Pergamon

Phytochemistry, Vol. 48, No. 8, pp. 1321–1328, 1998

Printed in Great Britain
0031-9422/98/$—see front matter

PII: S0031-9422(97)00847-9

METABOLISM OF THE TOMATO SAPONIN α-TOMATINE BY *GIBBERELLA PULICARIS*

KLAUS-MICHAEL WELTRING,* JUDITH WESSELS and GUIDO F. PAULI†

Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität, Schloßgarten 3, 48149 Münster, Germany; †Institut für Pharmazeutische Biologie und Phytochemie, Westfälische Wilhelms-Universität, Hittorfstr. 56, 48149 Münster, Germany

(*Received in final form* 18 *August* 1997)

Key Word Index—*Gibberella pulicaris*; *Fusarium*; fungi; metabolism; saponins; steroid alkaloids; tomatine; tomato.

Abstract—The ascomycete *Gibberella pulicaris* is able to metabolize the saponin α-tomatine within 2 h by first removing the complete lycotetraose moiety leading to the aglycone tomatidine. This product is further converted into 7α-hydroxy-tomatidine and the corresponding Δ^5-dehydro product 7α-hydroxy-tomatidenol. Structural evidence for the hydroxylated main metabolite as well as the unsaturated minor product is based on MS and NMR measurements. The latter include the application of modern soft pulse ^{1}H NMR techniques allowing the definitive localization of the position of hydroxylation even for the few milligram quantities that could be obtained.

INTRODUCTION

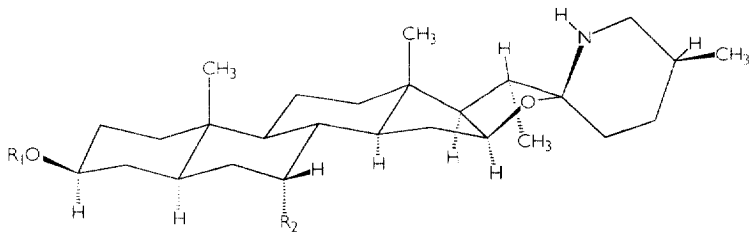
α-Tomatine (**1**) is a steroidal glycoalkaloid found in solanaceous plants, especially in a number of *Lycopersicon* and *Solanum* species [1]. It was first isolated and characterized almost 50 years ago as a fungal inhibitor from tomato leaf sap [2]. Besides its significant antifungal property, **1** like other saponins exhibits hemolytic and cholesterol binding activity [3]. The toxic potential is due to the disruption of sterol containing membranes by formation of complexes between the glycoalkaloids and membrane sterols [4, 5].

Both the antifungal activity and the significant accumulation of saponins in some plant tissues [6] has led to them being regarded as plant defence compounds called phytoanticipins [7, 8]. Proof of their function as deterrents in fungal infection has recently been provided by Osbourn et al. [9, 10] who studied the interaction between oat (*Avena sativa*) and the fungus *Gaeumannomyces graminis* var. *avenae*. The latter is able to metabolize and thereby detoxify the oat triterpenoid saponin avenacin by expression of the β-glucosidase avenacinase. This enzyme hydrolyzes β-1,2 and β-1,4-linked D-glucose molecules from the sugar moiety of avenacin [11, 12]. Mutants of *G. graminis* generated by transformation-mediated disruption of the avenacinase gene which were lacking the enzyme were no longer pathogenic on oat. However, they retained pathogenicity on wheat, which does not produce avenacin [9]. These results prove that the ability to detoxify a certain saponin can determine the host range of a fungus.

The metabolism of **1** has been studied in a variety of fungi pathogenic on tomato. *Fusarium oxysporum* f. sp. *lycopersici* [13] and *Botrytis cinerea* [14] are capable of hydrolysis of the complete sugar moiety leading to the aglycone tomatidine (**2**). *Alternaria* species also produce the aglycone by sequential release of all sugar molecules [15]. Only particular sugars such as β-1,3-linked L-xylose and β-1,2-D-glucose moieties are removed by *Botrytis cinerea* [16] and *Septoria lycopersici* [17] leading to β_1- and β_2-tomatine, respectively. In the latter case the enzyme, a 110-kDa protein with little or no activity on a variety of other glycosides, and the corresponding gene have been isolated [18]. In comparison to the enzyme of *S. lycopersici*, the tomatinase of *F. oxysporum* f. sp. *lycopersici* with a size of 50 kDA is much smaller [19], which indicates that fungi may have evolved different proteins for the detoxification of saponins.

In contrast to the knowledge of glycosidic cleavage, there are only few reports dealing with the hydroxylation of **2** by *Helicostylium piriforme*. The fungus is able to hydroxylate this substrate as well as the closely related steroids solasodine and diosgenin in positions 7α, 7β, and 11α [20–22] thereby improving the utility of these compounds as starting material for the production of steroid hormones. For the same reason the

* Author to whom correspondence should be addressed.



		R1	R2	
1	Xyl—^{3}(Glc—^{2}Glc)—^{4}Gal		H	α-tomatine
2		H	H	tomatidine
3		H	OH	7α-hydroxy-tomatidine
4		H	H	tomatidenole (Δ^5)
5		H	OH	7α-hydroxy-tomatidenole (Δ^5)

hydroxylation of progesterone by *Aspergillus* species [23, 24], *Rhizopus nigricans* [25], *Phycomyces blakesleeanus* [26], *Sepedonium ampullosporum* [27], *Botryospheria obtusa* [28] and *Cochliobolus lunatus* [29] has been studied. The hydroxylations are carried out by site selective cytochrome P-450 monooxygenases at various positions (6β-, 7α- and 7β-, 9α-, 11α- and 11β-, 14α-, 15α- and 15β-, 16α- and 17α-).

In this paper we report the metabolism of **1** by *Gibberella pulicaris* (anamorph *Fusarium sambucinum*) concerning both the deglycosidation and the modification of the steroid portion as part of our studies on the metabolism of saponins by this fungus. *G. pulicaris* has been isolated from soil and many plants, where it can cause root and seedling rots of cereals and storage rot of fruits and potatoes [30, 31]. In analogy to *F. oxysporum* f. sp. *lycopersici*, compound **1** is cleaved by *G. pulicaris* into the sugar moiety lycotetraose and **2**. The latter is further converted into 7α-hydroxy-tomatidine (**3**) and the corresponding Δ^5-unsaturated compound 7α-hydroxy-tomatidenol (**5**).

RESULTS AND DISCUSSION

The first result to be discussed is the time course of metabolism of **1**. This was monitored by TLC analysis in solvent system 1 using aliquots from a liquid culture of strain R-6380 in McIlvaine buffer supplemented with **1**. The substrate was metabolized within 2 h to give a product identical with **2** as shown by their identical R_f-values of 0.73 and molecular weight (*m/z* 416 $[M+H^+]$ due to $C_{27}H_{45}O_2N$). The aglycone **2** was then converted into a more polar product that could be detected after 4 h. The corresponding spot on TLC was centered at R_f 0.62 and was visualized as a deep blue color after spraying the pre-heated plate with 50% (v/v) H_2SO_4 in MeOH. Furthermore, this product was slowly metabolized to give at least three more compounds showing R_f-values of 0.54, 0.52, and 0.48, which were formed after about 8 h. All metabolites except **2** could still be detected after 48 h. In enzyme assays with crude protein extracts from culture fluid compound **1** was converted partially into **2**. No further metabolite could be detected up to 17 h of incubation.

Purification of the second, more polar metabolite was achieved by TLC-monitored preparative MPLC yielding 1.4 mg of the product from an incubation of 100 mg of **2** with *G. pulicaris*. The molecular weight of this compound was determined by DCI-MS: A base peak occurring at *m/z* 432 $[M+H^+]$ suggested the introduction of one equivalent of oxygen into its precursor **2**. This finding was supported by the occurrence of a typical hydroxylated carbon resonance at δ 68.25 in the ^{13}C NMR spectrum neighbouring the CH-OH signals of C-3 (δ 71.50) and C-16 (δ 79.89) as well as the bridgehead nucleus C-17 (δ 63.16). The shift value of δ 68.25 was indicative of an underivatized secondary alcoholic function. Attempts to assign the carbon resonances by comparison with literature data [32, 33, 34] only showed an excellent agreement for the carbons of rings E and F of the tomatidine skeleton, thus providing proof for the retention of the complete stereochemistry of this part of the molecule. Obviously, all of the 15 remaining CH and CH_2 resonances due to rings A–D falling into the narrow spectral window between δ 21 and 47 were affected by substituent chemical shifts indicating hydroxylation in a central position in either ring B or C. It also had to be recognized, that the MPLC fraction contained, in addition to the major component **3**, a second compound (**5**) giving rise to a set of weaker carbon resonances. Therefore due to practical limitations, such as the obtainable S/N ratio and lack of HMBC/HMQC data caused by the small sample available (1.4 mg), some carbon assignments of **3** given in the Experimental section remain tentative, although they support the final structure. On the other hand, the ^{13}C NMR and mass spectra results clearly indicated, that the main metabolite **3** consisted of a monohydroxyl-

ated derivative of **2** with the molecular formula $C_{27}H_{45}O_3N$.

The working hypothesis of **3** being a monohydroxylated **2** was sustained by the ^{1}H NMR spectrum. Comparison with the downfield resonances of **2** exhibited a prominent additional signal at δ 3.773 (named H-?eq in the following) due to a secondary alcohol function beside H-3ax (δ 3.527) and H-16α (δ 4.224). The splitting pattern showed only small J values clearly indicating the axial position of the adjacent hydroxy group located in either rings B or C. Therefore, the probable site of hydroxylation of **2** became either 6β-, 7α-, 11β-, or 12α-OH. Our further strategy to prove unequivocally the position of hydroxylation was directed towards the full exploration of the very complex proton spin system by applying 2D DQF-COSY, 2D 1-Step-Relay-COSY and TOCSY experiments, a methodology that for steroids only recently has been reported for *Xysmalobium undulatum* constituents [35]. The TOCSY spectra were performed as selective shaped pulse experiments in 1D mode allowing accumulation of all-in-phase subspectra with full digital resolution [36]. By application of increasing mixing times (t_{mix}) ranging from 5–140 ms, the relayed magnetization transfer could be incremented stepwise, hence allowing the subsequent identification of the coupling partners in α-position (t_{mix} = 5 ms, weak responses for β-protons), α- and β-positions (t_{mix} = 20 ms, weak responses for γ-protons), and α- through ε-positions (t_{mix} = 50–140 ms). As shown in Fig. 1, the 1D TOCSY experiments let us establish connectivity between H-3ax (δ 3.527) and H-?eq (δ 3.773) in the following manner: upon selective excitation of H-3ax, the *ddd* signal at δ 3.773 was the TOCSY response at t_{mix} = 50 ms, while at t_{mix} = 20 ms, only the signals for protons 1ax/1eq, 2ax/2eq, 4ax/4eq, 5, and (weak) 6ax/6eq were observed. Correspondingly, the signal of H-3ax was obtained in a t_{mix} = 100 ms TOCSY upon selective excitation of H-?eq at δ 3.773, whereas magnetization transfer was limited to protons 5, 6ax/eq, and 8 in the t_{mix} = 20 ms experiment. Thus, the newly introduced hydroxyl function was assigned to the 7α position and the signal at δ 3.773 was due to H-7eq (i.e. H-?eq). Interestingly, the essential connectivity relationship could not be established through a 2D 1-Step-Relay-COSY experiment. Although H-5 is representing the β-proton of both H-3ax and H-7eq, the cross peaks due to 1-step coherence transfer could not be distinguished because of the very close shifts of H-6ax and H-5. Accordingly, the resonance of H-5 could well be located through H-3ax, but the strong COSY correlation between H-7eq and H-6ax unfortunately overlapped with the relayed correlation H-7eq × H-5. Table 1 summarizes all relevant δ and J values of **3** proving that (i) the newly introduced secondary alcohol had three neighbouring protons, (ii) the important subsequent coupling positions were α-CH_2-6 and α-CH-8, β-CH-5, γ-CH_2-4 and δ-CH_{OH}-3ax, and (iii) the new substituent was in the axial position. Therefore, the structure of **3** was proved to be 7α-hydroxy-tomatidine.

Molecular mechanics calculations of **3** were performed and the dihedral angles of the minimized geometry were correlated with the ^{1}H coupling constants following the generalized Karplus relationship [37]. The mechanics clearly indicated the rigidity of the pentacyclic alkaloid skeleton in which the $^{22}C_{25}$-conformation was the preferred low energy conformation of ring E. This also was evident from the coupling pattern and constants of H-25 representing an axial proton (see Table 1). Finally, the measured J values showed good agreement with those from Karplus calculation after refinement of the coefficients to yield the equation $J = 6.3 - 0.9\cos\phi + 4.6\cos(2\phi)$. The probable low energy conformation of **3** is shown in Fig. 2.

As mentioned above, compound **3** was accompanied by a second metabolite (**5**) representing the minor component of the MPLC fraction (20% from the ^{1}H NMR integrals). Evidence for compound **5** was a prominent and well resolved *dd* signal at δ 5.456 indicating an unsaturated analogue. In accordance with a well known resolution problem in chromatography, the Δ^5-analogues have to be considered as impurities of isolated steroid alkaloids [32] and steroids [35]. In addition to the olefinic resonance, slightly shifted minor signals were observed for both H-3ax (*dddd* at δ 3.468 vs 3.527) and H-16α (*ddd* at δ 4.248 vs 4.224) as well as the introduced secondary alcohol geminal proton H-?eq (δ 3.779 vs 3.773). The latter was not only slightly deshielded, but also showed a considerably different splitting pattern. Proof for the structure of **5** being 7α-hydroxy-tomatidenol, the Δ^5-analogue of **3**, came from various directions (Fig. 3). Firstly, the 1-Step-Relay-COSY map showed a cross peak between H-3ax (δ 3.468) and the olefinic resonance at δ 6.456, while the latter exhibited regular COSY correlations with H-?eq, H-4ax/eq, and H-8. Consequently, there had to be a strong allylic coupling between H-4ax and the olefinic proton = H-6 (^4J = 1.9 Hz) which allowed the coherence transfer between H-3ax and H-?eq = H-7eq. Secondly, the signal of H-7eq at δ 3.779 was obtained in a t_{mix} = 140 ms TOCSY upon selective excitation of H-3ax.

It is noteworthy to mention a few special observations made during structure elucidation. The usual ^{1}H resonance behaviour in cyclohexane rings [δ(H-ax) < δ(H-eq)] is reversed in the case of the methylene protons 2H-6 as given in Table 1. This reversal is probably due to the introduction of a new asymmetric center in the α-position (C-7) giving rise to an anisotropic substituent chemical shift. Furthermore, Tables 1 and 2 carry the 2.2 Hz W-type couplings between H-2eq and H-4eq, indicating a high degree of planarity along the four bonds mediating this ^4J long-range coupling. Another long-range connectivity affects $H\text{-}6_{olef}$ and H-4ax. Finally, the full accordance of the 6 nuclei spectral simulation of H3···H-8 in **5** with the

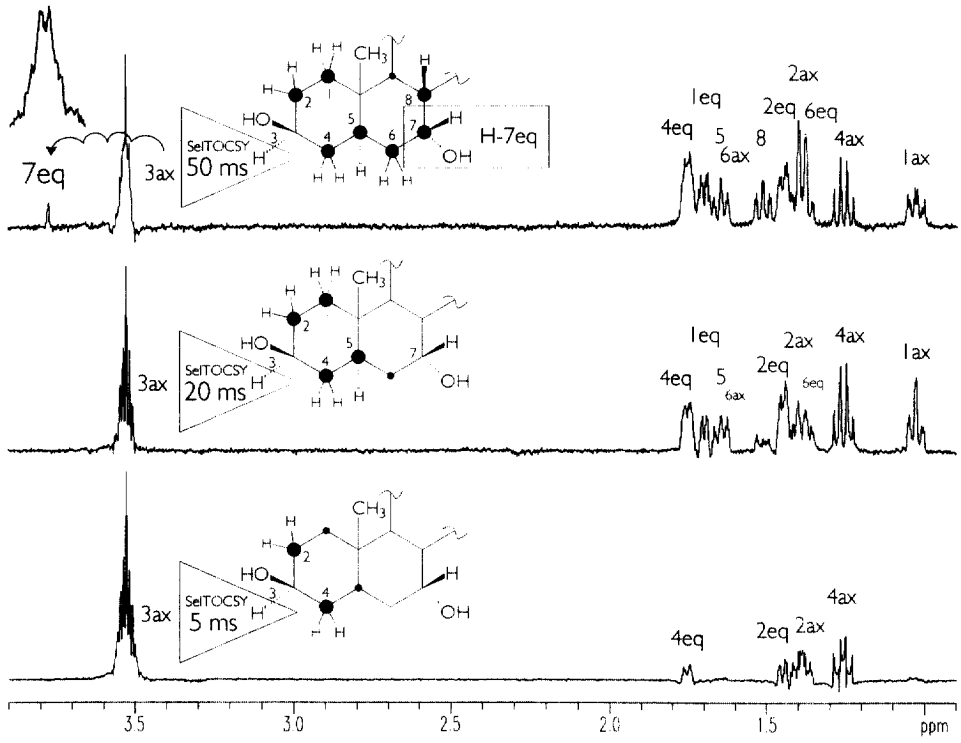


Fig. 1. Definitive proof for both stereochemistry and position of hydroxylation in **3** came from a series of 1D selective TOCSY experiments applying mixing times (t_{mix}) of 5, 20, and 50 ms. In case of the 50 ms delay, selective excitation of H-3ax led to the response of the newly introduced hydroxy function corresponding to the signal at δ 3.773 due to H-7eq. The stepwise increment of the relayed magnetization transfer (dots indicate the intensity) also allowed the subsequent identification of the protons involved in the spin system of rings A and B (see text for further details).

measured signals has to mentioned. The calculation included the homoallylic ^5J between H-4eq and H-7eq and allowed the refinement of the coupling constants as given in Table 2.

In conclusion, our findings have to be discussed in relation to the few reports on the metabolism of **1** by microorganisms. Up to now, observations are limited to modifications and/or removal of the sugar residues and only deal with fungi that are pathogenic on tomato plants. The reactions carried out by these fungi are thought to be detoxification mechanisms allowing these organisms to overcome this chemical defence barrier in tomatoes [38, 39]. *G. pulicaris* has occasionally been isolated from ripe tomatoes [30, 31], which do not contain detectable amounts of **1**. Nevertheless, the fungus readily metabolizes the saponin into the aglycone and the lycotetraose, which is the same mechanism found in *F. oxysporum* f. sp. *lycopersici*. Interestingly, the structurally closely related saponins α-chaconine and α-solanine found in potatoes are metabolized by *G. pulicaris* by removing single sugar molecules from their side chains [40]. In the case of α-chaconine, this also leads to the production of the aglycone solanidine, whereas α-solanine is only converted to the solanidine-galactoside. Consequently, the galactosidase hydrolyzing the bond between **2** and the sugar moiety is not able to cleave the bond between solanidine and the attached galactose. This indicates that the saponinases of *G. pulicaris* involved in the metabolism of different saponins might be as substrate specific as the tomatinase of *S. lycopersici* and the avenacinase of *G. graminis* [18, 41].

Like the other saponin metabolizing enzymes looked at so far, the tomatinase of *G. pulicaris* cleaving **1** into the aglycone and the lycotetraose is secreted into the medium, as shown by the conversion of the substrate by a protein extract of the culture fluid. In this experiment the aglycone is the only product. This could indicate that the 7α-hydroxylation is carried out by a membrane bound cytochrome P-450 monooxygenase as is the case in the fungal hydroxylation of progesterone. Results obtained with this substrate suggest that the monooxygenases involved might be position specific as indicated for example by inhibition experiments with microsomal preparations of *A. fumigatus* catalyzing the 7β- and 11α/15β-hydroxylation of this steroid [24]. Attempts are under way to isolate the hydroxylase and the corresponding gene of *G. pulicaris* in order to study its specificity and regulation. In addition, the conversion of the monohydroxylated metabolites into several unknown products will prompt us to investigate further the metabolism of **1**.

Table 1. Proton NMR data of compound **3** (600 MHz, CD_3OD) extracted from regular 1D ^1H NMR spectra, 1D Selective TOCSY and 2D [DQF-]COSY/RELAY-COSY experiments and aided by spectral simulation calculations

Proton	δ [ppm]	Multiplicity	J [Hz] (coupling proton)
1eq	1.701	*ddd*	2.5 (2eq), 4.3 (2ax), 12.8 (1ax)
1ax	1.030	*ddd*	3.7 (2eq), 13.1 (1eq), 13.5 (2ax)
2eq	1.450	*ddddd*	2.2(4eq!), 2.5 (1eq), 3.7 (2eq), 4.8 (3ax), 13.1 (2ax)
2ax	1.389	*dddd*	4.3 (1eq), 11.2 (3ax), 13.1 (2eq), 13.5 (1ax)
3ax	3.527	*dddd*(=*tt*)	4.8 (2eq & 4eq), 11.2 (2ax & 4ax)
4eq	1.753	*dddd*	2.2 (2eq!), 3.7 (5), 4.8 (3ax), 12.8 (4ax)
4ax	1.264	*ddd*	11.2 (3ax), 12.8 (4eq), 13.8 (5)
5	1.646	*ddd*	3.0 (6eq), 3.7 (4eq), 13.1 (6ax), 13.8 (4ax)
6eq	1.393	*ddd*	2.9 (7), 3.0 (5), 13.8 (6ax)
6ax	1.614	*ddd*	2.9 (7), 13.1 (5), 13.8 (6eq)
7eq	3.773	*ddd*(=*q*)	2.9 (6eq, 6ax, 8)
8	1.515	*ddd*(=*dt*)	2.9 (7), 13.5 (9, 14)
14	1.500		
15a = α	2.115		
15b = β	1.275		
16	4.224	*ddd*	7.9 (15a), 6.7 (15b), 7.3 (17)
17	1.705		
18 (CH_3)	0.841	*s*	
19 (CH_3)	1.018	*s*	
20	1.83	*dq*	7.0 (17, 21)
21	0.993	*d*	7.0 (20)
25	1.595		
26eq	2.742	*m*	
26ax	2.688	*t*	10.6 (26eq, 25)
27 (CH_3)	0.864	*d*	6.7 (25)

EXPERIMENTAL

Strains and media

G. pulicaris strain R-6380 isolated from potato was obtained from A. Desjardins [42]. For long term storage and to produce spores the fungus was grown on V-8 juice agar [43]. Liquid shake cultures were grown by incubating spores from a 7–10-day-old agar plate in 100 ml Mantle medium containing 10 g glucose instead of 100 g sucrose [44] for 2 days at 26° on a rotary shaker.

Compounds **1** and **2** were purchased from Fluka. For metabolism assays **1** was dissolved in 10% (v/v) HOAc at a concentration of 100 μg μl^{-1} and added to 20 mM McIlvaine buffer (NaH_2PO_4, Na-citrate, pH 5.0) at a final concentration of 50 μg ml^{-1}. Compound **2** was dissolved in DMSO–$CHCl_3$ (3:1) at a concentration of 5 mg ml^{-1} and added to 150 ml 20 mM McIlvaine buffer at a final concentration of 66 μg ml^{-1}.

Metabolism assays

Mycelium from liquid culture was separated from the culture fluid by filtration through a nylon membrane and washed 2 × with 20 mM McIlvaine buffer. The time course of metabolism of **1** was monitored by incubating 1 g mycelium in 10 ml McIlvaine buffer containing 50 μg ml^{-1} substrate at 26° on a rotary shaker and taking 1 ml samples at various time points. After making the samples alkaline with NH_4OH, the remaining substrate and the products were extracted with EtOAc, separated on a TLC plate, which was developed in solvent system 1 consisting of EtOAc–HOAc–MeOH–H_2O (30:20:10:1), and visualized under UV-light (365 nm) after spraying the TLC plate with 50% (v/v) H_2SO_4 and incubation at 110° for 5–10 min.

Protein extract of culture medium

Proteins from 1 l culture filtrate were precipitated by cooling on ice and adding NH_4SO_4 to a final concentration of 90%. After centrifugation for 30 min at 12,000 *g* the pellet was resuspended in 8 ml 20 mM McIlvaine buffer and dialyzed at 4° overnight against H_2O. The sample was lyophilized and resuspended in 1.5 ml 20 mM McIlvaine buffer. 10 μl of this protein solution was added to 1 ml McIlvaine buffer containing 50 μg **1**. After incubation at 26° for 4 and 17 h the enzyme assay was alkalized with NH_4OH, extracted with EtOAc, and analyzed by TLC in solvent system 1.

Characterization of metabolites

For MS analysis the metabolites were extracted with EtOAc from 500 ml McIlvaine buffer incubated with 50 g fr. wt of mycelium and 50 mg **1** for 24 h at 26° on a rotary shaker. The extract was concentrated *in vacuo* and applied to preparative TLC-plates. The products were eluted with $CHCl_3$ from the silica gel, dried under a stream of N_2, and subjected to DCI-MS analysis with NH_3 as a reactant gas using a Finnigan MAT 44S instrument. The same instrumentation was applied for the pure samples described in the following.

In order to obtain sufficient amounts for NMR structure determination analysis, larger quantities of the hydroxylated steroidal metabolites were produced by incubating ten 500 ml Erlenmeyerflasks each containing 10 g fr. wt mycelium in 150 ml McIlvaine buffer and 10 mg **2** for two days at 26° on a rotary

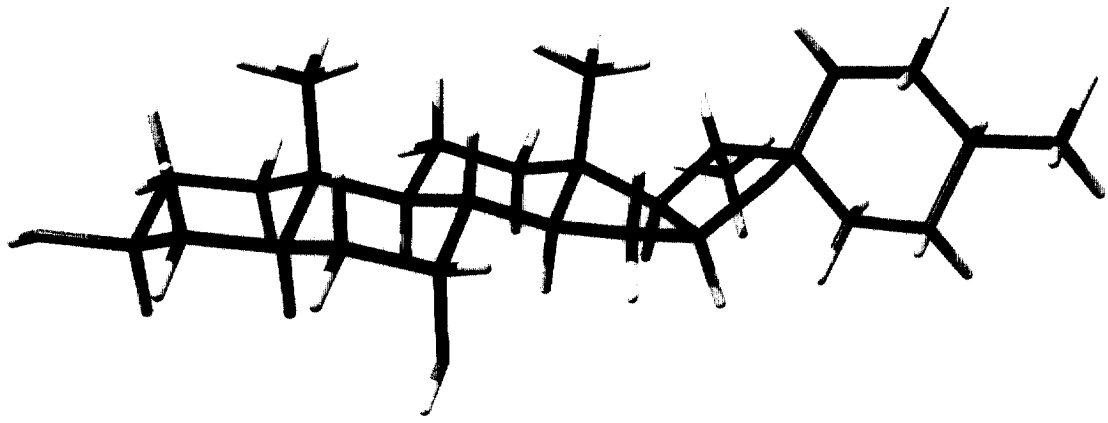


Fig. 2. Conformational view of 7α-hydroxy-tomatidine (**3**) resulting from torsionforcing calculations and supported by the Karplus dihedral relationships derived from H,H coupling patterns (calc. see text).

shaker. The culture filtrate was alkalized with NaOH (pH 12) and partitioned 3 times with EtOAc. The combined organic layers were evaporated to dryness *in vacuo* yielding 45 mg of an oily residue. TLC analysis in solvent system 2 (*n*-hexane : EtOAc-25% : NH_3 (10 : 90 : 1) upon detection with 50% (v/v) methanolic H_2SO_4 mainly revealed one intensive but diffuse spot at R_f 0.20 much more polar than **2** (R_f 0.52). Preparative purification by MPLC was carried out on a self-made glass column (14 × 500 mm) packed with 24 g LiChroprep 60 RP-18, 15–25 μm particle size using Waters 510 pumps. UV detection at 207 nm was applied in order to record impurities and metabolites with weak chromophores such as double bonds. A continuous solvent gradient from 35–100% aq. MeCN formed by a system of corresponding vessels as described in Ref. [45] was used at a flow rate of 6.0 ml min^{-1} and 15 ml fractions were collected. The latter were additionally monitored by TLC in solvent system 2 where compound **3** (R_f 0.22) eluted between 53–56% MeCN and represented the main component of the combined fractions 18–20 (containing 20% of **5**) yielding 1.4 mg of an amorphous white powder upon evaporation. This material was subjected to NMR and MS analysis.

For NMR-spectroscopy, the samples were taken up

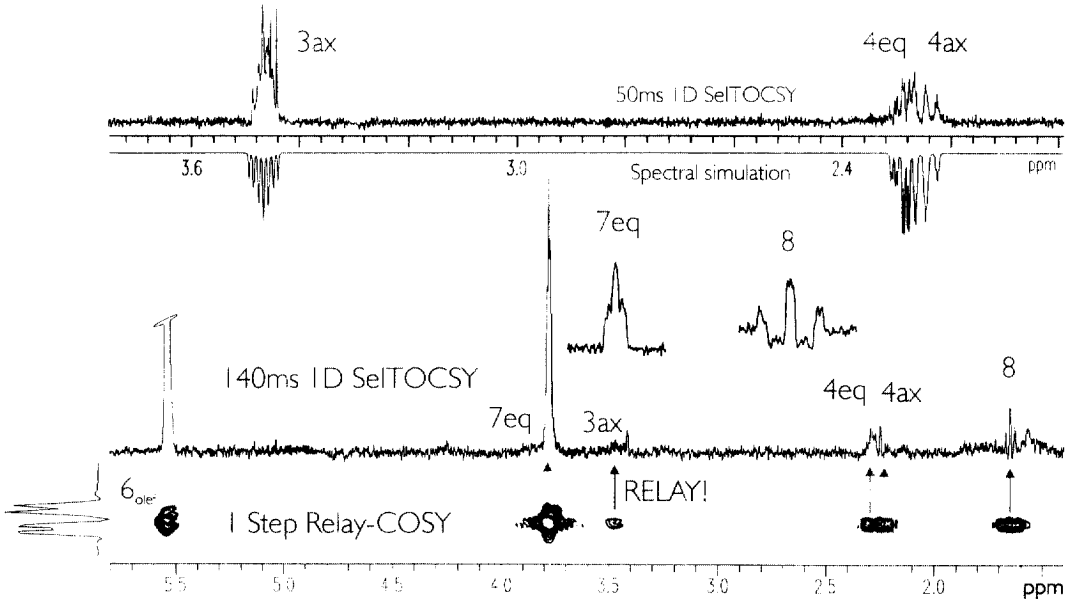


Fig. 3. Identification of 7α-hydroxy-tomatidenol (**5**) as the Δ^5 unsaturated minor metabolite of α-tomatine. Structural proof was obtained using only 300 μg of sample from a 1-step Relayed COSY linking H-3ax with H-7eq and from 1D selective TOCSY experiments elucidating the proton spin systems of rings A and B. Upon selective excitation of H-6_{olef} (t_{mix} = 50 ms), the response signals were due to both the metabolic H-7ax and the genuine H-3eq OH function. Spectral simulations were carried out to refine the *J* values in Table 2 and to support the assignment of the very close AB-type resonances of the methylene protons 2H-4 (upper slices).

Table 2. Proton NMR data of compound **5** (600 MHz, CD_3OD) extracted from regular 1D ^1H NMR spectra, 1D Selective TOCSY and 2D [DQF-]COSY/RELAY-COSY experiments and aided by spectral simulation calculations

Proton	δ [ppm]	Multiplicity	J [Hz]
1eq	1.85		
1ax	1.147	*ddd*	3.7 (2eq), 13.1 (2ax), 13.8 (1eq)
2eq	1.76		
2ax	1.40		
3ax	3.468	*dddd*	4.8 (2eq & 4eq), 11.2 (2ax & 4ax)
4eq	2.292	*ddd*	2.1 (2eq!), 5.1 (3ax), 13.1 (4ax)
4ax	2.249	*dddd*	1.5 (7eq!), 1.9 (6!), 11.2 (3ax), 13.1 (4eq)
6	5.639	*dd*	1.9 (4ax!), 5.3 (7eq)
7eq	3.779	*dddd*	0.5 (4eq!), 1.5 (4ax!), 3.8 (8), 5.1 (6)
8	1.630	*ddd(=dt)*	4 (7), 12 (9, 14)
16	4.248	*ddd*	7.9 (15a), 6.7 (15b), 7.3 (17)
17	1.705		
18 (CH_3)	0.890	*s*	
19 (CH_3)	0.962	*s*	
20	1.83	*dq*	7 (17, 21)
21	0.988	*d*	7.0 (20)
25	1.595		
26eq	2.742	*m*	
26ax	2.688	*t*	10.6 (26eq, 25)
27 (CH_3)	0.877	*d*	6.7 (25)

in methanol-d_4 with an isotopic purity of 99.8% D (Aldrich, Milwaukee, no. 15194) by first dissolving them in 0.2 ml of the solvent and clearing the concentrated soln by multiple filtration. More solvent was then added to the final sample volume of 0.7 ml corresponding to a filling height of 40 mm in 5 mm tubes (Wilmad 528-7).

The ^1H NMR spectra were recorded on Bruker AM360 (5mm dual probe) and Varian Unity 600 (5mm multi nuclear probe) instruments operating at 360/600 MHz for ^1H, and 90/150 MHz for ^{13}C, respectively. Chemical shifts are reported in ppm on the δ scale with the solvent as internal standard (δ 3.300 and δ 49.00, resp.), the coupling constants (*J*) are given in Hz. The spectra were obtained under the following conditions: ^1H 30° pulse, delay after acquisition (D1) 1.5 s, processed with 0.2 Hz line broadening (LB) or with Lorentz-Gauss resolution enhancement and zero-filling. Acquisition using 16k (SI) (32k at 600 MHz) data points in a spectral window of ca δ 6 yielded a digital resolution better than 0.2 Hz (or δ 0.0004). Water peak suppression was achieved by presaturation setting the HDO signal on resonance (O1). ^1H 2D COSY SW δ 6, D 1.2 s, 1k × 512 or 256 increments, 90° shifted sinebell-squared apodization, zero-filled in t_1 dimension during processing. The 1D Selective TOCSY experiments were performed at 600 MHz using the eburp1/25 selective pulse shape program (pws = 200, trimpwr = 54) and acquiring 16K data points (sw = 3900 Hz, aq = 1.9 sec). Offline data processing was done with the manufacturers NMR data processing software (Varian VNMR and Bruker DISNMR and WinNMR packages). Molecular mechanics calculations (dynamics and minimization) were performed using the MOBY program (DOS version) of Dr U. Höweler, Münster.

Physical data

7α-Hydroxy-tomatidine (**3**) $C_{27}H_{45}O_3N$ [purity approx. 80%, containing 20% 7α-Hydroxy-tomatidenol (**5**) see text]; R_f 0.22 in solvent system 2. UV: no absorption above 200 nm. DCI-NH_3-MS *m/z* (rel. int.): 432 (100) $[M+H]^+$, 414 (7) $[M-H_2O+H]^+$, 396 (7) $[M-2H_2O+H]^+$; ^1H NMR: see Table 1; ^{13}C NMR (CD_3OD, 90 MHz, ambiguous assignments are marked [a]): δ 37.68[a] (C-1), 31.76 (C-2), 71.50 (C-3), 38.20[a] (C-4), 40.23[a] (C-5), 32.56 (C-6), 68.25 (C-7), 37.95[a] (C-8), 46.70 (C-9), 35.64 (C-10), 21.70 (C-11), 40.59 (C-12), 41.68 (C-13), 50.59 (C-14), 37.53[a] (C-15), 79.89 (C-16), 63.16 (C-17), 17.10 (C-18), 11.63 (C-19), 43.50 (C-20), 15.94 (C-21), 99.66 (C-22), 27.34 (C-23), 28.96 (C-24), 31.36 (C-25), 50.49 (C-26), 19.95 (C-27).

Acknowledgements—Financial support from Fonds der Chemischen Industrie, Frankfurt am Main (G.F.P.) and Deutsche Forschungsgemeinschaft (J.W. and K.M.W.) is gratefully acknowledged. We are also grateful to Dr Volker Kluy, Düsseldorf, for helpful discussions.

REFERENCES

1. Roddick, J. G., *Phytochemistry*, 1974, **13**, 9.
2. Fontaine, T. D., Irving, G. W., Ma, R. M., Poole, J. B. and Doolittle, S. P., *Archives of Biochemistry*, 1948, **18**, 467.
3. Price, K. R. and Fenwick, G. R., *CRC Critical Reviews in Food Science and Nutrition*, Vol. 26, ed T. E. Furia. CRC Press, Inc., Boca Raton, FL, 1987, p. 27.
4. Keukens, E. A. J., de Vrije, T., Fabrie, C. H. J. P., Demel, R. A., Jongen, W. M. F. and de Kruijff, B. E., *Biochimica et Biophysica Acta*, 1992, **1110**, 127.
5. Keukens, E. A. J., de Vrije, T., van den Boom, C., de Waard, P., Plasman, H. H., Thiel, F., Chupin, V., Jongen, W. M. F. and de Kruijff, B., *Biochimica et Biophysica Acta*, 1995, **1240**, 216.
6. Jadhav, S. J., Sharma, R. P.and Salunkhe, D. K., *Critical Reviews in Toxicology*, 1981, **11**, 21.
7. Arneson, P. A. and Durbin, R. D., *Phytopathology*, 1968, **58**, 536.
8. Vanetten, H. D., Mansfield, J. W., Bailey, J. A. and Farmer, E. E., *The Plant Cell*, 1994, **6**, 1191.

9. Bowyer, P., Clarke, B. R., Lunness, P., Daniels, M. J. and Osbourn, A. E., *Science*, 1995, **267**, 371.
10. Osbourn, A. E., Clarke, B. R., Lunnes, P., Scott, P. R. and Daniels, M. J., *Physiological and Molecular Plant Patholgy*, 1994, **28**, 187.
11. Crombie, W. M. L., Crombie, L., Green, J. B. and Lucas, J. A., *Phytochemistry*, 1988, **25**, 2075.
12. Osbourn, A. E., Clarke, B. R., Dow, J. M. and Daniels, M. J., *Physiological and Molecular Plant Patholgy*, 1991, **38**, 301.
13. Ford, J. E., McCance, D. J. and Drysdale, R. B., *Phytochemistry*, 1977, **16**, 545.
14. Verhoeff, K. and Liem, J. I., *Phytopathologische Zeitschrift*, 1975, **83**, 333.
15. Schlösser, E., *Acta Phytopathologica*, 1975, **10**, 77.
16. Quidde, T. and Tudzynski, P., *Fungal Genetics Newsletter*, 1996, **43B**, 40.
17. Arneson, P. A. and Durbin, R. D., *Phytopathology*, 1967, **57**, 1358.
18. Sandrock, R. W., DellaPenna, D. and VanEtten, H. D., *Molecular Plant-Microbe Interactions*, 1995, **8**, 960.
19. Lairini, K., Perez-Espinosa, A., Pineda, M. and Ruiz-Rubio, M., *Applied and Environmental Microbiology*, 1996, **62**, 1604.
20. Sato, Y. and Hayakawa, S., *Journal of Organic Chemistry*, 1963, **28**, 2739.
21. Sato, Y. and Hayakawa, S., *Journal of Organic Chemistry*, 1963, **28**, 2742.
22. Sato, Y. and Hayakawa, S., *Journal of Organic Chemistry*, 1964, **29** 198.
23. Ghosh, D. K. and Samanta, T. B., *Journal of Steroid Biochemistry*, 1981, **14**, 1063.
24. Smith, K. E., Ahmed, F., Williams, R. A. D. and Kelly, S. L., *Journal of Steroid Biochemistry and Molecular Biology*, 1994, **49**, 93.
25. Breskvar, K., and Hudnik-Plevnik, T., *Journal of Steroid Biochemistry*, 1981, **14**, 395.
26. Smith, K. E., Latif, S. A., Kirk, D. N., *Journal of Steroid Biochemistry and Molecular Biology*, 1991, **38**, 249.
27. Smith, K. E., White, K. A. and Kirk, D. N., *Journal of Steroid Biochemistry*, 1989, **33**, 81.
28. Smith, K. E., Latif, S. A. and Kirk, D. N., *Journal of Steroid Biochemistry*, 1989, **33**, 927.
29. Vitas, M., Smith, K., Rozman, D., and Komel, R., *Journal of Steroid Biochemistry and Molecular Biology*, 1994, **49**, 87.
30. Harwig, J., Scott, P. M., Stoltz, D. R. and Blanchfield, B. J., *Applied and Environmental Microbiology*, 1979, **38**, 267.
31. Domsch, K. H., Gams, W. and Anderson, T.-H., *Compendium of Soil Fungi*, Vol. 1. Academic Press, London, New York, 1980, p. 361.
32. Kluy, V., PhD. Thesis, 1994. Heinrich Heine-Universität, Düsseldorf.
33. Radeglia, R., Adam, G. and Rippberger H., *Tetrahedron Letters*, 1977, **11**, 903.
34. Weston, R. J., Gottlieb, H. E., Hagaman, E. W. and Wenkert, E., *Australian Journal of Chemistry*, 1977, **30**, 917.
35. Pauli, G. F. and Schiller, H., *44th Annual Meeting of the Society for Medical Plant Research*, September 3–7, Prague, ed. T. Vanek and I. Valterova. Acad. Sci. Czech. Repub., Prague, 1996, p. 113.
36. Pauli, G. F., Nauman, M. and Fischer, N. H., *Spectroscopy Letters*, 1995, **28**, 903.
37. Karplus, M., *Journal of the American Chemical Society*, 1963, **85**, 2870.
38. Osbourn, A. E., *The Plant Cell*, 1996, **8**, 1821.
39. VanEtten, H. D., Sandrock, R. W., Wasmann, C. C., Soby, S. D., McCluskey, K. and Wang, P., *Canadian Journal of Botany*, 1995, **73**, 518.
40. Weltring, K.-M., Wessels, J. and Geyer, R., *Phytochemistry*, 1997, **46**, 1005.
41. Osbourn, A. E., Bowyer, P., Lunness, P., Clarke, B., Daniels, M., *Molecular Plant-Microbe Interactions*, 1995, **8**, 971.
42. Beremand, M. N., Desjardins, A. E., Hohn, T. M. and VanMiddlesworth, F. L., *Phytopathology*, 1991, **81**, 1452.
43. Stevens, R. B. *Mycology Guidebook*. University of Washington Press, Seattle, 1974, p. 703.
44. Mantle, P. G. and Nisbet, L. J., *Journal of General Microbiology*, 1976, **93**, 321.
45. Pauli, G. F., PhD. Thesis, 1993. Heinrich Heine-Universität, Düsseldorf.