"Blacklite" blue fluorescent lamp (FT15T8BLB, 15 W), placed at 30-cm distance.

Revertant colonies were determined on agar prepared with semienriched medium (SEM) agar (minimum medium agar (MMA) fortified with 0.1 mg/mL of Difco nutrient broth) after 48 h of incubation at 37 °C.

For dark mutagenesis, various amounts of compounds were added to molten top agar (containing 0.5 mM L-hystidine and 0.5 mM biotine, 0.1 mL of an overnight culture of *Salmonella typhimurium* TA 98) and then poured onto plates prepared with Vogel-Bonner agar.[30] Plates were incubated in the dark at 37 °C for 48 h.

For dark mutagenesis determined after metabolic activation, the *Salmonella typhimurium* TA 100 strain was used. The metabolic activation was carried out by means of S-9 Mix,[21] and the angelicins were tested in a concentration range between 5 and 20 μg/mL.

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Registry No. **1a**, 90369-99-6; **1b**, 90370-00-6; **1c**, 90370-01-7; **2a**, 90370-02-8; **2b**, 90370-03-9; **2c**, 90370-04-0; **3a**, 90370-05-1; **3b**, 90370-06-2; **3c**, 90370-07-3; **4a**, 90370-08-4; **4b**, 90370-09-5; **4c**, 90370-10-8; **5a**, 90370-11-9; **5b**, 90370-12-0; **5c**, 90370-13-1; **6a**, 90370-14-2; **6b**, 90370-15-3; **6c**, 90370-16-4; **7a**, 53811-55-5; **7b**, 90370-17-5; **7c**, 90370-18-6; **8a**, 90370-19-7; **8b**, 90370-20-0; **8c**, 90370-21-1; **9a**, 90370-22-2; **9b**, 90370-23-3; **9c**, 90370-24-4; **10a**, 90370-25-5; **10b**, 90370-26-6; **10c**, 90370-27-7; **11a**, 90370-28-8; **11b**, 90370-29-9; **11c**, 90370-30-2; 6-methyl-7-hydroxycoumarin, 53811-57-7; 4,6-dimethyl-7-hydroxycoumarin, 1484-98-6; 5,6-dimethyl-7-hydroxycoumarin, 90370-31-3; ethyl bromoacetate, 105-36-2.

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Design, Synthesis, and Testing of Potential Antisickling Agents. 4. Structure-Activity Relationships of Benzyloxy and Phenoxy Acids

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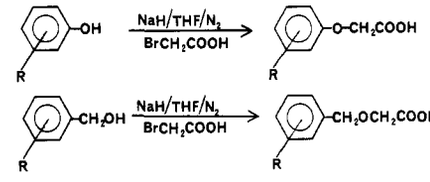
In this paper we further establish the activity of two classes of small molecules, benzyloxy and phenoxy acids, as potent inhibitors of hemoglobin S (HbS) gelation. Structural modifications with a large number of each class confirm our earlier work that the highest activity is observed with compounds that contain dihalogenated aromatic rings with attached polar side chains. We have also found a halogenated aromatic malonic acid derivative to be quite active. Compounds reported in this paper are compared with other antigelling agents studied in our laboratory. Comments are made concerning the antigelling activity and binding sites of four derivatives and their effect on the allosteric mechanism of hemoglobin (Hb) function.

Several groups have investigated the effects of aromatic compounds as antigelling agents.[1-5] During the last several years we also initiated a program to design, synthesize, and test small molecules that might be suitable as therapeutic agents in the treatment of sickle cell anemia. In our search for active agents we have employed three different methods of design and all have produced active compounds. One approach involved the design of agents modeled to bind stereospecifically to the surface of hemoglobin (Hb) at or near important contact areas in the hemoglobin S (HbS) polymer as indicated from the X-ray crystal and fiber structures of HbS. This approach produced the very active meta-disubstituted benzoic acids.[6] A second approach that was also successful in producing very active molecules involved determination of the binding sites of weakly active antigelling agents with use of X-ray diffraction studies[7] and subsequent redesign of agents to better fit that site using the binding information. The phenoxyacetic acids were discovered by using this approach.[8] The third method involved modification of a moderately active but insoluble antigelling agent, *p*-bromobenzyl alcohol,[9] by addition of a polar side chain to increase its solubility. This idea produced the very active (benzyloxy)acetic acids.[8] All three methods produced compounds with some overlapping structural features that appear to impart strong antigelling activity. Specifically, it has been shown that a mono- or dihalogenated aromatic acid with a polar side chain contains the appropriate moieties to bind effectively to HbS at sites that destabilize the HbS gel. Significant antigelling activity of other halogenated aromatics similar to ours has also been reported by three groups.[10-12]

Scheme I



This paper expands our earlier work[8] and attempts to rationalize the antigelling activity of the benzyloxy and phenoxy acids with our more recent X-ray studies as well as gives a hypothesis about a mechanism to explain their

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effects on the oxygen equilibrium properties of Hb.

Chemistry. The benzyloxy and phenoxy acids listed in Tables I and II were synthesized according to Scheme I except for **2**, **6**, **7**, and **19** (which were purchased from Aldrich) and those reported in the Experimental Section. Gemfibrozil (**54**) and bezafibrate (**61**) (Table III) were kindly provided by Dr. Max Perutz of the MRC Laboratory of Molecular Biology in Cambridge, England. The miscellaneous compounds (Table III) were synthesized as described in the Experimental Section.

Structure–Activity Correlations. Both the phenoxyacetic acids (Table I) and the (benzyloxy)acetic acids (Table II) contain some very active derivatives as shown from the results of the solubility assay developed by Hofrichter et al.[13] and modified for our work.[8] Since this assay is performed under anaerobic conditions, it monitors activity that must arise from a stereochemical distortion in Hb tertiary or quaternary structure, which in turn destabilizes polymer formation. Compounds that compete for any of the intermolecular contacts in the HbS fibre without necessarily binding strongly to individual Hb molecules would also be active under these assay conditions. The ratio values in the activity tables signify the solubility of HbS in the presence of the compound to the solubility of HbS without compound (i.e., control). Therefore, the higher the ratio, the higher the activity of the drug at that concentration. Because a measurement is made for each of four concentrations (5, 10, 20, 40 mM), it may be somewhat difficult to compare small differences in activities from compound to compound. Therefore, we have listed the compounds in order of their activity ratios as measured at 5 mM. Sunshine et al. have published the solubility ratios and kinetic parameters (under these assay conditions) for correlation of clinical severity with inhibition of sickle cell hemoglobin gelation.[14] Compounds with ratios above 1.06 at low concentrations meet the minimum ratio estimated by Sunshine et al. for the observation of an improved clinical course. Such compounds would be of interest for other antigelling or antisickling assays as well as be candidates for further molecular modification. As can be seen in Tables I–III, several of our most active compounds (at 5 mM) fit this criteria. The saturation effects of the more active molecules can be observed in the tables by looking at the solubility ratios vs. the concentration of drug.

The most active derivatives in both the phenoxy and (benzyloxy)acetic classes of compounds are dihalogenated acids (compounds **1**, **2**, **3**, **33–35**). The most active molecule in the miscellaneous structures listed in Table III is a halogenated aromatic malonic acid derivative (**52**). Methylated aromatics are not as potent as halogenated derivatives, and polar groups (OMe, NO_2) on the aromatic ring do not enhance activity. The sudden increase in activity of the nitro compound **32** at 20 mM is probably due to the formation of MetHb as the solution turned brown in the gelling assay. The addition or substitution of hydrophobic groups to the polar side chain (S for O or branched Me groups) decreases activity and the addition of a CH_2 unit to extend the chain as shown in compound **37** does not improve activity dramatically.

In general, the (benzyloxy)acetic acids are more potent than the phenoxyacetic acids (see ratios at 5 mM in Tables I and II). However, we caution against attempting a more detailed correlation of structure–activity relationships based solely on the structures and activities as shown in the tables. The reason for this caution arises from the X-ray crystallographic analysis of the binding of a number of our compounds to Hb. As will be seen later, weakly active molecules have multiple binding sites while the more active derivatives with similar structures, **33** and **40**, bind predominantly to single sites but at different locations on the Hb molecules. Such diversity in binding produces differences in HbS biological function (polymerization and allosteric behavior). Therefore, classical SAR treatment is not warranted.

Toxicity. A literature search was performed[15] to obtain data on the toxicity of our compounds. Two of the very active phenoxy acids have been studied and the following information was obtained.

1. (3,4-Dichlorophenoxy)acetic Acid (2). In mice the LD50 is > 1989 mg/kg. Mutagenicity tests in eight strains of *Salmonella* were negative. The 1989 mg/kg given to pregnant mice does cause some embryo toxicity.[16,17]

2. (2,4-Dichlorophenoxy)acetic Acid (3). Compound **3** is the well-known herbicide 2,4-D. Its toxicity in the most susceptible species, the dog, is LD50 = 100mg/kg.[18] In man, an iv dose of 3.6 g has caused acute illness. The patient suffered coma, fibrillary twitching of some muscles, hyporeflexia, and urinary incontinence. Seven hours after infusion, the patient could be roused but lapsed back into deep sleep. Twenty-four hours after infusion, the patient was eating well; however, a profound muscular weakness persisted. After another 24 h, all effects of the herbicide had dissipated. The same patient given 18 intravenous doses of **3** over a period of 33 days showed no side effects, even though the last 12 of those doses were over 800 mg and the final dose 2000 mg.[19] Another man consumed 500 mg of purified **3** for 21 days without ill effect.[20,21] The herbicide is relatively rapidly excreted from the body, mostly as unchanged compound. For a review of the literature in this area, see Hayes.[22]

We have also obtained preliminary toxicity data on our most active benzyloxy compound **33** in mice and find it to be in the range of the antilipidemic drug clofibric acid[23] (normal daily adult human dose is 2.0 g). It has been pointed out that[24] these low toxicities are very encouraging for the entire class of compounds.

Binding Sites and X-ray Studies. The double-stranded dimer structure of HbS molecules in the crystal form[25–27] has been directly related to the double-stranded

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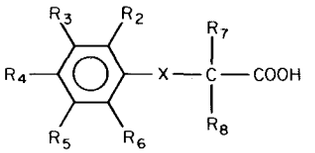
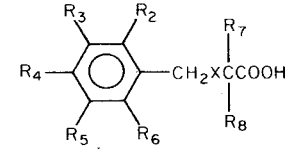
Table I. Phenoxyacetic Acids[a]



compd[f]	R_2	R_3	R_4	R_5	R_6	R_7	R_8	X	recryst sol[b]	mp, °C (ref)	[HbS drug]/[HbS control][d] 5 mM	10 mM	20 mM	40 mM	no. of runs	initial HbS concn, g/dL	dHbS control, g/dL
1	Br	H	Me	H	Br	H	H	O	2	161-163[50]	1.077	1.043	1.139	1.284	1	23.07	17.07
2	H	Cl	Cl	H	H	H	H	O	1	138-140[51]	1.061	1.117	1.234	1.339	3	23.88, 23.88, 23.88	17.00, 17.29, 17.29
3	Cl	H	Cl	H	Cl	H	H	O	4	181-183[54]	1.057	1.123	1.228	1.390	1	24.29	16.70
4	H	Me	Cl	H	H	H	H	O	1	177-179[51]	1.050	1.080	1.171	1.275	1	23.41	16.89
5	H	H	Br	H	H	H	H	O	2	157-159[53]	1.049	1.093	1.180	1.317	1	23.07	16.63
6	Cl	H	Cl	H	H	H	H	O	1	136-140[52]	1.049	1.083	1.151	1.313	2	23.83, 23.88	17.36, 17.29
7	H	H	Cl	H	H	H	H	O	1	157-159[53]	1.040	1.080	1.163	1.287	4	24.02, 23.82, 23.83, 23.88	17.11, 17.22, 17.07, 17.14
8	H	H	I	H	H	H	H	O	2	156-158[53]	1.040	1.046	1.090	1.176	1	23.07	16.63
9	Me	Me	H	Me	H	H	H	O	2	125-127[55]	1.035	1.086	1.180	1.351	1	23.41	16.70
10	H	Me	H	Me	H	H	H	O	2	108-110[56]	1.035	1.048	1.141	1.310	1	23.41	16.56
11	H	H	SMe	H	H	H	H	O	2	111-113[61]	1.035	1.027	1.160	1.223	2	24.24, 23.97	19.05, 17.55
12	H	Me	Cl	Me	H	H	H	O	2	147-149[51]	1.033	1.054	1.124	1.216	1	23.87	17.80
13	Me	H	H	Me	H	H	H	O	2	113-114[50]	1.031	1.055	1.114	1.248	1	23.41	16.67
14	Br	H	Me	H	H	H	H	O	2	141-143[51]	1.031	1.035	1.102	1.189	1	23.41	16.63
15	H	OMe	H	OMe	H	H	H	O	1	142-144[57]	1.030	1.052	1.120	1.232	1	24.15	17.07
16	H	Me	Me	H	H	H	H	O	2	158-160[68]	1.027	1.060	1.118	1.243	1	23.41	16.59
17	H	H	Cl	H	H	H	H	S	2	103-104[50]	1.026	1.069	1.168	1.296	1	23.07	17.07
18	F	F	F	F	F	H	H	S		oil[50]	1.025	1.053	1.117	1.236	1	24.81	18.02
19	Cl	Cl	H	H	H	H	H	O	1	172-174[60]	1.025	1.057	1.090	1.135	1	24.24	17.95
20	Cl	H	H	H	Cl	H	H	O	1	133-135[58]	1.025	1.047	1.087	1.199	1	23.33	17.29
21	H	H	Br	H	H	H	H	S	2	113-114[59]	1.023	1.063	1.113	1.261	1	23.07	17.44
22	H	H	NMe_2·HCl	H	H	H	H	O	5	200-203[50]	1.022	1.044	1.011	1.057	1	23.33	16.78
23	Br	H	Br	H	Br	H	H	O	4	195-198[54]	1.018	1.077	1.145	1.248	1	24.29	16.26
24	H	H	Cl	H	H	Me	Me	O	2	118-120[69]	1.018	1.064	1.155	1.302	3	24.50, 25.36, 24.68	17.07, 16.78, 17.51
25	Cl	H	Cl	Cl	H	H	H	S	3	110-111[50]	1.018	1.032	1.070	1.122	2	24.24, 23.97	18.65, 17.44
26	OMe	OMe	H	H	H	H	H	O	1	102-104[50]	1.017	1.036	1.064	1.144	1	24.15	17.07
27	H	H	H	H	H	H	H	O		oil[53,e]	1.017	1.009	1.047	1.097	1	24.68	17.36
28	H	H	*t*-Bu	H	H	H	H	O	2	86-88[62]	1.004	1.012	1.058		1	23.33	17.66
29	H	H	NH_2	H	H	H	H	O	5	215-220 (dec)[57]	1.000	1.010	1.113	1.145	1	23.87	17.22
30	H	Me	Br	Me	H	H	H	O	2	158-159[50]	0.996	1.004	1.063	1.092	1	23.87	17.51
31	H	OMe	OMe	OMe	H	H	H	O	1	110-112[63]	0.987	1.013	1.015	1.047	1	24.15	17.07
32	H	H	NO_2	H	H	H	H	O	3	183-185[57]	0.983	1.043	1.329[c]	1.344	1	24.01	17.14
Phe[g]											1.036	1.048	1.093	1.178	2	23.74, 24.50	17.32, 17.09

[a] All compounds were prepared as outlined under Experimental Section except for compounds **2**, **7**, **6**, and **19**, which were purchased from Aldrich. [b] Recrystallization solvent 1 = diethyl ether, 2 = diethyl ether/hexane, 3 = MeOH/H_2O, 4 = benzene, 5 = acetic acid. [c] High activity believed to be due to met formation in the tubes. During the course of the assay the hemoglobin turned brown, which seems to verify this. [d] The ratios are calculated as (sol HbS drug (g/dL)/(sol HbS control (g/dL)). [e] The sample was an oil with acceptable NMR and mass spectral data. The compound reported in ref 53 has mp 99-100 °C. [f] All compounds were dissolved in 0.15 M phosphate buffer, pH 7.4, with 1 equiv of sodium bicarbonate to make the sodium salt, at a concentration of 0.18 M. Appropriate aliquots of this solution (10, 20, 40, and 80 μL) were mixed with buffer to equal 90 μL. The 90-μL solutions were added to 250 μL of HbS (0.15 M phosphate), usually around 35 g %, and then 20 μL of dithionite (1.06 M) was added before sealing the EPR tubes. Final concentrations of drug were 5, 10, 20 and 40 mM in four separate tubes. A set of six tubes was spun on each run, which included the four drug concentrations, one dHbS control (90 μL of buffer, no acid), and a 40 mM phenylalanine control. The above dilution procedure produces identical HbS initial concentrations for all six tubes. The initial HbS concentrations (in grams per deciliter) for each after addition of the acids and dithionite and the respective solubility of deoxyhemoglobin S (in grams per deciliter) for each control run (no acid) appear in the table above. [g] Phenylalanine (Phe).

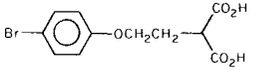
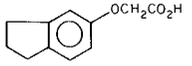
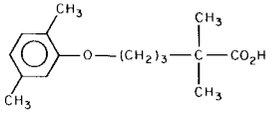
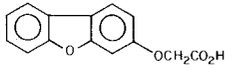
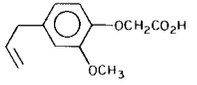
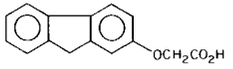
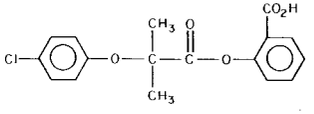
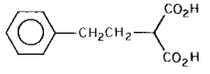
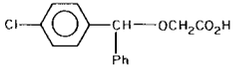
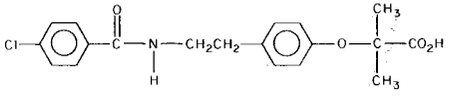
Table II. (Benzyloxy)acetic Acids[a]



compd[e]	R_2	R_3	R_4	R_5	R_6	R_7	R_8	X	recryst solv[b]	mp, °C (ref)	[HbS drug]/[HbS control][c] 5 mM	10 mM	20 mM	40 mM	no. of runs	initial HbS concn, g/dL	dHbS control, g/dL
33	H	Cl	Cl	H	H	H	H	O	1	58-60[3]	1.083	1.162	1.309	1.320	6	24.02, 23.82, 23.83, 24.24	17.07, 18.39, 17.00
																23.97, 23.14	18.39, 17.58, 16.85
34	Cl	H	Cl	H	H	H	H	O	1	88-90[50]	1.079	1.130	1.295	1.246	1	23.87	17.25
35	H	Cl	H	Cl	H	H	H	O	1	105-107[50]	1.076	1.149	1.330	1.382	1	23.87	16.96
36	H	H	Cl	H	H	H	H	O	1	74-76[64]	1.065	1.104	1.206	1.396	1	24.84	17.00
37	H	Cl	Cl	H	H	H	H	OCH_2	1	70-72[50]	1.062	1.142	1.305	1.395	2	23.25, 24.63	17.14, 17.22
38	OMe	H	Cl	H	H	H	H	O	2	83-85[50]	1.062	1.121	1.233	1.311	2	23.31, 23.97	17.84, 17.29
39	H	Me	Me	H	H	H	H	O	4	48-49[50]	1.060	1.086	1.142	1.289	1	23.87	17.00
40	H	H	Br	H	H	H	H	O	1	79-80[3]	1.059	1.118	1.233	1.398	4	22.85, 24.68, 24.99	15.93, 17.00, 12.51
																24.84	17.14
41	Me	H	Me	H	H	H	H	O	1	72-74[50]	1.050	1.054	1.163	1.325	1	23.87	17.58
42	H	H	Br	H	H	Me	Me	O	1	111-113[3,50]	1.048	1.086	1.193	1.379	4	22.85, 24.68, 24.99	16.70, 17.07, 16.41
																24.84	17.22
43	H	H	I	H	H	H	H	O	1	83-86[50]	1.039	1.090	1.236	1.350	1	24.15	17.07
44	H	H	OPh	H	H	H	H	O	2	59-61[50]	1.034	1.088	1.165	1.162	2	23.87, 24.24	17.66, 18.61
45	H	OMe	Me	H	H	H	H	O	4	52-54[50]	1.034	1.081	1.145	1.248	2	24.24, 23.97	18.28, 17.51
46	Me	H	Me	H	Me	H	H	O	2	114-116[50]	1.031	1.054	1.163	1.310	1	23.87	17.51
47	H	H	Br	H	H	H	H	S	1	72-75[50,65,d]	1.024	1.053	1.131	1.307	2	23.83, 23.88	17.36, 17.44
48	H	H	Me	H	H	H	H	O	1	49-51[50]	1.017	1.059	1.137	1.304	1	24.15	17.36
49	H	Me	H	Me	H	H	H	O	4	49-51[50]	1.010	1.031	1.129	1.315	1	23.87	17.55
50	H	H	NH_2	H	H	H	H	S	3	147-149[50,f,66]	1.006	1.031	1.076	1.174	1	23.87	17.03
51	H	H	OMe	H	H	H	H	O	1	48-49[64]	0.996	1.012	1.054	1.149	1	23.87	17.73
Phe[g]											1.036	1.048	1.093	1.178	2	23.74, 24.50	17.32, 17.09

[a] All compounds were prepared as outlined in the Experimental Section. [b] Recrystallization solvent 1 = H_2O, 2 = MeOH/H_2O, 3 = EtOH, 4 = petroleum ether. [c] Ratios calculated as shown in Table I, footnote *d*. [d] Different melting point than reported (62-63 °C) but we have a microanalysis and spectral data. [e] All compounds were assayed as described in Table I, footnote *f*. [f] Different melting point than reported (155-156 °C) but we have a microanalysis and spectral data. [g] Phenylalanine (Phe).

Table III. Miscellaneous[a]

compd[d] no.	structure	recryst solv[b]	mp, °C (ref)	[HbS drug]/[HbS control][c] 5 mM	10 mM	20 mM	40 mM	no. of runs	initial HbS concn, g/dL	dHbS control, g/dL
52		1	164-166[50]	1.057	1.134	1.323	1.360	2	24.63, 23.82	17.07, 17.62
53		2	154-157[50]	1.054	1.134	1.247	1.322	1	23.33	17.51
54		3	58-70[70]	1.040	1.073	1.117	1.234	3	24.99, 24.99, 24.84	16.34, 16.56, 17.22
55		5	163-164[50]	1.033	1.054	1.090	1.185	1	23.31	17.80
56		1	95-97[67]	1.033	1.043	1.141	1.245	2	23.31, 23.97	17.80, 17.40
57		2	143-144[50]	1.029	1.042	1.067	1.113	1	23.31	17.44
58		1	132-134[50]	1.022	1.044	1.153	1.109	1	24.99	16.45
59		1	122-123[72]	1.007	1.058	1.101	1.263	2	23.82, 23.88	16.92, 17.44
60			oil[50]	1.002	1.032	1.103	1.250	1	24.02	17.14
61		3	184-186[71]	0.954	0.932	0.981	1.198	3	24.80, 24.81, 24.81	18.24, 17.29, 17.22
Phe				1.036	1.048	1.093	1.178	2	23.74, 24.50	17.32, 17.09

[a] All compounds were prepared as outlined in the Experimental Section except for 54 and 61. [b] Recrystallization solvent 1 = diethyl ether/hexane, 2 = MeOH/H_2O, 3 = $CHCl_3$/diethyl ether, 4 = H_2O, 5 = MeOH/petroleum ether. [c] Ratios calculated as shown in Table I, foodnote *d*. [d] All compounds were assayed as described in Table I, footnote *f*.

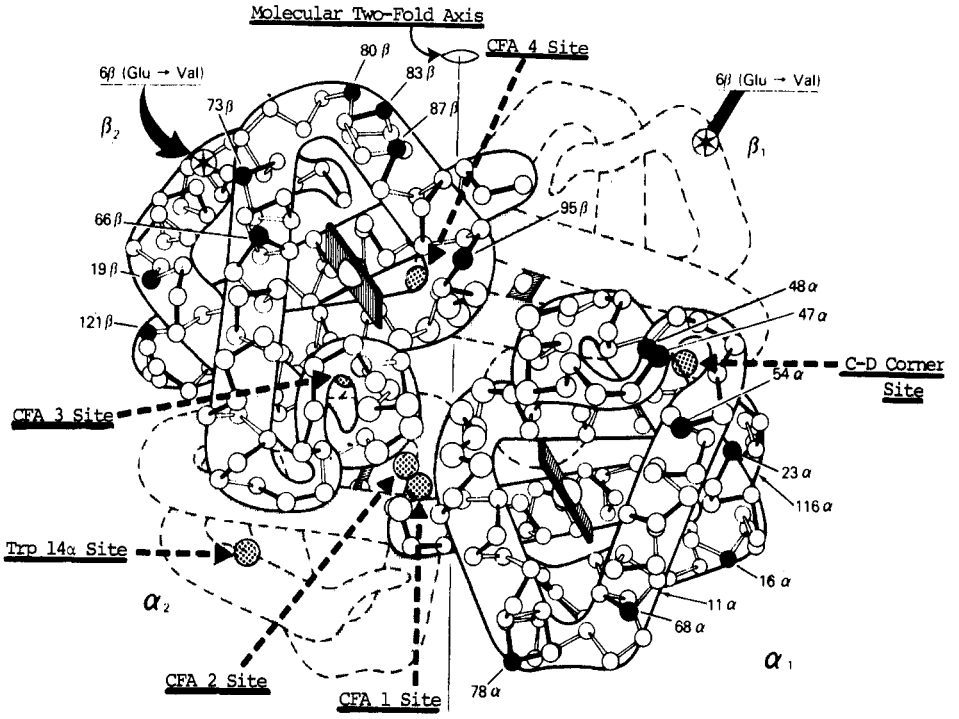


Figure 1. Schematic of hemoglobin S indicating the Trp-14α, C–D corner, and the central water cavity binding sites. The binding sites are indicated with dashed arrows. With the exception of the asymmetric C–D corner binding site, each binding site has another site on the Hb tetramer that is not indicated on the figure but is related by the molecular twofold. The drawing shows the helical outline of each chain and the α-carbon polypeptide backbone of one α and one β chain. The $\beta^{6\beta Glu\text{-}Val}$ substitutions are indicated with a heavy arrow and a heavy line. Amino acid residues in the region of probable polymer contacts are denoted with a filled circle and their number in the amino acid sequence. Arrows indicate residues on the "back side" of the molecule. The diagram has been simplified in that the identification of all the mutants is shown in the $\alpha_1\beta_2$ dimer whereas their exact location is still ambiguous. This figure is based on Figure 5 from an article by Dean and Schechter[5] with the permission of Dr. Alan Schechter.

structure of the HbS polymer found in vivo.[28-36] Love et al.[37,38] have compared six different human deoxy-Hb crystal forms, four grown in high salt and two in low salt (polyethylene glycol). All six crystal forms are built in the same way. Low- or high-salt conditions do not appear to have unusual effects on the Hb crystal structure or on the interactions in the strands.

We have been able to make good use of deoxy-HbA and -HbCO crystals grown in concentrated salt solution to monitor the binding of several of our agents.[7,39] Since our assays are run under anaerobic conditions, we will concentrate in this paper on the observations derived from the deoxy-Hb studies. Six binding sites of interest in this study are shown in Figure 1. These sites were uncovered during the X-ray crystallographic studies of four halogenated acids: clofibric acid, [(*p*-bromobenzyl)oxy]acetic acid, [(3,4-dichlorobenzyl)oxy]acetic acid, and bezafibrate. Four of the sites are located in the water-filled central cavity, and all four sites were found to be occupied by molecules of clofibric acid (CFA), **24**. Therefore, these sites have been designated as CFA1, CFA2, CFA3, and CFA4. Two CFA sites, CFA1 and CFA2 (major with high occupancy), were reported in an earlier publication[7] and are shown in Figure 2a. Two CFA sites, CFA3 and CFA4 (minor with low occupancy), nearer the α–β subunit interface, are also of interest. The electron difference density for these sites are depicted in Figure 2b.

Two other major sites are located near the surface of the molecule and are designated the Trp-14α site and the C–D corner site (see Figure 1). In deoxy-HbA crystals, [(3,4-dichlorobenzyl)oxy]acetic acid, **33**,[39] the most active compound in Table II, is exclusively bound at the Trp-14α site. Dichloromethane[41] has also been found in the Trp-14α site. A careful contouring of the clofibric acid difference map shows a very weak low occupancy peak at Trp-14α in deoxy-Hb. The Trp-14α site is the preferred site in HbCO crystals for iodobenzene, *p*-bromobenzyl alcohol, and clofibric acid.[7] The C–D corner site is occupied by [(*p*-bromobenzyl)oxy]acetic acid, **40** (one major asymmetric

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(37) Love, W. E.; Fitzgerald, P. M. D.; Hanson, J. C.; Royer, W. E., Jr. "Intermolecular Interactions in Crystals of Human Deoxy Hemoglobin A, C, F and S"; Rosa, J., Beuzard, Y., Hercules, J., Eds.; Elsevier, North-Holland Biomedical Press: Amsterdam, 1979; pp 65–75.
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(40) Perutz, M. F.; Abraham, D. J.; Luisi, B.; Poyart, C., unpublished results.
(41) Schoenborn, B. P. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 4195–4199.

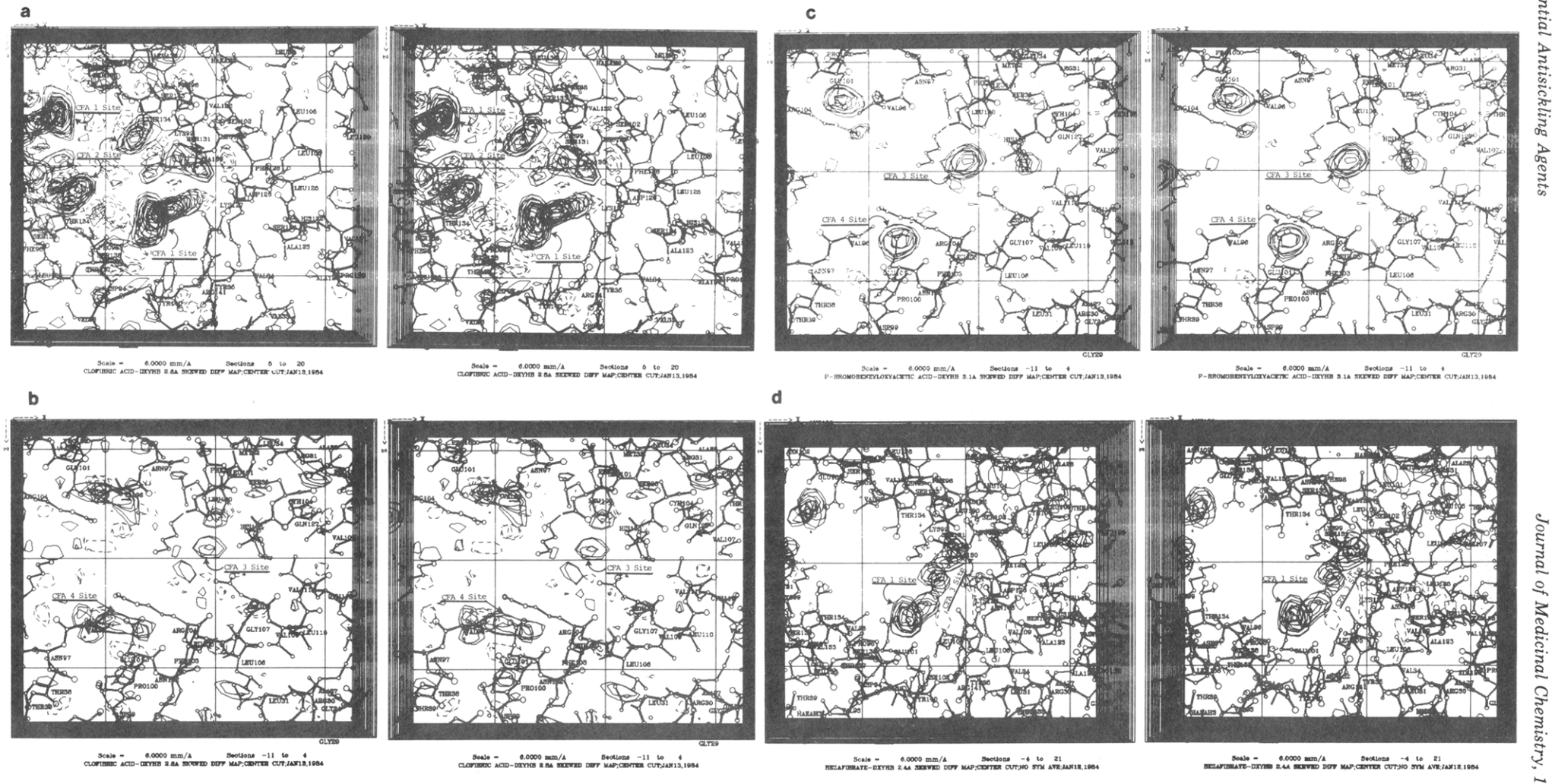


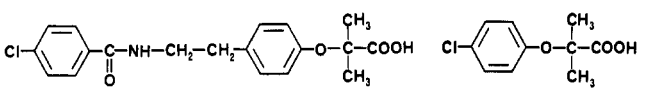
Figure 2. Electron density difference maps of the central water cavity binding sites. The binding sites of [(*p*-bromobenzyl)oxy]acetic acid and bezafibrate are correlated with respect to the major and minor clofibric acid binding sites. (a) The two major pairs of CFA sites (CFA1 and CFA2) are depicted. Notice that the twofold symmetry axis is perpendicular and centered between the sites. CFA2 almost makes an interaction with itself across the symmetry axis.[7] (b) The two minor CFA sites are designated CFA3 and CFA4. The occupancy of CFA in these sites is greatly reduced. (c) The minor [(*p*-bromobenzyl)oxy]acetic acid sites are shown to overlap with the CFA3 and CFA4 sites. (d) This is a view of the entire benzafibrate electron density which shows it to overlap the CFA1 and CFA3 sites. The bezafibrate molecule has been fitted to this density and the details will be published shortly.[40]

Table IV. Oxygen Equilibrium Curves of Compounds Studied by X-ray Crystallographic Analyses[a]

compd	run no.	P_{50} control, mmHg	5 mM, ΔP_{50}, mmHg	10 mM, ΔP_{50}, mmHg	20 mM, ΔP_{50}, mmHg
BZF (61)	1	15.0	6.0	12.0	24.0
	2	17.0	5.0	11.5	21.0
	3	17.0	6.0	13.0	20.0
			av 5.7	12.2	21.7
CFA (24)	1	15.5	2.5	4.5	9.0
	2	17.0	2.5	4.5	9.0
	3	16.5	2.5	4.5	8.0
			av 2.5	4.5	8.7
40	1	15.5	0.0	1.5	3.5
	2	18.0	0.5	1.0	2.0
	3	17.0	0.5	1.0	1.5
			av 0.3	1.2	2.3
33	1	17.0	0.0	1.0	1.5
	2	17.0	1.0	2.0	3.0
	3	17.0	1.0	1.0	2.0
			av 0.7	1.3	2.2

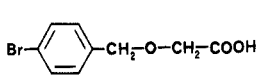
[a] The ΔP_{50} values for BZF, CFA, **40**, and **33** are calculated from P_{50}(drug) − P_{50}(control). The Aminco Hem-O-Scan P_{50} values are reproducible to about ±1 mmHg.

binding site with high occupancy). The apparent reason for the asymmetric binding of **40** (eighth in activity in the benzyloxy series) is that the CD2 (Pro-44), CD3 (His-45), and CD4 (Phe-46) residues of the C-D corner in the $\alpha 1$ subunit of Hb make a strong intermolecular contact in the crystal while the $\alpha 2$ subunit has no intermolecular contacts in this region.[42] This strong intermolecular contact may dislodge the drug from the $\alpha 1$ subunit, giving rise to the asymmetric binding. Three minor low occupancy sites for **40** were also observed in the central water cavity and correspond to the minor CFA3 and CFA4 sites (see Figure 2c) and CFA1 (not shown). A molecule that causes sickling at low concentration in the gelling assay, bezafibrate (BZF), **61** (a known antilipidemic drug marketed in Europe), has

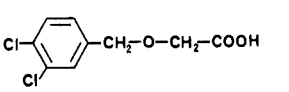


BEZAFIBRATE (BZF)

CLOFIBRIC ACID (CFA)



p-BROMOBENZYLOXYACETIC ACID (40)



3,4-DICHLOROBENZYLOXYACETIC ACID (33)

recently been found[40] to occupy both the CFA1 and CFA3 sites as shown in Figure 2d. BZF has been shown to have a profound effect in lowering the oxygen affinity of HbA (right shifting the oxygen equilibrium curve).[43] The determination of the binding site for BZF proved quite valuable in identifying sites that regulate Hb oxygenation.

Oxygen Equilibrium Studies. We have measured under identical conditions the oxygen equilibrium properties of the four compounds discussed above. The results are shown in Figure 3 and Table IV. They clearly demonstrate that bezafibrate has the greatest effect in shifting the oxygen equilibrium curve to the right. This decrease in oxygen affinity of hemoglobin is a result of the stabilization of the deoxy or T (tensed) structure by the specific binding of BZF to Hb.[40,43] Clofibric acid (**24**) shows the next strongest effect followed by [(*p*-bromobenzyl)oxy]-acetic acid (**40**) and [(3,4-dichlorobenzyl)oxy]acetic acid (**33**), with small or no effect on the oxygen equilibrium.

Conclusions. The binding and oxygen equilibrium studies on the phenoxy and benzyloxy derivatives studied to date show a very interesting combination of effects. The small molecules that shift the oxygen equilibrium the most are found predominantly in the central water cavity, while the most active antigeling compounds that do not have such a profound effect on the oxygen release properties of Hb are found near the surface of the molecule. So far molecules with multiple binding sites in the central water cavity have moderate to no antigelling activity but exhibit an effect on the allosteric equilibrium, producing a Hb with lower oxygen affinity (right shift of the oxygen equilibrium curve).

We cannot at this time unequivocally attribute the antigelling action to the surface sites even though Trp-14α has its indole ring displaced toward the surface away from the cavity that is near an intermolecular contact site in the HbS polymer (His-20α).[26,44] However, it does seem reasonable that the binding sites in the central water cavity do regulate the oxygen equilibrium properties of Hb. This conclusion comes from the observation of overlapping sites occupied by CFA and BZF (see Figures 2a, 2b, and 2d) and the fact that they both right shift the oxygen equilibrium curve (see Table IV). Therefore, we propose that these internal water cavity sites are additional sites that can regulate the allosteric properties of Hb. The normal allosteric site for Hb in vivo involves the binding of DPG across the twofold axis at the top of the β subunits[45] (see Figure 1).

Baldwin and Chothia[46] have shown that the quaternary structure change in going from T to R Hb involves a movement of α–β dimers that alters the size of this cavity. It can easily be envisioned that the occupancy of these new sties by our small halogenated aromatic molecules can hinder the movement of the $\alpha 1$–$\beta 1$ dimer with respect to the $\alpha 2$–$\beta 2$ dimer, producing the observed decrease in oxygen affinity (see Figure 4). The greater oxygen equilibrium effects of BZF as compared to clofibric acid may arise from the fact that one molecule of BZF occupies the place of two CFAs, lowering the entropy for binding.

A comparison of the antigelling activity at 5 mM of the four compounds whose X-ray binding sites have been determined shows the solubility ratios to be **33** (1.083) ≅ **40** (1.059) > CFA (1.018) > BZF (0.954), while a comparison of the oxygen regulation properties at 5 mM (see Table IV) shows the reverse with ΔP_{50} values: BZF (5.7) > CFA (2.5) > **40** (0.3) ≅ **33** (0.7 mmHg). Therefore it seems reasonable that the most active compound in each sequence occupies a primary site that is responsible for their function, i.e., BZF, oxygen regulation and **33** and **40**, antigelling activity. The intermediate compound CFA is bound at multiple sites and shows both oxygen regulation properties and antigelling activity. This multiplicity in binding and function for the moderately active compounds is the reason for our earlier caution against attempting traditional structure–activity correlations (QSAR) with use of the data in Tables I–III.

An interesting observation concerning the binding of these small molecules to Hb is that almost all contacts with the protein are hydrophobic and very little hydrogen bonding or salt bridge formation is observed.[7,39,40] However, potential surface binding sites that would occur

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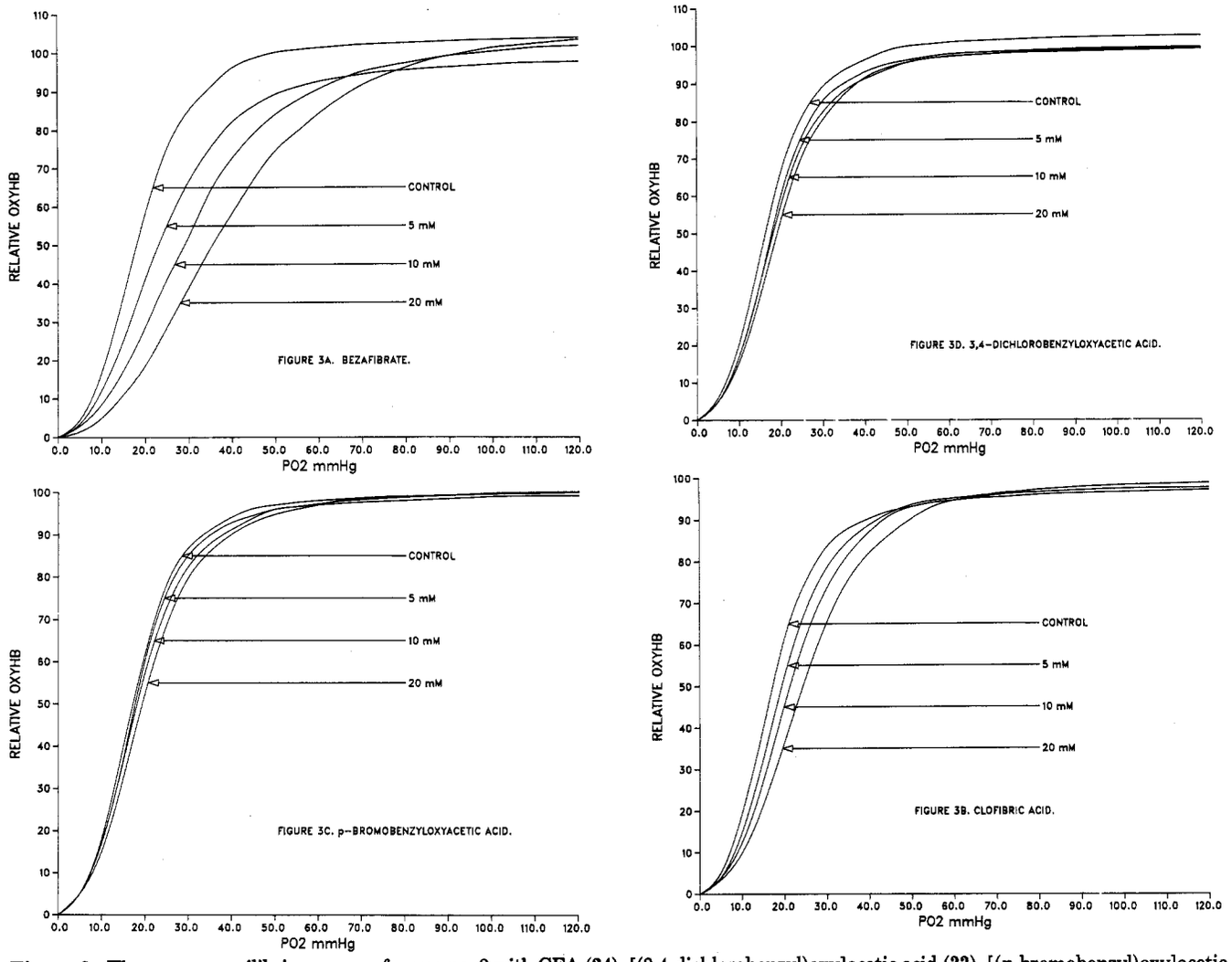

Figure 3. The oxygen equilibrium curves for run no. 3 with CFA (**24**), [(3,4-dichlorobenzyl)oxy]acetic acid (**33**), [(*p*-bromobenzyl)oxy]acetic acid (**40**), and bezafibrate (**61**) are shown. Curves were determined on an Aminco Hem-O-Scan under conditions stated in the text. P_{50} values were taken at half the height of the curve and appear in Table IV. (a) The oxygen equilibrium curve for BZF (**61**) shows a large right shift of the P_{50} at 20 mM drug (20.0 mmHg). The average for three runs was 21.7 mmHg. (b) The oxygen equilibrium curve for CFA (**24**) shows a right shift of the P_{50} of 8.0 mmHg at 20 mM drug. The average for three runs was 8.7 mmHg. (c) The oxygen equilibrium curve for [(*p*-bromobenzyl)oxy]acetic acid (**40**) shows only a small right shift (1.5 mmHg) of the P_{50} at 20 mM drugs. The average for three runs was 2.3 mmHg. (d) The oxygen equilibrium curve for [(3,4-dichlorobenzyl)oxy]acetic acid (**33**) also shows only a small (2.0 mmHg) effect on the P_{50} at 20 mM drug. The average for three runs was 2.2 mmHg.

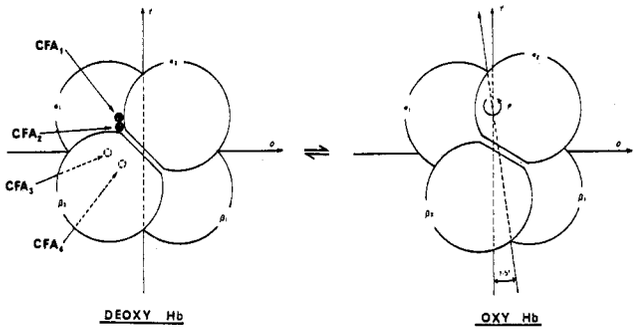

Figure 4. Schematic diagram illustrating the rotation of the $\alpha_2\beta_2$ dimer relative to $\alpha_1\beta_1$ that occurs in the quaternary structure change from deoxy- to oxyhemoglobin. The molecule is viewed along the rotation axis (P), which intersects the molecular dyad of deoxyhemoglobin (Y) at the point shown. The binding sites for CFA1, CFA2, CFA3, and CFA4 (the latter two are hidden, see arrow) only appear to have room to bind in the deoxy (T) state.

primarily from ionic interactions between the drug and protein would not be expected to be observed in high-salt X-ray structures. Since crystals in this study are grown from over 2 M solutions of sulfate or phosphate,[47] the high-salt concentration should competitively inhibit external surface binding of drug to protein via ionic interactions. Therefore, we cannot unequivocally attribute the antigelling activity to the near-surface sites at Trp-14α and the C–D corner. Binding experiments are currently being performed in our laboratory under low- and high-salt conditions to provide information on the question of ion concentration and binding.

As for the detailed binding interactions between the drugs and protein, more extensive hydrogen bonding between the phenoxy and benzyloxy polar groups (carboxyl and ether oxygen) with the peptide backbone or side chains were thought to be likely, a priori. However, in the structures determined thus far, such interactions are few. A detailed analysis of the X-ray work is planned for another paper.[39] Thus far we have found that the primary binding forces that govern these small-molecule drug interactions with Hb arise predominantly from weak dipolar van der Waals and hydrophobic forces.

These studies also suggest that the binding of a small molecule to a large one cannot always be envisioned from

(47) Perutz, M. F. *J. Cryst. Growth* **1968**, *2*, 54–56.

observing the surface of the large molecule. The ability of Hb to bind a variety of small molecules in pockets not even exposed to the surface (for example, the Trp at 14α must move to permit entry to that cavity) should provide a challenge to those writing drug design programs for molecular graphics and modeling. Also our studies as well as others[41,48,49] have not yet exhausted the potential use of the 3-D structure of Hb for disease states that might be sensitive to oxygen regulation. Finally, it should be noted that Hb is an ideal molecule to study as a model for drug–receptor interactions.

Experimental Section

Oxygen Equilibrium. Oxygen equilibrium curves were determined on a Minco HEM-O-SCAN oxygen dissociation analyzer (Travenol Laboratories).

HbA solution was prepared as follows: 20 mL of whole blood from a nonsmoking donor was drawn into a heparinized Vacutainer. The blood was immediately packed in ice (to prevent MetHb formation) and then centrifuged (10 min at 2500 rpm) to separate the plasma and buffy coat from the packed erythrocytes. The plasma and buffy coat were removed by aspiration and the cells washed three times with 0.9% NaCl (40 mg of EDTA/L) and then once with 1.0% NaCl (40 mg of EDTA/L). The cells were lysed by the addition of one to two volumes of deionized water containing 40 mg of EDTA/L. This was allowed to stand for 30 min with occasional mixing before being centrifuged for 2 h at 10000 rpm at 4 °C. The supernatant was decanted into a 50-mL tube. NaCl (60 mg/mL of Hb supernatant) was added, mixed, and centrifuged as described above to remove any remaining cell stroma. The supernatant was further purified by gel filtration with Sephadex G-25 (Sigma) and equilibrated with 0.05 M Bis-Tris, pH 7.4, containing 0.1 M Cl ion. Concentration of the Hb solution was accomplished by first using an Amicon Stirred Cell (Amicon Corp.) and then a Schleicher and Schuell Collodion Bag Apparatus (Schleicher and Schuell Inc.). The Hb solution was concentrated to 6 mM for the experiment. Less than 5% methemoglobin was noted even after several days at 4 °C.

All compounds were dissolved in the Bis-Tris buffer described above to give 0.2 M solutions. Aliquots of this solution (0.05, 0.10, and 0.20 mL) were diluted with the buffer to 1.00 mL to give the three stock solutions. Just prior to running the O_2 equilibrium curve, the Hb (6 mM) and the drug were mixed in a 1:1 ratio (50 μL of Hb + 50 μL of drug) to give 3 mM Hb with drug concentrations of 5, 10, and 20 mM. The control was prepared by the addition of 50 μL of Hb to 50 μL of the Bis-Tris buffer.

Synthesis. Proton magnetic resonance (NMR) spectra were recorded either on a 60-MHz Hitachi Perkin-Elmer R-24 spectrometer or on a 90-MHz JEOL FX90Q spectrometer and are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me_4Si). Infrared (IR) spectra were recorded in a Perkin-Elmer 267 grating spectrophotometer and are reported in reciprocal centimeters. Spectra were taken either in solution between two balanced salt plates with a reported solvent or as a KBr pellet. Low-resolution mass spectra were recorded on a Varian-MAT CH-5, LKB 5000, or Finnigan 3200 spectrometer. Column chromatographies were performed on Merck-60 silica gel with a reported solvent. TLC analyses were performed on EM Reagent silica gel 60 (0.20-mm thickness) with aluminum-backed plates impregnated with a fluorescent indicator. Spots were visualized either by ultraviolet (UV) or by charring. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed for unknown compounds at Galbraith Laboratories, Inc., Knoxville, TN, and are within 0.4% of calculated values. Yields for most compounds were not optimized. THF was distilled from a purple Na or K/benzophenone ketyl. Reactions using NaH were run under nitrogen. Solvents were concentrated on a rotary evaporator. The petroleum ether used had a boiling point range of 30–60 °C.

General Procedure: Preparation of Phenoxyacetic and (Benzyloxy)acetic Acids. A solution of 0.01 mol of the appropriate phenol or benzyl alcohol and 0.01 mol of bromoacetic acid in 15 mL of dry THF was placed under a nitrogen atmosphere. NaH (0.022 mol) (as 50% oil dispersion) was added in portions and the reaction heated at reflux for approximately 12 h (the reaction was checked for completeness by TLC before workup). The solvent was removed in vacuo, and to the residue was added 7 mL of water. This was then extracted with 10 mL of ethyl ether to remove unreacted starting material and the oil liberated from NaH. After acidification of the aqueous layer with 37% HCl, the reaction was extracted with ethyl ether (3 × 20 mL), dried (Na_2SO_4), and concentrated in vacuo to give in most cases a white solid. The crude material was recrystallized from the appropriate solvent. Column chromatography was performed before recrystallization where needed.

[*p*-(Dimethylamino)phenoxy]acetic Acid Hydrochloride (22). In a Parr bottle were placed 3.94 g (2×10^{-2} mol) of (*p*-nitrophenoxy)acetic acid, 0.8 g (2×10^{-2} mol) of NaOH, 5.0 mL of 37% aqueous HCHO solution, 200 mL of methanol, and 0.2 g of Pd/C. This was hydrogenated until there was no further drop in pressure. The solution was filtered and methanol evaporated under reduced pressure. The residue was acidified with HCl, dried, and taken up in methanol. Undissolved salt was filtered off and evaporation of methanol under reduced pressure gave crude product. Recrystallizaton from acetic acid gave 3 g of **22** as a white solid: yield 64.8%; mp 200–203 °C; NMR (Me_2SO-d_6) δ 7.7 (d, 2 H), 7.0 (d, 2 H), 4.7 (s, 2 H), 3.1 (s, 6 H); IR (KBr) 3300–2400 (OH), 1715 (CO) cm^{-1}; MS, m/e (relative intensity) 195 (20.72, M^+ – HCl), 136 (100, M^+ – HCl – CH_2COOH). Anal. ($C_{10}H_{13}NO_3$·HCl) C, H, N.

[(3,4-Dichlorobenzyl)oxy]propionic Acid (37). To 1.17 g (5×10^{-1} mol) of [(3,4-dichlorobenzyl)oxy]acetic acid (**33**) was added 16.31 g (1.37×10^{-1} mol) of thionyl chloride. This was heated at 70 °C for 2 h and then excess of thionyl chloride was evaporated under reduced pressure. The residue was dissolved in 20 mL of dry ethyl ether. To this was added ice-cold solution of diazomethane in ethyl ether (prepared from distillation of 4.1

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g of Diazald, 1 g of KOH, 20 mL of ethyl alcohol, and 80 mL of dry ethyl ether) over a 15-min period. The mixture was kept overnight in the dark, and then ethyl ether was removed under reduced pressure. The residual diazo ketone was dissolved in 20 mL of dioxane and was added dropwise to the mixture of 0.5 g (3.16×10^{-3} mol) of $Na_2S_2O_3$, 0.5 g (4.71×10^{-3} mol) of Na_2CO_3, and 1.0 g (4.31×10^{-3} mol) of Ag_2O in 30 mL of water maintained at 60 °C. At the end of the addition, the mixture was heated at 90 °C for 3 h, cooled, and filtered and the filtrate acidified with dilute HNO_3. The solution was extracted twice with 20 mL of ethyl ether, and the combined extract was dried over Na_2SO_4. Ethyl ether was then evaporated under reduced pressure to give the crude product. Recrystallization from boiling water or benzene–petroleum ether gave 0.72 g of **37** as a white solid: yield 58%; mp 70–72 °C; NMR ($CDCl_3$) δ 7.5–7.07 (m, 3 H), 4.5 (s, 2 H), 3.77 (t, 2 H), 2.69 (t, 2 H); MS, *m/e* (relative intensity), 247.9 (17.19, M^+) (with peaks due to two Cl atoms at *m/e* 249.9 and 251.9), 174.9 (100, $M^+ - CH_2CH_2COOH$). Anal. ($C_{10}H_{10}O_3Cl_2$) C, H, Cl.

Diethyl 2-[2-(*p*-Bromophenoxy)ethyl]malonate (52a). To a 15-mL solution of anhydrous THF containing 0.57 g (3.56×10^{-3} mol) of diethyl malonate was added 0.18 g (3.75×10^{-3} mol) of NaH (50% oil dispersion). To this was added 1.00 g (3.57×10^{-3} mol) of 2-bromoethyl *p*-bromophenyl ether followed by heating at reflux overnight. The cooled solution was concentrated in vacuo on a rotary evaporator, and to the residue was added 10 mL of water, which was extracted with ethyl ether (3 × 20 mL). The organic layers were combined, dried (Na_2SO_4), and concentrated in vacuo to give 0.73 g (2.03×10^{-3} mol) of **52a**: 57% yield as a clear oil; NMR ($CDCl_3$) δ 7.35 (d, 2 H), 6.73 (d, 2 H), 4.16 (m, 6 H), 3.64 (t, 1 H), 2.42 (t, 2 H), 1.31 (t, 6 H); IR ($CHCl_3$) 1725 (CO) cm^{-1}; MS, *m/e* (relative intensity) 360 (1.20, M^+), 358 (1.11, M^+).

2-[2-(*p*-Bromophenoxy)ethyl]malonic Acid (52). To 0.73 g (2.03×10^{-3} mol) of **52a** was added 0.20 g (8.33×10^{-3} mol) of LiOH dissolved in 7 mL of water. This was heated at 90 °C for 12 h. The cooled solution was acidified with 37% HCl and extracted with ethyl ether (3 × 20 mL). The organic layers were combined, dried (Na_2SO_4), and concentrated in vacuo to give a white solid. This after recrystallization from ethyl ether–hexane gave 0.38 g of **52**: yield 62%; mp 164–166 °C; NMR (acetone-d_6) δ 7.41 (d, 2 H), 6.85 (d, 2 H), 4.11 (t, 2 H), 3.64 (t, 1 H), 2.40 (t, 2 H); IR ($CHCl_3$) 3300–2850 (OH), 1720 (CO) cm^{-1}; MS, *m/e* (relative intensity) 304 (0.21, M^+), 302 (0.23, M^+), 174 (43.74, C_6H_5OBr), 172 (48.78, C_6H_5OBr). Anal. ($C_{11}H_{11}O_5Br$) C, H, Br.

(Indanyl-5-oxy)acetic Acid (53). To 1.00 g (7.46×10^{-3} mol) of 5-indanol was added 0.30 g (7.50×10^{-3} mol) of NaOH in 3 mL of water. To this was added 1.04 g (7.48×10^{-3} mol) of bromoacetic acid dissolved in 3 mL of deionized water containing 0.30 g (7.50×10^{-3} mol) of NaOH. This was heated at reflux for several hours before being acidified and extracted with ethyl ether (3 × 20 mL), dried (Na_2SO_4), and concentrated in vacuo to give a white solid. Recrystallization from methanol–water gave 0.81 g (4.22×10^{-3} mol) of **53**: 57% yield; mp 154–157 °C; NMR ($CDCl_3$) δ 7.28–6.62 (m, 3 H), 4.63 (s, 2 H), 2.88 (m, 4 H), 2.12 (m, 2 H); IR (KBr) 3300–2600 (OH), 1720 (CO) cm^{-1}; MS, *m/e* (relative intensity) 192 (46.78, M^+), 147 (12.32, $M^+ - CO_2H$), 133 (100.00, $M^+ - CH_2CO_2H$), 117 (27.39, $M^+ - OCH_2CO_2H$). Anal. ($C_{11}H_{12}O_3$) C, H. O.

(Dibenzofuranyl-3-oxy)acetic Acid (55). To 1.00 g (5.43×10^{-3} mol) of 3-dibenzofuranol was added 0.23 g (5.75×10^{-3} mol) of NaOH in 10 mL of water. To this was added 0.75 g (5.40×10^{-3} mol) of bromoacetic acid dissolved in 3 mL of water containing 0.23 g (5.75×10^{-3} mol) of NaOH. This was heated at reflux for several hours before being acidified. The solution was evaporated to dryness under reduced pressure, the solid taken up in methanol, and the undissolved salt filtered off. The methanol was evaporated under reduced pressure to give the crude product. Recrystallization from methanol–petroleum ether gave 0.70 g (2.89×10^{-3} mol) of **55** as a white solid: 53.5% yield; mp 163–164 °C; NMR (Me_2SO-d_6) δ 8.21–6.20 (m, 7 H), 4.80 (s, 2 H); IR (KBr) 3300–2600 (OH), 1720 (CO) cm^{-1}; MS, *m/e* (relative intensity) 242 (76.54, M^+), 183 (100, $M^+ - CH_2COOH$). Anal. ($C_{14}H_{10}O_4$) C, H.

(Fluorenyl-2-oxy)acetic Acid (57). To 1.00 g (5.50×10^{-3} mol) of 2-fluorenol was added 0.23 g (5.75×10^{-3} mol) of NaOH in 10 mL of water. To this was added 0.76 g (5.47×10^{-3} mol) of bromoacetic acid dissolved in 3 mL of water containing 0.23 g (5.75×10^{-3} mol) of NaOH. This was heated at reflux for several hours before being acidified and extracted with ethyl ether (3 × 20 mL), dried (Na_2SO_4), and concentrated in vacuo to give a white solid. Recrystallization from methanol–water gave 0.75 g (3.13×10^{-3} mol) of **57** in 57% yield: mp 143–144 °C; NMR (Me_2SO-d_6) δ 9.50 (s, 1 H), 7.90–6.71 (m, 7 H), 4.70 (s, 2 H), 3.81 (s, 2 H); IR (KBr) 3150–2700 (OH), 1725 (CO) cm^{-1}; MS, *m/e* (relative intensity) 240 (M^+, 52.46), 181 ($M^+ - CH_2CO_2H$, 100). Anal. ($C_{15}H_{12}O_3$) C, H.

Benzyl *O*-[2-(*p*-Chlorophenoxy)-2-methylpropionyl]salicylate (58a). To 40 mL of methylene chloride were added 3.00 g (1.40×10^{-2} mol) of 2-(*p*-chlorophenoxy)-2-methylpropionic acid, 3.18 g (1.37×10^{-2} mol) of benzyl salicylate, and 5.13 g (4.20×10^{-2} mol) of 4-(dimethylamino)pyridine. After the reaction became homogeneous, 3.45 g (1.67×10^{-2} mol) of DCC (dissolved in 10 mL of CH_2Cl_2) was added and the reaction stirred at room temperature overnight. The DCU was filtered from the reaction, the solvent removed in vacuo, and the residue chromatographed on 15 g of silica gel, using a 5:95 ethyl ether–hexane solvent system. This gave 4.66 g (1.10×10^{-2} mol) of **58a** as a clear oil: yield 78%; NMR ($CDCl_3$) δ 8.07 (dd, 1 H), 7.42 (s, 5 H), 7.35 (m, 7 H), 5.36 (s, 2 H), 1.73 (s, 6 H); IR ($CHCl_3$) 1758 and 1720 (CO) cm^{-1}; MS, *m/e* (relative intensity) 424.5 (0.32, M^+).

***O*-[2-(*p*-Chlorophenoxy)-2-methylpropionyl]salicylic Acid (58).** To a 100-mL round-bottom flask were added 3.74 g (8.81×10^{-3} mol) of **58a** and 50 mL of ethyl acetate. The flask was purged with nitrogen, 0.30 g of 10% Pd/C was added, and the vessel was fitted with a balloon apparatus filled with hydrogen. After 2.5 h a TLC of the reaction indicated the reaction to be complete. Longer reaction times led to dehalogenation of the aromatic ring. The catalyst was filtered from the reaction and the solvent removed in vacuo to give a white solid. Recrystallization from ethyl ether–hexane gave 0.94 g (2.81×10^{-3} mol) of **58**: yield 32%; mp 132–134 °C; NMR (acetone-d_6) δ 8.01 (dd, 1 H), 7.32 (m, 7 H), 1.72 (s, 6 H); IR ($CHCl_3$) 3300–2800 (OH), 1750 and 1695 (CO) cm^{-1}; MS, *m/e* (relative intensity) 334 (2.12, M^+), 171 (14.02, $C_9H_{10}ClO$), 169 (44.52, $C_9H_{10}ClO$). Anal. ($C_{17}H_{15}O_5Cl$) C, H, Cl.

Diethyl 2-Phenethylmalonate (59a). To a 25-mL solution of dry THF containing 4.33 g (2.70×10^{-2} mol) of diethyl malonate was added 1.37 g (2.85×10^{-2} mol) of NaH (50% oil dispersion). To this was added 5.00 g (2.70×10^{-2} mol) of (2-bromoethyl)-benzene followed by heating the reaction overnight at reflux. The cooled reaction was concentrated in vacuo to give a tan oil to which was added 10 mL of deionized water followed by extraction with ethyl ether (2 × 25 mL). The organic layers were combined, dried (Na_2SO_4), and concentrated to give 8.10 g of a tan oil. This was chromatographed on 13 g of silica gel, using a solvent system of 10:90 ethyl ether–hexane, to give 6.94 g (2.63×10^{-2} mol) of **59a** as a clear oil: yield 97%; NMR ($CDCl_3$) δ 7.20 (s, 5 H), 4.21 (q, 4 H), 3.32 (t, 1 H), 2.51 (m, 2 H), 2.25 (m, 2 H), 1.25 (t, 6 H); IR ($CHCl_3$) 1725 (CO) cm^{-1}; MS, *m/e* (relative intensity) 264 (1.41, M^+), 91 (63.78, benzyl).

2-Phenethylmalonic Acid (59). To 5.94 g (2.25×10^{-2} mol) of **59a** was added 2.16 g (9.00×10^{-2} mol) of LiOH dissolved in 15 mL of deionized water. After heating at reflux for 8 h, the cooled reaction was acidified with 37% HCl, extracted with ethyl ether (2 × 25 mL), dried (Na_2SO_4), and concentrated in vacuo to give a white solid. This after recrystallization from ethyl ether–hexane gave 3.19 g (1.53×10^{-2} mol) of **59**: yield 57%; mp 122–123 °C; NMR ($CDCl_3$) δ 7.20 (s, 5 H), 3.39 (t, 1 H), 2.63 (m, 2 H), 2.12 (m, 2 H); IR ($CHCl_3$) 3350–2730 (OH), 1715 (CO) cm^{-1}; MS, *m/e* (relative intensity) 208 (3.21, M^+), 91 (41.53, benzyl).

2-[(*p*-Chlorophenyl)phenylmethoxy]acetic Acid (60). To 25 mL of dry THF were added 2.00 g (9.15×10^{-3} mol) of *p*-chlorobenzhydrol and 1.27 g (9.14×10^{-3} mol) of bromoacetic acid. To the reaction in portions was added 1.27 g (2.65×10^{-2} mol) of NaH (50% oil dispersion) followed by heating the reaction at reflux overnight. The cooled reaction was concentrated in vacuo and 6 mL of deionized water added to the flask. This was acidified, extracted with ethyl ether (3 × 20 mL), dried (Na_2SO_4), and concentrated in vacuo to give a brown oil. The crude product was chromatographed on 12 g of silica gel, using a solvent system of 90:10 hexane–ethyl acetate, to give 1.66 g (6.00×10^{-3} mol) of **60** as a clear oil: yield 66%; NMR (acetone-d_6) δ 7.32 (s, 9 H),

5.62 (s, 1 H), 4.07 (s, 1 H); IR ($CHCl_3$) 3500–2500 (OH), 1720 (CO) cm^{-1}; MS, m/e (relative intensity) 277 (8.13, $M^+ - 1$), 275 (28.87, $M^+ - 1$), 201 (45.48, $M^+ - OCH_2CO_2H$). Anal. ($C_{15}H_{13}O_3Cl$) C, H, Cl.

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Registry No. 1, 38206-97-2; [1] (phenol), 2432-14-6; 2, 588-22-7; 3, 575-89-3; 3 (phenol), 88-06-2; 4, 588-20-5; 4 (phenol), 59-50-7; 5, 1878-91-7; 5 (phenol), 106-41-2; 6, 94-75-7; 7, 122-88-3; 8, 1878-94-0; 8 (phenol), 540-38-5; 9, 74592-71-5; 9 (phenol), 697-82-5; 10, 5406-14-4; 10 (phenol), 108-68-9; 11, 15267-49-9; 11 (phenol), 1073-72-9; 12, 19545-95-0; 12 (phenol), 88-04-0; 13, 7356-41-4; 13 (phenol), 95-87-4; 14, 25181-66-2; 14 (phenol), 6627-55-0; 15, 19728-23-5; 15 (phenol), 500-99-2; 16, 13335-73-4; 16 (phenol), 95-65-8; 17, 3405-88-7; 17 (thiophenol), 106-54-7; 18, 90296-05-2; 18 (thiophenol), 771-62-0; 19, 2976-74-1; 20, 575-90-6; 20 (phenol), 87-65-0; 21, 3406-76-6; 21 (thiophenol), 106-53-6; 22, 90296-06-3; 23, 84998-84-5; 23 (phenol), 118-79-6; 24, 882-09-7; 24 (phenol), 106-48-9; 25, 21248-54-4; 25 (thiophenol), 3773-14-6; 26, 90296-07-4; 26 (phenol), 5150-42-5; 27, 122-59-8; 27 (phenol), 108-95-2; 28, 1798-04-5; 28 (phenol), 98-54-4; 29, 2298-36-4; 29 (phenol), 123-30-8; 30, 90296-08-5; 30 (phenol), 7463-51-6; 31, 65876-10-0; 31 (phenol), 642-71-7; 32, 1798-11-4; 32 (phenol), 100-02-7; 33, 82513-28-8; 33 (alcohol), 1805-32-9; 34, 90296-09-6; 34 (alcohol), 1777-82-8; 35, 90296-10-9; 35 (alcohol), 60211-57-6; 36, 35513-00-9; 36 (alcohol), 873-76-7; 37, 90296-11-0; 38, 90296-12-1; 38 (alcohol), 90296-27-8; 39, 90296-13-2; 39 (alcohol), 6966-10-5; 40, 82499-60-3; 40 (alcohol), 873-75-6; 41, 90296-14-3; 41 (alcohol), 16308-92-2; 42, 82499-61-4; 43, 90296-15-4; 43 (alcohol), 18282-51-4; 44, 90296-16-5; 44 (alcohol), 2215-78-3; 45, 90296-17-6; 45 (alcohol), 4685-50-1; 46, 90296-18-7; 46 (alcohol), 4170-90-5; 47, 17742-50-6; 47 (thiol), 19552-10-4; 48, 51934-40-8; 48 (alcohol), 589-18-4; 49, 90296-19-8; 49 (alcohol), 27129-87-9; 50, 90296-20-1; 50 (thiol), 90296-28-9; 51, 88920-24-5; 51 (alcohol), 105-13-5; 52, 90296-21-2; 52a, 90296-22-3; 53, 1878-58-6; 53 (phenol), 1470-94-6; 54, 25812-30-0; 55, 90296-23-4; 55 (phenol), 20279-16-7; 56, 6331-61-9; 57, 90296-24-5; 57 (phenol), 2443-58-5; 58, 52160-84-6; 58a, 90296-25-6; 59, 3709-21-5; 59a, 6628-68-8; 60, 90296-26-7; 60 (alcohol), 119-56-2; 61, 41859-67-0; diethyl malonate, 105-53-3; 2-bromoethyl *p*-bromophenyl ether, 18800-30-1; benzyl salicylate, 118-58-1; (2-bromoethyl)benzene, 103-63-9.

[(*E*)-1-[^{123}I]Iodo-1-penten-5-yl]triphenylphosphonium Iodide: Convenient Preparation of a Potentially Useful Myocardial Perfusion Agent

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A rapid iodination method has been developed for the synthesis of the new ^{123}I-labeled phosphonium cation [(*E*)-1-iodo-1-penten-5-yl]triphenylphosphonium iodide by I^+ treatment of the corresponding *trans*-vinylboronic acid. This new model myocardial perfusion agent is obtained after purification in 25–50% yield in <1 h. High myocardial uptake (5 min, 2.38% dose/g) with prolonged retention (3 h, 2.21% dose/g) was observed in rats. In addition, heart/blood ratios were high and continued to increase over a 1-day period (5 min, 17:1; 60 min, 23:1; 3 h, 27:1; 1 day, 158:1). In rats, the liver uptake was moderate (5 min, 1.40% dose/g; 60 min; 0.25% dose/g). Excellent myocardial images were obtained in a dog.

Thallium-201, which is the most widely used cationic radiopharmaceutical for the evaluation of coronary artery disease (CAD) and is a powerful tool for the differentiation of ischemia from irreversible myocardial damage,[1] has the disadvantages of inefficient detection of its low-energy X-rays and redistribution during the imaging period. A myocardial perfusion agent labeled with an isotope having more attractive radionuclidic properties would be an advantage. In addition, nuclear medicine techniques could be of even greater benefit to the cardiologist if agents were available for measuring early indices of myocardial disease as well as regional perfusion.

Several radioiodinated organic cations, including ammonium cations such as *m*-[^{125}I]iodobenzyldimethylammonium,[2,3] [^{131}I]iodomethyltrimethylammonium,[4] and 4-[^{125}I]iodophenyltrimethylammonium,[5] have been used for myocardial imaging in experimental animals. Although encouraging results were observed in these preliminary animal studies, these agents have evidently not been pursued further, and no human studies have been reported.

More recently, interesting in vitro physiological properties of organic phosphonium cations have indicated the potential use of radiolabeled cations for in vivo evaluation of heart disease, which now takes on a new importance. The in vitro cell uptake of a model agent, tetraphenylphosphonium bromide (TPP), has been shown to correlate with the resting transmembrane potential in neuroblastoma-glioma hybrid cells,[6] mouse neuroblastoma,[7] guinea pig brain synaptosomes,[8] and, more importantly, in cardiac membrane vesicles.[9] Woo and colleagues have demon-

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