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Triphenylmethane dye as a substrate for chymotrypsin

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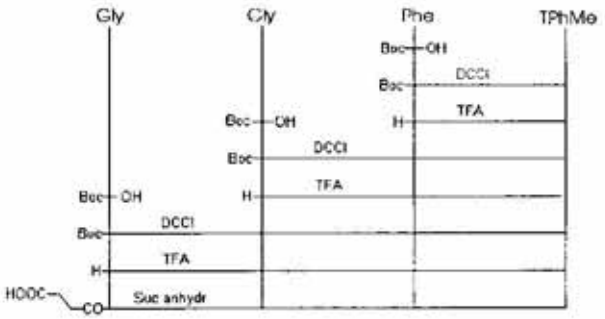
Hydrolase activity measurements are often used in clinical analysis as a supplementary diagnostic method, and also to monitor some diseases. Assays for hydrolases serve also as a basis for detection of particularly dangerous poisons such as phosphoroorganic compounds, known as industrial and military poisons, which are inhibitors of these enzymes. The chromogenic substrates most often used for this purpose give a coloured product as a result of enzymic reaction [1, 2]. Chromogenic substrates of hydrolases, such as nitrophenyl esters and nitroanilides, are characterized by relatively low product absorption coefficient and thus are applicable mainly in instrumental determinations, since it is rather difficult to assess visually the yellow colour of nitrophenol or nitroanilides [3, 4]. Application of the usually proposed chromogenic groups absorbing in the upper visual region is rather limited because of their low stability [5–8]. Methods in which a colourless product of the enzymic reaction is transformed in a subsequent reaction into a coloured compound have been applied in a paper-test to determine leucine aminopeptidase and γ-glutamyltranspeptidase activities [9, 10]. However, due to further reactions, these methods appeared somewhat erroneous and often display colour background [11].

Triphenylmethane dyes are characterized by an exceptionally high molar absorption coefficients, e.g. crystal violet has ε about 10^5 M^{-1} cm^{-1} and show a distinct change of colour on elimination of one electron donor group, e.g. crystal violet, malachite green.

We made a search for substrates for the hydrolases having in the active centre a serine involved in the catalytic process. Such substrates could be of use for detection on paper of the activity and inhibition of those enzymes. For this purpose we synthesized and assayed the following compounds: bis-(4-*N*,*N*-dimethylaminophenyl)-4-aminophenylmethane (ATPhMe)[1], forming after oxidation a dye analogous to crystal violet, and its peptidyl derivative — 4-*N*-(succinyl-glycyl-glycyl-phenylalanyl)amidophenyl-bis-(4-dimethylaminophenyl)methane (SucGly-Gly-Phe-TPhMe); the later after oxidation gives an analogue of malachite green. Syntheses of ATPhMe, bis-(4-*N*,*N*-dimethylaminophenyl)-4-acetamidophenylmethane (AcTPhMe), bis-(4-*N*,*N*-dimethylaminophenyl)-4-succinylamidophenylmethane) (Suc-TPhMe) were performed as described elsewhere [12]. Synthesis of Suc-Gly-Gly-Phe-TPhMe was performed by standard methods as summarized in Scheme 1. When the intermediate products were not sufficiently pure, they were purified on a silica gel column using the same developing system as for thin-layer chromatography (Table 1). Thin-layer chromatography was performed on GF_{254} silica gel plates (Merck). The chromatograms were observed under u.v. or visual light after spraying with about 0.01 M $KMnO_4$ solution (Table 1).

[1] Abbreviations used: Suc, 3-carboxypropionyl; Boc, *tert*-butyloxycarbonyl; DCCI, *N*,*N*'-dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; pNA, 4-nitroanilide; ATPhMe, bis-(4-*N*,*N*-dimethylaminophenyl)-4-aminophenylmethane; TPhMe, bis-(4-*N*-dimethylaminophenyl)-4-amidophenylmethane.



Scheme 1. Synthesis of Suc-Gly-Gly-Phe-TPhMe. Boc, *tert*-butyloxycarbonyl group, DCCI, *N,N'*-dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; Suc anhydr, succinic anhydride.

Table 1

Thin-layer chromatography of derivatives of bis-(4-N,N-dimethylaminophenyl)-4-aminophenylmethane (ATPhMe) on silica gel GF_{254} plates.

Solvent systems: 1, ethyl acetate/petroleum ether (4:6, v/v); 2, 2-propanol/acetic acid (9:1, v/v); 3, ethyl acetate/petroleum ether/acetic acid (3:7:1, by vol.); 4, dichloromethane/methanol/acetic acid (10:3:1, by vol.).

Peptidyl derivatives	R_F	Solvent
Boc-Phe-TPhMe	0.55	1
	0.56	2
Phe-TPhMe	0.00	3
	0.20	2
Boc-Gly-Phe-TPhMe	0.00	3
	0.59	2
Gly-Phe-TPhMe	0.00	3
	0.06	2
Boc-Gly-Gly-Phe-TPhMe	0.10	2
	0.54	4
Gly-Gly-Phe-TPhMe	0.05	2
	0.40	4

The obtained triphenylmethane derivatives were oxidized with chloranil in a methanol solution; under these conditions amido-derivatives turn green while free amine is violet. Molar absorption coefficients of the dyes obtained from triphenylmethane derivatives differ in acidic and alkaline solutions in visual and u.v. region, however, the coefficients remain constant over the pH range of 4.6–5.8 (Table 2).

The dissociation constants of oxidized triphenylmethane derivatives were determined by absorbance measurement at longwave peaks in 0.05 M buffers: KCl/HCl, sodium citrate/citric acid, glycine/NaOH, containing 30% methanol. For a dye resulting from ATPhMe oxidation pK values of 2.28 and 10.11, and for oxidized acylated derivatives, independently of the acyl group, pK of 2.18 and 6.89 were found.

The rate constant of the oxidation reaction of Suc-Gly-Gly-Gly-Phe-TPhMe with chloranil was found to be 9.5×10^{-2} $dm^3 mol^{-1} s^{-1}$ (S = 0.05) in methanol solution at 25°C. To study enzyme kinetics Suc-Gly-Gly-Phe-TPhMe was oxidized overnight in those conditions. The dye was crystallized from methanol and acetic acid.

The substrate dissociation constant was measured with the use of a point method at the oxidized Suc-Gly-Gly-Phe-TPhMe concentrations of: 2 µM; 5 µM; 1 µM; 50 µM; 100 µM and 200 µM in 0.1 M Tris/HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$ and methanol at final concentration of 30%. The reaction was started by adding chymotrypsin (Merck, Cat. No. 2307) solution to a final concentration of 0.2 µg/ml. After 5 and 10 min the reaction was terminated by adding an equal volume of 2 M

Table 2

Absorbance data of triphenylmethane dyes obtained from ATPhMe and Suc-Gly-Gly-Phe-TPhMe measured in 0.05 M acetate buffer, pH 5.0.

Dye obtained from			
ATPhMe		Suc-Gly-Gly-Phe-TPhMe	
λ_{max}	ε_{max}	λ_{max}	ε_{max}
nm	$M^{-1}cm^{-1}$	nm	$M^{-1}cm^{-1}$
241	3.07×10^5	238	3.07×10^5
297	1.83×10^4	300	1.32×10^4
344	6.90×10^4	450	2.15×10^4
587	5.58×10^4	617	7.24×10^4

acetate buffer, pH 4.6 and, after 30 min of further incubation at 25°C, concentration of the product was measured by following increase of absorbance at 520 nm. The measurements with the use of succinyl-L-phenylalanine-4-nitroanilide (Suc-Phe-pNA) as a substrate was performed in the same digestion conditions. The substrate dissociation constant, K_S for the oxidized Suc-Gly-Gly-Phe-TPhMe and Suc-Phe-pNA was calculated [13] as 2×10^{-4} mol dm^{-3} and 7×10^{-4} mol dm^{-3}, respectively. V_{max} for the oxidized Suc-Gly-Gly-Phe-TPhMe is about 300 times higher than for Suc-Phe-pNA.

The chymotrypsin substrate obtained from Suc-Gly-Gly-Phe-TPhMe is at least as good as other known substrates for this enzyme with regard to affinity for the enzyme and catalytic constant. The respective molar absorption coefficient for the hydrolysis product is several times higher than that for the conventional substrates.

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