Available online at www.sciencedirect.com
SCIENCE DIRECT
Phytochemistry 67 (2006) 561–569
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Natural and directed biosynthesis of communesin alkaloids

Lucy J. Wigley [a], Peter G. Mantle [a,*], David A. Perry [b]

[a] Department of Biochemistry, Imperial College of Science, Technology and Medicine, South Kensington, London SW7 2AZ, UK
[b] Pfizer Global Research and Development, Groton Laboratories, Eastern Point Road, Groton, CT 06340, USA

Received 10 August 2005; received in revised form 6 October 2005
Available online 1 December 2005

Abstract

A role for tryptophan, acetate, mevalonate and methionine in the biosynthesis of communesins A and B, novel structurally-related and biologically-active *Penicillium* metabolites, has been established by isotopic labelling techniques. The incorporation of ^{14}C-tryptamine has also been demonstrated. DL-2-^{13}C-tryptophan specifically enriched two carbon atoms in the ^{13}C NMR spectrum, thereby defining the intra-molecular arrangement of the two tryptophan-derived moieties. Feeding differentially labelled precursors during communesin production showed that tryptophan and methionine are involved early in the biosynthesis and that mevalonate provides an isoprene which is added later. A biosynthetic pathway involving an early precursor based on tryptophan is proposed. Indole-*N*-(^{13}C-methyl) tryptophan was not incorporated into communesins implying that *N*-methylation of tryptophan is not the first step of the communesin biosynthetic pathway. During deamination of indole-*N*-(^{13}C-methyl) tryptophan to 1-^{13}C-methylindole-3-carboxylic acid communesin biosynthesis was inhibited. Of several halogenated indoles tested for directed biosynthesis, only DL-6-fluoro-tryptophan and 6-fluoro-tryptamine caused accumulation of the corresponding monofluoro-analogues of communesins A and B.

Keywords: Communesin; Alkaloids; Biosynthesis; Radiolabel precursor feeding; Directed biosynthesis; Fluoro-communesin

1. Introduction

The structures of two fungal alkaloids communesin A and B (**1** and **2**), isolated from a *Penicillium*. sp possessing activity against cultured P-388 lymphocytic leukemia cells, have been described (Numata et al., 1993). Previously in the laboratories of Pfizer UK, structurally related compounds were discovered in an antihelminthic screen exhibiting activity against the free-living nematode *Caenorhabditis elegans*. The name ''commindolines'' was used for these metabolites partly on the basis of initial characterization of the producing fungus as *Penicillium commune* and partly due to the dominant indolic moiety. The structures of the principal compounds, ''commindolines'' B and A, equated to that of communesins A and B, respectively.

Communesins are of mixed biosynthetic origin, predictably derived from tryptophan, mevalonate, acetate and a methyl group from methionine. A biosynthetic relationship between communesins and the plant-derived calycanthaceous alkaloids is implied by the presence of a 1,2-diphenylethane group and two aminals. Studies on the biogenesis of calycanthaceous alkaloids (Robinson and Teuber, 1954; Henrickson et al., 1964; Kirby et al., 1969) may have relevance to that of the communesins. The present biosynthetic study was undertaken to seek any biosynthetic features in common with the plant alkaloids and to provide evidence for generating communesin analogues with potentially increased or altered biological activity by directed biosynthesis in microbial fermentation.

2. Results and discussion

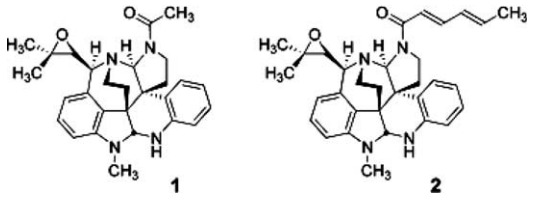
2.1. Typical fungal growth and communesin production in submerged fermentation

Communesin accumulation commenced during biomass accumulation (Fig. 1). However, decrease in biomass after 70 h is probably through respiration of assimilated reserve

* Corresponding author. Tel.: +44 207 594 5245; fax: +44 207 225 0960.
E-mail address: p.mantle@imperial.ac.uk (P.G. Mantle).

0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.10.011

carbohydrate. Replicatory growth alone is probably complete by 24 h, which is therefore the time point after which it is advantageous to add radiolabelled putative precursors of communesins that are also key intermediates in primary metabolism. The phase during which communesin concentration in broth continued to increase apparently lasted until around 200 h for both **1** and **2**. HPLC analysis of cell and broth extracts suggested that **2** is more cell-associated than **1**, possibly attributable to the latter's lipophilic sorbyl side chain constraining its cellular release.



1 **2**

2.2. Communesin biosynthesis: radiochemical experiments

Addition of ^{14}C-tryptophan, ^{14}C-acetate, ^{3}H-mevalonate, (methyl-^{14}C)-methionine and ^{14}C-tryptamine to separate submerged fermentations consistently gave rise to radiolabelled **1** and **2**, as determined by scintillation counting of HPLC-resolved **1** and **2**, indicating that these compounds are precursors of communesins (Table 1). The similar incorporation values recorded for tryptophan, methionine, mevalonate and tryptamine into **1** and **2** (mean of 8.25%, 1.0%, 0.59% and 1.75%, respectively), is consistent with a common structure in these communesins and also provides confidence for the experimental procedure employed. The incorporation of acetate into **2**, 2.3%, was approximately double that into **1** and equates closely to the requirement for three acetates in the sorbyl side chain of **2** as compared to the single acetate of **1**, particularly when the isoprene, common to both, is taken into account. The high efficiency of the incorporation of radiolabel from tryptophan contrasted with that of methionine. 2-^{14}C-Tryptophan theoretically only contributes twice as much radiolabel to **1** and **2** as methyl-^{14}C-methionine and therefore the sevenfold greater incorporation of tryptophan inversely reflects the relative metabolic demands for tryptophan and methionine in primary metabolism. The results suggest that the *N*-methyl group of methylated communesins might arise via donation from S-adenosyl methionine. Reduced competition from other pathways predictably makes tryptamine an even more efficient experimental biosynthetic precursor than tryptophan. However, the percentage incorporation of ^{14}C-tryptamine into **1** and **2** was much lower than that of ^{14}C-tryptophan (3.5% and 16.5%, respectively), possibly reflecting vacuole sequestration (Songstad et al., 1990) or poor uptake of exogenous tryptamine. Alternatively, if communesin biosynthesis is catalysed by a multienzyme complex, the low incorporation could be associated with problems of exogenous tryptamine gaining access to the site of catalysis, especially if biosynthetic intermediates are at some stage enzyme-bound.

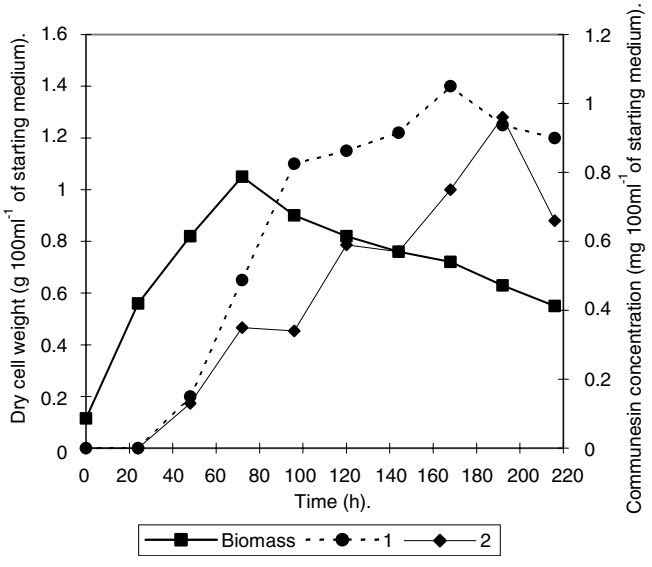


Fig. 1. Changes in total communesin yields and biomass over the period of feeding differentially labelled precursors.

2.3. ^{13}C NMR experiment: incorporation of DL[2-^{13}C]-tryptophan into 1 and 2

Natural abundance ^{13}C NMR spectra of **1** and **2** in acetone-$d_6$showed well-separated signals so that the ^{13}C NMR spectrum of each communesins isolated from a fermentation treated with [2-^{13}C]-tryptophan-showed specific enrichment in the signals at 36.9 and 44.6 ppm, corresponding to C-23 and C-17, respectively. This is consistent with biosynthesis of communesins from two tryptophan-derived moieties, the precise orientation of which was thus confirmed. The intensity ratios of signals corresponding to

Table 1
Comparison of percentage incorporation of ^{14}C-tryptophan, [1-^{14}C]acetic acid, RS-[2-^{3}H]mevalonic acid, L-(methyl-^{14}C)methionine and L-^{14}C-tryptamine into **1** and **2**

	^{14}C-trp (1 μCi)	^{14}C-acetate(1 μCi)	^{3}H-mev (8.5 μCi)	^{14}C-met (10 μCi)	^{14}C-tryptamine (0.14 μCi)
1	8.10	1.20	1.10	0.58	2.00
2	8.40	2.30	0.90	0.60	1.50
Total	16.50	3.50	2.00	1.18	3.50

C-23 and C-17 in the natural abundance spectra of **1** (1.2:1) and **2** (1.1:1) persist in the enriched spectra, suggesting that the enrichment of each carbon atom occurred in an even-handed manner. Using background signals C-3 and C-29 as a reference, with intensity similar to that of C-17 and C-23 in the natural abundance ^{13}C NMR spectrum, it was estimated that enrichment in excess of 30-fold for **1** and approximately 18-fold for **2** had been achieved. Such disparate enrichments could have resulted from different rates of accumulation of **1** and **2** during the period of isotope addition (up to ca. 100 h, Fig. 1).

2.4. Dual label experiments, exploring biosynthetic sequence via the mixtures ^{14}C-tryptophan: ^{3}H-methionine or ^{14}C-tryptophan: ^{3}H-mevalonate

If two precursors are involved concurrently in biosynthesis of any molecule throughout product accumulation, a mixture of the two precursors, given at any stage, will become incorporated into end product in an approximately constant ratio (Mantle and Shipston, 1987). Therefore, to gain information on the sequence of biosynthetic events, the incorporation of two precursor permutations ^{14}C-tryptophan: ^{3}H-methionine or ^{14}C-tryptophan: ^{3}H-mevalonate into **1** and **2** at various stages in the fermentation was investigated.

The approximately constant ratio, 1:1, of ^{14}C-tryptophan- and ^{3}H-methionine-derived label incorporation in **1** and **2** throughout the fermentation indicated that these precursors were involved in the biosynthesis at about the same time (Fig. 2), and demonstrated validity in this case of the principle upon which experiments on temporal involvement of precursors rely. Similar values for **1** and **2** follows from their identical structure in the regions derived from tryptophan and methionine. However, the skewed ratio of label specific activities in communesins following ^{14}C-tryptophan and ^{3}H-mevalonate administration at various stages of the fermentation implies the existence of temporal separation between the biogenic events involving these precursors (Fig. 2). Until 32 h into the fermentation there was an overwhelming preference for tryptophan incorporation into communesin biosynthesis, but later there was a swing in favour of mevalonate, coincident with the first appearance of end product (Fig. 1); the ratio of ^{14}C: ^{3}H decreased between 0 and 48 h from 0.83 to 0.33 in **1** and from 0.75 to 0.33 in **2** (Fig. 2). Isolation of ^{14}C-labelled **1** and **2** from cultures fed with differentially labeled precursors before communesins were detectable, i.e., before 32 h, signifies the involvement of an early, tryptophan-based, biosynthetic intermediate. These results suggest that during the early part of the fermentation there is a build up of 'early tryptophan-based intermediates' which later give rise to recognisable communesins via completion steps, e.g., prenylation. Synthesis of enzymes catalysing later steps in the biogenesis of these secondary metabolites might be in response to accumulation of critical substrate levels (i.e., accumulation of specific biosynthetic intermediates) and may account for the sudden appearance of communesins, coinciding with the preferential incorporation of mevalonate. Results of administration of differentially-labelled precursors to *Penicillium* sp. isolate N934-53 therefore indicates that tryptophan and methionine are involved early in communesin biosynthesis and that mevalonate provides the isoprene which is added later.

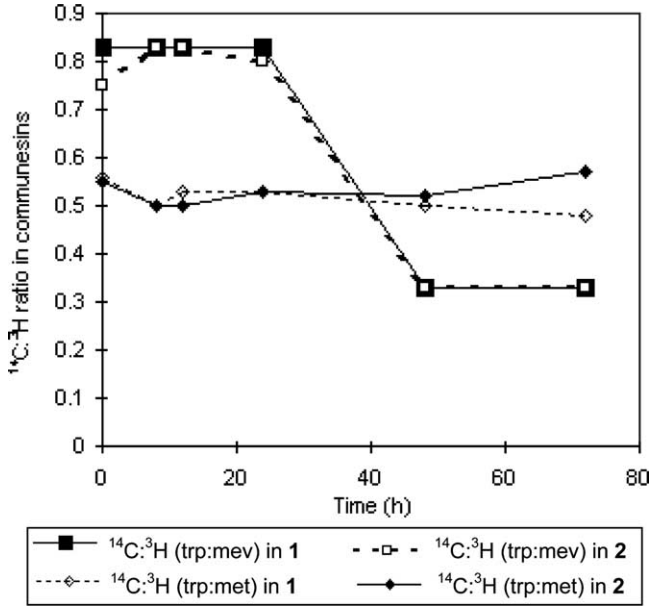
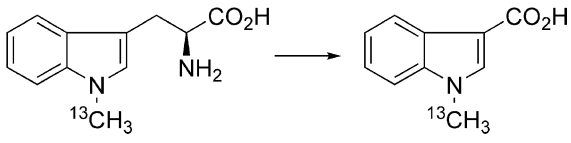


Fig. 2. Changes in the ^{14}C: ^{3}H ratios in **1** and **2**, attributed to radiolabelled communesin precursors from standard ^{14}C-tryptophan: ^{3}H-methionine or ^{14}C-tryptophan: ^{3}H-mevalonate mixtures, measured at 186 h (solid lines) and 192 h (dotted lines), respectively, following addition to individual cultures at various stages through the fermentation.

2.5. Biotransformation of indole-N-(methyl-^{13}C) tryptophan

Indole-*N*-(methyl-^{13}C) tryptophan was not incorporated into **1** or **2**, which implied at first sight that methylation of tryptophan was not the first biosynthetic step, consistent with the dual-label experiments. However, exogenous indole-*N*-(methyl-^{13}C) tryptophan was transformed to a compound identified as 1-methylindole-3-carboxylic acid (Fig. 3), as determined by MS and NMR spectroscopy (Wigley, 1995). Also communesins were not biosynthesised in the presence of indole-*N*-(methyl-^{13}C) tryptophan as suggested by the absence of radiolabeled communesins in



Indole-N-(^{13}C-methyl)-trp **Indole-N-(^{13}C-methyl)-3-carboxylic acid**

Fig. 3. Biotransformation of indole-*N*-(^{13}C-methyl)-tryptophan to indole-*N*-(^{13}C-methyl)-3-carboxylic acid.

cultures treated with indole-*N*-(methyl-^{13}C) tryptophan spiked with ^{14}C-tryptophan.

Plant hormones, indolacetic acid (IAA), 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) down-regulate transcription of the tryptophan decarboxylase (TDC) gene in cultured *Catharanthus roseus* cells. Thus negative regulation by IAA has been suggested as a mechanism controlling terpenoid indole alkaloid biosynthesis in plants (Goddijn et al., 1992). It is therefore possible that the structurally related 1-methylindole-3-carboxylic acid could have a similar affect in fungi and may explain why communesins were not produced in the culture fed with indole-*N*-(methyl-^{13}C) tryptophan. The cellular uptake, fungal transformation capacity and effect upon communesin production of this foreign compound was however, demonstrated.

The accumulated evidence concerning communesin biosynthesis suggests that the decarboxylation of tryptophan to yield tryptamine is the first biogenic step (rather than methylation of tryptophan), complementary to reports by various authors that tryptophan decarboxylase forms a regulatory link between primary and secondary metabolism (Aerts et al., 1992 and Noe et al., 1984). The subsequently formed tryptamine is then methylated either before or after ββ′-oxidative dimerisation with a second tryptamine moiety. Prenylation and acetylation of the putative di-tryptamine intermediate takes place at later stages (Fig. 4). Formation of a di-tryptamine moiety (by ββ′-oxidative dimerisation of *N*-methyl tryptamine, hydrolysis of which gives rise to tetra-aminodialdehyde) prior to methylation, analogous to the biosynthesis of the related calycanthaceous alkaloids (Robinson and Teuber, 1954), is more plausible because methylation does not appear to be a crucial step for the biosynthesis of all communesins.

In the biosynthesis of the catharanthaceous alkaloids two asymmetric centres (at the β-indolic positions) are formed upon dimerisation and thus two diastereomers of tetra-aminodialdehyde are possible; one is *meso* and the other racemic or optically active, each giving rise to the five isomers (α-ε) with corresponding optical activity (Robinson and Teuber, 1954). In the *meso* series however, only the disymmetric ε isomer has potential optical activity (Henrickson et al., 1964). α, β and δ are the only isomers that have previously been isolated from natural sources, and identified as (+)-calycanthine, (*rac*)-chimonanthine and *iso*-calycanthine, respectively (Adjibade et al., 1992). Communesins possibly represent the ε isomer isolated, for the first time to our knowledge in this form, from a natural source.

2.6. Suppression by ethionine of the methylation step in communesin biosynthesis to reveal an unmethylated communesin

Ethionine, as an exogenous competitive inhibitor of methylation, was used to alter flow of the communesin pathway to **1** and **2** and thus potentially to select for a demethyl-communesin. Preliminary experiments showed incorporation of ^{14}C from ^{14}C-methionine into **1** and **2**, and other methionine-derived metabolites (e.g., ergosterol) in

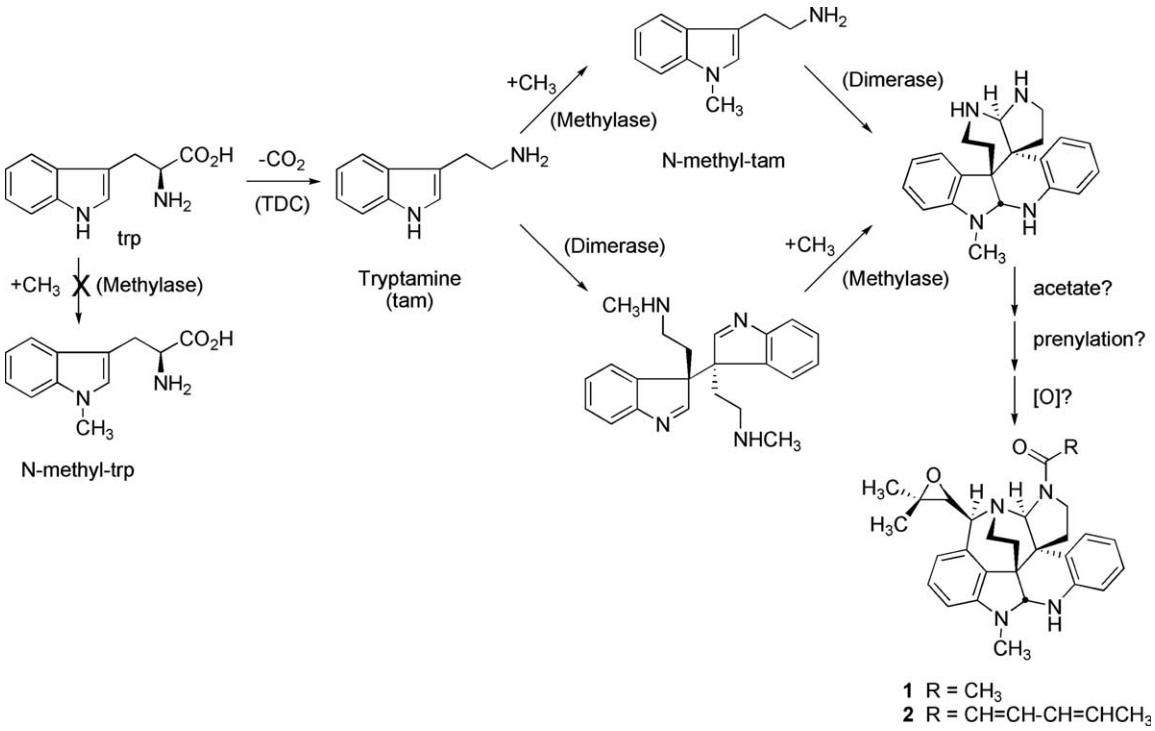


Fig. 4. Partial biosynthetic pathway for communesin alkaloids proposed on the basis of the present experimental findings and known biosynthesis of Catharanthaceous alkaloids.

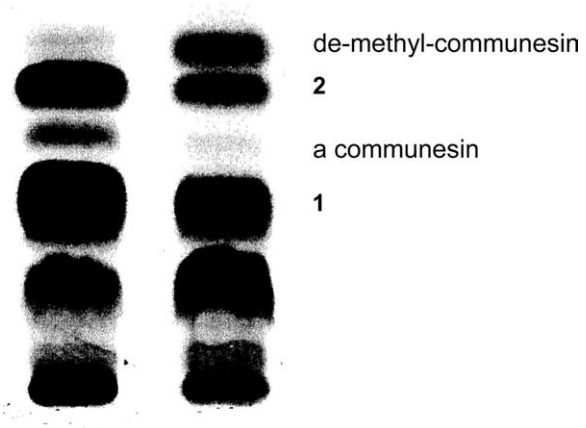


Fig. 5. Autoradiograph showing effect of ethionine (right), added to a fermentation at 48 h, on incorporation of ^{14}C-tryptophan into metabolites at 72 h, when labelled tryptophan was added 30 min after the ethionine and to a control fermentation (left).

cell extract, was markedly reduced after treating fermentations with ethionine (50 mg per 100 ml^{-1}) in early idiophase and adding ^{14}C-methionine 30 min after the ethionine (Wigley, 1995). A similar experiment, substituting ^{14}C-tryptophan for ^{14}C-methionine so as to detect previously unrecognised indole alkaloids according to their biosynthesis from a tryptophan precursor, clearly showed reduced yields of **1** and **2** in broth extract (Fig. 5). Similar reduction in the trace amount of an unidentified compound (b) would be consistent with it being a closely related communesin. However, another minor compound (a), that was not evident on TLC chromatograms under UV_{254nm} light and had only been recognised on account of the radioactivity in the control extract, was much increased in response to ethionine, implying that it may be a de-methyl communesin.

2.7. Directed biosynthesis of communesin analogues

Ability of *Penicillium* sp. N934-53 to accept biosynthetic precursor analogues that could direct biosynthesis towards communesin analogues was explored first using ^{14}C labelled tryptophan analogues as qualitative probes. Since such were not available commercially, sufficient of several probes (^{14}C-bromotryptophan substituted in the 4 or 5 position, ^{14}C-fluorotryptophan substituted in the 4 or 6 position, ^{14}C-5-nitrotryptophan) was prepared by an *E. coli* expressing high tryptophan synthetase activity (see Section 3.2.8). Radiolabelled 4-fluorotryptophan, 4-bromotryptophan and 5-nitrotryptophan were not incorporated into **1** and **2**, showing that the biosynthetic enzymes were too specific for these substrates. However, for the halogenated analogues, 8% and 11%, respectively, of the ^{14}C given was found in fungal protein. Additional amounts were in non-communesin metabolites, indicating uptake and acceptance of these tryptophan analogues into other metabolic pathways. In contrast, 6-fluorotryptophan and 5-bromotryptophan were incorporated into analogues of **1** and **2** and this finding prompted experiments on a larger scale with these halogenated indoles unlabelled. Also tested similarly were 6-fluoro-tryptamine hydrochloride, 5-fluorotryptamine and 5-methyl tryptophan.

The mass spectra of **1** and **2** that had been generated in the presence of 6-fluoro-tryptamine hydrochloride contained, in addition to ions characteristic of native **1** and **2**, ions corresponding to the communesin molecular ion plus 18 amu (*m/z* 474 and 526 for **1** and **2**, respectively). Similar data was obtained for **2** from culture fed DL-6-fluorotryptophan. The elemental composition of these new ions, deduced from accurate mass measurement, was $C_{28}H_{32}N_4O_2F$ (*m/z* 474) and $C_{32}H_{36}N_4O_2F$ (*m/z* 526), consistent with the incorporation of only one fluorinated-indole moiety, thus demonstrating the feasibility of generating an analogue of the various communesins by substrate feeding. Absence of ions 36 amu greater than the native communesin molecular ion, implies that di-fluorinated communesins were not formed. Presence of fragment ions in the EI mass spectra corresponding to fluorination of specific native communesin fragment ions, but not of others, is in accordance with the fluoro-group being located exclusively on the "non-prenylated" side of the molecule (Fig. 6). It would seem that the 6-fluorotryptophan and -tryptamine derivatives are unacceptable substrates for insertion into the ultimately-prenylated moiety of communesins. However, insertion specificity could be a useful tool in generating new communesin analogues biosynthetically.

The position of fluorine in the 'tryptophan-ring' moiety of fluoro-communesins derived from fluoro-tryptophan was assumed to be the same as in the parent precursor. The absence of a fluorine atom on the prenylated indole moiety suggests enzyme specificity at some stage in the biosynthesis, e.g., specificity of the dimerase, methylase or enzymes involved in prenylation whereby DL-6-fluorotryptophan or 6-fluoro-tryptamine cannot be accepted as sole indolic substrates for communesin biosynthesis. Since many secondary metabolite biosynthetic pathways are catalysed by multienzyme complexes (Gutierrez et al., 1991; Kleinkauf and von Dohren, 1990; Beck et al., 1990), it is conceivable that a metabolite precursor remains bound to the enzyme complex during a number of sequential biosynthetic steps. For example, tryptophan, implicated early in the biosynthesis, could be the starter unit, enzyme-bound to a very specific active site via interactions involving the aromatic ring. It seems rational therefore that tryptophan analogues can not be tolerated at this stage due to enzyme specificity, but this does not eliminate the possible acceptance of a second, modified tryptophan. It is probable that the presence of a highly electronegative fluorine atom would alter some chemical properties of communesins. However, low enrichment of native communesins with co-chromatographing 3-fluoro-communesins generated by directed biosynthesis made accurate biological assessment impossible, a problem also encountered in the generation of some squalestatin analogues via directed biosynthesis (Cannell et al., 1993).

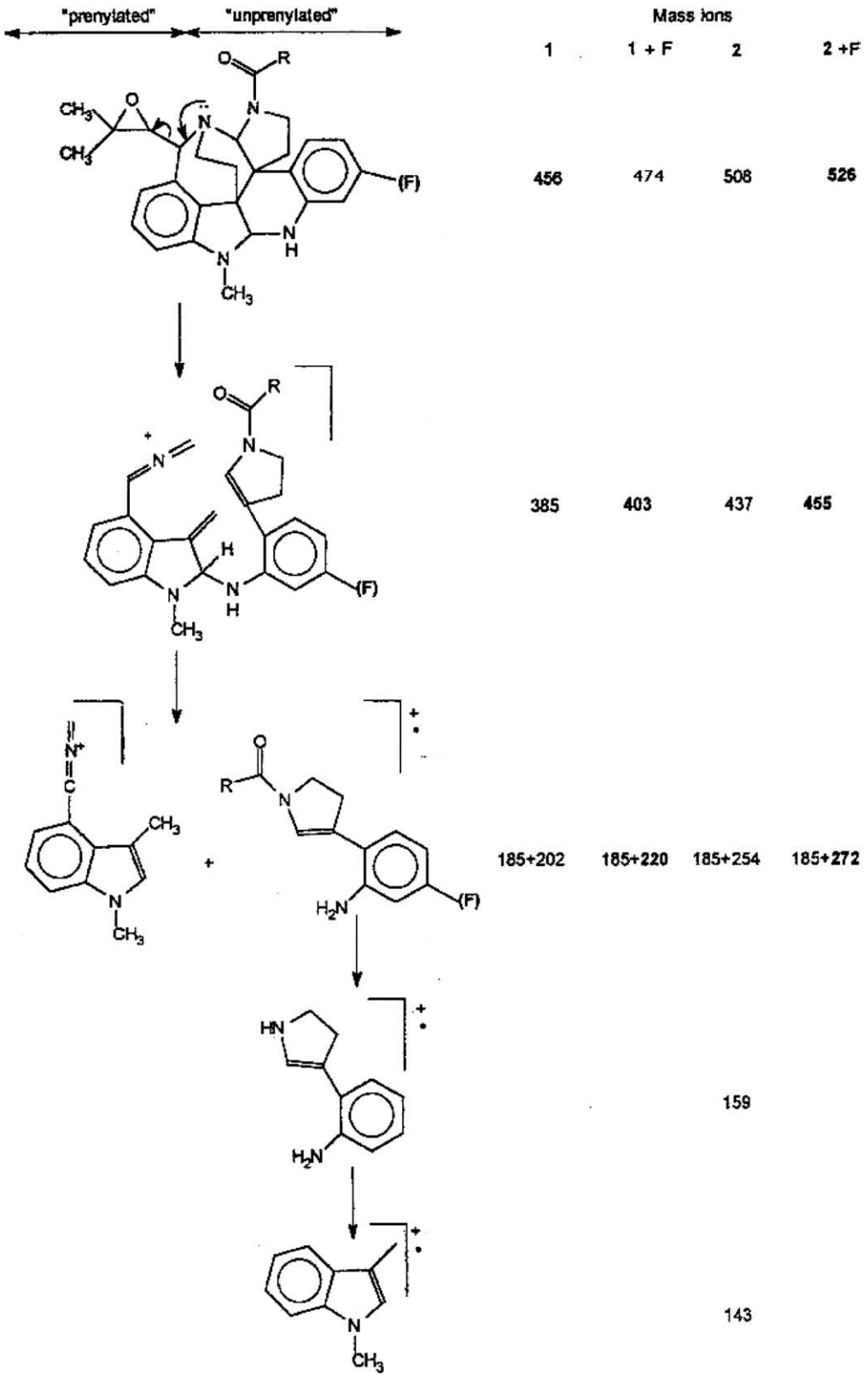


Fig. 6. Interpretation of EI mass spectrum fragmentation patterns of mono-fluorinated **1** and **2**.

The incorporation of 6-fluoro-tryptamine into communesins provides further evidence that tryptamine is a biosynthetic precursor.

Mass spectra of communesins biosynthesised in the presence of DL-5-bromo-tryptophan, 5-chloro-tryptamine and DL-5-methyl-tryptophan only contained signals associated

with native communesins (Wigley, 1995), suggesting that these putative precursor analogues can not be incorporated into communesins. The preferential incorporation of fluorine reported in the directed biosynthesis of squalestatin analogues, was attributed to the almost isosteric nature of hydrogen and fluorine atoms (Cannell et al., 1993) and possibly accounts for the same phenomenon observed during communesin analogue generation.

3. Experimental

3.1. Fungal isolate, media and fermentation conditions

Sporulating communesin-producing cultures of *Penicillium* sp. isolate N934-53 (from soil, Miyazaki, Japan; Pfizer culture collection) were stored at −20 °C following growth at 25 °C for 7–10 days on Bacto potato dextrose agar (PDA) slopes. Spores were transferred, as an opaque green suspension in a 0.01% Tween 80 solution, 2 ml, into baffled Erlenmeyer flasks (500 ml) containing seed-stage medium, (g/l: Corn starch 20, Pharmamedia 15, yeast extract 5, $CaCO_3$ 2, pH 7.0) 100 ml. Flasks were incubated at 27 °C on a rotary shaker (200 rpm, 10 cm eccentric throw) for approximately 36 h, giving a dense mycelial suspension, when 4% (v/v) transfer was made to production media, (ARM; g/l: Soluble starch 7.5, Trusoy flour 7.5, sodium chloride 2, yeast extract 0.05, Pharmamedia 2, pH 6.5), 100 ml in 500 ml un-baffled Erlenmeyer flasks, and incubated as above for between 96 and 144 h.

3.2. Isotopically-labelled-precursor feeding experiments

3.2.1. Radiolabelled putative precursor addition

L-[side chain (methylene)-3-^{14}C] tryptophan (aqueous solution containing 2% ethanol): specific activity, 53.80 mCi mmol^{-1} (New England Nuclear), 1 μCi; [1-^{14}C] acetic acid, sodium salt (aqueous solution): specific activity, 60 mCi mmol^{-1} (Amersham International), 1 μCi; RS-[2-^{14}C] mevalonic acid lactone (benzene solution): specific activity 50–60 mCi mmol^{-1} (Amersham International), DL-[2-^{3}H] mevalonic acid lactone (toluene solution): specific activity 1 Ci mmol^{-1}, 8.5 μCi; L-[methyl-^{14}C]met (freeze-dried, under nitrogen): specific activity, 50–60 mCi mmol^{-1} (Amersham International), 10 μCi; L-14C tryptamine, 0.14 μCi (see below for preparation): specific activity, ca. 7.2 μCi mmol^{-1}. All compounds in sterile distilled water, 1 ml, were delivered to separate cultures of *Penicillium* sp. N934-53 in four equal sub-doses at 48, 64, 68 and 72 h. The tryptamine and tryptophan-fed cultures were harvested at 80 and 144 h respectively; all others were harvested at 96 h.

3.2.2. Preparation of ^{14}C-tryptamine by decarboxylation of 14C L-tryptophan by L-phenylalanine decarboxylase

Decarboxylation of trp was achieved using L-phenylalanine decarboxylase (EC 4.1.1.53) from *Streptococcus faecalis* (Sigma). Dry cells 1 mg, were added to DL-tryptophan, 0.8 mg plus ^{14}C-L tryptophan, 5 μCi, in 50 mM Tris–HCl, containing 0.1 mM pyridoxal phosphate, 1 ml (Taylor and Wightman, 1987), at pH 5.5, incubated at 37 °C for 48 h. Cells were removed by centrifugation and tryptamine extracted from the supernatant, basidified to pH > 12 with NaOH, by partition into diethyl ether and purified by thin layer chromatography (as in 3.2.X), mobile phase: methyl acetate: propan-2-ol: 25% ammonium hydroxide, 9:3:1. ^{14}C-tryptamine: specific activity (7.2 μCi mmol^{-1}), 0.36 μCi total, was isolated.

3.2.3. Addition of differentially labelled precursors at various stages in the submerged culture fermentation

(a) Delivery of a mixture of ^{14}C-tryptophan and ^{3}H-methionineA solution of ^{14}C-tryptophan and L-[methyl-^{3}H] methionine (aqueous solution containing 0.2% 2-mercaptoethanol, sterilised): specific activity, 70–85 Ci mmol^{-1} (Amersham International); in the ratio of 1:8 counts per minute (cpm), was delivered to production stage cultures at either time 0, 8, 24, 32, 48, 72 or 96 h. All cultures were harvested at 168 h.

(b) Delivery of a mixture of ^{14}C-tryptophan and RS-(2-^{3}H)mevalonic acidA solution of ^{14}C-tryptophan and DL-[2-^{3}H]mevalonic acid lactone (toluene solution): specific activity 1 Ci mmol^{-1}, in the ratio of 1:30 (cpm), was delivered to production stage cultures at either time 0, 12, 24, 48, 72, 96, 120 or 144 h. All cultures were harvested at 192 h. The ratio (cpm) of ^{14}C: ^{3}H in **1** and **2** (measured in the total of each compound extracted) was determined by scintillation counting (see the following section).

3.2.4. Metabolite extraction and analysis

Fungal mycelium, separated under vacuum from broth by filtration (Whatman 541, 9 cm diameter) was extracted twice with methanol (25 ml per 100 ml of culture) for 12 h. Metabolites in broth (filtrate) that are soluble in organic solvent were isolated by partition extraction twice with an equal volume of ethyl acetate. Combined methanol and ethyl acetate extracts were taken to dryness under reduced pressure prior to analysis by HPLC and/or TLC. TLC employed silica gel, 0.25 mm thick on plastic backed plates, (Polygram SIL G/UV_{254}, Camlab). In the mobile phase chloroform:acetone (6:1), **1** and **2** had R_f values 0.3 and 0.48, respectively. HPLC was performed on a Waters Z-module C-18 reverse phase "Novapak" (0.8 cm by 10 cm) HPLC column controlled by an Apple II GS computer using the Gilson HPLC system manager with detection at 268 nm. Communesins were purified and analysed using methanol:water 80:20, flow 0.75 ml min^{-1}, R_t for **1** and **2** 10 and 14 min, respectively.

Radiolabel incorporation was determined quantitatively by scintillation counting (Kontron Intertechnique) of the

HPLC-purified communesins [sample:scintillant (Ecolume™ ICN) ca. 8:1]. Counting efficiency for ^{14}C and ^{3}H was 90–93% and ca. 40%, respectively. Semi-quantitative/qualitative analysis was by autoradiography of TLC-resolved extracts (Fuji X-ray film NIF RX developed automatically in a Fuji FPM 2100 processor).

Mass spectrometry (MS) used either a VG Micromass 7070E instrument, operated at 70 eV for electron impact (EI) or a VG Auto Spec instrument at 70 or 100 eV for EI and chemical ionisation (CI), respectively. Ammonia was the reagent gas for CI.

^{1}H and ^{13}C NMR spectroscopy used a Brucker AM 500 MHz instrument. Chemical shift values were all relative to tetramethylsilane.

3.2.5. Dry cell weight

Dry cell weight as a measure of fungal growth was determined by lyophilisation of filtered mycelium in pre-weighed flasks to constant weight.

3.2.6. Administration of DL-2-^{13}C-tryptophan to submerged fermentations

Preliminary experiments, measuring incorporation efficiency of ^{14}C-tryptophan into communesins in the presence of significant additions of tryptophan (Wigley, 1995), showed less than a 3-fold reduction when 10 mg tryptophan was added to a 100 ml fermentation. With 20 mg the efficiency was correspondingly halved, and thus there was no advantage in adding more than 10 mg ^{13}C-tryptophan for ^{13}C NMR enrichment. Further, maximum incorporation into communesins of a single probe-dose of tryptophan, added to a fermentation at 96 h was only reached after a further 24 h. Therefore, near-optimal administration of a ^{13}C-tryptophan probe was designed. DL-2-^{13}C-tryptophan (99 atom% ^{13}C, C/D/N Isotopes INC), 10 mg, was dissolved in distilled water, 25 ml, and filter sterilised (Acrodisc®, 0.2 μm). Aliquots were added to a 100 ml culture on 5 evenly-spaced occasions during the 3rd and 4th day of fermentation. The probe was therefore added between 46.5 and 75.5 h, and there were four replicate 100 ml cultures. The cultures were harvested at 78.5 h to prevent undue dilution of ^{13}C-communesins by new unlabelled compound. ^{13}C-tryptophan-enriched 1 and 2, 9.9 and 7.3 mg, respectively, were purified, dissolved in acetone-d_6, analysed by ^{13}C NMR and the spectra compared to the natural abundance ^{13}C NMR spectra of **1** and **2**.

3.2.7. Administration of indole-N-(methyl-^{13}C) tryptophan to submerged fermentations

Indole-*N*-(methyl-^{13}C)tryptophan, 50 mg per 100 ml culture (pH 5.0) (prepared by G. Goodwin, Pfizer, Sandwich by the method of Yamada et al., 1965) was administered to ten production stage cultures, 100 ml, in eight 1 ml aqueous aliquots between 27.5 and 48 h of the fermentation. One culture flask also received 1 μCi ^{14}C-tryptophan. Control cultures were fed distilled water (pH 5.0) or were not treated in any way. All cultures were harvested at 120 h and organic solvent extracts were analysed by HPLC and TLC.

3.2.8. Biosynthesis of tryptophan analogues

Escherichia coli, W 3110 trpAEdel2, containing the plasmid pHP3 encoding tryptophan synthetase, can convert L-serine and indole to L-tryptophan (Matthews et al., 1992). Provision also of plasmid encoded marker genes for ampicillin and tetracycline resistance facilitates selection and maintenance of bacteria containing plasmids (Enger-Valk et al., 1980). The following protocol for bacterial growth and the incubation procedure for tryptophan synthesis was modified from Matthews et al. (1992).

The *E. coli* strain was grown and maintained in medium 2TY (Maniatis et al., 1989); Bacto tryptone 16 g/l, Bacto yeast extract 10 g/l, NaCl 5 g/l. pH was adjusted to 7.0 with 5 M NaOH. Filter-sterilised ampicillin (Acrodisc, 0.2 μm), 50 μg/ml, was added to the sterilised medium. For solid medium Bacto agar, 15 g/l, was added. Liquid cultures were grown for 15 h at 37 °C. Sterile glycerol was added (10%) and 1 ml aliquots dispensed into sterile eppendorff tubes. Tubes were cooled slowly to −20 °C before storage in liquid nitrogen. For fermentations, the contents of one thawed tube was used to inoculate 200 ml of 2TY/ampicillin medium and incubated on a rotary shaker for 15 h at 37 °C. Cells were harvested by centrifugation and the pellet re-suspended in 100 mM potassium phosphate buffer, pH 7.8, supplemented with pyridoxal phosphate, 10 mg/l, $(NH_4)_2SO_4$, 15.6 g/l, $Na_2SO_3 \cdot 7H_2O$, 1.25 g/l. To cells from ca. 50 ml culture, suspended in the supplemented buffer, serine (151 mg) and 4-bromo-indole (36 mg) were added. Since the bromo-indole is only slightly soluble in water it was 'trickle-fed' in 18 drops of DMSO during the first 3 h of incubation. Incubation was for 6 h at 37 °C and, after centrifugation, the supernatant was treated with 5 volumes of ethanol (96%) to precipitate salts. Evaporation of the supernatant left a residue rich in tryptophan which was purified by PLC on 1 mm silica gel plates (Polygram SIL G/UV_{254}, Camlab) in *n*-butanol:acetic acid:water (12:3:5, R_f values for tryptophan and analogues in the range 0.7–0.8): yield 66%. 4-Bromotryptophan structure was confirmed (NMR, UV and EIMS) by Prof C. Moody (Loughborough University, UK).

Similar protocol on a smaller scale was used to make radiolabelled tryptophan analogues from 4-bromo-indole, 4-fluoro-indole, 5-bromo-indole, 6-bromo-indole and appropriate L-serine by adding 5–10 μCi ^{14}C-serine. Products, purified by TLC, were added (total 0.1–0.4 μCi according to availability) to *Penicillium* fermentations in aliquots at 52.5, 53, 69.5, 70.25, and 72 h and the cultures harvested at 96 h. Communesins were isolated, separated by TLC and the chromatograms autoradiographed (Fuji NIF RX).

3.2.9. Preparation of halogenated and methylated communesins by directed biosynthesis

DL-6-Fluorotryptophan (Aldrich) 11.9 mg, DL-5-bromotryptophan (Sigma) 11.9 mg, 6-fluorotryptamine (Sigma)

10 mg, 6-chlorotryptamine (Sigma) 10.6 mg and DL-5-methyltryptophan (Aldrich) 10 mg, were 'trickle-fed', each in 25 ml sterile distilled water, to separate culture flasks of production medium, 100 ml, frequently between 40 and 69 h of the fermentation, which continued until 96 h. The ethyl acetate broth extracts and methanol cell extracts were purified by TLC, and compounds co-chromatographing with **1**, **2** and other selected bands were eluted from the silica and investigated by CIMS. Accurate mass measurements were obtained for any important ions in the spectra that did not correspond to either **1** or **2**.

3.2.10. Probing for un-methylated communesins with ^{14}C-tryptophan following ethionine addition to fermentations

DL-Ethionine (Sigma), 50 mg in 10 ml sterile distilled water, was added to a 100 ml fermentation at 38 h. 10 ml water was added to a control fermentation. 30 min later ^{14}C-tryptophan (1 μCi) in 1 ml sterile water was added to each culture. Fermentations were harvested at 72 h and the broth extracted with ethyl acetate. Extract was chromatographed and the TLC plate autoradiographed for one month.

Acknowledgements

We thank R. Shepherd and J. Barton (Chemistry Department, Imperial College) for obtaining NMR and MS data, G. Goodwin (Pfizer, UK) for synthesis of indole-*N*-(^{13}C-methyl) tryptophan, and Prof. C. Moody, Loughborough University, UK, for confirming our tryptophan synthesis. We thank BBSRC for a research studentship (LJW) in collaboration with Pfizer Central Research (Sandwich, Kent, UK) and acknowledge the advice of Dr. M. Haxell (Pfizer, UK) concerning communesin fermentations.

References

Adjibade, Y., Weniger, B., Quirion, J.C., Kuballa, B., Cabalion, P., Anton, R., 1992. Dimeric alkaloids from *Psychotria fosteriana*. Phytochemistry 31, 317–319.

Aerts, R.J., Alarco, A.-M., De Luca, V., 1992. Auxins induce tryptophan decarboxylase activity in radicles of *Catharanthus* seedlings. Plant Physiology 100, 1014–1019.

Beck, J., Ripka, S., Siegner, A., Schiltz, E., Schweizer, E., 1990. The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Its gene structure relative to that of other polyketide synthases. European Journal of Biochemistry 192, 487–498.

Cannell, R.J.P., Dawson, M.J., Hale, R.S., Hall, R.M., Nobel, D., Lynn, S., Taylor, N.L., 1993. The squalestatins, novel inhibitors of squaline synthase produced by a species of *Phoma* 1V. Preparation of fluorinated squalestatins by directed biosynthesis. Journal of Antibiotics 46, 1381–1389.

Enger-Valk, B.E., Heyneker, H.L., Oosterbaan, R.A., Pouwells, P.H., 1980. Construction of new cloning vehicles with genes of the tryptophan operon of *Escherichia coli* as genetic markers. Gene 9, 69–85.

Goddijn, O.J.M., De Kam, R.J., Zanetti, A., Schilperoort, R.A., Hoge, J.H.C., 1992. Auxin rapidly down-regulates transcription of the tryptophan decarboxylase gene from *Cantharanthus roseus*. Plant Molecular Biology 18, 1113–1120.

Gutierrez, S., Diez, B., Montenegro, E., Martin, J.F., 1991. Characterisation of the *Cephalosporium acremonium* pcbAB gene encoding α-aminoadipyl-cysteinyl-valine synthetase, a large multidomain peptide synthetase: Linkage to the pcbC gene as a cluster of early cephalosporin biosynthetic genes and evidence of multiple functional domains. Journal of Bacteriology 173, 2354–2365.

Henrickson, J.B., Goschke, R., Rees, R., 1964. Total synthesis of the calycanthaceous alkaloids. Tetrahedron 20, 565–579.

Kirby, G.W., Shah, S.W., Herbert, E.J., 1969. Biosynthesis of chimonanthine from [2-^{3}H] tryptophan and [2-^{3}H] tryptamine. Journal of the Chemical Society (C), 1916–1919.

Kleinkauf, H., von Dohren, H., 1990. Nonribosomal biosynthesis of peptide antibiotics. European Journal of Biochemistry 192, 1–15.

Maniatis, T., Fritsch, E.L., Sambrook, J., 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor, New York.

Mantle, P.G., Shipston, N.F., 1987. Temporal separation of steps in the biosynthesis of verruculogen. Biochemistry International 14, 1115–1120.

Matthews, S., Jandu, S.K., Leatherbarrow, R.J., 1992. ^{13}C NMR study of the effects of mutation on the tryptophan dynamics in chymotrypsin inhibitor 2: correlations with structure and stability. Biochemical Journal 32, 657–662.

Noe, W., Mollenschott, C., Berlin, J., 1984. Tryptophan decarboxylase from *Catharanthus roseus* cell suspension cultures: purification, molecular and kinetic data of the homogeneous protein. Plant Molecular Biology 3, 281–288.

Numata, A., Takahashi, C., Ito, Y., Takada, T., Kawai, K., Usami, Y., Matsumura, E., Imachi, M., Ito, T., Hasegawa, T., 1993. Communesins, cytotoxic metabolites of a fungus isolated from a marine alga. Tetrahedron Letters 34, 2355–2358.

Robinson, Sir R., Teuber, H.J., 1954. Calycanthine and calycanthidine. Chemistry and Industry, 783–784.

Songstad, D.D., De Luca, V., Brisson, N., Kurz, W.G.W., Nessler, C.L., 1990. High levels of tryptamine accumulation in transgenic tobacco expressing tryptophan decarboxylase. Plant Physiology 94, 1410–1413.

Taylor, D.C., Wightman, F., 1987. Metabolism of D,L-chloro-phenylalanines by phenylalanine aminotransferase isozymes purified from bushbean shoots. Phytochemistry 26, 1279–1288.

Wigley, L.J. 1995. Biosynthesis of communesin alkaloids. Ph D thesis, University of London.

Yamada, S., Shioiri, T., Itaya, T., Hara, T., Matsueda, P., 1965. Ind.-N-alkylation of tryptophan and synthesis of 1-alkyltryptophan hydrazides. Chemical and Pharmaceutical Bulletin 13, 88–93.