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Synthesis and Characterization of Peptides Containing a Cyclic Val Adduct of Diepoxybutane, a Possible Biomarker of Human Exposure to Butadiene

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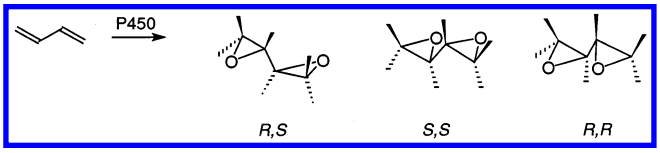
1,3-Butadiene, a potential human carcinogen widely used in industry, is oxidized by cytochrome P450 to diepoxybutane (DEB), which is the most mutagenic of the known butadiene metabolites. Assessment of the toxicological significance of DEB formation in humans and animals requires identification of a biomarker uniquely associated with DEB for use in molecular dosimetry studies. We wished to develop a specific and sensitive assay for one such suitable marker, the cyclic adduct 2-(3,4-dihydroxypyrrolidin-1-yl)-3-methylbutyramide (pyr-V), which is formed from addition of DEB to the terminal Val of the α- and *β*-chains of hemoglobin. We needed to prepare a pure, rigorously characterized DEB-modified *N*-terminal oligopeptide for raising antibodies both to use in an immunoaffinity purification step and to standardize the assay. In addition, we needed a pure isotopomer to serve as an internal standard for quantitation by LC-MS. Direct modification of the globin sequences by reaction with DEB in vitro proved to be unproductive. We therefore opted to synthesize the cyclic Val adduct and incorporate it by FMOC chemistry into the appropriate oligopeptide sequences. In vitro and in vivo, butadiene is oxidized to enantiomeric and *meso* forms of DEB. A priori, all three DEB isomers are expected to form pyr-V adducts, resulting in three diastereomeric *N*-terminal peptides. We therefore synthesized a mixture of the cyclic Val diastereomers as their methyl esters by reaction of DEB with L-Val methyl ester hydrochloride. After protection as the di-*O*-*tert*-butyl derivatives, the mixture of 2-(3,4-di-*t*-butoxypyrrolidin-1-yl)-3-methylbutyric acid diastereomers was incorporated as the *N*-terminal residue into the 1−11 human globin α-chain sequence VLSPADKTNVK. The presence of the three diastereomers was confirmed by two-dimensional correlation NMR spectroscopy and temperature-dependent ^{1}H NMR. This strategy enabled us to obtain pure, rigorously characterized haptens in quantity for the preparation of polyclonal antibodies. Use of FMOC-protected $^{2}H_{3}$-Leu in the automated oligopeptide synthesis provided the required isotopomers for use as internal standard.

Introduction

1,3-Butadiene (BD) is extensively used in the manufacture of synthetic rubber and thermoplastic resins and has been identified as an environmental contaminant in cigarette smoke, automobile exhaust, and gasoline vapor. BD has been classified as a probable human carcinogen by the International Agency for Research on Cancer and as a known human carcinogen in the Ninth Report on Carcinogens 2000 by the U.S. National Toxicology Program, based on a relationship between occupational exposure and excess mortality from lymphatic and hematopoietic cancers (*1*).

Among the metabolites of BD, diepoxybutane (DEB) is considered to be the most mutagenic (*2*). Further assessment of the toxicological significance of DEB in vivo, as well as the measurement of DEB formation in humans, rests on identification of a biomarker uniquely associated with the DEB metabolites. Although there is considerable interspecies variability in the metabolism of BD to DEB, both rodent and human cytochrome P450 isoforms oxidize BD to enantiomeric and *meso* forms of DEB in varying proportions but with all forms generated as significant fractions of total DEB yield (*3*−*5*).



Relative toxicities of the pure enantiomeric and *meso* forms of DEB have been investigated and found to vary depending on the test system (*6*, *7*). DEB adds to the terminal Val of the α- and *β*-chains of hemoglobin (*8*). In vitro, reaction of DEB with valinamide leads to prefer-

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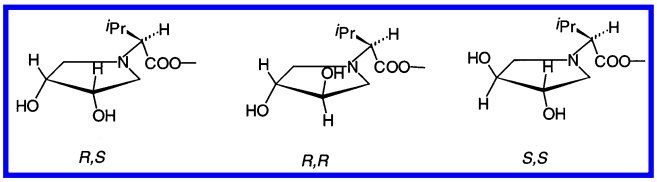
ential formation of the cyclic adduct, 2-(3,4-dihydroxypyrrolidin-1-yl)-3-methylbutyramide (pyr-V) (*8*). In vivo, pyr-V has also been identified in the globin of rodents exposed to BD (*5*, *10*).



While the cyclic adduct is potentially a highly specific marker for DEB exposure, the tertiary structure of nitrogen precludes analysis by the modified Edman reaction of Törnqvist et al. (*9*). Currently, a sensitive method for quantitation of the cyclic adduct has not been refined to the point of practical application to the routine analysis of pyr-V. However, a procedure published by Kautiainen et al. (*10*) and Swenberg et al. (*5*) provides the basis for a more sensitive isotope dilution mass spectrometric assay for quantitative measurement in globin from both rodents and humans exposed to BD. In the Kautiainen procedure, globin of mice injected intraperitoneally with DEB was trypsinized and the modified terminal heptapeptide of the α-chain was quantitated in the digest by LC-MS/MS using as an internal standard a DEB-treated (1-8)-d_8-octapeptide α-chain sequence ($^2H_8$-V)LSGEDKS of mouse globin. Prior to analysis, the analyte along with the internal standard was concentrated from the trypsin digest by semipreparative HPLC. One shortcoming of this procedure is the relatively low and only approximately quantifiable yield of internal standard from direct alkylation of the octapeptide. The reported use of semipreparative HPLC (*10*) to concentrate the analyte and internal standard is also cumbersome for large-scale use of the analysis. An LC-MS/MS analysis used by Swenberg (*5*) omitted the use of semipreparative HPLC. In both cases, highly alkylated globin could be measured, but neither procedure had adequate sensitivity to measure pyr-V in animals or humans exposed to BD.

Key to adapting the trypsin digestion procedure for application to human samples is the availability of a pure, isotopomeric DEB-modified *N*-terminal sequence of human globin α-chain to serve as an internal standard and a convenient, reliable method for concentration of the analyte and internal standard in the digestion matrix. In our laboratory, attempts to obtain an internal standard via modification of the 1−11 peptide sequence VLSPADKTNVK of the human globin α-chain at the terminal Val by reaction with DEB were complicated by alkylation of the free amino groups of the lysines and internal Val as well as the carboxylate of aspartic acid. As an alternative strategy for obtaining a pure internal standard, we investigated automated synthesis of a terminal sequence larger than seven residues, incorporating modified Val at the *N*-terminal position. The availability of well-characterized synthetic peptide in quantity has an additional utility in providing haptens to raise polyclonal antibodies for concentration of analyte and standard in trypsin digests by immunoaffinity chromatography. The immunochemical procedure offers the advantages of speed and convenience desirable in sample preparation. A priori, all three DEB metabolites are expected to form pyr-V adducts, resulting in three diastereomeric *N*-terminal peptides following digestion. Thus, the ability to complex all three peptides is a requirement for an immunochemical method. As a consequence, polyclonal antibodies must be raised against all three possible diastereomeric peptides. We report here the synthesis of a mixture of the cyclic Val diastereomers, the di-*O*-*tert*-butyl-protected derivatives for automated oligopeptide synthesis, the synthesis of the natural abundance isotopomers of the diastereomeric 1−11 peptide sequences of the human globin α-chain as haptens, and the $^2H_3$-Leu isotopomers of the diastereomeric peptides for use as internal standard.

Experimental Procedures

Materials and Methods. L-Val methyl ester hydrochloride, L-Leu-5,5,5-d_3, BD-1,3-diepoxide, and isobutene were purchased from Aldrich and used as received. The ^{1}H NMR spectrum of the DEB indicated the presence of ∼1% *meso* diepoxide in addition to *R*,*R* and *S*,*S* compound. ^{1}H NMR spectra were recorded on a Varian INOVA 500 spectrometer at 500 MHz at ambient temperature, unless otherwise stated. Coupling constants were determined by spectral simulation using MestRe-C 2.3 software (mestrec@usc.es). Mass spectra were obtained on a Finnigan LCQ^{DECA} quadrupole ion trap mass spectrometer with an electrospray source. Exact mass measurements on the modified valines were performed on a VG70-250SEQ mass spectrometer or a JEOL HX110HF mass spectrometer using a FAB source. The exact mass measurement on the peptide was obtained by MALDI/TOF on a Bruker Reflex III mass spectrometer.

Methyl 2-(3,4-Dihydroxypyrrolidin-1-yl)-3-methylbutyrate Diastereomers (1). An aqueous solution (9 mL) of Val methyl ester hydrochloride (955 mg, 5.72 mmol), triethylamine (0.9 mL), glacial acetic acid (0.3 mL), and BD-1,3-diepoxide (500 mg, 5.82 mmol) was stirred in a stoppered flask at 50 °C for 7 days. After it was cooled to room temperature, the reaction was neutralized with dilute ammonium hydroxide and **1** was extracted into chloroform. The crude product was purified by preparative TLC on a 1000 μm × 20 cm silica plate developed with 9:1 chloroform/methanol to yield 281 mg (22%) of a pale yellow oil. ESI-MS *m*/*z* (rel intensity): 218 (100) MH^+, 159 (12) (MH − methoxycarbonyl). Exact mass calcd for $C_{10}H_{20}NO_4$ (MH^+), 218.1392; found, 218.1376. ^{1}H NMR (chloroform-*d*): 4.34 (dd, 0.2 H, J = 10.0, 6.1 Hz, pyrrolidine carbinyl H, *meso* diastereomer), 4.05−4.02 (m, 1.8H, J_{CH-CH_2} = 4.5, 3.5, $J_{CH-CH_2'}$ = 4.6, 1.2 Hz, overlapping pyrrolidine carbinyl H, *R*,*R* and *S*,*S* diastereomers), 3.73 (s, 0.3H, ester CH_3), 3.70 (s, 2.7H, ester CH_3), 3.60 (ψ triplet, 0.2 H, J_{CH_2-CH} = 10.0, J_{gemH} = 8.5 Hz, pyrrolidine CH_2 *meso* diastereomer), 3.34 (ψ triplet, 0.2 H, J_{gemH} = 8.5 Hz, J_{CH_2-CH} = 6.0, pyrrolidine CH_2, *meso* diastereomer), 3.21 (dd, 0.9 H, J_{gemH} = 10.5, J_{CH_2-CH} = 4.5 Hz, pyrrolidine CH_2, *R*,*R* or *S*,*S* diastereomer), 3.09 (dd, 0.9 H, J_{gemH} = 10.0, J_{CH_2-CH} = 4.6 Hz, pyrrolidine CH_2, *R*,*R* or *S*,*S* diastereomer), 3.05 (d, 0.45 H, J = 9.1 Hz, H^α, *R*,*R* or *S*,*S* diastereomer), 3.01 (d, 0.45 H, J = 8.4 Hz, H^α, *R*,*R* or *S*,*S* diastereomer), 2.95 (d, 0.1 H, $J_{i\text{-Pr}-CH_3-H^\alpha}$ = 7.6 Hz, H^α, *meso* diastereomer), 2.81 (dd, 0.9 H, J_{gemH} = 10.0, J_{CH_2-CH} = 1.2 Hz, pyrrolidine CH_2, *R*,*R* or *S*,*S* diastereomer), 2.58 (dd, J_{gemH} = 10.5, J_{CH_2-CH} = 3.5 Hz, pyrrolidine CH_2 *R*,*R* or *S*,*S* diastereomer), 2.03 (m, 0.9 H, $J_{i\text{-Pr}-CH-H^\alpha}$ = 9.1, $J_{i\text{-Pr}-CH_3-i\text{-Pr}-CH}$ = 7.1, $J_{i\text{-Pr}-CH_3-i\text{-Pr}-CH}$ = 6.3 Hz, *i*-Pr-H *R*,*R* or *S*,*S* diastereomer), 1.89 (m, 0.1 H, $J_{i\text{-Pr}-CH-H^\alpha}$ = 8.4, $J_{i\text{-Pr}-CH_3-i\text{-Pr}-CH}$ = 7.0, $J_{i\text{-Pr}-CH_3-i\text{-Pr}-CH}$ = 6.5 Hz, *i*-Pr-H *meso* diastereomer), 1.00 (d, 2.7 H, $J_{i\text{-Pr}-CH_3-i\text{-Pr}-CH}$ = 6.3 Hz, diastereotopic *i*-Pr-CH_3 *R*,*R* or *S*,*S* diastereomer), 0.99 (d, 2.7H, $J_{i\text{-Pr}-CH_3-i\text{-Pr}-CH}$ = 7.0 Hz, diastereotopic *i*-Pr-CH_3 *R*,*R* or *S*,*S* diastereomer), 0.97 (overlapping d, 0.3 H, diastereotopic *i*-Pr-CH_3 *meso* diastereomer), 0.91 (d, 2.7H, $J_{i\text{-Pr}-CH_3-i\text{-Pr}-CH}$ = 7.1 Hz, diastereotopic *i*-Pr-CH_3 *R*,*R* or *S*,*S* diastereomer), 0.90 (d, 2.7H, $J_{i\text{-Pr}-CH_3-i\text{-Pr}-CH}$ = 6.5 Hz, diastereotopic *i*-Pr-CH_3 *R*,*R* or *S*,*S* diastereomer), 0.88 (d, 0.3H, $J_{i\text{-Pr}-CH_3-i\text{-Pr}-CH}$ = 6.6 Hz, diastereotopic *i*-Pr-CH_3 *meso* diastereomer) ppm.

Methyl 2-(3,4-Di-*t*-butoxypyrrolidin-1-yl)-3-methylbutyrate Diastereomers (2). A suspension of **1** (535 mg, 2.47 mmol) in methylene chloride (30 mL) in a 100 mL round bottom flask equipped with a magnetic stir bar was sonicated for 10 min with concentrated sulfuric acid (0.75 mL) at 0 °C under argon. A stream of isobutene was passed into the reaction flask until ~20 mL had collected. The reaction, with a stopper clamped in place, was stirred at 0 °C for 4 h and at ambient temperature for 4 days. The stopper was removed (after cooling the reaction in an ice bath), and the isobutene was evaporated under a stream of nitrogen. The reaction mixture was poured into 2 M ammonium hydroxide (30 mL) and extracted with methylene chloride. Evaporation of the methylene chloride yielded a yellow oil, 494 mg (61%), which was used without purification for the next step. ESI-MS *m*/*z* (rel intensity): 330 (100) MH^+. ^1H NMR (chloroform-*d*): 4.04−3.99 (m, 0.2 H), 3.92−3.84 (m, 2 H), 3.83−3.79 (m, 0.2H), 3.67 (s, 3 H), 3.07−2.98 (m, 0.9 H), 2.94−2.79 (m, 1.9 H), 2.57−2.49 (m, 0.9 H), 2.47−2.39 (m, 0.9 H), 2.04−1.90 (m, 1 H), 1.25 (s, 1.8 H), 1.16 (s, 16.2 H) ppm.

2-(3,4-Di-*t*-Butoxypyrrolidin-1-yl)-3-methylbutyric Acid Diastereomers (3). Ester **2** (304 mg, 0.924 mmol) from the previous reaction was refluxed with 2 M KOH in 2:1 methanol/water for 3 h. Concentration of the solution at reduced pressure yielded a precipitate, collected by decantation. Purification by TLC on 1000 μm × 20 cm silica plates developed with 2:2:1 methanol/chloroform/ammonium hydroxide yielded microcrystalline **3**, 276 mg (95%). ESI-MS *m*/*z* (rel intensity): 316 (100) MH^+, 260 (30) (MH − isobutene). Exact mass calcd for $C_{17}H_{34}NO_4$ (MH^+), 316.248784; found, 316.247816. ^1H NMR (D_2O + K_2CO_3): 4.18−4.13 (m, 0.2H, pyrrolidine carbinyl H *meso* diastereomer), 4.04 (ψt, 0.9 H, 1.8H, J_{CH-CH_2} = 6.0, 5.6, pyrrolidine carbinyl H *R*,*R* or *S*,*S* diastereomer), 403 (ψt, 0.9 H, 1.8H, J_{CH-CH_2} = 6.2, 5.8, pyrrolidine carbinyl H *R*,*R* or *S*,*S* diastereomer), 3.66−3.61 (m, 0.3H, pyrrolidine CH_2 *meso* diastereomer), 3.24 (dd + low amplitude overlapping m, 1.0 H, J_{gemH} = 10.0, J_{CH-CH_2} = 6.0 Hz, pyrrolidine CH_2 *R*,*R* or *S*,*S* and *meso* diastereomer), 2.98 (dd, 0.9 H, J_{gemH} = 10.0, J_{CH-CH_2} = 6.2 Hz, pyrrolidine CH_2 *R*,*R* or *S*,*S* diastereomer), 2.92 (d, 0.1 H, $J_{i\text{-}Pr-CH-H^\alpha}$ = 5.3 Hz, H^α *meso* diastereomer), 2.73 (d, 0.5 H, $J_{i\text{-}Pr-CH-H^\alpha}$ = 9.2 Hz, H^α *R*,*R* or *S*,*S* diastereomer), 2.69 (d, 0.5 H, $J_{i\text{-}Pr-CH-H^\alpha}$ = 7.9 Hz, H^α *S*,*S* and *R*,*R* diastereomer), 2.62 (dd, 0.9 H, J_{gemH} = 10.0, J_{CH-CH_2} = 5.8 Hz, pyrrolidine CH_2 *R*,*R* or *S*,*S* diastereomer), 2.48 (dd, 0.9 H, J_{gemH} = 10.0, J_{CH-CH_2} = 6.2 Hz, pyrrolidine CH_2 *R*,*R* or *S*,*S* diastereomer), 2.02−1.92 (m, 1H, *i*-Pr-C*H*), 1.27 (bs, 16.2 H, *t*-OBu H), 1.25 (bs, 1.8 H, *t*-OBu H), 1.01 (d, 1.5 H, $J_{i\text{-}Pr-CH_3-i\text{-}Pr-CH}$ = 6.9 Hz, unresolved diastereotopic *i*-Pr-CH_3 *meso* + *R*,*R* or *S*,*S* diastereomers), 0.99 (d, 1.5 H, $J_{i\text{-}Pr-CH_3-i\text{-}Pr-CH}$ = 5.9 Hz, unresolved diastereotopic *i*-Pr-CH_3 *meso* + *R*,*R* or *S*,*S* diastereomers), 0.95 (d, 1.4 H, $J_{i\text{-}Pr-CH_3-i\text{-}Pr-CH}$ = 6.9 Hz, diastereotopic *i*-Pr-CH_3 *R*,*R* or *S*,*S* diastereomer), 0.93 (d, 1.4 H, $J_{i\text{-}Pr-CH_3-i\text{-}Pr-CH}$ = 6.87 Hz, diastereotopic *i*-Pr-CH_3 *R*,*R* or *S*,*S*), 0.91 (d, 0.6 H, $J_{i\text{-}Pr-CH_3-i\text{-}Pr-CH}$ = 6.8 Hz, diastereotopic *i*-Pr-CH_3 *meso* diastereomer), 0.90 (0.2 H, $J_{i\text{-}Pr-CH_3-i\text{-}Pr-CH}$ = 6.1 Hz, diastereotopic *i*-Pr-CH_3 *meso* diastereomer) ppm.

Mixture of Diastereomeric 11-mers with Modified *N*-Terminal Val. The modified 1−11 globin α-chain sequence was synthesized by the UNC Protein Sequence and Peptide Synthesis Core Facility on a Rainin Symphony multiple peptide synthesizer using FMOC chemistry. The mixture of diastereomers **3** was added manually at the final synthesis cycle along with activator (HBTU) and base (NMM). After cleavage and deprotection, the peptide was purified by reverse phase HPLC and appropriate fractions were pooled and lyophilized. Exact mass calcd for $C_{55}H_{97}N_{14}O_{19}$ (MH^+), 1257.7054; found, 1257.7026. ^1H NMR and two-dimensional correlation spectroscopy (2D COSY) are discussed in the text.

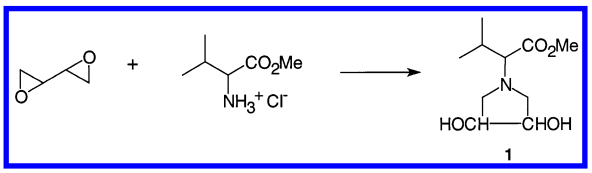
L-Leu-5,5,5-*d*$_3$-FMOC. L-Leu-5,5,5-d_3 was derivatized according to the procedure described in ref *11*. 9-Fluorenylchloroformate (585 mg, 2.26 mmol) was added to a solution of L-Leu-5,5,5-d_3 (301 mg, 2.25 mmol) in 15 mL of 10% Na_2CO_3 and 10 mL of dioxane. After brief sonication to form a clear solution, the reaction mixture was stirred overnight at ambient temperature. Following the addition of 20 mL of distilled water, the reaction mixture was extracted with 2 × 40 mL ether. The aqueous layer was cooled to 0 °C and carefully acidified with concentrated HCl to pH 3. The resulting white precipitate was extracted with 2 × 30 mL ethyl acetate, and the combined organic extracts were washed with 100 mL of distilled water, dried over Na_2SO_4, and evaporated under reduced pressure to give a syrup. The syrup was dissolved in a minimum quantity of ether, concentrated by boiling briefly, and then cooled to ambient temperature, yielding 542 mg (63%) of colorless crystals. ESI-MS *m*/*z* (rel intensity): 379 (100) MNa^+, 357 (12) MH^+. ^1H NMR (500 MHz, Me_2SO-d_6): 12.55 (bs, 1H, −COO*H*), 7.89 (d, 2H, *J* = 7.0 Hz, fluorene H-4,5), 7.73 (d, 2H, *J* = 7.7, fluorene H-1,8), 7.62 (d, 1H, $J_{NH-C\alpha H}$ = 8.2 Hz, N*H*), 7.42 (dd, 2H, *J* = 7.0, 7.6 Hz, fluorene H-3,6), 7.33 (dd, 1H, *J* = 7.0, 7.6 Hz, fluorene H-7), 7.32 (dd, 1H, *J* = 7.0, 7.6 Hz, fluorene H-2), 4.29 (m, 1H, *J* = 7.6, *J* = 10 Hz, diastereotopic fluorenyl 9-methylene H), 4.28 (m, 1H, *J* = 5.9, *J* = 10 Hz, diastereotopic fluorenyl 9-methylene H), 4.22 (dd, 1H, *J* = 5.9, *J* = 7.6 Hz, fluorenyl 9-H), 3.98 (m, 1H, $J_{C\alpha H-C\beta H_2}$ = 3.0, $J_{C\alpha H-C\beta H_2}$ = 7.0, $J_{NH-C\alpha H}$ = 8.2 Hz, $C^\alpha H$), 1.63 (m, 1H, $J_{i\text{-}Pr-CH-C\beta H_2}$ = 3.5, $J_{i\text{-}Pr-H-i\text{-}Pr-CH_3}$ = 4.9, $J_{i\text{-}Pr-H-i\text{-}Pr-CH_3}$ = 5.9, $J_{i\text{-}Pr-H-C\beta H_2}$ = 6.5, *i*-Pr-H), 1.58 (m, 1H, $J_{gemC\beta H_2}$ = 9.0, $J_{i\text{-}Pr-H-C\beta H_2}$ = 7.0, gem$C^\beta H_2$), 1.47 (m, 1H, $J_{i\text{-}Pr-CH-\beta H_2}$ = 9.0, $J_{i\text{-}Pr-H-C\beta H_2}$ = 3.5, gem$C^\beta H_2$), 0.89 (d, 1.5 H, $J_{i\text{-}Pr-H-i\text{-}Pr-CH_3}$ = 4.9, 1/2−*i*-Pr-CH_3), 0.85 (d, 1.5 H, $J_{i\text{-}Pr-H-i\text{-}Pr-CH_3}$ = 5.9, 1/2−*i*-Pr-CH_3) ppm.

Mixture of Diastereomeric 11-mer Isotopomers with Modified *N*-Terminal Val. The Leu-d_3 isotopomeric mixture of modified 1−11 globin α-chain sequences was synthesized as described above but incorporating L-Leu-5,5,5-d_3−FMOC and the mixture of diastereomers **3**. ESI-MS *m*/*z* (rel intensity): 1283 (47) MNa^+, 1261 (100) MH^+, 1076 (10) M-(pyr-V).

Results and Discussion

Automated synthesis of the mixture of diastereomeric 11-mers required synthesis of di-*O*-protected dihydroxypyrrolidin-1-yl butyric acid diastereomers. The starting point for the synthetic route was based on a reported synthesis of the cyclic adduct from reaction of DEB with valinamide (*3*). However, for the purpose of preparing the mixture of diastereomers for automated peptide synthesis, alkylation of Val seemed more direct and therefore preferable. A mixture of the three diastereomeric parent dihydroxypyrrolidinyl butyric acids was obtained in 30% total yield by condensation of DEB with L-Val. Attempts to obtain the protected diastereomers directly from the pyrrolidinyl butyric acid mixture were unsuccessful. Acetylation with acetic anhydride and triethylamine yielded no isolable products. The standard procedure for *tert*-butylation of the hydroxy groups with isobutene (*12*, *13*) yielded only traces of partially protected compounds. A reported butylation with isobutene catalyzed by sulfuric acid (*14*), when modified by sonication of the reaction mixture to improve dispersion of the pyrrolidinylbutyric acid, yielded the *tert*-butyl ester of the target di-*t*-butoxy compounds. However, the *tert*-butyl ester proved surprisingly resistant to hydrolysis. Extended refluxing with 2 M methanolic NaOH resulted in no change. Treatment at 0 °C with 1:1 trifluoroacetic acid/methylene chloride resulted in nonselective removal of both ester and *O*-*tert*-butyl groups, to give a mixture of mono- and di-*tert*-butyl derivatives along with some completely dealkylated product. To avoid formation of the *tert*-butyl ester, methyl pyrrolidinyl butyrate was investigated as the starting point for the protection reaction using the sulfuric acid-catalyzed procedure. Substitution

Scheme 1



of L-Val methyl ester hydrochloride for L-Val in the reaction with DEB gave a mixture of diastereomeric methyl esters in 25% total yield (Scheme 1), collected as a single band by preparative TLC in >90% purity, estimated from the ^{1}H NMR spectrum by comparison of the total integrated product signal with that of the total integrated impurity signals. The presence of the three diastereomeric Val adducts with *R*,*R*, *S*,*S*, and *meso* pyrrolidine carbinol centers was established by 2D COSY and nuclear Overhauser enhancement spectroscopy (NOESY) experiments. In the COSY spectrum (Figure 1), three separate sets of connectivities are observed. On the basis of proton integrations of the one-dimensional (1D) ^{1}H NMR spectrum, the COSY spectrum is consistent with one minor and two major diastereomers. From integration of the clearly resolved H$^\alpha$ signals in the 1D spectrum, a 45:45:10 ratio of diastereomers was determined. Because the DEB used as the starting material for adduct formation was predominantly a mixture of enantiomers by ^{1}H NMR analysis, the major diastereomers are assigned as having *R*,*R* and *S*,*S* optical configurations at the pyrrolidine carbinol carbons, and the minor diastereomer is assigned a *meso* configuration. The ~10-fold enrichment of the diastereomer mixture in the *meso* component relative to starting DEB suggests that the cyclic adduct forms more readily from *meso*-DEB. The set of signals assigned to the minor diastereomer consists of the carbinol protons at 4.3 ppm, which have the expected cross-peaks with two sets of proton signals at 3.60 and 3.34 ppm, corresponding to the diastereotopic pyrrolidine methylene resonances. H$^\alpha$ of the minor Val diastereomer at 2.95 ppm has a cross-peak with the methinyl isopropyl proton at 1.89 ppm, which, in turn, shows cross-peaks with two methyl signals of the diastereotopic isopropyl methyl groups. The upfield isopropyl methyl signal is well-resolved; however, the lower field methyl resonance is partially obscured by methyl signals of the major diastereomers.

The two major diastereomers have a similar pattern of connectivities overall. The pyrrolidine carbinol proton signals of the two compounds overlap at 4.03 ppm. The resulting multiplet carbinol signal has cross-peaks with two sets of methylene signals: one set at 3.21 and 2.58 ppm and a second at 3.09 and 2.81 ppm. Each set is defined by cross-peaks between signals of the geminal methylene protons. The Val H$^\alpha$ resonances of the major diastereomers appear at 3.05 and 3.01 ppm. NOESY cross-peaks show that the H$^\alpha$ signal at 3.05 ppm is associated with the set of pyrrolidine methylene resonances at 3.21 and 2.58 ppm, while H$^\alpha$ at 3.01 ppm is associated with the set at 3.09 and 2.81 ppm. The H$^\alpha$ protons couple with overlapping isopropyl methinyl proton multiplets at 2.03 ppm, and this multiplet has cross-peaks with four methyl resonances between 1.00 and 0.90 ppm, as expected from the diastereotopic character of isopropyl methyl groups. Weak NOESY cross-peaks are evident in Figure 1 between the H$^\alpha$ resonances and the diastereotopic methyls. These cross-peaks allow the H$^\alpha$ signal at 3.05 ppm to be assigned to a diastereotopic methyl pair at 1.00 and 0.91 ppm and the H$^\alpha$ signal at 3.01 ppm with a diastereotopic methyl pair at 0.99 and 0.90 ppm. The small range of the methyl shifts and the absence of definitive NOESY cross-peaks with any of the pyrrolidine or Val H$^\alpha$ signals preclude more specific assignment of sets of methyl resonances to one or the other of the diastereomers.

The pyrrolidine hydroxy groups of the methyl pyrrolidinyl butyrate diastereomers were *tert*-butylated under the same conditions described for synthesis of the pyrrolidine adduct of L-Val. Forcing conditions were required to hydrolyze the methyl ester, refluxing with 2 M methanolic KOH for 3 h (Scheme 2). Nevertheless, the di-*t*-butoxy-protected mixture was obtained quantitatively by this treatment. The mixture of diastereomers chromatographed as a single band by preparative TLC on silica, and this mixture was collected and used for the synthesis of the diastereomeric 1−11 sequence of the human globin α-chain.

Because concentration by immunoaffinity chromatography requires antibodies to all three diastereomeric peptides, the presence of the three diastereomeric peptides in the hapten mixture for antibody production is crucial. The peptide mixture could not be resolved by HPLC analysis. The presence of three diastereomeric peptides was therefore established by variable temperature 1D ^{1}H NMR and 2D COSY NMR using the unmodified 1−11 sequence as a reference. Figure 2 shows the 2D COSY NMR spectrum of the mixture of diastereomers at ambient temperature. Connectivities have been drawn between proton signals that can be assigned to the modified terminal Val residues of the diastereomers. A multiplet at 4.89 ppm integrating to ~0.2 H (Figure 2, inset) is assigned to the pyrrolidine carbinol protons of the minor (*meso*) diastereomer. This resonance has cross-peaks with signals at 3.90 and 3.92 ppm embedded in a complex of peaks between 4.05 and 3.80 ppm. The pattern of the cross-peaks and the shifts of the (unresolved) protons for which coupling is indicated are consistent with those expected for the methylene protons of the pyrrolidine ring.

Doublets at 3.87 and 3.82 ppm, with a combined integral of 0.9 H, have cross-peaks with two broad, overlapping multiplets at 2.34 ppm integrating to a total of 0.9 protons. These multiplets have a cross-peak with closely overlapping doublets integrating to 2.3 protons at 1.03 ppm in the isopropyl methyl region and also a cross-peak with a signal in a cluster of methyl signals at ~0.90 ppm. On the basis of the COSY connectivities and integrated signal areas, the doublets at 3.87 and 3.82 ppm are assigned to H$^\alpha$ signals of the modified valines of the two major diastereomers: the overlapping multiplets at 2.34 ppm, to the isopropyl methinyl protons and the signals at 1.03 and 0.90, to the four diastereotopic isopropyl methyl groups.

A broad, incompletely resolved resonance at 3.47 ppm in Figure 2 appears at a chemical shift appropriate for the methylene resonances of the pyrrolidine rings of the major diastereomeric adducts. The absence of connectivities for this signal at ambient temperature may be reasonably explained by signal breadth and low signal-to-noise ratio. The signal shape suggests rotational averaging on a time scale comparable to the NMR sampling time. Rotation of the pyrrolidine ring around the pyrrolidine N−Val C$^\alpha$ bond is consistent with this behavior. In support of this interpretation, variable

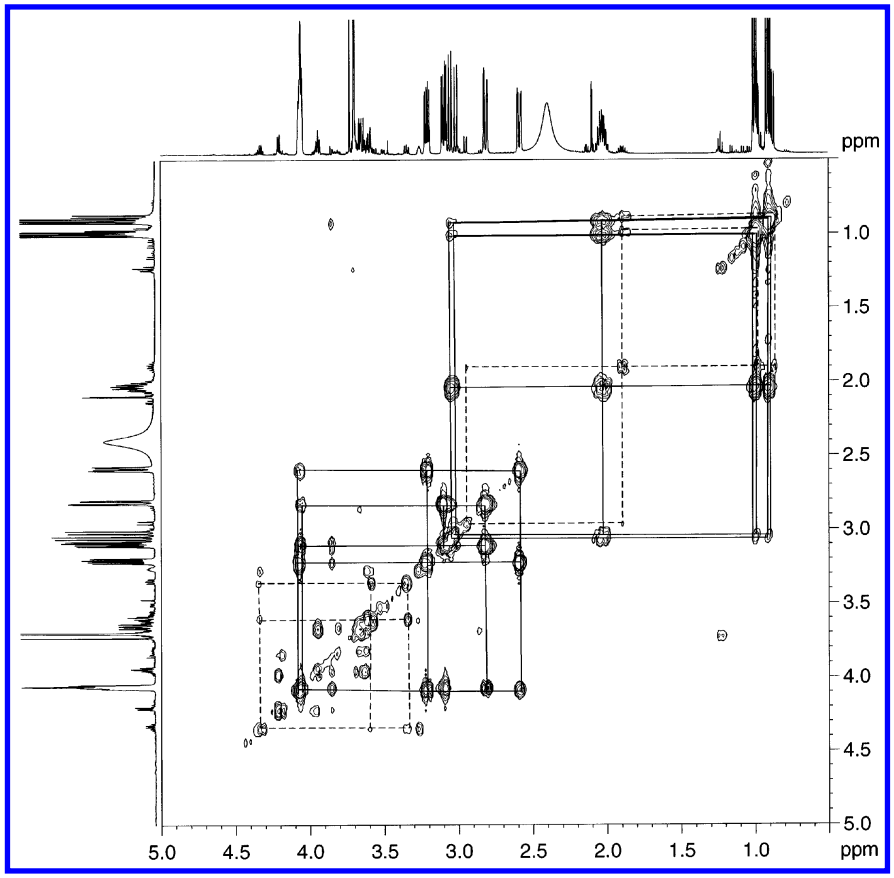
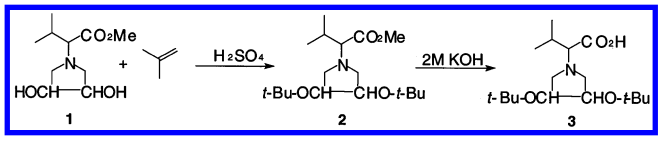


Figure 1. Connectivities in 2D COSY ^{1}H NMR (500 MHz, chloroform-*d*) spectrum of the mixture of methyl 2-(3,4-dihydroxypyrrolidin-1-yl)-3-methylbutyrate diastereomers. Cross-peaks between protons of the major diastereomers are indicated by solid lines, and cross-peaks between protons of the minor diastereomer are indicated by broken lines.

Scheme 2



temperature NMR spectra in Figure 3 show incipient resolution of two sets of signals as temperature increases or decreases from ambient. In the 2D COSY spectrum at 70 °C, a weak cross-peak appears between an emerging doublet centered at 3.45 ppm with $J \approx 10$ Hz and a broadened signal at 4.33 ppm, on the trailing edge of the water peak. Incomplete resolution from the water signal reduces the accuracy of the integral, which is ~2 protons. A second weak cross-peak is present between the signal at 4.33 ppm and a signal at 3.78 ppm, within a complex 5.5 proton multiplet. The low signal-to-noise ratio of the incompletely resolved signals would be expected to give rise to weak cross-peaks, as observed. The shifts of the signals and pattern of connectivities are consistent with pyrrolidine carbinol protons at 4.33 ppm coupled to pyrrolidine methylene protons of one major diastereomer at 3.45 and 3.78 ppm. In Figure 3, a second set of signals at 3.30 and 3.48 ppm with similar relaxation times and rotational averaging is clearly evident in the 50 and 70 °C traces. As a result of the low signal-to-noise ratio and dispersion of these signals, the absence of detectable cross-peaks with the resonance at 4.33 ppm is not surprising. However, this set can reasonably be identified as the methylene resonances of the pyrrolidine ring of the second major diastereomer. The value of the integrated signal at 4.33 ppm (~2 protons), which would then correspond to overlapping carbinol signals for the two major diastereomers, is consistent with this interpretation. The coupling pattern developing in the temperature-dependent traces of the modified peptide mixture is similar to that observed for the methylene and carbinol proton signals of the mixture of diastereomeric Val adducts (Figure 1), although the shifts are different, as expected for the different environment.

For the mixture of isotopomeric peptides to serve as an internal standard, an increase in mass of at least 3 amu over the natural abundance diastereomers is desirable. In the case of the globin α-chain 11-mer, this criterion is most efficiently met by incorporation of a multiply deuterated residue in the peptide. An appropriate deuterium-labeled amino acid was not available as an FMOC derivative. However, L-Leu-5,5,5-d_3 is available in quantity and, for incorporation into the peptide, requires only the single FMOC derivatization procedure, which was accomplished in high yield to give a pure product. Mass spectrometry and ^{1}H NMR confirmed the purity and deuterium content of the derivative. An interesting feature of the NMR spectrum is the diastereotopic character of the aromatic fluorenyl protons at the C2 and C7 peripheral positions and the 9-methylene protons, resulting from conjugation of the 9-fluorenylmethyloxycarbonyl moiety with the L-Leu.

The availability of suitable internal standards is crucial to development of microanalytical procedures to quantitate protein adduct biomarkers in biological matrices. While direct modification of proteins or peptide fragments is often relied upon to generate such standards, this approach cannot usually provide standards of unambigu-

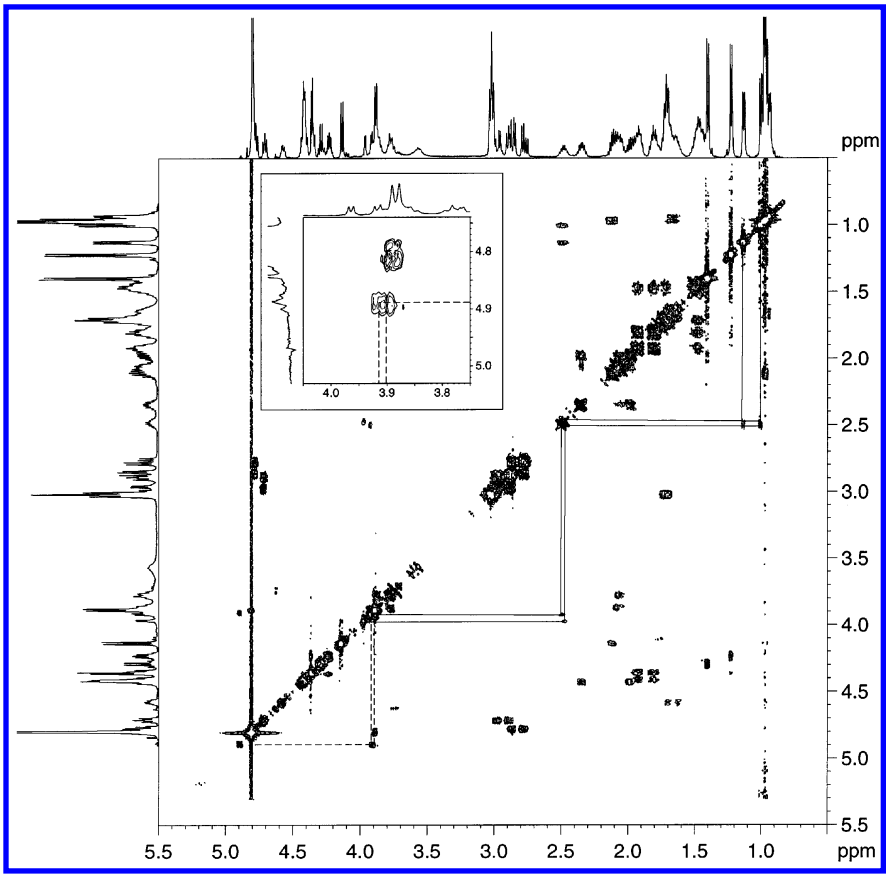


Figure 2. Two-dimensional COSY NMR spectrum (500 MHz, D_2O) of mixture of (pyr-V)LSPADKTNVK 11-mer diastereomers. Connectivities between protons of the major diastereomers are indicated by solid lines, and connectivities between protons of the minor diastereomer are indicated by broken lines. The cross-peak in the inset shows the expected form for coupling between the carbinyl and the pyrrolidine methylene protons of minor diastereomer.

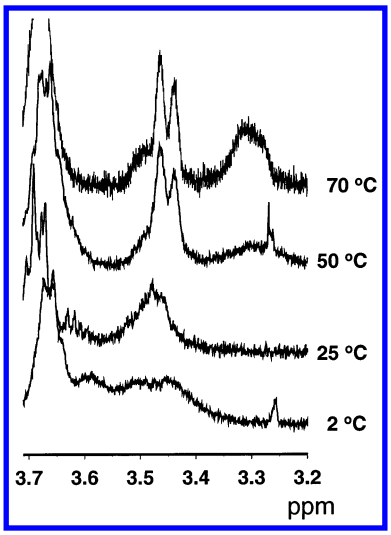


Figure 3. Temperature-dependent ^1H NMR traces (500 MHz, D_2O) of the mixture of diastereomeric modified peptides (pyr-V)LSPADKTNVK. The region shown contains signals of the pyrrolidine $-CH_2-$ protons of the major diastereomers.

ous structure and purity. The work reported here describes a synthesis approach to producing modified oligopeptide standards that should be generally applicable and offers the potential for both specificity and accuracy in quantitation. In addition, the availability of modified peptides in quantity allows exploitation of immunoaffinity chromatography for concentration of biomarkers in biological matrices. Application of immunoaffinity techniques has the potential of greatly enhancing sensitivity with little sample preparation and avoiding generation of artifacts that may accompany other methods of analyte concentration.

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Supporting Information Available: 1D ^1H NMR traces of methyl 2-(3,4-di-*t*-butoxypyrrolidin-1-yl)-3-methylbutyrate diastereomer mixture (**2**) and 2-(3,4-di-*t*-butoxypyrrolidin-1-yl)-3-methylbutyric acid diastereomer mixture (**3**). This material is available free of charge via the Internet at http://pubs.acs.org.

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