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Epicocconone, A Novel Fluorescent Compound from the Fungus *Epicoccum nigrum*

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In biological assays involving fluorescence, separation of emission from Rayleigh-scattered excitation is necessary and facilitated by a large Stokes' shift, which also minimizes quenching when two or more fluorophores are within the Förster radius.[1] Although fluorescent molecules have a wide range of in vitro and in vivo applications including labeling of specific proteins inside cells,[2] chemosensing,[3,4] fluorescence resonance energy transfer (FRET),[5] molecular beacons,[6] or optical projection tomography,[7] few small molecules have been found that emit red by irradiation in the UV-blue (long Stokes' shift) even though such a property has been identified as having great importance in dual staining methods.[8] Specifically, a dye that can be irradiated by the common Ar laser (488 nm) and have little overlap with the emission of fluorescein (520 nm) would allow dual color staining using a single excitation frequency. Some Eu and Ru complexes do fulfill these criteria, but because they are luminescent, quantum yields tend to be low, and, being inorganic, cell permeability and toxicity are problematic. Herein, we report the isolation, characterization, and application of a novel fluorescent natural product epicocconone (**1**), from the fungus *Epicoccum nigrum*, with a large Stokes' shift and cell permeability that secure its unique biological utilities.

Earlier studies on the fungus *Epicoccum nigrum* resulted in the isolation of many yellow-red pigments including several carotenoids,[9] flavipin,[10] and phenylalanine derived dimers such as 3,6-dibenzyl-2,5-dioxopiperazine[11] and epicorazine A,[12] and the polyketide orevactaene.[13] Fortuitous infection of an agar plate used for the routine culturing of yeast by the fungus *E. nigrum* stained the yeast cells growing near the fungus orange. Viewed under UV light, the yeast cells fluoresced red, but their growth and viability were not affected. To isolate the compound responsible for the observed fluorescence, we developed an assay based on the ability of fractions to stain yeast. The active principle was isolated by chromatography on cellulose followed by size exclusion (Sephadex LH-20), where the active component eluted as a deep purple band, and then HPLC.[14]

The molecular formula of **1** was determined by high-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometry to be $C_{23}H_{22}O_7$.

Initial analysis of the ^{1}H and ^{13}C NMR data indicated the presence of only 22 carbons and 20 unexchangeable protons including two aliphatic methyl groups, a methylene and a methine substituted by oxygen, an aliphatic methylene, and nine aromatic or olefinic protons. Interpretation of the TOCSY and HSQC data allowed the assignment of two subunits (Figure 1A), one of which contained the doublet methyl at 1.84 ppm. This was coupled to an olefinic proton (δ_H 5.97) that was, in turn, coupled to five further olefinic protons. The magnitude of the coupling constants ($^3J_{HH}$ alternating 15, 11 Hz) indicated a 1,3,5-heptatriene unit with all *E*-stereochemistry. The other subunit consisted of two methylenes linked by a methine (δ_H 4.39). One methylene (C6CH_2) was coupled to an exchangeable proton at 1.89 ppm (long-range TOCSY). The

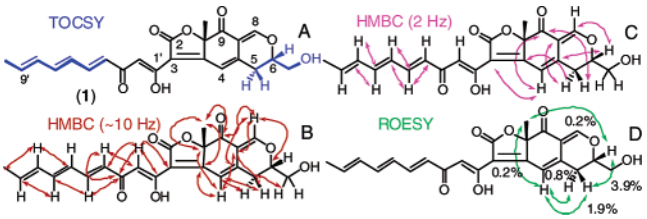

Figure 1. Key correlations from (A) TOCSY (blue), (B/C) HMBC ($^3J_{CH}$ 10 Hz red; 2 Hz magenta), and (D) ROESY experiments (green).

two subunits were further elaborated by interpretation of the HMBC data (Figure 1B). H4′ and H5′ were coupled to a carbonyl (δ_C 185.0), which was also coupled to another olefinic proton (δ_H 6.79; δ_C 101.0). This rather shielded carbon indicated that it must be between two strongly electron-withdrawing groups, suggesting an enolized 1,3-diketone in conjugation with the triene system. This was supported by the mass spectrum that showed the loss of 163 amu from the molecular ion in the negative ion ESI spectrum and prominent fragments in the MS/MS (positive ion ESI) of the parent ion at 163 ($C_{10}H_{11}O_2$) and 121 (base peak; C_8H_9O). This also accounted for the strongly H-bonded OH at 16 ppm. In the second fragment, $(H5)_2$ were both coupled to C4 (δ_C 113.4), and H6 was coupled to C8 (δ_C 159.0) in the HMBC spectrum. Both H4 and H8 were coupled to a quaternary carbon (C8*a*; δ_C 112.2), indicating that the second fragment was part of a five- or six-membered ring system. H8 was also coupled to an α,*β*-unsaturated ketone (δ_C 190.0; ν_{max} 1712 cm^{-1}) and another quaternary olefinic carbon (δ_C 140.9) that was also coupled to H6 (HMBC, Figure 1C). A strong coupling existed between the singlet methyl and the ketone, which was also coupled to C9*a* and C3*a*. The coupling between H4 and C9*a* completed a putative dihydroisopyran fragment.[14] At this point, a C_2O_2 fragment remained to be assigned. The IR spectrum suggested a *γ*-lactone (ν_{max} 1744 cm^{-1}), but final structure interpretation was confounded by the coincidence of C3*a* and C2 in the ^{13}C NMR spectrum. Coincidence of C3*a* and C2 has been observed in 7-(D-Ala)-monascorubrin, a related azaphilone pigment from *Monascus* sp.[15] Couplings in the HMBC spectra between H2′ and C3, and H4 and C3 completed the structure. The structure of epicocconone was also similar to that of the antimicrobial and cytotoxic bulgarialactone isolated from the ascomycete *Bulgaria inquinas*.[16] Bulgaralactone B has the same nucleus as **1** but a different side chain. Azaphilones have also been isolated from *Penicillium* spp[17−19] and *Pithomyces* sp.,[20] but none have been described as fluorescent.

The relative stereochemistry was based on molecular modeling and one-dimensional gradient selective ROESY spectra (Figure 1D).[14] In particular, there was an ROE between C9*a*-Me and H6 but not H5*β*. This suggested both C9*a*-Me and H6 were axial, which was supported by a trans diaxial coupling between H5α and H6 ($^3J_{HH}$ = 11.5 Hz). In addition, a large ROE between H4 and H5*β* supported a 6*S**, 9*aS** stereochemistry. In contrast, molecular

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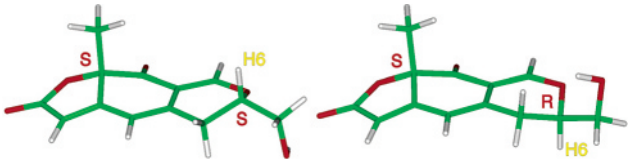

Figure 2. Lowest energy conformations (HF/6-31g**) of (6*S*, 9*aS*) and (6*R*, 9*aS*)-epicocconone based on molecular dynamics/mechanics followed by ab initio geometry optimization.[14]

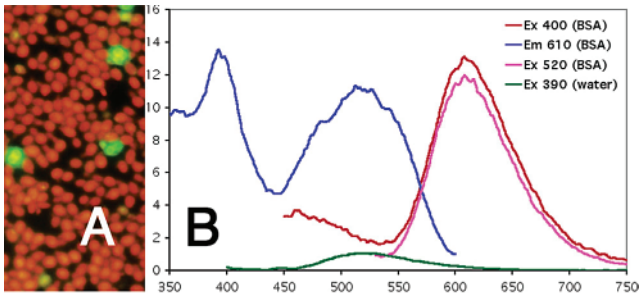

Figure 3. (A) Differentiation of vegetative (red) and sporulation (green) yeast cells using 1 and 5(6)-carboxyfluorescein diacetate as detected by epifluorescence microscopy (λ_{ex} 488 nm). (B) Fluorescence excitation (blue; λ_{em} 620 nm) and emission (red; λ_{ex}) 390 nm profiles of **1** in the presence of protein (BSA) in comparison to the emission profile (green; λ_{ex} 430 nm) in water alone.

modeling of the 6*R**, 9*aS** azaphilone nucleus (Figure 2) indicated a long C9*a*Me−H6 distance and a relatively short C9*a*Me−H5β distance.

Yeast cells stained with **1** became highly fluorescent, and the compound could be used for cell tracking and two-color (multiplex) staining (Figure 3) emitting orange/red when excited with a 488 nm Argon laser. The same frequency can be used to excite fluorescein that emits at 520 nm. This apparent long Stokes' shift was investigated further, and it was found that in water **1** was only weakly fluorescent but addition of protein (BSA) resulted in strong emission at 610 nm (Figure 3). The fluorescence spectrum in the presence of protein has two excitation maxima at ∼400 and 500 nm, which did not substantially overlap with the emission at 610 nm, suggesting that this compound could be used for staining of proteins in protein electrophoresis (SDS-PAGE, Figure 4) or in two-color applications with fluorescein. Protein amounts down to 2 ng/band were detectable, as sensitive as currently used fluorescent stains such as SYPRO Ruby.[8] The change in emission maximum (from 520 to 610 nm) in the presence of protein is indicative of an increase in the dipole moment of the excited state.[1] This is consistent with Schiff base formation with the pyranone as has been observed for related azaphilones when reacted with ammonia.[21] It is thus possible that **1** reacts with lysine residues in proteins to become highly fluorescent.

Epicocconone fills a gap in the large arsenal of fluorophores currently available: it has a large Stokes' shift (∼100 nm), excitable by common lasers such as argon (488 nm), Nd:YAG (532 nm), or transilluminators and with a large extinction coefficient (ϵ 10 000). This compound should find utility in cell tracking, flow cytometry, microscopy, or protein detection and multicolor staining (multiplex) applications. Work is currently underway to improve its detection limits and assess mass spectrometry compatibility for proteomics staining.

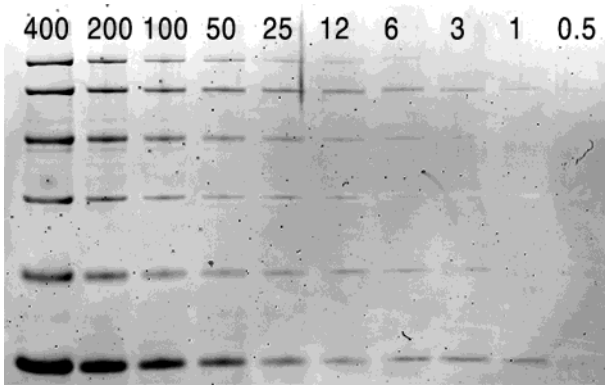

Figure 4. Two-fold dilution of standard MW markers (400−0.5 ng/band) stained with epicocconone.[14] λ_{ex} 532 nm, 560 nm LP filter.

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Supporting Information Available: Experimental section, full spectral data, table of intra-atomic distances derived from ab initio calculations, NMR, UV, and CD spectra of **1** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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