

## Alkaloids and Plant Metabolism

### VI. O-METHYLATION *IN VITRO* OF NORBELLADINE, A PRECURSOR OF AMARYLLIDACEAE ALKALOIDS\*

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Norbelladine is a precursor of Amaryllidaceae alkaloids (1) such as belladine, crinamine, lycorine (2), and galanthamine (3). We have found and partially purified an enzyme in *Nerine bowdenii* which catalyzes the conversion of norbelladine to its monomethyl ether, *N*-isovanillyltyramine. The latter is itself a precursor of the alkaloid haemanthamine (4), the methoxyl epimer of crinamine (Fig. 1).

The plant enzyme is analogous to the catechol *O*-methyltransferase of rat liver (5) in that both catalyze *O*-methylations of catechols, yet it differs from the catechol *O*-methyltransferase because it catalyzes methylation predominately *para* rather than *meta* to the alkyl substituent.

A survey of potential methyl donors for the reaction catalyzed by the *Nerine* enzyme has demonstrated that (–)-*S*-adenosyl-L-methionine is the preferred methyl donor. Other sulfonium compounds and diastereoisomers of (–)-*S*-adenosyl-L-methionine are less effective. A preliminary report on some of these findings has appeared (6).

#### EXPERIMENTAL PROCEDURE

(–)-*S*-Adenosyl-L-methionine-<sup>14</sup>CH<sub>3</sub> was synthesized enzymatically (7). Nonradioactive diastereoisomers of this compound were obtained by enzymatic resolution of chemically synthesized material (8). Norbelladine was prepared as previously described (2).

Bulbs of *N. bowdenii*<sup>1</sup> obtained from Pearce Seed Company, Moorestown, New Jersey, were planted in a light vermiculite-enriched soil and permitted to flower. Since the roots and stalks contained relatively small amounts of enzyme, they were discarded. The dead outer layers of the bulbs were removed, and the firm inner flesh was used for isolation of the enzyme.

**Purification of Norbelladine Methyltransferase**—The inner flesh of *Nerine* bulbs was minced with shears and ground for 1 minute in a Waring Blendor or Omni-mixer with an equal amount (w/v) of 0.05 M Tris buffer (pH 7.4). This and all subsequent operations were performed at 4°. In some experiments thiourea (1 mM) was added to the extracting buffer to minimize formation of brown pigments. The extracts were filtered through cheesecloth and centrifuged for 20 minutes at 5000 × *g*. Fractionation

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<sup>1</sup> We have obtained an apparently identical enzyme from bulbs of Dawn-Gold *Narcissus*.

between 40 and 70% saturation with ammonium sulfate yielded a 5-fold purified preparation with 91% recovery. The precipitate was dissolved in a small volume of 0.05 M potassium phosphate buffer (pH 7.4) and passed over a column of Sephadex G-25. Dialysis for 12 hours against 0.5 M sucrose-0.05 M potassium phosphate (pH 7.4) could be substituted for Sephadex treatment. Purified preparations catalyzed the *O*-methylation of approximately 0.5 μmole of norbelladine per minute per mg of protein (extrapolated to optimal conditions).

At all stages of purification, the enzyme could be stored at –20° when brought to a final sucrose concentration of 0.5 M. Repeated freezing and thawing caused only slight loss of activity.

**Assay Method 1**—An aliquot of enzyme was incubated for 15 minutes at 35° with 6 μmoles of (–)-*S*-adenosyl-L-methionine-<sup>14</sup>CH<sub>3</sub> (specific activity, 9.2 mc per mmole), 100 μmoles of norbelladine or other methyl acceptor, and 50 μmoles of Tris buffer (pH 8.1), in a final volume of 0.20 ml. The reaction was stopped by the addition of 0.20 ml of 0.25 M sodium borate (adjusted to pH 10 by addition of solid sodium carbonate) and 5.0 ml of ethyl acetate. The tubes were shaken and centrifuged, and 4.0 ml of the organic layer were removed for determination of radioactivity in a scintillation counter.

**Assay Method 2**—In order to test potential methyl donors, 0.7 ml of enzyme was incubated with 100 μmoles of Tris buffer (pH 8.1), 2 μmoles of norbelladine-1-<sup>14</sup>C (specific activity, 4 mc per mmole), various levels of methyl donor, and 1 μmole of sodium cyanide, in a final volume of 0.90 ml. After 30 minutes at 35°, the reaction was stopped by the addition of 0.5 ml of borate-carbonate buffer, and the procedure was continued as described above.

**Rat Liver Catechol *O*-Methyltransferase**—This preparation was made as described by Axelrod and Tomchick (5) through the precipitation between 30 and 50% saturation with ammonium sulfate. This procedure was followed by treatment with Sephadex G-25.

#### RESULTS

**Products of Reaction**—A large scale modification of assay Method 1 was used with a Sephadex-treated enzyme preparation. After incubation for 5 hours at 26° the reaction mixture was adjusted to pH 10 and extracted three times with ethyl acetate.

Synthetic compounds *N*-isovanillyltyramine and *N*-vanillyltyramine were added to separate aliquots of the ethyl acetate extract, crystallized, and degraded (6). Of the total radioactiv-

ity extracted into ethyl acetate after incubation with *Nerine* enzyme, 78% was found in the *p*-*O*-methylated product and 3.5% in the *m*-*O*-methylated product.

This procedure was carried out also with the methylated products of norbelladine resulting from the action of rat liver catechol *O*-methyltransferase;  $5 \times 10^{-4}$  M magnesium chloride was present during this incubation. The results indicated that approximately 83% of the extractable radioactivity was found in the *meta* product and 22% in the *para* product. This *meta* to *para* ratio is consistent with those previously found for this enzyme acting upon other catecholic substrates (9).

**Characteristics of *Nerine* and Rat Liver Enzymes**—The norbelladine methyltransferase from *N. bowdenii* exhibits maximal activity at pH 8.1. The  $K_m$  for (–)-*S*-adenosyl-L-methionine is 1 to  $3 \times 10^{-5}$  M. Norbelladine produces severe substrate inhibition; the approximate  $K_m$  for this compound is  $1 \times 10^{-4}$  M. The  $K_m$  of 3,4-dihydroxyphenylethylamine, an alternative methyl acceptor, is  $1 \times 10^{-3}$  M.

Both the plant and the rat liver enzyme are stimulated by cyanide ion (Table I). This stimulation is found when the *Nerine* enzyme is assayed by either Method 1 or 2. The stimulation by cyanide ranged from 80 to 404% in different preparations of enzyme at different stages of purification. Thiourea (10 mM) stimulates both the plant and the rat liver enzymes. Stimulation by cyanide and by thiourea is not additive. Mercaptoethanol inhibits the plant enzyme at all concentrations tested ( $10^{-5}$  to  $10^{-3}$  M).

EDTA (1 mM) and 8-hydroxyquinoline (10 mM) inhibit both enzymes (Table I). Inhibition by EDTA is not always complete with crude extracts, but either dialysis or increase in EDTA concentration permits complete inhibition. The inhibition cannot be prevented by higher catechol concentrations.

The effects of other chelating agents (Table I) are less striking and somewhat variable.

Rat liver catechol *O*-methyltransferase is stimulated by a number of divalent cations (5). When tested under comparable conditions, the *Nerine* enzyme was essentially insensitive to metal salts, although the animal enzyme responded as expected (Table II). *Nerine* enzyme preparations were tested at all stages of purity, but no significant stimulation was detected.

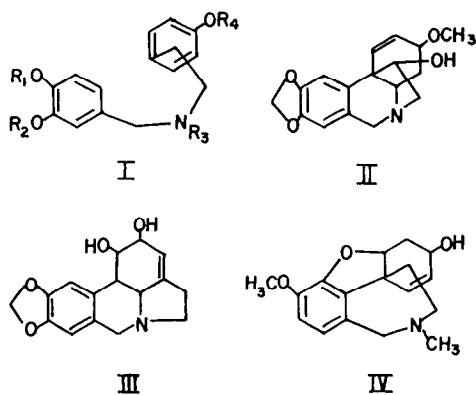


FIG. 1. *Ia*, R<sub>1</sub> = R<sub>2</sub> = R<sub>3</sub> = R<sub>4</sub> = H, norbelladine. *Ib*, R<sub>1</sub> = R<sub>2</sub> = R<sub>3</sub> = R<sub>4</sub> = CH<sub>3</sub>, belladine. *Ic*, R<sub>1</sub> = R<sub>3</sub> = R<sub>4</sub> = H, R<sub>2</sub> = CH<sub>3</sub>, *N*-vanillyltyramine. *Id*, R<sub>2</sub> = R<sub>3</sub> = R<sub>4</sub> = H, R<sub>1</sub> = CH<sub>3</sub>, *N*-isovanillyltyramine. *Ie*, R<sub>1</sub> = R<sub>2</sub> = CH<sub>3</sub>, R<sub>3</sub> = R<sub>4</sub> = H, *N*-veratryltyramine. *IIf*, methoxyl *cis* to ethano bridge, haemanthamine. *III*, lycorine. *IV*, galanthamine.

TABLE I

## Effect of metal-chelating agents

*Nerine* enzyme "A" was a crude extract; enzyme "B" had been purified by ammonium sulfate fractionation followed by treatment with Sephadex G-25 and with Dowex A-1. The rat liver enzyme was dialyzed after ammonium sulfate fractionation. Assay Method 1 was used. Change in the efficiency of extraction was ruled out as a cause of any of the observed results.

Chelating agent	Concentration	Relative rate of methylation		
		<i>Nerine</i> A	<i>Nerine</i> B	Rat liver
		%	%	%
None		100	100	100
Sodium cyanide	1		404	119
Sodium cyanide	10		214	119
Thiourea	10	117	319	179
EDTA	1	30	2	0
8-Hydroxyquinoline	10	34	31	31
Dithizone	1	63	135	76
$\alpha, \alpha$ -Dipyridyl	1	89	152	
Salicylate	10	92	89	99

TABLE II

## Effect of metal salts on methyltransferase activity

The plant enzyme preparation used in Experiment 1 was the ammonium sulfate precipitate treated with Sephadex. The rat liver enzyme of Experiments 2 and 3 was obtained as described in the text. The enzyme preparation used in Experiments 3c and 3d was passed through a column (0.5  $\times$  2 cm) of Dowex A-1; the first few milliliters were discarded to avoid dilution of enzyme by the bed volume of the column. The assay Method 1 was used.

Enzyme source	Metal	Concentration	Methylation rate	Relative rate
		mM	c.p.m.	%
<i>Experiment 1:</i> <i>Nerine</i>	None		3190	(100)
	BaCl <sub>2</sub>	0.2	3090	96
	CaCl <sub>2</sub>	0.15	2890	90
	CdCl <sub>2</sub>	0.15	3970	123
	CuCl <sub>2</sub>	0.15	2040	63
	MgCl <sub>2</sub>	0.15	2780	86
	ZnCl <sub>2</sub>	0.15	2370	73
	MnCl <sub>2</sub>	0.15	1130	36
<i>Experiment 2:</i> Rat liver, dialyzed	None		590	(100)
	MgCl <sub>2</sub>	0.50	1150	193
	ZnCl <sub>2</sub>	0.15	1040	176
<i>Experiment 3:</i> a. Rat. liver, dialyzed b. Rat. liver, dialyzed c. Rat. liver, Dowex A-1 d. Rat. liver, Dowex A-1	None		810	(100)
	MgCl <sub>2</sub>	0.50	1940	238
	None		770	95
	MgCl <sub>2</sub>	0.50	980	120

In an attempt to remove any tightly bound metals, preparations of *Nerine* and of rat liver enzymes were passed over columns of chelating resin Dowex A-1. This treatment did not significantly lower the methylation rate catalyzed by the enzyme in the absence of added metals. However, rat liver enzyme treated in this manner no longer responded to added magnesium chloride (Table II, Experiment 3). These findings with Dowex A-1 may have been due to leaching of chelating material from the ion

TABLE III

Steric specificity of *Nerine bowdenii* enzyme for diastereoisomers of *S*-adenosylmethionine

A Sephadex-treated enzyme fraction was used, with assay Method 2. The values given have been corrected for a boiled enzyme blank.

Methyl donor	Concentration	Methylation rate
	<i>mm</i>	<i>c.p.m.</i>
<i>Experiment 1:</i>		
None		196
(-)- <i>S</i> -Adenosyl-L-methionine	0.024	5150
(+)- <i>S</i> -Adenosyl-L-methionine	0.058	591
Adenosyldimethylsulfonium salt	0.058	66
Decarboxylated <i>S</i> -adenosylmethionine (adenosyl(3-aminopropyl)methylsulfonium salt)	0.058	0
<i>Experiment 2:</i>		
<i>S</i> -Adenosylethionine	1.45	22
(-)- <i>S</i> -Adenosyl-L-methionine	0.24	1780
(±)- <i>S</i> -Adenosyl-L-methionine	1.36	1480
(±)- <i>S</i> -Adenosyl-D-methionine	1.32	496

TABLE IV

Methyl acceptors for *Nerine* *O*-methyltransferase

Sephadex-treated enzyme preparations and assay Method 1 were used. A boiled enzyme blank has been subtracted. No correction has been made for possible differences in partition coefficients of the various methylated products.

Compound	Methylation rate
	<i>c.p.m.</i>
None	5
Norbelladine	1140
<i>N</i> -Vanillyltyramine	0
<i>N</i> -Isovanillyltyramine	8
<i>N</i> -Veratryltyramine	6
Dopamine*	897
None	4
Norbelladine	9790
Catechol	752
2,3-Naphthalenediol	5540
2,4-Dihydroxydiphenyl	2440
Dopamine	7430

\* 3,4-Dihydroxyphenylethylamine.

exchange resin. We suggest that results obtained by the use of this potentially useful resin should be interpreted with caution.

*Methyl Donors for Nerine Enzyme*—The availability of radioactive norbelladine permitted use of various potential methyl donors to test the specificity of the purified enzyme. The assay Method 2 was sufficiently sensitive to detect a rate of methylation 5% or more of that given by (-)-*S*-adenosyl-L-methionine.

Enzymatically resolved (+)-*S*-adenosyl-L-methionine was approximately 10% as effective as the (-)-diastereoisomer (Table III). Even this low activity may be due to incomplete removal of (-)-*S*-adenosyl-L-methionine from the mixture of (±)-diastereoisomers (8).

Less specificity was shown with regard to the configuration of the α-amino carbon, since (±)-*S*-adenosyl-D-methionine was

one-third as effective as (±)-*S*-adenosyl-L-methionine. Decarboxylated (±)-*S*-adenosyl-methionine, adenosyldimethylsulfonium salt, and *S*-adenosylethionine were not effective.

In addition, the following compounds were tested and found not to be active with the *Nerine* enzyme: *S*-methyl-L-methionine, *S*-methyl-DL-methionine, *N,N*-dimethylglycine, betaine, choline, dimethylpropiothetin, dimethylacetothetin, L-methionine sulfide, L-methionine sulfone, and a mixture of *S*-methyl-DL-methionine and *S*-adenosylhomocysteine. The compounds were used at final concentrations of approximately 0.2 mM, with the exception of *S*-methylmethionine, which was tested at 0.03, 0.3, and 3 mM.

*Methyl Acceptors for Nerine Enzyme*—The plant *O*-methyltransferase catalyzes the methylation of a number of catechols in addition to norbelladine (Table IV). Neither crude nor purified preparations, however, reacted with either *N*-isovanillyl- or *N*-vanillyltyramine.

## DISCUSSION

The partially purified preparation of norbelladine *O*-methyltransferase from *N. bowdenii* is the first reported cell-free system from a higher plant that catalyzes *O*-methylation, rather than *N*-methylation. The predominant product of the reaction is the *p*-*O*-methylated derivative, a compound that has recently been shown to be a precursor of haemanthamine when fed to intact daffodil bulbs (4) and that has been isolated from a natural source.<sup>2</sup>

Barton has shown that the *p*-*O*-methyl group of *N*-isovanillyltyramine is converted *in vivo* to the methylenedioxy group of haemanthamine (4). (-)-*S*-Adenosyl-L-methionine is therefore a probable source of the methylenedioxy groups commonly found in this and in other alkaloids of the Amaryllidaceae; this same compound has been demonstrated as a source of methylene bridge carbons of bacterial cyclopropane fatty acids (10, 11). The methylenedioxy group of protopine, an alkaloid of the Papaveraceae, is derived from the methyl group of L-methionine (12), presumably by way of (-)-*S*-adenosyl-L-methionine.

The specificity of the isolated *Nerine* enzyme in synthesizing the *para* rather than the *meta* isomer of *O*-methylnorbelladine suggests that the *para* isomer is the natural precursor of haemanthamine and, presumably, of many other Amaryllidaceae alkaloids. Since the isolated enzyme catalyzes methylation of a wide range of catechols, it is possible that phenol coupling may precede catechol *O*-methylation.

A significant finding is that only *S*-adenosylmethionine can serve as methyl donor for this enzyme, whereas *S*-methylmethionine and other sulfonium compounds are not effective. The D isomer of *S*-adenosylmethionine is one-third as effective as the L isomer. A similar degree of specificity is found for the methionine-activating enzyme of yeast, for which D-methionine supports 25% of the rate given by L-methionine.<sup>3, 4</sup>

<sup>2</sup> Professor Battersby has informed us that he and S. W. Breuer have recently isolated *N*-isovanillyltyramine from double *Narcissus* plants (private communication).

<sup>3</sup> S. H. Mudd, unpublished results.

<sup>4</sup> Note Added in Proof—B. J. Finkle and R. F. Nelson have recently informed us that in work now in press in *Biochimica et Biophysica Acta* they report on an enzyme extracted from cambial tissue of apple which catalyzes the *m*-*O*-methylation of caffeic acid, *S*-adenosylmethionine being the methyl donor.

## SUMMARY

An enzyme that has been extracted from flowering bulbs of *Nerine bowdenii* catalyzes the *p*-*O*-methylation of norbelladine. Other catechols are also methylated. (-)-*S*-Adenosyl-L-methionine is the best methyl donor; neither *S*-methylmethionine nor any other potential methyl donor tested is utilized. The reaction proceeds optimally at pH 8.1, and is not stimulated by divalent cations.

*N*-Isovanillyltyramine, the product of norbelladine methylation, is known to be a precursor of haemanthamine in at least one species of the Amaryllidaceae. (-)-*S*-Adenosyl-L-methionine is probably the source of methylenedioxy carbons in alkaloids of this group.

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