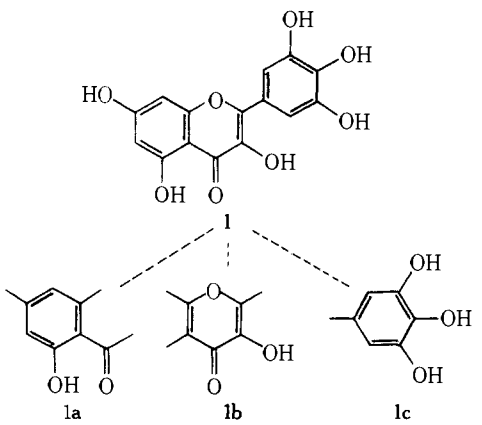
(13) S. S. Yang, F. M. Herrera, R. G. Smith, M. S. Reitz, G. Lancini, R. C. Ting, and R. C. Gallo, *J. Nat. Cancer Inst.*, **49**, 7 (1972).
(14) T. S. Papas, L. Sandhaus, M. A. Chirigos, and E. Furusawa, *Biochem. Biophys. Res. Commun.*, **52**, 88 (1973).
(15) M. A. Apple, *Annu. Rep. Med. Chem.*, 8, 251 (1973).
(16) P. Chandra, A. Di Marco, F. Zunino, A. M. Casazza, D. Gericke, F. Giuliani, C. Soranzo, R. Thorbeck, A. Götz, F. Arcamone, and M. Ghione, *Naturwissenchaften*, **59**, 448 (1972).
(17) M. C. Scrutton, C. W. Wu, and D. A. Goldthwait, *Proc. Nat. Acad. Sci. U. S.*, **68**, 2497 (1971).
(18) M. J. Chamberlin and J. Ring, *Biochem. Biophys. Res. Commun.*, **49**, 1129 (1972).
(19) J. S. Krakow, *Biochim. Biophys. Acta*, **95**, 532 (1965).
(20) W. Wehrli, F. Knüsel, K. Schmid, and M. Staehelin, *Proc. Nat. Acad. Sci. U. S.*, **61**, 667 (1968).
(21) R. H. Adamson, *Lancet*, 398 (1971).
(22) H. W. Toolan and N. Ledinko, *Nature (London), New Biol.*, **237**, 200 (1972).
(23) W. K. Roberts and W. H. Coleman, *Biochemistry*, **10**, 4304 (1971).
(24) T. Blumenthal and T. Landers, *Biochem. Biophys. Res. Commun.*, **55**, 680 (1973).
(25) R. R. Burgess, *J. Biol. Chem.*, **244**, 6160 (1969).
(26) D. Baltimore and D. Smoler, *Proc. Nat. Acad. Sci. U. S.*, **68**, 1507 (1971).
(27) O. W. Jones and P. Berg, *J. Mol. Biol.*, **22**, 199 (1966).

Catechol *O*-Methyltransferase. 5. Structure–Activity Relationships for Inhibition by Flavonoids

Ronald T. Borchardt*,† and Joan A. Huber

Department of Biochemistry, McCollum Laboratories, University of Kansas, Lawrence, Kansas 66045. *Received August 16, 1974*

Flavonoids have been long recognized as inhibitors of the enzyme catechol *O*-methyltransferase (COMT,‡ E.C. 2.1.1.6).[1-4] These compounds represent an interesting class of COMT inhibitors because of their multifunctional features which individually would be expected to have the potential of binding to the active site of this enzyme. As an example, myricetin (1) possesses structural features (1a-c) which are present in the COMT inhibitors salicylaldehyde,[5] 3-hydroxy-4-pyrone,[6] and pyrogallol.[7] In an attempt to show the potential involvement of the structural components 1a-c of myricetin (1) in its binding to COMT, we have evaluated as inhibitors of COMT various model compounds in which these three important structural features have been isolated. In this way we have been able to show that there exist three sites on myricetin, which would have the potential to bind to the active site of COMT. The present paper reports the results of this study.



1a 1b 1c

Results and Discussion

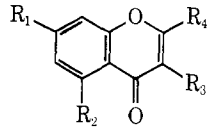
Table I shows the degree of COMT inhibition produced by the parent compound myricetin (1) and the various model compounds prepared in this study. These model compounds, in which we were able to isolate the various important structural features of myricetin (1), can be divided into three general types: (a) β-hydroxycarbonyl compounds [salicylaldehyde (7), 3,5-dihydroxyflavone (4), and 5-hydroxychromone (5)]; (b) α-hydroxycarbonyl compounds [3-hydroxy-4-pyrone (8), 3-hydroxyflavone (2), and 3-hydroxychromone (3)]; and (c) polyphenolic compounds (pyrogallol). As would be expected chromone (6), which has the basic skeletal structure of myricetin (1), is completely inactive as an inhibitor of COMT. The data in Table I show that the α-hydroxycarbonyl compounds, both 3-hydroxyflavone (2) and 3-hydroxychromone (3), are potent inhibitors of COMT and in fact appear to be more active than 3-hydroxy-4-pyrone (8). This difference in activity probably results from the fact that 3-hydroxy-4-pyrone (8) would show a greater tendency than compounds 2 and 3 to exist in a diketo tautomeric structure.[8] This diketo tautomeric structure would be expected to be inactive as a COMT inhibitor. For the β-hydroxycarbonyl compounds [salicylaldehyde (7), 3,5-dihydroxyflavone (4), and 5-hydroxychromone (5)] relatively weak inhibitory activity toward COMT was observed. Therefore, it would appear that replacement of one of the hydroxyl groups of catechol by a carbonyl function [*e.g.*, salicylaldehyde (7) and 5-hydroxychromone (5)] results in β-hydroxycarbonyl compounds which still have a potential to bind to COMT but provide less than an optimal fit to the enzyme site. In contrast, systems such as tropolone,[9] 3-hydroxy-4-pyridone,[6] and 3-hydroxy-4-pyrone,[6] which are α-hydroxycarbonyl compounds and biochemically isosteric to catechol, appear to bind more tightly to the enzyme. Similarly, systems in which one of the hydroxyl groups of catechol has been replaced by another heteroatom (*e.g.*, 8-hydroxyquinoline[10]) also show a greater affinity for the active site of this enzyme.

Using reciprocal velocity *vs.* reciprocal substrate plots, the kinetic patterns for COMT inhibition by myricetin (1), 3-hydroxychromone (3), 5-hydroxychromone (5), salicylaldehyde (7), and 3-hydroxy-4-pyrone (8) were determined. In order to more accurately compare the inhibitory properties of these compounds with previously reported COMT inhibitors,[5,6,9,10] DHB was used as the catechol substrate rather than *l*-NE. Use of DHB rather than *l*-NE in these kinetic studies also eliminated any possible formation of tetrahydroisoquinolines by reaction of *l*-NE and

†Established Investigator of the American Heart Association.

‡Abbreviations used are COMT, catechol *O*-methyltransferase; SAM, *S*-adenosyl-L-methionine; DHB, 3,4-dihydroxybenzoate; *l*-NE, *l*-norepinephrine; K_{is}, inhibition constant for the slope; K_{ii}, inhibition constant for the intercept.

Table I. *In Vitro* Inhibition of COMT[a]



Compd no.	Inhibitor[b] R_1	R_2	R_3	R_4	% inhibition[c] [I] = 0.1 mM	[I] = 1.0 mM
1	OH	OH	OH	3,4,5-Trihydroxyphenyl	64	89
2	H	H	OH	Phenyl	55	87
3	H	H	OH	H	59	86
4	OH	OH	H	Phenyl	19	59
5	H	OH	H	H	21	65
6	H	H	H	H	0	0
7	Salicylaldehyde				15	40
8	3-Hydroxy-4-pyrone				15	52
9	Pyrogallol				34	76

[a]COMT was purified and assayed as previously described.[5,6,9,10] SAM-$^{14}CH_3$, 0.05 μCi; SAM concentration, 1.0 m*M*; *l*-NE concentration, 2.0 m*M*; phosphate buffer, pH 7.60; Mg^{2+} concentration, 1.2 m*M*. Incubation was carried out for 30 min at 37° and the reaction stopped using 0.25 ml of borate buffer, pH 10.0. The assay mixture was extracted with 10 ml of toluene–isoamyl alcohol (3:2) and after centrifugation a 5-ml aliquot of the organic phase was measured for radioactivity. The results were corrected using the appropriate *l*-NE blank. [b]Inhibitors added from aqueous stock solution of 5.0 μmol/ml. [c]Expressed as per cent inhibition of O-methylation of *l*-NE.

salicylaldehyde (**7**), as had previously been observed with *l*-NE and pyridoxal 5′-phosphate.[5] As shown in Table II, linear competitive patterns of inhibition were observed when DHB was the variable substrate and either 3-hydroxychromone (**3**) or 3-hydroxy-4-pyrone (**8**) was the inhibitor. Of particular interest is the potent inhibitory effect of 3-hydroxychromone (**3**) as compared to 3-hydroxy-4-pyrone (**8**), which would suggest that appropriate modifications of 3-hydroxychromone (**3**) might provide a new class of potent COMT inhibitors. Linear noncompetitive patterns of inhibition were observed when myricetin (**1**), 5-hydroxychromone (**5**), or salicylaldehyde (**7**) was the inhibitor. Both 5-hydroxychromone (**5**) and salicylaldehyde (**7**) were relatively weak COMT inhibitors consistent with the previously reported data for other β-hydroxycarbonyl compounds.[5] These noncompetitive patterns of inhibition, coupled with the fact that both **5** and **7** show uncompetitive patterns of inhibition with varying SAM, would indicate these compounds are inhibiting COMT by a similar mechanism and binding to similar sites as tropolone[9] and 8-hydroxyquinoline.[10]

In summary, we have shown that a flavonoid like myricetin (**1**) has the potential of binding to COMT through

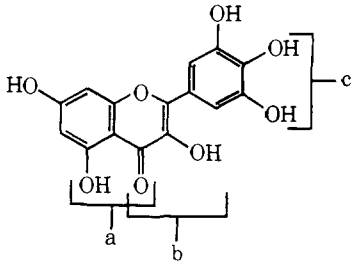


Table II. Inhibition Constants for Myricetin (**1**), 5-Hydroxychromone (**5**), 3-Hydroxychromone (**3**), Salicylaldehyde (**7**), and 3-Hydroxy-4-pyrone (**8**)[a]

Inhibitor	Inhibition pattern[b]	Inhibition constants, m*M*[c] K_{is}	K_{ii}
Myricetin (**1**)	NC	0.033 ± 0.010	0.182 ± 0.43
3-Hydroxychromone (**3**)	C	0.048 ± 0.008	
5-Hydroxychromone (**5**)	NC	0.322 ± 0.29	1.28 ± 0.15
Salicylaldehyde (**7**)	NC	0.552 ± 0.07	1.87 ± 0.24
3-Hydroxy-4-pyrone (**8**)	C	0.360 ± 0.019	

[a]COMT was assayed as described in Table I except SAM concentration, 1.0 m*M*; DHB concentration, 40–400 μ*M*; and reaction stopped with 0.10 ml of 1.0 *N* HCl and assay mixture extracted with 10 ml of toluene–isoamyl alcohol (7:3). [b]NC, noncompetitive; C, competitive. [c]Inhibition constants calculated as previously described.[5,6,9,10,14]

three structural features on the molecule: (a) a β-hydroxycarbonyl structure like that found in salicylaldehyde (site a); (b) an α-hydroxycarbonyl structure similar to that found in 3-hydroxy-4-pyrone (site b); and (c) a polyphenolic structure like that found in pyrogallol (site c). The structural features of myricetin (**1**), which appear to produce the most favorable interaction with the active site of COMT, are the polyphenolic site (site c) and the α-hydroxycarbonyl site (site b). The polyphenolic site (site c) probably contributes to the inhibitory properties of myricetin (**1**) by its inherent affinity for the active site of COMT and its potential for being methylated like pyrogallol,[7] therefore acting as an alternate substrate type inhibitor. The α-hydroxycarbonyl site (site b) also appears to have a high affinity for the active site of COMT and is probably contributing significantly to myrecitin's (**1**) inhibitory properties by acting like a dead-end inhibitor. The β-hydroxycarbonyl group (site a) appears to produce a less favorable interaction and is probably not important in the binding of myricetin (**1**) to COMT. However, the binding of myricetin (**1**), as well as other flavonoids to COMT, represents a rather unique ligand–enzyme interaction, because of the fact that the ligand possesses multifunctional features, each capable of binding with the same site on the enzyme surface.

Experimental Section

Materials. Materials used in the enzyme purification and assay were similar to those described in earlier publications.[5,6,9,10] Compounds **1**, **2**, **4**, **6**, **7**, **9**, 4-chromanone, and 2,6-dihydroxyacetophenone were available from commercial sources (Aldrich, Eastman). Compound **8** was prepared by a procedure previously described by Bickel.[11]

5-Hydroxychromone (5). 5-Hydroxychromone (**5**) was prepared by the reaction of 2,6-dihydroxyacetophenone (5.0 g, 33 mmol) with sodium ribbon (3.8 g, 165 mmol) in ethyl formate (58 g). Dehydration of the resulting condensation product was accomplished by refluxing in 30 ml of glacial acetic acid and 2 ml of concentrated HCl, according to the procedure of Murata, *et al.*[12] The product was isolated by steam distillation and recrystallized (EtOH–H_2O) affording 2.83 g (53%), mp 125.5–127.5° (lit.[12] mp 125.5–127.5°).

3-Hydroxychromone (3). Since difficulty was encountered in the preparation of 3-hydroxychromone (**3**) by the previously published procedures,[13] the following modification was used for the synthesis of the desired product. To a solution of 4-chromanone (1.08 g, 7.3 mmol) in 3.0 ml of methanol and 1.0 ml of concentrated HCl at 0° was added dropwise freshly distilled *n*-amyl nitrite

(1.58 g, 13.5 mmol). The solution was allowed to stand (under N_2) at 0° for 24 hr. A tlc (10% EtOH-$CHCl_3$, silica gel) of this reaction mixture showed the presence of a new spot with a lower R_f, but still indicated the presence of starting material. Therefore, 1.0 ml of additional concentrated HCl was added, followed by the dropwise addition of 1.58 g of *n*-amyl nitrite (13.5 mmol). The reaction mixture was maintained at 0–4° for an additional 24 hr after which 10 ml of H_2O was added and the aqueous solution extracted thoroughly with Et_2O. The Et_2O solution was washed with 5% NaOH; the NaOH solution was filtered and then acidified with 5% HCl. The acidic solution was washed with $CHCl_3$ and the $CHCl_3$ solution dried ($MgSO_4$), and the solvent was removed under reduced pressure to yield ~0.5 g (31%) of the desired product. This solid was sublimed (1.5 mm, 70–90° bath temperature) to give pale yellow crystal, mp 178.5–179.5° (lit.[13e] mp 178–179.5°).

Enzyme Purification and Assay. COMT was purified from rat liver (male, Sprague–Dawley, 180–200 g) and assayed using the substrates SAM-$^{14}CH_3$ and 3,4-dihydroxybenzoic acid or *l*-norepinephrine as previously described.[5,6,9,10] Processing of the kinetic data was achieved by plotting reciprocal velocities against reciprocals of the substrate concentrations. Inhibition constants were calculated using Cleland's equations[14] as described in earlier publications from this laboratory.[5,6,9,10]

Acknowledgment. The authors gratefully acknowledge support of this project by a Research Grant from the National Institutes of Neurological Disease and Stroke (NS-10918). The excellent technical assistance of Bi-Shia Wu is gratefully acknowledged.

References

(1) G. Kroneberg, K. Schlossmann, and G. Haberland, *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol.*, **241**, 522 (1961).
(2) S. B. Ross and O. Haljasmaa, *Acta Pharmacol. Toxicol.*, 21, 205, 215 (1964).
(3) K. P. Schwabe and L. Flohe, *Hoppe-Seyler's Z. Physiol. Chem.*, **353**, 476 (1972).
(4) R. Gugler and H. J. Dengler, *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol.*, **276**, 223 (1973).
(5) R. T. Borchardt, *J. Med. Chem.*, **16**, 387 (1973).
(6) R. T. Borchardt, *J. Med. Chem.*, **16**, 581 (1973).
(7) (a) J. R. Crout, C. R. Creveling, and S. Udenfriend, *J. Pharmacol. Exp. Ther.*, **132**, 269 (1960); (b) J. Axelrod and M. J. Laroche, *Science*, **130**, 800 (1959).
(8) G. Ittrich, *Z. Physiol. Chem.*, **312**, 1 (1958).
(9) R. T. Borchardt, *J. Med. Chem.*, **16**, 377 (1973).
(10) R. T. Borchardt, *J. Med. Chem.*, **16**, 382 (1973).
(11) A. F. Bickel, *J. Amer. Chem. Soc.*, **69**, 1801, 1803, 1805 (1947).
(12) A. Murata, T. Suzuki, and T. Ito, *Bunseki Kagaku*, **14**, 630 (1965).
(13) (a) H. Perkin and R. Robinson, *J. Chem. Soc., London*, **91**, 1073 (1967); (b) D. Caunt, W. Crow, R. D. Haworth, and C. A. Vodoz, *J. Chem. Soc.*, 1631 (1950); (c) T. A. Geissman and A. Armen, *J. Amer. Chem. Soc.*, **77**, 1623 (1955); (d) F. Meyer and L. van Zutphen, *Chem. Ber.*, **57B**, 202 (1924); (e) A. Murata, T. Ito, K. Fujiyasu, and T. Suzuki, *Bunseki Kagaku*, **15**, 145 (1966).
(14) W. W. Cleland, *Nature (London)*, **198**, 463 (1963).

Book Reviews

The Hydrophobic Effect: Formation of Micelles and Biological Membranes. By Charles Tanford. Wiley-Interscience, New York, N.Y. 1973. viii + 200 pp. 16 × 24 cm. $12.50.

Hydrophobic "bonding" has been one of the most widely used concepts in biophysical chemistry. Much of the original interest can be attributed to the very clear description of the forces holding proteins together written by Kauzmann in 1959. Since that time researchers have sought and found ample evidence for a large number of entropy driven association phenomena of biological import. Dr. Tanford has had a major role in the development of the quantitative thermodynamics of hydrophobic interactions, and he here explores a number of interesting systems. The book contains 19 short chapters. The first four focus on the underlying thermodynamic results that form the main framework for a systematic treatment of hydrophobic effects. There follows a brief summary of current views on the structure of water and then a moderately detailed treatment of micelles (six chapters). The remainder of the book is primarily devoted to protein–lipid interactions of various types with the final two chapters specifically dealing with biological membranes.

The book is well written. The thermodynamic material is neatly and concisely developed and should be of use to a wide range of readers. The relationships between experimental results and idealized models are clearly set out. The unified viewpoint is one of the main strengths of the book. The discussion of micelles is particularly nice. Some general principles for micelle shape are summarized and the long debated question of whether micelle formation should be treated, formally, as a phase change is resolved.

My main criticism of the book is its length. It is too short to present anything approaching a "comprehensive" treatment of the complex topics that are taken up. Some important areas given too brief a discussion are: X-ray diffraction results, the general question of mobility (although the spin resonance work of McConnell and collaborators is described), and perturbations of hydrophobic interactions in the presence of electrolytes. Specialists in various areas will almost certainly be disappointed in the depth of treatment compared with, for example, Tanford's articles on protein denaturation. References are highly selected, providing good contact with major review articles through 1972.

In sum, this book offers an interesting overview. It certainly provides a provocative starting point for students and active researchers alike.

Department of Pharmaceutical Chemistry **Irwin D. Kuntz, Jr.**
University of California
San Francisco, California 94143