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The Biosynthesis of the Mould Metabolites Roquefortine and Aszonalenin from *L*-[2,4,5,6,7-$^2H_5$]Tryptophan

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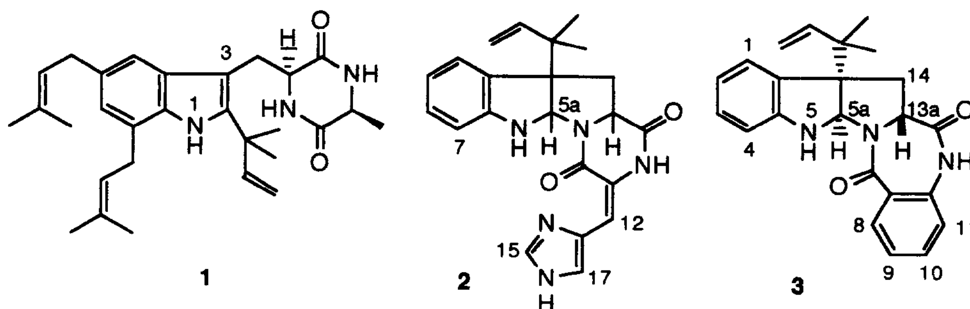
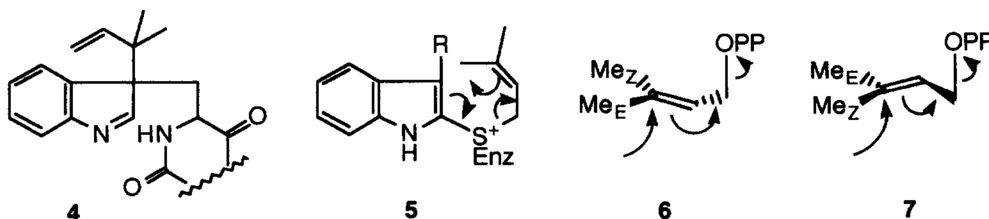
Abstract *L*-[2,4,5,6,7-$^2H_5$]Tryptophan was incorporated into roquefortine **2** by *Penicillium roqueforti* and aszonalenin **3** by *Aspergillus zonatus* with retention in each case of five deuterium atoms, the 5a-hydrogen of both metabolites is derived from the 2-hydrogen of tryptophan

INTRODUCTION

Much interest has been shown in recent decades in the biosynthesis of tryptophan-derived fungal metabolites that contain a 1,1-dimethylallyl substituent It is likely that 2-(1,1-dimethylallyl)indoles such as echinulin[1] **1** and 3a-(1,1-dimethylallyl)hexahydropyrrolo[2,3-b]indoles exemplified by roquefortine[2,3] **2** and aszonalenin[4,5] **3**, arise from a common type of precursor **4** *via* Wagner-Meerwein rearrangement of the prenyl group[6] or ring closure respectively Several proposals have been made concerning the manner in which the 1,1-dimethylallyl substituent is initially attached to the indole moiety The simplest, namely direct allylic displacement of pyrophosphate from dimethylallyl pyrophosphate by the indole moiety, has received scant attention, whilst the suggestion that the putative precursor **4** arises *via* aza-Cope rearrangement of a 1-(3,3-dimethylallyl)tryptophan derivative was not supported by our early experiments on the biosynthesis of echinulin [1]



Alternatively Bycroft and Landon suggested that the putative precursor **4** of echinulin arises from thio-Claisen rearrangement of an enzyme-bound sulphonium salt **5** followed by reductive removal of sulphur [7] This interesting proposal, for which chemical models have been described,[5,7,8] would imply that the 5a-proton of roquefortine **2** is not derived from the 2-proton of tryptophan In this connection Barrow *et al* reported that isotopic label from [2,4,5,6,7-$^2H_5$]tryptophan was incorporated by a tryptophan-auxotrophic mutant of *Penicillium roqueforti* into the benzenoid ring of roquefortine **2** but not into the 5a-



hydrogen of the latter,[9] in apparent support of Bycroft's proposal, though the former authors also considered the possibility that a 2-(1,1-dimethylallyl)indole was an intermediate in the biosynthesis of roquefortine However the use of ^{1}H-NMR in that study, for assay of deuterium by difference, is likely to be insensitive to the presence of biosynthetically significant traces of deuterium at the 5a-position of roquefortine Accordingly we have investigated the incorporation of [$^2H_5$]tryptophan into both roquefortine **2** and the related mould metabolite aszonalenin **3**, with direct assay of deuterium by ^{2}H-NMR, and now report[10] that the 5a-hydrogen atoms of both metabolites are derived from the 2-hydrogen of tryptophan, contrary to the earlier claim

RESULTS AND DISCUSSION

L-Tryptophan was labelled with deuterium in the aromatic positions by exchange with a mixture of deuteriated water and deuteriated trifluoroacetic acid,[11] followed by back-exchange of labile deuterium with water The resultant deuterium enrichment at positions 2, 4, 5, 6, and 7 was estimated as 97, 86, 99, 96, and 93% respectively by integration of residual aromatic protons against the unexchanged methylene and methine protons in the ^{1}H-NMR spectrum The isotopic composition of the sample was estimated by FAB mass spectrometry as 71 5% d_5, 24% d_4, 2 5% d_3 and 2% d_2

L-[2,4,5,6,7-$^2H_5$]Tryptophan (153 mg) was supplied to a 5-day-old surface culture of *Penicillium roqueforti* grown on a 2% yeast extract medium (2 5 l) supplemented with sucrose (15%) The mycelium was harvested after growth for a further 21 days at 24°C The CI mass spectrum of the roquefortine **2** (25 mg) that was isolated revealed the presence of both d_4- and d_5-species (*ca* 3% and 8% respectively) The ^{2}H-NMR spectrum of a $CHCl_3$ solution of this sample of roquefortine (**Figure 1a**) showed a sharp resonance at δ 7 25 for C^2HCl_3 at natural abundance, superimposed upon a broad envelope, *ca* δ 6 to 8, due to the four overlapping benzenoid resonances, and a broad, partially resolved resonance at δ 5 6 Reference to the ^{1}H-NMR spectrum of roquefortine (**Figure 1c**) revealed that the latter resonance could only be assigned to the deuterium-labelled 5a-proton, notwithstanding the poor resolution achieved in the ^{2}H-NMR spectrum The computer-simulated ^{2}H-NMR spectrum of the biosynthetically labelled roquefortine (**Figure 1b**) shows a satisfactory correspondence to the observed spectrum

Similarly *L*-[2,4,5,6,7-$^2H_5$]tryptophan was incorporated into aszonalenin **3** by *Aspergillus zonatus* The $[M-C_5H_9]^+$ cluster in the EI mass spectrum of the [^{2}H]aszonalenin that was isolated contained d_4- and d_5-species (*ca* 3% each), as well as traces of d_3-, d_6-, d_7-, d_8-, and d_9-species (each <1%) Comparison of the ^{2}H-NMR spectrum of the [^{2}H]aszonalenin (**Figure 2a**) with the ^{1}H-NMR spectrum (**Figure 2b**), revealed that the 5a-proton of the labelled aszonalenin is enriched with deuterium It is also noteworthy that the 8- and 10-protons were weakly labelled and that the extent of deuterium enrichment at the 5a-position of aszonalenin is only about 70% of the average for positions 1 to 4, these observations may be accounted for by the metabolism of some of the [$^2H_5$]tryptophan to [$^2H_4$]anthranilic acid, which could furnish protons 8 to 11 of aszonalenin, or which could be re-incorporated into tryptophan and thence furnish [1,2,3,4-$^2H_4$]aszonalenin A similar sequence might also explain the earlier report that the 5a-proton of roquefortine is not derived from the 2-proton of tryptophan [9] It may be significant that the [$^2H_5$]tryptophan in the latter study was supplied at the start of the fungal culture,[12] thus increasing the opportunity for its degradation to

Figure 1

(a) 55 MHz Resolution-enhanced proton-noise decoupled ^{2}H-NMR spectrum (61004 transients) of [^{2}H]roquefortine in $CHCl_3$ at 25°C

(b) *Lower Trace* Computer simulation of individual ^{2}H-NMR resonances for labelled sites in roquefortine, assuming Lorenzian lineshapes, each with $w_{1/2}$ 22 Hz, at δ 5 64, 6 60, 6 77, 7 10, and 7 18 (for ^{2}H-enriched H-5a, H-7, H-9, H-8, and H-10 respectively), with the same relative abundances of deuterium as those determined for the labelled precursor, and a Lorenzian resonance at δ 7 25, with $w_{1/2}$ 2 7 Hz (for C^2HCl_3 at natural abundance)

Upper Trace Computer simulated ^{2}H-NMR spectrum of roquefortine as the sum of the six Lorenzian resonances shown

(c) 360 MHz ^{1}H-NMR spectrum of roquefortine **2** in C^2HCl_3 at 25°C

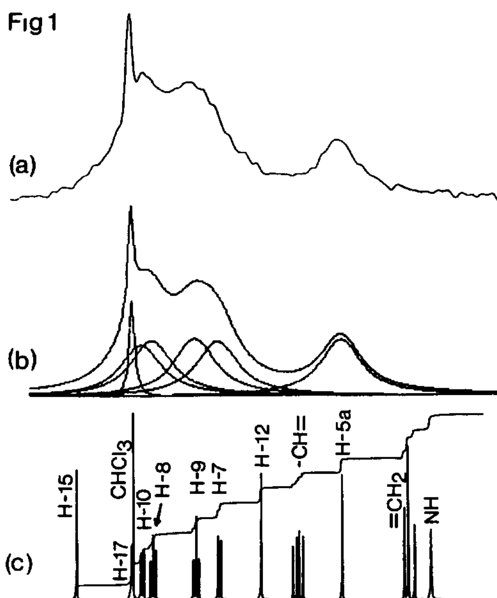
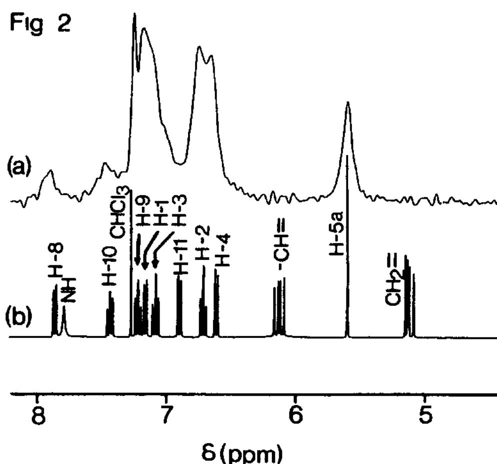


Figure 2

(a) 55 MHz Resolution-enhanced proton-noise decoupled ^{2}H-NMR spectrum of [^{2}H]aszonalenin in $CHCl_3$ (56000 transients) at 58°C The positions and areas of the recorded bands are consistent with the following distribution of the ^{2}H-label in **3** H-8, H-9, H-10, H-11 *ca* 5% each, H-1, H-2, H-3, H-4 ca 17% each, H-5a *ca* 12% The resonance at δ 7 25 is due to C^2HCl_3 at natural abundance

(b) 360 MHz ^{1}H-NMR spectrum of aszonalenin **3** in C^2HCl_3 at 58°C



[$^2H_4$]anthranilic acid before the onset of roquefortine biosynthesis

Our observation that the 5a-proton of both roquefortine and aszonalenin derives from *L*-tryptophan has important implications for the mechanism of introduction of the 1,1-dimethylallyl substituent during the biosynthesis of these compounds In particular these results preclude the involvement of a free 2-substituted indole[9,13] as an intermediate in the biosynthesis of either metabolite Furthermore the enzyme-bound 2-substituted indole[7] **5** now appears implausible as an intermediate in the absence of a mechanism which could account for overall retention of the 2-proton of tryptophan It now seems likely that the introduction of the 1,1-dimethylallyl substituent into roquefortine, echinulin, and related compounds, proceeds by direct alkylation of C-3 of the indole by dimethylallyl pyrophosphate It was reported that both diastereotopic methyls of the 1,1-dimethylallyl group in roquefortine[13] and echinulin[14] are furnished, to unequal extents,

by the methyl of mevalonic acid These observations do not preclude a high degree of stereochemical control by the alkylating enzyme For example, allylic displacement of pyrophosphate by a single face of the indole, proceeding with 100% *anti* stereochemistry, could lead to the observed results if the enzyme displayed poor discrimination between two alternative orientations (**6** and **7**) of 3,3-dimethylallyl pyrophosphate

EXPERIMENTAL

Organic extracts were routinely dried over $MgSO_4$ and evaporated to dryness *in vacuo* TLC was performed on 0 25 mm thick silica gel layers containing a fluorescent indicator precoated on plastic sheets, supplied by Camlab, Cambridge, compounds were visualised by fluorescence under UV light (254 nm), or by spraying with a cerium (IV) preparation [$Ce(SO_4)_2$ (10 g) and trichloroacetic acid (100 g) in boiling water (400 ml), clarified by the addition of conc sulphuric acid] followed by heating to 100°C Melting points were recorded on a Reichert hot stage m.p apparatus and are uncorrected

Except where stated otherwise, ^{1}H-NMR spectra were recorded at 360 MHz with a Bruker WH 360 spectrometer for $CDCl_3$ solutions ^{2}H-NMR spectra were recorded at 55 3 MHz, using the same spectrometer, for $CHCl_3$ solutions EI Mass spectra were recorded using a Kratos MS 80 spectrometer with 70 eV ionising electron beam, CI mass spectra utilised ammonia as reagent gas FAB Mass spectra were recorded on the same instrument for a glycerol/water matrix

Assignments of ^{1}H-N.M.R. Spectra

L-Tryptophan

δ_H (360 MHz, d_6-dmso) 2 97 (1H, dd, J 8 8 and 15 1 Hz) and 3 31 (1H, dd, J 4 0 and 15 1 Hz) ($C\underline{H_2}CH$) and 3 46 (1H, dd, J 4 1 and 8 8 Hz, $CH_2C\underline{H}$), superimposed on very broad resonance, δ~2 5 to 4 0 (NH_3^+), 6 97 (1H, ddd, J 1 0, 7 0, and 7 8 Hz, H-5), 7 06 (1H, ddd, J 1 1, 7 0, and 8 0 Hz, H-6), 7 22 (1H, d, J 2 3 Hz, H-2), 7 35 [1H, d, J 8 0 Hz, (collapsed to s on decoupling at δ 7 06), H-7], 7 57 [1H, d, J 7 8 Hz, (sharpened on decoupling at δ 7 06), H-4], 10 96 (1H, br s, H-1), irrad at δ 10.96 gave NOEs at 7 22 (~3 5%, H-2) and 7.35 (~4%, H-7) only

Roquefortine **2**

δ_H (360 MHz, $CDCl_3$) 1 03 (3H, s, Me), 1 15 (3H, s, Me), 2 47 (1H, dd, J 11 4 and 12 4 Hz) and 2 60 (1H, dd, J 6 0 and 12 4 Hz) ($C\underline{H_2}CH$, simplified to AB quartet on decoupling at δ 4 06), 4 06 (1H, dd, J 6 0 and 11 4 Hz, $CH_2C\underline{H}$), 4 96 (1H, s, NH), 5 11 (dd, J 17 2 and 1 1 Hz) overlapping with 5 15 (dd, J 10 8 and 1 1 Hz) (total 2H, $C\underline{H_2}$=CH), 5.64 (1H, s, H-5a), 5 98 (1H, dd, J 17 2 and 10 8 Hz, CH_2=C$\underline{H}$), 6 28 (1H, s, H-12), 6 59 [1H, d, J 7 8 Hz, (sharpened on decoupling at δ 6 77), H-7], 6 77 (1H, dt, J 1 0 and 7 4 Hz, H-9), 7 10 [1H, dt, J ~1 1 and 7 6 Hz, (collapsed to dd, J ~1 0 and 7 5 Hz, on decoupling at δ 6 77), H-8], 7 18 [1H, d, J 7 5 Hz, (collapsed to s on decoupling at δ 6 77), H-10], 7 26 (1H, br s, H-17), 7 69 (1H, s, H-15), 8 99 (1H, br s, NH), 12 93 (1H, br s, NH)

Aszonalenin **3** (*cf* assignments for (-)-dihydroaszonalenin[5])

δ_H (360 MHz, $CDCl_3$) 1 08 (3H, s, Me), 1 14 (3H, s, Me), 2 41 (1H, dd, J 8 9 and 14 0, H-14 *pro-R*), 3 47 (1H, dd, J 7 6 and 14 0 Hz, H-14 *pro-S*), 3 97 (1H, dd, J 7 7 and 8 9 Hz, H-13a), 5 10 (dd, J 1 2 and 17 3 Hz) overlapping with 5 13 (dd, J 1 2 and 10 9 Hz) (overall 2H, $C\underline{H_2}$=CH), 5 59 (1H, s, H-5a), 6 11 (1H, dd, J 10 9 and 17 3 Hz, CH_2=C$\underline{H}$), 6 60 (1H, d, J 7 8 Hz, H-4), 6 70 (1H, dt, J 1 0 and 7 5 Hz, H-2), 6 88 (1H, d, J 7 6 Hz, H-11), 7.06 (1H, dt, J 1 0 and 7 6 Hz, H-3), 7 14 (1H, d, J 7 5 Hz, H-1), 7 20 (1H, dt, J 1 2 and 7 6 Hz, H-9), 7 42 (1H, dt, J 1 6 and 7 7 Hz, H-10), 7 77 (1H, br s, NH), 7 85 (1H, dd, J 1 5 and 7 9 Hz, H-8)

[2,4,5,6,7-$^2H_5$]-*L*-Tryptophan[11]

A solution of *L*-tryptophan (5.0 g) in a mixture of deuterium oxide and ($^2H_1$)trifluoroacetic acid [prepared by the dropwise addition of deuterium oxide (7 5 ml) to ice-cold trifluoroacetic anhydride (15 6

ml)] was allowed to stand for 7 days in the dark at room temperature after which the solvent was removed by rotary evaporation under high vacuum The crude product was incubated four more times under the same conditions with fresh deuterium oxide and ($^2H_1$)trifluoroacetic acid, then the solvent was removed *in vacuo* The product was freed from labile deuterium by dissolution in water and re-isolation by evaporation Crystallisation from water and ethanol then furnished the title compound (4 2 g); δ_H (360 MHz, d_6-dmso) 2 98 (1H, dd), 3 33 (1H, dd), 3.46 (1H, dd), superimposed on very broad resonance, δ~2 to 4 5 (NH_3^+), 6 97 (0 013H, s, H-5), 7 06 (0 041H, s, H-6), 7 23 (0 034H, s, H-2), 7 35 (0 067H, s, H-7), 7 57 (0 135H, s, H-4), 10 96 (1H, s), m/z (FAB) 207 (3 1%), 208 (6 2%), 209 (39 9%), 210 (100%), 211 (19 0%), 212 (5 4%), 213 (1 1%), 214 (0 6%) {unlabelled tryptophan gave m/z (FAB) 204 (6 6%, M^+), 205 (100%, $[M+H]^+$), 206 (17 5%), 207 (4 1%), 208 (0 6%)}

Organisms

Aspergillus zonatus (Strain IFO 8817) supplied by Dr Y Kimura, and *Penicillium roqueforti* (Strain 111275) supplied by Dr R Vleggaar, were each maintained on potato-dextrose agar at 4°C For inoculation of liquid media, each fungus was grown at 25°C for between nine and fifteen days in petri dishes containing potato-dextrose agar Two such agar plates were macerated with water (150 ml) in a Waring blender, and 1 ml of the resulting suspension was added as inoculum to each flask of liquid medium All operations were performed under sterile conditions

Feeding of *L*-[2,4,5,6,7-$^2H_5$]Tryptophan to *Pencillium roqueforti*

A solution of Oxoid yeast extract (50 g) and sucrose (375 g) in tap water (2500 ml) was distributed evenly between ten 1-litre culture flasks The flasks were gently plugged with non-absorbent cotton wool and sterilised in the autoclave for 20 minutes at 120°C After cooling to room temperature, the flasks were each inoculated with *Penicillium roqueforti*, then maintained at 24°C in the dark After five days, by which time the mycelial mat had covered the surface of the liquid, a solution of [2,4,5,6,7-$^2H_5$]tryptophan (15 3 mg) in water (2 5 ml) was applied to each flask, below the mycelium, using a pipette The flasks were gently swirled, with minimal disturbance of the mycelium, then incubated at 24°C for 11 more days The mycelium was collected by filtration, washed with water, and dried to constant weight The finely powdered mycelium (11 2 g) was extracted twice with acetone (300 ml) by stirring for two days at room temperature The combined extracts were evaporated to dryness *in vacuo*, dissolved in chloroform (100 ml), and extracted 3 times with 0 5 *M* hydrochloric acid The combined acid extracts were brought to pH 10 with conc aqueous ammonia, and extracted with chloroform (3 x 50 ml) Removal of the solvent *in vacuo* furnished a impure sample of roquefortine which showed a major spot on silica gel TLC (Rf 0 60), with two minor contaminants (Rf 0 54 and 0 45), when eluted with chloroform, methanol, and 28% aqueous ammonia in the ratio 95 10 1 by volume Flash chromatography, eluted with chloroform, methanol, and 28% aqueous ammonia (in the ratio 200·10:1 by volume) and crystallisation from wet methanol furnished [^{2}H]roquefortine (25 mg), m p 196-200°C (lit [2] 195-200°C), m/z (CI, NH_3) 389 (25%), 390 (100%), 391 (40%), 392 (11%), 393 (5%), 394 (7%), 395 (16%), {unlabelled roquefortine gave m/z (CI, NH_3) 389 (9%, M^+), 390 (100%, $[M+H]^+$), 391 (28%), 392 (12%), 393 (2%), 394 (1%)}, m/z (EI) 320 (100%), 321 (26%), 322 (4%), 323 (1 5%), 324 (7%), 325 (8%), 389 (33%), 390 (5 5%), 391 (1%), 392 (1%), 393 (2%), 394 (1%), {unlabelled roquefortine gave m/z (EI) 320 (100%, $[M-C_5H_9]^+$), 321 (20%), 322 (3%), 389 (33%, M^+), 390 (6%), 391 (1%)}

Feeding of *L*-[2,4,5,6,7-$^2H_5$]Tryptophan to *Aspergillus zonatus*

Finely ground malt (125 g) was boiled with tap water (2500 ml) for 30 minutes and the cooled mixture was filtered at the pump The filtrate was supplemented with peptone (7 5 g) and glucose (75 g), and distributed equally between ten 1-litre culture flasks The flasks were gently plugged with non-absorbent cotton wool and sterilised in the autoclave for 20 minutes at 120°C After cooling to room temperature, the flasks were each inoculated with *Aspergillus zonatus* and maintained at 25°C in the dark

After six days, by which time the mycelial mat had covered the surface of the liquid, a solution of [2,4,5,6,7-$^2H_5$]tryptophan (15 mg) in water (2 5 ml) was applied to each flask, below the mycelium, using a pipette The flasks were gently swirled, with minimal disturbance of the mycelium, then incubated at 25°C for a further 16 days

The mycelium from the culture flasks was harvested, washed with water, and dried *in vacuo* The finely powdered mycelium (47 01 g) was extracted with acetone (300 ml) for 3 days at room temperature to furnish an oil (5 784 g) The latter was digested with a mixture of ethyl acetate and benzene (3 7 by volume) and the soluble fraction (5 405 g) was subjected to a preliminary chromatography on silica gel, with the same solvent, to give a fraction (1 146 g) which contained aszonalenin The latter fraction was submitted to "flash" chromatography on silica gel, with benzene-ethyl acetate (8 2, by volume); careful re-chromatography of the appropriate fraction (180 mg) gave aszonalenin (98 mg) Recrystallisation from chloroform and ethanol gave [^{2}H]aszonalenin (38 5 mg), mp 233-237°C (lit.[4] 244-247°C), which gave a single spot on TLC (R_f 0 29) and whose ^{1}H-NMR spectrum showed no extraneous resonances, m/z (EI) 303 (16 9%), 304 (100%), 305 (20 4%), 306 (3 0%), 307 (1.4%), 308 (3.6%), 309 (3 9%), 310 (1 1%), 311 (0 5%), 312 (1 0%), 313 (1 1%), 373 (18 1%), 374 (4 4%), 375 (1 0%), 376 (0.1%), 377 (0 7%), 378 (0 8%), 382 (0 1%) {unlabelled aszonalenin showed m/z (EI) 303 (10 6%), 304 (100%, $[M-C_5H_9]^+$), 305 (20 3%), 306 (3 1%), 307 (0 4%), 373 (15 2%, M+), 374 (4.4%)}

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