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Preparation of novel (*Z*)-4-ylidenebenzo[*b*]furo[3,2-*d*][1,3]oxazines and their biological activity ☆

Yukako Tabuchi [a], Yuko Ando [a], Hidemi Kanemura [a], Ikuo Kawasaki [a], Takahiro Ohishi [b], Masao Koida [c], Ryo Fukuyama [d], Hiromichi Nakamuta [d], Shunsaku Ohta [e], Kiyoharu Nishide [a,*], Yoshitaka Ohishi [a,f,*]

[a] *School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyubancho, Nishinomiya, Hyogo 663-8179, Japan*
[b] *Science of Environment and Mathematical Modeling, Graduate School of Engineering, Doshisha University, Kyotanabe, Kyoto 610-0394, Japan*
[c] *Department of Pharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotoge-cho, Hirakata, Osaka 573-1010, Japan*
[d] *Laboratory of Pharmacology, Department of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Hiroshima International University, 5-1-1 Hirokoshinkai, Kure, Hiroshima 737-0112, Japan*
[e] *Kyoto Pharmaceutical University, Misasagi, Yamashinaku, Kyoto 607-8412, Japan*
[f] *Taisho Pharm. Ind., Ltd, Research & Development Dept., 3, Oharaichiba, Koka-cho, Koka, Shiga 520-3433, Japan*

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ABSTRACT

A reaction of 2-acetyl-3-acylaminobenzo[*b*]furans (**9d**–**o**) with Vilsmeier (VM) reagent afforded a mixture of (*E*)- and (*Z*)-{(*E*)-2-aralkenylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene}acetaldehydes (**5**) with a characteristic *exo*-formylmethylene group on the oxazine ring. The *Z*-isomer was more stable than the *E*-isomer. The *Z*-isomers ((*Z*)-**5**) were reacted with phosphonate reagents under two different conditions to obtain various butadiene derivatives (**12**) containing benzo[*b*]furo[3,2-*d*][1,3]oxazine skeleton. Typical compounds (**5** and **12**) were evaluated for their anti-osteoclastic bone resorption activity, antagonistic activity for the cysLT1 receptor and growth inhibitory activity for MIA PaCa-2 and MCF-7. Compounds **12f** and **12j** showed potent anti-osteoclastic bone resorption activity comparable to E_2 (17β-estradiol).

1. Introduction

4*H*-3,1-Benzoxazine derivatives with fused aromatic rings showed various bioactivities such as anti-human coronavirus activity,[2] ICAM-1 expression inhibition activity,[2] inhibition of human leukocyte elastase,[3] inhibition of human cathepsin G,[4] inhibition of chymotrypsin,[4,5] inhibition of C1r serine protease of the complement system,[6] inhibition of thrombin[7] and inhibition of human cytomegalovirus protease.[8] Studies have been advancing on several oxazine ring cyclization reactions and the preparation of oxazine derivatives.[9] Over the past decade, oxazine ring cyclization has been examined for aromatic carbonylamines (**1**) having a carbonyl functional group at the *ortho*(*o*)-position,[10] representative example one shown in Scheme 1.[3b,11] Thus, these aromatic carbonylamines (**1a**) having carboxylic acid, ester, amide, and alcohol groups at the *o*-position were subjected to cyclization between these adjacent group pairs to form aromatic ring fused oxazines.

What was lacking was the examination of oxazine cyclization from aromatic carbonylamines (**1b**) having a ketone group at the *o*-position. Here we report a novel oxazine cyclization reaction of 2-acetyl-3-acylaminobenzo[*b*]furans, as representative of **1b**, under the Vilsmeier–Haack–Arnold reaction conditions and the preparation of novel (*Z*)-4-ylidene-benzo[*b*]furo[3,2-*d*][1,3]oxazine derivatives.

Recently, substantial efforts have been made toward the discovery of selective estrogen receptor modulators (SERMs). Several are currently on the market (tamoxifen for treatment of breast cancer[12] and raloxifene for the prevention and treatment of osteoporosis[13a,b]) or are in advanced clinical trials (lasofoxifene and bazedoxifene). SERMs are characterized by at least two common structural features, a phenolic hydroxyl group and a phenoxyethylamino group (phenyl-OCH_2CH_2N–).[14a,b] The phenoxyethylamino group has been postulated to be important for binding to the central core of the estrogen receptor.[14c] It has also been suggested to influence the endometrial properties of SERMs in women by the antiestrogenic side chain.[14a,b,d] We recently reported that the compound **4**[15] prepared in our current studies displayed very potent anti-bone resorption activity in vitro and exhibits a potent anti-osteopenic effect in vivo. This compound showed equivalent activity in vitro and in vivo assays for estrogen 2 and raloxifene.

☆ See Ref. 1.

* Corresponding authors. Tel.: +81 798 45 9953; fax: +81 798 41 2792 (Y.O.).
E-mail addresses: ikuo_k@mukogawa-u.ac.jp (I. Kawasaki), nishide@mukogawa-u.ac.jp (K. Nishide), ymohishi@kcc.zaq.ne.jp (Y. Ohishi).

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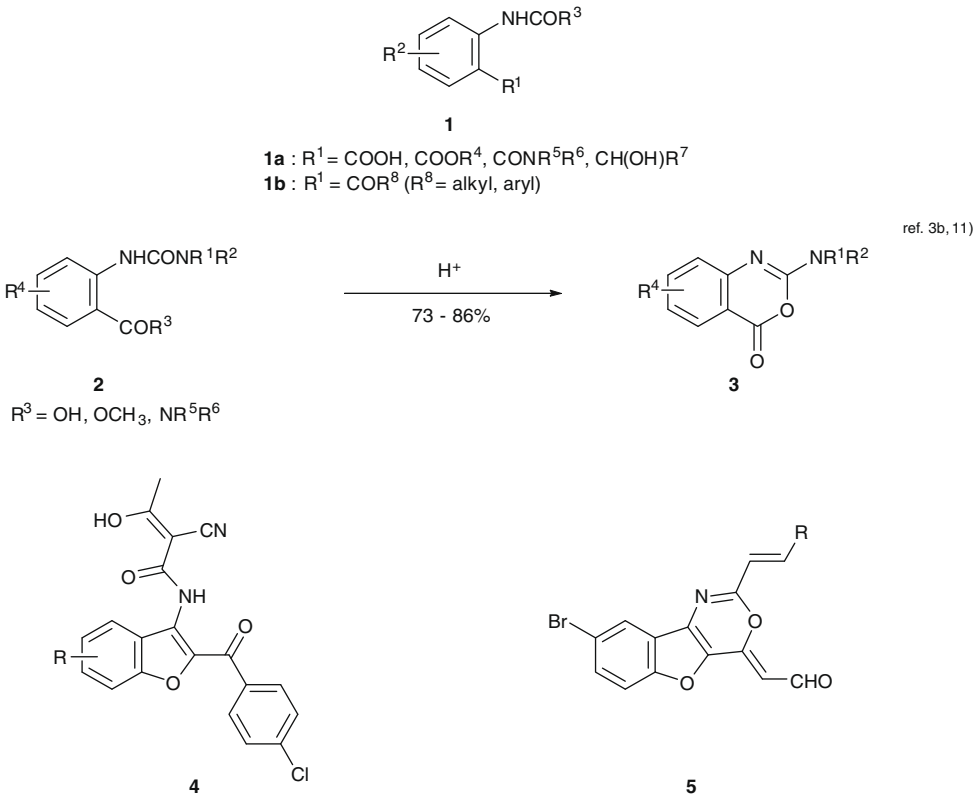


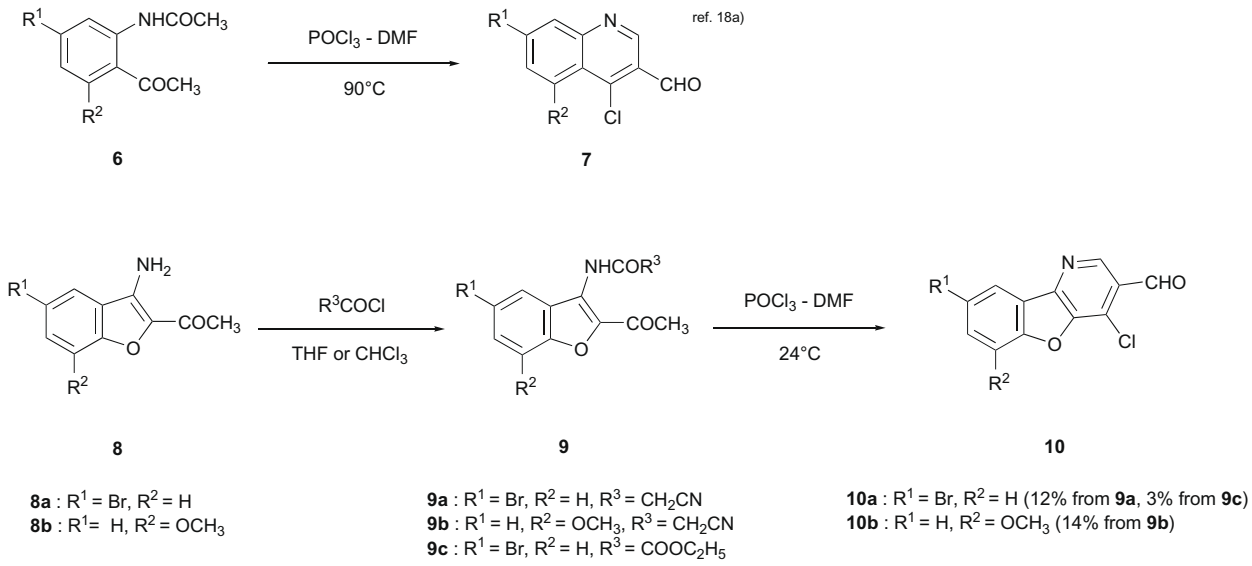
Scheme 1.

It is currently being examined for SERM activity and possible development as a new medicine to treat osteoporosis.[16] Both the compound **4**[15] and (*E*)-(8-bromo-(*E*)-2-aralkenylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)acetaldehydes (**5**) possess the phenoxyethenylamino moiety through the furan oxygen atom and the nitrogen atom. This suggests the value of preparing derivatives from **5** and evaluating their inhibition activity for osteoclasts. We therefore planed preparation of derivatives of **5** to evaluate their biological activities.

2. Results and discussion

Halomethyleniminium salts (VM Reagent) have found extensive use as formylating, halogenating and dehydroxylating reagents.[17] In addition, many kinds of heterocyclic compounds such as pyridines, quinolines, thienopyridines, quinolones, isoquinolones, naphthyridines, pyrans and furans can be efficiently prepared by ring closure reaction from acylamides under the VM conditions.[18] Although *N*-(2-acetylphenyl)acetamides (**6**) afforded 4-chloro-3-formylquinolines (**7**), not oxazine compounds, by reaction with VM reagent at 90 °C,[18a] we expected the reaction of 2-acetyl-3-cyanomethylcarbonylaminobenzo[*b*]furans (**9a**, **9b**) and 2-acetyl-3-ethoxycarbonylaminobenzo[*b*]furan (**9c**) prepared from 2-acetyl-3-aminobenzo[*b*]furans (**8a**, **8b**)[15] to give some oxazine compounds. However, both reactions of **9a** and **9c** with VM reagent at low temperature (24 °C) afforded 8-bromo-4-chloro-3-formylbenzo[*b*]furo[3,2-*b*]pyridine (**10a**), accompanied by loss of cyanomethylcarbonyl and ethoxycarbonyl groups, respectively, as shown in Scheme 2. The reaction of **9b** with VM reagent also afforded 4-chloro-3-formyl-6-methoxybenzo[*b*]furo[3,2-*b*]pyridine (**10b**). These results were similar to the case of benzene derivative **6**.

We predicted that the 2-acetyl-3-(2-aralkenylcarbonylamino)benzo[*b*]furan derivatives (**9d**–**o**) having a stable conjugating carbonyl group on the nitrogen at 3-position would be favorable for cyclization between the 2-acetyl group and the 3-acylamino group. Some kinds of 2-acetyl-3-acylaminobenzo[*b*]furans (**9d**,[15] **9e**–**g**, **9h**,[15] **9i**–**o**) were prepared by reactions of **8a** and **8b** with various acid chlorides such as (*E*)-5-phenylpenta-2-enonyl chloride,[19] cinnamoyl chlorides,[20] (*E*,*E*)-5-phenylpenta-2,4-dienoyl chlorides,[21] crotonyl chloride, 2-hexenoyl chloride and (*E*)-2-methylbut-2-enoyl chloride. The physical and spectral data of 2-acetyl-3-acylaminobenzo[*b*]furans (**9**) were listed in Table S1, see Supplementary data. To a VM reagent prepared from $POCl_3$ with dry *N*,*N*-dimethylformamide (DMF) at 6 °C was added 2-acetyl-5-bromo-(*E*)-3-cinnamoylaminobenzo[*b*]furan (**9d**). The reaction mixture was stirred at 25 °C for 30 h, and an orange precipitate was deposited. Purification of the orange precipitate (presumed to be the immonium salt)[1] was difficult because of its chemical instability. An orange suspension of this precipitation in water was treated with 10% NaOH aqueous solution with vigorous stirring, and an orange powder was obtained (Method A). The orange powder could also be obtained by treating the orange suspension with triethylamine (Method B). Recrystallization of each orange powder from $CHCl_3$–ethyl acetate (5:1) gave orange needles (representative **5a**, mp 213–216 °C, 46% (Method B)) (Scheme 3). ^{1}H NMR (HMBC, HMQC), MS and elemental analysis data of **5a** supported cyclization of oxazine ring fused at the 2- and 3-position of the benzo[*b*]-furan ring. Compound **5a** was presumed to be a novel (*E* or *Z*)-(8-bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)acetaldehyde with a characteristic *exo*-formylmethylene group on the oxazine ring. These data were, however, insufficient to confirm the structure of **5a**. Attempts to prepare single crystals of **5a** for X-ray analysis were unsuccessful. Thus, **5a** was treated with diethyl 2-(diethylamino)-2-oxoethylphosphonate (**11a**) under Horner–Wadsworth–Emmons (HWE) reaction conditions to afford a butadiene derivative (**12a**) of which single crystals were successfully prepared. X-ray analysis of **12a** demonstrated it to be (*Z*)-4-



Scheme 2.

(8-bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)-*N*,*N*-diethylbut-(*E*)-2-enamide (Fig. 1).[1] Consequently, a novel oxazine compound **5a** was determined to be (*Z*)-(8-bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)acetaldehyde ((*Z*)-**5a**) on the basis of physical data of **5a** and X-ray analysis of **12a**.

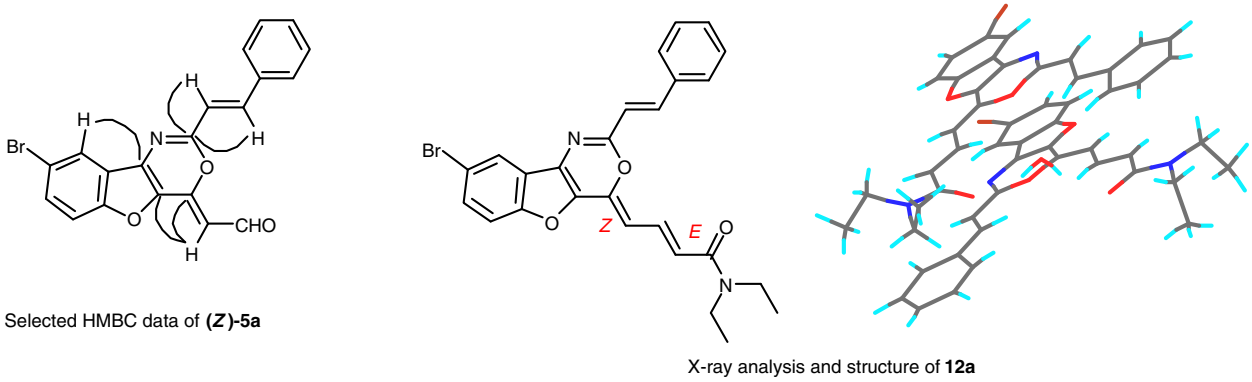
The reaction of **9d** with VM reagent gave a mixture of (*Z*)-**5a** and (*E*)-**5a** in a ratio of (*Z*)-**5a**:(*E*)-**5a** = 98:2 (by ^{1}H NMR). Both the oxazine ring closure mechanism to **5** from **9** and the reason for predominant production of the *Z*-isomer (**5**) were discussed in the preliminary communication.[1] Also, eleven 2-acetyl-(*E*)-3-aralkenylcarbonylaminobenzo[*b*]furans (**9e**–**o**) afforded corresponding mixtures of (*Z*)- and (*E*)-{(*E*)-2-aralkenylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene}acetaldehydes (**5b**–**l**) under the same reaction conditions. The *Z*-isomer was preferentially produced in all of these reactions. Five predominant *Z*-isomers ((*Z*)-**5d**, **5e**, **5i**, **5k**, **5l**) were isolated. Results and the physical data of **5e–l** were listed in Table S2, see Supplementary data.

The ring closure reaction of 2-acetyl-(*E*)-3-aralkenylcarbonylaminobenzo[*b*]furans (**9**) with VM reagent generated two different fused-rings, that is, the oxazine ring of **5** and the pyridine ring of **10**, depending on the functional group at the 3-position. The 2-acetyl group of **9** is indispensable for these ring closure reactions,[22] because no cyclization reaction of 2-(4-chlorobenzoyl)-5-bromo-(*E*)-3-cinnamoylaminobenzo[*b*]furan[15] occurs by treatment with the VM reagent under the above reaction conditions.

Isomerization of the *Z*-isomers ((*Z*)-**5**) to corresponding the *E*-isomer occurred in their solution. The (*Z*)-isomer ((*Z*)-**5a**) in DMSO-d_6 solution isomerized to (*E*)-**5a** in a time-dependent manner reaching a constant ratio of (*Z*)-**5a**:(*E*)-**5a** = 5:2 after 48 h according to ^{1}H NMR analysis. The isomerization of two mixtures, **A** ((*Z*)-**5a**:(*E*)-**5a** = 92:8) and **B** ((*Z*)-**5a**:(*E*)-**5a** = 62:38), was also examined in toluene solution and found to reach a constant equilibrium at the ratio of (*Z*)-**5a**:(*E*)-**5a** = 5:2 after 15 h by HPLC analysis (Fig. 2).[23] These results suggested that (*Z*)-**5a** would be more thermodynamically stable than (*E*)-**5a**. The heat of formation of (*Z*)-**5a** was calculated to be 0.5 kcal/mol lower than that of (*E*)-**5a**.[1]

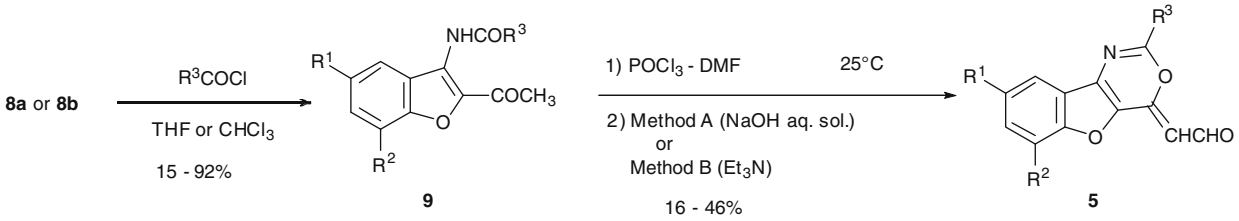
The isomerization between (*Z*)-**5a** and (*E*)-**5a** would be caused by the formyl group which conjugated with the *exo*-methylene. This was supported by the absence of isomerization of (*Z*)-2-((*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)ethanols ((*Z*)-**13a** and (*Z*)-**13b**) prepared by $NaBH_4$ reduction of the respective (*Z*)-**5a** and (*Z*)-**5c**.

We prepared thirty derivatives using the *exo*-formylmethylene group of **5** by Method C (NaH) and Method D ($TiCl_4$), as shown in Scheme 4. The compounds (**5**) were reacted with 11 phosphonate reagents (**11**)[24] in the presence of NaH under HWE reaction condi-



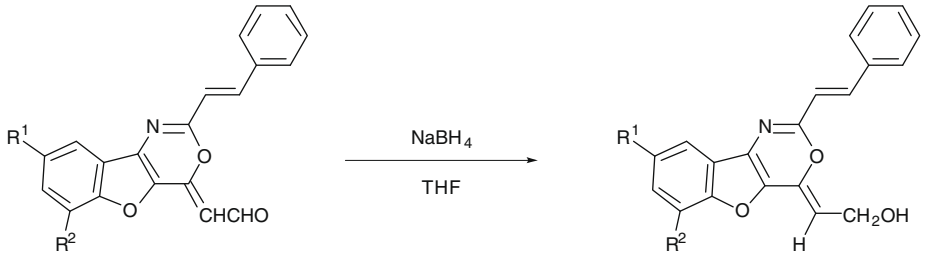
Selected HMBC data of **(*Z*)-5a**

Figure 1. Selected HMBC data of (*Z*)-(8-bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene) acetaldehyde ((*Z*)-**5a**) and X-ray analysis of (*Z*)-(8-bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)-*N*,*N*-diethylbut-(*E*)-2-enamide (**12a**).



	R^1	R^2	R^3
9d	Br	H	$CH{=}CHC_6H_5$
9e	Br	H	$CH{=}CHC_6H_4(4\text{-}OCH_3)$
9f	H	OCH_3	$CH{=}CHC_6H_5$
9g	H	OCH_3	$CH{=}CHC_6H_4(4\text{-}OCH_3)$
9h	Br	H	$CH{=}CHCH{=}CClC_6H_4(4\text{-}F)$
9i	H	OCH_3	$CH{=}CHCH{=}CClC_6H_4(4\text{-}F)$
9j	Br	H	$CH{=}CHCH{=}CClC_6H_4(4\text{-}Cl)$
9k	H	OCH_3	$CH{=}CHCH{=}CClC_6H_4(4\text{-}Cl)$
9l	Br	H	$CH{=}CHCH{=}CHC_6H_5$
9m	Br	H	$CH{=}C(CH_3)C_6H_4(4\text{-}OCH_3)$
9n	Br	H	$CH{=}C(C_6H_5)_2$
9o	Br	H	$CH{=}CHCH_3$

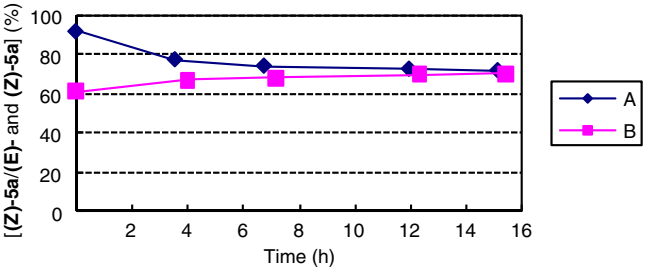
	R^1	R^2	R^3
5a	Br	H	$CH{=}CHC_6H_5$
5b	Br	H	$CH{=}CHC_6H_4(4\text{-}OCH_3)$
5c	H	OCH_3	$CH{=}CHC_6H_5$
5d	H	OCH_3	$CH{=}CHC_6H_4(4\text{-}OCH_3)$
5e	Br	H	$CH{=}CHCH{=}CClC_6H_4(4\text{-}F)$
5f	H	OCH_3	$CH{=}CHCH{=}CClC_6H_4(4\text{-}F)$
5g	Br	H	$CH{=}CHCH{=}CClC_6H_4(4\text{-}Cl)$
5h	H	OCH_3	$CH{=}CHCH{=}CClC_6H_4(4\text{-}Cl)$
5i	Br	H	$CH{=}CHCH{=}CHC_6H_5$
5j	Br	H	$CH{=}C(CH_3)C_6H_4(4\text{-}OCH_3)$
5k	Br	H	$CH{=}C(C_6H_5)_2$
5l	Br	H	$CH{=}CHCH_3$



5a: R^1 = Br, R^2 = H
5b: R^1 = H, R^2 = OCH_3

13a : R^1 = Br, R^2 = H (60%)
13b : R^1 = H, R^2 = OCH_3 (77%)

Scheme 3.

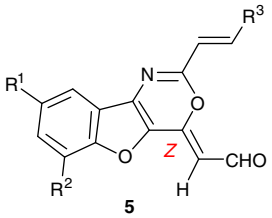


Isomeric mixture comp.	Ratio of **(*Z*)-5a** : **(*E*)-5a**	
	original ratio	ratio after 15h
A	92 : 8	5 : 2
B	62 : 38	5 : 2

Figure 2. Isomerization progress of mixture of (*Z*)-**5a** and (*E*)-**5a**.

tions to afford the corresponding butadiene derivatives (**12**) (Method C). The reactions of (*Z*)-**5a** with N-substituted dialkyl 2-amino-2-oxoethylphosphonates (**11a–d**) gave the corresponding (*Z*)-4-(8-bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)-*N*- or *N*,*N*-(mono- or di)substituted but-(*E*)-2-enamides (**12a–d**). α-Substituted diethyl methylphosphonates (**11e–g, 11k**) were reacted with (*Z*)-**5a** to afford the corresponding (*Z*)-4-(8-bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)but-(*E*)-2-enoic acid esters (**12e–g, 12o**). Reactions of (*Z*)-**5a** with diethyl cyanomethylphosphonate (**11h**) and tetraethyl methylenediphosphonate (**11i**) gave the butadiene derivatives (**12h** and **12i**), respectively. Only a NOE correlation between H_a and H_c was observed among the three olefinic H of **12a–i** (Scheme 4).

This suggested that the carbon–carbon double bond introduced by the HWE reaction has an *E*-form and the two carbon–carbon double bonds of the butadiene moiety are oriented with the s-*trans* conformation. This result was compatible with the structure of **12a** identified by X-ray analysis (Fig. 1). Physical and spectral data of the butadiene derivatives (**12**) were listed in Table S3, see Supplementary data. An aldehyde (*Z*)-**5c** was treated with **11b**, **11e**, **11f**, **11g**, **11h**, **11i** and **11k** to afford the butadiene compounds **12p**, **12q**, **12r**, **12s**, **12t**, **12u** and **12v**, respectively. Reaction of (*Z*,*E*)-**5g** with **11f** and reaction of (*Z*)-**5i** with **11i** gave **12w** and **12x**, respectively. Reaction of (*Z*)-**5a** with diethyl-4-chlorobenzylphosphonate (**11j**) afforded the unexpected phosphonate **12j** which appeared likely to have been formed by dehydration instead of dephosphonation, similar to the Knoevenagel condensation mechanism. The terminal carbon–carbon double bond of **12j** has the *E*-form because of the observation of only NOE between H_a and the hydrogen of the phenyl ring (Scheme 4). Because we expected enhancement of the binding to bone hydroxyapatite, the phosphonate group was introduced to the molecule of **12**. An aldehyde (*Z*)-**5a** was reacted with **11b**, **11f**, **11h**, **11i** in the presence of titanium tetrachloride ($TiCl_4$) and *N*-methylmorpholine (Method D) to produce the corresponding butadiene derivatives (**12k–n**) bearing a phosphonate group



5

(*Z*)-**5a** R^1 = Br, R^2 = H, R^3 = C_6H_5
(*Z*)-**5c** R^1 = H, R^2 = OCH_3, R^3 = C_6H_5
(*Z,E*)-**5g** R^1 = Br, R^2 = H, R^3 = CH=CClC$_6$H$_4$(4-Cl)
(*Z*)-**5i** R^1 = Br, R^2 = H, R^3 = CH=CHC$_6$H$_5$

\+ $R^1CHR^2PO(OR^3)_2$

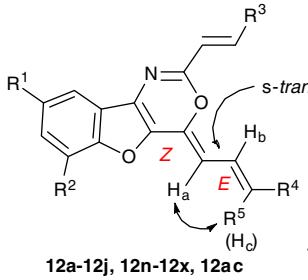
11

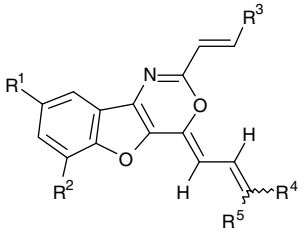
11a R^1 = CON$(C_2H_5)_2$, R^2 = H, R^3 = C_2H_5
11b R^1 = CONCH$_2$CH$_2$OCH$_2$CH$_2$, R^2 = H, R^3 = C_2H_5

11c R^1 = CONHC$_6$H$_4$(3-OCH$_3$), R^2 = H, R^3 = C_2H_5
11d R^1 = CONH$(CH_2)_2$C$_6$H$_3$(3, 4-OCH$_3$), R^2 = H, R^3 = C_2H_5
11e R^1 = $COOCH_3$, R^2 = H, R^3 = CH_3
11f R^1 = $COOC_2H_5$, R^2 = H, R^3 = C_2H_5
11g R^1 = $COOC(CH_3)_3$, R^2 = H, R^3 = C_2H_5
11h R^1 = CN, R^2 = H, R^3 = C_2H_5
11i R^1 = $PO(OC_2H_5)_2$, R^2 = H, R^3 = C_2H_5
11j R^1 = C_6H_4(4-Cl), R^2 = H, R^3 = C_2H_5
11k R^1 = $COOC_2H_5$, R^2 = CH_3, R^3 = C_2H_5

Method C (NaH / THF, 6 - 79%)

Method D ($TiCl_4$, H_3C–N⟨⟩O / THF, 4 - 60%)





12a-12j, 12n-12x, 12ac **12k-12m, 12y-12ab, 12ad**

12a R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = CON $(C_2H_5)_2$, R^5 = H
12b R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = CONCH$_2$CH$_2$OCH$_2$CH$_2$, R^5 = H

12c R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = CONHC$_6$H$_4$(3-OCH$_3$), R^5 = H
12d R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = CONH$(CH_2)_2$C$_6$H$_3$(3, 4-OCH$_3$), R^5 = H
12e R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = $COOCH_3$, R^5 = H
12f R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = $COOC_2H_5$, R^5 = H
12g R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = $COOC(CH_3)_3$, R^5 = H
12h R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = CN, R^5 = H
12i R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = $PO(OC_2H_5)_2$, R^5 = H
12j R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = $PO(OC_2H_5)_2$, R^5 = C_6H_4(4-Cl)
12k R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 and R^5 = $PO(OC_2H_5)_2$, CONCH$_2$CH$_2$OCH$_2$CH$_2$

12l R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 and R^5 = $PO(OC_2H_5)_2$, $COOC_2H_5$
12m R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 and R^5 = $PO(OC_2H_5)_2$, CN
12n R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = R^5 = $PO(OC_2H_5)_2$
12o R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 = $COOC_2H_5$, R^5 = CH_3
12p R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 = CONCH$_2$CH$_2$OCH$_2$CH$_2$, R^5 = H

12q R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 = $COOCH_3$, R^5 = H
12r R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 = $COOC_2H_5$, R^5 = H
12s R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 = $COOC(CH_3)_3$, R^5 = H
12t R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 = CN, R^5 = H
12u R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 = $PO(OC_2H_5)_2$, R^5 = H
12v R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 = $COOC_2H_5$, R^5 = CH_3
12w R^1 = Br, R^2 = H, R^3 = CH=CClC$_6$H$_4$(4-Cl), R^4 = $COOC_2H_5$, R^5 = H
12x R^1 = Br, R^2 = H, R^3 = CH=CHC$_6$H$_5$, R^4 = $PO(OC_2H_5)_2$, R^5 = H
12y R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 and R^5 = $PO(OC_2H_5)_2$, CONCH$_2$CH$_2$OCH$_2$CH$_2$

12z R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 and R^5 = $PO(OCH_3)_2$, $COOCH_3$
12aa R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 and R^5 = $PO(OC_2H_5)_2$, $COOC_2H_5$
12ab R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 and R^5 = $PO(OC_2H_5)_2$, CN
12ac R^1 = H, R^2 = OCH_3, R^3 = C_6H_5, R^4 = R^5 = $PO(OC_2H_5)_2$
12ad R^1 = Br, R^2 = H, R^3 = C_6H_5, R^4 and R^5 = $PO(OC_2H_5)_2$, COOH

c. HCl / THF

Scheme 4.

at the terminal carbon.[25] An aldehyde (*Z*)-**5c** was also reacted with **11b**, **11e**, **11f**, **11h** and **11i** to afford the butadiene derivatives **12y**, **12z**, **12aa**, **12ab**, **12ac**, respectively, under the conditions of Method D. Tetraethyl methylenediphosphonate (**11i**) produced diphosphonate compounds **12n** and **12ac** slowly by reaction with **5a** and **5c**, respectively, while the reaction of ethyl diethylphosphonoacetate **11f** with **5a** and **5c** proceeded smoothly to give monophosphonate **12l** and **12aa**, respectively. These reactions might proceed via a cyclic titanium complex.[25e]

4*H*-3,1-Benzoxazines showed various kinds of physiology activity.[2–8] Therefore we performed two kinds of small scale bioactive assays to find novel bioactivity for compounds prepared here. First, we evaluated the anti-osteoclastic bone resorption in vitro of seven representative compounds ((*Z*)-**5a**, (*Z*)-**5l**, **12a**, **12b**, **12d**, **12f**, **12j**) prepared in this work. By coculture of fresh bone marrow preosteoclasts expressing the receptor activator of NF-κB (RANK) with calvarial osteoblasts that express the ligand for RANK (RANKL), bone resorbing osteoclasts developed and formed resorption pits on a dentin slice. PGE_2 stimulated pit formation, and estrogens (e.g., estrogen 2 (E_2)) inhibited PGE_2-stimulated pit formation by suppressing the RANKL effect.[26] Among the compounds tested, the ethyl ester **12f** and phosphonate **12j** showed potent inhibition comparable to E_2, whereas the amide (**12a**, **12b**, **12d**) and *exo*-formylmethylene compounds ((*Z*)-**5a**, (*Z*)-**5l**) were inactive (Fig. 3).[27] The butadiene moiety with an ester or a phosphonate functional group might play an important role in inhibiting osteoclasts.

Several estrogenic agents such as 2-methoxyestradiol and E_2 were reported their growth inhibitory activity against human pancreatic carcinoma (MIA PaCa-2) and breast cancer (MCF-7).[28] Therefore, the aldehyde **5f** and butadiene derivatives **12p** and **12w** were selected as representative compounds and evaluated growth inhibitory activity in vivo against MIA PaCa-2 and MCF-7, and the results are shown in Table 1.[29] The butadiene amide (**12p**) inhibited MIA PaCa-2 more than 5-FU. The *exo*-formylmethylene compound ((*Z*)-**5f**) showed more inhibitory activity against MCF-7 than 5-FU. The butadiene ester (**12w**) was inactive against both types of cancer cells. Thus, two series of selective MIA Paca-2 inhibitory new compounds were found, and examinations of their inhibition mechanism of these compounds is preparing.

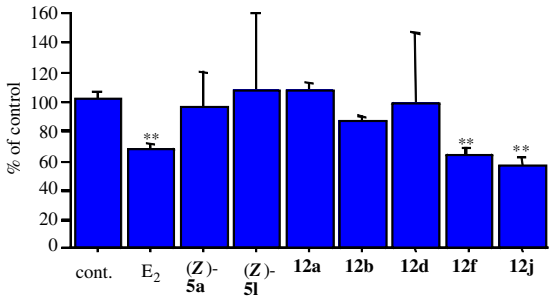


Figure 3. Anti-osteoblastic bone resorption activities of the oxazine derivatives ((*Z*)-**5a**,(*Z*)-**5l**, **12a**, **12b**, **12d**, **12f**, **12j**). All data were expressed as the means and SEs($n = 5$). cont.: control, E_2: 17β-estradiol, **: significant difference ($p < 0.05$) versus control.

Table 1
In vitro cell growth inhibitory activities of **12p**, **5f**, **12w** and 5-FU

Compd	GI_{50}[a] (μM)	
	MIA PaCa-2	MCF-7
12p	5.34	>10
5f	>10	7.14
12w	>10	>10
5-FU	>10	>10

[a] GI_{50} shows the concentration of the compound which affords 50% inhibition in cell growth compared to the negative control.

In conclusion, we established a new oxazine ring formation method using the reaction of 2-acetyl-3-(2-aralkenylcarbonylamino)benzo[*b*]furans with VM reagent. This led to a novel application of the Vilsmeier reaction for heterocyclization. (*E* and *Z*-(8-bromo-(*E*)-2-aralkenylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)acetaldehydes (**5**) bearing the characteristic *exo*-formylmethylene group at the 4-position were prepared by this reaction.[30] Unsaturated aldehydes (*Z*)-**5** were reacted with several phosphonate reagents under two reaction conditions to afford the butadiene derivatives (**12**) having an ester or phosphonate or amide group on the terminal carbon in the butadiene moiety. The butadiene ester and phosphonate compound (**12f**, **12j**) showed potent anti-osteoclastic bone resorption activity comparable to E_2 (17β-estradiol), and the evaluation of these activities of most of all prepared compounds is under way. These results including detail mechanism of biological activities will be reported elsewhere in due course. The *exo*-formylmethylene compound (*Z*)-**5f** and the butadiene amide **12p** inhibited cell growth of MIA PaCa-2 and MCF-7, respectively. In vivo studies for two kinds of biological activities are in progress, aimed at developing new drugs for osteoporosis and pancreatic cancer.

3. Experimental

All melting points were determined using a Yanaco microscopic hot-stage apparatus and are uncorrected. ^1H NMR, ^{13}C NMR and HMBC, HMQC spectra were obtained on a JEOL JNM-ECP400, JEOL JNM-ECP500 or JEOL PMX60FT spectrometer with tetramethylsilane as an internal standard. MS spectra (MS, HRMS) were obtained using a JEOL JMS-700 EIMS spectrometer. Elemental analyses were performed on a CHN CORDER MT-3 (Yanaco). All organic extracts were dried over anhydrous $MgSO_4$. Column chromatography was carried out on Wakogel C-200. Thin layer chromatography was performed on an E. Merck silica gel plate (0.5 mm, 60F-254).

3.1. 2-Acetyl-5-bromo-(*E*)-3-(4-methoxycinnamoylamino)-benzo[*b*]furan (9e) and general procedure for 9a–d, 9f–o

To a solution of **8a** (5.0 g, 19.7 mmol) in dry THF (120 ml) was added 4-methoxycinnamoyl chloride (7.72 g, 39.3 mmol) in dry THF (45 ml). The mixture was stirred at 68 °C for 6.0 h. The solution was poured into water and a brown precipitate was deposited. The precipitate was dissolved in chloroform. The organic layer was washed with a saturated sodium bicarbonate solution, brine, and dried. The solvent was evaporated to give a residue. The residue was washed with hexane–ethyl acetate (5:1) and recrystallized from ethyl acetate–chloroform (5:1) to afford **9e** (3.94 g, 48%) as pale brown needles.

3.2. 8-Bromo-4-chloro-3-formylbenzo[*b*]furo[3,2-*b*]pyridine (10a) from 9a

A mixture of phosphoryl chloride (0.79 ml, 8.48 mmol) and dry DMF (2 ml) was stirred at 6 °C for 0.5 h under an N_2 atmosphere. The mixture was added dropwise to a solution of **9a** (0.69 g, 2.15 mmol) in dry DMF (20 ml) at 6 °C and then stirred at 24 °C for 2.5 h. Additional phosphoryl chloride (0.79 ml, 8.48 mmol) was added to the mixture and stirred at 25 °C for 43 h. A solution was poured into ice water and extracted with ethyl acetate. The organic layer was washed with brine and dried. The solvent was evaporated off. The residue was purified with silica gel column chromatography [$CHCl_3$–ethyl acetate (5:1)] and [hexane–ethyl

acetate (5:2)] and then recrystallized from ethyl acetate to give **10a** (0.08 g, 12%) as colourless plates: mp 214–215 °C. Calcd for $C_{12}H_5BrClNO_2$: C, 46.41; H, 1.62; N, 4.51. Found: C, 46.26; H, 1.53; N, 4.41. δ_H (400 MHz; $CDCl_3$) 7.61 (1H, d, J = 8.8 Hz, 6-H), 7.80 (1H, dd, J = 8.8 and 2.2 Hz, 7-H), 8.41 (1H, d, J = 1.9 Hz, 9-H), 9.10 (1H, s, 2-H), 10.59 (1H, s, CHO); δ_C (125 MHz; $CDCl_3$) 114.32, 117.94, 124.59, 125.28, 125.88, 129.41, 134.33, 146.61, 147.58, 147.79, 157.67, 187.61; *m/z* (EI) 313 (M+4, 25), 311 (M+2, 100), 309 (M^+, 76), 284 (3), 282 (9), 280 (7), 247 (4), 245 (4).

3.3. 8-Bromo-4-chloro-3-formylbenzo[*b*]furo[3,2-*b*]pyridine (10a) from 9c

A similar reaction to that described above using **9c** gave **10a** (0.042 g, 3%): mp 210–212 °C.

3.4. 4-Chloro-3-formyl-6-methoxybenzo[*b*]furo[3,2-*b*]pyridine (10b)

Phosphoryl chloride (1.03 ml, 11.1 mmol) was added to dry DMF (2 ml) under a N_2 atmosphere at 6 °C with stirring. The mixture was added dropwise to a solution of **9b** (1.0 g, 3.67 mmol) in dry DMF (30 ml) at 6 °C and then stirred at 24 °C for 24 h. Additional phosphoryl chloride (0.5 ml, 5.36 mmol) was added to the mixture, which was stirred at 25 °C for 24 h. A solution was poured into ice water and extracted with ethyl acetate. The organic layer was washed with brine and dried. The solvent was evaporated off. The residue was purified with silica gel column chromatography [hexane–ethyl acetate (7:1)] and then recrystallized from hexane–ethyl acetate (1:5) to give **10b** (0.13 g, 14%) as colourless needles: mp 191–193 °C. Calcd for $C_{13}H_8ClNO_3$: C, 59.67; H, 3.08; N, 5.35. Found: C, 59.65; H, 2.92; N, 5.27. δ_H (400 MHz; $CDCl_3$) 4.11 (3H, s, OCH_3), 7.20 (1H, dd, J = 8.0 and 0.7 Hz, 7-H or 9-H), 7.44 (1H, t, J = 7.9 Hz, 8-H), 7.84 (1H, dd, J = 8.0 and 1.1 Hz, 7-H or 9-H), 9.10 (1H, s, 2-H), 10.59 (1H, s, CHO); δ_C (100 MHz; $CDCl_3$) 56.46, 113.27, 113.97, 124.27, 125.46, 125.56, 129.43, 146.03, 146.09, 147.36, 148.61, 149.23, 187.87; *m/z* (EI) 263 (M+2, 33), 261 (M^+, 100), 248 (3), 246 (9), 232 (2), 219 (3), 217 (5).

3.5. (*Z*)-(8-Bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)acetaldehyde ((*Z*)-5a) and general procedure for (*Z*)-5d, -5e, -5i, -5k, -5l; (*E*/*Z*)-5b, -5c, -5f, -5g, -5h, -5j, -5k

A mixture of phosphoryl chloride (1.2 ml, 12.9 mmol) and dry DMF (2.0 ml) was stirred under a N_2 atmosphere at 6 °C for 40 min. The mixture was added dropwise to a solution of **9d** (2.5 g, 6.51 mmol) in dry DMF (40 ml) at 6 °C and stirred at 25 °C for 30 h. The orange precipitate deposited in the mixture was filtrated off. The precipitate was treated by *Method A* or *B*. *Method A*: To a suspension of the orange precipitate in water (250 ml) was added dropwise 10% NaOH aqueous solution at 25 °C to adjust the pH at 10–11, and the mixture was stirred at 25 °C for 30 min to obtain an orange precipitate as a powder. *Method B*: A suspension of the orange precipitate in water (250 ml) was added dropwise to a solution of triethylamine (1.82 ml, 13.1 mmol) in chloroform. The mixture was vigorously stirred at 25 °C for 25 min and then extracted with chloroform. The organic chloroform layer was washed with brine, then dried. The solvent was evaporated off to afford an orange powder. Recrystallization of each orange powder from ethyl acetate–chloroform (5:1) gave (*Z*)-**5a** as orange needles. It was very difficult to isolate pure (*Z*)- **5b, -5c, -5f, -5g, -5h, -5j, -5k**: δ_C (125 MHz; $CDCl_3$) 98.7 (OHC–*C*H=), 114.1 (C-5a), 118.0 (C-8), 118.1 (Ph–CH=*C*H), 124.0 (C-9a), 128.2 (C-2′, 6′), 129.2 (C-3′, 5′), 130.7 (C-4′), 132.2 (C-7), 132.4 (C-9b), 132.9 (C-9), 134.6 (C-1′), 138.0 (C-4a), 142.5 (Ph–*C*H=CH), 153.1 (C-4), 155.8 (C-5a), 156.7 (C-2), 185.8 (CHO).

3.6. Ethyl (*Z*)-4-(8-bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]-oxazin-4-ylidene)but-(*E*)-2-enoate (12f): (Method C) and general procedure for 12a–j, 12o–x

To a mixture of ethyl diethylphosphonoacetate (**11f**) (0.3 ml, 1.50 mmol) and NaH (60% in oil, 0.076 g, 1.90 mmol) in anhydrous THF (2.0 ml) with stirring was added dropwise a solution of (*Z*)-**5a** (0.5 g, 1.27 mmol) in anhydrous THF (90 ml) at 3 °C under a N_2 atmosphere. The mixture was stirred at 27 °C for 2 h. The mixture was quenched with H_2O and concentrated under reduced pressure. The residue was added to a saturated NH_4Cl solution and extracted with chloroform. The organic layer was washed with brine and dried. The solvent was evaporated off to give a residue which was recrystallized from ethyl acetate–hexane (5:1) to give **12f**[1] (0.42 g, 71%) as red needles. Physical and spectral data of **12b**–**12e, 12g**–**12j, 12o**–**12x** are shown in Supplementary data.

3.7. Diethyl 3-{(*Z*)-4-(8-bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)-1-(*N*-morpholino-carbonyl)-(*E*)-1-propenylphosphonate (12k): (Method D) and general procedure for 12k–n, 12y, 12z, 12aa, 12ab, 12ac

To a yellow suspension of $TiCl_4$ (0.11 ml, 1.00 mmol) in carbon tetrachloride (10 ml) was added dropwise a mixture of (*Z*)-**5a** (0.2 g, 0.51 mmol) and **11b** (0.12 g, 0.45 mmol) in anhydrous THF (40 ml) at −10 to −4 °C, and the mixture was stirred at the same temperature for 0.5 h. *N*-Methylmorpholine (0.45 ml, 4.09 mmol) was added to the solution at −7 to −4 °C. The mixture was stirred at −10 °C for 2 h and 25 °C for 12 h and poured into water. A chloroform extraction was washed with brine and dried over $MgSO_4$. The solvent was evaporated off to give a residue which was purified with silica gel column chromatography [$CHCl_3$–ethyl acetate (20:1)] and recrystallized from hexane–ethyl acetate (5:1) to give **12k** (0.07 g, 22%) as red needles. Physical and spectral data of **12k**–**n**, **12y**, **12z**, **12aa**, **12ab**, **12ac** are shown in Supplementary data.

3.8. 2-{(*Z*)-(8-Bromo-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)}ethanol (13a)

To a solution of (*Z*)-**5a** (3.0 g, 7.61 mmol) in THF (320 ml) was added sodium borohydride (0.35 g, 9.25 mmol), and the mixture was stirred for 30 min at 45 °C. The mixture was poured into water and concentrated by evaporation under reduced pressure to give a precipitate. The precipitate was filtrated off and recrystallized from THF–chloroform (1:1) to afford **13a** (1.82 g, 60%) as yellow needles: mp 200–203 °C. Calcd for $C_{20}H_{14}BrNO_3 \cdot 1/2\ H_2O$: C, 59.28; H, 3.73; N, 3.46. Found: C, 59.44; H, 3.37; N, 3.43. δ_H (400 MHz; DMSO-d_6) 4.34 (2H, dd, J = 7.1 and 5.7 Hz, CH_2OH), 4.83(1H, t, J = 5.7 Hz, CH_2OH), 5.16 (1H, t, J = 7.2 Hz, =$CH$$CH_2OH$), 6.85 (1H, d, J = 16.1 Hz, CH=CHC_6H_5), 7.40–7.47 (3H, m, 3′-, 4′-, 5′-H), 7.59 (1H, dd, J = 8.8 and 2.2 Hz, 7-H), 7.61 (1H, d, J = 16.1 Hz, CH=CHC_6H_5), 7.68 (1H, d, J = 8.8 Hz, 6-H), 7.75–7.78 (2H, m, 2′-, 6′-H), 7.85 (1H, d, J = 2.2 Hz, 9-H); *m/z* (EI) 397 (M+2, 24), 395 (M^+, 26), 380 (12), 378 (12), 299 (2), 131 (100), 103 (68), 77 (50).

3.9. 2-{(*Z*)-(6-Methoxy-(*E*)-2-styrylbenzo[*b*]furo[3,2-*d*][1,3]oxazin-4-ylidene)}ethanol (13b)

Aldehyde (*Z*)-**5c** was treated with sodium borohydride in a similar manner to (*Z*)-**5a** to afford pale yellow prisms (**13b**): mp 186–190 °C. Calcd for $C_{21}H_{17}NO_4$: C, 72.61; H, 4.93; N, 4.03. Found: C, 72.44; H, 4.89; N, 4.04. δ_H (400 MHz; DMSO-d_6) 3.97 (3H, s, OCH_3), 4.33 (2H, d, J = 6.6 Hz, CH_2OH), 4.81 (1H, br

s, CH_2OH), 5.12 (1H, t, J = 7.2 Hz, =$CH$$CH_2OH$), 6.87 (1H, d, J = 16.1 Hz, CH=CHC_6H_5), 7.10 (1H, dd, J = 7.3 and 1.5 Hz, 7-H), 7.27–7.34 (2H, m, 8-, 9-H), 7.39–7.47 (3H, m, 3′-, 4′-, 5′-H), 7.58 (1H, d, J = 16.2 Hz, CH=CHC_6H_5), 7.75–7.77 (2H, m, 2′-, 6′-H); m/z (EI) 347 (M^+, 100), 330 (86), 303 (17), 244 (4), 217 (11), 131 (31), 103 (36).

4. Evaluation of anti-bone resorption activity

Calvarial osteoblasts precultured to preconfluent from 1 to 2 day old ddY mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) and fresh bone marrow cells from 5 week-old ddY male mice (Shizuoka Laboratory Animal Center) were cocultured in α-MEM (pH 7.0)(Sigma Chemical Co., St Louis, MO, USA) containing 10% fetal calf serum (FCS, Moregate, Australia and New Zealand),10 nM calcitriol (Wako Pure Chemical Ind., Osaka, Japan) and 1.0 μM prostaglandin E_2 (PGE_2, Sigma Chemical Co.)(13) for 7 days on a 100 mm dish (Greiner, Tokyo, Japan) precoated with collagen (Cell matrix Type I-A, Nitta Gelatin Inc., Osaka, Japan) for the development of osteoclasts. Cells were then resuspended by collagenase digestion and plated over dentin slices (10 mm in diameter and 0.64 mm in height) in α-MEM containing 10 nM E_2 or the oxazine derivatives on a 24-well plate (Greiner) for 2 days pit formation. Slices were dipped in 0.01 N NaOH, treated with ultrasonic waves to remove the cells and then dried and stained with 0.1% toluidine blue in 1.0% sodium borate for pit counting. The decrease of the number of pits on slice indicates anti-bone resorption activity of test compound.

5. Materials and methods for measurement of growth inhibitory activity on cancer cell lines

5.1. Reagents

5-Fluorouracil (5-FU) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. Stock solutions of the prepared compounds or 5-FU were prepared by dissolving each compound into DMSO at 10 μM. Some of the dilutions were subsequently prepared in growth medium (D-MEM or E-MEM). The final concentration of DMSO in growth medium was made to be 0.25% or less.

5.2. Cell Lines

MIA Paca-2 'human pancreatic carcinoma' and MCF-7 'human adenocarcinoma of the breast' were purchased from the Japan Health Sciences Foundation. MCF-7 was grown in E-MEM. MIA Paca-2 was grown in D-MEM. Each medium was supplemented with 10% of fetal calf serum (MultiSer™) and 6 ml of antibiotic-antimycotic 100× (GIBCO).

5.3. AlamarBlue™ assay for cell cytotoxicity

An alamarBlue™ (Biosource) assay was used to measure cell cytotoxicity. The human cells were seeded at 1×10^4 cells in 200 μl of growth medium/well in 96-well flat bottom tissue culture plates (Nunc). The cells were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Next, the growth media in plates were eliminated, and then 180 μl of growth medium containing drug was added to triplicate wells. The cells were incubated continuously for 72 h. Following incubation of the plates, 20 μl of alamarBlue™ was added to all wells, and the plates were set in an incubator for an additional 3 h. The live cells were counted on a microplate reader (Spectra Max M5, Molecular Devices), using an excitation wavelength of 530 nm and emission wavelength of 590 nm.

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Supplementary data

Supplementary data (physical and spectral data of **5**, **9** and **12**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.04.017.

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