5-(5,6-Dichloro-2-indolyl)-2-methoxy-2,4-pentadienamides: Novel and Selective Inhibitors of the Vacuolar H^+-ATPase of Osteoclasts with Bone Antiresorptive Activity

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The vacuolar H^+-ATPase (V-ATPase), located on the ruffled border of the osteoclast, is a proton pump which is responsible for secreting the massive amounts of protons that are required for the bone resorption process. With the aim to identify new agents which are able to prevent the excessive bone resorption associated with osteoporosis, we have designed a novel class of potent and selective inhibitors of the osteoclast proton pump, starting from the structure of the specific V-ATPase inhibitor bafilomycin A_1. Compounds **3a**−**d** potently inhibited the V-ATPase in chicken osteoclast membranes (IC_{50} = 60−180 nM) and were able to prevent bone resorption by human osteoclasts in vitro at low-nanomolar concentrations. Notably, the EC_{50} of compound **3c** in this assay was 45-fold lower than the concentration required for half-maximal inhibition of the V-ATPase from human kidney cortex. These results support the validity of the osteoclast proton pump as a useful molecular target to produce novel inhibitors of bone resorption, potentially useful as antiosteporotic agents.

Postmenopausal osteoporosis, the most common and widespread metabolic bone disease, is characterized by a net loss of bone mass which results from an imbalance of the bone-remodeling process, with bone resorption exceeding bone formation: bone resorption is a multistep process which ultimately requires secretion of hydrochloric acid by osteoclasts.[1] The lowering of pH in the sealed microcompartment which underlies osteoclasts at their site of attachment to the bone surface is necessary both to dissolve the bone mineral and to provide the acidic environment required by collagenases to degrade the bone matrix.[2] Protons are extruded by vacuolar-type H^+-ATPases[3] (V-ATPases) that are present in large numbers on the ruffled border of the osteoclast, while the chloride counterions diffuse passively through a chloride channel.[4]

The osteoclast V-ATPase is, therefore, a novel potential target for reducing osteoclast activity. The macrolide antibiotic bafilomycin A_1 is a very potent and specific inhibitor of V-ATPases[5] which does not affect significantly other important ATPases such as the H^+/K^+-ATPase[6] found in gastric parietal cells and which is responsible for maintaining gastric acidity. Indeed, bafilomycin A_1 is able to inhibit bone resorption both in vitro[7] and in vivo;[8] however, since it is not selective for any particular type of V-ATPases, its administration to animals causes inhibition of all the essential V-ATPases. This leads to systemic alteration of cellular physiology and, ultimately, to death.

Proton-translocating V-ATPases[9] are located in most intracellular organelles of both constitutive and specialized secretory pathways of eukaryotic cells and play an important role in membrane trafficking and protein sorting as well as in protein degradation.[10−12] In kidney, plasma membrane V-ATPases participate in urinary acidification.[13]

It is still controversial whether the osteoclast plasma membrane possesses a unique proton pump isoform: sequence differences between the chicken osteoclast enzyme and V-ATPases in other cell types have been reported,[14−16] as well as a peculiar sensitivity of chicken osteoclast V-ATPase to vanadate and nitrate.[17] The latter findings, however, have not been confirmed by other investigators using either chicken[18] or mammalian[19,20] osteoclasts.

To verify if different types of V-ATPases can be distinguished pharmacologically, we have performed a series of chemical modifications of the specific V-ATPase inhibitor bafilomycin A_1. As reported in a previous paper,[21] some key features required for biological activity have been identified, and these include the vinylic methoxy at position 2, the dienic systems, a free hydroxy group at position 7, and a portion of the side chain. Very interestingly, a slight differential effect of some bafilomycin derivatives on V-ATPase-driven proton transport in membrane vesicles from chicken osteoclasts and bovine chromaffin granules was observed suggesting that selective modulation of different V-ATPases may be possible.

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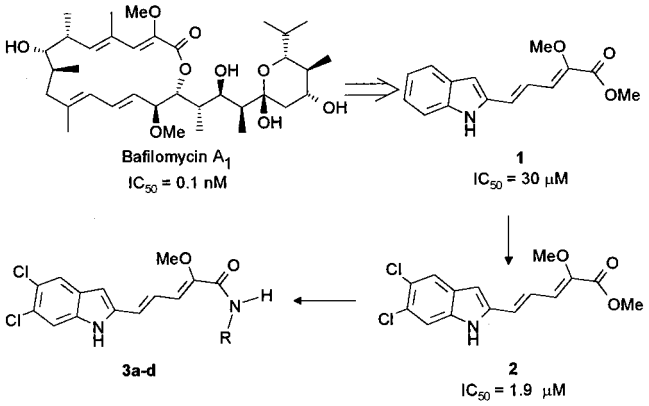
Chart 1

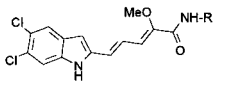


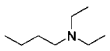
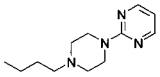
Encouraged by these preliminary results, we have continued our search for new analogues endowed with a higher degree of selectivity for the osteoclast enzyme. The inhibition of bafilomycin-sensitive ATPase activity was measured in membrane vesicle preparations from chicken osteoclasts (cOc) from the medullary bone of calcium-starved egg-laying hens.[22] Our strategy for identifying selective inhibitors entailed the comparison of the potency of the new derivatives in cOc against their potency on V-ATPases from different origins. For this purpose the enzyme from chicken adrenal glands (cAG)[23] was used since this V-ATPase is philogenetically related to the cOc proton pump. Potency in human tissues was preliminarily assessed using membranes from human kidney cortex (hK) since a high concentration of proton secretory V-ATPases is present in the renal intercalated cells where they regulate urinary acidification and bicarbonate resorption.[24]

Since chemical modifications of bafilomycin are limited by its high complexity and low chemical stability, a novel series of simplified derivatives was designed which contained the essential features for V-ATPase-inhibitory activity of bafilomycin, i.e., a vinylic methoxy group at position 2 of the 5-(2-indolyl)-2,4-pentadienoyl system in which the indole NH can possibly mimic the necessary hydroxy group at position 7 of bafilomycin A_1. Thus, as indicated in Chart 1, methyl (2*Z*,4*E*)-5-(2-indolyl)-2-methoxy-2,4-pentadienoate (**1**) was prepared which was a moderate inhibitor of the chicken osteoclast V-ATPase ($IC_{50} = 30\ \mu M$). Optimization of the aromatic substitution led to the identification of lipophilic and electron-withdrawing substituents at positions 5 and 6 of the indole ring as necessary requirements for high potency. Conversely, introduction of any type of substituent at other positions of the indole ring, i.e., 1, 3, 4, or 7, was deleterious for activity. Thus, the 5,6-dichloro derivative **2** showed a 15-fold increase in potency, with an IC_{50} of 1.9 μM in the cOc ATPase assay. A further increase of potency could be obtained by converting the carbomethoxy group of **2** into the corresponding carboxamides bearing a strongly basic nitrogen group separated by a three-carbon atom spacer from the amidic nitrogen.

Initially, (2*Z*,4*E*)-5-(5,6-dichloro-2-indolyl)-*N*-[3-(diethylamino)propyl]-2-methoxy-2,4-pentadienamide (**3a**) was obtained which displayed a submicromolar potency with an IC_{50} of 180 nM in cOc and a 15-fold selectivity against the vacuolar enzyme in chicken adrenal glands. Conformationally constrained analogues **3b**,**c**, where the distance and the relative orientation of the two nitrogen atoms were fixed, were even more potent (80 and 100 nM, respectively) with a similar degree of selectivity. The 2-pyrimidylpiperazine derivative **3d**, maintaining the linear amidic chain of **3a** but with a lipophilic basic head, was the most potent compound in the V-ATPase assay with an IC_{50} of 60 nM (Table 1).

Table 1. ATPase Inhibition of the Vacuolar ATPase from Different Enzyme Sources and in Vitro Inhibition of Bone Resorption by Isolated Human Osteoclastoma Cells

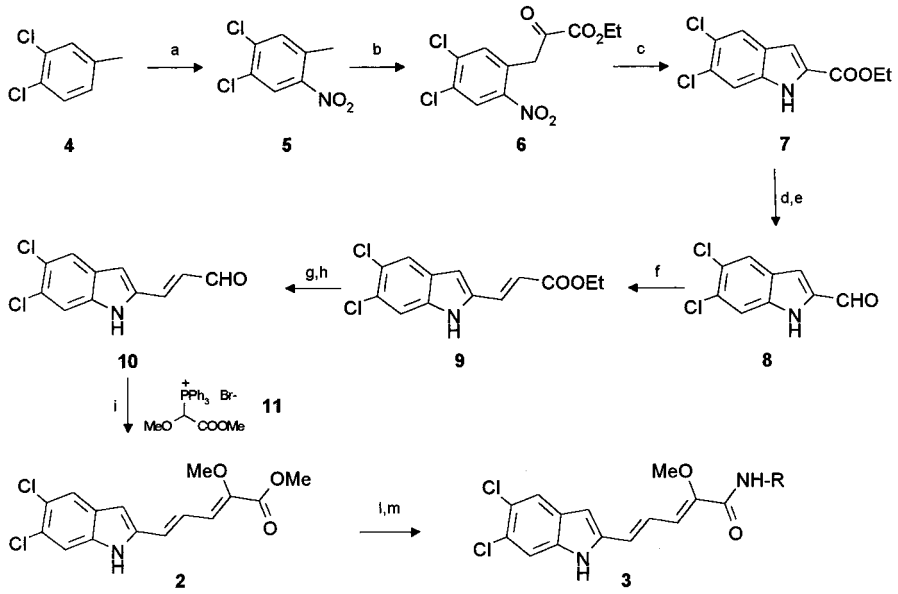


		IC_{50} (μM)				
		V-ATPase assays			Human osteoclast resorption assay	
	R	cOc	cAG	hK	readout: pits/bs	readout: ELISA
1	Bafilomycin A_1	0.1×10^{-3}	0.2×10^{-3}	0.1×10^{-3}	0.1×10^{-3}	0.01×10^{-3}
3a		0.18	2.80	3.00	0.18	0.14
3b		0.08	0.82	0.44	0.04	0.03
3c		0.1	2.50	1.32	0.03	0.03
3d		0.06	0.93	0.37	0.03	0.01

The synthesis for the preparation of compounds **3a**−**d** is described in Scheme 1. Briefly, nitration of 3,4-dichlorotoluene[25] followed by reaction with diethyl oxalate in the presence of potassium ethoxide and subsequent reductive cyclization gave ethyl 5,6-dichlorindole-2-carboxylate (**7**). This intermediate was reduced with $LiAlH_4$, oxidized with MnO_2, and homologated via a Horner−Emmons reaction to give ethyl (*E*)-3-(5,6-dichloro-2-indolyl)-2-propenoate (**9**). This compound was reduced with DIBAH and oxidized with MnO_2 to afford (*E*)-3-(5,6-dichloro-2-indolyl)-2-propenaldehyde (**10**). Wittig reaction of **10** with the phosphonium salt **11**[26] gave the dichloroindole ester **2** that was subsequently hydrolyzed and condensed with the appropriate amine affording derivatives **3a**−**d**.

The bone antiresorptive activity of these novel inhibitors was functionally evaluated in vitro using isolated human osteoclasts obtained from giant cell tumors of bone.[27] Both the number of excavations (pits/bone slice) and the generation of type I collagen telopeptides (ELISA)[28] produced after 48-h incubation were measured. As shown in Table 1, compounds **3a**−**d** were potent inhibitors of bone resorption by human osteoclasts with potencies that were comparable to their ability to inhibit chicken osteoclast V-ATPase, although all compounds were slightly more potent in the human system. In the ELISA assay, the most potent compounds were **3d**,**c**, with IC_{50}'s of 10 and 30 nM, respectively, and a selectivity of about 40-fold if compared with the potency data from human kidney V-ATPase assay. Selectivity is only indicative since potency on different human enzymes should be compared in the same assay. Unfortunately, however, it has not yet been possible to produce a viable preparation

Scheme 1. Preparation of (2*Z*,4*E*)-5-(5,6-Dichloroindol-2-yl)-2-methoxypentadienamides **3a**−**d**[a]



[a] Reagents: (a) nitric acid/sulfuric acid (95%); (b) diethyl oxalate, potassium ethoxide (59%); (c) Fe/AcOH reduction (92%); (d) $LiAlH_4$ in THF (80%); (e) MnO_2 in Et_2O (85%); (f) triethylphosphonoacetate, NaH in THF (89%); (g) DIBAH (100%); (h) MnO_2 in EtOAc (92%); (i) DBU in THF (89%); (l) NaOH (aq.) in MeOH/THF (97%); (m) R-NH_2, HOBt/*N*′-[3-(dimethylamino)propyl]-*N*-ethylcarbodiimide hydrochloride (56−80%).

of human osteoclast membrane vesicles containing a bafilomycin-sensitive ATPase of suitable purity.

In conclusion a novel class of compounds endowed with potent and selective osteoclast V-ATPase inhibitory activity has been identified and characterized in in vitro models of bone resorption. The results obtained clearly support the validity of the proton pump located on the ruffled border of the osteoclast as a useful molecular target to produce novel inhibitors of bone resorption which have potential as antiosteoporotic agents.

A more detailed description of our investigations within this novel chemical class will be reported in subsequent manuscripts, along with our studies aimed at further optimizing the potency, therapeutic efficacy, and selectivity of these new osteoclast V-ATPase inhibitors.

Experimental Section

Biochemistry. Preparation of Chicken Osteoclast Membranes (cOc). Bone microsomes were isolated from medullary bones of calcium-starved egg-laying hens as described previously.[22] The crude preparation was collected as 100 000g_{max} fraction and resuspended in 10 mM Hepes buffer, pH 7.4, 0.2 M sucrose, 50 mM KCl, 1 mM EGTA, and 2 mM DTT. This fraction was further purified using a three-step 15%, 30%, 45% (w/w) sucrose gradient spun at 280 000g_{max}. An amount of membrane proteins (20−40 μg/mL) was used in the ATPase assay.

Preparation of Membranes from Chicken Adrenal Glands (cAG). The whole adrenal glands were used. Crude microsomal membranes collected as 12 000g_{max} fraction were resuspended in 0.3 M sucrose, 10 mM Hepes-Tris, 5 mM EGTA, 1 mM DTT, 1 mM $ATPNa_2$, 2 μg/mL pepstatin A, and 2 μg/mL leupeptin buffered at pH 7.5. This fraction was further purified by a two-step 35%, 50% (w/w) sucrose gradient and processed as previously described.[23] An amount of membrane proteins (20−40 μg of protein/mL) was used in the ATPase assay.

Preparation of Membranes from Human Kidney Cortex (hKc). Brush border membranes were obtained from the cortex of human kidney frozen immediately after surgery, according to the method reported for bovine kidney.[24] Membrane proteins (about 70 μg of protein/mL) were used in the ATPase assay.

Bafilomycin-Sensitive ATPase Assay. The reaction medium contained 0.2 M sucrose, 50 mM KCl, 10 mM Hepes-Tris, pH 7.4 (cAG and hKc) or pH 8 (cOc), 1 mM CDTA, 1 mM ATP, 0.1 mM ammonium molybdate, 5 μM valinomycin, and 5 μM nigericin. The reaction was started by addition of 5 mM $MgSO_4$. After 30 min of incubation at 37 °C, the release of phosphate was measured using a colorimetric assay (malachite green) as described in the literature.[29]

Preparation of Human Osteoclasts. Tissue from human osteoclastoma tumor was used for the preparation of osteoclasts.[27] The tissue was chopped into small pieces and placed into a sterile 50-mL centrifuge tube. The pieces were disaggregated by incubation at 37 °C for 1 h in serum-free RPMI-1640 medium, supplemented with 3 mg/mL (w/v) type I collagenase. A cell suspension was obtained by gentle homogenization of the remaining tissue with a plunger from a 20-mL syringe. The cell suspension was washed twice in medium supplemented with 10% fetal calf serum (FCS). The cells were frozen in cryotubes at a concentration of 5 million/mL in liquid N_2.

Human Osteoclast Bone Resorption Assay (HOcA). The osteoclasts were negatively selected using Dynabeads Pan Human HLAII (supplied by Dynal) and settled on bone slices placed in a 96-well multiplate (4000 cells/well). After 30 min the bone slices were transferred into a 24-well multiplate (4 bone slices/well). After 1 h, different concentrations of the test compound were added (8 bone slices/each concentration). The bone slices were incubated for 48 h in D-MEM culture medium and then fixed by using 2% glutaraldehyde in 0.2 M cacodylate buffer (15 min at 4 °C). After the buffer was removed and the sample washed three times with distilled water, TRAP (tartrate-resistant acid phosphatase) staining solution[30] was added and incubated for 60 min at 37 °C. The bone slices were rinsed twice with distilled water, dried, and mounted on a glass slide using bio-adhesive tape. After the number of osteoclasts was read, the fixed-stained cells were removed by 5-min incubation in 5% bleach. The slices were washed, dried, and sputter-coated with gold. Cellular activity was quantitated by counting the number of pits formed during the resorption using a light microscope, and the results were expressed as number of pits/bone slice. The osteoclast activity was further evaluated using the experimental procedure

described in the literature[27] utilizing in vitro ELISA kit[28] for quantification of type I collagen fragments released into the bone cell culture supernatants during bone resorption.

Chemistry. Melting points were determined with a Büchi 530 hot stage apparatus and are uncorrected. Proton NMR spectra were recorded on a Bruker ARX 300 spectrometer at 303 K unless otherwise indicated. Chemical shifts were recorded in parts per million (δ units) downfield from tetramethylsilane (TMS). IR spectra were recorded in Nujol mull or in KBr with a Perkin-Elmer 1420 spectrometer. Mass spectra were obtained on a Finnigan MAT TSQ-700 spectrometer. Negative or positive electrospray (ESI NEG or ESI POS, respectively) was recorded with the following conditions: solvent methanol, spray 4.5 kV, skimmer ±60 V, capillary temperature 220 °C. Experimental conditions for electronic impact (EI) were source 180 °C, 70 eV, 200 μA. Silica gel used for flash column chromatography was Kiesegel 60 (230−400 mesh; E. Merk AG, Darmstadt, Germany). Solvent evaporation was performed at reduced pressure, and oily products were dried at 0.1 mbar for 16 h. 3,4-Dichlorotoluene (**4**) was supplied by Acros and Florisil by Fluka. Activated manganese-(IV) oxide (MnO_2; <5 μm, ca. 85%) was supplied by Aldrich Chemical and used without further activation. Data of the combustion elemental analyses were within 0.4% of theoretical values.

Ethyl 2-Oxo-3-(2-nitro-4,5-dichlorophenyl)propanoate (6). To a suspension of potassium (24.5 g, 0.63 ga) in anhydrous Et_2O (245 mL) was added a solution of EtOH (158 mL) and anhydrous Et_2O (126 mL) dropwise under nitrogen during 4 h. The resulting solution was diluted with Et_2O (600 mL), and then diethyl oxalate (85.5 mL, 0.63 mol) was added dropwise in about 30 min. A solution of **5**[25] (130 g, 0.63 mol) dissolved in anhydrous Et_2O (225 mL) was added dropwise to the resulting yellow mixture in 1 h. Stirring was continued for an additional 3 h, and the dark-brown mixture was allowed to settle at room temperature for 2 days. The potassium salt was filtered, washed with anhydrous Et_2O (200 mL), and dried to give 210 g of a dark-brown powder. The solid was suspended in a mixture of water (200 mL) and EtOAc (400 mL) and then acidified with 10% HCl. The organic layer was washed with a saturated solution of $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and evaporated to give ethyl 2-oxo-3-(2-nitro-4,5-dichlorophenyl)propanoate (**6**) (115.1 g, 59.7%) as a light-brown solid, mp 104−107 °C. IR (Nujol): 3400, 1750, 1710 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.41 (s, 1H); 8.32 (s, 1H); 6.89 (s, 2H); 4.41 (q, 2H); 1.41 (t, 3H). MS (ESI NEG): 304 (M − H). Anal. ($C_{11}H_9NO_5Cl_2$) C, H, N, Cl.

Ethyl 5,6-Dichloroindole-2-carboxylate (7). A mixture of ethyl 2-oxo-3-(2-nitro-4,5-dichlorophenyl)propanoate (**6**) (20 g, 65 mmol) and iron powder (32 g, 0.57 ga) in EtOH (125 mL) and glacial acetic acid (125 mL) was refluxed for 2 h. After cooling, the resulting mixture was evaporated under vacuum. THF (200 mL) was added to the solid residue, and the suspension was filtered on Florisil (100 g) eluting with a large amount of THF (1000 mL). The solvent was evaporated obtaining ethyl 5,6-dichloroindole-2-carboxylate (**7**) (15.5 g, 92%) as a light-brown powder, mp 216−218 °C. IR (Nujol): 3310, 1695 cm^{-1}. ^1H NMR ($CDCl_3$): δ 9.01 (s, 1H); 7.78 (s, 1H); 7.14 (s, 1H); 4.43 (q, 2H); 1.43 (t, 3H). MS (ESI POS): 258 (MH^+). Anal. ($C_{11}H_9NO_2Cl_2$) C, H, N, Cl.

5,6-Dichloroindole-2-carboxaldehyde (8). To an ice-cold 1 M solution of $LiAlH_4$ in anhydrous THF (1.6 L, 1 M) under Ar was added ethyl 5,6-dichloroindole-2-carboxylate (**7**) (150 g, 581 mmol), dissolved in anhydrous THF (1 L), dropwise under stirring, keeping the temperature below 5 °C. After stirring for 2 h at room temperature, the reaction was quenched by successive addition of water (75 mL), 15% NaOH (75 mL), and water (150 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated under vacuum affording (5,6-dichloro-2-indolyl)methanol (100 g, 80%) which was suspended in 3 L of Et_2O, treated with MnO_2 (200 g, 2.3 mol), and stirred at room temperature for 2 days. The suspension was filtered on a Celite pad washing with Et_2O and warm acetone. The combined organic phases were concentrated to give 5,6-dichloroindole-2-carboxaldehyde (**8**) (84 g, 85%), mp 205−208 °C. IR (Nujol): 3250, 1660 cm^{-1}. ^1H NMR (DMSO-d_6): δ 12.26 (s, 1H); 9.89 (s, 1H); 8.09 (s, 1H); 7.66 (s, 1H); 7.41 (s, 1H). MS (ESI NEG): 212. Anal. ($C_9H_5NOCl_2$) C, H, N, Cl.

Ethyl (*E*)-3-(5,6-Dichloro-2-indolyl)-2-propenoate (9). To a solution of triethylphosphonoacetate (32.9 g, 147 mmol) in THF (150 mL) was added a 60% oil dispersion of NaH (5.95 g, 149 mmol) portionwise in 30 min under nitrogen maintaining the temperature between 0 and 5 °C. A solution of 5,6-dichloroindole-2-carboxaldehyde (**8**) (29 g, 135.5 mmol) in THF (200 mL) was added dropwise maintaining the internal temperature at about 20 °C. The solvent was evaporated under reduced pressure, and the residue was treated with H_2O (200 mL) and EtOAc (500 mL). The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was triturated with hexane, filtered, and dried under vacuum to afford ethyl (*E*)-3-(5,6-dichloro-2-indolyl)propenoate (**9**) (34.5 g, 89.6%) as a light-brown powder, mp 188−190 °C. IR (Nujol): 3300, 1690, 1610 cm^{-1}. ^1H NMR (DMSO-d_6): δ 11.89 (s, 1H); 7.76 (d, 1H); 7.60 (s, 1H); 6.92 (s, 1H); 6.59 (d, 1H); 4.20 (q, 2H); 1.27 (t, 3H). MS (EI): 283 (M^+), 237, 211, 174. Anal. ($C_{13}H_{11}NO_2Cl_2$) C, H, N, Cl.

(*E*)-3-(5,6-Dichloro-2-indolyl)-2-propenaldehyde (10). To a solution of ethyl (*E*)-3-(5,6-dichloro-2-indolyl)-2-propenoate (**9**) (34.5 g, 121 mmol) in dry THF (500 mL) was added DIBAH (1 M solution in hexane, 242 mL, 2.4 mequiv) dropwise under nitrogen maintaining the temperature below −20 °C. Stirring was continued for 1 h; then the reaction was quenched with 10% H_2SO_4 (400 mL) and a saturated solution of potassium sodium tartrate tetrahydrate (400 mL). The organic layer was separated, and the aqueous phase was extracted with Et_2O (200 mL). The combined organic phases were dried over $MgSO_4$, filtered, and evaporated to dryness to yield 29 g of (*E*)-3-(5,6-dichloro-2-indolyl)-2-propen-1-ol (100%). ^1H NMR (DMSO-d_6): δ 11.51 (s, 1H); 7.70 (s, 1H); 7.48 (s, 1H); 6.60 (d, 1H); 6.45 (m, 1H); 6.41 (s, 1H); 4.98 (t, 1H); 4.15 (t, 2H). Crude (*E*)-3-(5,6-dichloro-2-indolyl)-2-propen-1-ol (29 g, 119.8 mmol) was suspended in EtOAc (800 mL), and activated MnO_2 (50 g, 574 mmol) was added. The mixture was stirred at room temperature for 2 days and then filtered on a Celite pad, washing with EtOAc (400 mL). The combined organic phases were evaporated to give (*E*)-3-(5,6-dichloro-2-indolyl)-2-propenaldehyde (**10**) (26.7 g, 92.8%) as a brown powder, mp 216−218 °C. IR (Nujol): 3210, 1685, 1620−1610 cm^{-1}. ^1H NMR (DMSO-d_6): δ 11.95 (s br, 1H); 9.65 (d, 1H); 7.89 (s, 1H); 7.71 (d, 1H); 7.61 (s, 1H); 7.00 (s, 1H); 6.78 (dd, 1H). MS (ESI NEG): 238 (M − H^-), 210. Anal. ($C_{11}H_7NOCl_2$) C, H, N, Cl.

Methyl 2-Methoxy-2-(triphenylphosphonium)acetate Bromide (11). A mixture of methyl methoxyacetate (88.2 g, 847 mmol), *N*-bromosuccinimide (151 g, 847 mmol), benzoyl peroxide (375 mg), and 420 mL of CCl_4 was refluxed overnight (**WARNING:** the reaction mixture must be warmed slowly and carefully because the reaction is very vigorous). The mixture was filtered and the solvent concentrated to dryness. The residue was distilled under vacuum (40−45 °C/0.1 mbar) to afford 134 g (86.4%) of methyl 2-bromo-2-methoxyacetate, which was dissolved in 1 L of Et_2O, added dropwise to a solution of triphenylphosphine (192 g, 732 mmol) in Et_2O (500 mL), and stirred at room temperature overnight. The precipitate was collected by filtration, washed with Et_2O, and dried in vacuo to give methyl 2-methoxy-2-(triphenylphosphonium)acetate bromide (**11**) (289 g, 88.6%) as a white powder, mp 143−145 °C. IR (Nujol): 2740, 2620, 1720 cm^{-1}. ^1H NMR (DMSO-d_6): δ 7.95 (m, 3H); 7.85−7.75 (m, 12H); 6.90 (d, 1H); 3.62 (s, 3H); 3.59 (s, 3H). MS (ESI POS): 365 (M^+), 277, 262, 103. Anal. ($C_{22}H_{22}BrO_3P$) C, H, Br, P.

Methyl (2*Z*,4*E*)-5-(5,6-Dichloro-2-indolyl)-2-methoxy-2,4-pentadienoate (2). To a solution of (*E*)-3-(5,6-dichloro-2-indolyl)-2-propenaldehyde (**10**) (11.5 g, 47.9 mmol) dissolved in 200 mL of THF were added methyl 2-methoxy-2-(triphenylphosphonium)acetate bromide (**11**) (23.5 g, 52.7 mmol) and DBU (7.84 mL, 1.1 equiv). The mixture was refluxed for 1.5 h. The solvent was removed under reduced pressure, and

the crude compound was purified by flash chromatography (hexane/EtOAc, 7/3). After trituration with $^{i}Pr_2O$, methyl (2*Z*,4*E*)-5-(5,6-dichloro-2-indolyl)-2-methoxy-2,4-pentadienoate (**2**) (14 g, 89.6%) was obtained as a yellow powder, mp 202−204 °C. IR (Nujol): 3400, 1700, 1610 cm^{-1}. ^1H NMR (DMSO-d_6): δ 11.82 (s, 1H); 7.77 (s, 1H); 7.52 (s, 1H); 7.21 (dd, 1H); 7.00 (d, 1H); 6.89 (d, 1H); 6.62 (s, 1H); 3.76 (s, 1H); 3.73 (s, 1H). MS (ESI NEG): 324 (M − H). Anal. ($C_{15}H_{13}NO_3Cl_2$) C, H, N, Cl.

(2*Z*,4*E*)-5-(5,6-Dichloro-2-indolyl)-2-methoxy-2,4-pentadienoic Acid. A suspension of methyl (2*Z*,4*E*)-5-(5,6-dichloro-2-indolyl)-2-methoxy-2,4-pentadienoate (**2**) (7.5 g, 23 mmol) and 20% NaOH (10 mL, 50 mmol) in MeOH (20 mL) and THF (40 mL) was heated at 50 °C for 1 h. After cooling to room temperature, the organic solvent was evaporated. The residue was acidified with 20% HCl, and the precipitate was collected by filtration, washed with water, and dried at 60 °C under vacuum to obtain (2*Z*,4*E*)-5-(5,6-dichloro-2-indolyl)-2-methoxy-2,4-pentadienoic acid (7 g, 97.5%) as a yellow powder, mp 249−250 °C. IR (Nujol): 3390, 1675, 1610 cm^{-1}. ^1H NMR (DMSO-d_6): δ 11.80 (s, 1H); 7.76 (s, 1H); 7.52 (s, 1H); 7.19 (dd, 1H); 6.95 (s, 1H); 6.84 (d, 1H); 6.60 (s, 1H); 3.75 (s, 3H); 3.37 (s, 1H). MS (ESI NEG): 310 (M − H). Anal. ($C_{14}H_{11}NO_3Cl_2$) C, H, N, Cl.

3-[4-(2-Pyrimidinyl)piperazin-1-yl]propylamine. A mixture of 4-(2-pyrimidinyl)piperazine (1.2 g, 7.3 mmol), CH_3CN (15 mL), K_2CO_3 (3 g), and 3-bromo-1-[(*tert*-butoxycarbonyl)-amino]propane (1.9 g, 8 mmol) was refluxed for 4 h. After cooling, the suspension was filtered, washed with CH_3CN (10 mL), and evaporated at reduced pressure. The residue was triturated with $^{i}Pr_2O$ (10 mL) and filtered to give 1.02 g (43%) of 3-[4-(2-pyrimidinyl)piperazin-1-yl]-1-[(*tert*-butoxycarbonyl)-amino]propane as a white powder, mp 99−100 °C. IR (Nujol): 3340, 1695 cm^{-1}. A solution of 3-[4-(2-pyrimidinyl)-piperazin-1-yl]-1-[(*tert*-butoxycarbonyl)amino]propane (4 g, 12.4 mmol) in CH_2Cl_2 (20 mL) was treated dropwise with TFA (20 mL) at 10 °C. The mixture was allowed to reach room temperature and stirred for 1 h. The solvent was removed at reduced pressure, and the residue was dissolved in water (10 mL), treated with solid K_2CO_3, extracted with EtOAc (50 mL), washed with brine (20 mL), and dried over Na_2SO_4. The solvent was evaporated to dryness to give the 3-[4-(2-pyrimidinyl)piperazin-1-yl]propylamine (2.3 g, 83%) as an oil. IR (film): 3370, 1681 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.30 (d, 2H); 6.46 (dd, 1H); 3.81 (m, 4H); 2.86 (t, 2H); 2.70 (s br, 2H); 2.50 (m, 4H); 2.48 (t, 2H); 1.71 (dt, 2H). MS (ESI POS): 222 (MH^+).

General Procedure for the Preparation of Amides 3a−d. A mixture of (2*Z*,4*E*)-5-(5,6-dichloro-2-indolyl)-2-methoxy-2,4-pentadienoic acid (0.4 g, 1.28 mmol), 1-hydroxybenzotriazole (HOBt) (0.173 g, 1.28 mmol), and *N*′-[3-(dimethylamino)propyl]-*N*-ethylcarbodiimide hydrochloride (WSC) (0.245 g, 1.28 mmol) in CH_3CN (3 mL) and THF (1 mL) was heated at 40 °C under nitrogen for 1 h. After the mixture warmed to 60 °C, the appropriate amine (2 mmol) was added, and the mixture was refluxed for 1 h. After cooling, the solvent was removed under reduced pressure, and the residue was treated with 10% aqueous NaOH, extracted with EtOAc, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was triturated with CH_3CN to afford pure compounds **3a−d**.

(2*Z*,4*E*)-5-(5,6-Dichloro-2-indolyl)-*N*-[3-(diethylamino)-propyl]-2-methoxy-2,4-pentadienamide (3a): prepared according to the general procedure using commercially available (Aldrich) 3-(diethylamino)propylamine to give a yellow powder (70%), mp 171−173 °C. IR (Nujol): 3350, 3228, 1640, 1604 cm^{-1}. ^1H NMR (DMSO-d_6): δ 11.70 (s, 1H); 8.27 (t, 1H); 7.74 (s, 1H); 7.51 (s, 1H); 7,16 (dd, 1H); 6.84 (d, 1H); 6.63 (d, 1H); 6.58 (s, 1H); 3.73 (s, 3H); 3.20 (dt, 2H); 2.45 (q, 4H); 2.41 (t, 2H); 1.59 (m, 2H); 0.96 (t, 6H). MS (ESI POS): 424 (MH^+). Anal. ($C_{21}H_{27}Cl_2N_3O_2$) C, H, N, Cl.

(2*Z*,4*E*)-5-(5,6-Dichloro-2-indolyl)-2-methoxy-*N*-[4-(2,2,6,6-tetramethyl)piperidinyl]-2,4-pentadienamide (3b): prepared according to the general procedure by using commercially available (Aldrich) 4-amino-1,1,6,6-tetramethylpiperidine to give a yellow powder (80%), mp 212−214 °C. IR (Nujol): 3358, 3298, 1664, 1620 cm^{-1}. ^1H NMR (DMSO-d_6): δ 11.70 (s br, 1H); 7.82 (d, 1H); 7.73 (s, 1H); 7.51 (s, 1H); 7.18 (dd, 1H); 6.82 (d, 1H); 6.58 (d, 1H); 6.58 (s, 1H); 4.23−4.10 (m, 1H); 3.70 (s, 3H); 1.60 (dd, 2H); 1.18 (dd, 2H); 1.17 (s, 6H); 1.00 (s, 6H). MS (ESI POS): 450 (MH^+). Anal. ($C_{14}H_{11}NO_3Cl_2$) C, H, N, Cl.

(2*Z*,4*E*)-*exo*-5-(5,6-Dichloro-2-indolyl)-2-methoxy-*N*-[8-methyl-8-azabicyclo[3.2.2]oct-3-yl]-2,4-pentadienamide (3c): prepared according to the general procedure by using 3-amino-8-methyl-8-azabicyclo[3.2.1]octane,[31] to give a yellow powder that was converted into the hydrochloride salt (56%), mp >250 °C. IR (Nujol): 3610, 3404, 3296, 3150, 2722, 1658, 1612 cm^{-1}. ^1H NMR (DMSO-d_6): δ 11.80 (s, 1H); 9.88 (s br, 1H); 8.12 (s br, 1H); 7.75 (s, 1H); 7.51 (s, 1H); 7.19 (dd, 1H); 6.86 (d, 1H); 6.61 (d br, 1H); 6.58 (s, 1H); 4.20−4.05 (m, 1H); 3.90−3.80 (m, 2H); 3.71 (s, 3H); 2.70−2.60 (m, 3H); 2.29−2.11 (m, 2H); 2.05−190 (m, 6H). MS (EI): 433 (M^+), 402, 262, 235, 188, 124, 96, 82. Anal. ($C_{14}H_{11}NO_3Cl_2$·HCl) C, H, N, Cl.

(2*Z*,4*E*)-5-(5,6-Dichloro-2-indolyl)-2-methoxy-*N*-[3-[4-(2-pyrimidinyl)piperazin-1-yl]propyl]-2,4-pentadienamide (3d): was prepared according to the general procedure by using the 3-[4-(2-pyrimidinyl)piperazin-1-yl]propylamine to give a yellow powder (75%), mp 227−228 °C. IR (Nujol): 3380, 3212, 1642, 1606 cm^{-1}. ^1H NMR (DMSO-d_6): δ 11.72 (s, 1H); 8.34 (d, 2H); 8.25 (t br, 1H); 7.73 (s, 1H); 7.50 (s, 1H); 7.18 (dd, 1H); 6.84 (d, 1H); 6.66 (d, 1H); 6.60 (dd, 1H); 6.58 (s, 1H); 3.72 (m, 7H); 3.22 (dt, 2H); 2.40 (m, 4H); 2.35 (t, 2H); 1.72−1.62 (m, 2H). MS (ESI POS): 515 (MH^+). Anal. ($C_{25}H_{28}Cl_2N_6O_2$) C, H, N, Cl.

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