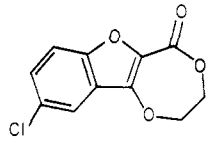
Synthesis and Pharmacological Evaluation of a Clofibrate-Related Tricyclic Spirolactone, 5-Chloro-4′,5′-dihydrospiro[benzofuran-2(3*H*),3′(2′*H*)-furan]-2′-one[1]

Donald T. Witiak,* Richard C. Cavestri, Howard A. I. Newman, John R. Baldwin, Christine L. Sober, and Dennis R. Feller

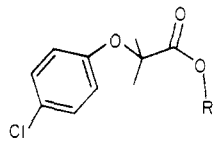
Divisions of Medicinal Chemistry and Pharmacology, College of Pharmacy, and the Department of Pathology, Division of Clinical Chemistry, College of Medicine, The Ohio State University, Columbus, Ohio 43210. *Received April 27, 1978*

The chemistry and pharmacology of the title compound, spirolactone 4, are reported. The synthesis represents a new approach to the preparation of spiro compounds. The pharmacological profiles of 4 are compared to that of clofibrate in Triton-induced hyperlipidemic, sucrose-fed, and normal Sprague–Dawley rat models. Clofibrate was effective in all animal models, but the spirolactone 4 exhibited antitriglyceridemic activity only in the Triton model. The inactivity of 4 in sucrose- and chow-fed rats could not be attributed to a resistance to hydrolysis by serum esterases. Comparative studies revealed that inhibition of hepatic HMG-CoA reductase activity may not be an index of hypocholesterolemic action in sucrose-fed rats. Additionally, only clofibrate exhibited significant changes in components of the hepatic microsomal monooxygenase system.

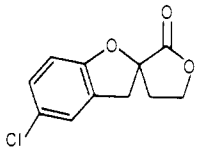
We previously reported the synthesis and antilipidemic properties for enol lactone 1, an analogue found to be



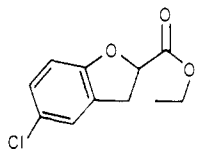
1



2, R = Et
3, R = H



4

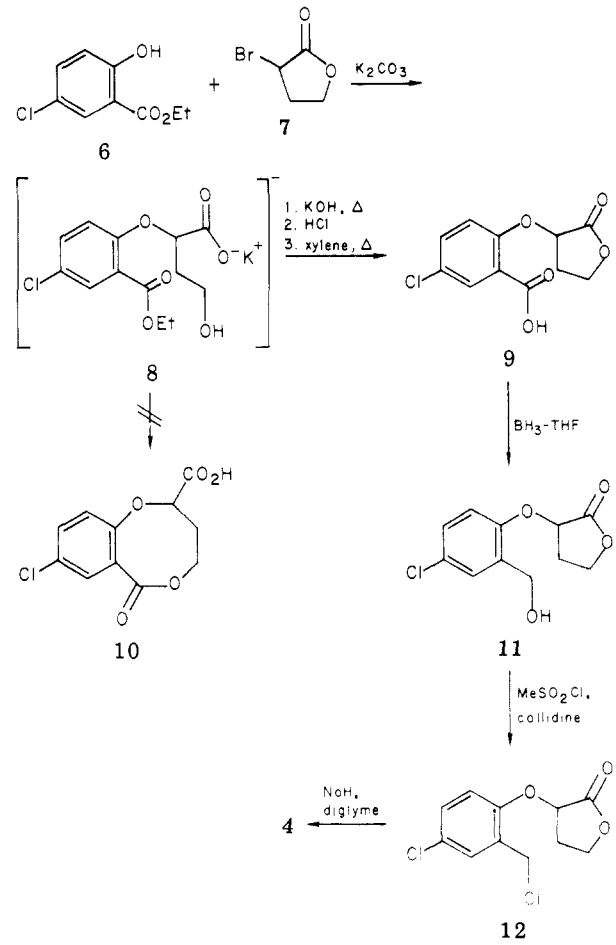


5

considerably more resistant to serum esterase hydrolysis than clofibrate (2) in vitro.[2] Tricyclic analogue 1 compared favorably[2] with 2 in reducing serum cholesterol and triglyceride levels in the Triton WR-1339 induced hyperlipidemic rat model.[3] However, in normolipemic rats 1 had no hypocholesterolemic activity, whereas 2 was active in such animals.[4] Since it was proposed that the antilipidemic properties of 2 are related to the rapid formation in vivo of the hydrolysis product 3,[5] the differential effects of 1 in the two animal models may reflect a resistance to hydrolysis in vivo. To further assess this possibility we synthesized the novel tricyclic spirolactone 4. Spirolactone 4 is not only structurally related to 2 but also may be envisioned as being derived from anticholesterolemic dihydrobenzofuran 5[6] through bonding of the β carbon of the ethyl group to the 2 position of the dihydrobenzofuran ring.

In this article we discuss the pharmacological properties of 4 and 2 in Triton,[3] sucrose-fed,[7] and normal[4] rat models. Assessment of pharmacological properties of new analogues employing more than one animal model was considered to be important since (a) there are major differences in drug dosing and in mechanisms associated with Triton- and sucrose-induced hyperlipidemia,[8-10] (b) we have previously observed differential effects of various analogues among the Triton, sucrose-fed, and normal rat models,[4,11,12] and (c) such investigations are a prerequisite to studies of structural influences on mechanisms of drug action.

Synthetic Aspects. Spirolactone 4 represents the 5-chloro analogue of a previously unreported tricyclic ring system.[13] Intermediate 5-chloro-2-[(tetrahydro-2-oxo-3-furanyl)oxy]benzoic acid (9) was prepared in 84% yield by condensation of α-bromo-γ-butyrolactone (7) with ethyl 5-chlorosalicylate (6) in the presence of K_2CO_3. Butyrolactone 9 was obtained by saponification (KOH–H_2O), followed by acidification and lactonization (refluxing xylene) of crude salt 8. Isomeric eight-membered lactone 10 could not be detected during formation of 9. The carbonyl-stretching absorbance of 1800 cm^{-1} for 9 is characteristic of a γ-butyrolactone.[14] Reduction (BH_3-THF)[15] afforded the expected benzyl alcohol 11 in 73% yield. Formation of benzyl chloride 12 (88% yield) was best accomplished utilizing methanesulfonyl chloride in dry collidine. This product likely forms via slow displacement by the chloride ion of the expected, but unisolated, intermediate mesylate.[16] Using gaseous HCl and $CaCl_2$ in benzene, no product 12 could be isolated. Chlorination using thionyl chloride afforded 12 which could not be separated from a contaminating product. Intramolecular chloride displacement induced by NaH in dry diglyme afforded the target tricyclic spirolactone 4 in 74%



0022-2623/78/1821-1198$01.00/0

Table I. Comparative Effects of Clofibrate (2) and Spirolactone 4 on Plasma Cholesterol and Triglyceride Levels (mg/dL) in Triton-Induced Hyperlipidemic Male Sprague-Dawley Rats

lipid analyzed	compd[a]	control group (I)[b]	drug-treated control (II)[b]	Triton hyperlipidemic (III)[b]	drug-treated Triton hyperlipidemic (IV)[b]
cholesterol	clofibrate (2)	88.7 ± 6.9	87.0 ± 8.1	166 ± 17.8	93.2 ± 8.9[c,d]
	spirolactone 4	90.1 ± 8.8	91.8 ± 8.9	183 ± 101	131 ± 26.5[d,e]
triglycerides	clofibrate (2)	20.8 ± 5.4	24.1 ± 7.4	98.5 ± 16.9	22.3 ± 8.3[c,d]
	spirolactone 4	20.9 ± 11.3	18.5 ± 7.1	156 ± 184	20.6 ± 6.3[c,d]

[a] All animals were given a total screening dose of 0.124 mmol/kg of compound. [b] Mean ± SD; ten rats per group. [c] Statistically significant; $p < 0.05$; comparison of groups III and IV. [d] Comparison of groups III and IV for statistical significance using the rank-sum nonparametric statistical treatment. This treatment for clofibrate-cholesterol shows $T_1 = 55$, $T_2 = 155$, $\alpha = 0.000$; clofibrate-triglycerides show $T_1 = 55$, $T_2 = 155$, $\alpha = 0.000$. Spirolactone 4-cholesterol shows $T_1 = 91$, $T_2 = 119$, $\alpha = 0.157$ (nonsignificant); spirolactone 4-triglycerides show $T_1 = 56$, $T_2 = 154$, $\alpha = 0.000$. [e] Statistically significant; $p < 0.05$; comparison of groups I and IV.

isolated yield. Intramolecular spirolactone formation seems to be solvent selective since under a variety of conditions employing many different solvents little or no reaction took place. Virtual quantitative conversion to spirolactone 4 is important since benzyl chloride 12 cannot easily be separated from 4 by physical methods.

Results

Hydrolysis of 2 and 4 in Rat Serum. The hydrolysis observed for clofibrate (2) and spirolactone 4 during incubation with rat serum at 37 °C was >90% after 5 min. Hydrolysis in serum was significantly faster for both 2 and 4 when compared to the chemically catalyzed reaction in Ringer bicarbonate buffer. In the presence of this buffer (pH 7.4) both 2 and 4 underwent partial hydrolysis (<20%) after 60 min.

Triton-Induced Hyperlipidemic Rat Model.[3] Whereas clofibrate (2) previously had been observed in two separate determinations[6,11] to lower both elevated serum cholesterol and triglyceride levels in Triton WR-1339 induced hyperlipidemic rats, spirolactone 4 only reduced elevated serum triglyceride levels (Table I) at an equivalent dose (0.124 mmol/kg). Both 2 and 4 reduced elevated serum triglyceride levels to those found in control animals. Neither 2 nor 4 showed antilipidemic activity in normal animals when this 48-h starved rat model was employed (Table I).

Antilipidemic and Hepatic Effects in the Sucrose-Fed Rat Model.[7] The effects of clofibrate (2) and spirolactone 4 (0.4 mmol/kg twice daily for 7 days) on serum cholesterol and triglyceride levels in sucrose-fed male Sprague–Dawley rats are summarized in Table II. The hepatic effects for these analogues in the sucrose-fed model are presented in Table III. Only 2 was found to be hypocholesterolemic and hypotriglyceridemic in this animal model in agreement with previously reported results. When assessed for hepatic effects (Table III), 2 was observed to significantly reduce liver triglyceride concentrations and HMG-CoA reductase activity. Additionally, 2 increased liver weight, microsomal protein, cytochrome P-450 level, and NADPH–cytochrome *c* reductase activity. Of the hepatic parameters studied (Table III), spirolactone 4 only caused an increase in the liver microsomal protein concentration.

Antilipidemic and Hepatic Effects in the Normal Rat Model. The effects of 4 (observed for two separate experiments) on serum cholesterol and triglyceride concentrations and various parameters of hepatic drug metabolism in this model are found in Table IV. Experimental procedures are identical with those described in previous publications.[4,11] In this animal model spirolactone 4 was essentially inactive except that during experiment 1 an increase in serum triglyceride concentrations was observed; this increase was not confirmed in experiment 2. In experiment 2, liver cholesterol concentrations were significantly increased, but no such increase was observed in the replicate experiment.

Table II. Comparative Effects of Clofibrate (2) and Spirolactone 4 on Serum Cholesterol and Triglyceride Levels (mg/dL) in Sucrose-Fed Male Sprague-Dawley Rats

	serum cholesterol[a]		serum triglycerides[a]	
compd	0 day	7 day	0 day	7 day
control	69 ± 9	92 ± 22	157.1 ± 77.8	115.6 ± 19.4
2	74 ± 16	36 ± 7[b]	153.0 ± 74.9[b]	50.2 ± 14.6[b]
4	67 ± 13	103 ± 21[c]	142.0 ± 48.0	121.5 ± 45.1[c]

[a] Values reported as mean ± SD; $n = 10$. [b] Statistically significant, $p < 0.05$ (response of drug vs. 0- or 7-day control). [c] Statistically significant, $p < 0.05$ (comparison of clofibrate group vs. drug group).

Discussion

Serving as a prototype compound, clofibrate (2) has been shown to exhibit significant hypocholesterolemic and hypotriglyceridemic activities in the Triton-induced,[6,11] sucrose-fed,[12] and normolipemic[4] animal models. At half the dosing schedule (0.062 mmol/kg) we noted that 1 and 2 exhibited a selective antitriglyceridemic action in the Triton model, suggesting that their activities for serum cholesterol and triglyceride lowering are mediated at different pharmacological sites.[2] In this study spirolactone 4 was effective as an antitriglyceridemic agent only in the Triton WR-1339 induced hyperlipidemic animal model (see Tables I, II, and IV). This observation does not rule out the possibility that spirolactone 4, at higher doses (>0.124 mmol/kg), would give both anticholesterolemic and antitriglyceridemic activities in this model. Additional studies with 4 are unwarranted owing to the lack of significant activity in both the sucrose-fed and normal rat models. These models employed both higher doses of 4 and longer pretreatment intervals. It would appear that the lack of activity for 4 cannot be attributed to either a lack of absorption or hydrolysis by serum since 4 is active orally in the Triton model and undergoes hydrolysis in vitro. It is also of interest to note that two ethyl esters of 6-phenyl- and 6-cyclohexylchroman-2-carboxylate, which were found to be active in the Triton hyperlipidemic and normal rat models,[17] are also inactive in the sucrose-fed model.[18] These differential drug effects in the various animal models are likely a reflection of differing modes of action since hyperlipidemia induced by Triton WR-1339 and by sucrose feeding is proposed to be mediated through a blockade of lipoprotein catabolism[8] and increased triglyceride synthesis,[9,10] respectively.

A mechanism proposed for the hypocholesterolemic action of clofibrate (2) is associated with an inhibition of hepatic cholesterol biosynthesis, specifically the blockade of HMG-CoA reductase activity and subsequent lowering

Table III. Comparative Effects of Clofibrate (2) and Spirolactone 4 on Various Hepatic Parameters after Chronic Administration to Sucrose-Fed Male Sprague-Dawley Rats

parameters[a]	experimental group		
	control[b]	clofibrate[c] (2)	spirolactone[c] 4
liver cholesterol, mg/g	1.81 ± 0.27	1.76 ± 0.36	
	2.17 ± 0.26	2.28 ± 0.11	2.32 ± 0.10
liver triglycerides, mg/g	7.26 ± 1.73	3.44 ± 1.42[d]	
	8.29 ± 2.24	3.05 ± 0.82	10.02 ± 1.86
HMG-CoA reductase, (nmol/mg)/h	8.3 ± 2.2	2.7 ± 1.2[d]	
	8.27 ± 1.18	3.93 ± 1.14[d]	7.10 ± 2.89
liver body wt, %	3.88 ± 0.11	5.52 ± 0.43[d]	
	4.06 ± 0.25	5.01 ± 0.50[d]	4.18 ± 0.34
liver wt, g	11.30 ± 0.75	16.15 ± 1.67[d]	
	13.16 ± 0.61	14.32 ± 1.35	13.16 ± 1.95
microsomal protein, mg/g	27.2 ± 3.0	30.0 ± 1.5[d]	
	25.0 ± 2.5	29.2 ± 3.0[d]	27.8 ± 1.9[d]
ethylmorphine *N*-demethylase, (nmol of HCHO formed/mg)/min	6.40 ± 0.63	7.91 ± 1.73	
	9.36 ± 0.50	8.13 ± 1.40	9.80 ± 1.89
cytochrome b_5, nmol/mg of protein	0.185 ± 0.020	0.183 ± 0.030	
	0.213 ± 0.032	0.169 ± 0.044	0.173 ± 0.024
cytochrome P-450, nmol/mg of protein	0.477 ± 0.076	0.806 ± 0.133[d]	
	0.593 ± 0.059	0.844 ± 0.189[d]	0.550 ± 0.035
NADPH-cytochrome *c* reductase, (nmol/mg)/min	202.5 ± 27.9	308.3 ± 13.1[d]	

[a] Values of the parameters are reported as the mean ± SD; $n = 5$. These animals were randomly selected from the treatment groups indicated in Table II. Values were determined after completion of a 7-day drug treatment. [b] Control animals received a requisite volume of vehicle. [c] The drug was given orally twice daily (0.4 mmol/kg). [d] Significant differences from the control ($p < 0.05$) for drug treated vs. control animals.

Table IV. Effects of Spirolactone 4 on Serum Cholesterol and Triglyceride Levels and Hepatic Parameters in Normal Male Sprague-Dawley Rats

parameter[a]	expt no.	control group[b]	treated group[b]
serum cholesterol, mg/dL	1	81.6 ± 13.8	68.3 ± 9.2
	2	59.1 ± 14.1	58.6 ± 12.3
serum triglycerides, mg/dL	1	69.1 ± 23.0	113.3 ± 30.1[c]
	2	61.2 ± 20.2	66.5 ± 19.7
liver cholesterol, mg/g	1	2.73 ± 0.26	3.18 ± 0.69
	2	3.28 ± 0.48	4.44 ± 1.08[c]
liver triglycerides, mg/g	1	2.73 ± 0.33	3.95 ± 1.68
	2	3.36 ± 0.73	3.17 ± 0.97
HMG-CoA reductase, (nmol/mg)/h	1		
	2	49.1 ± 6.7	45.0 ± 7.9
liver/body wt, %	1	4.21 ± 0.31	4.50 ± 0.30
	2	4.59 ± 0.59	4.58 ± 0.24
liver wt, g	1	6.73 ± 0.95	7.87 ± 0.73
	2	8.71 ± 1.70	9.19 ± 1.07
microsomal protein, mg/g of liver	1	29.0 ± 1.7	33.6 ± 3.0
	2	25.4 ± 2.2	27.3 ± 4.0

[a] Measurements were determined after a 7-day drug-treatment period. [b] Drug was administered orally twice daily for a total dose of 0.4 mmol/kg. Control animals received a requisite volume of vehicle. Values represent the mean ± SD; $n = 5$. [c] Statistically significant from control, $p < 0.05$.

of liver and serum cholesterol levels.[19] In a previous communication[12] we reported that administration of clofibrate and the cis,syn photodimer of antilipidemic ethyl 5-chlorobenzofuran-2-carboxylate to sucrose-fed rats did not exhibit a correlation between serum cholesterol lowering and an inhibition of hepatic HMG-CoA reductase activity. Like the photodimer,[12] ethyl 6-phenylchroman-2-carboxylate produced a significant inhibition of hepatic cholesterol biosynthesis but did not lower serum cholesterol levels.[18] Thus, a blockade of hepatic HMG-CoA reductase activity does not necessarily serve as an index of the serum cholesterol lowering action of clofibrate and related analogues in sucrose-fed rats. These data are consistent with the view that the serum cholesterol lowering action of clofibrate may be related to an increased catabolism of lipoproteins in rats[20] as well as humans.[21,22]

Spirolactone 4, unlike clofibrate, did not produce any changes in parameters associated with hepatic microsomal drug metabolism in sucrose-fed rats. This was not expected since hepatic effects of clofibrate analogues have been previously correlated to the presence of a chloro group on the aromatic ring.[23] Additionally, hypolipidemic activity in normal[4] and sucrose-fed[12] rats is independent of changes in the activity of the hepatic microsomal monooxygenase system. Hepatic peroxisomal proliferation has also been associated with halogen (Cl, Br, and I but not F) substitution but is not necessarily correlated with antilipidemic activity.[24] Whereas chloro-substituted spirolactone 4 did not alter hepatic parameters and is, therefore, an exception to these structural requirements for hepatic enzyme induction, this analogue is of no further interest to us because of its lack of activity in chronic animal models.

Experimental Section

Infrared spectra were recorded on a Beckman 4230 spectrophotometer. NMR spectra were recorded on a Varian A-60A spectrometer with tetramethylsilane as internal standard. Melting points were taken in open capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. Thin-layer chromatograms were carried out with silica gel GF (Analtech, Inc.) and the chromatograms were visualized using UV light or 10% H_2SO_4 containing 5% $Ce(SO_4)_2$. Tetrahydrofuran (THF) was purified and dried by distillation from $LiAlH_4$. Diglyme was dried[25] and used immediately. Enzymatic hydrolysis studies were carried out utilizing a Beckman DB spectrophotometer. Tracings were scanned using a Houston XY 2000 recorder. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

A. Synthetic Methods. 5-Chloro-2-[(tetrahydro-2-oxo-3-furanyl)oxy]benzoic Acid (9). A mixture of ethyl 5-chlorosalicylate[26] (6, 40.0 g, 0.2 mol), powdered anhydrous K_2CO_3 (71.8 g, 0.52 mol), and α-bromobutyrolactone (7, 42.9 g, 0.26 mol) in 400 mL of dry reagent-grade acetone was stirred at ambient conditions for 24 h. After 24 h additional 7 (8.5 g, 0.05 mol) and anhydrous K_2CO_3 (11.5 g, 0.084 mol) were added, and the mixture was stirred (24 h) and filtered (Celite). The solids were washed several times with acetone and the combined filtrates were concentrated under reduced pressure affording a brown viscous

oil. The oily residue was dissolved in 300 mL of Et_2O and extracted with H_2O (100 mL). The Et_2O layer was extracted (50-mL portions of 10% KOH) until no white precipitate formed when neutralized with concentrated HCl. The alkaline washings were combined and acidified (concentrated HCl), yielding an oily semisolid which solidified with scratching and cooling. The solid was removed by filtration, washed (50 mL of cold H_2O), and dissolved in saturated $NaHCO_3$ solution (200 mL). The solution was extracted (Et_2O) and KOH (16 g) was added. The mixture was heated on a steam bath for 2.5–3 h, cooled, extracted (Et_2O), and made acidic with concentrated HCl. After standing at 0–4 °C overnight the white solid was filtered, air-dried, and relactonized by recrystallization from boiling xylene, affording 43.2 g (84%) of **9**: mp 157–159 °C; IR ($CHCl_3$) 1800 (lactone C=O), 1750 (carboxyl C=O) cm^{-1}; NMR δ (Me_2SO-d_6) 2.3–3.2 (2 H, m, OCH_2CH_2–), 4.3–4.7 (2 H, m, –OCH_2CH_2), 5.33 (1 H, t, methine H, J = 8.5 Hz), 7.3–7.9 (3 H, m, Ar–H). Anal. ($C_{11}H_9O_5Cl$) C, H.

3-[4-Chloro-2-(hydroxymethyl)phenoxy]dihydro-2(3*H*)-furanone (11). To a stirred solution of lactone **9** (25.6 g, 0.10 mol) in dry THF maintained at 0–4 °C borane–THF complex[15] [105 mL (0.10 mol), 0.94 M solution; Ventron] was added dropwise over 2 h. After the addition the mixture was stirred for 3 h at 0–4 °C and at room temperature overnight. Saturated $NaHCO_3$ solution (300 mL) was added and the mixture stirred for 30 min. The aqueous phase was separated and extracted with Et_2O. The Et_2O extract and THF phase were combined, dried ($MgSO_4$), and concentrated under reduced pressure, affording a crude syrup which was purified by chromatography on silicic acid (240 g, grade II, active[27]) using benzene–CH_2Cl_2 (1:1) as the eluting solvent. All homogeneous fractions (monitored by TLC) were concentrated and afforded 18.4 g (73%) of a clear syrup which yielded a white solid (mp 79–81 °C) on standing. An analytical sample (mp 79–81 °C) was obtained by sublimation at 90 °C (oil bath) (0.005 mm): IR ($CHCl_3$) 1795 (lactone C=O), 3500 (br OH), 3610 (sh OH) cm^{-1}; NMR δ ($CDCl_3$) 2.1–3.0 (2 H, m, –OCH_2CH_2–), 3.3 (1 H, s, –OH), 4.1–5.2 (5 H, m, –OCH_2CH_2–, –CH_2OH, methine H), 6.8–7.4 (3 H, m, Ar–H). Anal. ($C_{11}H_{11}ClO_3$) C, H, Cl.

3-[4-Chloro-2-(chloromethyl)phenoxy]dihydro-2(3*H*)-furanone (12). To a vigorously stirred solution of lactone **11** (9.7 g, 0.04 mol) in 40 mL of dry collidine, maintained at 0–4 °C, methanesulfonyl chloride (3.42 g, 0.03 mol) was added dropwise over a 10-min period. The mixture was stirred at 0–4 °C for 6–8 h and at room temperature overnight. The mixture was diluted with 200 mL of anhydrous Et_2O. After stirring for 30 min the precipitated salts were rapidly filtered (Celite) and the solids were washed with Et_2O (5 × 100 mL). The filtrates were washed with 10% H_2SO_4 solution (until the aqueous washes were acidic to litmus) and saturated NaCl solution (2 × 50 mL), dried ($MgSO_4$), and concentrated under reduced pressure. The residual oil, which solidified on standing, was chromatographed on silicic acid (active;[27] grade 1) with benzene. All homogeneous fractions [TLC, benzene–CH_2Cl_2 (1:1)] were concentrated under reduced pressure affording 8.62 g (88%) of **12**: mp 92–94 °C (cyclohexane); IR ($CHCl_3$) 1800 (lactone C=O) cm^{-1}; NMR δ ($CDCl_3$) 2.2–3.0 (2 H, m, –OCH_2CH_2–), 4.1–4.6 (4 H, m, –OCH_2CH_2–, –CH_2Cl), 4.96 (1 H, t, methine H, J = 8.0 Hz), 6.9–7.4 (3 H, m, Ar–H). Anal. ($C_{11}H_{10}Cl_2O_3$) C, H, Cl.

5-Chloro-4',5'-dihydrospiro[benzofuran-2(3*H*),3'(2'*H*)-furan]-2'-one (4). To a vigorously stirred solution of lactone **12** (9.92 g, 0.038 mol; specially dried in an Abderhalden apparatus) at 63 °C (0.005 mm) in 190 mL of freshly distilled dry diglyme[25] was added NaH (1.82 g, 0.076 mol; 50% dispersion in mineral oil) in one portion. The mixture was stirred for 24–48 h at 55–60 °C. After the reaction was complete [monitored by TLC, benzene–CH_2Cl_2 (1:1)], the mixture was added with stirring to 380 mL of a cooled (0–4 °C) aqueous solution containing 1.5% HCl. Stirring was continued at 0–4 °C for an additional 1–5 h during which time spirolactone **4** precipitated as an amorphous solid. The dried solid was chromatographed on silicic acid (150 g; active,[27] grade 2) with benzene. All homogeneous fractions (TLC) were combined and concentrated. The residue was recrystallized from cyclohexane affording 6.3 g (74%) of **4**, mp 116.5–118.5 °C. An analytical sample was obtained by sublimation at 94 °C (oil bath) (0.005 mm): IR ($CHCl_3$) 1795 (lactone C=O) cm^{-1}; NMR δ ($CDCl_3$) 2.1–2.9 (2 H, m, –OCH_2CH_2–), 4.3–4.6 (2 H, m, –OCH_2CH_2–), 6.6–7.3 (3 H, m, Ar–H), with benzylic *gem*-CH_2 exhibiting δ_A 3.58, δ_B 3.22, and J_{AB} = –16.2 Hz. Anal. ($C_{11}H_9O_3Cl$) C, H, Cl.

B. Biological Methods. Determination of Antilipidemic Activity. (1) Triton WR-1339 Induced Hyperlipidemic Rat Model.[3] Methods utilized were identical with those previously reported.[11] Serum triglycerides were determined by the method of Eggstein,[28] and serum cholesterol was analyzed by the method of Holub and Galli.[29]

(2) Sucrose Rat Model.[7] Methods utilized were identical with those previously reported.[12] Serum triglycerides were determined by the method of Eggstein,[28] and serum cholesterol was determined by the method of Allain et al.[30]

(3) Normolipemic Rat Model. Methods utilized were identical with those previously reported.[4]

Preparation of Microsomes. Animals from the sucrose-fed and normolipemic rat model were anesthetized (Et_2O) to permit removal of blood from the abdominal aorta. Livers were excised immediately thereafter. Homogenization of livers and preparation of microsomes were carried out as described previously.[4,11]

HMG-CoA Reductase Assay. The assay for mevalonic acid formation from DL-3-hydroxy-3-methylglutaryl-3-^{14}C-CoA was carried out by procedures identical with those described previously.[11]

Ethylmorphine *N*-Demethylase Assay. The assay of formaldehyde liberated from ethylmorphine was carried out by procedures described previously.[31] Incubation mixtures contained 5 mg of microsomal protein, 10 μmol of ethylmorphine, a NADPH generating system,[31] and 60 μmol of Tris (pH 7.4) in a final volume of 0.3 mL. Reactions were terminated after 10 min of incubation at 37 °C.

NADPH–Cytochrome *c* Reductase Assay. The assay of cytochrome *c* reduction in liver microsomes was carried out by the method of Phillips and Langdon.[32]

Methods of Analysis in Liver. Hepatic microsomal cytochrome P-450 and cytochrome b_5 were estimated by the procedure of Kinoshita and Horie.[33] Microsomal protein was assayed by the method of Lowry et al.[34] Cholesterol and triglycerides were extracted from the liver by the method of Abell et al.[35] Cholesterol levels were measured by the method of Parekh and Jung.[36] Triglyceride content was determined by the method of Soloni.[37] Student's t test was used to make comparisons between means of treatment groups.

Hydrolysis of Spirolactone 4 by Rat Serum Esterase in Vitro.[2] Blood obtained from the abdominal aorta of anesthetized (Et_2O) male Sprague–Dawley rats (300–350 g) was collected into plastic tubes containing a few crystals of EDTA. Once clotted the blood was immediately centrifuged (15–20 min) and the supernatant serum was separated and pooled. A 0.05 M solution of spirolactone 4 in EtOH (0.1 mL) was added to 6.0 mL of rat serum and the mixture was incubated in 25-mL Erlenmeyer flasks at 37 °C on a Dubnoff metabolic shaker. Samples (1.0 mL) of serum were removed at 0-, 5-, 10-, 30-, and 60-min intervals and assayed for the presence of spirolactone 4 as follows. The serum samples were added to a tube containing 1.0 mL of saturated NaCl solution and 5.0 mL of an isooctane–absolute EtOH mixture (95:5 v/v) and immediately shaken for 30 s. The disappearance of spirolactone 4 in the organic layer was monitored at 250 nm. Nonenzymatic ester hydrolysis was conducted in an identical manner except that 6.0 mL of Ringer bicarbonate buffer (pH 7.4) was utilized in place of serum. In the absence of serum and buffer at any time, and in all systems at zero time, essentially 100% of **2** and **4** could be found in the organic layer.

References and Notes

(1) The authors gratefully acknowledge support for this work through U.S. Public Health Service Grant HL 12740 from the National Heart, Lung and Blood Institute.
(2) D. T. Witiak, G. K. Poochikian, D. R. Feller, N. A. Kenfield, and H. A. I. Newman, *J. Med. Chem.*, **18**, 992 (1975).
(3) P. E. Schurr, J. R. Schultz, and T. M. Parkinson, *Lipids*, **7**, 68 (1972).
(4) A. P. Goldberg, W. S. Mellon, D. T. Witiak, and D. R. Feller, *Atherosclerosis*, **27**, 15 (1977).
(5) J. M. Thorp, *Lancet*, 1, 1323 (1962).
(6) H. A. I. Newman, W. P. Heilman, and D. T. Witiak, *Lipids*, **8**, 378 (1973).

(7) E. L. Tolman, H. M. Tepperman, and J. Tepperman, *Am. J. Physiol.*, **218**, 1313 (1970).
(8) M. S. Schotz, A. Scanu, and I. H. Page, *Am. J. Physiol.*, 188, 399 (1957).
(9) E. L. Bierman and D. Porte, Jr., *Ann. Intern. Med.*, 68, 926 (1968).
(10) J. E. M. Groener and L. M. G. van Golde, *Biochim. Biophys. Acta*, 487, 105 (1977).
(11) D. T. Witiak, E. Kuwano, D. R. Feller, J. R. Baldwin, H. A. I. Newman, and S. K. Sankarapa, *J. Med. Chem.*, **19**, 1214 (1976).
(12) D. T. Witiak, H. A. I. Newman, G. K. Poochikian, S. W. Fogt, J. R. Baldwin, C. L. Sober, and D. R. Feller, *J. Med. Chem.*, **21**, 833 (1978).
(13) R. C. Cavestri and D. T. Witiak, presented to the Division of Medicinal Chemistry, 8th Central Regional Meeting of the American Chemical Society, Akron, Ohio, May 21, 1976.
(14) J. R. Dyer in "Application of Absorption Spectroscopy of Organic Compounds", Prentice-Hall Foundations of Modern Organic Chemistry Series, K. L. Rinehart, Jr., Ed., Prentice-Hall, Englewood Cliffs, N.J., 1965, p 34.
(15) N. M. Yoom, C. S. Pak, H. C. Brown, S. K. Krishnamurthy, and T. P. Stocky, *J. Org. Chem.*, 38, 2786 (1973).
(16) E. W. Collington and A. I. Meyers, *J. Org. Chem.*, **36**, 3044 (1971).
(17) D. T. Witiak, W. P. Heilman, S. K. Sankarappa, R. C. Cavestri, and H. A. I. Newman, *J. Med. Chem.*, 18, 934 (1975).
(18) H. A. I. Newman, D. R. Feller, and D. T. Witiak, unpublished results.
(19) L. W. White, *Biochem. Pharmacol.*, **178**, 361 (1971).
(20) P. Segal, R. S. Roheim, and H. A. Eder, *J. Clin. Invest.*, **51**, 1632 (1972).
(21) K. W. Walton, P. J. Scott, J. V. Jones, R. F. Fletcher, and T. Whitehead, *J. Atheroscler. Res.*, **3**, 396 (1963).
(22) P. J. Scott and P. J. Hurley, *J. Atheroscler. Res.*, **9**, 25 (1969).
(23) N. J. Lewis, D. R. Feller, G. K. Poochikian, and D. T. Witiak, *J. Med. Chem.*, 17, 41 (1974).
(24) D. L. Azarnoff, J. Reddy, C. Hignite, and T. Fitzgerald, *Proc. Int. Congr. Pharmacol., 6th, 1975*, 4, 137–147 (1976).
(25) L. F. Fieser and M. Fieser, "Reagents for Organic Synthesis", Vol. 1, Wiley, New York, N.Y., 1968, p 255.
(26) J. S. McIntyre, *Can. J. Chem.*, **45**, 767 (1967).
(27) The silicic acid used for adsorption column chromatography was prepared by the method of V. Suchy (personal communication) from Bratislava, Czechoslovakia. One pound of commercial silicic acid (100 mesh) was suspended in 4 L of H_2O. After standing for 1 h the suspended fines were removed by decanting. The settled solids were resuspended in 4 L of H_2O by stirring (in such a way as to avoid a vortex) and allowed to settle for 1 min. The suspended material in the upper one-third volume of water was siphoned into a second beaker. This was repeated (six to eight times) until the upper one-third volume was essentially free of solids, affording grade I. The contents of the second beaker were resuspended and allowed to settle for 2 min (vide supra), affording grade II. The method may be repeated four additional times. With settling times of 4, 8, 16, and 32 min, grades III–VI are obtained, respectively. Grades I–III are useful for crude separations, whereas grades IV and V are most suited for high-resolution chromatography. Grade VI is useful for thin-layer chromatography. All six grades were filtered, air-dried, and activated at 110–130 °C for 12–24 h. The silicic acid can be deactivated by empirically adding small percentages (up to 10% w/w) of H_2O.
(28) M. Eggstein, *Klin. Wochenschr.*, 44, 267 (1966).
(29) W. R. Holub and F. A. Galli, *Clin. Chem. (Winston-Salem, N.C.)*, 18, 239 (1972).
(30) C. C. Allain, L. Poon, S. G. Chan, W. Richmond, and P. Fu, *Clin. Chem. (Winston-Salem, N.C.)*, **20**, 470 (1974).
(31) N. J. Lewis, D. T. Witiak, and D. R. Feller, *Proc. Soc. Exp. Biol. Med.*, **145**, 281 (1974).
(32) A. H. Phillips and R. G. Langdon, *J. Biol. Chem.*, **237**, 2652 (1963).
(33) T. Kinoshita and S. Horie, *J. Biochem. (Tokyo)*, 61, 26 (1967).
(34) O. Lowry, N. Rosenbrough, A. Farr, and R. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
(35) L. L. Abell, B. B. Levy, B. B. Brodie, and F. E. Kendall, *J. Biol. Chem.*, **195**, 357 (1952).
(36) A. C. Parekh and D. H. Jung, *Anal. Chem.*, **42**, 1423 (1970).
(37) F. G. Soloni, *Clin. Chem., (Winston-Salem, N.C.)*, **17**, 529 (1971).

Inhibition of Four Human Serine Proteases by Substituted Benzamidines

Judith M. Andrews,* Daniel P. Roman, Jr., David H. Bing,

Center for Blood Research, Boston, Massachusetts 02115

and Michael Cory

Wellcome Research Laboratory, Research Triangle Park, North Carolina 27709. *Received May 11, 1978*

A series of substituted benzamidines has been examined for their inhibitory activity against the human serine proteases—trypsin, thrombin, plasmin, and C1s, a subunit of the first component of complement. The inhibition constants obtained for each enzyme were correlated with physical–chemical properties of the substituent group using the quantitative structure–activity relationship approach. This analysis indicated that plasmin and C1s are very similar in their interactions with substituted benzamidines. The binding of benzamidines in both enzymes was affected by electron donation from the substituent and its hydrophobicity. Thrombin–benzamidine interaction was affected only by the hydrophobicity of the substituent. Trypsin displayed a complex interaction with substituted benzamidines, and interaction was dependent on molar refractivity and molecular weight. Certain substituents deviated significantly from the interactions predicted by the analysis. These compounds, the (*m*- and *p*-amidinophenyl)pyruvic acids, when analyzed by computer modeling, suggested that direct interaction between the substituent and the enzyme surface is important in assessing the effect of substituent groups on inhibitory activity.

A central feature of blood coagulation, complement activation, fibrinolysis, and digestion is the activation of serine proteases[1] which specifically hydrolyze protein substrates. Although the mechanism of proteolysis by these enzymes has been extensively investigated,[2-4] the structural basis for substrate-binding specificity is poorly understood. We have undertaken studies to delineate the chemical basis of the substrate-binding specificity of four human serine proteases—trypsin (E.C. 3.4.4.4), thrombin (E.C. 3.4.4.13), plasmin (E.C. 3.4.4.14), and the complement enzyme C1s.[5] Three of the four enzymes, trypsin, thrombin, and plasmin, bind to protein substrates at lysine

0022-2623/78/1821-1202$01.00/0