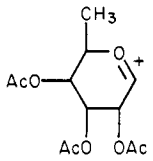
(m, SCH_2), 2.70 (t, J = 6.5 Hz, 2 H, SCH_2CH_2), 2.62 (t, J = 6.5 Hz, 4 H, SCH_2CH_2); $[\alpha]_D^{27}$ −17.3° (c 1.5, H_2O). Anal. ($C_{39}H_{68}N_4S_3O_{21}\cdot H_2O$) C, H, N, S.

2-Carboxyethyl 2,3,4-Tri-*O*-acetyl-1-thio-β-L-fucopyranoside (28). A solution of 2,3,4-tri-*O*-acetyl-1-thio-β-L-fucopyranose[35] (1.49 g, 4.87 mmol) and freshly crystallized 3-iodopropionic acid (0.97 g, 4.86 mmol) in CH_2Cl_2 (20 mL) containing Et_3N (1.35 mL) was kept for 16 h at room temperature. Hydrochloric acid (2.5 N, 20 mL) and CH_2Cl_2 (10 mL) were added, and the solution was washed with H_2O, dried, and evaporated in vacuo to a residue, which was put on a column of silica gel and eluted with $CHCl_3$–MeOH–H_2O (92.5:7.5:0.75, v/v). Compound **28** was isolated in 87% yield (1.6 g), and used, without further purification, for the preparation of **29**.

***p*-Nitrophenyl 3-(2,3,4-Tri-*O*-acetyl-1-thio-β-L-fucopyranosyl)propionate (29).** *p*-Nitrophenol (2.6 g, 18.7 mmol) was added to a solution of **28** (7.0 g, 18.5 mmol) and DCC (3.8 g, 18.5 mmol) in CH_2Cl_2 (20 mL), and the mixture was stirred for 3 h at room temperature and diluted with Et_2O (50 mL). The resulting mixture was poured through a sintered funnel packed with silica gel, and the filtrate was evaporated in vacuo to dryness. The residue was purified by means of PrepPak 500/silica on a Waters Associates Prep LC/System 500 at 250 mL/min using Et_2O–CH_2Cl_2 4:96 (v/v) as a liquid phase. Compound **29** was isolated in 38% yield (3.5 g). An analytical sample was crystallized from Et_2O: mp 96–97 °C; $[\alpha]_D^{27}$ +29.9 ± 0.5° (c 1.06, $CHCl_3$); MS, *m/e* 469 ($M^{+\cdot}$ − NO·), 439 ($M^{+\cdot}$ − HOAc), 379 ($M^{+\cdot}$ − 2HOAc), 361 ($M^{+\cdot}$ − ·$OC_6H_4NO_2$-*p*), 273 (ii). Anal. ($C_{21}H_{25}NSO_{11}$) C, H, N, S.



ii

***N*²-[*N*²,*N*⁶-Bis[3-[(2,3,4-tri-*O*-acetyl-β-L-fucopyranosyl)thio]propionyl]-L-lysyl]-*N*⁶-[3-[(2,3,4-tri-*O*-acetyl-β-L-fucopyranosyl)thio]propionyl]-L-lysine (32).** A solution of L-lysyl-L-lysine bis(trifluoroacetate) salt (0.6 g, 1.32 mmol) and **29** (2.0 g, 4 mmol) in DMF (10 mL) containing Et_3N (900 μL, 6.5 mmol) was stirred overnight at room temperature. The reaction mixture was worked up in the same manner as for **13** to give the title compound (1.55 g, 87%): $[\alpha]_D^{27}$ +12.3 ± 1.2° (c 0.83, $CHCl_3$). Anal. ($C_{57}H_{86}N_4S_3O_{27}\cdot 0.5\ Et_3N$) C, H, N, S.

***N*²-[*N*²,*N*⁶-Bis[3-(β-L-fucopyranosylthio)propionyl]-L-lysyl]-*N*⁶-[3-(β-L-fucopyranosylthio)propionyl]-L-lysine (33).** A solution of **32** (500 mg) in MeOH–H_2O–Et_3N (5:4:1, v/v; 5 mL) was kept for 3 h at room temperature and worked up in the same manner as for **14** to give the title compound (300 mg, 83%): $[\alpha]_D^{27}$ +28.4 ± 0.9° (c 1.0, H_2O); NMR (D_2O) δ 4.48 (d, $J_{1,2}$ = 9.5 Hz, 1 H, H-1), 4.46 (d, $J_{1,2}$ = 9.5 Hz, 2 H, H-1), 4.17 and 4.32 (2 q, α-CH), 3.22 (m, ε-CH_2), 2.98 (m, SCH_2), 2.69 (t, 1 H), 2.61 (t, 2 H) (SCH_2CH_2), 1.34–1.90 (m, $CCH_2CH_2CH_2C$), 1.25 (d, J = 6.0 Hz, CH_3-6). Anal. ($C_{39}H_{68}N_4S_3O_{18}\cdot 0.5\ Et_3N\cdot 1.5H_2O$) C, H, N, S.

***N*-[6-[(*tert*-Butyloxycarbonyl)amino]hexyl]-*N*²-[*N*²,*N*⁶-bis[3-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)thio]propionyl]-L-lysyl]-*N*⁶-[3-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)thio]propionyl]-L-lysinamide (34).** 6-[(*tert*-Butyloxycarbonyl)amino]hexylamine (0.432 g, 2 mmol) was added to a stirred solution of **30** (3.06 g, 2 mmol) and DCC (0.45 g, 2.2 mmol) in CH_2Cl_2 (30 mL). After 5 h at room temperature, the mixture was processed in the same manner as for **20**. The title compound was isolated in 66% yield (2.26 g): mp 50 °C (softened, CH_2Cl_2–Et_2O); $[\alpha]_D^{27}$ −4.1 ± 0.9° (c 1.05, $CHCl_3$). Anal. ($C_{74}H_{114}N_6S_3O_{34}$) C, H, N, S.

***N*-(6-Aminohexyl)-*N*²-[*N*²,*N*⁶-bis[3-(β-D-galactopyranosylthio)propionyl]-L-lysyl]-*N*⁶-[3-[(β-D-galactopyranosyl)thio]propionyl]-L-lysinamide Trifluoroacetate Salt (35).** A solution of **34** (1.2 g, 0.69 mmol) in 90% CF_3COOH (2 mL) was kept for 10 min at room temperature and evaporated in vacuo to dryness. Methanol–water–triethylamine (5:4:1, v/v; 20 mL) was added, and the solution was kept overnight at room temperature and evaporated to a residue, which was put on a column of silica gel and eluted with $CHCl_3$–MeOH–H_2O (2:2:1, v/v), followed by MeOH–NH_4OH (1:1, v/v). Fractions containing **35** were combined and evaporated to dryness. Methanol was added, the solution was filtered, and the filtrate was evaporated in vacuo to give **35** (0.33 g, 42%): $[\alpha]_D^{27}$ −8.4 ± 1.2° (c 0.8, H_2O; NMR (D_2O) δ 4.52 (d, $J_{1,2}$ = 10.0 Hz, 1 H, H-1), 4.50 (d, $J_{1,2}$ = 10.0 Hz, 2 H, H-1), 4.25 (m, α-CH), 3.99 (d, $J_{4,3}$ = 2.5 Hz, H-4), 3.22 (m, ε-CH_2), 3.01 (m, SCH_2), 2.72 (t, 2 H), 2.62 (t, 4 H) (SCH_2CH_2), 1.32–1.88 ($CCH_2CH_2CH_2C$).

Acknowledgment. The authors thank C. S. Tripp and M. H. Lam for their technical assistance, Dr. B. H. Arison and H. Flynn for recording 300-MHz spectra, and J. P. Gilbert and his associates for microanalyses.

Influence of Fluorine Substitution on the Site of Enzymatic O-Methylation of Fluorinated Norepinephrines

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The extent of meta- and para-O-methylation by catechol *O*-methyltransferase of 2-fluoro-, 5-fluoro-, and 6-fluoronorepinephrine (FNE) at pH 7 and 9 was determined. The rank order of preference for para-O-methylation is 5FNE ≫ NE > 6FNE > 2FNE. In all cases, increasing the pH to 9 results in an increase in para-O-methylation. Results with 2F- and 5FNE demonstrate the importance of ionization in the methyltransferase reaction when fluorine is situated ortho to one of the phenolic groups. To establish unequivocally the identities of the products, the isomeric, monofluorinated vanillins and isovanillins were synthesized and directly related to the products formed enzymatically from the monofluorinated norepinephrines.

Recently we have reported that substitution of fluorine in the 2, 5, or 6 position of the aromatic ring of norepinephrine dramatically affects the affinity of these analogues for α- and β-adrenergic receptors.[1-7] In a continuing effort to explore the effects of fluorine substitution on the interaction of such analogues with macromolecules, we have investigated their specificity and affinity for the catabolic enzyme catechol *O*-methyltransferase (COMT;

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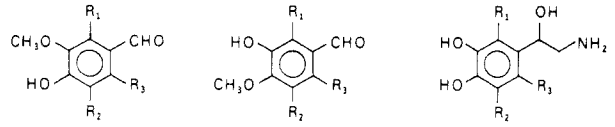
EC 2.1.1.6).[8] COMT catalyzes the transfer of the active methyl group of S-adenosyl-L-methionine to one of the two phenolic groups of the catechol, yielding a stoichiometric amount of O-methylated catechol and S-adenosylhomocysteine. The extent of methylation directed toward the meta or para phenolic group has been shown to be altered by the nature of other substituents on the aromatic ring[9,10] and by the pH of the reaction mixture.[9] The mechanism of O-methylation by catechols catalyzed by COMT has been shown to involve S_N2 attack on S-adenosyl-L-methionine by the catechol substrate.[11,12] If the substrate is involved in the rate-limiting step as the nucleophile, electronic perturbation of the attacking nucleophile (O^- or OH) resulting from fluorine substitution should affect the rate of this displacement. This would be reflected in altered kinetic constants and a change in the meta vs. para methylation of the substituted catechols. In preliminary work reported elsewhere, we observed significant variations in the kinetic parameters (K_M and V_{max}) of the fluorine-substituted norepinephrine for COMT.[13] However, because the identities of the O-methylated products were based on somewhat tenuous grounds (the Gibbs color reaction[13]) and because interpretations of our results depended critically on product identification, we felt it necessary to establish, unequivocally, the structure of the products. To this end we have synthesized the isomeric, monofluorinated vanillins (**1a–c**) and isovanillins (**2a–c**) and have related them directly to the products formed enzymatically through the action of COMT on the three monofluorinated norepinephrines (**3a–c**).



1a $R_1 = R_3 = H$, $R_2 = F$; b $R_2 = R_3 = H$, $R_1 = F$; c $R_1 = R_2 = H$, $R_3 = F$

2a $R_1 = R_3 = H$, $R_2 = F$; b $R_2 = R_3 = H$, $R_1 = F$; c $R_1 = R_2 = H$, $R_3 = F$

3a $R_1 = R_3 = H$, $R_2 = F$; b $R_2 = R_3 = H$, $R_1 = F$; c $R_1 = R_2 = H$, $R_3 = F$

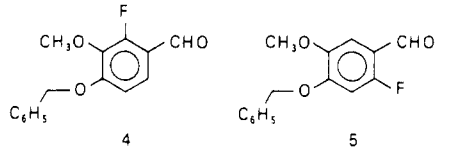
Chemistry. The ready availability of the three isomeric mononitrovanillins prompted us to consider direct conversion of the corresponding amines to the fluorinated vanillins (**1a–c**) by our photochemical fluorination procedure. This regiospecific approach would also allow unambiguous product identification. 6-Nitroisovanillin was also chosen as starting material for the preparation of 6-fluoroisovanillin (**2c**). Synthesis of the two remaining members of the series would then be approached by nonregiospecific routes, with identification of 5-fluoroisovanillin (**2a**) and 2-fluoroisovanillin (**2b**) depending in part upon their nonidentifies to **1a** and **1b**.

Table I. Yields and Physical Data

no.	yield, %	mp (purifn), °C	formula	anal.
1a	26	111–113 (subl)	$C_8H_7FO_3$	C, H
1b	14	115–117 (subl)	$C_8H_7FO_3$	C, H
1c	6	149–150 (cyclohexane/EtOAc)	$C_8H_7FO_3$	C, H
2a	6	94–97 (subl)	$C_8H_7FO_3$[a]	
2b	4	180–195 (subl)	$C_8H_7FO_3$[a]	
2c	13	133–136 (subl)	$C_8H_7FO_3$	C, H
4	8	73–74.7 (subl)	$C_{15}H_{13}FO_3$	C, H
5	2	65–76 (cyclohexane/EtOAc	$C_{15}H_{13}FO_3$	C, H

[a] Not analyzed; mass spectral data agreed with calculated molecular weights.

Direct conversion of 5-nitrovanillin to 5-fluorovanillin (**1a**) through reduction, after protection of the aldehyde as the dimethyl acetal, diazotization, and photochemical decomposition of the corresponding diazonium fluoroborate in fluoroboric acid by the usual procedure[2] proceeded smoothly. Similarly, 6-nitroisovanillin was converted smoothly to 6-fluoroisovanillin (**2c**), albeit in lower yield. Repeated attempts to reduce 2-nitrovanillin dimethyl acetal led to unstable products. Workup of the reduction invariably yielded mixtures of colored products, which were not identified. Attempts to prepare 2-fluorovanillin from the crude reduction product by diazotization and irradiation failed. To circumvent the apparent instability of the intermediate 2-amino-4-hydroxy-3-methoxybenzaldehyde acetal, protection of the phenolic group by benzylation was carried out. Here, additional problems were encountered in that the benzylated reduction product as well as the corresponding diazonium fluoroborate were only sparingly soluble in fluoroboric acid. While this represents an obvious limitation to our in situ photochemical fluorination procedure, we nonetheless were able to carry out the diazotization and irradiation by decreasing the ratio of amine to fluoroboric acid. In addition to **4**, the desired product, **1b**, was produced directly by debenzylation during the photolysis. The reduction of 6-nitrovanillin led to the same problems encountered with 2-nitrovanillin. Benzylation again led to solubility problems during the fluorination procedure, circumvented again by lowering the amine concentration. Photolysis gave 6-fluorovanillin (**1c**) directly, as well as **5**. While the synthesis of **1b** and **1c** by these procedures are adequate for our needs, the preparation of larger quantities of the materials will clearly require improvement in yields or alternate routes.



The remaining members of the series, **2a** and **2b**, were prepared by nonregiospecific syntheses. Thus, demethylation of 2-fluoroveratraldehyde with a limited amount of boron tribromide produced, in low yield, a monodemethylated product isomeric with **1b** and thus identified as **2b**. Finally, alkylation of 3-fluoro-4,5-dihydroxybenzaldehyde with less than an equivalent of dimethyl sulfate produced **1a** and its isomer **2a**. No attempts were made

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Table II. Proton NMR Parameters[a]

	aromatic		nonaromatic	
	δ	J	δ	J
1a	7.28 (m, H, H6)	unresolved	4.03 (s, CH_3)	
		ABX multiplet	9.80 (s, CHO)	
1b	6.82 (dd, H5)	J_{HHo} = 8.6	4.03 (d, CH_3)	J_{HF} = 1.7
	7.50 (dd, H6)	J_{HFm} = 7.1 J_{HFp} = 1.4	10.14 (s, CHO)	
1c	6.68 (d, H5)	J_{HFo} = 11.2	3.92 (s, CH_3)	
	7.25 (d, H2)	J_{HFm} = 6.4	10.16 (s, CHO)	
2a	7.24 (m, H2, H6)	unresolved	4.18 (d, CH_3)	J_{HF} = 1.8
		ABX multiplet	9.84 (s, CHO)	
2b	6.76 (dd, H5)	J_{HHo} = 8.7	4.00 (s, CH_3)	
	7.42 (dd, H6)	J_{HFm} = 7.1 J_{HFp} = 1.5	10.20 (s, CHO)	
2c	6.62 (d, H5)	J_{HFo} = 11.6	3.86 (s, CH_3)	
	7.32 (d, H2)	J_{HFm} = 7.1	10.18 (s, CHO)	
4	6.79 (dd, H5)	J_{HHo} = 8.8	3.94 (s, CH_3)	
	7.52 (dd, H6)	J_{HFm} = 7.2 J_{HFp} = 1.6	5.19 (s, CH_2) 10.16 (s, CHO)	
	7.16 (m, C_6H_5)			
5	6.59 (d, H5)	J_{HFo} = 11.6	3.88 (s, CH_3)	
	7.26 (d, H2)	J_{HFm} = 6.4	5.18 (s, CH_2)	
	7.36 (m, C_6H_5)		10.18 (s, CHO)	

[a] Spectra measured in $CDCl_3$ at 100 MHz. Chemical shifts are relative to $(CH_3)_4Si$. Superscript abbreviations used are: *o*, ortho; *m*, meta; *p*, para.

to optimize yields in these conversions (Table I).

The structures of **1a–c**, as well as **2c**, are firmly established by the synthetic routes. These assignments, as well as assignments for **2a** and **2b**, are confirmed by examination of the ^1H NMR spectra (Table II). The position of the fluorine in **1b**, **1c**, **2b**, and **2c** is unambiguously established by aromatic proton–proton and proton–fluorine coupling constants. In addition, in **1b** and **2a**, coupling of the fluorine to the protons of the methoxy substituent requires these groups to be ortho situated. This is particularly valuable for the assignment of structure **2a**, where nonidentity to **1a** would otherwise be the sole basis for assigning the position of the *O*-methyl group.

Biology. Previous studies have demonstrated that interactions between catechol substrates and COMT which involve polar (charged) substituents on the catechol ring are more important than electronic changes in the nucleophilicity of the catechol moiety in determining the extent of meta- and para-O-methylation. It has been clearly demonstrated that catechols bearing an anionic carboxylate function, like 3,4-dihydroxyphenylacetic acid, or a cationic, ionized ammonium function, like norepinephrine, give rise predominantly to the meta-O-methylated product.[9,10] However, this preference for meta-O-methylation is greatly diminished in the case of neutral, nonpolar derivatives of these catechols. Thus, the methyl ester of 3,4-dihydroxyphenylacetic acid yields equal amounts of both meta- and para-O-methylated products, while with the *N*-acetal or *N*-benzoyl derivatives of norepinephrine the preference for para-O-methylation is increased approximately threefold to 33%.[10] Further, with norepinephrine, which is almost completely in the cationic form at pH 7 (pK_a = 9.7)[14] and gives rise predominately to the meta-O-methylated product with only 10% of the para isomer being formed[10] (Table III), the extent of para-O-methylation increases as the pH is increased toward the pK_a[10] (Table III). The substitution of the electronegative fluorine on the aromatic ring of norepinephrine imposes marked electronic effects upon the phenolic hydroxyls of norepinephrine. The inductive effects of fluorine at the 2 position increases the acidity of the meta-hydroxyl group.[2] Substitution at the 5 position, on the other hand, should enhance the ionization of the para-hydroxyl group. These inductive effects should have little, if any, effect on polarity at the terminal amine moiety of the ethanolamine side chain. Thus, the changes in the extent of meta- and para-O-methylation relative to the parent compound should be attributable to changes in the nucleophilicity of the catechol moiety.

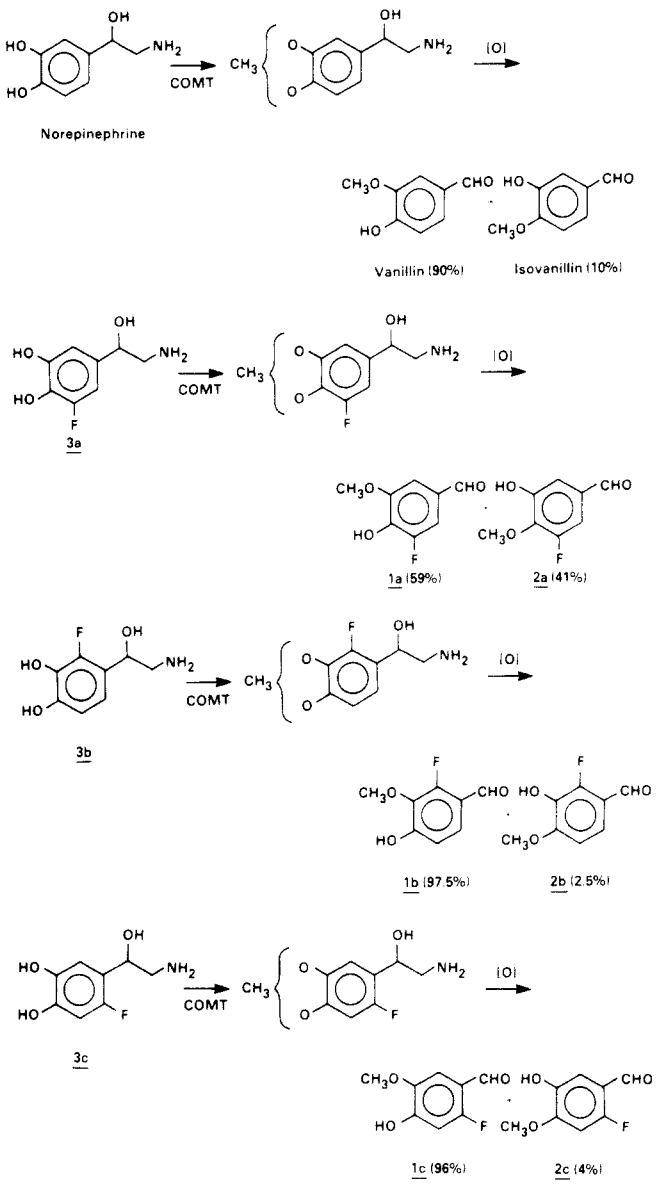
The enzymatic O-methylations were carried out at both pH 7 and 9 with partially purified hepatic COMT,[8] using optimum concentrations of all components and isotopically labeled *S*-adenosyl-L-methionine as the methyl donor. The products, mixtures of the *m*- and *p*-*O*-methylfluoronorepinephrines, were cleaved with periodate to yield the respective monofluorovanillins and isovanillins (**1a–c** and **2a–c**). After addition of the authentic aldehydes as carriers, the reaction mixtures were acidified, and the aldehydes were extracted into ethyl acetate. Following removal of the ethyl acetate under N_2, the aldehydes were successfully separated by thin-layer chromatography. The procedure and structures are schematically outlined in Scheme I. As shown in Table III the extent of para-O-methylation was significantly altered by the presence of a fluorine substituent on **3a–c**. The greatest increase in para-O-methylation occurred with 5-fluoronorepinephrine (**3a**) where, at pH 7, the extent of para-O-methylation was 41% or fourfold greater than in the parent compound. At pH 9 the extent of para-O-methylation increased to 66%, markedly exceeding the extent of O-methylation at the meta hydroxyl. The presence of fluorine at the 6 and 2 positions in **3c** and **3b**, at pH 7, reduced the extent of para-O-methylation by two- and fourfold, respectively, as compared to the parent compound. Thus, the rank order of preference for para-O-methylation is 5FNE ≫ NE > 6FNE > 2FNE. In all cases, increasing the pH of the reaction mixture from 7 to 9 led to an increase in the extent of para-O-methylation. The results obtained with 2- and 5-FNE clearly indicate that ionization plays an important role in enzymatic O-methylation when fluorine is situated ortho to one of the phenolic groups. A rationale for the decrease in para-O-methylation observed with **3c** is not readily apparent.

Table III. Influence of Fluorine Substituents on the Extent of Enzymatic Para O-Methylation of Norepinephrine[a]

	O-Methyl Isomers Produced					
	pH 7			pH 9		
substrate	meta, nmol	para, nmol	para, %	meta, nmol	para, nmol	para, %
5-FNE (**3a**)	2.4 ± 0.2	1.7 ± 0.1	41 ± 4	1.3 ± 0.05	2.7 ± 0.4	66 ± 4
NE	5.3 ± 1.8	0.6 ± 0.03	11 ± 3	4.7 ± 1.1	1.2 ± 0.2	20 ± 2
6-FNE (**3b**)	6.1 ± 0.1	0.3 ± 0.03	4.1 ± 0.5	5.7 ± 1.1	0.7 ± 0.2	12 ± 5
2-FNE (**3a**)	4.0 ± 5	0.10 ± 0.01	2.5 ± 0.03	3.9 ± 0.8	0.16 ± 0.01	4 ± 0.6

[a] Enzymatic O-methylation by COMT and measurement of the meta- and para-O-methylated products were determined as described under Experimental Section. [b] The total amounts of both products formed from each substrate are given in nanomoles. The values reported are the means plus or minus the range for two separate experiments.

Scheme I[a]



[a] Values given in parentheses denote the distribution (%) of the enzymatic products of pH 7.

From these results, it is clear that our initial product identifications of the *O*-methylfluorobenzaldehydes reported elsewhere were in error and reversed.[13] This was in part due to ambiguity in the Gibbs color reaction[15] on which the identification was based and to oxidative decomposition of products during thin-layer chromatography. In our earlier work, the chromatography involved several developments of a thin-layer plate with and intermediate drying of the plate. The decomposition was related to the time the dry plate was exposed to air.[16] Our present results and interpretations are in agreement with those of Firnau who demonstrated a fluorine-induced increase in the enzymatic para-O-methylation of 5-fluorophenylalanine.[17] However, in this study, as in our own earlier work,[13] direct proof of the structure of the O-methylated products by comparison to authentic compounds was lacking.

Experimental Section

Microanalyses, NMR spectra (JEOL FX100), and mass spectra (Finnigan 1015 mass spectrometer, chemical-ionization mode) were provided by the Microanalytical Services and Instrumentation Section of the Laboratory of Chemistry, NIAMDD, under the direction of Dr. David F. Johnson. In all cases, mass spectra data agreed with calculated molecular weights.

5-Fluorovanillin (1a). To 9.85 g of 5-nitrovanillin (Aldrich Chemical Co.) dissolved in 250 mL of dry methanol was added 1 g of Dowex 50W-X8 (acid form) to yield the acetal intermediate. After the mixture was stirred at room temperature overnight, the Dowex resin was removed by filtration, 1 mL of triethylamine and 300 mg of PtO_2 were added, and the solution was hydrogenated at 40 psi. When uptake of hydrogen ceased, the catalyst was removed and the methanol was removed by evaporation. The residue was dissolved in 500 mL of cold 50% fluoroboric acid, diazotized, and irradiated as described for the preparation of the fluorinated veratraldehyde.[12] Extraction of the neutralized (pH 6–7) reaction mixture with ethyl acetate and chromatography of the crude product (silica gel, 20% acetone in CCl_4) gave 2.24 g (26%) of 5-fluorovanillin (**1a**).

6-Fluorovanillin (1c). Using the same procedure, 4-(benzyloxy)-5-methoxy-2-nitrobenzaldehyde[18] (2.0 g, 6.97 mmol) was converted to the acetal, reduced, diazotized, and irradiated. Isolation and purification by preparative thin-layer chromatography (silica gel GF; petroleum ether–ether, 70:30) gave 42 mg (2%) of **5** and 73 mg (6%) of 6-fluorovanillin (**1c**). In addition to intractable material, there was isolated 158 mg (9%) 4-(benzyloxy)-2-hydroxy-5-methoxybenzaldehyde and 114 mg (7%) 4-(benzyloxy)-3-methoxybenzaldehyde. These products were identified by mass spectrometry but not characterized further.

6-Fluoroisovanillin (2c). 6-Nitroisovanillin (8.2 g, 0.042 mol)[18] was converted to the dimethyl acetal with Dowex 50W-X8 as above. The methanolic solution containing 1 mL of triethylamine was hydrogenated (PtO_2) for 5 h. The amine was dissolved in fluoroboric acid, diazotized, and irradiated as usual. The crude product was chromatographed on grade I alumina packed in chloroform. Elution with increasing concentrations of methanol in chloroform (0 to 10%) gave chromatographically pure **2c** (950 mg, *13%*).

2-Fluorovanillin (1b). 4-(Benzyloxy)-3-methoxy-2-nitrobenzaldehyde[18] (2.0 g, 6.97 mmol) was converted to the dimethyl acetal as above. Hydrogenation (PtO_2) of the methanolic solution of the acetal containing 0.3 mL of triethylamine produced the corresponding amine which, without purification, was dissolved in 500 mL of fluoroboric acid (dissolution was incomplete). The suspension was subjected to diazotization and photolysis as before. In this case, considerable polymeric material was apparent after the photolysis. The crude product was isolated by extraction of the neutralized solution (pH 6–7) with ethyl acetate and purified by preparative thin-layer chromatography (silica gel GF; petroleum ether–ether, 70:30) to give 171 mg (14%) of 2-fluorovanillin (**1b**) and 150 mg (8%) benzylated 2-fluorovanillin (**4**).

5-Fluoroisovanillin (2a). To a solution of 200 mg (1.28 mmol) of 3,4-dihydroxy-5-fluorobenzaldehyde (prepared by boron tribromide demethylation of 5-fluoroveratraldehyde) in 1.1 mL of 10% NaOH was added, with stirring, 138 mg (1.1 mmol) of dimethyl sulfate. After stirring overnight at room temperature, the solution was washed with ether (to remove 5-fluoroveratraldehyde). The aqueous phase was adjusted to pH 6.5 with dilute acid and extracted with ether. Preparative TLC (silica gel GF; pentane–ether, 1:1) gave 8 mg of 5-fluoroisovanillin (**2a**) in addition

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(16) When thin-layer plates were purposely allowed to stand overnight following the first solvent development and again redeveloped, comparison of the distribution of radioisotope (TLC scanner) revealed that approximately 50% of each peak remained stationary, thus markedly altering the apparent distribution of the two products. This presumed oxidative decomposition was not observed when the plate was replaced in the development tank immediately upon drying.
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to 5-fluorovanillin (1a), identical with the material described above.

2-Fluoroisovanillin (2b). To 200 mg (1.28 mmol) of 2-fluoroveratraldehyde dissolved in 3 mL of methylene chloride and cooled to −78 °C was added 0.05 mL (1.08 mmol) of BBr_3 in one portion with rapid stirring. The solution was stirred at room temperature for 15 h, cooled to 0 °C, and treated with excess H_2O. Ether was added, and the organic layer was separated and extracted with 2 N NaOH. The alkaline extract was acidified with dilute HCl and extracted with ether, and the ether extract was dried (Na_2SO_4) and evaporated. The residue was purified by preparative TLC (silica gel GF; pentane–ether, 1:1, developed 3 times) to give 9 mg of pure 2-fluoroisovanillin (2b).

Enzymatic O-Methylation. Stock solutions (10 mM) of 3a–c and (±)-norepinephrine were prepared in 0.001 N HCl and stored at −4 °C until used. Enzymatic reaction mixtures were prepared in ice, containing the following components (final concentrations) in a final volume of 0.25 mL: catechol (2 mM); magnesium chloride (2.4 mM); *S*-adenosyl-L-methionine iodide (Sigma Chemical Co.) (0.1 mM); *S*-adenosyl-L-[*methyl*-^{14}C]methionine (New England Nuclear Corp., sp act. = 55 mCi/mmol), 0.1 μCi; freshly prepared dithiothreitol (2 mM); Tris buffer, pH 7 or 9 (25 mM); and partially purified COMT (calcium phosphate eluate step[8]), 4 mg. Reaction was initiated by the addition of the catechol, incubated at 37 °C for 30 min, and terminated by the addition of 5 N ammonium hydroxide (0.5 mL). Control reactions were carried by omission of (1) the catechol or (2) replacement of COMT by bovine serum albumin. These control mixtures did not yield components which interfered with the thin-layer separation of the final products (Scheme I). The efficiency of the ethyl acetate extraction was determined by extraction of standard mixtures of the respective *O*-methylbenzaldehydes. Quantitation was achieved by HPLC analysis [stationary phase; Waters μ-Bondapac C_{18} reverse phase; mobile phase; methanol/water, 1:2, containing PIC (0.005 M) (Waters; 1-heptanesulfonic acid)]. In all cases, base-line separations of the respective aldehydes was achieved. No significant differences between the meta and para isomers were found.

Periodate Oxidation. To the reaction mixture (above) was added freshly prepared 2% aqueous $NaIO_4$ (0.2 mL) with vigorous mixing at room temperature; after 5 min, a freshly prepared solution of aqueous 10% sodium metabisulfate (0.2 mL) was added, and the reaction was cooled in ice bath. Carrier amounts of the authentic aldehydes 1a and 2a, 1b and 2b, 1c and 2c, and vanillin and isovanillin were added to the appropriate tubes. The reaction mixtures were acidified by the dropwise addition of 5 N HCl (0.5 mL) with continuous cooling and extracted two times with ethyl acetate (10 mL). The extracts were clarified by centrifugation, transferred to conical tubes, and concentrated under a stream of N_2, and the final residue was dissolved in MeOH (0.025 mL).

Chromatographic Separation. The methanol solutions (above) were spotted on silica gel plates (Analtech, GF silica gel, 250 μm, 5 × 20 cm) and immediately developed in the appropriate solvent system.[16] The solvent system used for the products derived from 3a was petroleum ether–diethyl ether (1:1); for products from 3b, 3c, and (±)-norepinephrine, benzene–acetic acid (1 N)–*p*-dioxane (90:1:1) was used. The compounds were visualized by UV absorption, Gibbs reagent and scanning with a gas-flow TLC-scanner (Berthold LB2760, gas phase, methane). Quantitation was achieved by quantitative transfer of the silica gel (3-mm strips) and measurement of radioactivity by scintillation counting in hydroflor (National Diagnostic, Inc.). The mobility of the authentic aldehydes corresponded in each case to the mobility of the isotopically labeled products. The enzymatic conversion to O-methylated products was approximately 40% for each catecholamine.

5-Fluoro- and 5-Chlorocyclophosphamide: Synthesis, Metabolism, and Antitumor Activity of the Cis and Trans Isomers

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Received February 6, 1981

In seeking analogues of cyclophosphamide (1) having improved antitumor activity by virtue of accelerated formation of the cytotoxic metabolite phosphoramide mustard, cis and trans isomers of 5-fluoro- and 5-chlorocyclophosphamide (9, 10, 11 and 12, respectively) were synthesized by condensation of the appropriate 3-amino-2-halopropan-1-ol (13 or 26) with *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (14). The metabolism of the halocyclophosphamides by rat liver microsomes was stereoselective; the cis isomers (9 and 11) were poorly metabolized, whereas the trans isomers (10 and 12) were metabolized with efficiency comparable to that of cyclophosphamide. However, there was no evidence that the yield of phosphoramide mustard produced by the trans analogues was significantly greater than that from cyclophosphamide following microsomal 4-hydroxylation. Hence, the halogen substituents did not accelerate β-elimination of acrolein from the acyclic aldehydo tautomers. As expected, the poorly metabolized *cis*-5-fluoride (9) had little activity against the ADJ/PC6 tumor in mice. However, the *cis*-5-chloride (11) was as active as the trans isomer (12) and each had approximately half the therapeutic index of 1. The *trans*-5-fluoride (10) was much less active, having an ED_{90} value some 16-fold that of 1.

Cyclophosphamide (1, 2-[bis(2-chloroethyl)amino]-tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide) is one of the most widely used of the alkylating-type anticancer drugs. The drug itself has little, if any, cytotoxic activity and requires metabolic activation[1] presumably by the cytochrome P-450 system and mainly in the liver. The principle features of the metabolic profile[2] (Scheme I) involves the initial formation of 4-hydroxycyclophosphamide (2), which is assumed to equilibrate rapidly with the acyclic tautomer, aldophosphamide (3). Structures 2 and 3 can undergo further enzymatically mediated oxidation–dehydrogenation to give the weakly cytotoxic metabolites 4-ketocyclophosphamide (4) and carboxyphosphamide (5), respectively. In competition with the metabolic reaction 3 → 5 there is an apparently nonenzymatic β-elimination which yields phosphoramide mustard (PAM, 6) and acrolein (7). It is the reactive alkylating product PAM (6) which is now generally regarded as the main source of the cytotoxicity of cyclophosphamide.

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0022-2623/81/1824-1399$01.25/0