Gly[9]]SP, did not confer any overall resistance to degradation. In order to confer an increased resistance toward the enzyme, all three cleavage sites in SP had to be protected. This was most successfully achieved by substitution of *N*-methyl amino acid residues in positions 8 and 9 (Figure 4). Thus, [(Me)Phe[8],(Me)Gly[9]]SP exhibited complete resistance to cleavage at any of the three normal sites when incubated with purified SP-degrading enzyme.[29] However, although [(Me)Phe[8],(Me)Gly[9]]SP retained biological activity, it was still degraded by N-terminal cleavages when incubated with rat hypothalamic slices, albeit more slowly than native SP.[45] It was, therefore, decided to attempt to design an enzyme-resistant analogue of SP based on a C-terminal fragment of the peptide. The most successful analogue, [Glp[5],(Me)Phe[8],(Me)Gly[9]]SP-(5–11) ("DiMe-C7") was equipotent with SP in the rat brain [^{3}H]SP binding assay and completely resistant to degradation when incubated with purified SP-degrading enzyme or with rat brain slices or synaptic membrane preparations[29,45] (Figure 4). Furthermore, this analogue seems to be protected against degradation in the intact rat brain in vivo. When [^{3}H]DiMe-C7 was microinfused into rat brain (ventral tegmentum), very little degradation was observed even after 50 min, compared with [^{3}H]SP, which was completely degraded[45] within 10 min. When DiMe-C7 was injected into the ventral tegmental area of the rat brain, it elicited a hyperactivity response similar to that produced by injecting SP, but of prolonged duration.[46,47] Thus, DiMe-C7 represents the first SP analogue that is protected against degradation in the mammalian CNS; as such, it should prove a useful new research tool.

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Conclusions

Although SP pharmacology is still in its infancy, there has clearly been some progress in recent years. We can begin to discern the general properties of SP receptors and to appreciate the possible existence of multiple subclasses in peripheral tissues and in the CNS—a phenomenon that clearly has a potentially important bearing on the possibility of designing agonists or antagonists with tissue-selective actions. The first useful SP antagonists are now available and will certainly be improved upon in the near future. Such compounds will undoubtedly play a crucial role in furthering our understanding of the functions of SP in the periphery and the CNS.

Drugs based on peptide structures, however, have a number of intrinsic disadvantages as potential therapeutic agents: they are not easily absorbed, they do not penetrate readily into the CNS, they are liable to rapid degradation, and they are costly to manufacture. An ideal would be to discover nonpeptide structures with agonist or antagonist properties at SP receptors. This goal, however, is still beyond even the most sophisticated resources of modern medicinal chemistry and is most likely to be achieved by serendipity. In more general terms, the pursuing of SP pharmacology offers many stimulating challenges, as there are few precedents to guide one to the most suitable strategies. It may be that the lessons learned from work on SP will prove in the future to have a more general application to the development of "peptide pharmacology" for the 2 dozen or more other neuropeptides now known to exist in the mammalian nervous system.

Communications to the Editor

Design, Synthesis, and Testing of Potential Antisickling Agents. 1. Halogenated Benzyloxy and Phenoxy Acids

Sir:

Many classes of organic compounds[1] have been investigated with the hope that a viable treatment for sickle cell anemia could be found. The cause of this disease is a mutation of Glu in normal hemoglobin (HbA) to Val in sickle hemoglobin (HbS) on the β subunits at position 6. This structural change results in the polymerization of HbS in the red blood cell under anaerobic conditions, giving rise to the disorder known as sickle cell anemia. As of yet, no known agents are approved for general use.

We have attempted[2,3] to design antigelling agents in a logical manner by determining their binding sites in the hemoglobin molecule by X-ray analysis. Using carboxyhemoglobin A crystals, we discovered that iodobenzene and *p*-bromobenzyl alcohol bind in a hydrophobic cavity located on the α subunits of Hb in the region of Trp-14α. Both of these agents possess some antigelling activity.[2-4] Previously, Schoenborn[5] has shown that the primary binding site of the antisickling agent dichloromethane is in this cavity. These results prompted us to continue to explore simple halogenated aromatic molecules for their ability to hinder hemoglobin S polymerization.

Our first attention toward further structural modifications of aromatic halogen molecules drew us to consider making derivatives of *p*-bromobenzyl alcohol. Ross and Subramanian[4] have shown that *p*-bromobenzyl alcohol, although very active at 5 mM, has questionable utility because of its limited solubility in aqueous medium. We therefore initiated a study to solubilize *p*-bromobenzyl alcohol via addition of various hydrophilic organic moieties to the alcohol function. The first of these modifications

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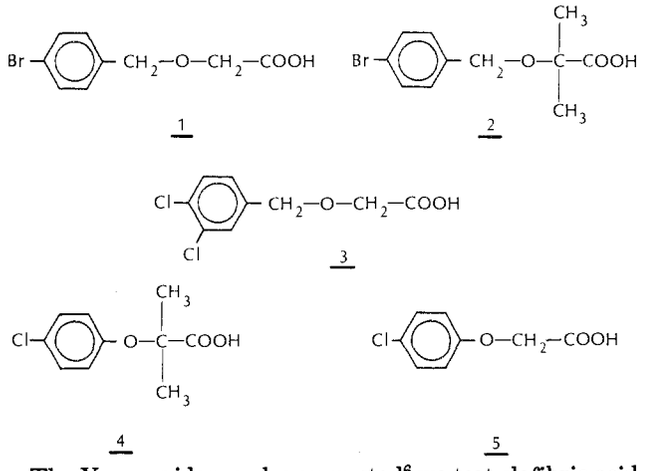
Table I. Comparison of Solubility Ratios for Benzyloxy and Phenoxy Acids with Phenylalanine[a]

no.[b]	$[HbS]_{acid}/[HbS]_{control}$[c] 5 mM acid	10 mM acid	20 mM acid	40 mM[d] acid	no. of runs
1	1.059 (0.023)	1.118 (0.036)	1.233 (0.045)	1.398 (0.034)	4[e]
2	1.048 (0.026)	1.086 (0.047)	1.193 (0.073)	1.379 (0.098)	4[f]
3	1.100 (0.012)	1.176 (0.021)	1.320 (0.019)	1.371 (0.043)	3[g]
4	1.018 (0.013)	1.064 (0.014)	1.155 (0.023)	1.302 (0.022)	3[h]
5	1.044 (0.024)	1.077 (0.035)	1.162 (0.054)	1.276 (0.051)	3[i]
6	1.036 (0.009)	1.048 (0.009)	1.093 (0.021)	1.200 (0.018)	2[j,k]
				1.178 (0.030)	18[l]

[a] All compounds were dissolved in 0.15 M phosphate buffer, pH 7.4, with 1 equiv of sodium bicarbonate to make the sodium salt, at a concentration of 0.18 M. Appropriate aliquots of this solution (10, 20, 40, and 80 μL) were mixed with buffer to equal 90 μL. The 90-μL solutions were added to 250 μL of HbS (0.15 M phosphate), usually around 35 g %, and then 20 μL of dithioniate (1.06 M) was added before sealing of the EPR tubes. Final concentrations of drug were 5, 10, 20, and 40 mM in four separate tubes. A set of six tubes was spun on each run, which included the four drug concentrations, one dHbS control (90 μL of buffer, no acid), and a 40 mM phenylalanine control. The above dilution procedure produces identical HbS initial concentrations for all six tubes. The initial HbS concentrations (in grams per deciliter) for each run after addition of the acids and dithionite and the respective solubility of deoxyhemoglobin S (in grams per deciliter) for each control run (no acid) follow for each compound studied. [b] 1 = [(*p*-bromobenzyl)oxy]acetic acid; 2 = 2-[(*p*-bromobenzyl)-oxy]-2-methylpropionic acid; 3 = [(3,4-dichlorobenzyl)oxy]acetic acid; 4 = clofibric acid; 5 = (*p*-chlorophenoxy)acetic acid; 6 = phenylalanine. [c] The average deoxyhemoglobin S solubility at 35 °C for 18 control assays is 16.97 g/dL (SD 0.36). [d] The nonlinearity of the highest measurements is probably due to a greater error in these measurements than at lower concentration as well as saturation effects as suggested by Ross and Subramanian.[4] [e] Initial HbS concentrations in g/dL: 22.85, 24.68, 24.99, and 24.84; respective solubility of dHbS controls for each run at 35 °C, in g/dL: 15.93, 17.00, 16.70, and 17.14. [f] 22.85, 24.68, 24.99, and 24.84 g/dL; 16.70, 17.07, 16.41, and 17.22 g/dL. [g] 24.02, 23.82, and 23.83 g/dL; 17.07, 16.93*, and 17.00 g/dL. *This control value was assumed from other runs, since the control for this set was erroneously high. [h] 24.50, 25.36, and 24.68 g/dL; 17.07, 16.78, and 17.51 g/dL. [i] 24.02, 23.82, and 23.83 g/dL; 17.11, 17.22, and 17.07 g/dL. [j] 23.74 and 24.50 g/dL; 17.32 and 17.09 g/dL. [k] The reported phenylalanine ratios[10] as calculated from a straight line through the points are as follows: 5 mM, 1.033; 10 mM, 1.049; 20 mM, 1.082; 40 mM, 1.147. The highest concentration run in ref 10 was 32 mM, so the extrapolated 40 mM value is only an estimate. [l] For 18 of the 19 runs a phenylalanine 40 mM control was also run, as well as the dHbS control (no acid).

synthesized was [(*p*-bromobenzyl)oxy]acetic acid (1). Subsequent benzyloxy analogues included in this study were 2-[(*p*-bromobenzyl)oxy]-2-methylpropionic acid (**2**) and [(3,4-dichlorobenzyl)oxy]acetic acid (**3**).



The X-ray evidence also suggested[6] we test clofibric acid (**4**), the active constituent of the antilipidemic drug clofibrate,[7] since it has a similar halogenated aromatic structure, as well as the phenoxy acid, (*p*-chlorophenoxy)acetic acid (**5**). This communication reports the synthesis of the benzyloxy acids **1**–**3** and the biological testing of compounds **1**–**5**.

Compounds **1**–**3** were synthesized as shown in Scheme I. Compounds **1** and **3** were synthesized via heating for 6–8 h of 1 equiv of the halogenated acid with 1 equiv of the properly substituted benzyl alcohol in the presence of 2 equiv of NaH in THF. The THF was removed by rotary evaporation. Addition of water and ice dissolved any solid material, and extraction of the aqueous phase with $CHCl_3$ removed any unreacted alcohol and mineral oil liberated from the NaH dispersion. Acidification of the aqueous layer produced the desired acids, which were recrystallized from boiling water. Compound **1** was obtained in 70% yield and had mp 79–80 °C (lit.[8] mp 80 °C). Compound **3** was obtained in 83% yield, mp 58–60 °C. Anal. ($C_9H_8O_3Cl_2$) C, H, Cl. Compound **2** was prepared by heating for 6–8 h 1 equiv of 2-hydroxyisobutyric acid, 2 equiv of NaH, and 1 equiv of *p*-bromobenzyl bromide in THF. A similar workup as described above produced a 31% yield of **2**, mp 111–113 °C, after recrystallization from boiling water. Anal. ($C_{11}H_{13}O_3Br$) C, H, Br. High-resolution mass spectrum: calcd (Br^{79}), 272.0048; found, 272.0029. Compounds **4** and **5** can be purchased from Aldrich Chemical Co. and recrystallized from boiling water. All compounds were shown to be pure via thin-layer chromatography on silica gel, with 3% acetic acid in chloroform or ethyl acetate–hexane–acetic acid mixture (50:50:1) as the mobile phase.

Biological testing of these materials was performed using the assay developed by Hofrichter et al.[9] This assay involves deoxygenation of concentrated HbS with dithionite in the presence of different concentrations of drug. Samples are then sealed in quartz EPR tubes under anaerobic conditions and spun at about 150000*g* for 2.5 h at 35 °C in an ultracentrifuge. This procedure pellets the polymerized HbS to the bottom of the tubes and the supernatant (soluble HbS) is measured in our laboratory as the cyanmethemoglobin derivative. The more active the compound, the greater the solubility of HbS and the smaller the pellet size. Activity is reported as a ratio of

(6) Suggested by Professor D. T. Witiak, Department of Medicinal Chemistry, Ohio State University, Columbus, OH, personal communication, Nov 25, 1981.

(7) For a comprehensive review, see D. T. Witiak, H. A. I. Newman, and D. R. Feller, Clofibrate and Related Analogs. A Comprehensive Review", Medical Research Series, Vol. 7, Marcel Decker, New York, 1977, pp 1–287.

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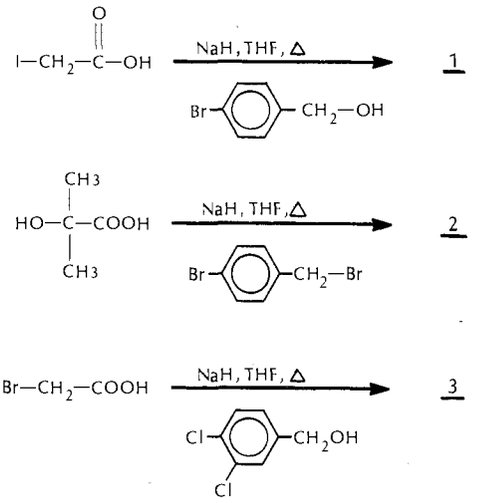
Scheme I



HbS solubility with drug to HbS solubility without drug, $[HbS]_{drug}/[HbS]_{control}$; i.e., the higher the ratio, the greater the activity. Table I shows the average ratios obtained, with standard deviations in parentheses, for multiple runs of 1–5 and phenylalanine[10] (a known antigelling amino acid that was used for comparison).

As can be seen from Table I, compound **3** is the most active, followed by the other bromobenzyloxy derivatives **1** and **2**. This study indicates that the bromobenzyloxy substituent is better than the chlorophenoxy group in preventing gelation of HbS. In addition, when **1** is compared with **2** and **4** is compared with **5**, it is clear that the dimethyl function next to the carboxyl group does not add to the antigelling action, whereas an increase in hydrophobicity (an extra chloro group) on the aromatic ring at the 3 and 4 positions does appear to significantly enhance activity. Other unpublished results in our laboratory confirm that increased hydrophobicity in the acid chain does not give rise to an increase in activity and that the benzyloxy group is superior to the phenoxy group for reducing gelation.

In order to evaluate the antigelling results of potential drugs in terms of possible clinical significance, Sunshine, Hofrichter, and Eaton[11] have published the solubility ratios and kinetic parameters (under these assay conditions) for correlation of clinical severity with inhibition of sickle cell hemoglobin gelation. These authors report the solubility ratios that result from mixtures of HbS and fetal hemoglobin (HbF) or HbS and HbA. In the double heterozygous conditions of S/B^+ thalassemia (15–30% HbA), which are clinically somewhat less severe than homozygous HbS disease, ratios of [(HbS/B^+ thal)/(HbS/S)] are from 1.06 to 1.17. For conditions that are much less severe, as in the double heterozygous condition of sickle/hereditary persistence of fetal hemogloin (S/HPFH) in which red blood cells contain 20–30% hemoglobin F, the solubility ratios obtained [(HbS/HPFH)/(HbS/S)] range from 1.19 to 1.26.

As can be seen from Table I, the ratios needed for a clinically less severe condition [(S/B^+ thal)/(HbS/S)], 1.06–1.17, are reached with the use of **1** and **3** as low as 5 to 10 mM. In these tests the average HbS concentration for 19 assays before gelation was 3.76 mM (24.25 g/dL) (see Table I, footnotes *e–j*). This indicates that only about 1 to 2 mol of drug are needed for every 1 mol of HbS to affect polymerization significantly. In red blood cells, the concentration of Hb is usually around 5 mM, and with a typical hematocrit for homozygous S/S patients of 30%, the concentration of Hb in whole blood is approximately 1.5 mM. Therapeutically then, the activities of these benzyloxy derivatives, especially **1** and **3**, if as nontoxic and membrane permeable as the structurally similar phenoxy antilipidemic drug, clofibrate (dosage, 2 g/day; plasma levels around 1 mM[12]), are near or in the dosage range needed for observation of possible positive clinical effects.

Further studies, which we hope to report shortly, involve the affect of these agents on Hb stability, Hb oxygen equilibrium, binding capability, transport across red blood cell membranes, and X-ray crystallographic location of binding sites on hemoglobin. During the submission of this work, Poillion reported[13] the interesting high activity of a halogenated amino acid, 5-bromotryptophan. To date we have avoided close structural modifications of known amino acids, since such agents might act as inhibitors of enzyme systems, resulting in toxicity. We intend to use current X-ray data to help design more potent compounds with high affinity for Hb.

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