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FORMATION OF *N*-MALONYL-L-TRYPTOPHAN IN WATER-STRESSED TOMATO LEAVES

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Key Word Index *Lycopersicon esculentum*; Solanaceae; tomato; biosynthesis; *N*-malonyl-L-tryptophan; amino acid malonylation.

Abstract—Excised tomato (*Lycopersicon esculentum* Mill. cv. Castlemart) leaves subjected to water loss of 20 to 30% of their fresh weight increased dramatically both tryptophan and *N*-malonyl-L-tryptophan (M-L-Trp) content. The identity of M-L-Trp was confirmed by chiral TLC, by oxidation of the released Trp residue with L-amino acid oxidase, and by mass spectrometry. Wilted leaves administered exogenous D- or L-Trp formed the corresponding malonyl-conjugate, *N*-malonyl-D-tryptophan (M-D-Trp) or M-L-Trp, respectively. Crude leaf extracts were able to *N*-malonylate both L- and D-Trp *in vitro*. The activity was higher in mature leaves than in immature leaves and was increased by water stress. Since most of the previous reports have assumed that all endogenous MTrp is M-D-Trp without actual chiral identification, our present results suggest that the current literature concerning the natural occurrence of M-D-Trp needs to be re-examined.

INTRODUCTION

Malonyltryptophan (MTrp) was first identified in plants by Good and Andreae [1] as a metabolite that was formed after spinach leaves or pea epicotyls were fed a tryptophan-containing solution, presumed to be a racemic mixture of D,L-Trp. They also reported that MTrp occurred naturally in tomato fruits and etiolated pea epicotyls [1]. The enantiomeric identity of MTrp was determined when Zenk and Scherf [2] demonstrated that naturally occurring MTrp from apple fruits and *Caragana arborescens* seeds is in the D-stereoconfiguration. This was done by hydrolysing the MTrp with acid and then oxidizing the tryptophan residue with D-amino acid oxidase. After administering D-Trp to 148 different angiosperm species, *N*-malonyl-D-tryptophan (M-D-Trp) was formed in 134 species. There was no MTrp formed after L-Trp incubation [2]. Following this report, all the investigators who have identified MTrp have assumed it to be the D-enantiomer without confirming the true chiral configuration, regardless of whether L-Trp and or D-Trp was administered to the tissues. M-D-Trp has been reported to occur in a wide range of higher plants. The ability to conjugate exogenous D-Trp into M-D-Trp has been reported in some species that do not accumulate MTrp [1, 3–6]. The enzyme that is responsible for this activity is D-Trp malonyltransferase. Matern *et al.* [7] demonstrated a D-Trp malonyltransferase from peanut that was specific for D-Trp and did not malonylate L-Trp.

There has been much recent interest in M-D-Trp metabolism following reports that M-D-Trp and D-Trp may serve as indole-3-acetic acid (IAA) precursors and that M-D-Trp levels increase during wilting. It has been proposed that L-Trp is first racemized to D-Trp, which is then *N*-malonylated to form M-D-Trp. This process would create a storage pool of D-Trp and prevent the use of D-Trp in protein synthesis or in the synthesis of Trp-derived secondary metabolites. At the appropriate developmental time, the M-D-Trp would be cleaved back to D-Trp which would be metabolized into IAA [8–13].

Rekoslavskaya *et al.* [14] have reported that the content of L-Trp and M-D-Trp increases greatly during wilting (water stress) of excised tomato leaves. Since they employed conventional chromatography, which did not distinguish between the enantiomers of MTrp, M-D-Trp and *N*-malonyl-L-tryptophan (M-L-Trp), the absolute configuration of malonyltryptophan could not be assigned. They assumed the MTrp was M-D-Trp on the basis of their observation that D-Trp was converted in soybean callus tissue into M-D-Trp, but L-Trp was not conjugated with malonic acid. Since M-D-Trp might be an IAA precursor, its accumulation during water stress would prepare the plant for auxin synthesis upon re-watering [14].

Originally, Marumo and Hattori reported the isolation from immature pea seeds of 4-chlorotryptophan,

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which they assigned to be in the D-stereoconfiguration based on its ability to rotate the plane of polarized light. However, they recently reported that the 4-chlorotryptophan from immature pea seeds is in fact the L-stereoisomer. They also reported that *N*-malonyl-chlorotryptophan is also the L-stereoisomer. The chiral configuration was determined by hydrolysing the MTrp and analysing the Trp residue using an HPLC equipped with a chiral column or using cellulose TLC [15–17]. There has been only one report describing the occurrence of M-L-Trp: Song *et al.* isolated M-L-Trp from cell suspension culture of *Ephedra distachya* [18].

In this investigation, we studied the accumulation and chiral property of malonyltryptophan, which had been reported to be M-D-Trp, in tomato leaves under water stress [9]. We also examined the ability of the tissue to *N*-malonylate exogenously administered L- and D-Trp, as only D-Trp had been reported to be malonylated.

RESULTS

Incorporation of D- or L-[^{14}C]-Trp in vivo

Either D- or L-[^{14}C]-Trp was administered to water-stressed tomato leaves, and the leaves were extracted as described. After the radioactive compounds were separated by paper chromatography (PC), two major peaks were revealed (Fig. 1). One ($R_f = 0.31$) co-migrated with free Trp while the other ($R_f = 0.71$) with MTrp. Both spots reacted with Ehrlich's reagent and gave the same colour as the standards. The spot corresponding to Trp reacted chromogenically with ninhydrin, indicating a free amino group, but the spot corresponding to MTrp did not. As shown in Fig. 1, both D- and L-Trp were metabolized into MTrp in tomato leaves. However, in wheat leaves only D-Trp was malonylated.

Cation-exchange chromatography was employed to measure rapidly and accurately the amount of MTrp formed under a variety of conditions. When the extract from tomato leaves incubated with either D- or L-Trp was passed through a cation-exchange column, the free Trp was retained on the column, but the MTrp was recovered in the effluent fraction. Thus, the amount of radiolabel incorporated into MTrp could be quantified easily by assaying the radioactivity in the effluent fraction. In tomato leaves administered with D-[^{14}C]-Trp, less than 20% of the radioactivity taken up was found in the EtOH-insoluble fraction after 72 hr. In contrast, leaves administered L-[^{14}C]-Trp had greater than 50% of their radioactivity in the EtOH-insoluble fraction. It is not surprising that so much of the L-[^{14}C]-Trp is insolubilized, as it should be readily incorporated into proteins. When equivalent amounts of D- or L-[^{14}C]-Trp were fed to the tomato leaves, more MTrp was formed from D-Trp than from L-Trp (Fig. 1).

Identification of the naturally occurring malonyltryptophan

As described above, when either D- or L-Trp was administered to tomato leaves, MTrp was formed. Previous reports had indicated that only D-Trp could be converted into MTrp in apple fruit [2], wheat seedling [3] and tomato leaves [18]. We wished to determine whether L-Trp was malonylated directly or whether it was racemized into D-Trp before being malonylated. Notably, the MTrp purified from L-Trp administered leaves did not migrate with authentic M-D-Trp on chiral TLC plates (Table 1), suggesting that the metabolite might be M-L-Trp. We have, therefore, characterized the MTrp produced in tomato leaves administered exogenous L-Trp.

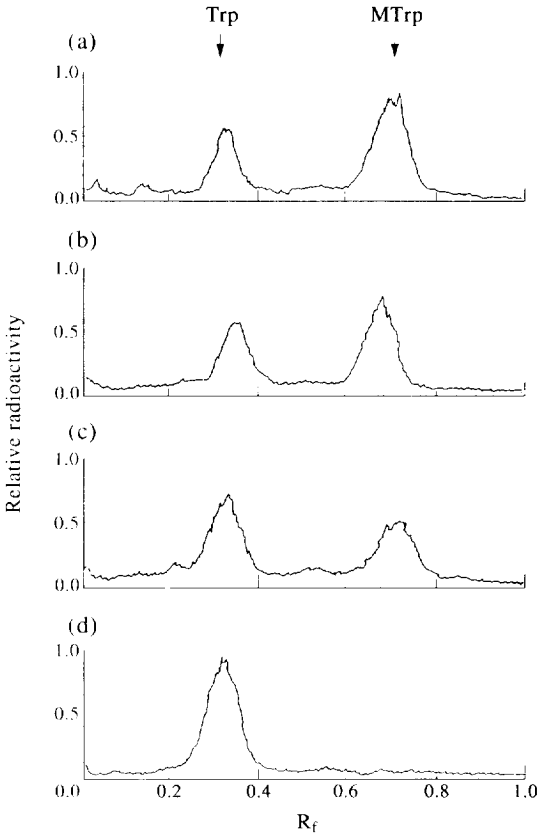

Fig. 1. Radioactivity scans of paper chromatograms of extracts from water-stressed tomato leaves following administration for 96 hr with (A) D-[^{14}C]-Trp or (B) L-[^{14}C]-Trp or from wheat leaves after administering for 96 hr with (C) D-[^{14}C]-Trp or (D) L-[^{14}C]-Trp; the amounts of radioactive tryptophan administered were all 185 nmol and 370 kBq.

As we did not have authentic M-L-Trp to use as a standard, we took several approaches to prove that our compound was M-L-Trp. First, we showed that it was an *N*-malonyl-Trp. Using M-D-Trp as a standard, we found that the putative M-L-Trp co-migrated with M-D-Trp on PC, and on silica gel TLC, using two solvent systems: 1-BuOH–HOAc–H_2O (60:15:25) and 1-PrOH–NH_4OH (7:3) (see Table 1). In addition, the putative M-L-Trp reacted chromogenically with Ehrlich's reagent, which detects indole compounds, but not with ninhydrin. The identity as MTrp was further confirmed by mass

Table 1. R_f of D-Trp, L-Trp, M-D-Trp, and the putative MTrp produced by enzymic reaction or extracted from leaf tissue following feeding with D- or L-Trp

	Authentic compounds			Metabolites after feeding	
	D-Trp	L-Trp	M-D-Trp	D-Trp	L-Trp
Paper chromatography					
1-BuOH–HOAc–H_2O (4:1:5)	0.31	0.31	0.71	0.71	0.71
TLC, silica gel					
1-BuOH–HOAc–H_2O (60:15:25)	0.42	0.42	0.50	0.50	0.50
1-PrOH–NH_4OH (7:3)	0.43	0.43	0.26	0.26	0.26
TLC, chiral					
MeOH–CH_3CN–H_2O (50:200:50, v/v)	0.51	0.61	0.71	0.71	0.66
1 mM $Cu(OAc)_2$ in 5% MeOH	0.16	0.16	0.83	0.83	0.62

Table 2. Oxidation by L-amino acid oxidase of free Trp fraction and the hydrolysate of M-Trp isolated from tomato leaves*

	Radioactivity (Bq)		
	Before oxidation	After oxidation	Oxidation (%)
Standard L-Trp	1443	113	92
Standard D-Trp	1382	1484	0
Free Trp fraction	571	53	91
Hydrolysate of M-Trp	469	33	93

* The radioactive Trp and M-Trp in extract of L-[^{14}C]-Trp-administered tomato leaves were separated by PC. The M-Trp fraction was hydrolysed with $Ba(OH)_2$. The standard Trp, free Trp fraction and the hydrolysate of M-Trp were treated with L-amino acid oxidase and the resulting solutions were subjected to PC again. The radioactive spot corresponding to Trp in each sample (before and after oxidation) was measured

spectrometry (MS). The authentic M-D-Trp was compared to both the M-D-Trp isolated from tomato leaves which had been administered D-Trp, and to the putative M-L-Trp from leaves which had been administered L-Trp. The presence of MTrp was verified by MS of the methyl ester of MTrp using the solid-probe introduction technique. Analysis by capillary GC–MS was also attempted, but this approach was unsuccessful because the ester derivative decomposed during GC separation even when cold on-column injection was employed. Although MS analysis cannot distinguish the stereoconfiguration of the compound, the presence of MTrp was confirmed by the coincident appearance of key ions at m/z 318 [M^+], 201 and 130 (data not shown). This mass spectrum is essentially identical to that reported by Elliott [3] for MTrp. All three compounds (the authentic M-D-Trp, the M-D-Trp isolated after incubation with D-Trp and the putative M-L-Trp isolated after incubation with L-Trp) had identical mass spectra (data not shown), leading us to conclude that all three were malonyltryptophan.

Chiral TLC plates are able to separate enantiomers of amino acids. Using two different solvent systems, we found that the M-D-Trp from D-Trp-administered leaves co-migrated with our authentic M-D-Trp. However, the conjugated tryptophan from L-Trp-administered leaves migrated slower than the M-D-Trp (Table 1). Furthermore, after base-catalysed hydrolysis of the putative M-L-Trp with $Ba(OH)_2$, the released free amino acid was confirmed to be Trp by PC. Finally, this released tryptophan was confirmed to be the L-enantiomer based on treatment with L-amino acid oxidase followed by paper radiochromatography (Table 2). We conclude that the malonyltryptophan formed when tomato leaves are incubated with L-Trp is *N*-malonyl-L-tryptophan.

Appearance and accumulation of N-malonyl-L-tryptophan in water-stressed tomato leaves

Having demonstrated the formation of M-D-Trp from D-Trp and M-L-Trp from L-Trp after exogenous tryptophan administration *in vivo*, the question arises as to which form of MTrp exists in the water-stressed tomato leaf. To answer this question, tomato leaves, harvested from plants grown in the field, were excised and either immediately extracted with EtOH (turgid control) or allowed to wilt at room temperature for one day until they had lost 23% of their fresh weight before EtOH extraction. The free Trp and the MTrp were analysed as described.

The free tryptophan in both unstressed and stressed leaves was the L-enantiomer as indicated by chiral TLC, with the L-Trp content significantly higher in stressed leaves (Table 3). MTrp accumulated to high levels (397 nmol g^{-1}) in stressed leaves, while there was relatively little (0.3 nmol g^{-1}) in the unstressed controls. The mass spectrum of the methylated MTrp isolated from stressed leaves was identical to that of the methylated authentic M-D-Trp (data not shown). When the MTrp was subjected to chiral TLC, its mobility was different from that of authentic M-D-Trp, but indistinguishable from that of M-L-Trp formed after administering L-Trp. However, no detectable D-Trp or M-D-Trp was observed in either unstressed or stressed leaves. These results demonstrate that water stress significantly increased L-Trp content and resulted in a massive accumulation of M-L-Trp in tomato leaves.

We have compared, therefore, the capability of excised turgid leaves and water-stressed tomato leaves to malonylate exogenously administered, radioactive L- or D-Trp. The results in Table 4 show that water-stressed leaves were more effective in malonylating both D- and L-Trp than turgid leaves. Although the M-L-Trp accumulated at a lower level than the M-D-Trp, the accumulation of M-L-Trp could be underestimated because the presence of endogenous L-Trp could have diluted the incorporation. Also, L-Trp has many metabolic fates, whereas D-Trp is primarily *N*-malonylated.

Table 3. The influence of water stress on L-Trp and M-L-Trp content in excised tomato leaves

	Free L-Trp*	M-L-Trp*
	(nmol g^{-1} fr.wt)	
Turgid	1.5	0.3
Water-stressed	22.9	397

* Excised leaves were allowed to lose 23% of their fresh weight for 1 day (water-stressed) and then were incubated in a humid chamber for 3 days. The L-Trp and M-L-Trp were then extracted and quantified by reacting with Ehrlich's reagent.

Table 4. The incorporation of radiolabelled D-Trp into M-D-Trp and radiolabelled L-Trp into M-L-Trp in turgid or water-stressed tomato leaves

	M-L-Trp*	M-D-Trp*
	(Bq)	
Turgid	338	1270
Water-stressed	457	1970

* Excised tomato leaves were incubated at room temperature until they lost 30% of their fresh weight (water-stressed). They were then incubated with 370 kBq of either L-[^{14}C]-Trp or D-[^{14}C]-Trp, the resulting malonyl conjugate was extracted, and the radioactivity was measured.

In vitro *Trp malonyltransferase activity*

Because the tomato leaves were capable of malonylating both D- and L-Trp provided exogenously, and because L-Trp and M-L-Trp accumulated naturally, we have investigated the enzyme system responsible for the Trp malonyltransferase activity. A crude enzyme preparation was made from water-stressed tomato leaves and its activity assayed according to that employed for 1-aminocyclopropane-1-carboxylic acid (ACC) malonyltransferase [20, 21]. This enzyme used malonyl-CoA as the malonyl donor. The enzyme preparation converted both D- and L-Trp into MTrp (Table 5). The product of the enzymic reaction had a mass spectrum identical to that of the authentic M-D-Trp. The tryptophan malonyltransferase activity was constitutively present in tomato leaf tissue, but it was further promoted by water stress, and the enzyme activity was higher in the mature leaf than in the young one (Table 6). Purification and characterization of this enzyme are described in the accompanying paper.

Table 5. D-Trp, L-Trp and ACC malonyltransferase activities of various preparations isolated from water-stressed tomato leaves

Fractionation	D-Trp	L-Trp	ACC
	(nmol mg^{-1} h^{-1})		
Crude extract	16.7	27.8	< 0.05
30% $(NH_4)_2SO_4$ ppt.	9.8	15.0	< 0.05
30–60% $(NH_4)_2SO_4$ ppt.	39.0	58.8	0.05
Supernatant	0	0	0

Inhibition by ACC

ACC, the immediate precursor of the plant hormone ethylene, is not chiral. In plant tissues, ACC is widely *N*-malonylated into 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) [22, 23]. Since the conversion of ACC into MACC is competitively inhibited by many

Table 6. The influence of tomato leaf maturity and water-stress condition (20% loss of fresh weight for 1 day) on the extractable D- or L-Trp malonyltransferase activity

Tissue	Treatment	Enzyme specific activity (nmol $mg^{-1} h^{-1}$) D-Trp	L-Trp
Expanding leaves	Turgid	12.4	23.8
	Water-stressed	31.6	77.2
Fully grown leaves	Turgid	29.8	48.4
	Water-stressed	44.6	84.1

D-amino acids and the conversion of D-amino acids into *N*-malonyl-D-amino acids is competitively inhibited by ACC, it is generally believed that ACC malonyltransferase is a D-amino acid malonyltransferase [20, 21, 24, 25]. However, ACC failed to interfere with the conjugation of either D- or L-Trp in the present *in vivo* and *in vitro* (see the accompanying paper) tomato system, indicating that different enzymes are responsible for the malonylations of Trp and ACC.

DISCUSSION

Although *N*-malonyl conjugates of D-amino acids (especially Trp) have been reported in a diverse variety of plants (for reviews see refs [5, 26]), either as natural constituents or after administer and metabolism of exogenously supplied D-amino acids, the chiral identity of many of these malonyl-amino acids was not examined. It has often been assumed the MTrp was M-D-Trp. We confirm here that D-Trp is malonylated when it is administered to tomato or wheat leaves. However, in tomato leaves under water stress, we did not find any M-D-Trp, and therefore surmise that D-Trp is not formed. In the present study using tomato leaf tissue, we have shown that exogenously administered L-Trp can be malonylated to form *N*-malonyl-L-tryptophan, and this activity was also demonstrated *in vitro*. Rekoslavskaya and co-workers reported the formation of M-D-Trp in water-stressed tomato leaves [6, 14, 27]. However, they did not confirm the absolute configuration. We found that M-L-Trp accumulates in water-stressed tomato leaves. Therefore, we believe that the MTrp which they identified was M-L-Trp, and not M-D-Trp.

No MTrp could be measured in wheat leaves which had been administered L-Trp, indicating that the capability to malonylate L-Trp may be limited to certain plant species or organs or to certain developmental stages. Recently, Song *et al.* [18] isolated M-L-Trp from cell suspension cultures of *Ephedra distachya*. Therefore, the existence and distribution of M-L-Trp in other plants should be examined. Indeed, MTrp was observed in wheat roots by Elliott after he had administered radiolabelled L-Trp, but he did not investigate the stereoconfiguration of this MTrp [3].

In the present study, using chiral TLC, the identification of the enantiomers of MTrp was simple and rapid. The chiral TLC appears to be a useful technique for examining the stereoconfiguration of MTrp in different tissues.

Recently, Rekoslavskaya and co-workers [5, 19, 28] reported that Trp and M-D-Trp increased dramatically in the leaf tissue of many plant species during wilting, and the amount of M-D-Trp accumulated was correlated with the degree of leaf dehydration and the duration of the water stress. However, the stereoconfiguration of MTrp was not determined. On the basis that the conjugate is M-D-Trp, they have hypothesized that L-Trp is racemized to D-Trp before conjugation with malonic acid. Therefore, they have advanced the view that the regulatory step in water-stress-induced MTrp synthesis is the formation of D-Trp from L-Trp. They found that the malonyltransferase activity is constitutively present in both wilted and turgid leaves [29]. Our results indicate that, in tomato leaves, only M-L-Trp is formed naturally, and this is greatly promoted by water stress by increasing the level of L-Trp malonyltransferase. However, in Rekoslavskaya's study, all species accumulated L-Trp. If the tryptophan malonyltransferase were present constitutively, then we would expect an increase in M-L-Trp in all the plants which accumulated L-Trp. This was not the case. A thorough study of which enantiomers are accumulated and the stereospecificity of the respective Trp malonyltransferase in different species will help to understand this complex phenomenon.

It is interesting that M-L-Trp accumulated only during wilting, although the malonyltransferase activity is constitutive in tomato leaves. Several factors may be responsible for this phenomenon. Aside from an increase in Trp malonyltransferase activity by water stress, another factor is that the K_m value for L-Trp is high for tryptophan malonyltransferase (1.9 mM; see accompanying paper). Since the content of L-Trp in the turgid tissue is normally low, but significantly increases during water stress (see Table 3), this would result in the promoted malonylation and accumulation of M-L-Trp. It is to be noted that the increase in enzyme activity, however, is small compared to the rise in M-L-Trp concentration. The compartmentalization of either L-Trp or the malonyltransferase might be affected by water stress as might the utilization of L-Trp by other enzymes. The physiological role of M-L-Trp remains to be elucidated.

It is interesting to note that the accumulation of malonyl conjugates of L-Trp and ACC are similarly enhanced by wilting in leaves [30–32]. The MACC accumulation is caused by a large increase in its precursor ACC, while the ACC malonyltransferase is present constitutively. Similarly, the increase in the Trp malonyltransferase activity is small relative to the increase in M-L-Trp concentration (Table 3). In addition to being incorporated into protein, tryptophan is thought to be a precursor to the plant hormone auxin. Recently, Ludwig-Müller and Hilgenberg [33] reported a membrane enzyme preparation from Chinese cabbage seedlings that catalysed the conversion of radiolabelled M-D, L-Trp

into indole-3-acetaldoxime (IAOX), a possible precursor of IAA. As the incorporation of radioactivity into IAOX was inhibited by cold M-L-Trp, but not cold M-D-Trp, they concluded that M-L-Trp was the substrate. Therefore, M-L-Trp might be important for IAA synthesis after re-watering and might aid in the recovery from water stress.

EXPERIMENTAL

Plant materials and chemicals. Tomato (*Lycopersicon esculentum* Mill. cv. Castlemart) plants were grown in the greenhouse or the field. Wheat (*Triticum aestivum* L.) seedlings were grown in a growth chamber (20°, 16 hr day) for 7 days, and the first leaves were used [32]. For water-stress induction, tomato and wheat leaves were cut and allowed to lose water under ambient room conditions and then incubated in a humid chamber as described by Rekoslavskaya and Gamburg [28]. The water-stressed leaves were used for extraction of Trp and MTrp and for prepn of the enzyme. For the administration of exogenous Trp to wilted leaves, the petiolar end of tomato leaves or the cut end of wheat leaves was immersed in a test soln containing radioactive L-Trp, D-Trp or ACC (370 kBq) in a total volume of 50–100 μl. After absorbing the soln, the leaves were incubated in a humid chamber for the indicated time period.

Malonyl-CoA was purchased from Sigma. L-[*methylene*-^{14}C]-Trp (1.98 GBq mmol^{-1}) was from DuPont. D-[*methylene*-^{14}C]-Trp (1.98 GBq mmol^{-1}) was from Amersham. [2,3-^{14}C]-ACC (2.93 GBq mmol^{-1}) was from CEA. *N*-Malonyl-D-tryptophan was synthesized by Chem Biochem Research.

Extraction of malonyltryptophan. The extraction procedure was as described by Rekoslavskaya and Gamburg [27] and Elliott [3]. Briefly, the leaves were extracted with hot 80% EtOH containing 0.02% Na diethyldithiocarbamate. The EtOH was evaporated *in vacuo*. The remaining aq. soln was adjusted to pH 9 with $NaHCO_3$ and the pigments were removed by extracting with EtOAc. The resulting aq. soln was then adjusted to pH 2 with HCl, and extracted with 1/2 volume (×10) of EtOAc. The EtOAc extracts were combined and concd *in vacuo* at 30°. The concentrated EtOAc solution was extracted with 1/2 volume (×4) of 5% $NaHCO_3$. The $NaHCO_3$ soln were combined and acidified to pH 2 and re-extracted with EtOAc. MTrp was extracted from the final acidified solution by Et_2O. The Et_2O fraction was concd to dryness *in vacuo*, and the residue was dissolved in 80% MeOH for further purification.

The aq. soln (pH 9) after extraction with EtOAc, was passed through a Dowex 50 × 8 – 100 cation-exchange column, H^+ form, which adsorbed the free Trp. The free Trp was eluted from the column with 2 N NH_4OH and concd to dryness *in vacuo*. The residue was dissolved in 80% MeOH for further purification.

In the metabolism experiments, the leaves were extracted with 80% EtOH as described above. The extract was concd to dryness *in vacuo*. The residue was dissolved in H_2O (0.5–1.0 ml), and the soln was analysed directly by PC and TLC, or it was passed through a column (5 × 100 mm) of Dowex 50 × 8 – 100 cation-exchange resin, H^+ form. The effluent containing the MTrp was concentrated to dryness *in vacuo*. The residue was dissolved in 80% MeOH for radioactive measurement and further study.

Chromatography. For PC, Whatman No. 1 and No. 3 papers were used, and developed in 1-BuOH–HOAc–H_2O (4:1:5). For TLC, silica gel plates with fluorescent indicator were developed in 1-BuOH–HOAc–H_2O (60:15:25) or 1-PrOH–conc. NH_4OH (7:3). The chiral TLC plates manufactured by Macherey-Nagel were purchased from Dychrom. The solvent systems used were MeOH–CH_3CN–H_2O (50:200:50) [34], or 1 mM $Cu(OAc)_2$ in 5% MeOH [35]. The Trp and MTrp spots from PC and TLC were visualized under UV or by spraying with Ehrlich's reagent for indole-containing compounds (1% *p*-dimethylaminobenzaldehyde in HCl–MeOH (1:3)) [36], and free Trp was identified by spraying with ninhydrin (0.5% in 60% EtOH).

Hydrolysis and oxidation. The appropriate spots containing free Trp or MTrp, following TLC or PC, were removed from the chromatograms and eluted with 80% MeOH. The MeOH extracts were evapd *in vacuo* and the residue was dissolved in a small volume of water. The aq. soln was hydrolysed with $Ba(OH)_2$ as described by Elliott [3]. After removing the $Ba(OH)_2$ by bubbling with CO_2 followed by centrifugation, the solution was incubated with L-amino acid oxidase and catalase in 100 mM K-Pi buffer (pH 7) at 30° overnight. Before and after oxidation the solutions were subjected to PC.

MS analysis. The authentic M-D-Trp and the purified MTrp from PC or TLC were methylated with CH_2N_2, and the resulting methyl esters were analysed by MS using the solid-probe introduction technique. A 2 μl aliquot of an ether soln containing the methyl ester was added to a glass capillary tube and the solvent was allowed to evaporate at 25°. The tubes were introduced into a Trio-2 mass spectrometer (VG Masslab) and heated at 300° min^{-1} to a final temp. of 350°. Mass spectra were obtained using 70 eV electron ionization.

Enzyme prepn and assay. Extraction of the enzyme was carried out at 4°. Tomato leaves were homogenized with an extraction buffer containing 0.4 M K-Pi (pH 8.0), 100 mM KCl, 1% β-mercaptoethanol, 10 mM EDTA, and 5% PVPP. The homogenate was passed through 4 layers of cheese cloth and centrifuged for 30 min at 28 000 *g*. The supernatant was subjected to $(NH_4)_2SO_4$ pptn. The pellet from 30–60% saturation was resuspended in 25 mM Tris buffer (pH 8.0) containing 0.1% β-mercaptoethanol, 10 mM EDTA, and 10% glycerol. The solution was then dialysed against the same buffer overnight. The dialysed prepn was used for the enzyme activity assay.

For the enzyme assay, a standard reaction mixture consisted of 0.1 M K-Pi buffer (pH 8.0), 0.1 M KCl, 2 mg ml^{-1} BSA, 0.8 mM malonyl-CoA, 0.5 mM D- or

L-Trp or ACC (370 kBq) in a total volume of 50 μl. Incubation was carried out at 40° for 1 hr. To stop the reaction, 50 μl of 0.2 N HOAc was added and the soln was passed through a small column (bed volume = 0.5 ml) of Dowex 50 × 8 — 100 cation-exchange resin, H^+ form, to absorb the free unreacted Trp or ACC. The radioactivity in the effluent containing the malonylated amino acid was measured in a liquid scintillation counter (Beckman LS 6000IC).

For chemical identification of the reaction products, the enzyme reaction mixture was passed through a column of Dowex 50 × 8 — 100 cation-exchange resin, H^+ form, and the effluent was collected and evapd to dryness. The residue was dissolved in 80% MeOH and analysed by PC and TLC as described above.

Determination of radioactivity in TLC and paper chromatograms. The zones of radioactivity on paper or thin-layer chromatograms were located by using a Radiochromatogram Scanner (Packard Instrument). For quantitative determination, the radioactive zones were removed from the chromatograms and assayed by a liquid scintillation counter.

Determination of tryptophan and malonyltryptophan. The content of Trp or MTrp was determined colorimetrically as described by Yang [37]. Ehrlich's reagent (100 μl), which was prepd by mixing 0.2 ml 3% *p*-dimethylaminobenzaldehyde in 2 N H_2SO_4 and 2.8 ml 18 N H_2SO_4, was added to the sample solution in a total volume of 1 ml. After incubation at room temp. for 30 min, 20 μl of 0.04% $NaNO_2$ was added. After 20 min, the absorbance at 600 nm was read.

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