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A new 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) precursor isolated from Riesling grape products: Partial structure elucidation and possible reaction mechanism*

by

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S u m m a r y : A heteroside, which produces 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) by acid hydrolysis, was isolated from Riesling grapes by retention on Amberlite XAD-2 resin, followed by preparative TLC and HPLC techniques. It was partially identified by NMR spectroscopic procedures. The presence of a megastigm-4-en-9-one structure with an enol-ether function in the C_4 position and a OH/OR function in the C_6 position was ascertained. The sugar part should be constituted of two or three glucose moieties with the same NMR characteristics. The linkage of these moieties to the megastigmane structure in the C_4 position and possibly also in the C_6 position remains to be determined. The isolated conjugated form produced only a TDN-d_4 isomer when reacted at 50 °C in D_2O at different acid pH values. A possible reaction mechanism was proposed, considering the kinetics of TDN-d_4 formation during the hydrolysis of the raw glycosidic fraction from two differently aged Riesling wines at pH 2, and comparing it with the kinetics of TDN formation as well. The latter may correspond to the mechanism proposed by Winterhalter (1991). Thus, the presence of at least two different TDN precursors in grape products at different concentrations was proved.

K e y w o r d s : 1,1,6-trimethyl-1,2-dihydronaphthalene glycosidic precursor, 4,6-dihydroxymegastigm-4-en-9-one derivative, Riesling grapes.

Introduction

1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN) is considered a major contributor to the typical bottle-aged kerosene-like character of older Riesling wines, in which the highest levels of this compound were found (Simpson 1978; Di Stefano 1985; Rapp *et al.* 1985; Marais *et al.* 1992 b). This C_{13}-norisoprenoid may develop in higher concentrations in wines from viticultural areas with specific climatic characteristics (Marais *et al.* 1992 c), and may become detrimental to wine quality when present at very high concentrations. It is also responsible for a hydrocarbon off-flavour in young wine distillates (Vidal *et al.* 1990). The development of TDN in wine is dependent on the concentration of its precursors in grapes which increases with sugar accumulation during grape ripening (Strauss *et al.* 1987; Marais *et al.* 1992 a). A possible contribution of yeast strain to the formation of TDN in wine has also been hypothesized by Sponholz and Hühn (1995).

Norisoprenoid aroma precursors have been isolated and identified in different fruits. For example, different megastigmane structures for the precursors of b-damascenone (Sefton *et al.* 1989), and the vitispiranes (Strauss *et al.* 1984; Winterhalter and Schreier 1988; Waldmann and Winterhalter 1992; Full and Winterhalter 1994) have been identified in grapes and wines. The above mentioned norisoprenoids may contribute to the aroma of young and aged wines. Winterhalter *et al.* (1990 a) demonstrated the presence of a number of precursors of these compounds in grapes, which may differ not only in the aglycon, but probably also in the glucose moieties. TDN was produced by acid hydrolysis (pH 1) and heating of the isolated precursor fraction (Williams *et al.* 1982). Winterhalter *et al.* (1990 b) showed that the separated glycosidic fractions able to produce TDN were mainly of intermediate polarity and remained partially in the solvent-extracted droplet countercurrent chromatographic residual stationary phase from which they were removed by dichloromethane, thus suggesting the presence of low polar non-glycosidic precursors.

Megastigma-5,7-dien-3,4,9-triol, isolated from grape juice, yielded TDN as a minor component upon heat treatment at pH 3 (Strauss *et al.* 1984). Di Stefano (1985) identified 4-hydroxy-1,1,6-trimethyl-1,2,3,4-tetrahydronaphthalene in older Riesling wines, but it was never found as an aglycon after enzymatic hydrolysis of grape and wine glycosides. Therefore, it may be considered an intermediate product of TDN biosynthesis or a hydroxylation product of TDN. A hydroxylated TDN (3-hydroxy-1,1,6-trimethyl-1,2,3,4-tetrahydronaphthalene), produced enzymatically from isolated glycosides of purple passion fruit juice, can also be regarded as a precursor of TDN (Winterhalter 1990), and Winterhalter (1991) identified 2, 6, 10, 10-tetramethyl-1-oxaspiro[4.5]dec-6-ene-2,8-diol after enzymatic hydrolysis of the glycosides, and suggested its bound compounds to be natural precursors of TDN in

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Riesling wine. 3,4-Dihydroxy-7,8-dihydro-b-ionone-b-D-glucopyranoside and (R)-3-hydroxy-b-ionone were also proposed as natural precursors of TDN in red currant leaves and quince fruit, respectively (HUMPF *et al.* 1991; GÜLDNER and WINTERHALTER 1991).

Our studies led to the isolation of another TDN precursor in Riesling grapes and to the structural characterisation of the megastigmane and sugar moieties in the molecule. Furthermore, reaction mechanisms for the formation of TDN from at least two TDN precursors in grapes were demonstrated.

Material and methods

Mature grapes of two Riesling clones (Geisenheim 239 and 49) collected in the vineyards of the Istituto Agrario of San Michele all'Adige, Italy, were used. Of these grapes, 10 l of free-run juice were clarified with 2 g/l bentonite and 0.1 g/l gelatine before being submitted to the TDN precursor isolation. Additionally, 1976 and 1993 Deidesheimer (Weingut Bassermann Jordan, Pfalz, Germany) Riesling wines were used.

Isolation and purification of the bound compounds: The TDN precursors were extracted from the clarified juice by the method of GUNATA *et al.* (1985), as adapted by VERSINI *et al.* (1987). The 20 l of juice were passed through five glass columns (4 l per column, 1 l at a time). The columns (18 cm x 3.5 cm i.d.) contained Amberlite XAD-2 resin (0.1 - 0.25 mm; Serva-Heidelberg), which had previously been purified with 400 ml MeOH and 1 l pentane/dichloromethane (2:1, v/v), activated with 150 ml MeOH and 250 ml diethyl ether, and washed with distilled water. After rinsing with 0.7 l distilled H_2O per column and eluting the free compounds with distilled 0.8 l pentane/dichloromethane (2:1, v/v), the precursors were recovered by elution with 1 l ethyl acetate (EtAc). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was redissolved in EtOH, divided into two parts, each of which was passed through a PVPP column (5 cm x 1 cm i.d.) under vacuum, then rinsed with EtOH (4 x 10 ml) and the combined fractions concentrated to dryness. This residue was chromatographed on a silica gel 60 (0.2 - 0.5 mm) column (25 cm x 2 cm i.d.), using 250 ml EtAc/EtOH (3:1, v/v) as eluent. Upon filtration through a 0.2 mm Millipore filter and concentration to dryness, about 400 mg of a glassy residue was obtained.

To test for residual TDN precursors on the XAD resin as well as on silica gel 60, further fractions were obtained by elution with MeOH, followed by acid hydrolysis at pH 1 in a waterbath (50 °C, 4 h) (MARAIS *et al.* 1992 b). The quoted glassy residue was washed twice with 3 ml chloroform (stabilised with 0.75 % EtOH). These combined fractions contained no TDN precursors.

Fractionation by preparative thin-layer chromatography (PTLC): The glassy residue, obtained after silica gel chromatography, was applied as a narrow band on PTLC plates (silica gel 60 F_{254}; 20 cm x 20 cm x 2 mm layer thickness; about 50 mg per plate) and eluted with EtAc/EtOH (85:15, v/v). Each 1 cm layer - or smaller ones, when different UV light sensitivities occurred - was scraped, extracted with EtOH (3 x 20 ml), the corresponding bands from different plates combined and tested for TDN development as described before. Fractions containing precursors were collected (about 115 mg) and subjected to further PTLC (similar plates; EtAc/EtOH, 65:35, v/v). Three close bands producing TDN precursors were collected (about 50 mg) and extracted with EtOH as above. The presence of sugars in the bands was detected by spraying a separate plate with concentrated H_2SO_4 + 1 % vanillin and heating at 80 °C for a few minutes. A grey-violet colour was obtained, which later changed to orange-brown.

Fractionation by preparative high-performance liquid chromatography (HPLC): Preparative HPLC was performed on the isolated precursor fraction (50 mg), using the following experimental conditions:

- HPLC system configuration: Milton Roy CM 4000 semipreparative pump; SM 4000 UV-Vis. Detector (280 mm); PROMIS II Autosample; Jaytee Biosciences 5512 fraction collecting system; Columns: Lichrospher 100 RP-18 (Merck) (25 cm x 10 mm i.d., film thickness: 10 mm); Lichrocart 25-4 (Merck) as precolumn.
- Chromatographic conditions: Solvents: A = H_2O, B = MeOH; linear gradient from 0 to 100 % of B in 30 min, 100 % of B for 5 min.

The precursor fraction was dissolved in 500 ml distilled H_2O and repeated injections of 90 ml of this solution were carried out with the "ml Pick up" method, so avoiding sample leaks. Fractions were collected every 12 seconds and each fraction tested for TDN production as described before; the chromatogramme was monitored at 280 nm (Fig. 1). The TDN precursor here considered was found at elution time from 23.4 to 24.6 min being a more pronounced TDN formation toward the tail of a peak. The fraction collected toward higher elution time (ca. 0.8 mg) was used for further study.

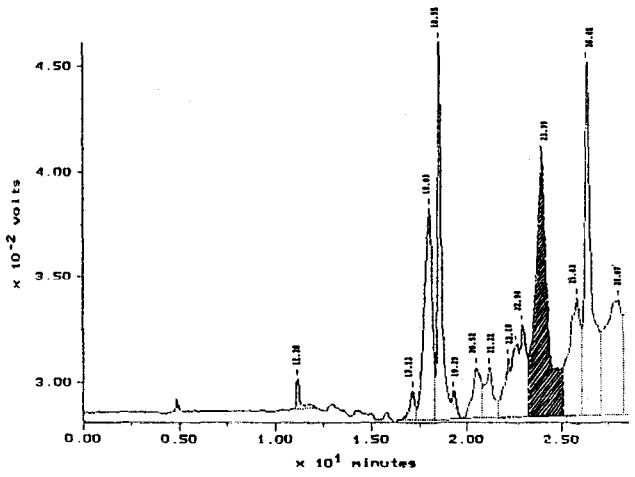

Fig. 1: Preparative HPLC profile. The collected part containing TDN precursor is indicated.

Gas chromatography: GC-EIMS (70 ev) and GC-SIM/MS analyses of the organic extracts were performed to control the TDN production by acid reaction of the PTLC and HPLC fractions, as well as to quantify TDN and deuterated TDN forms in the study of the formation mechanism, respectively. A HP 5890 gas chromatograph, equipped with an apolar PS-264 fused silica capillary column (Mega, Milan; 25 m x 0.25 mm i.d., film thickness = 0.15 mm) and coupled with a HP 5979 Mass Detector was used. Other instrumental conditions were reported earlier (VERSINI *et al.* 1994).

High-performance anion exchange (HPAE) / pulsed amperometric detection (PAD): Sugar monomers produced after chemical hydrolysis of the heteroside were analysed according to PASTORE *et al.* (1993).

Nuclear magnetic resonance (NMR) spectroscopy: The ^{1}H-NMR spectra were recorded on a Bruker AM 500 instrument. Use was also made of proton homonuclear decoupling, ^{13}C inverse detection, ^{1}H-^{13}C inverse long range correlation, TOCSY and ROESY techniques to indicate through-bond ^{1}H-^{13}C and ^{1}H-^{1}H connectivities as well as ^{1}H-^{1}H through-space connectivities.

Results and discussion

1. Structure elucidation

Structures of the megastigmane and glucose moieties of the TDN precursor are shown in Fig. 2, and the ^{1}H and ^{13}C NMR spectral results summarised in Tab. 1 and 2.

Megastigmane moiety: The proposed structural attribution, namely that of megastigm-4-en-9-one with an enol-ether function in the C_4 position and an OH/OR function in the C_6 position is the only one consistent with the spectral data and the observed connectivities (Tab. 1). This configuration is not stabilised through neighbouring groups, as in the case of 2,5-dimethyl-4-hydroxy-3(2H)-furanone glucoside, isolated from strawberry juice (MAYERL

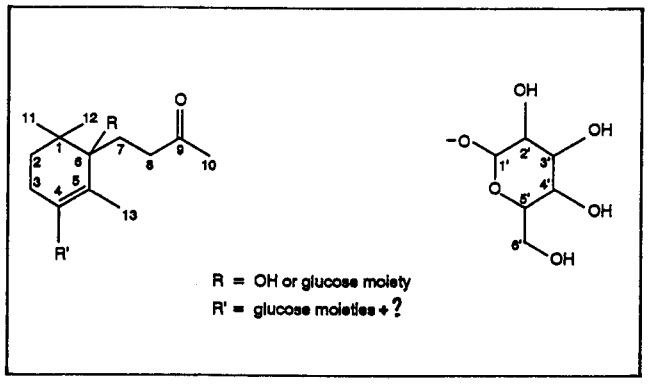

Fig. 2: Structures of megastigmane and sugar moieties identified in the TDN precursor molecule.

Table 1

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data (D_2O, δ: ppm) of the megastigmane moiety of the TDN precursor

Position	^{1}H	^{13}C	Experiments
1	-	38.0	inv.long range corr.
2	1.62, 2H, ddd, $J_{2,3ax-ax}$ = 12.3 Hz; $J_{2,3ax-eq}$ = 4.9 Hz; $J_{2,3eq-eq}$ = 2.3 Hz	39.3	inv.detect.
3	1.78, 2H, virt.br t, J_{gem} = $J_{3,2ax-ax}$ = 12.7 Hz; J_{gem} (H-2 decoupled) = 13.0 Hz	39.3	inv.detect.
4	-	142.9	inv.long range corr.
5	-	126.4	inv.long range corr.
6	-	69.6	inv.long range corr.
7	H_a, 2.27, 1H,dd, $J_{7-Ha,7-Hb}$ = 17 Hz (H-8 decoupled)	22.7	inv.detect.
	H_b, 2.32, 1H,dd, $J_{7-Ha,8}$ = 7.2 Hz		
8	2.67, 2H,m or br t at 400 MHz	43.9	inv.detect.
9	-	217.0	inv.long range corr.
10	2.22 , 3H,s,	30.0	inv.detect.
11	1.065, 3H,s,	27.0	inv.detect.
12	1.06 , 3H,s,	29.0	inv.detect.
13	1.73 , 3H,s,	18.0	inv.detect.

The H_2O signal was positioned at 4.75 ppm.
inv. detect. = inverse detection.
inv. long range corr. = inverse long range correlation.

et al. 1989). Therefore, it cannot exist after the OR linkage hydrolysis, because it is likely transformed into a compound with a keto-group. Thus, after an enzymatic or chemical hydrolysis of the enol-ether linkage and eventually the ether linkage at the C_6 position, 6-hydroxy-megastigmane-4,9-dione and thereafter 4-oxo-7,8-dihydro-b-ionone should originate. Such compounds have not yet been identified in fruit or in grape products as chemically or enzymatically liberated aglycons. 4-Oxo-b-ionol and 4-oxo-7,8-dihydro-b-ionol were found among the enzymatically liberated compounds, the latter being a partially reduced form of the corresponding ionone mentioned above, and also found in oak extracts where also other compounds from the same possible precursors, like 4,5-dihydro-4-oxo-theaspirane, were detected (SEFTON *et al.* 1990). A further reduced derivative, namely 4-hydroxy-7,8-dihydro-b-ionol, was involved in the formation of the theaspiranes, which are important aroma compounds of quince juice (WINTERHALTER and SCHREIER 1988). Another substance with similar structure, namely 4-oxo-b-ionone, was found as one of the main degradation products of b-carotene, when heated under oxygen at 60 °C, pH 3 and for 3 h (MARAIS *et al.* 1990; MARAIS 1992 b).

Two main compounds were produced by acid hydrolysis (pH 1, 50 °C, 4 h) of the investigated precursor fraction, namely TDN and a compound whose spectrum corresponded to that of 4-(2,3,6-trimethylphenyl)-2-butanone (WINTERHALTER 1991). Furthermore, when the precursor was reduced with $NaBH_4$ in water and then subjected to the same acid-catalysed hydrolysis, an equivalent quantity of two vitispirane isomers and an unknown compound were produced.

Glucose moieties: The presence of two or three chemically and magnetically equivalent glucose units was deduced from the ^{1}H NMR spectral data and integrals (Tab. 2). Their ^{1}H and ^{13}C chemical shifts were typical for b -D-glucose moieties linked only through the $C_{1'}$ posi-

tion (VOIRIN *et al.* 1990). Other chemical shifts typical for monohydroxylated sugar-like carbons, but with fractionated integration values less than 1, remained unassigned and they probably belonged to glycosidic impurities.

Table 2

^1H (500 MHz) and ^{13}C (125 MHz) NMR spectral data (D_2O, δ: ppm) of the glucose moieties of the TDN precursor

Position	^1H		^{13}C	Experiments
1'	4.65	d, $J_{1',2'}$ = 8.0 Hz	103.3	inv.detect.
2'	3.31	dd, $J_{2',1'}$ = 8.0 Hz; $J_{2',3'}$ = 9.4 Hz	73.8	inv.detect.
3'	3.51	dd, $J_{3',2'}$ = 9.2 Hz; $J_{3',4'}$ = 8.9 Hz	76.4	inv.detect.
4'	3.41	dd, $J_{4',3'}$ = 8.7 Hz; $J_{4',5'}$ = 9.8 Hz	70.4	inv.detect.
5'	3.45	ddd, $J_{5',4'}$ = 9.4-10.5 Hz; $J_{5',6'a}$ = 5.55 Hz; $J_{5',6'b}$ = 2.1 Hz	76.6	inv.detect.
6'	6'a 3.73	dd, $J_{6'a,5'}$ = 5.6 Hz; $J_{6'a,6'b}$ = 12.2 Hz	61.3	inv.detect.
	6'b 3.91	dd, $J_{6'b,5'}$ = 2 ca Hz; $J_{6'b,6'a}$ = 12.3 Hz		

The H_2O signal was positioned at 4.75 ppm.
inv. detect. = inverse detection.

A signal at d (^1H) 4.07 ppm (ca. 4H, broad singlet or very close double singlet) was interesting. It showed ROESY connectivities with H_1 (glucose), H_{11}, H_{12}, H_{13} and, less intensively, with H_2 and H_3, but no TOCSY connectivities. The corresponding directly correlating ^{13}C signal was found at 69.8 ppm.

Acid hydrolysis of a small part of the raw glycosidic fraction (under N_2 in a sealed vial, at pH 0 and at 100 °C for 2 h), followed by HPAE-PAD analysis, revealed almost exclusively the presence of glucose, probably a small quantity of apiose and traces of rhamnose.

Clarification of the exact configuration of the glucose moieties in the C_4 and C_6 positions was not possible. Support for the molecular weight investigation could not be obtained by FAB techniques because of the low quantity and impurity of the sample.

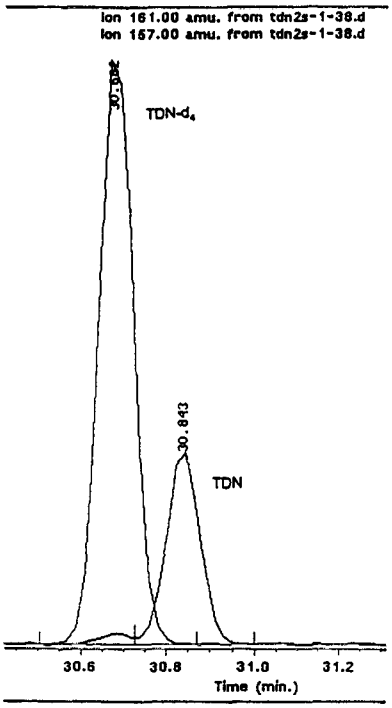

Fig. 3: Part of a GC-SIM/MS profile with TDN (m/z=157) and TDN-d_4 (m/z=161) peaks.

2. Proposed reaction mechanism

Hydrolysis of the isolated precursor at 80 °C in D_2O, acidified to pH 1 and pH 2.5 by H_2SO_4, for 1 and 10 h, respectively, produced one tetradeuterated TDN isomer only, well separated from TDN in the SIM/GC analysis (Fig. 3). On the basis of the MS spectrum and fragmentation proposal shown in Figs. 4 and 5, respectively, the 1,1-dimethyl-6-methyl-d_3-1,2-dihydro-7-deuteronaphthalene structure was proposed.

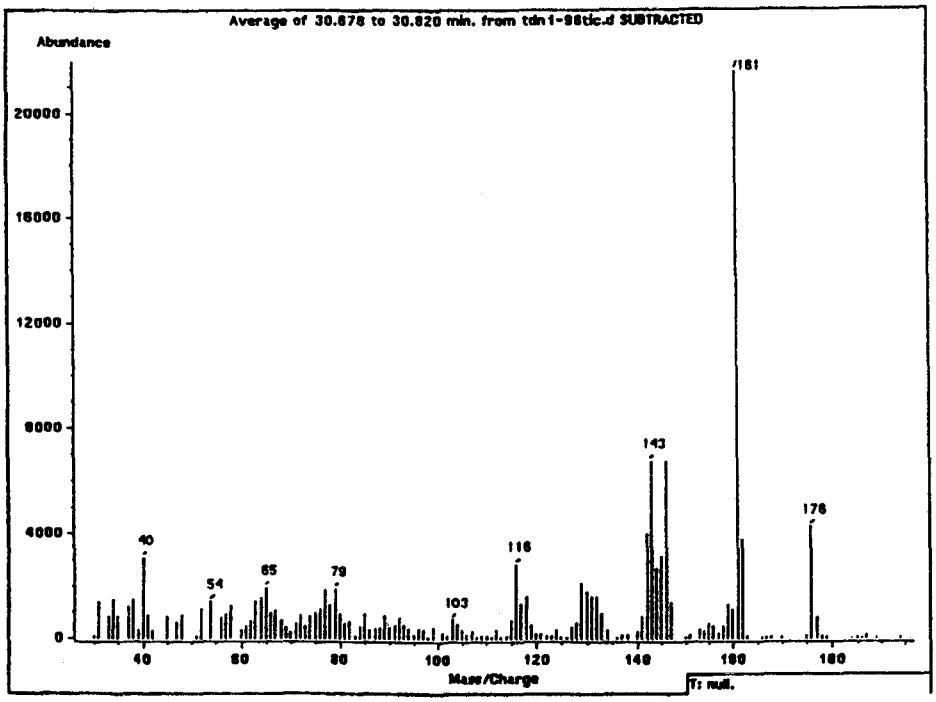

Fig. 4: MS spectrum of TDN-d_4.

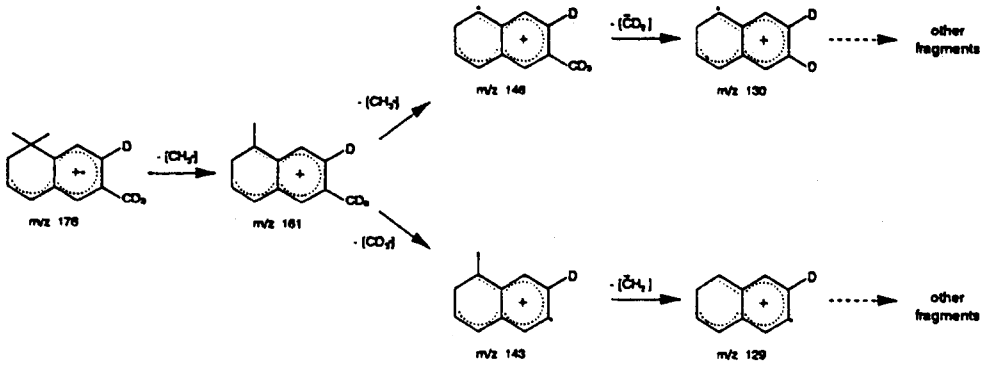

Fig. 5: Partial fragmentation proposal for the MS spectrum in Fig. 4.

Hydrolysis of the raw Riesling heteroside fraction, eluted from the XAD-2 resin under similar conditions, produced almost only the tetradeuterated TDN at pH 1, while at pH 2.5 a mixture of TDN and TND-d_4 was formed. The difference in behaviour prompted an investigation into the presence of two possible reaction mechanisms at a pH closer to that at which the natural products are formed. Both heteroside fractions were obtained on a preparative scale from 1l of the two German Riesling wines each. These fractions were dissolved in 10 ml D_2O (pH 2, acidified by H_2SO_4, was chosen as discussed later) and then divided into 1 ml lots (in 1 ml vials). Each lot was heated at 50 °C for different periods (between 4 and 176 h) and then extracted by 100 ml pentane/dichloromethane (2:1, v/v) by shaking for 5 min in the vial. Extracts (1.5 ml) were analysed in duplicate for the two isotopic isomers by splitless GC-SIM/MS (external standard method). The formation curves for both isomers of one wine and the evolution of the TDN (m/z 157)/TDN (m/z 161) ratio for both wines are shown in Figs. 6 and 7, respectively.

Different kinetics were observed, i.e. probably a first-order reaction (A®B) in the case of the TDN synthesis and a series first-order reaction (A'®C'; C'®B') (Frost and Pearson 1961) with the typical initial induction phase for TDN-d_4. A similar evolution curve of the quoted isomers ratio was found for both wines, but with a dominance of one isomer over the other (or vice versa) at advanced reaction time. These results suggested that at least two TDN-precursors at different concentrations have to be present in the wine extracts used. Under these experimental conditions, the precursor identified by Winterhalter (1991), probably produced only TDN, while the precursor investigated in this study produced the TDN-d_4.

The kinetic mechanisms proposed are shown in Fig. 8. The first one, producing TDN, can be in accordance to that of Winterhalter (1991). The other mechanism prob-

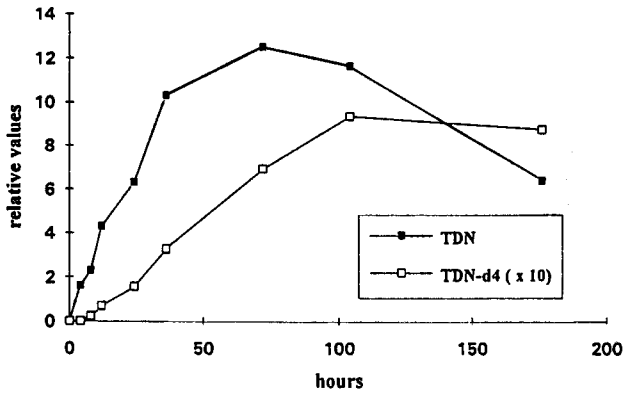

Fig. 6: Example of formation curves of TDN and TDN-d_4 by reacting raw heteroside fraction of a '76 Riesling wine in D_2O at pH 2 and 50 °C.

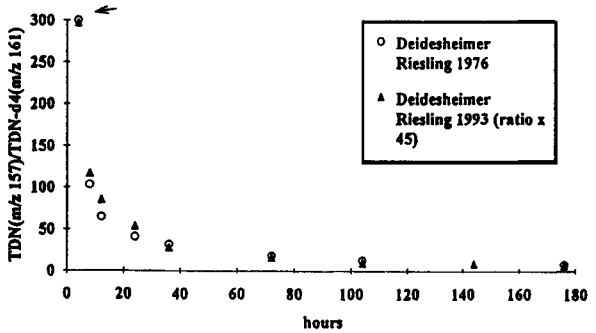

Fig. 7: Evolution of the ratio TDN(m/z=157)/TDN-d 4 (m/z=161) during the kinetics (see Fig. 6) for two Riesling wines (the arrow at 4 h indicates a value >300 in the kinetics for the '76 wine).

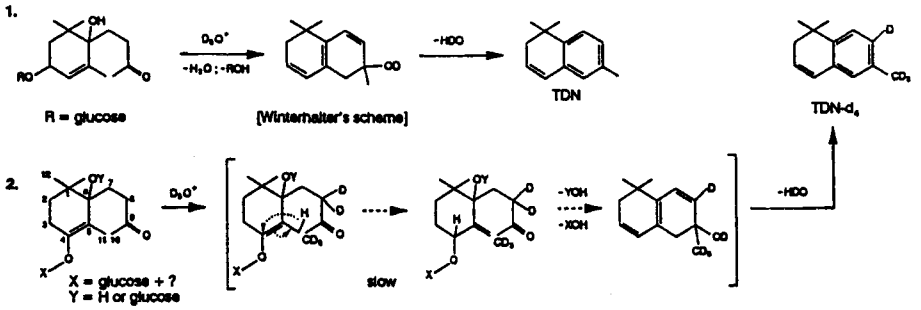

Fig. 8: Kinetic mechanisms proposed for TDN formation from the two possible precursors.

ably implies the rearrangement of the precursor through a slow sigmatropic-type reaction (WOODWARD and HOFFMANN 1970). This may involve a shift of the hydrogen atom from the C_{11} to the C_4 position and that of the double bond from C_4-C_5 to C_5-C_{11}. This fact proves that no addition of a deuterium atom in the C_4 position can happen through the addition of D_2O to the double bond C_4-C_5. Simultaneously, a rapid keto-enolic equilibrium causes the substitution of all five deuterated hydrogen atoms in the C_8 and C_{10} positions. It is reasonable to hypothesize that the keto-enolic equilibrium is so rapidly reached at pH 1 that the TDN-d_4 is also produced from the precursor identified by WINTERHALTER (1991). Thus, a pH of 2 was chosen as a compromise to obtain a faster reaction of the precursor in this study, and simultaneously avoiding the tetradeuterated keto-enolic equilibrium during the first mechanism. This fact can be proved by the very low production of TDN-d_4 in the first phase of the observed reaction. Maximum levels of TDN and TDN-d_4 were reached at about 80 and 100 h, respectively (Fig. 6). Both compounds then decreased slowly, as shown previously for TDN in wine stored at 30 °C (MARAIS *et al.* 1992 b). Furthermore, the remarkably different values for the TDN/TDN-d_4 ratio at maximum formation (about 0.3 and 18 for the 1993 and 1976 wine, respectively) indicate different contents of the corresponding precursors in the wines. The induction period for the TDN formation from the precursor investigated in this study, suggested that likely TDN is firstly produced from the precursor of WINTERHALTER (1991) during wine ageing.

Conclusions

Specific NMR procedures enabled us to characterize the uncommon chemical structure of the aglyconic part of another glycosidic TDN precursor after suitable isolation, purification and fractionation procedures of conjugated aroma precursors in Riesling grape products. This precursor produced in D_2O at different acid pH values only a TDN-d_4 isomer, whose structure was proposed by means of MS fragmentation. The investigated kinetics of TDN and TDN-d_4 formation in D_2O (at pH 2 and 50 °C) from the raw heterosides of two differently aged Riesling wines support the hypothesis of the existence of at least two TDN precursors at different concentrations in wine. A rather close maximum production time of TDN at pH 2 was found for both reaction mechanisms.

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