MAGNETIC RESONANCE IN CHEMISTRY, VOL. 35, 191–198 (1997)

NMR Characterization of 3,6-Dioxa-8-azabicyclo[3.2.1]octanes and *N*-[Tris(hydroxymethyl)methyl]alanine Formed from Methylglyoxal in Tris Buffer Solutions

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The reactions of methylglyoxal in aqueous solutions of Tris [tris(hydroxymethyl)aminomethane] buffer were examined at 600 MHz by two-dimensional NMR experiments selected to circumvent the difficulties associated with spectral assignment in the presence of intense resonances due to both solvent and buffer. Methylglyoxal, generated *in situ* from glyceraldehyde 3-phosphate, reacted with Tris to yield principally a mixture of 3,6-dioxa-8-azabicyclo[3.2.1]octanes. ^{1}H and ^{13}C NMR parameters are reported for three compounds containing this little-known ring system and structural influences compared with those in related compounds. A much slower reaction led to the ultimate formation of *N*-[tris(hydroxymethyl)methyl]alanine. Implications for various enzyme assays are considered and the results are compared with previous observations of reactions of α-dicarbonyl compounds with α-amino alcohols.

Magn. Reson. Chem. **35**, 191–198 (1997) No. of Figures: 5 No. of Tables: 3 No. of References: 45

Keywords: NMR; ^{1}H NMR; ^{13}C NMR; 3,6-dioxa-8-azabicyclo[3.2.1]octanes; methylglyoxal; tris(hydroxymethyl)aminomethane; *N*-[tris(hydroxymethyl)methyl]alanine

Received 6 August 1996; revised 10 September 1996; accepted 20 September 1996

INTRODUCTION

The extent to which reactions of aldehydes with amine buffers may compromise studies of certain enzyme-catalysed reactions is of continuing concern to biochemists.[1] Many of these buffers remain in common use in assays in which they might be expected to interfere but complex NMR spectra observed from their reactions with aldehydes have rarely been investigated in detail.

Inhibition by Tris [tris(hydroxymethyl)aminomethane] buffer of the glyceraldehyde phosphate dehydrogenase-catalysed oxidation of glyceraldehyde 3-phosphate was attributed, on the basis of ultraviolet spectroscopic studies,[2] to reversible formation of an imine. Subsequently, we presented NMR evidence[3] suggesting initial formation of 2-(β-phosphoglycolyl)-4,4-bis(hydroxymethyl)-1,3-oxazolidine which decomposed to give a similar product mixture to that formed between Tris and methylglyoxal, which is known[4,5] to form readily from glyceraldehyde 3-phosphate in aqueous solutions.

Characterization of the complex mixture of products apparently formed between methylglyoxal and Tris proved elusive in our earlier work but has been pursued because of several issues of specific biochemical and chemical interest. Thus, the inclusion of 2-amino alcohols such as Tris is recommended[6,7] for assays of 2-oxoaldehyde dehydrogenase, which converts methylglyoxal into pyruvate, while Tris has been used[8] in assays of a methylglyoxal reductase which converts methylglyoxal into lactaldehyde. Moreover, the reaction of α-dicarbonyl compounds with 2-amino alcohols has attracted the attention of chemists because of competition between different possible pathways,[9,10] some of which may contravene selection rules.[11] Here we report the use of selected two-dimensional NMR experiments to characterize the relatively stable intermediates and ultimate product of the reaction of Tris with methylglyoxal derived from glyceraldehyde 3-phosphate in neutral aqueous solutions.

RESULTS AND DISCUSSION

Characterization of buffer solutions

Solutions of glyceraldehyde 3-phosphate and Tris, which had been previously shown[3] to initially form 2-(β-phosphoglycolyl)-4,4-bis(hydroxymethyl)-1,3-oxazolidine, changed during *ca.* 60 h at ambient temperature to consistently yield a mixture characterized by a ^{1}H NMR spectrum as shown in Fig. 1. The high-frequency region [Fig. 1(a)] of this spectrum showed a triplet due to ethanol (as the glyceraldehyde 3-phosphate was prepared from its diethylacetal), three singlet resonances in the approximate ratio of 2:2:1 whose intensity diminished during several weeks at ambient temperature and

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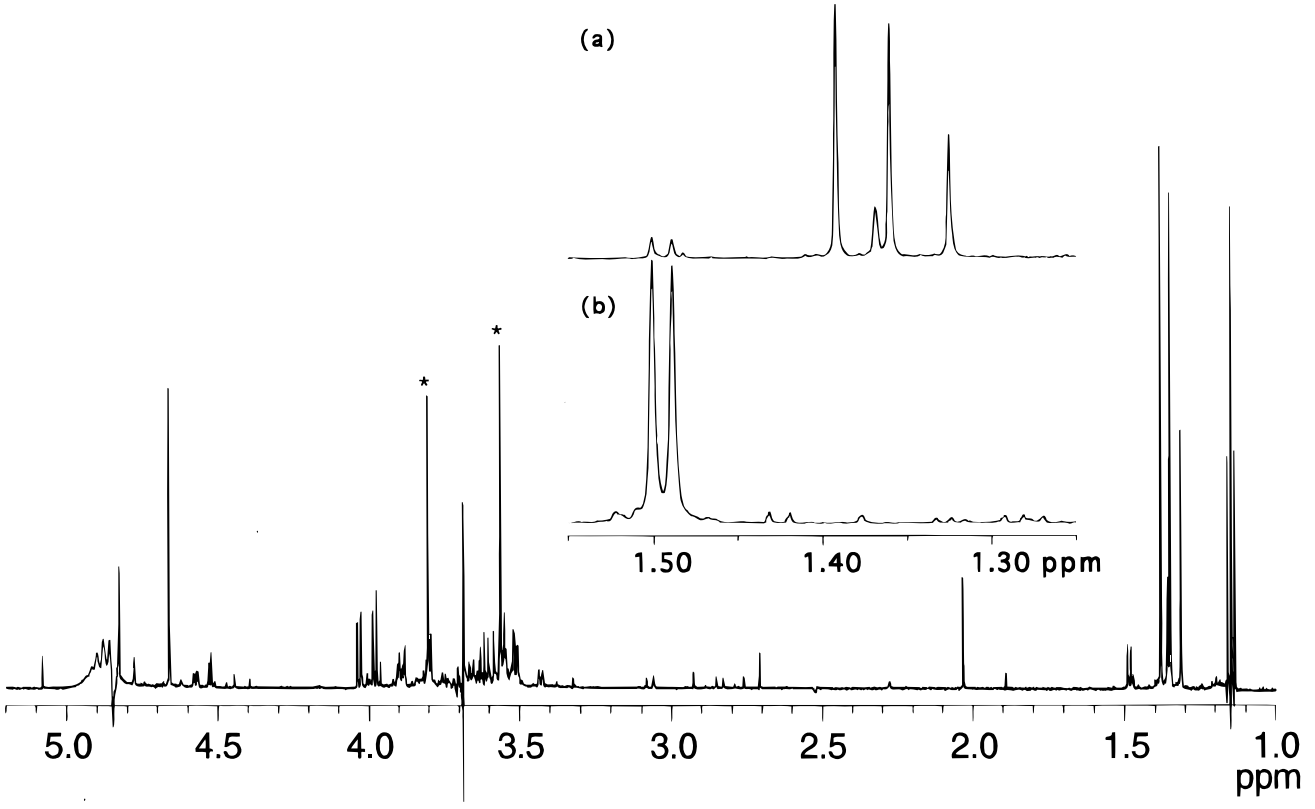


Figure 1. ^{1}H NMR spectrum of a solution of glyceraldehyde 3-phosphate in H_2O containing Tris buffer, after standing for 60 h at ambient temperature. The solvent and buffer resonances were suppressed by method (a). Peaks marked with asterisks are due to the ^{13}C satellites of the Tris CH_2 resonance. The insets show (a) an expansion of the signals assigned to the methyl groups of **4**–**6** and (b) the same region after the sample had been allowed to stand for several weeks at ambient temperature.

a doublet which became predominant [Fig. 1(b)] during this time. Products associated with each of the major methyl resonances accounted for most of the remainder of the spectrum as described below. Structures could be inferred for the broad peak at δ 1.36 and more reliably for the singlet at δ 2.03 but the low intensity of expected associated resonances prevented complete assignments.

Proton correlations could not be readily established by conventional COSY experiments owing to bleaching associated with presaturation of the solvent and buffer resonances, extensive overlap and proximity to the diagonal of product resonances, and the involvement, evident from 1-D spectra, of relatively small couplings. However, a heteronuclear multiple quantum coherence (HMQC[12]) experiment (Fig. 2) revealed six pairs of geminally coupled protons with ^{2}J either 7.5 or 11 Hz.

The interrelationship of the methylene groups was ascertained from a double quantum spectrum[13] (Fig. 3) which readily overcame the limitations encountered with double quantum filtered COSY. The double quantum spectrum, optimized for 2 Hz couplings, shows direct connectivity (Type I[13]) cross peaks between each pair of geminal protons identified in the HMQC experiment and additionally between half of the protons with 7.5 Hz geminal couplings and partners with an 11 Hz geminal coupling. Remote connectivity (Type III[13]) cross peaks indicated in Fig. 3 confirm the assignments for each of the three distinct spin systems.

Geminal couplings of 7.5 and 11 Hz are consistent with values reported[14] for protons next to oxygen in five- and six-membered rings, respectively. Consideration of the possible ways in which methylglyoxal might react with Tris present in large excess leads to four possible structures (Scheme 1, **4**–**7**) which contain the requisite ring systems with a distinct 'W' pathway[15] between the *exo*-protons in each ring to account for the small coupling.

Some discrimination between the structures **4**–**7** was possible from heteronuclear multiple-bond correlation (HMBC[16]) experiments, results from which are summarized in Table 1. Thus, H_{7endo} in each compound has distinct three-bond correlations to C_2 and C_5. Since C_5 is a protonated carbon for the minor product but quaternary for the two major products, the latter must have the methyl group at the bridgehead (**4** and **5**). These compounds can in turn be distinguished by correlations to the stereochemically distinct H_4 for which the resonances were coincident at 303 K but separated by 0.007 ppm at 283 K. Comparable long-range ^{13}C–^{1}H correlations were observed at each temperature but are shown for 283 K in Fig. 4 which indicates an appreciable coupling between H_4 and C_2 in one major isomer but between H_4 and C_5 in the other.

Models indicate that the 'W' pathway noted above requires a slightly flattened chair which suggests that the vicinal coupling between H_4 and C_2 should[17] be large (even after allowing for some reduction due to non-bonded interactions of the axial OH[18]) for **5** in which the dihedral angle between the C_4—H_4 and C_2—O_3 bonds is *ca.* 180° but near zero in **4** where the

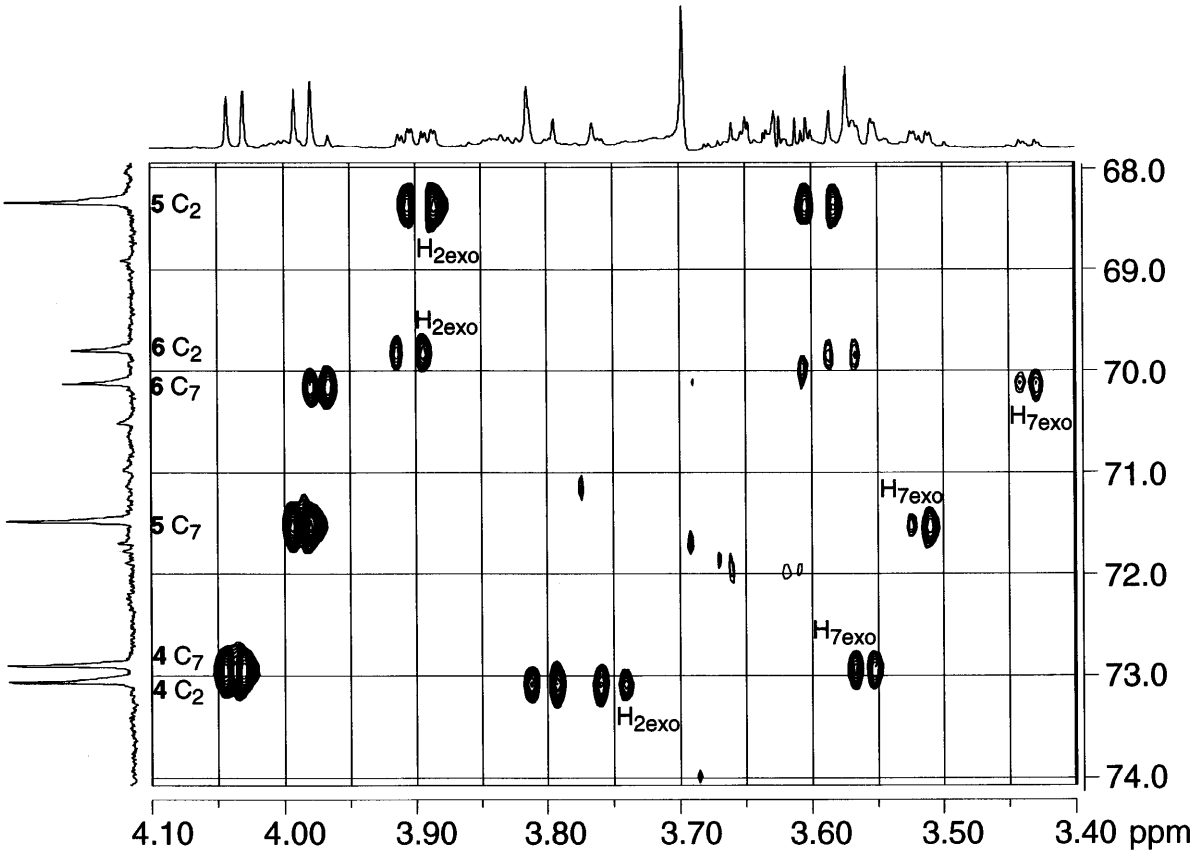


Figure 2. Part of an HMQC spectrum obtained for a solution of glyceraldehyde 3-phosphate which had been allowed to equilibrate in ^2H_2O containing Tris buffer. The solvent and buffer resonances were suppressed by method (b). The spectrum was processed by subtraction of a band of representative t_1 noise and only the positive contours are shown. The projections are from 1-D spectra. Assignments for carbon resonances are given at the left edge of the contour plot and attached *exo*-protons are identified next to the relevant contours.

Table 1. Long-range ^{13}C–^1H correlations observed for 3,6-dioxa-8-azabicyclo[3.2.1]octanes

	Correlation						
Compound	H_{2endo}	H_{2exo}	H_4	H_5	H_{7endo}	H_{7exo}	CH_3
4	C_4	C_7	C_5	—	C_2, C_5	C_2	C_4, C_5
5	C_4	C_1, C_7	C_2	—	C_2, C_5	C_2	C_4, C_5
6	C_4	C_1, C_7	—	C_1, C_4, C_7	C_2, C_5	C_2	C_4, C_5

angle between these bonds is *ca.* 90°. The two-bond correlation between H_4 and C_5 is thus observed in **4** but not **5**, which is consistent with predictions from either the vector resultant[19] or projection sum[20] methods for calculation of similar couplings in carbohydrates (provided the contribution of the less electronegative nitrogen atom is discounted compared with that of oxygen, as has been assumed[21] to rationalize $^2J_{C1'H2'}$ values in an oligonucleotide). A cross peak between H_4 and H_{2exo} for **4** but not **5** in a NOESY experiment at 283 K confirmed the assignment of **4** as the major isomer with the axial proton at C_4.

Differentiation between **6** and **7** as the structure of the minor product is not as straightforward owing to comparable stereochemical factors in each isomer for all long-range C–H correlations observed (Table 1). However in the NOESY experiment, the methyl protons showed a cross peak to H_5 but not to H_{2exo} which models indicated would be closer than H_5 to the *exo*-methyl group of **7**. Moreover in bicyclo[3.2.1]octanols **9** with *exo*-OH groups,[22,23] the γ-carbons have significant low-frequency shifts compared with their *endo* counterparts **10**, irrespective of the presence of a geminally substituted methyl group.[22] Here, shifts of a similar magnitude are seen (Table 2) for C_2 of both **5** and the minor product compared with C_2 in **4**, consistent with *exo*-OH groups at C_4 in **5** and the

Table 2. ^{13}C chemical shifts for 3,6-dioxa-8-azabicyclo[3.2.1]octanes

	Chemical shift (ppm)						
Compound	C_1	C_2	C_4	C_5	C_7	CH_2OH	CH_3
4	66.2	73.1	97.4	96.7	72.9	62.5[a]	19.9
5	67.1	68.3	95.2	97.1	71.5	62.2[a]	20.9
6	64.9	69.8	96.9	93.1	70.1	62.4	25.1

[a] Assignments could be reversed.

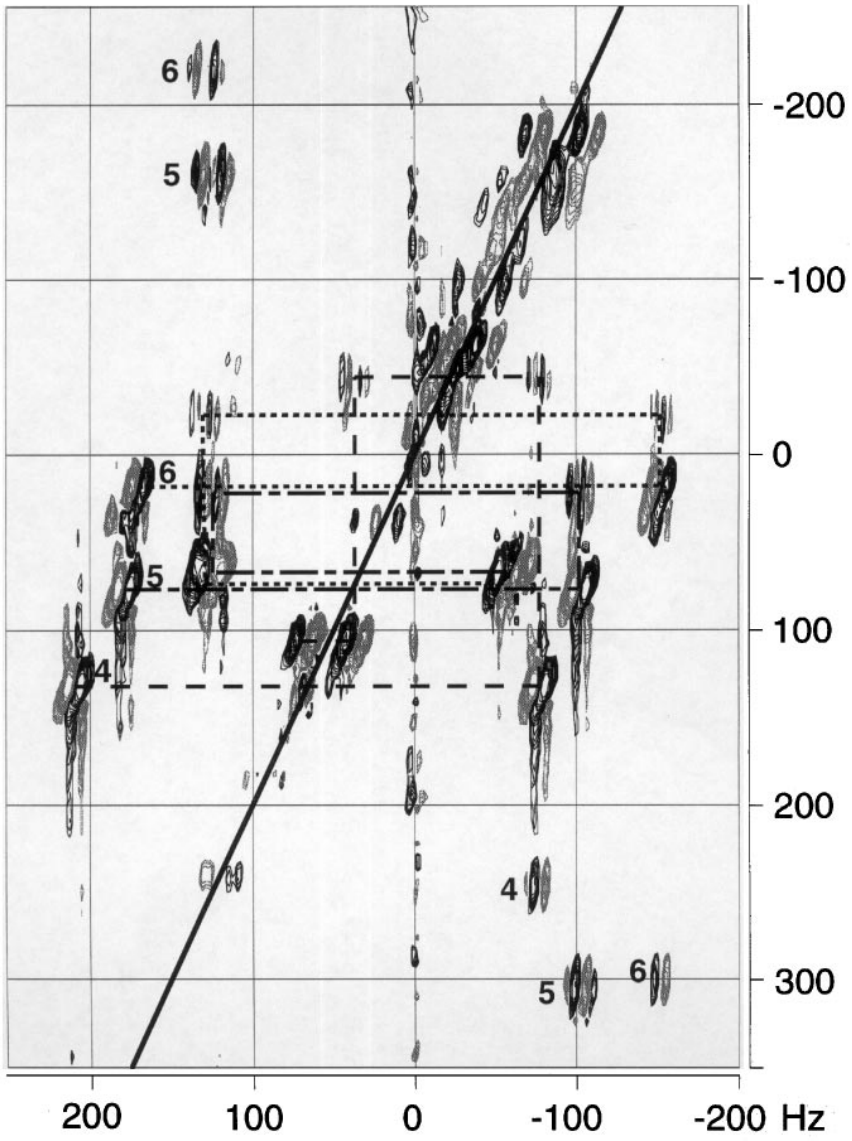
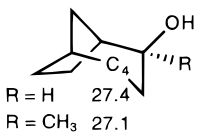


Figure 3. Part of a double quantum spectrum obtained for a solution of glyceraldehyde 3-phosphate which had been allowed to equilibrate in ^2H_2O containing Tris buffer. The solvent and buffer resonances were suppressed by method (a). Negative contours are shown in lighter shading and each spin system is linked by a distinctive dashed line for which the relevant structure is given next to the resonance assigned to H_{7endo}. Remote connectivity peaks (located away from the dashed lines) are identified with their appropriate structure numbers.

minor product but an *endo*-OH in **4**. Thus the minor product has the structure **6** as suggested by the NOE data.

^{1}H NMR data for **4**–**6** are summarized in Table 3. Only the CH_2OH protons could not be assigned in these structures because of severe overlap with the Tris



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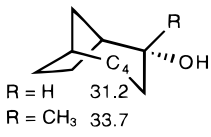

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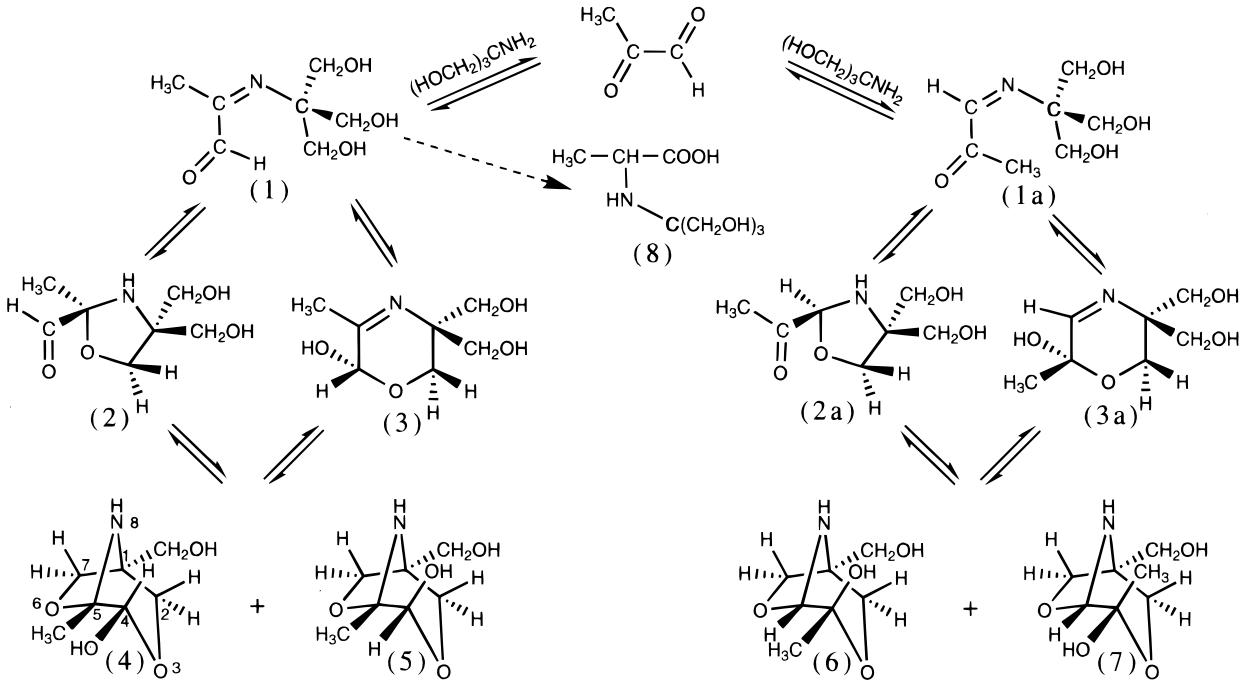
Table 3. ^{1}H NMR data for 3,6-dioxa-8-azabicyclo[3.2.1]octanes

	Chemical shift (ppm)							Coupling constants (Hz)		
Compound	H_{2endo}	H_{2exo}	H_4	H_5	H_{7endo}	H_{7exo}	CH_3	$J_{2exo,2endo}$	$J_{7exo,7endo}$	$J_{2exo,7exo}$
4	3.80	3.75	4.66	—	4.04	3.56	1.35	11	7.4	1.8
5	3.59	3.90	4.66	—	3.99	3.52	1.38	10.7	7.5	1.8
6	3.58	3.90	—	4.77	3.97	3.43	1.31	10.7	7.4	1.8
11a[a]	4.22	4.16	—	—	4.01	3.40	1.49	10	8	2.5
11b[b]	4.26	4.26	—	—	3.96	3.60	1.53	~15	8	

[a] From Ref. 28 for a solution in acetone-d_6 but assignments based on the present work whose numbering system (used by *Chemical Abstracts*) has been adopted.
[b] From Ref. 26 for a solution in CD_3CN but using the numbering system of the present work.



Scheme 1

resonance in the heteronuclear experiments. However, evidence for their presence is seen in the unassigned AB quartets along the skew diagonal of the double quantum spectrum (Fig. 3).

The ^{1}H resonance at δ 2.03 had similar intensity in all samples and is probably due to the 5,6-dihydro-2*H*-1,4-oxazine (**3**), as directly observed and indirectly detected chemical shifts are comparable to those reported for methyl protons[10] and methyl, imine and acetal carbons[24] in similar molecules. Moreover, the NOESY experiments showed chemical exchange cross peaks between the resonance at δ 2.03 and the methyl groups of both **4** and **5**. It was not possible to assign the ring methylene protons of **3** because of the proximity of their putative ^{13}C resonance to that of Tris.

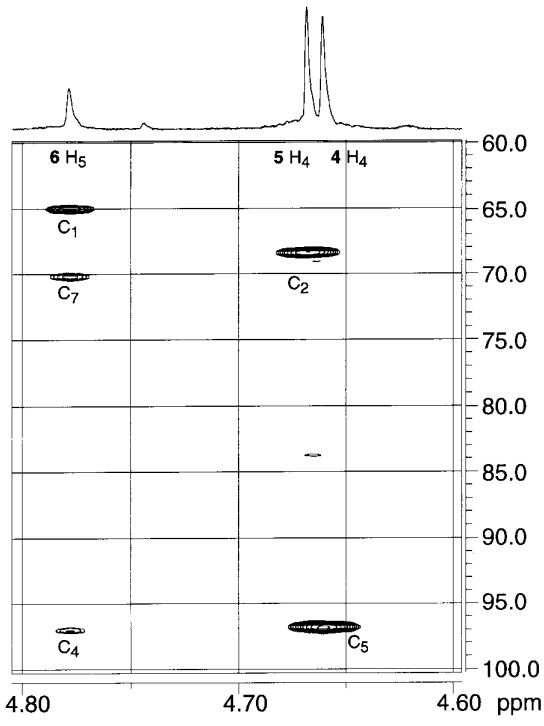


Figure 4. Part of an HMBC spectrum acquired at 283 K for a solution of glyceraldehyde 3-phosphate which had been allowed to equilibrate in H_2O containing Tris buffer. The solvent and buffer resonances were suppressed by method (c). The spectrum is presented in absolute value mode and the projection is taken from a one-dimensional spectrum recorded at 283 K. Assignments of cross peaks are given. At 303 K the resonances assigned to H_4 in **4** and **5** were coincident (Fig. 1).

The NOESY experiments also showed cross peaks indicating chemical exchange between both the methyl group of **6** and unassigned signals at δ 1.36 with a resonance at δ 2.28. The latter resonances were not of sufficient intensity to allow for their unambiguous assignment to specific structures, but their chemical shifts would be consistent with resonances at δ 1.36 being due to methyl groups in **3a** or **7** and that at δ 2.28 to methyl groups in **1a** or **2a**. That is, chemical exchange amongst species on the right-hand side of Scheme 1 would account for the observed cross peaks.

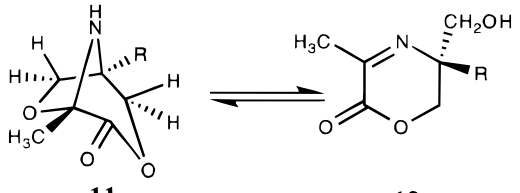
Over time, the methyl resonances associated with bicyclooctanes **4–6** slowly diminished in intensity while the doublet at δ 1.50 increased, until the compound associated with the latter resonance accounted for essentially the entire spectrum [Fig. 1(b)]. This compound was identified as *N*-[tris(hydroxymethyl)methyl] alanine (**8**) from fully assigned and correlated ^{1}H and ^{13}C spectra. Literature data[25] for a DMSO solution of **8** gives only a doublet at δ 1.40 resolved from an envelope of seven protons at δ 3.55, while the recently proposed[26] formation of the derived lactone was not supported by NMR data. The observation of a single AB quartet for the hydroxymethylene protons, with no coupling to the carbonyl carbon, strongly suggests that the species obtained here is the free acid (**8**), the formation of which was accelerated by heating but no attempt was made to optimize the conversion.

Structural influences on NMR parameters

The only previous reports of the ring system represented by **4–6** have been of a rearranged adduct of nitrosocyclohexene and maleic anhydride[27] (which contained an additional ring) and the bicyclooctanones **11**,[26,28] which were obtained as mixtures with their tautomeric oxazinones **12**. Relevant ^{1}H NMR data for the bicyclooctanones are reported in Table 3. Allowing for solvent differences and the effects of the carbonyl group, chemical shifts and coupling constants observed for **4–6** agree satisfactorily with those reported for **11a**. Analysis of the spectrum of **11b**, shown here as the mirror image of that previously reported[26] to facilitate comparison with the present data, was hindered by severe overlap with resonances from the oxazinone. In particular, the value of approximately 15 Hz reported for $J_{2exo,\,2endo}$ was estimated from an 'unresolved AB quartet' and is therefore probably unreliable.

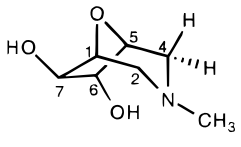
The 'W' coupling between H_{2exo} and H_{7exo} in **4–6** and **11a** can be unequivocally assigned because of the lack of spectral complexity in the individual structures and since there are no alternative pathways to give couplings of similar magnitude. By contrast, a value of 1.8 Hz was reported[29] for $^4J_{H2endo,\,H4endo}$ in the bridged morpholine derivative **13** but splittings of 1.8 and 2 Hz in H_{6exo} were not assigned as they were not mirrored in other resonances. Couplings of 0.8 and 2.2 Hz have also been observed[30] for $^4J_{H2endo,\,H4endo}$ in cocaine (**14a**) and allococaine (**14b**), respectively. Nevertheless, in the present work, the linewidth of H_{4exo} in **4** is comparable to that of H_{4endo} in **5** (Fig. 4, projection) suggesting that the conformation which favours 'W' coupling between H_{2exo} and H_{7exo} may inhibit such coupling between H_{2endo} and H_{4endo}.

Couplings of *ca.* 1.0 Hz were also observed[30] for $^4J_{H1,\,H5}$ in **14a** and $^4J_{H3exo,\,H5}$ in **14b**, but no long-range proton–proton couplings were reported[31] for **15** or other cocaine analogues which were said,[32] on the basis of vicinal couplings, to have a flattened boat conformation. In view of the multiplicity of 'W' pathways in the bicyclo[3.2.1]octane ring system, it is clear that both ends of the coupling network must be identified before drawing inferences regarding conformations. However, this identification may be especially useful in cocaine analogues since it was considered[32] that conformational preferences may be relevant to the efficacy with which these compounds bind to the dopamine transporter.



11 **12**

(a) R = CH_3 (b) R = CH_2OH



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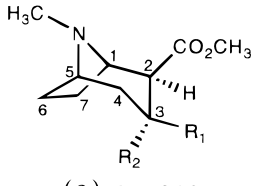

(a) R_1 = OCOPh, R_2 = H

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(b) R_1 = H, R_2 = OCOPh

15 R_1 = Ph, R_2 = H

Chemical and biochemical implications

Ring–chain tautomerism leading to the bicyclic system **11a** was reported[28] as unprecedented in the literature, as was[9] the combination in a single process of the hydroxyketone–hemiacetal and hydroxyimine–1,3-oxazolidine tautomeric equilibria. The system depicted in Scheme 1 and characterized in this work represents a combination and further extension of these phenomena. Both allowed 6-*exo-trig* and forbidden 5-*endo-trig* processes[10,11] are required for the double cyclization essential to the formation of **4–6**. Although it is not possible to state which ring forms first, it is evident from the product distribution that the second cyclization is strongly favoured. The predominance of products derived from (**1**) rather than (**1a**) may reflect stereochemical inhibition of cyclisation in the latter.

The condensation of α-diketones with aminoalcohols and aminophenols has been studied extensively,[10,24,33] with the wide variety of products being strongly influenced by the reaction conditions and nature of the reagents. Reactions of glyoxal[34–37] and phenylglyoxal[38] with similar reagents have also been studied but there do not appear to be any prior reports of reactions of a substituted glyoxal with a polyfunctional primary amine as represented by the reaction of methylglyoxal with Tris. However, the ultimate production of *N*-[tris(hydroxymethyl)methyl]alanine (**8**) is similar to the formation[36] of glycine derivatives (amongst other products) from the reaction of glyoxal with *N*-alkylethanolamines during 2–3 days at ambient temperature. A mechanism involving rearrangement of an epoxide, formed from a vicinal diol analogous to hydrated **3**, was proposed for the reaction which was subsequently adapted[37] to produce *N*-(2-hydroxyethyl)-*N*-alkylglycine derivatives in high yield. Although the latter procedure failed for *N*-unsubstituted ethanolamine, that limitation may not apply to a relatively hindered amine such as Tris. Alternatively, it had been shown previously[39] that reaction of morpholine and piperidine with glyoxal yielded glycinamides, hydrolysis of which gave morpholinoacetic acid and piperidinoacetic acid, respectively. A set of equilibria involving enolization was proposed to account for glycinamide formation and a similar mechanism cannot be excluded here.

From NMR characterization of the products, all of which can be rationalized as derivatives of methylglyoxal shown in Scheme 1, it is evident that the reaction between glyceraldehyde 3-phosphate and Tris is most definitely not reversible. Furthermore, the presence in a NOESY experiment of cross peaks due to chemical exchange confirmed that the spectrum shown in Fig. 1(a) represents a complex equilibrium. As with the bicyclooctanones **11**,[26,28] the bicyclooctanes **4–6**

may not be isolable as discrete species. However, as there was no evidence[3] of significant quantities of free methylglyoxal as the equilibrium was established, it is apparent that the bicyclooctanes **4–6** form rapidly in Tris solutions, even at pH 7. Evaluation of enzyme inactivation observed for 2-oxoaldehydes should therefore take into consideration the buffer which has been used for the assay as well as the recognized importance of order of addition.[40] The role of aminoalcohols in facilitating 2-oxoaldehyde dehydrogenase activity remains unclear but it is evident that the observation of optimal activity in Tris buffer[41] at pH 8.0 is not due to polymerization or failure of methylglyoxal to react with Tris in neutral solutions.

EXPERIMENTAL

Samples

Glyceraldehyde 3-phosphate, as the diethylacetal, was obtained from Sigma Chemical (St Louis, MO, USA). Samples for NMR analysis were prepared by the addition of glyceraldehyde 3-phosphate (final concentration *ca.* 65 mM) to solutions of Tris (final concentration *ca.* 1.7 M) constituted in either H_2O or ^2H_2O (Australian Institute for Nuclear Science and Engineering, Lucas Heights, NSW, Australia), adjusted to a glass electrode/pH meter reading of 7.0–7.2 with either NaO^2H, ^2HCl, NaOH or HCl. Although all products were ultimately characterized as derived from methylglyoxal, preparation of samples from glyceraldehyde 3-phosphate avoided the lengthy purification procedure required for commercial methylglyoxal which is known[42] to be seriously contaminated.

Parameters for NMR experiments

All spectra were obtained with a Bruker AMX-600 NMR spectrometer, using a broadband inverse-detection probe at 600.14 MHz (^1H) or 150.92 MHz (^{13}C) with the variable-temperature unit set to 303 K, except for the HMBC and NOESY experiments, which were performed at both 283 and 303 K. Spectral widths for the 2D experiments were routinely 2645 Hz for ^1H and 13 889 Hz for ^{13}C, but regions covering 13.4 ppm for ^1H and 237 ppm for ^{13}C were explored.

^1H-detected spectra were acquired with presaturation of both the H_2O/HO^2H and Tris CH_2 resonances. This was achieved by (a) combination of a low-power transmitter pulse (2 s) at the frequency of the Tris CH_2 with simultaneous CW irradiation at the water frequency through a directional coupler; (b) a train of ten square pulses of 100 ms duration applied to the Tris CH_2 through the transmitter channel interleaved with a train of ten similar pulses of 100 ms at the water frequency through the decoupler channel; and (c) a train of 20 modulated square pulses (100 ms) generated with the program MULE (modify shape for MULtiple Excitation, Bruker version 950516) to partition the pulse energy between the water (70%) and Tris resonances (30%), the ratio being determined empirically. Generally (a) was preferred for the one-dimensional experiments but (b) and (c) produced much less t_1 noise in the two-dimensional spectra.

^{13}C NMR spectra were acquired with proton decoupling (WALTZ-16[43]). Pulse lengths ($\pi/2$) were typically 10 μs for ^{13}C and 17–22 μs for ^1H, the longer pulses being encountered when using the directional coupler. ^1H and ^{13}C chemical shifts are given for spectra obtained in H_2O and referenced to sodium 3-trimethylsilyl-2,2,3,3-tetradeuteriopropionate (TSP-d_4, sodium salt) (Commissariat à l'Énergie Atomique, Gif-sur-Yvette, Cedex, France) in ^2H_2O which was contained within a capillary placed in the sample.

The following specific conditions were employed in standard Bruker (UXNMR 930601) pulse programs modified to provide the appropriate presaturation. HMQC[12] experiments, optimized for $^1J_{CH}$ of 155 Hz, were acquired for 512 increments over 1024 points, with GARP-1[44] decoupling during acquisition, and processed with a $\pi/2$ shifted sine bell and zero-filling in each dimension. HMBC[16] experiments were optimized for $^nJ_{CH}$ of 6.25 Hz and acquired for 256 increments over 1024 points and the data were examined in absolute value mode after zero-filling and processing with a sine-bell in F_2 and 20° shifted sine-bell in F_1. Some of the cross peaks reported in Table 1 were only evident after subtraction of a projection representative of the t_1 noise. Uncertainties introduced by use of this technique were minimized by obtaining the HMBC spectra on several samples, at two temperatures and by using both methods (b) and (c) for presaturation. Double quantum spectra[13] employed a fixed delay, $1/4J$, of 120 ms and were obtained for spectral widths of 3448 Hz over 2048 points in F_2 and 6896 Hz over 1024 increments in F_1 and were processed with a $\pi/2$ shifted sine-bell in each dimension. The NOESY[45] experiments employed a 1 s mixing time, were acquired for 400 increments over 2048 points, and were processed with zero-filling in F_1 and a $\pi/2$ shifted sine-bell in each dimension.

Spectral data

The NMR data for bicyclooctanes reported in the tables were obtained from first-order analysis of 1-D spectra (5000 Hz over 29 999 points zero-filled to 64K for ^1H and 35 714 Hz over 76 921 points zero-filled to 128K for ^{13}C) except for the chemical shifts of H_{2endo} for all compounds and H_{2exo} for **4**, and $J_{2exo,\,2endo}$ for **4**, which were obtained from 2-D spectra, calibrated with parameters determined from 1-D experiments. Otherwise, all samples contained Tris, ^1H, 3.69 (s); ^{13}C, 61.8 (CH_2), 63.3 (quat.) and a singlet of relatively low intensity at δ 2.03 for which partial assignments were consistent with chemical shifts and connectivities expected for **3**: ^1H, 2.03 (s, 3 H), 5.08 (s, 1 H); ^{13}C, 23.8, 60.7, 88.5, 172.9; long range ^1H–^{13}C correlations, 2.03 (88.5, 172.9), 5.08 (60.7). A broad signal at δ 1.36 was present in all samples and shown by resolution enhancement to comprise at least two singlets, while the remaining unassigned signals had lower and slightly variable intensity in different samples. The intensities of all resonances assigned to methyl protons were diminished in samples dissolved in ^2H_2O.

Samples were generally stored at 5 °C but when it became evident that this involved slow changes in composition, some samples were allowed to stand at ambient temperature in order to determine if the change was accelerated. During periods of several months at 5 °C or several weeks at ambient temperature, all solutions showed an increase in the intensity of the methyl resonance at δ 1.50. At equilibrium the product associated with this resonance was predominant and characterized as *N*-[tris(hydroxymethyl)methyl]alanine (**8**): ^{1}H, 1.50 (d, 3 H, J = 7.2 Hz), 3.95 (q, 1 H, J = 7.2 Hz), 3.71, 3.74 (6 H, AB quartet, J = 12.5 Hz); coupled ^{13}C, 19.5 (dq, J = 4.3, 130.4 Hz), 55.2 (d, J = 148.1 Hz), 61.0 (t, $J \approx$ 145 Hz), 68.1 (quat.), 178.1 (quat.); long range ^{1}H–^{13}C correlations, 1.50 (55.2, 178.1), 3.71/3.74 (61.0, 68.1), 3.95 (19.5, 68.1, 178.1). Heating a sample, previously stored at 5 °C and then ambient temperature, to 60 °C for 10 h caused an increase in the ratio of intensities of the doublet at δ 1.50 to each of the singlets at δ 1.35 and δ 1.38 from *ca.* 8:1 to *ca.* 30:1.

Acknowledgement

Bill Lowe is thanked for preparation of the samples.

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