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PARTIAL PURIFICATION AND CHARACTERIZATION OF TRYPTOPHAN *N*-MALONYLTRANSFERASE FROM TOMATO LEAVES

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; biosynthesis; malonyltryptophan; amino acid malonylation.

Abstract—An enzyme preparation that catalyses the malonyl-CoA-dependent malonylation of both D- and L-Trp to form the corresponding *N*-malonyltryptophan (MTrp) was partially purified over 500-fold from tomato leaves. The ratio of the two enzyme activities using D- or L-Trp as the substrate remained roughly constant during the purification steps, indicating that the same enzyme is likely to be responsible for both reactions. While the enzyme utilizes some D- and L-amino acids as its substrates or inhibitors, the ethylene precursor 1-aminocyclopropane-1-carboxylate did not serve as a substrate or an inhibitor. The molecular weight was estimated by gel filtration to be 48 kDa. The K_m values for L- and D-Trp were 1.9 and 1.0 mM, respectively. The K_m values for malonyl-CoA were 1.3 and 0.5 mM, respectively, when L- and D-Trp were employed as the substrates. The enzyme kinetics suggest a 'ping-pong' mechanism.

INTRODUCTION

Since *N*-malonyltryptophan (MTrp) was identified by Good and Andreae [1] in spinach leaves first incubated with a racemic tryptophan solution and then as a naturally occurring metabolite, there has been sporadic interest in MTrp. However, interest has intensified during the last 10 years stemming from reports that MTrp may be an auxin precursor [2–5] and that it may be a stress metabolite, because it accumulates during wilting [6–8].

Zenk and Scherf isolated naturally occurring MTrp from apple fruits and *Caragana arborescens* seeds, and showed that it is in the D-stereoconfiguration, by hydrolysing the MTrp with acid and then oxidizing the Trp residue with D-amino acid oxidase. They then analysed 148 different angiosperm species which had been administered D-Trp and found *N*-malonyl-D-tryptophan (M-D-Trp) in 134 of them [9]. Most subsequent reports have assumed that MTrp was M-D-Trp without reconfirming the absolute configuration [4, 5, 8, 10–15].

Rekoslavskaya and co-workers found that D-Trp and M-D-Trp had auxin-like activity in a number of different tissue culture systems, including soybean, tomato and blackberry. When D-Trp was incubated with these cell cultures, it was converted into M-D-Trp. Furthermore, when radiolabelled M-D-Trp was administered to soybean cell cultures, it was incorporated into indole-3-acetic acid (IAA), indicating that M-D-Trp could serve as a precursor to IAA [4, 5, 16].

Rekoslavskaya *et al.* [17] studied the effects of wilting (water stress) on M-D-Trp accumulation in 33 different species (from 12 different families). M-D-Trp accumulated in 20 of the species, and those in which it did not, nevertheless, had the capability to malonylate exogenous D-Trp.

Markova *et al.* [13] reported that there was a marked increase in free L-Trp and in M-D-Trp in excised tomato leaves subjected to water stress. Furthermore, they found a correlation between the amount of M-D-Trp accumulated and the extent of wilting. In addition, the amount of free L-Trp increased. Moreover, they reported that radiolabelled L-Trp was incorporated into M-D-Trp [13]. However, the absolute stereoconfiguration of MTrp was not determined.

We have recently found that when exogenous D- or L-Trp was applied to tomato leaves, the corresponding malonyl-conjugate, M-D- or M-L-Trp, was formed. Furthermore, when tomato leaves were subjected to wilting, it was M-L-Trp, not M-D-Trp, which accumulated (see accompanying paper). We also demonstrated that extracts from tomato leaves are capable of catalysing the malonylation of D- or L-Trp into M-D- or M-L-Trp, respectively. Although there have been reports describing various malonyltransferases [18], to date there has been no report describing an enzymic *N*-malonylation of L-Trp. In the present study we undertook a partial purification and characterization of this enzyme, tryptophan malonyltransferase.

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Table 1. Partial purification of tryptophan malonyltransferase from 1.2 kg of stressed tomato leaves

Purification steps	Proterin (mg)	Total activity (μmol h^{-1})		Specific activity (μmol mg^{-1} h^{-1})		Purification fold		Ratio of specific activity
		D-Trp	L-Trp	D-Trp	L-Trp	D-Trp	L-Trp	D-Trp : L-Trp
Crude extract	9266	100	166	0.011	0.018	1.0	1.0	0.60
$(NH_4)_2SO_4$ ppt.	4718	96	153	0.020	0.032	1.9	1.8	0.63
Phenyl Sepharose	868	53	102	0.061	0.12	5.7	6.6	0.52
DEAE Sepharose	74.8	35	64	0.47	0.86	43	48	0.55
Econo-Pac Blue	7.81	34	53	4.4	6.8	403	380	0.64
Superose 12	1.19	6	12	5.0	10.1	470	560	0.50

RESULTS

Partial purification of tryptophan malonyltransferase

We undertook a six-step purification, as summarized in Table 1. After homogenization and $(NH_4)_2SO_4$ precipitation, we employed two steps of conventional chromatography (Phenyl Sepharose and DEAE Sepharose) followed by two fast protein liquid chromatography (FPLC) steps, using an Econo-Pac Blue (Cibacron blue F3GA dye) cartridge and a gel filtration sizing column (Superose 12).

Since a crude enzyme preparation is capable of catalysing the *N*-malonylation of both D- and L-tryptophan (see accompanying paper), the enzyme activity, using both enantiomers as substrates, was determined at each step. The ratio of the specific activity of D-Trp malonylation to L-Trp malonylation remained roughly constant (0.50–0.64), indicating that the same enzyme is likely responsible for malonylating both substrates (Table 1). This conclusion was further supported by the observations that the activity peaks for the malonylations of L- and D-Trp co-migrated on an FPLC gel filtration column with an estimated molecular weight of 48 kDa (data not shown), and that D-Trp was able to significantly inhibit the malonylation of L-Trp, and vice versa (Table 2). After six steps, the enzyme was purified 560-fold with a yield of about 7%. The final specific activity was 10.1 μmol h^{-1} mg^{-1} protein for M-L-Trp formation and 5.0 μmol h^{-1} mg^{-1} protein for M-D-Trp formation. The enzyme preparation from the final step revealed three major bands of 49, 46 and 43 kDa on SDS–PAGE (data not shown).

Table 2. Effect of the addition of various amino acids on the activity of D- and L-Trp malonyltransferase

Addition	Relative enzyme acivity (%)*	
	D-Trp	L-Trp
None	100	100
ACC	100	94
D-Ala	69	78
L-Ala	94	84
D-Asp	72	72
L-Asp	88	81
D-Met	69	71
L-Met	86	84
D-Phe	39	35
L-Phe	93	91
D-Ser	77	90
L-Ser	77	94
D-Trp	34	34
L-Trp	40	35
M-D-Trp	10	6

* For each assay, a sample of an enzyme preparation after $(NH_4)_2SO_4$ precipitation was incubated with 0.8 mM malonyl-CoA and 1 mM of D- or L-Trp in the absence or presence of 5 mM various amino acids or M-D-Trp as indicated. The enzyme activities in the absence of added amino acids were 34 and 53 nmol h^{-1}, respectively, when D- and L-Trp were employed as substrates.

Reaction kinetics and substrate specificity

Using cation-exchange resin chromatography, we can separate Trp from MTrp. The Trp binds to the column, while the anionic MTrp passes through. Using this simple assay we determined that MTrp formation required both active enzyme and malonyl-CoA in the incubation mixture. We confirmed the identity of MTrp by mass spectrometric (MS) analysis and chiral TLC. The methyl ester of our enzyme product exhibited characteristic ion peaks of *m/z* 318, 201 and 130, identical to those of the methyl ester of authentic M-D-Trp. On chiral TLC, the R_f values of malonylated products (MTrp) using D-Trp as substrate were identical to the authentic MD-Trp, but different from that using L-Trp as the substrate in two solvent systems (data not shown, see accompanying paper).

As the malonylation of Trp to MTrp is a bi-substrate (tryptophan and malonyl-CoA) reaction, the kinetic properties were examined with variable concentrations of one substrate at fixed concentrations of the other substrate to determine the K_m values for the substrates. Because malonyl-CoA at a concentration higher than

1 mM was inhibitory (data not shown), the concentrations of malonyl-CoA employed in the K_m determination was kept below 1 mM. Figures 1a and 2a show that the double-reciprocal plots of v^{-1} versus either [malonyl-CoA]$^{-1}$ or [D-Trp]$^{-1}$ yielded parallel lines of nearly identical slopes, regardless of the other substrate's concentration. Using L-Trp and malonyl-CoA as the substrates, the double-reciprocal plots of v^{-1} versus [malonyl-CoA]$^{-1}$ or [L-Trp]$^{-1}$ also yield parallel lines (data not shown). From these data, the K_m values for L- and D-Trp were estimated to be 1.9 and 1.0 mM, respectively (Fig. 1b). Although the enzyme exhibited higher affinity for D-Trp than for L-Trp, the V_{max} for L-Trp was more than twice that for D-Trp (Fig. 1b). The K_m values for malonyl-CoA were 1.3 and 0.5 mM, respectively, when L- and D-Trp were employed as the substrates (Fig. 2b).

1-Aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of the plant hormone ethylene, is widely *N*-malonylated into 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC) in plant tissues [19, 20]. Since MACC formation is competitively inhibited by many D-amino acids and ACC can competitively inhibit D-amino acid malonylation, it is generally thought that ACC malonyltransferase is a D-amino acid malonyltransferase even though ACC is not chiral [21, 22]. This notion is supported by the observations that those chiral analogues of ACC (stereoisomers of 2-ethyl-ACC) which have a D-amino acid configuration are more efficient substrates for malonylation than those which correspond to L-amino acids [23]. Since ACC malonyltransferase converts only D-amino acids into their *N*-malonyl conjugates, and tryptophan malonyltransferase from peanut [18] has activity only for D-Trp and not L-Trp, we compared the effectiveness of both enantiomers of various amino acids in inhibiting the tomato Trp malonyltransferase activity. Table 2 shows that, in general, D-amino acids inhibited malonylation of both D- and L-Trp more than L-amino acids. D-Phenylalanine (Phe) was the most potent inhibitor among those examined. ACC failed to inhibit the enzyme activity significantly, reinforcing our previous demonstration that ACC is not malonylated by this enzyme (see accompanying paper).

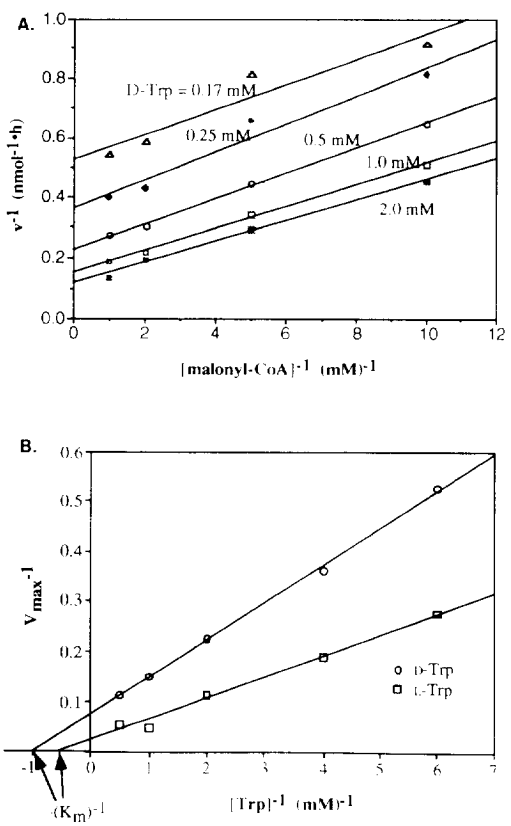

Fig. 1. Double reciprocal plots for malonyltransferase-catalysed reactions. (A) v^{-1} vs [malonyl-CoA]$^{-1}$ (0.1, 0.2, 0.5 and 1 mM) at 5 fixed D-Trp concentrations (0.17, 0.25, 0.5, 1.0 and 2.0 mM). For each assay 20 μl of enzyme solution purified though the Superose 12 FPLC step were used. (B) Secondary plots of V_{max}^{-1} axis intercept vs [D-Trp]$^{-1}$. Similar experiments were conducted using L-Trp as the substrate and the results are presented in (B).

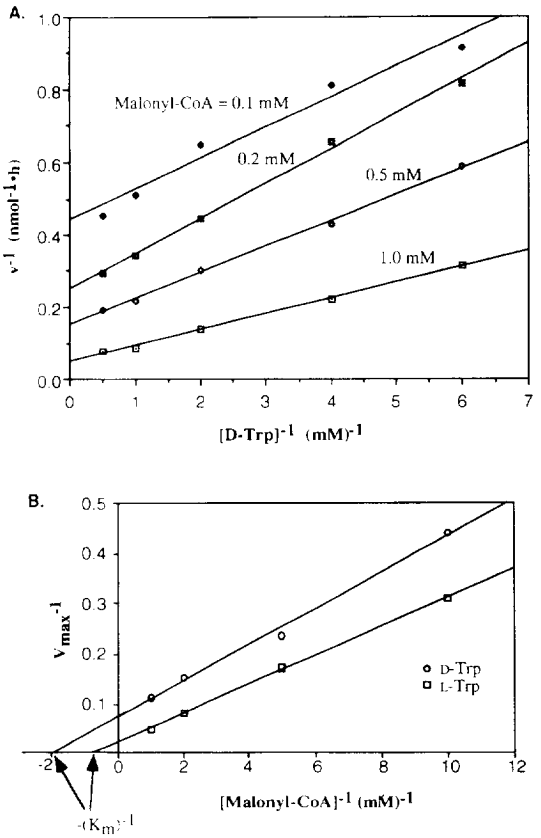

Fig. 2. Double reciprocal plots for malonyltransferase-catalysed reactions. (A) v^{-1} vs [D-Trp]$^{-1}$ (0.17, 0.25, 0.5, 1.0 and 2.0 mM) at 4 fixed malonyl-CoA concentrations (0.1, 0.2, 0.5 and 1.0 mM). For each assay 20 μl of enzyme solution purified though the Superose 12 FPLC step were used. (B) Secondary plots of V_{max}^{-1} axis intercept vs [malonyl-CoA]$^{-1}$. Similar experiments were conducted using L-Trp as the substrate and the results are presented in (B).

As Phe is an aromatic amino acid and is similar in structure to Trp, we assayed the enzyme activity employing D- or L-Phe as the amino acid substrate; their K_m values (measured at malonyl-CoA = 0.8 mM) were determined to be 3.4 and 22 mM, respectively (Fig. 3). D-Methionine, a non-aromatic amino acid, was able to serve as a substrate, but the activity was too low to be accurately estimated. There was no detectable activity for L-methionine. The product of the D-Trp malonyltransferase reaction, M-D-Trp, inhibited the malonylation of both D- and L-Trp (Table 2).

DISCUSSION

N-Malonic acid conjugates of D-amino acids have been isolated from a wide variety of higher plants [1, 6, 9, 24, 25], either as natural constituents or after administering D-amino acids. However, in only a few of these reports was the stereoconfiguration of the malonyl-amino acid determined. We have demonstrated the *N*-malonylation of L-Trp, which occurs under water stress in tomato leaves (see accompanying paper). ACC malonyltransferase has been isolated from mungbean hypocotyls [21, 23, 26] and other tissues [19, 27, 28]. It catalyses the malonylation of ACC, as well as several D-amino acids, including D-Trp [21, 22, 29], but it cannot malonylate L-amino acids. Therefore, it was suggested that ACC malonyltransferase could be classified as: malonyl-CoA: D-amino acid *N*-malonyltransferase [23]. Matern *et al.* [18] isolated a tryptophan *N*-malonyltransferase activity from peanut seedlings, which malonylates only D-Trp but not L-Trp. However, this peanut enzyme was capable of malonylating ACC. Since the present tomato enzyme is capable of malonylating both D- and L-tryptophan, but lacks the capability to malonylate ACC, it is clearly distinct from ACC malonyltransferase isolated from mung bean [21, 23, 26, 29] and D-Trp malonyltransferase from peanut [18].

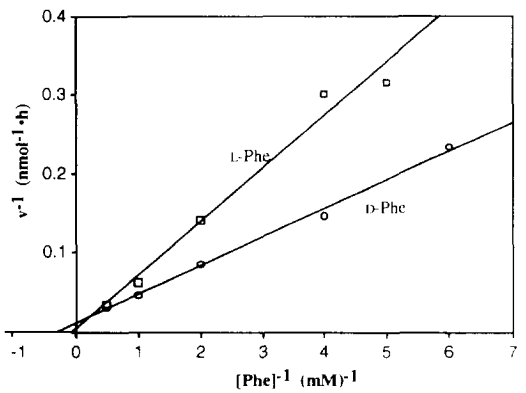

Fig. 3. Double reciprocal plots of Trp malonyltransferase activity vs variable concentrations of D-Phe (0.17, 0.25, 0.5, 1.0 and 2.0 mM) or L-Phe (0.2, 0.25, 0.5, 1.0 and 2.0 mM) at 0.8 mM malonyl CoA. An enzyme preparation (15 μl) purified through Econo-Pac Blue FPLC was used for each assay.

As the activities for both L- and D-Trp were purified together in our isolation procedure, and each stereoisomer is able to inhibit the malonylation of the other, it appears that the same enzyme catalyses the malonylation of both enantiomers. If the same enzyme catalyses the malonylation of both L- and D-Trp, then inhibitors should interfere with both activities. Table 2 shows that each inhibitory amino acid does reduce the D-Trp malonyltransferase activity as much as it reduces the L-Trp malonyltransferase activity. Interestingly, D-amino acids appear to inhibit both activities more than do L-amino acids. This is not unexpected because the K_m for D-Trp is lower than that for L-Trp, indicating that the affinity of the enzyme is greater for D-Trp than for L-Trp (Fig. 1b). Therefore, the enzyme may have an overall higher affinity for D-amino acids than for L-amino acids. The enzyme reported here appears to be the first described activity that catalyses *N*-malonylation of both L- and D-Trp. Since the enzyme catalyzes the transfer of the malonyl moiety from malonyl-CoA to the amino group of both D- and L-tryptophan, it can be classified as malonyl-CoA: D-/L-tryptophan *N*-malonyltransferase.

The double reciprocal plots of v^{-1} versus $[S]^{-1}$ yielded parallel lines when different concentrations of the fixed substrate were used (Figs 1 and 2), suggesting that the enzyme reaction proceeds via a 'ping-pong' mechanism [30]. Our observations that the reaction product MTrp was an effective inhibitor of the enzyme activity (Table 2) are in agreement with such a notion. Thus, the reaction mechanism for Trp malonylation can be described as shown below, similar to those proposed for ACC malonyltransferase [23], where Enz-SH stands for the enzyme:

$$\text{Enz} - \text{SH} + \text{Malonyl-CoA} \rightarrow \text{Malonyl-S-Enz} + \text{CoA}$$

$$\text{Malonyl} - \text{S} - \text{Enz} + \text{Trp} \rightarrow \text{Malonyl} - \text{Trp} + \text{Enz} - \text{SH}$$

The only other plant enzyme identified that is able to act on both L- and D-amino acid is an arginase from jack bean (*Canavalia ensiformis*), which can hydrolyse both D- and L-Arg [31]. Most other arginases from plants or animals did not exhibit this lack of enantiomeric discrimination. This enzyme also hydrolyses L-canavine, the most abundant free amino acid in jack bean leaves. The authors suggested that when the enzyme evolved a broader substrate specificity it lost some of its substrate stereospecificity. It may be possible that a similar situation has occurred with Trp malonyltransferase.

Future work on Trp malonyltransferase will delineate its role in water stress of tomato leaves and its relationship to ACC malonyltransferase and D-Trp malonyltransferase.

EXPERIMENTAL

Plant materials

Tomato (*Lycopersicon esculentum* Mill. cv. Castlemart) plants were grown in the field. The leaves were excised and subjected to partial dehydration by allowing them to

wilt at room temperature until they had lost 30% of their fr. wt. These wilted leaves were used as the enzyme source.

Chemicals

L-[*methylene*-^{14}C]-Trp (1.98 GBq mmol^{-1}) was purchased 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. Malonyl-CoA was from Sigma. M-D-Trp was synthesized by Chem Biochem Research.

Enzyme purification

All purification steps were carried out at 0–4°, except the FPLC, which was done at room temp. Water-stressed tomato leaves (1.2 kg) were homogenized in a blender for three 30-sec intervals in 2-fold excess of 400 mM K-Pi buffer (pH 8.5) containing 0.1 M KCl, 10 mM EDTA, 20 mM Na_2HSO_3, 0.5% β-mercaptoethanol (buffer A) and 0.5% PVPP. The homogenate was filtered through four layers of cheesecloth and centrifuged at 28 000 *g* for 40 min. The protein that pptd between 30–60% $(NH_4)_2SO_4$ satn was collected by centrifugation (28 000 *g*, 40 min) and resuspended in 100 ml 25 mM Tris–HCl buffer (pH 8.5) containing 1 mM EDTA, 0.1% β-mercaptoethanol and 10% glycerol (buffer B). The soln was adjusted to 0.4 M $(NH_4)_2SO_4$, and was loaded on a Phenyl Sepharose column (10 × 10 cm), which had been equilibrated with buffer B containing 0.4 M $(NH_4)_2SO_4$. After washing the column thoroughly with buffer B containing 0.4 M $(NH_4)_2SO_4$, the column was eluted with buffer B containing 0.1 M $(NH_4)_2SO_4$. The eluate (800 ml) was brought to 65% $(NH_4)_2SO_4$ satn and allowed to stand overnight. After centrifugation, the pellet was resuspended in buffer B (20 ml) and dialysed against buffer B overnight.

The dialysed solution was diluted with buffer B (to a total volume of 100 ml) and loaded on a DEAE Sepharose CL 4B column (5 × 13 cm), which had been equilibrated with buffer B. The column was washed with buffer containing 50 mM KCl (200 ml). The column was then eluted with 100 mM KCl in buffer B, the frs with high enzyme activity were combined, concd with the buffer next changed to buffer C (25 mM Tris–HCl at pH 8.0, 0.4 mM EDTA, 0.4 mM DTT, 10% glycerol) using a microsep concentrator (Microsep Filtron Technology). The soln was loaded on an Econo-Pac Blue cartridge (Bio-Rad) attached to a Pharmacia FPLC system. After washing with 27 ml buffer C, protein was eluted from the column by first using 300 mM KCl in buffer C (20 ml), followed by a linear gradient of 0.3 to 1.0 M KCl in buffer C (30 ml) at a flow rate of 0.5 ml min^{-1}. After several runs, the frs with high Trp malonyltransferase activity were pooled, concd, and had the buffer changed to 25 mM Tris–HCl (pH 8.0) containing 100 mM KCl (buffer D), using a microsep concentrator. Size determination and the final purification step were carried out using a Superose 12 Hr 10/30 gel filtration column on the FPLC. The column was equilibrated with buffer D. The sample soln (total 2 ml) was loaded on the column in 200 μl aliquots. The active fractions were collected, combined, and stored at −20° for further investigation.

Enzyme assay

The enzyme assay was conducted as described in the accompanying paper. A standard incubation mixture contained 0.1 M K-Pi buffer (pH 8.0), 0.1 M KCl, 0.8 mM malonyl-CoA, 2 mg ml^{-1} BSA, 0.5 mM Trp, containing 185–370 Bq of radiolabelled Trp, and enzyme extract in a total volume of 50 μl. The mixture was incubated at 40° for 1 hr, and the reaction was terminated by the addition of 50 μl of 0.2 N HOAc. Subsequently, the mixture was passed through a small (500 μl bed volume) Dowex 50 × 8–200, H^+ form, cation-exchange column. The Trp is retained on the column and the MTrp passes through in the effluent. The enzyme activity was calcd based on the radioactivity in the effluent. The amount of radioactivity was measured using a trough in the effluent. The enzyme activity was calcd based on the radioactivity in the effluent. The amount of radioactivity was measured using a Beckman Liquid Scintillation Counter (model LS 6000IC) using Ecolume liquid scintillation cocktail (ICN).

Protein was measured by the method of Bradford [32] using the Bio-Rad protein assay reagent.

MS analysis

The putative MTrp from the enzymic reactions was separated by cation-exchange resin chromatography as described above and methylated with CH_2N_2. The presence of MTrp was confirmed by MS using solid-probe introduction of the methyl ester as described in the accompanying paper.

Chiral TLC

As described in accompanying paper, the chiral TLC plates (Macherey-Nagel) were purchased from Dychrom. The solvent systems used were MeOH–CH_3CN–H_2O (50:200:50) and 1 mM $Cu(OAc)_2$ in 5% MeOH. The Trp and Mtrp spots were visualized under UV or by spraying with Ehrlich's reagent for indole-containing compounds, and free Trp was identified by spraying with ninhydrin (0.5% in 60% EtOH).

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