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Microbiological production of omeprazole metabolites by *Cunninghamella elegans*

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Abstract

Incubation of *Cunninghamella elegans* ATCC 9245 and the anti ulcer drug omeprazole allowed putative fungal metabolites to be isolated in sufficient quantities for structural elucidation. Three metabolites produced by the fungi were isolated using semi-preparative HPLC and their structures identified by a combination of LC/MS(n) and NMR experiments. These isolates will be used as reference standards in the confirmatory analysis of mammalian metabolites of this drug.

Keywords: *Cunninghamella elegans*; Omeprazole; Reduction; Metabolites; NMR

1. Introduction

In the last 30 years a family of proton pump inhibitors, with substituted 2-pyridyl-methylsulphinyl benzimidazoles as the common structural feature, has been developed successfully for the treatment of gastritis, gastric duodenal ulcers and heartburn [1]. Furthermore, in combination with antimicrobial agents, these drugs are capable of preventing the progression of such disease states into gastric cancer [2]. From this group of compounds, omeprazole (Prilosec®) stands out as being the most widely used and extensively studied; others in the group include lansoprazole, esomeprazole, pantoprazole and rabeprazole. Their mode of action requires the formation of tetracyclic sulphenamide as active intermediates to bind covalently with (H^+, K^+)-ATPase at the surface of the gastric parietal cells. As a result, cell secretion is inhibited for 24–72 h while new proton pumps are synthesized [3,4].

Studies of omeprazole metabolism in humans have shown complete and rapid first phase metabolism via CYP450 enzymes, leading mainly to the oxidised products omeprazole sulphone and hydroxyomeprazole. Other structurally related drugs from this family are also reported to be metabolised in this way and so close monitoring of patients is recommended when there is concomitant use with other medications affecting liver metabolism [5–7]. Furthermore, omeprazole is widely used in veterinary practice and the application of the drug is under regulation in sport.

Various animal metabolism studies have reported omeprazole metabolites as a range of conjugated and unconjugated oxidation and reduction products of the parent drug [8–10]. There are also reports concerning the development of residue methods for detecting omeprazole in biological fluids, such as serum, plasma and urine [11,12], but information on the synthesis of omeprazole metabolites and subsequent use as reference compounds in analytical procedures is lacking.

The use of microbial models to mimic mammalian metabolism is well known [13,14]. Such biotransformations provide an efficient and environmentally friendly means for achieving large-scale metabolite production of a range of drugs. The filamentous fungi *Cunninghamella elegans* are considered to be useful biocatalysts for bringing about a number of important transformations, including hydroxylation of aromatic rings, reduction of ketones, and various oxidations and demethylations.

Earlier we detected several omeprazole metabolites in the horse that appeared to be reproduced by microbial transformations with *C. elegans* [15]. Here we use this organism to produce metabolites in quantities sufficient for ^1H and ^{13}C NMR experiments to provide, in turn, final proof of structure.

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2. Experimental

2.1. General procedures

LC–MS and LC–MS/MS experiments were carried out on a Thermo-Finnigan LCQ Classic instrument. The spectrometer was operated in positive ion electrospray (ESI) mode with a source temperature of 250 °C and a spray voltage of 4.0 kV; the chromatographic conditions were as described below. MS/MS experiments involved collision-induced dissociation (CID), employing nitrogen as the nebulizing and collision gas with the relative collision energy set at 35%.

^{1}H and ^{13}C NMR determinations were performed on a Bruker DRX500 using a 5 mm cryoprobe facility. ^{1}H and ^{13}C spectra were acquired in CD_3CN and $CDCl_3$ at 500.13 and 125.74 MHz, respectively, using standard pulse sequences. HMQC and HMBC experiments were used in making assignments.

2.2. Material

Omeprazole was obtained from Sigma–Aldrich (Dorset, UK) and other chemicals were also supplied by commercial sources. All were used without further purification.

C. elegans ATCC 9245, was purchased from the American Type Culture Collection (Rockville, MD, USA). The fungal culture was grown on Sabouraud dextrose agar (Sigma) on standard 9 cm plates for 5 days and stored at 4 °C until required. All works were carried out under aseptic conditions.

2.3. Chromatographic procedures

The LC–MS and LC–MS/MS analysis performed during the course of this work utilised a Waters 2690 chromatograph (Milford, USA) coupled to the mass spectrometer defined above. Components were separated by reversed-phase HPLC on a Hypersyl HyPurity^{TM} column (150 mm × 4.6 mm; particle size 5 μm). The mobile phase consisted of 10 mM ammonium hydroxide in water (Solvent A) and 1 mM ammonium hydroxide in 90% methanol (solvent B). A linear, one-step solvent gradient was applied, changing an initial composition of 85% A to a final composition of 10% A in 14 min. The injection volume was 10 μl, the solvent flow rate was 0.25 ml min^{-1} and the total run time for each sample was 15 min.

Metabolite isolation was carried out by reversed-phase HPLC on a semi-preparative scale. The chromatographic system comprised a Hewlett-Packard 1050 system, equipped with a variable wavelength detector, a Gilson sample injector, and a Gilson FC204 fraction collector. A Hypersil HyPurity^{TM} Elite C18 column (150 mm × 10 mm; particle size 5 μm) was used. The mobile phase consisted of 10 mM ammonium hydroxide in water (solvent A) and 1 mM ammonium hydroxide in 90% methanol (solvent B). A linear, three-step solvent gradient was applied, maintaining an initial composition of 75% solvent A for one min, changing to 40% solvent A over the next 15 min, then to 30% solvent A in the next 2 min and finally to 10% solvent A in the next 2 min. The injection volume was 2.0 ml, the solvent flow rate 1.0 ml min^{-1}, the UV detection wavelength 302 nm and the total run time for each sample loading was 21 min.

2.4. Microbial transformation

2.4.1. Culture and screening procedures

Fungi were grown at 25 °C in Sabouraud dextrose broth (Sigma), containing (l^{-1}) mycological peptone (10 g) and dextrose (20 g). The spores and mycelia from two agar plates were transferred aseptically to a sterile blender cup containing physiological saline solution (150 ml) and then homogenised. For screening experiments, 250-ml Erlenmeyer flasks containing sterile liquid medium (30 ml) were inoculated with a prepared *C. elegans* suspension (5 ml) and then orbitally shaken (150 rpm) at 25 °C for 48 h. Omeprazole was then added as a dimethylformamide (DMF) solution (100 μl) to give a final concentration of 0.3 mg ml^{-1}. Transformations were continued for 24 h.

Control experiments were performed where fungi were incubated in the absence of the substrate, i.e. omeprazole. These served to exclude from consideration any extraneous microbial products and were defined as Blank controls. Further control experiments were used to evaluate whether omeprazole degraded in the presence of the culture-growing media alone. These experiments, where the drug was dispersed in un-inoculated media under otherwise identical experimental conditions, were defined as Substrate controls. Aliquots (1 ml) were withdrawn aseptically after 24 h, centrifuged and the supernatants microfiltered (0.45 μm) before analysis by LC–MS.

2.4.2. Preparation and isolation of main metabolites

Solutions of omeprazole (10 mg) in dimethylformamide (0.1 ml) were added to each of four growing cultures of *C. elegans* (35 ml) in Erlenmeyer flasks (250 ml) and the resultant mixtures incubated with orbital shaking (150 rpm) at 25 °C for 24 h. The biotransformations were terminated by the addition of methanol (30 ml). Aliquots of the mixtures were centrifuged and the supernatants extracted with chloroform. The residual aqueous solutions were adjusted to pH 8.5 and then each was repeatedly extracted with chloroform. After drying with sodium sulphate, the pooled chloroform extracts were evaporated in vacuo and the oily residue dissolved in a solvent mixture (2 ml), comprising methanol and 10 mM ammonium hydroxide in a ratio of 13:100. Final purification was by semi-preparative HPLC and the yields of the products were determined on the basis of the peak areas recorded on the chromatograms. The separated fractions showing an absorbance at 302 nm were analysed by LC–MS/MS.

The following products were isolated as amorphous colourless solids (relative chromatographic yields in parenthesis): **M1** (34.3%), **M2** (9.1%), **M3** (26.8%), **M4** (4.1%), **M5** (0.6%), **M** (25.1%). Mass spectrometric data can be summarised as follows (ion abundance and designation in parenthesis): **M1**, MS-ESI, *m/z* 346 (100, $M+H^+$); MS/MS, *m/z* 313 (100, $M+H^+$-33), 198 (50), 149 (10); **M2**, MS-ESI, *m/z* 316 (100, $M+H^+$); MS/MS, *m/z* 283 (100, $M+H^+$-33), 168 (36), 149 (28); **M3**, MS-ESI, *m/z* 316 (100, $M+H^+$); MS/MS, *m/z* 283 (70, $M+H^+$-33), 182 (100);

M4, MS-ESI, *m/z* 332 (100, $M+H^+$); MS/MS, *m/z* 299 (100, $M+H^+$-33), 198 (72); **M5**, MS-ESI, *m/z* 302 (100, $M+H^+$); MS/MS *m/z* 269 (100, $M+H^+$-33), 168 (69), 135 (19); and **M**, MS-ESI, *m/z* 330 (100, $M+H^+$); MS/MS, *m/z* 297 (100, $M+H^+$-33), 182 (92), 149 (17).

3. Results and discussion

3.1. Preliminary analysis

The products **M1**, **M2**, **M3**, **M4**, **M5** and **M** are the outcome of a biotransformation of omeprazole by *C. elegans* over a 24 h period. Their structures are shown in Fig. 1 and were first proposed from preliminary work on the basis of LC–MS and LC–MS/MS data alone. However, it was noted that three of the compounds, **M1**, **M2**, and **M3**, corresponded to metabolites previously identified in the horse [15] and therefore these compounds were of sufficient interest to be isolated as reference compounds. For this it was necessary to scale-up and refine the in vitro procedure that had been used thus far. Accordingly, the incubation of 40 mg of omeprazole, and the application of a modified chromatographic procedure on a semi-preparative scale, afforded the metabolites in sufficient yield and purity.

With **M1**, **M2** and **M3** isolated in larger amounts, mass spectrometric analysis was used to consolidate previous findings, prior to examination by NMR. Hence, positive-ion ESI mass spectrometry re-affirmed a molecular weight of 345 Da for **M1** with two structural changes from omeprazole, namely a sulphide rather than a sulphoxide bridging group and hydroxylation of one of the methyl groups of the substituted pyridine. Similarly, **M2** and **M3** were also shown to be sulphides, both with a molecular weight of 315 Da. Further analysis re-affirmed that demethylation of a different methoxy group had occurred in each case, thereby producing positional isomers. A demethylated pyridine moiety was indicated for **M2** and a demethylated benzimidazole for **M3**. Mass spectra from both the preliminary and the scaled-up work were consistent. All the metabolites, **M** and **M1**–**M5**, gave strong pseudo-molecular ions with a common loss of *m/z* (33) corresponding to the release of a sulphydryl radical.

3.2. Identification by NMR

^{1}H NMR data of omeprazole and metabolites **M1**, **M2**, **M3** are presented in Tables 1 and 2, respectively. The numbering system applied here is based on the numbering system used for omeprazole by Claramunt et al. [16]. Initial experiments were acquired in $CDCl_3$ but CD_3CN was subsequently found to be a better NMR solvent for studying metabolite solutions because the interfering solvent signals were moved away from the diagnostic aromatic region. Unexpectedly, the spectrum of omeprazole in $CDCl_3$ proved useful for the interpretation of the metabolite spectra in the other solvent.

In CD_3CN, the eighteen carbon-bound protons gave rise to ten sharp signals (the diastereotopic H-8 protons were strongly

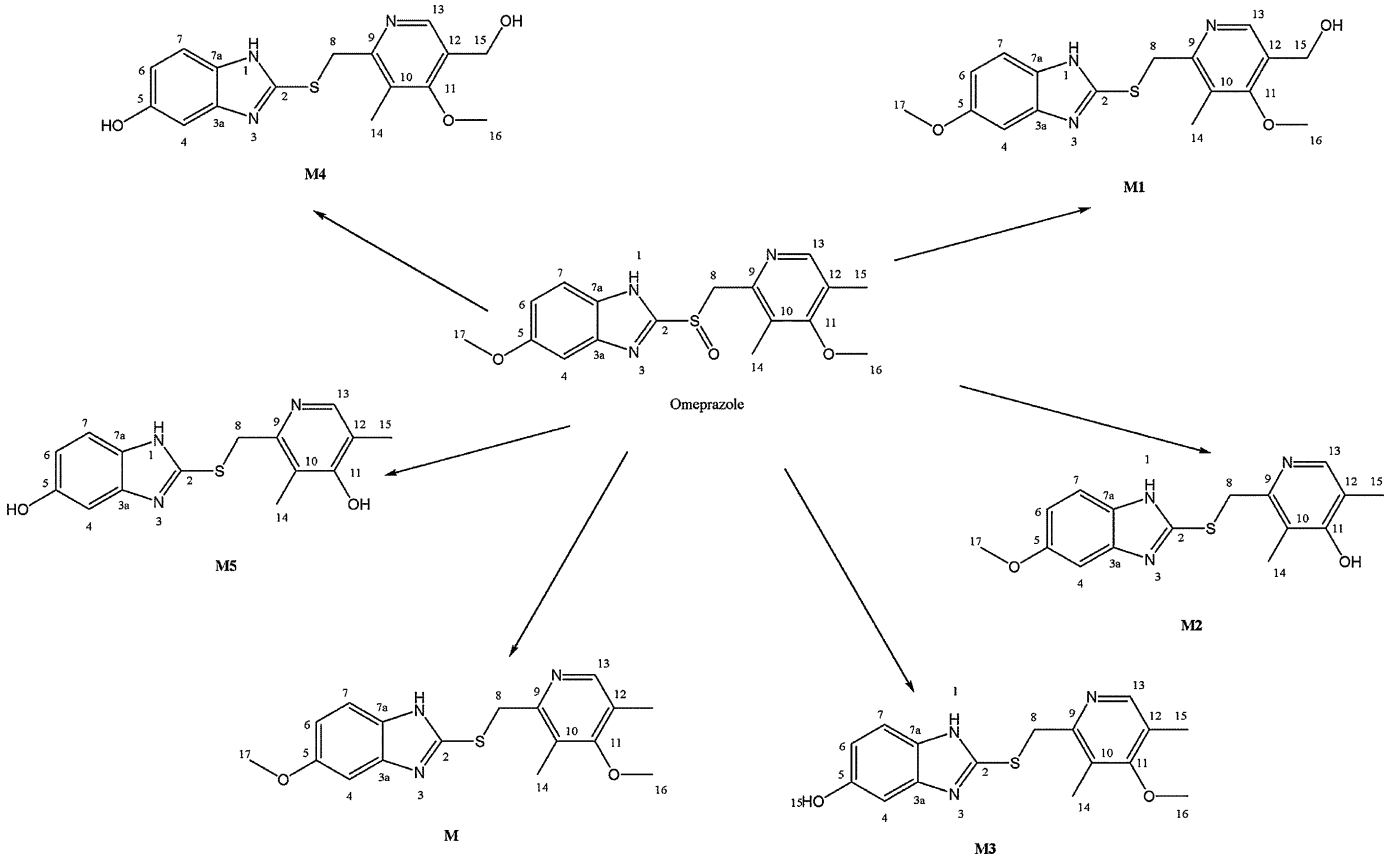

Fig. 1. Biotransformation pathway of omeprazole induced by *C. elegans* ATCC 9245.

Table 1
^{1}H NMR data for omeprazole in CD_3CN and $CDCl_3$ at 500 MHz

Position	CD_3CN, δ_H (multiplicity, *J*/Hz)[a]	$CDCl_3$ δ_H (multiplicity, *J*/Hz)[a]
NH	11.25 (v br s)	11.55 & 11.38 (br s/br s)
H-13	8.16 (s)	8.22 (s)
H-7	7.54 (d, 8.9)	7.65 (br d, 8.8)
H-4	7.13 (d, 2.4)	7.35 (br d, 8.8)
H-6	6.95 (dd, 8.9, 2.4)	6.94 (br m)
H-8	4.64 (d, 13.7)	4.74 (br d, 13.5)
H-8′	4.60 (d, 13.7)	4.65[b] (br d, 13.5)
16-OMe	3.84 (s) or 3.70 (s)	3.84 (br s) or 3.66 (br s)
17-OMe	3.84 (s) or 3.70 (s)	3.84 (br s) or 3.66 (br s)
14-Me	2.22 (s) or 2.16 (s)	2.23 (s) or 2.17[b] (br s)
15-Me	2.22 (s) or 2.16 (s)	2.23 (s) or 2.17[b] (br s)

[a] Multiplicity abbreviations: br, broad; d, doublet; m, multiplet; s, singlet; v br, very broad.
[b] Presence of shoulder on main peak.

coupled) and the benzimidazole NH was seen as a very broad and diffuse signal, centring around δ 11.25. Under essentially the same instrument conditions, the spectrum in $CDCl_3$ showed the broad features characteristic of chemical exchange. Claramunt et al. reported an almost complete second set of ^{1}H signals and a complete second set of ^{13}C signals for omeprazole in THF-d_8 when the spectrum was acquired at very low temperature (195 K). These observations were attributed to the well-known tautomerisation of benzimidazoles, the low temperature causing the two tautomeric forms to be in slow exchange with each other on the NMR timescale. Our NMR experiments were performed at room temperature (298 K) but still there was clear evidence of chemical exchange in the $CDCl_3$ spectrum. Most signals were broad, with some showing marked distortion or shoulders; and the benzimidazole NH, gave rise to two distinct, broad signals.

The structure of M1 was confirmed by its ^{13}C NMR spectrum and by the results of HMBC and HMQC experiments that are summarised in Table 3. Thirteen of the molecule's 17 carbons were revealed and the HMQC and HMBC experiments identified the ^{1}H signal at δ 4.68 as being due to the H-15 protons of the postulated hydroxymethyl group. This assignment was key and confirmed the site of metabolism in this molecule.

Table 2
^{1}H NMR spectral data for metabolites (**M1**, **M2** and **M3**) in CD_3CN at 500 MHz

Position	**M1**, δ_H (multiplicity, *J*/Hz)[a]	**M2**, δ_H (multiplicity, *J*/Hz)[a]	**M3**, δ_H (multiplicity, *J*/Hz)[a]
NH	11.8 (v br s)	11.3 & 10.6 (br s/br s)	11.73 & 11.60 (br s/br s)
H-13	8.49 (s)	Not observed	8.29 (s)
H-7	7.42 (d, 8.7)	7.48 (br m)	7.35 (br m)
H-4	7.10 (d, 2.4)	7.11 (br s)	6.92 (br s)
H-6	6.83 (dd, 8.7, 2.4)	6.88 (dd, 8.8, 2.5)	6.73 (br m)
H-15	4.68 (s)		
H-8	4.56 (s)	4.42 (s)	4.51 (s)
16-OMe	3.85 (s)	3.86 (s)	3.79 (s)
17-OMe	3.85 (s)		
15-OH	3.27 (br s)		
14-Me	2.35 (s)	2.17 (br s)	2.33 (s) or 2.28 (s)
15-Me		2.17 (br s)	2.33 (s) or 2.28 (s)

[a] Multiplicity abbreviations: br, broad; d, doublet; m, multiplet; s, singlet; v br, very broad.

Table 3
^{1}H–^{13}C correlation data for metabolite (**M1**) in CD_3CN from HMQC and HMBC experiments

Position	δ	HMQC (^{1}H detected)	HMBC (^{1}H detected)
2	151.5 (broad)		H-8
3a	Not seen		
4	130.5 or 126.2		
5	156.9		H-4, H-6, H-7, 17-OMe
6	111.7	H-6	H-4
7	130.5 or 126.2		
7a	Not seen		
8	36.5	H-8	
9	157.9		H-8, H-13, 14-Me
10	Not seen		H-8, H-13, 14-Me
11	165.0		H-13, H-15, 16-OMe, 14-Me
12	Not seen		H-13, H-15
13	148.3	H-13	H-15
14	11.4	14-Me	
15	58.2	H-15	H-13
16	61.9 or 56.2	16-OMe	
17	61.9 or 56.2	17-OMe	

The ^{1}H spectra of **M2** and **M3** in CD_3CN were unlike the spectrum of **M1** in that they were complicated significantly by exchange broadened signals, analogous to what had been observed previously with omeprazole in $CDCl_3$. Most obvious were the shoulders on the aromatic signals, the complete absence of a signal for H-13 in **M2** and the two broad signals for the benzimidazole NH in **M3**. It is not clear why slow exchange should occur with these metabolites when it did not occur with **M1** under the same conditions. Nevertheless, from the fully assigned ^{1}H spectrum of **M1**, it was possible to complete the assignments in **M2** and **M3**, despite their complicated features.

3.3. Biotransformation pathways

The predominant metabolic pathway observed in the biotransformation of omeprazole by *C. elegans* ATCC 9245 involved the reduction of the sulphoxide group to a sulphide and the hydroxylation of the molecule's aromatic rings and side chain (Fig. 1). Compound **M** was also considered a biotransformation product despite being observed in substrate control experiments. In the microbiological conditions it was one of the major products observed whereas in the control experiments it was formed in much smaller amounts. Therefore, the reaction rate was clearly accelerated by the presence of the microorganism. All the other biotransformations introduced polar hydroxy groups (**M1**–**M5**) without evidence for the formation of conjugated products.

Interestingly, a recently reported microbial biotransformation of a related compound, pantoprazole, with C*unninghamella blakesleeana* AS 3.153, gave rise to two glucoside conjugates amongst three unconjugated sulphide (thioether) metabolites and one sulphone [17]. The difference in the metabolic profile

could be due to the different enzymes present in the two strains of fungi, but, equally, differences between work-up procedures could be responsible.

4. Conclusion

The metabolic activity of *C. elegans* ATCC 9245 provides a facile means of obtaining hydroxylated metabolites of omeprazole in amounts and purities that permit their use as analytical reference materials. Of the six significant metabolites observed in our experiments, three corresponded to known equine metabolites and hence were selected for full characterisation by mass spectrometry and NMR. Their structures were assigned as 5(6)-methoxy-2-[[(5-hydroxymethyl-4-methoxy-3-methyl-2-pyridinyl)methyl]thio]-1H-benzimidazole (**M1**), 5 (6)-methoxy-2-[[(3,5-dimethyl-4-hydroxy-2-pyridinyl)methyl] thio]-1H-benzimidazole (**M2**), and 5(6)-hydroxy-2-[[(3,5-dimethyl-4-methoxy-2-pyridinyl)methyl]thio]-1H-benzimidazole (**M3**) and shortly they will be used in confirmatory methods supporting drug surveillance in equine sport.

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