Viewpoint

Effects of acidity and molecular size on bacteriostatic properties of beer hop derivates

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Isohumulones exert bacteriostatic effects on most gram-positive bacteria. Hop compounds can cross cytoplasmic membranes in undissociated form, being dissociated internally. Transport pathway across cell membranes requires a reasonably hydrophobic solute of moderate size.

Hydrophobicities of solutes can readily be determined by measuring partition coefficients, P.

Therefore, in order to evaluate the antimicrobial activity of isohumulones and structurally similar molecules, a free energy correlation has been used which allows estimation of $\log P$ values. Moreover, the maximum molecular diameter of these molecules has been calculated by using an AM1 semiempirical calculation. A comparative analysis of the antimicrobial potential of the hop derived molecules which might be present in beer is then established.

Introduction

Hydrophobic interactions are of critical importance in many areas of food technology. These include enzyme—ligand interactions, the assembly of lipids in biomembranes, aggregation of surfactants, coagulation, and detergency (Israelachvili & Wennerstrom, 1992).

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The integrity of biomembranes and the tertiary structure of proteins in solution are determined by apolar-type interactions (Rose, Geselowitz, Lesser, Lee, & Zehfus, 1985). The hydrophobicities of solutes can readily be determined by measuring partition coefficients designated as P. Partition coefficients deal with neutral species, whereas distribution ratios incorporate concentrations of charged and/or polymeric species as well. By convention, P is the ratio of concentration of the solute in octanol to its concentration in water (Leo & Hansch, 1999). It was fortuitous that octanol was chosen as the solvent most likely to mimic the biomembrane (Fig. 1). Extensive studies over the last years have failed to dislodge octanol from its secure perch (Leo & Hansch, 1999).

Lipophilicity is a major determinant of pharmacokinetic and pharmacodynamic properties of drug molecules (Mannhold & van de Waterbeemd, 2001). In the case of hop compounds they behave as weak acids, which can cross cytoplasmic membranes in undissociated form (Sakamoto, Van Veen, Saito, Kobayashi, & Konings, 2002). The iso-α-acids, derived from the flowers of the hop plant (*Humulus lupulus* L.), give beer a bitter taste and exert bacteriostatic effects on most gram-positive bacteria due to their ability to dissipate the proton motive force (Sakamoto, Margolles, Van Veen, & Konings, 2001). Due to the higher internal pH, hop compounds dissociate internally (Blanco et al., 2006), thereby dissipating the pH gradient across the membrane (Sakamoto et al., 2002). Hop constituents were found to cause leakage in the cytoplasmic membrane, resulting in the inhibition of active transport of sugar and amino acids (Teuber & Schmalreck, 1973).

On the other hand *Lactobacillus brevis*, which showed limited decrease of intracellular pH during exposure to iso-α-acids, also showed better ability for growth in the presence of these compounds as well as in commercial beer products (Simpson & Fernandez, 1994; Yansanjav et al., 2004).

In order to evaluate the toxicity of hop derivatives in human cells, it seems that bitter acids inhibited *in vitro* formation of vascular endothelial cell and significantly prevented *in vivo* angiogenesis (Shimamura, Hazato, & Ashino, 2001). Humulone, 100 μg;/ml, induced apoptosis in the premyocytic leukemia cell line (Tobe, Kubota, Yamaguchi, Kocha, & Aoyagi, 1997). Data suggested that there was a correlation between the apoptosis-inducing activity of humulone and its antioxidative activity (Tobe et al., 1997).
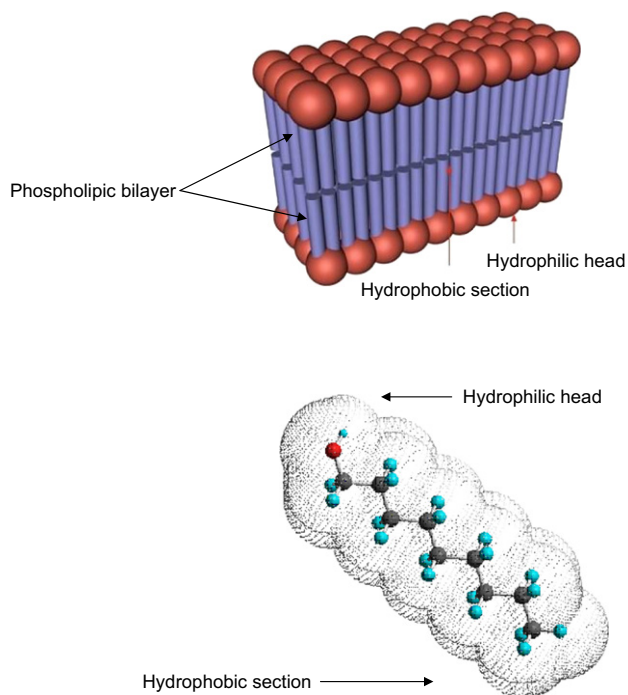
Fig. 1. Phospholipid bilayer scheme and octanol AM1 structure.

Semi-empirical calculations, such as AM1 (Dewar, Zoebisch, Heafely, & Stewart, 1985), include methods which make serious approximations to the quantum mechanical laws and then employ a few empirical parameters to patch things up. The proliferation of methodologies and programs to calculate partition coefficients continues unabated. These programs are based on substructure or whole molecule approaches (Mannhold & van de Waterbeemd, 2001; van de Waterbeemd & Mannhold, 1996), and they run on different platforms (e.g., Mac, PC, Unix, VAX, etc.) and use different calculation procedures.

Our previous studies have often dealt with correlation, some of them to estimate approximate stability constants and to predict overall equilibrium constants (Blanco, 1998). More recently, the relationship between structurally similar compounds and their physicochemical properties has been carried out (Blanco, Rojas, Verdú, Ronda, & Caballero, 2003; Rojas, Pérez-Encabo, Herraiz-Sierra, & Blanco, 2001). Furthermore, some works even focus on effective control of production processes in the brewing industry (Blanco, Caballero, Rojas, Gómez, & Alvarez, 2003; Blanco et al., 2006).

In this work, partition coefficients of *iso*-α-acids and derivates, including hydrogenated molecules such as dihydro-, tetrahydro- or hexahydro, as well as their molecular sizes have been used in order to establish bacteriostatic effects of referred species.

Ionization equilibrium

Bacteriostatic effects of *H. lupulus* derivates are usually attributed to ionization equilibrium (Sakamoto *et al.*, 2002),

this effect increased when decreasing pH (Simpson & Smith, 1992). Ionization mechanism in β-dicarbonyl compounds is closely related to keto−enol equilibrium (Rojas *et al.*, 2001). The structures of keto and enol forms, and the intramolecular O−H⋯O hydrogen bond formed by the enol tautomer have been extensively studied by a remarkable variety of methods, including H NMR, Raman and IR spectroscopy, X-ray and neutron diffraction, and theoretical calculations (Gilli, Bellucci, Ferretti, & Bertolasi, 1989). High-resolution NMR spectra yield a wide variety of information about a tautomeric system: the chemical and steric structures of the tautomers, the position and strengths of hydrogen bonds, the amounts of different tautomers, and the rate and mechanism of their interconversion (Hoek, Hermans-Lokkerbol, & Verpoorte, 2001; Verzele & de Keukeleire, 1991).

In keto−enol equilibria, in which the tautomers are only partially interconverted at room temperature, the carbon signal of individual isomers may be easily distinguishable (Rojas *et al.*, 2001). The *trans*-enolic form is rarely observed, as the *cis*-enolic form is more stable (Bolvig & Hansen, 1996). The NMR spectra of the *cis*-enolic tautomer are weighted averages of the internal enolic pairs. Assignment of the ^{13}C NMR chemical shifts was made by means of substituent effects (Bolvig & Hansen, 1996).

Chemical shifts ($\delta_{\exp}$) of β carbon relative to carbonyl groups in parts per million have been determined for a variety of representative compounds (Table 1). NMR chemical shift analysis allows us to conclude that diketones are present in keto and enol forms, whereas triketones are completely enolised. NMR information is key to know the dominant species in solution which will then be used to carry out AM1 calculations.

Hydrophobic evaluation

Hydrophobicities can be determined by partition coefficients (log *P*). Over 130 partition coefficient experimental values (Bouchard *et al.*, 2003; De Villiers, Vanhoenacker, Lynen, & Sandra, 2004; Gulaboski *et al.*, 2004; Hansch, Leo, & Hoekman, 1995), including: amino acids like valine, serine and proline; aromatic molecules such as toluene or benzene; alcohols such as 1-propanol and ethanol; organic acids such as dodecanoic acid and benzoic acid; ketones such as 2,4-pentanedione and 3,5-heptanedione; *iso*-α-acids and derivates have been represented in Fig. 2.

In order to evaluate hydrophobicities, empirical methods such as the Atomic Solvation Parameter (ASP) method often provide simple and quick ways to evaluate solvation energy (Platts, Abraham, Butina, & Hersey, 2000; Platts, Butina, Abraham, & Hersey, 1999). The ASP method assumes that the overall solvation free energy is the sum of all atomic solvation contributions (Pei, Wang, Zhou, & Lai, 2004; Tao, Wang, & Lai, 1999) (Eq. (1)):

$$\Delta G = \sum \sigma_i A_i \qquad (1)$$

Table 1. ^{13}CNMR chemical shift (δ/ppm), maximum calculated diameter (d_{max}/nm) and calculated partition coefficient (clog P)

Compound	δ/ppm	d_{max}/nm	Clog P
2-Acetyl-1,3-cyclohexanedione	110.0	0.6649	
2-Acetyl-1,3-cyclopentanedione	112.0	0.6537	
Humulinic acids	111.6[a]		
Isocohumulone		1.1664	
Isohumulone	110.7[b]	1.3620	2.90
Isoadhumulone		1.3830	
Dihydroisohumulone	110.5[b]	1.3536	2.80
Tetrahydroisohumulone		1.3851	3.00
Hexahydroisohumulone		1.3609	3.70
Cohulupone	121.0[b]	1.2300	4.15
Hulupone	121.0[b]	1.2720	4.52
2,4-Pentanedione	100.4	0.6859	0.40
2,4-Hexanedione	99.0	0.8115	0.54
3,5-Heptanedione	97.6	0.9338	1.12
2,6-Dimethyl-3,5-heptanedione	94.6	0.8435	2.22
2,2,6,6-Tetramethyl-3,5-heptanedione	90.7	0.8718	2.78
6-Methyl-2,4-heptanedione		0.8256	
2,4-Octanedione		0.9032	
2,4-Nonanedione		1.0363	
2-Acetylcyclohexanone	101.0	0.7405	1.23
2-Acetylcyclopentanone	109.0	0.6381	0.86
Phenyllacetic acid		0.8540	1.27
Sorbic acid		0.9430	1.15
3-Hydroxydodecanoic acid		1.5170	3.24

[a] Verzele and de Keukeleire (1991).
[b] Hoeck, Hermans-Lokkerbol, and Veerporte (2001).
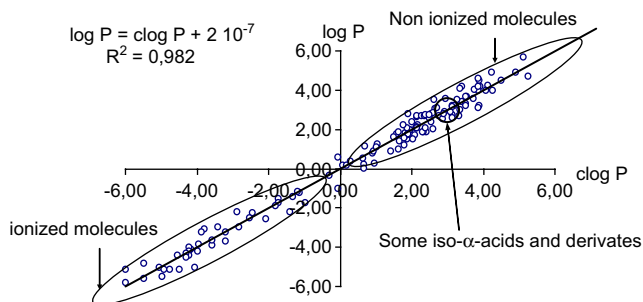
Table 2. A_iS_i contribution for molecular fragments

Molecular fragment	A_iS_i
$-CH_3$	0.3699
$-CH_2CH_3$	1.2872
Benzyl	1.8618
Phenyl	1.5027
RCOOH	−0.7516
COOMe	−0.2847
CH_2CH_2OH	−0.8329
Cl	0.5053
Nitro	−0.0755
NH_2	−0.9852
Naphthalene	2.8662
CH_2OH	−0.5991
CH_3O	−0.5286
CO	−0.6793
Pyridine	4.3771
Sulphate	−1.1987
OH	−1.1074
Phenantrene	3.9771
Ethene	0.5067
SO	−1.6542
Amide	−1.1322
F	−1.1660
Br	0.9454
I	1.0656
CN	−0.9005
NH_3 (correction for amino acids)	−3.3441
S	0.2840
O (ionized forms)	−7.8238
CO_2 (ionized forms)	−8.3786

where ΔG is the solvation free energy, σ_i is the solvation energy for each atom and A_i is the solvent-accessible surface area for each atom.

Taking into account Eq. (2), the following equation may be developed for each molecular fragment:

$$\log P = C + \sum A_iS_i \qquad (2)$$

where C is a constant, and S_i and A_i are, respectively, the solvation parameter and the solvent-accessible surface area parameter for each fragment. The Austin Model method (AM1) can be used for calculating A_i parameters. This procedure enables calculation of S_i parameters for

each fragment as well as the C constant value, which is equal to 0.5229. Table 2 shows A_iS_i fragment contributions.

The agreement between bibliographic log P (Bouchard et al., 2003; Gulaboski et al., 2004; Hansch et al., 1995) and calculated octanol/water values clog P, by using Eq. (2), is satisfactory. Fig. 2 shows a very good agreement with excellent correlation coefficients, where calculated values (clog P) are represented against bibliographic log P values.

Size effects

Transport through membranes requires a reasonably lipophilic solute of moderate size, and numerous studies indicate that the vast majority of well absorbed drugs are transported passively across cell membranes (Stenberg, Luthman, & Artursson, 2000). It is very important to know the maximum diameter of molecules, d_{max} (Table 1), is closely related to bioconcentration (Dimitrov, Dimitrova, Walker, Veith, & Mekenyan, 2002). The higher the molecular length, the smaller the chances of the molecule reaching the cell membrane at an appropriate angle (Dimitrov et al., 2002). The threshold of maximum diameter is comparable with the half thickness of a leaflet constituting the lipid bilayer of the cell membrane (Horton, Moran, Ochs, Rawb, & Scrimgeour, 1992). It seems that diffusion through the cell membrane is limited to molecules having a length not exceeding the threshold of about 1.5 nm

Fig. 2. Experimental partition coefficient, log P, vs. calculated partition coefficient, clog P.

(Dimitrov *et al.*, 2002). Molecular length of isomerised *H. lupulus* derivates (Table 1) indicated a great tolerance of the cell membrane.

The contribution of the partition coefficients to the central triketonic structure (Fig. 3) was determined by using the experimental value obtained chromatographically by De Villiers *et al.* (2004), log $P = 2.9$, from which a value of $A_iS_i = -1.13$ for the core has been obtained (Fig. 3). On the basis of this core-structure value, the partition coefficient for a series of humulone derivatives has been calculated (Fig. 2). Calculated values (clog P) in Table 1 point out that the lipophilia of hexahydroderivates is higher than that of the tetra-, which is, in turn, higher than that of non-hydrogenated derived (isohumulones), which present a higher lipophilic degree than the di-hydrogenated derived.

The two-step partitioning process can be rationalized by considering the insertion of a polar, but lipophilic, solute into a phospholipid membrane (Jacobs & White, 1989). Lipophilicity constitutes the major driving force of solute accommodation into the region of the phospholipid head groups (Jacobs & White, 1989). The anisotropic nature of the membrane has been included in a model in which membrane partitioning is considered to be a two-step process, and this model has been successfully applied to describe drug transport (Burton, Conradi, Hilgers, Ho, & Maggiora, 1992). Clog P results (Table 1) appear to indicate that *iso*-β-acids (hulupones) are more lipophilic than *iso*-α-acids (isohumulones).

In contrast, transfer of solute into the interior of the phospholipid bilayer depends mainly on energetically unfavourable interactions between the bilayer and the polar parts of the solute.

Since *iso*-β-acids present the highest partition coefficients, they will be the ones most capable of crossing the phospholipid bilayer, although, once inside, they will also be the best retained due to their high number of apolar chains. On the other hand, molecular size seems also to affect membrane crossing rates: the larger the size of the humulone derivative (Table 1), the more they will be retained by the membrane. *iso*-α-Acids present a smaller partition coefficient and longer diameter (Fig. 4) due to which they

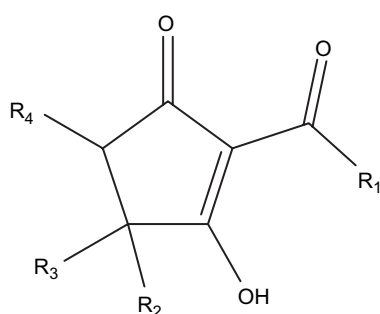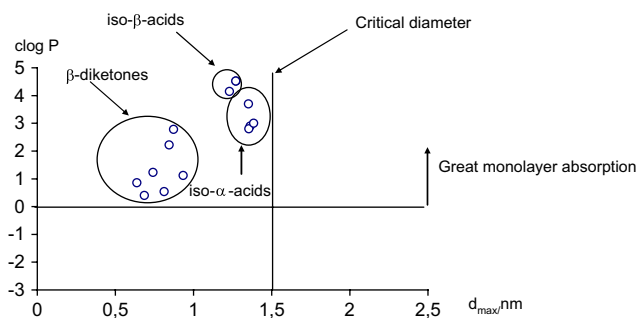**Fig. 4.** Calculated partition coefficient *vs.* maximum diameter of molecules.

will cross the bilayer in a small proportion whenever *iso*-β-acids are not ionized.

The importance of partition coefficients and pK_a value to bacteriocidal activity is not limited to hop compounds. It is generally applicable to a number of antimicrobial compounds used in foods (Table 1), sorbic acid, 3-hydroxydodecanoic acid and phenyllactic acid which prove to have a related mode of action (Corsetti, Gobbetti, Rossi, & Damiani, 1998; Lavermicocca *et al.*, 2000; Lowe & Arendt, 2004).

Minimum inhibitory concentration

The concentration of *iso*-α-acids in beer ranges from about 10 up to ca. 100 ppm, hulupones and α-acids are a few parts per million and for β-acids parts per billion (Benitez *et al.*, 1997). When the concentration of *iso*-α-acids (*trans*-isohumulone) exceeded 72 ppm, the proton motive force is dissipate for hop-sensitive *Lactobacillus*, although when the hop-resistant *Lactobacillus* is exposed to 72 and 144 ppm *iso*-α-acid, the force is reduced but not abolished (Simpson & Fernandez, 1994). It seems that the relative sensitivity of hop-sensitive and hop-resistant bacteria to *trans*-isohumulone was maintained at different pH values although, as expected, the MICs were lower at lower pH values (Simpson & Fernandez, 1994). The minimal concentrations needed to prevent growth of hop-sensitive microorganisms were about two to four times higher than the 50%
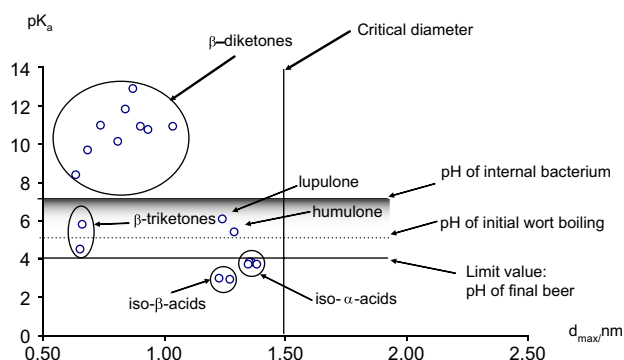
Fig. 3. The triketo core structure.

Fig. 5. Ionization constant *vs.* maximum diameter of molecules.

values for both humulone (α-acids) and isohumulone (*iso-α*-acids) at pH = 7 (Teuber, 1970). However, in the 3.8–4 pH range, the antibacterial activity of *trans*-isohumulone was ca. 20 times greater than that of humulone (Simpson & Smith, 1992), since the proportion of undissociated molecules increased when decreasing pH values (Simpson & Smith, 1992).

In order to explain these effects it is important to take into account that the degree of ionization depends on the pH of the solution. In beer systems pH varies from 4 to 5, and since pK_a is lower than 5 for isomerised derivatives (Fig. 5) and higher than 5 for non-isomerised (Blanco *et al.*, 2006), under these conditions isohumulone will not be ionized, and its derivatives will be ionized in a small extent, so they will all be able to cross the membrane easily. However, *iso-β*-acids, which present pK_a values close to 3, will be totally ionized and it would be quite difficult for them to cross the membrane (Fig. 5). Humulone (α-acids) and lupulone (β-acids) can cross cytoplasmic membranes in undissociated form and dissociate into the cell (Fig. 5) but they dissolve to a lesser degree in beer and water.

Conclusions

A quick look at Fig. 4 allows us to conclude that *iso-β*-acids are the hop derivatives with the greatest ability to cross cellular membranes, since they present the highest value of the partition coefficient and a relatively small maximum diameter value. However, Fig. 5 shows that *iso-α*-acids present the appropriate pK_a to cross membranes in their molecular form.

Once inside the bacteria in a pH range 7.6–7.8 (Bouhss, Crouvoisier, Blanot, & Mengin-Lecreulx, 2004; Harold & Maloney, 1996), both *iso-α*-acids and *iso-β*-acids ionize modifying the proton concentration inside and outside the cell, affecting cellular metabolism in an important way.

Moreover, since *iso-β*-acids are present in beer in very small amounts since they are degraded during the wort boiling, it could be concluded that *iso-α*-acids would present much better bacteriostatic properties, conclusion which is consistent with previous bibliographic studies (Simpson & Fernandez, 1994; Simpson & Smith, 1992). Among *iso-α*-acids potentially present in beer, hexaderivatives present the longest diameter, although their length does not exceed the threshold of about 1.5 nm. Further, hexahydroisohumulone presents a partition coefficient almost 10 times higher than isohumulona which would make this hexahydrogenated derivative quite a good bacteriostatic agent when present in beer.

Acknowledgement

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