Exploring the biosynthetic origin of fluoroacetate and 4-fluorothreonine in *Streptomyces cattleya*

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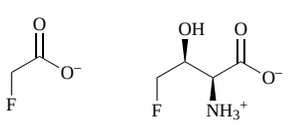
The biosynthesis of the fluorometabolites, fluoroacetate and 4-fluorothreonine, has been investigated in cell suspensions of *Streptomyces cattleya* using both carbon-13 and deuterium labelled precursors. The extent of incorporation of label into each metabolite and the distribution of label within each metabolite has been determined by GC–MS and $^{19}F\{^{1}H\}$ NMR spectral analyses. Efficient metabolism of glycine, serine and pyruvate into the fluorometabolites is observed, the results being consistent with metabolism *via* oxaloacetate to an intermediate in the glycolytic pathway between phosphoenolpyruvate and dihydroxyacetone phosphate, from which the substrate for fluorination is derived. The striking similarity between the labelling patterns within the two fluorometabolites recorded in every experiment demonstrates that there is a single fluorination enzyme in *S. cattleya*.

Introduction

Despite the diversity of organochlorine compounds in nature[1] organofluorine compounds are of very limited occurrence in living organisms.[2] One reason for this scarcity is the insoluble nature of fluorine-containing minerals which renders the element biologically unavailable. However, probably the most important factor restricting the biosynthesis of organofluorine compounds is the high redox potential required for oxidation of fluoride which does not permit activation of fluoride ion by reduction of hydrogen peroxide, thus precluding the incorporation of fluorine by the haloperoxidase reaction, the main route for formation of other organohalogen compounds in nature.[3] The mechanism by which fluorine is introduced biologically into organic compounds is therefore of some biochemical interest and an understanding of the process may reveal exciting biotechnological possibilities.

The most widespread fluorinated natural product is fluoroacetate **1** which is found, often in high concentrations, in the



1 **2**

leaves and seeds of a variety of tropical plant species.[2,4] Although several investigators have utilised plants in studies attempting to elucidate the biochemistry of fluorination[5] and several speculative theories as to the mechanism have been proposed,[6] little progress has been achieved to date toward an understanding of the nature of the process in higher plants or in identifying the metabolic intermediates involved.[2] However, the discovery by Sanada *et al.*[7] that the thienamycin-producing organism *Streptomyces cattleya* is capable of producing both fluoroacetate **1** and 4-fluorothreonine **2** as secondary metabolites has provided a more tractable biological system in which to study the mechanism of biological fluorination. Both Tamura *et al.*,[8] a Japanese group, and ourselves[9,10] have studied the incorporation of ^{14}C-labelled precursors into fluoroacetate by resting cell suspensions of *S. cattleya*. Generally the levels of incorporation have been disappointingly low. Whereas we[10] obtained the highest level of ^{14}C incorporation with glycolate as a precursor, the Japanese group[8] showed maximum incorporation with glycerol and β-hydroxypyruvate and demonstrated incorporation of [2-^{13}C]glycerol into C-1 of fluoroacetate. They concluded that the carbon skeleton of fluoroacetate was derived from C-1 and C-2 of glycerol *via* β-hydroxypyruvate.

The biosynthetic origin of 4-fluorothreonine has also been the subject of some debate. Initially Sanada *et al.*[7] suggested that fluoroacetate was the immediate product of fluorination and that 4-fluorothreonine was derived by condensation of fluoroacetaldehyde and glycine. Tamura *et al.*[8] have concluded that since fluoroacetate **1** is detectable in the culture medium before 4-fluorothreonine **2**, the amino acid must be formed from fluoroacetate **1**. However work in this laboratory[9,10] has indicated very low levels of interconversion of **1** and **2** signifying that the carbon skeleton of neither compound is derived by metabolism of the other.

In this paper we present our studies on the incorporation into the fluorinated metabolites **1** and **2** of labelled (^{13}C and/or ^{2}H) glycolate, glycine, serine, pyruvate, alanine, aspartate, succinate and acetate. The extent of incorporation was established by two complementary methods, GC–MS and ^{19}F NMR spectroscopy. A GC–MS method developed for fluoroacetate involving conversion to its *p*-phenylphenacyl derivative allowed accurate assessment of ^{13}C incorporation into both C-1 and C-2 of fluoroacetate **1** and ^{2}H incorporation onto C-2 of the compound. 4-Fluorothreonine **2** was per-trimethylsilylated by treatment with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA). On GC–MS this derivative undergoes cleavage α to the amino group to give two characteristic ions at 218 and 236 as shown in Fig. 1. Monitoring of these fragments allows ^{13}C and ^{2}H incorporations into [C-1 + C-2] and [C-2 + C-3 + C-4] of 4-fluorothreonine **2** to be studied.

Both ^{13}C and ^{2}H incorporations into C-1 and C-2 of fluoroacetate **1** and C-3 and C-4 of 4-fluorothreonine **2** could be determined directly by $^{19}F\{^{1}H\}$ NMR of the supernatants from *S. cattleya* cultures. Proton decoupling proved advantageous in simplifying the complexity of the ^{19}F NMR signals for fluoro-

Table 1 Incorporation of ^{13}C-label determined by GC–MS from various ^{13}C-labelled glycines into (i) fluoroacetate **1** and (ii) 4-fluorothreonine **2** by cell suspensions of *S. cattleya*

(i) Incorporation into fluoroacetate **1**

Precursor (10 mM)	Incubation time/h	^{13}C-Incorporation (%)[a,b] Single C-1	Single C-2	Double
[1,2-$^{13}C_2$]glycine	24	6.6	4.1	41.0
[1,2-$^{13}C_2$]glycine	48	8.1	6.1	33.7
[1-^{13}C]glycine	24	2.0	<2.0	<0.5
[1-^{13}C]glycine	48	1.3	2.4	<0.5
[2-^{13}C]glycine	24	5.8	4.4	39.2
[2-^{13}C]glycine	48	8.3	5.1	31.9

(ii) Incorporation into 4-fluorothreonine **2**

Precursor (10 mM)	Incubation time/h	^{13}C-Incorporation (%)[a,c] (C-1 + C-2) Single	(C-1 + C-2) Double	(C-2 + C-3 + C-4) Single	(C-2 + C-3 + C-4) Double	(C-2 + C-3 + C-4) Triple
[1,2-$^{13}C_2$]glycine	24	7.4	18.3	17.6	29.4	9.2
[1,2-$^{13}C_2$]glycine	48	5.8	15.4	19.0	27.6	7.0
[1-^{13}C]glycine	24	7.9	<1.0	3.0	<1.0	<1.0
[1-^{13}C]glycine	48	6.0	<1.0	3.5	<1.0	<1.0
[2-^{13}C]glycine	24	13.2	11.7	17.7	28.4	9.2
[2-^{13}C]glycine	48	10.7	9.7	18.8	26.3	6.7

[a] Values are means of duplicate samples. [b] Average standard deviation ± 0.71. [c] Average standard deviation ± 0.40.

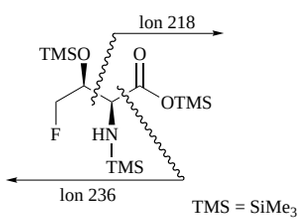


Fig. 1 Schematic representation of the fragmentation ions used to determine isotopic incorporations into 4-fluorothreonine **2** by GC–MS

acetate **1** (−215 ppm) and 4-fluorothreonine **2** (−229 ppm). Incorporations of ^{13}C into C-1 and C-2 of fluoroacetate **1** and C-3 and C-4 of 4-fluorothreonine **2** are readily diagnosed from the ^{19}F–^{13}C coupling patterns. An intact ^{19}F–^{13}C–^{13}C combination appears as a doublet of doublets ($^1J_{^{19}F-^{13}C}$ = 165 Hz, $^2J_{^{19}F-^{13}C}$ = 18 Hz) and intact ^{19}F-^{13}C–^{12}C and ^{19}F–^{12}C–^{13}C combinations appear as doublets with coupling constants of 165 and 18 Hz respectively. The presence of deuterium induces a shift to lower frequency for each of the fluorine signals in the ^{19}F{^{1}H} NMR spectra. The effect is additive, with a single deuterium atom geminal to fluorine (*viz*. FDHC-) inducing a shift of *ca*. 0.6 ppm in magnitude, and two geminal deuteriums (*viz*. FD_2C-) inducing a shift of *ca*. 1.2 ppm in magnitude. The resultant spectra are shown in the relevant Figs. 2–5. Incorporations into C-1 and C-2 of 4-fluorothreonine **2** could not however be determined by ^{19}F{^{1}H} NMR spectroscopy as the isotope-induced shifts on fluorine were no longer observable; therefore incorporations into these carbons were determined by GC–MS only. The results of experiments on incorporation of isotopically labelled glycine and pyruvate obtained by ^{19}F NMR spectroscopy have been reported in a preliminary communication.[11] Here we report all of our labelling studies to date employing both GC–MS and ^{19}F NMR spectroscopy to monitor incorporation of a variety of possible metabolic precursors into fluoroacetate **1** and 4-fluorothreonine **2**. These studies have enabled us to elucidate the pathways channelling metabolites towards fluorometabolite biosynthesis and to shed fresh light on the metabolic relationship between fluoroacetate **1** and 4-fluorothreonine **2**.

Results and discussion

Incorporation of labelled glycine and glycolate

Our previous work[10] had shown that, of a range of radiolabelled compounds tested, glycolate was the most effective precursor of fluoroacetate **1** in *S. cattleya*. Metabolically related compounds such as glycine and L-serine also gave significant levels of incorporation. Hence initial experiments with ^{13}C-labelled precursors involved incubation of labelled glycine and glycolate with resting cell suspensions of *S. cattleya*.

The rates of production of fluoroacetate and 4-fluorothreonine by washed cell suspensions were not significantly affected by the presence of either precursor and remained constant at 77 and 52 nmol (g wet wt)$^{-1}$ h^{-1} respectively over the 48 h duration of the experiments. Table 1 shows the incorporation of ^{13}C-label from [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-glycine into fluoroacetate and 4-fluorothreonine by *S. cattleya* as determined by GC–MS. Several interesting features are apparent. Whereas [1-^{13}C]glycine is very poorly incorporated, the label from [1,2-$^{13}C_2$]- and [2-^{13}C]-glycine exhibited high and similar magnitudes of overall incorporation into C-1 and C-2 of fluoroacetate **1** and also into [C-2 + C-3 + C-4] of 4-fluorothreonine **2**. Remarkably the patterns of ^{13}C-incorporation from these two labelled glycines are also very similar with a strikingly high level of double incorporation (*ca*. 40%) into fluoroacetate **1**, a finding which can only be interpreted in terms of the utilization of C-2 of glycine for synthesis of both carbon atoms of fluoroacetate **1**. Furthermore the patterns of ^{13}C incorporation of [1,2-$^{13}C_2$]- and [2-^{13}C]-glycine into [C-2 + C-3 + C-4] of 4-fluorothreonine **2** are essentially the same, indicating that both C-3 and C-4 of fluorothreonine are also labelled by C-2 of glycine. The precise extent of recombination of C-2 of glycine into C-3 and C-4 of 4-fluorothreonine and the exact degree of analogy between the labelling pattern in C-3 and C-4 of 4-fluorothreonine **2** and that in C-1 and C-2 of fluoroacetate **1** is not possible to assess from the mass spectrum of the silylated derivative of 4-fluorothreonine, as no ion is formed containing only C-3 and C-4 carbon atoms. This uncertainty was resolved by inspection of the ^{19}F{^{1}H} NMR spectra [Figs. 2(a) and (b)] of the supernatants from these experiments. These spectra con-

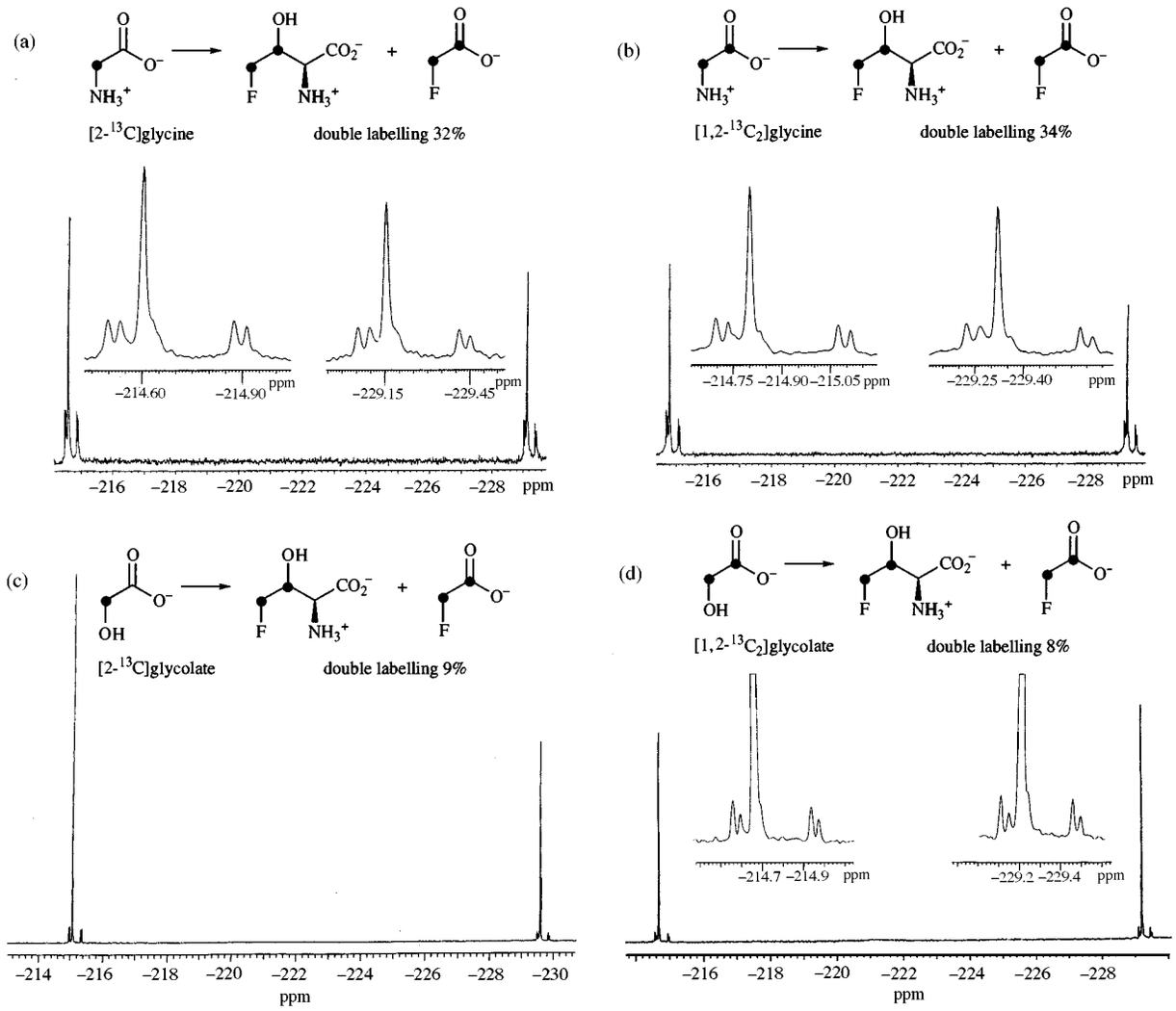


Fig. 2 $^{19}F\{^{1}H\}$ NMR spectra of fluoroacetate **1** and 4-fluorothreonine **2** in the supernatant after incubation of resting cells of *S. cattleya* for 48 h with (a) [2-^{13}C]glycine; (b) [1,2-$^{13}C_2$]glycine; (c) [2-^{13}C]glycolate and (d) [1,2-$^{13}C_2$]glycolate. Percentage incorporations are those determined by GC–MS analysis and a schematic representation of the predominant isotope distribution in **1** and **2** is illustrated.

firm the high incorporations from both [2-^{13}C]- and [1,2-$^{13}C_2$]-glycine into both fluorometabolites. The predominant doublet of doublets obtained with both labelled glycines indicates that the majority of labelled fluoroacetate **1** and 4-fluorothreonine **2** contains two ^{13}C atoms verifying the high level of recombination of isotope from the C-2 atom of glycine demonstrated by GC–MS. The striking similarity between the fluorometabolites in the patterns of the ^{19}F NMR signals both in terms of multiplicity and relative magnitude is readily apparent and demonstrates that the analogy between the labelling patterns in C-3 and C-4 of 4-fluorothreonine, and that in C-1 and C-2 of fluoroacetate, is almost exact—neatly complementing the GC–MS data. The most plausible explanation of this observation is that both sets of carbon atoms have an identical biosynthetic origin and that *S. cattleya* possesses a single fluorinating enzyme. As the direct interconversion of one fluorometabolite to the other has been discounted in previous work[10] the only possible conclusion is that each fluorometabolite is derived by metabolism of a common intermediate yet to be identified.

The observed recombination of C-2 of glycine into both fluorometabolites can be rationalised if their biosynthesis proceeds *via* serine or a metabolite of serine such as pyruvate. In a pathway which has been well delineated in bacteria,[12] C-2 of glycine is converted by a glycine cleavage system to N^5,N^{10}-methylenetetrahydrofolate which is then condensed in a reaction mediated by serine hydroxymethyl transferase with another molecule of glycine to form serine. Thus one glycine molecule contributes C-1 and C-2 of serine, and C-3 of serine is derived from C-2 of another. During this process C-1 of the second glycine molecule is lost. Furthermore if C-1 of serine is lost by decarboxylation during further metabolism to fluorometabolites the incorporation patterns produced from [1,2-$^{13}C_2$]- and [2-^{13}C]-glycine will be identical.

The incorporation patterns into fluoroacetate from [2-^{13}C]- and [1,2-^{13}C]-glycolate [see Fig. 2(c) and (d) for ^{19}F NMR spectra] were similar to those for [2-^{13}C]- and [1,2-$^{13}C_2$]-glycine respectively but lower in magnitude (9 and 8% *versus* 32 and 34% double labelling found in fluoroacetate after 48 h by GC–MS). The common labelling patterns suggest that glycolate is metabolized in a similar manner to glycine presumably *via* the latter compound after oxidation to glycolate and transamination. Further support for this hypothesis is provided by a comparison of the retention of deuterium atoms from C-2 of [$^{2}H_2$]-glycine and [$^{2}H_2$]-glycolate in fluoroacetate. In the former case one or both deuterium atoms can be retained (FDHC, 6%; FD_2C, 0.6% by GC–MS) in the fluoromethyl group of **1** and **2** as there is no mechanistic requirement to lose a hydrogen atom from C-2 of glycine if glycine is converted directly to serine by serine hydroxymethyl transferase. In the latter case however no more than a single deuterium is ever retained (FDCH, 1%) in **1** and **2**, a finding consistent with the obligatory oxidation of glycolate to glyoxalate prior to its conversion to glycine.

It is noteworthy that in these experiments a much more efficient incorporation of [2-^{13}C]- and [1,2-$^{13}C_2$]-labelled compounds was obtained with glycine than with glycolate. This observation was in marked contrast to our earlier [^{14}C] labelling

Table 2 Effect of cell density and precursor concentration on the incorporatioin of ^{13}C-label measured by GC–MS from [2-^{13}C]glycolate and [2-^{13}C]glycine into fluoroacetate **1** by cell suspensions of *S. cattleya* after 48 h

Precursor	Conc. (mM)	Cell density	^{13}C-Incorporation into fluoroacetate (%)[a] Single	Double
[2-^{13}C]glycine	2	Low	5.4	8.9
[2-^{13}C]glycine	10	Low	11.3	38.2
[2-^{13}C]glycolate	2	Low	4.4	13.1
[2-^{13}C]glycolate	10	Low	2.5	9.2
[2-^{13}C]glycine	2	High	2.8	1.0
[2-^{13}C]glycine	10	High	10.1	8.6
[2-^{13}C]glycolate	2	High	5.4	8.0
[2-^{13}C]glycolate	10	High	4.0	8.4

[a] Values are means of duplicate samples. Average standard deviation ±0.29.

study[10] where glycolate with an incorporation of 7% over 24 h emerged as a four-fold better precursor than glycine. We took some effort to determine the cause of this apparent anomaly and have found that the incorporation of glycine by a resting cell suspension of *S. cattleya* is particularly sensitive to precursor concentration and cell density. In Table 2 the incorporations of label from [2-^{13}C]glycine and [2-^{13}C]glycolate into fluoroacetate **1** by *S. cattleya* suspensions are compared at 2 and 10 mM precursor concentrations and at high and low cell densities. Some dramatic differences are revealed. Incorporation of [2-^{13}C]glycolate is relatively stable regardless of cell density and precursor concentration ranging between 8 and 13% double labelling. By contrast, incorporation of [2-^{13}C]-glycine shows a spectacular variation between 1 and 38% double labelling. The lowest incorporation occurs under conditions of low precursor concentration and high cell density whereas the highest value is observed at high precursor concentration and low cell density. This remarkable dependence of incorporation of label from glycine on the conditions of incubation is probably attributable to the rapid utilization of the compound as a carbon source under conditions of high cell density, drastically reducing the period for which it is available for incorporation into fluorometabolites. Glycolate on the other hand would appear to be only slowly utilized reflecting either low permeability of the cells to the compound or sluggish metabolism of the compound and so remains available for incorporation into fluorometabolites throughout the incubation period. This difference accounts for our earlier observations during the ^{14}C precursor incorporation study[10] of the apparent superiority of glycolate over glycine as a precursor. Those experiments were conducted under conditions of low precursor concentration and high cell density whereas the ^{13}C studies described in this paper were performed under conditions of high precursor concentration and low cell density.

Sanada *et al.*[7] tentatively suggested that 4-fluorothreonine **2** may be derived *via* condensation of fluoroacetaldehyde and glycine. This idea was based largely on the logic of an aldol condensation to generate **2** rather than any experimental evidence. From the incorporation patterns of [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C]-glycines into C-1 and C-2 of 4-fluorothreonine (Table 1) it is now clear that glycine *is not* incorporated as an intact unit into C-1 and C-2 of 4-fluorothreonine **2**. The GC–MS data allows a determination of incorporation into C-1 + C-2 of **2** but the fragmentation pattern does not allow an evaluation of the distribution of isotope between these sites. Nevertheless it is clear that there is a significant double label from [2-^{13}C]- and [1,2-$^{13}C_2$]-glycines but not from [1-^{13}C]glycine at these sites. Therefore the major pathway for glycine incorporation into C-1 and C-2 of **2** must be *via* serine produced from glycine by the action of serine hydroxymethyl transferase and not by direct incorporation. Thus a metabolite of serine must be delivering C-1 and C-2 of 4-fluorothreonine although a definitive conclusion as to its identity is not possible from the current data.

Incorporation of serine

The incorporation results reported above implicate L-serine as an intermediate in the biosynthesis of fluorometabolites from glycine. To further elucidate the role of serine the incorporation of L-[3-^{13}C]serine into **1** and **2** was investigated. The ^{19}F{^{1}H} NMR spectrum of supernatant from cell suspensions incubated for 48 h in the presence of labelled serine is shown in Fig. 3(a). It is clear from the doublet (J = 165 Hz) associated with each fluorometabolite signal that the majority of the isotope from [3-^{13}C]serine is regiospecifically incorporated (14% labelling in fluoroacetate by GC–MS after 48 h) in the fluoromethyl carbons of fluoroacetate **1** and 4-fluorothreonine **2**. This finding is consistent with L-serine acting as an intermediate in fluorometabolite biosynthesis, C-2 and C-3 of serine contributing C-1 and C-2 of fluoroacetate and C-3 and C-4 of 4-fluorothreonine.

Incorporation of pyruvate and L-alanine

The widespread presence of serine dehydratase in microorganisms ensures the rapid conversion of serine to pyruvate. Therefore the possible role of pyruvate in fluorometabolite biosynthesis was investigated using GC–MS by examining the incorporation of label from [1-^{13}C]-, [2-^{13}C]- and [3-^{13}C]-pyruvate into **1** and **2** by cell suspensions of *S. cattleya* (Table 3). The rates of production of fluoroacetate and 4-fluorothreonine were not significantly affected by the presence of pyruvate and remained constant at 66 and 60 nmol (g wet wt)$^{-1}$ h^{-1} [nmol product per g of *S. cattleya* (wet weight) per h] respectively over the 48 h duration of the experiment. Little incorporation of label from [1-^{13}C]pyruvate was observed implying that C-1 is lost during the biosynthetic process. However high incorporation of label from [2-^{13}C]- and [3-^{13}C]-pyruvate into both fluoroacetate **1** and 4-fluorothreonine **2** was found with label from C-2 of pyruvate entering mainly C-1 of **1** and label from C-3 of pyruvate entering C-2 of **1**. Together these observations furnish strong evidence that C-2 and C-3 of pyruvate are incorporated as a unit into fluoroacetate.

The incorporation from C-2 and C-3 of pyruvate into (C-2 + C-3 + C-4) of 4-fluorothreonine **2** is very similar in terms of magnitude and proportion of double to single label to that into C-1 and C-2 of fluoroacetate. Bearing in mind that the incorporation into C-2 of 4-fluorothreonine **2** must of necessity be low because the combined (C-1 + C-2) label in 4-fluorothreonine **2** is quite small it therefore follows that incorporation into C-3 and C-4 of 4-fluorothreonine **2** must closely parallel that into C-1 and C-2 of fluoroacetate signifying that very probably these units arise by a similar mechanism.

The similarity of the labelling in these positions in the two compounds is confirmed by the ^{19}F{^{1}H} NMR spectra of the supernatants from the [2-^{13}C]- and [3-^{13}C]-pyruvate incorporation experiments [Figs. 3(b) and (c)]. The spectrum obtained using [2-^{13}C]pyruvate has a predominant doublet (J = 18 Hz) associated with the fluorine signal of each fluorometabolite showing that C-2 of pyruvate contributes the carboxylate carbon of fluoroacetate **1** and C-3 of 4-fluorothreonine **2**. There is a minor doublet (J = 165 Hz) which indicates that some label has entered the fluoromethyl groups. This is explicable in terms of the condensation of a small proportion (*ca.* 20%) of the [2-^{13}C]oxaloacetate formed from [2-^{13}C]pyruvate by pyruvate carboxylase with unlabelled acetyl CoA to generate [3-^{13}C]-citrate which is processed *via* the citric acid cycle to yield [2-^{13}C]- and [3-^{13}C]-oxaloacetate (see below). The spectra of both fluorometabolites obtained using [3-^{13}C]pyruvate clearly show a doublet (J = 165 Hz) characteristic of incorporation into the fluoromethyl groups. Together therefore the GC–MS and ^{19}F{^{1}H} NMR results indicate unequivocally that ^{13}C incorporations from labelled pyruvates into C-1 and C-2 of **1**

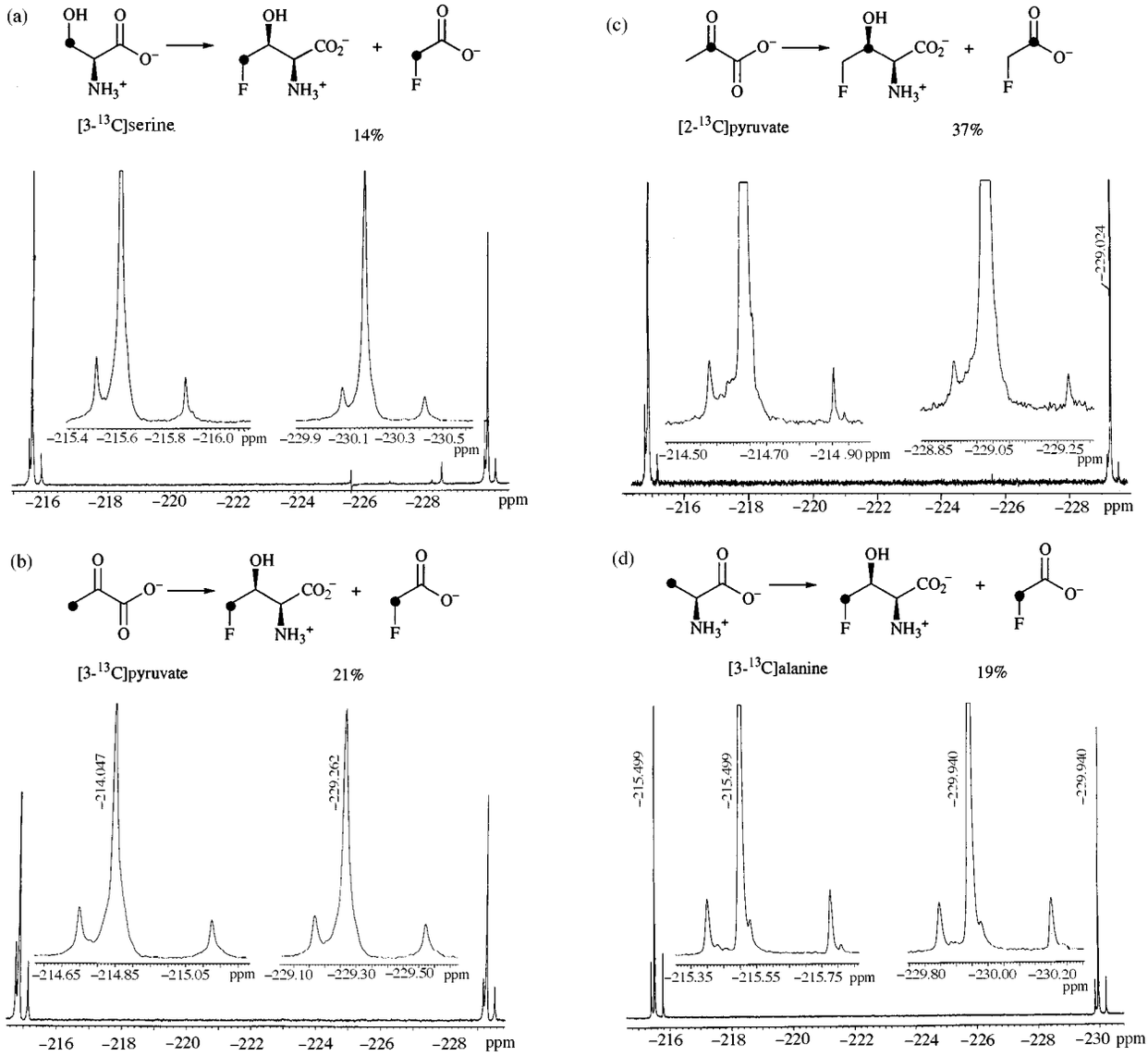


Fig. 3 $^{19}F\{^{1}H\}$ NMR spectra of fluoroacetate **1** and 4-fluorothreonine **2** in the supernatant after incubation of resting cells of *S. cattleya* for 48 h with (a) [3-^{13}C]serine; (b) [2-^{13}C]pyruvate; (c) [3-^{13}C]pyruvate and (d) L-[3-^{13}C]alanine. Percentage incorporations are those determined by GC–MS analysis and a schematic representation of the predominant isotope distribution in **1** and **2** is illustrated.

mirror those into C-3 and C-4 of **2** in terms of magnitude and regiochemistry.

The amino acid L-alanine can be considered a pyruvate surrogate as transamination will release pyruvate *in vivo*. An experiment in which cell suspensions were incubated with L-[3-^{13}C]alanine was conducted and the results are shown in Fig. 3(d). A substantial amount of label (19% labelling in fluoroacetate by GC–MS after 48 h) was incorporated into C-1 of **1** and C-4 of **2**, similar to that observed after incubation with [3-^{13}C]pyruvate.

Incorporation of aspartate and succinate

In order to delineate further the pathway of incorporation of pyruvate into the fluorometabolites it is necessary to consider the various potential metabolic fates for pyruvate. Pyruvate can be oxidatively decarboxylated by the pyruvate dehydrogenase complex to yield acetyl-CoA. There is some evidence from the experiments discussed above using [2-^{13}C]pyruvate that this metabolic route is operating in *S. cattleya* albeit to a comparatively minor extent. However, this metabolic option appears an unlikely route toward the fluorometabolites, as the methyl group is no longer activated for a potential fluorination reaction. Alternatively pyruvate could be converted by pyruvate carboxylase to oxaloacetate prior to activation to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase. Entry to the glycolytic cycle by this route would ensure that the potential fluoromethyl carbon is activated as a phosphomethyl carbon, a feature which is intuitively attractive.

In order to assess the plausibility of this route to the fluorometabolites, the efficacy of oxaloacetate as a precursor was explored by incubating *S. cattleya* cell suspensions with DL-[$^{2}H_3$]aspartate and [$^{2}H_4$]succinate both of which are readily metabolized to oxaloacetate *in vivo*. The incorporation of deuterium from DL-[$^{2}H_3$]aspartate was low (6.5% onto C-2 of fluoroacetate after 48 h as measured by GC–MS) but nevertheless it is clear from the mass spectral data that a significant proportion (2%) of the two fluorometabolites retained both deuterium atoms in their fluoromethyl groups. This is also apparent in the $^{19}F\{^{1}H\}$ NMR spectrum in Fig. 4(a) where a signal at 215.8 ppm represents the population of fluoroacetate molecules bearing a FD_2C-fluoromethyl group. The incorporation of label from [$^{2}H_4$]succinate into both fluorometabolites is considerably higher (20% label on C-2 of fluoroacetate after 48 h by GC–MS). The resultant $^{19}F\{^{1}H\}$ NMR is shown in Fig. 4(b). However it is significant that in this experiment only a single deuterium atom is incorporated. There was no detectable population of molecules containing two deuterium atoms attached to the fluoromethyl groups of **1** and **2**. The results of

Table 3 Incorporation of ^{13}C-label determined by GC–MS from various ^{13}C-labelled pyruvates into (i) fluoroacetate **1** and (ii) 4-fluorothreonine **2** by cell suspensions of *S. cattleya*

(i) Incorporation into fluoroacetate **1**

Precursor (10 mM)	Incubation time/h	^{13}C-Incorporation (%)[a,b] Single C-1	Single C-2	Double
[1-^{13}C]pyruvate	24	1.2	1.0	<1.0
[1-^{13}C]pyruvate	48	1.1	4.0	<1.0
[2-^{13}C]pyruvate	24	41.5	3.8	<1.0
[2-^{13}C]pyruvate	48	36.9	5.6	<1.0
[3-^{13}C]pyruvate	24	8.3	31.4	3.3
[3-^{13}C]pyruvate	48	6.8	20.7	2.3

(ii) Incorporation into 4-fluorothreonine **2**

Precursor (10 mM)	Incubation time/h	^{13}C-Incorporation (%)[a,c] (C-1 + C-2) Single	(C-1 + C-2) Double	(C-2 + C-3 + C-4) Single	(C-2 + C-3 + C-4) Double	(C-2 + C-3 + C-4) Triple
[1-^{13}C]pyruvate	24	2.8	<1.0	<1.0	<1.0	<1.0
[1-^{13}C]pyruvate	48	4.6	<1.0	4.6	<1.0	<1.0
[2-^{13}C]pyruvate	24	10.5	<1.0	43.7	1.4	<1.0
[2-^{13}C]pyruvate	48	11.6	<1.0	42.1	2.0	<1.0
[3-^{13}C]pyruvate	24	6.6	<1.0	38.2	4.9	<1.0
[3-^{13}C]pyruvate	48	4.4	<1.0	27.8	3.0	<1.0

[a] Values are means of duplicate samples. [b] Average standard deviation ± 0.50. [c] Average standard deviation ± 0.39.

both these experiments are consistent with a role for oxaloacetate as an intermediate in fluorometabolite biosynthesis. Aspartate only requires to be transaminated to generate oxaloacetate and therefore up to two deuterium atoms can be retained whilst succinate is processed *via* fumarate and malate to oxaloacetate which results in the retention of a maximum of one deuterium atom.

Incorporation of acetate

The distribution of isotope in fluoroacetate **1** and 4-fluorothreonine **2** after incubation of *S. cattleya* cell suspensions with [2-^{13}C]acetate is summarised in Fig. 5. There is substantial overall incorporation into both fluorometabolites (26% labelling in fluoroacetate **1** after 48 h by GC–MS) but it is not regiospecifically located, a significant level of isotopic recombination (11%) being observed. The pattern of incorporation can be explained in terms of the entry of acetate into the citric acid cycle by condensation of [2-^{13}C]acetyl CoA with oxaloacetate and subsequent processing of [2-^{13}C]citrate through the cycle to ultimately generate both [2-^{13}C]- and [3-^{13}C]-oxaloacetate. This pool of oxaloacetate can either be converted to phosphoenolpyruvate or be recycled by combination with another molecule of [2-^{13}C]acetyl CoA to generate a pool of [2,3-$^{13}C_2$]oxaloacetate. There is no evidence from this experiment that acetate is incorporated directly into either fluorometabolite. Rather the results serve to reinforce the importance of oxaloacetate as an entry point into the metabolic sequence leading to the formation of the carbon substrate for fluorination.

Conclusions

Glycine has been identified as a highly effective precursor of fluoroacetate **1** and 4-fluorothreonine **2** in *S. cattleya*. Studies involving incubation of cell suspensions with various ^{13}C-labelled glycines demonstrate that C-1 and C-2 of fluoroacetate **1** and C-3 and C-4 of fluorothreonine **2** can originate from the α-carbon of glycine. No label is contributed by the carboxylate carbon of glycine which is lost in the biosynthetic process. This incorporation pattern is consistent with the cleavage of glycine to generate N^5,N^{10}-methylenetetrahydrofolate which is then condensed with another molecule of glycine to form serine in a reaction catalysed by serine hydroxymethyl transferase. Serine is subsequently converted to pyruvate by serine dehydratase. Evidence for this pathway is provided by the observation that C-2 and C-3 of pyruvate are efficiently incorporated into both fluorometabolites, C-3 contributing the fluoromethyl carbon. Furthermore [3-$^2H_3$]alanine which readily transaminates *in vivo* to [3-$^2H_3$]pyruvate generates fluorometabolites bearing up to two deuterium atoms on their fluoromethyl groups. Ensuing activation of pyruvate to oxaloacetate by the action of pyruvate carboxylase is implicated by the efficient incorporation of deuterium from [$^2H_4$]succinate. A single deuterium atom was retained in the fluoromethyl group of each fluorometabolite, an observation consistent with succinate being processed *via* the citric acid cycle through fumarate and malate prior to oxidation to oxaloacetate. The role of oxaloacetate as an intermediate is further reinforced by the retention of up to two deuterium atoms in the fluoromethyl groups from [$^2H_3$]aspartate which is transaminated *in vivo* to oxaloacetate. The decarboxylation of oxaloacetate to phosphoenolpyruvate provides an entry into the glycolytic pathway. The results from the current study therefore trace the metabolic origin of both fluorometabolites to an intermediate in the glycolytic pathway. Tamura *et al.*[8] identified glycerol as a good precursor for fluoroacetate biosynthesis in *S. cattleya* and we have recently established[13] that *pro-R*- and not *pro-S*-[1-$^2H_2$]glycerol labelled the fluoromethyl groups of both **1** and **2**. This stereochemical constraint is consistent with glycerol entering the glycolytic pathway by the action of glycerol kinase yielding glycerol-3-phosphate with the phosphate group ultimately being replaced by fluoride. The observations of the current study taken in the context of these previous findings indicate a role for an intermediate in the glycolytic pathway between dihydroxyacetone phosphate and phosphoenolpyruvate as the substrate for the fluorination process. The metabolic overview presented in Scheme 1 rationalises and integrates the results of all labelling studies to date on the origin of fluorometabolites. An important corollary arises from our interpret-

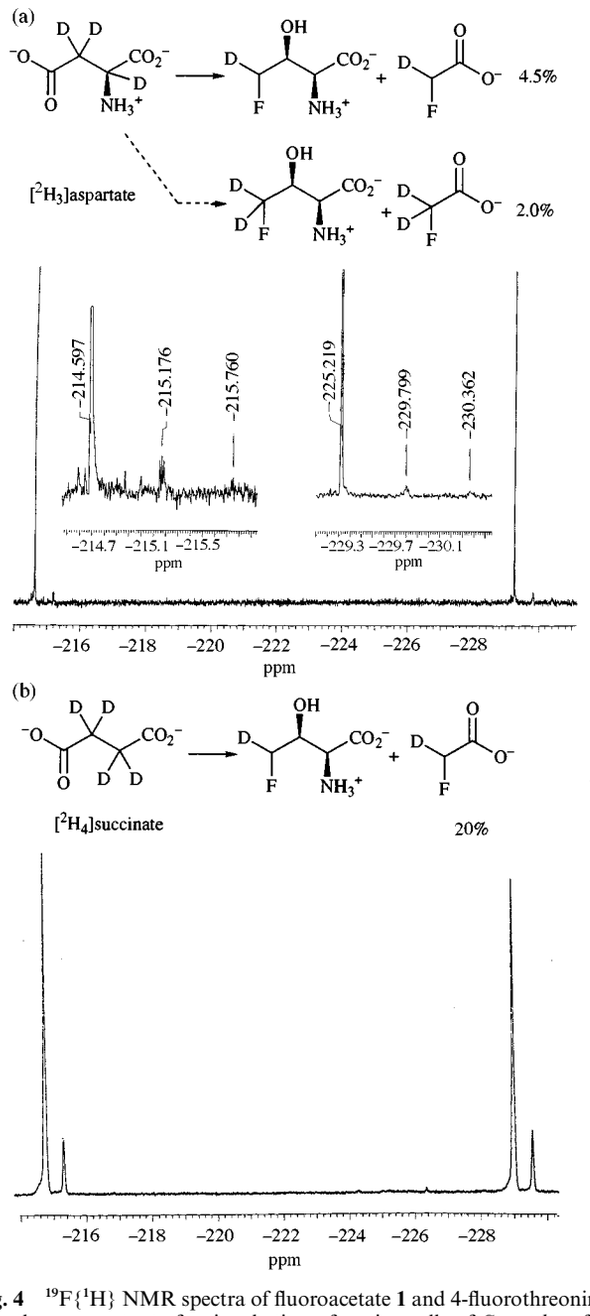


Fig. 4 $^{19}F\{^{1}H\}$ NMR spectra of fluoroacetate **1** and 4-fluorothreonine **2** in the supernatant after incubation of resting cells of *S. cattleya* for 48 h with (a) DL-[$^{2}H_3$]aspartate; (b) [$^{2}H_4$]succinate. Percentage incorporations are those determined by GC–MS analysis and a schematic representation of the predominant isotope distribution in **1** and **2** is illustrated.

ation. The extent to which labelling in C-1 and C-2 of fluoroacetate parallels that in C-3 and C-4 of 4-fluorothreonine in the results of experiments with *every* precursor examined in this study renders inescapable the conclusion that both fluorometabolites arise from a common fluorinated intermediate which implies that a single fluorinating enzyme exists in *S. cattleya*. Our current studies are now focussed on identifying the glycolytic intermediate most closely associated with the fluorination event and the immediate product of the fluorination process. In parallel with these investigations we intend to explore the mechanism by which *S. cattleya* protects itself from the toxic effects of fluoroacetate which is lethal to most organisms as a result of the synthesis of fluorocitrate, an inhibitor of the citric acid cycle enzyme aconitase,[14] and of citrate transport more generally.[15] Clearly *S. cattleya* must possess several unusual metabolic adaptions in order to survive the synthesis of such a toxic secondary metabolite.

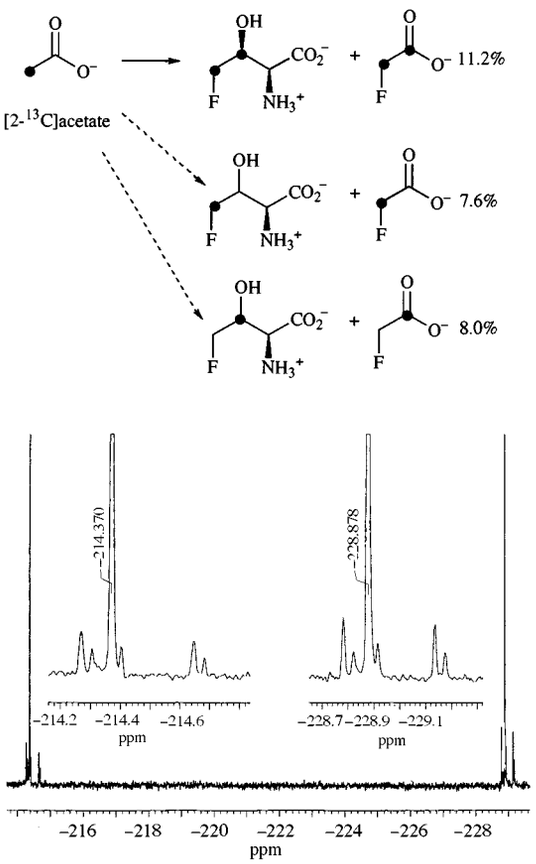


Fig. 5 $^{19}F\{^{1}H\}$ NMR spectra of fluoroacetate **1** and 4-fluorothreonine **2** in the supernatant after incubation of resting cells of *S. cattleya* for 48 h with [2-^{13}C]acetate. Percentage incorporations are those determined by GC–MS analysis and a schematic representation of the predominant isotope distribution in **1** and **2** is illustrated.

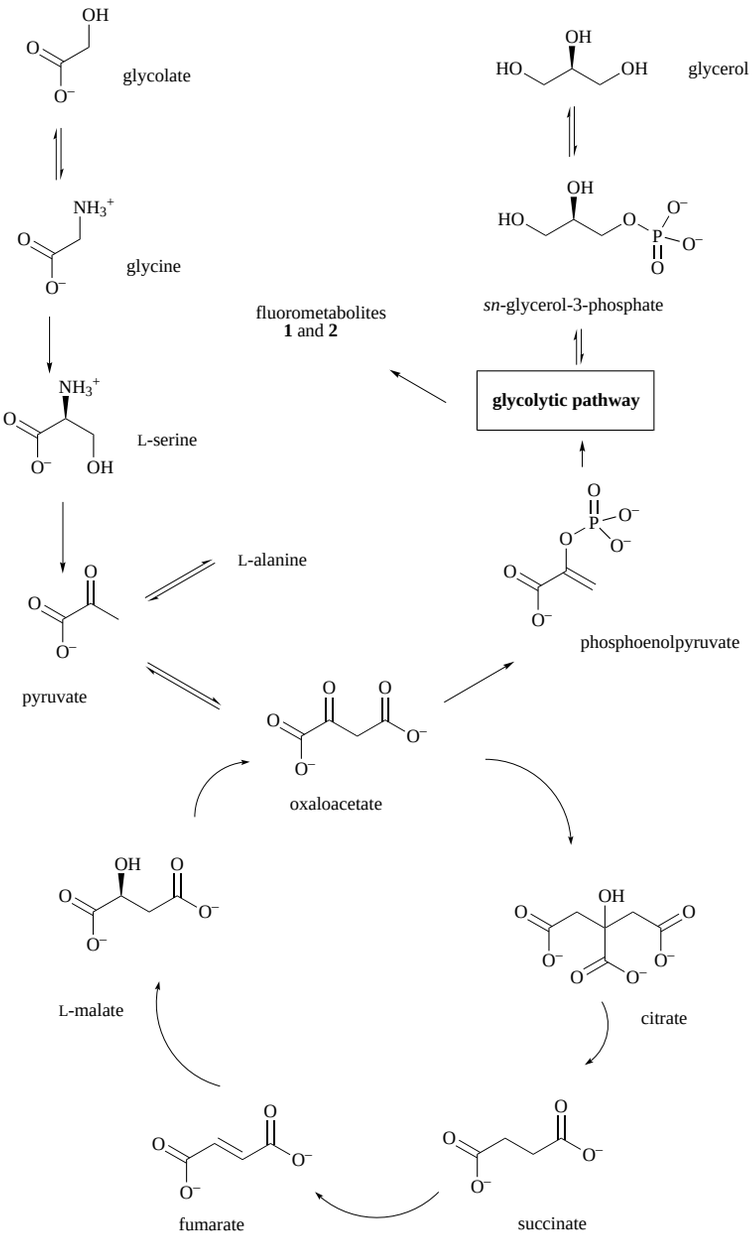
Experimental

Isotopically labelled compounds

[1-^{13}C]Glycine (99 atom% ^{13}C), [2-^{13}C]glycine (99 atom% ^{13}C), [1,2-$^{13}C_2$]glycine (99 atom% ^{13}C), [$^{2}H_5$]glycine (97 atom% ^{2}H), [2-^{13}C]acetic acid (99 atom% ^{13}C) and [$^{2}H_4$]succinic acid (98 atom% ^{2}H) were acquired from Sigma-Aldrich, Gillingham, Dorset, UK. Sodium [1-^{13}C]pyruvate (99.5 atom% ^{13}C), sodium [2-^{13}C]pyruvate (99 atom% ^{13}C), sodium [3-^{13}C]pyruvate (99 atom% ^{13}C), L-[3-^{13}C]alanine (99.1 atom% ^{13}C) and DL-[$^{2}H_3$]-aspartate (98 atom% ^{2}H) were acquired from CDN, Isotopes, Quebec, Canada. L-[3-^{13}C]Serine (99 atom% ^{13}C) was obtained from Cambridge Isotopic Laboratories, Cambridge, UK. Sodium [1,2-$^{13}C_2$]glycolate (>99 atom% ^{13}C) and sodium [2-^{13}C]glycolate (>99 atom% ^{13}C) were prepared from [1,2-$^{13}C_2$]bromoacetic acid and [2-^{13}C]bromoacetic acid respectively by hydrolysis with aqueous KOH in a sealed tube at 120 °C for 3 h as described by Tamura *et al.*[8] Calcium [$^{2}H_2$]glycolate (99 atom% ^{2}H) was prepared by the method of Hughes *et al.*,[16] from [2-$^{2}H_3$]acetic acid.

Organism and cultural conditions

S. cattleya NRRL8057 was obtained from the Northern Utilization Research and Development Division, US Department of Agriculture, Peoria, Ill and maintained on agar slants containing 2% (wt/vol) soyabean flour and 2% (wt/vol) mannitol. Batch cultures were grown at 29 °C in 2 l conical flasks plugged with cotton wool and containing 150 ml of medium of the following composition (g l^{-1}): KH_2PO_4, 2.0; NH_4Cl, 1.5; $MgSO_4 \cdot 7H_2O$, 0.05; NaCl, 0.5; glycerol, 10; inositol, 0.4; monosodium L-glutamate monohydrate, 5.0; NaF, 0.084; $FeSO_4 \cdot 7H_2O$, 0.025; $ZnSO_4 \cdot 7H_2O$, 0.01; $CoCl_2 \cdot 6H_2O$, 0.01; $CaCO_3$, 0.25; *p*-aminobenzoic acid, 0.0001. The pH of the medium



Scheme 1

was adjusted to 7.0 and each flask inoculated with 1 ml of a suspension of vegetative mycelium prepared from a 6 day old culture. Cultures were incubated on an orbital shaker at 140 rpm.

Preparation and utilization of resting cell suspensions

After growth for 6 days, cells of *S. cattleya* were harvested by centrifugation, washed three times with 50 mM 2-morpholinoethanesulfonic acid (MES) buffer pH 6.5 using a volume equivalent to that of the original culture volume and then suspended in this buffer by placing the appropriate weight of cells in a known volume of buffer. Except where otherwise indicated, in experiments involving incubation with various ^{13}C- or ^{2}H-labelled precursors, cell suspensions (23 ml, 133 mg wet weight) containing 2 mM NaF and the precursor (10 mM) were incubated at 29 °C with shaking in 100 ml conical flasks stoppered with cotton wool bungs. In experiments where incorporation of [2-^{13}C]glycolate was compared with that of [2-^{13}C]glycine, precursor concentrations of both 2 and 10 mM were employed and cell suspension densities of 33 mg wet weight ml^{-1} (low density) and 133 mg wet weight ml^{-1} (high density) were utilized. In all experiments after periods of 24 and 48 h the contents of duplicate flasks representing each treatment were centrifuged and the supernatants stored at −15 °C pending GC–MS analysis. $^{19}F\{^{1}H\}$ NMR spectra were also recorded for all 48 h samples.

GC–MS Determination of ^{13}C or ^{2}H incorporation into fluoroacetate 1

Fluoroacetate in culture supernatants was derivatized as the phenylphenacyl derivative and analysed by GC–MS in the selected ion monitoring mode. Culture supernatant (1 ml) was frozen, lyophilized and the dried powder was suspended in toluene–acetonitrile (1:1 v/v, 1 ml), 1,4,7,10,13,16-hexaoxacyclooctadecane (10 mg) and 4-(bromoacetyl)biphenyl (10 mg) were added and the mixture heated at 75 °C for 12 h. The derivatized samples were cooled and analysed by GC–MS on a Hewlett Packard 5890 gas chromatograph linked to an HP 5970B mass selective detector controlled by an HP300 series computer. The gas chromatograph was fitted with an autosampler and equipped with an Ultra 1 fused-silica wall-coated open tubular capillary column (12 × 0.22 mm) with 5% biphenyl, 95% dimethyl polysiloxane as the bonded phase. Helium was used as the carrier gas at a flow rate of 0.8 ml min^{-1}

and samples (1 μl) were injected employing a split ratio of 80:1 except where concentrations in samples were less than 0.2 mM when injection was performed splitless. After injection the oven temperature was held at 130 °C for 1 min then programmed at 10 °C min^{-1} to 300 °C.

The mass spectrometer was operated in the selected ion monitoring mode. For determination of a ^{13}C-label incorporated into (C-1 + C-2), ion currents at *m*/*z* 272 (molecular ion, *M*), 273 (M + 1) and 274 (M + 2) were monitored. After correction of peak areas for natural isotropic abundances, the percentage of fluoroacetate mono- and di-labelled with ^{13}C was calculated by dividing the peak area for ions 273 and 274 respectively by the sum of the peak areas for ions 272, 273 and 274. The percentage of fluoroacetate with a ^{13}C-label present in C-2 of fluoroacetate was calculated from the peak areas for ions 33 and 34 (after correction for natural isotopic abundances) by dividing the peak area for ion 34 by the sum of the peak area for ion 33 and 34. The percentage of fluoroacetate that was monolabelled with ^{13}C-label in C-2 was obtained by subtracting the percentage of fluoroacetate dilabelled with ^{13}C from the percentage of fluoroacetate with a ^{13}C-label in position 2. The percentage of fluoroacetate with ^{13}C-label in C-1 was obtained by subtraction of the percentage of fluoroacetate with ^{13}C label in C-2 from the percentage of monolabelled fluoroacetate. The incorporation of ^{2}H-label on to C-2 of fluoroacetate was determined in a similar manner.

GC–MS Determination of ^{13}C or ^{2}H incorporation into 4-fluorothreonine 2

4-Fluorothreonine in culture supernatants was derivatized as the trimethylsilyl compound and incorporation of label was determined by GC–MS in the selected ion monitoring mode. Culture supernatant (0.25 ml) was lyophilized and 4-fluorothreonine derivatized by addition of MSTFA (0.25 ml) to the dried powder followed by heating at 100 °C for 1 h. The sample was subjected to GC–MS under conditions similar to those employed for determination of incorporation into fluoroacetate. Samples (1 μl) were injected in the split mode using a split ratio of 50:1 for 4-fluorothreonine concentrations above 0.2 mM. Below this concentration a splitless injection was performed. Since the silylated derivative of 4-fluorothreonine yielded no significant molecular ion, monitoring of ^{13}C-incorporation into the intact molecule was not possible. However, the molecule undergoes cleavage α to the amino group to give two characteristic fragment ions. Cleavage between C-1 and C-2 yields a major ion at *m*/*z* 236 containing C-2, C-3 and C-4 of 4-fluorothreonine whilst cleavage between C-2 and C-3 gives an intense ion at *m*/*z* 218 combining C-1 and C-2 of the 4-fluorothreonine. Thus monitoring of ions 218, 219 and 220 will allow ^{13}C incorporation into (C-1 + C-2) to be calculated and monitoring of ions 236, 237, 238 and 239 will enable ^{13}C incorporation into (C-2 + C-3 + C-4) to be studied. After correction for natural isotopic abundances the percentage of each fragment mono-, di- and tri-labelled with ^{13}C was calculated by dividing the area for the appropriate ion by the sum of the areas for all the ions of the fragment. The incorporation of a ^{2}H-label on to the (C-1 + C-2) and (C-2 + C-3 + C-4) fragments of 4-fluorothreonine was determined in a similar manner.

$^{19}F\{^{1}H\}$ NMR Analysis

The culture supernatant was lyophilized and dissolved in D_2O (1 ml) and the resulting solution filtered through glass wool to remove particulate matter. $^{19}F\{^{1}H\}$ NMR spectra were recorded on a Bruker Spectrospin AMX-500 instrument operating at 471.54 MHz and with a proton decoupling frequency of 500.14 MHz. Chemical shifts were measured relative to the fluoride signal (−119 ppm) and spectra were typically acquired over 10 h.

Acknowledgements

We thank BNFL Company Research Laboratories (CRL), Springfields, Preston for financial support. We also thank Mr Ian H. McKeag of the University of Durham for recording $^{19}F\{^{1}H\}$ NMR spectra and Dr Roy Bowden, BNFL Fluorochemicals and Drs Peter Binks and Harry Eccles, BNFL CRL for their close interest in the project.

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Paper 7/06554J
Received 8*th September* 1997
Accepted 6*th November* 1997