N^{α}-TOSYL-L-ARGININE-*p*-NITRANILIDE AS SUBSTRATE FOR TRYPSIN

E.KASAFÍREK[a], M.CHAVKO[b] and M.BARTÍK[b]

[a] *Research Institute for Pharmacy and Biochemistry, Prague 3,*
[b] *Institute of Experimental Biology,*
Slovak Academy of Sciences, Košice

Received April 10th, 1970

The use of chromogenic substrates in biochemical research and clinical diagnostics has considerably simplified the procedure for the determination of enzymatic activities[1]. Among chromogenic substrates employed for the determination of the activity of proteolytic enzymes are *p*-nitranilides of free or, alternatively, N-acylated amino acids (*cf.*, *e.g.* ref.[2-5]). Their use is based on the release of a color product, *p*-nitroaniline, by the enzymatic digestion, whose absorption spectrum is different from the spectra of *p*-nitranilides of amino acids. The advantage of using these chromogenic substrates lies in the possibility of following the digestion by direct spectrophotometry and in their considerably high molar extinction coefficient.

The substrates* generally used for trypsin are TAME (ref.[6]) and BAME (ref.[7]) respectively, and of the chromogenic substrates DL-BAPA (ref.[8,9]). Since the effect of the tosyl residue has been studied[10] in detail both from the kinetic and thermodynamic viewpoint and since it favorably affects — compared to the benzoyl residue — the rate of digestion both by trypsin[7] and also, *e.g.* by thrombin[11], we have synthetized a chromogenic substrate of trypsin containing a tosyl group, *i.e.* N^{α}-tosyl-L-arginine-*p*-nitranilide (L-TAPA). The synthesis of *p*-nitranilides of amino acids and of their derivatives has been reported by many authors[12-15]. In the present study we made use of the phosphoazo method[16] which is known to afford *p*-nitranilides in high yields and optically pure[17]. The synthesis was also effected by the method using phosphorus pentoxide[18]. In the first case we started with N^{α}-tosyl-L-arginine hydrochloride which had been prepared by the same approach as N^{α}-benzoyl-L-arginine[19]. In the latter case we used N^{α}-tosyl-L-arginine[6]. The tryptic digestion of L-TAPA is compared with the known enzymatic cleavage of DL-BAPA (ref.[8]).

EXPERIMENTAL AND RESULTS

The melting points were determined on a Kofler block and were not corrected. Samples for analysis were dried *in vacuo* at 0·5 mm Hg over phosphorus pentoxide at 105°C. Optical rotation measurements were carried out in a photoelectric polarimeter. Spectrophotometric measurements were performed in Unicam SP 500 spectrophotometer in quartz cells.

N^{α}-Tosyl-L-arginine Hydrochloride

Concentrated hydrochloric acid (2·5 ml) was added to a suspension of N^{α}-tosyl-L-arginine[6] (9·55 g) in acetone (60 ml). The reaction mixture was allowed to stand 1 h at room temperature and then evaporated *in vacuo*. The yield of the reaction was 9·0 g (99%) of crystalline hydro-

* The following abbreviations will be used in this paper: TAME, N^{α}-tosyl-L-arginine methyl ester; BAME, N^{α}-benzoyl-L-arginine methyl ester; L-TAPA, N^{α}-tosyl-L-arginine *p*-nitranilide, DL-BAPA, N^{α}-benzoyl-DL-arginine *p*-nitranilide.

chloride, m.p. 184—186°C. The sample for analysis was crystallized from a mixture of methanol and ether. The m.p. remained unaltered, $[\alpha]_D^{20}$ +28·2 (*c* 0·43; methanol). For $C_{13}H_{20}N_4O_4S$. .HCl (364·9) calculated: 42·79% C, 5·80% H, 15·36% N, 9·72% Cl; found: 43·07% C, 5·94% H, 14·92% N, 9·88% Cl.

N^{α}-Tosyl-L-arginine *p*-Nitranilide

Phosphoazo method: A solution of phosphorus trichloride (0·23 ml) in anhydrous pyridine (5 ml) chilled to −20°C was added to a solution of *p*-nitroaniline (0·7 g; 5 mmol) in anhydrous pyridine (25 ml) at −20°C. After 30 min of standing at room temperature, a solution of N^{α}-tosyl-L-arginine (1·85 g; 5 mmol) in phosphoric acid hexamethyltriamide (10 ml) was added to the reaction mixture. The latter was allowed to stand three days at room temperature, then evaporated *in vacuo*, and the oily residue dissolved in 2-propanol (15 ml). To this solution 1M-HCl (125 ml) was added until the solution became permanently turbid. The reaction mixture was then inoculated with crystals of N^{α}-tosyl-L-arginine *p*-nitranilide hydrochloride. After 12 h of standing at 0°C, the crystalline product was filtered off and crystallized from 20% acetic acid (65 ml). The product was set aside for 12 h at 0°C, then filtered off, and washed with a small volume of cold water. The yield was 0·9 g (37%) of a product of m.p. 238—242°C. The sample for analysis was crystallized in the same manner. The m.p. remained unaltered, $[\alpha]_D^{20}$ −17·4° (*c* 0·41; 50% acetic acid). For $C_{19}H_{24}N_6O_5S$. .HCl (485·0) calculated: 47·05% C, 5·20% H, 17·33% N, 7·31% Cl; found: 47·10% C, 5·20% H, 17·18% N, 7·60% Cl.

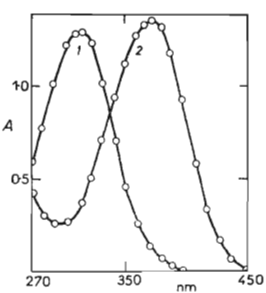

FIG. 1

Absorption Spectra of 10^{-4}M L-TAPA (*1*) and 10^{-4}M *p*-Nitroaniline (*2*) in 0·05 Veronal buffer, pH 8·2

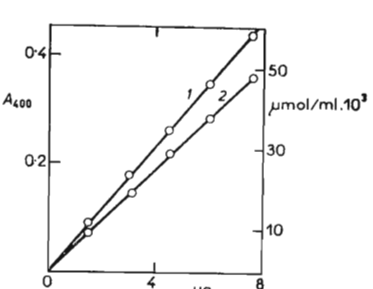

FIG. 2

Rate of Hydrolysis of DL-BAPA and L-TAPA as Function of Concentration of Trypsin

Incubation period 10 min. Substrate concentration at the beginning of the reaction *1* 0·83 . 10^{-3}M L-TAPA, *2* 0·83 . 10^{-3}M DL-BAPA in 0·042M Tris buffer containing 0·017M-$CaCl_2$, pH 8·2, 25°C. Concentration of trypsin: μg/ml, concentration of hydrolyzed substrate: μmol/ml . 10^3.

Phosphorus pentoxide method: L-TAPA was prepared by the same method as N^{α}-benzoyl-DL-arginine *p*-nitranilide[8] in a yield of 44%, m.p. 239—240°C. No depression of the m.p. was observed when the product was mixed with a sample of the product prepared as described under *a*) $[\alpha]_D^{20}$ −15·6° (*c* 0·39; 50% acetic acid).

Fig. 1 shows the absorption spectra of *p*-nitroaniline and L-TAPA, at 10^{-4}M concentration in 0·05M veronal buffer at pH 8·2. The absorption maximum of L-TAPA lies at 315 nm. Since *p*-nitranilides also absorb at 380 nm where *p*-nitroaniline shows maximum absorption, all the remaining photometric measurements were carried out at 400 nm.

Enzymatic Hydrolysis of L-TAPA and DL-BAPA

Measurement of enzymatic activity. The enzymatic digestion of the substrates was followed by two procedures. The first one (procedure *I*) was based on the measurement of the quantity of products released after a certain constant period.

The stock solution of the substrate (10^{-3}M DL-BAPA) was prepared by dissolving 43·94 mg of the substrate in 1 ml of dimethyl sulfoxide. The volume of the solution was made up to 100 ml with 0·05M Tris buffer at pH 8·2, containing 0·02M $CaCl_2$. The temperature of this solution was maintained at 25°C. The solution of L-TAPA (10^{-3}M) was prepared by a similar procedure by dissolving 48·5 mg of the substrate. The stock solution of the enzyme contained 90—450 μg

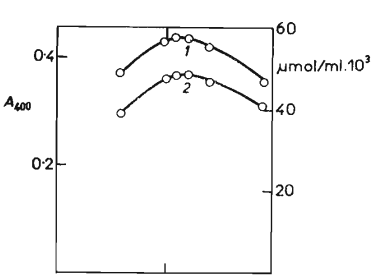

FIG. 3

Tryptic Hydrolysis of DL-BAPA and L-TAPA as Function of pH

Concentration of trypsin 7·5 μg/ml, incubation period 10 min. Substrate concentration at the beginning of the hydrolysis: *1* $0{\cdot}83 \cdot 10^{-3}$M L-TAPA, *2* $0{\cdot}83 \cdot 10^{-3}$M DL-BAPA in 0·042M. Tris buffer containing 0·017M-$CaCl_2$, pH 8·2, temperature 25°C. Concentration of hydrolyzed substrate: μmol : ml . 10^3.

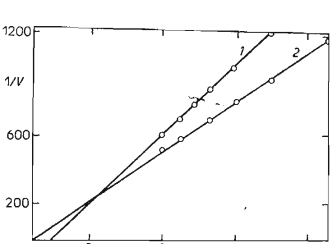

FIG. 4

Determination of K_m-value for Hydrolysis of DL-BAPA (*1*) and L-TAPA (*2*) by Trypsin.

See methods (procedure *II*) for details. $1/V$ in $s/\Delta A_{400}$, $1/S \cdot 10^{-3}$ in l/mol.

of trypsin in 1 ml of 0·001M-HCl. (Trypsin was a twice crystallized product containing 2850 NFU/mg.) The measurement itself was carried out with 5 ml of the substrate solution to which 0·9 ml of water was added. The solution was set aside in an ultrathermostat at 25°C until its temperature was the same as that of the thermostat (approximately 5 min). The substrate was digested at the same temperature. At zero time, 0·1 ml of the trypsin solution (9—45 μg of trypsin) was added to the incubation mixture, the reaction was allowed to proceed 10 min, and then discontinued by the addition of 1 ml of 30% acetic acid. The quantity of liberated *p*-nitroaniline was measured photometrically at 400 nm in 1 cm cells. In control experiments 0·1 ml of 0·001M-HCl was used instead of the enzyme solution and no digestion was observed in any case.

The second procedure (procedure *II*) was based on the determination of the rate of digestion of the substrates by continuous measurement of liberated *p*-nitroaniline in a temperature-controlled cell (Spekol, Zeiss Jena). The 10^{-3}M stock solutions of the substrates were prepared as described for procedure *I*, save for the exception that 0·05M veronal buffer at pH 8·2 containing 0·02M-$CaCl_2$ was used. The stock solution of the enzyme contained 200 μg of trypsin per ml of 0·001M-HCl. In this procedure the stock solution of the buffer was added to the cell and made up to 1·8 ml with the buffer. After the temperature of the cell had been equilibrated at 25°C, 0·2 ml of the enzyme solution was added. The extinction of the solution was read every 10 seconds for 4 min. The initial hydrolysis rate was determined graphically.

Dependence of rate of hydrolysis of substrates on enzyme concentration. Fig. 2 shows the rate of hydrolysis of DL-BAPA and L-TAPA in the presence of different enzyme concentrations. As can be seen from the figure, the rate increases linearly at least in the range of trypsin concentrations 0—7·5 μg/1 ml. The rate of hydrolysis of L-TAPA is approximately by 15% higher than the rate of hydrolysis of DL-BAPA.

Effect of pH *on rate of hydrolysis of substrates.* The dependence of tryptic hydrolysis of DL-BAPA and L-TAPA on pH in buffer systems is shown in Fig. 3. The profile of this plot is identical for both substrates, with a maximum between 8·1 and 8·2 and thus identical with recorded data for DL-BAPA (ref.[8]).

Dependence of rate of hydrolysis on concentration of substrates. The kinetic constants K_m for L-TAPA and DL-BAPA were determined by procedure *II* and calculated according to Lineweaver and Burk[20] (Fig. 4). The thus obtained K_m-value for L-TAPA is $0{\cdot}625 \cdot 10^{-3}$M and for DL-BAPA $0{\cdot}95 \cdot 10^{-3}$M. Since the K_m-value for racemic DL-BAPA is by itself of rather discutable importance, we present for reasons of exact comparison the K_m-value for L-BAPA $0{\cdot}939 \cdot 10^{-3}$M according to Erlanger, Kokowski, and Cohen[8] obtained under similar conditions.

The results of the measurement of the rate of hydrolysis of DL-BAPA and L-TAPA permit us to conclude that L-TAPA is hydrolyzed by trypsin at a higher rate than DL-BAPA. The sensitivity of the reaction of L-TAPA to trypsin is high and can be used for the determination of the enzymatic activity at concentrations lower than 1 μg 1 ml. L-TAPA represents a more convenient substrate for trypsin than DL-BAPA.

We wish to thank Dr I. Rychlík for kindly supplying us with trypsin, Dr I. Frič for the measurement of optical rotations, Mrs J. Komancová and Mr K. Havel for the analyses, and Mrs A. Roubalová, Mrs I. Schořálková, and Miss E. Sameková for technical assistance.

REFERENCES

1. Hoffmann K. H.: Pharmazie *23*, 679 (1968).
2. Bartík M., Michnová E.: Vet. Med. *12*, 143 (1967).
3. Rybák M., Petáková M., Simonianová E.: This Journal *32*, 1051 (1966).

4. Wachsmuth E. D., Fritze I., Pfleiderer G.: Biochemistry *5*, 169 (1966).
5. Barth T., Pliška V., Rychlík I., Šorm F.: This Journal *32*, 2327 (1967).
6. Hummel B. C. W.: Can. J. Biochem. Physiol. *37*, 1393 (1959).
7. Schwert G. W., Neurath H., Kaufman S., Snoke J. E.: J. Biol. Chem. *172*, 221 (1948).
8. Erlanger B. F., Kokowsky N., Cohen W.: Arch. Biochem. Biophys. *95*, 271 (1961).
9. Haverback B. J., Dyce B., Edmondson H. A.: Am. J. Med. *29*, 424 (1960).
10. Bechet J. J.: J. Chim. Phys. *61*, 584 (1964).
11. Seegers W. H., Henry R. L.: Tromb. Diath. Haemorrhag. *9*, 227 (1963).
12. Tuppy H., Wiesbauer U., Wintersberger E.: Z. Physiol. Chem. *329*, 278 (1962).
13. Bundy H. F.: Arch. Biochem. Biophys. *102*, 416 (1963).
14. Nagel W., Willig F., Peschke W., Schmidt F. H.: Z. Physiol. Chem. *340*, 1 (1965).
15. Ramenskij E. V., Botvinik M. M., Bejsembaeva R. U.: Chimia Prir. Soedin. *23* (1968).
16. Goldschmidt S., Rosculet G.: Ber. *93*, 2387 (1960).
17. Kasafírek E., Rudinger J.: Unpublished results.
18. Erlanger B. F., Kokowsky N.: J. Org. Chem. *26*, 2534 (1961).
19. Riedel A., Wünsch E.: Z. Physiol. Chem. *316*, 61 (1959).
20. Lineweaver H., Burk D.: J. Am. Chem. Soc. *56*, 658 (1934).

Translated by V. Kostka.

NEUROTROPIC AND PSYCHOTROPIC SUBSTANCES. XLIX.*

3,8-DIAZABICYCLO[3.2.1]OCTYL DERIVATIVES OF DIBENZO[*b*,*f*]THIEPIN

J. O. JÍLEK, J. METYŠOVÁ and M. PROTIVA

Research Institute of Pharmacy and Biochemistry, Prague 3

Received November 25th, 1970

3,8-Diazabicyclo[3.2.1]octane as a bridged analogue of piperazine may be considered as a component of molecules of potential psychotropic substances in which it would replace the previously proven piperazine fragment. Recently, the synthesis and pharmacology of 3,8-diazabicyclo[3.2.1]-octyl derivatives derived from phenothiazine and 10,11-dihydro-5*H*-dibenzo[*a*,*d*]cycloheptene were reported[1]. The results obtained pointed to a considerable degree of psychotropic activity of the products.

In connection with our finding of a pronounced neuroleptic activity of some derivatives of 10-piperazinodibenzo[*b*,*f*]thiepin, particularly of 8-chloro-10-(4-methylpiperazino)-10,11-dihydrodibenzo[*b*,*f*]thiepin ("octoclothepin")[2] and of its 8-methylthio-analogue ("methiothepin")[3]

* Part XLVIII: This Journal *36*, 3300 (1971).