Pergamon 0031-9422(94)00907-4 Phytochemistry, Vol. 39, No. 2, pp. 315-322, 1995
Copyright © 1995 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0031-9422/95 $9.50 + 0.00

TIGLOYL-CoA:PSEUDOTROPINE ACYL TRANSFERASE—AN ENZYME OF TROPANE ALKALOID BIOSYNTHESIS

SYLVIE RABOT, ABIGAEL C. J. PEERLESS* and RICHARD J. ROBINS*†

UEPSD, Bâtiment 440, INRA, Centre de Recherches de Jouy, F-78352 Jouy-en-Josas Cedex, France; *Institute of Food Research, Norwich Laboratory, Norwich Research Park, Colney, Norwich NR4 7UA, U.K.

(*Received in revised form* 20 *October* 1994)

Key Word Index—*Datura stramonium*; Solanaceae; acyl transferase; pseudotropine; transformed root culture; tropane alkaloid.

Abstract—Tigloyl-CoA:pseudotropine acyl transferase esterifies the 3β-hydroxyl group of pseudotropine (3β-hydroxytropane) with the tiglic acid moiety of tigloyl-Coenzyme A. This enzyme was purified to near electrophoretic homogeneity—about 330-fold—from transformed root cultures of *Datura stramonium*. The protocol involved the sequential use of ammonium sulphate precipitation, hydrophobic interaction, anion-exchange, chromatofocusing and gel filtration chromatography. The enzyme has a M_r of 65 000, as determined by gel filtration chromatography on Sepharose 6 and SDS–PAGE electrophoresis, indicating it to be active as a monomer. Maximal activity occurs at pH 9.0 but the enzyme is still about 30% active at pH 7.0. The purified protein shows simple Michaelis–Menten kinetic behaviour, with K_m values of 0.36 and 1.31 mM for pseudotropine and tigloyl-CoA, respectively. The enzyme is specific for the acyl group receptor. Of a range of potential acceptors tested, only pseudotropine and 4-hydroxy-1-methylpiperidine (14%) were used: tropine (3α-hydroxytropane) and norpseudotropine were not acylated. In contrast, the enzyme possesses the ability to transfer the acyl moiety to pseudotropine from a wide range of aliphatic acyl-CoA thioesters. Tigloyl-CoA and acetyl-CoA act reciprocally as competitive inhibitors, suggesting that they compete for the same active site on the enzyme. With acetyl-CoA the K_m is 0.33 mM, indicating that the enzyme has a higher affinity for this acyl donor than for tigloyl-CoA. Neither CoA nor any of the alkamine acceptors tested were able to inhibit the acylation of pseudotropine.

INTRODUCTION

A number of *Datura* species and hybrids are cultivated for the production of the pharmaceuticals hyoscyamine and hyoscine [1]. These tropane alkaloids are esters between tropine and scopine, derived from arginine/ornithine, and tropic acid, derived from phenylalanine. *Datura* and several other solanaceous genera produce a wide range of alkaloids that are esters of tropanols [2]. They also form esters of the 3β-isomer of tropine, pseudotropine, which can be dominant in some species.

In *D. stramonium* transformed root cultures, hyoscyamine is the predominant product [3]. 3α-Tigloyloxytropane and 3α-acetoxytropane constitute minor alkaloids in the natural spectrum [4]. If, however, tropine or tropinone is fed, 3α-acetoxytropane accumulates to a high level [3]. Tropinone feeding also greatly stimulates the accumulation of pseudotropine, leading to elevated levels of the acetyl- and tigloyl-pseudotropine esters [5]. This effect is more marked if tropinone reductase I, the tropine-forming enzyme of the pathway, is inhibited at the same time [5]. Separable enzyme activities responsible for forming 3α-acetoxytropane and 3β-acetoxytropane have been identified [6].

The purification and properties of the enzyme that forms 3β-tigloyloxytropane (Fig. 1) are reported here. A summary of some of these results has been presented previously [7].

RESULTS

Activity in root cultures

The levels of tigloyl-CoA:pseudotropine acyl transferase and acetyl-CoA:pseudotropine acyl transferase are both higher during rapid growth than in stationary phase roots (Fig. 2). This is particularly marked if the activity is related to the fresh mass of tissue present. The ratios of the two activities, on a protein basis, are similar at all ages of culture. These profiles do not show the sharp maximum activity found for some other enzymes of tropane alkaloid formation in these cultures [3].

†Author to whom correspondence should be addressed: Laboratoire de RMN-RC, URA-CNRS 472, Faculté des Sciences, 2 rue de la Houssinière, F-44072 Nantes Cedex 03, France.

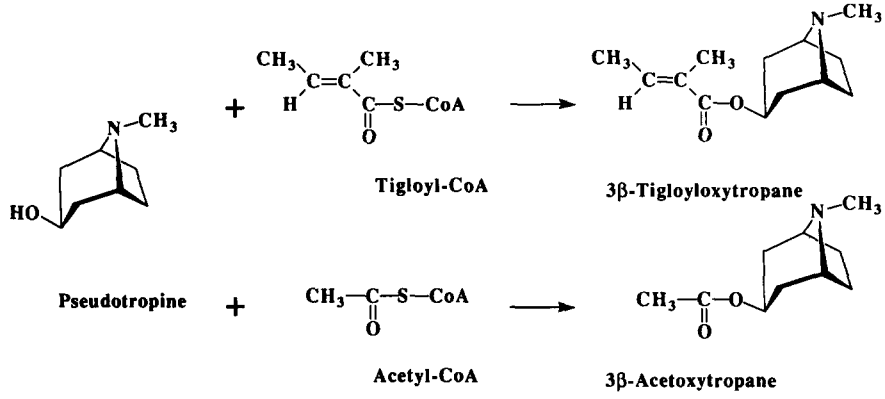


Fig. 1. Scheme showing the formation of 3β-tigloyloxytropane and 3β-acetoxytropane.

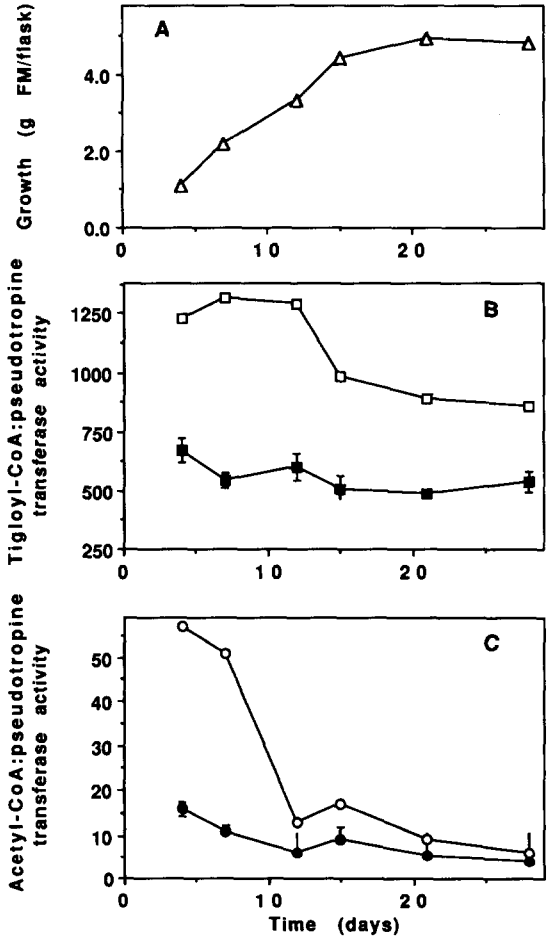


Fig. 2. The levels of tigloyl-CoA:pseudotropine acyl transferase and acetyl-CoA:pseudotropine acyl transferase activities in root cultures of different ages. (A) growth; (B) tigloyl-CoA:pseudotropine acyl transferase (■, (□); (C) acetyl-CoA:pseudotropine acyl transferase (●, ○). Closed symbols are activity expressed as pkat mg^{-1} protein; open symbols are activity expressed as pkat g^{-1} FM. Each assay was performed in duplicate on extracts from duplicate flasks.

Enzyme purification

Tigloyl-CoA:pseudotropine acyl transferase has been purified about 330-fold by the procedure outlined in Table 1 and described in the Experimental. Gel filtration on Superose 6®, indicated the M_r of the native protein to be 65 ± 6000. When the active fractions were examined by SDS–PAGE (Fig. 3), the major band occurred at M_r 66 ± 3000, with minor bands at *ca* 42000 and 24000. These smaller molecular weight bands occur in fractions that do not have activity, indicating that they are not associated with the enzyme. The isoelectric point of the protein, estimated by chromatofocusing chromatography, is 5.4.

The enzyme is stable at −20° for at least 18 months. The activity is linear with time for up to 75 min, provided the protein concentration is below 0.15 mg per assay. At protein concentrations above this level, linearity is lost after about 45 min. The assay is linear up to about 20% conversion of pseudotropine to product.

pH optimum

Maximal activity was found with glycine buffer at pH 9.0 (Fig. 4). About 30% maximal activity was determined at pH 7: above pH 9.5, activity is rapidly lost. Activity was highest in glycine buffer; Tris and borate (data not shown) both giving lower activity at pH 9.

Alkaloid substrate requirement

The purified enzyme showed a typical Michaelis–Menten-type saturation curve with pseudotropine, with a K_m of 0.36 mM (4 mM tigloyl-CoA) and a V_{max} of 481 pkat mg^{-1} protein. Transferase activity with tigloyl-CoA (3 mM) was determined for a number of other potential acyl group acceptors (8 mM) having some structural analogy with pseudotropine (Table 2). Of the other compounds tested, only 4-hydroxyl-1-methylpiperidine was acylated. No activity was found with tropine, indicating that a 3β-hydroxyl group is required.

Table 1. Purification of tigloyl-CoA:pseudotropine acyl transferase

Purification step	Total volume (ml)	Total protein (mg)	Total activity (nkat)	Specific activity (nkat mg^{-1} protein)	Yield (%)	Activity enrichment (-fold)	Ratio (tigloyl: acetyl)
Crude extract	2298	4411	2838.0	0.64	100.0	1.0	19.8
AmS* 30–80%	371	2182	2120.0	0.97	74.7	1.5	18.2
Phenyl-Sepharose: 0% AmS	238	456	380.0	0.83	13.4	1.3	14.2
Mono Q®: †0.04–0.11 M KCl	40	29.5	312.5	10.6	11.0	16.5	23.9
First Mono P®: ‡pH 6.86–5.87	4	3.54	140.4	39.6	5.0	61.6	39.6
Second Mono P®: ‡pH 5.58–5.13	2	0.49	28.1	57.4	1.0	89.1	23.8
Superose 6®§	1.75	0.09	18.9	210.0	0.67	328.1	n.d‖

*AmS, % saturation ammonium sulphate (4°).
†Anion exchange column (Pharmacia).
‡Chromatofocusing column (Pharmacia).
§Gel filtration column (Pharmacia).
‖n.d., Not determined.

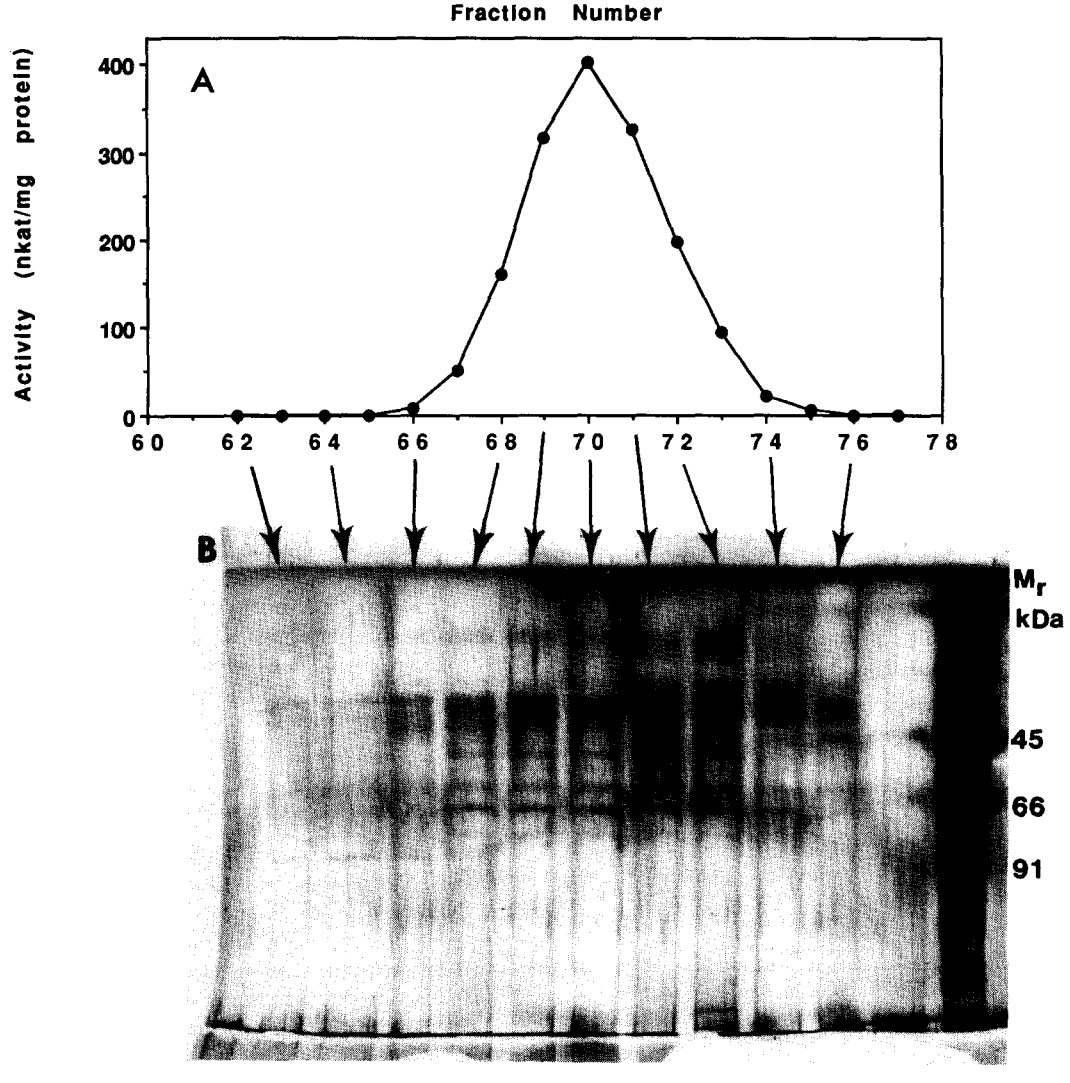
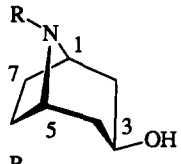
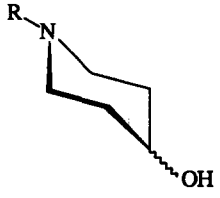


Fig. 3. The elution of tigloyl-CoA:pseudotropine acyl transferase from a Superose 6® gel filtration column. Fractions are 0.25 ml volume: (A) the profile of activity; (B) the SDS–PAGE from the fractions.

Table 2. Substrate specificity of pseudotropine: tigloyl-CoA acyl transferase: alkamine moieties

Alkaloid		Tigloyl-CoA transferase activity*
	Pseudotropine	R=Me 100
	Norpseudotropine	R=H 0
	Tropine	R=Me 0
	Nortropine	R=H 0
	4-Hydroxy-1-methylpiperidine	R=Me 14
	4-Hydroxypiperidine	R=H 0
	3-Pyrrolidinol	0
	3-Quinuclidinol	0
	Scopine	0
	Methylecgonine	0
	N-Cyanonorpseudotropine	0
	8-Thiabicyclo[3.2.1] octan-3β-ol	0
	cis-4-Methylcyclohexanol	0
	Tetrahydro-4H-pyran-4-ol	0
	7β-Hydroxytropinone	0

*Activity expressed relative to that with pseudotropine (100% = 57 nkat mg^{-1} protein).
All alkamine substrates at 8 mM. Tigloyl-CoA at 3 mM. All products confirmed by GC-MS.

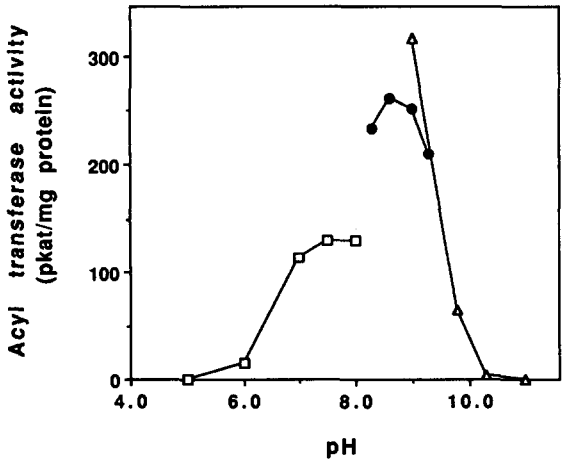


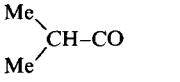
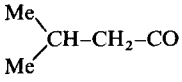
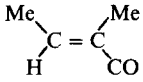
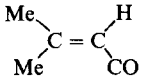
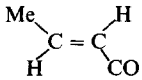
Fig. 4. The effect of the pH of the incubation buffer on the activity of tigloyl-CoA:pseudotropine acyl transferase activity. Buffers used are (□), phosphate; (●), Tris; (△), glycine.

A requirement for a *N*-methyl group was also indicated by the lack of activity with both norpseudotropine and 4-hydroxypiperidine. The enzyme was inactive with 7β-hydroxytropinone, indicating that the activity is specific to the 3-position.

Coenzyme A thioester requirement

The purified enzyme also showed a typical Michaelis–Menten-type saturation curve with tigloyl-CoA, with a K_m of 1.31 mM (4 mM pseudotropine). Transferase activity was determined with pseudotropine (4 mM) as acceptor and a range of CoA thioesters (3 mM). The enzyme was found able to transfer an acyl group to pseudotropine from a wide range of CoA thioesters, including acetyl-CoA (Table 3). With acetyl-CoA, a K_m of 0.33 mM (4 mM pseudotropine) was determined. When enzyme extract was incubated with pseudotropine and both tigloyl-CoA and acetyl-CoA, it was demon-

Table 3. Substrate specificity of pseudotropine:tigloyl-CoA acyl transferase: thioester moieties

Coenzyme A thioester		Pseudotropine transferase activity*
Me–CO	Acetyl	4
Me–CO–CH_2–CO	Acetoacetyl	0
Me–CH_2–CO	*n*-Propionoyl	5
	*iso*-Butyroyl	10
	*iso*-Valeroyl	2
	Tigloyl	100
	β-Methylcrotonoyl	10
	Crotonoyl	0

*Activity expressed relative to that with tigloyl-CoA (100% = 57 nkat mg^{-1} protein).
All CoA substrates at 3 mM. Pseudotropine at 8 mM. All products confirmed by GC-MS.

strated that these thioesters act as acyl donors in a competitive manner (Fig. 5).

Purification of acetyl-CoA:pseudotropine acyl transferase

Acetyl-CoA:pseudotropine acyl transferase was monitored throughout the purification of tigloyl-CoA:pseudotropine acyl transferase. As can be seen from Table 1, the two activities purified in parallel, a constant ratio of activity nearly being maintained during a 90-fold increase in specific activity. These data, and the competitive inhibition reported above, strongly suggest that the acetyl-CoA and tigloyl-CoA transferase are two functions of the same enzyme.

Inhibition of tigloyl-CoA:pseudotropine acyl transferase

The ability of a number of other compounds to inhibit the activity of tigloyl-CoA:pseudotropine acyl transferase at equimolar concentration was tested. None of these (norpseudotropine, tropine, nortropine, tropinone, 4-hydroxy-1-methylpiperidine, 4-hydroxypiperidine, 3-quinuclidinol, 8-thiabicyclo[3.2.1] octan-3β-ol, ecgonine methyl ester, acetate, tiglate, CoA) had the ability to depress the tigloylation of pseudotropine to any significant extent. This was true even for 4-hydroxy-1-methylpiperidine, the only compound tested that is a substrate of the enzyme.

DISCUSSION

Tigloylated secondary products have been reported from a wide range of plants. Alkaloids esterified with tiglic acid have been described from a number of solanaceous genera [2] and from *Lupinus* species [8–10]. Recently, tigloylated esters of the quassinoids, chaparrin and chaparrinone, have been identified [11]. Acetylation is also well known, as, for example in the tropanes [2] and the indole alkaloids of *Catharanthus* [12, 13].

Acyl transferases able to tigloylate alkaloids have previously been described in crude extracts of seedlings of both *Lupinus albus* [8] and *L. hirsutus* [10]. To date, however, none of these tigloylating enzymes has been purified. In this present report, a tigloyl-CoA:pseudotropine acyl transferase from transformed roots of *D. stramonium* is identified, characterized and purified to near homogeneity. From studying the properties of the purified enzyme, it is apparent that the activity seen in a crude extract which acylates pseudotropine with a broad range of acyl-CoA thioesters can be assigned to the single protein. The demonstration that the acylation of pseudotropine with acetyl-CoA and tigloyl-CoA are competitive confirms this deduction. Whether the similar broad specificity reported for the crude enzyme of *L. albus* [8] is also due to a single protein is yet to be clarified. However, the ability of a single enzyme to transfer an acyl moiety from a group of structurally related acyl-CoA thioesters might be relatively common. For example, cell-free extracts of *Capsicum annuum* can transfer a range of fatty acids from their CoA thioesters to vanillylamine [14]. Whether the acetyl-CoA:tropine acyl transferase activity [6] has similar properties remains to be determined.

With the alkamine acyl acceptor, however, tigloyl-CoA:pseudotropine acyl transferase shows a high degree of specificity. The *Lupinus* tigloyl transferases [8, 10] and the acetyl-CoA:deacetylvindoline 17-*O*-acetyltransferase from *Catharanthus* [12, 13] similarly show high degrees

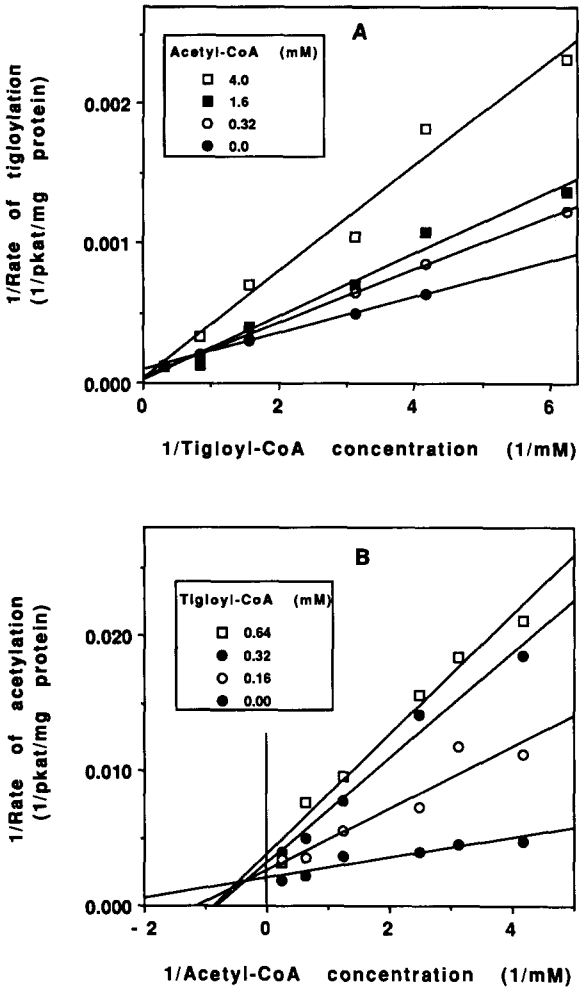


Fig. 5. Inhibition of acyl-CoA:pseudotropine acyl transferase activity by acyl CoA thioesters: (A) effect of acetyl-CoA on the rate of tigloyl-CoA:pseudotropine acyl transferase activity; (B) effect of tigloyl-CoA on acetyl-CoA:pseudotropine acyl transferase activity.

of selectivity for the alkaloidal acceptor. Other than pseudotropine, the *Datura* enzyme will only accept 4-hydroxy-1-methylpiperidine, in which the C6–C7 bridge is missing (Table 3). A substituent at the nitrogen is required, norpseudotropine being unmetabolized. Recently, we have shown [15, 16] that *N*-ethyl-, *N*-fluoroethyl- and *N-iso*-propyl-norpseudotropine can also be metabolized, but less effectively than pseudotropine. It has already been shown in *Datura* [6] that separate enzymes are responsible for the acetylation of tropine and pseudotropine. The formation of these two compounds by the reduction of the common intermediate, tropinone, also requires separate enzymes [16–18]. The structure–function relationships described for the tropinone reductases of *Datura* [17, 18] closely parallel those described here for acyl transfer. Thus, it appears that the catalytic sites of these tropane-metabolizing enzymes may well have similar three-dimensional structures.

Crude extracts from a number of *Datura* species contain both tigloyl-CoA:pseudotropine and phenylacetoyl-CoA:pseudotropine acyl transferase activities [7]. In both *L. albus* and *L. hirsutus*, the ability to acylate alkaloids with an aromatic acid from the respective CoA thioester was also demonstrated [8, 10]. Similarly, activities that used 4-coumaroyl-CoA and feruloyl-CoA could be separated from the transferase that used tigloyl-CoA [10]. The protocol described here completely frees the tigloylating activity from the aromatic acyl transferase [7], indicating that a distinct enzyme is responsible for the aromatic acylation of pseudotropine.

The tigloyl-CoA:pseudotropine acyl transferase activity is demonstrated to have a pH optimum of 9.0, considerably higher than the cytoplasmic pH of the root cultures [19]. The acyl transferases discussed above from *Lupinus* [8–10], *Catharanthus* [12, 13] and *Capsicum* [14] also show optimal activities around pH 9. With the acetylation of tropine [6], also optimal at pH 9.0, we suggested [6] that this property might be due to the uncharged alkaloid acting as substrate. Strong support for this proposal has recently been presented by Portsteffen *et al.* [18] who showed that the effect of pH on the K_m of tropinone reductase can be interpreted as due to the effect of pH on the degree of protonation of tropinone.

It might be argued that these pseudotropine-acylating activities in *D. stramonium* are due to the non-specific activity of an enzyme intended to perform an entirely different role. However, this suggestion is refuted by the evidence presented that the purified enzyme is highly specific for pseudotropine as acyl acceptor. Nevertheless, the role of this enzyme is not at all clear. The products are not on the direct pathway to hyoscyamine and the tropan-3β-ols tend to constitute very minor bases in the alkaloidal extract. Indeed, usually only traces of 3β-acetoxytropane can be detected, 3β-tigloyloxytropane being absent. It would be valuable in this respect to examine the levels of acyl transferase activity in species known to accumulate substantial amounts of tropan-3β-ol esters. Evidence has been presented [5] that it is, at least partially, the relative availability of the tropan-3-ols that determines the final spectrum of products in the culture, rather than the ability to esterify them. As tropinone reduction in *D. stramonium* root cultures strongly favours tropine formation [17, 18], the lack of any accumulation of the tropan-3β-ol esters can readily be explained. Although the level of activity of acetyl-CoA:tropine acyl transferase in *Datura stramonium* is lower than the level of acetyl-CoA:pseudotropine acyl transferase, there is still sufficient activity to account for the amount of 3α-acetoxytropane that accumulates [6, 7]. The preferred acylation with acetate, rather than tiglate, presumably reflects the combined influence of both the greater availability of acetyl-CoA and the higher affinity of the enzyme for this acyl donor.

EXPERIMENTAL

Chemicals. Coenzyme A thioesters were purchased from Sigma. Tropine was from Aldrich. Pseudotropine

was synthesized essentially as described in ref. [20]. Nortropine, *N*-cyanonorpseudotropine and norpseudotropine were the kind gifts of Dr B Dräger, Institut für Pharmazeutische Biologie, Münster, Germany. 8-Thiabicyclo [3.2.1]octan-3β-ol was prepared as described in ref. [5] and kindly supplied by Dr P. McCabe, Chemistry Department, Glasgow, U.K. 3α-Acetoxytropane and 3β-acetoxytropane were prepared by T. Robinson as described in ref. [6]. All other chemicals were purchased from either Sigma or Aldrich and were of the highest grade available. Chromatography media were purchased from Pharmacia.

Transformed root cultures. Root cultures of *Datura stramonium* L. D15/5 were initiated and subcultured as described in ref. [3]. All experiments were performed using roots growing in the absence of added antibiotics. Roots used for purifying enzyme activities were grown in 15 l fermenters, essentially as described in ref. [21].

Buffers. Buffer I: 200 mM potassium phosphate, 20 mM EDTA, 125 mM sucrose, 3 mM DTT, pH 7.0 (at 20°);

Buffer II: 50 mM potassium phosphate, 5 mM EDTA, 100 mM sucrose, 2 mM DTT, pH 7.0 (at 20°);

Buffer III: 50 mM potassium phosphate, 5 mM EDTA, 100 mM sucrose, 2 mM DTT, 860 mM ammonium sulphate, pH 7.0 (at 20°);

Buffer IV: 50 mM potassium phosphate, 5 mM EDTA, 100 mM sucrose, 2 mM DTT, 430 mM ammonium sulphate, pH 7.0 (at 20°);

Buffer V: 100 mM ethanolamine, 5 mM EDTA, 100 mM sucrose, 2 mM DTT, pH 9.0 (at 20°);

Buffer VI: 125 mM ethanolamine, 100 mM sucrose, 2 mM DTT, pH 9.0 (at 20°);

Buffer VII: 125 mM ethanolamine, 100 mM sucrose, 2 mM DTT, 1 M KCl pH 9.0 (at 20°);

Buffer VIII: 25 mM imidazole, 100 mM sucrose, 2 mM DTT, pH 7.4 (at 20°);

Buffer IX: 10% (v/v) Polybuffer 74® (Pharmacia, U.K.), 100 mM sucrose, 2 mM DTT, pH 3.9 (at 20°);

Buffer X: 50 mM potassium phosphate, 100 mM sucrose, 2 mM DTT, pH 7.0 (at 20°);

Preparation of enzyme extract and ammonium sulphate fractionation. Root cultures were harvested 10–14 days from subculture, blotted on absorbent towel, aliquoted into 60 g packets and frozen in liquid nitrogen. Tissue was stored at − 40° or − 70° until required. Under these conditions, activity was retained at a constant level for at least 18 months.

Extraction and precipitation was performed in a 4° cold room, using pre-chilled equipment. Approximately 800 g of frozen tissue was crushed, mixed (1 g per 10 g fr. wt tissue) with insoluble polyvinylpyrrolidone (Sigma) and ground in a small grinder (Moulinex) for about 10 sec. The powder was dispersed into 2.75 l buffer I in a 5 l beaker; and left to stir gently for about 30 min. The liquid was removed by filtering through nylon in a manual fruit press and clarified at 15 000 *g* (20 min, 4°). The clear supernatant (crude extract) was brought to 30% saturation (4°) by slowly adding powdered ammonium sulphate, stirred gently for 20 min then clarified at 17 700 *g* (20 min, 4°). The supernatant was brought to 80% saturation with powdered ammonium sulphate, stirred gently for 20 min then clarified at 17 700 *g* (20 min, 4°). The pellet was re-suspended in 180 ml buffer II and frozen in liquid nitrogen.

Hydrophobic interaction chromatography. Performed at 4°. The thawed extract was loaded at 6 ml^{-1} on to a phenyl-Sepharose® column (Pharmacia XK50/30, 5 × 25 cm) previously equilibrated with buffer III. The column was sequentially eluted with 1 l volumes of buffers III, IV and V. Activity principally eluted in the buffer V wash.

Anion exchange chromatography. The active fraction (about 750 ml) from phenyl-Sepharose was concentrated at 4° to about 100 ml by ultrafiltration on a MINITAN® concentrator (Millipore) fitted with PM10 membranes and buffer exchanged by 4 sequential 5-fold dilutions with buffer VI. The extract was brought to room temp. and loaded onto a Mono Q® (8 ml), column at 2 ml min^{-1} and eluted at a flow rate of 0.5 ml min^{-1} with a gradient from 0 to 0.35 M KCl using buffer VII. Activity eluted between 0.15 and 0.3 M KCl.

Chromatofocusing chromatography. The anion-exchanger active fractions were pooled and concd at 4° to about 5 ml by ultrafiltration on an Amicon® concentrator 8150 (Amicon), fitted with a PM10 membrane. They were buffer exchanged on PD-10® columns pre-equilibrated in buffer VIII, brought to room temp. and loaded on to an HR5/20 Mono-P® column, pre-equilibrated in buffer VIII. Activity was eluted with a pH gradient generated by running buffer IX into the column at 0.5 ml min^{-1}. Activity eluted between pH 6.8 and 5.1. This step was performed twice, the activity eluting at a more acidic pH and in a tighter band from the second column.

Gel filtration chromatography. The active fractions from the Mono-P column were pooled and buffer exchanged on PD-10 columns at 4° pre-equilibrated with buffer X, concentrated to 0.2 ml, brought to room temp. and loaded on to a Superose 6® column which was eluted in buffer X.

Protein determination. Protein was measured by the dye-binding method [22] using the BioRad dye reagent (BioRad Laboratories, U.K.) as described in their literature. Bovine serum albumin (Sigma, fraction V) was used as standard.

Molecular mass determination. The M_r of the native acyl transferase was estimated by gel filtration on a Superose 6 column, calibrated with a mixt. of proteins (Sigma, U.K.) ranging from 49 to 200 000. The M_r of the denatured acyl transferase monomeric unit was estimated by SDS–PAGE in a 7.5% acrylamide gel, cross-linked with 0.4% piperazine diacrylamide, using a tris–tricine buffer essentially as described in ref. [23]. Molecular weight markers covering the range 110 000–23 000 were obtained from Gibco.

Enzyme activity determination. Tigloyl-CoA:pseudotropine acyl transferase activity was measured by determining the formation of 3β-tigloyloxytropane by GC. Typically, the assay mixt. (200–300 μl total volume) con-

tained 0.3 M glycine buffer pH 9.4 mM pseudotropine, 1.4 mM tigloyl-CoA and enzyme extract (5–150 μg protein). The reaction was started by the addition of tigloyl-CoA. The reaction was incubated at 30° for 60 min and stopped by rapid freezing in liquid nitrogen.

To measure the amount of product formed, the incubation mixture was made basic with 100 μl 35% (v/v) ammonia solution and applied to 1 g Extrelute® (Merck, U.K.). Hydrophobic material was desorbed from the solid matrix with 12 ml $CHCl_3$ followed by 4 ml $CHCl_3$–MeOH (19:1). Following evapn of the solvent to dryness, the residue was dissolved in 1.0 ml EtOAc and the alkaloid content determined by GC as described previously [5] except with a ramp rate of 15° min^{-1}.

Activity with other acyl acceptors or acyl donors was determined as described above, using concentrations of substrates as indicated in the Tables. In competitive experiments, both products were determined in the same incubation mixt. In all cases, the identity of the product was confirmed by GC/MS as described previously [5].

Acknowledgements—Dr Rabot gratefully acknowledges the assistance of an INRA/AFRC Fellowship, held at IFR for 6 months. For supplies of substrates and substrate analogues we wish to thank Dr B. Dräger (Münster, Germany) and Dr P. McCabe (Glasgow, U.K.); for assistance with producing the fermenter cultures we thank Martin Hilton, Chris Waspe and Dr P. Wilson (IFR); for much advice and discussion we are indebted to our colleagues Drs M.J.C. Rhodes, A.J. Parr and N.J. Walton.

REFERENCES

1. Evans, W. C. (1990) *Pharmaceutical J.* **244**, 651.
2. Lounasmaa, M. and Tamminen, T. (1993) *The Alkaloids* **44**, 1.
3. Robins, R. J., Parr, A. J., Bent, E. G. and Rhodes, M. J. C. (1991) *Planta* **183**, 185.
4. Parr, A. J., Payne, J., Eagles, J., Chapman, B., Robins, R. J. and Rhodes, M. J. C. (1990) *Phytochemistry* **29**, 2545.
5. Dräger, B., Portsteffen, A., Schaal, A., McCabe, P. H., Peerless, A. C. J. and Robins, R. J. (1992) *Planta* **188**, 581.
6. Robins, R. J., Bachmann, P., Robinson, T., Rhodes, M. J. C. and Yamada, Y. (1991) *FEBS Letters* **292**, 293.
7. Robins, R. J., Bachmann, P., Peerless, A. C. J. and Rabot, S. (1995) *Plant Cell Tiss. Org. Cult.* **38**, 241.
8. Wink, M. and Hartmann, T. (1982) *Planta* **156**, 560.
9. Strack, D., Becher, A., Brall, S. and Witte, L. (1991) *Phytochemistry* **30**, 1493.
10. Saito, K., Suzuki, H., Takamatsu, S. and Murakoshi, I. (1993) *Phytochemistry* **32**, 87.
11. Carter, C. A. G., Tinto, W. F., Reynolds, W. F. and McLean, S. (1993) *J. Nat. Prod.* **56**, 130.
12. De Luca, V., Balsevich, J. and Kurz, W. G. W. (1985) *J. Plant Physiol.* **121**, 417.
13. Fahn, W. and Stöckigt, J. (1990) *Plant Cell Rep.* **8**, 613.
14. Fujiwake, H., Suzuki, T., Oka, S. and Iwai, K. (1980) *Agric. Biol. Chem.* **44**, 2907.
15. Bolwell, H. D., Parr, A. J., Robins, D. J., Robins, R. J. and Walton, N. J. (in preparation).
16. Robins, R. J., Bolwell, H. D., Dräger, B., McLauchlan, W. R., Portsteffen, A., Robins, D. J. and Walton, N. J. (in preparation).
17. Portsteffen, A., Dräger, B. and Nahrstedt, A. (1992) *Phytochemistry* **31**, 1135.
18. Portsteffen, A., Dräger, B. and Nahrstedt, A. (1995) *Phytochemistry* **37**, 391.
19. Ford, Y.-Y., Fox, G. G., Ratcliffe, R. G. and Robins, R. J. (1994) *Phytochemistry* **36**, 333.
20. Nickon, A. and Fieser, L. F. (1952) *J. Am. Chem. Soc.* **74**, 5565.
21. Hilton, M. and Rhodes, M. J. C. (1993) *Planta Med.* **59**, 340.
22. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.
23. Schägger, H. and von Jagow, G. (1987) *Analyt. Biochem.* **166**, 369.