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Discovery of non-competitive thrombin inhibitor derived from competitive tryptase inhibitor skeleton: Shift in molecular recognition resulted from skeletal conversion of carboxylate into phosphonate

Hiroshi Aoyama [a,*], Ryosuke Ijuin [a], Jun-ya Kato [a], Sarasa Urushiyama [a], Masashi Tetsuhashi [b], Yuichi Hashimoto [b], Tsutomu Yokomatsu [a]

[a] School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji-city, Tokyo 192-0392, Japan
[b] Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

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ABSTRACT

A novel series of terminal and internal phosphonate esters based on our previously developed aryl carboxylate-type tryptase selective inhibitor **1** was synthesized. The potency of these synthesized compounds was assessed in vitro with an enzyme inhibition assay using three available serine proteases, that is, tryptase, trypsin, and thrombin. The internal phosphonate derivative **6** showed potent thrombin inhibitory activity with an IC_{50} value of 1.0 μM, whereas it exhibited no or only weak tryptase and trypsin inhibition at 10 μM. The Lineweaver–Burk plot analysis indicates that the inhibition pattern of thrombin with **6** is non-competitive in spite of the fact that the lead carboxylate compound **1** is competitive inhibitor. Therefore, the skeletal conversion of the carboxylate into a phosphonate alters the mode of molecular recognition of these inhibitors by thrombin.

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Trypsin-like serine proteases belong to one of the two serine protease subfamily including trypsin, chymotrypsin, tryptase, plasmin, thrombin, Factor Xa, and so on. These enzymes play pivotal and multiple roles in various biological phenomena including protein digestion,[1,2] blood coagulation,[3,4] apoptosis,[5,6] the factors in allergic condition such as asthma and rhinitis,[7,8] and the innate immune response to pathogen infection.[9]

Recently, we have developed tryptase inhibitors **1** and **2** that showed moderate to potent inhibitory activity and good enzyme selectivity (Fig. 1).[10] In addition, several research groups have shown that peptidyl diphenyl phosphonate derivatives exhibit inhibitory activity against various trypsin-like serine proteases.[11–15] Meanwhile, since the end of the 20th century, we have been engaged in developing sphingomyelinase (SMase) inhibitors based on the replacement of the phosphodiester moiety of sphingomyelin with isosteric difluoromethylenephosphonic acid. Using this strategy, we have successfully developed phosphonate-containing inhibitors against lysosomal acid sphingomyelinase (A-SMase) and membrane-bound neutral sphingomyelinase (N-SMase).[16,17] Furthermore, Mansat et al. reported that some serine protease inhibitors can block N-SMase activation stimulated by daunorubicin.[18] Although serine protease inhibitory activity of our phosphonate-containing SMase inhibitors was yet to be identified, these findings and reports led us to make a hypothesis that phosphonate structure have a potential to have molecular recognition for serine protease(s). Then, we designed two kinds of phosphonate derivatives based on the structure of **1**: one with a terminal phosphonate moiety (compounds **3** and **4**) and second with an internal phosphonate moiety (compounds **5** and **6**) (Fig. 2). Although the lead compound **1** was developed as TFA salt in our previous study,[10] we planned to synthesize phosphonates **3**–**6** as HCl salt in this study.

Syntheses of terminal phosphonates **3** and **4** and internal phosphonates **5** and **6** are outlined in Schemes 1 and 2, respectively. Briefly, carboxylic acid **7**[10] was converted to a Weinreb amide, followed by reduction of the amide group to afford aldehyde **8**. α-Aminophosphonates **10** were constructed by magnesium perchlorate catalyzed three-component reaction of the aldehyde **8**, aminophthalimides **9**, and diphenyl phosphite.[19] Finally, deprotection of aminopyridyl group of **10** with TFA and the resulting amine TFA salt were treated with HCl/EtOAc gave terminal phosphonate derivatives **3** and **4**, respectively. On the other hand, diethyl phosphonates **13** were obtained by Arbuzov reaction using triethyl

* Corresponding author. Tel.: +81 42 676 3251; fax: +81 42 676 3243.
E-mail address: aoyamahr@toyaku.ac.jp (H. Aoyama).

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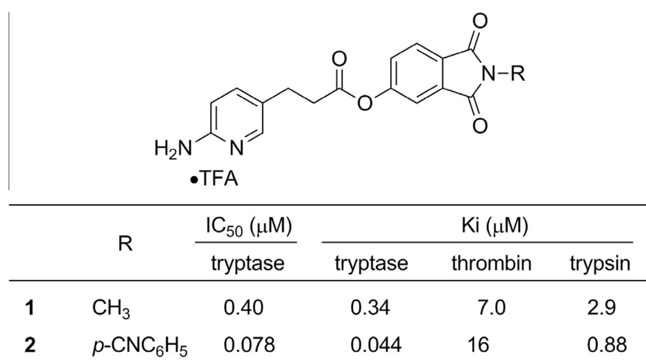


	R	IC_{50} (μM)	Ki (μM)		
		tryptase	tryptase	thrombin	trypsin
1	CH_3	0.40	0.34	7.0	2.9
2	*p*-CNC_6H_5	0.078	0.044	16	0.88

Figure 1. Tryptase selective inhibitors **1** and **2** with aryl carboxylate skeleton.

phosphite and either picolyl bromide **12a**[20] or (2-bromoethyl)pyridine **12b**, prepared from pyridylethanol **11**.[21] The resulting diethyl esters **13** were then treated with oxalyl chloride to generate phosphoryl chloride monoethyl ester intermediates followed by the addition of 5-hydroxy-*N*-methylphthalimide[10] to obtain phosphoric acid alkyl aryl diesters **14**. Finally, the nitro group on the pyridyl group of **14** was reduced to an amino group using Pd-catalyzed hydrogenation under H_2 atmosphere in the presence of TFA. The resulting amine TFA salts were treated with HCl/EtOAc afforded internal phosphonate derivatives **5** and **6**. The HCl salt of **1** (**1**·HCl) was obtained from the corresponding TFA salt by the same procedure as mentioned above.

The effects of synthesized compounds **1**–**6** on enzyme activity were determined by incubating each enzyme with its appropriate peptide substrates in Tris–HCl buffer in the absence or presence of synthesized compounds. In the case that calculation of IC_{50} values of some compounds were possible, their values were determined by 4- to 5-dose titration of test compounds in the presence of appropriate peptide substrates. First, we measured the enzyme inhibition rate of **1**·HCl and newly synthesized compounds (**3**–**6**) at a fixed concentration of 10 μM against three serine proteases summarized in Table 1. The carboxylate-type **1**·HCl showed potent tryptase inhibitory activity, that is, almost completely inhibited the activity at 10 μM. The IC_{50} value of **1**·HCl was estimated to be 0.19 μM, that is, almost equal to our previous synthesized its corresponding TFA salt (IC_{50} = 0.40 μM).[10] On the other hand, the internal phosphonate derivatives **5** and **6** indicated weak tryptase inhibitory activity at 10 μM, the inhibition rate of 5.3% and 11%, respectively. The terminal phosphonate derivatives **3** and **4** displayed completely no activity against tryptase inhibition at all. Concerning trypsin inhibitory activity, all examined compounds were denoted the same tendency of tryptase inhibition. In fact that the phosphonate derivatives showed no trypsin inhibition, while the carboxylate **1**·HCl exhibited good inhibitory activity (Table 1); the inhibition rate of **1**·HCl at 10 μM was 97% with an IC_{50} value of 1.2 μM. Meanwhile, the behavior of thrombin

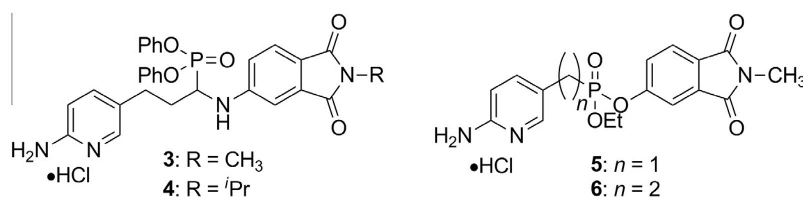
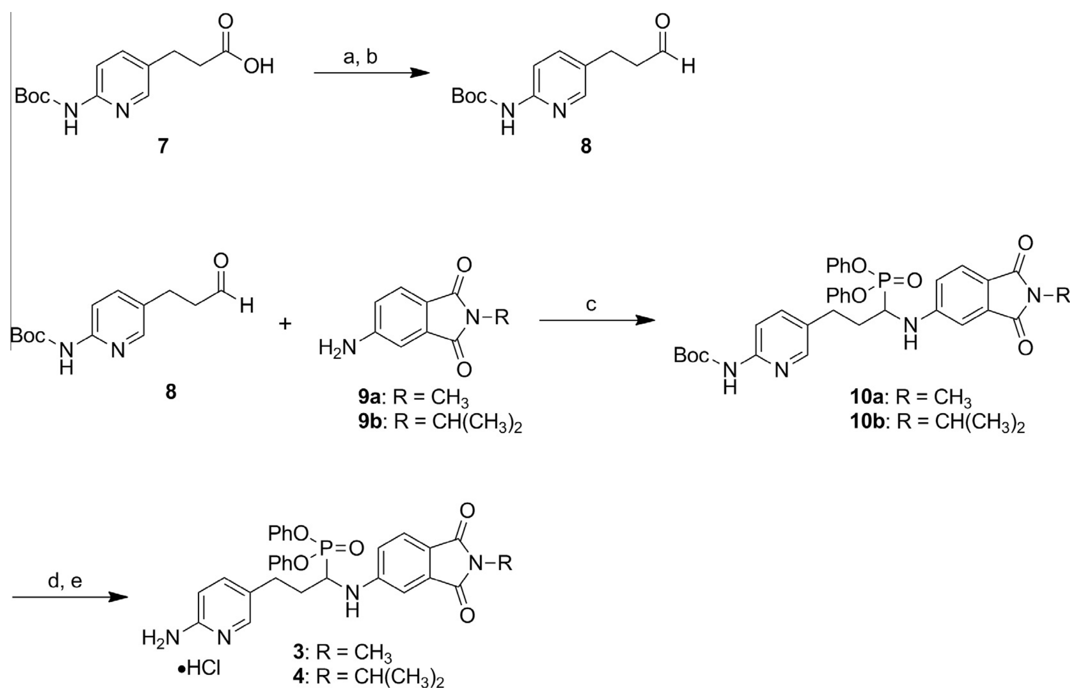


Figure 2. Design of terminal phosphonate derivatives **3** and **4** and internal phosphonate derivatives **5** and **6**.



Scheme 1. Reagents and conditions: (a) MeO(Me)NH·HCl, EDCI·HCl, Et_3N, CH_2Cl_2, rt, 67%; (b) DIBAL-H, THF, −78 °C, 83%; (c) $(PhO)_2P(O)H$, $Mg(ClO_4)_2$, neat, rt, 8–25%; (d) TFA, CH_2Cl_2, rt; (e) HCl, EtOAc, rt, 34–69% (2 steps).

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