Design, Synthesis, and Testing of Antisickling Agents. 2.[1a,b] Proline Derivatives Designed for the Donor Site

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We have used a three-dimensional model of deoxyhemoglobin to design potential antisickling agents with an intended binding site in the vicinity of the β-6 mutation (donor site). Two proline derivatives, (4*S*)-1-butyryl-4-[(carboxymethyl)amino]-L-proline (**9a**) and its 1-benzoyl analogue (**9b**), were designed to interact, via ionic or hydrogen bonds, with polar residues β His-2, β Thr-4, and β Lys-132 of hemoglobin S (HbS). Two other proline derivatives containing a salicylate leaving group, (4*S*)-1-butyryl-4-[(carboxymethyl)methylamino]-L-proline, 2-ester with salicyclic acid (**14a**), and its 1-benzoyl analogue (**14b**), were designed to bind covalently to β Lys-132, as well as to interact with β His-2 and β Thr-4 via ionic and hydrogen bonds. This paper describes the synthesis of these agents, beginning with natural L-hydroxyproline methyl ester, and the testing of their ability to increase or decrease the solubility of dHbS by using a standard solubility assay. The covalent derivatives **14a,b** were found to be inactive, while the noncovalent compounds **9a,b** showed weak antigelling activity, below that observed for phenylalanine. The presence of only weak activity does not invalidate this approach, since only one structural parameter has been investigated.

Our principal method of finding a potential drug to treat sickle cell anemia has been via the synthesis and testing of compounds that were designed to interact stereospecifically with sickle hemoglobin (HbS, which contains the mutation β-6 Glu → Val). In an earlier publication[2] it was pointed out that drugs of the future might be tailor-made by using a combination of the three-dimensional structural knowledge of proteins derived from X-ray crystallographic studies and the hydrophobic transport qualities (Hansch relationships) of organic molecules. Recently, we have successfully obtained active antigelling compounds utilizing information concerning an aromatic binding site found via X-ray crystallographic difference Fourier synthesis.[1a-3] Several other investigators[5-9] have attempted the design of stereospecific inhibitors of gelation as a primary approach to finding suitable drugs for treating sickle cell anemia. As yet, no compounds have been approved for therapeutic purposes.

A significant amount of structural information concerning the polymerization of HbS has appeared in recent years, and this has enabled us to initiate a detailed approach to drug design. For example, the 5-Å[10] and 3-Å[11] single-crystal X-ray structures of deoxyhemoglobin S indicate the presence of tetrameric hemoglobin dimers in which the β-6 mutation site, valine (donor site), of one molecule inserts itself into a hydrophobic cavity (acceptor site) near β Phe-85 and β Leu-88 of the second molecule. Figure 1 depicts the donor site–acceptor site interaction with a hydrogen bond between β Thr-4 of the donor site with β Asp-73 of the acceptor site as found in the Wishner–Love model.[11] Magdoff–Fairchild and workers[12,13] have also shown that the X-ray diffraction patterns of the fiber and crystalline structures of dHbS are sufficiently similar in spacings and in intensity distributions to suggest, with some assurance, that the use of the high-resolution X-ray crystal structures might prove beneficial for drug-design purposes. This paper deals with molecules designed to interact at the donor site.

Noncovalent Derivatives. Two proline derivatives, **9a,b**, were designed to bind stereospecifically to the area near the mutation site (donor site) on the β chain (see Figure 1). Figure 2 illustrates a composite picture of regional hydrophobic and hydrophilic amino acid residues. The profile of this area suggested the design of agents that would interact in a complementary fashion with the polar trapezoidal area residues (β Glu-7, β Thr-4, β His-2, and β Lys-132). It was also envisioned that stereospecific orientation of the proline molecules into this trapezoidol area would be enhanced by the addition of a hydrophobic group that would project into the adjacent nonpolar area (Figure 3). Since Thr-4 is involved in a cross-polymer contact, a disruption of this bond with β Asp-73 (Figure 1) should destabilize polymer formation. In addition to the above, it was envisioned that the hydrophobic moiety of the proline derivatives might provide steric incompatibilities for donor–acceptor interaction and, thus, produce further polymer destabilization. It was also hoped that binding of agents at this site would show no interference with the oxy–deoxy equilibrium mechanism,[14] since this area is removed from subunit interface movements.

Covalent Derivatives. Aspirin, salicylate esters, or aspirin derivatives have been shown to be effective as Hb-acetylating agents by several workers.[15-22] For exam-

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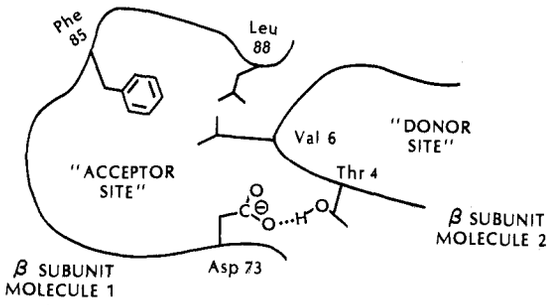


Figure 1. Representation of the donor site–acceptor site interaction between HbS molecules in the deoxy state.

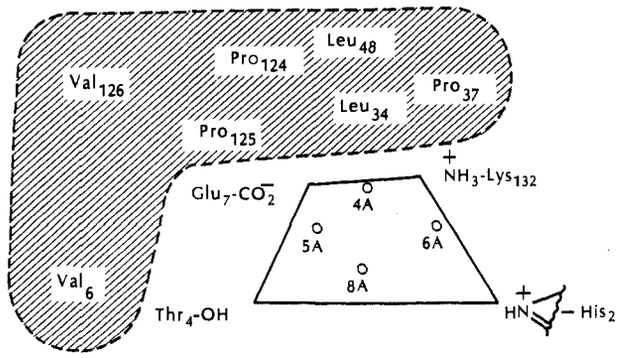


Figure 2. Representation of the regional hydrophobic and hydrophilic amino acid residues about the mutation (donor) site.

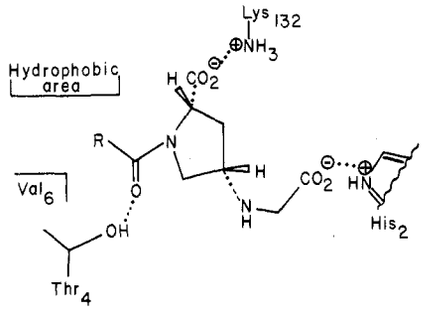


Figure 3. Representation of a noncovalent proline derivative binding near the mutation (donor) site (R = n-C_3H_7, C_6H_5).

ple, aspirin acetylates a variety of sites on both the α and β chains of hemoglobin S.[22] The vast majority of acetyl groups were attached at three loci: β Lys-59, β Lys-144, and α Lys-90. As described above, our proline derivatives were designed to interact at a trapezoidal area of polar groups. Therefore, to further increase potential antisickling activity, we synthesized compounds **14a** and **14b**, wherein we added a salicylate group to the α-carboxy group of both proline derivatives **9a** and **9b**, thus hopefully placing the ester in the vicinity of β Lys-132. Reaction of β Lys-132 with the salicylate ester would release salicylic acid and covalently attach the proline derivative to HbS (Figure 4).

Chemistry. The common intermediate required for the synthesis of **9a,b**, as well as **14a,b**, was the azide **5**. Compound **5** had the required cis geometry and thus allowed

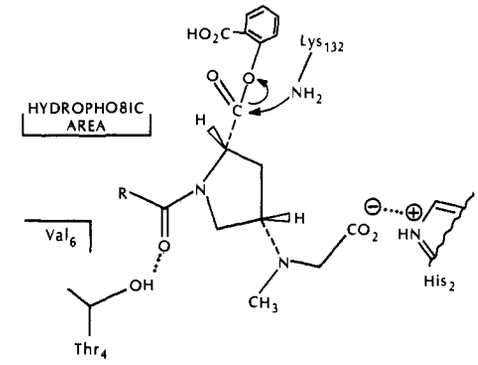
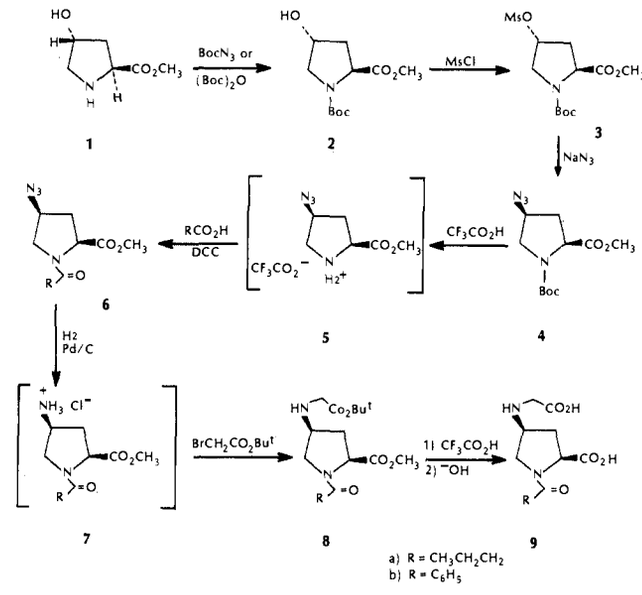


Figure 4. Representation of a potential covalent proline derivative interacting near the mutation (donor) site (R = n-C_3H_7, C_6H_5).

Scheme I[a]



[a] Boc = $(CH_3)_3COOC$; Ms = CH_3SO_2; Bu^t = $(CH_3)_3C$.

the construction of the needed cis dicarboxylic acid functionalities found in **9a,b** and **14a,b**. The nitrogen of commercial L-hydroxyproline methyl ester (**1**) was protected as its urethane derivative **2** by reaction with *tert*-butoxycarbonyl azide, according to the method of Schwyzer et al.,[23] or more recently by the use of di-*tert*-butyl dicarbonate. The *trans*-4-hydroxy function was activated for stereochemical replacement by reaction with methanesulfonyl chloride by using the conditions of Lednicer et al.,[24] affording the mesylate **3** in 91% yield. The appearance of the sulfonyl methyl group at δ 2.98 in the ^{1}H NMR was indicative of structure **3**. Inversion of configuration at carbon 4 was accomplished by using sodium azide,[25] affording **4** as an oil. The analytical sample of **4** was pure on TLC on three different solvent systems, indicating a single isomer and presumed to be the desired cis structure, i.e., 2*S*,4*S*. Analogously, it is known[26] that the tosylate of 1-acetyl-L-hydroxyproline methyl ester can be converted to 1-acetyl-L-allohydroxyproline upon alkaline

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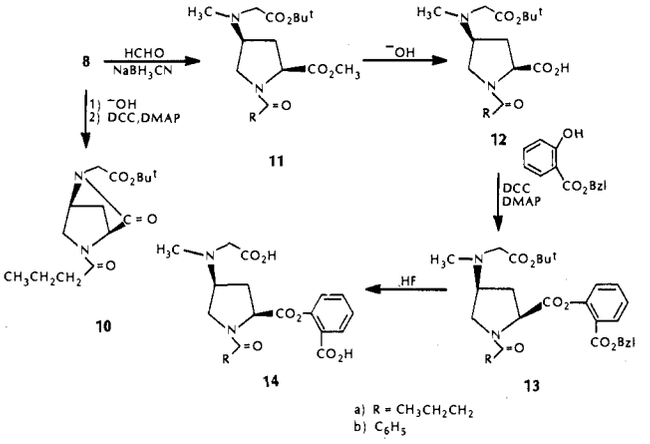
Scheme II[a]



[a] $Bu^t = (CH_3)_3C$; Bzl = $C_6H_5CH_2$.

treatment, i.e., inversion of configuration of the hydroxy group from 4*R* to 4*S*. Acidolytic cleavage of the *tert*-butoxycarbonyl group gave the common intermediate **5**, which was isolated as its trifluoroacetate salt without further purification (Scheme I).

For the amidation of the amine **5**, a general procedure,[27] utilizing dicyclohexylcarbodiimide (DCC) as the coupling agent, was used that was suitable for the preparation of **6a,b**. Thus, butanoic acid was coupled with **5** to give **6a** in a nearly quantitative yield. Catalytic reduction of the azide **6a** afforded the correspoding amine **7a** as its hydrochloride salt. Due to the instability of the latter compound, it was not isolated but reacted directly with *tert*-butyl bromomoacetate to give the diester **8a** in a 30% yield (for the two steps). Cleavage of the *tert*-butyl ester with trifluoroacetic acid (TFA), followed by base hydrolysis of the methyl ester, afforded the desired diacid **9a** in a 43% yield. This compound was pure according to high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC) and thus must be a single isomer (2*S*,4*S*). Inspection of the ^1H NMR spectrum of **9a** at 600 MHz[28] revealed two triplets centered at δ 0.89 and 0.91, which we have assigned to the terminal methyl of the butyramide chain existing in both the cis and trans conformations. Cis/trans isomerism about the amide bond involving the nitrogen of proline is a well-recognized phenomenom.[29] Further proof was obtained by examining the ^1H NMR spectrum of known[30] 1-butyryl-L-proline (see Experimental Section). It too showed the two triplets for the methyl group at nearly identical chemical shifts with that of **9a**.

In a similar fashion, benzoic acid was coupled to **5** with DCC to give **6b** in a 63% yield. Catalytic reduction of the azide group and alkylation of the resulting amino group with *tert*-butyl bromoacetate gave the diester **8b** in a yield of 34%. Two-step cleavage of the *tert*-butyl ester with TFA and the methyl ester with base, followed by HPLC purification, gave the *N*-benzoyl diacid **9b** in a 45% yield. Inasmuch as **9b** was one component according to TLC and HPLC, we have assigned it as the desired 2*S*,4*S* isomer.

Table I. Solubility Assay: Proline Derivatives and Phenylalanine[a]

compd	[HbS drug]/[HbS control] 5 mM	10 mM	20 mM	40 mM
9a[b]	1.000	1.011	1.021	1.047
9b[c]	0.991	1.019	1.056	1.114
14a[d]	0.965	0.960	0.896	0.848
14b[e]	0.967	0.974	0.924	0.896
Phe[f]	1.036	1.048	1.093	1.178

[a] All compounds were dissolved in 0.15 M phosphate buffer, pH 7.4, at a concentration of 0.18 M, with 1 equiv of sodium bicarbonate added to make the sodium salt. Appropriate aliquots of this solution (10, 20, 40, and 80 μL) were mixed with buffer using Hamilton automatic syringes to equal 90 μL. The 90-μL solutions were added to EPR tubes containing 250 μL of HbS (0.15 M phosphate), usually around 35 to 36 g %, then 20 μL of dithionite (1.06 M) was added under anaerobic conditions in a glove box, and the EPR tubes were sealed. 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 concentration tubes, one HbS control (90 μL of buffer), and a 40 mM phenylalanine control. The above dilution procedure produces identical HbS initial concentrations for all six tubes. The initial deoxyhemoglobin (dHbS) concentrations (in grams per deciliter) for each run after addition of the test compounds and dithionite (0 °C) and the respective solubility of dHbS (in grams per deciliter) for each control run (35 °C) follow for each compound studied. [b] Initial dHbS concentration in grams per deciliter, 25.08; respective solubility of dHbS control in grams per deciliter, 17.00. [c] 25.08 and 17.07 g/dL. [d] 25.08 and 16.59 g/dL. [e] 25.08 and 16.85 g/dL. [f] Values for Phe taken from ref 1a. For comparison, the reported phenylalanine ratios as calculated from a straight line through the points in ref 5 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 5 was 32 mM.

Further proof of the cis geometry of diacids **9a,b** was obtained by selectively hydrolyzing the methyl ester of **8a** and then effecting an intramolecular cyclization with the aid of DCC and 4-(dimethylamino)pyridine (DMAP).[31] The resulting bicyclic lactam **10**, obtainable only from a cis compound, had physical data supporting its structure (Scheme II).

Scheme II outlines the procedures used to obtain the proline derivatives **14a,b**, which were designed to interact covalently at the donor site. Due to the ease of intramolecular cyclization of the monoacid derived from **8a** upon DCC treatment, it was necessary to convert the amino group of **8** to a tertiary amine in order to prevent this cyclization at a later stage. Thus, **8a,b** were N-alkylated with formaldehyde and $NaBH_3CN$ via the method of Borch and Hassid,[32] affording the tertiary amines **11a,b** in yields of 96 and 74%, respectively. Inasmuch as *tert*-butyl esters are resistant[33] to mild alkali, we were able to selectively hydrolyze the methyl esters **11a,b** to their corresponding monoacids **12a,b** in 78 and 63% yields, respectively. DCC coupling of benzyl salicylate to these acids, promoted by the catalyst DMAP,[34] gave the corre-

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sponding triesters **13a,b** in yields of 83 and 66%, respectively. Acidolytic cleavage of the benzyl and *tert*-butyl esters was simultaneously accomplished by treatment of **13a,b** with liquid HF,[35] crystalline amino acids **14a,b** thus being obtained in yields of 43 and 55%, respectively.

Biological Results

Four compounds, **9a,b** and **14a,b**, were tested by the solubility assay described by Hofrichter, Ross, and Eaton.[36] This assay involves the incubation of the drugs to be tested (at several concentrations) with HbS in EPR tubes in the presence of a deoxygenating substance, as previously described.[1a] Activity is reported as a ratio of HbS concentration that is soluble in the presence of a given concentration of drug to HbS solubility in the absence of the drug, [HbS drug]/[HbS control]. Ratios of 1.06 to 1.17 are estimated[37] to be necessary for the observance of a clinical improvement in sickle cell anemia.

For comparative purposes, we have included phenylalanine, an antigelling amino acid,[5] in Table I along with the results obtained for **9a,b** and **14a,b**. As can be seen from Table I, the two noncovalent agents (**9a,b**) are well below the activity ratio needed for therapeutic interest. The covalent compounds (**14a,b**) appear to have an enhanced gelling behavior, the cause of which is unknown. It is interesting, however, that some activity was obtained by the noncovalent compounds considering that these agents were designed by a purely stereochemical approach.

In addition, Chang and Nagel[38] also measured the calorimetry of compound **9a** and found it to give exothermic heats of 0.73 kcal/mol of hemoglobin at 1 mM drug and 2.55 kcal/mol of hemoglobin at 10 mM drug. This is in the same order of magnitude as the alkylureas, whereas 2,3-DPG is about 10 kcal/mol at 1 mM.

The fact that these new molecules (which were designed for the donor site) show only weak or nonexistent activity does not invalidate the present theoretical approach. This series only monitors one class of compounds as a single parameter in our exploration of this approach. Forthcoming work from our laboratory will show that a series of molecules designed for this donor site possess high activity. The presence of minimum activity with **9a,b** indicates that these agents do cause some structural perturbations on hemoglobin even with weak binding, as suggested by the calorimetry results mentioned above. In order to minimize the enormous amount of work effort needed to test this hypothesis, we have opted not to pin down the binding site of these weaker acting agents via X-ray crystallography nor to investigate their oxygen equilibrium in the presence of hemoglobin, but we expect to do so with our more active agents designed for this site.

Experimental Section

Melting points were determined either on a Fisher-Johns or Thomas-Hoover apparatus and are uncorrected. Infrared spectra were recorded on either a Perkin-Elmer 137 or 267 spectrometer as KBr pellets (solids) or as neat films (oils). ^{1}H NMR spectra were recorded at 60 MHz on a Hitachi Perkin-Elmer R-24 spectrometer or at 600 MHz on the Carnegie-Mellon University Biomedical Facility. The spectra are reported in parts per million (δ) downfield from internal standards tetramethylsilane ($CDCl_3$) or sodium 3-(trimethylsilyl)proprionate-d_4 (D_2O). Low-resolution mass spectra were recorded on a Varian-MAT CH-5, LKB 5000, or Finnigan 3200 spectrometer, while high-resolution data[39] (HRMS) were obtained from the Massachusetts Institute of Technology Mass Spectrometry Facility on a Varian MAT 731 spectrometer, either in the electron-impact (EI) or field-desorption (FD) mode. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. HPLC purifications were performed with a Waters Associates setup (U-6K injector, M6000 pump, Model 440 254-nm absorbance detector, Model 450 variable-wavelength detector) by using either of the two following semipreparative columns, μBondapak C_{18} 0.78 × 30 cm (column 1) or Bondapak C_{18}/Porasil B 0.78 × 61 cm (column 2). Optical rotations were determined with a Perkin-Elmer Model 241 automatic polarimeter. The apparatus and method for conducting the liquid HF reactions were as previously described.[40] TLC analyses were carried out on either silica gel GF glass plates (Analtech) or on aluminum sheets (EM Reagents), unless stated otherwise. Preparative TLC was carried out on Analtech plates (500, 1000, or 2000-μm thick, 20 × 20 cm). The following TLC systems were used: 1, 2% $CH_3OH/CHCl_3$; 2, 4% $CH_3OH/CHCl_3$; 3, 10% $CH_3OH/CHCl_3$; 4, $CHCl_3$; 5, $CH_3OH/EtOAc/NH_3$ (4:94:2); 6, 2-propanol/H_2O (7:3); 7, 5% CH_3OH/0.2 M NaCl; 8, $CH_3OH/EtOAc/NH_3$ (3:96.5:0.5); 9, 1-propanol/H_2O (7:3). All evaporations were performed in vacuo on a rotary evaporator. Organic solutions that had been previously extracted with aqueous solution were dried over anhydrous Na_2SO_4 prior to evaporation, except where noted. The petroleum ether used had bp 30–60 °C, unless stated otherwise.

(4*R*)-1-(*tert*-Butoxycarbonyl)-4-hydroxy-L-proline Methyl Ester (2). (a) Methyl ester hydrochloride 1 (Vega Biochemical Corp.; 2.00 g, 11.0 mmol) and Et_3N (1.7 mL, 12.3 mmol) were stirred in dry pyridine (20 mL) for 30 min at room temperature, after which *tert*-butyloxycarbonyl azide (Aldrich Chemical Co.; 1.7 mL, 12.0 mmol) was added. After 12 h, the solution was concentrated to dryness, and the residue was dissolved in EtOAc (25 mL) and washed successively with 1 M citric acid, 1 M $NaHCO_3$, and H_2O. Reduction of the solvent volume to 1–2 mL and addition of petroleum ether gave, upon cooling in the refrigerator, an oil. The oil was washed with petroleum ether and dried under high vacuum, yielding 2.22 g (82%) of **2** as a colorless, viscous oil, which we could not induce to crystallize. The product was pure on TLC in systems 1 (R_f 0.18), 2 (R_f 0.31), and 3 (R_f 0.55): NMR ($CDCl_3$) δ 3.68 (s, 3 H, OCH_3), 1.40 [s, 9 H, $C(CH_3)_3$]; MS, *m/e* 245 (M^+), 227 ($M^+ - H_2O$), 186 ($M^+ - CO_2CH_3$), 144 [$M^+ - CO_2C(CH_3)_3$], 130 [186 – $(CH_3)_2C{=}CH_2$, metastable at 90.9], 86 [$M^+ - CO_2\,C(CH_3)_3 - CO_2CH_3 + H$]; $[\alpha]^{28}_D$ –75.2° (*c* 1, CH_3OH) [lit.[41] $[\alpha]^{22}_D$ –79 ± 0.5° (*c* 1.03, CH_3OH); mp 97–98 °C].

(b) Methyl ester hydrochloride 1 (Vega Biochemical Corp.; 1.00 g, 5.5 mmol) was dissolved in dioxane (3 mL), and diisopropylethylamine (1.2 g, 9.3 mmol) was added. Di-*tert*-butyl dicarbonate (1.60 g, 7.3 mmol) in dioxane (7 mL) was then added, and vigorous effervescence was observed. The reaction was allowed to proceed at room temperature for 2 h, and then DMAP (0.014 g, 0.11 mmol) was added. The reaction mixture was stirred for a further 0.5 h and then concentrated to dryness, and the residue was dissolved in EtOAc (120 mL) and washed successively with 1 M citric acid, 1 M $NaHCO_3$, and H_2O. The EtOAc solution was dried ($MgSO_4$) and evaporated to give a light yellow oil (1.27 g, 94%), which was identical with **2** prepared above. This oil was used without further purification.

(4*R*)-1-(*tert*-Butoxycarbonyl)-4-[(methylsulfonyl)oxy]-L-proline Methyl Ester (3). Hydroxy compound **2** (1.83 g, 7.47 mmol) was dissolved in dry pyridine (10 mL) and cooled in ice. Methanesulfonyl chloride (1.2 mL, 15.5 mmol) was added in one portion, and the reaction mixture was stirred for 4 h in ice and

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then for 12 h at room temperature. Evaporation of the solvent gave a residue that was dissolved in EtOAc (10 mL) and washed successively with 1 M $NaHCO_3$, 1 M citric acid, and H_2O. Evaporation of the solvent and crystallization of the residue from EtOAc/petroleum ether (bp 60–90 °C) afforded 2.20 g (91%) of **3** as colorless needles: mp 85–86 °C; ^1H NMR ($CDCl_3$) δ 5.20 (m, 1 H, C*H*OS), 2.98 (s, 3 H, CH_3SO_3); $[\alpha]^{25}_D$ −52.3° (*c* 1.6, $CHCl_3$). Anal. ($C_{12}H_{21}NO_7S$) C, H, S.

(4*S*)-1-(*tert*-Butoxycarbonyl)-4-azido-L-proline Methyl Ester (4). Mesylate **3** (2.92 g, 9.04 mmol) and NaN_3 (3.00 g, 46.2 mmol) were stirred in dry DMF (10 mL) at 45–55 °C overnight. The solvent was evaporated, and the residue was mixed with EtOAc (25 mL) and H_2O (15 mL). After separation of the layers, the organic phase was washed with H_2O until neutral and then with 0.1 N HCl and H_2O and dried. Evaporation of the EtOAc gave the product **4** as a tan oil, 2.42 g (crude 99%), which was suitable for the next step. For analytical purposes, a portion was purified by preparative TLC (system 4, programmed multiple development[42]). The analytical sample, an oil, was pure according to TLC in systems 1–3 (programmed multiple development (50, 100, and 150 mm): IR 4.85 μm (azide); $[\alpha]^{25}_D$ −36.6° (*c* 2.8, $CHCl_3$). Anal. ($C_{11}H_{18}N_4O_4$) C, H, N.

(4*S*)-1-Butyryl-4-azido-L-proline Methyl Ester (6a). Azide **4** (0.72 g, 2.67 mmol) was dissolved in CH_2Cl_2 (4 mL) and then treated with anhydrous TFA (2 mL). After the mixture was stirred at room temperature for 25 min, the solvents were evaporated, and the residue was dried under high vacuum. The resulting TFA salt **5** was mixed with CH_2Cl_2 (40 mL) and Et_3N (0.74 mL, 5.34 mmol); after stirring for 20 min, *n*-butyric acid (0.24 mL, 2.62 mmol) was added, and the reaction was cooled in ice. DCC (0.60 g, 2.90 mmol) was then added to the cooled mixture and stirred for 48 h. After the dicyclohexylurea (DCU) was removed by filtration, the volume of the solution was reduced to about 15 mL and washed successively with 1 M citric acid, 1 M $NaHCO_3$, and H_2O. The CH_2Cl_2 solution was decolorized with Norit and concentrated to a colorless viscous oil, 0.67 g (crude weight greater than theory due to DCU contamination). A portion of the oil was purified by preparative TLC (system 3), affording the analytical sample **6a**: IR 4.75 (azide), 5.73 (ester), 6.05 μm (amide); $[\alpha]^{25}_D$ −74.0° (*c* 1.5, CH_3OH). Anal. ($C_{10}H_{16}N_4O_3$) C, H, N.

(4*S*)-1-Benzoyl-4-azido-L-proline Methyl Ester (6b). TFA salt **5** (0.605 g, 2.13 mmol) and Et_3N (0.33 mL, 2.39 mmol) were stirred at room temperature in CH_2Cl_2 (5 ml) for 20 min, benzoic acid (0.261 g, 2.14 mmol) was added, and the mixture was cooled in ice. DCC (0.486 g, 2.36 mmol) was added, and the mixture was stirred in the cold for 48 h. Workup as described above for **6a** gave 0.584 g of crude oily product. Purification by preparative TLC (system 1) afforded a 63% yield of **6b** as a colorless oil: IR 4.75 (azide), 5.75 (ester), 6.1 μm (amide); MS, *m/e* (relative intensity) 274 (1, M^+), 231 (5, $M^+ - HN_3$), 215 (38, $M^+ - CO_2CH_3$), 186 (10), 141 (14), 105 (100, C_6H_5CO), 77 (81, C_6H_5); $[\alpha]^{25}_D$ −72.0° (*c* 2.5, CCl_4). Anal. ($C_{13}H_{14}N_4O_3$) C, H, N.

(4*S*)-1-Butyryl-4-[(carboxymethyl)amino]-L-proline 4-*tert*-Butyl Methyl Ester (8a). Azide **6a** (0.40 g, 1.67 mmol) was dissolved in CH_3OH (55 mL), and concentrated HCl (1.0 mL) was added, followed by 10% Pd/C (0.13 g). The resulting mixture was hydrogenated at atmospheric pressure for 2 h and filtered, and the filtrate was concentrated to dryness. The resulting crude amine HCl **7a** was dried under vacuum and dissolved in dioxane (4 mL), Et_3N (1.00 g, 9.9 mmol) was added, and the mixture was stirred at room temperature for 10 min. *tert*-Butyl bromoacetate (0.60 g, 3.07 mmol) was dissolved in dioxane (2 mL) and added dropwise over a short period. This mixture was then heated at 50 °C for 48 h, diluted with dioxane (15 mL), and filtered, and the filtrate was concentrated to dryness. The entire residue was purified on preparative TLC plates (system 5), and the desired band (R_f 0.44–0.56) was located by spraying an edge with ninhydrin, thus affording **8a** as a light yellow oil (0.17 g, 30%): one spot on TLC (R_f 0.60, system 5). The analytical sample was obtained by rechromatographing, affording **8a** as a pale yellow oil, which slowly solidified: mp 49–51 °C; MS, *m/e* (relative intensity) 328 (0.2, M^+), 272 [10, $M^+ - (CH_3)_2C{=}CH_2$], 227 [32, $M^+ - CO_2C(CH_3)_3$]; $[\alpha]^{25}_D$ −50.3° (*c* 1.1, $CHCl_3$); HRMS (EI) calcd, 328.19983; found 328.20103. Anal. ($C_{16}H_{28}N_2O_5 \cdot 0.5H_2O$) C, H, N.

(4*S*)-1-Benzoyl-4-[(carboxymethyl)amino]-L-proline 4-*tert*-Butyl Methyl Ester (8b). In a similar manner as for **8a**, the azide **6b** (2.10 g, 7.66 mmol) was hydrogenated over palladium on charcoal (10%, 0.59 g) at 35 psi in a solution of methanol (110 mL) and concentrated HCl (0.67 mL) for 2.5 h. The mixture was worked up as for **8a** and then dissolved in a mixture of dioxane (20 mL) and DMF (2 mL). Triethylamine (3.9 g, 38.6 mmol) and *tert*-butyl bromoacetate (2.14 g, 10.8 mmol) were added to the mixture, which was then heated at 50 °C for 2 days, worked up, and purified as for **8a**, affording **8b** as a light yellow oil (0.952 g, 34%) which crystallized spontaneously on standing. Recrystallization from ether–petroleum ether afforded **8b** as white crystals, mp 102–103 °C; $[\alpha]^{25}_D$ −97.1° (*c* 1.1, $CHCl_3$). Anal. ($C_{19}H_{26}N_2O_5$) C, H. N.

(4*S*)-1-Butyryl-4-[(carboxymethyl)amino]-L-proline (9a). Diester **8a** (0.16 g, 0.49 mmol) was dissolved in TFA (5.0 mL), stirred at ambient temperature for 20 min, and concentrated to dryness. The resulting oil was triturated with anhydrous Et_2O, evaporated 3 times, and then triturated with anhydrous Et_2O; the solvent was then removed with a pipet. This TFA salt of the monoacid was then dried in vacuo over P_2O_5 and NaOH, affording a tan solid (0.17 g, 90%). A portion of the TFA salt (0.096 g, 0.25 mmol) was dissolved in H_2O (2.0 mL) and treated with a solution of NaOH (0.035 g, 0.87 mmol) in H_2O (1.0 mL). The disappearance of starting material and appearance of product was followed every 15 min by HPLC (column 1, isocratic with 15% CH_3OH/0.02 N AcOH). The reaction was terminated after 80 min by the addition of dilute HCl (pH 3.5). After lyophilization, the crude solid product was dissolved in 3.0 mL of the above mobile phase and purified via semipreparative HPLC (column 1, above mobile phase, 2.0 mL/min, monitored at 215 and 254 nm, 0.25–mL injections). The combined fractions were lyophilized, redissolved in H_2O, and lyophilized to give **9a** as a powder (0.032 g, 43% for the two steps), which was homogeneous upon HPLC (as above, visible at 215 nm only), TLC systems 6 and 7 (reversed-phase plates, Analtech, R_f 0.59). The analytical sample was obtained by crystallization from 2-propanol: mp 158–160 °C; ^1H NMR (D_2O, 600 MHz) δ 0.89 and 0.91 (2 t, CH_2CH_3, J = 7.35 Hz, cis and trans), 1.59 (m, $CH_2CH_2CH_3$), 2.40 (m, $NCOCH_2$), irradiation at 1.59 collapses the two triplets to singlets and the multiplet at δ 2.40 to an AB quartet, 3.81 (AB quartet, $N^+CH_2CO_2^-$, J_{AB} = 16.55), 4.41 and 4.52 (2 q, X portion of AMX, C_α-H, cis and trans); $[\alpha]^{28}_D$ −37.2° (*c* 0.75, H_2O); HRMS (FD) calcd, 259.1294; found, 259.1290 (MH^+). Anal. ($C_{11}H_{18}N_2O_5 \cdot 0.25H_2O$) C, H, N.

(4*S*)-1-Benzoyl-4-[(carboxymethyl)amino]-L-proline (9b). In a similar manner as for **9a**, the diester **8b** (0.17 g, 0.47 mmol) was selectively cleaved with TFA (affording 0.17 g, 84%), and the corresponding monoester (0.096 g, 0.23 mmol) was then hydrolyzed with NaOH (0.032 g, 0.81 mmol) as above. After two purifications by HPLC, **9b** was obtained as a pure white powder (0.036 g, 44%, for the two steps), which was homogeneous upon HPLC (visible at 215 and 254 nm) and TLC (as above, R_f 0.58 on reversed-phase plate). The analytical sample was obtained by crystallization from 2-propanol: mp 160–162 °C; ^1H NMR (D_2O, 600 MHz) δ 3.70 (AB quartet, $N^+CH_2CO_2^-$, J_{AB} = 16.5 Hz), 4.44 and 4.61 (2 q, X portion of AMX, C_α-H, cis and trans), 7.4–7.6 (m, 5 H, aromatic); $[\alpha]^{28}_D$ −42.3° (*c* 0.65, H_2O); HRMS (FD) calcd, 293.1137; found, 293.1146 (MH^+). Anal. ($C_{14}H_{16}N_2O_5 \cdot 0.25H_2O$) C, H, N.

1-Butyryl-L-proline. L-Proline benzyl ester (0.50 g, 2.44 mmol), *n*-butyric acid (0.215 g, 2.44 mmol), and DMAP (0.030 g, 0.24 mmol) were dissolved in CH_2Cl_2 (20 mL). To this solution was added DCC (0.554 g, 2.68 mmol), and the mixture was stirred at ambient temperature for 4 h (TLC indicated reaction complete). After filtration of the precipitated DCU, the solvent was concentrated to a smaller volume and chromatographed on silica gel (15 g; hexane/EtOAc, 1:1), affording 0.30 g (45%) of 1-butyryl-L-proline benzyl ester. A portion (0.25 g, 0.91 mmol) of the latter compound was dissolved in EtOAc (250 mL), 10% Pd/C (0.050 g) was added, and the mixture was hydrogenated at 35 psi in a Parr hydrogenator. After filtration and evaporation of the solvent, a white solid was obtained, which was dissolved in warm H_2O,

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filtered free of DCU, and lyophilized. The lyophilized powder was crystallized from EtOAc–petroleum ether, giving the desired product, 0.105 g (28% for the two steps): mp 115–116.5 °C; $[\alpha]^{25}_D$ −102.1° (*c* 1.02, H_2O) [lit.[30] mp 114–115.5 °C; $[\alpha]^{18}_D$ −101° (*c* 1, H_2O)]; ^1H NMR (D_2O, 600 MHz) δ 0.90 and 0.94 (2 t, CH_2CH_3, J = 7.35 Hz, cis and trans), 1.60 (m, $CH_2CH_2CH_3$), 2.38 (m, $NCOCH_2$), irradiation at δ 1.60 collapses the 2 t to nearly singlets and the multiplet at δ 2.38 to an AB quartet, 4.25 and 4.35 (2 q, X portion of AMX, C_α-H, cis and trans).

***tert*-Butyl (1*S*,4*S*)-5-Butyryl-3-oxo-2,5-diazabicyclo-[2.2.1]heptane-2-acetate (10).** Diester **8a** (0.10 g, 0.30 mmol) was dissolved in methanol (1.5 mL) and then cooled to 0 °C. NaOH (0.15 N, 2.0 mL) was added, and the resulting mixture was stirred at 15 °C for 1 h. The reaction mixture was adjusted to pH 6.9 with 0.2 N HCl and lyophilized. The crude product was purified by HPLC (column 2, 30% CH_3OH in 0.02 N AcOH), affording the corresponding *tert*-butyl monoester as a lyophilized powder. This monoacid (0.11 g, 0.35 mmol) was dissolved in dichloromethane (8 mL) and DCC (0.08 g, 0.39 mmol) and DMAP (0.0035 g, 0.029 mmol) was added. The mixture was stirred at room temperature for 18 h, filtered, and purified by preparative TLC (system 2), affording **10** as a clear oil (0.08 g, 93%): MS, *m/e* (relative intensity) 296 (0.9, M^+), 240 [15, $M^+ - (CH_3)_2C{=}CH_2$], 170 [42.9, $M^+ - (CH_3)_2C{=}CH_2 - C_4H_6O$], 69 (100); $[\alpha]^{25}_D$ −19.7° (*c* 1.1, $CHCl_3$). Anal. ($C_{15}H_{24}N_2O_4$) C, H, N.

(4*S*)-1-Butyryl-4-[(carboxymethyl)methylamino]-L-proline 4-*tert*-Butyl Methyl Ester (11a). To **8a** (0.08 g, 0.24 mmol) in acetonitrile (1 mL) was added formalin (37%, 0.18 mL, 2.4 mmol), followed by $NaBH_3CN$ (0.045 g, 0.79 mmol) in portions. The reaction mixture was stirred at room temperature for 15 min, and glacial AcOH was added to maintain the pH near neutrality. After 2 h, the solvents were removed by evaporation, and the residue was purified by preparative TLC (system 8), affording **11a** as an oil (0.08 g, 96%): MS, *m/e* (relative intensity) 342 (1.4, M^+), 286 [3.5, $M^+ - (CH_3)_2C{=}CH_2$], 241 [73.7, $M^+ - CO_2C(CH_3)_3$], 68 (100); ^1H NMR ($CDCl_3$, 600 MHz) δ 2.44 (s, N-CH_3), 3.73 (s, O-CH_3); $[\alpha]^{25}_D$ −69.8° (*c* 1.35, $CHCl_3$); HRMS (EI) calcd, 342.21547; found, 342.21268. Anal. ($C_{17}H_{30}N_2O_5$) C, H, N.

(4*S*)-1-Benzoyl-4-[(carboxymethyl)methylamino]-L-proline 4-*tert*-Butyl Methyl Ester (11b). In a similar manner as for **11a**, **8b** (0.72 g, 1.99 mmol) in acetonitrile (7 mL) was treated with formalin and $NaBH_3CN$ and then subjected to preparative TLC (system 8). Thus, **11b** was obtained as a light yellow oil (0.56 g, 75%), which was homogeneous on TLC (systems 1 and 8): MS, *m/e* (relative intensity) 376 (2.2, M^+), 320 [6.3, $M^+ - (CH_3)_2C{=}CH_2$], 275 [74.8, $M^+ - CO_2C(CH_3)_3$], 105 (100, C_6H_5CO); ^1H NMR ($CDCl_3$, 600 MHz) δ 2.38 (s, N-CH_3), 3.78 (s, OCH_3); $[\alpha]^{25}_D$ −97.2° (*c* 1.1, $CHCl_3$). HRMS (EI) calcd, 376.19983; found, 376.20279. Anal. ($C_{20}H_{28}N_2O_5$) C, H, N.

(4*S*)-1-Butyryl-4-[(carboxymethyl)methylamino]-L-proline 4-*tert*-Butyl Ester (12a). Compound **11a** (0.50 g, 1.46 mmol) was dissolved in CH_3OH (3.8 mL) and H_2O (1.9 mL). A solution of NaOH (0.5 N, 4.2 mL, 2.1 mmol) and then added dropwise with stirring. After 3 h at room temperature the reaction mixture was adjusted to pH 6.0 with 1 N HCl and then lyophilized. The product was purified by HPLC (column 1, 35% CH_3OH in 0.02N AcOH) and afforded **12a** as a white lyophilized powder (0.37 g, 77%). This powder was homogeneous on both TLC (system 9) and HPLC and was used as such in the next step.

(4*S*)-1-Benzoyl-4-[(carboxymethyl)methylamino]-L-proline 4-*tert*-Butyl Ester (12b). In a similar manner as for **12a**, **11b** (0.47 g, 1.25 mmol) in CH_3OH (3 mL) was treated with a NaOH solution (0.5 N, 5.27 mL, 2.6 mmol). Purification by HPLC (column 2, 40% methanol in 0.02 N acetic acid) afforded **12b** as a lyophilized powder (0.28 g, 62%), homogeneous on both TLC (system 9) and HPLC, and was used as such in the next step.

(4*S*)-1-Butyryl-4-[(carboxymethyl)methylamino]-L-proline 4-*tert*-Butyl Ester 2-Ester with Benzyl Salicylate (13a). Compound **12a** (0.26 g, 0.79 mmol) was dissolved in CH_2Cl_2 (6.5 mL) and mixed with benzyl salicylate (0.71 g, 3.11 mmol). DCC (0.32 g, 1.55 mmol) and DMAP (0.018 g, 0.15 mmol) were added, and the mixture was stirred at room temperature overnight. After filtration and concentration to dryness, the residue was purified by preparative TLC (system 1), affording **13a** as a clear oil (0.35 g, 83%). The purified sample was homogeneous on TLC analysis (systems 1 and 8); $[\alpha]^{25}_D$ −57.6° (*c* 0.9, $CHCl_3$). Anal. ($C_{30}H_{38}N_2O_7$) C, H, N.

(4*S*)-1-Benzoyl-4-[(carboxymethyl)methylamino]-L-proline 4-*tert*-Butyl Ester 2-Ester with Benzyl Salicylate (13b). In a similar manner as for **13a**, **12b** (0.23 g, 0.64 mmol) dissolved in CH_2Cl_2 (9 mL) was reacted with benzyl salicylate (0.47 g, 2.06 mmol), DCC (0.19 g, 0.92 mmol), and DMAP (0.016 g, 0.13 mmol). Preparative TLC (system 1) purification afforded **13b** as a clear oil (0.24 g, 66%), which was homogeneous on TLC analysis (systems 1 and 3) and solidified spontaneously on standing. Recrystallization from ether–petroleum ether gave the analytical sample: mp 78–79 °C; $[\alpha]^{25}_D$ −132.0° (*c* 1.28, $CHCl_3$). Anal. ($C_{33}H_{36}N_2O_7$) C, H, N.

(4*S*)-1-Butyryl-4-[(carboxymethyl)methylamino]-L-proline 2-Ester with Salicyclic Acid (14a). Ester **13a** (0.16 g, 0.30 mmol) was mixed with anisole (0.3 mL) and stirred with anhydrous HF (ca. 5 mL) at 0 °C for 50 min. The HF was evaporated in vacuo, and the residue remaining was dissolved in water (25 mL) and washed with ether (3 × 25 mL). The aqueous layer was lyophilized, and the powder was purified by HPLC (column 1, 35% methanol in 0.02 N acetic acid) to give a white powder (0.10 g). Repeated crystallizations from acetonitrile–acetone afforded **14a** as white crystals (0.05 g, 43%): mp 161–162 °C; ^1H NMR (D_2O, 600 MHz) δ 0.92 (t, CH_2CH_3), 1.62 (m, $CH_2CH_2CH_3$), 2.43 (m, $NCOCH_2$), 3.05 (s, $CH_3{}^+NCH_2CO_2^-$), 7.21 (d, J = 8.1 Hz, arom H ortho to ester O), 7.46 (t, arom H meta to carboxy), 7.65 (t, arom H para to carboxy), 7.90 (d, J = 7.4 Hz, arom H ortho to carboxy); $[\alpha]^{25}_D$ −97.6° (*c* 0.7, H_2O). Anal. ($C_{19}H_{24}N_2O_7$) C, H, N.

(4*S*)-1-Benzoyl-4-[(carboxymethyl)methylamino]-L-proline 2-Ester with Salicylic Acid (14b). Ester **13b** (0.10 g, 0.18 mmol) was mixed with anisole (0.25 mL) and stirred with anhydrous HF (ca. 4 mL) at 0 °C for 50 min. The HF was evaporated in vacuo, and the residue was dissolved in water (50 mL) and washed with ether (3 × 40 mL). The aqueous layer was lyophilized, and the powder was crystallized from THF to afford **14b** as white crystals (0.059 g, 55%): mp 150–152 °C; ^1H NMR (D_2O, 600 MHz) δ 3.00 (s, $CH_3{}^+NCH_2CO_2^-$), 7.22 (d, J = 8.1 Hz, arom H ortho to ester O), 7.4–7.7 (m, 7 H, benzoyl, arom H meta and para to carboxy), 7.87 (d, J = 7.4 Hz, arom H ortho to carboxy); $[\alpha]^{25}_D$ −109.6° (*c* 0.5, Me_2SO). Anal. ($C_{22}H_{22}N_2O_7 \cdot THF \cdot 0.5H_2O$) C, H, N.

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Registry No. 1·HCl, 40216-83-9; **2**, 74844-91-0; **3**, 84520-67-2; **4**, 84520-68-3; **5**, 84520-70-7; **6a**, 84520-71-8; **6b**, 84520-72-9; **7a**, 84520-73-0; **8a**, 84520-74-1; **8a** demethyl ester, 84520-75-2; **8a** de-*tert*-butyl ester TFA, 84520-77-4; **8b**, 84520-78-5; **8b** de-*tert*-butyl ester, 84520-79-6; **8b** de-*tert*-butyl ester TFA, 84520-80-9; **9a**, 84520-81-0; **9b**, 84520-82-1; **10**, 84520-83-2; **11a**, 84520-84-3; **11b**, 84520-85-4; **12a**, 84520-86-5; **12b**, 84520-87-6; **13a**, 84520-88-7; **13b**, 84520-89-8; **14a**, 84520-90-1; **14b**, 8452091-2; *tert*-butyloxycarbonyl azide, 1070-19-5; di-*tert*-butyl dicarbonate, 24424-99-5; *n*-butyric acid, 107-92-6; benzoic acid, 65-85-0; *tert*-butyl bromoacetate, 5292-43-3; 1-butyryl-L-proline, 23500-13-2; L-proline benzyl ester, 41324-66-7; 1-butyryl-L-proline benzyl ester, 84520-92-3; benzyl salicylate, 118-58-1.