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Note

Chromatographic separation of preparative quantities of the stereoisomers of *trans*-2,3-*cis*-3,4-dihydroxy-L-proline

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Many studies of collagen metabolism have examined the synthesis of monohydroxylated forms of proline (4-hydroxy-L-proline and 3-hydroxy-L-proline)*, since these imino acids are considered markers of collagen biosynthesis. Additional studies have used a variety of proline analogues both *in vivo* and *in vitro*, in attempts to modulate collagen deposition[1,2]. This has resulted in a requirement for methods to quantitate these imino acids[3–5]. During the development of a high-performance liquid chromatographic (HPLC) method for quantitating hydroxy-L-prolines[6], the need arose for a non-physiologic hydroxylated imino acid to be used as an internal standard. This requirement was met by *trans*-2,3-*cis*-3,4-dihydroxy-L-proline which has been synthesized by Hudson *et al.*[7] using alkaline potassium permangenate oxidation of 3,4-dehydroproline. This procedure yields good recoveries of the racemate; however, the separation of the two *cis* isomers was not complete using standard separation procedures. This paper describes the complete synthesis and separation of the stereoisomers of this dihydroxylated proline using C_{18} reversed-phase chromatography. Other hydroxylated imino acids may be isolated in preparative quantities using this rapid and simple chromatographic technique.

EXPERIMENTAL

HPLC was performed on a Hewlett-Packard Model 1084-B instrument using 1% acetic acid and acetonitrile (HPLC grade). Mass spectra were obtained following gas chromatographic separation of trifluoroacetylamino ester derivatives of the compounds[8], using a Finnigan Model 4000 gas chromatography–mass spectrometry (GC–MS) instrument. Separation was on a 91.4 × 0.2 cm I.D. column of OV-17 (3%) at 100°C. Melting point determinations were made with a Meltek instrument.

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* The nomenclature employed in this paper represents the classical system for naming amino acid analogues of proline. IUPAC nomenclature rules yield the following names for these compounds: L-proline = L-2-pyrrolidine carboxylic acid; 4-hydroxy-L-proline = L-4-hydroxy-2-pyrrolidine carboxylic acid; *trans*-2,3-*cis*-3,4-dihydroxy-L-proline = L-*t*-4-dihydroxy-*r*-2-pyrrolidine carboxylic acid; *cis*-2,3-*cis*-3,4-dihydroxy-L-proline = L-*c*-3,*c*-4-dihydroxy-*r*-2-pyrrolidine carboxylic acid; L-3,4-dehydroproline = L-3-pyrroline-2-carboxylic acid.

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The dihydroxy analogues were synthesized according to Hudson *et al.*[7] with several modifications. An iced solution (50 ml) containing 0.095 *M* potassium permanganate was added with stirring to an equal volume of 0.25 *M* sodium hydroxide containing 0.09 *M* L-3,4-dehydroproline (Hoffmann-La Roche, Compound Number RO 20-5933). The mixture was vigorously stirred on ice for 1 min, followed by reduction of excess potassium permanganate with a rapid stream of sulfur dioxide for 2 min. The solution was acidified to pH 1.5 and kept on ice. This solution was applied to a 14 × 2.5 cm I.D. column of AG 50W-X8 cation-exchange resin overlaying a 14.5 × 2.5 cm bed of P-2 polyacrylamide gel (Bio-Rad, Richmond, CA, U.S.A.). The column was washed with 300 ml of 0.005 *M* sulfuric acid and eluted with 1 *M* ammonium hydroxide. The appropriate fractions were evaporated to dryness and recrystallized three times from ethanol–water. The yield of crystals was 40.0% (0.26 g) with a m.p. = 237–239°C (uncor, dec). Mass spectrum (30 eV), *m/e* (relative intensity): 390 (77), 335 (100), 276 (20), 222 (85), 178 (15), 59 (18).

Separation of the two isomers was accomplished by C_{18} semi-preparative HPLC. Operating conditions were: temperature, 50°C; flow-rate, 1.00 ml/min; mobile phase, acetonitrile–1% acetic acid solution (70:30) with detection at 200 nm. The stereoisomeric configuration of each diol was determined by HPLC analysis of Dns derivatives[4] of proline analogues using cyanopropylsilane-bonded silica. Instrument conditions: temperature, 50°C; flow-rate, 2.0 ml/min; 0.33%/min gradient of acetonitrile beginning at 3% (against 1% acetic acid) with detection at 285 nm.

RESULTS AND DISCUSSION

A number of modifications were made to the synthetic protocol for *trans*-2,3-*cis*-3,4 dihydroxy-L-proline. These changes included the elimination of an ether extraction and the development of an alternative ion-exchange procedure to separate the dihydroxylated proline from other reaction components. The extraction could be eliminated since GC–MS analysis of the reaction products following ion-exchange chromatography failed to demonstrate pyrrole-2-carboxylic acid. Ion-exchange chromatography as originally described did not consistently retain dihydroxyproline if an exhaustive water wash was performed at the indicated pH. In addition, under the alkaline conditions necessary to elute the dihydroxyprolines, manganese compounds eluted concurrently. This interference was minimized by employing a large ion-exchange bed along with a polyacrylamide gel layer. It must be noted that hydroxylated prolines will epimerize under acidic conditions[9] with dihydroxylated compounds having a greater rate of epimerization. Consequently, the initial reactant solutions and all subsequent procedures under acidic conditions should be kept at reduced temperatures.

The *cis*-2,3 and *trans*-2,3 isomers of *cis*-3,4-dihydroxyproline are extremely difficult to separate with only a partial separation of these isomers having been reported[7]. Standard techniques of preparative thin-layer chromatography (silica gel) and a modified ion-exchange chromatography method[10] failed to separate these compounds. The use of boric acid gel[11,12], a *cis*-diol-specific affinity chromatography material, also failed to produce separation, although the isomers were bound by the gel. Complete resolution was obtained by the use of a reversed-phase C_{18} semi-pre-

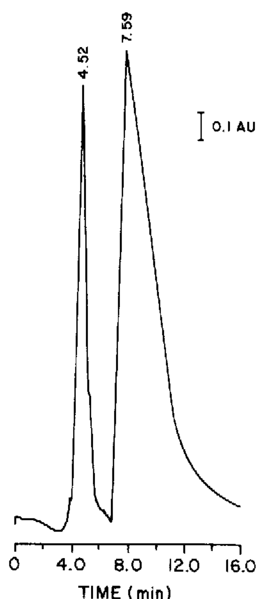

Fig. 1. Chromatogram of *cis* dihydroxylated prolines separated by HPLC using a C_{18} reversed-phase semi-preparative column. The chromatography conditions are described in the Experimental section. Complete resolution was obtained within 14.0 min with *trans*-2,3-*cis*-3,4-dihydroxy-L-proline eluting first. Detection was at 200 nm.

ative HPLC column (Fig. 1). The two isomers separate with no overlap, and retention time differences of 3.20 min. Using this method, several milligrams of the *trans* isomer may be prepared per hour.

The use of HPLC also enables one to identify stereoisomers of isolated fractions. Table I presents relative retention times for Dns-proline analogues employing a 6-μm cyanopropylsilane-bonded silica packing (Zorbax-CN®, DuPont). It is apparent that *cis*-hydroxy-L-proline analogues have a less polar behavior on this packing than the corresponding *trans* isomer. The diol analogues should maintain this *cis* to *trans* elution relationship, although they would elute more rapidly due to the in-

TABLE I

RETENTION TIMES FOR Dns-L-PROLINE ANALOGUES

Compound	*Retention time**	
	Absolute (min)	*Relative***
L-Proline	21.73	3.16
trans-4-Hydroxy-L-proline	7.50	1.09
cis-4-Hydroxy-L-proline	10.46	1.52
trans-3-Hydroxy-L-proline	8.39	1.22
trans-2,3-*cis*-3,4-Dihydroxy-L-proline	5.70	0.83
cis-2,3-*cis*-3,4-Dihydroxy-L-proline	6.88	1.00

* Retention time using a cyanopropylsilane-bonded silica column (25.0 cm × 4.6 mm I.D.).
** Relative values are based on threonine (retention time = 6.88).

creased polarity of the molecule. This approach was used to designate the earlier eluting compound as the *trans* configuration. As an additional confirmation of these designations, the ratio of these two isomers under acidic conditions was shifted from 50:50 to 15:85 following heating at 50°C for 4 h. This represents the epimerization from L to D which would effectively change the *trans*-L configuration to a *cis*-D with a resultant shift in retention time.

This method of separation enables one to isolate pure geometric isomers of hydroxylated imino acids without derivatization or chiral column packings. It should be applicable to other isomers of proline which may possess anticollagen activity.

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REFERENCES

1 G. C. Fuller, in U. Gerlach, G. Pott, J. Rautenberg and B. Voss (Editors), *Connective Tissue of the Normal and Fibrotic Human Liver*, Georg Thieme Verlag, Stuttgart, 1982, p. 219.
2 M. Chvapil, in J. B. Weiss and M. I. V. Jayson (Editors), *Collagen in Health and Disease*, Churchill Livingstone, London, 1982, p. 206.
3 D. J. Prockop and S. Udenfriend, *Anal. Biochem.*, 1 (1960) 228.
4 G. J. Schmidt, D. C. Olson and W. Slavin, *J. Liquid Chromatogr.*, 2 (1979) 1031.
5 A. Bisker, V. Pailler, A. Randoux and J. P. Borel, *Anal. Biochem.*, 122 (1982) 52.
6 W. J. Lindblad and R. F. Diegelmann, *Anal. Biochem.*, 138 (1984) 390.
7 C. B. Hudson, A. V. Robertson and W. R. J. Simpson, *Aust. J. Chem.*, 21 (1968) 769.
8 R. A. Cruickshank and J. C. Sheehan, *Anal. Chem.*, 36 (1964) 1191.
9 D. D. Dziewiatkowski, V. C. Hascall and R. L. Riolo, *Anal. Biochem.*, 49 (1972) 550.
10 A. Kaplan, B. Witkop and S. Udenfriend, *J. Biol. Chem.*, 239 (1964) 2559.
11 M. Uziel, L. H. Smith and S. A. Taylor, *Clin. Chem.*, 22 (1976) 1451.
12 S. Higa, T. Suzuki, A. Hayashi, I. Tsuge and Y. Yamamura, *Anal. Biochem.*, 77 (1977) 18.