HPLC analysis of the freeze-dried residue on a TSK AX-5 column showed the presence of a number of PITC-labeled peaks (Figure 1A). In a control experiment in which the N-GLYCANASE enzyme was omitted, no PITC-labeled peaks eluted between 13 and 18 min. Digestion of the PITC-labeled mixture with jack bean hexosaminidase followed by HPLC analysis demonstrated that the majority of peaks were indeed oligosaccharide derivatives (Figure 1B).

These experiments demonstrate the feasibility of derivatizing (oligosaccharyl)amines obtained from glycoproteins by enzymatic treatment with PNGase F. Reaction with an electrophilic reagent containing an appropriate chromophore provides a simple route to stable oligosaccharide derivatives suitable for chromatographic fractionation and subsequent structural characterization. A variety of reagents have been developed to label the amino group of amino acids. Experiments are in progress to evaluate the relative merits of a number of these methods for preparing derivatives of (oligosaccharyl)amines. One obvious extension of this work is to employ bifunctional electrophilic reagents to produce oligosaccharide derivatives that are suitable for coupling to solid supports, proteins, or other molecules of interest.

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Supplementary Material Available: ^{1}H NMR spectra of the experiments described in the text (3 pages). Ordering information is given on any current masthead page.

Stereospecific Assignment of Leucine Methyl Groups with ^{13}C in Natural Abundance or with Random ^{13}C Labeling†

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The precision of structures of peptides and proteins is increased if the stereochemical assignments of diastereotopic atoms or groups are known.[1] Methods for the measurement of χ_1 angles using a combination of homonuclear H_β,H_α couplings from E.COSY[2,3] type experiments and $^{15}N,H_\beta$[4,5] or $^{13}C',H_\beta$ coupling constants[5-7] have been developed. A general method for the stereochemical assignment of methyl groups in leucines or valines has been presented which is based on diastereoselective carbon labeling.[1]

In this communication we present a heteronuclear NMR method for the stereospecific assignment of leucine methyl groups. This method is applicable both to polypeptides with carbon in natural abundance or randomly labeled polypeptides with ^{13}C at least in the δ positions. The method requires the stereochemical assignment of the H_β protons. Also the signals of the two methyl groups must be resolved either in the proton or in the carbon

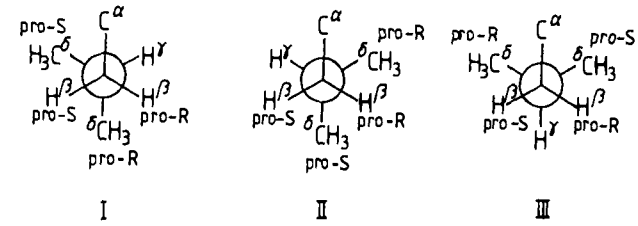

Figure 1. Staggered conformations around the C_β–C_γ bond of leucine.

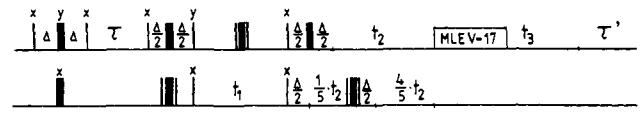

Figure 2. BIRD-3D-HSQC-TOCSY for molecules with ^{13}C in natural abundance. For the cyclolinopeptide spectrum, $t_3 + \tau' = 472$ ms, $\tau = 170$ ms, which fulfills the equation, $1 - \exp[-(t_3 + \tau')/T_1) = \exp(\tau/T_1) - 1$, for the shortest proton T_1 in the molecule of 260 ms. A mixing time of 120 ms was used in the TOCSY to enhance multistep proton–proton cross peaks. Scaling of the heteronuclear coupling to 0.6 of its value in t_2 was performed to avoid overlap of cross peaks from geminal protons in other regions of the spectrum (not shown). $90_x180_y90_x$ pulses were used as inversion pulses for longitudinal magnetization. Eight scans per t_1 (64 experiments, 6037 Hz) and t_2 (384 experiments, 4424 Hz) were recorded with 3072 points in t_3. The spectrum was Fourier transformed with Felix[17] to 64 × 512 × 2k real points.

domain. The measurement of the $^3J(H_\beta,C_\delta)$ and, if available, the $^3J(H_\beta,H_\gamma)$ couplings leads to the stereochemical assignment of the δ methyl groups. Assuming the staggered conformations about the C_β–C_γ bond (Figure 1) are in equilibrium, the relevant coupling constants reflecting the populations p_i are given by the Pachler equations.[8,9] $J' = (J - J_{\text{synclinal}})/(J_{\text{antiperiplanar}} - J_{\text{synclinal}})$ is a dimensionless reduced coupling:

$$J'(H_\gamma,H_\beta^{pro\text{-}S}) = J'(C_\delta^{pro\text{-}S},H_\beta^{pro\text{-}R}) = p_I$$

$$J'(H_\gamma,H_\beta^{pro\text{-}R}) = J'(C_\delta^{pro\text{-}R},H_\beta^{pro\text{-}S}) = p_{II}$$

$$J'(C_\delta^{pro\text{-}R},H_\beta^{pro\text{-}R}) = J'(C_\delta^{pro\text{-}S},H_\beta^{pro\text{-}S}) = p_{III}$$

The stereochemical assignment of the methyl groups can be achieved whenever at least one of the conformations is populated differently than the others. This is no restriction to the generality of the method since NMR parameters such as NOEs or couplings of the two methyl groups show no difference if all populations are equal.

At least three coupling constants are necessary for the diastereospecific assignment: $J(H_\gamma,H_\beta^{pro\text{-}S})$, $J(H_\gamma,H_\beta^{pro\text{-}R})$, and one of the $J(C_\delta,H_\beta)$ couplings. The heteronuclear $J(C_\delta,H_\beta)$ couplings must reflect one of the conformations that is distinct in population from the others. If the homonuclear couplings are not available, all heteronuclear couplings need to be determined, and the stereochemical assignment follows from the requirement:

$$J(C_\delta^{pro\text{-}R},H_\beta^{pro\text{-}R}) = J(C_\delta^{pro\text{-}S},H_\beta^{pro\text{-}S})$$

The pulse sequence employed, HSQC-TOCSY either in two or three dimensions, has the same basic principles as the 3D HMQC-NOESY sequence introduced for the measurement of $^{15}N,H_\beta$ couplings in proteins.[4] Three dimensions are necessary if the diastereotopic δ methylprotons are not resolved or lie in a congested region.

We demonstrate the method with leucine[8] in cyclolinopeptide A, cyclo(Pro^1-Pro^2-Phe^3-Phe^4-Leu^5-Ile^6-Ile^7-Leu^8-Val^9). The proton signals of the two δ methyl groups overlap completely. Therefore we employed the 3D version of the experiment. We combined the pulse sequence with a BIRD[10] pulse (Figure 2) to

† Dedicated to Professor G. Quinkert on the occasion of his 65th birthday.

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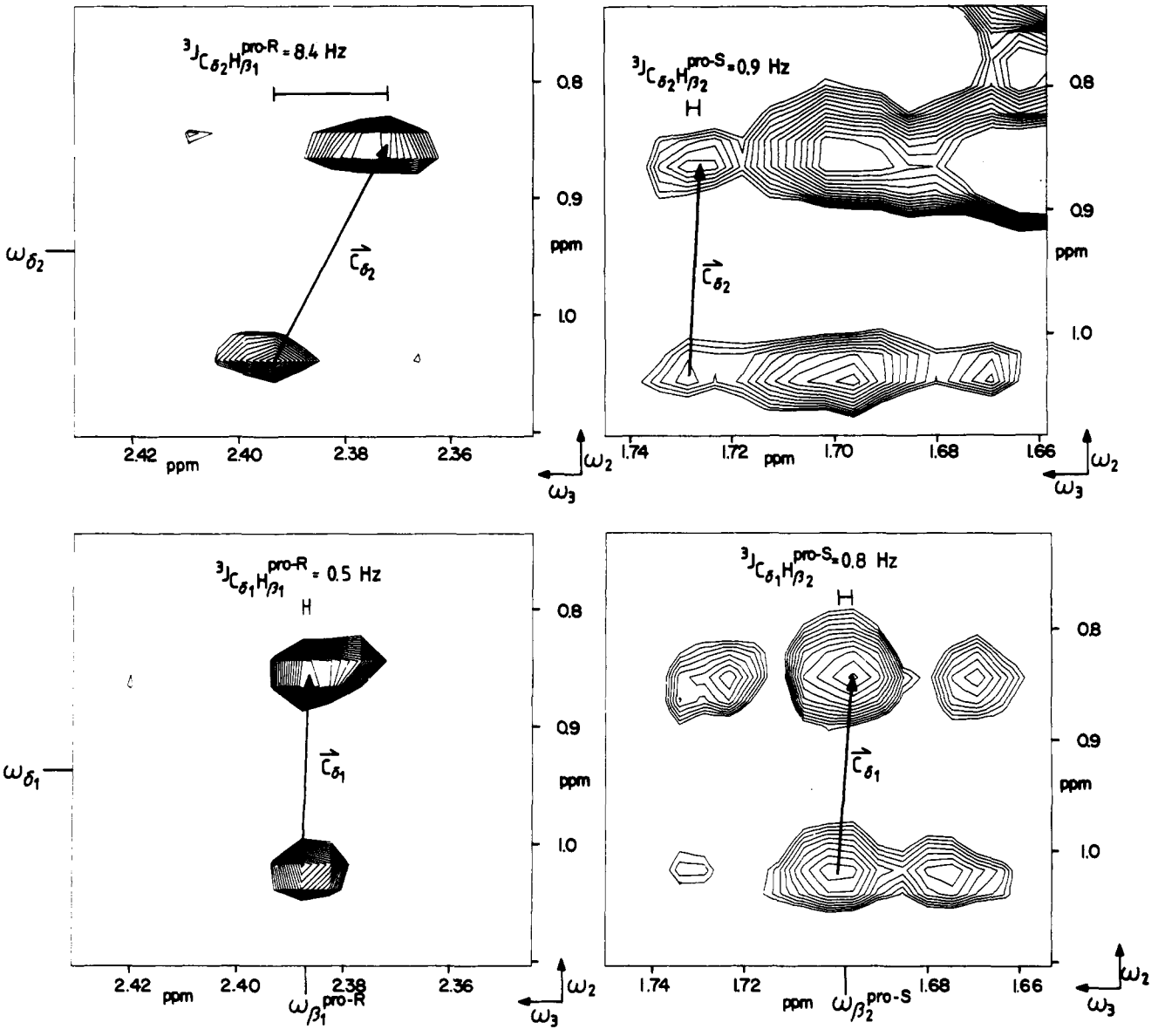

Figure 3. Slices through the 3D HSQC–TOCSY showing the four $C_\delta,H_\delta,H_\beta$ cross peaks with the displacement vectors due to the C_δ's. The two lower traces are taken at $\omega_1 = \delta(C_{\delta 1}) = 23.6$ ppm and the two upper traces at $\omega_1 = \delta(C_{\delta 2}) = 20.2$ ppm.

suppress magnetization of protons bound to $^{12}C^{11}$ in the isotopically unlabeled peptide. The relevant cross peaks $C_{\delta 1/2},H_\beta^{pro\text{-}R/S}$ are shown in Figure 3. (The numerical indices 1 and 2 refer to the low-field and the high-field resonances.) The following coupling constants can be extracted: $J(C_{\delta 2},H_{\beta 1}^{pro\text{-}R})$ = 8.4 Hz; $J(C_{\delta 1},H_{\beta 1}^{pro\text{-}R})$ = 0.5 Hz; $J(C_{\delta 2},H_{\beta 2}^{pro\text{-}S})$ = 0.9 Hz; $J(C_{\delta 1},H_{\beta 2}^{pro\text{-}S})$ = 0.8 Hz. From a P.E.COSY spectrum[2,3,12] we obtain the following: $J(H_\gamma,H_{\beta 2}^{pro\text{-}R})$ = 3.6 Hz and $J(H_\gamma,H_{\beta 1}^{pro\text{-}S})$ = 12.7 Hz. The stereochemical assignment of the H_β protons had been obtained from $J(H_\alpha,H_\beta^{pro\text{-}R})$ = 12.6 Hz, $J(H_\alpha,H_\beta^{pro\text{-}S})$ = 3.6 Hz, and the very small $J(C',H_\beta^{pro\text{-}R})$ couplings observed in an HMBC[13] experiment ($\chi_1 = -60°$).[5,16]

The values of the $J(H_\gamma,H_\beta)$ coupling constants indicate that conformation I prevails, so the large $J(C_{\delta 2},H_\beta^{pro\text{-}R})$ coupling is sufficient to assign the $C_{\delta 2}$ to the *pro-S* position. Even if the H_γ,H_β coupling constants were not known, δ_1 could be assigned to *pro-R* and δ_2 could be assigned to *pro-S* using the fact that $J(C_{\delta 1},H_\beta^{pro\text{-}R})$ and $J(C_{\delta 2},H_\beta^{pro\text{-}S})$ are of equivalent size (0.5 Hz and 0.9 Hz, respectively) but $J(C_{\delta 2},H_\beta^{pro\text{-}S})$ = 8.4 Hz and $J(C_{\delta 1},H_\beta^{pro\text{-}R})$ = 0.5 Hz are of different size.

For proteins, the BIRD–HSQC–TOCSY would be replaced by an HSQC–NOESY experiment since the transfer via NOE is more efficient than via scalar couplings for proteins. For completely ^{13}C-labeled proteins the homonuclear H_β,H_γ couplings would be measured by an INEPT–constant time-C,C-COSY–β-INEPT experiment[14,15] instead of an E.COSY type experiment.

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