Thymidine-5'-carboxylic Acid Methyl Ester (11). A solution of 1.85 g (7.2 mmol) of **10** in 10 ml of DMF was titrated with ethereal diazomethane (prepared from 3 g of *N*-methyl-*N*-nitrosourea in 40 ml of ether) until the yellow color persisted (after *ca.* 2 min). Excess CH_2N_2 was destroyed with 0.5 ml of AcOH, and the precipitate was washed with Et_2O to give pure methyl ester: 1.78 g (91%); mp 247° dec. *Anal.* ($C_{11}H_{14}N_2O_6$) C, H, N.

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Catechol *O*-Methyltransferase. 4. *In Vitro* Inhibition by 3-Hydroxy-4-pyrones, 3-Hydroxy-2-pyridones, and 3-Hydroxy-4-pyridones

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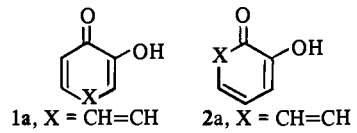
Tropolones[1,2] have been shown to be reversible dead-end inhibitors of the enzyme catechol *O*-methyltransferase (COMT) (E.C. 2.1.1.6).† Tropolone appears to bind to a similar site as the catechol substrate, but it itself is not O-methylated. If one looks at the tropolone molecule as shown in structure **1a** or **2a**, it becomes readily apparent that any functional group or atom (X) capable of maintaining these keto tautomeric structures should show similar inhibitory properties toward COMT. Possible examples include 3-hydroxy-4-pyridone (**1b**), 3-hydroxy-4-pyrone (**1c**), 3-hydroxy-2-pyridone (**2b**), and 3-hydroxy-2-pyrone (**2c**). All have been shown to exist predominatly in these tautomeric forms[3-5] and thus would be isosteric to tropolone and



1a, X = CH=CH **2a**, X = CH=CH
b, X = NH b, X = NH
c, X = O c, X = O

catechol. This communication reports the preliminary results of a study on the *in vitro* inhibition of COMT by these heterocyclic systems.

Results and Discussion

Table I shows the degree of COMT inhibition produced by derivatives of 4-pyrone and 4-pyridone, as well as by 3-hydroxy-2-pyridone (**2b**) and tropolone (**1a**). The inhibitory activity of 3-hydroxy-4-pyridone (**1b**), 3-hydroxy-4-pyrone (**1c**), *N*-methyl-3-hydroxy-4-pyridone (**4**), meconic acid (**7**), kojic acid (**8**), and 3-hydroxy-2-pyridone (**2b**) clearly illustrates that these heterocyclic systems are biochemically isosteric to tropolone (**1a**) with respect to their ability to inhibit COMT. Complete absence of inhibitory activity for the 3-methoxylated compounds **3**, **5**, and **6** illustrates the importance of the free hydroxyl group for inhibition. The lower inhibitory activity of the 3-hydroxy-4-pyrones **1c**, **7**, and **8** probably reflects the fact that these systems can also exist in a diketo tautomeric structure,[5] which would be expected to be inactive as a COMT inhibitor.

Since these heterocyclic systems are isosteric to catechol, they could conceivably serve as substrates for COMT. However, incubation of **1b**, **1c**, or **2b** in the presence of the enzyme under conditions which were optimal for the O-methylation of *l*-NE resulted in no detectable methylation products being formed.

Using reciprocal velocity *vs.* reciprocal substrate plots, the kinetics patterns for 3-hydroxy-4-pyridone (**1b**), 3-hydroxy-2-pyridone (**2b**), and kojic acid (**8**) inhibition of the COMT-catalyzed O-methylation of DHB were determined. As shown in Table II linear competitive patterns of inhibition were observed when DHB was the variable substrate and either **1b**, **2b**, or **8** was the inhibitor. When SAM was the variable substrate, uncompetitive patterns of inhibition were observed as summarized in Table II. Except for the competitive rather than noncompetitive pattern observed with varying DHB, the inhibitory kinetics of these heterocyclic systems (**1b**, **2b**, and **8**) are quite similar to those observed for tropolone (**1a**).[2]

In an attempt to determine if tropolone and these heterocyclic systems are truly isosteric with respect to COMT, a study of the kinetics of multiple inhibition of COMT by 3-hydroxy-4-pyridone (**1b**) and tropolone (**1a**) was conducted using the procedures of Yonetani and Theorell.[6] These studies were carried out at pH 7.24 where both tropolone (**1a**)[2] and 3-hydroxy-4-pyridone (**1b**) show pure competitive kinetics with respect to DHB. As shown in Figure 1, a series of parallel straight lines were obtained when reciprocal velocities were plotted *vs.* tropolone (**1a**) concentrations at varying concentrations of 3-hydroxy-4-pyridone (**1b**). The slope of the lines remained constant; however, the intercepts were a linear function of the concentration of **1b**. These data, as well as the inhibitory kinetic data, provide strong evidence that tropolone and

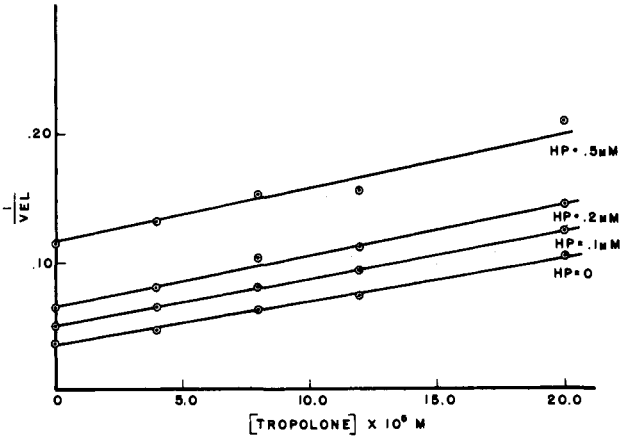
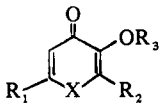


Figure 1. Reciprocal velocity *vs.* tropolone concentration with varying 3-hydroxy-4-pyridone (HP). Assay conditions are the same as in Table II except pH 7.24. Vel = nmol of product/mg of N Kjeldahl/min.

†Abbreviations used are: SAM, *S*-adenosyl-L-methionine; DHB, 3,4-dihydroxybenzoic acid; *l*-NE, *l*-norepinephrine; COMT, catechol *O*-methyltransferase (E.C. 2.1.1.6); K_{is}, inhibition constant for the slope; K_{ii}, inhibition constant for the intercept.

Table I. Inhibition of COMT by 3-Hydroxy-4-pyrones, 3-Hydroxy-4-pyridones, and 3-Hydroxy-2-pyridones[a]



Compd no.	Inhibitor[b] X	R_1	R_2	R_3	% inhibition[c] [I] = 0.1 m*M*	[I] = 1.0 m*M*
1a	HC=CH	H	H	H	60	96
1b	NH	H	H	H	61	95
1c	O	H	H	H	15	52
3	NH	H	H	CH_3	0	0
4	NCH_3	H	H	H	42	90
5	NCH_3	H	H	CH_3	0	0
6	O	H	H	CH_3	0	0
7	O	CO_2H	CO_2H	H	34	81
8	O	CH_2OH	H	H	12	62
2b	3-Hydroxy-2-pyridone				22	81

[a]COMT was purified and assayed as previously described.[2] SAM concentration, 1.0 m*M*. *l*-NE concentration, 2.0 m*M*. Mg^+ concentration, 1.2 m*M*. TES buffer concentration, 40 m*M*; pH 7.60. Incubation time, 20 min. [b]Inhibitors added from stock solution of 5.0 μmol/ml. [c]Expressed as per cent inhibition of O-methylation of *l*-NE.

Table II. Inhibition Patterns and Constants for Tropolone (1a), 3-Hydroxy-4-pyridone (1b), 3-Hydroxy-2-pyridone (2b), and Kojic Acid (8)[a]

Inhibitor	Substrate concn, m*M*[b] DHB	SAM	Inhibition[b]	Inhibition constants, μ*M*[c] K_{is}	K_{ii}
Tropolone (1a)	Var	1.0	NC	22 ± 1.0	247 ± 16
	2.0	Var	U		128 ± 4
3-Hydroxy-4-pyridone (1b)	Var	1.0	C	41 ± 4.2	
	2.0	Var	UC		349 ± 23
3-Hydroxy-2-pyridone (2b)	Var	1.0	C	243 ± 32	
	2.0	Var	UC		1916 ± 248
Kojic acid (8)	Var	1.0	C	126 ± 9.2	
	2.0	Var	UC		1821 ± 145

[a]Kinetic conditions are similar to those previously described[2,7,8] TES buffer, pH 7.60; Mg^{2+} concentration, 1.2 m*M*. [b]Var indicates the variable substrate. DHB concentration, 40–400 μ*M*. SAM concentration, 24–210 μ*M*. [c]UC, uncompetitive and data fitted to eq 3; NC, noncompetitive and data fitted to eq 4.

3-hydroxy-4-pyridone (**1b**) are competing for the same site on the enzyme and are thus biochemically isosteric with respect to COMT.

In summary, we have shown that 3-hydroxy-4-pyrones, 3-hydroxy-2-pyridones, and 3-hydroxy-4-pyridones are COMT inhibitors and inhibit by a mechanism similar to that of other dead-end inhibitors studied in our laboratory (*e.g.*, tropolones,[2] 8-hydroxyquinolines,[7] and salicylaldehydes[8]). These heterocyclic systems represent a new class of dead-end inhibitors of COMT and will be the subject of future communications.

Experimental Section

Materials. SAM-$^{14}CH_3$ (New England Nuclear, 55.0 mCi/mmol) was diluted to a concentration of 10 μCi/ml and stored at −20°F. SAM iodide (Calbiochem) was stored as a 0.01 *M* solution in 0.001 *N* HCl at −20°F. *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES, Sigma) buffer was prepared as a 0.02 *M* stock solution.

The following compounds were commercially available from the indicated sources: **1a**, **2b**, **8** (Aldrich); **7** (Chemical Procurement Laboratories).

Compounds **1b**, **1c**, **3**, **4**, **5**, and **6** were prepared by procedures previously outlines by Bickel.[9] The intermediates were purified to analytical purity by sublimation or recrystallization prior to testing as inhibitors.

Purification and Assay of COMT. COMT was purified from rat liver (male, Sprague–Dawley, 180–200 g) according to the methods previously described.[2] Enzyme preparations utilized for kinetic experiments had a specific activity of 50.4 nmol of product/mg of N Kjeldahl/min for DHB as substrate.

Data Processing. Reciprocal velocities were plotted graphically against reciprocals of the substrate concentrations. In all cases a reasonably linear relationship was obtained. These data were then fitted to eq 1 using a least-squares method and assuming equal variance for the velocities.[10]

$$v = VA/(K + A) \quad (1)$$

All calculations were performed on a Hewlett-Packard 2100 A digital computer using a FORTRAN IV program[11,12] which provided values of K, V, K/V, $1/V$, and the standard errors of their estimates. Slopes (K/V) and intercepts ($1/V$) were then plotted graphically against inhibitor concentrations. All replots were linear. Data fitting linear competitive inhibition were fitted to eq 2, for linear uncompetitive inhibition to eq 3, and linear noncompetitive inhibition to eq 4.

$$v = VA/[K(1 + I/K_{is}) + A] \quad (2)$$

$$v = VA/[K + A(1 + I/K_{ii})] \quad (3)$$

$$v = VA/[K(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (4)$$

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A New Method for Nitrosation of Proline and Related *sec*-α-Amino Acids to *N*-Nitrosamino Acids with Possible Oncogenic Activity

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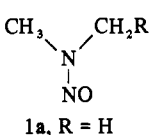
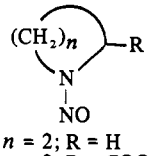
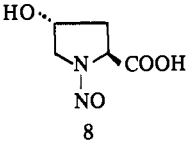
The potent oncogenicity of dimethylnitrosamine (**1a**) and other *N*-nitroso-*sec*-amines to rodent species presents disquieting ramifications of environmental hazards to man,[1,2] inasmuch as **1a** has been detected in tobacco smoke condensate[3] and in nitrite and nitrate-treated smoked fish, as well as in untreated raw and smoked fish.[4] Structure-activity correlations suggest that a wide series of *N*-nitroso-*sec*-amines with diverse molecular structures (straight or branched chain, alicyclic or heterocyclic) is oncogenic to rodents to some degree and elicits a variety of tumors in a number of different organs.[5-8] Recently, Lijinsky and Epstein[1] as well as others[9-11] have implicated nitrosamines in the etiology of human cancer on the basis of their wide occurrence and multipotency.



1a, R = H
b, R = COOH



2a, *n* = 2; R = H
b, *n* = 2; R = COOH
3a, *n* = 3; R = H
b, *n* = 3; R = COOH
4a, *n* = 4; R = H
b, *n* = 4; R = COOH
5a, *n* = 5; R = H
b, *n* = 5; R = COOH
6a, *n* = 6; R = H
b, *n* = 6; R = COOH
7a, *n* = 7; R = H
b, *n* = 7; R = COOH



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The suggestion has been made[1] that ingested nitrites (or nitrates which can be reduced to nitrites by intestinal bacterial flora) may interact in the gastrointestinal tract (a) with amino acids such as proline and hydroxyproline taken in as food, (b) with orally administered drugs,[2] or (c) with secondary amines added as flavoring agents or formed in the cooking of protein and protein products to produce 1-nitrosoprolines (**3b**, **8**) and other *N*-nitroso-*sec*-amines such as 1-nitrosopyrrolidine (**3a**) and 1-nitrosopiperidine (**4a**) which may be tumorigenic to man. Indeed, the latter two are proven oncogens in rodents.[7] *N*-Nitrososarcosine (**1b**), the simplest of the nitrosamino acids, produces esophageal tumors in rats on chronic prolonged administration in their drinking water,[12] 1-nitrosoproline (**3b**), however, did not increase the lung adenoma incidence in mice on short-term treatment (26 weeks) when compared to normal controls.[13] Long-term studies on the oncogenicity of a series of *N*-nitroso-*sec*-amino acids appear to be in progress,[13,14] although these results have not yet appeared in the literature.

Chemistry. Such long-term oncogenicity tests of nitrosamino acids require large quantities of samples of extremely high purity, since the presence of impurities may completely obscure the results of experiments where high doses are administered over the lifetime of the animals. The ease of decomposition of nitrosamino acids by thermal or photolytic mechanism[14,15] requires also that mild conditions be used for their preparation and purification.

N-Nitroso derivatives of those naturally occurring amino acids that are secondary amines, *viz.*, *N*-nitrososarcosine (**1b**), 1-nitroso-L-azetidine-2-carboxylic acid (**2b**), 1-nitroso-L-proline (L-**3b**), 1-nitroso-4-hydroxy-L-proline (**8**), and 1-nitrosopipecolic acid (**4b**), have previously been prepared by treatment of the respective amino acids in aqueous solutions with nitrous acid.[14,16-18] The large discrepancies reported in the melting points and other properties of these *N*-nitroso-*sec*-amino acids prepared in different laboratories have been reconciled by Lijinsky, *et al.*,[14] as being due to syn-anti isomerism of the *N*-nitroso group on the basis of detailed analyses of their nmr spectra. The identity of mass spectral fragmentation patterns and elemental compositions of preparations with different melting points constituted confirmatory evidence. Similar rotational isomerism of ester derivatives of **3b** and ester and amide derivatives of **1b** has been reported by Stewart.[19]

We wish to describe a facile new method for the nitrosation of secondary amino acids in an *aprotic solvent* utilizing nitrosyl tetrafluoroborate and pyridine.[20] This reagent combination readily nitrosated proline and its analogs in acetonitrile (or ethyl acetate) at 0° generally in good yields to give the corresponding *N*-nitrosamino acids in consistant high purity (Table I). Although nitrosyl tetrafluoroborate will itself N-nitrosate morpholine and piperidine in the presence of excess amine,[21] nitrosation of proline or pipecolic acid did not proceed to completion without the addition of pyridine; hence, the active nitrosating agent here may be the species *N*-nitrosopyridinium tetrafluoroborate known to be formed under these conditions.[20]

The physicochemical properties of these *N*-nitrosamino acids prepared in this manner were in good agreement with those reported (Table I). The *negative* Cotton effect observed in the circular dichroism curves for 1-nitroso-2(*R*)-proline (D-**3b**) is in line with the sector rules recently proposed by Gaffield, *et al.*,[22] for these compounds.

The present procedure is especially attractive for the preparation of nitrosamino acids of high water solubility such as **2b**, **3b**, and **8**, as the reaction is conducted in a non-aqueous solvent. This procedure may likely find application in the nitrosation of other biologically important secondary amines such as substituted aminopurine and aminopyrimidines, as well as their nucleosides.

The present work and the recent synthetic availability of

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