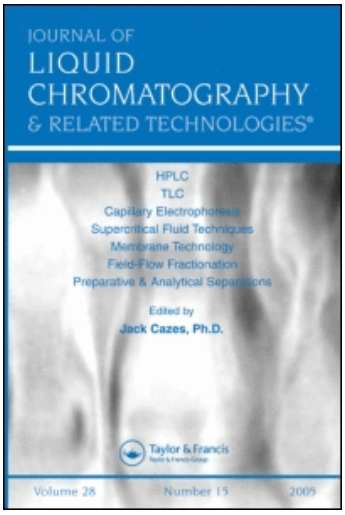

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ENANTIOSELECTIVITY OF AZALANSTAT AND ITS KETAL TOSYLATE INTERMEDIATE IN CHIRAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATIONS

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ABSTRACT

Separation of the stereoisomers of azalanstat (RS-21607-197), a substituted imidazolyl-1,3-dioxolane which acts as a potent inhibitor of lanosterol 14α-demethylase in cholesterol biosynthesis, and its ketal tosylate intermediate was achieved by chiral high performance liquid chromatography. Resolution of all four stereoisomers of azalanstat was accomplished by normal phase separation of the diastereomers on a short silica gel column coupled on-line to a Chiralpak AS amylose carbamate phase chiral column for resolution of the enantiomers.

A reversal in elution order of the *cis*-(2S,4S) and *cis*-(2R,4R) enantiomers of the ketal tosylate intermediate of azalanstat was exhibited by an Ultron ES-OVM ovomucoid protein bonded-

phase column with a change in organic modifier from ethanol to acetonitrile. This unusual enantioselectivity in the chiral reverse phase separation of the ketal tosylate was attributed to a putative change in binding domains or recognition sites on the ovomucoid protein as a function of the organic modifier of the mobile phase. Resolution of all four stereoisomers of the ketal tosylate was achieved by use of a Chiralpak AD amylose carbamate phase column in the normal-phase mode.

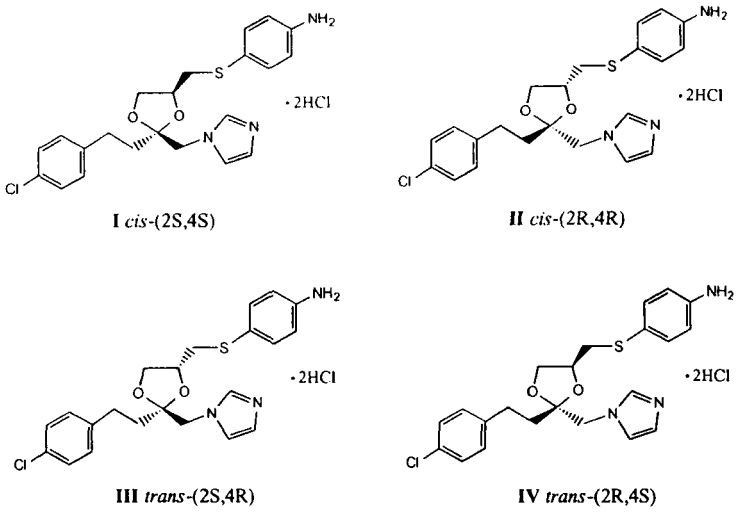
INTRODUCTION

Lanosterol 14α-demethylase is a cytochrome P_{450}-dependent enzyme that catalyzes the first step in conversion of lanosterol to cholesterol in mammals.[1] Selective inhibition of this enzyme represents a possible strategy for cholesterol lowering. Azalanstat (Figure 1) has been shown to be selective inhibitor of mammalian lanosterol 14α-demethylase.

The *cis*-(2S,4S) enantiomer of azalanstat is approximately two orders of magnitude more effective than the *cis*-(2R,4R) enantiomer for inhibition of cellular cholesterol biosynthesis using human fibroblasts in tissue cultures.[2]

Synthesis of azalanstat (RS-21607-197) involves the trans-ketalization of 1-(imidazol-1-yl)-4-(4-chlorophenyl)-butan-2-one with (S)-solketal tosylate to produce the *cis*-(2S,4S) and *trans*-(2R,4S) ketal tosylate intermediates of azalanstat (Figure 2). Use of (R)-solketal tosylate yields the corresponding *cis*-(2R,4R) and *trans*-(2S,4R) ketal tosylate intermediates. Coupling of the ketal tosylate intermediates with 4-aminothiophenol gives (2S, 4S)-4-[(4-aminophenylthio)-methyl]-2-[2-(4-chlorophenyl)-ethyl]-2-[(1H-imidazol-1-yl)-methyl]-1,3-dioxolane (azalanstat) and its corresponding stereoisomers. High performance liquid chromatography (HPLC) using chiral bonded phase columns was utilized to establish controls for the stereochemical purity of the ketal tosylate intermediates and of azalanstat during synthesis.

The purpose of this study was to elucidate the enantioselectivity of azalanstat and its ketal tosylate intermediates in chiral HPLC separations. As a biologically active azole, azalanstat shares several structural features (ketal functionality, imidazole heterocycle, substituted phenyl ring) with many azole antifungal agents which inhibit the biosynthesis of ergosterol from squalene.[3] The enantioselectivity observed for azalanstat, therefore, may provide insight on stereoisomer separations of other chiral azoles and of related compounds with similar structural characteristics.



I *cis*-(2S,4S) II *cis*-(2R,4R)

III *trans*-(2S,4R) IV *trans*-(2R,4S)

Figure 1. Structure of azalanstat (**I**) and its stereoisomers: **I** *cis*-(2S,4S) stereoisomer; **II** *cis*-(2R,4R) stereoisomer; **III** *trans*-(2S,4R) stereoisomer; **IV** *trans*-(2R,4S) stereoisomer. The *cis* and *trans* designations of the stereoisomers indicate the relative configuration of the aminophenylthio)methyl substituent at C_4 and the (imidazolyl)-methyl substituent at C2 in the 1,3-dioxolane ring.

Resolution of the enantiomers of azalanstat and its ketal tosylate intermediates was accomplished using an Ultron ES-OVM ovomucoid protein bonded-phase column and a Chiralcel OD-R cellulose carbamate phase column in the reversed-phase mode. Normal phase separation of the enantiomers of azalanstat and its ketal tosylate intermediates was achieved by employing a Chiralpak AS and a Chiralpak AD amylose carbamate phase column, respectively. Other chiral columns were also employed in this study in an effort to detail the enantioselectivity exhibited for azalanstat and its ketal tosylate intermediates in chiral HPLC separations.

MATERIALS

A Varian Series 9010 solvent delivery system, a Polychrom 9065 diode-array detector with the LC Star 9021 workstation, Polyview, and 3DView chromatographic and spectral processing software, and a 9095 autosampler were employed for chromatographic separations and data collection. A Varian

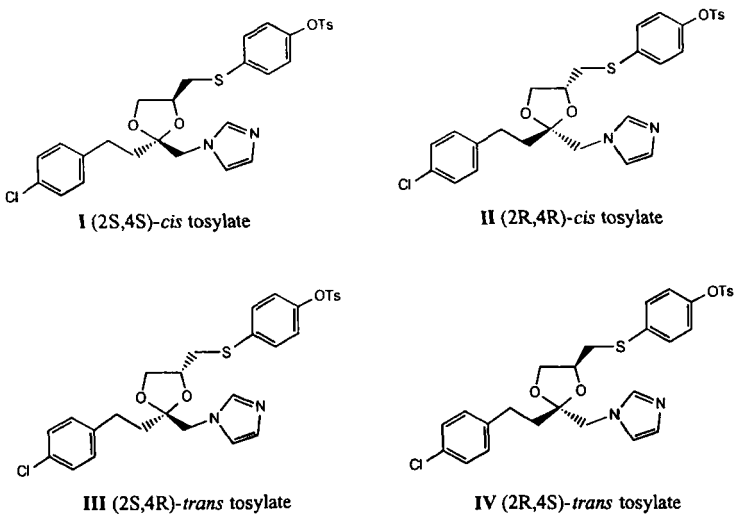


Figure 2. Structures of the ketal tosylates of azalanstat and their stereoisomers: **I** (2S,4S)-*cis* tosylate; **II** (2R,4R)-*cis* tosylate; **III** (2S,4R)-*trans* tosylate; **IV** (2R,4S)-*trans* tosylate.

Model 5000 HPLC system with a Kratos Model 783 UV absorbance detector and a Spectra-Physics Chromjet integrator were also used in this work. A Waters Mod-1 HPLC system was also employed for some separations. A Shimadzu CT-6A HPLC column oven was used for control of column temperature where required. A Rheodyne automated fixed loop injector equipped with a 20μL sample loop was used in these studies.

A Zorbax Rx C8 (Mac-Mod Analytical) 5μm column (250mm x 4.6mm i.d.) was employed for reverse phase separation of the diastereomers of azalanstat and its ketal tosylate intermediate. Normal phase separations of the diastereomers of azalanstat were carried out with a Supelcosil LC-Si (Supelco Inc.) 3μm column (33mm x 4.6mm i.d.).

Reversed-phase chiral HPLC separations were carried out with an Ultron ES-OVM (Mac-Mod Analytical) ovomucoid protein bonded-phase column (5μm, 150mm x 4.6mm i.d.) and with a Chiralcel OD-R (Chiral Technologies Inc.) cellulose tris-(3,5-dimethylphenyl carbamate) phase column (10μm, 250mm x 4.6mm i.d.). Normal phase chiral separations utilized Chiralcel OD cellulose tris-(3,5-dimethylphenyl carbamate) phase and Chiralcel OJ cellulose

tris-(4-methyl benzoate) phase columns as well as Chiralpak AD amylose tris-(3,5-dimethylphenyl carbamate) phase and Chiralpak AS amylose tris-[(S)-1-phenylethyl carbamate] phase columns (Chiral Technologies Inc., 10μm, 250mm x 4.6mm i.d.).

In addition, a Pirkle-type Chirex 3019 phase (Phenomonex, Inc.) 5μm column (250mm x 4.6mm i.d.) with a bonded phase consisting of a (S)-tert-butyl-leucine moiety coupled to (S)-1-(α-naphthyl)ethylamine via a urea linkage was employed for normal phase separation of the ketal tosylate stereoisomers.

B & J Brand (Baxter Healthcare, Burdick and Jackson Division) high-purity acetonitrile, methanol, 1,2-dichloroethane, and 2-propanol as well as HPLC-grade water from a Milli-Q water purification system (Millipore Corp.) and punctillious grade ethanol (Quantum Chemical Co.) were employed for chromatographic studies. Potassium dihydrogen phosphate and sodium perchlorate, reagent grade, were obtained from J. T. Baker Company. Heptane sulfonic acid was purchased from Eastman-Kodak Chemical Company.

Azalanstat (RS-21607), its stereoisomers, its ketal tosylate intermediates, and related compounds were synthesized by previously reported procedures (see ref. 2).

METHODS

All chromatographic analyses were carried out in a similar manner. Azalanstat and its ketal tosylate intermediates were dissolved in a minimal volume of either isopropanol, methanol or acetonitrile and diluted to volume with mobile phase for HPLC separations. Solutions of approximately 0.05mg/mL of the analyte were freshly prepared just prior to analysis. Unless otherwise stated, a detection wavelength of 220nm was employed in these studies.

A Zorbax Rx C8 column with a 0.02M phosphate buffer/0.01M heptane sulfonic acid-methanol (41:59) mobile phase at a flow rate of 1.0mL/min was employed for reverse phase HPLC separations of the diastereomers of the ketal tosylate intermediate with a detection wavelength of 220nm. For analysis of azalanstat diastereomers, a Zorbax Rx C8 column was employed with a 0.05M phosphate buffer (pH 3.0)-methanol (45:55) mobile phase at a flow rate of 1.5mL/minute. Normal phase separations of the diastereomers of azalanstat were carried out with a Supelcosil LC-Si column using a hexane/2-propanol (78:32) mobile phase at a flow rate of 1mL/minute.

Chiral HPLC separations of azalanstat and its ketal tosylate intermediate were carried out with several reversed-phase and normal-phase chiral columns. An Ultron ES-OVM ovomucoid protein column with a 0.02M phosphate buffer (pH 5.5)-ethanol (73:27 for *cis* stereoisomers and 67:33 for *trans* stereoisomers) mobile phase at a flow rate of about 0.5 mL/minute and column temperature of 35 °C was used for reverse phase chiral analysis of the ketal tosylate intermediates.

An Ultron ES-OVM ovomucoid protein chiral column with a 0.02M phosphate buffer (pH 4.6)-ethanol (78:22) mobile phase at a flow rate of about 0.8mL/minute and column temperature of 35°C was used for reverse phase chiral analysis of azalanstat.

A Chiralcel OD-R cellulose carbamate phase column with a 0.5M perchlorate buffer-acetonitrile (40:60) mobile phase at a flow rate of 1mL/minute and a column temperature of 35 °C was employed for reverse phase chiral HPLC separations of the ketal tosylate intermediates. The Chiralcel OD-R column was utilized with a 0.5M perchlorate buffer-methanol (5:95) mobile phase at a flow rate of 1 mL/minute for separation of the enantiomers of azalanstat.

Chiralpak AD and AS amylose carbamate phase columns as well as a Chiralcel OD cellulose carbamate phase column and a Chiralcel OJ cellulose ester phase column were utilized for normal phase chiral separations of azalanstat and the ketal tosylate intermediates.

A 2-propanol/hexane mobile phase (20:80) at a flow rate of 1 mL/minute was employed separation of the enantiomers of the ketal tosylate intermediates using the Chiralpak AD column. A 2-propanol-hexane mobile phase (32:68) at a flow rate of 1 mL/minute was employed for separation of the enantiomers of azalanstat using a 3.3cm LC-Si silica gel column coupled on-line to the Chiralpak AS column.

Various mixtures of 2-propanol-hexane and ethanol-hexane were utilized in methods development work with the Chiralcel OD and OJ cellulose based columns and with the Chiralpak AD and AS amylose based columns for separation of the stereoisomers of azalanstat and its ketal tosylate intermediates. In addition, a Chirex 3019 phase column employing a (S)-tert-leucine with a urea linkage to (S)-1-(α-naphthyl)ethylamine as an electron-donor bonded phase with an ethanol-dichloroethane-hexane (10:2:1) mobile phase at a flow rate of 1.0mL/min was utilized for normal phase separation of the ketal tosylate enantiomers.

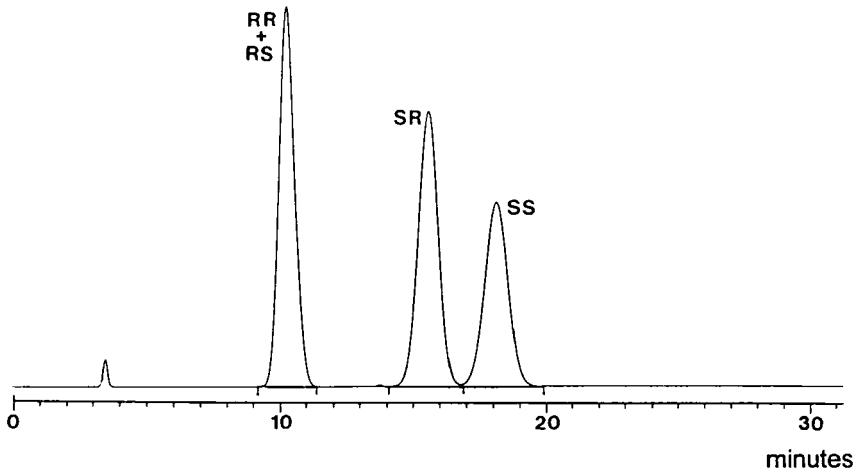


Figure 3. Chiral HPLC separation of the stereoisomers of azalanstat: **SS** *cis*-(2S,4S) stereoisomer (azalanstat); **RR** *cis*-(2R,4R) stereoisomer; **SR** *trans*-(2S,4R) stereoisomer; **RS** *trans*-(2R,4S) stereoisomer. Conditions: Ultron ES-OVM column, 78% 20mM KH_2PO_4 (pH 4.6)/22% EtOH, 0.8mL/min, 35°C, 220nm.

RESULTS AND DISCUSSION

Initial studies of the separation of the stereoisomers of azalanstat and its ketal tosylate intermediates employed an Ultron ES-OVM ovomucoid protein bonded-phase column. Although the *cis*-(2R,4R)/*cis*-(2S,4S) and *trans*-(2S,4R)/*trans*-(2R,4S) enantiomeric pairs could be separately resolved using different ethanol-buffer mobile phases for both compounds, one of the *cis*-stereoisomers and one of the *trans* stereoisomers coeluted on the ovomucoid protein chiral column. Off-line reverse phase HPLC separation of the *cis* and *trans* diastereomers using a C8 column, together with resolution of enantiomers on the ovomucoid bonded-phase column, afforded control of the stereochemical purity of azalanstat during synthesis.

Investigation of chiral separations of azalanstat, its ketal tosylate intermediates, and related compounds was initiated in order to examine the enantioselectivity of several HPLC chiral columns in detail.

Chiral HPLC Separations of the Stereoisomers of Azalanstat

An Ultron ES-OVM ovomucoid protein column with a 0.02M phosphate buffer (pH 4.6)-ethanol mobile phase (78:22 for *cis* stereoisomers and 70:30 for *trans* stereoisomers) at a flow rate of 0.5 mL/minute and column temperature of 35 °C was used for reversed-phase chiral analysis of azalanstat. Figure 3 displays the separation of the stereoisomers of azalanstat using an Ultron ES-OVM chiral column with a 0.02M phosphate buffer (pH 4.6)-ethanol (78:22) mobile phase at a flow rate of 0.8mL/minute and column temperature of 35 °C. As can be seen, under these conditions the *cis*-(2R,4R) stereoisomer and the *trans*-(2R,4S) stereoisomer of azalanstat coelute.

Results of chiral separations of several analogs of azalanstat have shown that N-methylation of the imidazole ring along with attachment of the imidazole side chain at the 5-position increases the stereoselectivity obtained with the Ultron ES-OVM chiral column relative to azalanstat. For example, resolution of all four stereoisomers of the N-methylated imidazole analog of azalanstat was achieved employing a mobile phase identical to that used for the parent compound (Figure 4).

In contrast, the des-amino analog of azalanstat, which differs in structure from the parent compound only by the absence of the amino substituent of the thiophenol ring, yields stereoselectivity on the ovomucoid column very similar to that of the parent compound. Based on these results, it appears that neither hydrogen bonding nor protonation of the amino substituent of the aniline group (pK_a=4.1) is critical to the stereoselectivity observed for azalanstat with the Ultron® ES-OVM ovomucoid bonded-phase column. N-methylation of the imidazole ring of azalanstat apparently enhances the stereoselectivity of the column for these isomers.

A Chiralcel OD-R reversed-phase cellulose carbamate based column was also employed for separation of the stereoisomers of azalanstat. A mobile phase of methanol-0.5M sodium perchlorate buffer (95:5) was found to yield separation of the *cis* enantiomers (α=1.5) and of the *trans* enantiomers (α=1.6). However, the *cis*-(2S,4S) stereoisomer coeluted with the *trans*-(2S,4R) stereoisomer under these conditions.

Several normal-phase chiral HPLC columns were investigated for separation of the stereoisomers of azalanstat. Chiralcel OD and OJ functionalized cellulose phase columns, as well as Chiralpak AD and AS functionalized amylose phase columns, were examined. The Chiralcel OD and OJ cellulose phase columns were found to exhibit a higher degree of retention, but lower stereoselectivity, for the isomers of azalanstat compared to the Chiralpak AD

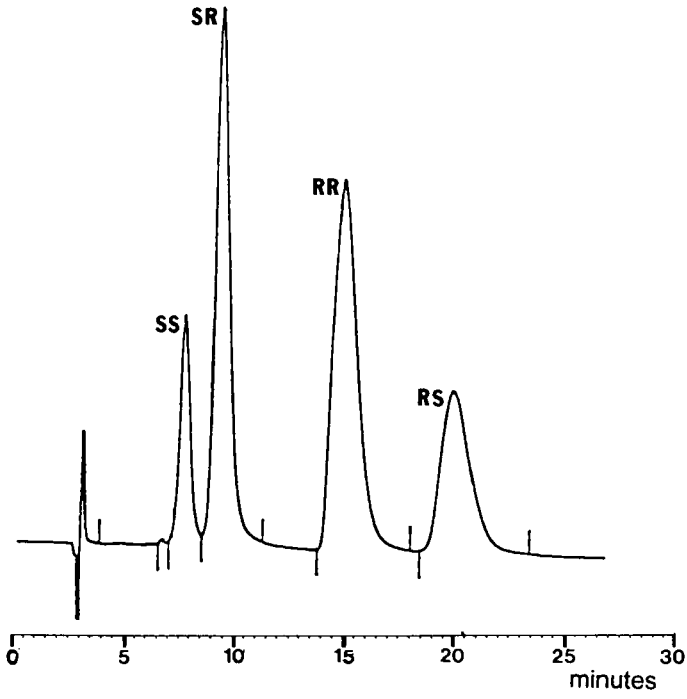


Figure 4. Chiral HPLC separation of the stereoisomers of the N-methylated imidazole analog of azalanstat: **SS** *cis*-(2S,4S) stereoisomer; **RR** *cis*-(2R,4R) stereoisomer; **SR** *trans*-(2S,4R) stereoisomer; **RS** *trans*-(2R,4S) stereoisomer. Conditions: Ultron ES-OVM column, 78% 20mM KH_2PO_4 (pH 4.6)/22% EtOH, 0.8mL/min, 35°C, 220nm.

and AS amylose phase columns. Konishi, et al.[4] also observed a high degree of retention ($k' \geq 20$) for sulconazole, a related imidazole, with the Chiralcel OD and OJ columns.

Although the Chiralpak AD amylose carbamate phase column provided high enantioselectivity (α=3.5) for the *trans* enantiomers of azalanstat, coelution was observed for the *cis* enantiomers. Optimum overall selectivity for the *cis* and *trans* enantiomers of azalanstat was displayed by the Chiralpak AS amylose carbamate phase column using a 2-propanol - hexane mobile phase (32:68) at a flow rate of 1 mL/minute (α=1.7 and 1.2, for the *cis* and the *trans*

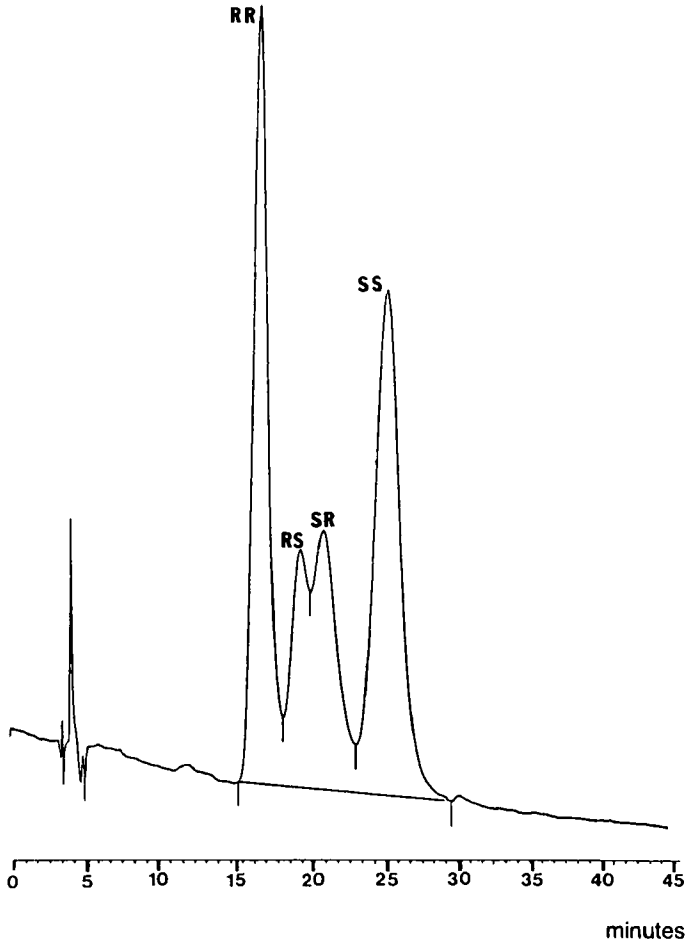


Figure 5. Coupled column HPLC separation of the stereoisomers of azalanstat: **SS** *cis*-(2S,4S) stereoisomer (azalanstat); **RR** *cis*-(2R,4R) stereoisomer; **SR** *trans*-(2S,4R) stereoisomer; **RS** *trans*-(2R,4S) stereoisomer. Conditions: Supelcosil LC-Si column (3.3cm x 4.6mm i.d.) coupled on-line with a Chiralpak AS column (25cm x 4.6mm i.d.), 2-propanol-hexane (32:68), 1mL/min, 220nm.

enantiomers, respectively). However, as was seen for the Chiralcel OD-R reversed-phase chiral column, the *cis*-(2S,4S) stereoisomer coeluted with the *trans*-(2S,4R) stereoisomer under these conditions.

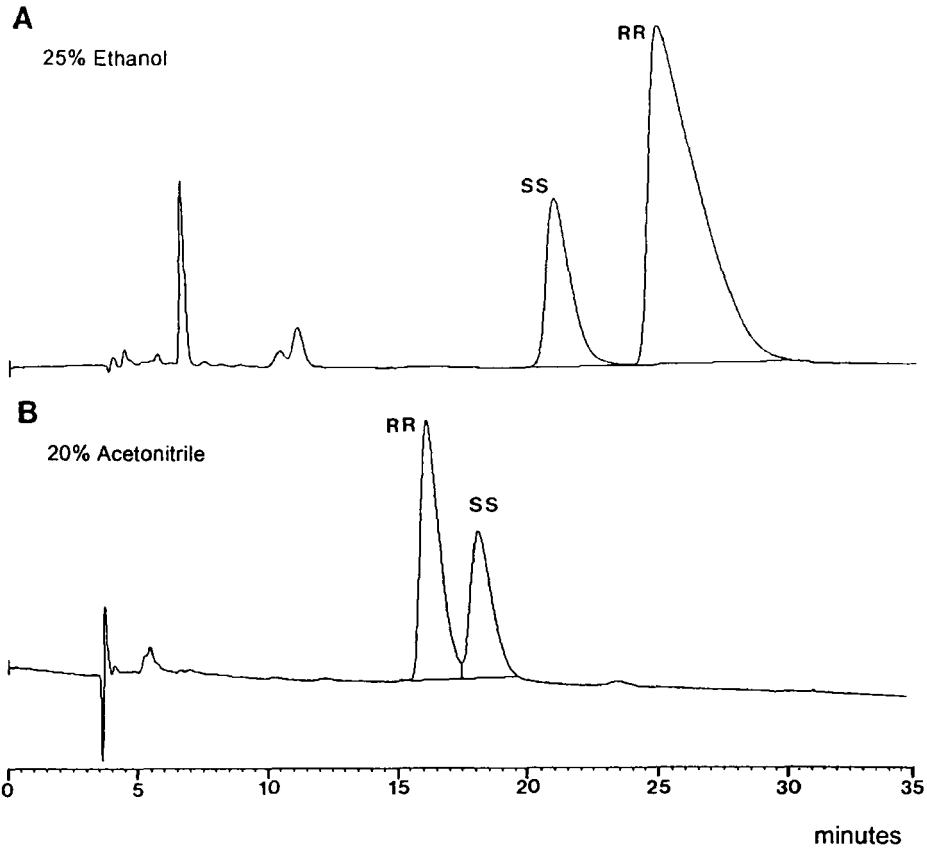


Figure 6. Chiral HPLC separation of the (2S,4S)-*cis* tosylate and (2R,4R)-*cis* tosylate enantiomers of the ketal tosylate intermediate of azalanstat as a function of organic solvent modifier with an ovomucoid bonded-phase column. **RR** (2R,4R)-*cis* tosylate enantiomer; **SS** (2S,4S)-*cis* tosylate enantiomer. Conditions: Ultron ES-OVM column, 35°C, 0.5mL/min, 220nm; (A) 75% 20mM KH_2PO_4 (pH 5.5)/25% ethanol; (B) 80% 20mM KH_2PO_4 (pH 5.5)/20% acetonitrile.

Resolution of all four stereoisomers of azalanstat was achieved by separation of the diastereomers on a 3.3cm silica gel column, coupled on-line with a Chiralpak AS amylose carbamate phase column, for separation of the enantiomers in the normal phase mode (Figure 5). A coupled-column approach using a silica gel column and a Chiralcel OD column with a switching valve was employed by McKean, et al.[5] for normal-phase separation of the stereoisomers

of an anti-emetic quinuclidine dibenzofurancarboxamide with multiple chiral centers. Rizzi[6] also utilized a coupled column approach for reversed-phase separation of the stereoisomers of 5-phenyltetrahydrooxazol-2-one and related compounds with an octylsilica-cellulose triacetate column combination.

Chiral HPLC Separations of the Stereoisomers of the Ketal Tosylate Intermediates of Azalanstat

Reverse phase chiral analysis of the ketal tosylate intermediates was carried out using an Ultron ES-OVM ovomucoid bonded-phase column with a 0.02M phosphate buffer (pH 5.5)-ethanol mobile phase (73:27 for *cis* stereoisomers and 67:33 for *trans* stereoisomers) at a flow rate of 0.5 mL/minute and a column temperature of 35 °C. Analogous to results observed for the stereoisomers of azalanstat with the Ultron ES-OVM column, the *cis*-(2S,4S) stereoisomer and the *trans*-(2S,4R) stereoisomer of the ketal tosylate intermediate coeluted using these ethanol-phosphate buffer mobile phases.

A reversal in elution order of the *cis*-(2S,4S) and *cis*-(2R,4R) enantiomers of the ketal tosylate intermediate of azalanstat was exhibited by the Ultron ES-OVM column with a change in organic modifier from ethanol to acetonitrile (Figure 6). A plot of log k' (the logarithm of the capacity factor) of the *cis*-(2S,4S) and the *cis*-(2R,4R) enantiomers as a function of ethanol/acetonitrile mixtures used as organic modifiers is shown in Figure 7.

The enantiomers coeluted at a mobile phase organic modifier content of about 10% ethanol/12% acetonitrile with 78% phosphate buffer. Higher amounts of acetonitrile caused the *cis*-(2R,4R) enantiomer to elute before the *cis*-(2S,4S) enantiomer. Control of the enantioselectivity of this separation was important in order to allow the undesired enantiomer [in this case the *cis*-(2R,4R) enantiomer] to elute *before* the desired enantiomer thereby providing a more accurate assessment of enantiomeric purity.

This unusual enantioselectivity in the reverse phase separation of the *cis*-ketal tosylates was attributed to a putative change in binding domains or recognition sites in the ovomucoid protein as a function of the organic modifier of the mobile phase. These results suggest that use of acetonitrile in conjunction with alkanol modifiers may be exploited to affect changes in selectivity with this chiral column. A similar reversal in enantiomer elution order as a function of the addition of organic solvents was observed by Haginaka, et al.[7] in studies of the enantioselectivity of propanolol and its ester

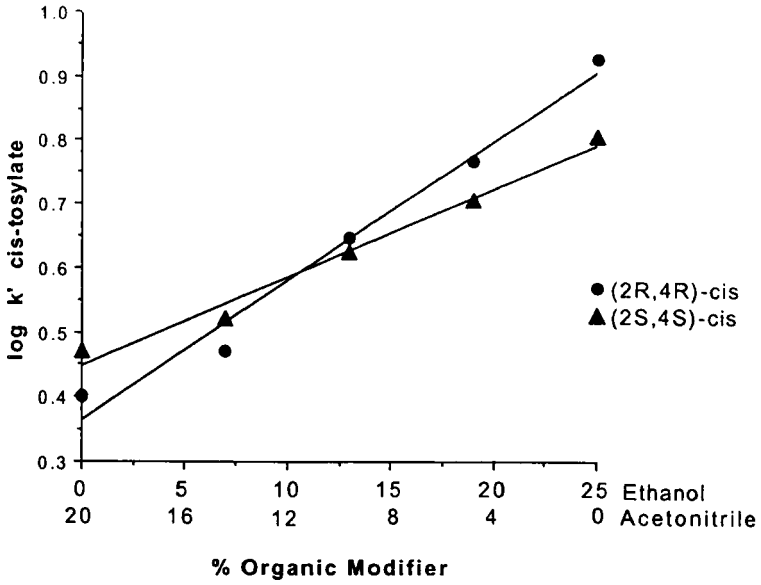


Figure 7. Plot of log k' of the (2R,4R)-cis and (2S,4S)-cis enantiomers of the ketal tosylate intermediate of azalanstat versus ethanol/acetonitrile mixtures as organic modifiers in the mobile phase with an ovomucoid bonded-phase column. Conditions: Ultron ES-OVM column, 20 mM KH_2PO_4 (pH 5.5) buffer with ethanol/acetonitrile mixtures used as organic modifiers in percentages as listed on the plot, 0.5 mL/min, 35°C, 220 nm.

derivatives on an ovomucoid bonded-phase column. Multiple binding sites on the ovomucoid protein phase column and/or at least two chiral recognition mechanisms, along with possible conformational changes in the ovomucoid bonded phase structure, were postulated for the observed effect with propanolol.

A Chiralcel OD-R reverse phase column was also employed for separation of the stereoisomers of the ketal tosylates. A mobile phase of 0.5M perchlorate buffer-acetonitrile (40:60) at a flow rate of 1mL/minute, and a column temperature of 35°C, was utilized for reverse phase chiral HPLC separations of the ketal tosylate intermediates.

Although the *cis*-(2S,4S) and the *cis*-(2R,4R) enantiomers can be readily resolved (α=1.5) under these conditions, the *trans*-(2S,4R) and *trans*-(2R,4S) enantiomers coelute.

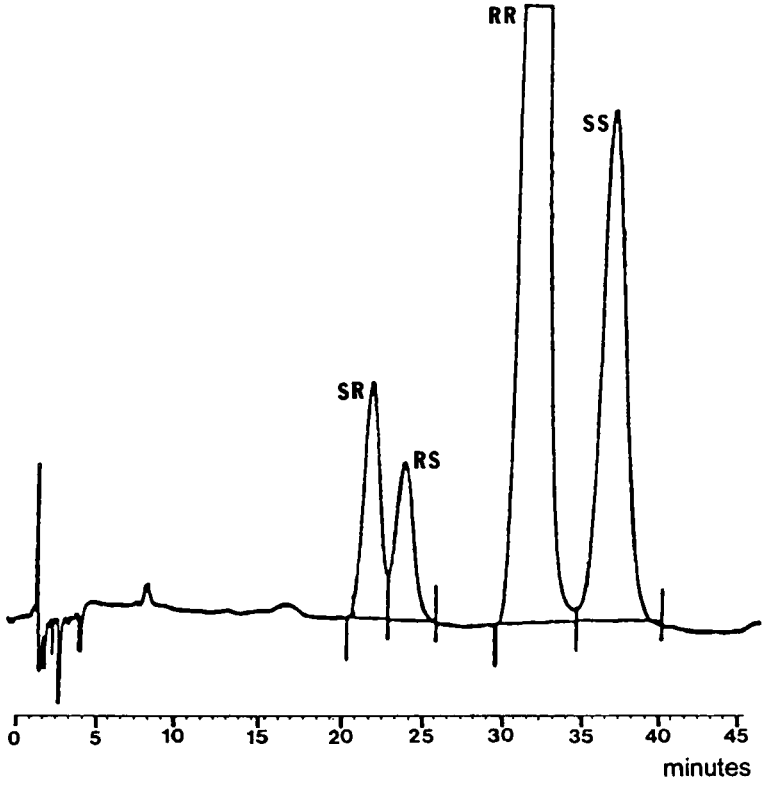


Figure 8. Chiral HPLC separation of the stereoisomers of the ketal tosylate intermediates of azalanstat: **SS** (2S,4S)-*cis* tosylate; **RR** (2R,4R)-*cis* tosylate; **SR** (2S,4R)-*trans*tosylate; **RS** (2R,4S)-*trans* tosylate. Conditions: Chiralpak AD column, 2-propanol-hexane (80:20), 1mL/min, 220nm.

A Chirex 3019 phase Pirkle-type chiral HPLC column, employing a (S)-tert-leucine with a urea linkage to (S)-1-(α-naphthyl)ethylamine as a bonded phase, was investigated for separation of the stereoisomers of the ketal tosylate intermediate. This column has been shown to afford resolution of the stereoisomers of several structurally-related azole compounds.[8] Poor selectivity ($\alpha \leq 1.1$) for the enantiomers of the ketal tosylates was exhibited by this column using a mobile phase of 2-propanol-dichloroethane-hexane. Cleveland[9] has recently published a summary of several Pirkle-type chiral stationary phases of this nature for the HPLC separation of pharmaceutical racemates.

A comparison of the Chiralpak AD and AS amylose carbamate phase columns for the separation of the stereoisomers of the ketal tosylate, indicated that the Chiralpak AS column yields higher retention (about a two-fold increase in k') but lower enantioselectivity ($\alpha < 1.1$ for the cis-tosylate enantiomers) than the Chiralpak AD column. Resolution of all four stereoisomers of the ketal toslylate intermediate was achieved with a Chiralpak AD column using a 2-propanol-hexane (20:80) mobile phase (Figure 8). Since the only difference between the Chiralpak AS and AD columns is the functionality of the derivatized amylose phase, the tris-(3,5-dimethylphenyl carbamate) phase of the Chiralpak AD column apparently yields higher selectivity for the stereoisomers of the ketal tosylate than the tris-[(S)-1-phenylethyl carbamate] phase of the Chiralpak AS column.

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