Characterization of the Interaction between Human α-Thrombin and Methyl 3-(2-Methyl-1-oxopropoxy)[1]benzothieno[3,2-*b*]furan-2-carboxylate (LY806303) Using Electrospray Mass Spectrometry and Tandem Mass Spectrometry

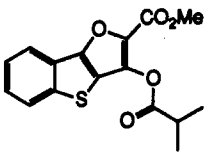
Daniel J. Sall* and Raymond E. Kaiser, Jr.

Divisions of Cardiovascular Research and of Bioprocess Research and Development, Lilly Research Laboratories, Indianapolis, Indiana 46285

Received March 15, 1993

Electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) have been used for the first time to study the interaction of human α-thrombin with methyl 3-(2-methyl-1-oxopropoxy)[1]benzothieno[3,2-*b*]furan-2-carboxylate (LY806303; 1), a potent and selective inhibitor whose mechanism of action was never fully defined. Using ESI-MS, it is shown that inhibitor 1 covalently modifies human α-thrombin as evidenced by a shift in the molecular weight of the native protein by 72 Da, which is consistent with isobutyrylation (C_4H_7O; 71 Da) of the enzyme at a single site. Tryptic digestion of the modified protein and tandem mass spectral analysis of isolated peptide fragments indicate that compound 1 acylates Ser-205 of the heavy chain of α-thrombin. Ser-205, along with His-43 and Asp-99 make up the catalytic triad within the active site of thrombin.

Thromboembolic diseases remain the leading cause of morbidity and mortality in developed societies. Thrombin, a trypsin-like serine protease, is a key mediator in such disease states, primarily through platelet activation and fibrin formation.[1] In response to the well-documented liabilities[2] associated with current antithrombotic therapies (i.e., heparin and warfarin),[3-5] the effort to develop safe and effective, direct-acting inhibitors of thrombin has intensified. Recently, methyl 3-(2-methyl-1-oxopropoxy)[1]benzothieno[3,2-*b*]furan-2-carboxylate (LY806303; 1) has been characterized as a potent and highly



LY806303; 1

selective inhibitor of thrombin.[6] While preliminary biochemical studies suggest that this inhibitor reacts irreversibly with thrombin to generate a catalytically inactive acyl/enzyme complex, no definitive proof for either the formation or structural nature of the acyl/enzyme complex or the site(s) of modification has been generated. A clear understanding of the interaction between inhibitor 1 and thrombin, in conjunction with the recently published thrombin X-ray crystallography data,[7-10] could facilitate the design of even more potent and selective agents.

Electrospray mass spectrometry (ESI-MS)[11] has recently emerged as a valuable tool for characterizing both covalent and noncovalent enzyme/inhibitor and receptor/ligand complexes.[12-19] ESI-MS, however, has never been applied to the characterization of thrombin, a high molecular weight glycoprotein with a susceptibility to autoproteolysis. We were interested in utilizing mass spectrometry to characterize the irreversible nature of the interaction between thrombin and inhibitor 1 and to determine the exact site of modification within the enzyme. Characterization of the inhibitor-modified thrombin complex represents the application of previously defined ESI-MS techniques[20,21] to a novel target. However, using mass spectrometry to determine the exact site of inhibitor interaction is not straightforward. In theory, identifying the site of covalent modification should be feasible if the size and complexity of the modified protein is reduced through enzymatic proteolysis and subsequent mass analysis of the resulting peptide fragments. Assuming that the covalent modification survives proteolysis, a peptide whose mass increases relative to the control should be discernible. Subsequent tandem mass analysis (MS/MS) or Edman sequence analysis of the isolated modified peptide(s) could pinpoint the exact residue(s) being modified, thereby yielding a microscopic view of the complexation between enzyme and inhibitor. This methodology would be restricted to covalent modifications since noncovalent interactions should be disrupted upon proteolysis. This paper reports the use of ESI-MS and MS/MS to characterize the nature and site of interaction of compound 1 with human α-thrombin.

A. Characterization of the Thrombin/Inhibitor 1 Adduct by Electrospray Ionization Mass Spectrometry (ESI-MS)

ESI-MS is capable of providing accurate molecular weights of biopolymers to about 1 in 10 000 Da. In contrast to traditional methods for determining molecular weights of proteins, such as SDS polyacrylamide gel electrophoresis, ESI-MS is an ideal tool for probing enzyme–inhibitor interactions because of its better mass accuracy and higher mass resolving power. Figure 1 illustrates the ESI mass spectrum of commercially obtained purified human α-thrombin, prepared from activation of homogeneous human prothrombin by factor Va, factor Xa, phospholipid, and calcium. The top spectrum (Figure 1a) shows the multiplicity of charging which is typically observed for proteins while the bottom spectrum (Figure 1b) shows a deconvoluted spectrum of the top and represents the experimentally derived molecular weight of the enzyme.[22] The control human α-thrombin sample shows at least three predominant species, with the major component having a molecular weight of 36 029 ± 3.1 Da, which is proposed

* Author to whom correspondence should be addressed.

0022-2623/93/1836-2350$04.00/0

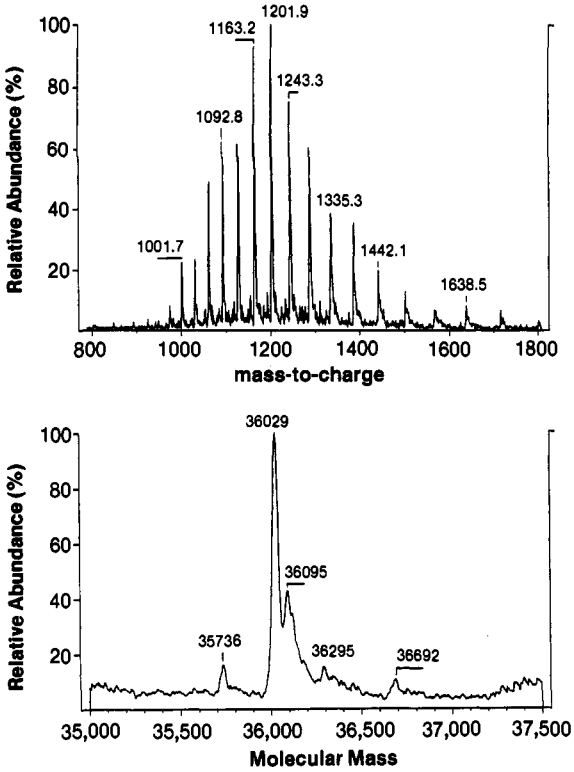


Figure 1. Native human α-thrombin: (a) ESI mass spectrum, (b) deconvoluted mass spectrum.

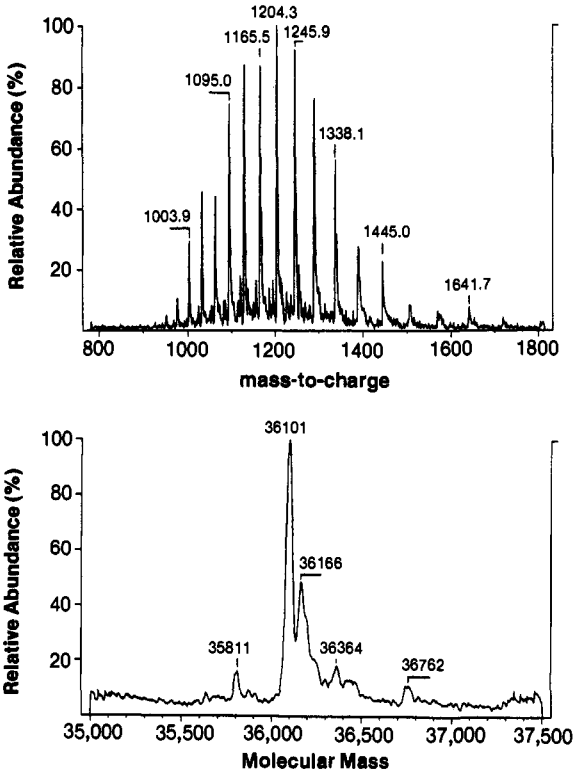


Figure 2. LY806303-treated thrombin: (a) ESI mass spectrum, (b) deconvoluted mass spectrum.

to correspond to the native form of human α-thrombin. α-Thrombin is a glycoprotein made up of two polypeptide chains of 36 (A-chain) and 259 amino acid residues (B-chain) which are covalently linked by a single disulfide bond.[7] The calculated molecular weight of the primary sequence of human α-thrombin is 33 821 Da (average isotopic MW), making the molecular weight contribution from the single glycosylation site approximately 2208 Da, which is consistent with a proposed oligosaccharide structure of a typical Man_3-$GlcNAc_2$ core, with an outer chain of NeuAcα2→6Galβ1→4GlcNAc.[23] Surprisingly, thrombin's asparagine-linked oligosaccharide appears to be rather homogeneous, which is often not the case for other glycoproteins.[24] A small degree of microheterogeneity is observed in the spectrum from a related protein at 35 736 ± 3.2 Da, which is believed to correspond to native thrombin less sialic acid. The observance of this glycoform is expected since sialic acid is most commonly incorporated during the last step of physiological protein glycosylation.[25] Another related product is observed at 36 095 ± 1.8 Da; however, it is not possible to ascertain whether this is a gas-phase adduct resulting from the experimental conditions[21,26] or a protein impurity in the human α-thrombin sample. Other minor components of unknown origin are observed at 36 295 ± 2.8 and 36 692 ± 3.1 Da. It is conceivable that other related proteins are present and that the resolution of this technique does not allow their mass separation.

Thrombin was treated with 20 equiv of inhibitor 1 and the mixture analyzed by ESI-MS (Figure 2). The deconvoluted spectrum (Figure 2b) shows a single major component at 36 101 ± 2.0 Da with no detection of native protein (36 029 Da). The molecular weight of this component (thrombin + 72 Da) is consistent (within experimental error) with protein incorporation of a single isobutyryl group (C_4H_7O; 71 Da). Compound 1 does have a labile isobutyryloxy functionality at C-3 which could undergo nucleophilic addition and acyl transfer. Conceivably, covalent modification of thrombin could also result from enzymatic attack at the C-3 methyl ester. However, no protein component consistent with the transfer of the substituted benzothieno[3,2-*b*]furan nucleus ($C_{15}H_{11}O_4S$, 287 Da) to thrombin is detected in the ESI spectrum.

Since ESI-MS is probably the "softest" of all ionization techniques currently available, noncovalent complexes have been shown to survive the ionization process for subsequent mass analysis.[13-15,27] Under these conditions, it is possible that isobutyric acid, resulting from *in situ* inhibitor hydrolysis, could noncovalently coordinate with thrombin and that the molecular weight of this adduct (thrombin + 88 Da) is indistinguishable from the isobutyrylated protein (thrombin + 71 Da). Denaturing conditions, however, such as the high concentrations of organic solvents used in these experiments for both sample desalting by reverse-phase HPLC and for operation of the ESI, is reported to cause protein denaturation,[28-30] making it unlikely that the acyl–enzyme complex is noncovalent. In fact, incubation of thrombin with 20 equiv of isobutyric acid under standard conditions leads to no change in the molecular weight of the native enzyme. The most plausible explanation is that inhibitor 1 binds to thrombin and covalently modifies the enzyme through isobutyrylation of a single residue.

It is interesting that, even after prolonged incubation periods (30 min) in the presence of a 20-fold excess of inhibitor, only the monoacylated enzyme adduct is detected, suggesting that ester 1 is not a general acylating agent, but binds selectively to a single site before acyl transfer occurs. Selective binding is certainly consistent with the thrombin specificity that inhibitor 1 displays relative to other trypsin-like serine proteases.[6]

Light Chain

TFGSGEADCGLRPLFEKKSLEDKTERELLESYIDGR

Heavy Chain

IVEGSDAEIGMSPWQVMLFRKSPQELLCGASLISDRWVLT

AAHCLLYPPWDKNFTENDLLVRIGKHSRTRYERNIEKISM

LEKIYIHPRYNWRENLDRDIALMKLKKPVAFSDYIHPVCL

PDRETAASLLQAGYKGRVTGWGNLKETWTANVGKGQPSVL

QVVNLPIVERPVCKDSTRIRITDNMFCAGYKPDEGKRGDA

CEGDSGGPFVMKSPFNNRWYQMGIVSWGEGCDRDGKYGFY

THVFRLKKWIQKVIDQFGE

Figure 3. Primary sequence of human α-thrombin: (a) lysine and arginine residues are indicated by an (●) and represent the potential sites of C-terminal cleavage by trypsin, (b) peptides 2 and 3 as they occur in the native enzyme are underlined.

B. Tryptic Digestion of Isobutyrylated Thrombin and Characterization of the Fragments by On-Line Liquid Chromatography/Electrospray Ionization Mass Spectrometry (LC–ESI-MS)

Thrombin, a trypsin-like serine protease which cleaves Arg-Gly bonds in both the Aα and Bβ chains of fibrinogen, contains the catalytic triad Ser-205, His-43, and Asp-99. It is conceivable that inhibitor 1 covalently modifies the active site of the enzyme through isobutyrylation of Ser-205. However, it would be difficult to rationalize the thrombin specificity of this agent relative to trypsin since the active site topographies of trypsin and thrombin are nearly identical. *N*-butyrylimidazole, for instance, is thought to acylate thrombin at either His-43 or Ser-205 of the catalytic triad and shows very little selectivity for thrombin over other trypsin-like serine proteases.[31] Because of the high thrombin specificity reported for agent 1,[6] we were interested in identifying the exact site of modification on the enzyme with the potential that this information could be used for the design of more potent and selective agents.

To identify the site of isobutyrylation in the thrombin/inhibitor adduct, it was necessary to reduce the complexity of the modified protein by digestion into smaller, more manageable peptides. Trypsin is an arginine/lysine-specific endopeptidase which could conceivably generate 36 different peptides upon complete digestion of native thrombin (Figure 3a).[32] Separation and analysis of the tryptic peptides by on-line LC–ESI-MS[33] analysis could simplify the isolation and identification of a residue-modified peptide. Subsequent sequence analysis could lead to characterization of the exact amino acid which has been isobutyrylated.

Native human α-thrombin and inhibitor-modified thrombin were subjected to tryptic digestion and the reaction samples were analyzed by LC–ESI-MS. Figure 4 shows the total ion chromatograms for both the digested native human α-thrombin and the inhibitor-treated thrombin samples (Figure 4a,b respectively). Visual inspection of the chromatograms between 31 and 32 min shows that at least two peptides in the digest of the native enzyme (A and B, Figure 4a) are not present in the inhibitor-treated sample (Figure 4b). Upon comparison of the molecular weights of the peptides observed in Figure 4a to those in Figure 4b (molecular weight data not shown), two unique peptides (C and D) were observed in Figure 4b which happen to coelute with other tryptic peptides found in both digests.

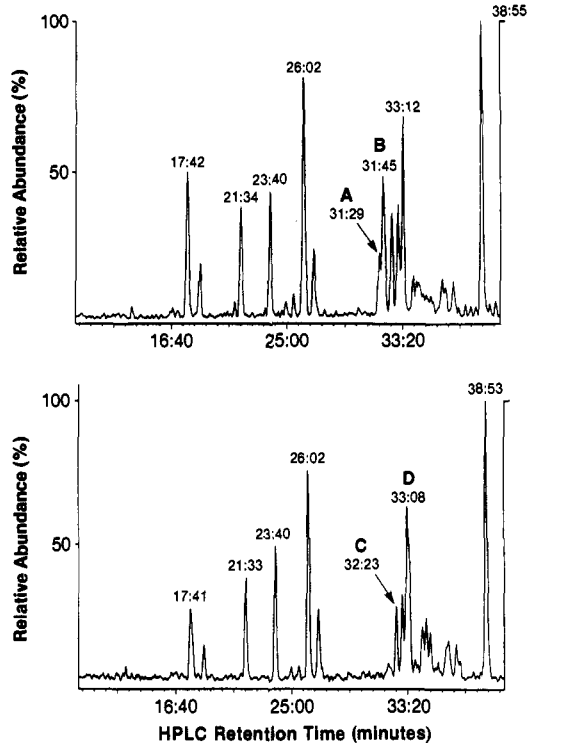


Figure 4. LC–ESI total ion chromatogram of tryptic digested thrombin: (a) native human α-thrombin; the molecular weights of components A and B are 3709 ± 0.4 and 3553 ± 0.4 Da, respectively; (b) LY806303-treated human α-thrombin; the molecular weights of components C and D are 3779 ± 0.4 and 3633 ± 0.3 Da, respectively.

The ESI-MS-derived molecular weights assigned to peaks A and B (Figure 4a) are 3709 ± 0.4 Da (A; 31 min, 29 s) and 3553 ± 0.4 Da (B; 31 min, 45 s), respectively. Comparison of the molecular weights calculated for the protein fragments expected from tryptic digestion of thrombin (Figure 3a) with the ESI-MS-derived molecular weights of components A and B suggests that A and B correspond to structures 2 and 3. Peptide 2 is a disulfide-

H_2N-RGDACEGDSGGPFVMK$_{(197-212)}$

S——S

H_2N-WYQMGIVSWGEGCDR$_{(219-233)}$

2 (A)

H_2N-GDACEGDSGGPFVMK$_{(196-212)}$

S——S

H_2N-WYQMGIVSWGEGCDR$_{(219-233)}$

3 (B)

linked peptide consisting of residues 197–212 and 219–233 of the heavy chain of α-thrombin (Figure 3b). Peptide 3 is identical to compound 2 with the exception of an N-terminal Arg-197. (Note that residue 196 is lysine.) Peaks C and D that appear in the tryptic digest of inhibitor-modified thrombin (Figure 4b) have ESI-MS-determined molecular weights of 3779 ± 0.4 Da (C; 32 min, 23 s) and 3623 ± 0.3 Da (D; 33 min, 8 s), respectively. These peptides have masses which are 70 Da higher than peptides 2 and 3, respectively, suggesting that peptides C and D are isobutyrylated derivatives of the native peptide fragments 2 and 3. Peptides C and D are tentatively assigned structures 4 and 5, respectively, in which the site of isobutyrylation is as yet undetermined.

H_2N-RGDACEGDSGGPFVMK$_{(197-212)}$
S——S C_4H_7O
H_2N-WYQMGIVSWGEGCDR$_{(219-233)}$

4 (C)

H_2N-GDACEGDSGGPFVMK$_{(198-212)}$
S——S C_4H_7O
H_2N-WYQMGIVSWGEGCDR$_{(219-233)}$

5 (D)

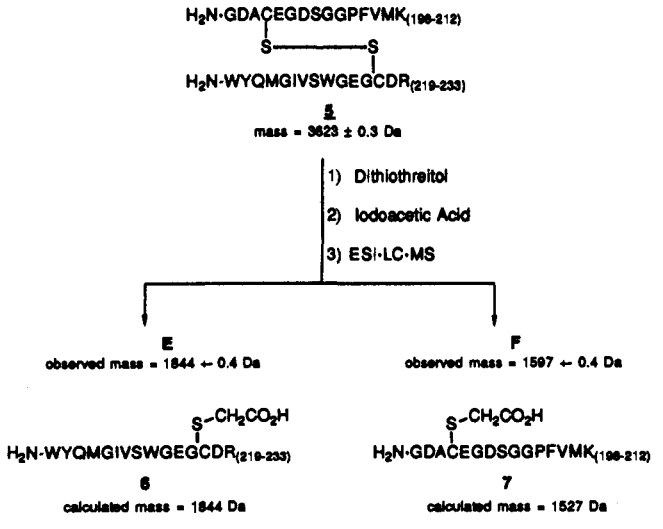
All of the other peptide fragments detected in the HPLC chromatograms of the tryptic digests of both the control and the inhibitor-modified thrombin samples were assigned molecular weights. There were no other peptides in the inhibitor-treated sample that showed mass increases relative to the control. The mass-modified peptides 4 and 5 were isolated by HPLC fractionation for further analysis. We chose to concentrate on the structural identity of peptide 5 because of the greater quantities of material obtained from HPLC purification relative to peptide 4.

To further reduce the size and complexity of peptide 5, the disulfide bond was reduced with dithiothreitol, and the resulting thiols were carboxymethylated with iodoacetic acid.[34] The reaction was analyzed by LC–ESI-MS, which showed two constituents with masses of 1844 ± 0.4 and 1597 ± 0.4 Da (E and F, respectively; Scheme I). The observed mass of component E is consistent with the calculated molecular weight of the carboxymethylated peptide fragment containing residues 219–233 of thrombin (6; Scheme I), indicating the absence of any modified amino acid. The observed mass of component F, on the other hand, is 70 Da higher than the mass calculated for the carboxymethylated fragment containing residues 198–212 (7; 1527 Da; Scheme I), suggesting that peptide F contains the isobutyrylated amino acid. The molecular weight of the isolated peptide F was reconfirmed by direct infusion ESI-MS analysis (Figure 5).

C. Characterization of Modified Tryptic Peptide F by Tandem Mass Spectrometry (MS/MS)

From the ESI-MS data, isobutyrylated peptide F is assigned structure 8. The amino acid composition of peptide 8 contains a number of residues which could be isobutyrylated at their side chains, including Ser-205 of

Scheme I



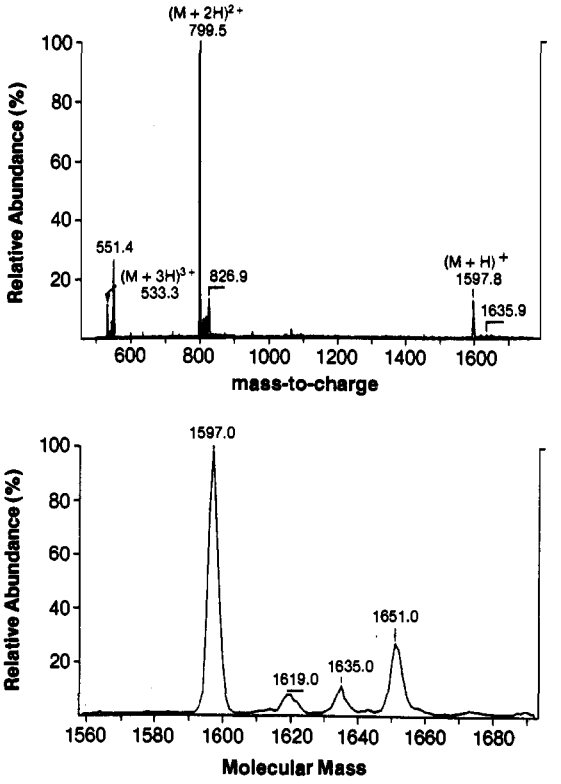


Figure 5. ESI mass spectrum of isobutyrylated peptide F (8).

H_2N-GDACEGDSGGPFVMK$_{(198-212)}$ · (C_4H_7O)

8

the thrombin active site. In theory, sequencing of this peptide without disrupting the modified amino acid could pinpoint the exact site of isobutyrylation. Automated Edman sequence analysis is routinely used to sequence the first 20–30 N-terminal residues of a peptide or protein, and the first 10 residues of peptide 8 were confirmed using this technique. No information regarding the exact site of modification was provided during sequencing, however. A slight boost in serine and dehydroalanine levels were observed at cycle 8, though yields were very low due to apparent degradation. It is known that Edman degradation, coupled with amino acid analysis and HPLC analysis, has serious limitations with regard to characterizing structurally modified proteins and peptides.[35-37] Many commonly occurring modifications are lost or destroyed by the harsh cleavage and derivatization conditions employed during Edman degradation and amino acid analysis.[34,38,39] Even if the modified amino acid is not destroyed, it may be easily misassigned or go unrecognized since Edman sequencing is based entirely on chromatographic retention times relative to one of the 20 commonly occurring amino acids or derivatives thereof. We turned our efforts toward the utilization of tandem mass spectrometry (MS/MS), which in contrast to Edman degradation, provides information on an intrinsic property of the molecule, its molecular mass. In fact, tandem mass spectrometry (MS/MS) has been used to yield partial to complete sequence information for peptides containing up to 25 amino acid residues.[20,40]

Tandem mass spectrometry consists of selectively passing the ionized peptide of interest through the first quadrupole of the triple-quadrupole mass spectrometer. The ion population then passes through the second quadrupole, which is pressurized with argon, thereby causing energetic collisions and subsequent fragmentation

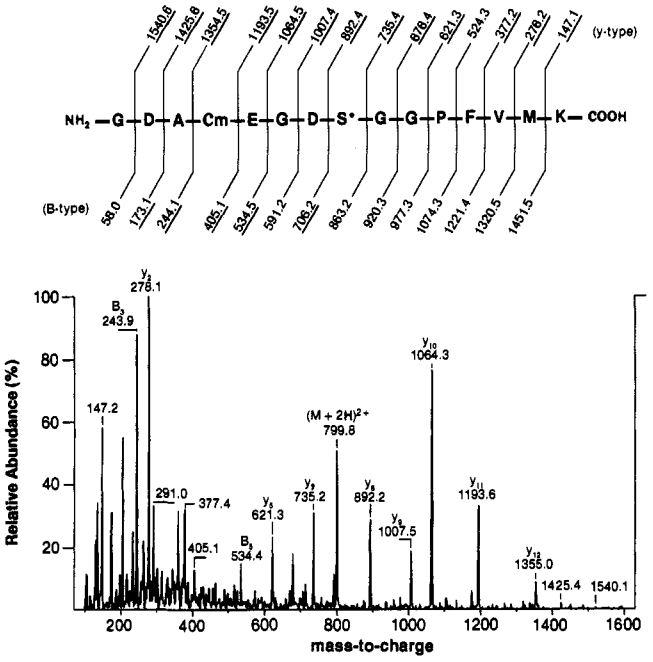


Figure 6. ESI MS/MS spectrum of the doubly protonated peptide 8 (m/z 799.5) showing the sequential loss of amino acids from the N-terminus of the peptide. Underlined fragment masses indicate that the ions were observed in the MS/MS spectrum.

of the incident ion. Fragmentation occurs from either the amino or carboxyl terminus, producing an incremental mass reduction of the peptide by the residue weight of the terminal amino acid. The degree of fragmentation is dependent upon the collisional energy.[20] The third quadrupole acts as the mass analyzer to measure the molecular weights of the sequence specific fragment ions.

Because trypsin cleaves at the C-terminus of lysine and arginine, it yields peptides with at least two basic sites for protonation by electrospray ionization: the N-terminal amine and the side chain of the C-terminal lysine (in the case of peptide 8).[41,42] The doubly protonated tryptic peptide undergoes sequential cleavage of the amide bonds beginning at either the amino or carboxyl terminus, or in some instances from both directions. Given a peptide whose primary sequence is known and which has one of its constituent amino acids modified, it should be possible to use MS/MS to sequence the modified peptide and identify the exact amino acid whose molecular weight has increased.

Tandem mass spectrometric analysis of the doubly protonated form of peptide 8 (m/z 799.7) is shown in Figure 6. As expected, the sequential loss of amino acid residues from the N-terminus of the peptide, often referred to as y-type ions,[20] is observed. For example, the starting molecular weight of peptide 8 is 1597 ± 0.4 Da so that the sequential loss of glycine (57 Da) and aspartic acid (115 Da) from the N-terminus results in ions at m/z 1540 and 1425, respectively. Further loss of alanine (71 Da) yields a fragment at m/z 1355. Sequential loss of the expected amino acids is observed until loss of Ser-205 (87 Da), where one would expect to observe an ion at m/z 805. However, the amino acid lost during this cycle has a mass of 157 Da, giving rise to a peak at 735 Da. No naturally occurring amino acid has a mass of 157 Da. The molecular weight of this residue does, however, correspond to [Ser (MW 87) + 70 Da], which is consistent with isobutyrylated serine. Following cleavage of the isobutyrylserine residue, the sequential loss of ions corresponding to the remainder of peptide 8 was observed. In addition to observing predominantly y-type ions, some b-type ions are observed. No other modified amino acids were detected.

While we cannot rule out the possibility that agent 1 acylates an alternate site on thrombin and that acyl migration to Ser-205 occurs during processing of the various proteins, this data strongly suggests that Ser-205 is the site of inhibitor modification. In light of the present mass spectrometry data, the mechanistic details of thrombin modification by compound 1 become clear. Namely, inhibitor 1 engages in very specific, tight-binding interactions at the active site of thrombin before transacylation occurs at Ser-205.

In this study, we have used electrospray ionization mass spectrometry to show that ester 1, a potent and highly selective inhibitor of thrombin, covalently modifies the enzyme through isobutyrylation. Extending the scope of the preexisting state-of-the-art technology in mass spectrometry, we have used MS/MS to determine that inhibitor 1 is an active-site-directed agent and acylates Ser-205 of the catalytic triad. To answer unequivocally whether acylation is solely responsible for the ability of compound 1 to inhibit thrombin, the extent of protein acylation must be correlated with the degree of enzyme inhibition. Studies are currently underway to address this question.

The strategy outlined in this paper can be used to probe the site of covalent modifications without significant effort. Since mass spectrometry is becoming a more widely used tool for protein characterization, learning more about the irreversible nature of new enzyme inhibitors has become more accurate and less time-consuming than conventional methods. This new understanding of the binding orientation of inhibitor 1, coupled with recently published X-ray crystallography data on human α-thrombin, should aid in the design of more potent and selective inhibitors.

D. Experimental Section

The reaction of human α-thrombin (Enzyme Research Laboratories, South Bend, IN) with methyl 3-(2-methyl-1-oxopropoxy)[1]benzothieno[3,2-*b*]furan-2-carboxylate (LY806303; 1; Eli Lilly and Co., Indianapolis, IN) was carried out by adding a solution of 54 nmol of the inhibitor in 50 μL of DMSO to 2.7 nmol (100 μg) of human α-thrombin in 0.5 mL Tris buffer, pH 7.4. The reaction was allowed to proceed at 25 °C for 15 min and was frozen at −78 °C to reduce enzymatic activity. A thrombin control was prepared by following the same procedure, less the addition of the inhibitor. The samples were later thawed and prepared for ESI-MS analysis by desalting the material on an Applied Biosystem RP-4 NEWGUARD column. The samples were loaded and washed at 20% acetonitrile/0.1% TFA for 1 min followed by a step elution from 20–80% acetonitrile/0.1% TFA at a flow rate of 1 mL/min. The eluant giving an UV absorption at 280 nm was collected and vacuum evaporated to dryness. The sample was then reconstituted in 5% acetic acid/deionized water and 50% acetonitrile to a final concentration of approximately 10 pmol/μL for ESI-MS analysis.

Electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) were performed on a Finnigan MAT TSQ700 triple quadrupole mass spectrometer. For mass spectral analysis, a Harvard apparatus syringe pumping system was used to infuse the protein samples directly into the ESI source at a flow rate of 1 μL/min. An optimum voltage of 4200 V was maintained on the ESI-MS electrode for multiple protonation of the protein. The mass spectrometer (calibrated with horse heart apomyoglobin, Sigma Chemical) was scanned from 500 to 2000 mass-to-charge units in 5 s when in the profile mode during direct infusion sampling. Twelve scans were averaged to obtain the mass-to-charge spectra. Finnigan's computer algorithm was utilized to interpret the mass-to-charge spectra to yield a deconvoluted spectrum indicating the molecular

weight(s) of the protein. Tandem mass spectrometric studies were done at a collision offset of -20 V and at a collision cell pressure of 1 mTorr of argon.

The trypsin digest was accomplished using sequencing-grade trypsin (Sigma) at a 1:100 molar ratio to thrombin in 0.25 M Tris acetate, pH 7.5. The mixture was incubated at 37 °C for 18 h and was quenched by adjusting the pH to 1 with 1 N aqueous HCl. The tryptic digest was analyzed via high-performance liquid chromatography (HPLC), which was interfaced to the ESI source. A Vydac C-18 HPLC column (0.46 cm × 25 cm × 5 μm) was used for separation, using a linear gradient of 5–60% B over 55 min (solvent A, 10% acetonitrile/0.1% TFA; solvent B, 90% acetonitrile/0.1% TFA) at a flow rate of 1 mL/min. The HPLC column eluant was split approximately 500:1 in order to deliver approximately 2 μL/min to the ESI source. 2-Methoxyethanol (Aldrich) was used as a "sheath liquid" and delivered at 2 μL/min for mixing with the HPLC eluant for more efficient desolvation prior to ionization. Nitrogen was used as a "sheath gas" to aid in nebulizing the HPLC eluant/2-methoxyethanol mixture at a pressure yielding an optimum signal-to-noise ratio in the mass spectrum. The remainder of the eluant from the HPLC column was directed to a UV detector, set at 220 nm, in order to provide a UV chromatogram with the total ion chromatogram.

For reduction and carboxymethylation studies, the peptide was dissolved at 1 mg/mL in a solution containing 6 M guanidinium chloride, 0.1 M Tris, and 1 mM EDTA, adjusted to pH 8.3 with HCl. Dithiothreitol was added to a concentration of 2 mM. Argon was passed through the solution, sealed, and the solution was incubated at 37 °C for 1 h. After incubation, a solution of 50 mM iodoacetic acid was added to yield a final concentration of 2 mM. Argon was again passed through the sample, which was then sealed and incubated in the dark at 37 °C for 1 h.

Edman sequence analysis was performed on a Porton (Beckman) LF-3000 gas-phase protein sequencer.

References

(1) Machovich, R. Thrombin and Hemostasis. In *The Thrombin*, 1st ed.; Machovich, R., Ed.; CRC Press Inc.: Boca Raton, FL, 1984; V1, pp 1–22.

(2) Amerena, J.; Mashford, M. L.; Wallace, S. Adverse Effects of Anticoagulants. *Adverse Drug React. Acute Poisoning Rev.* **1990**, *9*, 1–36.

(3) Rosenberg, R. D. Chemistry of the Hemostatic Mechanism and its Relationship to the Action of Heparin. Molecular Basis of Heparin Action. *Fed. Proc.* **1977**, *36*, 10–18.

(4) Smith, G. F.; Sundboom, J. L. Heparin and Protease Inhibition II. The Role of Heparin in the alpha-2-Antithrombin Inactivation of Thrombin, Plasmin and Trypsin. *Thromb. Res.* **1981**, *22*, 115–133.

(5) Smith, G. F.; Neubauer, B. L.; Sundboom, J. L.; Best, K. L.; Goode, R. L.; Tanzer, L. R.; Merriman, R. L.; Frank, J. D.; Herrmann, R. G. Correlation of the In Vivo Anticoagulant, Antithrombotic, and Antimetastatic Efficacy of Warfarin in the Rat. *Thromb. Res.* **1988**, *50*, 163–174.

(6) Sall, D. J.; Berry, D. R.; Coffman, W. J.; Craft, T. J.; Denney, M. L.; Gifford-Moore, D. S.; Kellam, M. L.; Smith, G. F. Characterization of LY806303 as a Potent and Selective Inhibitor of Thrombin. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1025–1028.

(7) Bode, W.; Mayr, I.; Baumann, U.; Huber, R.; Stone, S. R.; Hofsteenge, J. The Refined 1.9 Å Structure of Human α-Thrombin: Interaction with D-Phe-Pro-Arg Chloromethylketone and Significance of the Tyr-Pro-Pro-Trp Insertion Segment. *EMBO J.* **1989**, *8*, 3467–3475.

(8) Banner, D. W.; Hadváry, P. Crystallographic Analysis at 3.0 Å Resolution of the Binding to Human Thrombin of Four Active Site-directed Inhibitors. *J. Biol. Chem.* **1991**, *266*, 20085–20093.

(9) Bode, W.; Turk, D.; Stürzebecher, J. Geometry of Binding of the Benzamidine- and Arginine-based Inhibitors N^{α}-(2-Naphthylsulfphonyl-glycyl)-DL-*p*-amidinophenylalanyl-piperidine (NAPAP) and (2R,4R)-4-Methyl-1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulphonyl)-L-arginyl]-2-piperidine Carboxylic Acid (MQPA) to Human α-Thrombin. *Eur. J. Biochem.* **1990**, *193*, 175–182.

(10) Rydel, T. J.; Tulinsky, A.; Bode, W.; Huber, R. Refined Structure of the Hirudin-Thrombin Complex. *J. Mol. Biol.* **1991**, *221*, 583–601.

(11) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. Electrospray Ionization for Mass Spectrometry of Large Biomolecules. *Science* **1989**, *246*, 64–71.

(12) Stevenson, D. E.; Feng, R.; Storer, A. C. Detection of Covalent Enzyme-Substrate Complexes of Nitrilase by Ion-Spray Mass Spectroscopy. *FEBS* **1990**, *277*, 112–114.

(13) Ganem, B.; Li, Y.-T.; Henion, J. D. Detection of Noncovalent Receptor-Ligand Complexes by Mass Spectrometry. *J. Am. Chem. Soc.* **1991**, *113*, 6294–6296.

(14) Ganem, B.; Li, Y.-T.; Henion, J. D. Observation of Noncovalent Enzyme-Substrate and Enzyme-Product Complexes by Ion-Spray Mass Spectrometry. *J. Am. Chem. Soc.* **1991**, *113*, 7818–7819.

(15) Katta, V.; Chait, B. T. Observation of the Heme-Globin Complex in Native Myoglobin by Electrospray-Ionization Mass Spectrometry. *J. Amer. Chem. Soc.* **1991**, *113*, 8534–8535.

(16) Ashton, D. S.; Bedell, C. R.; Cooper, D. J.; Green, B. N.; Oliver, R. W. A.; Welham, K. J. Some Electrospray Mass Spectrometric Evidence for the Existence of Covalent O-acyl Enzyme Intermediates. *FEBS* **1991**, *292*, 201–204.

(17) Edmonds, C. G.; Loo, J. A.; Barinaga, C. J.; Udseth, H. R.; Smith, R. D. Capillary Electrophoresis-Electrospray Ionization-Mass Spectrometry. *J. Chromatogr.* **1989**, *474*, 21–37.

(18) Shneier, A.; Kleanthous, C.; Deka, R.; Coggins, J. R.; Abell, C. Observation of an Imine Intermediate on Dehydroquinase by Electrospray Mass Spectrometry. *J. Am. Chem. Soc.* **1991**, *113*, 9416–9418.

(19) Loo, J. A.; Ogorzalek Loo, R. R.; Light, K. J.; Edmonds, C. G.; Smith, R. D. Multiply Charged Negative Ions by Electrospray Ionization of Polypeptides and Proteins. *Anal. Chem.* **1992**, *64*, 81–88.

(20) Biemann, K. Mass Spectrometry of Peptides and Proteins. *Annu. Rev. Biochem.* **1992**, *61*, 977–1010.

(21) Smith, R. D.; Loo, J. A.; Ogorzalek Loo, R. R.; Busman, M.; Udseth, H. R. Principles and Practice of Electrospray Ionization Mass Spectrometry for Large Polypeptides and Proteins. *Mass Spectrom. Rev.* **1991**, *10*, 359–451.

(22) Mann, M.; Meng, C. K.; Fenn, J. B. Interpreting Mass Spectra of Multiply Charged Ions. *Anal. Chem.* **1989**, *61*, 1702–1708.

(23) Mizuochi, T.; Yamashita, K.; Fujikawa, K.; Kisiel, W.; Kobata, A. The Carbohydrate of Bovine Prothrombin. *J. Biol. Chem.* **1979**, *254*, 6419–6425.

(24) Kornfeld, R.; Kornfeld, S. Assembly of Asparagine-Linked Oligosaccharides. *Annu. Rev. Biochem.* **1985**, *54*, 631–664.

(25) Hubbard, S. C.; Ivatt, R. J. Synthesis and Processing of Asparagine-Linked Oligosaccharides. *Ann. Rev. Biochem.* **1981**, *50*, 553–583.

(26) Chowdhury, S. K.; Viswanatham, K.; Beavis, R. C.; Chait, B. T. Origin and Removal of Adducts (Molecular Mass = 98*n*) Attached to Peptide and Protein Ions in Electrospray Ionization Mass Spectra. *J. Am. Soc. Mass Spectrom.* **1990**, *1*, 382–388.

(27) Smith, R. D.; Light-Wahl, K. J.; Winger, B. E.; Loo, J. A. Preservation of Non-covalent Associations in Electrospray Ionization Mass Spectrometry: Multiply Charged Polypeptide and Protein Dimers. *Org. Mass Spectrom.* **1992**, *27*, 811–821.

(28) Timasheff, S. N. Protein-Solvent Interactions and Protein Conformation. *Acc. Chem. Res.* **1970**, *3*, 62–68.

(29) Franks, R.; Eagland, D. *CRC Critical Rev. Biochem.* **1975**, *3*, 165.

(30) Loo, J. A.; Udseth, H. R.; Smith, R. D. *Biomed. Environ. Mass Spectrom.* **1988**, *17*, 411–414.

(31) Lundblad, R. L. The Reaction of Bovine Thrombin with *N*-Butyrylimidazole. Two Different Reactions Resulting in the Inhibition of Catalytic Activity. *Biochemistry* **1975**, *14*, 1033–1037.

(32) Friezner Degen, S. J.; MacGillivray, R. T. A.; Davie, E. W. Characterization of the Complementary Deoxyribonucleic Acid and Gene Coding for Human Prothrombin. *Biochemistry* **1983**, *22*, 2087–2097.

(33) Suter, M. J. F.; DaGue, B. B.; Moore, W. T.; Shen-Nan, L.; Caprioli, R. M. Recent Advances in Liquid Chromatography-Mass Spectrometry and Capillary Zone Electrophoresis-Mass Spectrometry for Protein Analysis. *J. Chromatogr.* **1991**, *553*, 101–116.

(34) Allen, G. *Sequencing of Proteins and Peptides*; Elsevier Science Publishers B.V.; New York, 1981.

(35) Tsunasawa, S.; Sakiyami, F. Amino-Terminal Acetylation of Proteins: An Overview. *Methods Enzymol.* **1984**, *106*, 165–170.

(36) Sharon, N.; Lis, H. In *The Proteins*, 3rd ed.; Neurath, H.; Hill, R. L.; Eds.; Academic Press: New York, 1982; Vol. 5, pp 1–144.

(37) Yazdanparast, R.; Andrews, P. C.; Smith, D. L.; Dixon, J. E. Assignment of Disulfide Bonds in Proteins by Fast Atom Bombardment Mass Spectrometry. *J. Biol. Chem.* **1987**, *262*, 2507–2513.

(38) Wold, F.; Moldave, K., Eds. *Methods in Enzymology*; Academic Press: New York, 1984, Vols. 106, 107.

(39) Glazer, A. N.; DeLange, R. J.; Sigman, D. S. *Chemical Modifications of Proteins: Selected Methods and Analytical Procedures*; Elsevier Science Publishers B.V.: New York, 1982.

(40) Hunt, D. F.; Yates, J. R., III; Shabanowitz, J.; Winston, S.; Hauer, C. R. Protein Sequencing by Tandem Mass Spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6233–6237.

(41) Covey, T. R.; Bonner, R. F.; Shushan, B. I.; Henion, J. D. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 249–256.

(42) Covey, T. R.; Huang, E. C.; Henion, J. D. Structural Characterization of Protein Tryptic Peptides via Liquid Chromatography/Mass Spectrometry and Collision-Induced Dissociation of Their Doubly Charged Molecular Ions. *Anal. Chem.* **1991**, *63*, 1193–1200.