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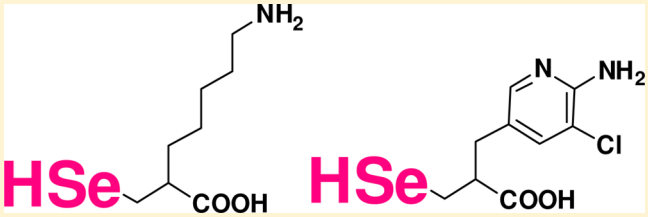
Design and Characterization of a Selenium-Containing Inhibitor of Activated Thrombin-Activatable Fibrinolysis Inhibitor (TAFIa), a Zinc-Containing Metalloprotease

Nobuko Yoshimoto,[†,‡] Tomoyuki Sasaki,[†,§] Katsuyoshi Sugimoto,[†,§] Hidemi Ishii,[†,§] and Keiko Yamamoto*[,†,‡]

[†]High Technology Research Center, [‡]Laboratory of Drug Design and Medicinal Chemistry and [§]Laboratory of Molecular and Cellular Pathophysiology, Showa Pharmaceutical University, 3-3165 Higashi-Tamagawagakuen, Machida, Tokyo 194-8543, Japan

ABSTRACT: Available therapies for thromboembolic disorders include thrombolytics, anticoagulants, and antiplatelets, but these are associated with complications such as bleeding. To develop an alternative drug which is clinically safe, we focused on activated thrombin-activatable fibrinolysis inhibitor (TAFIa) as the target molecule. TAFIa is a zinc-containing carboxypeptidase that significantly inhibits fibrinolysis. Here we designed and synthesized selenium-containing compounds **5**−**13** to discover novel TAFIa inhibitors having a superior zinc-coordinating group. Compounds **5**−**13** significantly inhibited TAFIa activity (IC_{50} 2.2×10^{-12} M − 2.6×10^{-6} M). We found that selenol is a better functional group than thiol for coordinating to zinc at the active site of TAFIa. Furthermore, compound **12**, which has an amino-chloro-pyridine ring, was found to be a potent and selective TAFIa inhibitor that lacks carboxypeptidase N inhibitory activity. Therefore, compound **12** is a promising candidate for the treatment of thromboembolic disorders. This is the first report of a selenium-containing inhibitor for TAFIa.



INTRODUCTION

Thromboembolic disorders are a major cause of morbidity and mortality in developed countries. Available therapies include thrombolytics, anticoagulants, and antiplatelets, although these therapies are associated with complications such as bleeding. To develop a clinically safe drug, we focused on thrombin-activatable fibrinolysis inhibitor (TAFI) as the target molecule. TAFI is an important regulator of fibrinolysis[1] and is also termed plasma procarboxypeptidase B, R, or U.[2] TAFI is an inactive 60 kDa zymogen glycoprotein that is produced by the liver and is present in plasma.[3] In the coagulation cascade, cleavage by thrombin at Arg92 converts TAFI from its inactive form to activated TAFI (TAFIa). TAFIa, a labile enzyme with a half-life of less than 10 min at 37 °C,[4] is a zinc-containing basic carboxypeptidase found in plasma that cleaves the C-terminal arginine and lysine residues from peptide and protein.

Fibrin is initially degraded by plasmin to produce fibrin containing newly exposed surface C-terminal lysine and arginine residues. These basic residues function as binding sites for tissue plasminogen activator (t-PA) and its substrate, plasminogen, therefore positioning t-PA and plasminogen in proximity and increasing plasmin production.[5] TAFIa stabilizes clots by eliminating these binding sites; consequently, TAFIa significantly inhibits fibrinolysis.

Inhibition of TAFIa should modulate clotting and lysis, without resorting to direct action on the coagulation cascade. A TAFIa inhibitor would therefore likely diminish the risk of side effects compared with other potential mechanisms for treating thrombotic disease. At present, TAFIa inhibitors in combination with t-PA appear to enhance the efficiency of thrombolysis, enabling lower dosing with t-PA and consequently fewer bleeding problems.[6,7] Therefore, the development of TAFIa inhibitors as profibrinolytic agents is an attractive concept.

The research of TAFIa inhibitors is active worldwide, but none of these inhibitors are currently used as therapeutic agents. Several groups have reported synthetic small molecules that act as a TAFIa inhibitor.[7−13] All of these have three characteristic functional groups (Figure 1). Because TAFIa recognizes the C-terminal basic amino acid on the surface of fibrin, it is reasonable that the inhibitors would have (i) a basic group corresponding to the lysine side chain to bind to Asp256 at the bottom of the S1′ pocket, (ii) a carboxylic acid corresponding to the lysine C-terminal carboxylic acid, and (iii) a functional group to coordinate to the catalytic Zn (Figure 1). Indeed, the amine group mimicking the arginine or lysine side chain has been used as a basic group to bind to Asp256. Various zinc-coordinating groups have been reported, including carboxylic acid, thiol, imidazole, phosphonic acid, phosphinic acid, hydroxamic acid, sulfonamide, and α-hydroxyketone. We focused on a zinc-coordinating group and aimed to design novel TAFIa inhibitors having a superior zinc-coordinating group. Here, we report the design, synthesis, and biological evaluation of novel TAFIa inhibitors bearing a potential zinc-coordinating functional group.

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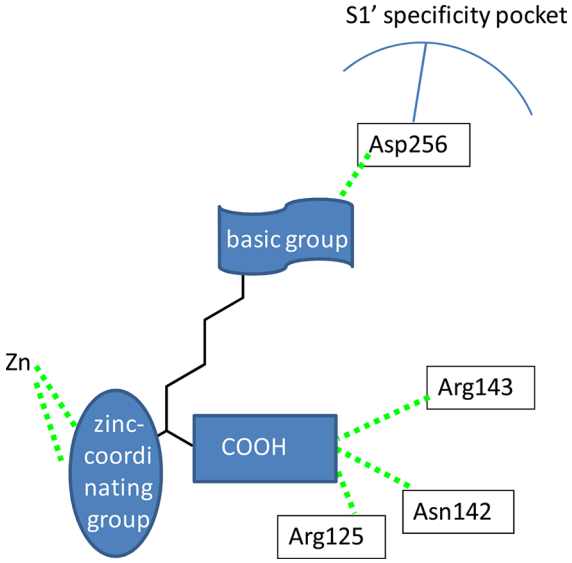

Figure 1. Consensus structure of TAFIa inhibitor and its interactions with TAFIa. Coordinate bonds to the zinc, and intermolecular salt bridges and hydrogen bonds, are shown as dotted green lines.

DESIGN

Although thiol effectively coordinates zinc in the active site of various zinc metalloproteases, we chose to use the selenol rather than the thiol group. Previously, Suzuki et al. reported that (*S*)-7-amino-2-[[[(*R*)-2-methyl-1-(3-phenylpropanoylamino) propyl]hydroxyphosphinoyl]methyl]heptanoic acid (EF6265), which contains a phosphinic acid, is a potent inhibitor of TAFIa.[7] EF6265 specifically inhibits TAFIa activity, with an IC_{50} value of 8.3 nM, and enhances t-PA-mediated clot lysis concentration dependently. However, EF6265 is orally inactive. We hypothesized that oral inactivity is due to the presence of a phosphinic acid moiety because phosphinic acid is hydrophilic not hydrophobic. We modified the phosphinic acid moiety to a common functional group, thiol, and also to an innovative group, selenol. Figure 2 shows compounds **1**−**13**, designed using EF6265 as a lead compound. Note that the thiol and selenol compounds shown in Figure 2 are homologues of cysteine and selenocysteine, respectively.

Selenium is an essential element for humans and animals. For instance, selenocysteine is recognized as the 21st naturally occurring amino acid in proteins, and glutathione peroxidases, which are selenium-containing enzymes, are well-known for playing an important role in redox processes.[14−16] Various small organoselenium compounds have been studied as biological models.[17−21] Of these, the most promising compound is 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one, ebselen, which has been demonstrated to act as a glutathione peroxidase mimic and as a scavenger of peroxynitrite.[19,20] The sulfur analogue of ebselen shows 15-fold lower activity than ebselen, indicating that ebselen holds a promise as an antioxidant drug. As reviewed by Nogueira and Rocha,[20b] ebselen was apparently not toxic for humans after short-term intake, whereas dietary overexposure to selenium compounds can increase the incidence of chronic degenerative diseases such as type 2 diabetes, amyothrophic lateral sclerosis, and some types of cancer. We should note that because potential therapeutic use of organoselenium compounds has not yet been sufficiently explored, detailed toxicological studies of selenium are needed.

Carboxypeptidase N (CPN) is a plasma zinc-containing metalloprotease that cleaves C-terminal arginines and lysines from peptides found in the bloodstream such as vasoactive peptide hormones, growth factors, and cytokines.[22] By cleaving only one basic residue, CPN can change peptide activity and receptor binding. CPN has an important function as an inactivator of complement anaphylatoxines. From these functions of CPN, it is important to design inhibitors that are selective for TAFIa over CPN. Compounds **10**−**13** were designed as inhibitors with good selectivity for TAFIa but not for CPN. CPN has a spatially narrower active site cavity than TAFIa,[23] so CPN would likely have difficulty accommodating an aromatic ring such as an amino-chloro-pyridine ring. A 2-aminopyridine ring instead of aniline was selected because 2-aminopyridine is more basic than aniline.

SYNTHESIS

The synthetic scheme for thiol compounds **1**−**4** is shown in Scheme 1. 5-Amino-1-pentanol derivative **14** was derived from commercially available 5-amino-1-pentanol by the reported procedure.[24] Mesylate **14** was converted to alkyl malonate **15** by condensation with diethyl malonate under basic conditions. One of the two ester moieties of **15** was saponified with potassium hydroxide in ethanol to provide half ester **16** in excellent yield. Half ester **16** was subjected to a Mannich reaction in the presence of formaldehyde and diethylamine to afford enoate **17**. The key intermediate **17** was subjected to Michael addition: **17** was treated with thioacetic acid in the presence of triethylamine to afford conjugate addition product **18** in good yield. Thioacetate **18** was refluxed in conc HCl to provide desired product **1**, and **18** treated with trifluoroacetic acid (TFA) provided **2**. Furthermore, treatment of thioacetate **18** with lipase PS gave racemic thiol **19**, which was then treated with TFA to give ester **3**. Target compound **4** was obtained by hydrolysis of ester **17** followed by Michael addition using thioacetic acid and deprotection of the *N*-Boc amine.

Selenium was introduced using a method recently developed by Knapp and Darout:[25] They prepared selenocarboxylic acid from the corresponding carboxylic acid and Woollins's reagent[26,27] in toluene and used the selenocarboxylic acids in substitution, addition, and amidation reactions. We treated enoate **17** with selenopropionic acid **23** prepared in situ from propionic acid and Woollins's reagent **22** to give the Michael addition product, selenoester **24** in 32% yield. Selenoester **24** was refluxed in conc HCl to provide a single compound, **5**, which is a dimer and an oxidized form of selenol produced by hydrolysis. Treatment of ester **24** with TFA provided *N*-Boc-deprotected product **6** (Scheme 2).

Selenoesters **7**−**9** were prepared by a Michael addition to alkenoic acid **20** (Scheme 3). Treatment of **20** with selenopropionic acid **23** afforded selenoester **25** in 44% yield. Selenoester **25** was treated with TFA to provide *N*-Boc-deprotected product **7**. Seleno-4-phenylbutyric acid ester **27** was obtained using seleno-4-phenylbutyric acid **26** prepared from the corresponding carboxylic acid and Woollins's reagent **22**. Seleno-3-phenylpropionic acid ester **29** was also obtained using a procedure similar to that used for the preparation of **27**. Compounds **27** and **29** were treated with TFA to provide **8** and **9**, respectively.

Alkenoic acid ester with an aminopyridine-ring in the side chain, **30**, and its chloro-analogue, **32**, were prepared by the method reported previously.[9] Selenylation followed by deprotection were accomplished using a procedure similar to

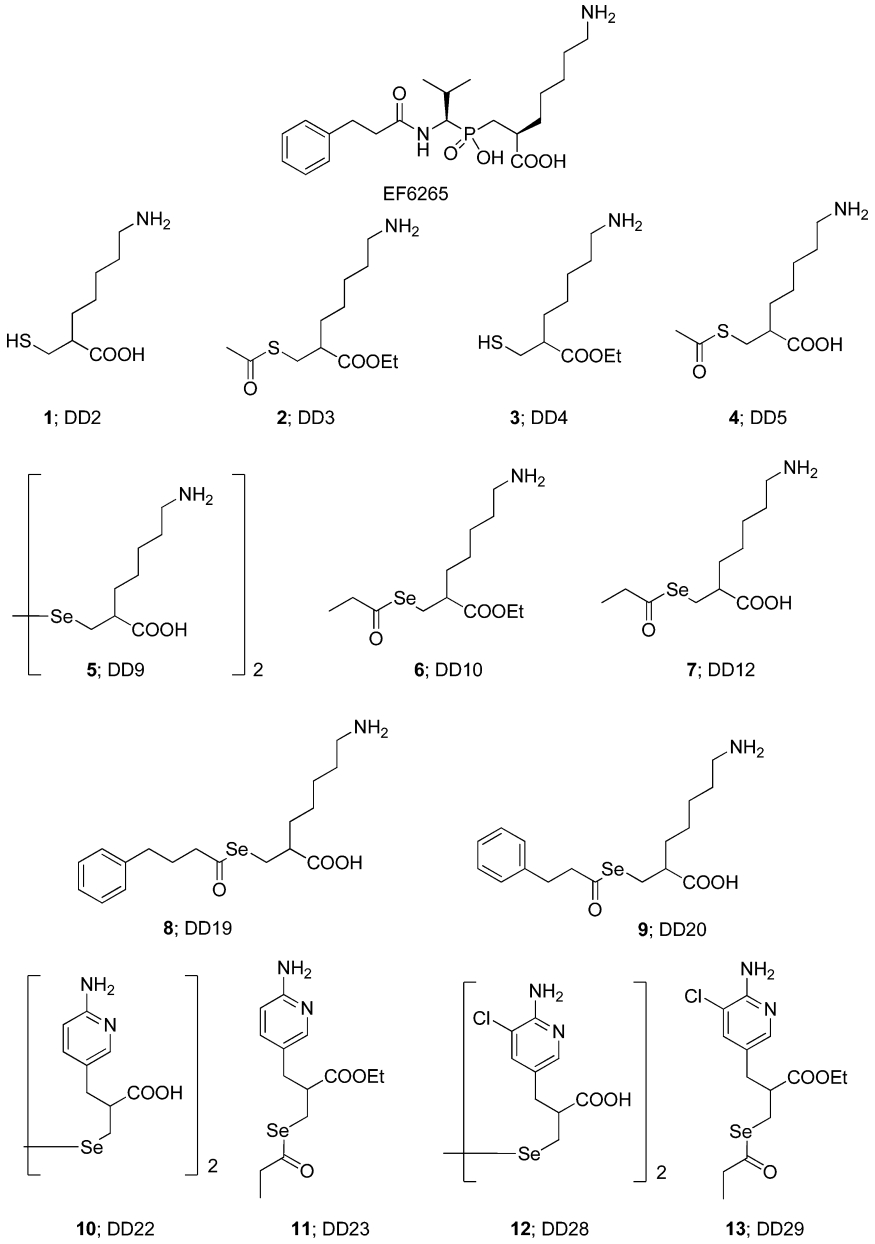

Figure 2. Structures of EF6265 and TAFIa inhibitor candidates.

that described above (Scheme 4). Thus, we synthesized all target compounds **10**–**13** in excellent yields.

ESI-mass and ^{13}C NMR spectra confirmed that thiol compounds **1** and **3** exist as monomers whereas selenol compounds **5**, **10**, and **12** exist as dimers. The dimer form of selenol compounds is reasonable because selenol is susceptible to oxidation in air.

■ TAFIa INHIBITION ACTIVITY

The inhibition of human TAFIa, porcine pancreatic carboxypeptidase B (ppCPB), and human CPN were assayed using a procedure similar to that reported previously.[7] ppCPB was used instead of human pancreatic CPB, which is a digestive basic carboxypeptidase. Pancreatic CPB is closely related to TAFIa; indeed, TAFIa is also known as plasma CPB. Transient inhibition of pancreatic CPB has been thought to hardly have side effects. Inhibition of pancreatic CPB alone should not diminish intestinal absorption due to the presence of alternate active proteases available for digestion.[11] CPN is a basic carboxypeptidase circulating in plasma that is critical for regulating the complement system.[22] Inhibition of CPN results in undesirable side effects. Therefore, it is critical to design TAFIa selective inhibitors that have no CPN inhibition activity.[11]

The assay results for enzyme inhibition by thiocompounds **1**–**4** are shown in Table 1. Compounds **1**–**4** inhibited TAFIa activity in order of **1**, **4**, **3**, and **2**. These compounds inhibited ppCPB activity with potency similar to their inhibition of TAFIa, and compounds **1**, **3**, and **4** inhibited CPN activity with 40, 0.4, and 40 times less potency compared to their inhibition of TAFIa, respectively.

Because diselenide is an oxidized form of the selenol compound, we tested the TAFIa inhibition activity of selenium-containing compounds in the presence of 10 μM dithiothreitol (DTT). DTT is a strong reducing agent because, once oxidized, it forms a stable six-membered ring with an internal disulfide

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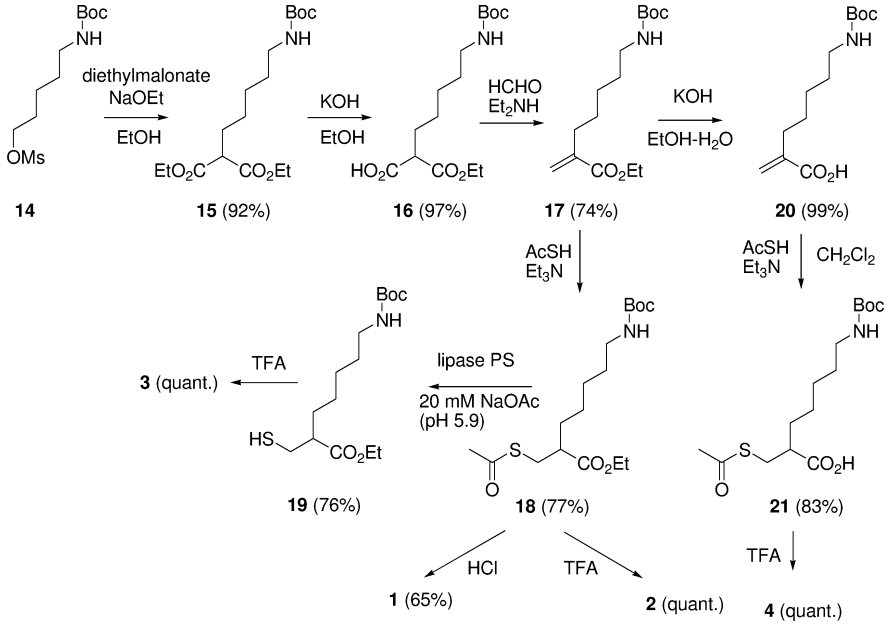
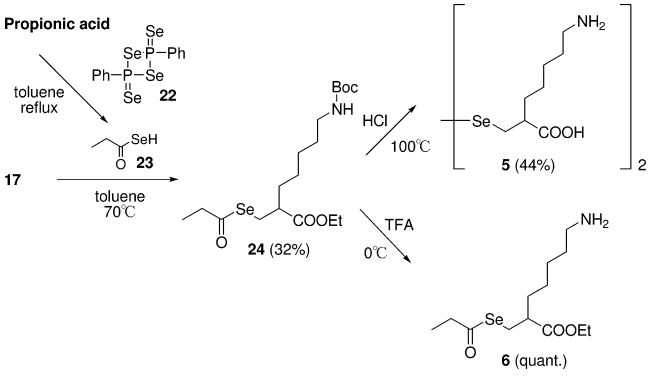
Scheme 1. Synthetic Scheme of Thio-Acyl and Thiol Compounds 1−4



Scheme 2. Synthetic Scheme of Selenium-Containing Compounds 5 and 6



bond. In animal and human cells, glutathione acts as reducing agent in a similar manner to that of DTT. Prior to the inhibition test, we verified that 10 μM DTT does not affect TAFIa activity (data not shown). All selenium-containing compounds (**5**−**13**) showed potent TAFIa inhibition activity, with IC_{50} values of 10^{-12}−10^{-6} M (Table 2). In particular, diselenide compounds **5**, **10**, and **12** showed strong activity, with IC_{50} values in the range 10^{-9}−10^{-12} M. In the absence of DTT, compounds **5**, **10**, and **12** showed 10^5 times less potent activity than in the presence of DTT. Selenoesters **6**−**9**, **11**, and **13** showed strong/moderate TAFIa inhibition activity. They also showed weaker activity in the absence of DTT.

Compounds with a lysine mimic side chain (**5**−**9**) inhibited ppCPB activity with potency similar to their inhibition of TAFIa, while compounds with an aminopyridine ring in the side chain, **10**−**13**, showed 3−90 times less potent inhibition of ppCPB. As described above, it is not disadvantageous if TAFIa inhibitors also inhibit pancreatic CPB.

All compounds with a lysine mimic side chain (**5**−**9**), and two compounds with an aminopyridine ring in the side chain (**10** and **11**), significantly inhibited CPN. Interestingly, compounds with an amino-**chloro**-pyridine ring in the side chain, **12** and **13**, showed no inhibition activity against CPN. Therefore, compounds **12** and **13** exhibited desirable inhibition profiles for the enzymes tested.

■ DISCUSSION

Here we presented the design and synthesis of novel TAFIa inhibitors containing an original element, "**Se**", and provide the TAFIa inhibition activity of these synthetic compounds. Synthetic compounds **1**−**13** showed significant inhibition activity against TAFIa. Carboxylic acid compounds **1** and **7** showed stronger activity than the corresponding carboxy ester compounds **3** and **6**, indicating that the carboxylic acid group is superior to the corresponding ester group. This is reasonable because two Arg residues in TAFIa recognize a carboxylic acid in the substrate or inhibitor and form a salt bridge. This also explains the lower activity of ethyl ester compounds **11** and **13**.

Comparison of sulfur- and selenium-compounds (**1** versus **5**, **2** versus **6**, **4** versus **7**−**9**) demonstrated the more potent activity of the selenium-containing compounds, indicating that "**Se**" is superior to "**S**" at coordinating to zinc in enzyme. In addition, selenoesters **7**−**9** showed activity comparable to selenol **5**, indicating that selenoesters **7**−**9** function after hydrolysis to selenol **5**. Indeed, selenoesters **7**−**9** in stock solution gradually decomposed into hydrolysis product **5**. Quite recently, we obtained the preliminary result that Se of selenol compound coordinates to zinc at the active site of CPB by the X-ray crystal structural analysis (data not shown). After the more detailed study, we will publish the experimental results.

Selenium is an essential trace element. In the form of selenocysteine, the 21st amino acid, selenium is incorporated cotranslationally into selenoproteins. As a homologue of sulfur in the periodic table of the elements, the chemistry of selenium has some resemblance to that of sulfur.[28] Biology uses the differences between sulfur and selenium, particularly lower pK_a value of the selenol/selenolate couple compared to that of the thiol/thiolate couple.[28] In the present study, the strong nucleophilicity of selenol might contribute to strong inhibition activity. As described in the Design section, detailed toxicological studies of our selenium compounds are needed in the next step.

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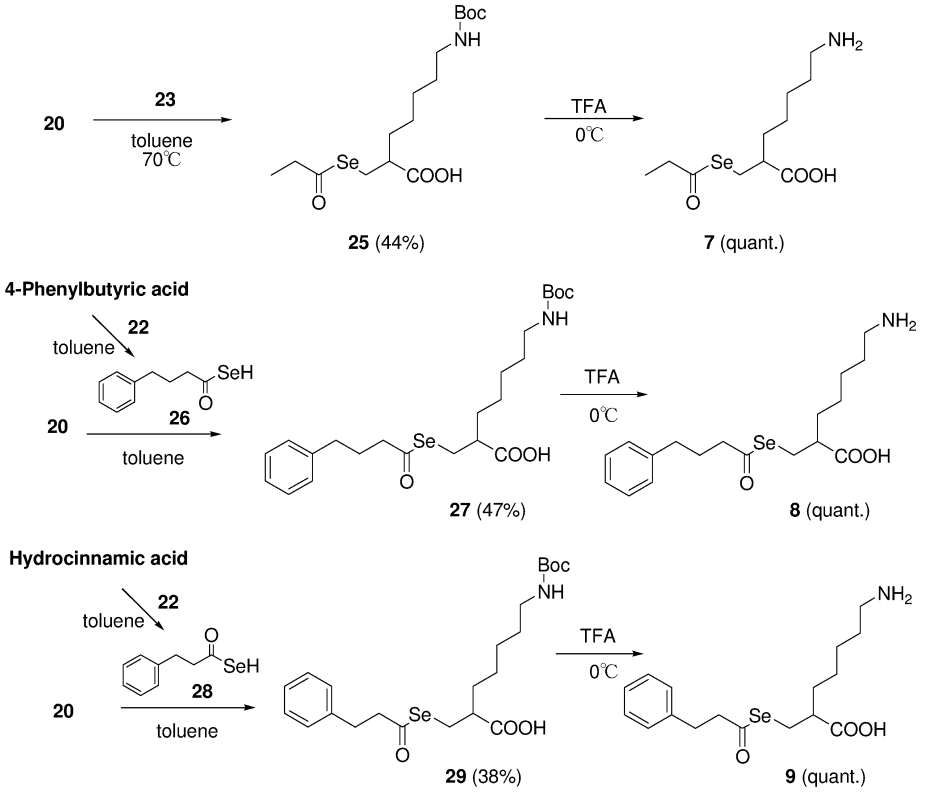
Scheme 3. Synthetic Scheme of Selenium-Containing Compounds 7−9



Compounds with an aminopyridine side chain, **10** and **12**, showed 10^5 times stronger activity than their esters, **11** and **13**, indicating that "SeH" and "COOH" are desirable functional groups. In terms of selectivity for TAFIa, compound **12** exhibits desirable specificity. Similar selectivity has been reported for the corresponding sulfur compound,[9] but the inhibitory activity of the sulfur compound is more than 10^5 times weaker than that of selenium compound **12**. Therefore, compound **12** is a promising candidate as a TAFIa inhibitor that lacks CPN inhibition activity.

The crystal structures of human and bovine TAFI demonstrated that the TAFI pro-domain is partially rotated away from the active site, exposing the catalytic residues to the solvent.[29,30] In addition, the salt-bridge in pro-CPB, located between the pro-domain and the substrate binding site in the catalytic cavity, was missing in TAFI, thereby exposing the active site.[29,30] Enghild's group showed that TAFI itself exhibits continuous and stable carboxypeptidase activity against substrates, and they suggested that the activity down-regulates fibrinolysis in vivo.[31] Furthermore, they recently determined the crystal structure of bovine TAFI complexed with tick carboxypeptidase inhibitor (TCI) and suggested that the inhibitors, such as TCI, interact with TAFI in a substrate-like manner.[32] Therefore, inhibitors of TAFI but not of TAFIa might be effective for fibrinolysis. Small molecules like our synthetic inhibitors may be effective if they can easily enter to the active site of zymogen TAFI. In particular, compound **12** is promising candidate for the treatment of thrombosis.

CONCLUSIONS

We have designed and synthesized a novel type of TAFIa inhibitor, compounds **5**−**13**, that contain selenium to coordinate to zinc in the active site of enzymes. In particular, compound **12**, which has an amino-**chloro**-pyridine ring at the side chain, is a promising and practical candidate drug for fibrinolysis because it is a selective TAFIa inhibitor that lacks CPN inhibitory activity. This is the first report of a selenium-containing inhibitor for TAFIa which is a zinc-containing metalloprotease. The present study demonstrated the potential for developing novel inhibitors for other zinc-containing metalloproteases.

EXPERIMENTAL SECTION

All reagents were purchased from commercial sources. Silica gel column chromatography was carried out using 100−210 μm silica gel (KANTO CHEMICAL silica gel 60 N). Unless otherwise stated, NMR spectra were recorded at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR in $CDCl_3$ solution with TMS as an internal standard, and the chemical shifts are given in δ values. Signals arising from the trifluoroacetate counteranions were not listed. Low-resolution mass spectra (LRMS) and high-resolution mass spectra (HRMS) were recorded on a JEOL AccuTOF LC-plus JMS-T100LP using electrospray positive ionization. All air and moisture sensitive reactions were carried out under argon or nitrogen atmosphere. Purity was determined by HPLC (CAPCELL PAK UG120, 4.6 mm × 150 mm, 0.2% TFA−methanol/milliQ (1:1−3:7), flow rate 1.0 mL/min), and was >95% for all compounds whose biological activity was tested.

Diethyl {5-[(*tert*-Butoxycarbonyl)amino]pentyl}-propanedioate (15). To a suspension of NaOEt (30.19 g, 0.44 mol) in dry EtOH (300 mL) was added diethyl malonate (67.5 mL, 0.44 mol) at 0 °C, and the mixture was stirred at room temperature for 10 min. A solution of mesylate **14** (24.93 g, 88.70 mmol) in dry EtOH (120 mL) was added to the mixture, and the mixture was stirred for 17 h at 40 °C. The mixture was evaporated, and the residue was poured into aq NH_4Cl and the mixture was extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated. The residue was chromatographed on silica gel (400 g, hexane/AcOEt = 7/3) to give **15** (28.05 g, 91.7%) as a colorless oil. ^{1}H NMR δ 1.27 (6 H, t, J = 7.1 Hz, $COOCH_2CH_3$ × 2), 1.2−1.6 (4 H, m, CH_2 × 2), 1.44 (9

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Scheme 4. Synthetic Scheme of Selenium-Containing Compounds 10−13

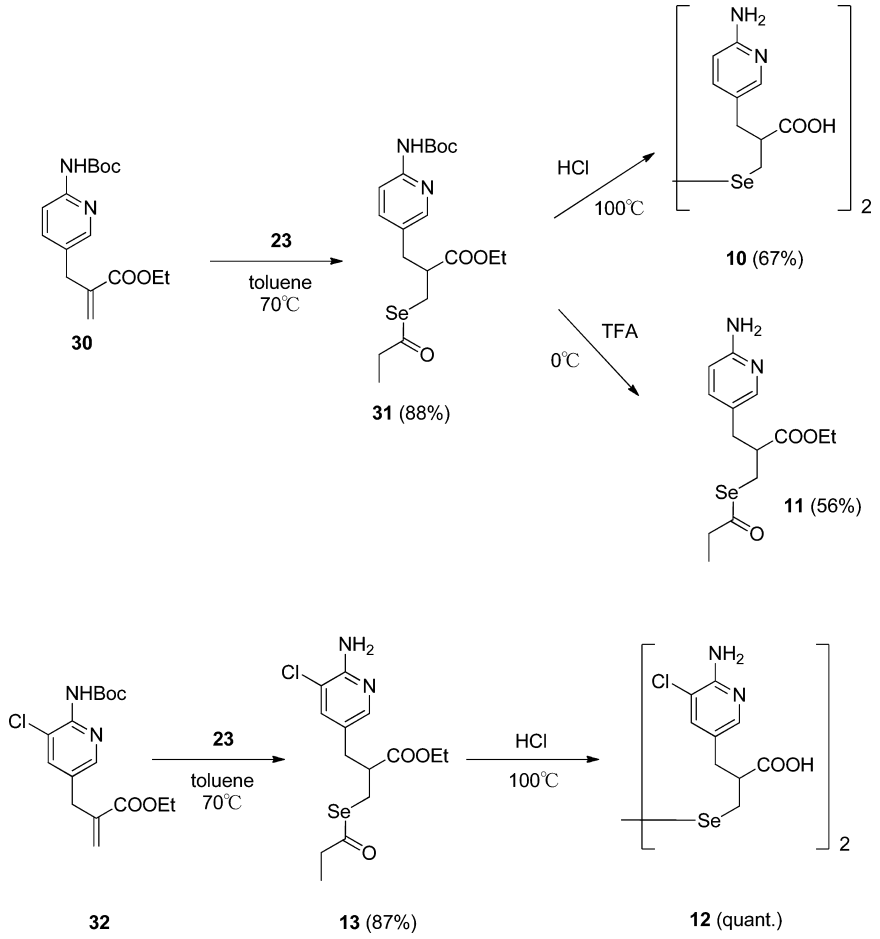

Table 1. Enzyme Inhibition Data on Compounds 1−4

	IC_{50} (M)		
compd	TAFIa	CPB	CPN
EF6265	5.5×10^{-9}	4.9×10^{-9}	6.7×10^{-6}
1; DD2	3.4×10^{-8}	3.6×10^{-8}	1.4×10^{-6}
2; DD3	3.1×10^{-4}	1.9×10^{-5}	$>3.8 \times 10^{-3}$
3; DD4	6.2×10^{-5}	2.6×10^{-4}	2.5×10^{-5}
4; DD5	3.8×10^{-6}	1.4×10^{-6}	1.6×10^{-4}

Table 2. Enzyme Inhibition Data on Compounds 5−13[a]

	IC_{50} (M)		
compd	TAFIa	CPB	CPN
5; DD9	2.0×10^{-9} ($>3.8 \times 10^{-3}$)	1.1×10^{-9} (1.1×10^{-4})	8.6×10^{-9} ($>3.8 \times 10^{-3}$)
6; DD10	3.0×10^{-8} (1.4×10^{-6})	3.3×10^{-8} (9.1×10^{-4})	1.6×10^{-7} (1.4×10^{-5})
7; DD12	1.1×10^{-9} (7.6×10^{-7})	1.1×10^{-9} (1.9×10^{-6})	1.8×10^{-8} (2.0×10^{-6})
8; DD19	4.1×10^{-9} (1.6×10^{-6})	3.2×10^{-9} (1.5×10^{-6})	1.6×10^{-8} (6.0×10^{-6})
9; DD20	5.3×10^{-9} (2.0×10^{-6})	3.2×10^{-9} (1.4×10^{-6})	1.4×10^{-8} (1.7×10^{-6})
10; DD22	2.2×10^{-12} (2.0×10^{-7})	1.9×10^{-10} (2.8×10^{-7})	2.9×10^{-10} (4.2×10^{-6})
11; DD23	8.9×10^{-7} (1.8×10^{-4})	2.5×10^{-6} ($>3.8 \times 10^{-3}$)	1.7×10^{-6} (1.5×10^{-5})
12; DD28	2.5×10^{-11} (3.3×10^{-6})	1.5×10^{-9} (3.7×10^{-4})	$>3.8 \times 10^{-3}$ ($>3.8 \times 10^{-3}$)
13; DD29	2.6×10^{-6} ($>3.8 \times 10^{-3}$)	1.3×10^{-4} ($>3.8 \times 10^{-3}$)	$>3.8 \times 10^{-3}$ ($>3.8 \times 10^{-3}$)

[a]Parentheses indicate IC_{50} values in the absence of DTT.

H, s, Boc), 1.65 (2 H, m, CH_2), 1.89 (2 H, m, CH_2), 3.10 (2 H, q, J = 6.5 Hz, CH_2NHBoc), 3.30 (1 H, t, J = 7.5 Hz, CH), 4.20 (4 H, q, J = 7.1 Hz, $COOCH_2CH_3 \times 2$), 4.51 (1 H, bs, NH). ^{13}C NMR δ 14.1 (2 carbons), 26.4, 27.0, 28.4 (3 carbons), 28.6, 29.8, 40.4, 51.9, 61.3 (2 carbons), 79.0, 155.9, 169.5 (2 carbons). LRMS: m/z 368.2 (MNa^+). HRMS calcd for $C_{17}H_{31}NNaO_6$ 368.2049, found 368.2082.

7-[(*tert*-Butoxycarbonyl)amino]-2-(ethoxycarbonyl)-heptanoic Acid (16). To a solution of alkyl malonate **15** (27.82 g, 80.63 mmol) in EtOH (100 mL) was added a solution of KOH (6.01 g, 107.08 mmol) in EtOH (140 mL), and the mixture was stirred at room temperature for 13 h. The mixture was concentrated under reduced pressure and the residue dissolved in water. The solution was washed with AcOEt, acidified by addition of 1N HCl, and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated. The residue was dried in vacuo to afford half ester **16** (24.82 g, 97.1%) as a colorless oil. ^{1}H NMR (CD_3OD) δ 1.26 (3 H, t, J = 7.2 Hz, $COOCH_2CH_3$), 1.34 (4 H, m, $CH_2 \times 2$), 1.43 (9 H, s, Boc), 1.46 (2 H, m, CH_2), 1.85 (2 H, m, CH_2), 3.01 (2 H, t, J = 6.8 Hz, CH_2NHBoc), 3.34 (1 H, m, CH), 4.18 (2 H, q, J = 7.2 Hz, $COOCH_2CH_3$). ^{13}C NMR (CD_3OD) δ 14.5, 27.7, 28.3, 28.9 (3 carbons), 30.0, 30.9, 41.4, 53.2, 62.5, 80.0, 158.7, 171.6, 173.0. LRMS: m/z 340.2 (MNa^+). HRMS calcd for $C_{15}H_{27}NNaO_6$ 340.1736, found 340.1746.

Ethyl 7-[(*tert*-Butoxycarbonyl)amino]-2-methylideneheptanoate (17). A solution of half ester **16** (24.03 g, 75.80 mmol), diethylamine (30 mL), and 36% formaldehyde (30 mL) was stirred at room temperature for 15 h. The mixture was poured into H_2O and extracted with AcOEt. The organic layer was dried over $MgSO_4$ and evaporated. The residue was chromatographed on silica gel (120 g,

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hexane/AcOEt = 9/1) to give **17** (16.00 g, 74.1%) as a colorless oil. ^{1}H NMR δ 1.30 (3 H, t, J = 7.1 Hz, COOCH$_2$C*H*$_3$), 1.31 (4 H, m, C*H*$_2$ × 2), 1.44 (9 H, s, Boc), 1.49 (2 H, m, C*H*$_2$), 2.30 (2 H, t, J = 7.1 Hz, C*H*$_2$), 3.11 (2 H, m, C*H*$_2$NHBoc), 4.20 (2 H, q, J = 7.1 Hz, COOC*H*$_2$CH$_3$), 4.52 (1 H, bs, N*H*), 5.51 and 6.13 (each 1 H, s, C═C*H*$_2$). ^{13}C NMR δ 14.2, 26.3, 28.1, 28.4 (3 carbons), 29.9, 31.7. 40.5, 60.6, 79.0, 124.4, 140.8, 155.9, 167.3. LRMS: m/z 308.2 (MNa$^+$). HRMS calcd for $C_{15}H_{28}NO_4$ 286.2018, found 286.2029.

Ethyl 2-[(Acetylsulfanyl)methyl]-7-[(*tert*-butoxycarbonyl)-amino]heptanoate (18). Triethylamine (320 μL, 2.30 mmol) was added to a solution of enoate **17** (542.7 mg, 1.90 mmol) in thioacetic acid (3 mL) at 0 °C, and the mixture was stirred at room temperature for 45 h. The reaction mixture was poured into satd $NaHCO_3$ and ice−water, and the mixture was extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated. The residue was chromatographed on silica gel (40 g, benzene/AcOEt = 19/1) to give **18** (525.8 mg, 76.5%) as a colorless oil. ^{1}H NMR δ 1.26 (3 H, t, J = 7.1 Hz, COOCH$_2$C*H*$_3$), 1.2−1.8 (8 H, m, C*H*$_2$ × 4), 1.44 (9 H, s, Boc), 2.33 (3 H, s, SAc), 2.56 (1 H, m, C*H*), 3.00 (1H, dd, J = 13.5, 8.6 Hz, C*H*$_2$SAc), 3.11 (2 H, m, C*H*$_2$NHBoc), 3.12 (1 H, dd, J = 13.5, 5.5 Hz, C*H*$_2$SAc), 4.15 (2 H, q, J = 7.1 Hz, COOC*H*$_2$CH$_3$), 4.52 (1 H, bs, N*H*). ^{13}C NMR δ 14.2, 26.5, 26.6, 28.4 (3 carbons), 29.8, 30.3, 30.5, 31.8, 40.4, 45.5, 60.6, 79.0, 155.9, 174.2, 195.4. LRMS: m/z 384.2 (MNa$^+$). HRMS calcd for $C_{17}H_{31}NNaO_5S$ 384.1821, found 384.1859.

7-Amino-2-(sulfanylmethyl)heptanoic Acid (1). A solution of thioacetate **18** (341.6 mg, 0.95 mmol) in conc HCl (1 mL) was stirred at 100 °C for 1 h. The mixture was allowed to cool to room temperature and evaporated. The residue was purified by MPLC (ODS-SM, 26 mm × 300 mm, 50 μm, 50% MeOH/Milli-Q + 0.2% TFA, 12 mL/min) to give target compound **1** as TFA salt (187.1 mg, 64.8%). ^{1}H NMR (D$_2$O) δ 1.39 (4 H, m, C*H*$_2$ × 2), 1.62 (4 H, m, C*H*$_2$ × 2), 2.65 (1 H, m, C*H*), 2.73 (2 H, m, C*H*$_2$SH) 2.98 (2 H, t, J = 7.6 Hz, C*H*$_2$NH$_2$). ^{13}C NMR δ 25.3, 25.7, 26.4, 29.2, 31.2, 39.3, 45.9, 179.2. LRMS: m/z 192.1 (MH$^+$). HRMS calcd for $C_8H_{18}NO_2S$ 192.1058, found 192.1090.

Ethyl 2-[(Acetylsulfanyl)methyl]-7-aminoheptanoate (2). A solution of **18** (29.5 mg, 0.082 mmol) in TFA/H$_2$O (19:1, 1 mL) was stirred at 0 °C for 1 h, and then the mixture was concentrated in vacuo to afford compound **2** as the TFA salt (33.1 mg, quantitative yield). ^{1}H NMR (CD$_3$OD) δ 1.25 (3 H, t, J = 7.1 Hz, COOCH$_2$C*H*$_3$), 1.40 (4 H, m, C*H*$_2$ × 2), 1.62 (4 H, m, C*H*$_2$ × 2), 2.31 (3 H, s, SAc), 2.56 (1 H, m, C*H*), 2.91 (2 H, t, J = 7.5 Hz, C*H*$_2$NH$_2$), 3.01 (1 H, dd, J = 13.6, 5.4 Hz, C*H*$_2$SAc), 4.14 (2 H, q, J = 7.1 Hz, COOC*H*$_2$CH$_3$). ^{13}C NMR δ 14.7, 27.2, 27.6, 28.4, 30.5, 31.3, 32.6, 40.7, 47.0, 62.0, 176.0, 197.0. LRMS: m/z 262.2 (MH$^+$). HRMS calcd for $C_{12}H_{24}NO_3S$ 262.1477, found 262.1521.

Ethyl 7-[(*tert*-Butoxycarbonyl)amino]-2-(sulfanylmethyl)-heptanoate (19). A solution of **18** (35.3 mg, 0.098 mmol) and Amano Lipase PS (immobilized on diatomite) (21.3 mg) in 20 mM NaOAc (pH 5.9, 1.5 mL) was stirred at 55 °C for 17 h. The reaction mixture was poured into water, and the mixture was extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated. The residue was chromatographed on silica gel (10 g, hexane/AcOEt = 3/1) to give **19** (23.8 mg, 76.2%) as a colorless oil. ^{1}H NMR δ 1.1−1.8 (8 H, m, C*H*$_2$ × 4), 1.26 (3 H, dt, J = 7.0, 1.1 Hz, COOCH$_2$C*H*$_3$), 1.42 (9 H, s, Boc), 2.60 (2 H, m, C*H*$_2$SH), 2.74 (1 H, m, C*H*), 3.08 (2 H, m, C*H*$_2$NHBoc), 4.16 (2 H, dq, J = 7.0, 1.1 Hz, COOC*H*$_2$CH$_3$), 4.54 (1 H, bs, N*H*). ^{13}C NMR δ 14.3, 26.0, 26.5, 26.7, 28.4 (3 carbons), 29.8, 31.3, 40.4, 49.2, 60.5, 79.0, 155.9, 174.2. LRMS: m/z 342.2 (MNa$^+$). HRMS calcd for $C_{15}H_{29}NNaO_4S$ 342.1715, found 342.1747.

Ethyl 7-Amino-2-(sulfanylmethyl)heptanoate (3). In a similar manner to that for the synthesis of **2** from **18**, target compound **3** was obtained as the TFA salt (23.6 mg) from **19** (22.1 mg, 0.069 mmol) in quantitative yield. ^{1}H NMR (CD$_3$OD) δ 1.26 (3 H, t, J = 7.2 Hz, COOCH$_2$C*H*$_3$), 1.38 (4 H, m, C*H*$_2$ × 2), 1.64 (4 H, m, C*H*$_2$ × 2), 2.56 (1 H, m, C*H*), 2.68 (2 H, m, C*H*$_2$SH), 2.91 (2 H, t, J = 7.6 Hz, C*H*$_2$NH$_2$), 4.17 (2 H, q, J = 7.2 Hz, COOC*H*$_2$CH$_3$). ^{13}C NMR (CD$_3$OD) δ 14.8, 26.7, 27.4, 27.8, 28.5, 32.4, 40.7, 50.7, 61.9, 176.2. LRMS: m/z 220.2 (MH$^+$). HRMS calcd for $C_{10}H_{22}NO_2S$ 220.1371, found 220.1414.

7-[(*tert*-Butoxycarbonyl)amino]-2-methylideneheptanoic Acid (20). A solution of enoate **17** (542.2 mg, 1.90 mmol) in 5% KOH/EtOH-H$_2$O (19:1, 5 mL) was stirred at 50 °C for 3.5 h. The mixture was evaporated, and the residue was dissolved in water. The mixture solution was washed with AcOEt, acidified by addition of satd citric acid, and extracted with AcOEt. The organic layer was dried over $MgSO_4$ and evaporated. The residue was chromatographed on silica gel (50 g, hexane/AcOEt = 1/1) to give **20** (482.2 mg, 98.8%) as a colorless oil. ^{1}H NMR (CD$_3$OD) δ 1.2−1.6 (6 H, m, C*H*$_2$ × 3), 1.43 (9 H, s, Boc), 2.29 (2 H, m, C*H*$_2$), 3.02 (2 H, t, J = 6.8 Hz, C*H*$_2$NHBoc), 5.57 and 6.12 (each 1 H, d, J = 1.3 Hz, C═C*H*$_2$). ^{13}C NMR (CD$_3$OD) δ 27.6, 28.9 (3 carbons), 30.0, 30.9, 33.0, 41.4, 79.9, 125.5, 142.8, 158.7, 170.7. LRMS: m/z 280.2 (MNa$^+$). HRMS calcd for $C_{13}H_{23}NNaO_4$ 280.1525, found 280.1552.

2-[(Acetylsulfanyl)methyl]-7-[(*tert*-butoxycarbonyl)amino]-heptanoic Acid (21). A solution of **20** (453.5 mg, 1.76 mmol), thioacetic acid (500 μL), and triethylamine (295 μL, 2.12 mmol) in dry CH_2Cl_2 (3 mL) was stirred at room temperature for 22 h. The reaction mixture was poured into ice−water and extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated. The residue was chromatographed on silica gel (70 g, hexane/AcOEt = 3/2) to give compound **21** (490.8 mg, 83.4%) as a colorless oil. ^{1}H NMR (CD$_3$OD) δ 1.2−1.6 (6 H, m, C*H*$_2$ × 3), 1.43 (9 H, s, Boc), 1.61 (2 H, mC*H*$_2$), 2.31 (3 H, s, SAc), 2.52 (1 H, m, C*H*), 2.99 (3 H, m, C*H*$_2$NHBoc and C*H*$_2$SAc), 3.11 (1 H, dd, J = 13.5, 5.5 Hz, C*H*$_2$SAc). ^{13}C NMR (CD$_3$OD) δ 27.8, 27.9, 28.9 (3 carbons), 30.6, 30.9, 31.4, 33.1, 41.4, 47.1, 80.0, 158.7, 178.1, 197.1. LRMS: m/z 356.2 (MNa$^+$). HRMS calcd for $C_{15}H_{27}NNaO_5S$ 356.1508, found 356.1550.

2-[(Acetylsulfanyl)methyl]-7-aminoheptanoic Acid (4). In a similar manner to that for the synthesis of **2** from **18**, target compound **4** was obtained as the TFA salt (33.6 mg) from **21** (31.2 mg, 0.094 mmol) in quantitative yield.. ^{1}H NMR (CD$_3$OD) δ 1.42 (4 H, m, C*H*$_2$ × 2), 1.66 (4 H, m, C*H*$_2$ × 2), 2.31 (3 H, s, SAc), 2.54 (1 H, m, C*H*), 2.92 (2 H, t, J = 7.5 Hz, C*H*$_2$NH$_2$), 3.00 (1 H, dd, J = 13.6, 8.5 Hz, C*H*$_2$SAc), 3.12 (1 H, dd, J = 13.6, 5.5 Hz, C*H*$_2$SAc). ^{13}C NMR (CD$_3$OD) δ 27.3, 27.6, 28.4, 30.6, 31.3, 32.7, 40.7, 46.9, 180.0, 197.2. LRMS: m/z 234.1 (MH$^+$). HRMS calcd for $C_{10}H_{20}NO_3S$ 234.1164, found 234.1206.

Ethyl 7-[(*tert*-Butoxycarbonyl)amino]-2-[(propanoylselanyl)-methyl]heptanoate (24). A heterogeneous mixture of Woollins's reagent **22** (225.8 mg, 0.42 mmol) and propionic acid (190 μL, 2.55 mmol) in dry toluene (1 mL) was refluxed for 2 h. The resulting yellow solution containing the selenopropionic acid **23** was cooled to room temperature, and then to this solution methylene compound **17** (240.6 mg, 0.84 mmol) in dry toluene (1 mL) was added. The mixture was stirred at 70 °C for 41 h. The reaction mixture was allowed to cool to room temperature and chromatographed on silica gel (30 g, hexane/AcOEt = 3/1) to give selenoester **24** (114.5 mg, 32.1%) as a colorless oil. ^{1}H NMR δ 1.17 (3 H, t, J = 7.5 Hz, SeCOCH$_2$C*H*$_3$), 1.26 (3 H, t, J = 7.1 Hz, COOCH$_2$C*H*$_3$), 1.2−1.8 (8 H, m, C*H*$_2$ × 4), 1.44 (9 H, s, Boc), 2.63 (3 H, m, C*H* and SeCOC*H*$_2$CH$_3$), 3.08 (4 H, m, C*H*$_2$NHBoc and C*H*$_2$Se), 4.14 (2 H, q, J = 7.1 Hz, COOC*H*$_2$CH$_3$), 4.53 (1 H, bs, N*H*). ^{13}C NMR (CDCl$_3$) δ 9.7, 14.5, 26.3, 26.8, 26.9, 28.6 (3 carbons), 30.1, 33.0, 40.7, 41.7, 46.5, 60.8, 79.3, 156.2, 174.9, 202.6. LRMS: m/z 446.1 (MNa$^+$). HRMS calcd for $C_{18}H_{33}NNaO_5{}^{80}Se$ 446.1422, found 446.1419.

2,2′-(Diselane-1,2-diyldimethanediyl)bis(7-aminoheptanoic Acid) (5). In a similar manner to that for the synthesis of **1** from **18**, target compound **5** was obtained as the HCl salt from **24** (36.9 mg, 0.087 mmol). Purification was performed with ion exchange chromatography (adande:l PCX5006) using methanol/28% ammonia (1:1) as an elution solvent. To the eluent containing target compound was added 4 M HCl (495 μL), and the mixture was dried in vacuo to give **5** as the HCl salt (21.1 mg, 44.1%). ^{1}H NMR (D$_2$O) δ 1.34 (8 H, m, C*H*$_2$ × 4), 1.61 (8 H, m, C*H*$_2$ × 4), 2.80 (2 H, m, C*H* × 2), 2.93 (4 H, t, J = 7.5 Hz, C*H*$_2$NH$_2$ × 2), 3.08 (4 H, dd, J = 7.2, 2.5 Hz, C*H*$_2$Se × 2). ^{13}C NMR (D$_2$O) δ 25.3 (2 carbons), 25.7 (2 carbons), 26.4 (2

carbons), 30.2, 30.3, 31.65, 31.74, 39.4 (2 carbons), 46.7 (2 carbons), 179.4 (2 carbons). LRMS: m/z 477.1 (MH^+). HRMS calcd for $C_{16}H_{33}N_2O_4{}^{80}Se_2$ 477.0771, found 477.0764.

Ethyl 7-Amino-2-[(propanoylselanyl)methyl]heptanoate (6). In a similar manner to that for the synthesis of **2** from **18**, target compound **6** was obtained as the TFA salt (19.9 mg) from **24** (19.2 mg, 0.045 mmol) in quantitative yield. ^{1}H NMR (CD_3OD) δ 1.13 (3 H, t, J = 7.5 Hz, $SeCOCH_2CH_3$), 1.24 (3 H, t, J = 7.1 Hz, $COOCH_2CH_3$), 1.40 (4 H, m, $CH_2 \times 2$), 1.65 (4 H, m, $CH_2 \times 2$), 2.63 (1 H, m, CH), 2.65 (2 H, q, J = 7.5 Hz, $SeCOCH_2CH_3$), 2.91 (2 H, t, J = 7.5 Hz, CH_2NH_2), 3.07 (2 H, m, CH_2Se), 4.13 (2 H, q, J = 7.1 Hz, $COOCH_2CH_3$). ^{13}C NMR ($CDCl_3$) δ 9.9, 14.7, 26.8, 27.3, 27.8, 28.5, 33.6, 40.8, 42.4, 47.7, 62.0, 176.3, 203.6. LRMS: m/z 324.1 (MH^+). HRMS calcd for $C_{13}H_{26}NO_3{}^{80}Se$ 324.1078, found 324.1084.

7-[(*tert*-Butoxycarbonyl)amino]-2-[(propanoylselanyl)-methyl]heptanoic Acid (25). In a similar manner to that for the synthesis of **24** from **17**, target compound **25** (235.3 mg) was obtained from **20** (346.3 mg, 1.35 mmol) in 44.3% yield. ^{1}H NMR δ 1.18 (3 H, t, J = 7.5 Hz, $SeCOCH_2CH_3$), 1.2−1.8 (8 H, m, $CH_2 \times 4$), 1.44 (9 H, s, Boc), 2.64 (3 H, m, CH, $SeCOCH_2CH_3$), 3.09 (4 H, m, CH_2NHBoc, CH_2Se), 4.60 (1 H, bs, NH). ^{13}C NMR δ 9.7, 25.9, 26.7, 26.8, 28.6 (3 cabons), 30.0, 32.8, 40.6, 41.7, 46.3, 79.4, 156.3, 179.6, 202.6. LRMS m/z 418.1 (MNa^+). HRMS calcd for $C_{16}H_{29}NNaO_5{}^{80}Se$ 418.1109, found 418.1088.

7-Amino-2-[(propanoylselanyl)methyl]heptanoic Acid (7). In a similar manner to that for the synthesis of **2** from **18**, target compound **7** was obtained as the TFA salt (25.3 mg) from **25** (24.3 mg, 0.062 mmol) in quantitative yield. ^{1}H NMR (CD_3OD) δ 1.14 (3 H, t, J = 7.5 Hz, $SeCOCH_2CH_3$), 1.42 (4 H, m, $CH_2 \times 2$), 1.66 (4 H, m, $CH_2 \times 2$), 2.58 (1 H, m, CH), 2.65 (2 H, q, J = 7.5 Hz, $SeCOCH_2CH_3$), 2.92 (2 H, t, J = 7.5 Hz, CH_2NH_2), 3.06 (2 H, m, CH_2Se). ^{13}C NMR (CD_3OD) δ 9.8, 26.6, 27.1, 27.6, 28.3, 33.4, 40.6, 42.2, 47.4, 178.2, 203.7. LRMS: m/z 296.0 (MH^+). HRMS calcd for $C_{11}H_{22}NO_3{}^{80}Se$ 296.0765, found 296.0754.

7-[(*tert*-Butoxycarbonyl)amino]-2-{[(4-phenylbutanoyl)-selanyl]methyl}heptanoic Acid (27). In a similar manner to that for the preparation of selenopropionic acid **23**, selenocarboxylic acid **26** was prepared. To the solution containing **26**, compound **20** (153.6 mg, 0.60 mmol) in dry toluene (1 mL) was added and the mixture was stirred at 70 °C for 18 h. The reaction mixture was allowed to cool to room temperature and chromatographed on silica gel (15 g, hexane/AcOEt = 7/3) to give compound **27** (135.7 mg, 46.9%) as a colorless oil. ^{1}H NMR (CD_3OD) δ 1.2−1.8 (8 H, m, $CH_2 \times 4$), 1.42 (9 H, s, Boc), 1.95 (2 H, m, CH_2CH_2Ph), 2.56 (1 H, m, CH), 2.63 (4 H, m, CH_2Ph, CH_2CO), 3.03 (4 H, m, CH_2NH, CH_2Se), 7.16 (3 H, m, Ph-H), 7.26 (2 H, m, Ph-H). ^{13}C NMR (CD_3OD) δ 27.1, 27.8, 28.0, 28.5, 29.0 (3 carbons), 30.9, 33.9, 35.8, 41.4, 47.6, 48.2, 80.0, 127.2, 129.6 (2 carbons), 130.0 (2 carbons), 142.7, 158.7, 178.4, 202.8. LRMS: m/z 508.2 (MNa^+). HRMS calcd for $C_{23}H_{35}NNaO_5{}^{80}Se$ 508.1578, found 508.1578.

7-Amino-2-{[(4-phenylbutanoyl)selanyl]methyl}heptanoic Acid (8). In a similar manner to that for the synthesis of **2** from **18**, target compound **8** was obtained as the TFA salt (19.3 mg) from **27** (17.2 mg, 0.036 mmol) in quantitative yield. ^{1}H NMR (CD_3OD) δ 1.42 (4 H, m, $CH_2 \times 2$), 1.66 (4 H, m, $CH_2 \times 2$), 1.94 (2 H, m, CH_2CH_2Ph), 2.58 (1 H, m, CH), 2.64 (4 H, m, CH_2Ph, CH_2CO), 2.91 (2 H, m, CH_2NH_2), 3.07 (2 H, m, CH_2Se), 7.16 (3 H, m, Ph-H), 7.26 (2 H, m, Ph-H). ^{13}C NMR (CD_3OD) δ 26.8, 27.2, 27.6, 28.3, 28.4, 33.4, 35.7, 40.6, 47.4, 48.0, 127.1, 129.45 (2 carbons), 129.49 (2 carbons), 142.5, 178.1, 202.8. LRMS: m/z 386.1 (MH^+). HRMS calcd for $C_{18}H_{28}NO_3{}^{80}Se$ 386.1234, found 386.1259.

7-[(*tert*-Butoxycarbonyl)amino]-2-{[(3-phenylpropanoyl)-selanyl]methyl}heptanoic Acid (29). In a similar manner to that for the preparation of selenopropionic acid **23**, selenocarboxylic acid **28** was prepared from hydrocinnamic acid and Woollins's reagent. Using selenocarboxylic acid **28**, target compound **29** (108.4 mg) was obtained from **20** (158.1 mg, 0.61 mmol) in 37.5% yield. ^{1}H NMR (CD_3OD) δ 1.2−1.8 (8 H, m, $CH_2 \times 4$), 1.43 (9 H, s, Boc), 2.56 (1 H, m, CH), 2.94 (4 H, m, CH_2Ph, CH_2CO), 3.03 (4 H, m, CH_2NHBoc, CH_2Se), 7.17 (3 H, m, Ph-H), 7.25 (2 H, m, Ph-H). ^{13}C NMR (CD_3OD) δ 27.1, 27.8, 27.9, 29.0 (3 carbons), 30.9, 32.4, 33.8, 41.4, 47.5, 50.4, 80.0, 127.5, 129.5 (2 carbons), 129.6 (2 carbons), 141.3, 158.6, 178.3, 202.1. LRMS: m/z 494.2 (MNa^+). HRMS calcd for $C_{22}H_{33}NNaO_5{}^{80}Se$ 494.1422, found 494.1433.

7-Amino-2-{[(3-phenylpropanoyl)selanyl]methyl}heptanoic Acid (9). In a similar manner to that for the synthesis of **2** from **18**, target compound **9** was obtained as the TFA salt (29.4 mg) from **29** (27.1 mg, 0.058 mmol) in quantitative yield. ^{1}H NMR (CD_3OD) δ 1.39 (4 H, m, $CH_2 \times 2$), 1.60 (4 H, m, $CH_2 \times 2$), 2.59 (1 H, m, CH), 2.92 (2 H, m, CH_2NH_2), 2.95 (4 H, m, CH_2Ph, CH_2CO), 3.06 (2 H, m, CH_2Se), 7.17 (3 H, m, Ph-H), 7.26 (2 H, m, Ph-H). ^{13}C NMR (CD_3OD) δ 26.8, 27.1, 27.6, 28.3, 32.3, 33.4, 40.6, 47.3, 50.3, 127.4, 129.4 (2 carbons), 129.5 (2 carbons), 141.2, 178.1, 202.1. LRMS: m/z 372.1 (MH^+). HRMS calcd for $C_{17}H_{26}NO_3{}^{80}Se$ 372.1078, found 372.1110.

Ethyl 3-{6-[(*tert*-Butoxycarbonyl)amino]pyridin-3-yl}-2-[(propanoylselanyl)methyl]propanoate (31). Compound **30** (150.7 mg, 0.49 mmol) was subjected to selenylation reaction in a similar manner to that for the synthesis of **24** from **17**. The reaction mixture was allowed to cool to room temperature and chromatographed on silica gel [25 g, CH_2Cl_2/28% ammonia solution−MeOH (1:9) = 39/1] to give compound **31** (192.6 mg, 88.3%) as a white solid. ^{1}H NMR δ 1.16 (3 H, t, J = 7.1 Hz, $COOCH_2CH_3$), 1.17 (3 H, t, J = 7.5 Hz, $COCH_2CH_3$), 1.53 (9 H, s, Boc), 2.63 (2 H, q, J = 7.5 Hz, $COCH_2CH_3$), 2.80 (1 H, m, CH), 2.91 (2 H, m, Py-CH_2), 3.07 (2 H, d, J = 6.2 Hz, CH_2Se), 4.07 (2 H, q, J = 7.1 Hz, $COOCH_2CH_3$), 7.48 (1 H, dd, J = 8.6, 2.3 Hz, Py-4), 7.88 (1 H, d, J = 8.6 Hz, Py-5), 8.02 (1 H, bs, NH), 8.08 (1 H, d, J = 2.3 Hz, Py-2). ^{13}C NMR δ 9.7, 14.4, 26.0, 28.6 (3 carbons), 35.5, 41.7, 48.0, 61.1, 81.1, 112.2, 128.4, 138.9, 148.2, 151.1, 152.7, 173.7, 202.0. LRMS: m/z 445.1 (MH^+). HRMS calcd for $C_{19}H_{29}N_2O_5{}^{80}Se_1$ 445.1242, found 445.1251.

2,2′-(Diselane-1,2-diyldimethanediyl)bis[3-(6-aminopyridin-3-yl)propanoic Acid] (10). A solution of compound **31** (24.3 mg, 0.055 mmol) in conc. HCl (1 mL) was stirred at 100 °C for 1 h. The reaction mixture was evaporated. The residue was purified by ion exchange chromatography (OASIS MCX) using methanol/28% ammonia (13:1) as an elution solvent. To the eluent containing target compound was added 4 M HCl (900 μL), and the mixture was dried in vacuo to give **10** as the HCl salt (10.8 mg, 66.7%). ^{1}H NMR (CD_3OD) δ 2.90 (4 H, d, J = 6.7 Hz, Py-$CH_2 \times 2$), 3.13 (6 H, m, $CH \times 2$, $CH_2Se \times 2$), 7.01 (2 H, d, J = 9.1 Hz, Py-4 × 2), 7.71 (2 H, s, Py-5 × 2), 7.87 (2 H, d, J = 9.1 Hz, Py-2 × 2). ^{13}C NMR (CD_3OD) δ 31.5, 31.6, 34.5, 34.6, 48.8, 48.9, 114.9 (2 carbons), 125.0 (2 carbons), 135.1 (2 carbons), 147.0 (2 carbons), 154.7 (2 carbons), 176.2 (2 carbons). LRMS: m/z 519.0 (MH^+). HRMS calcd for $C_{18}H_{23}N_4O_4{}^{80}Se_2$ 519.0050, found 519.0060.

Ethyl 3-(6-Aminopyridin-3-yl)-2-[(propanoylselanyl)methyl]-propanoate (11). A solution of compound **31** (21.6 mg, 0.049 mmol) in TFA/H_2O (19:1, 1 mL) was stirred at 0 °C for 1 h. The reaction mixture was evaporated, to which H_2O was added. The aqueous solution was washed with hexane and concentrated in vacuo to afford **11** as the TFA salt (12.5 mg, 56.1%). ^{1}H NMR (CD_3OD) δ 1.14 (3 H, t, J = 7.4 Hz, $COCH_2CH_3$), 1.19 (3 H, t, J = 7.1 Hz, $COOCH_2CH_3$), 2.66 (2 H, q, J = 7.4 Hz, $COCH_2CH_3$), 2.79 (1 H, m, CH), 2.82 and 2.90 (each 1 H, d, J = 6.1 Hz, Py-CH_2), 3.10 (2 H, m, CH_2Se), 4.09 (2 H, q, J = 7.1 Hz, $COOCH_2CH_3$), 6.98 (1 H, d, J = 9.1 Hz, Py-5), 7.67 (1 H, d, J = 2.1 Hz, Py-2), 7.84 (1 H, dd, J = 9.1, 2.1 Hz, Py-4). ^{13}C NMR (CD_3OD) δ 9.7, 14.5, 26.1, 34.7, 42.2, 48.2, 62.1, 114.8, 124.8, 135.3, 147.0, 154.9, 174.6, 203.1. LRMS: m/z 345.1 (MH^+). HRMS calcd for $C_{14}H_{21}N_2O_3{}^{78}Se$ 343.0725, found 343.0754.

Ethyl 3-(6-Amino-5-chloropyridin-3-yl)-2-[(propanoylselanyl)methyl]propanoate (13). Compound **32** (88.4 mg, 0.26 mmol) was subjected to selenylation reaction in a similar manner to that for the synthesis of **24** from **17**. The mixture was allowed to cool to room temperature and chromatographed on silica gel [30 g, CH_2Cl_2/28% ammonia solution−MeOH (1:9) = 99/1] to give compound **13** (84.9 mg, 86.5%) as a colorless oil. ^{1}H NMR δ 1.18 (3 H, t, J = 7.5 Hz, $COCH_2CH_3$), 1.19 (3 H, t, J = 7.1 Hz, $COOCH_2CH_3$), 2.65 (2 H, q, J = 7.5 Hz, $COCH_2CH_3$), 2.78 (1 H, m, CH), 2.86 (2 H, m, Py-CH_2), 3.07 (2 H, d, J = 6.1 Hz, CH_2Se), 4.09 (2

H, q, J = 7.1 Hz, $COOCH_2CH_3$), 4.78 (2 H, bs, N*H*), 7.35 (1 H, d, J = 2.0 Hz, Py-4), 7.79 (1 H, d, J = 2.0 Hz, Py-2). ^{13}C NMR δ 9.4, 14.2, 25.7, 34.6, 41.5, 47.8, 60.9, 114.8, 124.9, 137.5, 146.3, 153.5, 173.4, 201.8. LRMS: m/z 379.0 (MH^+). HRMS calcd for $C_{14}H_{20}ClN_2O_3{}^{80}Se$ 379.0328, found 379.0331.

2,2′-(Diselane-1,2-diyldimethanediyl)bis[3-(6-amino-5-chloropyridin-3-yl)propanoic Acid] (12). In a similar manner to that for the synthesis of **5** from **24** followed by the purification, target compound **12** was obtained as the HCl salt (30.0 mg, quantitative yield) from **13** (32.6 mg, 0.086 mmol). ^1H NMR (CD_3OD) δ 2.94 (4 H, d, J = 7.0 Hz, $CH_2CH \times 2$), 3.09 (2 H, m, $CH \times 2$), 3.19 (4 H, m, $CH_2Se \times 2$), 7.81 (2 H, s, Py-4 × 2), 8.14 (2 H, d, J = 1.7 Hz, Py-2 × 2). ^{13}C NMR (CD_3OD) δ 31.6, 31.7, 34.37, 34.41, 48.71, 48.74, 119.8 (2 carbons), 125.8 (2 carbons), 134.6 (2 carbons), 145.9 (2 carbons), 152.0 (2 carbons), 176.06, 176.08. LRMS: m/z 586.9 (MH^+). HRMS calcd for $C_{18}H_{20}\ Cl_2N_4O_4{}^{78}Se^{80}Se$ 583.9200, found 583.9239.

TAFIa Inhibition. TAFI was purified from normal human plasma using plasminogen-depleted plasma as described previously.[33] Thrombomodulin (TM) was purchased from American Diagnostica, (Greenwich, CT, USA). Human plasma thrombin, hippuryl-arginine (Hip-Arg), and hippuryl-lysine (Hip-Lys) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Porcine pancreas carboxypeptidase B (ppCPB) was purchased from Worthington Biochemical (Freehold, NJ, USA). Human plasma carboxypeptidase N (CPN) was purchased from Elastin Products (St. Louis, MO, USA).

To activate TAFI, TAFI from human plasma (4.3 μg/mL TAFI, 20 μL) was mixed with 20 μL of 300 ng/mL TM in buffer A (50 mM Tris-HCl buffer, pH 7.4, supplemented with 0.1% BSA and 0.15 M sodium chloride) and incubated at 25 °C for 3 min. The mixture was additionally mixed with 20 μL of 3 U/mL thrombin in buffer A and TAFI in the mixture was activated by incubation at 25 °C for 30 min. A part of prepared TAFIa solution (25 μL) was transferred into another tube and incubated with 25 μL of 3.2 mM Hip-Arg substrate in buffer B (0.1 M Tris-HCl buffer containing or not containing a final concentration of 10 μM DTT, pH 7.6) at 25 °C for 30 min (the final volume was 80 μL). The reaction was stopped by adding 100 μL of 0.2 M PIPES−NaOH buffer, pH 7.6, containing 12.5% Tween 20. Color was developed by adding 100 μL of 1% cyanuric acid in 2-methoxyethanol, followed by measurement of absorbance at 405 nm.[34]

For determination of inhibitor activities of various compounds against TAFIa and ppCPB activity, 25 μL of 20 U/L of TAFIa or ppCPB was transferred into other tubes and then incubated with 30 μL of various concentrations of compounds at 25 °C for 10 min. TAFIa and ppCPB activities were measured by same method as described above. In measurement of inhibitor activity of various compounds against CPN, 50 U/L CPN was transferred into other tubes and incubated with 30 μL of various concentrations of inhibitors at 37 °C for 10 min. The mixture was incubated with 25 μL of 3.2 mM Hip-Lys substrate at 37 °C for 30 min in buffer B. Stopping of the reaction, development of color, and measurement of absorbance were same as described above. IC_{50} (50% inhibitory concentration) values were calculated from the sigmoidal inhibition curves by using Microsoft Excel software.

AUTHOR INFORMATION

Corresponding Author

*Phone: +81 42 721 1580. Fax: +81 42 721 1580. E-mail: yamamoto@ac.shoyaku.ac.jp.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated thrombin-activatable fibrinolysis inhibitor; t-PA, tissue plasminogen activator; CPN, carboxypeptidase N; CPB, carboxypeptidase B; TFA, trifluoroacetic acid; DTT, dithiothreitol

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