A Highly Stereoselective Michael Addition to an α,β-Unsaturated Ester as the Crucial Step in the Synthesis of a Novel β-Amino Acid-Containing Fibrinogen Receptor Antagonist

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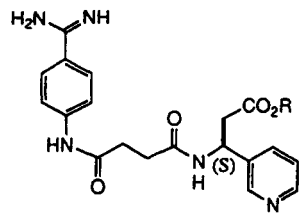
Background

Convenient large-scale syntheses of chiral β-amino acids are needed for preparation of various compounds from natural or synthetic origin endowed with interesting biological activities.[1] For example, (*S*)-ethyl β-[[4-[[4-(aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-3-pyridinepropanoate (**1**), a peptidomimetic for the Arg-Gly-Asp-Phe sequence of fibrinogen,[2] is an orally active antiplatelet agent that may be useful in preventive therapy for myocardial infarction and unstable angina.[3]

The ester derivative **1**, is a prodrug form of the active agent (*S*)-β-[[4-[[4-(aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-3-pyridinepropanoic acid (**2**). To carry on biological characterization *in vivo*, multigram quantities of **1** were required along with several grams of the derived acid **2**.

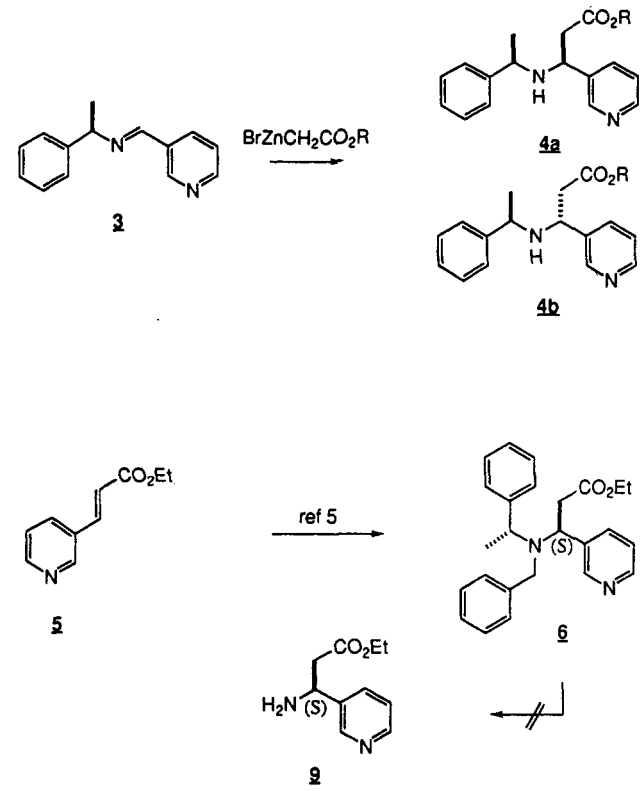


1 R = Et

2 R = H

The major problem in the preparation of large quantities of **1** was the access to the chiral β-amino ester. A stereoselective preparation of (*S*)-β-amino-3-pyridinepropionic acid had not been previously reported in the literature although the racemic material had been described.[4] Attempts to use the modified Reformatsky reaction[5] with the chiral imine **3** derived from pyridinecarbaldehyde and (*R*)-1-phenylethylamine gave mediocre selectivity (Scheme I; diastereoselectivity 57:43) although separation of the diastereoisomers **4a** and **4b** allowed access to the desired **4a**.[6] However, access to multigram quantities required a more practical procedure.

Scheme I



Results and Discussion

The excellent diastereoselectivity recently reported by Davies for Michael addition of a homochiral lithium amide derivative to an α,β-unsaturated ester[7] prompted us to examine that approach for our synthesis. This reaction is a significant improvement over the addition of chiral amines to carbon–carbon double bonds.[8] Using (*R*)-*N*-(1-phenylethyl)benzylamine lithium amide according to the Davies method, excellent diastereoselectivity was achieved in the Michael addition to ethyl *trans*-3-pyridineacrylate[9] (**5**) easily obtained by esterification of *trans*-3-pyridineacrylic acid (Scheme I). Unfortunately the resulting tertiary amine **6** resisted our attempts to debenzylate with $Pd(OH)_2$ and H_2 using a variety of reaction conditions. Perhaps catalyst poisoning by the pyridine moiety was responsible. Other palladium cat-

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(1) For reviews: (a) Barrett, G. C., Ed. *Chemistry and Biochemistry of the Amino Acids*; Chapman and Hall: London New York, 1985; p 25. (b) Griffith, O. W. *Annu. Rev. Biochem.* **1986**, *55*, 855–78. For some additional examples, see: (c) Konopelski, J.; Chu, K. S.; Negrete, G. R. *J. Org. Chem.* **1991**, *56*, 1355–1357. (d) Kato, S.; Hamada, Y.; Shioiri, T. *Tetrahedron Lett.* **1988**, *29*, 6465–6. (e) Baldwin, J. E.; Adlington, R. M.; O'Neil, I. A.; Schofield, C.; Spivey, A. C.; Sweeney, J. B. *J. Chem. Soc. Chem. Commun.* **1989**, 1852. See also ref 7.

(2) Tjoeng, F. S.; Fok, K. F.; Zupec, M. E.; Garland, R. B.; Miyano, M.; Panzer-Knodle, S. G.; King, L. W.; Nicholson, N. S.; Taite, B. B.; Feigen, L. P.; Adams, S. P. In *Peptides: Chemistry, Structure and Biology (Proceedings of the 12th American Peptide Symposium*; Smith, J. A., Rivier, J. E., Eds.; Escom: Leiden, The Netherlands, 1992; p 752.

(3) (a) Bovy, P. R.; Garland, R. B.; Tjoeng, F. S.; Zupec, M. E.; Zablocki, J. A.; Rico, J. G.; Rogers, T. E.; Lindmark, R. J.; Panzer-Knodle, S. G.; Nicholson, N. S.; Taite, B. B.; Miyano, M.; Feigen, L. P.; Adams, S. P. *J. Cell. Biochem. Suppl. C* **1993**, L 308. (b) Bovy, P. R.; Rico, J. G.; Rogers, T. E.; Tjoeng, S. F.; Zablocki, J. PCT Int. Appl. WO 93/07867, 1993.

(4) Secor, H. V.; Edwards, W. B. *J. Org. Chem.* **1979**, *44*, 3136–3140.

(5) (a) Bellasoued, M.; Arous-Chtara, R.; Gaudemar, M. *J. Organometal. Chem.* **1982**, *231*, 185–89. (b) Furukawa, M.; Okarawa, T.; Noguchi, Y.; Trawaki, Y. *Chem. Pharm. Bull.* **1978**, *26*, 260–63.

(6) Madigan, D. L.; Walters, D. E.; Culberson, J. C.; Dubois, G. E.; Carter, J. S.; Nagarajan, S.; Klix, R. C.; Ager, D. J.; Klade, C. A. EP 355819, 1990.

(7) Davies, S. G.; Ichihara, O. *Tetrahedron:Asymmetry* **1991**, *2*, 183–186.

(8) Furukawa, M.; Okarawa, T.; Terawaki, Y. *Chem. Pharm. Bull.* **1977**, *25*, 1319–1325.

(9) The trans geometry for the carbon double bond is supported by the coupling constant for the vinylic protons (J = 15 Hz).

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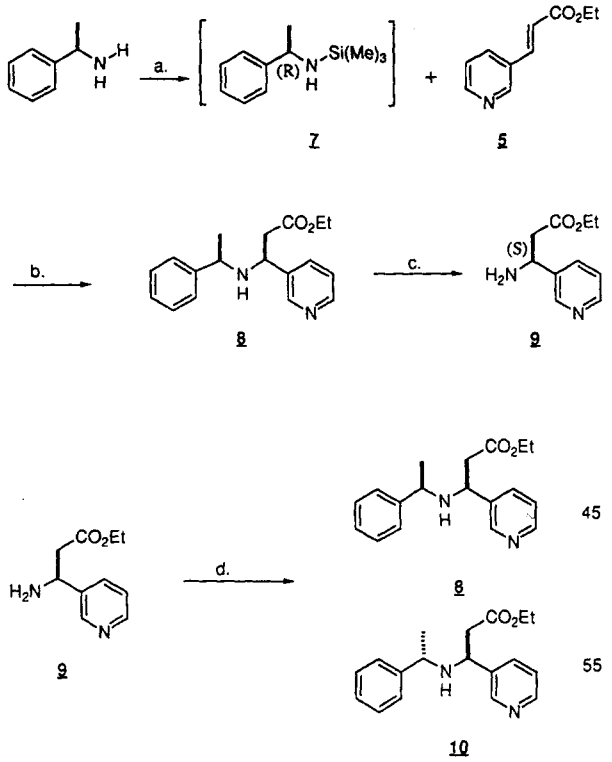
Scheme II[a]



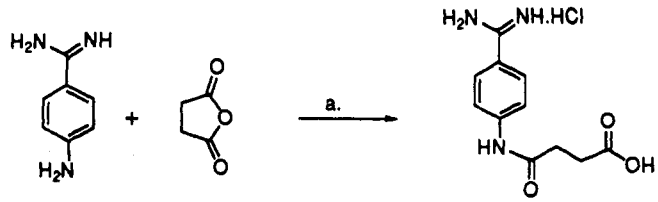
7 5 8 9 8 9 10

[a] (a) NEt_3, TMSCl, THF; (b) butyllithium, THF, 0 °C; −78 °C to rt; (c) Pd/C, NH_4CO_2H or Pd/C/1,3-cyclohexadiene; (d) acetophenone, $NaBH_3CN$, MeOH.

alysts[10] under hydrogen atmosphere only gave starting material or unidentified species.

Parallel investigations with the novel chiral lithium silylamide generated from *N*-(trimethylsilyl)-(*R*)-1-phenylethylamine (**7**) gave good nucleophilic addition. In this adduct, a single benzylic bond needs to be cleaved and easier deprotection to the desired amino acid was anticipated. We focused our efforts on this novel homochiral nucleophile (Scheme II). *N*-(Trimethylsilyl)-(*R*)-1-phenylethylamine (**7**) was prepared *in situ* and after filtration allowed to react with butyllithium to generate the lithium amide **7a**. Subsequent addition at low temperature to ethyl *trans*-3-pyridineacrylate gave **8** as the major product after allowing the reaction to warm to room temperature and aqueous workup. The diastereoselectivity was verified by HPLC and proton NMR and was found to be greater than 25:1 by these criteria. The debenzylation of this adduct still afforded a very low yield using $Pd(OH)_2$. However, catalytic transfer hydrogenation using ammonium formate or, better, 1,4-cyclohexadiene and palladium on carbon[11] (5%) gave the desired product in 65% isolated yield. Under these conditions, the debenzylation produced ethyl (*S*)-β-amino-3-pyridinepropionate in enantiomeric ratio of 95:5 with its *R* enantiomer[12] as evidenced by chiral chromatography.[13] The enantiomeric purity appears somewhat lower than predicted from the highly stereoselective Michael addition. We have verified that the

Scheme III[a]



11

[a] (a) DMF, pyr, cat. DMAP; HCl

diastereoselectivity of the Michael addition was indeed very high. We have independently synthesized the enantiomer **10** of the undesired diastereoisomer potentially formed during the synthesis of **8** (Scheme II). With the chromatographic and spectroscopic (^1H NMR) characteristics of **10** in hand, we have examined the composition of the crude reaction mixture and found no evidence for the epimer of **8** within the limits of detectability (1–2%). We concluded that the Michael addition of **7** occurs with high diastereoselectivity (>95%) and identified the origin of the undesired enantiomer to the presence of the undesired (*S*)-1-phenylethylamine as a small impurity in the chiral auxiliary used.[14] In addition, some scrambling may occur from hydrogen exchange at the surface of the metal during the transfer hydrogenation.

With sufficient quantities of β-amino ester of satisfactory chiral purity in hand, we pursued the synthesis of **1**. p-Aminobenzamidine was reacted with succinic anhydride in a mixture of dimethylformamide and pyridine at 100 °C for 1 h to give 4-[[4-(aminoiminomethyl)phenyl]-amino]-4-oxobutanoic acid in isolated yields exceeding 80% (Scheme III). Under these conditions, the undesirable formation of the succinimide is kept to a minimum. The hydrochloride salt **11** was used in the subsequent coupling step (Scheme IV). The best results were obtained when mixed anhydride formation was limited to 5 min at room temperature. This avoided the competitive cyclization to the succinimide. No protection was required for the amidine function when the carboxylic acid was activated by this method. Purification by RPHPLC provided **1** in 50–60% yield. The acid **2** could be easily obtained by hydrolysis of the ester with aqueous lithium hydroxide at room temperature.

This sequence illustrates the large-scale asymmetric synthesis of a novel chiral β-amino acid. The use of the lithium *N*-(trimethylsilyl)-α-methylbenzylamide (**7a**) for the nucleophilic addition shows excellent diastereoselectivity. The bulky trimethylsilyl group which facilitated removal of the chiral auxiliary preserves excellent reactivity and diastereoselectivity, similar to the *N*-benzylamine in Davies's work. We speculate that the silicon group stabilizes the intermediate enolate **8a** resulting from the anion addition[15,16] and forces an addition–elimination equilibrium toward the desired addition product. Removal of the chirality transfer agent, the *N*-α-methylbenzyl, ne-

(10) (a) Pearlman, W. M. *Tetrahedron Lett.* **1967**, 1663. (b) Bernotas, R.; Cube, R. C. *Synth. Commun.* **1990**, *20*, 1209–12. (c) Adger, B. M.; O'Farrell, C.; Lewis, N. J.; Mitchell, M. B. *Synthesis* **1987**, 53.

(11) Khan, S. A.; Sivanandaiah, K. M. *Synthesis* **1978**, 750.

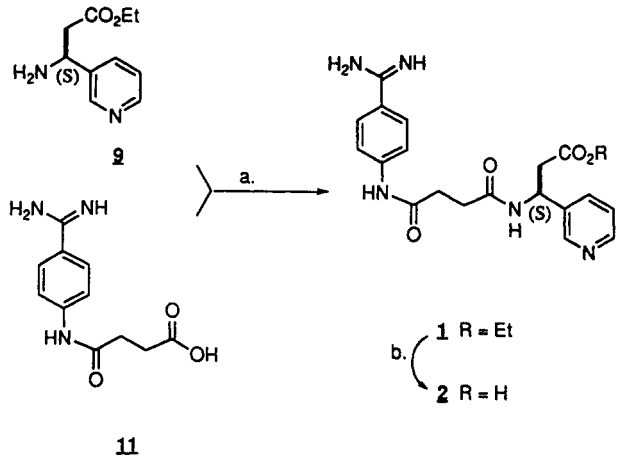
(12) The absolute configuration was assessed unambiguously by comparison with an authentic sample provided by Nutrasweet Co. (ref 6). The high biological activity of the material prepared further sustain this determination (ref 2, 3) which also fits the induction as predicted in literature (ref 7).

(13) The chiral chromatography columns used in this work were purchased from Chiral Technologies, Inc. Two types of column were used: Daicel ChiralPak WH and Crown Pak CR(+). The latter was the most advantageous. Base line resolution of the acids as well as the esters were obtained when eluted at 0 °C with 10% methanol/90% $HClO_4$ pH = 1 at a flow rate of 0.5 mL/min.

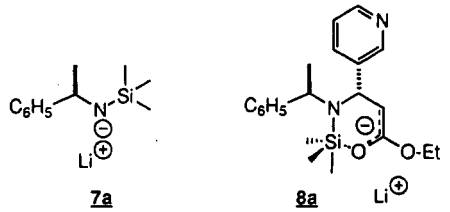
(14) Commercially available (*R*)-(+)-1-phenylethylamine from Aldrich contained up to 2–3% of the (*S*)-(−) enantiomer as observed using the chiral chromatography column Daicel Crown Pak CR(+).

(15) Lalonde, M.; Chan, T. H. *Synthesis* **1985**, 817–845.

Scheme IV[a]



[a] (a) i-Butyl chloroformate, DMF, *N*-methylmorpholine; (b) aqueous LiOH, pH 12.



cessitated a thorough investigation of various debenzylation methods. Only the hydrogen transfer conditions gave satisfactory results. The synthesis of the desired multifunctional polar compounds **1** and **2** was performed according to a convergent scheme requiring few protecting groups which allowed ready access to multigram quantities. We are currently investigating the scope of the Michael addition to acrylate derivatives using the new readily prepared nucleophilic chiral reagent **7**/**7a**.

Experimental Section

The reagents and solvents were commercially available (Aldrich Chemical Co.) and of synthetic grade. The analytical TLC plates and silica gel (230–400 mesh) were purchased from EM Reagents. Melting points were taken using a Mettler FP80/81 apparatus and are uncorrected.

^{1}H NMR spectra were routinely obtained on a Varian VXR-300 at 300 MHz in $CDCl_3$, DMSO-d_6, or CD_3OD. Mass spectra were obtained using a Finnigan MAT 90 or a VG Model 250T spectrometer with either DCI or FAB ionization. Elemental analysis for C, H, N was obtained from Galbraith Laboratories, Inc. or Searle Physical Methods. RP-HPLC column: Waters Delta Pak C, 100 Å, 15 μm, 50 mm × 30 cm. Mobile phases: A, water (0.05% TFA); B, acetonitrile (0.05% TFA); gradient 5–70% B over 30 min; flow rate 70 mL/min; detection at 215 nm.

Ethyl *trans*-3-(3-Pyridyl)acrylate (5). *trans*-3-(3-pyridyl)-acrylic acid (Aldrich, P6,620-3) was esterified with dry HCl in ethanol refluxed for 24 h. After removal of the solvent *in vacuo*, the residue was partitioned between aqueous potassium carbonate and methylene chloride. The organic phase was dried and concentrated to provide the ester as a clear yellowish oil (yield >95%) and used without further purification: ^{1}H NMR (DMSO-d_6) δ 1.34 (t, 3H, J = 7.2 Hz), 4.27 (q, 2H, J = 7.2 Hz), 6.5 (d, 1H, J = 15 Hz), 7.32 (m, 1H), 7.67 (d, 1H, 15 Hz), 7.83 (m, 1H), 8.6 (m, 1H), 8.74 (bs, 1H).

Ethyl *N*-[(*R*)-1-Phenylethyl]-(*S*)-β-amino-3-Pyridinepropanoate (8). Trimethylsilyl chloride (33.5 g, 0.33 mol) was added to (*R*)-(+)-α-methylbenzylamine (34 g, 0.28 mol) and triethylamine (40 g, 0.4 mol) in 100 mL of tetrahydrofuran. This mixture was allowed to stir for 1 h at 25 °C. The triethylamine hydrochloride was removed by filtration through a medium sintered glass funnel under a blanket of nitrogen. The resulting clear silylamine in tetrahydrofuran solution was cooled to −78 °C, and *n*-BuLi (84 mL, 0.21 mol) was added via cannula and stirred for 15 min at the same temperature. Ethyl *trans*-3-(3-pyridyl)acrylate (25 g, 0.14 mol) was added in 50 mL of tetrahydrofuran and the mixture stirred for 15 min at −78 °C before quenching with saturated ammonium chloride (100 mL). The mixture was allowed to warm to room temperature and extracted with diethyl ether. This solution was concentrated to 60 mL and then 1 N HCl was added and extracted with ether again. The ether extracts were discarded, and the acidic aqueous solution was made basic with solid K_2CO_3. The product was extracted with methylene chloride, and the extracts were dried over Na_2SO_4. The solvent was removed under reduced pressure to a red oil. The product was purified on a plug of silica gel (150 g, 30% ethyl acetate in hexane) to give 27 g (64%) of chiral amine as an amber oil: ^{1}H NMR (DMSO-d_6) δ 1.17 (t, 3H, J = 7.5 Hz), 1.35 (d, 3H, J = 7.5 Hz), 2.63 (dd, 1H, J = 6.5 Hz, 15 Hz), 2.75 (dd, 1H, J = 9 Hz, 15 Hz), 3.66 (q, 1H, J = 7.5 Hz), 4.07 (dd, 2H, J = 6.5 Hz, 9 Hz), 4.19 (m, 1H), 7.2 (m, 6H), 7.59 (m, 1H), 8.45 (m, 2H); MS (FAB) *m*/*e* 299.1 $[\alpha]^{25}_{Na}$ +7.5° (*c* = 1.05, $CHCl_3$).

Ethyl *N*-[(*R*)-1-Phenylethyl]-(*S*)-β-amino-3-pyridinepropanoate (8) and Ethyl *N*-[(*S*)-1-Phenylethyl]-(*S*)-β-amino-3-Pyridinepropanoate (10). In a Dean–Stark apparatus acetophenone (2 g, 16.7 mmol) and ethyl (*S*)-β-amino-3-pyridinepropanoate (2.5 g, 13 mmol) were added to benzene (100 mL) and heated to reflux for 3 h. After complete reaction the benzene was removed under reduced pressure, methanol (70 mL) was added followed by $NaCNBH_3$ (0.9 g, 15 mmol), and the solution was stirred for 20 h at 25 °C. The methanol was removed *in vacuo*. The resulting oil was taken up in 10% aqueous HCl and extracted with diethyl ether. The ether layer was discarded and the aqueous acid solution was made basic with K_2CO_3. The product was extracted with methylene chloride dried over Na_2SO_4, and volatiles were removed using a rotary evaporator to leave 2.1 g, 54% yield, of a 1:1 mixture of diastereomers which could be separated by RP-HPLC. Data for the diastereoisomer **10**: ^{1}H NMR (DMSO-d_6) δ 1.21 (t, 3H, J = 7.4 Hz), 1.48 (d, 3H, J = 6.7 Hz), 2.5 (dd, 1H, J = 6.5, 15 Hz), 2.63 (dd, 1H, J = 7.9, 15 Hz), 3.4 (q, 1H, J = 6.7 Hz), 4.07 (q, 2H, J = 7.4 Hz), 4.43 (dd, 1H, J = 6.5, 7.9 Hz), 7.2 (m, 6H), 7.6 (m, 1H), 8.5 (m, 1H), 8.48 (m, 1H), MS (FAB) *m*/*e* 299.2. Anal. (C, H, N).

(*S*)-Ethyl β-Amino-3-Pyridinepropanoate (9) Procedure A. The chiral amino ester from above (6 g) was added to ethanol (150 mL) followed by the addition of ammonium formate (6 g) and 10% palladium on carbon (6 g). Additional ammonium formate and/or catalyst may have to be added if reduction slows. The mixture was refluxed for 4 h. The progress of the reaction was monitored by TLC (chloroform/methanol 10:1; R_f of product ~ 0.1) or HPLC. After complete reaction the mixture was filtered through a Celite pad and the ethanol removed under reduced pressure. The product was purified by reverse-phase chromatography (RP-HPLC, gradient water/acetonitrile as specified in general methods) to result in 1.5 g (38% yield) of amino ester TFA salt.

Procedure B. A solution of the chiral amino ester (8.2 g) in 25 mL of 1,4-cyclohexadiene and 100 mL of glacial acetic acid was treated with 8 g of 5% Pd on carbon. The mixture was stirred for 4 h at 70–75 °C under nitrogen. The reaction was monitored by HPLC. After the mixture was cooled, it was filtered through a Celite pad and was concentrated *in vacuo*. The resulting clear oil was triturated with 3 × 50 mL diethyl ether which was then decanted. The oil was dissolved in 250 mL of water and 4 mL of trifluoroacetic acid and the solution purified as above. The appropriate fractions were combined to give 8.1 g (68%) of the di-TFA salt. The ratio of the enantiomer was determined to be 5.3:94.7 *R*/*S* using a CrownPak CR(+) column: ^{1}H NMR (DMSO-d_6) δ 1.08 (t, 3H, J = 7.10 Hz), 3.05 (dd, 2H,

(16) Possible d-orbital participation in the formation of σ-bonds, stabilization of reaction intermediates and transition states, and the formation of internal π-bonds have been proposed. (a) Onan, K. D.; McPhail, A. T.; Yoder, C. H.; Hillard, R. W. *J. Chem. Soc. Chem. Commun.* **1978**, 209. (b) Perozzi, E. F.; Martin, J. C. *J. Am. Chem. Soc.* **1979**, *101*, 1591.

J = 8.5, 16.5 Hz), 3.15 (dd, 2H, J = 6.3, 16.5 Hz), 4.04 (m, 2H), 4.75 (m, 1H), 7.55 (m, 1H), 8.09 (m, 1H), 8.65 (m, 2H), 8.76 (m, 1H); $[\alpha]^{25}_{Na}$ +3.3° (c = 10, DMF). The dihydrochloride salt was obtained as a solid by trituration in ether/dioxane/dry HCl(g). Anal. (C, H, N, Cl).

4-[[4-(Aminoiminomethyl)phenyl]amino]-4-oxobutanoic Acid Hydrochloride (11). Aminobenzamidine bishydrochloride (25 g, 120 mmol) was added to dry DMF (100 mL). To this solution dry pyridine (100 mL) and succinic anhydride (12 g, 120 mmol) were added, followed by dimethylaminopyridine (DMAP 1.5 g 0.012 mmol). The product precipitated after heating for 0.5 h at 100 °C. The product was filtered, washed with water, acetonitrile, and ether. The white solid was suspended in ether, 4 N HCl in dioxane (100 mL) was added, and the suspension was stirred for 1 h, filtered, and dried in a desiccator to give 28 g, 88%, of 4-[[4-(aminoiminomethyl)phenyl]amino]-4-oxobutanoic acid hydrochloride as a white yellow solid which decomposes between 270 and 290 °C: ^{1}H NMR (DMSO-d_6) δ 2.55 (m, 4H), 7.8 (s, 4H), 8.95 (bs, 2H), 9.4 (bs, 2H), 10.55 (s, 1H).

(*S*)-Ethyl β-[[4-[[4-(Aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-3-pyridinepropanoate (1). In a flask under nitrogen, 4-[[4-(aminoiminomethyl)phenyl]amino]-4-oxobutanoic acid hydrochloride (11, 5.0 g, 18 mmol) was added to dry DMF (200 mL) followed by *N*-methylmorpholine (2.0 g, 18 mmol) and isobutyl chloroformate (2.3 g, 18 mmol) at 25 °C. The mixture was stirred for 5 min. β-Amino-3-pyridinepropionic acid ethyl ester bistrifluoroacetate (9) (7.73 g) was added in a solution of 100 mL of DMF, 4 mL of *N*-methylmorpholine, and 100 mg of DMAP all at once. After stirring for 1 h at room temperature, water was added and the reaction was concentrated *in vacuo*. The residue was triturated with ether (3 × 25 mL) and then dissolved in water and the pH adjusted to 6.7–7.0 with $NaHCO_3$. A precipitate was filtered off. The filtrate's pH was adjusted to 1.0 with TFA and was purified by reverse-phase chromatography (RP-HPLC, water/acetonitrile gradient as specified in general method) to give 7.3 g of white solid (52% yield). A portion of the solid (3.5 g) was dissolved in water and the trifluoroacetate ion was exchanged for acetate using 10 equiv. of resin AG1X-8 (acetate form, Biorad). The aqueous solution resulting from the exchange was lyophilized to a white powder (2.5 g): ^{1}H NMR (DMSO-d_6) δ 1.1 (t, 3H, J = 7 Hz), 1.7 (s, 3H), 2.4 (m, 2H), 2.5 (m, 4H), 2.8 (d, 1H, J = 7 Hz), 3.95 (q, 2H, J = 7 Hz), 5.2 (m, 1H), 7.3 (m, 1H), 7.7 (m, 4H), 8.4 (dd, 1H, $J1$ = 8 Hz, $J2$ = 2.5 Hz), 8.5 (d, 1H, J = 2.5 Hz), 8.55 (d, 1H, J = 8 Hz); $[\alpha]^{25}_{Na}$ −1.3° (c = 0.5, H_2O). Anal. (C, H, N).

(*S*)-β-[[4-[[4-(Aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-3-pyridinepropanoic Acid (2). A sample (100 mg) of the ester isolated above was dissolved in water and 2 N aqueous LiOH was added to pH 12. After stirring 1 h at 25 °C, the resulting mixture was purified by reverse-phase high pressure chromatography (retention time 8 min on linear gradient 5–70% acetonitrile in water over 30 min). After lyophylization, 90 mg of a white solid were obtained: ^{1}H NMR (DMSO) δ 2.5 (m, 2H), 2.55 (m, 2H), 2.8 (m, 2H), 5.25 (m, 1H), 7.40 (m, 2H), 7.5 (bs, 1H), 7.75 (s, 4H), 7.95 (bs, 1H), 8.15 (m, 2H), 8.6 (m, 2H), 8.95 (bs, 2H), 9.15 (bs, 2H), 10.45 (s, 1H); MS (FAB) m/e 384(M + H$^+$); $[\alpha]^{25}_{Na}$ −1.14° (c = 1.0, DMSO). Anal. (C, H, N).

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