Phytochemistry, 1977, Vol. 16, pp. 229–233, Pergamon Press, Printed in England.

^{13}C-NMR STUDIES ON GRISEOFULVIN BIOSYNTHESIS AND ACETATE METABOLISM IN *PENICILLIUM PATULUM*

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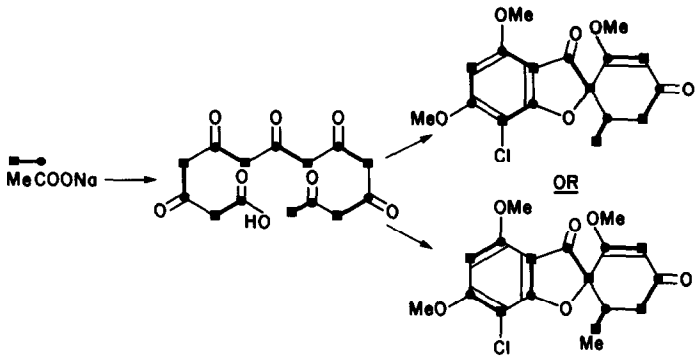
(*Received* 12 *May* 1976)

Key Word Index—*Penicillium patulum*; Ascomycetes; biosynthesis; fungal polyketide; griseofulvin.

Abstract—Incorporations of singly and doubly-labelled acetate-[^{13}C] into griseofulvin by a mutant strain of *Penicillium patulum* confirm its origin from simple folding of a single heptaketide chain. An acetate 'starter' effect is observed in the ^{13}C-NMR spectra of griseofulvin enriched from acetate-[^{13}C], and analysis of the ^{13}C–^{13}C spin–spin couplings observed indicate a rapid metabolic turnover of added acetate. Methyl, but not carboxyl, of acetate is efficiently metabolised into the C_1 pool.

INTRODUCTION

Griseofulvin (**1**), an important antifungal antibiotic, was first isolated from the mycelium of *Penicillium griseofulvum*, and has since been recognised as a metabolic product of many species of *Penicillium* [1]. Its biosynthesis has been a subject of several studies and much speculation. The polyketide origin of griseofulvin was indicated by incorporation studies with acetate-[1-^{14}C] by Birch and co-workers [2], who suggested derivation of griseofulvin from a single chain of seven acetate units as shown in Scheme 1. Studies by Rhodes *et al.* [3] have established that griseophenone C(**2**), and griseophenone B(**3**) but not griseophenone A(**4**) are likely intermediates between acetate and griseofulvin and they have proposed that the final stage in griseofulvin biosynthesis involves the binding of griseophenone B to a multienzyme complex which can effect oxidation, reduction, and methylation of enzyme bound intermediates. The co-occurrence of griseofulvin and fulvic acid (**5**) in the same organism, as well as the co-occurrence of other heptaketides in other fungi, has led to the proposal of several theories of biogenesis which aim to account for the formation of all fungal heptaketides from common intermediates, which would be formed either by condensation of two shorter polyketide chains [4, 5], or by ring closure and subsequent ring fission of a single heptaketide chain [6]. As ^{13}C-NMR has proved useful for providing information on the nature of the biosynthetic intermediates between acetate and malonate and the final polyketide metabolites [7], incorporations of singly and doubly-labelled acetate-[^{13}C], as well as malonate-[^{14}C] into griseofulvin using a high-yielding mutant strain of *Penicillium patulum* have been carried out in an attempt to solve some of the uncertainties in griseofulvin biosynthesis. The ^{13}C-NMR spectrum of griseofulvin has recently been fully assigned [8].



Scheme 1. Alternate foldings of the precursor heptaketide chain in griseofulvin.

RESULTS AND DISCUSSION

Preliminary incorporation experiments were carried out with sodium acetate-[^{14}C] in order to determine the minimum amount of acetate-[^{13}C] required to give sufficiently low dilution values for significant enrichments to be observed in the resultant ^{13}C-NMR spectra

Table 1. Dilution of sodium acetate-[1-^{14}C] on incorporation into griseofulvin by cultures of *P. patulum*

Experiment	Acetate (mg/ml)	μCi	Griseofulvin mg	dpm/mg	Dilution
1	0	—	176	—	—
2	0.5	10	209	4569	53.6
3	1.5	10	207	3768	32.5
4	2.0	10	168	4054	15.1

[7]. These results, summarized in Table 1, indicated that at least 2 g/l of acetate was required to give a doubling ot ^{13}C-abundance at each labelled position. Accordingly fermentations supplemented with 4 g/l of sodium acetate-[1-^{13}C], -[2-^{13}C], and -[1,2-^{13}C] respectively were carried out. The ^{13}C-NMR spectra of the acetate-[1-^{13}C], and acetate-[2-^{13}C] enriched griseofulvin samples are summarized in Table 2.

The alternate labelling pattern anticipated for the polyketide origin of griseofulvin was generally observed but, the apparent enrichment of labelled sites is very much lower with acetate-[2-^{13}C] than with acetate-[1-^{13}C] particularly C-7 which does not show the anticipated enrichment from acetate-[2-^{13}C]. On the other hand, MS studies (see below) indicate that the total incorporation of acetate-[2-^{13}C] is at least as high as that of acetate-[1-^{13}C]. The most likely explanation of this apparent discrepancy is that the label of acetate-[2-^{13}C] becomes randomised into the 1-position via operation of the Krebs' Cycle. The method of calculating enrichments [9] automatically compensates for any excess ^{13}C-abundance arising in this way, therefore an apparently low enrichment of acetate-[2-^{13}C] is observed.

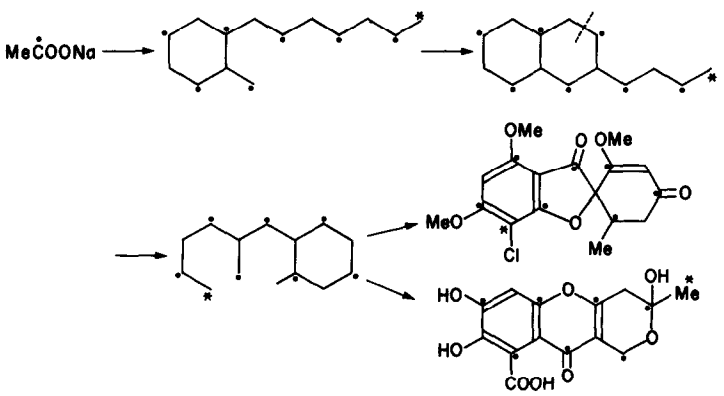
The degree of enrichment at positions C-1 and C-2 is significantly higher than in the remaining acetate-derived carbons. Differential labelling of a polyketide has not been observed previously in ^{13}C-biosynthetic studies. However, Birch *et al* [10] have observed a small preferential incorporation of acetate-[^{14}C], in tracer experiments into the methyl terminal acetate unit of both griseofulvin and curvularin and on feeding sodium acetate-[1-^{14}C], along with unlabelled diethyl malonate, to cultures of *Penicillium urticae* the resultant 2-hydroxy-6-methylbenzoic acid showed a 12–15% excess isotopic

Table 2. ^{13}C-chemical shifts (downfield from Me_4Si), enrichments in acetate-[1-^{13}C] and acetate-[2-^{13}C] labelled griseofulvin, and ^{13}C–^{13}C coupling constants (Hz) observed in acetate-[1,2-^{13}C] labelled griseofulvin

Carbon	δ(ppm)	Enrichment* acetate-[1-^{13}C]	acetate-[2-^{13}C]	$J^{13}C-^{13}C$
4	197.1	2.4‡	1.1	—
8	192.3	2.1‡	0.9	—
6	170.9	2.4‡	1.1	—
12	169.5	1.7‡	0.9	—
14	164.5	1.8‡	1.0	—
10	157.7	2.0‡	1.0	—
9	105.0	1.1	1.3‡	—
5	104.7	1.1	1.3‡	75, 60
13	97.1	0.9	1.4‡	—
7	90.6	1.0	1.1‡	41
11	89.7	1.0	1.7‡	77, 75
MeO	57.3	1.1	1.5	—
MeO	56.9	1.0	1.5	—
MeO	56.9	1.0	1.5	—
3	40.0	1.0	1.5‡	†
2	36.4	2.7	0.9	32
1	14.3	1.1	2.1	34
Average for anticipated malonate derived carbons (‡)		2.1(±0.4)	1.4(±0.3)	

* See experimental for details of calculation. † Complex pattern. ‡ Carbons anticipated to be derived from malonate.

abundance in the 'starter' acetate unit, no difference being observed in the absence of added malonate. This suggests that in *P. patulum* the acetate-[^{13}C] derived malonate is diluted by an endogenous metabolic pool of malonate with no corresponding dilution of the acetate-[^{13}C] so causing a clear acetate 'starter' effect to be observed. No other acetate unit shows this enhanced incorporation, so establishing that griseofulvin is formed from a single heptaketide chain and ruling out the ring-fission pathway which requires that the methyl group at position C-1 is not part of the chain initiating acetate being formed in the ring-fission process (Scheme 2). Kuhn–Roth oxidation of griseofulvin-[^{14}C] obtained

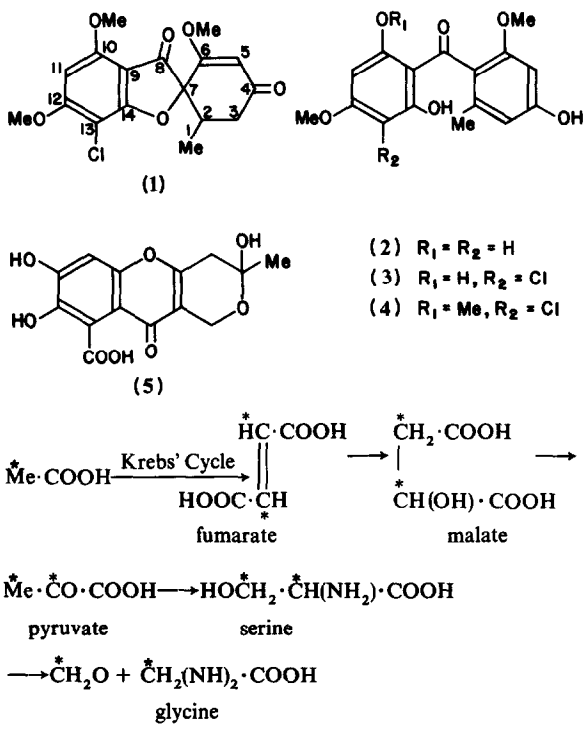


Scheme 2. Postulated ring-fission pathway to griseofulvin and fulvic acid.

from fermentations of *P. patulum* supplemented with diethyl malonate-[2-^{14}C], produces acetic acid with only 9.8% of the total activity of griseofulvin (even labelling of a heptaketide would require 14.3%) so confirming that the methyl at position C-1 is part of a chain initiating acetate unit, the observed incorporation into this position being due to decarboxylation of malonate to acetate.

It may be seen, Table 2, that incorporation of acetate-[2-^{13}C], but not acetate-[1-^{13}C] results in substantial enrichment of the methoxyl groups of griseofulvin. These have previously been shown to arise from the usual biochemical C_1 donor systems [11]. Enrichment of carbons derived from the C_1 pool has been observed in biosynthetic studies with both acetate-[1-^{14}C]- and acetate-[2-^{14}C] but usually at a low level and possibly it occurs *via* catabolism of acetate to CO_2 and subsequent re-incorporation. The specific enrichment from the methyl of acetate observed in this case could arise from conversion of acetate *via* the Krebs Cycle and pyruvate into serine. It has been shown *inter alia* in *Torulopsis* yeast grown on acetate that the α and β carbons of serine are derived from the methyl carbon only of acetate [12]. Subsequent dehydroxymethylation of serine would give rise to glycine-[2-^{13}C] and a [^{13}C]-enriched C_1 pool (Scheme 3). Incorporations of serine-[3-^{13}C] and glycine-[2-^{13}C] in *Serratia marcesens* [13] and *Streptomyces longisporus rubber* [14] result in substantial enrichments of the methoxyl group in the prodigiosins, though no enrichment of this group was observed from acetate-[^{13}C].

The ^{13}C-NMR spectrum of griseofulvin enriched from acetate-[1,2-^{13}C] is shown in part in Fig. 1. The anticipated characteristic triplets normally observed in doubly labelled spectra arising from the natural abundance signal with satellites on either side due to ^{13}C–^{13}C coupling between carbons derived from acetate units incorporated intact are seen only for C-1 and C-2. C-3, C-5 and C-11 show more complex patterns of satellites and the remaining carbons are of such low intensity that no satellites can be readily distinguished above the spectral noise level. The methoxyl carbons are again significantly enriched, presumably from the acetate methyl carbon only. The relatively high intensity of the satellites on the resonances due to C-1 and C-2 again indicate the preferential incorporation of acetate into this unit. The complex satellite patterns observed for the



Scheme 3. Conversion of the methyl carbon of acetate into formaldehyde and glycine.

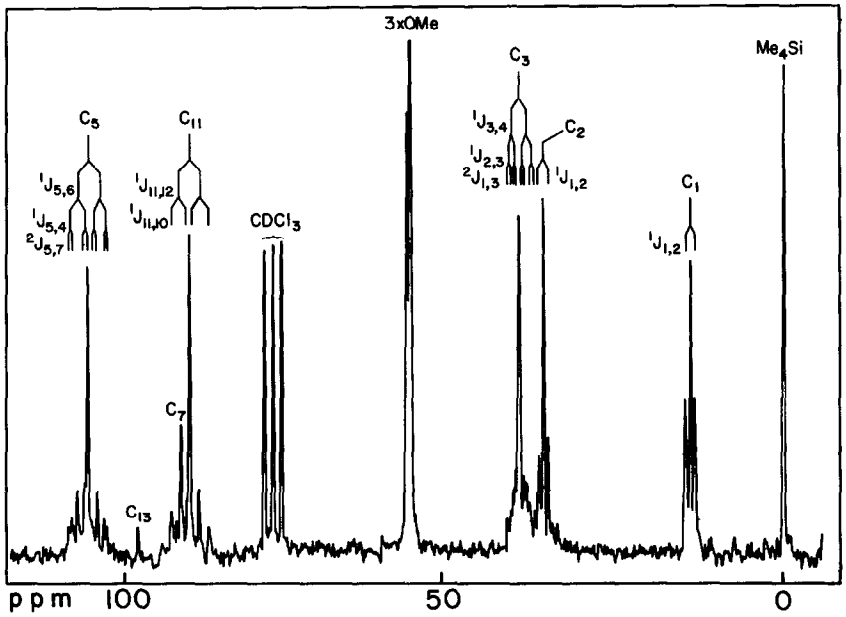


Fig. 1. ^{13}C NMR spectrum (0–110 ppm) of acetate-[1,2-^{13}C] enriched griseofulvin.

remaining carbons can only be accounted for by substantial ^{13}C–^{13}C coupling between adjacent doubly-labelled acetate units. This further coupling reduces the intensity of the satellites arising from incorporation of intact acetate units and further satellites appear due to one-bond ^{13}C–^{13}C coupling between adjacent acetate units as is indicated for C-11 in Fig. 1. The innermost satellites in this case are obscured by the large natural abundance resonance. An additional complicating factor is the possibility of two-bond ^{13}C–^{13}C couplings, which owing to the use of doubly-labelled acetate in which there is greater than 90% ^{13}C at both positions, is almost as likely as one-bond ^{13}C–^{13}C coupling between adjacent units. Two-bond ^{13}C–^{13}C couplings are usually small, typically 0–15 Hz, so are not always resolvable. However, the satellite pattern for C-5 shows an additional small splitting, 9.4 Hz, due to the two-bond coupling between C-5 and C-7, or possibly C-3. The unusual shape of the C-3 signal is partly explained by the fact that the one-bond couplings between the aliphatic C-3 and C-2 are comparatively smaller than those for the aromatic carbons above. However, no significant satellites corresponding to the one bond coupling between C-3 and C-4 is observable. It has already been established that acetate is incorporated into C-1 and C-2 to a greater extent than in the remainder of the molecule, so it is likely that for every griseofulvin molecule in which C-3 and C-4 are derived biosynthetically from added precursor acetate, so also will be C-1 and C-2, though the reverse obviously is not true. The result of this is that essentially all the carbons at position C-3 which are coupled to C-4 are further split by both C-2 and C-1 to give the observed pattern. The reciprocal couplings on C-1 and C-2 are not readily observable due to the high proportion of these atoms not further coupled in this way. Confirmation of the two-bond coupling between C-1 and C-3 is found in the acetate-[2-^{13}C] derived ^{13}C-NMR spectrum where the C-3 signal appears to be broader than the remaining signals. Redetermination of the spectrum on a narrow sweep width, readily allows a coupling of 12.2 Hz to be resolved. Two-bond couplings arising from incorporation of singly-labelled acetate-[^{13}C] into aflatoxin B have been reported by Hseih *et al.* [15], but in this case, very high overall enrichments (20–30%) of the labelled carbons were obtained.

Extensive inter-acetate unit coupling of this sort can only occur when there is a high probability of two or more acetate units being incorporated into adjacent positions in any one molecule. This has been recognised as a problem with organisms where the incorporation of acetate is very efficient, giving very low dilution values, and dilution of the labelled acetate with unlabelled acetate before feeding has been used to reduce the possibility of two labelled units coming together [16], although the actual validity of doing this has been questioned by a recent detailed statistical treatment [17]. This problem was not anticipated in *P. patulum*, however, due to the comparatively high dilution values observed in the preliminary ^{14}C experiments and indeed the overall average ^{13}C-enrichment observed is much too low to allow this, and suggests there is only an approx. 4% probability of adjacent units being labelled.

MS studies can be used to estimate the excess ^{13}C abundance, and Table 3 shows the ion intensities in the molecular ion region for unlabelled, and both acetate-[1-^{13}C] and acetate-[2-^{13}C] enriched griseofulvin. Although accurate estimations are complicated by the isotope pattern expected for a mono-chloro-substituted compound it is evident that the overall enrichment is low but that a substantial proportion of the biosynthetically enriched molecules are multiply labelled, there even being a significant number of molecules in which all seven acetate units are derived from the added precursor, so accounting for the extensive ^{13}C–^{13}C coupling. [The rather high proportion of M+1 ions in the acetate-[2-^{13}C] relative to acetate-[1-^{13}C] enriched sample is difficult to account for but is due, in part at least to the C_1 pool enrichment.]

The above results indicate that for a short period most of the griseofulvin produced by *P. patulum* is derived from the added acetate-[^{13}C], with some dilution by endogenous malonate as discussed above. This phase is followed or possibly preceded by production of unlabelled griseofulvin to give the low overall enrichment observed, though for a period an exceedingly high enrichment has been obtained. Whitlock has noted the occurrence of extra couplings in the spectrum of acetate-[1,2-^{13}C] enriched islandicin. These satellites, though of low intensity, are stronger than would be expected from the average overall enrichment: the presence of a substantial preformed pool of islandicin, or a lag period after precursor addition during which no added precursor is incorporated but islandicin is formed, was suggested to account for this [18]. Time studies with *P. patulum* indicate that only a small amount of griseofulvin is produced before precursor addition. It appears likely that addition of a large amount of exogenous acetate represses the production of endogenous acetate by a feedback inhibition mechanism so that, until this pool falls below a, probably very low, threshold level and endogenous acetate production recommences, most of the metabolite is produced from exogenous precursor. Subsequently, predominantly unenriched griseofulvin is produced to give the low average enrichment observed.

The unexpected pattern of acetate metabolism observed in *P. patulum* is probably due to two factors. Firstly a highly developed, mutant strain was used, which is probably capable of rapid utilisation of any readily available carbon source. Secondly, it is due to the use of elevated amounts of precursor, in contrast to the trace

Table 3. Ion intensities in the molecular ion region of the mass spectra of natural abundance, and acetate-[1-^{13}C] and acetate-[2-^{13}C]-enriched griseofulvin

Sample	M^+(352)	M+1	M+2	M+3	M+4	M+5	M+6	M+7	M+8	M+9
Natural abundance	100	19	37	7	0.4	—	—	—	—	—
Acetate-[1-^{13}C] enriched	100	21	37	7	0.5	0.3	0.4	0.3	0.2	0.1
Acetate-[2-^{13}C] enriched	100	25	39	9	0.7	0.3	0.3	0.3	0.2	0.1

amounts used in classical radioisotope studies, and these results serve to emphasise that this factor must always be taken account of in interpretation of ^{13}C-biosynthetic studies. The effects observed in this study provide an excellent illustration of the problems and factors that can arise through using non-tracer amounts of precursor, and will probably be encountered more frequently as ^{13}C-biosynthetic studies become more commonplace. Great care must be exercised when, for example, using assumedly unenriched carbons of different biosynthetic origin for normalisation of spectra and calibration of enrichments. However, it also serves to illustrate the power of ^{13}C methods in elucidating the overall metabolic patterns in an organism.

EXPERIMENTAL

^{13}C-NMR spectra were determined for saturated solns in $CDCl_3$, with Me_4Si as internal reference on either a Varian XL-100-15FT spectrometer operating at 25.197 MHz or a Jeol JNM FX60 spectrometer operating at 15.04 MHz. The enrichment values quoted in Table 2 were calculated from spectra determined in the presence of 0.1 molar chromium Tris–acetoacetonate [19] as relaxation agent under GATED-2 decoupling conditions [20]. These conditions eliminate the wide range of resonance intensities arising from variable relaxation times and nuclear Overhauser enhancements so facilitating measurement of intensities. The natural abundance and enriched spectra were each determined three times, and the average intensities after normalization were used to calculate the enrichments using the method reported previously [9]. The actual values are the ratio of the intensities in the enriched spectrum to the same intensities in the natural abundance spectrum and are consistent within a range of ±20%. ^{14}C-Radiochemical assays were carried out by scintillation counting. Counting efficiencies were measured with [^{14}C]hexadecane as internal standard using Butyl-PBD (10 g) in toluene (1 l.) as scintillator solution.

Culture conditions. A Glaxo mutant strain of *P. patulum* was grown in 100 ml shake flasks each containing 30 ml of Glaxo standard shake flask medium with 10% lactose. Each flask was innoculated with 1.5 ml of pre-grown mycelium. A time study indicated that griseofulvin production commenced at about day 2 and had reached a conc. of approx. 200 mg per flask after 7 days growth.

Incorporation studies. (a) *Sodium acetate-*[1-^{14}*C*]. Increasing amounts of sodium acetate, as indicated in Table 1, each spiked with sodium acetate-[1-^{14}C] (10 μCi), in 1 ml H_2O were added to 2 day old cultures of *P. patulum*. After 7 days the mycelium was collected by filtration, stirred in Me_2CO (75 ml) together with added lime (200 mg) for 10 min. The Me_2CO extract was then filtered off and the Me_2CO removed *in vacuo* at 60–70°. The resulting hot aq. suspension was filtered and the crude product approx. 200 mg) washed with H_2O (60°). Recrystallisation from Me_2CO petrol (bp) 60–80°) gave essentially pure griseofulvin which was crystallised to constant specific radioactivity. The minimum dilution value obtained was 15. On this basis it would be anticipated that equivalent feedings of 0.2% acetate-[1-^{13}C] (90%) would give griseofulvin with an excess of 0.8% of ^{13}C-label over natural abundance at each labelled position, assuming a total of 7 labelled positions in the molecule.

(b) *Diethyl malonate* [2-^{14}*C*]. Diethyl malonate-[2-^{14}C] (25 μCi) was added as above to a 2 day old culture flask and the product isolated after 7 days growth. Recrystallization to constant specific radioactivity gave griseofulvin-[^{14}C] (108 mg, 5.00×10^7 dpm/mmol). After dilution with unlabelled griseofulvin (150 mg, 8.34×10^6 dpm/mmol) it was subjected to Kuhn–Roth oxidation. The resultant HOAc was isolated and converted to the *p*-bromophenacyl derivative (35 mg) by standard procedures. Recrystallization to constant specific radioactivity gave *p*-bromophenacyl acetate with an activity of 8.21×10^5 dpm/mmol. Thus the terminal unit contains 9.8% of the total radioactivity of griseofulvin.

(c) *Sodium acetate-*[^{13}*C*]. To each of 3 flasks containing a 2 day old culture of *P. patulum* was added sodium acetate-[1-^{13}C], -[2-^{13}C], or -[1, 2-^{13}C] (120 mg, 90% enriched). After a further 5 days growth, the enriched griseofulvin, 56, 65 and 82 mg respectively after recrystallization, was isolated as above.

Acknowledgements—We wish to thank Glaxo Laboratories Ltd., Ulverston, Cumberland for the use of cultures, culture facilities and technical assistance. We thank Dr R. D. Lapper for ^{13}C-NMR spectral determinations.

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