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ARTICLE

Mechanisms of Hop Inhibition: Hop Ionophores

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In this work, the mechanism of hop inhibition toward (beer spoiling) bacteria is revised. The mode of action of iso-α-acids was investigated via bilayer lipid membrane (BLM) measurements and growth challenges of hop-sensitive and -resistant *Lactobacillus brevis* strains in the presence of uncouplers of class I and II or a H^+/Mn^{2+} exchanger. The antibacterial action of iso-α-acids as proton ionophores could be confirmed by the BLM measurements; however, the reported ionophore properties, as electroneutral H^+/Mn^{2+} exchangers, could not be verified. Potentiometric measurements indicated the manganese-dependent enhancement of transmembrane charge permeation. The origin of high membrane potentials in the presence of manganese, as well as the strongly elevated membrane conductivity with concomitant increase in effectiveness of an uncoupler, suggest a different origin of charge transfer under these conditions. The mode of antibacterial action of hop ionophores can be described as proton ionophores of class I/II, which are capable transporting protons within a wide range of pH due to their inherent complexity of chemical composition. However, growth challenges in the presence of both types of ionophore classes in combination with the measured unusual high BLM potentials in the presence of manganese and at low pH indicate an additional mechanism of inhibition by hop compounds. The latter may be due to the nature/properties of hop compounds, which are known to be highly reactive substances. As a consequence, hop resistance of bacteria can be described as multiple resistance to a heterogeneous mixture of compounds comprising different known and yet unknown charge transport mechanisms, which were dependent on several factors, for example, compound concentrations, cation composition, and pH value. Thus, only specialists such as some *L. brevis* strains, which can cope with unusually low intracellular manganese levels, can survive hop stress. Accordingly, cross-resistance to single proton ionophores or H^+/Mn^{2+} exchangers was not detectable and cannot be expected.

KEYWORDS: *Lactobacillus brevis*; hop inhibition; bilayer lipid membranes; ionophores

INTRODUCTION

The tradition of using the inflorescences of the hop plant *Humulus lupulus* for beer brewing was documented first in the sixth century B.C. (*1*). In addition to the comfortable bitterness (*2*) and flavor of hop compounds in beer (*3*, *4*), they exhibit an inhibitory effect on bacteria. The antibiotic and bacteriostatic properties of hops were discovered about 70 years ago. From Shimwell in 1937 to Simpson in the 1990s (*5*, *6*), several factors of inhibitory mechanisms of hop constituents were uncovered, namely, permeability changes of the bacterial cell wall (*5*), leakage of the cytoplasmic membrane and a subsequent inhibition of respiration and protein, DNA and RNA synthesis (*7*) as well as changes in leucine uptake and proton ionophore activity (*6*). This antibacterial activity was found to be restricted to Gram-positive bacteria, whereas Gram-negatives were nearly not inhibited, probably as a result of their hop-shielding outer membrane (*8*). A dependence of the inhibitory effect of hops on pH value and the decrease and increase of antibacterial activity in the presence of divalent cations, such as Mn^{2+} and monovalent cations such as K^+, respectively, was found by Shimwell and Simpson (*5*, *6*).

In summary of these literature data, hop inhibition has been described as the effect of hop compounds to act as ionophores, which dissipate the pH gradient across the cytoplasmic membrane and reduce the proton motive force (pmf). Consequently, the low intracellular pH interferes with essential enzyme reactions, and the pmf-dependent nutrient uptake is hampered, resulting in cell death of hop-sensitive strains (*1*, *9*, *10*). The proton influx is therefore described to be mediated by a proton/divalent cation exchange across the bacterial cytoplasmic membrane (*9*). However, the expected cross-resistance to other proton ionophores could not be detected, and no antibiotic tested until now could simulate the stress induced by hop compounds in bacteria (*11*). Accordingly, the antibacterial mechanisms of hop compounds, derived from measurements in biological systems (*9*, *12*−*14*), cannot fully explain the hop inhibition in bacteria. This may be related to the fact that, under these circumstances, the influence of bacterial stress response or decay

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[e.g., hop transport by HorA, possible manganese transport by HitA (*15*, *16*) or the loss of bacterial membrane integrity] on the results is outside the control of the experimenter. Consequently, an approach for better understanding the stress on bacteria committed by hop compounds is required. For the latter, a phenomenological investigation of the mode of antibacterial action of hop compounds was carried out. In this context, the term "phenomenological investigation" points to the fact that an empirical relationship of occurring phenomena (e.g., proton ionophore action and manganese binding of hop compounds) on account of definite experimental conditions is developed. These measurable phenomena may result from several parallel molecular events (complexity of chemical composition of hops), which are basically unknown and for which up to now no theory exists. This analytical approach is based on electrochemical investigations in reconstituted planar bilayer lipid membranes (BLMs) (*17*−*19*). As the inhibitory action of hop compounds on bacteria was found to be associated with the bacterial membrane (*9*), the investigation of hop inhibition with membrane participation is required. Life processes taking place in cell membranes (e.g., membrane-associated electron and proton transfer in bioenergetics) comprise complex processes and, thus, the employment of simpler membranes to evaluate function and membrane participation of the latter is necessary (*17*, *20*). BLMs are used as such "simpler" models for biological membranes (e.g., cytoplasmic membranes of bacteria) as they exhibit similar structures and physicochemical properties. They allow studies on parameters and functions of membrane components (e.g., phospholipids), as well as on the impact of biologically effective substances on membranes. The disciplines of BLM measurements allow several parameters of the membrane, for example, their thickness and area for, for example, BLM quality control (capacitance methods), membrane potentials (potentiometry), and conductance (amperometry) (*17*) to be measured. The information provided by measurements in "simpler" membranes makes biological data more meaningful (*21*). Such measurements were published, for example, for ionophores such as calcimycin, nigericin, CCCP, and the ion channel gramicidin (*18*, *22*−*24*), which are commonly used for biological experiments. Consequently, several characteristics of membrane-active substances can be investigated, which as a result can undoubtedly contribute to the understanding of biological membrane processes (*17*).

MATERIALS AND METHODS

Characterization of Isomerized Hop Extract. The chemical composition of the isomerized hop extract Isohop (precipitated and dissolved in methanol; Joh. Barth & Sohn GmbH & Co., Nuernberg, Germany) was determined by Roland Schmidt via HPLC-DAD (Nateco2 GmbH u. Co. KG, Mainburg, Hallertau, Germany). A titration curve of the isomerized hop extract at a concentration of 3.5 mM iso-α-acids in 0.2 M KCl in bidistilled water (set to initial pH of 2.6) was generated by a conventional method under nitrogen atmosphere (*25*). The changes of pH were measured with a pH-meter (761 calimatic, Knick GmbH, Berlin, Germany) under stirring. As titrant 0.1 M NaOH was used. The degree of protonation was calculated as P = (protonated iso-α-acids/total iso-α-acids) = $1 - [(\Delta v_{ba} \times N)/V_{total})$/total iso-α-acids concentration], where Δv_{ba} = titrant volume$_{\text{iso-α-acids titration}}$ − titrant volume$_{\text{blank titration}}$ and N is the molarity of titrant and total titration volume $V_{total} = V_{titration} + v_{titrant}$ (*26*).

Phenomenological Investigation of Mode of Action of Hop Compounds via Bilayer Lipid Membrane Techniques. Hop-resistant *L. brevis* TMW 1.465 (*27*, *28*) was grown in 10 L mMRS4 for 10 days at 25 °C to a transient to stationary growth phase. Daily the pH of the growth medium was adjusted to 6.0. The cells were harvested by centrifugation at 25 °C and 2500*g* for 90 min and washed twice with phosphate buffer (50 mM, pH 7.0). The natural membrane lipids from *L. brevis* TMW 1.465 cells were extracted and purified as previously described (*29*−*31*). Aliquots of 1 mg of *L. brevis* membrane lipids were stored under nitrogen at −80 °C. Further egg phosphatidylcholine (E PC S, Lipoid GmbH, Ludwigshafen, Germany) and synthetic phosphatidyl glycerol 16:0/16:0 (DPPG, Lipoid GmbH, Ludwigshafen, Germany) were used. Membrane-forming mixtures contained 20 mg of lipids and 20 mg of cholesterol (*32*) per 1 mL of decane. The bilayer lipid membrane (BLM) was formed in a 1.1 mm hole in a 0.2 mm thick Teflon partition by the Mueller−Rudin method (*32*) using a symmetrical 2 mL Teflon BLM chamber with glass windows on both sides. Sintered Ag/AgCl electrodes (In Vivo Metric, Healdsburg, CA) were connected to the electrolyte solution through 10 μL pipet tips filled with 2% agarose in 0.2 M KCl. Capacitance, open circuit, and chronoamperometric measurements were performed with a custom-made microchip-controlled two- or four-electrode capacitance measurement and potentiostat/galvanostat system (measurement setups for BLM experiments are available as Supporting Information Figure S1) built on the basis of previous descriptions of Kalinowski et al. (*18*, *19*). The formation of the membrane was controlled visually by a microscope and by the measurement of its electric capacitance. The measurements were performed thermostatically (Multitemp III, Amersham Biosciences Europe GmbH, Freiburg, Germany) at 23 °C. Isomerized hop extract Isohop (Joh. Barth & Co., Nuernberg, Germany), ionophores A23187 (calcimycin) and CCCP, and salts of divalent cations $CaCl_2$, $MgCl_2$, and $MnCl_2$ were used. Unless otherwise stated, KCl (0.2 M) was used as supporting electrolyte. Electrolyte solutions were buffered with Tris, Mes,and citric acid (5 mM). pH gradients were set by the addition of 1 M HCl. All solutions were stored under nitrogen.

Correlation of Hop Action in BLMs to Hop Resistance in Bacteria. Growth challenges with hop-sensitive *L. brevis* TMW 1.6 and 1.1369 and hop-resistant TMW 1.313 and 1.465 were done in mMRS4 medium (*33*) without cysteine at pH 4.3 (for the calcimycin and hop compound MIC assays the divalent cation content of mMRS4 was reduced to 98 mg/L magnesium and 0.16 mg/L manganese according to the divalent cation content of a pilsner lager beer (personal communication, Anton Piendl, Institut für Brauereitechnologie and Mikrobiologie der Technischen Universität München, Germany) and additions of various concentrations of antibiotics CCCP, DNP, or A23187 (calcimycin) and hop compounds. The concentrations were varied from 0.02 to 0.2 mM, from 0.1 to 1.0 mM, and from 2 to 20 μM in 10% steps for CCCP, DNP, or calcimycin and hop compounds, respectively. Controls were performed without the addition of antibiotics. Growth challenges were carried out in microtiter plates sealed with parafilm using resazurin as metabolic growth indicator (*34*). The inoculation density was set to $OD_{590\ nm} = 0.10$. The metabolic tests were analyzed after a 3 days of incubation at 30 °C via the addition of 80 mM Tris-HCl (pH 8.8) to each well to achieve a pH in the range of pH 5.5−11.0 (highest pH tested), at which resazurin shows a blue color in oxidized form and a pink color in its reduced form, resorufin.

RESULTS

Characterization of Isomerized Hop Extract. The chemical composition of the isomerized hop extract is shown in **Table 1**. For dosage of hop compounds the molarity of the isoextract, which contained 98.4% (w/v) iso-α-acids, was calculated on the basis of the molecular weight of *trans*-isohumulone. To understand the role of solution pH and acid/base properties of hop compounds, from an isomerized hop extract a titration curve was established. The degree of protonation P = (protonated iso-α-acids/total iso-α-acids) as a function of the pH value was calculated (Supporting Information Figure S2). The isomerized hop extract showed buffer capacities between pH 2.6 (P set to 1) and pH 3.6 (P = 0.25) and between 4.4 (P = 0.23) and 6.4 (P = 0.11) in accordance with the published pK_a values for, for exmaple, *trans*-isohumulone (3.1), humulone (5.0), and colupulone (6.1) (*14*). A second buffer range was determined between pH 9.0 (P = 0.08) and 11.4 (P = −0.14; the negative value is due to unknown buffering substances, which were part of the hop extract). As a drift of measured pH was observed (*14*) and precipitations at low pH could not be avoided at given hop

Table 1. Determination of Iso-α-acids and α-Acids in Isomerized Hop Extract via HPLC-DAD Analysis

	% (w/v)		
	isohumulone	humulone	lupulone
cocompound	6.4	0.1	0.1
n-compound	14.3	0.2	0.0
ad-compound	3.6	0.0	0.0
sum	24.3	0.3	0.1

compound concentrations, an influence of chemical conversions due to the high reactivity of hop compounds (*35*) or shifts in concentrations of hop fractions on the titration result cannot be excluded.

Optimization and Control Experiments for Reproducible Bilayer Lipid Membrane Measurements. To obtain basic information on the mode of action of hop compounds in biological membranes, a bilayer lipid membrane measurement system was set up according to Kalinowski et al. (*18*, *19*). Capacitance measurements during the membrane formation ensured similar initial conditions for all experiments. The influence of variation of the supporting electrolyte on the measurement result was negligible for NaCl, KCl, and choline chloride. As the solubility of high concentrations of hop compounds was best in KCl solution, it was chosen as electrolyte at a concentration of 0.2 M. The influence of buffer composition was assessed for the buffer compounds Hepes, Tris, Mes, histidine, phosphate, and citric acid, which are generally used for BLM experiments. The choice of buffer did not significantly influence the measurement result (e.g., conductance data at pH 7.0 for 60 μM iso-α-acids in Hepes buffer, 1.4 e^{-10} Ohm^{-1}; in histidine buffer, 1.7 e^{-10} Ohm^{-1}). For large-range pH variation experiments the composition Tris, Mes, and citric acid (5 mM each) was chosen (*22*). Divalent cations were added as chlorides and sulfates, resulting in no measurable influence of the counteranion at given concentrations. As lipids, natural lipids isolated from *L. brevis* TMW 1.465, egg phosphatidylcholine (PC), and synthetic phosphatidylglycerol 16:0/16:0 (GP; Lipoid GmbH, Ludwigshafen, Germany) were used. The mode of action of hop compounds in all lipids was consistent, but with higher markedness for PC/GP and PC. PC was favored for experiments, when a change in membrane surface potential mediated by a screening effect of divalent cations on charged membranes (GP and possibly *L. brevis* lipid) should be avoided (*36*). To assess the role of mono- and divalent cations on membrane potential in the presence of hop compounds, NaCl, KCl, $MnCl_2$, $MgCl_2$, and $CaCl_2$ were used. The experiments were recorded at pH 7.0 (Tris, Mes, choline chloride) and a salt gradient of 1/100 in the presence of 86 μM iso-α-acids. Monovalent cations Na^+ and K^+ caused membrane potentials of 0.3 ± 1.6 and 1.0 ± 0.3 mV, respectively. Divalent cations Mn^{2+}, Mg^{2+}, and Ca^{2+} resulted in potentials of 65.1 ± 5.5, 28.2 ± 3.8, and 10.8 ± 2.2 mV, correspondingly. As Mn^{2+} showed the greatest effect, only $MnCl_2$ was used for further investigations.

Comparison of Isomerized Hop Extract and A23187 (Calcimycin). As initial measurement, a comparison of the mode of action of hop ionophores, which have been described as proton/manganese exchangers (*9*), with the well-characterized proton/divalent cation exchanger calcimycin was recorded (*24*, *37*, *38*). Calcimycin exhibits a high affinity for the divalent cations $Mn^{2+} > Ca^{2+} = Mg^{2+}$ (*39*). The nonelectrogenic divalent cation/proton exchange can be monitored by equalizing the proton transport of the exchange by the addition of a proton ionophore as TTFB or CCCP (*37*, *40*). Thus, the divalent cation transport results in a potential that is positive on the side of lower divalent cation salt concentration. The change in membrane potential

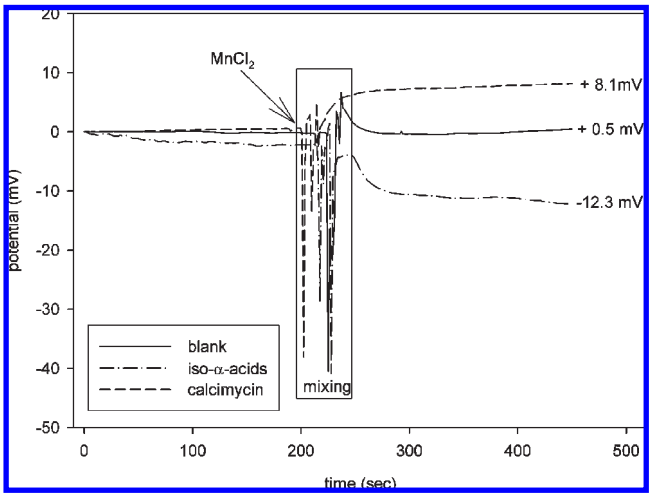

Figure 1. Potential generation on BLM (*L. brevis* lipid) upon addition of $MnCl_2$ (5 mM) in one compartment of the electrolytic cell in the presence of 10 μM CCCP and 86 μM iso-α-acids or 5 μM calcimycin or no addition of an additional ionophore (blank). Composition of aqueous solutions: Tris, Mes, KCl (pH 7.0).

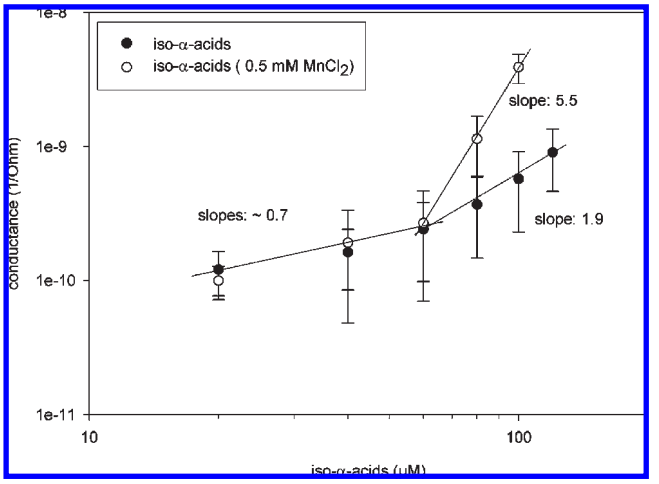

Figure 2. Dependence of membrane (*L. brevis* lipid) conductivity at 50 mV upon the variation of the aqueous concentration of hop compounds with and without $MnCl_2$ (0.5 mM) addition to both compartments of the electrolytic cell. The slopes change in log(conductance) per log(iso-α-acids concentration) are indicated. Composition of aqueous solutions: Tris, Mes, KCl (pH 7.0).

after the addition of $MnCl_2$ is shown in **Figure 1**. Under the same experimental conditions hop compounds cause a potential difference opposite in sign in comparison to calcimycin.

Conductivity Data from BLMs in the Presence of Hop Compounds. Conductance data of BLMs, which will deliver information concerning the general mechanism of charge transport through the BLM, as the class of an ionophore, were recorded principally according to the method of Foster et al. (*41*) upon variation of the hop compound concentration, pH values, the presence and absence of $MnCl_2$ (information about the dependence of BLM conductance in the presence of hop compounds upon variation of $MnCl_2$ concentrations is available as Supporting Information Figure S3) in aqueous solutions, and lipid composition. **Figure 2** shows the dependence of membrane conductivity upon the variation of the aqueous concentration of hop compounds with and without $MnCl_2$ addition at a pH of 7.0. A linear dependence of conductance versus log(hop compound concentration) is observed, which

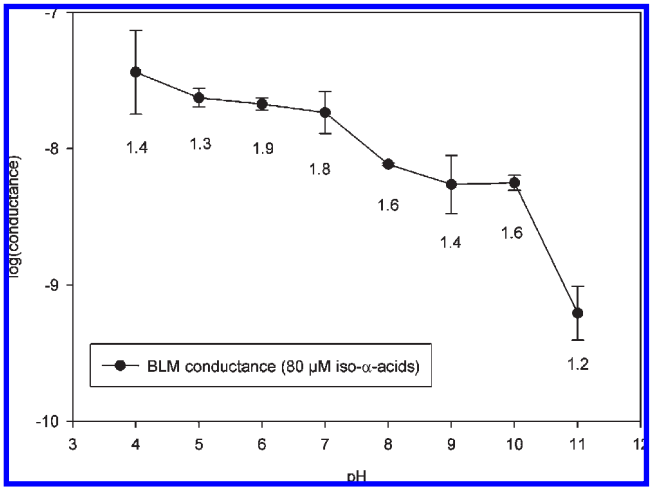

Figure 3. Dependence of membrane (PC) conductivity at 25 mV upon the variation of pH value calculated for 80 μM of hop compounds. The slopes change in log(conductance) per log(iso-α-acids concentration are indicated. Composition of aqueous solutions: Tris, Mes, citrate, KCl at pH indicated.

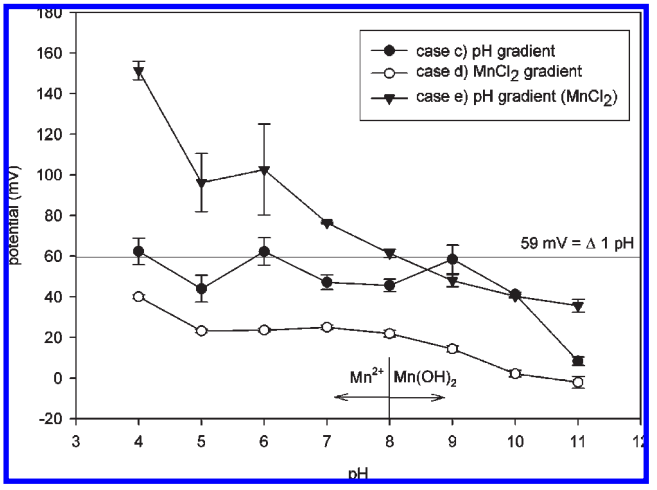

Figure 4. Potential generation on BLM (PC) upon the setup of a ΔpH (1 pH unit; case c), a $MnCl_2$ gradient (case d), and a ΔpH (1 pH unit) in the presence of $MnCl_2$ (250 μM in both compartments; case e) between the compartments of the electrolytic cell in the presence of 10 μM iso-α-acids in both compartments of the cell at the pH indicated. The potential is positive on the side of higher pH (cases c and e). The potential is positive on the side of $MnCl_2$ addition (case d). Composition of aqueous solutions: Tris, Mes, citrate, KCl at the pH indicated.

changes slope at a given hop compound concentration of 60 μM iso-α-acids for both conditions. The slope from 20 to 60 μM iso-α-acids indicates a direct proportionality of the charge transport process across the BLM to the hop compound concentration. Above 60 μM iso-α-acids the conductance increases to a higher order of magnitude, particularly in the presence of Mn^{2+}. The dependence of conductivity on the pH value of the aqueous solution is shown in **Figure 3**. The conductance was calculated for identical concentrations of iso-α-acids (80 μM) at respective pH. A decrease of BLM conductance over pH in the range from pH 4.0 to 7.0, followed by a more distinct decrease in the range from pH 7.0 to 11.0 (except for the conductance calculated for pH 10.0), was found. The slope of change in BLM conductance due to the hop compound concentration in dependence of solution pH was >1 [1 = direct proportionality (class I) and 2 = quadratic proportionality (class II) of ionophore concentration and charge transport across the BLM (*42*,*43*)] for any pH measured. The slope was about 1.4 between pH 4.0 and 5.0 and between pH 8.0 and 11.0 and nearly 2 (1.9) for pH 6.0 and 7.0. The influence of the lipid composition on BLM conductance, which will allow the charge of the translocator species (*36*) to be assessed, was recorded. To obtain BLMs with different surface charges, *L. brevis* lipid was mixed with the zwitterionic, but net neutral, PC in a ratio of 2:3 [PC/*L. brevis* lipid (w/w)] and PC was mixed with negatively charged GP [1:1(w/w)]. The conductance in the presence of hop compounds decreased with higher content of GP or *L. brevis* lipid with regard to the PC content of the membrane. The conductance of PC-doped *L. brevis* lipid membranes increased by 49%. The conductance of GP/PC [1:1(w/w)] compared to PC membranes decreased by 33 ± 4%.

Membrane Potential Data from BLMs in the Presence of Hop Compounds. Membrane potential data, which will deliver information concerning the driving forces of charge permeation through the BLM and characterize the charge transport capabilities of an ionophore under different experimental conditions, were recorded upon variation of the hop compound concentration, pH values, and $MnCl_2$ concentration. The membrane potentials were generated as follows: the solutions separated by the membrane exhibited (a) the same pH and the hop compound concentration gradient was varied; (b) the same pH and the hop compound concentration and the $MnCl_2$ concentration gradient was varied; (c) the same hop compound concentration, but differing in a fixed ΔpH; (d) the same pH and hop compound concentration, but differing in a fixed $MnCl_2$ concentration gradient; and (e) the same hop compound and $MnCl_2$ concentration, but differing in a fixed ΔpH. For case a the potential generated shows a linear dependence of potential versus log (hop compound concentration). The potential increased for each order of magnitude of hop compound concentration by 3.5 ± 0.4 mV at pH 4.0, by 5.2 ± 2.2 mV at pH 7.0, and by 5.1 ± 1.4 mV at pH 10.0 (Supporting Information Figure S4). For case b the potential was linearly dependent on log($MnCl_2$ concentration) with an increase of 37.5 ± 6.1 mV for pH 4.0 and and an increase of 43.8 ± 3.9 mV for pH 7.0 per power of 10. At pH 10.0 a nonlinear behavior of $MnCl_2$-dependent membrane potential was monitored. An elevated hop compound concentration shifted the pH 10.0 potential curve toward the results derived from the other pH values (Supporting Information Figure S5). The data for case c are depicted in **Figure 4**. A pH gradient of 1 pH unit was set up for each pH and the iso-α-acids concentration was kept constant at 10 μM. From pH 4.0 to 9.0 the hop-doped membrane worked nearly perfectly as pH electrode. An average signal of 53.3 ± 9.3 mV (theoretic value = 59 mV) was generated by a ΔpH of 1.0. Above pH 9.0 the potential decreased to the final value of 8.4 ± 2.0 mV at a pH of 11.0. Case d is shown in **Figure 4**. At pH 4.0 a high membrane potential of 40.0 ± 1.0 mV was monitored. From pH 5.0 to 8.0 the potential was constant at 23.5 ± 1.3. Above pH 8.0 it decreased to 2.0 ± 2.9 mV. The highest membrane potentials were measured for case e and are shown in **Figure 4**. It has to be stressed that case e is not an addition of the potentials measured for conditions c and d. The aqueous solutions separated by the membrane contained each 10 μM iso-α-acids and 250 μM $MnCl_2$, which will not cause any potential difference across the membrane. The measured high potentials are due to the same ΔpH as in condition c and the presence of low amounts of $MnCl_2$ on both sides of the membrane. The ΔpH = 1 caused potential across the membrane was 121.7 ± 46.4 mV at pH 4.0 and decreased nearly linearly over pH up to the pH 8.0 condition. From pH 8.0 to 10.0 the measured potential coincides with the data gained for case c, indicating that the $MnCl_2$ addition does not influence the proton gradient driven charge transport process

Table 2. Determination of Minimum Inhibitory Concentrations (MIC in Micromolar) of Hop Compounds and Different Classes of Antibiotics[a] for Hop-Sensitive *Lactobacillus brevis* TMW 1.6 and 1.1369 and Hop-Resistant TMW 1.465 and 1.313 in mMRS4 pH 4.3[b,c]

strain	hop compound	CCCP[d]	DNP[d]	calcimycin
TMW 1.6	4	20/40	200/200	4
TMW 1.1369	6	180/180	500/600	6
TMW 1.465	20	>200/80	500/400	6
TMW 1.313	20	180/60	400/300	8

[a] CCCP, class I proton ionophore; DNP, class II proton ionophore; calcimycin, proton/divalent cation exchanger. [b] For calcimycin and hop compounds MIC assays the divalent cation content was reduced to 98 mg/L magnesium, 0.16 mg/L manganese. [c] The data are representative of two independent experiments. [d] The number following the slash is the MIC in medium used for calcimycin and hop compounds assays.

in this range. Only for case e were high standard deviations obtained in the acidic measurement range. ΔpH as sole source of potential generation cannot explain the high measurement values observed at low pH.

Correlation of Hop Action in BLMs to Hop Resistance in Bacteria. Growth challenges with hop-sensitive *L. brevis* TMW 1.6 and 1.1369 and hop-resistant TMW 1.313 and TMW 1.465 were done in the presence of antibiotics CCCP (class I proton ionophore), DNP (class II proton ionophore), calcimycin (proton/divalent cation exchanger), or hop compounds. The minimum inhibitory concentrations (MIC) are given in **Table 2**. Hop-sensitive strains of *L. brevis* exhibited a MIC of 4 or 6 μM hop compounds, whereas hop-resistant strains showed a >3 times higher MIC of 20 μM. The resistance of hop-sensitive strains to a class I uncoupler CCCP strongly varied. *L. brevis* TMW 1.6 showed a MIC of 20 μM, wheeras TMW 1.1369 as well as hop-resistant TMW 1.313 exhibited a MIC of 180 μM CCCP. Only TMW 1.465 grew under concentrations of 200 μM CCCP. For uncouplers of class II a similar result was found, whereas the highest MIC was switched between the hop-resistant strains. The resistance to an electroneutral proton/divalent cation exchanger calcimycin was identical to the hop resistance for hop-sensitive strains TMW 1.6 (4 μM) and TMW 1.1369 (6 μM), but did not correspond to hop resistance in hop-resistant strains TMW 1.465 (6 μM) and TMW 1.313 (8 μM).

DISCUSSION

Mode of Action of Hop Compounds in Bilayer Lipid Membranes. To improve the understanding of the inherent complexity of the antibacterial action of hop compounds, a phenomenological investigation on the mode of action of hop compounds in BLM was realized. BLM measurements were performed with regard to pH gradients or manganese gradients in solutions separated by the membrane. A comparison of membrane potential formation due to a $MnCl_2$ gradient in the presence of hop compounds or A23187 (calcimycin) indicated that, under the same experimental conditions, hop compounds cause a potential difference opposite in sign in comparison to calcimycin. This observation clearly pointed to a mode of action of hop compounds, which is different from that of a nonelectrogenic proton/divalent cation exchanger.

The membrane conductivity will deliver information related to the general mechanism of charge transport through the BLM, as the class of an uncoupler. Such ionophores can be divided into at least two classes, which exhibit different mechanisms of charge permeation. Uncouplers of class I exhibit a linear dependence of membrane conductance on the ionophore concentration (*42*). For uncouplers of class II the dependence is quadratic, as determined for, for example, DNP conductance (*43*). In this case the transfer of charge is attributed to a proton hopping or a bimolecular process, where a complex is formed between a neutral molecule and an uncoupler anion (*21*). The conductance measurements in dependence of the hop compound concentration indicated a class I uncoupler behavior at low iso-α-acids concentrations, which switches to a class II uncoupler characteristic above 60 μM at pH 7.0. The same characteristics were observed in the presence of additional $MnCl_2$ with a higher order of magnitude. As divalent cation binding was proposed to be essential for the antibacterial action of hop compounds (*9*), a formation of complexes, also known for nigericin, which can form a trimer (*44*), is likely. This suggests a charge transport, which is altered by aggregation of hop compounds within the membrane (*38*). Above a certain definite uncoupler concentration, a self-exchange process of hop compounds in the membrane can mediate the charge transport (*45*).

Membrane conductivity of BLMs recorded upon variation of solution pH is described by nonlinear decrease with pH. The slope of change in BLM conductance due to the hop compounds concentration in dependence of solution pH was >1 for any pH measured, indicating a more or less pronounced class I/II uncoupler behavior. Associated with the pH dependent degree of protonation of an uncoupler, the highest BLM conductivities are often found in the neighborhood of the pK_a of the substance investigated (*21*, *42*). The known pK_a values for hop compounds, for example, *trans*-isohumulone (3.1), humulone (5.0), and colupulone (6.1) (*14*), cover a wide range. In addition to that, a buffer range of hop compounds was detected between pH 9.0 and 11.4. These observations clarify why no bell-shaped conductance/pH curve, which is characteristic for investigations of a single uncoupler (with one pK_a), was obtained. The variation of charge transport class upon pH can be attributed to the complex chemical composition of hop-derived extracts and, furthermore, may point to the formation of complexes of different hop-derived uncoupler species, as determined for mixtures of DTFB and DNP (*41*). The influence of lipid composition with respect to lipid charge on BLM conductance characterizes the charge of the translocated uncoupler species. For charged molecules their concentration at the charged membrane−solution interface, with regard to the bulk concentration, varies according to the Boltzmann expression (*46*, *47*). If it is assumed that the mobility and the partition coefficient of the uncoupler are the same for charged and neutral membranes, a difference in the respective membrane conductance will allow the charge of the translocator species to be assessed (*36*). Therefore, the measured decrease in membrane conductance in negatively charged (GP containing) membranes points at negatively charged translocator species. This observation is substantiated by the measured degree of protonation of hop compounds and their known pK_a values (*14*), because, for example, half of these compounds are already dissociated ($P = 0.5$) at a pH of 3.0 and, thus, could exhibit negative charges. The decrease in BLM conductance in pure *L. brevis* lipid BLMs in comparison to the PC-doped *L. brevis* lipid BLMs indicates that the *L. brevis* lipid contains negatively charged phospholipids [confirmed by electrostriction analysis in the presence of divalent cations (*36*, *48*); data not shown]. These observations point to the model for charge transfer via complexes of negatively charged and neutral hop compounds as established for DNP (*43*) or DTFB and DNP (*41*).

Membrane potentials at BLMs were recorded upon the setup of ionophore, $MnCl_2$, and pH gradients. The potentials developed upon sole iso-α-acids gradients in the solutions separated by the BLM were small with respect to those measured in the presence of $MnCl_2$ or pH gradients. These results are consistent with data gained for other uncouplers (*21*, *41*). Still, this observation is of great importance for the understanding of the mode of

action of hop compounds, because it rules out the possibility that a removal of hop compounds by manganese complexation (and/or precipitation) on one side of the BLM is responsible for the potentials formed in the presence of $MnCl_2$ gradients. These potentials show a strict dependence on the $MnCl_2$ concentration gradient at pH 4 and pH 7. At high pH the potentials generated were low and exhibited a nonlinear shape, which might be attributed to the $Mn^{2+}/Mn(OH)_2$ conversions taking place above a pH of 8 (*49*). An elevated hop concentration shifted the results toward that obtained at lower pH values. This indicates the reduced membrane solubility of dissociated iso-α-acids at high pH. The major point of interest in this experiment is the sign of potential generated upon a setup of a $MnCl_2$ gradient. If Mn^{2+} is transferred through the membrane and released to the aqueous solution on the opposite side of the BLM, one would expect to receive a potential positive in sign on the side of lower (or zero) $MnCl_2$ concentration (*24*). As the opposite was monitored in the presence of iso-α-acids and $MnCl_2$ gradients, a different origin of charge transfer is suggested under these conditions, which is the target of current investigations. The data gained from BLMs in the presence of iso-α-acids and pH gradients showed that in the range from pH 4 to 9, the hop-doped membrane worked nearly perfectly as a pH electrode. This indicates that the hop compounds behaved as proton ionophores according to the Nernst expression and no divalent cations are required to drive the proton ionophore action of hop compounds. Only at high pH values, where the solubility of iso-α-acids in the membrane is reduced, were the potentials below the theoretical ones ($V = RT/F \ln\{[H^+]_{cis}/[H^+]_{trans}\}$; V = potential measured between the two aqueous solutions separated by the BLM, R = gas constant, F = Faraday, T = temperature) (*41*). Normally, both pH gradients and manganese gradients are present at the same time in vivo. Measurements upon the setup of both manganese and pH gradients resulted in potentials beyond the breakdown voltage of the BLMs. Accordingly, the potential formation upon a BLM in the presence of iso-α-acids and $MnCl_2$ and a pH gradient was recorded. Under these conditions the highest membrane potentials were observed. From pH 4 to 6 the potentials formed by a gradient of 1 pH unit were approximately twice as high as compared to those formed without $MnCl_2$ in the solutions. From pH 8 to 10 the recorded potential coincided with the values from the sole pH gradient measurements. This indicates that the influence of the $MnCl_2$ addition on both sides of the BLM is restricted to the pH values below 7. The origin of these high membrane potentials in the presence of $MnCl_2$ caused by a pH gradient cannot be assigned to a sole manganese or proton flux.

Taken together, the described mode of action of iso-α-acids as proton ionophores could be confirmed by the BLM measurements; however, the reported ionophore properties, as electroneutral H^+/Mn^{2+} exchangers, could not be verified. Potentiometric measurements indicated the manganese-dependent enhancement of the resulting effect (membrane potential or conductance). However, cation/proton exchange processes could not explain the latter effect. Rather, the mode of antibacterial action of hop compounds can be described as manganese (accelerated) triggered antibiotics, which are capable transporting protons within a wide range of pH due to their inherent complexity of chemical composition. The dramatic high membrane potentials measured at low pH and in the presence of manganese clearly point to an additional new antibacterial mechanism of hop compounds, beyond that committed by hop proton ionophores, and is currently investigated.

Relevance of This Study To Understanding the Antibacterial Action of Hop Compounds. Antibacterial hop compounds, mainly iso-α-acids, have been described as ionophores, which exchange H^+ for cellular divalent cations, for example, Mn^{2+}, and thus dissipate ion gradients across the cytoplasmic membrane (*1*, *9*). In several studies it was demonstrated that the main factors affecting the antibacterial activity of hop compounds are the pH value and their ability to bind to cations such as Mn^{2+} (*9*, *13*, *14*). A low pH value is suggested to contribute to an elevated amount of membrane-soluble undissociated iso-α-acids, acting as weak acids and thus mediating the proton influx (*5*, *6*). The function of manganese was assigned to enable the dissociated iso-α-acids to return from the inside of the bacterial membrane to the outside, release the divalent cation, and transport another proton into the bacterial cell. Thus, the antibacterial activity was dedicated to proton ionophore action of iso-α-acids, which was found to be stimulated by monovalent cations (*14*, *50*). Consequently, the low intracellular pH interferes with essential enzyme reactions and the pmf-dependent nutrient uptake is hampered, resulting in cell death of hop-sensitive strains (*1*, *9*, *10*). In this study, the mode of action of iso-α-acids as proton ionophores could be confirmed by the BLM measurements. However, the therefore expected cross-resistance to other proton ionophores such as CCCP of uncoupler class I (*11*) as well as to uncouplers of class II (DNP) could not be detected. The proton ionophore action of hop compounds was found to be not restricted to low pH values and distinct up to pH 9 and more. These findings are not in contradiction to the results of Simpson and colleagues (*5*, *6*), because it is known that the cellular proton gradient is dispensable at pH values in the range of the normal intracellular pH of the bacterium (about 6.5 for *L. brevis*), if other ion gradients, such as potassium or sodium take over its function as a driving force (*51*). In this context, the role of extracellular monovalent cations, beyond their cooperative binding to hops (*50*) can reside in the change in ion gradients upon the bacterial membrane, needed for survival under low proton gradients. The change from the proton gradient to, for example, sodium or potassium gradients as driving force under hop stress is supported by the fact that the membrane potential ($\Delta\Psi$) in the presence of hop compounds (*9*) was altered to a lesser extent in comparison to the proton gradient. The properties of hop compounds, which were described as electroneutral proton/Mn^{2+} exchangers (*1*), could not be verified by BLM experiments. This was substantiated by the fact that a cross-resistance to calcimycin, a proton/divalent cation exchanger, with a high affinity for the divalent cations $Mn^{2+} > Ca^{2+} = Mg^{2+}$ (*39*), could not be detected in hop-resistant strains. Whenever the participation of manganese in the mode of action of hop compounds was substantiated, the origin of high membrane potentials in the presence of $MnCl_2$ as well as the strongly elevated membrane conductivity, with concomitant increase in effectiveness of an uncoupler (*41*), suggests a different origin of charge transfer under these conditions.

In conclusion, the mode of antibacterial action of hop ionophores can be described as proton ionophores of classes I/II, which are capable transporting protons within a wide range of pH due to their inherent complexity of chemical composition. However, growth challenges in the presence of both types of ionophore classes in combination with the measured unusual high BLM potentials in the presence of manganese and at low pH indicate an additional mechanism of inhibition by hop compounds. The latter may be due to the nature/properties of hop compounds, which are known to be highly reactive substances (*12*, *35*). As a consequence, hop resistance of bacteria can be described as the multiple resistance to a heterogeneous mixture of compounds comprising different known and yet unknown charge transport mechanisms, which were dependent on several factors such as compound concentrations, cation composition, and pH value. Thus, only specialists such as some *L. brevis* strains, which can

cope with unusually low intracellular manganese levels, can survive hop stress. Because this implies a complex bacterial response (*28*), a cross-resistance to single proton ionophores or H^+/Mn^{2+} exchangers was not detectable and cannot be expected.

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Supporting Information Available: Figures S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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