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Principal Investigator: Thomas G. Burke, Ph.D.
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SUMMARY

Pharmacological approaches to the optimization of the oxygen affinity of liposome-encapsulated hemoglobin (LEH), a potential blood replacement fluid, are being developed. LR16 and L35 are recently-synthesized drugs which are known to be potent modulators of the P_{50} of human hemoglobin [Lalezari et al. *Biochemistry* 29:1515-1523 (1990)]. In this report we describe the synthesis of several related 2-methylpropionic acid-derived modifiers of human hemoglobin. Structural modifications to the LR16 molecule include an analogue containing the addition of a quaternary ammonium salt, as well as drugs containing an amino group, trifluoromethyl moieties, and fluorine substituents. A thiourea analogue was also synthesized. Several of the new compounds were found to be biologically-active when tested on human hemoglobin, the most potent compound shifting the P_{50} value of 200 μM human hemoglobin to 18 mm Hg using a drug concentration of 2 mM. Analogues that exhibit both potency as well as low membrane permeability (i.e. high retentivity of drug within the liposomal particle) are being sought.

DRUG SYNTHESIS

2-[4-[[(aryl)amino]carbonyl]amino]phenoxy-2-methyl propionic acid derivatives **26-37** were prepared by a two step reaction starting from commercial 4-aminophenol (Scheme 1). Its condensation in pyridine with isocyanates **2-13** led to intermediates **14-25**. To avoid a formation of by-products the isocyanates were added to the reaction mixture at 0°C and the reactions were carried out at this temperature for a period of about 15 min. Then, the reaction was continued at room temperature. In most cases high yields were obtained (over 90%). Urea derivatives **14-25**, while reacted with acetone-chloroform in the presence of NaOH followed by hydrolysis led to the sodium salts of the final products. The water suspension after reaction was washed with ethyl acetate to give solution of pure sodium salts. That modification omits a filtration, as it was described before - which is long and problematic. The final products were precipitated with 12% HCl.

Scheme 1

Attempts to prepare some thiourea analogues of knowr allosteric effectors according above reaction sequence failed. Condensation of corresponding 1-aryl-3-(4-hydroxyphenyl)-thiourea derivatives in acetone-chloroform-NaOH led to a complicated mixture of undefined products. Hence, thio analogues of **26** and **27** were finally prepared by a reaction of lithium salt of 2-(4-aminophenoxy)-2-methyl propionic acid (**41**) with 3-chloro- and 3,4-dichlorophenyl isothiocyanates (**38,39**) in pyridine with 58% and 52% yield, respectively (Scheme 2). This

Ohio State Univ., Columbus

method was also successfully applied for the synthesis of 3,5-bis(trifluoromethyl) derivative **44** (44% yield), which could not be obtained previously according procedure shown on scheme 1, because of a partial hydrolysis of CF_3 groups in the reaction with acetone-chloroform, while an intermediate type **14-25** was refluxed in a strong alkaline conditions.

Scheme 2

Amino analogues of allosteric effectors of hemoglobin, possessing strong basic center on NH_2 group, can be easily accessible from a corresponding nitro derivatives by a catalytic hydrogenation (under 10 psi) on Pd/C in methanolic solution. Using this method compounds **45-49** were prepared with 44-90% yield. Till now, they were not reported in the literature and their effects on human hemoglobin were not investigated. Methyl ester of 2-[4-[[(3-aminophenyl)amino]carbonyl]amino]-phenoxy-2-methyl propionic acid (**50**), which was obtained as a by-product, while a crude amine (**46**) was filtered through silica gel in acidic methanolic solution, indicated much lower effect on hemoglobin as compared with compounds with free carboxylic group. Corresponding permethylated compound on amino group (**51**) was synthesized (83% yield) according similar procedure as it was described in the literature, using methyl iodide as an methylated agent. As an acceptor of evolved hydroiodide diethyl aniline was used. In this case high purity of the starting amine and longer reaction time are required to obtain salt in a pure crystalline form.

EXPERIMENTAL METHODS

TLC was performed on precoated plastic sheets (0.2 mm) of silica gel 60 F-254 (E. Merck AG, Darmstadt, Germany); compounds were detected by UV lamps (254 nm). Hydrogenations were carried out in Parr Apparatus. Melting points were determined with a Buchi 530 apparatus and are uncorrected. NMR spectra were recorded for solution in DMSO-d_6 or $CDCl_3$/DMSO-d_6) (internal standard TMS) with a QE-300 (300 MHz) and Bruker AM-400 (400 MHz) spectrometers. Most of synthesized compounds has high tendency to associate water, hence for some of them elemental analysis was assessed for hydrates and subsequent high resolution-mass spectrometry confirmed empirical formula. Isocyanates and isothiocyanates were commercially available (Carbolabs - Bethany, Connecticut 06525; Aldrich - Milwaukee, Wisconsin 53201; Lancaster - Windham, New Hampshire 03087). 2-(4-Aminophenoxy)-2-methyl propionic acid (**41**) was obtained according a procedure described in the literature.[a,c]

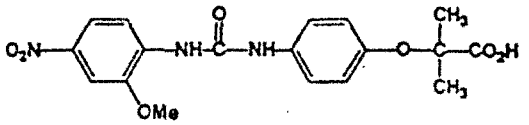
Synthesis of 1-aryl-3-(4-hydroxyphenyl)urea derivatives (14-25). General Procedure.

Aryl isocyanate (**2-13**, 30 mmol) was added as a pure substance or as a solution in pyridine (1-2 mL) to a vigorously stirred solution of 4-aminophenol (3.3 g, 30 mmol) in pyridine (8-10 mL) at 0°C. The reaction was kept at this temperature for 15 min. Then, the ice bath was removed and the reaction continued for another 15 min at room temperature. Then, water (250 mL) was added, and the pyridine was neutralized with a small excess of 12% HCl (~180

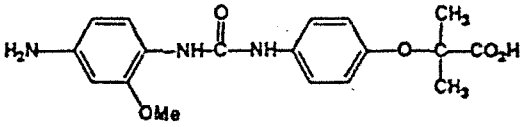
mL). The suspension was left with stirring for 0.5 h, the precipitate was separated by filtration, washed with water, and dried to give products **14-25**. If the product was not pure enough (TLC:$CHCl_3$/MeOH - 10:1) it was dissolved in methanol and the insoluble white solid (products of polymerization of isocyanates) were filtered off. The filtrate was concentrated and dried *in vacuo*. Analytical samples were recrystallized (or chromatographed and recrystallized) from acetone, methanol or methanol/chloroform mixture.

Drug Structures Synthesized.

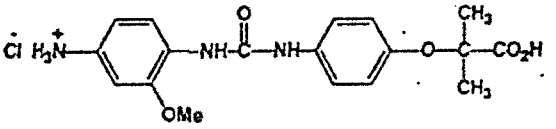
O1 - 36



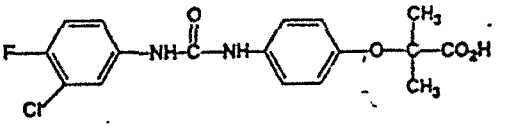
O2 - 25



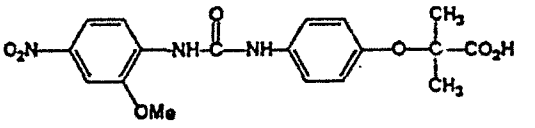
O2 - 24



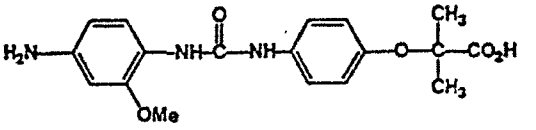
O2 - 26



O1 - 36



O2 - 25



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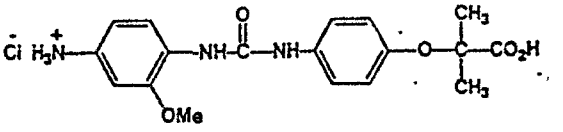
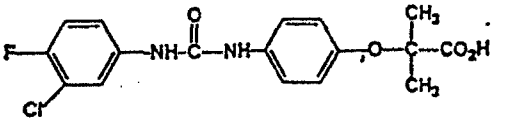
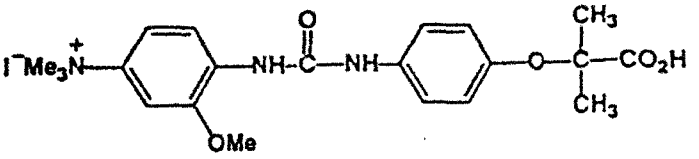


O2 - 26



O2-31



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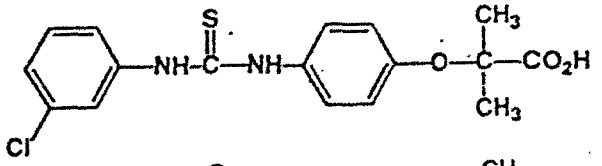
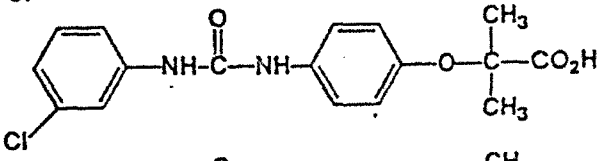
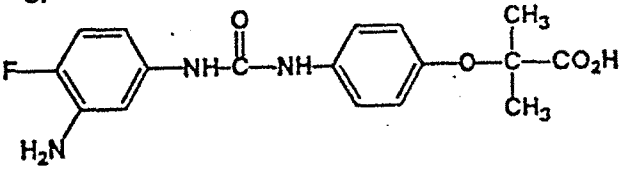


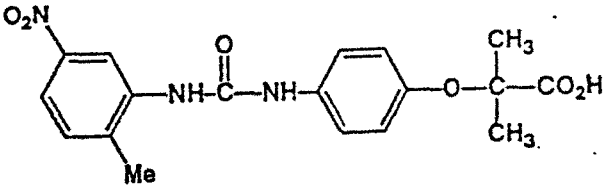
O2-30



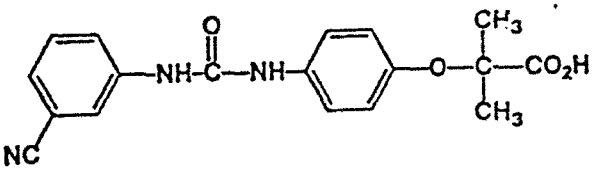
O1-22



O1-43



O2-39



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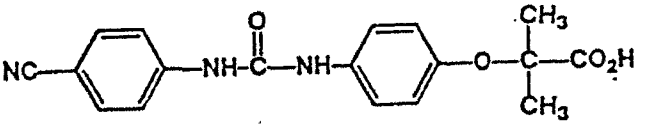
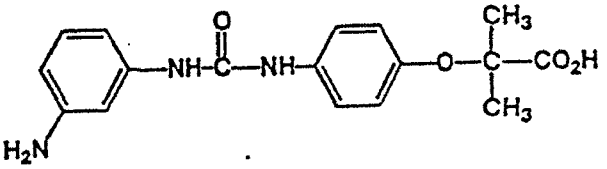


O1-37-2



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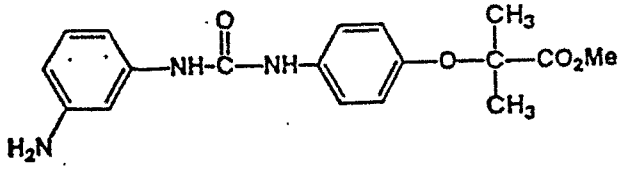

Preparation of Hemolysate.

Hemoglobin solutions are prepared from outdated humna blood. To accomplish this, blood was washed with saline (0.9% NaCl), centrifuged for 10 minutes at 3,000 rpm, and lysed with cold distilled water. The sample was then centrifuged at high speed (16,000 rpm for 30 minutes) and dialyzed with 30 mM sodium phosphate (pH 7.4) at 4 °C. This buffer was changed four times during the procedure.

The hemoglobin was concentrated by pressure ultrafiltration using a 30,00 dalton cut-off filter. The hemoglobin concentration reached 17.7 % (2.75 mM), a substantial increase from the original concentration prior to ultrafiltration of 5.6 %.

Sample	P_{50}	P_{50ch}	P_{50}/P_{50ch}
Fresh human blood	25.5	-	-
Human Hb solution	-	5	-
LR16	27.5	5	5.5
L35	26.0	5	5.2
01-22	7	5	1.4
01-35	5	5	1.0
01-38	10	5	2.0
01-40	9	5	1.8
01-42	7.5	5	1.5
01-43	8	5	1.6
01-45	7.5	5	1.5
01-37-2	8.5	5	1.7
02-30	12.5	5	2.5
02-31	5.5	5	1.1
02-38	5.5	5	1.1
02-39	8	5	1.5
02-50	19	5	3.8
02-51	6	5	1.2
01-36	5	4	1.3
02-24	6.25	4	1.6
02-25	5.75	4	1.4
02-26	10.5	4	2.6

Effect of the Allosteric Effectors on the Oxygen Affinity of Human Hemoglobin.

Drug concentrations of 1.5 mM were employed. P_{50} refers to the partial oxygen pressure at which purified hemoglobin solution is half-saturated in the presence of 1.5 mM drug; P_{50cb} refers to the partial oxygen pressure at which purified hemoglobin solution is half-saturated in the absence of drug. The P_{50cb} value was either 4 or 5 mm Hg for all of the samples. P_{50} values were determined from oxygen dissociation curves taken on a Hemox Analyzer from TCS, Southhampton, PA. Experiments were conducted at 37 °C.

The effects of the allosteric effectors on human hemoglobin are summarized on the previous page. Of the new compounds tested, 02-50 was the most potent at modulating the oxygen binding pressure of hemoglobin. This analogue was somewhat less effective than LR16 and L35 at modulating the oxygen binding properties of hemoglobin. Studies evaluating relative drug retention in the liposomal particles are in progress.

Meeting Abstracts/Submitted Manuscripts

Burke, T.G., Ostrowski, S., Rahbar, S., and Priebe, W. "Liposome-Encapsulated Hemoglobin as a Blood Substitute: Synthesis and Evaluation of Allosteric Effectors for the Optimization of Oxygen Affinity", Pharmaceut. Res. 9: S-74 (1992).

Ostrowski, S., Burke, T.G., and Priebe, W. "^{13}C NMR Spectra of Allosteric Effectors of Hemoglobin", Journal of Magnetic Resonance, submitted.