Inhibition of Myeloperoxidase Release from Rat Polymorphonuclear Leukocytes by a Series of Azachalcone Derivatives

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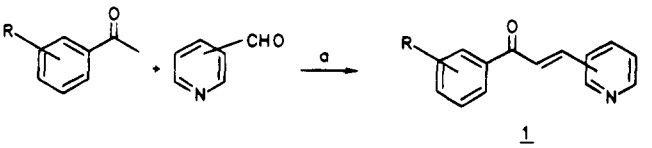
A series of azachalcones was evaluated for ability to affect secretion of myeloperoxidase by rat polymorphonuclear leukocytes stimulated by fMLP. The compounds were found to interfere with cellular uptake of extracellular calcium. Structure–activity relationships are discussed.

Polymorphonuclear leukocytes (PMN) are the body's first line of defense against infection by microorganisms. When activated, the PMN engulf microorganisms by phagocytosis and destroy them by the release of tissue destructive enzymes (myeloperoxidase (MPO), proteases) and reactive oxygen species (hydrogen peroxide, superoxide anion) onto the phagocytized microorganism.[1] These robust defense mechanisms may have adverse effects when mistakenly directed against the host[2] resulting in tissue destruction such as that seen in rheumatoid arthritis. Similarly, the host-directed response by PMN may be a component of the tissue destruction observed in adult respiratory distress syndrome and reperfusion injury.[2] While modulation of the PMN response would be of use in these disease states, few reports of compounds with such activity exist.[3–5]

To identify compounds that can suppress their activation, rat PMN were stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)[6] and the release of myeloperoxidase was measured. Colchicine has been reported to be inhibitory in this assay.[7] While colchicine has been used as an antiarthritic[8] and an inhibitor of microtubule polymerization,[9,10] the two effects demonstrate divergent structure-activity relationships (SAR).[11] This was of interest to us, for we had synthesized a series of chalcones with potent microtubule polymerization inhibition[12] which bind to the colchicine-binding site of microtubules. Testing of this series of compounds in the PMN assay led to the observation that azachalcones (**1**) were effective inhibitors of MPO release from PMN activated by addition of fMLP. As with colchicine analogs, a divergent SAR was observed. The azachalcones were the most potent of the chalcone series as inhibitors of MPO release from rat PMN but were not especially effective inhibitors of microtubule polymerization. It was this selectivity that led us to develop the azachalcones.

It was also observed that the azachalcones selectively inhibited PMN myeloperoxidase release; they inhibited MPO secretion stimulated by zymosan but showed little effect on the zymosan-induced production of superoxide anion. For rat PMN, release of MPO requires elevation of intracellular Ca^{2+} levels[13] while no increase in intracellular Ca^{2+} is required for superoxide anion generation.[14,15] Results are presented from experiments with one of these compounds (**33**) in which intracellular calcium concentrations were measured[16,17] during activation by fMLP, and it was found that compound **33** directly inhibited the influx of extracellular calcium which results from fMLP activation. This influx is reported to be through a receptor-mediated calcium channel,[18,19] and we investigated the possibility that these compounds were inhibitors of this mechanism. Consistent with the role of PMN in acute inflammation, compound **33** demonstrated activity against carrageenan-induced inflammation in the rat hind limb.

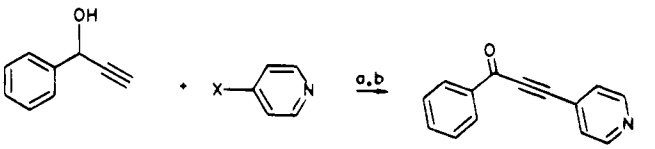
Scheme 1 Method A[a]



1

[a] (a) $Co(OAc)_2$, α,α-dipyridyl, DMF, 85 °C.

Scheme 2. Method B[a]



2

[a] (a)Bis(triphenylphosphine)palladium chloride, CuCl, $HN(C_2H_5)_2$; (b) DMSO, oxalyl chloride.

Chemistry

In general, the synthesis of analogs followed the route of Scheme 1. The initial series of azachalcones was synthesized by condensation of an aromatic ketone with a pyridine carboxaldehyde. While various reaction conditions have been described in the literature,[20,21] we found the procedure of Watanabe et al.[22] which used a cobalt 2,2′-dipyridyl complex as catalyst to be generally useful. These conditions allowed synthesis of all three pyridyl isomers and accommodated a wide variety of substituents on the aromatic ketone nucleus with yields (nonoptimized) of 4–57%.

The acetylenic analog **2** was synthesized by the palladium-catalyzed reaction of phenylpropynol and 4-bromopyridine hydrochloride[23] followed by oxidation of the acetylenic alcohol with DMSO/oxalyl chloride (Scheme 2). Synthesis of the pyridylbenzophenones is illustrated in Scheme 3. Diethylpyridylborane (**3**) was coupled with the appropriate bromobenzoic acid ester (**4**) to give the azabiphenyl product (**5**).[24,25] The ester functionality of **5** was converted to an acid halide, and a palladium-catalyzed coupling of this acid halide with phenyltrimethylstannane was performed to give the

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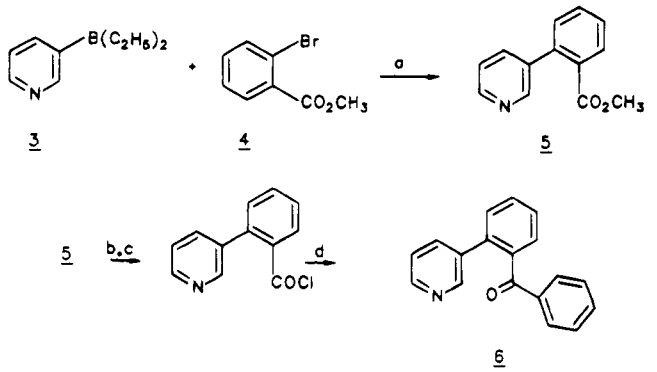
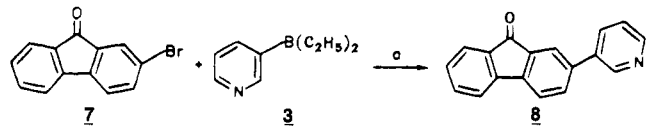
Scheme 3. Method C[a]



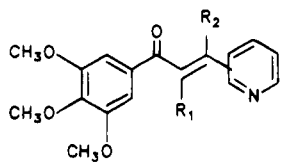
[a] (a) $Bu_4N^+Br^-$, KOH. $(PPh_3)_4Pd$; (b) KOH, EtOH; (c) oxalyl chloride; (d) $C_6H_5Sn(CH_3)_3$, tris(dibenzylideneacetone)palladium.

Scheme 4. Method D[a]



[a] (a) KOH, $(Bu)_4N^+Br^-$, $(PPh_3)_4Pd$.

Table 1. Inhibition of fMLP-Activated MPO Release from Rat PMN by Analog[a]



compd no.	R_1, R_2	Py substitution	IC_{50}, μM	mp, °C
9	H, H	4	7	128–9
10	H, H	3	30	137–8
11	H, H	2	18	94–6
12	CH_3, H	4	>100	128–9
13	H, CH_3	4	>100	119–20

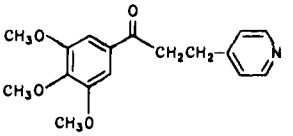
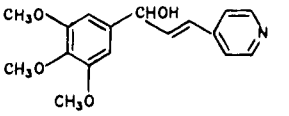
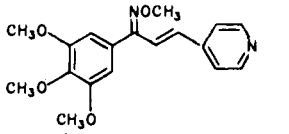
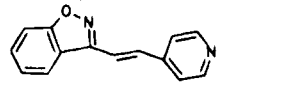
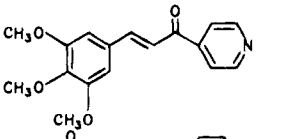
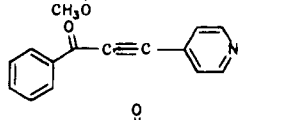
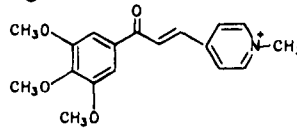
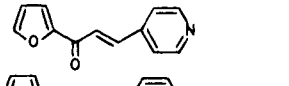
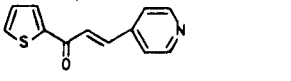
[a] For experimental details, see Biological Methods.

pyridylbenzophenone (**6**). Diethylpyridylborane was reacted with a variety of other halogenated aromatic systems such as 2-bromofluorenone (**7**) to give the pyridyl-coupled products (Scheme 4).

Results and Discussion

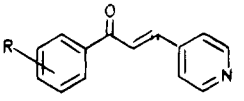
Inhibition of fMLP-activated MPO release from rat PMN was the preliminary screen for all compounds. Evaluation of the previously reported series of chalcone derivatives[12] led to the observation that azachalcones such as compound **9** were effective inhibitors in this assay. The data in Table 1 demonstrate that the three pyridyl isomers (**9**–**11**) were all active, while analogs with a methyl group substitution on the double bond of the α,β-unsaturated carbonyl (**12**, **13**) were inactive. The importance of the α,β-unsaturated carbonyl is further reinforced by the data in Table 2. When the ene or ketone groups were reduced, the resulting compounds (**14**, **15**) were inactive in this assay. Replacement of the carbonyl with *O*-methyl oxime (**16**) or incorporating the oxime into a fused heterocyclic ring (**17**) gave inactive compounds. The reversed eneone system (**18**) was weakly active while the ynone analog (**2**) was active in this assay. Quaternization of the pyridine system gave

Table 2. Inhibition of fMLP-Activated MPO Release from Rat PMN by Analog[a]

compd no.	structure	mp, °C	IC_{50}, μM
14		142-43	>100
15		123-24	>100
16		89-92	>100
17		143-44	>100
18		135-36	28% @ 50
2		74-5	16
19		210-11 (dec)	>50
20		122-24	50
21		150.5-52	30

[a] For experimental details, see Biological Methods.

Table 3. Inhibition of fMLP-Activated MPO Release from Rat PMN by Analog[a]



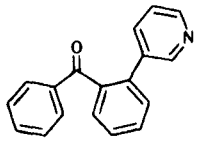
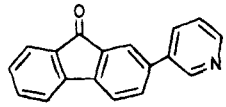
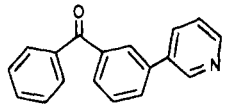
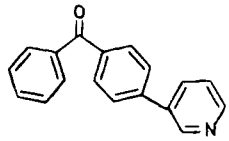
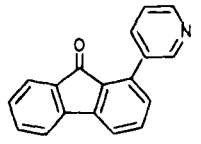
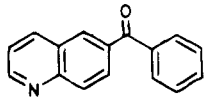
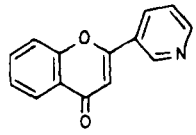
compd. no.	R	mp, °C	IC_{50}, μM
22	4-$N(CH_3)_2$	150–1	11
23	4-Cl	148–9	60
24	3-Cl	106–9	69
25	4-*tert*-butyl	119–20	47
26	3-CF_3	89–90	41
27	3-$N(CH_3)_2$	98–100	19
28	3-CH_3	113–4	39
29	4-CF_3	139–40	204
30	4-SCH_3	133–4	33
31	3-CN	163	85
32	4-CN	202.5–3.5	123
33	H	77–8	49

[a] For experimental details see Biological Methods.

an inactive compound (**19**) while compounds in which the benzene ring was replaced with other electron rich rings (**20**, **21**) were active in this test.

Variation of substituents on the aromatic ring (Table 3) gave a series of compounds with IC_{50} values between 11 and >200 μM. From these, compound **33** was selected as the lead compound for further testing (see

Table 4. Inhibition of fMLP-Activated MPO Release from Rat PMN by Analogs

compd no.	structure	mp, °C	IC_{50}, μM
6		106-07	>100
7			>100
34		63-63	~100
35		77-78	>100
36		151-52	>100
37		60-61	>100
38		142-43	~100

below). For the series of compounds in Table 4, the enone system is incorporated into an aromatic ring. These analogs were designed to maintain the essential geometry of the azachalcone system in a fixed arrangement. Unfortunately, these compounds were uniformly inactive in this assay. These data suggest that the biological activity depends on an intact α,β-unsaturated carbonyl system, perhaps capable of reaction with some functionality at the active site. Although no quantitative comparisons of reactivity between analogs were made, there seems to be an optimal level of reactivity of the system; strongly electron-withdrawing substituents gave less active analogs.

To investigate the mechanism by which azachalcones inhibit rat PMN secretion, the pharmacology of compound **33** was investigated in detail. Compound **33** showed comparable concentration-dependent inhibition of MPO release from rat PMN stimulated with either fMLP or opsonized zymosan (OZ) while ionomycin stimulation was unaffected (Figure 1). PMN myeloperoxidase production induced by all three agents results from increased intracellular calcium.[26,27] The calcium level increase produced by ionomycin differs in that a receptor-mediated calcium channel is not involved. The lack of activity of **33** on ionophore stimulation suggests that the activity observed involves a step in the signal transduction pathway between receptor activation and the responding elevation of intracellular calcium.

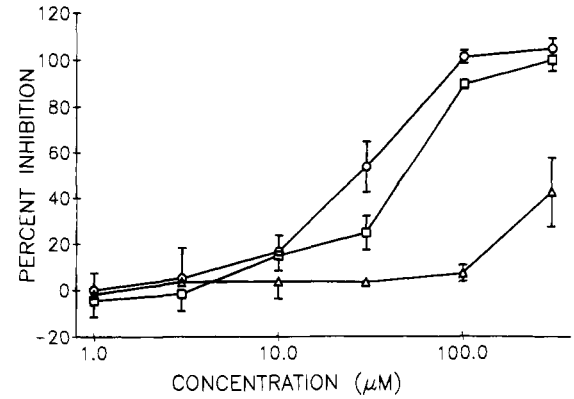


Figure 1. Effect of compound **33** on myeloperoxidase release from rat PMN. Rat PMN in suspension (2×10^7 cells/mL) were activated with either fMLP (○ 0.1 μM), opsonized zymosan (□ 2.6 μg/mL) or ionomycin (△, 10 μM) for 30 min at 37 °C in the presence of different concentrations of **33**. The supernatants were then assayed for myeloperoxidase released from the PMN ($n = 8$).

Figure 2A shows the biphasic calcium level response by rat PMN to fMLP stimulation. The uptake of Mn^{2+} has been used to assess divalent cation uptake into Fura-2-loaded PMN,[28] as manganese that enters the cytosol displaces calcium from Fura-2, quenching the fluorescence. This is illustrated in Figure 2B, where loaded PMN show a slow decline in fluorescence in the presence of extracellular Mn^{2+} in the absence of any stimulation. When Fura-2-loaded PMN are activated with fMLP in the presence of extracellular Mn^{2+}, there is an initial rise in fluorescence indicating an increase in cytosolic calcium levels presumably due to release of intracellular bound calcium. This response corresponds in time to the first phase of fMLP-induced intracellular calcium increase (see Figure 2A). Gradually the initial increase in fluorescence declines and falls below basal level, in a time course which parallels the second wave of fMLP-induced calcium level increase. This suggests the first wave represents a release of bound calcium and the second an influx of extracellular calcium. When cells pretreated with **33** were stimulated with fMLP in the presence of external Mn^{2+}, the second phase decrease of fluorescence levels was not observed (Figure 2C). Instead, there is a plateau of fluorescent signal after the initial increase, suggesting that extracellular Mn^{2+} cannot enter the cells pretreated with compound **33**.

These data demonstrate that **33** blocks the uptake of extracellular calcium by rat PMN. Diltiazem, verapamil, and nifedipine all block the voltage-sensitive calcium channel. In rat PMN stimulated with fMLP, none of these three inhibited the increase in intracellular calcium when used at levels up to 100 μM (data not shown). These results suggest that another calcium uptake process is the target of the azachalcones.

The activity of compound **33** in the carrageenan-induced paw edema model was studied to assess whether the inhibition of PMN by this compound would result in activity in an in vivo model. In this assay, carrageenan injected into the rat hind limb produces an acute inflammation accompanied by the infiltration of PMN. The activation of these PMN is believed to be a major factor in the acute inflammatory process.[29] When compound **33** was given ip, at a dose of 100 mg/kg 1 hour prior to the carrageenan injection, a reduction in

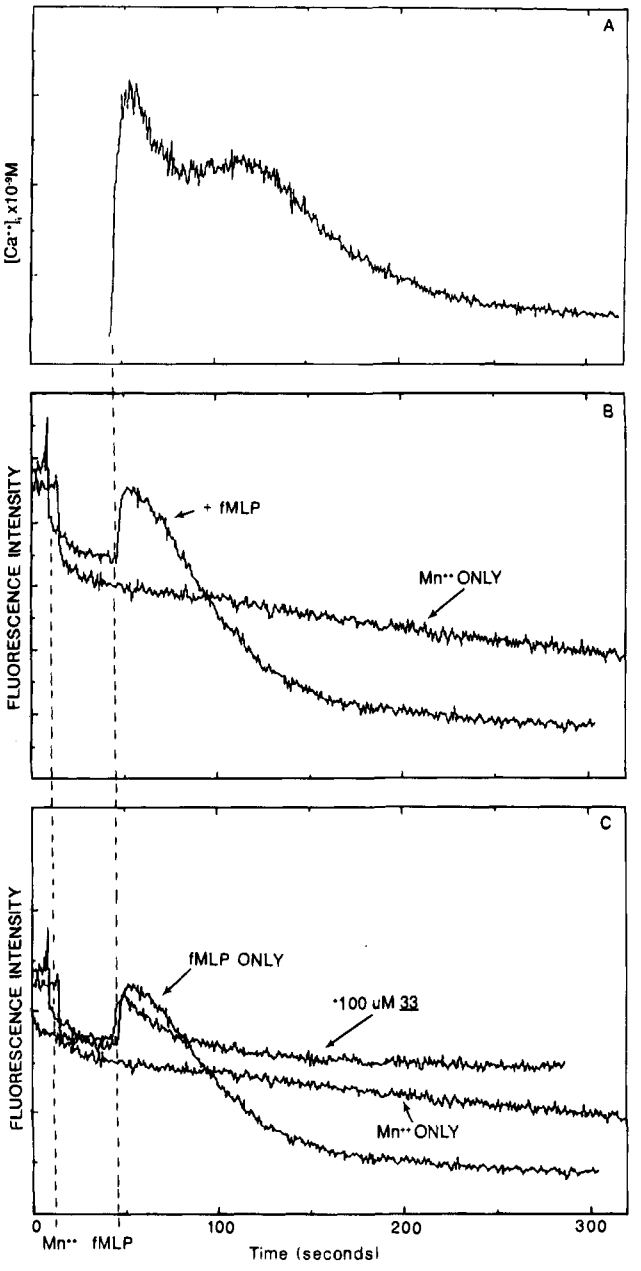


Figure 2. Measured fluorescence changes on rat PMN which have incorporated the intracellular calcium indicator Fura-2. (A) Intracellular calcium concentration $[Ca]_i$ determined after stimulation with fMLP (0.1 μM). (B) Fluorescence changes seen at 480 nm in the presence of extracellular manganese chloride (100 μM). (C) Same experiment as in B in the presence of 100 μM **33**. Experiment shown is representation of three replications.

paw swelling of 51 ± 8% was observed. This is comparable to the reduction reported for this model when PMN activation was blocked by other agents.[29]

In summary, we report a series of azachalcones which inhibit PMN responses caused by a variety of stimulants. A detailed study of the effects of a member of the series (**33**) on the responses of these cells to various stimuli is reported. Activity paralleled the ability of the compound to inhibit the uptake of extracellular calcium, a novel mechanism for modulation of PMN response. Consistent with its ability to significantly inhibit PMN activation in vitro, compound **33** inhibited a PMN-associated acute inflammation in an in vivo model. The chalcones have proven a useful source of biologically interesting compounds, with activity as antimitotic agents[12] and antibacterial agents[32] and inhibitors of lipoxygenase/cyclooxygenase[33] and IL-1 biosynthesis.[34]

Experimental Section

General Methods. Melting points were determined in a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by the Marion Merrell Dow Analytical Department and, unless otherwise indicated, agree with theory within ±0.4%. NMR spectra were obtained on a Varian VXR-300 or EM 360L spectrometer. Chemical shifts are reported downfield from TMS in spectra obtained in $CDCl_3$. IR spectra were obtained on a Perkin-Elmer 1800 FT IR spectrometer. All spectra were consistent with the proposed structure. Thin layer chromatography (TLC) was developed on Merck silica gel F254 analytical plates, visualized with I_2, UV light, or $KMnO_4$. All reagents used in the biological experiments were obtained from Sigma unless otherwise stated.

Biological Methods. See the supplementary material for detailed description of test procedures.

Isolation of Rat Peritoneal PMN. PMN were obtained from Harlan Sprague–Dawley rats (150–250 g). Cell viability by trypan blue exclusion was greater than 90%, and greater than 90% of the cells were rat PMN by Wright-Giemsa stain.

Myeloperoxidase Release. MPO release was determined using the MPO-catalyzed oxidation of o-dianisidine, using 96-well microtiter plates.[30] Opsonized zymosan was prepared by the method of Ward et al.[31]

Measurement of Intracellular Calcium. Intracellular calcium levels were obtained using Fura-2 as fluorescent indicator.[16] Intracellular calcium concentration, $[Ca^{2+}]_i$, was calculated by the method of Grynkiewicz,[17] assuming K_D for Fura-2 to be 240 nM. Each experiment was performed and repeated on three separate occasions, and the pattern of calcium changes shown in the figures is representative of these experiments. To observe effects of inhibitors on divalent cation influx into PMN, the method of Merritt et al.[19] was used.

Carrageenan-Induced Rat Paw Edema. Charles River male Sprague–Dawley rats weighing 90–100 g were used. Inflammation of the left hind paw was induced by a 1% carrageenan solution (0.05 mL) injected into the plantar surface of the paw. Each group measurement consisted of the average of four rats, each with three separate measurements.

Chemical Preparations. 1-[4-(Dimethylamino)phenyl]-3-(4-pyridyl)propeneone (22) (Method A). Cobalt acetate hydrate (3.5 g, 20 mmol) was vacuum-dried, and the anhydrous material was added to a solution of α,α′-dipyridyl (3.0 g, 20 mmol) in DMF (500 mL). The mixture was stirred for 30 min, 4-(dimethylamino)acetophenone (50 g, 0.306 mol) and pyridine-4-carboxaldehyde (25 g, 0.23 mol) were added, and the mixture was heated at 85 °C for 20 h. The mixture was evaporated, and the residue was chromatographed (toluene/ethyl acetate, 2/1) to give the product (2.1 g, 4.2%) as a bright yellow solid, mp 150–1 °C. IR (KBr): 1650, 1612, 1594, 1582, 1544, 1532, 1376, 1344, 1308, 1244, 1236, 1198, 808, 560 cm^{-1}. NMR ($CDCl_3$): δ 3.1 (s, 6H), 6.72 (d, J = 15 Hz, 2H), 7.48 (d, J = 7.5 Hz, 2H), 7.62–7.78 (m, 2H), 8.0 (d, J = 15 Hz, 2H), 8.76 (d, J = 7.5 Hz, 2H). MS (CI/CH_4): m/z 253 (M + H). Anal. Calcd for $C_{16}H_{16}N_2O$: C,H,N.

1-Phenyl-3-(4-pyridyl)propynone (2) (Method B). To a solution of 4-bromopyridine hydrochloride (2.3 g, 12 mmol) and 1-phenyl-2-propyn-1-ol (1.9 g, 15 mmol) in diethylamine (25 mL) were added bis(triphenylphosphine)palladium chloride (105 mg, 0.15 mmol) and copper(I) chloride (59 mg, 0.6 mmol), and the mixture was stirred for 3 h at ambient temperature. The reaction mixture was evaporated, and the residue was suspended in ethyl acetate (50 mL). Insoluble material was filtered off, the filtrate was evaporated, and the residue was chromatographed (ethyl acetate/hexane 4/3). The product was recrystallized from hexane/chloroform to give the product (0.4g, 19%), mp 93–96 °C. NMR ($CDCl_3$) δ 3.05 (d, J =7.5 Hz, 1H), 5.7 (d, J =7.5 Hz, 1H), 7.2–7.45 (m, 5H), 7.55–7.62 (m, 2H) and 8.5–8.55 (m, 2H). MS (EI) m/z 209 ($M^{+\cdot}$).

A solution of oxalyl chloride in dichloromethane (0.5 mL, 2M) was chilled in a dry ice-acetone bath and DMSO (0.14

mL, 2 mmol) was added. The solution was stirred for 10 min at −70 °C, and a solution of 1-phenyl-3-(4-pyridyl)-propynol (0.18 g, 0.86 mmol) in dichloromethane (1 mL) was added. The mixture was stirred for 20 min at −78 °C, triethylamine (0.56 mL, 4 mmol) was added, the bath was removed, and the mixture was stirred for 15 min. The reaction mixture was poured onto a silica gel column and eluted with 60% ethyl acetate/hexane. The product was recrystallized from hexane/chloroform to give a tan solid (58 mg, 32%), mp 74−5 °C. NMR ($CDCl_3$): δ 7.5−7.7 (m, 5H), 8.2−8.25 (m, 2H), 8.7−8.8 (m, 2H). MS (CI/CH_4): *m/z* 208 (M + H). Anal. Calcd for $C_{14}H_9NO$: C,H,N.

2-(3-Pyridyl)benzophenone (6) (Method C). A mixture of 3-(diethylboryl)pyridine (4.8 g, 24 mmol), methyl 2-bromobenzoate (9.6 g, 36 mmol), powdered KOH (4 g, 72 mmol), tetrabutylammonium bromide (3.8 g, 12 mmol), and tetrakis(triphenylphosphine)palladium (1 g) in THF (700 mL) was heated at reflux for 18 h. The reaction mixture was evaporated, and the residue was taken up in ethyl acetate/H_2O. The organic layer was separated, dried, and evaporated, the residue was chromatographed (toluene/ethyl acetate, 2/1), and the product was distilled to give **5** (4.4 g, 86%), bp 137−9 °C (0.7 mmHg). IR (film): 1726, 1290, 1258, 1092, 760, 714 cm^{-1}. NMR ($CDCl_3$): δ 3.68 (s, 3H), 7.25−7.7 (m, 5H), 7.93−7.98 (m, 1H), 8.55−62 (m, 2H). MS (CI/CH_4): *m/z* 214 (M + H).

Compound **5** (1.2 g, 5 mmol) was dissolved in ethanol (4 mL), and the solution was added to ethanolic KOH (5 mL, 2 M). The mixture was stirred for 18 h and evaporated, and the residue was triturated with 2-propanol. The solid was vacuum-dried overnight at 50 °C. The dried solid was suspended in dichloromethane (50 mL), the mixture was chilled in an ice bath, and oxalyl chloride (0.76 g, 6 mmol) was added. The mixture was stirred for another hour, the ice bath was removed, and the mixture was stirred overnight at ambient temperature. The reaction mixture was evaporated, the residue was redissolved in THF (40 mL), and phenyltrimethylstannane (1.2 g, 5 mmol) and tris(dibenzylideneacetone)-palladium (20 mg) were added. The mixture was heated at reflux for 4 h and evaporated, and the residue was chromatographed (toluene/ethyl acetate, 2/1). The product was recrystallized from hexane/dichloromethane to give **6** (0.37 g, 28%) as a white solid, mp 106−7 °C. IR (KBr): 1654, 1312, 1284, 766, 704 cm^{-1}. NMR ($CDCl_3$): δ 7.1−7.7 (m, 11H), 8.4−8.45 (m, 1H), 8.52−8.55 (m, 1H). MS (CI/CH_4): *m/z* 259 (M + H). Anal. Calcd for $C_{18}H_{13}NO$: C,H,N.

2-(3-Pyridyl)-9-fluorenone (8) (Method D). A mixture of 2-bromo-9-fluorenone (200 mg, 0.77 mmol), diethylpyridylborane (113 mg, 0.77 mmol), powdered KOH (86 mg, 1.54 mmol), tetrabutylammonium bromide (124 mg, 0.39 mmol), and tetrakis(triphenylphosphine)palladium (20 mg) in THF (50 mL) was heated at reflux for 18 h. The reaction mixture was evaporated, and the residue was chromatographed (toluene/ethyl acetate, 1/1). The product was recrystallized from methanol to give **8** (40 mg, 20%) as a yellow solid, mp 131−2 °C. IR (KBr): 1722, 1002, 1456, 764, 738, 708 cm^{-1}. NMR ($CDCl_3$): δ 7.26−7.75 (m, 7H), 7.9−7.95 (m, 2H), 8.6−8.7 (m, 1H), 8.9−8.95 (m, 1H). MS (CI/CH_4): *m/z* 258 (M + H). Anal. Calcd for $C_8H_{11}NO$: C,H,N.

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Supplementary Material Available: Detailed description of Biological Methods (2 pages). Ordering information is given on any current masthead page.

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