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Synthesis of Photoactivable Inhibitors of Osteoclast Vacuolar ATPase

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Abstract—Amides of (2*Z*,4*E*)-5-[(5,6-dichloroindol-2-yl)]-2-methoxy-*N*-[3-[4-[3-(carboxymethoxy)phenyl)] piperazin-1-yl]propyl]-2,4-pentadienamide (**1**) and of 5-(5,6-dichloro-2-indolyl)-2-methoxy-2,4-pentadienoic acid (**2**) are strong inhibitors of the vacuolar ATPase located on the plasma membrane of osteoclasts. In order to understand which V-ATPase subunit is involved in the interaction with these novel inhibitors, analogues containing a photoactivable group and an iodine atom were designed. A series of alcohols or amines containing the photoactivable trifluoroaziridinophenyl or benzophenone moiety and an iodine atom were linked to the above acids via an ester or amide group. These compounds could be thereafter used as a radioactive photoprobe to label the protein. Whereas the compounds containing the photoactivable groups maintained good inhibitory activity, the introduction of the bulky iodine atom was generally detrimental, decreasing potency significantly. Better results were obtained by linking 3-(4-aminopiperidinomethyl)-3′-iodobenzophenone to 3-ethoxy-4-(2-(5,6-dichlorobenzimidazolyl))benzoic acid to give the corresponding amide **27**, that inhibited vacuolar ATP-ase with a $IC_{50} = 140$ nM. The feasibility of introducing a radioactive ^{125}I atom was ascertained by exchanging the iodine with a tributylstannyl group, that was again substituted by iodine.

Introduction

Osteoporosis is a widespread metabolic bone disease, particularly among postmenopausal women, characterised by low bone mass and microarchitectural deterioration of bone with consequent bone fragility and an increased risk of vertebral, wrist and hip fractures. It 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[1] from osteoclasts into the resorption compartment. Protons are extruded by vacuolar-type ATPases[2] (V-ATPases) that are present in large number on the ruffled border of the osteoclasts, while the chloride counter-ions diffuse passively through a chloride channel to dissipate the membrane potential generated in the resorption lacuna by proton transport.[3]

Inhibitors of the osteoclast proton pump may therefore represent useful agents able to prevent the excessive bone resorption associated with osteoporosis.[4] Among them, natural macrolide antibiotics such as bafilomycins and concanamycins are potent but completely unselective inhibitors of V-ATPases,[5] ruling out any possible therapeutic utility of these compounds. It has been reported that chemical modifications of the backbone of bafilomycin A_1 may be able to differentiate potency and selectivity against different vacuolar-type ATPases[6] as assessed by measuring the effects of novel bafilomycin analogues on the V-ATPase-driven proton transport in membrane vesicles from different tissues.

Extensive SAR studies on bafilomycin A_1[7] led to the identification of the key structural requirements for biological activity and allowed the design and synthesis of novel indole derivatives as simpler and more easily accessible inhibitors of the osteoclast V-ATPase.[8]

SB-242784 (Fig. 1), the most representative compound of this class, was able to inhibit bone resorption in vitro in the human osteoclast (hOc) resorption assay[9] (Table

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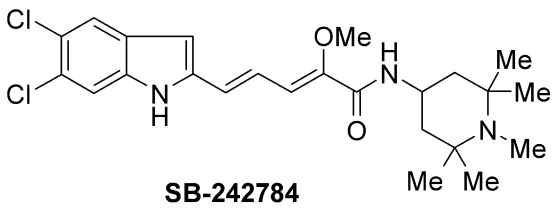


SB-242784

Figure 1. Structure of SB-242784.

Table 1. In vitro activity of SB-242784 (IC_{50} nM)

	V-ATPase assay[a]		Human osteoclast resorption assay	
	COc	hOc	Pits/bone slices	ELISA
SB-242784	26.5±1.5	22±0.9	13.5±2.0	3.4±1.9

[a]cOc, chicken osteoclasts; hOc, human osteoclasts.

1) and to completely prevent the bone loss induced by ovariectomy in rats after 6 months treatment at 10 mg/kg/d po, thus suggesting a possible utility of selective inhibition of the osteoclast V-ATPase as a novel approach for the prevention of bone loss in humans.[10]

V-ATPases are complex, heteromultimeric enzymes, composed of at least 20 different subunits. The osteoclast V-ATPase is characterized by the presence of a specific isoform of the 116 kDa subunit (subunit *a*) which is predominantly expressed in these multinucleated cells.[11] Together with the 16 kDa proteolipid (*c* subunit) and some accessory subunits, subunit *a* makes up the V_0 portion of the V-ATPase, which contains the proton channel. It has been shown that both bafilomycin and SB-242784 do inhibit V-ATPase activity by binding to the V_0 subcomplex.[12] However, whilst bafilomycin and concanamycin were shown, very recently, to bind to the ubiquitous 16 kDa (*c*) subunit,[13,14] it is not known whether SB-242784 and related inhibitors bind the same subunit *c* or do indeed interact with the osteoclast-specific subunit *a*, thus providing a mechanistic rationale for osteoclast selectivity of these inhibitors.

To try to answer this question, we started a chemical approach to obtain novel analogues of SB-242784 and/or analogues bearing photoactivable groups, a suitable radiolabel and, at the same time, maintaining a significant inhibitory potency against osteoclast V-ATPase. The availability of such compounds, would then allow photoaffinity labelling experiments to identify the primary binding proteins to which they bind. In a second level of identification, the tagged protein target can be fragmented into short peptides to localize the binding region within the biopolymer.[15]

This approach entailed the preparation of the compounds with a 'cold' label, assessment of their biological activity and, finally, optimization of the conditions for the synthesis of the 'hot' molecules. Among the possible photoreactive groups that have been mostly used because of their reliable and reproducible high-efficiency labelling of target proteins, such as tetrafluorophenylazide, trifluoromethylphenyldiazirine and benzophenone photophores,[16,17] we selected the two latter groups, which in a first instance were inserted without the cold iodine label. Among the photolabelled inhibitors thus obtained, those retaining the most significant activity at the molecular target were iodinated to verify that the presence of this large substituent was not detrimental for V-ATPase inhibitory activity.

Results

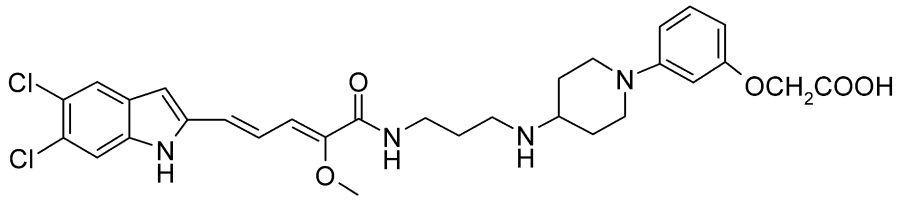
As the active compounds were esters or amides of the acids **1** and **2** (Fig. 2),[8] amines or alcohols containing the selected photophores were prepared, and coupled with the appropriate acid. Acid **1** was obtained from **2** by coupling with the appropriate amine. The esters and the amides were prepared by a standard procedure which requires coupling of the acid with the appropriate alcohol or amine in the presence of WSC and HOBt.

The synthesis of meta-trifluoromethyldiazirinoaniline **8** was performed according to Scheme 1 starting from 3-nitrotrifluoroacetophenone **3**[18,19] following the general procedure of Brunner and Semenza[20] for the synthesis of the trifluoromethyldiazirino group. A synthesis of **8**, differing in some steps from ours, was recently reported by Darrow et al.[21] but without experimental details.

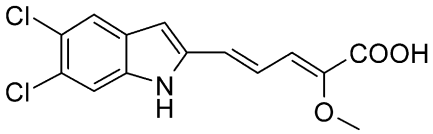
The other ligands used to obtain products reported in Table 2 were prepared according to the literature or following standard methods.

Table 2 reports the value of the inhibition of V-ATPase (IC_{50}, nM) of some derivatives of acid **1**, and of their analogues containing the trifluoroazirino or benzophenone photoactive group, or this group and an iodine atom. The amide **8** from aniline exhibited an acceptable IC_{50} of 340 nM and the introduction of the trifluoroazirinyl group did not change the activity substantially (**9**, IC_{50} = 380 nM). Therefore the effect of the presence of the necessary, but bulky iodine atom was assessed by testing the ester **10**. The potency of this compound, unfortunately, decreased significantly to an IC_{50} of 1200 nM. The possibility that such an effect was due to the replacement of the amide group by an ester, was excluded by comparing the activity of the corresponding amide **11**, which showed an even higher IC_{50}. Again the possible effect of the benzylamino versus the anilino group was excluded by comparing the potencies of the amides with benzylamine (**12**) and with *m*-iodoaniline (**13**). The resulting conclusion was that the introduction of a bulky iodine atom in the photolabelling group was detrimental to the activity. The introduction of the benzophenone photophoric group instead of the trifluoroazirino led to a compound **(14)** with unsufficient potency (IC_{50} = 800 nM) even if iodine was not present. Consequently, the insertion of the halogen on this group was not attempted.

Our attention then shifted toward the amides of the shorter acid **2** with 4-aminopiperidine (Table 3). Amide **15** exhibited an acceptable IC_{50}, but introduction of the photophore and of iodine (**16**) strongly decreased the activity. A similar, but less marked, effect was shown by iodobenzophenone-derived amides (**18**–**19**), where a

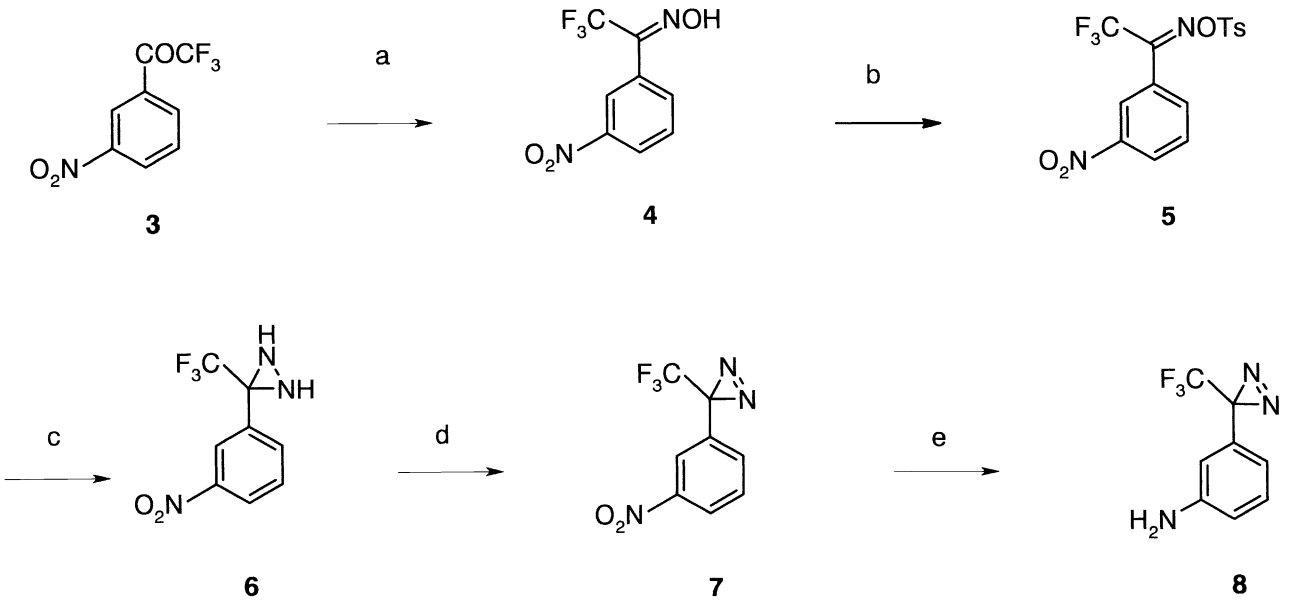


1



2

Figure 2. Structure of acids **1** and **2**.



Scheme 1. (a) $NH_2OH{\cdot}HCl$, Py, EtOH, 80 °C; (b) TsCl, DMAP, Et_3N, CH_2Cl_2, rt; (c) liq NH_3, Et_2O/CH_2Cl_2; (d) I_2, Et_3N, EtOH, rt; (e) $Na_2S_2O_4$, Bu_4NBr, NaOH, THF.

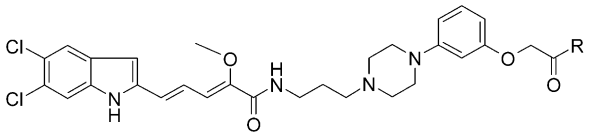
7–10-fold increase of the IC_{50} with respect to **17** was observed, depending on the position of the iodine atom.

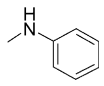
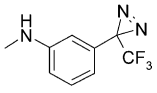
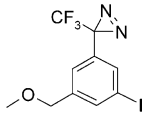
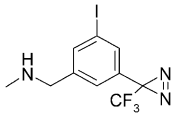
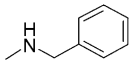
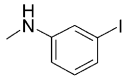
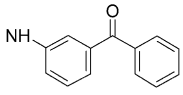
Compounds possessing the dienic chain, such as SB-242784, undergo photo-induced isomerisation of the double bonds when they are in solution. This liability led us to evaluate the possibility to replace the dienic chain with a phenyl ring. Potency was increased when a methoxy group was inserted in the position close to the indole ring. The replacement of the indole ring by benzimidazole improved water solubility while retaining the same inhibitory potency. Finally substitution of the methoxy by an ethoxy group gave the most potent inhibitor (**25**, Scheme 2), with an IC_{50} in hOc ATPase assay of 8 nM.[22] On the basis of these data it was decided to produce photoaffinity derivatives also in this new class of ATPase inhibitors. Amide **26** maintained a reasonable potency and therefore an iodinated benzoylbenzylpiperidine (**27**) was synthesised. In this case, the activity (140 nM) was high enough, and compound **27** was chosen for the photoaffinity test (Table 4).

In order to allow the introduction of radioactive ^{125}I, compound **27** had to be stannylated, and the tributylstannyl group exchanged again with iodine. The reaction of **27** with hexabutylditin to give **28**, followed by iodination with chloramine-T and sodium iodide[23] proceeded satisfactorily (Scheme 3), so that this procedure can be used for the preparation of the radioactive derivative.

In conclusion, we have obtained a iodinated strong inhibitor of vacuolar ATPase containing a photoactivable benzophenone group, that, once labelled with ^{125}I, can be used as a photoprobe for the photoaffinity labelling of this enzyme.

Table 2. In vitro activity (V-ATPase inhibition in human osteoclasts) of derivatives of compound **1**



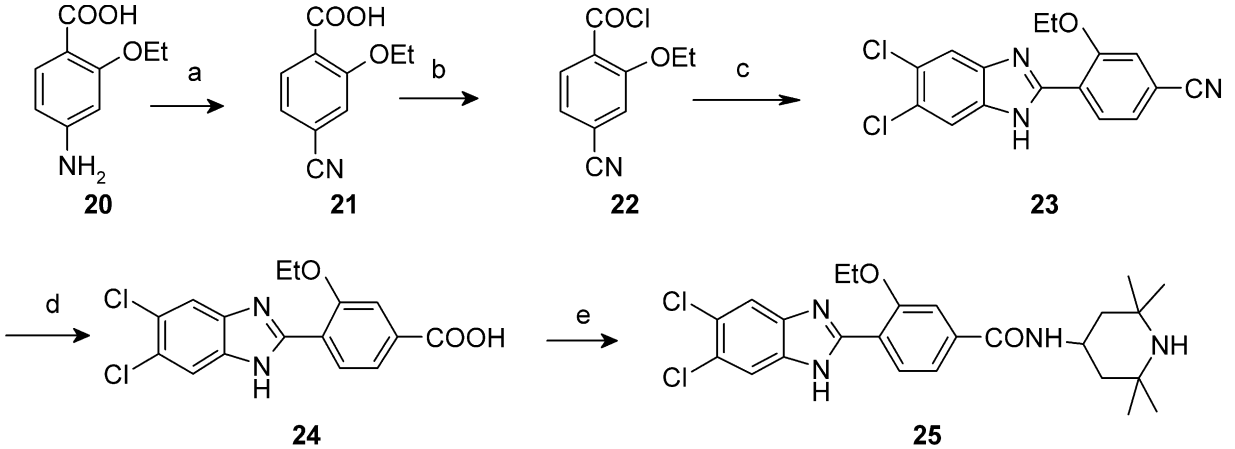
Compd	R	Mp (°C)	Yield (%)	IC_{50} (nM)
1	–OH	240–242	Ref 8	84
	–OEt	184–185	Ref 8	45
8		100–105	27	340
9		90–92	28	380
10		113–116	30	1200
11		80–85	48	2400
12		130–133	61	120
13		216–220	34	490
14			63	800

Experimental

All reagents and solvents were reagent grade or were purified by standard methods before use. Melting points were determined in open capillaries on a Büchi melting point apparatus and are uncorrected. Column chromatography was carried out on flash silica gel (Merck 230–400 mesh). TLC analysis was conducted on silica gel plates (Merck $60F_{254}$). NMR spectra were recorded at 300 MHz with a Bruker instrument, in $CDCl_3$, if not otherwise stated. Chemical shifts (δ values) and coupling constants (*J* values) are given in ppm and Hz, respectively. Mass spectra were recorded at an ionizing voltage of 70 eV on a Finnigan TQ70 spectrometer. The relative intensities of mass spectrum peaks are listed in parentheses. Solvents were routinely distilled prior to use; anhydrous tetrahydrofuran (THF) and ether (Et_2O) were obtained by distillation from sodium-benzophenone ketyl; dry methylene chloride was obtained by distillation from phosphorus pentoxide. All reactions requiring anhydrous conditions were performed under a positive nitrogen flow, and all glassware was oven-dried and/or flame dried.

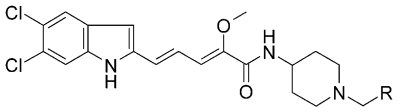
(2*Z*,4*E*)-5-[(5,6-Dichloroindol-2-yl)]-2-methoxy-*N*-[3-[4-[3-(carboxymethoxy)phenyl)] piperazin-1-yl]propyl]-2,4-pentadienamide hydrochloride (1). (a) 4-(3-Hydroxyphenyl)piperazine (9 g, 50.5 mmol) was dissolved in CH_3CN and Et_3N (18 mL, 130 mmol) and 3-bromo-*N*-*t*-butoxycarbonylpropylamine (18 g, 75.6 mmol) were added. The solution was refluxed for 5 h. The organic solvent was evaporated to dryness and the residue was re-dissolved in EtOAc, washed with water, brine and dried over Na_2SO_4. After solvent evaporation an oil was obtained (17 g) that was used in the next step without further purification.

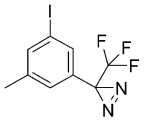
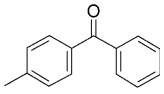
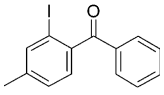
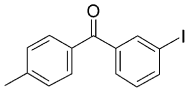
The crude compound was dissolved in DMF (170 mL) and solid K_2CO_3 (51 g) was added. The suspension was stirred at room temperature and ethyl bromoacetate (6.21 mL, 56 mmol) was added dropwise. Stirring was

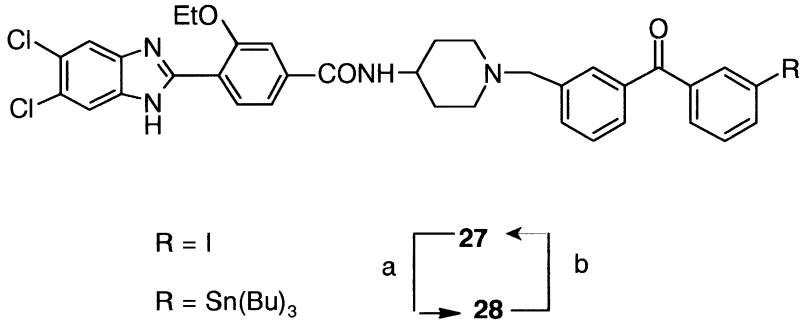


Scheme 2. (a) $NaNO_2$, HCl, CuCN–NaCN; (b) $SOCl_2$; (c) *o*-$NH_2C_6H_2Cl_2NH_2$, Et_3N, then P_2O_5; (d) NaOH; (e) $SOCl_2$, then 4-aminotetramethylpiperidine.

Table 3. In vitro activity (V-ATPase assay in human osteoclasts) of derivatives of compound **2**



Compd	R	Mp (°C)	Yield (%)	IC_{50} (nM)
15		255–258	71	93
16		235–240	28	> > 1000
17		241–249	10	100
18		218–220	37	1000
19		184–188	60	710

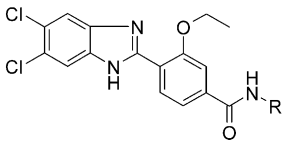


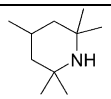
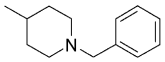
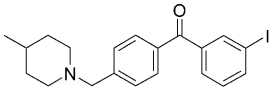
Scheme 3. (a) $(Sn(Bu)_3)_2$, $Pd(PPh_3)_4$, Et_3N; (b) chloramine-T, NaI.

continued for 24 h. Et_2O (200 mL) was added and the suspension was filtered and evaporated to dryness. The residue was re-dissolved in EtOAc and washed with water, brine and dried over Na_2SO_4. The residue was triturated with hexane and filtered obtaining 12.4 g of 3-[4-[3-(ethoxycarbonylmethoxy)phenyl)]piperazin-1-yl]-*N*-*t*-butoxycarbonylpropylamine, mp 93–97 °C. The protective group was removed dissolving the compound in CH_2Cl_2 (50 mL) and adding TFA (25 mL). After stirring 3 h at room temperature, the solvent was evaporated to dryness obtaining 14 g of 3-[4-[3-(ethoxycarbonylmethoxy)phenyl)]piperazin-1-yl]propylamine as a trifluoroacetate salt, NMR $\delta = 7.15$ (dd, $J = 7.4$, 7.4 Hz, 1H, 5ArH); 6.90 (s br, 3H, NH_3^+); 6.57 (dd, $J = 7.4$, 1.2 Hz, 1H, 4(6) ArH); 6.49 (dd, $J = 1.2$, 1.2 Hz, 1H, 2ArH); 6.38 (dd, $J = 7.4$, 1.2 Hz, 1H, 6(4) ArH); 4.59 (s, 2H, OCH_2CO); 4.27 (q, $J = 7.2$ Hz, 2H, $COCH_2$); 3.21–3.10 (m, 6H, $ArN(CH_2)_2 + CH_2NH_3^+$); 2.80–2.68 (m, 6H, $N(CH_2)_3$); 1.92 (m, 2H, NCH_2–CH_2); 1.30 (t, $J = 7.2$, 3H, CH_3).

(b) Acid **2**[8] (3.6 g, 11.5 mmol), dissolved in a mixture of acetonitrile and THF (3:1), was coupled with 3-[4-[3-(ethoxycarbonylmethoxy)phenyl)]piperazin-1-yl]propylamine using HOBT (1.55 g) and WSC (2.21 g). The reaction mixture was refluxed for 1 h. Solvent was evaporated to dryness and the residue was dissolved in EtOAc and washed with water, $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4, filtered and concentrated to give a solid that was triturated with Et_2O. The solid (5.1 g) was filtered and then re-dissolved in THF. 20% NaOH was added (12 mL) and the reac-

Table 4. In vitro activity (V-ATPase assay on human osteoclasts) of 5,6-dichlorobenzimidazole derivatives



Compd	R	IC_{50} (nM)
25		8
26		90
27		140

tion was stirred at 50 °C for 1 h. After evaporating to dryness, 10% HCl was added and the solid obtained was filtered, washing twice with water. Crude compound was triturated with acetonitrile to give 3.7 g of **1**, mp 240–242 °C, NMR (DMSO-d_6 and TFA) δ 11.81 (s br, 1H); 10.36 (s br, 1H); 8.34 (t br, $J=6.0$ Hz, 1H); 7.74 (s, 1H); 7.53 (s, 1H); 7.20 (dd, $J=15.5$,11.6 Hz, 1H); 7.15 (dd, $J=8.5$, 8.5 Hz, 1H); 6.86 (d, $J=15.5$ Hz, 1H); 6.69 (d, $J=11.6$ Hz, 1H); 6.60 (dd, $J=8.2$, 1.9 Hz, 1H); 6.58 (d, $J=1.6$ Hz, 1H); 6.54 (dd, $J=2.2$, 1.9 Hz, 1H); 6.41 (dd, $J=8.2$, 2.2 Hz, 1H); 4.64 (s, 2H); 3.88–3.88 (m, 2H); 3.76 (s, 3H); 3.55 (m, 2H); 3.27 (m, 2H); 3.20–3.04 (m, 6H); 2.00–1.89 (m, 2H), *m/z* 587; 555; 351; 319; 262; 236. Anal. calcd for $C_{30}H_{34}Cl_2N_4O_5$: C, 59.90; H, 5.70, N, 9.31. Found: C, 59.78; H, 5.82; N, 9.21.

3-Nitrotrifluoroacetophenone oxime (4). Compound **3** (1.44 g, 6.57 mol) was dissolved in 8 mL of dry ethanol, added with 8 mL of pyridine and 1.37 g (19.7 mmol) of hydroxylamine·HCl, and refluxed 2.5 h. Evaporation, taking up with ether, washing with acid water (pH 5), drying, evaporation and chromatography (hexane–AcOEt 4:1) gave 2.58 g of **4**,[17] mp 48–51 °C.

3-Nitro-(1-tosyloxyimino)trifluoroethylbenzene (5). Oxime **4** (2.46 g, 10.5 mmol) in 25 mL of CH_2Cl_2 was cooled at 0 °C, added with Et_3N (2.2 mL, 15.8 mmol), DMAP (0.64 g, 5.25 mmol) and tosyl chloride (2.2 g, 11.6 mmol), stirred 24 h at room temperature, evaporated, taken up with AcOEt, washed with water and brine, evaporated and the crude product crystallised from CH_2Cl_2/hexane to give 3.06 g (75%) of **5** as a white solid, mp 95–98 °C, NMR δ 2.50 (s, CH_3), 7.40 (m, 2H, H-3′ + H-5′), 7.65–7.78 (m, H-5 and H-6), 7.9 (m, H-2′ and H-6′), 8.22 (m, H-2), 8.40 (m, H-4). Anal. calcd for $C_{15}H_{11}F_3N_2O_5S$: C, 46.39; H, 2.86, N, 7.21. Found: C, 46.54; H, 2.81; N, 7.28.

3-(3-Nitrophenyl)-3-trifluoromethyldiaziridine (6). To 3 mL of liquid ammonia at −78 °C was added a suspension of 2.62 g (6.75 mmol) of **5** in 30 mL of CH_2Cl_2, the mixture stirred in a closed vessel 2 days at room temperature, then cooled at −78 °C, gaseous ammonia bubbled for 5 min, left 2 days at room temperature. Evaporation of ammonia, filtration and evaporation of the solvent gave a red-orange oil (1.67 g), NMR δ 2.4 (NH), 2.95 (NH), 7.63 (t, $J=7$, H-5), 7.97 (dd, $J=7$,1.5, H-4), 8.30 (dd, $J=7$,1.5, H-6), 8.48 (s, H-2), that was used without further purification.

3-(3-Nitrophenyl)-3-trifluoromethyldiazirine (7). Diaziridine **6** (0.8 g, 3.43 mmol) in 10 mL of MeOH was treated with 0.96 mL (6.86 mmol) of Et_3N and with 0.87 g (3.43 mmol) of iodine, the mixture stirred in the dark for 40 min, then ether (20 mL) was added and the mixture washed with satd aq citric acid, satd aq sodium thiosulfate, and water, dried and evaporated to give 0.64 g of crude **7**. Chromatography with hexane/CH_2Cl_2 4:1 gave 0.59 g (74%) of **7**, NMR δ 7.5–7.7 (m, 2H Ar), 8.05 (s, 1H Ar), 8.3 (d, $J=7$, 1H Ar). Anal. calcd for $C_9H_8F_3N_3O_2$: C, 43.73; H, 3.26, N, 17.00. Found: C, 43.87; H, 3.29; N, 17.18.

3-(3-Aminophenyl)-3-trifluoromethyldiazirine (8). Diazirine **7** (101 mg, 0.44 mmol) in 6 mL of THF was added with 3 mL of aq NaOH at pH 10, then with 229 mg (1.32 mmol) of sodium dithionite and 14 mg (0.04 mmol) of tetrabutylammonium bromide, and heated in an oil bath. After 15 min, when pH was 6 and temperature 40 °C, the mixture was added with further 229 mg of dithionite, refluxed 6.5 h, keeping the solution basic by addition of 6 M NaOH every 30 min, and adding dithionite (115 mg) every h. The mixture was cooled, treated with $NaHCO_3$ and extracted with ether. Drying, evaporation and chromatography with hexane/CH_2Cl_2 1:1 gave 41.6 mg (47%) of **8**, mp 68–70 °C, NMR δ 6.45 (s, H-2), 6.52 (d, $J=7$, H-4), 6.69 (d, $J=7$, H-6), 7.17 (t, $J=7$, H-5), *m/z* 201(77), 173(76), 83(28), 49(100). Anal. calcd for $C_9H_{10}F_3N_3$: C, 49.77; H, 4.64, N, 19.35. Found: C, 49.88; H, 4.73; N, 19.18..

General method for the synthesis of esters or amides of (1) and (2)

The acid (0.5 mmol) was suspended in 12 mL of CH_3CN–THF 2:1 in the dark and under nitrogen. WSC (0.75 mmol) and HOBt (0.75 mmol) were added and the mixture heated at 40 °C. After 1–2 h, if the acid was still detectable by TLC, WSC (0.35 mmol) and HOBt (0.35 mmol) were added. After 2 h, the appropriate alcohol or amine (0.55 mmol) was added and the mixture refluxed 2–3 h. Then water and AcOEt were added, and the mixture stirred overnight at room temperature. Addition of 10% NaOH, extraction with AcOEt, and chromatography afforded the products.

2-Ethoxy-4-aminobenzoic acid (20). A suspension of methyl 2-ethoxy-4-acetamidobenzoate (50 g, 211 mmol) in aqueous solution of NaOH (15% w/w, 200 mL) was gently refluxed for 16 h. The resulting pale brown solution was allowed to cool to room temperature and then further cooled in an ice water bath. Concentrated HCl (37% w/w) was added until the solution reached pH 6. The solid precipitated from the solution was filtered under vacuum, dried at 50 °C obtaining 38.3 g (yield

100%) of **20**, mp 152–154 °C, NMR (DMSO-d_6) δ 1.33 (t, J = 6.9 Hz, Me), 4.01 (q, J = 6.9 Hz, CH_2), 5.79 (br s, 2H exch. with D_2O), 6.12 (dd, J = 8.5, 2.2 Hz, ArH), 6.20 (d, J = 2.2 Hz, ArH), 7.48 (d, J = 8.5 Hz, ArH), 11.98 (br s, 1H exch. with D_2O).). Anal. calcd for $C_9H_{11}NO_3$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.48; H, 6.05; N, 7.65.

2-Ethoxy-4-cyanobenzoic acid (21). CuCN (12 g, 134 mmol) was suspended in 100 mL of distilled water. NaCN (18.3 g, 373 mmol) was added with vigorous stirring and the internal temperature was kept below 40 °C until all the CuCN went into solution. A suspension of 2-ethoxy-4-aminobenzoic acid (20 g, 110 mmol), in water (200 mL) and concentrated HCl (33 mL) was stirred and cooled in an ice bath. When the temperature reached 5 °C, a solution of $NaNO_2$ (9.7 g, 140 mmol) in water (30 mL) was added dropwise at such a rate as to maintain the temperature below 5 °C. When all the $NaNO_2$ was added, the solution was slowly introduced through an ice-cooled dropping funnel into the reactor containing the NaCN/CuCN solution. A reaction took place with the vigorous formation of N_2. A few drops of 1-octanol were added to keep the foaming under control. Stirring was continued for 4 h. The resulting suspension was then extracted with ethyl acetate (3×100 mL) and the organic phase dried over $MgSO_4$ and evaporated under vacuum obtaining 15 g of the title compound (yield 71.1%) as a light brown powder, mp 170–172 °C. NMR (DMSO-d_6) δ 1.60 (t, J = 6.9 Hz, Me), 4.37 (q, J = 6.9 Hz, CH_2), 7.30 (d, J = 0.9 Hz, ArH), 7.41 (dd, J = 8.2, 0.9 Hz, ArH), 8.27 (d, J = 8.2 Hz, ArH), 10.42 (s br, 1H exch. with D_2O).). Anal. calcd for $C_{10}H_9NO_3$: C, 62.82; H, 4.74, N, 7.33. Found: C, 62.93; H, 4.82; N, 7.22.

2-Ethoxy-4-cyanobenzoyl chloride (22). 2-Ethoxy-4-cyanobenzoic acid (10 g, 52.3 mmol) and thionyl chloride (50 mL) were refluxed in CH_2Cl_2 (80 mL) for 5 h. Solvent was removed under vacuum to leave 10.9 g of an off white solid (52 mmol, yield 99%) that was used without further purification.

5,6-Dichloro-2-(4-cyano-2-ethoxyphenyl)benzimidazole hydrochloride (23). A solution of 2-ethoxy-4-cyanobenzoyl chloride (10.9 g, 52 mmol) in dichloromethane (109 mL) was added dropwise in 5 h to a solution of 4,5-dichlorophenylenediamine (18.5 g, 104.5 mmol) and triethylamine (53 g = 72.6 mL, 523 mmol) in dichloromethane (550 mL). Stirring was continued for additional 2 h. The solvent was removed under vacuum and the residue was triturated with water (100 mL), filtered and dried at 50 °C under vacuum. The solid was suspended in diethyl ether (200 mL), stirred for 1 h, filtered and dried under vacuum to give 19 g of *N*-(2-amino-4,5-dichlorophenyl)-2-ethoxy-4-cyanobenzamide (yield 51%), mp 195–198 °C. A suspension of *N*-(2-amino-4,5-dichlorophenyl)-2-ethoxy-4-cyanobenzamide (19 g, 54.2 mmol) and P_2O_5 (19 g, 134 mmol) in xylene (380 mL) was refluxed for 24 h. Additional P_2O_5 (9.5 g, 67 mmol) was added and the mixture was refluxed for 48 h. Solvent was removed under reduced pressure. The residue was treated with 30% NaOH (80 mL) and water (100 mL) and then acidified with 37% HCl. The solid was filtered, washed with water and dried under vacuum at 50 °C to give 16.5 g of the title compound as a light brown powder (yield 82.6%), mp > 250 °C, NMR (DMSO-d_6) δ 1.45 (t, J = 6.9 Hz, Me), 4.43 (q, J = 6.9 Hz, CH_2), 7.55 (dd, J = 8.2, 0.3 Hz, ArH), 7.79 (d, J = 0.3 Hz, ArH), 7.91 (s, ArH benzimidazole), 8.36 (d, J = 8.2 Hz, ArH), 11.62 (s, 1H). Anal. calcd for $C_{16}H_{11}Cl_2N_3O$: C, 57.85; H, 3.34, N, 12.65. Found: C, 57.87; H, 3.26; N, 12.58.

5,6-Dichloro-2-(4-carboxy-2-ethoxyphenyl)benzimidazole hydrochloride (24). A mixture of 5,6-dichloro-2-(4-cyano-2-ethoxyphenyl)benzimidazole hydrochloride (16.3 g, 44.2 mmol) and 32% NaOH (40 mL) in ethanol (100 mL) was refluxed for 8 h. After cooling to room temperature the organic solvent was removed under vacuum and the aqueous phase was acidified with 37% HCl, and stirred for 1 h. The solid was filtered, washed with water (100 mL) and dried at 50 °C under vacuum to give 12 g as a light brown powder (yield 70%), mp > 250 °C, NMR (DMSO-d_6) δ 8.70 (s br, 3H, exch. with D_2O); 1.45 (t, J = 6.9 Hz, Me), 4.44 (q, J = 6.9 Hz, CH_2), 7.69 (dd, J = 8.2, 0.2 Hz, ArH), 7.71 (d, J = 0.2 Hz, ArH), 8.02 (s, ArH benzimidazole), 8.29 (d, J = 8.2 Hz, ArH). Anal. calcd for $C_{16}H_{13}Cl_3N_2O_3$: C, 49.57; H, 3.38; N, 7.23. Found: C, 49.77; H, 3.20; N, 7.38.

4-(5,6-Dichlorobenzimidazol-2-yl)-*N*-(2,2,6,6-tetramethylpiperidin-4-yl)-3-ethoxybenzamide (25). A mixture of 5,6-dichloro-2-(4-carboxy-2-ethoxyphenyl)benzimidazole hydrochloride (1 g, 2.58 mmol) and of thionyl chloride (8 mL) in dichloromethane (20 mL) was refluxed for 3 h. After cooling to room temperature the solvent was removed under vacuum and the crude residue was used in the following reaction without further purification. The acid chloride was added portionwise to a solution of 4-amino-2,2,6,6-tetramethylpiperidine (0.612 g, 3.87 mmol), triethylamine (5 mL, 36 mmol) in dichloromethane (50 mL). Stirring was continued at room temperature for 2 h. The solvent was removed under vacuum and the residue was suspended in water (50 mL) and filtered. The solid was dried at 50 °C under vacuum and then crystallised with ethanol to give 0.6 g of the title compound as a light brown powder (yield 47.5%), mp > 280 °C, ^{1}H NMR (DMSO-d_6) δ 1.05 (s, 6H), 1.20 (dd, 2H), 1.05 (s, 6H), 1.20 (s, 6H), 1.50 (t, 3H), 1.73 (d, 2H), 4.35–4.22 (m, 1H), 4.40 (q, 2H), 7.59 (d, 1H), 7.64 (s, 1H), 7.90 (s, 2H), 8.30 (m, 2H), 12.20 (s br, 1H), m/z 488 (MH^+); 473; 124. Anal. calcd for $C_{25}H_{30}Cl_2N_4O_2$: C, 61.35; H, 6.18; N, 11.45. Found: C, 61.22; H, 6.27; N, 11.58.

***N*-4-Phenylmethylpiperidinamide of 3-ethoxy-4-(5,6-dichlorobenzimidazol-2-yl)benzoic acid (26).** Prepared following the general procedure described above, for example, for **25**. Obtained in 63% yield after trituration with abs. EtOH. Mp 295 °C, NMR δ 1.48 (t, CH_3), 1.54–1.69 (CH_2), 1.80 (CH_2), 2.03 (CH_2), 2.84 (CH_2), 3.48 (s, ArylCH_2N), 3.87–3.82 (m, C*H*NH), 4.28 (q, 2H, OCH_2), 6.12 (d, NHCO), 7.2–8.0 (11H arom), 8.12 (s, 1H), 8.59 (d, 1H, J = 8), 11.63 (1H, NH). m/z 522 (MH^+). Anal.

calcd for $C_{28}H_{28}Cl_2N_4O_2$: C, 64.25; H; 5.39; N, 10.70. Found: C, 64.42; H, 5.48; N, 10.58.

***N*-4-(3-Iodobenzoyl)-phenylmethylpiperidinamide of 3-ethoxy-4-(5,6-dichlorobenzimidazol-2-yl)benzoic acid (27).** Prepared following the general procedure described above, for example, for **25**. Obtained in 25% yield after chromatography with CH_2Cl_2–MeOH–NH_4OH 80:15:1, mp 137–139 °C, NMR δ 1.65 (t, CH_3), 1.75–1.85 (CH_2), 2.1–2.3 (CH_2), 2.2–2.35 (CH_2), 2.9–3.1 (CH_2), 3.64 (s, ArylCH_2N), 4.0–4.15 (m, C*H*NH), 4.46 (q, 2H, OCH_2), 6.12 (d, NHCO), 7.2–8.0 (11H arom), 8.12 (s, 1H), 8.59 (d, 1H, $J = 8$ Hz), 10.7 (1H, NH) *m*/*z* 754(55), 753(29), 752(62), 751(15), 403(50), 82(100). Anal. calcd for $C_{35}H_{31}Cl_2IN_4O_3$: C, 55.79; H, 4.15; N, 7.44. Found: C, 55.92; H, 4.27; N, 7.53.

***N*-4-(3-Tributylstannylbenzoyl)-phenylmethylpiperidinamide of 3-ethoxy-4-(5,6-dichlorobenzimidazol-2-yl)benzoic acid (28).** Compound **27** (20 mg, 0.026 mmol) was dissolved in 0.3 mL of THF, triethylamine (0.5 mL, 0.052 mmol), hexabutylditin (0.02 mL, 0.04 mmol) and tetrakis(triphenylphosphine)Pd (6 mg, 0.005 mmol) were added, and the mixture refluxed 12 h. Evaporation and chromatography with CH_2Cl_2/MeOH 100:2 gave 10 mg of **28**, NMR δ 0.7–0.9 (9H, 3 CH_3), 0.9–1.65 (20H), 1.95–2.05 (CH_2), 2.1–2.25 (CH_2), 2.75–2.90 (CH_2), 3.53 (s, ArylCH_2N), 3.90–4.05 (m, 1H, C*H*NH), 4.33 (q, 2H, OCH_2), 6.06 (d, NHCO), 7.15–7.80 (12H arom), 8.51 (d, 1H, $J = 8$ Hz), 10.65 (s, 1H, NH). Anal. calcd for $C_{47}H_{58}Cl_2N_4O_3Sn$: C, 61.59; H, 6.38; N, 6.11. Found: C, 61.82; H, 6.17; N, 6.44.

Synthesis of 27 from 28. To a solution of **28** (10 mg, 0.011 mmol) in 10 mL of EtOH were added 1.1 mg of NaI in 1.1 mL of 0.01 M NaOH, then HCl 0.05 N to pH 4–5, then chloramine-T (6.2 mg, 0.022 mmol) in 6.2 mL of H_2O. After 15 min, sodium metabisulfite (125 mg in 12.5 mL of H_2O) was added, then, after 10 min, 30 mL of satd aq $NaHCO_3$, and the solution extracted with CH_2Cl_2. Drying, evaporation and taking up with ether gave 8 mg (75%) of **27**. Anal. calcd for $C_{35}H_{31}Cl_2IN_4O_3$: C, 55.79; H, 4.15; N, 7.44. Found: C, 55.97; H, 4.09; N, 7.33.

ATPase inhibition activity. ATPase inhibition data were obtained using chicken osteoclast membrane preparation or human osteoclastic tumor cell membrane preparation following the procedure described in refs 7 or 24, respectively. All the data are the average of two independent measurements.

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