Bioorganic & Medicinal Chemistry 17 (2009) 7290–7300

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and evaluation of benzo[*b*]thiophene derivatives as inhibitors of alkaline phosphatases

Lina Li [a,b,c,d,e,f], Lei Chang [a,b,c,d,e], Stéphane Pellet-Rostaing [a,b,c,d,e,*], François Liger [a,b,c,d,e], Marc Lemaire [a,b,c,d,e,*], René Buchet [a,b,c,d,e,*], Yuqing Wu [f]

[a] *Université de Lyon, Villeurbanne F-69622, France*
[b] *Université Lyon 1, Villeurbanne F-69622, France*
[c] *INSA de Lyon, Villeurbanne F-69621, France*
[d] *CPE Lyon, Villeurbanne F-69616, France*
[e] *CNRS UMR 5246 ICBMS, Villeurbanne F-69622, France*
[f] *State Key Laboratory for Supramolecular Structure and Materials, Jilin University, Changchun 130012, China*

ARTICLE INFO

Article history:
Received 13 March 2009
Revised 17 August 2009
Accepted 21 August 2009
Available online 3 September 2009

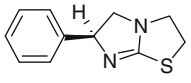
Keywords:
Tetramisole
Benzo[*b*]thiophene
TNAP
Inhibitor

ABSTRACT

Presence of basic calcium phosphate in knee joints of osteoarthritis patients could be prevented by inhibiting tissue non-specific alkaline phosphatase (TNAP) activity. Levamisole or the L stereoisomer of tetramisole (a known TNAP inhibitor) has been used as a treatment for curing rheumatoid arthritis but its therapeutical use is limited due to side effects. We report the synthesis and the TNAP inhibition property of benzo[*b*]thiophene derivatives, among which benzothiopheno-tetramisole and benzothiopheno-2,3-dehydrotetramisole, which could be involved in a drug therapy for osteoarthritis. Two water soluble racemic benzothiopheno-tetramisole and -2,3-dehydrotetramisole with apparent inhibition constants K_i = 85 ± 6 μM and 135 ± 3 μM (n = 3) comparable to that of enantiomeric levamisole 93 ± 4 μM were found. Several novel derivatives showed more pronounced inhibition properties towards intestinal alkaline phosphatase than TNAP.

1. Introduction

Calcium-containing crystals are present in synovial fluid extracted from the knee joints of up to 70% of osteoarthritis patients, indicating that pathological calcification occurs in the majority of osteoarthritis.[1–4] Calcified diseases associated with osteoarthritis are correlated with the presence of calcium pyrophosphate dihydrate (CPPD) crystals (25–55% of the time) and/or of the occurence of basic calcium phosphate (BCP) crystals (35–70% of the time) consisting of carbonate-substituted hydroxyapatite (HA) and octacalcium phosphate.[5–8] The origin of CPPD crystals is associated with the increase in inorganic pyrophosphate (PP_i) concentration. Upregulation of nucleotide pyrophosphatase phosphodiesterase 1 (NPP1) and of ankylosis protein (ANK, a PP_i transporter) expressions in articular cartilage can contribute to an extracellular PP_i excess,[9] leading possibly to CPPD deposition. Consistent with this mechanism, some mutations affecting *ank* gene, upregulating ANK activity, cause chondrocalcinosis in humans.[10,11] In addition, matrix vesicles (MVS) of affected cartilage in osteoarthritis increase tissue non-specific alkaline phosphatase (TNAP) activity as much as 30-fold and induce HA deposition.[12] TNAP is among the first functional genes expressed in the process of calcification. The crucial role of TNAP in the mineralization process is evidenced in the case of hypophosphatasia patients, whose disease results from mutations in the gene coding TNAP leading to a decreased or absent TNAP activity.[13,14] Therefore, the formation of BCP crystals could be prevented by inhibiting TNAP activity. Levamisole (Fig. 1) or the L stereoisomer of tetramisole (a known TNAP inhibitor[15]) have been used as a treatment for curing rheumatoid arthritis.[16–18] However, skin rashes and agranulocytosis reported as side effects for Levamisole[19,20] have limited its therapeutical use. Although numerous levamisole analogs (6-aryl,heteroarylimidazo[2,1-*b*]thiazole) have been described in the literature as anthelmintics,[21–33] few of them have been tested as inhibitors of alkaline phosphatase[22,30] and, as far as we know, none of these contained heterocyclic moieties (thiophen, pyridine, benzofuran...). More recently, Sidique et al. described the screening test of a library of pyrazolo derivatives as potent and selective

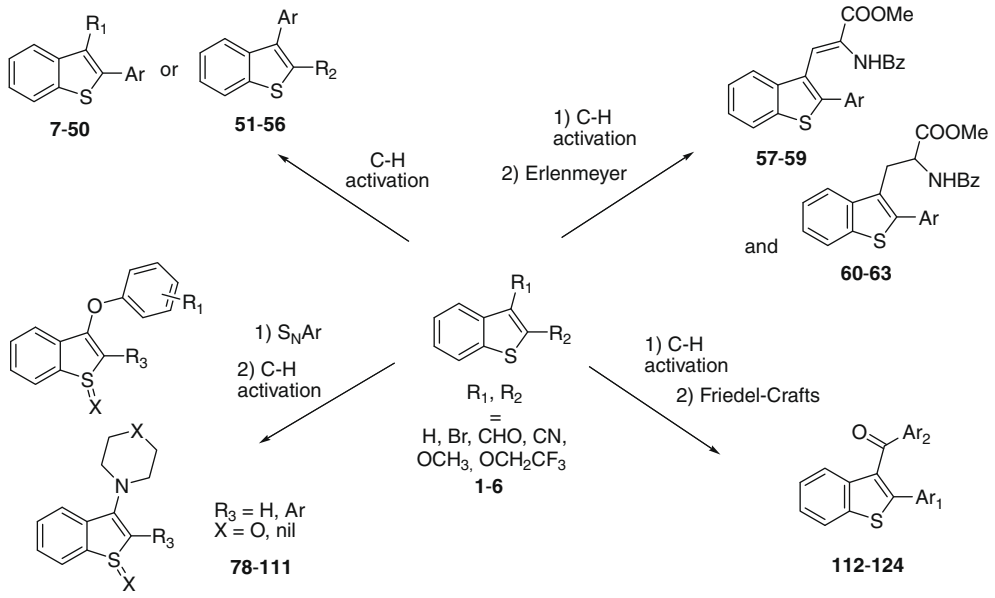


Levamisole (L-tetramisole)

Figure 1. Structure of levamisole.

* Corresponding authors. Tel.: +33 0 72 44 85 07; fax: +33 0 4 72 43 14 08.
E-mail address: pellet@univ-lyon1.fr (S. Pellet-Rostaing).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.08.048



Scheme 1. General synthesis of benzo[*b*]thiophene derivatives[35–40] **7–63** and **78–124**.

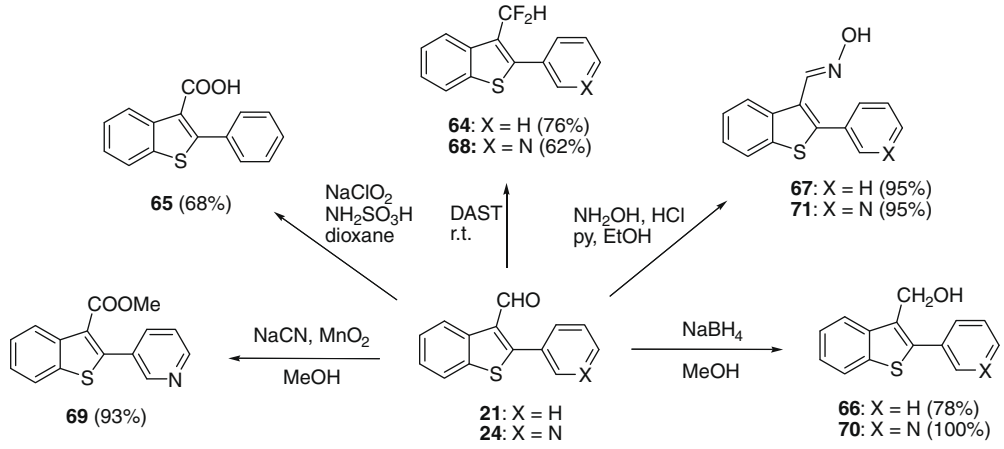
inhibitors of TNAP[34] demonstrating the great interest in the research of new active heterocycles. Recently we developed several strategies to synthesize benzothiophene derivatives,[35–40] which we could derivative easily, and having a large library, we firstly checked their inhibitory properties to several alkaline phosphatases. Some of them had significant inhibitory properties. Here, we present the synthesis and the BIAP (bovine intestinal alkaline phosphatase)/TNAP (porcine kidney tissue non-specific alkaline phosphatase and chicken femur tissue non-specific alkaline phosphatase) inhibition properties of a library of benzothiophene derivatives among which the 6-benzothiopheno-imidazo[2,1-*b*]thiazole derivatives (benzothiopheno-tetramisole and benzothiopheno-2,3-dehydrotetramisole), which could be implemented in a drug therapy for osteoarthritis.

2. Results and discussion

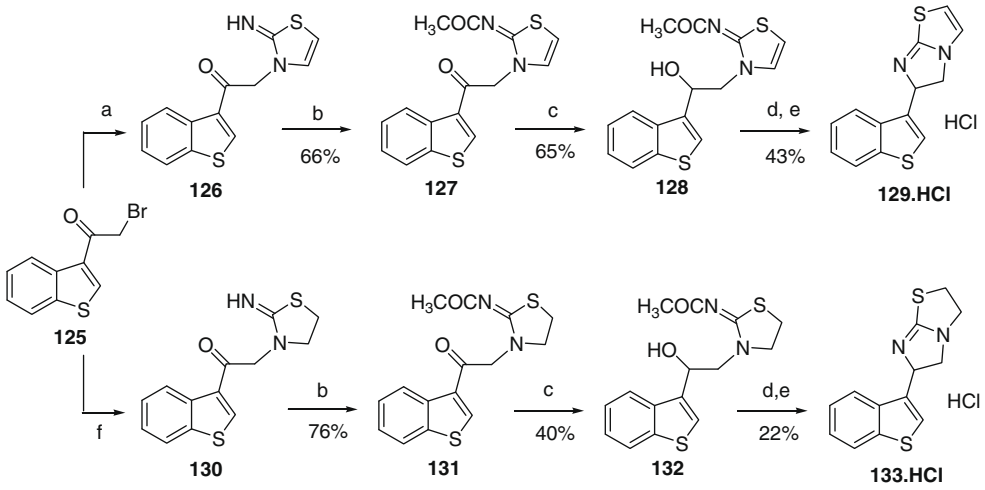
2.1. Chemistry

The synthetic chemistry used for the preparation of the benzothiophene derivatives **7–124** is shown in Schemes 1–3. Some of them were synthesized as previously reported in the literature.[35–40] Thus, 2- and 3-aryl-Benzo[*b*]thiophenes **7–56** (Tables 1 and 2) were obtained from the corresponding benzo[*b*]thiophene and 2- or 3-cyano, methoxy, carbaldehyde, (2,2,2-trifluoroethoxy)-benzo[*b*]thiophene by a one-step palladium coupling with various aryl bromides as described by us in previous works[35,39,40](Scheme 1). Compounds **57–63** were obtained as described[40] from the corresponding 2-arylbenzo[*b*]thiophenes-3-carbaldehydes involving an Erlenmeyer condensation with hippuric acid, followed by an electrophilic opening of azalactone intermediates and a hydrogenation of the alkenes. 2-Aryl-3-amino or phenoxybenzo[*b*]thiophenes **78–111**[36] were obtained from the starting 3-bromo-benzo[*b*]thiophene 1-oxide **77** (Table 3) by using an aromatic nucleophilic substitution reaction affording the 3-amino and 3-phenoxybenzo[*b*]thiophenes 1-oxide followed by a palladium coupling involving the corresponding aryl-bromides. Aroyl-Benzo[*b*]thiophenes **112–124** were synthesized by a direct acylation of benzo[*b*]thiophene or 2-aryl-benzo[*b*]thiophene.[37]

Finally, 2-aryl-3-methylamino-benzo[*b*]thiophene type-intermediates **72–76** (Table 2) were obtained by the reduction of the corresponding 2-aryl-3-cyano-benzo[*b*]thiophene derivatives with



Scheme 2. Synthesis of compounds **65–71**.



Scheme 3. Reagents and conditions: (a) 2-aminothiazole, 2-propanol, reflux, (78%); (b) Ac_2O, pyridine, chloroform, reflux; (c) $NaBH_4$, MeOH, rt; (d) $SOCl_2$, Ac_2O, reflux; (e) HCl, MeOH (f) 2-aminothiazoline, CH_3CN, reflux, (89%).

BH_3–THF.[38] New 2-aryl-benzothiophenes **65**–**71** were synthesized from the 2-phenyl-benzo[*b*]thiophene-3-carbaldehyde **21** and the 2-(3′-pyridine)-benzo[*b*]thiophene-3-carbaldehyde **24** (Scheme 2). Fluorination with DAST and amination with hydroxylamine afforded benzo[*b*]thiophenes **64, 67, 68** and **71**. Reduction of **21** and **24** with sodium borohydride gave the corresponding hydroxymethyl derivatives **66**[41] and **70**. Although it was already synthesized from thianaphthen-2-one,[42] the carboxylic acid **65** was easily obtained by oxidation of **21** with sodium chlorite and sulfamic acid. Finally, the compound **24** was oxidized[43] to a methyl ester **69** with manganese dioxide in the presence of sodium cyanide.

Benzo[*b*]thiopheno-2,3-dehydrotetramisole **129** and -tetramisole **133** were obtained in five steps from the known 3-(2-bromoacetyl)benzo[*b*]thiophene **125** previously synthesised from benzo[*b*]thiophene as reported in the literature[44] (Scheme 3). Following the synthetic methodology already described in the literature,[21,22] **129** was prepared in an overall yield of 14% by condensation of **125** with the 2-aminothiazole followed by the acylation of **126**, sodium borohydride reduction of **126** and ring closure of **128** with thionyl chloride and acetic anhydride. Following the similar strategy, the condensation of **125** with the 2-aminothiazoline gave the intermediate **130** which was converted into the levamisole derivative **133**.

2.2. Evaluation of BIAP and TNAP inhibition

The library of benzothiophene compounds **1**–**124** (0.1 mM in bovine intestinal alkaline phosphatase (BIAP) reactive medium containing *p*-nitrophenyl phosphate (*p*NPP) as substrate with final dimethyl sulfoxide (DMSO) 1% (v/v) and 0.4 mM in porcine kidney TNAP reactive medium with final DMSO 4% (v/v), respectively) was tested for the potential inhibition activity of the bovine intestinal alkaline phosphatase BIAP and the porcine kidney TNAP, respectively (Tables 1–4). As observed in Figure 2A and B, most of benzothiophene derivatives were ineffective or tend to inhibit towards BIAP and were totally ineffective towards TNAP. Among the 2-aryl-benzo[*b*]thiophenes **7**–**50** (Table 1 and Fig. 2A), **14** and **23** presented the more interesting effect with 95% and 91% inhibition of BIAP (corresponding, respectively to a residual activity of 5% and 9% as stated in Table 1), no inhibition was observed with the 3-H derivatives **43**–**50** and only the 2-(3'-pyridine)-benzo[*b*]thiophene **35** was the best porcine kidney TNAP inhibitor with a residual activity of 46% (Table 1 and Fig. 2B) whereas all of the other 2-(2' or 3'-pyridine)-benzo[*b*]thiophene are totally inefficient towards BIAP and porcine kidney TNAP.

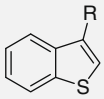
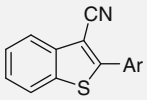
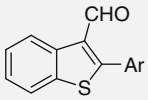
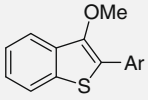
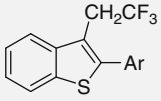
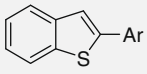
Although 2-aryl-benzo[*b*]thiophene-3-carbaldehydes are globally more efficient than the 3-H, 3-cyano, 3-methoxy and 3-OCH_2-CF_3 analogs, 2-phenyl-benzo[*b*]thiophen-3-oxime **67** (Table 2 and Fig. 2A) was one of the most promising. 0.1 mM of it inhibited almost totally the *p*NPP hydrolysis by BIAP (Fig. 3A) and 0.4 mM of it caused a residual TNAP-activity of 68% (Table 2). Figure 3B shows the inhibition of **67** on the bone TNAP activity of matrix vesicles (MVs). The inhibition was found to be dependent on the concentration of DMSO (data not shown), while DMSO (up to 4% v/v) alone had nearly no effect on BIAP, porcine kidney TNAP or bone TNAP activities. In this series (Table 2), no significant inhibition was observed with 3-aryl-benzo[*b*]thiophene derivatives **51**–**56** towards BIAP or porcine kidney TNAP and 3-aminomethyl compounds **72, 74** and **76** increased the activity of the BIAP.

Whatever the nature of the phenolic or amino moieties incorporated in the benzo[*b*]thiophene-sulfoxide **78**–**86** (Table 3) and 2-aryl-benzo[*b*]thiophene **100**–**110**, no significant inhibition was observed on BIAP and porcine kidney TNAP with compound concentrations of 0.1 mM and 0.4 mM, respectively. Only the cyano derivative **81** exhibited a relative residual porcine kidney TNAP-activity of 67% and the carbaldehyde **90** induced a residual BIAP-activity of 9%. Finally, in the series of 3-aroyl compounds **112**–**124** (Table 4), only 2-H derivatives **112** and **113** promoted, respectively 43% and 40% residual BIAP activities. No significant inhibition was observed on porcine kidney TNAP with compounds **112**–**124**.

As well as for the most promising compounds **35** and **81** (inhibiting specifically porcine kidney TNAP) or **4** and **67** (inhibiting both BIAP and porcine kidney TNAP), the solubilities of benzothiophene derivatives **1**–**124** were not high enough in aqueous buffer and DMSO (up to 4% v/v) was generally added to solubilize them. Addition of 4% v/v DMSO into aqueous mineralization medium induced spontaneously hydroxyapatite formation[45] as in the case of matrix vesicles which are released from hypertrophied chondrocytes during physiological endochondral ossification[46–48] or from osteoarthritic articular chondrocytes.[49–52]

DMSO is very often used as solvent for water- insoluble drugs and in several human therapeutic situations.[53] Although DMSO has some beneficial effects,[53] several reports indicate that care must be taken in the experiments with DMSO[54,55] or using DMSO as a drug vehicle.[56] DMSO induced hydroxyapatite formation in synthetic cartilage lymph.[45] Although we found that several water-insoluble benzothiophene molecules could inhibit TNAP, their effects were dependent on the DMSO concentration. Whether, DMSO could take the place of the inhibitor or alter the accessibility of the active site is

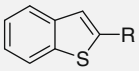
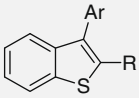
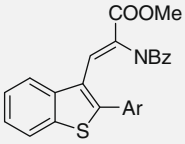
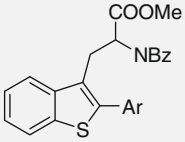
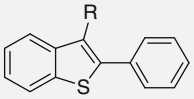
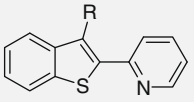
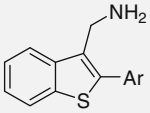
Table 1
Inhibition effects of benzo[*b*]thiophene derivatives **1–3** and **7–50** on BIAP and on porcine kidney TNAP activities at pH 10.4 and at 37 °C

General structures	Compound number	BIAP + 0.1 mM inhibitor with 1% DMSO Relative activity (%)	TNAP + 0.4 mM inhibitor with 4% DMSO Relative activity (%)
	CN: **1**	92	92
	CHO: **2**	120	85
	OMe: **3**	90	93
	Ph: **7**	76	N.T.
	4-OMe–Ph: **8**	94	N.T.
	4-CF_3–Ph: **9**	96	97
	2-Napht: **10**	18	99
	3-py: **11**	106	N.T.
	2-py: **12**	94	N.T.
	2-CN–Ph: **13**	90	N.T.
	2-Me–Ph: **14**	5	85
	3-Quinoline: **15**	95	N.T.
	3-Cl–Ph: **16**	115	N.T.
	4-Cl–Ph: **17**	69	N.T.
	3-OMe–Ph: **18**	97	N.T.
	3,4,5-OMe–Ph: **19**	88	91
	4-$N(Me)_2$–Ph: **20**	82	83
	Ph: **21**	41	77
	4-OMe–Ph: **22**	37	99
	4-Cl–Ph: **23**	9	N.T.
	3-Py: **24**	119	93
	2-CN–Ph: **25**	110	79
	3-Quinoline: **26**	110	N.T.
	4-CN–Ph: **27**	92	105
	2-NO_2–Ph: **28**	116	78
	4-CF_3–Ph: **29**	30	80
	3-Cl–Ph: **30**	62	95
	2-Cl–Ph: **31**	63	97
	Ph: **32**	80	87
	2-CN–Ph: **33**	57	79
	4-OMe–Ph: **34**	78	97
	3-Py: **35**	112	44
	2-Napht: **36**	102	N.T.
	2-Me–Ph: **37**	73	96
	3-Quinoline: **38**	64	86
	2-Py: **39**	99	90
	2-CN–Ph: **40**	70	87
	3-Py: **41**	122	84
	4-OMe–Ph: **42**	107	94
	2-Py: **43**	119	94
	2-NO_2–Ph: **44**	121	86
	2-NH_2–Ph: **45**	93	70
	2-NO_2-4-Me–Ph: **46**	127	97
	2-NO_2-4-Cl–Ph: **47**	107	93
	2-NH_2-4-Me–Ph: **48**	107	93
	3-Cl–Ph: **49**	95	96
	2-NO_2-4-OMe–Ph: **50**	99	96

not yet clear. Such behavior has been previously reported in the case of diethanolamine affecting the inhibition of a 2,3,4-trichlorophenyl derivative of pyrazole on TNAP activity.[34] Since DMSO is a promoter of mineralization, water-soluble benzothiophene derivatives have to be synthesized. We reasoned that water-soluble tetramisole could be derivatized with benzothiophene compounds to increase the solubility of benzothiophene moiety in water and to increase the inhibition effect having two active sites in one molecule. Therefore, we designed and synthesized water-soluble benzothiophene compounds.

As levamisole, **129** and **133** hydrochloride salt did not inhibit intestinal alkaline phosphatase (Table 5). However, levamisole chlorhydrate (Fig. 4), **129·HCl** (Fig. 5) and **133·HCl** (Fig. 6) inhibited the porcine kidney TNAP. Both samples as levamisole inhibited, in an uncompetitive manner, porcine kidney TNAP activity, being consistent with the fact that the location of the binding site of the inhibitor is controlled by the tetramisole moiety. Their plots of v_{max}^{-1} versus inhibitor concentration (Figs. 4B, 5B and 6B) allowed us to determine their apparent inhibition constants K_i (pH 7.8; 37 °C and without DMSO). Small differences in their apparent inhibitor constants (K_i) were observed. A better stabilization of the interactions between inhibitor and porcine kidney TNAP was evidenced for **129·HCl** (K_i = 85 μM) as compared with **133·HCl** (K_i = 135 μM) (Table 6).

Table 2
Inhibition effects of benzo[*b*]thiophene derivatives **4–6** and -**51–76** on BIAP and on porcine kidney TNAP activities at pH 10.4 and at 37 °C

General structures	Compound number	BIAP + 0.1 mM inhibitor with 1% DMSO Activity (%)	TNAP + 0.4 mM inhibitor with 4% DMSO Activity (%)
	I: **4**	41	54
	OCH_2CF_3: **5**	116	100
	CN: **6**	103	105
	R = H, Ar =4-OMe–Ph: **51**	100	N.T.
	R = CN, Ar = 4-CF_3–Ph: **52**	115	N.T.
	Ar = 3-Py: **53**	120	N.T.
	R = OCH_2CF_3, Ar = 2-CN–Ph: **54**	101	87
	Ar = 3-Py: **55**	102	97
	R = 2-NO_2–Ph Ar = 2-NO_2–Ph: **56**	108	88
	4-CN–Ph: **57**	108	N.T.
	4-CF_3–Ph: **58**	35	87
	4-OMe–Ph: **59**	77	91
	4-CN–Ph: **60**	115	N.T.
	4-CF_3–Ph: **61**	63	N.T.
	4-OMe–Ph: **62**	91	86
	H: **63**	116	74
	CF_2: **64**	94	90
	COOH: **65**	113	78
	CH_2OH: **66**	103	87
	C=N–OH: **67**	1	68
	CF_2: **68**	103	79
	COOMe: **69**	117	98
	CH_2OH: **70**	104	106
	C=N–OH: **71**	103	71
	Ph: **72**	148	76
	3-Py: **73**	105	111
	2,3,4-(OMe)3-Ph: **74**	142	94
	2-NH_2-4-OMe–Ph: **75**	103	103
	3-$N(CH_3)_2$: **76**	134	84

2.3. Comparison of inhibition effects of levamisole and benzothiopheno-tetramisole derivatives

Although porcine kidney TNAP activity is usually measured at alkaline pH as to screen putative inhibitors (Table 5), the inhibition property of samples and kinetic parameters were determined at pH 7.8 and at 37 °C to match physiological conditions.[57] The apparent K_i of porcine kidney TNAP for levamisole was 93 μM at pH 7.8 and at 37 °C (Table 6). The K_i of human TNAP for levamisole amounted to 16 μM or to 136 μM for that of chicken TNAP measured at pH 9.8.[57] The difference in K_i of human TNAP and that of chicken TNAP was attributed to the presence of His-434 residue.[58] The source of alkaline phosphatase (tissues/organs) could modify the inhibition effects of inhibitors.[59] The K_i of racemic **129z·HCl** (K_i = 85 μM) and that of racemic **133·HCl** (K_i = 135 μM) were slightly distinct from that of enantiomeric levamisole (K_i = 93 μM) indicating that there is some potential to synthesize and optimize enantiomeric levamisole derivatives. Although **129·HCl** and **133·HCl** had similar inhibition effects than levamisole, their side effects could be entirely different than those of levamisole. The strategy to develop drug-like-TNAP soluble inhibitors for therapeutic use for treating pathological soft tissue mineralization disorders[34,57,58] looks promising.

3. Conclusion

Some benzothiophene derivatives showed more pronounced inhibition properties towards BIAP than porcine kidney TNAP. Such compounds may have a clinical application, since the intestinal type of alkaline phosphatase increased in the urine of patients with renal disease.[60] On the other hand, 6-benzothiopheno-imidazo[2,1-*b*]thiazole derivatives (benzothiopheno-tetramisole and benzothiopheno-2,3-dehydrotetramisole), proved to be efficient in the porcine kidney TNAP and in chicken femur TNAP inhibitions, which could be implemented in a drug therapy for osteoarthritis. Two water-soluble racemic benzothiopheno-tetramisole and -2,3-dehydrotetramisole (**129·HCl and 133·HCl)** with apparent inhibition constants K_i = 85 ± 6 μM and 135 ± 3 μM (n = 3) comparable to that of enantiomeric levamisole 93 ± 4 μM were found (as determined with porcine kidney TNAP), indicating some potential to synthesize and optimize enantiomeric benzothiopheno-tetramisole.

4. Experimental section

4.1. Materials

Reactants and solvents were supplied by Aldrich, Acros, Lancaster, Alfa Aeser and Fluka and purchased at the highest commercial quality and used without further purification. Porcine kidney tissue non-specific alkaline phosphatase (TNAP), bovine intestinal alkaline phosphatase (BIAP) and levamisole hydrochloride were purchased from Sigma and used without further purification. *p*-Nitrophenylphosphate was obtained from Fluka.

All reactions were carried out under an argon atmosphere with dry solvents under anhydrous conditions, unless otherwise stated. NMR spectra were recorded on a Bruker DPX-300 (^{1}H: 300 MHz; ^{13}C: 300 MHz) instrument using $CDCl_3$ and DMSO as solvents. The chemical shifts (δ ppm) and coupling constants (Hz) are reported in the standard fashion. The following abbreviations are used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Electrospray ionization (ESI) mass spectrometry (MS) experiments were performed on a thermo Finnigan LCQ Advantage mass. High-resolution mass spectra (HRMS) were recorded on a Finnigan Mat 95xL mass spectrometer using CI. Analytical thin-layer chromatography was effected on silica gel Merck 60 D254 (0.25 mm). Flash chromatographies were performed on Merck Si 60 silica gel (40–63 μm) Merck aluminum oxide 90 active neutral (63–200 μm).

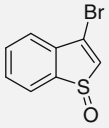
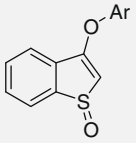
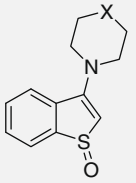
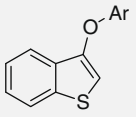
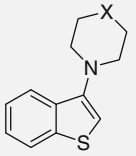
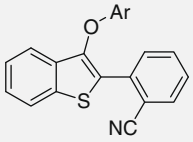
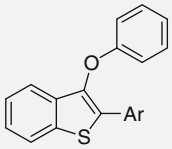
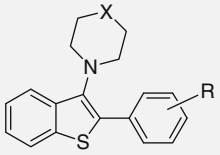
4.2. Chemistry. General procedure of 2-aryl-3(difluoromethyl)-benzo[*b*]thiophenes 64 and 68

DAST (3.08 mL, 25.2 mmol) was added dropwise to the solid 2-aryl-benzo[*b*]thiophene-3-carboxaldehyde (0.84 mmol) **21** or **24** and the resulting red solution was stirred at room temperature for 16 h. under argon. The mixture was then poured dropwise into ice-cold water (50 mL) and extracted with CH_2Cl_2 (2 × 50 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated. The residue was purified by flash column chromatography (SiO_2) to obtain the pure desired compound.

4.2.1. 2-Phenyl-3-(difluoromethyl)-benzo[*b*]thiophene 64

Eluent: cyclohexane. Yield: 75%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.16 (dd, J = 0.9 Hz, 6.8 Hz, 1H), 7.85 (dd, J = 0.9 Hz, 6.8 Hz, 1H),

Table 3
Inhibition effects of benzo[*b*]thiophene derivatives **77–111** on BIAP and on porcine kidney TNAP activities at pH 10.4 and at 37 °C

General structures	Compound number	BIAP + 0.1 mM inhibitor with 1% DMSO Relative activity (%)	TNAP + 0.4 mM inhibitor with 4% DMSO Relative activity (%)
	**77**	105	100
	Ph: **78**	102	104
	4-OMe–Ph: **79**	107	100
	4-Cl–Ph: **80**	122	103
	4-CN–Ph: **81**	106	67
	4-F–Ph: **82**	109	79
	3,4,5-$(OMe)_3$-Ph: **83**	127	101
	CH_2: **84**	110	101
	N–CH_3: **85**	102	95
	O: **86**	97	111
	Ph: **87**	104	89
	4-OMe–Ph: **88**	96	82
	4-F–Ph: **89**	104	78
	4-CHO–Ph: **90**	9	N.T.
	4-Cl–Ph: **91**	107	94
	t-Bu–Ph: **92**	113	100
	4-CH_2NH_2–Ph: **93**	105	83
	4-CN–Ph: **94**	87	85
	3,4,5-$(OMe)_3$-Ph: **95**	129	101
	N-(2-OH)-Ph: **96**	49	109
	N–CH_3: **97**	107	97
	O: **98**	108	99
	CH_2: **99**	92	80
	4-F–Ph: **100**	66	85
	Ph: **101**	110	91
	2,3,4-$(OMe)_3$-Ph: **102**	121	106
	4-Cl–Ph: **103**	101	79
	4-OMe–Ph: **104**	106	98
	4-CN–Ph: **105**	82	97
	4-OMe-Ph: **106**	105	94
	Ph: **107**	104	91
	X = CH_2; R=2-CN: **108**	99	100
	X = CH_2; R = 4-OMe: **109**	106	91
	X = CH_2; R = H: **110**	99	81
	X = O; R = 2-CN: **111**	107	93

7.53–7.38 (m, 7H), 6.77 (t, J = 54 Hz, 1H, CF_2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 147.1, 139.1, 136.9, 132.1, 129.9, 129.5, 129.1, 125.2, 124.9 (t, J = 24.5 Hz), 124.0, 122.2, 112.7 (t, J = 232 Hz). ^{19}F NMR ($CDCl_3$): −108.9. MS (EI^+): m/z 260($[M+H]^+$).

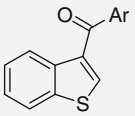
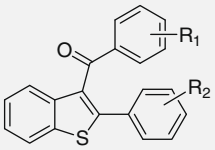
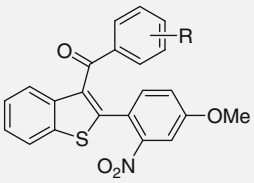
4.2.2. 2-(3′-Pyridine)-3-(difluoromethyl)-benzo[*b*]thiophene 68

Eluent: cyclohexane/AcOEt (8:2). Yield: 62%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.77 (d, br, 2H), 8.16 (dd, J = 1.1 Hz, 6.6 Hz, 1H), 7.81 (dd, J = 1.0 Hz, 6.6 Hz, 1H), 7.75 (ddd, J = 1.7 Hz, 1.7 Hz, 7.9 Hz 1H), 7.47–7.34 (m, 3H), 6.73 (t, J = 53.7 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 150.4, 149.8, 142.5 (t, J = 9.82 Hz), 139.1, 136.9, 136.6, 128.3, 125.9 (t, J = 25.1 Hz), 125.6, 125.4, 123.8 (t, J = 2.2 Hz), 123.5, 122.1, 112.0 (t, J = 234 Hz). ^{19}F NMR ($CDCl_3$): −109.2. MS (EI^+): m/z 261($[M+H]^+$).

4.3. General procedure of 2-aryl-3(hydroxymethyl)-benzo[*b*]thiophenes 66 and 70

$NaBH_4$ (0.6 mmol) was added to a solution of **21** or **24** (0.4 mmol) in MeOH (2 mL) at 0 °C under argon. After stirring for

Table 4
Inhibition effects of benzo[*b*]thiophene derivatives **112–124** on BIAP and on porcine kidney TNAP activities at pH 10.4 and at 37 °C

General structures	Compound number	BIAP + 0.1 mM inhibitor with 1% DMSO Relative activity (%)	TNAP + 0.4 mM inhibitor with 4% DMSO Relative activity (%)
	Ph: **112**	43	80
	4-OMe-Ph: **113**	40	96
	R_1 = H, R_2 = 2-CN: **114**	91	92
	R_1 = 4-OMe, R_2 = 4-OMe: **115**	70	108
	R_1 = 2-OMe, R_2 = 2-NO2: **116**	92	98
	R_1 = 3-OMe,R_2 = 2-NO_2: **117**	98	96
	R_1 = 4-OMe,R_2 = 2-NO_2: **118**	107	101
	H: **119**	114	97
	3-OMe: **120**	116	100
	4-OMe: **121**	95	100
	2-OMe: **122**	117	111
	3, 5-$(OMe)_2$-4-OH: **123**	125	95
	3,4,5-(OMe)3: **124**	124	92

2 h at 0 °C, the reaction mixture was quenched with acetone, poured into NH_4Cl 10% (10 mL) and extracted with CH_2Cl_2 (2 × 10 mL). The organic layers were collected, dried over $MgSO_4$, filtered and concentrated. The residue was purified by flash column chromatography (SiO_2) to obtain the pure desired compound.

4.3.1. 2-Phenyl-3-(hydroxymethyl)-benzo[*b*]thiophene 66[41]

Eluent: cyclohexane/AcOEt (9:1). Yield: 78%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.96 (d, J = 7 Hz, 1H), 7.86 (d, J = 7 Hz, 1H), 7.61 (dd, J = 1.7 Hz, 6.6 Hz, 2H), 7.51–7.35 (m, 5H), 4.91 (s, 2H). ^{13}C NMR (75 MHz, CDCl^3) δ 143.1, 104.1, 139.2, 133.8, 130.4, 129.8, 128.9, 128.6, 124.7, 122.4, 122.3, 56.97. MS (EI$^+$): m/z 240([M+H]$^+$).

4.3.2. 2-(3'-Pyridine)-3-(hydroxymethyl)-benzo[*b*]thiophene 70

Eluent: cyclohexane/AcOEt (7:3). Yield: 100%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.86 (d, J = 2.2 Hz, 1H), 8.66 (dd, J = 1.5 Hz, 4.7 Hz, 1H), 8.08–8.00 (m, 3H), 7.58 (ddd, J = 0.8 Hz, 4.7 Hz, 4.7 Hz, 1H), 7.46 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 149.4, 149.3, 140.0, 138.4, 136.8, 136.7, 133.4, 129.6, 125.1, 124.7, 123.9, 123.3, 122.3, 54.85. MS (EI$^+$): m/z 241([M+H]$^+$).

4.4. General procedure of 2-aryl-3-oxime-benzo[*b*]thiophenes 67 and 71

Pyridine (1.26 mmol) was added to a solution of **21** or **24** (0.353 mmol) and NH_2OH·HCl (1.47 mmol) in EtOH (1 mL). The mixture was refluxed for 1.5 h. After cooling to room temperature, CH_2Cl_2 (15 mL) was added and the solution was poured into cold water (30 mL). After addition of CH_2Cl_2 (15 mL), the organic phase was separated, dried over $MgSO_4$ and concentrated under vacuum at 20 °C. Purification by chromatography (SiO_2) provided pure products.

4.4.1. 2-Phenyl-3-oxime-benzo[*b*]thiophene 67

Eluent: cyclohexane/AcOEt (9:1). Yield: 85%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.63 (d, J = 7.7 Hz, 1H), 8.40 (s, 1H), 7.85 (d, J = 7.7 Hz, 1H), 7.55–7.39 (m, 8H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 147.8, 147.0, 138.8, 137.5, 133.1, 130.2, 129.1, 128.9, 125.6, 125.5, 125.2, 123.7, 122.0. MS (EI$^+$): m/z 253([M+H]$^+$).

4.4.2. 2-(3'-Pyridine) -3-oxime-benzo[*b*]thiophene 71

Eluent: cyclohexane/AcOEt (7:3). Yield: 95%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.49 (s, 1H), 8.75 (s, 1H), 8.70 (d, J = 4.4 Hz, 1H), 8.58 (d, J = 7.4 Hz, 1H), 8.18 (s, 1H), 8.06 (d, J = 7.7 Hz, 1H), 7.99 (d, J = 7.7 Hz, 1H), 7.60–7.45 (m, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 150.0, 149.7, 143.5, 141.0, 138.3, 137.3, 136.9, 128.9, 125.6, 125.5, 125.4, 125.3, 123.9, 122.5. MS (EI$^+$): m/z 254([M+H]$^+$).

4.4.3. 2-Phenyl-benzo[*b*]thiophene-3-carboxylic acid 65

Compound 21 (0.09 g, 0.377 mmol) was dissolved in 5.4 mL of dioxane/H_2O (7:3). $NaClO_2$ (0.045 g, 0.5 mmol) and NH_2SO_3H (0.209 g, 2.15 mmol) was added and the mixture was stirred for 2 h at room temperature. After addition of 10% $NaHCO_3$ and extraction with AcOEt, the organic layer was washed with 2 N HCl. The organic layer was separated, dried over $MgSO_4$, filtered and evaporated. Purification by flash chromatography (SiO_2, cyclohexane/AcOEt 8:2) produced pure **65** in 68% yield (0.065 g). ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.38 (s, 1H), 8.51 (d, J = 8.1 Hz, 1H),

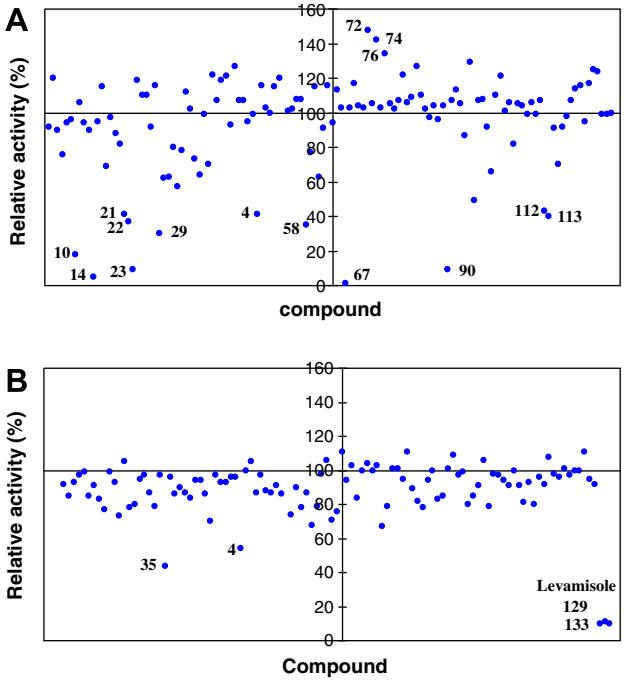


Figure 2. Inhibition effects of benzo[*b*]thiophene derivatives **1–124** on bovine intestinal BIAP (A) and on porcine kidney TNAP (B) activities at pH 10.4 and at 37 °C.

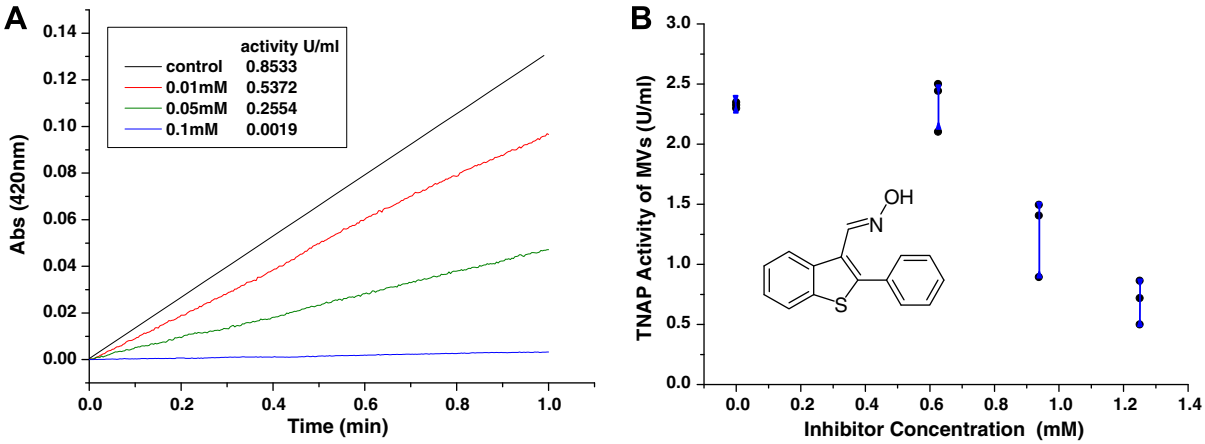


Figure 3. Inhibition of *p*NPP hydrolysis by 0.2 μg ml^{-1} bovine intestinal BIAP with 0–0.1 mM benzothiophenyl compound **67** in 1% DMSO (v/v) (A). Inhibition of *p*NPP hydrolysis by 10 μg protein ml^{-1} matrix vesicles containing bone TNAP with 0–1 mM compound **67** in 1% DMSO (v/v) at pH 10.4 and at 37 °C (B). *p*NPP concentration was 0.1 mM. Three independent measurements were made.

7.84 (d, J = 8.1 Hz, 1H), 7.60–7.40 (m, 7H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.2, 162.4, 155.1, 138.7, 138.2, 133.8, 129.8, 129.3, 128.3, 125.8, 125.2, 121.8. MS (EI$^+$): *m/z* 254([M+H]$^+$).

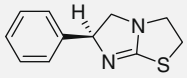
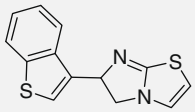
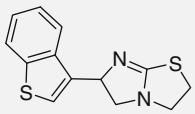
4.4.4. 2-(3'-Pyridine)-benzo[*b*]thiophene-3-carboxylic acid methyl ester 69

NaCN (0.195 g, 4 mmol) and MnO_2 (1.38 g, 15.8 mmol) were added to a solution of **24** (0.19 g, 0.8 mmol) in MeOH (30 mL). The mixture was stirred at room temperature overnight. CH_2Cl_2 (100 mL) was then added and the resulting precipitate filtered on Celite. The filtrate was concentrated and dissolved in H_2O/CH_2Cl_2. The organic phase was washed with water, dried over $MgSO_4$ and evaporated. Purification by flash chromatography (SiO_2, cyclohexane/AcOEt 9:1) gave pure **69** in 93% yield (0.2 g). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.73 (s, 1H), 8.62 (d, J = 4.0 Hz, 1H), 8.41 (d, J = 7.9 Hz, 1H), 7.79 (d, J = 7.9 Hz, 2H), 7.49–7.31 (m, 3H), 3.74 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.7, 149.7, 147.9, 138.7, 138.2, 136.8, 130.3, 125.7, 125.4, 125.0, 123.8, 122.8, 121.8, 51.7. MS (EI$^+$): *m/z* 269 ([M+H]$^+$).

4.4.5. Compound 126

Benzo[*b*]thiophene **125** (9.7 mmol, 2.48 g) was refluxed for 1 h with 2-aminothiazole (9.7 mmol, 0.97 g) in 25 ml of 2-propanol. The resulting solid was filtered, triturated with a 10% Na_2CO_3 solution then filtered and dried. The crude product was purified by flash chromatography (EtOAc) to give **127** (2.1 g, 78%) as a milky solid. Data for compound **126:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.63 (s, 1H), 9.16 (s, 1H), 8.53 (dd, 1H, J = 1.5 Hz, 6.8 Hz), 8.17 (dd, 1H, J = 0.75 Hz, 7.1 Hz), 7.55–7.5 (m, 2H), 7.44 (d, 1H, J = 4.5 Hz), 7.1 (d, 1H, J = 4.5 Hz), 5.88 (s, 2H); ^{1}C NMR (75 MHz, DMSO-d_6) δ 185.9, 169.5, 162.2, 141.4, 139.5, 136.5, 131.3, 131.1, 126.5, 126.1, 124.7, 107.8, 55.5; MS(ESI): 275[M+H]$^+$; HR ESIMS calcd for $C_{13}H_{11}OS_2N_2^+$ = 275.0313; found = 275.03121.

Table 5
Inhibition effects of levamisole derivatives on BIAP and on porcine kidney TNAP activities at pH 10.4 and at 37 °C

General structures	Compound number	BIAP + 0.1 mM inhibitor with 1% DMSO relative activity (%)	TNAP + 0.4 mM inhibitor with 4% DMSO Relative activity (%)
	**Levamisole·HCl**	99	10
	**129·HCl**	99	11
	**133·HCl**	100	19

4.4.6. Compound 127

To a mixture of **126** (7.6 mmol, 2.1 g) and 5 ml of pyridine in 50 ml of chloroform was added 2 ml of acetic anhydride. The mixture was refluxed for 1.5 h and the chloroform was removed to leave oil. Then the residue was washed by ethyl ether and purified by flash chromatography (EtOAc) to afford **127** (1.6 g, 66%) as a brown solid. Data for compound **127:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.3 (s, 1H), 8.54 (dd, 1H, J = 0.9 Hz, 2.8 Hz), 8.15 (dd, 1H, J = 1.9 Hz, 7.0 Hz), 7.53–7.50 (m, 2H), 7.48 (d, 1H, J = 4.7 Hz), 7.0 (d, 1H, J = 4.7 Hz), 5.82 (s, 2H), 2.02 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 187.7, 178.7, 167.2, 141.0, 139.6, 136.4, 131.8, 128.2, 126.4, 126.1, 124.8, 123.4, 108.3, 54.8, 27.0; MS(ESI): 317 [M+H]$^+$; HR ESIMS calcd for $C_{15}H_{13}O_2S_2N_2^+$ = 317.0418; found = 317.04179.

4.4.7. Compound 128

To a solution of **127** (3 mmol, 1 g) in 25 ml of methanol maintained at 10 °C was added in small portions 5 mmol of $NaBH_4$. The solution was stirred at room temperature for 2 h, solvent was removed under vacuum, and the residue was suspended in water and extracted with DCM. The DCM layer was dried with $MgSO_4$ and the solvent was removed to leave a solid which was purified by flash chromatography (EtOAc/MeOH = 95/5) to afford **128** (0.65 g, 65%) as a white solid. Data for compound **128:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.45 (d, 1H, J = 7.4 Hz), 8.00 (d, 1H, J = 8.5 Hz), 7.64 (s, 1H), 7.47–7.40 (m, 3H), 6.92 (d, 1H, J = 4.7 Hz), 5.95 (d, 1H, J = 5.1 Hz), 5.38 (s, 1H), 4.63 (dd, 1H J = 2.4 Hz, 13.4 Hz), 4.12 (dd, 1H J = 9.0 Hz, 13.4 Hz), 2.20 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 178.5, 166.5, 140.5, 138.2, 137.5, 129.1, 124.9, 124.5, 123.5, 123.4, 122.8, 107.6, 66.8, 54.7, 27.0; MS(ESI): 319 [M+H]$^+$; HR ESIMS calcd for $C_{15}H_{15}O_2S_2N_2^+$ = 319.0575; found = 319.05759.

4.4.8. Compound 129

Compound **128** (0.63 mmol, 0.2 g) was added in small portion to 2 ml of thionyl chloride at 5 °C over a period of 30 min. The mix-

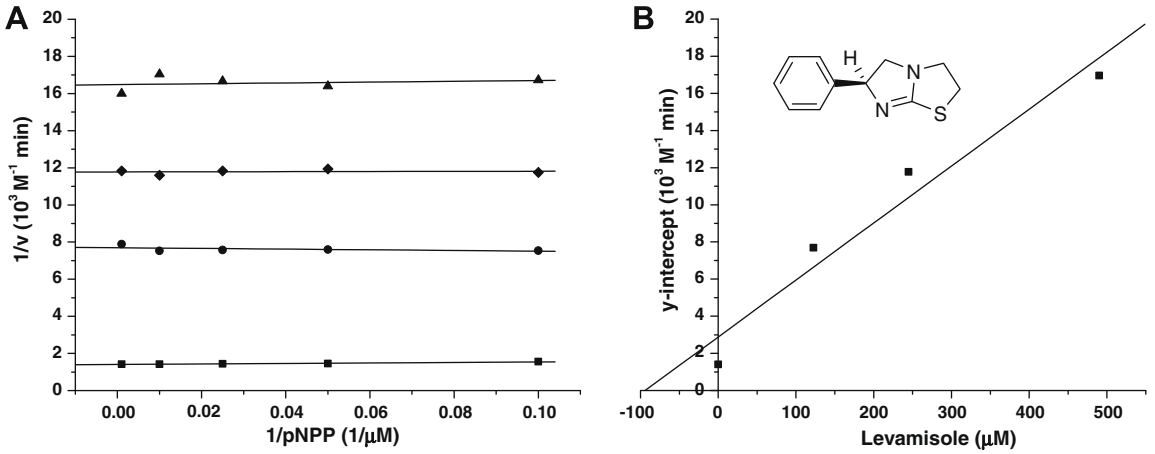


Figure 4. Inhibition of *p*NPP hydrolysis of porcine kidney TNAP by levamisole. HCl at pH 7.8 and at 37 °C without DMSO. (A) Linewaever–Burk plot in the presence of 6 μg ml^{-1} TNAP with 10, 20, 40, 100 and 1000 μM *p*NPP and increasing concentration of 0 μM (■), 122.5 μM (●), 245 μM (♦) and 490 μM (▲) levamisole. (B) Plot of v_{max}^{-1} versus levamisole concentration that enabled us to determine apparent K_i. The *x* intercept gives the negative value of K_i.

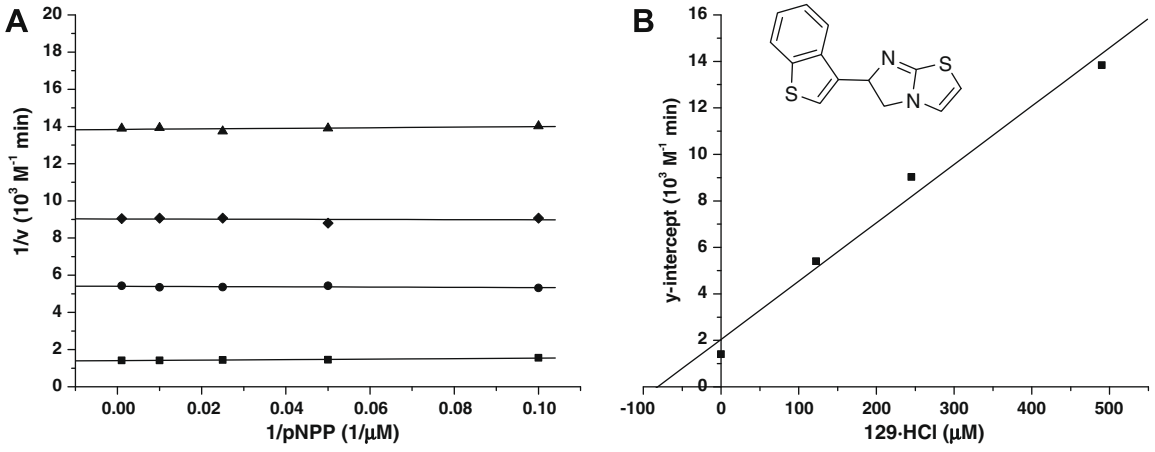


Figure 5. Inhibition of *p*NPP hydrolysis of porcine kidney TNAP by **129·HCl** at pH 7.8 and at 37 °C without DMSO. (A) Linewaever–Burk plot in the presence of 6 μg ml^{-1} TNAP with 10, 20, 40, 100 and 1000 μM *p*NPP and increasing concentration of 0 μM (■), 122.5 μM (●), 245 μM (♦) and 490 μM (▲) μM **129·HCl**. (B) Plot of v_{max}^{-1} versus **129·HCl** concentration that enabled us to determine apparent K_i. The *x* intercept gives the negative value of K_i.

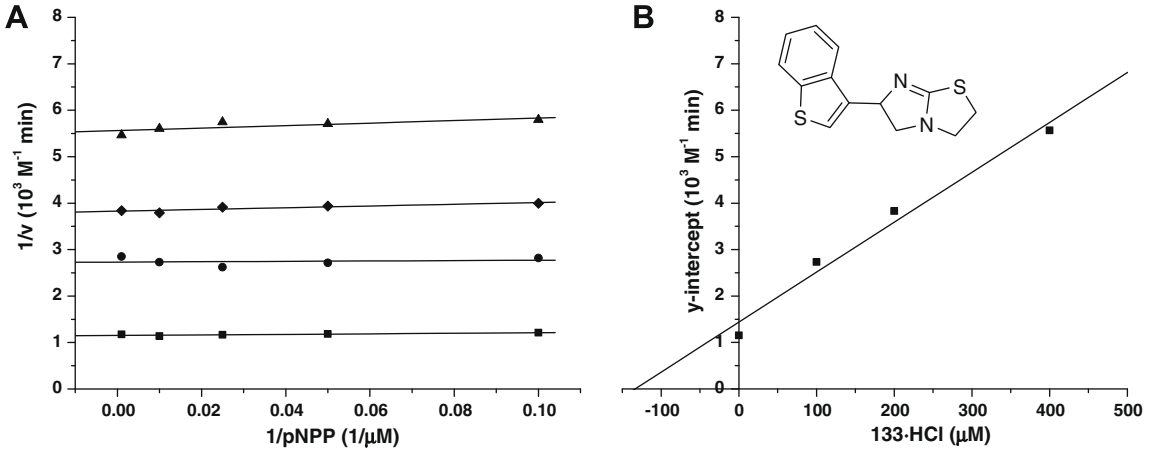


Figure 6. Inhibition of *p*NPP hydrolysis of porcine kidney TNAP by **133·HCl** at pH 7.8 and at 37 °C without DMSO. (A) Linewaever–Burk plot in the presence of 6 μg ml^{-1} TNAP with 10, 20, 40, 100 and 1000 μM *p*NPP and increasing concentration of 0 μM (■), 100 μM (●), 200 μM (♦) and 400 μM (▲) **133·HCl**. (B) Plot of v_{max}^{-1} versus **133·HCl** concentration that enabled us to determine apparent K_i. The *x* intercept gives the negative value of K_i.

ture was stirred at room temperature for 1 h, Ac_2O (10 ml) was added, and the acetyl chloride which formed was removed under vacuum. The mixture was refluxed for another 0.5 h and the excess Ac_2O was removed under vacuum. The residue was washed by 10% Na_2CO_3 solution and extracted with DCM. The DCM layer was dried with $MgSO_4$ and the solvent was removed to leave a solid which

Table 6
Apparent inhibition constants (K_i) of porcine kidney TNAP activity determined at pH 7.8 and at 37 °C without DMSO

Inhibitors	Type	K_i (μM) ± SD
Levamisole·HCl	Uncompetitive	93 ± 4 (n = 3)
129·HCl	Uncompetitive	85 ± 6 (n = 3)
133·HCl	Uncompetitive	135 ± 3 (n = 3)

was purified by flash chromatography (EtOAc) affording **129** (0.07 g, 43%) as a white solid. Then **129** was directly dissolved in 2 ml methanol and 37% hydrochloric acid in water was added. The mixture was stirred overnight and the solvent was removed to leave a slight green solid. After washing with acetone and dried, the hydrochloride salt of **129** was obtained. Data for compound **129·HCl:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.87 (dd, 1H, J = 2.1 Hz, 7.7 Hz), 7.70 (dd, 1H, J = 2.5 Hz, 6.6 Hz), 7.45 (s, 1H), 7.41–7.32 (m, 2H), 6.48 (d, 1H, J = 4.5 Hz), 5.90 (t, J = 8.7 Hz, 1H), 5.77 (d, J = 8.7 Hz, 1H), 4.31 (t, J = 8.7 Hz, 1H), 3.78 (t, J = 8.7 Hz 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.6, 141.6, 138.2, 137.5 124.8, 124.5, 123.6, 123.2, 122.8, 121.9, 102.1, 71.1 53.2; MS(ESI): 259 $[M+H]^+$; HR ESIMS calcd for $C_{13}H_{11}S_2N_2^+$ = 259.0364; found = 259.03637.

4.4.9. Compound 130

2-Aminothiazoline (11.8 mmol, 1.2 g) was dissolved in 25 ml acetonitrile and small portions of 3-(2-bromoacetyl)benzo[*b*]thiophene **125** (11.8 mmol, 3 g) was added. The mixture was stirred at rt for 2 h and filtered. The precipitate was macerated with a 10% Na_2CO_3 solution then filtered and dried. The crude product was purified by flash chromatography (EtOAc) to afford **130** (2.88 g, 88.6%) as a white solid. Data for compound **130:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.08 (s, 1H), 8.59 (dd, 1H, J = 1.5 Hz, 7.7 Hz), 8.10 (dd, 1H, J = 1.1 Hz, 8.7 Hz), 7.55–7.44 (m, 2H), 4.85 (s, 2H), 3.72 (t, J = 8.7 Hz, 2H), 3.23 (t, J = 8.7 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 191.0, 161.9, 139.8, 139.5, 136.6, 132.5, 126.2, 125.8, 124.9, 123.3, 52.5, 52.0, 27.2; MS(ESI): 277 $[M+H]^+$; HR ESIMS calcd for $C_{13}H_{13}OS_2N_2^+$ = 277.0469; found = 277.04684.

4.4.10. Compound 131

To a mixture of **130** (10.4 mmol, 2.88 g) and 5 ml of pyridine in 50 ml of chloroform was added 2 ml of acetic anhydride. The mixture was refluxed for 1.5 h and the chloroform was removed to leave an oil. Then the residue was washed by ethyl ether and purified by flash chromatography (EtOAc) to afford **131** (2.52 g, 76%) as a brown solid. Data for compound **131:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.15 (s, 1H), 8.57 (dd, 1H, J = 1.5 Hz, 8.7 Hz), 8.12 (dd, 1H, J = 1.3 Hz, 8.5 Hz), 7.55–7.46 (m, 2H), 5.20 (s, 2H), 3.76 (t, J = 8.7 Hz, 2H), 3.22 (t, J = 8.7 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 188.8, 180.7, 170.0, 140.6, 139.5, 136.5, 132.0, 126.3, 125.9, 124.8, 123.4, 54.0, 50.4, 27.5, 27.0; MS(ESI): 319 $[M+H]^+$; HR ESIMS calcd for $C_{15}H_{15}O_2S_2N_2^+$ = 319.0575; found = 319.05751.

4.4.11. Compound 132

To a solution of **131** (4.7 mmol, 1.5 g) in 25 ml of methanol maintained at 10 °C was added in small portions 5 mmol of $NaBH_4$. The solution was stirred at room temperature for 2 h, solvent was removed under vacuum, and the residue was suspended in water and extracted with DCM. The DCM layer was dried with $MgSO_4$ and the solvent was removed to leave a solid which was purified by flash chromatography (EtOAc/MeOH = 95/5) to afford **132** (0.6 g, 40%) as a yellowish solid. Data for compound **132:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.31 (d, J = 7.2 Hz, 1H), 7.99 (d, J = 7.2 Hz, 1H), 7.63 (s, 1H), 7.45–7.35 (m, 2H), 5.89 (d, J = 7.2 Hz, 1H), 5.35 (t, *br*, 1H), 4.12 (dd, J = 2.4, 13.5 Hz 1H), 3.95 (m, 1H) 3.70 (m, 1H), 3.47 (m 1H) 3.15–3.06 (m, 2H), 2.18 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 180.4, 169.9, 140.5, 138.8, 137.5, 124.8, 124.3, 123.3, 123.2, 122.9, 67.5, 54.0, 51.7, 27.4, 27.1; MS(E-SI): 321 $[M+H]^+$; HR ESIMS calcd for $C_{15}H_{16}O_2S_2N_2Na^+$ = 343.0551; found = 343.05518.

4.4.12. Compound 133

A solution of **132** (0.63 mmol, 0.2 g) in 15 ml of chloroform was added to 2 ml of thionyl chloride at 5 °C over a period of 30 min. The mixture was stirred at room temperature for 2 h, 20 ml of NaOH solution (1 M) was added, and the mixture was refluxed for 1 h. Organic layer was dried by $MgSO_4$, then filtered and the solvent removed. The residue was purified by flash chromatography (DCM/EtOAc = 1/1) to afford **133** (35 mg, 21.5%) as a milky solid. Then **133** was dissolved in 2 ml methanol and 37% hydrochloric acid in water was added. The mixture was stirred overnight and the solvent was removed to leave a slight grey solid. After washing with acetone and dried, the hydrochloride salt of **133** was obtained. Data for compound **133·HCl:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.86 (dd, 1H, J = 2.8 Hz, 7.0 Hz), 7.74 (dd, 1H, J = 2.1 Hz, 6.0 Hz), 7.46 (s, 1H), 7.40–7.32 (m, 2H), 5.85 (t, J = 8.7 Hz, 1H), 3.85 (t, J = 8.7, 1H), 3.74–3.56 (m, 2H), 3.44 (t, J = 8.7 Hz,1H), 3.23(q, J = 8.7 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 138.6, 123.3, 123.2, 122.5, 121.4, 47.8, 33.6, 30.9, 28.7, 28.6, 28.4, 21.7, 13.1; MS(ESI): 261 $[M+H]^+$; HR ESIMS calcd for $C_{13}H_{12}S_2N_2$ = 261.0520; found = 261.05207.

4.5. Screening test

To screen putative inhibitors, activity of BIAP as well as that of porcine kidney TNAP were measured in 25 mM piperazine, 25 mM glycylglycine, 5 mM $MgCl_2$, 5 μM $ZnCl_2$ at pH 10.4 and at 37 °C.[61] The mixtures containing the buffer, BIAP (0.1–0.3 μg mL^{-1}), or TNAP (4–6 μg mL^{-1}), and the inhibitors (0.1 mM for BIAP with final DMSO 1% (v/v), 0.4 mM for TNAP with final DMSO 4% (v/v)) were incubated for 10 min at 37 °C without *p*NPP. Then, 0.05 mM *p*NPP was added at the last minute to initiate the reaction. The activity was quantified at 420 nm, using a molar absorption coefficient of 18.6 cm^{-1} mM^{-1} at pH 10.4. The activity of each sample containing the inhibitor was compared with the control sample (without inhibitor). A relative activity of 100 ± 5% indicated that the inhibitor has no effects. The experimental errors were 5% as determined by the sample to sample assays. An higher relative activity (greater than 105%) indicated that the tested compound has an activation effect on the alkaline phosphatase activities, while a relative value lower than 95% indicated the the tested compound is a inhibitor.

4.6. Inhibition of the best benzothiophene inhibitor on BIAP and matrix-vesicle TNAP activity

From the screening test, the best benzothiophene inhibitor was selected. Its activity was measured in 25 mM piperazine, 25 mM glycylglycine, 5 mM $MgCl_2$, 5 μM $ZnCl_2$ at pH 10.4 and at 37 °C in the presence of either 0.2 μg mL^{-1} BIAP or 10 μg mL^{-1} matrix vesicles (MVs) with 0.1 mM *p*NPP. The concentrations of inhibitor (0–1.2 mM) are indicated in Figure 1. MV-protein concentration was determined by the method of Bradford.[62] MV extracellular organelles produced by chondrocytes, osteoblasts and odontoblasts[47,63] initiate normal skeletal calcification and are characterized by high TNAP activity.[13,14] Collagenase released MVs were isolated from bone and epiphyseal cartilage slices of 17-day-old chicken embryos according to Balcerzak et al.[64]

4.7. Determination of the inhibition constant in physiological pH

To determine the inhibition constant K_i of the soluble inhibitors, porcine kidney TNAP activity was measured in 0.1 M Tris–HCl buffer

with 5 mM $MgCl_2$ and 5 μM $ZnCl_2$ at pH 7.8 and at 37 °C. The mixtures containing the buffer, TNAP (6 μg ml^{-1}), and the inhibitors (from 100 to 500 μM) were incubated for 10 min at 37 °C without *p*NPP. Then, *p*NPP was added at the last minute to initiate the reaction. The concentrations of *p*NPP were 10 μM, 20 μM, 40 μM, 100 μM and 1000 μM, respectively. The change in absorbance of released *p*-nitrophenolate chromophore was monitored at 420 nm, using a molar absorption coefficient of 9.2 cm^{-1} mM^{-1} at pH 7.8. In all cases, one unit of the alkaline phosphatase activity (U) was defined as the amount of enzyme hydrolysing 1 μmol of *p*NPP per min under described conditions. All the experiments were repeated three times in an independent manner.

Acknowledgements

We thank Dr. John Carew for the English correction. Ms. Lina Li was a recipient of a scholarship awarded by the China-France Doctoral College.

References and notes

1. Sun, Y.; Anley, E. N. J. *Curr. Opin. Orthop.* **2007**, *18*, 472.
2. Rosenthal, A. K. *Curr. Opin. Rheumatol.* **2007**, *19*, 158.
3. Molloy, E. S.; McCarthy, G. M. *Curr. Opin. Rheumatol.* **2006**, *18*, 187.
4. Rosenthal, A. K. *Rheum. Dis. Clin. North. Am.* **2006**, *32*, 401.
5. Gordon, G. V.; Villanueva, T.; Schumacher, H. R.; Gohel, V. *J. Rheumatol.* **1984**, *11*, 681.
6. Carroll, G. J.; Stuart, R. A.; Armstrong, J. A.; Breidhahl, P. D.; Laing, B. A. *J. Rheumatol.* **1991**, *18*, 861.
7. Derfus, B. A.; Kurian, J. B.; Butler, J. J.; Daft, L. J.; Carrera, G. F.; Ryan, L. M.; Rosenthal, A. K. *J. Rheumatol.* **2002**, *29*, 570.
8. Nalbant, S.; Martinez, J. A.; Kitumnuaypong, T.; Clayburne, G.; Sieck, M.; Schumacher, H. R. J. *Osteoarthritis Cartilage* **2003**, *11*, 50.
9. Johnson, K.; Terkeltaub, R. *Front Biosci.* **2005**, *10*, 988.
10. Williams, C. J. *Curr. Opin. Rheumatol.* **2003**, *15*, 326.
11. Netter, P.; Bardin, T.; Bianchi, A.; Richette, P.; Loeuille, D. *Joint Bone Spine* **2004**, *71*, 365.
12. Ali, S. Y. *Eur. J. Rheumatol. Inflamm.* **1978**, *1*, 115.
13. Golub, E. E.; Boesze-Battalgia, K. *Curr. Opin. Orthop.* **2007**, *18*, 444.
14. Balcerzak, M.; Hamade, E.; Zhang, L.; Pikula, S.; Azzar, G.; Radisson, J.; Bandorowicz-Pikula, J.; Buchet, R. *Acta Biochim. Pol.* **2003**, *50*, 1019.
15. Van Belle, H. *Clin. Chem.* **1976**, *22*, 158.
16. Schuermans, Y. *Lancet* **1975**, *1*, 111.
17. McGill, P. E. *Lancet* **1976**, *2*, 149.
18. Miller, B.; de Merieux, P.; Srinivasan, R.; Clements, P.; Fan, P.; Levy, J.; Paulus, H. E. *Arthritis Rheum.* **1980**, *23*, 172.
19. Rosenthal, M.; Breysse, Y.; Dixon, A. S.; Franchimont, P.; Huskisson, E. C.; Schmidt, K. L.; Schuermans, Y.; Veys, E.; Vischer, T. L.; Janssen, P. A.; Brugmans, J.; de Crepe, J.; Symoens, J.; Macnair, A. L.; Amery, W. K.; Veys, E. *Lancet* **1977**, *1*, 904.
20. Mielants, H.; Veys, E. M. *J. Rheumatol.* **1978**, *5*, 77.
21. Raeymaekers, A. H. M.; Allewinjn, F. T. N.; Vandenberk, J.; Demoen, P. J. A.; Van Offenwert, T. T. T. *J. Med. Chem.* **1966**, *9*, 545.
22. Bhargava, K. K.; Lee, M. H.; Huang, Y.-. M.; Cunningham, L. S.; Agrawal, K. C.; Sartorelli, A. C. *J. Med. Chem.* **1977**, *20*, 563.
23. Collington, E. W.; Middlemiss, D.; Panchal, T. A.; Wilson, D. *Tetrahedron Lett.* **1981**, *22*, 3675.
24. Kumar, D. S.; Rashmi, R.; Satyavan, S. *Monatsh. Chem.* **1981**, *112*, 1387.
25. Nielek, S.; Tadeusz, L. *Chem. Ber.* **1982**, *115*, 1247.
26. Jones, G. S.; Hanson, R. N.; Davis, M. A. *J. Heterocycl. Chem.* **1983**, *20*, 523.
27. Gouesnard, J.-P. *J. Chem. Soc., Perkin Trans. 1* **1986**, 1901.
28. Brewer, M. D.; Dorgan, J. J.; Manger, B. R.; Mamalis, P.; Webster, A. B. *J. Med. Chem.* **1987**, *30*, 1848.
29. Amarouch, H.; Loiseau, P. R.; Bonnafous, M.; Caujolle, R.; Payard, M.; Loiseau, P. M.; Bories, C.; Gayral, P. *Farm. Ed. Sci.* **1988**, *43*, 421.
30. Metaye, T.; Mettey, Y.; Lehuede, J.; Vierfond, J.-. M.; Lalegerie, P. *Biochem. Pharm.* **1988**, *37*, 4263.
31. Weikert, R. J.; Bingham, S.; Emanuel, M. A.; Fraser-Smith, E. B.; Loughhead, D. G.; Nelson, P. H.; Poulton, A. L. *J. Med. Chem.* **1991**, *34*, 1630.
32. Comley, J. C. W.; Gutteridge, W. E.; Hudson, A. T.; Jenkins, D. C.; Miller, D. D.; Nicol, R. H.; Randall, A. W.; Stables, J. N.; Thornton, R. *Eur. J. Med. Chem.* **1992**, *27*, 503.
33. Kaugars, G.; Martin, S. E.; Nelson, S. J.; Watt, W. *Heterocycles* **1994**, *38*, 2593.
34. Sidique, S.; Ardecky, R.; Su, Y.; Narisawa, S.; Brown, B.; Millan, J. L.; Sergienko, E.; Cosford, D. P. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 222.
35. Fournier Dit Chabert, J.; Joucla, L.; David, E.; Lemaire, M. *Tetrahedron* **2004**, *60*, 3221.
36. David, E.; Perrin, J.; Pellet-Rostaing, S.; Fournier Dit Chabert, J.; Lemaire, M. *J. Org. Chem.* **2005**, *70*, 3569.
37. Fournier Dit Chabert, J.; Chatelain, G.; Pellet-Rostaing, S.; Bouchu, D.; Lemaire, M. *Tetrahedron Lett.* **2006**, *47*, 1015.
38. David, E.; Rangheard, C.; Pellet-Rostaing, S.; Lemaire, M. *Synlett* **2006**, 2016.
39. David, E.; Pellet-Rostaing, S.; Lemaire, M. *Tetrahedron* **2007**, *63*, 8999.
40. Fournier Dit Chabert, J.; Marquez, B.; Neville, L.; Joucla, L.; Broussous, S.; Bouhours, P.; David, E.; Pellet-Rostaing, S.; Marquet, B.; Moreau, N.; Lemaire, M. *Bioorg. Med. Chem.* **2007**, *15*, 4482.
41. Nakamura, I.; Sato, T.; Tamamoto, Y. *Angew. Chem., Int. Ed.* **2006**, *45*, 4473.
42. Conley, R. A.; Heindel, N. D. *J. Org. Chem.* **1976**, *41*, 3743.
43. Corey, E. J.; Gilman, N. W.; Ganem, B. E. *J. Am. Chem. Soc.* **1968**, *90*, 5616.
44. Al Nakib, T.; Meegan, M. J.; Burke, M. L. *J. Chem. Res.* **1994**, 1037.
45. Li, L.; Buchet, R.; Wu, Y. *Anal. Biochem.* **2008**, *381*, 123.
46. Kirsch, T.; Nah, H. D.; Shapiro, I. M.; Pacifici, M. *J. Cell. Biol.* **1997**, *137*, 1149.
47. Anderson, H. C. *Clin. Orthop. Relat. Res.* **1995**, *314*, 266.
48. Anderson, H. C.; Garimella, R.; Tague, S. E. *Front Biosci.* **2005**, *10*, 822.
49. Pritzker, K. P. *J. Am. Geriatr. Soc.* **1980**, *28*, 439.
50. Ali, S. Y. *Scan Electron Microsc.* **1985**, *4*, 1555.
51. Anderson, H. C. *Rheum. Dis. Clin. North. Am.* **1988**, *14*, 303.
52. Anderson, H. C. *Curr. Rheumatol. Rep.* **2003**, *5*, 222.
53. Santos, N. C.; Figueira-Coelho, J.; Martins-Silva, J.; Saldanha, C. *Biochem. Pharmacol.* **2003**, *65*, 1035.
54. Pérez-Pastén, R.; Martinez-Galero, E.; Garduno-Siciliano, L.; Conde Lara, I.; Chamorro Cevallos, G. *Toxicol. Lett.* **2006**, *161*, 167.
55. Mereghetti, I.; Cagnotto, A.; Mnnini, T. J. *Neurosci. Methods* **2007**, *160*, 251.
56. Fossum, E. N.; Lisowski, M. J.; Macey, T. A.; Ingram, S. L.; Morgan, M. M. *Brain Res.* **2008**, *1204*, 53.
57. Narisawa, S.; Harmey, D.; Yadav, M. C.; O'Neill, W. C.; Hoylaerts, M.; Millan, J. L. *J. Bone Miner. Res.* **2007**, 1700.
58. Kozlenkov, A.; Le Du, M. H.; Cuniasse, P.; Ny, T.; Hoylaerts, M. F.; Millan, J. L. *J. Bone Min. Res.* **2004**, *19*, 1862.
59. Martins, M. J.; Negrão, M. R.; Hipólito-Reis, C. *Clin. Biochem.* **2004**, *34*, 463.
60. Tsumura, M.; Ueno, Y.; Kinouchi, T.; Koyama, I.; Komoda, T. *Clin. Chim. Acta* **2001**, *312*, 169.
61. Cyboron, G. W.; Wuthier, R. E. *J. Biol. Chem.* **1981**, *256*, 7262.
62. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
63. Anderson, H. C. *Curr. Opin. Orthop.* **2007**, *18*, 428.
64. Balcerzak, M.; Radisson, J.; Azzar, G.; Farlay, D.; Boivin, G.; Pikula, S.; Buchet, R. *Anal. Biochem.* **2007**, *361*, 176.