Photo-isomerization of Azadirachtin Studied by High Performance Liquid Chromatography Coupled to High Field Proton NMR Spectroscopy

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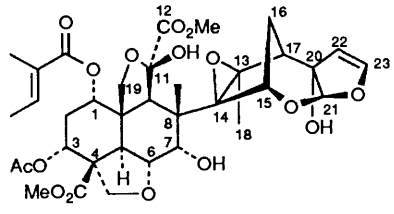
Azadirachtin was irradiated with UV light, in both oxygen and nitrogen atmospheres. The reactions were followed by supercritical fluid chromatography (SFC) and the chromatograms showed that one photo-product was formed in both cases. High performance liquid chromatography coupled to high field proton NMR spectroscopy (HPLC–NMR) was used to identify the photo-product. The HPLC–NMR data indicated that the (*E*)-2-methylbut-2-enoate ester group had been converted into a (*Z*)-2-methylbut-2-enoate ester group. Confirmation was obtained by isomerizing methyl (*E*)-2-methylbut-2-enoate with UV light to form a mixture of geometric isomers and then recording the ^{1}H NMR shift for the alkene proton. Additional structural information was obtained from the isolated product, confirmed to be the (*Z*)-2-methylbut-2-enoate geometric isomer of azadirachtin.

As public concern about the use of synthetic chemicals for crop protection increases, there is a growing need for the development of more environmentally acceptable bioinsecticides. Of these bioinsecticides, neem extracts enriched in azadirachtin **1**,



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a powerful insect antifeedant and growth-regulating substance isolated from the seeds of the neem tree (*Azadirachta indica* A. Juss),[1] show great potential. One such neem based insecticide, Azatin® (Agridyne Tech. Inc., Salt Lake City, USA), has recently been approved for use on food crops by the US Environmental Protection Agency. However, the use of azadirachtin based insecticides may be limited by the acid and base sensitivity of the compound and its susceptibility to photo-degradation.

Previous studies on the photo-decomposition of azadirachtin **1** have involved the use of either natural sunlight[2] or UV light[3] using both HPLC and biological activity to monitor the degradation. In this study we have determined the effects of UV radiation on azadirachtin and used the linked technique of HPLC–NMR in the identification of the product.

The coupling of a liquid chromatograph (LC) to a mass or IR spectrometer has become a widely used technique, whereas the coupling of a chromatograph to an NMR spectrometer has been more difficult due to the problems of the relatively low sensitivity of NMR spectrometry and the interference caused by solvent signals. With the increased availability of higher field strength spectrometers with consequently greater sensitivity, and improvements in solvent suppression techniques, many of these problems are being overcome, which should allow HPLC–NMR to become widely used. The use of HPLC–NMR has already been described for polymer analysis[4] and the analysis of drug metabolites from urine.[5]

In this study, we use the technique to identify the photo-isomerization product of azadirachtin **1** and to demonstrate how structural information can be obtained on such a complex product without having to isolate it.

Results and Discussion

The use of supercritical fluid chromatography (SFC) for the analysis of azadirachtin **1** has been shown to be a simple and fast technique.[6] The course of the UV irradiation experiments with azadirachtin was followed by periodic analysis by SFC as shown in Fig. 1. The reaction was carried out in both oxygen and nitrogen atmospheres for 24 h and in both cases, similar chromatographic profiles were obtained. Under both conditions, after 3 h, an equilibrium mixture of an approximately 2:1 ratio of azadirachtin to photo-isomerized product was formed. In the oxygen environment, the yield of the photo-product and the amount of recovered azadirachtin was low (< 5%). Most of the azadirachtin was converted into a mixture of more polar material from which no products could be isolated. When oxygen was excluded from the reaction, there was an improved yield of the photo-product (21.8%) and a large amount of azadirachtin was recovered (56.7%) with none of the polar material being formed.

Before the photo-isomerization of azadirachtin **1** was carried out, a test mixture containing nimbin **2**, salannin **3** and azadirachtin was analysed by HPLC–NMR to assess the feasibility of the technique for the analysis of the photo-product. The three compounds were separated using a mobile phase of acetonitrile–deuterium oxide, which restrict the regions of lost information on account of solvent proton resonances to a lesser extent than other common LC eluents. Fig. 2 shows the UV chromatogram and the response of the NMR spectrometer as the compounds were eluted through the system. The contour plot clearly reveals the ^{1}H NMR spectra of the three compounds,[7–9] with azadirachtin eluting after 5.1, nimbin after 16.1 and salannin after 20.2 min.

The broad signals in the plot in Fig. 2 around δ 2.0 and 4.2 correspond to the Me group of acetonitrile and HOD, respectively, the latter formed by exchange between D_2O in the mobile phase and hydroxy protons in the mixture. It is interesting to note that using an acetonitrile–water solvent system, the elution order of nimbin and salannin was reversed compared to a methanol–water solvent system. The results obtained from the test mixture clearly demonstrated that the technique of HPLC–NMR could be useful in the structural identification of the photo-product.

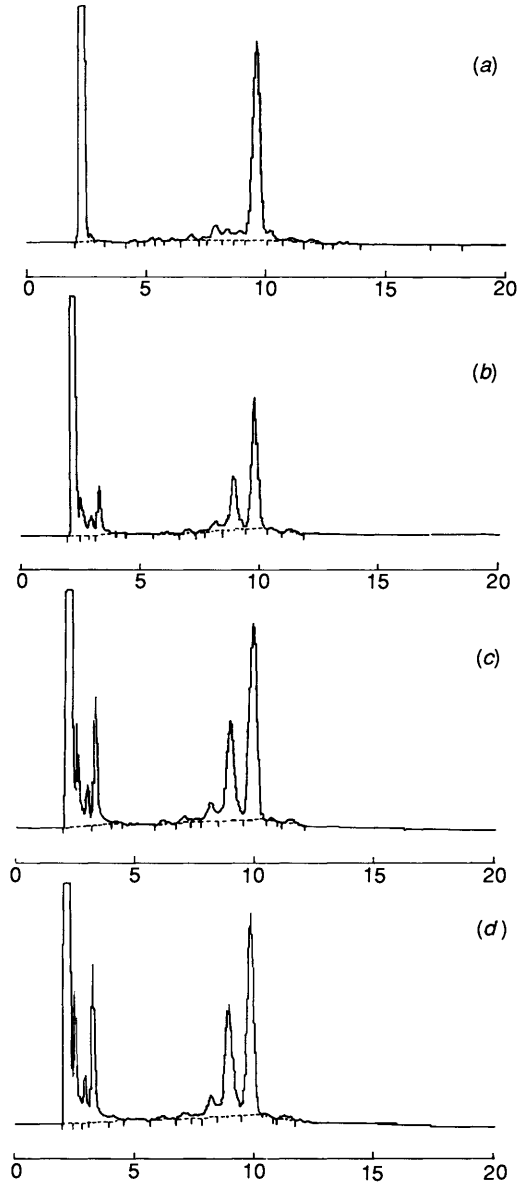


Fig. 1 SFC chromatograms from azadirachtin irradiation after (*a*) zero time, (*b*) 1 h, (*c*) 2 h, (*d*) 3 h

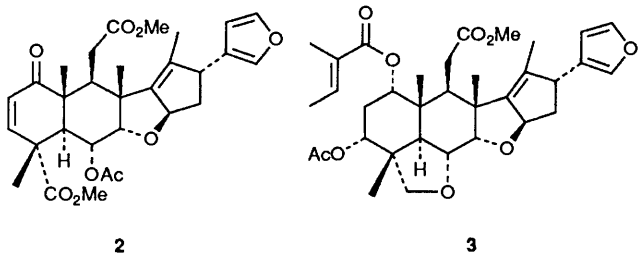


Fig. 3(*a*) shows the LC separation of the photo-isomerized product. Figs. 3(*b*) and (*c*) show the ^{1}H NMR spectra of the compounds eluting after 13.5 and 15.6 min, respectively. By co-injection of a pure standard and comparison of literature NMR data,[7] the peak eluting after 13.5 min was confirmed to be azadirachtin **1** (Table 1), although there were several signals of the spectrum of azadirachtin that were not visible. The C-7, C-11 and C-20 OH signals were removed by D_2O exchange. Many of the Me signals were masked by the acetonitrile signal and the C-6 proton signal was lost under the HOD signal. The signals

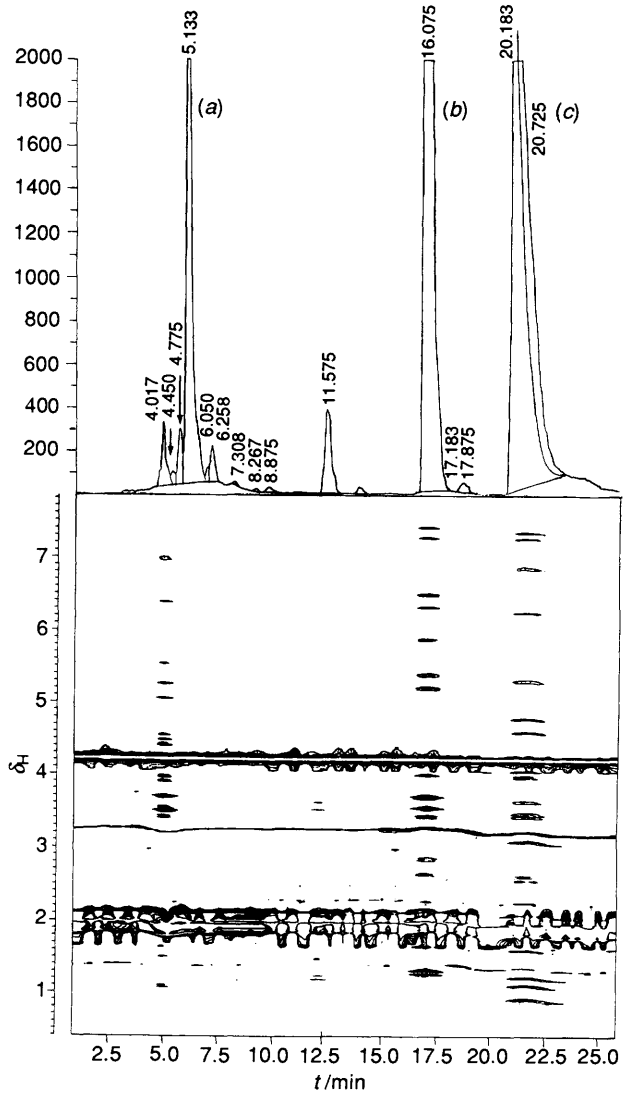


Fig. 2 HPLC–UV (at 217 nm) *vs.* ^{1}H NMR profile of test mixture for (*a*) azadirachtin, (*b*) nimbin, (*c*) salannin

from the C-2, C-5, C-9 and C-17 protons were not visible although they were in regions of the spectrum where the solvent did not interfere. To confirm that the loss of these signals was due to an effect of the mobile phase, a ^{1}H NMR spectrum of pure azadirachtin in MeCN–D_2O was obtained. Again the C-2, C-5, C-9 and C-17 proton signals were not observed, indicating that their signals were shifted to regions of the spectrum which were masked by either the HOD or acetonitrile. The C-3 and C-22 signals were also affected by the solvent; the C-3 signal was shifted upfield by 0.19 ppm and the C-22 signal was shifted downfield by 0.08 ppm in MeCN–D_2O as compared to $CDCl_3$.

The compound eluting after 15.6 min gave a very similar spectrum to azadirachtin **1**, with one major difference in that the alkene proton of the (*E*)-2-methylbut-2-enoate ester in azadirachtin was shifted upfield by 0.85 ppm in the photo-product and again, the signals from the C-2, C-5, C-9 and C-17 protons were missing. In both spectra, the signals at δ 1.38 and 3.26 are due to an impurity in the mobile phase. The shift in the alkene proton signal was thought to be the result of isomerizing the (*E*)-2-methylbut-2-enoate group to the (*Z*)-2-methylbut-2-enoate group. This shift between the *Z*- and *E*-isomers was confirmed by comparison with literature NMR data [10] and by irradiating a sample of methyl (*E*)-2-methylbut-2-enoate **4** with UV light. After 72 h, an approximate 4 : 1 ratio of the *E*-**4** to *Z*-**5** isomers was formed. A ^{1}H NMR spectrum of the mixture of

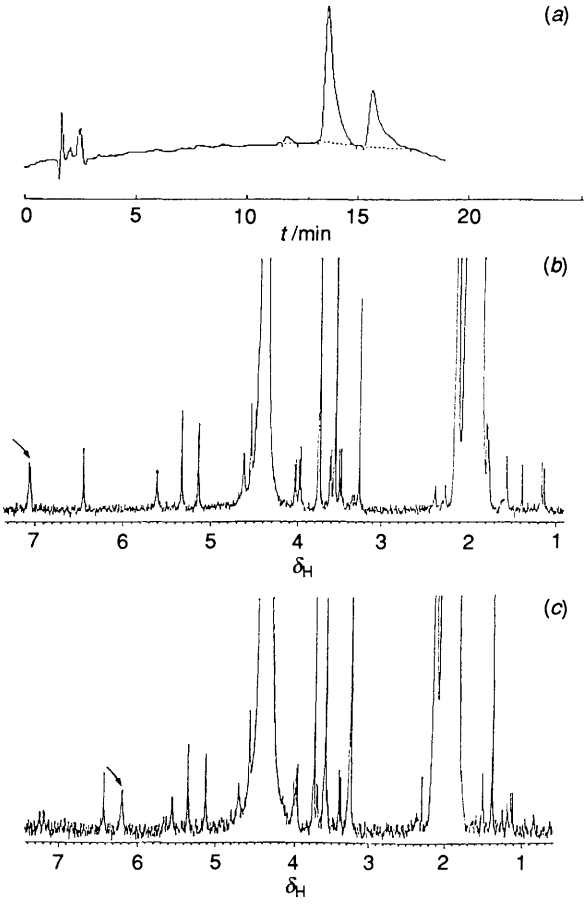
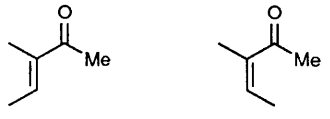


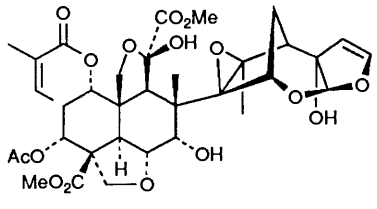
Fig. 3 (*a*) LC of separation of azadirachtin and photo-product, (*b*) ^{1}H NMR spectrum of compound eluting after 13.5 min, (*c*) ^{1}H NMR spectrum of compound eluting after 15.6 min. Arrow indicates shift of 3′-H.

isomers gave a signal at δ 6.86 corresponding to the *E* alkene proton and a signal at δ 6.06 corresponding to the *Z* alkene proton.



4 **5**

To confirm the conclusions from HPLC–NMR studies, we then isolated the photo-product and from structural studies with IR, NMR and mass spectrometry, we confirmed the compound as being the (*Z*)-2-methylbut-2-enoate analogue of azadirachtin **6**. The ^{1}H NMR spectrum showed two Me



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groups at δ 1.96 and 2.06. The ^{13}C NMR spectrum showed the shift in carbon resonances expected for a (*Z*)-2-methylbut-2-enoate ester (Table 2). The IR spectrum displayed an absorption at 805 cm^{-1} due to the *Z* double bond, and an accurate mass spectrum gave *m*/*z* at 743.2527 (M^+ + Na) corresponding to M of $C_{35}H_{44}O_{16}$.

Table 1 Summary of 500 MHz ^{1}H NMR spectral data obtained by HPLC–NMR analysis

Group	Azadirachtin	Photo-product
1-H	4.62	4.70
2-H_a	—[a]	—[a]
2-H_b	—[a]	—[a]
3-H	5.32	5.33
5-H	—[a]	—[a]
6-H	—[b]	—[a]
7-H	4.62	4.70
9-H	—[a]	—[a]
15-H	4.54	4.56
16-H_a	—[a]	—[c]
16-H_b	1.15	1.12
17-H	—[a]	—[a]
18-H	—[c]	—[c]
19-H_a	3.49	3.40
19-H_b	4.03	4.01
21-H	5.60	5.55
22-H	5.12	5.11
23-H	6.45	6.44
28-H_a	3.98	3.96
28-H_b	3.60	3.70
30-H	—[c]	—[c]
7-OH	—[d]	—[d]
11-OH	—[d]	—[d]
20-OH	—[d]	—[d]
12-OCH_3	3.55	3.60
29-OCH_3	3.75	3.75
CH_3CO_2	—[c]	—[c]
3′-H	7.05	6.20
4′-H	—[c]	—[c]
5′-H	—[c]	—[c]

[a] Not observed in spectrum. [b] Masked by HOD signal. [c] Masked by methyl group in acetonitrile. [d] Hydroxy proton exchanged with D_2O from the mobile phase.

Conclusion

In azadirachtin **1** there are two major UV absorbing chromophores, the vinyl ether group and the α,β-unsaturated ester [(*E*)-2-methylbut-2-enoate] group. In the presence of UV radiation and oxygen, both groups might undergo photochemical reactions, such as the formation of peroxides, hydroperoxides and the possible dimerization and polymerization of the molecule, which may explain the formation of a large proportion of polar products. In an inert environment, the only reaction that occurred was the isomerization of the (*E*)-2-methylbut-2-enoate group.

The results obtained by HPLC–NMR analysis show that the technique can be applied to the study of biologically complex molecules; the results obtained for the photo-product show that it can also be used in the identification of an unknown compound.

Experimental

Solvents.—Acetonitrile was of HPLC grade (Rathburn Chemicals, UK and Riedel de Haen, Germany), benzene was spectroscopic grade (Fisons Scientific Equipment, UK), deuterium oxide was 99.9 atom% pure (Cambridge Isotopes Laboratory), methanol was redistilled GPR grade and water was glass distilled. All other solvents were GPR grade and were used as received.

Isolation of Nimbin **2**, *Salannin* **3** *and Azadirachtin* **1**.—Neem seeds (2 kg) were defatted by grinding the seeds with light

Table 2 Comparison of ^1H NMR shifts (at 270 MHz) and ^{13}C NMR shifts (at 67.89 MHz) for compounds **1**, **6**, **4** and **5** (in $CDCl_3$)

	1		**6**		**4**		**5**	
Position	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
1′		166.30		166.71		168.38		168.26
2′		128.55		127.73		128.80		128.06
3′	6.93	137.89	6.06	138.68	6.86	137.05	6.06	137.97
4′	1.78	14.33	2.06	15.75	1.80	14.26	1.99	15.74
5′	1.85	11.91	1.96	20.45	1.84	12.03	1.89	20.64

petroleum (b.p. 60–80 °C) in a high speed mixer (Linshear Mixers Ltd, Stourbridge, UK) and then reground with methanol (3 × 2 dm^3). The methanol extract was concentrated to 500 cm^3, then coated onto Florex RVM (Whitecourt Ltd, London, UK), and eluted through a Florex RVM column, with light petroleum–diethyl ether (4:1). Early eluting fractions contained nimbin which was further purified by recrystallizing from methanol. Later eluting fractions contained salannin. The column was then eluted with diethyl ether (100%) to remove the intermediate polarity impurities, then with diethyl ether–acetone (4:1) to elute impure azadirachtin. This was rechromatographed through a smaller column eluting with diethyl ether–acetone (49:1) to give azadirachtin of single peak purity (2 g) as determined by SFC.

UV Irradiation of Azadirachtin **1**.—A solution of azadirachtin in benzene (360 mg in 45 cm^3) was irradiated with UV light (Hanovia, 90 W) whilst bubbling oxygen through the reaction mixture. A sample was removed every 15 min and analysed by SFC. The reaction was also carried out using the same conditions as above, except nitrogen was bubbled through the solution.

Chromatographic Equipment.—An LDC analytical (Stone, Staffordshire, UK) supercritical fluid chromatograph, consisting of a cooler (Haake C), liquid CO_2 pump (CP 3000), modifier pump (ConstaMetric 3000), gradient programmer (GM-4000), high pressure mixer, pulse dampener (Negretti, Southampton, UK), variable wavelength UV detector (SpectroMonitor 3100) set at 217 nm and an oven containing a model 7125 loop injector fitted with a 20 mm^3 loop (Rheodyne, Cotati, USA), backpressure regulator (Tescom, Elk River, USA) and the analytical column was used to monitor the irradiation of azadirachtin.

An HPLC system consisting of an Altex model 100 pump (Pye Unicam, Cambridge, UK), injection valve fitted with 20 mm^3 loop (Negretti, Southampton, UK) and a variable wavelength UV detector (Pye Unicam, Cambridge, UK) was used for initial determination of chromatographic conditions prior to LC–NMR analysis. For both SFC and HPLC, an integrator (Shimadzu C-R3A) was used for chromatographic data collection.

For HPLC–NMR analysis, a Bruker HPLC system consisting of a pump (LC22C) and a variable wavelength UV detector (LC313) set at 217 nm was connected *via* a PEEK capillary (0.25 mm inside diameter) to the probehead of a Bruker NMR spectrometer (AMX-500), equipped with a Bruker ^1H-flow probe, with an inner diameter of 2 mm and an internal volume of 60 mm^3.

Chromatographic Conditions.—For SFC analysis, a Spherisorb cyanopropyl silica column (150 mm × 4.6 mm inside diameter) of 5 μm particle size (HPLC Technology, Macclesfield, UK) was used. Separation was performed under isocratic conditions at a flow rate of 2 cm^3 min^{-1}, using a mobile phase of CO_2–methanol (47:3), a pressure of 3000 psi,* and an oven temperature of 55 °C.

For HPLC analysis a Spherisorb ODS column (250 mm × 4.6 mm inside diameter) of 5 μm particle size (HPLC Technology) was used. Separation of the test mixture was performed under isocratic conditions at a flow rate of 1 cm^3 min^{-1}, using a mobile phase of acetonitrile–water (2:3). Separation of the photo-product was performed under isocratic conditions at a flow rate of 1 cm^3 min^{-1} using a mobile phase of acetonitrile–water (7:13).

For HPLC–NMR analysis, a Spherisorb ODS II column (250 mm × 4.6 mm inside diameter) of 5 μm particle size (Muder and Wochele, Berlin, Germany) was used. The mobile phase was acetonitrile–deuterium oxide (7:13). All other conditions were as for HPLC separation.

Isomerization of Methyl (E)-2-*Methylbut-2-enoate* **4**.—Ester **4** (methyl tiglate, Lancaster Synthesis, Lancashire, UK) was irradiated in a quartz cuvette with UV light. The reaction was monitored by gas chromatography (GC) and was stopped after 72 h when equilibrium appeared to have been reached.

Isolation of the Photo-product.—Isolation of the photo-product was performed on a Florex RVM column eluting with diethyl ether–acetone (99:1).

* 1 psi = 6.894 kPa.

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