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BIOSYNTHESIS OF THE TIGLOYL ESTERS IN *DATURA*: THE ROLE OF 2-METHYLBUTYRIC ACID*

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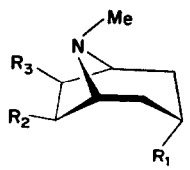
Key Word Index—*Datura innoxia*; Solanaceae; biosynthesis; 3α,6β-ditigloyloxytropane; 3α,6β-ditigloyloxytropan-7β-ol; L-isoleucine, 2-methylbutyric and 2-hydroxy-2-methylbutyric acids as precursors.

Abstract—*Datura innoxia* plants were wick fed with (±)-2-methylbutyric acid-[1-^{14}C] and harvested after 7 days. The root alkaloids 3α,6β-ditigloyloxytropane and 3α,6β-ditigloyloxytropan-7β-ol were isolated and degraded. In each case the radioactivity was located in the ester carbonyl group indicating that this acid is an intermediate in the biosynthesis of tiglic acid from L-isoleucine. On the other hand, (±)-2-hydroxy-2-methylbutyric acid-[1-^{14}C], which was fed to hydroponic cultures of *Datura innoxia* alongside isoleucine-[U-^{14}C] positive control plants, is not an intermediate.

INTRODUCTION

APART FROM the well known poisonous tropoyl esters, the roots of all *Datura* species (Solanaceae) so far examined contain a number of minor alkaloids which are esters of tiglic acid and either tropine (I), tropan-3α,6β-diol (II), or teloidine (III).[1] L-Isoleucine was first shown to be a precursor for the tigloyl moiety of 3α-tigloyloxytropane (IV), 3α,6β-ditigloyloxytropane (V), meteloidine (VI) and 3α,6β-ditigloyloxytropan-7β-ol (VII) in *D. innoxia* Miller, and *D. meteloides* D.C. ex Dunal some time ago,[2] a result later confirmed by Leete who was able to demonstrate that the radioactivity of L-isoleucine-[2-^{14}C] specifically incorporated into the ester carbonyl of meteloidine in *D. meteloides*.[3] Although it is



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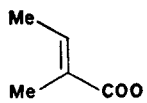


(I) Tropine	R_1 = OH;	R_2 = H;	R_3 = H
(II) Tropan-3α,6β-diol	R_1 = OH;	R_2 = OH;	R_3 = H
(III) Teloidine	R_1 = OH;	R_2 = OH;	R_3 = OH
(IV) 3α-Tigloyloxytropane	R_1 = Tig;	R_2 = H;	R_3 = H
(V) 3α,6β-Ditigloyloxytropane	R_1 = Tig;	R_2 = Tig;	R_3 = H
(VI) Meteloidine	R_1 = Tig;	R_2 = OH;	R_3 = OH
(VII) 3α,6β-Ditigloyloxytropane-7β-ol	R_1 = Tig;	R_2 = Tig;	R_3 = OH

FIG. 1. THE STRUCTURES OF THE TIGLOYL ESTERS OF *Datura*.

* Part III in the series "The biosynthesis of the tigloyl esters in *Datura*". For Part II see Ref. 2.

[1] EVANS, W. C., GHANI, A. and WOOLLEY, V. A. (1972) *Phytochemistry* **11**, 2527; and Refs. 2–19 cited therein.
[2] WOOLLEY, J. G. (1966) *Abhandl. Deut. Akad. Wiss. Berlin Kl. Chem. Geol. Biol.* (3), 531.
[3] LEETE, E. and MURRILL, J. B. (1967) *Tetrahedron Letters* 1233.

known that in animal tissues isoleucine is degraded to tiglate via 2-methylbutyric acid,[4-6] preliminary feeding experiments with this latter acid under a variety of conditions failed to show any incorporation into the tigloyl esters.[2]

Because of these failures, and particularly in the light of more recent research into the metabolism of isoleucine in other plants,[7,8] it was decided to re-examine the role of 2-methylbutyric acid and other related acids in the biosynthesis of the tigloyl esters.

RESULTS AND DISCUSSION

Of the two acids used, it is clear that only 2-methylbutyric is an intermediate in the biosynthesis of the tigloyl esters (Tables 1 and 2). In this context we are firmly of the opinion that negative incorporations should always be qualified by a positive control. The 2-hydroxy-2-methylbutyrate, tiglate and angelate residues are frequently encountered as parts of the necic acids in the pyrrolizidine ester alkaloids of *Senecio* and related genera.[9] All are derivable from L-isoleucine[7,10,11] although it is not known at what stage the hydroxylation of the C(3) of isoleucine takes place (the 6, 5, 10 or even 12 carbon level). It would be of immense interest to discover if either of the acids used here can serve as precursors of the necic acids. Similarly, the biosynthesis of the cyanogenetic glycoside lotaustralin from isoleucine involves C(3) hydroxylation, but in this case there is a wealth of evidence to indicate that this occurs after decarboxylation.[8]

TABLE 1. DISTRIBUTION OF RADIOACTIVITY FROM 2-METHYLBUTYRIC ACID-[1-^{14}C] FEEDING EXPERIMENT IN *Datura*

Alkaloid	% Specific incorporation*	Sp. act. diluted picrate (dpm/mM $\times 10^{-4}$)	Sp. act. tiglic acid (dpm/mM $\times 10^{-4}$) with % recovery in parenthesis*	Sp. act. $BaCO_3$ (dpm/mM $\times 10^{-4}$) with % recovery in parenthesis*
V	2·4	5·25	4·57 (87)	4·82 (92)
VII	1·6	5·79	5·20 (89)	5·20 (89)

* Calculated as: (Specific activity of product/Specific activity of precursor or starting material) ($\times$ 100).

Loss of water at a sheltered tertiary hydroxyl like the C(3) of isoleucine (or the C(2) of 2-methylbutyrate) to give a double bond is an unusual step, but by no means without precedent—e.g. the conversion of citrate to *cis*—aconitate in the TCA cycle or nearer at hand perhaps, the conversion of 3-hydroxy-3-carboxy-isocaproate to dimethylcitraconate

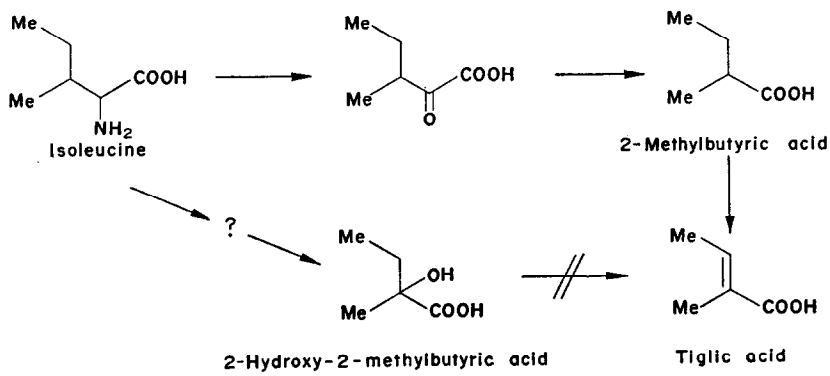
[4] COON, M. J., ABRAHEMSON, N. S. B. and GREEN, G. S. (1952) *J. Biol. Chem.* **195**, 805.
[5] ROBINSON, W. G., BACHHAWAT, B. K. and COON, M. J. (1956) *J. Biol. Chem.* **218**, 391.
[6] COON, M. J., ABRAHEMSON, N. S. B. and GREEN, G. S. (1952) *J. Biol. Chem.* **199**, 75.
[7] CROUT, D. H. G., DAVIES, N. M., SMITH, E. H. and WHITEHOUSE, D. (1972) *J. Chem. Soc. Perkin I*, 671.
[8] CONN, E. E. and BUTLER, G. W. (1969) *The Biosynthesis of the Cyanogenetic Glycosides in Perspectives in Phytochemistry* (HARBORNE, J. B. and SWAIN, T., eds.), p. 47, Academic Press, New York.
[9] SANTAVY, F. (1966) *Abhandl. Deut. Akad. Wiss. Berlin Kl. Chem. Geol. Biol.* (3), 43.
[10] CROUT, D. H. G., BENN, M. H., IMASEKI, H. and GEISSMAN, T. A. (1966) *Phytochemistry* **5**, 1.
[11] CROUT, D. H. G. (1967) *J. Chem. Soc.* C, 1233.

in the biosynthesis of leucine from valine.[12] In *Datura* there is no evidence that the double bond of tiglate is formed by dehydration and it is likely that it is formed by direct dehydrogenation of 2-methylbutyrate (Scheme 1). The ability of *Datura* to dehydrate 3-hydroxy-2-methylbutyrate has not been tested yet. Certain organisms are able to produce tiglate by dehydration of this acid which in turn is formed by condensation of acetate and propionate under anaerobic conditions.[13] However, in *Datura* neither acetate nor propionate incorporates into the tigloyl esters.[2]

TABLE 2. COMPARATIVE FEEDING EXPERIMENTS IN *Datura*

Precursor	Isoleucine-[U-^{14}C]			2-Hydroxy-2-methylbutyric acid -[1-^{14}C]		
Alkaloid	IV	V	VII	IV	V	VII
Wt base (mg)	0·18	0·08	1·85	0·25	Trace	0·49
Spec. act. diluted picrates (dpm/mM $\times 10^{-4}$)	6·93	5·07	31·2	0	0	0
Calc. Spec. act. original bases (dpm/mM $\times 10^{-6}$)	3·87	3·94	2·19	0	0	0
% Specific incorporation	0·02	0·02	0·01	0	0	0

It has always been assumed that tiglic acid itself esterifies with tropine (I), tropan-3α,6β-diol (II) and teloidine (III) to give the corresponding esters, but this is not necessarily the case. Biosynthetic evidence so far collected could equally well allow 2-methylbutyric to be the esterifying acid, the dehydrogenation taking place at the ester level. As with previous data, the results are not consistent with our theory that the hydroxytropine nucleus may be



SCHEME 1. THE BIOSYNTHESIS OF TIGLIC ACID IN *Datura*.

[12] MATTOON, J. R. (1967) *The Biosynthesis of Amino Acids in Biogenesis of Natural Products* (BERNFELD, P., ed.), p. 24, Pergamon Press, Oxford.
[13] SAZ, H. J. and WEIL, A. (1960) *J. Biol. Chem.* **235**, 914.

formed by progressive hydroxylation of 3α-tigloyloxytropane at the C(6) and/or C(7) positions.[2,14] In the present series of experiments which also included the feeding of non-labelled 2-methylbutyrate, no toxicity symptoms were reported, as distinct from the now well-known leaf damage apparent in daturas fed with tiglate.

EXPERIMENTAL

Counting procedure. Duplicate samples, bases as picrates, acids as Na salts, were counted at finite thickness in a Labgear D4126 gas-flow counter making appropriate corrections for self-absorption and geometry.

Tracer compounds. L-Isoleucine-[U-^{14}C], Ba-[$^{14}CO_3$] and Na-[^{14}CN] were purchased from the Radiochemical Centre, Amersham.

Synthesis of (±)-2-*methylbutyric acid*-[1-^{14}C]. A mixture of 2-bromobutane (6 g) and Mg turnings (1·07 g) in dry THF (40 ml) was gently heated. The Grignard reagent thus produced was cooled in ice, rapidly stirred and carbonated in an enclosed, previously evacuated apparatus using silica-gel dried $^{14}CO_2$ produced by the gradual addition of 5 N HCl (50 ml) to 4 g Ba-[$^{14}CO_3$] (1 mCi). After 1 hr, N HCl (20 ml) was added to the complex and the 2-methylbutyric acid-[1-^{14}C] was extracted with 5% KOH (6 × 20 ml). The alkaline solution was washed ($CHCl_3$), acidified (conc. HCl), and extracted with Et_2O, evaporation of which gave 1·29 g 2-methylbutyric acid-[1-^{14}C] sp. act. 8·35 × 10^7 dpm/mM (48% radiochemical yield), b.p. 175° IR (film) identical with authentic compound.

Synthesis of (±)-2-*hydroxy*-2-*methylbutyric acid*-[1-^{14}C]. EtCOMe (0·73 g) and 0·5 g Na-[^{14}CN] (250 μCi) in 2 ml H_2O were cooled to −10° then cooled 95% H_2SO_4 (0·52 g) in 2 ml H_2O was added drop-wise with stirring, followed by 1 ml N NaOH. After 30 min vigorous stirring, the upper layer was separated and refluxed with conc. HCl (20 ml) for 3 hr. The hydrolysate was cooled, made alkaline with KOH pellets, washed with Et_2O, re-acidified with 50% H_2SO_4 and extracted into Et_2O. Evaporation of the solvent gave 2-hydroxy-2-methylbutyric acid-[1-^{14}C], which was recrystallized from acetone–light petrol., yield 0·7 g (58%) fine silver needles m.p. and m.m.p. 68°, sp. act. 3·39 × 10^7 dpm/mM.

Hydroponic culture. 3-week-old *Datura innoxia* plants were transferred to individual sand beds and fed from a central reservoir with solutions of the following composition per gallon: Solution A, $Ca(NO_3)_2$-(anhyd.) 14 g; Solution B, KH_2PO_4 6 g, $MgSO_4.7H_2O$ 10·8 g, $(NH_4)_2SO_4$ 1·8 g; Solution C, H_3BO_4 1·6 g, $MnSO_4$ 1·6 g, $ZnSO_4.7H_2O$ 1·6 g; Solution D, $FeSO_4.7H_2O$ 0·4 g; Final mixture: A 500 ml: B 500 ml: C 0·5 ml and D 5 ml.[15]

Administration of tracers. Twelve 3-month-old *Datura innoxia* plants, grown from seed in pots under glass were fed via cotton wicks inserted into the stems close to the soil with a solution of 48·4 mg 2-methylbutyric acid-[1-^{14}C], sp. act. 8·35 × 10^7 dpm/mM, previously neutralized with 0·1 N $NaHCO_3$ in 50 ml H_2O. The tracer was quickly taken up and the plants were watered through the wicks for 2 days, then normally until the termination of the experiment 5 days later. The roots and aerial parts were separately dried at 60° for 24 hr (wt of root 174 g). The culture fluid was drained from 10 three-month-old *Datura innoxia* plants, which had been grown in hydroponic culture from the age of 3 weeks. 2-Hydroxy-2-methylbutyric acid-[1-^{14}C] (119 mg) sp. act. 3·39 × 10^7 dpm/mM was neutralized with 0·1 N NaOH, diluted to 100 ml with H_2O and distributed to 5 plants. The remaining 5 plants were similarly fed via the roots with a solution containing L-isoleucine-[U-^{14}C] (25 μCi) sp. act. 1·93 × 10^{10} dpm/mM. Seven days later, during which time the plants were freely watered, the roots and aerial parts were collected and separately dried (wt isoleucine root 15·7 g; wt 2-hydroxy-2-methylbutyrate root 12·7 g).

Isolation of alkaloids. The powdered, moistened roots made alkaline with $Ca(OH)_2$ were extracted with Et_2O, and the extract, evaporated to *ca.* 2 ml was transferred to a kieselguhr column (10 g) containing 0·5 M phosphate buffer (5 ml) pH 6·8. Development of the column with light petrol. (120 ml) gave a mixture of 3α,6β-ditigloyloxytropane and 3α,6β-ditigloyloxytropan-7β-ol, as shown by TLC on aluminium oxide G (Merck) using Et_2O and I_2 in CCl_4 for detection. The ditigloyl esters were resolved by further column chromatography on kieselguhr (10 g) at pH 5·6, development with light petrol. giving the former base, Et_2O the latter. The bases were estimated by titration and converted to their picrates.[16] Continued development of the pH 6·8 column with Et_2O gave (in order), hyoscine, 3α-tigloyloxytropane; and $CHCl_3$ gave hyoscyamine. All were isolated as the picrates and recrystallized from EtOH–H_2O.

Degradation of the alkaloids from the 2-*methylbutyric acid*-[1-^{14}C] *feed.* 3α,6β-Ditigloyloxytropane base, sp. act. 5·25 × 10^4 dpm/mM, recovered from the picrate m.p. 151° (23·73 mg) with NH_4OH–$CHCl_3$, was dissolved in *ca.* 1 ml EtOH and hydrolysed by boiling with 5% $Ba(OH)_2$ (5 ml) in a sealed tube for 3 hr, when after cooling and acidification with 50% H_2SO_4, the tiglic acid was obtained by extraction with Et_2O and counted as the Na salt, sp. act. 4·57 × 10^4 dpm/mM (87% recovery). The remaining acid hydrolysate

[14] LEETE, E. (1972) *Phytochemistry* **11**, 1713.
[15] YOUNGKEN, H. W. and SCIUCHETTI, L. A. (1958) *J. Am. Pharm. Assoc. Sci. Educ.* **47**, 803.
[16] EVANS, W. C. and WOOLLEY, J. G. (1965) *J. Chem. Soc.* 910.

was neutralized ($BaCO_3$), centrifuged and the supernatant liquid evaporated to dryness. The residue was redissolved in *ca.* 0·5 ml H_2O and addition of Na picrate gave crude tropan-3α,6β-diol picrate which was recrystallized from H_2O, m.p. and m.m.p. 240° (inactive).

Sodium tiglate (2 mg) in H_2O (3 ml) was acidified with a little 10% H_2SO_4 and hydrogenated over Pt_2O at room temp. and pressure. The solution was basified (KOH), filtered, re-acidified (50% H_2SO_4) and extracted with Et_2O, which after evaporation to *ca.* 0·5 ml was cooled in ice and mixed with 5 ml 95% H_2SO_4. Sodium azide (5 mg) was added and the temp. was gradually raised to 80° over a 1 hr period, during which time a slow stream of CO_2 free N_2 was drawn through the apparatus.[17] The liberated CO_2 from the decarboxylation was collected in freshly prepared 5% $Ba(OH)_2$ and the Ba CO_3 ppt washed 3× with CO_2 free H_2O, 2× with Me_2CO and gently dried giving 2·09 mg $BaCO_3$ (74%) sp. act. $4{\cdot}82 \times 10^4$ dpm/mM (92% radiochemical yield based on the original picrate).

The 3α,6β-ditigloyloxytropan-7β-ol was treated in a similar manner. The base, sp. act. $5{\cdot}79 \times 10^4$ dpm/mM, recovered from 19·76 mg diluted picrate m.p. 184°, was hydrolysed to tiglic acid ($5{\cdot}2 \times 10^4$ dpm/mM) and inactive teloidine, picrate m.p. 223° (8 mg). Sodium tiglate (3 mg) was decarboxylated and the resultant CO_2 collected as $BaCO_3$ (3·06 mg) sp. act. $5{\cdot}2 \times 10^4$ dpm/mM (89% yield based on the sp. act. original picrate).

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[17] FINLAYSON, A. J. (1966) *Can. J. Biochem.* **44,** 397.