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Research Article

LUPULON AND HUMULON—ANTIBIOTIC CONSTITUENTS OF HOPS[1]

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Commercial hops, the dried cones of the hop vine (*Lupulus humulus*), contain two antibacterial agents, lupulon and humulon. Methods of preparation of the compounds, their chemical structures, and their inhibitive action against Gram-positive bacteria have been known for many years; nevertheless, their antibiotic properties have been largely ignored in relation to animal infections. Their relatively high content in such an accessible source, together with the availability of simple methods for their isolation, prompted a survey of the antibiotic spectra of the substances. Lupulon and humulon were supplied to Dr. H. H. Anderson of the University of California Medical School for the determination of their activity toward *Mycobacterium tuberculosis*. The ensuing demonstration by Chin *et al.* (1) that lupulon inhibits the growth of a virulent strain of this pathogen *in vitro* and exerts a pronounced effect on experimental tuberculosis infections in mice has quickened interest in the microbiological, pharmacological, and therapeutic investigation of these agents.

The Western Regional Research Laboratory has been engaged recently in the determination of the antibiotic spectra of lupulon and humulon, development of methods by which substantial quantities of each can be readily prepared, determination of a number of their physical and chemical properties, preparation of certain derivatives, and development of methods of assay.

CHEMICAL AND PHYSICAL PROPERTIES

Structure. The formulas given in Figure 1 for lupulon and humulon had been established by 1926 through the investigations of Wieland (2) and Wöllmer (3) on the basis of degradative reactions summarized in von Richter's Organic Chemistry (4). The agents have not been synthesized.

Solubility and stability. Both lupulon and humulon are fairly soluble in polar and non-polar organic solvents such as methanol, ethanol, petroleum ether, hexane, and iso-octane. Both are only slightly soluble in neutral or acidic aqueous solution but are readily soluble as the sodium salts.

Lupulon is moderately stable to both acid and alkali. At room temperature in the presence of air it is very labile. In the crystalline form, it may become yellow and amorphous within a few days. This change is much slower at 5° C. in air; storage for several months is accompanied by development of a characteristic odor, though color development is not marked. Lupulon crystals appear perfectly stable *in vacuo* even at 60° C. Recently, Lundin (5) has reported that oxidation of lupulon (and humulon) is promoted by daylight and by metal oxides and that the oxidation is much more rapid in petroleum ether than in alcohol. F. Stitt and G. F. Bailey of this Laboratory have found that the ultra-violet absorption spectra of very dilute solutions of lupulon in petroleum ether or in iso-octane change very rapidly on standing at room temperature, but similar solutions in methanol or water have more stable spectra.

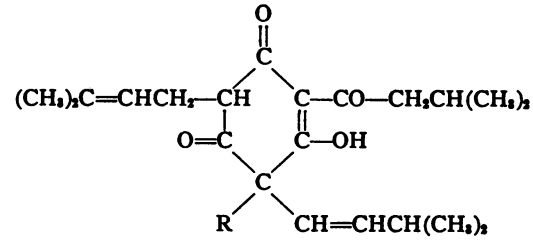

FIG. 1

Humulon: R = —OH
$C_{21}H_{30}O_5$, MW 362.3, m.p. 55°, $[\alpha]_D$ − 232°, monobasic acid.

Lupulon: R = —CH=CHCH(CH$_3$)$_2$
$C_{26}H_{38}O_4$, MW 414.3, m.p. 92°, optically inactive, monobasic acid.

[1] Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11–12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

[2] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

Michener and Andersen (6) of this Laboratory found that the addition of 0.1% of ascorbic acid exerted a marked protective action on the bacteriostatic activity of lupulon steamed or autoclaved at a concentration of 4 ppm. in phosphate buffers at pH 6.5 and 8.5.

Humulon is relatively stable to acid. The lead salt is stable, but the *o*-phenylenediamine salt slowly turns brown at room temperature in air, but not *in vacuo*. In aqueous solution at neutrality, or particularly in alkaline solution, humulon is transformed by boiling to an unidentified product which is not precipitable by lead acetate but which is believed by Walker (7) to retain some antibacterial activity. Quite recently, Verzele and Govaert (8) have reported the chromatographic separation of the immediate transformation product of humulon ("isohumulon"). They state that on boiling in methanol solution, humulon is quantitatively converted to isohumulon. On boiling with aqueous alkali, they found isohumulon to be converted to humulinic acid. Humulinic acid has been prepared in this Laboratory by boiling humulon with 1 *N* NaOH (2). It proved to be antibiotically inactive.

Michener and Andersen (6) found no loss of bacteriostatic potency against *Staphylococcus aureus* when 40 ppm. of humulon in phosphate buffer at pH 6.5 or 8.5 was autoclaved. However, the presence of low concentrations of ascorbic acid extended the duration of bacteriostatic action of humulon as well as that of lupulon.

METHODS AND RESULTS

Assay

Lupulon in simple solutions may be determined by its inhibitive action on Gram-positive bacteria in turbidistatic or cup-plate tests. A reduction of the bacteriostatic action of lupulon by blood serum, the mechanism of which is being investigated at this Laboratory by L. E. Sacks, prevents the use of such a method for determination of lupulon in blood and other tissues.

A tentative spectrophotometric method of analysis for the lupulon and humulon content of hop extracts has been developed by F. Stitt and G. F. Bailey of this Laboratory and will be described in a coming publication.

Isolation of hop antibiotics

Lupulon. Lupulon has been isolated on a kilogram scale in this Laboratory. The method is essentially that devised by Bungener in 1886. High-quality hops are necessary, since lupulon is unstable and since quantitative yields are not obtained by this process.

The hops, ground with an equal weight of dry ice to reduce the stickiness of their resinous content, are extracted with petroleum ether (30–60° C.) in a column, or in countercurrent fashion in several columns. The petroleum ether extract is concentrated without delay *in vacuo* to a thin syrup, which is then placed at approximately − 15° C. to crystallize. A rich extract may form a single porous cake so that the mother liquor can be drained off directly, or the crude crystals are filtered on a cold Büchner funnel. The crude crystals are then dissolved in warm petroleum ether (approx. 150 g. per l. at 40° C.), and recrystallized by chilling with stirring in a dry ice bath. The process is repeated twice, and then the lupulon is dried and dissolved in methanol (approx. 150 g. per l. at 20–25° C.). A white insoluble impurity is filtered off, and the lupulon is recrystallized by the slow addition of 1/10 volume of water and overnight storage at 0° C. The recrystallization from 90% methanol is repeated twice. High-quality hops yielded 3% of once-recrystallized and 1.5% of 6 × recrystallized lupulon, compared to the weight of the air-dry hops.

The product consists of fine white crystals, optically inactive, with theoretical C and H contents, and melting at 92–94° C. The lupulon crystals are stored *in vacuo* in the cold. Samples are distributed in evacuated ampules.

Humulon. The conventional method for the isolation of humulon by precipitation as the lead salt has been greatly simplified in this Laboratory by making the first precipitation with *o*-phenylenediamine from the crude petroleum ether extract of hops (usually from the mother liquor remaining after the crystallization of lupulon). *o*-Phenylenediamine was first used in 1916 by Wöllmer (3) for the purification of humulon lead salt. To our knowledge it has not been used previously for the direct isolation. Eighty % recovery is obtained. Free humulon can be obtained by acidification and extraction with an immiscible organic solvent such as ethyl ether.

Antibiotic spectra of humulon and lupulon

The antibiotic spectra of humulon and lupulon given in Table I have been determined in this Laboratory by the quantitative agar-streak dilution method of Waksman and Reilly (9). The antibiotics were dissolved in 1% concentration in 95% ethanol, and by means of aqueous dilutions, decreasing amounts were added to a series of 10 cm. Petri dishes, *i.e.*, 1.0, 0.3, 0.16, 0.1, 0.03, etc., ml. per dish. The lupulon used was a preparation recrystallized three times. Humulon was tested in two forms, namely the *o*-phenylenediamine salt and humulon prepared from the above salt two hours before the test.

The test medium for bacteria and yeasts was nutrient agar (0.5% Difco peptone, 0.3% Difco meat extract, 0.5% NaCl, and 1.5% Difco agar in tap water, pH 7.0, autoclaved at 121° C. for 20 minutes). For fungi other than yeasts, potato dextrose agar was employed. Melted and cooled (45° C.) 10 ml. portions of the medium were added to plates, which were immediately and thoroughly

TABLE I

Antibiotic spectra

(Maximum dilution for complete inhibition on agar)

Type	Name	Lupulon	Humulon
Gram-Positive Bacteria	*Bacillus anthracis*	300,000	100,000
	B. cereus v. mycoides	1,000,000	100,000
	B. subtilis	1,000,000	50,000
	Corynebacterium diphtheriae gravis	100,000	10,000
	Diplococcus pneumoniae Type I	300,000	20,000
	Micrococcus lysodeikticus	300,000	60,000
	M. pyogenes v. aureus	500,000	30,000
	Sarcina lutea	100,000	30,000
	Streptococcus faecalis	500,000	30,000
Acid-Fast Bacteria	*Mycobacterium phlei*	300,000	30,000
	M. tuberculosis v. hominis (607)	100,000	10,000
Actinomycetes	*Streptomyces coelicolor*	50,000	3,000
Gram-Negative Bacteria	13 species*	<3,000	<3,000
Yeasts	4 species†	<3,000	<3,000
Fungi	7 species‡	<3,000	<3,000

* *Aerobacter aerogenes, Alcaligenes faecalis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella enteritidis, Salmonella schottmuelleri, Salmonella typhosa, Serratia marcescens, Shigella dysenteriae, Shigella paradysenteriae.*

† *Cryptococcus neoformans, Saccharomyces cerevisiae, Torulopsis dattila, Zygosaccharomyces mandschuricus.*

‡ *Alternaria citri, Aspergillus niger, Fusarium solani v. pisi, Penicillium citrinum, Rhizoctonia solani, Rhizopus nigricans, Trichoderma koenigi.*

rocked to disperse the antibiotics. After about 30 minutes, cell suspensions from 24-hour-old cultures of the test organisms were streaked onto the solidified plates, each within a designated sector. Each sector received three streaks made with an L-shaped needle without recharging.

With bacteria-streaked plates, incubation was carried out at 35° C. for 18–20 hours, except for *Sarcina lutea*, the mycobacteria and the actinomycete, which were incubated for two days. Yeasts and other fungi were incubated at 30° C. for three days. After incubation, the results of growth were recorded and compared with control plates lacking the antibiotic. The inhibitory endpoint was taken as that dilution of the antibiotic which completely or nearly completely inhibited growth of the test organism. To reduce error, each spectrum was run five to ten times on different days, with replicate dilution plates each day.

Humulinic acid was found to be inactive at 0.1% for *Escherichia coli, Micrococcus conglomeratus, Micrococcus pyogenes v. aureus, S. lutea,* and *M. tuberculosis v. hominis.*

The inhibitive action of hop extracts towards bacteria has long been recognized and turned to practical use in brewing and in preservation of unpasteurized beer. In 1937 Shimwell (10) pointed out that Gram-negative bacteria would grow as readily in hopped as in unhopped beer worts, whereas Gram-positive bacteria grew not at all or very poorly in hopped wort. Walker and Parker (11) presented data which showed that 0.8 ppm. of humulon or 0.2 ppm. of lupulon give 50% inhibition of acid production by a strain of *Lactobacillus bulgaricus* inoculated into grain wort at pH 4.5. Hansen (12) found pure humulon and lupulon to exert no inhibitive action at 50 ppm. on several species of *Saccharomyces,* on *E. coli, Eberthella typhosa,* and *Salmonella paratyphus* A and B. Both *Staph. aureus* and *Bacillus mesentaricus* were inhibited at 12 and 2 ppm. of humulon and lupulon, respectively, while germination of spores of *Bacillus subtilis* was inhibited by 4 and 0.5 ppm., respectively. Michener, Snell, and Jansen (13) found that humulon and lupulon possess a low order of antibiotic activity against a panel of plant pathogenic fungi. Yeasts were largely unaffected.

The tuberculostatic action of lupulon was first noted by Chin *et al.* (1) and confirmed by Salle *et al.* (14) and by ourselves (see above). Chin found lupulon active against the H37Rv strain of *M. tuberculosis* at 1:40,000 in Dubos' medium, while Salle found the same strain inhibited by 1:200,000 in Long's medium and in Proskauer and Beck's medium, and by 1:90,000 in Dubos' medium. Humulon was much less active, and was not tested further.

The possibility of the development of lupulon-fast strains of Gram-positive and acid-fast bacteria has not been investigated as yet, but the brewing literature contains references to the "acclimatization" of Gram-positive bacteria to beer wort.

Serum has been found by Salle *et al.* (14), by Chin *et al.* (15), and by ourselves to reduce the bacteriostatic action of lupulon *in vitro.* In serial dilution tests with *Staph. aureus* and *M. conglomeratus* in broth containing 0.7 or 7% of serum, we found approximately 50 μg. of lupulon to be inactivated per ml. of undiluted serum. In cup-plate tests with *Mycobacterium phlei* and *B. subtilis,* 9 ppm. of lupulon in 10% serum gave zones equivalent to those given by 2 ppm. of lupulon in water.

Although inactivation by serum *in vitro* might by present concepts eliminate an antibiotic from further consideration for internal and other applications, such an effect does not prove a lack of therapeu-

tic value. In fact, the positive influence of lupulon administered orally or intramuscularly in oil on mouse tuberculosis as described elsewhere (1) suggests that otherwise promising tuberculostatic drugs if discarded solely because of inactivation by serum *in vitro* should be reevaluated by animal infection tests.

SUMMARY

The isolation, chemistry, assay, and antibiotic spectra of lupulon and humulon have been briefly discussed. A Bureau of Agricultural and Industrial Chemistry Circular of Information entitled "Antibacterial Agents from Hops," in which are summarized the findings of past and present studies of these substances, is now available for distribution.

The Western Regional Research Laboratory is preparing lupulon and humulon in amounts which will allow for distribution on a limited scale to parties interested in their evaluation.

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