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Chemical and biological modification of cynaropicrin and grosheimin: a structure–bitterness relationship study

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Abstract: This work is part of a plan to investigate the structure–bitterness relationship in sesquiterpene lactones of dietary origin. The major guaianolides from artichoke (*Cynara scolimus* L) were chosen for this study because of their exceedingly bitter taste and well-proven safety at concentrations currently employed in alcoholic beverages. Moreover they are available from horticultural left-overs and amenable to a wide range of chemical modifications. We isolated cynaropicrin and grosheimin from artichoke leaves and used either chemical modification or bioconversion by basidiomycetes to prepare a number of derivatives which were submitted to a panel test for sensory evaluation. Bitterness variations appeared to be related to changes in molecule polarity. Bitter taste was markedly abated by either the loss of exomethylenes or the opening of the lactone ring.

Keywords: bitter taste; artichoke; cynaropicrin; grosheimin; basidiomycetes

INTRODUCTION

Vegetable foods and drinks that contain such bitter compounds (BCs) as isoflavones, polyphenols, catechins and flavonoids, may be found unpalatable by consumers. Food industries have tackled the problem by removing BCs, at least partially, using a variety of processes such as plant breeding and filtration, or by adding substances that mask the undesirable taste. Bitterness may also be an indicator of spoilage, eg in dairy products when bitter peptides are produced by the proteolysis of casein.[1] However, bitterness is desired in certain kinds of foods and beverages, whether BCs are originally present (as naringin in grapefruit and other citrus juices) or added to spike up the flavour (as quinine to soft drinks).

From comparison of a large number of alkaloids, peptides and glycosides, structural requisites for a compound to taste bitter have been identified in an electrophilic group (often an ammonium group) and a hydrophobic side chain.[2]

The perception of bitterness involves one of the most complex transduction mechanisms. It has been ascertained that different taste modalities, formerly held to reside in different zones of the tongue surface, are distributed over the whole population of taste buds.[3] Within each bud, however, they are thought to be encoded in different types of taste receptor cells (TRCs), each of which expresses different receptor genes.[4] Most (though probably not all) taste receptors are formed by a few specific families of G-protein-coupled receptor molecules (GPCRs), found in different associations on the plasma membrane of each TRC type. Bitter tastants are detected by a family of about 30 GPCRs (the T2Rs),[5–7] most of which are co-expressed in the same subset of TRCs,[5] suggesting that these cells are capable of responding to a broad array of bitter compounds. More than one transduction system for bitter stimuli must be involved, because intercorrelation of individual sensitivities to different chemical classes of bitter tastants has shown that they fall into two non-exhaustive general groups.[8]

Sesquiterpene lactones, that function as repellents for mammalian herbivores and show a pleiotropic pattern of biological activities *in vitro*,[9] qualify as appropriate leads for a study of structure–bitterness relationships thanks to a combination of intense bitterness (surpassing alkaloids like caffeine and quinine)[10] and proven safety at current concentrations in bitter alcoholic beverages. Among them, guaianolides from artichoke (*Cynara scolimus* L), available in bulk from horticultural left-overs, are easily amenable to

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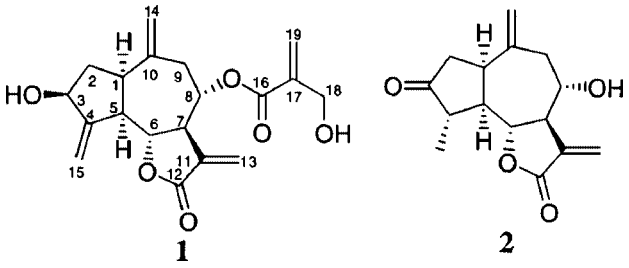


Figure 1. Cynaropicrin (**1**) and grosheimin (**2**).

chemical modifications as they are polyfunctional compounds.

Artichoke (*Cynara scolymus* L, Compositae) is widely cultivated in Mediterranean countries, particularly in Italy, the sprout being consumed as a vegetable. The alcoholic extract of the leaves, currently used for the production of bitter liqueurs (about 10 g of dried leaves per litre), has been documented from old as a traditional folk remedy for dyspeptic disorders. In clinical trials the leaves extracts have proved effective against the irritable bowel syndrome,[11] hyperlipoproteinaemia[12] and hyperlipidaemia.[13] They also exert a choleretic effect[14] and, owing to their high content in polyphenols (cynarin, caffeic acid, chlorogenic acid, luteolin), they possess a marked antioxidant activity.[15] Cynaropicrin (**1**), the major guaianolide found in the extracts, contributes approximately 80% to the bitter taste,[16] followed by grosheimin (**2**), present in much smaller amounts (Fig 1).

In the present study we used both chemical and microbiological transformations to prepare a number of cynaropicrin and grosheimin derivatives, which we submitted to sensorial evaluation in order to ascertain how selected structural changes would affect taste.

Chemical modifications such as esterification (giving compound **6**) and lactone ring opening (giving compound **14**) were promoted by microwave irradiation, a technique that can operate without solvent on reagents either neat or supported on an inert substrate,[17] usually achieving better yields in much shorter reaction times.[18]

The ability of fungi to metabolize foreign substrates is also of considerable interest in organic chemistry because bio-reactions proceed under mild conditions and show a high degree of regio- and/or stereospecificity.[19,20] We investigated the biotransformations of cynaropicrin and grosheimin by three selected basidiomycetes (*Collybia velutipes, Trametes hirsuta* and *Schyzophyllum commune*) in the hope of isolating products that could not easily be obtained by chemical modification.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Carlo Erba Reagenti (Rodano-MI, Italy) and Acros Organics (Geel, Belgium); pure cynaropicrin and grosheimin were isolated from the extract of *Cynara scolimus* L. Reactions were monitored by TLC on Fluka (St Louis, MO, USA) F_{254} (0.25 mm) plates, which were visualized by UV inspection or by spraying with a molybdic acid solution and heating. Silica gel Merck 60 (Whitehouse Station, NJ, USA) was used for column chromatography (CC). Melting points (uncorrected) were determined on a Büchi SMP-20 instrument (Assago-MI, Italy). IR: spectra were obtained on a Shimadzu FT-IR 8001 spectrophotometer (Duisburg, Germany). NMR: spectra were obtained on a Bruker 300 Advance instrument (Milano, Italy) (300 MHz and 75 MHz for ^{1}H and ^{13}C, respectively). For ^{1}H NMR, $CDCl_3$ and CD_3OD were used as solvents, $CHCl_3$ at $\delta = 7.27$, CD_3OH at $\delta = 3.31$ as reference. For ^{13}C NMR, $CDCl_3$ and CD_3OD were as solvents, $CDCl_3$ at $\delta = 76.9$, CD_3OD at $\delta = 49.0$ as reference. Chemical shifts (δ) are given in ppm, coupling constants (J) in Hertz. Low-resolution mass spectra (LRMS) were obtained on a Finnigan-MAT TSQ70 instrument (Thermo Electro Corporation, Rodano-MI, Italy) in chemical ionization mode with isobutane as reactant gas. HPLC analysis was on a Thermo-Quest Spectra Series P200 (Bremen, Germany) and Gilson 305 set-up (Middleton, WI, USA) with UV/VIS Jasco 875-UV (Easton, MD, USA) and Gilson 133 refractive index detectors and a Millipore 740 Waters (Milford, MA, USA) integrator. Microwave-promoted reactions were carried out in a modified domestic oven (Candy MSA 20M, Brugherio-MI, Italy).

Mycelia were obtained from the Department of Plant Biology, University of Turin, Italy.

Isolation of cynaropicrin and grosheimin from dry artichoke leaves

Dry artichoke leaves (500 g) were pulverized in a blender and extracted three times with acetone. The extract was concentrated under vacuum to about 50 ml, diluted with 500 ml of ethanol and treated with 3% lead diacetate (500 ml) to precipitate chlorophyll. After standing for 4 h, the mixture was filtered on a Buchner funnel and the filtrate concentrated under vacuum to half the original volume, diluted with water (100 ml) and extracted twice with ethyl acetate (250 ml each). The organic phase was dried over anhydrous Na_2SO_4 and evaporated, yielding 43 g of raw material; one-fifth of this was purified by silica gel CC using hexane/EtOAc as eluent (gradient 9:1 to 6:4) yielding cynaropicrin 3.6 g (41.9%), grosheimin 0.2 g (2.3%) and 0.4 g of their mixture (4.7%).

Synthesis

Diacetylcynaropicrin (3)

In a 25-ml two-necked round-bottomed flask, equipped with a magnetic stirrer and a nitrogen inlet, cynaropicrin (196 mg, 0.56 mmol), acetic anhydride (0.54 ml, 5.72 mmol) and a catalytic amount of *N*,*N*-dimethylaminopyridine (DMAP) were dissolved in anhydrous pyridine (8 ml). The mixture was stirred

for 5 h under nitrogen at room temperature and the reaction monitored by TLC (eluent hexane/EtOAc 1:1, R_f **3** = 0.55). For work-up, the reacted mixture was diluted with EtOAc, washed with cold 5% HCl, with $NaHCO_3$ and with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. CC (eluent hexane/EtOAc 9:1) yielded 170 mg of **3** (70%)[21,22] as a yellow oil; IR (liquid film, cm^{-1}): ν = 1778, 1456, 1404, 1374, 1269, 1045; ^1H NMR ($CDCl_3$): δ = 5.58 (1H, br t, $\jmath$ = 7.1 Hz, H-3α and H-15, overlapped), 4.86 (2H, br s, H-18), 2.11 (6H, br s, 2 Ac); ^{13}C NMR ($CDCl_3$): δ = 170.6 (C-1′), 170.1 (C-1′), 168.7, 164.2, 146.9, 141.1, 137.2, 135.0, 129.1, 122.5, 118.5, 116.1, 77.8, 74.5, 74.3, 62.2, 51.6, 47.4, 45.5, 37.0, 36.2, 21.1 (C-2′), 20.7 (C-2′); CIMS *m/z* 431 $[M+1]^+$ $[C_{23}H_{26}O_8+H]^+$.

*Disuccinylcynaropicrin (**4**)*
Using the same procedure, 566 mg of succinic anhydride (5.72 mmol) were added instead of the acetic anhydride. After stirring for 20 h the reaction was monitored by TLC (eluent $CHCl_3$/methanol 9:1, R_f **4** = 0.50). For work-up, the reacted mixture was diluted with EtOAc, washed with cold 5% HCl, H_2O and brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. CC (eluent $CHCl_3$/methanol 98:2) yielded 156 mg of **4** (51%) as a yellow oil; IR (liquid film, cm^{-1}): ν = 1732, 1126, 1074, 964, 912; ^1H NMR ($CDCl_3$): δ = 5.62 (1H, br t, $\jmath$ = 7.0 Hz, H-3α, overlapped to H-15 and H-18), 5.51 (2H, br s, H-18), 2.67 (8H, m, 2 H-2′ and 2 H-3′); ^{13}C NMR ($CDCl_3$): δ = 176.7, 176.4, 174.3, 171.6, 167.2, 166.5, 149.9, 143.8, 140.0, 139.7, 128.7, 123.1, 119.2, 116.1, 79.9, 76.8, 76.1, 60.5, 53.5, 47.4, 42.4, 37.7, 36.0, 30.7, 30.4, 30.1, 29.0; CIMS *m/z* 529 $[M-H_2O+1]^+$ $[C_{27}H_{30}O_{12}—H_2O+H]$.

*Dibutyrylcynaropicrin (**5**)*
Using the same procedure, 1 ml of butyric anhydride (5.72 mmol) was added instead of the acetic anhydride. The reaction was monitored by TLC (eluent hexane/EtOAc 8:2, R_f **5** = 0.30). CC (eluent hexane/EtOAc 9:1) yielded 114 mg of **5** (42%) as a yellow oil; IR (liquid film, cm^{-1}): ν = 1714, 1269, 1022, 964, 914, 816; ^1H NMR ($CDCl_3$): δ = 5.59 (1H, br t, $\jmath$ = 7.7 Hz, H-3α), 4.86 (2H, br s, H-18), 2.32 (4H, m, 2 H-2′) 1.68 (4H, m, 2 H-3′), 0.97 (6H, t, $\jmath$ = 9.0 Hz, 2 H-4′); ^{13}C NMR ($CDCl_3$): δ = 173.2 (C-1′), 172.8 (C-1′), 168.8, 164.2, 147.1, 141.1, 137.2, 135.2, 128.8, 122.6, 118.4, 115.9, 77.8, 74.3, 74.2, 61.9, 51.6, 47.4, 45.5, 37.1, 36.3, 36.2 (C-2′), 29.6 (C-3′), 18.3 (C-4′); CIMS *m/z* 487 $[M+1]^+$ $[C_{27}H_4O_8+H]^+$.

*Dihept-6-enylcynaropicrin (**6**)*
In a pressure-resistant tube (Pyrex) cynaropicrin (100 mg, 0.29 mmol), dioxane (2 ml), hept-6-enoic acid (0.24 ml, 1.8 mmol), *N*,*N*′-dicyclohexilcarbodiimide (DCC) (340 mg, 1.7 mmol), DMAP (42 mg, 0.34 mmol) and a small amount of anhydrous Na_2SO_4 were added. The mixture was irradiated with microwave (MW) for 7 minutes (400 W), a 30-s pause being interposed after every minute. The reaction was monitored by TLC (eluent hexane/EtOAc 4:1, R_f **6** = 0.52). For work-up; the reacted mixture was diluted with EtOAc and filtered to remove Na_2SO_4, washed with $NaHCO_3$, 1 N HCl and brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. CC (eluent hexane/EtOAc 98:2 to 9:1) yielded 52 mg of **6** (32%) as a yellow oil; IR (liquid film, cm^{-1}): ν = 2361, 1771, 1738, 1642, 1263, 1150, 1028, 912, 806; ^1H NMR ($CDCl_3$): δ = 5.75 (2H, m, 2 H-6′), 5.53 (1H, br t, $\jmath$ = 7.7 Hz, H-3α), 4.98 (4H, m, 2 H-7′), 4.85 (2H, br s, H-18), 2.33 (4H, m, 2 H-2′) 2.07 (4H, m, 2 H-5′), 1.66 (4H, m, 2 H-3′), 1.44 (4H, m, 2 H-4′); ^{13}C NMR ($CDCl_3$): δ = 173.2 (C-1′), 172.8 (C-1′), 168.7, 164.2, 147.0, 141.1, 138.0 (C-6′). 137.2, 135.1, 128.8, 122.5, 118.4, 116.0, 114.7 (C-7′), 77.8, 74.3, 74.2, 61.9, 51.2, 47.2, 45.3, 37.1, 36.2, 34.2, 33.1, 28.1, 24.2; CIMS *m/z* 567 $[M+1]^+$ $[C_{33}H_{42}O_8+H]^+$.

Perhydroxylated cynaropicrin (mixture of diastereomers) (7)
In a 25-ml round-bottomed flask, equipped with a magnetic stirrer, cynaropicrin (210 mg, 0.60 mmol) was dissolved in acetone–water 4:1 (8 ml). OsO_4 (30 µl 0.2 M in toluene, 0.006 mmol) and 4-methylmorpholine-N-oxide (NMMO) (210 mg, 1.79 mmol) were added to the solution. The mixture was stirred for 2 h at room temperature and the reaction monitored by TLC (eluent hexane/EtOAc 1:9, R_f **7** = 0.1). For work-up, the reacted mixture was diluted with ether, extracted with water, washed with ether and freeze-dried to obtain 80 mg of **7** (28%) as a whitish powder; mp = 110 °C; IR (KBr, cm^{-1}): ν = 3547, 1773, 1736, 1458, 1223, 1153 1067, 1034; ^1H NMR (D_2O) = 4.13 (2H, br s), 3.88 (2H, br s), 3.69 (2H, br s), 3.53 (2H, br s) (the disappearance of olefinic signals was diagnostic); CIMS *m/z* 483 $[M+1]^+$ $[C_{19}H_{30}O_{14}+H]^+$.

*Deacylcynaropicrin (**8**)*
In a 25-ml round-bottomed flask 67 mg of cynaropicrin (0.19 mmol) were dissolved in 1.75 ml of 2 N NaOH. The solution was heated to 100 °C under magnetic stirring and refluxed for 10 min. The reaction was monitored by TLC (eluent hexane/EtOAc 1:1, R_f **8** = 0.30). For work-up, the solution was brought to pH 8 with 2 N H_2SO_4, extracted three times with EtOAc, washed with saturated brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. 10 mg of **8** were obtained (20%). The compound was identified on the basis of published data.[23]

*11,13-Dihydrocynaropicrin (mixture of 2 diastereomers) (**9**) and 11,13,17,19-tetrahydrocynaropicrin (mixture of 4 diastereomers) (**10**)*
In a 50-ml round-bottomed flask 500 mg of cynaropicrin (1.44 mmol) were dissolved in 10 ml of methanol.

109 mg of $NaBH_4$ (2.87 mmol) were added and the mixture was magnetically stirred for 24 h at room temperature. The reaction was monitored by TLC, eluent hexane/EtOAc 3:7 (R_f **9** = 0.40, R_f **10** = 0.30). For work-up, the reacted mixture, diluted with H_2O and acidified with 2 N H_2SO_4, was transferred to a separatory funnel and extracted with $CHCl_3$. The organic phase was washed with H_2O and saturated brine, dried over anhydrous Na_2SO_4 and evaporated to dryness. Products were purified by HPLC, eluent hexane/EtOAc 3:7. 70 mg of **9** (14%) and 61 mg of **10** (12%) were obtained.

Compound **9**: transparent oil; IR (liquid film, cm^{-1}): $\nu = 3084, 1790, 1771, 1456, 1172, 964, 912, 727$; ^1H NMR ($CDCl_3$): $\delta = 2.54$ (1H, m, H-11), 2.58 (1H, m, H-7), 1.27 (3H, d, $\mathcal{J} = 6.9$ Hz, H-13); ^{13}C NMR ($CDCl_3$): $\delta = 177.5, 165.1, 152.5, 141.9, 139.1, 126.3, 117.3, 112.3, 78.7, 76.2, 73.4, 62.1, 53.2, 50.4$ (C-11), 44.1, 41.1 (C-7), 40.3, 38.6, 15.3 (C-13);-CIMS *m/z* 349 $[M + 1]^+$ $[C_{19}H_{24}O_6 + H]^+$.

Compound **10**: yellow oil; IR (liquid film, cm^{-1}): $\nu = 3084, 1732, 1296, 1269, 1034, 912$; ^1H NMR ($CDCl_3$): $\delta = 3.76$ (2H, br s, H-18), 2.70 (1H, m, H-17, H-9α overlapped), 2.50 (1H, m, H-11), 2.57 (1H, m, H-7), 1.30 (3H, d, $\mathcal{J} = 7.02$ Hz, H-13), 1.21 (3H, d, $\mathcal{J} = 7.2$ Hz, H-19); ^{13}C NMR ($CDCl_3$): $\delta = 181.8, 175.2, 153.1, 142.5, 117.8, 112.9, 79.4, 76.4, 74.0, 65.0, 53.4, 51.1$ (C-11), 44.7, 42.4 (C-17), 41.7 (C-7), 40.5, 39.2, 15.9 (C-13), 13.6 (C-19); CIMS *m/z* 351 $[M + 1]^+$ $[C_{19}H_{26}O_6 + H]^+$.

*3,11,13-Tetrahydrogrosheimin (isolipidiol) (mixture of 2 diastereomers) (**12**) and 3,11,12,13-hexahydrogrosheimin (mixture of 2 diastereomers) (**13**)*

In a 50-ml round-bottomed flask 310 mg of grosheimin (1.18 mmol) were dissolved in 10 ml of methanol. 89.3 mg of $NaBH_4$ (2.36 mmol) were added and the mixture was magnetically stirred for 18 h at room temperature. The reaction was monitored by TLC, eluent hexane/EtOAc 3:7 (R_f **12** = 0.40, R_f **13** = 0.50). For work-up, the procedure was the same as that for cynaropicrin reduction. The products were purified by HPLC, eluent hexane/EtOAc 3:7. 62 mg of **12** (20%), identified on the basis of published data,[24,25] and 56 mg of **13** (18%) were obtained. Compound **13**: amorphous foam; IR (KBr, cm^{-1}): $\nu = 3350, 2961, 2361, 1637, 1076, 1053, 974, 900$; ^1H NMR ($CDCl_3$): $\delta = 2.10$ (1H, m, H-11 overlapped to H-2 and H-9β), 3.61 (1H, m, H-3 overlapped to H-6β and H-8β), 4.50 (1H, d, $\mathcal{J} = 2.1$ Hz, H-12), 1.23 (3H, d, $\mathcal{J} = 4.5$ Hz, H-13); ^{13}C NMR ($CDCl_3$): $\delta = 145.1, 114.2, 110.8$ (C-12), 81.0, 78.9 (C-3), 76.4, 55.0, 51.0, 47.9, 47.8, 42.8, 38.9 (C-11), 30.1, 20.1 (C-13), 18.6; CIMS *m/z* 269 $[M + 1]^+$ $[C_{15}H_{24}O_6 + H]^+$.

*Potassium 2-(4,6-dihydroxy-3-methyl-8-methylene-2-oxo-decahydroazulen-5-yl) acrylate (Grosheimin potassium salt; **14**)*

To a pressure-resistant tube (pyrex) grosheimin (54 mg, 0.21 mmol), THF (1 ml) and 0.85 ml of 0.25 M KOH (0.21 mmol) were added. The mixture was irradiated with MW for 1 min (300 W) and the reaction monitored by TLC (eluent hexane/EtOAc 1:1, R_f **14** = 0). The reacted mixture was evaporated to dryness to yield 55 mg of **14** (84%) as a yellow oil; IR (KBr, cm^{-1}): $\nu = 3490, 2361, 1769, 1743, 1649, 1561, 1388, 1179, 1072, 1050, 903$; ^1H NMR (D_2O): $\delta = 3.65$ (1H, m, H-6β); ^{13}C NMR (D_2O): $\delta = 228.9$ (C-3), 176.8 (C-12), 146.1, 145.3, 122.9, 114.0, 73.2, 71.4, 59.1, 53.8, 47.3, 44.2, 43.8, 40.1, 16.3; CIMS *m/z* 319 $[M + 1]^+$ $[C_{15}H_{19}KO_5 + H]^+$.

Mycelia

Basidiomycetes belonging to the families of Tricholomataceae (*Collybia velutipes*) and Polyporaceae (*Trametes hirsuta*, *Schizophyllum commune*) were chosen on the basis of previous work which had proved their ability to biotransform unusual substrates (Nano & Binello, 2001, unpublished results).

Mycelia, obtained from the Department of Plant Biology, University of Turin, Italy, have been registered by MUT (Mycotheca Universitatis Taurinensis) with the following MUT accession numbers: *Ganoderma applanatum* 3637, *Collybia velutipes* 3638, *Trametes hirsuta* 3639.

Bioconversion by submerged cultures

Mycelia were grown on Petri dishes for 14 days at 24 °C to provide precultures. The culture medium contained 20 g of glucose (Merck), 20 g malt extract (Merck) and 20 g of agar (Merck) per liter of distilled water, pH unadjusted.

In order to isolate fungal metabolites in sufficient amounts for identification (at least 10 mg), four 1-l Erlenmeyer flasks containing fragmented disks (1.6 cm in diameter) of mycelia suspended in 500 ml of liquid medium (20 g l^{-1} glucose and 20 g l^{-1} malt extract) were inoculated with 250 mg of substrate previously solubilized in the smallest volume of EtOH (about 5 ml) and were kept at 24 °C for 7 days on a rotary shaker (Dubnoff BSD) at 90 rev min^{-1}. Each experiment was repeated three times. Blanks were run in parallel without mycelia, to rule out the occurrence of spontaneous chemical transformations.

Extraction and identification of metabolites

At the end of incubations the cultures were filtered, filtrates were extracted with $CHCl_3$ and the solvent was evaporated to yield crude extracts.

Each extract was chromatographed first on a Silica gel 60 (Merck) column with a petroleum ether/EtOAc gradient, and then by semipreparative HPLC to complete the separation of metabolites, using a MicroPorasil Waters 7.8 × 300 mm column with 70/30 hexane/EtOAc isocratic elution. On the basis of NMR spectra and of published data,[25,26] the main products were identified as 11,13-dihydrocynaropicrin (**9**) and 3-dehydro-4,15,11,13-tetrahydrocynaropicrin (**11**) (new product) from cynaropicrin bioelaborations,

isolipidiol (**12**), isoamberboin (**15**) and grosheiminol (**16**) from those of grosheimin.

*3-Dehydro-4,15,11,13-tetrahydrocynaropicrin (**11**)*
Yellow oil; R_f (hexane/EtOAc 3:7): 0.43; IR (liquid film, cm^{-1}): $\nu = 3453, 2106, 1649, 1298, 1261, 1167, 1062, 1028$; ^1H NMR ($CDCl_3$): $\delta = 2.53$ (1H, m, H-11 overlapped to H-2α and H-2β), 2.27 (1H, m, H-4 overlapped to H-9β), 1.33 (3H, d, $\mathcal{J} = 6.3$ Hz, H-13), 1.26 (3H, d, $\mathcal{J} = 6.4$ Hz, H-15) (the disappearance of H-3α was diagnostic);^{13}C NMR ($CDCl_3$): $\delta = 218.8$ (C-3), 177.8, 165.5, 143.0, 139.4, 127.0, 116.7, 83.1, 76.8, 62.7, 52.4, 51.7 (C-11), 47.7 (C-4), 44.8, 43.9, 40.9, 40.0,16.3 (C-13), 14.8 (C-15); CIMS *m/z* 349 $[M + 1]^+$ $[C_{19}H_{24}O_6 + H]^+$.

Sensory evaluation

Twelve volunteers, six males and six females, 23–42 years old (mean age 26.4 years), previously trained to evaluate the sensation of bitterness by comparison with standard caffeine solutions,[27] tested 0.001% solutions of compounds **1–16**. All compounds used in the present study were tested in ethanol–water (8:92 v/v). First of all, dilutions of cynaropicrin (**1**) were prepared and tested. The panelists were asked to score bitterness on a six-point scale ranging from 'like water' (0) to 'exceedingly bitter' (5). When the sensation fell in between two steps, the panelists were told to use half-point values. Six-millilitre aliquots to be tasted were presented to the panelists in 90-ml PET cups under codes which were unknown to them. The sample was not swallowed but swirled for about 10 s inside the oral cavity, which was subsequently rinsed with mineral water. The order of presentation was from the least concentrated solution to the most concentrated one, and the inter-stimulus interval was 10 min. Cynaropicrin concentrations and corresponding bitterness values are shown in Table 1. The low SD figures suggest that the panel was quite homogeneous in bitterness perception and did not include insensitive subjects. The aim of this preliminary step was to identify a cynaropicrin concentration (to be adopted for all subsequent tests) whose bitterness value would conveniently fall in the middle of the scale, ie near 3; the results pointed to 0.001%. The same concentration was identified for grosheimin (**2**) and adopted for all subsequent tests. Compounds **3**–**16** were made up to 0.001% in water (EtOH 96% was used to favour dissolution, its final concentration being 0.3%). Samples under testing were presented randomly and a 0.001% cynaropicrin solution was available to panelists who wanted to recall its taste. Results, when subjected to the one-way ANOVA procedure (Excel, Office 2000) with a p value of 0.05, revealed no significant difference among panelists in regard to the present sensorial tests (F_s (1.842) = 0.012). Tables 1, 3 and 4 list the results (averages of two separate runs conducted at a one week's interval) as mean values, side-by-side with standard deviations (SD).

Table 1. Cynaropicrin solutions and corresponding bitterness values

Cynaropicrin concentration (%)	Bitterness[a] (mean ± SD)
0.0016	4.5 ± 0.2
0.0012	3.7 ± 0.3
0.0010	3.0 ± 0.4
0.0005	1.5 ± 0.4

[a] Perceived taste : 0 = like water; ? = differs from water but taste is indeterminate; 1 = very faintly bitter; 2 = faintly bitter; 3 = definitely bitter; 4 = strongly bitter; 5 = exceedingly bitter.

RESULTS AND DISCUSSION

Systematic chemical modification of cynaropicrin (**1**) and grosheimin (**2**), carried out to provide a library of compounds for our sensorial investigation, comprised a series of esterifications, redox reactions on the oxygenated functions, chemoselective reduction of the exomethylene double bonds and opening of the lactone ring.

From bioconversions of cynaropicrin and grosheimin, five major metabolites were isolated and identified, four of which were produced by all of our three mycelia in different ratios, as shown in Table 2.

The highest yields were obtained with *T hirsuta*. Only *S commune* yielded both reduction and oxidation products. Although products **9** and **12** can also be prepared by chemical reduction of substrates with $NaBH_4$, biocatalysis yielded compounds that cannot be obtained by conventional reactions used in the present work. Minor metabolites, particularly from *S commune*, are still under investigation.

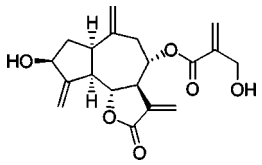
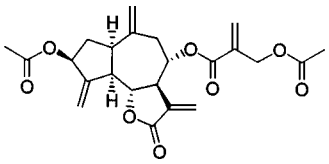
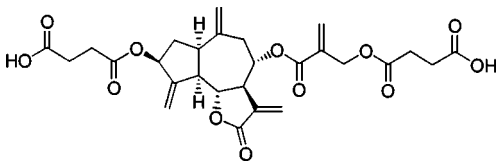
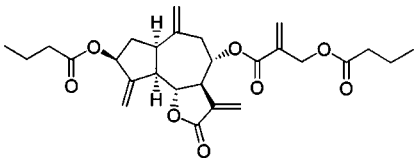
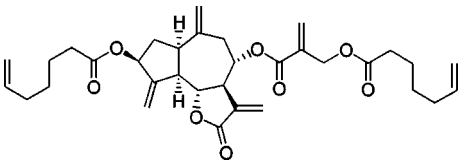
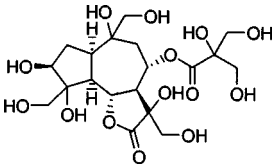
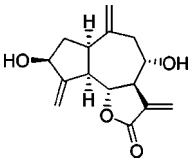
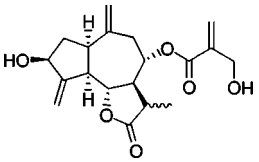
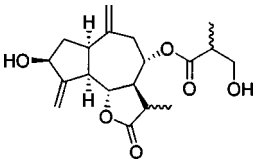
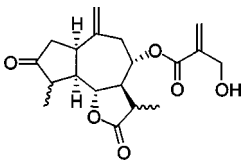
From sensory evaluation of our cynaropicrin and grosheimin derivatives the following structure–bitterness relationships emerged:

(A) bitterness decreases when the polarity of the molecule is increased. Acylation of both hydroxyls in cynaropicrin affected bitterness only slightly

Table 2. Products from bioelaboration by basidiomycetes: yields are given as percentages of starting materials

	Bioelaboration of cynaropicin		Bioelaboration of grosheimin		
Mycelia	11,13-Dihydro-cynaropicrin (**9**)	3-Dehydro-4,15,11,13-tetrahydrocynaropicrin (**11**)	Grosheiminol (**16**)	Isolipidiol (**12**)	Isoamberboin (**15**)
Trametes hirsuta	50%	—	10%	20%	50%
Collybia velutipes	32%	—	5%	10%	50%
Schizophillum commune	13%	10%	5%	10%	25%

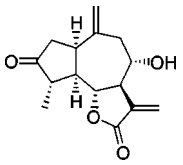
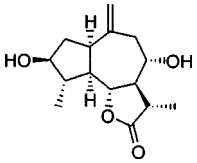
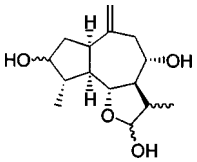
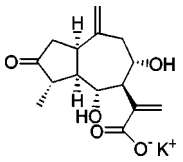
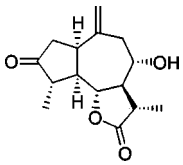
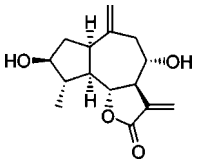
Table 3. Panel tests on cynaropicrin and their derivatives

Compd	Cynaropicrin (1) and derivatives	Bitterness (mean ± SD)	Reaction
1		3.0 ± 0.2	—
3		3.3 ± 0.5	Acylation (acetic anhydride)
4		1.0 ± 0.7	Acylation (succinic anhydride)
5		3.2 ± 0.3	Acylation (butyric anhydride)
6		3.0 ± 0.4	Acylation (6-heptenoic acid)
7		0.0 ± 0.5	Perhydroxylation (OsO_4, NMMO)
8		0.9 ± 0.4	Hydrolysis (NaOH aq)
9		0.8 ± 0.4	Reduction ($NaBH_4$) or bioelaboration
10		0.5 ± 0.4	Reduction ($NaBH_4$)
11		4.2 ± 0.7	Bioelaboration

The same results were obtained for compound **9** whether prepared by chemical or biochemical modification.

(compounds **3, 5, 6**) unless the acyl group contained a hydrophilic function such as the carboxyl (**4**), in which case bitterness was considerably abated. The same effect resulted when cynaropicrin was made more polar by deacylation (**8**). Further confirmation was provided by **11**, a compound that tested more strongly bitter than parent cynaropicrin owing to the replacement of

Table 4. Panel tests on grosheimin and their derivatives

Compd	Grosheimin (2) and derivatives	Bitterness (mean ± SD)	Reaction
2		3.3 ± 0.3	—
12		1.7 ± 0.5	Reduction ($NaBH_4$) or bioelaboration
13		1.3 ± 0.4	Reduction ($NaBH_4$)
14		0.4 ± 0.3	Lactone opening (KOH, MW)
15		2.9 ± 0.4	Bioelaboration
16		0.7 ± 0.3	Bioelaboration

The same results were obtained for compound **12** whether prepared by chemical or biochemical modification.

the hydroxyl in **3** with a carbonyl group, in spite of its having lost by reduction two out of three exomethylene groups, a structural change that has the opposite effect, as shown below (relationship C).

(B) The opening of the lactone ring to yield compound **14** drastically suppressed the bitter taste. We can safely rule out the possibility that the effect was due to the bitterness-masking power of the K^+ counterion, as the solution submitted to the sensory test was less than 5×10^{-5} M in K^+, at least two orders of magnitude less than would be expected to exert a significant bitterness-masking effect.

(C) Exomethylene groups play a significant role in determining bitterness. This effect was found to decrease in parallel with the number of exomethylenes converted to methyl groups by reduction; the effect seemed to be especially marked with the exomethylene placed on the γ-lactone ring (compounds **9, 10**). The taste of grosheimin was not so markedly affected by the methylene reduction (**15**) as by reduction to hydroxyl of carbonyls 12 and/or 3 (**12, 13, 16**), a finding that further confirms the above-stated relationship between polarity and bitterness.

The objection can be raised that **7, 9, 10, 12** and **13**, as prepared by chemical modification, were not stereochemically pure, and consisted of diastereomeric mixtures. On these grounds point C may be disputed. The same conclusion was however found to hold for **9** and **12** even when they were prepared by bioconversions that can be assumed to be stereoselective.

CONCLUSIONS

A sensorial study of fourteen compounds obtained by structural modifications of cynaropicrin and grosheimin shed some light on structure–taste relationships concerning these sesquiterpene lactones. Bitterness appeared to be strongly dependent on the presence of oxygenated polar groups and was markedly abated by the opening of the lactone ring and, probably, by the reduction of exomethylenes.

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