Identification and Enantiomeric Separation of (+)- and (-)-(*E*)-3-[5-(2,4-Diaminopyrimidin-5ylmethyl)-2,3-dimethoxyphenyl]-1(1-propyl-1*H*-phthalazin-2-yl)propenone by Supercritical Fluid Chromatography

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The objective of this study was to develop technology and validate a method for the identification and separation of enantiomers of (±)-(*E*)-3-[5-(2,4-diaminopyrimidin-5-ylmethyl)-2,3-dimethoxyphenyl]-1-(1-propyl-1*H*-phthalazin-2-yl)propenone. Separation of enantiomers was achieved with a CHIRALPAK® ADH® 4.6 x 100 mm column using a Minigram Supercritical Fluid Chromatography (SFC) unit. Enantiomeric excesses were 99.2% and 99.4% for the (*S*)-isomer and (*R*)-isomer, respectively. This is an important separation since it has recently been shown that the (*S*)-isomer forms an inhibitory complex with dihydrofolate reductase [DHFR] in *Bacillus anthracis* which is a bacteria causing anthrax.

INTRODUCTION

Anthrax is a well known pathogen (Jones SD, Teigen PM. 2008) clearly recognized as a major threat to security around the world and a possible weapon for bioterrorism (Inglesby TV. and others. 1999; Inglesby TV. and others. 2002). The enzyme dihydrofolate reductase [DHFR] present in *Bacillus anthracis* is a viable drug target but it is resistant to commercially available anti-bacterials (Barrow EW. and others, 2007; Barrow EW. and others, 2004). Discovery of the strong inhibition of the activity of this enzyme when treated with (±)-*E*)-3-[5-(2,4-diaminopyrimidin-5-ylmethyl)-2,3-dimethoxyphenyl]-1-(1-propyl-1*H*-phthalazin-2-yl) propenone (1) resulted in the identification in the enzyme-substrate complex of the (S)-isomer which was preferentially extracted b the enzyme from the racemic mixture (Bourne CR. and others. 2009). Insights into the strong inhibition of activity of DHFR when treated with 1 were obtained from an X-ray crystal structure in which the (S)-isomer-DHFR complex crystallized from the racemic mixture (Bourne CR. and others. 2009) of racemic 1. The potential anthrax threat and the significant value of the inhibitory data prompted our investigation into the chemistry of 1. Consequently, this important finding necessitated determining the parameters for the identification, separation, and purification of the enantiomers. Although 1 and the two enantiomers have been recorded (Guerrp P. and others, 1998), structural details were lacking and no activity against DHFR was noted. In the present study, the identification and separation of the individual enantiomers from racemic 1 have been accomplished (See Figure 1).

EXPERIMENTAL

Chemicals-Racemic 1 was prepared as described previously(Bourne, CR. and others. 2009.

Instrumentation-A Minigram SFC unit was employed with a packed CHIRALPAK® ADH 4.6 x 100 mm column. The

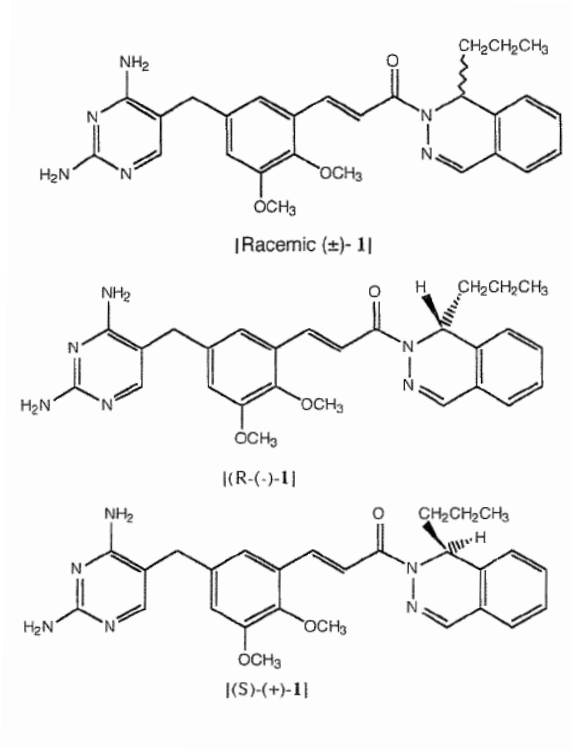

Figure 1. The structures of recemic (±)-1, (R)-(-)-1, and (S)-(+)-1.

particle size was 5 microns (pore diameter is proprietary), and the temperature of the Daicel column was 35° C. The sample was dissolved in ethanol and 0.2% diethylamine (DEA), and the solution was kept warm during the experiment. The mobile phase was 40% ethanol in CO_2 plus 0.2% DEA. All solutions of racemic 1 were maintained in a slightly warm condition for the experiments. Injections were made by an autosampler with the volume of injection being 45 microliters. A concentration of 15 mg/mL was employed. The flow rate was 10 mL/minute at 100 bar. No temperature program was necessary to initiate the separations. Values for kl' and k2' were 15.3 and 27.8, respectively, as determined from the retention of non-retained 1,3,5-tri-*tert*-butylbenzene. The SFC unti was manufactured by Berger Company, 130 Executive Drive, Suite 2A, Newark, DE. However, the company was purchased by Thar Instruments, 575 Epsilon Drive,Pittsburgh, PA 15238. The UV-VIS dectector was a Knauer, model K-2501 [Knauer, Wisseenschaftliche Geratebau, Attn: Dr. Ing. Herbert Kneur BmbH., Hegauer Weg 38, 14163 Berlin, Germany] with a range of 190-740 nm. Chiral Techonologies, Inc., 800 North Five Points Road, West Chester, PA 19380, provided the Minigram SFC unit to effect the separation. All rotational data on the enantiomers in solution were obtained by direct digital readout on an Autopol V polarimeter, manufactured by Rudolph Research Analytical, Hackettstown, NJ. The light source was a sodium lamp (589 nm), and the sample tube was 100 mm in length. The melting points of the isomers were taken on samples placed in capillary tubes using

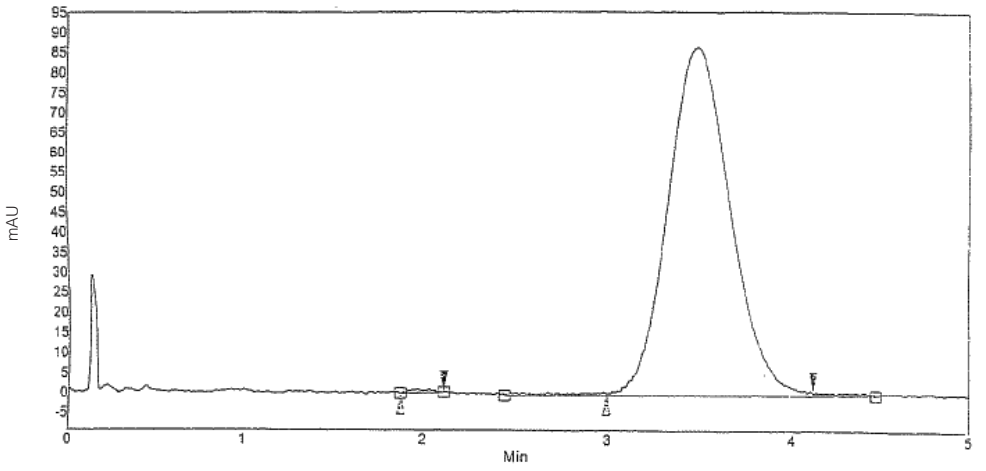

Figure 2. Chromatogram for (R)-(-)-1.

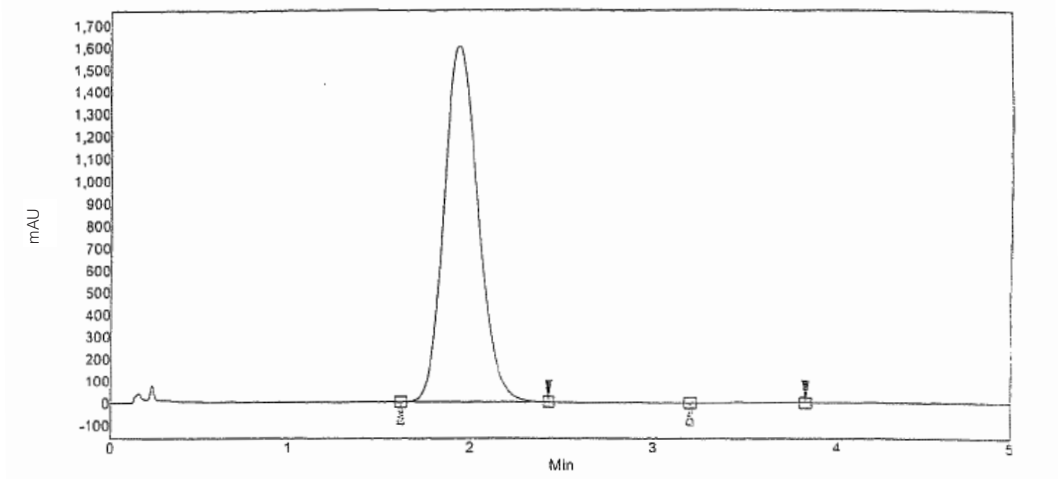

Figure 3. Chromatogram for (S)-(+)-1.

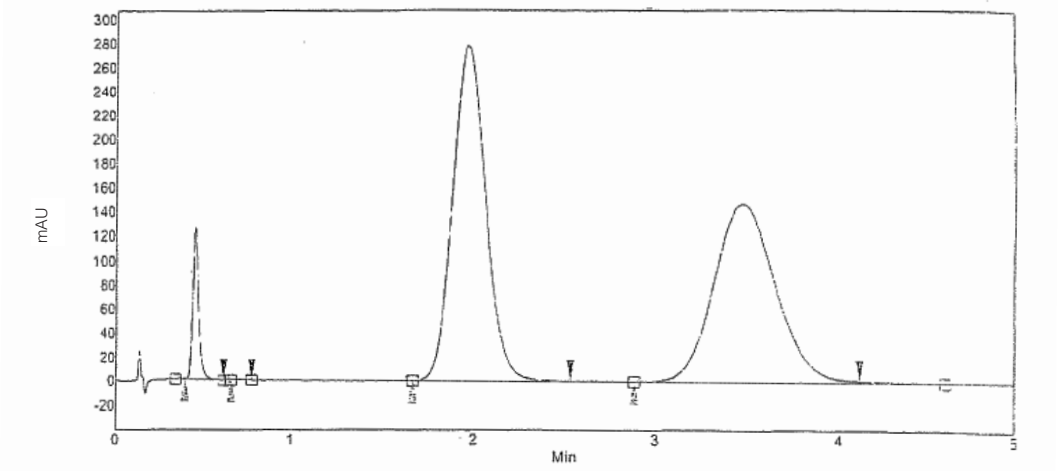

Figure 4. Chromatogram for the original mixture of enaniomers.

a Melt-Temp melting point apparatus and were uncorrected. NMR data were collected on a Varian Gemini 300 MHz [300.082 MHz for H-1] and on a Varian INOVA 600 MHz unit [598.724 MHz for H-1]. Chemical shifts for H-1 were recorded on solutions of racemic 1 and for (S)-(+) in $DCCI_3$. A drop of a saturated solution of racemic 1 in $DCCI_3$ was placed on a salt plate, and IR data were taken on a thin film resulting from evaportion of the $DCCI_3$ using a Perkin-Elmer 2000 FTIR unit.

RESULTS

The best results from the HPLC unit described above to separate (+)-1 and (-)-1 required the eluent to be 40% ethanol and 0.2% DEA / 60% CO_2 at 35° C with UV detection at 220 nm. A 51-mg sample was resolved and provided 20 mg of the (S)-isomer and 24 mg of the (R)-isomer. Enantiomeric excesses were 99.2% and 99.4% with elution times of 1.94 minutes and 3.94 minutes obtained for the (S)-isomer and (R)-isomer, respectively. The chromatograms are found in Figures 2, 3, and 4.

At the D line of sodium, the molecular rotations obtained for (*S*)-(+)-1 and (*R*)-(-)-1 were $[a]^{20}$ +775 degrees (c = 0.12, methanol) and $[a]^{20}$ -854 degrees (c = 0.14, methanol), respectively. The concentrations were in

g/100 mL of solvent. To confirm the structures of racemic 1 and the enantiomers, the following analyses were performed. The melting points for our samples of (S)-(+)-1 and (S)-(-)-1 were 121-123° C (shrank to a glass-like bead) and 125-128° C (shrank to a glass-like bead), respectively. No melting points were previously reported for these enantiomers. IR peaks of racemic 1 appeared at 3473, 3341, and 3182 (NH_2), 1645 (C=O), and 1608 (C=C) cm^{-1}. The proton NMR analysis of (S)-(+)-1 in $DCCI_3$ possessed a large number of signals as anticipated. Chemical shifts occurred at: δ 0.87 (t, 3H, J = 7.1), 1.27 (m, 2H), 1.64 (m, 2H), 3.68 (s, 2H), 3.80 (s, 3H), 3.84 (s, 3H), 4.66 (brs, 2H), 4.84 (brs, 2H), 5.91 (t, 1H, J = 6.8), 6.66 (d, 1H, J = 1.9), 7.13 (d, 1H, J = 1.9), 7.18 (dd, 1H, J = 7.3, 0.4), 7.28 (dd, 1H, J = 7.5, 1.5), 7.36 (td, 1H, J = 7.5, 1.3), 7.45 (td, 1H, J = 7.3, 1.5), 7.64 (d, 1H, J = 16.1), 7.66 (s, 1H), 7.80 (s, 1H), 8.07 (d, 1H, J = 16.1). Of course, the H-1 NMR spectrum of (R)-(-)-1 is identical to that of (S)-(+)-1. Thus, all spectral data support the structures of racemic 1 and (S)-(+)-1.

DISCUSSION AND CONCLUSIONS

In summary, we have found a useful and powerful method for the resolution of (±)-1 using supercritial fluid chromatography (SFC) to separate the individual enantiomers. To the best of our knowledge, this is the first such approach employed for separation of enantiomers from the racemic target system (±)-1 of which the (S)-(+)-1 enantiomer has been shown to complex with DHFR present in *Bacillus anthrax* (Bourne, CR. and others. 2009). The spectral information and melting point data, coupled with the X-ray analysis (Bourne, CR. and others. 2009) of (S)-(+)-1, have now firmly established these molecular systems. The high purity of the enantiomers resulting from the separation enables these isomers to serve as models for reference with respect to related systems. This application will be valuable for the separation and identification of the other members of this family of important chiral heterocycles possessing useful biological activity.

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