COMMUNICATION

ChemComm
www.rsc.org/chemcomm

A novel regiospecific *N* to *O*-methyl transferase activity in the biotransformation of a thebaine derivative with *Cunninghamella echinulata* NRRL 1384

Anthony M. Abel,[a] Graham R. Allan,[a] Andrew J. Carnell*[a] and J. Alf Davis[b]

[a] *Department of Chemistry, Robert Robinson Laboratories, University of Liverpool, UK L69 7ZD*

[b] *Reckitt Benckiser Healthcare (UK) Ltd, Hull, UK HU8 7DS*

Received (in Cambridge, UK) 15th May 2002, Accepted 1st July 2002

First published as an Advance Article on the web 12th July 2002

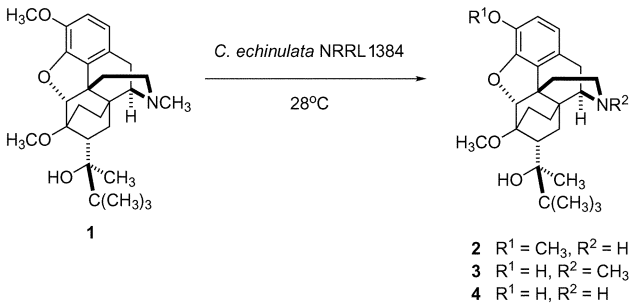
A novel regiospecific *N*- to *O*-methyl transfer reaction has been characterised in the biotransformation of an *N*-CD_3-thebaine derivative with the fungus *Cunninghamella echinulata* NRRL 1384.

The ability to regioselectively *N*- and *O*-demethylate compounds possessing two or more methyl groups has attracted considerable interest. A number of filamentous fungi have been shown to possess enzymes capable of catalysing *N*- and *O*-demethylation of alkaloids. *Mucor piriformis* selectively *N*-demethylates thebaine,[1] *Streptomyces* strains and *Cunninghamella echinulata* *N*-demethylate the indole alkaloid lergotrile[2] and various *Cunninghamella* and *Fusarium* strains *N*-demethylate codeine.[3]

We have previously identified and characterised filamentous fungi capable of the whole cell biotransformation of thebaine derivatives to generate key intermediates for the synthesis of buprenorphine.[4,5] These biotransformations result in the demethylation of compound **1** to form the *N*-demethylated, *O*-demethylated and *N*,*O*-didemethylated compounds **2**, **3** and **4** respectively (Scheme 1). We have also determined that this process is dependent upon the constitutive expression of cytochrome P450 hydroxylase enzymes[5] that generate the *N*-demethylated compound **2** as the major biotransformation product during batch fermentations. We now report a unique and unexpected direct methyl group translocation that converts compound **3** to compound **2** upon incubation with *Cunninghamella echinulata* NRRL 1384. Through the use of an isotopically labelled substrate we show that the origin of the new phenolic *O*-methyl that appears in **2** is the *N*-methyl group in compound **3**.

Using compound **1** as a substrate under standard biotransformation conditions[6] the yields of the *N*-demethylated product **2** and the *N*,*O*-didemethylated product **4** were 46% and 13% respectively (Table 1), and are in broad agreement with previous studies. When incubated as a substrate the *N*-demethylated compound **2** is relatively stable and up to 70% of the substrate can be recovered after 7 days. However, a small amount of the didemethylated product, compound **4**, is formed (*ca*. 7%).

Compound **4** is completely stable under the biotransformation conditions and is clearly not a substrate for enzymatic methylation which might regenerate any of the other intermediates.



1

Scheme 1

We have previously postulated that compound **3** is a possible intermediate in the pathway leading to the formation of product **4**.[4] It would be expected therefore that intermediate **3** should be converted exclusively to product **4** by a simple *N*-demethylation reaction. However, as Table 1 shows, intermediate **3** is converted to both product **4** (18%) and product **2** (71%), with the latter compound being the major product. Since we have established that product **4** is not a substrate for further modification, the formation of product **2** cannot be *via* a sequential pathway that *N*-demethylates intermediate **3** and then methylates product **4** on the phenolic hydroxy group to form product **2**. Two alternative routes would be: (1) regeneration of substrate **1** from intermediate **3** followed by *N*-demethylation to form the product **2** or (2) *via* a transmethylation that directly converts intermediate **3** into product **2**.

In order to distinguish between these two mechanisms the interconversion of the *O*-demethylated compound **3** into the *N*-demethylated product **2** was investigated by performing a biotransformation using the deuterated substrate **5** (Scheme 2) in which the *N*-methyl is fully deuterated. This substrate was synthesized from compound **4** by reaction with d^3-methyl iodide in DMF under basic conditions.

The deuterated compound **5** was converted into the expected didemethylated product **4** (yield 10–15%) and the *N*-demethylated compound **6** (yield 60–65%). HPLC, mass spectroscopic and ^{13}C NMR analysis of this purified biotransformation product **6** indicated that the CD_3 group present had been transferred to the phenolic oxygen.

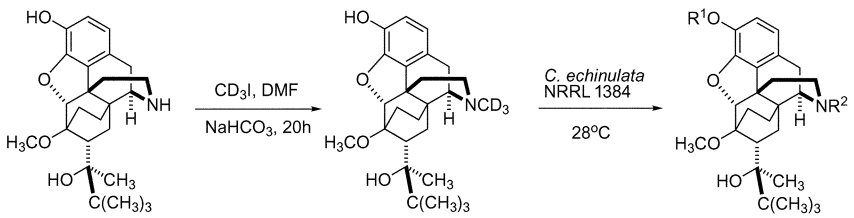
HPLC analysis (ODS reverse phase column, flow rate 1 ml min^{-1}, eluent 63 : 1 MeOH : H_2O) of compound **6** showed it to have the same retention time (7.5 min.) as the non-deuterated compound **2**. Mass spectra of these compounds showed peaks at *m*/*z* 428 for **2** and 431 for trideuterated **6**. In the ^{13}C spectra, comparison of the deuterated and non-deuterated pairs **3** and **5** and **2** and **6** showed the expected absence of the CD_3 signal in compounds **5** and **6** due to the multiplicity (7 lines) and broadening expected for this weak signal. The signals for the *C*HNR^2 carbon are diagnostic for the presence or absence of the *N*-methyl group. This signal appears at 61.8 and 61.7 ppm for the *N*-methyl compounds **3** and **5** and at 54.7 and 54.5 ppm for the compounds **2** and **6** which lack a methyl on the nitrogen. All other signals for the *N*- and *O*-methyl compounds **3**/**5** and **2**/**6** are very similar.

The *N*- to *O*-methyl transfer reaction is unlikely to be mediated by a cytochrome P_{450} enzyme since the CD_3 group would not remain intact. *N*- and *O*-demethylations are thought to proceed by hydroxylation to give the hydroxymethyl

Table 1 Whole cell biotransformation properties and substrate preferences of *Cunninghamella echinulata* NRRL 1384

	Percent product formation	
Biotransformation substrates	**2**	**4**
Compound-**1**	46 ± 5	13 ± 4
Compound-**2**	69 ± 4	7 ± 2
Compound-**3**	71 ± 10	18 ± 5
Compound-**4**	0	34 ± 7

DOI: 10.1039/b204697k



4 **5** **4** $R^1 = H, R^2 = H$ **6** $R^1 = CD_3, R^2 = H$

Scheme 2

derivative which spontaneously decomposes to release formaldehyde. A number of methyl transferase enzymes are known in which the cofactor colbalamin can transfer methyl groups *via* a methyl cob(III)alamin (d^6) complex.[7] This can be formed by demethylation of protonated amines such as N^5-methyltetrahydrofolate and other methylamines[8] by the so-called supernucleophile cob(I)alamin. The methyl group of cob(II)alamin can then be transferred to nucleophiles such as sulfur (*e.g.* homocysteine, coenzyme M) or reductively carboxylated to give acetyl CoA in methanogenic bacteria. The overall process constitutes a double S_N2 process. It is therefore possible that a cobalamin-dependent methyl transferase is able to demethylate *N*-protonated compounds **3** and **5** and transfer the methyl to the phenolic oxygen to give compounds **2** and **6** respectively either directly or *via* another methylated intermediate such as *S*-adenosyl methionine. We are currently isolating the enzyme responsible and investigating the mechanism of this novel methyl transfer reaction in order to determine whether the methyl transfer is intra or intermolecular. If it is the latter we envisage the possibility of developing the use of a surrogate methyl donor for regioselective enzymatic *O*-methylation reactions.

Notes and references

1 K. M. Madyastha and G. V. B. Reddy, *J. Chem. Soc., Perkin Trans. 1*, 1994, 911.

2 P. J. Davis, J. C. Glade, A. M. Clark and R. V. Smith, *Appl. Environ. Microbiol.*, 1979, **38**, 891.

3 M. Gibson, G. J. Spoer, R. T. Parfitt and G. J. Sewell, *Enzyme Microb. Technol.*, 1984, **6**, 471.

4 A. M. Abel, A. J. Carnell and J. A. Davis, *Biotechnol Lett.*, 2002, in press.

5 A. M. Abel, A. J. Carnell and J. A. Davis, *J. Mol. Catal., Sect B*, submitted.

6 Standard biotransformation procedure: All organisms were grown in liquid fermentation media (50 ml) at 25 °C for 7–10 days with vigorous shaking (250 rpm) in a New Brunswick Scientific orbital incubator. The fungal fermentation medium consisted of 2% (w/v) glucose, 0.5% (w/v) Corn Steep Solids, pH 6.2. After all biotransformations were complete the medias were sampled (1 ml) and extracted with an equal volume of ethyl acetate. The extent of alkaloid demethylation was assessed by thin layer chromatography (TLC) in a solvent system comprising ethyl acetate–triethylamine (19 : 1) and developed with a CAM dip. Quantitative analysis of putative positive results was carried out by HPLC (Waters 2690 separations module, 996 photo-diode array). Samples were prepared as above, dried under a stream of N_2 gas and re-dissolved in a methanol based mobile phase (methanol, 600 ml; ammonium acetate, 1 g; distilled water, 160 ml; 0.1 M acetic acid, 1 ml). Reverse phase HPLC was performed on an ODS-A column (250 mm × 4.6 mm, YMC Co. Ltd., Japan). Sample size injected was 20 μl. Flow rate and operating pressure was 0.6 ml and *c*.1900 psi, respectively. Detection was at 288 nm.

7 B. T. Golding and W. Buckel, *Corrin-Dependent Reactions, Comprehensive Biological Catalysis*, Academic Press Limited, 1998, 240.

8 K. Sauer, U. Harms and R. K. Thauer, *Eur. J. Biochem.*, 1997, **243**, 670.