

Figure 6. Kinetics of EGF receptor kinase autophosphorylation. The kinetic results at two temperatures are depicted. Experimental details are given in the text.

0.05% TEMED, and 0.46% ammonium persulfate). The gel was dried and autoradiography was performed with Agfa Curix RP2 X-ray film. The relevant radioactive bands were cut and counted in the Cerenkov mode. The fast phase of autophosphorylation continued for another 10 min (data not shown). The extent of phosphorylation completed in the first 10 s at 15 °C comprises $\frac{1}{3}$ of the total autophosphorylation signal and probably reflects the phosphorylation of the first site on the receptor (Figure 6). The 10-s interval was therefore chosen for use in subsequent autophosphorylation experiments.

ATP and EGF Dependence of Autophosphorylation. WGA-purified EGF receptor from A431 cells (0.5 $\mu\text{g}/\text{assay}$) was activated with EGF (0.85 μM) for 20 min at 4 °C. The assay was performed at 15 °C and initiated by addition of $\text{Mg}(\text{Ac})_2$ (60 mM), Tris-Mes buffer, pH 7.6 (50 mM), [^{32}P]ATP (carrier free, 5 $\mu\text{Ci}/\text{assay}$), and increasing concentrations of nonradioactive ATP. The assay was terminated after 10 s by addition of SDS sample buffer (see above). The samples were run on a 6% SDS polyacrylamide gel (see above). The gel was dried and autoradiographed as described above (Figure 6). The relevant radioactive bands were cut and counted in the Cerenkov mode. The K_m for ATP determined in this fashion was found to be 7.2 μM ⁵¹ (data

not shown). With use of the 10-s assay protocol, the EGF concentration dependence of EGFRK autophosphorylation was determined and found to be similar to that shown in Figure 5 (data not shown).⁵¹

Inhibition of EGFR Autophosphorylation. WGA-purified EGF receptor from A431 cells (0.5 $\mu\text{g}/\text{assay}$) was activated with EGF (0.85 μM) for 20 min at 4 °C. The assay was performed at 15 °C and initiated by addition of $\text{Mg}(\text{Ac})_2$ (60 mM), Tris-Mes buffer, pH 7.6 (50 mM), and [^{32}P]ATP (10 μM , 5 $\mu\text{Ci}/\text{assay}$) and various inhibitors at increasing concentrations. The assay was terminated after 10 s by the addition of SDS-PAGE sample buffer.

Inhibition of Copoly(Glu₄Tyr) Phosphorylation by Insulin-Receptor Kinase (InsRK). Rat liver membranes were prepared from the livers of 6-week-old rats as described by Cuatrecasas.⁵² WGA-purified insulin receptor was prepared according to Zick et al.⁵³ WGA-purified rat liver InsRK (1.25 μg) was preincubated with or without 330 nM insulin in 50 mM Tris-Mes buffer, pH 7.6, for 30 min at 22 °C. The assay was performed at 22 °C and initiated by addition of a mixture which contained $\text{Mg}(\text{Ac})_2$ (60 mM), NaVO_3 (40 μM), [^{32}P]ATP (125 μM , 3–5 $\mu\text{Ci}/\text{assay}$), and poly(GT) [poly(Glu₄Tyr)] at three concentrations: whenever an inhibitor was tested, it was added at the proper concentration as indicated. The concentrations referred to are the final concentration in the assay. The final concentration of insulin in the assay was 125 nM. The total volume of the assay was 40 μL . After 20 min, aliquots of 30 μL were applied on Whatman 3-mm paper and were soaked in cold 10% TCA, containing 0.01 M sodium pyrophosphate. After being washed overnight, the papers were dried and counted, measuring Cerenkov radiation. The InsRK-catalyzed phosphorylation of poly(GT) obeys Michaelis-Menten kinetics¹⁴ and the inhibitors are purely competitive as was observed from the well-behaved Dixon³⁹ plots¹⁴ (data not shown).

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(53) Zick, Y.; Kasuga, M.; Kahn, R.; Roth, J. *J. Biol. Chem.* **1983**, *258*, 75.

Synthesis and Investigation of Effects of 2-[4-[[[(Arylamino)carbonyl]amino]phenoxy]-2-methylpropionic Acids on the Affinity of Hemoglobin for Oxygen: Structure-Activity Relationships

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A series of 2-[4-[[[(substituted-phenyl)amino]carbonyl]amino]phenoxy]-2-methylpropionic acids and other substituted phenoxyacetic acids were synthesized and tested for their ability to reduce the affinity of hemoglobin for oxygen. 2-[4-[[[(3,4,5-trichlorophenyl)amino]carbonyl]amino]phenoxy]-2-methylpropionic acid was found to be the most potent compound known. Structure-activity relationships of the compounds synthesized are discussed.

Perutz and Poyart in 1983 reported that the antihyperlipidemic agent bezafibrate lowered the affinity of hemoglobin for oxygen.¹ We have reported that 2-[4-[[[(3,4-dichlorophenyl)amino]carbonyl]amino]phenoxy]-2-methylpropionic acid (**8g4**, LR-16), a newly synthesized compound related to clofibrate, has an even higher affinity for hemoglobin and causes a larger shift of the hemoglobin-oxygen-dissociation curve to the right.² X-ray

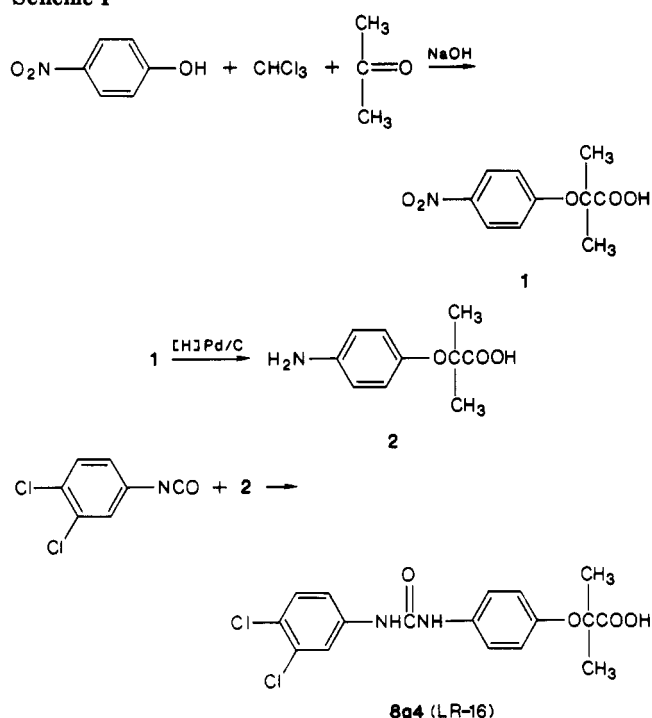
crystallography demonstrated that the site in the central cavity of hemoglobin occupied by **8g4** was the same as that occupied by bezafibrate and adjacent to that of the natural allosteric agent 2,3-diphosphoglycerate.²

In the present paper we report the synthesis of a series of 2-[4-[[[(arylamino)carbonyl]amino]phenoxy]-2-methylpropionic acids and their structure-allosteric activity re-

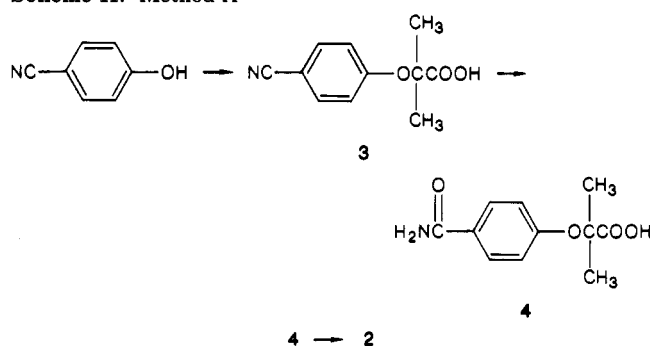
(1) Perutz, M. F.; Poyart, C. *Lancet* **1983**, 881-882.

(2) Lalezari, I.; Rahbar, S.; Lalezari, P.; Fermi, G.; Perutz, M. F. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6117-6121.

Scheme I



Scheme II. Method A



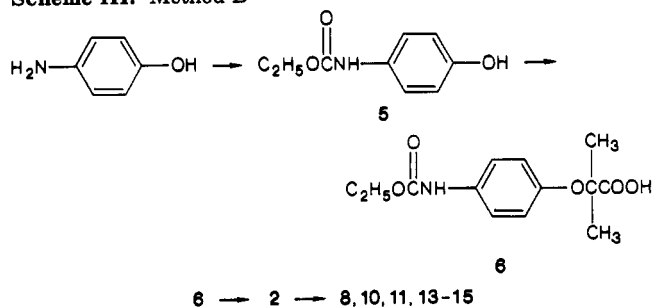
relationships. This study has led to identification of new derivatives with exceptionally powerful effects in reducing the affinity of hemoglobin for oxygen.

Chemistry

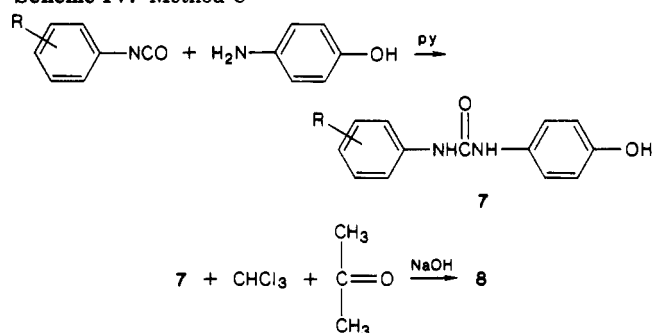
LR-16 (8g4) was prepared by a multistep reaction starting from 4-nitrophenol² with an overall yield of 25–28% (see Scheme I). In view of the biological importance of this new class of compounds and the need for larger quantities and evaluation of the role of different substituents, we have found the three independent synthetic methods outlined in Schemes II–IV. In these simplified methods (A–C), the yields were ranged between 55 and 75%. In method A, 4-cyanophenol was converted to 2-(4-cyanophenoxy)-2-methylpropionic acid (3). Base-catalyzed hydrolysis of 3 in the presence of H₂O₂ gave a high yield of 2-(4-carbamoylphenoxy)-2-methylpropionic acid (4). Compound 4, upon treatment with sodium hypobromite, gave 2-(4-aminophenoxy)-2-methylpropionic acid (2). The amino acid 2 was reacted with an appropriate aryl isocyanate to give compounds 8.

In method B, 4-aminophenol was converted to 4-[(ethoxycarbonyl)amino]phenol (5). The latter was reacted with acetone–chloroform in the presence of solid NaOH to give 2-[4-[(ethoxycarbonyl)amino]phenoxy]-2-methylpropionic acid (6). Base hydrolysis of 6 gave 2 in high yield. In method C, an aryl isocyanate was reacted with 4-aminophenol to give 4-[[arylamino]carbonyl]amino]phenol (7).

Scheme III. Method B



Scheme IV. Method C



Phenols 7 were easily converted to asymmetrical ureas 8 reported in Table I. In addition to these compounds 8, the series reported in Table II were also synthesized and tested. Compound 9 was prepared starting from 4-phenylphenol through the chloroform–acetone–sodium hydroxide reaction. Compounds 10a, 10b, 14, and 15 were obtained by acylation of 2. Compounds 11 and 13 were obtained by reacting 2 with *S*-methyl-(4-chlorophenyl)-thiourea and α -toluenesulfonyl chloride, respectively. Compounds 16 were prepared according to the literature³ (see Chart I).

Biological Activities

The effects on the affinity of hemoglobin for oxygen were evaluated by determination of oxygen-dissociation curves in the presence and absence of the test compounds, using a Hemox analyzer (ISC Medical Products, Huntington Valley, PA). In this test, P_{50} represents the partial pressure of oxygen (P_{O_2} in mmHg) at which 50% of the oxyhemoglobin is deoxygenated. Compounds which reduce the affinity of hemoglobin for oxygen shift the dissociation curve to the right and increase the P_{50} .

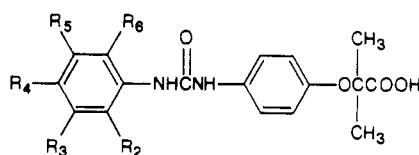
The synthesized compounds were initially screened in duplicates and at 1 mM concentration against both the intact red cells and membrane-free hemoglobin solution. Compounds which produced P_{50} values greater than twice the control were retested at lower concentrations. Since the more potent compounds at 1 mM concentration interfered with full oxygenation of hemoglobin, even at 200 mmHg P_{O_2} , the P_{50} values were considered accurate only in the lower concentrations at which hemoglobin oxygenation was complete.

The mean P_{50} values obtained in the presence of various 2-[4-[[arylamino]carbonyl]amino]phenoxy]-2-methylpropionic acids, are summarized in Table I. Compounds structurally different in the urea moiety, synthesized, and similarly tested are listed in Table II.

Asymmetrical ureas 8 varied in their effectiveness in modifying the affinity of hemoglobin for oxygen (Table I).

(3) Witte, E. C. U.S. Patent 3,781,328, 1973.

(4) Howard, C. *Ber. Dtsch. Chem. Ges.* **1897**, *30*, 545.

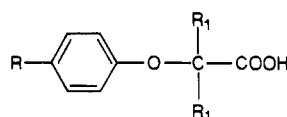
Table I. 2-[4-[[[(Substituted-phenyl)amino]carbonyl]amino]phenoxy]-2-methylpropionic Acids; Effects on the Affinity of Hemoglobin for Oxygen

compd	R ₂	R ₃	R ₄	R ₅	R ₆	yield	mp, °C	formula ^a	concn, mM	hemoglobin		whole blood ^d	
										P ₅₀ ^b	P ₅₀ /P _{50c} ^c	P ₅₀	P ₅₀ /P _{50c} ^c
8a	H	H	H	H	H	94	195–196	C ₁₇ H ₁₈ N ₂ O ₄	1.0	10.0	1.42	10.0	1.05
8b1	H	H	CH ₃	H	H	96	191–193	C ₁₈ H ₂₀ N ₂ O ₄	1.0	26.0	2.88	15.5	1.72
8b2	H	CH ₃	CH ₃	H	H	80	153–155	C ₁₉ H ₂₂ N ₂ O ₄	1.0	27.25	4.05	18.5	2.05
									0.5	16.50	2.53	14.5	1.61
8b3	CH ₃	H	CH ₃	H	CH ₃	58	133–135	C ₂₀ H ₂₄ N ₂ O ₄	1.0	6.5	1.0	12.0	1.26
8c1	H	H	CH ₃ O	H	H	87	156–158	C ₁₈ H ₂₀ N ₂ O ₅	1.0	19.0	2.11	12.5	1.25
8c2	H	CH ₃ O	CH ₃ O	H	H	92	111–112	C ₁₉ H ₂₂ N ₂ O ₆	1.0	14.5	2.07	8.5	1.0
8c3	H	CH ₃ O	CH ₃ O	CH ₃ O	H	80	96–98	C ₂₀ H ₂₄ N ₂ O ₇	1.0	11.5	1.64	9.5	1.05
8d	H	-OCH ₂ O-		H	H	85	165–166	C ₁₈ H ₁₈ N ₂ O ₆	1.0	14.0	2.15	9.5	1.05
8e1	H	H	F	H	H	75	200–202	C ₁₇ H ₁₈ FN ₂ O ₄	1.0	18.0	2.0	12.5	1.38
8e2	H	F	H	F	H	92	181–183	C ₁₇ H ₁₆ F ₂ N ₂ O ₄	1.0	15.25	1.99	21.0	2.21
									0.5	12.25	1.60	15.0	1.57
8f1	Cl	H	H	H	H	86	196–198	C ₁₇ H ₁₇ ClN ₂ O ₄	1.0	14.16	1.75	16.2	1.42
8f2	H	Cl	H	H	H	80	156–158	C ₁₇ H ₁₇ ClN ₂ O ₄	1.0	22.0	2.89	17.75	1.5
8f3	H	H	Cl	H	H	86	222–223	C ₁₇ H ₁₇ ClN ₂ O ₄	1.0	21.83	2.70	19.5	1.64
8g1	Cl	Cl	H	H	H	78	188–189	C ₁₇ H ₁₆ Cl ₂ N ₂ O ₄	0.5	16.0	2.46	30.0	3.3
									0.2	12.5	1.68	18.5	2.02
8g2	Cl	H	Cl	H	H	88	189–190	C ₁₇ H ₁₆ Cl ₂ N ₂ O ₄	0.5	15.5	2.38	21.25	2.04
									0.2	9.75	1.50	14.75	1.42
8g3	Cl	H	H	Cl	H	73	181–183	C ₁₇ H ₁₆ Cl ₂ N ₂ O ₄	0.5	18.0	2.77	23.75	2.56
									0.2	11.5	1.77	15.83	1.70
8g4	H	Cl	Cl	H	H	80	184–185	C ₁₇ H ₁₆ Cl ₂ N ₂ O ₄	0.5	28.5	4.38	23.5	2.29
									0.2	14.25	2.19	15.5	1.5
8g5	H	Cl	H	Cl	H	88	182–183	C ₁₇ H ₁₆ Cl ₂ N ₂ O ₄	0.5	44.5	5.23	51.0	4.43
									0.2	20.5	2.75	22.75	2.19
8h1	Cl	H	Cl	Cl	H	96	216–218	C ₁₇ H ₁₅ Cl ₃ N ₂ O ₄	0.2	16.75	2.3	31.0	3.87
									0.1	12.0	1.62	17.75	2.0
8h2 ^e	H	Cl	Cl	Cl	H	90	233–235	C ₁₇ H ₁₅ Cl ₃ N ₂ O ₄	0.05	9.0	1.22	14.25	1.61
									0.1	28.5	3.94	35.0	4.37
									0.05	16.25	2.19	27.5	3.43
8i	H	H	NO ₂	H	H	78	201–203	C ₁₇ H ₁₇ N ₃ O ₆	1.0	19.0	2.92	26.5	2.79
									0.5	13.0	2.0	21.0	2.21
8j	Cl	H	H	NO ₂	H	65	214–216	C ₁₇ H ₁₆ ClN ₃ O ₆	1.0	23.5	3.61	40.0	4.21
8k	H	NO ₂	Cl	H	H	79	182–183	C ₁₇ H ₁₆ ClN ₃ O ₆	1.0	43.5	5.43	52.5	5.52
									0.5	30.0	3.75	40.0	4.21
8l1	OH	H	H	H	H	72	193–194	C ₁₇ H ₁₈ N ₂ O ₅	1.0	12.5	1.91	10.0	1.42
8l2	H	H	OH	H	H	70	173–174	C ₁₇ H ₁₈ N ₂ O ₅	1.0	11.0	1.68	8.5	1.21
8m	H	H	CH ₂ COO	H	H	98	155–156	C ₁₉ H ₂₀ N ₂ O ₆	1.0	11.5	1.64	16.0	1.77
8n ^f	H	H	OHC	H	H	89	189–190	C ₁₈ H ₁₈ N ₂ O ₅	1.0	15.0	2.30	20.5	2.15
8o	H	H	HOOC	H	H	88	>300	C ₁₈ H ₁₈ N ₂ O ₆	1.0	8.0	1.14	9.0	1.0
8p	H	H	C ₂ H ₅ OOCNH	H	H	48	191–193	C ₂₀ H ₂₃ N ₃ O ₆	1.0	10.5	1.61	11.5	1.27
16c (bezafibrate)									1.0	14.25	1.84	17.0	1.63
									0.5	9.0	1.38	14.5	1.39
control										7.26 ± 0.88	1.0	9.62 ± 1.53	1.0
										(N = 16)		(N = 8)	

^a All compounds in this table gave satisfactory analysis for C, H, and N (±0.4%). ^b P₅₀ is the partial pressure of O₂ (mmHg) at which 50% of oxyhemoglobin is converted to hemoglobin. ^c The mean of P₅₀ values obtained in the presence of the test compound divided by the mean of the respective P₅₀ of the controls. ^d 30-μL aliquots of whole blood diluted in 5 mL of HEPES buffer containing the test compounds. The final concentration of hemoglobin was 12.5 ± 0.5 μM when either whole blood or hemoglobin solution was used. The molar ratio of the compounds (at 1 mM) to hemoglobin was 80 ± 3:1. ^e Intermediary compound 3,4,5-trichlorophenyl isocyanate was prepared by dropwise addition of a solution of 3,4,5-trichloroaniline (1.965 g, 10 mmol) in ethyl acetate (15 mL) to a 20% solution of phosgene in toluene (30 mL). After 1/2 h of stirring at room temperature, the reaction mixture was refluxed for 4 h. Following evaporation of the solvents and the excess phosgene under reduced pressure, a solid was obtained which was recrystallized from hexane, mp 54–55 °C (81%). Anal (C₇H₃Cl₃NO) C, H, N. ^f Similar to 3,4,5-trichlorophenyl isocyanate, the intermediary 4-formylphenyl isocyanate was prepared using 4-aminobenzaldehyde, bp 123–125 °C/16 mm (yield 72%). Anal (C₈H₅NO₂) C, H, N.

In general, these compounds were more effective when tested with purified hemoglobin as compared to the intact red cells. Several factors may be responsible for these differences. These include incomplete permeability across the red-cell membrane, the presence of contaminating serum proteins in the aliquots of the whole blood used to provide intact red cells, and possible reaction of the test compounds with intracellular structures other than hemoglobin. Exceptions to these observations were higher

effects of compounds 8h1, 8h2, 8j, and 8k on intact red cells. Unsubstituted 8 had slight activity when tested with purified hemoglobin but none in intact red cells. Substitution of hydrogen in the benzene ring had a great impact on the potency of the compounds: 4-methyl and 3,4-dimethyl substitutions increased the potency, but effectiveness was markedly suppressed in the trimethyl derivative with the methyl groups in the 2-, 4-, and 6-positions. Similar reduction in relative potency of the

Table II. Substituted Phenoxyacetic Acids; Effects of the Affinity of Hemoglobin for Oxygen

compd	R	R ₁	concn, mM	hemoglobin		whole blood	
				P ₅₀	P ₅₀ /P _{50c}	P ₅₀	P ₅₀ /P _{50c}
9	C ₆ H ₅	CH ₃	1.0	7.0	1.0	15.5	1.63
10a	4-ClC ₆ H ₄ CONH	CH ₃	1.0	39.0	6.0	37.5	5.35
			0.5	27.0	4.15	19.0	2.71
10b	3,5-Cl ₂ C ₆ H ₃ COHN	CH ₃	1.0	16.0	2.34	25.33	2.96
			0.5	12.16	1.78	19.66	2.30
11	4-ClC ₆ H ₄ NH(C=NH)NH	CH ₃	1.0	9.0	1.14	16.0	1.39
12	3,4-Cl ₂ C ₆ H ₃ NHCONH	H	0.5	10.0	1.42	13.0	1.13
			0.2	8.0	1.14	11.5	1.0
13	C ₆ H ₅ CH ₂ SO ₂ NH	CH ₃	1.0	9.16	1.34	11.5	1.64
14	4-ClC ₆ H ₄ OCH ₂ CONH	CH ₃	1.0	9.25	1.27	17.5	1.84
15	4-ClC ₆ H ₄ CONHCH ₂ CONH	CH ₃	1.0	8.5	1.14	12.0	1.26
16a	3,5-Cl ₂ C ₆ H ₃ CONH(CH ₂) ₂	CH ₃	1.0	12.5	1.78	22.75	2.33
			0.5	10.0	1.42	17.75	
16b	4-ClC ₆ H ₄ CONHCH(COOH)CH ₂	CH ₃	1.0	7.5	1.07	12.0	1.26
16c (bezafibrate)			1.0	14.25	1.84	17.0	1.63
			0.5	9.0	1.38	14.5	1.39
control				7.26 ± 0.88 (N = 16)	1.0	9.62 ± 1.53 (N = 8)	1.0

^a All compounds in this table gave satisfactory analysis for C, H, and N (±0.4%). ^b P₅₀ is the partial pressure of O₂ (mmHg) at which 50% of oxyhemoglobin is converted to hemoglobin. ^c The mean P₅₀ values obtained in the presence of the test compound by the mean of the respective P₅₀ of the controls. ^d 30-μL aliquots of whole blood diluted in 5 mL of HEPES buffer containing the test compounds. The final concentration of hemoglobin was 12.5 ± 0.5 μM when either whole blood or hemoglobin solution was used. The molar ratio of the compounds (at 1 mM) to hemoglobin was 80 ± 3:1.

trimethoxy (8c3) compound was noticed as compared to those of the mono- and dimethoxy derivatives (8c1, 8c2). Of interest was the observation that the presence of oxygen-containing groups such as methoxy, methylenedioxy, hydroxy, and acetoxy reduced or abolished the activity, particularly when the intact red cells were used. Whereas an aldehyde moiety was associated with a moderate potency, substitution by a carboxylic group totally eliminated the activity. Halogens, specially chlorine, and a nitro group or their combination increased the activity. Dichloro and trichloro compounds were very active. The position of the chlorine atoms played an important role. In the monochloro series, the order of potency in three isomers was 3 > 4 > 2. In the dichloro series, the 3,5-dichloro derivative was the most active and the 2,4 derivative had the least potency. The 3,4,5-trichloro compound was more potent than the 2,4,5 derivative and so far it is the most powerful compound synthesized. High polarity, electron-distribution property, and partial double bond character of the urea moiety, which confers a certain degree of rigidity to the molecule, seemed to be important in the activity of the molecules, even though there is partial free rotation about the N-Ar bonds in these structures. Representative compounds lacking a urea moiety were found to be either inactive or had relatively reduced potency (compounds 9, 13–16). Interestingly 11, the guanidine analogue of 8, and compound 16b, which contains a carboxylic group, were basically inactive. We speculate that in these compounds the presence of positively or negatively charged moieties in the middle of the molecule interferes with access to the central cavity of hemoglobin. Compounds with a longer distance between the two benzene rings (14–16), including bezafibrate, also appear to be less potent. Compound 16a, an analogue of 8g5 was found to be only 1/5 as active as the latter. An unexpected and thus far unexplained finding was the difference between the high and the moderate activities observed for compounds 10a and 10b. It is also of interest that replacement of the terminal isobutyric moiety by an acetic acid group significantly reduced the potency as seen in compound 12. It should be

added that 2-(4-aminophenoxy)-2-methylpropionic acid (2), used in the synthesis of compounds 8, 10a, 10b, 11, and 13–15, was found to lack any allosteric effect.

Experimental Section

Melting points (uncorrected) were measured using a Fisher-Johns apparatus. NMR spectra were determined with a Varian M-360 spectrophotometer using TMS as internal standard. Analysis of the compounds for C, H, and N were within ±0.4% of the calculated values.

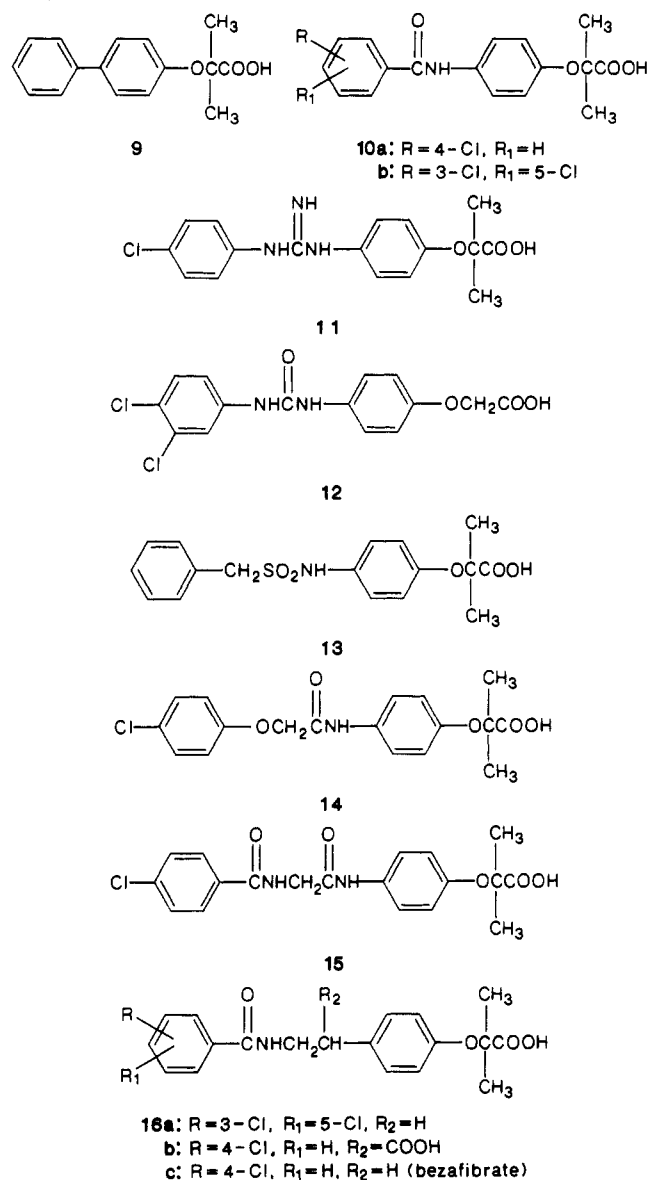
2-(4-Cyanophenoxy)-2-methylpropionic Acid (3). A stirring mixture of 4-cyanophenol (5.95 g, 0.05 mol) and sodium hydroxide (10 g, 0.25 mol) in dry acetone (44 mL, 0.6 mol) was heated to reflux. Chloroform (4 mL, 0.05 mol) was then added dropwise and the mixture was stirred over a 20-min period. After 4 h of refluxing, the reaction mixture was concentrated under reduced pressure. The solid residue was dissolved in water (100 mL), charcoaled, and filtered. The solution was acidified (HCl). The precipitate was recrystallized from alcohol to give 3 (8g, 78%) as a white crystalline powder, mp 118–120 °C. Anal. (C₁₁H₁₁NO₃) C, H, N.

2-(4-Carbamoylphenoxy)-2-methylpropionic Acid (4). The cyano compound 3 (11.3 g, 0.055 mol) was added to a solution of 3% hydrogen peroxide (280 mL) containing KOH (8 g) dissolved in water (32 mL). The mixture was stirred until the exothermic reaction and evolution of gas ceased. After cooling, the solution was acidified (HCl) to give 4 (10.8 g, 88%). It was recrystallized from ethanol to give 10.3 g of small white crystals, mp 202–204 °C. Anal. (C₁₁H₁₃NO₄) C, H, N.

Method A: 2-(4-Aminophenoxy)-2-methylpropionic Acid (2). The amide 4 (11.15 g, 0.05 mol) was gradually added to 180 mL of a stirred, ice-cold, aqueous solution of bromine (8.8 g, 0.055 g-atom) and NaOH (22 g, 0.55 mol). The solution obtained was warmed to above 75 °C and kept at this temperature for 1/2 h. After cooling, it was acidified with acetic acid, giving an almost colorless, crystalline 2 (8.84 g, 90%) mp 214–216 °C dec. The compound was identical (TLC, mp) with a sample prepared by an independent method.²

Method B: 2-[4-[(Ethoxycarbonyl)amino]phenoxy]-2-methylpropionic Acid (6). Ethyl chloroformate (10 mL, 0.1 mol) was dropwise added to a stirring solution of 4-aminophenol (10.9 g, 0.1 mol) in 40 mL of 10% NaOH. The reaction mixture was heated at about 80 °C for 1/2 h and then cooled. Acidification (HCl) gave a solid (16.65 g, 92%). After recrystallization from

Chart I



aqueous 2-propanol, **5** was obtained as small plates, mp 118–120 °C. Anal. (C₉H₁₁NO₃) C, H, N. To a refluxing mixture of **5** (6.335 g, 0.035 mol) and NaOH (8 g, 0.2 mol) in dry acetone (30 mL, 0.4 mol) was added dropwise chloroform (4 mL, 0.03 mol) and the mixture was refluxed for 4 h as described for **3**. The oily product rapidly solidified. Recrystallization from ethanol gave white crystals (4.5 g, 48%): mp 82–83 °C; NMR (CDCl₃) δ 1.25 (t, 3 H, CH₃), 1.54 (s, 6 H, 2 CH₃), 4.21 (q, 2 H, CH₂), 7.12 (q, 4 H, aromatics), 7.51 (br, 1 H, NH), 9.60 (s, 1 H, COOH). Anal. (C₁₃H₁₇NO₆) C, H, N.

Compound **6** (13.35 g, 0.05 mol) was boiled in 10% KOH (120 mL) for 1/2 h. After cooling, the solution was acidified with acetic acid to give **2** (8.51 g, 87%) identical with a sample prepared by method A.

2-[4-[[[(3,5-Dichlorophenyl)amino]carbonyl]amino]phenoxy]-2-methylpropionic Acid (8g5). A solution of 3,5-dichlorophenyl isocyanate (4.7 g, 0.025 mol) in THF (25 mL) was gradually added to a stirring solution of **2** (4.875 g, 0.025 mol) in 2 N NaOH (12.5 mL, 0.025 mol) and THF (30 mL) cooled to ice-salt-bath temperature. After 1/2 h, the bath was removed and stirring was continued for an additional hour. Water (50 mL) was added and most of THF was evaporated under reduced pressure. The solution was charcoaled, filtered, and acidified (HCl). The solid was recrystallized from aqueous acetone to give **8g5** as small plates (8.42 g, 88%): mp 182–183 °C dec; NMR (DMSO-*d*₆) δ 1.49 (s, 6 H, 2 CH₃), 6.81–7.22 (m, 7 H, aromatics), 8.16 (s, 1 H, NH), 8.22 (s, 1 H, NH), 9.3 (s, 1 H, COOH). Anal. (C₁₇H₁₆Cl₂N₂O₄) C, H, N.

Method C: 2-[4-[[[(3,4,5-Trimethoxyphenyl)amino]carbonyl]amino]phenoxy]-2-methylpropionic Acid (8c3). 1-(4-Hydroxyphenyl)-3-(3,4,5-trimethoxyphenyl)urea (**7**) was prepared as follows: 3,4,5-trimethoxyphenyl isocyanate (2.09 g, 0.01 mol) was reacted with 4-aminophenol (1.09, 0.01 mol) dissolved in pyridine (10 mL) by stirring for 1/2 h at room temperature. Cold water (80 mL) was added to the reaction mixture, and the pyridine was neutralized with 10% HCl. The solid separated was recrystallized from methanol to give **7** (2.4 g, 75%): mp 203–204 °C; NMR (CF₃COOH) δ 3.65 (s, 3 H, 4-CH₃O), 3.69 (s, 6 H, 2 CH₃O), 6.34 (s, 2 H, aromatics), 6.58–7.03 (q, 4 H, aromatics). Anal. (C₁₆H₁₈N₂O₅) C, H, N.

To a refluxing mixture of the above urea (**7**) (2.1 g, 6.6 mmol) and NaOH (1.32 g, 0.033 mol) in acetone (15 mL) was dropwise added CHCl₃ (0.78 mL, 0.033 mol). After 4 h of refluxing, the mixture was treated as described for **3**. An oil was obtained which rapidly solidified. Recrystallization from aqueous acetone gave **8c3** (2.135 g, 80%) as white crystals: mp 96–98 °C; NMR (CDCl₃) δ 1.55 (s, 6 H, 2 CH₃), 3.61 (s, 3 H, 4-CH₃O), 3.71 (s, 6 H, 2 CH₃O), 6.68 (m, 6 H, aromatics), 6.91 (s, 1 H, NH), 7.18 (s, 1 H, NH), 7.91 (s, 1 H, COOH). Anal. (C₂₀H₂₄N₂O₇) C, H, N.

All other compounds in series **8** were prepared by methods B and C.

2-(4-Phenylphenoxy)-2-methylpropionic Acid (9). This compound was prepared with 4-phenylphenol according to the method described for preparation of compounds **1**, **3**, and **6**, and it was recrystallized from acetone as transparent plates: mp 171–173 °C; yield 53%; NMR (CDCl₃) δ 1.75 (s, 6 H, 2 CH₃), 7.01–7.28 (m, 9 H, aromatics), 9.01 (s, 1 H, COOH). Anal. (C₁₆H₁₆O₃) C, H.

2-[4-(4-Chlorobenzamido)phenoxy]-2-methylpropionic Acid (10a). To a stirring solution of **2** (0.975 g, 0.005 mol) in 2 N NaOH (10 mL) was added dropwise a solution of 4-chlorobenzoyl chloride (0.7 mL, 0.005 mol) in THF (5 mL). Finally, additional 2 N NaOH (5 mL) was added. After 1 h, the solution was acidified (HCl) and the precipitate was recrystallized from 2-propanol to give **10a** (1.51 g, 90%) as plates: mp 179–180 °C; NMR (DMSO-*d*₆) δ 1.65 (s, 6 H, CH₃), 7.10–7.22 (m, 8 H, aromatics), 8.81 (s, 1 H, NH), 9.33 (s, 1 H, COOH). Anal. (C₁₇H₁₆ClNO₄) C, H, N.

Compound 10b was prepared as described for **10a**: yield 78%, mp 206–207 °C; NMR (DMSO-*d*₆) δ 1.61 (s, 6 H, 2 CH₃), 7.10–7.28 (m, 7 H, aromatics), 8.96 (s, 1 H, NH), 9.29 (s, 1 H, COOH). Anal. (C₁₇H₁₅Cl₂NO₄) C, H, N.

Compound 15. With 4-chlorohippuryl chloride, this compound was prepared (yield 66%) as described for **10a**, mp 196–197 °C. Anal. (C₁₈H₁₉ClN₂O₅) C, H, N.

2-[4-[[[(4-Chlorophenyl)amino]imino]methyl]phenoxy]-2-methylpropionic Acid (11). A mixture of *S*-methyl-(4-chlorophenyl)isothiourea (2 g, 0.01 mol) and **2** (1.95 g, 0.01 mol) in 2-propanol (50 mL) containing 2 drops of piperidine was refluxed for 24 h during which time the methyl mercaptane formed was evaporated. At the end, the volume was reduced to one-half. The solid formed by cooling was separated. It was redissolved in 5% NaHCO₃ solution, charcoaled, and reprecipitated by the addition of citric acid. The compound was recrystallized from aqueous 2-propanol to give **11** (2.5 g, 72%): mp 236–238 °C dec; NMR (DMSO-*d*₆) δ 1.57 (s, 6 H, 2 CH₃), 7.11–7.31 (m, 8 H, aromatics), 9.11 (s, 1 H, COOH), 9.33–9.52 (br m, 3 H, 3 NH). Anal. (C₁₇H₁₈ClN₃O₃) C, H, N.

[4-[[[(3,4-Dichlorophenyl)amino]carbonyl]amino]phenoxy]acetic Acid (12). (4-Aminophenoxy)acetic acid⁴ was reacted with 3,4-dichlorophenyl isocyanate as described for the synthesis of compounds **8**: yield 65%; mp 202–204 °C; NMR (DMSO-*d*₆) δ 4.83 (s, 2 H, CH₂), 7.11–7.41 (m, 7 H, aromatics), 9.15 (s, 1 H, COOH), 9.45 (br s, 1 H, NH), 9.66 (br s, 1 H, NH). Anal. (C₁₅H₁₂Cl₂N₂O₄) C, H, N.

2-(4-Benzenesulfonamidophenoxy)-2-methylpropionic Acid (13). α -Toluenesulfonyl chloride (0.953 g, 0.005 mol) was dissolved in THF (10 mL) and added dropwise to a stirring solution of **2** (0.975 g, 0.005 mol) in 1 N NaOH (5 mL) cooled to ice-salt-bath temperature. Additional 1 N NaOH (5 mL) was added and the stirring was continued for 1 h. THF was evaporated, water (20 mL) was added, and the mixture was acidified (HCl). The solid obtained was recrystallized from aqueous acetone, giving **13** (1.08 g, 62%) as small, white crystals mp 161–162

°C. NMR (DMSO- d_6) 1.55 (s, 6 H, 2 CH₃), 4.42 (s, 2 H, CH₂), 6.71–7.22 (q, 4 H, aromatics), 7.32 (s, 5 H, aromatics), 7.69 (s, 1 H, NH), 4.70 (s, 1 H, COOH). Anal. (C₁₅H₁₂Cl₂N₂O₄) C, H, N.

Compounds 16a–c. Bezafibrate (16c), its 3,5-dichloro analogue (16a), and its α -carboxylic derivative (16b) were prepared by using methods described in the literature.³

Oxygen-Dissociation Curves. For determination of the effects of various compounds on the affinity of hemoglobin for oxygen in intact red cells, 30- μ L aliquots of freshly drawn, heparinized, normal whole blood were added to 5 mL of the test compounds and diluted to the desired concentrations in 0.1 M HEPES buffer (pH 7.4). The final concentration of hemoglobin in these red-cell suspensions was $12.5 \pm 0.5 \mu\text{M}$ as measured spectrophotometrically.⁵ The mixtures were equilibrated for 30 min at 37 °C before being placed in the instrument chamber for oxygenation. A new method was devised for preparation of membrane-free hemoglobin: erythrocytes were washed five times in 20 volumes of 0.15 M NaCl solution, and the packed cells free of leukocytes, platelets, and serum proteins were diluted with 5 volumes of distilled water. The hemolysates were then passed sequentially through a double layer of Whatman filter paper #1 and a single layer of Whatman #5, followed by filtration through 1.2, 0.8, 0.45, and 0.22 μm -pore size Millipore filters all placed in a Swinex-47 Millipore filter holder. For the study of the effects of various compounds on hemoglobin in solution, the test compounds were first dissolved in 5-mL aliquots of 0.1 M HEPES buffer (pH 7.4), hemoglobin was added at a final concentration

of $12.5 \pm 0.5 \mu\text{M}$, and then oxygen-dissociation curves were determined after 30-min incubation at 37 °C. In both the intact red-cell suspensions and membrane-free hemoglobin solutions, the molar ratio of hemoglobin to the test compound was $80 \pm 3:1$, when the test compound was at 1 mM.

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(5) Dacie, J. V.; Lewis, S. M. *Practical Haematology*, 6th ed.; Churchill Livingstone: London, 1984; p 22.

The Xanthene-9-spiro-4'-piperidine Nucleus as a Probe for Opiate Activity

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A series of novel 1'-methylxanthene-9-spiro-4'-piperidines has been prepared in the search for opiate analgesics with improved pharmacological properties. It has been found that introduction of a hydroxyl group into the 4-position of the xanthenespiropiperidine nucleus produces a potent μ -opiate agonist. The structure-activity relationship of the series has been explored by use of isosteric replacements of the phenolic hydroxyl group. Moreover, the effect of altering the conformation of the piperidine ring has been studied. It was interesting to note that, in compounds lacking the phenolic hydroxyl group, opiate activity could be produced by introduction of the (phenylamino)ethyl group instead of methyl at the 1'-position.

It has long been the objective of medicinal chemists to prepare analgesics with the efficacy of morphine (1; see Chart I) as pain killers while lacking the serious and use-limiting side effects of nausea, respiratory depression, and addictive liability.^{1,2} A wide variety of compounds related to the natural opiates or of completely novel structure have been investigated, and interesting hypothetical models of the receptor have been advanced in an attempt to explain the structure-activity data.³⁻⁷ The discovery of different types of opiate receptor^{8,9} has shown

why in the past it was so difficult to construct a single model that would satisfactorily explain all the results. Our knowledge of the existence of μ , δ , and κ receptor subtypes of opiate receptors allows a reevaluation of earlier hypotheses of structure activity. This is particularly relevant in the role of the phenolic hydroxyl group in contributing to μ -opiate properties in compounds related to morphine.^{10,11}

In the search for novel compounds with opiate properties, we have studied the xanthenespiropiperidine as a nucleus. Substitution in the aromatic rings produces compounds with a variety of pharmacological properties.^{12,13} In this paper we describe the synthesis of sub-

(1) Eddy, N. B.; May, E. L. *Science* **1973**, *181*, 407.

(2) Hahn, E. F. *Drugs Future* **1984**, *9*, 443. Isbell, H. *Clin. Pharmacol. Ther.* **1977**, *22*, 377.

(3) Beckett, A. H.; Casy, A. F. *J. Pharm. Pharmacol.* **1954**, *6*, 986.

(4) Bentley, A. K.; Cowan, A.; Lewis, J. W. *Annu. Rev. Pharmacol.* **1971**, *11*, 241.

(5) Portoghese, P. S. *Acc. Chem. Res.* **1978**, *11*, 21.

(6) Feinberg, A. P.; Creese, I.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 4215.

(7) Galt, R. H. B. *J. Pharm. Pharmacol.* **1977**, *29*, 711.

(8) Martin, W. R. *Pharmacol. Rev.* **1983**, *35*, 283.

(9) Zukin, R. S.; Zukin, S. R. *Trends Neurosci.* **1984**, *7*, 160.

(10) Portoghese, P. S.; Alreja, B. D.; Larson, D. L. *J. Med. Chem.* **1981**, *24*, 782.

(11) Reden, J.; Reich, M. F.; Rice, K. C.; Jacobson, A. E.; Brossi, A.; Streaty, R. A.; Klee, W. A. *J. Med. Chem.* **1979**, *22*, 256.

(12) Galt, R. H. B.; Pearce, R. J. U.K. Patent 1,447,583, 1974.