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FORMATION OF π, τ-DIMETHYLHISTIDINE ON ALKYLATION OF TRYPSIN WITH ACTIVE-SITE-DIRECTED SULFONIC ACID METHYL ESTERS

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The possibility of synthesizing stable alkyl analogues of acyl trypsins by introducing the alkyl residue by means of active-site-directed sulfonic acid esters was studied. Nine amidino- or guanidino-substituted sulfonic acids of different geometries and their methyl esters were prepared. The time-dependent inhibition of bovine trypsin by these esters, indicating modification at the active site of the enzyme, was followed. With the exception of *p*-guanidinobenzenesulfonic acid methyl ester, all the esters acted as irreversible inhibitors. The site of methylation, Ser-195 or His-57 (chymotrypsinogen numbering), was determine by analyzing for *O*-methylserine and methylhistidines. With four of the esters indications of a possible formation of, at most, 0.1 residue of *O*-methylserine per inactivated trypsin molecule were obtained. τ-Methylhistidine (but no π-methylhistidine) was, however, always observed as the main product of the modification reaction. A further product, hitherto not yet described in active site methylations of serine proteinases, was π,τ-dimethylhistidine (1,3-dimethylhistidine). The failure of an attempted synthesis of the *N*-acetyl-ethanolamine ester of *p*-toluene-sulfonic acid reported in the literature is shown to be due to the high instability of this ester.

KEY WORDS: Acyl enzyme analogues, serine proteinases, trypsin methylation, histidine methylation, serine methylation, *N*-acetyl-ethanolamine tosyl ester

INTRODUCTION

Whereas the rate of hydrolysis of a "specific" ester substrate such as Ac-Gly-ArgOMe by the serine proteinase trypsin amounts[1] to 412 s^{-1}, the rate of deacylation of the Ac-Gly-Gly- acyl enzyme intermediate of an "unspecific" substrate has been determined[2] as 1.26 s^{-1}. Comparative studies of such acyl enzymes with methods such as X-ray diffraction or NMR might contribute to an understanding of the causes of this 330-fold rate difference, but seem possible only with analogues with increased stability. A stable acyl enzyme analogue devoid of additional bulk is obtained by substituting two hydrogen atoms for the carbonyl oxygen of the acyl residue. In the case of the analogue of Ac-Gly-trypsin, active site Ser-195 (chymotrypsinogen numbering) is thus

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Abbreviations: Ac, acetyl; Bz, benzoyl; Mp, melting point; RT, retention time; Tos, *p*-toluenesulfonyl.

alkylated by an Ac-NHCH$_2$CH$_2$-grouping. Such a residue could possibly be introduced by means of a sulfonic acid ester of N-acetyl-ethanolamine. In this way, methylation in the active site of trypsin, accompanied by stoichiometric inhibition of the enzyme, has been accomplished with methyl esters of *m*-guanidinobenzenesulfonic acid[3,4] or *p*-amidinophenylmethanesulfonic acid.[5] However, both these reagents methylated one histidine residue, probably active site His-57 of the enzyme. Nevertheless, selective alkylation of Ser-195 of trypsin has been achieved with bromoacetamidoalkylamines and -guanidines[6] or *p*-guanidino- and *p*-amidino-phenacyl bromide,[7] though other halo-ketones as Tos-Lys-chloromethyl-ketone alkylated His-57.[8] Which of the two nearby amino acids becomes modified appears to be determined by the exact geometry of the reagent-enzyme complex.[7] Therefore, it seemed worthwhile to examine whether esters of active-site-directed sulfonic acids of similar type to those mentioned, but of somewhat different structure, could alkylate the Ser-195 residue of trypsin.

As regards the synthesis of sulfonic acid esters of *N*-Ac-ethanolamine, an earlier attempt to obtain the tosyl ester failed.[9] It is shown below that this ester can be synthesized, but is quite unstable. Presumably, analogous esters appropriate as alkylating reagents for trypsin can nevertheless be prepared. It was decided, however, to use for the present study a series of methyl esters as model compounds. These esters are conveniently prepared from the sulfonic acids by means of trimethyloxonium fluoroborate.[3,4] The site of methylation in the trypsin molecule was determined by automated amino acid analysis for *O*-methylserine and methylhistidines. Selective methylation of active site serine could not be achieved. However, all the inhibitors generated besides τ-methylhistidine[4] a further product, π,τ-dimethylhistidine (1,3-dimethylhistidine). This finding should be of concern in studies on the role of His-57 of serine proteinses in catalysis and inhibitor binding by specific methylation of this residue as well as in the application of the restricted activity of methylated serine proteinases in peptide synthesis.[10–12]

MATERIALS AND METHODS

Materials

N-Acetyl-ethanolamine, 3-aminopropanesulfonic acid, 3- and 4-(bromomethyl)-benzonitrile, S-ethylisothiourea hydrobromide, 3- and 4-nitrobenzylbromide, and trimethyloxonium tetrafluoroborate were from Aldrich, Steinheim. *p*-Cyanobenzenesulfonyl chloride[13] was generously provided by Dr. H. Vieweg, Leipzig. *p*-Amidinophenylmethanesulfonyl fluoride was obtained from Calbiochem, Bad Soden, *O*-methyl-D,L-serine and N^{α}-acetylhistidine from Sigma, Deisenhofen, and π- and τ-methylhistidine from Serva, Heidelberg. 4 N Methanesulfonic acid/0.2% 3-(2-aminoethyl)indole hydrolysis reagent and amino acid calibration standard were purchased from Pierce Europe. Trypsin from bovine pancreas, cryst. 40 U/mg (Bz-ArgOEt, pH 8, 25°C) for biochemical purposes, and molecular sieve 0.3 nm were products from Merck, Darmstadt.

N-Acetyl-ethanolamine tosyl ester. *N*-Acetyl-ethanolamine, redistilled *in vacuo*[14] and stored over molecular sieves 0.3 nm, was reacted with tosylchloride in dry pyridine, at 1/20th of the scale reported.[9] Aliquots were analyzed in HPLC system I. Alternatively, 242 mg (1.27 mmol) TosCl was dissolved in 1 ml acetone and added to a mixture of 59 μl (0.64 mmol) *N*-Ac-ethanolamine and 37 μl (0.64 mmol) ethanol in 0.70 ml water and 0.64 ml 2N NaOH. After shaking for 1 min a sample was analyzed by HPLC.

Tosyl-2-hydroxyethylamide[15]. 138 mg (0.72 mmol) *p*-toluenesulfonyl chloride was dissolved in 215 μl pyridine, cooled in ice, and 65 μl (1.08 mmol) of ethanolamine were added. A sample was withdrawn after 5 min and dissolved immediately in buffer A for HPLC in system I. Tosylchloride had been nearly quantitatively converted to the amide with RT 10.6 min.

Tosyl-2-acetoxyethylamide. To the above reaction mixture was added an equivalent amount of acetic anhydride. After 30 min at room temperature, 13% of the tosyl-2-hydroxyethylamide had been converted to a new substance with RT 12.6 min in HPLC system I.

m-Amidinobenzenesulfonic acid. Metanilic fluoride (free base) was prepared by analogy to Steinkopf *et al.*[16] from the hydrochloride hydrate and NaOH. Mp 29°C (crude product); (Ref. 16) 29–30°C. *m*-Cyanobenzenesulfonyl fluoride[16] was synthesized from metanilic fluoride by a modified Sandmeyer reaction[17]. Mp 69°C (crude product); (Ref. 16) 69–70°C. The sodium salt of *m*-cyanobenzenesulfonic acid was obtained by hydrolysis of the sulfonyl fluoride with aqueous NaOH at room temperature. After concentrating the solution on the rotary evaporator, the sulfonic acid salt was precipitated in an ice bath by the addition of a saturated solution of NaCl. *m*-Amidinobenzenesulfonic acid was prepared from this sulfonic acid salt in analogy to the synthesis of *p*-amidino-phenylmethanesulfonic acid outlined below, using as solvent for the HCl step anhydrous methanol instead of THF/methanol.

p-Amidinobenzenesulfonic acid[18] was synthesized in an analogous way from the sodium salt of *p*-cyanobenzenesulfonic acid obtained by alkaline hydrolysis of *p*-cyanobenzenesulfonyl chloride.

m-Amidinophenylmethanesulfonic acid. The sodium salt of *m*-cyanophenylmethanesulfonic acid[19] was synthesized from 3-(bromomethyl)benzonitrile as described and the cyano group converted to the amidino group as in the synthesis of *p*-amidinophenylmethanesulfonic acid outlined in the following.

p-Amidinophenylmethanesulfonic acid[5,20] was prepared as described via the sodium salt of *p*-cyanophenylmethanesulfonic acid[19] obtained from 4-(bromomethyl) benzonitrile. Though the product has been called ammonium *p*-amidinophenylmethanesulfonate hydrochloride, its composition as a mixture of ammonium chloride and the sulfonic acid zwitter ion salt seems possible as well.

m-Guanidinobenzenesulfonic acid[4] was prepared essentially as described from metanilic acid obtained by alkaline hydrolysis of metanilic fluoride.

p-Guanidinobenzenesulfonic acid was obtained from sulfanilic acid in analogy to the aforementioned synthesis of *m*-guanidinobenzenesulfonic acid.

m-Guanidinophenylmethanesulfonic acid. The sodium salt of *m*-nitrophenylmethane sulfonic acid[21] was prepared from 3-nitro-benzylbromide as outlined in the reference. *m*-Aminophenylmethanesulfonic acid[21] was obtained by reduction with ammonium sulfide in the manner described for the preparation of 4-amino-α-amyl-cinnamic acid.[22] The amino group was reacted with cyanamide to obtain *m*-guanidinophenylmethanesulfonic acid by analogy to the synthesis of *m*-guanidinobenzenesulfonic acid mentioned above.

p-Guanidinophenylmethanesulfonic acid was synthesized starting from 4-nitrobenzylbromide in the same way as outlined for the meta-compound.

γ-Guanidinopropanesulfonic acid[23] was prepared from 3-aminopropanesulfonic acid as described. The product obtained was located in column eluates with the sodium nitroprusside/potassium hexacyano-ferrate (III) spray reagent no. 113 for cyanamide and derivatives.[24]

Sulfonic acid methyl esters were synthesized by analogy to the preparation of *m*-guanidinobenzenesulfonic acid methyl ester.[3,4] 30 to 300 μmol of the well dried sulfonic acid was thoroughly mixed with double the molar amount of trimethyloxonium tetrafluoroborate. The mixture was heated for 4 min at 155°C in an oil bath with continuous stirring and protection against moisture by a phosphorus pentoxide tube. After cooling to room temperature, the reaction mixture was dissolved in 0.1 M Tris/HCl buffer, pH 8.0, to a total concentration of 0.1 M for all the sulfonic acid products. Samples were taken for determination of purity and yield in HPLC system II. Hydrolytic stability of the methyl esters under the conditions used for the methylation of trypsin was determined by diluting an aliquot with 0.1 M Tris/HCl buffer, 20 mM Ca^{2+}, pH 7.4, and analyzing the samples taken after appropriate times at room temperature by HPLC.

π,τ-Dimethylhistidine[25,26] was synthesized from commercially available N^{α}-acetylhistidine, and its ninhydrin colour value was determined. A solution 1 M in N^{α}-acetylhistidine was prepared in 1 M NaOH. A sample of 500 μl was made up with water to 100 ml. Another 500 μl-sample, containing 0.5 mmol Ac-His, was mixed with 200 μl 10 N NaOH, and 200 μl (2 mmol) dimethyl sulfate was added. Shaking was continued, and two further 200 μl portions of dimethyl sulfate were added after 5 and 15 min, respectively. Alkaline conditions were restored with addition of another 200 μl of 10 N NaOH. After 45 min, the whole reaction mixture was again made up with water to 100 ml. 20 μl-aliquots of the two dilutions were hydrolyzed in triplicate in ampoules with 300 μl 6 N HCl *in vacuo* for 24 h at 110°C. The content of the ampoules was dried down in a desiccator, and 10 nmol-samples were analyzed in the dimethylhistidine system of the amino acid analyzer. The preparation of π,τ-dimethylhistidine contained 1.3% of τ-methylhistidine, but no detectable histidine or π-methylhistidine.

Inactivation of Trypsin with Sulfonic Acid Methyl Esters

20 mg bovine trypsin was dissolved in 100 ml 0.1 M Tris/HCl buffer, pH 7.4, containing 20 mM $CaCl_2$[5], and thermostated at 25.0°C. Typically, 2 ml of a nominally 0.1 M solution of the respective sulfonic acid methyl ester in Tris buffer, prepared as stated before, was added. This solution had been kept at room temperature for 10 min to hydrolyze excess trimethyloxonium tetrafluoroborate[4,27] and was analyzed by HPLC for its content of methyl ester and other products. The enzymatic activity of trypsin was measured on samples taken before and immediately after the addition of the inhibitor solution and during the course of inactivation. The inactivation reaction was stopped, usually after 20 h, by adding 0.3 ml of 6 N HCl to produce a pH of about 3. The solution was dialyzed at 4°C for 24 h against three changes of 5 l 1 mM HCl and lyophilized. For amino acid analysis, 0.5 mg of methylated trypsin was hydrolyzed *in vacuo* with 0.50 ml commercial 4 N methanesulfonic acid/0.2% 3-(2-aminoethyl)indole hydrolysis reagent[28] for 24 h at 110°C. A solution of NaOH (0.5 ml, 3.5 N) was added, and the solution was centrifuged.

Trypsin activity was determined at 25°C on 50 μl-samples of the trypsin solution in 3 ml 0.1 M Tris/HCl, pH 8.0, and 0.38 mM Bz-Arg-*p*-nitroanilide. The reaction was usually followed for 6 min at 405 nm.

Amino Acid Analyses

Amino acid analyses were performed on a Biotronik LC 5000 amino acid analyzer equipped with a Shimadzu C-R2AX integrator. To separate π- and τ-methylhistidine from histidine, the last alkaline buffer of the standard elution system recommended for protein hydrolyzates by the manufacturer of the amino acid analyzer was substituted by a buffer containing 0.90 M Na^+ and 0.20 M citrate, pH 4.30 ("methylhistidine system"). This relatively high concentration of citrate[29] was essential for successful separation of histidine and its derivatives, though lysine and ammonia were only partially resolved. π-Methylhistidine was eluted immediately after ammonia. π,τ-Dimethylhistidine appeared in both this methylhistidine system and the standard elution system immediately after histidine, only partially resolved. Changing the last buffer to 0.84 M Na^+, 0.54 M citrate, pH 4.30 ("dimethylhistidine system") effected a good separation of histidine, τ-methylhistidine and π,τ-dimethylhistidine in this order, but lysine was co-eluted with ammonia and π-methylhistidine with histidine. For the separation of *O*-methylserine from threonine, addition of propanol to the first elution buffer has been recommended.[30,31] With our ion exchange resin, a buffer 0.1 M in Na^+ and citrate, pH 3.00, containing 19% (v/v) methanol was finally found to elute methylserine between aspartic acid and threonine, but serine was co-eluted with glutamic acid. These analyses were run separately, using solely this buffer ("methylserine system").

HPLC

A Hewlett-Packard model 1084 B liquid chromatograph with variable wavelength detector, integrator and automatic sampling system was used. The prepacked reversed-

phase Nucleosil $5C_8$, 4 × 200 mm column (Macherey-Nagel, Düren) was run at 1.0 ml/min at 30°C. Detection wavelength was 200 nm and reference wavelength 430 nm. Elution buffer A consisted of 0.1 M NaH_2PO_4 adjusted to pH 3.0 with concentrated orthophosphoric acid.[32] Eluant B of HPLC system I used for tosyl derivatives consisted of a mixture of 70% (v/v) acetonitrile with 30% buffer A. Elution started with 10% B (and 90% A) for 5 min, followed by linear gradients from 10 to 50% B for 1 min and from 50 to 90% B for 7 min, and 90% B for further 5 min. In HPLC system II for sulfonic acids and derivatives with positively charged substituents, eluant B contained 20% (v/v) acetonitrile in buffer A. 10% B was run for 5 min, a linear gradient from 10 to 90% B for 10 min, and 90% B for further 2 min.

RESULTS AND DISCUSSION

Synthesis and Stability of N-Acetyl-Ethanolamine Tosyl Ester

Incorporation of an acylamidoethyl group into the active site of trypsin in the manner outlined in the introduction will require a sulfonic acid ester of the respective *N*-acyl-ethanolamine. An attempt to synthesize such an ester of *N*-acetyl-ethanolamine is described in the literature.[9] On treating *N*-Ac-ethanolamine with tosyl chloride in pyridine, no ester, but a low yield (recalculated as 20%) of the product of a rearrangement, Tos-2-acetoxyethylamide, had been obtained after 24 h. Strangely enough, a mixed melting point with what must have been Tos-2-hydroxyethylamide was used as one argument for the identity of the compound. Repetition of the synthesis under the conditions described in the literature and analysis of an aliquot of the 24 h reaction mixture in HPLC system I (where TosOMe is eluted at 13.2 min and TosOEt at 13.9 min) showed, besides the sulfonic acid TosOH (RT 5.0 min; 64%) and some residual TosCl (14.8 min; 16%), two additional minor peaks A (10.6 min; 5%) and C (12.2 min; 15%). These were identified as Tos-2-hydroxyethylamide and Tos-2-acetoxyethylamide, respectively, by co-chromatography with authentic materials. However, at a reaction time of only 5 min, a new main product B (11.6 min; 40%) appeared, besides 34% TosOH, 3% of A, 18% of C, and 5% TosCl. After 4 h, the amount of B had decreased to 6%. On treatment with aqueous alkali of an aliquot of a short-time reaction mixture, B (but not A or C) disappeared quickly under formation of TosOH. B (32%) was also obtained, besides 32% TosOH, 3% TosOEt, and 33% TosCl, but no Tos-2-acetoxyethylamide, by the NaOH-catalyzed reaction of TosCl with an equimolar mixture of *N*-Ac-ethanolamine and ethanol in aqueous acetone. Ethanol had been added to verify ester formation under these conditions. Evidently, the reaction of TosCl with *N*-Ac-ethanolamine occurs much faster than that with ethanol. Though its instability precluded isolation and further characterization, syntheses, retention time and behaviour on hydrolysis leave no doubt that peak B is due to the tosyl ester of *N*-Ac-ethanolamine. Its half life in aqueous solution (HPLC buffer A, pH 3) at room temperature was determined by HPLC as only 15 min, similar to the 9 min of TosCl. The instability of the tosyl ester of *N*-Ac-ethanolamine is probably due to oxazoline formation.[33] Hydrolysis of the oxazoline to yield *O*-Ac-ethanolamine that

reacts with a second molecule of TosCl is an alternative to the pathway of formation of the *O*-acetylated sulfonamide previously suggested.[9]

The present experiments show that the tosyl ester of *N*-Ac-ethanolamine can be obtained, but its instability leaves little hope for using such esters as alkylating agents for serine proteinases. However, tosyl esters of *N*-benzyloxycarbonyl-ethanolamine and *N*-phenoxyacetyl-ethanolamine have been synthesized and isolated in good yields.[34] Evidently, more electronegative acyl substituents impart a higher stability to such esters, which even prevented oxazoline formation from the benzyloxycarbonyl compound.[34] Workable alkylating reagents of the required type might consequently be obtained by the choice of an appropriate *N*-acyl residue. Therefore, it seemed useful to examine the possibility of a specific alkylation of Ser-195 of trypsin by active-site-directed sulfonic acid esters. The easily accessible methyl esters were chosen for this purpose.

Synthesis and Stability of Methyl Esters of Sulfonic Acids with Positive Substituents

The eight *p*- and *m*-isomers of amidino- or guanidino-substituted benzenesulfonic acid and phenylmethanesulfonic acid and the γ-guanidinopropanesulfonic acid were synthesized. On HPLC of the sulfonic acids obtained, the main peak with retention times given in Table 1 contained 95 to 99% of the UV-absorling material detected, with a single exception. This exception was *m*-guanidinophenylmethanesulfonic acid, where two additional peaks containing about 5% each appeared. The starting materials for the guanidino-substituted sulfonic acids, the corresponding amino-substituted compounds, were eluted still earlier and were always present only in negligible quantities. The size of the main peaks per nmol of the sulfonic acids containing a benzene ring was roughly similar and similar to that of commercially available *p*-amidinophenyl-methanesulfonyl fluoride and of the sulfonic acid obtained from this fluoride by hydrolysis with alkali. *m*-Guanidinophenylmethanesulfonic acid was again an exception with a peak size of only about 30% of the average ones, which indicates a relatively high content of salt in this preparation.

When the sulfonic acids were reacted with two equivalents of trimethyloxonium fluoroborate,[4] HPLC analysis showed that the methyl esters were always obtained as the main product (Table 1). Except for some residual sulfonic acid, the four amidino-substituted esters contained only negligible UV-absorbing impurities. The content of such side products was much higher with the guanidino-substituted compounds, especially with the aliphatic γ-guanidinopropanesulfonic acid (Table 1). Here, 13% of a peak eluting at 2.6 min and 29% of one at 5.6 min were the main additional components. When the hydrolytic stability of the methyl ester was tested as described under Materials and Methods, the peak at 5.6 min decreased as well, at a similar rate as the methyl ester, and the 2.6 min peak increased correspondingly. These observations suggest that the 5.6 min peak is also a methyl ester and the 2.6 min peak is the corresponding acid. Under milder methylation conditions with only 1.5 equivalents of trimethyloxonium fluoroborate, the yield of the 2.6 min peak was reduced to 3% and that of the 5.6 min peak to 10%, but the residual γ-guanidino-propanesulfonic acid had increased to 31%. The 2.6 and 5.6 min peaks are therefore probably due to

TABLE 1
HPLC retention times (in system II) of positively substituted sulfonic acids and their methyl esters, yields of the methyl esters and their hydrolytic stabilities (0.1 M Tris/HCl, 20 mM Ca^{2+}, pH 7.4, room temperature).

Sulfonic acids	Retention times (min)		Yields (%)			$t_{1/2}$ (min)
	Sulfonic acid	Methyl ester	Sulfonic acid	Methyl ester	Side products	Methyl ester
Benzenesulfonic acids:						
m-Amidino-	2.4	7.2	25	75	<1	100
p-Amidino-	2.4	6.9	3	93	4	60
m-Guanidino-	2.7	8.8	21	71	8	180
p-Guanidino-	2.4	9.0	9	62	29	180
Phenylmethanesulfonic acids:						
m-Amidino-	3.2	8.0	4	95	1	80
p-Amidino-	3.2	7.6	5	94	1	100
m-Guanidino-	3.6	10.3	18	69	13	180
p-Guanidino-	3.3	10.2	30	59	11	180
γ-Guanidinopropane-sulfonic acid	2.2	3.8	18	34	48	330

products of methylation at the guanidino group. This supposition is in accordance with their being eluted later than the corresponding non *N*-methylated acid, respectively methyl ester. Analogous observations were made in the course of this work with other guanidino-substituted sulfonic acids. A lower content of the presumptive guanidino-methylated products could always be achieved only at the expense of a higher content of the starting sulfonic acid. No important adverse effect on the methylation of trypsin was expected from the presence of such side products. In fact, the probable presence of a further methyl ester increases the efficiency of screening for an ester that will alkylate Ser-195 of the enzyme. The methyl ester preparations specified in Table 1 were therefore used for trypsin modification. Table 1 also shows the hydrolytic half lives, determined by HPLC, of the esters in aqueous solution under conditions as used for their reaction with trypsin. The lower stability of the amidino- in eomprison to the guanidino-substituted esters is in accordance with σ_p-values of +0.65 and -0.02, respectively, for these two substituents.[35] All the esters were sufficiently stable to allow long reaction times on trypsin modification.

Alkylation of Trypsin by the Active-Site-Directed Sulfonic Acid Methyl Esters

The course of the modification in the active site by the methyl esters of trypsin was monitored by the convenient assay with Bz-Arg-*p*-nitroanilide. The competitive effect

of the esters and other products present during the assay usually caused a decrease of the activity measured in trypsin samples taken even immediately after addition of the inhibitors. However, this decrease amounted generally to less than 25% of the initial activity and did not interfere with monitoring the progress of the reaction. The increase of the absorbance due to substrate hydrolysis remained constant during the 6 min period of activity measurements, and no progressive inhibition by the esters was notable during the assay. The values of $t_{1/2}$ of the irreversible inhibition of trypsin by the methyl esters given in Table 2 were determined from the initial time-dependent decrease of the enzymatic activity according to a first order rate law. At longer reaction times, the decrease of activity generally slowed down, probably due to a decrease in ester concentration caused by hydrolysis.

Only the *p*-guanidinobenzenesulfonic acid methyl ester did not measurably inactivate trypsin, even after 20 h at 10 mM concentration. This compound has already been reported not to irreversibly inhibit trypsin,[3] but was included in the present study, as the slowness of inhibition might have indicated reaction with Ser-195. The other eight esters inactivated trypsin with greatly differing rates (Table 2). These rates might in part be influenced by the relatively high content of sulfonic acid and side products of several of the methyl ester preparations. For the two trypsin inhibitors of this type described previously, the presently determined $t_{1/2}$ agrees reasonably with values of 7.9 min calculated for an inhibitor concentration of 1.4 mM from the reported kinetic constants for *m*-guanidinobenzenesulfonic acid methyl ester[4] or of 142 min found for the inhihition of β-trypsin by 0.25 mM *p*-amidinophenylmethanesulfonic acid methyl ester.[5] *m*-Amidinobenzenesulfonic acid methyl ester proved a still more rapidly acting inactivator than the first mentioned ester (Table 2). The final degree of inhibition observed did not decrease on dialysis of the samples of modified trypsin, providing evidence for the irreversible nature of the inactivation.

Identification of the Sites of Methylation

To identify possibly formed *O*-methylserine in the samples of methylated trypsin by means of automated amino acid analysis, hydrolysis of the protein was performed with 4 N methanesulfonic acid/0.2% tryptamine,[28,30,31] as methylserine is largely destroyed on hydrolysis with 6 N HCl.[30] The commercial methanesulfonic acid/tryptamine mixture was found to yield under hydrolysis conditions constant amounts of a ninhydrin positive contaminant co-eluting with glycine, and variable amounts of a component overlapping arginine on elution with the alkaline final buffer of the standard system. To test for possible losses of methylserine on hydrolysis in the presence of an excess of trypsin, this compound was added to samples in an amount corresponding to 0.4 residues per trypsin molecule. 68 to 78% of the added methylserine was recovered after hydrolysis. Increasing the time of hydrolysis of partially methylated trypsin from 24 to 48 h did not release additional τ-methylhistidine. Spiking experiments showed that, including the hydrolytic losses, 0.10 residues of *O*-methylserine per trypsin molecule could be safely detected in trypsin hydrolyzates in the methylserine system and 0.05 residues of π-methylhistidine in the methylhistidine system.

TABLE 2
Rates of inactivation of bovine trypsin (0.1 M Tris/HCl, 20 mM Ca^{2+}, pH 7.4, 25.0°C) and products of histidine modification by active-site-directed sulfonic acid methyl esters.

Sulfonic acid methyl ester	Initial concentration (mM)	$t_{1/2}$ of inhibition	Total reaction time	Final degree of inactivation (%)	Methylhistidine system*		Dimethylhistidine system*			
					His + DHis	MHis	His	MHis	DHis	MHis + DHis
Without Inhibitor			20 h	(15)	2.88	0.00	2.71	0.00	0.00	0.00
m-Amidinobenzene-	1.5	5 h	20 h	46	2.57	0.35	2.34	0.40	0.03	0.43
p-Amidinobenzene-	1.9	2.5 min	3 min	57	2.35	0.44	2.23	0.47	0.03	0.50
	1.8	2.5 min	8 min	89	2.08	0.68	2.11	0.75	0.08	0.83
	1.9	2.5 min	12 min	97	2.08	0.71	1.81	0.73	0.14	0.87
	1.5	3.0 min	75 min	100	2.61	0.24	2.00	0.23	0.58	0.81
m-Guanidinobenzene-	1.4	10 min	20 h	100	2.44	0.48	2.13	0.50	0.11	0.61
p-Guanidinobenzene-	10	>200 h	20 h	3						
m-Amidinophenylmethane-	1.9	5.5 h	20 h	93	2.19	0.74	1.94	0.81	0.04	0.85
p-Amidinophenylmethane-	1.9	2 h	20 h	91	2.43	0.40	2.14	0.49	0.24	0.73
m-Guanidinophenylmethane-	1.2	6 h	20 h	57	2.45	0.38	2.20	0.38	0.11	0.49
p-Guanidinophenylmethane-	2.3	15 h	20 h	41	2.63	0.15	2.26	0.20	0.10	0.30
γ-Guanidinopropane-	0.7	4.5 h	20 h	67	2.43	0.46	2.01	0.52	0.11	0.63

*Histidine, τ-methylhistidine (MHis) and π,τ-dimethylhistidine (DHis) as determined with the respective buffer-system on the amino acid analyzer are given as residues per trypsin molecule, related to the content of Asp, Tyr and Phe and the number of these residues in trypsin.[36]

Only hydrolyzates of trypsin methylated with *m*-amidinobenzene-, *p*- and *m*-amidinophenylmethane- or γ-guanidinopropane sulfonic acid esters displayed in the methylserine system small humps in a position coinciding with that of *O*-methylserine. These might have been due to at most 0.1 residue of *O*-methylated serine per trypsin molecule. However, the main product of methylation of trypsin was in each case τ-methylhistidine (Table 2), whereas no π-methylhistidine was observed in the methylhistidine system. The latter finding is in accord with what has been reported for trypsin methylated with *m*-guanidinobenzenesulfonic acid methyl ester.[4]

In several of the methylated trypsin samples, much less τ-methylhistidine was found than would be expected from their degree of inactivation (Table 2). Trypsin treated with *p*-amidinobenzenesulfonic acid methyl ester for 75 min contained distinctly less τ-methylhistidine than samples treated for a shorter period. The peak of histidine from the 75 min-sample was strongly asymmetric in the methylhistidine system, displaying a trailing shoulder. These observations suggested that this shoulder could be due to π,τ-dimethylhistidine formed by further methylation of τ-methylhistidine. A good separation of authentic π,τ-dimethylhistidine from other amino acids required special adjustment of the final buffer of the amino acid analyzer. In this dimethylhistidine system, the material of the shoulder migrated indeed to the position of π,τ-dimethylhistidine. A ninhydrin colour value of 96.4% of that of histidine was determined for π,τ-dimethylhistidine. This value differs appreciably from that of 80% reported by Cowgill,[26] though it seems plausible in comparison to values of 96.6% and 93.4% given for π- and τ-methylhistidine, respectively.[29] Analysis of the methylated trypsins in the dimethylhistidine system showed the occurrence of notable amounts of π,τ-dimethylhistidine in all the samples (Table 2). The content of τ-methylhistidine was in reasonable agreement with that determined in the melhylhistidine system, but the values for histidine were lower, as dimethylhistidine was now separated. On the average, 2.85 residues of histidine plus its methylated derivatives per molecule of trypsin were found in the methylhistidine and 2.74 residues in the dimethylhistidine system, in acceptable agreement with the occurrence of three histidine residues in bovine trypsin.[36] Overlap of π,τ-dimethylhistidine with another peak might be the reason why this compound has not been described before as product of active site methylation of serine proteinases.

In the case of chymotrypsin it has been shown explicitly that a serine proteinase methylated at His-57 retains only a low level of enzymatic activity.[10,37,38] In the present work, the lowest measured activity of methylated trypsin amounted to <0.5% of the native enzyme with both the *m*-guanidinobenzenesulfonic ester product containing the modified histidine mainly as τ-methylhistiine and the 75 min *p*-amidinobenzenesulfonic ester product containing mainly π,τ-dimethylhistidine (Table 2). Both, mono- and dimethylated trypsins, seem therefore largely devoid of enzymatic activity. The sum of the methylated histidine residues determined per molecule of trypsin (Table 2, last column) was always somewhat lower than one residue for 100% inactivation. This seems a reasonable value for a trypsin preparation certainly containing enzymatically inactive protein that will also resist methylation and therefore contribute to a higher content of unmodified histidine. Most probably, the single histidine residue modified in a molecule of enzymatically active trypsin is active site His-57.

Formation of π,τ-dimethylhistidine had already happened when only part of the susceptible histidine residues of the enzyme had become monomethylated. Methyltrypsin (and possibly any other methylated serine proteinase as well) evidently is a mixture of mono- and dimethylated molecular species. These may differ in properties, a fact to be considered in all work with active site methylated enzymes. Introduction of the second methyl group is roughly only ten-fold slower than formation of τ-methylhistidine, the exact relative rates depending on the nature of the methylating agent. Because of the much lower residual activities of monomethylated serine proteinases, the second methylation step does not seem to require a fully functional catalytic triad.

Most probably, the initial rates measured for the irreversible inhibition of trypsin by the active-site-directed sulfonic acid esters (Table 2) all pertain to the same reaction, formation of τ-methylhistidine from His-57. Rather subtle differences in the geometry of the reagents seem to play a decisive role in determining the rate of the reaction. This is illustrated by the surprising lack of reactivity of *p*-guanidinobenzenesulfonic acid methyl ester, though *p*-amidino- and *m*-guanidino-phenylmethanesulfonic acid methyl esters — where the lack of an interposed NH-group or the shift of the guanidino group closer to the sulfonic acid residue is compensated by the additional methylene group — do modify trypsin. The methyl esters of *p*-amidino- and *m*-guanidinobenzenesulfonic acid appear similar in their dimensions in the sense just mentioned and are indeed by far the most reactive methylating agents. Here the difference in activities as modifiers of trypsin is even similar to the difference in intrinsic reactivities as indicated by their hydrolytic rates (Table 1), though this coincidence might well be fortuitous.

Since the inactivation of trypsin occurred mainly by methylation of a histidine residue, it did not seem rewarding to confirm the possible formation of some *O*-methylserine or to investigate which serine residue has become modified. Whether selective alkylation of Ser-195 of trypsin can in principle not be achieved with sulfonic acid esters, or whether a more serendipitous choice of the structure of the sulfonic acid would lead to such a reaction, remains an open question.

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References

1. Fiedler, F. (1987) *Eur. J. Biochem.*, **163**, 303–312.
2. Fujioka, T., Tanizawa, K. and Kanaoka, Y. (1981) *J. Biochem.*, **89**, 637–643.
3. Jackson, M.B. and Bender, M.L. (1970) *Biochem. Biophys. Res. Commun.*, **39**, 1157–1162.
4. Magnotti, R.A., Jr. (1987) *Biochem. Biophys. Acta*, **915**, 46–52.
5. Wong, S.-C. and Shaw, E. (1976) *Arch. Biochem. Biophys.*, **176**, 113–118.
6. Lawson, W.B., Leafer, M.D. Jr., Tewes, A. and Rao, G.J.S. (1968) *Hoppe-Seyler's Z. Physiol. Chem.*, **349**, 251–262.
7. Schroeder, D.D. and Shaw, E. (1971) *Arch. Biochem Biophys.*, **142**, 340–350.
8. Shaw, E. and Springhorn, S. (1967) *Biochem. Biophys. Res. Commun.*, **27**, 391–397.

9. McQuillin, F.J. and Stewart, J. (1955) *J. Chem. Soc.*, 2966–2967.
10. West, J.B., Scholten, J., Stolowich, N.J., Hogg, J.L., Scott, A.I. and Wong, C.-H. (1988) *J. Am. Chem. Soc.*, **110**, 3709–3710.
11. West, J.B., Hennen, W.J., Lalonde, J.L, Bibbs, J.A., Zhong, Z., Meyer, E.F., Jr. and Wong, C.-H. (1990) *J. Am. Chem. Soc.*, **112**, 5313–5320.
12. Zhong, Z., Bibbs, J.A., Yuan, W. and Wong, C.-H. (1991) *J. Am. Chem. Soc.*, **113**, 2259–2263.
13. Wagner, G., Vieweg, H and Kühmstedt, H. (1973) *Pharmazie*, **28**, 288–292.
14. Temin, S.C. (1956) *J. Org. Chem.*, **21**, 250–251.
15. Slotta, K.H. and Behnisch, R. (1932) *J. Prakt. Chem. N.F.*, **135**, 225–236.
16. Steinkopf, W. (1927) *J. Prakt. Chem. N.F.*, **117**, 1–82.
17. *Organikum. Organisch-chemisches Grundpraktikum.*, p. 664, VEB Deutscher Verlag der Wissenschaften, Berlin 1977.
18. Andrewes, C.H., King, H. and Walker, J. (1946) *Proc. R. Soc.*, London, Series B, **133**, 20–62.
19. Wong, S.-C. and Shaw, E. (1974) *Arch. Biochem. Biophys.*, **161**, 536–543.
20. Laura, R. and Bing, D.H. (1981) US Patent 4303592.
21. Purgotti, A. and Monti, C. (1900) *Gazzetta Chim. It.*, **30**, part 2, 246–260.
22. Schröter, R. and Möller, F. (1957) in *Methoden der organischen Chemie* (Houben-Weyl), Vol. XI/1: *Stickstoffverbindungen II* (Müller, E., ed.), p. 414, Thieme-Verlag, Stuttgart.
23. Fujii, A. and Cook, E.S. (1975) *J. Med. Chem.*, **18**, 502–505.
24. Waldi, D. (1962) in *Dünnschicht-Chromatographie* (Stahl, E., ed.), pp. 496–515, Springer-Verlag, Berlin, Göttingen, Heidelberg.
25. Madgwick, J.C., Ralph, B.J., Shannon, J.S. and Simes, J.J. (1970) *Arch. Biochem. Biophys.*, **141**, 766–767.
26. Cowgill, R.W. (1957) *J. Am. Chem. Soc.*, **79**, 2249–2251.
27. Rawn, J.D. and Lienhard, G.E. (1974) *Biochem. Biophys. Res. Commun.*, **56**, 654–660.
28. Simpson, R.J., Neuberger, M.R. and Liu T.-Y. (1976) *J. Biol. Chem.*, **251**, 1936–1940.
29. Hamilton, P.B. (1963) *Anal. Chem.*, **35**, 2055–2064.
30. Hodges, R.S. and Merrifield, R.B. (1974) *J. Org. Chem.*, **39**, 1870–1872.
31. Hodges, R.S. and Merrifield, R.B. (1975) *J. Biol. Chem.*, **250**, 1231–1241.
32. Fiedler, F. and Geiger, R. (1988) *Meth. Enzymol.*, **163**, 257–262.
33. Boyd, R.N. and Hansen, R.H. (1953) *J. Am. Chem. Soc.*, **75**, 5896–5897.
34. Ginsburg, S. and Wilson, I.B. (1964) *J. Am. Chem. Soc.*, **86**, 4716–4720.
35. Wang, C.-C. and Shaw, E. (1972) *Arch. Biochem. Biophys.*, **150**, 259–268.
36. Mikes, O., Holeysovsky, V., Tomasek V. and Sorm, F. (1966) *Biochem. Biophys. Res. Commun.*, **24**, 346–352.
37. Henderson, R. (1971) *Biochem. J.*, **124**, 13–18.
38. Byers, L.D. and Koshland, D.E. (1978) *Bioorg. Chem.*, **7**, 15–33.