Pergamon

PII: S0031-9422(98)0017-94

Phytochemistry, Vol. 49, No. 8, pp. 2315–2322, 1998

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

IN-VITRO PRENYLATION OF AROMATIC INTERMEDIATES IN THE BIOSYNTHESIS OF BITTER ACIDS IN *HUMULUS LUPULUS*

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(*Received in revised form* 9 *February* 1998)

Key Word Index—*Humulus lupulus*; Cannabaceae; hop; biosynthesis; bitter acid intermediates; prenylation of phloroglucinol derivatives; prenyltransferase.

Abstract—Bitter acids are accumulated in the glandular hairs of hop cones. The first step of the biosynthesis of the bitter acids, namely formation of the aromatic intermediates, is catalysed by a chalcone synthase-like enzyme. In the present paper two sequential prenylation steps, leading to first the production of compounds X (4-prenylphlorisovalerophenone) or co-X (4-prenylphlorisobutyrophenone) and thereafter to the production of deoxyhumulone or deoxycohumulone, respectively, are described. An HPLC assay for these prenylation reactions has been set up, and enzyme activities were measured in protein extracts from hop flowerbuds, flowers and young cones. Furthermore, preliminary characterization experiments of the prenyltransferases were performed.

INTRODUCTION

The ripe cones of the hop plant, *Humulus lupulus* (Cannabaceae), are covered with glandular hairs which contain essential oil and bitter acids (α- and β-acids). These compounds show antimicrobial, antifungal and antifeedant activities [1, 2] and are therefore believed to be involved in the defence mechanism of the plant. Additionally, the bitter acids have commercial value for the brewing industry. Especially the α-acids, which are transformed into iso-α-acids during the brewing process, are important for the bitter taste of beer.

Recently, the biosynthesis of the hop bitter acids has been partly elucidated [3–5]. The first step, formation of phloroglucinol derivatives from malonyl-CoA and isovaleryl-CoA or isobutyryl-CoA (Figure 1), is catalysed by a chalcone synthase-like enzyme [4], which is purified and named valerophenone synthase (VPS) (Paniego, N.B., unpublished results). Thereafter, two sequential *C*-prenylation steps, catalysed by one or different prenyltransferases, lead to first the production of compounds X (**3**, new suggested name: 4-prenylphlorisovalerophenone, PPIVP) or co-X (**4**, new suggested name: 4-prenylphlorisobutyrophenone, PPIBP) and then to the production of deoxyhumulone (**5**) or deoxycohumulone (**6**), respectively (present paper). Starting with **5** it is possible to synthesize the α-acid humulone (**7**) both chemically as well as catalysed by protein extracts from hop glandular hairs [5].

In the present paper, the *in vitro* activities in hop protein extracts of the two sequential prenylation steps Fig. 1 are described. These steps are the formation of **3** and **4** from DMAPP plus phlorisovalerophenone (PIVP, **1**) or phlorisobutyrophenone (PIBP, **2**), respectively, and the formation of **5** and **6** from DMAPP plus **3** or **4**, respectively. These enzyme activities were studied in relation to incubation time, protein concentration, type of cofactors (Mg^{2+}/Mn^{2+}) and substrates (IPP/DMAPP).

RESULTS AND DISCUSSION

HPLC assays for the detection of the prenylated products have been set up (see Experimental) in a similar way as for the assay of hop VPS [4, 6]. Two different HPLC systems were used: one for the detection of **3** and **4** and the bitter acids and the other one for the detection of **5** and **6**. With these assays we were able to show the *in vitro* formation of **3** and **4** from DMAPP plus **1** or **2**, respectively, using protein extracts from hop young cones (Figure 2 A + B). The formation of **5** and **6** from DMAPP plus **3** or **4**, respectively, could also be detected (Figure 2 C + D). The identities of the products were confirmed by com-

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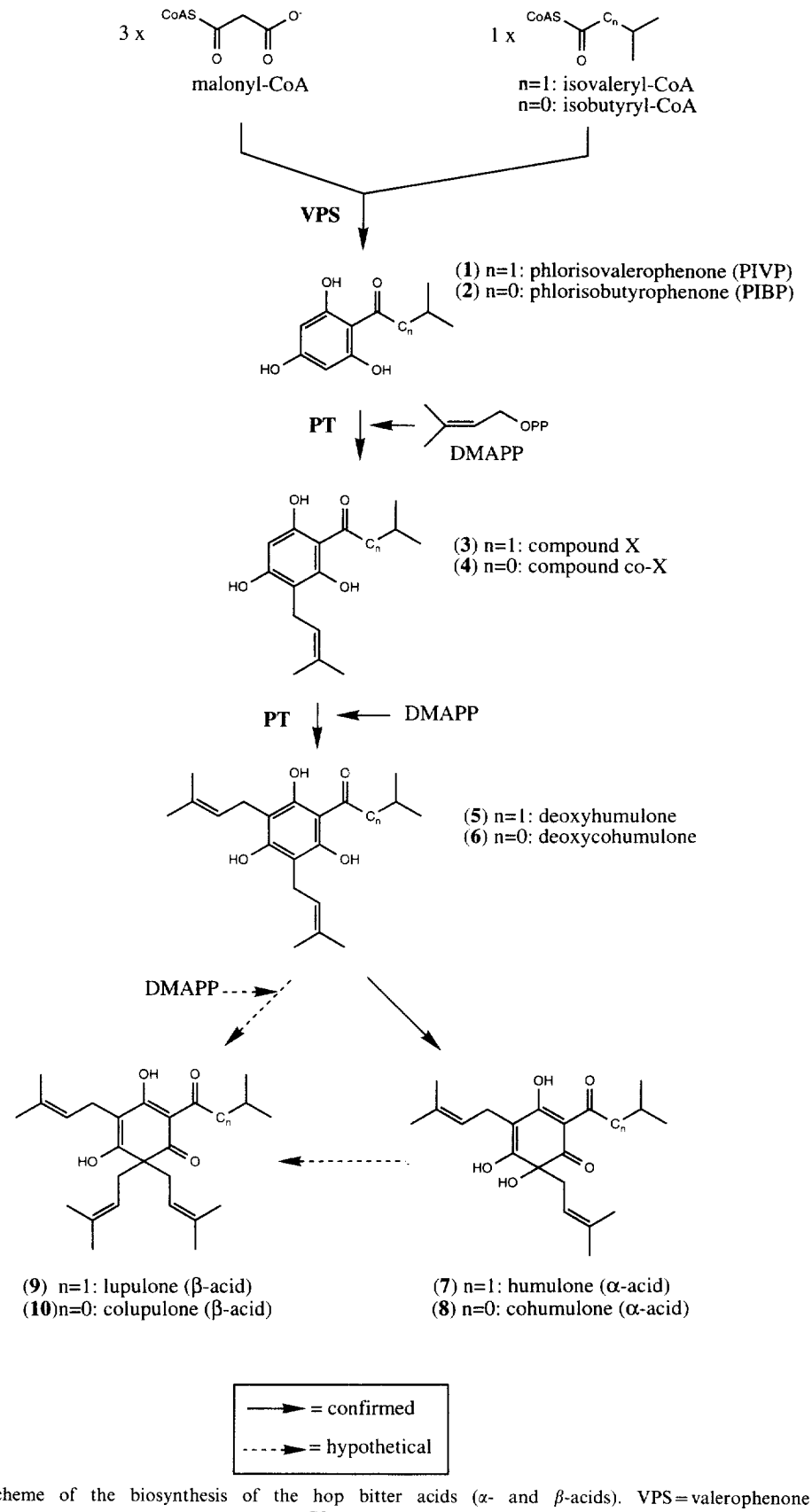

Fig. 1. Scheme of the biosynthesis of the hop bitter acids (α- and β-acids). VPS = valerophenone synthase, PT = prenyltransferase.

paring the retention times and the UV absorption spectra with those of authentic samples. The control experiments in the absence of substrate, DMAPP or protein extract showed no activity. It should be noted, however, that in addition to the products described above also some (unidentified) products with other retention times were produced.

The majority (more than 90%) of each prenylating activity was present in the soluble fraction and not in the microsomal fraction of the hop protein extract, meaning that the enzymes involved were not membrane-bound. However, all known prenyltransferases that catalyse similar reactions in the biosynthesis of secondary metabolites are not soluble. Examples of enzymes only present in the microsomal fractions are found for both the *C*-prenylation [7, 8] and 7-*O*-prenylation [8] of the coumarin umbelliferone in cell suspension cultures from *Ammi majus* [8] and *Ruta graveolens* [7], for the prenylations of 3,9-dihydroxypterocarpan and 3,6a,9-trihydroxypterocarpan in elicited soybean seedlings and cell suspension cultures [9, 10] and for the prenylations at three different positions of the isoflavones genistein and 2′-hydroxygenistein in white lupin (*Lupinus albus*) radicles and cell suspension cultures [11, 12].

Under the assay conditions studied, the formation of **3** is linear with time up to *ca* 30 min(Figure 3A) and that of **4** to *ca* 100 min(Figure 3B). Thereafter the reactions slow down and stop. In case of **3** it even seems that the formed product is degraded. This degradation could be the result of the further conversion of the formed **3** into **5**. In order to check this possibility, the same incubations at 15, 60 and 120 min were repeated and tested for the formation of **5** and **6** with the other HPLC system. It indeed appeared that **5** was produced, as was detected after incubation for 60 and 120 min, whereas no or hardly any **6** could be detected at these incubation times.

In a separate experiment (results not shown) the relationship between incubation time and the formation of **5** and **6** (from the added substrates **3** and **4**) was determined. The reactions appeared to be linear until *ca* 60 min.

In Fig. 4 the amount of hop protein that is used for

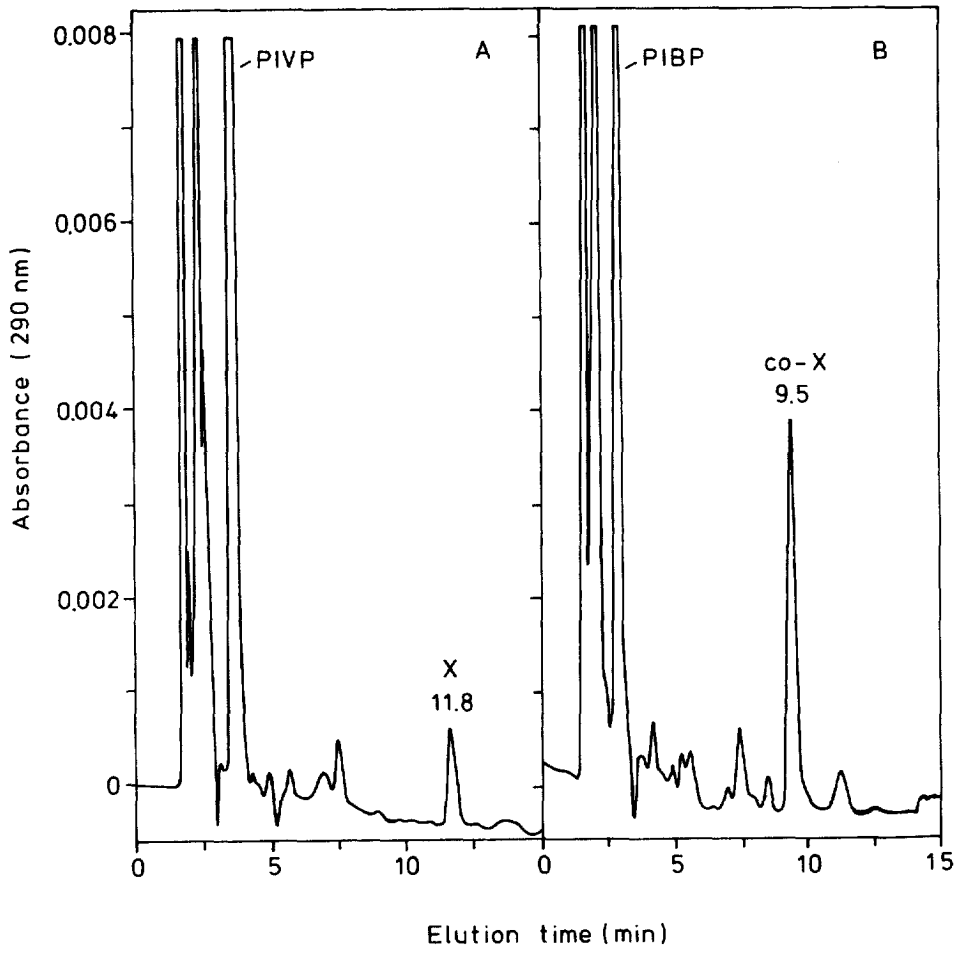

Fig. 2. HPLC elution profiles obtained from assays of hop prenyltransferase activities in a protein extract from "Olympic" young cones using various substrates. In combination with DMAPP the following substrates were used: **1** (A), **2** (B), **3** (C) or **4** (D). The products formed were **3** (A), **4** (B), **5** (C) or **6** (D). For assay and HPLC conditions, see Experimental.

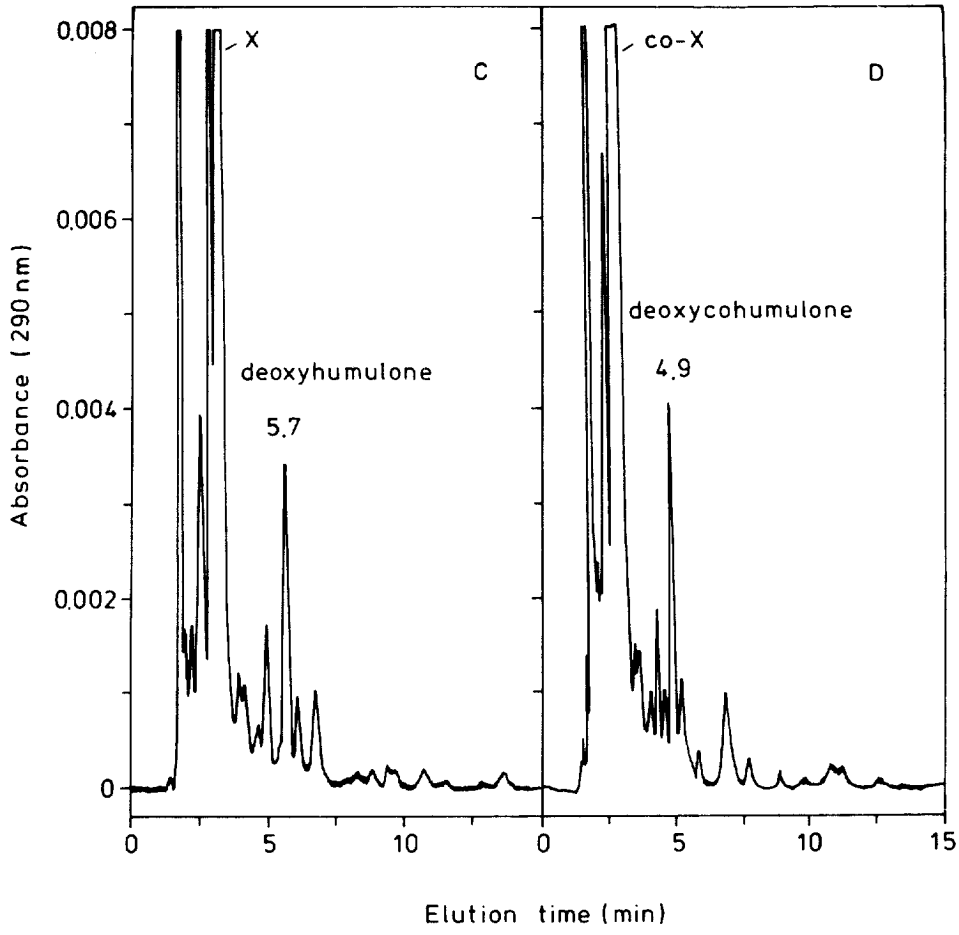

Fig. 2.—*Continued.*

Table 1. Effects of different assay conditions on the activity percentages of the hop prenyltransferase reactions.

product formed	A standard	B boiled extract	C IPP	D KF	E $MnCl_2$
3	100%	6%	0%	31%	≳25%
4	6%	6%	0%	89%	≳40%
5	14%	14%	0%	80%	385%
6	15%	15%	0%	73%	960%

The formation of **3** and **4** (from the substrates **1** and **2**) and **5** and **6** (from the substrates **3** and **4**) was measured under standard conditions (A, see Experimental), with denaturated (5 min 100°C) protein extract (B), with 13 μl 10 mM IPP instead of DMAPP (C), plus 30 mM KF (D) or with 10 mM $MnCl_2$ instead of cofactor $MgCl_2$ (E). The average values of duplo experiments are given.

the incubation is plotted against the formation of the products. The reactions are linear until *ca* 7.5 μg protein (formation of **3**, Fig. 4A and **6**, Fig. 4D) and 15 μg protein (formation of **4**, Fig. 4B and **5**, Fig. 4C). Enzyme activities calculated from Fig. 4 are: 78 (production of **3**), 38 (production of **4**), 68 (production of **5**) and 91 (production **6**) μkat. kg^{-1}. These values are higher but in the same order of magnitude as compared to the activity of kaempferol 8-dimethylallyl transferase from *Epimedium diphyllum* cell suspension cultures [13].

In Table 1 the results of some additional experiments are shown. Denaturation of the hop proteins by boiling for 5 min resulted in a major loss of all activities. A total loss of production appeared in the incubations with IPP instead of DMAPP, meaning

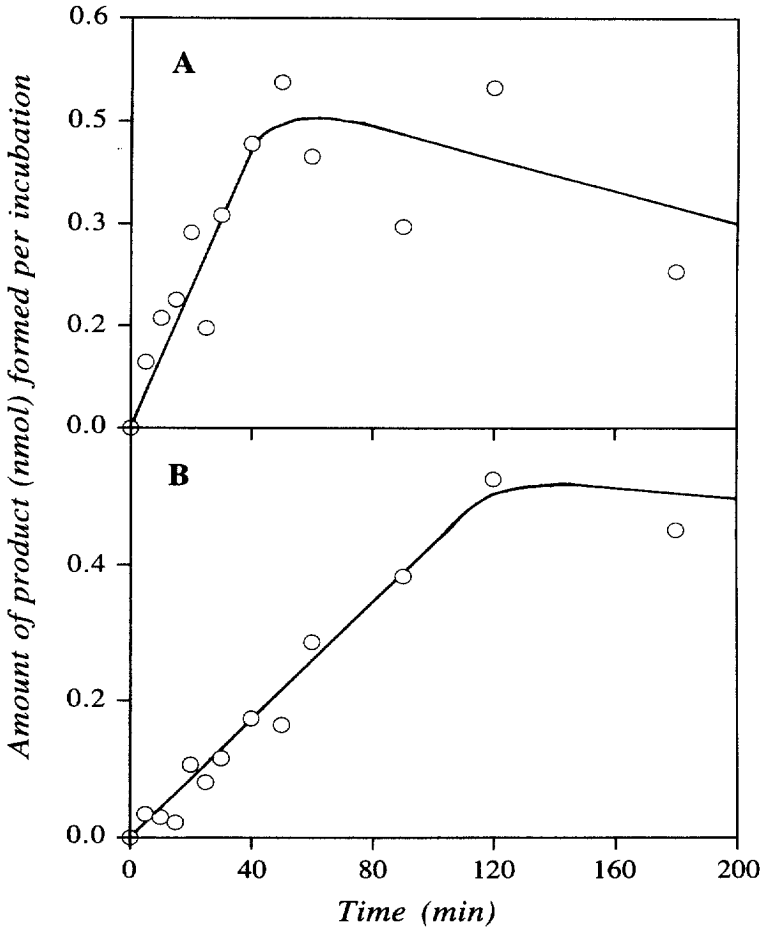

Fig. 3. Amounts of **3** (A) or **4** (B) formed as a function of the incubation time in the hop prenyltransferase HPLC assay using 9 μl protein extract (6.7 μg protein) from hop "Yeoman" flowers (see Experimental). In combination with DMAPP the following substrates were used: **1** (A) and **2** (B). All incubations were performed in duplicate. The average values are presented.

that no isomerase was present or active to convert IPP into DMAPP, essential for prenyltransferase activity. KF was added to determine whether any possible phosphatase reaction, which hydrolyses DMAPP, could be inhibited leading to possibly higher prenylating activities. As is clear from Table 1 this was not the case. When $MnCl_2$ instead of $MgCl_2$ was used as a cofactor, the second prenylation step was increased four (**5** formation) to ten (**6** formation) times in activity. The first prenylation might also be increased, but because of the higher activity of the second prenylation the products formed were immediately used for the second reaction and the first products could therefore not be detected (Table 1). In a separate experiment indeed higher amounts of **5** and **6** were detected after incubation of DMAPP plus **1** and **2**, respectively, in the presence of $MnCl_2$. The literature about the effects of these cofactors on the prenylations of umbelliferone in different plants is contradictory. Ellis and Steward [7] described maximum enzyme activity, concerning *C*-prenylation of umbelliferone in cell suspension cultures from *Ruta graveolens*, in the presence of Mn^{2+}, whereas Hamerski *et al.* [8] found that Mg^{2+} stimulated *C*-prenylations and Mn^{2+} stimulated prenylations at the OH-group (*O*-prenylations) of umbelliferone in *Ammi majus* cell suspension cultures. The latter authors also observed that in both cases the *O*-prenylation was the major reaction. It can not be excluded that *O*-prenylation, in addition to the shown *C*-prenylations, also occurs in our experiments. We are not able to test this possibility, since the suitable reference compounds for determination of the retention times and UV spectra are not available.

Until now we could not detect the third prenylation step, namely the production of the β-acids lupulone (**9**) and colupulone (**10**). One explanation for this could be the instability of the substrates **5** and **6**, since most of the substrate was degraded or converted, possibly by other hop enzymes, after incubation with hop protein extract, even in the absence of DMAPP. In case of incubation with **5** the α-acid **7** was formed together

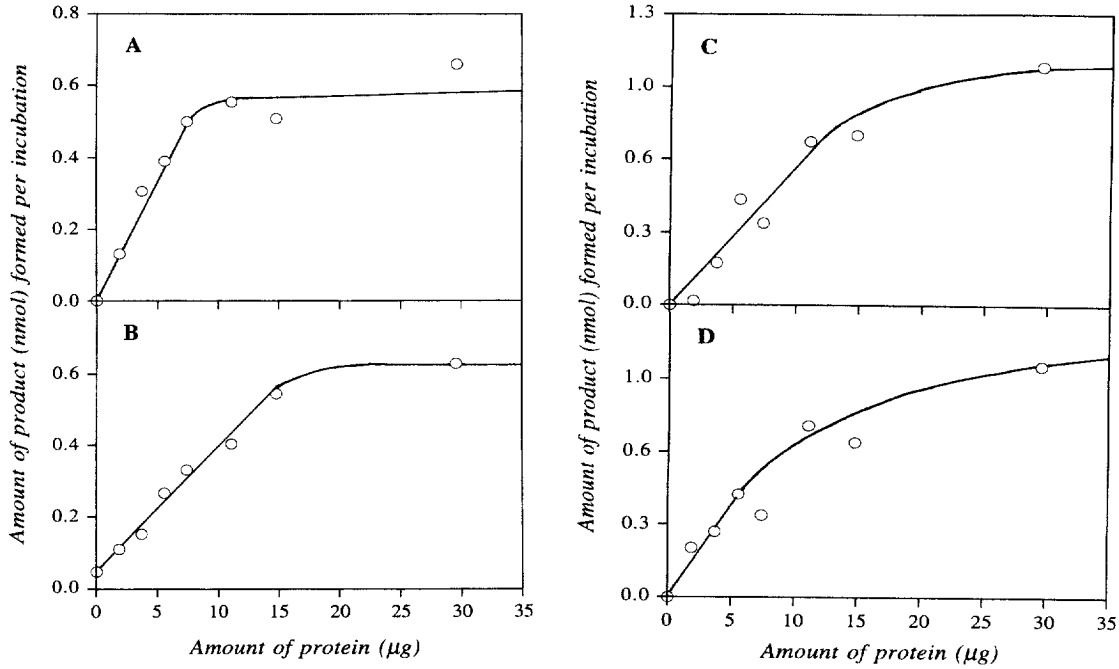

Fig. 4. Amounts of **3** (A), **4** (B), **5** (C) or **6** (D) formed with respect to the amount of hop protein added in the hop prenyltransferase HPLC assay (incubation time 15 min) using protein extracts from hop "Yeoman" flowers (see Experimental). In combination with DMAPP the following substrates were used: **1** (A), **2** (B), **3** (C) or **4** (D). All incubations were performed in duplicate. The average values are presented.

with other, not yet identified, products. This formation could be detected both in presence and in absence of DMAPP. **9** and **10** formation could not be detected. Further purification of the prenyltransferases could solve these problems. Another explanation could be that the third prenylation step in the biosynthesis of the bitter acids does not take place via the intermediates **5** and **6**, but that in hop the β-acids are synthesized from the α-acids.

In conclusion, assays using two suitable HPLC systems has been set up for measuring the prenylation of the aromatic intermediates of the hop bitter acids. Using these assays with protein extracts from hop flowers and cones, it was, for the first time, possible to detect two sequential prenylations of **1** and **2** leading to **5** and **6**, respectively, the direct precursors of the hop bitter acids. It has been shown before [5] that the α-acid **7** can be produced from **5** both enzymatically, in the presence of hop protein extracts, as well as chemically, in the absence of hop protein extracts. This means that all enzymatic steps involved in the biosynthesis of the bitter acid **7** from the start precursors malonyl-CoA and isovaleryl-CoA can be performed *in vitro* using hop protein extracts. The final reactions leading to the β-acids **9** and **10** still have to be elucidated. In comparison with other prenyltransferases known from literature [7–12] the main difference is that the presently described hop prenyltransferases are soluble and not microsomal. Whether the two sequential prenylations are catalysed by the same or by different prenyltransferases has still to be determined. In the literature some examples are described where prenylations at different positions of the aromatic structure were catalysed by different enzymes [8, 12, 14]. In hop, other prenylations of aromatic structures should occur as well, since the presence of prenylated flavonoids was detected in hop cones [15, 16]. These prenylated flavonoids appear to have antifungal activities [17]. It is also known [11, 14, 17, 18] that prenylation of isoflavonoids in general increases the antifungal and insecticidal activities. From structure-antifungal activities tests [2] it was shown that a prenylated **1** possesses relatively high antifungal activities due to both its prenyl- and acyl-sidechains. The prenylations of **1**, **2**, **3** and **4**, as presented in this paper, may thus result in increased antifungal activity. The prenyl groups can be considered as interesting farmacophores. The prenyltransferases are thus of interest to be further explored for prenylation of other aromatic ring systems.

For the commercial aspects of the hop bitter acids it will be interesting to determine whether the hop enzymes accept related substrates, thus leading to new "bitter compounds" which could possibly further improve the quality of beer or give new interesting tastes or flavours to beer.

EXPERIMENTAL

Plant material. Humulus lupulus "Yeoman" and "Olympic" were grown in our garden (Division of Pharmacognosy, Leiden). Flowers and young cones

were harvested, frozen in liquid N_2 and stored at $-80°$.

Chemicals. The reference compounds **1**, **2**, **3** and **4** were prepd according to ref. [3]. Supercritical carbon dioxide hop extracts and **5** were gifts of Heineken Technical Services. Pure α- and β-acids were isolated from the carbon dioxide extracts by Mr A. C. Hoek using centrifugal partition chromatography [19]. **6** was obtained by Mr A. C. Hoek after irradiation of **10** according to ref. [20]. DMAPP was prepd by Dr R. van der Heijden according to ref. [21]. Reagents and solvents used were of analytical grade.

Protein extraction from "Yeoman" flowers. All steps were carried out at 0–4°. Frozen flowers (4.9 g) were ground using a pestle and mortar in the presence of sea sand, liquid N_2 and 50% (w/w) polyvinylpolypyrrolidone. The frozen powder was mixed with extraction buffer (0.5 M KP_i buffer pH 8.0, 10% (w/v) sucrose, 1.5% (w/v) polyethyleneglycol 4000, 200 mM ascorbic acid, 50 mM cysteine, 1 mM EDTA and 2% (w/v) Dowex 1 × 2). After thawing, the homogenate was filtered using a Miracloth filter. The filtrate (total volume 26 ml) was centrifuged twice for 30 min at 10 000 g and 13 000 g, respectively. The pellets and the supernatant were tested for the described hop prenyltransferase activities. The pellets appeared to have no activities. The supernatant was frozen in liquid N_2 and stored at $-80°$ for further experiments. The protein concentration was 740 $\mu g.ml^{-1}$ as determined according to ref. [22].

Preparation of microsomal and soluble fractions from "Yeoman" flowerbuds. 5 g of frozen flowerbuds were used. The protein extraction method used was similar as the one described above for the "Yeoman" flowers. The protein concentration, determined according to ref. [22], of the resulting supernatant was 1.2 $mg.ml^{-1}$. A 100 µl sample of this supernatant was centrifuged for 1 hr at 100 000 g leading to a microsomal (pellet) and a soluble (supernatant) fraction. Afterwards these fractions were tested for prenyltransferase activities (formation of **3** and **4**, **5** and **6**).

Protein extraction from "Olympic" young cones. The glandular hairs were removed from the young cones (16 g) by mixing the cones with 50% H_2O/50% extraction buffer using a vortex at maximum speed. Then the glandular hair fraction (supernatant fraction containing the hairs) and the cone fraction (cones at the bottom of the tube) were separated from each other and filtrated. In this way *ca* 0.9 g glandular hairs (including Dowex from buffer) and 30 g cones (including retained H_2O) were obtained. It should be mentioned however that the majority of the glandular hairs retained to the cones, and that therefore no complete separation was possible between hairs and cones. The protein extraction of both fractions was performed in a similar way as described above for "Yeoman" flowers. After the second centrifugation step the supernatant was desalted using a PD 10 column (Sephadex G-25M, Pharmacia) according to the manufacturer's directions for use. The desalting buffer used was 5 mM KP_i buffer pH 7.5 plus 10% (w/v) sucrose. The protein concentration of the "glandular hair" fraction was 0.4 $mg.ml^{-1}$ and that of the "cone" fraction 1.3 $mg\,ml^{-1}$ as determined according to ref. [22].

Prenyltransferase assay. Protein extracts ("glandular hair" as well as "cone" fraction) of "Olympic" were used for the experiments described in Fig. 2 and that of "Yeoman" flowers for Figs. 3–4 and Table 1. Protein extract (35 µl for Fig. 2 and Table 1, 9 µl for Fig. 3 or other volumes as mentioned in Fig. 4) was incubated with 100 µl (Fig. 2) or 125 µl assay buffer (0.1 M Tris-HCl pH 7.5, 10 mM $MgCl_2$) plus 13 µl stock soln(0.15 mg/ml in MeOH; Fig. 2) or 3 µl stock soln (3.3 mM in MeOH) of **1**, **2**, **3** or **4**. The reaction was started by the addition of 13 µl 10 mM DMAPP. After incubation at 30° for 15 min (Fig. 4), 20 min (Fig. 2), 30 min (Table 1) or other incubation times as mentioned in Fig. 3, the reaction was stopped by the addition of 5 µl 6 N HCl and 265 µl EtOAc. For the experiments in Figs. 3–4 also an internal standard (recoveries are mentioned at the end of this paragraph) was added of 5 µl 15 $\mu g\,ml^{-1}$ **3** (in case of **4** formation), **4** (in case of **3** formation), **5** (in case of **6** formation) or **6** (in case of **5** formation). Product extraction was performed by thoroughly mixing (using a vortex) the sample and isolation of the EtOAc layer, which contained the products, after centrifugation for at least 2 min(using a microcentrifuge). Thereafter the EtOAc layer was collected and evaporated to dryness using a vacuum concentrator. The residue was then dissolved in 25 µl MeOH and analysed by HPLC. The efficiencies of the product extractions by EtOAc were *ca* 72% for **3**, 78% for **4**, 90% for **5** and 85% for **6** as determined from the added internal standards in the assays for Figs. 3–4.

HPLC analysis. The system consisted of an LKB Model 2150 HPLC pump, an LKB LCC 2252 controller, an autoinjector (Gilson 234) equipped with a 100 µl syringe, an LKB Model 2151 variable wavelength monitor and a chromatographic data processor Chromatopac Model CR 501 (Shimadzu). A guard column packed with octadecyl silica (30 µm particle size) and a reversed phase column (Hypersil 5 µm C18, 250 × 4.6 mm (i.d.)) were used. The injection volume was 15 µl plus 2 µl of air gap, using center loop filling of a 20 µl loop. Detection was performed at 290 nm. The mobile phase for HPLC analysis of **3**, **4**, and the bitter acids consisted of 0.05 M triethanolamine in MeOH-H_2O (13:7), pH set at 6.85 using 85% H_3PO_4 [23]. A flow rate of 1.75 ml min^{-1} was used and the HPLC column was heated to 37° using a water bath in order to decrease the pressure (final pressure was 230 bar). **5** and **6** could not be detected with this mobile phase since they were retained strongly and eluted as broad "peaks" which were even difficult to be recognized as peaks. For the analysis of these compounds MeOH-H_2O-85% H_3PO_4 (85:17:0.25) [24, 25] was used as mobile phase using a flow rate of

1.5 ml min^{-1}. In this system the HPLC column was used at room temp.

HPLC photo diode array detection. The system consisted of a Waters 712 WISP injector, a Waters 600 E system controller and a Waters 991 photo diode array detector. All other conditions were as described above.

Acknowledgements—We thank Mr W. Snoeijer for growing the hop plants in our experimental garden. Mr L. C. Verhagen from Heineken Technical Services is kindly acknowledged for the gift of hop supercritical carbon dioxide extracts and **5**. **6** and the pure α- and β-acids were supplied by Mr A. C. Hoek and a sample of DMAPP by Dr R. van der Heijden. This study was financed by the Technology Foundation from the Netherlands.

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