Structure−Activity Relationships in a Series of Substituted Indolocarbazoles: Topoisomerase I and Protein Kinase C Inhibition and Antitumoral and Antimicrobial Properties

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A series of compounds structurally related to staurosporine, rebeccamycin, and corresponding aglycones was synthesized, and their activities toward protein kinase C and topoisomerases I and II were tested together with their *in vitro* antitumor efficiency against murine B16 melanoma and P388 leukemia cells. Their antimicrobial activities were also examined against a Gram-negative bacterium (*Escherichia coli*), a yeast (*Candida albicans*), and three Gram-positive bacteria (*Bacillus cereus, Streptomyces chartreusis,* and *Streptomyces griseus*). To avoid side effects expected with protein kinase C inhibitors, we introduced substitution on the maleimide nitrogen and/or a sugar moiety linked to one of the indole nitrogens to obtain specific inhibitors of topoisomerase I with minimal activities on protein kinase C. As expected, these structures were inefficient on topoisomerase II, and some of them exhibited a strong activity against topoisomerase I. Generally, dechlorinated compounds were found to be more active than chlorinated analogues against both purified topoisomerase I and protein kinase C. On the other hand, opposite results were obtained in the cell antiproliferative assays. These results suggest lack of cell membrane permeability in the absence of the chlorine residue or cleavage of carbon−chlorine bonds inside the cell.

Introduction

Protein kinase C (PKC) plays a key role in cell signal transduction, controlling a large variety of cell responses including gene expression and cell proliferation.[1] PKC consists of at least 12 isoenzymes requiring different cofactors and showing differential tissue distribution and substrate specificities. Altered expression of PKC isoenzymes has been reported in a wide range of neoplastic and preneoplastic tissues. In these tissues, some of the PKC isoforms concerned are overexpressed and some others underexpressed.[2]

Among the known PKC inhibitors possessing an indolocarbazole moiety and interacting with the ATP binding site of the enzyme are microbial metabolites such as staurosporine, K-252a, and UCN-01 together with the K-252a derivative KT6006 and related aglycones[3−9] (Chart 1). However, these nonselective PKC inhibitors, compared with other kinases and different PKC isoforms, may be responsible for serious side effects. In this series, the K-252a derivatives KT6124 and KT6528[4,10] (Chart 1), substituted on the nitrogen of the upper heterocycle with a hydroxy or an amino group, are much less potent PKC inhibitors. They exert a broad spectrum of antiproliferative activity against human tumor cell lines *in vitro* and are also inhibitors of topoisomerase I.

Rebeccamycin (**1**) (Chart 2), a structurally related antitumor antibiotic,[11] in which the sugar moiety is linked to only one indole nitrogen, is not a PKC inhibitor. Its antitumor activity seems rather to be correlated to its inhibitory potency against topoisomerase I.[12] ED-110 and NB-506, semisynthetic derivatives of antibiotic BE-13793C, and a rebeccamycin derivative **2** (Chart 2) were reported to exhibit antitumoral properties.[13−15] ED-110, NB-506, and **2**, like rebeccamycin, bear a sugar unit attached to one indole nitrogen. In addition, NB-506 and **2** bear a substituent on the maleimide nitrogen. The cumulative toxicity of NB-506 was found to be much lower than those of some anticancer drugs on the market, and a phase I trial has shown reduction of tumors resistant to taxol.[16]

Chart 1

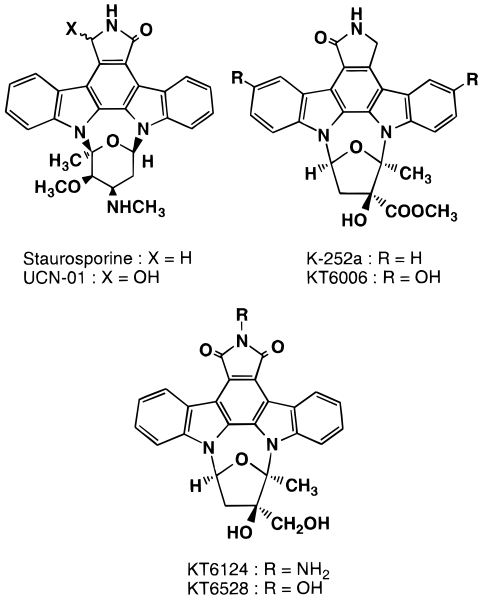

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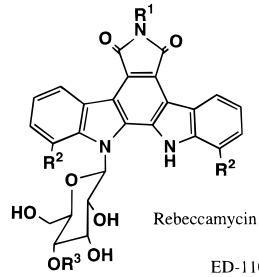
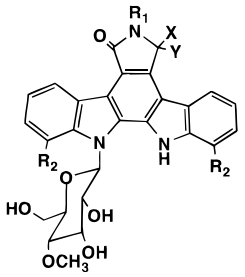
Chart 2

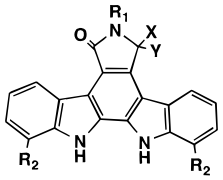


Rebeccamycin **1** $R^1 = H, R^2 = Cl, R^3 = CH_3$
2 $R^1 = NH_2, R^2 = Cl, R^3 = CH_3$
ED-110 $R^1 = R^3 = H, R^2 = OH$
NB-506 $R^1 = NHCHO, R^2 = OH, R^3 = H$

Chart 3



1 rebeccamycin : $R_1 = H; R_2 = Cl; X, Y = O$
2 $R_1 = NH_2; R_2 = Cl; X, Y = O$
3 $R_1 = OH; R_2 = Cl; X, Y = O$
4 $R_1 = NHCHO; R_2 = Cl; X,Y = O$
5 $R_1 = H; R_2 = H; X, Y = O$
6 $R_1 = NH_2; R_2 = H; X, Y = O$
7 $R_1 = OH; R_2 = H; X, Y = O$
8 $R_1 = NHCHO; R_2 = H; X,Y = O$
9 $R_1 = H; R_2 = Cl; X = Y = H$
10 $R_1 = H; R_2 = H; X = Y = H$



11 $R_1 = H; R_2 = H; X = Y = H$
12 $R_1 = H; R_2 = Cl; X = Y = H$
13 $R_1 = H; R_2 = Cl; X, Y = O$
14 $R_1 = H; R_2 = H; X, Y = O$
15 $R_1 = H; R_2 = Cl; X = H, Y = OH$
16 $R_1 = H; R_2 = H; X = H, Y = OH$
17 $R_1 = NH_2; R_2 = H; X, Y = O$
18 $R_1 = OH; R_2 = H; X, Y = O$
19 $R_1 = NHCHO; R_2 = H; X, Y = O$
20 $R_1 = CH_2CH_2OH; R_2 = H; X, Y = O$
21 $R_1 = NHCONHNH_2; R_2 = H; X, Y = O$
22 $R_1 = NH_2; R_2 = Cl; X, Y = O$
23 $R_1 = NHCHO; R_2 = Cl; X, Y = O$

From these literature data, it appears that in maleimide indolocarbazole compounds the presence of a sugar moiety linked to one of the two indole nitrogens, with or without a substitution on the maleimide nitrogen with a functional group bearing a labile hydrogen, may induce a weaker or absence of PKC inhibitory effect and a marked topoisomerase I inhibitory potency. The lower toxicity of these compounds may be due to their more specific action against topoisomerase I.

To extend knowledge in this field, we prepared a range of maleimide and maleamide indolocarbazoles with and without sugar moieties and with and without substitution on the nitrogen of the upper heterocycle by substituents bearing a labile hydrogen (Chart 3).

Chemistry

Rebeccamycin (**1**) was isolated from cultures of *Saccharotrix aerocolonigenes* (ATCC 39243).[11] **5**, **9**, **10, 12**, **13**, and **15** were obtained from rebeccamycin by structural modifications.[9,17] **11**, **14**, and **16** were synthesized from 2,3-dibromo-*N*-methylmaleimide and indolyl-MgBr.[7,8,18] **2** and **3** were prepared from rebeccamycin and **6** and **7** from dechlorinated rebeccamycin by reaction with hydrazine hydrate and hydroxylamine hydrochloride, respectively. **4** and **8** were prepared from **2** and **6**, respectively, by reaction with dimethylformamide and hydrochloric acid (Scheme 1).

22 was obtained from **13** by reaction with hydrazine hydrate and **23** from **22** by reaction with dimethylformamide and hydrochloric acid. **17**−**21** were prepared from the corresponding anhydride by reaction with hydrazine hydrate,[19] hydroxylamine hydrochloride,[19] formic hydrazide, ethanolamine, and carbohydrazide, respectively (Scheme 2). For the preparation of the chlorinated analogue of **18**, our classical method by reaction of hydroxylamine hydrochloride on imide **13** failed even though it was applied successfully to rebeccamycin (**1**). To have a more reactive starting material, we tried, unsuccessfully, to prepare the corresponding anhydride from **13** in a basic medium followed by ring closure by acidic treatment.[20]

Results and Discussion

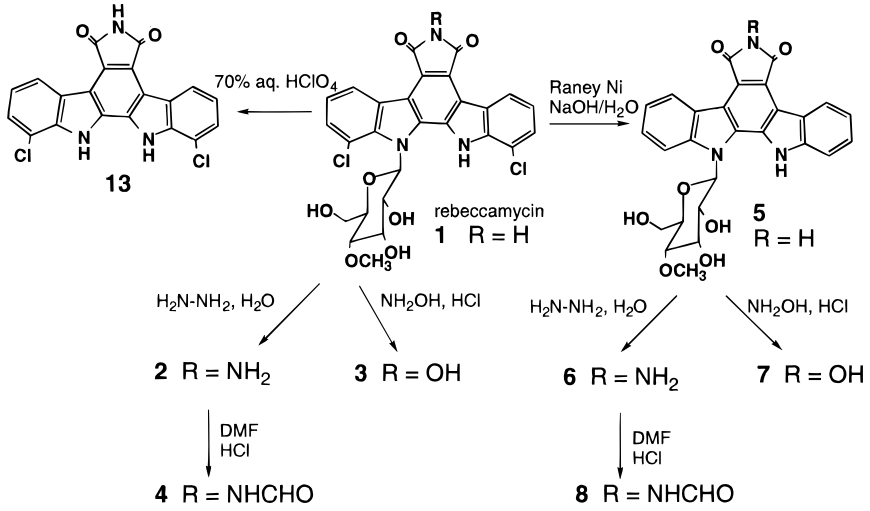
The inhibitory activities of compounds **1**−**23** toward PKC, topoisomerase I, and topoisomerase II were determined. The inhibition of protein kinase A (PKA) was examined for some of them to evaluate their selectivity. The antiproliferative activities against two different murine cell lines, B16 melanoma cells and P388 leukemia cells, were also examined *in vitro*. The results are reported in Table 1.

Inhibitory Potencies toward PKC. Maleimide indolocarbazoles bearing a sugar linked to one of the indole nitrogens were inactive against PKC except for compounds **3** and **7** substituted with a hydroxy group. **9** and **10** bearing an amide function in the upper heterocycle such as staurosporine were found to be PKC inhibitors. In the aglycone series, substitution on the upper heterocycle produced an activity in the same range as or lower than that of the unsubstituted analogue; in series with the sugar moiety, substitution with a hydroxy group enhanced activity toward PKC. Except for **17** and **18**, the compounds tested against both PKC and PKA exhibited a stronger activity against PKC. Dechlorinated compounds, bearing a sugar moiety or in the aglycone series, were always more active against PKC than their chlorinated analogues (except when both were inactive).

Topoisomerase Inhibition. As expected from literature data,[4,10] compounds **1**−**23** were inactive toward topoisomerase II. Concerning topoisomerase I inhibition, the same results were obtained as for PKC inhibition:Dechlorinated compounds had an identical or greater activity than the dichloro analogues. Compared with the corresponding aglycones, the presence of the sugar moiety enhanced the activity toward topoisomerase I. Compared with **5**, analogues **6**−**8** substituted on the maleimide nitrogen were 10 times more active. Amide **10** was the most active toward topoisomerase I but was also a strong PKC inhibitor.

***In Vitro* Antiproliferative Activity**. Compared with reference compounds **1** and **2**, no further improve-

Scheme 1



Scheme 2

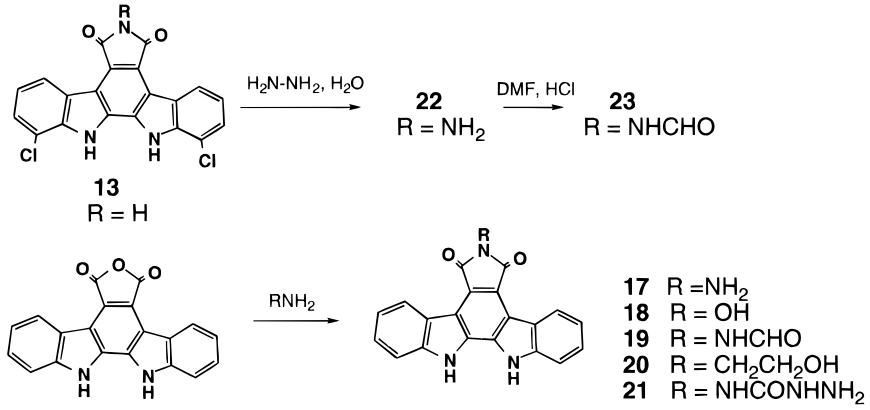


Table 1. Inhibitory Activities of Compounds **1**−**23** toward PKC, PKA, Topoisomerase I, and Topoisomerase II and Antiproliferative Activities *in Vitro* against Murine B16 melanoma and P388 leukemia Cells

	IC_{50} (μM)				MIC (μg/mL)	
compd	PKC	PKA	B16	P388	topoisomerase I	topoisomerase II
1	> 100[a]	> 100	0.48	0.3	1	>10
2	>100[b]	nd	0.3	0.1	0.1	>10
3	68[b]	nd	0.7	0.5	0.3	>10
4	>100[b]	nd	nd	0.3	1	>10
5	>100[a]	>100	17.5	3.5	1	>10
6	>100[b]	nd	4.1	0.3	0.1	>10
7	40[b]	nd	5.8	3	0.1	>10
8	>100	nd	nd	0.3	0.1	>10
9	28.8[a]	>100	>125	3	1	>10
10	3.7[a]	nd	nd	3	0.01	>10
11	2.45[a]	25.7	4.8	4	10	>10
12	>100[a]	>100	n.d.	3.5	>10	>10
13	>100[a]	>100	24	>10	>10	>10
14	44.7[a]	60	>100	4	10	>10
15	>100[a]	>100	8.4	9	>10	>10
16	22.1[a]	34	nd	0.3	1	>10
17	43.5[a]	44.6	>250	>10	10	>10
18	79.2[a]	41.5	4.6	>10	>10	>10
19	>100[b]	nd	>100	3.5	1	>10
20	>100[b]	nd	>150	7	>10	>10
21	78[b]	nd	75−15*	>10	>10	>10
22	>100[b]	nd	17	>10	>10	>10
23	>100	nd	nd	>10	>10	>10

[a] PKC and PKA inhibition measured according to procedure a described in the Experimental Section. [b] PKC inhibition measured according to procedure b. ***21** partially precipitated in DMSO. The IC_{50} value for **11** determined by procedure b was 0.64 μM.

ment of the cytotoxic properties against B16 melanoma or P388 leukemia was found for the new derivatives synthesized. Cytotoxic activity was found to be weaker for dechlorinated compounds than for the dichloro analogues in the series bearing the sugar, which corresponds to an opposite structure−activity relationship (SAR) compared to *in vitro* results against topoisomerase I and PKC.

Table 2. *In Vivo* Efficacy Study on B16 Melanoma and P388 Leukemia Cells[a]

dose (mg/kg/day, 1−5−9)	mean survival in days	T/C × 100
1. *In Vivo* Efficacy Study on B16 Melanoma Cells		
control	24.5	100
1, 2	30.5	124
16	27.5	112
64	26	106
11, 2	23	94
16	30	122
64	26	106
18, 2	27.5	112
16	31.5	128
64	27.5	112
2. *In Vivo* Efficacy Study on P388 Leukemia Cells		
control	11	100
1, 2	15	136
16	16.5	150
64	18	164
11, 2	11	100
16	11	100
64	11.5	104
18, 2	11.5	104
16	11	100
64	11	100

[a] Drugs were administred intraperitoneally on days 1, 5, and 9. Antitumor activity was determined by comparing the median survival time of treated animals (T) with that of controls (C) and is expressed as an oncostatic index: T/C × 100.

In all cases, for maleimide indolocarbazoles, compounds bearing the sugar moiety (**1**, **2**, **4**−**10**) were more active than their corresponding aglycones (**13**, **22**, **23**, **14**, **17**−**19**, **12**, **11**). For the dechlorinated analogues of rebeccamycin, substitution on the maleimide nitrogen afforded a stronger activity (**6**−**8** compared to **5**). For **10** and **11**, which were the strongest PKC inhibitors, and **10**, which was the strongest topoisomerase I inhibitor, there was no evidence for any additive effect that might enhance antiproliferative activity in cells.

***In Vivo* Antitumor Assay**. The antitumor activities of **1**, **11**, and **18** were examined *in vivo* on mice inoculated with B16 melanoma cells (Table 2.1) and P388 leukemia cells (Table 2.2). DL_{50} values also determined for **1** and **11** were found to be >120 mg/kg. The antiproliferative activity obtained *in vitro* on B16 melanoma cells was not confirmed by the *in vivo* tests. Only **1** exhibited a significant activity on mice injected with P388 leukemia cells. None of the three compounds was significantly active on mice bearing B16 cells. Some marginal activity was observed with **18** at 16 mg/kg against B16 melanoma (T/C × 100 = 128) but was not confirmed against P388 leukemia. This discrepancy between *in vivo* and *in vitro* tests may be due to poor membrane crossing or a metabolic process inactivating these drugs. The nonsignificant results obtained *in vivo* could also be due to the poor solubility of the compounds (soluble in DMSO), which could prevent the transport of the drug. We are investigating the synthesis of analogues of **18** bearing lipophilic substituents to enhance the membrane-crossing ability.

Antimicrobial Activity. Antibiogram tests on a Gram-negative bacterium (*Escherichia coli*), a yeast (*Candida albicans*), and three Gram-positive bacteria (*Bacillus cereus*, *Streptomyces chartreusis*, and *Streptomyces griseus*) (data not shown) showed that **1**−**23** were all inactive on *E. coli*. All were inactive against yeast *C. albicans*, except aglycones **12**, **13**, and **16**. Aglycones **13**−**15**, **17**, **18**, and **20** were inactive on the Gram-positive bacteria tested. **11** was only active on *B. cereus*, and **23** was only active on *S. griseus*. The other compounds exhibited moderate to strong antibacterial activities on the Gram-positive bacteria tested.

Table 3. MIC Values (μg/mL) Determined on *B. cereus*

compd	*B. cereus*	compd	*B. cereus*	compd	*B. cereus*
1	6.25	**9**	3.1	**17**	>50
2	0.8	**10**	12.5	**18**	>50
3	3.1	**11**	>50	**19**	50
4	3.1	**12**	6.25	**20**	>50
5	>50	**13**	>50	**21**	50
6	50	**14**	>50	**22**	nd[a]
7	25	**15**	>50	**23**	>50
8	25	**16**	6.25		

[a] The insolubility of **22** prevented the determination of its MIC value.

MIC values were determined on *B. cereus* (Table 3). The activity of **22** could not be determined because of its insolubility. As observed on melanoma B16 and leukemia P388 cells, chlorinated compounds were more active than their dechlorinated analogues. In the maleimide series, MIC values for the aglycones were >50 mM, while a strong antimicrobial effect was observed for the chlorinated analogues of rebeccamycin.

These results raise the question of the role of the chlorine. It may induce the lipophilicity necessary to facilitate the membrane crossing. Enzymatic carbon−chlorine bond cleavage may subsequently occur inside the cells.

Experimental Section

Chemistry. IR spectra were recorded on a Perkin-Elmer 881 spectrometer (ν in cm^{-1}) and NMR spectra on a Bruker AC 400 spectrometer (^1H, 400 MHz; ^{13}C, 100 MHz) (chemical shifts δ in ppm, abbreviations: singlet (s), doublet (d), triplet (t), multiplet (m), tertiary carbons (C tert), quaternary carbons (C quat)). Mass spectra (EI and FAB+) were determined at CESAMO (Talence, France) on a high-resolution FISONS Autospec-Q spectrometer. Chromatographic purifications were performed with flash Geduran SI 60 (Merck) 0.040−0.063 mm. For purity tests (compounds **19**−**23**), TLC was performed on fluorescent silica gel plates (60 F_{254} from Merck) and visualized by UV light.

6-Amino-1,11-dichloro-12-(4-*O*-methyl-β-D-glucopyrannosyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (2). To rebeccamycin (352 mg, 0.618 mmol) in THF (2 mL) was added H_2N-NH_2, H_2O (2.1 mL, 43.2 mmol). The mixture was stirred at room temperature for 3 h. Water (140 mL) was then added, and the mixture was stirred overnight. The precipitate was collected and dried in a desiccator to give **2** as a dark yellow powder (211 mg, 0.361 mmol, 58% yield). IR (KBr): $\nu_{NH,OH}$ 3300−3600 cm^{-1}, $\nu_{C=O}$ 1720 cm^{-1}. Mp: 254−257 °C. HRMS (FAB+): calcd for $C_{27}H_{23}Cl_2N_4O_7$, 585.0944; found, 585.0896. ^1H NMR (400 MHz, DMSO-d_6): 3.55−4.20 (6H, m, $C_{2'}$-H, $C_{3'}$-H, $C_{4'}$-H, $C_{5'}$-H, $C_{6'}$-H_2), 3.65 (3H, s, OCH_3), 5.08 (3H, s, NH_2, OH), 5.40 (1H, s, OH), 5.50 (1H, s, OH), 6.99 (1H, d, J = 9.2 Hz, $C_{1'}$-H), 7.51 (2H, t, J = 7.8 Hz), 7.75 (1H, d, J = 7.8 Hz), 7.78 (1H, d, J = 7.8 Hz), 9.14 (1H, d, J = 7.8 Hz), 9.34 (1H, d, J = 7.8 Hz), 10.67 (1H, s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 59.5 ($C_{6'}$), 60.1 (OCH_3), 72.1, 77.3, 79.2, 80.2, 84.4 ($C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{1'}$), 116.2, 116.3, 117.6 (2C), 119.4, 119.7, 123.1, 125.0, 129.6 (2C), 137.2, 137.7 (C quat arom), 122.1, 122.6, 123.4, 124.0, 127.1, 130.1 (C tert arom), 167.9, 168.1 (C=O).

1,11-Dichloro-6-hydroxy-12-(4-*O*-methyl-β-D-glucopyrannosyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]-carbazole-5,7-dione (3). To rebeccamycin (200 mg, 0.35 mmol) in DMF (2 mL) were added NH_2OH, HCl (1.70 g, 24.5 mmol), and NEt_3 (3.4 mL, 24.5 mmol). The mixture was heated at 80 °C for 4 h. It was then poured into water and extracted with AcOEt. The organic phase was washed with

brine and dried over $MgSO_4$. After removal of the solvent and purification by flash chromatography (eluent, cyclohexane−AcOEt, 30:70), compound **3** was isolated as a yellow solid (120 mg, 0.21 mmol, 60% yield). IR (KBr): $\nu_{NH,OH}$ 3200−3600 cm^{-1}, $\nu_{C=O}$ 1670 cm^{-1}. Mp: >300 °C. ^1H NMR (400 MHz, DMSO-d_6): 3.35−4.12 (6H, m, $C_{2'}$-H, $C_{3'}$-H, $C_{4'}$-H, $C_{5'}$-H, $C_{6'}$-H_2), 3.62 (3H, s, OCH_3), 5.15 (1H, s, OH), 5.40 (2H, s, OH), 6.97 (1H, d, J = 9.1 Hz, $C_{1'}$-H), 7.50 (2H, t, J = 7.8 Hz), 7.75 (1H, d, J = 7.8 Hz), 7.79 (1H, d, J = 7.7 Hz), 9.08 (1H, d, J = 7.9 Hz), 9.27 (1H, d, J = 7.9 Hz), 10.65 (1H, s, N_{indole}-H), 10.88 (1H br s, N-OH). ^{13}C NMR (100 MHz, DMSO-d_6): 60.0 ($C_{6'}$), 60.2 (OCH_3), 72.4, 77.5, 79.2, 80.2, 84.3 ($C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{1'}$), 116.0, 116.1, 116.6, 117.8, 118.8, 119.3, 123.0, 124.9, 129.3, 129.5, 137.2, 137.6 (C quat arom), 121.8, 122.3, 123.1, 123.8, 127.0, 130.0 (C tert arom), 166.1, 166.5 (C═O).

1,11-Dichloro-6-formamido-12-(4-*O*-methyl-*β*-D-glucopyrannosyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (4). A mixture of **2** (183 mg, 0.312 mmol), DMF (2 mL), and concentrated HCl (0.06 mL) was stirred at 60 °C for 4 h. A further 0.03 mL of concentrated HCl was added and the mixture warmed at 37 °C for 16 h. AcOEt was added and the mixture washed successively with 2% aqueous bicarbonate solution and brine. The organic phase was dried over $MgSO_4$, the solvent was removed, and the residue was purified by flash chromatography (eluent, AcOEt) to yield **4** as a yellow powder (146 mg, 0.238 mmol, 76% yield). IR (KBr): $\nu_{NH,OH}$ 3200−3550 cm^{-1}, $\nu_{C=O}$ 1710, 1720 cm^{-1}. Mp: .210−212 °C. HRMS (FAB+): calcd for $C_{28}H_{23}N_4O_8Cl_2$, 613.0892; found, 613.0985. ^1H NMR (400 MHz, DMSO-d_6): 3.57−4.11 (6H, m, $C_{2'}$-H, $C_{3'}$-H, $C_{4'}$-H, $C_{5'}$-H, $C_{6'}$-H_2), 3.65 (3H, s, OCH_3), 5.05 (1H, m, OH), 5.55 (2H, m, 2 OH), 6.95 (1H, d, J = 9.2 Hz, $C_{1'}$-H), 7.46 (1H, t, J = 7.0 Hz), 7.48 (1H, t, J = 6.5 Hz), 7.72 (1H, d, J = 7.6 Hz), 7.78 (1H, d, J = 7.7 Hz), 8.56 (1H, s, CHO), 9.03 (1H, d, J = 8.0 Hz), 9.21 (1H, d, J = 7.9 Hz), 10.77 (1H, s, NHCHO), 10.97 (1H, s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 59.9 (OCH_3), 60.2 ($C_{6'}$), 72.1, 77.4, 79.1, 80.3, 84.5 ($C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{1'}$), 116.4, 116.5, 117.1, 119.1, 119.8, 122.9, 124.8 (2C), 130.1, 130.2, 137.3, 138.0 (C quat arom), 122.4, 122.9, 123.2, 123.8, 127.5, 130.3 (C tert arom), 160.7 (CHO), 165.8, 166.0 (C═O).

6-Amino-12-(4-*O*-methyl-*β*-D-glucopyrannosyl)-6,7,12,-13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (6). To compound **5** (100 mg, 0.200 mmol) was added H_2N-NH_2, H_2O (0.7 mL, 14 mmol). The mixture was stirred at 50 °C for 1.5 h and then poured into water and extracted with AcOEt. The organic phase was dried over $MgSO_4$, the solvent was removed, and the residue was purified by flash chromatography (eluent, cyclohexane−AcOEt, 10:90) to give **6** as a red solid (94 mg, 0.183 mmol, 91% yield). IR (KBr): $\nu_{NH,OH}$ 3330, 3420 cm^{-1}, $\nu_{C=O}$ 1750 cm^{-1}. Mp: 260−262 °C. HRMS (FAB+): calcd for $C_{27}H_{25}N_4O_7$, 517.1723; found, 517.1680. ^1H NMR (400 MHz, DMSO-d_6): 3.55−4.10 (6H, m, $C_{2'}$-H, $C_{3'}$-H, $C_{4'}$-H, $C_{5'}$-H, $C_{6'}$-H_2), 3.71 (3H, s, OCH_3), 5.05 (2H, s, NH_2), 5.10 (1H, d, J = 7.7 Hz, OH), 5.36 (1H, d, J = 5.5 Hz, OH), 6.22 (1H, s, OH), 6.35 (1H, d, J = 8.9 Hz, $C_{1'}$-H), 7.43 (2H, t, J = 7.5 Hz), 7.64 (2H, t, J = 7.5 Hz), 7.77 (1H, d, J = 8.1 Hz), 8.01 (1H, d, J = 8.4 Hz), 9.17 (1H, d, J = 7.9 Hz), 9.23 (1H, d, J = 7.9 Hz), 11.65 (1H, s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 58.5 ($C_{6'}$), 60.1 (OCH_3), 73.2, 76.3, 77.1, 77.3, 84.1 ($C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{1'}$), 111.8, 112.3, 120.5, 120.7, 124.4 (2C), 126.9, 127.1 (C tert arom), 116.6, 117.1, 118.3, 118.5, 121.0, 121.4, 128.2, 129.6, 140.9, 142.2 (C quat arom), 168.7, 168.8 (C═O).

6-Hydroxy-12-(4-*O*-methyl-*β*-D-glucopyrannosyl)-6,7,-12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (7). To **5** (100 mg, 0.200 mmol) in DMF (2 mL) were added NH_2OH, HCl (970 mg, 13.9 mmol), and NEt_3 (1.40 g, 13.9 mmol). The mixture was stirred at 70 °C for 2 h and then treated as for **3**. Purification by flash chromatography (eluent, AcOEt−MeOH, 90:10) gave **7** as an orange solid (103 mg, 0.194 mmol, 99% yield). IR (KBr): $\nu_{NH,OH}$ 3200−3550 cm^{-1}, $\nu_{C=O}$ 1710 cm^{-1}. Mp: 298−300 °C. HRMS (FAB+): calcd for $C_{27}H_{24}N_3O_8$, 518.1563; found, 518.1551. ^1H NMR (400 MHz, DMSO-d_6): 3.50−4.15 (6H, m, $C_{2'}$-H, $C_{3'}$-H, $C_{4'}$-H, $C_{5'}$-H, $C_{6'}$-H_2), 3.71 (3H, s, OCH_3), 5.10 (1H, s, OH), 5.38 (1H, s, OH), 6.25 (1H, s, OH), 6.35 (1H, d, J = 8.9 Hz, $C_{1'}$-H), 7.37 (2H, t, J = 7.3 Hz), 7.64 (2H, t, J = 7.6 Hz), 7.83 (1H, d, J = 8.1 Hz), 8.03 (1H, d, J = 8.5 Hz), 9.11 (1H, d, J = 8.0 Hz), 9.18 (1H, d, J = 7.9 Hz), 10.82 (1H, s, N-OH), 11.65 (1H, s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 58.6 ($C_{6'}$), 60.2 (OCH_3), 73.4, 76.5, 77.3 (2C), 84.2 ($C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{1'}$), 111.9, 112.4, 120.5, 120.7, 124.5 (2C), 127.1, 127.3 (C tert arom), 115.3, 117.1, 117.3, 118.6, 121.0, 121.4, 128.2, 129.7, 141.0, 142.3 (C quat arom), 166.9, 167.0 (C═O).

6-Formamido-12-(4-*O*-methyl-*β*-D-glucopyrannosyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (8). A mixture of **6** (124 mg, 0.240 mmol), DMF (1.5 mL), and concentrated HCl (0.04 mL) was stirred at 60 °C for 4 h. A further 0.02 mL of concentrated HCl was added and the mixture warmed at 37 °C for 16 h. AcOEt was added, and the same workup as for **4** yielded **8** as a yellow powder (53 mg, 0.098 mmol, 41% yield). IR (KBr): $\nu_{NH,OH}$ 3300−3550 cm^{-1}, $\nu_{C=O}$ 1710, 1720 cm^{-1}. Mp: 265−267 °C. HRMS (FAB+): calcd for $C_{28}H_{24}N_4O_8$, 544.1594; found, 544.1591. ^1H NMR (400 MHz, acetone-d_6): 3.75 (3H, s), 3.87−4.25 (6H, m), 4.53 (1H, d, J = 4.8 Hz), 4.59 (1H, d, J = 4.0 Hz), 5.35 (1H, s), 6.34 (1H, d, J = 8.5 Hz), 7.28 (1H, t, J = 7.5Hz), 7.32 (1H, t, J = 7.5 Hz), 7.50 (1H, t, J = 7.5 Hz), 7.53 (1H, t, J = 7.5 Hz), 7.72 (1H, d, J = 8.2 Hz), 7.88 (1H, d, J = 8.4 Hz), 8.53 (1H, s, CHO), 9.07 (1H, d, J = 8.1 Hz), 9.18 (1H, d, J = 8.1 Hz), 9.77 (1H, s, NHCHO), 11.55 (1H, s, N_{indole}-H). ^{13}C NMR (100 MHz, acetone-d_6): 59.3 (OCH_3), 60.1 ($C_{6'}$), 73.8, 77.3, 77.4, 78.0, 84.9 ($C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{1'}$), 111.3, 112.1, 120.3, 120.5, 121.7, 121.8, 124.7, 124.8 (C tert arom), 116.4, 118.0, 118.2, 119.4, 120.9, 121.0, 128.9, 130.5, 141.4, 142.5 (C quat arom), 159.9 (CHO), 166.1, 166.6 (C═O).

6-Formamido-6,7,12,13-tetrahydro-5,7-dioxoindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (19). To 6,7,12,13-tetrahydro-5,7-dioxo-5*H*-indolo[2,3-*a*]furo[3,4-*c*]carbazole[18] (100 mg, 0.306 mmol) in DMF (10 mL) was added formic hydrazide (184 mg, 3.06 mmol). The mixture was stirred at 140 °C for 1 h. After cooling, addition of water (12 mL) caused the precipitation of an orange solid that was collected and washed successively with water and ethyl ether to give **19** (106 mg, 0.280 mmol, 94% yield). **19** was isolated as a single compound. Only one spot was obtained by TLC (R_f = 0.28; eluent, cyclohexane−AcOEt, 30:70). IR (KBr): $\nu_{NH,OH}$ 3200−3500 cm^{-1}, $\nu_{C=O}$ 1690, 1760 cm^{-1}. Mp: >300 °C. HRMS (EI): calcd for $C_{21}H_{12}N_4O_3$, 368.0909; found, 368.0913. ^1H NMR (400 MHz, DMSO-d_6): 7.44 (2H, t, J = 7.3 Hz), 7.65 (2H, t, J = 7.3 Hz), 7.88 (2H, d, J = 8.0 Hz), 8.51 (1H, s), 9.01 (2H, d, J = 7.8 Hz), 10.82 (1H, s), 11.96 (2H, s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 112.3, 120.5, 124.0, 127.2 (C tert arom), 115.8, 116.4, 121.3, 129.4, 140.5 (C quat arom), 160.7, 166.9 (C═O).

6-(2-Hydroxyethyl)-6,7,12,13-tetrahydro-5,7-dioxoindolo-[2,3-*a*]pyrrolo[3,4-*c*]carbazole (20). A mixture of 6,7,12,-13-tetrahydro-5,7-dioxo-5*H*-indolo[2,3-*a*]furo[3,4-*c*]carbazole[18] (100 mg, 0.306 mmol) in ethanolamine (1.3 mL, 1.29 g, 21.2 mmol) was stirred at room temperature for 1 h and then poured into water and extracted with AcOEt. The organic phase was washed with brine and dried over $MgSO_4$. Removal of the solvent yielded **20** as a yellow solid (85 mg, 0.230 mmol, 75% yield). TLC (R_f = 0.66; eluent, cyclohexane−AcOEt, 30:70) revealed there was only a single compound. IR (KBr): $\nu_{NH,OH}$ 3300−3500 cm^{-1}, $\nu_{C=O}$ 1750 cm^{-1}. Mp: >300 °C. HRMS (EI): calcd for $C_{22}H_{15}N_3O_3$, 369.1113; found, 369.1110. ^1H NMR (400 MHz, DMSO-d_6): 3.70 (2H, t, J = 5.4 Hz), 3.78 (2H, t, J = 5.4 Hz), 4.90 (1H br s, OH), 7.35 (2H, t, J = 7.6 Hz), 7.54 (2H, t, J = 7.4 Hz), 7.79 (2H, d, J = 8.2 Hz), 9.02 (2H, d, J = 8.0 Hz), 11.75 (2H, br s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 40.1 (CH_2), 58.4 (CH_2OH), 112.1, 120.2, 124.2, 126.8 (C tert arom), 115.6, 118.8, 121.5, 128.9, 140.4 (C quat arom), 169.9 (C═O).

6-(4-Semicarbazido)-6,7,12,13-tetrahydro-5,7-dioxoindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (21). A mixture of 6,7,-12,13-tetrahydro-5,7-dioxo-5*H*-indolo[2,3-*a*]furo[3,4-*c*]carbazole[18] (100 mg, 0.306 mmol), DMF (5 mL), and carbohydrazide (160 mg, 1.77 mmol) was stirred at 80 °C for 3 h. After cooling, addition of water (12 mL) caused the precipitation of an orange solid. After collection and washing with water and then petroleum ether, **21** was isolated (57 mg, 0.143 mmol, 47% yield). TLC (R_f = 0.13; eluent, cyclohexane−AcOEt, 30:70)

revealed there was only a single compound. IR (KBr): ν_{NH} 3200−3400 cm^{-1}, $\nu_{C=O}$ 1720, 1760 cm^{-1}. Mp: >300 °C. HRMS (EI): calcd for $C_{20}H_{12}N_4O_2$ (M − (CO-NH-NH_2) + H), 340.0960; found, 340.0951. ^{1}H NMR (400 MHz, DMSO-d_6): 4.45 (2H, br s, NH_2), 7.40 (2H, t, J = 7.4 Hz), 7.64 (2H, t, J = 7.2 Hz), 7.85 (2H, d, J = 8.1 Hz), 8.04 (1H, br s), 9.02 (3H, d, J = 7.8 Hz, br s), 12.45 (2H, s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 111.9, 120.3, 124.1, 127.0 (C tert arom), 115.4, 116.7, 121.3, 129.4, 140.2 (C quat arom), 158.4, 168.0 (C=O).

6-Amino-1,11-dichloro-6,7,12,13-tetrahydro-5,7-dioxoindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (22). To **13** (200 mg, 0.5 mmol) in DMF (20 mL) was added H_2N-NH_2, H_2O (1.72 mL, 35.5 mmol). The mixture was stirred at 70 °C for 1 h. After cooling, addition of water (25 mL) caused the precipitation of a yellow solid which was collected and washed with water and ethyl ether to give **22** (186 mg, 0.454 mmol, 90% yield). **22** was isolated as a single compound. Only one spot was obtained by TLC (R_f = 0.23; eluent, cyclohexane−AcOEt, 50:50). IR (KBr): ν_{NH} 3340 cm^{-1}, $\nu_{C=O}$ 1710 cm^{-1}. Mp: >300 °C. HRMS (EI): calcd for $C_{20}H_{10}Cl_2N_4O_2$, 408.0181; found, 408.0179. ^{1}H NMR (400 MHz, DMSO-d_6): 4.96 (2H, s, NH_2), 7.37 (2H, t, J = 7.9 Hz), 7.65 (2H, d, J = 7.6 Hz), 8.89 (2H, d, J = 7.9 Hz), 11.85 (2H, br s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6): 121.3, 123.1, 126.1 (C tert arom), 115.6, 115.8, 117.8, 123.0, 128.7, 137.0 (C quat arom), 168.5 (C=O).

6-Formamido-1,11-dichloro-6,7,12,13-tetrahydro-5,7-dioxoindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (23). To **22** (100 mg, 0.244 mmol) in DMF (10 mL) was added concentrated HCl (0.05 mL), and the mixture was stirred at 60 °C for 4 h. Concentrated HCl (0.02 mL) was then added and the mixture stirred at 37 °C for 16 h. After cooling, water was added, and the solid was collected, washed with water and then Et_2O, and dried in a desiccator to yield **23** (100 mg, 0.228 mmol, 93% yield) as a yellow powder. **23** was isolated as a single compound. Only one spot was obtained by TLC (R_f = 0.26; eluent, cyclohexane−AcOEt, 50:50). IR (KBr): ν_{NH} 3300 cm^{-1}, $\nu_{C=O}$ 1660, 1720 cm^{-1}. Mp: >300 °C. HRMS (EI): calcd for $C_{21}H_{10}N_4O_3Cl_2$, 436.0123; found, 436.0123. ^{1}H NMR (400 MHz, DMSO-d_6): 7.40 (2H, t, J = 7.8 Hz), 7.68 (2H, d, J = 7.6 Hz), 8.51 (1H, s, CHO), 8.84 (2H, d, J = 7.9 Hz), 10.85 (1H, s, NH), 11.90 (2H, s, N_{indole}-H). **23** was too insoluble in DMSO to get the ^{13}C NMR spectrum.

Biological Tests. Rebeccamycin was from our laboratory stock sample.

Topoisomerase Inhibition. Topoisomerases I and II were prepared from calf thymus as already described.[21,22] Topoisomerase I or II inhibitions were evaluated using the DNA cleavage assay carried out according to the procedure previously described.[23] Each compound was evaluated for its minimal inhibitory concentration (MIC), corresponding to the lowest concentration (μg/mL) that would produce a detectable stimulation of the DNA cleavage reaction.

Growth Inhibition Assays: 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 according to a published procedure.[24] The concentrations of drugs giving 50% of growth inhibition (IC_{50}) were determined.

B16 Cell Culture. B16, a mouse melanoma cell line derived from spontaneous skin tumor in C57BI/6 mice, was supplied by the Institut de Cancérologie et Immunogénétique, Villejuif, France. Stock cell cultures were maintained as monolayers in 25 cm^3 culture flasks in Eagle's minimum essential medium (Gibco, Paisly, Scotland) supplemented with 10% fetal calf serum (Sigma Chemical Co.), vitamin solution (100×; Gibco), 100 mM sodium pyruvate (Gibco), nonessential amino acids (100×; Gibco), 200 μM L-glutamine, and gentamycin (Schering-Plough). The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. In these conditions, the doubling time was 15 h.

B16 Cell Cytotoxic Assay. B16 cells were plated into 60 mm Petri dishes (200 cells/dish) and allowed to adhere for 20 h before treatment. Culture medium containing increasing concentrations of drugs was added, and incubation was conducted for 24 h at 37 °C in a CO_2 incubator. After this time, the drug-containing medium was discarded, and the cells were washed with PBS. Fresh medium was added, and incubation was performed at 37 °C in a CO_2 incubator for 12 days. Dishes were then rinsed with PBS, fixed with methanol, and stained with 0.2% crystal violet solution, and colonies (>50 cells) were counted.

The antiproliferative activity is expressed as IC_{50} (inhibiting concentration 50%), the drug concentration giving a 50% cloning efficiency compared to untreated cells.

Protein Kinase Inhibition. (a) Measurements of PKC and PKA Inhibition (Procedure a). Histones IIIs and IIa, phosphatidylserine, and diacylglycerol were purchased from Sigma; [γ^{32}P]ATP was from Amersham. PKA was purchased from Sigma and PKC from Calbiochem.

PKC phosphorylation assays were performed in a reaction mixture (80 μL) containing histone IIIs (2.4 mg/mL), $MgCl_2$ (10 mM), $CaCl_2$ (0.1 mM), phosphatidylserine (10 mg/mL), diacylglycerol (10 mg/mL), ATP (10 μM), [γ^{32}P] ATP (10^6 cpm/80 μL), Tris buffer (50 mM, pH 7.5), PKC (0.5 μg/mL), and inhibitors at different concentrations. PKA phosphorylation assays were performed in a reaction mixture (80 μL) containing histone IIa (1 mg/mL), $MgCl_2$ (5 mM), ATP (10 μM), [γ^{32}P]-ATP (10^6 cpm/80 μL), Tris buffer (50 mM, pH 7.0), PKA (1 μg/mL), and inhibitors at different concentrations. For each kinase, reactions were run at 30 °C for 12 min and stopped with trichloroacetic acid (12%, w/v) in the presence of bovine serum albumin (0.9 mg) as a carrier protein. After centrifugation (10 min at 3000 rpm), the pellet was dissolved in 1 M NaOH and precipitated a second time with trichloroacetic acid. Radioactivity incorporated into histones was counted by scintillation spectrometry (Tri-Carb 4530, Packard). All experiments were carried out in triplicate.

(b) Measurements of PKC-α Inhibition (Procedure b). 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 PKC 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.[25] Stock solutions of compounds (in DMSO) were diluted in serial 10-fold dilutions using DMSO/water (v/v 50:50) as the solvent. PKC isoenzyme activity was assayed using protamine sulfate as a substrate in the absence of phosphatidylserine and diacylglycerol.[26] Incorporation of γ-^{33}P onto protamine sulfate was determined by spotting 50 μL aliquots on P81 chromatography paper (Whatman).[27] Compounds were tested on PKC-α in two independent experiments. Data show IC_{50} values (half-maximum inhibitory concentrations) expressed in micromolar.

***In Vivo* Antitumor Assay**. Male DBA/2Jco mice and male B6D2F1/Jco mice were purchased from IFFA CREDO (L'Arbresle, France). Murine P388 leukemia cells, obtained from ICIG, Villejuif, were maintained by weekly transplantation of the tumor cells into the peritoneal cavity of male DBA/2 mice. For antitumor testing, 1×10^6 cells were intraperitoneally injected into male B6D2F1 mice on day 0. The B16 melanoma cell line was maintained in culture in MEM medium;[28] 0.5×10^6 cells were subcutaneously implanted on the dorsum of B6D2F1 mice on day 0. Each treated group comprised six and the control group 12 mice. Drugs were administered intraperitoneally on days 1, 5, and 9. Control animals were treated with solvent (DMSO−olive oil). Median survival times (MST) were determined for the respective groups. Antitumor activity is expressed as an oncostatic index: T/C × 100.[29,30]

Antibiogram Tests and MIC Determinations. Five strains were tested, three Gram-positive bacteria (*B. cereus* ATCC 14579, *S. chartreusis* NRRL 11407, *S. griseus* ATCC 23345), 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-Hilton broth (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. Compounds **1**−**23** were dissolved in DMSO, and a paper disk containing each one (300 μg) was placed on Petri dishes. Growth inhibition was examined after 24 h incubation at 27 °C.

MIC values of **1**−**23** were determined classically on *B. cereus* ATCC 14579 in Mueller−Hilton broth, pH 7.4 (Difco), after 24 h incubation at 27 °C. The compounds diluted in DMSO were added to 12 tubes; the concentration range was from 100 to 0.05 μg/mL.

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