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## Accessing low-oxidation state taxanes: is taxadiene-4(5)-epoxide on the taxol biosynthetic pathway?†

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We have shown for the first time that taxadiene (**3**) can be epoxidised in a regio- and diastereoselective manner to provide taxadiene-4(5)-epoxide (**12**) as a single diastereoisomer, and that this epoxide can be rearranged to give taxa-4(20),11(12)-dien-5 $\alpha$ -ol (**4**). Furthermore, the epoxide **12** rearranges under acidic conditions to give taxa-4(20),11(12)-dien-5 $\alpha$ -ol (**4**), the known bridged ether OCT (**5**) and the new oxacyclotaxane (OCT2) **15**. Contrary to previous speculation, taxadiene-4(5)-epoxide (**12**) is susceptible to rearrangement when exposed to an iron<sup>III</sup> porphyrin, and these observations justify consideration of epoxide **12** as a chemically competent intermediate on the taxol biosynthetic pathway.

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## Introduction

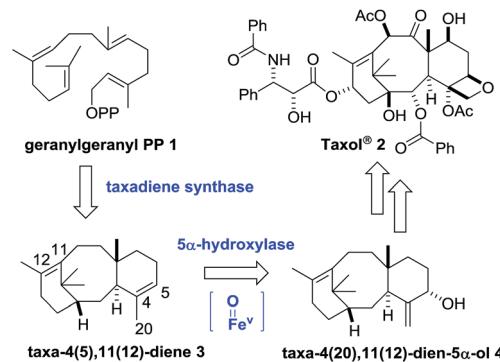
Since its isolation from the pacific yew (*Taxus brevifolia*), and subsequent FDA approval in 1992, taxol and its close derivatives continue to be used as frontline drugs for the treatment of cancer.<sup>1</sup> Its effectiveness in the clinic, coupled with an intriguing tricyclic structure, has ensured that taxol has endured as a molecule of interest to scientists for nearly 50 years.<sup>2</sup> In this paper we show that a combination of metabolic engineering and synthetic chemistry can be used to give ready access to low oxidation state taxanes, giving new insight into the early stages of the 'oxidase-phase' of the taxol biosynthetic pathway.<sup>3</sup>

The first committed step in the taxol biosynthetic pathway (Scheme 1) is the taxadiene synthase-catalysed cyclisation of geranylgeranyl pyrophosphate **1** to produce taxa-4(5),11(12)-diene (**3**).<sup>4</sup> The remaining biosynthetic steps involve a series of oxidation, and functional group interconversion processes, the first of which is the taxadiene-5 $\alpha$ -hydroxylase-mediated oxidation of **3** into taxa-4(20),11(12)-dien-5 $\alpha$ -ol (**4**).<sup>5</sup>

A number of research groups have reported the over-production of taxa-4(5),11(12)-diene (**3**) in a variety of chassis organisms (yeast,<sup>6</sup> tobacco,<sup>7</sup> *E. coli*,<sup>8</sup> tomato<sup>9</sup>), and the incorporation of both taxadiene synthase and its 5 $\alpha$ -hydroxylase

(tobacco,<sup>7</sup> *E. coli*<sup>8a</sup>) has also been described. In 2008 Rontein showed that overexpression of both taxadiene synthase and taxa-4(5),11(12)-diene 5-hydroxylase (CYP725A4) in tobacco (*Nicotiana sylvestris*) did not produce taxa-4(20),11(12)-dien-5 $\alpha$ -ol (**4**) as expected, but instead led to the production of 5(12)-oxa-3(11)-cyclotaxane (OCT) **5** (Scheme 2).<sup>7</sup>

In 2010 Stephanopoulos reported a significant improvement in this area using *E. coli* as the chassis organism.<sup>8a</sup> Under their optimised conditions, taxa-4(20),11(12)-dien-5 $\alpha$ -ol (**4**) could be produced, but unfortunately the desired product **4** was obtained as a 1 : 1 mixture with OCT (**5**), thus severely limiting the amount of **4** being produced. These two studies clearly demonstrate that the presence of both taxadiene synthase and taxadiene-5 $\alpha$ -hydroxylase in a metabolically engineered chassis organism does not guarantee satisfactory production of taxadien-5-ol **4**, and the catalytic promiscuity and multispecificity of taxadiene-5 $\alpha$ -hydroxylase has attracted recent attention.<sup>10</sup>



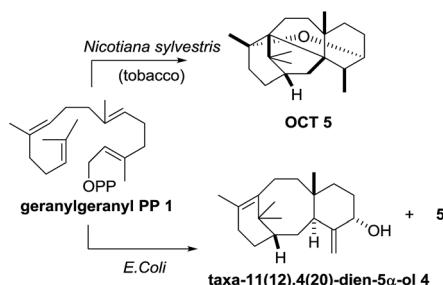
Scheme 1 Biosynthesis of Taxol® from geranylgeranyl-pyrophosphate, via taxadiene.

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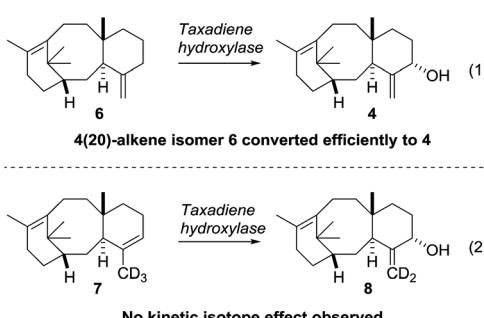




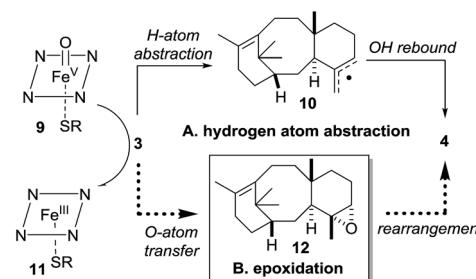
**Scheme 2** Production of oxidised taxanes in metabolically engineered tobacco and *E. coli* containing both taxadiene synthase and taxadiene hydroxylase.

Our current understanding of the taxadiene-5 $\alpha$ -hydroxylase oxidation mechanism is derived from experiments performed by Williams and Croteau (Scheme 3).<sup>5</sup> The observation that taxadiene-containing microsomes could convert both the 4(5)-3 (Scheme 1) and the 4(20)-6 alkene isomers of taxadiene to taxadien-5-ol 4 with equal efficiency (Scheme 3, eqn (1)), lead Williams and Croteau to suggest an H-atom abstraction/oxygen rebound mechanism, *via* the allylic radical 10, as being the most likely (path A, Scheme 4).

An alternative pathway involving epoxidation of 3 to give 12, followed by rearrangement to give 4 (path B, Scheme 4) was also considered, but was eventually discounted by the fact that the 4(20)-alkene isomer 6 is also converted to 4 by taxadiene hydroxylase (*via* a process unlikely to involve 12).<sup>5</sup> This conclusion was further supported by the fact that the epoxide 12 has not been observed as an oxidation product of 3 in any studies reported thus far. In order to provide further evidence for the H-atom abstraction/oxygen rebound mechanism (path A, Scheme 4), Williams *et al.* prepared deuterium-labelled [C20-<sup>2</sup>H<sub>3</sub>]-taxadiene (7) and subjected this to taxadiene hydroxylase. However, under these conditions, the expected kinetic isotope effect was not observed for the transformation of 7 to 8 (Scheme 3, eqn (2)),<sup>5</sup> which is at odds with the proposed H-atom abstraction process. Furthermore, Williams *et al.* report that their experiment ‘unexpectedly revealed that the deuterated substrate yielded slightly more taxa-4(20),11(12)-dien-5 $\alpha$ -ol than did the unlabeled substrate’,<sup>5b</sup> thus indicating a small inverse isotope effect. This experimental observation actually



**Scheme 3** Elucidating the taxadiene hydroxylase mechanism (Williams and Croteau).



**Scheme 4** Taxadiene hydroxylase mediated oxidation of taxa-4(5),11(12)-diene (3) to taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4).

supports the epoxide/rearrangement route for the conversion of 3 to 4, as small inverse secondary isotope effects are observed in epoxidation reactions,<sup>11</sup> but no further experiments have been reported to examine this possibility.

The production of OCT 5, along with additional oxidation products, in engineered taxadiene synthase/taxadiene hydroxylase-containing organisms<sup>7,8a</sup> lead us to question whether epoxide 12 could be an intermediate in the taxadiene hydroxylase mechanism as we could envisage viable pathways for the production of both 4 and 5 from epoxide 12. Therefore, we decided to synthesise 12 and study its chemistry in the context of the early stages of the taxol biosynthetic pathway.

## Results and discussion

### Epoxidation of taxadiene

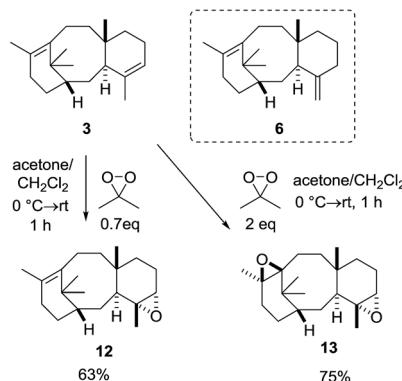
Our studies began by isolating taxadiene from our previously described taxadiene synthase-containing tomatoes,<sup>9</sup> using a slightly modified protocol that allows extraction directly from fresh fruit (see ESI† for details). This procedure afforded taxa-4(5),11(12)-diene (3) and taxa-4(20),11(12)-diene (6) as an inseparable 17 : 1 (3 : 6) mixture. With ready access to taxadiene we next turned our attention to epoxidation of 3, with DMDO being selected as the oxidant due to its ease of use.<sup>12</sup> As we were concerned with the potential over-epoxidation of taxadiene, we performed the reactions with substoichiometric quantities of oxidant. Pleasingly, when taxa-4(5),11(12)-diene (3) was treated with 0.7 equivalents of DMDO, the desired epoxide 12 was obtained as the major new product (95% purity as judged by <sup>1</sup>H NMR; see ESI†) and unreacted taxadiene was recovered (Scheme 5).

Whilst the epoxide derived from 6 was not observed, the recovered taxadiene (20%) was significantly enriched in 6 (1 : 2; 3 : 6) compared to the starting material (17 : 1; 3 : 6), thus indicating that 3 is much more reactive towards epoxidation than 6. Care had to be taken during chromatography on silica gel as the epoxide 12 was acid sensitive (*vide infra*). Treatment of taxadiene (3) with excess DMDO (2 equivalents), produced the bis-epoxide 13 in 75% yield, and this epoxide was found to be much more stable than 12 to chromatography on silica gel (Scheme 5).

### Synthesis of taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4)

With a reliable route to the key epoxide 12 secured, we next wanted to assess its ability to act as a precursor to



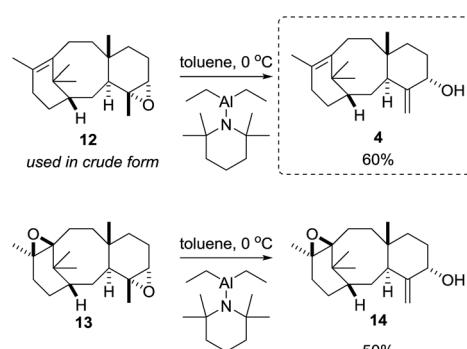
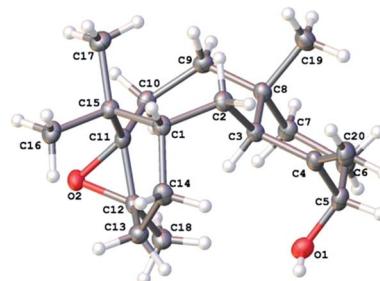


Scheme 5 DMDO epoxidation of taxadiene-4(5),11(12)-diene.

taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4). Before examining conditions of relevance to the biosynthesis, we first reacted 12 with Yamamoto's aluminium amide reagent (TMPAIEt<sub>2</sub>) to produce 4 as a reference sample (Scheme 6).<sup>13</sup> As the epoxide 12 was prone to decomposition during column chromatography (*vide infra*), we used the epoxide in crude form directly from the DMDO oxidation. Thus, treatment of unpurified 12 with freshly prepared Yamamoto's reagent (BuLi, TMP, ClAlEt<sub>2</sub>, 0 °C, PhMe) gave taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4) in 60% isolated yield over the two steps from taxadiene (3). The spectroscopic data for 4 matched that reported by Williams for the 5 $\alpha$ -stereoisomer,<sup>5</sup> and this enabled us to confirm that epoxidation (3 → 12) must have occurred on the  $\alpha$ -face of taxadiene. Having prepared bis-epoxide 13, we next examined its behaviour under the same rearrangement conditions. Thus, treatment of 13 with Yamamoto's reagent provided epoxy-alcohol 14 as the major isolable product (50%). It is interesting to note that the 11(12)-epoxide moiety is also observed in natural taxanes such as taxinine A 11(12)-epoxide.<sup>14</sup> Fortunately, 14 was obtained as a crystalline solid and we were able to determine an X-ray crystal structure (Fig. 1) to confirm the stereochemistry of the 11(12)-epoxide, and also show that the C5-hydroxyl was on the  $\alpha$ -face.

### Rearrangements of taxadine-4(5)-epoxide 12

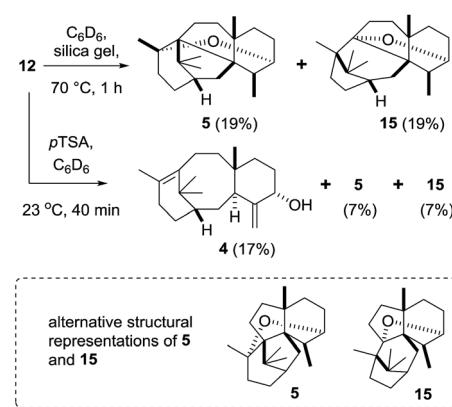
Encouraged by the successful conversion of epoxide 12 to taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4) using Yamamoto's reagent, we next

Scheme 6 Synthesis of taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4) via rearrangement of epoxide 12.Fig. 1 X-ray crystal structure of the taxadiene derived epoxyalcohol 14.<sup>†</sup>

explored the behaviour of 12 under conditions of more relevance to the biosynthesis. We speculated that if taxadiene hydroxylase acts as a monooxygenase and epoxidises taxadiene 3 to produce 12, then this would initially leave a mild Lewis acidic iron centre in close proximity to the epoxide, which could catalyse subsequent rearrangement reactions. Therefore, we decided to examine the behaviour of 12 under a range of acidic conditions.

In order to simulate the acid-mediated decomposition encountered during silica gel chromatography, the epoxide 12 was treated with silica gel in C<sub>6</sub>D<sub>6</sub> at 70 °C. Reaction progress was monitored by <sup>1</sup>H NMR (see ESI<sup>†</sup>), and we determined that 12 converts into OCT (5), the molecule that had previously been produced in metabolically engineered tobacco by Rontein (Scheme 7),<sup>7</sup> and the new isomeric oxacyclotaxane 15 (OCT2). Complete conversion of epoxide 12 was observed, as judged by the loss of the C19 methyl <sup>1</sup>H NMR signal at 0.58 ppm, and the isomeric bridged ethers 5 and 15 were produced in an approximately 3 : 2 ratio (<sup>1</sup>H NMR). Chromatographic separation gave isolated samples of 5 (19%) and 15 (19%), which were then fully characterised.

Treatment of epoxide 12 with a stronger acid (pTSA, C<sub>6</sub>D<sub>6</sub>) gave taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4) as the major new product, with OCT (5) and OCT2 (15) being produced as minor products (isolated yields: 4 (17%); 5 (7%); 15 (7%)). The formation of 4(20),11(12)-dien-5 $\alpha$ -ol (4) from the epoxide 12 under these



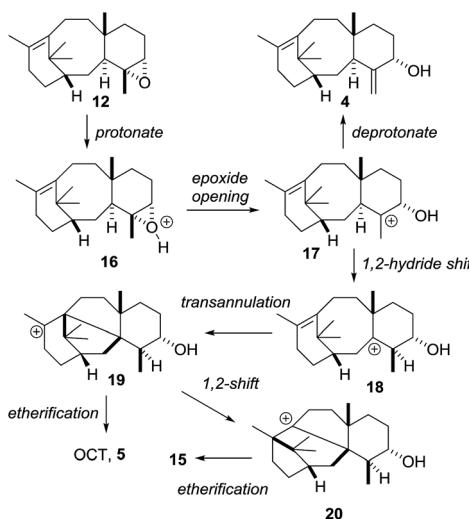
Scheme 7 Rearrangement of taxadiene-4(5)-epoxide (12) under acidic conditions.



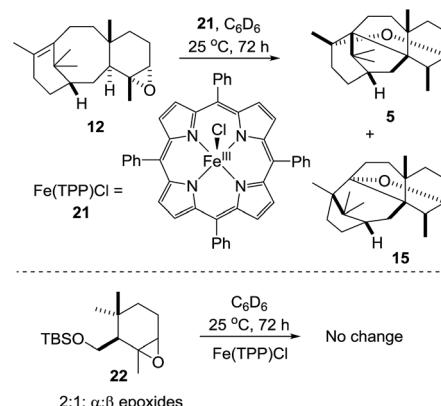
strongly acidic conditions is readily explained by invoking protonation of the epoxide **12** to produce **16** (Scheme 8). Ring-opening then affords the cation **17**, and loss of a proton from the C20 methyl group installs the *exo*-methylene group in **4** (Scheme 8). The formation of OCT (**5**) also implicates the cation **17** as an intermediate. A 1,2-hydride shift first produces the new tertiary cation **18**, which next undergoes trans-annulation with the C11(12)-alkene leading to the cation **19**. Etherification, involving trapping the cation **19** with the secondary hydroxyl, then gives OCT (**5**). Similarly, the formation of OCT2 (**15**) can be rationalised by invoking a 1,2-alkyl shift of the tertiary cation **19**, leading to the new tertiary cation **20**, which is then trapped as the ether **15** by reacting with the C5-hydroxyl (Scheme 8).

As the biological oxidant (taxadiene hydroxylase<sup>5</sup>) acting upon taxadiene is a cytochrome P450, it is tempting to speculate that the reduced iron<sup>III</sup> porphyrin (**11**, Scheme 4) is capable of facilitating a Lewis acid-catalysed rearrangement of the epoxide *in vivo*. Rontein, however, discounted this proposal<sup>17</sup> on the basis that previous work on very different chemical systems has shown that iron<sup>III</sup> porphyrins are poor catalysts for the rearrangement of epoxides.<sup>15</sup> As we had access to the epoxide **12**, we could test this hypothesis experimentally, and we decided to treat **12** with an iron<sup>III</sup> porphyrin.

Contrary to the literature hypothesis, we were pleased to find that treatment of **12** with Fe<sup>III</sup>(TPP)Cl (2 equiv.) in C<sub>6</sub>D<sub>6</sub> at 25 °C for 72 hours, lead to epoxide rearrangement, with the production of OCT (**5**) and OCT2 (**15**) as the main new products in a 1 : 1 ratio (<sup>1</sup>H NMR). Formation of taxa-4(20),11(12)-dien-5 $\alpha$ -ol (**4**) was not observed under these Lewis acidic conditions (Scheme 9). As a control experiment, we exposed the similarly-substituted cyclogeraniol-derived epoxide **22**<sup>16</sup> to the same Fe(TPP)Cl rearrangement conditions,<sup>17</sup> and as expected from previous reports,<sup>15</sup> no rearrangement was observed, thus highlighting the propensity of **12** to rearrange.



Scheme 8 Proposed mechanisms for the formation of taxa-4(20),11(12)-dien-5 $\alpha$ -ol (**4**), OCT (**5**) and OCT2 (**15**) from taxadiene-4(5)-epoxide (**12**).

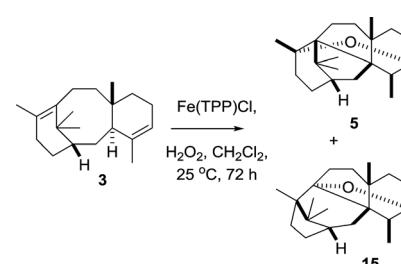


Scheme 9 Iron<sup>III</sup> porphyrin mediated rearrangement of taxadiene-4(5)-epoxide (**12**).

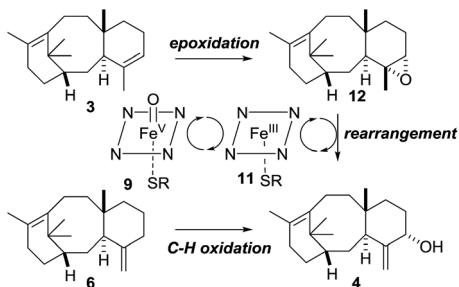
Having shown that the two step epoxidation/Fe<sup>III</sup> induced rearrangement mimics that seen *in vivo* (tobacco) mediated by taxa-4(5),11(12)-diene 5-hydroxylase (CYP725A4), we wondered if the initial oxidation of taxadiene could also be achieved using the Fe<sup>III</sup>(TPP)Cl catalyst and a suitable stoichiometric oxidant (Scheme 10). Thus, treatment of taxadiene (**3**) with Fe<sup>III</sup>(TPP)Cl (10 mol%) and hydrogen peroxide (1 equiv.)<sup>18</sup> lead to complete consumption of starting material (as judged by t.l.c. and <sup>1</sup>H NMR), and the subsequent production of oxidation products. Although the isolated yields were low, <sup>1</sup>H NMR of the crude reaction mixture showed that the two major products were OCT (**5**) and the OCT2 (**15**). The production of taxa-4(20),11(12)-dien-5 $\alpha$ -ol (**4**) was not observed under these conditions (Scheme 10).

### Implications for the taxol biosynthetic pathway

As discussed in the introduction (Scheme 2), the current proposal for the biosynthesis of **4** from taxadiene **3** is that taxadiene hydroxylase performs an H-atom abstraction from the C20 methyl group of the 4(5)-alkene isomer of **3** to form the allyl radical **10**, and involvement of the epoxide **12** was rejected. Further support for the involvement of a common allyl radical **10** came from the fact that the 4(20)-alkene isomer **6** was also converted to taxa-4(20),11(12)-dien-5 $\alpha$ -ol (**4**) by taxadiene hydroxylase. However, our experiments, coupled with the previously published kinetic isotope effect data,<sup>5</sup> demonstrate that the epoxide **12** cannot be discounted as an intermediate on



Scheme 10 Iron<sup>III</sup> porphyrin mediated oxidation of taxa-4(5),11(12)-diene (**3**).



Scheme 11 Proposal for the role of epoxide 12 in the biosynthesis of taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4).

the taxol biosynthetic pathway. We have shown that the major, naturally occurring, 4(5)-alkene isomer of taxadiene 3 can be converted to taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4) *via* the epoxide 12, and this suggests that the 4(5)-3 and 4(20)-6 alkene isomers of taxadiene are processed differently by taxadiene hydroxylase (Scheme 11).<sup>19</sup>

It is possible that 4(5)-alkene isomer 3 is epoxidised to produce 12, which is then rearranged to 4, by the action of the reduced form of the hydroxylase 11. In contrast, the 4(20)-alkene isomer 6 could be converted directly to 4 *via* the accepted H-atom abstraction mechanism. The involvement of epoxide 12 in the pathway provides an explanation for the lack of a significant primary kinetic isotope effect and the presence of an inverse secondary isotope effect when deuterium labelled [C20- $^2$ H<sub>3</sub>]-taxadiene (7) was oxidized by taxadiene hydroxylase. The labelled C20 methyl likely plays only a small role in the epoxidation process (*i.e.* leads to small inverse isotope effect), and loss of a proton from C20 in an intermediate such as 19 (Scheme 8) is unlikely to be rate-limiting.

## Conclusions

In this study, we have shown that taxa-4(5),11(12)-diene (3) can be isolated from the fruit of metabolically engineered tomatoes using our new optimised procedure. Furthermore, we have shown that taxadiene (3) can be epoxidised in a regio- and diastereoselective manner to provide taxadiene-4(5)-epoxide (12), and that this epoxide can be rearranged to give taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4) in 60% over the two chemical steps. We have shown that the epoxide 12 is sensitive to acids, and that both taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4), the known bridged ether OCT (5) and the new oxacyclotaxane (OCT2) 15 can be obtained from this material. We have shown that contrary to previous speculation, taxadiene-4(5)-epoxide (12) is susceptible to rearrangement when exposed to an iron<sup>III</sup> porphyrin, and these observations combine to warrant reconsideration of the epoxide 12 as a chemically competent intermediate on the taxol biosynthetic pathway.

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19 Whilst this manuscript was under review, a complementary study by Stephanopoulos *et al.* has been reported that also proposes taxadiene epoxidation by taxadiene-5 $\alpha$ -hydroxylase as being a step on the taxol biosynthetic pathway. Please see: S. Edgar, K. Zhou, K. Qiao, J. R. King, J. H. Simpson and G. Stephanopoulos, *ACS Chem. Biol.*, 2016, DOI: 10.1021/acschembio.5b00767.

