^1H NMR (400 MHz, DMSO-d_6) δ 3.58 (1H, m), 3.59 (3H, s, OCH_3), 3.68 (1H, dd, J_1 = 10.8 Hz, J_2 = 4.0 Hz), 4.07 (1H, m, $H_{5'}$), 4.19 (1H, dd, $H_{4'}$, J_1 = 9.9 Hz, J_2 = 3.0 Hz), 4.64 (1H, t, $OH_{6'}$, J = 5.0 Hz), 4.77 (1H, d, $H_{2'}$, J = 4.0 Hz), 4.94 (1H, pt, $H_{3'}$, J = 3.5 Hz), 7.11 (1H, s, $H_{1'}$), 7.41 (1H, t, J = 7.9 Hz), 7.48 (1H, t, J = 7.9 Hz), 7.50 (1H, broad s, OH), 7.72 (1H, d, J = 7.4 Hz), 7.74 (1H, dd, J_1 = 7.9 Hz, J_2 = 1.0 Hz), 9.08 (1H, d, J = 7.9 Hz), 9.40 (1H, dd, J_1 = 7.9 Hz, J_2 = 1.0 Hz), 11.30 (1H, s, N_{imide}-H), 12.00 (1H, s, N_{indole}-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 57.3 (OCH_3), 59.9 ($C_{3'}$); 60.5 ($C_{6'}$); 69.3 ($C_{2'}$); 73.6 ($C_{4'}$); 78.1 ($C_{5'}$); 84.0 ($C_{1'}$); 115.5, 115.8, 117.0, 118.4, 119.4, 122.2, 122.5, 125.4, 130.5, 130.6, 136.4, 137.0 (C quat. arom.); 121.0, 122.7, 123.5, 124.0, 126.3, 129.7 (C tert. arom.); 170.4, 170.7 (C=O).

Compound **4**: mp 296–298 °C; IR(KBr): $\nu_{C=O}$ 1700, 1760 cm^{-1}, $\nu_{NH,OH}$ 3200–3600 cm^{-1}; HRMS (FAB+) M^+: calcd for $C_{27}H_{19}Cl_2N_3O_6$ 551.0650 found 551.0647; ^1H NMR (400 MHz, DMSO-d_6) δ 3.46 (3H, s, OCH_3), 3.47 (3H, m, $2H_{6'} + H_{4'}$), 3.75 (1H, m, $H_{5'}$), 4.53 (1H, d, J = 7.1 Hz, $OH_{3'}$), 4.61 (1H, m, $H_{3'}$), 4.92 (1H, t, J = 4.5 Hz, $OH_{6'}$), 5.48 (1H, dd, J_1 = 6.3 Hz, J_2 = 3.2 Hz, $H_{2'}$), 6.39 (1H, d, J = 6.3 Hz, $H_{1'}$), 7.29 (1H, t, J = 8.0 Hz), 7.32 (1H, t, J = 8.0 Hz), 7.52 (1H, d, J = 7.9 Hz), 7.57 (1H, d, J = 7.9 Hz), 8.45 (1H, d, J = 7.9 Hz), 8.51 (1H, d, J = 7.9 Hz), 10.98 (1H, s, N_{indole}-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 62.1 ($C_{6'}$); 56.6 ($C_{2'}$), 57.1 (OCH_3), 68.7 ($C_{3'}$), 77.4 ($C_{5'}$), 78.3 ($C_{4'}$), 80.7 ($C_{1'}$); 111.0, 111.1, 116.5, 117.5, 120.1, 120.9, 126.1, 126.5, 128.0, 129.7, 136.2, 136.8 (C quat. arom.); 122.2, 122.6, 122.9, 123.5, 127.7, 127.9 (C tert. arom.); 170.3 (C=O).

12,13-[1,2-(4-*O*-Methyl-D-mannopyranosyl)]-6,7,12,13-tetrahydro(5*H*)-indolo[2,3-*a*]-pyrrolo[3,4-*c*]-carbazole-5,7-dione (5). A mixture of compound **4** (472 mg, 0.855 mmol), palladium 5% on activated carbon (570 mg) and ammonium formate (570 mg) in methanol (200 mL) was stirred at room temperature for 48 h. The mixture was filtered off over celite and the solid washed with THF. The solvent was removed and the residue purified by flash chromatography (eluent, cyclohexane:EtOAc, 40:60) to give **5** (342 mg, 0.708 mmol, 83% yield). Melting point 284–286 °C; IR(KBr): $\nu_{C=O}$ 1710, 1750 cm^{-1}, $\nu_{NH,OH}$ 3200–3600 cm^{-1}; HRMS (FAB+) $(M+H)^+$: calcd for $C_{27}H_{22}N_3O_6$ 484.1508, found 484.1516. ^1H NMR (400 MHz, DMSO-d_6) δ 3.40–3.56 (3H, m, $2H_{6'}$, $H_{4'}$), 3.63 (3H, s, OCH_3), 3.83 (1H, m, $H_{5'}$), 4.60 (2H, m, $H_{3'}$, $OH_{6'}$), 5.23 (1H, s, $H_{2'}$), 6.76 (1H, d, J = 5.0 Hz, $OH_{3'}$), 6.85 (1H, s, $H_{1'}$), 7.46 (1H, t, J = 7.4 Hz), 7.49 (1H, t, J = 7.4 Hz), 7.64 (1H, t, J = 8.3 Hz), 7.67 (1H, t, J = 7.4 Hz), 7.98 (1H, d, J = 8.3 Hz), 8.71 (1H, d, J = 7.9 Hz), 8.84 (1H, d, J = 7.9 Hz), 8.91 (1H, d, J = 8.3 Hz), 11.10 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 59.9 (OCH_3); 60.0 ($C_{6'}$); 63.2, 71.8, 76.1, 78.7, 80.2 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$); 111.8, 115.2, 120.8, 121.7, 124.4, 124.5, 126.8, 126.9 (C tert. arom.); 112.3, 112.7, 120.5, 120.9, 123.6, 123.8, 130.2, 131.0, 140.6, 142.5 (C quat. arom.) 171.0, 171.2 (C=O).

12,13-[1,2-(4-*O*-Methyl-D-mannopyranosyl)]-6,7,12,13-tetrahydro-5-oxo(5*H*)-indolo[2,3-*a*]-pyrrolo[3,4-*c*]-carbazole (6) and 12,13-[1,2-(4-*O*-methyl-D-mannopyranosyl)]-6,7,12,13-tetrahydro-7-oxo(5*H*)-indolo[2,3-*a*]-pyrrolo-[3,4-*c*]-carbazole (6′). Zinc-amalgam (1 g) was added to **5** (100 mg, 0.207 mmol) in a solution of ethanol (14 mL) and 6 N HCl (2.3 mL). The mixture was refluxed for 4 h then filtered off and the solid washed with EtOAc. After identical work up as for **4**, purification by flash chromatography (eluent, EtOAc) gave the isomeric mixture of **6** and **6′** (62 mg, 0.132 mmol, 64% yield) as an off-white powder. IR(KBr): $\nu_{C=O}$ 1670 cm^{-1}, $\nu_{NH,OH}$ 3200–3600 cm^{-1} HRMS (FAB+) $(M+H)^+$: calcd for $C_{27}H_{24}N_3O_5$ 470.1716, found 470.1730. ^1H NMR (400 MHz, DMSO-d_6) δ 3.44–3.56 (6H, m), 3.62 (3H, s, OCH_3), 3.63 (3H, s, OCH_3), 3.79 (2H, m), 4.53 (1H, t, J = 6.3 Hz, $OH_{6'}$), 4.57 (1H, t, J = 6.3 Hz, $OH_{6'}$), 4.63 (2H, m), 4.98 (4H, m, 2 CH_2), 5.12 (1H, m), 5.17 (1H, m), 6.76 (1H, d, J = 4.8 Hz, OH), 6.82 (3H, m, 1 OH + $2H_{1'}$), 7.34 (1H, t, J = 7.1 Hz), 7.39 (1H, t, J = 7.1 Hz), 7.41 (1H, t, J = 7.1 Hz), 7.43 (1H, t, J = 7.1 Hz), 7.51 (1H, t, J = 7.9 Hz), 7.53 (1H, t, J = 8.0 Hz), 7.55 (1H, t, J = 7.1 Hz), 7.58 (1H, t, J = 7.2 Hz), 7.90 (1H, d, J = 8.7 Hz), 7.94 (1H, d, J = 8.7 Hz), 8.14 (2H, d, J = 7.9 Hz), 8.58 (2H, s, 2NH), 8.86 (1H, d, J = 8.0 Hz), 8.90 (1H, d, J = 8.0 Hz), 8.96 (1H, d, J = 8.7 Hz), 9.04 (1H, d, J = 8.0 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 45.5, 45.6 (CH_2); 60.0 ($C_{6'}$); 60.1 (OCH_3); 63.5, 63.8, 72.5, 76.1, 76.2, 78.8, 78.9, 80.4 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$); 111.3, 111.4, 114.9, 115.4, 119.7, 120.4, 120.7, 121.3, 121.7, 122.1, 125.1, 125.2 (C tert. arom.); 111.5, 111.6, 112.6, 112.9, 119.7, 120.1, 124.6, 124.8, 125.0, 125.3, 127.1, 127.6, 129.4, 130.0, 133.4, 133.7, 139.7, 141.4, 141.5 (C quat. arom.), 172.0, 172.2 (C=O).

Absorption and fluorescence measurements. Absorption spectra were recorded on a Uvikon 943 spectrophotometer. The fluorescence emission spectra were recorded on a Perkin±Elmer LS50B fluorometer and are uncorrected. In both cases, the cell holder (10 mm pathlength) was thermostated with a Neslab RTE 111 cryostat. The DNA from calf thymus (highly polymerized sodium salt from Sigma Chemical Co.) was deproteinized twice with sodium dodecyl sulphate. DNA concentrations were determined applying a molar extinction coefficient of 6600 $M^{-1}cm^{-1}$. Titrations of the drugs with DNA were performed by adding aliquots of a concentrated DNA solution to a dilute drug solution (10 μM).

Topoisomerase I inhibition. Topoisomerase I was prepared from calf thymus as already described.[16,17] Topoisomerase I inhibition was evaluated using the DNA cleavage assay carried out according to the previously described method.[18] The MIC values correspond to the lowest concentration (μg/mL) producing a detectable DNA cleavage.

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

Protein kinase C inhibition. Protamine sulphate 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 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* strains. Paper disks impregnated with solutions of **1**–**6** 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**–**6** 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 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 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 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 RT assay as already described.[19] The 50% inhibitory concentration (IC_{50}) was derived from the computer-generated median effect plot of the dose–effect data.[20] 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 deshydrogenase 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).[21] 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.

Acknowledgements

The authors are grateful to Nicole Grangemare for technical assistance in microbiology and to Jacques Guyot for molecular modeling experiments.

References

1. Nettleton, D. E.; Doyle, T. W.; Krishnan, B.; Matsumoto G. K.; Clardy. J. *Tetrahedron Lett*. **1985**, *26*, 4011.

2. Bush, J. A.; Long, B. H.; Catino, J. J.; Bradner, W. T.; Tomita, K. J. *Antibiotics* **1987**, *40*, 668.

3. Yoshinari, T.; Yamada, A.; Uemura, D.; Nomura, K.; Arakawa, H.; Kojiri, K.; Yoshida, E.; Suda, H.; Okura, A. *Cancer Res*. **1993**, *53*, 490.

4. Arakawa, H.; Iguchi, T.; Morita, M.; Yoshinari, T.; Kojiri, K.; Suda, H.; Okura, A.; Nishimura, S. *Cancer Res*. **1995**, *55*, 1316.

5. Kojiri, K.; Kondo, H.; Yoshinari, T.; Arakawa, H.; Nakajima, S.; Satoh, F.; Kawamura, K.; Okura, A.; Suda, H.; Okanishi, M. J. *Antibiotics* **1991**, *44*, 723.

6. Tamaoki, T.; Nomoto, H.; Takahashi, I.; Kato, Y.; Morimoto, M.; Tomita, F. *Biochem. Biophys. Res. Commun*. **1986**, *135*, 397.

7. Rodrigues-Pereira, E.; Belin, L.; Sancelme, M.; Prudhomme, M.; Ollier, M.; Rapp, M.; Sevère, D.; Riou, J. F.; Fabbro, D.; Meyer, T. *J. Med. Chem*. **1996**, *39*, 4471.

8. Toullec, D.; Pianetti, P.; Coste, H.; Bellevergue, P.; Grand-Perret, T.; Ajakane, M.; Baudet, V.; Boissin, P.; Boursier, E.; Loriolle, F.; Duhamel, L.; Charon, D.; Kirilovsky, J. *J. Biol. Chem*. **1991**, *266*, 15771.

9. Fabre, S.; Prudhomme, M.; Sancelme, M.; Rapp, M. *Bioorg. Med. Chem*. **1994**, *2*, 73.

10. Wilkinson, S. E.; Parker, P. J.; Nixon, J. S. *Biochem. J*. **1993**, *294*, 335.

11. Bailly, C.; Riou, J. F.; Colson, P.; Houssier, C.; Rodrigues Pereira, E.; Prudhomme, M. *Biochemistry* **1997**, *36*, 3917.

12. Bailly, C.; Colson, P.; Houssier, C.; Rodrigues Pereira, E.; Prudhomme, M.; Waring, M. C. *Mol. Pharmacol*. **1998**, *53*, 77.

13. Anizon, F.; Belin, L.; Moreau, P.; Sancelme, M.; Voldoire, A.; Prudhomme, M.; Ollier, M.; Sevère, D.; Riou, J. F.; Bailly, C.; Fabbro, D. ; Meyer, T. *J. Med. Chem*. **1997**, *40*, 3456.

14. Takahashi, H.; Matsuda, M.; Kojima, A.; Sata, T.; Andoh, T.; Kuruta, T.; Nagashima, K.; Hall, W. W. *Proc. Natl. Acad. Sci. U.S.A* **1995**, *92*, 5694.

15. Pommier, Y.; Poddevin, B.; Gupta, M.; Jenkins, J. *Biochem. Biophys. Res. Commun*. **1994**, *205*, 1601.

16. Halligan, B. D.; Edwards, K. A.; Liu, L. F. *J. Biol. Chem*. **1985**, *260*, 2475.

17. Riou, J. F.; Helissey, P.; Grondard, L.; Giorgi-Renaud, S. *Mol. Pharmacol*. **1991**, *40*, 699.

18. Riou, J. F.; Fosse, P.; Nguyen, C. H.; Larsen, A. K.; Bissery, M. C.; Grondard, L.; Saucier, J. M.; Bisagni, E.; Lavelle, F. *Cancer Res*. **1993**, *53*, 5987.

19. Moog, C.; Wick, A.; Le Ber, P.; Kirn, A.; Aubertin, A. M. Bicyclic imidazo derivatives, a new class of highly selective inhibitors for the human immunodeficiency virus type 1. *Antiviral Res*. **1994**, *24*, 275.

20. Chou, J.; Chou, T. C. *Elsevier-Biosoft, Elsevier Science, Cambridge, U.K*. **1985**.

21. Mosmann, T. *J. Immunol. Meth*. **1983**, 65, *55*.