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Synthesis and evaluation of isoxazole derivatives as lysophosphatidic acid (LPA) antagonists

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Abstract—A series of isoxazole derivatives were synthesized and their antagonistic activities against LPA stimulation on both LPA_1/CHO cells and rHSC cells were evaluated. Among them, 3-(4-{4-[1-(2-chloro-cyclopent-1-enyl)-ethoxycarbonylamino]-isoxazol-3-yl}-benzylsulfanyl)-propionic acid (**34**) showed the most potent activities.
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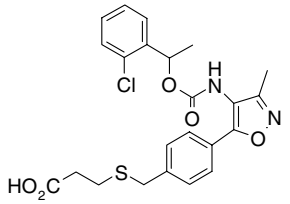
Lysophosphatidic acid (LPA; 1- or 2-*O*-acyl-*sn*-glycero-3-phosphate) is a bioactive lipid mediator with diverse physiological and pathological actions including platelet aggregation,[1] proliferation and migration of various tumor cell types[2] and smooth muscle cell contraction.[3] Four receptors of the cell membrane G protein-coupled receptors (GPCR) were demonstrated to mediate the cellular signals induced by LPA, of which LPA_1/Edg2, LPA_2/Edg4 and LPA_3/Edg7 belong to the EDG (endothelial cell differentiation gene) superfamily and LPA_4 was classified as p2y9/GPR23.[4] Recently, the nuclear transcription factor peroxisome proliferator-activator receptor-γ (PPARγ) was reported to be an intracellular receptor of LPA.[5]

It was also reported that LPA promotes cell proliferation in rat hepatic stellate cells (HSC) and migration in hepatic myofibroblast, both of which play an important role in liver fibrosis, via the Rho-signaling pathway.[6,7] Moreover, Rho-kinase inhibitor Y-27632 demonstrated the therapeutic effect on the rat liver fibrosis model.[8] Therefore, LPA may be an important factor in pathophysiology of HSC and hepatic myofigroblast during fibrogenesis by modulating cell morphology, attachment to the extracellular matrix and contraction,[9] and a compound with LPA antagonist activity is expected to be a potent drug for liver fibrosis. Among the LPA receptors, LPA_1 was thought to play an important role in the pathological state of liver fibrosis, since it had Rho-signaling pathway in its downstream and its expression was found in liver.[9]

To date, many compounds with LPA antagonistic activity were reported, though most of which were LPA analogues or had lipids-like structures which seems to have poor oral bioavailability.[2a,10,11] Recently, small molecule LPA antagonist Ki16425 (**1**) was reported with oral activity.[12]



Ki16425 (**1**)

In this article, we wish to report the synthesis and structure-activity relationship (SAR) studies based on Ki16425 for the improved antagonistic activity against LPA to find more potent and effective drug candidates for liver fibrosis. The LPA_1 antagonist activities of

Keywords: Lysophosphatidic acid; Lysophosphatidic acid receptor (LPA1); LPA antagonists; Isoxazole derivative; Liver fibrosis.
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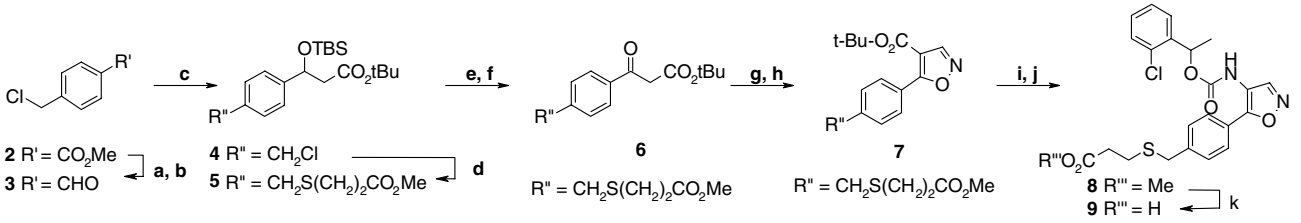
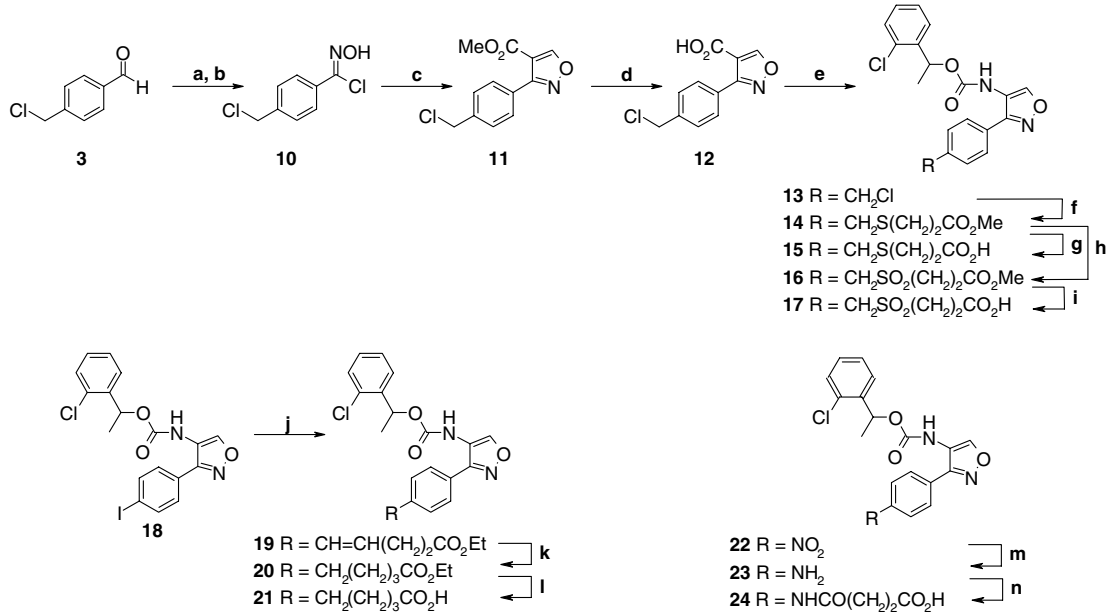
compounds were examined with CHO-K1 cells stably transfected with LPA_1 receptors. The inhibitory activities against LPA stimulation were also tested with rHSC for evaluating the efficacy of the compounds.

3-Unsubstituted isoxazolyl carboxylic acid **9** was synthesized from commercially available 4-chloromethyl benzoic acid methyl ester **2** as shown on Scheme 1. Compound **2** was reduced with BH_3–THF following oxidation to provide 4-chloromethyl benzaldehyde **3**. Following Mukaiyama Aldol addition[13] with (1-*tert*-butoxy vinyloxy) *tert*-butyl dimethylsilane gave 3-phenylproponic acid derivative **4**, which was condensed with 3-mercaptopropionic acid methyl ester to afford **5**. Deprotection of **5** followed by PDC oxidation yielded ketone **6**. Formilation of **6** using dimethoxymethyl dimethyl amine at 100 °C following hydroxylamine treatment gave isoxazole derivative **7** in moderate yield (65% for 2 steps). Since the ester of isoxazole-4-carboxylate was revealed to be unstable under basic condition to afford α-cyanoenol derivative via ring opening reaction,[14] the hydrolysis of **7** was carefully preceded under acidic condition to give carboxylic acid. Following coupling reaction under Curtius condition with 1-(2-chlorophenyl) ethanol led to isoxazole-4-carbamate **8**. Following hydrolysis of methyl ester afforded desired compound **9**.

The other isoxazole derivative, 5-unsubstituted isoxazolyl carboxylic acid **15** was obtained using **3** as a starting material (Scheme 2). Reaction of **3** with hydroxylamine following *N*-chloro succinimide treatment under acidic condition gave chloroxime **10**. Following Hetero Diels-Alder reaction with 3-methoxyacryl acid methyl ester yielded 5-unsubstituted isoxazole-4-carboxylic acid methyl ester **11** in moderate yield (49%), which leaded to carboxylic acid **12** via hydrolysis. Compound **12** were reacted with 1-(2-chlorophenyl) ethanol under Curtius condition to afford carbamate **13** (71% yield), which condensed with 3-mercaptopropionic acid methyl ester to give **14**. Following hydrolysis of ester gave desired product **15**. The sulfonic acid **32** was synthesized using



Scheme 1. Reagents and conditions: (a) BH_3, THF, rt, 2 h, 98%; (b) MnO_2, CH_2Cl_2, rt, 24 h, 83%; (c) (1-*tert*-butoxy vinyloxy) *tert*-butyl dimethyl silane, $Bu_2Sn(OTf)_2$, CH_2Cl_2, −78 °C, 4 h, quant.; (d) 3-mercaptopropionic acid methyl ester, NEt_3, TBAI, $CHCl_3$, 50 °C, overnight, 25%; (e) TBAF, THF, rt, 1 h, 92%; (f) PDC, AcOK, CH_2Cl_2, rt, overnight, 71%; (g) $Me_2NCH(OMe)_2$, 100 °C; (h) $HONH_2$, EtOH, 70 °C, 2 h, 65% for 2 steps; (i) HCl, dioxane, 100 °C, 21 h, 98%; (j) 1-(2-chloro-phenyl)-ethanol, DPPA, NEt_3, toluene, 90 °C, 40 min. 20%; (k) HCl, EtOH, 50 °C, 2 h, 47%.



Scheme 2. Reagents and conditions: (a) $HONH_2$, EtOH, 50 °C, 5 h, 99%; (b) NCS, HCl, dioxane, rt, 1 h, quant.; (c) $MeOC{=}CCO_2Me$, NEt_3, CH_2Cl_2, rt, 19 h, 49%; (d) 1 N LiOH, THF, rt, 1 h, quant.; (e) 1-(2-chlorophenyl) ethanol, DPPA, NEt_3, toluene, 90 °C, 2 h, 71%; (f) 3-mercaptopropionic acid methyl ester, NEt_3, CH_2Cl_2, rt, 26 h, 66%; (g) 1 N LiOH, THF, rt, 1 h, 86%; (h) mCPBA, CH_2Cl_2, rt, 2 h, 95%; (i) 1 N LiOH, THF, rt, 1 h, 30%; (j) 4-pentenoic acid ethyl ester, $Pd(OAc)_2$, *o*-tol_3P, 1,4-dioxane, DIEA, reflux, 4 h, 64%; (k) H_2, Pd/C, EtOAc, 1 h, 92%; (l) 1 N LiOH, THF, rt, 1 h, 73%; (m) H_2, Pd/C, EtOAc, 5days, 56%; (n) succinic anhydride, acetone, overnight, 81%.

the same method as described for **15**. The method was also applied for phenethylsulfanyl acetic acid **33** which was obtained using 4-(2-chloro-ethyl) benzaldehyde and mercaptoacetic acid methyl ester as the starting materials. The sulfone **16** was obtained by the mCPBA treatment of **14**. Following hydrolysis gave a 3-sulfonyl propionic acid derivative **17**.

Heck reaction using 4-pentenoic acid ethyl ester and 4-(*p*-iodophenyl)-isoxazole derivative **18**, which was prepared from 4-iodobenzaldehye, gave **19**. The hydrogenation of **19** followed by hydrolysis gave **21**. 3,3-Dimethyl-4-pentanoic acid derivative **34** were prepared by the same method from 3,3-dimethyl-pent-4-enoic acid ethyl ester.

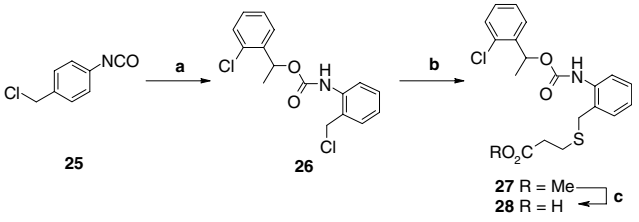
4-(*p*-Nitrophenyl)-isoxazole derivative **22** was synthesized by same method used for **13** from 4-nitro benzaldehyde. Subsequent hydrogenation gave aniline derivative **23**. Succinamic acid derivative **24** was obtained via condensation of **23** with succinic anhydride. (2-Chloromethylphenyl) carbamate **26** was prepared from *p*-chloromethyl phenylisocyanate **25** and 1-(2-chlorophenyl) ethanol. Condensation with 3-mercaptopropionic acid methyl ester followed by hydrolysis gave {2-(2-carboxyethylsulfanyl) methyl-phenyl} carbamate **28** (Scheme 3).

The cycloheptenone **29** was easily converted to 2-chlorocyclopent-1-ene carbaldehyde **30** in the presence of DMF and phosphorus oxychloride. Following methyl magnesium bromide treatment yielded alcohol **31**, which led to 1-(2-chlorocyclopent-1-enyl) ethyl carbamate **34**. 1-(2-Chlorocyclohex-1-enyl) ethyl carbamate **35** was synthesized with same method from cyclohexanone (Scheme 4).

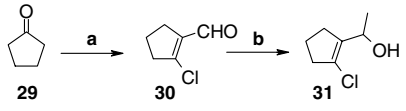
The compounds were tested for their inhibitory activities of LPA stimulated $[Ca^{2+}]_i$ influx in CHO-K1 cells which stably transfected with human LPA_1 receptor (LPA_1/CHO cells).[15] The antagonist activities against LPA stimulation was also tested on the rHSC cells, which is thought to play an important role in the pathology of liver fibrosis.[16]

We started our investigation to modify 3-methyl-isoxazole ring of Ki16425 to 3- and 5-unsubstituted-isoxazolyl derivatives **9** and **15** (Table 1). Interestingly, both of them proved to have potent inhibitory activities for LPA stimulation. Introduction of phenyl ring instead of isoxazole ring completely lost the activity (**28**). Among them, the IC_{50} value of **15** (IC_{50} = 0.22 μM on LPA_1/CHO cells and 0.049 μM on rHSC cells) were the highest and more potent than Ki16425 (IC_{50} = 0.51 μM on LPA_1/CHO cells and 0.16 μM on rHSC cells) in both of assays based on the LPA_1/CHO cells and the rHSC cells. Therefore, we preformed further optimizations on **15** as shown in Tables 2 and 3.

The modification started from the substitution of terminal carboxylic acid of **15** with sulfonic acid (**32**) (Table 2). However, this led to decrease the activities (IC_{50} = 0.43 μM on LPA_1/CHO cells and 0.074 μM on rHSC cells). The result implied that carboxylic acid at the terminal position was necessary to have a good potency. We next investigated on the modifications of 2-thioalkyl-propionic acid moiety of **15**. The sulfone derivative (**17**) and alkyl derivative (**21**) showed equivalent activities to **15** in the LPA_1/CHO assay (IC_{50} = 0.28 and 0.20 μM, respectively), but in the rHSC assay, their activities were decreased (IC_{50} = 0.11 and 0.072 μM, respectively). The β,β-dimethyl carboxylic acid derivative (**34**) was also tested, but its activities were also

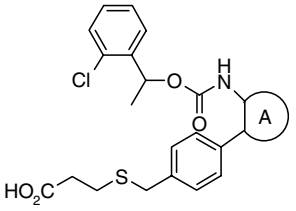


Scheme 3. Reagents and conditions: (a) 1-(2-chlorophenyl) ethanol, DMAP, toluene, 50 °C, 1 h, 89%; (b) 3-mercaptopropionic acid methyl ester, NEt_3, CH_2Cl_2, rt, overnight, 36%; (c) 1 N LiOH, THF, rt, 1 h, 82%.



Scheme 4. Reagents and conditions: (a) $POCl_3$, DMF, toluene, rt, 1 h, 82%; (b) MeMgBr, THF, rt, 30 min, 42%.

Table 1. SAR table of the derivatives with LPA stimulated $[Ca^{2+}]_i$ influx inhibition in $hLPA_1$/CHO-K1 cells



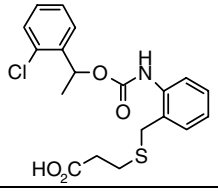
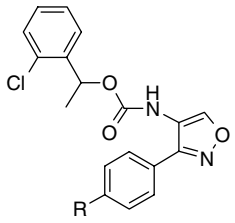
Compound	A	LPA_1/CHO IC_{50} (μM)	rHSC IC_{50} (μM)
Ki16425		0.51	0.16
9		0.26	0.081
15		0.22	0.048
28		>40	>20

Table 2. SAR table of the derivatives with LPA stimulated $[Ca^{2+}]_i$ influx inhibition in $hLPA_1$/CHO-K1 cells



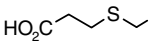
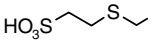
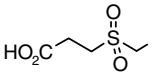
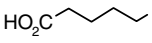
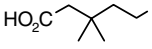
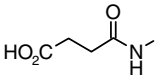
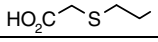
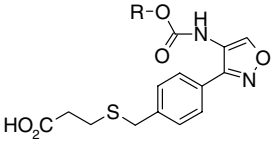
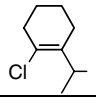
Compound	R	LPA_1/CHO IC_{50} (μM)	rHSC IC_{50} (μM)
15		0.22	0.049
32		0.43	0.074
17		0.28	0.11
21		0.20	0.072
34		0.37	0.12
24		0.14	0.073
33		0.13	0.10

Table 3. SAR table of the derivatives with LPA stimulated $[Ca^{2+}]_i$ influx inhibition in $hLPA_1$/CHO-K1 cells



Compound	R	LPA_1/CHO IC_{50} (μM)	rHSC IC_{50} (μM)
15		0.22	0.049
34		0.13	0.029
35		0.17	0.056

found to be less potent than that of **15** (IC_{50} = 0.37 μM on the LPA_1/CHO cells and 0.12 μM on the rHSC cells). The succinamic acid derivative (**24**) showed good activity in the LPA_1/CHO assay (IC_{50} = 0.14 μM) with decreased IC_{50} value in the rHSC assay (IC50 = 0.073 μM). Finally, thioacetic acid derivative (**33**) showed improved activity than **15** in the LPA_1/CHO assay (IC_{50} = 0.13 μM). However, its activity in the rHSC assay was decreased (IC_{50} = 0.10 μM). Thus, among all of these derivatives with modified terminal carboxylic acid moiety, the lead compound **15** showed the best IC_{50} value in the assay with rHSC cells, and its activity in the LPA_1/CHO assay was still one of the best. Therefore, we performed the further optimization on carbamate moiety of **15** (Table 3).

In the second-phase modification of **15**, the phenyl ring of 1-(2-chlorophenyl)-ethyl carbamate was substituted by cyclopentene and cyclohexene rings (**34** and **35**). The cyclohexene derivative (**35**) faild to show the increased potency in the rHSC assay (IC_{50} = 0.056 μM), though it showed good activity in the assay with LPA_1/CHO cells (IC_{50} = 0.17 μM). However, the activities of the cyclopentene derivative (**34**) showed superior IC_{50} values to that of **15** (IC_{50} = 0.13 μM in the LPA_1/CHO assay and 0.029 μM in the rHSC assay, respectively).

In summary, as a result of the modification of Ki16425 based on the activities in both the LPA_1/CHO and rHSC assays, we found the promising LPA antagonist **15**. Subsequent substitution on carbamate moiety, we found promising 3-(4-{4-[1-(2-chloro-cyclopent-1-enyl)-ethoxycarbonylamino]-isoxazol-3- yl}-benzylsulfanyl)-propionic acid (**34**) which possessed the most potent inhibitory activity against LPA_1 receptor as well as on rHSC cells with LPA stimulation. The activity of **34** showed 5 and 5.5 times more potency than that of Ki16425 in the LPA_1/CHO and rHSC assays, respectively. Because of the high inhibitory activities at the LPA_1 receptor and on the rHSC cells against LPA stimulation, **34** was expected to be a potent drug candidate for liver fibrosis.

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14. 5-(4-Methyl-phenyl)-isoxazole-4-carboxylic acid methyl ester, model compound of the ester of isoxazole-4-carboxylate, was easily decomposed into 3-(4-methyl-phenyl)-2-cyano-3-hydroxy-acrylic acid with overnight treatment of 0.1 M NaOH aq/MeOH. Similar ring opening reaction was known in anti-inflammatory drug leflunomide kalgutkar, A. S.; Nguyen, H. T.; Vaz, A. D. N.; Doan, A.; Dalvie, D. K.; McLeod, D. D.; Murray, J. C. *Drug Metab. Dispos.* **2003**, *31*(10), 1240, and references herein.
15. Measurement of LPA_1 receptor inhibitory activity was as follows: CHO-K1 cells stably transfected with LPA_1 receptor were cultured in αMEM/F12 (α-minimum essential medium:Ham's F12) 1:1 mixture containing 400 μg/ml Zeocin, 7% FBS (fetal bovine serum) and Penicillin-Streptomycin. The cells were loaded with the Ca^{2+} indicator dye Fluo-3,AM (Biotium) in assay buffer (20 mM Hepes, 1 × HBSS, 2.5 mM Probenecid) for 1 h at 37 °C. Cells were washed with assay buffer and resuspended in assay buffer containing 0.1% BSA and seeded into 384-well plates (24,000 cells/well). After cells were attached to the bottom of the well, the plates were placed into a FLIPR (Fluorometric Imaging Plate Reader, Molecular Devices). Cell fluorescence (excitation = 488 nm, emission = 540 nm) was monitored before and after the addition of test compounds in the presence or absence of 1 μM LPA. Ki16425 and compound **15** were used as a positive control throughout the assay (IC_{50} = 510 ± 150 nM, n = 25 for Ki16625 and 220 ± 70 nM, n = 29 for **15**, respectively) and IC_{50} values represent the mean of not less than two different runs.
16. Measurement of inhibitory activity against LPA stimulation with rat hepatic stellate (rHSC) cells was as follows: A stellate cell fraction was obtained from the liver of male Wister rat according to a conventional method Kawada, N.; Tran-Thi, T. A.; Klein, H.; Decker, K. *Eur. J. Biochem.* **1993**, *213*(2), 815, The cells were seeded overnight in a 384 well plate at 24,000 cells/well and the medium was removed. An assay buffer (0.1% BSA, 20 mM Hepes, 1 × HBSS, 2.5 mM Probenecid) containing 4 μM of Fluo-3, AM was added, and the cells were stained at 37 °C for 1 h. Following the buffer containing the dye reagent was removed, and an assay buffer was added, intracellular Ca^{2+} concentration was measured with FLIPR. Adding a test substance and LPA at the final concentration of 5 μM, the inhibitory action of the test substance on the increase in the intracellular Ca^{2+} concentration by LPA was examined. The increase in the intracellular Ca^{2+} concentration by LPA addition without test substance was taken as 100%, that without the addition of LPA was taken as 0%, and the substrate's concentration (IC_{50}) at inhibiting increase in the intracellular Ca^{2+} concentration by 50% was determined. Ki16425 and compound **15** were used as a positive control throughout the assay (IC_{50} = 160 ± 33 nM, n = 13 for Ki16625 and 49 ± 12 nM, n = 5 for **15**, respectively) and IC_{50} values represent the mean of not less than two different runs.