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Low molecular weight coloured compounds formed in xylose–lysine model systems†

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Aqueous solutions of xylose (1 M) and lysine monohydrochloride (1 M), initial pH 5·27, were refluxed for 1 h, either by control of the pH at 5 (by the addition of sodium hydroxide solution during heating) or without pH control (final pH 2·83). The ethyl acetate-extractable components were separated from each model system and represented 0·41 and 0·36 ± 0·05% (m/m), respectively, of the initial reactants for the systems heated with and without pH control. Analysis of the ethyl acetate-extractable components by TLC and HPLC (with diode array detection) showed some similarities, but also many differences between the two systems. Two and seven coloured peaks were analysed, respectively, in the systems heated with and without pH control, but only one was detected in both systems. A novel coloured Maillard reaction product (detected only in the model system heated without pH control) was isolated and purified by repeated semi-preparative TLC and HPLC, prior to analysis by electronic absorption and NMR spectroscopy and by low and high resolution FAB MS.

INTRODUCTION

The development of colour is the most obvious effect of the Maillard reaction, but very little is known about the chemical nature of the coloured compounds formed (Nursten, 1986). Coloured Maillard reaction products may be divided into two classes: the melanoidins, which are brown and possess molecular weights of several thousand daltons, and the low molecular weight structures which typically comprise 2–4 linked rings (Ames & Nursten, 1989).

Much of the information available on the low molecular weight coloured Maillard reaction products is the result of studies carried out by Ledl and his co-workers in Munich and Stuttgart (Ledl, 1990; Ledl & Schleicher, 1990). However, several of Ledl's studies have been carried out in methanol or ethanol, rather than in aqueous solution, or using amines rather than amino acids. In fact, very few studies have reported structural data for non-volatile coloured compounds formed from aqueous sugar–amino acid mixtures and the structures of only five such compounds have been elucidated worldwide (see Table 1) (Severin & Kronig, 1972; Ledl & Severin, 1978; Nursten & O'Reilly, 1983, 1986; Banks *et al.*, 1988). An additional 29 compounds were isolated from an aqueous xylose–glycine model system and analysed by mass spectrometry only (O'Reilly, 1982). They possessed relative molecular masses in the region 149–448 D, and a furfuryl group was evident in several of them. No doubt, the lack of structural information available for coloured compounds formed in sugar–amino acid model systems is due to the highly complex mixtures of reaction products obtained, and the consequent problems encountered in isolating individual components in adequate amounts for analysis by IR, NMR and mass spectrometry.

The Maillard reaction is known to be greatly influenced by the pH of the medium but, again, very little information is available regarding the nature of the coloured compounds formed in model systems heated at different pH values. A comparison of the *volatile* reaction products formed in a xylose–lysine model system heated either with the pH controlled at 5 or without pH control (final pH 2·83) revealed the formation of

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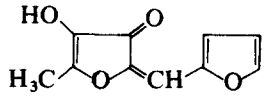
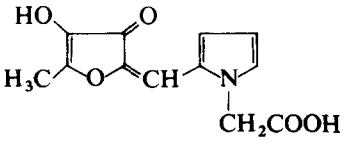
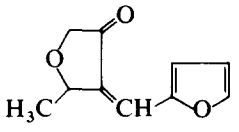
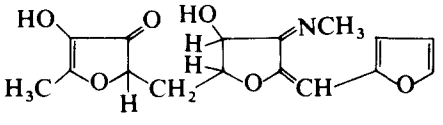
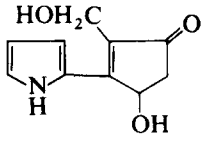
Table 1. Coloured, low molecular weight reaction products identified in aqueous sugar–amino acid model systems

Reactants	Product	Ref.
Xylose or arabinose + glycine or lysine	 1	Severin & Kronig (1972)
Xylose + glycine	 2	Ledl & Severin (1978)
Xylose + glycine	 3	Nursten & O'Reilly (1983)
Xylose + glycine	 4	Nursten & O'Reilly (1986)
Xylose + lysine	 5	Banks *et al.* (1988)

a lower level of furfural, but higher levels of nitrogen-containing components, in the system heated with pH control (Apriyantono & Ames, 1990). The current paper reports a comparison of selected fractions of *coloured* reaction products formed in this system, heated with and without pH control.

MATERIALS AND METHODS

Materials

All chemicals used were the purest available. Analytical grade solvents were obtained from BDH Chemicals Ltd, Poole, UK, and HPLC grade methanol was obtained from Rathburn Chemicals Ltd, Walkerburn, UK. HPLC grade water was prepared in the laboratory using a Purite Labwater RO50 unit (Purite Ltd, High Wycombe, UK).

Methods

A separation scheme for the total browning products is given in Fig. 1.

Preparation of model systems and isolates of the ethyl acetate-extractable components

D-(+)-Xylose, 0·5 mol (99+%, gold label, Aldrich Chemical Co. Ltd, Gillingham, Dorset, UK) and L-lysine monohydrochloride, 0·5 mol (chromatographically homogeneous, BDH Chemicals Ltd, Poole, UK), were dissolved in 500 ml degassed distilled water to give solutions with an initial pH of 5·27. Solutions were refluxed for 1 h either without pH control or by controlling the pH at 5·0 by the addition of 3 M sodium hydroxide solution, with continuous monitoring of the pH using an autoclavable pH electrode (Russell pH Ltd, Auchtermuchty, UK) connected to a Kent EIL 7045/46 pH meter (Kent Industrial Measurement Ltd, Stonehouse, UK).

The total browning products thus formed were extracted with ethyl acetate (6 × 100 ml) and the solvent was removed on a rotary evaporator (<40°C). Removal of trace amounts of water was achieved by placing the concentrates in a vacuum desiccator for 2–3 h. Residues were dissolved in methanol and stored at −20°C.

Electronic absorption spectroscopy

Absorbance measurements and spectra were obtained using a Perkin Elmer Lambda 5 instrument (Perkin Elmer, Beaconsfield, UK).

HPLC

Analytical HPLC was performed using a 250 mm × 4·6 mm i.d. Spherisorb ODS2 (particle size 5 μm) column connected to a 50 mm × 4·6 mm Spherisorb ODS2 guard column (Hichrome Ltd, Theale, UK), and a linear gradient of 100% water to 100% methanol over

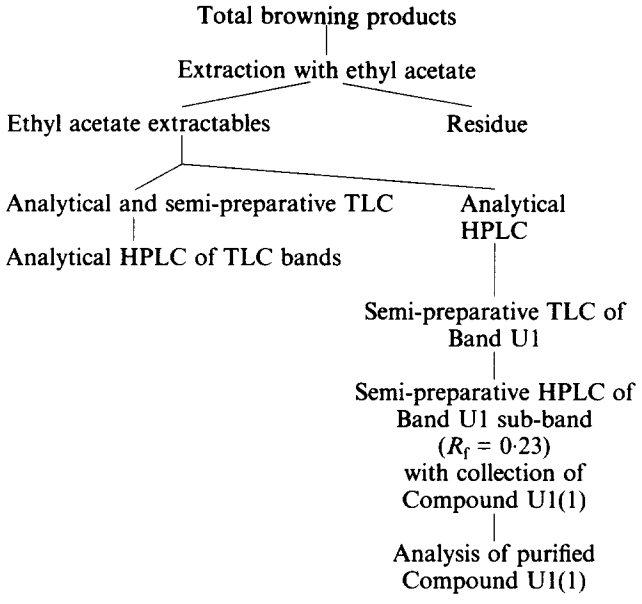


Fig. 1. Separation scheme for the total browning products obtained from the model systems.

60 min. The flowrate was 1 ml min^{-1} and the injection volume was 20 μl. Analyses were carried out using two HPLC systems. The first comprised a Perkin Elmer binary (Model 250) pump (Perkin Elmer Ltd, Beaconsfield, UK) linked to a Kratos Analytical Model 757 absorbance detector with detection at 420 or 280 nm (Kratos Analytical, Manchester, UK), and a Hewlett Packard Model 3396A integrator (Hewlett Packard, Wokingham, UK). The second HPLC system comprised a Philips quaternary (Model PU4100M) pump, linked to a Philips PU4120 diode array detector with scanning over the range 190–390 nm (Philips Scientific, Cambridge, UK) and a Dell Systems 310 computer (Dell Computer Corporation, Bracknell, UK), loaded with PU6003 diode array software (Philips Scientific, Cambridge, UK).

Semi-preparative HPLC was carried out by injecting 200 μl aliquots of sample on to a 250 mm × 8 mm i.d. Spherisorb ODS2 (particle size 10 μm) column connected to a 50 mm × 8 mm i.d. Spherisorb ODS 2 guard column (Hichrome Ltd, Theale, UK) and the first HPLC system described above. The solvent gradient used was the same as that employed for analytical HPLC. The flowrate was 1·0 ml min^{-1}.

TLC

Analytical and semi-preparative separations were carried out, respectively, on 10 cm × 2·5 cm and 20 cm × 20 cm aluminium plates coated with a 0·2 mm layer of unmodified silica gel G60 (BDH Chemicals Ltd, Poole, UK). Mobile phases were developed and optimised for each fraction. Total ethyl acetate extractables from both model systems were separated using chloroform: ethyl acetate: 95% ethanol (15:2:2). Further separation of the components of one band (Band U1), obtained from the ethyl acetate-extractable components of the model systems heated without pH control, was achieved by silica gel TLC using methyl acetate: water (8 : 1) as the mobile phase. Separated components were detected in daylight and by using a UV lamp. For semi-preparative work, 2 ml aliquots were applied to each plate, and separated bands were recovered from the stationary phase by exhaustive elution with methanol, prior to concentration on a rotary evaporator (<40°C) and storage at −20°C.

Mass spectrometry

Low resolution EI spectra were obtained using a Kratos MS80 RFA mass spectrometer (Kratos Analytical, Manchester, UK) equipped with a Kratos DS90 data system. Samples were introduced into the ion source on a probe, heated from 40 to 350°C, at a rate of 35°C min^{-1}. The mass range was 30–500 amu. Low resolution FAB spectra were obtained using a Finnigan TSQ70 mass spectrometer (Finnigan-MAT, San Jose, CA, USA) equipped with a standard FAB ion source and with an Ion Tech gun supplied with xenon gas. The matrix was thioglycerol and the mass range was from 200 to 1000 amu in 1 s. High resolution FAB spectra were obtained using a VG ZAB-E instrument (VG Analytical, Altrincham, UK). 3-Nitrobenzyl alcohol was the matrix and a matrix ion, at 460·135 60 amu, was used as the reference.

NMR spectroscopy

PMR spectra were obtained at 600 MHz, using d_6-dimethylsulphoxide as the solvent and a Bruker AMX 600 instrument (Bruker, Karlsruhe, Germany).

IR spectroscopy

Samples were examined as KBr pellets using a Perkin Elmer Model 881 instrument (Perkin Elmer, Beaconsfield, UK).

RESULTS AND DISCUSSION

This study has focused on the development of procedures (mainly TLC and HPLC) for the fractionation of coloured Maillard reaction products, and for the isolation and purification of selected compounds from a heated xylose–lysine model system. Some comparisons of the components formed in the systems heated with and without pH control have also been made and, in this respect, HPLC with diode array detection has been invaluable.

During heating, both model systems changed colour from pale yellow to dark brown. The final pH of the system heated without pH control was 2·83 ± 0·14. Dilutions of each model system (1 : 1250 in distilled water) gave absorbance readings (at 420 nm) of 0·160 and 0·102, respectively, for the systems heated with and without pH control. Yellowish-brown extracts were produced on extracting the model systems with ethyl acetate, and the masses of browning products obtained represented 0·41 and 0·36 ± 0·05% (m/m), respectively, of the initial reactants for the systems heated with and without pH control.

The ethyl acetate-extractable components of both model systems were analysed by HPLC with diode array detection. Ten and 11 major peaks, respectively, were detected with and without pH control (see Table 2). Absorption spectra were reconstructed from the diode array data and scrutinised carefully. Where it was apparent that absorbance occurred in the visible region, the component in question was considered to be coloured. Two and four of the separated compounds were coloured, for the systems heated with and without pH control, respectively. Based on their retention times and diode array data, only one of the components observed in the chromatograms was common to both solvent extracts, i.e. that with a retention time of 40·10 and 41·15 min, for the systems heated with and without pH control, respectively (see Table 2). Additional analysis of the ethyl acetate-extractable fraction prepared from the model system heated without pH control, using a more sensitive single wavelength detector at 420 nm, revealed the presence of a further eight coloured components.

The ethyl acetate-extractable components of both model systems were analysed by silica gel TLC using chloroform : ethyl acetate : 95% ethanol as the mobile phase. Four and seven bands, respectively, were resolved from the model systems heated with and without pH control (see Table 3). Each band from both model systems was recovered into methanol and analysed by HPLC with diode array detection. The reconstructed spectra obtained from the diode array data of the coloured TLC bands indicated the presence of two coloured components for the system heated with pH control, while seven coloured compounds, including three components not listed in Table 2, were present in the system heated without pH control (see Table 4). The presence of still further coloured components was evident in the system heated without pH control, when monitoring using the more sensitive single wavelength detector at 420 nm.

A comparison of the literature electronic absorption data for 2-furfurylidene-4-hydroxy-5-methyl-3(2*H*)-furanone (1) (Ledl & Severin, 1978; O'Reilly, 1982) with those given in Table 4 suggests the presence of this compound in the model system heated without pH control (retention times obtained equal 38·87 and 38·77 min, respectively, for the samples obtained before and after TLC) (see Tables 2 and 4). This supposition was confirmed by carrying out semi-preparative HPLC, collecting the relevant peak and analysing by EI MS and IR spectroscopy. The spectra obtained matched those reported in the literature for (1) (Ledl & Severin, 1978; Nursten & O'Reilly, 1983). This compound was the first coloured low molecular weight Maillard reaction product to be isolated and characterised (Severin & Kronig, 1972), and was identified in subsequent studies of coloured compounds formed in xylose–

Table 2. HPLC retention times and wavelengths of maximum absorption of major peaks of the ethyl acetate-extractable components

Model system			
With pH control		Without pH control	
t_R (min)[a]	λ_{max} (nm)[b]	t_R (min)[a]	λ_{max} (nm)[b]
11·22	296	8·77	235
11·75[c]	292	12·97	294
14·37	221, 292	14·25	294
19·10	297, 242	17·35[g]	283, 234
23·30	234, 284	22·42	250, 308
25·57[d,e]	363	25·75[d,e]	353
30·12	293	27·50	316
35·90	262, 311	31·52	317, 252
38·87	313	32·75[d,e]	>390, 272, sh. 312
40·10[d,e,f]	357	38·87[d,e]	367
		41·15[d,e,f]	359

[a] Retention times quoted are the averages obtained from two runs (standard deviation ± 0·30 min).
[b] λ_{max} values are the averages obtained from two runs (standard deviation ± 2 nm).
[c] A comparison of the retention time and diode array data with those for the authentic compound indicates that this component is 4-hydroxy-5-methyl-3(2*H*)-furanone.
[d] The peak is coloured.
[e] The peak is also given in Table 4.
[f] Peaks with this superscript in common are present in both model systems.
[g] A comparison of the retention time and diode array data with those for the authentic compound indicates that this component is furfural.

Table 3. R_f values and colours of bands obtained by TLC of the ethyl acetate-extractable components

Model system					
With pH control			Without pH control		
Code	R_f value	Colour	Code	R_f value	Colour
C1	0·32	Colourless	U1	0·09	Yellow
C2	0·55	Yellow	U2	0·15	Colourless
C3	0·60	Dark yellow	U3	0·23	Yellow
C4	0·65	Colourless	U4	0·30	Light brown
			U5	0·43	Yellow
			U6	0·54	Yellow
			U7	0·66	Light yellow

Table 4. HPLC retention times and wavelengths of maximum absorption of the coloured peaks obtained from the coloured TLC bands of the ethyl acetate-extractable components

Model system			
With pH control		Without pH control	
t_R (min)[a]	λ_{max} (nm)[b]	t_R (min)[a]	λ_{max} (nm)[b]
25·57[c]	363	25·79[c]	354
39·92[c,d]	357	32·75[c]	>390, 270, sh. 236, 311
		33·80	>390, 270, sh. 239, 314
		36·42	340, >390
		38·77[c]	366
		40·62	338, >390
		41·59[c,d]	356

[a,b] See Table 2.
[c] The peak is also given in Table 2.
[d] Peaks with this superscript in common are present in both model systems.

glycine and xylose–lysine model systems (Nursten & O'Reilly, 1983; Ames, unpublished results). (1) is clearly formed by the condensation of furfural with 4-hydroxy-5-methyl-3(2*H*)-furanone and its presence indicates that the Amadori Rearrangement Product is able to partly degrade via the 1-deoxyosone, even when the pH of the medium is relatively low. (The pH of the model system heated without pH control decreased very quickly on heating, and a measurement of 3·73 was obtained after refluxing for 15 min.)

One TLC band obtained from the model system heated without pH control, Band U1 (see Table 3), was analysed in more detail. HPLC analysis of this band using the single wavelength detector at 420 nm, showed the presence of three main compounds, while about 20 components were detected when monitoring at 280 nm. The major coloured compound (accounting for 75·7% of Band U1, based on detection at 420 nm but only 8·3% based on detection at 280 nm) was that with a retention time of 40·62 min in Table 4. It was not detected in fractions prepared from the model system heated with pH control, and was called Compound U1(1). A cleaned-up fraction containing the coloured components of Band U1 was obtained by further silica gel TLC. All the coloured reaction products were recovered in one band (R_f = 0·23) and represented 1·7% (m/m) of the ethyl acetate extractable components. Semi-preparative HPLC of this band resulted in the collection of sufficient quantities of the major peak, Compound U1(1) (freed from the majority of the UV-absorbing compounds), for analysis by UV–visible, NMR and mass spectrometry.

Compound U1(1) was yellow in methanol and possessed a λ_{max} of 406 nm, with a subsidiary maximum at 334 nm. This is the first compound isolated from an aqueous sugar–amino acid model system with the wavelength of maximum absorption in the visible region, and a structure possessing extensive conjugation is suggested. Attempts to obtain a mass spectrum for Compound U1(1) using EI were unsuccessful, and it appeared that the compound was insufficiently volatile for analysis, even using a probe temperature of 350°C. Analysis by low resolution FAB MS suggested a molecular weight of 445 D, and subsequent high resolution analysis revealed a mass of 446·124 00 D for the MH^+ ion. Possible empirical formulae were $C_{28}H_{18}O_4N_2$ and $C_{25}H_{20}O_7N$. Since the mass of the compound is 445 D, it must possess an odd number of nitrogen atoms and so the only possible formula is $C_{25}H_{19}O_7N$. Based on this formula, the number of rings plus double bonds equals 17, and this, coupled with the empirical formula, suggests a cyclic structure, four rings possibly being involved.

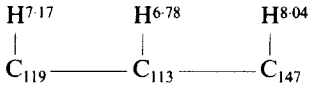
Proton NMR gave signals for 16 of the 19 protons. All the signals were decoupled and the results are shown in Table 5. It was not possible to obtain a ^{13}C-NMR spectrum due to the small sample size. However, a discrete ^1H-^{13}C inverse correlation was carried out and the chemical shifts for some of the carbons thus obtained. It was also possible to obtain the chemical shifts of some quaternary carbons and the relationships between some groups from long range coupling data. Some sequences of atoms could thus be established, as shown in Fig. 2. A comparison of the NMR data for Compound U1(1) with that for similar components suggests that Compound U1(1) probably possesses two terminal furan rings, each substituted in the 2-position (see Table 6). Sequence 3 in Fig. 2 suggests that a propenyl alcohol residue is also present, possibly attached to a ring (Williams & Fleming, 1989). Thus, in common with (1), (3) and (4), Compound U1(1) possesses a terminal furan ring substituted in the 2-position; in common with (2), (4) and (5) it contains one nitrogen atom, and in common with (5), it

Table 5. PMR data for Compound U1(1)

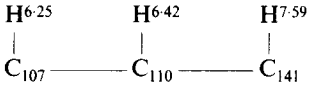
Chemical shift (ppm)	Integral	Multiplicity[a]	Coupling constant *J* (Hz)	Inference
2·5				DMSO
3·2				Water from DMSO
3·9	3	s		No vicinal H, could be OCH_3 coming from interaction with methanol
4·05	1	dd	1, 4	Coupled with OH and H at 5·85
4·23	1	dd	1, 4	Coupled with OH and H at 5·85
5·25	1	t	4	Corresponds to H of an OH group
5·85	1	dt	1, 2	Coupled with H at 7·34 and H at 4·05 and 4·23 Indicates a CH_2OH group bound to a CH group
6·25	1	d	3·2	Coupled with H at 6·42
6·42	1	dd	1·9, 3·2	Coupled with H at 6·25 and 7·59
6·78	1	dd	1·8, 4	Coupled with H at 7·17 and 8·04
7·17	1	d	4	Coupled with H at 6·78
7·34	1	d	2	Coupled with H at 5·85
7·45	1	s		No vicinal H
7·59	1	d	1·9	Coupled with H at 6·42
7·95	1	s		No vicinal H
8·04	1	d	1·8	Coupled with H at 6·78

[a] s, singlet; d, doublet; t, triplet; dd, double doublet; dt, double triplet.

Sequence 1



Sequence 2



Sequence 3

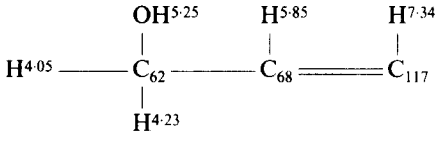


Fig. 2. Sequences of atoms derived from the NMR data for compound U1(1). (Chemical shifts for carbon and hydrogen atoms are given as subscripts and superscripts, respectively.)

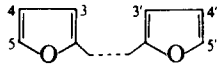
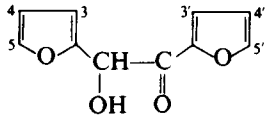
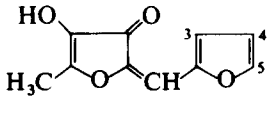
possesses an alcohol chain. Therefore, although the structure of Compound U1(1) could not be completely elucidated, it shares certain structural features with the compounds listed in Table 1. An extensive search of the literature showed Compound U1(1) to be novel. It has not previously been isolated from any carbonyl-amino compound model system. Further sample will need to be purified in order to obtain the high resolution MS and ^{13}C-NMR data required to completely elucidate the structure of this compound.

CONCLUSION

The complexity of the coloured low molecular weight compounds formed on heating an aqueous xylose–lysine model system has been illustrated. In addition, similarities and differences on heating with control of the pH at 5 and without pH control have been shown. Analysis of the ethyl acetate-extractable components, accounting for <0·5% (m/m) of the initial reactants, by TLC and HPLC resulted in different separation patterns for the two model systems. A total of two coloured peaks were analysed from the model system heated with pH control, while seven coloured peaks were studied from the system heated without pH control, using HPLC with diode array detection. Only one peak was common to both systems.

The empirical formula and some preliminary structural data for a novel Maillard reaction product

Table 6. Selected NMR data for compound U1(1), and for terminal furan rings (substituted in the 2-position) of some related components

Compound	Nucleus	Chemical shift (ppm)	Multiplicity[a]	Coupling constant $J_{H,H}$ (Hz)	Assignment[b]	Ref.[c]
	H	6·78	dd	1·84, 4	C4	1
	H	7·17	d	4	C3	1
	H	8·04	d	1·8	C5	1
	C	113			C4	1
	C	119			C3	1
	C	147			C5	1
	H	6·42	dd	1·9, 3·2	C4'	1
	H	6·25	d	3·2	C3'	1
	H	7·59	d	1·9	C5'	1
	C	110			C4'	1
	C	107			C3'	1
	C	141			C5'	1
	H	6·37	—	—	C4	2
	H	6·40	—	—	C3	2
	H	7·38	—	—	C5	2
	H	6·55	—	—	C4'	2
	H	7·27	—	—	C3'	2
	H	7·62	—	—	C5'	2
	H	6·58	dd	2·0, 3·5	C4	3
	H	7·00	d	3·5	C3	3
	H	7·58	d	2·0	C5	3
	C	110			C4	4
	C	143			C5	4

— indicates data not quoted.

[a] d, doublet; dd, double doublet.

[b] C3, C4, C5, C3', C4' and C5' refer to carbon atoms 3, 4, 5, 3', 4' and 5', respectively, on the appropriate furan ring.

[c] Ref. 1, Table 5. Ref. 2, Bhacca *et al.* (1962). Ref. 3, O'Reilly (1982). Ref. 4, Williams & Fleming (1989).

have been obtained, and this compound possesses a wavelength of maximum absorption at 406 nm (in the visible region). Work is currently in progress to completely elucidate the structure of this compound and to isolate further selected reaction products in sufficient quantities for structural analysis.

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