extraction medium (consisting of Triton X-100, 16 mM, potassium phosphate, 50 mM, and EDTA, 1 mM)[29] and allowed to stand in the cold for 1 h. The suspension was then centrifuged at 105000g for 60 min, and the supernatant liquid was collected and used directly as the enzyme source.

Assay Methods. Enzyme Specific Activity. For the determination of the specific activity of the enzyme, the assay (see below) was conducted with 10 μmol of L-proline. One unit of activity is defined as that amount of enzyme that catalyzes the reduction of 1 μmol of INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride] per minute. The specific activities of the enzyme preparations were usually 0.6–0.7 unit/mg, with one preparation giving 2.9 units/mg.

Determination of K_m and V_{max} Values. Proline oxidase activity was assayed by following the reduction of INT by added substrate at 500 nm and at 30 °C by using a molar extinction coefficient of 11.5×10^6 for reduced INT.[30] The assay medium consisted of 100 μmol of potassium phosphate bufer (pH 7.5), 1.5 μmol of KCN, 0.39 μmol of stabilized menadione, 0.5 mg of INT, varying amounts of substrate, 0.1 mL of the solubilized enzyme preparation, and water to give a final volume of 3.0 mL. The reaction was initiated by adding the enzyme preparation last. Blanks were made up identically but without substrates. The data were analyzed on a Hewlett-Packard desk-top computer programmed to graphically display the best straight line fit of Lineweaver–Burk plots by weighted regression analysis using Michaelis–Menten kinetics. The K_m and V_{max} values were then calculated.

With compounds **2a** and **6**, no observable changes in the absorbance at 500 nm were discernible even after 5 min of incubation. However, with compounds **1b** and **7**, this absorbance increased progressively with time even when an equal volume of H_2O was substituted for the enzyme (Figure 2). This nonenzymatic color production could be mimicked by substituting L-cysteine for the thiazolidinecarboxylic acid in the assay medium.

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Registry No. (2*R*,4*R*)-**1b**, 88855-01-0; (2*S*,4*R*)-**1b**, 88855-02-1; (2*R*,4*R*)-[^{14}C]**1b**, 88867-03-2; (2*S*,4*R*)-[^{14}C]**1b**, 88867-04-3; (2*R*,4*R*)-**1c**, 88855-05-4; (2*S*,4*R*)-**1c**, 88855-06-5; (2*R*,4*R*)-**1d**, 88904-06-7; (2*S*,4*R*)-**1d**, 88904-07-8; (2*R*,4*R*)-**1e**, 88855-07-6; (2*S*,4*R*)-**1e**, 88855-08-7; (2*R*,4*R*)-**1f**, 64970-78-1; (2*S*,4*R*)-**1f**, 59999-67-6; (2*R*,4*R*)-**1g**, 88855-09-8; (2*S*,4*R*)-**1g**, 88855-10-1; (2*R*,4*S*)-**2b**, 88855-03-2; (2*S*,4*S*)-**2b**, 88855-04-3; (*R*)-**6**, 72778-00-8; (*S*)-**6**, 22916-26-3; L-cysteine, 52-90-4; D-cysteine, 921-01-7; acetaldehyde, 75-07-0; [1,2-^{14}C]acetaldehyde, 1632-97-9; L-(+)-penicillamine, 1113-41-3; D-penicillamine, 52-67-5; formaldehyde, 50-00-0; butanal, 123-72-8; benzaldehyde, 100-52-7; propanal, 123-38-6; hexanal, 66-25-1; 4-pyridinecarboxaldehyde, 872-85-5; acetaminophen, 103-90-2.

(29) Kramar, R. *Enzymologia* **1967**, *33*, 33.
(30) Johnson, A. B.; Strecker, H. J. *J. Biol. Chem.* **1962**, *237*, 1876.
(31) Mitchell, J. R.; Jollow, D. J.; Potter, W. Z.; Davis, D. C.; Gillette, J. R.; Brodie, B. B. *J. Pharmacol. Exp. Ther.* **1973**, *187*, 185.

Synthesis of Pyridine Derivatives of L-Phenylalanine as Antisickling Reagents

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Several bicyclic agents composed of L-phenylalanine coupled to various pyridines were synthesized: 2-, 3-, and 4-(L-phenylalanylamino)pyridine. All three compounds at 3 mM gave positive morphological antisickling effects on homozygous SS cells under reduced O_2 tension. Studies on two of these compounds, 2- and 3-(L-phenylalanylamino)pyridine, showed that these agents increase the deoxy-HbS solubility ratio, C_s/C_s^0, by 14% at 20 mM. Observed changes in the mean corpuscular hemoglobin concentration (MCHC) values of treated cells ranged from 4% at 1.3 mM to 15% at 5.6 mM in compound concentration. Very minor lytic activity was found for treated cells, indicating water uptake is responsible for changes in the MCHC. Further, exposure of sickle cells to a 3 mM concentration of these agents also increased by 6- to 7-fold cellular deformability of a treated erythrocyte population as compared to an untreated one at the same total O_2 saturation of 47%. These agents demonstrate the potential of bicyclic compounds composed of a common constituent, L-Phe, in the development toward a viable therapeutic agent.

Deoxygenated sickle cell hemoglobin, deoxy-HbS, has been shown to have increased solubility in the presence of aromatic amino acids,[1,2] oligopeptides,[3-5] alkylureas,[6] different phenyl derivatives,[7-12] aryl-substituted alanines,[13] and small aromatic di- and tripeptides.[14] Little is known concerning the mechanism by which noncovalent interactions betwen these compounds and the HbS tetramer result in increased HbS solubility. However, hydrophobicity, a measure of the nonpolar content of these noncovalent antigelling agents, is an important chemical feature. It is related to their antigelling activity as measured by the concentration of the compound required in solution to delay gelation or increase the minimum gelling concentration of deoxy-HbS. With respect to aromatic compounds, bicyclic agents have been shown[13,14] to be more effective than monocyclic members. Increasing the polarizability of the ring system via halogenation also plays a role in enhancing the antigelation activity of aromatic compounds.[10,13] Two different approaches have been re-

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(1) Dean, J.; Chechter, A. N. *N. Engl. J. Med.* **1978**, *299*, 863.
(2) Noguchi, C. T.; Schechter, A. N. *Biochemistry* **1978**, *17*, 5455.
(3) Kubota, S.; Change, C. T.; Samejima, T.; Yang, J. T. *J. Am. Chem. Soc.* **1976**, *96*, 2677.
(4) Kubota, S.; Yang, J. T. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5431.
(5) Votano, J.. R.; Gorecki, M.; Rich, A. *Science* **1977**, *196*, 1216.
(6) Elbaum, D.; Nagel, R. L.; Bookchin, R. M.; Herskovita, T. T. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4718.
(7) Ross, P. D.; Subramanian, S. *Biochem. Biophys. Res. Commun.* **1977**, *77*, 1217.
(8) Adhikary, P. K.; Haynes, J. K.; Patthey, H. L.; Rhodes, R. S. *Experientia* **1978**, *34*, 804.
(9) Behe, M. J.; Englander, S. W. *Biochemistry* **1979**, *18*, 4196.
(10) Abraham, D. J.; Pertuz, M. F.; Phillips, S. E. V. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 324.
(11) Kneen, G.; White, R. D. *Br. J. Pharmacol.* **1981**, *74*, 965.
(12) Abraham, D. J.; Mehanna, A. S.; Williams, F. L. *J. Med. Chem.* **1982**, *25*, 1015.
(13) Poillon, W. N. *Biochemistry* **1982**, *21*, 1400.

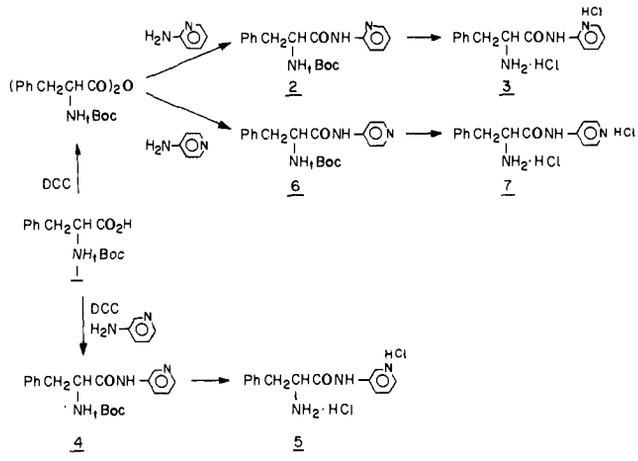
0022-2623/84/1827-0596$01.50/0

Table I. Data for Dihydrochlorides

compd	mp, °C	R_f[a]	formula	anal.
3	178-180	0.62	$C_{14}H_{17}N_3OCl_2 \cdot H_2O$	C, H, N, Cl
5	163-164	0.49	$C_{14}H_{17}N_3OCl_2 \cdot H_2O$	C, H, N, Cl
7	209-214	0.29	$C_{14}H_{17}N_3OCl_2$	C, H, N, Cl

[a] On silica gel with fluorescent indicator, 10 × 20 cm, with AcOH-*n*-BuOH-H_2O (4:4:1) as the eluent.

Scheme I



ported to enhance a potential antisickling agent's uptake by the red cell; one involves the use of liposome-mediated transport,[15] and the other uses a derivatization of an aromatic amino acid with an aromatic group, such as a benzyl ester.[16] In the latter approach, phenylalanyl benzyl ester was found to be quite permeable to the red cell and showed morphological antisickling activity. However, the compound is of limited use due to its low solubility and its degradation due to hydrolysis. In order to increase solubility and eliminate the hydrolysis problem of benzyl esters but still maintain a two-ring system, we have replaced the ester by an amide linkage via synthesis of phenylalamine derivatives containing a pyridine moiety.

In this study we describe the synthesis of these phenylalanine derivatives of aminopyridines and demonstrate the morphological antisickling effects of these three compounds. Further, results for two of their members, 2- and 3-(L-phenylalanylamino)pyridine, include their effects on deoxy-HbS solubility, changes in the mean corpuscular hemoglobin concentration (MCHC) of treated erythrocytes, and their influence on homozygous sickle cell deformability. These pyridines were chosen because their pharmacological properties are known and some of them are used as intermediates in drug production.[17,18]

Results and Discussion

Chemical Synthesis. Acylation of aminopyridines with simple aliphatic and aromatic acids is well known;[19] however, no such derivatives with an amino acid have been described. We found that amide formation of N-protected phenylalanine with different aminopyridines depends on the basicity of the amino group on the pyridine ring. Thus, 2- and 4-aminopyridine could react only with symmetrical *N*-(*tert*-butyloxycarbonyl)-L-phenylalanine (*t*-Boc-Phe) anhydride (in 44 and 63% yield), while the 3-aminopyridine could be coupled directly with *t*-Boc-Phe in the presence of dicyclohexylcarbodiimide (DCC) in high (88%) yield (see Scheme I).

The *t*-Boc protecting group was removed by treatment with dry HCl in methanol and required a prolonged reaction period. It seems that the presence of the pyridine (hydrochloride) moiety of the molecule affords shielding of the protecting group and slows the speed of its decomposition. The dihydrochlorides **3**, **5**, and **7** are easy to handle. All these compounds, as well as their precursors, were characterized by TLC, elemental analysis, and NMR and found to be homogeneous (see Table I).

Table II. Percentage of Cells Sickled after Incubation in 3 mM Solutions of Phenylalanine Derivatives under Different Oxygen Contents

	% cells sickled		
compd	O_2 = 20.1%[a]	O_2 = 4.06%[b]	O_2 = 0.0%
control	9.4	87.5	91.8
3	9.1	32.6	71.1
5	11.4	23.6	59.0
7	10.2	25.2	70.8

[a] Normal atmospheric pressure. [b] 30.8 mmHg (4.06%).

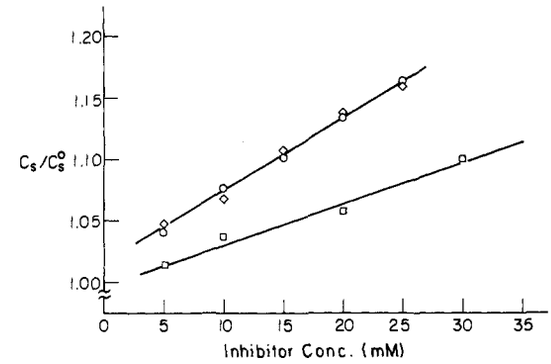


Figure 1. Deoxy-HbS solubility ratio, C_s/C_s^0, as a function of inhibitor concentration: (○) 2-(L-phenylalanylamino)pyridine, (◇) 3-(L-phenylalanylamino)pyridine, (□) L-phenylalanine.

Morphological Antisickling Activity. The antisickling activity of these materials was checked morphologically (see Table II). This assay involves the partial or total deoxygenation of red cells at 37 °C at different oxygen pressures as measured by a blood gas analyzer. The cells were mounted on slides and examined under a microscope. Two hundred or more cells were counted in four zones per slide, and the results are summarized in Table II. All the compounds have very good visual antisickling properties at partial deoxygenation; P_{O_2} = 30.8 mmHg (4.06% O_2). Upon total deoxygenation, compounds **3**, **5**, and **7** showed about the same morphological antisickling activity, about 30–40%, given the ±10% variation in the number of cells appearing sickled in different zones.

Deoxy-HbS Solubilities. In Figure 1, the upper curve gives C_s/C_s^0 values of compounds **3** and **5** [2- and 3-(L-phenylalanylamino)pyridine] and the lower gives C_s/C_s^0 values of L-Phe. An average value of 17.0 ± 0.3 g %, obtained from six independent samples, was used for C_s^0, the solubility of pure HbS. The influence of the N atom

(14) Gorecki, M.; Votano, J. R.; Rich, A. *Biochemistry* **1980**, *19*, 1546.
(15) Kumpati, J. *Biochem. Biophys. Res. Commun.* **1982**, *105*, 482.
(16) Gorecki, M.; Acquaye, C. T. A.; Wilchek, M.; Votano, J. R.; Rich, A. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 181.
(17) Coutts, R. T.; Casy, A. F. *Heterocycl. Compd.* **1975**, *14* (suppl) 445.
(18) Bowman, W. C. *Trends Pharmacol. Sci.* **1982**, *4*, 183.
(19) Grant, C. S. *Heterocycl. Compd.* **1974**, *14* (suppl) 69.

Table III. Changes in the Mean Corpuscular Hemoglobin Concentration Ratio, $MCHC_0/MCHC$, as a Function of the Concentration of Two (Phenylalanylamino)pyridine Compounds and Estimates of Percent Water Gain, W

compd	concn, mM	$MCHC_0/MCHC$	W^a
2-(L-phenylalanyl-amino)pyridine (3)	1.35	1.042	6.2
	2.55	1.080	11.5
	3.65	1.107	15.4
	4.80	1.147	21.1
3-(L-phenylalanyl-amino)pyridine (5)	1.55	1.05	7.2
	2.60	1.075	10.8
	3.95	1.128	18.4
	5.55	1.170	24.5

[a] The estimated percent water gain, W, is given by $W = 1 - Y$; $Y = [A + (A - 1)G_{Hb}](0.92/G_W)$, where $A = MCHC_0/MCHC$, G_{Hb} and G_W (=0.72) are the grams of Hb and H_2O per milliliter of cells, and the specific volumes of Hb and H_2O used were 0.92 and 1.0 cm^3/g, respectively. The $MCHC_0$ of treated cells = 0.344 g/cm^3.

ring position in pyridine appears to have no effect on their antigelling acty. Changes in the N atom's ring position in pyridine are not expected to alter the nonpolar nature of the heterocyclic ring nor to promote differences in the conformation of these compounds in solution. These stereochemical considerations may account for lack of differences in their C_s/C_s^0 values. However, an increase in the hydrophobic content of an aromatic agent, barring ring-substituent effects, has been found[13] to increase the agents' antigelation capacity, in some cases[14] linearly. This effect is apparent in the difference of slopes in Figure 1. The bicyclic agents have a rate change of 0.006 mM^{-1} in C_s/C_s^0, while that of L-Phe is only 0.0028 mM^{-1}.

MCHC Values of Treated Erythrocytes. Erythrocyte suspensions incubated with compounds **3** and **5** at varying concentrations produced a decrease in MCHC as seen in Table III in terms of their average $MCHC_0/MCHC$ values. From the data in Table III, an average rate change in $MCHC_0/MCHC$ for both compounds is 0.031 per millimolar change in concentration. A small amount of lytic activity, about 1–2% v/v, above 4 mM was found with these compounds, but none was found below this concentration when compared to untreated red cells. At a higher concentration it may be that increased cell swelling can cause some lysis in older, more fragile cells. The reduction in $MCHC_0$, at least for compound **5**, could be based in its high permeability (to be published) toward the red cell, and we suspect that compounds **3** and **7** [2- and 4-(L-phenylalanylamino)pyridine] are also permeable given their similar stereochemical features. Uptake of any one of these compounds could change the cellular osmotic water balance due to an influx of an accompanying counterion to maintain solution electroneutrality. Estimates of the percent water gain, W, determined from changes in MCHC, are given in Table III and can explain the reduction in morphological sickling in Table II by an analogous antisickling effect found with the tricyclic ester Cetiedil. The latter has morphological antisickling activity[20,21] and has been shown[22] to increase the measured intracellular water content anywhere from 10 to 20%, depending upon its exposure time and concentration.

Table IV. Effects of Two Inhibitors at 3 mM Concentration on the Filtration Recovery Ratio, R, of Treated Homozygous Sickel Cells at a Total O_2 Saturation of 47% and Estimates of the Volume Fraction, f_p, of HbS Polymer Present

compd	R,[a] %	f_p (est)
2-(L-phenylalanyl-amino)pyridine (3)	41 ± 7	0.24
3-(L-phenylalanyl-amino)pyridine (5)	48 ± 5	0.24
control	7 ± 1	0.37

[a] R = (weight of deoxygenated treated cells)/(weight of oxygenated treated cells) collected after passing through a 5-μm pore membrane. R has the same formulation for control (untreated) cells. The total O_2 saturation of 47% was obtained from the O_2 affinity curve of the sickle cell suspension in PBS, pH 7.392, and the percent O_2 (4.06%) used to deoxygenate cells. The O_2 affinity curve was obtained with an Aminco Hem-O-Scan O_2 analyzer (Silver Springs, MD). P_{50} values of treated normal cells at 3 mM concentration in compounds 3 and 5 showed no alteration, shift, in this parameter.

Cetiedil does not penetrate the cell nor have antigelling activity. At 3 mM in the phenylalanyl derivatives the estimated water gain is around 13%, which falls in the range of Cetiedil-treated red cells. The observed antisickling activities of compounds **3**, **5**, and **7** in Table II suggest that these effects have the same underlying mechanism, water uptake.

Sickle Cell Deformability and HbS Polymer Content. Cellular flexibility of treated SS erythrocytes in the presence of compounds **3** and **5** is expressed here as the recovery ratio, R, which is defined in Table IV as the ratio of the weight of treated deoxygenated to oxygenated sickle cell recovered in order to eliminate the small effect on filterability due to red cell swelling. As seen in Table IV, treated erythrocytes have R values 6 to 7 times larger than the control (untreated) cells at the same total O_2 saturation. The basis of improved deformability of treated SS erythrocytes, putting aside compound permeability with its small attendant increase in HbS solubility, is a result more attributable to the reduction in the mole fraction of HbS polymer, f_p. An evaluation of f_p is straightforward if one neglects the small increase in HbS solubility by these agents if both are assumed fully permeable. The expression for f_p is $f_p = (1 - V_s)C_p/C_t$. V_s, the volume fraction of Hb in the monomer phase, is $(C_p - C_t)/(C_p - C_s)$; C_t used here is the measured $MCHC_0$, and C_p = 0.69 g/cm^3 is the HbS concentration in the polymer phase.[23] A value of 0.164 g/cm^3 was used for C_s^0 for pure deoxy-HbS solubility at 37 °C. C_s, the Hb concentration in the monomer phase at a given O_2 saturation, can be obtained from an empirical formulation, and a quantitative description, based on the two-state allosteric model for Hb, can be found to describe O_2 binding in the HbS polymer phase.[24] A comparison of the f_p values in Table IV shows a 35% decrease in f_p for treated cells as opposed to untreated cells. This change may be sufficient to account for a major portion of the observed increase in deformability, since viscosity of homozygous erythrocytes has been shown[25] to vary hyperbolically with O_2, which is consistent with the fact that f_p has also been shown to vary in a similar hyperbolic manner with variation in the total O_2 saturation.[26]

(20) Asakura, T.; Ohnishi, S. T.; Adachi, K.; Ozguc, M.; Hashimoto, K.; Singer, M.; Rusell, M. O.; Schwartz, E. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2955.
(21) Benjamin, L. J.; Kokkini, G.; Peterson, C. M. *Blood* **1980**, *55*, 265.
(22) Schmidt, W. F.; Asakura, T.; Schwartz, E. *Blood Cells* **1982**, *8*, 269.
(23) Hofrichter, J. *J. Mol. Biol.* **1979**, *128*, 335.
(24) Sunshine, H. R.; Hofrichter, J.; Ferrone, F. A.; Eaton, W. *J. Mol. Biol.* **1982**, *158*, 251.
(25) Ohnishi, S. T. *Blood Cells* *8*, 79 (1982).
(26) Noguchi, C. T.; Torchia, D. A.; Schechter, A. N. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5487.

Conclusions

The ability of these phenylalanyl bicyclic agents to decrease the intracellular Hb concentration of the erythrocyte is their most important property. A small reduction in the MCHC of 1 to 2 g % translates into longer delay times[27] for intracellular HbS gelation of homozygous sickle cells. This effect on delay time would be even more pronounced at various partial O_2 saturations. Although several of these agents have antigelling activity, their effect on cellular deformability of reversible SS erythrocytes as opposed to the irreversible subpopulation of cells[28] is more a function of reducing f_p than any small increase in HbS solubility, at least at a 3 mM compound concentration. We feel that singly charged bicyclic agents characterized by L-Phe as a constituent group offer a new avenue toward the development of similar agents aimed at a viable agent for the treatment of sickle cell diseases.

Experimental Section

Chemical Methods. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on a Varian FT 80A NMR spectrometer. IR were obtained on a Perkin-Elmer 467 spectrophotometer. Elemental analyses were performed by the Microanalysis Laboratory of the Weizmann Institute of Science and are within ±0.3% of calculated values.

Synthetic Methods. The conjugation of various aminopyridines with *N*-(*tert*-butyloxycarbonyl)-L-phenylalanine was carried out through its anhydride (method A), by a direct coupling with DCC (method B) or by using *N*-hydroxysuccinimide ester (method C). All compounds subjected to biological evaluation gave the expected NMR resonances, absorbed in the appropriate regions of the IR, and had correct elemental analyses.

2-[[*N*-(*tert*-Butyloxycarbonyl)-L-phenylalanyl]amino]-pyridine (2). Method A. *N*-(*tert*-Butyloxycarbonyl)-L-phenylalanine (2.13 g, 8 mmol) was dissolved in dry acetonitrile (30 mL) and cooled in an ice bath. DCC (824 mg, 4 mmol) dissolved in acetonitrile (20 mL) was slowly added. The mixture was stirred for 1 h at 0 °C and for 2 h at room temperature and then filtered from cyclohexylurea. The IR absorption at 1820 cm^{-1} indicated the presence of anhydride. 2-Aminopyridine (370 mg, 4 mmol) and pyridine (1 mL) were added; the mixture was left overnight and then evaporated. The residue was dissolved in ethyl acetate, washed with saturated $NaHCO_3$, dried, and concentrated. The crude product was chromatographed on a silica column (Merck, 60 g) and eluted with methylene chloride to yield 590 mg (44%): mp 91–93 °C (from methylene chloride–hexane); NMR ($CDCl_3$) δ 9.0 (1, s, NH), 8.28–6.94 (9, m, aromatic), 5.34 (1, br, NH), 4.54 (1, m, CH), 3.3–3.0 (2, m, CH_2), 1.38 (9, s, CH_3). Anal. ($C_{19}H_{23}N_3O_3$) C, H. N.

4-[[*N*-(*tert*-Butyloxycarbonyl)-L-phenylalanyl]amino]-pyridine (6) was prepared as above by method A. The crude product was chromatographed on a column filled with first with silica (100 g) and then with alumina (50 g, neutral, Woelm Pharma). Upon elution with ethyl acetate–methylene chloride (1:4), *N*-acylurea was eluted. The same solvents in a 2:3 ratio eluted dicyclohexylurea. The pure product was obtained by elution with ethyl acetate in 63% yield: mp 96 °C; NMR ($CDCl_3$) δ 8.42 and 7.32 (4, AA'BA' pattern, heterocyclic), 7.30 (5, s, aromatic), 5.4 (1, d, NH), 4.53 (1, q, CH), 3.9 (2, t, CH_2), 1.25 (9, s, CH_3). Anal. ($C_{19}H_{23}N_3O_3$) C, H, N.

3-[[*N*-(*tert*-Butyloxycarbonyl)-L-phenylalanyl]amino]-pyridine (4). Method B. 3-Aminopyridine (11.2 g, 0.13 mol) and DCC (25.9 g, 0.126 mol) were dissolved in dry THF (350 mL) and cooled in an ice bath. *N*-(*tert*-butyloxycarbonyl)-L-phenylalanine (31.8 g, 0.12 mol) dissolved in THF (150 mL) was slowly added during 3 h. The mixture was stirred for 2 h at 0 °C and overnight at room temperature and then filtered from urea; the filtrate was concentrated and chromatographed on a column filled first with silica (360 g) and then with neutral alumina (100 g). The product was eluted with a mixture of ethyl acetate–methylene chloride (1:4) to yield 30.8 g (82%). Ethyl acetate–hexane (3:7) is an alternative solvent system for chromatography: mp 122–123 °C (from ethyl acetate–hexane); NMR ($CDCl_3$) δ 8.4–7.8 (5, m, heterocyclic and NH), 7.25 (5, s, atomatic), 4.51 (1, g, CH), 3.12 (2, d, CH_2), 1.40 (9, s, CH_3). Anal. ($C_{19}H_{23}N_3O_3$) C, H, N.

Removal of the Protecting Group (General Method). The corresponding Boc derivative (450 mg) is dissolved in dry methanol (5 mL). Methanol saturated with HCl up to 15% (4 mL) is added, and the mixture is stirred overnight at room temperature. At the beginning, the hydrochloride is precipitated; as the decarboxylation progresses, the dihydrochloride goes into solution. The product is precipitated with dry ether and left to stand a few hours. The solvent is decanted, and the crystals are washed several times with dry ether and finally purified by dissolving in dry methanol and precipitating with ether. The yields are quantitative (see Table I).

Biology. Sickle Cell Erythrocytes and HbS Preparation. Both the morphological and filterability assays used homozygous sickle cell erythrocytes with MCHC values of 33.5 and 33.9 g %, respectively, as determined by a Coulter hemoglobinometer. The percentage of HbF was less than 8% as determined FTEST (Isolab, Akron, OH). Sickle cells were used within 24 h and kept at 4 °C. HbS was prepared from homozygous SS erythrocytes by previously described procedures,[14] except the HbS solution was dialyzed against 50 mM sodium phosphate, pH 7.2. The HbF content was 3.2%.

Morphological Sickling Assay. The washed erythrocytes were incubated at a 1.0% hematocrit in phosphate buffer saline, pH 7.2 (PBS), containing a 3 mM concentration of a given phenylalanyl derivative. The osmolality of PBS used was adjusted to 295 mosmol/kg and contained, besides the compound, 0.5% (w/v) bovine serum albumin (Sigma Chemical Co.). Incubation of sample suspension plus compound was for 1 h at 37 °C with continual inversion by a circular rotor operating at 40 rpm. After 1 h, and aliquot was removed and exposed to air for 10 min with frequent mixing, and then the erythrocytes were examined for their morphology. The remaining portion of the cells were deoxygenated by the method of Clark et al.[29] utilizing either a water-saturated gas mixture (4.06% O_2, 4.4% CO_2, N_2 balance) or N_2 gas alone. Just prior to fixation of the SS erythrocytes, the P_{O_2} of the cell suspension was determined (Radiometer BMS MKS 2). Cells were fixed under an anaerobic condition and remained for 30 min at room temperature prior to a morphological examination with a Zeiss phase contrast microscope (1250×). Two hundred or more cells were counted and were considered sickled or unsickled by established criteria.[30,31]

Deoxy-Hb Solubility Assay. Equilibrium solubilities of deoxy-HbS were determined according to method of Poillon[13] with modifications. The HbS solutions were deoxygenated by an anaerobic addition of 20% (w/v) $Na_2S_2O_4$ in 0.35 N NaOH, followed by continuous inversion for 5 min at 4 °C of the stoppered HbS solution contained in an 0.8-mL cellulose centrifuge tube (Beckmann), and then overlaid with mineral oil. The final HbS concentration was 28.0 g % in 50 mM $Na_2S_2O_4$ and 85 mM sodium phosphate buffer, pH 7.2. The sample was centrifuged in an SW 50.1 rotor at 240000*g* for 75 min at 28 ± 0.5 °C. The deoxy-HbS solubilities of treated and untreated solutions, C_s and C_s^0, were determined with HbS in the cyanomet form with 11.0 cm^{-1} per heme as the millimolar extinction coefficient. C_s^0 values were determined with each centrifugation run.

MCHC Evaluation. Erythrocytes were incubated in the presence of a compound identical with that described in the morphological assay, except a 10% hematocrit was used. Triplicate measurements were made on each of three aliquots (1.5 mL) withdrawn at 1 to 3 h after red cell exposure to compound at 37 °C. A small variation of only 0.4–0.6% was found in the value

(27) Coletta, M.; Hofrichter, J.; Ferrone, F. A.; Eaton, W. *Nature (London)* **1982**, *300*, 194.
(28) Fabry, M. E.; Nagel, R. L. *Blood Cells* **1982**, *8*, 9.
(29) Clark, W. T.; Gautelli, J. C.; Mohandas, N.; Shohet, S. B. *Blood* **1980**, *55*, 823.
(30) Jensen, W. N.; Rucknagel, D. L.; Taylor, W. J. *J. Clin. Med.* **1960**, *56*, 854.
(31) Serjeant, G. R.; Serjeant, B. E.; Milner, P. F. *Br. J. Haematol.* **1969**, *17*, 527.

of the heamtocrit at a given compound concentration. The intracellular Hb concentration was determined by a Coulter hemoglobinometer and Diluter 2. Red cell lysis and cyanomet-Hb conversion was done with Zapolgobin (Coulter). The average MCHC values (nine measurements at a given compound concentration) are given in Table III.

Filterability Assay. The apparatus was previously described,[16] except an effective membrane diameter of 2.3 cm was used with a 5-μm pore size polycarbonate membrane (Bio-Rad). A sickle cell suspension (Hct = 1%) was deoxygenated at 37 °C with the same gas mixture used in the morphological assay above. The equilibrium P_{O_2} of each sample suspension was checked just prior to filtration by an anaerobic withdrawal and placement of a 100-μL portion into an O_2 analyzer (Radiometer BMS MK2). This same gas was used to push 2.5 mL of treated or untreated cell suspension through the 5-μm pore size membrane at a pressure of 3.1 cmHg as measured on Croft pressure gauge. The cells were always exposed to this gaseous environment during filtration. After 5-6 min, SS cells that had passed through the membrane into the lower chamber of the filter assembly were harvested and weighed, and a correction for their weight due to intersitial solvent was made with ^{3}H-labeled methoxyinulin (NEN).

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Registry No. 1, 13734-34-4; 1 (anhydride), 33294-54-1; 2, 88932-67-6; 3, 88932-68-7; 3 (free base), 88932-73-4; 4, 88932-69-8; 5, 88932-70-1; 5 (free base), 88932-75-6; 6, 88932-71-2; 7, 88932-72-3; 7 (free base), 88932-74-5; 2-aminopyridine, 504-29-0; 3-aminopyridine, 462-08-8; 4-aminopyridine, 504-24-5.

Methotrexate Analogues. 19. Replacement of the Glutamate Side Chain in Classical Antifolates by L-Homocysteic Acid and L-Cysteic Acid: Effect on Enzyme Inhibition and Antitumor Activity

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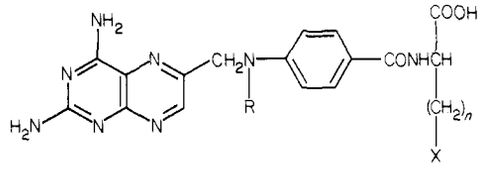
Methotrexate (MTX) and aminopterin (AMT) analogues containing L-homocysteic acid or L-cysteic acid in place of L-glutamic acid were synthesized and tested as inhibitors of dihydrofolate reductase from L1210 cells and folyl polyglutamate synthetase from mouse liver. The ID_{50} against dihydrofolate reductase was comparable for the MTX and AMT analogues (0.04–0.07 μM), whereas the ID_{50} against folyl polyglutamate synthetase was 3- to 4-fold lower for the AMT analogues (40–60 μM) than for the MTX analogues (100–200 μM). Thus, N^{10}-substitution has a greater effect on binding to folyl polyglutamate synthetase than dihydrofolate reductase. The cytotoxicity of these compounds was assayed in vitro against L1210 cells, and the AMT analogues again proved more potent (ID_{50} = 0.03–0.05 μM) than the MTX analogues (ID_{50} = 0.1–0.4 μM). A similarly increased potency was observed for the AMT analogues against L1210 leukemia in vivo. Though differential cell uptake cannot be ruled out as the basis of increased potency, it is possible that part of the activity of the AMT analogues involves interference with the intracellular polyglutamation of reduced folate cofactors, i.e., that they are "self-potentiating antifolates". Of the four compounds reported, the most active was *N*-(4-amino-4-deoxypteroyl)-L-homocysteic acid, which produced a 138% increase in life span (ILS) in L1210 leukemic mice when given on a modified bid × 10 schedule at a dose of 2 mg/kg. A comparable ILS was obtained with AMT itself at 0.24 mg/kg. Thus, replacement of γ-CO_2H by γ-SO_3H in the side chain does not decrease therapeutic effect. However, a higher dose is required, presumably to offset pharmacological differences reflecting the inability of the sulfonate group to be polyglutamated.

As part of a larger program on side-chain modified antifolates related to methotrexate (MTX) and aminopterin (AMT),[1-4] we were interested in preparing and evaluating the biological activity of compounds in which the γ-carboxyl of the glutamate moiety is replaced by another acidic group. Replacement of CO_2H by SO_3H seemed attractive because of the ready availability of L-homocysteic acid. Our rationale for this structural modification was that it would yield analogues that are incapable of forming polyglutamate conjugates inside the cell, while retaining a good affinity for dihydrofolate reductase. Polyglutamation of MTX and other classical antifolates has become widely recognized as an important determinant of toxicity and selectivity.[5-9] In this context we postulated that non-



	R	n	X
MTX	Me	2	COOH
AMT	H	2	COOH
1	Me	2	SO_2OH
2	Me	1	SO_2OH
3	H	2	SO_2OH
4	H	1	SO_2OH

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(1) Rosowsky, A.; Yu, C.-S. *J. Med. Chem.* **1983**, *26*, 1448 (paper 18 in this series).
(2) Rosowsky, A.; Forsch, R.; Uren, J.; Wick, M.; Kumar, A. A.; Freisheim, J. H. *J. Med. Chem.* **1983**, *26*, 1719 (paper 20 in this series).
(3) Rosowsky, A.; Moran, R. G.; Forsch, R.; Colman, R.; Wick, M. *Biochem. Pharmacol.*, in press (paper 17 in this series).
(4) Rosowsky, A.; Forsch, R. *J. Med. Chem.* **1982**, *25*, 1454 (paper 16 in this series).
(5) Poser, R. G.; Sirotnak, F. M.; Chello, P. L. *Cancer Res.* **1981**, *41*, 1757.

0022-2623/84/1827-0600$01.50/0