Pergamon

PII: S0031-9422(96)00671-1

Phytochemistry, Vol. 44, No. 6, pp. 1047–1053, 1997

0031-9422/97 $17.00+0.00

CONVERSION OF DEOXYHUMULONE INTO THE HOP α-ACID HUMULONE

SUEN-YING FUNG, KARIN W. M. ZUURBIER, NORMA B. PANIEGO, JOHANNES J. C. SCHEFFER and ROBERT VERPOORTE

Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Leiden University, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands

(*Received* 12 *July* 1996)

Key Word Index—*Humulus lupulus*; Cannabaceae; hop; biosynthesis; bitter acids; deoxyhumulone; humulone; bovine serum albumin.

Abstract—In the final step of the biosynthesis of hop bitter acids an oxidation leads to the formation of α-acids. A method was set up to analyse the bitter acids in the presence of proteins, since proteins from a crude hop extract were found to interact with the phenolic bitter acids and interfere with the extraction and assay of these acids. After examination of the interaction of bovine serum albumin with hop bitter acids, concentration of the samples by lyophilization and subsequent analysis by HPLC were used to study the conversion of deoxyhumulone into humulone. Two types of oxidation were found to take place *in vitro*: a chemical reaction and a reaction catalysed by an unknown factor from hop protein extracts. The results of this study indicate that this factor, isolated from the glandular hairs of hop cones, is an enzyme that is involved in the biosynthesis of α-acids. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

The cones of the hop plant, *Humulus lupulus* L. (Cannabaceae), are an important raw material for the beer brewing industry; after brewing, the essential oil and the bitter acids from the cones contribute to the specific aroma and taste of the final product beer. The bitter acids consist of so-called α- and β-acids. The essential oil and these acids are stored in the glandular hairs of the ripe cones.

Recently, we initiated a study on the biosynthesis of the hop α- and β-acids. Three types of reaction take place in this biosynthesis: the formation of aromatic intermediates, the introduction of several prenyl side chains into the aromatic ring and the oxidation of a carbon atom from the phenolic nucleus. The first reaction is similar to the initial step in the biosynthesis of flavonoids; the successive condensations of three molecules of malonyl-CoA to isobutyryl-CoA or isovaleryl-CoA lead to the formation of phloroglucinol intermediates [1, 2]. The aromatic nucleus is then prenylated at several positions to yield a deoxy-α-acid and finally a β-acid; in the final stage of the biosynthesis an α-acid is hypothesized to be formed after oxidation of a α-acid or of the intermediate deoxy-α-acid. The two possibilities for the formation of the main α-acid humulone are shown in Fig. 1. It is not known whether the oxidation is a photochemical reaction influenced by sunlight or a conversion catalysed by an enzyme.

In order to study the conversion of the β-acid lupulone or of deoxyhumulone into humulone, we chose HPLC as a method to analyse the samples, since in this way the two possible substrates and the product could be determined in a mixture. Using lyophilization and HPLC, we analysed the *in vitro* formation of humulone, both in the presence and absence of protein extracts prepared from the glandular hairs of hop cones.

RESULTS AND DISCUSSION

Recovery of hop bitter acids from aqueous solutions in the presence of bovine serum albumin

Initially, the mixtures of the substrates and hop protein extracts were acidified after incubation and then extracted with ethyl acetate; after separation the organic layer was concentrated and analysed by HPLC, using a system developed for the measurement of hop bitter acids [3, 4]. The results of these experiments showed that in some cases the amount of lupulone decreased without any apparent formation of new products. Since phenolic compounds can form complexes with proteins [5], which might influence the solvent extraction, we decided to study the possible interaction between bitter acids and proteins. We used bovine serum albumin (BSA) as a model protein and

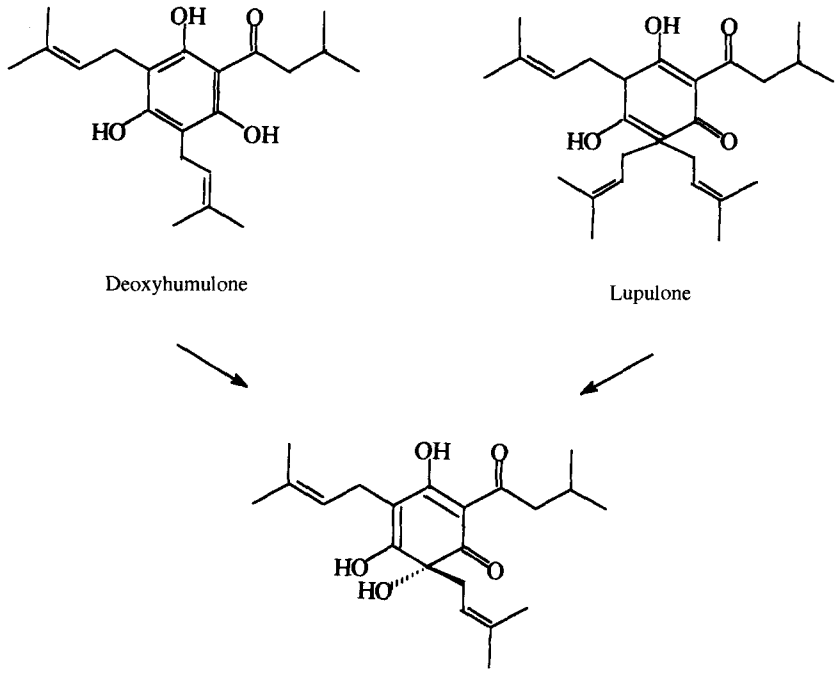

Fig. 1. Two possible ways for the formation of humulone in hop.

added it in various amounts to 0.1 M potassium phosphate (K-Pi) (pH 7.0) solutions containing different bitter acids or their precursors, and analysed their contents in the concentrated organic phase.

It is clear from Fig. 2(A) (showing data for the α-acid cohumulone, the β-acid colupulone and their precursors) and Fig. 2(B) (showing data for humulone, lupulone and their precursors) that the amounts of the α-acids cohumulone and humulone and the β-acids colupulone and lupulone extracted by ethyl acetate from the acidified solution decreased when increasing amounts of BSA had been added. Since bitter acids are phenolic compounds and therefore weak acids, it is expected that their extraction from aqueous solutions by organic solvents will be most efficient at pH < 3.

In a second set of experiments, the aqueous solutions with bitter acid were extracted with ethyl acetate at different pH, both with and without BSA, to test at which pH the loss in recovery occurs. The extraction of deoxyhumulone is optimal at pH 7–8; this is an indication that an ion pair extraction is involved in the case of this compound (Fig. 3(A)). In the presence of 2.5 μg BSA nmol^{-1} bitter acid, the extraction of deoxyhumulone and lupulone by ethyl acetate was found to be poor at low pH (Fig. 3(A) and 3(C)), which indicates that the loss of the bitter acids in the organic phase is due to a hydrophobic interaction of these non-polar phenolics with the protein in the aqueous layer. In this set of experiments the addition of BSA only led to a small decrease of humulone recovery in the organic phase at low pH (Fig. 3(B)); other experiments with humulone, however, showed

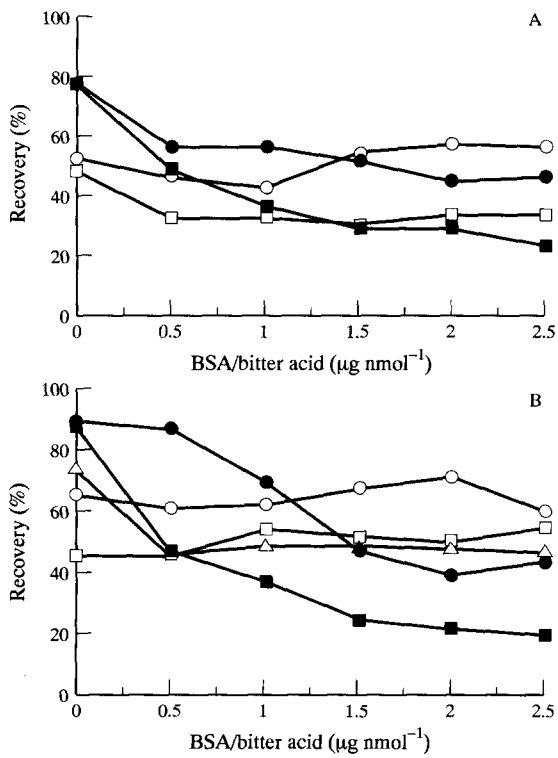

Fig. 2. Influence of BSA on the recovery of cohumulone intermediates (A) (○, phloroglucinol derivative PIBP; □, prenylated intermediate Co-X; ●, cohumulone; ■, colupulone) and of humulone intermediates (B) (○, phloroglucinol derivative PIVP; □, prenylated intermediate X; △, deoxyhumulone; ●, humulone; ■: lupulone) (B) after extraction with ethyl acetate.

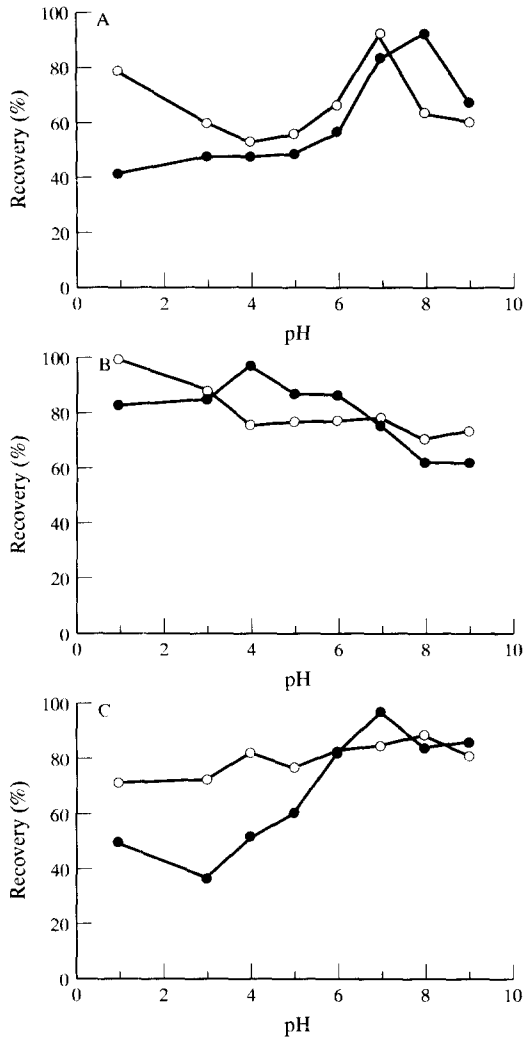

Fig. 3. Influence of pH on the recovery of (A) deoxyhumulone, (B) humulone and (C) lupulone in absence or presence of BSA (2.5 μg nmol^{-1} bitter acid) after extraction (○, without BSA; ●, with BSA).

a strong decrease in recovery in the presence of BSA (Fig. 2(B) and Table 1). There is a variation in the recovery of the bitter acids after replicate treatments in different experiments; the variation could be caused by decomposition of the labile bitter acids during the concentration of the extracts.

Since extraction after acidification in the presence of BSA, led to a considerable loss of bitter acids, we tested a different method to concentrate and purify the samples before analysis by HPLC. After incubation and acidification, which was necessary to prevent oxidation of the phenolic compounds at high pH, the samples were freeze-dried and resuspended in methanol. The recovery of the bitter acids after extraction or after lyophilization and resuspension is shown in Table 1. Except for the labile compound deoxyhumulone, the latter method led to an improved recovery of the bitter acids in the presence of 2.5 μg BSA nmol^{-1} bitter acid. When hop protein extracts were used instead of BSA, similar high recoveries were found for the bitter acids after lyophilization and resuspension in methanol (data not shown); therefore, this method was used in our further experiments.

Extraction of proteins from glands of hop cones

We expect that the final step in the formation of α-acids takes place in the glandular hairs of the cones, which is the storage site of the essential oil and bitter acids. Therefore, we isolated proteins from these glands and tested them on humulone-forming activity. Since we harvest hop material once a year and store it at −20 or −80°, the structure of the glands is partly disrupted after thawing and the glands can be isolated using a cell homogenizer, as described for a study of glandular hairs from *Mentha* sp. [6].

The isolated glands contain a large amount of phenolic compounds, which can interfere with the proteins during the extraction and the assays on enzymic activity. Inactivation of the proteins by phenolics was circumvented by using a buffer we employed for earlier work with hop protein extracts [2, 7]. Hop protein extracts were prepared from the glands and fractionated in a supernatant and pellet by centrifugation at 18 000 *g*.

Activity of humulone and cohumulone formation in hop protein extracts

In a series of experiments either lupulone or deoxyhumulone was incubated with several hop protein extracts; the use of lupulone did not lead to humulone

Table 1. Recovery of bitter acids (%) without or with BSA (2.5 μg nmol^{-1} bitter acid) after acidification and extraction with ethyl acetate (I) or after acidification and lyophylization (II)

	I		II	
Compound	EtOAc	BSA + EtOAc	Lyophil.	BSA + lyophil
Deoxyhumulone	50	22	34	33
Cohumulone	89	19	92	81
Humulone	56	16	85	100
Colupulone	73	16	90	86
Lupulone	74	20	66	96

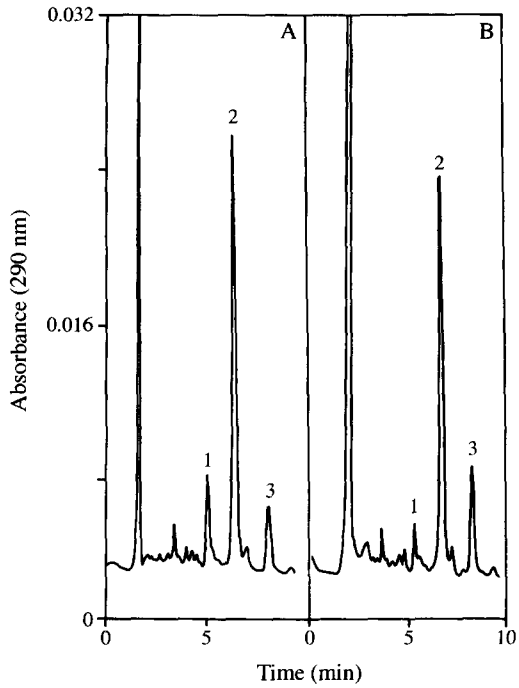

Fig. 4. HPLC showing (A) chemical conversion into humulone and (B) conversion into humulone in the presence of a gland extract from cones of hop cultivar 'Brewer's Gold' containing 18 μg of soluble protein, after incubation of 0.094 nmol μl^{-1} deoxyhumulone in 0.1 M K-Pi (pH 7.0) at 30° for 20 min. 1, Unknown product; 2, deoxyhumulone; 3, humulone.

formation, whereas this was sometimes the case for deoxyhumulone. In view of these results no further experiments with lupulone were pursued. The gland protein extracts from the young cones of the hop cultivars 'Brewer's Gold', 'Hersbrucker Spät', 'Olympic' and 'Yeoman' were active in forming humulone from deoxyhumulone. Further tests were only conducted with young cones of the hop cultivar 'Brewer's Gold', as they were available in a larger quantity than the cones of the other varieties.

In the synthesis of humulone, deoxyhumulone undergoes an oxidation to yield a racemic mixture of the α-acid [8]. HPLC showed that incubation of deoxyhumulone in 0.1 M K-Pi (pH 7.0) at 30°, acidification and subsequent concentration by lyophilization led to the formation of two products (Fig. 4A); HPLC-PDA and liquid chromatography-mass spectrometry/mass spectrometry showed that they are humulone and an unknown product. The second product is a derivative of deoxyhumulone that is probably oxidized at one carbon atom of one of the side chains, as concluded from its UV maximum at 290 nm (identical with that of deoxyhumulone) and its M_r of 362 m/z (identical with that of humulone). The amount of humulone found after incubation of deoxyhumulone with hop gland protein extracts was higher than that found after incubation of deoxyhumulone alone (Fig. 4(B)), indicating that the conversion of deoxyhumulone into humulone is enhanced by an as yet unknown factor from the hop protein extracts. There are probably two types of oxidation taking place during the incubation: a chemical reaction yielding humulone and the second product (both compounds seem to be formed in equal amounts, see Fig. 4(A)), and a conversion into humulone catalysed by the plant protein extract. The chemical reaction seems to be suppressed in the presence of hop proteins, as the formation of the second product is lower after the incubation with hop protein extracts. After extraction of proteins from the hop glands, the humulone-forming activity can be found both in the pellet and in the supernatant after centrifugation. In a separate experiment we found that solubilization of the pellet with 0.5 M K-Pi (pH 7.0) buffer yields a supernatant which contains humulone-forming activity.

When the deoxycohumulone (tested at *ca* 0.04 nmol $\mu l)^{-1}$, precursor of the α-acid cohumulone, was incubated with the pellet of a gland protein extract, an increase in the cohumulone peak was noticed on HPLC. No cohumulone was formed after incubation of desoxycohumulone with the desalted supernatant, indicating that less or no activity was present in this fraction.

Stability of humulone-forming activity in hop protein extracts

No difference in the activity was observed for young hop cones stored at −20 or −80° during six months. The activity of hop protein extracts, however, was affected by storage at −80°, since storage for two to four weeks led to a decrease in activity. The pellets were more stable than the desalted supernatant during storage. When the pellet or the supernatant was heated at 100° for 3 min, loss of activity was observed. Part of the activity of the pellet was retained, whereas the activity of the supernatant was completely lost, leaving only the chemical oxidation to humulone to be detected. Binding of the bitter acids to proteins from the pellet possibly prevents a complete loss of activity caused by denaturation after heating.

Effect of light, pH and temperature on humulone formation

Light did not influence the two types of reactions occurring in the presence or absence of hop protein extracts, since incubation with or without illumination by TL-light led to the same results. The temperature and pH during incubation were varied in seeking the optimal conditions for the following experiments. Under the conditions employed in this study, the reaction catalysed by the hop protein extracts was optimal at 25–30° and at pH 7 (Figs 5 and 6). The chemical formation of humulone was more pronounced at pH 5 than at pH 6–8 (Fig. 6).

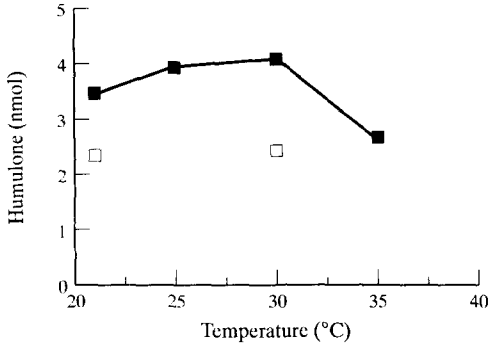

Fig. 5. Influence of temperature on humulone formation (0.1 nmol μl^{-1} deoxyhumulone, 11 μg hop soluble protein, 20 min incubation, 0.1 M K-Pi, pH 7.0) (□, without protein; ■, with protein).

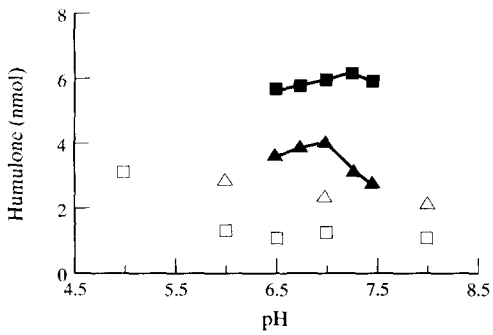

Fig. 6. Influence of pH on humulone formation (0.1 nmol μl^{-1} deoxyhumulone, 20 min incubation at 30°, 0.1 M K-Pi) (□, without pellet protein; ■, with 19 μg pellet protein; △, without soluble protein; ▲, with 11 μg soluble protein).

Influence of chemical compounds on humulone formation

The effect of oxygen on the humulone formation was studied by partial exclusion of air by flushing the buffer and the vials with argon before incubation, and by preincubation during 10 min with an oxygen-consuming system consisting of glucose, glucose oxidase and catalase. The amount of humulone formed decreased after both treatments: a decline of 22–78% was found for the chemical oxidation, while a decrease of 34–63% was noted after incubation with the hop soluble protein extract.

Enzymes with hydroxylating activities, namely peroxidases [9, 10] and α-acid oxidase [11], have been reported for hop. Hydroxylations of flavonoids are usually catalysed by cytochrome P-450 monooxygenases [12] and 2-oxoglutarate-dependent dioxygenases [13]. We found no indications that one of these enzymes was involved in the conversion into humulone by the hop proteins as the separate addition of the cofactors H_2O_2, Mn^{2+}, NADPH/NADH, or Fe^{2+}, 2-oxoglutaric acid and sodium ascorbate did not lead to more product formation. The chemical conversion of deoxyhumulone into humulone, however, was enhanced in the presence of H_2O_2 or Mn^{2+}.

CONCLUSIONS

During this study we found that hop bitter acids, being phenolic compounds, can interact with proteins in several ways. Using BSA as a model protein, it seems that in aqueous solutions the bitter acids bind to proteins at low pH, causing a loss of the phenolic compounds in the organic phase after extraction. The complex formation of the bitter acids and proteins is probably due to a hydrophobic interaction and is reversible, as lyophilization of the aqueous solutions and resuspension in methanol lead to a good recovery of the bitter acids.

During cone maturation α- and β-acids are accumulated in the glandular hairs of hop. Initially, we hypothesized that the β-acids as end products of the biosynthesis could also serve as precursors for the α-acids. In this study we found no evidence in support of this hypothesis. More probably α-acids are formed by oxidation of the intermediates deoxy-α-acids.

Although this oxygen-dependent oxidation can proceed chemically *in vitro*, the reaction is enhanced in the presence of hop gland protein extracts. The results of this study show that the conversion of deoxyhumulone into humulone is catalysed by an unknown factor from hop protein extracts. Several characteristics of this factor (e.g. extraction from glands of hop cones, loss of activity after prolonged storage and after heating, solubilization from pellets, presence in protein fraction after size exclusion, optimum at 25–30° and pH 7) indicate that they are due to a protein which is involved in the final step of the biosynthesis of hop bitter acids.

EXPERIMENTAL

Chemicals. Supercritical CO_2 hop extracts and deoxyhumulone (synthesized by Dr Brandsma, University of Utrecht) were gifts of Heineken Technical Services. Pure α- and β-acids were isolated from the CO_2 hop extracts by Mr A. C. Hoek using centrifugal partition chromatography (in preparation). Deoxycohumulone was obtained by Mr A. C. Hoek after irradiation of colupulone [8]. Other precursors of the bitter acids were synthesized as described in ref. [1]. Reagents and solvents used were of analytical grade. Mobile phases were filtered through a 0.45 μm membrane filter before use in HPLC.

Plant material. Several hop cvs were grown in our experimental garden. Young cones of hop cvs 'Brewer's Gold' (voucher no. 880500 A), 'Hersbrucker Spät' (voucher no. 880500 F), 'Olympic' (voucher no. 880500 J) and 'Yeoman' (voucher no. 910044 BB) were used. Plant material was collected in August 1995 and kept at −20 or −80° until use.

Preparation of protein extracts. All protein work took place at 4°. Glands were isolated from cones by vortexing cone bracts or by using a cell disrupter (Bead-Beater, Biospec Products, Bartlesville, U.S.A.). The second method was more efficient and resulted in

a higher yield of glands. The polycarbonate chamber of the disrupter was filled with 10–15 g cones, 75 g glass beads (diameter 0.5 mm) and Millipore H_2O. The Bead-Beater was operated for 1 min at 100 V. Plant material was then washed several times until it was almost free from glands. The glands were obtained by filtration on filter paper and stored at −20° until extraction.

Proteins extracts were prepd from glands using a pestle and mortar; 50% (w/w of amount of glands isolated) PVPP and sea sand were added, the mixt. was frozen with liquid N_2 and then extracted with 0.5 M K-Pi buffer (*ca* 10 ml g^{-1} of glands) [7], which was slightly modified (BSA and PVPP were omitted and 2% instead of 10% Dowex was used). The homogenate was filtered over a nylon filter and centrifuged at 18 000 *g* during 30 min. Both supernatant and pellet contained bitter acids. The pellet was washed with 0.05 M K-Pi (pH 7.0) containing 10% sucrose and centrifuged again for 20 min. The supernatant was desalted by gel filtration (PD-10 column, Pharmacia Biotech, Uppsala, Sweden). Elution with 3.3 ml of 0.05 M K-Pi (pH 7.0) with 10% sucrose resulted in a soluble protein fr. free from antioxidants from the extraction buffer, but which still contained traces of bitter acids. Pellets were resuspended in a small vol. of the buffer as above, and the desalted supernatant were frozen in liquid N_2 and stored at −80° until use. The amount of proteins in the extracts was determined after ref. [14].

Assays and concentration of samples. All assays and subsequent analyses were performed in duplicate; the data in the figures represent the mean of these duplicate analyses. For study of the interaction between BSA (Sigma) and hop bitter acids, 10 μl 2 mM bitter acid soln in MeOH and 50 μl 0.2–1 mg ml^{-1} BSA were added to 150 μl 0.1 M K-Pi (pH 7.0) and incubated at 20° for 15 min. After incubation, the soln was acidified by adding 10 μl 6 M HCl. The soln was then extracted ×2 with 200 μl EtOAc. The organic layers were combined and concd by using a vacuum centrifuge, operating at ambient temp. Another method of concn consisted of lyophilization of acidified samples. The residues were taken up in 100 μl MeOH. In the first experiments on humulone formation, 10 μl 2 mM lupulone or deoxyhumulone in MeOH were incubated in 150 μl 0.1 M K-Pi (pH 7.5) at 30° for 20 min, with either 50 μl protein supernatant, 25 μl pellet suspension or 50 μl H_2O. Samples were acidified after incubation. In later experiments the pH of the buffer was changed to pH 7. The amount per assay of the different tested chemicals were: 10 μmol glucose, 40 U glucose oxidase and 2 U catalase; 0.05 μmol H_2O_2; 0.01 μmol $MnCl_2$; 0.2 μmol NADPH and NADH; 5 μmol $FeSO_4$, 5 μmol 2-oxoglutaric acid and 5 μmol Na ascorbate. Sample pretreatment for HPLC analysis consisted of lyophilization, resuspension in 100 μl MeOH and centrifugation prior to injection.

HPLC analysis. Except for the deoxy-acids, precursors of the bitter acids precursors were analysed by a HPLC system described in ref. [15]. The mobile phase for HPLC analysis of deoxyhumulone and the α- and β-acids was MeOH–H_2O–85% H_3PO_4 (85:17:0.25); [3, 4]. The flow rate was 1.5 ml min^{-1}. An autoinjector (Gilson 234, Villiers le Bel, France) equipped with a 100 μl syringe was used; 14 μl were injected using centre loop filling with 3 μl of air gap. The HPLC system consisted further of an LKB 2150 pump (Bromma, Sweden), an LKB LCC 2252 controller, a precolumn (C18, 30 μm, Upchurch, Harbor, U.S.A.), a reversed phase column (Hypersil 5 C18, 250 cm × 4.6 mm, Phenomenex, Torrance, USA), an LKB 2151 variable wavelength detector and a Shimadzu CR 501 (Kyoto, Japan) data processor (set at attenuation 2^5 or 2^6). The compounds were detected at 290 nm. Analysis by HPLC-PDA was as described previously [1]. For analysis of hop bitter acids by LC-MS an electrospray interface was used; more details are provided in a separate communication (B. Hofte, in preparation).

Acknowledgements—The hop plants in our experimental garden were grown by Mr W. Snoeijer. Mr L. C. Verhagen from Heineken Technical Services is kindly acknowledged for the gift of hop supercritical carbon dioxide extracts and deoxyhumulone. Deoxycohumulone and the pure α- and β-acids were supplied by Mr A. C. Hoek. This study was financed by the Technology Foundation from The Netherlands.

REFERENCES

1. Fung, S.-Y., Brussee, J., van der Hoeven, R. A. M., Niessen, W. M. A., Scheffer, J. J. C. and Verpoorte, R., *Journal of Natural Products*, 1994, **57**, 452.
2. Zuurbier, K. W. M., Fung, S.-Y., Scheffer, J. J. C. and Verpoorte, R., *Phytochemistry*, 1995, **38**, 77.
3. Verzele, M., Dewaele, C., Van Kerrebroeck, M., Strating, J. and Verhagen, L., *Journal of the American Society of Brewing Chemists*, 1983, **41**, 36.
4. Verzele, M., Dewaele, C., Van Kerrebroeck, M., Strating, J. and Verhagen, L., *Journal of the American Society of Brewing Chemists*, 1984, **42**, 94.
5. Loomis, W. D., *Methods in Enzymology*, 1974, **31**, 528.
6. Gershenzon, J., McCaskill, D., Rajaonarivony, J. I. M., Mihaliak, C., Karp, F. and Croteau, R., *Analytical Biochemistry*, 1992, **200**, 130.
7. Claudot, A.-C. and Drouet, A., *Phytochemistry*, 1992, **31**, 3377.
8. Verzele, M. and De Keukeleire, D., *Developments in Food Science*, Vol. 27, *Chemistry and Analysis of Hop and Beer Bitter Acids*. Elsevier, Amsterdam, 1991.

9. Robins, R. J., Furze, J. M. and Rhodes, M. J. C., *Phytochemistry*, 1984, **24**, 709.
10. Fowler, M. W., Stafford, A., Martin, P. A., Stewart, G. G. and Kenber, R. M. J., in *Proceedings of the 21st Congress of the EBC*, Madrid, 1987, p. 313.
11. Menary, R. C., Williams, E. A. and Doe, P. E., *Journal of the Institute of Brewing*, 1983, **89**, 200.
12. Bolwell, G. P., Bozak, K. and Zimmerlin, A., *Phytochemistry*, 1994, **37**, 1491.
13. De Carolis, E. and De Luca, V., *Phytochemistry*, 1994, **36**, 1093.
14. Peterson, G. L., *Analytical Biochemistry*, 1977, **83**, 346.
15. Hermans-Lokkerbol, A. C. J. and Verpoorte, R., *Journal of Chromatography*, *A*, 1994, **69**, 65.