Articles

Synthesis of NO-Donor Bisphosphonates and Their in-Vitro Action on Bone Resorption

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A new class of bisphosphonates containing nitrooxy NO-donor functions has been developed. The products proved to display affinity for hydroxyapatite. Injection of ^{99m}Tc-labeled derivatives **11** and **18** into male rats showed a preferential accumulation of the compounds in bone as compared to blood and muscles. The products were found to inhibit the differentiation of pre-osteoclasts to functional osteoclasts induced by receptor activator of NF-κB ligand (RANKL), through a prevalent NO-dependent mechanism.

Introduction

Nitric Oxide (NO•) is a key signaling molecule involved in the regulation of many physiological processes such as vascular relaxation, neurotransmission, platelet aggregation, and events of the immune system.[1,2] It is produced from L-arginine under the action of a family of enzymes called NO synthase (NOS).[2,3] At present, three different isoforms of this enzyme are known, of which two, the endothelial NOS (eNOS) and the neuronal NOS (nNOS), are constitutively expressed, while a third is an inducible isoform (iNOS).[3] In the organism, NO• exerts a wide-ranging number of actions in the cardiovascular, central, and peripheral nervous systems. These events are triggered through the intermediate activation of the soluble guanylate cyclase (sGC).[4] Nitric oxide is also one of the final effectors in the immune system where it is produced in particular by the macrophages.[3] It diffuses into the parasite cell and destroys it both by producing reactive compounds and by inhibiting enzymes essential for the survival of the parasite. In past years, a large body of literature has emerged indicating that nitric oxide has important effects on bone cell functions.[5] The skeleton undergoes continuous remodeling, and the two main cells involved in this process are the osteoclasts and osteoblasts. While the former are responsible for degrading old bone, osteoblasts are replacing it again. In general, bone resorption is determined by recruitment and subsequent activation of osteoclasts from cells of the hemopoietic compartment, specifically from the granulocyte-macrophage colony-forming unit (GM-CFU). Both events are largely controlled by cells of the osteoblastic lineage.[6] Recently, it was demonstrated that eNOS is expressed both in osteoclasts and in osteoblasts.[5] It is not clear, however, whether osteoclasts express iNOS as it is the case in osteoblasts which synthesize the enzyme in response to inflammatory processes. The nitric oxide effect on osteoclasts is biphasic.[5] At low concentration, NO• induces bone resorption, while at high doses osteoclast formation and activity become inhibited. The same was found to be the case on osteoblasts, where small NO• amounts support osteoblast proliferation, while high concentrations inhibit it.

The implication of nitric oxide as signaling molecule in numerous biological systems has prompted investigators to engineer compounds with NO-releasing capability. Thus, NO-donors have become an important class of substances which have been proven to be rather useful for the treatment of pathological conditions that are associated with nitric oxide deficency.[7] Classically these compounds are used to manage various cardiovascular diseases.[8] Glyceryl trinitrate (GTN) may be considered as prototype of these products. Currently, many new therapeutic applications for NO-donors are emerging. These include treatment of nervous, sexual, respiratory, and gastrointestinal disorders, enhanced immunological response, and regulation of tumor growth.[3,9] Furthermore, a number of NO-donors were found to affect osteoclastic and osteoblastic activity,[10−14] bone healing,[15] and to reduce ovariectomy-induced bone loss.[16] The targeting of NO-donors is one of the major problems because NO exerts an array of pharmacological actions and therefore may also induce cellular toxicity if produced in high amounts.[17] A strategy could be to link suitable NO-donor moieties to carriers which display tropism for specific tissues. Bisphosphonates (BPs) (**1**, Chart 1) are interesting compounds from this point of view. These products are considered analogues

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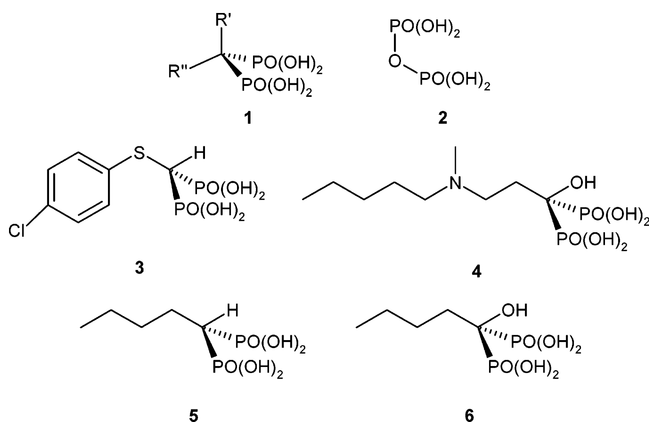
Chart 1



of pyrophosphate (**2**, Chart 1). They contain the P−C−P substructure, which is resistant to chemical and enzymatic hydrolysis, while P−O−P is a susceptible hydrolysis moiety.[18] As a result of their ability to chelate calcium ions by bidentate or tridentate binding, bisphosphonates display a high affinity to the bone mineral surface[19] where they accumulate because they cannot be degraded metabolically. Therefore, BPs are believed to remain in the skeleton for life. Recently, methods for synthesizing biodegradable bisphosphonates have been published.[20]

A number of BPs, among them *Tiludronate* and *Ibandronate* (**3**, **4**, Chart 1), have been developed as potent drugs to inhibit bone resorption caused by a variety of disorders of mineral metabolism, such as Paget's disease, hypercalcemia of malignancy, and postmenopausal or glucocorticosteroid induced osteoporosis. Bisphosphonates, apart from estrogens, have proven to be very effective to inhibit age-related osteoporosis. As mentioned above, nitric oxide has been shown to inhibit osteoclast-mediated resorption. However, to efficiently make use of this, compounds that release nitric oxide have to be targeted. Recently, bisphosphonates have been employed as tools for targeting a number of drugs to bone.[21] This sort of approach has prompted us to design a series of NO-donor bisphosphonates (NO−BPs) in which the bisphosphonic substructure is linked to nitrooxy NO-donor moieties. In the present Article, we report on the synthesis and the structural characterization of these products (der.s **10**, **11**, **17**, **18**, Scheme 1) and of the analogues **5**, **6** (Chart 1) deprived of the nitrooxy function(s). Furthermore, experiments were designed to determine their biological activity to inhibit the differentiation of pre-osteoclasts to functional osteoclasts. The tropism of compounds **11**, **18** for bone mineral is also described.

Results and Discussion

Chemistry. The synthetic pathways followed to obtain the final BPs are reported in Scheme 1. The preparation of the final 1-hydroxybisphosphonate derivatives **10**, **11** required the preliminary synthesis of the carboxylic acids **8**, **9** bearing respectively one and two nitrooxy groups. The former product was obtained by the action of $AgNO_3$ on the parent bromide according to a procedure reported in the literature.[22] The latter was obtained by the action of $AgNO_3$ and I_2 on 5-hexenoic acid (**7**) in acetonitrile solution. The reaction of these two acids with thionyl chloride generated the intermediate acyl chlorides **A**, which, without further purification, were treated with trimethyl phosphite in dry THF to yield the corresponding acylphosphonates **B** (Michaelis−Arbuzov reaction). The products were immediately transformed into the expected 1-hydroxybisphosphonates methyl esters **C** by action of dimethyl phosphite in dry THF in the presence of diethylamine. These intermediates were purified by flash chromatography and due to their instability were immediately used for the next synthetic step. Thereafter, they were hydrolyzed through the action of trimethylsilyl bromide to produce the final BPs. The nitrooxy BPs **17**, **18** were obtained by hydrolysis of the corresponding esters **13**, **16**. Ester **13** was obtained by nitration with a mixture of HNO_3/H_2SO_4 in methylene chloride of the parent alcohol **12** according to a procedure reported in the literature.[23] Ester **16** was prepared by the action of $AgNO_3$ and I_2 on the unsaturated ester **15**, the product derived from the action of the appropriate Grignard reagent on the tetraethyl vinylidenebisphosphonate (**14**).[24] All final bisphosphonic acids (**10**, **11**, **17**, **18**) and the two bisphosphonate ethyl esters (**13**, **16**) were characterized by ^{1}H, ^{13}C, ^{31}P NMR spectra (see Experimental Section). Spectral assignments were given on the basis of chemical shifts, signal multiplicity, coupling constant values, and literature data.[24,25]

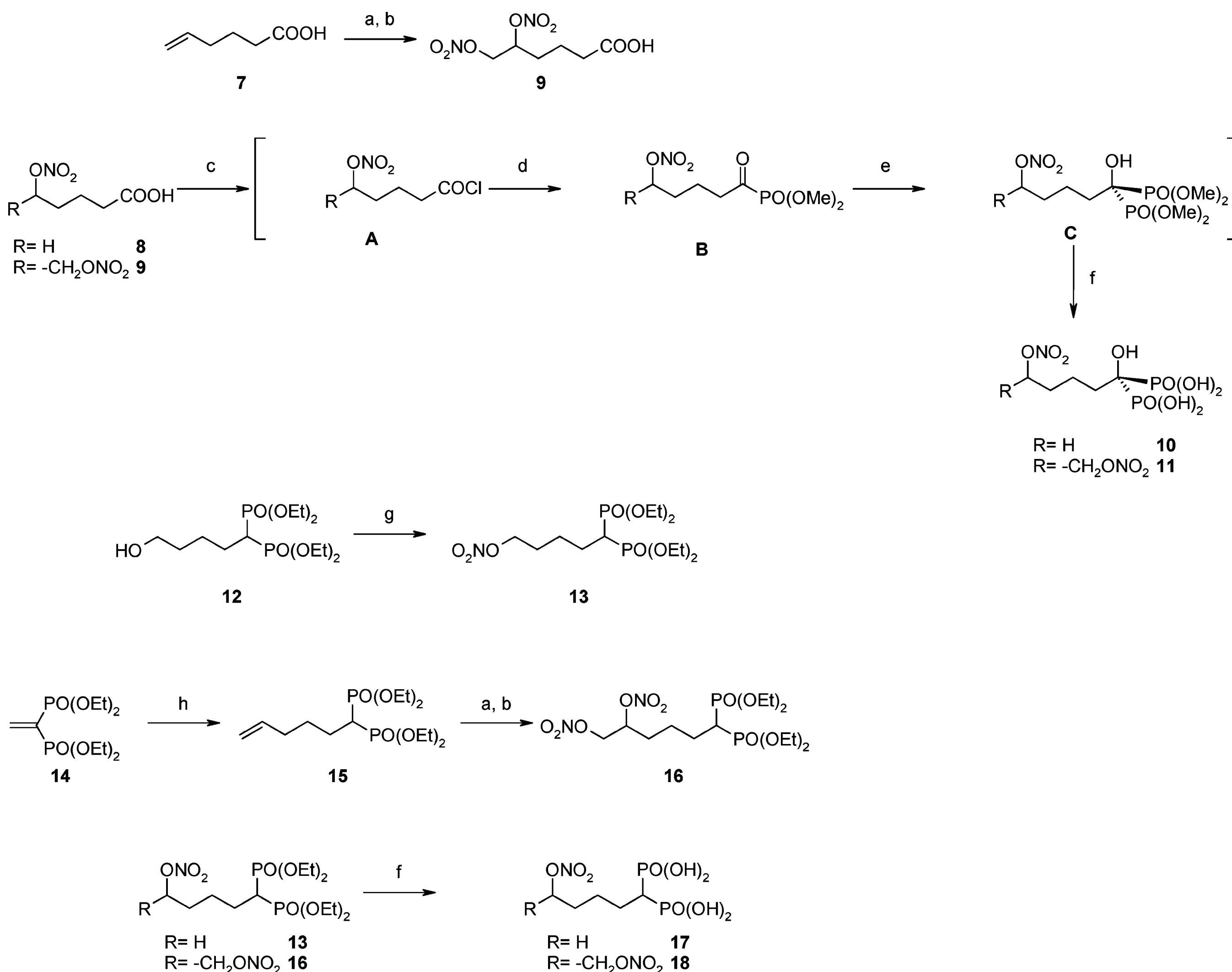
^{1}H NMR and proton-decoupled ^{13}C NMR spectra show rather complex patterns due to coupling with the two phosphorus nuclei, often resulting in second-order multiplets and/or in diastereotopic effects. The protonic pattern of the −CH group bearing the two phosphonic groups is peculiar in all of the spectra of derivatives **13**, **15**, **16**, **17**, and **18**. It occurs in the region 2.1−2.3 ppm and appears as a triplet of triplets due to proton coupling with the two chemically equivalent phosphorus atoms ($^2J_{HP}$ range: 23−24 Hz) and with the vicinal CH_2 protons ($^3J_{HH}$ about 6 Hz). Analogously, in the case of hydroxyl derivatives **10**, **11**, a diagnostic triplet at 73.6 ppm ($^1J_{CP}$ range: 145−148 Hz) is observed in the proton-decoupled ^{13}C NMR spectrum for the corresponding C−OH.

Proton-decoupled ^{31}P NMR spectra display only one signal, ranging between 20 and 24 ppm with respect to external phosphoric acid (85% v/v). At times, the peak appears broad possibly due to either the equilibrium between species in different electrical states (**10**, **11**, **17**, **18**) or the diastereotopic effects (**11**, **16**, **18**).[26]

Dissociation Constants. The dissociation constants (pK_a's) of the new products, determined by the pH-metric method using a Sirius GLpK_a instrument, are listed in Table 1. The consecutive values correspond to the successive ionizations of the two phosphonic groups. The first pK_a values are not detectable by this method because they are too low. The values are in line with those found for other similar BPs.[27] The strong hydrophilicity of the compounds prevented us from determining the lipophilic−hydrophilic balance.

Hydroxyapatite (HAP) and Bone Affinity. The effects of BPs on calcium-related disorders are principally due to their interaction with osteoclasts and osteoblasts after the formation of stable complexes with calcium of HAP present at the sites of new bone formation.[18] To evaluate the affinity for hydroxyapatite,

Scheme 1[a]



[a] (a) $AgNO_3$, I_2, CH_3CN, 0 °C, 2.5 h; (b) $AgNO_3$, CH_3CN, reflux, 2 h; (c) $SOCl_2$; (d) $P(OMe)_3$, dry THF, 0 °C, 12 h; (e) $HPO(OMe)_2$, Et_2NH (40%), dry THF, 0 °C, 5 h; (f) TMSBr, CH_2Cl_2, 0 °C, 24 h; MeOH, 0 °C; (g) CH_2Cl_2, HNO_3/H_2SO_4, −15° C; (h) 3-butenylmagnesium bromide, dry THF, −15 °C.

Table 1. pK_a Values of the Compounds **3**, **10**, **11**, **17**, and **18**

compound	pK_{a1}	pK_{a2} ± SD	pK_{a3} ± SD	pK_{a4} ± SD
3	<1.5	2.27 ± 0.04	6.31 ± 0.05	10.60 ± 0.10
10	<1.5	2.85 ± 0.02	6.83 ± 0.01	11.30 ± 0.03
11	<1.5	3.11 ± 0.02	6.69 ± 0.01	11.47 ± 0.11
17	<1.5	3.08 ± 0.02	6.87 ± 0.01	11.46 ± 0.11
18	<1.5	2.71 ± 0.02	6.60 ± 0.01	11.19 ± 0.03

the BPs were dissolved in 0.05 M Tris buffer and subsequently incubated with HAP, according to a procedure described elsewhere.[28] After 24 h incubation, BPs left in the solution were determined with reverse phase HPLC. *Tiludronate* (**3**) was used as reference bisphosphonate. The results obtained are shown in Table 2. As expected, both 1-hydroxy-substituted nitrooxy derivatives (**10**, **11**), due to their ability to form Ca^{2+}-tridentate complexes, display significantly higher affinities for HAP than compounds **17** and **18**, which lack the hydroxyl group.

These findings were confirmed by an in-vivo study employing ^{99m}Tc-labeled complexes of products **11** and **18**. As shown in Table 2, a clear accumulation of the products in the bone with respect to blood and muscles occurred after 4 h. The accumulation of the 1-hydroxy-substituted structure **11** is greater than that of structure **18**, which is deprived of the hydroxyl group. These findings are in good agreement with those obtained in vitro.

Biochemical Evaluation. Numerous reports in the literature indicate that bisphosphonates act directly on osteoclasts where they induce apoptosis caused by inhibiting the mevalonate pathway which is required for the posttranslational prenylation of GTP-binding proteins.[29−31] Previously, others and we have reported that BPs inhibit the formation of resorption pits by osteoclasts cultured on dentine mineral surface.[32−34] Moreover, it was found that the reduction in pit number was not due to a decrease in osteoclast resorption activity but osteoclast number, suggesting an action of BPs on osteoclast recruitment.[33,35] Such considerations have also been forwarded by a previous study.[36] Hence, these and our findings motivated us to assess whether nitrooxy BPs may also affect the differentiation of pre-osteoclasts into functional osteoclasts. As target cell, we used RAW264.7 cells, a murine monocyte/macrophage cell line with pre-osteoclastic attributes.[37,38] Thus, it has been shown that RAW264.7 cells differentiate into

Table 2. Affinity for HAP of the Compounds **3**, **10**, **11**, **17**, and **18** (1 mM) and Biodistribution of ^{99m}Tc-Labeled Complexes of Compounds **11** and **18** (0.1 mg)

	affinity to HAP[a] %[b]	biodistribution[a]			
		bone/blood[c]		bone/muscle[c]	
compound	24 h	2 h	4 h	2 h	4 h
3	58.4 ± 0.6	*d*	*d*	*d*	*d*
10	43.4 ± 0.7	*d*	*d*	*d*	*d*
11	46.3 ± 0.2	15.2 ± 3.1	54.4 ± 4.8	66.8 ± 9.2	271.8 ± 34.7
17	65.0 ± 0.6	*d*	*d*	*d*	*d*
18	68.1 ± 0.7	10.2 ± 2.9	21.2 ± 3.8	59.0 ± 17.2	154.4 ± 36.4

[a] All values are mean ± SEM. [b] Percentage of BP left in solution. [c] Uptake ratios at different times after injection of ^{99m}Tc-complex. [d] Not tested.

Table 3. Effect on Osteoclasts Formation of Compounds **4**, **5**, **10**, and **17** (100 nM) in the Presence of RANKL (50 ng/mL) and in the Absence or in the Presence of ODQ (10 μM)

	number of osteoclasts[a,b]
control	25.6 ± 4.01
RANKL	62.4 ± 3.88
5 + RANKL	61.2 ± 4.57
5 + RANKL + ODQ	62.4 ± 6.45
10 + RANKL	32.8 ± 2.56***
10 + RANKL + ODQ	59.6 ± 4.62
17 + RANKL	40.2 ± 2.53**
17 + RANKL + ODQ	58.9 ± 4.44
4 + RANKL	39.8 ± 5.02**
4 + RANKL + ODQ	34.2 ± 2.00

[a] Data represent the mean ± SEM of five individual wells. [b] Clonal RAW264.7 cells were employed. (**) $p < 0.01$; (***) $p < 0.001$ vs **5**.

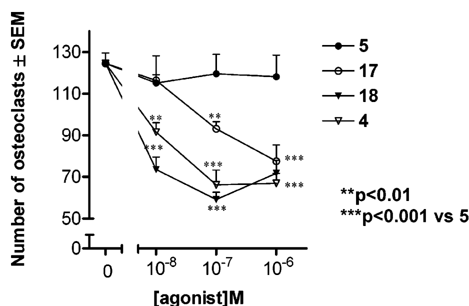


Figure 1. Effect of compounds **5**, **17**, **18**, and **4** on osteoclasts formation from RAW264.7 cells treated with receptor activator of NF-kB ligand (RANKL).

osteoclasts following treatment with receptor activator of NF-κB ligand (RANKL). Osteoclastogenesis progresses along a stringent and well-defined developmental pathway where RANKL, an osteoblast membrane associated cytokine, assumes a crucial role both in regulating osteoclast differentiation and in expression of resorption activity.[39] Consecutive cultivation of RAW264.7 cells with RANKL and nitrooxy BPs, as shown in Table 3 and Figures 1 and 2, rendered a substantial inhibition on osteoclast maturation. Analysis of the data shows that while the bisphosphonate which lacks the nitrooxy group(s) (compound **5**) produced no effect, both BPs which carry the nitrooxy-donor group(s) (compounds **17** and **18**) inhibit osteoclast differentiation in a dose-dependent manner. The dinitrooxy derivative is more potent than its mononitrooxy analogue and, at the lower concentration tested, also is more potent than *Ibandronate* (**4**), a well-studied bisphosphonate. At concentrations greater than 10^{-7} M, the inhibitory activity leveled off, a phenomenon that may possibly be the result of receptor down regulation.

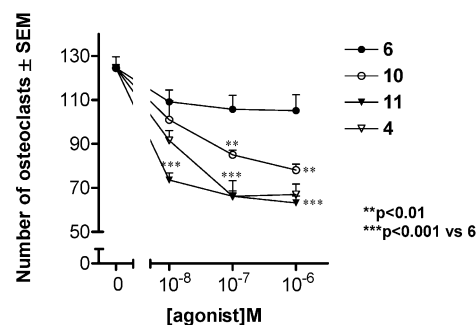


Figure 2. Effect of compounds **6**, **10**, **11**, and **4** on osteoclasts formation from RAW264.7 cells treated with receptor activator of NF-kB ligand (RANKL).

As illustrated in Figure 2, the 1-hydroxyl-substituted nitrooxy-BP derivatives display inhibitory activities similar to those which are devoid of the hydroxyl-group. Again, the reference compound (compound **6**) appears to be inactive on the osteoclast maturation. Both BPs (compounds **10** and **11**) decreased the RANKL induced differentiation of RAW264.7 to multinuclear osteoclast in a dose-related manner, while the dinitrooxy derivative once more was more potent than *Ibandronate* (**4**). These results clearly indicate that the presence of nitrooxy group(s) caused the inhibition on osteoclast differentiation and that nitric oxide might possibly set it off. To confirm that such indeed is the case, we cultured RAW264.7 cells with 100 nM of the BPs **10**, **17**, of the reference compound **5**, and of *Ibandronate* (**4**) with or without 10 μM 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a well-known inhibitor of the soluble guanylate cyclase.[40] Previously, it was demonstrated that growth stimulatory and apoptosis-inducing effects of nitric oxide on primary osteoblasts are mediated by the second messenger cGMP because both effects were abolished by the guanylate cyclase inhibitor ODQ.[11] As depicted in Table 3, the reference compound **5** was inactive both in the presence and in the absence of ODQ, while the inhibitory activity of **4** on osteoclast differentiation was not abolished by the presence of ODQ. By contrast, the two nitrooxy BPs that were found to inhibit osteoclasts maturation when tested alone lost their effectiveness in the presence of ODQ. These results support the notion that NO•, by acting on guanylate cyclase, appears to inhibit the differentiation of pre-osteoclasts into functional osteoclasts. Finally, to assess whether the NO-bisphosphonates may act on authentic osteoclasts, we allowed pre-and mature osteoclasts, isolated from 1-day-old rat femurs, to settle onto dentine slices and submitted them for 24 h to compounds **17** and **18** and salmon calcitonin (10^{-12} M), a potent inhibitor

Table 4. Effect of Compounds **17** and **18** (100 nM) and Calcitonin (10^{-12} M) on Osteoclasts Number and Osteoclast Resorption Activity

compounds	number of osteoclasts[a]	ratio pits/osteoclast
control	31.99 ± 2.44	0.97 ± 0.08
17	21.88 ± 1.23[b]	1.18 ± 0.14
18	18.88 ± 1.59[b]	1.26 ± 0.19
calcitonin	27.50 ± 2.55	0.01 ± 0.01

[a] Data represent the mean ± SEM of osteoclasts scored on 16 individual dentine slices. [b] $p < 0.001$ versus control.

of osteoclast resorption activity, as internal control. As summarized in Table 4, both nitrooxy-bisphosphonates but not calcitonin significantly reduced the number of TRAP + MNC on the dentine mineralized surfaces. The calculated ratios pits formed per osteoclasts, a measure of osteoclast resorption activity, indicate that compounds **17** and **18** act on osteoclast number while calcitonin clearly inhibited osteoclast activity. These results together with the data reported in Figures 1 and 2 suggest that the NO-BPs may indeed act on osteoclast development, although an NO-BP-induced apoptosis in osteoclasts cannot be entirely ruled out. In summary, we have successfully been able to attach nitrooxy NO-donor functions to bisphosphonates. The resulting molecules display affinity for the bone and inhibit the differentiation of pre-osteoclastic cells to functional osteoclasts by a prevalent NO-mediated action. In the future, a development of this work could be the design of new NO-donor BPs whose ability to inhibit such differentiation should depend both on their bisphosphonic structures and on NO-donor properties in a more well-balanced manner.

Experimental Section

Chemistry. All of the compounds were routinely checked via IR (Shimadzu FT-IR 8101 M), ^{1}H and ^{13}C NMR (Bruker AC-200 and Bruker Avance 300), and mass spectrometry (Finnigan-Mat TSQ-700). ^{31}P chemical shifts are referred to external 85% w/v H_3PO_4. The following abbreviations were used to indicate the peak multiplicity: *s* = singlet; *d* = doublet; *t* = triplet; *qt* = quartet; *qi* = quintet; *n* = nine lines; *m* = multiplet. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230−400 mesh ASTM) using the indicated eluents. MPLC chromatography was carried out on octadecyl-functionalized silica gel (Sigma Aldrich) using the indicated eluents. Thin-layer chromatography (TLC) was carried out on 5 × 20 cm plates with a layer thickness of 0.25 mm. When necessary, they were developed with iodine, $KMnO_4$, and ammonium thiocyanate-iron(III) chloride reagent.[41] HPLC analyses were performed using a diode array UV detector (Shimadzu LC10A). Anhydrous magnesium sulfate was used as drying agent for the organic phases. Analysis (C, H, N) of the new compounds was performed by REDOX (Monza), and the results are within ±0.4% of the theoretical unless otherwise stated. Structures **5**,[42] **6**,[43] **7**,[44] **8**,[22] **12**,[24] and **14**[45] were synthesized according to methods described in the literature. 3-Butenylmagnesium bromide was synthesized according to a method described elsewhere[46] and was titrated immediately before use.[47] Tetrahydrofuran (THF) was distilled immediately before use from Na and benzophenone under a positive atmosphere of N_2. When needed, the reactions were performed in flame- or over-dried glassware under a positive pressure of dry N_2 or Ar. All reactions were carried out three times without any attempts to optimize the yields.

5,6-Dinitrooxyhexanoic Acid (9). To a stirred solution of 5-hexenoic acid (**7**) (1.40 g, 11.39 mmol) and $AgNO_3$ (4.16 g, 22.78 mmol, 2 equiv) in CH_3CN (15 mL) kept at 0 °C was added dropwise a solution of iodine (3.11 g, 11.39 mmol) in CH_3CN (50 mL). At the end of the addition, the mixture was allowed to reach room temperature (rt). $AgNO_3$ (8.32 g, 45.56 mmol, 4 equiv) was added, and the mixture was heated at reflux for 2 h. After cooling, a saturated solution of KBr was added to precipitate the excess of $AgNO_3$, and the mixture was filtered through Celite. The filtrate was concentrated under reduced pressure, dissolved in water (30 mL), and extracted with CH_2Cl_2 (2 × 50 mL). The combined organic layers were extracted with a saturated solution of $NaHCO_3$ (3 × 30 mL). After acidification, the aqueous layers were extracted with CH_2Cl_2 (3 × 50 mL). The latter combined organic phases were dried and evaporated under vacuum at room temperature, and the resulting residue was purified by flash chromatography (CH_2Cl_2/EtOAc 95/5 v/v) yielding the pure compound as a white solid. Yield 69%. ^{1}H NMR ($CDCl_3$) δ 1.86 (*m*, 4H, $-CH_2CH_2CH_2COOH$), 2.50 (*m*, 2H, $-CH_2CH_2CH_2$-COOH), 4.50−5.36 (*m AMX like*, 3H, $O_2NOCH_2CH(ONO_2)$-CH_2-); ^{13}C NMR ($CDCl_3$) δ 20.4 ($-CH_2CH_2COOH$), 29.0 ($-CH_2CH_2COOH$), 33.5 ($-CH(ONO_2)CH_2CH_2-$), 71.4 ($O_2NOCH_2CH(ONO_2)CH_2-$), 79.1 ($O_2NOCH_2CH(ONO_2)$-CH_2-), 179.1 ($-CH_2CH_2COOH$); MS (CI) *m/z* 239 (M + 1)$^+$; drying conditions: rt; 15 days, pressure < 10 mmHg.

Tetraethyl (5,6-Dinitrooxyhexylidene)bisphosphonate (16). A solution of iodine (314 mg, 2.46 mmol, 2 equiv) in CH_3CN (20 mL) was slowly added to a magnetically stirred solution of tetraethyl (5-hexenylidene)bisphosphonate (**15**) (440 mg, 1.23 mmol) and $AgNO_3$ (420 mg, 2.46 mmol, 2 equiv) in CH_3CN (30 mL) kept at 0 °C. Thereafter, $AgNO_3$ (420 mg, 2.46 mmol, 2 equiv) was added, and the mixture was refluxed for 24 h. After cooling, a saturated solution of KBr was added to precipitate the excess of $AgNO_3$ and the mixture was filtered through Celite. The filtrate was concentrated under reduced pressure. The obtained residue was dissolved in CH_2Cl_2 (30 mL), and the resulting solution was washed twice with H_2O (20 mL), dried, and then concentrated under reduced pressure. The crude material was purified by flash chromatography (CH_2Cl_2/EtOAc 95/5 v/v to CH_2Cl_2/MeOH 98/2 v/v) to give the pure compound as a pale yellow oil. Yield 68%. ^{1}H NMR ($CDCl_3$) δ 1.35 (*t*, 12H, $-PO(OCH_2CH_3)_2$), 1.7−2.1 (*m*, 6H, $-(ONO_2)CHCH_2CH_2CH_2-$), 2.24 (*tt*, 1H, $^2J_{HP}$ = 23.2 Hz, $^3J_{HH}$ = 6 Hz, $-CH(PO(OCH_2CH_3)_2)_2$), 4.1−4.3 (*m*, 8H, $-PO(OCH_2$-$CH_3)_2$), 4.4−5.3 (*m AMX like*, 3H, $O_2NOCH_2CH(ONO_2)$-CH_2-); ^{13}C NMR ($CDCl_3$) δ 16.2 (*d*, $^3J_{CP}$ = 6 Hz, −PO-$(OCH_2CH_3)_2$), 24.0 (*s, broad*)/25.0 (*s, broad*) ($-CH_2CH_2CH_2$-$CH(PO(OCH_2CH_3)_2)_2$), 28.8 ($O_2NOCH_2CH(ONO_2)CH_2-$), 36.3 (*t*, $^1J_{CP}$ = 133 Hz, $-CH(PO(OCH_2CH_3)_2)_2$), 62.5 (*m*, $-PO(OCH_2$-$CH_3)_2$), 71.0 ($O_2NOCH_2CH(ONO_2)CH_2-$), 78.5 (O_2NOCH_2CH-$(ONO_2)CH_2-$); ^{31}P NMR ($CDCl_3$) δ 23.9 *broad*; MS (CI) *m/z* 481 (M + 1)$^+$; drying conditions: rt; 8 h, pressure < 1 mmHg.

Tetraethyl (5-Nitrooxypentylidene)bisphosphonate (13). A solution of tetraethyl (5-hydroxypentylidene)bisphosphonate (**12**) (1.75 g, 4.96 mmol) in CH_2Cl_2 (10 mL) was added dropwise to a stirring solution of HNO_3 65% v/v (1.35 mL, 19.44 mmol, 4 equiv) and H_2SO_4 98% v/v (1.1 mL, 19.44 mmol, 4 equiv) in CH_2Cl_2 (40 mL) kept at −15 °C. The mixture was allowed to reach room temperature and was then stirred for an additional 12 h. The mixture was poured into water (20 mL) and extracted with CH_2Cl_2 (50 mL). The organic layer was dried and concentrated under reduced pressure. The crude material was purified by flash chromatography (CH_2Cl_2/^{i}PrOH 95/5 v/v) yielding the pure compound as a pale yellow oil. Yield 69%. ^{1}H NMR ($CDCl_3$) δ 1.35 (*t*, 12H, $-PO(OCH_2CH_3)_2$), 1.7−2.0 (*m*, 6H, $ONO_2CH_2CH_2CH_2CH_2-$), 2.27 (*tt*, 1H, $^2J_{HP}$ = 24.0 Hz, $^3J_{HH}$ = 6 Hz, $-CH(PO(OCH_2CH_3)_2)_2$), 4.1−4.3 (*m*, 8H, $-PO(OCH_2CH_3)_2$), 4.46 (*t*, 2H, $^3J_{HH}$ = 6 Hz, $O_2NOCH_2CH_2$-CH_2-); ^{13}C NMR ($CDCl_3$) δ 16.4 (*d*, $^3J_{CP}$ = 6 Hz, −PO-$(OCH_2CH_3)_2$), 24.9−25.1 (*m*, $-CH_2CH_2CH(PO(OCH_2CH_3)_2)_2$), 26.4 (*m*, $-CH_2CH_2CH_2CH(PO(OCH_2CH_3)_2)_2$), 30.7 (*m*, $-CH_2CH_2CH_2CH(PO(OCH_2CH_3)_2)_2$), 36.6 (*t*, $^1J_{CP}$ = 132 Hz, $-CH(PO(OCH_2CH_3)_2)_2$), 62.4 (*t*, $^2J_{CP}$ = 8 Hz, $-PO(OCH_2CH_3)_2$), 76.9 ($O_2NOCH_2CH_2CH_2-$); ^{31}P NMR ($CDCl_3$) δ 24.1; MS (CI) *m/z* 406 (M + 1)$^+$; drying conditions: 40 °C; 80 h, pressure < 1 mmHg.

Tetraethyl (5-Hexenylidene)bisphosphonate (15). A solution of 3-butenylmagnesium bromide (13.9 mL, 0.574 M,

1.2 equiv) in dry THF was slowly added to a magnetically stirred solution of tetraethyl vinylidenebisphosphonate (**14**) (2 g, 6.66 mmol) in dry THF (26 mL) kept at −15 °C under Ar. The mixture was allowed to reach room temperature and then was slowly poured into a saturated solution of NH_4Cl (30 mL). The mixture was extracted with Et_2O (2 × 30 mL), and the combined dried organic layers were concentrated under reduced pressure. The crude residue was purified by flash chromatography (CH_2Cl_2/MeOH 98/2 v/v) to yield the pure compound as a colorless oil. Yield 63%. ^{1}H NMR ($CDCl_3$) δ 1.37 (*t*, 12H, $-PO(OCH_2CH_3)_2$), 1.7−2.1 (*m*, 6H, $CH_2{=}CHCH_2CH_2CH_2-$), 2.29 (*tt*, 1H, $^2J_{HP}$ = 30 Hz, $^3J_{HH}$ = 6 Hz, $-CH(PO(OCH_2CH_3)_2)_2$), 4.2−4.3 (*m*, 8H, $-PO(OCH_2CH_3)_2$), 5.0 (*m*, 2H, $CH_2{=}CHCH_2CH_2CH_2-$), 5.8 (*m*, 1H, $CH_2{=}CHCH_2CH_2CH_2-$); ^{13}C NMR ($CDCl_3$) δ 16.8 (*dd*, $^3J_{CP}$ = 6.4 Hz, $^5J_{CP}$ = 2 Hz, $-PO(OCH_2CH_3)_2$), 25.5/28.6 ($-CH_2CH_2CH(PO(OCH_2CH_3)_2)_2$), 33.8 ($CH_2{=}CHCH_2CH_2-$), 37.1 (*t*, $^1J_{CP}$ = 131 Hz, $-CH(PO(OCH_2CH_3)_2)_2$), 62.9 (*dd*, $^2J_{CP}$ = 10.7 Hz, $^4J_{PC}$ = 6.8 Hz, $-PO(OCH_2CH_3)_2$), 115.3 ($CH_2{=}CHCH_2CH_2CH_2-$), 138.5 ($CH_2{=}CHCH_2CH_2CH_2-$); ^{31}P NMR ($CDCl_3$) δ 24.6; MS (EI) *m/z* 356 (M)$^+$; drying conditions: 40 °C; 24 h, pressure < 10 mmHg.

General Procedure for the Preparation of Bisphosphonic Acids 17, 18. Trimethylsilyl bromide (2.5 equiv) was slowly added to a stirred solution of the appropriate tetraethyl ester (**13**, **16**) (500 mg) in dry CH_2Cl_2 (10 mL) and kept under N_2 for 18 h. The reaction was followed via RP-18 TLC (CH_3CN/H_2O 5/5 v/v). After cooling at 0 °C, dry MeOH (10 mL) was added, and the resulting mixture was allowed to reach rt. The solution was then concentrated under reduced pressure. The residue was dissolved in dry MeOH (10 mL) and subsequently concentrated under reduced pressure twice. The crude material was purified by MPLC (RP-18, CH_3CN/H_2O 2/8 v/v, flow 15 mL/min, λ 217 nm, injection 500 μL, solution 100 mg/mL) to give the pure product as a white solid.

(5-Nitrooxypentylidene)bis(phosphonic acid) (17). Yield 78%. ^{1}H NMR (D_2O) δ 1.5−1.8 (*m*, 6H, $ONO_2CH_2CH_2CH_2CH_2-$), 2.1 (*tt*, 1H, $^2J_{HP}$ = 23.6 Hz, $^3J_{HH}$ = 6 Hz, $-CH(PO(OH)_2)_2$), 4.4 (*t*, 2H, $O_2NOCH_2CH_2CH_2-$); ^{13}C NMR (D_2O) δ 25.0 (*t*, $^2J_{CP}$ = 4.6 Hz, $ONO_2CH_2CH_2CH_2CH_2-$, tentatively assigned), 25.2 (*t*, $^3J_{CP}$ = 7.0 Hz, $ONO_2CH_2CH_2CH_2CH_2-$, tentatively assigned), 26.1 ($ONO_2CH_2CH_2CH_2CH_2-$), 37.6 (*t*, $^1J_{CP}$ = 126 Hz, $-CH(PO(OH)_2)_2$), 74.1 ($O_2NOCH_2CH_2CH_2-$); ^{31}P NMR (D_2O) δ 23.1 *broad*; drying conditions: rt; 24 h, pressure < 1 mmHg.

(5,6-Dinitrooxyhexylidene)bis(phosphonic acid) (18). Yield 41%. ^{1}H NMR (D_2O) δ 1.66 (*m*, 6H, $-(ONO_2)CHCH_2CH_2CH_2-$), 2.11 (*broad t*, 1H, $^2J_{HP}$ = 23 Hz, $-CH(PO(OH)_2)_2$), 4.5−5.4 (*m AMX like*, 3H, $O_2NOCH_2CH(ONO_2)CH_2-$); ^{13}C NMR (D_2O) δ 24.4 (*t*, $^3J_{CP}$ = 7 Hz, $-CH_2CH_2CH_2CH(PO(OH)_2)_2$, tentatively assigned), 25.1 (*t*, $^2J_{CP}$ = 4.6 Hz $-CH_2CH_2CH_2CH(PO(OH)_2)_2$, tentatively assigned), 25.4 ($-CH_2CH_2CH_2CH(PO(OH)_2)_2$), 34.6 (*t*, $^1J_{CP}$ = 125 Hz, $-CH(PO(OH)_2)_2$), 69.0 ($O_2NOCH_2CH(ONO_2)CH_2-$), 77.3 ($O_2NOCH_2CH(ONO_2)CH_2-$); ^{31}P NMR (D_2O) δ 22.6/22.8; drying conditions: rt; 48 h, pressure < 1 mmHg.

General Procedure for the Preparation of Bisphosphonic Acids 10, 11. A solution of the appropriate nitrooxy acid (**8**, **9**) (2 g) in thionyl chloride (7 mL, fresh distilled) was constantly stirred at rt for 3 h under Ar. Thionyl chloride was then distilled under reduced pressure, and the system was refilled with Ar. Thereafter, dry THF (10 mL) was added to the residue and then distilled under reduced pressure. The procedure was repeated three times. The residue was dissolved in dry THF (10 mL), and to the resulting solution, magnetically stirred under Ar at 0 °C, was finally added trimethyl phosphite (1 equiv). The mixture was then allowed to reach rt and was stirred for 12 h. The resulting solution containing the acylphosphonate (**B**) was slowly added to a stirred solution of dimethyl phosphite (2 equiv) and diethylamine (0.4 equiv) in dry THF (40 mL) under Ar and kept at 0 °C. The mixture was stirred at 0 °C for 3 h, allowed to reach rt, and then poured into HCl 0.5 M (50 mL) and Et_2O (60 mL). The ethereal fraction was extracted with HCl 0.5 M (10 × 50 mL). The collected aqueous layers were extracted with CH_2Cl_2 (13 × 80 mL). The organic layers were dried and concentrated under reduced pressure. The crude material obtained was purified by flash chromatography (CH_2Cl_2/EtOAc 9/1 v/v to CH_2Cl_2/MeOH 9/1 v/v) to yield the unstable tetramethylesters as pale yellow oils. The compounds were immediately used for the next synthetic step. Trimethylsilyl bromide (2.5 equiv) was slowly added to a magnetically stirred solution of the appropriate tetramethylester (**C**) (2 g) in dry CH_2Cl_2 (40 mL) kept under N_2 for 20 h. The reaction was followed via RP-18 TLC (CH_3CN/H_2O 5/5 v/v). After the mixture was cooled to 0 °C, dry MeOH (20 mL) was added and the mixture was allowed to reach room temperature. The solution was then concentrated under reduced pressure. The residue was dissolved in dry MeOH (10 mL) and concentrated under reduced pressure twice. The crude material was purified by preparative HPLC (Lichrospher 250-25 C_{18}, MeOH/H_2O 2/8 v/v, flow 39 mL/min, λ 217 nm, injection 3 mL, solution 60 mg/mL) to give the pure product as a white solid.

(5-Nitrooxy-1-hydroxypentylidene)bis(phosphonic acid) (10). Yield 9%. ^{1}H NMR (D_2O) δ 1.61 (*m*, 4H, $ONO_2CH_2CH_2CH_2CH_2-$), 1.89 (*m*, 2H, $ONO_2CH_2CH_2CH_2CH_2-$), 4.43 (*t*, 2H, $O_2NOCH_2CH_2CH_2-$); ^{13}C NMR (D_2O) δ 20.1 (*t*, $^3J_{CP}$ = 6.4 Hz, $ONO_2CH_2CH_2CH_2CH_2-$), 27.0 ($ONO_2CH_2CH_2CH_2CH_2-$), 33.5 ($ONO_2CH_2CH_2CH_2CH_2-$), 73.6 (*t*, $^1J_{CP}$ = 145 Hz, $-C(OH)(PO(OH)_2)_2$), 74.4 ($O_2NOCH_2CH_2CH_2CH_2-$); ^{31}P NMR (D_2O) δ 20.1 *broad*; drying conditions: rt; 15 days, pressure < 10 mmHg.

(5,6-Dinitrooxy-1-hydroxyhexylidene)bis(phosphonic acid) (11). Yield 21%. ^{1}H NMR (D_2O) δ 1.7−2.0 (*m*, 6H, $-(ONO_2)CHCH_2CH_2CH_2-$), 4.6−5.4 (*m*, 3H, $O_2NOCH_2CH(ONO_2)CH_2-$); ^{13}C NMR (D_2O) δ 19.3 (*t*, $^3J_{CP}$ = 6.4 Hz, $-(ONO_2)CHCH_2CH_2CH_2-$), 29.3/33.5 ($-CH_2CH_2CH_2C(OH)(PO(OH)_2)_2$), 72.2 ($O_2NOCH_2CH(ONO_2)CH_2-$), 73.6 (*t*, $^1J_{CP}$ = 148 Hz, $-C(OH)(PO(OH)_2)_2$), 80.6 ($O_2NOCH_2CH(ONO_2)CH_2-$); ^{31}P NMR (D_2O) δ 20.2 *broad*; drying conditions: rt; 15 days, pressure < 10 mmHg.

pH-Metric Approach To Obtain pK_a. Potentiometric titrations of compounds were performed with the GlpKa apparatus (Sirius Analytical Instruments Ltd., Forrest Row, East Sussex, UK). Ionization constants were determined as outlined in the literature: the aqueous titrations were carried out under N_2 at 25.0 ± 0.1 °C, and final data were obtained by the Multiset approach.[48]

Adsorption of BPs to HAP. HAP (150 mg, Aldrich) was equilibrated in 50 mL of 0.05 M Tris-HCl pH 7.4 for 24 h at 37 °C according to the literature.[28] The BPs (1 mM) were incubated in HAP suspension at 37 °C and magnetically stirred. After 24 h, 1 mL of suspension was centrifuged (10 000 rpm, 10 min), and the concentrations of the BPs in the surnatant were determined by RP-HPLC.

Chromatographic Conditions. The HPLC system consisted of a Shimadzu LC-10AS chromatograph system equipped with a diode-array detector (SPD-M10A, Shimadzu) and a Lichrospher 60 RP-select B column (250 × 4 mm, 5 μm, Merck). The mobile phase consisted of methanol/50 mM phosphate buffer pH 7.4 (10/90 v/v containing 1 mM tetrabutylammonium hydrogen sulfate (Fluka) for compounds **10**, **17**; 15/85 v/v containing 1 mM tetrabutylammonium hydrogen sulfate for compounds **11**, **18**; 10/90 v/v for *Tiludronate* (**3**)). The flow-rate was 1 mL/min, and the injection volume was 50 μL (Rheodyne, Cotati, CA). The peaks were monitored at 226 nm for compounds **10**, **11**, **17**, **18** and at 254 nm for **3**.

Complexes of BPs with ^{99m}Tc. A solution (4 mL) of sodium pertechnetate ($^{99m}TcO_4^-$, 40 mCi) was added to 1 mL of a 0.2 M solution of sodium acetate (pH = 6) containing 2.5 mg of the appropriate bisphosphonate, $SnCl_2{\cdot}2H_2O$ (0.3 mg), and sodium gentisate (5 mg). The absence of free $^{99m}TcO_4^-$ and the yield in the reduced $^{99m}Tc^{4+}$ complexes so obtained were detected by paper chromatography. The absence of hydrolyzed ^{99m}Tc colloid compounds was controlled by size exclusion chromatography on biogel P6 (Bio-Rad). Male Norvegian black rats, each weighing 150−200 g, were used to determine the organ distribution of $^{99m}Tc^{4+}$-complexes. The rats were sacrificed at 2 or 4 h after injection of 0.2 mL of $^{99m}Tc^{4+}$-complexes via the caudal vein. Samples of muscle, stomach, small intestine, kidney, liver, and bone (femur) were taken and

weighed. In addition, a sample of blood was drawn from the heart immediately after sacrifice. Samples of different organs were counted in a well-type gamma counter to calculate resident activity in different organs. Tissue concentrations were calculated and expressed as percent uptake of injected dose per gram or per milliliter (% ID/g). Bone-to-blood and bone-to-muscle uptake ratios were determined from the % ID/g values for the organs.

Cultivation of RAW264.7 Cells and Assessment of Nitric Oxide Donor Substances on Osteoclast Formation. *Ibandronate* (**4**) was a gift of Boehringer Mannheim GmbH, Germany (the company was later sold to the Roche AG, Basel Switzerland). RAW264.7 cells (kindly provided by Dr. M. J. Rogers, University of Aberdeen, UK) were seeded at 2×10^3 cells per well using 48 well plastic tissue culture plates (Becton & Dickenson, USA) and cultured for 24 h in αMEM, (Invitogen, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS). Thereafter, media were changed and replaced by one containing RANKL (50 ng/mL) and various concentrations of tested compounds. Some cultures media were supplemented with 10 μM 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). Cells were grown to confluence in phenol red-free α MEM supplemented with 10% FBS and antibiotics. At confluence, media were removed and cells were washed with phosphate buffered saline (PBS) and subsequently fixed and stained for tartrate-resistant acid phosphatase (TRAP) (Sigma, Buchs Switzerland) according to the recommendation of the manufacturer. The action of nitric oxide was assessed by its effect on the RANKL-induced TRAP+ multinucleated cell (osteoclast) formation. The assessment of the NO-bisphosphonates on freshly isolated rat osteoclasts was carried out with the "Pit Assay".[32,33] Freshly isolated osteoclasts were allowed to adhere onto mineralized elephant dentine for 40 min. Nonadherent cells were then removed, and the remaining osteoclastic cells were cultivated with or without 100 nM of compound **17** or **18** or 10^{-12} M calcitonin for 24 h.

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Supporting Information Available: Elemental analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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