Articles

Synthesis and X-ray Studies of Chiral Allosteric Modifiers of Hemoglobin

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This study was designed to investigate the effect of chirality on the allosteric activity of a series of Hb allosteric modifiers. The chiral analogues were based on the lead compound (**4**), JP7, {1-[4-(((3,5-dimethylanilino)carbonyl)methyl]phenoxy]cyclopentanecarboxylic acid} with different D- and L-amino acids conjugated to the JP7 acid moiety. The D-isomers were the most potent in vitro effectors in Hb solutions as well as with whole blood. In general, this study demonstrated that the chirality of extended amino acid side chains in JP7 conjugates plays an important role in observed degree of allosteric activity. The binding site interactions for four analogues were determined by single crystallographic diffraction studies. Conclusions show that the chiral configuration of some of the D-isomers enable the effectors to bind with a greater number of interactions with the protein residues. D- and L-isomers with equivalent or near equivalent allosteric activity did not show any significant differences or interactions between their amino acid side chains and the protein. The most potent effectors, in vitro, were compounds **15** and **19**, D-isomers of leucine and phenylalanine, respectively. Compounds **21**, **22**, **30**, and **32** were more potent in vitro in Hb solutions than JP7.

Introduction

Hemoglobin (Hb) is a tetrameric allosteric protein that consists of two α chains and two β chains arranged around a central water cavity that is bisected by a molecular 2-fold axis. It exists in two states: a relaxed (R) conformation when oxygenated and a tense (T) conformation in the deoxygenated state. The allosteric equilibrium can be shifted in either direction by allosteric effectors. When the allosteric equilibrium is shifted toward the R-state in the oxygen binding curve (OBC), a high-affinity Hb is obtained that more readily binds and holds oxygen, whereas a shift toward the T-state produces the converse: a low-affinity Hb that more readily releases oxygen. We have had a long-standing interest in designing agents that produce low-affinity Hb's. Such agents have several potential clinical applications including radiosensitization of tumors, prolongation of stored blood half-life, and treatment of ischemic diseases, i.e., stroke, trauma, angina, cardiopulmonary bypass surgery, etc.[1,2]

Several synthetic agents have been reported to lower the oxygen affinity of Hb (Figure 1). During the search for an antisickling agent, Abraham and co-workers[3,4] discovered that the antilipidemic drug clofibrate (**1**) lowered the oxygen affinity of Hb. Perutz and Poyart[5]
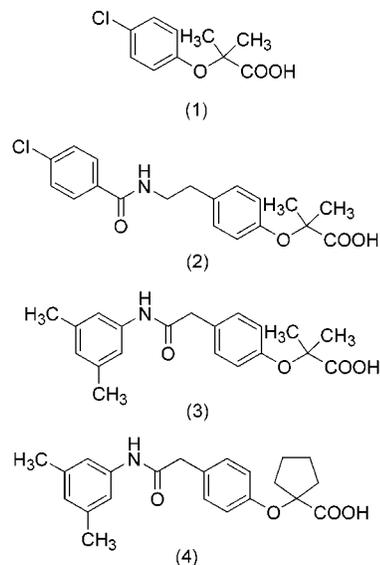

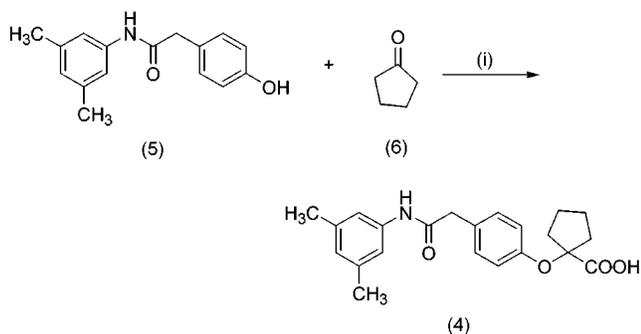**Figure 1.** Structures of allosteric modifiers that decrease oxygen affinity.

found that bezafibrate (BZF, **2**), another antilipidemic agent, more potently right-shifted the OBC. Lalezari et al.[6−8] developed several urea analogues of BZF with a urea linkage replacing the four-atom bridge between the two benzene rings in BZF. These urea analogues did not have clinical utility due to their strong affinity for serum proteins (albumin, etc.) at physiological concentrations. Structure-based drug design by Abraham et

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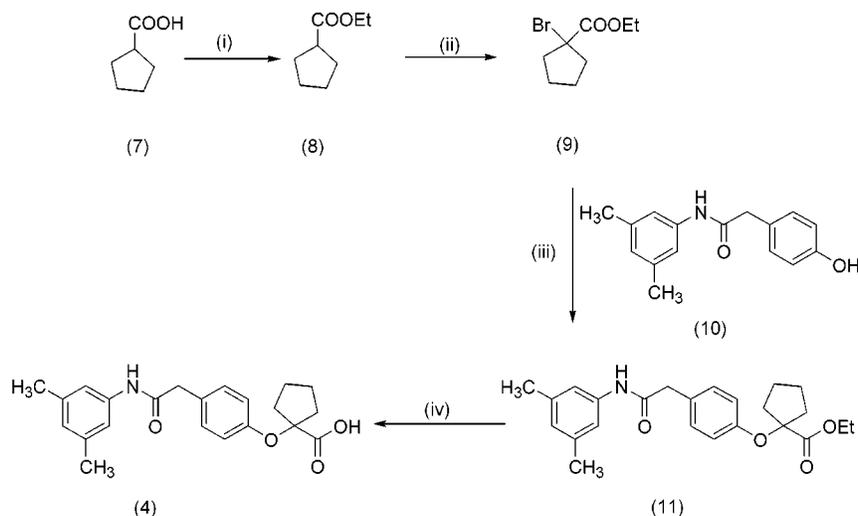　‡ INSERM.

Scheme 1[a]

a Reagents and conditions: (i) CHCl$_3$, NaOH.

Synthesis

The desired compounds were synthesized from the precursor molecule 4-[[(3,5-dimethylanilino)carbonyl]-methyl]phenol (**5**) as shown in Scheme 1. The synthesis of compound **5** has been reported previously.[16] Two different procedures were used to obtain compound **4** (JP7) from amidophenol **5**. Scheme 1 involved the reaction of amidophenol **5** with cyclopentanone in the presence of sodium hydroxide and chloroform. This reaction is assumed to involve the preliminary formation of a dichloroepoxide[17,18] which, in turn, reacts with the phenolate ion to give **4**. An alternate synthetic route (Scheme 2) was devised for large-scale synthesis. As shown in Scheme 2, bromination of ethyl cyclopentane-carboxylate with *N*-bromosuccinimide in refluxing carbon tetrachloride under an IR lamp for 6 h afforded the corresponding bromoester. Convergence of the ami-dophenol **5** with ethyl 2-bromocyclopentanecarboxylate in the presence of potassium carbonate and a catalytic amount of potassium iodide (Williamson ether synthesis) produced **4**. The final steps in the synthesis of **4** involved the hydrolysis of the ester under basic conditions using potassium hydroxide, followed by protonation to produce the subsequent free acid. Compound **4** was then coupled with several D- and L-amino acid methyl ester hydrochlorides in the presence of 1-hydroxybenzotriazole hydrate, 1-(3-dimethylaminopropyl)3-ethylcarbodiimide hydrochloride (DEC), and *N*-methyl-morpholine. The corresponding amino acid methyl esters obtained were hydrolyzed in aqueous ethanol using lithium hydroxide. The residue was dissolved in water and acidified to obtain the corresponding amino acids (Scheme 3). In the case of L-glutamate and L-aspartate-*tert*-butoxycarbonyl ester, the hydrolysis was accomplished using trifluoroacetic acid (Scheme 4).

In the synthesis of N^ϵ-benzyloxycarbonyl-L- and D-lysine, the corresponding lysines were obtained by catalytic hydrogenation using 10% palladium on carbon. The mixture was hydrogenated in a Parr-Shaker apparatus until absorption of the hydrogen gas was stopped. The pure product was obtained upon recrystallization (Scheme 5).

al.[9,11] produced a series of fibrate analogues that replaced the urea link with an amide methylene bridge. RSR13 {2-[4-(((3,5-dimethylanilino)carbonyl)methyl)-phenoxy]-2-methylpropionic acid} (**3**) showed the highest activity in whole blood as well as in Hb solution assays. RSR13 was also found to shift the oxygen equilibrium curve to the right when administered in vivo in rats, mice, and cats.[12−14] Preclinical evaluations of RSR13 demonstrated that the effector enhances the effectiveness for radiation treatment of hypoxic tumors by increasing tumor oxygenation and oxygen radical formation.[15] RSR13 is currently in phase III clinical trials as a radiosensitizing agent for metastatic brain cancer and for the treatment of glioblastoma multiforme brain tumors.

This paper describes a new series of potent allosteric effectors that represent potential candidates for in vivo animal studies. Compound **4** (JP7), a potent allosteric effector,[16] was used as a template for modification by conjugation with D- and L-amino acids. To determine the effect of the amino acid stereocenter on allosteric activity, several amino acid conjugates of JP7 were prepared. The D-, L-JP7 conjugates were evaluated with Hb solutions as well as with whole blood in vitro. X-ray crystallographic studies on deoxy Hb−allosteric effector complexes were initiated to correlate enantiomeric allosteric effector potencies with differences in stereospecific binding.
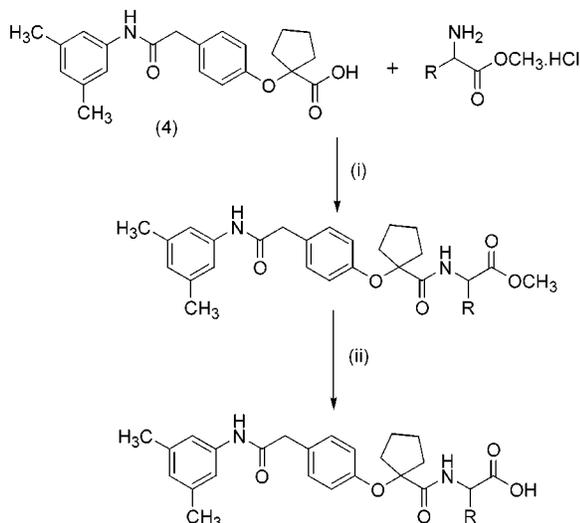
Scheme 2[a]

a Reagents and conditions: (i) EtOH, H$^+$; (ii) *N*-bromosuccinimide (NBS), CCl$_4$ reflux under IR; (iii) K$_2$CO$_3$, EtOH; (iv) KOH.

Scheme 3[a]

(12) R = H
(13) R = CH$_3$ (D)
(14) R = CH$_3$ (L)
(15) R = CH$_2$CH(CH$_3$)$_2$ (D)
(16) R = CH$_2$CH(CH$_3$)$_2$ (L)
(17) R = CH(CH$_3$)$_2$ (D)
(18) R = CH(CH$_3$)$_2$ (L)
(19) R = CH$_2$C$_6$H$_5$ (D)
(20) R = CH$_2$C$_6$H$_5$ (L)
(21) R = CH$_2$indole (D)
(22) R = CH$_2$indole (L)

(23) R = (CH$_2$)$_2$SCH$_3$ (D)
(24) R = (CH$_2$)$_2$SCH$_3$ (L)
(25) R = CH$_2$OH (D)
(26) R = CH$_2$OH (L)
(27) R = (CH$_2$)$_4$NHCOOCH$_2$C$_6$H$_5$ (D)
(28) R = (CH$_2$)$_4$NHCOOCH$_2$C$_6$H$_5$ (L)
(29) R = CH(CH$_3$)C$_2$H$_5$ (L)
(30) R = CH$_2$C$_6$H$_4$OH (L)
(31) R = (CH$_2$)$_3$
(32) R = CH$_2$S CH$_2$C$_6$H$_5$ (L)
(33) R = CH(OH)CH$_3$ (L)

[a] Reagents and conditions: (i) DEC, HOBt, NMM, DMF; (ii) LiOH, EtOH.

Scheme 4[a]
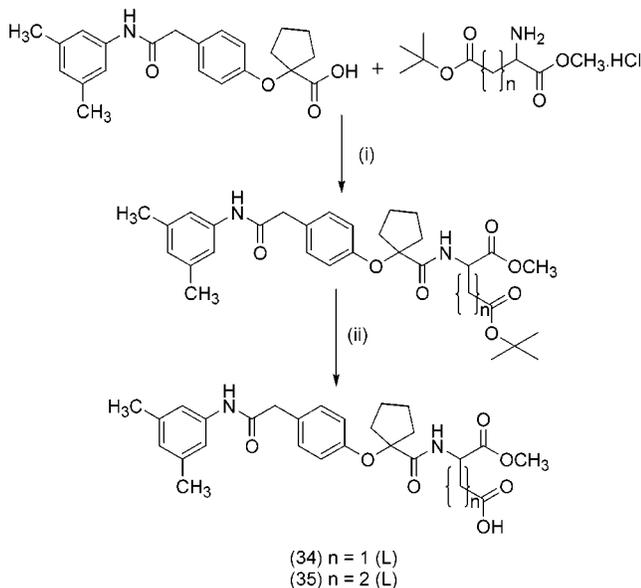
(34) n = 1 (L)
(35) n = 2 (L)

[a] Reagents and conditions: (i) DEC, HOBt, NMM, DMF; (ii) TFA, CH$_2$Cl$_2$.

Biological Evaluation

Biological evaluation for structure–activity relationships was conducted with hemoglobin solutions to exclude serum protein binding and transport differences. The new analogues were tested for their ability to right-shift the oxygen equilibrium curves (OEC's) and quantified by their ability to increase in P_{50} (partial pressure of oxygen at 50% Hb saturation). P_{50} is the

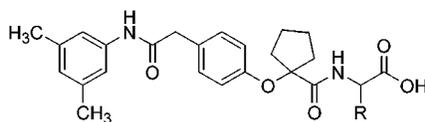**Scheme 5**[a]
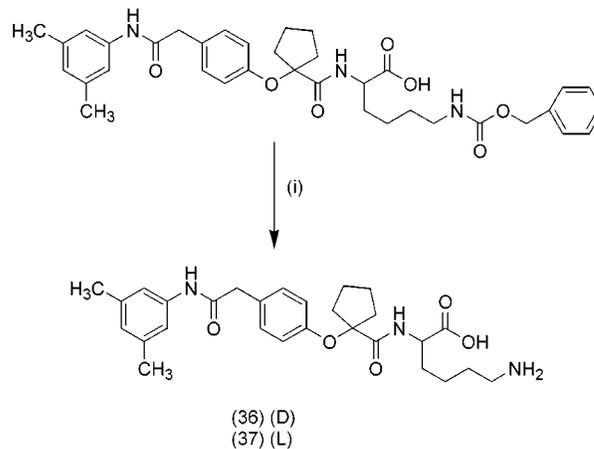
(36) (D)
(37) (L)

[a] Reagents and conditions: (i) H$_2$, Pd, C.

oxygen pressure in mmHg at which Hb is 50% saturated with oxygen in the presence (P_{50} compound) or absence (P_{50} control) of an effector. The Hb OEC's were recorded using purified-stripped human adult Hb (Hb A) with a Hemox analyzer (TCS, Southampton, PA) under the following conditions: pH 7.2, 100 mM NaCl, 50–60 μM heme, and 50 mM bis-Tris buffer at 25 °C.[19,20] The results of OEC studies in the presence of 0.5 mM of compounds are described in Table 2 and the Discussion section. Preparation and purification of Hb A was performed as described earlier.[10] It involved separation of RBC's from plasma by centrifugation followed by lysing cells with NaCl to obtain a mixture of hemoglobin solution. The Hb mixture was then purified by column chromatography over DEAE-Sephacel ion-exchange resin using pH 8. 6 Tris buffer. The purity of Hb A fractions was checked by electrophoresis, and pure fractions were concentrated using Schleicher and Schuell collodoin bag and used for OEC studies as desired.

Biological evaluation for potential clinical applications of the new allosteric effectors were run in whole blood to monitor any loss of effector activity due to poor transport into red cells and/or serum protein binding. The whole blood OEC's were obtained using multi-point tonometry (Instrumentation Laboratories, Inc., Lexington, MA). Compounds with the highest degree of allosteric activity produce the largest right shift in P_{50} relative to control P_{50}. The slope of the log of the oxygen binding curves is known as the Hill coefficient (n_{50}). The Hill coefficient measures the degree of cooperativity in ligand binding to an allosteric protein; the normal range for hemoglobin in human blood being 2.7–3.2. High cooperativity is important to oxygen binding for efficient oxygen delivery at normal capillary oxygen pressures (pO$_2$ ~ 40 mmHg) since the step portion of the sigmoidal-binding curve occurs near this pO$_2$. The whole blood results are summarized in Table 1 and discussed below.

Results and Discussion

Hb Solution Studies. The D-isomer conjugates that showed enantiomeric differences were found to be more potent than L-isomer conjugates in Hb solutions. Compounds **21** and **22** exhibited a strong shift in the Hb A OEC's (P_{50} compound/P_{50} control (absence of compound) = 7), retaining high cooperativity (n_{50} = 2.3 and 2.1, respectively).
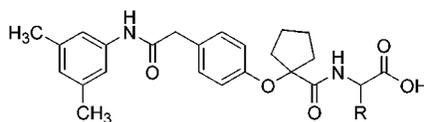
Table 1. Results of in Vitro Whole Blood Studies[a]

compound	configuration	R	$P_{50}c^b$	$P_{50}e^c$	$\Delta P_{50} \pm SD^d$	$n_{50}e^e$
3			29.8	82.6 80.0	51.5 ± 1.8	1.5
4			28.9	74.7 67.7	42.3 ± 4.9	1.7
12	Gly	H	26.2	52.6 50.0	25.1 ± 1.8	2.1
13	D-Ala	CH_3	26.8	46.2 42.8	17.6 ± 2.5	2.1
14	L-Ala	CH_3	29.4	52.1 50.3	21.9 ± 1.3	1.7
15	D-Leu	$CH_2CH(CH_3)_2$	29.4	71.3 70.6	41.5 ± 0.5	1.8
16	L-Leu	$CH_2CH(CH_3)_2$	27.1	42.6 41.9	15.2 ± 0.6	2.3
17	D-Val	$CH(CH_3)_2$	25.7	54.8 53.9	28.6 ± 0.4	2.0
18	L-Val	$CH(CH_3)_2$	26.7	57.3 56.6	30.2 ± 0.5	1.9
19	D-Phe	$CH_2C_6H_5$	27.4	67.5 64.1	38.4 ± 2.4	1.8
20	L-Phe	$CH_2C_6H_5$	28.7	49.8 48.1	20.3 ± 1.2	1.9
21	D-Trp	$CH_2indole$	27.9	48.8 47.2	20.2 ± 1.1	2.0
22	L-Trp	$CH_2indole$	28.7	49.9 47.5	20.0 ± 1.6	2.1
23	D-Met	$(CH_2)_2SCH_3$	27.1	56.8 50.7	26.6 ± 4.3	2.3
24	L-Met	$(CH_2)_2SCH_3$	24.5	45.5 44.8	20.6 ±0.5	2.1
25	D-Ser	CH_2OH	26.2	44.7 38.7	15.5 ± 4.2	2.3
26	L-Ser	CH_2OH	27.1	34.0 31.1	5.4 ± 2.0	2.1
27	N^ϵ-benzyloxycarbonyl-D-lys	$(CH_2)_4NHCOOCH_2C_6H_5$	28.3	45.0 39.5	14.0 ± 3.9	2.1
28	N^ϵ-benzyloxycarbonyl-L-lys	$(CH_2)_4NHCOOCH_2C_6H_5$	27.5	28.2 27.6	0.4 ± 0.5	2.5
29	L-Ile	$CH(CH_3)C_2H_5$	26.5	40.6 38.9	13.2 ± 1.2	2.3
30	L-Tyr	$CH_2C_6H_4OH$	27.2	49.0 47.0	20.8 ± 1.4	2.0
31	L-Pro	$(CH_2)_3$	24.8	42.5 41.1	17.0 ± 1.0	2.3
32	S-benzyl-L-cys	$CH_2SCH_2C_6H_5$	24.8	33.3 30.6	7.2 ± 1.9	2.3
33	L-Thr	$CH(OH)CH_3$	27.5	41.1 39.7	12.9 ± 1.0	2.2
34	L-Asp	CH_2COOH	26.2	55.3 50.2	26.6 ± 3.6	1.9
35	L-Glu	CH_2CH_2COOH	28.3	51.4 50.1	22.5 ± 0.9	2.0
36	D-Lys	$(CH_2)_4NH_2$	28.3	29.0 27.2	−0.2 ± 1.3	2.7
37	L-Lys	$(CH_2)_4NH_2$	28.3	28.6 26.7	−0.6 ± 1.3	2.7

[a] Whole blood analyses were carried out at a final effector concentration of 5 mM. All stock solutions were prepared in DMSO. [b] P_{50} control value in mmHg. [c] P_{50} value in the presence of the effector in mmHg. [d] $\Delta P_{50} = (P_{50}$ effector $- P_{50}$ control) in mmHg. [e] The Hill coefficient at 50% saturation (n_{50}) in the presence of effector (average n_{50} control value = 2.7, $n = 8$).

Compounds **30** and **32** exhibited an even greater shift in the OEC, the P_{50} ratio (drug to control) increasing by a factor around 10 (P_{50} 48.6 and 43 mmHg, respectively). The average control P_{50} of pure Hb solution in the absence of an allosteric effector was consistent, near 4.7 mmHg. Both **30** and **32** also retained good cooperativity (see Hill coefficient, Table 2). In summary, these effectors appear to be strong candidates for further toxicological and pharmacological studies to measure their in vivo efficacy in animals. RSR13, currently in clinical trials, and JP7, on the other hand, only increased the P_{50} ratio (effector/control) by a factor of 5.

Whole Blood Studies. In general, the enantiomers that showed equivalent activity in hemoglobin solutions

Table 2. Results of Hemoglobin Solution Studies[a]

compound	configuration	R	$P_{50}e$[b]	$P_{50}e/P_{50}c$[c]	n_{50}[d]
3			23.4	4.7	2.3
4			25.7	5.1	2.4
12	Gly	H	12.0	2.4	2.6
13	D-ALa	CH_3	10.5	2.1	2.7
14	L-Ala	CH_3	13.8	2.8	2.6
15	D-Leu	$CH_2CH(CH_3)_2$	21.3	4.3	2.7
16	L-Leu	$CH_2CH(CH_3)_2$	9.4	1.9	2.6
17	D-Val	$CH(CH_3)_2$	13.0	2.6	2.7
18	L-Val	$CH(CH_3)_2$	13.9	2.8	2.6
19	D-Phe	$CH_2C_6H_5$	12.4	2.6	2.7
20	L-Phe	$CH_2C_6H_5$	8.2	1.7	2.6
21	D-Trp	$CH_2indole$	37.0	7.4	2.3
22	L-Trp	$CH_2indole$	37.1	7.4	2.1
23	D-Met	$(CH_2)_2SCH_3$	18.1	3.6	2.6
24	L-Met	$(CH_2)_2SCH_3$	9.5	1.9	2.8
25	D-Ser	CH_2OH	17.1	3.4	2.6
26	L-Ser	CH_2OH	11.2	2.3	2.6
27	N^ϵ-benzyloxycarbonyl-D-lys	$(CH_2)_4NHCOOCH_2C_6H_5$	29.5	5.9	2.4
28	N^ϵ-benzyloxycarbonyl-L-lys	$(CH_2)_4NHCOOCH_2C_6H_5$	22.4	4.5	2.5
29	L-Ile	$CH(CH_3)C_2H_5$	12.3	2.5	2.6
30	L-Tyr	$CH_2C_6H_4OH$	48.6	9.7	2.3
31	L-Pro	$(CH_2)_3$	13.5	2.7	2.7
32	S-benzyl-L-cys	$CH_2SCH_2C_6H_5$	43.0	8.6	2.1
33	L-Thr	$CH(OH)CH_3$	14.3	2.9	2.6
34	L-Asp	CH_2COOH	16.1	3.2	2.6
35	L-Glu	CH_2CH_2COOH	16.8	3.4	2.6
36	D-Lys	$(CH_2)_4NH_2$	5.8	1.2	2.3
37	L-Lys	$(CH_2)_4NH_2$	8.1	1.6	2.4

[a] All studies were carried out at 50−60 μM heme concentration and in the presence of 0.5 mM effector. All compound solutions were prepared in 100 mM bis-Tris buffer, pH 7.2, except compounds **19** and **20** that were prepared in 30% DMSO. [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 $P_{50}e/P_{50}c$ (P_{50} control value with no effector present, 5.0 mmHg, in case of DMSO is 4.7 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).

also showed equivalent activity in the whole blood studies. Effectors with differences in enantiomeric activity in Hb solutions also exhibited the same trends in the whole blood studies, the D-isomer conjugates being more potent than the L-isomer conjugates. All of the new D- and L-analogues, with the exception of **28** (L), **36** (D), and **37** (L), increased whole blood P_{50} values by varying degrees (Table 1).

One disappointing observation occurred when it was found that enantiomers **21/22** and compounds **30** and **32** did not show a significant rise in the P_{50} in the whole blood studies probably due to the propensity of the tryptophan, tyrosine, and benzyl-cys moieties to bind to human serum albumin and plasma protein or due to their poor red cell membrane permeability. To explore whether such factors are responsible for the poor allosteric activities of enantiomers **21/22** and compounds **30** and **32**, we have initiated plasma protein binding and red cell binding partitioning studies with these compounds.

It appears that the lack of allosteric effector potency of compounds **28**, **36**, and **37** is probably related to their long and polar amino acid side chain groups, which could deter transport into red blood cells. Compounds **15** {1-[4-(((3,5-dimethylanilino)carbonyl)methyl)phenoxy]-cyclo-pentanecarbonyl-D-leucine} and **19** {D-phenylalanine} were found to be the most active allosteric effectors, right-shifting the oxygen dissociation curve in whole blood by 42 and 38 mmHg, respectively. Compounds **15** and **19** are equipotent in whole blood with JP7. Further pharmacokinetic evaluation of active analogues is underway to determine if any differences in plasma protein binding capability can be attributed to allosteric activity variations.

Crystallographic Binding Studies. Hb crystallographic binding studies were conducted on two pairs of allosterically active enantiomers, constituting **17** and **18** as one pair and enantiomers **19** and **20** as the second pair. We asked the question, does the stereochemistry of effector side chains play a crucial role in binding the effectors to the Hb water cavity? Therefore, we selected enantiomeric pairs (**17/18** and **19/20**) for crystallographic studies. Enantiomers (**17** and **18**), possessing small alkyl side chains, are equipotent in their allosteric activities and should show no major differences in binding, while enantiomers **19** and **20** with bulky aromatic side chains exhibit significant differences in their allosteric properties and should show significant differences in binding. In efforts to evaluate these differences in binding interactions resulting from the extended amino acid side chains that can be correlated to their differences in allosteric activities, enantiomers (**17/18** and **19/20**) were selected for the crystallographic studies. The difference electron density maps show all four compounds bind to the Hb central water cavity in symmetry related pairs, similar to that found for JP7
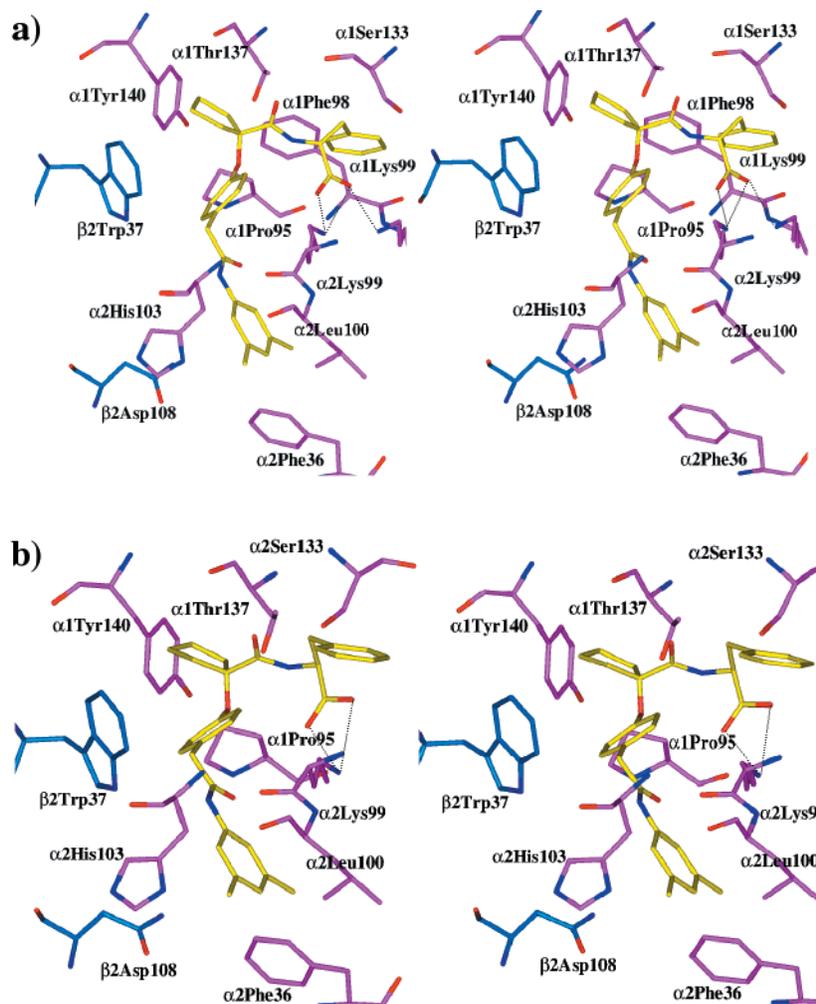
Figure 2. Stereoview of the allosteric site of Hb complexes of enantiomers **19** (a) and **20** (b). The effector (yellow) interacts with $\alpha1$ and $\alpha2$ subunits (magenta) and $\beta2$ subunit (blue). Atoms are shown in stick representation, with oxygen and nitrogen atoms colored red and blue, respectively. Hydrogen bonds are shown in black dashed lines.

(Abraham et al., unpublished results) and RSR13.[11] However, further analysis indicates that unlike JP7 and RSR13 effectors, the symmetry related pair of each enantiomer (**17**, **18**, **19**, and **20)** cannot be simultaneously accommodated in the central water cavity due to the steric interactions between each. As also found previously with JP7 and RSR13, each bound effector interacts with three different subunits (two α and one β).

The JP7 portion of each enantiomer−Hb complex exhibited similar interactions with Hb as observed for the unconjugated JP7 molecule, except for Lys 99α. With RSR-13 and JP7, the bridging amide carbonyl oxygen of the effectors points toward the terminal side chain ammonium of Lys 99α_2 forming a hydrogen bond. On the other hand, the conjugated amino acid carboxylate of the new conjugates makes a hydrogen bond with Lys 99α_2. For all complexes, 3,5-dimethyl phenyl ring is nestled in a pocket surrounded by the following hydrophobic residues Phe 36α_2, Lys 99α_2, Leu 100α_2, and His 103α_2 (Figures 2 and 3). As previously indicated, the hydrophobic interaction between the 3,5-dimethyl group and the residues Lys 99α_2, Leu 100α_2, and His 103α_2 constrains the T-state of deoxy Hb by preventing subunit rotation during the allosteric transition. Similar to JP7 and RSR13 complexes, only one of

the two 3,5-dimethyl groups on the terminal phenyl ring was oriented toward these hydrophobic residues. The other methyl was directed into the water cavity. The amide group of Asn 108β_2 forms an interaction with the π electron cloud of the 3,5-dimethyl phenyl group via a Lewis acid−base like interaction. Perutz et al.[21] proposed that the π cloud of the benzene ring of the structurally similar BZF interacts with the electron-deficient side chain of Asn 108β_2. This novel type of aromatic/polar hydrogen bond has about one-half the strength of a normal hydrogen bond and may make a significant contribution to protein stability.[22] Hydrophobic interactions were also observed between the phenoxy ring and Trp 37β_2. The cyclopentane ring made hydrophobic interactions with Tyr 140α_1, Pro 95α_1, and Thr 137α_1. By engaging in interdimer interactions with three of the four protein subunits, the effector stabilizes the T-state deoxy Hb and thus is able to shift the allosteric equilibrium to the right.

Unlike the enantiomeric pair **17** and **18**, the extended amino acid side chains of the enantiomer−Hb complexes of **19** and **20** are oriented differently from each other resulting in additional but different interactions. In the case of **19** (Figure 2a), the phenyl group of the amino acid side chain of the effector makes hydrophobic interactions with Pro 95α_1, Phe 98α_1, Lys 99α_1, and Ser
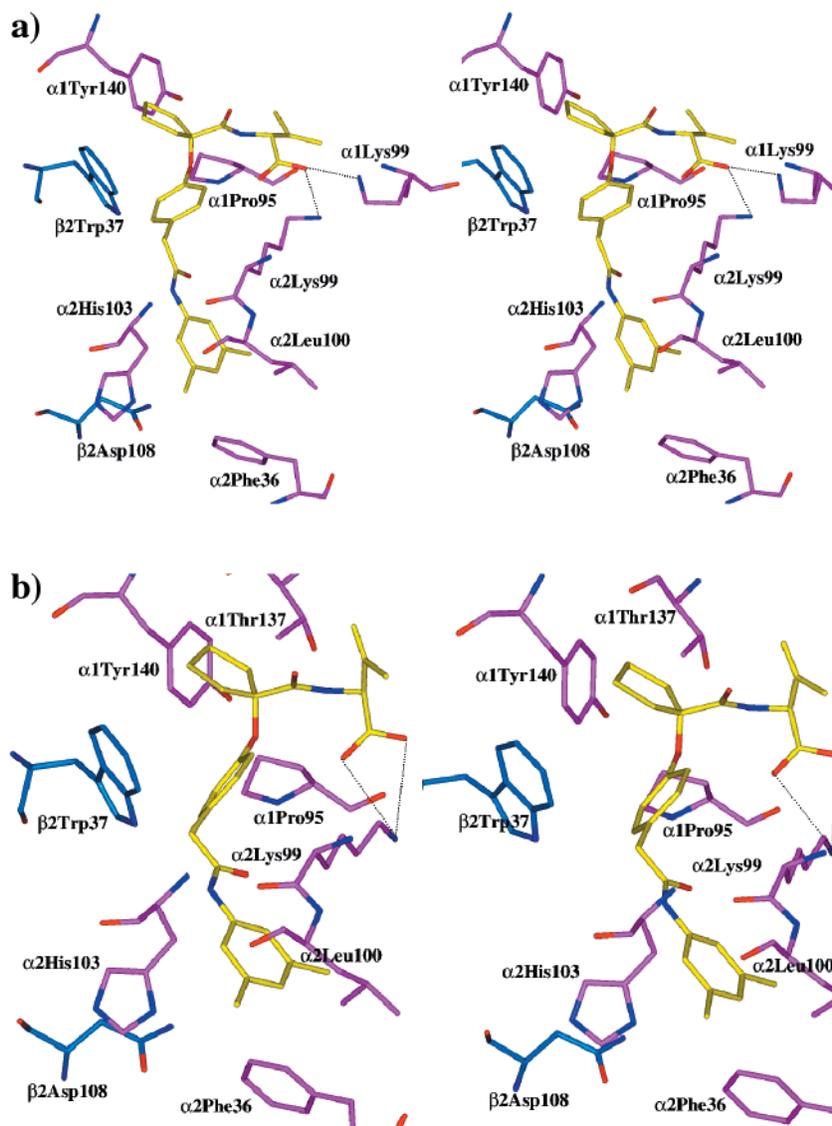
Figure 3. Stereoview of the allosteric site of Hb complexes of enantiomers **17** (a) and **18** (b). The effector (yellow) interacts with α1 and α2 subunits (magenta) and β2 subunit (blue). Atoms are shown in stick representation, with oxygen and nitrogen atoms colored red and blue, respectively. Hydrogen bonds are shown in black dashed lines.

133α$_1$. On the other hand, enantiomer **20**, which is a weaker acting effector than **19**, exhibited hydrophobic interactions with only Ser 133α$_2$ (Figure 2b). We believe this accounts for the lower activity observed for **20**.

The binding sites of enantiomers **17** and **18** with Hb are shown in parts a and b of Figure 3, respectively. The best fit to the difference electron density maps for each showed that the amino acid conjugate in both complexes does not make strong interactions with the protein. The lack of differential contact would account for the similar activity observed for both enantiomers. The crystallographic studies therefore suggest that the amino acid conjugate derivatives of JP7 reported here play a crucial role in contributing to the allosteric activity.

Summary and Conclusions

The chiral allosteric amino acid conjugates of JP7 synthesized in this study decreased the oxygen affinity of hemoglobin and exhibit a wide range of allosteric activities. The orientation of the chiral center deter-mines the observed allosteric activity. SARs demon-strated that, in general, D-isomers are more potent than the corresponding L-isomers. D-Orientation enables greater polar hydrophobic side chain interactions be-tween the effectors and binding site residues. Within the Hb solution data, the amino acid conjugates con-taining a heteroatom or polar group such as hydroxyl are more active than effectors with branched chain alkyl and arylalkyl susbtituents. However, in whole blood studies, the activity profile is reversed where effectors with branched chain alkyl and arylalkyl susbtituents are more active than amino acid conjugates containing a heteroatom or polar group. The loss of activity by the polar effector conjugates is probably due to serum protein binding and lack of transport into erythrocytes. Studies are underway to test this hypothesis. The X-ray studies indicate that the orientation and interactions of the chiral center with the protein plays an important role in determining the observed allosteric activity. In general, all effectors function by preventing the subunit rotation during the allosteric transition. The effectors

stabilize the T-state to varying degrees, and this is reflected in the observed allosteric activities.

Experimental Section

Chemistry. All reagents and starting materials used in the synthesis were purchased from Aldrich Chemical Co., Sigma, Fluka, and Advanced-Chem-Tech and were used directly without further purification. Specifically, glycine methyl ester, D- and L-alanine, leucine, valine, phenylalanine, tryptophan, methionine, and serine methyl ester hydrochlorides in 99% purity were purchased from Aldrich. N^{ϵ}-benzyloxycarbonyl-D- and L-lysine, L-tyrosine, L-isoleucine, L-proline, *S*-benzyl-L-cysteine, L-threonine methyl ester hydrochlorides, L-aspartyl-γ-*tert*-butyl, L-glutamate-γ-*tert*-butyl ester hydrochlorides in 98% purity were purchased from Advanced-Chem-Tech. All solvents were purchased from Fischer Scientific Co. and Aldrich Chemical Co. Silica gel coated plates (0.25 mm thickness) were purchased from Analtech Inc. and used for thin-layer chromatography (TLC). The separations were spotted by visualizing under UV light ($\lambda = 254$ nm) or iodine chamber. Flash chromatographic separations were done using Merck, grade 9385, 230−400 mesh, 60 Å silica gel. The proton nuclear magnetic magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Gemini 300 MHz spectrophotometer and are reported in parts per million ($\delta = $ ppm) using tetramethylsilane (TMS) as an 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 by TLC and ^{1}H NMR but are not reported. Melting points (mp) were determined on Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were determined using a Digital Polarimeter (DIP 1000) Ver. 1.31.00 (Jasco Corp.).

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarboxylic Acid (4, JP7). Scheme 1. To a tetrahydrofuran (25 mL) solution of 4-[[(3,5-dimethylanilino)-carbonyl]methyl]phenol (2.0 g, 7.84 mmol) stirred at 0 °C were added pulverized sodium hydroxide (3.14 g, 78.4 mmol) and cyclopentanone (6.59 g, 78.4 mmol). To this mixture was added 3.74 g (31.4 mmol) of chloroform dropwise over a 20 min period. The reaction mixture was stirred and maintained below 15 °C for the first hour and then stirred at room temperature overnight. Tetrahydrofuran was evaporated under reduced pressure to give a solid residue that was dissolved in water and extracted with ethyl acetate, the aqueous layer was acidified with 37% hydrochloric acid, and the organic materials re-extracted with ethyl acetate.

The title compound was extracted into aqueous sodium bicarbonate, acidified, and re-extracted with ethyl acetate. The organic layer was dried over anhydrous magnesium sulfate, filtered, and evaporated under reduced pressure. The analytically pure product was obtained upon recrystallization from ethyl acetate and hexane; yield 1.88 g, 65%. Mp: 170−171 °C. ^{1}H NMR (CDCl$_3$): δ 1.75−1.81 (m, 4H), 2.17−2.4 (m, 10H), 3.60 (s, 2H), 6.75 (s, 1H), 6.84 (d, 2H, $J = 8$ Hz), 7.1 (s, 2H), 7.18 (d, 2H, $J = 8$ Hz). Anal. (C$_{22}$H$_{25}$NO$_4$) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarboxylic Acid (4, JP7). Scheme 2. The title compound was synthesized in four steps:

(1) A mixture of cyclopentanecarboxylic acid (15.0 g, 131.6 mmol), ethanol (70 mL, 200% proof), and concentrated sulfuric acid (1 mL, 98%) was refluxed for 5 h using a water condenser. The reaction can be monitored by TLC (hexane:ethyl acetate, 9:1, developed in iodine chamber) because the starting compound as well as the product are not visible under UV light. After completion of the reaction, ethanol was evaporated from the reaction mixture under reduced pressure. The pale yellow liquid obtained was cooled to room temperature and then slowly poured over crushed ice under stirring. The product separated as an oil and was isolated using a separatory funnel, and the aqueous portion was extracted with chloroform (2 × 50 mL). The combined organic layer was washed with water, 100 mL of saturated solution of sodium bicarbonate, followed

by water. It was then dried over anhydrous magnesium sulfate. The removal of the solvent afforded a pale yellow residue, ethyl cyclopentanecarboxylate. The analytical pure sample was obtained by flash chromatography using hexane as eluent; yield 17.6, 94%. ^{1}H NMR (CDCl$_3$): δ 1.22 (t, 3H, $J = 7$ Hz), 1.54−1.89 (m, 8H), 2.69 (m, 1H), 4.09 (q, 2H, $J = 7$ Hz).

(2) A mixture of ethyl cyclopentanecarboxylate (15.0 g, 105.6 mmol), *N*-bromosuccinimide (19.7 g, 110.7 mmol), and carbon tetrachloride (120 mL) was heated to reflux under the infrared lamp for 6 h. The yellow reaction mixture turned red after initiation and then slowly became almost colorless. Separation of white solid, succinimide, was observed during this time. The reaction progress was monitored by TLC (hexane:ethyl acetate, 9:1). The product can be detected only by the difference in color after complexation with iodine. After completion of the reaction, the reaction mixture was filtered while hot. Evaporation of solvent furnished a yellow liquid residue, which was cooled to 5 °C and kept refrigerated for an additional 16 h. It was then filtered, and the organic layer was evaporated under reduced pressure to get a pale yellow liquid, ethyl-2-bromocyclopentanecarboxylate. The analytically pure sample was obtained by flash chromatography using hexane as eluent; yield 21.3 g, 91.4%. ^{1}H NMR (CDCl$_3$): δ 1.33 (t, 3H, $J = 7$ Hz), 1.8 (m, 2H), 1.98 (m, 2H), 2.28 (t, 4H, $J = 6$ Hz), 4.24 (q, 2H, $J = 7$ Hz).

(3) A mixture of *N*-(3,5-dimethylphenyl)-4-hydroxyphenylacetamide (5.0 g, 19.6 mmol), ethyl-2-bromocyclopentanecarboxylate (15.2 g, 68.8 mmol), 325 mesh potassium carbonate (8.1 g, 58.7 mmol), potassium iodide (0.01 g, 0.06 mmol), and ethanol (50 mL) was heated to reflux for 25 h. The reaction mixture was filtered, and the residue was washed with ethanol (30 × 50 mL). The combined filtrate was evaporated under reduced pressure to get a dark liquid, which solidified on standing. The pure ester, ethyl 1-[4-(((3,5-dimethylanilino)-carbonyl)methyl)phenoxy]cyclopentanecarboxylate was obtained by flash chromatography using hexane−chloroform mixture in 5% increments up to 50% chloroform maximum as eluent; yield 7.1 g, 92%. Mp: 80−81 °C. ^{1}H NMR (CDCl$_3$) δ 1.2 (t, 3H, $J = 7$ Hz), 1.81 (m, 4H), 2.2−2.4 (m, 10H), 3.6 (s, 2H), 4.15 (q, 2H, $J = 7$ Hz), 6.6 (s, 1H), 6.78 (d, 2H, $J = 8$ Hz), 7.03 (s, 2H), 7.18 (d, 2H, $J = 8$ Hz).

(4) The ethyl ester (6.0 g, 15.2 mmol) was then dissolved in ethanol (60 mL). To this solution were added potassium hydroxide (2.13 g, 38.0 mmol) and water (3 mL). The mixture was heated to reflux for 50 h. The solvent was evaporated under reduced pressure, and the solid residue obtained was dissolved in water (70 mL). The resultant solution was filtered, and the filtrate was acidified using concentrated hydrochloric acid, filtered, and washed with water.

The pure product **4**, JP7, was obtained upon recrystallization from ethyl acetate and hexane; yield 5.1 g, 91%. Mp and ^{1}H NMR data were the same as the compound prepared in Scheme 1. Anal. (C$_{22}$H$_{25}$NO$_4$) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentane Carbonyl Glycine (12). Scheme 3. To a stirring solution of 1-[4-(((3,5-dimethylanilino)carbonyl)methyl)phenoxy]cyclopentanecarboxylic acid (2.21 g, 6.0 mmol), glycine methyl ester hydrochloride (0.75 g, 6.0 mmol), and 1-hydroxybenzotriazole hydrate (0.88 g, 6.5 mmol) in dimethylformamide (30 mL) were added *N*-methylmorpholine (0.9 g, 8.9 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.36 mg, 7.1 mmol) under nitrogen at room temperature. After being stirred for another 24 h, the reaction mixture was diluted with ethyl acetate (100 mL) and washed with water (40 mL). The ethyl acetate solution was further washed with 10% potassium hydrogen sulfate solution (2 × 50 mL), brine (50 mL), saturated sodium bicarbonate solution (2 × 50 mL), and brine (50 mL). The organic phase was dried over anhydrous magnesium sulfate, filtered, and evaporated under reduced pressure. The pure ester product was obtained by flash chromatography using hexane:ethyl acetate (1:1) as eluent; yield 2.32 g, 88.5%. Mp: 142−143 °C. ^{1}H NMR (CDCl$_3$): δ 1.71−1.8 (m, 4H), 2.1−2.34 (m, 10H), 3.63 (s, 2H),

3.68 (s, 3H), 4.04 (d, 2H, $J = 5.8$ Hz), 6.7 (s, 1H), 6.84 (d, 2H, $J = 8$ Hz), 7.1 (s, 2H), 7.18 (d, 2H, $J = 8$ Hz).

The corresponding glycine methyl ester (1.0 g, 2.3 mmol) in ethanol (30 mL) and lithium hydroxide (0.11 g, 4.6 mmol) dissolved in water (10 mL) were stirred at room temperature overnight. The solvent was evaporated on a rotavap at room temperature. The residual product was dissolved in water (100 mL) and extracted with ethyl acetate (2 × 50 mL). The aqueous phase was acidified with hydrochloric acid and extracted with ethyl acetate (4 × 40 mL). The organic phase was washed with brine (2 × 50 mL), dried over anhydrous magnesium sulfate, filtered, and evaporated to give a pure product; yield 0.8 g, 82.5%. Mp: 160−161 °C. ^{1}H NMR (CDCl$_3$): δ 1.71−1.8 (m, 4H), 2.1−2.34 (m, 10H), 3.63 (s, 2H), 4.04 (d, 2H, $J = 5.8$ Hz), 6.7 (s, 1H), 6.84 (d, 2H, $J = 8$ Hz), 7.1 (s, 2H), 7.18 (d, 2H, $J = 8$ Hz). Anal. (C$_{24}$H$_{28}$N$_2$O$_5$) C, H, N.

Compounds **13**−**33** were prepared using the same procedure as described above for compound **12**.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-D-alanine (13). JP7 (2.21 g, 6.0 mmol) was reacted with D-alanine methyl ester hydrochloride (0.84 g, 6.0 mmol), 1-hydroxybenzotriazole hydrate (0.88 g, 6.5 mmol), N-methylmorpholine (0.9 g, 8.9 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.36 mg, 7.1 mmol). The crude product obtained after workup was purified by flash chromatography using hexane:ethyl acetate (1:1); yield 2.35 g, 86.7%. Mp: 117−118 °C. ^{1}H NMR (CDCl$_3$): δ 1.35 (d, 3H, $J = 7.2$ Hz), 1.68−1.76 (m, 4H), 2.04−2.14 (m, 2H), 2.2−2.4 (m, 8H), 3.6 (s, 2H), 3.65 (s, 3H), 4.59 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, $J = 8$ Hz), 7.1 (s, 2H), 7.18 (d, 2H, $J = 8$ Hz).

The final compound was prepared using the D-alanine methyl ester (1.04 g, 2.3 mmol). The product was obtained upon recrystallization from ether and hexane; yield 0.9 g, 89.1%. Mp: 168−169 °C. $[\alpha]_D$ +23.8° ($c = 0.5$, methanol). ^{1}H NMR (CDCl$_3$): δ 1.35 (d, 3H, $J = 7.2$ Hz), 1.68−1.76 (m, 4H), 2.04−2.14 (m, 2H), 2.2−2.4 (m, 8H), 3.6 (s, 2H), 4.59 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, $J = 8$ Hz), 7.1 (s, 2H), 7.18 (d, 2H, $J = 8$ Hz). Anal. (C$_{25}$H$_{30}$N$_2$O$_5$) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-alanine (14). This compound was synthesized similarly to the previous reaction using L-alanine methyl ester hydrochloride (0.84 g, 6 mmol). The crude product was purified by flash chromatography using hexane:ethyl acetate (1:1) as eluent; yield 2.0 g, 73.8%. Mp: 117−118 °C.

The title compound was obtained on hydrolysis of the corresponding L-alanine methyl ester (1.04 g, 2.3 mmol). A pure final product was obtained upon recrystallization from ether and hexane; yield 0.92 g, 91.1%. Mp: 169−171 °C. $[\alpha]_D$ −25.0° ($c = 0.4$, methanol). Anal. (C$_{25}$H$_{30}$N$_2$O$_5$) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-D-leucine (15). This compound was synthesized similarly to the previous reaction using D-leucine methyl ester hydrochloride (1.1 g, 6.0 mmol). The crude ester was recrystallized using ether−hexane mixture; yield 2.22 g, 75%. Mp: 119−120 °C. ^{1}H NMR (CDCl$_3$): δ 0.82 (2d, 6H, $J = 5.3$ Hz), 1.42−1.6 (m, 3H), 1.74−1.8 (m, 4H), 2.04−2.12 (m, 2H), 2.21−2.34 (m, 8H), 3.6 (s, 2H), 3.65 (s, 3H), 4.6 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, $J = 8$ Hz), 7.1 (s, 2H), 7.18 (d, 2H, $J = 8$ Hz).

The end product was produced by hydrolysis of the corresponding D-leucine methyl ester (1.14 g, 2.3 mmol); yield 0.94 g, 84.7%. Mp: 87−88 °C. $[\alpha]_D$ +37.3° ($c = 1.05$, methanol). ^{1}H NMR (CDCl$_3$): δ 0.82 (2d, 6H, $J = 5.3$ Hz), 1.42−1.6 (m, 3H), 1.74−1.8 (m, 4H), 2.04−2.12 (m, 2H), 2.21−2.34 (m, 8H), 3.6 (s, 2H), 4.6 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, $J = 8$ Hz), 7.1 (s, 2H), 7.18 (d, 2H, $J = 8$ Hz). Anal. (C$_{28}$H$_{36}$N$_2$O$_5$) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-leucine (16). This compound was synthesized similarly to the previous reaction using L-leucine methyl ester hydrochloride (1.1 g, 6.0 mmol). The crude ester was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.31 g, 78%. Mp: 120−122 °C.

The final acid was prepared similarly to the previous reaction subjecting the corresponding l-leucine methyl ester for hydrolysis (1.14 g, 2.3 mmol); yield 0.99 g, 89.2%. Mp: 89−90 °C. $[\alpha]_D$ −37.7° ($c = 1.03$, methanol). Anal. (C$_{28}$H$_{36}$N$_2$O$_5$) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-D-valine (17). This compound was synthesized similarly to the previous reaction using D-valine methyl ester hydrochloride (1.0 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent. The product was recrystallized using ether and hexane; yield 2.44 g, 85%. Mp: 104−105 °C. ^{1}H NMR (CDCl$_3$): δ 0.73 (d, 3H, $J = 6.8$ Hz), 0.81 (d, 3H, $J = 6.8$ Hz), 1.71−1.81 (m, 4H), 2.04−2.18 (m, 2H), 2.21−2.4 (m, 9H), 3.6 (s, 2H), 3.65 (s, 3H), 4.5 (dd, 1H, $J = 5,10$), 6.7 (s, 1H), 6.84 (d, 2H, $J = 8$ Hz), 7.1 (s, 2H), 7.18 (d, 2H, $J = 8$ Hz).

Compound **17** was synthesized similarly to the previous reaction using the corresponding D-valine methyl ester (1.1 g, 2.3 mmol) for hydrolysis; yield 1.0 g, 93.5%. Mp: 81−82 °C. $[\alpha]_D$ +14.5° ($c = 1$, methanol). ^{1}H NMR (CDCl$_3$): δ 0.73 (d, 3H, $J = 6.8$), 0.81 (d, 3H, $J = 6.8$ Hz), 1.71−1.81 (m, 4H), 2.04−2.18 (m, 2H), 2.21−2.4 (m, 9H), 3.6 (s, 2H), 4.5 (dd, 1H, $J = 5,10$ Hz), 6.7 (s, 1H), 6.84 (d, 2H, $J = 8$ Hz), 7.1 (s, 2H), 7.18 (d, 2H, $J = 8$ Hz). Anal. (C$_{27}$H$_{34}$N$_2$O$_5$·0.25H$_2$O) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-valine (18). l-Valine methyl ester hydrochloride (1.0 g, 6.0 mmol) was used to prepare compound **19**. The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.64 g, 92%. Mp: 104−106 °C.

The L-valine methyl ester (1.1 g, 2.3 mmol) was then hydrolyzed as before to yield 0.92 g, 86% of compound **19**. Mp: 81−82 °C. $[\alpha]_D$ −18.2° ($c = 0.5$, methanol). Anal. (C$_{27}$H$_{34}$N$_2$O$_5$·0.5H$_2$O) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-D-phenylalanine (19). This compound was synthesized similarly to the previous reaction using D-phenylalanine methyl ester hydrochloride (1.29 g, 6.0 mmol). The crude ester obtained after workup was recrystallized using ether−hexane mixture; yield 2.88 g, 91.1%. Mp: 106−108 °C. ^{1}H NMR (CDCl$_3$): δ 1.7−1.81 (m, 4H), 1.9−2.35 (m, 10H), 2.97 (dd, 1H, $J = 7.6,14.2$ Hz), 3.11 (dd, 1H, $J = 5.3,14.2$ Hz), 3.6 (s, 2H), 3.65 (s, 3H), 4.85 (m, 1H), 6.65 (s, 1H), 6.71 (d, 2H, $J = 8$ Hz), 6.95−7.18 (m, 9H).

The final acid was obtained using the corresponding D-phenylalanine methyl ester (1.21 g, 2.3 mmol) for hydrolysis; yield 1.1 g, 93.2%. Mp: 87−88 °C. $[\alpha]_D$ +8.2° ($c = 1.05$, methanol). ^{1}H NMR (CDCl$_3$) δ 1.7−1.81 (m, 4H), 1.9−2.35 (m, 10H), 2.97 (dd, 1H, $J = 7.6,14.2$ Hz), 3.11 (dd, 1H, $J = 5.3,-14.2$ Hz), 3.6 (s, 2H), 4.85 (m, 1H), 6.65 (s, 1H), 6.71 (d, 2H, $J = 8$ Hz), 6.95−7.18 (m, 9H). Anal. (C$_{31}$H$_{34}$N$_2$O$_5$·0.25H$_2$O) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-phenylalanine (20). This compound was prepared using L-phenylalanine methyl ester hydrochloride (1.29 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.52 g, 79.7%. Mp: 107−109 °C.

The hydrolysis of L-phenylalanine methyl ester (1.21 g, 2.3 mmol) afforded **20**; yield 1.0 g, 84.7%. Mp: 87−88 °C. $[\alpha]_D$ −9.9° ($c = 0.88$, methanol). Anal. (C$_{31}$H$_{34}$N$_2$O$_5$·0.25H$_2$O) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-D-tryptophan (21). This compound was synthesized as above using D-tryptophan methyl ester hydrochloride (1.53 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.72 g, 80%. Mp: 83−84 °C. ^{1}H NMR (DMSO-d_6): δ 1.6−1.8 (m, 4H), 2.16−2.2 (m, 2H), 2.21−2.4 (s, 8H), 3.08 (dd, 1H, $J = 8.7,15$ Hz), 3.3 (dd, 1H, $J = 4, 15$ Hz), 3.5 (s, 2H), 3.55 (s, 3H), 4.76 (m, 1H), 6.6 (s, 1H), 6.7 (d, 2H, $J = 8$ Hz), 6.79 (s, 1H), 6.9 (t, 1H, $J =$

7.5 Hz), 7.01 (t, 1H, J = 7.5 Hz) 7.1 (s, 2H), 7.2 (d, 2H, J = 8 Hz), 7.25 (d, 1H, J = 7.5 Hz), 7.46 (d, 1H, J = 7.5 Hz).

The hydrolysis of D-tryptophan methyl ester (1.3 g, 2.3 mmol) produced **22**; yield 1.2 g, 94.5%. Mp: 107−108 °C. [α]$_D$ +62.8° (c = 0.5, methanol). ^{1}H NMR (DMSO-d_6): δ 1.6−1.8 (m, 4H), 2.16−2.2 (m, 2H), 2.21−2.4 (s, 8H), 3.08 (dd, 1H, J = 8.7,15 Hz), 3.3 (dd, 1H, J = 4, 15 Hz), 3.5 (s, 2H), 4.76 (m, 1H), 6.6 (s, 1H), 6.7 (d, 2H, J = 8 Hz), 6.79 (s, 1H), 6.9 (t, 1H, J = 7.5 Hz), 7.01 (t, 1H, J = 7.5 Hz) 7.1 (s, 2H), 7.2 (d, 2H, J = 8 Hz), 7.25 (d, 1H, J = 7.5 Hz), 7.46 (d, 1H, J = 7.5 Hz). Anal. (C$_{33}$H$_{35}$N$_3$O$_5$) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-tryptophan (22). This compound was obtained from L-tryptophan methyl ester hydrochloride (1.53 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.95 g, 86.8%. Mp: 86−87 °C.

The ester (1.3 g, 2.3 mmol) was then hydrolyzed as before to furnish **23**; yield 1.2 g, 94.5%. Mp: 107−108 °C. [α]$_D$ −63.1° (c = 0.5, methanol). Anal. (C$_{33}$H$_{35}$N$_3$O$_5$·0.75H$_2$O) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-D-methionine (23). This compound was synthesized by using D-methionine methyl ester hydrochloride (1.2 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.8 g, 96%. Mp: 54−55 °C. ^{1}H NMR (CDCl$_3$): δ 1.76−1.81 (m, 4H), 1.91−2.0 (m, 4H), 2.1−2.4 (m, 13H), 3.6 (s, 2H), 3.7 (s, 3H), 4.66 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1 (s, 2H), 7.18 (d, 2H, J = 8 Hz).

The final acid was obtained from the corresponding D-methionine methyl ester (1.64 g, 2.3 mmol) on hydrolysis; yield 1.28 g, 80%. Mp: 62−63 °C. [α]$_D$ +24.9° (c = 0.3, methanol). ^{1}H NMR (CDCl$_3$): δ 1.76−1.81 (m, 4H), 1.91−2.0 (m, 4H), 2.1−2.4 (m, 13H), 3.6 (s, 2H), 4.66 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1 (s, 2H), 7.18 (d, 2H, J = 8 Hz). Anal. (C$_{27}$H$_{34}$N$_2$O$_5$S) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-methionine (24). This compound was prepared using L-methionine methyl ester hydrochloride (1.2 g, 6.0 mmol). As usual, the crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.74 g, 93.8%. Mp: 55−57 °C.

The final compound was obtained by hydrolysis of the corresponding L-methionine methyl ester (1.64 g, 2.3 mmol); yield 1.33 g, 83.1%. Mp: 64−65 °C. [α]$_D$ −25.5° (c = 0.25, methanol). Anal. (C$_{27}$H$_{34}$N$_2$O$_5$S) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-D-serine (25). This compound was synthesized using D-serine methyl ester hydrochloride (0.93 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using ethyl acetate as eluent; yield 2.52 g, 94.7%. Mp: 58−59 °C. ^{1}H NMR (DMSO-d_6): δ 1.64−1.81 (m, 4H), 2.16−2.2 (m, 2H), 2.21−2.4 (m, 8H), 3.5 (s, 2H), 3.55 (s, 3H), 3.6 (dd, 1H, J = 3, 11 Hz), 3.72 (dd, 1H, J = 4, 11 Hz), 4.24 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1 (s, 2H), 7.2 (d, 2H, J = 8 Hz).

The ester D-serine methyl ester (1.08 g, 2.3 mmol) was then hydrolyzed to afford **25**; yield 0.94 g, 89.5%. Mp: 73−75 °C. [α]$_D$ +19.2° (c = 0.5, methanol). ^{1}H NMR (DMSO-d_6): δ 1.64−1.81 (m, 4H), 2.16−2.2 (m, 2H), 2.21−2.4 (m, 8H), 3.5 (s, 2H), 3.6 (dd, 1H, J = 3, 11 Hz), 3.72 (dd, 1H, J = 4, 11 Hz), 4.24 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1 (s, 2H), 7.2 (d, 2H, J = 8 Hz). Anal. (C$_{25}$H$_{30}$N$_2$O$_6$·0.5H$_2$O) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-serine (26). This compound was synthesized using corresponding L-isomer of D-serine methyl ester hydrochloride (0.93 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using ethyl acetate as eluent; yield 2.51 g, 94.4%. Mp: 60−62 °C.

The corresponding ester (1.08 g, 2.3 mmol) was then hydrolyzed to furnish **26**, yield 0.93 g, 88.6%. Mp: 73−74 °C. [α]$_D$ −20.3° (c = 0.4, methanol). Anal. (C$_{25}$H$_{30}$N$_2$O$_6$·0.25H$_2$O) C, H, N.

N^{α}-1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]cyclopentane-carbonyl-N^{ϵ}-benzyloxycarbonyl-D-lysine (27). This lysine derivative was synthesized using N^{ϵ}-benzyloxycarbonyl-D-lysine methyl ester hydrochloride (1.99 g, 6.0 mmol). The crude ester was purified by flash chromatography using hexane:ethyl acetate (1:1) as eluent; yield 3.6 g, 93.5%. Mp: 53−55 °C. ^{1}H NMR (CDCl$_3$): δ 1.1−1.35 (m, 4H), 1.76−1.85 (m, 4H), 2.02−2.4 (m, 10H), 2.95−3.05 (m, 4H), 3.6 (s, 2H), 3.65 (s, 3H), 4.55 (m, 1H), 5.15 (s, 2H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1−7.35 (m, 9H).

The title compound was obtained after base hydrolysis of the corresponding N^{ϵ}-benzyloxycarbonyl-D-lysine methyl ester (1.48 g, 2.3 mmol); yield 1.34 g, 92.4%. Mp: 61−62 °C. [α]$_D$ +18.5° (c = 3, methanol). ^{1}H NMR (CDCl$_3$): δ 1.1−1.35 (m, 4H), 1.76−1.85 (m, 4H), 2.02−2.4 (m, 10H), 2.95−3.05 (m, 4H), 3.6 (s, 2H), 4.55 (m, 1H), 5.15 (s, 2H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1−7.35 (m, 9H). Anal. (C$_{36}$H$_{43}$N$_3$O$_7$) C, H, N.

N^{α}-1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)-phenoxy]cyclopentane-carbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysine (28). The corresponding L-isomer was obtained by reacting N^{ϵ}-benzyloxycarbonyl-L-lysine methyl ester hydrochloride (0.93 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (1:1) as eluent; yield 3.28 g, 85.2%. Mp: 53−55 °C.

Hydrolysis of N^{ϵ}-benzyloxycarbonyl-L-lysine methyl ester (1.48 g, 2.3 mmol) produced **28**; yield 1.3 g, 87.9%. Mp: 63−65 °C. [α]$_D$ −19.9° (c = 2.4, methanol). Anal. (C$_{36}$H$_{43}$N$_3$O$_7$·0.5H$_2$O) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-isoleucine (29). This compound was prepared by using L-isoleucine methyl ester hydrochloride (1.1 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.31 g, 78%. Mp: 127−128 °C. ^{1}H NMR (CDCl$_3$): δ 0.82 (t, 3H, J = 7.3 Hz), 0.9 (d, 3H, J = 6.8 Hz), 0.98−1.4 (m, 2H), 1.69−1.81 (m, 4H), 2.02−2.18 (m, 2H), 2.2−2.45 (m, 9H), 3.6 (s, 2H), 3.65 (s, 3H), 4.48 (dd, 1H, J = 6, 8.4 Hz), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1 (s, 2H), 7.2 (d, 2H, J = 8 Hz).

The ester L-isoleucine methyl ester (1.14 g, 2.3 mmol) was further hydrolyzed to give **29**; yield 0.97 g, 87.4%. Mp: 72−73 °C. [α]$_D$ −11.6° (c = 0.5, methanol). ^{1}H NMR (CDCl$_3$): δ 0.82 (t, 3H, J = 7.3 Hz), 0.9 (d, 3H, J = 6.8 Hz), 0.98−1.4 (m, 2H), 1.69−1.81 (m, 4H), 2.02−2.18 (m, 2H), 2.2−2.45 (m, 9H), 3.6 (s, 2H), 4.48 (dd, 1H, J = 6, 8.4 Hz), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1 (s, 2H), 7.2 (d, 2H, J = 8 Hz). Anal. (C$_{28}$H$_{36}$N$_2$O$_5$·0.5H$_2$O) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-tyrosine (30). This derivative was synthesized using L-tyrosine methyl ester hydrochloride (1.39 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.84 g, 87.1%. Mp: 74−75 °C. ^{1}H NMR (CDCl$_3$): δ 1.7−1.81 (m, 4H), 2.03−2.17 (m, 2H), 2.2−2.34 (m, 8H), 2.75 (dd, 1H, J = 9.5, 13.5 Hz), 3.15 (dd, 1H, J = 4.3, 13.5 Hz), 3.6 (s, 2H), 3.65 (s, 3H), 4.6 (m, 1H), 6.4 (s, 1H), 6.45 (d, 2H, J = 8 Hz), 6.65 (d, 2H, J = 8.5 Hz), 6.7 (d, 2H, J = 8.5 Hz), 6.8 (s, 2H), 7.0 (d, 2H, J = 8 Hz).

L-Tyrosine methyl ester (1.25 g, 2.3 mmol) was then hydrolyzed to give **31**; yield 1.1 g, 90.2%. Mp: 97−98 °C. [α]$_D$ −26.2° (c = 0.52, methanol). ^{1}H NMR (CDCl$_3$): δ 1.7−1.81 (m, 4H), 2.03−2.17 (m, 2H), 2.2−2.34 (m, 8H), 2.75 (dd, 1H, J = 9.5, 13.5 Hz), 3.15 (dd, 1H, J = 4.3, 13.5 Hz), 3.6 (s, 2H), 4.6 (m, 1H), 6.4 (s, 1H), 6.45 (d, 2H, J = 8 Hz), 6.65 (d, 2H, J = 8.5 Hz), 6.7 (d, 2H, J = 8.5 Hz), 6.8 (s, 2H), 7.0 (d, 2H, J = 8 Hz). Anal. (C$_{31}$H$_{34}$N$_2$O$_6$·0.25H$_2$O) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-proline (31). The proline derivative was synthesized using the corresponding L-proline methyl

ester hydrochloride (0.99 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.3 g, 80.1%. Mp: 71−72 °C. ^{1}H NMR (CDCl$_3$): δ 1.69−1.81 (m, 4H), 1.89−2.43 (m, 14H), 3.6 (s, 2H), 3.64−3.7 (m, 5H), 4.5 (dd, 1H, J = 4.3, 7.3 Hz), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1 (s, 2H), 7.2 (d, 2H, J = 8 Hz).

The final acid was obtained by hydrolysis of the corresponding L-proline methyl ester (1.1 g, 2.3 mmol); yield 0.94 g, 93.1%. Mp: 90−91 °C. [α]$_D$ −57.9° (c = 1, methanol). ^{1}H NMR (CDCl$_3$): δ 1.69−1.81 (m, 4H), 1.89−2.43 (m, 14H), 3.6 (s, 2H), 3.65 (m, 2H), 4.5 (dd, 1H, J = 4.3, 7.3 Hz), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1 (s, 2H), 7.2 (d, 2H, J = 8 Hz). Anal. (C$_{27}$H$_{33}$N$_2$O$_5$·0.25H$_2$O) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-*S*-benzyl-L-cysteine (32). This compound was synthesized using *S*-benzyl-L-cysteine methyl ester hydrochloride (1.57 g, 6.0 mmol). The crude ester was separated as oil and purified by flash chromatography using hexane:ethyl acetate (1:2) as eluent; yield 3.12 g, 90.7%. ^{1}H NMR (CDCl$_3$): δ 1.78−1.81 (m, 4H), 2.08−2.18 (m, 2H), 2.21−2.4 (m, 8H), 2.74 (dd, 1H, J = 7, 14 Hz), 2.84 (dd, 1H, J = 5, 14 Hz), 3.6 (s, 2H), 3.65 (s, 3H), 3.85(s, 2H), 4.72 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 6.97(s, 2H), 7.03 (d, 2H, J = 8 Hz), 7.14−7.27 (m, 5H).

The ester *S*-benzyl-L-cysteine methyl (1.32 g, 2.3 mmol) was further hydrolyzed to produce **32**; yield 1.23 g, 95.3%. Mp: 130−132 °C. [α]$_D$ −40.1° (c = 0.79, methanol). ^{1}H NMR (CDCl$_3$): δ 1.78−1.81 (m, 4H), 2.08−2.18 (m, 2H), 2.21−2.4 (m, 8H), 2.74 (dd, 1H, J = 7, 14 Hz), 2.84 (dd, 1H, J = 5, 14 Hz), 3.6 (s, 2H), 3.85(s, 2H), 4.72 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 6.97(s, 2H), 7.03 (d, 2H, J = 8 Hz), 7.14−7.27 (m, 5H). Anal. (C$_{32}$H$_{36}$N$_2$O$_5$S) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-threonine (33). This compound was synthesized similar to the previous reaction using L-threonine methyl ester hydrochloride (1.02 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.7 g, 93.4%. Mp: 64−66 °C. ^{1}H NMR (CDCl$_3$): δ 1.02(d, 3H, J = 6.5 Hz), 1.76−1.81 (m, 4H), 2.07−2.18 (m, 2H), 2.21−2.4 (m, 8H), 3.6 (s, 2H), 3.65 (s, 3H), 4.32 (m, 1H), 4.47 (dd, 1H, J = 2.4, 8.5 Hz), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1 (s, 2H), 7.2 (d, 2H, J = 8 Hz).

The L-threonine methyl ester (1.11 g, 2.3 mmol) was hydrolyzed to give **33**; yield 0.84 g, 83.2%. Mp: 82−83 °C. [α]$_D$ −12.3° (c = 0.5, methanol). ^{1}H NMR (CDCl$_3$) δ 1.02(d, 3H, J = 6.5 Hz), 1.76−1.81 (m, 4H), 2.07−2.18 (m, 2H), 2.21−2.4 (m, 8H), 3.6 (s, 2H), 4.32 (m, 1H), 4.47 (dd, 1H, J = 2.4, 8.5 Hz), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 7.1 (s, 2H), 7.2 (d, 2H, J = 8 Hz). Anal. (C$_{26}$H$_{32}$N$_2$O$_6$) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-aspartate (34). Scheme 4. Following the procedure as described above, JP7 (2.21 g, 6.0 mmol) was reacted with L-aspartate-γ-*tert*-butyl ester hydrochloride (1.44 g, 6.0 mmol), 1-hydroxybenzotriazole hydrate (0.88 g, 6.5 mmol), *N*-methylmorpholine (0.9 g, 8.9 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.36 mg, 7.1 mmol). The crude product was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.31 g, 70%. Mp: 47−48 °C. ^{1}H NMR (CDCl$_3$): δ 1.4 (s, 9H), 1.75−1.81 (m, 4H), 2.02−2.19 (m, 2H), 2.21−2.34 (m, 8H), 2.82 (dd, 2H, J = 4.5, 7.8 Hz), 3.61 (s, 2H), 3.68 (s, 3H), 4.6 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 6.97(s, 2H), 7.03 (d, 2H, J = 8 Hz).

Trifluoroacetic acid (2 mL) was then added to the corresponding tertiarybutoxycarbonyl ester (1.27 g, 2.3 mmol) in dry dichloromethane (30 mL) at 0 °C. The mixture was stirred at room temperature overnight. After completion of the reaction, the mixture was diluted with dichloromethane (40 mL) and washed with water (3 × 30 mL) and brine (30 mL). The organic phase was dried over anhydrous magnesium sulfate, filtered, and evaporated under reduced pressure.

The pure product was obtained by flash chromatography using ethyl acetate as eluent; yield 1.0 g, 87.7%. Mp: 63−65 °C. [α]$_D$ −11.2° (c = 0.65, methanol). ^{1}H NMR (CDCl$_3$) δ 1.75−1.81 (m, 4H), 2.02−2.19 (m, 2H), 2.21−2.34 (m, 8H), 2.82 (dd, 2H, J = 4.5, 7.8 Hz), 3.61 (s, 2H), 3.68 (s, 3H), 4.6 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 6.97(s, 2H), 7.03 (d, 2H, J = 8 Hz). Anal. (C$_{27}$H$_{32}$N$_2$O$_7$) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-glutamate (35). This compound was synthesized by following a previous reaction but using l-glutamate-γ-*tert*-butyl ester hydrochloride (1.52 g, 6.0 mmol). The crude ester obtained after workup was purified by flash chromatography using hexane:ethyl acetate (2:1) as eluent; yield 2.38 g, 70%. Mp: 50−51 °C. ^{1}H NMR (CDCl$_3$): δ 1.41 (s, 9H), 1.7−1.9 (m, 4H), 2.02−2.4 (m, 14H), 3.6 (s, 2H), 3.7 (s, 3H), 4.55 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 6.97(s, 2H), 7.03 (d, 2H, J = 8 Hz).

The ester L-glutamate-γ-*tert*-butyl ester (1.27 g, 2.3 mmol) was hydrolyzed as before to afford **35**. The crude product was purified by flash chromatography using ethyl acetate as eluent; yield 0.94 g, 80.3%. Mp: 60−61 °C. [α]$_D$ −14.2° (c = 0.46, methanol). ^{1}H NMR (CDCl$_3$): δ 1.7−1.9 (m, 4H), 2.02−2.4 (m, 14H), 3.6 (s, 2H), 3.7 (s, 3H), 4.55 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 6.97(s, 2H), 7.03 (d, 2H, J = 8 Hz). Anal. (C$_{28}$H$_{34}$N$_2$O$_7$) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-D-lysine (36). Scheme 5. To the corresponding *N*^{ε}-benzyloxycarbonyl-D-lysine (**18**) (0.5 g, 0.79 mmol) in ethanol (10 mL) was added 10% palladium on carbon. The mixture was hydrogenated in the Parr-Shaker until absorption of the hydrogen gas was stopped. The catalyst was filtered and washed with ethanol (2 × 25 mL), and the combined filtrate was evaporated under reduced pressure. The pure product was obtained upon recrystallization from chloroform; yield 0.33 g, 84.6%. Mp: 124−125 °C. [α]$_D$ +30.0° (c = 0.5, methanol). ^{1}H NMR (CDCl$_3$): δ 1.1−1.35 (m, 4H), 1.76−1.85 (m, 4H), 2.02−2.4 (m, 10H), 2.95−3.05 (m, 4H), 3.6 (s, 2H), 4.55 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 6.97(s, 2H), 7.03 (d, 2H, J = 8 Hz). Anal. (C$_{28}$H$_{37}$N$_3$O$_5$.0.5C$_2$H$_5$OH) C, H, N.

1-[4-(((3,5-Dimethylanilino)carbonyl)methyl)phenoxy]-cyclopentanecarbonyl-L-lysine (37). This compound was synthesized as described above using the corresponding *N*^{ε}-benzyloxycarbonyl-L-lysine (**19**) (0.5 g, 0.79 mmol). The final product was obtained upon recrystallization from chloroform; yield 0.35 g, 89.7%. Mp: 124−125 °C. [α]$_D$ −29.6° (c = 0.5, methanol). ^{1}H NMR (CDCl$_3$): δ 1.1−1.35 (m, 4H), 1.76−1.85 (m, 4H), 2.02−2.4 (m, 10H), 2.95−3.05 (m, 4H), 3.6 (s, 2H), 4.55 (m, 1H), 6.7 (s, 1H), 6.84 (d, 2H, J = 8 Hz), 6.97(s, 2H), 7.03 (d, 2H, J = 8 Hz). Anal. (C$_{28}$H$_{37}$N$_3$O$_5$.0.5 C$_2$H$_5$OH) C, H, N.

Oxygen Equilibrium Studies. 1. Whole Blood Analysis. A multi-point tonometry was used to measure the OEC of whole blood. This technique measures the Hb saturation using a co-oximeter on individual blood samples after equilibrium of each sample with a gas of known oxygen concentrations. The whole blood samples were collected in heparinzed tubes from healthy volunteers and stored over ice. A 200 mM stock solution of the compound was prepared in DMSO. Just before tonometry, 50 μL of the compound stock solution was mixed with 1950 μL of whole blood to achieve a final concentration of 5 mM. OEC study controls included the addition of 50 μL of DMSO (the same amount of solvent used to dissolve the effectors). Each sample was run in duplicate. The samples were incubated in tonometers (IL 237 Instrumentation Laboratories, Inc., Lexington, MA) for approximately 10 min at 37 °C against gas mixtures containing 2.95%, 5.85%, and 8.75% concentrations of O$_2$, a fixed concentration of 5.8% CO$_2$, and a balance of N$_2$. The samples were allowed to equilibrate at three separate concentrations of O$_2$ mixtures 20%, 40%, and 60%, respectively. After equilibration at each concentration of O$_2$, a sample was removed via syringe and aspirated into the blood gas analyzer (IL 1420 Instrumentation Laboratories, Inc., Lexington, MA) and co-oximeter (IL 482 and IL 682 Instru-

mentation Laboratories, Inc., Lexington, MA) to determine the pH, pCO_2, and pO_2 and to obtain the oxygen saturation values (sO_2), respectively. The measured values for pO_2 and sO_2 at each oxygen saturation point was then subjected to the nonlinear regression analyses to calculate P_{50} and n_{50} using the program Scientist (Micromath, Salt Lake City, UT). The control was prepared by adding 1950 μL of whole blood to 50 μL of DMSO.

2. Hb Solution Studies. All of the effectors (except compound **19** and **20**) 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 and back-titrated carefully to pH 7.7 at 25 °C prior to use. Compounds **19** and **20** were prepared in 30% DMSO because of their poor solubility in NaCl bis-Tris buffer. Oxygen equilibrium measurements were performed with the HEMOX analyzer (TCS Medical Products, Southampton, PA) using purified stripped human adult hemoglobin as described previously.[19,20] A total of 4 mL of buffer (100 mM NaCl, 50 mM bis-Tris at pH 7.2 at 25 °C) is added to a cuvette in the HEMOX, followed by 200 μL of the 10 mM effector stock solution to achieve 0.5 mM of the effectors. Hb is then added to have a final Hb concentration of 50−60 μM in 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. 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 P_{50} and n_{50} values were calculated by linear regression analysis from data points comprised between 40% and 60% oxygen saturation.

X-ray Data Analysis. Crystals of deoxyHb complexed with the effectors **17**, **18**, **19**, and **20** were obtained under similar conditions as previously described for the RSR13−Hb complex.[23] As expected, all Hb complexes yielded crystals that are isomorphous to that of the native Hb crystal.[24] The crystals belong to the space group $P2_1$ and have one tetramer in their asymmetric unit. X-ray diffraction data were collected using R-axis II image plate detector equipped with a Rigaku RU-200 generator operating at 50 kV and 180 mA. All data sets were processed using the Molecular Structure Corporation (MSC) Biotex software and the CCP4 program suite.[25]

Binding of the effectors to deoxyHb were ascertained from difference Fourier electron density maps, computed from [from F_{obs}(complex) − F_{obs}(native)] amplitude at 2.0 Å resolutions, where F_{obs}(complex) is the observed structure factors of the effector−Hb complexes and F_{obs}(native) is the observed structure factors from the previously determined structure of native T-state Hb.[24] For all complexes, a model of the effector molecule was built with the program Sybyl[26] and fitted to the observed effector electron density.

Supporting Information Available: Atomic coordinates for four unrefined hemoglobin−allosteric effector complexes, deposited as D-valine-17.pdb, L-valine-18.pdb, D-phenylalanine-19.pdb, and L-phenylalanine-20.pdb. This material is available free of charge via the Internet at http://pubs.acs.org. [The coordinates are also available from the corresponding author (dabraham@hsc.vcu.edu).]

References

(1) Hirst, D. G.; Wood, P. J.; Schwartz, H. C. The Modification of Hemoglobin Affinity for Oxygen and Tumor Radiosensitivity by Antilipedimic Drugs. *Radiat. Res.* **1987**, *112*, 164−172.
(2) Siemann, D. W.; Macler, L. M. Tumor Radiosensitization Through Reductions in Hemoglobin Affinity. *Int. J. Radiat. Oncol. Biol. Phys.* **1986**, *12*, 1295−1297.
(3) Abraham, D. J.; Perutz, M. F.; Phillips, S. E. V. Physiological and X-ray Studies of Potential Antisickling Agents. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 324−328.
(4) Abraham, D. J.; Kennedy, P. E.; Mehanna, D. C.; Patwa, D. C.; Williams, F. L. Design, Synthesis, and Testing of Potential Antisickling Agents. 4. Structure−Activity Relationships of Benzyloxy and Phenoxy Acids. *J. Med. Chem.* **1984**, *27*, 967−978.
(5) Perutz, M. F.; Poyart, C. Bezafibrate Lowers Oxygen Affinity of Haemoglobin. *Lancet* **1983**, *15*, 881−882.
(6) Lalezari, I.; Rahbar, S.; Lalezari, P.; Fermi, G.; Perutz, M. F. LR16, A Compound with Potent Effects on the Oxygen Affinity of Hemoglobin, on Blood Cholesterol, and on Low-Density Lipoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6117−6121.
(7) Lalezari, I.; Lalezari, P. Synthesis and Investigation of Effects of 2-[4-[[(Arylamino)carbonyl]amino]phenoxy]-2-methylpropionic Acids on the Affinity of Hemoglobin for Oxygen: Structure−Activity Relationships. *J. Med. Chem.* **1989**, *32*, 2352−2357.
(8) Lalezari, I.; Lalezari, P.; Poyart, C.; Marden, M.; Kister, J.; Bohn, B.; Fermi, G.; Perutz, M. F. New Effectors of Human Hemoglobin: Structure and Function. *Biochemistry* **1990**, *29*, 1515−1523.
(9) Abraham, D. J.; Wireko, F. C.; Randad, R. S.; Poyart, C.; Kister, J.; Bohn, B.; Liard, J.; Kunert, M. P. Allosteric Modifiers of Hemoglobin: 2-[4-[[(3,5-disubstitutedanilino)carbonyl] amino]-phenoxy]-2-methylpropionic Acid Derivatives that Lower the Oxygen Affinity of Hemoglobin in Red Cell Suspension, in Whole Blood and in Vivo in Rats. *Biochemistry* **1992**, *31*, 9141−9149.
(10) Randad, R. S.; Mahran, M. A.; Mehanna, A. S.; Abraham, D. J. Allosteric Modifiers of Hemoglobin. 1. Design, Synthesis, Testing and Structure-Allosteric Activity Relationship of Novel Hemoglobin Oxygen Affinity Decreasing Agents. *J. Med. Chem.* **1991**, *34*, 752−757.
(11) Abraham, D. J.; Kister, J.; Joshi, G. S.; Wireko, F. C.; Safo, M. K.; Marden, M. C.; Poyart, C. Structure/Function of Allosteric Effectors of Hemoglobin. *Med. Chem. Res.* **1998**, *8*, 478−486.
(12) Kunert, M. P.; Liard, J. F.; Abraham, D. J. RSR-13, An Allosteric Effector of Hemoglobin, Increases Systemic and Iliac Vascular Resistance in Rats. *Am. J. Physiol.* **1996**, *271*, H602−H613.
(13) Khandelwal, S. R.; Randad, R. S.; Lin, P.-S.; Meng, H.; Pittman, R. N.; Kontos, H. A.; Choi, S. C.; Abraham, D. J.; Schmidt-Ullrich, R. Enchanced Oxygenation in Vivo by Allosteric Inhibitors of Hemoglobin Saturation. *Am. J. Physiol.* **1993**, *265*, H1450−H1453.
(14) Wei, E. P.; Randad, R. S.; Levasseur, J. E.; Abraham, D. J.; Kontos, H. A. Effect of Local Change in Oxygen Saturation of Hemoglobin on Cerebral Vasodilation from Hypoxia and Hypotension. *Am. J. Physiol.* **1993**, *265*, H1439−H1443.
(15) Teicher, B. A.; Ara, G.; Emi, Y.; Kakeji, Y.; Ikebe, M.; Maehara, Y.; Buxton, D. RSR13: Effects on Tumor Oxygenation and Response to Therapy. *Drug Dev. Res.* **1996**, *38*, 1−11.
(16) Abraham, D. J.; Joshi, G. S.; Randad, R. S.; Panikker, J. Allosteric Modifiers of Hemoglobin Useful for Decreasing Oxygen Affinity and Preserving Oxygen Carrying Capability of Stored Blood. U.S. Patent 5,731,454 (March 24, 1990).
(17) Weizman, C.; Sulzbacher, M.; Bergman, E. The Synthesis of α-Alkoxyisobutyric Acids and Alkyl Methacrylates from Acetonechloroform. *J. Am. Chem. Soc.* **1948**, *70*, 1153−1158.
(18) Reeve, W. Reactions of Aryl Trichloromethyl Carbinols with Nucleophiles. *Synthesis* **1971**, 131−138.
(19) Poyart, C.; Marden, M. C.; Kister, J. Bezafibrate Derivatives as Potent Allosteric Effectors of Hemoglobin. *Methods Enzymol.* **1994**, *232*, 496−513.
(20) Kister, J.; Poyart, C.; Edelstein, S. J. An Expanded Two-state Allosteric Model for Interactions of Human Hemoglobin A with Nonsaturating Concentrations of 2,3-Diphosphoglycerate. *J. Biol. Chem.* **1987**, *262*, 12085−12091.
(21) Perutz, M. F.; Fermi, G.; Abraham, D. J.; Poyart, C.; Bursaux, E. Hemoglobin as a Receptor of Drugs and Peptides: X-ray Studies of the Stereochemistry of Binding. *J. Am. Chem. Soc.* **1986**, *108*, 1064−1078.
(22) Burley, S. K.; Petsko, G. A. Amino-Aromatic Interactions in Proteins. *FEBS* **1986**, *203*, 139−143.
(23) Safo, M. K.; Moure, C. M.; Burnett, J. C.; Joshi, G. S.; Abraham, D. J. High-Resolution Crystal Structure of Deoxy Hemoglobin Complexed with a Potent Allosteric Effector. *Protein Science* **2001**, *10*, 951−957.
(24) Fermi, G.; Perutz, M. F.; Shannon, B.; Fourme, R. The Crystal Structure of Human Deoxyhemoglobin at 1.7 Å Resolution. *J. Mol. Biol.* **1984**, *175*, 159−174.
(25) The CCP4 Suite: Programs for Protein Crystallography. *Acta Crystallogr.* **1994**, *D50*, 760−763.
(26) Sybyl Molecular Modeling System (version 6.6), Tripos, Inc., 1699 Hanley Road, St. Louis, MO 63144.