Biochemistry

 Volume 5, Number 7 July 11, 1966

# Production, Isolation, and Characterization of Sirenin*

Leonard Machlis, William H. Nutting, M. Wyn Williams, and Henry Rapoport

ABSTRACT: Sirenin is a sperm attractant produced by the female gametes of the water mold *Allomyces*. The growth methods used for the production of approximately 2.5 g of sirenin and the apparatus for the continuous extraction from aqueous solution with methylene chloride are described.

The crude methylene chloride solution was partially purified by alumina chromatography and the sirenin-enriched fractions then treated with 4-(4-nitrophenylazo)benzoyl chloride (NABS-Cl) to form NABS-sirenin and bis-NABS-sirenin. These were detected by the development of a thin layer chromatographic technique. Analysis and molecular weight determinations of pure bis-NABS-sirenin, sirenin, and NABS-sirenin indicate the molecular formula of sirenin to be $C_{15}H_{24}O_2$ with a molecular weight of 236. Sirenin is thus shown to be an oxygenated sesquiterpene with four degrees of unsaturation and with both of its oxygen functions capable of forming esters.

Sirenin (Machlis, 1958a,b,c) is a powerful attractant for the male gametes of the water mold *Allomyces*. This substance is produced by the female gametes, synthesis beginning before their emergence from the gametangia, and is released into the ambient liquid environment. It is one of a large number of chemotactic and chemotropic agents demonstrated to operate in plant systems that serve as the prelude for fertilization (Machlis and Rawitscher-Kunkel, 1963).

No plant hormones or pheromones controlling sexual reproduction in plants are yet fully described chemically. This report covers the work we have done the past several years on the production, isolation, and characterization of sirenin whose complete structure will be the subject of a future report.

## Methods

*The Organisms. Allomyces* is a genus of water molds normally found in moist or intermittently moist conditions such as ditches, drains, edges of ponds, etc. The definitive systematic and general biological treatment of the genus was provided by Emerson (1941). Pertinent to this investigation are the species in the subgenus *Euallomyces* which are characterized by independent haploid, gametophytic and diploid, sporophytic generations (Figure 1). The vegetative body of both generations is a dichotomously branching mycelium which, when grown on nutrient agar plates, rapidly spreads over the surface of the agar with rhizoids penetrating into the agar. The sexual, gametophytic generation appears orange because the hyphae bear large numbers of male and female gametangia which tend to occur in pairs, with the male gametangia orange and smaller than the colorless female gametangia. When these gametangia are placed in water or when the plates are flooded with water, the motile male and female gametes emerge from their respective gametangia about 2 hr later and subsequently fuse to form motile zygotes. The zygote eventually comes to rest and develops into the asexual, sporophytic generation which bears two kinds of sporangia and is brown. The colorless sporangia in the presence of water release zoospores that develop into additional sporophytic plants the same as those from which they came. The brown, thick-walled sporangia release zoospores which develop into new gametophytic plants. The work reported in

* Contribution from the Department of Botany (L. M.) and Department of Chemistry (W. H. N., M. W. W., H. R.), University of California, Berkeley, California. *Received April 4, 1966.* We are pleased to acknowledge support for this research from the National Science Foundation.

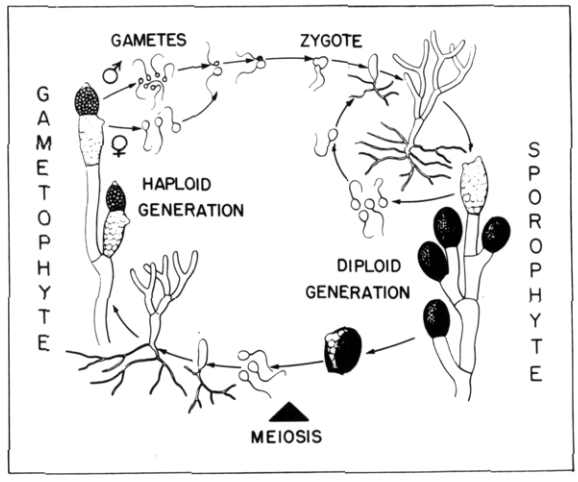


FIGURE 1: Life history of *A. macrogynus*.

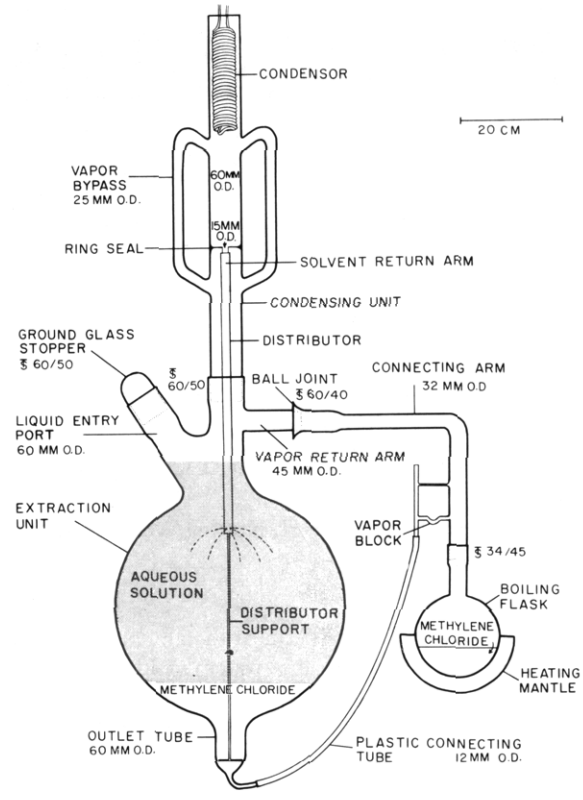


FIGURE 2: Diagrammatic scale drawing of continuous extractor for use with solvents heavier than water. See text for details and operation.

this paper was done with hybrids derived from crosses between *Allomyce sarbuscula* and *A. javanicus* (Emerson and Wilson, 1954). These two species differ in chromosome number and in the arrangement of the gametangia. The hybrids (Machlis, 1958a) bear either 95% or better male gametangia or 95% or better female gametangia.

*Bioassay for Sirenin.* The early phases of the work were dependent on the use of the bioassay for sirenin fully described by Machlis (1958a,c). Briefly, solutions to be tested are placed on one side of a membrane whose other side is in contact with a suspension of male gametes. The male gametes attach to the membrane in proportion to the concentration of sirenin above the membrane.

*Extraction of Sirenin.* The biological production of sirenin, which will be described later, results in aqueous solutions containing under the best of production conditions about $10^{-6}$ M sirenin. The production facility yielded 80 l. of such solution/day. Since sirenin is soluble in nonpolar solvents (Machlis, 1958a), two large continuous extractors, using methylene chloride, were constructed on the basis of much smaller units already developed in the Department of Chemistry. The apparatus is diagrammed to scale in Figure 2. It consists of six major components: the extraction unit, the condenser, the condensing unit, the distributor, the connecting arm, and the boiling flask.

The extraction unit which rests on a cork ring supported by a tripod is fabricated from a standard 50-l. round-bottom flask. The neck end is provided with a ground glass joint into which the condensing unit fits, an oblique liquid entry port closed with a ground glass stopper, and a vapor return arm to which the connecting arm is attached. The bottom of the flask is equipped with an outlet tube to which the plastic connecting tube is attached. Before the condensing unit is put in place, the distributor is positioned in the extraction unit and the condensing unit is then inserted so that the solvent return arm fits inside the stainless steel distributor. This is made of 1-in. o.d., $^1/_{16}$-in. thick, stainless steel tubing and has five $^1/_{32}$-in. perforations evenly spaced around the circumference 1 in. from the bottom of the distributor. The support consists of a stainless steel tube 0.25 in. in diameter welded to the distributor. The other end is internally threaded to take a $^3/_{16}$-in. fully threaded bolt 3 in. long which terminates in a perforated, plastic disk 2 in. in diameter held in place by appropriate nuts and which rests on the bottom of the extraction flask. The bolt at the end permits adjustment of the height of the distributor.

The construction of the condensing unit is self-evident from the drawing. Into the upper end is inserted a coiled, stainless steeel condensor, 2 in. in diameter, with the inlet and outlet leads soldered to a steel disk which rests loosely on top of the condensing unit. These leads are plumbed with loosely coiled copper tubing (to prevent any strain on the glass) to the house cold-water line and the house drain system.

The vapor return arm of the extraction unit is joined to the boiling flask by the connecting arm which has a vapor block sealed to its vertical portion. The vapor block is connected to the extraction unit by solvent-resistant, slightly flexible plastic tubing firmly attached at each end. The boiling flask consists of a 3-l. round-bottom flask the bottom half of which is encased in a heating mantle controlled by a rheostat. The flask and mantle rests on a jack for ready removal and insertion of the boiling flask into the connecting arm.

The apparatus is placed in operation by adding about 4 l. of methylene chloride to the extraction unit through the liquid entry port. Then, using a small motor-driven pump, the aqueous, sirenin-containing solution is added. This forces methylene chloride through the plastic connecting tube and vapor block into the boiling flask. When the level of the liquid in the extraction unit is approximately 1 in. below the vapor return arm and no more methylene chloride is running into the boiling flask, the level of methylene chloride in the extraction unit is marked for future reference. The level of methylene chloride in the boiling flask is then adjusted by addition or removal to approximately one-third to one-half full. With the liquid entry port closed and the condenser water turned on the unit is ready to operate. Two or three boiling chips are added to the boiling flask, the heating mantle is turned on, and the methylene chloride is brought to a boil. The vapor passes through the connecting arm unit to the condensing unit, around the ring seal *via* the vapor bypass arms, to where it condenses on the lower part of the condenser. The liquid then drops into the distributor unit and sprays out through the perforations. The heat input into the boiling flask has to be enough to keep a sufficient hydrostatic head in the distributor to result in a vigorous spray from the perforations.

The unit as diagrammed holds approximately 40 l. of aqueous solution. Sirenin and other substances soluble in methylene chloride are removed in 24 hr. The aqueous solution and some of the scum at the liquid–liquid interface are then removed by siphon through a 0.5-in. plastic tube and the lost methylene chloride made up to the mark. Another load of solution is then extracted. Once each week the content of the boiling flask is removed and reduced to 50 ml under vacuum. With two extractors in continuous operation, 28 carboys (see under production) of sirenin-containing solution are handled each week, the total volume being approximately 425 l.

*Purification.* Using the bioassay as the only criterion for the presence of sirenin, a variety of methods was applied to the crude methylene chloride solution of sirenin described in the extraction procedure. Chromatography on alumina was the most effective and convenient for large-scale work and this was incorporated into the final procedure. Since the sirenin-enriched fractions from alumina chromatography all showed strong hydroxyl absorption in the infrared, they were treated with 4-(4-nitrophenylazo)benzoyl chloride (Hecker, 1955) (NABS-Cl).[1] Thin layer chromatography (tlc)[2] on silica gel (chloroform) revealed six red spots in the products, two very intense spots at $R_F$ 0.2 and 0.5, and four weak spots at $R_F$ 0.3, 0.4, 0.6, and 0.7. The spots were eluted with benzene, and the resulting NABS esters were hydrolyzed (Hecker, 1955). Only the NABS esters with $R_F$ 0.2 and 0.5 yielded biologically active material. Treatment of the $R_F$ 0.2 NABS ester with NABS-Cl converted it to the one with $R_F$ 0.5. In addition both NABS esters were produced when sirenin, regenerated from either NABS ester, was treated with NABS-Cl. These esters were subsequently shown to be mono and bis esters and are designated NABS-sirenin ($R_F$ 0.2) and bis-NABS-sirenin ($R_F$ 0.5), respectively.

It was now possible to establish a tlc procedure to detect the presence of sirenin in the fractions from alumina chromatography. A small amount of each fraction was treated with NABS-Cl and the product analyzed by silica gel (chloroform) tlc. When compared to the silica gel (methanol) thin layer chromatogram of each fraction before NABS-Cl treatment, it was found that the fractions from alumina chromatography that had a major spot at $R_F$ 0.6–0.7, visualized with iodine vapor and immediately preceded by a spot visible only under ultraviolet light, corresponded to the fractions that showed the presence of both NABS-sirenin esters. Therefore, tlc on silica gel (methanol) was used to establish which fractions from alumina chromatography contained sirenin.

*Bis-NABS-Sirenin.* For a large-scale isolation, 250 ml of crude sirenin methylene chloride solution was concentrated *in vacuo* at 32° to give 16.1 g of brown, viscous oil. This was diluted with 10 ml of chloroform and applied to 505 g of alumina (neutral, activity grade 3), using chloroform for development and elution. A total of 550 ml was collected in the first seven fractions which gave 9.6 g of residue after evaporation, while the last six fractions totaled 700 ml and gave 2.9 g of residue. These last six fractions contained sirenin as shown by silica gel (methanol) tlc.

To a 100-ml flask covered with aluminum foil[3] and in a nitrogen atmosphere was added the sirenin-enriched material in 100 ml of anhydrous benzene, 7.2 ml of pyridine (distilled from *p*-toluenesulfonyl chloride), and 6.5 g of NABS-Cl. The reaction mixture was stirred for 17 hr at room temperature and then filtered, washing the precipitate with benzene until the washings were colorless. The combined filtrate and washings were evaporated *in vacuo* at 32°, leaving 3.5 g of red product which was chromatographed on 324 g of alumina (neutral, activity grade 2.5). Elution was started with benzene and continued with ether and then chloroform. The only sirenin-derived material was shown to be in the benzene fraction by tlc, and this material (2.4 g) was chromatographed two additional times on alumina (630 and 480 g) to yield 1.14 g (1.53 mmoles) of pure bis-NABS-sirenin. This material was recrystallized from benzene–cyclohexane and melted at 186–187°. The molecular weight was determined from its extinction coefficient in methanol–chloroform (9:1, v/v), $\lambda_{max}$ 330 m$\mu$, using 32,000 asthe s tandardvalue per NABS residue (Hecker, 1955).[3]

[1] The abbreviation NABS comes from the German name for this compound (4′-nitroazobenzolcarbonsaure-4) and is already established in the literature.

[2] tlc = thin layer chromatography.

[3] The effect of the light-induced *cis–trans* isomerization and solvent composition on the extinction coefficient of NABS esters is significant and will be reported in the near future.

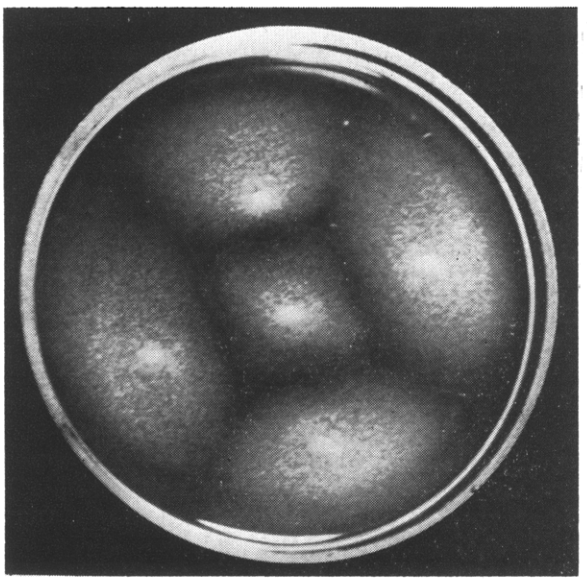

FIGURE 3: Plates of female plants used to begin the production cycle.

*Anal.* Calcd for $C_{41}H_{38}N_6O_8$: C, 66.3; H, 5.2; N, 11.3; mol wt, 742.8. Found: C, 66.2; H, 5.2; N, 11.1; mol wt, 744.

*Sirenin.* Hydrolysis of bis-NABS-sirenin was carried out in a nitrogen atmosphere by treating a solution of bis-NABS-sirenin (314 mg, 0.42 mmole) in benzene (63 ml) with 8.8 ml of a solution of 4% potassium hydroxide in 2-methoxyethanol. The reaction mixture was stirred at room temperature for 28 hr and filtered, and the precipitate was then washed with two 10-ml portions of benzene. The combined filtrate and washings were washed with water (5 ml), several portions of saturated sodium bicarbonate solutions (3 ml each) until the washings were colorless, and saturated sodium chloride solution (3 ml). The benzene solution was then dried over sodium sulfate, filtered, and evaporated *in vacuo* at 32°, leaving 97.5 mg (98% yield) of sirenin as a pale yellow, viscous liquid. A small amount of colored impurity (4%) can be removed by chromatography on alumina (37 g, neutral, activity 3), eluting successively with benzene (100 ml), methylene chloride (60 ml), and chloroform (100 ml), from which sirenin is obtained as a faintly yellow oil, $[\alpha]_D^{22}$ −45° (*c* 1.0, chloroform).

*Anal.* Calcd for $C_{15}H_{24}O_2$: C, 76.2; H, 10.2; mol wt, 236. Found: C, 75.6; H, 10.1; mol wt, 236.

The slightly low carbon values were due to a trace of residual chloroform (presence of chlorine) which was extremely difficult to remove. The molecular weight was established by mass spectral analysis of sirenin and of its oily diacetate (calcd 320, found 320), prepared by treating sirenin with acetic anhydride and pyridine.

*NABS-Sirenin.* To a 50-ml centrifuge tube covered with aluminum foil was added sirenin (47 mg, 0.2 mmole) dissolved in benzene (6 ml), pyridine (37 μl), and NABS-Cl (67 mg, 0.23 mmole) dissolved in benzene (6 ml). After standing under nitrogen for 22 hr at room temperature, the reaction mixture was centrifuged and the supernatant was removed. The residue was rinsed thrice with 2-ml portions of benzene and the combined supernatants were washed with 3 ml of water and twice with 3 ml of saturated sodium chloride solution. After being dried over sodium sulfate, the solution was filtered and then evaporated *in vacuo* at 32° to give 95.5 mg of orange-red solid which was chromatographed on alumina (47 g, neutral, activity 3) with benzene to separate the bis-NABS-sirenin (36 mg, second fraction) from the NABS-sirenin (29.5 mg, fourth fraction). In this manner chromatographically pure NABS-sirenin was obtained. After crystallization from benzene–cyclohexane it melted at 115–116°. The molecular weight was determined from its extinction coefficient in 95% ethanol, $\lambda_{max}$ 330 mμ, using 31,400 as the standard value per NABS residue (Hecker, 1955).[3]

*Anal.* Calcd for $C_{28}H_{31}N_3O_5$: C, 68.7; H, 6.4; N, 8.6; mol wt, 489.6. Found: C, 68.7; H, 6.3; N, 8.4; mol wt, 490.

## Results

*Production.* The production facility and procedures described herein were planned to yield 80 l. of sirenin-containing aqueous solution each day. It replaced part of the methods previously described (Machlis, 1963) which ultimately failed because of excessive problems with contamination.

The process began with stock cultures of the female strain of *Allomyces* maintained in slants on Difco Emerson YpSs agar prepared according to the manufacturer's directions. From the stock culture, master plate cultures were started by transferring 1-mm square agar blocks containing vegetative mycelium to the center of agar plates (100 × 16 mm) containing 40 ml of the YpSs solidified medium. The master plates were incubated at 25° for 7 days in darkness and then used over the next 3–4 days. From the growing edge of the master plates or, if they were lost by contamination, from a stock culture, two master plates were started every 3–4 days. Twice a week transfers were made from the growing front of the master plates to secondary plates, five transfers per plate, and these were also grown for 7 days at 25° in the dark (Figure 3) and then used over the next 3–4 days. Enough secondary plates were grown to provide two mature, contaminant-free plates daily.

The contents of two secondary plates, agar and plants, were transferred to a sterile Waring blendor cup (semimicro, monel metal with cover), 75 ml of sterile distilled water was added, and the mixture was then fragmented for 30 sec. This material was used to inoculate liquid cultures consisting of 300 ml of medium (0.2% w/v glucose and 1.2% w/v Anheuser–Busch Basaminbact autolyzed yeast extract) contained in 500-ml Erlenmeyer flasks plugged with cotton and capped with paper cups. To keep contamination to a

minimum, the transfer of the fragmented material was made using sterile 30-ml hypodermic syringes fitted with 3.5-in. no. 13 guage needles. After picking up 20 ml of the blend, the needle was inserted between the cotton plug and the glass wall of the flask and then emptied. Six flasks were so inoculated daily and then incubated at 25° on a shaker for 56 hr. The now greatly increased amount of material consisting of numerous small plants bearing only a few gametangia was used to inoculate carboys.

The carboy cultures consisted of standard 5-gal Pyrex carboys containing 10 l. of 0.15% w/v glucose in tap water plus 20 ml of 3.7 M sodium acetate adjusted to pH 7.5 with NaOH. The neck of each carboy was wrapped with several layers of cheesecloth held in place with stainless steel wire so that when the closure was put in place a close but not excessively tight fit resulted. The closure consisted of a standard $4 \times 1/8$ in. diameter commercial can enameled inside and out which was cut to a length of $3 \times 0.5$ in. A hole was drilled in the end of the can to take a $7/16$-in. electrical grommet through which was passed the glass aerating tube (Figure 4). A plastic (autoclavable) standard 8-in., single-bulb, drying tube filled with cotton was attached to the aerating tube by rubber tubing. The carboy cultures were autoclaved for 3 hr, 14 at a time, in a large autoclave made available by the Department of Bacteriology.

Each day there was added aseptically to each of four carboys the contents of one liquid culture. The carboys were incubated at 25° with vigorous aeration for 48 hr. For this part of the process a portion of a room was equipped with outlets from the building compressed air supply to make possible the growth of up to 16 carboys at one time. The air was passed through charcoal and cotton filters before passing through the gas drying tube attached to each carboy. The vigor of the aeration was such as to keep the plants in constant motion throughout the liquid in the carboy. At the end of the incubation each of the original plants had increased in size, was spherical in form, and was coated with gametangia.

The final step in the biological part of the process was the only one in which aseptic procedures were not used. After 56 hr of incubation, the carboys were filled to within 3 in. of the top with tap water by hose directly from the house water supply. The incubation with aeration was then continued for 18 hr. Within 2–4 hr the previously clear liquid would become milky as a result of the release of gametes. Assays for sirenin at intervals after adding the tap water showed that the highest level of sirenin was reached after 18 hr and then decreased in concentration.

At the conclusion of the gamete discharge the contents of the carboys were poured through cheesecloth, thereby removing the plants, and the filtrate was then pumped into the extractors described earlier. The weekly production represented by 50 ml of methylene chloride solution was assayed biologically and became the beginning point for the subsequent purification procedures. The average concentration of sirenin in the final carboy solutions was approximately $10^{-6}$ M. Production was discontinued when crude methylene chloride extracts containing approximately 2.5 g of sirenin had been accumulated.

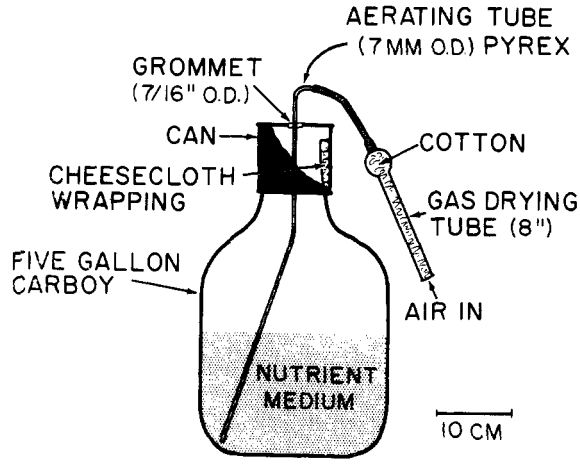


FIGURE 4: Diagrammatic scale drawing of carboy culture apparatus for production of sirenin.

Certain details of the production process deserve some explanation. It might appear possible to inoculate the liquid flask cultures with fragmented material prepared from liquid flask cultures, thus eliminating the master and secondary plate cultures on solid medium. This cannot be done because the female strain is not pure female but always contains a small proportion of male gametangia. A continuous liquid culture system would soon result in the buildup of sporophytic plants which do not bear gametangia and do not give rise to sirenin. For the same reason, the primary and secondary plates must be surface dry before inoculation. If not, the moisture can result in the discharge of what few gametangia might be present on the transfer mycelium resulting in the development of unwanted sporophytic plants. The details for the growth of the liquid flask cultures were arrived at through a series of experiments having the objective of rapid growth with a minimal development of gametangia. If these cultures are allowed to grow under the specified conditions for much longer than 56 hr there is then a profuse appearance of gametangia. This must be avoided because on adding these plants to the carboys, the gametangia will discharge, fertilization will occur, and the carboy cultures will then contain an undesirably high number of sporophytic plants.

Acetate was added to the carboy cultures as a simple means of controlling the pH. In the absence of the acetate, the pH of the carboy cultures drops to a value <4.0 which prevents the formation of gametangia and greatly reduces the growth. The drop in pH is presumably caused by the production of lactic acid (Ingraham and Emerson, 1954). Machlis (1953) had shown that *Allomyces* could utilize acetate with a concomitant increase in pH of the medium. Appropriate experiments with the carboy cultures established the concen-

tration of acetate necessary to keep the pH from dropping below 6.5 during 48 hr of incubation.

The obviously deficient medium in the carboys, supplemented only by the partially spent medium from the liquid flask cultures, was developed by direct experiment with the carboys. The objective was to obtain the maximum growth in 48 hr but with each plant coated with gametangia. The latter do not develop in rich media unless sufficient time is provided for the medium to become deficient.

*Isolation.* The sirenin-containing aqueous solution from the carboys was extracted for 24 hr with methylene chloride in the continuous extractors described earlier. The methylene choloride extract of 28 carboys (1 week of production) was reduced to 50 ml.

A variety of methods was explored for further purification of sirenin, analyzing the results of a given method by the bioassay technique. While countercurrent distribution and vapor phase chromatography did not increase the purity of crude sirenin, short-path distillation at 100° (0.01 mm) and thin layer chromatography afforded a 5- to 10-fold increase in activity. Paper chromatography (butanol–acetic acid–water, 4:1:1, v/v) gave a 20–50-fold increase in activity but was not as suitable for preparative scale purification as was chromatography on alumina which produced a comparable increase in activity. Further attempts to purify sirenin by alumina chromatography produced little or no increase in activity although impurities could be detected by tlc.

Since solubility behavior and infrared spectra had indicated the presence of hydroxyl functions, the sirenin-enriched fractions from one alumina chromatography were treated with NABS-Cl. This has been shown to be an excellent derivatizing agent for alcohols (Hecker, 1955), usually yielding colored, crystalline esters with favorable chromatographic and solubility properties. From the resulting mixture of at least six NABS esters, only two were found to yield biologically active material when hydrolyzed. The two NABS-sirenin esters at $R_F$ 0.2 and 0.5 on silica gel (chloroform) could be regenerated from sirenin, obtained from the hydrolysis of either, and the NABS ester at $R_F$ 0.2 could be converted to the one at $R_F$ 0.5 by treatment with NABS-Cl. Thus sirenin formed two esters with NABS-Cl which are designated NABS-sirenin ($R_F$ 0.2) and bis-NABS-sirenin ($R_F$ 0.5) on the basis of combustion analyses and ultraviolet absorption.

For preparative scale isolation of the NABS-sirenin esters it was convenient to convert the sirenin-enriched material from one alumina chromatography to bis-NABS-sirenin with an excess of NABS-Cl since impurities could not be removed easily from the NABS-sirenin by alumina chromatography. Also, a tlc procedure was developed to analyze directly the fractions from alumina chromatography for the presence of sirenin. This was done simply by comparing the silica gel–methanol thin layer chromatogram of the fractions from alumina chromatography before treatment with NABS-Cl with the silica gel–chloroform thin layer chromatogram of the resulting NABS esters. In the former system a spot at $R_F$ 0.6–0.7 visible after exposure to iodine vapor and immediately preceded by a whitish spot visible under ultraviolet light corresponded to the production of the NABS-sirenin esters. Once the fractions from alumina chromatography had been analyzed for sirenin and the sirenin-enriched fractions converted to bis-NABS-sirenin, three to four chromatographies on alumina yielded chromatographically pure bis-NABS-sirenin from which sirenin could be obtained. NABS-sirenin was obtained from sirenin along with bis-NABS-sirenin which was separated by chromatography on alumina. From 250 ml of crude methylene chloride extract, a yield of 360 mg of sirenin could be obtained. The crude extract used was accumulated prior to the full stabilization of the production process; hence it is not possible to relate the yield of sirenin to the original concentration in the carboys.

*Characterization.* Sirenin is a colorless, optically active, viscous liquid with a molecular weight of 236 assigned from its mass spectrum and from the mass spectrum of the diacetate of sirenin, mol wt 320. The molecular weights of NABS-sirenin (490) and of bis-NABS-sirenin (744) were determined from the ultraviolet absorption of the NABS chromophore and fully support the assigned molecular weight. The molecular formulas for NABS-sirenin ($C_{28}H_{31}N_3O_5$) and bis-NABS-sirenin ($C_{41}H_{38}N_6O_8$) support the formula of $C_{15}H_{24}O_2$ for sirenin, as does its analysis. Sirenin is thus an oxygenated sesquiterpene with four degrees of unsaturation and with both of its oxygen functions capable of forming esters.

## Acknowledgments

The biological production of sirenin was made possible by a series of undergraduate and graduate students who performed the daily routines as well as the many different types of experiments and the numerous bioassays. Early exploratory experiments on the isolation were carried out by Peter Sheldrick.

## References

Emerson, R. (1941), *Lloydia 4*, 77.

Emerson, R., and Wilson, C. M. (1954), *Mycologia 46*, 393.

Hecker, E. (1955), *Ber. 88*, 1666.

Ingraham, J. L., and Emerson, R. (1954), *Am. J. Botany 41*, 146.

Machlis, L. (1953), *Am. J. Botany 40*, 460.

Machlis, L. (1958a), *Physiol. Plantarum 11*, 181.

Machlis, L. (1958b), *Nature 181*, 1790.

Machlis, L. (1958c), *Physiol. Plantarum 11*, 845.

Machlis, L. (1963), *in* Physiology of Reproduction, Hisaw, F. L., Jr., Ed., Corvallis, Ore., Oregon State University.

Machlis, L., and Rawitscher-Kunkel, E. (1963), *Intern. Rev. Cytol. 15*, 97.