Biochemical Pharmacology, Vol. 26, pp. 377–380. Pergamon Press, 1977. Printed in Great Britain.

N-METHYLATION OF BENZIMIDAZOLE BY CATECHOL-*O*-METHYLTRANSFERASE*†

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(*Received* 26 *February* 1976; *accepted* 19 *July* 1976)

Abstract—The substrate specificity of catechol-*O*-methyltransferase (COMT) has been extended in the present study to include benzimidazole, a non-catechol compound. Gas chromatography-mass spectrometry, thin-layer chromatography, and reverse isotope dilution analysis were used to verify the identity of the product of this reaction as 1-methylbenzimidazole. This is the first demonstration of an *N*-methylation by COMT. The conclusion is reached that benzimidazole and catechol are bioisosteric molecules. The implication of this in the pharmacology of adrenergic systems is discussed.

Catechol-*O*-methyltransferase (COMT; *S*-adenosyl-L-methionine; catechol-*O*-methyltransferase; EC 2.1.1.6) is known to have a broad substrate specificity. The only structural requirement for substrates appears to be a catechol (*ortho* dihydroxy substituted benzene) ring [1]. Since COMT is the enzyme primarily responsible for the metabolic inactivation of extraneuronal catecholamines, the development of specific inhibitors of the enzyme is important for the pharmacological study of adrenergic systems.

A logical approach to the design of inhibitors for COMT is a search for compounds which are isosteric with known substrates. On the basis of COMT inhibition studies, Belleau and Burba [2] concluded that the tropolone ring is bioisosteric with the catechol ring. Tropolones are not substrates, but are non-competitive inhibitors of COMT [3]. Evidence *in vivo* for bioisosterism between these molecules had been suggested by earlier studies demonstrating adrenergic receptor blockade by 4-methyltropolone [4]. Tyrosine hydroxylase, an enzyme in the biosynthetic pathway of catecholamines, is inhibited by 4-isopropyltropolone, an effect attributed to biochemical isosterism between the tropolone and catechol rings [5].

Recent findings in our laboratory have suggested the possibility of bioisosterism between benzimidazole and catechol. Benzimidazole-5(6)-DL-alanine (BA) has been shown to be a potent inhibitor of tyrosine hydroxylase, both *in vitro* [6] and *in vivo* [7]. A possible decarboxylation product of BA, 2-[5(6)-benzimidazolyl]ethylamine, was first ascribed hypertensive properties by Maron [8] in 1914. The pressor activity of this analogue of dopamine has recently been confirmed [9]. R,S-1-[5(6)-Benzimidazolyl]-2-aminoethanol, a benzimidazole analogue of norepinephrine, exhibits direct activity at the alpha-adrenergic receptor controlling blood pressure in the rat [10].

The present study was undertaken to determine if benzimidazole can serve as a substrate for COMT. Gas chromatography–mass spectrometry (GC–MS), thin-layer chromatography (t.l.c.), and reverse isotope dilution analysis (RIDA) are powerful analytical tools which can be used independently to confirm the chemical structure of sub-microgram quantities of compounds. These techniques were utilized to determine the structure of the product of this reaction.

MATERIALS AND METHODS

Materials. Benzimidazole and 3,4-dihydroxybenzoic acid (DHBA) were obtained from Aldrich Chemical Co., Inc. Tropolone was obtained from Pfaltz & Bauer, Inc. Histamine dihydrochloride was obtained from Fisher Scientific Co. The free base and hydrochloride salt of 1-methylbenzimidazole were synthesized by the treatment of benzimidazole with methyl iodide according to the method of Fischer [11]. *S*-adenosyl-L-methionine (SAM) was obtained as the iodide salt from CalBiochem. *S*-adenosyl-L-methionine [methyl-^{14}C] ([^{14}C]SAM; 0.5 mCi/m-mole) was obtained as a specially purified substrate for enzyme assays from Amersham/Searle Corp. A higher specific activity *S*-adenosyl-L-methionine [methyl-^{14}C] (51.8 mCi/m-mole) was purchased from ICN for the reverse isotope dilution analysis. The COMT used in this study was obtained from Miles Laboratories, Inc., in the form of a partially purified freeze-dried powder containing dithiothreitol as stabilizer. The enzyme had been purified from bovine liver through the second ammonium sulfate precipitation step of Axelrod [12]. It was stored at −40° and reconstituted immediately before use. The reconstituted enzyme contained 100 mg protein/ml. Deionized glass-distilled water and spectrophotometric grade solvents were used throughout.

Incubation conditions. Each tube contained 100 μmoles potassium phosphate buffer (pH 7.8), 0.2 μmole (122 nCi) [^{14}C]SAM, 5.0 μmoles $MgCl_2 \cdot 6H_2O$, 2.0 μmoles of the appropriate substrate (the

* Presented in part at the Seventh Central Regional Meeting of the American Chemical Society, Morgantown, W. VA, May 28–30, 1975.

† Taken in part from a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of Maryland, 1976.

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blank contained buffer in place of substrate), and an aliquot of the reconstituted COMT containing the equivalent of 20 mg protein in a final volume of 1.0 ml. The reaction was started by adding enzyme, and the tubes were incubated for 60 min at 37°. The reaction was stopped by adding 100 μl of either concentrated ammonium hydroxide solution (blank and benzimidazole tubes) or 4 N HCl (DHBA tube), and the solutions were immediately extracted with 10 ml of ethyl acetate. A 5.0-ml aliquot of each extract was evaporated to dryness and redissolved in 1.0 ml of 2-ethoxyethanol. Ten ml of Aquasol liquid scintillation solution (New England Nuclear) was added, and the solutions were counted on a Packard model 2002 liquid scintillation spectrometer. The counting efficiency, determined by using standard [^{14}C] toluene, was 65 per cent. The remainder of the ethyl acetate extract was used for radiochromatographic analysis as with the reverse isotope dilution analysis (see below for details).

GC–MS analysis of reaction product. For the GC–MS study, higher concentrations of enzyme (50 mg protein/ml) and unlabeled SAM (1.0 μmole/ml) were used. The ethyl acetate extracts from two tubes were combined, evaporated to dryness, and the residue was dissolved in 10 μl of methanol. A 1.0-μl aliquot of this solution was injected into the gas chromatograph. The instrument used was a DuPont 21-490 mass spectrometer equipped with a Varian Series 1400 gas chromatograph, GC–MS interface consisting of a heated glass jet separator, and a DuPont 21-094 Data System. The GC conditions were: 6 ft by $\frac{1}{8}$ in glass column packed with 3% OV-17 on Chromosorb W.H.P., carrier gas He (30 ml/min), injector temp 255°, and oven temp programmed 90–260° (10°/min). The source temp of the MS was 190°, and the ionizing voltage was 70 eV.

Reverse isotope dilution analysis of reaction product. The incubation concentrations were the same as with the initial study, except a higher specific activity of [^{14}C]SAM (51.8 mCi/m-mole) was used at a lower concentration (0.09 μmole/ml), and the COMT concentration was 50 mg protein/ml. The dried ethyl acetate extract from the incubation was dissolved in 10 μl methanol. A 5-μl sample of this was spotted on a t.l.c. plate (silica gel G) and developed with a solvent mixture consisting of toluene, ethyl acetate, ethanol and ammonium hydroxide (60:10:10:2) [13]. The resulting radiochromatogram was scanned with a Packard model 7201 Radiochromatogram Scanner System. The radioactive spot was scraped off and eluted with 10 ml of 0.1 N HCl. The eluate was evaporated to dryness, and 200 mg of 1-methylbenzimidazole hydrochloride was added to the residue. The resulting solid was crystallized three times from ethanol–acetone (1:4). A 20-mg sample from each crystallization was dissolved in 1.0 ml of H_2O, 16 ml ACS liquid scintillation solution (Amersham/Searle Corp.) was added, and the solutions were counted as before. The counting efficiency was determined for each vial by adding internal standard [^{14}C]toluene and recounting.

RESULTS AND DISCUSSION

The results of the initial study are listed in Table 1. Since the experiment was designed to demonstrate product formation, and quantitative extraction efficiencies were not determined, the reported values may not represent initial reaction rates, and valid quantitative comparison of substrate reactivities must await more extensive kinetic analysis. Tropolone, at a 2 mM concentration, caused a 61 per cent reduction of the enzymatic methylation of benzimidazole.

To rule out the possibility that histamine-*N*-methyltransferase was contaminating the COMT used in this study, benzimidazole was incubated as in the initial study in the presence of 2 mM histamine. No inhibition of the methylation of benzimidazole was observed. Radiochromatographic analysis (butanol–acetic acid–water: 4:1:1) of the ethyl acetate extract from this incubation mixture showed greater than 90 per cent of the total radioactivity associated with a position isographic with authentic 1-methylbenzimidazole (R_f = 0.50). No radioactivity was seen in the region corresponding to the R_f of 1-methylhistamine (0.10) in this solvent system [14]. The high activity toward DHBA, the use of partially purified enzyme, the inhibition of tropolone, and the lack of inhibition by histamine indicate that the enzyme active in this study was COMT and not some other methyl-transferring enzyme (e.g. histamine-*N*-methyltransferase).

Since SAM has been shown to methylate free carboxyl residues of various proteins non-enzymatically [15], and since Hsu and Mandell [16] reported evidence for non-enzymatic *N*-methylation of tryptamine and *N*-methyltryptamine by SAM, a second blank was run containing benzimidazole, [^{14}C]SAM, and no enzyme. A radiochromatogram of the extract from this blank showed greater than 95 per cent of the total radioactivity was associated with the origin. No peak was observed in the region with the same R_f as 1-methylbenzimidazole, indicating no non-enzymatic methylation of benzimidazole had occurred. On the other hand, the radiochromatogram of the extract from the complete benzimidazole tube showed at least 80 per cent of the total radioactivity associated with a position isographic with authentic 1-methylbenzimidazole.

Table 1. Results of incubating various substrates with COMT and [^{14}C]SAM

Substrate	Extracted radioactivity* (cpm)
Blank	2,254
Benzimidazole (2.0 mM)	11,144
Benzimidazole (2.0 mM) + tropolone (2.0 mM)	5,682
2,4-Dihydroxybenzoic acid (2.0 mM)	148,066

* Averages of duplicate determinations. See text for incubation conditions.

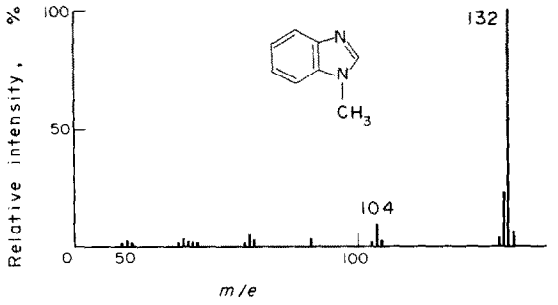

Fig. 1. Background-corrected mass spectrum of authentic 1-methylbenzimidazole measured by a GC–MS computer system.

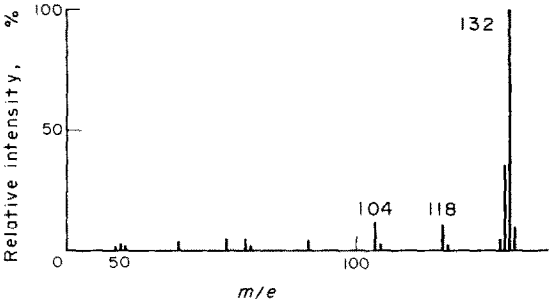

Fig. 2. Background-corrected mass spectrum of an extract of the COMT-benzimidazole reaction taken at the same GC retention time as authentic 1-methylbenzimidazole and measured by a GC–MS computer system.

GC–MS analysis. Figure 1 shows a background-corrected electron impact mass spectrum obtained for authentic 1-methylbenzimidazole. The molecular ion (m/e = 132) is the base peak in the spectrum. The fragmentation to give ions of m/e = 131 (M–H) and *m/e* = 104 (M-CH_2N) has been described elsewhere [17]. Figure 2 is a mass spectrum obtained from GC–MS analysis of the reaction product. Mass chromatography [18] was used to locate the elution position of 1-methylbenzimidazole in the gas chromatographic analysis of the reaction extract. Each of the mass chromatograms for ions m/e = 132, 131 and 104 showed a maximum at the scan used to obtain this spectrum. Automatic background correction was then used to produce a mass spectrum (Fig. 2) essentially identical to that of the authentic compound (Fig. 1). A significant ion at m/e = 118, which was not eliminated by the background correction, may be due to

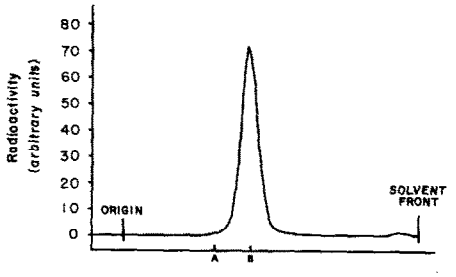

Fig. 3. Radiochromatogram of an extract from the reaction of benzimidazole, COMT and [^{14}C]SAM. Migration positions of benzimidazole (A, R_f = 0.31) and 1-methylbenzimidazole (B, R_f = 0.43) were determined with authentic compounds.

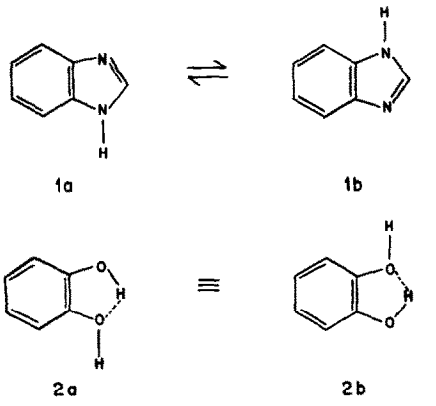

Fig. 4. Comparison of the two tautomers of benzimidazole (1a and 1b) with the two stable conformations of catechol (2a and 2b).

substrate benzimidazole. Benzimidazole itself undergoes very little fragmentation in the electron impact mass spectrometer [17].

Reverse isotope dilution analysis. RIDA was used to confirm the identity of the reaction product. Methyl-^{14}C-labeled SAM was incubated with benzimidazole and COMT to produce a radioactive product which was isographic with authentic 1-methylbenzimidazole on t.l.c. analysis (Fig. 3). The t.l.c.-purified product was converted to its hydrochloride salt, mixed with unlabeled 1-methylbenzimidazole hydrochloride, and the mixture recrystallized to constant specific activity and melting point. The specific activities of the three crops of 1-methylbenzimidazole hydrochloride were 4792, 4777 and 4612 dis./min respectively. The average counting efficiency was 79 per cent. Each of the three crops melted at 231–232° (cf. literature [19] melting point of 228°). Of the total radioactivity eluted from the radiochromatogram, 88 per cent was recovered as 1-methylbenzimidazole hydrochloride.

The importance of this demonstration is seen in light of the proposed analogy between benzimidazole and catechol. The preferred conformation of the catechol molecule is such that intramolecular hydrogen bonding forms a five-membered planar ring of the substituent hydroxyl groups [29]. This places the acidic proton (the one not involved in forming the hydrogen bond) in a position sterically similar to that of the acidic proton [21] of one tautomer of benzimidazole (Fig. 4). Conformational flexibility allows rotation of the carbon–oxygen bonds of catechol in such a way as to cause interconversion of the acidic and hydrogen-bonded protons. This is analogous to the tautomeric shift of the acidic proton of benzimidazole between the two nitrogens of the imidazole ring. It must be pointed out, however, that while the five-membered ring formed by the catechol hydroxyls is coplanar with the benzene ring, it is not aromatic. The imidazole ring of benzimidazole is, of course, aromatic. This aromaticity confers a high degree of chemical stability on the benzimidazole nucleus [21].

If bioisosterism between benzimidazoles and catechols extends to adrenergic receptor binding, as indicated by the direct agonistic activity of the previously

mentioned benzimidazole analogue of norepinephrine [10], a new class of pharmacologically important adrenergic agents may be provided by substituting the chemically stable benzimidazole ring system for the relatively unstable ring system of known drugs.

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