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INHIBITORS AND DIPEPTIDE SUBSTRATES FOR A MICROSOMAL TYROSYLSULFOTRANSFERASE FROM RAT BRAIN

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The sulfotransferase associated with a microsomal fraction from rat brain was previously shown to transfer sulfate groups from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to peptides derived from the cholecystokinin (CCK) molecule. Three tyrosine-containing dipeptide derivatives, i.e., Cbz-Glu-Tyr, Cbz-Gly-Tyr and Ac-Phe-Tyr are shown here to accept the [^{35}S] sulfate group from [^{35}S] PAPS under the action of this sulfotransferase. The sulfotransferase activity evaluated with either any of these dipeptide derivatives or CCK-8 as acceptors is similarly inhibited by a series of compounds, i.e., lipophilic polycyclic compounds like fluphenazine, tyrosine derivatives like Boc-*O*-benzyl-tyrosine and phenolsulfotransferase inhibitors like 4,4-di-isothiocyano 2′,2′-disulfonic acid stilbene.

KEY WORDS: Tyrosylsulfotransferase, Inhibition, Dipeptide substrates, fluphenazine, 4,4-di-isothiocyano 2′,2′-disulfonic acid stilbene.

INTRODUCTION

Sulfation on tyrosine (Tyr) residues is a post-translational modification of several secretory proteins like fibrinogens[1,2] gastrin,[3] cholecystokinin (CCK)[4] immunoglobulin G[5] of fibronectin.[6]

In general the role of Tyr sulfation is poorly understood but, in the case of CCK, it appears essential for its recognition by receptors mediating its various hormonal or neuronal actions.[7,8]

For a long time, very little was known about the enzyme(s) catalyzing this protein modification except that it was distinct from cytosolic sulfotransferases (EC 2.8.2) like phenolsulfotransferase (EC 2.8.2.1). The latter are unable to transfer the sulfate group from the universal donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS)[9] to N-substituted Tyr residues.[10,11]

However, a membrane-bound enzyme activity, designated tyrosylprotein sulfotransferase, and catalyzing the transfer of sulfate to Tyr residues of unidentified proteins, was first detected in a rat pheochromocytoma cell-line.[12] Recently sulfotransferase activities associated with microsomal and vesicular fractions from rat brain[13,14] and Golgi membrane fractions from bovine adrenal medulla[15] were characterized by using synthetic substrates, i.e., cholecystokinin fragments or derivatives of non-sulfated (n.s.) CCK-8 and an acidic random polymer containing Tyr (Glu^{62}, Ala^{30}, Tyr^{8})$_n$, respectively.

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In addition to their similar subcellular localisations, several analogous properties of these two enzyme activities suggest their possible identity, e.g., presence of dicarboxylic amino acid residues in acceptors, acidic optimal pH, micromolar apparent affinity of PAPS, activation by $MnCl_2$. However definitive identification of these enzymes and delineation of their functional roles requires their purification and the development of inhibitors.

We describe here the use of substituted Tyr-containing dipeptides as substrates and the inhibitory activity of a wide variety of compounds for the tyrosylsulfotransferase activity of a crude microsomal fraction of rat cerebral cortex.

MATERIALS AND METHODS

The materials used were obtained from the following cources: non-radioactive PAPS, carbobenzoxy-glutamyl-tyrosine (Cbz-Glu-Tyr), carbobenzoxy-glycine-tyrosine (Cbz-Gly-Tyr), 2,6-dichloro 4-nitrophenol (Sigma Chemical Co.); Boc-CCK-8 (n.s.), and other peptides and derivatives (Bachem, Budendorf, Switzerland); polystyrene bead resin, 100–200 mesh (Porapak Q, Waters Assoc., France); organic solvents and other analytical grade chemicals (Prolabo, France).

[^{35}S]PAPS (1–5 Ci/mmol) was purchased from NEN Chemicals and ACS scintillation mixture from Radiochemical Centre (Amersham, U.K.). Wistar male rats (180–220 g) were obtained from Iffa-Credo (France).

Microsomal Fraction from Rat Brain

Immediately after decapitation, the whole cerebral cortex was dissected out and homogenized in 20 volumes of ice-cold 0.32 M sucrose using a teflon-glass Potter (0.15 mm clearance, 8 up and down strokes). The microsomal fraction was obtained at 3–5°C, essentially as described.[16] The homogenate was centrifuged (Lourdes model A, Inst. Corp. Brooklyn, N.Y.) at $4 \times 10^5 g \times$ min and the resulting supernatant then centrifuged (Beckman Ultracentrifuge model L) at $6 \times 10^6 g \times$ min. The pellet was rinsed and resuspended in 10 mM Tris-maleate, pH 7.0, and recentrifuged at $3 \times 10^6 g \times$ min. The washed microsomal fraction was resuspended in buffer of the same composition and this suspension was used within 1–3 h for tyrosyl sulfotransferase activity assays.

Assays of Tyrosyl Sulfotransferase Activity

The incubations were usually performed for 30 min at 37°C in 0.2 ml of a mixture containing 75 mM K-phosphate buffer pH 5.8, 125 mM NaCl, 25 mM NaF and 0.2 mM $MnCl_2$. The two substrates, [^{35}S]-PAPS (usually at a 0.2 μM concentration) and Boc-CCK-8 (n.s.) or a substituted dipeptide like Ac-Phe-Tyr, Cbz-Glu-Tyr or Cbz-Gly-Tyr) were added to start the incubations, after a 3 min preincubation of the microsomal fraction (usually 80 μg protein) in the presence of the inhibitor.

The reaction was stopped by heat denaturation (95°C, 4 min), tubes were chilled and then centrifuged for 30 s in a table microcentrifuge. The [^{35}S]-sulfated peptide formed was isolated from an aliquot of the supernatant by chomatography on a polystyrene bead column (0.4 × 3 cm). The column was washed with 5 × 2 ml of K-phosphate buffer (75 mM, pH 5.8) and the [^{35}S]-sulfated peptide eluted into a liquid

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scintillation vial with 1 ml of pure ethanol. The eluted radioactivity was quantified by liquid scintillation spectrometry in a Packard TRI-CARB. The recovery of Boc-CCK-8, as evaluated spectrophotometrically (λ = 295 nm) with a known standard, was 90%. In contrast, contamination by [^{35}S]-PAPS (or $^{35}SO_4$) in the final eluate was less than 0.1%. Blanks, obtained in parallel incubations in the presence of [^{35}S]-PAPS but in the absence of any peptide acceptor, represented about 5–7% of control values obtained under optimal conditions and were substracted. When substrates other than Boc-CCK-8 (n.s.) were used in the sulfotransferase assay their recoveries in the polystyrene bead column chromatographic procedure were evaluated as follows. Standards of the substrates were passed down the column and their concentration in the final eluate estimated either by U.V. spectrophotometry or using Lowry's reagent.[17] In most cases a 80–90% recovery was obtained and results corrected accordingly.

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RESULTS

The tyrosylsulfotransferase activity was progressively and completely inhibited by compounds like fluphenazine or imipramine (Figure 1). The effects of some inhibitors on tyrosylsulfotransferase activity were studied varying the concentrations of either the peptide acceptors or the sulfate donor PAPS under conditions of initial velocity measurement. In the presence of 0.3 μM [^{35}S]-PAPS, the tyrosylsulfotransferase activity first increased with the concentration of the acceptor Boc-CCK-8 (n.s.) and was, then, inhibited in the presence of an excess of this peptide, thus leading to a biphasic Lineweaver–Burk representation (Figure 2). Kinetic data were obtained taking only into account data from acceptor concentrations below inhibition, i.e., the initial linear

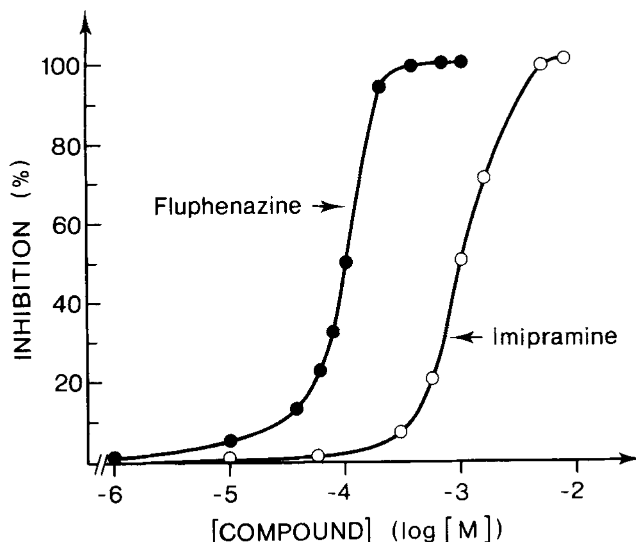

FIGURE 1 Inhibition of tyrosylsulfotransferase activity by fluphenazine and imipramine. The enzyme activity was determined in 30 min incubations in the presence of 0.2 μM [^{35}S]-PAPS and 0.15 mM Boc-CCK-8 (n.s.). The non-inhibited tyrosylsulfotransferase activity represented 0.42 $\pm$ 0.03 pmol/mg protein/min.

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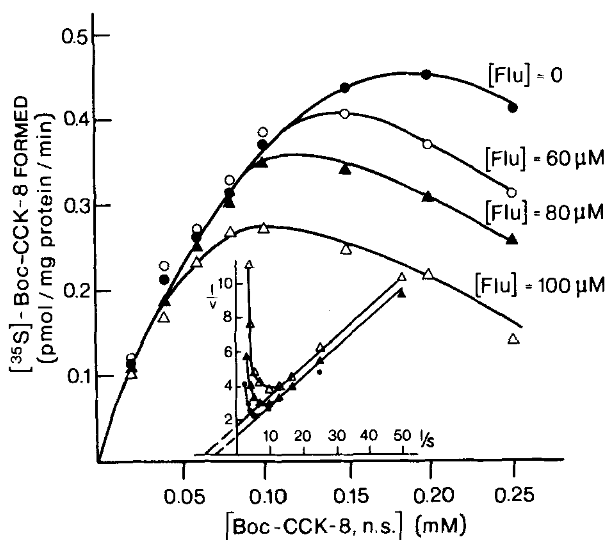

FIGURE 2 Inhibition of tyrosylsulfotransferase activity by fluphenazine (Flu) at increasing concentrations of the substrate Boc-CCK-8 (n.s.). Initial rates were determined in 30 min incubations in the presence of 0.3 μM [^{35}S]-PAPS. Inset: double reciprocal plot of the data.

portion of the double-reciprocal line. This procedure was previously used by others[18,19] for the soluble phenolsulfotransferase for which a similar inhibition by excess substrate also occurred. Apparent K_M and V_{max} values and their standard errors were obtained by least square analysis of these data. Fluphenazine (60–100 μM) inhibited the tyrosylsulfotransferase activity in a complex and apparently uncompetitive manner. At low concentrations of the peptide acceptor its effect was hardly detectable whereas at higher concentrations the saturation curve was progressively shifted (Figure 2). Thus at 100 μM fluphenazine the K_M for the peptide decreased from 0.14 to 0.045 mM whereas the V_{max} decreased from 0.85 $\pm$ 0.06 to 0.39 $\pm$ 0.03 pmol/min/mg protein. When PAPS was the varying substrate at 0.15 mM Boc-CCK-8 (n.s.), fluphenazine apparently acted as a non-competitive inhibitor, with V_{max} decreasing from 1.26 $\pm$ 0.08 to 1.06 $\pm$ 0.05 and 0.74 $\pm$ 0.07 pmol/min/mg protein at 80 and 100 μM fluphenazine, respectively with no significant change in K_M (0.28 $\pm$ 0.02 μM) (Figure 3).

The K_i value of fluphenazine (0.10 $\pm$ 0.02 mM) was obtained from these data assuming purely non-competitive inhibition and calculating the slopes of the double-reciprocal lines as described by Segel[20] (Table I).

Using the same acceptor, tyrosylsulfotransferase activity was progressively inhibited by Boc-*O*-benzyl-Tyr, also in an uncompetitive manner toward Boc-CCK-8 (V_{max}), with apparent K_M values of the latter compound decreasing from 0.85 $\pm$ 0.05 to 0.46 $\pm$ 0.04 pmol/min/mg protein (at 200 μM of the inhibitor). As in the case of fluphenazine this compound was uncompetititve toward Boc-CCK-8 and non-competitive toward PAPS, its K_i value in the latter case being 0.20 $\pm$ 0.04 mM (Table I).

In addition, the inhibitory potency of various compounds was established by determining their IC_{50} value in the standard tyrosylsulfotransferase assay, i.e., using

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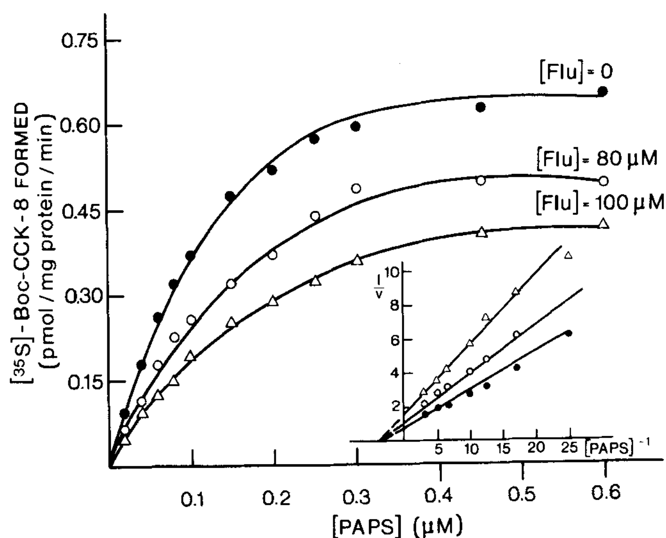

FIGURE 3 Inhibition of tyrosylsulfotransferase activity by fluphenazine at increasing concentrations of the sulfate donor PAPS.

TABLE I
Apparent kinetic constants of substrates and inhibitors for the microsomal tryosylsulfotransferase activity

Substrates	Apparent kinetic constants		K_I (mM) of inhibitors	
	K_M (mM)	V_{max} (pmol/mg prot/min)	Fluphenazine	Boc-*O*-benzyl-Tyr
Boc-CCK-8 (n.s.)	0.14 ±0.02	0.67 ±0.11	0.10 ±0.02	0.20 ±0.04
Ac-Phe-Tyr	0.81 ±0.06	0.33 ±0.08	0.14 ±0.03	0.10 ±0.02
Cbz-Glu-Tyr	0.54 ±0.10	0.28 ±0.05	0.16 ±0.01	0.12 ±0.04
Cbz-Gly-Tyr	0.95 ±0.22	0.31 ±0.06	0.13 ±0.02	N.E.

N.E.: not estimated.

The tyrosylsulfotransferase activity of the microsomal fraction was measured in the presence of 0.3 μM [^{35}S]-PAPS and peptide acceptors in varying concentration. Boc-*O*-benzyl-tyrosine and fluphenazine were tested at 12 different concentrations in the presence of peptide acceptors at a concentration corresponding to their K_M value. Apparent kinetic constants were calculated from Lineweaver–Burk plots and the slopes of the double-reciprocal lines as described.[20] Values are the means ± standard deviations from 3 different experiments.

0.15 mM Boc-CCK-8 (n.s.) as acceptor and 0.2 μM [^{35}S] PAPS as donor (Table II). A series of lipophilic drugs like imipramine, chlorimipramine, chlorpromazine and haloperidol were active in the submillimolar range whereas apomorphine, phentolamine, theophylline, ouabain and verapamil were poorly active. 2,6-dichloro-4-nitrophenol and 4,4-di-isothiocyano-2,2′-disulfonic acid stilbene were quite active, the latter with an IC_{50} of 10 μM.

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TABLE II
Inhibition of tyrosylsulfotransferase activity by various compounds

Compound	IC_{50} (mM)
Chlorimipramine	0.40 ± 0.06
Imipramine	0.85 ± 0.10
Chlorpromazine	0.14 ± 0.03
Haloperidol	0.40 ± 0.05
Apomorphine	> 1.0
Phentolamine	> 1.0
Theophylline	> 5
Ouabain	> 5
Verapamil	> 1.0
Boc-3,5-dibromo Tyr	0.50 ± 0.02
4,4-Di-isothiocyano 2,2′-Disulfonic acid stilbene	0.010 ± 0.002
Arsenazo	0.130 ± 0.010
2,6-Dichloro-4-nitrophenol	0.64 ± 0.08

The tyrosylsulfotransferase activity of the microsomal fraction was measured in the presence of 0.15 mM Boc-CCK-8 (n.s.) 0.2 μM [^{35}S]-PAPS and the various compounds in increasing concentrations. The activity was completely abolished in the presence of compounds with IC_{50} values lower than 1 mM.

In order to identify simplified peptide acceptors, a series of *N*-protected dipeptides with *C*-terminal Tyr were tested. Sulfation occured on Cbz-Glu-Tyr, Cbz-Gly-Tyr and Ac-Phe-Tyr. Optimal pH for sulfation of these compounds was 6.2, i.e., slightly less acidic than for Boc-CCK-8. With all compounds tyrosyl sulfotransferase activity was inhibited by an excess of substrate, as shown in the case of Cbz-Glu-Tyr (Figure 4). All three dipeptide derivatives displayed similar apparent V_{max} values, with

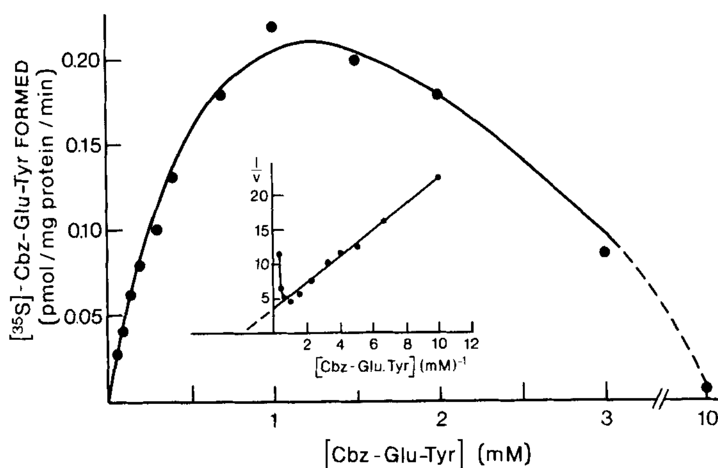

FIGURE 4 Tyrosylsulfotransferase activity of the microsomal fraction evaluated with Cbz-Glu-Tyr as substrate. Thirty min incubations in the presence of 75 mM phosphate buffer (pH 6.2), 125 mM NaCl, 25 mM NaF and 0.2 mM $MnCl_2$. [^{35}S]-PAPS was present at 0.3 μM concentration.

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Cbz-Glu-Tyr displaying a slightly lower apparent K_M value (Table I). The K_i values of fluphenazine and Boc-*O*-benzyl-Tyr did not differ when the acceptor was either one of these substituted dipeptides or Boc-CCK-8 (Table I). In addition the IC_{50} value of 2,6-dichloro-4-nitrophenol was similar with the four acceptors (not shown).

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DISCUSSION

The present study has allowed the identification of simplified substrates and of several classes of inhibitors for the tyrosylsulfotransferase activity previously found in a crude microsomal fraction from rat brain.[13,14] Apparent kinetic data for these various substances are summarized in Tables I and II; however the approximate character of these values (calculated as indicated in Results) should be emphasised. Like those of other sulfotransferases,[21] the kinetics of the microsomal tyrosylsulfotransferase are complex since the reactions they catalyze involve two substrates, with one of them exerting inhibition at high concentrations. Preliminary analysis of the reaction mechanism of the microsomal tyrosylsulfotransferase have suggested that it is similar to that of soluble rat brain phenolsulfotransferase.[19] The inhibition patterns of the product PAP (adenosine 3′,5-biphosphate) or the dead-end inhibitor APS (adenosine 5′-phosphosulfate) were consistent with a steady state ordered bi-bi kinetic mechanism with PAPS binding first to the enzyme and PAP being the last product to leave it.[14]

The starting point for the identification of simplified substrates was the observation that Boc-Asp-Tyr, the protected dipeptide corresponding to the *N*-fterminal end of CCK-8, was detectably sulfated.[13] This observation is now extended to the related dipeptides Cbz-Glu-Tyr, Cbz-Gly-Tyr and Ac-Phe-Tyr which all displayed inhibitory activity at high substrate concentration and similar apparent kinetic constants. There was only a slightly higher apparent affinity for Cbz-Glu-Tyr, possibly related to the favourable role of dicarboxylic amino acid resides close to the Tyr residue for binding to the active site, as already suggested in the series of CCK- fragments.[13,14] However this role might have been minimized by the presence of the hydrophobic protecting group which may interact with a domain of the active site recognizing the Asp-Arg sequence corresponding to the *N*-terminal extension of CCK-8 in its precursor. In addition, all three substituted dipeptides appeared less efficient substrates, in terms of both apparent affinity and catalytic activity, than Boc-CCK-8, further illustrating the role of the *C*-terminal amino acid sequence of the CCK-8 molecule in its recognition by the tyrosylsulfotransferase.

It is important to note that the tyrosylsulfotransferase activity of the microsomal preparation, as measured with any of these substituted dipeptides or with Boc-CCK-8 as acceptors, was similarly inhibited by the various compounds tested, indicating presumably that a single enzyme was involved.

The various effective inhibitors belong to three distinct groups. The first is constituted by a series of lipophilic polycyclic compounds, among which the phenothiazines fluphenazine and chlorpromazine were the most potent and which also comprizes compounds like imipramine, chlorimipramine and haloperidol.

Fluphenazine acted in a complex manner i.e., mainly as an uncompetitive inhibitor with respect to peptide acceptor and as a non-competitive inhibitor with respect to the sulfate donor. In addition that action of fluphenazine was mainly apparent at high concentrations of the peptide acceptor (Figure 2) which might be related to an

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interference with the mechanism of inhibition by an excess of the acceptor. This phenothiazine derivative also inhibits with similar potency cyclic AMP phosphodiesterase[22] and Na^+–K^+-adenosine triphosphatase[23] from brain tissues.

The second group consists of the two tyrosine derivatives Boc-*O*-benzyl-Tyr and Boc 3,5-dibromo Tyr, both active in the 0.1 mM range. In spite of some analogy with the simplified substrates, Boc-*O*-benzyl-Tyr was an uncompetitive inhibitor with respect to the peptide acceptor, possibly because the bulkiness of the benzyl group prevented adequate interaction with the Tyr recognition subsite in the enzyme.

Finally both 2,6-dichloro-4-nitrophenol and, even more, 4,4-di-isothiocyano 2′,2′-disulfonic acid stilbene quite potently inhibited the tyrosylsulfotransferase. These two compounds are also inhibitors of the soluble phenolsulfotransferase[24,25] although 2,6-dichloro-4-nitrophenol is approximately 100 times more potent on this enzyme than on the tyrosylsulfotransferase[13] whereas the other compound displays similar potency towards both enzymes (not shown).

In conclusion, the present identification of several new substrates and inhibitors of the microsomal tyrosylsulfotransferase should be helpful for purification of the enzyme, comparison of enzyme activities in various tissues and delineation of their functional roles.

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