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Short Communication

Behaviour of Dehydroalanine Derivatives under Hydrazinolysis Conditions. Possible Relevance to Glycoprotein Hydrazinolysis

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Abstract: Reactions that are relevant to the cleavage, by hydrazinolysis, of *O*-linked oligosaccharides from glycoproteins were studied using dehydroalanine derivatives as models of the intermediates formed from *O*-glycosylated serine residues. Conjugate addition of hydrazine followed by cyclisation to form pyrazolidinones, if occurring during glycoprotein hydrazinolysis, could reduce the yield of released oligosaccharide. However, *N*-acetyldehydroalanine amide derivatives, which modelled the dehydroalanine derivatives believed to be intermediates in the hydrazinolysis of glycoproteins containing *O*-linked oligosaccharides, underwent conjugate addition but no cyclisation. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: hydrazinolysis; dehydroalanine; glycoprotein

Hydrazinolysis is a useful reaction for the release of oligosaccharides from glycoproteins prior to their characterisation [1]. In the case of asparagine-linked oligosaccharides (*N*-linked glycans) the γ-amide linkage is cleaved and the reducing monosaccharide, usually 2-acetamido-2-deoxy-D-glucopyranosyl amine, in the released oligosaccharide reacts further with hydrazine to form the glycosyl hydrazine [2]. In the case of serine- and threonine-linked oligosaccharides (*O*-linked glycans), hydrazinolysis probably induces β-elimination to release the free oligosaccharides that react further with hydrazine to form the glycosyl hydrazines. The degradation that was originally reported [3] for the oligosaccharides released from serine or threonine was later shown by Parekh and co-workers [4] to be avoidable when a sufficiently low temperature was used for hydrazinolysis.

The fate of the alkene derivatives that are presumed to be formed in the hydrazinolysis of *O*-linked glycans is of interest. They would be expected to react with hydrazine by conjugate addition [5]. Although amides are relatively unreactive acylating agents, the addition product, e.g. **1**, could in principle undergo an intramolecular reaction to form pyrazolidinone derivatives **2** with concomitant cleavage of the peptide chain. If the released *N*-terminal amino acid was a serine or threonine residue, which still had an oligosaccharide attached to the side chain, this could result in incomplete release of the oligosaccharides due to the absence of the activating *N*-acyl substituent [6]. Some mucus glycoproteins contain highly glycosylated regions with many contiguous serine and/or threonine residues [7]. This communication reports the behaviour of substituted dehydroalanine derivatives towards hydrazine under different conditions.

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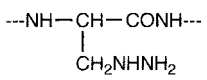

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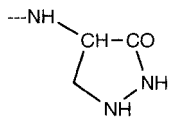

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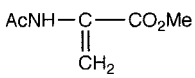

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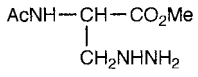

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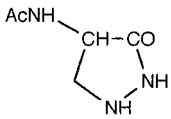

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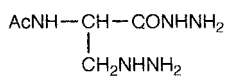

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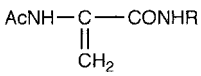
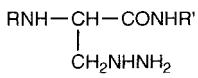


7 R = ^{i}Pr
10 R = CH_2Ph



8 R = Ac, R' = ^{i}Pr
9 R = H, R' = ^{i}Pr
11 R = Ac, R' = CH_2Ph

N-Acetyldehydroalanine isopropyl and benzyl amide derivatives were prepared [8] in pure crystalline form. The isopropyl amide, with branching α to the nitrogen, provides a steric environment somewhat similar to that in a neighbouring serine or threonine residue. The benzyl amide, a less sterically hindered amide, was studied for comparison.

The reactions of the dehydroalanine derivatives with hydrazine were first studied on a small scale in different solvents and monitored by NMR. Reaction of *N*-acetyldehydroalanine methyl ester (methyl 2-acetamidopropenoate) **3** with hydrazine (one equivalent) was first studied in order to provide NMR data for the expected addition **4** and cyclisation **5** products. The reaction in $^{2}H_4$-MeOH was relatively rapid, the conjugate addition product **4** being detected in 10 min (methylene protons: two d at δ_H 2.95 and 3.11, $^{2}J = 12.8$ Hz). This adduct cyclised to form the pyrazolidinone **5**, which was isolated pure in a separate experiment. A similar slower reaction sequence occurred in dimethylsulfoxide solution.

The stability of the pyrazolidinone under hydrazinolysis conditions was next examined. Half the starting material was unchanged after heating with anhydrous hydrazine at 60°C for 4 h, conditions used to release *O*-linked glycans from glycoproteins [4]. The main product was the hydrazide **6**.

The experiments that are most relevant to the hydrazinolysis of glycoproteins are those that involve *N*-acetyl dehydroalanine amides. Reaction of the *N*-isopropyl amide **7** with hydrazine (one equivalent) in $^{2}H_4$-MeOH was much slower than that of the corresponding methyl ester, and NMR analysis showed that no pyrazolidinone formation had occurred. The addition product **8** was provisionally identified as the major product (70%) (δ_H 2.85, d and 2.97, d, $^{2}J = 12.9$ Hz, C$\underline{H}_2$CD; δ_C 56.80, $\underline{C}H_2$). A similar reaction with DMSO as solvent (at 60°C) was slower than for MeOH, two-thirds of the starting material being unchanged after 3 h. After 6 h at 60°C, the starting material constituted 30% of the mixture and the adduct **8** was the major product. Characteristic amide proton signals were detected for the addition product (δ_H 7.91, d, *J* 8.3, 7.86, d, *J* 7.6) and the starting material (δ_H 8.10, d, *J* 7.7, N$\underline{H}$CH; 9.03, bs, N$\underline{H}$C=CH_2). In the key experiment, no starting material was present after hydrazinolysis of the amide **7** in anhydrous hydrazine at 60°C for 4 h. ^{13}C-NMR measurements of the hydrazine solution indicated that the addition product **8** was the major product ($\sim$85%). This identification was supported by the ^{1}H-NMR spectrum of the crude product, isolated after removal of the hydrazine. The methylene protons had chemical shifts similar to those of the corresponding ester **4**. Crystallisation of the crude product gave the addition product **8**, which was characterised by mass spectrometry, but it was found to be unstable on storage. The *N*-deacetylated product **9** was provisionally identified as a minor product by NMR data.

Hydrazinolysis of the dehydroalanine benzyl amide **10** was similar to that of the isopropyl amide. No starting material was present after reaction in hydrazine (60°C for 4 h); the major product was shown by NMR to be **11**. The solid product was characterised by mass spectrometry but decomposed during purification attempts.

The results imply that dehydroalanine moieties formed during the hydrazinolysis of glycoproteins or glycopeptides are likely to undergo conjugate addition of hydrazine, but subsequent cyclisation to form pyrazolidinone derivatives with concomitant cleavage of the peptide chain is unlikely under the conditions (60°C for 4 h) used to release *O*-linked oligosaccharides.

EXPERIMENTAL PART

^{1}H- (400 MHz) and ^{13}C-NMR (62.9 MHz) spectra were measured on a Bruker AC400 spectrometer. Unless otherwise stated the deuterated solvent was

$CDCl_3$ and all chemical shifts were measured from $SiMe_4$. *J* values are given in Hz; the digital resolution in proton spectra was 0.25 Hz/point. Low resolution mass spectra (EI and CI) were measured using Fisons VG Quattro II or VG12-253 spectrometers with NH_3 as reagent gas for CI. Assignments of fragment ions are provisional. High resolution mass spectra were measured on a VG ZAB E spectrometer. Column chromatography was carried out with silica gel 60 (230–400 mesh). TLC was achieved on Merck silica gel 60 F_{254} fluorescent plates. Melting points were determined in capillary tubes and are uncorrected. Hydrazinolyses were carried out in sealed teflon containers.

N-Acetyldehydroalanine Isopropylamide

N-Acetyldehydroalanine (1 g, Aldrich), *N*,*N*′-dicyclohexylcarbodiimide (1.6 g), *N*-hydroxysuccinimide (0.9 g) and isopropylamine (0.46 g) in dry EtOAc (25 ml) were stirred at −15°C for 1 h, then at room temperature for 1 day. The precipitated *N*,*N*′-dicyclohexyl urea (1.8 g) was filtered off and the filtrate evaporated to a foam (1.9 g). Column chromatography (eluants: CH_2Cl_2–EtOAc) followed by two recrystallisations from CH_2Cl_2–EtOAc gave the pure amide, 0.55 g (42%), m.p. 117.5–119.5°C; δ_H 8.13 (1H, bs, NHC=C), 6.42 (1H, d, *J* 1.5, C=CH), 5.90 (1H, bs, N*H*CH), 5.12 (1H, s, C=CH), 4.13 (1H, m, C*H*Me_2), 2.12 (3H, CH_3CO), 1.22 (6H, d, *J* 6.5, CH(CH_3)$_2$); *m*/*z* (EI) 170.1055 (M^+, 100%, $C_8H_{14}N_2O_2$ requires 170.1055), 128 (60%), 170 − CH_2CO), 113 (29%, 128 − CH_3), 100 (22%, 128 − CO), 85 (16%, 128 − CH_3CHNH), 58 (72%, $AcNH^+$).

N-Acetyldehydroalanine Benzylamide

The previous preparation was repeated using benzylamine instead of isopropylamine, and the benzylamide was isolated pure in 54% yield after column chromatography (eluants: CH_2Cl_2–EtOAc) and two recrystallisations from CH_2Cl_2–petroleum ether; m.p. 123–124°C, δ_H 8.13 (1H, bs, NHC=C), 7.31 (5H, ArH), 6.62 (1H, bs, N*H*CH_2), 6.44 (1H, s, CH=C), 5.22 (1H, s, CH=C), 4.52 (2H, d, *J* 5.7, CH_2Ph), 2.10 (3H, s, CH_3CO); δ_C 169.15, 163.91 (2C=O), 137.35, 134.21 (*C*=CH_2 and *C* of Ph), 128.85, 127.83, 127.74 (3*C*H of Ph), 100.99 (*C*H_2=C), 44.16 (*C*H_2Ph), 24.71 (CH_3); *m*/*z* (EI) 218.1055 (7%, M^+, $C_{12}H_{14}N_2O_2$ requires 218.1055), 175 (14%, M − Ac), 106 (100%, $PhCH_2NH$), 91 (70%, C_7H_7).

Hydrazinolysis Experiments

***N*-Acetyldehydroalanine methyl ester**. A solution of the ester (18 mg, 0.126 mmol, Aldrich) and hydrazine (4 mg, 0.125 mmol) in [$^2H_6$-DMSO] (0.5 ml) was kept at room temperature and monitored by ^{1}H-NMR. The first product detected after 10 min was the addition product **4**; δ_H 8.20 (d, *J* 7.4, NHCO), 4.46 (dt, *J* 5.3, 7.4, CH), 3.61 (s, OCH_3), 2.87 (dd, *J* 5.3, 12.5, *H*CHCH), 2.82 (dd, *J* 7.4, 12.5, HC*H*CH), 1.86 (s, CH_3CO). Signals for the pyrazolidinone **5** were detected (see data below) after 1.5 h. A similar experiment with $^2H_4$-MeOH as solvent (ester, 49 mg, 0.34 mmol and hydrazine, 10.9 mg, 0.30 mmol) showed the reactions were faster than in DMSO; data for the addition product **4**, δ_H 3.73 (s, OCH_3), 3.11 and 2.95 (each d, *J* 12.8, CH_2CD), 2.01 (s, CH_3CO). Methanol released in the cyclisation was detected at δ_H 3.35. The cyclisation product, 4-acetamido-3-pyrazolidinone, **5**, was the major product. It was prepared in a scaled-up experiment as follows. A solution of *N*-acetyldehydroalanine methyl ester (2.5 g, 17.5 mmol) and hydrazine (0.56 g, 15.6 mmol) in dry MeOH (10 ml) was stirred for 1 day, the reaction being monitored by TLC (eluants: CH_2Cl_2:MeOH, 2:1; product **5**, R_f 0.3, was detected as a white spot with alkaline potassium permanganate solution; starting material had R_f 0.8, and several minor unidentified by-products were also detected). Evaporation of the reaction mixture gave the crude product as a foam (2.4 g), which was crystallised from MeOH–diethyl ether to give the pure pyrazolidinone, 0.25 g, m.p. 158–160°C. Column chromatography gave a further 0.5 g (after recrystallisation) of product (total 35%); δ_H ($^2H_4$-MeOH) 4.68 (1H, dd, *J* 8.1, 10.7, CH), 3.67 (1H, dd, *J* 8.1, 11.4, 1H of CH_2), 3.09 (1H, dd, *J* 10.7, 11.4, 1H of CH_2), 2.00 (3H, s, CH_3CO); δ_C 175.51 (*C*OCH_3), 173.5 (CONH), 52.38 (CH_2), 52.34 (CH), 22.43 (*C*H_3CO); *m*/*z* (CI) 144.0773 ($M^+ + 1$); calc. for $C_5H_9N_3O_2$ 144.0773.

4-Acetamido-3-pyrazolidinone. The pyrazolidinone (0.12 g) was heated with hydrazine (1 ml) at 60°C for 4 h. After removal of the hydrazine *in vacuo* over concentrated H_2SO_4, the product was examined by NMR with $^2H_4$-MeOH as solvent. Starting material (∼50%) was present and the ring-opened hydrazide **6** was the major product; δ_H 4.57 (dd, *J* 5.5, 8.0, CH_2CH), 3.02 (dd, *J* 5.5, 12.8, *H*CHCH), 2.91 (dd, *J* 8.0, 12.8, HC*H*CH), 2.00 (s, CH_3CO).

***N*-Acetyldehydroalanine isopropylamide**. A solution of the amide (31 mg, 0.18 mmol) and hydrazine (5.8 mg, 0.16 mmol) in $^2H_6$-DMSO (0.5 ml) was heated at

60°C for 3 h. The addition product **8** was shown by NMR to constitute ~30% of the mixture, and starting material was the major constituent. After another 3 h at 60°C there was still 30% of starting material present. No pyrazolidinone or isopropylamine could be detected. Data for product **8**: δ_H 7.91 (1H, d, *J* 8.3, NHCO), 7.86 (1H, d, *J* 7.6, NHCO), 4.41 (1H, m, C*H*CH_2), 3.80 (1H, m, C*H*Me_2), 2.78 (1H, dd, *J* 5.7, 12.4, 1H of CH_2), 2.65 (1H, dd, *J* 7.8, 12.4, 1H of CH_2), 1.85 (3H, s, CH_3CO), 1.038, 1.022 [2 × 3H, 2d, *J* 6.6, (CH_3)$_2$CH]; δ_C (DEPT) 50.7 (CH), 40.39 (*C*HMe_2), 23.93 (CO*C*H_3), 22.29 (*C*H_3CO). A similar reaction in $^2H_4$-MeOH (with amide, 40 mg, 0.235 mmol and hydrazine, 7.5 mg, 0.21 mmol) was faster, the hydrazide being the major product (~70% of the mixture) after 3 h at 50°C; δ_H 3.94 (m, C*H*Me_2), 2.97 (d, *J* 12.9, *H*CHCD), 2.85 (d, *J* 12.9, HC*H*CD), 2.01 (s, CH_3CO), 1.15 and 1.14 [2d, *J* 6.6, CH(CH_3)$_2$]. Starting material was a minor constituent of the mixture. ^{13}C-NMR studies (using $^2H_6$-acetone in a coaxial tube for the field frequency lock) of the reaction of *N*-acetyldehydroalanine isopropylamide (0.2 g) and hydrazine (2 ml) at 60°C for 4 h revealed the presence of a major product [85%; δ_c 58.050 (CH_2), 53.209 (CH), 42.869 (*C*HMe_2)] and a minor product [10%; δ_c 57.577 (CH_2), 55.547 (CH), 42.634 (*C*HMe_2)]. Removal of the hydrazine *in vacuo* over concentrated H_2SO_4 gave an oil (0.19 g), which crystallised from CH_2Cl_2–Et_2O to give *N*-acetyl-β-hydrazino-DL-alanine isopropylamide **8** (0.15 g), m.p. 99–101°C; δ_H ($^2H_4$-MeOH) 4.54 (1H, dd, *J* 4.8, 8.5, CHCO), 3.95 (1H, septet, *J* 6.6, C*H*Me_2), 2.98 (1H, dd, *J* 4.8, 12.9, 1H of CH_2), 2.85 (1H, dd, *J* 8.5, 12.9, 1H of CH_2), 2.01 (3H, s, CH_3CO), 1.15, 1.14 [6H, 2d, *J* 6.6, (CH_3)$_2$CH)]; δ_C 56.70 (CH_2), 52.29 (*C*HCO), 42.59 (*C*HMe_2), 22.71 (*C*H_3CO), 22.62 [(CH_3)$_2$CH); *m*/*z* (CI) 203.1508, calc. for $C_8H_{19}N_4O_2$, 203.1509 (13%, $M^+ + 1$), 173 (100%, $203 - N_2H_2$). Weak signals in the ^{1}H-NMR spectrum of the crude product were assigned to the *N*-deacetylated hydrazine derivative **9** [3H 3.95 (m, C*H*Me_2, overlapping), 3.39 (m, H_2NC*H*CO), 2.83, 3.00 (2 m, partly obscured, CH_2), 1.12 (d, C*H*Me_2)] and acetylhydrazine [δ_H 1.90 (CH_3); δ_C 20.45 (CH_3)].

***N*-Acetyldehydroalanine benzylamide**. *N*-Acetyldehydroalanine benzylamide (0.1 g) and hydrazine (1 ml) were heated at 60°C for 4 h, and excess hydrazine was removed *in vacuo* over concentrated H_2SO_4 to give a solid. NMR data indicated that starting material was absent and the major product was the addition product **11**; δ_H 8.51 (t, *J* 6.0, N*H*CH_2), 8.03 (d, *J* 8.0, N*H*CH), 7.2–7.3 (m, ArH), 4.50 (m, C*H*CH_2), 4.28 (d, *J* 6.0, PhCH_2CH), 2.88 (dd, *J* 5.8, 12.3, *H*CHCH), 2.74 (dd, *J* 7.7, 12.3, *H*CHCH), 1.87 (s, CH_3CO); *m*/*z* (CI) 251.1508, calc. for $C_{12}H_{19}N_4O_2$, 251.1509 (3%, $M^+ + 1$), 233 (5%, $M - NH_3$), 221 (100%, $251 - N_2H_2$). Attempted purlfication involving column chromatography and recrystallisation was unsuccessful since the compound decomposed.

Acknowledgements

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