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Metabolism of *N*-alkyldiamines and *N*-alkylnortropinones by transformed root cultures of *Nicotiana* and *Brugmansia* ☆

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Abstract

A range of analogues of *N*-methylputrescine and tropinone were fed to transformed root cultures of *Nicotiana rustica* and/or a *Brugmansia candida* × *aurea* hybrid. These cultures were made by the transformation of the relevant plant species with *Agrobacterium rhizogenes*. A number of the metabolites, notably those showing a relatively modest alteration in the *N*-alkyl substituent, were metabolized in vivo to form homologues of the normal alkaloids biosynthesized by these roots. These products were identified by GC/MS and comparison with some synthetic reference materials. Analogues with major alterations in the size of the *N*-alkyl substituent were not metabolized at all. In the *N. rustica* cultures, the analogues fed at 1 mM significantly affected the profile of normal alkaloids, with up to a 4-fold diminution in nicotine being found in the presence of *N*-*n*-propylputrescine. The ratio between alkaloids of the pyrrolidine series and the piperideine series was also affected. In contrast, the presence of the analogues in the *B. candida* × *aurea* hybrid culture at 1 mM did not inhibit or substantially interfere with the accumulation of the normal spectrum of alkaloids. The potential for using these cultures to make complex novel products from simple precursors is discussed.

Keywords: Alkaloid (analogue); Biotransformation; *Brugmansia candida* × *aurea*; Solanaceae; Hybrid; *Nicotiana rustica*; Transformed root culture

1. Introduction

The pyrrolidine alkaloid, (*S*)-nicotine **23**, and the tropane alkaloids, (*S*)-hyoscyamine **18** and (*S*)-scopolamine **19**, are produced by members of the Solanaceae and share in part a common biosynthetic pathway (Fig. 1) from L-arginine or L-ornithine, via putrescine **1** and *N*-methylputrescine **2**, to *N*-methylpyrrolinium (Leete, 1983). In *Nicotiana*, nicotine **23** is formed by the direct decarboxylative condensation of *N*-methylpyrrolinium with nicotinic acid (Leete, 1983). Anabasine **21**, a minor alkaloid in many nicotine-producing species, is formed from L-lysine via cadaverine **6** (Leete, 1983). This diamine is directly oxidised, apparently by the same diamine oxidase as *N*-methylputrescine, *N*-methylputrescine oxidase (MPO) (Walton & McLauchlan, 1990) and the product, Δ^1-piperideine **20**, condensed with nicotinic acid (Fig. 1). In the biosynthesis of the tropanes (Fig. 1), metab-

☆ Part 1 in the series 'Biosynthesis of novel alkaloids'.

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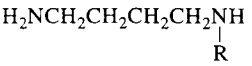
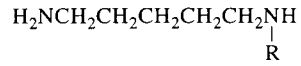
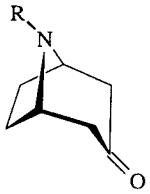


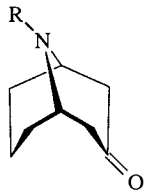


1 Putrescine R=H
2 *N*-Methylputrescine R=CH_3
3 *N*-Ethylputrescine R=CH_2CH_3
3a *N*-(2-Fluoroethyl)putrescine R=CH_2CH_2F
4 *N*-*n*-Propylputrescine R=$(CH_2)_2CH_3$
4a *N*-*iso*-Propylputrescine R=$CH(CH_3)_2$
5 *N*-*n*-Butylputrescine R=$(CH_2)_3CH_3$

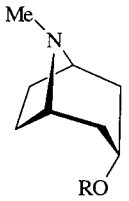
6 Cadaverine R=H
7 *N*-Methylcadaverine R=CH_3
8 *N*-Ethylcadaverine R=CH_2CH_3



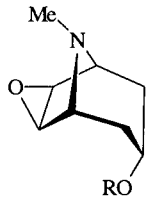
9 Tropinone R=CH_3
10 *N*-Ethylnortropinone R=CH_2CH_3
11 *N*-(2-Fluoroethyl)nortropinone R= CH_2CH_2F
12 *N*-*iso*-propylnortropinone R=$CH(CH_3)_2$



13 Norpseudopelletierine R=H
14 Pseudopelletierine R=CH_3
15 *N*-Ethylnorpseudopelletierine R=CH_2CH_3
16 *N*-*iso*-Propylnorpseudopelletierine R=$CH(CH_3)_2$



17 Littorine R= $COCH(OH)CH_2Ph$
18 Hyoscyamine R= $COCHPh$ with CH_2OH



19 Scopolamine R= $COCHPh$ with CH_2OH

olism is now understood to proceed via a formal condensation of *N*-methylpyrrolinium with activated acetoacetate (Robins, Abraham, Parr, Eagles & Walton, 1997), followed by conversion to tropinone **9**, reduction to the alcohol, tropine, and esterification with a phenyllactoyl residue to produce littorine **17**, the tropane precursor of hyoscyamine **18** (Robins & Walton, 1993; Robins et al., 1995). Many species that accumulate tropane alkaloids also accumulate the pyrrolidine alkaloids, hygrine **25** and cuscohygrine **26**, also derived from *N*-methylpyrrolinium (Fig. 1).

Much of the enzymology of these pathways remains poorly characterized (Robins, 1998). The mechanism of the condensation reaction to produce nicotine **23** remains to be elucidated and only a very low activity in vitro has been reported (Friesen & Leete, 1990). The enzymological characterization of the steps of the tropane pathway between *N*-methylpyrrolinium and tropinone **9** remain to be determined, although recent studies have helped to redefine the probable intermediates, notably 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate **24** (Robins et al., 1997). Recently, the *S*-adenosylmethionine-stimulated skeletal rearrangement of nonnatural (*S*)-littorine by a cell-free extract of *Datura stramonium* has been reported (Ollagnier, Kervio & Rétey, 1998). Whether the same activity will be observed with the natural isomer, (*R*)-littorine, remains to be confirmed.

It has been known for some years that the pyrrolidine alkaloid pathway in *Nicotiana* will accept and metabolize nonnatural derivatives of putrescine or nicotinic acid (Leete, 1983). Methylated pyrrolinium ions were incorporated into nicotine analogues by *N. glutinosa* plants (Rueppel & Rapoport, 1971) and wick-feeding 5-fluoronicotinic acid to plants of *N. glauca* led to the formation of 5-fluoronicotine and 5-fluoroa-

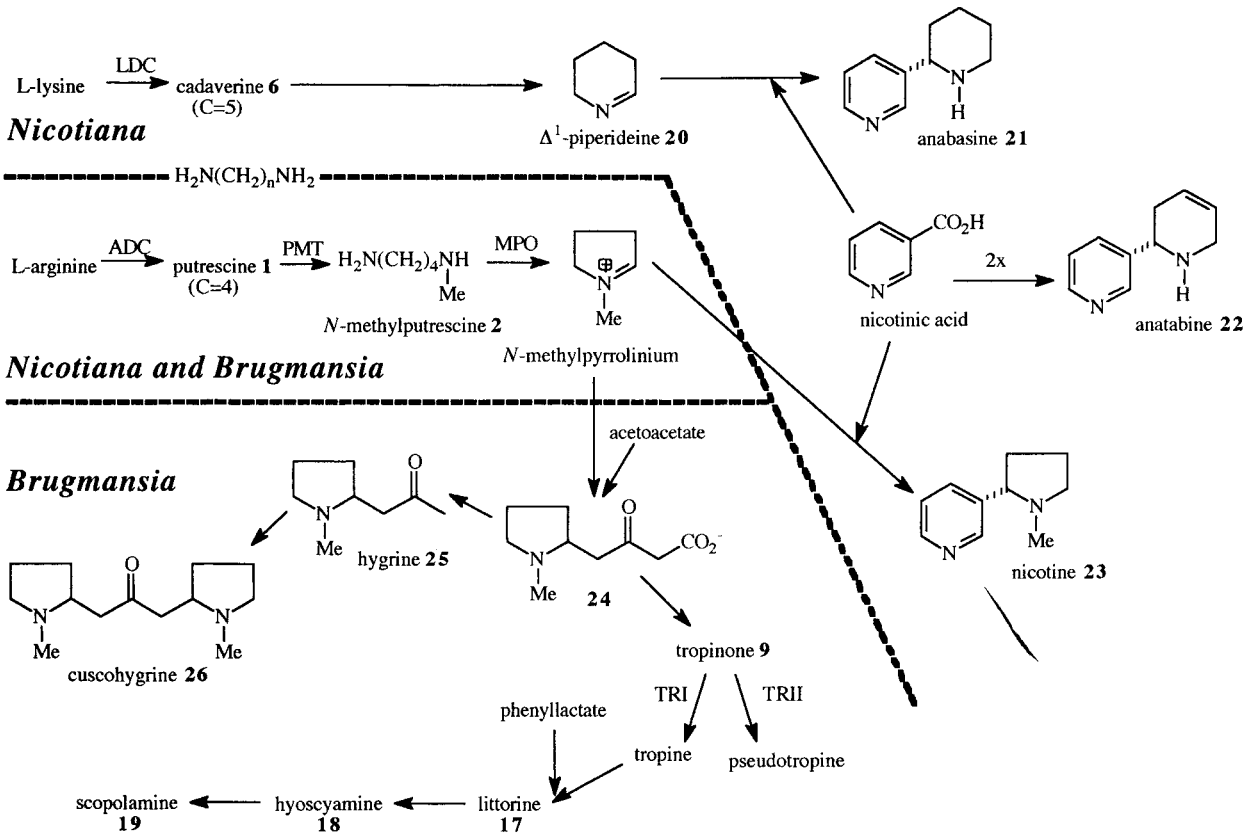


Fig. 1. Schematic pathway for the biosynthesis of various alkaloids in *Nicotiana* and *Brugmansia*. Note that (i) arrows do not necessarily imply a single step and (ii) the exact form in which acetoacetate and phenyllactate are supplied is not known.

nabasine (Leete, 1979; Leete, Bodem & Manuel, 1971). Similarly, feeding *N*-methyl-Δ^1-piperideine to *N. glauca* caused the accumulation of *N*-methylanabasine (Leete & Chedekel 1972), a known natural alkaloid that normally occurs in only trace amounts (Demole & Berthet 1972). The more distal precursor, cadaverine **6**, is actively biotransformed to anabasine **21** when supplied to transformed root cultures of *Nicotiana* spp. (Walton & Belshaw, 1988; Walton, Robins & Rhodes, 1988), while its isomer, *N*-ethylputrescine **3**, is readily converted to the nonnatural (*S*)-*N*-ethylnornicotine (Boswell, Watson, Walton & Robins, 1993).

Such studies are of value on two counts. First, they can demonstrate the degree of plasticity of alkaloid biosynthetic pathways, illustrating in the absence of detailed enzymological characterization the extent to which analogues of the normal substrates might be utilised in successive reaction steps. These data can be valuable in establishing the substrate specificity and giving some mechanistic information about as yet unidentified enzymes. Secondly, they enable the potential for biotransformations to be assessed, using cultures or extracted enzymes as a means to generate biologically-active novel compounds from chemically-synthesized nonnatural substrates.

Root cultures of *Nicotiana rustica* — established by genetic transformation with *Agrobacterium rhizogenes* LBA9402 — accumulate nicotine **23** as the major alkaloid, with minor amounts of nornicotine, anatabine **22** and anabasine **21** (Hamill, Parr, Robins & Rhodes, 1986). Transformed root cultures of a *Brugmansia (ex. Datura) candida* × *aurea* hybrid (line DB5) accumulate a large range of tropane and pyrrolidine alkaloids, the profile being dominated by the aromatic esters of tropine, littorine **17**, hyoscyamine **18** and scopolamine **19** (Robins, Parr, Payne, Walton & Rhodes, 1990). In this paper we report the ability of these cultures to metabolize nonnatural analogues of intermediates to form novel alkaloids. As a group of precursors distal to the final products, lower *N*-alkylputrescines and *N*-alkylcadaverines were synthesized and supplied to growing cultures as potential substrates. Cultures of the *B. candida* × *aurea* hybrid were also supplied with tropinone analogues in order to evaluate the relative efficiency of supplying distal or proximal precursors of the desired products. In a further paper (Boswell et al., 1999), we describe the in vitro activity of relevant enzymes of the tropane alkaloid pathway extracted from the *B. candida* × *aurea* hybrid culture with these substrates.

Table 1
GC/MS retention times and fragmentation characteristics for the major alkaloids and the major alkaloid analogues identified from transformed root cultures of *Nicotiana rustica* fed with *N*-alkyldiamines

N-Alkyldiamine supplied ⇒ *N*-alkylnornicotine produced	Relative R_t[a]	M^+ ion (% abundance)	Characteristic fragments (% abundance)
None/nicotine (control)	1.00	162(20)	161(30), 133(46), 119(10), 84(100), 78(8)
N-*n*-Propylputrescine ⇒ *N*-*n*-propylnornicotine	1.35	190(4)	189(1), 162(11), 161(100), 147(2), 132(11), 130(17), 117(11), 112(8), 78(1)
N-*n*-Butylputrescine ⇒ *N*-*n*-butylnornicotine	1.65	204(3)	203(1), 162(8), 161(100), 147(3), 132(13), 130(23), 117(15), 78(4)
None/anabasine (control)	1.00	162(33)	161(41), 133(79), 119(70), 106(76), 105(100), 92(20), 84(64), 78(26)
N-Methylcadaverine ⇒ *N*-methylanabasine	0.93	176(14)	175(11), 147(14), 133(21), 119(50), 105(11), 98(100), 92(6), 78(8)
N-Ethylcadaverine ⇒ *N*-ethylanabasine	0.98	190(10)	189(7), 175(49), 161(11), 132(18), 119(23), 112(100), 105(19), 92(15), 84(9), 78(8)
N-Propylcadaverine ⇒ *N*-propylanabasine	1.04	204(3)	203(7), 176(12), 175(100), 161(3), 132(30), 126(26), 119(12), 92(21), 84(5), 78(8)

[a] Relative to normal alkaloid. Nicotine = 15.9 min; anabasine = 24.7 min.

Table 2
Production of major natural alkaloids and analogues of nicotine or anabasine by transformed root cultures of *Nicotiana rustica* fed with *N*-alkylated putrescine or cadaverine analogues

	Alkaloid yield (% of total alkaloid)[a]					
Additive (1 mM)	None ($N = 4$)[b]	*N*-*n*-propylputrescine	*N*-*n*-butylputrescine	*N*-methylcadaverine	*N*-ethylcadaverine	*N*-propylcadaverine
Alkaloid or alkaloid analogue produced						
Nicotine	77.4 ± 5.0	19.8	71.4	33.1	63.4	81.7
Nornicotine	3.3 ± 1.9	0.7	1.7	2.1	2.7	0.3
Anabasine	1.4 ± 0.5	2.8	0.8	20.6	2.4	1.1
Anatabine	18.2 ± 3.6	43.3	17.9	20.1	21.4	15.2
Analogue of nicotine	–	33.5	8.2	–	–	–
Analogue of anabasine	–	–	–	24.0	10.1	1.7
Total normal alkaloid (mg)[c]	7.5 ± 2.3	1.8	6.4	12.6	7.3	4.1
Total novel alkaloid (mg)[c]	–	0.9	0.6	4.0	0.8	0.1

[a] Each feeding represents the mean of between 5 and 10 flasks, pooled and extracted together.
[b] The control represents the average of 4 separate experiments, each representing the mean of between 5 and 10 flasks, pooled and extracted together. Values given are the average ± range.
[c] Some minor alkaloids are not included.

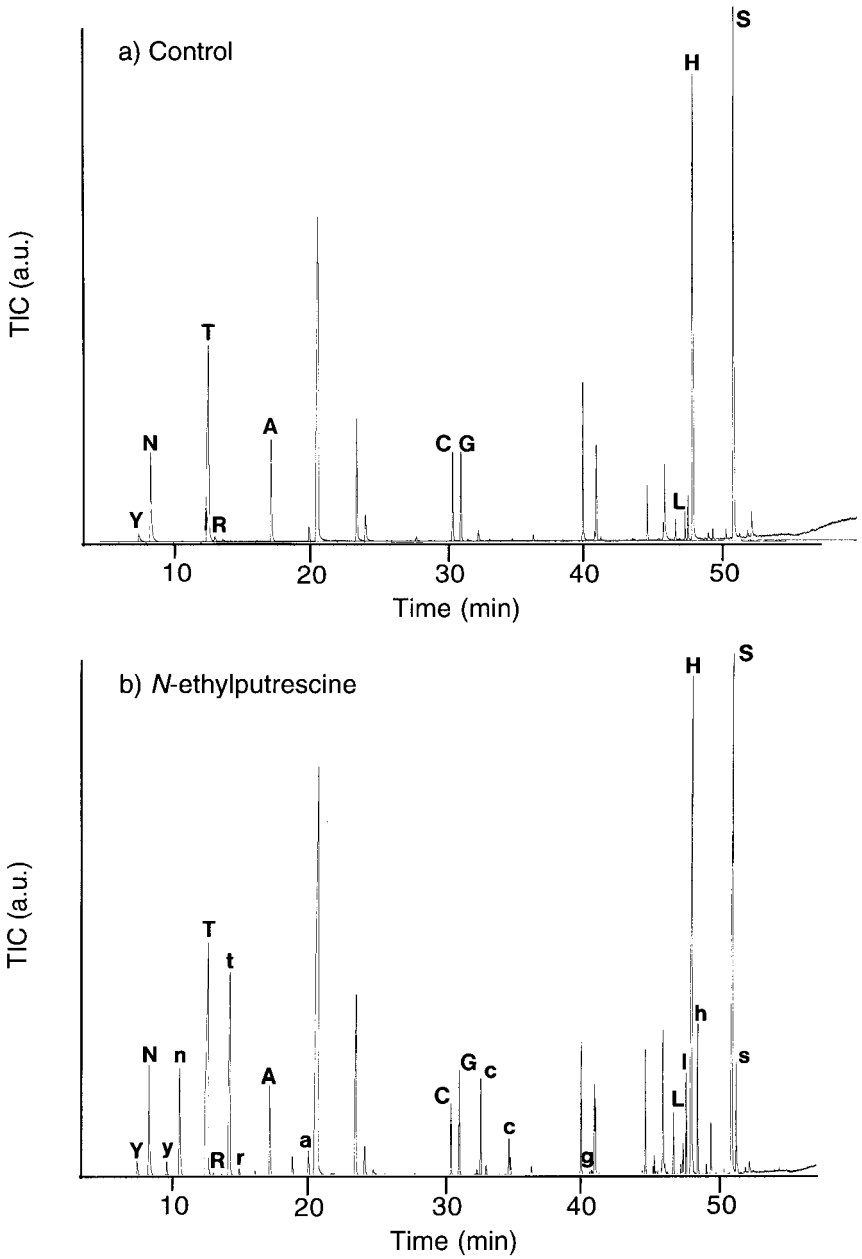


Fig. 2. Gas chromatograms of alkaloidal extracts from cultures of a *Brugmansia candida* × *aurea* hybrid supplied with (a) no alkylated diamine; (b) *N*-ethylputrescine; (c) *N*-(2-fluoroethyl)putrescine; (d) *N*-*n*-propylputrescine; (e) cadaverine; (f) *N*-methylcadaverine. Gas chromatography conditions were as described in the Experimental. The following abbreviations are used: Y: hygrine; N: oxohygrine; C: cuscohygrine; R: tropinone; T: tropine; A: acetyltropine; G: tigloyltropine; L: littorine; H: hyoscyamine; S: scopolamine; these symbols in lower case (y, n, u,...) denote analogues of these alkaloids. TIC (a.u.) = total ion current, (arbitrary units).

2. Results

2.1. N-alkyldiamine metabolism by Nicotiana rustica root cultures

N. rustica root cultures supplied with simple *N*-alkylputrescines and *N*-alkylcadaverines at 1 mM gave rise to new alkaloidal products. These were characterized by GC and GC/MS (Table 1). Each of these exhibited a mass ion and fragmentation pattern consistent with the mass increment of the *N*-alkyl substrate relative to *N*-methylputrescine **2**, the precursor of nicotine **23**, or to cadaverine **6**, the precursor of anabasine **21** (Fig. 1). These can be assigned to homologous series of alkaloids. The extent of conversion (Table 2) declined with increasing chain length of the *N*-alkyl substituent. Thus, *N*-*n*-propylputrescine **4** was actively converted to a novel alkaloid (*N*-*n*-propylnor-

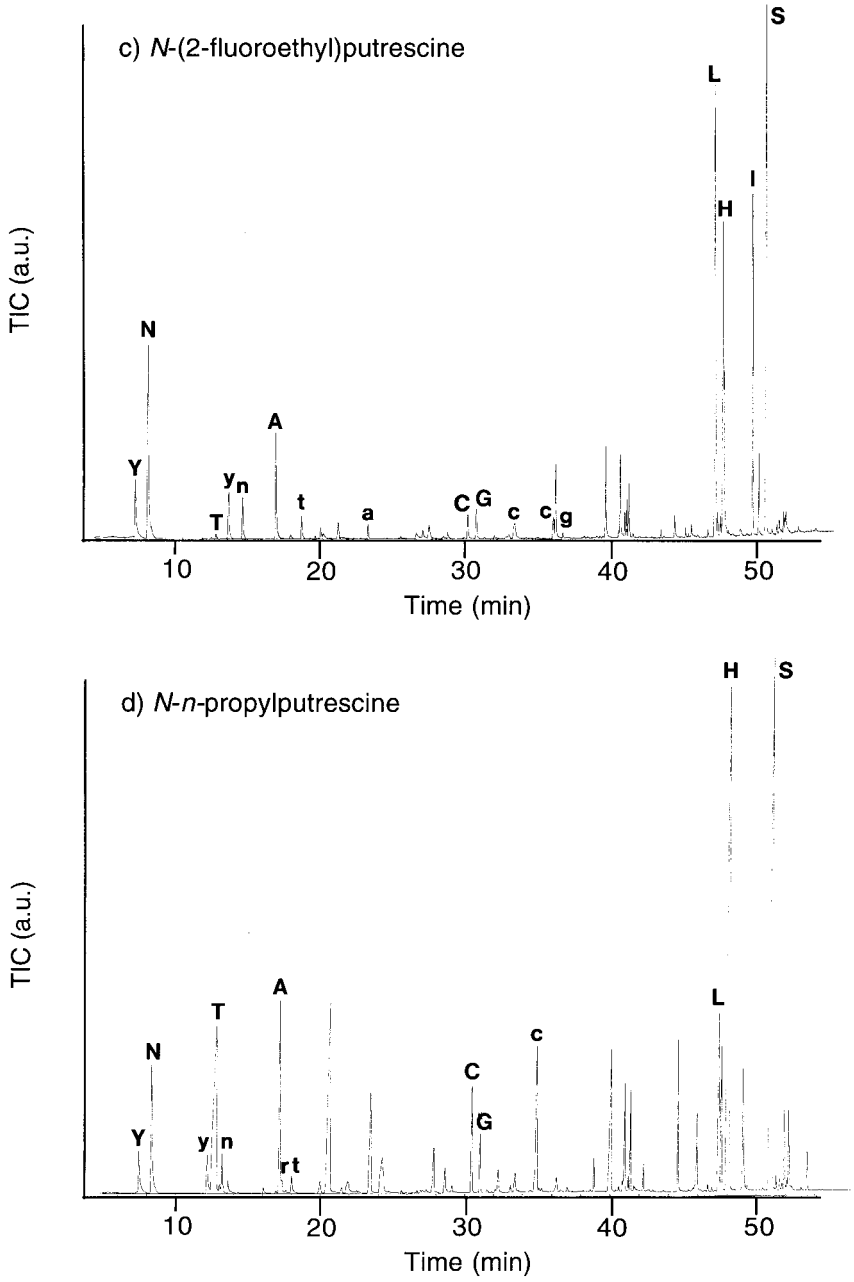


Fig. 2 (*continued*)

nicotine) which accounted for about 33% of the total alkaloid produced. In contrast, *N*-*n*-butylputrescine **5** was relatively poorly metabolized and its alkaloidal product accounted for only 8% of the total alkaloid. It should be noted, however, that much the same yield of product was obtained, the total alkaloid content being markedly decreased in the presence of *N*-*n*-propylputrescine. No metabolism was detected when *N*-benzylputrescine, *N*-phenylethylputrescine or *N*-cyclohexylputrescine were supplied.

In the *N*-alkylcadaverine series, only *N*-methylcadaverine **7** and *N*-ethylcadaverine **8** were substantially metabolized (Table 2); conversion of *N*-*n*-propylcadaverine was feeble and there was no detectable product from *N*-*iso*-propylcadaverine, *N*-*n*-butylcadaverine or *N*-benzylcadaverine.

With *N*-*n*-propylputrescine **4** and *N*-methylcadaverine **7**, where the novel alkaloid accounted for 20% or more of the total alkaloid, nicotine accounted for a reduced proportion of the total — 20% in the case of *N*-*n*-propylputrescine **4** and 33% in the case of *N*-methylcadaverine **7**. *N*-*n*-Propylputrescine **4** also

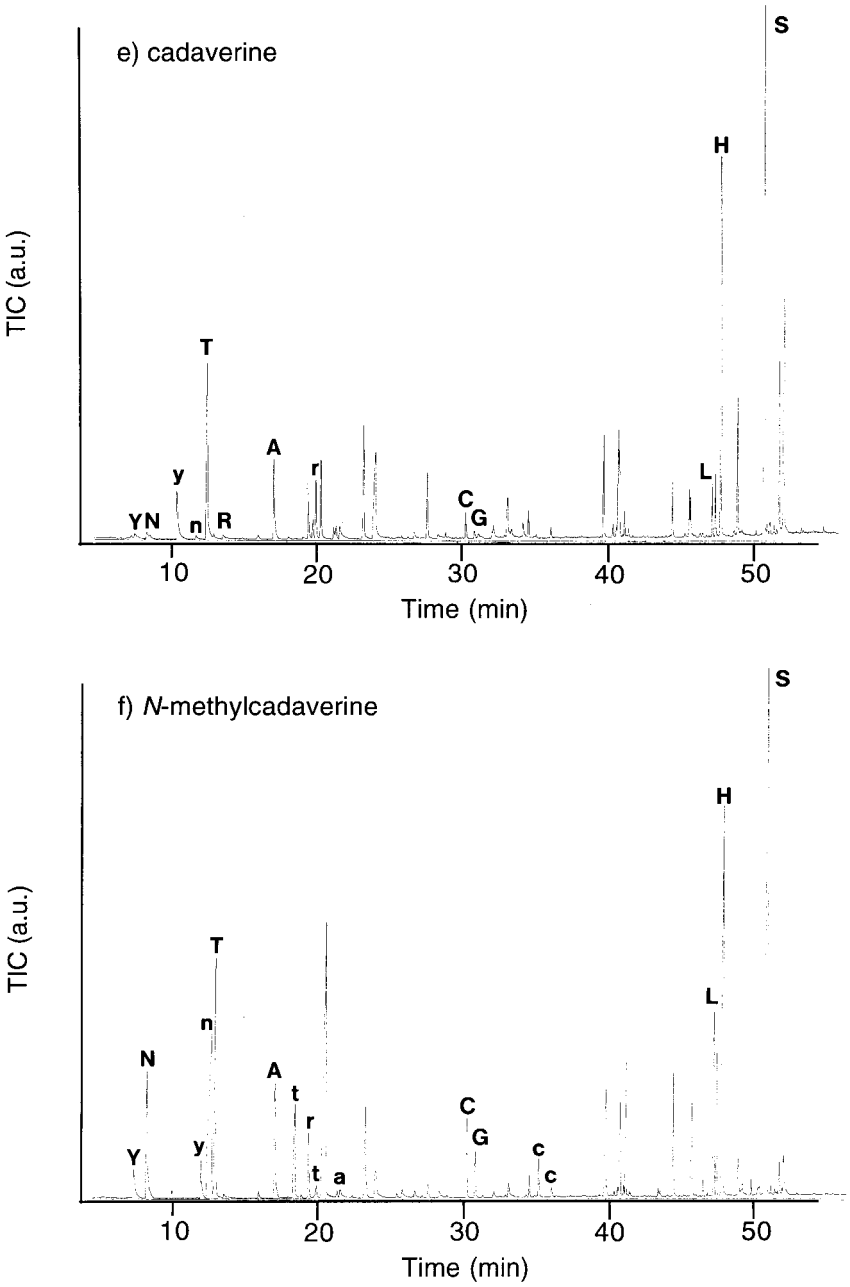


Fig. 2 (*continued*)

increased the proportion of anatabine **22**, though this was not observed when *N*-methylcadaverine **7** was supplied. In contrast, *N*-methylcadaverine-feeding was associated with a large rise in the proportion of anabasine **21**. Those compounds found not to be metabolised to novel alkaloids (*N*-benzylputrescine, *N*-phenylethylputrescine, *N*-cyclohexylputrescine) did not influence the proportions of the major alkaloids present.

2.2. N-alkyldiamine metabolism by Brugmansia candida × aurea root cultures

Corresponding experiments were also performed in which *N*-alkyldiamines were fed at 1 mM to *B. candida × aurea* root cultures and the degree of metabolism determined by GC/MS. In this species, these compounds also represent analogues of the initial substrates of the pathway but, in contrast, are more

Table 3
GC/MS Fragmentation characteristics for the series of alkaloids identified from transformed root cultures of *Brugmansia* fed with *N*-ethylputrescine

Novel alkaloid	Relative R_t[a]	M^+ ion (% abundance)	Characteristic fragments (% abundance)
(A) Pyrrolidine alkaloid			
N-Ethylnorhygrine	1.287	155(1)	140(4), 126(2), 112(2), 98(83), 84(8), 82(17), 70(22), 55(15), 43(100)
N-Ethylnoroxohygrine	1.259	171(1)	156(4), 114(3), 110(1), 98(100), 84(5), 82(5), 41(16)
N-Ethyl-*N′*-methylnorcuscohygrine	1.045	238(<1)	223(2), 140(5), 110(4), 98(50), 84(100), 82(64), 68(33), 55(34), 42(95)
N,N′-Diethylnorcuscohygrine	1.085	252(<1)	98(100), 84(5), 82(45), 68(43), 55(31), 43(71), 41(88)
(B) Tropane alkaloids			
N-Ethylnortropinone	1.140	153(14)	138(9), 124(11), 110(20), 96(95), 95(48), 82(21), 69(89), 56(80), 42(72), 41(100)
N-Ethylnortropine	1.123	155(21)	138(37), 126(21), 110(54), 97(100), 96(94), 82(40), 69(76), 56(85), 41(79)
3-Acetyl-*N*-ethylnortropine	1.096	197(11)	182(4), 154(11), 138(100), 108(42), 96(44), 82(17), 67(43), 56(42), 43(95)
3-Tigloyl-*N*-ethylnortropine	1.036	237(2)	222(4), 194(6), 154(12), 138(76), 110(100), 98(52), 80(62), 68(72), 55(95), 41(91)
N-Ethylnorlittorine	1.011	303(3)	154(8), 138(100), 110(10), 108(25), 97(28), 96(31), 67(28), 56(16)
N-Ethylnorhyoscyamine	1.009	303(4)	154(10), 138(100), 110(9), 108(22), 97(28), 96(26), 67(21), 56(17)
N-Ethylnorscopolamine	1.005	317(8)	302(6), 168(42), 152(100), 150(31), 122(51), 108(76), 103(39), 56(78)

[a] Relative to the normal alkaloid (at $R_t = 1.00$). Hygrine = 7.77 min; oxohygrine = 8.72 min; cuscohygrine = 27.90 min; tropine = 13.13 min; tropinone = 13.47 min; 3-acetyltropine = 17.62 min; 3-tigloyltropine = 28.27 min; littorine = 39.29 min; hyoscyamine = 39.79 min; scopolamine = 42.47 min.

distal from the final products (Fig. 1). GC traces of a control extract and extracts of roots supplied with various *N*-alkyldiamines are presented in Fig. 2. The spectra of extracts from fed roots contain, to varying extents, additional compounds that are absent from the control. These were shown by GC/MS to have the expected mass increment relative to the normal alkaloids and to give corresponding fragmentation patterns (Table 3). The precursors were found to be extensively metabolized into alkaloids of both the monocyclic/bismonocyclic pyrrolidine series and the bicyclic [3.2.1]tropane alkaloid types (Table 4). Within the former category, peaks corresponding to the higher *N*-alkyl-series for hygrine, oxohygrine (an uncharacterized oxidation product of hygrine) and the *N*-methyl,*N′*-alkyl- and *N*-alkyl,*N′*-alkyl- cuscohygrine analogues are present. These alkaloids constitute the major metabolic products in these feeds and may readily be distinguished on the basis of the ratio of the mass fragments at 84 and 98 m.u. With the exception of *N*-ethylputrescine **3**, the yield of analogues of the tropane alkaloids was poor, the yield broadly diminishing as the *N*-alkyl constituent increased in mass. The majority of the products that accumulated were of the *endo*/α configuration, i.e. analogues of tropine derivatives. No products could be detected when *N*-cyclohexylputrescine or *N*-phenylethylputrescine were fed.

When the higher homologous series was fed, both cadaverine **6** and *N*-methylcadaverine **7** were found to be metabolized. Although cadaverine **6** was only converted to pelletierine (the higher homologue of hygrine) and norpseudopelletierine **13**, *N*-methylcadaverine **7** proved to be a good substrate, producing both the cuscohygrine-like bispiperidines and a number of esters of reduced pseudopelletierine. Apart from some slight oxidation of *N*-ethylcadaverine **8** to an uncharacterized homologue of oxohygrine, however, higher homologues were not metabolized and no products were found with *N*-benzylcadaverine, *N*-butylcadaverine, *N*-*n*-propylcadaverine or *N*-*iso*-propylcadaverine.

In contrast to the *N. rustica* cultures, the feeding of these putative precursors at 1 mM had a minimal effect on the profile and yield of normal alkaloids. As shown in Table 5, where the profile is compared to the control grown in parallel, the yields of both tropane and nontropane alkaloids is comparable and is overall still strongly dominated by littorine **17**, hyoscyamine **18** and scopolamine **19**.

2.3. *N*-alkylnortropinone metabolism by *Brugmansia candida* × *aurea* root cultures

In a further series of feeds, *N*-alkyl analogues of tropinone **9** or its higher homologue, pseudopelletierine **14**, were fed to examine the difference between providing distal and proximal precursors to the same root cultures.

As can be seen (Table 6), all three of *N*-ethyl- **10**, *N*-(2-fluoroethyl)- **11** and *N*-*iso*-propylnortropinone **12** were effectively metabolized to a range of aliphatic and aromatic esters. No analogues of the pyrrolidine-type alkaloids were observed (data not shown). The yield was in all cases superior to that found with the

Table 4
Production of alkaloid analogues by transformed root cultures of *Brugmansia* fed with *N*-alkylated putrescine or cadaverine analogues

Diamine additive (1 mM)[c]	Analogue alkaloid yield (% of total alkaloid)[a]					
	N-ethylputrescine ($N = 2$)	*N*-(2-fluoroethyl)putrescine	*N*-*n*-propylputrescine	*N*-*iso*-propyl-putrescine	cadaverine	*N*-methylcadaverine ($N = 2$)
Alkaloid produced as an analogue of						
(A) Pyrrolidine alkaloids						
Hygrine	0.4 ± 0.1	1.1	1.3	0.3	13.6	2.2 ± 0.9
Oxohygrine	6.1 ± 2.2	2.0	1.7	0.5	2.1	11.5 ± 0.7
Cuscohygrine (*N*-alkyl-*N*′-methyl)	4.8 ± 0.4	3.5	12.2	0.8	0.0	2.2 ± 0.1
Cuscohygrine (*N*,*N*′-dialkyl)	7.7 ± 5.3	2.2	1.4	0.0	0.0	0.3 ± 0.1
(B) Tropane alkaloids						
Tropine	10.3 ± 1.3	0.6	0.0	0.0	0.0	4.6 ± 0.5
Pseudotropine	0.05 ± 0.05	0.3	0.8	0.0	0.0	0.3 ± 0.1
Tropinone	0.45 ± 0.15	0.0	< 0.1	0.0	9.1	3.6 ± 0.7
3-Acetyltropine	0.35 ± 0.25	0.9	0.0	0.0	0.0	0.4 ± 0.0
3-Acetylpseudotropine	0.0	0.0	0.0	0.0	0.0	0.0
3-Tigloyltropine	0.3 ± 0.0	0.9	0.2	< 0.1	0.0	0.15 ± 0.05
3-Tigloylpseudotropine	0.15 ± 0.05	0.0	0.0	0.0	0.0	0.0
Littorine	7.3 ± 0.5	7.2	0.5	5.9	0.0	0.0
Hyoscyamine	1.45 ± 0.45	1.8	0.0	0.0	0.0	0.0
Scopolamine	1.0 ± 0.1	0.0	0.0	0.0	0.0	0.0
Analogue as % total alkaloid	40.2 ± 2.6	20.4	18.5	7.5	24.8	25.7 ± 1.2
Total normal alkaloid (mg)[b]	36.1 ± 1.6	25.4	64.5	63.2	26.1	40.1 ± 4.4
Total novel alkaloid (mg)[b]	24.2 ± 1.6	6.5	14.6	5.1	7.5	14.1 ± 2.3

[a] The values represent the average of N separate experiments, each representing the mean of between 5 and 10 flasks, pooled and extracted together. Values given are the average $\pm$ range.
[b] Some minor alkaloids are not included.
[c] No identifiable products were found with the following compounds: *N*-cyclohexylputrescine; *N*-phenylethylputrescine; *N*-ethylcadaverine; *N*-*n*-propylcadaverine; *N*-*iso*-propylcadaverine; *N*-butylcadaverine; *N*-benzylcadaverine.

Table 5
Effect of *N*-alkylated putrescine or cadaverine analogues on the production of normal alkaloids by transformed root cultures of *Brugmansia*

	Alkaloid yield (% of total normal alkaloid) diamine additive (1 mM)[a]											
	N-ethylputrescine ($N = 2$)		*N*-(2-fluoroethyl)putrescine		*N*-*n*-propylputrescine		*N*-*iso*-propylputrescine		cadaverine		*N*-methylcadaverine ($N = 2$)	
	control	etPut-fed	control	FetPut-fed	control	prPut-fed	control	iprPut-fed	control	Cad-fed	control	meCad-fed
Alkaloid produced												
(A) Pyrrolidine alkaloids												
Hygrine	1.0 ± 0.7	0.6 ± 0.2	0.4	2.6	0.1	2.4	0.0	1.2	0.0	1.0	1.6 ± 0.7	3.2 ± 1.5
Oxohygrine	10.9 ± 5.2	12.0 ± 3.1	2.2	25.4	9.5	14.6	0.0	7.2	< 0.1	0.3	15.2 ± 5.6	13.4 ± 0.3
Cuscohygrine	8.2 ± 3.3	3.3 ± 0.3	2.3	0.0	9.2	4.3	3.3	2.8	5.0	2.8	5.4 ± 2.1	4.2 ± 1.4
(B) Tropane alkaloids												
Tropine	20.6 ± 5.0	28.9 ± 9.5	0.0	0.0	1.6	1.9	0.0	0.2	0.1	0.0	20.6 ± 6.5	22.2 ± 7.4
Tropinone	0.7 ± 0.4	0.7 ± 0.3	0.5	0.5	0.5	0.8	0.2	0.4	0.2	0.5	0.8 ± 0.0	0.0 ± 0.0
3-Acetyltropine	7.6 ± 3.0	6.4 ± 1.9	11.6	8.4	6.6	0.8	9.8	22.4	4.8	9.0	8.7 ± 2.0	6.7 ± 1.8
3-Tigloyltropine	3.3 ± 0.3	3.9 ± 0.7	4.7	1.7	1.5	1.6	5.1	2.6	3.6	0.6	3.1 ± 0.0	1.6 ± 0.2
Littorine	6.4 ± 2.3	7.4 ± 4.1	22.7	13.7	18.5	17.5	19.4	15.2	22.0	9.5	7.2 ± 3.3	6.9 ± 2.5
Hyoscyamine	10.8 ± 1.2	10.5 ± 5.2	21.7	13.3	15.5	17.6	20.9	18.2	23.2	14.2	10.2 ± 0.3	8.5 ± 0.6
Scopolamine	13.6 ± 2.9	16.7 ± 0.8	16.2	19.9	23.6	21.9	22.1	16.0	21.2	22.9	14.3 ± 1.9	18.2 ± 1.7
Total normal alkaloid (mg)[b]	25.7 ± 10.3	36.1 ± 1.6	27.4	25.4	51.4	64.5	32.0	63.2	50.3	26.1	24.0 ± 1.2	40.1 ± 4.4
Total novel alkaloid (mg)[b]	–	24.2 ± 1.6	–	6.5	–	14.6	–	5.1	–	7.5	–	14.1 ± 2.3

[a] The values represent the average of N separate experiments, each representing the mean of between 5 and 10 flasks, pooled and extracted together. Values given are the average $\pm$ range.
[b] Some minor alkaloids are not included.

Table 6
Production of alkaloid analogues by transformed root cultures of *Brugmansia* fed with *N*-alkylated nortropinones

Tropinone additive (1 mM)[a]	Analogue alkaloid yield (% of total alkaloid)[a]			
	N-ethylnortropinone ($N = 2$)	*N*-(2-fluoroethyl)-nortropinone	*N-iso*-propyl-nortropinone	*N*-ethylnor-pseudopelletierine
Analogue of				
Tropine	20.4 ± 10.0	21.6	19.8	10.4
Tropinone (substrate)	8.0 ± 7.4	0.0	0.7	15.4
Pseudotropine	0.55 ± 0.55	4.1	0.0	0.9
3-Acetyltropine	11.8 ± 3.2	6.2	0.9	2.9
3-Acetylpseudotropine	0.0 ± 0.0	0.0	0.0	0.0
3-Tigloyltropine	0.45 ± 0.05	0.6	0.2	0.0
3-Tigloylpseudotropine	0.35 ± 0.15	0.0	0.1	0.0
Phenylacetyltropine	0.15 ± 0.15	0.0	0.0	0.0
Littorine	13.3 ± 6.8	6.2	9.7	0.0
Hyoscyamine	1.9 ± 1.4	0.0	0.0	0.0
Scopolamine	4.9 ± 4.0	0.0	0.0	0.0
Analogue as % total alkaloid	62.0 ± 1.8	48.5	32.6	48.2
Total normal alkaloid (mg)[b]	4.8 ± 1.0	15.5	12.7	11.6
Total novel alkaloid (mg)[bc]	7.2 ± 1.6	14.6	6.1	2.6

[a] The values represent the average of N separate experiments, each representing the mean of between 5 and 10 flasks, pooled and extracted together. Values given are the average $\pm$ range.
[b] Some minor alkaloids are not included.
[c] Corrected for the presence of unmetabolised substrate.

equivalent *N*-alkyldiamine (Table 4) but, in the same manner, showed a diminution with the mass of the *N*-alkyl substituent. Metabolism of the *N*-ethyl analogue **10** was particularly strong, the *N*-ethyl products exceeding 60% of the total alkaloid. While the *N*-(2-fluoroethyl)- **11** and *N-iso*-propyl- **12** nortropinone analogues were very efficiently reduced — leaving negligible quantities of the substrate in the extract — the products of reduction were less effectively esterified. Again, the reduction strongly favoured the formation of the *endo*/α-tropan-3-ol. The majority of the esterified alkaloid accumulated with all three substrates was the phenyllactoyl ester and only with *N*-ethylnortropinone **10** was there any measurable rearrangement to the tropoyl ester (hyoscyamine plus scopolamine analogues).

With the higher homologues of pseudopelletierine [3.3.1], reduction and acetylation of the *N*-ethyl analogue **15** was observed but no aromatic products could be detected. No metabolism of *N*-(2-fluoroethyl)norpseudopelletierine or *N-iso*-propylnorpseudopelletierine **16** occurred.

The presence of the *N*-alkylnortropinones qualitatively had no obvious effect on the accumulation of the normal alkaloids (Table 7). Quantitatively, it is difficult to assess accurately their impact, in part due to overlapping peaks in some of the GC spectra. Overall, there appears to be no significant diminution of normal alkaloid accumulation, with the possible exception of *N*-ethylnortropinone-treated cultures. This aspect of the analogue metabolism merits further attention.

3. Discussion

These studies clearly show that a considerable degree of plasticity exists in the substrate specificity of many of the enzymes in the alkaloid biosynthetic pathways of *N. rustica* and *B. candida* × *aurea*. In this, they extend considerably previous studies in which nonnatural precursors were fed (Boswell et al., 1993; Demole & Berthet, 1972; Leete, 1983; Leete, 1979; Leete & Chedekel, 1972; Leete et al., 1971; Rueppel & Rapoport, 1971; Walton & Belshaw, 1988; Walton et al., 1988). Nevertheless, it was found that only with a precursor analogue closely similar to the natural intermediate could the yield of analogue-derived alkaloids in the culture exceed that of the natural alkaloids. With more structurally-divergent analogues, only small amounts (if any) of novel alkaloids were produced. Effectively, therefore, the potential to produce novel alkaloids was limited to relatively minor structural variations of the normal alkaloid repertoire. With the *B. candida* × *aurea* cultures, it was found that the yield of analogues of hyoscyamine and scopolamine was greatest when pre-

Table 7
Effect of *N*-alkylated tropinone analogues on the production of normal alkaloids by transformed root cultures of *Brugmansia*

Tropinone additive (1 mM)	Alkaloid yield (% of total normal alkaloid)[a]							
	N-ethylnortropinone ($N = 2$)		*N*-(2-fluoroethyl)nortropinone		*N-iso*-propylnortropinone		*N*-ethylnorpseudopelletierine	
	control	etNT-fed	control	FetNT-fed	control	iprNT-fed	control	etNP-fed
Alkaloid produced								
Tropine	22.3 ± 1.7	18.0 ± 16.4	14.4	10.8	20.6	11.2	24.0	43.3
Tropinone	0.9 ± 0.5	0.3 ± 0.3	1.2	1.4	0.4	0.5	1.4	0.0
Pseudotropine	0.05 ± 0.05	0.2 ± 0.2	0.1	0.1	< 0.1	0.2	0.0	0.0
3-Acetyltropine	5.3 ± 0.6	5.9 ± 3.5	10.3	15.4	4.6	14.9	5.9	5.4
3-Tigloyltropine	3.2 ± 0.4	5.4 ± 3.0	3.2	2.7	3.6	5.7	2.7	1.9
Phenylacetyltropine	2.1 ± 1.2	0.1 ± 0.1	0.3	0.8	3.3	0.1	0.8	0.7
Littorine	12.7 ± 7.8	3.8 ± 1.8	12.9	9.7	20.5	15.8	4.9	4.1
Aposcopolamine	1.0 ± 0.5	0.6 ± 0.6	0.3	0.7	0.5	3.3	1.5	1.0
Hyoscyamine	15.5 ± 1.3	9.0 ± 7.2	10.8	12.6	14.3	14.0	16.8	16.9
Scopolamine	21.5 ± 5.1	13.1 ± 7.7	13.9	10.4	16.4	12.4	26.6	19.2
Total normal alkaloid (mg)[b]	7.8 ± 3.5	4.8 ± 1.0	12.0	15.5	22.5	12.7	4.3	11.6
Total novel alkaloid (mg)[bc]	–	7.2 ± 1.6	–	14.6	–	6.1	–	2.6

[a] The values represent the average of N separate experiments, each representing the mean of between 5 and 10 flasks, pooled and extracted together. Values given are the average $\pm$ range.
[b] Some minor alkaloids are not included.
[c] Corrected for the presence of unmetabolised substrate.

cursor analogues close to these tropane alkaloids were supplied; with more distant precursor analogues (*N*-alkyldiamines), there was substantial metabolism via a competing pathway leading to pyrrolidine alkaloids.

An efficient conversion of *N*-ethylputrescine by *N. rustica* transformed root cultures to the anticipated higher homologue of nicotine, *N′*-ethyl-(*S*)-nornicotine has already been reported (Boswell et al. 1993). This product accounted for about 40% of the total alkaloid present in the roots. The results presented here suggest that *N*-*n*-propylputrescine is metabolised similarly and to a comparable extent, although the small quantity of this and other precursors available for feeding did not permit characterization of the alkaloidal product by NMR or by CD. *N-iso*-propylnornicotine is known as a trace natural metabolite in Burley tobacco (Leete, 1983). Three *N*-alkylcadaverines (*N*-methyl-, *N*-ethyl- and *N*-*n*-propyl-) were examined for bioconversion; each gave rise to a novel alkaloid, but only in the case of *N*-methylcadaverine **7** did this account for a substantial (ca. 20%) proportion of total alkaloid content. This new alkaloid exhibited a mass spectrum consistent with *N′*-methylanabasine, a known natural product of *N. tabacum* where it occurs as the (*S*)-isomer (Demole & Berthet, 1972). It would be valuable to establish whether, in common with *N′*-methylanabasine isolated from *N. glauca* and *N. tabacum* fed *N*-methyl-Δ^1-piperideine, the biotransformation product is the (*S*)-isomer or whether, like naturally occuring anabasine **21** from *Nicotiana* species (Leete, 1983) and anabasine extracted from transformed root cultures of *N. rustica* and *N. tabacum* fed with cadaverine **6** (Watson, Brown, Colquhoun, Walton & Robins, 1990), it is a racemic mixture.

The inhibitory effects of cadaverine **6** on the formation of nicotine **23** in transformed root cultures of *Nicotiana* spp. have been discussed elsewhere (Walton & Belshaw, 1988; Walton & McLauchlan, 1990; Walton et al., 1988; Walton et al., 1994) and were similarly observed here with *N*-methylcadaverine **7**. Whether or not this is a direct or indirect effect needs to be tested with labelled substrates. If there were a direct effect, since the *N*-methylcadaverine was free of cadaverine, the stimulation of anabasine **21** formation by *N*-methylcadaverine **7** must result from the demethylation either of the compound itself or of *N*-methylpiperideine: *N*-methylanabasine has already been ruled out as an intermediate in anabasine formation by wick-feeding experiments with *N. glauca* plants (Leete & Chedekel, 1972). Feeding of *N*-*n*-propylputrescine, though not of the other compounds tested, caused an increase in the proportion of anatabine **22**. Although anatabine arises by the condensation of two molecules of nicotinic acid (Leete, 1983), increased anatabine formation both in response to cadaverine-feeding of transformed root cultures

(Walton & Belshaw, 1988; Walton et al., 1988) and to the expression of a bacterial lysine decarboxylase gene in *N. glauca* (Fecker et al., 1992) has been reported previously. While the effect of excess cadaverine could be due to an aberrant route to anatabine **22** from cadaverine (Mothes, Schütte, Simon, & Weygand, 1959), for example via anabasine or the condensation of two piperideine units, this effect could equally well be indirect. Certainly, it is hard to explain the influence of *N*-*n*-propylputrescine as other than indirect. Since these compounds are both substrates for MPO (Boswell et al., 1999; Walton and McLauchlan, 1990) the diminution could perhaps result from inhibition of the formation of *N*-methylpyrrolinium, with a consequent stimulation of the condensation of two molecules of decarboxylated nicotinic acid to give rise to anatabine. This interpretation finds support in the observed overall diminution of alkaloid yield in cultures fed *N*-*n*-propylputrescine (Table 2).

In the *B. candida* × *aurea* cultures, all four *N*-alkyldiamines tested and cadaverine **6** were metabolized beyond the *N*-methylpyrrolinium level to analogues of hygrine and cuscohygrine (Table 4). No such compounds arose in the cultures fed *N*-alkylnortropinones (**10**, **11**, **12**), confirming that the pyrrolidine alkaloids diverge from the tropanes before ring closure. Cadaverine **6** has previously been shown to be an effective substrate for MPO of *N. tabacum* (Walton & McLauchlan, 1990) and *Hyoscyamus niger* (Hashimoto, Mitani, & Yamada, 1990) and both cadaverine **6** and *N*-methylcadaverine **7** are good substrates for the MPO from *B. candida* × *aurea* cultures (Boswell et al., 1999). These data indirectly confirm the wide substrate range for this enzyme.

Conversion of the *N*-alkyldiamines into the tropane pathway metabolites, however, was limited. *N*-Ethylputrescine **3** was metabolized through the entire length of the tropane biosynthetic pathway, leading to an appreciable production of analogues of tropine esters including littorine and, to a lesser extent, hyoscyamine and scopolamine. *N*-(2-Fluoroethyl)putrescine **3a** and *N*-*iso*-propylputrescine **4a** were also metabolized as far as the littorine analogue, but only the former was rearranged to an hyoscyamine analogue and neither gave any scopolamine analogue. A similar pattern occurred with the *N*-alkylnortropinones with the notable difference that much higher levels of the reduction products — analogues of tropine and pseudotropine — accumulated. Almost no tropane alkaloids arose from *N*-*n*-propylputrescine **4**, although this was an effective substrate for analogues of the pyrrolidine alkaloid, cuscohygrine. Thus, it appears that the linear C_3 unit may interfere with acetoacetylation or ring closure to form the tropane skeleton. In contrast, both cadaverine **6** and *N*-methylcadaverine **7** were metabolized to norpseudopelletierine **13** and pseudopelletierine **14** respectively, showing that the enzyme(s) responsible for the ring-closure of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate **24** can apparently catalyse this reaction with either a 5- or 6-membered ring.

In view of the established requirement for an *N*-alkyl substituent on the tropane ring for tropinone reductase (TR) I and TRII activity (Portsteffen, Dräger, & Nahrstedt, 1994), it is not surprising that reduction only occurs with the *N*-alkylated substrates. The reduction products, in particular the analogues of tropine (3α-ol), accumulated much more effectively from the *N*-alkylnortropinone feeds than from *N*-alkyldiamines. Notably, also, the more proximal substrates gave a wider range of esterified tropines. Although with all of *N*-ethyl- **10**, *N*-(2-fluoroethyl)- **11** and *N*-*iso*-propyl- **12** nortropinones only one peak is found in the GC/MS chromatogram with the appropriate mass for the acetyl ester, *N*-ethyl- and *N*-*iso*-propyl- nortropinones show two peaks for the tigloyl ester, indicating products of both the *endo*/3α and *exo*/3β series. By analogy with the natural (*N*-methyl-) products, the earlier peak is assigned to the 3α-configuration. Similarly, in view of the 3α:3β ratio normally found in the root cultures (Boswell et al., 1999; Dräger et al. 1992; Robins et al., 1990) (Tables 5 and 7), the single acetyl ester is assigned to the 3α-configuration. It should be noted, however that this interpretation is in contrast to the in vitro enzyme activities (Boswell et al., 1999). Similarly, the accumulation of the (3α-ol) reduction product of *N*-ethylnorpseudopelletierine **15** in vivo is surprising in view of the apparent absence in vitro of any activity of TRI with this substrate (Boswell et al., 1999).

Phenyllactoyl ester formation (littorine analogue) occurred fairly readily with the *N*-alkylnortropanes but the complete absence of a littorine analogue from *N*-methylcadaverine **7** or *N*-ethylnorpseudopelletierine **15** suggests that the phenyllactoylation reaction is restricted to the [3.2.1]tropane ring-containing structures, the [3.3.1]system failing to act as a substrate. Thus, it appears that for phenyllactoylation the structural requirements for the *N*-alkyl group are less stringent than for the ring structure. Similarly, rearrangement to hyoscyamine occurred with *N*-ethyl- and *N*-(2-fluoroethyl)- norlittorines (Tables 4 and 6), but not with the *N*-*n*-propyl- or *N*-*iso*-propyl- norlittorines. Only with *N*-ethylputrescine **3** was a *N*-ethylnorscopolamine analogue detected. This is in accord with the report that *N*-acetylnoratropine but not *N*-butanoylnoratropine are accepted as substrate by purified hyoscyamine 6β-hydroxylase (Hashimoto & Yamada, 1987). The present findings imply that this enzyme may similarly accept a *N*-

ethyl substituent, though not a *N*-(2-fluoroethyl) or *N*-propyl group.

In conclusion, it would appear that the most stringent structural specificity in tropane alkaloid formation is shown by the enzymes catalysing the phenyllactoylation of tropine to littorine **17** and the subsequent rearrangement of littorine **17** to hyoscyamine **18**. These restrictions inevitably limit the biotransformation capacity of the cultures and their ability to accumulate large amounts of novel alkaloids. However, limited alterations to structure, such as the replacement of the *N*-methyl of tropinone **9** with an *N*-ethyl can be effectively exploited.

4. Experimental

4.1. Transformed root cultures

Root cultures of *Nicotiana rustica* and of the *Brugmansia candida* × *aurea* hybrid, transformed with *Agrobacterium rhizogenes*, were grown as described elsewhere (Hamill et al., 1986; Robins et al., 1990). In general, 0.2 g fresh mass of roots was subcultured into 50 ml of B50 medium and grown in the absence of antibiotics.

4.2. Synthetic substrates

Analogues of putrescine and cadaverine were prepared following published procedures (Alonso Garrido, Buldain & Frydman, 1984). *N*-Alkyl analogues of tropinone were prepared by a modified Robinson synthesis (Robinson, 1917), involving the acid-catalysed condensation (pH 4.8; 25°C; 48 h) of butan-1,4-dialdehyde, 3-ketoglutaric acid and the appropriate alkylamine. The unstable tropan-2,4-dicarboxylate product was decarboxylated by treatment with HCl (6 M; 70°C; 1 h) and the product worked up by solvent extraction and crystallisation as either the HCl or the oxalate salt. Higher homologues were similarly prepared but with pentan-1,5-dialdehyde. Purity was assessed by GC/MS (Dräger et al., 1992) and by NMR spectroscopy. Standards for the identification of products by GC and GC/MS were prepared by the chemical reduction with sodium borohydride of *N*-alkylnortropinones and *N*-alkylnorpseudopelletierines to a mixture of the 3α- and 3β-isomers. This mixture was in turn used to synthesize the phenyllactoyl-esters by heating (140°C; 48 h) a fused mix of the *N*-alkylnortropine with (*R*)-phenyllactic acid under an HCl atmosphere.

4.3. Alkaloid extraction

Crude alkaloid extracts were prepared as described previously (Dräger et al., 1992).

4.4. GC and GC/MS

Alkaloids were analysed by GC and GC/MS as described previously (Dräger et al., 1992).

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