^1H and ^{13}C NMR characterization of new oleuropein aglycones

Rita Limiroli,[a] Roberto Consonni,[b] Gianluca Ottolina,[c] Vincenzo Marsilio,[d] Giorgio Bianchi[d] and Lucia Zetta*,[b]

[a] *Istituto Sperimentale per l'Elaiotecnica, Pescara, c/o Istituto di Chimica delle Macromolecole, Lab. NMR, CNR, Milano, Italy*
[b] *Istituto di Chimica delle Macromolecole, Lab. NMR, CNR, Milano, Italy*
[c] *Istituto di Chimica degli Ormoni, CNR, Milano, Italy*
[d] *Istituto Sperimentale, per l'Elaiotecnica, Pescara, Italy*

Enzymatic hydrolysis of oleuropein carried out in a NMR tube, gave two diastereoisomeric aglycones, which have been identified and characterized by proton and carbon NMR experiments in the same aqueous reaction medium. The stereostructures, assigned to the aglycones by NOESY spectroscopy and molecular dynamics calculation are those of two diastereoisomers, **4a** and **4b**.

Introduction

Phenolic compounds present in olives are well known to undergo qualitative and quantitative seasonal variations during the development and maturation of the fruits.[1] In unripe olives one of the main phenolic compounds is oleuropein **1**, the ester of elenolic acid and 3,4-dihydroxyphenylethanol, which is known to undergo transformation during fruit ripening.

Many substances isolated from olive have been considered to originate from oleuropein, *via* the aglycone, opening of the elenolic acid ring and final rearrangement to secoiridoid compounds **2**.[2-4] Although the mechanism of formation of the latter compounds has been extensively investigated, some facets of the reaction scheme need to be further defined.

NMR analyses of aqueous extracts of the olive (varieties Dritta, Cipressino and Leccino) harvested at different times (June, August, October and November) show a highly variable oleuropein content which is at a maximum in August and is almost zero in winter (work in preparation). In particular, in the crude aqueous extracts collected in the period August–December, we observed and characterized, in addition to **1**, a number of phenolic compounds, thought to derive from **1**, and already described in the literature; namely, secoiridoids and dialdehydes of types **2** and **3**, respectively.[2-4]

In order to understand the oleuropein transformation pathway, several groups have investigated the enzymatic degradation of **1** *in vitro*, using β-glucosidases[5,6] or yeasts.[7] Such studies, especially those with yeasts, were aimed at detecting the possible antimicrobial activity of the hydrolysis products of oleuropein affecting the olive fermentation.[8,9] However, except for the studies with yeasts, in which the enzymatic activity of different yeasts was checked in aqueous medium, in all cases the hydrolysis products were isolated by extraction with organic solvents in order to obtain pure samples for further studies.

In the present paper results are reported on the enzymatic degradation of **1** with β-glucosidase, carried out directly in the NMR tube. The structure of the products formed was studied *in situ*, in the same aqueous reaction medium, avoiding any further work-up that might affect the nature of the initial intermediate in the reaction.

In this study ^1H and ^{13}C NMR spectroscopy was used to characterize the hydrolysis products. It was thus possible to detect and structurally define two compounds **4a** and **4b** not yet reported in the literature. They are derived from enzymatic hydrolysis of the glycosidic linkage in **1** and we consider them to be the first observable intermediates in the process of formation of the secoiridoids from oleuropein.

Results and discussion

Characterization of the extracts

In order to characterize thoroughly the reaction products, the extracts of the enzymatic reaction, were dissolved in the three following solvents. (a) The extract was dissolved in deuteriated chloroform and the ^1H NMR spectrum recorded a few minutes after dissolution; this showed signals indicating the presence of more than one compound, as deduced from the presence of several resonances in the range 9.2–9.8 ppm, attributable to aldehydic protons. The NMR spectrum carried out 1 month later showed an increase in one component, identified as secoiridoid **2** by comparison with data reported in the literature.[2-4]

(b) The extract was dissolved in perdeuteriated acetone and the ^1H NMR spectrum, run immediately after dissolution; this showed the presence of a number of compounds, all readily transformed into the same secoiridoid **2** detected in chloroform.

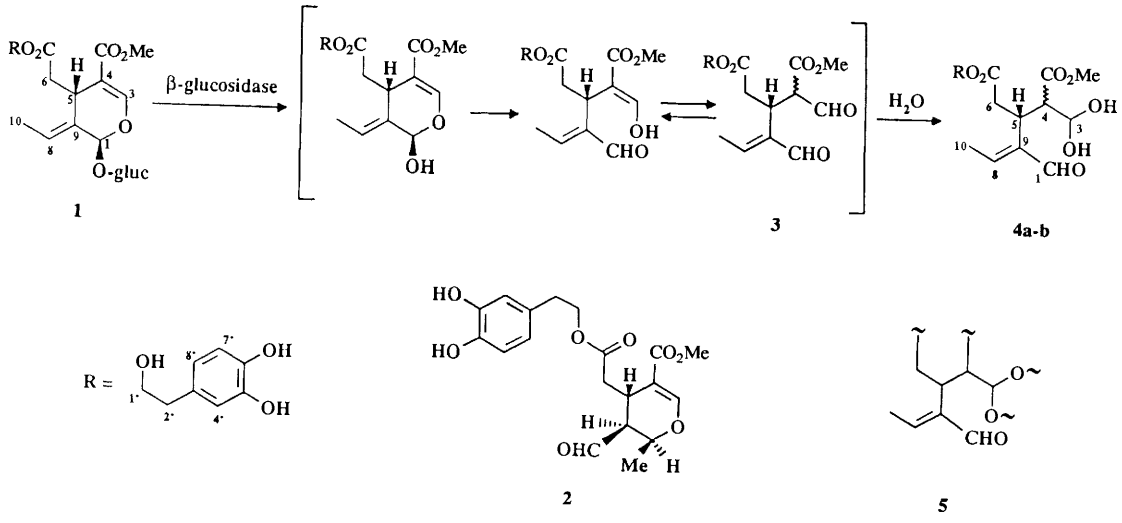
(c) The NMR spectrum of the extract dissolved in perdeuteriated water (Fig. 1) indicated the presence of two secoiridoids already found in organic solvents,[2-4] but not previously reported for an aqueous solution, together with the two new compounds **4a** and **4b**.

In situ characterization of the principal aglycones from oleuropein treated with β-glucosidase

The compounds first obtained by enzymatic degradation of oleuropein in water possess varying life times in the different solvents used, that is chloroform, acetone and water, before being transformed into the final stable compounds. This observation, reported here for the first time, suggests that transformation of oleuropein aglycones occurs during the extraction steps following the enzymatic reaction. The fast conversion into secoiridoid **2** observed in acetone and the presence of several compounds in chloroform, represented a serious difficulty for a complete characterization of the intermediates in organic solvents.

These data and findings prompted us to plan experiments in which the original reaction products were stable enough to be characterized. Thus, the enzymatic reaction was performed in water, in a NMR tube, and the products were studied *in situ*, without any chemical manipulation.

The sample was kept in the probe at 27 °C for 30 min before the first NMR spectrum was recorded. After 2 h, the NMR spectrum (Fig. 2) showed the resonances of (i) unchanged oleuropein (O), (ii) free β-glucose (G), indicating the hydrolytic action of the enzyme on the glucosidic bond and (iii) new compounds (A). The enzymatic reaction was followed by



Scheme 1

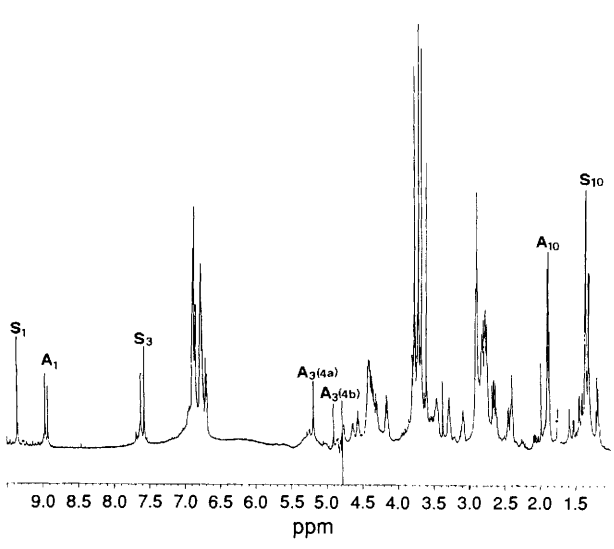


Fig. 1 Aglycone extracts from the enzymatic reaction of **1** with β-glucosidase: 500 MHz ^{1}H NMR spectrum in perdeuteriated water. Signals from secoiridoids **2** are indicated by letter S, from **4a** and **4b** are indicated by letter A.

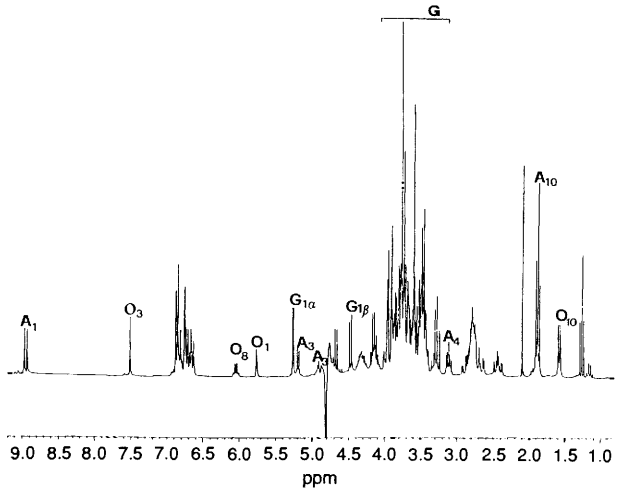


Fig. 2 270 MHz ^{1}H NMR spectrum of reaction products obtained from **1** after 2 h incubation with β-glucosidase in H_2O, at pH = 5.2. Signals from reaction product protons are indicated by letter A, those of unchanged **1** by letter O and those from free glucose by letter G.

observing the disappearance of the oleuropein signals at 1.55 (10-H), 5.73 (1-H), 6.02 (8-H) and 7.48 (3-H) ppm and the appearance of new signals at 1.86 and 1.88 ppm (doublets, J 7.17 Hz) and 8.92 and 8.95 ppm (doublets, J 1.76 Hz). Since each new pair of signals appeared at the same time and always had the same relative area, it seemed likely that they arose either from a single compound or a mixture of two similar constituents, present in roughly equal concentrations. On the basis of the chemical shifts, the two pairs of signals were tentatively assigned to two methyl (higher field) and two aldehydic moieties (lower field), respectively. After a few hours hydrolysis of the β-glucosidic linkage was complete, as deduced from complete disappearance of the oleuropein signals.

An analysis of the whole spectrum revealed that all signals or groups of signals were doubled: in particular, in addition to the previously mentioned resonances, two sets of aromatic systems were observed together with two sets of signals at 4.8 and 5.2 ppm, characteristic of protons on a carbon directly bonded to two oxygens, such as a gem diol. The signals of the two sets of protons present in a 1:1 ratio suggested the co-existence of two slightly different compounds. These compounds remained stable for many days, a period of time long enough to carry out a series of 2D NMR homo- and hetero-correlated experiments.

By combining COSY and TOCSY experiments, it was possible to identify the two following separate spin systems MeCH= and $CH_2CH(CH)C$–CHO. A 2D NOESY experiment showed a correlation between the olefinic and the aldehydic protons, which allowed us to deduce the molecular moiety shown in **5**.

In order to prove the specificity of β-glucosidase, we took tyrosol (4-hydroxyphenylethanol) as a model to compare the free alcoholic moiety of **1**, namely the 3,4-dihydroxyphenylethanol. By comparing the spectra of **1** and of the two new compounds with that of tyrosol (not reported), the hydrolysis of the C-7 ester linkage can be ruled out on the following basis: in tyrosol the $CH_{2(1')}CH_{2(2')}$ group is an A_2X_2 spin system, in which the two A (3.80 ppm) and the two X (2.80 ppm) protons are degenerate; in contrast, in **1** and in the two compounds the comparable group of signals shows up as an ABX_2 system, with resonances at 4.14 ppm for A, 4.37 ppm for B and 2.82 ppm for both X protons. Hence, on the basis of the reasonable assumption that β-glucosidase hydrolyses only the sugar linkage, possible structures for the two compounds that suggest themselves derive from the opening of the elenolic ring, through

Table 1 ^{1}H NMR data of **4a** and **4b** (500 MHz, H_2O, TSP as internal standard). Chemical shifts are in ppm, coupling constants in Hz

	4*S*,5*S* **4a**	4*R*,5*S* **4b**
1-H	8.92 (d, $J_{1,5}$ 1.76)	8.95 (d, $J_{1,5}$ 1.76)
3-H	5.18 (d, $J_{3,4}$ 6.3)	4.89 (d, $J_{3,4}$ 5.10)
4-H	3.08 (dd, $J_{4,5}$ 4.80)	3.12 (dd, $J_{4,5}$ 3.70)
5-H	3.48 (m)	3.47 (m)
6a-H	2.79 (dd, $J_{6a,6b}$ 15.06, $J_{5,6a}$ n.d.)	2.38 (dd, $J_{6a,6b}$ 14.87, $J_{5,6a}$ 4.30)
6b-H	2.88 (dd, $J_{5,6b}$ 5.09)	2.78 (dd, $J_{5,6b}$ n.d.)
8-H	6.72 (q)	6.74 (q)
10-H	1.88 (d, $J_{8,10}$ 7.17)	1.86 (d, $J_{8,10}$ 7.17)
1′a-H	4.14 (m)	4.14 (m)
1′b-H	4.37 (m)	4.37 (m)
2′-H	2.82	2.82
4′-H	6.75–6.76 (d, $J_{4,8}$ 2.15)	6.75–6.76 (d, $J_{4,8}$ 2.15)
7′-H	6.86 (d, $J_{7,8}$ 8.05)	6.86 (d, $J_{7,8}$ 8.05)
8′-H	6.66–6.67 (dd)	6.66–6.67 (dd)
OMe	3.78 (s)	3.60 (s)

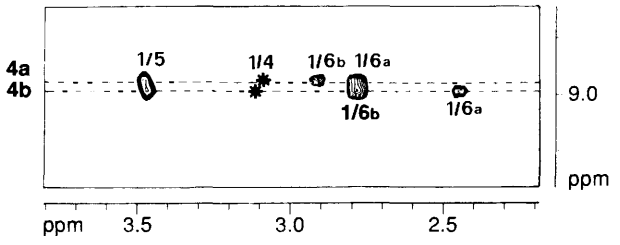


Fig. 3 500 MHz TOCSY spectrum of **4a** and **4b** in perdeuteriated water, at pH = 5.2. Correlations of the aldehydic proton 1-H with 5-H, 6a-H and 6b-H are shown for each diastereoisomer. Asterisks indicate missing correlations between 1-H and 4-H.

the formation of an unstable enolic species, which, on addition of one molecule of water, gives rise to the formation of hydrated compounds (gem diols) (Scheme 1).

While the absolute configuration of C-5 in **1** is known to be *S*[10,11] and remains unchanged in the new compounds, keto-enol tautomerism causes racemization at C-4, with formation of the two diastereoisomers **4a** and **4b**. Accordingly, the two gem diols showed two sets of NMR signals with the same spin patterns and the 2D spectra were characterized by two sets of signals with identical correlations. Fig. 3 shows the expanded region of the TOCSY spectrum in which the aldehydic correlations are clearly shown for **4a** and **4b**. In order better to characterize the structures of the two diastereoisomers, in addition to COSY and TOCSY experiments, a series of ^{13}C NMR spectra was acquired. ^{1}H and ^{13}C spectral data are shown in Tables 1 and 2.

By comparing the ^{13}C chemical shifts of **1** with those of the two diastereoisomers [Fig. 4(*a*) and 4(*b*) and Table 2] it appears that (i) the C-1 anomeric signal at 98.4 ppm in **1** [Fig. 4(*a*)] is substituted by two CHs at 200.9 and 201.1 ppm in **4a** and **4b** [Fig. 4(*b*)], assigned to the aldehydic carbons; and (ii) the olefinic carbons C-3 and C-4 at 158.1 and 111.6 ppm in **1** are replaced by two CHs at 92.2 and 92.8 ppm, assigned to the gem diols moieties (C-3), and two CHs (C-4) at 56.2 and 56.5 ppm, respectively, in **4a** and **4b** [Fig. 4(*b*)], derived from the hydration of the aldehydic group in position 3 in **3**. The multiplicity of the two last carbons guarantees that C-4, being a CH and not a CH_2, still carries the CO_2Me group. This finding indicates that, in water, the reaction proceeds through the opening of the elenolic ring with no decarboxymethylation.[4] Indeed, the carboxyl resonance, which, in **1** [Fig. 4(*a*)], is at 172 ppm due to the conjugation with the double bond, in the two diastereoisomers [Fig. 4(*b*)] moves, as expected,[12] to lower fields around 177 ppm, close to the C-7 resonances. Interestingly, in

Table 2 ^{13}C NMR chemical shifts in ppm of **4a** and **4b** (67 MHz, H_2O, TSP as internal standard)

C-1	200.9/201.1
C-3	92.2/92.8
C-4	56.2/56.5
C-5	35.6
C-6	36.2
C-7	176.9/177.4
C-8	162.2/162.4
C-9	142.9/143.5
C-10	17.7
C-1′	68.57/68.65
C-2′	38.1
C-3′	133.83
C-4′	119.4
C-5′	145.2/145.4
C-6′	146.8/146.9
C-7′	119.1
C-8′	124.0
CO_2Me	176.9/177.4
OMe	55.1/55.3

The stereochemical assignment was possible only for the C-3 signal. Only a few carbons show distinct signals for each stereoisomer.

4a and **4b** the effect of conjugation with the double bond of the aldehydic moiety influences the chemical shifts of the olefinic C-8 and C-9, which change from 128 and 131 in **1** into 162.2/162.4 and 142.9/143.5 ppm in **4a** and **4b**, as expected[13] for olefinic carbons in position β and α, respectively, to an aldehydic carbonyl function.

2D Heteronuclear shift-correlated experiments allowed two ambiguities in the assignment of the carbon resonances to be solved: in particular, (i) C-2′/C-6 showed an inversion of chemical shifts with respect to the values reported for **1** in chloroform[4] (assignments to the two carbons were equivocal both in dimethyl sulfoxide[2] and in methanol[3]), and (ii) the aromatic C-4′/C-7′ were assigned on the basis of the proton multiplicities, being for 4′-H a 2.15 Hz doublet at 6.76 ppm, and for 7-H a 8.05 Hz doublet at 6.86 ppm. All the NMR data were consistent with the structures **4a** and **4b** proposed for the two compounds.

Finally, the same enzymatic reaction was performed in perdeuteriated water. As a result of keto-enol tautomerism a deuterium atom is present at position 4; consequently, as shown in Fig. 5(*b*), signals for 4-H were absent in the proton NMR spectrum and those for 3-H were singlets (the coupling constant between the proton and deuterium being of the order of the linewidth). Further, the 4-H correlation was absent in the TOCSY spectrum (see Fig. 3).

At 67 °C the two stereoisomers **4a** and **4b** were transformed

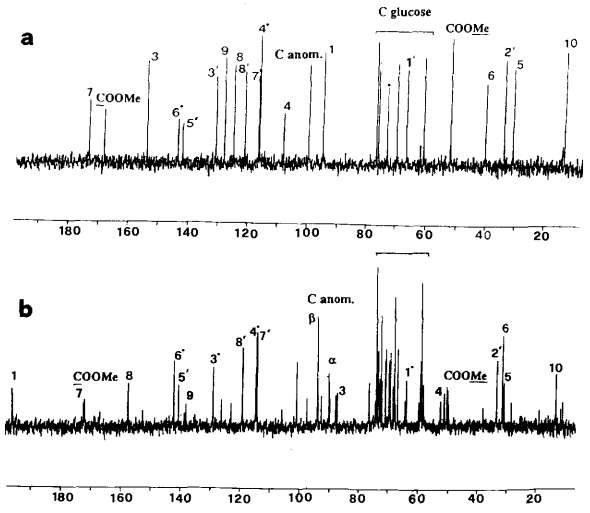


Fig. 4 67 MHz ^{13}C NMR spectra of (*a*) **1** and (*b*) **4a** and **4b** in perdeuteriated water at pH 5.2. ^{13}C signals of free glucose are also observed. The assignments are in agreement with data reported by Montedoro *et al.*[4]

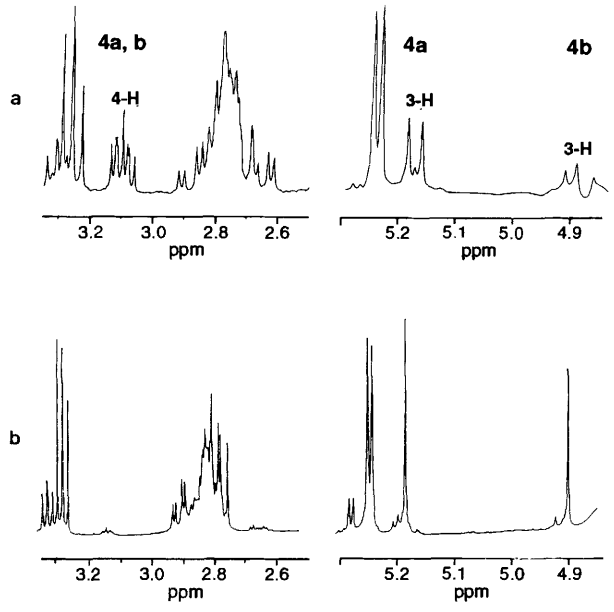


Fig. 5 270 MHz ^1H NMR spectra of **4a** and **4b**: 3-H and 4-H regions in (*a*) H_2O and (*b*) perdeuteriated water

readily into the secoiridoids previously described in the literature and observed when the enzyme reaction products were taken up in organic solvents.

Computational analysis was carried out to correlate the molecular stereochemistry with proton signals. Distance restraints were obtained from NOE data. Each molecule was subjected to an unrestrained energy minimization of 300 cycles using a conjugated gradient process. Then a distance and chiral restraint file was associated with each structure obtained by energy minimization. A file of 12 distance restraints, common to both structures, was used for the starting simulated annealing calculations, then a new file including the 3-H/5-H restraint was applied.

Only the isomer with the assigned stereochemistry 4*S*,5*S* **4a** showed no violation of the 3-H/5-H distance. In this way, it was possible to distinguish the 3-H signal of the 4*S*,5*S* stereoisomer from the 4*R*,5*S* **4b**. By reconsidering the COSY, TOCSY and NOESY correlations, the 3-H signal was the starting point for the stereochemical assignment of most of the proton signals. After simulated annealing calculations, each structure showed a hydrogen bond between the aldehydric oxygen and one of the OH groups in the 3-position (Fig. 6). The hydrogen bond contribution may contribute to the relative stability of the intermediates **4a** and **4b** in aqueous solution.

Conclusions

Data available in the literature concerning the enzymatic and chemical hydrolysis of oleuropein are related to various aglycones with the cyclic secoiridoid structure. The described aglycones were thought to originate from the freed hemiacetal moiety of the secoiridoid present in oleuropein, and their basic skeleton is that shown in compound **2**.

We were interested in exploring the possibility of characterizing intermediates of the reaction steps through which the hemiacetal aglycone from **1** rearranges to compounds of type **2**. The intermediate was expected to possess the basic skeleton of the dialdehyde **3** in tautomeric equilibrium with the enolic species shown in square brackets.

Our NMR study has succeeded in uncovering and detecting (i) the formation and the intermediacy of the dialdehyde **3** as the hydrates (gem diol) **4a** and **4b**; (ii) the relative stability of **4a** and **4b** at temperatures up to 67 °C; (iii) the formation of compounds **2** from **1** *via* **3** and **4**; and (iv) the occurrence of hydrogen exchange at C-4. In contrast to that reported in recent papers,[4,14] we detected no compounds deriving from demethyloleuropein, a compound expected from esterase activity.[1]

Experimental

Preparation of the samples

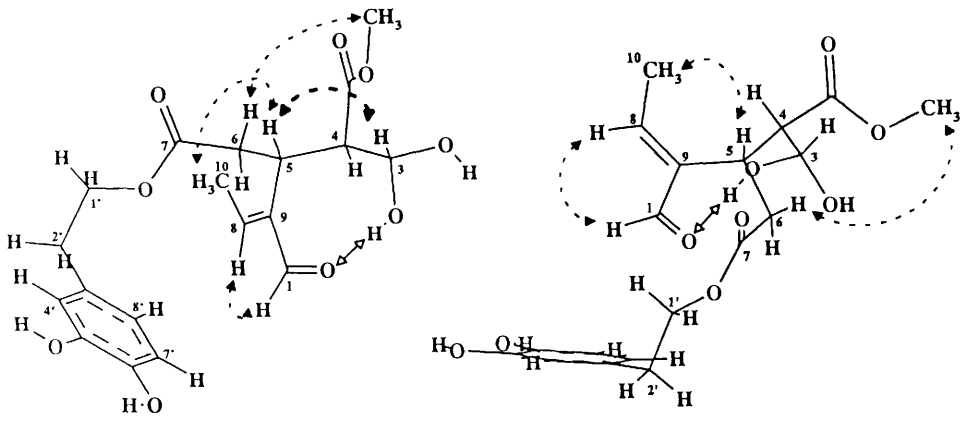
β-Glucosidase (EC 3.2.1.21; 5.7U mg^{-1}, Fluka) from almonds was added to a solution of oleuropein (130 mg) in acetate buffer (0.05 mol dm^{-3}, pH 5.2; 10 cm^3) at a final concentration of 1 mg cm^{-3}. The mixture was gently stirred and incubated at 32 °C for *ca.* 3–4 h. The sample, after centrifugation at 12 000 *g* for 10 min, was extracted by shaking for 1 min, with $CHCl_3$–MeOH 2:1, then centrifugated at 3500 *g* for 5 min. The organic fraction was evaporated under a stream of nitrogen and then dissolved, separately, in C^2HCl_3, [$^2H_6$]acetone or ^2H_2O.

For the *in situ* NMR characterization **1** (23 mg) was dissolved in water ($H_2O/^2H_2O$ 85:15; 0.5 cm^3), at pH 5.2 (acetate buffer) and β-glucosidase (10 mg) was added to the solution. This was then incubated at 27 °C for 30 min before recording of the NMR spectra.

NMR experiments

^1H and ^{13}C NMR spectra were obtained on Bruker AM-500 and AM-270 spectrometers, respectively, controlled by Aspect 3000 computers.

DQF-COSY[15,16] (Double-Quantum filtered COSY), TOCSY[17–19] (Total Correlation Spectroscopy) and NOESY[20] (Nuclear Overhauser Effect Spectroscopy) experiments were acquired by the time-proportional phase-increment method.[21] The TOCSY was performed by using the MLEV-16 composite pulse cycle for the generation of the spin-lock field of γB_2 = 10 KHz, which was applied during 149 ms. At the beginning and the end of the mixing period two 'trim' pulses of 2.5 ms each were applied. In the NOESY experiment a mixing time of 700 ms was used. In all the homonuclear 2D experiments, the water signal was suppressed by presaturation. The spectra were recorded at 27 °C, with 2048 data points in the acquisition domain and 512 data points in t_1. Prior to Fourier transformation, the data were zero-filled to 1024 points in the t_1 dimension. In the TOCSY experiment Gaussian windows in



4S 5S **4a** 4R 5S **4b**

Fig. 6 Structures **4a** and **4b** obtained after restrained molecular dynamics. Dotted lines with filled arrows show NOE relationships. Solid lines with open arrows show H bonds. These projections allow the best view of correlations.

both dimensions were used, while in the NOESY and DQF-COSY experiments $\pi/2$ shifted sine windows in both dimensions were employed. A baseline correction was performed in both dimensions using a polynomial function. Data were processed on a Bruker X-32 station using a UXNMR program.

The heteronuclear shift-correlated 2D spectrum[22] was obtained with quadrature detection in both dimensions, using polarization transfer from ^1H to ^{13}C *via* $J_{^1H^{13}C}$, without ^1H–^1H decoupling. Chemical shifts were referred to sodium trimethylsilyl[2,2,3,3-$^2H_4$]sulfonate (TSP) in water and to tetramethylsilane in organic solvents. Deuteriated solvents were purchased from Merck and used without further purification.

Computational analysis

Calculations were carried out on a Silicon 4D/30/GT work station. Distance restraints were obtained from NOE data. A cross peak volume calculation was performed with the Aurelia program package from Bruker, using a X32 work station. The distance between the hydrogen atoms of the 1′-methylene group of **4a** and **4b** was used for calibration. Where no stereospecific assignment was possible, a pseudoatom was considered and a correction of ± 1 Å for distance was used. In all other cases a correction of ± 0.5 Å was employed.

Programs InsightII and Discover from Biosym were used to build starting structures and to carry out calculations. Restrained molecular dynamics was carried out at 300 K for 30 ps; it was preceded and followed by two minimization procedures using two different algorithms (steepest descents and conjugated gradients).

Acknowledgements

The Italian MI.R.A.A.F. (Ministero delle Risorse Agricole, Alimentari e Forestali) is thanked for the fellowship to R. Limiroli. The authors thank F. Greco for skilful technical assistance.

References

1 M.-J. Amiot, A. Fleuriet and J.-J. Macheix, *Phytochemistry*, 1989, **28**, 67.
2 P. Gariboldi, G. Jommi and L. Verotta, *Phytochemistry*, 1986, **25**, 865.
3 H. Kuwajima, T. Uemura, K. Takaishi, K. Inoue and H. Inouye, *Phytochemistry*, 1988, **27**, 1757.
4 G. Montedoro, M. Servili, M. Baldioli, R. Selvaggini, E. Miniati and A. Macchioni, *J. Agric. Food Chem.*, 1993, **41**, 2228.
5 L. Panizzi, M. L. Scarpati and G. Oriente, *Gazz. Chim. Ital.*, 1960, **90**, 1449.
6 A. Bianco, R. Lo Scalzo and M. L. Scarpati, *Phytochemistry*, 1993, **32**, 455.
7 R. Materassi, N. Miclaus and O. Pelagatti, *Ann. Ist. Sper. Elaiot.*, 1975, **V**, 53.
8 W. M. Walter Jr., H. P. Fleming and J. L. Etchelles, *Appl. Microbiol.*, 1973, **26**, 773.
9 M. De Caro and M. G. Ligori, *Rend. Ist. Sup. Sanita*, 1959, **22**, 223.
10 H. Inouye, T. Yoshida, S. Tobita, K. Tanaka and T. Nishioka, *Tetrahedron Lett.*, 1970, 2459.
11 H. Inouye, T. Yoshida, S. Tobita, K. Tanaka and T. Nishioka, *Tetrahedron*, 1974, **30**, 201.
12 E. Breitmaier and W. Voelter in *Carbon-13 NMR Spectroscopy*, ed. H. F. Ebel, 1987, VCH, Germany, p. 114.
13 F. W. Weherli, A. P. Marchand and S. Wehrli in *Interpretation of Carbon-13 NMR Spectra*, 1988, J. Wiley and Sons, p. 275.
14 R. Lo Scalzo and M. L. Scarpati, *J. Nat. Prod.*, 1993, **56**, 621.
15 U. Piantini, O. W. Sorensen and R. R. Ernst, *J. Am. Chem. Soc.*, 1982, **104**, 6800.
16 A. J. Shaka and R. Freeman, *J. Magn. Reson.*, 1983, **51**, 169.
17 L. Braunschweiler and R. R. Ernst, *J. Magn. Reson.*, 1983, **53**, 521.
18 D. G. Davis and A. Bax, *J. Am. Chem. Soc.*, 1985, **107**, 2821.
19 A. Bax and D. G. Davis, *J. Magn. Reson.*, 1985, **63**, 207.
20 S. Macura and R. R. Ernst, *Mol. Phys.*, 1980, **41**, 95.
21 D. Marion and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 1983, **113**, 967.
22 A. Bax and G. Morris, *J. Magn. Reson.*, 1981, **42**, 501.

Paper 5/00367I
Received 23rd *January* 1995
Accepted 7th *March* 1995