Isolation of β -Amyrin from the Fungus *Aspergillus nidulans*

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The pentacyclic triterpene alcohol β -amyrin, which is commonly found in plants, was isolated from wild-type cultures of the ascomycete fungus *Aspergillus nidulans*. The isolated β -amyrin was characterized by TLC, GLC, and HPLC and produced identical mass and ^1H NMR spectra to those of authentic β -amyrin. This material was isolated from static (non-shaking) cultures.

INTRODUCTION

While the sterols are the most common eukaryotic biochemical consequence of squalene cyclization, other end-products have been observed. These include tetracyclic compounds such as euphol and euphol-like materials in plants and pentacyclic compounds such as tetrahymanol in *Tetrahymena pyriformis*, hopene-I in ferns and lichens, and the amyryns (α - and β -) in several plant species (Boar & Allen, 1973; Nes & McKean, 1977). The presence (or absence) of these synthetic products in an organism has been used as an aid in taxonomic categorization; this characterization presumes that the spectrum of genetic capabilities is being expressed at the time of examination. The finding of specific compounds in uncharacteristic species may indicate either convergent evolutionary processes or the maintenance of unexpressed genetic potential across vast phylogenetic distances.

In a previous report (Shapiro & Gealt, 1982), we identified lanosterol as the first cyclization product of squalene with ergosterol as the ultimate sterol in *A. nidulans*. We report here that this organism is capable of producing a second cyclization product, β -amyrin (Fig. 1), under stationary growth conditions in a defined medium. This is the first report of this compound in the aspergilli and one of the rare reports of it in fungi.

METHODS

Organism and growth conditions. *Aspergillus nidulans* FGSC-4 was grown in a defined glucose/salts medium described previously (Adler *et al.*, 1981). Stationary (non-shaking) cultures were grown for 8 d at 30 °C using an inoculum of 10^6 conidia ml^{-1} . Cells were collected by filtration through Miracloth (Chicopee Mills, Inc., N.Y.), as described previously (Gealt *et al.*, 1976) and extracted immediately.

Lipid extraction. Cells were extracted with methanol/chloroform (2:1, v/v) as described by Kates (1975). During the extraction, cells were homogenized in a Sorvall Omni-Mixer for 1 min at maximum speed to ensure complete removal of lipid material. The extracts were fractionated by TLC on Silica Gel 60 using a mobile phase of benzene/ethyl acetate (80:20, v/v). Materials co-migrating with authentic ergosterol (a 4-desmethyl sterol) and lanosterol (a 4,4-dimethyl sterol) were extracted twice from the silica with chloroform and once with anhydrous diethyl ether. The major component of the dimethyl sterol fraction was further purified by preparative HPLC on a C-18 reverse-phase column (μ -Bondapak, Waters, Inc.) using a mobile phase of acetonitrile/methanol (90:10, v/v).

Analytical methods. GLC was performed on XE-60 (1%) on Chromosorb Q, 100–200 mesh, at 240 °C using a Perkin Elmer gas chromatograph, model 3B, with a flame-ionization detector. All retention times are given relative to cholesterol [relative retention time (RRT) = 1.00]. Analytical HPLC was performed on a C-18 reverse-phase column (Zorbax ODS, Du Pont, Inc.) at 45 °C using a UV detector (either an LDC Spectromonitor III, Laboratory Data Control, or an LC-75, Perkin Elmer Co., equipped for UV wavelength scanning). Retention values are reported as α_c [$\alpha_c = (k'_{\text{sample}})/(k'_{\text{cholesterol}})$]. Direct-probe mass spectral (MS) analysis was performed on a Finnegan Series 4021 GLC-MS instrument with a Series 6000 data system. Proton nuclear magnetic resonance

Fig. 1. The cyclization products of squalene in *A. nidulans*. Squalene (a) is converted to the pentacycle β -amyrin (b) and to the tetracycle lanosterol (c). The lanosterol is converted to ergosterol (d), the predominant sterol of this organism.

(^1H NMR) spectra were obtained at 360 MHz at ambient temperature on a Bruker instrument, model WH-360, in CDCl_3 with $\text{Si}(\text{CH}_3)_4$ as internal standard, at the National Institutes of Health sponsored NMR regional facility at the University of Pennsylvania, Philadelphia, PA, U.S.A. Authentic β -amyrin (Pierce Chemical Co.) was the gift of W. R. Nes (Drexel University).

RESULTS

Lipids from 8-day static cultures of *A. nidulans* were extracted and separated by TLC. In addition to a dense band of material co-migrating with authentic 4-desmethyl sterol (ergosterol), a second band was observed in the sterol region of the TLC co-migrating with authentic 4,4-dimethyl sterol (lanosterol). Both regions were collected from the TLC and the lipids were extracted from the silica for further examination.

The 4-desmethyl material exhibited characteristics consistent with authentic ergosterol: a GLC RRT = 1.33; an HPLC α_c = 0.72; the MS fragmentations at m/e 396 (M^+ , 72%), 378 ($\text{M}^+ - \text{H}_2\text{O}$, 6%), 363 ($\text{M}^+ - \text{H}_2\text{O}$, $-\text{CH}_3$, 94%), 337 ($\text{M}^+ - \text{H}_2\text{O}$, $-\text{C}_3\text{H}_5$, 57%), 253 ($\text{M}^+ - \text{H}_2\text{O}$, $-\text{side chain}$, 100%), and 211 ($\text{M}^+ - \text{H}_2\text{O}$, $-\text{side chain}$, $-\text{C}_3\text{H}_6$, 94%); and ^1H NMR absorptions, in p.p.m. shift (δ) from $\text{Si}(\text{CH}_3)_4$ at 0.63 [C-18(s)], 0.82 and 0.84 [C-26,27(d), J = 7 Hz], 0.91 [C-28(d), J = 7 Hz], 0.94 [C-19(s)] and 1.03 [C-21(d), J = 6 Hz].

The 4,4-dimethyl region contained a major peak with a GLC retention at RRT = 1.63 (72% of the total material) and minor peaks at 1.25 (1%), 1.39 (1%) and 1.86 (27%). The major band was further purified by preparative HPLC. This HPLC-purified material was characterized by GLC, which indicated that the component at RRT = 1.86 was removed, resulting in a sample composition of components at RRT = 1.63 (92%), 1.25 (4%), and 1.39 (4%). Authentic β -amyrin also had an RRT = 1.63, while lanosterol, the dimethyl sterol precursor of ergosterol in non-photosynthetic organisms, has an RRT = 1.48. Another triterpene pentacycle, α -amyrin, has an RRT = 1.82.

When the purified *A. nidulans* material with RRT = 1.63 was examined by analytical HPLC, it exhibited an α_c = 0.91. Authentic β -amyrin also had an α_c = 0.91 (α -amyrin had an α_c = 0.99).

The MS of the amyryns is characterized by an M^+ of m/e 426 and a major fragmentation yielding a component of m/e 218 (Budzikiewicz *et al.*, 1964). The fragmentation of the *A. nidulans* component was: m/e 426 (M^+ , 1%), 275 (1%), 257 (1%), 218 (100%), 203 (47%), 189 (33%), 175 (20%) and 161 (20%). Authentic β -amyryn yielded fragments of m/e 426 (M^+ , 2%), 411 ($M^+ - CH_3$, 0.75%), 275 (4.5%), 257 (2%), 218 (100%), 203 (21%), 189 (20%), 175 (12%) and 161 (20%). The 1H NMR at 360 MHz of both the *A. nidulans* component and the authentic β -amyryn produced singlet peaks at δ : 0.79, 0.83, 0.87, 0.93, 0.96, 0.99 and 1.13 p.p.m. The peak at 0.87 was the strongest, probably representing two singlets from the compound.

Quantitative GLC analysis of lipid extracted from statically grown *A. nidulans* indicated that the material at $RRT = 1.63$ accounted for 0.5% of the cellular dry weight while ergosterol, as the free sterol, was present as 1.8% of the cellular dry weight. Previous extraction of shaking cultures grown in this medium indicated no component at $RRT = 1.63$ (Shapiro & Gealt, 1982).

DISCUSSION

Static cultures of the fungus *A. nidulans* grown on a defined glucose/salts medium produced the pentacyclic triterpenoid alcohol β -amyryn. While TLC, GLC, and HPLC retention characteristics were very suggestive, the MS fragmentation coupled with the 1H NMR spectrum provide compelling evidence for our identification. The presence of β -amyryn in *A. nidulans* was unexpected because, although common in plants, reports of its presence in the fungi are extremely rare. It has been reported as a constituent of the parasitic coffee fungus *Balanophora indica* (Narayanan *et al.*, 1976), but may be a product of the plant host. The biochemical mechanism of synthesis of this compound has been demonstrated in the pea, *Pisum sativum* (Baisted *et al.*, 1962; Capstack *et al.*, 1965) indicating that β -amyryn is an alternative cyclization product of squalene, in photosynthetic organisms to cycloartenol and in non-photosynthetic organisms to lanosterol.

The role of β -amyryn in *A. nidulans* remains unknown. It has been reported as a membrane component in plants (Kemp & Mercer, 1968; Janiszowska & Kasprzyk, 1977), although it is generally sequestered in the resins, latex and waxes (Robinson, 1963). An attempt to substitute it for a sterol in the membranes of anaerobic *Saccharomyces cerevisiae*, which cannot produce sterols, was unsuccessful (Sekula, 1979) although it can replace sterols as a growth stimulant in the fungus *Phytophthora cactorum* (Nes & Heftmann, 1981). It has been shown that many triterpenoids, especially triterpene glycosides, have antifungal activity (Anisimov *et al.*, 1979). Although β -amyryn was not tested by these authors, it might serve as an antifungal agent or a precursor of such an agent. Production of such a factor may give *A. nidulans* a competitive advantage in the natural environment.

Synthesis of both lanosterol and β -amyryn from squalene probably requires two different enzymes with which the precursor may interact. Since β -amyryn was not observed when flasks were shaken during the culture period (Shapiro & Gealt, 1982), we suggest that the enzyme responsible for this alternative squalene cyclization was induced by some environmental factor(s). Investigation of these factors and of their effect on cellular lipid metabolites and protein components is being pursued.

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