Articles

Synthesis and Structure−Activity Relationships of Chiral Allosteric Modifiers of Hemoglobin[#]

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Received May 8, 2000

A series of allosteric effectors of hemoglobin, 2-(aryloxy)-2-alkanoic acids, was prepared to investigate the effect of the stereocenter on allosteric activity. The chiral analogues were based on the lead compound, RSR13 (**3b**), with different alkyl/alkanoic and cycloalkyl/cycloalkanoic groups positioned at the acidic chiral center. Of the 23 racemic molecules synthesized, 5 were selected for resolution based on structure−activity relationships. One chiral analogue, (−)-(1*R*,2*R*)-1-[4-[[(3,5-dimethylanilino)carbonyl]methyl]phenoxy]-2-methylcyclopentanecarboxylic acid (**11**), exhibited greater in vitro activity in hemoglobin solutions than its antipode, racemate, and RSR13. Compound (−)-(1*R*,2*R*)-**11** was equipotent with RSR13 in whole blood, is a candidate for in vivo animal studies, and if efficacious and safe has a potential for use in humans. In general, it was found that chirality affects allosteric effector activity with measurable differences observed between enantiomers and the racemates.

Introduction

Human hemoglobin (Hb) is a tetrameric allosteric protein composed of two α-chains and two *β*-chains. The tetramer equilibrates between the deoxy tense (T) state and oxy relaxed (R) state. The subunits are bisected by a molecular 2-fold axis of symmetry creating a central water cavity, which narrows in the R state. The allosteric equilibrium is influenced by allosteric modifiers. The major regulatory allosteric effector in vivo in humans and most mammals is 2,3-diphosphoglycerate (2,3-DPG). Other naturally occurring Hb effectors include chloride ions, protons, and temperature. Modifiers that decrease the oxygen affinity such as 2,3-DPG act by adding constraints (intersubunit salt bridge interactions) to the T state.[1] Oxygen affinity decreasing agents have several potential clinical applications in the treatment of diseases or illnesses associated with hypoxia, i.e., stroke, angina, trauma, etc., as well as for the radiosensitization of hypoxic tumors and to increase the shelf life of stored blood.[2,3]

Several synthetic agents have been reported to lower the oxygen affinity of Hb (Figure 1). In the search for an antisickling agent, Abraham and co-workers discovered that the antilipidemic drug clofibric acid (**1**) lowered the oxygen affinity of Hb.[4,5] Perutz and Poyart followed with a report that bezafibrate (**2**), another

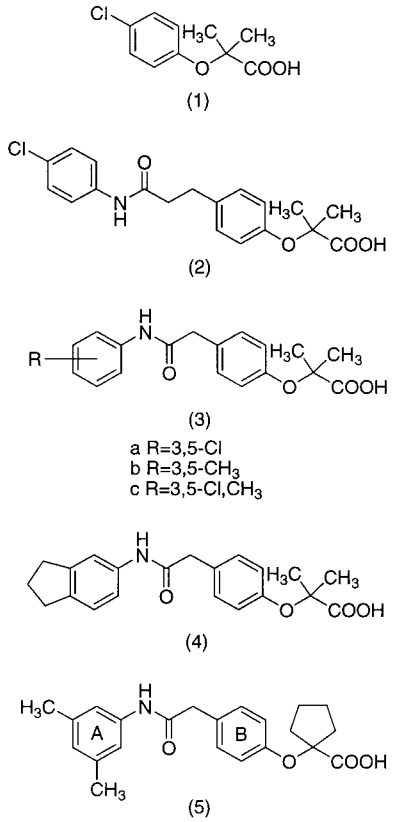

Figure 1. Structures of allosteric modifiers that decrease oxygen affinity. Phenyl rings have been denoted A and B.

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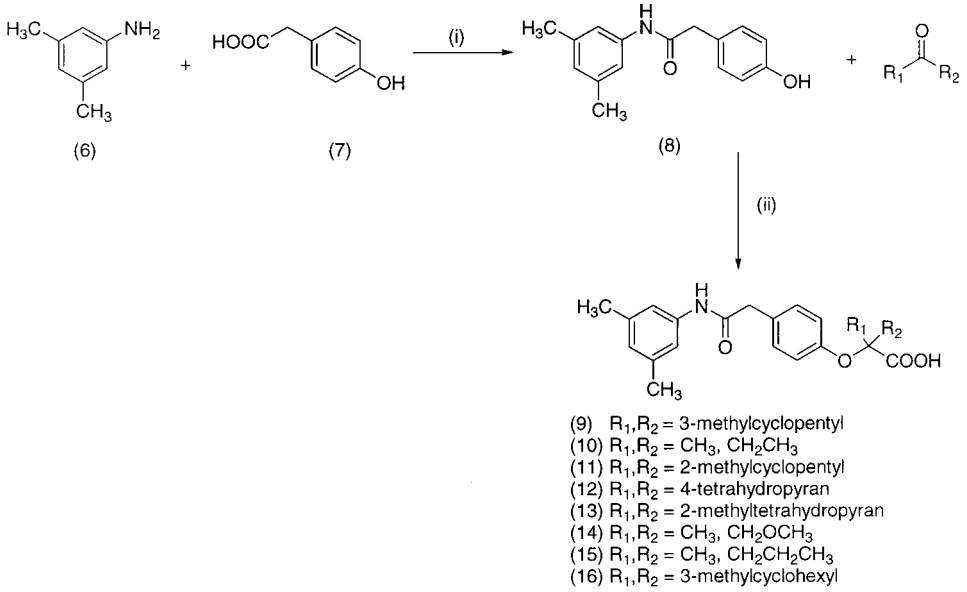
[#] Abbreviations: Hb, hemoglobin; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; SAR, structure−activity relationships.

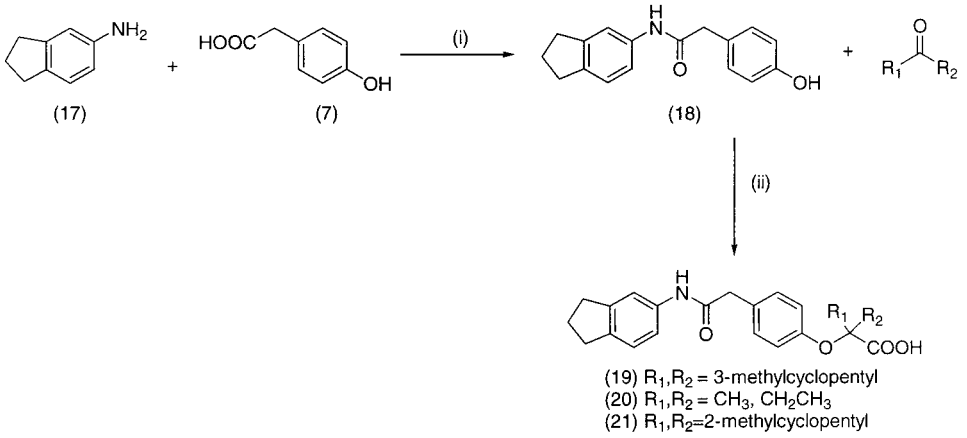
10.1021/jm000199q CCC: $19.00
Published on Web 11/18/2000

Scheme 1[a]



[a] Reagents and conditions: (i) xylene, reflux, 3 days; (ii) $CHCl_3$, NaOH.

Scheme 2[a]



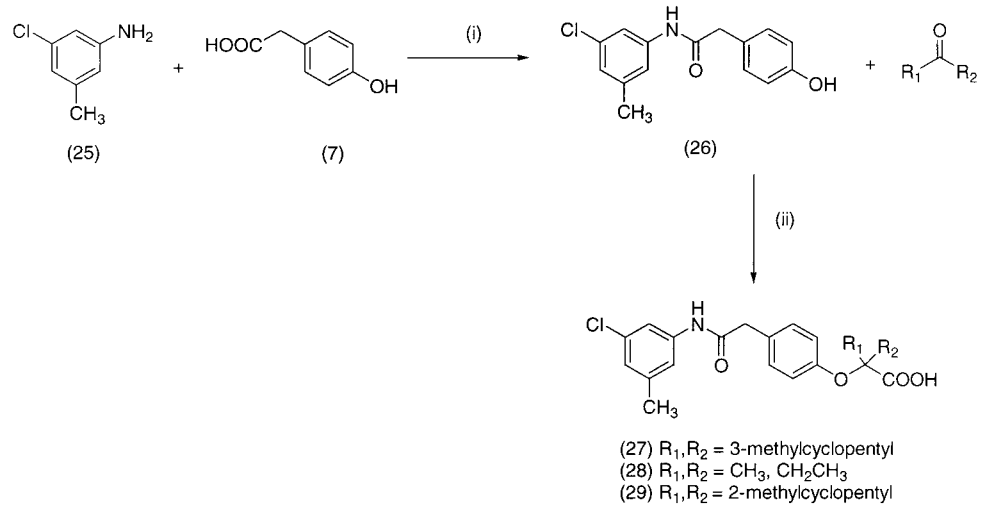
[a] Reagents and conditions: (i) xylene, reflux, 3 days; (ii) $CHCl_3$, NaOH.

antilipidemic agent, produced a greater decrease in oxygen affinity than clofibric acid.[6] X-ray crystallography studies of bezafibrate complexed with Hb by Perutz, Abraham, and colleagues revealed that two symmetrically related molecules bind to the protein near the top of the α-subunits and extend to the α,β-interface.[7,8] Lalezari and co-workers showed that shortening the four-atom bridge to a three-atom urea bridge produced even more potent allosteric modifiers, but their potential as clinical agents was limited due to loss of activity in the presence of serum albumin and plasma proteins.[9−11] Abraham et al. designed and synthesized a series of fibrate analogues that replaced the urea linkage with an amide bridge and a variety of substitutions on ring A (Figure 1, structure **3**).[12] Compounds RSR4 (**3a**) and RSR13 (**3b**) retained strong allosteric effector activity in the presence of serum albumin and plasma proteins making them candidates for clinical trials. RSR4 was a stronger effector than RSR13 in Hb solution studies and about equipotent in whole blood studies.[13−17] However, RSR13 was chosen as the candidate for phase I clinical trials with the rationale that the phenyl methyl substitution might be better tolerated in vivo in animal studies. This proved to be correct. Currently, RSR13 is in phase III clinical trials for the treatment of brain metastasis in conjunction with radiation therapy.

We have continued to search for a more potent molecule than RSR13 by modifying (1) the phenyl ring A, (2) the three-atom bridge between aromatic rings, and (3) the *gem*-dimethyl group. Prior to this study, the most potent analogues of RSR13 included a 3-methyl-5-chloro derivative (KDD86, **3c**), an indanyl derivative (RSR46, **4**), and a cyclopentyl derivative (JP7, **5**).[18,19] The effect of an asymmetric center on the allosteric activity of Hb has not been investigated.

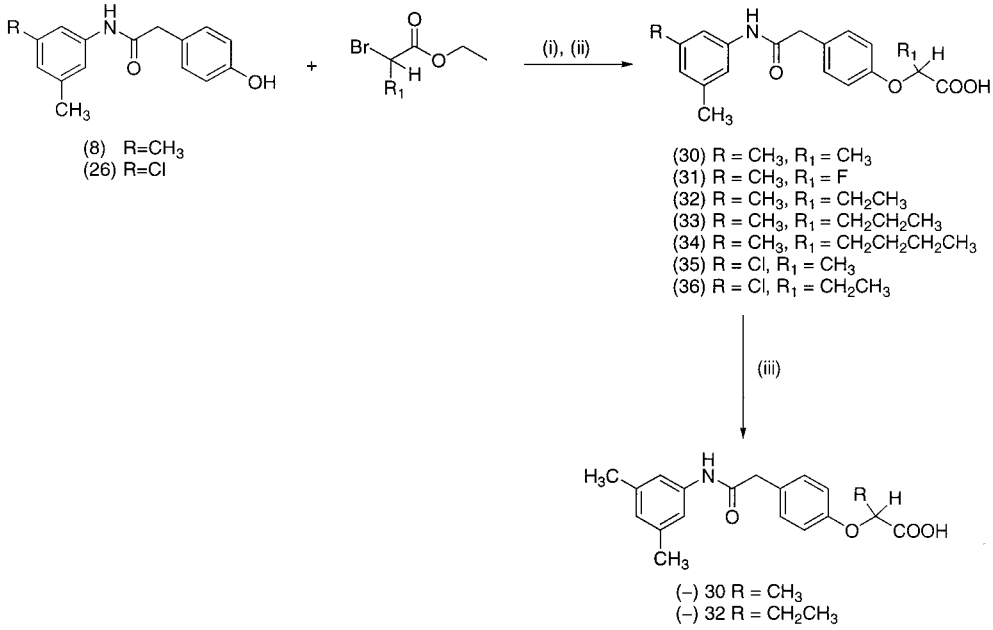
To determine the effect of a stereocenter on allosteric activity, several chiral analogues of RSR13, RSR46, JP7, and KDD86 were designed by replacing the *gem*-dimethyl group with alkyl groups, substituted cycloalkyl groups, and cycloalkyl groups with a heteroatom in the ring. This study describes the synthesis of the chiral allosteric modifiers, their resolution into enantiomers, and measurement of their ability to shift the oxygen-binding curve of Hb toward the low-affinity state. Separation into enantiomers was based on degree of activity and chemical diversity among the racemates.

Scheme 3[a]



[a] Reagents and conditions: (i) EDAC, HOBt, 12 h, rt; (ii) $CHCl_3$, NaOH.

Scheme 4[a]



[a] Reagents and conditions: (i) K_2CO_3, DMF; (ii) 10% NaOH, EtOH; (iii) cinchonidine, EtOH.

Racemates and enantiomers were subjected to in vitro testing with Hb solutions and in whole blood for structure−activity relationships (SARs).

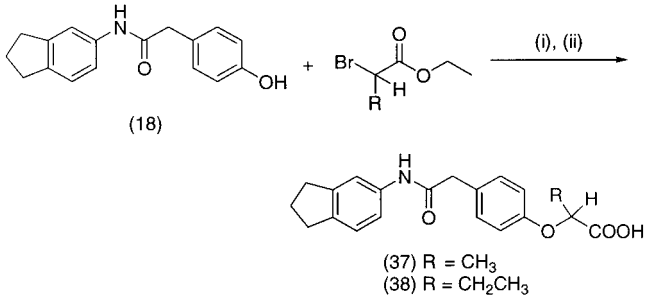
Results and Discussion

Synthesis. The synthesis of the proposed compounds involved central intermediate amidophenols **8**, **18**, and **26**. Schemes 1 and 2 were followed to produce amidophenols **8** and **18** where 3,5-dimethylaniline or 5-aminoindan was condensed with 4-hydroxyphenylacetic acid in refluxing xylene over a 3-day period. While 3,5-dimethylaniline and 5-aminoindan were both readily available, 3,5-chloromethylaniline (**25**) was prepared by following a literature procedure. Thus, a chlorination of 4-methyl-2-nitroaniline with *N*-chlorosuccinimide, followed by diazotization to remove the amino group, gave 3-chloro-5-nitrotoluene, **24**. Reduction of **24** using tin(II) chloride dihydrate yielded the desired 3-chloro-5-methylaniline, **25**. 1Hydroxybenzotriazole (HOBt) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDAC) were used to form the amide bond between **25** and the 4-hydroxyphenyl acetic acid (Scheme 3).

Schemes 1, 2, and 3−5 were utilized to produce the racemates. The previously reported α-aryloxyisobutyric acid analogues were obtained via reaction of amidophenols with acetone−chloroform in the presence of sodium hydroxide.[12,20,21] In this process, the appropriate ketone was substituted for acetone in tetrahydrofuran to obtain the proposed compounds **9**−**16**, **19**−**21**, and **27**−**29** (Schemes 1−3). The alternate method to prepare compounds **30**−**38** followed Schemes 4 and 5. The latter method employed condensation of the corresponding amidophenol with an α-bromo ester in the presence of base followed by base hydrolysis of the ester to give the desired α-aryloxy acid product.[22]

Resolution and Stereochemistry. Crystallization from ethanol of (±)-**30** cinchonidine salt, the monomethyl analogue of RSR13, gave (−)-**30**. The optically pure

Scheme 5[a]



[a] Reagents and conditions: (i) K_2CO_3, DMF; (ii) 10% NaOH, EtOH.

antipode (+)-**30** was recovered from the mother liquor by crystallization. The same method was used to obtain the monoethyl RSR13 analogue (−)-**32**. Enriched (+)-**32** was obtained from the mother liquor by crystallization. Enantiomers of JP7 analogues 2-methylcyclopentyl (**11**) and 2-methyltetrahydrofuran (**13**) and the methylethyl RSR13 analogue **10** were separated and isolated using a Chiracel OD semipreparative HPLC column.[23] The column was also used in the purification of enriched (+)-**32**.

Analysis of the alkyl-substituted analogues **10**, **30**, and **32** on the Chiracel OD column revealed that the (−)-isomer eluted first and the (+)-isomer second (Figure 2). Cycloalkyl racemates **11** and **13** showed the opposite pattern with the (+)-isomer eluting first and the (−)-isomer second. Furthermore, HPLC chromatograms showed that racemates **11** and **13** were composed of only two of the four possible stereoisomers. Sharp melting points, optical rotation measurements, and proton NMR of the purified enantiomers confirmed the presence of only one set of diastereomers.

The absolute configurations of the enantiomers were not established. Configurational studies on 2-phenoxypropionic acids have shown that isomers with (−)-rotation have the (*S*)-configuration and the (+)-isomers have the (*R*)-configuration.[24,25] However, it is not possible by direct comparison of the optical rotation of the structurally similar phenoxy acids to unequivocally establish the stereochemical assignments of the alkyl-substituted RSR13 analogues as (+)-(*R*) and (−)-(*S*).

The appearance of only one set of diastereomers for the methylcyclopentyl or methylcyclotetrahydrofuran derivatives **11** and **13** was rationalized in the following way. Attack by the phenoxide ion (Figure 3) on the least sterically hindered dichloroepoxide intermediates of **11** and **13** with the configuration (1*R*,2*S*) and (1*S*,2*R*) (Figure 4) suggests that one set of diastereomers would be favored (Figure 5). Crystallographic studies to be published elsewhere confirm the stereochemistry of the (±)-**11** diasteromeric pair suggested by the mechanism.

Biological Evaluation. Allosteric SARs were determined for the synthetic chiral effectors comparing the shift in the oxygen-binding curve of Hb solutions using a Hemox analyzer. In vitro biological activity testing in the presence of plasma proteins was also performed on selected enantiomers and racemates (multipoint tonometry and blood gas analysis) to screen for candidates with potential clinical use. SAR studies compared the degree in shift in P_{50} values, i.e., the partial pressure of molecular oxygen necessary to half-saturate Hb. An

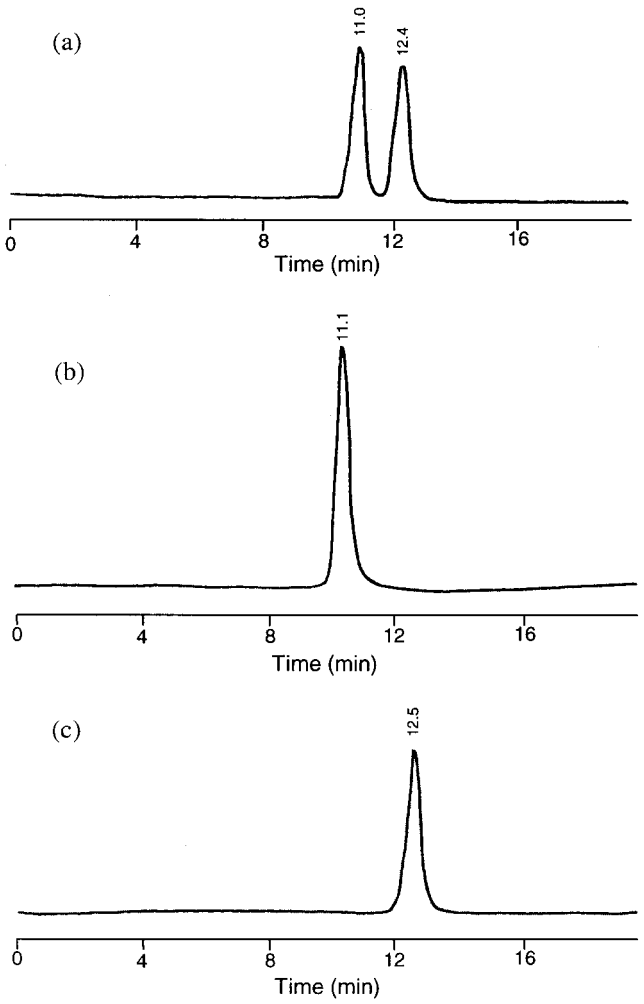


Figure 2. HPLC enantioseparation of (a) racemic mixture (±)-**10**, (b) (−)-**10**, and (c) (+)-**10**. Conditions: Chiracel OD (0.46 × 25 cm); mobile phase 90:10 heptane/ethanol with 1% TFA; flow rate 1.0 mL/min; detection wavelength 254 nm.

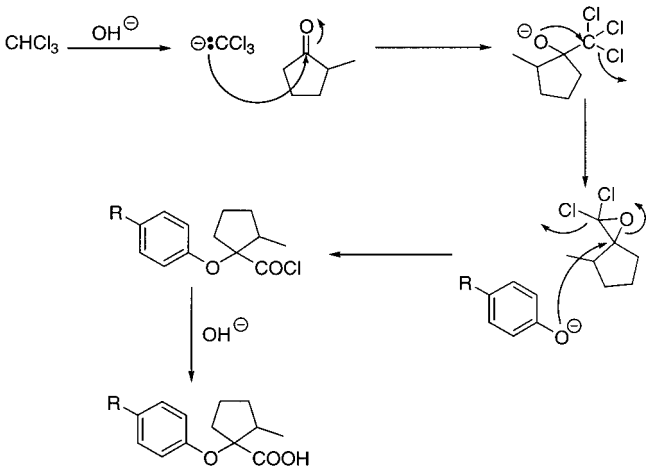


Figure 3. Mechanism for the ketone−chloroform reaction in the synthesis of compound **11**.

effector that decreases Hb oxygen affinity increases the P_{50} value relative to the control. Thus, the activity or potency of each analogue could be expressed by the ratio P_{50}(effector)/P_{50}(control). Tables 1 and 2 summarize the P_{50} and the Hill coefficient values (n_{50}) at saturation. The slope of the logarithmic Hill plot is known as the Hill coefficient. The Hill coefficient measures the degree

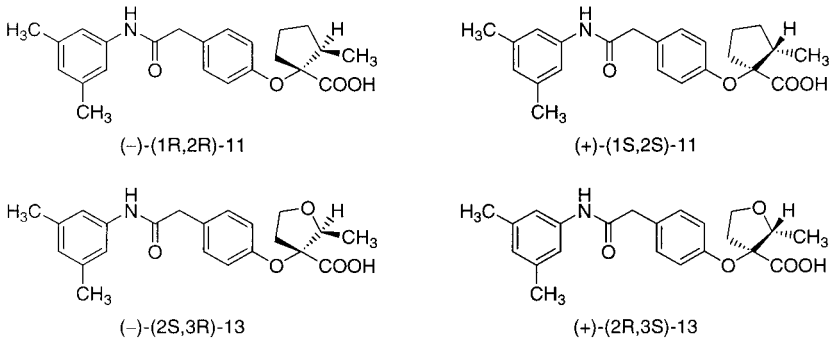


Figure 4. Structure of proposed configurations for the enantiomers of compounds **11** and **13**.

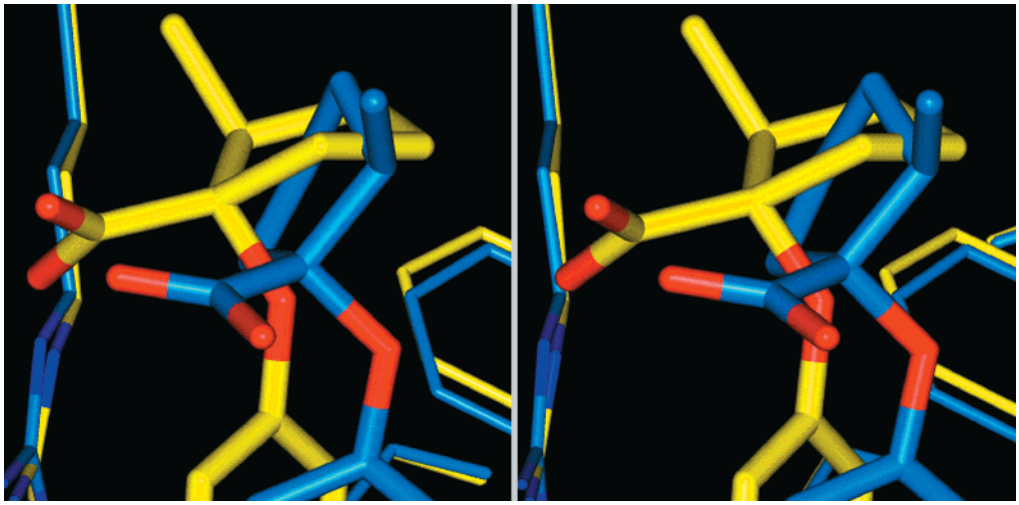


Figure 5. Close-up stereoview of the superimposed binding sites for (+)-(1*S*,2*S*)-**11** and (−)-(1*R*,2*R*)-**11** Hb complexes showing the stereochemistry at the chiral centers of the effectors. The effectors (+)-(1*S*,2*S*)-**11** and (−)-(1*R*,2*R*)-**11** and their binding site residues are shown in light blue and yellow, respectively. Oxygen and nitrogen atoms are dark blue and red, respectively.

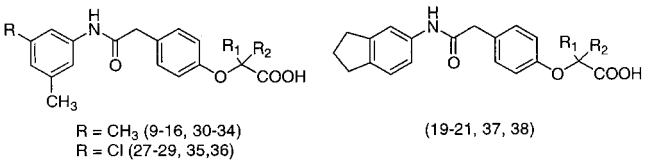
of cooperativity of ligand binding to an allosteric protein; the normal range for human blood is 2.7−3.2. Tables 1−3 present the results from Hb solution and whole blood studies given as P_{50}, ΔP_{50}, and Hill coefficient (n_{50}) values.

SARs. The new analogues differ in their substitution at the *gem*-dimethyl position α to the carboxylate group. All of the synthesized derivatives from this study increased P_{50} exhibiting a wide range of allosteric effector activity (Table 1). In general, similarly substituted compounds from the 3,5-dimethylphenyl and 3,5-chloromethylphenyl series appeared to be equal in potency. Corresponding compounds with the indanyl group substitution were less active. The majority of the compounds showed good cooperativity in Hb solutions, $n_{50} = 2.3-2.7$ (Table 1).

Removal of one of the methyl groups from the *gem*-dimethyl position, **30**, significantly reduced activity. Substitution of fluoro group for methyl resulted in further decreased activity. The length of the monoalkyl group enhances activity, as exhibited in compounds **30** and **32**−**34**. The activity of compounds with a monoalkyl substitution tended to increase with the size of the group (butyl ≥ propyl > ethyl > methyl). The effect seemed to plateau going from the propyl to the butyl group with only a slight increase in the P_{50}. Replacement of one of the methyl groups with an ethyl group decreased activity by one-half, and increasing the length of the chain further reduced P_{50} (Table 1). The position of the methyl substitution on the cyclopentyl ring appears to be important, 2-methyl being > 3-methyl. The 2-methylcyclopentyl compound (**11**) was nearly twice as active as the 3-methyl derivative (**9**). A slight loss in activity was observed when the size of the cycloalkyl ring was increased to a six-membered ring. Oxygen substitutions to alkyl chains and cycloalkyl groups reduced activity. This observation was most apparent with compound **13**. Compounds **11** and **13** are structurally similar except for an ether oxygen substitution in the cyclopentyl ring, yet **11** was nearly 3 times more potent than **13**. Compound **11** was the most potent compound from the study exhibiting activity comparable to that of RSR13 and JP7.

In general, the results of the enantiomers showed that the stereocenter does have an effect on allosteric activity (Table 2). A small difference in the P_{50} values was observed between the enantiomers of **30**: (+)-**30** was slightly more active than (−)-**30**. While the specific activity for compound **30** is not high and the differences in shifting the Hb solution oxygen-binding curve between enantiomers and the racemate are small, the whole blood studies show the same trend: i.e., the (+)-enantiomer exhibited stronger activity than the (−)-enantiomer. The same trend was observed for the isomers of **32**. The (+)-isomer was nearly twice as active as its mirror image, (−)-isomer. (+)-**32** was also more potent than (+)-**30**, which further indicated that the longer alkyl group enhances activity. A similar difference in activity between the enantiomers of **10** was also observed: (+)-**10** > (−)-**10**. The difference in activity

Table 1. Results of Hb Solution Studies for Synthesized Analogues[a]



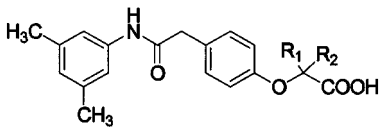
no.	R_1	R_2	P_{50}e[b]	P_{50}e/P_{50}c[c]	n_{50}[d]
3b (RSR13)	CH_3	CH_3	23.4	4.7	2.3
3c (KDD86)	Cl	CH_3	21.0	4.4	2.4
4 (RSR46)	CH_3	CH_3	15.3	3.0	2.6
5 (JP7)	cyclopentyl		25.7	5.1	2.4
9	3-methylcyclopentyl		12.9	2.6	2.5
10	CH_3	CH_2CH_3	13.3	2.7	2.4
11	2-methylcyclopentyl		21.6	4.3	2.3
12	4-tetrahydropyran		7.6	1.5	2.4
13	2-methyltetrahydrofuran		7.6	1.5	2.4
14	CH_3	CH_2OCH_3	8.4	1.7	2.4
15	CH_3	$CH_2CH_2CH_3$	11.7	2.3	2.7
16	3-methylcyclohexyl		10.3	2.1	2.5
19	3-methylcyclopentyl		12.7	2.5	2.6
20	CH_3	CH_2CH_3	10.6	2.1	2.3
21	2-methylcyclopentyl		13.8	2.8	2.6
27	3-methylcyclopentyl		14.0	2.8	2.4
28	CH_3	CH_2CH_3	16.6	3.3	2.5
29	2-methylcyclopentyl		21.9	4.4	2.4
30	CH_3	H	9.3	1.9	2.6
31	F	H	5.9	1.2	2.5
32	CH_2CH_3	H	13.0	2.6	2.7
33	$CH_2CH_2CH_3$	H	16.1	3.2	2.5
34	$CH_2CH_2CH_2CH_3$	H	17.0	3.4	2.5
35	CH_3	H	9.4	1.9	2.6
36	CH_2CH_3	H	13.1	2.6	2.6
37	CH_3	H	6.3	1.3	2.7
38	CH_2CH_3	H	9.1	1.8	2.6

[a] All studies were carried out at 50−60 mM heme concentration in the presence of 0.5 mM effector concentration. All solutions were prepared in 100 mM bis-Tris buffer, pH 7.2. See Experimental Section for more details. [b] P_{50}e is the oxygen pressure in mmHg at which Hb is 50% saturated with oxygen in the presence of the effector. [c] Ratio of P_{50}e to P_{50}c (P_{50} control value with no effector present, 5.0 mmHg). [d] The Hill coefficient at 50% saturation (n_{50}) is calculated from the Hill equation by linear regression analysis of data points between 40% and 60% oxygen saturation (n_{50} control value with no effector present, 2.7).

between the enantiomers of compound **13** did not appear to be as significant. The most interesting observation among the enantiomers involved compound **11**. The results showed that (−)-(1*R*,2*R*)-**11** was more potent than the (+)-(1*S*,2*S*)-**11** isomer, and furthermore, (−)-**11** was more potent in Hb solutions than both RSR13 and JP7 with a P_{50} of 30.7 mmHg (Table 2). The activity observed for some of the racemates was not an average of the P_{50} value for the enantiomeric pair. The difference in activity may be related to an intrinsic activity factor discussed in one of our earlier papers.[26]

The racemates and the enantiomers of compounds **11** and **30** were also analyzed in vitro using human whole blood. Results from the whole blood study revealed the same general trend in activity as observed in Hb solutions. The compounds lowered the n_{50} which means to a small extent all of the compounds reduce the cooperativity of Hb. Generally, compounds with a high P_{50} value cause the n_{50} to decrease. (+)-**30** was more potent than (−)-**30**. In addition, (−)-**11** was significantly more active than (+)-**11** and equipotent to RSR13 and JP7. However, the whole blood results revealed that the

Table 2. Results of Hb Solution Studies for Resolved Enantiomers[a]



no.	R_1	R_2	P_{50}e[b]	P_{50}e/P_{50}c[c]	n_{50}[d]
(+)-**10**	CH_3	CH_2CH_3	17.4	3.5	2.4
(−)-**10**	CH_3	CH_2CH_3	10.5	2.1	2.3
(+)-(1*S*,2*S*)-**11**	2-methylcyclopentyl		19.9	4.0	2.6
(−)-(1*R*,2*R*)-**11**	2-methylcyclopentyl		30.7	6.1	2.4
(+)-**13**	2-methyltetrahydrofuran		7.7	1.5	2.8
(−)-**13**	2-methyltetrahydrofuran		9.3	1.9	2.6
(+)-**30**	CH_3	H	9.8	2.0	2.6
(−)-**30**	CH_3	H	6.6	1.3	2.5
(+)-**32**	CH_2CH_3	H	18.0	3.6	2.7
(−)-**32**	CH_2CH_3	H	9.8	2.0	2.8

[a] All studies were carried out at 50−60 mM heme concentration in the presence of 0.5 mM effector concentration. All solutions were prepared in 100 mM bis-Tris buffer, pH 7.2. See Experimental Section for more details. [b] P_{50}e is the oxygen pressure in mmHg at which Hb is 50% saturated with oxygen in the presence of the effector. [c] Ratio of P_{50}e to P_{50}c (P_{50} control value with no effector present, 5.0 mmHg). [d] The Hill coefficient at 50% saturation (n_{50}) is calculated from the Hill equation by linear regression analysis of data points between 40% and 60% oxygen saturation (n_{50} control value with no effector present, 2.7).

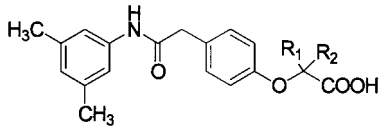
more active isomer from both **11** and **30** was only slightly more active than the racemic mixture. This suggests that there are pharmacokinetic and/or bioavailability factors involved, possibly enantioselective plasma protein binding. Enantioselective plasma protein binding has been observed for several drugs including ibuprofen, warfarin, and propranolol where the enantiomers differ in their affinity for plasma proteins resulting in differing free fractions of the isomers.[27−30] Further studies need to be initiated to investigate this possibility for these chiral allosteric effectors.

Summary and Conclusions

The chiral allosteric effectors synthesized in this study decreased the oxygen affinity of Hb. SARs showed that modifying the *gem*-dimethyl group of RSR13 with various alkyl groups reduced activity. The 2-methylcyclopentyl derivative of RSR13 was the most potent allosteric effector from this study demonstrating that methyl substitution on the cyclopentyl ring was better tolerated in the 2-position than the 3-position. Furthermore, ether oxygen substitutions for methylene groups significantly decreased activity.

Most importantly, this investigation revealed that enantiomeric selectivity produces differences in allosteric activity. Enantiomers were observed to possess different degrees of potency. The replacement of the *gem*-dimethyl group on RSR13 (**3b**) with alkyl substituents showed that the (+)-isomer was more potent than the (−)-isomer. In whole blood, (−)-(1*S*,2*S*)-**11** exhibited strong allosteric activity, making it one of the most potent chiral allosteric effector reported to date. Since the Hb solution studies do not include DPG, a natural allosteric effector, they show a large change in P_{50} compared to the whole blood. Therefore, the amplitude of the P_{50} effects cannot be compared directly with whole

Table 3. Results of in Vitro Whole Blood Studies[a]



no.	R_1	R_2	P_{50}c[b]	P_{50}e[c]	$\Delta P_{50} \pm$ SD[d]	$n_{50} \pm$ SD[e]
(±)-**11**	2-methylcyclopentyl		26.5	66.0		
				67.2	40.1 ± 0.9	1.6 ± 0.09
(+)-(1*S*,2*S*)-**11**	2-methylcyclopentyl		25.7	49.7		
				46.8		
				42.4	20.6 ± 3.6	1.8 ± 0.12
(−)-(1*R*,2*R*)-**11**	2-methylcyclopentyl		27.9	78.5		
				72.5	47.6 ± 4.2	1.6 ± 0.04
(±)-**30**	CH_3	H	27.2	47.2		
				43.5	18.2 ± 2.6	2.0 ± 0.03
(+)-**30**	CH_3	H	27.3	47.4		
				48.7	20.7 ± 0.7	1.9 ± 0.04
(−)-**30**	CH_3	H	27.8	39.7		
				40.5	12.3 ± 0.6	2.2 ± 0.05
3b (RSR13)	CH_3	CH_3	29.8	76.5		
				73.4	45.2 ± 2.2	1.47 ± 0.05
5 (JP7)	cyclopentyl		28.9	74.7		
				67.7	42.3 ± 4.9	1.66 ± 0.3

[a] All studies were carried out at 2.5 mM Hb concentration in the presence of 5.0 mM effector concentration. All solutions were prepared in DMSO. See Experimental Section for more details. [b] P_{50}c is the control value in mmHg (average P_{50}c value = 27.6 ± 1.3, n = 8). [c] P_{50}e is the value in the presence of the effector in mmHg. [d] $\Delta P_{50} = (P_{50}\text{e} - P_{50}\text{c})$ in mmHg. [e] The Hill coefficient at 50% saturation (n_{50}) in the presence of effector (average n_{50} control value = 2.7 ± 0.1, n = 8).

blood studies. Plasma protein binding could be a significant factor in whole blood studies since that affects the red cell membrane permeability of racemates and enantiomers and hence the Hb allosteric activity. SARs and differences in binding interactions based on high-resolution X-ray crystallography studies with enantiomers bound to Hb have been investigated and will be discussed in detail in a future manuscript.

Experimental Section

Chemistry. All reagents and starting materials used in the syntheses were purchased from Aldrich, Fluka, or Sigma and used without purification. All solvents were purchased from Aldrich or Fisher. Silica gel-coated plates (0.25-mm thickness) from Analtech, Inc. were used for thin-layer chromatography (TLC). Separations were visualized by ultraviolet (UV) lamp or iodine exposure. Column chromatography was performed on silica gel (Merck, grade 9385, 230−400 mesh). Melting points (mp) were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Varian Gemini 300-MHz spectrophotometer and are reported in parts per million (δ, ppm) with tetramethylsilane as the internal standard. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA), and results are within ±0.4% of the theoretical value. All intermediate compounds were analyzed but are not reported. Their purity was determined by TLC and ^{1}H NMR.

4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenol (8). A mixture of 4-hydroxyphenylacetic acid (20.0 g, 131 mmol) and 3,5-dimethylaniline (15.9 g, 131 mmol) in xylene (100 mL) was stirred for 3 days at 160 °C with a Dean−Stark trap. The mixture was cooled to room temperature and filtered. The solid product obtained was washed with hexane (200 mL), 10% sodium bicarbonate solution (250 mL), water (200 mL), 10% hydrochloric acid (200 mL), and then water (200 mL). The beige solid was air-dried to yield 27.7 g, 82.7%. Mp: 183−185 °C. ^{1}H NMR ($CDCl_3$): δ 2.25 (s, 6H), 3.60 (s, 2H), 6.71 (s, 1H), 6.82 (d, 2H, J = 8.5 Hz), 7.05 (s, 2H), 7.13 (d, 2H, J = 8.4 Hz).

1-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-3-methylcyclopentanecarboxylic Acid (9). Sodium hydroxide (1.8 g, 45 mmol) was added to a stirred solution of 4-[[(3,5-dimethylanilino)carbonyl]methyl]phenol (1.27 g, 5 mmol) in anhydrous tetrahydrofuran (30 mL). After 15 min, 3-methylcyclopentanone (4.9 g, 50 mmol) was added, the mixture was cooled to 0 °C and anhydrous chloroform (2.4 g, 20 mmol) was added dropwise. The reaction mixture was maintained at 0 °C for 2 h and then allowed to come to room temperature while stirring overnight. Tetrahydrofuran was removed under reduced pressure. The residue was dissolved in water (150 mL) and washed with ethyl acetate (2 × 30 mL). The aqueous layer was acidified (pH 2) with concentrated HCl and extracted with ethyl acetate (3 × 40 mL). The combined organic fractions were washed with brine, dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The brown oil was purified by flash chromatography (eluent: hexane/ethyl acetate, 2:1) to afford a pale yellow powdery solid, 0.89 g, 47%. Mp: 148−153 °C. ^{1}H NMR ($CDCl_3$): δ 1.03 (d, 3H, J = 6.6 Hz), 1.27−2.31 (m, 6H), 2.24 (s, 6H), 2.40−2.66 (m, 1H), 3.60 (s, 2H), 6.73 (s, 1H), 6.78 (d, 2H, J = 8.5 Hz), 7.06 (s, 2H), 7.18 (d, 2H, J = 8.4 Hz). Anal. ($C_{23}H_{27}NO_4 \cdot 0.25H_2O$) C, H, N.

Compounds **10**−**16** were prepared using the same procedure as described above for **9**.

2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-2-methylbutanoic Acid (10). Compound **10** was prepared by reacting **8** (1.50 g, 5.88 mmol) and 2-butanone (4.23 g, 58.8 mmol) to yield a brown oil. The crude product was purified by flash chromatography (eluent: hexane/ethyl acetate 2:1) to obtain a yellow oil. Recrystallization from methylene chloride and hexane gave yellow crystals, 0.59 g, 28%. Mp: 131−133 °C. ^{1}H NMR (CD_3OD): δ 1.02 (t, 3H, J = 7.3 Hz), 1.51 (s, 3H), 1.99 (m, 2H), 2.27 (s, 6H), 3.63 (s, 2H), 6.75 (s, 1H), 6.92 (d, 2H, J = 7.0 Hz), 7.09 (s, 2H), 7.22 (d, 2H, J = 7.1 Hz). Anal. ($C_{21}H_{25}NO_4$) C, H, N.

1-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-2-methylcyclopentanecarboxylic Acid (11). 2-Methylcyclopentanone (3.46 g, 35.3 mmol) and **8** (1.0 g, 3.9 mmol) were reacted to yield a brown oil. The oil was purified by flash chromatography (eluent: hexane/ethyl acetate 3:1) to give a yellow solid. Recrystallization from methylene chloride and hexane gave a white solid, 0.30 g, 20%. Mp: 184−186 °C. ^{1}H NMR (CD_3OD): δ 1.02 (d, 3H, J = 7.2 Hz), 1.43−2.46 (m, 6H), 2.25 (s, 6H), 2.48−2.54 (m, 1H), 3.56 (s, 2H), 6.53 (s, 1H), 6.55 (d, 2H, J = 8.3 Hz), 6.94 (s, 2H), 7.00 (d, 2H, J = 8.4 Hz). Anal. ($C_{23}H_{27}NO_4 \cdot 0.25H_2O$) C, H, N.

4-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-tetrahydro-2*H*-4-pyrancarboxylic Acid (12). Tetrahydro-

4*H*-pyran-4-one (4.5 g, 45 mmol) and **8** (1.3 g, 5 mmol) were reacted to yield a yellow-brown oil. The product was purified by flash chromatography (eluent: hexane/ethyl acetate, 2:1) to afford a pale yellow solid. Recrystallization with methylene chloride and hexane gave a white solid, 0.85 g, 45%. Mp: 186−188 °C. ^{1}H NMR (CD_3OD): δ 2.05−2.23 (m, 4H), 2.29 (s, 6H), 3.63 (s, 2H), 3.79 (m, 4H), 6.79 (s, 1H), 6.92 (d, 2H, J = 8.6 Hz), 7.19 (s, 2H), 7.30 (d, 2H, J = 8.6 Hz). Anal. ($C_{22}H_{25}NO_5 \cdot 0.25H_2O$) C, H, N.

3-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-2-methyltetrahydro-3-furancarboxylic Acid (13). 2-Methyltetrahydrofuran-3-one (3.5 g, 35 mmol) and **8** (1.0 g, 3.9 mmol) were reacted to give an orange oil. Purification by column chromatography (eluent: hexane/ethyl acetate 3:1→1:2) afforded a yellow oil which upon recrystallization from methylene chloride and hexane gave a white solid, 0.47 g, 32%. Mp: 187−190 °C. ^{1}H NMR (DMSO-d_6): δ 1.16 (d, 3H, J = 6.5 Hz), 2.15 (m, 1H), 2.21 (s, 6H), 2.75 (m, 1H), 3.51 (s, 2H), 3.73 (q, 1H, J = 7.5 Hz), 3.97−4.14 (m, 2H), 6.67 (s, 1H), 6.71 (d, 2H, J = 8.6 Hz), 7.20 (s, 2H), 7.22 (d, 2H, J = 8.3 Hz). Anal. ($C_{22}H_{25}NO_5$) C, H, N.

2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-3-methoxy-2-methylpropanoic Acid (14). Methoxyacetone (10.0 g, 113 mmol) and **8** (3.22 g, 12.6 mmol) were reacted together as described for compound **9** to yield an orange-brown semisolid. The product was recrystallized from methylene chloride and hexane to give a pale yellow solid, 2.27 g, 48%. Mp: 170−172 °C. ^{1}H NMR (CD_3OD): δ 1.46 (s, 3H), 2.25 (s, 6H), 3.37 (s, 3H), 3.59 (s, 2H), 3.66 (s, 2H), 6.74 (s, 1H), 6.95 (d, 2H, J = 8.5 Hz), 7.15 (s, 2H), 7.24 (d, 2H, J = 8.5 Hz). Anal. ($C_{21}H_{25}NO_5$) C, H, N.

2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-2-methylpentanoic Acid (15). Using **8** (1.5 g, 5.9 mmol) and 2-pentanone (4.55 g, 52.9 mmol), compound **15** was prepared as described for compound **9**. The brown semisolid obtained was purified by flash chromatography (eluent: hexane/ethyl acetate 3:1→1:1). Recrystallization from methylene chloride and hexane gave a white amorphous solid, 0.70 g, 32%. Mp: 145−147 °C. ^{1}H NMR (CD_3OD): δ 0.94 (t, 3H, J = 7.3 Hz), 1.46 (s, 3H), 1.47 (m, 2H), 1.88 (m, 2H), 2.25 (s, 6H), 3.58 (s, 2H), 6.74 (s, 1H), 6.87 (d, 2H, J = 8.6 Hz), 7.15 (s, 2H), 7.23 (d, 2H, J = 8.6 Hz). Anal. ($C_{22}H_{27}NO_4$) C, H, N.

1-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-3-methylcyclohexanecarboxylic Acid (16). 3-Methylcyclohexanone (4.39 g, 39.2 mmol) and **8** (1.0 g, 3.9 mmol) were reacted together to give a light brown oil. The product was purified by flash chromatography (eluent: hexane/ethyl acetate 2:1), followed by recrystallization from methylene chloride and hexane to obtain white fluffy crystals, 0.08 g, 5%. Mp: 175−177 °C. ^{1}H NMR (CD_3OD): δ 0.93 (d, 3H), J = 6.3 Hz), 1.20 (m, 1H), 1.45−1.80 and 2.37 (m, 8H), 2.26 (s, 6H), 3.58 (s, 2H), 6.75 (s, 1H), 6.90 (d, 2H, J = 8.6 Hz), 7.15 (s, 2H), 7.22 (d, 2H, J = 8.6 Hz). Anal. ($C_{24}H_{29}NO_4$) C, H, N.

4-[[(5-Indanyl)carbonyl]methyl]phenol (18). Using 5-aminoindan (10.0 g, 75.2 mmol) and 4-hydroxyphenylacetic acid, the amide was synthesized as described for **8** to give a brown solid, 18.1 g, 90%. Mp: 148−151 °C. ^{1}H NMR ($CDCl_3$): δ 2.04 (m, 2H), 2.84 (m, 4H), 3.60 (s, 2H), 6.83 (d, 2H, J = 8.5 Hz), 7.13 (s, 2H), 7.15 (d, 2H, J = 8.5 Hz), 7.35 (s, 1H).

Compounds **19**−**21** were prepared using the same procedure as described above for **9.**

1-[4-[[(5-Indanyl)carbonyl]methyl]phenoxy]-3-methylcyclopentanecarboxylic Acid (19). 3-Methylcyclopentanone (5.9 g. 60 mmol) and **18** (1.6 g, 6.0 mmol) were reacted to yield a brown oil. Purification by flash chromatography (eluent: hexane/ethyl acetate 2:1) afforded a yellow oil. Recrystallization with ether and hexane gave yellow crystals, 0.87 g, 37%. Mp: 148−151 °C. ^{1}H NMR (CD_3OD): δ 1.06 (d, 3H, J = 6.6 Hz), 1.32−2.32 (m, 6H), 2.05 (m, 2H), 2.42−2.67 (m, 1H), 2.84 (m, 4H), 3.56 (s, 2H), 6.78 (d, 2H, J = 8.5 Hz), 7.12 (s, 2H), 7.20 (d, 2H, J = 8.5 Hz), 7.37 (s, 1H). Anal. ($C_{24}H_{27}NO_4$) C, H, N.

2-[4-[[(5-Indanyl)carbonyl]methyl]phenoxy]-2-methylbutanoic Acid (20). 2-Butanone (5.4 g, 75 mmol) and **18** (2.0 g, 7.5 mmol) were reacted to yield a brown oil. The product was purified by flash chromatography (eluent: hexane/ethyl acetate, 2:1) to give a yellow solid. Recrystallization from methylene chloride and hexane afforded a pale yellow solid, 0.85 g, 31%. Mp: 160−161 °C. ^{1}H NMR ($CDCl_3$): δ 1.01 (t, 3H, J = 7.5 Hz), 1.49 (s, 3H), 1.95 (m, 2H), 2.04 (m, 2H), 2.85 (m, 4H), 3.60 (s, 2H), 6.91 (d, 2H, J = 8.5 Hz), 7.12 (s, 2H), 7.21 (d, 2H, J = 8.5 Hz), 7.37 (s, 1H). Anal. ($C_{22}H_{25}NO_4$) C, H, N.

2-[4-[[(5-Indanyl)carbonyl]methyl]phenoxy]-2-methylcyclopentanecarboxylic Acid (21). 2-Methylcyclopentanone (3.30 g, 33.7 mmol) and **18** (1.0 g, 3.7 mmol) were reacted together as described for **9**. The product was recrystallized from acetone and ether to yield a tan amorphous solid, 0.40 g, 27%. Mp: 191−193 °C. ^{1}H NMR (CD_3OD, free acid): δ 1.02 (d, 3H, J = 7.1 Hz), 1.43−2.46 (m, 6H,), 2.05 (m, 2H), 2.48−2.55 (m, 1H), 2.84 (m, 4H), 3.57 (s, 2H), 6.75 (d, 2H, J = 8.7 Hz), 7.13 (s, 2H), 7.22 (d, 2H, J = 8.7 Hz), 7.41 (s, 1H). Anal. ($C_{24}H_{27}NO_4 \cdot 0.25H_2O$) C, H, N.

2-Chloro-4-methyl-6-nitroaniline (23). A mixture of 4-methyl-2-nitroaniline (50.0 g, 329 mmol) and *N*-chlorosuccinimide (70.3 g, 526 mmol) was refluxed in acetonitrile (200 mL) overnight. Removal of acetonitrile under reduced pressure, afforded a dark red crude oil mixture. The mixture was purified by flash chromatography (eluent: hexane/ethyl acetate 7:1→6:1) to obtain a dark orange solid, 17.6 g, 29%. Mp: 55−58 °C. ^{1}H NMR ($CDCl_3$): δ 2.28 (s, 3H), 6.41 (s, 2H), 7.38 (s, 1H), 7.90 (s, 1H).

3-Chloro-5-nitrotoluene (24). A solution of **23** (17.5 g, 94.4 mmol) in ethanol (100 mL) was allowed to cool to 0 °C and concentrated sulfuric acid was added (20 mL). Sodium nitrite (40 wt % solution in water, 13.0 g, 189 mmol) was added dropwise to the solution, and the reaction mixture was allowed to warm to room temperature and stirred for 30 min. Then the mixture was refluxed until the evolution of nitrogen gas ceased. The reaction mixture was concentrated under reduced pressure and then diluted with water (100 mL) and extracted with ethyl acetate (4 × 75 mL). The combined organic fractions were washed with brine, dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure to afford a dark red oil. The product was purified by flash chromatography (eluent: hexane/ethyl acetate 7:1) to give an orange solid, 11.2 g, 69%. Mp: 55−57 °C. ^{1}H NMR ($CDCl_3$): δ 2.46 (s, 3H), 7.50 (s, 1H), 7.94 (s, 1H), 8.03 (s, 1H).

3-Chloro-5-methylaniline (25). To a solution of **24** (7.0 g, 41 mmol) in ethanol (100 mL) was added tin(II) chloride dihydrate (45.8 g, 204 mmol) and the mixture was refluxed for 3 h. Upon cooling, ice was added to the reaction mixture and basified with 2 N NaOH. The mixture was filtered and the filtrate was concentrated under reduced pressure. The aqueous solution was extracted with ethyl acetate (4 × 50 mL). The organic layers were combined, washed with brine, dried over anhydrous $MgSO_4$ and the solvent was removed under reduced pressure to obtain a yellow-orange liquid, 5.4 g, 93%. ^{1}H NMR ($CDCl_3$): δ 2.22 (s, 3H), 3.66 (s, 2H), 6.36 (s, 1H), 6.48 (s, 1H), 6.56 (s, 1H).

4-[[(3-Chloro-5-methylanilino)carbonyl]methyl]phenol (26). To a solution of 4-hyroxyphenylacetic acid (5.9 g, 39 mmol) and 1-hydroxybenzotriazole hydrate (5.8 g, 43 mmol) in dimethylformamide (40 mL) were added 3-chloro-5-methylaniline (5.5 g, 39 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (9.0 g, 47 mmol). The reaction mixture was stirred overnight at room temperature, then diluted with ethyl acetate (100 mL). The mixture was washed with water (3 × 50 mL) and 10% potassium hydrogen sulfate (3 × 50 mL). The organic layers were combined and washed with brine, then dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure to give a brown oil. Recrystallization from methylene chloride and hexane afforded a beige solid, 6.26 g, 58%. Mp: 176−178 °C. ^{1}H NMR ($CDCl_3$): δ 2.26 (s, 3H), 3.54 (s, 2H), 6.74 (d, 2H, J = 8.6 Hz), 6.91 (s, 1H), 7.14 (d, 2H, J = 8.6 Hz), 7.23 (s, 1H), 7.52 (s, 1H).

Compounds **27**−**29** were prepared using the same procedure as described above for **9**.

1-[4-[[(3-Chloro-5-methylanilino)carbonyl]methyl]phenoxy]-3-methylcyclopentanecarboxylic Acid (27). 3-Methylcyclopentanone (3.57 g, 36.4 mmol) and **25** (1.0 g, 3.6 mmol) were reacted together to give a brown oil. The impure product was purified by flash chromatography (eluent: hexane/ethyl acetate 3:1→2:1) to give a yellow semisolid. Recrystallization from methylene chloride and hexane yielded a white solid, 0.38 g, 26%. Mp: 164−166 °C. ^{1}H NMR (CD_3OD): δ 1.04 (d, 3H, J = 6.6 Hz), 1.36−2.32 (m, 6H), 2.29 (s, 3H), 2.43−2.67 (m, 1H), 3.58 (s, 2H), 6.73 (d, 2H, J = 8.6 Hz), 6.91 (s, 1H), 7.20 (d, 2H, J = 8.7 Hz), 7.22 (s, 1H), 7.51 (s, 1H). Anal. ($C_{22}H_{24}ClNO_4$) C, H, Cl, N.

2-[4-[[(3-Chloro-5-methylanilino)carbonyl]methyl]phenoxy]-2-methylbutanoic Acid (28). 2-Butanone (4.64 g, 64.4 mmol) and **25** (1.77 g, 6.44 mmol) were reacted together to give a brown oil. Purification by flash chromatography (eluent: hexane/ethyl acetate 3:1→2:1) followed by recrystallization from methylene chloride and hexane afforded a beige solid, 0.44 g, 18%. A small portion of the product was purified for analytical purposes via esterification which was purified by flash chromatography (eluent: hexane/ethyl acetate 4:1) then hydrolyzed to the acid to give white fluffy crystals. Mp: 101−103 °C. ^{1}H NMR (CD_3OD): δ 0.99 (t, 3H, J = 7.4 Hz), 1.45 (s, 3H), 1.96 (m, 2H), 2.29 (s, 3H), 3.59 (s, 2H), 6.87 (d, 2H, J = 8.6 Hz), 6.92 (s, 1H), 7.22 (d, 2H, J = 8.5 Hz), 7.24 (s, 1H), 7.51 (s, 1H). Anal. ($C_{20}H_{22}ClNO_4$) C, H, Cl, N.

1-[4-[[(3-Chloro-5-methylanilino)carbonyl]methyl]phenoxy]-2-methylcyclopentanecarboxylic Acid (29). 2-Methylcyclopentanone (3.57 g, 36.4 mmol) and **25** (1.0 g, 3.6 mmol) were reacted together to give a brown oil. Purification by flash chromatography (eluent: hexane/ethyl acetate 3:1→2:1) gave a yellow solid. Recrystallization from methylene chloride and hexane yielded a white solid, 0.15 g, 10%. Mp: 180−182 °C. ^{1}H NMR (CD_3OD): δ 1.02 (d, 3H, J = 7.1 Hz), 1.43−2.48 (m, 6H), 2.52−2.57 (m, 1H), 3.58 (s, 2H), 6.76 (d, 2H, J = 8.6 Hz), 6.92 (s, 1H), 7.21 (d, 2H, J = 8.7 Hz), 7.24 (s, 1H), 7.52 (s, 1H). Anal. ($C_{22}H_{24}ClNO_4 \cdot 1.0H_2O$) C, H, Cl, N.

2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-propionic Acid (30). Ethyl 2-bromopropionate (1.8 g, 10 mmol) was added to a stirred mixture of **8** (1.27 g, 5.00 mmol) and potassium carbonate (1.4 g, 10 mmol) in dry dimethylformamide (30 mL). The mixture was heated overnight at 80 °C, cooled to room temperature, and diluted with ethyl acetate (100 mL). The mixture was washed with water (3 × 40 mL) followed by brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to afford a yellow oil. Without further purification the ester was hydrolyzed using 10% sodium hydroxide (10 mL) in ethanol (25 mL) and allowing the reaction to stir overnight at room temperature. Ethanol was removed under reduced pressure at room temperature. The residual product was dissolved in water (100 mL) and washed with ethyl acetate (3 × 40 mL). The aqueous layer was acidified (pH 2) with concentrated hydrochloric acid and extracted with ethyl acetate (3 × 40 mL), dried over anhydrous $MgSO_4$, and evaporated to dryness to obtain a yellow solid. The product was recrystallized from a mixture of methylene chloride and hexane to yield a white powder, 1.07 g, 66%. Mp: 183−187 °C. ^{1}H NMR ($CDCl_3$): δ 1.55 (d, 3H, J = 6.9 Hz), 2.17 (s, 6H), 3.52 (s, 2H), 4.65 (q, 1H, J = 6.7 Hz), 6.65 (s, 1H), 6.81 (d, 2H, J = 8.6 Hz), 6.98 (s, 2H), 7.15 (d, 2H, J = 8.6 Hz). Anal. ($C_{19}H_{21}NO_4$) C, H, N.

(−)-2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]propionic Acid (30). A solution of cinchonidine (4.50 g, 15.3 mmol) in hot ethanol (70 mL) was added to a solution of (±)-**30** (5.00 g, 15.3 mmol) in hot ethanol. The mixture was cooled to room temperature and a portion of the solvent was removed under reduced pressure. Crystals obtained were collected by filtration, 4.6 g. Mp: 198−200 °C. Optical rotation measured at 25 °C: $[\alpha]_D$ −69.8° (c = 0.2, methanol). The salt was recrystallized from ethanol to give pale yellow crystals, 2.86 g. Mp: 200−202 °C. Optical rotation measured at 25 °C: $[\alpha]_D$ −71.0° (c = 0.2, methanol). 2.7 g of the salt was dissolved in warm methanol (65 mL) and acidified to pH 2 with 1 N HCl. The solution stirred for 1 h and then the majority of the methanol was removed by rotavap. A white solid precipitated and was collected by filtration to obtain 1.3 g. Mp: 169−171 °C. Optical rotation measured at 21 °C: $[\alpha]_D$ −25.6 (c = 1, methanol). Anal. ($C_{19}H_{21}NO_4 \cdot 0.5H_2O$) C, H, N.

(+)-2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]propionic Acid (30). The enriched mother liquor obtained after the first crystallization of (−)-**30** was concentrated under reduced pressure. The residue was neutralized as described for (−)-**30** to give a white solid, 2.3 g of optically pure (+)-**30**. Mp: 169−171 °C. Optical rotation measured at 21 °C: $[\alpha]_D$ +25.1 (c = 1, methanol). Anal. ($C_{19}H_{21}NO_4$) C, H, N.

Compounds **31**−**38** were prepared using the same procedure as described above for **30**.

2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-2-fluoroacetic Acid (31). Using ethyl bromofluoroacetate (2.9 g, 16 mmol), **8** (2.0 g, 7.8 mmol), and potassium carbonate (2.16 g, 15.6 mmol), compound **31** was prepared as described for **30**, except the reaction went for 2 days. The brown oil obtained was purified by flash chromatography (eluent: hexane/ethyl acetate, 2:1) to afford a yellow oil. Ester hydrolysis afforded a yellow oil. The product was recrystallized from methylene chloride and hexane to yield a pale yellow powder, 0.47 g, 18%. Mp: 123−127 °C. ^{1}H NMR (CD_3OD): δ 2.26 (s, 6H), 3.64 (s, 2H), 6.10 (d, 1H, J = 59.7 Hz), 6.75 (s, 1H), 7.10 (d, 2H, J = 8.6 Hz), 7.16 (s, 2H), 7.34 (d, 2H, J = 8.6 Hz). Anal. ($C_{18}H_{18}FNO_4$) C, H, N.

2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-butanoic Acid (32). Ethyl 2-bromobutyrate (3.9 g, 20 mmol) and **8** (2.5 g, 10 mmol) were reacted to yield the ethyl ester of **32**, a yellow oil. Ester hydrolysis afforded a yellow solid, which upon recrystallization from methylene chloride and hexane gave a white solid, 2.86 g, 84%. Mp: 173−175 °C. ^{1}H NMR ($CDCl_3$): δ 1.08 (t, 3H, J = 7.5 Hz), 1.95 (m, 2H), 2.25 (s, 6H), 3.56 (s, 2H), 4.61 (t, 1H, J = 7.0 Hz), 6.74 (s, 1H), 6.86 (d, 2H, J = 8.6 Hz), 7.15 (s, 2H), 7.25 (d, 2H, J = 8.6 Hz). Anal. ($C_{20}H_{23}NO_4$) C, H, N.

(−)-2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]butanoic Acid (32). Following the same procedure as described for (−)-(**30**), cinchonidine (8.61 g, 29.3 mmol) in hot ethanol (175 mL) was added to a solution of (±)-**32** (10.0 g, 29.3 mmol) in hot ethanol. The solution was allowed to cool to room temperature and a portion of the solvent was removed under reduced pressure. Crystals obtained were collected by filtration, 6.6 g. Mp: 204−205 °C. Optical rotation measured at 21 °C: $[\alpha]_D$ −73.3° (c = 0.5, methanol). The salt was recrystallized from ethanol to give fluffy white crystals, 3.7 g. Mp: 206−207 °C. Optical rotation measured at 21 °C: $[\alpha]_D$ −74.2° (c = 0.5, methanol). The acid was recovered from salt, as described previously for (−)-**30**, to obtain a white solid, 1.7 g. Mp: 150−151 °C. Optical rotation measured at 21 °C: $[\alpha]_D$ −28.4° (c = 1.2, methanol). Anal. ($C_{20}H_{23}NO_4$) C, H, N.

2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-pentanoic Acid (33). Ethyl 2-bromovalerate (4.2 g, 20 mmol) and **8** (2.5 g, 10 mmol) were reacted in the presence of potassium carbonate (2.76 g, 20.0 mmol) as described for compound **30** except that the solvent used was ethanol (50 mL). The mixture was refluxed overnight. The potassium carbonate was removed by filtration and washed with ethyl acetate. Concentration under reduced pressure afforded a light-brown oil. Hydrolysis afforded a yellow solid. Recrystallization from methylene chloride and hexane gave a white solid, 1.38 g, 39%. Mp: 164−166 °C. ^{1}H NMR (CD_3OD): δ 0.98 (t, 3H, J = 7.3 Hz), 1.55 (m, 2H), 1.89 (m, 2H), 2.25 (s, 6H), 3.57 (s, 2H), 4.64 (t, 1H, J = 7.4 Hz), 6.74 (s, 1H), 6.86 (d, 2H, J = 8.6 Hz), 7.15 (s, 2H), 7.24 (d, 2H, J = 8.6 Hz). Anal. ($C_{21}H_{25}NO_4 \cdot 0.25H_2O$) C, H, N.

2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-hexanoic Acid (34). Ethyl 2-bromohexanoate (1.95 g, 10.0 mmol) and **8** (1.3 g, 5.0 mmol) were reacted together to give a yellow oil. A white solid was obtained by flash chromatography (eluent: hexane/ethyl acetate 5:1). Ester hydrolysis yielded a white amorphous solid, 1.2 g, 65%. Mp: 153−155 °C. ^{1}H NMR (CD_3OD): δ 0.94 (t, 3H, J = 7.3 Hz), 1.36−1.54 (m, 4H), 1.91

(m, 2H), 2.25 (s, 6H), 3.57 (s, 2H), 4.64 (t, 1H, J = 6.1 Hz), 6.74 (s, 1H), 6.85 (d, 2H, J = 8.7 Hz), 7.15 (d, 2H), 7.24 (d, 2H, J = 8.7 Hz). Anal. ($C_{22}H_{27}NO_4$) C, H, N.

2-[4-[[(3-Chloro-5-methylanilino)carbonyl]methyl]phenoxy]propionic Acid (35). Ethyl 2-bromopropionate (1.2 g, 6.5 mmol) and **26** (0.90 g, 3.27 mmol) were reacted together to yield the ethyl ester, a yellow-orange oil. Hydrolysis of the ester was carried out as described to obtain a yellow semisolid. Recrystallization from methylene chloride and hexane yielded a yellow solid, 0.86 g, 76%. Mp: 163−165 °C. ^{1}H NMR (CD_3OD): δ 1.56 (d, 3H, J = 6.9 Hz), 2.29 (s, 3H), 3.59 (s, 2H), 4.78 (q, 1H, J = 6.7 Hz), 6.86 (d, 2H, J = 8.7 Hz), 6.92 (s, 1H), 7.22 (s, 1H), 7.24 (d, 2H, J = 8.7 Hz), 7.50 (s, 1H). Anal. ($C_{18}H_{18}ClNO_4$) C, H, Cl, N.

2-[4-[[(3-Chloro-5-methylanilino)carbonyl]methyl]phenoxy]butanoic Acid (36). Following the procedure given for **30**, ethyl 2-bromobutyrate (1.4 g, 7.3 mmol) and **26** (1.0 g, 3.6 mmol) were reacted together to give a yellow oil. The ester was hydrolyzed as described for **13** to give a white powder, 0.90 g, 69%. Mp: 168−170 °C. ^{1}H NMR (CD_3OD): δ 1.07 (t, 3H, J = 7.6 Hz), 1.95 (m, 2H), 2.29 (s, 3H), 3.59 (s, 2H), 4.60 (t, 1H, J = 6.9 Hz), 6.86 (d, 2H, J = 8.7 Hz), 6.92 (s, 1H), 7.23 (s, 1H), 7.24 (d, 2H, J = 8.7 Hz), 7.51 (s, 1H). Anal. ($C_{19}H_{20}ClNO_4$) C, H, Cl, N.

2-[4-[[(5-Indanyl)carbonyl]methyl]phenoxy]propionic Acid (37). Ethyl 2-bromopropionate (1.8 g, 10 mmol) and **18** (1.3 g, 5.0 mmol) were reacted to yield a brown oil. A brown oil was obtained from the hydrolysis, which upon recrystallization from methylene chloride and hexane yielded a white solid, 0.77 g, 45.5%. Mp: 133−136 °C. ^{1}H NMR ($CDCl_3$): δ 1.64 (d, 3H, J = 6.9 Hz), 2.04 (m, 2H), 2.85 (m, 4H), 3.63 (s, 2H), 4.74 (q, 1H, J = 6.9 Hz), 6.89 (d, 2H, J = 8.6 Hz), 7.12 (s, 2H), 7.24 (d, 2H, J = 8.6 Hz), 7.37 (s, 1H). Anal. ($C_{20}H_{21}NO_4$) C, H, N.

2-[4-[[(5-Indanyl)carbonyl]methyl]phenoxy]butanoic Acid (38). Ethyl 2-bromobutyrate (3.9 g, 20 mmol) and **18** (2.67 g, 10.0 mmol) were reacted to yield a dark brown oil. Purification by column chromatography (eluent: hexane/ethyl acetate, 4:1→2:1) gave a beige solid. Hydrolysis was carried out to yield a tan oil. Recrystallization from methylene chloride and hexane gave a white solid, 1.77 g, 50%. Mp: 160−162 °C. ^{1}H NMR (CD_3OD): δ 1.07 (t, 3H, J = 7.3 Hz), 1.95 (m, 2H), 2.05 (m, 2H), 2.84 (m, 4H), 3.57 (s, 2H), 4.60 (t, 1H, J = 7.4 Hz), 6.86 (d, 2H, J = 8.6 Hz), 7.12 (s, 2H), 7.25 (d, 2H, J = 8.6 Hz), 7.40 (s, 1H). Anal. ($C_{21}H_{23}NO_4\cdot 0.25H_2O$) C, H, N.

Enantiomeric Resolution by HPLC. The resolution of compounds **10**, **11**, and **13** and the purification of **32** were performed using a chiral semipreparative HPLC column (Chiracel OD, 1 cm × 25 cm) packed with cellulose tris(3,5-dimethylphenylcarbamate) on a silica gel substrate. The samples were injected using a Waters 712 WISP automated injector system and detected with a Waters Lambda Max (model 481) variable wavelength detector. All of the compounds were detected at 254 nm. The solvent delivery was controlled with a Waters automated gradient controller (model 660). A Hewlett-Packard integrator (HP 3393A) was used to integrate the peaks and to plot the chromatograms. The peak fractions were collected using a Spectrum CF-1 fraction collector. All solvents used for HPLC separation were purchased from Aldrich Chemical Co. as HPLC grade and filtered prior to use.

(±)-2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-2-methylbutanoic Acid (10). The compound was eluted with a mobile phase of heptane/ethanol with 1% TFA (89:11) at a flow rate of 2.0 mL/min. A stock solution (1 mg/mL) of the compound was prepared in ethanol/mobile phase (1:1). The injection sample volume was 250 μL. Under these conditions, the (−)-isomer eluted at 20.7 min and the (+)-isomer eluted at 22.9 min. The collected fractions were concentrated under reduced pressure at room temperature. (−)-**10** was collected as a yellow solid, 0.11 g. Mp: 125−127 °C. Optical rotation measured at 20 °C: $[\alpha]_D$ −11.6° (c = 0.3, methanol). ^{1}H NMR (CD_3OD): δ 0.99 (t, 3H, J = 7.4 Hz), 1.45 (s, 3H), 1.96 (m, 2H), 2.25 (s, 6H), 3.58 (s, 2H), 6.74 (s, 1H), 6.87 (d, 2H, J = 8.6 Hz), 7.15 (d, 2H), 7.24 (d, 2H, J = 8.5 Hz). Anal. ($C_{21}H_{24}NO_4\cdot 0.75H_2O\cdot 0.17$TFA) C, H, F, N. (+)-**10** was collected as a yellow solid, 0.10 g. Mp: 127−129 °C. Optical rotation measured at 20 °C: $[\alpha]_D$ +11.0 (c = 0.3, methanol). ^{1}H NMR (CD_3OD): δ 0.99 (t, 3H, J = 7.4 Hz), 1.45 (s, 3H), 1.96 (m, 2H), 2.25 (s, 6H), 3.58 (s, 2H), 6.74 (s, 1H), 6.87 (d, 2H, J = 8.6 Hz), 7.15 (d, 2H), 7.24 (d, 2H, J = 8.6 Hz). Anal. ($C_{21}H_{24}NO_4\cdot 0.5H_2O\cdot 0.25$TFA) C, H, F, N.

(+)-2-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]butanoic Acid (32). The mother liquor from the recrystallization of (−)-**32** cinchonidine salt was concentrated under reduced pressure. The enriched (+)-**32** isomer was obtained from the mother liquor by neutralizing the salt to obtain the free acid, as described previously for compound **30**. The optical rotation showed that the isomer was approximately 80% enantiomeric excess. Optical rotation measured at 21 °C: $[\alpha]_D$ +18.2° (c = 1.2, methanol). A stock solution (1 mg/mL) of the racemic mixture (**32**) was prepared in ethanol/mobile phase (1:1). The injected sample volume was 250 μL. The compound was eluted with a mobile phase of heptane/ethanol with 1% TFA (88:12) at a flow rate of 2.5 mL/min. Under these conditions, the (−)-isomer eluted at 15.8 min and the (+)-isomer eluted at 18.2 min retention times. The collected fractions were concentrated under reduced pressure at room temperature. (+)-**32** was collected as a white solid, 0.14 g. Mp: 146−148 °C. Optical rotation measured at 21 °C: $[\alpha]_D$ +25.3° (c = 0.5, methanol). ^{1}H NMR (CD_3OD): δ 1.07 (t, 3H, J = 7.4 Hz), 1.95 (m, 2H), 2.25 (s, 6H), 3.57 (s, 2H), 4.60 (t, 1H, J = 6.7 Hz), 6.74 (s, 1H), 6.87 (d, 2H, J = 8.6 Hz), 7.15 (d, 2H), 7.24 (d, 2H, J = 8.5 Hz). Anal. ($C_{20}H_{23}NO_4\cdot 0.75H_2O$) C, H, N.

(±)-3-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-2-methyltetrahydro-3-furancarboxylic Acid (13). A stock solution (1 mg/mL) of the compound **13** was prepared in ethanol/mobile phase (1:1). The racemic compound after loading on HPLC was eluted with a mobile phase of heptane/ethanol with 1% TFA (85:15) at a flow rate of 2.75 mL/min. The injection sample volume was 1000 μL. Under these conditions, the (+)-isomer eluted at 11.4 min and the (−)-isomer eluted at 17.5 min retention times. The collected fractions were concentrated under reduced pressure at room temperature. (+)-**13**, a pale yellow solid, was collected by filtration and washed with ether, 0.11 g. Mp: 164−167 °C. Optical rotation measured at 21 °C: $[\alpha]_D$ +67.5° (c = 0.5, methanol). ^{1}H NMR (CD_3OD): δ 1.26 (d, 3H, J = 6.5 Hz), 2.25 (s, 6H), 2.28 (m, 1H), 2.85 (m, 1H), 3.57 (s, 2H), 3.85 (q, 1H, J = 7.3 Hz), 4.07 and 4.19 (m, 1H), 6.74 (s, 1H), 6.78 (d, 2H, J = 8.6 Hz), 7.15 (s, 2H), 7.24 (d, 2H, J = 8.6 Hz). Anal. ($C_{22}H_{25}NO_5\cdot 0.75H_2O\cdot 0.125$TFA) C, H, F, N. (−)-**13** was collected by filtration and washed with ether to give a beige solid, 0.10 g. Mp: 168−171 °C. Optical rotation measured at 21 °C: $[\alpha]_D$ −70.6° (c = 0.3, methanol). ^{1}H NMR (CD_3OD): δ 1.26 (d, 3H, J = 6.5 Hz), 2.25 (s, 6H, ArCH_3), 2.28 (m, 1H), 2.87 (m, 1H), 3.57 (s, 2H), 3.85 (q, 1H, J = 8.1 Hz), 4.07 and 4.19 (m, 1H), 6.74 (s, 1H), 6.78 (d, 2H, J = 8.6 Hz), 7.15 (s, 2H), 7.24 (d, 2H, J = 8.4 Hz). Anal. ($C_{22}H_{25}NO_5\cdot 0.75H_2O\cdot 0.125$TFA) C, H, F, N.

(±)-1-[4-[[(3,5-Dimethylanilino)carbonyl]methyl]phenoxy]-2-methylcyclopentanecarboxylic Acid (11). A stock solution (1 mg/mL) of the compound was prepared in ethanol/mobile phase (1:1). The injection sample volume was 500 μL. The mixture was eluted with a mobile phase of heptane/ethanol with 1% TFA (90:10) at a flow rate of 2.0 mL/min. Under these conditions, the (+) isomer eluted at 18.9 min and the (−)-isomer eluted at 26.2 min. retention times. The collected fractions were concentrated under reduced pressure at room temperature. (+)-**11**, a white solid, was collected by filtration and washed with ether, 0.11 g. Mp: 186−187 °C. Optical rotation measured at 21 °C: $[\alpha]_D$ +64.8° (c = 0.5, methanol). ^{1}H NMR (CD_3OD): δ 1.02 (d, 3H, J = 7.2 Hz), 1.43−2.48 (m, 6H), 2.25 (s, 6H), 2.51−2.57 (m, 1H), 3.57 (s, 2H), 6.74 (s, 1H), 6.77 (d, 2H, J = 8.6 Hz), 7.15 (s, 2H), 7.22 (d, 2H, J = 8.6 Hz). Anal. ($C_{23}H_{27}NO_4\cdot 0.25H_2O$) C, H, N. The fractions for (−)-**11** were collected and concentrated under reduced pressure. The white solid was collected by filtration

and washed with ether, 0.11 g. Mp: 184−186 °C. Optical rotation measured at 21 °C: $[\alpha]_D$ −60.2° (c = 0.5, methanol). ^{1}H NMR (CD_3OD): δ 1.02 (d, 3H, J = 7.1 Hz), 1.42−2.47 (m, 6H), 2.25 (s, 6H), 2.49−2.57 (m, 1H), 3.56 (s, 2H), 6.74 (s, 1H), 6.76 (d, 2H, J = 8.6 Hz), 7.15 (s, 2H), 7.22 (d, 2H, J = 8.6 Hz). Anal. ($C_{23}H_{27}NO_4 \cdot 0.25H_2O$) C, H, N.

Oxygen Equilibrium Studies. 1. Hb Solution Studies. The effectors were prepared as 10 mM stock solutions in 100 mM NaCl bis-Tris buffer, pH 7.2. After the addition of an excess of $NaHCO_3$, the solution was warmed to 60 °C and stirred for several hours. The solutions were back-titrated carefully to pH 7.2 at 25 °C prior to use. Oxygen equilibrium measurements were performed with the Hemox analyzer (TCS Medical products, Southampton, PA) using purified stripped human adult Hb as described previously.[11,32] 4 mL of buffer, 100 mM NaCl, 50 mM bis-Tris at pH 7.2, was added to a cuvette in the Hemox, followed by 200 μL of the 10 mM effector stock solution. Hb was then added to achieve a final Hb concentration of 60−70 μM on heme basis. Catalase (20 μg/mL) and 50 mM EDTA were added to limit oxidation of the hemes. The solution was then fully oxygen-saturated using 95% carbogen gas mixture. The oxygen pressure was gradually decreased to record the curve continuously from the right to the left. The saturation of Hb was determined spectrophotometrically with a dual wavelength spectrophotometer (577 and 586.2 nm). The solution was stirred constantly during the 45−60-min recordings. The P_{50} and n_{50} values were calculated by linear regression analysis from data points comprised between 40% and 60% oxygen saturation. The solution binding experiments (Tables 1 and 2) were single measurements using a Hemox analyzer (see Results). It is a standard practice to run each effector with an individual control and the results accepted if the controls are within ±1 mmHg. Conformational efficacy evidence for each run was obtained at least in duplicate in the whole blood study (Table 3) for both racemic and all enantiomers. The whole blood studies confirmed the trend in activity observed in the solution-binding curves.

2. Whole Blood Studies. The whole blood samples were collected in heparinized tubes from healthy volunteers and stored over ice. The sodium salts of the compounds were prepared as described earlier. A 200 mM stock solution of the effector was prepared in DMSO. A 5.0 mM test solution was prepared from 50 μL of the 200 mM test solution and 1950 μL of whole blood. The blood samples were incubated in IL 237 tonometers (Instrumentation Laboratories, Inc., Lexington, MA) for approximately 10−12 min at 37 °C and equilibrated at three separate concentrations of O_2 (20%, 40%, and 60%). After equilibration at each concentration of O_2, a sample was removed via syringe and aspirated into a IL 1420 automated blood gas analyzer (Instrumentation Laboratories, Inc., Lexington, MA) and a IL 482 and IL 682 co-oximeter (Instrumentation Laboratories, Inc., Lexington, MA) to determine the pH, pCO_2, pO_2 and the Hb oxygen saturation values (sO_2), respectively. The measured values for pO_2 and sO_2 at each oxygen saturation level were then subjected to a nonlinear regression analysis using the program Scientist (Micromath, Salt Lake City, UT) to calculate the P_{50} and Hill coefficient (n_{50}) values. In all Hb solution-binding curves, carbogen (95% oxygen, 5% carbon dioxide) was used to fully saturate samples. The P_{50} values for human whole blood control samples were also identical to that reported as standard for humans (27 mmHg).

Molecular Modeling. Molecular modeling was performed using the modeling software package SYBYL on a Silicon Graphics Indigo 2 workstation.[33] The models were built using the SYBYL sketch mode and each model was minimized using the Powell method to a derivative of 0.05 kcal mol^{-1} Å^{-1} and the Tripos force field with Gasteiger−Huckel charges.

Acknowledgment. This work was supported by a grant from Allos Therapeutics, Inc. We also gratefully acknowledge the AFPE.

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JM000199Q