Synthesis and Antitumor Activity of Several New Analogues of Penclomedine and Its Metabolites

Anita Tiwari,* James M. Riordan, William R. Waud, and Robert F. Struck

Drug Discovery and Drug Development Divisions, Southern Research Institute, 2000 Ninth Avenue South, Birmingham, Alabama 35255-5305

Analogues of penclomedine (PEN, 3,5-dichloro-4,6-dimethoxy-2-(trichloromethyl)pyridine) and its metabolites have been synthesized and evaluated as potential antitumor agents. PEN and 4-DMPEN (3,5-dichloro-4-hydroxy-6-methoxy2-(trichloromethyl)pyridine (**3a**)), the major plasma metabolite in patients, were modified at 4- and 6-positions with different alkyl, aryl, and ester groups. All of the analogues and many of the intermediates were evaluated against the PEN-sensitive MX-1 human breast tumor xenograft in vivo, and several analogues of PEN and 4-DMPEN showed modest to curative activity.

Introduction

PEN[1] (3,5-dichloro-4,6-dimethoxy-2-(trichloromethyl)-pyridine) is a chemical alkylating agent as revealed by its facile reaction with 4-(*p*-nitrobenzyl)pyridine in the Epstein test[2] (unpublished results). Plowman et al.[3] reported that, of 28 agents active against breast tumor lines in vivo, 23 of the 28 were known alkylating agents, and PEN was one of the five other active agents, suggesting that it may be functioning as a biological alkylating agent. In addition, Harrison et al.[4] observed cross resistance in vivo to P388 leukemia lines resistant to clinical DNA−DNA cross-linking agents but sensitivity to lines resistant to antimetabolites, topoisomerase II inhibitors, and a mitotic inhibitor, consistent with PEN functioning as a DNA cross-linking agent in vivo. PEN is a lipophilic, highly substituted 2-methylpyridine derivative synthesized by Dow Chemical Company as an insecticide and was selected for clinical development by the National Cancer Institute because of evidence of good antitumor activity against mouse CD8F$_1$ mammary carcinoma and human MX-1 mammary tumor xenograft.[3,4] PEN is more active in vivo than in vitro,[5,6] suggesting that it may be metabolized to a more potent compound.

The antitumor activity of PEN against intracranially implanted MX-1 xenografts was up to 4 times greater than that of carmustine, the most commonly used clinical agent for the treatment of primary brain tumors, but in all clinical trials, dose limiting neurotoxicity[7−9] was observed after both iv and oral administration. 4-DMPEN, as reported[10,11] earlier, was shown to be an excellent antitumor active metabolite of PEN in vivo when evaluated against the PEN-sensitive MX-1 human tumor xenograft without neurotoxicity. Synthesis and analytical data for 4-DMPEN are reported here in detail, and studies on 4-DMPEN are still in progress.

On the basis of these considerations, we have undertaken the synthesis of analogues of PEN and 4-DMPEN with modifications in both 4- and 6-positions with different alkyl or aryl groups to determine whether any

of these agents are superior to PEN in their activity, whereas previous studies from our laboratory had demonstrated the absolute dependence on the trichloromethyl substituent for antitumor activity in vivo.[11] Since the antitumor activity of 4-DMPEN in vivo indicated that it was on the metabolic activation pathway, although it was inactive in vitro,[11] we were interested in determining if metabolic dealkylation or dearylation of groups other than methyl at the 4-postion might yield a more active agent than PEN. In earlier studies, Reid et al.[5] had observed demethylation of PEN by liver microsomes but did not determine if demethylation occurred at the 4- or 6-position or both, while later studies from our laboratory indicated that both 6-DM-PEN and DDM-PEN (3,5-dichoro-4,6-dihydroxy-2-(trichloromethyl)pyridine) were inactive in vivo.[11] All of the analogues were evaluated against MX-1 human breast carcinoma xenografts in vivo.

Results and Discussion

Chemistry. The pyridine **1**[12,13] was prepared by a sequence of reactions starting from pentachloropyridine, and PEN (**2a**) was prepared by a reported method.[1] Compounds **2b−d** (Scheme 1) were prepared by the same method as PEN by refluxing with two or more molar equivalents of sodium hydroxide and the corresponding alcohols, while **2e** and **2f** were prepared from sodium hydride and trifluoroethanol in dioxane and from sodium phenoxide in dioxane, respectively. Removal of the alkyl or aryl groups in the 4-position of compounds **2a−f** was attempted with DMSO at 150 °C as reported for 4-DMPEN,[10] but only compounds **3a−c** were prepared, the others failing to react. For this reason **2e** was first converted to **6** and then demethylated to give **7**. Compounds **3b** and **3c** were methylated by trimethylsilyldiazomethane to produce **4a** and **4b** in good overall yields. Acetylation of **3a** and **3b** with acetic anhydride or methoxyacetyl chloride, respectively, yielded **5a−c**. Treatment of **2b** with AlCl$_3$ in CH$_2$Cl$_2$ afforded the 4,6-dihydroxy compound (DDM-PEN) **8** which on methylation gave **3d** and on acetylation gave **9**. Treatment of **1** with sodium hydroxide and ethanol at room temperature yielded **10b** (Scheme 2). On methylation

* To whom correspondence should be addressed. Tel: (205) 581-2427. Fax: (205) 581-2877. E-mail: a.tiwari@sri.org.

Scheme 1
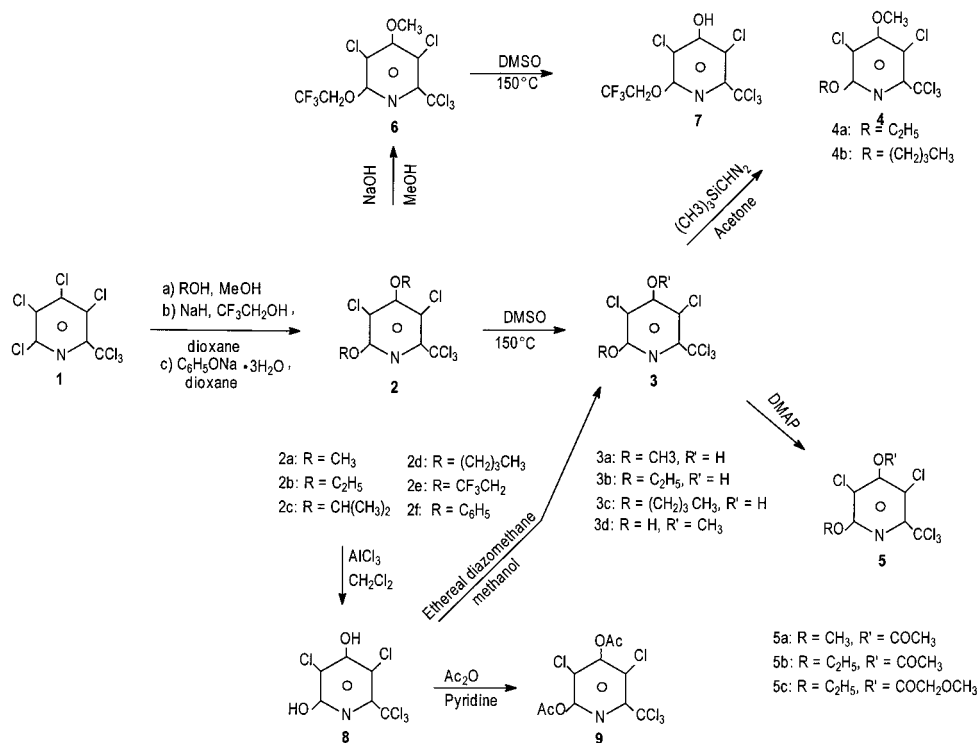
Scheme 2
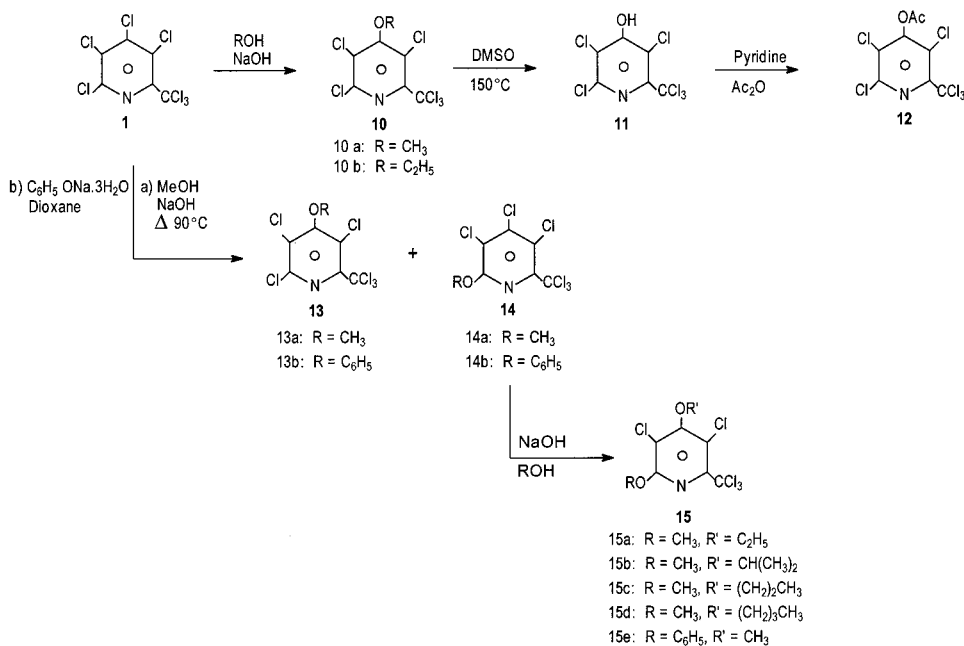
with 1 molar equiv of NaOH at room temperature, **1** gave **10a**, which on demethylation and acetylation yielded **11** and **12**, but when methylation was accomplished with 1 mol of NaOH at 90 °C, isomers **13a** and **14a** were obtained. Similarly, equimolar amounts of sodium phenoxide and **1** in dioxane at 100 °C produced isomers **13b** and **14b**. Compounds **14a** and **14b** were treated with NaOH and different alcohols to yield **15a**−**e**. Compound **16**[12] (Scheme 3), prepared through a series of reactions as described,[12] was chlorinated to give **17** that on methylation afforded **18**.

Distinguishing the 4- and 6-isomeric forms of mono DMPEN was solved by NMR studies on PEN, on each

monodemethylated PEN derivative and on a ^{13}C-methyl-labeled, methylated derivative of the synthetic mono-demethylated product that was identical to the major plasma metabolite in patients, using ^{13}C-diazomethane prepared from *N*-methyl-^{13}C-diazald (Aldrich). [1]H and ^{13}C chemical shifts and coupling constants are shown

Scheme 3
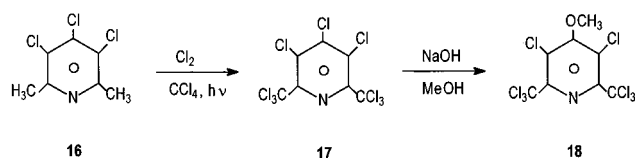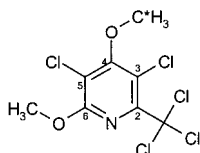
Figure 1. ^{1}H NMR (CDCl$_3$) δ 4.02 ppm (d, 3, 4-OCH$_3$, $^1J_{C,H}$ = 147.0 Hz); 4.10 (s, 3, 6-OCH$_3$). ^{13}C NMR (CDCl$_3$) δ 55.24 ppm (s, 6-OCH$_3$, $^1J_{C,H}$ = 147.5 Hz); 61.13 (br s, 4-OCH$_3$, $^1J_{C,H}$ = 147.0 Hz); 96.07 (s, -CCl$_3$); 114.27 (d, C-5 or C-3, $^3J_{C,C}$ = 1.5 Hz); 118.23 (d, C-3 or C-5, $^3J_{C,C}$ = 1.1 Hz); 147.66 (s, C-2); 155.86 (s, C-6, $^3J_{C,H}$ = 3.8 Hz); 162.40 (d, C-4, $^2J_{C,C}$ = 3.6 Hz, $^3J_{C,H}$ = 3.7 Hz). The multiplicities shown are from the hydrogen decoupled spectrum. The $J_{C,H}$'s are from the hydrogen coupled spectrum.

in Figure 1 for ^{13}C-PEN along with the description of how the structure for the major plasma metabolite was assigned.

The ^{13}C labeling of one of the methyl groups allowed the determination of the structure of monodemethyl-penclomedine corresponding to the major plasma metabolite and also allowed the assignments of the methyl groups in the ^{1}H spectrum of PEN. It is well-known that ortho and para carbons of the pyridine ring will be the most downfield of the pyridine carbon atoms (larger chemical shifts). The meta carbons of the pyridine ring, the ones with attached chlorine, are the most upfield. ^{13}C labeling of one of the methyl groups with a ^{13}C carbon atom allowed the direct observation of long-range carbon−carbon coupling constants, $^nJ_{c,c}$. Four and five bond carbon−carbon couplings, $^4J_{c,c}$ and $^5J_{c,c}$, are very rare. $^3J_{c,c}$ and $^2J_{c,c}$ are the most commonly observed couplings, and both are generally larger than $^4J_{c,c}$ and $^5J_{c,c}$ values. The above ^{13}C NMR data show that both meta carbons of pyridine have $^3J_{c,c}$ values of 1.1 and 1.5 Hz and para carbon has corresponding $^2J_{c,c}$ of 3.6 Hz. Therefore, the methyl with the label is in the para position of the pyridine ring.

Biological Evaluation. All of the analogues and some of the intermediates were evaluated against staged, subcutaneously implanted human breast tumor xenograft MX-1 in vivo with intraperitoneal treatment on a successive 5 day schedule, which was shown previously to be optimal for PEN and 4-DMPEN[11] (Table 1). While most of the analogues were inactive, the limited SAR study revealed that only minor variation from the parent structure was permissible at the 4- and 6-positions for retention of high or even modest activity. Previous investigations had established the absolute necessity of the 2-trichloromethyl moiety and allowed demethylation of the 4- but not the 6-position.[11]

In the present study, the 4-methyl group could be replaced with ethyl **15a**, isopropyl **15b**, or butyl **15d** for curative activity, although the percentage of tumor-free survivors decreased with increasing number of carbons. The ethyl analogue **15a** was comparably active to PEN itself, yielding 100% tumor-free survivors. Replacement of the 6-methyl group was more restrictive, since activity was observed only for the ethyl analogue **4a** but not for the trifluoroethyl **6**, butyl **4b**, and phenyl **15e** analogues. Three separate experiments with the ethyl analogue **4a** indicated modest activity in two experiments and some curative activity in the third experiment (40% tumor-free survivors). Replacement of both methyl groups was similarly restrictive, with activity being observed only

Table 1. Summary of the In Vivo Antitumor Activity of Penclomedine and Its Metabolites and Analogues against Subcutaneously Implanted Human Mammary Tumor Xenograft MX-1 on a Five-Successive Day Schedule

compd	optimal ip dosage ($<$LD$_{10}$) (mg/kg/dose/day)	$T - C$ (days)	tumor-free (survivors/total)
2a	90	>49.0	5/5
2b	135	>41.0	4/5
2c	135	−0.7	0/5
2d	135	−0.6	0/5
2e	135	0.4	0/5
2f	135	0.2	0/5
3a	90	>37.2	3/5
3b	60	5.2	0/5
3c	135	−0.9	0/5
3d	135	0.8	0/5
4a	200	>38.6	2/5
4b	135	−0.8	0/5
5a	90	>41.0	5/5
5b	90	1.4	0/5
5c	135	17.8	0/5
6	90	1.2	0/5
7	90	−0.2	0/5
8	40	−0.5	0/5
9	40	−0.5	0/5
11	50	2.0	0/5
12	toxic		
13a	50	4.3	0/5
14a	135	−1.4	0/5
15a	90	>42.7	5/5
15b	60	>35.2	2/5
15c	60	5.4	0/5
15d	90	20.5	1/5
15e	135	2.3	0/5
18	135	−0.8	0/5

for the diethyl analogue **2b** but not for **2c−f**. In four separate experiments, **2b** produced either modest to high noncurative activity in two experiments and curative activity in the other two, yielding 60% and 80% tumor-free survivors, respectively.

Of interest was the observation that the 4-hydroxy-6-alkoxy analogue **7** was inactive and the 4-hydroxy-6-ethoxy analogue **3b** was only modestly active. Our proposed mechanism of action of PEN shown in Figure 2 includes metabolic demethylation at the 4-position in the liver to generate 4-DMPEN, followed by metabolic activation of the trichloromethyl group to an intermediate capable of alkylating tumor cell DNA to produce a DNA adduct. The adduct is then capable of keto−enol tautomerism at the 4,5-positions to produce an α-chloroketone moiety capable of completing a DNA−DNA cross-link, which is presumably the cytotoxic lesion. The proposed mechanism includes conversion of the trichloromethyl group to a free radical, based on studies by Reid et al.[5] in which the major product of incubation of PEN with liver microsomes resulted from free radical coupling of two PEN molecules through their trichloromethyl groups and also based on studies by Benvenuto et al.[6] in which incubation of [^{14}C] PEN with rat liver S-9 fraction in the presence of calf thymus DNA resulted in the stable transfer of radioactivity to DNA. Addition of butylated hydroxytoluene, a free radical scavenger, to the incubation mixture inhibited the binding of drug to DNA, implicating free radicals in the formation of the reactive species. These data suggest that PEN can be metabolized to free radical, DNA-reactive products. Analogues **3b** and **7** would similarly be able to activate the 5-chloro group, but their modest
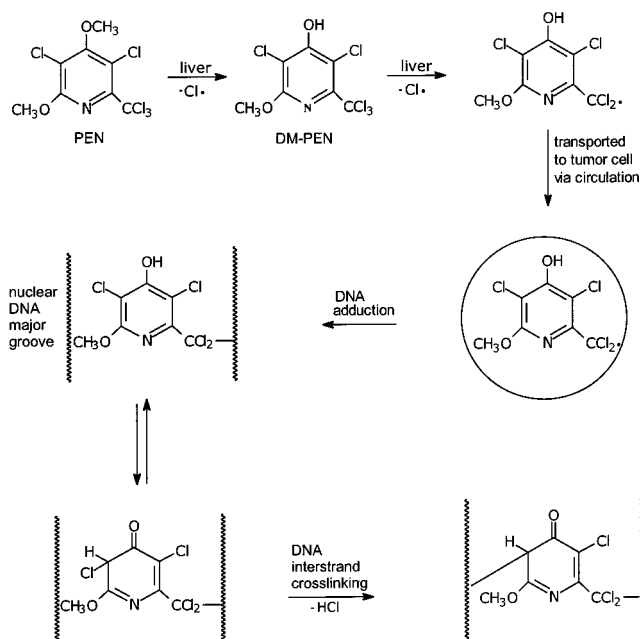
Figure 2. Proposed mechanism of action of penclomedine (PEN).

activity to inactivity demonstrates a pharmacologic role for the 6-substituent in these two analogues, although analogues **2b** and **4a** are presumably metabolized to **3b** in vivo. Didemethyl-PEN **8** was previously reported to be inactive,[11] and its diacetyl derivative **9** was similarly inactive. In addition, the analogues in which a chloro group replaced the 4- or 6-methoxy group (**13a**, **14a**) were inactive. Finally, a single analogue **18** in which the 6-methoxy group was replaced with a trichloromethyl group was also inactive.

Synthesis and evaluation of a series of 4-acyl derivatives of 4-DMPEN illustrated by **5a** revealed consistent and high activity[14] whereas the 6-ethyl analogues **5b** and **5c** were inactive or only modestly active, respectively.

Experimental Section

Melting points were determined on a Mel-Temp apparatus and are uncorrected. TLC was carried out on Analtech precoated (250 μm) silica gel (GF) plates. All chromatographic separations were carried out by flash chromatography using 230−400 mesh silica gel from E. Merck. Analytical results indicated by element symbols were within ±0.4% of the theoretical values, and where solvents were indicated in the formula, their presence was confirmed by ^{1}H NMR. The ^{1}H NMR spectra and ^{13}C NMR spectra in Me$_2$SO-d_6 or CDCl$_3$ with tetramethylsilane as an internal reference were determined with a Nicolet NT 300 NB spectrometer at 300.635 MHz for ^{1}H NMR spectra and at 75.6 MHz for ^{13}C NMR spectra. Chemical shifts (δ) quoted for multiplets were measured from the approximate centers, and relative integrals of peak areas agreed with those expected for the assigned structures. Mass spectra were recorded on a Varian/MAT 311A mass spectrometer in the fast atom bombardment (FAB) mode.

3,4,5,6-Tetrachloro-2-(trichloromethyl)pyridine (1). This compound was prepared by a reported method as described in refs 12a−c and 13.

3,5-Dichloro-4,6-dimethoxy-2-(trichloromethyl)pyridine (PEN) (2a). This compound was prepared by a reported method.[1] To a solution of NaOH (598 mg, 14.95 mmol) in anhydrous methanol (25 mL) was added compound **1** (2.00 g, 5.98 mmol), and the reaction mixture was heated to reflux for 2 h. The reaction mixture was cooled and concentrated to

dryness. The compound was extracted with CH$_2$Cl$_2$ (2 × 20 mL) and washed with water. The organic layer was dried over MgSO$_4$, filtered, and concentrated to dryness. The crude product was purified by column chromatography (silica gel 230−400 mesh, elution with hexanes). The desired fractions were combined, concentrated, and dried in vacuo over P$_2$O$_5$: yield 1.53 g (78.86%); mp 72−74 °C; MS m/z 324 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 4.02 (s, 3H, 4-CH$_3$), 4.09 (s, 3H, 6-CH$_3$).

3,5-Dichloro-4,6-diethoxy-2-(trichloromethyl)pyridine (2b). Compound **2b** was prepared from compound **1** (450 mg, 1.34 mmol), sodium hydroxide (216 mg, 5.4 mmol), and ethanol (4.5 mL) using the same procedure described for the preparation of **2a**: yield after column chromatography (97:3 hexanes:methylene chloride) 286 mg (60.2%); mp 27−28 °C; MS m/z 352 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.46 (t, 3H, 4-CH$_3$), 1.51 (t, 3H, 6-CH$_3$), 4.24 (q, 2H, 4-CH$_2$), 4.53(q, 2H, 6-CH$_2$); Anal. (C$_{10}$H$_{10}$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-4,6-diisopropoxy-2-(trichloromethyl)pyridine (2c). The same procedure as described for **2a** was used to prepare **2c** from compound **1** (500 mg, 1.49 mmol), sodium hydroxide (220 mg, 5.5 mmol), and 2-propanol (15 mL): yield after column chromatography (hexanes) 326 mg, liquid (57.2%); MS m/z 380 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.42 (d, 6H, 4-CH$_3$, $J = 6.3$ Hz), 1.43 (d, 6H, 6-CH$_3$, $J = 6.3$ Hz), 4.83 (m, 1H, 4-CH), 5.37 (m, 1H, 6-CH); Anal. (C$_{12}$H$_{14}$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-4,6-dibutoxy-2-(trichloromethyl)pyridine (2d). The same procedure previously described for **2a** was used to prepare **2d** from compound **1** (800 mg, 2.39 mmol), sodium hydroxide (352 mg, 8.8 mmol), and n-butanol (25 mL): yield after column chromatography (hexanes) 511 mg, liquid (52.14%); MS m/z 408 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 0.98 (t, 3H, 4-CH$_3$), 1.00 (t, 3H, 6-CH$_3$), 1.43−1.63 (m, 4H, CH$_2$-CH$_3$), 1.77−1.90 (m, 4H, CH$_2$−CH$_2$), 4.15 (t, 2H, 4-OCH$_2$), 4.46 (t, 2H, 6-OCH$_2$); Anal. (C$_{14}$H$_{18}$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-4,6-ditrifluoroethoxy-2-(trichloromethyl)-pyridine (2e). To a stirred suspension of NaH (287 mg, 11.95 mmol) in 10 mL of anhydrous dioxane was added compound **1** (1.00 g, 2.99 mmol). After being stirred for 20 min, the solution was treated with 2,2,2-trifluoroethanol (1.21 g, 0.87 mL, 11.95 mmol) and heated at 110 °C for 1.5 h. The reaction mixture was cooled and concentrated to dryness. The compound was extracted with CH$_2$Cl$_2$ (2 × 20 mL) and washed with water. The organic layer was dried over MgSO$_4$, filtered, and concentrated to dryness. The resulting oil was purified by column chromatography (silica gel 230−400 mesh) using hexanes. The desired fractions were combined, concentrated, and dried in vacuo over P$_2$O$_5$ to give an oil that solidified in the freezer: yield 315 mg (22.82%); mp 50−52 °C; MS m/z 460 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 4.58 (q, 2H, 4-OCH$_2$), 4.88 (q, 2H, 6-OCH$_2$); Anal. (C$_{10}$H$_4$F$_6$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-4,6-diphenoxy-2-(trichloromethyl)pyridine (2f). Compound **1** (800 mg, 2.39 mmol) was dissolved in 1 mL of anhydrous dioxane. Sodium phenoxide trihydrate (1.22 g, 7.17 mmol) was added, and the solution was heated to reflux for 3 h. The reaction mixture was cooled and concentrated to dryness. The compound was extracted with CH$_2$Cl$_2$ (2 × 30 mL) and washed with water. The organic layer was dried over MgSO$_4$, filtered, and concentrated to dryness. The residue was purified by column chromatography (silica gel 230−400 mesh, elution with hexanes). The desired fractions were collected, concentrated, and dried in vacuo over P$_2$O$_5$ to give a white solid: yield 778 mg (72.71%); mp 72−74 °C; MS m/z 448 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 6.91 (bd, 2H, 4-C$_6$H$_5$ (2,6)), 7.14 (bt, 1H, 4-C$_6$H$_5$ (4)), 7.31−7.23 (m, 3H, 6-C$_6$H$_5$ (2,4,6)), 7.37 (m, 2H, 4-C$_6$H$_5$ (3,5)), 7.43 (bt, 2H, 6-C$_6$H$_5$ (3,5)); Anal. (C$_{18}$H$_{10}$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-4-hydroxy-6-methoxy-2-(trichloromethyl)-pyridine (4-DMPEN) (3a). Compound **2a** (6.00 g, 18.43 mmol) was dissolved in 20 mL of anhydrous DMSO and heated at 150 °C for 45 min. The reaction mixture was cooled and lyophilized. The residue was treated with 50 mL of ether with stirring. The ether layer was decanted from an insoluble syrup and treated with 100 mL of hexanes, allowed to stand until the supernate was clear, and decanted from a precipitated oil.

The supernate was evaporated to dryness, and the compound was dried in vacuo over P_2O_5: yield 3.00 g (52.26%); mp 52–54 °C; MS m/z 310 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 4.09 (s, 3H, 6-OCH$_3$), 7.00 (bs, 1H, OH); Anal. (C$_7$H$_4$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-6-ethoxy-4-hydroxy-2-(trichloromethyl)-pyridine (3b). The general procedure previously described for **3a** was used to prepare **3b** using **2b** (1.48 g, 4.18 mmol) and DMSO (15 mL), and the reaction mixture was heated for 2.5 h. Starting material remained but since the solution was getting darker, heating was stopped. The product was purified by column chromatography (95:5 chloroform:methanol) to give an oil that solidified upon freezing: yield 371 mg (27.28%); mp 57–59 °C; MS m/z 324 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.45 (t, 3H, CH$_3$), 4.54 (q, 2H, CH$_2$), 6.65 (s, 1H, OH); Anal. (C$_8$H$_6$-Cl$_5$NO$_2$·0.15C$_2$H$_5$OH) C, H, N.

3,5-Dichloro-6-butoxy-4-hydroxy-2-(trichloromethyl)-pyridine (3c). The same procedure as described for **3a** was used to prepare **3c** from **2d** (2.00 g, 4.88 mmol) and DMSO (18 mL), and the reaction mixture was heated for 5.5 h. Starting material remained but since the solution was getting darker, heating was stopped. The product was purified by column chromatography (chloroform) to give an oil: yield 442 mg (25.7%); MS m/z 352 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 0.93 (t, 3H, CH$_3$), 1.42 (m, 2H, CH$_2$CH$_3$), 1.73 (m, 2H, CH$_2$CH$_2$), 4.41 (t, 2H, OCH$_2$); Anal. (C$_{10}$H$_{10}$Cl$_5$NO$_2$·0.3C$_2$H$_5$OH) C, H, N.

3,5-Dichloro-6-hydroxy-4-methoxy-2-(trichloromethyl)-pyridine (6-DMPEN) (3d). DidemethylPEN **8** (2.00 g, 6.72 mmol) in 10 mL of methanol was treated dropwise with stirring at room temperature with 30 mL of ethereal diazomethane prepared from Diazald as reported. The solution was stirred 10 min and evaporated in vacuo. The residue was separated on an 8 in. 2 mm silica gel plate (Analtech) in dichloromethane:methanol (9:1), and elution of the UV–visible band at R_f 0.7 with methanol and evaporation gave a solid (345 mg); bands at R_f 0.9 and R_f 0.5 were extracted and identified by co-TLC as PEN and 4-DMPEN, respectively. The solid was separated again by preparative TLC in the same solvent, and elution with methanol and evaporation gave a white solid (250 mg). The solid was dissolved in 5 mL of ether followed by 20 mL of hexanes. Concentration in vacuo to 5 mL and filtration gave a white crystalline solid, which was homogeneous upon analytical TLC and whose R_f was different from the R_fs of PEN and 4-DMPEN upon co-TLC in CH$_2$Cl$_2$: MeOH (9:1): yield 170 mg (8.13%); MS m/z 310 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 4.02 (s, 3H, 4-OCH$_3$), 2.50 (vbs, 1H, OH).

3,5-Dichloro-6-ethoxy-4-methoxy-2-(trichloromethyl)-pyridine (4a). To a solution of compound **3b** (824 mg, 2.53 mmol) in 20 mL of anhydrous acetone was added trimethyl-silyldiazomethane solution (2 M solution in hexanes, 2.53 mL), and the reaction mixture was stirred for 45 min at room temperature. The reaction mixture was evaporated to dryness. The residual product was purified by column chromatography (silica gel 230–400 mesh) and was eluted with hexanes. The desired fractions were combined, concentrated, and dried in vacuo over P_2O_5 to give an oil that solidified in the freezer: yield 764 mg (88.94%); mp 42–44 °C; MS m/z 338 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.46 (t, 3H, 6-CH$_3$), 4.02 (s, 3H, 4-CH$_3$), 4.54 (q, 2H, CH$_2$); ^{13}C NMR (CDCl$_3$) δ 14.38 (CH$_3$), 61.10 (OCH$_3$), 64.21 (OCH$_2$), 96.21 (CCl$_3$), 114.29, 117.83 (3-C, 5-C), 147.62 (2-C), 155.61 (6-C), 162.41 (4-C); Anal. (C$_9$H$_8$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-6-butoxy-4-methoxy-2-(trichloromethyl)-pyridine (4b). The same procedure as described for **4a** was used to prepare **4b** from **3c** (798 mg, 2.25 mmol) and tri-methylsilyldiazomethane solution (2 M solution in hexanes, 2.24 mL). After column chromatography (hexanes), an oil was obtained: yield 718 mg (86.6%); MS m/z 366 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 0.98 (t, 3H, 6-CH$_3$), 1.49 (m, 2H, CH$_2$CH$_3$), 1.82 (m, 2H, CH$_2$CH$_2$), 4.02 (s, 3H, 4-CH$_3$), 4.47 (t, 2H, OCH$_2$); Anal. (C$_{11}$H$_{12}$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-6-methoxy-4-acetoxy-2-(trichloromethyl)-pyridine (5a). To a solution of compound **3a** (1.00 g, 3.21 mmol) in 25 mL of anhydrous CH$_2$Cl$_2$ was added DMAP (375 mg) and Ac$_2$O (327 mg, 3.21 mmol). The reaction mixture was stirred at room temperature for 2 days and concentrated to dryness. The reaction did not go to completion. The product was purified by column chromatography (silica gel 230–400 mesh, elution with CHCl$_3$). The desired fractions were collected, concentrated, and dried in vacuo over P_2O_5: yield 477 mg (42.21%); MS m/z 352 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 2.45 (s, 3H, 4-CH$_3$), 4.18 (s, 3H, 6-CH$_3$); ^{13}C NMR (CDCl$_3$) δ 20.20 (s, CH$_3$ of Ac), 55.44 (OCH$_3$), 95.69 (CCl$_3$), 114.78, 117.40 (3-C, 5-C), 147.68 (2-C), 154.39 (4-C), 155.60 (6-C), 165.86 (C=O); Anal. (C$_9$H$_6$Cl$_5$NO$_3$·0.1CHCl$_3$) C, H, N.

3,5-Dichloro-6-ethoxy-4-acetoxy-2-(trichloromethyl)-pyridine (5b). Compound **5b** was prepared from **3b** (400 mg, 1.22 mmol), DMAP (148 mg), and Ac$_2$O (137 mg, 1.34 mmol). After column chromatography (9:1 hexanes:CH$_2$Cl$_2$), a solid was obtained: yield 220 mg (48.78%); mp 63–65 °C; MS m/z 366 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.47 (t, 3H, CH$_2$CH$_3$), 2.45 (s, 3H, COCH$_3$), 4.56 (q, 2H, OCH$_2$); Anal. (C$_{10}$H$_8$Cl$_5$NO$_3$) C, H, N.

3,5-Dichloro-6-ethoxy-4-methoxyacetoxy-2-(trichloro-methyl)pyridine (5c). The same procedure as described for **5a** was used to prepare **5c** from **3b** (240 mg, 0.73 mmol), DMAP (90 mg), and methoxyacetyl chloride (96 mg, 0.88 mmol). After column chromatography (6:4 hexanes:dichloromethane), an oil was obtained: yield 220 mg (75.08%); MS m/z 396 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.48 (t, 3H, 6-CH$_2$CH$_3$), 3.58 (s, 3H, 4-OCH$_3$), 4.46 (s, 2H, 4-COCH$_2$), 4.57 (q, 2H, 6-OCH$_2$); Anal. (C$_{11}$H$_{10}$Cl$_5$NO$_4$) C, H, N.

3,5-Dichloro-4-methoxy-6-trifluoroethoxy-2-(trichloro-methyl)pyridine (6). A mixture of compound **2e** (545 mg, 1.18 mmol), NaOH (54 mg, 1.35 mmol), and anhydrous methanol was stirred at room temperature for 4 h. The reaction mixture was concentrated to dryness. The compound was extracted with CH$_2$Cl$_2$ (2 × 20 mL) and washed with water. The organic layer was dried over MgSO$_4$, filtered, and concentrated to dryness. The residue was purified by column chromatography (silica gel 230–400 mesh, elution with hexanes). The desired fractions were collected, concentrated, and dried in vacuo over P_2O_5 to give an oil that solidified in the freezer: yield 444 mg (95.68%); mp 48–50 °C; MS m/z 392 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 4.06 (s, 3H, 4-OCH$_3$), 4.87 (q, 2H, OCH$_2$); Anal. (C$_9$H$_5$F$_3$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-4-hydroxy-6-trifluoroethoxy-2-(trichloro-methyl)pyridine (7). The same procedure previously described for **3a** was used to prepare **7** from compound **6** (540 mg, 1.37 mmol) and DMSO (18 mL) by heating for 1.5 h at 150 °C. After column chromatography (7:1 chloroform:methanol), an oil was obtained: yield 555 mg (99.46%); MS m/z 378 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.8 (bs, 1H, OH), 4.86 (q, 2H, OCH$_2$); Anal. (C$_8$H$_3$F$_3$Cl$_5$NO$_2$·0.1H$_2$O) C, H, N.

3,5-Dichloro-4,6-dihydroxy-2-(trichloromethyl)pyri-dine (8). Compound **2b** (1.00 g, 2.8 mmol) was dissolved in 20 mL of anhydrous dichloromethane. To this solution was added AlCl$_3$ (1.00 g). The reaction mixture was stirred for 1 h at room temperature and evaporated to dryness. The crude product was purified by column chromatography (silica gel 230–400 mesh, elution with 5:1 CHCl$_3$:MeOH). The desired fractions were collected, concentrated, and dried in vacuo over P_2O_5: yield 818 mg (97%); MS m/z 295 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 3.42 (bs, 2H, OH); ^{13}C NMR (CDCl$_3$) δ 92.71 (CCl$_3$), 107.07, 109.24 (3-C, 5-C), 142.54 (2-C), 155.93 (6-C), 158.67 (4-C).

3,5-Dichloro-4,6-diacetoxy-2-(trichloromethyl)pyri-dine (9). Compound **8** (950 mg, 3.19 mmol) in 2 mL of Ac$_2$O and 2 drops of pyridine was heated at 80 °C for 15 min, allowed to stand 30 min at room temperature, and stored at −20 °C overnight. The solvent was removed, and the residue was dried in vacuo. The product was purified by column chromatography (silica gel 230–400 mesh, elution with CH$_2$Cl$_2$). The desired fractions were collected, concentrated, and dried in vacuo over P_2O_5: yield 600 mg (49.58%); mp 122–124 °C; MS m/z 380 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 2.42 (s, 3H, 4-OCH$_3$), 2.47 (s, 3H, 6-OCH$_3$); ^{13}C NMR (CDCl$_3$) δ 20.13 (CH$_3$ of 4-Ac), 20.57 (CH$_3$ of 6-Ac), 94.45 (CCl$_3$), 121.42, 123.72 (3-C, 5-C), 149.36, 149.90

(2-C, 6-C), 155.58 (4-C), 165.39 (C=O of 4-Ac), 166.98 (C=O of 6-Ac); Anal. ($C_{10}H_6Cl_5NO_4$) C, H, N.

4-Methoxy-3,5,6-trichloro-2-(trichloromethyl)pyridine (10a). Compound **10a** is the same as **13a** but it was prepared under different reaction conditions. To a solution of NaOH (90 mg, 2.25 mmol) in anhydrous methanol (10 mL) was added compound **1** (749 mg, 2.24 mmol), and the reaction mixture was stirred at room temperature overnight. No isomer was obtained. Isolation of **10a** was the same as described for **13a**: yield 497 mg (67.25%).

4-Ethoxy-3,5,6-trichloro-2-(trichloromethyl)pyridine (10b). The procedure was the same as reported above for **10a** using **1** (1.5 g, 4.48 mmol), NaOH (179 mg, 4.48 mmol), and anhydrous ethanol (24 mL). In this case, a small amount of the 6-ethoxy isomer was also obtained. After column chromatography (hexanes), a solid was obtained: yield 1.21 g (78.5%); mp 42−44 °C; MS *m/z* 342 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.53 (t, 3H, 4-CH$_3$), 4.28 (q, 2H, 4-OCH$_2$); Anal. (C$_8$H$_5$Cl$_6$NO) C, H, N.

4-Hydroxy-3,5,6-trichloro-2-(trichloromethyl)pyridine (11). Compound **11** was prepared by the same procedure as described for the preparation of **3a** using **10a** (786 mg, 2.38 mmol) and DMSO (15 mL). After column chromatography (7:3 chloroform:methanol), a foamy solid was obtained: yield 678 mg (90.15%); MS *m/z* 314 (M + H)$^+$; ^{1}H NMR (Me$_2$SO-*d*$_6$) δ 7.8 (bs, 1H, OH); Anal. (C$_6$H$_1$Cl$_6$NO·1.0CH$_3$OH) C, H, N.

4-Acetoxy-3,5,6-trichloro-2-(trichloromethyl)pyridine (12). Compound **11** (656 mg, 2.07 mmol) in 2 mL of Ac$_2$O and a few drops of pyridine was heated at 80 °C overnight. The reaction mixture was cooled and evaporated to dryness. The residual product was purified by column chromatography (silica gel 230−400 mesh) by elution with 97:3 hexanes: dichloromethane. The desired fractions were combined, concentrated, and dried in vacuo over P$_2$O$_5$ to give a solid: yield 332 mg (44.6%); mp 96−98 °C; MS *m/z* 356 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 2.48 (s, 3H, CH$_3$); Anal. (C$_8$H$_3$Cl$_6$NO$_2$) C, H, N.

4-Methoxy-3,5,6-trichloro-2-(trichloromethyl)pyridine (13a) and 6-Methoxy-3,4,5-trichloro-2-(trichloromethyl)pyridine (14a). Compound **1** (5.00 g, 14.9 mmol) and sodium hydroxide pellets (600 mg, 15 mmol) in 60 mL of anhydrous methanol in a round-bottom flask were heated at 90 °C for 45 min. The reaction mixture was cooled and concentrated to dryness. The compound was extracted with CH$_2$Cl$_2$ (2 × 70 mL), and the extract was washed with water. The organic layer was dried over MgSO$_4$, filtered, and concentrated to dryness. The crude product was purified by column chromatography. The column (packed with silica gel 230−400 mesh) was eluted with hexanes. The desired fractions were collected, concentrated, and dried over P$_2$O$_5$ in vacuo yielding two isomers. F1 (**13a**): yield 4.17 g (84.5%); mp 69−70 °C; MS *m/z* 328 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 4.07 (s, 3H, 4-CH$_3$); Anal. (C$_7$H$_3$Cl$_6$NO) C, H, N. F2 (**14a**): yield 462 mg (9.37%); mp 78−80 °C; MS *m/z* 328 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 4.12 (s, 3H, 6-CH$_3$); Anal. (C$_7$H$_3$Cl$_6$NO) C, H, N.

4-Phenoxy-3,5,6-trichloro-2-(trichloromethyl)pyridine (13b) and 6-Phenoxy-3,4,5-trichloro- 2-(trichloromethyl)pyridine (14b). Compound **1** (1.6 g, 4.78 mmol) was dissolved in 18 mL of anhydrous dioxane. To this solution was added sodium phenoxide trihydrate (814 mg, 4.78 mmol). The reaction mixture was slowly heated to 100 °C and kept at 100 °C for 1.5 h. The reaction mixture was cooled and concentrated to dryness. The compound was extracted with CH$_2$Cl$_2$ (2 × 60 mL), and the extract was washed with water. The organic layer was dried over MgSO$_4$, filtered, and concentrated to dryness. The residue was purified by column chromatography (silica gel 230−400 mesh, elution with hexanes). The desired fractions were collected, concentrated, and dried in vacuo over P$_2$O$_5$ to give two isomers and other products. F1(**13b**): yield 810 mg (43.31%); mp 93−95 °C; MS *m/z* 390 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 6.84 (bd, 2H, C$_6$H$_5$ (2,6)), 7.15 (bt, 1H, C$_6$H$_5$ (4)), 7.36 (bt, 2H, C$_6$H$_5$ (3,5)); Anal. (C$_{12}$H$_5$Cl$_6$NO) C, H, N. F2 (**14b**): yield 370 mg (19.78%); mp 113−115 °C; MS *m/z* 390 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 7.22−7.27 (m, 3H, C$_6$H$_5$ (2,4,6)), 7.39−7.44 (m, 2H, C$_6$H$_5$ (3,5)); Anal. (C$_{12}$H$_5$Cl$_6$NO) C, H, N.

3,5-Dichloro-4-ethoxy-6-methoxy-2-(trichloromethyl)-pyridine (15a). Sodium hydroxide (88 mg, 2.2 mmol) was dissolved in 12 mL of anhydrous ethanol. After 20 min, **14a** (730 mg, 2.2 mmol) was added. The reaction mixture was stirred at room temperature for 7 days and concentrated to dryness. The compound was extracted with CH$_2$Cl$_2$ (2 × 20 mL), and the extract was washed with water. The organic layer was dried over MgSO$_4$, filtered, and concentrated to dryness. The resulting oil was purified by column chromatography (hexanes) to give an oil: yield 525 mg (69.9%); MS *m/z* 338 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.51 (t, 3H, 4-CH$_3$), 4.09 (s, 3H, 6-CH$_3$), 4.24 (q, 2H, CH$_2$); ^{13}C NMR (CDCl$_3$) δ 15.58 (CH$_3$), 55.22 (OCH$_3$), 70.50 (OCH$_2$), 96.20 (CCl$_3$), 114.40, 118.48 (3-C, 5-C), 147.61 (2-C), 155.85 (6-C), 161.86 (4-C); Anal. (C$_9$H$_8$Cl$_6$NO$_2$) C, H, N.

3,5-Dichloro-4-isopropoxy-6-methoxy-2-(trichloromethyl)pyridine (15b). The same procedure used to prepare **15a** was used to prepare **15b** except in this case, after 1 day of stirring at room temperature, the reaction mixture was heated at 65 °C for 2 days using **14a** (600 mg, 1.81 mmol), NaOH (72 mg, 1.8 mmol), and 2-propanol (20 mL). After column chromatography (hexanes), an oil was obtained that solidified upon freezing: yield 358 mg (55.67%); mp 26−28 °C; MS *m/z* 352 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.42 (d, 6H, CH-(CH$_3$)$_2$, *J* = 6.2 Hz), 4.09 (s, 3H, 6-OCH$_3$), 4.85 (m, 1H, CH); Anal. (C$_{10}$H$_{10}$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-4-propoxy-6-methoxy-2-(trichloromethyl)-pyridine (15c). Compound **15c** was prepared by the same procedure as reported for **15b** except the temperature was kept at 55 °C using **14a** (600 mg, 1.81 mmol), NaOH (72 mg, 1.8 mmol), and *n*-propanol (20 mL). After column chromatography (hexanes), an oil was obtained: yield 428 mg (66.56%); MS *m/z* 352 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.10 (t, 3H, CH$_2$CH$_3$), 1.92 (m, 2H, CH$_2$CH$_3$), 4.09 (s, 3H, 6-OCH$_3$), 4.12 (t, 2H, 4-OCH$_2$); Anal. (C$_{10}$H$_{10}$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-4-butoxy-6-methoxy-2-(trichloromethyl)-pyridine (15d). The procedure described for **15b** was used to prepare **15d** from **14a** (500 mg, 1.51 mmol), NaOH (60 mg, 1.5 mmol), and *n*-butanol (15 mL). After column chromatography (hexanes), an oil was obtained that solidified upon freezing: yield 217 mg (39%); mp 33−35 °C; MS *m/z* 366 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 1.00 (t, 3H, CH$_2$CH$_3$), 1.56 (m, 2H, CH$_2$CH$_3$), 1.87 (m, 2H, CH$_2$CH$_2$), 4.09 (s, 3H, 6-OCH$_3$), 4.16 (t, 2H, 4-OCH$_2$); Anal. (C$_{11}$H$_{12}$Cl$_5$NO$_2$) C, H, N.

3,5-Dichloro-4-methoxy-6-phenoxy-2-(trichloromethyl)-pyridine (15e). Compound **15e** was prepared from **14b** (510 mg, 1.3 mmol), NaOH (62 mg, 1.55 mmol), and methanol (15 mL) by refluxing for 2.5 h using the procedure described for the preparation of **6**. After column chromatography (hexanes), a white solid was obtained: yield 358 mg (71%); mp 91−93 °C; MS *m/z* 386 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 4.09 (s, 3H, 4-CH$_3$), 7.20−7.26 (m, 3H, C$_6$H$_5$ (2,4,6)), 7.38−7.43 (m, 2H, C$_6$H$_5$ (3,5)); ^{13}C NMR (CDCl$_3$) δ 61.25 (OCH$_3$), 95.58 (CCl$_3$), 115.11 (3-C), 119.59 (5-C), 121.54 (C2, C6, Ph), 125.34 (C4, Ph), 129.24 (C3, C5, Ph), 147.82 (C1, Ph), 152.69 (2-C), 154.94 (6-C), 163.11 (4-C); Anal. (C$_{13}$H$_8$Cl$_5$NO$_2$) C, H, N.

3,4,5-Trichloro-2,6-dimethylpyridine (16). This compound was prepared by a reported method.[12]

3,4,5-Trichloro-2,6-bis(trichloromethyl)pyridine (17). A slow stream of chlorine gas was passed through a refluxing solution of **16** (2.00 g, 9.5 mmol) in CCl$_4$ (100 mL) illuminated with a 500 W incandescent lamp for 4 h. Evaporation of the solvent afforded **17**, which was purified by column chromatography using hexanes for elution: yield 1.62 g (41%); mp 104−106 °C; MS *m/z* 414 (M + H)$^+$; Anal. (C$_7$H$_9$Cl$_9$N) C, H, N.

3,5-Dichloro-4-methoxy-2,6-bis(trichloromethyl)pyridine (18). The procedure is the same as reported above for **10a** using **17** (500 mg, 1.19 mmol), NaOH (56 mg, 1.4 mmol), and MeOH (15 mL). After column chromatography (hexanes), a white solid was obtained: yield 450 mg (91.09%); mp 115−117 °C; MS *m/z* 410 (M + H)$^+$; ^{1}H NMR (CDCl$_3$) δ 4.09 (s, 3H, CH$_3$); Anal. (C$_8$H$_3$Cl$_8$NO) C, H, N.

Antitumor Evaluation in Vivo. Athymic NCr-nu/nu mice were obtained from various suppliers under contract with NCI

and housed in sterile, filtered-capped microisolater cages in a barrier facility. Human MX-1 breast tumor was obtained from the NCI Tumor Repository (Frederick, MD). For intraperitoneal (ip) injection into mice, the analogues were prepared as a suspension in aqueous hydroxypropyl cellulose. For subcutaneous implants, tumor fragments (30−40 mg) from in vivo passage were implanted into the axillary region of the mice.

Treatment of groups of five mice each was initiated when the tumors reached approximately 300 mg in mass and was continued for 5 days for all treatment groups. Each tumor was measured by caliper in two dimensions twice weekly and converted to tumor mass. Antitumor activity was assessed on the basis of tumor growth delay in comparison to a vehicle-treated control ($T - C$, i.e., the difference in the median time poststaging for tumors of the treated (T) group to double twice in mass compared to the median of the control (C) group), tumor regression (partial and complete), and tumor-free survivors, and experiments were terminated when the control tumors attained a mass of 1 g, which was typically 57−61 days.

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