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# Heterocyclic derivatives of sugars: the formation of 1-glycosyl-3-methylpyrazol-5-ones from hydrazones

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**Abstract**

Conditions to effect the conversion of monosaccharide and disaccharide hydrazones to 1-glycosyl-3-methylpyrazol-5-ones were examined. The sugar pyrazolone derivatives were sensitive to oxidation, but high yields were achieved with 2,2,2-trifluoroethyl acetoacetate in mildly acidic solution. Azo coupling of the pyrazolones produced highly coloured azopyrazolone derivatives that prevented further degradation, and these may prove useful labels for chromatographic analysis of carbohydrates. 

*Keywords:* Sugar hydrazone; Pyrazolone; Azopyrazolone; Carbohydrate labelling

## 1. Introduction

Previously, the feasibility of preparing pyrazole derivatives of sugars from saccharide hydrazones was demonstrated [1] as part of a search for alternatives to common procedures for the analysis of glycans [2,3]. The derivatisation exploited the tautomerism of acyclic saccharide hydrazones to cyclic glycosylhydrazines [4], which enabled the use of familiar hydrazine chemistry to generate the heterocyclic ring. The pyrazoles were formed in a simple two-step process from glycan hydrazones, but a major disadvantage was that dimethylpyrazole absorbs in the near-UV spectrum and has only a modest extinction coefficient. Modification of the heterocyclic ring may offer better detection characteristics and result in improved separations, and we have now investigated the use of methyl pyrazolones. Furthermore, pyrazolones open up possibilities for further derivatisation using methods that have been developed during their long history in the preparation of dyes and colourants [5,6].

The archetypal pyrazolone synthesis is the condensation of a β-ketoester, such as ethylacetoacetate, with a hydrazine [5,6]. Previous attempts at the formation of glycosylpyrazolones in this manner yielded an intractable mixture [7], but we have been able to produce pyrazolone derivatives of selected mono- and disaccharides in good yield by optimising the reaction conditions and azo-coupling the pyrazolones to improve their stability. The highly coloured azo-pyrazolone derivatives may prove useful for the labelling of glycans, which appear to be especially attractive for the analysis of the products of hydrazinolysis of a glycoprotein, or β-elimination in the presence of hydrazine [8–10].

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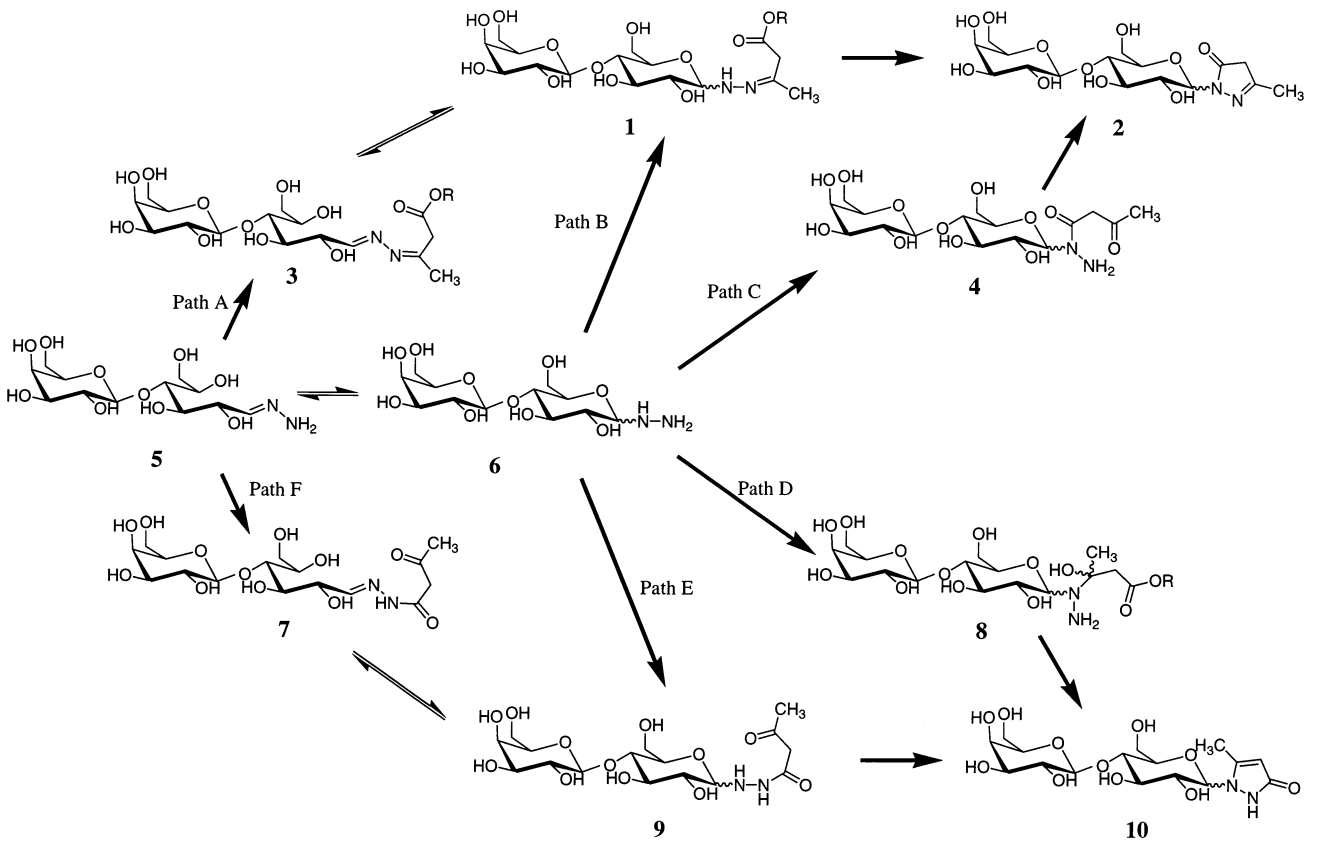
## 2. Results

*Reactions with ethyl acetoacetate.*—The addition of ethyl acetoacetate to freshly prepared aqueous solutions of lactose hydrazone produced deep red-coloured and complex mixtures. Analysis by HPLC using porous graphitic carbon (PGC) or hydrophilic interaction chromatography with diode array detection or ESIMS ($[M+H]^+ = 423$ $m/z$; $[M-H]^- = 421$ $m/z$) enabled the detection of the 1-lactosylpyrazolone products. The reaction was not instantaneous, and a reaction intermediate was observed as described below.

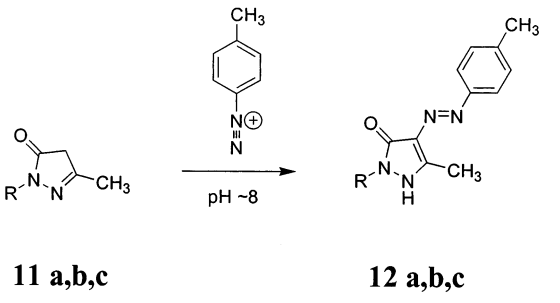
Both isomers of the pyrazolone ring were detected. Each isomer can exist in more than one tautomeric form, with the proportions depending on the solvent [6,11]. In aqueous solution, the main form of 5-pyrazolones is the NH tautomer, which has an absorption maximum in the range of 250–260 nm, while 3-pyrazolones exist predominantly as the OH tautomer with an absorption maximum at about 220 nm. The absorption spectra of the glycosylpyrazolones separated by HPLC indicated that the principal species was the 5-pyrazolone isomer **2** as expected (Scheme 1), but small amounts of the 3-pyrazolone isomer, **10**, were also present.

The glycosylpyrazol-5-one derivatives degraded rapidly after chromatographic isolation and had to be further derivatised using azo coupling before they could be characterised. Crude preparations of lactosylpyrazolones and fucosylpyrazolones were reacted at moderately alkaline pH with diazotised 4-methyl aminobenzene and yellow 1-glycosyl-4-(4-methylbenzeneazo)-3-methylpyrazol-5-ones, and1-glycosyl-4-(4-methylbenzeneazo)-5-methyl-pyrazol-3-ones (Scheme 2) formed almost instantly. The products were purified by reversed-phase HPLC and characterised by visible and NMR spectroscopy and collision-induced dissociation ESIMS/MS.

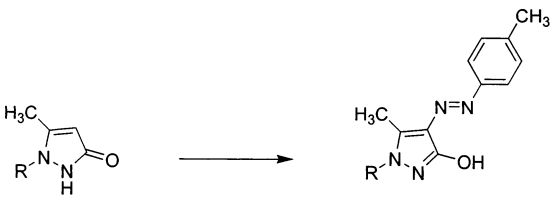
The mass spectra contained peaks corresponding to the expected pseudo-molecular



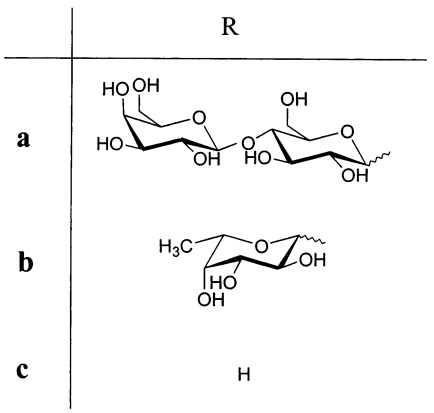

Scheme 1. Reaction pathways for the formation of 1-lactosylpyrazol-5-one (**2**) and 1-lactosylpyrazol-3-one (**10**) from lactose hydrazone. The azine intermediate **1** must adopt the *Z* conformation to enable heterocyclic ring closure [17].




11 a,b,c 12 a,b,c



13 a,b 14 a,b




Scheme 2. Formation of azopyrazolones.

Table 1
Proportions of tolylazopyrazol-5-one isomers from six aldoses and a ketose

| | Isomer proportions [a] |
|---|---|
| Glucose | 94:2:1:3 |
| Galactose | 69:6:7:18 |
| Mannose | 2:4:93:1 |
| Ribose | 6:36:37:21 |
| Fructose | 74 [b]:13:13 |
| Lactose | 97:3 |
| Melibiose | 92:4:3:1 |

[a] The proportions were determined by reversed-phase HPLC, and the isomers are listed in order of elution.

[b] The major peak appears to be two incompletely resolved isomers.

ions, and the principal collision-induced fragments of these ions had lost mass equivalent to the glycan moiety and an abstracted hydrogen. The lactosyl derivatives gave additional ions corresponding to loss of (i) the galactose residue and (ii) loss of the azo groups. The UV absorption maximum of the major isomer was at 418 nm, about a 70 nm longer wavelength than that for the other isomer, confirming that the predominant product was the tolylazopyrazol-5-one isomer [11].

The predominant sugar ring form was β-pyranosyl for both the glucosyl and fucosyl derivatives, as shown by the chemical shift and coupling constant of the anomeric proton signal in the $^1$H NMR spectra of the compounds. The spectrum of the lactosyl compound, recorded in $D_2O$, displayed broad resonances until base was added. The broadening was probably the result of interconversion between four tautomers [11], which was eliminated when the pyrazolone was converted to the anion [12,13]. The $^1$H NMR spectrum of the fucosyl azopyrazolone derivative was acquired in chloroform solution and was not broadened, presumably because a single tautomer is favoured in this solvent.

The distribution of the other sugar ring forms produced by selected mono- and disaccharide hydrazones was determined after conversion to azopyrazolone derivatives (Table 1). The reaction mixtures were separated by reversed-phase HPLC with diode array detection, and the pyrazolone ring isomers were identified by their characteristic UV–Vis absorption spectra. The number of isomers and their proportions were determined, but no attempt was made to establish the configuration of each isomer. Previously, we showed that the distribution of sugar-ring isomers in pyrazole derivatives [1] was determined by a complex equilibrium involving intermediates produced by the first carbonyl condensation step. It was, therefore, expected that the distribution of ring forms for the pyrazolone derivatives would be similar, and the isomeric proportions were broadly similar to those for the pyrazoles, with a slightly greater preponderance of the major isomer.

*Optimisation of pyrazolone formation.*—The effects of using other β-ketoesters that provided a systematic variation in the reactivities of the ester moieties and of varying reaction

conditions were evaluated by derivatising lactose hydrazone. The reaction products were analysed by HPLC using two separation modes, PGC [14,15] and hydrophilic interaction media [16]. Peak areas were determined with diode-array detection, while on-line ES-IMS was used for peak identification.

The reaction of lactose hydrazone with either methyl acetoacetate or ethyl acetoacetate produced an intermediate that was detected by hydrophilic interaction chromatography. The amount of intermediate decreased with time in a first-order manner, while the amount of lactosylpyrazol-5-one product increased. The intermediate formed from methyl acetoacetate decayed with a half-life of approximately 5 h, whilst the half-life for that formed from ethyl acetoacetate was 7.5 h. The intermediates are believed to be the cyclic azines, **1** (R = methyl or ethyl), and not the open-chain diazines **3**, because ketone condensation with the outer nitrogen of a saccharide hydrazone (Scheme 1, path A) has been shown to lead to rapid sugar ring closure [1]. Heterocyclic ring closure of these azines is much slower than reported for similar azines formed from alkyl and phenyl hydrazines [17].

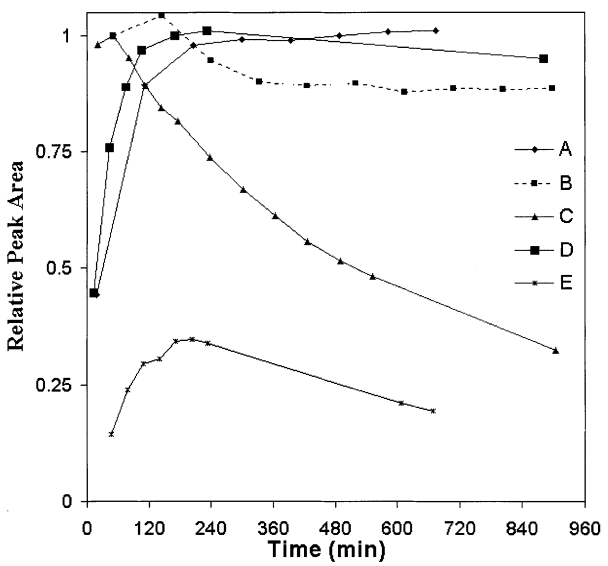



Fig. 1. Time course of the formation of **11a** from lactose hydrazone by reaction with 2,2,2-trifluoroethyl acetoacetate at room temperature in (A) 30:70 MeOH–water; (B) 30:70 MeOH–0.2 M $CH_3CO_2Na$ pH 4.6; (C) 30:70 MeOH–0.2 M $Na_2HPO_4$ PH 6; (D) 30:70 MeOH–25 mM $Na_2HPO_4$ pH 6; (E) 30:70 MeOH–25 mM $Na_2B_4O_7$ pH 8. Yields are expressed relative to the maximum yield observed. The initial concentration of lactose hydrazone was 1 mM. HPLC analysis was performed on a PGC column.

The reaction of 2,2,2-trifluoroethyl acetoacetate with lactose hydrazone was much faster, and the yield was sensitive to the buffer used (Fig. 1). At longer reaction times, degradation of the product became more noticeable, and when the derivatisation was repeated with lactose hydrazone at 100 times greater concentration in methanol–water solvent, 50% of the lactosylpyrazolone had degraded within 4 h. It is believed that oxidation of the pyrazolone ring is responsible [18–21]. Under optimal conditions (0.2 M $CH_3CO_2Na$ buffer, pH 4.6), the reaction was complete within 2 h (Fig. 1) and degradation was negligible.

Precise quantitation of the amount of pyrazol-3-one was not possible, but assuming that the extinction coefficient at 227 nm for the pyrazol-3-one derivative is approximately half that for the pyrazol-5-one derivative at 246 nm [11], the proportion of the pyrazol-3-one isomer formed was less than 1% for all three alkyl esters.

When *N*-hydroxysuccinimidyl acetoacetate was reacted with lactose hydrazone, approximately equal amounts of the pyrazol-5-one and pyrazol-3-one isomers were formed. It is likely that the increased reactivity of the *N*-hydroxysuccinimidyl moiety [22] resulted in acylation of the saccharide hydrazone (Scheme 1, paths D–F) rather than enamine formation. Hence, 2,2,2-trifluoroethyl acetoacetate is a better reagent for our purposes as it provides a compromise between the two objectives of increasing ester reactivity to effect fast pyrazolone ring closure and the formation of a single pyrazolone isomer.

*Derivatisation efficiency.*—An estimate of the derivatisation efficiency was obtained for the formation of the tolylazopyrazolone derivative from lactose by using HPLC analysis with detection at 418 nm. For calibration, the N-unsubstituted analogue, 4-(4-methylbenzeneazo)-1-*H*-3-methylpyrazol-5-one (**12c**), was prepared, purified and checked by NMR and HPLC. In view of the strong qualitative similarities between the visible absorption spectra of the glycosylated and non-glycosylated azopyrazolones, it was assumed that the extinction coefficients at 418 nm for the glycosyl derivatives were the same as that for the standard compound.

The HPLC peak area was a linear function of the amount of 4-(4-methylbenzeneazo)-1-*H*-3-methylpyrazol-5-one injected, over the range of 20–5000 pmol. The peak areas for the tolylazo derivative **12a**, obtained from derivatising amounts of lactose in the range of 4–200 nmol were fitted by least-squares analysis, and a straight line was obtained with a slope that was $80 \pm 7\%$ of the slope obtained for the calibrant solution [21].

## 3. Discussion

Veibel and co-workers have shown that 5-pyrazolones are susceptible to oxidation to bispyrazolones and 4-hydroxypyrazolones [18–21]. The reaction occurs via a free-radical mechanism, and the kinetic order was found to vary according to the substrate [18,23]. Oxidation was faster in alkali and was more complex in aqueous solution, resulting in uncharacterised oils [19]. Pyrazol-4,5-diones and hydroxy-bispyrazolones have also been identified as oxidation products of pyrazolones [23]. Although the pyrazolone ring is normally stable in either strong acid or base [5], pyrazol-4,5-diones have been shown to undergo pyrazolone ring opening to generate oxobutanoic acid hydrazones [23].

Glycosylpyrazolones appear to be especially susceptible to oxidation. Indeed, in a previous study, ethyl acetoacetate produced an intractable mixture when added to galactose hydrazone [7]. It seems likely that the conditions used in that study (basic conditions with extended time at reflux) caused extensive oxidation of the galactosylpyrazolone initially formed. In the present investigation we were able to use high-resolution chromatography with mass spectrometry to show that the pyrazolones were present and were able to convert them to stable azopyrazolones.

Hence, it has been shown that saccharide hydrazones can be converted efficiently to 1-glycosyl-3-methylpyrazol-5-one derivatives under conditions suitable for use in routine analysis. In order to maintain high yields, however, the pyrazolones must be further derivatised as quickly as possible, for example, by azo coupling. The key requirements for high efficiency are the use of an active ester like 2,2,2,-trifluoroethyl acetoacetate [24] and the suppression of oxidation in mildly acidic solution [21]. We have avoided more forcing conditions that might accelerate heterocyclic ring closure, for fear that this might compromise acid-sensitive substituents like sialic acids.

## 4. Experimental

*General.*—2,2,2-trifluoroethyl acetoacetate [25] and 1,3-dimethylpyrazol-5-one [26] were synthesised according to published procedures. 4-(4-Methylbenzeneazo)-1,3-dimethylpyrazol-5-one and 4-(4-methylbenzeneazo)-1-*H*-3-methylpyrazol-5-one was prepared by coupling diazotised 4-methyl aminobenzene in the usual manner [27] followed by recrystallisation of the products from EtOAc. No significant impurities were detected in the products using either $^1$H NMR spectroscopy or reversed-phase HPLC with multiwavelength detection. Glycosylpyrazole derivatives were prepared as described previously [1]. All other materials were purchased from Sigma-Aldrich (Sydney, Australia). All commercial products were used without prior purification.

NMR spectra were acquired at 27 °C on a Varian XL-400 spectrometer operating at 400 MHz for $^1$H and 100 MHz for $^{13}$C observation. Proton decoupling was used to assist in spectral assignments. Chemical shifts are reported downfield from $Me_4Si$, which was used as internal reference in deuterochloroform solutions, or from sodium 3-(trimethylsilyl)propionic acid, which was used in $D_2O$ solutions.

The HPLC system was comprised of two LC-10 pumps, SGU-104 solvent degassing unit, SPD-10MA photodiode array detector, 10Axl autosampler, manual Rheodyne injector (Rheodyne, Cotari, USA), and FRC-10 fraction collector. The pumps were configured for either binary high-pressure mixing or quaternary low-pressure mixing as required. Data acquisition and instrument control was achieved using Shimadzu CLASS 5000 software.

Electrospray-ionisation mass spectrometry (ESIMS) was performed with a Fisons VG Quattro spectrometer (VG Analytical, Manchester, UK). Data acquisition and instrument control was achieved using Mass Lynx software (VG Analytical). The Shimadzu HPLC was coupled with the mass spectrometer for LC/MS experiments. A 10:1 split ratio between the detector effluent and the mass spectrometer inlet was achieved using a T-piece.

*Preparation of 1-β-D-lactosyl-4-(4-methylbenzeneazo)-3-methylpyrazol-5-one* (**12a**).—Lactose (1 g, 3 mmol) was converted to the hydrazone as described previously [1]. The residue was dissolved in 1:3 MeCN–water (20 mL) that had been purged with argon, and 2,2,2-trifluoroethyl acetoacetate (2 mL) was added. Argon was bubbled through the mixture for a further 3 h, and the sample was lyophilised to yield 2.2 g of a yellow powder containing mostly 3-methylpyrazol-5-one (**11c**) and 1-lactosyl-3-methylpyrazol-5-one (**11a**). A portion of the crude product (0.5 g, corresponding to approximately 0.7 mmol of **11a**) was dissolved in 0.5 M $NaHCO_3$ (50 mL). Diazotised 4-methyl aminobenzene (5 mmol) was added with stirring, the solution turned yellow immediately, and a yellow precipitate began to deposit. After 10 min, 2,4-pentanedione (1 mL) was added, and the solution was stirred for a further 10 min before the pH was adjusted to pH 6 by the dropwise addition of glacial AcOH. The mixture was washed with $CHCl_3$ ($3 \times 30$ mL), and the washings were discarded. The product was purified from the aq liquor by HPLC chromatography on a $100 \times 10$ mm polymeric Jordi column using repetitive injection on a BIOCAD chromatography workstation (Perseptive Biosystems, MA, USA). The gradient was 3:17 MeCN–0.026 M $HCO_2H$ for 5 min, followed by a linear gradient to 35:65 MeCN–0.026 M $HCO_2H$ at 25 min, then to 6:4 MeCN–0.026 M $HCO_2H$ at 27.5 min where the composition was maintained for 3 min. The flow rate was 1.8 mL/min, and the eluted species were monitored at 420 and 250 nm. Fractions corresponding to 1-lactosyl-4-(4-methylbenzeneazo)-3-methylpyrazol-5-one were pooled, concd on a rotary evaporator, and lyophilised to give a yellow gum (70 mg; 0.13 mmol; 15%). $^1$H NMR: ($\sim$0.02 M $ND_3$ in $D_2O$): $\delta$ 2.37 (s, 6 H, 2 methyl groups), 3.55 (dd, 1 H, H-2′, $J_{2'3'}$ 9.7 Hz), 3.65–4.00 (m, 10 H, H-3–H-6 and H-3′- H-6′), 4.10 (dd, 1 H, H-2, $J_{23}$ 9.0 Hz), 4.52 (d, 1 H, H-1′, $J_{1'2'}$ 7.9 Hz), 5.35 (d, 1 H, H-1, $J_{12}$ 9.2 Hz), 7.33 (d, 2 H, $J$ 5.9 Hz), 7.55 (d, 2H, $J$ 6.3 Hz). Positive-ion ESIMS/MS: $[M + H]^+ = m/z$ 541. Daughters of $m/z$ 541 (CID, 16 V): $m/z$ 379 (relative intensity (RI) = 90%, loss of galactose with hydrogen abstraction), $m/z$ 259 (RI = 60%, loss of azo moiety and two hydrogens from $m/z$ 379), $m/z$ 217 (RI = 100%, loss of lactose with hydrogen abstraction). Negative-ion ESIMS: $[M - H]^- = m/z$ 539.

*Preparation of 1-β-lactosyl-4-(4-methylbenzeneazo) - 5 - methylpyrazol - 3 - one* (**14a**).—Freshly prepared lactose hydrazone (0.25 g, 0.75 mmol) was dissolved in 3:7 dioxane–water (5 mL), and NHS acetoacetate (0.5 g, 2.5 mmol) was added. After 30 min the sample was diluted with water (5 mL) and applied to a $50 \times 20$ mm column packed with Hypercarb (Alltech Associates, Sydney, Australia). The column was washed with 9:1 MeOH–13 mM TFA (300 mL at 10 mL/min) to elute most of the 3-methylpyrazol-5-one formed from excess hydrazine. The lactosylpyrazolones were eluted with 5:2:13 MeCN–MeOH–13 mM TFA (50 mL) and lyophilised. The residue was dissolved in 0.5 M $Na_2CO_3$ (5 mL), diazotised 4-methyl aminobenzene (2 mmol) was added, and the solution was stirred for 30 min. Methyl acetoacetate (0.4 mL) was added, and stirring was continued for a further 10 min. The pH was adjusted to 6, and the mixture was applied to a preconditioned 1-g $C_{18}$ solid-phase extraction cartridge, which was then washed with water (10 mL). The lactosylazopyrazolones were eluted with 3:7 MeCN–water (6 mL) and lyophilised. The product was analysed by HPLC on a $150 \times 4.6$ mm Hypersil ODS column. The gradient was 3:17 MeCN–0.026 M $HCO_2H$ for 1 min, followed by a linear gradient to 7:13 MeCN–0.026 M $HCO_2H$ at 21 min. The eluent was monitored at 350 and 420 nm. The mixture contained mostly 1-lactosyl-4-(4-methylben-

zeneazo)-3-methylpyrazol-5-one, but 1-lactosyl-4-(4-methylbenzeneazo)-5-methylpyrazol-3-one was also present, eluting 0.8 min later than the major isomer and having a visible absorption maximum at 348 nm. This peak was collected and analysed by ESIMS/MS. $[M+H]^+ = m/z$ 541. Daughters of $m/z$ 541 (collision voltage 16 V): $m/z$ 379 (RI = 16%, loss of galactose with hydrogen abstraction), $m/z$ 259 (RI = 13%, loss of tolylazo moiety and two hydrogens from $m/z$ 379), $m/z$ 217 (RI = 100%, loss of lactose with hydrogen abstraction), $m/z$ 163 (RI = 18%, galactose residue), $m/z$ 145 (RI = 22%, loss of water from galactose residue). Negative-ion ESIMS: $[M-H]^- = m/z$ 539.

*Time-course studies of the formation of lactosylpyrazolones*

*Analysis by HPLC on PGC.* The reaction mixtures were separated on a Shandon Hypersil 5 μm, 100 × 4.6 mm PGC column (Alltech Associates) using a flow rate of 1 mL/min. Quaternary or ternary gradients (formed by configuring the Shimadzu LC-10 for low-pressure mixing) were used depending on the acidic component of the eluent. For mass spectrometry the volatile eluents were: A., water; B., MeCN; C., 0.26 M trifluoroacetic acid; and D., MeOH. For quantitative analysis the eluents were A., 8 mM $H_2SO_4$; B., MeCN; C., MeOH. Aliquots (20 μL) of the sample were withdrawn at intervals by the autosampler. The column was maintained at 35 °C, the flow rate was 1 mL/min and the detection wavelengths were 230 and 250 nm.

*Analysis by hydrophilic interaction HPLC.* Either a PolyLC 5 μm, 100 × 2.1 mm polyethylaspartamide column (PolyLC Inc., Columbia, USA) or a Lichrosorb diol 5 μm, 200 × 2.1 mm column (Alltech Associates) was used. Injections of 10 μL were made at intervals using an autosampler. Gradients of MeCN and water (formed by configuring the Shimadzu LC-10 for binary high-pressure mixing) were used at a flowrate of 0.22 mL/min. The columns were maintained at 30 °C, and the detection wavelengths were 210, 230 and 250 nm. For some experiments an evaporative light scattering detector (Alltech Associates) was used to detect lactose. This detector was operated at 110 °C, with a flow of nitrogen at 2 L/min and was placed in-line after the diode-array detector.

*(i) Preparation of lactose hydrazone standard.*—Two stock solutions containing 7.36 mM lactose in 2:3 EtOH–water were prepared, with one also containing approximately 1 mg/mL 1-D-glucosyl-3,5-dimethylpyrazole as internal standard, and were stored at −20 °C. Aliquots (100 μL) of either solution were placed into autosampler vials, hydrazine monohydrate (20 μL) was added, and the samples were incubated at rt overnight. After drying in a centrifugal evaporator, toluene (0.5 mL) was added, and the samples were re-evaporated. Finally, water (100 μL) was added, and the samples were evaporated again before capping and storage at 4 °C for up to 10 days.

*(ii) Formation of lactose pyrazolones using β-ketoesters.*—The formation using 2,2,2-trifluoroethyl acetoacetate was performed as follows. The procedure for other esters was similar. A sample of lactose hydrazone (0.75 μmol), prepared as in (i), was dissolved in water (160 μL). Aliquots (50 μL) were placed into two 800-μL crimp-top autosampler vials and buffer or solvent (300 μL) was added. A solution of 1:4 2,2,2-trifluoroethyl acetoacetate–2,2,2-trifluoroethanol (15 μL) was added, the vial capped and placed into the HPLC autosampler. HPLC analysis was performed using either a PGC column or a PolyLC column. The PGC column was eluted with a ternary gradient at 1 mL/min. The gradient was 90:3:7 8 mM $H_2SO_4$–MeCN–MeOH for 1 min, then changed to 79:14:7 at 14 min, then to 43:50:7 at 18 min. The PolyLC column was eluted at 0.22 mL/min with a gradient comprised of 47:3 MeCN–water for 1 min, then changed linearly to 7:3 at 18 min, and then to 3:7 at 21 min. The columns were equilibrated between analyses for 6 min (PGC) or 10 min (PolyLC).

*(iii) Monitoring the degradation of lactosylpyrazolones in concentrated solutions.*—Lactose hydrazone (25 mg, 80 μmol) was dissolved in 3:7 MeOH–water (1 mL), 2,2,2-trifluoroethyl acetoacetate (20 μL) was added, and the vials were capped and placed into the autosampler. The autosampler was pro-

grammed to dilute an aliquot of this sample at intervals, immediately prior to analysis. Aliquots (10 μL) were added to water (1.0 mL) and mixed, and 10-μL samples were injected. Analysis was performed using PGC as described above for lactosylpyrazolones. Peak areas were measured relative to that of 3-methyl-1*H*-pyrazolone.

*Estimation of the yield of 1-lactosyl-4-tolylazopyrazol-5-one.*—An aliquot (2–100 μL) of a stock solution of lactose containing between 1.6 and 400 nmol was placed into an autosampler vial and evaporated to dryness. Hydrazine hydrate (25 μL) and water (25 μL) were added, and after 4 h the sample was evaporated to dryness. Toluene was added (50 μL), and the samples were evaporated, dissolved in water (25 μL), and re-evaporated before being dissolved in 3:7 MeOH–0.2 M sodium acetate pH 4.6 (100 μL). After the addition of 1:4 2,2,2-trifluoroethyl acetoacetate–2,2,2 trifluoroethanol (20 μL), the vial was capped and incubated at rt for 1 h. The sample was evaporated to dryness (centrifugal evaporator), redissolved in water (25 μL), and re-evaporated before being dissolved in satd $NaHCO_3$ (0.5 mL), followed by the addition of diazotised aminotoluene (2–20 μmol). After 10 min, the reaction was quenched by adding acetylacetone (20 μL), the sample was left for 10 min, then it was quantitatively transferred to appropriate volumetric flasks to produce solutions containing between 0.3 and 20 nmol/mL of the lactose derivative. The insoluble precipitates formed from reagent excess were observed to redissolve upon dilution. Replicate samples were prepared.

The solutions were analysed by HPLC using a Keystone $C_{18}$ column, 5μm, 150 × 4.6 mm. The gradient was 16:85 MeCN–(1:19 MeCN–0.02 M ammonium acetate pH 5.9) for 1 min, increasing linearly to 7:18 at 17 min, then increased 3:2 at 20 min and maintained for 3 min. Aliquots (25 μL) of each sample were injected using an autosampler, and the detection wavelength was 420 nm.

*Estimation of the number of isomers formed for selected monosaccharides and disaccharides.*—Approximately 1.4 μmol of sugar dissolved in water (100 μL) was placed into a vial, 20 μL of hydrazine monohydrate was added and incubated at rt overnight. After drying in a centrifugal evaporator, 0.5 mL of toluene was added, and the samples were re-evaporated. The residue was dissolved in water (200 μL), and an aliquot (50 μL, 350 nmol) was taken for conversion to the azopyrazolone derivative and HPLC analysis in the manner described for the estimation of yield (above).

*Preparation of 1-β-L-fucopyranosyl-4-(4-methylbenzeneazo) - 3 - methylpyrazol - 5 - one* (**12b**).—Fucose hydrazone was prepared according to the method of Williams [4]. Fucose hydrazone (100 mg, 0.56 mmol) was dissolved in 1:3 MeCN–water (5 mL) and 2,2,2-trifluoroethyl acetoacetate (0.2 mL) was added. After 3 h, the sample was lyophilised, the residue was dissolved in 0.5 M $NaHCO_3$ (10 mL), and diazotised 4-methyl aminobenzene (1 mmol) was added in aliquots (4 × 0.5 mL) within 2 min. After 5 min, 2,4-pentanedione (0.2 mL) was added, and the mixture stirred for a further 10 min. The mixture was acidified with AcOH to pH 6 and washed with $CHCl_3$ (2 × 10 mL). It was purified by reversed-phase HPLC as described above for 1-lactosyl-4-(4-methylbenzeneazo)-3-methylpyrazol-5-one, concd on a rotary evaporator, and lyophilised to give a yellow gum (50 mg; 0.14 mmol; 25%). $^1$H NMR ($CDCl_3$): 1.34 (d, 3 H, H-6), 2.26 (s, 1 H, methyl), 2.24 (s, 1 H, methyl), 3.61 (dd, 1 H, H-3, $J_{3,4}$ 3.5 Hz), 3.67 (d, 1 H, H-4, $J_{4,5}$ 0 Hz), 3.82 (q, 1 H, H-5, $J_{5,6}$ 6.5 Hz), 4.17 (dd, 1 H, H-2, $J_{2,3}$ 9.2 Hz), 5.07 (d, 1 H, H-1, $J_{1,2}$ 9.2 Hz), 7.16 (d, 2 H, H-2′, $J_{2'3'}$ 8.4 Hz), 7.26 (d, 2 H), 13.3 (br s, O–H). $^{13}$C NMR ($CDCl_3$): 11.8, 16.5 and 21.0 (3 Me), 68.0, 72.0, 73.2 and 75.0 (Fuc C-2, C-3, C-4 and C-5), 82.1 (Fuc C-1), 115.9 (Ph C-2/6), 127.2 (Ph C-4), 130.2 (Ph C-3/5), 136.2 (Ph C-1), 138.7 (Pyr C-4), 148.8 (Pyr C-3), 159.3 (Pyr C-5). Positive-ion ESIMS: $[M + H]^+ = m/z$ 363. Negative-ion ESIMS: $[M + H]^+ = m/z$ 363.

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