Microsome-mediated transformation of *O*-methylandrocymbine to demecolcine and colchicine

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Abstract Microsomal preparations from immature seeds of *Colchicum autumnale* L. catalyse the ring expansion reaction of *O*-methylandrocymbine to demecolcine in the presence of NADPH and O_2. In addition evidence is given for further transformation of demecolcine to colchicine in the presence of acetyl-CoA and NADPH.

Key words: Colchicine biosynthesis; Demecolcine; Ring expansion reaction; *Colchicum autumnale*

1. Introduction

Colchicine, the main alkaloid of *Colchicum autumnale* L. (Liliaceae), is a useful agent in the treatment of acute attacks of gout. As well, derivatives of colchicine are under development as anticancer agents. The biosynthesis of this alkaloid containing an unusual tropolone ring has so far not been fully established [1]. It is known that the first biosynthetic step comprises the condensation of dopamine with 3-(4-hydroxyphenyl) propanal to a phenethylisoquinoline [2]. By further hydroxylation and methylation steps, the sequence of which is not yet completely clear, autumnaline (Fig. 1) is formed. Autumnaline has been shown to be the substrate for the cytochrome P-450-catalysed oxidative coupling reaction leading to isoandrocymbine [3]. Enzymatic methylation to *O*-methylandrocymbine precedes a ring expansion that has been postulated, on the basis of results of feeding experiments with mixtures of ^{3}H- and ^{14}C-labelled autumnaline, to involve the loss of its C-3 carbon [4,5] (Fig. 1). This loss of the carbon atom was further substantiated by feeding experiments with 3,9-^{13}C-double-labelled autumnaline where only the ^{13}C signal from carbon atom 9 could be identified in colchicine while carbon atom 3 was lost [6]. The ring expansion was previously postulated [4] to involve a hydroxylation step at position C-12 of the *O*-methylandrocymbine molecule. C-ring expansion should occur by an ionic mechanism and the resulting demecolcine is first *N*-demethylated and subsequently *N*-acetylated to lead to colchicine [4,6] (Fig. 1).

We now present results giving evidence for the involvement of microsomal-bound cytochrome P-450 enzyme systems in the ring expansion reaction.

2. Materials and methods

2.1. Plant material

Young fruits (2–3 cm size) of *C. autumnale* L. were collected locally and deep-frozen with liquid nitrogen.

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2.2. Preparation of microsomes

Seeds (40–50 g fresh weight (fwt), 6–10 mg fwt/seed) of *C. autumnale* were separated from the frozen fruits and ground in a mortar and pestle with sea sand (4 g) and polyvinylpolypyrrolidone (4 g) in 100 ml 0.1 M tricine-NaOH buffer, pH 7.5 containing 20 mM β-mercaptoethanol at 4°C. The homogenate was filtered through four layers of cheesecloth and centrifuged at $3000\times g$. The resulting supernatant was passed through an XAD-2 (0.3–1 mm particle size) column (2.5×10 cm) and subsequently centrifuged for 30 min at $50\,000\times g$. The pellet was taken up in 5 ml extraction buffer. The protein concentration was determined according to [7], using BSA as standard.

2.3. Enzyme assays

The incubation mixture for detection of metabolites with microsomal preparations consisted of [6-*O*-$^{14}CH_3$]autumnaline (56 μCi/mmol), 30 000 cpm; 0.3 nmol), [$^{3}H_3$]*S*-adenosylmethionine (SAM) (80 mCi/μmol, 150 000 cpm; 2 pmol), NADPH (200 nmol), acetyl-CoA (5 nmol) and glycine-NaOH, pH 8.5 (50 μmol). Incubation was carried out for 5 h at 30°C in a total volume of 200 μl. For identification of the labelled products 6 ml of the incubation mixture was extracted three times with each 10 ml ethylacetate. The combined extracts were evaporated, taken up in 500 μl methanol and subjected to TLC.

The incubation mixture for testing the formation of demecolcine from *O*-methylandrocymbine contained in a total volume of 200 μl [10-*O*-$^{14}CH_3$]*O*-methylandrocymbine (6.3×10^{6} dpm/μmol, 10 000 cpm; 2 nmol), NADPH (100 nmol) glycine-NaOH, pH 8.5 (50 μmol) and protein (400 μg). After incubation for 120 min at 30°C the assay was extracted with 400 μl ethylacetate for 10 min, the organic layer was evaporated, taken up in 30 μl methanol and subjected to TLC.

2.4. TLC

TLC was performed on silica gel sheets (0.25 mm, F_{254} Macherey-Nagel).

Two different solvent systems were used: (1) Me_2CO-toluene-EtOH-NH_4OH (45:45:7:3), colchicine $R_f = 0.4$, demecolcine $R_f = 0.6$ and (2) $CHCl_3$-Me_2O-Et_2NH (7:2:1), colchicine $R_f = 0.6$, demecolcine $R_f = 0.8$.

2.5. Radiochemicals

[$^{3}H_3$]SAM was synthesised from [$^{3}H_3$]methionine (Amersham, 80 mCi/μmol) according to [8] and [6-*O*-$^{14}CH_3$]autumnaline synthesised according to [4]. [10-*O*-$^{14}CH_3$]*O*-Methylandrocymbine was obtained by feeding 1 μCi [6-*O*-$^{14}CH_3$]autumnaline (56 μCi/μmol) to 96 seeds in a 24-well multidish (Nunc); in each well four seeds were placed in 0.5 ml water for 2 days in the dark according to [6]. The seeds were extracted with hot methanol (20 ml), the methanol evaporated and the [10-*O*-$^{14}CH_3$]*O*-methylandrocymbine purified by TLC as described above (incorporation rate: 8%, 6.3×10^{6} dpm/μmol). The structure was verified by ^{1}H NMR on a Bruker AM 360 at 360.166 MHz in $CDCl_3$: δ 1.60 (1H, m, H_{eq}-12), 1.69 (1H, m, H_{ax}-6), 2.17 (1H, m, H_{eq}-6), 2.36 (1H, m, H_{ax}-5), 2.39 (NMe, 3H, s), 2.58 (1H, m, H_{eq}-13), 2.77 (1H, m, H_{ax}-13), 2.93 (1H, m, H_{eq}-5), 2.98 (1H, m, H_{ax}-12), 3.63 (OMe-10, s), 3.83 (2×OMe-2,3, s), 4.02 (OMe-4, s), 6.30 (1H, H-11, s), 6.32 (1H, H-8, s), 6.81 (1H, H-1, s).

3. Results and discussion

Isolated immature seeds of *C. autumnale* (6–10 mg fwt/seed) have been proven to be an excellent source for studying the

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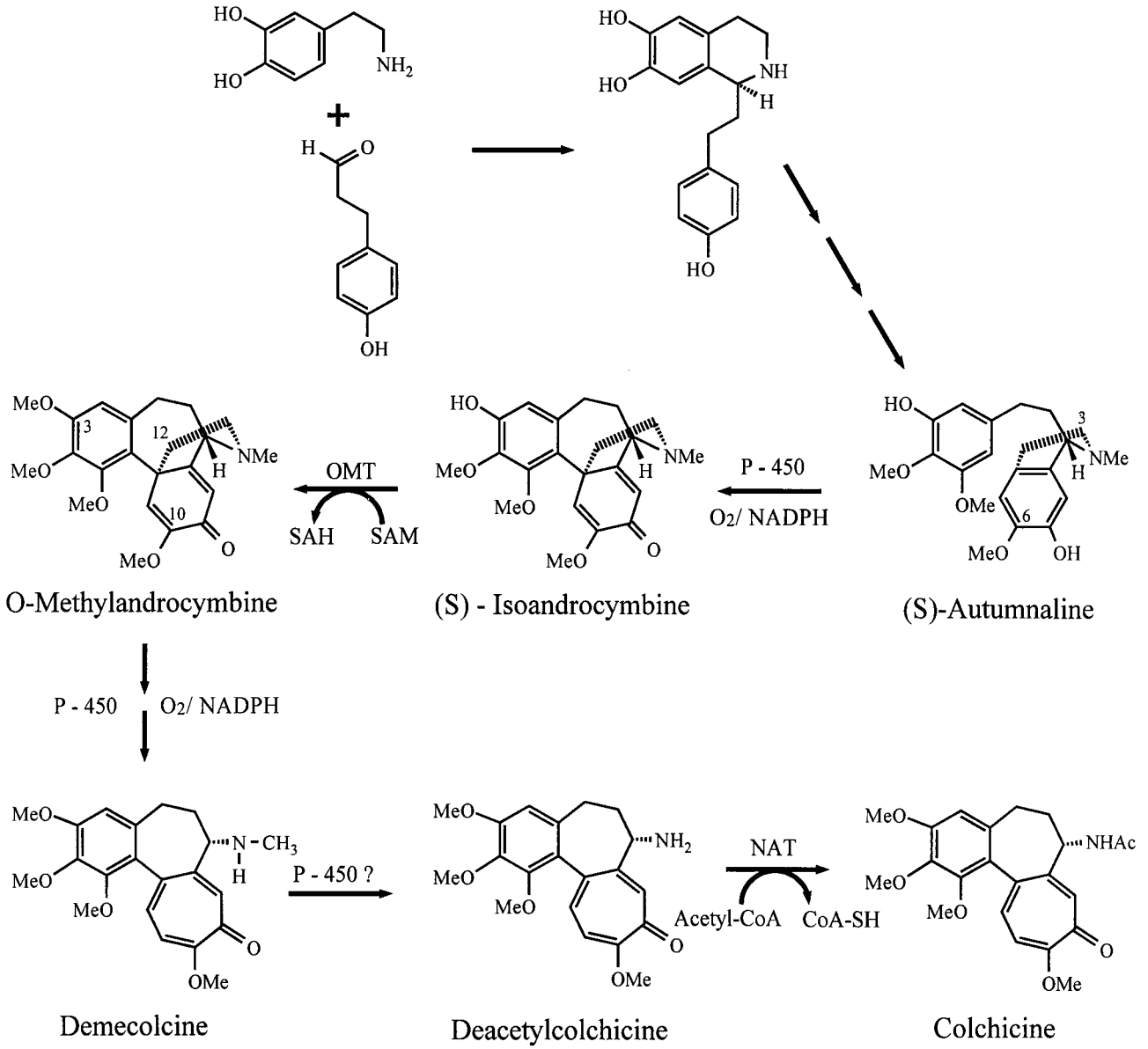

Fig. 1. Proposed biosynthesis of colchicine at the enzymatic level (OMT, *O*-methyltransferase; NAT, *N*-acetyltransferase; P-450: cytochrome P-450).

biosynthesis of colchicine at the enzymatic and precursor feeding level [2]. To investigate the ring expansion reaction we incubated crude microsomal preparations with ^{14}C-labelled *O*-methylandrocymbine and NADPH. A new labelled product was formed in 54% yield which showed the R_f value of demecolcine in two different solvent systems. 20 ml of the incubation mixture containing a total of 500 nmol unlabelled *O*-methylandrocymbine was incubated accordingly. The reaction mixture was extracted with ethylacetate and the major product formed purified by TLC. The EI mass spectrum showed a M^+ of 371, a characteristic base peak at *m/z* 207 and minor peaks at *m/z* 340, *m/z* 328 and *m/z* 312 in agreement with the data described for demecolcine [9]. The product formation was clearly dependent on NADPH (with 50% saturation at 75 μM) and on unlabelled *O*-methylandrocymbine (50% saturation at 60 μM concentration). Without NADPH or with boiled enzyme no transformation could be observed. The pH optimum of the reaction is pH 8.5 with glycine-NaOH buffer. The reaction could be nearly completely inhibited (90%) after gassing the mixture with $CO:O_2 = 9:1$. No inhibition occurred with a gas mixture of $N_2:O_2 = 9:1$ (Table 1). Since the inhibition could be reversed to 60% by white light we conclude that a cytochrome P-450 enzyme is involved. This conclusion is also supported by the fact that added cytochrome *c* is an effective inhibitor (50% inhibition at 40 μM concentration). From these experiments we propose that the ring expansion reaction is possibly preceded by a hydroxylation reaction at C-12 of *O*-methylandrocymbine, consistent with the mechanism formulated previously by Battersby et al. [4]. Based on our results, however, it is clear that this reaction is catalysed by a cytochrome P-450 enzyme system.

Subsequently the crude microsomal preparations used above were fortified with various combinations of cofactors in an attempt to detect further metabolic transformation of [6-*O*-$^{14}CH_3$]autumnaline. Since the postulated pathway from demecolcine to colchicine (Fig. 1) involves *N*-demethylation

Table 1
Effect of carbon monoxide on the microsomal transformation of *O*-methylandrocymbine to demecolcine

Assay condition	Demecolcine formed	
	nmol	% enzyme activity
Air, white light	1.1	100
Air, dark	1.1	100
N_2:O_2 (9:1), white light	1.1	100
N_2:O_2 (9:1), dark	1.1	100
CO:O_2 (9:1), dark	0.11	10
CO:O_2 (9:1), white light	0.7	60

The standard assay contained [10-*O*-$^{14}CH_3$]*O*-methylandrocymbine (10 000 cpm; 2 nmol), NADPH (100 nmol), glycine-NaOH, pH 8.5 (50 μmol) and protein (400 μg).

and subsequent acetylation and assuming that downstream enzymes were present in the crude cell free preparation, NADPH, [C^3H_3]SAM (as cofactor for the methylation from isoandrocymbine to *O*-methylandrocymbine) and acetyl-CoA were added to the incubation mixture. Indeed incubation of [6-*O*-$^{14}CH_3$]autumnaline, [C^3H_3]SAM, unlabelled acetyl-CoA and NADPH resulted in the formation of $^3H/^{14}C$-double-labelled colchicine (13.7% conversion of autumnaline, 260 580 dpm ^3H/170 778 dpm ^{14}C, ^3H:^{14}C = 1.5:1) and demecolcine (22.4% conversion of autumnaline 557 940 dpm ^3H/280 310 dpm ^{14}C, ^3H:^{14}C = 2.3:1). After chromatographic purification with two different solvent systems of the labelled compounds corresponding in their R_f values to colchicine and demecolcine, these products were diluted with the unlabelled alkaloids and recrystallised three times from chloroform/ether. The specific activities and the $^3H/^{14}C$ ratios remained constant in all three crystallisation steps (colchicine: 71 913 dpm/μmol ^3H, 37 248 dpm/μmol^{14}C, ^3H:^{14}C = 1.9:1, demecolcine 293 000 dpm/μmol ^3H, 125 909 dpm/μmol ^{14}C, ^3H:^{14}C = 2.3:1). These results indicate that according to the proposed biosynthetic scheme (Fig. 1) not only the phenol coupling to isoandrocymbine but also the methylation at C-3 to *O*-methylandrocymbine (shown by the labelling with [C^3H_3]SAM), the ring expansion to the tropolone ring and, in the case of colchicine, the *N*-demethylation and reacetylation, must have taken place in this incubation mixture with the endoplasmic reticulum-derived preparation. The *N*-demethylation of demecolcine may also be catalysed by a cytochrome P-450 enzyme in analogy with other reactions described in plants [10] or by a flavin enzyme of the berberine bridge type [11]. The slightly differing $^3H/^{14}C$ ratios of the products are most probably due to traces of alkaloidal precursors present in the microsomal preparations, which are also methylated by [C^3H_3]SAM.

Microsomal preparations from young seeds of *C. autumnale* appear to contain the whole enzymatic system required to catalyse the formation of colchicine from autumnaline in the presence of necessary cofactors. The phenol oxidative coupling of autumnaline to yield isoandrocymbine follows the same mechanistic pattern as shown in plant [12] and mammalian [13] systems for salutaridine formation from (*R*)-reticuline, which in both cases was shown to be catalysed by cytochrome P-450 systems. After *O*-methylation at C-3 by a SAM-dependent methyltransferase present in the microsomal preparation, *O*-methylandrocymbine is converted, most likely by a radical mechanism as shown in this contribution, to demecolcine. This latter reaction leading to the tropolone ring-containing product, demecolcine, is catalysed by cytochrome P-450 system or systems. Further enzymatic *N*-demethylation [10,11] could yield deacetylcolchicine. Clearly an acetyltransferase responsible for acetylating this intermediate at the expense of acetyl-CoA catalyses the last step in the biosynthesis of colchicine. This enzyme is already in hand.

Since cytochrome P-450 enzymes are exceedingly difficult to purify from plants a cloning strategy, which has previously been proven successful in identifying and isolating cryptic enzymes of this type, will be applied to the isolation of enzymes involved in this pathway [14].

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