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Article

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Macrocyclic Prodrugs of a Selective Non-peptidic Direct Thrombin Inhibitor Display High Permeability, Efficient Bioconversion but Low Bioavailability

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Abstract

The only oral direct thrombin inhibitors that have reached the market, ximelagatran and dabigatran etexilat, are double prodrugs with low bioavailability in humans. We have evaluated an alternative strategy - the preparation of a non-peptidic, polar direct thrombin inhibitor as a single, macrocyclic esterase-cleavable (acyloxy)alkoxy prodrug. Two homologous prodrugs were synthesized and displayed high solubilities and Caco-2 cell permeabilities, suggesting high absorption from the intestine. In addition, they were rapidly and completely converted to the active zwitterionic thrombin inhibitor in human hepatocytes.

Unexpectedly, the most promising prodrug displayed only moderately higher oral bioavailability in rat than the polar direct thrombin inhibitor, most likely due to rapid metabolism in the intestine or the intestinal wall. To the best of our knowledge, this is the first *in vivo* ADME study of macrocyclic (acyloxy)alkoxy prodrugs, and it remains to be established if the modest increase in bioavailability is a general feature of this category of prodrugs or not.

Introduction

Plasma coagulation, activated during vascular damage, aims at reducing blood loss. It is a cascade activation of trypsin-like serine proteases with delicate control due to feed-back loops, co-factors and inhibitors. In the final step of the cascade thrombin (coagulation factor IIa) cleaves fibrinogen to fibrin, which stabilizes the clot during the wound healing process. In addition, platelet aggregation helps plasma coagulation and fibrinolysis counteracts it. Plasma coagulation can also be activated in diseases like venous thrombosis, pulmonary embolism, stroke and myocardial infarction and oral anticoagulants are an important group of pharmaceuticals used in the prevention and treatment of these diseases. For many years vitamin K antagonists such as warfarin were the only oral anticoagulants, but recently direct thrombin inhibitors (DTIs) and direct coagulation factor Xa inhibitors have been approved.[1, 2] The two oral DTIs that have reached the market, ximelagatran[3] and dabigatran etexilate,[4] both have hydrophilic groups at the ends of the molecules; a benzamidine in the P1 position and a carboxylic acid attached to the P3 group. The result of this zwitterionic character is poor absorption from the gastrointestinal tract. Both DTIs are therefore prepared as double-prodrugs with a hydroxyl group or an O-*n*-hexylcarbamate on the amidine, while the acid is protected as an ethyl ester. Still, oral bioavailability in humans is in the range of 20 and 6 %

for ximelagatran and dabigatran etexilate, respectively.[1] Moreover, double-prodrugs are complicated to develop since it often happens that one of the prodrug arms is metabolized, but not both.[3] Another liability common to ximelagatran and dabigatran etexilate is their low selectivity against trypsin,[5, 6] which may result in GI-side effects.

In the search for novel DTIs with improved properties we developed a series of potent inhibitors based on a P2 dihydropyrid-2-one scaffold, designed from the unique 5,6-dihydroxyoctahydroindole-2-carboxylic acid core present in naturally occurring thrombin inhibitors like chlorodysinosin.[7] The dihydropyrid-2-ones were designed to bind in the P1-P3 pockets of thrombin and had low nano-molar potency against thrombin and high selectivity against trypsin. However, highly potent inhibitors of human thrombin such as **1** (Figure 1) had liabilities such as high metabolism in rat liver microsomes and some inhibition of CYP 3A4 (cf. Table 1).[8] We reasoned that introduction of a carboxylic acid in the meta position of the P3 moiety, which points towards solution in models of **1** and thrombin, would decrease lipophilicity and thus decrease both metabolism and CYP 3A4 inhibition, but zwitterions such as **2** require conversion into prodrugs to improve bioavailability. To avoid complications with double-prodrugs we decided to investigate if a macrocyclic prodrug that was transformed to an active DTI in only one enzymatic reaction could be an alternative and advantageous approach. The esterase-sensitive[9] (acyloxy)alkoxy linker has been developed for transformation of linear peptides into cyclic prodrugs, that have shown promising ADME properties *in vitro*, and was therefore chosen for use in our study (cf. **3** and **4**, Figure 1).[10-15] We were also interested in investigating the physicochemical properties and cell permeability of the macrocyclic prodrugs as compared to a linear analogue. Herein we describe the synthesis of zwitterionic direct thrombin inhibitor **2**, the corresponding macrocyclic prodrugs

3 and **4**, and acyclic analogue **5** (Figure 1). We also report the potency, serine protease selectivity as well as *in vitro* and *in vivo* ADME properties of these compounds.

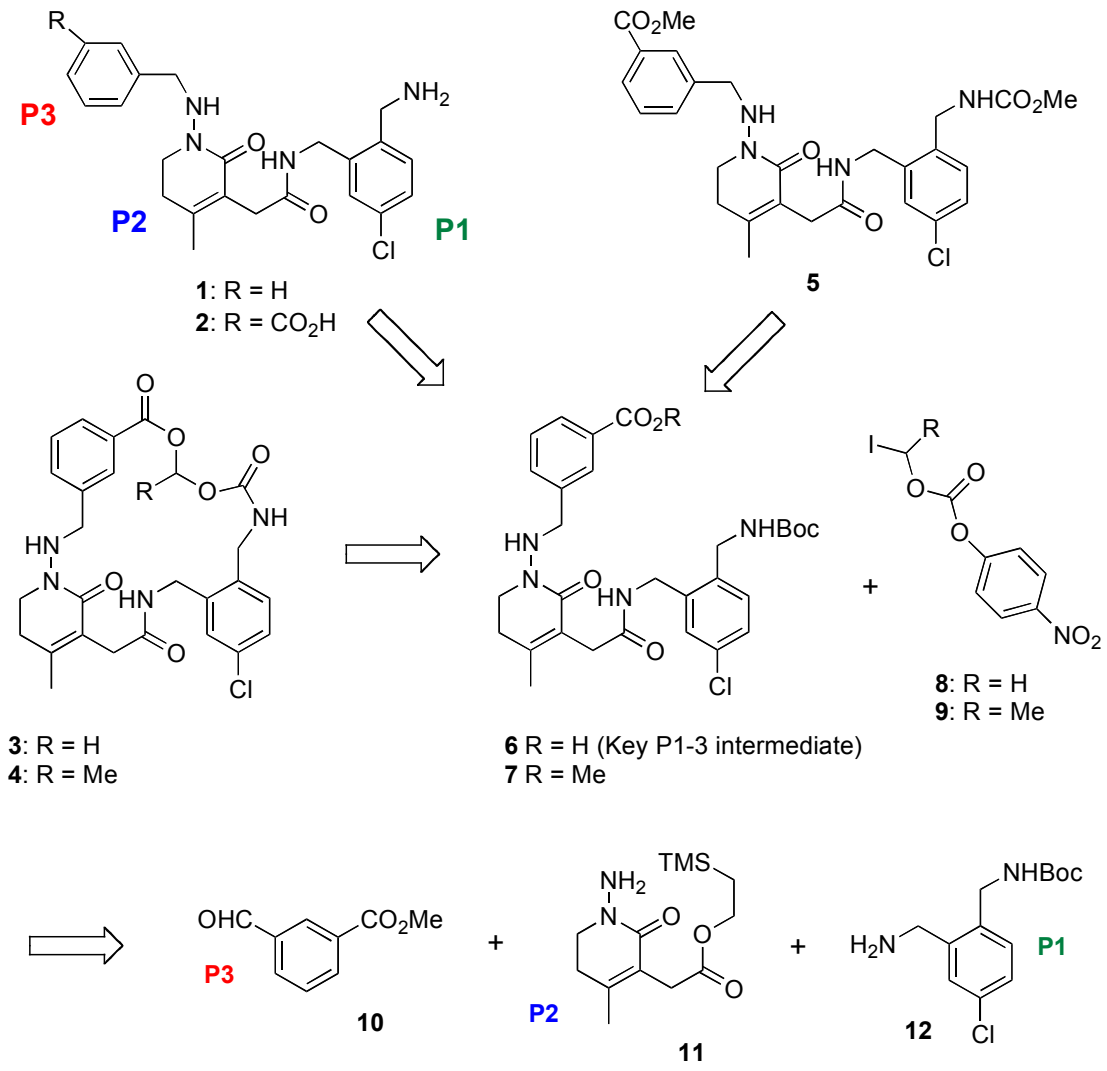


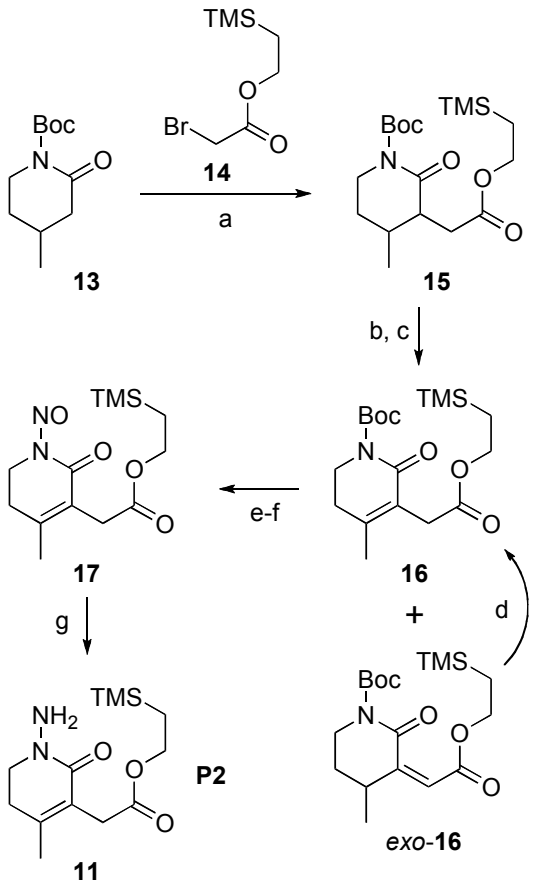
Figure 1. Structures of direct thrombin inhibitors **1** and **2**, and a retrosynthetic analysis of **2**, macrocyclic prodrugs **3** and **4**, and acyclic analogue **5.**

Results and Discussion

Retrosynthesis. We envisaged that macrocyclic prodrugs **3** and **4** could be synthesized from key P1-3 intermediate **6** and (acyloxy)alkoxy synthetic equivalents **8** and **9** through a one-pot cyclisation procedure (Figure 1). Thus, the carboxylic acid of **6** would first be alkylated with **8** and **9** while having the P3-amine protected with a Boc group. Cyclization would then be achieved by deprotection of the amine, basification and an intramolecular attack to form macrocycles **3** and **4**. Direct thrombin inhibitor **2** would be obtained by deprotection of **6**, while the acyclic analogue **5** of the two prodrugs would be obtained by exchange of the protective group on the P3-amine of **7**. Key intermediate **6** could, in turn, be prepared from P1-3 fragments **10**-**12.**

Synthetic chemistry. P3 fragment **10** is commercially available, whereas P1 fragment **12** was synthesized as previously reported.[16] P2 fragment **11** was prepared via a route similar to that developed for the corresponding ethyl ester.[7] This started from *N*-Boc-protected piperidone **13**, which was prepared using a three-step process starting from 4-methylpiperidine (Scheme 1).[17] Alkylation of **13** with **14** afforded TMS ester **15**, in 85 % yield. The dihydropyrid-2-one functionality was obtained by a Sharpless selenoxide elimination[18] involving step-wise α-addition with PhSeBr followed by an oxidative elimination with hydrogen peroxide. Initial attempts gave **16** with a poor regioisomeric distribution (1:4 *endo*:*exo*) and consequently the yield of desired product **16** was low. However, the unwanted *exo*-cyclic product could be rearranged with DBU in toluene at reflux to give **16** in 45 % yield, over three steps. Compound **16** was subsequently *N*-Boc deprotected with TFA and then reacted with *tert*-butyl nitrite to form the *N*-nitroso compound **17** in 89 % yield. The *N*-nitroso group was then reduced with Zn to give the hydrazidic P2 fragment **11** in 65 % yield.

Scheme 1. Synthesis of P2 fragment **11**[a]

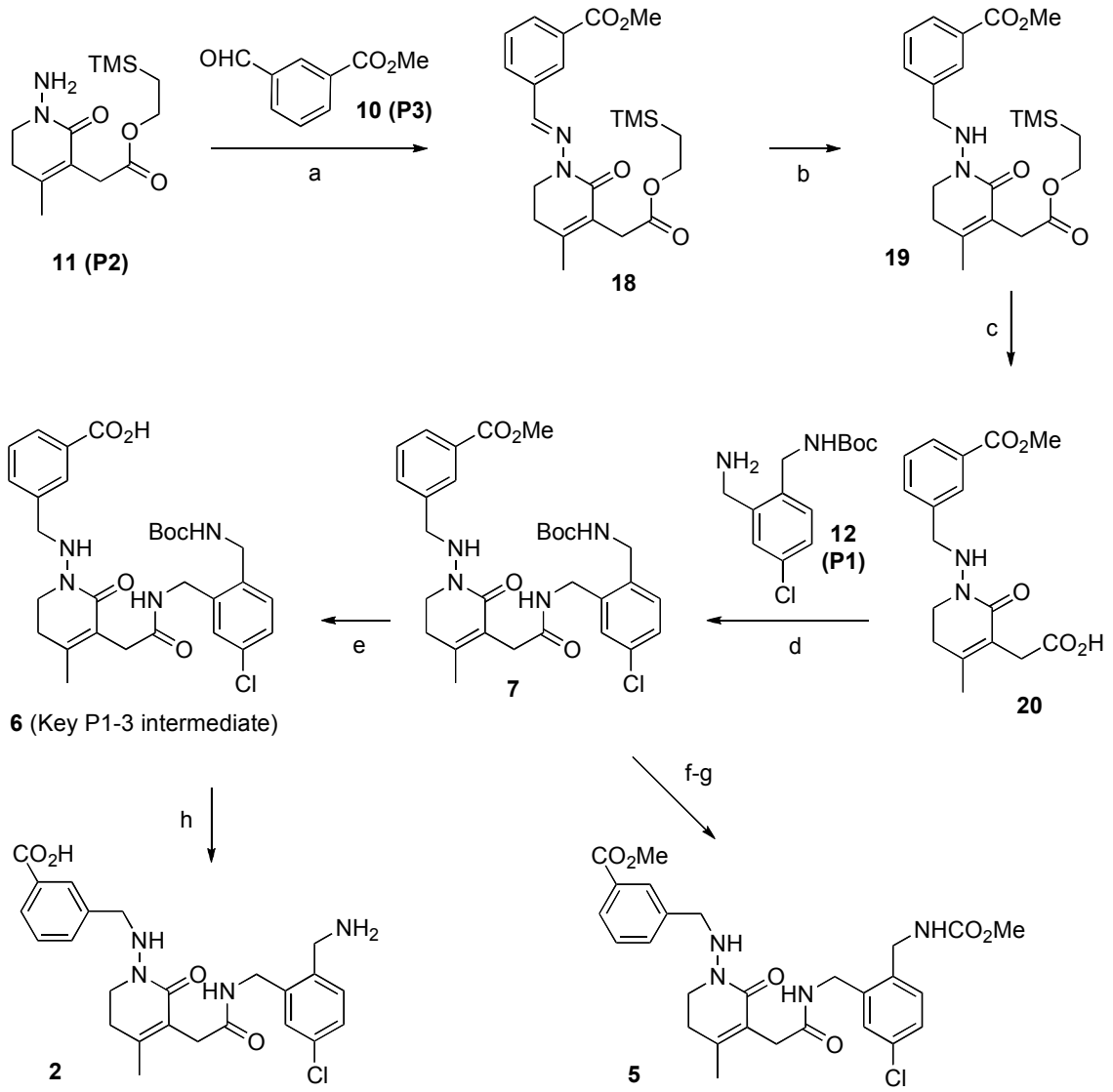


[a]Reagents and conditions: (a) **14**, LHMDS, THF, -78 to -20 °C, 90 min, 85%; (b) PhSeBr, LHMDS, THF, -78 to -20 °C, 210 min. (c) H_2O_2 in excess, CH_2Cl_2, 10 °C, 30 min; (d) DBU, toluene, reflux, 12 h, 45% over three steps; (e) TFA, CH_2Cl_2, rt, 8 h. (f) *tert*-BuONO, pyridine, Et_2O, reflux, 24 h, 89% over two steps; (g) Zn nanopowder, acetic acid, methanol, 0 to 25 °C, 65%.

An attempted, one-pot reductive amination of P2 fragment **11** with the aldehyde in P3 fragment **10** did not provide acceptable yields. Instead, imine **18** was first isolated and then sequentially reduced with $NaBH_3CN$, which gave **19** in 90 % yield, based on recovered

starting material (brsm, Scheme 2). Next, selective cleavage of the TMS ester in **19** with TBAF gave **20** in 93 % yield (brsm). Formation of the amide bond between carboxylic acid **20** and mono-protected di-amine **12** (P1 fragment) was performed using HATU as coupling reagent in the presence of DIPEA to give **7** in 72 % yield. Finally, a convenient ester hydrolysis with LiOH in 1,4-dioxane and water for 8 h provided key P1-3 intermediate **6** in 95 % yield.

Scheme 2. Synthesis of key P1-3 intermediate **6**, direct thrombin inhibitor **2** and acyclic prodrug analogue **5** from P1-P3 fragments **10**-**12**[a]



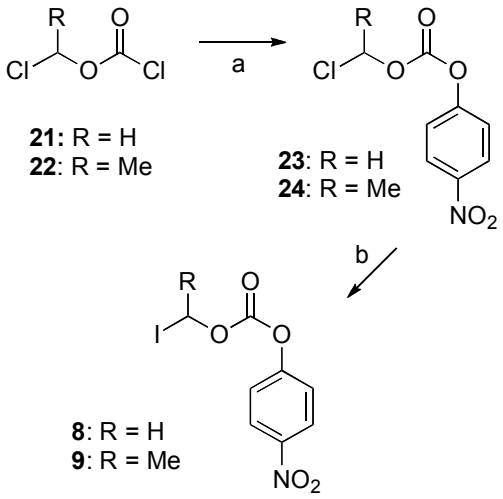
[a]Reagents and conditions: (a) methyl 3-formylbenzoate (P3-fragment), acetic acid, methanol, rt, 12 h, 81%; (b) $NaBH_3CN$, acetic acid, methanol, rt, 6 h, 90% based on recovered starting material (brsm), 66% conversion of **18**; (c) TBAF, THF, rt, 6 h, 93% brsm, 92 % conversion of **19**; (d) HATU, DIPEA, **12**, NMP/CH_2Cl_2, 40 °C, 6 h, 72%; (e) LiOH, methanol, 1,4-

dioxane/water, rt, 8 h, 95%. (f) TFA, CH_2Cl_2, rt, 6 h; (g) methyl carbonochloridate, CH_2Cl_2, 0 °C, 20 min, 71% over two steps. (h) TFA, CH_2Cl_2, rt, 40 min, 97%.

Acyclic prodrug analogue **5** was synthesized from **7** via an *N*-Boc deprotection with TFA followed by acylation of the liberated benzylic amino group with methyl carbonochloridate at low temperature to provide **5** in 71 % yield, over two steps. Compound **2** was prepared as a trifluoroacetate in almost quantitative yield by treatment of **6** with TFA.

Synthesis of (acyloxy)alkoxy linkers **8** and **9** was performed from commercially available chloroformates **21** and **22** (Scheme 3).[11] Reaction with *para*-nitrophenol in the presence of 4-methylmorpholine as base in CH_2Cl_2 gave **23** and **24** in 90 and 81 % yields, respectively. A Finkelstein halide exchange reaction with NaI in dry acetone at reflux then provided iodo-compounds **8** and **9** in 85 and 47 % yields, respectively. A competing E2-elimination of ethene to release carbon dioxide and reform *para*-nitrophenol was observed during the synthesis of **9**. For this reason, the reaction to give **9** needed to be carefully monitored and stopped at 75 % conversion, which led to a lower yield.

Scheme 3. Synthesis of (acyloxy)alkoxy linkers **8** and **9**[a]

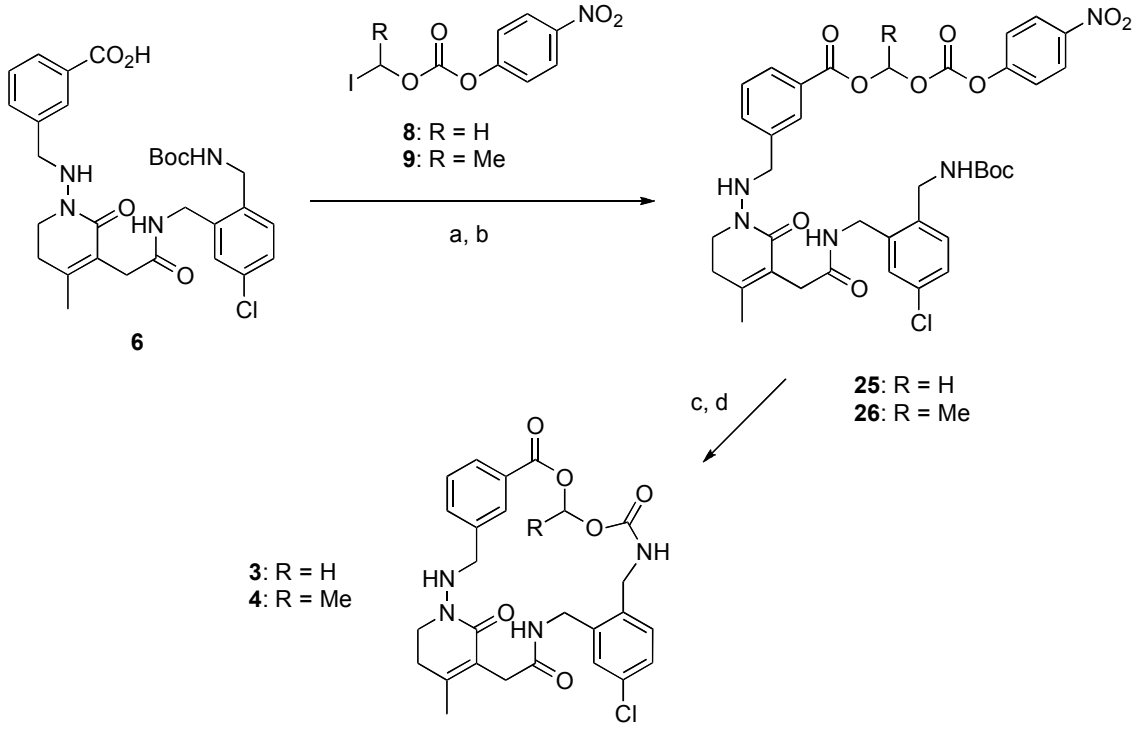


[a]Reagents and conditions: (a) p-nitrophenol, 4-methylmorpholine, CH_2Cl_2, 0 to 25 °C, 12 h, **23:** 90% and **24**: 81%; (b) NaI, acetone, 50 °C, 24 to 48 h, **8**: 85% and **9**: 47%.

Finally, formation of the macrocycles was done in a four-step procedure, the first step of which involved generation of a cesium carboxylate anion by mixing **6** and cesium carbonate (Scheme 4). Alkylation of the cesium carboxylate using one equivalent of either of **8** and **9** at 0 °C in DMF for 4 h gave approximately 49 and 25 % of alkylated intermediates **25** and **26**, respectively, according to LC-MS. Steric effects are a plausible explanation for the lower degree of reaction in the case of iodo-ethyl linker **9**. It has been reported that this type of activation with (acyloxy)alkoxy substrates (R = H) gives yields around 30% when using stoichiometric amounts of alkylating agents.[11] Other cations such as Ag^+ and Hg^+ have been reported to mediate substitutions with similar alkylating agent but with less functionalized acids in good yields.[19] Therefore attempts were performed using silver carbonate and **6**, but they showed little or no reaction in comparison with cesium carbonate. Dimethyl sulfoxide and toluene that have been used as solvents in the literature were also tested, unfortunately without any improvements. Alkylated intermediates **25** and **26** were then subjected to *in situ*

N-Boc deprotection with TFA in CH_2Cl_2 to give free amines as trifluoroacetates and the solution was subsequently diluted to 0.7 mM and basified with aqueous potassium carbonate after which it was left to macrocyclize for 72 h. An intramolecular attack of the primary amine on the acyloxy carbonyl group then leads to formation of macrocyclic prodrugs **3** and **4**. The overall isolated yields of **3** and **4** from **6** was 16 and 8 %, over 4 steps respectively, which connoted that the final macrocyclization proceeded with an effective yield of 32-33 %.

Scheme 4. Synthesis of macrocyclic prodrugs **3** and **4**[a]



[a]Reagents and conditions: (a) Cs_2CO_3, methanol, rt, 1 h; (b) **8** or **9**, DMF, 0 °C, 4 h. **25**: 49% and **26**: 25% over two steps based on LCMS analyses; (c) TFA, CH_2Cl_2, rt, 6 h; (d) CH_2Cl_2, K_2CO_3(aq), rt, 72 h, **3**: 33% and **4**: 32% over two steps. Overall yields of 16% and 8% for **3** and **4**, respectively, over four steps from **6**.

Structure verification. High-resolution mass spectrometry (HR-MS) showed a single molecular ion having the expected mass for each of **3** and **4** within the standard deviation, ±5 ppm. In theory, macrocyclization of activated intermediates **25** and **26** could have occurred at another position, for instance at the amide bond, and use of only mass spectrometry for structural confirmation was therefore not judged to be sufficient. Structural verification of macrocycles **3** and **4** by NMR spectroscopy was not straightforward since each of them generated two sets of signals at room temperature, suggesting slow interconversion of conformational isomers. Structural confirmation was, however, achieved through a complete assignment of the NMR spectra of **3** and **4** based on ^{1}H, ^{13}C, COSY, NOESY, HSQC, HMBC and N-HMBC experiments (cf. Supporting information). The HMBC spectrum of **4** provided the key information, i.e. the couplings between carbonyl carbon C21 and linker proton H34, between H34 and carbonyl carbon C36, and between C36 and proton H30 (Figure 2). These couplings verified that the macrocyclization had occurred at the correct position and that the cyclic structure was intact after purification. The structure of macrocycle **3** was elucidated and confirmed in the same manner.

4

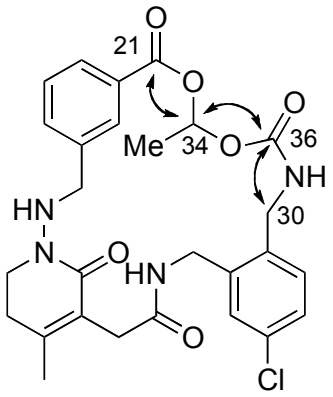


Figure 2. Arrows indicate the key couplings in the HMBC spectrum that confirmed the structure of **4**.

Potency, in vitro metabolism and selectivity towards serine proteases. As mentioned in the introduction direct thrombin inhibitor **1** had liabilities that included high metabolism in rat liver microsomes and inhibition of CYP3A4 (Table 1). Introduction of a carboxylic acid in the meta position **1**, to give compound **2**, reduced both of these liabilities. Satisfactorily, this was accomplished with only a minor loss in functional *in vitro* anticoagulant potency in human plasma, as measured by the concentration required to double the activated partial thromboplastin time potency (APTT), and the *in vivo* efficacy in prevention of venous thrombosis in the rat caval vein thrombosis model[20] (VT). Accordingly, compound **2** was somewhat less potent than **1** as an inhibitor of human thrombin *in vitro* (Table 2). The selectivity of **1** and **2** towards other serine proteases was also examined, and both compounds displayed a >1000-fold selectivity window against trypsin I, Factors VIIa, IXa, Xa and XIa as well as against plasmin. Prodrug **3** was a weak inhibitor of thrombin and did not show any detectable inhibition of trypsin I. Direct thrombin inhibitor **2** thus displayed high potency both *in vitro* and *in vivo*, excellent selectivity against a panel of serine proteases as well as satisfactory metabolic properties. The ADMET properties of the corresponding prodrugs **3** and **4** were therefore investigated further.

Table 1. Potency and *in vitro* metabolism of direct thrombin inhibitors **1** and **2**

Compound	RLM Cl_{int} (μL/min/mg)[a]	CYP_{3A4} IC_{50} (μM)[b]	APTT IC_{50} (μM, rat)[c]	VT IC_{50} (nM)[d]
1	150	13	0.68	72
2	<15	>50	1.2	97

[a]Metabolic stability in rat liver microsomes. [b]Inhibition of human CYP3A4 *in vitro*. [c]Activated partial thromboplastin time. [d]Venous thrombosis model in the rat.

Table 2. Selectivity of direct thrombin inhibitors **1** and **2**, and macrocyclic prodrug **3** towards serine proteases

Enzyme	Compound **1** K_i (nM) (SD, n)	Compound **2** K_i (nM) (SD, n)	Compound **3** K_i (nM)
Thrombin	0.5 (0.15, 2)	4.6 (2.3, 3)	200
Trypsin I[a]	>5.4 x10^3	>5.4 x10^3	>5.4 x10^3
FVIIa	>33 x10^3	>33 x10^3	-
FIXa	>29 x10^3	>29 x10^3	-
FXa	>17 x10^3	>17 x10^3	-
FXIa	>19 x10^3	>19 x10^3	-
Plasmin	>19 x10^3	>19 x10^3	-

[a]Based on two repeats.

Physicochemical properties and *in vitro* ADME. To investigate if prodrugs **3** and **4** had suitable *in vitro* ADME properties for further development their solubility, lipophilicity, cell permeability and in vitro metabolism was investigated. Data was also acquired for acyclic analogue **5** and direct thrombin inhibitor **2**. Solubility was determined in phosphate buffer at pH 7.4 using amorphous material, whereas stock solutions in DMSO were employed for determination of LogD using a modified version of the shake-flask method.[21] Caco-2 cells were used for determination of cell monolayer permeability with a pH gradient from 6.5 on the apical side of the cellular monolayer to 7.4 on the basolateral side. This is a common model for absorption of drugs from the small intestine into the bloodstream.[22] The passive

cell monolayer permeability was determined by inclusion of an inhibitor cocktail (quinidine, sulfasalazine and benzobromarone) that targets the three major efflux transporters in the intestinal epithelium: P-glycoprotein, (MDR1/P-gp; ABCB1), breast cancer resistance protein (BCRP; ABCG2) and multidrug-resistance associated protein 2 (MRP2, ABCC2). Caco-2 cell permeability was also determined in the absence of the inhibitor cocktail with a pH of 7.4 on both sides of the cellular monolayer to provide insight into the degree of efflux for compounds **2**-**5**.

Table 3. Solubility, lipophilicity and cell monolayer permeability for compounds **2**-**5**

Compound	MW	TPSA[a] ($Å^2$)	Solubility[b] (μM)	$LogD_{7.4}$ (SD, n)	Caco-2 P_{app} AB[c] (x 10^{-6} cm/s)	Efflux ratio (SD, n)
2	470	125	>100	-0.5 (0.1, 3)	0.10	2.4 (0.5, 4)
3	526	126	120	2.3 (0.1, 3)	48	5.6 (1.5, 4)
4	540	126	340	2.6 (0.1, 3)	45	4.8 (1.1, 4)
5	542	126	160	2.9 (0.1, 3)	54	3.3 (0.4, 4)

[a]TPSA was calculated by Molinspiration (www.molinspiration.com). [b]Based on three repeats for **3**-**5**, with SD ≤0.17 for Log solubility, and one measurement for **2**. [c]Based on three repeats for **2**-**5**, with SD ≤0.14 for Log [P_{app} AB].

As expected from its zwitterionic character (pK_a = 3.94 and 9.27) at physiological pH (7.4) direct thrombin inhibitor **2** had a low lipophilicity (Table 3). Consequently, the Caco-2 cell

permeability was in the range where the *in vivo* fraction absorbed is usually too low for oral administration (<1 x 10^{-6} cm/s). In contrast, cyclic prodrugs **3** and **4**, as well as acyclic analogue **5**, had lipophilicities that are optimal for cell permeability and oral delivery (LogD 1-3).[23] Accordingly, **3**-**5** had high solubilities (>100 μM) as well as high passive cell permeabilities (appr. 50 x 10^{-6} cm/s) that can be expected to translate into close to complete absorption from the intestine. Compounds **3**-**5** did display some transporter mediated efflux (efflux ratios: 2.4-5.6) but not to the extent where oral administration can be expected be severely compromised. In fact, all classes of macocyclic drugs and clinical candidates that are delivered orally for systemic use have been linked to efflux transporters, either as substrates or inhibitors.[24] Macrocyclization often results in increased cell permeability as compared to non-macrocyclic controls,[25, 26] but this was not observed for **3** and **4** as compared to acyclic analogue **5**, most likely because macrocyclization did not result in that polar moieties were shielded e.g by formation of intramolecular hydrogen bonds.

Prodrug **3** was stable when incubated in human plasma for 4 h, indicating that **3** and **4** are not substrates for human plasma esterases. In contrast, when incubated with human hepatocytes at 37 °C both **3** and **4** were rapidly and completely metabolized to direct thrombin inhibitor **2** in 40 min (Figure 3). Identical results were obtained with rat hepatocytes (data not shown). Metabolism of prodrug **3** results in formation of one equivalent of formaldehyde, which is a potential safety concern. However, even at a dose of 1 g of **3**/day the liberated formaldehyde is comparable to the daily intake from food sources and <0.001% of the daily endogenous turnover of formaldehyde.[27] The methyl ester of acyclic analogue **5** was also cleaved to a high extent in human hepatocytes, but the methyl carbamate turned out to be almost completely resistant to metabolism during these conditions. When incubated with human liver microsomes both **3** and **4** where degraded at equal rates in the presence and absence of

NADPH, strongly indicating that conversion to **2** was mediated by esterases but not by cytochrome P450 oxidases. In summary, prodrugs **3** and **4** had solubilities and permeabilities across Caco-2 cells that suggested that they would display high absorption on oral administration. Their rapid metabolism to direct thrombin inhibitor **2** in human and rat hepatocytes also supported that they were progressed to *in vivo* ADME studies in rat.

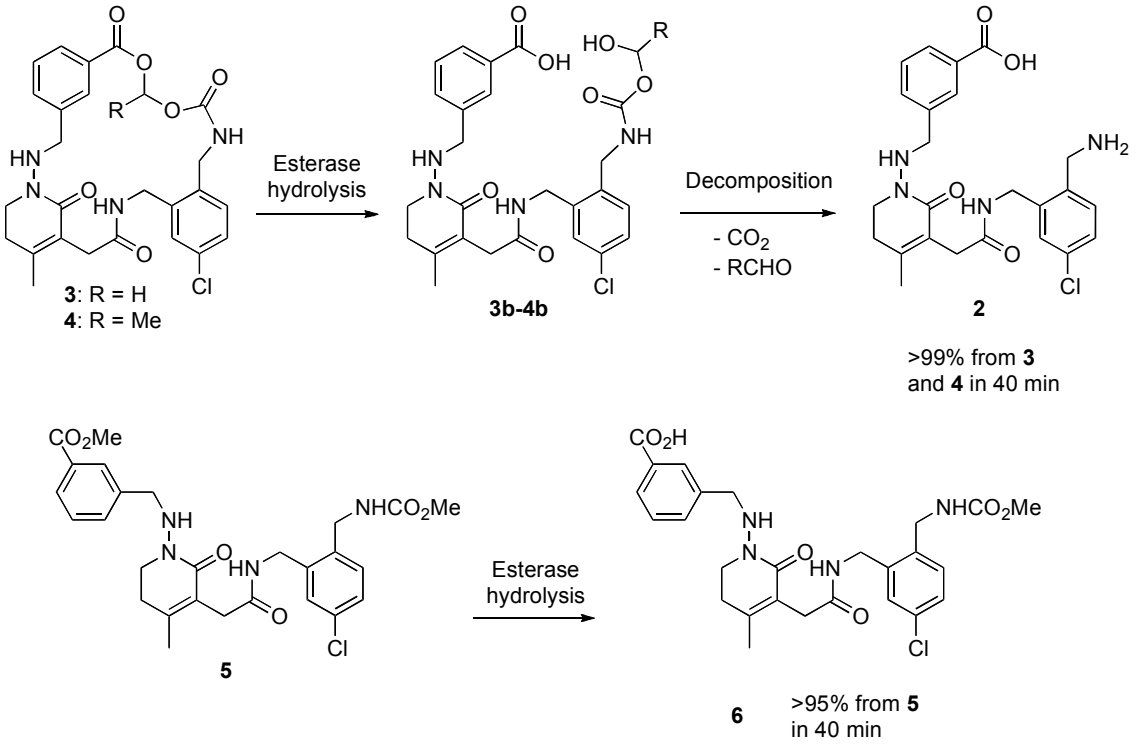


Figure 3. Esterase mediated cleavage of (acyloxy)alkoxy linked prodrugs **3** and **4** in human hepatocytes, followed by chemical decomposition to give direct thrombin inhibitor **2**.[9] The methyl ester of acyclic analogue **5** is also cleaved by esterases in the hepatocytes.

Bioavailability in rat. Prodrug **3** was selected for in vivo pharmacokinetic studies because homologue **4** displayed a higher acid lability during purification, indicating that it might undergo acid catalysed decomposition on oral administration. Pharmacokinetic data for direct

thrombin inhibitor **2** and prodrug **3** were determined in male Han Wistar rats after single 0.5 μmol/kg *i.v.* and 1 μmol/kg *p.o.* doses and calculated using non-compartmental analysis (Table 4 and Figure 4). Plasma levels of **2** declined rapidly after *i.v.* dosing with a terminal half-life of approximately 0.35 h. The total plasma clearance was 32 mL/min/kg, which is about half the liver blood flow. The volume of distribution at steady state was determined to be 0.23 L/kg, which is smaller than that of the exchangeable water and implies a low tissue distribution. The systemic bioavailability (*F*) after *p.o.* dosing of **2** was 0.5%. Prodrug **3** was not detected in plasma samples at any time point after *p.o.* dosing, which indicates a rapid conversion to **2**. The area under the plasma concentration-time curve at infinite time (AUC_{inf}) for **2** was approximately 5-fold higher after *p.o.* administration of **3** compared to *p.o.* administration of **2**. This increase in systemic exposure illustrates the potential of using a double prodrug macrocycle approach, but the absolute bioavailability of **2** after administration of **3** was only 2.5%. Although, this is higher than when **2** is administered it is still low and there could be several reasons for this. For instance, hydrolysis of the prodrug could occur in the acidic environment during passage through the stomach and thereby limit the bioavailability. However, administration of **3** directly in the duodenum in the rat did not show an increased bioavailability of **2**, and thus acid induced degradation does not explain the low bioavailability. Instead we speculate that rapid metabolic degradation of prodrug **3** occurs either in the duodenum, or during uptake across the intestinal wall, and that metabolism constitutes the major reason for the low bioavailabilities obtained on oral administration of prodrug **3**. In order to reduce the rate of metabolism, structurally different, but still esterase sensitive macrocyclic prodrugs could be explored, e.g. prodrugs based on coumarinic or *o*-hydroxyphenylpropionic acid derivatives.[28, 29]

Table 4. Pharmacokinetic parameters[a] for compound **2** after dosing of compounds **2** or **3** via different routes of administration.

	Intravenous administration of **2** (0.5 μmol/kg)	Per oral administration of **2** (1 μmol/kg)	Per oral administration of **3** (1 μmol/kg)	Intraduodenal administration of **3** (1 μmol/kg)
Rat CL (mL/min/kg)	32.4 (SD=1.3)	-	-	-
Rat Vss (L/kg)	0.23 (SD=0.05)	-	-	-
Terminal $t_{1/2}$ (h)	0.35 (SD=0.04)	0.56 (SD=0.006)	1.12 (SD=0.23)	0.11 (SD=0.01)
AUC_{inf} of **2** (h × μmol/L)	0.258 (SD=0.010)	0.0025 (SD=0.0005)	0.0127 (SD=0.0012)	0.0081 (SD=0.0026)
F of **2** (%)	-	0.5	2.5	1.6
C_{max} (μmol/L)	-	0.004 (SD=0.002)	0.011 (SD=0.002)	0.108 (SD=0.041)

[a]Values for all data except the bioavailability (F) are means from n=3 experiments. F is calculated from the average AUC_{inf} values.

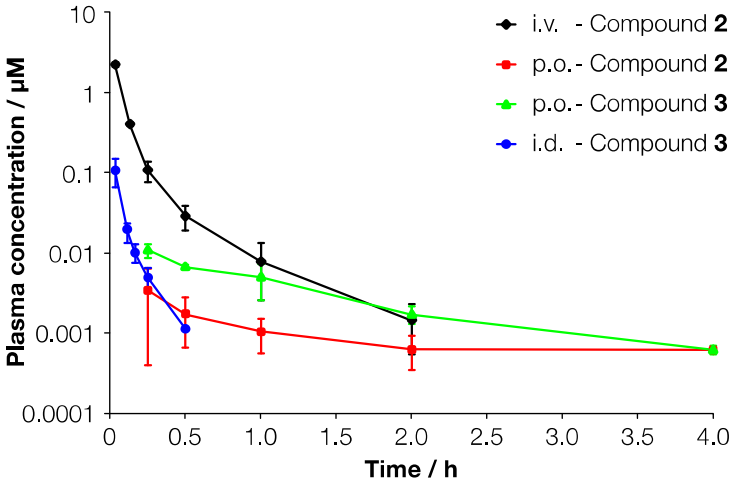


Figure 4. Plasma concentration over time for direct thrombin inhibitor **2** after intravenous (*i.v.*), peroral (*p.o.*) or intraduodenal (*i.d.*) administration of **2** or prodrug **3** to rat.

Conclusions

Drug targets that require zwitterionic or other bi-polar ligands are ¨difficult to drug¨ as such drug candidates often suffer from poor absorption from the gastrointestinal tract. In such cases bioavailability may be improved by conversion into double prodrugs, but they are often complicated to develop because of difficulties to obtain efficient cleavage of both prodrug moieties. With this in mind we developed a synthetic route to zwitterionic, non-peptidic direct thrombin inhibitor **2**, which displayed high antithrombotic potency, excellent selectivity against serine proteases and satisfactory *in vitro* metabolic properties. Inhibitor **2** was converted into macrocycles **3** and **4**, each of which carried an (acyloxy)alkoxy prodrug moiety[11] across the carboxyl and amino group of **2**. The two macrocyclic prodrugs displayed high solubilities and also had high cell monolayer permeabilities, in contrast to low-

permeable inhibitor **2**. Moreover, the prodrug moieties of **3** and **4** were cleaved rapidly in human hepatocytes, most likely by liver esterases. These *in vitro* studies suggested that both **3** and **4** would be highly absorbed from the intestine and then cleaved to liberate potent direct thrombin inhibitor **2** in the liver. Unfortunately, the bioavailability of inhibitor **2** after oral administration of prodrug **3** in rat was low (2.5%) and only moderately higher than on direct oral administration of **2** (0.5%). We conclude that the low bioavailability after administration of **3** most likely results from rapid metabolism of the (acyloxy)alkoxy prodrug moiety in the intestine, or during uptake across the intestinal wall. To the best our knowledge, only *in vitro* data has been reported in previous studies[10-15] of cyclic (acyloxy)alkoxy prodrugs. Thus, our study is the first that involves *in vivo* ADME studies and therefore we can not speculate if the modest increase in bioavailability is a general feature of this category of prodrugs. Furthermore, chemical developability at large scale constitutes a challenge for macrocyclic (acyloxy)alkoxy prodrugs as the macrocyclization step requires high dilution and proceeds in modest yields.

Experimental

General chemistry. Reagents and solvents used were of reagent grade and purchased from commercial suppliers and used as received. Unless stated otherwise, reactions were performed under nitrogen atmosphere, additions of reagents and/or solvents with sterilized disposable syringes and needles, Silicone Oil (550 Fluid) was used for heatings, stirring was made with magnetic bars, evaporation of solvents was done under vacuum with a rotavapor. Glassware were dried in oven (150 °C), when necessary. Building blocks for synthesis, i.e. aldehyde **10** and chloroformates **21-22** were from Sigma-Aldrich, while amine **12** was purchased from Syngene. Reactions were monitored with (i) thin-layer chromatography

(TLC), (ii) high-performance liquid chromatography mass spectrometry (HPLC/MS), (iii) ^{1}H NMR or combinations of (i), (ii) and (iii). (i) TLC analysis was carried out on silica gel plates (Merck 60 $_{F254}$). The plates were stained with basic $KMnO_4$ or CAM (ceric ammonium molybdate) manually prepared solutions from available protocols. (ii) Analytical HPLC/MS was conducted on a QTOF mass spectrometer using a UV detector monitoring either at (a) 210 nm with a BEH C18 column (2.1 × 100 mm, 1.7 μm, 0.7 mL/min flow rate), using a gradient of 2% v/v CH_3CN in H_2O (ammonium carbonate buffer pH 10) to 98% v/v CH_3CN in H_2O or at (b) 230 nm with an HSS C18 column (2.1 × 100 mm, 1.8 μm, 0.7 mL/min flow rate) or C18 CSH 1.7μm column (2.1x50mm, 0.7 mL/min flow rate), using a gradient of 2% v/v CH_3CN in H_2O (ammonium formate buffer pH 3) to 98% v/v CH_3CN in H_2O. Samples diluted in MeOH or CH_3CN, 2 or 4 min measurements. (iii) NMR measurements were recorded on a Bruker Avance DPX400 (400 MHz), AV500 (500 MHz) or AV600 (600 MHz) instrument at 25 °C as indicated. The chemical shifts (δ) are expressed as parts per million (ppm) relative to residual solvent signal as internal reference at 7.26 ppm ($CDCl_3$) or at 3.30 ppm (MeOD-d_4) and coupling constants (*J*) values are reported in Hertz (Hz). Splitting pattern abbreviations are as follows: s = singlet; d = doublet; t = triplet; q = quartet; p = pentet; m = multiplet; br = broad; om = overlapping multiplet. Silica gel column chromatography was carried out on pre-packed silica gel columns SNAP (KP-Sil) cartridges supplied by Biotage and using Biotage automated flash systems with UV detection. Preparative High-performance liquid chromatography (HPLC) was performed by a Waters Xbridge C18 OBD 5 μm column (19 × 150 mm, flow rate 30 mL/min, or 30 × 150 mm, flow rate 60 mL/min) using a varying gradient of CH_3CN with 0.2% NH_3 at pH 10 with UV detection. For purification of final compounds **3**-**4**, Waters Sunfire ODB MS-triggered fraction collector was utilized with a UV/VIS detector 155 on a C18 5 μm column (19 × 150 mm, flow rate 19 mL/min, or 50 × 250 mm, flow rate 100 mL/min) using a varying gradient

of CH_3CN and 0.1 M NH_4OAc. The purity of each compound tested was determined with analytical method (b), relative UV absorbance at 230 nm. All tested compounds were ≥ 95% pure. High-resolution mass spectrometry (HRMS) was determined as for (ii) on a Waters Xevo QTOF, except for compounds **8**, **23-24**, which were determined on a GC-FI-TOF-MS setup Waters GCT HP-5MS 0.25µm column (30 x 0.32 mm, 2 mL/min flow rate) using He as carrier gas.

3-((3-(2-(2-(Aminomethyl)-5-chlorobenzylamino)-2-oxoethyl)-4-methyl-2-oxo-5,6-dihydropyridin-1(*2H*)-ylamino)methyl)benzoic acid (2). TFA (5 mL, 66 mmol) was added to **6** (86 mg, 0.147 mmol) in CH_2Cl_2 (5 mL) and the solution was stirred at rt for 40 min. The solvents were removed in vacuo and to give pure **2** as a TFA salt (86 mg, 97 %). The product was used directly without further purification. ^{1}H NMR (500 MHz, DMSO) δ 1.81 (s, 3H, H11), 2.30 (t, *J* = 7.1 Hz, 2H, H2), 3.19 (s, 2H, H7), 3.30 (t, *J* = 7.2 Hz, 2H, H1), 3.96 (s, 2H, H14), 4.7-6.3 (br, 1H, H22), 4.11 (q, *J* = 5.7 Hz, 2H, H32), 4.29 (d, *J* = 6.0 Hz, 2H, H24), 7.46-7.40 (om, 3H, H28/29/26), 7.44 (t, *J* = 7.7 Hz, 1H, H19), 7.57 (dt, *J* = 1.2, 7.6 Hz, 1H, H20), 7.84 (dt, *J* = 1.4, 7.7 Hz, 1H, H18), 7.93 (s, 1H, H16), 8.15 (br s, 3H, H33/H10), 8.39 (t, *J* = 6.0 Hz, 1H, H9). ^{13}C NMR (126 MHz, DMSO) 170.5, 167.3, 164.7, 158.0, 147.5, 140.7, 138.7, 133.4, 131.5, 130.7, 130.4, 129.8, 128.5, 128.2, 127.0, 122.5, 53.0, 46.8, 33.4, 30.3, 20.2. HRMS (ESI) *m*/*z* calcd for $C_{24}H_{27}N_4O_4Cl$ $[M + H]^+$ 471.1799 ; found 471.1807.

25-Chloro-18-methyl-13,14,17,20,22,23-hexahydro-*1H*,*7H*-15,19-methano-8,12-(metheno)-4,6,2,14,15,22-benzodioxatetraazacyclopentacosine-3,7,21,28(*2H*,*16H*)-tetrone (3). Cesium carbonate (14 mg, 40 µmol) was added to a solution of **6** (40 mg, 70 µmol) in methanol (0.5 mL) and was stirred at rt for 1 h. The solvent was removed in vacuo to generate the cesium salt as a light green solid. The solid was redissolved in dry DMF (0.6

mL) and the solution was added to a solution of **8** (23 mg, 70 μmol) dissolved in dry DMF (0.6 mL) at 0 °C during 30 min. The reaction was monitored with LC-MS at pH 3 and was stirred at 0 °C for an additional 4 h. The solvent was removed in vacuo and the residue was redissolved in EtOAc (5 mL) and was then sequentially washed with H_2O (5 mL), brine (5 mL) and concentrated in vacuo to give a residue containing 49 % alkylated intermediate **25**. Crude **25** was dissolved in CH_2Cl_2 (3 mL), after which TFA (0.15 mL, 2.0 mmol) was added and the solution was stirred at rt for 6 h. The reaction was monitored with LC-MS at pH 3 which indicated complete *N*-Boc deprotection. The solution was diluted with CH_2Cl_2 (80 mL) to obtain a concentration of 0.7 mM of the deprotected intermediate. It was then washed with aqueous 10 % K_2CO_3 (5 mL), filtered with a phase separator and slowly stirred at rt for 72 h. The reaction was monitored with LC-MS at pH 10 which indicated that macrocycle **3** had been formed. The solvent was removed in vacuo and the residue was purified with preparative HPLC, (10 to 70 % acetonitrile in H_2O-NH_4OAc) to give macrocycle **3** (6 mg, 16 % from **6**) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$, contains set of two rotamers) δ 2.02 (s, 1.5H, H6), 2.07 (s, 1.5H, H6), 2.08 – 2.16 (m, 1H, H7), 2.26 (d, *J* = 7.0 Hz, 1H, H7), 2.94 (s, 1H, H8), 3.16 (d, *J* = 7.2 Hz, 1H, H8), 3.28 (s, 1H, H9), 3.33 (s, 1H, H9), 3.87 (s, 1H, H14), 3.89 (s, 1H, H14), 4.24 (d, *J* = 6.6 Hz, 1H, H30), 4.36 (d, *J* = 6.1 Hz, 1H, H22), 4.38 (d, *J* = 5.7 Hz, 1H, H30), 4.42 (bs, 1H, H22), 5.93 (s, 1H, H34), 5.96 (s, 1H, H34), 6.21 – 6.35 (m, 1H, H31), 6.93 – 6.97 (m, 0.5H, H26), 6.99 (s, 0.5H, H24), 7.06 – 7.1 (m, 0.5H, H26), 7.21 (s, 0.5H, H16), 7.22 – 7.29 (om, 2H, H16/24/27), 7.34 – 7.46 (m, 1H, H19), 7.54 (d, *J* = 7.5 Hz, 0.5H, H20), 7.59 (d, *J* = 7.6 Hz, 0.5H, H20), 7.64 – 7.68 (m, 0.5H, H11), 7.74 (d, *J* = 7.5 Hz, 0.5H, H18), 7.92 (d, *J* = 7.7 Hz, 0.5H, H18), 8.00 – 8.06 (m, 0.5H, H11); ^{13}C NMR (151 MHz, $CDCl_3$, contains set of two rotamers) δ 171.1 (C10), 171.0 (C10), 166.5 (C2), 166.0 (C2), 165.1 (C21), 164.8 (C21), 154.4 (C36), 154.0 (C36), 151.0 (C5), 149.0 (C5), 139.0 (C23), 138.6 (C23), 138.0 (C15), 137.8 (C15), 134.6 (C20/C28), 134.5 (C20),

134.2 (C25), 133.8 (C25), 133.3 (C28), 132.0 (C27), 131.9 (C27), 131.0 (C16), 129.9 (C16), 129.3(C17), 129.2 (C18), 128.9 (C18), 128.8 (C19), 128.7(C17), 128.6 (C24), 128.3(C24), 127.4 (C26), 127.2 (C26), 122.2 (C4), 121.3 (C4), 81.8 (C34), 81.2 (C34), 53.9 (C14), 53.7 (C14), 47.6 (C8), 47.5 (C8), 42.6 (C30), 42.0 (C30), 39.6 (C22), 38.9 (C22), 35.3 (C9), 35.3 (C9), 30.6 (C7), 30.4 (C7), 20.8 (C6), 20.6 (C6). HRMS (ESI) *m*/*z* calcd for $C_{26}H_{28}N_4O_6Cl$ $[M + H]^+$ 527.1697; found 527.1574.

25-Chloro-5,18-dimethyl-13,14,17,20,22,23-hexahydro-*1H*,*7H*-15,19-methano-8,12-(metheno)-4,6,2,14,15,22-benzodioxatetraazacyclopentacosine-3,7,21,28(*2H*,*16H*)-tetrone (4). Cesium carbonate (34 mg, 0.11 mmol) was added to a solution of **6** (100 mg, 0.18 mmol) in methanol (1 mL) and was stirred at rt for 1 h. The solvent was removed in vacuo to generate the cesium salt as a light green solid. The solid was redissolved in dry DMF (1.2 mL) and the solution was added to a solution of **9** (81 mg of 75% w/w, 0.18 mmol) dissolved in dry DMF (1.2 mL) at 0 °C during 30 min. The reaction was monitored with LC-MS at pH 3 and was stirred at 0 °C for an additional 4 h. The solvent was removed in vacuo and the residue was redissolved in EtOAc (10 mL) and was then sequentially washed with H_2O (10 mL), brine (10 mL) and concentrated in vacuo to give a residue containing 25 % alkylated intermediate **26**. Crude **26** was dissolved in CH_2Cl_2 (4 mL), after which TFA (0.15 mL, 2.0 mmol) was added and the solution was stirred at rt for 6 h. The reaction was monitored with LC-MS at pH 3 which indicated complete *N*-Boc deprotection. The solution was diluted with CH_2Cl_2 (150 mL) to obtain a concentration of 0.7 mM of the deprotected intermediate. It was then washed with aqueous 10 % K_2CO_3 (10 mL), filtered with a phase separator and slowly stirred at rt for 72 h. The reaction was monitored with LC-MS at pH 10, which indicated that macrocycle **4** (observed $[M + H]^+$: 497 – loss of CO_2) had been formed. The solvent was removed in vacuo and the residue was purified with preparative HPLC, (10

to 70 % acetonitrile in H_2O-NH_4OAc) to give macrocycle **4** (8 mg, 8 % from **6**) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$, contains set of two rotamers) δ 1.65 (d, *J* = 5.4 Hz, 1.5H, H38) 1.75 (d, *J* = 5.4 Hz, 1.5H, H38), 1.96 – 2.03 (om, 1.5H, H7/8), 2.05 (s, 1.5H, H6), 2.08 (s, 1.5H, H6), 2.53 (s, 1H, H7), 2.74 (s, 0.5H, H8), 3.02 – 3.15 (m, 1H, H8/9), 3.22 – 3.36 (om, 2H, H8/9), 3.38 – 3.45 (m, 0.5H, H9), 3.62 (d, *J* = 12.7 Hz, 0.5H, H14), 3.80 – 3.98 (m, 2.5H, H14/22), 4.10 – 4.19 (m, 0.5H, H30), 4.23 (s, 0.5H, H30), 4.44 (dd, *J* = 8.3, 15.0 Hz, 0.5H, H30), 4.57 (dd, *J* = 8.5, 14.7 Hz, 0.5H, H30), 4.82 (dd, *J* = 9.0, 14.9 Hz, 0.5H, H22), 5.01 – 5.10 (m, 0.5H, H22), 6.13 – 6.17 (m, 0.5H, H31), 6.19 (s, 0.5H, H31), 6.79 (d, *J* = 5.5 Hz, 0.5H, H34), 6.87 (s, 0.5H, H16), 6.94 – 7.01 (om, 1.5H, H26/34), 7.14 (d, *J* = 6.1 Hz, 0.5H, H26), 7.24 (d, *J* = 9.0 Hz, 0.5H, H16), 7.27 – 7.35 (om, 1.5H, H24/27), 7.39 – 7.48 (m, 1.5H, H19/27), 7.58 (d, *J* = 7.8 Hz, 0.5H, H20), 7.64 (d, *J* = 7.0 Hz, 0.5H, H20), 7.75 (s, 0.5H, H11), 7.88 (d, *J* = 7.8 Hz, 0.5H, H18), 7.91 (d, *J* = 7.5 Hz, 0.5H, H18), 8.23 (s, 0.5H, H11); ^{13}C NMR (151 MHz, $CDCl_3$, contains set of two rotamers) δ 171.2 (C10), 171.1 (C10), 166.5 (C2), 166.3 (C2), 164.7(C21), 154.3 (C36), 153.4 (C36), 151.8 (C5), 149.4 (C5), 138.7 (C23), 138.7 (C23), 138.1 (C15), 137.8 (C15), 135.1 (C28), 135.1 (C28), 135.0 (C20), 134.5 (C20), 134.1 (C17), 134.0 (C17), 132.9 (C27), 132.5 (C27), 130.6 (C16), 129.9 (C16), 129.7 (C25), 129.5 (C25), 129.4 (C18), 129.1 (C18), 128.9 (C19), 128.8 (C19), 127.6 (C26), 127.4 (C26), 122.1 (C4), 120.6 (C4), 91.4 (C34), 90.9 (C34), 54.7 (C14), 53.9 (C14), 48.2 (C8), 42.3 (C30), 41.9 (C30), 39.4 (C22), 39.0 (C22), 35.5 (C9), 35.4 (C9), 30.6 (C7), 30.3 (C7), 20.8 (C6), 20.7 (C6), 19.7 (C38), 19.5 (C38). HRMS (ESI) *m*/*z* calcd for $C_{27}H_{30}N_4O_6Cl$ $[M + H]^+$ 541.1854; found 541.1841.

Methyl 3-(((3-(2-((5-chloro-2-(((methoxycarbonyl)amino)methyl)benzyl)amino)-2-oxoethyl)-4-methyl-2-oxo-5,6-dihydropyridin-1(*2H*)-yl)amino)methyl)benzoate (5). TFA (80 μL, 1.0 mmol) was added to a solution of **7** (35 mg, 60 μmol) in CH_2Cl_2 (1.5 mL) and the

reaction was stirred at rt for 6h. The reaction was diluted with CH_2Cl_2 (5 mL) and sequentially washed with aqueous solution of 10 % K_2CO_3 (2 x 10 mL), H_2O (2 x 10 mL), brine (2 x 10 mL), dried (Na_2SO_4), filtered and concentrated in vacuo to give the deprotected amine and was redissolved in CH_2Cl_2 (0.8 mL). To this solution was added methyl carbonochloridate (4.7 µL, 60 µmol) dissolved in CH_2Cl_2 (1 mL) dropwise at 0 °C for 20 min. The reaction was carefully monitored with LC-MS at pH 10 and the reaction was quenched with an aqueous 5% HCl solution (15 mL) and sequentially washed with saturated aqueous $NaHCO_3$ (2 x 10 mL), H_2O (2 x 10 mL), brine (2 x 10 mL), dried (Na_2SO_4), filtered and concentrated in vacuo to give a 2:1 mixture of mono-acylated **5** and bi-acylated byproduct. The product was purified with preparative HPLC at pH10 (15 to 70 % acetonitrile in H_2O-NH_3OAc) to give **5** (23 mg, 71 %) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.01 (s, 3H, H11), 2.34 (t, *J* = 7.2 Hz, 2H, H2), 3.29 (s, 2H, H7), 3.37 (t, *J* = 7.2 Hz, 2H, H1), 3.63 (s, 3H, H37), 3.92 (s, 3H, H38), 4.00 (s, 2H, H14), 4.34 (d, *J* = 5.7 Hz, 2H, H32), 4.36 (d, *J* = 6.0 Hz, 2H, H24), 5.87 (br, 1H), 7.13 – 7.16 (m, 1H, H29), 7.19 (dd, *J* = 2.1, 8.1 Hz, 1H, H28), 7.30 (d, *J* = 8.1 Hz, 1H, H26), 7.36 (br, 1H), 7.39 (t, *J* = 7.7 Hz, 1H, H19), 7.51 – 7.56 (m, 1H, H20), 7.92 – 7.97 (m, 1H, H18), 8.04 (s, 1H, H16); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.1, 166.3, 157.3, 148.9, 138.5, 138.0, 135.1, 134.1, 133.7, 131.2, 130.7, 130.1, 129.2, 128.7, 128.4, 128.1, 127.7, 54.4, 52.8, 51.9, 48.2, 41.4, 40.3, 35.6, 31.8, 31.1. HRMS (ESI) *m*/*z* calcd for $C_{27}H_{32}N_4O_6Cl$ $[M + H]^+$ 543.2010; found 543.2025.

3-(((3-(2-((2-(((*Tert*-butoxycarbonyl)amino)methyl)-5-chlorobenzyl)amino)-2-oxoethyl)-4-methyl-2-oxo-5,6-dihydropyridin-1(*2H*)-yl)amino)methyl)benzoic acid (6). Lithium hydroxide (9 mg, 0.35 mmol) and a catalytic amount of methanol (1.2 µL, 30 µmol) were added to a solution of **7** (170 mg, 0.29 mmol) in 1,4-dioxane and water (1:1, 1 mL). The mixture was stirred at rt for 8 h. It was then diluted with EtOAc (5 mL), washed with aqueous

HCl (pH 5, 3 x 5 mL), dried (Na_2SO_4), filtered with a phase separator and concentrated in vacuo to give carboxylic acid **6** (157 mg, 95 %) as a white solid. The product was used directly in the next step without further purification. ^{1}H NMR (500 MHz, MeOD-d4) δ 1.44 (s, 9H, H38/39/40), 1.93 (s, 3H, H11), 2.37 (t, *J* = 7.2 Hz, 2H, H2), 3.34 (s, 2H, H7), 3.37 (t, *J* = 7.2 Hz, 2H, H1), 4.02 (s, 2H, H14), 4.26 (s, 2H, H32), 4.39 (s, 2H, H24), 7.20 – 7.24 (m, 1H, H28), 7.25 – 7.28 (m, 1H, H29), 7.28 – 7.30 (m, 1H, H26), 7.35 (t, *J* = 7.6 Hz, 1H, H19), 7.47 – 7.53 (m, 1H, H20), 7.86 – 7.90 (m, 1H, H18), 7.98 – 8.02 (m, 1H, H16); ^{13}C NMR (126 MHz, MeOD-d4) 173.6, 167.0, 158.4, 150.7, 145.6, 139.6, 138.9, 137.0, 134.1, 133.2, 131.5, 130.7, 129.7, 129.1, 128.9, 128.2, 123.6, 80.3, 55.1, 48.3, 41.9, 41.1, 38.5, 34.8, 31.7, 28.8, 20.6. HRMS (ESI) *m*/*z* calcd for $C_{29}H_{36}N_4O_6Cl$ $[M + H]^+$ 571.2323; found 571.2338.

3-(((3-(2-((2-(((*Tert*-butoxycarbonyl)amino)methyl)-5-chlorobenzyl)amino)-2-oxoethyl)-4-methyl-2-oxo-5,6-dihydropyridin-1(*2H*)-yl)amino)methyl)benzoate (7). HATU (1.10 g, 2.91 mmol), DIPEA (1.01 mL, 5.82 mmol) and benzylic amine **12** (578 mg, 2.13 mmol) were sequentially added to a solution of carboxylic acid **20** (645 mg, 1.94 mmol) in CH_2Cl_2 and NMP (1:1; 6 mL). The solution was stirred at 40 °C for 6 h then diluted with EtOAc (15 mL) and sequentially washed with saturated aqueous $NaHCO_3$ (20 mL), brine (20 mL), saturated aqueous NH_4Cl (20 mL) and brine (20 mL), dried (Na_2SO_4), filtered with a phase separator and concentrated in vacuo. The residue was purified with preparative HPLC at pH 10 (15 to 80 % acetonitrile in H_2O-NH_3) to give **7** (810 mg, 71 %) as a white solid. ^{1}H NMR (500 MHz, MeOD-d4) δ 1.44 (s, 9H, H38/39/40), 1.93 (s, 3H, H11), 2.38 (t, *J* = 7.2 Hz, 2H, H2), 3.34 (s, 2H, H7), 3.40 (t, *J* = 7.2 Hz, 2H, H1), 3.90 (s, 3H, H41), 4.03 (s, 2H, H14), 4.25 (s, 2H, H32), 4.39 (s, 2H, H24), 7.22 (dd, *J* = 1.7, 8.2 Hz, 1H, H28), 7.27 (d, *J* = 8.1 Hz, 2H, H26/29), 7.42 (t, *J* = 7.7 Hz, 1H, H19), 7.63 (d, *J* = 7.6 Hz, 1H, H20), 7.91 (d, *J* = 7.8 Hz, 1H, H18), 8.05 (s, 1H, H16); ^{13}C NMR δ_C (126 MHz, MeOD-d4) 173.3, 168.2, 166.9, 158.1,

150.4, 139.6, 139.5, 136.8, 135.1, 133.9, 131.3, 131.2, 130.6, 129.6, 129.5, 128.7, 128.1, 123.6, 80.2, 54.6, 52.7, 48.1, 41.9, 41.1, 34.9, 31.6, 28.8, 20.6. HRMS (ESI) *m*/*z* calcd for $C_{30}H_{38}N_4O_6Cl$ $[M + H]^+$ 585.2480; found 585.2497.

1-Iodomethyl (4-nitrophenyl) carbonate (8). Prepared as previously reported[30] with minor modifications. The ^1H NMR is in agreement with reported data and the product was used directly used without further purifications, see S.I. HRMS (FI) *m*/*z* calcd for $C_8H_6NO_5I$ $[M + H]^+$ 322.9291; found 322.9296.

1-Iodoethyl (4-nitrophenyl) carbonate (9). Sodium iodide (1.19 g, 7.94 mmol) was added to **24** (650 mg, 2.65 mmol) in dry acetone (4 mL). The reaction was monitored with ^1H NMR spectroscopy and was heated at 50 °C for 48 h in a capped vial. The reaction was stopped after 75% conversion to prevent the competing elimination of ethene. The solvent was removed in vacuo and redissolved in *tert*-butyl methyl ether (7.5 mL), sequentially washed with aqueous 10 % Na_2SO_3 (3 x 10 mL), H_2O (3 x 15 mL), brine (3 x 15 mL), dried (Na_2SO_4), filtered and concentrated in vacuo to give an inseparable mixture of **9** (560 mg, 47 %) and starting material (3:1) as a yellow oil. The yield is based on integration of the ^1H NMR spectrum, which is in agreement with reported data,[31] see S.I. The product was used directly without further purification. All attempts to obtain HRMS for this compound failed.

2-(Trimethylsilyl)ethyl 2-(1-amino-4-methyl-2-oxo-1,2,5,6-tetrahydropyridin-3-yl)acetate (11). Nanopowder zinc (927 mg, 14.2 mmol) and concentrated acetic acid (10 mL, 168 mmol) were added to a solution of *N*-nitroso compound **17** (1410 mg, 4.70 mmol) in dry methanol (10 mL) at 0 °C. The mixture was stirred at 0 °C for 20 min, after which the green-yellow colour had disappeared. The mixture was then allowed to attain rt, filtered through

celite and the filter cake was washed with methanol (3 x 5 mL). The combined organic phases were concentrated in vacuo, and the remaining acetic acid and solvent were azeotropically removed with toluene (3 x 10 mL) in vacuo to give hydrazide **11** (870 mg, 65 %) as a white solid. The product was used directly in the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.03 (s, 9H, H17/18/19), 0.99 (t, *J* = 8.5 Hz, 2H, H15), 1.91 (s, 3H, H11), 2.53 (t, *J* = 7.3 Hz, 2H, H2), 3.39 (s, 2H, H7), 3.66 (t, *J* = 7.3 Hz, 2H, H1), 4.17 (t, *J* = 8.5 Hz, 2H, H14); ^{13}C NMR (126 MHz, $CDCl_3$) 171.4, 166.2, 148.6, 122.4, 63.3, 48.6, 32.3, 30.8, 20.7, 17.5, -1.4. HRMS (ESI) *m*/*z* calcd for $C_{13}H_{23}N_2O_3Si$ $[M + H]^+$ 283.1478; found 283.1465.

***Tert*-butyl 4-methyl-2-oxo-3-(2-oxo-2-(2-(trimethylsilyl)ethoxy)ethyl)piperidine-1-carboxylate (15).** LHMDS (230 mL of 1M THF solution, 229.85 mmol) was added to a solution of **13** (43.0 g, 192 mmol) in THF (500 mL) at -78 °C. When the addition was complete, 2-(trimethylsilyl)ethyl 2-bromoacetate **14** (50.3 g, 192 mmol) dissolved in THF (50 mL), was slowly added to the solution and the temperature was kept below -60 °C. The solution was then allowed to reach -20 °C, for 1 h. It was then added to an ice-cooled solution of acetic acid (23 mL) and H_2O (150 mL), the THF was removed in vacuo after which the aqueous residue was extracted with EtOAc (200 mL). The organic layer was washed with aqueous 2 M $KHSO_4$ solution (100 mL), concentrated in vacuo and purified with silica gel column chromatography (30% EtOAc in heptane) to obtain **15** (60.6 g, 85 %), as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.03 (s, 9H, H26/27/28), 0.98 (t, *J* = 8.6 Hz, 2H, H24), 1.04 (d, *J* = 6.4 Hz, 3H, H11), 1.51 (s, 9H, H20/21/22), 1.54 – 1.57 (m, 1H, H3), 1.87 – 1.95 (m, 2H, H2), 2.41 (p, *J* = 5.5, 1H, H4), 2.69 (dd, *J* = 5.5, 17.0 Hz, 1H, H7), 2.80 (dd, *J* = 5.5, 17.0 Hz, 1H, H7), 3.65 (ddd, *J* = 3.8, 10.1, 13.0 Hz, 1H, H1), 3.74 (dt, *J* = 4.8, 13.0 Hz, 1H, H1), 4.17 (t, *J* = 8.6 Hz, 2H, H23); ^{13}C NMR (126 MHz, $CDCl_3$) 172.1, 172.0, 152.4, 82.3, 62.5,

47.7, 44.3, 33.3, 31.6, 31.0, 27.8, 20.3, 17.0, -1.7. HRMS (ESI) *m*/*z* calcd for $C_{18}H_{34}NO_5Si$ $[M + H]^+$ 372.2206; found 372.2240.

***Tert*-butyl 4-methyl-2-oxo-3-(2-oxo-2-(2-(trimethylsilyl)ethoxy)ethyl)-5,6-dihydropyridine-1(*2H*)-carboxylate (16).** LHMDS (47.8 mL of a 1M THF solution, 47.8 mmol) was added to a solution of **15** (14.7 g, 39.7 mmol) in THF (150 mL) and was allowed to stirr at -78 °C for 90 min. Phenyl selenylbromide (11.2 g, 47.6 mmol) dissolved in THF (20 mL) was then added to the solution which was stirred at -78 °C for 90 min and then stirred for an additional 2 h to reach -20 °C. The reaction was quenched with a saturated aqueous solution of NH_4Cl (300 mL) and extracted with EtOAc (3 x 300 mL) and the combined organic layers were dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was purified with silica gel column chromatography (20 to 100 % EtOAc in heptane) to give a regioisomeric mixture of α-substituted selenium intermediates as a dark-yellow oil. This mixture was dissolved in CH_2Cl_2 (150 mL), cooled to 10 °C, and excess of hydrogen peroxide (11.9 mL of a 30 % aqueous solution, 147.7 mmol) was added. The pH was adjusted to 7 with pyridine and after 20 min the reaction was quenched with a saturated aqueous solution of NH_4Cl (300 mL). The aqueous layer was extracted with CH_2Cl_2 (150 mL), then the combined organic phases were sequentially washed with H_2O (3 x 200 mL), brine (3 x 200 mL), dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was purified with silica gel column chromatography (10 to 100 % EtOAc in heptane) to give *endo*-cyclic **16** (1.3 g), *exo*-cyclic product (3.5 g), as well 2.1 g of an inseparable mixture (2:1, *exo*:*endo*), all as yellow oils. The undesired *exo*-cyclic product and the inseparable mixture were pooled (5.6 g, 15.2 mmol), dissolved in dry toluene (30 mL) and DBU (6.85 mL, 45.5 mmol) was added. The solution was heated at reflux for 12 h after which it was quenched with an aqueous solution of saturated NH_4Cl and brine (1:1, 150 mL) and extracted with CH_2Cl_2 (3 x

30 mL), dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was purified with silica gel column chromatography (35 to 100 % EtOAc in heptane) to give an additional 5.2 g of pure *endo*-cyclic **16** (in total 6.5 g, 45 %) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.03 (s, 9H, H26/27/28), 0.98 (t, J = 8.4 Hz, 2H, H24), 1.53 (s, 9H, H20/21/22), 1.93 (s, 3H, H11), 2.41 (t, J = 6.4 Hz, 2H, H2), 3.40 (s, 2H, H7), 3.82 (t, J = 6.4 Hz, 2H, H1), 4.16 (t, J = 8.4 Hz, 2H, H23); ^{13}C NMR (126 MHz, $CDCl_3$) 171.4, 164.1, 153.1, 151.3, 124.9, 82.9, 63.2, 42.8, 32.3, 31.2, 28.3, 21.0, 17.4, -1.4. HRMS (ESI) m/z calcd for $C_{18}H_{32}NO_5Si$ $[M + H]^+$ 370.2050; found 370.2086.

2-(Trimethylsilyl)ethyl 2-(4-methyl-1-nitroso-2-oxo-1,2,5,6-tetrahydropyridin-3-yl)acetate (17). TFA (1.5 mL, 19.5 mmol) was added to a solution of **16** (1.45 g, 3.92 mmol) in CH_2Cl_2 (15 mL). The solution was stirred at rt for 8 h, under air. The solvent and excess TFA were azeotropically removed with toluene (3 x 15 mL) in vacuo to generate the deprotected dihydropyrid-2-one as a yellow oil. The oil was used directly without further purification and was dissolved in dry diethyl ether (20 mL) to which *tert*-butyl nitrite (1.6 mL, 13.5 mmol) and pyridine (1.4 mL, 16.2 mmol) were added. The solution was allowed to reflux for 12 h, after which dry diethyl ether (10 mL) and an additional equivalent of *tert*-butyl nitrite was added. Then the solution was again allowed to reflux for 12 h, the solvent was removed in vacuo and the residue was purified with silica gel column chromatography (50% EtOAc in heptane) to give *N*-nitroso compound **17** (1.0 g, 89 %) as a green-yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.04 (s, 9H, H17/18/19), 1.01 (t, J = 8.62 Hz, 2H, H15), 2.06 (s, 3H, H11), 2.55 (t, J = 6.7 Hz, 2H, H2), 3.56 (s, 2H, H7), 3.88 (t, J = 6.7 Hz, 2H, H1), 4.21 (t, J = 8.66 Hz, 2H, H14); ^{13}C NMR (126 MHz, $CDCl_3$) 170.7, 162.7, 155.1, 123.2, 63.6, 37.7, 32.1, 30.0, 21.5, 17.5, -1.3. HRMS (ESI) m/z calcd for $C_{13}H_{23}N_2O_4Si$ $[M + H]^+$ 299.1427; found 299.1414.

(*E*)-Methyl 3-(((4-methyl-2-oxo-3-(2-oxo-2-(2-(trimethylsilyl)ethoxy)ethyl)-5,6-dihydropyridin-1(*2H*)-yl)imino)methyl)benzoate (18). Methyl 3-formylbenzoate **10** (286 mg, 1.74 mmol) and a catalytic amount of concentrated acetic acid (8.6 µL, 0.15 mmol) were added to hydrazide **11** (450 mg, 1.58 mmol) in methanol (4 mL) together with 4Å molecular sieves. The mixture was shaken at rt for 12 h during which the reaction was monitored with ^{1}H NMR spectroscopy. The mixture was then diluted with EtOAc (15 mL) and was sequentially washed with H_2O (25 mL) and brine (25 mL), dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was purified with preparative HPLC at pH10 (15 to 75 % acetonitrile in H_2O-NH_3) to give imine **18** (550 mg, 81 %) as a colourless solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.03 (s, 9H, H27/28/29), 1.00 (t, *J* = 8.45 Hz, 2H, H25), 1.98 (s, 3H, H11), 2.62 (t, *J* = 7.0 Hz, 2H, H2), 3.43 (s, 2H, H7), 3.91 (t, *J* = 7.0 Hz, 2H, H1), 3.93 (s, 3H, H30), 4.19 (t, *J* = 8.45 Hz, 2H, H24), 7.46 (t, *J* = 7.8 Hz, 1H, H16), 7.95 (dt, *J* = 1.3, 7.8 Hz, 1H, H19), 8.03 (dt, *J* = 1.3, 7.8 Hz, 1H, H20), 8.33 – 8.36 (m, 1H, H18), 8.91 (s, 1H, H14); ^{13}C NMR (126 MHz, $CDCl_3$) 171.5, 166.9, 163.2, 149.1, 147.7, 135.7, 131.6, 131.0, 130.8, 128.9, 128.8, 124.4, 63.2, 52.4, 47.6, 32.6, 31.0, 20.7, 17.5, -1.3. HRMS (ESI) *m*/*z* calcd for $C_{22}H_{31}N_2O_5Si$ $[M + H]^+$ 431.2002; found 431.2018.

Methyl 3-(((4-methyl-2-oxo-3-(2-oxo-2-(2-(trimethylsilyl)ethoxy)ethyl)-5,6-dihydropyridin-1(*2H*)-yl)amino)methyl)benzoate (19). Sodium cyanotrihydroborate (75 mg, 1.20 mmol) and a catalytic amount of acetic acid (2.74 µL, 0.05 mmol) were added to imine **18** (206 mg, 0.48 mmol) dissolved in methanol (4 mL). The reaction was monitored with ^{1}H NMR spectroscopy and was stirred at rt for 6 h. It was diluted with CH_2Cl_2 (7.5 mL), sequentially washed with aqueous 10 % $NaHCO_3$ solution (15 mL), H_2O (3 x 10 mL) and brine (3 x 10 mL), dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was

purified with silica gel column chromatography (35% EtOAc in heptane, isocratic) to give **19** (123 mg, 90 % based on recovery of 70 mg starting material) as a colourless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.04 (s, 9H, H27/28/29), 1.00 (t, *J* = 8.2 Hz, 2H, H25), 1.87 (s, 3H, H11), 2.31 (t, *J* = 7.2 Hz, 2H, H2), 3.34 (t, *J* = 7.2 Hz, 2H, H1), 3.40 (s, 2H, H7), 3.92 (s, 3H, H30), 4.03 (d, *J* = 5.5 Hz, 2H, H14), 4.18 (t, *J* = 8.2 Hz, 2H, H24), 5.53 (t, *J* = 5.5 Hz, 1H, H10), 7.41 (t, *J* = 7.7 Hz, 1H, H16), 7.57 – 7.61 (m, 1H, H19), 7.94 – 7.98 (m, 1H, H20), 8.05 – 8.09 (m, 1H, H18); ^{13}C NMR (126 MHz, $CDCl_3$) 171.6, 167.1, 165.7, 147.3, 138.4, 134.0, 130.5, 130.5, 128.9, 128.6, 122.9, 63.2, 54.7, 52.3, 47.8, 32.4, 31.1, 20.6, 17.5, -1.3. HRMS (ESI) *m*/*z* calcd for $C_{22}H_{33}N_2O_5Si$ $[M + H]^+$ 433.2159; found 433.2180.

2-(1-((3-(Methoxycarbonyl)benzyl)amino)-4-methyl-2-oxo-1,2,5,6-tetrahydropyridin-3-yl)acetic acid (20). TBAF (674 mg, 2.14 mmol) was added to **19** (770 mg, 1.78 mmol) dissolved in THF (5 mL) and the mixture was stirred at rt for 6 h. The solvent was removed in vacuo and the residue was redissolved in EtOAc (10 mL), then sequentially washed with aqueous 0.1 M HCl (3 x 15 mL), H_2O (3 x 15 mL) and brine (3 x 15 mL), dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was purified with silica gel column chromatography (0 to 20% methanol in EtOAc) to give carboxylic acid **20** (510 mg, 93 % based on recovery of 58 mg starting material) as a colourless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.98 (s, 3H, H11), 2.32 (t, *J* = 7.2 Hz, 2H, H2), 3.38 (s, 2H, H7), 3.40 (t, *J* = 7.2 Hz, 2H, H1), 3.93 (s, 3H, H24), 4.09 (s, 2H, H14), 7.44 (t, *J* = 7.7 Hz, 1H, H16), 7.57 – 7.61 (m, 1H, H19), 7.98 – 8.01 (m, 1H, H20), 8.05 – 8.08 (m, 1H, H18); ^{13}C NMR (126 MHz, $CDCl_3$) 170.6, 167.0, 167.0, 149.9, 137.3, 133.9, 130.7, 130.5, 129.4, 128.9, 120.8, 54.4, 52.4, 47.4, 35.3, 31.1, 20.5. HRMS (ESI) *m*/*z* calcd for $C_{17}H_{21}N_2O_5$ $[M + H]^+$ 333.1450; found 333.1458.

Chloromethyl (4-nitrophenyl) carbonate (23). Prepared as previously reported[30] with minor modifications. The ^{1}H NMR spectrum is in agreement with reported data and the product was used directly without further purifications, see S.I. HRMS (FI) *m*/*z* calcd for $C_8H_6NO_5Cl$ $[M + H]^+$ 230.9935; found 230.9933.

1-Chloroethyl (4-nitrophenyl) carbonate (24). Prepared as previously reported[30] with minor modifications. The ^{1}H NMR spectrum is in agreement with reported data and the product was used directly without further purifications, see S.I.HRMS (FI) *m*/*z* calcd for $C_9H_8NO_5Cl$ $[M + H]^+$ 245.0091; found 245.0101.

In vitro determination of thrombin inhibition. The thrombin inhibitor potency was measured with a chromogenic substrate method, in a Plato 3300 robotic microplate processor (Rosys AG, CH-8634 Hombrechtikon, Switzerland), using 96-well, half-volume microtitre plates (Costar, Cambridge, MA, USA; Cat No 3690). Stock solutions of test substances **1** and **2** in DMSO (72 μL, 0.1 - 1 mM) were diluted serially 1:3 (24 + 48 μL) with DMSO to obtain ten different concentrations, which are analyzed as samples in the assay. Each test sample (2 μL) was diluted with assay buffer (124 μL), then chromogenic substrate solution (12 μL of S-2366, Chromogenix, Mölndal, Sweden) in assay buffer and α-thrombin solution (12 μL, Human α-thrombin, Sigma Chemical Co. or Hematologic Technologies) in assay buffer were added and the samples mixed. The final assay concentrations were: test substance 0.00068 - 133 μM, S-2366 0.30 □M, α-thrombin 0.020 NIHU/mL. The linear absorbance increment during 40 minutes incubation at 37 °C was used for calculation of percentage inhibition for the test samples, as compared to blanks without inhibitor. The IC_{50} value, corresponding to the inhibitor concentration which causes 50% inhibition of the thrombin activity, is calculated from a log concentration vs. %-inhibition curve and K_M was determined to be 0.33 mM.

In vitro determination of inhibition of human serine proteases. The inhibition of each enzyme was studied at one substrate concentration and 10 concentrations for each inhibitor at 37 □C using a robotic system (FX Biomek, Beckman Coulter Inc, Nyon, Switzerland). The inhibitor dissolved in DMSO (2 µL, maximum 1.3% DMSO in assay at 133 µM final inhibitor concentration) was first added to the 96-wells plates (Costar 3695, Biostad, Saint-Julie, Québec, Canada) followed by assay buffer, which was specific for each enzyme (Table 5). FIXa was analysed in a buffer containing ethylene glycol as this was necessary for good activity.[32]

Table 5. Assay buffers for enzymes

Enzyme	Buffer
FIXa	50 mM Tris-HCl pH 7.4 (37 °C), 0.1 M NaCl, 5 mM $CaCl_2$ with 0.3% BSA and 33% ethylene glycol
Trypsin (Biotrend, Cat#9220-0604)	0.05 Tris-HCl, pH 7.4 (37 °C) with 40 mM NaCl and 20 mM $CaCl_2$ with 0.1% Tween 80
Plasmin (Chromogenix, ba 3982)	Stock buffer: 0.05 Tris-HCl, pH 7.4 (37 °C) with 0.1 M NaCl, 0.1% Tween 80 Glycerol-HCl: (1 mM HCl, 1% PEG 6000 and 43% glycerol). Assay buffer is stock buffer and Glycerol-HCl (2:1).
FVIIa/recombinant Tissue Factor (Novo®Seven, Novo Nordisk/Am.	50 mM Tris-HCl, pH 7.4 (37 °C) with 0.1 M NaCl, 5 mM $CaCl_2$ with 0.1% BSA.

Diag.4500B), FXIa (Haematologic Technol. Inc) and FXa (Haematologic Technologies Inc)	

The mixtures were incubated for 300 s, after which substrate (12 µL) was added. After 40 min the plates were read by determination of the absorption of light at 405 nm. Enzymes, substrates and their concentrations are given in Table 6. With these conditions, the absorption increases linearly for time >40 minutes. An IC_{50}-value was calculated for each inhibitor from the decrease in light absorption. The IC_{50} values were recalculated to inhibition constants (K_i) using the formula $IC_{50} = K_i * (1+(S/K_M))$ as all inhibitors are competitive and the K_M values were determined under the exact condition used in the assays. Substrate (S) concentrations and K_M values are give in Table 6.

Table 6. Enzymes, substrates at final concentrations in the determination of IC_{50}-values for inhibition of human enzymes.

Enzyme	Enzyme concentration	Substrate	Substrate concentration (mM)	K_M (mM)
FIXa	6 nM	Pefachrome FIXa® (Pefa-3107)	0.48	0.9
FVIIa + recombinant Tissue Factor	2.4 and 5.4 nM, resp.	Spectrozyme (Pefachrome 5979)	0.20	0.6
Plasmin	0.0018 CU/ml	S-2366 (Chromogenix)	0.3	0.23

FXIa	0.016 μg/ml	S-2366 (Chromogenix)	0.38	0.3
FXa	5 ng/mL	S-2765 (Chromogenix)	0.40	0.25
Trypsin	7 ng/mL	S-2222 (Chromogenix)	0.5	0.07

pK_a. The pKa values for direct thrombin inhibitor **2** was determined by pressure-assisted capillary electrophoresis and mass spectrometry, using the procedure reported previously.[33]

Solubility. Solutions of compounds **2-5** in DMSO (10 mM, 50 μL) were dried in glass vials using a Genevac vacuum evaporator (Genevac Ltd, Ipswich, UK). When dry, phosphate buffer (0.1 M, pH 7.4, 500 μL) was added to the vials to give a maximum compound concentration of 1 mM. The vials were put on an Eppendorf Thermomixer (Eppendorf AG, Hamburg, Germany) for 24 h at 25 °C. Precipitated and un-dissolved residues of dried compounds were removed by centrifugation in two steps, and the resulting supernatant was diluted 10-fold in CH_3CN/H_2O (1/1). Standards were prepared by a 10-fold dilution of 1 mM compound solutions in DMSO with CH_3CN/H_2O (1/1). Three further 10-fold dilution steps were applied to both the samples and standards. All samples were analyzed by LC/MS/MS using ultra-performance liquid chromatography (Waters ACQUITY UPLC, Milford, MA, USA) coupled to a Waters Xevo TQ-S instrument (Waters, Manchester, UK). Solubility was determined using the integrated peak areas of the samples in the linear MS response range. Reported values are the mean of N = 3 determinations.

$LogD_{7.4}$. LogD was determined using a modified version of the shake-flask method by partitioning of compounds **2-5** between 1-octanol and phosphate buffer (0.1 M, pH 7.4) at 20 °C.[21] Compounds **2-5** in DMSO solution were added to a 96-well plate containing 500 µL respectively of octanol and buffer. The plate was equilibrated on an Edmund Bühler shaker (Edmund Bühler GmbH, Hechingen, Germany) for 3 h at 20 °C and then centrifuged for 30 min to separate the phases before sampling. Aliquots of 5 µL octanol was transferred and diluted with 495 µL CH_3CN/H_2O (1/1). To avoid contamination in the buffer samples, the remaining octanol was removed before 150 µL of buffer samples were transferred. Octanol and buffer samples were diluted with CH_3CN/H_2O (1/1) in four steps of 10 times to yield octanol samples diluted 100 to 1,000,000 times and buffer samples diluted 1 to 10,000 times. All samples were analyzed by LC/MS/MS using ultra-performance liquid chromatography (Waters ACQUITY UPLC, Milford, MA, USA) coupled to a Waters Xevo TQ-S instrument (Waters, Manchester, UK). $LogD_{7.4}$ was calculated from the integrated peak areas of the samples in the linear MS response range. Reported values are the mean of N = 3 determinations.

Intrinsic permeability across Caco-2 cell monolayers. A monolayer of Caco-2 cells, cultured on semi-permeable polycarbonate surfaces, was used to study the permeability in the apical to basolateral direction. HTS Transwell-24 well permeable supports from Corning Corporation (Cambridge, MA, USA) were used. HBSS buffer (25 mM Hepes, pH 7.4, containing 50 µM quinidine, 30 µM benzbromarone and 20 µM sulfasalazine) was dispensed to the basal side of the monolayer. The assay was initiated by adding the test substrate at 10 µM in HBSS buffer (25 mM Hepes, pH 6.5, containing 50 µM quinidine, 30 µM benzbromarone and 20 µM sulfasalazine) to the apical side of the monolayer. Samples were withdrawn from the donor and acceptor sides before the addition of the test substrate and at

45 and 120 min post addition of the test substrate. The Transwell-24 plates were incubated in a shaking incubator at 37 °C between sampling. All samples were analyzed by LC/MS/MS using ultra-performance liquid chromatography (Waters ACQUITY UPLC, Milford, MA, USA) coupled to a Waters Xevo TQ-S instrument (Waters, Manchester, UK). The apparent permeability (Papp) was calculated from the compound concentration on the donor and receiver sides. Reported values are the mean of N = 3 determinations.

Efflux in Caco-2 cell monolayers. The effect of transporter interactions for cell monolayer permeability was studied using Caco-2 cells, and a very similar experimental setup as for the intrinsic permeability assay. The differences were that HBSS buffer pH 7.4 was used on both sides of the monolayer, and that the apparent permeability was determined in the apical to basolateral direction (P_{app} AB), as well as in the basolateral to apical direction and (P_{app} BA). The influence of transporter interactions on the permeability was described by the efflux ratio derived from the following equation: P_{app} BA / P_{app} AB. Reported values are the mean of N = 4 determinations.

Identification of in vitro metabolites in human hepatocytes. Cryopreserved human hepatocytes at a concentration of 10^6 viable cells/mL were used. Hepatocytes were thawed and then incubated for 10 min at 37 °C on an Eppendorf Thermomixer (Eppendorf AG, Hamburg, Germany). Test compounds **3**-**5**, dissolved in CH_3CN, were added to give a final concentration of 4 μM. To stop the reaction, 50 μL of samples were transferred to a plate containing 150 μL cold CH_3CN after 40 min incubations. Hepatocyte incubations without added compound were also sampled and used as blank samples during analysis. The samples were analyzed by ultra-performance liquid chromatography (Waters ACQUITY UPLC, Milford, MA, USA) coupled to a Waters Quattro Premier TOF instrument (Wythenshawe,

UK) equipped with an electrospray interface. The software package MetaboLynx (Waters) was used to process the data. Product ion spectra of major metabolites were acquired to allow interpretation and structural assignments.

In vivo rat PK. A week prior to dosing, male Han Wistar rats (Charles River Laboratories, Germany) were prepared by cannulation of the left carotid artery for blood sampling and by cannulation of the right jugular vein for intravenous administration. For the experiments using intraduodenal administration, rats were cannulated two days prior to dosing. The catheters were filled with heparin (100 IU/mL), exteriorized at the nape of the neck and sealed. The surgery was performed under isoflurane (Forene®, Abbott) anesthesia. After surgery the rats were housed individually and had free access to food and water. The rats had free access to drinking water and food during the experiment. On the experiment day, the test item formulation was administered orally by gavage, intravenously in the jugular vein and intraduodenally. Water with 5% mannitol and DMA:TEG:Water (1:1:1) was used as vehicles for compound **2** and **3**, respectively. At pre-defined time points, blood samples of about 0.150 mL were withdrawn from the carotid artery up to 24 h after dosing. The blood samples were collected in K2-EDTA plastic tubes and centrifuged, within 30 minutes, for five minutes at 10 000 g and 4 °C. The plasma was transferred to a 96 well plate and stored at –20 °C until analysis by LC-MS/MS. The studies were approved by the Göteborg Animal Research Ethical Board.

Bioanalytical method. In vivo rat plasma samples were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Waters Acquity UPLC chromatographic system coupled to a Waters Quattro Premier XE. The mobile phase consisted of (A) 0.2% formic acid in water and (B) 0.2% formic acid in LC-MS grade acetonitrile (Fisher

Scientific). Separation was performed on a 30×2.1 mm Acquity UPLC HSS T3 column with 1.8 μM particle size using a linear gradient of 5-95% B in 1.8 min, 95% B for 0.3 min, and returned to initial conditions for 0.01 min. The first 0.2 min containing salts and very polar compounds were diverted to waste and after that the effluent entered the MS. Before injection, 50 μL of plasma sample was protein-precipitated using 150 μL of acetonitrile containing internal standard. After vigorous shaking, the precipitated plasma samples were centrifuged at 2900 g and 4 °C for 20 min, and 75 μL of the supernatant was transferred and diluted with and equal volume of 0.2% formic acid in water. A standard curve from 0.5 nM to 10 μM were prepared in rat plasma and handled in the same way as the in vivo samples. The MS transitions from m/z = 470.94 to m/z = 301.01 and from m/z = 526.87 to m/z = 301.00 were recorded for compounds **2** and the **3**, respectively.

Pharmacokinetic data evaluation. Pharmacokinetic parameters were determined by standard non-compartmental methods using Phoenix® WinNonlin® Version 6.4 (Certara, L.P., Princeton, NJ). The maximum plasma concentrations (C_{max}) and the time at which it occurred (t_{max}) were determined. The area under the plasma concentration-time curve up to the last quantifiable sample (AUC_t) was calculated using the linear/logarithmic trapezoidal rule. The area under the plasma concentration-time curve extrapolated to infinite time (AUC_{inf}) was calculated as the sum of the AUC_t and the residual area (C_{last}/k), where C_{last} denotes the predicted plasma concentration at the last quantifiable sampling time and k is the terminal slope of the logarithmic plasma concentration-time plot. The apparent terminal half-life ($t_{1/2}$) was calculated as $ln2/k$, the plasma clearance (CL) as $Dose/AUC_{inf}$ and the volume of distribution at steady state (V_{SS}) as $Dose \times AUMC/AUC_{inf}^2$, where $AUMC$ denotes the area under the first moment of the plasma concentration-time curve. The extra-vascular bioavailability (F) was obtained from [$AUC_{extra\text{-}vascular}/Dose_{extra\text{-}}$

$_{vascular}$]/[$AUC_{intravenous}$/$Dose_{intravenous}$]. For extravascular administration of the prodrug **3**, the $AUC_{extra\text{-}vascular}$ used for determination of the bioavailability was that of compound **2**.

Associated Content

Supporting information. ^{1}H and ^{13}C NMR spectra for all compounds. Purity determination for tested compounds **2-5**.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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Abbreviations used

DTI, direct thrombin inhibitor; SD, standard deviation.

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