Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 853–862

BIOORGANIC & MEDICINAL CHEMISTRY

The Role of Dicarbonyl Compounds in Non-enzymatic Crosslinking: A Structure–Activity Study

Susie J. Meade, Antonia G. Miller and Juliet A. Gerrard*

Department of Plant and Microbial Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

Received 2 September 2002; accepted 5 November 2002

Abstract—The Maillard reaction is a complex network of reactions that has been shown to result in the non-enzymatic crosslinking of proteins. Recent attention has focussed on the role of α-dicarbonyl compounds as important in vivo contributors to protein crosslinking but, despite extensive research, the molecular mechanisms of the crosslinking reaction remain open to conjecture. In particular, no relationship between the structure of the carbonyl-containing compounds and their activity as crosslinking agents has been established. In an effort to elucidate a structure–reactivity relationship, a wide range of dicarbonyl compounds, including linear, cyclic, di-aldehyde and di-ketone compounds, were reacted with the model protein ribonuclease A and their crosslinking activity assessed. Methylglyoxal and glutaraldehyde were found to be the most efficient crosslinkers, whilst closely related molecules effected crosslinking at a much lower rate. Cyclopentan-1,2-dione was also shown to be a reactive crosslinking agent. The efficiency of methylglyoxal and glutaraldehyde at crosslinking is thought to be related to their ability to form stable heterocyclic compounds that are the basis of protein crosslinks. The reasons for the striking reactivity of these two compounds, compared to closely related structures is explained by subtle balances between competing pathways in a complex reaction network.

Introduction

The Maillard reaction is the term used to describe a complex network of reactions resulting from the initial condensation of a free amine, including those of proteins, and a carbonyl-containing compound, usually a sugar. These reactions are of interest in any system where amines and carbonyl-containing compounds are available to react, and have been shown to be of particular importance in the medical arena and in food science.[1] Although first described in 1912,[2] the complexity of the Maillard reaction has precluded full characterisation of the vast array of molecules that result from the later stages of this chemistry.[3] The Maillard reaction of proteins, also termed protein glycation, has been the subject of intense recent research, due to its importance in the pathophysiologies of many conditions associated with aging,[4–7] especially in diabetics, where sugar concentrations in the body are unusually high and glycation is more likely to occur.[8–10]

The compounds produced during the later stages of the Maillard reaction of proteins are referred to as advanced glycation end products (AGEs). In some diseases, such as diabetes and uremia, advanced lipoxidation end products (ALEs) have also been implicated. For example, increased levels of ALEs, such as malondialdehydelysine, have been observed in plasma proteins in uremia.[11] These ALEs are thought to result from increased plasma concentrations of small, reactive carbonyl precursors of AGEs and ALEs, such as glyoxal, methylglyoxal, 3-deoxyglucosone, dehydroascorbate, and malondialdehyde.[11]

One class of AGE compounds that has attracted particular literature scrutiny is the non-enzymatic crosslinks.[12] Several authors have suggested that prevention or reversal of these crosslinks in vivo may lead to novel therapeutic strategies for the treatment of conditions associated with aging.[13–15]

However, progress in this field is severely hindered by a lack of fundamental knowledge as to the nature of the crosslinks formed in vivo[16,17] and the mechanisms by which they are formed.[18–20]

Early research into protein crosslinking via the Maillard reaction focussed on the role of glucose as the crosslinking moiety. This work culminated in the proposal of

*Corresponding author. Tel.: +64-3-364-2987x7302; fax: +64-3-364-2083; e-mail: j.gerrard@botn.canterbury.ac.nz

0968-0896/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
PII: S0968-0896(02)00564-3

a structure for the crosslink by Vasan et al.[15] and, further, a suggestion that this structure could be specifically cleaved by *N*-phenacylthiazolium bromide. This suggestion proved very contentious[21,22] and more recent literature has focused on the role of α-dicarbonyl compounds in the non-enzymatic crosslinking of proteins facilitated by dicarbonyl compounds, with particular attention being paid to the role of methylglyoxal.[23–27] However, surprisingly little is known about why some dicarbonyl compounds crosslink proteins more rapidly than others. In a previous study we have noted that structurally related α-dicarbonyls such as, methylglyoxal, biacetyl and glyoxal crosslink proteins at vastly different rates.[27] Although not commonly considered in the Maillard field, other dicarbonyls such as glutaraldehyde are known to induce rapid crosslinking of proteins.[28,29] To our knowledge, the relationship between the structure of a dicarbonyl compound and its ability to crosslink proteins has not been systematically explored.

The molecular mechanisms of the crosslinking reaction, by those molecules, which have been studied in detail, remain open to conjecture. Some crosslinked structures resulting from dicarbonyl compound reactions with model proteins have been identified and possible mechanisms for their formation have been postulated. Many of these crosslinks include heterocyclic structures. Examples include, pentosidine, glucosepane, MOLD (methylglyoxal lysine dimer), GOLD (glyoxal lysine dimer), GODIC (glyoxal lysine arginine dimer), MODIC (methylglyoxal lysine arginine dimer), and DOGDIC (a lysine arginine dimer) (Fig. 1).[20,30–34]

Research to date has largely focussed on a small number of biologically important α-dicarbonyl compounds.

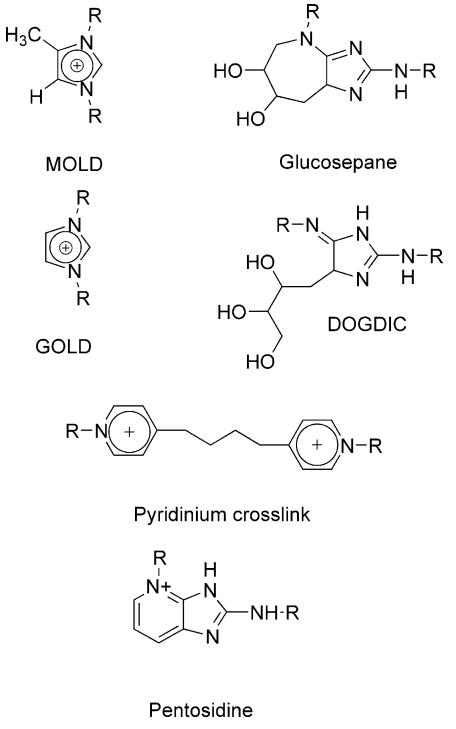


Figure 1. A selection of proposed heterocyclic crosslink structures.

Rather than focus only on those molecules known to exist in vivo, we have adopted a novel approach to the problem and assessed the crosslinking ability of a large series of simple dicarbonyl compounds in an effort to establish the effect of structure on crosslinking reactions. The goal of this study was to elucidate relationships between the structure of the dicarbonyl and its reactivity, and to use these relationships to provide insight into the mechanisms of crosslinking in vivo.

Results

Choice of model system

Ribonuclease A (RNase A) has been extensively used as a model protein for crosslinking studies in our own and other laboratories.[35–38] It is a well-characterised, commercially available protein that is both thermally and chemically stable and has a number of amino groups that have been shown to be reactive during protein crosslinking.[39] RNase A was thus selected as the protein of choice for a systematic exploration of the structural features required in dicarbonyl compounds to facilitate efficient protein crosslinking. A high concentration of dicarbonyl compounds was selected in order to compare a broad range of reactivities on a reasonable timescale.

Effect of chain length of dialdehyde

Dialdehydes constitute a class of difunctional compounds that contains some well known protein crosslinkers. Glutaraldehyde, a 1,5-dialdehyde, has been widely used in microscopy and for various biotechnological applications, notably conjugation of specific proteins to anti-bodies by enzyme-linked immunosorbent assay (ELISA).[40] Glyoxal, a 1,2-dialdehyde, has attracted much interest in medical research into diseases associated with the Maillard reaction, such as diabetes and cataracts. It has been identified as a breakdown product of glucose in vivo.[41] The ability of glyoxal to crosslink proteins has been widely reported and a heterocyclic crosslink structure for a lysine-lysine dimer (GOLD) has been proposed, which has been detected in vivo.[25]

Malondialdehyde, a 1,3-dialdehyde is reported to be a rapid crosslinker of proteins and heterocyclic crosslink structures have been proposed.[42] However, despite the literature on individual members of this series, no systematic investigation of linear dialdehydes of various chain lengths has been undertaken. The purpose of this part of our investigation was to assess the relative efficiency of crosslinking for this type of molecule as a function of chain length. The dialdehydes chosen for study were: glyoxal (ethanedial), malondialdehyde (propanedial), succinaldehdye (butanedial), glutaraldehyde (pentanedial) and hexanedial (Fig. 2).

Glyoxal and glutaraldehyde were obtained commercially. Malondialdehyde was simply generated from hydrolysis of the commercially available 1,1,3,3-tetramethoxypropane immediately prior to use[43] and

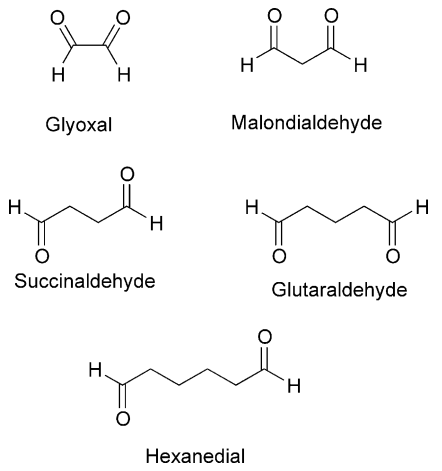


Figure 2. Structures of the dialdehydes studied (all structures are drawn as dicarbonyl compounds for clarity).

characterised by proton NMR spectrometry. In addition, incubations were carried out with the precursor molecule 1,1,3,3-tetramethoxypropane, and the resulting crosslinking was indistinguishable from that of malondialdehyde. Thus malondialdehyde could be successfully generated in situ as the reaction proceeded. Succinaldehyde was prepared immediately prior to reaction by acid hydrolysis of dimethoxydihydrofuran.[44] Hexanedial was prepared by ozonolysis of cyclohexene using a modification of the method of Hudlicky,[45] and characterised by proton NMR spectrometry. Typical SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis of the products of the incubations of glyoxal, malondialdehyde, succinaldehyde, glutaraldehyde and hexanedial with RNase A are shown in Figures 3 and 4. After a period of 5 days, glyoxal incubations with RNase A produced a significant amount of dimer and a trace of trimer. After this same time interval, incubations in the presence of malondialdehyde had resulted in numerous multimers visible by SDS-PAGE, indicating that malondialdehyde is a more effective crosslinker of RNase A than glyoxal. Incubations containing succinaldehyde and RNase A invariably resulted in the production of a gelatinous mixture. These samples were not soluble in water or gel

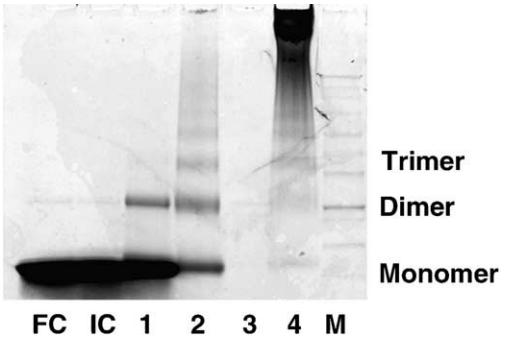


Figure 3. A typical SDS-PAGE showing the effect chain length of dialdehydes on crosslinking of RNase A (SDS-PAGE gel of samples @37 °C for 5 days FC = Frozen control RNase A, IC = Incubated control RNase A, 1 = RNase A + glyoxal, 2 = RNase A + malondialdehyde, 3 = RNase A + succinaldehyde, 4 = RNase A + glutaraldehyde, M = Marker).

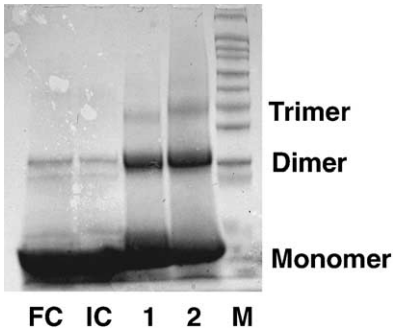


Figure 4. A typical SDS-PAGE showing the effect of varied chain length of dialdehydes on crosslinking of RNase A (SDS-PAGE gel of samples @37 °C for 5 days FC = Frozen control RNase A, IC = Incubated control RNase A, 1 = RNase A + glyoxal, 2 = RNase A + hexanedial, M = Marker).

treatment buffer and therefore these samples were not amenable to electrophoretic analysis. However, gelatinous reaction mixtures have been associated with compounds that are efficient crosslinkers, suggesting a high reactivity for this member of the series.[46,47] Gelatinous material was also occasionally observed in glutaraldehyde crosslinking reactions. Glutaraldehyde appeared to produce the most crosslinking with extensive formation of large aggregates which is shown by the large amount of material at the top of the gel. Analysis of incubations of hexanedial and RNase A after 5 days at 37 °C indicated the presence of dimer, trimer and a small amount of larger multimers. Thus it was concluded that hexanedial was more efficient at crosslinking than glyoxal. However, it appeared to be substantially less efficient than glutaraldehyde. The data are consistent with increasing chain length corresponding to an increase in reactivity until glutaraldehyde, with a sudden drop at hexanedial. This led us to hypothesise that the smaller members of the series crosslink via the efficient production of heterocyclic structures that are inaccessible for hexanedial.

Crosslinking with cyclic α-dicarbonyls: cyclopentane-1,2-dione and derivatives

Another class of compounds that attracted our attention as protein crosslinking agents is the cyclopentanediones. We have previously reported[37] the crosslinking of RNase A by cyclotene (3-methylcyclopentane-1,2-dione), a volatile flavour compound that has also been identified as a breakdown product of ascorbic acid.[48] Cyclotene was previously shown to crosslink RNase A over 15–30 days, and show unusually distinct dimers and trimers, as judged by SDS-PAGE. In this study, the crosslinking ability of cyclotene was compared to two structurally related cyclopentane dicarbonyls, cyclopentane-1,2-dione and 3,5-dimethylcyclopentane-1,2-dione (Fig. 5).

3,5-Dimethylcyclopentan-1,2-dione is commercially available, but cyclopentane-1,2-dione required synthesis. It was prepared by the method of Acheson[49] and characterised by proton NMR spectrometry and X-ray crystallography.[50] A representative SDS-PAGE analysis of the products of incubations of these dicarbonyls with

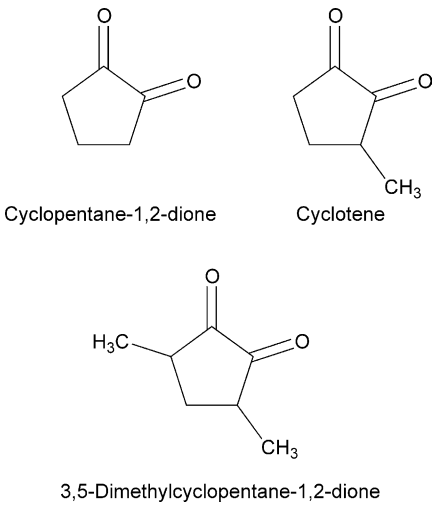


Figure 5. Structure of cyclic α-dicarbonyls: cyclopentane-1,2-dione and derivatives studied.

RNase A is shown in Figure 6. Strikingly, incubations after 5 days containing cyclopentane-1,2-dione showed extensive crosslinking, with multiple bands indicating the presence of dimer, trimer and aggregates formed that were too large to enter the gel and remained in the wells at the top of the gel. To our knowledge, this is the first time that the crosslinking ability of this molecule has been demonstrated, despite recent reports that cyclopentane-1,2-dione is present in vivo. In contrast, both cyclotene and 3,5-dimethylcyclopentane-1,2-dione produced only dimers of RNase A over the same time period. No significant crosslinking was observed in the controls. Thus, under these conditions, the least substituted molecule, cyclopentane-1,2-dione was the most efficient crosslinker of this series. This dramatic change in reactivity caused by a single methyl substitution may indicate that the rate determining step in the formation of the crosslink is sterically hindered in the case of cyclotene and 3,5-dimethylcyclopentane-1,2-dione.

Crosslinking with cyclic dicarbonyls— the effect of ring size

The efficient crosslinking of cyclopentane-1,2-dione prompted the investigation of other cycloalkane-1,2-diones to determine the effect of ring size on the crosslinking ability of cycloalkane-1,2-diones. The four-, five-, and six-membered rings were assessed (Fig. 7). Cyclobutane-1,2-dione and cyclohexane-1,2-dione were both obtained commercially. Typical results from incubations of cyclobutane-1,2-dione, cyclopentane-1,2-dione and cyclohexane-1,2-dione with RNase A are shown in Figure 8. Minimal crosslinking was evident for cyclobutane-1,2-dione after 7 days. Cyclohexene-1,2-dione produced both dimer and a small amount of trimer over 7 days under these conditions. Thus cyclopentane-1,2-dione was by far the most efficient crosslinker in the series. Interestingly, the efficiency of crosslinking correlates with the number of carbons in both linear series and the ring size. Glutaraldehyde and cyclopentane-1,2-dione are more efficient relative to cyclohexane-1,2-dione and hexanedial, suggesting that a five-carbon species is optimal for protein crosslinking reactions.

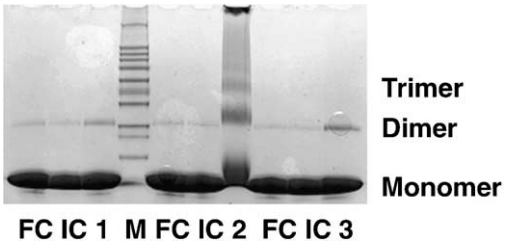


Figure 6. A typical SDS-PAGE showing the effect of methyl substitution on cyclopentanedione on crosslinking of RNase A (SDS-PAGE gel of samples @37 °C for 5 days FC = Frozen control RNase A, IC = Incubated control RNase A, 1 = RNase A + Cyclotene, M = Marker, 2 = RNase A + cyclopentane-1,2-dione, 3 = RNase A + 3,5-dimethylcyclopentane-1,2-dione).

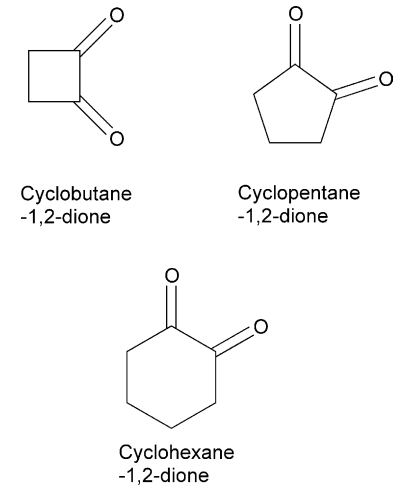


Figure 7. Structures of cyclic dicarbonyls studied.

Crosslinking with cyclic dicarbonyls— the effect of relative dicarbonyl position

The particular reactivity of cyclopentane-1,2-dione relative to either cyclobutane-1,2-dione or cyclohexane-1,2-dione led to the investigation of ring substitution

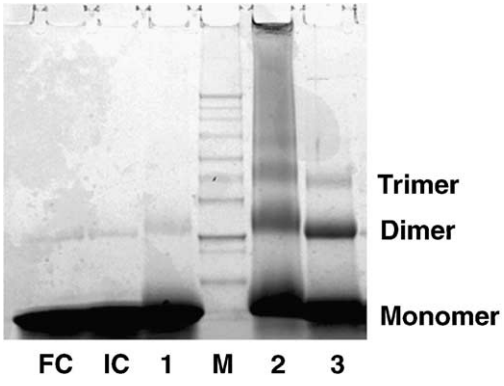


Figure 8. A typical SDS-PAGE showing the effect of ring size on the crosslinking ability of cycloalkane-1,2-diones for four-, five-, and six-membered rings (SDS-PAGE gel of samples @37 °C for 7 days FC = Frozen control RNase A, IC = Incubated control RNase A, 1 = RNase A + cyclobutane-1,2-dione, 2 = RNase A + cyclopentane-1,2-dione, 3 = RNase A + cyclohexane-1,2-dione).

patterns of the dicarbonyl ring compounds. All dicarbonyl ring substitution patterns were assessed for the five- and six-membered cycloalkane-diones. All these compounds, except cyclopentane-1,2-dione, were commercially available (Fig. 9).[49]

As above, 7-day incubations of cyclopentane-1,2-dione showed extensive crosslinking of RNase A (Fig. 10). In contrast, cyclopentane-1,3-dione incubations with RNase A over the same time period indicated that no crosslinking was evident. It was therefore concluded that cyclopentane-1,2-dione was much more efficient at crosslinking than cyclopentane-1,3-dione. Cyclohexane-1,2-dione incubations produced a small amount of dimer after 7 days. Similarly, cyclohexane-1,4-dione produced a minimal amount of dimer, whilst no crosslinking was observed in incubations containing cyclohexane-1,3-dione Cyclohexane-1,2-dione was more effective at crosslinking than either cyclohexane-1,3-dione or cyclohexane-1,4-dione. Thus the 1,2-dione substitution pattern for both five and six-membered cycloalkanes appeared to be the more efficient at crosslinking RNase A.

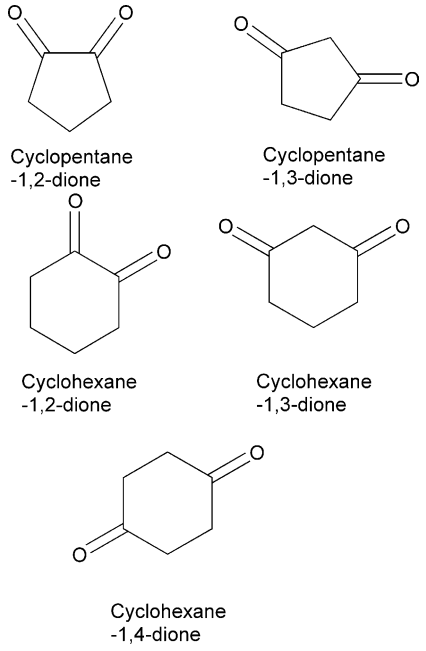


Figure 9. Structures of cyclopentane-diones and cyclohexane-diones studied.

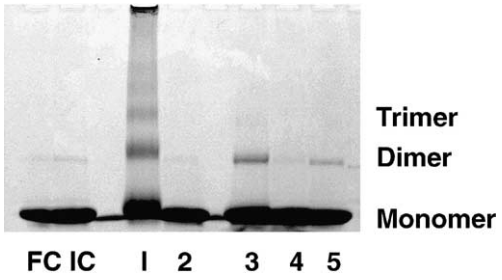


Figure 10. A typical SDS-PAGE showing the effect of ring substitution patterns of the dicarbonyl ring compounds on crosslinking (SDS-PAGE gel of samples @37 °C for 7 days FC = Frozen control RNase A, IC = Incubated control RNase A, 1 = RNase A + cyclopentane-1,2-dione, 2 = RNase A + cyclopentane-1,3-dione, 3 = RNase A + cyclohexane-1,2-dione, 4 = RNase A + cyclohexane-1,3-dione, 5 = RNase A + cyclohexane-1,4-dione).

Crosslinking with α-dicarbonyl compounds

Previous research had established the relative crosslinking reactivities of glyoxal, methylglyoxal and biacetyl and shown that methylglyoxal crosslinks RNase A significantly more rapidly than either glyoxal or biacetyl.[27] This large difference in crosslinking ability led us to examine the reactivity of some structurally related compounds. Methylglyoxal molecules contain both an aldehyde and a ketone moiety which suggested that perhaps both were required for efficient crosslinking. Thus, the following series of molecules (Fig. 11) was investigated, each an α-dicarbonyl compounds containing one aldehyde and one ketone moiety. These compounds were compared with α-dicarbonyl compounds that contained either two aldehyde or two ketone moieties, namely glyoxal and biacetyl, respectively.

Both methylglyoxal and phenylglyoxal were commercially available, whilst ethylglyoxal required synthesis. Although a literature method was found, the production of ethylglyoxal proved to be a synthetic hurdle that could not be overcome. Following literature precedent[51,52] a synthesis from butanal was attempted. Butanal was successfully chlorinated to form the α-dichloroaldehyde.[51] This was, in turn, treated with methoxide to form the methoxy-protected form of ethylglyoxal. Removal of the methoxy protecting groups proved somewhat challenging: the acetal was initially subjected to acid as described by Verhe,[52] but proved to be more resistant to acid hydrolysis than reported. A systematic investigation of acid hydrolysis conditions with increasing strength of acid led only to recovery of starting material on each occasion.

Another unsymmetrical dicarbonyl, phenylglyoxal, was studied under standard incubation conditions. When analysed by SDS-PAGE (Fig. 12), incubations of RNase and phenylglyoxal were indistinguishable from the incubated control. During the same period, crosslinking was observed in incubations containing methylglyoxal or glyoxal, with trimer and dimer clearly visible

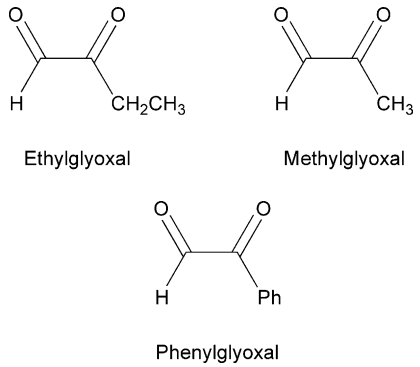


Figure 11. Structures of unsymmetrical α-dicarbonyl compounds studied.

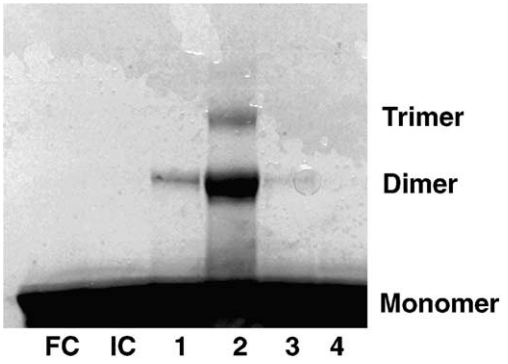
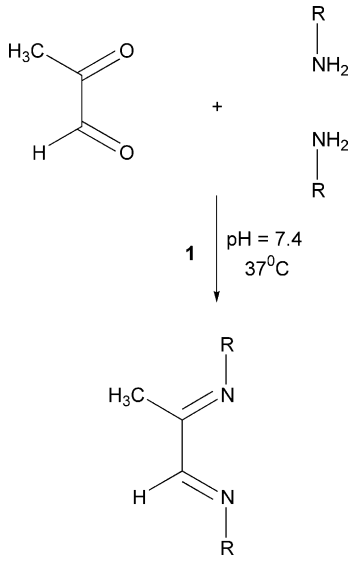


Figure 12. A typical SDS-PAGE showing the effect of symmetrical versus unsymmetrical α-dicarbonyls (SDS-PAGE gel of samples @37 °C for 1 day FC = Frozen control RNase A, IC = Incubated control RNase A, 1 = RNase A + glyoxal, 2 = RNase A + methylglyoxal, 3 = RNase A + biacetyl, 4 = RNase A + phenylglyoxal).



Scheme 1. Reaction of methylglyoxal with amine groups to form a diimine.

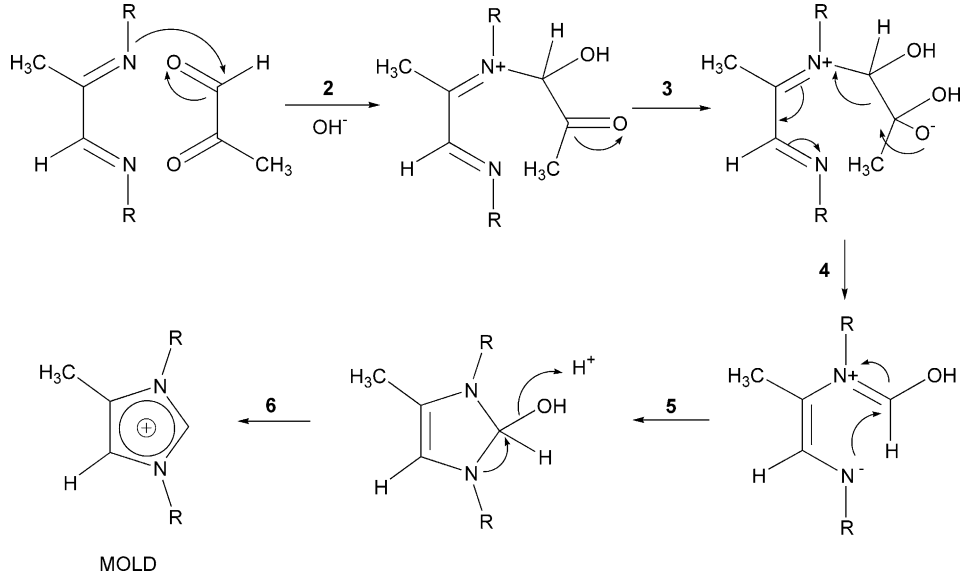
after 1 day. However, the failure of phenylglyoxal to crosslink may be related to its use as an arginine capper,[53,54] such that phenylglyoxal might be irreversibly capping arginine residues and not crosslinking; although why this should not occur with methylglyoxal is not clear. Thus, whether or not the unique reactivity of methylglyoxal is due to the presence of one aldehyde and one ketone moiety remains to be rigorously tested.

Discussion

Unlike previous research into the crosslinking of proteins via the Maillard reaction, which has focussed on a small number of biologically important α-dicarbonyl compounds, we sought to investigate a wider range of dicarbonyl compounds with a view to probing the relationship between the structure of the carbonyl containing compound and the crosslinking reactivity displayed. The goal of this study was to establish if there are structure–reactivity relationships for dicarbonyl compounds that are able to predict efficiency of protein crosslinking. These structure—activity relationships, should they exist, may provide insight into the structural features required for an effective crosslinking reagent and thence the mechanisms of these important reactions.

The literature consensus on the structure of protein crosslinks suggests that heterocyclic structures predominate.[36,31,55] The discussion within this paper is framed around lysine-lysine crosslinks, as the preceding paper clearly indicates the importance of lysine in this type of crosslinking.[27] However, many of the arguments are valid for arginine-lysine crosslinks.[31]

In recent years, a number of heterocyclic crosslink structures have been identified in crosslinking reactions. One of these crosslinked structures is MOLD and a reaction mechanism for its formation has been proposed by Brinkman et al.[56] (Schemes 1 and 2). The initial reaction to form a diimine is well characterised (Scheme 1).



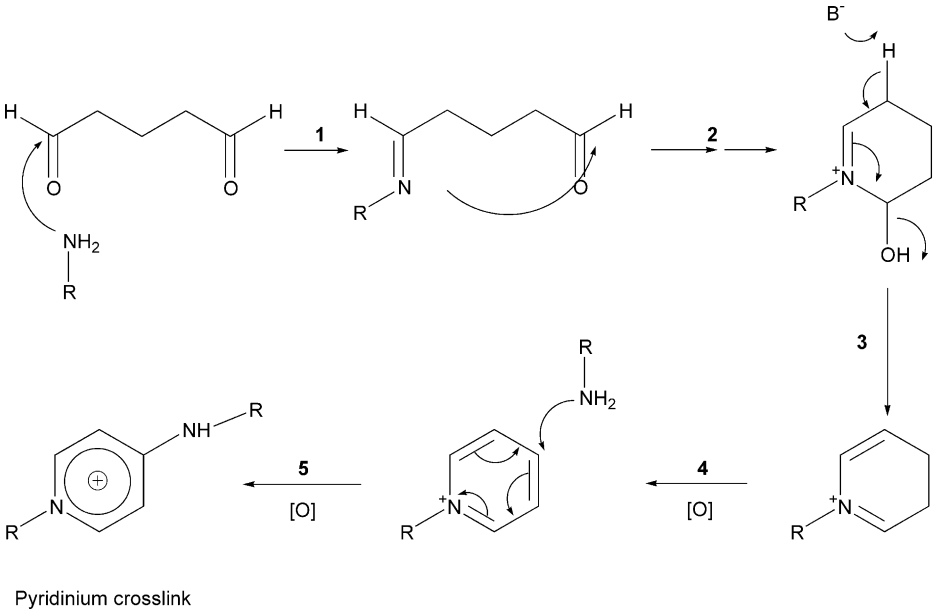
Scheme 2. Proposed mechanism for formation of MOLD.

Subsequent reaction has been proposed to proceed via an intermolecular Cannizzaro-type reaction, which after dehydration forms the imidazolium compound referred to as MOLD.

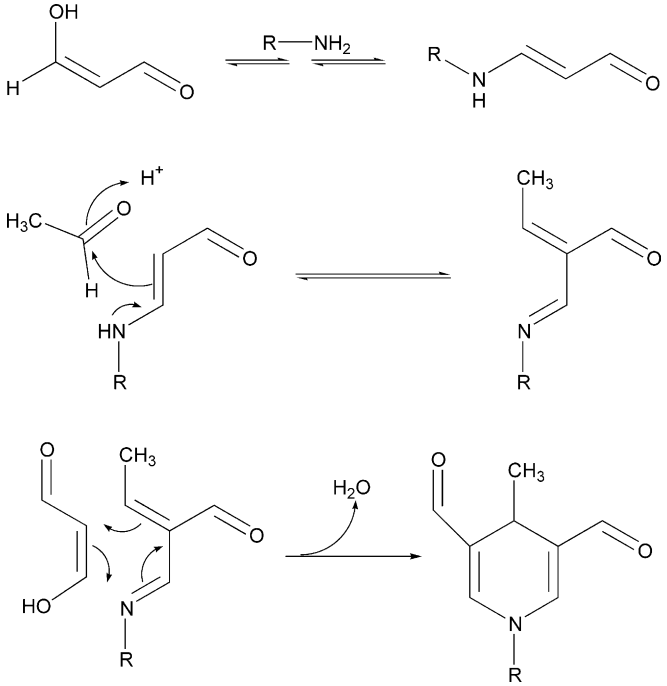
Imidazolium crosslinks, such as MOLD, are not the only proposed heterocyclic linkages between proteins. A similar pyridinium crosslink structure has recently been proposed to be derived from glutaraldehyde and proteins.[40] Glutaraldehyde is a well-studied protein crosslinker. One possible mechanism for the formation of a pyridinium crosslinks from glutaraldehyde is shown in Scheme 3.

The crosslinking ability of a number of straight chain dialdehydes from glyoxal to hexanedial can be interpreted in the context of these reaction mechanisms. Glyoxal is known to form GOLD imidazolium crosslinks, via a similar mechanism to the one shown for MOLD as shown in Schemes 1 and 2.

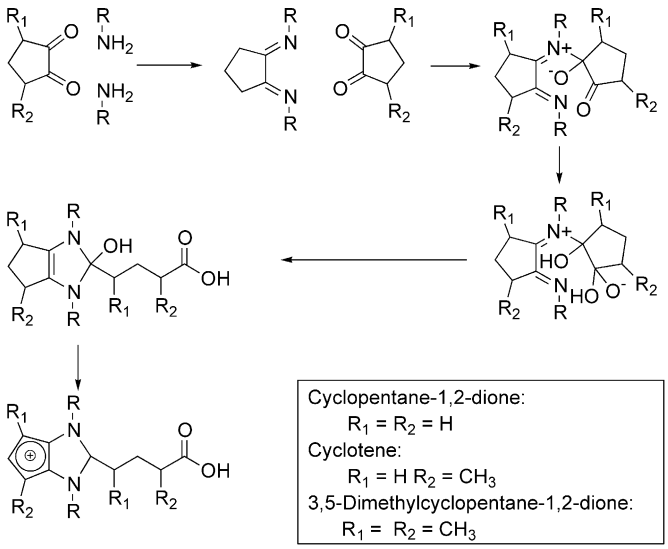
If malondialdehyde reacts via this mechanism, a stable pyrazine crosslink would be formed. An alternative mechanism has been proposed by Slatter et al. for the reaction of malondialdehyde and propylamine (Scheme 4),[42] but this requires the presence of acetal-



Scheme 3. Proposed reaction mechanism for formation of pyridinium crosslinks.



Scheme 4. Slatter's proposed mechanism for reaction of malondialdehyde with propylamine.[42]



Scheme 5. A proposed mechanism for crosslinking cycloalkane-1,2-diones.

dehyde, which is not necessarily present in our incubation mixture.

Unlike glyoxal and malondialdehyde, if succinaldehyde reacted via the mechanism shown in Scheme 2, no stable heterocyclic crosslink structure is simply accessible. However, succinaldehyde could react in a similar way to glutaraldehyde, to form a stable pyrrole compound. The pyrrole would not be expected to react further with nucleophiles as proposed for the pyridinium crosslink (step 5, Scheme 3). It is suggested, however, that two or more protein-bound pyrroles could form crosslinks via a similar route to the well characterised polymerisation of simple pyrrole structures.[57] This may explain the propensity of the succinaldehyde incubations to form gelatinous mixtures, in contrast to most other molecules tested.

Hexanedial can form a seven-membered ring heterocyclic crosslink.[58] If hexanedial reacts via a similar mechanism to glutaraldehyde (Scheme 3) we would expect the rate of crosslinking would be a less than for glutaraldehyde, due to the ease of formation of a seven- versus six-membered ring structure.[59,60] This is consistent with the observed results with hexanedial crosslinking significantly slower than glutaraldehyde. It may be that longer chain dialdehydes favour intramolecular reactions that do not directly result in crosslinking.

In summary, it appears that compounds in this series react and crosslink via several different routes, so the structure-reactivity relationship is complicated. However, we have empirically demonstrated that glutaraldehyde, with a five-carbon chain, reacts most effectively.

Crosslinking by cycloalkane dicarbonyls

Analysis of the results of incubations of cyclopentane-1,2-dione, cyclotene and 3,5-dimethylcyclopentane-1,2-dione with protein showed cyclopentane-1,2-dione was significantly faster at crosslinking. We propose that the cyclopentane-diones are able to react via a similar mechanism to methylglyoxal to form MOLD analogues (Scheme 5). This mechanism is consistent with a reduced efficiency of crosslinking for cyclotene and 3,5-dimethylcyclopentane-1,2-dione relative to cyclopentane-1,2-dione. This effect would be due to the increase steric hindrance due to the addition of adjacent methyl groups, suggesting that Schiff base formation is the rate limiting step for these reactions.

Interestingly, cyclopentane-1,2-dione was more effective at crosslinking proteins than either cyclobutane-1,2-dione or cyclohexane-1,2-dione. It is unlikely that cyclobutane-1,2-dione would react to form an imidazolium ion, as bicyclic ring structures containing a cyclobutane ring are relatively strained. These reaction mechanisms are multiple step reactions and a small change in structure may perturb the reaction rates sufficiently to lead to a large change in rate of observed crosslinking. A possible alternative explanation for the efficient crosslinking of cyclopentane-1,2-dione is that it ring opens to form glutaraldehyde. Glutaraldehyde is a rapid crosslinker and a possible mechanism for crosslinking is discussed earlier in this paper (Scheme 3).

The ring substitution patterns of cyclopentane-dione and cyclohexane-dione were also assessed for crosslinking reactivity. Reaction of the 1,2-diones resulted in the most crosslinking in both cases. It is possible for both cyclopentane-1,2-dione and cyclohexane-1,2-dione to form imidazolium crosslinks. However, since it is not possible for cyclopentane-1,3-dione, cyclohexane-1,3-dione or cyclohexane-1,4-dione to react via a similar mechanism to form imidazolium crosslinks this may account for their poor crosslinking ability. Therefore, the relative reactivity of this series of compounds correlates well with the predicted ease of forming the imidazolium moiety.

Unsymmetrical versus symmetrical dicarbonyls

The observation that glyoxal is a less efficient crosslinker of protein than methylglyoxal is chemically consistent with the proposed mechanism for imidazolium

formation. Reaction of methylglyoxal requires a fragmentation reaction (Scheme 2, step 4) which is predicted to be more favourable than for reaction of glyoxal, due to increased relative stability of the substituted leaving group. Biacetyl is also observed to be less reactive at crosslinking than methylglyoxal. Biacetyl contains two ketone moieties. Reaction of biacetyl to form an imidazolium crosslink would be hindered, as ketone groups would react more slowly than aldehydes in step 2 (Scheme 2), due a combination of steric hindrance and reduced electrophilic reactivity of the carbonyl centres.

Phenylglyoxal would be predicted to be an efficient crosslinker via this type of mechanism. The experimental observations that phenylglyoxal showed no detectable crosslinking ability was not consistent with the prediction. However, the lack of crosslinking observed for phenylglyoxal may be related to its known reactivity with arginine.[53,54]

The wide range of compounds tested has allowed us to explore the many complexities of the reaction, which must be unravelled before we can fully understand the mechanisms of protein crosslinking in vivo. Although the dicarbonyl compounds selected for this investigation were carefully chosen to explore specific structural features, the chemistry open to these relatively reactive compounds is still complex, and the balance of reactivities is easily perturbed. One small change in the structure may cause changes in competing reaction rates. Despite this difficulty, many structural features and their effect on crosslinking have been probed with a view to increasing our fundamental knowledge of crosslinking reactions.

Conclusions

It appears that structure–activity relationships do exist between series of structurally similar dicarbonyls. However, these relationships are complex and do not yet allow the general prediction of activity from structure due to the heterogeneous nature of the crosslinks produced.

Cyclopentane-1,2-dione, recently identified in vivo[61] was found for the first time, to be an efficient crosslinker. This molecule warrants further investigation under conditions closer to physiological.

In general the less hindered molecules were more reactive crosslinkers, although glyoxal is an exception here in that the substituted methyl glyoxal was substantially more reactive. This may be due to the complexities of the solution structure of glyoxal.

Methylglyoxal and glutaraldehyde were found to be the most efficient crosslinkers, whilst closely related molecules effected crosslinking at a much lower rate. The efficiency of methylglyoxal and glutaraldehyde at crosslinking is thought to be due to their ability to efficiently form stable heterocyclic compounds that crosslink proteins. The reasons for the particular reactivity of these two compounds, compared to closely related structures, remains unclear, but is consistent in most cases with subtle changes in the balance between competing pathways in a complex reaction network.

This paper has focussed on the reaction mechanisms of formation of heterocyclic crosslinks and assessed the potential of these mechanisms to explain the differences in efficiency. Interestingly, in many cases the efficiencies are well explained by simplistic analysis of the appropriate mechanism of formation of the proposed heterocyclic crosslinked product.

Experimental

Materials

Bovine pancreatic ribonuclease A Type XII-A (RNase A), methylglyoxal (40% aqueous solution), biacetyl (99% aqueous solution), glyoxal (trimer: dihydrate), phenylglyoxal, cyclobutane-1,2-dione, cyclopentane-1,3-dione, cyclohexane-1,2-dione, cyclohexane-1,3-dione, cyclohexane-1,4-dione, glutaraldehyde, wide-range marker for SDS-PAGE analysis, β-mercaptoethanol and glycerol were all purchased from Sigma-Aldrich (St Louis, MO, USA). Coomassie Brilliant Blue was purchased from Merck (Darmstadt, Germany). 4–20 and 8–16% Tris-glycine SDS-PAGE gels were obtained from Gradipore (NSW, Australia). 3,5-Dimethylcyclopentane-1,2-dione was purchased from Scientific Supplies Ltd (Wellington, New Zealand).

Cyclotene was kindly gifted by Dr. D. K. Weerasinghe, Firmenich Inc. Cyclopentane-1,2-dione was prepared using the method of Acheson.[49,50] Malondialdehyde was produced by hydrolysis of 1,1,3,3-tetramethoxypropane[43] which was obtained from ACROS organics (Geel, Belgium). Succinaldehyde was synthesised by acid hydrolysis from a mixture of *cis* and *trans* isomers of 2,5-dimethoxy-2,5-dihydrofuran[44] which was obtained from Sigma-Aldrich (St Louis, MO, USA). Hexane-dial was prepared by ozonolysis of cyclohexene, using the method Hudlicky described for ozonolysis of methylcyclohexene.[45] Cyclohexene was obtained from Sigma-Aldrich (St Louis, MO, USA). Synthesised compounds were characterised by standard methods and found to be consistent with the literature. Commercially available compounds were assessed by proton NMR spectrometry prior to use.

Incubations

For each of the replicate incubations of RNase A with each α-dicarbonyl, the following procedure was undertaken. Stock solutions were prepared by solubilising RNase A in dH_2O to give a final concentration of 50 mg/mL with the pH adjusted to 7 using 0.1 M NaOH. Aliquots of each solution were diluted 1:1 with a 260 mM α-dicarbonyl solution to give final concentrations of 25 mg/mL RNase A and 130 mM α-dicarbonyl. The resultant solution was aliquoted into Eppendorf tubes and incubated at 37 °C for the prescribed period of time then stored at −20 °C prior to analysis. Control solutions of RNase A were both frozen and incubated at 37 °C for the longest time interval in the incubation series. Samples were analysed by SDS-PAGE

using the method outlined by Fayle et al.[62] All RNase incubations were repeated at least in triplicate with triplicate analysis of each incubation.

Acknowledgements

This work was financially supported by the Royal Society of New Zealand, Marsden Fund. We would like to thank Prof. Peter J. Steel, Dr. Jonathan C. Morris and Dr. Richard M Hartshorn for useful discussions and proof reading. We would also like to acknowledge Andrew Muscroft-Taylor, Rebecca O'Loughlin and Jackie P. Healy for technical input.

References and Notes

1. Ledl, F.; Schleicher, E. *Angew. Chem.* **1990**, *29*, 565.
2. Maillard, L.-C. *C. R. Acad. Sci. Ser.* **1912**, *2*, 154 631.
3. Friedman, M. *J. Agric. Food Chem.* **1996**, *44*, 631.
4. Fu, M.-X.; Requena, J. R.; Jenkins, A. J.; Lyons, T. J.; Baynes, J. W.; Thorpe, S. R. *J. Biol. Chem.* **1996**, *271*, 9982.
5. Bailey, A. J.; Paul, R. G.; Knott, L. *Mech. Ageing. Dev.* **1998**, *106*, 1.
6. Paul, R. G.; Bailey, A. J. *Int. J. Biochem. Cell Biol.* **1999**, *31*, 653.
7. Munch, G.; Thome, J.; Foley, P.; Schinzel, R.; Riederer, P. *Brain Res. Rev.* **1997**, *23*, 134.
8. Adrian, J.; Billaud, C.; Potus, J. *Med. Nutr.* **2000**, *36*, 69.
9. Lee, K. W.; Simpson, C.; Ortwerth, B. *Biochim. Biophys. Acta-Mol. Basis Dis.* **1999**, *1453*, 141.
10. Monnier, V. M.; Glomb, M.; Elgawish, A.; Sell, D. R. *Diabetes* **1996**, *45*, S67.
11. Miyata, T.; Van Ypersele de Strihou, C.; Kurokawa, K.; Baynes, J. W. *Kidney Int.* **1999**, *55*, 389.
12. Sajithlal, G. B.; Chithra, P.; Chandrakasan, G. *Biochim. Biophys. Acta* **1998**, *1407*, 215.
13. Thome, J.; Rosler, M.; Johri, M.; Sakai, N.; Riederer, P.; Munch, G. *Drugs Future* **1999**, *24*, 411.
14. Liggins, J.; Rodda, N.; Iley, J.; Furth, A. *Spec. Publ. R. Soc. Chem.* **1998**, *223*, 327.
15. Vasan, S.; Zhang, X.; Zhang, X. N.; Kapurniotu, A.; Bernhagen, J.; Teichberg, S.; Basgen, J.; Wagle, D.; Shih, D.; Terlecky, I.; Bucala, R.; Cerami, A.; Egan, J.; Ulrich, P. *Nature* **1996**, *382*, 275.
16. Prabhakaram, M.; Katz, M. L.; Ortwerth, B. J. *Mech. Ageing Dev.* **1996**, *91*, 65.
17. Zyzak, D. V.; Wells-Kencht, K. J.; Blackledge, J. A.; Litchfield, J. E.; Wells-Knecht, M. C.; Fu, M. X.; Thorpe, S. R.; Feather, M. S.; Baynes, J. W. *Spec. Publ. R. Soc. Chem.* **1994**, *151*, 274.
18. Fu, M. X.; Wells-Knecht, K. J.; Blackledge, J. A.; Lyons, T. J.; Thorpe, S. R.; Baynes, J. W. *Diabetes* **1994**, *43*, 676.
19. Shipanova, I. N.; Glomb, M. A.; Nagaraj, R. H. *Arch. Biochem. Biophys.* **1997**, *344*, 29.
20. Brinkmann, E.; Wellsknecht, K. J.; Thorpe, S. R.; Baynes, J. W. *J. Chem. Soc., Perkin Trans. 1* **1995**, 2817.
21. Thornalley, P. J.; Minhas, H. S. *Biochem. Pharmacol.* **1999**, *57*, 303.
22. Price, D. L.; Rhett, P. M.; Thorpe, S. R.; Baynes, J. W. *J. Biol. Chem.* **2001**, *276*, 48967.
23. Chellan, P.; Nagaraj, R. H. *Arch. Biochem. Biophys.* **1999**, *368*, 98.
24. Brinkmann Fyre, E.; Degenhardt, T. P.; Thorpe, S. R.; Baynes, J. W. *J. Biol. Chem.* **1998**, *273*, 18714.
25. Odani, H.; Shinzato, T.; Usami, J.; Matsumoto, Y.; Brinkmann Frye, E.; Baynes, J. W.; Maeda, K. *FEBS Lett.* **1998**, *427*, 381.
26. Chellan, P.; Nagaraj, R. H. *J. Biol. Chem.* **2001**, *276*, 3895.
27. Miller, A. G.; Meade, S. J.; Gerrard, J. A. *Bioorg. Med. Chem.* (previous paper).
28. Richards, F.; Knowles, J. R. *J. Mol. Biol.* **1968**, *37*, 231.
29. Gerrard, J. A.; Brown, P. K.; Fayle, S. E. *Food Chem.* **2002**, *79*, 357.
30. Biemel, K. M.; Reihl, O.; Conrad, J.; Lederer, M. O. *J. Biol. Chem.* **2001**, *276*, 23405.
31. Biemel, K. M.; Buhler, H. P.; Reihl, O.; Lederer, M. O. *Nahrung* **2001**, *45*, 210.
32. Wells-Knecht, K. J.; Brinkmann, E.; Wells-Knecht, M. C.; Litchfield, J. E.; Ahmed, M. U.; Reddy, S.; Zyzak, D. V.; Thorpe, S. R.; Baynes, J. W. *Nephrol., Dial., Transplant* **1996**, *11*, 41.
33. Sell, D. R.; Monnier, V. M. *J. Biol. Chem.* **1989**, *264*, 21597.
34. Brown, P. K. Masters Thesis, University of Canterbury, 2001.
35. Eble, A. S.; Thorpe, S. R.; Baynes, J. W. *J. Biol. Chem.* **1983**, *258*, 9406.
36. Litchfield, J. E.; Thorpe, S. R.; Baynes, J. W. *Int. J. Biochem. Cell Biol.* **1999**, *31*, 1297.
37. Gerrard, J. A.; Fayle, S. E.; Sutton, K. H. *J. Agric. Food Chem.* **1999**, *47*, 1183.
38. Acharya, A. S.; Manning, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3590.
39. Baynes, J. W.; Ahmed, M. U.; Fisher, C. I.; Hull, C. J.; Lehman, T. A.; Watkins, N. G.; Thorpe, S. R. *Dev. Food Sci.* **1986**, *13*, 421.
40. Hermansson, G. T. *Bioconjugate Techniques*; Academic: San Diego, 1996.
41. Glomb, M. A.; Pfahler, C. *J. Biol. Chem.* **2001**, *276*, 41638.
42. Slatter, D. A.; Murray, M.; Bailey, A. J. *FEBS Lett.* **1998**, *421*, 180.
43. Nair, V.; Vietti, D. E.; Cooper, C. S. *J. Am. Chem. Soc.* **1983**, *103*, 3030.
44. Fakstrop, J.; Raleigh, D.; Schniepp, L. E. *J. Am. Chem. Soc.* **1950**, *72*, 869.
45. Hudlicky, T.; Ranu, B. C. *J. Org. Chem.* **1985**, *50*, 123.
46. Hill, S.; Easa, A. M. *Spec. Publ. R. Soc. Chem.* **1998**, *223*, 133.
47. Mitchell, J. R.; Hill, S. E. *Trends Food Sci. Technol.* **1995**, *6*, 219.
48. Vernin, G.; Chakib, S.; Rogacheva, S. M.; Obretenov, T. D.; Parkanyi, C. *Carbohydr. Res.* **1998**, *305*, 1.
49. Acheson, R. M. *J. Chem. Soc.* **1956**, *159*, 4232.
50. Gerrard, J. A.; Meade, S. J.; Morris, J. C.; Steel, P. J. *Acta. Crystallogr.* **2000**, *C56*, 1251.
51. De Buyck, L.; Verhe, R.; De Kimpe, N.; Courtheyn, D.; Schamp, N. *Bull. Soc. Chim. Belg.* **1980**, *89*, 441.
52. Verhe, R.; Courtheyn, D.; De Kimpe, N.; De Buyck, L.; Schamp, N. *Synthesis* **1982**, 667.
53. Takahashi, K. *J. Biol. Chem.* **1968**, *243*, 6171.
54. Gadda, G.; Negris, A.; Pilone, M. S. *J. Biol. Chem.* **1994**, *269*, 17809.
55. Brinkmann, E. F.; Degenhardt, T. P.; Thorpe, S. R.; Baynes, J. W. *J. Biol. Chem.* **1998**, *273*, 18714.
56. Brinkmann, E.; Wells-Knecht, K. J.; Thorpe, S. R.; Baynes, J. W. *J. Chem. Soc., Perkin Trans. 1* **1995**, 2817.
57. Joule, J. A.; Mills, K.; Smith, G. F. *Heterocyclic Chemistry*, 3rd ed.; Chapman and Hall: London, 1995.
58. Ruggiero-Lopez, D.; Lecomte, M.; Moinet, G.; Patereau, G.; Lagarde, M.; Wiernsperger, N. *Biochem. Pharmacol.* **1999**, *58*, 1765.
59. McMurray, J. *Organic Chemistry*, 2nd ed.; Brooks/Cole: CA, 1988.
60. Baldwin, J. E. *J. Chem. Soc. Chem. Commun.* **1976**, 734.
61. Tressl, R.; Piechotta, C. T.; Norbert, W., Eieter, R.; Eberhard, K., In *7th International Symposium on the Maillard Reaction*, Kumamoto, Japan. Elsevier Science: Amsterdam, 2001.
62. Fayle, S. E.; Healy, J. P.; Brown, P. A.; Reid, E. A.; Gerrard, J. A.; Ames, J. M. *Electrophoresis* **2001**, *22*, 1518.