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^{1}H NMR Study of Glycopeptides with the Triplet Sequence –Asn–Gly–Ser–

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ABSTRACT: The conformation and intramolecular interactions in solution were investigated for the glycopeptides, Boc–Asn(GlcNAc)–OMe, Boc–Asn(GlcNAc)–Gly–OMe, and Boc–Asn(GlcNAc)–Gly–Ser–OMe with the aids of ^{1}H NMR. There are no specific intramolecular interactions in glycopeptides and the hydrogen bond between the side chains of Asn and Ser was not found in Boc–Asn(GlcNAc)–Gly–Ser–OMe contrary to that in Boc–Asn–Gly–Ser–OMe. The side chain conformation of the triplet sequence is considerably affected by *N*-glycosylation.

KEY WORDS Glycopeptide / Conformation / Intramolecular Interaction / Triplet Sequence / NMR /

It is now well known that carbohydrates are attached to Asn side chains through *N*-glycosidic linkages in glycoproteins.[1] The Asn residue must be a part of the triplet sequence –Asn–X–Ser(or Thr)–, which is a prerequisite to enzymatic glycosyl transfer.[2,3] Here, X is an arbitrary amino acid residue except for Pro. It has been shown that glycosylated Asn residues are frequently located within tetrapeptides having a β-turn conformation.[4,5] The role of the hydroxyl amino acid in the triplet sequence has been studied by several authors. Marshall has proposed, using the space filling model of the sequence –Asn–Leu–Thr–, that there is a hydrogen bond between the donor hydroxyl proton of Thr and the carbonyl group of Asn.[1] Recently, conformational energy calculation has revealed that the same type of intramolecular interaction occurs in Ac–Asn–Ala–Thr–NH_2.[6] Bause has proposed another type of interaction within the triplet sequence on the basis of enzymatic *N*-glycosylation studies of peptides.[7] This interaction is the hydrogen bond between the amide hydrogen of the Asn side chain and the hydroxyl oxygen of the hydroxyl amino acid. This type of intramolecular interaction was found by NMR spectroscopy in the compound Boc–Asn–Gly–Ser–OMe by the present authors.[8]

Compared to the triplet sequence –Asn–X–Ser(or Thr)–, little is known about the conformation and intramolecular interactions of –Asn(Carb)–X–Ser(or Thr)–, *i.e.*, the glycosylated triplet sequence. Here, Carb represents the carbohydrate moiety. The possible conformational perturbation due to *N*-glycosylation has been evaluated for Asn and Asn-containing dipeptides.[9] It is of interest to investigate the conformational features of glycopeptides having the triplet sequence.

In this paper we report on the NMR conformational study of Boc–Asn(GlcNAc)–OMe, Boc–Asn(GlcNAc)–Gly–OMe, and Boc–Asn(GlcNAc)–Gly–Ser–OMe. Here, GlcNAc represents 2-acetamide 3,4,6-tri-*O*-acetyl β-D-glucopyranose. The structure of –Asn-(GlcNAc)– was shown in Figure 1. The last glycopeptide is a glycosylated derivative of Boc–Asn–Gly–Ser–OMe, whose conformation has been analyzed.[8]

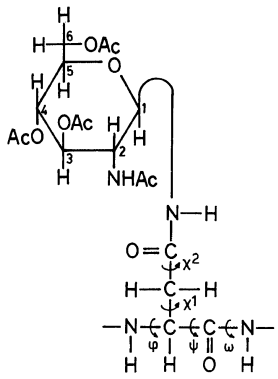

Figure 1. Structure and atom numbering of glycosylated Asn residue.

EXPERIMENTAL

Glycopeptide Syntheses

The glycopeptides were synthesized by coupling of the corresponding peptides containing Asp with 2-acetamide 3,4,6-tri-*O*-acetyl β-D-glucopyranosylamine.[10] The starting materials, Asp, Gly, and Ser were purchased from Kokusan Kagaku, Tokyo, Japan. Boc–Asp(OBzl) was prepared from Asp(OBzl)[11] with *tert*-butyl S-4,6-dimethylpyrimidin-2-ylthiolcarbonate. Gly–OMe and Ser–OMe were prepared by the procedure of Brenner and Huber.[12]

Boc–Asp–OMe

Boc–Asp(OBzl) (9.7 g; 30 mmol) was dissolved in tetrahydrofuran and cooled to −20°C. To this solution triethylamine (4.2 ml; 30 mmol) and isobutyl chloroformate (3.9 ml; 30 mmol) were added. After 2 min MeOH (10 ml) was added and stirred overnight at room temperature. The solvent was evaporated and the residue was dissolved in ethyl acetate. The organic layer was successively washed with 5% $NaHCO_3$, H_2O, 10% citric acid, and H_2O and dried over Na_2SO_4. The solvent was evaporated to leave Boc–Asp(OBzl)–OMe as a white crystal, which was recrystallized from MeOH. The benzyl group was removed by hydrogenation in the presence of Pd-charcoal.

Boc–Asp–Gly–OMe

To a dimethylformamide solution of Gly–OMe (2.5 g; 20 mmol), *N*-methylmorpholine (2.4 ml; 20 mmol), and Boc–Asp(OBzl) (6.46 g; 20 mmol), dicyclohexylcarbodiimide (4.9 g; 24 mmol) was added at 0°C and stirred overnight. After filtration of dicyclohexylurea, the solvent was evaporated and the residue was dissolved in ethyl acetate. The organic layer was successively washed with 5% $NaHCO_3$, H_2O, 10% citric acid, and H_2O and dried over Na_2SO_4. The solvent was evaporated to leave Boc–Asp(OBzl)–Gly–OMe, which was recrystallized from ethyl acetate–petroleum ether. The benzyl group was removed as described for Boc–Asp–OMe.

Boc–Asp–Gly–Ser–OMe

A dimethylformamide solution of Z–Gly (4.2 g; 20 mmol),[13] Ser–OMe (3.1 g; 24 mmol), *N*-methylmorpholine (2.4 ml; 20 mmol), and dicyclohexylcarbodiimide (4.9 g; 24 mmol) was stirred for 5 h at 0°C. The workup as described for Boc–Asp(OBzl)–Gly–OMe gave Z–Gly–Ser–OMe. The Z group was removed by hydrogenation in the presence of Pd–charcoal. To a dimethylformamide solution of Gly–Ser–OMe (2.5 g; 12 mmol), *N*-methylmorpholine (1.4 ml; 12 mmol), and Boc–Asp(OBzl) (3.9 g; 12 mmol), dicyclohexylcarbodiimide (3.0 g; 14 mmol) was added at 0°C and stirred overnight. The workup as described for Boc–Asp(OBzl)–Gly–OMe gave Boc–Asp(OBzl)–Gly–Ser–OMe, which was recrystallized from ethyl acetate. The benzyl group was removed as described for Boc–Asp–OMe.

Boc–Asn(GlcNAc)–OMe

To a dimethylformamide solution of Boc–Asp–OMe (4.0 g; 16 mmol) and *N*-hydroxysuccinimide (2.2 g; 19 mmol), dicyclohexylcarbodiimide (4.0 g; 19 mmol) was added at 0°C and stirred for 3 h. After filtration of the dicyclohexylurea, 2-acetamide 3,4,6-tri-*O*-

acetyl glucopyranosylamine (6.0 g; 16 mmol) was added to the solution and stirred for 2 days at room temperature. After removal of small amounts of impurities by filtration, the solvent was evaporated and the residue was dissolved in ethyl acetate. The organic layer was successively washed with 5% $NaHCO_3$, H_2O, 10% citric acid, and H_2O and dired over Na_2SO_4. The solvent was evaporated to leave crystals which were recrystallized from ethyl acetate-petroleum ether.

Boc–Asn(GlcNAc)–Gly–OMe

Boc–Asp–Gly–OMe (3.0 g; 9.3 mmol), *N*-hydroxysuccinimide (1.1 g; 9.3 mmol), and dicyclohexylcarbodiimide (2.2 g; 11 mmol) were stirred in dimethylformamide for overnight. After filtration of the dicyclohexylurea, 2-acetamide 3,4,6-tri-*O*-acetylglucopyranosylamine (3.5 g; 3 mmol) was added to the solution and stirred for 2 days. Small amounts of insoluble materials were filtered off and the solvent was evaporated. The obtained white solid was washed with ethyl acetate and then recrystallized from MeOH.

Boc–Asn(GlcNAc)–Gly–Ser–OMe

1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1.2 g; 4.9 mmol) was added to a $CHCl_3$ solution of Boc–Asp–Gly–Ser–OMe (2.0 g; 4.9 mmol) and 2-acetamide 3,4,6-tri-*O*-acetylglucopyranosylamine (1.8 g; 4.9 mmol) at 0°C. The mixture was stirred for overnight while slowly warming it to room temperature. The organic layer was successively washed with 5% $NaHCO_3$, H_2O, 10% citric acid, and H_2O, and dried over Na_2SO_4. The solvent was evaporated and the obtained white solid was washed with ethyl acetate, and then recrystallized from ethyl acetate–MeOH.

De-*O*-acetylation of the glycopeptides, Boc–Asn(GlcNAc)–NHMe, Boc–Asn(GlcNAc)–Gly–NHMe, and Boc–Gly–Asn(GlcNAc)–NHMe[9] was achieved by triethylamine in MeOH.

NMR Measurements

^{1}H NMR spectra of glycopeptides (0.03 M solution) were recorded on JEOL PS-100 and FX-270 spectrometers operating at 100 and 270 MHz, respectively. Hexadeuterated dimethylsulphoxide (DMSO-d_6) was used as the solvent. Chemical shifts are reported in ppm from internal tetramethylsilane with an accuracy of 0.001 ppm. The error in the coupling constants was ± 0.3 Hz. The assignments were carried out by selective homo-spin decoupling and a comparison of spectra with each other. We assigned two Asn β protons in a stereochemical sense, according to the customary assignment.[11]

RESULTS AND DISCUSSION

In Table I are tabulated the chemical shift (δ) and vicinal coupling constants (^3J) of the amide protons. The chemical shifts of N^1H and N^2H are comparable for all three glyco-

Table I. Chemical shifts (ppm) and the vicinal proton coupling constants (Hz) of glycopeptides in 0.03 M DMSO-d_6 solutions at 298 K

	N^2H		N^1H		Asn NH		Gly NH		Ser NH	
	δ	J	δ	J	δ	J	δ	J	δ	J
Boc–Asn(GlcNAc)–OMe	7.90_0	9.2_3	8.63_3	9.2_3	7.01_1	7.9_1				
Boc–Asn(GlcNAc)–Gly–OMe	7.88_8	8.9_0	8.53_4	9.2_2	6.80_2	8.2_4	8.12_9	5.6_0 5.2_8		
Boc–Asn(GlcNAc)–Gly–Ser–OMe	7.87_0	8.9_0	8.56_9	8.9_0	6.82_2	7.1_3	7.97_3	n.d.[a]	8.13_7	7.9_2

[a] Not determined.

Table II. Vicinal coupling constants (Hz) and rotamer populations[a] for Asn side chain of glycopeptides and peptide in 0.03 M DMSO-d_6 solutions at 298 K

	J_{AX}	J_{BX}	P_{g^-}	P_t	P_{g^+}
Boc–Asn(GlcNAc)–OMe	6.9_2	6.1_0	0.39	0.32	0.29
			(0.41)	(0.34)	(0.25)
Boc–Asn(GlcNAc)–Gly–OMe	8.5_6	4.2_8	0.54	0.15	0.31
			(0.62)	(0.17)	(0.21)
Boc–Asn(GlcNAc)–Gly–Ser–OMe	7.6_2	5.4_2	0.46	0.26	0.28
			(0.50)	(0.28)	(0.22)
Boc–Asn–Gly–Ser–OMe[b]	5.9_3	8.5_7	0.30	0.54	0.16
			(0.26)	(0.62)	(0.12)

[a] The population values were calculated using the approximations of Pachler and Feeney (in parentheses).
[b] From ref 8.

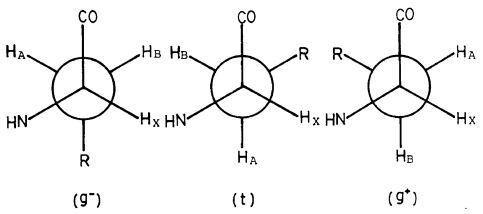

Figure 2. Three stable rotamers about the C^{α}–C^{β} bond. The values of the dihedral angle χ^1 are $-60°$, 180°, and 60° for (g^-)-, (t)-, and (g^+)-rotamers, respectively. R = CO-carbohydrate.

peptides. Here, N^1H and N^2H denote the amide groups at sugar carbons 1 and 2, respectively (for the numbering see Figure 1). The vicinal coupling constants ^3J (HNCH) of the glycopeptides are large for N^1H and N^2H protons. This is in good agreement with those of glycopeptides[9] and glycosylated Asn in aqueous solution.[15] The orientation of the carbohydrate may be determined by χ^1 and χ^2 rotational angles.

Side chain conformation of glycosylated Asn was estimated by the analysis of proton coupling constants. 270 MHz ^1H NMR resonances of α and β protons of Asn residue can be analyzed as an ABX-spin system. In Table II are tabulated the proton coupling constants ^3J ($HC^{\alpha}C^{\beta}H$) and relative populations of the three rotamers, P_{g^-}, P_t, and P_{g^+}, about the C^{α}–C^{β} bond of the Asn side chain (see Figure 2). The populations were calculated using the approximations of Pachler[16] and Feeney[17] (shown Table II, in parentheses). It is clear that the (g^-)-rotamer is the most preferred for the glycosylated Asn side chain in these glycopeptides. The predominance of (g^-)-rotamer is also observed for glycosylated peptides,[9] although it has been proposed that the most preferred conformer of glycosylated Asn is the (t)-rotamer[15] or (g^+)-rotamer.[18] The predominance of the (g^-)-rotamer may be due to the bulkiness of the side chain and/or the absence of any significant side chain-side chain interactions. There are some differences in rotamer populations. The values P_{g^+} are comparable for all three glycopeptides, while the values of P_{g^-} and P_t are dependent on the type of peptide sequence. It is unlikely that there is a specific stable conformation for the Asn side chain in the glycopeptide with the triplet sequence.

The side chain conformation of Asn in Boc–Asn(GlcNAc)–Gly–Ser–OMe differs from that in the corresponding nonglycosylated tripeptide, Boc–Asn–Gly–Ser–OMe.[8] In Boc–Asn(GlcNAc)–Gly–Ser–OMe, the (g^-)-rotamer is the most populated, whereas the largest value was observed for the (t)-rotamer in Boc–Asn–Gly–Ser–OMe. The side chain conformation was influenced by the glycosylation of Asn side chain in –Asn–Gly–Ser– sequence. The predominance of the (t)-rotamer for the Asn side chain has been in-

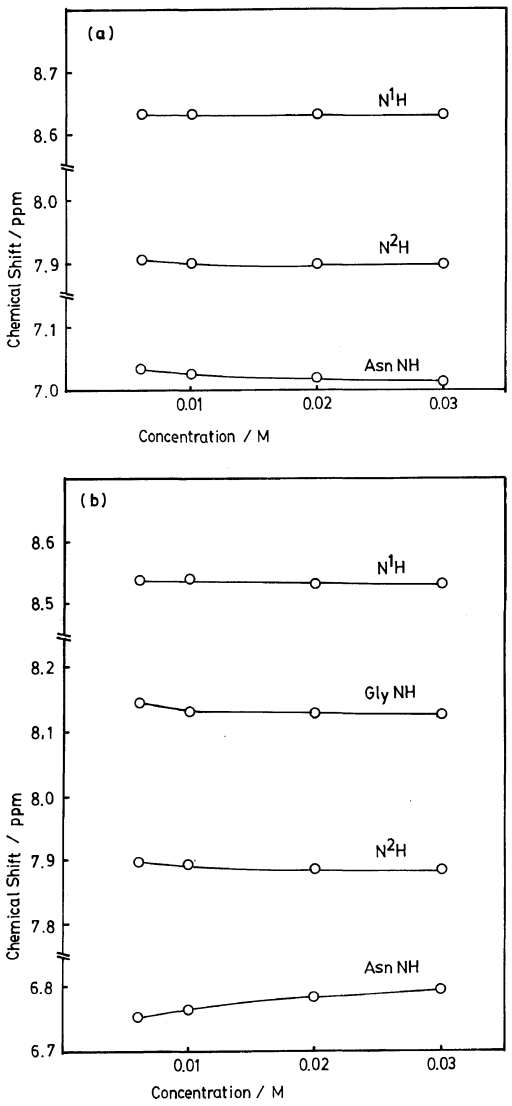

Figure 3.

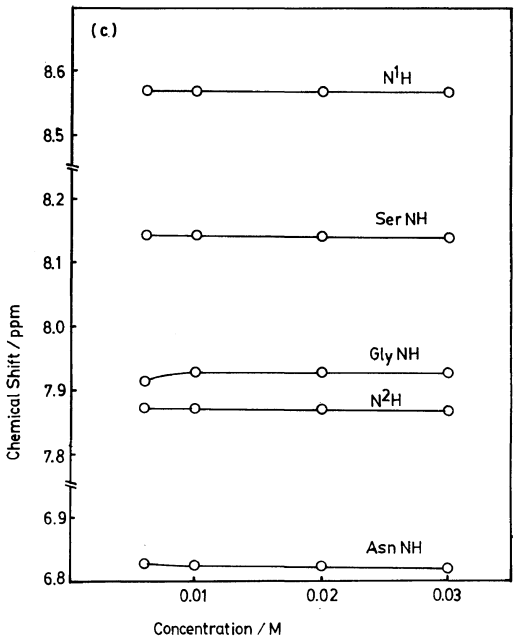

Figure 3. Concentration dependence of the amide proton chemical shifts (a) Boc–Asn(GlcNAc)–OMe, (b) Boc–Asn(GlcNAc)–Gly–OMe, (c) Boc–Asn(GlcNAc)–Gly–Ser–OMe.

terpreted in terms of the intramolecular interaction between the Asn and Ser side chains in Boc–Asn–Gly–Ser–OMe.[8] The results suggest that the intramolecular interactions between the side chains in Boc–Asn(GlcNAc)–Gly–Ser–OMe are not the same type as those in Boc–Asn–Gly–Ser–OMe. Since the coupling constants for Ser side chain protons were not observed due to spectral overlapping, we could not estimate the rotamer population for the Ser side chain.

Concentration and temperature dependence of the amide proton chemical shifts was measured to investigate the hydrogen bonding tendencies. In Figure 3 is shown the concentration dependence of the amide proton chemical shifts. Little change with dilution was observed for any of the amide protons, especially Boc–Asn(GlcNAc)–Gly–Ser–OMe. This indicates the absence of any specific intermolecular interactions involving amide protons. Thus, these glycopeptides exist without aggregation in DMSO-d_6.

The values of the temperature dependence, $d\delta/d(1/T)$, of the amide proton chemical shifts are tabulated in Table III. The $d\delta/d(1/T)$ values are adopted instead of the commonly used $d\delta/dT$ values because of the clear physical meaning of the former. The $d\delta/dT$ values are also tabulated (shown in parentheses) for convenience of the comparison with literature values.

Table III. Temperature dependence, $d\delta/d(1/T)$ (and $d\delta/dT$[a]) of the amide proton chemical shifts in DMSO-d_6 solutions at 0.03 M

Compound	$d\delta/d(1/T)$/ppm deg ($(d\delta/dT)/10^3$ ppm deg^{-1})				
	N^1H	N^2H	Asn N^zH	Gly N^zH	Ser N^zH
Boc–Asn(GlcNAc)–OMe	516 (−5.11)	589 (−5.83)	853 (−8.43)		
Boc–Asn(GlcNAc)–Gly–OMe	552 (−5.46)	587 (−5.80)	811 (−7.96)	574 (−5.63)	
Boc–Asn(GlcNAc)–Gly–Ser–OMe	569 (−5.64)	525 (−5.19)	684 (−6.76)	407 (−4.02)	538 (−5.32)

[a] Shown in parentheses are $d\delta/dT$.

For the N^1H amide proton, the $d\delta/d(1/T)$ value for Boc–Asn(GlcNAc)–Gly–Ser–OMe is comparable to those for Boc–Asn(GlcNAc)–OMe and Boc–Asn(GlcNAc)–Gly–OMe. Furthermore, the temperature dependence of N^1H protons is relatively large, *i.e.*, larger than the threshold value of -3.00×10^{-3} ppm deg^{-1} (in $d\delta/dT$) for hydrogen bonded amide protons.[19] This suggests that the N^1H protons are exposed to the solvent not only in Boc–Asn(GlcNAc)–OMe and Boc–Asn(GlcNAc)–Gly–OMe but also in Boc–Asn(GlcNAc)–Gly–Ser–OMe. The N^1H amide proton of Boc–Asn(GlcNAc)–Gly–Ser–OMe does not participate in intramolecular hydrogen bonding as was found to be the case for Boc–Asn–Gly–Ser–OMe. From this and the analysis of the rotamer populations, it is evident that the stable intramolecular hydrogen bond cannot be formed between the carboxyamide proton of Asn side chain and the hydroxyl oxygen of Ser in Boc–Asn(GlcNAc)–Gly–Ser–OMe. The glycosylation of Asn side chain affects the overall peptide conformation in Boc–Asn(GlcNAc)–Gly–Ser–OMe. This is in contrast to the case of terminally protected Asn and Asn-containing dipeptides[9] where little conformational change was observed upon *N*-glycosylation. The existence of the intramolecular hydrogen bond between the carbonyl oxygen of Asn and hydroxyl proton of Ser is now an open problem for the –Asn–Gly–Ser– sequence.

The $d\delta/d(1/T)$ values are large for all backbone amide protons, indicating that there is no intramolecular hydrogen bond which contributes to the folding of the peptide chain. The $d\delta/d(1/T)$ values for N^2H are comparable for the glycopeptides. There seems to be no interaction between the peptide chain and carbohydrate in the glycopeptides.

The use of the hydroxyl group-protected carbohydrate for the model of carbohydrate chain is not meaningless, since the position 4 of GlcNAc is a reduced form in native glycoproteins. We ascertained the conformational differences between the *O*-acetylated and de-*O*-acetylated glycopeptide models. In Tables IV and V are tabulated the temperature dependence of the amide proton chemical shifts and the rotamer populations of Asn side chain, respectively, for Boc–Asn(GlcNAc)–NHMe, Boc–Asn(GlcNAc)–Gly–NHMe, and Boc–Gly–Asn(GlcNAc)–NHMe[9] and their de-*O*-acetylated derivatives. Similar values of temperature dependence were observed for the amide protons in *O*-acetylated and de-*O*-acetylated glycopeptides, except for the amide proton of N-terminal residue in the dipeptides. The predominance of (g^-)-rotamer for Asn side chain was also observed for both *O*-acetylated and de-*O*-acetylated glycopeptides. From these results, it is most likely that *O*-

Table IV. Temperature dependence, $d\delta/d(1/T)$ (and $d\delta/dT$[a]) of the amide proton chemical shifts measured in DMSO-d_6 solution at concentration of 0.03 M

Compound[b]	$d\delta/d(1/T)$/ppm deg ($(d\delta/dT)/10^3$ ppm deg^{-1})				
	N^1H	N^2H	Asn $N^\alpha H$	Gly $N^\alpha H$	NHMe
Boc–Asn(GlcNAc)–NHMe[c]	513	527	779		456
	(−5.08)	(−5.22)	(−7.71)		(−4.51)
Boc–Asn(GlcNAc*)–NHMe	539	436	733		632
	(−5.34)	(−4.32)	(−7.26)		(−6.26)
Boc–Asn(GlcNAc)–Gly–NHMe[c]	430	486	848	553	274
	(−4.26)	(−4.81)	(−8.40)	(−5.48)	(−2.71)
Boc–Asn(GlcNAc*)–Gly–NHMe	515	323	495	520	217
	(−5.10)	(−3.20)	(−4.90)	(−5.15)	(−2.15)
Boc–Gly–Asn(GlcNAc)–NHMe[c]	556	556	465	695	511
	(−5.51)	(−5.51)	(−4.60)	(−6.88)	(−5.06)
Boc–Gly–Asn(GlcNAc*)–NHMe	505	377	409	485	419
	(−5.00)	(−3.73)	(−4.05)	(−4.80)	(−4.15)

[a] Shown in parentheses are $d\delta/dT$.
[b] GlcNAc and GlcNAc* represent the tri-*O*-acetylated and de-*O*-acetylated carbohydrates, respectively.
[c] From ref 9.

Table V. Vicinal coupling constants (Hz) and rotamer populations for glycosylated Asn side chain measured in DMSO-d_6 solutions at 0.03 M

Compound[a]	J_{AX}	J_{BX}	P_{g^-}	P_t	P_{g^+}
Boc–Asn(GlcNAc)–NHMe[b]	7.2_6	n.d.[c]	0.42		
Boc–Asn(GlcNAc*)–NHMe	8.5_0	n.d.[c]	0.54		
Boc–Asn(GlcNAc)–Gly–NHMe[b]	7.3_3	n.d.[c]	0.43		
Boc–Asn(GlcNAc*)–Gly–NHMe	7.6_3	5.8_0	0.46	0.29	0.25
Boc–Gly–Asn(GlcNAc)–NHMe[b]	8.1_0	n.d.[c]	0.50		
Boc–Gly–Asn(GlcNAc*)–NHMe	$(7.0_1)_{av}$		0.80[d]		0.20

[a] GlcNAc and GlcNAc* represent the tri-*O*-acetylated and de-*O*-acetylated carbohydrates, respectively.
[b] From ref 9.
[c] Not determined.
[d] Sum of $P_{g^-} + P_t$.

acetylation of hydroxyl group does not influence the intrinsic conformational feature of the glycopeptides, although the C-terminal protecting groups are not identical.

CONCLUSIONS

From the NMR study of the conformation of the glycopeptides, it is concluded that there is no specific intramolecular interaction either in Boc–Asn(GlcNAc)–OMe and Boc–Asn-(GlcNAc)–Gly–OMe or in Boc–Asn-(GlcNAc)–Gly–Ser–OMe. The intramolecular hydrogen bond between side chains observed in Boc–Asn–Gly–Ser–OMe was not formed in the corresponding glycopeptide, Boc–Asn(GlcNAc)–Gly–Ser–OMe. The side chain conformation of the triplet sequence was greatly influenced by *N*-glycosylation.

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