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High-performance liquid chromatographic enantiomeric separation of an enzyme inhibitor which possesses both a chiral center and tautomeric moieties

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Abstract

The enantiomeric separation using high-performance liquid chromatography (HPLC) on chiral stationary phases (CSPs) of a chiral compound which exists in solution in several tautomeric forms is described. 2,4-Dioxo-5-acetamido-6-phenylhexanoic acid is the most potent inhibitor known for peptidylamidoglycolate lyase (PGL, EC 4.3.2.5), an enzyme which plays an essential role in carboxyl-terminal amidation of many biological peptides. Synthesis of this inhibitor entails an alkaline hydrolysis step, under which condition the compound is racemized; thus, HPLC with a CSP was employed to obtain the individual enantiomers of this inhibitor. Since 2,4-dioxo-5-acetamido-6-phenylhexanoic acid exists in solution in several tautomeric forms, the strategy of first converting this compound from its multiple enol forms into a single diketo tautomer, which was then applied to various CSPs, was employed. Successful preparative scale enantiomeric separation of this compound was achieved using a Chiralpak AD CSP. Enantiomeric separation was also accomplished on a D-penicillamine column, but this CSP was found to be less satisfactory for preparative purposes.

Keywords: Enantiomer separation; Chiral stationary phases, LC; 2,4-Dioxo-5-acetamido-6-phenylhexanoic acid; Enzyme inhibitors

1. Introduction

Elucidation of stereochemistry of enzymatic reactions is a critical issue for detailed mechanistic studies and for the rational design of pseudo-substrates and inhibitors. We have established that peptidylglycine monooxygenase (PAM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PGL, EC 4.3.2.5) – enzymes which catalyze the formation of many α-amidated peptides from their glycine-extended precursors – exhibit tandem stereospecificities in carrying out the two sequential steps of carboxyl-terminal amidation (Scheme 1) [1]. In contrast, these enzymes differ dramatically in their subsite stereospecificities toward the residue at the penultimate position of their respective substrates and inhibitors [2]. Furthermore, a novel class of pyruvate-extended amino acid derivatives as highly potent inhibitors for these amidating enzymes has been developed [3].

This paper reports the separation of the enantio-

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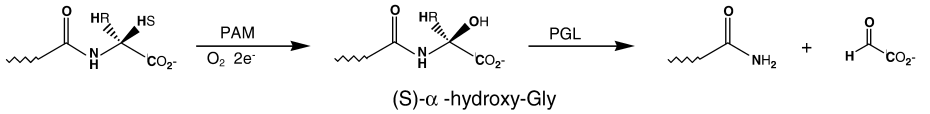

Scheme 1. PAM and PGL enzymatic reaction.

mers of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid, the most potent PGL inhibitor known, using several different types of chiral stationary phases (CSPs). As illustrated in Fig. 1, this inhibitor exists in solution as a mixture of tautomeric enol and diketo forms; under very acidic conditions, hydrate forms also exist [3]. It thus can be viewed as a model for other molecules which possess both a chiral center and a tautomeric moiety. Separation of such tautomeric chiral molecules using chiral chromatography is an issue which has not been widely addressed in the literature.

2. Experimental

2.1. Materials and equipment

All buffers, trifluoroacetic acid (TFA), L-ascorbic acid were from Aldrich (Milwaukee, WI, USA). High-performance liquid chromatography (HPLC)-grade methanol, ethanol and acetonitrile were from Burdick & Jackson (Muskegon, MI, USA). 2,4-Dioxo-5-acetamido-6-phenylhexanoic acid was synthesized as described elsewhere [3].

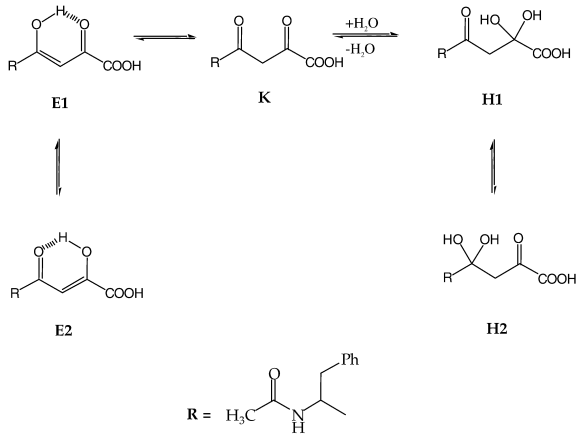

Fig. 1. Tautomerization of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid among enol (E), diketo (K), and hydrate (H). The hydrate forms are present only under very acidic conditions [3].

2.2. HPLC chiral chromatography

Chiral chromatography was performed on a Waters LC Module I Plus HPLC system that was controlled by the Millennium Chromatography Manager software package (Waters, Milford, MA, USA). The chiral columns used were: Chiralpak AD column (250 mm×4.6 mm; Chiral Technologies, Exton, PA, USA), D-penicillamine column (150 mm×4.6 mm; Phenomenex, Torrance, CA, USA), and Cyclobond I 2000 column (250 mm×4.6 mm; Astec, Whippany, NJ, USA). Appropriate guard columns were used when available. HPLC analyses were performed at room temperature with a typical flow-rate of 1.0 ml/min. Methanol–acetonitrile–aqueous buffer in various ratios was used as the mobile phase for the Cyclobond I 2000 column. Aqueous $CuSO_4$ solution–MeOH (from 85:15 to 70:30) was used as the mobile phase for the D-penicillamine column. The Chiralpak AD column was run either under normal mode using a mobile phase system of hexane–ethanol–TFA, or under polar organic mode using a mobile phase of acetonitrile–TFA. The detection wavelength was set at 300 nm. The ratio details of the eluent systems are given in the legends of Figs. 4 and 6.

2.3. Circular dichroism measurements

Circular dichroism (CD) measurements were performed on a Jasco J-720 spectropolarimeter at room temperature, using a 5-cm cylindrical quartz cell. The CD spectrum between 200 and 360 nm was measured for each sample. Resolution was set at 1 nm, bandwidth was set at 1 nm, response time was 2 s, scanning speed was 50 nm/min, and the accumulation number was 5.

2.4. Conversion from enol tautomer to diketo tautomer

Conversion of the enol tautomer of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid to the diketo tautomer was carried out by incubating 50 mg of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid in aqueous solution at 37°C under argon. The process was monitored using an analytical reversed-phase Spherisorb C_8 HPLC column (Alltech) with an LDC Constametric III system outfitted with an LDC Spectromonitor 3100 variable-wavelength detector. The eluting mobile phase used was acetonitrile–water–TFA (20:79.9:0.1). Flow-rate was 1.5 ml/min, and the detection wavelength was 300 nm. When the peak for the enol tautomer completely disappeared (usually after 5 days), the sample was lyophilized and stored at −20°C.

2.5. Enantiomer collection from chiral HPLC

Enantiomer collection was performed on appropriate analytical chiral columns. After the enantiomers were collected, 0.2 m*M* NaOAc, pH 9.0 solution was added immediately to neutralize the TFA in the mobile phase. The collected enantiomers were then concentrated by evaporation of acetonitrile under reduced pressure, followed by lyophilization to dryness. The resulting powder was reconstituted into aqueous solution before use, and the concentrations of the enantiomer solutions were determined spectrophotometrically using a Hewlett-Packard 8453 diode-array spectrophotometer.

2.6. Purification of diketo 2,4-dioxo-5-acetamido-6-phenylhexanoic acid

Diketo 2,4-dioxo-5-acetamido-6-phenylhexanoic acid was further purified using a semi-preparative C_8 column to remove any residual enol tautomer. The mobile phase used was acetonitrile–water–TFA (20:79.9:0.1); 0.2 *M* NaOAc was added immediately after the diketo tautomer was collected, and the acetonitrile was evaporated under reduced pressure. The resulting solution was lyophilized and dissolved in a minimum amount of water. The concentration of the solution was determined spectrophotometrically using a Hewlett-Packard 8453 diode-array spectrophotometer.

2.7. Inhibition kinetics of peptidylamidoglycolate lyase

Xenopus laevis PGL was obtained from an *Spodoptera frugiperda* (Sf9)/baculovirus expression system, and isolated as described previously [4]. PGL assays were performed at 37°C in 100 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.5, containing various concentrations of the substrate, trinitrophenyl-D-Tyr-Val-α-(OH)-Gly, and the inhibitor, *R*-, *S*-, or racemic 2,4-dioxo-5-acetamido-6-phenylhexanoic acid. Formation of the enzymatic product, trinitrophenyl-D-Try-Val-NH_2, was monitored by quantitative HPLC as previously described [3].

3. Results and discussion

3.1. Pyruvate-extended amino acids as PGL inhibitors

2,4-Dioxo-5-acetamido-6-phenylhexanoic acid is a novel highly potent amidation inhibitor developed in our laboratory, which was designed to resemble likely transient species along the reaction pathway of PGL catalysis [3]. Attempts to synthesize the individual enantiomers of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid were not successful because the final step of the synthesis involves an alkaline hydrolysis, wherein the chiral center is racemized. Thus, we turned to chiral chromatography in order to obtain individual enantiomers of this inhibitor.

3.2. Separation of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid

We first attempted to resolve the enol tautomer of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid using a Chiralpak AD column, which contains a stationary phase of silica gel coated with an amylose derivative. However, chiral separation was not obtained under either normal or polar organic modes (see below); a typical chromatogram is shown in Fig. 2. The enol tautomer of 2,4-dioxo-5-acetamido-6-phenylhexanoic

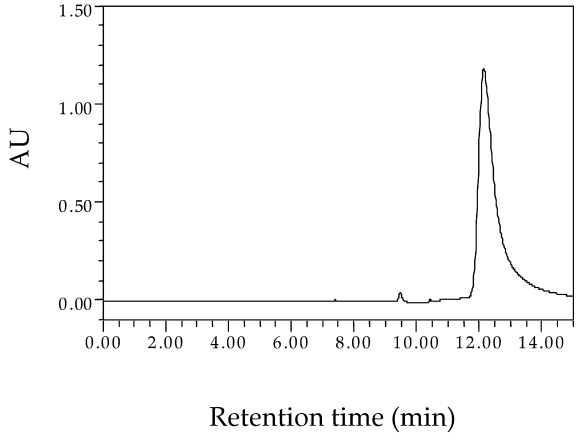

Fig. 2. Chromatogram of the enol tautomer of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid on Chiralpak AD column. Mobile phase: isopropanol–hexane–TFA (92.4:7.5:0.1); flow-rate: 1 ml/min.

acid was also applied to two other types of CSPs, a Cyclobond I 2000 column (Astec) and a D-penicillamine column (Phenomenex); however, chiral separation was not obtained in either case.

3.3. Conversion of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid to its diketo tautomer

As shown in Fig. 1, 2,4-diketo compounds exist in several tautomeric forms: enol (i.e., two possible enols, E1 and E2), diketo (K), and hydrate (i.e., two possible hydrates, H1 and H2), with the equilibrium distribution between these tautomers being dependent on pH and solvent [3,5,6]. Our nuclear magnetic resonance (NMR) and HPLC studies confirm that the hydrate appears only under acidic conditions [3]. When synthetic 2,4-dioxo-5-acetamido-6-phenylhexanoic acid is first dissolved in aqueous solution, it consists almost completely of the enol tautomer [3], and a chromatogram of this solution on a C_8 HPLC column gives a broad peak with a retention time of 9.6 min (Fig. 3A). Since our attempts to resolve the enol tautomer of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid were unsuccessful, we hypothesized that it might be less complex to resolve the enantiomers of the diketo tautomer, since the two enol forms give rise to four species in the racemic mixture (Fig. 1). Therefore, the enol tautomer was converted to the diketo tautomer by incubation under argon at 37°C for 5 days. HPLC analysis (Fig. 3B) confirmed the quantitative conversion of the 2,4-dioxo-5-acetamido-6-phenylhexanoic acid solution to the diketo form under these conditions.

3.4. Separation of diketo 2,4-dioxo-5-acetamido-6-phenylhexanoic acid on a D-penicillamine column

When the diketo form of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid was injected onto the D-penicillamine column, the chromatogram shown in Fig. 4A, with two major peaks of equal area with retention times of 46.8 min and 51.5 min, respectively, was obtained. When each of these peaks was

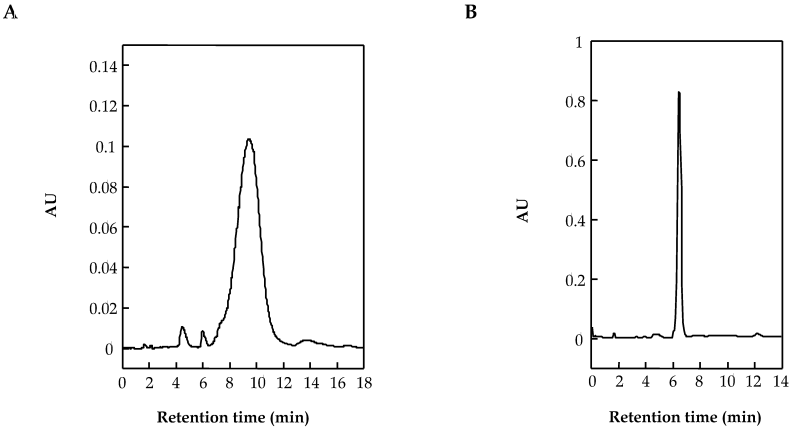

Fig. 3. Chromatograms of fresh (A) and 5-day-old (B) aqueous solution of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid on a C_8 reversed-phase column. Mobile phase: CH_3CN–water–TFA (20:79.9/0.1); flow-rate: 1.5 ml/min.

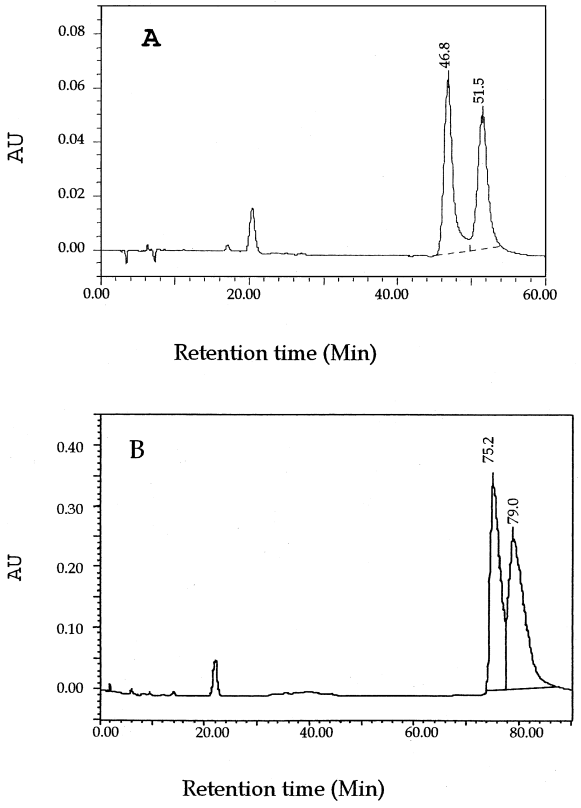

Fig. 4. Elution profile of diketo 2,4-dioxo-5-acetamido-6-phenylhexanoic acid from the D-penicillamine column. (A) On a new column. Mobile phase: 2 m*M* $CuSO_4$–methanol (75:25); flow-rate: 1 ml/min. The retention times for the two major peaks are 46.8 min and 51.5 min. (B) After multiple injections of the same compound. Mobile phase: 3 m*M* $CuSO_4$–MeOH (75:25); flow-rate: 1 ml/min. The retention times for the two peaks were 75.2 and 79.0 min.

collected and injected onto a C_8 HPLC column, each gave a single peak with a retention time of 6.5 min, corresponding to authentic diketo 2,4-dioxo-5-acetamido-6-phenylhexanoic acid. CD spectra of the two collected fractions exhibited completely opposite spectral features (Fig. 5), confirming that they are enantiomers of each other. The Cotton effects evident in the spectra indicate that the peak at 46.8 min represents the *S*-enantiomer, whereas the peak at 51.5 min represents the *R*-enantiomer [7,8].

It was noticed that both enantiomer peaks from the D-penicillamine column became increasingly broad after only a few injections, and the separation between the two peaks decreased significantly (Fig. 4B), from an *R* value of 0.97 to 0.65. Injection of the chromatographic standards, *R*- and *S*-aspartic acid, onto the D-penicillamine column also gave a much lower separation than that for a new column. Although washing the column overnight with 3 m*M* $CuSO_4$ solution restored the separation capacity, the combination of long retention times, non-baseline separation, and the relative instability of this stationary phase made it unsatisfactory in our hands for preparative purposes.

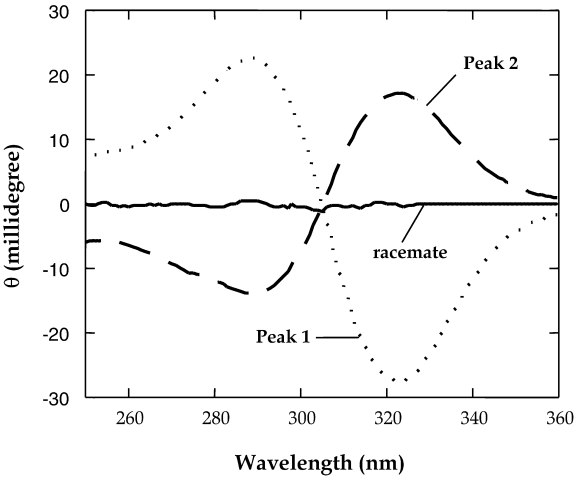

Fig. 5. CD spectra of racemic 2,4-dioxo-5-acetamido-6-phenylhexanoic acid and of the two enantiomers collected from the D-penicillamine column. The mobile phase of the D-penicillamine column (2 m*M* $CuSO_4$–MeOH, 75:25) was used as blank.

3.5. Separation of diketo 2,4-dioxo-5-acetamido-6-phenylhexanoic acid on a Chiralpak AD column

The Chiralpak AD column can be run under either normal mode, using hexane-based mobile phases containing various amounts of isopropanol or ethanol, or under polar organic mode, using acetonitrile-based mobile phases containing various amounts of isopropanol. TFA can be added to the mobile phase as a pH modifier. Because of its low solubility in both hexane and acetonitrile, 2,4-dioxo-5-acetamido-6-phenylhexanoic acid was dissolved in methanol first, followed by 1:1 dilution with the solvent of the appropriate mobile phase, before it was injected onto the Chiralpak AD column. A chromatogram with two equal-sized peaks was obtained under both

modes. However, when the column was used in the normal-phase mode, reinjection of the collected fractions on the Chiralpak AD column gave chromatograms with multiple peaks for each fraction, indicating decomposition. We therefore turned to the polar organic mode, and baseline separation of diketo 2,4-dioxo-5-acetamido-6-phenylhexanoic acid was successfully obtained with an *R* value of 1.8 using a mobile phase of acetonitrile containing 0.1% TFA (Fig. 6A). The retention times for the two peaks were 9.4 and 11.2 min, respectively. When each peak was collected and reinjected on the Chiralpak AD column, a major peak with the same retention time as before was obtained in each case (Fig. 6B and C). When each of these peaks was collected and then injected onto a C_8 HPLC column, each gave a single peak with a retention time of 6.5 min, corresponding to authentic diketo 2,4-dioxo-5-acetamido-6-phenylhexanoic acid. CD spectra similar to those shown in Fig. 5 were obtained, confirming that they were enantiomers of each other. Based on their spectral features, the peak eluting at 9.4 min was assigned to the *S*-enantiomer, and the peak eluting at 11.2 min was assigned to the *R*-enantiomer [7,8].

Overall, the polar organic eluent gave separation with the shortest retention time, highest resolution, and least tailing. We have also been able to use this solvent system to separate the enantiomers of a series of α-hydroxyglycine derivatives [9]. The chromatographic advantages of operating Chiralpak AD column under the polar organic elution system has also been demonstrated previously with other analytes [10].

3.6. Inhibition of PGL by the individual enantiomers of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid

Using the enantiomeric separation described above, milligram quantities of the individual enantiomers of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid were collected. Kinetic inhibition analysis was then carried out using *R*-, *S*- and racemic 2,4-dioxo-5-acetamido-6-phenylhexanoic acid, respectively. In all cases, pure competitive inhibition kinetics were obtained with respect to the substrate TNP-D-Tyr-Val-α-(OH)-Gly. K_I values of 2.2±0.2 μ*M*, 1.9±0.2 μ*M* and 1.7±0.2 μ*M* were obtained for the *S*-enantiomer, the *R*-enantiomer, and the racemate of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid, respectively. These results confirm our expectation that the binding site of PGL is able to accommodate both *R*- and *S*-residues at the penultimate position of

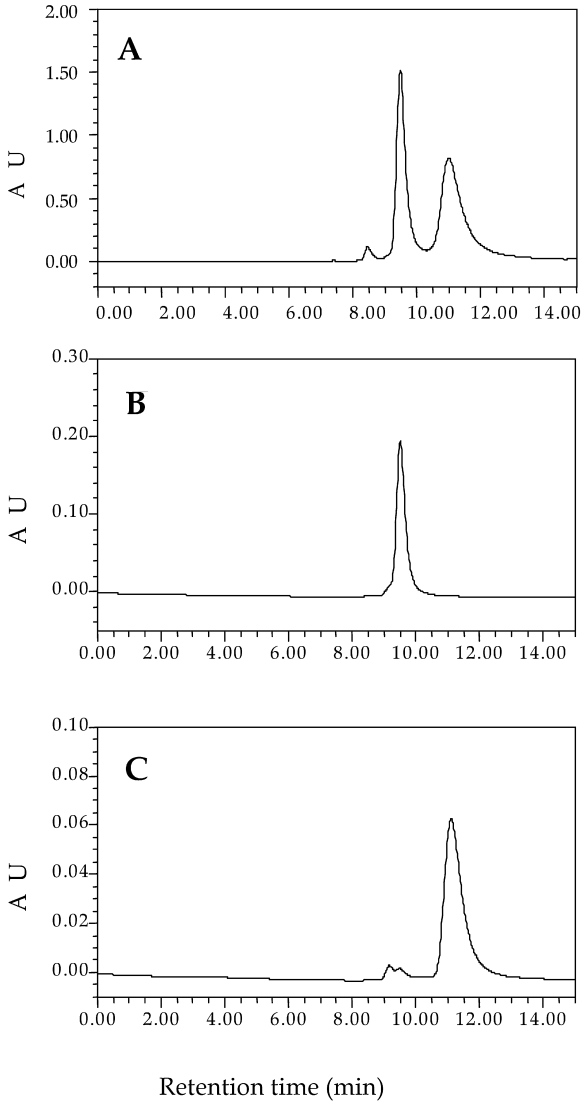

Fig. 6. Resolution of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid on a Chiralpak AD column. (A) Elution profile of diketo 2,4-dioxo-5-acetamido-6-phenylhexanoic acid on Chiralpak AD column under polar organic mode. The retention times for the two peaks are 9.5 min and 11.0 min. (B) and (C) are the chromatograms of the two fractions (shown in A) collected from the Chiralpak AD column. Mobile phase: acetonitrile–TFA (99.9:0.1); flow-rate: 1 ml/min.

competitive inhibitors, as is the case for PGL substrates [2,3].

4. Conclusions

Using a Chiralpak AD HPLC column, we have successfully separated the enantiomers of 2,4-dioxo-5-acetamido-6-phenylhexanoic acid, a chiral compound which exists in solution in several tautomeric forms. Our strategy entailed first converting this compound from its multiple enol forms into a single diketo tautomer, which was then successfully resolved using HPLC on a CSP. Enantiomeric separation was also accomplished on a D-penicillamine column, but this CSP was found to be less satisfactory.

Acknowledgements

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