June 1993 Chem. Pharm. Bull. 41(6) 1007—1011 (1993)

A Chemical Model of Catechol-*O*-methyltransferase. Methylation of 3,4-Dihydroxybenzaldehyde in Methanol Solution[1]

Setsuro SUGATA

Kyoritsu College of Pharmacy, Shibakoen 1–5–30, Minato-ku, Tokyo 105, Japan. Received November 13, 1992

The reaction of 3,4-dihydroxybenzaldehyde (LH_2) and dimethyl sulfate (DMS) in forming *m*- and *p*-*O*-methylated products (vanillin and isovanillin, respectively) in a methanol buffer solution was studied kinetically as a chemical model of catechol-*O*-methyltransferase (COMT). The *O*-methylations, especially *m*-*O*-methylations, were catalyzed by divalent metal ions such as Cu(II), Mg(II) and Zn(II). A clear Mg(II) catalysis was observed for the first time in this medium. As Mg(II) is an important metal in the COMT catalyzed reaction *in vivo*, this observation is very interesting. Kinetic analyses of the present data and recalculation of a part of the previous data offered the following evidence. In Cu(II) catalysis, a 1 : 2 complex(CuL_2) was more active than the 1 : 1 complex (CuL). On the other hand, in Mg(II) catalysis and Zn(II) catalysis, ML was more active than ML_2. These facts show that ML_2 is not always more active than ML, contrary to previous reports. Methanolysis of DMS, a significant side reaction of this model reaction, and dissociation of LH_2 were studied thoroughly as bases for these kinetic analyses.

Keywords catechol-*O*-methyltransferase; enzyme model; 3,4-dihydroxybenzaldehyde; dimethyl sulfate; metal chelate; *O*-methylation

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6), one of the important enzymes in catecholamine metabolism, occurs widely in mammalian tissues and catalyzes the transfer of the methyl group of *S*-adenosyl-L-methionine (SAM) to a hydroxyl group of catechol or substituted catechols.[2] The quantity of this enzyme is elevated in human breast cancers and catabolyzes catecholestrogens, which exert opposite effects on hormone-sensitive breast cancer cell growth and differentiation, into methoxy metabolites.[3] Mg^{2+} is required for the full activity of this enzyme, and can be substituted by other divalent metal ions such as Mn^{2+}, Co^{2+} and Zn^{2+}.[2,4,5] The *O*-methylation *in vivo* occurs almost exclusively on the *m*-hydroxyl group of the catechols,[6,7] but *in vitro* it occurs at one of the *m*- or *p*-hydroxyl groups.[5,7,8] The role of Mg^{2+} was proposed as bringing substrates and COMT together in the bridge complex[5] and so on,[9] but is not fully understood at present.

A chemical model of COMT, nonenzymatic *O*-methylation, was studied by Senoh *et al.*[5] They accounted for the catalytic effects of metal ions in terms of the formation of a 2 : 1 catechol–metal complex (this complex is expressed as ML_2 in this paper). However, because of the lack of an easy analytical method, sufficient kinetic studies have not been done.

We studied the reaction of 3,4-dihydroxybenzaldehyde (LH_2) and dimethyl sulfate (DMS) in forming the *m*- and *p*-*O*-methylated products (vanillin and isovanillin, respectively) in an aqueous buffer solution, which is one of the model reaction of Senoh *et al.*,[5] kinetically.[1] Kinetic studies showed that CuL_2 was an active species. This was the same result that Senoh *et al.* showed.[5] However, Zn-catalysis couldn't be analyzed thoroughly because of a lack of reliable stability constants of ZnL and ZnL_2. This medium had two defects. One was the presence of a significant side reaction, that is, the rapid hydrolysis of DMS.[1,10] Therefore, the amounts of *O*-methylated products were very small. The other was a low stability of the Mg(II) complex of LH_2, with Mg(II) showing only a small catalytic effect. To overcome these defects, the same reactions in a methanol solution were studied.

Experimental

The water content of methanol used was about 0.035% (w/v) (about 20 mM) by means of Karl Fischer's method. The methanol solution of hydrogen chloride was prepared as follows. Hydrogen chloride gas produced from conc. HCl–conc. H_2SO_4 was desiccated through conc. H_2SO_4 and dissolved in methanol. Other chemicals were the same as described in the previous paper.[1]

Kinetic runs were carried out in 0.2 M bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane(Bis-Tris)–0.02 M HCl methanol buffer solution unless otherwise stated. The kinetic procedures were described in the previous paper.[1] After removal of the solvent and the addition of water, the aqueous solution was treated in a similar manner to that described in the previous paper.[1]

Conditions of the HPLC measurement were essentially the same as described in the previous paper.[1]

Results and Discussion

Basic Study The effect of the concentration of HCl in the buffer was studied in the absence of metal ions. The concentration of *O*-methylated products at the completion of the reaction (S_∞) decreased with an increasing concentration of HCl, as shown in Fig. 1. The *m*-/*p*- ratio increased slightly. With an increasing concentration of HCl, the slope

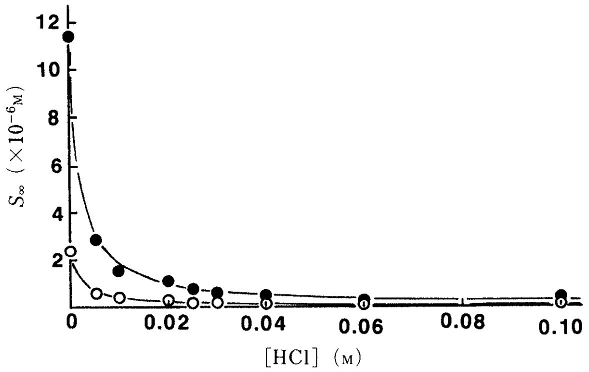

Fig. 1. *O*-Methylation of LH_2 as a Function of the Concentration of Added HCl at 37 °C in 0.2 M Bis-Tris Methanol Solution

Open circles, vanillin; closed circles, isovanillin. Lines are best fit curves of $y=a/(x+b)+c$. $[LH_2]_0=[DMS]_0=2.5$ mM.

Table I. S_∞ and First-Order Rate Constants (k_d) of Methanolysis of DMS Calculated from the Time Dependence of *O*-Methylation

T (°C)	$[LH_2]_0$ (mM)	$[DMS]_0$ (mM)	$[M(II)]_T$[a] (mM)	*n*	S_∞ (μM)		k_d[b] ($\times 10^{-5}\,s^{-1}$)
					Van	IVan	
30	5	2.5	0	2	0.60±0.03	2.16±0.25	3.73±0.48
37	2.5	2.5	0	2	0.30±0.02	1.10±0.01	7.74±0.86
	2.5	5	0	2	0.67±0.01	2.39±0.05	8.08±0.46
	5	2.5	0	2	0.51±0.06	1.97±0.17	7.98±1.02
	2.5	2.5	5 (Cu)	1	2.45	1.93	7.94±0.01
	2.5	2.5	10 (Cu)	1	2.71	1.96	7.21±0.95
	2.5	2.5	15 (Cu)	1	2.98	2.23	7.85±0.22
	2.5	2.5	5 (Mg)	1	1.87	1.82	8.02±0.31
	2.5	2.5	10 (Mg)	1	2.78	1.74	7.63±0.66
	2.5	2.5	15 (Mg)	1	3.05	2.14	8.56±0.91
	2.5	2.5	5 (Zn)	1	0.93	1.60	7.23±0.07
	2.5	2.5	10 (Zn)	1	2.18	1.76	6.85±0.26
	2.5	2.5	15 (Zn)	1	3.71	2.63	7.21±0.35
	2.5	2.5	20 (Zn)	1	5.33	2.88	7.25±0.75
						Av.[c]	7.71±0.79
	5	2.5	0	1[d]	0.43	1.47	8.10±0.91
	5	2.5	0	1[e]	0.48	1.77	8.37±0.38
	5	2.5	0	1[f]	0.55	1.69	8.18±0.83
44	5	2.5	0	3	0.55±0.03	2.22±0.13	15.06±2.26

Experiments were done in 0.2 M Bis-Tris–0.02 M HCl methanol buffer solution, unless otherwise stated. Values are mean ± S.D. *a)* The metal salts used were hexahydrates, $Cu(ClO_4)_2 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$ and $Zn(ClO_4)_2 \cdot 6H_2O$. *b)* As values of k_d were obtained from both Van and IVan formation, the number of data is two-fold *n*. *c)* The average value of k_d ($n=16$) at 37 °C is used for calculations in this paper. *d)* Concentration of HCl in the buffer solution is 0.03 M. *e)* Concentration of added H_2O is 60 mM. *f)* Concentration of added H_2O is 120 mM.

of the S_∞ curve decreased. This fact assured the constancy of the reactivity of LH_2 at a high HCl concentration. On the other hand, at a high HCl concentration, the catalytic effects of divalent metal ions decreased (data not presented). From these contrary facts, 0.02 M HCl was used as the appropriate buffer concentration.

The time dependence of the formation of *O*-methylated products was essentially the same as described in the previous paper,[1] both in the presence and in the absence of divalent metal ions. That is, the formation of vanillin (Van) and isovanillin(IVan) followed first-order kinetics. The observed first-order rate constants (k_{obs}) calculated from Van formations were almost the same as those of the IVan formation. The total yields of Van and IVan at the completion of the reaction were less than 1% of the amount expected from the yields of LH_2 and DMS. Although the value of k_{obs} is more than 10 times smaller than in aqueous solution, these phenomena were essentially identical with those in aqueous solution and were explained in terms of the rapid solvolysis of DMS. Therefore, k_{obs} can be presented by k_d, which is the first-order rate constant of the solvolysis of DMS.[1] The values of k_d and S_∞ are shown in Table I. Although the values of S_∞ and *m*-/*p*-ratios were dependent on the conditions, the values of k_d were almost completely consistent if the temperature remained constant. Because only a small effect was caused by the addition of a small amount of water, the main solvolysis was methanolysis. Temperature affected the values of k_d clearly, but affected the values of S_∞ ambiguously. The Arrhenius plot of the values of k_d was almost linear. The activation parameters of the methanolysis of DMS are shown in Table II.

Kolesnikov *et al.* studied the methanolysis of DMS and reported that methanolysis produced monomethyl sulfate (MMS) and dimethyl ether, but further methanolysis of MMS didn't occur.[11] The values of the first-order rate constant and the activation parameters at 37°C calculated from their data were almost the same as the values of this work, as shown in Table II. These facts support the validity of the interpretation of the time dependence of *O*-methylation, as mentioned above.

Table II. Values of k_d and Activation Parameters of Methanolysis of DMS at 37 °C

Source	k_d (s^{-1})	E_a ($kJ\,mol^{-1}$)	$\Delta H^{\neq}$ ($kJ\,mol^{-1}$)	$\Delta S^{\neq}$ ($JK^{-1}\,mol^{-1}$)
This work[a]	7.71×10^{-5}	79.7	77.1	−75.3
Ref. 11[b]	6.07×10^{-5}	83.2	80.7	−65.9

a) Reproduced and calculated from the data in Table I. *b)* Calculated from the data at 35 °C in ref. 11.

In the methanol solution, methanolysis of DMS, a significant side reaction, was observed. Therefore, kinetics were discussed in terms of the initial rate (V_0) of *O*-methylation calculated as follows.[1]

$$V_0 = S_\infty \cdot k_d \tag{1}$$

In the absence of metal ions, S_∞ was measured at various concentrations of LH_2 and DMS as shown in Fig. 2 at 37°C. The values of k_d are almost constant, and S_∞ is almost proportional to the initial concentration of these substances, $[LH_2]_0$ and $[DMS]_0$, except at excess $[DMS]_0$. Therefore, V_0 can be expressed as

$$V_0 = k_1[LH_2]_0[DMS]_0 \tag{2}$$

where k_1 indicates the second-order rate constant, and the values are listed in Table III. At excess $[DMS]_0$, the constancy of $[H^+]$ may not be maintained during the reaction. The values of k_1 at different temperatures were calculated from the data in Table I and Eqs. 1 and 2, and the results are shown in Table III. Although the Arrhenius

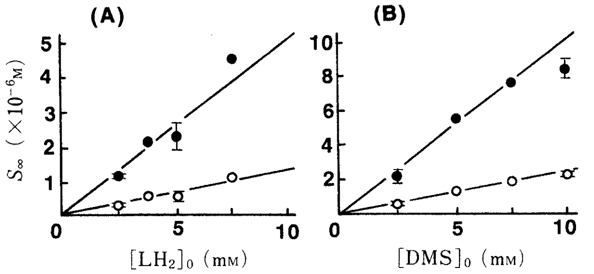

Fig. 2. *O*-Methylation of LH_2 as a Function of the Concentration of the Reactants, LH_2 (A) or DMS (B), at 37 °C in Methanol Buffer Solution

Open circles, vanillin; closed circles, isovanillin. Error bars represent S.D. of mean, and lines are best fit lines of $y = ax$. (A): $[DMS]_0 = 2.5$ mM. (B): $[LH_2]_0 = 5$ mM. Lines were obtained without the data of $[DMS]_0 = 10$ mM.

TABLE III. Apparent Second-Order Rate Constants (k_1)

T (°C)	k_1 ($\times 10^{-5}$ M^{-1} s^{-1})	
	Van	IVan
30	0.179	0.643
37	0.400	1.57
44	0.659	2.68

plots of the values were almost linear in both Van and IVan formation, activation parameters weren't calculated. Because, as k_1 is an apparent constant, the calculation is meaningless.

In this reaction system, the dissociation equilibrium can be written as follows.

$$LH_2 = LH^- + H^+$$

$$LH^- = L^{2-} + H^+$$

Dissociation constants and total concentration of LH_2 are defined as follows.

$$K_{a1} = [LH^-][H^+]/[LH_2] \quad (3)$$

$$K_{a2} = [L^{2-}][H^+]/[LH^-] \quad (4)$$

$$[LH_2]_T = [L^{2-}] + [LH^-] + [LH_2] \quad (5)$$

$[LH_2]_T$ is equal to $[LH_2]_0$ at $t = 0$, and is kept almost constant during the reaction. Therefore, Eq. 2 is replaced by Eq. 6.

$$V_0 = k_1[LH_2]_T[DMS]_0 \quad (6)$$

On the other hand, V_0 can be expressed using concentrations of three species of LH_2 as follows.

$$V_0 = (k_{a1}[L^{2-}] + k_{a2}[LH^-] + k_{a3}[LH_2])[DMS]_0 \quad (7)$$

In this equation, k_{a1}, k_{a2}, and k_{a3} are second-order rate constants of each species, respectively. Eq. 7 is replaced by Eq. 8 using Eqs. 3—5.

$$V_0 = \{(k_{a1}K_{a1}K_{a2} + k_{a2}K_{a1}[H^+] + k_{a3}[H^+]^2)/(K_{a1}K_{a2} + K_{a1}[H^+] + [H^+]^2)\}[LH_2]_T[DMS]_0 \quad (8)$$

From the comparison of Eqs. 6 and 8, k_1 is equal to the part of { } in Eq. 8. Therefore, k_1 is an apparent second-order rate constant. Determination of the exact concentration of $[H^+]$ in this methanol buffer solution is difficult technically. Therefore, determination of these constants in Eq. 8 by the method of least squares is difficult. Because the difference of HCl concentration in the buffer solution caused almost no effect to the values of k_d as shown in Table I, V_0 is inferred to be proportional to S_∞. The simulation of Eq. 8 accounts for Fig. 1, approximately, and shows $K_{a1} \gg K_{a2}$ and $k_{a1} \gg k_{a2}, k_{a3}$. This order of rate constants is accordant with the result of Senoh *et al.*[5] This simulation is well expressed by a more simple but less rational equation, $y = a/(x+b) + c$, which is fitted to the data by the method of least squares for a rough estimate of S_∞ variation.

Kinetics of the Metal Iron Catalysis S_∞ was measured in the concentrations of $[LH_2]_0 = [DMS]_0 = 2.5$ mM and various concentrations of metal ions, and V_0 was calculated according to Eq. 1. The results are shown in Fig. 3. The *O*-methylations, especially *m*-*O*-methylations, were catalyzed by divalent metal ions. Variations of V_0 in both Cu(II) and Zn(II) catalysis were almost the same as those in aqueous solution.[1] On the other hand, the Mg(II) catalysis was clearly observed for the first time in this medium. As Mg(II) is an important metal in the COMT catalyzed reaction *in vivo*, this observation is very interesting. These effects were explained in terms of the formation of the complexes, ML and ML_2^{2-}, as follows.

$$M^{2+} + L' = ML$$

$$ML + L' = ML_2^{2-}$$

In these equations, L′ is free L not combined with any metal, and denotes a mixture of L^{2-}, LH^- ane LH_2. In the following description, M^{2+} and ML_2^{2-} are abbreviated as M and ML_2, respectively. In a given buffered methanol solution, the conditional stability constants are defined as follows.

$$K_1 = [ML]/([M][L']) \quad (9)$$

$$K_2 = [ML_2]/([ML][L']) \quad (10)$$

Total concentration of M and L are expressed as follows.

$$[M]_T = [M] + [ML] + [ML_2] \quad (11)$$

$$[L]_T = [L^{2-}] + [LH^-] + [LH_2] + [ML] + 2[ML_2] = [L'] + [ML] + 2[ML_2] \quad (12)$$

$[L]_T$ is equal to $[LH_2]_0$ at $t = 0$, and is kept almost constant during the reaction. Therefore, in the metal ion-catalyzed reactions, V_0 can be expressed as follows,

$$V_0 = (k_1[L'] + k_2[ML] + k_3[ML_2])[DMS]_0 \quad (13)$$

where k_2 and k_3 are second-order rate constants, respectively.

It is necessary to know the values of K_1 and K_2 for the analyses of these kinetics. Although ultraviolet absorbance at about the 350 nm region increased with the formation of complexes ML and ML_2,[12] reliable stability constants were not obtained. Therefore, these metal ion catalyses were analyzed by the method of nonlinear least squares from the data in Fig. 3 using Eqs. 9—13 directly. Previously reported Zn(II) catalysis in aqueous solution[1] was inserted again in Fig. 4, in which reliable stability constants were not obtained, and was analyzed similarly. From Figs. 3A, 3B and 4, sets of values of K_1, K_2, k_2 and k_3 were obtained from Van formation, but not from IVan formation because of the relatively small variation and large deviation of V_0. Therefore, k_2 and k_3 of IVan formation were calculated using K_1 and K_2 obtained from Van formation. Results are shown in Table IV. In these figures, the calculated curves fitted well in the data, and these facts support the proposed mechanism mentioned above. In Cu(II) catalysis, the order of magnitude of the constants in *m*-*O*-methylation is $k_2 < k_3/2$, and a prominently convex V_0 curve is observed similar to that observed in aqueous solution.[1] Since k_3 is the constant of ML_2, $k_3/2$ was compared with k_2 of the constant of ML. In Mg(II) catalysis, the order is $k_2 > k_3/2$, and a slightly convex V_0 curve is observed. In Zn(II) catalysis in aqueous solution, the order is $k_2 \gg k_3/2$, and a sigmoidal V_0 curve is observed. These relationships between the order of rate constants and the shape of the V_0 curve can generally be simulated using Eqs. 9—13. On the other hand, from Fig. 3C, no set of these values was obtained from either Van or IVan formation because of a relatively large deviation. In Zn(II) catalysis in methanol solution, a sigmoidal V_0 curve was observed; the order is inferred to be $k_2 \gg k_3/2$.

In conclusion, the observation of clear Mg(II) catalysis in methanol solution is an important progress in the study of the COMT model, though the amounst of *O*-methylated products were very small similarly to the aqueous solution.[1] On the other hand, if metal complexes were formed, the difference of the solvents, water or methanol, was not considered to be large. In Cu(II) catalysis, ML_2 is more active than ML in *m*-*O*-methylation. As ML_2 is an anionic species, the nucleophilicity of the *m*-phenoxide ion[5] may be increased. On the other hand, in Mg(II) catalysis and Zn(II) catalysis, ML is more active than ML_2. In these cases an alternative interpretation is needed, but distinct interpretation is not possible because of the lack of sufficient data. This difference of metal ions shows that ML_2 is not always more active than ML, contrary to the conclusions of previous reports.[1,5] Further, we are studying the effect of another bidentate ligand(R) to this chemical model.[13] In the presence of R, the formation of the ternary complex MLR, which is a good model of a bridge complex[5] and so

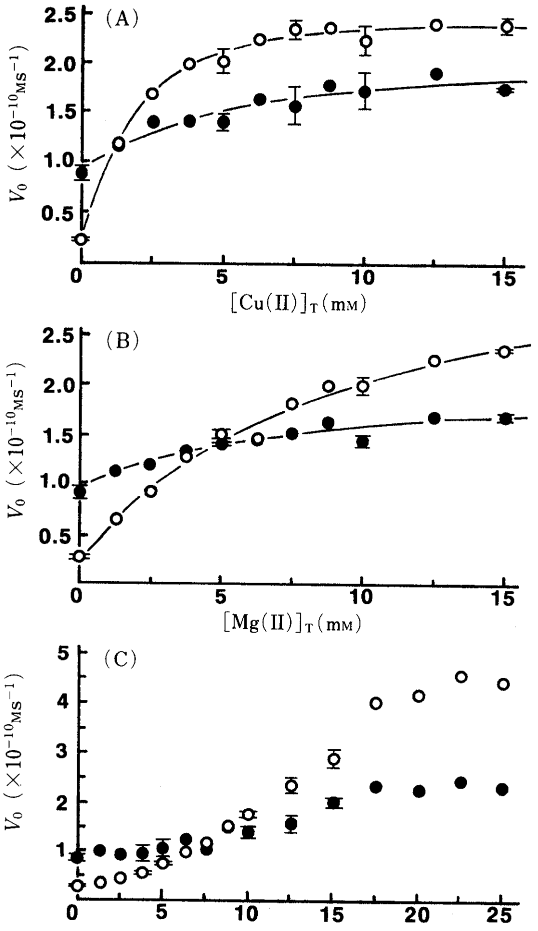

Fig. 3. M(II)-Catalyzed *O*-Methylation of LH_2 at 37 °C in Methanol Buffer Solution

Open circles, vanillin; closed circles, isovanillin. Error bars represent S.D. of mean, and lines are best fit curves calculated by Eqs. 9—13 with parameters in Tables III and IV. $[LH_2]_0 = [DMS]_0 = 2.5$ mM (A): Cu(II)-catalyzed (B): Mg(II)-catalyzed (C): Zn(II)-catalyzed. Best fit curves were not obtained.

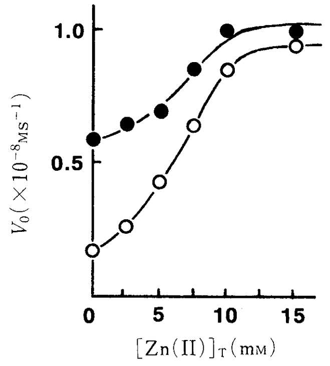

Fig. 4. Recalculation of Zn(II)-Catalyzed *O*-Methylation of LH_2 at 37 °C in Aqueous Buffer Solution[1]

Open circles, vanillin; closed circles, isovanillin. Lines are best fit curves calculated by Eqs. 9—13 with parameters in Table IV and other parameters in the previous report.[1] $[LH_2]_0 = [DMS]_0 = 10$ mM.

TABLE IV. Conditional Stability Constants (K_1, K_2) and Second-Order Rate Constants (k_2, k_3) at 37 °C

Metal	Medium	Source	Product	Stability const.		Rate const. (×10^{-5}M^{-1}s^{-1})	
				$\log K_1$	$\log K_2$	k_2	k_3
Cu(II)	Methanol	Fig. 3A	Van	2.53	2.04	3.73	26.4
			IVan	N.D.		3.36[a]	3.23[a]
Mg(II)	Methanol	Fig. 3B	Van	2.37	3.21	8.87	0.405
			IVan	N.D.		4.33[a]	3.10[a]
Zn(II)	Aqueous	Fig. 4	Van	4.22	1.93	9.66	0.117
			IVan	N.D.		10.37[a]	8.53[a]

These values were obtained by the method of non-linear least squares. N.D., not determined. *a*) Calculated using the values of K_1 and K_2 obtained from the data of Van formation.

on,[9] is expected. Details of the study will be reported shortly. In view of MLR, the R of ML is solvent. For a better chemical model of COMT, a combination of M(II) and R will be important.

Acknowledgement The author thanks Professor Y. Matsushima of Kyoritsu College of Pharmacy, for useful discussions. The author also thanks Miss A. Ozawa and Miss S. Katsumata for assistance in the preliminary study. This work was supported in part by the Nishi Cancer Research Fund.

References and Notes

1) Preliminary report: S. Sugata, S. Ishihara, Y. Watanabe, Y. Nagata and Y. Matsushima, *Chem. Pharm. Bull.*, **37**, 1143 (1989).
2) J. Axelrod and R. Tomchick, *J. Biol. Chem.*, **233**, 702 (1958).
3) M. Assicot, G. Contesso and C. Bohuon, *Eur. J. Cancer*, **13**, 961 (1977); B. Vandewalle and J. Lefebvre, *Mol. Cell. Endocrinol.*, **61**, 239 (1989).
4) J. Veser, *J. Bacteriol.*, **169**, 3696 (1987).
5) S. Senoh, Y. Tokuyama and B. Witkop, *J. Am. Chem. Soc.*, **84**, 1719 (1962).
6) J. Axelrod, S. Senoh and B. Witkop, *J. Biol. Chem.*, **233**, 697 (1958).
7) J. W. Daly, J. Axelrod and B. Witkop, *J. Biol. Chem.*, **235**, 1155 (1960).
8) C. R. Creveling, N. Dalgard, H. Shimizu and J. W. Daly, *Mol. Pharmacol.*, **6**, 691 (1970).
9) D. R. Jeffery and J. A. Roth, *Biochemistry*, **26**, 2955 (1987).
10) R. E. Robertson and S. E. Sugamori, *Can. J. Chem.*, **44**, 1728 (1966).
11) V. A. Kolesnikov, R. V. Efremov and S. M. Danov, *Kinet. Catal.*, **20**, 671 (1979).
12) S. Sugata, S. Ishihara and Y. Matsushima, *Ann. Report of Kyoritsu College of Pharmacy*, **33**, 1 (1988).
13) S. Sugata and Y. Matsushima, *J. Pharmacobio-Dyn.*, **14**, s-117 (1991).