Sprüht man das erhaltene Chromatogramm mit Kongorotlösung, so sind die an den Auftragungsstellen in der Salzsäurefront entstandenen Lücken deutlich erkennbar. Es ist auch möglich, durch Betrachten des entwickelten, nicht besprühten Chromatogramms im UV.-Licht sowohl Salzsäure- als Lösungsmittelfront als hellleuchtende Linien zu erkennen, die direkt nachgezeichnet werden können[3].

Die durch Sprühen mit Kongorot oder Zeichnen im UV.-Licht sichtbar gemachten Lücken werden mit einem Planimeter[4] gemessen[5]. Die in einem Chromatogramm erhaltenen Werte sind den aufgetragenen Basenmengen direkt proportional. Bei sorgfältigem Arbeiten liegt die Fehlergrenze bei ± 5%. Es lassen sich so zum Beispiel NaOH, $Na_2CO_3$, $NaHCO_3$ bestimmen. Die Salzsäure lässt sich durch andere Mineralsäuren ersetzen.

In den Tabellen I und II sind einige Kontrollversuche zusammengestellt, die die Brauchbarkeit der Methode belegen. Die Tabellen enthalten unter $cm^2/\varepsilon$ die aus den gemessenen Lückengrössen berechnete Fläche, die einem $\gamma$-Äquivalent ($= \varepsilon$) entspricht.

Über die Bestimmung kleinster Mengen anderer anorganischer Ionen nach dieser Methode wird später berichtet.

B. PRIJS und H. ERLENMEYER

*Anstalt für Anorganische Chemie der Universität Basel, den 13. November 1955.*

*Summary*

A new method, based on retention-paperchromatography, is developed for ultramicrodetermination of inorganic bases.

[3] Bei allen Versuchen wurde Whatman-Nr.-1-Papier verwendet.

[4] Firma J. AMSLER, Schaffhausen.

[5] Die absolute Grösse der erhaltenen Lücken hängt einmal von der $OH'$-Menge, dann aber auch von der HCl-Konzentration und daneben von Temperatur, Laufzeit und Feuchtigkeit ab, so dass nur gleichzeitig unter gleichen Bedingungen laufende Chromatogramme direkt verglichen werden können.

# Informations - Informationen - Informazioni - Notes

## STUDIORUM PROGRESSUS

### The Chemical Nature of the Antibacterial Substance Present in *Aucuba japonica* Thunbg.

By J. E. ROMBOUTS and J. LINKS[1]

#### I.—*Introduction*

Several compounds with antibiotic properties have been demonstrated to occur in green plants. The majority of them has been exclusively tested for growth-inhibiting activity against certain strains of common bacteria in order to detect antibacterial substances which might be effective against zoopathogens. In this respect an impressive amount of work has been done, among others, by OSBORN[2], CARLSON *et al.*[3], and COLLIER and VAN DER PIJL[4].

Although the occurrence of substances with antibacterial properties in Phanerogams was found to be a common feature, identification of these substances has been carried through in only a small number of cases. This report deals with the identification and biological characterization of the antibacterial compound first demonstrated to be present in leaves of *Aucuba japonica* by OSBORN[2], who found aqueous extracts of the variety with maculated leaves (var. *variegata* D'Ombr.) to be effective *in vitro* against *Staphylococcus aureus*, but not against *Escherichia coli*; similar extracts of the variety with entirely green leaves (var. *viridis* Hort.) were said to be ineffective to either one of these bacteria.

WINTER and WILLEKE[5] stated that juices expressed from leaves and roots of *A. japonica* strongly inhibited the growth of a population of the most different kinds of soil bacteria, and that *Bacillus subtilis* and both *S. aureus* and *E. coli* were very sensitive species. More recently FITZPATRICK[6] found that aqueous extracts of the leaves of *A. japonica* were effective *in vitro* against a human strain of *Mycobacterium tuberculosis*, activity being greater in autumn than in spring. These investigators do not reveal whether they used a special variety of *Aucuba*.

#### II.—*Experimental*

(1)—*Detection of an inactive precursor.* In the ordinary routine screening for the presence of antibiotic activity in crude plant juices, it was found that squeezed leaves of *A. japonica* var. *variegata* showed a marked activity against both the spores of *Bacillus subtilis* and the conidia of *Penicillium italicum*. When the fresh *Aucuba* leaves were immersed in boiling water for a few minutes before squeezing, the activity of leaf extracts against both micro-organisms was entirely destroyed; it could be restored, however, by mixing the inactive leaf juice with a minute amount of crushed fresh *Aucuba* leaves, an amount which was so small that it exerted no noticeable inhibition by itself. This observation led us to the assumption that in *Aucuba* leaves an antimicrobial substance is present in the form of an inactive, thermostable compound which, on mixing with an enzyme, is decomposed and gives rise to components, at least one of which is capable of inhibiting the growth of certain microorganisms. So far it was still uncertain whether one single component accounted for both the antibacterial and the antifungal activity.

(2)—*Isolation from leaves of an activating enzyme preparation.* An active crude enzyme mixture was prepared as follows: From fresh leaves of *A. japonica* var. *variegata* the petioles were removed. The laminae were frozen

[1] N. V. Philips-Roxane, Agro-Biological Laboratory "Bocksteyn", 's-Graveland, and Central Laboratory, Weesp, The Netherlands.

[2] E. M. OSBORN, Brit. J. exper. Pathol. *24*, 227 (1943).

[3] H. J. CARLSON, H. D. BRISSELL, and M. G. MUELLER, J. Bact. *52*, 155 (1946). – H. J. CARLSON and H. DOUGLAS, J. Bact. *55*, 235 (1948). – H. J. CARLSON, H. G. DOUGLAS, and J. ROBERTSON, J. Bact. *55*, 241 (1948).

[4] W. A. COLLIER and L. VAN DER PIJL, Chron. Naturae *106*, 73 (1950).

[5] A. G. WINTER and L. WILLEKE, Naturwissenschaften *38*, 262 (1951).

[6] F. K. FITZPATRICK, Antibiot. Chemother. *4*, 528 (1954).

with the aid of solid $CO_2$ and collected in a towel in which they were pounded to pieces of about 1 $cm^2$. These small pieces were ground in an ordinary household mincing machine, previously cooled with solid $CO_2$. The resulting product was hereupon extracted for 5 min at – 50°C in a Turmix with 8 times its weight of acetone. This extraction was repeated once with a fresh portion of acetone and finally completed with a same volume of ether, all at – 50°. If portions of the remaining light green powder were stirred for 20 min with about 30 times its amount of 0·01 N phosphate buffer, pH 7, and hereupon filtered, almost clear solutions were obtained which possessed an excellent enzyme activity. This justifies the conclusion that easily dissociating prosthetic groups were apparently absent and not needed for the enzyme's action, a property which pointed to a hydrolytic enzyme.

(3)–*Isolation and identification of the inactive precursor.* Work was now directed towards the isolation of the precursor of the antibacterial substance. We had noticed that its thermostability was increasingly affected by lowering the pH. This observation and the apparent hydrolytic nature of the activating enzyme made an easily hydrolyzable glycosidal structure very likely. Therefore the classical method of BOURQUELOT and HÉRISSEY[7] for the isolation of glycosides was applied at once.

These French investigators first used this method for the isolation of the main glucoside aucubin, then new, from the ripe seeds of *Aucuba japonica*. The glycoside we isolated from unripe seeds as the inactive precursor of the antibacterial substance turned out to be identical with aucubin.

Green fruits (berries) of *A. japonica* var. *variegata* were longitudinally cut. In this way the single central seed was cut as well. The green pericarp was peeled off by hand, whereupon the seed halves were immediately put into boiling 90% ethanol and left boiling on a reflux for 35 min in order to inactivate the enzymes. Subsequently the seed halves were removed from the alcohol and ground in a Peppink hammer mill.

The resulting seed powder was again extracted for 15 min with boiling 90% ethanol on a reflux. The first and second extracts were pooled and the volume drastically reduced *in vacuo*. After addition of an adequate amount of water and some $CaCO_3$, a little baker's yeast was added and the extract fermented at 25°C to remove free sugars. $CO_2$ production ceased after 4 days. The yeast was then killed by boiling, and the mixture centrifuged, decolourized with an active charcoal (Norit) and filtered. From the resulting clear, pale yellow sirupy liquid, almost white needles crystallized during slow evaporation in a desiccator over concentrated sulphuric acid. The crystals were collected on a filter and washed with 95% ethanol and ether. 390 g of fresh green fruits yielded 3·5 g of white crystals, m.p. 180°C (uncorrected). Repeated crystallization from 80% ethanol did not alter the melting point. With the aid of the *Aucuba* enzyme mixture it was easy to demonstrate that the crystalline compound was the inactive precursor.

The identity of our product with aucubin was established by:

(a) its solubility in different solvents: water, methanol, ethanol, ether, and chloroform;
(b) the formation of a brownish-black precipitate on mixing an aqueous solution with diluted mineral acids and gentle heating; prolonged heating of such acid solutions made them give off a penetrating, pleasant and characteristic odour (cp. BOURQUELOT and HÉRISSEY);
(c) the characteristic blue solution caused by reagent A (10 vol. of glacial acetic acid, 1 vol. of 0·2% aq. solution of $CuSO_4 \cdot 5\ H_2O$ and 0·5 vol. of conc. HCl) of HILL and VAN HEYNINGEN[8];
(d) a sugar analysis: calculated (as glucose) 52·4%, experimental 49·4%;
(e) its melting point: 180°C (uncorrected); BOURQUELOT and HÉRISSEY[9] 181°; KARRER and SCHMID[10] 182°–183°; TRIM and HILL[11] 180°–182°;
(f) its specific rotation in water: $[\alpha]_D^{21} = -163{\cdot}1$ (c = 1·6); BOURQUELOT and HÉRISSEY[9] $[\alpha]_D = -173{\cdot}1$ (c = 2·956); LEBAS[12] $[\alpha]_D = -164{\cdot}4$ (c = 2·007); BERGMANN and MICHALIS[13] $[\alpha]_D^{21} = -171{\cdot}4$ (c = ?); KARRER and SCHMID[10] $[\alpha]_D^{15} = -162{\cdot}0$ (c = 1·988) and $-162{\cdot}3$ (c = 1·128); TRIM and HILL[11] $[\alpha]_D^{18} = -164{\cdot}7$ (c = 1·5);
(g) its UV. absorption spectrum: we also found the unpronounced absorption maximum at about 270 m$\mu$ and the rapidly increasing absorption from about 250 m$\mu$ towards shorter wave lengths (cp. KARRER and SCHMID[10], and TRIM and HILL[11]).

(4)–*Chromatographic isolation of aucubin from the Aucuba plant.* Glycosides are adsorbed from aqueous plant extracts by charcoal. Many other adsorbed substances can be removed from the charcoal by washing with water, whereas the glycosides can be eluted afterward with diluted ethanol. TRIM and HILL[11] used this principle for the isolation of aucubin. They stirred the plant extracts with charcoal, filtered, washed with water, refiltered, washed with ethanol, and filtered again. We used a simple chromatographic method for the same fractionation.

The aqueous extract from the natural raw material (e.g. leaves) was poured onto a column of Norit (chromatographic quality P.K. 0·1–0·25). The column was hereupon washed with water until the reaction for chlorine in the eluate had become negative. Washing was then continued with another 30% of the volume of water used for removing the chlorine. At that time no aucubin had been removed as yet from the column; this was regularly checked by a simple colour reaction in samples of the eluate. Subsequently the column was eluted with 50% ethanol, which removed at once the major part of the adsorbed aucubin. After some time the amount of eluted aucubin decreases sharply, whereupon only minute amounts are further released by the ethanol. Evaporation *in vacuo* of the collected ethanol eluate yields a pale brown syrup from which white crystals of aucubin can easily be obtained by crystallization from water and one recrystallization from 80% ethanol.

(5)–*Antibiotic activity of aucubin.* Once we had crystalline preparations of aucubin to hand, it was easy to prove that the products of hydrolysis were responsible for the activity both against *P. italicum* and *B. subtilis*. We extended these observations with several other micro-organisms and found an activity spectrum which is presented in the Table. The activity was graded accord-

[7] E. BOURQUELOT and H. HÉRISSEY, C. r. Soc. biol. *104*, 695 (1902); C. r. Acad. Sci. Paris *134*, 1441 (1902).

[8] R. HILL and R. VAN HEYNINGEN, Biochem. J. *49*, 332 (1951).

[9] E. BOURQUELOT and H. HÉRISSEY, Ann. Chim. *4*, 289 (1905).

[10] P. KARRER and H. SCHMID, Helv. chim. Acta *29*, 525 (1946).

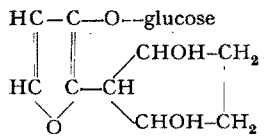
[11] A. R. TRIM and R. HILL, Biochem. J. *50*, 310 (1952).

[12] M. C. LEBAS, J. Pharm. Chim. [6] *30*, 390 (1909).

[13] M. BERGMANN and G. MICHALIS, Ber. deutsch. chem. Ges. *60*, 935 (1927).

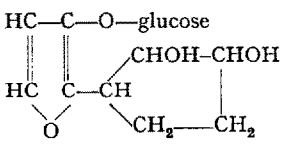
ing to the diameter of the zones of growth inhibition formed on nutrient agar around holes containing enzyme-activated aucubin solutions in three concentrations. The activity spectrum of crystalline aucubin exactly matches that which can be obtained with crude juices from *Aucuba* leaves, irrespective of whether these originate from the variety *variegata* or *viridis*. This indicates that the activity results from the formation of aucubigenin which is both antibacterial and, to a less extent, fungicidal. The fungicidal action is rather specific for only a small number of fungi, *Penicillium italicum* being by far the most sensitive species met with.

*Discussion.* Aucubin (synonyms: rhinanthin, aucuboside) was first found in the green parts of *Rhinanthus Crista-gallii* L. by LUDWIG[14] in 1868. It was first described from *Aucuba japonica* by BOURQUELOT and HÉRISSEY[7]. Its chemical nature was later investigated in detail by BERGMANN and MICHALIS[13], KARRER and SCHMID[10], NAKAMURA[15], and TRIM and HILL[11]. It was not until 1946 that KARRER and SCHMID[10] were capable of suggesting the structure formula (I) which explained all the established properties.

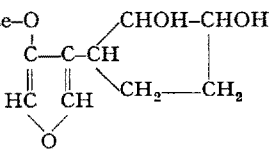


(I)

In 1950 NAKAMURA[15] gathered evidence for one of the two formula's [II] and [III].



(II)



(III)

Under the influence of $\beta$-glycosidases (e.g. emulsin) or an acid hydrolysis, the aglucone, aucubigenin, which has the substituted furan structure, is set free. Because of its $\beta$-hydroxy group this aglucone is extremely unstable and therefore it has never been isolated (KARRER and SCHMID[10]). Especially in acid solutions, it easily forms a derivate of succindialdehyde which in turn polymerizes readily, building poorly soluble dark coloured products. For this reason it was impossible for us to decide whether the aglucone itself possesses the antibiotic properties or whether this is due to the formation of a di- or polymere.

Aucubin has been demonstrated in many plant species belonging to taxonomically very different families. A recent review of the plants families in which this glycoside was found to be present is given by TRIM and HILL[11]. Because of its widespread occurrence, we were not surprised to find that, in contrast with the results obtained by OSBORN[2], both *Aucuba* varieties: *viridis* and *variegata*, yielded extracts with similar antibiotic properties which in both cases could be proved to be due to the aucubin content. Perhaps seasonal differences in the constituents of the leaves, as reported by FITZPATRICK[6], may be responsible for these contradictory results.

We found aucubin to be distributed throughout the whole *Aucuba* plant, including its roots. Though HATTORI[16] emphasized its absence in the ripe pericarp, we found it in that tissue of green fruits in quite appreciable amounts.

Antibiotic activity of crystalline aucubin after activation with a hydrolytic enzyme from *Aucuba* leaves

| Micro-organism | Concentration of aucubin: 0·1% | 1% | 3% |
|---|---|---|---|
| *Micrococcus aureus* | ± | + | ++ |
| *Escherichia coli* | ± | + | + |
| *Bacillus subtilis* | + | ++ | +++ |
| *Mycobacterium phlei* | + | ++ | +++ |
| *Pythium debaryanum* | – | – | – |
| *Phytophthora cactorum* | – | – | ± |
| *Phytophthora cinnamomi* | – | – | – |
| *Rhizopus nigricans* | – | – | – |
| *Sclerotinia fructigena* | – | – | ± |
| *Chaetomium globosum* | – | – | – |
| *Ophiostoma coerulescens* | – | – | ± |
| *Ophiostoma paradoxum* | – | ± | + |
| *Glomerella cingulata* | – | – | – |
| *Diaporthe alnea* | – | – | ± |
| *Gibberella pulicaris* | – | – | – |
| *Pleospora lycopersici* | – | – | – |
| *Mycosphaerella pinodes* | – | – | – |
| *Torulopsis utilis* | – | – | – |
| *Candida krusei* | – | – | – |
| *Ustilago nuda* | – | ± | + |
| *Phialophora heteroderae* | – | – | – |
| *Phoma betae* | – | – | ± |
| *Septoria lycopersici* | – | – | ± |
| *Colletotrichum lindemuthianum* | – | – | ± |
| *Penicillium italicum* | ± | ++ | +++ |
| *Penicillium martensii* | – | – | – |
| *Aspergillus flavipes* | – | – | – |
| *Aspergillus niger* | – | – | – |
| *Trichoderma viride* | – | – | ± |
| *Botrytis allii* | – | – | – |
| *Botrytis cinerea* | – | – | – |
| *Verticillium dahliae* | – | – | – |
| *Alternaria porri* | – | – | – |
| *Alternaria tenuis* | – | – | – |
| *Cercospora beticola* | – | – | ± |
| *Fusarium culmorum* | – | – | ± |

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*Résumé*

1° La substance antibactérielle des feuilles d'*Aucuba japonica* est identifiée comme étant l'aucubigénine. Ce furane substitué se forme à partir de la $\beta$-glucoside aucubine sous l'action d'un enzyme hydrolytique.

2° En dehors d'une action générale antibactérielle, l'aucubigénine a des propriétés antifongiques bien spécifiques.

3° L'aucubigénine se forme également dans les extraits crus des feuilles des deux variétés: *variegata* et *viridis*.

4° L'aucubine se trouve distribuée dans la plante entière, y compris le péricarpe des fruits verts.

5° A l'aide d'une colonne de charbon activé, l'aucubine est facilement isolée des feuilles d'*Aucuba* dans un état très pur par une méthode chromatographique.

[14] H. LUDWIG, Arch. Pharm. *186*, 64 (1868).

[15] Y. NAKAMURA, J. chem. Soc. Japan, Pure Chem. Sect. *71*, 123 (1950); *71*, 186 (1950).

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