Recl. Trav. Chim. Pays-Bas **114**, 321–324 (1995)
SSDI 0165-0513(95)00037-2

0165–0513/95/07321–04$09.50

The synthesis of pure Amadori rearrangement products

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(Received December 1, 1994)

Abstract. The term "Maillard reaction" is used to describe a complex set of reactions in foods leading to flavour generation and non-enzymatic browning. The first step in this process is the so-called Amadori rearrangement: a reducing saccharide and a peptide fragment react to form an Amadori Rearrangement Product (ARP). To be able to do model studies on flavour generation, gram amounts of pure ARP are required. In the present study, glucose-derived ARPs were synthesised from specifically protected and activated starting compounds. After deprotection and purification, pure ARPs were obtained. This is the first time that ARPs of dipeptides have been isolated.

Introduction

The term "Maillard reaction" is used to describe a complex set of reactions in foods leading to flavour generation and non-enzymatic browning[1–4]. This reaction, as described by *J. Mauron*[1], "may well be considered the characteristic feature that distinguishes between the feeding habits of the animal and those of man". In general, the Maillard reaction comprises the reactions of aldehydes and ketones with amines. More specifically in foods, the series of reactions starts with a condensation reaction of the carbonyl function of a (reducing) carbohydrate and the amino function of an amino acid to yield a very complex mixture of polymeric brown pigments (melanoidins) and volatile heterocyclic flavour compounds. The reactions are strongly accelerated by heat (cooking, baking) and are much more efficient when the water content is low (baking). The first step in this process is the Amadori rearrangement: an aldose is converted into a 1-amino-1-deoxy-2-ketose, the so-called Amadori Rearrangement Product (ARP) (Scheme 1). The reducing carbohydrate and a peptide fragment form an imine which is unstable and is protonated and deprotonated to yield the enol tautomer of a ketose which equilibrates to the ketose. The ARPs are relatively stable intermediates which can be isolated.

This is the starting point for several possible pathways of colour and flavour formation which make a thorough study of the Maillard reaction process rather complicated. Since ARP is an intermediate that is stable enough to be isolated, it offers the best place to start in-depth studies of the mechanism of flavour formation. In flavour generation minute amounts (ng/kg) of certain compounds can have serious influences on the perception of flavours. Therefore, extremely pure ARPs are required. Many studies have already been carried out to prepare ARPs Refs. 2–5 and references cited therein). The conventional synthesis consists of heating the amino acid of choice with a reducing sugar (mostly glucose) in dry methanol followed by extensive and tedious purification techniques[5]. Since on the one hand ARPs are only relatively stable, and on the other hand both carbohydrates and amino acids have more than one functional group, by-products are readily formed which are difficult to remove. Therefore, if pure compounds are required, only very small quantities will be obtained.

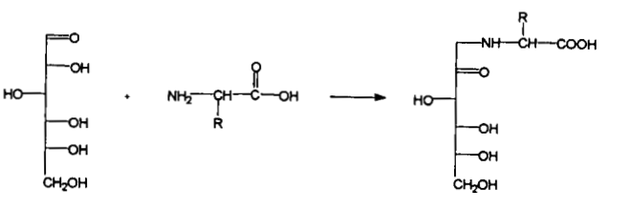
To obtain gram quantities of pure ARP, the use of proper protecting groups which will prevent unwanted reactions, seems the method of choice. As far as we are aware, this method has been described twice[6,7]. However, neither publication reports the full deprotection of ARP.

Synthesis

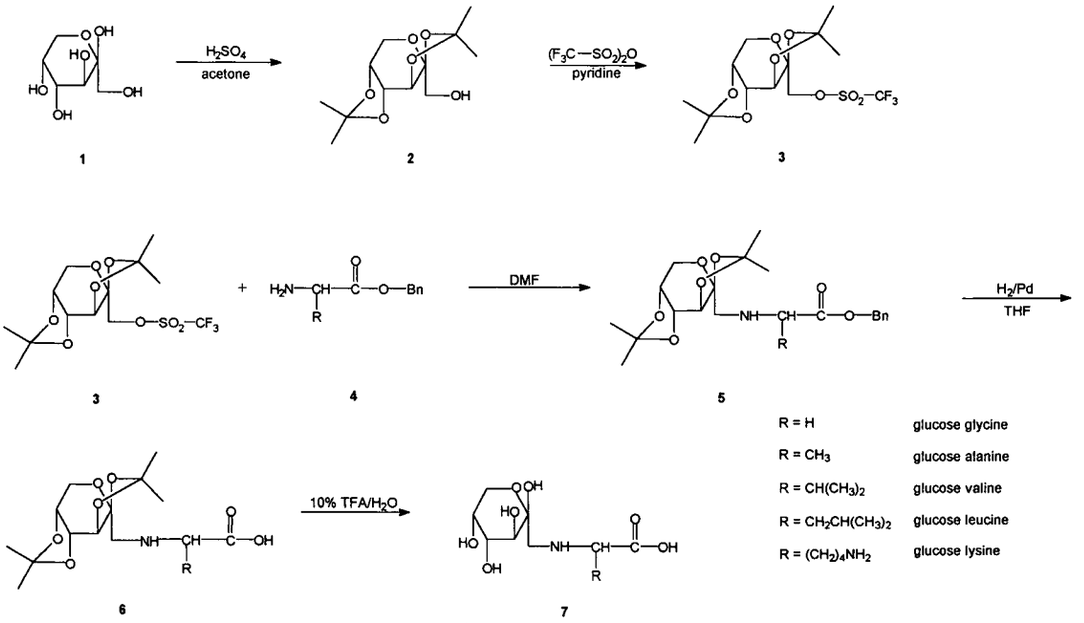
Preparation of fully protected ARP

Almost all studies on ARPs have been carried out with glucose. Since glucose is the most abundant carbohydrate this is a logical choice and all experiments described here deal with glucose-derived ARPs. During the Amadori rearrangement a glucose is converted into a fructose derivative (Scheme 1). *Xenakis* et al. coupled amino acids to C-1 of fructose, so bypassing the actual Amadori rearrangement but yielding the correct ARP (Scheme 2)[6].

In our laboratory the protected ARPs of glucose and the amino acids glycine, alanine, valine, leucine, and lysine and the dipeptides glycylglycine and alanylalanine have been produced successfully. As shown in Scheme 2, D-fructose (**1**) was converted with acetone and sulfuric acid



Scheme 1. Amadori rearrangement.



Scheme 2. Preparation of Amadori rearrangement product (ARP).

to yield 2,3 : 4,5-di-*O*-isopropylidene-β-fructopyranose (**2**), after work-up, as a white powder in 70% yield[8]. As shown in the NMR spectrum, about 10% of 1,2 : 4,5-di-*O*-isopropylidene-β-fructopyranose was also formed. This compound was inert to the subsequent triflic [a] anhydride treatment and was removed in the silica gel purification step. To activate the primary hydroxy group, compound **2** was reacted with triflic anhydride in pyridine at 0°C to obtain the active triflate derivative **3**. The yield was 80% after purification over silica gel. Triflic anhydride, purchased as a slightly brown coloured liquid, was used after distillation from a small amount of phosphorus pentoxide. After distillation it could be stored for months on phosphorus pentoxide at 4°C and remained colourless. Traces of pentoxide did not disturb the reaction.

Xenakis reported the triflate to be very unstable but this was found not to be true confirming the findings of others[7,10]. After purification by column chromatography, triflate **3** could be stored at −20°C for several weeks.

Benzyl esters of amino acids were purchased as hydrochlorides or 4-tosylates [b]. To prevent the ϵ-NH_2 group from interfering in the coupling reaction, lysine was obtained as the N^{ϵ}-benzyloxycarbonyl derivative. The dipeptides were only available in the free form and were converted into the benzyl ester hydrochlorides with benzyl alcohol and dry hydrogen chloride[9]. Since the free amino group is the reactive species, the hydrochlorides or 4-tosylates were neutralized in diethyl ether with dry ammonia. After filtration and careful evaporation under vacuum at 5–15°C, a quantitative yield was obtained. In general, the material was used immediately but could be stored for a few days at 4°C.

The actual coupling of sugar and amino acid is now the nucleophilic substitution reaction of the free amino group of the amino acid or dipeptide benzyl ester **4** at C-1 of triflate **3**. This reaction was carried out in DMF at 70°C. Fewer by-products were formed than under reflux conditions (150°C)[6]. Reaction times are shown in Table I.

Two equivalents of free amino acid were theoretically sufficient for complete reaction: one to couple with triflate **3** and one to neutralize the equivalent triflic acid formed. In fact, four equivalents of compound **4** turned out to be necessary to achieve this. Compound **5** was purified by column chromatography and a white/yellow solid or yellow syrup was obtained.

Deprotection of protected ARP

Quantitative removal of the benzyl group from fully protected ARP **5** was achieved by hydrogenation in THF at room temperature with a catalytic amount of palladium on active carbon (Scheme 2)[13]. Although the coupling between triflate **3** and *O*-benzyl-N^{ϵ}-(benzyloxycarbonyl) lysine could be achieved, hydrogenolysis to remove both protecting groups of the amino acid part was incomplete. Even addition of extra catalyst did not yield satisfactory results. Poisoning of the catalyst by the ϵ-NH_2 group was thought to be the cause of this. Subsequently, the isopropylidene groups were removed by treatment with aqueous trifluoroacetic acid (10%) at 70°C. The yields were higher than 90%. *López*[7] used 90% trifluoroacetic acid at room temperature for acetone removal. Both methods yielded satisfactory results.

When *tert*-butyl esters of amino acids were used, all protecting groups could be removed in one step by acid hydrolysis. This was proved to be the case for *O*-^{t}Bu-glycine and -alanine. However, since benzyl esters were more easily obtainable and removal of the benzyl group was quantitative, amino acids and dipeptides were used as their benzyl esters.

After evaporation of the solvent, the ARP trifluoroacetates were obtained as brown, highly viscous products.

Table I Reaction times and yields for coupling reactions of fructose triflate **3** *and peptide benzyl ester* **4**.

Amino acid/ dipeptide	Reaction time (min)	Yield (%)
glycine	90	65
glycylglycine	210	54
alanine	150	52
alanylalanine	300	38
valine	300	73
leucine	300	70
lysine	300	65

[a] triflic = trifluoromethane sulfonyl, F_3CSO_2-.
[b] tosylate = toluenesulfonate, $MeC_6H_4SO_3$-.

Table II NMR data from fully protected ARPs (amino acid part).

	Gluc-Gly	Gluc-Ala	Gluc-Gly-Gly	Gluc-Ala-Ala	Gluc-Val	Gluc-Leu	Gluc-Lys
CH_2NH	2.9 dd	2.8 dd	3.0 dd	2.9 dd	2.8 dd	2.8 s	2.8 dd
$NHCH$	3.6 dd	3.6 q	3.8 dd	3.2 q	3.2 d	3.4 t	3.4 t
$CNHCH$			3.4 s	4.5 q			
$CH(CH_3)_2$					2.0 m	1.7 m	
CH_3		1.2 d		1.4 2× d	0.9 2× d	1.0 2× d	
$CHCH_2CH$						1.6 m	
CH_2NHCO							3.1 dd
$(CH_2)_3$							1.6 m

Table III NMR-data of the ARPs after the removal of the benzyl ester (amino acid part).

	Gluc-Gly	Gluc-Ala	Gluc-Gly-Gly	Gluc-Ala-Ala	Gluc-Val	Gluc-Leu
CH_2NH	3.2 dd	3.0 dd	3.0 dd	3.0 dd	3.2 dd	2.8 dd
$NHCH$	3.6 dd	3.5 q	3.8 dd	3.6 q	2.9 d	3.2 t
$CNHCH$			3.5 s	4.4 q		
$CH(CH_3)_2$					2.2 m	1.7 m
CH_3		1.3 d		1.4 2× d	1.2 2× d	0.9 2× dd
$CHCH_2CH$						1.2 d

Table IV NMR-data from ARPs (amino acid part).

	Gluc-Gly	Gluc-Ala	Gluc-Gly-Gly	Gluc-Ala-Ala	Gluc-Val	Gluc-Leu
CH_2NH	3.2 dd	3.2 dd	3.4 dd	3.2 dd	3.3 s	3.8 dd
$NHCH$	4.0 dd	3.6 q	4.0 dd	4.0 q	3.6 d	4.2 t
$CNHCH$			4.0 dd	4.2 q		
$CH(CH_3)_2$					2.2 m	2.3 m
CH_3		1.5 d		1.4 2× d	1.0 2× d	1.5 2× dd
$CHCH_2CH$						1.7 t

Crystallization looked a promising technique for removal of the brown colour and the crystals would also be easily manageable for flavour studies. Crystallization, with ethanol as solvent, has been reported for several ARPs[5]. ARP ethyl esters crystallized upon addition of diethyl ether to the raw product[7]. The crude ARPs dissolved readily in ethanol. To obtain ARP as a solid, the crude sample was dissolved in a small amount of ethanol and added dropwise to propan-2-ol (for ARP of glycine and alanine derivatives) or acetonitrile (for ARP of valine and leucine) (Table VI). Yields were low, however, and differences in purity between crude and precipitated product could be found using NMR as the analytical technique. The ARP of lysine could not be isolated in measurable amounts. After precipitation the compounds were obtained as off-white powders, in high purity according to HPLC[11] (> 90%) NMR and MS.

Ion exchange chromatography using Dowex AG W-50 X-8 was investigated as an alternative method. Water was used as the mobile phase to remove the brown colour followed by trifluoroacetic acid (0.1 mol/l) to collect the ARP. Freeze drying of the appropriate fractions did not result in easily manageable solids. Moreover, recrystallization from ethanol or acetonitrile still did not yield crystalline material. Preparative HPLC could be an alternative method.

Spectroscopic characterization

NMR spectra of ARPs have previously been studied in detail to elucidate the conformation of the sugar part[5,12]. Therefore, only the data on the amino acid part are summarized here (Tabel II-IV). The data on the sugar part were in agreement with the earlier results whereas the signals of the amino acid part were only slightly different from the spectra of the free amino acids.

FAB (fast-atom bombardment) mass spectrometry in the positive mode with xenon as the reagent gas have been carried out to check the molecular mass. In all spectra the $[M + H]^+$ was the base peak (except for the peaks originating from the matrix) and the $[M + H]^+-H_2O$ was clearly present.

Conclusion

A series of glucose derived ARPs have been synthesised on a gram scale from specifically protected and activated starting compounds. After deprotection and purification pure ARPs have been obtained. Previously published recrystallization methods could not be reproduced in our hands. Characterization using HPLC, NMR and MS showed that the correct ARPs were obtained in pure (> 90%) form.

Experimental

Materials and methods

Amino acids and dipeptides were obtained from Bachem, Switzerland. Solvents and other chemicals were obtained from Janssen Chimica.

Column chromatography was carried out in the ratio 30/1 (silica gel/material) using silica gel 60, 70–320 mesh ASTM, ex Merck as the stationary phase. TLC(thin layer chromatography) was carried out on pre-coated TLC plates, silica gel 60 F-254 ex Merck and TLC plates, silica gel RP-18 $F_{254}S$ ex Merck.

Elution solvents (1) ethyl acetate/hexane 1/1 (v/v); (2) ethyl acetate/hexane 4/6 (v/v); (3) diethyl ether/petroleum ether 1/1 (v/v); (4) butanol/acetic acid/water 4/1/1 (v/v); (5) methanol/water 1/1 (v/v).

Detection methods: (1) phosphomolybdic acid test: a solution of 15% phosphomolybdic acid in 100% ethanol; detection of hydrocarbons; (2) UV_{254}: fluorescence of the plate; detection of aromatic groups; (3) ninhydrin test: a solution of 250 mg ninhydrin and 5 ml of collidine in 95 ml of methanol; detection of NH_2 groups.

NMR spectra were recorded on a Bruker AM 360 spectrometer (360 MHz). FAB mass spectra were obtained on a Fisons Instrument VG 7070 EF operating in FAB+ mode with xenon as reagent-gas. HPLC

chromatograms were recorded on a Waters 510 HPLC containing nucleosil 100–5 NH_2 using acetonitrile/phosphate buffer (5 mM, pH 2.75) 8/2, as the mobile phase.

2,3:4,5-Di-O-isopropylidene fructopyranose (**2**)

A mixture of concentrated sulfuric acid (35 ml; 0.66 mol) and acetone (700 ml) was cooled in ice. D-Fructose (**1**) (36.6 g; 0.2 mol) was added and stirred at room temperature for 90 min. The mixture was cooled in ice and a cold solution of sodium hydroxide (500 ml; 22% w/w in water; 2.75 mol) was added. The acetone was evaporated *in vacuo*. The residue was extracted with 3×100 ml of dichloromethane, the organic layers were washed with 2×100 ml of water, dried with magnesium sulfate and filtered. After evaporation of the dichloromethane a white solid was obtained in a yield of 38 g (70%).
TLC [elution solvent (1); detection method (1)] showed the presence of the 1,2:4,5-di-O-isopropylidenefructopyranose in minor amounts (7% according to NMR). NMR: δ 1.4–1.5 (4xs, 3H each, CH_3), 3.9 (dd, 2H, CH_2OH), 3.6–4.6 (m, 5H, sugar).

Amino acid benzyl ester hydrochlorides

A suspension of amino acid or dipeptide (3 g; *e.g.* 22 mmol H-Gly-Gly-OH) in benzyl alcohol (60 ml) was cooled in a salt/ice bath. The mixture was saturated with HCl gas. Toluene was added (20 ml) and evaporated *in vacuo* as an azeotrope with water formed during the reaction. This procedure was repeated until, according to TLC [elution solvent (1); detection method (1)] the starting material had disappeared. Usually five times were sufficient. The mixture was distilled (T 80°C; $p \leq 10^{-2}$ bar). The product was precipitated in diethyl ether, filtered, dried and stored at T −20°C. NMR: δ 3.9 (s, 2H, NH_2CH_2), 4.1 (s, 2H, $NHCH_2$), 5.2 (s, 2H, benzyl-CH_2), 7.3 (s, 5H, benzyl).

2,3:4,5-di-O-isopropylidene fructopyranose triflate (**3**)

A solution of protected fructose (4 g; 15 mmol) in dry pyridine (45 ml) was cooled in ice. Triflic anhydride (8 ml; 48 mmol) was added dropwise. After approximately 30 min the reaction was complete according to TLC (elution solvent (1); detection method (1)). Cold water (50 ml) and HCl (1 mol/l), (4 ml) were added. After extraction with dichloromethane (3×50 ml) the organic layers were dried with magnesium sulfate, filtered and evaporated *in vacuo*. The crude product was purified by column chromatography (elution solvent (2)). The fractions containing the triflate were evaporated *in vacuo*. The yield was 4.7 g (80%). NMR: δ 1.4–1.5 (4xs, 3H each, CH_3), 4.5 (dd, 2H, $CH_2OSO_2CF_3$), 3.9 (dd, 2H, CH_2O), 4.0–4.9 (m, 3H, sugar).

Neutralization of the tosylates and chlorides (**4**)

A suspension of amino acid or dipeptide salt (8 g; *e.g.* 32 mmol H-ala-OBz.HCl) in diethyl ether (150 ml) was cooled in ice. The mixture was saturated with gaseous ammonia for 15 min. After 15 min of stirring the suspension was allowed to stand for 15 min. The suspension was filtered and the filtrate was evaporated *in vacuo* at a water bath temperature of 5–15°C yielding 6.7 g (83%). NMR: δ 1.2 (d, 3H, CH_3), 3.6 (q, 1H, $NHCH$), 5.1 (s, 2H, benzyl-CH_2), 7.2 (s, 5H, benzyl).

Protected ARP (**5**)

Triflate **3** (4.7 g; 12 mmol) and the amino acid or dipeptide **4** (6.7 g; *e.g.* 31 mmol H-ala-OBz) were dissolved in DMF (30 ml). The mixture was heated (T 70°C) in an oil bath. The reaction was stopped when the triflate had disappeared on TLC [elution solvent see Table V, detection method (1,2)]. The mixture was quenched in water, extracted with dichloromethane (3×50 ml). The organic layers were washed with water (2×50 ml) and a saturated sodium chloride solution (50 ml). The organic layers were dried with magnesium sulfate, filtered and evaporated *in vacuo*. The crude product was purified by column chromatography (elution solvent see Table V). Yield 3.7 g (65%). NMR: δ 1.2–1.6 (4xs, 3H each, CH_3), 1.2 (d, 3H, CH_3-CH), 2.8 (dd, 2H, CH_2NH), 3.6 (q, 1H, $NHCH$), 3.6–4.6 (m, 5H, sugar).

Table V Reaction time of coupling reaction and analytical data.

Amino acid/ dipeptide	Reaction time (min)	TLC/Column eluent	R_f-value
glycine	90	1	0.5
glycylglycine	210	1	0.3
alanine	150	1	0.6
alanylalanined	300	1	0.3
valine	300	3	0.7
leucine	300	3	0.6
lysine	300	1	0.6

Table VI Overall yield, crystallization solvent and R_f values for ARPs.

Amino acids/ dipeptides	Overall yield (%)	Crystallization solvent	R_f-value
glycine	20	propan-2-ol	1.0
glycylglycine	13	propan-2-ol	0.8
alanine	21.5	propan-2-ol	1.0
alanylalanine		unsuccesfull	0.9
valine	16.5	acetonitrile	0.9
leucine	20.5	acetonitrile	0.8

2,3:4,5-Di-O-isopropylidene ARP (**6**)

The fully protected ARP (3 g; *e.g.* 6.5 mmol Gluc-Ala) was dissolved in THF (100 ml). The mixture was hydrogenated at room temperature with palladium on activated carbon as the catalyst. After the benzyl group had disappeared on TLC [elution solvent (4), detection method (1,2)] the reaction was stopped. The mixture was filtered through a bed of Celite 545 and the filtrate was evaporated *in vacuo*. Yield 2.4 g (95%). NMR: δ 1.2–1.6 (4xs, 3H each, CH_3), 1.3 (d, 3H, $CHCH_3$), 3.0 (dd, 2H, CH_2NH), 3.5 (q, 1H, $NHCH$), 3.6–4.6 (m, 5H, sugar).

ARP (**7**)

The protected ARP **6** (2 g; *e.g.* 5.5 mmol Gluc-Ala) was dissolved in 10% trifluoroacetic acid in water (100 ml) and stirred at T 60°C until the ARP was fully deprotected. The reaction was followed by reverse phase TLC [elution solvent (5), detection method (3)]. The mixture was evaporated *in vacuo*. To remove traces of trifluoroacetic acid, water was added and evaporated. To remove as much water as possible ethanol was added and evaporated. The crude product was a brown liquid with very high viscosity. Yield 1.6 g (90%). The product was crystallized in a variety of solvents (see Table VI). NMR: δ 1.5 (d, 3H, $CHCH_3$), 3.2 (dd, 2H, CH_2NH), 3.6 (q, 1H, $NHCH$), 3.5–4.2 (m, 5H, sugar).

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