# Synthesis and Cytotoxicity on Sensitive and Doxorubicin-Resistant Cell Lines of New Pyrrolo[2,1-*c*][1,4]benzodiazepines Related to Anthramycin

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A new 7,8-methylenedioxy analogue (**4**) of (+)-porothramycin B (**2**) and its water-soluble sodium bisulfite derivative (**15**) have been synthesized in high yields and have been shown to exhibit high cytotoxic activities against several tumor cell lines. The new pyrrolo[2,1-*c*][1,4]benzodiazepine **4** was as effective against the resistant cell lines as against the doxorubicin-sensitive cell lines tested.

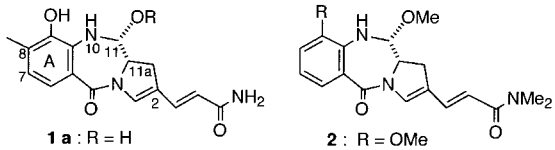
## Introduction

Several pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) with antibiotic and antitumor activities have been isolated from various *Streptomyces* species. Extensive studies have been devoted to the first member of this family, anthramycin **1a** or its methyl ether **1b** (Chart 1). The formation of a covalent bond in the minor groove of DNA by nucleophilic attack of the 2-amino group of a guanine base to form an aminal linkage to C-11 is responsible for the biological activities of PBDs. A selective DNA binding of PBDs with a preference for Pu-G-Pu sequences has been established. These molecules therefore have potential applications as tools in molecular biology (for example, as affinity cleavage reagents) and as drugs for the therapy of diseases involving modifications of cellular DNA such as cancers.[1] It is also known that the A-ring substituents of PBDs, particularly oxy substituents at C-8, may be involved in noncovalent binding with DNA probably through hydrogen-bonding interactions, which play a part in their fitting into the minor groove of DNA.[2] On the other hand, the possibility of quinone formation from a free phenolic hydroxyl group at C-7 or C-8 cannot be precluded and could generate cardiotoxicity, as was postulated for orthoquinone imines derived from anthramycin **1a**.[3]

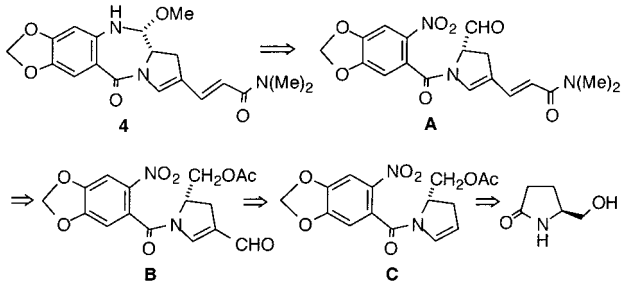
To design our synthetic PBD target, we took into account these various points as well as the classical requirements for a covalent DNA binding:[4] (1) 11a*S* configuration providing the suitable shape to fit into DNA, (2) imine or equivalent functional group at N10−C11, such as carbinolamine methyl ether, to allow the linking with guanine, (3) $sp^2$ carbon at C-2, which enhances DNA-binding affinity, together with the high cytotoxicity observed with (+)-porothramycin B **2**[5] and its 9-demethoxy analogue **3**[6] (Chart 1).

Thus, we have selected early the new PBD **4** as a good synthetic analogue that meets all these requirements for antitumor candidates. This compound constituted at this time the first example of cytotoxic PBD bearing a methylenedioxy group on aromatic ring A,[7] but recently, the preparation of a 7,8-methylenedioxy derivative of the simple PBD DC-81 has also been described.[8]

**Chart 1.** Structures of Anthramycin (**1a**), Its Methyl Ether (**1b**), Porothramycin B (**2**), and Its 9-Demethoxy Analogue (**3**)




**Scheme 1**




We report here the total synthesis of the bioactive enantiomer of **4** and a water-soluble derivative (**15**), starting from inexpensive (*S*)-pyroglutaminol, as well as the results of their bioassays.

## Synthesis

We prepared **4** according to the retrosynthetic route shown in Scheme 1, through reductive cyclization of the nitroaldehyde precursor **A** with Raney nickel, following the method developed in our laboratory.[6,7,9] The preparation of this nitroaldehyde involved as key steps a Horner−Wadsworth−Emmons reaction between a suitably functionalized phosphonate and the imide-vinylogous aldehyde **B**, itself derived from the enamide **C**, which could be prepared from (*S*)-pyroglutaminol.

**Enamide C Synthesis.** *o*-Nitropiperonylic acid was obtained from 6-nitropiperonal,[10] and its chloride was condensed with the anion of *O*-EVE-protected (*S*)-pyro-

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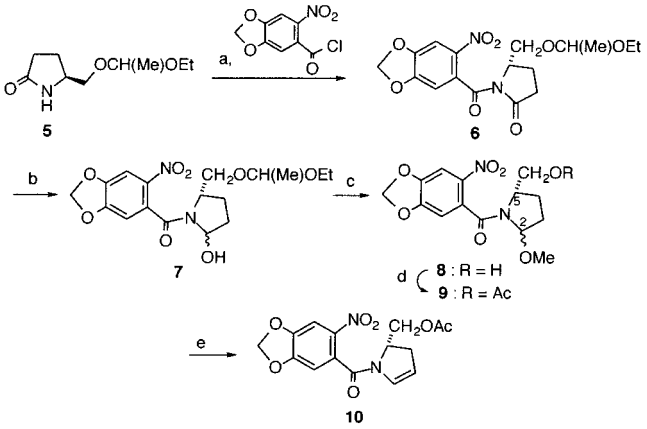
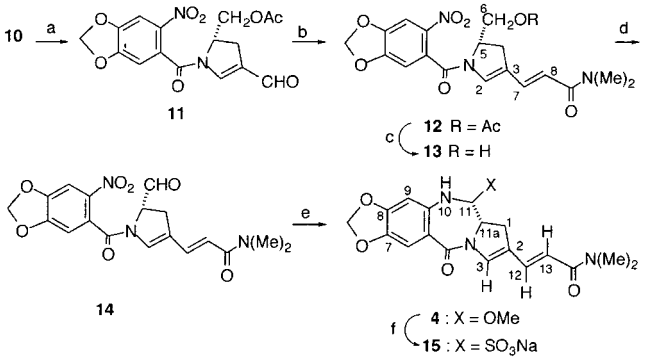
**Scheme 2**[a]



[a] Reagents: (a) NaH−KI, THF, rt (86%); (b) DIBAL-H, toluene, −78 °C (97%); (c) MeOH, $H^+$ (94%); (d): $Ac_2O$−Py (100%); (e): QCS, toluene, Δ (89%).

**Scheme 3**[a]



[a] Reagents: (a) DMF, $POCl_3$, $CH_2Cl_2$ (84%); (b) *n*-BuLi, $(EtO)_2P(O)CH_2CONMe_2$; (c) $Ba(OH)_2$, dioxane, rt (99% for two steps); (d) DMSO, $(COCl)_2$, $iPr_2NEt$ (99%); (e) (i) Raney Ni, (ii) MeOH, $H^+$ (68%); (f) $NaHSO_3$ (90%).

glutaminol **5**[11] to give rise to (5*S*)-5-(1-ethoxy-ethoxymethyl)-1-(4,5-methylenedioxy-2-nitrobenzoyl)pyrrolidin-2-one **6** in 86% yield (Scheme 2). The efficient partial reduction of the lactam carbonyl was performed with 2 equiv of DIBAL-H in toluene at −78 °C and was highly regioselective. Diastereomeric α-hydroxy-*o*-nitrobenzamides **7** (97%) were converted by methanol in acidic medium (TsOH) into the more stable α-methoxy derivatives **8** (94%), in which the primary alcohols at C-5 were deprotected. After quantitative acetylation to **9** to preclude the formation of byproducts in the next step, quinolinium camphorsulfonate (QCS) catalyzed the elimination reaction, leading to the enamide **10** (89%).[12]

**Nitroaldehyde A and New PBDs 4 and 15.** The enamide **10** was formylated at C-3 in 84% yield with a large excess of Vilsmeier−Haack reagent in dichloromethane at room temperature to provide **11** (Scheme 3). The lithiated anion of diethyl [2-(dimethylamino)-2-oxoethyl]phosphonate (prepared from *N,N*-dimethylbromoacetamide and triethyl phosphite by Michaelis−Arbuzov reaction)[13] was condensed with the unsaturated formyl derivative **11** to elaborate very efficiently the C-2 side chain present in porothramycin B **2**, affording **12**. Then, the appropriate transient acyl protection of the primary alcohol was removed by alkaline hydrolysis to afford **13** (99% for the two steps). Swern oxidation of **13** using Hünig's base allowed the preparation of the precursor of PBD **14** (99%) without racemization. Reduction of the aromatic nitro group of **14** by Raney nickel in excess gave rise to PBD imine, which was not purified at this stage but directly converted into the crystalline carbinolamine methyl ether **4** by a weak acid−methanol treatment, evaporation to dryness followed by crystallization from methanol, and cooling at −20 °C. Crystalline **4** was easily separated in 68% yield, the mother liquor containing more **4**. Sodium bisulfite adducts of synthetic PBDs have already been prepared to obtain water-soluble material for biological evaluation.[14] Applied to the new analogue **4**, this derivatization led to compound **15** in 90% yield.

**Table 1.** In Vitro Cytotoxicities of **3**, **4**, and **15** on Various Cell Lines

| cell lines | nature | $IC_{50}$ (nM) **3** | **4** | **15** | comparison $IC_{50}$ (nM) |
|---|---|---|---|---|---|
| KB (human) | standard line | 86 | 5 | 50 | mitomycin C (500) doxorubicin (100) |
| VERO | monkey kidney | 75 | 20 | 50 | doxorubicin (5000) |
| HCT 116 | human colon carcinoma | 50 | 20 | 25 | doxorubicin (100) |
| K562S | sensitive clone | 85 | 50 | | doxorubicin (50) |
| K562R | resistant clone | 120 | 30 | | doxorubicin (7000) |

## Biological Results: Cytotoxic Activities of Compounds 4 and 15 on Several Cell Lines

The cytotoxic activities of the new analogues **4** and **15** were evaluated in vitro on three human cancer cell lines (KB, HCT 116, and K562) and on a nonmalignant but immortalized monkey kidney cell line (VERO). These activities are expressed as nanomolar concentrations of the compound that inhibited 50% of cell proliferation ($IC_{50}$) and are compared with the $IC_{50}$ values in the same cell lines of 9-demethoxyporothramycin **3**, doxorubicin (DOX), or mitomycin C (Table 1). Compounds **4** and **15** exhibited strong cytotoxic activities on all cell lines examined. A comparison with 9-demethoxyporothramycin **3** showed that the substitution of the aromatic ring in **4** by a 7,8-methylenedioxy group enhanced the activities to some extent. In the KB cell line, analogue **4** was 100-fold more active than mitomycin C and 20-fold more active than doxorubicin, whereas its $IC_{50}$ value in the HCT 116 cell line was 5-fold lower than that of DOX. Furthermore, the activity of compound **4** on a cell line endowed with the MDR+ (multiple drug resistant) phenotype was of similar magnitude as that exerted on its MDR− counterpart, whereas doxorubicin was significantly less active on the resistant cell line (K562R) than on the sensitive cell line (K562S). These data warranted further studies described below.

The cytotoxic activity of compound **4** was compared with that of DOX on six human cancer cell lines. For each of these six cell lines, resistant cell lines were established by culturing them in the presence of increasing doses of DOX for several months, as detailed elsewhere.[15,16] The new PBD **4** and DOX-induced cytotoxic effects were assayed by means of the MTT colorimetric assay.[17,18] Table 2 indicates that the two DOX-resistant bladder cancer cell lines; i.e., J82-R and T24-R exhibited resistance to doxorubicin that was at least 3 logarithmic doses higher than for the sensitive J82-S and T24-S cell lines. Compound **4** induced cytotoxic

**Table 2.** Cytotoxicities of Compound **4** on Several Human Cancer Cell Lines (Sensitive or Resitant to Doxorubicin)

| | | $IC_{50}$ (nM) | |
|---|---|---|---|
| cell lines | variants | doxorubicin | **4** |
| J82 (bladder) | sensitive | 30 | 20 |
| | resistant | >10000 | 10 |
| T24 (bladder) | sensitive | 20 | 10 |
| | resistant | >10000 | 10 |
| HCT 15 (colon) | sensitive | 50 | 8 |
| | resistant | 80 | 7 |
| LoVo (colon) | sensitive | 8 | 9 |
| | resistant | 80 | 9 |
| U87 (glioblastoma) | sensitive | 60 | 40 |
| | resistant | 140 | 38 |
| U373 (glioblastoma) | sensitive | 10 | 30 |
| | resistant | 130 | 37 |

effects that were similar on both S and R bladder cancer cell lines, and this new PBD was as effective as DOX on the sensitive cell lines. The two resistant glioblastoma cell lines that we set up against doxorubicin exhibited about 1 log unit higher doxorubicin resistance compared to their sensitive counterparts. Once more, PBD **4** was as effective against the sensitive cell lines as against the doxorubicin-resistant glioblastoma cell lines. We failed, in our attempts, to set up significantly resistant colon cancer cell lines against doxorubicin. However, compound **4** killed these human colon cancer cells with the same magnitude of doses as it did for the two bladder and the two glioblastoma cell lines.

## Conclusions

The bioactive enantiomers of two new pyrrolo[2,1-*c*]-[1,4]-benzodiazepines **4** and **15** related to anthramycin were synthezised efficiently from (*S*)-pyroglutaminol (respectively in 38% and 34% overall yield), and the parent compound **4** crystallized from methanol in pure methyl ether form. This compound displayed very strong cytotoxic activities in vitro against a large number of human cancer cell lines of various histological origins and was slightly more active than 9-demethoxyporothramycin **3**. The water-soluble derivative **15** displayed also strong cytotoxicities on the cell lines examined, and it would be worthwhile to pursue the investigations of in vivo antitumor activities and to evaluate the cardiotoxicity of these analogues. The most interesting feature is the fact that **4** is equally active on doxorubicin-resistant cell lines as on sensitive cell lines.

## Experimental Section

**General.** General procedures have been described.[6b,9b]

**(5*S*)-5-(1-Ethoxy-ethoxymethyl)-2-hydroxy-1-(4,5-methylenedioxy-2-nitrobenzoyl)pyrrolidines (7) and (5*S*)-5-Hydroxymethyl-2-methoxy-1-(4,5-methylenedioxy-2-nitrobenzoyl)pyrrolidines (8).** The reduction of **6** (6.50 g, 17.1 mmol) was performed with DIBAL-H in toluene to give compound **7** (6.34 g, 97%). *p*-Toluenesulfonic acid (2.47 g, 14.4 mmol) was added under an inert atmosphere to a stirred solution of **7** (5.84 g, 15.3 mmol) in 9:1 $CH_2Cl_2$/MeOH (190 mL) cooled at 0 °C. An aqueous solution of $Na_2CO_3$ (10%) was added after 10 min, and the product was extracted with $CH_2Cl_2$ and purified by flash column chromatography (eluent: EtOAc) to afford α-methoxy-*o*-nitrobenzamides **8** (4.66 g, 94%). MS: *m*/*z* 324 ($M^{+\bullet}$), 293 [$(M - OCH_3)^{+\bullet}$, 100%], 262, 194, 120. HRMS Calcd for $C_{14}H_{16}N_2O_7$: 324.0957. Found: 324.0955.

**(5*S*)-5-Acetoxymethyl-2-methoxy-1-(4,5-methylenedioxy-2-nitrobenzoyl)pyrrolidines (9).** Alcohols **8** were quantitatively acetylated into **9**. MS: *m*/*z* 366 ($M^{+\bullet}$), 335, 293, 260, 194 (100%), 120.

**(*S*)-5-Acetoxymethyl-1-(4,5-methylenedioxy-2-nitrobenzoyl)-2-pyrroline (10).** The enamide **10** was prepared in 89% yield according to the efficient procedure of our laboratory.[12b] $[\alpha]_D^{30}$ −144° (*c* 0.84). MS: *m*/*z* 334 ($M^{+\bullet}$), 260, 194 (100%), 120. Anal. ($C_{15}H_{14}N_2O_7$) C, H, N.

**(*S*)-5-Acetoxymethyl-3-formyl-1-(4,5-methylenedioxy-2-nitrobenzoyl)-2-pyrroline (11).** Phosphorus oxychloride (3.62 mL, 38.8 mmol) was added at 0 °C under a nitrogen atmosphere to DMF (3.01 mL, 38.9 mmol); the mixture was stirred for 20 min at rt and evaporated to dryness under reduced pressure. To a solution of this reagent in $CH_2Cl_2$ (12 mL) stirred at 0 °C under nitrogen was added the enamide **10** (1.00 g, 2.99 mmol) in $CH_2Cl_2$ (12 mL). After being stirred for 2.5 h at rt, the reaction mixture was poured slowly into a solution of $Na_2CO_3$ (40%) at 0 °C, was stirred at the same temperature for 0.25 h, and then was extracted with $CH_2Cl_2$. The crude product obtained after the usual treatment was purified by chromatography on silica gel (eluent: $Et_2O$) to afford the aldehyde **11** (0.912 g, 84%). Mp: 70 °C. $[\alpha]_D^{35}$ −217° (*c* 0.53). MS: *m*/*z* 362 ($M^{+\bullet}$), 302, 195, 194 (100%), 120, 119. HRMS Calcd for $C_{16}H_{14}N_2O_8$: 362.0750. Found: 362.0751.

**(*S*)-3-[5-Acetoxymethyl-1-(4,5-methylenedioxy-2-nitrobenzoyl)-2-pyrroline-3-yl]-3-(*N*,*N*-dimethyl)acrylamide (12) and (*S*)-3-[5-Hydroxymethyl-1-(4,5-methylenedioxy-2-nitrobenzoyl)-2-pyrroline-3-yl]-3-(*N*,*N*-dimethyl)acrylamide (13).** To a solution of diethyl [2-(dimethylamino)-2-oxoethyl]phosphonate (0.410 g, 1.84 mmol) in anhydrous THF (13.0 mL), stirred at 0 °C under argon, was added *n*BuLi (1.5 M in hexane, 2.3 mL, 3.45 mmol). The mixture was stirred at 0 °C for 0.5 h before the addition of a solution of aldehyde **11** (0.666 g, 1.84 mmol) in anhydrous THF (13 mL). After complete reaction, a saturated aqueous solution of $NH_4Cl$ was added to the reaction mixture and the product was extracted with EtOAc. After the usual treatment, the crude compound **12** (0.824 g, containing small amounts of the corresponding primary alcohol **13**) was directly hydrolyzed ($Ba(OH)_2$) into the alcohol **13** (0.709 g, 99%). $[\alpha]_D$ −85° (*c* 0.29). MS: *m*/*z* 389 ($M^{+\bullet}$), 371, 345, 324, 252, 195, 194 (100%), 120, 119. HRMS Calcd for $C_{18}H_{19}N_3O_7$: 389.1223. Found: 389.1195.

**(*S*)-3-[5-Formyl-1-(4,5-methylenedioxy-2-nitrobenzoyl)-2-pyrroline-3-yl]-3-(*N*,*N*-dimethyl)acrylamide (14).** Swern oxidation of **13** (0.600 g, 1.54 mmol) was performed as previously described[7] to afford the aldehyde **14** (0.591 g, 99%). $[\alpha]_D$ −146° (*c* 0.40). MS: *m*/*z* 387 ($M^{+\bullet}$), 358, 252, 195, 194 (100%), 148, 120. HRMS Calcd for $C_{18}H_{17}N_3O_7$: 387.1066. Found: 387.1047. The absence of racemization was established by a Swern oxidation−$NaBH_4$ reduction sequence without loss of optical activity.

**(11*R*,11a*S*)-2-(*N*,*N*-dimethyl)acrylamide-1,10,11,11a-tetrahydro-11-methoxy-7,8-methylenedioxy-5*H*-pyrrolo-[2,1-*c*][1,4]benzodiazepine-5-one (4).** A solution of the aldehyde **14** (402.0 mg, 1.04 mmol) in a mixture 85:15 EtOAc−MeOH (20.8 mL) was treated with an excess of Raney nickel as described,[6b] to give the PBD **4** as pale-yellow crystals (262 mg in two crops, 68%, the mother liquor containing more **4**). Mp: 170 °C. $[\alpha]_D^{25}$ +390° (*c* 0.08). MS: *m*/*z* 339 $(M - CH_3OH)^{+\bullet}$, 337, 293 (100%), 265. HRMS Calcd for $C_{18}H_{17}N_3O_4$ $(M - CH_3OH)^{+\bullet}$: 339.1219. Found: 339.1223.

**Sodium Bisulfite Adduct (15).** A solution of sodium bisulfite (8.4 mg, 0.081 mmol) in $H_2O$ (3.3 mL) was added to a solution of **4** (29.9 mg, 0.08 mmol) in $CH_2Cl_2$ (devoid of EtOH, 1.6 mL), and the mixture was stirred at rt for 24 h. The aqueous layer was separated and evaporated to dryness to give **15** (32.1 mg, 90%). $^1H$ NMR (300 MHz, $D_2O$, HOD $\delta = 4.8$ ppm): $\delta$ 7.35 (d, 1H, $J_{12,13} = 15$ Hz, 12-H), 7.29, 7.00 (2s, 2H, 6-H, 3-H), 6.62 (s, 1H, 9-H), 6.30 (d, 1H, $J_{12,13} = 15$ Hz, 13-H), 5.99 (s, 2H, $OCH_2O$), 3.2−2.8 (2H masked, 1-$H_2$), 3.13, 2.98 (2s, 6H, $NMe_2$).

**Biological Evaluations. 1. Cytotoxicity Assays.** The in vitro activities of **4** and **15** were evaluated in several cell lines: KB (human epidermoid carcinoma of the mouth), VERO (African green monkey kidney), HCT-116 (human colon car-

cinoma), and K-562 (human erythroleukemia). Standard KB cells and VERO cells were from our own collection. The HCT-116 cell line was provided by Dr. M. C. Bissery (Rhône-Poulenc Rorer, Vitry-sur-Seine, France), and the K-562 cell lines were provided by Dr. H. Tapiero (Institut de cancérologie et d'immunogénétique, Villejuif, France). Standard KB cells and VERO cells were grown in medium 199 + 10% newborn calf serum, as monolayers in Costar 12-well plastic plates ($12 \times 10^4$ cells seeded in 2 mL of medium). For the assays, serial dilutions in the media of a stock solution of compounds **3** and **4** in DMSO and **15** in $H_2O$ were added to the cultures at the time of cell seeding (the final concentration of DMSO was less than 1%). The cultures were incubated at 37 °C in a 95% air/5% $CO_2$ incubator. After incubation (48 h) cell viability in the monolayers was determined by an additional 18 h of incubation in a medium containing neutral red, followed by lysis with sodium dodecyl sulfate and photometric quantification of the extracted dye at 540 nm.

**2. In Vitro Cytotoxicity of Compound 4 on Human Cancer Cell Lines Sensitive or Resistant to Doxorubicin.** Six human tumor cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These included two glioblastomas (U373 and U87), two colon (HCT-15 and LoVo) cancer models, and two bladder (J82 and T24) cancer models. The ATCC numbers of these cell lines are HTB 14 (U87), HTB 17 (U373), CCL225 (HCT-15), CCL229 (LoVo), HTB1 (J82), and HTB4 (T24). The cells were cultured at 37 °C in sealed (airtight) Falcon plastic dishes (Nunc, Gibco, Belgium) containing Eagle's minimal essential medium (MEM, Gibco) supplemented with 10% fetal calf serum (FCS). All the media were supplemented with a mixture of 0.6 mg/mL glutamine (Gibco), 200 IU/mL of penicillin (Gibco), 200 IU/mL of streptomycin (Gibco), and 0.1 mg/mL of gentamycin (Gibco). The FCS was heat-inactivated for 1 h at 56 °C. The six cell lines were cultured in increasing concentrations (starting at $10^{-14}$ M) of doxorubicin over several months as detailed elsewhere.[15,16] The maximum DOX dose in which these cell lines were cultured ranged between 10 and 500 nM of DOX. This experimental protocol was adopted in order to set up resistant cell lines to DOX and to compare to PBD-**4**-induced cytotoxic effects on sensitive versus resistant cell lines to doxorubicin. The 12 S and R cell lines were incubated for 24 h in 96-microwell plates (at a concentration of 10 000 cells/mL of culture medium) to ensure adequate plating prior to cell growth determination, which was carried out by means of the colorimetric MTT assay.[17,18] Nine concentrations ranging from $10^{-5}$ to $10^{-9}$ M were assayed for each of the two drugs under study. This dose range enabled us to determine the $IC_{50}$ value for each drug on each cell line (Table 2).

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**Supporting Information Available:** Details of experimental procedures, IR and NMR spectroscopic data of compounds **4**−**14**, and a table listing analytical and spectroscopic data of compounds **4**−**15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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