SYNTHESIS OF METHYL ETHERS OF N^{α}-ARYLSULFONYLVALYLARGININES — THROMBIN AND TRYPSIN SUBSTRATES

V. K. Kibirev, V. P. Romanova, and S. B. Serebryanyi

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Thrombin — the key enzyme of the blood-clotting system — hydrolyzes the Arg—Gly bond of fibrinogen dissolved in the blood plasma and thereby brings about the conversion of this substrate into fibrin — the monomer which is the basis of the thrombus that is formed. Thrombin possesses a trypsin-like action and belongs to the groups of serine proteinases [1, 2]. Possessing a considerable homology of primary structure with trypsin [1, 2], having a similarly constructed active center [2], and exhibiting a similarity in the mechanism of its action and its primary substrate specificity [3, 4], thrombin nevertheless differs from trypsin by the kinetics of its interaction with many substrates [4-9].

To elucidate the nature of the fine specificity of thrombin and to analyze the interconnection between the structure and reactivity of its substrates we previously synthesized esters of N^{α}-arylsulfonyl-L-arginine, so-called TAME analogs with the general formula $R_1-C_6H_4-SO_2-Arg-OCH_3$ and studied their properties [9, 10]. The kinetic characteristics of their hydrolysis by thrombin permitted the hypothesis to be forwarded [9] that the differences in the reactivities of these esters are due to a change in the strength of the hydrogen bond between the NH group of the arginine residue and the active center of the thrombin, the strength of which depends on the nature of the substituent of the arylsulfonyl radical. For a further study of the structure—reactivity relationship of thrombin substitutes, no little interest is presented by oligopeptides forming fragments of the α(A) chain of fibrinogen in the region of the Arg—Gly bond that is cleaved by thrombin [11]. Such extended substrates permit the mapping of the whole of the active center of the enzyme — both its catalytic and its binding sections — while TAME analogs are suitable for investigating interactions only in the catalytic center of thrombin.

In the present paper we describe the synthesis of methyl esters of N^{α}-arylsulfonylvalylarginines of the general formula

$$R_1-C_6H_4-SO_2-Val-Arg-OCH_3,$$

where

$$R_1 = -CH_3,\ -OCH_3,\ -Cl,\ -\text{iso-}C_3H_7,\quad XII-XV$$

i.e., compounds in which an additional amino acid — valine — has been "inserted" between the NH group of the arylsulfonyl radical. In this series of substrates, the arylsulfonyl radical should have no influence on the strength of the hydrogen bond of the NH group of the arginine with the active center of the enzyme.

For the synthesis of compounds (XII-XV) we tried several methods of condensing arylsulfonylamino acids: the dicyclohexylcarbodiimide method, the acid chloride method, and the p-nitrophenyl ester method, i.e., those methods that are used most frequently for the introduction of a N-terminal tosylamino acid residue into peptides. In these syntheses we used the methyl ester of nitroarginine with subsequent splitting off of the nitro groups by catalytic hydrogenation or the methyl ester of arginine itself. In the latter case, condensation took place poorly and a mixture of products was formed which could not be separated. On working with nitroarginine derivatives, the protected dipeptides were synthesized mainly by the carbodiimide method and were obtained in the homogeneous state, but after the splitting out of the nitro group by hydrogenolysis a mixture of reaction products that could not be resolved was formed. Thus, for the synthesis of compounds (XII-XV) the only suitable and effective method proved to be condensation of the N-hydroxysuccinimide esters of arylsulfonylvalines with un-

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TABLE 1. Physicochemical Properties of the Compounds Synthesized*

Formula	Yield, %	mp, °C	E_{Arg} pH 6,5	R_f in system		$[\alpha]_D^{25}$, solvent (c = 1)
				1	2	
I. CH_3—C_6H_4—SO_2—D—Val—OH	64	140	—		0,63	—24,5, ethanol
II. CH_3O—C_6H_4—SO_2—Val—OH	54	114—115	—	0,59	0,66	+38,2, dioxane
III. Cl—C_6H_4—SO_2—Val—OH	70	123—124	—	0,64	0,78	+47,5, dioxane
IV. iso-C_3H_7—C_6H_4—SO_2—Val—OH	76	135—136	—	0,71	0,69	+21,5, ethanol
V. CH_3—C_6H_4—SO_2—Val—ONSu	78	183—184	—	0,52†	0,74	+8,2, dioxane
VI. CH_3—C_6H_4—SO_2—D—Val—ONSu	77	181—182	—	0,50†	0,90	—9,0, dioxane
VII. CH_3O—C_6H_4—SO_2—Val—ONSu	68	166—167	—	0,30†	0,94	+16,0 THF
VIII. Cl—C_6H_4—SO_2—Val—ONSu	84	179—183	—	0,50†	0,82	+12,0, dioxane
IX. iso-C_3H_7—C_6H_4—SO_2—Val—ONSu	57	135—136	—	0,65†	0,69	+17,9, THF —25,0, methanol
X. CH_3—C_6H_4—SO_2—Val—Arg—OH	31		0,09			
XI. CH_3O—C_6H_4—SO_2—Val—Arg—OH	87	168 decomp.				
XII. CH_3—C_6H_4—SO_2—Val—Arg—OCH_3	51	100 decomp.	0,66	0,23	0,49	—45,0, methanol
XIII. CH_3O—C_6H_4—SO_2—Val—Arg—OCH_3	50	81—82	0,68	0,16	0,50	—38,5, methanol
XIV. Cl—C_6H_4—SO_2—Val—Arg—OCH_3	48	113—114	0,70	0,32	0,54	—15,6, methanol
XV. iso-C_3H_7—C_6H_4—SO_2—Val—Arg—OCH_3	52	100—102	0,65	0,21	0,56	—44,5, methanol

*Esters (XII-XV) in the form of the acetates.
†System 3 was used for these compounds.

TABLE 2. Results of the Elementary Analysis of the Compounds Synthesized

Empirical formula	Found, %				Calculated, %			
	C	H	N	other elements	C	H	N	other elements
II. $C_{12}H_{17}NO_5S$	50.00	6.04	4.66	S 11.41	50.16	5.96	4.88	S 11.16
III. $C_{11}H_{14}ClNO_4S$	45.15	4.89	4.60	Cl 12.24	45.28	4.85	4.80	Cl 12.15
IV. $C_{11}H_{21}NO_4S$	56.22	7.10	4.60	S 10.69	56.16	7.07	4.68	S 10.71
V. $C_{16}H_{20}N_2O_6S$	—	—	7.50	—	—	—	7.60	—
VI. $C_{16}H_{20}N_2O_6S$	—	—	7.56	S 8.68	—	—	7.60	S 8.70
VII. $C_{16}H_{20}N_2O_7S$	49.97	5.00	7.49	S 8.33	49.99	5.24	7.28	S 8.34
VIII. $C_{15}H_{17}ClN_2O_6S$	46.27	4.45	7.13	—	46.35	4.40	7.20	—
IX. $C_{18}H_{24}N_2O_6S$	54.88	6.10	7.21	—	54.53	6.09	7.07	—
XI. $C_{18}H_{29}N_5O_6S \cdot H_2O$	46.68	6.66	15.14	S 6.92	46.84	6.77	15.17	S 6.95
XII. $C_{21}H_{35}N_5O_7S \cdot CH_3COOH \cdot 2H_2O$	46.53	7.15	12.88	—	46.91	7.31	13.03	—
XIII. $C_{19}H_{31}N_5O_6S \cdot CH_3COOH \cdot H_2O$	47.17	6.94	13.12	—	47.09	6.96	13.08	—
XIV. $C_{18}H_{28}ClN_5O_5S \cdot CH_3COOH \cdot H_2O$	44.78	6.06	12.40	S 6.09	44.48	6.35	12.97	S 5.94
XV. $C_{23}H_{39}N_5O_6S \cdot CH_3COOH \cdot H_2O$	50.41	7.27	12.70	—	50.44	7.55	12.79	—

substituted arginine in mixtures of dimethylformamide with dioxane or of dioxane with water. The first mixture of solvents was used for the reaction with arginine methyl ester, and the second for condensation with completely unprotected arginine [11].

The possibility of synthesizing arginine-containing peptides with unprotected C-terminal arginine, which gives an intramolecular salt through the guanidine and carboxy groups of the arginine, was shown in the work of Virovets et al. [13] and has been widely used for the synthesis of bradykinin and its derivatives by V. T. Ivanov et al. [14, 15]. As was found, this method is also very suitable in the synthesis of methyl esters of N^{α}-arylsulfonylvalylarginines. Table 1 gives the formulas of the compounds synthesized and their physicochemical characteristics. The ester (XII-XV) included in this table were obtained by condensing N-hydroxysuccinimide esters and were purified on carboxymethylcellulose at pH 6.0. The purity of the peptides was checked by TLC in two solvent systems or by paper electrophoresis at pH 6.8. At this pH value, good separation was achieved of the components of the reaction mixture: arginine, arginine methyl ester, N^{α}-arylsulfonylvaline, N^{α}-arylsulfonylvalylarginine, and esters of N^{α}-arylsulfonylvalylarginine.

EXPERIMENTAL

L-(amino acid)s from the firm Reanal (Hungary) were used. The melting points were determined in open capillaries; they are given without correction. For TLC on Silufol plates (Czechoslovakia) we used the following solvent systems: 1) butan-1-ol—acetic acid—water (4:1:1); 2) butan-1-ol—pyridine—acetic acid—water (30:20:6:10), and 3) benzene—ethyl acetate (4:3). The buffer solution for electrophoresis was prepared by diluting a mixture of 100 ml of pyridine and 4 ml of acetic acid with distilled water to 1000 ml. Electrophoresis was carried out in an instrument with a cooled horizontal plate in a gradient of 60-80 V/cm at pH 6.5 or in a vertical instrument at 900 V (gradient 20 V/cm) in 0.1 M acetic acid (pH 2.4) on FN-16 paper. The electrophoretograms were revealed with the Sakaguchi reagent or by the Rydon—Smith method. The optical activities were determined on a Spectropol-1 spectropolarimeter (Sofica, France) in a thermostated (at 25°C) cell with an optical path length of 1 cm. The concentration of the solutions was 1%.

The N^{α}-Arylsulfonylvalines (I-IV). A solution of 0.03 mole of L- or D-valine in 40-55 ml of 1 N NaOH was treated with a 1.4-fold excess of the appropriate arenesulfonyl chloride; the mixture was stirred vigorously for 3 h with the periodic addition of 1 N alkali. The excess of arenesulfonyl chloride was filtered off or extracted with ether, and the filtrate was extracted with hydrochloric acid. The colorless crystalline precipitate that deposited was filtered off, washed with water, and recrystallized from water or aqueous ethanol. Table 1 gives the formulas and some characteristics of the colorless crystalline arylsulfonlylvalines.

N-Hydroxysuccinimide Esters of N^{α}-Arylsulfonylvalines (V-IX). A solution of 0.06-0.08 mole of an arylsulfonylvaline (I-IV) and 0.06-0.08 mole of N-hydroxysuccinimide in 20 ml of absolute tetrahydrofuran was cooled to 0°C, and a 1.1-fold molar excess of DCHC in tetrahydrofuran was added. The reaction mixture was stirred at 0°C for 1 h and was left overnight in the refrigerator, the resulting precipitate of dicyclohexylurea was filtered off, the filtrate was evaporated in a rotary evaporator, and the residue was crystallized from isopropanol. Compounds (V-IX) consisted of colorless needles; their formulas and some characteristics are given in Table 1.

N^{α}-Arylsulfonylvalylarginines (X, XI). A solution of 1.1 g (3 mmole) of the N-hydroxysuccinimide ester of a N-arylsulfonylvaline (V-IX) in 5 ml of dioxane was added to 0.5 g (3 mmole) of arginine in 2 ml of water, the mixture was stirred at room temperature for 3 h and was left overnight. The solvent was evaporated off in vacuum, the residue was dissolved in DMFA, ethyl acetate was added, and the mixture was left in the refrigerator. The amorphous product was filtered off, washed with ethyl acetate, and crystallized from n-butanol; the properties of compounds (X) and (XI) are given in Table 1.

Methyl Esters of N^{α}-Arylsulfonylvalylarginine (XII-XV). a) A suspension of 5 mmole of the dihydrochloride of arginine methyl ester [16] in 8 ml of DMFA was cooled to 0°C and was treated with 0.69 ml (5 mmole) of triethylamine, the mixture was stirred with cooling for 10 min, the triethylamine hydrochloride was filtered off, and the filtrate was treated with a solution of 5 mmole of the appropriate N-hydroxysuccinimide ester (V-IX) in 6 ml of dioxane. The mixture was stirred in the cold for 1 h and was left overnight in the refrigerator at 4°C,

after which the solvent was distilled off and the resulting oil was triturated with ethyl acetate and then with petroleum ether. The esters (XII-XV) consisted of amorphous colorless hygroscopic compounds. For further work, they were purified by ion-exchange chromatography on carboxymethylcellulose as described below.

b) A suspension of 2 mmole of a N^{α}-arylsulfonylvalylarginine (X or XI) in 3 ml of absolute methanol was cooled to −15°C, and with vigorous stirring 0.16 ml of thionyl chloride was added. The temperature was slowly allowed to rise to that of the room, after which the reaction mixture was kept at 40°C for 2 h. The methanol was evaporated off in a rotary evaporator and the resulting oil was worked up as described in a).

Ion-Exchange Chromatography of the Substrates Synthesized. A solution of 200-300 mg of a substrate in 3-4 ml of the initial buffer solution was added to a column with dimensions of 34 × 2 cm filled with carboxymethylcellulose (0.7 meq/g, Reanal, Hungary) and equilibrated with 0.005 M ammonium acetate buffer, pH 6.0. Elution was carried out with molarity gradient of the same buffer solution of from 0.005 M (300 ml) to 0.5 M (300 ml) at the rate of about 100 ml/h, fractions being collected every 2.5 min. The adsorption of the fractions at 260 nm were measured on an SF-4 A spectrophotometer. The material was collected and freeze-dried. The homogeneity of each fraction was confirmed by paper electrophoresis at pH 6.5.

SUMMARY

New thrombin substrates — methyl esters of N^{α}-arylsulfonylvalylarginines — have been synthesized by the condensation of N-hydroxysuccinimide esters of arylsulfonylvalines with arginines followed by the treatment of the reaction product with thionyl chloride in methanol. The same compounds have been obtained by condensing N-hydroxysuccinimide esters of arylsulfonylvalines with arginine methyl ester.

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