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The Isolation and Characterization of Asperenone, a New Phenylpolyene from *Aspergillus niger**

W. E. Jefferson, Jr.

ABSTRACT: Asperenone, a new pigment from the vegetative mycelium of *Aspergillus niger*, has been isolated as a crystalline solid by solvent extraction and chromatographic procedures. Nuclear magnetic resonance, mass, and infrared spectroscopy of asperenone and its decahydro derivative shows asperenone to be a methyl derivative of 13-phenyltrideca-4,6,8,10,12-pentaen-3-one. The methyl group is located on one of the positions 5–11 and is tentatively suggested to be at position 8 on the basis of the abundance of hydrocarbon fragments in the mass spectra. The occurrence of two other substances with absorption spectra suggesting polyene chromophores in extracts of *A. niger* mycelium is also reported.

An accompanying paper (Jefferson, 1967) describes the accumulation of asperenone, a yellow pigment, when *Aspergillus niger* mycelium in replacement cultures is treated with small amounts of certain steroids. A spectroscopically identical pigment accumulates when the organism is grown or replaced on glycerol medium without added steroids. The present communication describes the isolation and characterization of this new metabolite of *A. niger* for which the name asperenone is proposed.

Materials and Methods

The organism used was *A. niger* NRRL-3. Cultures used for the isolation of the pigment were either grown, replaced, and treated with estradiol as described by Jefferson (1967) or were grown in glycerol medium consisting of the following per liter of tap water; glycerol, 20 ml; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; NH_4NO_3, 2.5 g; $ZnCl_2$, 0.52 mg; $FeSO_4 \cdot 7H_2O$, 6.5 mg; $CuCl_2 \cdot 2H_2O$, 0.32 mg; yeast extract, 30 mg; and HCl (37%), 0.5 ml. For small-scale production, 500 ml of the glycerol medium in 2-l. culture flasks was sterilized by autoclaving and inoculated with conidia. Incubation was at 26° on a reciprocating shaking machine. For larger scale production, 16 l. of unsterilized medium in carboys was inoculated with 300 ml of 48-hr cultures grown on sterile sucrose medium. The larger cultures were vigorously aerated by means of sintered-glass cylinders.

After 3–4 days, the pigmented mycelium was filtered through cheesecloth in a Buchner funnel, washed several times with water, and partially dried by covering the funnel with a rubber sheet (dental dam) while continuing the aspiration. Drying was completed at 50° in an oven equipped with a blower. The dried mycelium was stored in a refrigerator and ground immediately prior to extraction.

Extracts and samples were stored at refrigerator temperatures. Exposure to bright light was avoided.

Thin layer chromatographic plates were prepared by the dipping method of Peifer (1962) using silica gel G as adsorbent. They were developed with 25% ethyl ether in petroleum ether (bp 30–60°). Spots were observed under ultraviolet light and after spraying with concentrated sulfuric acid and heating.

Florisil and silicic acid columns were prepared from petroleum ether slurries of the adsorbents. Samples dissolved in petroleum ether were added to the columns. Development was with varying amounts of U.S.P. ethyl ether (usually 20% v/v) in petroleum ether.

Countercurrent distribution employed a stationary phase of 85%, v/v, aqueous methanol equilibrated with a mobile phase of petroleum ether.

Visible and ultraviolet spectra obtained with a

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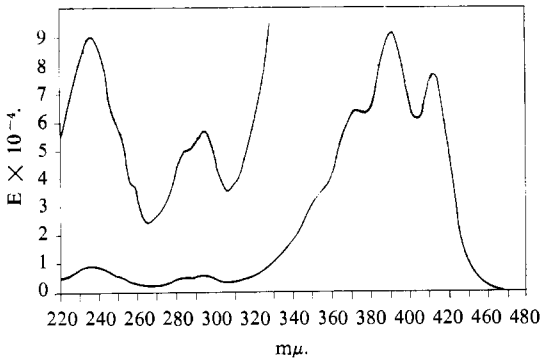

FIGURE 1: The visible and ultraviolet absorption spectra of asperenone in hexane. Values on the ordinate refer to the lower curve. The upper curve is ten times the concentration of the lower.

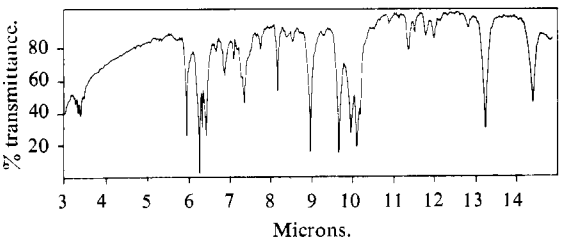

FIGURE 2: The infrared spectrum of asperenone. Concentration = 0.5% in KBr.

Beckman Model DK-2 spectrophotometer, are replotted on a linear wavelength scale. Evaporations were carried out in a rotary evaporator at water aspirator pressure and a bath temperature of 50° or less. Melting points were taken on a Fisher-Johns apparatus and are uncorrected.

Crude extracts were saponified in an atmosphere of N_2 by 40-min reflux with 0.1 N KOH in methanol. Nonsaponifiable material was extracted with ether after dilution with four volumes of water.

Results

The yellow pigment was found to be extractable with a variety of lipid solvents. An initial absorption spectrum was obtained after leaching small quantities of the pigmented mycelium with methanol at room temperature. After removal of the methanol by evaporation, a spectrum of the material dissolved in hexane was obtained. In addition to the strong absorption due to ergosterol in the 260–290-mμ region, peaks were visible at approximately 320, 338, 352, 371, 390, and 415 mμ. Some samples also had a shoulder at about 440 mμ. Subsequent fractionation indicated that these absorption maxima result from a mixture of three materials: asperenone which is described below; a substance we have called unknown A with absorption maxima at 338, 355, and 375 mμ in hexane; and unknown B absorbing at 376 (sh), 394, 413, and 437 mμ. A small quantity (1.7 mg) of unknown B has been crystallized. Its melting range is 117–121°. Unknown B is eluted from Florisil or silicic acid columns before asperenone.

Some crystalline asperenone was prepared by extracting the ground mycelium with methylene chloride in a Soxhlet extractor followed by saponification, digitonin precipitation of the sterols, countercurrent distribution, and chromatography on silicic acid. However better yields and greater convenience were achieved with the scheme described below which includes yield data from one preparation.

Dry mycelium (1500 g) was ground, mixed with an equal volume of Celite, and extracted by percolation with methylene chloride until most of the yellow pigment was removed. The extract was evaporated and the residue was taken up in petroleum ether. The resulting solution was extracted three times with one-third its volume of 85% methanol. The aqueous methanol extracts were combined, evaporated (15.3 g), saponified, and extracted with diethyl ether. The ether-soluble portion (2.66 g) was treated with digitonin to remove sterols, washed, taken up in petroleum ether, and applied to a 50 × 4 cm column containing Florisil. Elution was with 20% ethyl ether in petroleum ether. Most of the asperenone was contained in fractions consisting of the 630th–740th ml of eluate. These were combined, evaporated (636 mg), applied in petroleum ether to a 30 × 2 cm column of silicic acid, and eluted with 20% ethyl ether in petroleum ether. The 535th–580th ml of eluate was evaporated (482 mg) and taken up in a minimum volume of warm hexane. Crystals (96 mg, mp 129–132°) formed on slow cooling. Recrystallization from methanol yielded 75 mg of crystals (mp 132–134°). The melting point was unchanged after another recrystallization from hexane. The progress of purification was followed by thin layer chromatography. The final product gave only one spot.

Elemental analysis of the material isolated by this procedure gave: C, 86.32; H, 8.35; O, 5.37. Calcd for $C_{20}H_{22}O$: C, 86.29; H, 7.97; O, 5.75. The visible and ultraviolet absorption spectra of asperenone in hexane are shown in Figure 1. The positions of the

TABLE I: Position of the Major Absorption Maxima of Asperenone in Various Solvents.

Solvent	Wavelength (mμ)		
Hexane	373	391	414
Benzene	384 (sh)	400	421
Carbon disulfide	393 (sh)	411	431
Methanol		400[a]	
Chloroform	383 (sh)	402	425 (sh)
Isooctane	370	389	413

[a] Very broad.

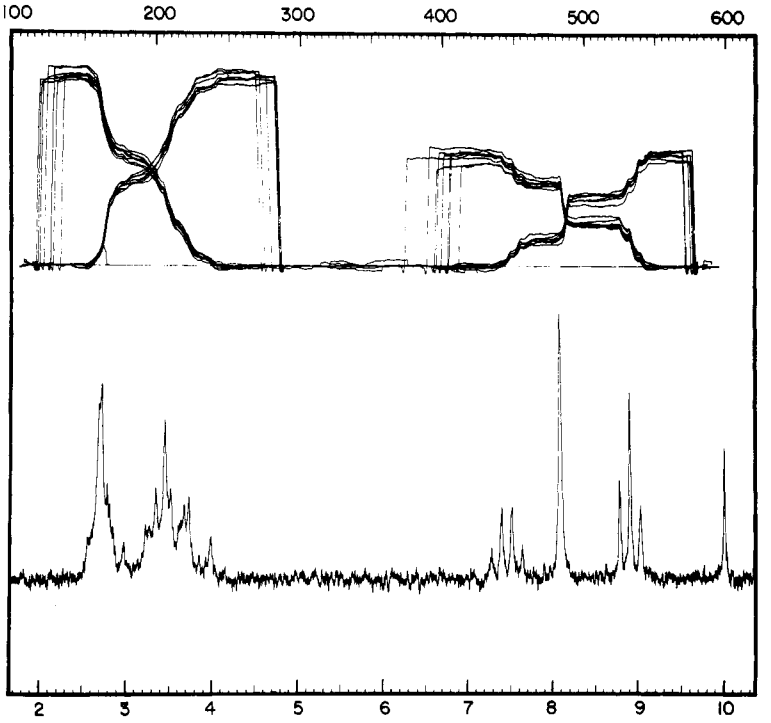

FIGURE 3: The 60-Mcycle nuclear magnetic resonance spectrum of asperenone in $CDCl_3$. The internal standard was tetramethylsilane.

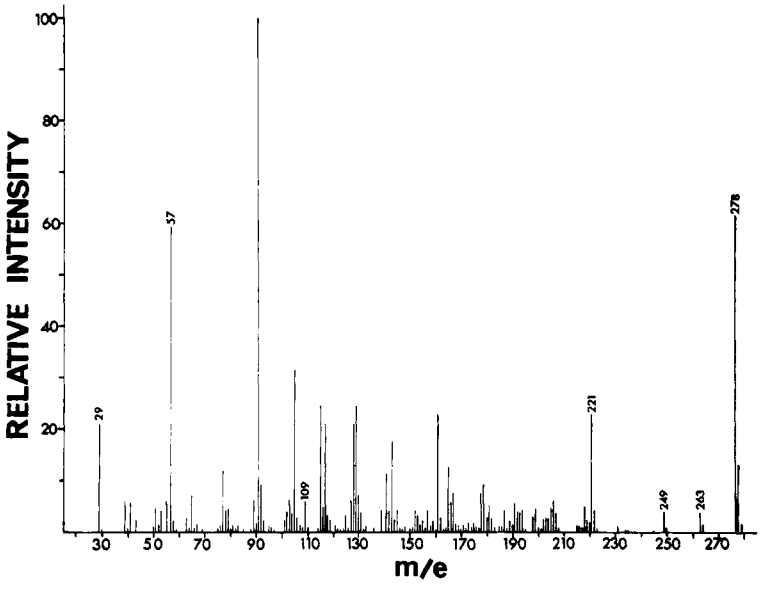

FIGURE 4: The mass spectrum of asperenone.

major maxima in a variety of solvents are given in Table I. The infrared spectrum in KBr is shown in Figure 2, the 60-Mcycle nuclear magnetic resonance spectrum in $CDCl_3$ is shown in Figure 3, and the mass spectrum in Figure 4.

That the compound must have the empirical formula $C_{20}H_{22}O$ is established by the parent peak in the mass spectrum at *m/e* 278, the elemental analysis, and the integration of the nuclear magnetic resonance spectrum which shows 22 protons if the sum of the aliphatic protons is taken to be eight, *i.e.*, one ethyl and one methyl group.

That the oxygen is part of an ethyl ketone conjugated with the rest of the chromophore is indicated by the following observations. The carbonyl peak at 5.95 μ is characteristic of α,β-unsaturated ketones. The promi-

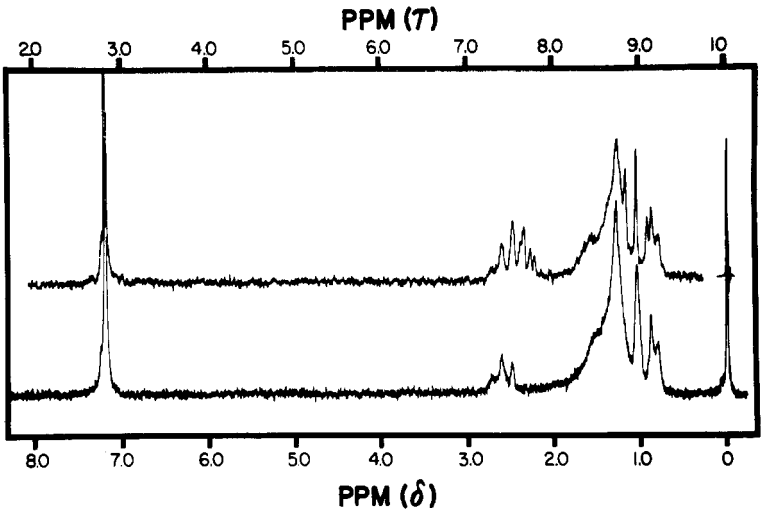

FIGURE 5: The 60-Mcycle nuclear magnetic resonance spectrum of tetrahydroasperenone in $CDCl_3$. The upper curve was taken before and the lower after deuterium exchange by the method of Lund *et al.* (1963). The internal standard was tetramethylsilane.

nent peaks in the mass spectrum at *m/e* 57 and 221 and 29 and 249 can be explained as α-cleavage of an ethyl ketone. The chemical shift (τ 7.45) of the methylene protons of the ethyl group (Figure 3) is consistent with this interpretation, and the contrast between the ultraviolet absorption in hexane and methanol (Table I) is characteristic of polyene chromophores conjugated with carbonyls.

The existence of a phenyl ring is clearly shown by the infrared, nuclear magnetic resonance, and mass spectra. The strong infrared absorption bands at 14.42 and 13.24 μ and the intensity of the peak at *m/e* 91 in the mass spectrum suggest that it is monosubstituted. The singlet at τ 8.1 in the nuclear magnetic resonance spectrum can only be a methyl group while the remaining protons (9 or 10) appearing between τ 3 and 4 must represent a polyene structure.

The above deductions indicate that asperenone is a methyl derivative of 13-phenyltrideca-4,6,8,10,12-pentaen-3-one. A partial confirmation of this assignment is obtained from the ultraviolet spectrum (Figure 1) which, within the limits possible from the published spectrum (Gansser and Zechmeister, 1957), is indistinguishable from that of 11-phenylundecapentaenal which has the same chromophore.

The position of the methyl group is more difficult to assign. In an attempt to locate its position, a 16-mg sample of crystalline asperenone was dissolved in methanol and reduced with H_2 at atmospheric pressure using 1.3 mg of 10% palladium on carbon as the catalyst. Hydrogenation was complete in about 20 min at room temperature with the consumption of approximately 5 moles of H_2/mole of asperenone. The product is a viscous oil with an ultraviolet spectrum essentially identical with that of toluene. An infrared spectrum of a drop of the oil between plates of NaCl has a carbonyl band at 5.84 μ. The nuclear magnetic resonance spectrum of this reduction product is shown by the upper curve in Figure 5 and the mass spectrum in Figure 6. The parent peak at *m/e* 288 confirms the existence of five olefinic double bonds. The methyl singlet at τ 8.1 in the nuclear magnetic resonance spectrum of asperenone has become a doublet centered at τ 9.1 (J = 4.6 cps) in the decahydro derivative. The methyl group must, therefore, be located on the side chain. This spectrum also shows a group of protons absorbing between τ 7 and 8 which must be the protons which are α to the carbonyl and α to the phenyl ring. Integration of this portion of the spectrum gave a value of 5.3 which was inadequate to determine whether or not the methyl group in question occupies one of these positions. That it does not was shown by exchanging the protons adjacent to the carbonyl for deuterium by the method of Lund *et al.* (1963). After deuterium exchange the spectrum changed to that shown by the lower curve in Figure 5. The absorption in the τ 7–8 region was reduced to a triplet centered at τ 7.32 (J = *ca.* 7 cps) which could only be due to the protons on the carbon atom adjacent to the ring. The doublet due to the methyl branch at τ 9.1 was unchanged while the spectrum of the terminal methyl became a single peak at τ 8.93. The methyl group can not be on a carbon α to the carbonyl since it still appeared as a doublet after deuterium exchange and it can not be either α or β to the phenyl ring since the protons α to the ring are split by two others rather than by the five expected for α substitution or the one for β substitution.

We are thus left with the conclusion that the methyl branch is on one of the similar positions (5–11). We have not succeeded in definitely establishing its location, but believe it to be on position 8 on the basis of the abundance of hydrocarbon fragments in the mass spectra. The abundance of hydrocarbon fragments corresponding to the sum of the series C_nH_n, C_nH_{n+1}, C_nH_{n-1}, and 3_nH_{n-2} reaches a minimum at n = 12. Peaks corresponding to C_7H_7, C_8H_9, $C_{10}H_9$, and $C_{11}H_{11}$

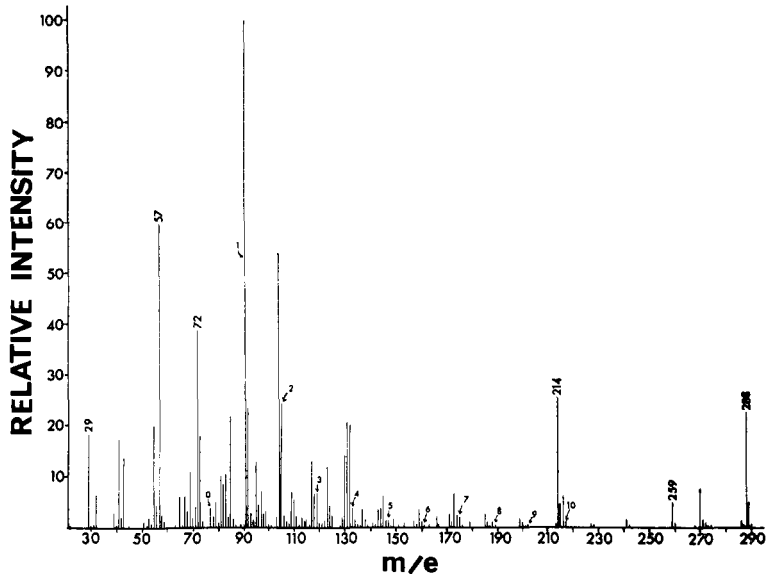

FIGURE 6: Mass spectrum of tetrahydroasperenone. Numbered arrows give values of n for the series $C_6H_5(CH_2)_n^+$.

are prominent while those containing 13 and 14 carbon atoms are less abundant, but exceed the 12-carbon fragments. A small peak at *m/e* 109, corresponding to C_7H_9O, also occurs and may represent a break adjacent to the methyl branch at C-8. Examination of the mass spectrum of decahydroasperenone (Figure 6) leads to a similar conclusion in that 12-carbon hydrocarbon fragments are less abundant than those containing 11 or 13 carbon atoms.

The most likely structure for asperenone is, therefore, 8-methyl-13-phenyltrideca-4,6,8,10,12-pentaen-3-one.[1] Comparison of the ultraviolet spectrum with that of 11-phenylundecapentaenal (Gansser and Zechmeister, 1957) suggests that the polyene portion of asperenone is all *trans*.

Discussion

Among fungal pigments, asperenone most closely resembles cortisalin, 15-*p*-hydroxyphenylpentadeca-2,4,-6,8,10,12,14-heptaenoic acid (Gripenberg, 1952). It would also appear to be related to the various phenylpolyenes and polyynes from higher plants and less strikingly to the larger class of nonisoprenoid polyenes.

The relationship of asperenone to unknowns A and B is not established, but their absorption spectra are compatible with similar structures containing shorter and longer chromophores, respectively. This interpretation appears particularly likely for unknown B since we see it only in extracts which also contain large amounts of asperenone.

The relationship between asperenone and unknowns A and B to other pigments from *A. niger* is also unestablished. They clearly are not flavasperone (Bycroft *et al.*, 1962), the only other yellow pigment from *A. niger* of known structure. We have not seen flavasperone in these extracts and conclude that its occurrence may be restricted to conidia. They also are distinct from the "acidic carotenoid" described by Zajic and Kuehn (1962) although there appears to be a correspondence between wavelength of maximum absorption of unknown B and one of the minor absorption peaks reported for this substance. They may be the fluorescent materials reported by Reid (1950), but we generally see more than one each of yellow and blue fluorescent substances on chromatograms of extracts of *A. niger* mycelium.

[1] A synthetic approach to a more definitive location of the branch-methyl group is being attempted.

Acknowledgment

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Added in Proof

Since the submission of this manuscript, a paper, "Asperyellone a New Yellow Pigment of *Aspergillus awamori* and *Aspergillus niger* Part II. The Chemical Structure of Asperyellone (Yu, J., Tamura, G., Takahashi, N., and Arima, K. (1967), *Agr. Biol. Chem.*

(*Tokyo*) *31*, 831), has appeared. Asperyellone is apparently identical with asperenone. These authors suggest the structure 7-methyl-13-phenyl-3-oxotrideca-4,6,8,-10,12-pentaene for asperyellone.

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Steroids and Other Factors Influencing the Accumulation of Asperenone and Fermentation Acids by *Aspergillus niger* in Replacement Cultures*

W. E. Jefferson, Jr.

ABSTRACT: Asperenone and unknown B accumulate together in nitrogen-free replacement cultures of *Aspergillus niger* with no evidence for a precursor–product relationship. There is an inverse relationship between the quantities of asperenone and the citric and oxalic acids produced.

The amount of asperenone accumulated is increased by some steroids, especially estradiol and estrone, while other steroids, especially testosterone and androstenedione, cause increases or decreases depending on the mineral content of the growth or replacement medium. Copper deficiency prevented the accumulation of asperenone and the effect of estradiol on the production of asperenone and citric and oxalic acids. Asperenone accumulation is decreased by cycloheximide and puromycin.

During an investigation of the effects of steroids on fungi, accumulations of an intramycelial yellow pigment accompanied by decreased accumulations of citric and oxalic acids in some estrogen-containing replacement cultures of *Aspergillus niger* were noted. The major yellow pigment was subsequently isolated and characterized as a methyl derivative of 13-phenyltrideca-4,6,8,10,12-pentaen-3-one and given the trivial name, asperenone (Jefferson, 1967). It is accompanied by smaller amounts of two other substances with similar absorption spectra and solubilities. These two uncharacterized materials have been designated as unknowns A and B. This paper reports the results of efforts to determine the factors responsible for the accumulation of asperenone.

Materials and Methods

Organism. A. niger NRRL-3 was employed in these experiments.

Cultural Conditions. The original observations were made using the cultural methods of Shu and Johnson (1948) for the production of citric acid. Except where it is stated otherwise in the text, subsequent cultures were grown as described by Jefferson and Sisco (1961).

Replacement cultures were prepared by pipetting ethyl ether solutions of the steroids, etc., into empty 300-ml erlenmeyer flasks. The ether was evaporated in an air stream prior to the addition of 50 ml of replacement medium which was 5% sucrose unless it is stated otherwise. Approximately 0.75-g portions of washed mycelium obtained by filtration in a Büchner funnel were weighed into each flask. The cultures were incubated at 26° on a reciprocating shaker for 48 hr.

Assays. The exact weight of mycelium in each flask was determined after filtration and drying at the end of the incubation. In most of the experiments the relative amount of asperenone was determined by extracting the dry mycelium with 95% ethanol at 60° for 1 hr.

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