Analysis of the Inhibition of the Ergosterol Pathway in Fungi Using the Atmospheric Solids Analysis Probe (ASAP) Method

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Fungal cells treated with various inhibitors to the ergosterol pathway were analyzed using an Orbitrap mass spectrometer equipped with an atmospheric solids analysis probe (ASAP). The technique is a rapid means for determining which of the multiple steps in the ergosterol pathway were interrupted by an inhibitor. Furthermore, in an inhibitor concentration study, the ASAP method was able to rapidly provide an estimate of the effectiveness of inhibition. In this method the cells are inserted directly into a hot nitrogen stream, thus eliminating extensive sample workup before analysis. Data indicating the point of pathway interruption are obtained in seconds. (J Am Soc Mass Spectrom 2007, 18, 1274–1278) © 2007 American Society for Mass Spectrometry

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources are used almost exclusively for introduction of samples in a liquid stream. These atmospheric pressure (AP) ion sources, however, are capable of ionization of samples introduced into the ionization region as vapors. We recently reported modifications of commercial liquid chromatograph/mass spectrometer ion sources to also accommodate gas chromatography/AP-mass spectrometry analyses and demonstrated the advantages of the additional capabilities this modification affords [1, 2]. Also introduced was a method we called atmospheric solids analysis probe (ASAP) for rapidly analyzing volatile or semivolatile liquid or solid materials using only slightly modified commercial ESI or APCI ion sources [3]. The technique is similar to mass spectrometry methods reported for the analysis of drugs [4] and explosives [5, 6] at atmospheric pressure, and overlaps significantly with the DART [7], DESI [8], and DAPCI [9] methods.

APCI ion sources have been used for the analysis of trace organic compounds in air [10–12]. Analysis of less volatile compounds such as drugs [4] and explosives [5] was achieved by introducing the samples into a stream of hot gas that is directed into the APCI ionization region. Using the more direct ASAP method, we have analyzed a variety of synthetic compounds, objects, and biological tissues. The heated nitrogen nebulizing gas from either an ESI or APCI probe is used as the energy source of vaporization. Therefore, the only modification

to a commercial APCI source is installation of a port for inserting the sample into the hot gas stream within the ion source region. Because vaporization and ionization occur at atmospheric pressure, the technique is a rapid sample introduction method and in most cases can replace vacuum solids probe analysis.

The DESI-MS technique has also been used to profile bacterial cells under ambient conditions [13], and DART-MS has been employed to study methyl esters of fatty acids generated under ambient conditions from bacterial whole cells [14]. Mass resolution can be important when analyzing complex mixtures such as those obtained from biological tissue using direct ionization methods because components having the same nominal mass but different elemental compositions can be resolved. In this study, we used an Orbitrap mass spectrometer in combination with ASAP-MS for the direct ambient analysis of ergosterol in a fungal pathogen with minimal sample preparation. Ergosterol is a necessary component of the cell wall of many fungi, and disruption of this biosynthetic pathway with small molecules continues to be a viable means of controlling both pharmaceutically and agriculturally relevant pathogens.

Experimental

All experiments reported herein used an Orbitrap mass spectrometer (Thermo-Fisher Scientific, Bremen, Germany) equipped with a flange that was machined to fit onto the port that is normally used for a photoionization (PI) lamp. The flange allowed a probe assembly to insert a melting point (mp) tube (VWR, Gibbstown, NJ) directly into the hot nitrogen gas stream flowing from the Orbitrap

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Scheme 1. Ergosterol pathway of some fungal pathogens.

APCI probe while simultaneously sealing the ion source volume so that vaporized sample exited the source through the vent tubing into a hood exhaust line. In these experiments the APCI probe was used without flow of

solvent. The nitrogen nebulizing/desolvation gas used for vaporization was heated to 300 °C in these experiments. All mass spectra were acquired in the positive ion mode at 30,000 mass resolution ($M/\Delta M$, 50% valley definition).

Figure 1. ASAP mass spectrum of (a) untreated fungal cells introduced into a hot nitrogen gas stream in an atmospheric pressure ion source with ionization by corona discharge and (b) cells treated with the triazole inhibitor of C14-demethylase (4 μ M) added during growth. The major component in the mass spectrum of the untreated cells is ergosterol and the treated cells eburicol as determined by accurate mass measurements of the molecular and MS/MS fragment ions (data not shown).

Figure 2. ASAP mass spectrum of fungal cells after treatment with inhibitor of (a) C24-methyltransferase (4 μ M) showing lanosterol as the most abundant sterol and (b) inhibition of C14-reductase by fenpropidin (4 μ M) and accumulation of intermediate with elemental composition $C_{28}H_{46}O$ (m/z 398.3777), which is ignosterol as determined by accurate mass MS/MS fragmentation (data not shown).

Ustilago maydis (ATCC14826) was cultured and maintained following established procedures [15]. Before treatment with the various compounds, an overnight culture was grown at 30 °C in yeast extract-phosphate medium (pH 6.5) supplemented with 1% (wt/vol) glucose. The cells were washed with phosphate buffer and inoculated into fresh medium (without yeast extract) to give an initial OD_{600} of 0.1. One mL aliquots of the fungal suspension were dispensed into individual wells of a 24 well culture plate, each one containing serially diluted concentrations of the compounds to be tested. After incubation overnight at 30 °C with shaking at 150 rpm, the cells were separated from the liquid medium using a micro-centrifuge (Digisystem Laboratory Instruments, Taipei Hsien, Taiwan) with subsequent removal of the medium using a pipette. Deionized water was added to the Eppendorf tubes containing the fungal cells to dilute any remaining supernatant before the cells were removed by insertion and removal of the closed end of a mp tube. The mp tube containing the cells was allowed to dry before being placed into the APCI probe hot nitrogen stream for 10 s using the ASAP probe described above. Alternatively, nearly identical results were obtained by freeze-drying the cells and simply scraping the closed end of a mp tube over the cells before insertion in the AP ion source.

Results and Discussions

Ascomycetes are a major class of fungi, many of which are agricultural and pharmaceutical pathogens of significance. Amongst the plant pathogens are those responsible for some significant and costly cereal diseases, such as leaf blotch, leaf rust, and powdery mildew. The ergosterol pathway (Scheme 1) is necessary for fungal cell wall integrity. Interruption of the pathway has provided a successful means of controlling these pathogens; therefore inhibitors of certain steps of the pathway are of potential commercial importance. There are multiple steps that offer numerous targets for inhibition (Scheme 1). A single, facile method that is able to look at valid targets in the biosynthetic pathway simultaneously is of interest as a means for identifying new inhibitors. We therefore tested the ASAP method against several established ergosterol inhibitors of ascomycete growth using the corn smut pathogen, *Ustilago maydis*.

Figure 1a shows the ASAP mass spectrum of untreated *Ustilago*. Under the dry conditions of the closed ion source and dry sample, sterols produce predominately odd-electron molecular ions and radical loss fragment ions. Addition of a slight amount of water vapor to the AP ionization region, however, produces predominately $[MH - H_2O]^+$ and less abundant MH^+

Figure 3. ASAP MS concentration study of inhibitors of ascomycetes with an inhibitor of C24-methyl transferase. The concentration of the inhibitor ranges from zero (control) to 4 μM . The abundances of the molecular ions of ergosterol (left arrows) decrease relative to those of lanosterol (right arrows) with increasing concentration of the inhibitor.

ions (data not shown). The ion observed at m/z 396 is M^{++} of ergosterol, which is clearly being produced by the growing fungal cells. Ions at m/z 397 are from the ^{13}C isotope of the m/z 396 ion as well as MH^+ ions of ergosterol. Figure 1b shows the change in the ASAP spectrum for cells in which the triazole inhibitor of the C14-demethylase step, flusilazole [15], was added during cell growth. It is obvious that the inhibition of ergosterol (m/z 396) occurs after the biosynthesis of eburicol (m/z 440 $[\text{M}]^+$, 425 $[\text{M}-\text{CH}_3]^+$) as this is the dominant sterol intermediate that accumulates most in the cells after treatment with flusilazole. Likewise, in Figure 2a an AZA-sterol analog of lanosterol [16] that inhibits C24-methyl transferase and thus the methylation of lanosterol, causes an increase in concentration of this specific substrate (m/z 426 $[\text{M}]^+$, 411 $[\text{M}-\text{CH}_3]^+$).

Similarly, Figure 2b shows inhibition of the C14-reductase step by fenpropidin and thus accumulation of ignosterol [15] (m/z 398 $[\text{M}]^+$).

It is interesting to note that ergosterol and ignosterol, which have no methyl groups on the "A" ring, show little loss of CH_3 radicals from the molecular ion, whereas lanosterol and eburicol, which have two methyl groups on the "A" sterol ring, show abundant loss of methyl radicals. It should also be noted that numerous other ions are observed in the ASAP mass spectra of cells. For example, other products from the fungal cells such as the saturated fatty acid at m/z 368 ($\text{C}_{24}\text{H}_{48}\text{O}_2$) are observed in all spectra. Also observed are dioctylphthalates (m/z 391, $\text{C}_{24}\text{H}_{39}\text{O}_4$) from the plastic used in preparing the samples. Because the temperature of the heated nitrogen gas can be con-

trolled through the APCI probe, it is possible to select a gas temperature that optimizes the vaporization of the desired products.

A concentration study using the inhibitor of the reaction catalyzed by C24-methyl transferase that causes the accumulation of lanosterol is shown in Figure 3. In the control and at the lowest inhibitor concentration (0.32 μ M), the lanosterol substrate is just detectable. At 0.8 μ M inhibitor concentration the substrate amount increases and by 4 μ M dominates, as determined from high-resolution and accurate mass measurement of the ergosterol ions at m/z 396/379 versus the lanosterol ions at m/z 426/411, and the inhibition of cell growth is essentially complete. The small amount of ergosterol that is detectable is from those cells used to initiate the dose response study. These data show, without any special precautions or sample workup, that estimates can be obtained for the relative effectiveness of a particular inhibitor after treatment of the cells at various concentrations. The ion source condition for this set of experiments was less dry than those discussed above, and loss H_2O from the MH^+ ion was observed for both ergosterol (m/z 379) and lanosterol (m/z 409).

As with certain other methods used to understand the mode of action of ergosterol inhibitors, ASAP-MS also allows both the detection of inhibition of ergosterol and determination of the target of the particular step in the biosynthetic pathway where inhibition occurs. However, a significant advantage is the ability to analyze cellular material directly without resorting to involved extraction procedures of the particular pathway intermediates that accumulate and the elimination of chromatographic steps.

Conclusions

The ASAP MS method allows rapid analysis of fungal cells for inhibition of individual steps of the ergosterol biosynthetic pathway. The only sample workup required was separation of the cells from the liquid medium to observe in-cell processes. In a few seconds per sample, the entire sterol pathway is screened for

inhibition. Observation of the relative ion current for ergosterol versus other sterols makes it possible to estimate the degree of inhibition versus inhibitor concentration.

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