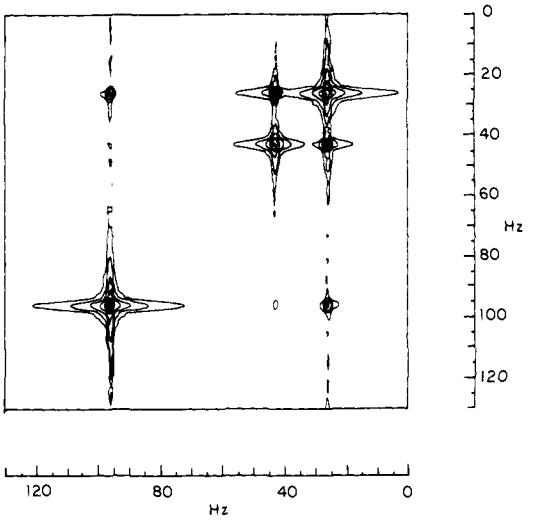

Figure 2. Two-dimensional ^2H–^2H magnitude mode optimized COSY spectrum of pyridine-d_5 under identical conditions. Optimal mixing time: 1.056 s.

spin lock is *in-phase*, while the transfer under INEPT, being *anti-phase*, would be partially cancelled out in the resulting unresolved multiplet.

These ideas were investigated on several perdeuterated molecules, including ethanol-d_6, THF-d_8, and pyridine-d_5. In each case, a neat sample was run; a COSY experiment optimized for small couplings was compared with a TOCSY experiment employing a WALTZ-16[15,16,17] mixing sequence, with the radio frequency (rf) field strength for mixing being in the range 1.5–2.5 kHz. It may be noted that the rf power employed during spin lock is typically between 0.5 and 1.0 W, which leads to no detectable heating at sensible repetition rates. The relative efficiencies of the laboratory-frame COSY experiment and the rotating-frame TOCSY experiment were quantified both with respect to the absolute intensities of the diagonal and cross peaks, as well as to the ratio of diagonal to cross peak intensities. In each case, both of these parameters were decidedly more favorable for the TOCSY experiment, typically by a factor of between 2 and 3 as expected from the theory outlined above.

Figure 1 shows the TOCSY spectrum of pyridine-d_5, for which the calculated magnitudes of the couplings[3,18] are as follows: J_{23} = 0.115 Hz, J_{34} = 0.181 Hz, and J_{24} = 0.045 Hz. While the data of Figure 1 were acquired in about 85 min, a run could in fact be performed in just about 10 min with no significant degradation of spectral quality. It is remarkable that the relay mechanism inherent in the spin lock experiment[5,6,7] results in a clear 2–4 cross-peak, although this coupling is less than 0.05 Hz! From the flip angle dependence of selective 1D pulsed spin lock experiments, we estimate that ROESY contributions to the cross-peak intensity under our conditions amount to less than 2%. This spectrum may be compared against the optimized COSY spectrum of Figure 2, displayed at a 4-fold intensity magnification with respect to Figure 1; in fact, both our optimized COSY and RELAY runs not only have much inferior sensitivity, but lead to a mere suspicion of the 2–4 cross-peak.

Given the fact that deuterium correlation spectroscopy exhibits no 2D spectral complexity owing to multiplet structure and given the further circumstance that isotropic mixing would be almost ideally behaved for this isotope, whose chemical shift range is just about 925 Hz on a 600-MHz machine, we believe that, the limited shift resolution notwithstanding, TOCSY studies on the perdeuterated species would be a most interesting possibility for structure elucidation of biomolecules, line widths permitting.

Acknowledgment. This work was motivated by the elegant research and interest of Professor H. Günther and his group, Siegen Univ. Gesamthochschule, FRG. N.C. also acknowledges the interest of Dr. D. Moskau, Spectrospin AG. A.R. gratefully acknowledges a CSIR fellowship.

Registry No. Pyridine-d_5, 7291-22-7.

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Identification and Derivatization of (Oligosaccharyl)amines Obtained by Treatment of Asparagine-Linked Glycopeptides with N-GLYCANASE Enzyme

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The initial steps in the structural analysis of the asparagine (Asn)-linked oligosaccharides of a glycoprotein generally consist of chemical[1] or enzymatic[2] hydrolysis of the *N*-acetylglucosaminylasparagine bond between each oligosaccharide chain and the polypeptide, derivatization of the released oligosaccharide chains with a radioactive or fluorescent tag, and chromatographic fractionation of the labeled oligosaccharides. The purified oligosaccharides can be further characterized by ^1H NMR spectroscopy, mass spectroscopy, methylation analysis, and exoglycosidase digestions. Current methods for labeling the released oligosaccharides involve derivatization of the hemiacetal moiety at the reducing terminus of the sugar chain, for example, by reduction with sodium borotritide[1] or reductive amination with a chromophore such as 2-aminopyridine[3] (Scheme I, pathway b). We report here an alternative approach to labeling Asn-linked oligosaccharides that is based on our observation that treatment of glycopeptides with a peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase (PNGase, EC 3.5.1.52) generates a set of (oligosaccharyl)amine intermediates that can be derivatized with electrophilic reagents (Scheme I, pathway a).

PNGase from almond emulsin (PNGase A) has been shown to be an amidase by ^1H NMR spectroscopic experiments.[4] The preferred PNGase for releasing Asn-linked oligosaccharides from glycoproteins is obtained from *Flavobacterium meningosepticum* (PNGase F, N-GLYCANASE enzyme).[5] PNGase F has a broader substrate specificity, a lower molecular weight (35 100 vs 68 000 Da), and a higher pH optimum (8.5 vs 5.0) than PNGase A.[6] It also seems likely that PNGase F would be a superior

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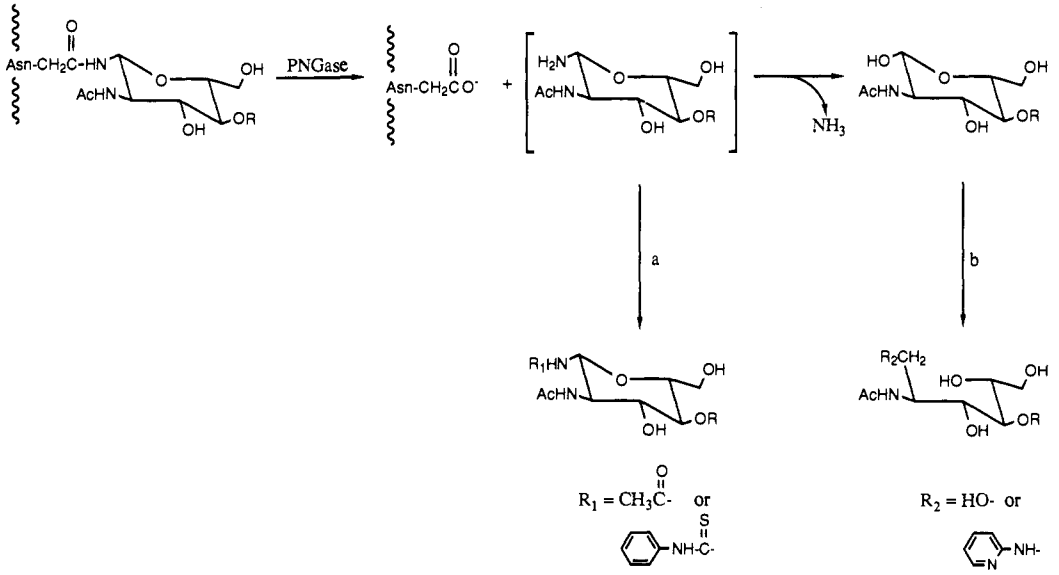
Scheme I



enzyme for generating a set of (oligosaccharyl)amine intermediates because it is known that glycosylamines are more stable at pH 8.5 than 5.0.[7]

In a set of preliminary experiments using techniques employed in the study of the PNGase A mechanism of action,[4] we confirmed that PNGase F is also an amidase. Thus, two units of N-GLYCANASE enzyme (Genzyme) catalyzed the hydrolysis of turkey ovomucoid glycopeptide[8] at p^{2}H 9.0 and 37.5 °C with a pseudo-first-order rate constant of 1.35×10^{-5} s^{-1}. Two ^{1}H NMR signals were used to follow the hydrolysis of the substrate—the *N*-acetyl group (2.000 ppm) and the anomeric proton (5.020 ppm) of the terminal, Asn-linked GlcNAc residue.[9] As the hydrolysis reaction proceeded the intensity of these two signals decreased, and a new signal for the *N*-acetyl group of the GlcNAc at the reducing end of the carbohydrate moiety appeared at 2.042 ppm. No NMR signal corresponding to the α-anomeric proton of the terminal GlcNAc residue of the fully hydrolyzed, free carbohydrate (5.188 ppm) was observed over a 48-h period. This result indicates that less than 10% of the (oligosaccharyl)amines were converted to ammonium ion and free oligosaccharides. At longer incubation times the presence of the free oligosaccharide became detectable. Acidification to p^{2}H 5.7 with 5 M [$^2H_4$]acetic acid completely hydrolyzed the mixture of (oligosaccharyl)amines within 2 h as judged by the sharpening of the *N*-acetyl peaks and the appearance of a signal for the α-anomeric proton of the terminal GlcNAc residue.

These results confirm that PNGase F is an amidase and, more importantly, that conditions can be chosen for which this enzyme generates a surprisingly stable group of (oligosaccharyl)amine intermediates at pH ranges near those where the enzyme exhibits its maximal activity.

It occurred to us that if the (oligosaccharyl)amines could be trapped with electrophilic reagents, then it should be possible to develop simple methods for derivatizing Asn-linked oligosaccharides for subsequent fractionation and analysis. In preliminary experiments it was found that incubation of the (oligosaccharyl)amines released from turkey ovomucoid glycopeptide with acetic anhydride gave a high yield of the corresponding *N*-acetyl (oligosaccharyl)amines.[10] In a subsequent experiment, a tryptic hen ovomucoid glycopeptide (10.3 mg) containing the Asn-53 glycosylation site[11] was incubated with 60 units of N-GLYCANASE enzyme in 0.6 mL of 0.2 M sodium phosphate, pH 7.5, for 4 h at 37 °C. At the end of this time the amidase reaction was 85% complete as judged by HPLC analysis. The reaction mixture was frozen and lyophilized. The lyophilized solid was dissolved in 500 μL of water and stirred with a solution of phenyl isothiocyanate (500 μL) in 3.5 mL of methanol at 20 °C for 1 h. The reaction mixture was extracted with four 6-mL portions of $CHCl_3$, and the aqueous layer was passed through a column of Amberlite 3 resin to remove salts and peptide species.

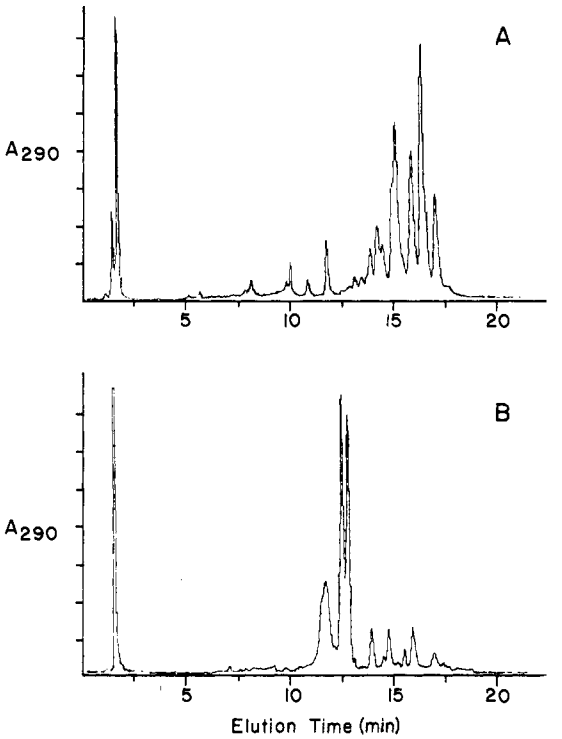


Figure 1. HPLC analysis of PITC-labeled (oligosaccharyl)amines derived from hen ovomucoid glycopeptide before (A) and after (B) digestion with jack bean hexosaminidase. Analysis was performed on a TSK AX-5 column. The mobile phase was a 20-min gradient beginning with 80% CH_3CN/20% H_2O and ending with 10% CH_3CN/90% H_2O. The flow rate was 2 mL/min, and the UV absorbance of the eluant was monitored at 290 nm.

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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

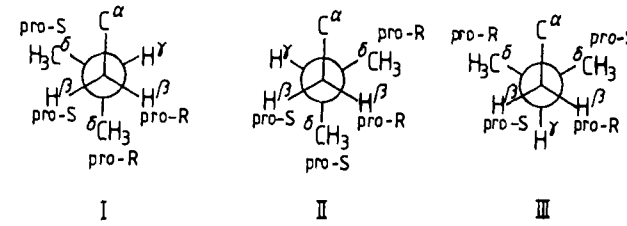

Figure 1. Staggered conformations around the C_β–C_γ bond of leucine.

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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