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Specificities of the enzymes of *N*-alkyltropane biosynthesis in *Brugmansia* and *Datura*☆

Henry D. Boswell[a], Birgit Dräger[b,1], W. Russell McLauchlan[c], Andreas Portsteffen[b], David J. Robins[a], Richard J. Robins[d,e,*], Nicholas J. Walton[c]

[a]*Chemistry Department, The University, Glasgow G12 8QQ, UK*
[b]*Institut für Pharmazeutische Biologie und Phytochimie, Westfälische Wilhelms-Universität, Hittorfstrasse 56, D-48149 Münster, Germany*
[c]*Institut für Pharmazeutische Biologie der Martin-Luther-Universität Halle-Wittenberg, Hoher Weg 8, D-06120, Halle, Germany*
[d]*Institute of Food Research, Norwich Laboratory, Norwich Research Park, Colney, Norwich NR4 7UA, UK*
[e]*Laboratoire d'Analyse Isotopique et Electrochimique de Métabolismes, CNRS ESA 6006, 2 rue de la Houssinière, F-44322 Nantes cedex 03, France*

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Abstract

The enzymes *N*-methylputrescine oxidase (MPO), the tropine-forming tropinone reductase (TRI), the pseudotropine-forming tropinone reductase (TRII), the tropine:acyl-CoA transferase (TAT) and the pseudotropine:acyl-CoA transferase (PAT) extracted from transformed root cultures of *Datura stramonium* and a *Brugmansia candida* × *aurea* hybrid were tested for their ability to accept a range of alternative substrates. MPO activity was tested with *N*-alkylputrescines and *N*-alkylcadaverines as substrates. TRI and TRII reduction was tested against a series of *N*-alkylnortropinones, *N*-alkylnorpelletierines and structurally related ketones as substrates. TAT and PAT esterification tests used a series of *N*-substituted tropines, pseudotropines, pelletierinols and pseudopelletierinols as substrates to assess the formation of their respective acetyl and tigloyl esters. The results generally show that these enzymes will accept alien substrates to varying degrees. Such studies may shed some light on the overall topology of the active sites of the enzymes concerned.

Keywords: Alkaloid; *Brugmansia candida* × *aurea* hybrid; Solanaceae; *Datura stramonium*; Root; Pseudotropine; Pseudopelletierine; Tropinone; Tropine

1. Introduction

One of the most interesting aspects of alkaloid biosynthesis concerns the degree to which the range of observed products is determined by the substrate specificity of the individual enzymes of the alkaloid pathway (Fig. 1). If a pathway receives an input of a metabolite analogue, to what extent can a range of novel alkaloids then be produced and are such compounds predictable variants of the normal alkaloids? In principle, stringent substrate specificity could be a property of each enzyme in the pathway; alternatively, the same spectrum of products could result from a similar degree of overall stringency but with individual enzymes possessing considerably broader substrate specificities. Under these conditions, the final yield of products reflects the resultant of the specificities of all the sequence of enzymes involved. In the case of stringent specificity, the formation of novel alkaloids as end products will be very limited; in the second case, considerable potential for biotransformation exists.

A good example of this flexibility in tropane alkaloid formation is the specificity of the enzymes esterifying tropine and pseudotropine with aliphatic acids from the appropriate acyl-CoA (Rabot, Peerless & Robins, 1995; Robins, Bachmann, Peerless & Rabot,

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* Corresponding author. Fax: +33-2-51-21-57-12.
E-mail address: richard.robins@chimbio.univ-nantes.fr (R.J. Robins)
[1] Previous address: Institut für Pharmazeutische Biologie und Phytochimie, Westfälische Wilhelms-Universität, Hittorfstrasse 56, D-48149, Münster, Germany.

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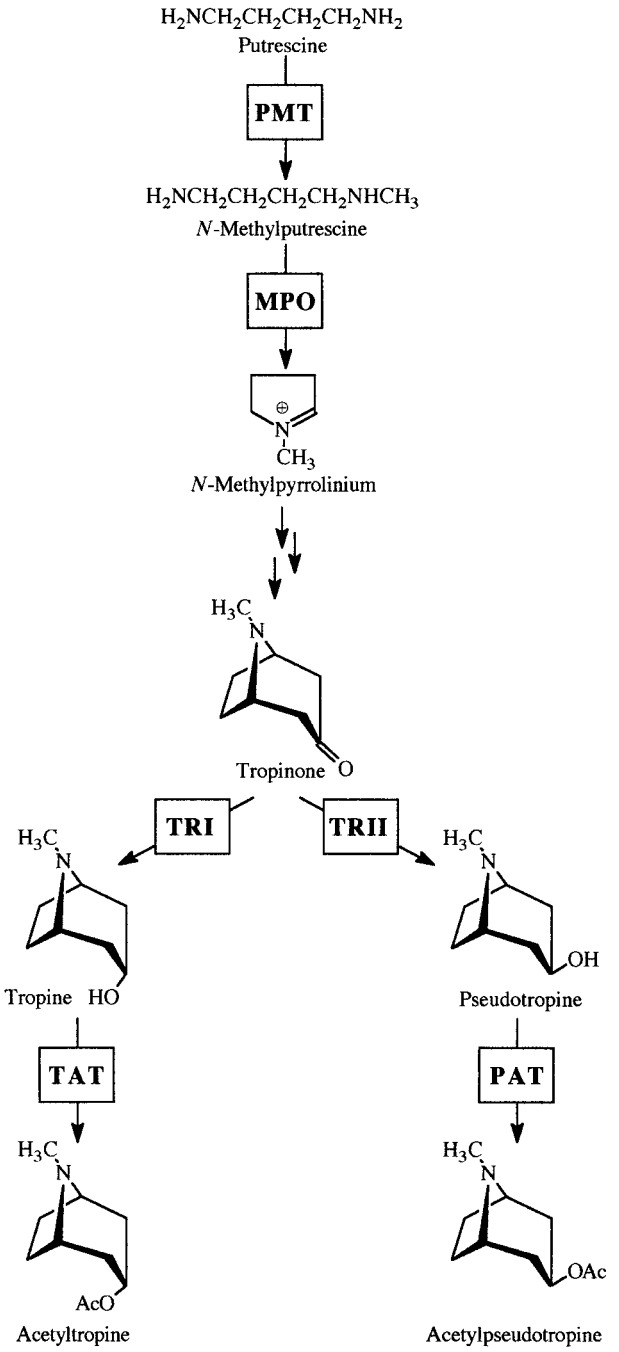


Fig. 1. Schematic pathway showing the intermediates for which analogues have been fed, the enzymes involved in their metabolism and the products of the reactions. Abbreviations: PMT = Putrescine:SAM *N*-methyltransferase; MPO = *N*-methylputrescine oxidase; TRI = tropine-forming tropinone reductase; TRII = pseudotropine-forming tropinone reductase; TAT = tropine:acyl-CoA acyl transferase; PAT = pseudotropine:acyl-CoA acyl transferase. The multiple arrows indicate undefined steps.

1994; Robins, Bachmann, Robinson, Yamada & Rhodes, 1991). The specificity of the enzyme for the alcoholic acceptor is very strong but the transferases esterify either tropine (TAT) or pseudotropine (PAT) respectively with a wide range of aliphatic esters. The profile of products obtained in vivo apparently reflects the availability of the acyl-CoA substrates more than the enzyme specificity.

Very simple examples of the exploitation of this flexibility to form nonnatural analogues of alkaloids are the incorporation of fluoronicotinic acids and substituted *N*-methylpyrrolidines into tobacco alkaloids (Leete, 1983). In a previous paper (Boswell et al., 1999), we have presented evidence for the metabolism of a range of *N*-alkyldiamines and *N*-alkylnortropanes fed to transformed root cultures of a *Brugmansia candida* × *aurea* hybrid. Some analogues of *N*-methylputrescine and tropinone altered either in the *N*-alkyl substituent or in the ring structure (pseudopelletierines) were found to be effectively metabolized in vivo by transformed root cultures of a *Brugmansia* (ex *Datura*) *candida* × *aurea* hybrid to a variety of products, identified by GC/MS. For example, *N*-ethylputrescine was efficiently incorporated into a wide range of both aliphatic and aromatic analogues of the natural tropane alkaloids found in this culture. Other analogues were poorly metabolized, or not metabolized at all. Novel pyrrolidine alkaloid analogues were also produced from the *N*-alkyldiamines, indicating extensive conversion of the substrates presented. The extent of metabolism to both classes of alkaloid was found to depend broadly on the size of the *N*-alkyl substituent. The higher homologues, *N*-alkylcadaverine and *N*-alkylnorpseudopelletierine, also showed limited conversion, notably to acetyl and tigloyl esters.

From these data it can be deduced that some enzymes of alkaloid formation in *B. candida* × *aurea* show relatively lax substrate specificity whilst others are much more specific for their substrates. In this paper we explore the relationships between the formation of novel alkaloids from analogues of biosynthetic intermediates and the substrate specificities of known enzymes of the tropane–alkaloid biosynthetic pathway. The availability of enzymes to undertake such a comparison is necessarily limited, given that the central (between *N*-methylpyrrolinium and tropinone) and final (between tropine and hyoscyamine) parts of the biosynthetic sequence remain enzymically entirely uncharacterised; therefore three categories of enzymes have been studied:

- the *N*-methylputrescine oxidase (MPO), responsible for the initial steps in the conversion of *N*-alkyldiamines into *N*-alkylnortropinones and *N*-alkylpyrrolidine alkaloids
- the tropine-forming tropinone reductase (TRI) and the pseudotropine-forming tropinone reductase (TRII)
- the tropine:- and pseudotropine: acyl-CoA transferases (TAT and PAT respectively).

Table 1
The activity of *N*-methylputrescine oxidase (MPO) from the *B. candida* × *aurea* hybrid with various putrescine analogues

Compound[a]	MPO % Activity[b]
Putrescine	56
N-Methylputrescine	100[c]
N-Ethylputrescine	100
N-(2-Fluoroethyl)putrescine	103
N-*n*-Propylputrescine	50
Cadaverine	15
N-Methylcadaverine	16
N-Ethylcadaverine	17

[a] All substrates at 1 mM.
[b] Extract was prepared essentially as in McLauchlan et al., 1993, concentrated by precipitation with 70% ammonium sulphate (4°C), resuspended in extraction buffer and desalted on Sephadex PD-10 (Pharmacia).
[c] Activity with *N*-methylputrescine as substrate was 5 pkat/mg protein (Robins et al., 1990).

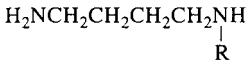
2. Results and discussion

2.1. The activity of N-methylputrescine oxidase with putrescine analogues

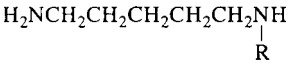
The properties of MPO purified from *Nicotiana tabacum* (McLauchlan, McKee & Evans, 1993; Walton & McLauchlan, 1990) or *Hyoscyamus niger* (Hashimoto, Mitani & Yamada, 1990) are very similar. The highest measured affinity of this enzyme was for *N*-methylputrescine. A number of related diamines were also oxidised if presented at a sufficiently high concentration, indicating a relatively high degree of flexibility in the catalytic capacity of this enzyme. When *N*-alkyl analogues of putrescine or cadaverine were fed to *Brugmansia* root cultures, alkaloidal products derived from these were formed (Boswell et al., 1999), indicating that the MPO of this species can also accept a range of substrates.

In agreement with this observation, MPO from *Brugmansia* is found to catalyse the deaminative oxidation of a number of *N*-alkyldiamines in vitro (Table 1). As previously reported for MPO (Hashimoto et al., 1990; Leete, 1983; McLauchlan et al., 1993; Walton & McLauchlan, 1990), the highest activity is found with *N*-methylputrescine, putrescine and cadaverine both being oxidised at a slower rate. The concentration used (1 mM) was, however, unlikely to be saturating, considering the very high K_m values reported previously (Leete, 1983; McLauchlan et al., 1993). Replacing the *N*-methyl- with *N*-(2-fluoroethyl)- or *N*-ethyl- did not affect the efficiency of oxidation of the substrate. Thus, a range of putrescine or cadaverine analogues could potentially act as precursors of novel alkaloids and the in vitro properties of MPO reported here are consistent with the observed metabolism of these compounds to a range of tropane and pyrrolidine alkaloid analogues (Boswell et al., 1999).

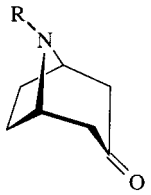
Not all the substrates for MPO are efficiently metabolized as far as the tropane skeleton, however, notably *n*-propylputrescine, indicating that the acetoacetylation of *N*-alkylpyrrolinium ion and subsequent ring closure are sensitive to the size of the *N*-alkyl substituent. The formation of norpseudopelletierine and pseudopelletierine indicates, however, that the enzymes responsible for these reactions can handle the higher homologues produced by the action of MPO. Furthermore, limited further metabolism of the pseudopelletierine analogues occurs (Table 3 of Boswell et al., 1999).



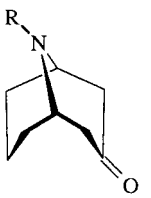
Putrescine	R=H
N-methylputrescine	R=CH_3
N-ethylputrescine	R=CH_2CH_3
N-(2-fluoroethyl)putrescine	R=CH_2CH_2F
N-*n*-propylputrescine	R=$CH_2CH_2CH_3$



Cadaverine	R=H
N-methylcadaverine	R=CH_3
N-ethylcadaverine	R=CH_2CH_3



Nortropinone	R=H
Tropinone	R=CH_3
N-ethylnortropinone	R=CH_2CH_3
N-(2-fluoroethyl)nortropinone	R=CH_2CH_2F
N-*iso*-propylnortropinone	R=$CH(CH_3)_2$



Norpseudopelletierine	R=H
Pseudopelletierine	R=CH_3
N-ethylnorpseudopelletierine	R=CH_2CH_3
N-(2-fluoroethyl)norpseudopelletierine	R=CH_2CH_2F
N-*iso*-propylnorpseudopelletierine	R=$CH(CH_3)_2$

Table 2
The substrate specificities of tropinone reductase I (TRI) and tropinone reductase II (TRII) from the *B. candida* × *aurea* hybrid and *Datura stramonium*[f]

Compound	Conc (mM)[a]	TR I[b]			TR II[b]		
		Datura		*Brugmansia*	*Datura*		*Brugmansia*
		K_m (mM)	(% Activity)	(% Activity)	K_m (mM)	(% Activity)	(% Activity)
Tropinone	2.5/5.0	1.3	100/100[c]	100/100[d,e]	0.11	100/100[c]	100/100[d,e]
Nortropinone	2.5	n.d.	3	n.d.	n.d.	23[e]	n.d.
7-Hydroxytropinone	5.0	5.5	15	17	n.d.	6	0
N-Methylpiperid-4-one	5.0	c. 20	25	26	1.4	178	156
N-*n*-Propylpiperid-4-one	5.0	n.d.	0	0	n.d.	73	133
3-Quinuclidinone	5.0	2.2	76	108	–	0	46
3-Methylcyclohexanone	5.0	n.d.	38	63	n.d.	33	54
4-Methylcyclohexanone	5.0	0.03	36	69	2.8	22	77
4-Ethylcyclohexanone	5.0	0.05	41	106	n.d.	45	122
N-Ethylnortropinone	2.5/5.0	2.5	65/65[e]	n.d./47	0.25	45/45[e]	n.d./60
N-(2-Fluoroethyl)nortropinone	2.5/5.0	3.4	79[e]/n.d	n.d/45	0.67	47[e]/n.d	n.d/56
N-*iso*-Propylnortropinone	2.5/5.0	c. 20	19[e]/55	n.d/30	c. 20	19[e]/n.d.	n.d/22
Norpseudopelletierine	2.5	n.d.	0	n.d.	n.d.	8	n.d.
Pseudopelletierine	2.5	n.d.	0	n.d.	n.d.	7	n.d.
N-Ethyl-norpseudopelletierine	5.0	n.d.	0	n.d.	n.d.	5	n.d.

[a] Substrates were used at 2.5 or 5 mM. For certain substrates, activity measurements were made at both concentrations and values before and after the oblique represent the 2.5 and 5.0 mM values respectively.
[b] Assays were conducted using fractions containing fully separated TR I and TR II enzymes.
[c] Activity with tropinone: TR I = 43.0 nkat/mg, TR II = 136 nkat/mg. All assays were performed at pH 6.4 (0.1 M K phosphate) with 0.2 mM NADPH. Some data from Portsteffen, 1994 and Portsteffen et al., 1994.
[d] Activity with tropinone: TR I = 1.63 nkat/mg protein, TR II = 0.13 nkat/mg protein. All assays were performed at pH 6.4 (0.1 M K phosphate) with 0.2 mM NADPH.
[e] Products were identified by GC/MS against synthetic standards.
[f] n.d. = not determined.

2.2. The activity of tropinone reductases with tropinone analogues

It has previously been reported (Boswell et al., 1999) that a number of *N*-alkylnortropinones, when fed to the *B. candida* × *aurea* hybrid root cultures, were completely reduced in vivo to the corresponding tropan-3-ols. Consistent with this, only trace amounts of the *N*-alkylnortropinones accumulated in roots fed *N*-alkylputrescines. In all cases, much more tropine (*endo*/3α) analogue was formed than pseudotropine (*exo*/3β) analogue (see Tables 4 and 6 of Boswell et al., 1999). Indeed, with *N*-*iso*-propylnortropinone and *N*-*iso*-propylputrescine, no pseudotropine analogue was detected. Thus, the profile of reduction in vivo of these fed analogues closely mimics the reduction profile found for endogenous tropinone, with the tropine series predominating.

Two tropinone reductase activities (TRI and TRII) stereospecifically reduce tropinone to tropine (tropan-3α-ol) or pseudotropine (tropan-3β-ol) respectively. These enzymes have been purified from *D. stramonium* and characterised (Portsteffen, 1994; Portsteffen, Dräger & Nahrstedt, 1992, 1994). They have been shown to catalyse the reduction in vitro of a range of substrates, indicating that, in vivo, they should metabolise analogues of tropinone. TRI and TRII were extracted from *D. stramonium* and *B. candida* × *aurea* root cultures and completely separated following the protocol described in Portsteffen et al., 1994. As can be seen from the data in Table 2, the substrate specificities of TRI and TRII from these two species are found to be very similar (Table 2 and Nakajima, Hashimoto & Yamada, 1993; Portsteffen et al., 1992, 1994). The values of K_m for the reduction of tropinone from *B. candida* × *aurea* — 1.56 mM for TRI and 0.16 mM for TRII — correspond well to the values from *D. stramonium* (Table 2). Kinetic data for the reduction of *N*-alkylnortropinones obtained from *D. stramonium* show a consistent pattern of a lower K_m for TRII than for TRI at pH 6.4. It should be borne in mind, however, that these values are likely to be highly sensitive to the pH, as shown for tropinone (Portsteffen et al., 1994), making it hard to relate these in vitro values to the effective relative affinities in vivo.

As found for *D. stramonium* (Portsteffen, 1994; Portsteffen et al., 1994) and *Hyoscyamus niger*

(Hashimoto, Nakajima, Ongena & Yamada, 1992), the TRI and TRII enzymes extracted from *B. candida* × *aurea* show a considerable flexibility of activity, reducing a range of model compounds lacking the tropane skeleton (Table 2). Similarly, *N*-ethylnortropinone, *N*-(2-fluoroethyl)nortropinone and *N*-*iso*-propylnortropinone were all found to be good substrates for both reductases in vitro (Table 2).This is consistent with the metabolism of these analogues in vivo. While at first sight the observed in vitro activity with TRII is not consistent with the virtual absence of *N*-alkylnortropan-3β;-ol products in the fed cultures, it must be borne in mind that in all cultures from the tribe Datureae (containing *Brugmansia* and *Datura*) which have been analysed, the activity of TRI greatly exceeds that of TRII. In the present experiments, TRI exceeds TRII in *D. stramonium* by ca. 25-fold (Portsteffen et al., 1992) and in *B. candida* × *aurea* by ca. 8-fold. Therefore a preponderance of the products with the 3α configuration is likely to be favoured, as is observed to be the case in vivo. Thus, the overall properties of the enzymes — substrate specificity combined with specific activity — are broadly consistent with the observed in vivo accumulation of products.

The norpseudopelletierines, in contrast, are found to be poor substrates for both TRI and TRII (Table 2). This observation is consistent with the lack of metabolism of cadaverine beyond norpseudopelletierine in vivo. It is not, however, consistent with the effective metabolism of pseudopelletierine and *N*-ethylnorpseudopelletierine to a mixture of 3α-ol and 3β-ol products which was observed in vivo (see Tables 4 and 6 of Boswell et al., 1999). This apparent contradiction between in vivo and in vitro findings might be explained either by the possibility that the time-integrated activities, though low, are nevertheless sufficient to account for the conversions in vivo or, alternatively, by the possibility that the enzymes of the *B. candida* × *aurea* hybrid (in which the in vivo work was carried out) show a somewhat broader substrate specificity than those of *D. stramonium*.

None of the *N*-alkylnortropinones showed significant inhibitory activity with TR I or TR II when incubated at 1 mM in the presence of 5 mM tropinone (Portsteffen, 1994). As the K_m values (Table 2) are at least double those for the endogenous substrate, this is not unexpected. In the in vivo experiments (Boswell et al., 1999), however, substrates were fed at 1 mM, a concentration likely to have given an intracellular concentration well in excess of that of tropinone, which is usually present at only low levels (probably due to high levels of TRI). The lack of inhibition of TRI by analogues is consistent with the observed lack of inhibition of the accumulation of normal alkaloids in cultures fed with these analogues (Tables 5 and 7 of Boswell et al., 1999).

2.3. The esterification of the products of the reductase activities by tropine:- and pseudotropine: acyl-CoA transferases

The *N*-alkyltropan-3-ols produced endogenously by the reduction of *N*-alkylnortropinones undergo considerable further metabolism (Boswell et al., 1999). While the major products are the aromatic phenyllactoyl and tropoyl esters, significant amounts of the aliphatic acetyl and tigloyl esters accumulate. Thus, as with reduction, esterification mimics the metabolism of the natural compounds. This metabolism has also been examined in vitro at the enzymatic level.

Two separate acyl transferase activities have been identified in *D. stramonium* (Rabot et al., 1995; Robins et al., 1991, 1994) that transfer an acyl unit from an acyl-CoA thioester to tropine (TAT) or pseudotropine (PAT) respectively. As with TRI and TRII, these enzymes show absolute specificity in vitro for the *endo*/3α and *exo*/3β configurations respectively but considerable flexibility in the structure of the substrate (Rabot et al., 1995; Robins et al., 1991). *N*-Substituted tropan-3-ols and norpseudopelletierines, the products of TRI and TRII on the respective ketones, were tested in vitro as substrates for the *B. candida* × *aurea* activities (Table 3). Substrate solutions, containing a mixture of both the 3α- and 3β-ols were incubated with a partially purified extract of *B. candida* × *aurea* roots containing both TAT and PAT activities. Products were separated and quantified by GC and their identities determined by GC/MS. TAT showed a very constrained substrate specificity range, only *N*-ethyltropan-3α-ol (besides tropine itself) proving to be a good substrate. In contrast, all three *N*-substituted tropan-3-ols proved to be good substrates for PAT, 3β-tigloylated and 3β-acetylated products being found for each substrate.

The observed properties of these acyl transferases in vitro with *N*-alkylnortropinones are broadly consistent with the accumulation of acetyl- and tigloyl-esters observed following in vivo feedings. With tropine and pseudotropine, tigloyl-CoA proved the better substrate in vitro, as found previously for the *D. stramonium* enzymes (Rabot et al., 1995; Robins et al., 1991, 1994), although a constant ratio was not observed. Nevertheless, in vivo, the spectrum of alkaloids (both natural and analogues) is dominated by the acetyl products (Boswell et al., 1999) and with all three *N*-substituted tropan-3-ols, only a single acetyl ester product was found. On the basis of the PAT and TAT enzymatic data alone, this might be expected to be 3β-isomer. In vivo, however, the tropine:pseudotropine ratio is very high and the normal ratio of acetyltropine:acetylpseudotropine is large. Thus, by analogy, the acetyl ester products are much more likely to be the 3α-isomers (see Boswell et al., 1999). Tigloylated products, in contrast, were found with both 3α- and 3β-configur-

Table 3
The activities of tropine:acyl transferase and pseudotropine:acyl transferase from the *B. candida* × *aurea* hybrid with tropine and pseudotropine *N*-acyl analogues

Compound[a]	TAT[b] (% Activity) Acetyl-CoA	Tigloyl-CoA	PAT[b] (% Activity) Acetyl-CoA	Tigloyl-CoA
Tropine	100[c,e]	100[c,e]	0	0
Pseudotropine	0	0	100[d,e]	100[d,e]
Nortropine	0	0	0	0
N-Ethylnortropan-3-ol (α:β = 0.6:1)	16[e]	76[e]	35[e]	81[e]
N-(2-Fluoroethyl)nortropan-3-ol (α:β = 0.9:1)	4	0	16[e]	74[e]
N-*iso*-Propylnortropan-3-ol (α:β = 1.0:1)	0	5[e]	1[e]	40[e]
Norpseudopelletierin-3-ol (α:β = 13.7:1)	0	0	9[e]	13[e]
Pseudopelletierin-3-ol (α:β = 120.6:1)	0	0	55[e,f]	40[e,f]
N-Ethylnorpseudopelletierin-3-ol (α:β = 9.8:1)	0	0	152[e,f]	5[e,f]
N-*iso*-Propylnorpseudopelletierin-3-ol (α:β = 11.6:1)	0	0	7[e,f]	2[e,f]

[a] All substrates at 4 mM total concentration.
[b] Assays were conducted using a partially purified concentrated preparation containing both acyl transferase activities.
[c] Activity of tropine:acetyl-CoA acyl transferase = 12.5 pkat/mg protein; activity of tropine:tigloyl-CoA acyl transferase = 16.7 pkat/mg protein.
[d] Activity of pseudotropine:acetyl-CoA acyl transferase = 59 pkat/mg protein; activity of pseudotropine:tigloyl-CoA acyl transferase = 423 pkat/mg protein.
[e] Products were identified by GC/MS and comparison with authentic standards.
[f] Products are assigned to the pseudotropine activity on the basis of the substrates being predominantly of the 3β-configuration, as determined by ^{1}H NMR.

ations when either *N*-ethylnortropinone or *N*-*iso*-propylnortropinone was fed. The accumulation of the 3*β*-isomer under feeding conditions is similar to that found when high levels of tropinone are fed (Dräger, Portsteffen, Schaal, McCabe, Peerless & Robins, 1992), presumably due to the 'overloading' of the TRI resulting in substrate being available for TRII. Why the 3β-acetyl esters do not accumulate is, however, not clear.

From the analysis of the activities with TRI and TRII in *D. stramonium* (Table 2), the *N*-substituted norpseudopelletierines are reduced by TRII but not TRI, making it possible that esterified derivatives could have arisen from the reaction products when these compounds were fed to cultures. As can be seen from Table 3, these products of TRII were indeed substrates for PAT. No activity was measured for these substrates with TAT, even though the 3α-isomer was present at between 9 and 120 times higher concentration in the substrate solution. Nevertheless, by comparison with the metabolism of the natural alkaloids and with the identified products of the reduction in vivo leading to a much higher availability of the 3α-ol (see Table 6 of Boswell et al., 1999), it seems probable that the acetyl product determined in vivo is also of the 3α-series. These data show a marked lack of agreement between the in vivo and in vitro experiments.

3. Conclusions

In a previous communication (Boswell et al., 1999), it has been shown that a broad range of non-natural tropane alkaloids can effectively be made by feeding analogues of the natural substrates to root cultures. It is now shown that at each step in the tropane alkaloid pathway, the pertinent enzymes show a considerable degree of flexibility. Indeed, the in vitro activity is broader than apparent from the range of in vivo products, indicating that in vivo the necessary substrates are probably absent. Thus, potentially, a range of other biotransformation products could be made that could not have been predicted from feeding experiments alone (Boswell et al., 1999). Notably, *N*-substituted pseudopelletierines are not made from *N*-substituted cadaverines yet some of them are substrates for enzymes that catalyse steps subsequent to the formation of the tropane ring. Conversely, although putrescine and cadaverine are both substrates for MPO, the reductase and acyl transferase activities are low or zero in the absence of an *N*-substituent on the derived [3.2.1] or [3.3.1] rings.

The acetyl and tigloyl esters are the major aliphatic esters present and their accumulation in vivo can be explained, at least qualitatively, by the observed in vitro activities of pertinent enzymes. These esters are, however, relatively minor constituents of the total alkaloidal fractions, the aromatic esters being far more plentiful (Boswell et al., 1999; Robins et al., 1994; Robins, Parr, Payne, Walton & Rhodes, 1990), in some cases accumulating to amounts comparable with the natural alkaloids. Although the enzymes responsible for aromatic esterification and rearrangement (O'Hagan & Robins, 1998) have yet to be described, a recent report (Ollagnier, Kervio & Rétey, 1998) of

the rearrangement of (*S*)-littorine, the non-natural isomer, has apparently determined the conditions for the rearrangement reaction. The study of the substrate specificity of this and other activities will help further to elucidate the flexibility of tropane alkaloid metabolism.

If there are multiple substrates at a biosynthetic step, then presumably the plant might 'fine tune' the response by altering their relative availability. This could in principle apply to the availability of CoA thioesters in this pathway. Such flexibility should help to maintain the diversity of tropane alkaloids in the Solanaceae, and the plethora of minor alkaloids present in any one plant, notwithstanding the fact that the spectrum is reproducibly dominated by a small number of major products. Thus, it appears that the plant may produce, in adequate concentrations, compounds which are effective in defence whilst maintaining a low-level potential to produce and evolve others.

4. Experimental

4.1. Transformed root cultures

Root cultures of the *Brugmansia candida* × *aurea* hybrid, transformed with *Agrobacterium rhizogenes*, were grown as described in Robins et al., 1990. Root cultures of *Datura stramonium* D15/5, transformed with *Agrobacterium rhizogenes*, were grown as described in Robins, Parr, Bent, & Rhodes, 1991. For most experiments, approximately 0.2 g fresh mass of roots was subcultured into 50 ml B50 medium and grown in the absence of antibiotics.

4.2. Synthetic substrates and products

Analogues of putrescine and tropinone were synthesised as described in Boswell et al., 1999. Purity was assessed by GC/MS (Dräger et al., 1992) and by NMR spectroscopy. Configuration was assigned by NMR by comparison with tropine and pseudotropine. *N*-Alkylnortropinones were chemically reduced to a mixture of the 3α- and 3β-isomers by reduction with sodium borohydride.

4.3. Enzyme and protein assays

N-Methylputrescine oxidase was extracted essentially as described in McLauchlan et al., 1993, concentrated by precipitation with 70% ammonium sulfate (4°C), resuspended in extraction buffer and desalted on Sephadex PD-10 (Pharmacia). This preparation was used without further purification. Oxidase activity was determined spectrophotometrically. The release of hydrogen peroxide was monitored by following the rate of change in A_{595} due to the indamine dye generated by the horseradish peroxidase-catalysed oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone and 3-(dimethylamino)-benzoic acid (Equi, Brown, Cooper, Ner, Watson & Robins, 1991).

Tropinone reductases were completely separated as described previously (Portsteffen, 1994; Portsteffen et al., 1992,1994). Reductase activity was assayed spectrophotometrically by following the rate of change of absorption of NADPH at 333 nm (Portsteffen et al., 1994).

Tropine:- and pseudotropine:acyl-CoA acyl transferases were assayed in a crude extract after concentration and desalting. Acyl transferase activity was determined by extracting the reaction products with Extrelute® (Merck, Germany) and quantification by GC as described in Rabot et al., 1995.

Protein was determined by the dye-binding method (Bradford, 1976), using the BioRad dye reagent.

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