Simple, Efficient Method for studying Biosynthetic Oxidation using P-450 Inhibitors: Late Oxidative Modification in Betaenone B Biosynthesis

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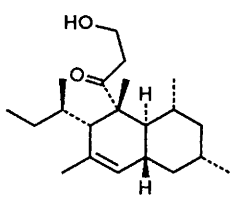
Later oxidation processes in biosynthesis of betaenone B (**2**) were examined by a simple method involving preparation of isotopically labelled probetaenone I (**1**) from [1-^{14}C]- and [1,2-^{13}C]-acetate and [*S*-$^{13}CH_3$]methionine in *Phoma betae* and their efficient conversion to (**2**); the results show that (**1**) is an intermediate for formation of (**2**).

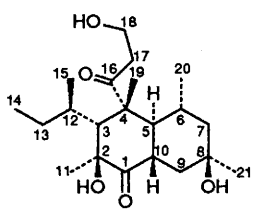
Cytochrome P-450 dependent oxidation commonly occurs in the biosynthesis of secondary metabolites, such as steroids,[1] terpenoids,[2] and flavonoids.[3] This type of oxidation usually takes place in the late stages of biosynthesis and is an important step for introducing biological activity. If a versatile inhibitor for P-450 were available, it would enable us to investigate these oxidative modifications. Recently, various inhibitors[4] have been developed as clinical drugs and plant growth regulators, *e.g.* S-3307D[5,6] and ancymidol.[5,7] They possess lipophilic and heteroaromatic moieties which reversibly bind the lipophilic domain and haem iron replacing the substrate and molecular oxygen.[8] Based on this rather simple mechanism, these compounds might inhibit a variety of P-450 oxidations. In fact, they have effectively suppressed the oxidative modification of several secondary metabolites, the less oxidized compounds accumulating.[5,9] Using a simple isotopically labelled precursor, these plausible intermediates could possibly be obtained in labelled form which could subsequently be subjected to oxidation. We now describe a simple, efficient method for studying late biosynthetic oxidation.

Probetaenone I (**1**),[5] a plausible intermediate in formation of the phytotoxin, betaenone B (**2**),[10,11] was isolated by treatment with a P-450 inhibitor of *Phoma betae*.† Its structure was elucidated by spectroscopic methods and was confirmed by asymmetric synthesis, which confirmed the absolute configuration.[12] To prove its intermediacy, ^{14}C-labelled (**1**) was prepared. To samples of a medium (2 × 150 ml) four days after inoculation with *Phoma betae*, sodium [1-^{14}C]acetate (100 μCi) and a solution in dimethyl sulphoxide of ancymidol (final concentration, 1.04 mM) were distributed equally and the cultures were incubated for a further twelve days. EtOAc extraction of the mycelium and purification by silica gel column chromatography gave radioactive (**1**) (12.2 mg, 11.8 μCi/mmol). A sample of this compound (2.0 mg in 4 ml of ethanol; 1.63 × 10^5 dpm) was fed to the medium (2 × 150 ml) six days after inoculation. Usual work-up[10] afforded (**2**) (9.4 mg; 9.83 × 10^3 dpm; 0.17 μCi/mmol; incorporation 6.02%).

In order to exclude the possibility of degradation–reincorporation, we examined the conversion of the stable isotopic-

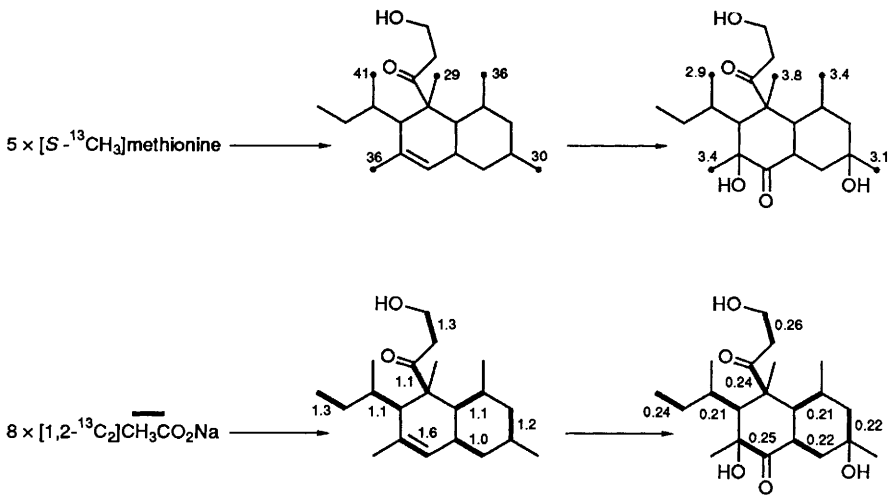


(1)



(2)

† Another inhibitor, metyrapone,[8] is commercially available and was effective at similar concentration (1 mM) for this oxidation.



Scheme 1. % Enrichments compared with natural abundance, and intensity ratios between the centre peak and the average of the satellite peaks.

ally labelled (**1**). In many cases, polyketide metabolites have some C_1-units derived from (*S*)-adenosylmethionine. These units thus can be specifically and efficiently labelled by a methionine precursor. Transformation of the intermediate labelled with sodium [$^{13}C_2$]acetate can be examined by double quantum coherence NMR techniques even for low incorporations. ^{13}C-Labelled (**1**) was prepared in a similar way except using S-3307D as an inhibitor with [*S*-$^{13}CH_3$]methionine (2 × 150 mg/150 ml broth) and sodium [$^{13}C_2$]acetate (2 × 150 mg/150 ml broth). In the former experiment, the ^{13}C NMR spectrum of the product (**1**) (15.3 mg) included five enhanced signals at δ 25.93 (C-11), 23.64 (C-20), 22.33 (C-21), 21.70 (C-15), and 17.78 (C-19).‡ % Enrichments for these positions are shown in Scheme 1. The latter experiment, which yielded (**1**) (7.7 mg), indicated high incorporation for eight pairs of coupled signals, δ 13.26 (C-14)–26.99 (C-13), 34.78 (C-12)–57.05 (C-3), 130.79 (C-2)–127.20 (C-1), 42.57 (C-10)–42.35 (C-9), 33.95 (C-8)–46.60 (C-7), 37.99 (C-6)–44.39 (C-5), 53.86 (C-4)–215.20 (C-16), and 41.31 (C-17)–58.12 (C-18),‡ the intensity ratios for which between the centre peak and the average of the satellite peaks are also shown in Scheme 1. From the feeding experiments with radioactive (**1**), the enrichments obtained should be sufficient for studying the subsequent oxidation. Labelled methionine and sodium acetate were administered independently as described above and in the ^{13}C NMR spectrum of compound (**2**) obtained [9.6 mg from (**1**) derived from methionine; 4.0 mg from (**1**) from acetate], the corresponding signals were enhanced or accompanied by satellite peaks, the enrichments and intensity ratios being summarized in Scheme 1. The enrichments for the two experiments were calculated to be 9.6 and 19.1%. Considering that different types of signals were observed, the difference between these figures could be significant. The high incorporation in these experiments confirms direct incorporation of ^{13}C-labelled (**1**) into (**2**). The high retention of satellite peaks and the similar intensity for both methionine- and acetate-derived signals in the latter experiment support this conclusion. The sequence of oxidation at the double bond and at C-8 is not clear from these data. However, the retention of configuration at C-8 agrees with results for other P-450 reactions.[13]

‡ The assignment of structure (**1**) was established from NMR data including INEPT, H–H and C–H COSY, and from the incorporation experiment with sodium [1,2-$^{13}C_2$]acetate.

The present method thus provides information on late biosynthetic processes and should be applicable for studies on complex molecules. The technique is not applicable to all oxidation processes and cannot replace conventional mutant studies. However, its simplicity and facility make it easy to obtain further detailed information.

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