Synthesis of Novel 2-Benzothiopyran and 3-Benzothiepin Derivatives and Their Stimulatory Effect on Bone Formation[1]

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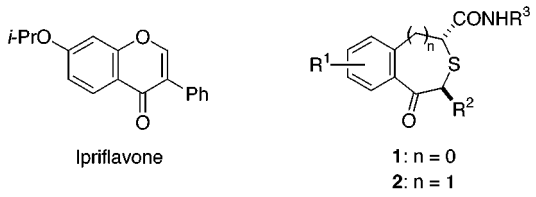
In a search for therapeutic agents for the treatment of osteoporosis and bone fracture, we found that 2-benzothiopyran-1-carboxamide derivatives **1**, derived from ipriflavone as a lead compound, increase cellular alkaline phosphatase activity in cultures of rat bone marrow stromal cells. Further modification of **1** has led to the discovery of more potent 3-benzothiepin-2-carboxamide derivatives **2**. Of these, 3-benzothiepin derivatives bearing a 4-(dialkoxyphosphorylmethyl)phenyl group on the 2-carboxamide moiety such as **2h** and **2q** exhibited significant improvement of activity compared to ipriflavone. Asymmetric synthesis of **2h** and **2q** revealed that the (−)-isomers possessed activities superior to those of the (+)-isomers. Further evaluation of these compounds using the mouse osteoblastic cell line MC3T3-E1 revealed that (−)-**2q** enhanced the effect of bone morphogenetic protein. In addition, application of a sustained-release agent containing **2q** increased the area of newly formed bone in a rat skull defect model. Based on these findings, (−)-**2q** was selected for further investigation as a new drug stimulating bone formation. Synthesis and structure−activity relationships for this novel series of 2-benzothiopyran and 3-benzothiepin derivatives are detailed.

Introduction

Metabolic bone diseases are disorders representative of the aging process. The associated decrease in bone mass frequently results in fractures in aged individuals who require prolonged periods of hospitalization.[2] Often this results in significant inactivity and morbidity, leading not only to a reduction in the overall physical and mental well-being of patients but also to negative economic effects on society.

In the mature skeleton, bone mass is maintained by a process of bone remodeling in which old bone is removed and then replaced by new bone. This process persists throughout life as a delicate balance between bone resorption by osteoclasts and bone formation by osteoblasts. An increment in osteoclastic activity and/or a decrease in osteoblastic activity can readily contribute to steady and progressive bone loss and consequent increase in risk of fracture.[2] Present therapeutic strategies are focused on preventing further bone loss using antiresorptive drugs, such as calcitonins,[3] bisphosphonates,[4] estrogens,[5] and selective estrogen receptor modulators (SERMs).[6] On the other hand, there is no satisfactory bone anabolic agent which restores lost bone mass and stimulates fracture healing. Although fluoride[7] and parathyroid hormone (PTH)[8] are known to increase bone mass, these drug therapies need special care of administration because of their possible side effects (clinical trials are ongoing with sustained-release agent of fluoride and with intermittent low-dose administration of PTH). In recent years, local growth factors including bone morphogenetic proteins (BMPs) have attracted particular interest in the field of fracture repair.[9] However, they are polypeptides, and have problems associated with their route of administration, cost of production, and systemic and local toxicity. Therefore, their clinical application may be limited.

Chart 1



We previously reported that ipriflavone (7-isopropoxyisoflavone, Chart 1) enhanced bone-like tissue formation in vitro due to stimulation of differentiation of rat bone marrow stromal cells into osteoblasts.[10] This finding led us to search for compounds with more potent effects on bone formation than ipriflavone. Among compounds designed based on the chemical structure of ipriflavone, 2-benzothiopyran-1-carboxamide derivatives (**1**, Chart 1) were found to increase cellular alkaline phosphatase (ALP) activity, one of the markers characteristic of the osteoblast phenotype, in cultures of rat bone marrow stromal cells. Various 2-benzothiopyran-1-carboxamide derivatives and the ring-expanded 3-benzothiepin-2-carboxamide derivatives (**2**, Chart 1) have been synthesized and evaluated for activity. In this article, we report the synthesis of this novel series of 2-benzothiopyran (**1**) and 3-benzothiepin (**2**) derivatives and structure−activity relationships (SARs) with regard to stimulation of ALP activity. The osteogenic activity of the active compounds including the in vivo effect in a rat skull defect model is also discussed.

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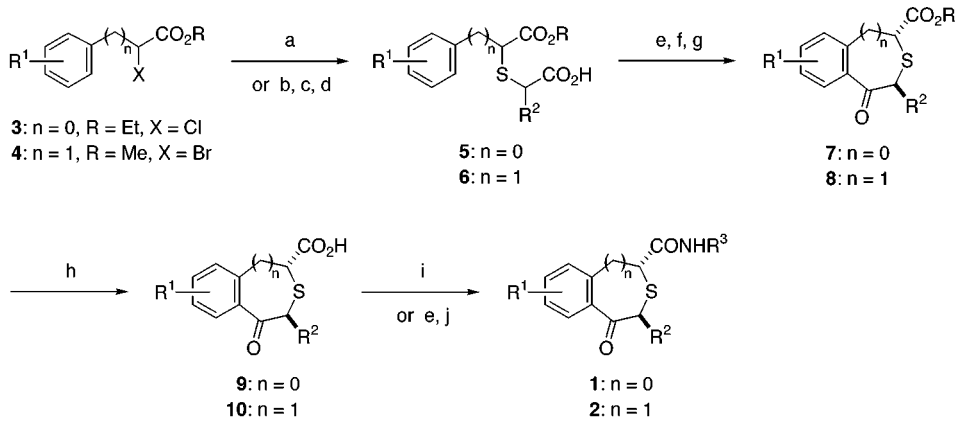
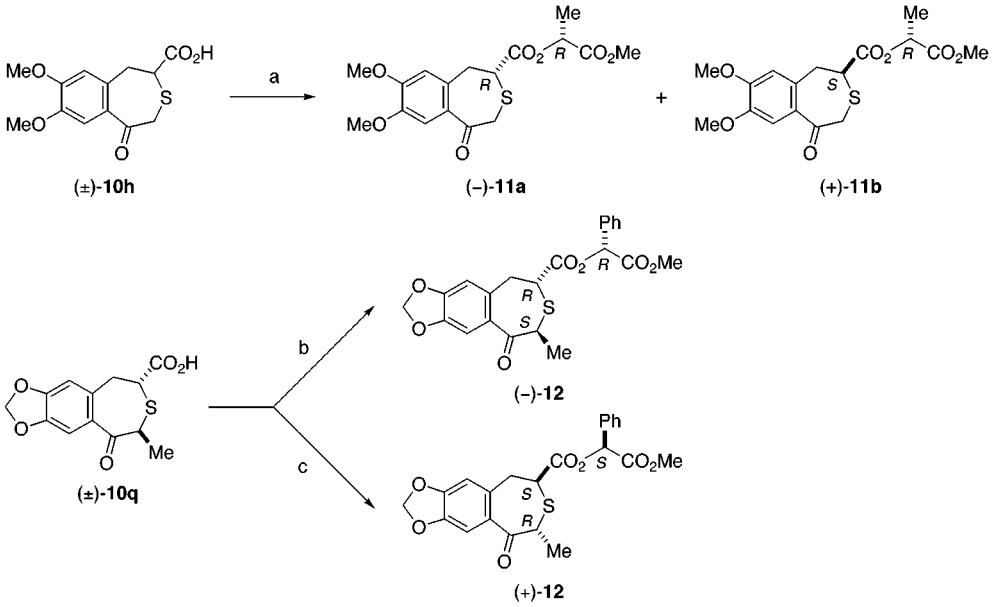
Scheme 1[a]



[a] Reagents: (a) $HSCH(R^2)CO_2H$, K_2CO_3 or Et_3N; (b) AcSK; (c) morpholine; (d) $BrCH(R^2)CO_2H$, K_2CO_3; (e) $(COCl)_2$, DMF or $SOCl_2$, pyridine; (f) $AlCl_3$ or $SnCl_4$; (g) NaOR ($R^2 \neq H$); (h) 2 N KOH; (i) R^3NH_2, DEPC, Et_3N; (j) R^3NH_2, Et_3N.

Scheme 2[a]



[a] Reagents: (a) methyl (*R*)-(+)-lactate, WSC, DMAP; (b) methyl (*R*)-(−)-mandelate, WSC, DMAP; (c) methyl (*S*)-(+)-mandelate, WSC, DMAP.

Chemistry

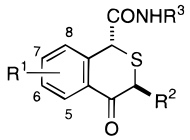
2-Benzothiopyran-1-carboxamide derivatives (**1**) and 3-benzothiepin-2-carboxamide derivatives (**2**) were synthesized starting from α-haloesters (**3**, **4**) as shown in Scheme 1. The sulfides (**5**, **6**) were prepared generally by coupling of **3** or **4** with α-mercaptocarboxylic acids in the presence of a base. An alternative three-step sequence involving conversion of the halides to thiols was used for variation of the substituent R^2. The sulfides (**5**, **6**) were converted into acyl chlorides and cyclized by intramolecular Friedel−Crafts reaction to give esters (**7**, **8**), which were then hydrolyzed to provide carboxylic acids (**9**, **10**). The amides (**1**, **2**) were prepared by straightforward coupling reactions of **9** or **10** with amines (R^3NH_2).[11] The intramolecular cyclization of the sulfides (**5**, **6**: when $R^2 \neq H$) gave a mixture of cis and trans products, which were treated with alkoxide to afford the more stable trans form as a single product.

Optical isomers of **10h** and **10q** were obtained through esterification with optically active glycolates (Scheme 2) followed by acid hydrolysis. The racemic **10h** was successfully resolved to two diastereomers (**11a**, **11b**)[12] using methyl (*R*)-(+)-lactate. The optical isomers of **10q** were similarly obtained. Esterification of (±)-**10q** with methyl (*R*)-(−)- and (*S*)-(+)-mandelate gave (2*R*,4*S*)-(−)-**12** and (2*S*,4*R*)-(+)-**12**, respectively.[12] Acid hydrolysis of the esters gave the corresponding carboxylic acids. These carboxylic acids were readily converted to the amides by coupling with diethyl 4-aminobenzylphosphonate using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC) and 1-hydroxybenzotriazole (HOBt).

Results and Discussion

The structures and physical constants of the 2-benzothiopyran-1-carboxamide and 3-benzothiepin-2-carboxamide derivatives synthesized are listed in Tables 1−4. The effects of the compounds on cellular alkaline phosphatase (ALP) activity, one of the markers characteristic of the osteoblast phenotype, were evaluated using stromal cells isolated from the femoral bone marrow of young rats.[10] The cells and the compounds

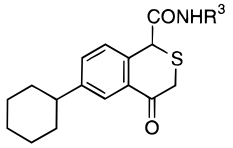
Table 1. Physical Data, Yield, and ALP Stimulatory Activity of 2-Benzothiopyran-1-carboxamide Derivatives **1**



compd	R^1	R^2	R^3	yield[a] (%)	mp (°C)	formula	anal.[b]	ALP (10^{-5} M)[c]
ipriflavone								1.6**[d]
1a	6-cyclohexyl	H	C_6H_4(4-Cl)	60	176−177	$C_{22}H_{22}NO_2SCl$	C, H, N	2.0**
1b	6-cyclohexyl	H	C_6H_4(4-Me)	87	181−182	$C_{23}H_{25}NO_2S$	C, H, N	1.7**
1c	6-cyclohexyl	H	C_6H_3[2,5-$(EtO)_2$]	52	117−118	$C_{26}H_{31}NO_4S$	C, H, N	2.3**
1d	6-cyclohexyl	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	87	171−172	$C_{27}H_{34}NO_5SP$	C, H, N	2.4**
1e	6-cyclohexylmethyl	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	78	167−168	$C_{28}H_{36}NO_5SP$	C, H, N	2.0**
1f	6-C_6H_4(4′-Cl)	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	54	188−189	$C_{27}H_{27}NO_5SPCl$	C, H, N	1.7**
1g	6,7-Me_2	H	3-pyridyl	61	198−199	$C_{17}H_{16}N_2O_2S$	C, H, N	1.3
1h	6,7-Me_2	H	pyrazinyl	52	228−229	$C_{16}H_{15}N_3O_2S$	C, H, N	1.3**
1i	6,7-Me_2	H	C_6H_4[4-$P(O)(OEt)_2$]	85	219−220	$C_{22}H_{26}NO_5SP$	C, H, N	1.5**
1j	6,7-Me_2	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	91	194−195	$C_{23}H_{28}NO_5SP$	C, H, N	1.5**
1k	6,7-$(CH_2)_3$-	H	C_6H_4(4-Cl)	80	205−206	$C_{19}H_{16}NO_2SCl$	C, H, N	1.3**
1l	6,7-$(CH_2)_3$-	H	C_6H_4[4-$P(O)(OEt)_2$]	71	187−188	$C_{23}H_{26}NO_5SP$	C, H, N	1.1
1m	6,7-$(MeO)_2$	H	C_6H_4(4-Cl)	74	218−219	$C_{18}H_{16}NO_4SCl$	C, H, N	1.3*
1n	6,7-$(MeO)_2$	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	77	173−174	$C_{23}H_{28}NO_7SP$	C, H, N	2.5**
1o	6,7-OCH_2O-	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	65	203−204	$C_{22}H_{24}NO_7SP$	C, H, N	1.2
1p	6,7-OCH_2O-	Me	4-phenyl-2-thiazolyl	42	131−132	$C_{21}H_{16}N_2O_4S_2$	C, H, N	1.3**
1q	6,7-OCH_2O-	Me	C_6H_4[4-$CH_2P(O)(OEt)_2$]	48	178−179	$C_{23}H_{26}NO_7SP$	C, H, N	1.2

[a] Yield from **9**. [b] All compounds gave satisfactory results (±0.4%). [c] The effect of compounds (10^{-5} M) on ALP activity in the culture of rat bone marrow stromal cells was evaluated according to the method of Maniatopoulos et al.[10,13] (see Biological Procedures) and expressed as the ratio value compared to the control group ($n = 5-10$). [d] Statistically significant at $*p < 0.05$ and $**p < 0.01$ (vs control), by Student's *t*-test.

Table 2. Physical Data, Yield, and ALP Stimulatory Activity of 6-Cyclohexyl-2-benzothiopyran-1-carboxamide Derivatives **1**



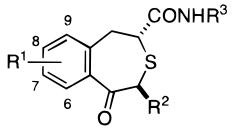
compd	R′	yield[a] (%)	mp (°C)	formula	anal.[b]	ALP (10^{-7} M)[c]
1d	C_6H_4[4-$CH_2P(O)(OEt)_2$]	87	171−172	$C_{27}H_{34}NO_5SP$	C, H, N	1.7**[d]
1r	C_6H_4[4-$P(O)(OMe)_2$]	74	210−211	$C_{24}H_{28}NO_5SP$	C, H, N	1.5**
1s	C_6H_4[4-$P(O)(OEt)_2$]	77	195−196	$C_{26}H_{32}NO_5SP$	C, H, N	1.6**
1t	C_6H_4[4-$P(O)(On\text{-}Pr)_2$]	71	174−175	$C_{28}H_{36}NO_5SP$	C, H, N	1.5**
1u	C_6H_4[4-$CH_2P(O)(OMe)_2$]	64	219−220	$C_{25}H_{30}NO_5SP$	C, H, N	1.7**
1v	C_6H_4[4-$CH_2P(O)(Oi\text{-}Pr)_2$]	80	179−180	$C_{29}H_{38}NO_5SP$	C, H, N	2.0**
1w	C_6H_4[4-$CH_2P(O)(On\text{-}Bu)_2$]	80	133−134	$C_{31}H_{42}NO_5SP$	C, H, N	1.7**
1x	C_6H_4[2-$CH_2P(O)(OEt)_2$]	84	119−120	$C_{27}H_{34}NO_5SP$	C, H, N	1.5**
1y	C_6H_4[4-$CH_2CH_2P(O)(OEt)_2$]	75	181−182	$C_{28}H_{36}NO_5SP$	C, H, N	1.5**
1z	$P(O)(OEt)_2$	36	163−164	$C_{20}H_{28}NO_5SP$	C, H, N	1.1*

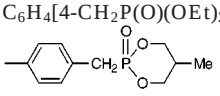
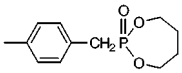
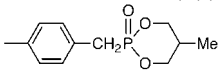
[a] Yield from **9**. [b] All compounds gave satisfactory results (±0.4%). [c] See the footnote of Table 1. [d] Statistically significant at $*p < 0.05$ and $**p < 0.01$ (vs control), by Student's *t*-test.

(10^{-5} M or 10^{-7} M) were cultured in the presence of β-glycerophosphate and dexamethasone according to the method of Maniatopoulos et al.[13] The results are shown in Tables 1−4, and the potency is expressed as fold increase of cellular ALP activity compared to the control value.

Our search for novel potent bone formation stimulants began with chemical modification of ipriflavone (Chart 1). Variation of the 1-benzopyran structure led to the finding of 2-benzothiopyran-1-carboxamide as a promising skeleton. As shown in Table 1, the 2-benzothiopyran-1-carboxamide derivatives with a phenyl moiety as the *N*-substituent (R^3) exhibited considerable ALP stimulatory activity equipotent to that of ipriflavone. No significant structure−activity relationships (SARs) were observed with regard to the substituent(s) on the benzene ring (R^1). However, the activity of compounds **1d**, **1e**, and **1n** suggests that a phosphonate moiety on the phenyl moiety is advantageous. The phosphonate moiety was introduced in order to increase the affinity of the molecule for the inorganic substrate of bone.[14] Although the possible affinity may be unrelated to their efficacy in the bone marrow cell culture assay involving no bone matrix, the phosphonate derivatives possessed the favorable activity. The effects of the dialkoxy function in the phosphonate moiety were examined with compounds in a series of 6-cyclohexyl-2-benzothiopyrans (Table 2). While most of these derivatives prepared in this analogue program had potency equivalent to that of **1d**, no significant improvement in activity was achieved. The methylene linker between the phenyl and the phosphonate moieties could be shortened (**1d** vs **1s**), lengthened (**1d** vs **1y**), or shifted to the ortho position of the phenyl moiety without affecting the activity (**1d** vs **1x**). Removal of the phenylene moiety resulted in a compound with decreased efficacy (**1d** vs **1z**).

Table 3. Physical Data, Yield, and ALP Stimulatory Activity of 3-Benzothiepin-2-carboxamide Derivatives **2**



compd	R^1	R^2	R^3	yield[a] (%)	mp (°C)	formula	anal.[b]	ALP (10^{-5} M)[c]
ipriflavone								1.6**[d]
2a	H	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	42	221−222	$C_{22}H_{26}NO_5SP$	C, H, N	1.5**
2b	7-Cl	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	58	240−241	$C_{22}H_{25}NO_5SPCl$	C, H, N	1.1
2c	7-Me	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	89	232−233	$C_{23}H_{28}NO_5SP$	C, H, N	1.1
2d	7-Me	Me	C_6H_4[4-$CH_2P(O)(OEt)_2$]	79	228−229	$C_{24}H_{30}NO_5SP$	C, H, N	1.7**
2e	7-Me	Ph	C_6H_4[4-$CH_2P(O)(OEt)_2$]	56	198−199	$C_{29}H_{32}NO_5SP$	C, H, N	1.2**
2f	7,8-Me_2	H	C_6H_4[4-$P(O)(OEt)_2$]	65	203−204	$C_{23}H_{28}NO_5SP$	C, H, N	2.2**
2g	7,8-$(MeO)_2$	H	C_6H_4[4-$P(O)(OEt)_2$]	51	171−172	$C_{23}H_{28}NO_7SP$	C, H, N	2.6**
2h	7,8-$(MeO)_2$	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	79	217−218	$C_{24}H_{30}NO_7SP$	C, H, N	4.3**
2i	7,8-$(MeO)_2$	H		27	218−219	$C_{24}H_{28}NO_7SP$	C, H, N	2.7**
2j	7,8-$(MeO)_2$	H		80	145−146	$C_{24}H_{28}NO_7SP$	C, H, N	2.8**
2k	7,8-$(MeO)_2$	Me	C_6H_4[4-$CH_2P(O)(OEt)_2$]	76	231−232	$C_{25}H_{32}NO_7SP$	C, H, N	2.3**
2l	7,8-$(MeO)_2$	Et	C_6H_4[4-$CH_2P(O)(OEt)_2$]	44	230−231	$C_{26}H_{34}NO_7SP$	C, H, N	1.5**
2m	7,8-$(MeO)_2$	*i*-Pr	C_6H_4[4-$CH_2P(O)(OEt)_2$]	61	239−240	$C_{27}H_{36}NO_4SCl$	C, H, N	1.4**
2n	7,8-OCH_2O-	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	81	192−193	$C_{23}H_{26}NO_7SP$	C, H, N	2.9**
2o	7,8-OCH_2O-	Me	C_6H_4[4-$P(O)(OEt)_2$]	60	185−186	$C_{23}H_{26}NO_7SP$	C, H, N	1.8**
2p	7,8-OCH_2O-	Me	C_6H_4[4-$CH_2P(O)(OMe)_2$]	65	218−219	$C_{22}H_{24}NO_7SP$	C, H, N	7.2**
2q	7,8-OCH_2O-	Me	C_6H_4[4-$CH_2P(O)(OEt)_2$]	82	220−221	$C_{24}H_{28}NO_7SP$	C, H, N	5.5**
2r	7,8-OCH_2O-	Me	C_6H_4[2-$CH_2P(O)(OEt)_2$]	69	165−166	$C_{24}H_{28}NO_7SP$	C, H, N	1.1
2s	7,8-OCH_2O-	Me	C_6H_4[3-$CH_2P(O)(OEt)_2$]	64	151−152	$C_{24}H_{28}NO_7SP$	C, H, N	1.8**
2t	7,8-OCH_2O-	Me	C_6H_4[4-$CH_2P(O)(Oi\text{-}Pr)_2$]	66	212−213	$C_{26}H_{32}NO_7SP$	C, H, N	3.9**
2u	7,8-OCH_2O-	Me	C_6H_4[4-$CH_2CH_2P(O)(OEt)_2$]	74	171−172	$C_{25}H_{30}NO_7SP$	C, H, N	1.9**
2v	7,8-OCH_2O-	Me		33	203−204	$C_{24}H_{26}NO_7SP$	C, H, N	3.0**
2w	7,8-OCH_2O-	Me	$CH_2CH_2P(O)(OEt)_2$	68	139−140	$C_{19}H_{26}NO_7SP$	C, H, N	1.1
2x	7,8-OCH_2O-	Et	C_6H_4[4-$CH_2P(O)(OEt)_2$]	63	193−194	$C_{25}H_{30}NO_7SP$	C, H, N	4.6**
2y	7,8-OCH_2O-	*i*-Pr	C_6H_4[4-$CH_2P(O)(OEt)_2$]	53	202−203	$C_{26}H_{32}NO_7SP$	C, H, N	3.3**
2z	7,8-OCH_2CH_2O-	H	C_6H_4[4-$CH_2P(O)(OEt)_2$]	93	245−246	$C_{24}H_{28}NO_7SP$	C, H, N	1.2

[a] Yield from **10**. [b] All compounds gave satisfactory results (±0.4%). [c] See the footnote of Table 1. [d] Statistically significant at $^{**}p < 0.01$ (vs control), by Student's *t*-test.

Table 4. ALP Stimulatory Activity of 3-Benzothiepin-2-carboxamide Derivatives **2h**, **2q**, and Their Optical Isomers in Rat Bone Marrow Stromal Cells

		ALP activity (nmol/min/well)[b]		
compd	config[a]	10^{-7} M	10^{-6} M	10^{-5} M
(±)-**2h**	racemic			1912.8 ± 96.2**[c]
(−)-**2h**	2*R*			2094.0 ± 94.7**
(+)-**2h**	2*S*			770.1 ± 39.3**
			(control: 408.6 ± 45.5)	
(±)-**2q**	racemic	185.4 ± 27.4**	411.0 ± 54.9**	1212.6 ± 88.6**
(−)-**2q**	2*R*, 4*S*	192.6 ± 19.5**	682.5 ± 123.2**	1636.5 ± 169.7**
(+)-**2q**	2*S*, 4*R*	165.6 ± 13.2**	159.6 ± 10.4**	566.0 ± 74.9**
			(control: 113.7 ± 8.1)	

[a] Determined by X-ray crystallographic analysis.[12] [b] The effect of compounds on ALP activity in the culture of rat bone marrow stromal cells was evaluated according to the method of Maniatopoulos et al.[10,13] (see Biological Procedures) and expressed as mean ± SE (n = 5−10). [c] Statistically significant at $^{**}p < 0.01$ (vs control), by Student's *t*-test.

Considering the SARs mentioned above, attempts were made to further improve activity by expanding the six-membered 2-benzothiopyran ring to a seven-membered 3-benzothiepin ring. The results are presented in Table 3. In general, activity could be maintained in this series of 3-benzothiepin-2-carboxamides. A sharp increase in activity was observed with compounds **2h**, **2p**, and **2q**. We therefore proceeded to explore the SARs around **2h**, **2p**, and **2q**, further introducing fine modification of the dialkoxy group of the phosphonate moiety and the substituent at the 4-position of the thiepin ring. The effect of the substituent at the 4-position (R^2) was studied for 7-methyl- (**2c**, **2d**, and **2e**), 7,8-dimethoxy- (**2h**, **2k**, **2l**, and **2m**), and 7,8-methylenedioxy- (**2n**, **2q**, **2x**, and **2y**) 3-benzothiepin derivatives. In general, introduction of a methyl substituent at the 4-position increased activity (**2d** vs **2c**; **2q** vs **2n**), although the unsubstituted compound (**2h** vs **2k**, **2l**, **2m**) was the most active in the 7,8-dimethoxy series. The X-ray analysis of (−)-**12** revealed that 2,4-trans form was a stable diastereomer of the 4-methyl-3-benzothiepin ring.[12] Increase of steric hindrance at the 4-position reduced activity (**2d** vs **2e**; **2k** vs **2l**, **2m**; **2q** vs **2x**, **2y**). These findings suggest that the substituent at the

Table 5. ALP Stimulatory Activity of Optical Isomers of **2h** and **2q** in MC3T3-E1 Cells

		ALP activity (A_{405})[a]	
compd	dose (M)	+BMP	−BMP
(±)-**2h**	10^{-5}	0.169 ± 0.002**	0.143 ± 0.003††
(−)-**2h**	10^{-5}	0.199 ± 0.006**	0.164 ± 0.004††
(+)-**2h**	10^{-5}	0.131 ± 0.002	0.120 ± 0.001
(±)-**2q**	10^{-6}	0.162 ± 0.008**	0.133 ± 0.002††
	10^{-5}	0.221 ± 0.008**	0.178 ± 0.004††
(−)-**2q**	10^{-6}	0.191 ± 0.003**	0.154 ± 0.003††
	10^{-5}	0.284 ± 0.010**	0.195 ± 0.008††
(+)-**2q**	10^{-5}	0.131 ± 0.001	0.115 ± 0.001
control	(0)	0.134 ± 0.002	0.118 ± 0.001

[a] The effect of the compounds was assessed by determining ALP activity in the culture of MC3T3-E1 in the presence (3 ng/mL, "+BMP") or absence ("−BMP") of BMP-4/7 heterodimer (see Biological Procedures) and expressed as the absorbance at 405 nm. Mean ± SE (n = 6). Statistically significant at $^{**}p < 0.01$ (vs control, +BMP) or $^{††}p < 0.01$ (vs control, −BMP), by Dunnett's test.

4-position of the benzothiepin ring might play an important role in exerting activity through a steric constraint of the thiepin ring. In studies of variation of the dialkoxy part of the phosphonate moiety, cyclic phosphonates did not potentiate activity (**2h** vs **2i**, **2j**; **2q** vs **2v**). Small alkoxies such as methoxy and ethoxy moieties appear to be best for activity.

From among the compounds with favorable activity obtained above, **2h** and **2q** were selected as candidates for further evaluation based on their chemical and biological profile. Comparison of the activities of their optical isomers was performed (Table 4). The ALP activity of the compound-treated cells increased significantly more than that of the control cells. In both cases, the (−)-isomers were superior to the (+)-isomers, and the potency of the (−)-isomers was much higher than that of ipriflavone.[10]

Several important observations were obtained in additional experiments using cultures of rat bone marrow stromal cells. The compounds **2h** and **2q** stimulated other markers characteristic of osteoblast phenotype besides ALP activity, including collagen and osteocalcin secretion, implying stimulation of bone matrix deposition.[15] Furthermore, continuous treatment with these compounds for 1−21 days resulted in an increase in the area of bone-like mineralized tissue.[15] These findings demonstrate that the compounds **2h** and **2q** have biological profiles similar to ipriflavone[10,16] with regard to osteogenic activity.

To clarify the relationship between the stimulatory effects of compounds **2h** and **2q** and the presence of dexamethasone in culture medium, we used the mouse osteoblastic cell line MC3T3-E1, which spontaneously exhibits osteoblast phenotype without steroid. Under the conditions of culture, the compounds (±)-**2h**, (−)-**2h**, (±)-**2q**, and (−)-**2q** also stimulated cellular ALP activity (Table 5). The (−)-isomers [(−)-**2h** and (−)-**2q**] were more potent than the corresponding (+)-isomers [(+)-**2h** and (+)-**2q**]. These findings were similar to those for rat bone marrow stromal cells, suggesting that the compound acts on committed osteoblast lineage cells, rather than modulating the effects of dexamethasone. Recent studies suggest that autocrine/paracrine factors including bone morphogenetic proteins (BMPs) or other growth factors regulate cellular growth and differentiation of osteoblasts. For this reason, the activities of these compounds were evaluated in the presence of BMP using MC3T3-E1 cells (Table 5). The BMP-4/7 heterodimer alone at a concentration of 3 ng/mL stimulated ALP activity of these cells.[17] The compounds (−)-**2h** and (−)-**2q** further enhanced the stimulatory effect of BMP. Similar results were also observed in rat bone marrow stromal cells (data not shown). These findings suggest that the enhancement of BMP activity by compound (−)-**2q** may have contributed to the stimulatory effect of these compounds on bone formation.

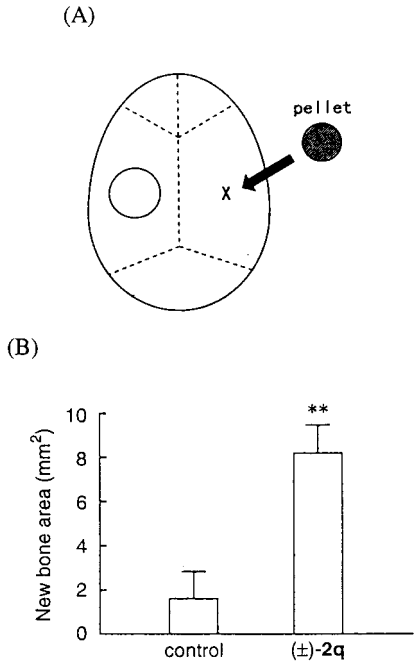


Figure 1. Effect of (±)-**2q** on Bone Formation in vivo. (A) A rat skull defect model (see Biological Procedures). (B) Area of newly formed bone in defect was calculated according to the following formula: [area of the initial defect created by trephination (12.6 mm^2)] − [area of the remaining defect]. Statistically significant at $^{**}p < 0.01$ (vs control), by Student's t-test.

The in vivo effect of compound **2q** was tested using a rat skull defect model.[18] An ethylene vinyl acetate (EVA) copolymer pellet containing 1 mg of **2q** was prepared. The pellet permits sustained release of various molecules[19] and allows evaluation of new bone formation with local application of test compound (Figure 1). The area of newly formed bone in the model was increased significantly by treatment with the sustained release pellet containing **2q** for 4 weeks, compared with that in the control group. Histological studies showed that the structure of calcified new bone formed in the defect was similar to that of the original bone.[15]

In conclusion, we found that a novel series of 3-benzothiepin-2-carboxamides with a *N*-[4-(dialkoxyphosphorylmethyl)phenyl] moiety designed based on the chemical structure of ipriflavone possessed potent ALP stimulatory activity. These derivatives were proven to have biological features similar to those of ipriflavone,[10,16] in addition to significantly improved efficacy. One of the active derivatives, **2q**, exhibited a promising effect in the rat skull defect model, suggesting the potential therapeutic application of the compound for treatment of osteoporosis and bone fracture. Compound (−)-**2q** (TAK-778) was selected as a candidate for further pharmacological evaluation.[15]

Experimental Section

Chemistry. Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations of $CHCl_3$, MeOH, or dimethyl sulfoxide (DMSO) solutions were measured on a Jasco DIP-370 digital polarimeter. ^{1}H NMR spectra of deuteriochloroform ($CDCl_3$) or DMSO-d_6 solutions (tetramethylsilane as an internal standard, δ 0) were recorded on a Varian Gemini-200 or a Varian EM-390 spectrometer, and the following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad singlet. IR spectra were recorded on a Jasco IR-810 spectrophotometer. Elemental analysis (C, H, and N) was carried out in Takeda Analytical Research Laboratories, Ltd. All compounds exhibited ^{1}H NMR, IR, and analytical data consistent with the proposed structures. Silica gel column chromatography was performed on E. Merck silica gel 60 (0.063−0.200 mm). A Hitachi 655A liquid chromatography system equipped with a Chiralcel OD column (Daicel Chemical Industries, Tokyo, Japan) was employed for chiral stationary phase HPLC.

General Procedure for Ethyl α-Chlorophenylacetates (3). Ethyl α-Chloro-4-cyclohexylphenylacetate (3d). Ethyl 4′-cyclohexylmandelate[20] (52.0 g, 198 mmol) was dissolved in thionyl chloride (100 mL) and refluxed for 1 h. The reaction mixture was concentrated in vacuo, diluted with H_2O (500 mL) and extracted with Et_2O. The extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo to give **3d** as an oil (56.0 g, quant.): ^{1}H NMR ($CDCl_3$) δ 1.23 (3H, t, J = 7.1 Hz), 1.23−1.87 (10H, m), 2.48−2.52 (1H, m), 4.21 (2H, q, J = 7.1 Hz), 5.30 (1H, s), 7.18 (2H, d, J = 8.8 Hz), 7.40 (2H, d, J = 8.8 Hz).

General Procedure for Methyl 2-Bromo-3-phenylpropionates (4). Methyl 2-Bromo-3-(3,4-dimethoxyphenyl)propionate (4h). A solution of $NaNO_2$ (12.6 g, 183 mol) in H_2O (20 mL) was added dropwise to a solution of 3,4-dimethoxyaniline (25.4 g, 166 mol) and 47% aqueous HBr (85.8 g, 498 mmol) in acetone (250 mL) at 5−10 °C. After the mixture was stirred at 5 °C for 30 min, methyl acrylate (85.8 g, 997 mmol) was added and the temperature was raised to 15 °C. Then Cu_2O (ca. 0.5 g) was added portionwise with vigorous stirring. An exothermic reaction occurred and the temperature was kept below 40 °C. After being stirred at ambient temperature for 1.5 h, the reaction mixture was concentrated in vacuo, diluted with H_2O (300 mL) and extracted with Et_2O. The extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The residual oil was distilled to give **4h** as an oil (23.2 g, 46%): bp 155−157 °C (0.37 mmHg); ^{1}H NMR ($CDCl_3$) δ 3.18 (1H, dd, J = 14.0, 6.8 Hz), 3.42 (1H, dd, J = 14.0, 8.6 Hz), 3.73 (3H, s), 3.86 (3H, s), 3.87 (3H, s), 4.37 (1H, dd, J = 8.6, 6.8 Hz), 6.73 (1H, s), 6.77−6.85 (2H, m).

Methyl 2-Bromo-3-(3,4-methylenedioxyphenyl)propionate (4q). The title compound was prepared according to the procedure described for **4h** in 62% yield as an oil: bp 146−148 °C (0.4 mmHg); ^{1}H NMR ($CDCl_3$) δ 3.15 (1H, dd, J = 14.2, 7.0 Hz), 3.38 (1H, dd, J = 14.2, 8.6 Hz), 3.74 (3H, s), 4.34 (1H, dd, J = 8.6, 7.0 Hz), 5.94 (2H, s), 6.66 (1H, dd, J = 8.0, 1.6 Hz), 6.69 (1H, d, J = 1.6 Hz), 6.75 (1H, d, J = 8.0 Hz).

General Procedure for Ethyl α-(Carboxymethylthio)-phenylacetates (5) and Methyl 2-(Carboxymethylthio)-3-phenylpropionates (6). Ethyl α-Carboxymethylthio-4-cyclohexylphenylacetate (5d). A mixture of **3d** (5.6 g, 20 mmol), mercaptoacetic acid (1.8 g, 20 mmol), K_2CO_3 (5.5 g, 40 mmol) and 2-butanone (70 mL) was refluxed for 5 h and concentrated in vacuo. The residue was diluted in H_2O (100 mL) and washed with Et_2O. The aqueous layer was acidified with concentrated HCl and extracted with AcOEt. The AcOEt extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo to give **5d** as an oil (5.7 g, 85%): ^{1}H NMR ($CDCl_3$) δ 1.23 (3H, t, J = 7.1 Hz), 1.30−2.00 (10H, m), 2.48−2.52 (1H, m), 3.07 (1H, d, J = 15.4 Hz), 3.30 (1H, d, J = 15.4 Hz), 4.16 (2H, q, J = 7.1 Hz), 4.83 (1H, s), 7.17 (2H, d, J = 8.8 Hz), 7.38 (2H, d, J = 8.8 Hz).

Methyl 2-Carboxymethylthio-3-(3,4-dimethoxyphenyl)propionate (6h). A solution of **4h** (23.2 g, 77 mmol) in *N*,*N*-dimethylformamide (DMF) (20 mL) was added dropwise to a solution of mercaptoacetic acid (7.8 g, 84 mmol) and triethylamine (17.0 g, 168 mmol) in DMF (100 mL) at 0 °C. After being stirred at 0 °C for 1 h, the reaction mixture was poured into H_2O (350 mL) and washed with Et_2O. The aqueous layer was acidified with concentrated HCl and extracted with AcOEt. The AcOEt extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo to give **6h** as an oil (22.1 g, 92%): ^{1}H NMR ($CDCl_3$) δ 2.94 (1H, dd, J = 14.0, 6.4 Hz), 3.17 (1H, dd, J = 14.0, 9.0 Hz), 3.35 (1H, d, J = 16.0 Hz), 3.49 (1H, d, J = 16.0 Hz), 3.68 (3H, s), 3.66−3.75 (1H, m), 3.85 (3H, s), 3.86 (3H, s), 6.72−6.83 (3H, m), 8.05 (1H, br s).

Methyl 2-(1-Carboxyethylthio)-3-(3,4-methylenedioxyphenyl)propionate (6q). The title compound was prepared according to the procedure described for **6h** in 99% yield as an oil: ^{1}H NMR ($CDCl_3$) major diastereomer δ 1.44 (3H, d, J = 7.2 Hz), 2.84−3.16 (2H, m), 3.70 (3H, s), 3.57−3.85 (2H, m), 5.92 (2H, s), 6.66−6.71 (3H, m); minor diastereomer δ 1.47 (3H, d, J = 7.2 Hz), 2.88−3.20 (2H, m), 3.45−3.85 (2H, m), 3.67 (3H, s), 5.93 (2H, s), 6.66−6.71 (3H, m).

General Procedure for Methyl 2-(Carboxyalkylthio)-3-phenylpropionates (6). Methyl 2-(1-Carboxypropylthio)-3-(3,4-dimethoxyphenyl)propionate (6l). A solution of **4h** (25.0 g, 85 mmol) in DMF (20 mL) was added dropwise to a mixture of potassium thioacetate (10.4 g, 91 mmol) and DMF (100 mL) at 0 °C. After being stirred at 0 °C for 1.5 h, the reaction mixture was poured into H_2O (500 mL) and extracted with Et_2O. The extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo to give methyl 2-acetylthio-3-(3,4-dimethoxyphenyl)propionate as an oil (23.7 g, 97%): ^{1}H NMR ($CDCl_3$) δ 2.33 (3H, s), 2.96 (1H, dd, J = 14.0, 7.2 Hz), 3.20 (1H, dd, J = 14.0, 8.2 Hz), 3.68 (3H, s), 3.86 (3H, s), 3.87 (3H, s), 4.43 (1H, dd, J = 8.2, 7.2 Hz), 6.73−6.82 (3H, m).

Morpholine (27.7 g, 318 mmol) was added dropwise to a solution of methyl 2-acetylthio-3-(3,4-dimethoxyphenyl)propionate (23.7 g, 80 mmol) in MeOH (110 mL) at room temperature. After being stirred at room temperature for 1 h, the reaction mixture was poured into H_2O (250 mL) and extracted with AcOEt. The extract was successively washed with H_2O, 1 N HCl, H_2O, and brine, dried over $MgSO_4$, and concentrated in vacuo. The residual oil was chromatographed on SiO_2 (270 g) with AcOEt−hexane (1:4 to 1:2, v/v) to give methyl 2-mercapto-3-(3,4-dimethoxyphenyl)propionate as an oil (15.2 g, 75%): ^{1}H NMR ($CDCl_3$) δ 2.12 (1H, d, J = 9.0 Hz), 2.97 (1H, dd, J = 14.0, 6.8 Hz), 3.20 (1H, dd, J = 14.0, 8.6 Hz), 3.58 (1H, dt, J = 6.8, 8.8 Hz), 3.70 (3H, s), 3.86 (3H, s), 3.87 (3H, s), 6.72 (1H, d, J = 1.8 Hz), 6.74 (1H, dd, J = 8.4, 1.8 Hz), 6.81 (1H, d, J = 8.4 Hz).

K_2CO_3 (6.0 g, 43 mmol) was added to a solution of methyl 2-mercapto-3-(3,4-dimethoxyphenyl)propionate (5.1 g, 20 mmol) and 2-bromobutyric acid (3.3 g, 20 mmol) in DMF (30 mL) at room temperature. After being stirred at room temperature for 1.5 h, the reaction mixture was poured into H_2O (200 mL) and washed with Et_2O. The aqueous layer was acidified with concentrated HCl and extracted with AcOEt. The AcOEt extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo to give **6l** as an oil (5.8 g, 86%): ^{1}H NMR ($CDCl_3$) major diastereomer δ 1.02 (3H, t, J = 7.2 Hz), 1.61−1.98 (2H, m), 2.98 (1H, dd, J = 6.6, 4.8 Hz), 3.12 (1H, t, J = 9.0 Hz), 3.41 (1H, t, J = 7.4 Hz), 3.68 (3H, s), 3.68−3.83 (1H, m), 3.85 (3H, s), 3.86 (3H, s), 6.72−6.77 (3H, m); minor diastereomer δ 0.99 (3H, t, J = 7.2 Hz), 1.61−1.98 (2H, m), 2.91 (1H, dd, J = 6.2, 4.8 Hz), 3.19 (1H, t, J = 9.0 Hz), 3.29 (1H, t, J = 7.4 Hz), 3.66 (3H, s), 3.68−3.83 (1H, m), 3.85 (3H, s), 3.86 (3H, s), 6.72−6.77 (3H, m).

General Procedure for Ethyl 3,4-Dihydro-4-oxo-1*H*-2-benzothiopyran-1-carboxylates (7) and Methyl 1,2,4,5-Tetrahydro-5-oxo-3-benzothiepin-2-carboxylates (8). Ethyl 6-Cyclohexyl-3,4-dihydro-4-oxo-1*H*-2-benzothiopyran-1-carboxylate (7d). A mixture of **5d** (45.0 g, 134 mmol), thionyl chloride (23.9 g, 200 mmol), pyridine (5 drops), and

Et_2O (300 mL) was stirred at room temperature for 2 h and refluxed for 20 min. The solvent and excess thionyl chloride were evaporated off. The residual oil was dissolved in CH_2Cl_2 (50 mL), and the solution was added dropwise to a mixture of $AlCl_3$ (37.5 g, 281 mmol) and CH_2Cl_2 (400 mL) at 0 °C. After being stirred at 0 °C for 3 h, the reaction mixture was poured onto ice−water (600 mL) followed by addition of AcOEt (60 mL). After the mixture was stirred at room temperature for 1 h, the organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The residual oil was chromatographed on SiO_2 (400 g) with AcOEt−hexane (1:10, v/v) to give **7d** as an oil (36.0 g, 84%): ^{1}H NMR ($CDCl_3$) δ 1.31 (3H, t, J = 7.2 Hz), 1.35−1.46 (5H, m), 1.84−1.88 (5H, m), 2.52−2.56 (1H, m), 3.26 (1H, dd, J = 16.4, 1.0 Hz), 4.24 (2H, q, J = 7.2 Hz), 4.27 (1H, d, J = 16.4 Hz), 4.41 (1H, s), 7.16 (1H, d, J = 8.0 Hz), 7.34 (1H, dd, J = 8.0, 2.0 Hz), 7.97 (1H, d, J = 2.0 Hz).

Methyl 1,2,4,5-Tetrahydro-7,8-dimethoxy-5-oxo-3-benzothiepin-2-carboxylate (8h). Oxalyl chloride (10.7 g, 84 mmol) was added dropwise to a solution of **6h** (22.1 g, 70 mmol) and DMF (1 drop) in tetrahydrofuran (THF) (100 mL) at room temperature. After the mixture was stirred at room temperature for 3 h, the solvent and excess oxalyl chloride were evaporated off. The residual oil was dissolved in CH_2Cl_2 (200 mL). $SnCl_4$ (40.2 g, 154 mmol) was added dropwise to the ice-cooled solution. After being stirred at 0 °C for 1 h and at room temperature for 1 h, the reaction mixture was poured onto ice−water (300 mL) followed by addition of AcOEt (30 mL). After the mixture was stirred at room temperature for 1 h, the organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The residual oil was chromatographed on SiO_2 (330 g) with AcOEt−hexane (1:3 to 1:1, v/v) to give crystals. Recrystallization from AcOEt−hexane gave **8h** as colorless prisms (12.1 g, 58%): mp 143−144 °C; ^{1}H NMR ($CDCl_3$) δ 3.18 (1H, dd, J = 14.6, 5.0 Hz), 3.41 (1H, d, J = 17.6 Hz), 3.47 (1H, dd, J = 14.6, 11.6 Hz), 3.70 (1H, dd, J = 11.6, 5.0 Hz), 3.81 (3H, s), 3.93 (3H, s), 3.95 (3H, s), 3.98 (1H, d, J = 17.6 Hz), 6.71 (1H, s), 7.51 (1H, s). Anal. ($C_{14}H_{16}O_5S$) C, H.

Methyl *trans*-1,2,4,5-Tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxylate (8q). Oxalyl chloride (39.5 g, 311 mmol) was added dropwise to a solution of **6q** (80.9 g, 259 mmol) and DMF (3 drops) in THF (320 mL) at room temperature. After the mixture was stirred at room temperature for 3 h, the solvent and excess oxalyl chloride were evaporated off. The residual oil was dissolved in CH_2Cl_2 (700 mL). $SnCl_4$ (148.5 g, 570 mmol) was added dropwise to the ice-cooled solution. After being stirred at 0 °C for 1 h and at room temperature for 1 h, the reaction mixture was poured onto ice−water (1100 mL) followed by addition of AcOEt (110 mL). After the mixture was stirred at room temperature for 1 h, the organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was suspended in MeOH (800 mL) followed by addition of a solution of sodium methoxide in MeOH (28%, 33 mL). The mixture was stirred at room temperature for 1 h and poured into 2 N HCl (800 mL). After the mixture was stirred at room temperature for 10 min, the crystals were collected by filtration and successively washed with H_2O, EtOH, and *i*-Pr_2O. Recrystallization from AcOEt gave **8q** as colorless prisms (57.0 g, 75%): mp 170−171 °C; ^{1}H NMR ($CDCl_3$) δ 1.51 (3H, d, J = 7.0 Hz), 3.17 (1H, dd, J = 14.0, 4.2 Hz), 3.44 (1H, dd, J = 14.0, 12.4 Hz), 3.57 (1H, dd, J = 12.4, 4.2 Hz), 3.82 (3H, s), 4.05 (1H, q, J = 7.0 Hz), 6.03 (2H, dd, J = 2.3, 1.3 Hz), 6.68 (1H, s), 7.53 (1H, s). Anal. ($C_{14}H_{14}O_5S$) C, H.

General Procedure for 3,4-Dihydro-4-oxo-1*H*-2-benzothiopyran-1-carboxylic Acids (9) and 1,2,4,5-Tetrahydro-5-oxo-3-benzothiepin-2-carboxylic Acids (10). 6-Cyclohexyl-3,4-dihydro-4-oxo-1*H*-2-benzothiopyran-1-carboxylic Acid (9d). 2 N Aqueous KOH (35 mL, 70 mmol) was added to a solution of **7d** (15.0 g, 47 mmol) in MeOH (80 mL) at room temperature. After being stirred at room temperature for 1 h, the reaction mixture was diluted with H_2O (100 mL) and washed with Et_2O. The aqueous layer was acidified with concentrated HCl and extracted with AcOEt. The AcOEt extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo to give crystals. Recrystallization from AcOEt−hexane gave **9d** as colorless prisms (10.4 g, 77%): mp 171−172 °C; ^{1}H NMR ($CDCl_3$) δ 1.30−1.52 (5H, m), 1.77−1.92 (5H, m), 2.53−2.57 (1H, m), 3.26 (1H, d, J = 16.6 Hz), 4.24 (1H, d, J = 16.6 Hz), 4.43 (1H, s), 6.51 (1H, br s), 7.18 (1H, d, J = 8.0 Hz), 7.37 (1H, dd, J = 8.0, 2.0 Hz), 7.99 (1H, d, J = 2.0 Hz). Anal. ($C_{16}H_{18}O_3S$) C, H.

1,2,4,5-Tetrahydro-7,8-dimethoxy-5-oxo-3-benzothiepin-2-carboxylic Acid (10h). The title compound was prepared according to the procedure described for **9d** in 94% yield as colorless prisms: mp 245−246 °C (AcOEt); ^{1}H NMR (DMSO-d_6) δ 3.23−3.40 (2H, m), 3.41 (1H, d, J = 17.0 Hz), 3.66 (1H, dd, J = 10.2, 6.0 Hz), 3.79 (3H, s), 3.84 (3H, s), 3.98 (1H, d, J = 17.0 Hz), 7.02 (1H, s), 7.36 (1H, s). Anal. ($C_{13}H_{14}O_5S$) C, H.

***trans*-1,2,4,5-Tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxylic Acid (10q).** The title compound was prepared according to the procedure described for **9d** in 79% yield as colorless needles: mp 209−210 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ 1.54 (3H, d, J = 7.0 Hz), 3.22 (1H, dd, J = 14.4, 5.0 Hz), 3.42 (1H, dd, J = 14.4, 12.2 Hz), 3.60 (1H, dd, J = 12.2, 5.0 Hz), 4.05 (1H, q, J = 7.0 Hz), 6.04 (2H, dd, J = 2.4, 1.0 Hz), 6.69 (1H, s), 7.53 (1H, s). Anal. ($C_{13}H_{12}O_5S$) C, H.

General Procedure for 3,4-Dihydro-4-oxo-1*H*-2-benzothiopyran-1-carboxamides (1). *N*-[4-(Diethoxyphosphorylmethyl)phenyl]-6-cyclohexyl-3,4-dihydro-4-oxo-1*H*-2-benzothiopyran-1-carboxamide (1d). Diethyl phosphorocyanidate (DEPC) (538 mg, 3.3 mmol) was added to a solution of **9d** (871 mg, 3.0 mmol) in DMF (10 mL) at 0 °C. After the mixture was stirred at 0 °C for 30 min, diethyl 4-aminobenzylphosphonate (803 mg, 3.3 mmol) and triethylamine (334 mg, 3.3 mmol) were added in that order. The reaction mixture was stirred at 0 °C for 1 h, poured into H_2O (100 mL), and extracted with AcOEt. The extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo to give crystals. Recrystallization from EtOH gave **1d** as colorless prisms (1.3 g, 87%): mp 171−172 °C; ^{1}H NMR ($CDCl_3$) δ 1.26 (6H, t, J = 7.1 Hz), 1.37−1.85 (10H, m), 2.50−2.54 (1H, m), 3.12 (2H, d, J = 21.4 Hz), 3.26 (1H, d, J = 16.2 Hz), 3.94−4.10 (4H, m), 4.27 (1H, d, J = 16.2 Hz), 4.67 (1H, s), 7.10−7.43 (6H, m), 7.97 (1H, d, J = 2.0 Hz), 9.62 (1H, s). Anal. ($C_{27}H_{34}NO_5SP$) C, H, N.

General Procedure for 1,2,4,5-Tetrahydro-5-oxo-3-benzothiepin-2-carboxamides (2). *N*-[4-(Diethoxyphosphorylmethyl)phenyl]-1,2,4,5-tetrahydro-7,8-dimethoxy-5-oxo-3-benzothiepin-2-carboxamide (2h). Oxalyl chloride (1.9 g, 15 mmol) was added to a solution of **10h** (3.4 g, 12 mmol) in THF (60 mL) at room temperature followed by addition of DMF (1 drop). The mixture was stirred at room temperature for 2 h, and the solvent and excess oxalyl chloride were evaporated off. The residue was dissolved in THF (25 mL), and the solution was added dropwise to a solution of diethyl 4-aminobenzylphosphonate (3.2 g, 13 mmol) and triethylamine (1.4 g, 13 mmol) in THF (90 mL) at room temperature. After being stirred at room temperature for 1 h, the reaction mixture was poured into H_2O (500 mL) and extracted with AcOEt. The extract was successively washed with 1 N HCl, H_2O, saturated aqueous $NaHCO_3$, H_2O, and brine, dried over $MgSO_4$ and concentrated in vacuo to give crystals. Recrystallization from $CHCl_3$−EtOH gave **2h** as colorless prisms (4.85 g, 79%): mp 217−218 °C; ^{1}H NMR ($CDCl_3$) δ 1.17 (3H, t, J = 7.0 Hz), 1.21 (3H, t, J = 7.0 Hz), 3.11 (2H, d, J = 21.6 Hz), 3.33−3.62 (3H, m), 3.93 (3H, s), 3.94 (3H, s), 3.91−4.04 (6H, m), 6.76 (1H, s), 7.20 (2H, dd, J = 8.4, 2.6 Hz), 7.39 (2H, d, J = 8.2 Hz), 7.56 (1H, s), 9.12 (1H, s). Anal. ($C_{24}H_{30}NO_7SP$) C, H, N.

***trans*-*N*-[4-(Diethoxyphosphorylmethyl)phenyl]-1,2,4,5-tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxamide (2q).** The title compound was

prepared according to the procedure described for **2h** in 82% yield as colorless prisms: mp 220−221 °C ($CHCl_3$−EtOH); ^{1}H NMR ($CDCl_3$) δ 1.17 (3H, t, J = 7.0 Hz), 1.20 (3H, t, J = 7.0 Hz), 1.55 (3H, d, J = 7.0 Hz), 3.11 (2H, d, J = 21.2 Hz), 3.33 (1H, dd, J = 14.8, 5.0 Hz), 3.53 (1H, dd, J = 14.8, 12.2 Hz), 3.82 (1H, dd, J = 5.0, 12.2 Hz), 3.88−4.04 (4H, m), 4.16 (1H, q, J = 7.0 Hz), 6.04 (2H, s), 6.74 (1H, s), 7.19 (2H, dd, J = 8.6, 2.4 Hz), 7.43 (2H, d, J = 8.4 Hz), 7.56 (1H, s), 9.41 (1H, s). Anal. ($C_{24}H_{28}NO_7SP$) C, H, N.

General Procedure for Cyclic Phosphonates. 2-(4-Aminobenzyl)-1,3,2-dioxaphosphepane-2-oxide. A mixture of 4-nitrobenzylphosphonic acid[11c] (54.2 g, 250 mmol), thionyl chloride (210 mL), and DMF (7 drops) was refluxed for 5 h and concentrated in vacuo. The residual oil was dissolved in THF (500 mL). A solution of 1,4-butanediol (22.5 g, 250 mmol) in acetonitrile (75 mL) was added dropwise to the solution at −78 °C. Pyridine (41.4 g, 524 mmol) was added dropwise to the mixture at the same temperature. After the mixture was stirred at room temperature for 15 h, the precipitate was removed by filtration. The filtrate was concentrated in vacuo, and the residue was chromatographed on SiO_2 (750 g) with AcOEt−$CHCl_3$−MeOH (10:10:1, v/v) to give crystals. Recrystallization from EtOH−hexane gave 2-(4-nitrobenzyl)-1,3,2-dioxaphosphepane-2-oxide as colorless needles (39.9 g, 59%): mp 136−137 °C; ^{1}H NMR ($CDCl_3$) δ 1.79−1.99 (4H, m), 3.33 (2H, d, J = 22.4 Hz), 3.79−3.94 (2H, m), 4.20−4.38 (2H, m), 7.51 (2H, dd, J = 8.8, 2.4 Hz), 8.20 (2H, d, J = 8.8 Hz). Anal. ($C_{11}H_{14}NO_5P$) C, H, N.

A solution of 2-(4-nitrobenzyl)-1,3,2-dioxaphosphepane-2-oxide (39.4 g, 145 mmol) in MeOH (900 mL) was hydrogenated in the presence of 5% Pd−C (50% wet) (14.0 g) at room temperature under ordinary pressure. After removal of the catalyst by filtration, the filtrate was concentrated in vacuo to give crystals. Recrystallization from EtOH−hexane gave 2-(4-aminobenzyl)-1,3,2-dioxaphosphepane-2-oxide as colorless prisms (33.6 g, 96%): mp 128−129 °C; ^{1}H NMR ($CDCl_3$) δ 1.68−1.91 (4H, m), 3.14 (2H, d, J = 21.2 Hz), 3.59−3.82 (4H, m), 4.10−4.29 (2H, m), 6.64 (2H, d, J = 8.0 Hz), 7.09 (2H, dd, J = 8.7, 2.5 Hz). Anal. ($C_{11}H_{16}NO_3P$) C, H, N.

(1′*R*)-1′-Methoxycarbonylethyl (2*R*)-(−)-1,2,4,5-Tetrahydro-7,8-dimethoxy-5-oxo-3-benzothiepin-2-carboxylate (11a) and (1′*R*)-1′-Methoxycarbonylethyl (2*S*)-(+)-1,2,4,5-Tetrahydro-7,8-dimethoxy-5-oxo-3-benzothiepin-2-carboxylate (11b). A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC) (4.9 g, 26 mmol) in CH_2Cl_2 (80 mL) was added to a solution of (±)-**10h** (6.0 g, 21 mmol) and methyl (*R*)-(+)-lactate (4.4 g, 43 mmol) in DMF (80 mL) at 0 °C followed by addition of 4-(dimethylamino)pyridine (DMAP) (1.3 g, 11 mmol). After being stirred at 0 °C for 1 h and at room temperature for 15 h, the reaction mixture was concentrated in vacuo, and the residue was dissolved in AcOEt−H_2O (1:1, 1 L). The organic layer separated was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The crystals were collected by filtration, washed with Et_2O, and recrystallized from AcOEt−hexane to give **11a** as pale yellow needles (2.2 g, 28%): mp 161−162 °C; $[\alpha]^{16}_D$ −194.1° (c = 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.56 (3H, d, J = 7.0 Hz), 3.26 (1H, dd, J = 15.0, 5.4 Hz), 3.42 (1H, d, J = 17.6 Hz), 3.47 (1H, dd, J = 15.0, 11.4 Hz), 3.78 (1H, dd, J = 11.4, 5.4 Hz), 3.80 (3H, s), 3.94 (3H, s), 3.96 (3H, s), 3.99 (1H, d, J = 17.6 Hz), 5.21 (1H, q, J = 7.0 Hz), 6.76 (1H, s), 7.51 (1H, s). Anal. ($C_{17}H_{20}O_7S$) C, H.

The filtrate was concentrated in vacuo to give crystals, which were collected by filtration and recrystallized from AcOEt−hexane to give **11b** as pale orange plates (1.6 g, 20%): mp 121−122 °C; $[\alpha]^{16}_D$ +234.3° (c = 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.57 (3H, d, J = 7.0 Hz), 3.26 (1H, dd, J = 14.8, 5.2 Hz), 3.44 (1H, d, J = 17.6 Hz), 3.49 (1H, dd, J = 14.8, 11.6 Hz), 3.74 (1H, dd, J = 11.6, 5.2 Hz), 3.79 (3H, s), 3.94 (3H, s), 3.96 (3H, s), 4.00 (1H, d, J = 17.6 Hz), 5.22 (1H, q, J = 7.0 Hz), 6.73 (1H, s), 7.52 (1H, s). Anal. ($C_{17}H_{20}O_7S$) C, H. The absolute configuration of **11b** was confirmed as (2*S*)-form by the X-ray crystallographic analysis.[12]

(α′*R*)-α′-Methoxycarbonylbenzyl (2*R*,4*S*)-(−)-1,2,4,5-Tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxylate [(−)-12]. A solution of WSC (12.6 g, 66 mmol) in CH_2Cl_2 (200 mL) was added to a solution of (±)-**10q** (15.3 g, 55 mmol) and methyl (*R*)-(−)-mandelate (18.2 g, 109 mmol) in DMF (200 mL) at 0 °C followed by addition of DMAP (3.3 g, 27 mmol). After being stirred at 0 °C for 1 h and at room temperature for 15 h, the reaction mixture was poured into H_2O (600 mL) and extracted with $CHCl_3$. The extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The residual oil was dissolved in AcOEt (600 mL), and the solution was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo to give crystals. Recrystallization from AcOEt−hexane gave (−)-**12** as colorless needles (4.1 g, 17%): mp 140−141 °C; $[\alpha]^{23}_D$ −244.2° (c = 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.50 (3H, d, J = 7.2 Hz), 3.28 (1H, dd, J = 14.9, 5.6 Hz), 3.45 (1H, dd, J = 14.9, 12.1 Hz), 3.73 (1H, dd, J = 12.1, 5.6 Hz), 3.78 (3H, s), 4.07 (1H, q, J = 7.2 Hz), 6.04 (3H, s), 6.72 (1H, s), 7.40−7.49 (5H, m), 7.52 (1H, s). Anal. ($C_{22}H_{20}O_7S$) C, H. The absolute configuration of (−)-**12** was confirmed as (2*R*,4*S*)-form by the X-ray crystallographic analysis.[12]

(α′*S*)-α′-Methoxycarbonylbenzyl (2*S*,4*R*)-(+)-1,2,4,5-Tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxylate [(+)-12]. The title compound was prepared from (±)-**10q** and methyl (*S*)-(+)-mandelate according to the procedure described for (−)-**12** in 20% yield as colorless needles: mp 141−142 °C (AcOEt−hexane); $[\alpha]^{23}_D$ +239.7° (c = 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.50 (3H, d, J = 7.2 Hz), 3.28 (1H, dd, J = 14.9, 5.6 Hz), 3.44 (1H, dd, J = 14.9, 12.1 Hz), 3.73 (1H, dd, J = 12.1, 5.6 Hz), 3.77 (3H, s), 4.06 (1H, q, J = 7.2 Hz), 6.04 (3H, s), 6.71 (1H, s), 7.39−7.47 (5H, m), 7.51 (1H, s). Anal. ($C_{22}H_{20}O_7S$) C, H.

(2*R*)-(−)-1,2,4,5-Tetrahydro-7,8-dimethoxy-5-oxo-3-benzothiepin-2-carboxylic Acid [(−)-10h]. A mixture of **11a** (0.50 g, 1.4 mmol), AcOH (2.5 mL), and concentrated HCl (2.5 mL) was refluxed for 30 min. The reaction mixture was poured into H_2O (50 mL) to give crystals, which were collected by filtration and successively washed with H_2O, EtOH, and Et_2O to give (−)-**10h** as colorless needles (0.20 g, 53%): mp 223−224 °C; $[\alpha]^{23}_D$ −190.0° (c = 0.50, DMSO); ^{1}H NMR (DMSO-d_6) δ 3.23−3.39 (2H, m), 3.41 (1H, d, J = 17.0 Hz), 3.67 (1H, dd, J = 10.2, 5.8 Hz), 3.79 (3H, s), 3.85 (3H, s), 3.98 (1H, d, J = 17.0 Hz), 7.02 (1H, s), 7.37 (1H, s). Anal. ($C_{13}H_{14}O_5S$) C, H.

(2*S*)-(+)-1,2,4,5-Tetrahydro-7,8-dimethoxy-5-oxo-3-benzothiepin-2-carboxylic Acid [(+)-10h]. The title compound was prepared according to the procedure described for (−)-**10h** in 56% yield as colorless needles: mp 223−224 °C; $[\alpha]^{22}_D$ +196.7° (c = 0.50, DMSO); ^{1}H NMR (DMSO-d_6) δ 3.23−3.39 (2H, m), 3.41 (1H, d, J = 17.0 Hz), 3.67 (1H, dd, J = 10.2, 5.8 Hz), 3.79 (3H, s), 3.85 (3H, s), 3.98 (1H, d, J = 17.0 Hz), 7.02 (1H, s), 7.37 (1H, s). Anal. ($C_{13}H_{14}O_5S$) C, H.

(2*R*,4*S*)-(−)-1,2,4,5-Tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxylic Acid [(−)-10q]. A mixture of (−)-**12** (4.2 g, 9.8 mmol), AcOH (45 mL) and concentrated HCl (30 mL) was refluxed for 30 min. The reaction mixture was poured into H_2O (800 mL) to give crystals, which were collected by filtration and dissolved in AcOEt (150 mL). The solution was washed with brine, dried over $MgSO_4$, and concentrated in vacuo to give crystals. Recrystallization from AcOEt−hexane gave (−)-**10q** as colorless needles (1.62 g, 59%): mp 194−195 °C; $[\alpha]^{23}_D$ −210.8° (c = 0.50, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.54 (3H, d, J = 7.0 Hz), 3.22 (1H, dd, J = 14.6, 5.1 Hz), 3.41 (1H, dd, J = 14.6, 12.1 Hz), 3.60 (1H, dd, J = 12.1, 5.1 Hz), 4.05 (1H, q, J = 7.0 Hz), 6.05 (2H, dd, J = 2.4, 1.4 Hz), 6.69 (1H, s), 7.52 (1H, s). Anal. ($C_{13}H_{12}O_5S$) C, H.

(2*S*,4*R*)-(+)-1,2,4,5-Tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxylic Acid [(+)-10q]. The title compound was prepared according to the procedure described for (−)-**10q** in 48% yield as colorless needles: mp 192−193 °C (AcOEt−hexane); $[\alpha]^{23}_D$ +212.9° (c = 0.50, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.54 (3H, d, J = 7.0 Hz), 3.22 (1H, dd, J = 14.6, 5.1 Hz), 3.41 (1H, dd, J = 14.6, 12.1 Hz), 3.60 (1H, dd, J

= 12.1, 5.1 Hz), 4.06 (1H, q, J = 7.0 Hz), 6.04 (2H, dd, J = 2.4, 1.4 Hz), 6.69 (1H, s), 7.52 (1H, s). Anal. ($C_{13}H_{12}O_5S$) C, H.

(2*R*)-(−)-*N*-[4-(Diethoxyphosphorylmethyl)phenyl]-1,2,4,5-tetrahydro-7,8-dimethoxy-5-oxo-3-benzothiepin-2-carboxamide [(−)-2h]. A solution of WSC (149 mg, 0.78 mmol) in CH_2Cl_2 (3 mL) was added to a solution of (−)-**10h** (183 mg, 0.65 mmol) and diethyl 4-aminobenzylphosphonate (158 mg, 0.65 mmol) in DMF (3 mL) at 0 °C followed by addition of 1-hydroxybenzotriazole (HOBt) (109 mg, 0.71 mmol). After being stirred at 0 °C for 1 h and at room temperature for 15 h, the reaction mixture was poured into H_2O (30 mL) and extracted with $CHCl_3$. The extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The residual oil was chromatographed on SiO_2 (10 g) with AcOEt−$CHCl_3$−MeOH (1:1:0 to 15:15:1, v/v) to give (−)-**2h** as a colorless amorphous solid (136 mg, 41%): $[\alpha]^{23}_D$ −155.0° (c = 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.18 (3H, t, J = 7.0 Hz), 1.19 (3H, t, J = 7.1 Hz), 3.11 (2H, dd, J = 21.5, 2.1 Hz), 3.38 (1H, dd, J = 15.0, 5.2 Hz), 3.52 (1H, d, J = 17.0 Hz), 3.56 (1H, dd, J = 15.0, 12.0 Hz), 3.93 (3H, s), 3.94 (3H, s), 3.83−4.06 (6H, m), 6.76 (1H, s), 7.22 (2H, dd, J = 8.7, 2.5 Hz), 7.40 (2H, d, J = 8.2 Hz), 7.56 (1H, s), 8.98 (1H, s). Anal. ($C_{24}H_{30}NO_7SP$) C, H, N. The optical purity of (−)-**2h** was found to be >99% ee by chiral stationary phase HPLC.

(2*S*)-(+)-*N*-[4-(Diethoxyphosphorylmethyl)phenyl]-1,2,4,5-tetrahydro-7,8-dimethoxy-5-oxo-3-benzothiepin-2-carboxamide [(+)-2h]. The title compound was prepared according to the procedure described for (−)-**2h** in 40% yield as a colorless amorphous solid: $[\alpha]^{23}_D$ +155.3° (c = 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.18 (3H, t, J = 7.0 Hz), 1.19 (3H, t, J = 7.1 Hz), 3.11 (2H, dd, J = 21.5, 2.1 Hz), 3.38 (1H, dd, J = 15.0, 5.2 Hz), 3.52 (1H, d, J = 17.0 Hz), 3.56 (1H, dd, J = 15.0, 12.0 Hz), 3.92 (3H, s), 3.94 (3H, s), 3.86−4.03 (6H, m), 6.75 (1H, s), 7.20 (2H, dd, J = 8.7, 2.5 Hz), 7.38 (2H, d, J = 8.2 Hz), 7.55 (1H, s), 9.10 (1H, s). Anal. ($C_{24}H_{30}NO_7SP$) C, H, N. The optical purity of (+)-**2h** was found to be >99% ee by chiral stationary phase HPLC.

(2*R*,4*S*)-(−)-*N*-[4-(Diethoxyphosphorylmethyl)phenyl]-1,2,4,5-tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxamide [(−)-2q]. A solution of WSC (0.39 g, 2.0 mmol) in CH_2Cl_2 (7 mL) was added to a solution of (−)-**10q** (0.47 g, 1.7 mmol) and diethyl 4-aminobenzylphosphonate (0.41 g, 1.7 mmol) in DMF (7 mL) at 0 °C followed by addition of HOBt (0.28 g, 1.8 mmol). After being stirred at 0 °C for 1 h and at room temperature for 15 h, the reaction mixture was poured into H_2O (50 mL) and extracted with $CHCl_3$. The extract was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo to give crystals. Recrystallization from MeOH-hexane gave (−)-**2q** as colorless prisms (0.37 g, 44%): mp 181−182 °C; $[\alpha]^{23}_D$ −187.4° (c = 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.17 (3H, t, J = 7.0 Hz), 1.18 (3H, t, J = 7.1 Hz), 1.54 (3H, d, J = 7.2 Hz), 3.11 (2H, dd, J = 21.5, 3.9 Hz), 3.31 (1H, dd, J = 14.8, 5.0 Hz), 3.54 (1H, dd, J = 14.8, 12.4 Hz), 3.83 (1H, dd, J = 12.4, 5.0 Hz), 3.83−4.02 (4H, m), 4.17 (1H, q, J = 7.2 Hz), 6.04 (2H, s), 6.73 (1H, s), 7.18 (2H, dd, J = 8.4, 2.6 Hz), 7.41 (2H, d, J = 8.0 Hz), 7.55 (1H, s), 9.51 (1H, s). Anal. ($C_{24}H_{28}NO_7SP$) C, H, N. The optical purity of (−)-**2o** was found to be >99% ee by chiral stationary phase HPLC.

(2*S*,4*R*)-(+)-*N*-[4-(Diethoxyphosphorylmethyl)phenyl]-1,2,4,5-tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxamide [(+)-2q]. The title compound was prepared according to the procedure described for (−)-**2q** in 41% yield as colorless prisms: mp 183−184 °C (MeOH−hexane); $[\alpha]^{23}_D$ +190.5° (c = 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.17 (3H, t, J = 7.0 Hz), 1.18 (3H, t, J = 7.1 Hz), 1.54 (3H, d, J = 7.2 Hz), 3.11 (2H, dd, J = 21.5, 3.9 Hz), 3.31 (1H, dd, J = 14.8, 5.0 Hz), 3.54 (1H, dd, J = 14.8, 12.4 Hz), 3.82 (1H, dd, J = 12.4, 5.0 Hz), 3.83−4.02 (4H, m), 4.16 (1H, q, J = 7.2 Hz), 6.03 (2H, s), 6.73 (1H, s), 7.17 (2H, dd, J = 8.4, 2.6 Hz), 7.41 (2H, d, J = 8.0 Hz), 7.55 (1H, s), 9.48 (1H, s). Anal. ($C_{24}H_{28}NO_7SP$) C, H, N. The optical purity of (+)-**2q** was found to be >99% ee by chiral stationary phase HPLC.

Biological Procedures. 1. Assay of Cellular Alkaline Phosphatase (ALP) Activity. (1) Rat Bone Marrow Stromal Cells.[10] Bone marrow stromal cells were obtained from the femoral bone marrow of 7-week-old male Sprague−Dawley rats (Japan Charles River, Tokyo, Japan).[13] The standard culture medium consisting of α-minimum essential medium (MEM) containing 10 mM HEPES (pH 7.0) was used. It contained 15% fetal bovine serum (FBS), 2 mM glutamine, 50 μg/mL of ascorbic acid, 10 mM β-glycerophosphate, 10^{-7} M dexamethasone, and antibiotics (80 μg/mL of gentamicin and 100 μg/mL of kanamycin). The cells were seeded in 100 mm culture dishes (Falcon 3003: Becton Dickinson and Co., Lincoln Park, NJ) containing 10 mL of culture medium and cultured at 37 °C in an atmosphere of 5% CO_2 in humidified air. After 1 week, confluent cells in the primary culture were harvested after treatment with 0.25% trypsin and 0.2% EDTA and then subcultured in a 6-well plate (Falcon 3046: Becton Dickinson and Co., Lincoln Park, NJ) at a cell density of 4×10^4 cells/well (day 0). The test compound was dissolved in a solution of EtOH−DMSO (1:1, v/v) or DMF at a concentration of 10 mM before use, diluted with culture medium to the designated concentrations, and added to cultures from day 1 to the end of the experiment (day 10−14). Culture medium was changed every other day. The test compound was freshly dissolved and diluted with culture medium at each medium change. The cellular ALP activity was determined by the method of Lowry et al.[21] using the supernatant of the cell lysate as previously described.[10]

(2) MC3T3-E1 cells. MC3T3-E1 cells were seeded in a 96-well plate (NUNCLON: Nunc, Roskilide, Denmark) at a cell density of 3000 cells/well and were cultured in α-MEM containing 10% FBS. Three days later, the medium was replaced with α-MEM containing 1% FBS and designated concentrations of the test compound with or without 3 ng/mL recombinant (*Xenopus*) BMP-4/7 heterodimer,[17] followed by further cultivation for 3 days. The ALP activity in the supernatant of the cell lysate was assayed.

2. Effect on a Rat Skull Defect Model. An EVA copolymer pellet containing the compound was prepared according to the procedure described by Wakamatsu et al.[19] Release of the compound from the pellet was confirmed by the increase of ALP activity in culture of rat bone marrow stromal cells.[19] Six-week-old male Splague−Dawley rats (Japan Charles River, Tokyo, Japan) were used.[22] With the rats under anesthesia by intraperitoneal injection (0.1 mL/100 g body weight) of 50 mg/mL pentobarbital sodium (Abott Laboratories, North Chicago, IL), an incision was made in the skin over the sagittal suture, and the parietal bones were exposed. A trephine defect, 4 mm in diameter, was drilled with a 4 mm sterile trephine under a continuous saline buffer irrigation using a low-speed dental drill. An EVA copolymer pellet containing the compound or an empty EVA copolymer pellet was placed onto the adjacent nondefected skull as illustrated in Figure 1 and the skin flaps were sutured. Four weeks after the operation, the animals were killed by carbon dioxide inhalation. The skulls were removed and the new bone formation was evaluated radiographically. Area of newly formed bone in defect was calculated according to the following formula: [area of the initial defect created by trephination (12.6 mm^2)] − [area of the remaining defect].

3. Statistical Analysis. All results are expressed as means ± standard errors of the mean (SE). Intergroup differences in means were statistically analyzed using Dunnett's multiple comparison method or Student's t-test. A p-value of less than 0.05 was considered significant.

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