Experimental Section[4]

The physical properties, yields, and analyses are listed in Table I.

2-Carboxy-6,8-dichloro-γ-chromone (I).—A mixture of 11.8 g (0.080 mole) of ethyl oxalate and 15.0 g (0.075 mole) of 3,5-dichloro-2-hydroxyacetophenone[5] in 200 ml of anhydrous Et_2O was added, over a period of 30 min, to a vigorously stirred suspension of NaOEt (13.6 g, 0.2 mole) in 100 ml of anhydrous Et_2O. The mixture was kept at 20–25° for 0.5 hr, heated under reflux for 2 hr, cooled, and filtered. The sodium salt was suspended in 360 ml of a mixture of AcOH–concentrated HCl (5:1) and heated under reflux for 2 hr. The reaction mixture was cooled and the insoluble solid was collected. Recrystallization from AcOH gave 10.5 g (56%) of white needles.

Esters. General Method.—Concentrated H_2SO_4 (2 ml) was added slowly to a suspension of I (2 g) in 20 ml of the appropriate alcohol. The mixture was refluxed for 4 hr. The ester, which precipitated on cooling, was collected and washed ($NaHCO_3$, H_2O).

6,8-Dichloro-γ-chromone-2-carbonyl Chloride (VII).—The acid I was suspended in a mixture of 5 g of $SOCl_2$ and 6.0 ml of 1,2-dichloroethane and heated with occasional shaking under reflux for 7–8 hr. The hot mixture was filtered and the residue was extracted twice with hot petroleum ether (60–80°). The filtrate and the ethereal extracts were pooled and evaporated *in vacuo*. The residue was recrystallized from petroleum ether; yield 240 mg (6%) of pale yellow prisms.

2-(N,N-Diethylcarbonamide)-6,8-dichloro-γ-chromone (VI).—Diethylamine (0.5 ml, excess) was added to a cold suspension of 140 mg of VII in 3 ml of anhydrous C_6H_6. The mixture was kept at room temperature for 30 min and then refluxed for 30 min. The solvent was removed, and the residue was treated with H_2O, filtered, and washed (H_2O). The solid, recrystallized from EtOH, gave 100 mg (64%) of white needles.

Acknowledgment.—The author wishes to thank Professor Dr. Armando Novelli for his many helpful suggestions. This work was carried out with a grant from the C. N. I. C. y T.

(4) All melting points were taken in capillaries and are uncorrected. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

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Experimentally Induced Phenylketonuria. III. Inhibitors of Phenylalanine Hydroxylase Related to Esculetin

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Received November 17, 1967

In the first paper of this series[1] we reported some observations concerning the *in vitro* inhibitory action of several *o*-dihydroxy compounds and various simple phenylalanine derivatives on the enzyme, phenylalanine hydroxylase. Esculetin (6,7-dihydroxycoumarin) and 4-fluorophenylalanine were found to be the most effective inhibitors, confirming the work of other investigators.[2,3] Phenylalanine-derived alkylating agents, designed to be irreversible inhibitors, were further explored without success as reported in a second communication.[4] The subject of this paper is a further investigation, both *in vitro* and *in vivo*, of hydroxylated coumarin compounds related to esculetin.

We began our structure–activity investigation by preparing various 3- and 4-substituted 6,7-dihydroxycoumarins. We found that, *in vitro*, the 4-methyl, 4-*n*-butyl, and 4-phenyl analogs were more potent inhibitors of phenylalanine hydroxylase than esculetin. The 4-ethyl-, *n*-propyl-, and isopropyl-substituted compounds were about as active as esculetin, while the activity was considerably diminished for the 3-methyl and 3,4-dimethyl analogs (Table I).

TABLE I

In Vitro INHIBITION OF RAT LIVER PHENYLALANINE HYDROXYLASE[a]

Substituted coumarin	% inhib	Ratio of substrate: inhibitor
6,7-Dihydroxy- (esculetin)	55	100:1
Esculin	16	1:1
4-Methyl-6,7-dihydroxy-	77	200:1
	34	1000:1
4-Ethyl-6,7-dihydroxy-	55	100:1
4-*n*-Propyl-6,7-dihydroxy-	58	100:1
4-Isopropyl-6,7-dihydroxy-	53	100:1
4-*n*-Butyl-6,7-dihydroxy-	64	200:1
4-Phenyl-6,7-dihydroxy-	54	200:1
3-Methyl-6,7-dihydroxy-	41	50:1
3,4-Dimethyl-6,7-dihydroxy-	54	50:1
5,6-Dihydroxy-	52	10:1
7,8-Dihydroxy-4-methyl-	44	10:1
6,7,8-Trihydroxy-4-methyl-	55	100:1
5,6,7-Trihydroxy-	50	5:1
5-Hydroxy-4-methyl-	16	1:1

[a] See ref 1 for biological procedures.

We also investigated the effects of varying the position and number of hydroxyl groups while retaining either hydrogen or methyl at the 4 position. Both 5,6-dihydroxycoumarin and 7,8-dihydroxy-4-methylcoumarin were poor inhibitors as was 5,6,7-trihydroxycoumarin. 6,7,8-Trihydroxy-4-methylcoumarin was as potent as esculetin, but considerably less than 4-methylesculetin. 5-Hydroxy-4-methylcoumarin was a very poor inhibitor.

Two of the more active compounds, namely, 4-methyl- and 4-phenylesculetin, were selected for *in vivo* studies (Table II). Since esculin (the 6-glycoside

TABLE II

In Vivo INHIBITION OF RAT LIVER PHENYLALANINE HYDROXYLASE[a]

Compd	% inhib	Time of sacrifice after oral administration, hr
Esculin	82	0.5–1
4-Methylesculetin	70	3
4-Phenylesculetin	68	5

[a] Three 42–50-g Sprague–Dawley rats were used for each compound. Compounds were given orally as aqueous suspensions (pH 8–9) at 2 mmoles/kg. Animals were sacrificed at various intervals. The specific activity of the control liver phenylalanine hydroxylase in several experiments ranged from 0.113 to 0.193 μmole of tyrosine formed/hr per mg of protein.

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(2) S. B. Ross and O. Haljasmaa, *Life Sci.*, **3**, 579 (1964).

(3) D. D. Watt and J. P. Vandervoorde, *Fed. Proc.*, **23**, 146 (1964).

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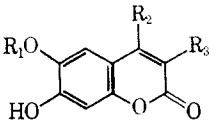
TABLE III
SUBSTITUTED COUMARINS



R_1	R_2	R_3	Reaction time, hr	Recrystn solvent	Mp, °C	Yield, %	Formula	Analyses
CH_3	C_2H_5	H	16	EtOH	185–186	53	$C_{12}H_{12}O_4$	C, H
CH_3	n-C_3H_7	H	16	EtOAc	144–146	55	$C_{13}H_{14}O_4$	C, H
CH_3	i-C_3H_7	H	72	Oil				
CH_3	n-C_4H_9	H	72	Oil				
CH_3	C_6H_5	H	24	EtOAc	195.5–197	37	$C_{16}H_{12}O_4$	C, H
CH_3	H	CH_3	1	Toluene	147–151[a]	45		
CH_3	CH_3	CH_3	18	EtOAc	191–193	33	$C_{12}H_{12}O_4$	C, H
H	C_2H_5	H	1	EtOAc	218–221	48	$C_{11}H_{10}O_4$	C, H
H	n-C_3H_7	H	2	EtOAc	210–213	21	$C_{12}H_{12}O_4$	C, H
H	i-C_3H_7	H	1	EtOAc	227.5–229	6.1[b]	$C_{12}H_{14}O_4$	C, H
H	n-C_4H_9	H	2	EtOAc	199–201	5.5[b]	$C_{13}H_{14}O_4$	C, H
H	C_6H_5	H	2	EtOAc	274–276[c]	46	$C_{15}H_{10}O_4$	C, H
H	H	CH_3	2	EtOH	252–255	58	$C_{10}H_8O_4$	C, H
H	CH_3	CH_3	2	EtOH	267–268[d]	89		

[a] D. G. Crosby and R. V. Berthold, *J. Org. Chem.*, **27**, 3083 (1962), report 151–152°. [b] Yield for two reactions based upon amount of 3-hydroxy-4-methoxyphenyl formate used at start. [c] V. K. Ahluwalia and T. R. Seshadri, *J. Chem. Soc.*, 970 (1957), report 267–268°. [d] S. Tamura, K. Ohkuma, and T. Hayashi, *J. Agr. Chem. Soc. Japan*, **26**, 410 (1952) [*Chem. Abstr.*, **48**, 2004*h* (1954)], report 280°.

of esculetin) was previously found to inhibit phenylalanine hydroxylase activity in the intact rat to about the same extent as esculetin on an equimolar basis,[1] it was used as a reference compound. Each compound (2 mmoles/kg) was administered orally to Sprague–Dawley rats (40–50 g) which were sacrificed at various intervals. The maximal inhibition of phenylalanine hydroxylase attained by these compounds was approximately the same. However, with esculin the maximum inhibition of the enzyme was observed at 0.5–1 hr; with 4-methylesculetin, 3 hr; and with 4-phenylesculetin, the maximum may not have been reached by 5 hr. One probable explanation for this observation is that the more lipid-soluble 4-phenyl analog is deposited in fatty depots and is released slowly from the depot, thus exerting a prolonged biological effect, while esculetin reaches the liver in greater quantity sooner and is excreted faster than the more lipid-soluble analogs. More detailed *in vivo* studies with the 4-phenyl analog are in progress.

The 3- and 4-substituted 6,7-dihydroxycoumarins were prepared by the general method of Crosby,[5] whereby an appropriate β-keto ester is condensed with 3-hydroxy-4-methoxyphenyl formate in H_3PO_4. The resultant 6-methoxy-7-hydroxycoumarins were cleaved with hot HBr to the 6,7-dihydroxycoumarins (Table III). 6-Hydroxylation[6] of 5,7-dimethoxycoumarin by $K_2S_2O_8$ followed by ether hydrolysis afforded 5,6,7-trihydroxycoumarin.

Experimental Section

Melting points were obtained with a Fischer-Johns apparatus and are corrected. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

5,6-Dihydroxy- and 6,7,8-trihydroxy-4-methylcoumarins were prepared by the method of Naik and Thakor.[7] 6,7-Dihydroxy-4-methyl-, 7,8-dihydroxy-4-methyl-, and 5-hydroxy-4-methylcoumarin were obtained from Aldrich Chemical Co.

5,7-Dimethoxy-6-hydroxycoumarin.—To 2.06 g (10 mmoles) of 5,7-dimethoxycoumarin (Aldrich Chemical Co.) was added 40 ml of 10% KOH and 5 drops of pyridine. The solution was chilled in an ice bath and 5.12 g (19 mmoles) of $K_2S_2O_8$ in 250 ml of H_2O was slowly added. The resulting solution was stirred for 16 hr at room temperature, acidified (HCl) to pH 3, and washed (three 50-ml portions of Et_2O) to remove starting material. Concentrated HCl (15 ml) and 1.0 g of $NaHSO_3$ was added and the resulting mixture was heated on a steam bath for 1 hr, chilled, and extracted (three 50-ml portions) with Et_2O. The extract was dried and evaporated *in vacuo* to yield a brown semisolid. Two recrystallizations (EtOAc) gave 0.112 g (5.1%), mp 172–173°. *Anal.* ($C_{11}H_{10}O_5$) C, H.

5,6,7-Trihydroxycoumarin.—A mixture of 0.134 g (0.60 mmole) of 5,7-dimethoxy-6-hydroxycoumarin and 5.0 ml of concentrated HBr was stirred at reflux for 2 hr, cooled, and diluted with H_2O (15 ml). The aqueous mixture was extracted with EtOAc (three 20-ml portions) and dried ($MgSO_4$) and the EtOAc was evaporated *in vacuo*, the product was recrystallized (EtOAc), yield 0.067 g (58%), mp 259–262°. *Anal.* ($C_9H_6O_5 \cdot 0.5H_2O$) C, H.

3-Hydroxy-4-methoxyphenyl Formate.—To a solution of 20.0 g (0.131 mole) of isovanillin in 250 ml of EtOAc at 35° was added a solution of 31.5 g (0.183 mole) of *m*-chloroperbenzoic acid in 350 ml of EtOAc. The resulting solution was stirred at 35° for 3 days. The reaction mixture was washed with 15% $NaHSO_3$ (three 100-ml portions), saturated $NaHCO_3$ (three 100-ml portions), and H_2O (three 100-ml portions). The EtOAc was dried ($MgSO_4$) and evaporated *in vacuo* to yield a yellow oil, which crystallized upon chilling; it was recrystallized (i-Pr_2O), yield 15.0 g (68%), mp 55–58°, lit.[3] mp 57–58°.

4-Ethyl-6-methoxy-7-hydroxycoumarin.—A solution of 10.0 mmoles of ethyl propionylacetate and 8.9 mmoles of 3-hydroxy-4-methoxyphenyl formate in 15 ml of 85% H_3PO_4 was stirred at room temperature for 16 hr and diluted with 20 ml of H_2O. The solid was collected and recrystallized. The physical properties of the 6-methoxy-7-hydroxycoumarins are listed in Table III. The dihydroxy coumarins were prepared by the procedure used for 5,6,7-trihydroxycoumarin.

Acknowledgment.—This work was supported by Public Health Service Grant HD 01972.

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