untersuchung mit $2n = 48$ angegeben worden. Er zählte aber auch 45, 42 und 40 Chromosomen, hielt diese Zahlen jedoch für Zählfehler. Es scheint nicht ausgeschlossen, dass in seinem Material mehrere Chromosomenzahlen vorlagen. Alle vier von RAINER[1] untersuchten Tiere hatten haploid 30 Chromosomen. In dem von ihm abgebildeten Chromosomensatz gibt es ein sonst bei *C. hortensis* nicht vorhandenes kleines Chromosom. Das 1. Chromosom ist nur wenig länger als das 2. im Gegensatz zum 1. Chromosom bei haploid 22 Chromosomen. Von 5 Untersuchern haben 2, vielleicht 3, Veränderungen am Chromosomensatz von *C. hortensis* gesehen, die sicher alle auf Bruchereignisse zurückgehen. *Cepaea* scheint relativ unempfindlich gegen Veränderungen des Karyotyps zu sein[6].

*Summary.* The karyotype of 9 *Cepaea hortensis* were analyzed. The haploid chromosome number was 22. 2 types of longest chromosomes were encountered. In 8 out of 9 animals, the longest chromosome had an arm ratio of 1.2. One animal showed a ratio of 4.9. This aberration is thought to have arisen by a pericentric inversion. Because both the normal and the aberrant chromosome coexisted in the same population, a chromosomal polymorphism seems to be present.

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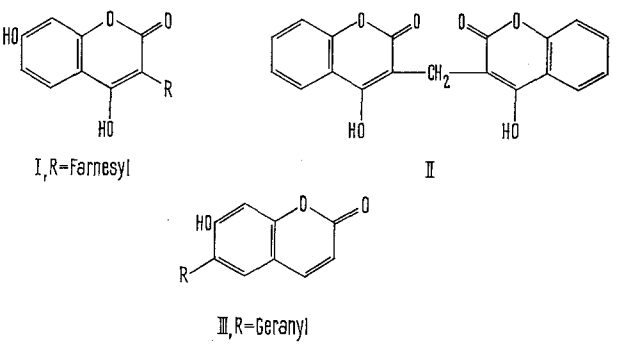
[6] Wir danken Fräulein ELISABETH RÄBER für die technische Assistenz.

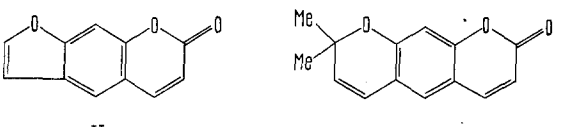
## The Effect of Alkylation on the Antimicrobial Activities of 7-Hydroxy- and 4-Hydroxycoumarins

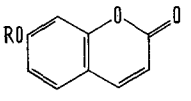
Although it has been reported that some natural coumarins possess antibacterial or antifungal properties[1,2], the effects of structural variations on microbial inhibition are not well established. In the case of bacteria, some relations between structure and antibacterial activity were recently proposed by DADÁK and HŎDÁK[3] on the basis of the effects of 15 natural coumarins and derivatives on bacterial growth. All were inactive against Gram-negative microorganisms (*Escherichia coli, Aerobacter aerogenes* and *Serratia marcescens*). However, growth of Gram-positive organisms belonging to the genera *Staphylococcus, Micrococcus* and *Bacillus* were inhibited by ammoresinol I, dicoumarol II, and ostruthin III, I being 2–5 times more effective than II or III. DADÁK and HŎDÁK pointed out that the activity of ammoresinol is dependent on the presence of the free hydroxyls at positions 4 and 7, since the dimethyl derivative of I was completely inactive. The particular importance of the free hydroxyl at position 4 for antibacterial activity was also indicated by the inhibitory effect of dicoumarol II. The antibacterial activity of ostruthin appears to require both the free 7-hydroxyl group and the carbon-linked, unsaturated geranyl group, since methylation of the hydroxyl and removal of the side chain (to give 7-hydroxycoumarin) resulted in partial or complete loss of activity.

We have now investigated the effects of *O*-alkylation and *O*-benzoylation on the inhibitory effects of simple monohydroxy-coumarins on microbial growth. With the exception of *Byssochlamys fulva* 7-hydroxycoumarin IVa was inactive against the variety of molds and yeasts shown in Table I. In contrast to the inactivity of IVa, the methyl ether IVb strongly inhibited the growth of 5 *Aspergillus* species, of *Byssochlamys fulva*, and of 8 of the 9 species of yeasts examined. The activity of 7-ethoxycoumarin IVc was similar to that of 7-methoxycoumarin and it strongly inhibited growth of all of these fungi.

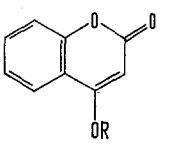
7-Benzyloxycoumarin IVf inhibited growth of *Candida chalmersi* and *Saccharomyces mellis*. With these two exceptions the antifungal properties of 7-geranyloxy- IVd, 7-allyloxy- IVe, 7-benzyloxy- IVf, and 7-benzoyloxycoumarin IVg were similar to those of the parent 7-hydroxycoumarin IVa, i.e. they inhibited *Byssochlamys fulva* but were ineffective against *Aspergillus* species and yeasts. Groupings such as allyloxy-, benzyloxy-, and geranyloxy- are structurally and chemically related in that, being $\alpha,\beta$-unsaturated, they are much more readily hydrolyzed or hydrogenolyzed than stable methoxy- or ethoxy-groups. The inactivity of these derivatives IVd–g may be due, therefore, to their facile enzymic hydrolysis or hydrogenolysis to the inactive 7-hydroxycoumarin.




IVa, R=H
IVb, R=Me
IVc, R=Et
IVd, R=Geranyl
IVe, R=Allyl
IVf, R=Benzyl
IVg, R=Benzoyl




VIIa, R=H
VIIb, R=Me
VIIc, R=Allyl
VIId, R=Benzoyl

Table I. Effect of coumarins on growth of fungi

| Coumarin | *Aspergillus glaucus* | *Aspergillus oryzae* | *Aspergillus niger* | *Byssochlamys fulva* | *Aspergillus flavus* NRRL 2999 | *Aspergillus flavus* NRRL 3145 | *Hanseniaspora melligeri* | *Zygosaccharomyces japonicus* | *Candida tropicalis* | *Pichia chodati* | *Hansenula anomala* | *Candida chalmersi* | *Saccharomyces rouxii* | *Saccharomyces mellis* | *Zygosaccharomyces barkeri* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IVa | — | — | — | + | — | — | — | — | — | — | — | — | — | — | — |
| IVb | + | + | + | + | + | + | + | + | + | + | — | + | + | + | + |
| IVc | + | + | + | | + | + | + | + | + | + | + | + | + | + | + |
| IVd | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| IVe | — | — | — | + | — | — | — | — | — | — | — | — | — | — | — |
| IVf | — | — | — | | — | — | — | — | — | — | — | + | — | + | — |
| IVg | — | — | — | + | — | — | — | — | — | — | — | — | — | — | — |
| VIIa | — | — | — | + | — | — | — | — | — | — | — | — | — | — | — |
| VIIb | + | + | + | + | — | — | + | + | + | + | — | + | + | + | + |
| VIIc | + | — | — | | — | — | — | — | — | — | — | — | — | — | — |
| VIId | — | — | — | + | — | — | + | + | + | + | + | + | + | + | + |

(+), effective inhibition of growth. (—), no growth inhibition.

The mechanism of inhibition of fungal growth by 7-methoxy- and 7-ethoxycoumarin is not known. It has been reported that psoralen and other methoxylated psoralens exert a photosensitizing effect on fungi after exposure to light[4]. We have determined, however, that 7-methoxycoumarin is effective against *Aspergillus glaucus* and *Aspergillus flavus* in both diffuse light and in complete darkness.

On the basis of these observations it would appear that the antifungal activity of a 7-hydroxycoumarin derivative is increased substantially when the phenolic group at position 7 is protected by a stable ether grouping. The activity of natural coumarins such as psoralen V and xanthyletin VI against *Curvularia lunata* and *Aspergillus niger*[5] may be accounted for by this generalization, since the cyclic alkoxy groupings (the furano- and chromeno-rings) present in these compounds are stable to hydrolytic ring fission.

The antifungal activity of 4-hydroxycoumarin VIIa was similar to that of 7-hydroxycoumarin IVa. It inhibited growth of *Byssochlamys fulva* but was ineffective against the 5 *Aspergillus* species and the 9 yeasts. 4-Methoxycoumarin VIIb, on the other hand, inhibited the growth of 12 of the 15 fungi examined. 4-Allyloxycoumarin VIIc inhibited *Aspergillus glaucus*. With this exception VIIc was ineffective against other fungi. The effect of alkylation on 4-hydroxycoumarin, therefore, closely parallels the alkylation effects observed with 7-hydroxycoumarin. It is noteworthy, however, that benzoylated 4-hydroxycoumarin differs from the corresponding 7-benzoyloxycoumarin in that it inhibited the growth of all 9 yeasts.

The pronounced and consistent contrast in the inhibitory effects on the growth of molds and yeasts by 7-methoxy- and 4-methoxycoumarins as compared with 7-hydroxy- and 4-hydroxycoumarins has not been observed with bacteria. Thus, whereas 7-hydroxycoumarin and its geranyl IVd, allyl IVe, benzyl IVf and benzoyl IVg derivatives proved ineffective against all of the bacteria shown in Table II, its methyl IVb and ethyl IVc derivatives inhibited the growth of at least some of these organisms, including the Gram-negative bacteria *Acaligenes faecalis* and *Escherichia coli*. In agreement with the findings of DADÁK and HŎDÁK, however, the hydroxyl group at position 4 of 4-hydroxycoumarin VIIe appears to be necessary for antibacterial action, since methylation or allylation of this hydroxyl resulted in complete loss of antibacterial activity. Although 4-hydroxycoumarin,

Table 2. Effect of coumarins on growth of bacteria

| Coumarin | *Bacillus cereus* | *Sarcina lutea* | *Staphylococcus aureus* | *Streptococcus lactis* | *Acaligenes faecalis* | *Escherichia coli* | *Pseudomonas aeruginosa* | *Salmonella typhimurium* | *Serratia marcescens* |
|---|---|---|---|---|---|---|---|---|---|
| IVa | — | — | — | — | — | — | — | — | — |
| IVb | + | + | — | — | + | — | — | — | — |
| IVc | + | — | — | + | + | + | — | + | — |
| IVd | — | — | — | — | — | — | — | — | — |
| IVe | — | — | — | — | — | — | — | — | — |
| IVf | — | — | — | — | — | — | — | — | — |
| IVg | — | — | — | — | — | — | — | — | — |
| VIIa | + | + | + | + | + | — | — | — | — |
| VIIb | — | — | — | — | — | — | — | — | — |
| VIIc | — | — | — | — | — | — | — | — | — |
| VIId | + | + | + | + | + | — | — | + | — |

(+), effective inhibition of growth. (—), no growth inhibition.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as indicated in Table I, is generally ineffective against fungi, it inhibits a number of bacteria. In these respects it is similar to the antibiotic novobiocin (a 4-hydroxy-coumarin derivative), which is active against bacteria but has no action on fungi[1,6].

The antimicrobial effectiveness of these coumarins was determined by comparison of pour plates containing about 200 bacterial or yeast cells and 500 ppm of the coumarin against control plates without added coumarin, after incubation at 28 °C for 1–4 days. Mold growth inhibition was observed by spot inoculation of the surface of the prepared medium. Bacteria were grown on plate count agar, and yeasts and molds on potato dextrose agar. LEDERBERG's replica plating technique[7] was also employed, master plates of bacteria and yeasts being used to inoculate the velveteen cloth from which the test plates were then prepared[8].

*Zusammenfassung.* Es wird der Einfluss einer Alkylierung auf die antimikrobielle Wirkung von 7-Hydroxy- und 4-Hydroxy-Cumarin dargestellt.

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*Western Regional Research Laboratory, Agricultural Research Service, US Department of Agriculture, Albany (California 94710, USA), 27 April 1970.*

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## PRO EXPERIMENTIS

### Tissue Sodium and Potassium: Direct Detection in the Electron Microscope

Previous attempts to localize sodium or potassium in biological tissue have usually depended on electron histochemical techniques[1,2]. These methods are unsatisfactory for two reasons. First it is reasonable to expect that most, if not all, of the sodium and potassium ions would be washed out during the tissue preparation techniques. Secondly there are doubts about the specificity of the histochemical reaction[3].

We have utilized a method which is more direct and should be capable of adaptation to a quantitative technique. We cut ultrathin frozen sections through corneal stroma and stored them freeze dried as described previously[4]. The sections were transported to the high resolution analytical electron microscope, A.E.I. EMMA-4 and analyzed for sodium and potassium. EMMA-4 operates in the following mode. High energy electrons in the microscope beam strike the atoms in the specimen section and a fraction undergo inelastic collisions which excite or ionize the atoms. As electrons drop back into the empty orbitals they emit characteristic X-rays. These emitted X-rays are analyzed in 2 spectrometers mounted on the side of the column. Scanned X-ray spectra for the K lines (quanta emitted due to the relaxation of electrons from the L shell into the K shell) of sodium and potassium emanating from our frozen sections are shown in Figures 1 and 2. A representative area of frozen section through corneal stroma is shown in Figure 3. Similar analysis of ultrathin sections through corneal stroma conventionally processed for electron microscopy[5] and embedded in Araldite showed no detectable quantities of sodium or potassium.

Stromal sodium and potassium concentrations are measured after acid extraction at 172 m*M* and 22 m*M* respectively[6]. It is reasonable to presume that as the

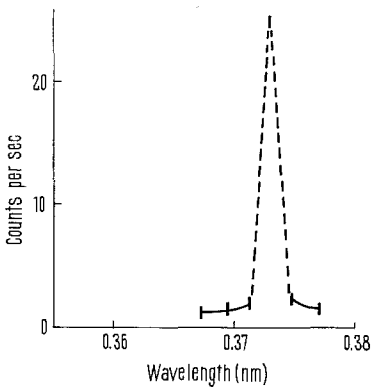


Fig. 1. Emission spectrum of the potassium Kα-line emanating from frozen sections through non-fixed corneal stroma. Section thickness estimated at 150 nm; probable potassium concentration in the stroma is 22 m*M*. Emitted X-rays were analyzed by a lithium fluoride crystal set at the appropriate Bragg angle, through a 3 μm thick Mylar window. Energy of electrons bombarding the section, 40 keV; probe size 0.8 μm. Vertical lines represent twice the standard error on the mean of each reading.

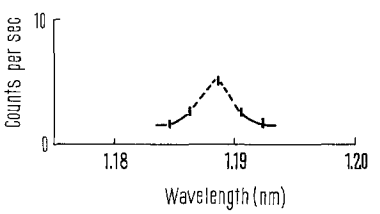


Fig. 2. Emission spectrum of the sodium Kα-line counted simultaneously with that in Figure 1. Probable sodium concentration in corneal stroma is 172 m*M*. Detecting crystal, potassium ammonium phthalate. Vertical lines represent twice the standard error on the mean of each reading.

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