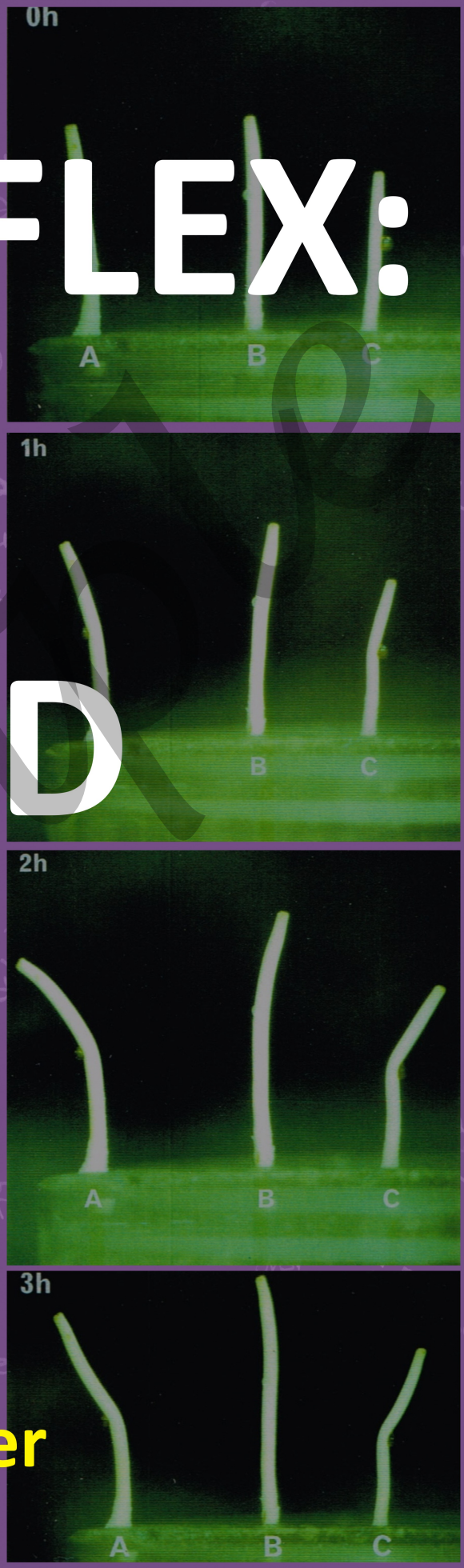
FUNGIFLEX: THE UNTOLD STORY



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FUNGIFLEX: THE UNTOLD STORY

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Fungiflex: the untold story

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Fungiflex: the untold story

Outline

In the latter half of the 1990s, and into the first couple of years of the new millennium, we carried out some ground-breaking research into communication within mushroom fruit bodies by chemical signals. Because we were hoping to commercialise the research, the work was never published beyond initial patent applications, which have since lapsed.

We believed at the time that we had accessed at least some of the controls of fungal morphogenesis by extracting compounds which caused mushroom stipes to bend at the point of application within hours of treatment.

Using standardised bioassays, we made a start on purifying and chemically characterising the extracted substances. We worked with two components which had recognisably different activities in the bioassay. These we called Fungiflex 1 and Fungiflex 2.

Our extracts caused (vertical) mushroom stipes to bend towards a drop of the extract within 1 hour of its application to the side of the stipe. This localised growth inhibition was Fungiflex 1 activity. Such stipes grew back to the vertical in about 9 hours, but then started bending in the opposite direction at the point of application of the drop. By 18 hours the stipe had bent to an angle of 90° from the vertical. This localised growth promotion was Fungiflex 2 activity.

We got close to understanding the structure of these first fungal hormonal compounds but were unable to secure sufficient funding to take the research any further.

Nonetheless, the scientific knowledge we revealed is still important and worthy of publication. Our research has not lost its uniqueness, nor has it been duplicated up to now.

In this publication, we report in full the research we accomplished nearly 20 years ago in the hope that it might stimulate future work in the topic and prompt further advances.

You could pick up the research where we left off.
You could make yourself a fortune!
And if you do, please remember where you got the idea!

David Moore and LilyAnn Novak-Frazer
June, 2017

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1. Introduction

Some years ago, we carried out some ground-breaking research into chemical communication within mushroom fruit bodies. Because we were hoping to commercialise the research, the work was never published beyond initial patent applications.

We believed at the time that we had devised methods to gain access to at least some of the chemical signals that control fungal morphogenesis, and developed a method (the basis of a patent application at the time) to extract compounds which caused mushroom stipes to bend at the point of application within hours of treatment.

Using standardised bioassays, we made a start on purifying and chemically characterising the extracted substances. We worked with two components which had recognisably different activities in the bioassay. These we called **Fungiflex 1** and **Fungiflex 2**. Our extracts caused (vertical) mushroom stipes to bend towards a drop of the extract within 1 hour of its application to the side of the stipe.This (essentially a **localised growth inhibition**) was **Fungiflex 1 activity**. Such stipes grew back to the vertical in about 9 hours, but then started bending in the opposite direction ***at the point of application of the drop***. By 18 hours the stipe had bent to an angle of 90° from the vertical. This (a **localised growth promotion**) was **Fungiflex 2 activity**.

Our research has not lost its uniqueness, ***nor has it been duplicated*** up to now, and the scientific knowledge we revealed is still important and worthy of publication. In these pages, we summarise our research in the hope that it might stimulate additional research and prompt further advances. This document is structured to give you successively greater amounts of detail about our discoveries. We start with a summary of the outcome of our research, and this includes sufficient background information for you to put our findings into their scientific context. In Appendix 1 we outline some earlier research (indeed, 30 years earlier) that now seems directly relevant. Appendix 2 is effectively our 'results' section, and details the experiments that were carried out, and shows how our interpretations developed. Finally, Appendix 3 displays our 'methods' in sufficient detail for you to replicate any of our experiments (and, where feasible in this section, we give cross-references to present day sources of materials).

As well as reading through this document, you may like to refer to the website ***David Moore's World of Fungi*** at http://www.davidmoore.org.uk. There's a lot of useful information on this website; but it's also a source of (free) PDF reprints of our published papers. You don't have to go searching through dusty old libraries to find 30-year old literature (some of which was only published on *paper*!). Go to a dusty old website and download the PDF in nice clean 21st century electrons.

2. Essential Fungiflex methods

Most of the methods and materials that we used in this research are detailed in the Appendices at the end of this report; but there are two aspects that we need to stress right at the start: the identity of the organism we used and the basic method for extracting the bioactive materials we called **Fungiflex**.

Organism

Most of the work was done with the ink cap mushroom *Coprinopsis cinerea*. The organism has previously been called (by us and by our colleagues) *Coprinus cinereus* and before that *Coprinus lagopus* and *Coprinus macrorhizus*. It is widespread in temperate climates around the world being a commonly-encountered member of the community that recycles leaf litter. Its life cycle, genetics and methods for *in vitro* cultivation have been described by Moore & Pukkila (1985).

Extraction methods

Numerous methods for extraction of bioactive materials, involving a variety of organic solvents, hot and cold, and a wide range of salt solutions, have been described in the literature. So, we want to stress at the outset that ***our*** method was the simplest of all; it simply consisted of steeping (or ***infusing***) freshly harvested mushroom tissue in ***sterile distilled water at room temperature***.

There was no vortexing, no mixing, no blending, no grinding; just stationary infusion of intact tissues in water.

After a few hours standing on the bench, the liquid was drained from the tissue, filtered and freeze-dried. The freeze-dried material was our bioactive extract.

Usually, the tissue we used was some part of a *Coprinopsis* mushroom fruit body; the nature and quantity of that tissue depended on the nature and purposes of the planned experiments, but in all cases the required mushrooms were freshly detached from the parent mycelium immediately prior to being placed in the steeping/infusing water. At various times and for different experiments the detached tissues may have been:

- at the stage of a young fruit body primordium, which was 1 to 3 mm in diameter and height, and harvested before the time of meiosis (= *pre-meiosis)*;

- an older, differentiated fruit body primordium (more than 3 mm in diameter and height), immature, and though cap and stipe tissues were clearly developed, these were also pre-meiosis;

- or a young fruit body, which was 20 to 50 mm in height, the cap of which was still cylindrical (that is, still enclosing the apex of the stipe, and not expanded like a coprinoid 'umbrella'); the basidia of these caps had completed the meiotic division (= post-meiosis), though the youngest in this class may not have yet formed spores, or spores, if formed, may not have developed their characteristic

'black' pigmentation (progress of meiosis, and spore formation and pigmentation were easily followed by light microscopy of squashes of tiny slivers of gill tissue).

Different extracts could be made by removing the caps from young fruit bodies, which could be done without damaging the stipe; in these experiments the caps and stipes were placed separately into pre-weighed and pre-sterilised 25 ml plastic tubes or pre-weighed, autoclaved 25 ml glass tubes.

Caps of primordia were left intact as removing them caused severe injury to both the cap and stipe and we wanted to avoid excessive leakage of intracellular chemicals. There were no differentiated zones in the youngest primordia and these were placed whole into pre-weighed sterilised plastic or glass tubes.

For each tissue sample used, the wet weight was determined and recorded. Sterilised distilled water was added to fill the tube completely (between 10 - 30 ml depending on the volume of tissue in the tube). Tubes were sealed tightly and kept in the dark at room temperature (21 - 23° C) for 1, 2 or 3 h.

As we were interested in gravitropism at the time, in some experiments comparisons were made between tubes that were kept vertical and those laid horizontal, but we found no difference in the outcome.

At the end of the infusion period the tissue(s) were removed from the tubes, the supernatant was filtered through Whatman No. 1 filter paper and then through a 0.45 µm cellulose nitrate membrane filter (Whatman) before being frozen at -20° C until needed.

Initially, when several tubes had been accumulated (volume more than 150 ml), they were frozen in liquid nitrogen individually and then set to freeze dry for 2-3 days. Later we found we could greatly reduce the volume of the pooled primary extract by rotary evaporation (water bath at less than 40° C) prior to being frozen in liquid nitrogen and freeze dried overnight.

Control experiments performed by infusing empty tubes with distilled water showed that there were no chemicals originating from the tubes or the filtration materials themselves which caused activities like those of our extracts.

Standard procedure

A standard procedure for analysis of a sample and development of the purification process can be outlined as follows:

- Samples are bioassayed for Fungiflex 1 and Fungiflex 2 to verify their activity.
- Samples are fractionated either by reverse phase HPLC, silica gel chromatography or Thin Layer Chromatography (TLC).
- All fractions collected by chromatography are bioassayed to identify fractions in which activity is localised.
- Active fractions are then fractionated again by HPLC and/or TLC.

- Secondary fractions are bioassayed again and the process repeated until the compounds of interest are purified.

Outline of routine extraction and purification of Fungiflex growth hormones

Step 1
Water extraction of caps or stipes
↓
Methanol extraction (extract contains about 60 compounds)
↓
Reversed phase chromatography with a Partisphere SAX HPLC C 18 strong anion exchange column (about 30 compounds detectable at 254 nm)

Water: methanol (solvent gradient from 100% water to 100% methanol)
↓
Fraction 1 (3 min) = substances with growth inhibition and stimulation activity (hydrophilic group)
Fraction 22+ (over 20 min) = substances with growth stimulation activity (this is the hydrophobic group)

Step 2
C 18 Fraction 1
↓
Amino column chromatography (about 30 compounds)
80% Methanol: 20%water isocratic solvent system
↓
Peak 1 (3 min) = peak detectable at 254 nm, not containing bioactive substances
Peak 2 (4 min) = hexose positive peak detectable by colorimetric assay, containing bioactive substances

Step 3
C 18 Fraction 1
↓
Amino column chromatography (about 8 compounds)
Acetonitrile: water (65%: 35% to 60%: 40%) solvent gradient
↓
Peak 2 (4 min) = D-glucose
Peak 3 (6 min) = up to 3 substances with growth stimulation activity

The experimental development of these procedures and all other methods and materials are recorded ***in detail*** in **Appendix 2** and **Appendix 3** at the end of this document.

3. Key aspects of Fungiflex research

Let's start with an overall summary of the key aspects of this research. Crude extracts from *Coprinopsis cinerea* stipes and caps were found to have growth factor-like activities in standardised vertical stipe bioassays. Importantly, only the gentlest extraction technique (infusion in distilled water) was used, thus avoiding release of the cytosolic components which may have degraded growth factor activity and/or grossly contaminated the extract with other irrelevant components.

The activities observed, one with 'contractile properties' named **Fungiflex 1**, and the other with 'extension-stimulating properties' called **Fungiflex 2**, were attributed to two different substances, which were eventually found to be physically and chemically separable and therefore distinct, though possibly related compounds.

Microscopic analysis of their effects on vertical stipes, like those used in the standard bioassays, revealed that the substances penetrated tissue laterally very quickly since hyphal shape changes were apparent after only a 10-minute exposure. Yet, their activities were separated significantly in time, with the stipe-bending effects of Fungiflex 1 observed first, and being evident after 1 h, and the effects of Fungiflex 2 apparent some hours later.

Fungiflex 1 caused hyphae in the stipe to become flaccid and produce a large amount of extracellular material, which might be an indication of a role in permeabilising the plasma membrane. However, this morphology would also be consistent with signs of cell stress; application of Fungiflex 1 in a highly-concentrated form probably caused many hyphae to collapse and eject their cytoplasmic contents. This was sometimes an inevitable pitfall of working with impure extracts in which the actual amount of applied growth factor was unknown.

Fungiflex 2 activity resulted in enhanced extension and not expansion of hyphae. Confirmation of the roles of Fungiflex 1 and 2 as growth factors responsible for hyphal inhibition and enhanced hyphal extension, respectively, requires further microscopic investigation of longitudinal stipe sections.

The specificity of the growth factor-like responses was confirmed when the two components responsible, Fungiflex 1 and Fungiflex 2, were separated by Thin Layer Chromatography (TLC) and their separate activities and molecular weights as well as spectral (IR) properties confirmed.

Similarity of the bending activities of some inorganic acids, bases and salts, as well as some amino acids to that of Fungiflex 1 was a coincidental outcome of the high concentrations that had to be applied causing an osmotic shock or a pH imbalance, rather than specific inhibition of hyphal elongation. Enhanced growth was not observed when stipes were treated with low concentrations of these inorganic compounds and amino acids. No bending activity was observed in stipes treated with various concentrations of mono- and disaccharides either.

Whereas many compounds elicited contraction, albeit at elevated concentration, significantly fewer exhibited Fungiflex 2-type activity: among those tested, just indole-3-acetic acid (IAA), ammonium formate and possibly cAMP.

Fungiflex 2 was determined to be different from these compounds by virtue of its greater molecular weight and its UV/visual and IR spectral patterns. However, the fact that these compounds, as well as the nicotinamide and/or ADP-ribose degradation products of NADase digestion of β-NAD, exhibited similar activity to Fungiflex 2 suggested that they may well all share chemical/functional groups which might have been at least partially responsible for the manner in which these substances activated enhanced extension; for example, ammonium formate and Fungiflex 2 shared carboxylic acid moieties.

Fungiflex activity was developmentally-regulated. The fact that Fungiflex 1 was found in extracts of all the developmental stages of *Coprinopsis cinerea* examined suggests that it was a constituent of multihyphal *C. cinerea* structures. Its production was possibly correlated with multihyphal development, although vegetative, dikaryotic mycelia were not analysed. Its activity was similar to the primordial cap inhibitor(s) extracted from *Coprinus congregatus* (= *Coprinellus congregatus*) (Robert & Bret, 1987; Robert, 1990), shown to be involved during the photoinhibition phases of mushroom fruit body development.

4. Fungal growth factors

The absence of Fungiflex 2 from undifferentiated primordia (in contrast to Fungiflex 1) and presence at all later stages of development suggested that its production was correlated with multihyphal differentiation. Again, there were similarities to the 'stimulator' described by Robert (1990) which caused stipe elongation and fruiting induction in *Coprinellus congregatus*. That inhibitory and stimulatory substances produced in coprinoid fungi are active at different stages of mushroom development suggests that there is also a temporal aspect to their regulation.

Unfortunately, the *C. congregatus* compounds were never purified or identified so their similarities, in structure and activity, with Fungiflexes 1 and 2 remain unknown.

The fruiting induction substance (FIS) extracted by Rusmin & Leonard (1978) from different developmental stages of *Agaricus bisporus* fruit bodies showed no temporal differences in activities; that is, all stages produced equivalent fruiting inducing activity. Technically, these two cases are not totally comparable since the first pertains to factors which enhance or inhibit stipe extension while the second deals with fruiting induction (so there is a danger we might be comparing apples with oranges).

Production of the Fungiflexes was not gravity-induced and therefore not specific to the gravitropic response since both compounds were extracted from vertical as well as horizontal stipes and their production was not meiosis-dependent, as gravicompetence seemed to be.

This does not preclude their possible roles in regulating the differential growth characteristic of gravitropic bending but suggests a general role in coordinating growth. It may be that the activity of the gravity-regulated growth factor receptors, if they exist, were only manifested after meiosis, the event which triggered gravicompetence and after which functional receptors would have become essential for establishing the vertical orientation of differentiated primordia or stipes.

The Fungiflexes were also different in terms of their activities in other mushroom species. Whereas Fungiflex 1 was non-species specific, its activity being observed in all the mushroom species examined, Fungiflex 2 activity was specific to *C. cinerea*, although no closely related species were tested.

This suggests that Fungiflex 1 might be a universal mushroom growth factor, like auxins and other growth hormones described in plants (Salisbury & Ross, 1985), although its activity in a much wider spectrum of mushroom species remains to be confirmed.

The universality of Fungiflex 1 activity might extend to filamentous hyphae, although this also needs to be confirmed with much purer Fungiflex 1 extracts than the ones used in the mycelial assays.

Crude stipe extracts (rich in Fungiflex 1 activity but poor in Fungiflex 2 activity) had different activities in taxonomically distinct species; specifically, extracts were *inhibitory* to other basidiomycete mycelia but *stimulatory* to ascomycete mycelia, which also exhibited a changed morphology, possibly indicative of a hormone/morphogen role for Fungiflex 1 in these fungi.

The inhibitory/stimulatory activities could not be assigned conclusively to either Fungiflex 1 or 2 and so further investigation would be needed to establish whether basidiomycete hyphae were *specifically* Fungiflex 1-sensitive and ascomycete hyphae Fungiflex 2-sensitive or whether the Fungiflexes had different activities in different species. These results had implications for the development of these fungal inhibitors/stimulators for products with agricultural, pharmaceutical and other applications.

Considering the fragmentary evidence for the presence of fungal growth factors (for review see Novak Frazer, 1996) and the divergent species used to prepare extracts, it was surprising that the Fungiflexes shared similarities with the various mushroom extracts described previously, including those extracts which were analysed for their ability to elicit fruit body induction/formation.

The Fungiflexes and extracts from *Agaricus bisporus*, *Coprinus macrorhizus*, *Hypholoma fasciculare*, *Armillaria matsutake* (Hagimoto & Konishi, 1960; Konishi, 1967; Urayama, 1969), *Lentinula edodes*, *Flammulina velutipes*, and *Pleurotus ostreatus* (Urayama, 1969; Gruen, 1982) were all found to be less than 12,000 MW, heat stable, acid/base stable and mostly soluble in polar solvents including water.

These substances also *all enhanced* stipe elongation with the notable exceptions of Fungiflex 1 and the substances extracted from *Coprinus congregatus* primordia (Robert & Bret, 1987; Robert, 1990), which inhibited stipe elongation. Although these growth-controlling substances are probably not identical, they possess remarkably similar characteristics and may be similar types of compounds.

They might comprise a family of hormones/growth factors with slightly different chemical properties in each species, but with enough similarities to be cross-reactive, as Fungiflex 1 activity seems to suggest and as in the case of the *Agaricus bisporus* extracts, which induced fruiting in *Schizophyllum commune* (Rusmin & Leonard, 1978).

This is not unlike the situation in plants where 'auxin' is actually a family of related compounds based on indole-3-acetic acid (Salisbury & Ross, 1985) and is active on a very wide variety of plants.

The similarity of the Fungiflexes to other fungal growth factors may again suggest a general role in controlling extension of the fruit body stipe. During rapid elongation, which occurs soon after meiosis in *Coprinopsis cinerea* (Hammad *et al.*, 1993a), the growth of the expanding cap and elongating stipe must be coordinated. The expansion

of the whole fruit body must also proceed accurately in a vertical direction so that the gill surface is properly oriented when sporulation occurs a few hours later.

The Fungiflex factors may be responsible for continually correcting the direction of growth of the mushroom as well as coordinating the expansion of the cap and elongation of the stipe during the latter stages of fruit body development.

Gravitropism as a case study for putative Fungiflex activity

Although the involvement of Fungiflexes in bringing about the differential growth characteristic of gravitropic bending has not been proved, the way these putative growth factors affected differential extension in vertical stipes paralleled events which normally occurred during the gravitropism of horizontal stipes.

Gravitropism consists of the upward bending of a horizontal stipe until the stipe apex reaches a minimum angle of about 35° (to the horizontal), which acts as a trigger for the activation of the compensation mechanism (Kher *et al.*, 1992; Moore *et al.*, 1994a; Moore *et al.*, 1996), initiated to dampen bending so that the stipes return to the vertical without overshooting.

The initial upward bending response in a horizontal stipe, is due to the selective inhibition of extension of hyphae in the upper flank of the bending stipe (Greening, 1995; Greening & Moore, 1996), and may be regulated by the activity of Fungiflex 1, which caused 'bending by contraction' in vertical stipes. Meanwhile, the bend compensation mechanism, because of resumption of growth on the upper side, may be regulated by the activity of Fungiflex 2, which caused enhanced hyphal extension in the bioassays with vertical stipes.

Whether the Fungiflexes functioned in this manner to bring about gravitropic bending requires further experimentation with purified compounds. Clearly, the fact that different regions of the same gravitropically responding stipe extend differently implies not only that the relative position of hyphae in the stipe is recognised but that there must be a mechanism by which differential growth is coordinated.

Models incorporating the information from studies of gravitropism and the observed activities of the Fungiflexes may explain how these growth control substances might function. But before we speculate about this mechanism we should define what we know about the chemistry of the Fungiflexes.

5. Chemistry of the Fungiflexes

We must confess here at the outset that our research was prematurely terminated by events outside our control. So, although we were tantalisingly close to our goals, we did not achieve purification to homogeneity, nor complete the chemical characterisation. Nevertheless, we did identify a string of facts about the active agents in our bioassays that narrow the chemical focus onto a small family of molecules which are candidates to be Fungiflex 1 and Fungiflex 2.

The function of the Fungiflexes is to co-ordinate and orchestrate the behaviour of multihyphal systems. Our bioassay identified two separate activities with contrasting behaviours and different timings:

- Fungiflex 1 activity was demonstrated when mushroom stipes bent towards the side on which a drop of the extract was applied within 1 h of its application;
- the stipe grew back to the vertical in about 6 to 9 h, but then started bending away from the point of application of the drop. By 18 h from application of the drop, the stipe had bent to an angle of 90° from the vertical away from the site of application. This was Fungiflex 2 activity.

These two activities are determined by two different chemicals, sufficiently different in their basic chemistries to be separated quite readily.

Both Fungiflex 1 and Fungiflex 2 were stable to boiling for 10 min. Both were small molecules of low molecular mass, certainly less than 12000 MW.

Both Fungiflex 1 and Fungiflex 2 were soluble in water and methyl alcohol but virtually insoluble in ethyl alcohol, acetone, ethyl acetate and less polar organic solvents.

- Fungiflex 1 was preferentially solubilised in methyl alcohol and was also more negatively charged than Fungiflex 2 (as determined with the positively charged resin diethylaminoethyl cellulose (DEAE) in ion-exchange column chromatography), suggesting that Fungiflex 1 is more polar than Fungiflex 2.
- The possibility exists that Fungiflex 1 may be derived from Fungiflex 2 (*perhaps* as a breakdown product or as the oxidised form of Fungiflex 2).

Biological activity of both Fungiflex 1 and Fungiflex 2 was unaffected by digestion with alpha-amylase or a variety of proteinases. These observations suggest, respectively, that neither substance is likely to depend for activity on 1,4-glycosidic bonds, or peptide bonds.

Similarly, activities of both Fungiflexes survived treatment with tyrosinase + catecholase (= neither substance is a polyphenol or contains tyrosine), and digestion with nuclease or RNase (= neither substance is likely to be a single stranded nucleoside/nucleotide sequence).

Both Fungiflex 1 and Fungiflex 2 bioassay activities were stable to basic conditions, but Fungiflex 2 was labile in acidic conditions, suggesting that it may contain a carboxylic or activated amine group (a feature also suggested by Fourier transform infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR) data). Both were also

stable to treatment with sodium dodecyl sulfate (cold or boiled for 5 minutes) suggesting that their activities do not depend on disulfide bonding.

Fungiflex 1 activity was stable to reducing conditions (treatment with 5 mM dithiothreitol (DTT)), but Fungiflex 2 was unstable in these conditions although it regained activity when the DTT solution was boiled for 5 min and then cooled prior to bioassay.

These results with DTT indicate, again, that Fungiflex 1 and 2 are not proteins or peptides but that Fungiflex 2 may have required an oxidative environment for activity. Extensive spectral analyses, particularly FTIR spectroscopy and NMR spectroscopy, have given useful results.

- There is evidence for hydrogen-bonded hydroxyl groups (-OH), amide (-CONH), and possibly amine ($-NH_{1-3}$) functionalities in the Fungiflexes.
- There are at least 4 different types of carbonyl (C=O) groups in Fungiflex, possibly including a carboxylic acid, a cyclic lactam (5-membered ring including N), a secondary amide or a salicylate.
- The presence of individual amino acids is also possible although not as part of a peptide chain.
- There are many hydroxyl groups and acidic protons suggesting the presence of sugar rings.
- There is no evidence for the presence of aromatic rings but there is evidence of the presence of double or triple bonds since the compound fluoresces at 415 nm when exposed to 260 nm UV.

Elemental microanalysis of crude Fungiflex suggested it contains phosphorus; there was spectral evidence for the presence of diphosphate groups. An interesting point is that P=O groups are hygroscopic and Fungiflex was also a very hygroscopic compound.

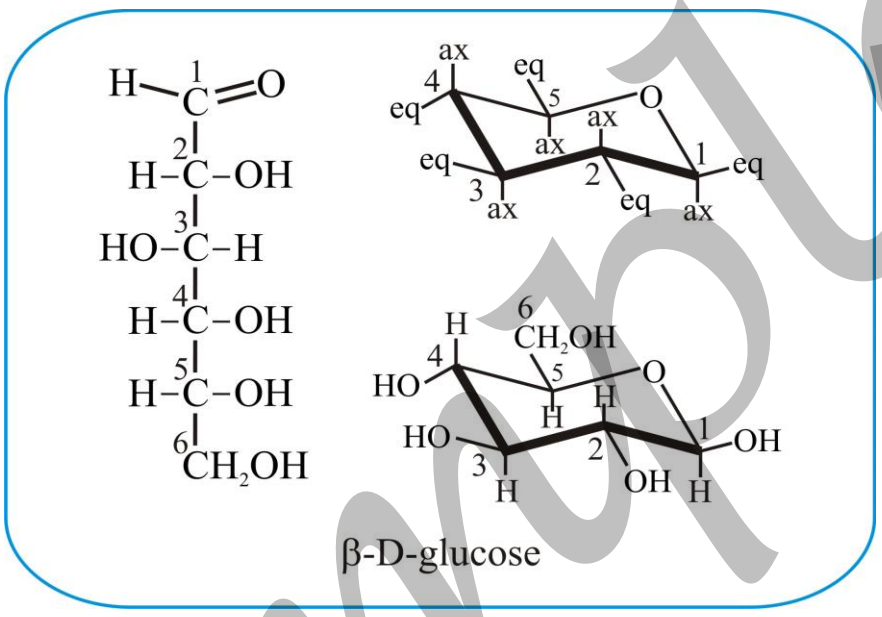
^{1}H-NMR confirmed the presence of an amide and sugar groups, with signals characteristic of sugar skeleton protons, including both α-anomeric and β-anomeric protons, so the preparations could have contained both isomeric forms (α and β) of one sugar molecule.

In particular, signals in the ^{1}H-NMR spectra were very characteristic of the C5 and C6 protons in the methyl pentoses, fucose (6-deoxy-L-galactose [https://en.wikipedia.org/wiki/Fucose]) and rhamnose (6-deoxy-L-mannose [https://en.wikipedia.org/wiki/Rhamnose]).

Signals in the ^{13}C-NMR spectra were also typical for sugar skeleton carbon atoms, with peaks typical of the carbon of a deoxysugar, and possibly peaks characteristic of N-acetate groups.

6. Some reminders about the structures of sugars

Without giving too much of the plot away, it's clear that sugar molecules are critically important aspects to our story. Some background information in this section will give you an in-depth understanding of the terminology used in sugar chemistry, and place our interpretations in context. However, if you want to skip this section (and maybe return to it later if you need), you could 'cut to the chase' without further ado, and look at section 8, entitled 'Meet the Fungiflexes' on page 20.



The images above depict the **glucose molecule**. On the left is the linear structure, or **Fischer projection**, of D-glucose, while on the right is the C1-chair form of the same molecule, demonstrating the five-carbon pyranose ring. The five carbon atoms of the pyranose sugar (as indicated by the numerals) are connected into a six-atom ***heterocyclic*** ring through formation of a stable hemiacetal or hemiketal (called a lactol).

This occurs when a spontaneous intramolecular reaction takes place between the OH group of carbon-5 (C5) and the oxygen of the carbonyl group (>C=O) of carbon-1 (C1) in this example. The ring is planar (that is, it's fairly flat) and the figure above tries to depict this in three-dimensions by showing the bonds closest to you, the viewer, emphasised with thickened lines. In an aqueous solution of glucose about 97% of the glucose molecules are in this ring form.

A few important structural features of sugar molecules need to be defined. **Chiral molecules** are those whose mirror images cannot be superimposed. They contain at least one carbon atom with four non-identical substituents. In the Fischer projection above, there are four (4) chiral carbons (C2-C5) and one non-chiral carbon (C6). C1, the carbonyl carbon, is called the anomeric centre as it reacts with C5 to form the cyclic form of glucose.

In the cyclic glucopyranose molecule depicted above, C1, which is now a chiral centre, has four different substituents in this form: the adjacent carbon 2 (C2), the oxygen that's part of the ring, a proton [H] and the hydroxyl [OH]). More importantly, it is also an **anomeric centre** because in this cyclised form, the molecule can take up two different geometries or **anomers**,

named the alpha and beta forms. Alpha and beta glucopyranose molecules differ only in the direction that -H and -OH groups point on the anomeric carbon (or C1) when in the ring or cyclic form. Alpha (**α**) glucopyranose has its C1-OH (hydroxyl) group on the **axial bond** (i.e. pointing downwards, away from the plane of the ring), whereas the C1-OH of beta (**β**) glucopyranose is attached through the **equatorial bond** and is held slightly above the ring. The axial (labelled **ax**) and equatorial (**eq**) bonds are shown in the skeletal structure at top right of the panel on page 12.

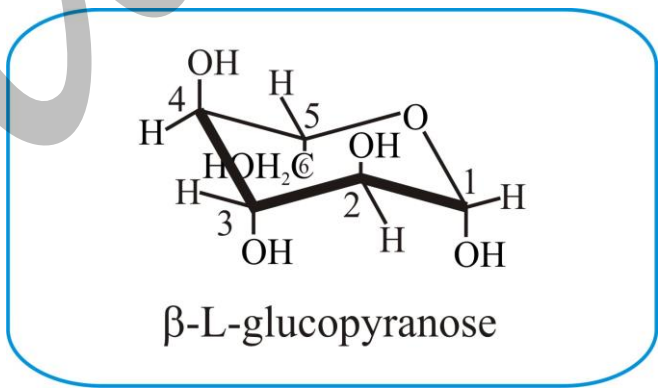
The anomeric carbon is particularly important as it is the carbon involved in linking sugar monomers together to form **polysaccharides**. Considering the Fischer projection of glucose shown above, a **D or L designation** is denoted by the configuration at the chiral centre furthest from the anomeric centre.

In glucose, the aldehyde group is C1, also the anomeric centre, and the most distant chiral carbon is C5. The rule is 'if the chiral centre furthest from the anomeric centre has the hydroxyl group on the right, it is a **D-sugar**; if on the left, it is an **L-sugar**'. The open chain molecular representations of D & L sugars are mirror images of one another.

- The sources of the D- and L- labels were the Latin words **dexter** (on the right) and **laevus** (on the left) as when this scheme was devised (in the early years of the twentieth century by the German organic chemist Emil Fischer) the form of the molecule (glyceraldehyde being the object of study) that rotated plain polarized light clockwise (also designated '+') was arbitrarily labelled 'D' (for **dextrorotatory**). The other form of the molecule was found to rotate polarized light anticlockwise (also designated '-') and was called 'L' for **laevorotatory**.

In the ring-form representation in the panel above (page 12), the D-sugar has carbon-6 (C6) directed above the ring, while the L-sugar has that carbon directed below the ring. Generally, the highest numbered chiral carbon (typically to the left of the oxygen in chair or Haworth projections) determines whether the structure has a D- or L-configuration.

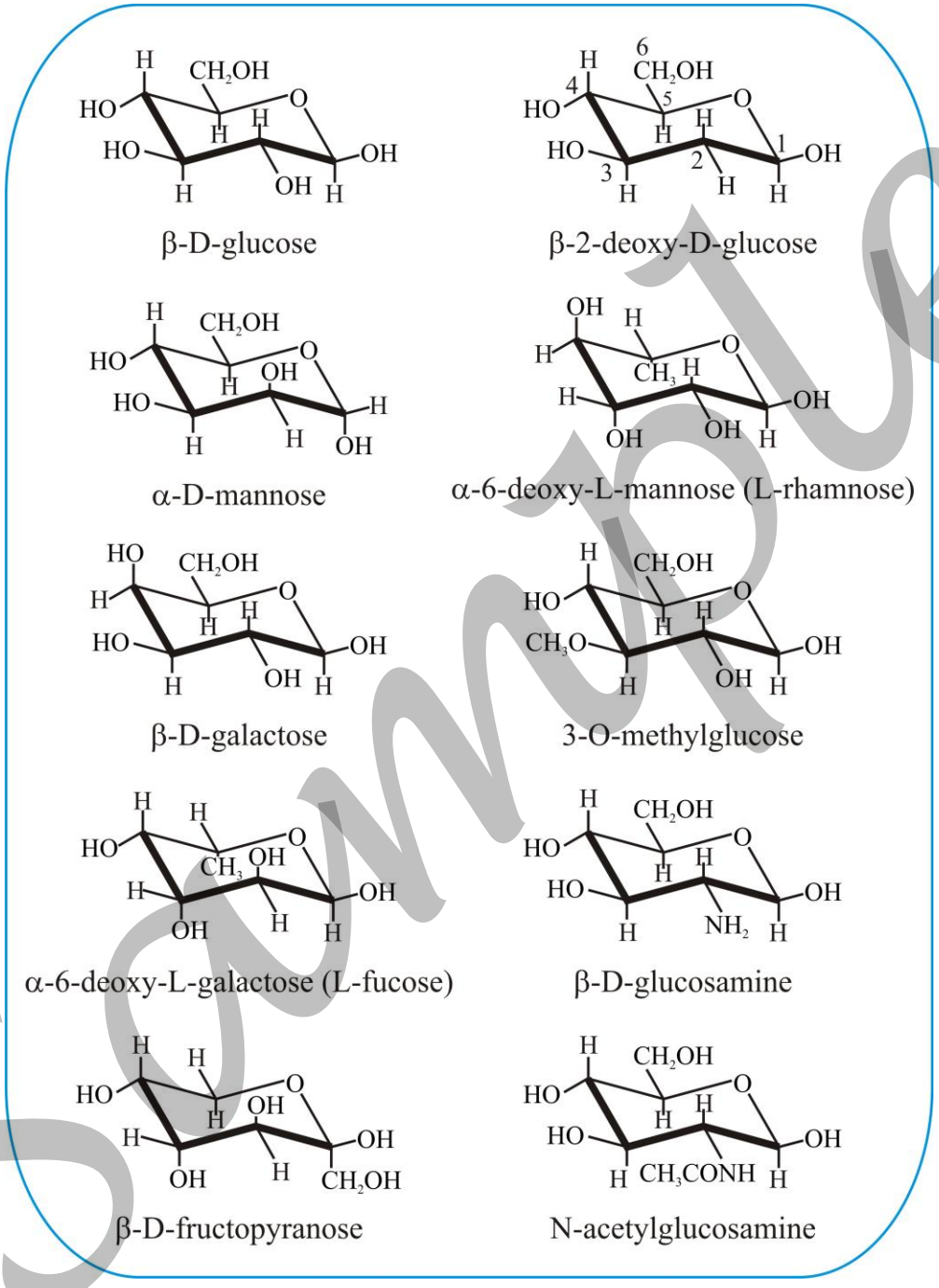
D-glucose is depicted in the illustration ***above*** (page 12), and is the most common form of the molecule in nature (and this is true for most other naturally occurring sugars); **L-glucose** is depicted immediately ***below***.



β-L-glucopyranose

In **L-glucose** the hydroxymethyl group (- OCH_3) and the proton (-H) are interchanged on C5. L-Glucose is rarely found in nature, but can be chemically-synthesized and its taste is indistinguishable from D-glucose. However, L-glucose cannot be metabolised by living

organisms as a source of energy because it cannot be phosphorylated by hexokinase, the first enzyme in glycolytic metabolism.



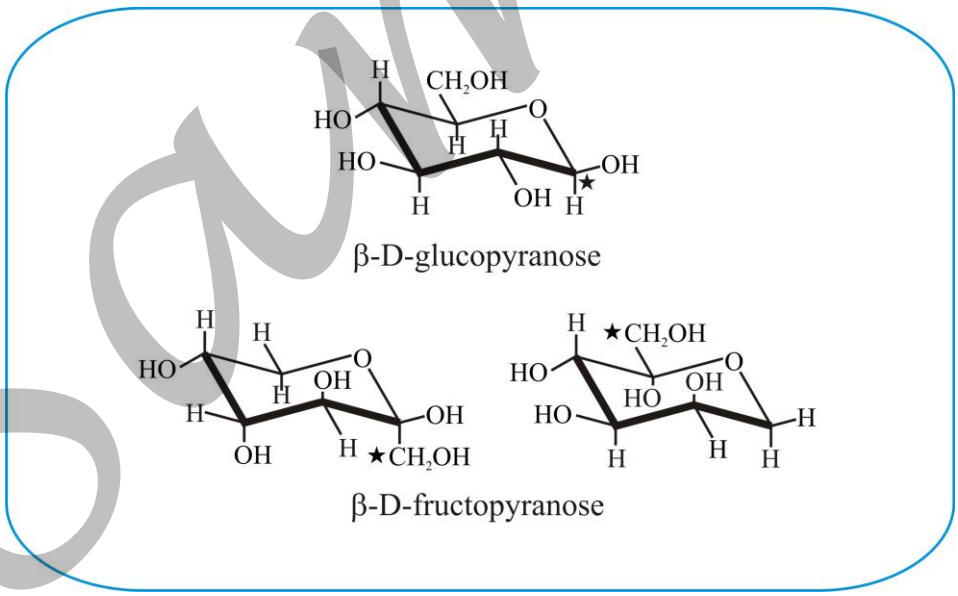
In the panel of sugar molecular structures immediately **above** we show the molecular structures of a selection of commonly-encountered sugars and some chemically-modified sugars (three deoxysugars, methylglucose, glucosamine and acetylglucosamine). Our motive here is to illustrate just a little of the enormous diversity represented among the hexose sugars. We ***also*** want to illustrate how tiny differences in the geometry of the molecules are

sufficient to distinguish between completely different sugars. Can you spot the difference(s) between glucose, mannose and galactose in the left-hand column of the illustration above?

And then there's **fructose** (also called beta-D-fructopyranose), shown at the bottom of the left-hand column in the panel on page 14; compare it with the glucose molecule at the top of that column. Doesn't look much like glucose, does it? And yet fructose is also called **fruit-sugar**. It's paired with glucose to make the common plant disaccharide **sucrose** (so every sucrose molecule you digest produces a molecule of fructose and glucose). Fructose is sweeter than either glucose or sucrose and ***high-fructose corn syrup*** (HFCS) industrially produced from sucrose is widely used in soft drinks and other 'sweet treats'. Its consumption has risen in parallel with the epidemic of obesity, which suggests a relation, and implicates dietary fructose as a potential risk factor for diabetes and cardiovascular disease.

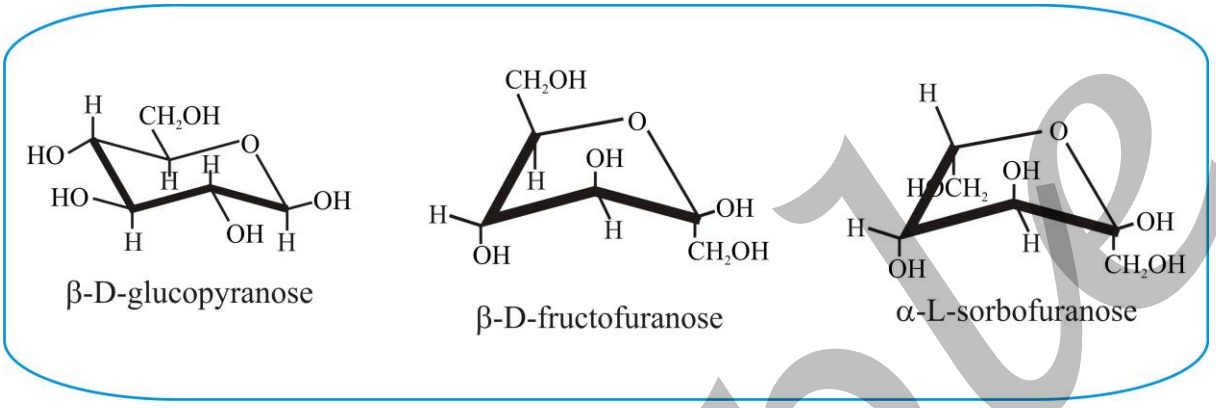
- If you want more information about the dangers of fructose in the human diet, you could start with the website of the Journal of the American Society for Clinical Nutrition (http://ajcn.nutrition.org/content/86/4/895.full.pdf+html) and the (free) PDF you can download from the site.

You might well wonder how a sugar structure that looks so different from that of glucose can be a dietary concern. The answer is that fructose is an essential component of the glycolytic metabolism of glucose. Fructose 6-phosphate is produced by isomerisation of glucose 6-phosphate, which is in turn further phosphorylated to fructose-1,6-bisphosphate. So, although there is no biological need for dietary fructose, it will be metabolised as a source of carbon and energy. Fructose is metabolized by phosphorylation on the 1-position and how this might be accomplished by normal glycolytic enzymes is indicated in the next panel.



This comparison of the ***pyranose ring*** forms of β-D-glucose and β-D-fructose demonstrates the important concept of the conformational flexibility of monomeric sugars. The fructopyranose ring is shown on the left in the conventional orientation and, in the right-hand diagram above, the molecular structure has been inverted and rotated to present a view which

provides a closer comparison with glucose. In each diagram in the panel above, the **C1-carbon atom** is indicated with a star (★). The structural similarity with glucose is evidently now sufficient for enzymes that normally add phosphate groups to carbon-6 of glucose to add phosphate groups to carbon-1 of fructose.



Another example of conformational flexibility is shown in the panel immediately ***above***, which compares the ***pyranose*** ring form of glucose with the ***furanose*** ring forms of fructose and sorbose. The furanose structure is a five-membered heterocyclic ring, consisting of four carbon atoms and one oxygen atom. The conventional diagram shows the anomeric carbon to the right of the oxygen.

The furanose sugar will be either an alpha (α) or beta (β) anomer, depending on which direction the anomeric hydroxyl group is pointing. In a **D-configuration** furanose, **α (alpha) configuration has the hydroxyl pointing down, and β (beta) has the hydroxyl pointing up**. It is **the opposite in an L-configuration furanose**. In aqueous solution, there will be an equilibrium mixture of α and β configurations due to **mutarotation** at the anomeric carbon.

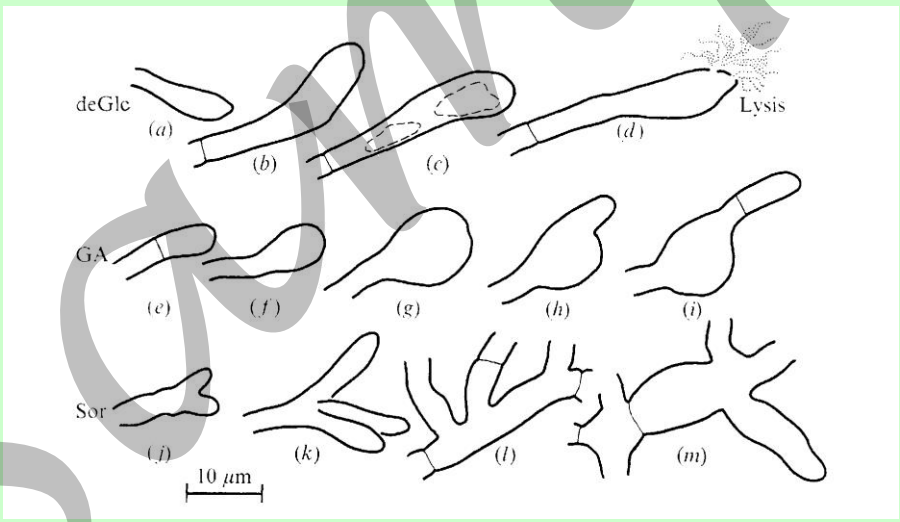
7. Sugars as regulatory molecules

The data in Section 5 (*Chemistry of the Fungiflexes*) demonstrate that these 'fungal growth co-ordinating factors' that we have called 'Fungiflexes' are probably specifically-modified sugars. That sugars should play multidisciplinary roles in fungal development and extracellular communication, as well as being metabolic intermediates is significant and may not be too surprising.

More surprising to the senior author (David Moore, that is, and in this context the word 'senior' should be taken to mean 'more ancient') is that the findings of this Fungiflex research, which was done close to the **end** of his career, echoed resoundingly research on carbohydrate metabolism in *Coprinopsis cinerea* that was done at the **start** of his research career some 30 years earlier. David Moore has reviewed his research career in the book '*Coprinopsis: an Autobiography*' (Moore, 2013b), but he has described his researches in these distant days for us and we present some extracts of this here.

In this section, we ***summarise*** David Moore's own account of his research on fungal carbohydrate metabolism in the 1960s and 1970s. If you want to read the complete original, it is shown as the first Appendix of this document.

David's first interest upon taking up his first job as Assistant Lecturer in Genetics at the University of Manchester was to initiate a study of sugar metabolism in *Coprinopsis cinerea* (named *Coprinus lagopus* at the time) using sugar analogues (Moore, 1969).



Shapes assumed by terminal and intercalary compartments of hyphae of *Coprinopsis cinerea* grown on media containing inhibitory concentrations of hexose analogues. Drawings are tracings from photographs of individual hyphal compartments observed with oil immersion microscope objectives. The figures are arranged in a likely developmental series but individual hyphae were not followed through the development of these aberrations. Note the vacuolation and eventual lysis of swollen terminal cells which occurred on deGlc; the extreme swelling of terminal compartment on GA; and the frequent branching and giant mycelial cells seen on Sor. The following media were used: (a), (b), (d), 5 mM Glucose + 20 mM-deGlc; (c), 5 mM-Acetate + 0.1 mM-deGlc; (e), 5 mM Glucose + 15 mM GA; (f), (h), 5 mM Fructose + 0.2 mM GA; (g), (i), 40 mM Glucose + 80 mM GA; (j), (m), 5 mM Acetate + 2 mM Sor: (k), (i), 5 mM Glucose + 75 mM Sor. Figure and legend from Moore & Stewart, 1972.

Sugar analogues were reported to inhibit fungal growth because they replaced D-glucose in metabolism. One such analogue was **2-deoxy-D-glucose** (deGlc), which when phosphorylated, inhibited the activity of glycolytic enzymes. This in turn caused downstream effects, eroding polysaccharide, and eventually, blocking cell wall synthesis. Another sugar analogue shown to affect polysaccharide synthesis was **L-sorbose**, and although its mechanism of action was unknown, growth with this sugar caused formation of an abnormally thick cell wall, with the mycelium exhibiting an abnormal growth form in which cells are much shorter and branching frequency is increased. Because these sugar analogues were shown to inhibit growth of *Coprinopsis cinerea* and caused concurrent morphological changes (Moore & Stewart, 1971a), they were called **paramorphogens**.

The morphologies of *Coprinopsis cinerea* hyphae grown on media containing inhibitory concentrations of the hexose analogues **2-deoxy-D-glucose** (deGlc), **D-glucosamine** (GA), and **L-sorbose** (Sor) were illustrated by Moore & Stewart (1972) and are shown in the figure on page 17. In summary: the drastic effects of deGlc, GA and Sor illustrate clearly the morphogenetic changes in hyphal growth, and led the authors to conclude that ***'processes which control the shape of the [hyphal] compartment must be very closely related to the processes which determine the chemical composition of the hyphal wall.***'

Having shown that sugar analogues inhibited *Coprinopsis cinerea* hyphal growth, 388 ***allelic*** mutants were generated. All of these showed cross-resistance among deGlc, GA and L-sorbose (Moore & Stewart, 1971b; Moore, 1973). More importantly this research led to the discovery of **two transport systems for hexose sugars:** an allosteric ATP-binding cassette hexose transporter, which:

- couples hydrolysis of adenosine triphosphate (ATP) to the translocation of hexose across the hyphal membrane in the high-affinity configuration, and
- enables hexose transport by facilitated diffusion over a proton gradient in its low affinity configuration.

None of the hundreds of mutants obtained in this study could utilise fructose as a sole source of carbon due to a defect in sugar transport, though all had approximately normal levels of activity of enzymes involved in intracellular sugar metabolism. The gene symbol ***ftr***, signifying fructose transport, was assigned to the gene because the loss of fructose transport across the membrane, from the extracellular environment to the cytoplasm, was the most easily demonstrated phenotype. Subsequent detailed kinetic analysis of sugar transport in the wild type showed that the **ftr gene-product** is a complex allosteric transport protein which is alone responsible for the two hexose transport systems referred to above.

Using current terminology, the **ftr gene product** is predicted by the kinetic data obtained to be an ***allosteric ATP-binding cassette hexose transporter***, which (a) couples hydrolysis of adenosine triphosphate (ATP) to the translocation of hexose across the hyphal membrane in the *high-affinity* configuration, which it assumes when sugar is in low supply; and (b) facilitates hexose transport as a *glucose-proton symport* by *facilitated diffusion* over a proton gradient in its low affinity configuration, which it assumes when extracellular glucose is abundant.

More detail about the mechanisms of sugar transport in *Coprinopsis cinerea* can be found in this publication: Moore & Devadatham, 1979.

Such **carrier proteins** are generally integral membrane proteins; meaning that they exist within, and span, the membrane across which they transport their substrates.

All these transporters have α-(alpha) helical structures in their membrane-spanning domains that contribute to substrate translocation across the membrane and it is tempting to suggest that the mutant clusters observed in the **ftr** gene map correspond to the membrane-spanning domains of the **ftr** gene product, as regions of these would be responsible for, or at least take part in, the molecule-specific substrate binding. Such an interpretation would explain why:

- the kinetic characters of *ftr* mutants show they were defective in V_{max} rather than K_m for sugar uptake (that is, in rate of translocation rather than affinity);
- ***ftr*** mutants selected for resistance to different inhibitory sugar analogues mapped consistently to different positions within the gene.

8. Meet the Fungiflexes

The indications are, therefore that these '*fungal growth co-ordinating factors*' are specifically-modified intermediary metabolites, and in particular, modified sugar molecules.

That they are no more exotic than this would fit in well with the claim that septum formation in the most primitive fungal hypha was the process that 'invented' eukaryotic multicellularity (Moore, 2013a).

Moore (2013a) argued that multicellular fungal hyphae were the first successful multicellular eukaryotic tissue structures, appearing about 1.5 billion years ago. The development of the septum across the filament has the immediate selective advantage of protecting the filament against the disaster of wall damage opening the cell to the environment.

But it also simultaneously invents compartment ('cell') differentiation because the filament extends by growth at its apex. So when a septum is formed, the 'front' compartment is the growing apex and the sub-terminal compartment is no longer part of the growing apex. It is, specifically, sub-terminal; and may be capable of intercalary wall growth, but is not engaged in apical growth.

Moore claims that it was the fungal life style that began the ancestral eukaryote's tendency towards multicellularity, possibly at a time even before kingdom Fungi was fully delineated. Those early eukaryotes first worked out how to make and use multicellular filaments (now called hyphae), then evolved the mechanisms to control those key features of multicellularity (now known as developmental biology or morphogenesis). The key features being differentiation of the compartments ('cell differentiation'), patterns of distribution in space and time, and establishment of 'body plans' that feature in the development of multicellular structures like fungal fruit bodies; and which will feature later in geological time in plants and, eventually, animals too (Moore, 2013a).

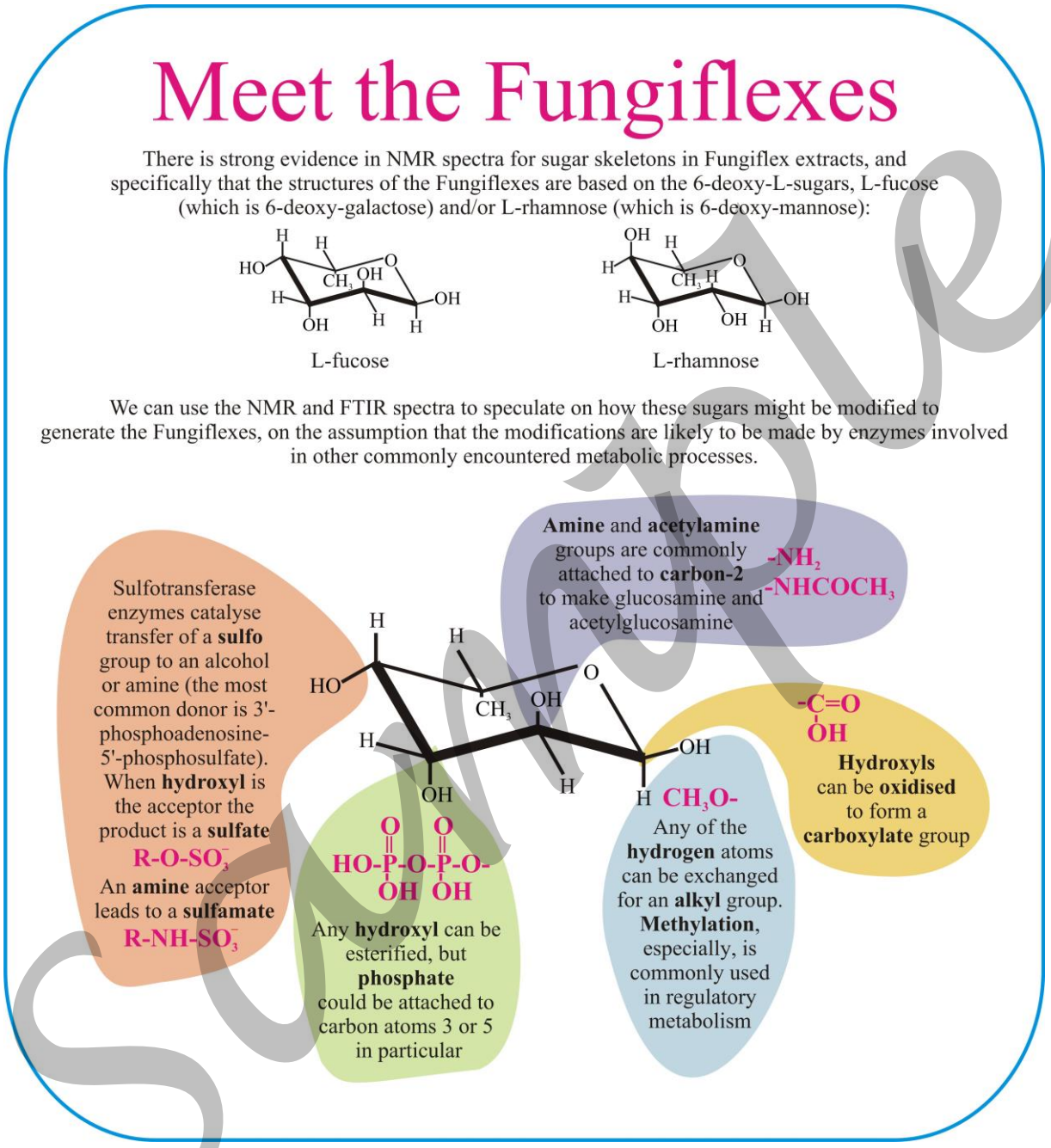
If the simplicity of form and function suggested by distant evolutionary events is even partially accurate, then the chemicals most readily available for recruitment as growth control agents in the primitive metabolism of the time were probably the intermediary metabolites themselves. Metabolism was already focussed on hexose sugars and featured sugar phosphates, amino sugars, sugar alcohols and sugar acids, and, of course, synthesis of chitin for the fungal walls made use of repeating units of N-acetylglucosamine. Before the need for signalling molecules arose, the enzymes and pathways existed to form sugar derivatives that could eventually serve as specific regulators of the enzyme systems involved in wall structure and/or architecture to control the shape and form of hyphae and the morphogenesis of fungal tissues.

Distilling all these hints and suggestions brings us to the conclusion that the **Fungiflex** activities we extract so readily by ***infusing*** intact tissues in water at room temperature, and detect so readily in our 'stipe bending' bioassay exhibit the following characteristics:

- a family of molecules, certainly two, possibly more;
- most probably 6-deoxy hexoses (and therefore also interpretable as methyl-pentoses);
- probably substituted with amino and/or amido groups;
- probably N-acetylated;
- possibly phosphorylated, and maybe sulfated.

The NMR spectra of Fungiflex extracts are strongly characteristic of a laevorotatory 6-deoxy hexose sugar, probably **L-fucose**, possibly **L-rhamnose**. Using the evidence for substituents

of the molecules shown earlier (see sections 5 and 6, above), we can illustrate our speculations about likely Fungiflex molecular structures in the following panel.



The sugar's anomeric signals are weak, but the FTIR spectrum of Fungiflex shows evidence for a **carboxylate ion**. Monosaccharides with a carboxyl group are known as sugar acids, and are the result of oxidation of one or more hydroxyl groups. An interesting example, and perhaps relevant considering the weak anomeric signals detected, is ***galactonic acid*** in which the carboxylate group is at C1 of galactose. Other aspects of the FTIR spectra could provide evidence for the presence of a **diphosphate group** and the elemental analyses are not inconsistent with the presence of **sulfur**.

9. And now some speculations

So where does all this lead us? In the 1990s we showed that what we now believe to be **modified sugars** could be extracted by the simplest procedure of water-infusion of fruit body stipes or caps. When applied asymmetrically to isolated stipes, hyphal cells in the immediate region of the application changed their growth pattern in response to at least two components, one promoting immediate hyphal lengthwise contraction (**Fungiflex 1**) and the second (**Fungiflex 2**), after some hours promoting hyphal lengthwise extension.

One obvious interpretation is that the Fungiflex molecules are produced in the vicinity of the stipe apex, perhaps in what constitutes cap tissue near or at the junction of stipe and cap. They are then released into the extracellular matrix of the stipe to diffuse downwards to regulate hyphal extension progressively. The expectation is that Fungiflex 1 inhibits stipe extension while the cap forms and, following-on several hours later, Fungiflex 2 enhances the extension of stipe hyphae to raise the cap above surrounding vegetation to facilitate the eventual spore release.

In the 1970s we demonstrated that modified sugars caused grossly abnormal construction of the hyphal wall; the exact nature of the effect was dependent on the molecular nature of the modified sugar. The wall abnormalities themselves caused mycelial growth inhibitions and all mutants selected for resistance to those growth inhibitions were found to have mutations in the organism's one and only hexose transporter.

The clear-cut interpretation of these facts is that normal wall construction is controlled by endogenously produced sterically or chemically modified sugars. When such sugars are applied externally experimentally (under which circumstances, location and concentration are undoubtedly abnormal), they interfere with wall growth, resulting in an altered, and also abnormal, hyphal growth response.

Arguably, the modified sugars must be transported into the ***intracellular*** environment ***to be effective***; in normal circumstances, it is the single hexose transporter that translocates normal and sterically or chemically-modified hexose sugars.

Which all leads to the overall conclusion: the ***Fungiflexes are examples of the endogenously-produced modified sugars*** that function as control molecules for normal hyphal wall construction.

We think Fungiflexes are specifically-modified sugar molecules that are externalised at source (presumed to be the apex of the stipe) and diffuse through the extracellular matrix surrounding the hyphae. They are effective in modifying the activity of the hyphal wall construction apparatus after being translocated to the intracellular environment. We showed above that Fungiflex 1 was present at all stages of mushroom

development and the appearance of Fungiflex 2 activity was correlated with differentiation of the cap region in primordia (preceding meiosis).

We interpret the normal function of Fungiflex 1 as an *immediate* down-regulator of the extension growth of stipe hyphae to allow time for the cap to differentiate.

Fungiflex 2 activity **promotes hyphal extension growth** and appears some hours after Fungiflex 1; remember, though, this late Fungiflex 2 activity is from the ***same extract application*** in which the more immediate Fungiflex 1 activity appears. There are a number of plausible implications of this:

- Fungiflex 2 (the 'up-regulator') could be a derivative, produced spontaneously or enzymically, of Fungiflex 1 (the 'down-regulator');
- Fungiflex 2 could be a separate molecule that is transported with such a low V_{max} for translocation across the membrane that it takes several hours for the intracellular concentration to reach activation levels;
- Plausibly, both Fungiflex 1 and Fungiflex 2 may be transported equally into the intracellular compartment but their targets for regulation are separated in time. That is, the target of Fungiflex 1 is immediately accessible to down-regulation, but the regulatory target of Fungiflex 2 may need to be freshly synthesised or enzymatically modified, either to overcome the down-regulation of Fungiflex 1 or to remove/replace permanently repressed Fungiflex 1 targets.

This interpretation would make ***Fungiflex 2*** (specifically) a candidate for ***the signal that coordinates hyphal cell inflation across the fruit body as a whole*** (Hammad, Ji, Watling & Moore, 1993a; Hammad, Watling & Moore, 1993b).

The stipe of *Coprinopsis cinerea* comprises two cell populations: large and small diameter hyphae. Cell inflation is accentuated in cells occupying a specific zone just beneath the 'epidermis' of the stipe; differential expansion of cells in this zone readily explains how the fruit body stipe changes from a solid cylinder to a hollow tube during its development.

Cell length does not increase during early stages (that is, between a 3 mm and an 8 mm tall fruit body; both of which are at pre-meiotic developmental stages). Presumably any stipe elongation occurring at these stages is due primarily to cell proliferation rather than cell elongation. In contrast, there is a large increase in cell length between stipes of an 8 mm pre-meiotic fruit body and that of a 25 mm fruit body undergoing meiosis. Initially the cells in the basal and middle regions of the stipe lengthen. Cells in the extreme basal and apical regions are always shorter than those in other regions of elongated stipes. For example, cells near the cap/stipe junction at the extreme apex of an 83 mm fruit body (fully elongated) have a typical length of 150 µm compared to an average of 313 µm for the whole of the apical section examined (about 10 mm long).

The most elongated cells are found in the upper mid-region of the stipe. Ratios of length to width are about 2 in pre-meiotic stipes (3 mm and 8 mm fruit bodies), but increase after meiosis, particularly in the upper middle regions, to 10, 20 and approximately 35 in 48 mm, 55 mm and 83 mm tall fruit bodies respectively. Overall, therefore, developmental stipe extension of *C. cinerea* involves increase in length ***and*** cross-sectional area of inflated hyphae and recruitment of narrow hyphae into the inflated population (Hammad, Ji, Watling & Moore, 1993a; Hammad, Watling & Moore, 1993b).

These two studies were the first (and so far, only) ***quantitative*** hyphal analyses in which computer-aided image analysis was used to measure and enumerate cell types at different stages of development in light microscope sections of fruit bodies of *Coprinopsis cinerea*. The quantitative analyses enabled a dynamic understanding of fruit body development in this organism. Essentially, early development involves cell proliferation and late development involves cell elongation and expansion (see the information box above for details).

Significant for the present discussion, ***expansion of all the different cell types*** in the fruit body cap as well as inflation of cells of the fruit body stipe ***coincides with completion of meiosis***.

Hammad *et al*. (1993a) suggested that ***coordination between cap and stipe*** could be achieved by some sort of signalling system that 'reports' the end of meiosis to spatially distant parts of the fruit body.

The extracellular matrix, which surrounds all the cells of the fruit body, could provide an aqueous continuum through which such a reporter molecule could diffuse and provide signalling over both short (less than μm-length) and long (more than mm-length) distances (Moore, 1993). ***We suggest that Fungiflex 2 may be, or may generate, that cell-expansion-coordinating reporter signal.***

10. Fungiflex in the context of fungal gravitropism

The emphasis of the interpretation so far is very much on ***uniform chemical signalling diffusing*** through the whole of the *Coprinopsis* fruit body, from a source near the cap-stipe junction. Yet our experiments that resulted in the discovery of the Fungiflexes were initiated after our earlier studies on gravitropism which suggested a **differential** distribution of signalling molecules might be the cause of gravitropic curvature of *Coprinopsis* stipes.

Application of Fungiflex extracts does indeed mimic gravitropically-induced stipe curvature, *but the distribution of the Fungiflex is entirely in the hands of the experimenter*. How do our interpretations of the normal biological activity of Fungiflex fit to the biology of a disoriented stipe responding to a changed gravity vector?

We know that gravitropic bending results ***solely*** from hyphae of the lower side of the stipe preferentially extending in length significantly more than hyphae of the upper side of the disoriented stipe (Table 1; Greening & Moore, 1996; Greening, Sánchez & Moore, 1997). None of the other parameters measured differed significantly between the upper and lower flanks of the bending stipe (Table 1).

Table 1. Cell morphometrics in sections of gravitropically-responding stipes of *Coprinopsis cinerea* at the point of maximum curvature

	Upper flank of bend	Lower flank of bend
Mean width of hyphae (μm)	19.9	20.9
% narrow hyphae	28.8-41.5	30.5-39.1
Packing density	0.47	0.44
Cell length (μm)	116	542

Data from Greening, Sánchez & Moore, 1997.

By attaching inert markers to the stipe, we found that the outer flank of the bend initially had a faster rate of extension, although the inner flank matched this growth rate later in the response. Thus, bending resulted from **differential *enhancement of growth rates*** rather than sustained differences.

In a horizontal stipe, all hyphae experience the same force of the gravitational field and so the problem of coordinating hyphal extension growth to generate a bend in the right direction becomes two-fold:

- what is the nature of the signal; and
- how is a **differential** growth impulse generated across the **diameter** of the **horizontal** stipe?

If we are content with the notion that the Fungiflex molecules provide the ultimate signal, we must understand how the activity of freely-diffusing molecules can be ***expressed asymmetrically across the diameter of the stipe***.

After a considerable amount of analysis of the gravitropic responses of both *Coprinopsis cinerea* and *Flammulina velutipes* it was concluded (Moore, Hock, Greening, Kern, Novak Frazer & Monzer, 1996) that gravity perception in agarics depended on the nuclei acting as statoliths and exerting tension on the actin filament system that surrounds them. See the following information box for more details.

Size determinations in *Coprinopsis* demonstrated that cells of the stipe increase in length, not diameter, to produce the growth differential. In *Flammulina* a unique population of highly electron-transparent microvacuoles changes in distribution, decreasing in upper cells and increasing in the lower cells in a horizontal fruit body within a few minutes of disorientation. These are thought to contribute to vacuolar expansion which accompanies/drives cell elongation.

Application of a variety of metabolic inhibitors indicates that the secondary messenger calcium is also involved in regulating the ***growth differentials*** of gravimorphogenesis, but that gravity ***perception is unaffected*** by inhibitors of calcium signalling.

In both *Flammulina* and *Coprinopsis*, gravity perception seems to be dependent on the actin cytoskeleton since cytochalasin treatment suppresses gravitropic curvature in *Flammulina* and, in *Coprinopsis*, significantly delays curvature without affecting stipe extension.

This, together with altered nuclear motility observed in living hyphae during reorientation, suggests that gravity perception involves **statoliths** (and the dikaryon nuclei, which are paired together in an actin cage are the best candidates for this function) **acting on the actin cytoskeleton** and triggering specific vesicle/microvacuole release from the endomembrane system (Monzer & Haindl, 1994; Monzer, Haindl, Kern & Dressel, 1994; Monzer, 1995; Novak Frazer & Moore, 1993, 1996).

Moore *et al*. (1996) suggested a plausible mechanism for ***gravity* perception *in agarics***:

> *'…nuclei act as statoliths, their displacement within the cytoskeleton surrounding them being communicated by some of those actin microfilaments to the endomembrane system, and maybe by similar means directly to the plasma membrane … An important point is that the initial event does not need to be a major displacement. The whole point of having a signal transduction chain is to provide for amplification of the primary input… in nature the system must be constantly monitoring orientation and correcting small disturbances to maintain vertical growth. In these circumstances statolith movement may be extremely restricted… We would envisage that statolith-activation of microfilament membrane connections could prompt export of a signalling molecule through the plasma membrane, and/or positional-dependent amplification of vesicle/microvacuole production by the endomembrane systems.'*

We suggest a ***two-part model for fungal gravitropism*** that accounts for all these details and includes the concept of Fungiflex.

Fungiflex can be the uniformly diffusible proto-effector for the gravitropic response if it is assumed that the microfilament cage connections apply stress asymmetrically to the transporter molecules in the cell membrane. This asymmetry is the result of the gravity sensor (believed to be the paired nuclei within their actin cage) reacting to the direction of the gravity vector and so changing the pattern of stresses exerted by the microfilaments connected to the plasma membrane.

The important point about the Fungiflexes is that they diffuse through the extracellular matrix surrounding the hyphae, but are effective in modifying the activity of the hyphal wall construction apparatus ***only after translocation into the intracellular environment***.

Control could be as mechanically direct as **tension in the microfilaments connected to the transporter's membrane-spanning domains**. Distortions to those domains could alter the substrate affinities of the normal hexose transporter (that is, the ***ftr***-gene-product).

Consequently, the Fungiflex molecules can be uniformly distributed, but in a disoriented stipe the activities of hexose transporter molecules are differentially regulated by the cytoskeleton across the diameter of the stipe.

- Transporters located on the 'upper' hyphal membrane may be activated to transport the ***extension-inhibiting Fungiflex 1***;
- Transporters located on the 'lower' hyphal membrane may be activated to transport the ***extension-promoting Fungiflex 2*** without delay.

So, this is our two-part model for gravitropism in *Coprinopsis*, and perhaps in other agarics: (a) an asymmetry in transporter kinetics is caused by gravity-vector-induced changes in tensions in the cytoskeleton, which (b) generates an asymmetry in uptake of **Fungiflex** from an otherwise uniform diffusion field of that modified sugar. All we need now is for somebody to prove it!

Our thanks are due to **Dr Rebecca J. Moore** for interpretation of FTIR spectra and **Dr Bryan Eastwood** for interpretation of NMR spectra. However, responsibility for the *speculations* about Fungiflex structures implicit in the figures and discussion above rests with the authors.

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Fungiflex: the untold story by
David Moore and LilyAnn Novak Frazer

In the latter half of the 1990s, and into the first couple of years of the new millennium, we carried out some ground-breaking research into communication within mushroom fruit bodies by chemical signals. Because we were hoping to commercialise the research, the work was never published beyond initial patent applications, which have since lapsed.

We believed at the time that we had accessed at least some of the controls of fungal morphogenesis by extracting compounds which caused mushroom stipes to bend at the point of application within hours of treatment.

Using standardised bioassays, we made a start on purifying and chemically characterising the extracted substances. We worked with two components which had recognisably different activities in the bioassay. These we called **Fungiflex 1** and **Fungiflex 2**.

Our extracts caused (vertical) mushroom stipes to bend towards a drop of the extract within 1 hour of its application to the side of the stipe. This localised growth inhibition was Fungiflex 1 activity. Such stipes grew back to the vertical in about 9 hours, but then started bending in the opposite direction at the point of application of the drop. By 18 hours the stipe had bent to an angle of 90° from the vertical. This localised growth promotion was Fungiflex 2 activity.

We got close to understanding the structure of these first fungal hormonal compounds but were unable to secure sufficient funding to take the research any further. Nonetheless, the scientific knowledge we revealed is still important and worthy of publication. Our research has not lost its uniqueness, **nor has it been duplicated up to now**.

In this publication, we report in full the research we accomplished nearly 20 years ago in the hope that it might stimulate future work in the topic and prompt further advances.
You could pick up the research where we left off.
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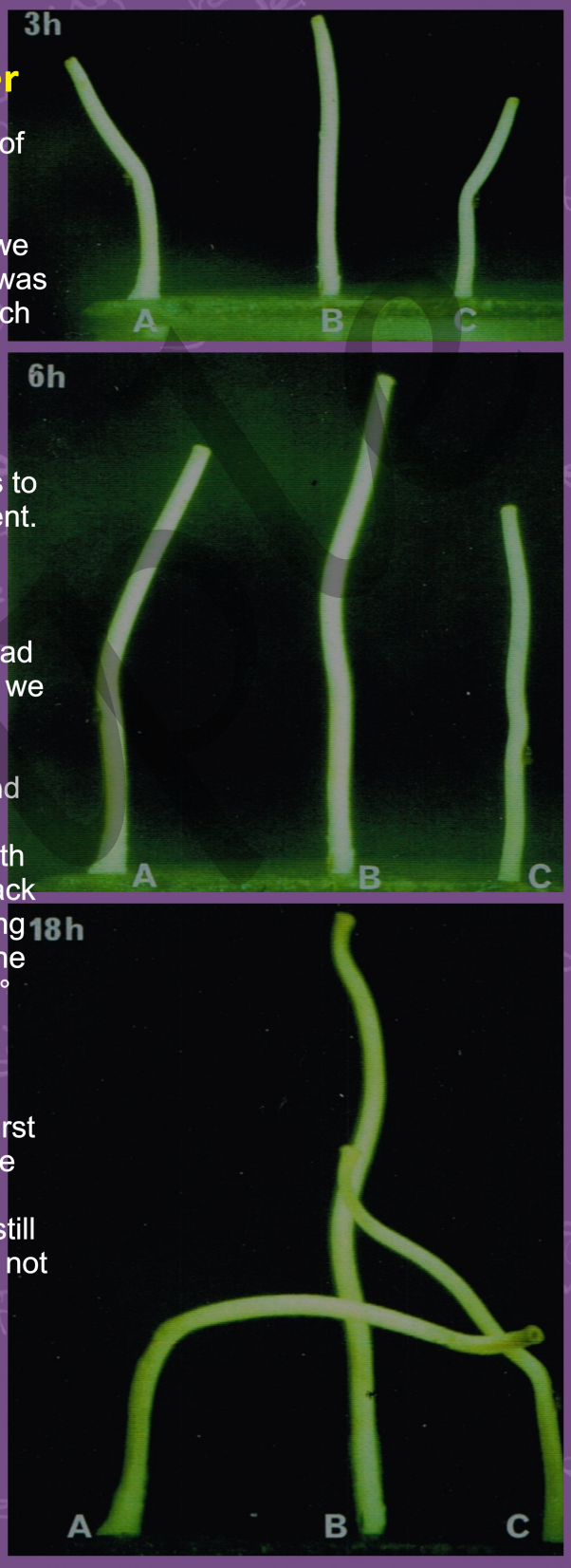

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