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Discovery of novel prostaglandin analogs as potent and selective EP2/EP4 dual agonists

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ABSTRACT

To identify potent EP2/EP4 dual agonists with excellent subtype selectivity, a series of γ-lactam prostaglandin E analogs bearing a 16-phenyl ω-chain were synthesized and evaluated. Structural hybridization of **1** and **2**, followed by more detailed chemical modification of the benzoic acid moiety, led us to the discovery of a 2-mercaptothiazole-4-carboxylic acid analog **3** as the optimal compound in the series. An isomer of this compound, the 2-mercaptothiazole-5-carboxylic acid analog **13**, showed 34-fold and 13-fold less potent EP2 and EP4 receptor affinities, respectively. Structure activity relationship data from an in vitro mouse receptor binding assay are presented. Continued evaluation in an in vivo rat model of another 2-mercaptothiazole-4-carboxylic acid analog **17**, optimized for sustained compound release from PLGA microspheres, demonstrated its effectiveness in a rat bone fracture-healing model following topical administration.

1. Introduction

Prostaglandin E_2 (PGE_2) is an oxidative metabolite produced by the action of cyclooxygenase on arachidonic acid that is produced in a multitude of significant physiological processes. Receptors for PGE_2 (EPs) can be classified into four subtypes (EP1, EP2, EP3 and EP4), each of which mediates different effects in various tissues and cells.[1] The EP4 receptor is distributed in the thymus, lung, heart, kidney, bone, womb and other organs, and mediates an increase in intracellular cyclic AMP concentration. Various biological actions of PGE_2, such as cytoprotection, improvement of blood flow, regulation of inflammatory cytokine production and bone resorption/formation, are thought to be mediated by the EP4 subtype. In our previous study, we introduced the potent and orally available EP4 agonist **1**, which potently inhibited the production of TNF-α in LPS-induced rats.[2] Current literature suggests that EP4 agonists restore bone mass and strength normally lost in rats subjected to ovariectomy or immobilization.[3]

Recently, EP2 receptor agonists have also been shown to have an anabolic effect on bone formation in various animal models.[4] These findings led us to develop a novel EP2/EP4 dual agonist that would combine these actions on bone structure, and perhaps lead to a putative novel therapeutic agent for bone diseases such as osteoporosis and bone fracture healing in humans.

Several research groups have investigated strategies for improving the pharmacological properties of PGE_2.[5–8] Efforts to improve its subtype selectivity and chemical stability have focused mainly on two general chemical modifications: replacement of the α-alkenyl side chain with a phenylethyl group; and replacement of the 11-hydroxy cyclopentanone moiety with 2-pyrrolidinone.

Because of the presumed therapeutic potential of EP2 and EP4 agonists for the treatment of bone diseases, our aim was to modify PGE_2 to produce analogs with high subtype selectivity and high potency for both EP2 and EP4 receptor subtypes. In our previous reports, we described a 2-pyrrolidinone prostanoid **1** (Fig. 1 and Table 2) that was an EP4 receptor agonist with high selectivity and potency.[2] Compound **2** (Fig. 1 and Table 1) was also reported as an EP4-selective agonist.[5] We focused on the activity profiles of **2** because it also displayed weak-to-moderate binding affinity for the EP2 subtype (K_i = 340 nM for mEP2; in-house data in Table 1), in addition to potent affinity for EP4 and moderate affinity for EP3. Based on the information described above, structural hybridization of **1** and **2**, followed by more detailed chemical modification of the benzoic acid moiety, was expected to generate a new structure with the desired activity profiles: an EP2/EP4 dual agonist with high selectivity and potency.

In this report, we describe the development of a new PGE analog of structure **3** (Fig. 1 and Table 2) consisting of the ω-chain (beneficial for EP4 receptor affinity), a γ-lactam ring as a backbone and a novel 2-mercaptothiazole-4-carboxylic acid substitution of the α-alkenyl side chain. In vivo assessment of 2-mercaptothiazole-4-carboxylic acid analog **17** further optimized for sustained release is also presented.

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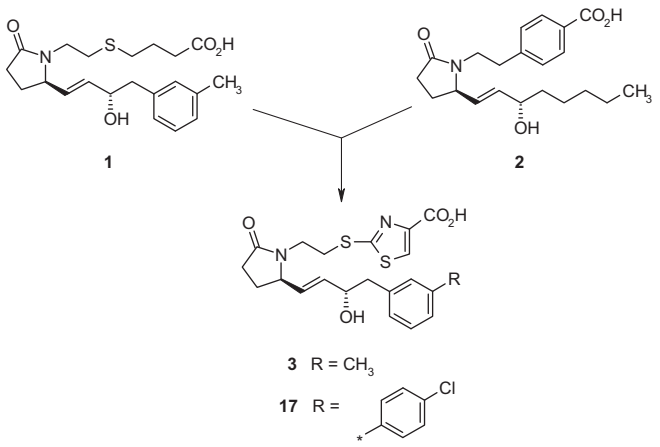


Figure 1. Molecular design of a γ-lactam PGE analogs bearing ethyl-linked 2-mercaptothiazole-4-caboxylic acid as its α-chain.

2. Chemistry

Synthesis of the test compounds listed in Tables 1–3 is described in Schemes 1–9. Compounds **3** and **13**–**15** were synthesized as described in Scheme 1a. Ethanolysis of **19**, followed by *S*-arylation with ethyl 2-bromothiazole-4-carboxylate in the presence of potassium carbonate in ethanol, produced **20**.[10] Deprotection of **20** with tetrabutylammonium fluoride (TBAF) provided an alcohol **21a**. Oxidation of **21a** with dimethyl sulfoxide (DMSO) in the presence of sulfur trioxide–pyridine complex (SO_3–Py) and *N,N*-diisopropylethylamine produced an aldehyde **22a**, which was converted to enones **23a**–**c** by Horner–Emmons olefination using **29a**–**c** (Scheme 1c) as phosphonates. Stereoselective reduction of the 15-ketones of **23a**–**c** with (*R*)-Me-CBS produced 15(*S*)-alcohols **24a**–**c**.[11] Alkaline hydrolysis of **24a**–**c** provided **3** and **14**–**15**. Ethanolysis of **19**, followed by *S*-arylation with ethyl 2-bromothiazole-5-carboxylate, produced **25**. Deprotection of **25** with TBAF yielded an alcohol **26**. Oxidation of **26** with DMSO in the presence of SO_3–Py and *N,N*-diisopropylethylamine, followed by Horner–Emmons olefination, produced an enone **27** using **29a** (Scheme 1c) as a phosphonate. Stereoselective reduction of **27** with (*R*)-Me-CBS resulted in 15(*S*)-alcohol **28**, alkaline hydrolysis of which resulted in **13**.

Synthesis of **16**–**18** is outlined in Scheme 1b. Ester exchange reaction of the above-described **20**, followed by deprotection with TBAF, provided an alcohol **21b**. Oxidation of **21b** with DMSO in the presence of SO_3–Py and *N,N*-diisopropylethylamine produced an aldehyde **22b**, Horner–Emmons olefination of which using **29e**–**g** (Scheme 1c) afforded enones **23d**–**f**. Stereoselective reduction of **23d**–**f** with (*R*)-Me-CBS afforded **24d**–**f**. Alkaline hydrolysis of **24d**–**f** afforded **16**–**18**.

The phosphonates **29a**–**e** were prepared according to conventional methodology.[2,10,12] The preparation of phosphonates **29f**–**g** (Scheme 1c) is described in Scheme 1c. Condensation of (3-bromophenyl)acetic acid with *N,O*-dimethylhydroxylamine hydrochloride in the presence of EDC–HCl produced the corresponding Weinreb amide, palladium-catalyzed cross-coupling reaction of which with 4-chlorophenylboronic acid and 2-naphthaleneboronic acid gave **30a**–**b**. Reaction of Weinreb amides **30a**–**b** with the lithium anion of dimethyl methylphosphonate provided **29f**–**g**.

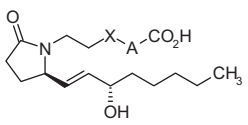
Synthesis of **7** is described in Scheme 2. *O*-Protection of the commercially available D-pyroglutaminol with methoxymethyl chloride, followed by palladium-catalyzed *N*-phenylation with methyl 3-(3-bromophenyl)acrylate, provided **31**. Sodium borohydride reduction of **31** in the presence of nickel dichloride provided saturated *N*-phenylpropionate **32**. Compound **32** was converted to **7** by the conventional procedure of ω-chain synthesis of prostaglandin (PG), as described above.

Compounds **8** and **10b**–**c** were synthesized as described in Scheme 3. *N*-Alkylation of *O*-protected D-pyroglutaminol **33** with 1,3-bis(bromomethyl)benzene in the presence of sodium hydride provided **34a**.[10] Palladium-catalyzed insertion of carbon monoxide into the benzyl bromide moiety of **34a** provided **34b**, which was converted to **8** via **35** by the usual procedure of ω-chain synthesis of PG, as described above. *N*-Alkylation of **33** with methyl 5-(3-bromopropyl)thiophene-2-carboxylate in the presence of sodium hydride produced **34c**, deprotection of which with TBAF provided **36**. Compound **36** was converted to **10b**–**c** by the usual procedure for prostaglandin synthesis.

To synthesize **9**, we developed an alternative synthetic method of 8-aza PGE analogs starting from *N*-Boc D-glutaminol **37** as described in Scheme 4. Oxidation of *N*-Boc D-glutaminol **37** with DMSO in the presence of oxallyl chloride and *N,N*-diisopropylethylamine afforded an aldehyde **38**.[13] Horner–Emmons olefination of **38** with the phosphonate anion prepared from **29d** followed by the usual (*R*)-Me-CBS-reduction of the formed enone resulted in an allyl alcohol **39** stereoselectively. Acidic deprotection of **39** with trifluoroacetic acid afforded **40**. Cyclization of **40** followed by *N*-alkylation of the formed γ-lactam with a bromide **42** prepared as described below gave **41**, alkaline hydrolysis of which afforded **9**.

Synthesis of **10a** is outlined in Scheme 5. Horner–Emmons olefination of an aldehyde **38** with the phosphonate **29a** in the presence of sodium hydride, followed by stereoselective reduction with (*R*)-Me-CBS, produced an allyl alcohol **43**. Acidic deprotection of **43** provided **44**. Reductive alkylation of the amine hydrochloride **44** with 3-(4-bromophenyl)propionaldehyde in the presence of sodium triacetoxyborohydride, followed by intramolecular cyclization and then *O*-protection as a TBS–ether, produced **45**. Carbonyl insertion reaction into the phenylbromide **45** in ethanol provided an ethyl ester **46**. Acidic deprotection of **46** followed by alkaline hydrolysis resulted in **10a**.

Table 1
Effect of the α-chain structure on the activity profiles of the γ-lactam prostanoids bearing the natural ω-chain



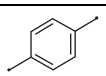
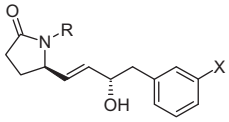
Compound	X	A	Binding assay (K_i, nM)				Functional assay (EC_{50}, nM)	
			mEP1	mEP2	mEP3	mEP4	rEP2	rEP4
2	Bond		>10^4	340	86	1.7	1800	3.1
4	CH_2	$-(CH_2)_3-$	>10^4	>10^4	39	9.2	>10^4	8.4
5	S	$-(CH_2)_3-$	>10^4	2500	26	2.0	>10^4	2.0
	PGE_2		6.0	22	5.0	3.1	19	3.5

Table 2
Effect of the α-chain structure on the activity profiles of the γ-lactam prostanoids bearing the 16-(3-methylphenyl) or 16-(3-chlorophenyl) ω-chains



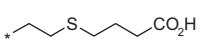
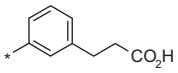
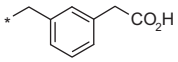
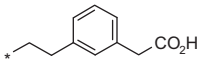
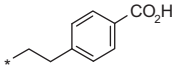
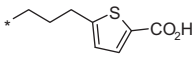
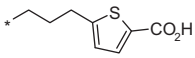
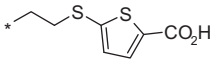
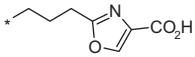
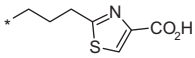
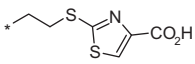
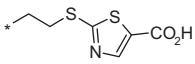
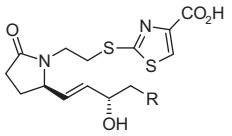
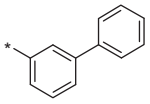
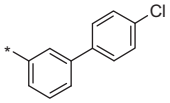
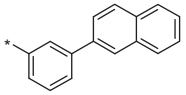
Compound	X	R	Binding assay (K_i, nM)			
			mEP1	mEP2	mEP3	mEP4
1	CH_3		>10^4	>10^4	5800	1.8
6	Cl		>10^4	6900	>10^4	9.0
7	Cl		>10^4	6100	>10^4	3000
8	CH_3		>10^4	>10^4	>10^4	78
9	Cl		5100	>10^4	>10^4	53
10a	CH_3		>10^4	410	>10^4	0.9
10b	Cl		>10^4	190	350	0.8
10c	CH_3		>10^4	127	1600	0.6
10d	CH_3		>10^4	91	570	0.8
11	CH_3		>10^4	240	>10^4	35
12	CH_3		>10^4	84	>10^4	12
3	CH_3		>10^4	9.3	540	0.41
13	CH_3		>10^4	320	>10^4	5.5

Table 3
Effect of the ω-chain structure on the activity profiles of the γ-lactam prostanoids bearing ethyl-linked 2-mercaptothiazole-4-carboxylic acid as the most highly optimized α-chain



Compound	R	Binding assay (K_i, nM)			
		mEP1	mEP2	mEP3	mEP4
14	$-(CH_2)_3CH_3$	3400	3.0	15	0.5
15		>10^4	19	770	1.5
16		4200	3.0	4300	0.94
17		6300	1.7	>10^4	0.81
18		>10^4	22	>10^4	0.012

5-Mercaptothiophene-2-carboxylic acid analog **10d** was synthesized as outlined in Scheme 6. *O*-Methanesulfonylation of **47a** followed by substitution reaction with sodium iodide provided **47c**, which was converted to **48** with sulfur (S_8) and the anion prepared from ethyl thiophene-2-carboxylate and lithium diisopropylamine (LDA).[10] Deprotection of **48** produced **49**, which was converted to **10d** by the usual procedure for ω-chain synthesis of PG, as described above.

Compound **11** was synthesized as described in Scheme 7. *N*-Acylation in the presence of EDC–HCl of DL-serine methyl ester with 4-*tert*-butyldiphenylsilyl(TBDPS)oxybutyric acid, which was prepared by *O*-protection of sodium 4-hydroxybutyrate with TBDPS chloride in the presence of imidazole, provided **50**. Treatment of **50** with diethylaminosulfur trifluoride (DAST) in the presence of potassium carbonate, followed by treatment with bromotrichloromethane and DBU, provided an oxazole **51**, deprotection of which resulted in **52**.[14] Reductive alkylation in the presence of sodium triacetoxyborohydride of **44** (Scheme 5) with an aldehyde, which was prepared by the DMSO oxidation of **52** in the presence of SO_3–Py and diisopropylethylamine, produced **53**. Alkaline hydrolysis of **53** produced **11**.

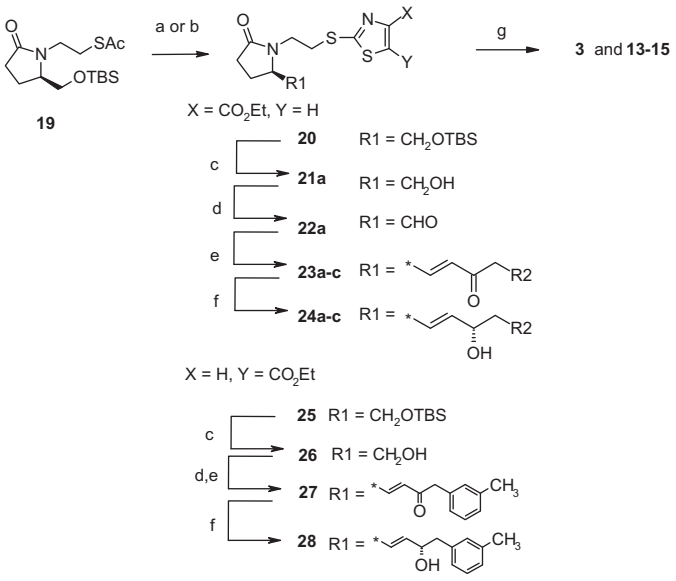
Compound **12** was synthesized as described in Scheme 8. 4,4-Dimethoxybutyronitrile was converted to **54** by the following sequential reactions: (1) NaSH in the presence of $MgCl_2$–$6H_2O$ in DMF; (2) ethyl bromopyruvate in DMF; (3) 2 N HCl in DME. Reductive alkylation of **44** (Scheme 5) with the aldehyde **54** in the presence of sodium triacetoxyborohydride, followed by alkaline hydrolysis, provided **12**.

Compounds **2** and **5** were synthesized as outlined in Scheme 9. Horner–Emmons olefination of **38** with the phosphonate **29b** in the presence of sodium hydride, followed by stereoselective reduction with (*R*)-Me-CBS, produced **55**, acidic deprotection of which provided **56**. Reductive alkylation of **56** with 4-methoxycarbonyl phenylacetaldehyde or **57**, followed by alkaline hydrolysis, resulted in **2** and **5**, respectively.[15]

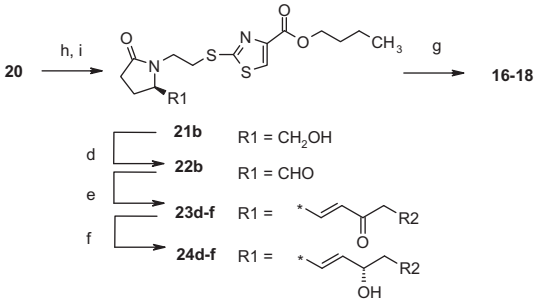
3. Results and discussion

The compounds listed in Tables 1–3 were evaluated for their binding affinity using membrane fractions of CHO cells expressing the mouse EP-receptor. K_i values were determined by a competitive binding assay, which was performed according to the method of Kiriyama et al. with minor modification.[16]

To investigate the effect of the α-chain structure on the activity profiles of the compounds, α-chain congeners **2**, **4**, and **5** (each bearing a natural ω-chain) were compared with natural PGE_2 for their subtype selectivity and receptor affinity. The results are summarized in Table 1. Compound **4**, which possesses an *N*-heptanoic acid moiety, showed moderate and potent binding affinity for EP3 and EP4 subtypes, respectively, but showed no affinity for the EP1 and EP2 subtypes at a concentration of up to 10 μM. The corresponding 5-thia analog **5** showed moderate and highly potent affinity for EP3 and EP4 subtypes, respectively, very weak affinity for EP2 and no affinity for EP1 at a concentration of up to 10 μM. Compared with congeners **4** and **5**, our in-house data suggested that compound **2** has increased affinity for EP2 and slightly more reduced affinity for EP3, while retaining its potent EP4 receptor affinity. As a result, all of these γ-lactam analogs were found to show no affinity for the EP1 subtype at concentrations up to 10 μM. Rat functional assay data for these chemical leads are also listed in Table 1. The potent mouse EP4 binding affinities of **2** and **4**–**5** were reflected in the rat EP4 agonist assay, while they tended to show weaker EP2 functional activities than those expected from

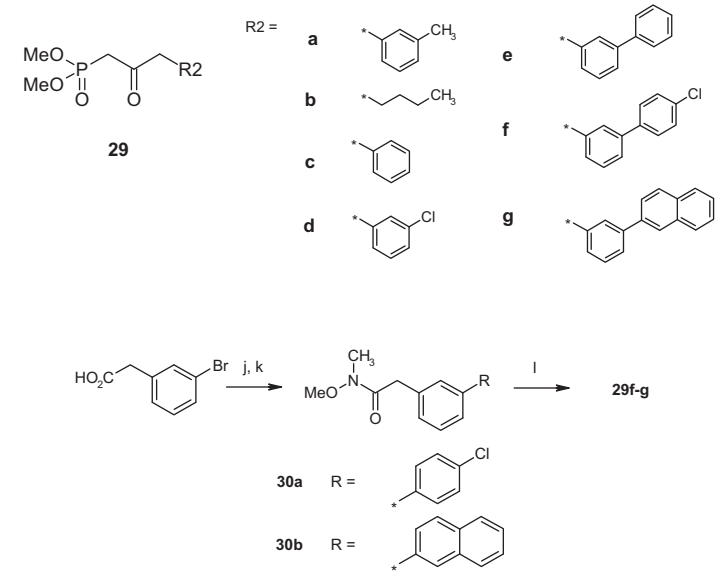


(a) Synthesis of **3**, and **13-15**



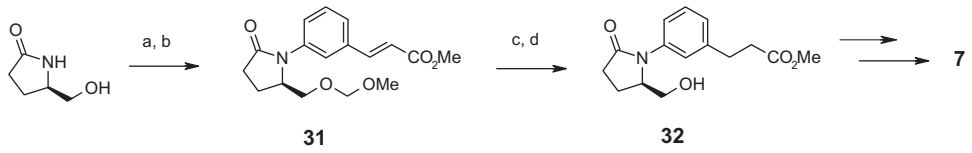
(b) Synthesis of **16-18**

Phosphonates:

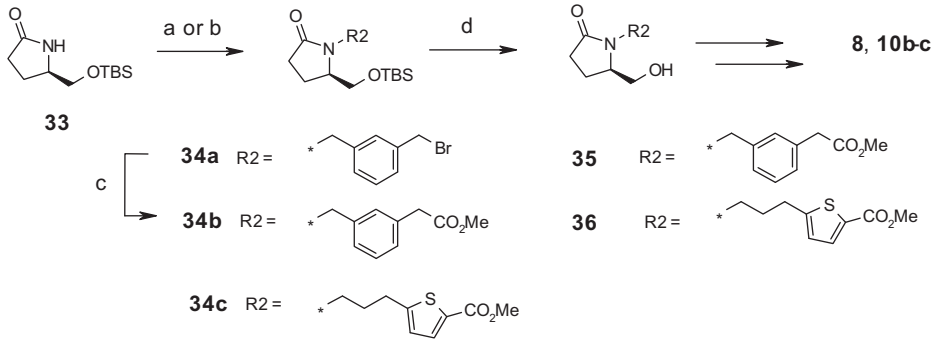


(c) Preparation of phosphonates **29f-g**

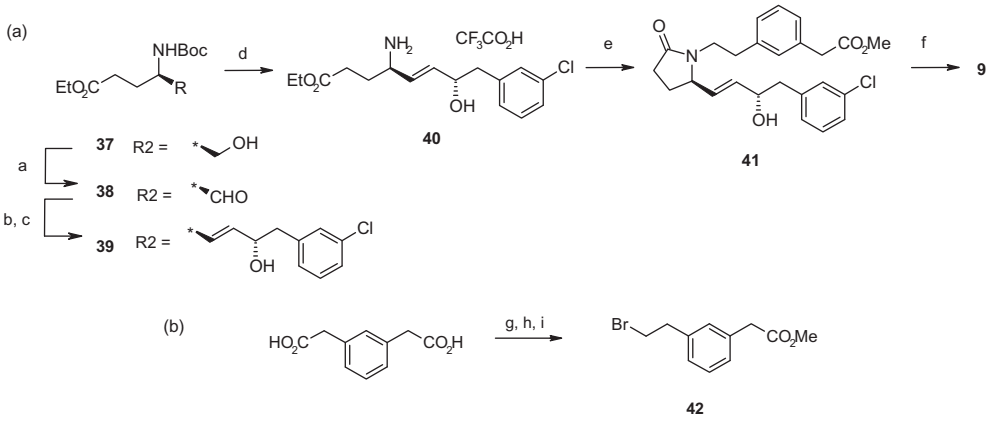
Scheme 1. Synthesis of **3**, **13–18** and preparation of phosphonates **29f–g**. Reagents: (a) ethyl 2-bromothiazole-4-carboxylate, K_2CO_3, EtOH; (b) ethyl 2-bromothiazole-5-carboxylate, K_2CO_3, EtOH; (c) TBAF, THF; (d) SO_3–Py, *i*-Pr_2NEt, DMSO, AcOEt; (e) **29a–c**, **29e–g**, NaH, THF; (f) (*R*)-Me-CBS, BH_3–THF, THF; (g) aq NaOH, DME; (h) K_2CO_3, *n*-BuOH; (i) TBAF, THF; (j) *N*,*O*-dimethylhydroxylamine hydrochloride, EDC–HCl, Et_3N, CH_3CN; (k) 4-chlorophenylboronic acid, $Pd(PPh_3)_4$, aq Na_2CO_3, DME or 2-naphthaleneboronic acid, $Pd(OAc)_2$, (*S*)-BINAP, aq Na_2CO_3, DME; (l) dimethyl methylphosphonate, *n*-BuLi, toluene.



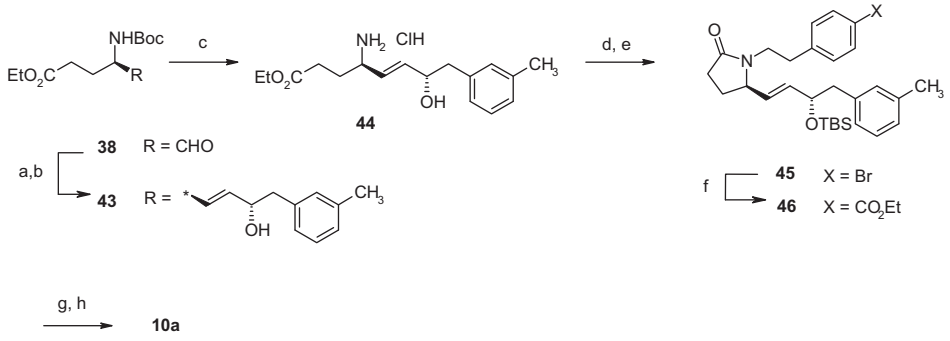
Scheme 2. Synthesis of **7**. Reagents and conditions: (a) $MeOCH_2Cl$, trifluoromethanesulfonic acid, CH_2Cl_2, 80%; (b) methyl 3-(3-bromophenyl)acrylate, $Pd(OAc)_2$, 1,1-bis(diphenylphosphino)ferrocene, KO^{t}Bu, xylene, 49%; (c) $NaBH_4$, $NiCl_2$–$6H_2O$, MeOH, THF; (d) 4 N HCl, dioxane, MeOH, 64% in two steps.



Scheme 3. Synthesis of **8** and **10b–c**. Reagents: (a) 1,3-bis(bromomethyl)benzene, NaH, DMF; (b) methyl 5-(3-bromopropyl)thiophene-2-carboxylate, NaH, DMF, 32%; (c) CO, $PdCl_2(PPh_3)_2$, K_2CO_3, THF, MeOH; (d) TBAF, THF.



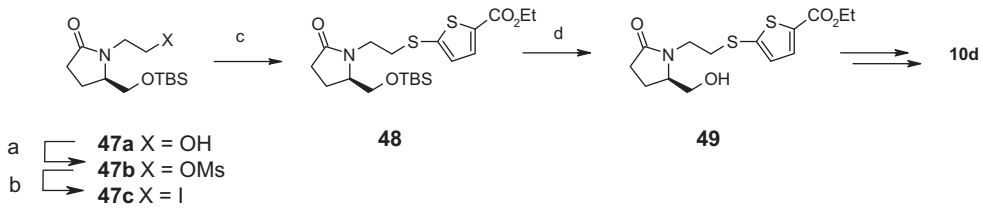
Scheme 4. Synthesis of **9**. Reagents and conditions: (a) $(COCl)_2$, *i*-Pr_2NEt, DMSO, CH_2Cl_2; (b) phosphonate **29d**, NaH, THF; (c) (*R*)-Me-CBS, BH_3–THF, THF; (d) trifluoroacetic acid, CH_2Cl_2; (e) **42**, K_2CO_3, DMF; (f) 2 N NaOH, MeOH, THF; (g) $TMSCHN_2$, MeOH; (h) BH_3–SMe_2, THF, 47%; (i) PBr_3, CH_2Cl_2, 28%.



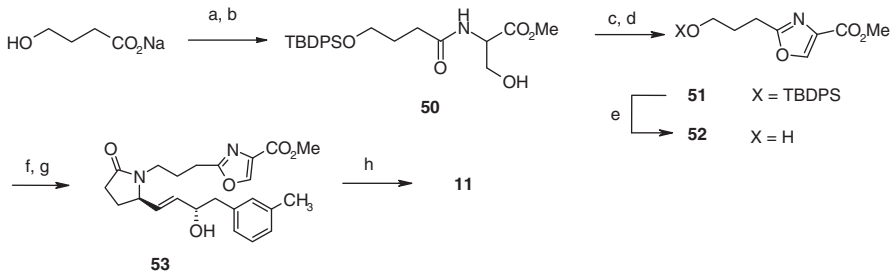
Scheme 5. Synthesis of **10a**. Reagents and conditions: (a) **29a**, NaH, THF; (b) (*R*)-Me-CBS, BH_3–THF, THF, 51% in two steps; (c) HCl in dioxane, EtOH, 100%; (d) 3-(4-bromophenyl)propionaldehyde, $NaBH(OAc)_3$, THF; (e) TBSCl, imidazole, DMF, 72% in two steps; (f) CO gas, $PdCl_2(PPh_3)_2$, 1,1-bis(diphenylphosphino)ferrocene, Et_3N, DMSO, MeOH, 94%; (g) 2 N HCl, MeOH, DME; (h) 2 N NaOH, MeOH, DME, 76% in two steps.

their mouse binding affinities. Native PGE_2 demonstrated non-specific affinities for all subtypes, but showed potent functional activities for both the rat EP2 and EP4 subtypes.

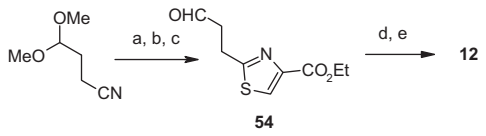
Based on the results described above, structural hybridization of **1** (Fig. 1 and Table 2) and **2** was considered a rational starting point for the discovery of an EP2/EP4 dual agonist with high selectivity



Scheme 6. Synthesis of **10d**. Reagents and conditions: (a) MsCl, Et_3N, THF; (b) NaI, CH_3CN; (c) ethyl 2-thiophene carboxylate, S_8, LDA, THF, 84%; (d) TBAF, THF, 83%.



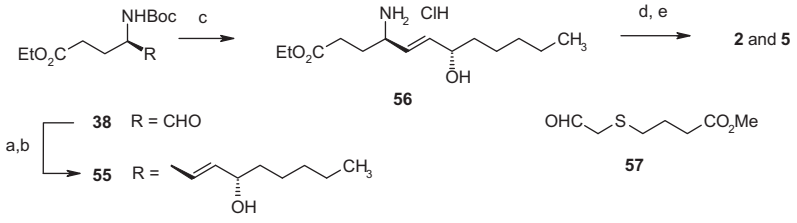
Scheme 7. Synthesis of **11**. Reagents: (a) TBDPSCl, imidazole, DMF; (b) DL-serine methyl ester hydrochloride, EDC–HCl, Et_3N, CH_3CN, 32% in two steps; (c) diethylaminosulfur trifluoride, K_2CO_3, CH_2Cl_2; (d) bromotrichloromethane, DBU, CH_2Cl_2, 54%; (e) TBAF, THF, 79%; (f) SO_3–Py, *i*-Pr_2NEt, DMSO, AcOEt; (g) **44**, $NaBH(OAc)_3$, THF, 74%; (h) aq NaOH, MeOH, DME, 70%.



Scheme 8. Synthesis of **12**. Reagents and conditions: (a) NaSH, $MgCl_2$–$6H_2O$, DMF; (b) ethyl bromopyruvate, DMF; (c) 2 N HCl, DME, 12% in three steps; (d) **44**, $NaBH(OAc)_3$, THF, 60%; (e) 2 N NaOH, DME, MeOH, 66%.

and potency. As shown in Table 2 and 5-thia analogs **1** and **6** bearing a 16-(*meta*-substituted)aryl ω-chain were found to show highly selective and potent EP4 receptor affinity, without a concomitant increase in the EP3 receptor affinity, relative to the corresponding natural ω-chain analog **5** (Table 1).[2] Thus, the 16-(*meta*-substituted)aryl ω-chain was found to be beneficial for reducing EP3 receptor affinity while retaining potent EP4 receptor affinity. Our next focus was placed on the optimization of the α-chain of **1** and **6** toward EP2/EP4 dual selectivity.

According to the reported data and our results described above, we synthesized and evaluated the biological activity of compound **10a**, which bears a benzoic acid moiety in its α-chain.[5] As shown in Table 2, compound **10a** exhibited very potent affinity for the EP4 receptor, and moderate affinity for EP2 receptor, without EP1 and EP3 receptor affinities at concentrations up to 10 μM. On the basis of the data described above, we synthesized and assessed compounds **7**–**9**, which bear a *meta*-substituted phenylene moiety in their α-chains. Compound **7**, in which the *meta*-substituted phenylpropionic acid moiety is directly attached to the nitrogen of the γ-lactam, showed weak receptor affinity for EP2 and EP4 subtypes, with no affinity for EP1 and EP3 at concentrations up to 10 μM. Compound **8**, in which the *meta*-substituted phenyl acetic acid moiety is attached to the nitrogen of the γ-lactam through a methylene moiety, displayed moderate affinity for the EP4 subtype without showing any affinity for EP1, EP2 and EP3 subtypes at concentrations up to 10 μM. Compound **9**, in which the *meta*-substituted phenyl acetic acid moiety is attached to the nitrogen of the γ-lactam through an ethylene moiety, retained the moderate EP4 receptor affinity without losing its EP4 subtype selectivity. As a result, compound **10a** was found to show the most desired result for EP2/EP4 dual selectivity and potency among the tested α-phenylene analogs **7**–**10a**. The corresponding five-membered heteroaryl analogs **10b**–**c** and **11**–**12** were synthesized as their *N*-propyl linked analogs to adjust the length between the γ-lactam moiety and the carboxylic acid function. Replacement of the *N*-ethyl linked benzoic acid α-chain of **10a** with *N*-propyl linked thiophene carboxylic acid provided **10c**, which exhibited EP2/EP4 selectivity and slightly increased EP3 affinity. The corresponding 16-(3-chlorophenyl) analog **10b** exhibited potent affinity for the EP4 subtype, and moderate affinity for the EP2 subtype with a tendency toward increased EP3 affinity. Replacement of the *N*-propyl linker of **10c** with the *N*-ethylmercapto linker provided **10d**, which showed potent affinity for the EP4 subtype, and moderate affinity for the EP2 subtype together with weak affinity for EP3. Activity profiles of the



Scheme 9. Synthesis of **2** and **5**. Reagents: (a) phosphonate **29b**, NaH, THF; (b) (*R*)-Me-CBS, BH_3–THF, THF; (c) HCl in dioxane, EtOH; (d) 4-methoxycarbonyl phenylacetaldehyde or **57**, $NaBH(OAc)_3$, THF; (e) 2 N NaOH, MeOH, DME.

thiophene analogs **10b–d** indicated that they were not suitable as highly selective EP2/EP4 dual agonists because of their insufficient EP2 receptor affinity and increased affinity for the EP3 subtype.

Second, *N*-propyl linked oxazole-4-carboxylic acid analog **11** was synthesized and evaluated. Compound **11** showed a 1.7-fold increase, and a 39-fold reduction, in EP2 and EP4 receptor affinity relative to **10a**, respectively. The corresponding thiazole-4-carboxylic acid analog **12** showed increased affinity for both receptors while retaining good EP2/EP4 dual selectivity. Replacement of the *N*-propylene moiety attached to the 2-position of the thiazole moiety of **12** with a *N*-ethylmercapto moiety was predicted to be beneficial for our experimental aim. 2-Mercaptothiazole-4-carboxylic acid analog **3**, which was designed based on the concept described above, resulted in excellent activity profiles. Its isomeric compound 2-mercaptothiazole-5-carboxylic acid **13** demonstrated 34-fold less potent EP2 receptor affinity, and 13-fold less potent EP4 receptor affinity, relative to **3**, although its EP2/EP4 dual selectivity was still maintained. The significant difference in the EP2/EP4 dual receptor affinity between **3** and **13** was of great interest because of their structural similarity. Compounds **11** and **12**, both of which bear a nitrogen atom in the α-position of the carboxylic acid group, tended to show increased receptor affinity for the EP2 subtype in comparison to **10a**, which does not bear the corresponding nitrogen atom. These α-nitrogen atoms of **11** and **12** may be important for their interaction with the EP2 receptor through a predicted hydrogen bond, whereas the corresponding interaction is not expected in the thiazole-5-carboxylic acid derivative **13**. Also the nitrogen atom at the α-position of the carboxylic acid function in **3** was estimated to strengthen the acidity of the carboxylic acid function more than the isomer **13**. Conversely, compounds **11** and **12** tended to show reduced EP4 receptor affinity relative to **10a** that was effectively recovered by the chemical modifications leading to the 2-mercaptothiazole-4-carboxylic acid derivative **3**, which displayed a remarkable increase in both EP2 and EP4 receptor affinity. Thus, introduction of a sulfur atom into the 2-position of the thiazole moiety was effective in increasing the EP2/EP4 dual subtype selectivity.

The benzoic acid derivative **10a**, oxazole carboxylic acid derivative **11** and thiazole carboxylic acid derivatives **3**, **12** and **13**, had higher affinity for EP2/EP4 subtypes than **7–9**. In compounds **3**, **10a**, and **11–13**, but not in compounds **7–9**, the carboxylic acid group, and the carbon or sulfur atom directly attached to the aryl moiety, occupy the same plane. This structural feature of **3**, **10a** and **11–13** was considered to be a beneficial factor for EP2/EP4 dual selectivity, in addition to the above-described nitrogen atom in the α-position of the carboxylic acid group and the 2-mercapto moiety of the thiazole-4-carboxylic acid analog **3**. Additionally, the 2-mercapto moiety of **3** may have some effects on the three dimensional distance between the γ-lactam nitrogen and the carboxylic acid function.[9] Thus, compound **3**, which bears *N*-ethyl linked 2-mercaptothiazole-4-carboxylic acid as an α-chain and 16-(3-methyl)phenyl as a ω-chain, was found to have the most optimal activity profiles among the tested compounds.

Because evaluation of the EP2/EP4 dual agonist in the bone fracture-healing model in rats requires four weeks, its sustained release from the carrier after the topical injection is considered to be another important determinant of efficacy. Further structural optimization of compound **3** was performed to enable its sustained release from the poly lactide-co-glycolide (PLGA) microsphere formulation.[17] Chemical modification was conducted to concurrently optimize its lipophilicity (*c*Log*P*) and activity profiles so that sustained release of the compound from PLGA microsphere could be achieved. Maintaining the optimal α-chain, we continued further chemical modification of the ω-chain of **3** to investigate SAR in more detail. The results are shown in Table 3. Compound **14**, substituted with 16-(*n*-butyl), and unsubstituted 16-phenyl analog **15**, were synthesized and evaluated by SAR studies. Compound **14** showed potent affinity for EP2, EP3 and EP4 subtypes, with negligible affinity for the EP1 subtype. Replacement of the 16-alkyl moieties of **14** with 16-phenyl moieties provided **15** with a clear tendency of EP2/EP4 dual selectivity, or with clearly reduced affinity for the EP3 receptor subtype. Analogous SAR was also observed by the transformation of **2** to **10a**. Thus, the improved activity profile of 16-phenyl analog **15** relative to the natural ω-chain analog **14** was confirmed. In our previous report, introduction of a substituent into the *meta*-position of the 16-phenyl moiety was reported to be acceptable for compound optimization.[2,12] Based on this information, we introduced phenyl, 4-chlorophenyl and naphth-2-yl moieties into the *meta*-position of the 16-phenyl moiety of **15** to produce **16–18**. Compounds **16** and **17** displayed excellent activity profiles as EP2/EP4 dual agonists because of the increased EP2 affinity and the reduced EP3 affinity relative to **15**. Compound **18** also showed EP2/EP4 dual selectivity as a result of the retained EP2 affinity, 18-fold more potent EP4 affinity and reduced EP3 affinity relative to **15**.

Prior to in vivo evaluation in rats, the activities of compounds **3** and **16–18** in an EP2/EP4 functional assay, and their *c*Log*P* values, were determined. The results are summarized in Table 4. Compounds **3** and **16–18** showed, respectively, 1.9-fold, 8.5-fold, 33-fold and 4×10^5-fold less potency relative to the corresponding binding affinity (rEC_{50}/mK_i). Greater reduction of the agonist activity of **18** relative to the other compounds was considered mainly due to its increased lipophilicity, which promotes strong protein binding in the cell-based assay. The rat EP2 agonist activities, of these compounds were reduced (9.7-fold, 600-fold, 153-fold and >455-fold less potent rEC_{50} relative the mouse K_i value) compared to the rat EP4 agonist activities. These results indicate that the rEP2 agonist activity was affected more than the rEP4 agonist activity in the cell-based assay system. Compounds **16–18** showed higher lipophilicity relative to **3**, which is necessary for their sustained release from the PLGA microsphere. Among compounds **16–18**, compound **17** showed the most balanced profiles regarding dual activity profiles in the rat functional assay and the *c*Log*P* value. Based on the results described above, compound **17** was selected as an EP2/EP4 dual agonist for in vivo evaluation because compounds **16** and **18** demonstrated extremely reduced rEP2 and/or rEP4 functional activities.

Because our aim was to identify a compound that exhibits efficacy in the rat bone fracture-healing model following a single topical injection, we initially investigated the sustained release of compound **17** from PLGA microspheres in vitro. As shown in Table 5, the in vitro sustained release of **17** was observed by measuring its remaining percentage in the PLGA microspheres after 28 days.

Table 4
Functional activities of compounds **3** and **16–18** on rat EP2/EP4 receptors

Compound	Functional assay (EC_{50}, nM)		*c*log*P*
	rEP2	rEP4	
3	90	0.79	3.0
16	1800	8.0	4.4
17	260	27	5.1
18	$>10^4$	480	5.6

Table 5
In vitro release profile of compound **17** from PLGA microspheres

Compound	% Remaining (days)			
	0	7	21	28
17	100	97.9	79.0	48.0

Table 6
Biological evaluation of compound **17**/PLGA in the rat bone fracture-healing model

Parameters	Vehicle	**17**/PLGA (μg/kg)			
		30	100	300	1000
Breaking strength (of % intact)	56.9	68.7	73.2	85.9	105.0

The remaining percentage of **17** in PLGA microspheres was 97.9% after 7 days and 48% after 28 days. These results gave us the confidence to test **17** in PLGA microspheres (**17**/PLGA) in an in vivo rat model of bone healing.

Using the **17**/PLGA microsphere formulation described above, the efficacy of **17** in the rat bone fracture-healing model was investigated. Table 6 shows the changes in the mechanical properties of the healing fibulae. Seventeen days after the topical injection of **17** (30, 100, 300, 1000 μg/kg), the mechanical breaking strength of the fractured fibulae increased in a dose-dependent manner. In the fibulae treated with a topical injection of 1000 μg/kg of **17**/PLGA, the breaking strength reached 105% of nonfractured fibulae, while it was 56.9% in the vehicle-treated fibulae. Based on the results described above, compounds with EP2/EP4 dual agonist activity are promising drug candidates for the treatment of bone fracture.

4. Conclusion

In order to optimize EP2/EP4 subtype selectivity, we have generated ethyl-linked 2-mercaptothiazole-4-carboxylic acid analogs **3** and **15**–**18** bearing a 16-aryl moiety as the ω-chain. These compounds were shown to be putative novel EP2/EP4 dual agonists with excellent subtype selectivity and potency in a mouse receptor assay. We have also discussed compound optimization by SAR, which was initiated by structural hybridization of 5-thia γ-lactam PGE_1 analog **1** and the benzoic acid prototype **2**.

Further structural optimization was performed to enable the sustained release of a test compound from PLGA microspheres. Compound **17**, which exhibited excellent in vitro activity profiles as an EP2/EP4 dual agonist, displayed dose-dependent efficacy in the rat bone fracture-healing model. Compound **17**, and other structurally related compounds, will now undergo full pharmacological and pharmacokinetic evaluation, the results of which will be reported in due course.

5. Experimental

5.1. Chemistry

Analytical samples were homogeneous as confirmed by TLC, and afforded spectroscopic results consistent with the assigned structures. Proton nuclear magnetic resonance spectra (^{1}H NMR) were taken on a Varian Mercury 300 spectrometer, Varian GEMINI-200 or VXR-200s spectrometer using deuterated chloroform ($CDCl_3$), deuterated methanol (CD_3OD) and deuterated dimethylsulfoxide (DMSO-d_6) as the solvent. Fast atom bombardment (FAB-MS, HRMS) and electron ionization (EI) mass spectra were obtained on a JEOL JMS-DX303HF spectrometer. Atmospheric pressure chemical ionization (APCI) mass spectra were determined on a HITACHI MI200H spectrometer. Infrared spectra (IR) were measured in a Perkin-Elmer FT-IR 1760X spectrometer. Melting points and results of elemental analyses were uncorrected. Column chromatography was carried out on silica gel [Merck Silica Gel 60 (0.063–0.200 μm), Wako gel C-200, or Fuji Silysia FL60D]. Thin layer chromatography was performed on silica gel (Merck TLC or HPTLC plates, Silica Gel 60 F_{254}). The following abbreviations for solvents and reagents were used; diethyl ether (Et_2O), *N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethanol (EtOH), ethyl acetate (EtOAc), methanol (MeOH), tetrahydrofuran (THF), methanol (MeOH), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), dimethoxyethane (DME), acetonitrile (CH_3CN), *tert*-butyl methyl ether (MTBE), sulfur trioxide/pyridine complex (SO_3–Py), tetrabutylammonium fluoride (TBAF).

5.1.1. Ethyl 2-({2-[(2*R*)-2-({*tert*-butyldimethylsilyloxy}methyl)-5-oxo-1-pyrrolidinyl]ethyl}thio)-1,3-thiazole-4-carboxylate (20)

To a stirred solution of **19** (1.66 g, 5.00 mmol) in EtOH (25 mL) were added potassium carbonate (897 mg, 6.50 mmol) and ethyl 2-bromothiazole-4-carboxylate (1.42 g, 6.00 mmol) at room temperature under argon atmosphere. After being stirred at room temperature for 16 h, the reaction mixture was diluted with EtOAc, washed with H_2O (×2), brine, and dried over $MgSO_4$. The organic layer was evaporated to give a sulfide **20** as a yellow oil.

5.1.2. Ethyl 2-({2-[(2*R*)-2-(hydroxymethyl)-5-oxo-1-pyrrolidinyl]ethyl}thio)-1,3-thiazole-4-carboxylate (21a)

A solution of **20** (1.58 g, 3.56 mmol) in THF (3 mL) was treated with a solution of TBAF (1.0 M in THF, 4.27 mL, 4.27 mmol) at room temperature under argon atmosphere for 1 h. After addition of brine, the reaction mixture was extracted with EtOAc repeatedly (×5). The combined organic layers were dried over $MgSO_4$, and then evaporated. The resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 1:1–0:1) to give **21a** as a pale yellow oil (1.05 g, 63% in 2 steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.00 (s, 1H), 4.39 (q, J = 7.2 Hz, 2H), 3.93–3.65 (m, 5H), 3.61–3.40 (m, 2H), 3.33 (m, 1H), 2.56–2.25 (m, 2H), 1.12 (m, 1H), 0.90 (m, 1H), 0.40 (t, J = 7.2 Hz, 3H).

5.1.3. Ethyl 2-({2-[(2*R*)-2-formyl-5-oxo-1-pyrrolidinyl]ethyl}thio)-1,3-thiazole-4-carboxylate (22a)

To a stirred solution of the alcohol **21a** (460 mg, 1.39 mmol) in EtOAc (5 mL) and *N*,*N*-diisopropylethylamine (1.46 mL, 8.36 mmol) was added a solution of SO_3–Py (665 mg, 4.18 mmol) in DMSO (2.5 mL) at 0 °C under argon atmosphere. After being stirred at the same temperature for 20 min, the reaction was quenched with 1 N HCl. The reaction mixture was diluted with EtOAc, washed with brine, and dried over $MgSO_4$. The organic layer was evaporated to yield an aldehyde **22a** as a pale yellow oil.

5.1.4. Ethyl 2-[(2-{(2*R*)-2-[(1*E*)-4-(3-methylphenyl)-3-oxo-1-buten-1-yl]-5-oxo-1-pyrrolidinyl}ethyl)thio]-1,3-thiazole-4-carboxylate (23a)

To a stirred solution of dimethyl 3-(3-methylphenyl)-2-oxopropanephosphonate **29a** (166 mg, 0.649 mmol) in THF (5 mL) was added sodium hydride (62.5% in mineral oil, 21.5 mg, 0.556 mmol) at 0 °C under argon atmosphere and stirring was further continued at ambient temperature for 90 min. To the stirred suspension was added a solution of the above-described aldehyde **22a** in THF (2 mL) at 0 °C and stirring was continued at room temperature for 1 h. The reaction was quenched with saturated aqueous NH_4Cl. The reaction mixture was diluted with EtOAc, washed with water, then brine, and dried over $MgSO_4$. The organic layer was evaporated to give an enone **23a** as a pale yellow oil.

5.1.5. Ethyl 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-3-hydroxy-4-(3-methylphenyl)-1-buten-1-yl]-5-oxo-1-pyrrolidinyl}ethyl)thio]-1,3-thiazole-4-carboxylate (24a)

To a stirred solution of **23a** in THF (2.3 mL) was added a solution of (*R*)-2-methyl-CBS-oxazaborolidine (1.0 M in toluene, 0.116 mL, 0.116 mmol) at room temperature under argon atmosphere. To the reaction mixture was added dropwise a solution of borane–THF complex (1.0 M in THF, 0.278 mL, 0.278 mmol) over 5 min. The resulting solution was stirred for 20 min, then treated with

MeOH and stirring was continued for 5 min. The reaction mixture was diluted with EtOAc, washed with 1 N HCl, water, saturated aqueous $NaHCO_3$, brine, and dried over Na_2SO_4. The organic layer was evaporated and the resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 1:2–0:1) to give an alcohol **24a** as a colorless oil (106 mg, 50% in 3 steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.01 (s, 1H), 7.18 (m, 1H), 7.08–6.93 (m, 3H), 5.81 (dd, J = 15.3, 5.7 Hz, 1H), 5.50 (ddd, J = 15.3, 8.7, 1.0 Hz, 1H), 4.43–4.30 (m, 3H), 4.20 (m, 1H), 3.72 (m, 1H), 3.40 (m, 2H), 3.20 (m, 1H), 2.80–2.73 (m, 2H), 2.45–2.10 (m, 7H), 1.75 (m, 1H), 1.38 (t, J = 7.2 Hz, 3H).

5.1.6. 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxy-4-(3-methylphenyl)but-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)sulfanyl]-1,3-thiazole-4-carboxylic acid (3)

To a stirred solution of **24a** (105 mg, 0.229 mmol) in DME (0.5 mL) was added 2 N NaOH (0.229 mL, 0.458 mmol) and stirring was continued at ambient temperature for 2 h. After acidification with 2 N HCl under cooling, the reaction mixture was extracted with EtOAc (×3). The combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 100:1–9:1) to afford **3** as a colorless oil (79 mg, 80%). IR (film): 3390, 3118, 3007, 2921, 1714, 1666, 1494, 1421, 1360, 1325, 1216, 1100, 1029, 975, 751 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.08 (s, 1H), 7.20 (m, 1H), 7.08–6.95 (m, 3H), 5.80 (dd, J = 15.3, 5.7 Hz, 1H), 5.50 (dd, J = 15.3, 8.7 Hz, 1H), 4.40 (m, 1H), 4.12 (m, 1H), 3.70 (m, 1H), 3.50–2.95 (m, 5H), 2.85–2.78 (m, 2H), 2.50–2.19 (m, 6H), 1.77 (m, 1H); MS (APCI) *m/z*: 431 (M−H)$^-$; HRMS-FAB (*m/z*): [M−H]$^-$ calcd for $C_{23}H_{23}N2O_4S_2$, 431.1099; found, 431.1113.

5.1.7. Ethyl 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-3-hydroxy-1-octen-1-yl]-5-oxo-1-pyrrolidinyl}ethyl)thio]-1,3-thiazole-4-carboxylate (24b)

Compound **24b** was prepared from **22a** using a phosphonate **29b** instead of **29a** according to the same procedure as described for the preparation of **24a** from **22a** as a colorless oil (99 mg, 51% from **21a**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02 (s, 1H), 5.78 (dd, J = 15.3, 5.7 Hz, 1H), 5.54 (dd, J = 15.3, 9.0 Hz, 1H), 4.39 (q, J = 6.9 Hz, 2H), 4.21 (m, 1H), 4.10 (m, 1H), 3.79 (m, 1H), 3.50–3.38 (m, 3H), 2.50–2.10 (m, 3H), 1.95 (br s, 1H), 1.77 (m, 1H), 1.66–1.20 (m, 11H), 0.87 (t, J = 7.2 Hz, 3H).

5.1.8. 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxyoct-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)sulfanyl]-1,3-thiazole-4-carboxylic acid (14)

Compound **14** was prepared from **24b** according to the same procedure as described for the preparation of **3** from **24a** as a colorless oil (78 mg, 85%). IR (film): 3402, 3121, 2929, 2858, 1714, 1665, 1505, 1459, 1422, 1357, 1325, 1214, 1146, 1027, 975, 753 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.10 (s, 1H), 5.80 (dd, J = 15.6, 6.0 Hz, 1H), 5.55 (dd, J = 15.6, 8.7 Hz, 1H), 4.30–3.77 (m, 5H), 3.60–3.29 (m, 3H), 2.58–2.20 (m, 3H), 1.80 (m, 1H), 1.62–1.21 (m, 8H), 0.88 (t, J = 7.5 Hz, 3H); MS (APCI) *m/z*: 397 (M−H)$^-$; HRMS-FAB (*m/z*): [M+H]$^+$ calcd for $C_{18}H_{27}N_2O_4S_2$, 399.1412; found, 399.1421.

5.1.9. Ethyl 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-3-hydroxy-4-phenylbut-1-enyl]-5-oxo-1-pyrrolidinyl}ethyl)thio]-1,3-thiazole-4-carboxylate (24c)

Compound **24c** was prepared from **22a** using a phosphonate **29c** instead of **29a** according to the same procedure as described for the preparation of **24a** from **22a** as a colorless oil (101 mg, 49% from **24a**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02 (s, 1H), 7.33–7.12 (m, 5H), 5.80 (dd, J = 15.6, 6.0 Hz, 1H), 5.49 (ddd, J = 15.6, 8.4, 1.0 Hz, 1H), 4.42–4.30 (m, 3H), 4.20 (m, 1H), 3.70 (m, 1H), 3.39 (m, 2H), 3.20 (m, 1H), 2.85–2.78 (m, 2H), 2.45–2.08 (m, 4H), 1.72 (m, 1H), 1.38 (t, J = 7.2 Hz, 3H).

5.1.10. 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxy-4-phenylbut-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)sulfanyl]-1,3-thiazole-4-carboxylic acid (15)

Compound **15** was prepared from **24c** according to the same procedure as described for the preparation of **3** from **24a** as a colorless oil (80 mg, 85%). IR (film): 3398, 3005, 2923, 1715, 1664, 1496, 1421, 1325, 1216, 1099, 1029, 976, 750, 702 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.09 (s, 1H), 7.38–7.14 (m, 5H), 5.80 (dd, J = 15.3, 6.0 Hz, 1H), 5.47 (dd, J = 15.3, 8.7 Hz, 1H), 4.40 (m, 1H), 4.21–3.61 (m, 4H), 3.38–3.16 (m, 3H), 2.97–2.79 (m, 2H), 2.52–2.18 (m, 3H), 1.76 (m, 1H); MS (APCI) *m/z*: 417 (M−H)$^-$; HRMS-FAB (*m/z*): [M+H]$^+$ calcd for $C_{20}H_{23}N_2O_4S_2$, 419.1099; found, 419.1105.

5.1.11. Ethyl 2-({2-[(2*R*)-2-(hydroxymethyl)-5-oxo-1-pyrrolidinyl]ethyl}thio)-1,3-thiazole-5-carboxylate (26)

Compound **26** was prepared from **19** according to the same procedure as described for the preparation of **21a** from **19** as a pale yellow oil (358 mg, 64% from **19**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.19 (s, 1H), 4.35 (q, J = 7.2 Hz, 2H), 4.40–3.89 (m, 7H), 3.15–3.02 (br s, 1H), 2.58–2.33 (m, 2H), 2.19–2.05 (m, 1H), 1.90–1.78 (m, 1H), 1.38 (t, J = 7.2 Hz, 3H).

5.1.12. Ethyl 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxy-4-(3-methylphenyl)but-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)sulfanyl]-1,3-thiazole-5-carboxylate (28)

Compound **28** was prepared from **26** according to the same procedure as described for the preparation of **24a** from **21a** as a colorless oil (248 mg, 50% in three steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.18 (s, 1H), 7.20 (t, J = 8.0 Hz, 1H), 7.10–6.90 (m, 3H), 5.74 (dd, J = 15, 6.0 Hz, 1H), 5.51 (dd, J = 15, 9.0 Hz, 1H), 4.41–4.33 (m, 1H), 4.36 (q, J = 7 Hz, 2H), 4.12 (q, J = 7 Hz, 1H), 3.75–3.6 (m, 1H), 3.40 (t, J = 7 Hz, 2H), 3.3–3.2 (m, 1H), 2.8–2.7 (m, 2H), 2.5–2.1 (m, 6H), 2.0–1.95 (br, 1H), 1.35 (t, J = 7 Hz, 3H).

5.1.13. 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxy-4-(3-methylphenyl)but-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)sulfanyl]-1,3-thiazole-5-carboxylic acid (13)

Compound **13** was prepared from **28** according to the same procedure as described for the preparation of **3** from **24a** as a colorless viscous oil (44 mg, 93%). IR (film): 3387, 2922, 1701, 1520, 1418, 1359, 1241, 1158, 1097, 1035, 975, 755 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.17 (s, 1H), 7.14 (t, J = 8 Hz, 1H), 7.0–6.9 (m, 3H), 5.68 (dd, J = 15, 7 Hz, 1H), 5.35 (dd, J = 15, 9 Hz, 1H), 4.31 (q, J = 7 Hz, 1H), 4.25–4.1 (m, 1H), 3.7–3.55 (m, 1H), 3.4–3.2 (m, 2H), 3.05–2.9 (m, 1H), 2.88 (dd, J = 13, 6 Hz, 1H), 2.63 (dd, J = 13, 7 Hz, 1H), 2.4–2.25 (m, 5H), 2.25–2.1 (m, 1H), 1.75–1.6 (m, 1H); MS (APCI) *m/z*: 431 (M−H)$^-$; HRMS-FAB (*m/z*): [M+H]$^+$ calcd for $C_{21}H_{25}N_2O_4S_2$, 433.1256; found, 433.1250.

5.1.14. Butyl 2-({2-[(2*R*)-2-(hydroxymethyl)-5-oxo-1-pyrrolidinyl]ethyl}thio)-1,3-thiazole-4-carboxylate (21b)

To a stirred solution of **20** (1.71 g, 3.85 mmol) in *n*-butanol (39 mL) was added potassium carbonate (53 mg, 0.385 mmol) under argon atmosphere. After being stirred at 90 °C for 3 h, the reaction mixture was cooled to room temperature, diluted with EtOAc, washed with water, brine, and dried over Na_2SO_4. The organic layer was evaporated to give an ester as a brown oil. A solution of the ester in THF (3.9 mL) was treated with TBAF (1.0 M in THF, 3.85 mL, 3.85 mmol) at room temperature. After being stirred for 2 h, the reaction mixture was diluted with EtOAc, washed with water, brine, and dried over Na_2SO_4. The organic layer was evaporated and the resulting residue was purified by column chromatography on silica gel (EtOAc/MeOH, 100:0–20:1) to afford **21b** as a brownish orange oil (820 mg, 59% from **23**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.99 (s, 1 H). 4.33 (t, J = 6.8 Hz, 2 H), 3.30–3.96 (m, 7H), 2.57–2.23

(m, 2H), 2.23–2.06 (m, 1H), 1.98–1.82 (m, 1H), 1.81–1.65 (m, 2H), 1.54–1.35 (m, 2H), 0.97 (t, J = 6.0 Hz, 3H), 0.75–0.35 (m, 1H).

5.1.15. Butyl 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-4-(3-biphenylyl)-3-hydroxy-1-buten-1-yl]-5-oxo-1-pyrrolidinyl}ethyl)thio]-1,3-thiazole-4-carboxylate (24d)

Compound **24d** was prepared from **21b** using a phosphonate **29e** instead of **29a** according to the same procedure as described for the preparation of **24a** from **21a** as a colorless oil (67 mg, 29% from **21b**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.98 (s, 1H), 7.64–7.29 (m, 8H),7.19–7.08 (m, 1H), 5.85 (dd, J = 15.4, 5.8 Hz, 1H), 5.52 (dd, J = 15.4, 8.8 Hz, 1H), 4.49–4.03 (m, 4H), 3.84–3.57 (m, 2H), 3.33 (t, J = 6.6 Hz, 2H), 3.26–3.11 (m, 1H), 2.94–2.80 (m, 2H), 2.45–2.06 (m, 3H), 1.80–1.49 (m, 3H), 1.50–1.33 (m, 2H), 0.94 (t, J = 7.2 Hz, 3H).

5.1.16. 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-4-(1,1'-Biphenyl-3-yl)-3-hydroxy but-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)sulfanyl]-1,3-thiazole-4-carboxylic acid (16)

Compound **16** was prepared from **24d** according to the same procedure as described for the preparation of **3** from **24a** as a colorless amorphous oil (46 mg, 78%). IR (film): 3421, 2925, 1716, 1662, 1504, 1479, 1455, 1421, 1324, 1214, 1100, 1027, 975, 921, 853, 799, 758 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): 8.07 (s, 1H), 7.35 (m, 9H), 5.82 (dd, J = 15.4, 5.8 Hz, 1H), 5.51 (dd, J = 15.4, 8.8 Hz, 1H), 4.47 (m, 1H), 4.11 (m, 1H), 3.68 (m, 1H), 3.06–2.96 (m, 7H), 2.30–2.15 (m, 3H), 1.72 (m, 1H); MS (APCI) m/z: 493 $(M-H)^-$; HRMS-FAB (m/z): $[M-H]^-$ calcd for $C_{26}H_{25}N_2O_4S_2$, 493.1256; found, 493.1230.

5.1.17. Butyl 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-4-(4'-chloro-3-biphenylyl)-3-hydroxy-1-buten-1-yl]-5-oxo-1-pyrrolidinyl}ethyl)thio]-1,3-thiazole-4-carboxylate (24e)

Compound **24e** was prepared from **21b** using a phosphonate **29f** instead of **29a** according to the same procedure as described for the preparation of **24a** from **21a** as a colorless oil (65 mg, 26% from **21b**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.99 (s, 1H), 7.55–7.30 (m, 7H), 7.21–7.12 (m, 1H), 5.85 (dd, J = 15.4, 5.8 Hz, 1H), 5.53 (dd, J = 15.4, 8.5 Hz, 1H), 4.48–4.07 (m, 4H), 3.79–3.58 (m, 1H), 3.35 (t, J = 6.9 Hz, 2H), 3.29–3.15 (m, 1H), 2.91–2.81 (m, 2H), 2.47–2.07 (m, 3H), 1.78–1.60 (m, 3H), 1.49–1.34 (m, 2H), 0.94 (t, J = 7.4 Hz, 3H).

5.1.18. 2-[(2-{(2*R*)-2-[(1*E*,3*S*)-4-(4'-Chloro-1,1'-biphenyl-3-yl)-3-hydroxybut-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)sulfanyl]-1,3-thiazole-4-carboxylic acid (17)

Compound **17** was prepared from **24e** according to the same procedure as described for the preparation of **3** from **24a** as a colorless amorphous oil (49 mg, 83%). IR (film): 3115, 2923, 2691, 2581, 2510, 1716, 1661, 1498, 1478, 1421, 1396, 1372, 1323, 1260, 1212, 1091, 1026, 1012, 974, 921, 833, 791, 748 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.07 (s, 1H), 7.35 (m, 8H) 5.83 (dd, J = 15.4, 5.8 Hz, 1H), 5.52 (dd, J = 15.4, 8.8 Hz, 1H), 4.46 (m, 1H), 4.11 (m, 1H), 3.48–3.11 (m, 6H), 2.90–2.85 (m, 2H), 2.30–2.11 (m, 3 H), 1.69 (m, 1H); FAB-MS (m/z): 529 $(M+H)^+$; HRMS-FAB (m/z): $[M+H]^+$ calcd for $C_{26}H_{26}ClN_2O_4S_2$, 529.1023; found, 529.1025.

5.1.19. Butyl 2-({2-[(2*R*)-2-{(1*E*,3*S*)-3-hydroxy-4-[3-(2-naphthyl) phenyl]-1-buten-1-yl}-5-oxo-1-pyrrolidinyl]ethyl}thio)-1,3-thiazole-4-carboxylate (24f)

Compound **24f** was prepared from **21b** using a phosphonate **29g** instead of **29a** according to the same procedure as described for the preparation of **24a** from **21a** as a colorless oil (102 mg, 40% from **21b**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02 (s, 1H), 7.95–7.82 (m, 4H), 7.76–7.69 (m, 1H), 7.62–7.46 (m, 3H), 7.41 (t, J = 7.6 Hz, 2H), 7.18 (m, J = 7.4 Hz, 1H), 5.87 (dd, J = 15.4, 5.8 Hz, 1H), 5.53 (dd, J = 15.4, 8.8 Hz, 1H), 4.50–4.39 (m, 1H), 4.29 (t, J = 7.1 Hz, 2H), 4.32–4.17 (m, 2H), 3.73–3.60 (m, 1H), 3.42–3.14 (m, 3H), 2.96–2.86 (m, 2H), 2.44–2.10 (m, 4H) 1.77–1.63 (m, 2H), 1.46–1.33 (m, 2H), 0.92 (t, J = 7.4 Hz, 3H).

5.1.20. 2-{[2-((2*R*)-2-{(1*E*,3*S*)-3-Hydroxy-4-[3-(2-naphthyl) phenyl]but-1-enyl}-5-oxopyrrolidin-1-yl)ethyl]sulfanyl}-1,3-thiazole-4-carboxylic acid (18)

Compound **18** was prepared from **24f** according to the same procedure as described for the preparation of **3** from **24a** as a colorless amorphous oil (49 mg, 83%). IR (film): 3114, 3053, 2923, 1717, 1663, 1507, 1490, 1456, 1421, 1324, 1213, 1101, 1028, 975, 891, 859, 821, 790 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.05–8.00 (m, 2H), 7.93–7.82 (m, 3H), 7.71 (dd, J = 8.4, 1.8 Hz, 1H), 7.61 (d, J = 7.8 Hz, 1H), 7.56–7.47 (m, 4H), 7.42 (t, J = 7.8 Hz, 1H), 7.19 (d, J = 7.8 Hz, 1H), 5.84 (dd, J = 15.3, 5.7 Hz, 1H), 5.52 (dd, J = 15.3, 8.7 Hz, 1H), 4.49 (q, J = 6.0 Hz, 1H), 4.15–4.05 (m, 1H), 3.75–3.65 (m, 1H), 3.35–3.05 (m, 3H), 2.95 (dd, J = 7.2, 3.3 Hz, 2H), 2.50–2.10 (m, 3H), 1.80–1.60 (m, 1H); FAB-MS (m/z): 545 $(M+H)^+$; HRMS-FAB (m/z): $[M+H]^+$ calcd for $C_{30}H_{29}N_2O_4S_2$, 545.1569; found, 545.1573.

5.1.21. 2-(4'-Chloro-3-biphenylyl)-*N*-methoxy-*N*-methylace tamide (30a)

To a stirred solution of *N*,*O*-dimethylhydroxylamine hydrochloride (3.41 g, 35.0 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (5.81 g, 30.3 mmol) and triethylamine (4.88 mL, 35.0 mmol) in CH_3CN (40 mL) was added a solution of (3-bromophenyl)acetic acid (5.00 g, 23.3 mmol) in CH_3CN (17 mL) at room temperature under argon atmosphere. After being stirred for 1 h, the reaction was quenched with water. The reaction mixture was diluted with EtOAc, washed with 2 N HCl, water, then brine, and dried over $MgSO_4$. The organic layer was evaporated to give a Weinreb amide as a colorless oil.

To a stirred solution of above-described Weinreb amide (1.20 g, 4.66 mmol) in DME (15 mL) and H_2O (10 mL) were added 4-chlorophenylboronic acid (1.09 g, 7.00 mmol), sodium carbonate (742 mg, 7.00 mmol) and tetrakis(triphenylphosphine)palladium (60 mg, 0.052 mmol) at under argon atmosphere. After being stirred at 90 °C for 5 h, the reaction mixture was cooled to room temperature. The resulting mixture was filtered through a pad of Celite, and the filtrate was evaporated and purified by column chromatography on silica gel (hexane/EtOAc, 3:1–1:1) to afford **30a** as a pale yellow solid (1.58 g, 100%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.54–7.47 (m, 3H), 7.44–7.35 (m, 4H), 7.32–7.27 (m, 1H), 3.84 (s, 2H), 3.65 (s, 3H), 3.21 (s, 3H).

5.1.22. Dimethyl [3-(4'-chloro-3-biphenylyl)-2-oxopropyl] phosphonate (29f)

To a stirred solution of dimethyl methylphosphonate (0.76 mL, 7.00 mmol) in toluene (15 mL) was added dropwise a solution of *n*-BuLi (1.57 M in hexane, 5.04 mL, 7.92 mmol) at −78 °C under argon atmosphere, and stirring was continued for 1 h at the same temperature. To the reaction mixture was added a solution of **30a** (1.58 g, 4.66 mmol) in toluene (4 mL), and stirring was continued for additional 2 h at the same temperature. The reaction was quenched with acetic acid. The reaction mixture was allowed to warm up to room temperature with stirring, diluted with EtOAc, washed with water, then brine, and dried over $MgSO_4$. The organic layer was evaporated and the resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 2:1–0:1) to give a phosphonate **29f** as a colorless oil (1.07 g, 65%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.57–7.32 (m, 7H), 7.22 (d, J = 7.1 Hz, 1H), 3.97 (s, 2H), 3.81 (s, 3H), 3.78 (s, 3H), 3.15 (d, J = 23.3 Hz, 2H).

5.1.23. *N*-Methoxy-*N*-methyl-2-[3-(2-naphthyl)phenyl]acetamide (30b)

To a stirred solution of the Weinreb amide (1.81 g, 7.00 mmol) described in the experimental of **30a** in DME (15 mL) and 2 M aqueous sodium carbonate (10.5 mL, 21.0 mmol) were added naphthalene-2-boronic acid (2.49 g, 14.0 mmol), (*S*)-BINAP (871 mg, 1.4 mmol) and palladium acetate (157 mg, 0.7 mmol) at under argon atmosphere. After being stirred at 80 °C for 12 h, the reaction mixture was cooled to room temperature. The resulting mixture was filtered through a pad of Celite. The filtrate was evaporated and purified by column chromatography on silica gel (hexane/EtOAc, 4:1–1:1) to afford **30b** as a pale yellow solid (1.61 g, 75%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.08–8.01 (m, 1 H) 7.94–7.83 (m, 3H), 7.75 (dd, J = 8.5, 1.9 Hz, 1H), 7.68–7.57 (m, 2H), 7.54–7.41 (m, 3H), 7.35–7.29 (m, 1H), 3.88 (s, 2H), 3.65 (s, 3H), 3.23 (s, 3H).

5.1.24. Dimethyl {3-[3-(2-naphthyl)phenyl]-2-oxopropyl}phosphonate (29g)

Compound **29g** was prepared from **30b** according to the same procedure as described for the preparation of **29f** from **30a** as a colorless oil (1.18 g, 61%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.03 (s, 1H), 7.95–7.83 (m, 3H), 7.73 (dd, J = 8.7, 1.8 Hz, 1H), 7.65 (d, J = 8.7 Hz, 1H), 7.58 (s, 1H), 7.54–7.43 (m, 3H), 7.28–7.22 (m, 1H), 4.01 (s, 2H), 3.82 (s, 3H), 3.78 (s, 3H), 3.17 (d, J = 22.8 Hz, 2H).

5.1.25. Methyl (2*E*)-3-(3-{(2*R*)-2-[(methoxymethoxy)methyl]-5-oxo-1-pyrrolidinyl}phenyl)acrylate (31)

To a stirred solution of (*R*)-5-(hydroxymethyl)-2-pyrrolidinone (1.50 g, 13.0 mmol) in CH_2Cl_2 (12 mL) were added dimethoxymethane (12 mL) and trifluoromethanesulfonic acid (1.0 mL) at room temperature under argon atmosphere. After being stirred for 6 h, the reaction mixture was poured into cold saturated aqueous NH_4Cl, and extracted with EtOAc (×6). The combined organic layers were washed with brine, dried over $MgSO_4$, and evaporated to give an methoxymethoxy(MOM) ether as a pale yellow oil (1.65 g, 80%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.07 (br s, 1H), 4.63 (s, 2H), 3.86 (m, 1H), 3.60 (dd, J = 9.9, 3.6 Hz, 1H), 3.38 (m, 1H), 3.37 (s, 3H), 2.44–2.14 (m, 3H), 1.75 (m, 1H).

To a stirred solution of the above-described MOM ether (300 mg, 1.89 mmol) in xylene (4.7 mL) were added methyl 3-(3-bromophenyl)acrylate (682 mg, 2.83 mmol), palladium acetate (32 mg, 0.141 mmol) and 1,1-bis(diphenylphosphino)ferrocene (627 mg, 1.13 mmol) and potassium *tert*-butoxide (317 mg, 2.83 mmol) at room temperature under argon atmosphere. After being stirred at 140 °C for 6 h, the reaction mixture was cooled to room temperature and filtered through a pad of Celite. The filtrate was evaporated and purified by column chromatography on silica gel ($CHCl_3$/MeOH, 140:1–100:1) to afford **31** as a brown viscous oil (296 mg, 49%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.74–7.32 (m, 5H), 6.44 (d, J = 16.2 Hz, 1H), 4.56 (d, J = 6.6 Hz, 1H), 4.49 (d, J = 6.6 Hz, 1H), 4.39 (m, 1H), 3.81 (s, 3H), 3.64–3.50 (m, 2H), 3.25 (s, 3H), 2.75 (m, 1H), 2.54 (m, 1H), 2.35 (m, 1H), 2.16 (m, 1H).

5.1.26. Methyl (2*E*)-3-{3-[(2*R*)-2-(hydroxymethyl)-5-oxo-1-pyrrolidinyl]phenyl}acrylate (32)

To a stirred solution of **31** (280 mg, 0.877 mmol) in THF (4 mL) and MeOH (4 mL) were added nickel chloride hexahydrate (208 mg, 0.877 mmol), and then sodium borohydride (133 mg, 3.51 mmol) in several portions at 0 °C under argon atmosphere. After being stirred for 1 h, the reaction mixture was diluted with Et_2O and filtered through a pad of Celite. The filtrate was poured into cold aqueous NH_4Cl, extracted with Et_2O (×2). The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to give the corresponding saturated ester as a brown viscous oil (278 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36–7.16 (m, 3H), 7.05 (m, 1H), 4.56 (d, J = 6.3 Hz, 1H), 4.49 (d, J = 6.3 Hz, 1H), 4.35 (m, 1H), 3.67 (s, 3H), 3.56–3.50 (m, 2H), 3.26 (s, 3H), 2.95 (t, J = 7.2 Hz, 2H), 2.82–2.16 (m, 6H).

A mixture of the above-described saturated ester (278 mg, 0.877 mmol) in MeOH (3.5 mL) and 4 N HCl in dioxane (0.88 mL) was stirred at room temperature for 5 h. The reaction mixture was poured into ice-water, extracted with EtOAc (×2), washed with brine, dried over $MgSO_4$ and evaporated. The resulting residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 170:–30:1) to give **32** as a brown viscous oil (156 mg, 64% in two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.38–7.20 (m, 3H), 7.07 (m, 1H), 4.28 (m, 1H), 3.76–3.54 (m, 2H), 3.67 (s, 3H), 2.96 (t, J = 7.5 Hz, 2H), 2.80–2.46 (m, 4H), 2.38–2.08 (m, 2H).

5.1.27. 3-(3-{(2*R*)-2-[(1*E*,3*S*)-4-(3-Chlorophenyl)-3-hydroxybut-1-enyl]-5-oxopyrrolidin-1-yl}phenyl)propanoic acid (7)

Compound **7** was prepared from **32** according to the same procedure as described for the preparation of **3** from **21a** as a colorless oil (34 mg, 18% form **32**). IR (film): 3404, 3013, 2360, 1681, 1600, 1491, 1455, 1395, 1217, 1081, 1035, 971, 882, 756, 701 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.60–6.80 (m, 8H), 5.70–5.50 (m, 2H), 5.20–4.00 (m, 2H), 4.61 (m, 1H), 4.33 (m, 1H), 3.04–2.20 (m, 8H), 1.84–1.60 (m, 2H).; MS (APCI) *m/z*: 412 $(M-H)^-$; HRMS-FAB (*m/z*): $[M+H]^+$ calcd for $C_{23}H_{25}ClNO_4$, 414.1472; found, 414.1470.

5.1.28. (5*R*)-1-[4-(Bromomethyl)benzyl]-5-({*tert*-butyldimethylsilyloxy}methyl)-2-pyrrolidinone (34a)

To a stirred solution of **33** (1.0 g, 4.40 mmol) in DMF (20 mL) was added sodium hydride (62% in mineral oil, 200 mg, 5.17 mmol) at 0 °C under argon atmosphere. Stirring was continued at room temperature for 1 h and 50 °C for additional 1 h. To the resulting suspension was added 1,4-bis(bromomethyl)benzene (1.40 ml, 5.20 mmol) at room temperature, and stirring was continued at room temperature for 1.5 h. The resulting pale brown solution was poured into saturated aqueous NH_4Cl, extracted with EtOAc, washed with H_2O, brine, and dried over $MgSO_4$. The organic layer was evaporated and the resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 5:1–3:1) to give **34a** as a colorless oil (499 mg, 27%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.38–7.34 (m, 2H), 7.24–7.20 (m, 2H), 4.96 (d, J = 15.4 Hz, 1H), 4.48 (d, J = 15.4 Hz, 1H), 3.65 (m, 1H), 3.57–3.50 (m, 2H), 2.56 (m, 1H), 2.48 (m, 1H), 2.07 (m, 1H), 1.89 (m, 1H), 0.89 (s, 9H), 0.04 (s, 6H).

5.1.29. Methyl (3-{[(2*R*)-2-({*tert*-butyldimethylsilyloxy}methyl)-5-oxo-1-pyrrolidinyl]methyl}phenyl)acetate (34b)

To a stirred solution of **34a** (491 mg, 1.19 mmol) in THF (3.0 mL) and MeOH (1.5 mL) were added potassium carbonate (197 mg, 1.43 mmol) and bis(triphenylphosphine)palladium dichloride (17 mg, 0.020 mmol) under argon atmosphere, and the argon gas was replaced with carbon monooxide gas repeatedly. After being stirred at room temperature for 20 h, the reaction mixture was cooled to room temperature. The resulting mixture was evaporated and purified by column chromatography on silica gel (hexane/EtOAc, 5:1–1:1) to afford **34b** as a colorless oil (291 mg, 62%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25–7.19 (m, 4H), 4.98 (d, J = 15.0 Hz, 1H), 4.02 (d, J = 15.0 Hz, 1H), 3.68 (s, 3H), 3.64 (m, 1H), 3.61 (s, 2H), 3.57–3.48 (m, 2H), 2.56 (m, 1H), 2.37 (m, 1H), 2.05 (m, 1H), 1.91 (m, 1H), 0.89 (s, 9H), 0.04 (s, 6H).

5.1.30. Methyl (3-{[(2*R*)-2-(hydroxymethyl)-5-oxo-1-pyrrolidinyl]methyl}phenyl)acetate (35)

A solution of **34b** (288 mg, 0.740 mmol) in THF (1.5 mL) was treated with a solution of TBAF (1.0 M in THF, 0.81 mL, 0.81 mmol) at room temperature under argon atmosphere for 1 h. The reaction mixture was diluted with EtOAc, washed with H_2O, brine, and

dried over Na_2SO_4. The organic layer was evaporated and the resulting residue was purified by column chromatography on silica gel (EtOAc/MeOH, 1:0–20:1) to give **35** as a colorless oil (158 mg, 77%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25–7.19 (m, 4H), 4.82 (d, J = 15.1 Hz, 1H), 4.24 (d, J = 15.1 Hz, 1H), 3.75 (m, 1H), 3.68 (s, 3H), 3.62 (s, 2H), 3.60–3.47 (m, 2H), 2.56 (m, 1H), 2.41 (m, 1H), 2.12–1.93 (m, 2H).

5.1.31. [3-({(2*R*)-2-[(1*E*,3*S*)-3-Hydroxy-4-(3-methylphenyl)but-1-enyl]-5-oxopyrrolidin-1-yl}methyl)phenyl]acetic acid (8)

Compound **8** was prepared from **35** according to the same procedure as described for the preparation of **3** from **21a** as a colorless oil (76 mg, 58% form **21a**). IR (film): 3399, 2922, 1726, 1660, 1488, 1418, 1251, 1173, 1096, 1037, 974, 916, 784, 746, 702, 664, 609 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.27–6.97 (m, 8H), 5.62 (dd, J = 15.4, 5.8 Hz, 1H), 5.41 (ddd, J = 15.4, 8.8, 1.1 Hz, 1H), 4.74 (d, J = 14.6 Hz, 1H), 4.36 (m, 1H), 3.87 (m, 1H), 3.81 (d, J = 14.6 Hz, 1H), 3.60 (s, 2H), 2.78 (d, J = 6.6 Hz, 2H), 2.55–2.35 (m, 2H), 2.32 (s, 3H), 2.15 (m, 1H), 1.69 (m, 1H); MS (APCI) m/z: 392 $(M-H)^-$; HRMS-FAB (m/z): $[M+H]^+$ calcd for $C_{24}H_{28}NO_4$, 394.2018; found, 394.2018.

5.1.32. Methyl 5-{3-[(2*R*)-2-({*tert*-butyldimethylsilyloxy}methyl)-5-oxo-1-pyrrolidinyl]propyl}-2-thiophenecarboxylate (34c)

To a stirred solution of the lactam 33 (922 mg, 4.03 mmol) in DMF (10 mL) was added sodium hydride (62% in mineral oil, 183 mg, 4.80 mmol) at 0 °C under argon atmosphere. Stirring was continued at room temperature for 1 h, and 60 °C for additional 1 h. To the resulting suspension was added methyl 5-(3-bromopropyl)thiophene-2-carboxylate (1.05 g, 4.00 mmol) at 0 °C, and stirring was continued at 80 °C for additional 1.5 h. The resulting pale brown solution was poured into saturated aqueous NH_4Cl, extracted with EtOAc, washed with H_2O, brine, and dried over $MgSO_4$. The organic layer was evaporated, and the resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 2:2–1:1) to give **34c** as a colorless oil (530 mg, 32%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.62 (d, J = 3.6 Hz, 1H), 6.81 (d, J = 3.6 Hz, 1H), 3.86 (s, 3H), 3.72–3.54 (m, 4H), 3.11 (m, 1H), 2.85 (t, J = 7.5 Hz, 2H), 2.48–2.23 (m, 2H), 2.08–1.70 (m, 4H), 0.87 (s, 9H), 0.04 (s, 3H), 0.03 (s, 3H).

5.1.33. Methyl 5-{3-[(2*R*)-2-(hydroxymethyl)-5-oxo-1-pyrrolidinyl]propyl}-2-thiophenecarboxylate (36)

A solution of **34c** (530 mg, 1.29 mmol) in THF (5 mL) was treated with a solution of TBAF (1.0 M in THF, 2.60 mL, 2.60 mmol) at room temperature under argon atmosphere for 1 h. After addition of brine, the reaction mixture was extracted with EtOAc repeatedly. The combined organic layers were dried over $MgSO_4$, and evaporated. The resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 1:1–0:1) to give **36** as a pale yellow oil (394 mg, 100%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.62 (d, J = 3.9 Hz, 1H), 6.81 (d, J = 3.9 Hz, 1H), 3.86 (s, 3H), 3.80–3.61 (m, 4H), 3.12 (m, 1H), 2.86 (t, J = 7.5 Hz, 2H), 2.48 (m, 2H), 2.32 (m, 1H), 2.15–1.86 (m, 4H), 1.74 (br s, 1H).

5.1.34. 5-(3-{(2*R*)-2-[(1*E*,3*S*)-4-(3-Chlorophenyl)-3-hydroxybut-1-enyl]-5-oxopyrrolidin-1-yl}propyl)thiophene-2-carboxylic acid (10b)

Compound **10b** was prepared from **34c** using a phosphonate **29d** instead of **29a** according to the same procedure as described for the preparation of **3** from **21a** as a colorless amorphous powder (52 mg, 45% from **21a**). IR (KBr): 3417, 2930, 2623, 1660, 1599, 1574, 1540, 1463, 1422, 1377, 1266, 1167, 1097, 1031, 975, 910, 819 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.68 (d, J = 3.6 Hz, 1H), 7.23–7.18 (m, 3H), 7.08 (m, 1H), 6.83 (d, J = 3.6 Hz, 1H), 5.71 (dd, J = 15.3, 6.0 Hz, 1H), 5.48 (ddd, J = 15.3, 8.7, 0.9 Hz, 1H), 4.39 (m, 1H), 4.02 (m, 1H), 3.53 (m, 1H), 3.40 (br s, 1H), 2.90–2.70 (m, 5H), 2.50–2.10 (m, 3H), 1.90–1.60 (m, 3H); FAB-MS (m/z): 434 $(M+H)^+$; HRMS-FAB (m/z): $[M+H]^+$ calcd for $C_{22}H_{25}ClNO_4S$, 434.1193; found, 434.1185.

5.1.35. 5-(3-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxy-4-(3-methylphenyl)but-1-enyl]-5-oxopyrrolidin-1-yl}propyl)thiophene-2-carboxylic acid (10c)

Compound **10c** was prepared from **34c** using the phosphonate **29a** according to the same procedure as described for the preparation of **3** from **21a** as a colorless oil (60 mg, 59% from **21a**). IR (KBr): 3391, 2924, 1666, 1540, 1463, 1421, 1378, 1264, 1097, 1033, 975, 820, 754, 701, 666 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.68 (d, J = 3.8 Hz, 1H), 7.19 (t, J = 7.4 Hz, 1H), 7.07–6.96 (m, 3H), 6.83 (d, J = 3.8 Hz, 1H), 5.75 (dd, J = 15.4, 6.0 Hz, 1H), 5.47 (ddd, J = 15.4, 8.8, 1.1 Hz, 1H), 4.38 (m, 1H), 4.02 (m, 1H), 3.53 (m, 1H), 2.90–2.76 (m, 5H), 2.46–2.37 (m, 2H), 2.33 (s, 3H), 2.21 (m, 1H), 1.90–1.65 (m, 3H). FAB-MS (m/z): 414 $(M+H)^+$; HRMS-FAB (m/z): $[M+H]^+$ calcd for $C_{23}H_{28}NO_4S_2$, 414.1752; found, 414.1739.

5.1.36. Methyl [3-(2-bromoethyl)phenyl]acetate (42)

A solution of 1,3-phenylenediacetic acid (1.78 g, 9.20 mmol) in MeOH (3 mL) and EtOAc (3 mL) was treated with a solution of trimethylsilyldiazomethane (2.0 M in hexane, 4,6 mL, 9.2 mmol) at 0 °C for 5 min. The resulting mixture was evaporated to give a mixture of the corresponding half ester and diester. To a stirred solution of a mixture of the half ester and the diester in THF (9 mL) was added dropwise a solution of diborane–dimethylsulfide complex (2.0 M in THF, 9.0 mL, 18 mmol) at 0 °C under argon atmosphere. After being stirred at room temperature for 16 h, the reaction was quenched with MeOH. The reaction mixture was diluted with EtOAc, washed with saturated aqueous $NaHCO_3$, brine, and dried over Na_2SO_4. The organic layer was evaporated, and the resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 1:1–1:2) to give the corresponding 1,3-bisphenethyl alcohol as a colorless oil (834 mg, 47%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.45–7.08 (m, 4H), 3.86 (t, J = 7.2 Hz, 2H), 3.70 (s, 3H), 3.62 (s, 2H), 2.86 (t, J = 7.2 Hz, 2H), 1.56–1.45 (br s, 1H).

To a stirred solution of the above-described 1,3-bispenethyl alcohol (834 mg, 4.30 mmol) in CH_2Cl_2 (10 mL) was added phosphorous tribromide (0.75 mL, 5.20 mmol) at 0 °C under argon atmosphere. After being stirred for 30 min, the reaction was poured into ice-water. The resulting mixture was extracted with EtOAc, washed with water, brine, and dried over Na_2SO_4. The organic layer was evaporated, and the resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 4:1) to give **42** as a pale brown oil (251 mg, 23%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.45–7.12 (m, 4H), 3.70 (s,3H), 3.62 (s, 2H), 3.57 (t, J = 8.0 Hz, 2H), 3.18 (t, J = 8.0 Hz, 2H).

5.1.37. Ethyl (4*R*)-4-({2-*tert*-butoxycarbonyl}amino)-5-oxopentanoate (38)

To a stirred solution of oxalyl chloride (0.49 mL, 5.64 mmol) in CH_2Cl_2 (9 mL) was slowly added a solution of DMSO (0.50 mL, 7.05 mmol) in CH_2Cl_2 (3 mL) at −70 °C in 10 min. The resulting solution was stirred for an additional 5 min at the same temperature. To the reaction mixture was added a solution of **37** (1.23 g, 4.70 mmol) in CH_2Cl_2 (5 mL). The resulting suspension was allowed to warm up to −40 °C over 30 min. After being stirred for an additional 10 min, the reaction mixture was treated with *N*,*N*-diisopropylethylamine (2.0 mL, 11.3 mmol) and the resulting suspension was allowed to warm up to −5 °C over 30 min. The reaction was quenched with water and then poured into ice-cold 1 N HCl. The reaction mixture was extracted with EtOAc. The organic layer was washed with 1 N HCl, water, brine, dried over $MgSO_4$ and evaporated to give an

aldehyde **38** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.60 (s, 1H), 5.25–5.20 (br s, 1H), 4.34–4.20 (m, 1H), 4.15 (q, J = 7.2 Hz, 2H), 2.42 (t, J = 7.2 Hz, 2H), 2.45–2.22 (m, 1H), 2.05–1.89 (m, 1H), 1.43 (s, 9H), 1.25 (t, J = 7.2 Hz, 3H).

5.1.38. Ethyl (4*R*,5*E*)-8-(3-chlorophenyl)-4-({[(2-methyl-2-propanyl)oxy]carbonyl}amino)-7-oxo-5-octenoate (39)

To a stirred solution of the phoshonate **29d** (1.56 g, 5.64 mmol) in THF (50 mL) was added sodium hydride (63% in mineral oil, 197 mg, 5.17 mmol) in several portions at 0 °C under argon atmosphere. Stirring was continued at ambient temperature for 30 min. To the stirred suspension was added a solution of the above-described aldehyde **38** in THF (10 mL) at room temperature and stirring was continued for 2 h. The reaction was quenched with acetic acid. The reaction mixture was diluted with EtOAc, washed with water, then brine, dried over $MgSO_4$. The organic layer was evaporated. The resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 5:1–3:1) to give an enone as a white solid (1.40 g, 73% in 2 steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.31–7.00 (m, 4H), 6.77 (dd, J = 15.6 , 6.0 Hz, 1H), 6.23 (d, J = 15.6 Hz, 1H), 4.81-.466 (m, 1H), 4.47–4.20 (m, 1H), 4.14 (q, J = 7.0 Hz, 1H), 3.82 (s, 2H), 2.38 (t, J = 7.2 Hz, 2H), 2.15–1.75 (m, 2H), 1.41 (s, 9H), 1.24 (t, J = 7.2 Hz, 3H).

To a stirred solution of the above-described enone (1.31 g, 3.20 mmol) in THF (10.0 mL) was added a solution of (*R*)-2-methyl-CBS-oxazaborolidine (1.0 M in toluene, 0.90 mL, 0.90 mmol) at room temperature under argon atmosphere. To the reaction mixture was added dropwise a solution of diborane–THF complex (1.0 M in THF, 1.90 mL, 1.90 mmol) in 5 min. The resulting solution was stirred for 1 h, then treated with MeOH and stirring was continued for 5 min. The reaction mixture was diluted with EtOAc, washed with 1 N HCl, water, saturated $NaHCO_3$, brine, and dried over Na_2SO_4. The organic layer was evaporated. The resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 3:1–2:1) to give **39** as a pale yellow oil (1.13 g, 86%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25–7.22 (m, 3H), 7.15–7.05 (m, 1H), 5.69 (dd, J = 15.6, 6.0 Hz, 1H), 5.55 (d, J = 15.6, 9.0 Hz, 1H), 4.61–4.55 (m, 1H), 4.45–4.32 (m, 1H), 4.22–4.10 (m, 3H), 2.95–2.74 (m, 2H), 2.32 (t, J = 7.2 Hz, 2H), 1.89–1.70 (m, 2H), 1,44 (s, 9H), 1.24 (t, J = 7.2 Hz, 3H).

5.1.39. Ethyl (4*R*,5*E*,7*S*)-4-amino-8-(3-chlorophenyl)-7-hydroxy-5-octenoate trifluoroacetate (salt) (40)

A solution of **39** (930 mg, 2.26 mmol) in CH_2Cl_2 (20 mL) was treated with trifluoroacetic acid (2.3 mL) at 0 °C under argon atmosphere. After being stirred for 90 min at room temperature, the trifluoroacetic acid was removed by the repeated evaporation with benzene to give **40** as a pale red oil. ^{1}H NMR (300 MHz, CD_3OD): δ 7.40–7.0 (m, 4H), 5.93 (dd, J = 15.3, 6.0 Hz, 1H), 5.56 (dd, J = 15.3, 8.8 Hz, 1H), 4.49–4.37 (m, 1H), 4.21–4.10 (m, 2H), 3.87–3.70 (m, 1H), 2.92–2.74 (m, 2H), 2.35–2.25 (m, 2H), 2.11–1.95 (m, 1H), 1.90–1.75 (m, 1H), 1.27 (t, J = 7.2 Hz, 3H).

5.1.40. Methyl [3-(2-{(2*R*)-2-[(1*E*,3*S*)-4-(3-chlorophenyl)-3-hydroxy-1-buten-1-yl]-5-oxo-1-pyrrolidinyl}ethyl)phenyl]acetate (41)

To a stirred solution of **40** (255 mg, 0.60 mmol) in DMF (3 mL) were added sodium bicarbonate (101 mg, 1.20 mmol) and the bromide **42** (185 mg, 0.720 mmol) at room temperature under argon atmosphere. After being stirred at 90 °C for 8 h, the reaction mixture was cooled to room temperature, and diluted with EtOAc, washed with water, then brine, and dried over Na_2SO_4. The organic layer was evaporated. The resulting residue was purified by column chromatography on silica gel (EtOAc/MeOH, 30:1) to give **41** as a pale yellow oil (90 mg, 34%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.30–7.00 (m, 8H), 5.61–5.35 (m, 2H), 4.44–4.38 (m, 1H), 3.80–3.50 (m, 7H), 3.04–2.77 (m, 5H), 2.46–2.22 (m, 2H), 2.21–2.00 (m, 1H), 1.89–1.70 (m, 1H).

5.1.41. [3-(2-{(2*R*)-2-[(1*E*,3*S*)-4-(3-Chlorophenyl)-3-hydroxybut-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)phenyl]acetic acid (9)

Compound **8** was prepared from **41** according to the same procedure as described for the preparation of **3** from **24a** as a pale yellow amorphous (62 mg, 73%). IR (film): 3387, 2932, 1724, 1660, 1422, 1257, 1164, 1033, 976, 783, 757, 705 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25–7.0 (m, 8H), 5.51 (dd, J = 15, 6.0 Hz, 1H), 5.25 (dd, J = 15, 8 Hz, 1H), 4.4–4.3 (m, 1H), 3.75–3.65 (m, 1H), 3.62 (s, 2H), 3.65–3.55 (m, 1H), 3.3–2.4 (br), 3.0–2.7 (m, 5H), 2.4–2.2 (m, 2H), 2.1–1.95 (m, 1H), 1.65–1.5 (m, 1H); MS (APCI) m/z: 428 (M+2−H)$^-$, 426 (M−H)$^-$; HRMS-FAB (m/z): [M+H]$^+$ calcd for $C_{24}H_{27}ClNO_4$, 428.1629; found, 428.1617.

5.1.42. Ethyl (4*R*,5*E*,7*S*)-8-(3-Methylphenyl)-7-hydroxy-4-({*tert*-butyoxycarbonyl}amino)-5-octenoate (43)

Compound **43** was prepared from **38** according to the same procedure as described for the preparation of **39** from **38** as a colorless oil (4.55 g, 84% from **38**).

5.1.43. Ethyl (4*R*,5*E*,7*S*)-4-amino-7-hydroxy-8-(3-methylphenyl) -5-octenoate hydrochloride (44)

A solution of **43** (3.82 g, 9.87 mmol) in EtOH (10 mL) was treated with 4 N HCl in dioxane (7.5 mL) at room temperature under argon atmosphere. After being stirred for 8 h, the reaction mixture was evaporated to give **44** as a brown oil (3.20 g, 100%). ^{1}H NMR (300 MHz, CD_3OD): δ 7.19–7.10 (m, 1H), 7.07–6.96 (m, 3H), 5.91 (dd, J = 15.7, 4.7 Hz, 1H), 5.54 (ddd, J = 15.5, 8.9, 1.5 Hz, 1H), 4.43–4.32 (m, 1H), 4.22–4.06 (m, 2H), 3.80–3.64 (m, 1H), 2.90–2.67 (m, 2H), 2.39–2.19 (m, 5H), 2.16–1.92 (m, 2H), 1.88–1.70 (m, 1H), 1.26 (t, J = 7.1 Hz, 3H).

5.1.44. (5*R*)-1-[2-(4-Bromophenyl)ethyl]-5-[(1*E*,3*S*)-3-{*tert*-butyldimethylsilyloxy}-4-(3-methylphenyl)-1-buten-1-yl]-2-pyrrolidinone (45)

To a stirred solution of the amine hydrochloride **44** (200 mg, 0.61 mmol) in THF (5 mL) was added 3-(4-bromophenyl)propionaldehyde (134 mg, 0.670 mmol) at room temperature under argon atmosphere. After being stirred for 30 min, to this reaction mixture was added sodium acetoxyborohydride (194 mg, 0.92 mmol) at 0 °C. After being stirred at room temperature for additional 16 h, the reaction mixture was diluted with EtOAc, washed with water, then brine and dried over Na_2SO_4. The organic layer was evaporated to afford a lactam alcohol as a yellow oil.

To a stirred solution of the above-described lactam alcohol in DMF (3 mL) and imidazole (244 mg, 3.60 mmol) was added *tert*-butyldimethylsilyl chloride (184 mg, 2.40 mmol) at room temperature under argon atmosphere. After being stirred for 6 h at 50 °C, the reaction mixture was poured into water, and extracted with EtOAc, washed with H_2O twice, brine, and dried over Na_2SO_4. The organic layer was evaporated. The resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 2:1) to give **45** as a pale yellow oil (237 mg, 72% in steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.39 (d, J = 8.3 Hz, 2H), 7.18–7.08 (m, 1H), 7.06–6.89 (m, 5H), 5.57 (dd, J = 15.3, 5.9 Hz, 1H), 5.25 (dd, J = 15.4, 8.5 Hz, 1H), 4.34–4.22 (m, 1H), 3.81–3.63 (m, 2H), 2.95–2.60 (m, 5H), 2.43–2.18 (m, 5H)), 2.16–2.01 (m, 1H), 1.69–1.56 (m, 1H), 0.89–0.77 (m, 9H), −0.10 (s, 3H), −0.17 (s, 3H).

5.1.45. Ethyl 4-(2-{(2*R*)-2-[(1*E*,3*S*)-3-(*tert*-butyldimethylsilyloxy)-4-(3-methylphenyl)-1-buten-1-yl]-5-oxo-1-pyrrolidinyl} ethyl)benzoate (46)

To a stirred solution of **45** (237 mg, 0.437 mmol) in DMSO (2.0 mL) and EtOH (3.0 mL) were added triethylamine (0.073 mL,

0.52 mmol), palladium acetate (9.0 mg, 0.04 mmol) and 1,1-bis(diphenylphosphino)ferrocene (32 mg, 0.08 mmol) under argon atmosphere, and the argon gas was replaced with carbon monoxide gas. After being stirred at 80 °C for 8 h, the reaction mixture was cooled to room temperature, and filtered through a pad of Celite. The filtrate was diluted with EtOAc, washed with water (×2), then brine, and dried over Na_2SO_4. The organic layer was evaporated. The resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 2:1) to give **46** as a pale yellow oil (220 mg, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.96 (d, J = 8.0 Hz, 2H), 7.20 (d, J = 8.0 Hz, 2H), 7.15–7.10 (m, 2H), 7.04–6.87 (m, 2H), 5.56 (dd, J = 15.3, 5.9 Hz, 1H), 5.27 (d, J = 8.8 Hz, 1H), 4.37 (q, J = 7.1 Hz, 2H), 4.28 (m, J = 6.0 Hz, 1H), 3.83–3.65 (m, 3H), 3.00–2.61 (m, 5H), 2.38–2.18 (m, 6H), 1.67–1.60 (m, 1H), 1.40 (t, J = 7.0 Hz, 3H), 0.92–0.77 (m, 9H), −0.10 (s, 3 H), −0.18 (s, 3H).

5.1.46. 4-(2-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxy-4-(3-methylphenyl)but-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)benzoic acid (10a)

To a stirred solution of **46** (220 mg, 0.410 mmol) in DME (1 mL) and MeOH (1 mL) was added 2 N HCl (0.5 mL) under argon atmosphere. After being stirred at 50 °C for 8 h, the reaction mixture was cooled to room temperature, and then treated with 2 N NaOH (2 mL) for 2 h. The reaction mixture was acidified with 1 N HCl and extracted with EtOAc. The organic layer was washed with water, then brine, and dried over Na_2SO_4. The organic layer was evaporated and the resulting residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 100:1–30:1) to give **10a** as a beige amorphous (122 mg, 76%). IR (film): 2927, 1666, 1261, 755 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02 (d, J = 8.1 Hz, 2H), 7.30–7.15 (m, 3H), 7.10–6.97 (m, 3H), 5.64 (dd, J = 15.6, 6.3 Hz, 1H), 5.37 (dd, J = 15.6, 8.7 Hz, 1H), 4.41–4.32 (m, 1H), 3.83–3.70 (m, 2H), 3.09–2.95 (m, 1H), 2.95–2.75 (m, 4H), 2.48–2.25 (m, 5H), 2.20–2.13 (m, 1H), 1.72–1.58 (m, 1H); MS (APCI) m/z: 392 (M−H)$^-$; HRMS-FAB (m/z): [M+H]$^+$ calcd for $C_{24}H_{28}NO_4$, 394.2018; found, 394.2023.

5.1.47. 2-[(2*R*)-2-({*tert*-Butyldimethylsilyloxy}methyl)-5-oxo-1-pyrrolidinyl]ethyl methanesulfonate (47b)

To a stirred solution of the alcohol **47a** (11.8 g, 43.3 mmol) and triethylamine (9.07 mL, 65.1 mmol) in THF (50 mL) was added methanesulfonyl chloride (3.68 mL, 47.7 mmol) at 0 °C under argon atmosphere. After being stirred at the same temperature for 10 min, the reaction was quenched with H_2O, diluted with EtOAc, washed with brine, and dried over $MgSO_4$. The organic layer was evaporated to yield methanesulfonate **47b** as a yellow oil.

5.1.48. (5*R*)-5-({*tert*-Butyldimethylsilyloxy}methyl)-1-(2-iodoethyl)-2-pyrrolidinone (47c)

To a stirred solution of the methanesulfonate **47b** in CH_3CN (120 mL) was added sodium iodide (19.5 g, 130 mmol) at room temperature under argon atmosphere, and stirring was continued at 80 °C for 15 h. The resulting yellow solution was diluted with EtOAc, and washed with water, then brine, and dried over $MgSO_4$. The organic layer was evaporated to afford **47c** as a yellow oil (11.3 g, 67%).

5.1.49. Ethyl 5-({2-[(2*R*)-2-({*tert*-butyldimethylsilyloxy}methyl)-5-oxo-1-pyrrolidinyl]ethyl}thio)-2-thiophenecarboxylate (48)

To a stirred suspension of ethyl thiophene-2-carboxylate (936 mg, 6.0 mmol) and sulfur (S_8, 240 mg, 7.5 mmol) in THF (50 mL) was added dropwise lithium diisopropylamide (2.0 M in THF/heptane/ethyl benzene, 4.0 mL: 8.0 mmol) at −78 °C under argon atmosphere. Stirring was continued for 35 min. To the stirred suspension was added a solution of **47c** (1.92 g, 5.0 mmol) in THF (5.0 mL), and stirring was continued at room temperature for additional 1.5 h. The resulting mixture was diluted with MTBE, poured into saturated aqueous NH_4Cl and extracted with EtOAc. The organic layer was washed with H_2O, brine, dried over $MgSO_4$ and evaporated. The resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 3:1–1:1) to give **48** as a brown oil (1.86 g, 84%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.65 (d, J = 3.9 Hz, 1H), 7.09 (d, J = 3.9 Hz, 1H), 4.32 (q, J = 7.5 Hz, 2H), 3.86–3.61 (m, 3H), 3.55 (m, 1H), 3.32 (m, 1H), 3.22–3.00 (m, 2H), 2.50–2.21 (m, 2H), 2.10 (m, 1H), 1.80 (m, 1H), 1.36 (t, J = 7.5 Hz, 3H), 0.86 (s, 9H), 0.03 (s, 3H), 0.02 (s, 3H).

5.1.50. Ethyl 5-({2-[(2*R*)-2-(hydroxymethyl)-5-oxo-1-pyrrolidinyl]ethyl}thio)-2-thiophenecarboxylate (49)

Compound **49** was prepared from **48** according to the same procedure as described for the preparation of **21a** from **20** as a colorless oil (1.15 g, 83%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.64 (d, J = 3.9 Hz, 1H), 7.10 (d, J = 3.9 Hz, 1H), 4.33 (q, J = 6.9 Hz, 2H), 3.80–3.68 (m, 3H), 3.60 (m, 1H), 3.40 (m, 1H), 3.17 (t, J = 7.0 Hz, 2H), 2.58–2.28 (m, 2H), 1.10 (m, 1H), 1.98–1.80 (m, 2H), 1.37 (t, J = 7.2 Hz, 3H).

5.1.51. 5-[(2-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxy-4-(3-methylphenyl)but-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)sulfanyl]thiophene-2-carboxylic acid (10d)

Compound **10d** was prepared from **49** according to the same procedure as described for the preparation of **3** from **21a** as a colorless oil (67 mg, 20% from **49**). IR (film): 3097, 3009, 2921, 1792, 1676, 1637, 1524, 1487, 1457, 1420, 1318, 1244, 1159, 1096, 1035, 975, 884, 820, 754 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.69 (d, J = 3.8 Hz, 1H), 7.19 (t, J = 7.3 Hz, 1H), 7.10–7.02 (m, 4H), 5.72 (m, 1H), 5.47 (m, 1H), 4.38 (m, 1H), 4.12 (m, 1H), 3.66 (m, 1H), 3.15–3.05 (m, 3H), 2.88–2.77 (m, 2H), 2.43–2.38 (m, 2H), 2.33 (s, 3H), 2.20 (m, 1H), 1.72 (m, 1H); FAB-MS (m/z): 432 (M+H)$^+$; HRMS-FAB (m/z): [M+H]$^+$ calcd for $C_{22}H_{26}NO_4S_2$, 432.1303; found, 432.1299.

5.1.52. Methyl 3-hydroxy-2-[(4-{*tert*-butyldiphenylsilyloxy}butanoyl)amino]propanoate (50)

To a stirred solution of sodium 4-hydroxybutanonate (1.54 g, 12.2 mmol) in DMF (20 mL) and imidazole (1.25 g, 18.3 mmol) was added *tert*-butyldiphenylsilyl chloride (4.03 g, 14.6 mmol) at room temperature under argon atmosphere. After being stirred for 1 h, the reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with water (×2), brine, and dried over $MgSO_4$. The organic layer was evaporated to give a silyl ether as a colorless oil.

To a stirred solution of DL-serine methylester hydrochloride (2.85 g, 18.3 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC: 3.05 g, 15.9 mmol) and triethylamine (2.55 mL, 18.3 mmol) in CH_3CN (30 mL) was added a solution of the above-described silyl ether in CH_3CN (5 mL) at room temperature under argon atmosphere. After being stirred for 1 h, the reaction was quenched with water. The reaction mixture was diluted with EtOAc, washed with 1 N HCl, water, then brine, and dried over $MgSO_4$. The organic layer was evaporated and the resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 1:1–0:1) to give **50** as a colorless oil (1.73 g, 32% in two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.65–7.50 (m, 4H), 7.41–7.30 (m, 6H), 6.41 (m, 1H), 4.67 (m, 1H), 4.05–3.97 (m, 2H), 3.79 (s, 3H), 3.85–3.72 (m, 2H), 2.42 (t, J = 7.2 Hz, 2H), 2.01–1.93 (m, 2H), 1.05 (s, 9H).

5.1.53. Methyl 2-(3-{*tert*-butyldiphenylsilyloxy}propyl)-1,3-oxazole-4-carboxylate (51)

To a stirred solution of **50** (1.72 g, 3.88 mmol) in CH_2Cl_2 (15 mL) was added *N*,*N*-diethylaminosulfur trifluoride (0.56 mL, 4.27 mmol) at −78 °C under argon atmosphere and stirring was

continued for 2 h. To the reaction mixture was added potassium carbonate (1.60 g, 11.6 mmol), and the reaction mixture was allowed to warm up to −20 °C under stirring. After being stirred for additional 1 h, the reaction was quenched with water, extracted with CH_2Cl_2, and dried over Na_2SO_4. The organic layer was evaporated. To a stirred solution of the resulting residue in CH_2Cl_2 (19.4 mL) were added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU: 1.74 mL, 11.6 mmol) at 0 °C under argon atmosphere and then dropwise a solution of bromotrichloromethane (0.80 mL, 8.14 mmol) in CH_2Cl_2 (4 mL). The reaction mixture was allowed to warm up to room temperature under stirring. After being stirred for 10 h, the reaction mixture was diluted with EtOAc, washed with 1 N HCl, water, saturated aqueous $NaHCO_3$, brine, and dried over Na_2SO_4. The organic layer was evaporated, and the resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 9:1–1:1) to give **51** as a colorless oil (886 mg, 54% from **53**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.13 (s, 1H), 7.70–7.58 (m, 4H), 7.47–7.32 (m, 6H), 3.91 (s, 3H), 3.72 (t, J = 6.0 Hz, 2H), 3.01–2.90 (m, 2H), 2.11–1.98 (m, 2H), 1.08–0.99 (m, 9H).

5.1.54. Methyl 2-(3-hydroxypropyl)-1,3-oxazole-4-carboxylate (52)

A solution of **51** (886 mg, 2.09 mmol) in THF (3 mL) was treated with TBAF (1.0 M in THF, 4.27 mL, 4.27 mmol) at room temperature under argon atmosphere for 1 h and then with brine. The reaction mixture was extracted with EtOAc repeatedly (×5). The combined organic layers were dried over $MgSO_4$, and evaporated. The resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 1:4–0:1) to give **52** as a pale yellow oil (304 mg, 79%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.16 (s, 1H), 3.91 (s, 3H), 3.79–3.66 (m, 2H), 2.96 (t, J = 7.4 Hz, 2H), 2.14–1.97 (m, 2H), 1.76 (m, 1H).

5.1.55. Methyl 2-(3-{(2*R*)-2-[(1*E*,3*S*)-3-hydroxy-4-(3-methyl phenyl)-1-buten-1-yl]-5-oxo-1-pyrrolidinyl}propyl)-1,3-oxa zole-4-carboxylate (53)

To a stirred solution of the alcohol **52** (85 mg, 0.459 mmol) in DMSO (2 mL) and *N*,*N*-diisopropylethylamine (0.48 mL, 2.75 mmol) was added SO_3–Py (219 mg, 1.38 mmol) at 0 °C under argon atmosphere. After being stirred at room temperature for 20 min, the reaction was quenched with 1 N HCl. The reaction mixture was diluted with EtOAc, washed with brine, and dried over $MgSO_4$. The organic layer was evaporated to yield the corresponding aldehyde as a pale yellow oil (98 mg).

To a stirred solution of the above-described aldehyde in THF (0.5 mL) was added a solution of the amine hydrochloride **44** (100 mg, 0.306 mmol) in THF (0.5 mL) at room temperature under argon atmosphere and stirring was continued for 1 h at 50 °C. To the stirred reaction mixture was added sodium acetoxyborohydride (117 mg, 0.550 mmol) at 0 °C under argon atmosphere. After being stirred at room temperature for additional 15 h, the reaction mixture was diluted with EtOAc, washed with water, saturated aqueous $NaHCO_3$, brine, and dried over Na_2SO_4. The organic layer was evaporated and the resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 1:4–0:1) to give **53** as a pale yellow solid (93 mg, 74%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.14 (s, 1H), 7.24–7.14 (m, 1H), 7.10–6.93 (m, 3H), 5.75 (dd, J = 15.1, 5.2 Hz, 1H), 5.50 (ddd, J = 15.1, 8.8, 1.1 Hz, 1H), 4.40 (m, 1H), 4.13–3.99 (m, 1H), 3.87 (s, 3H), 3.55–3.41 (m, 1H), 2.97–2.67 (m, 5H), 2.47–2.11 (m, 7H), 2.03–1.85 (m, 2H), 1.72 (m, 1H).

5.1.56. 2-(3-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxy-4-(3-methylphenyl)but-1-enyl]-5-oxopyrrolidin-1-yl}propyl)-1,3-oxazole-4-carboxylic acid (11)

Compound **11** was prepared from **53** according to the same procedure as described for the preparation of **10a** from **46** as a white powder (66 mg, 70% from **53**). IR (KBr): 3422, 2933, 1727, 1656, 1586, 1459, 1421, 1280, 1161, 1110, 1036, 980, 747 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.19 (s, 1H), 7.20 (m, 1H), 7.06–6.97 (m, 3H), 5.78 (dd, J = 15.3, 6.0 Hz, 1H), 5.50 (ddd, J = 15.3, 9.0, 1.2 Hz, 1H), 4.40 (m, 2H), 4.07 (m, 1H), 3.47 (m, 1H), 2.94 (m, 1H), 2.83–2.75 (m, 4H), 2.50–2.10 (m, 6H), 2.05–1.83 (m, 2H), 1.64 (m, 1H); MS (APCI) *m/z*: 397 $(M-H)^-$; HRMS-FAB (*m/z*): $[M+H]^+$ calcd for $C_{22}H_{27}N_2O_5$, 399.1920; found, 399.1903.

5.1.57. Ethyl 2-(3-oxopropyl)-1,3-thiazole-4-carboxylate (54)

To a stirred solution of 4,4-dimethoxybutyronitrile (5.0 g, 38.7 mmol) in DMF (50 mL) was added portionwise magnesium chloride hexahydrate (10.2 g, 50.3 mmol) at 0 °C under argon atmosphere. To the resulting suspension was successively added sodium hydrosulfide (4.02 g, 50.3 mmol) at 0 °C under argon atmosphere. After being stirred at room temperature for 16 h, the reaction mixture was diluted with EtOAc, washed with H_2O, and dried over $MgSO_4$. The organic layer was evaporated to give the corresponding thioamide as a colorless oil.

To a stirred solution of the above-described thioamide (300 mg, 1.81 mmol) in DMF (2 mL) was added ethyl bromopyruvate (422 mg, 2.17 mmol) at room temperature under argon atmosphere. After being stirred for 2 h, the reaction was quenched with H_2O. The reaction mixture was diluted with EtOAc, washed with saturated aqueous $NaHCO_3$, brine and dried over $MgSO_4$. The organic layer was evaporated to give ethyl 2-(3,3-dimethoxypropyl)-1,3-thiazole-4-carboxylate as a brown oil.

A solution of the above-described dimethylacetal in DME (4 mL) was treated with 2 N HCl at room temperature for 2 h. The homogeneous reaction mixture was diluted with EtOAc, washed with H_2O, and dried over Na_2SO_4. The organic layer was evaporated, and the resulting residue was purified by column chromatography on silica gel (hexane/EtOAc, 2:1–1:1) to give **54** as a brown oil (225 mg, 12% in 3 steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.85 (s, 1H), 8.06 (s, 1H), 4.41 (q, J = 6.9 Hz, 2H), 3.37 (t, J = 6.9 Hz, 2H), 3.08 (t, J = 6.9 Hz, 2H), 1.40 (t, J = 6.9 Hz, 3H).

5.1.58. 2-(3-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxy-4-(3-methylphenyl)but-1-enyl]-5-oxopyrrolidin-1-yl}propyl)-1,3-thiazole-4-carboxylic acid (12)

Compound **12** was prepared from **54** using **44** according to the same procedure as described for the preparation of **11** from **52** as a white powder (66 mg, 70% form **54**). IR (KBr): 3423, 2924, 1718, 1661, 1488, 1459, 1421, 1376, 1322, 1216, 1100, 1037, 975, 783, 748, 702 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.12 (s, 1H), 7.18 (m, 1H), 7.05–6.97 (m, 3H), 5.75 (dd, J = 15.3, 5.7 Hz, 1H), 5.60–5.20 (m, 3H), 4.40 (m, 1H), 4.07 (m, 1H), 3.51 (m, 1H), 3.07–2.85 (m, 3H), 2.79 (d, J = 6.6 Hz, 2H), 2.50–2.12(m, 6H), 2.04–1.90 (m, 2H), 1.70 (m, 1H); MS (APCI) *m/z*: 413 $(M-H)^-$; HRMS-FAB (*m/z*): $[M+H]^+$ calcd for $C_{22}H_{27}N_2O_4$, 415.1692; found, 415.1686.

5.1.59. Ethyl (4*R*,5*E*,7*S*)-7-hydroxy-4-({*tert*-butoxycarbonyl} amino)-5-dodecenoate (55)

Compound **55** was prepared from **38** according to the same procedure as described for the preparation of **39** from **38** as a colorless oil (2.97 g, 83% from **38**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.71–5.47 (m, 2H), 4.51 (br s, 1H), 4.20–4.02 (m, 3H), 2.38 (t, J = 7.4 Hz, 2H), 1.95–1.73 (m, 2H), 1.54–1.49 (m, 2H), 1.46–1.30 (m, 11H), 1.29 (s, 9H), 0.89 (t, J = 6.6 Hz, 3H).

5.1.60. Ethyl (4*R*,5*E*,7*S*)-4-amino-7-hydroxy-5-dodecenoate hydrochloride (56)

Compound **56** was prepared from **55** according to the same procedure as described for the preparation of **44** from **43** as a colorless oil (2.49 g, 100%). ^{1}H NMR (300 MHz, CD_3OD): δ 5.87 (dd, J = 15.4, 6.0 Hz, 1H), 5.57 (dd, J = 17.0, 9.6 Hz, 1H), 4.13 (m, 3H), 3.83–3.69

(m, 1H), 2.49–2.28 (m, 2H), 2.09 (m, 1H), 1.88 (m, 1H), 1.57–1.12 (m, 11H), 0.90 (t, J = 6.0 Hz, 3H).

5.1.61. 4-(2-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxyoct-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)benzoic acid (2)

Compound **2** was prepared from **56** using 4-methoxycarbonyl phenylacetaldehyde according to the same procedure as described for the preparation of **11** from **44** as a white powder (10 mg, 50% from **56**). IR (KBr): 3222, 2953, 2930, 2857, 1715, 1658, 1612, 1509, 1459, 1425, 1415, 1363, 1327, 1307, 1244, 1204, 1174, 1154, 1107, 1064, 1019, 977, 910, 867, 837, 749, 702.93, 612 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 7.84 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 5.62 (dd, J = 15.6, 6.3 Hz, 1H), 5.33 (dd, J = 15.6, 8.7 Hz, 1H), 4.71 (d, J = 4.8 Hz, 1H), 4.00–3.84 (m, 2H), 3.60 (m, 1H), 2.99 (m, 1H), 2.89–2.66 (m, 2H), 2.30–2.00 (m, 3H), 1.60 (m, 1H), 1.50–1.15 (m, 8H), 0.81 (t, J = 6.3 Hz, 3H); MS (APCI) m/z: 358 $(M-H)^-$; HRMS-FAB (m/z): $[M+H]^+$ calcd for $C_{21}H_{30}NO_4$, 360.2175; found, 360.2173.

5.1.62. 4-[(2-{(2*R*)-2-[(1*E*,3*S*)-3-Hydroxyoct-1-enyl]-5-oxopyrrolidin-1-yl}ethyl)sulfanyl]butanoic acid (5)

Compound **5** was prepared from **56** using aldehyde **57** according to the same procedure as described for the preparation of **11** from **44** as a white powder (11 mg, 45% from **56**). IR (KBr): 3214, 2928, 2857, 1730, 1660, 1460, 1369, 1320, 1269, 1210, 1173, 1150, 1022, 982, 910, 668, 570 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 5.73 (dd, J = 15.3, 5.7 Hz, 1H), 5.53 (ddd, J = 15.3, 8.4, 1.2 Hz, 1H), 4.25–4.18 (m, 2H), 3.63 (m, 1H), 3.11 (m, 1H), 2.78–2.20 (m, 10H), 2.00–1.70 (m, 3H), 1.62–1.21 (m, 8H), 0.90 (t, J = 6.6 Hz, 3H); MS (APCI) m/z: 356 $(M-H)^-$; HRMS-FAB (m/z): $[M+H]^+$ calcd for $C_{18}H_{32}NO_4S$, 358.2052; found, 358.2051.

5.2. PLGA formulation

5.2.1. Preparation of PLGA microspheres containing compound 17

Microspheres were prepared by the emulsion-solvent evaporation method.[17] Compound **17** (10 mg) and PLGA 75–65 (90 mg) were dissolved in 1 mL of dichloromethane in the oil phase. The oil phase was gradually added into an aqueous 0.1% PVA (polyvinyl alcohol) solution stirred with a turbine-shaped mixer (Homomixer) at 6000 rpm to obtain o/w emulsion. The emulsion was continuously stirred gently with a magnetic stirrer for 3 h to remove CH_2Cl_2. After organic solvent evaporation, the PLGA microspheres were suspended in the PVA solution. The suspension was centrifuged at 3000 rpm for 10 min to precipitate the microspheres and remove any unreacted compound **17**. The supernatant was then discarded and replaced with fresh water or aqueous medium containing 0.2% Tween 80. This washing procedure was performed repeatedly. The washed microsphere precipitation was lyophilized to remove residual organic solvent and water, and to recover compound **17** microspheres in solid form.

5.2.2. Drug loading and encapsulation efficiency of compound 17 PLGA microspheres

The appropriate quantity of compound **17**-loaded PLGA microspheres was dissolved with *p*-hydroxy benzoic acid *n*-nonyl ester in acetonitrile. The concentration of compound **17** in the solution was analyzed by high performance liquid chromatography (HPLC). The drug loading and the encapsulation efficiency were calculated as follows:

$$\text{Drug loading}(\%, \text{w/w}) = (\text{M/W}) \times 100$$

where M = measured amount of compound **17** in the compound **17**-loaded-PLGA microspheres (μg), W = weight of compound **17**-loaded-PLGA microspheres (μg), Encapsulation efficiency (%, w/w) = (Lm/Lt) × 100, where Lm = measured drug loading (%, w/w) and Lt = theoretical drug loading (%, w/w).

5.2.3. In vitro release profile of compound 17 PLGA microsphere

Compound **17**-loaded PLGA microspheres were suspended in phosphate-buffered saline (PBS) containing 0.2% Tween 80 to adjust the concentration of compound **17** to 100 μg/ml. The microspheres were then completely dispersed by vortex mixing and sonication. The solution was divided into 1 ml samples and incubated at 37 °C. At various time intervals, the aliquots were centrifuged for 5 min at 12,000 rpm (n = 3 for each). The supernatant was removed and the pellet was dissolved with *p*-hydroxy benzoic acid *n*-nonyl ester in acetonitrile. The concentration of compound **17** in the solution was analyzed by high performance liquid chromatography (HPLC).

The remaining percentage of compound **17** was calculated as follows:

$$R_x = P_x/(S_0 + P_0) \times 100$$

where R_x = Remaining percentage of compound 17 at x days (%), P_x = Amount of compound **17** in the pellet of 1 mL sample at x days (μg), S_0 = Amount of compound **17** in the supernatant of 1 mL sample at 0 day (μg) and P_0 = Amount of compound **17** in the pellet of 1 mL sample at 0 day (μg).

5.3. Biological evaluation

Animals: Sprague–Dawley male rats (Crj:CD(SD)IGS, 8-weeks old, Charles River Laboratories (Atsugi, Japan), were maintained in a controlled environment of 12 h light/12 h dark at 24 ± 2 °C, humidified at 55 ± 15%. The animals were housed in polycarbonate cage. All animal experiments in this study were performed in accordance with ethical guidelines established by the Experimental Animal Care and Use Committee of ONO Pharmaceutical Co., Ltd.

Fracture models: Fibulae were fractured according to the method of Kawaguchi et al. with minor modification.[18] In brief, after anesthetizing the animals with sodium pentobarbital (30–50 mg/kg), the midshaft of each left fibula was exposed and cut sharply with bone cutter.

Determination of bone strength: Fibulae were removed from anesthetized rats to estimate mechanical strength 17 days after fracture. The three-point destructive bending test was performed with a bone-testing machine (Instron 5544 model, Instron Japan). Breaking strength was expressed as percentage of the mean value of nonfractured fibulae harvested from intact rats.

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