SUPPORTING INFORMATION

FOR

Lovastatin Nonaketide Synthase (LNKS) Catalyses an Intramolecular Diels Alder Reaction of a Substrate Analog

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Cell free protein extracts from *A. nidulans* containing *lovB* gene; assay and purification of LNKS. Generation and fermentation of *A. nidulans* containing both *lovB* and *lovC* genes or *lovB* gene only has been previously described.[1] The method used to prepare cell free extracts is similar to a literature procedure.[2] The fully grown culture (1 L) of *A. nidulans* (containing either only the *lovB* gene or both the *lovB* and *lovC* genes) was gravity filtered through Miracloth and the mycelia were washed with purifed water (3.5 L). The wet mycelia (~15 g) was frozen in a dry ice/acetone bath and freeze-dried overnight. All the following steps were done at 4 °C. A portion of the dried mycelia (~ 3 g) was pulverised in extraction buffer (25 mL, 20 mM Tris-HCl pH 8.0, 10% glycerol, 1 M NaCl, 5 mM sodium ascorbate, 1 mM EDTA, 1 mM DTT, 3.8 μg/mL of leupeptin, 17.7 μg/mL of chymostatin, 2 μg/mL of pepstatin, 42 μg/mL of trypsin inhibitor, 0.2 mM phenylmethylsulfonylfluoride (PMSF), 2.2% polyvinyl polypyrrolidone) using a mortar

and pestle for at least 10 min. PMSF is used here to accelerate precipitation of both DNA and RNA. The broken cell suspension was diluted with more extraction buffer (175 mL) and briefly stirred on an orbital shaker at 75 rpm to complete the extraction. The cell debris was separated by centrifugation at 10,000 × *g* for 30 min. The supernatant was gravity filtered through 4 layers of cheesecloth to afford the cell free extract.

LNKS was purified from the cell free extract (prepared as above) of *A. nidulans* having *lovB* over-expressed (1.1 L fermentation). The extract (150 mL) was subjected to dialysis using the Amicon 200 apparatus (100000 MW cut-off YM100 membrane) until the solution volume was concentrated by about 7 times (~20 mL). This was loaded onto a Sephadex G-200 column (700 mL) equilibrated at 4 °C in elution buffer consisting of: 20 mM Tris-HCl, 10% glycerol, 0.1 M NaCl, 1 mM sodium ascorbate, 1 mM EDTA, 1 mM DTT, and 0.1% Tween 80. Fractions of 8.0 mL were collected at 0.8 mL/min. The protein concentration was monitored at 280 nm. The fractions were pooled and the protein was precipitated by adding ammonium sulfate to 60% saturation and centrifuging twice at 10000 × *g* for 30 min. The collected proteins were resuspended in pellet buffer (20 mM Tris pH 8.0, 1 mM DTT, and 1 mM sodium ascorbate). Bio-Rad 5% Tris-HCl SDS-PAGE Ready Gels were run at 150 V for 80 min and stained with Coomassie Blue. The first protein fraction of the size exclusion column contained a protein of about 600 kDa. The second protein fraction contained the desired protein based on its predicted molecular weight of 335 kDa. Variable amounts of a minor protein (~ 260 kDa) observed in the latter is believed to arise from decomposition of the target protein during precipitation since it is not observed in the crude extract. Although the instability of the pigments limits the accuracy of analysis, preliminary kinetic

evaluation of LNKS was done in Tris-HCl buffer at pH 8.0 at an enzyme concentration of 0.07 nM and a specific activity of 10.9 μmol (of pyrone generated)min^{-1} mg^{-1} with the acetyl CoA concentration fixed at 0.60 mM. The direct linear plot method[3] was employed to give an apparent Km for malonyl CoA of 0.5 mM and V_{max} of $1x10^{-7}$ $M{\cdot}min^{-1}$.

Cell-free production of dihydromonacolin L. A cell free extract (40 mL) of *A. nidulans* having both the *lovB* and *lovC* genes over-expressed was prepared as above. To this was added the cofactor solution [0.5 mL of 2.5% (w/v) of each NADPH, FAD, SAM], acetyl CoA (5 mg) and malonyl CoA (20 mg). The solution was kept on an orbital shaker at 20 °C and 50 rpm overnight. The reaction mixture was quenched by acidification to pH 2 using 2N HCl and extracted using EtOAc (4 × 40 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated *in vacuo*. In order to ensure that all dihydromonacolin L was lactonised, toluene (25 mL) was added to the residue and the solution was heated under reflux through a Soxhlet containing calcium hydride for 45 min. The toluene was removed *in vacuo* and the product was purified by HPLC. The compound obtained (0.1 mg) had the same ^1H NMR and mass spectra, HPLC retention time and TLC behavior as the 4a,5-dihydromonacolin L standard isolated from whole cell fermentation.[1]

Characterisation of 4-hydroxy-6-[(1*E*,3*E*,5*E*)-1-methylhepta-1,3,5-trien-1-yl]-2-pyrone (3) and 4-hydroxy-6-[(1*E*,3*E*,5*E*,7*E*)-3-methylnona-1,3,5,7-tetraen-1-yl]-2-pyrone (4). Due to the instability of **3** and **4**, all procedures should be completed as rapidly as possible (≤ 24 h) and the product should be stored as a dry solid under an Ar atmosphere in the dark at ≤4 °C. Purification of **4** and **5** was carried out by HPLC using a

μBondapak radial compression RP C18, 40 × 100 mm, 10 μm, 125 Å column. The flow rate was kept at 35 mL/min, and the gradient was raised from 30% acetonitrile in water + 0.1% TFA, to 50% over 12 min, kept at 50% for 5 min, from 50% to 70% over 6 min, and from 70% to 100% over 2 min. The UV detector was set at 360 nm. Retention times were 13 min for **3** and 17 min for **4**. Spectral Data for **3**: mp (dec) 185-195 °C; UV (MeOH) λmax 356, 268, 218 nm; IR (CH_2Cl_2 cast) 3100 (br), 1683 (br s), 1662 (s), 1621 (s), 1549 (s) cm^{-1}; ^1H NMR (600MHz, CD_2Cl_2) δ 7.07 (d, 1H, J = 10.4 Hz, H-2'), 6.42-6.58 (m, 2H, H-3' and H-4'), 6.23 (tdd, 1H, J ~ 15, 9, 2 Hz, H-5'), 6.13 (d, 1H, J = 1.6 Hz, H-5), 5.88-5.95 (m, 1H, H-6'), 5.55 (d, 1H, J = 1.6 Hz, H-3), 1.95 (s, 3H, 1'-C$\underline{H}_3$), 1.82 (d, 3H, J = 5.7 Hz, H-7'); ^{13}C NMR for 70% ^{13}C-labeled sample (125 MHz, CD_3OD) δ 173.5 (dt, J_{CC} ~ 65, 29 Hz, C-4), 167.5 (d, J_{CC} ~ 81 Hz, C-2), 163.5 (m, C-6), 140.5 (br t, J_{CC} ~ 63 Hz, J_{CH} = 154 Hz, C-4'), 134.0 (br t, J_{CC} ~ 45 Hz, J_{CH} = 154 Hz, C-6'), 133.0 (J_{CH} = 150 Hz, C-2'), 132.0 (J_{CH} = 154 Hz, C-5'), 126.0 (br t, J_{CC} = 63 Hz, J_{CH} = 154 Hz, C-3'), 99.5 (m, J_{CH} = 170 Hz, C-5), 90.5 (m, J_{CH} ~ 145 Hz, C-3 and C-1'), 18.5 (d, J_{CC} = 43 Hz, J_{CH} ~ 126 Hz, C-7'), 12.0 (weak d, J_{CC} = 50 Hz, J_{CH} ~ 170 Hz, 1'-$\underline{C}H_3$); MS (ES) calcd for $C_{13}H_{15}O_3$ (MH^+) 219.10212, found 219.10232. Spectral Data for **4**: mp (dec) 165-170 °C, UV (MeOH) λmax 378, 288, 282, 232 nm; IR (CH_2Cl_2 cast) 3050 (br), 1675 (br s), 1651 (s), 1625 (s), 1541 (s) cm^{-1}; ^1H NMR (600 MHz, CD_2Cl_2) δ 7.15 (d, 1H, J = 15.5 Hz, H-2'), 6.47 (dd, 1H, J ~ 15, 2 Hz, H-5'), 6.38 (m, 1H, H-6'), 6.32 (m, 1H, H-4'), 6.21 (ddd, 1H, J ~15, 12, 2 Hz, H-7'), 6.09 (d, 1H, J = 15.5 Hz, H-1'), 6.01 (d, 1H, J = 1.8 Hz, H-5), 5.86 (m, 1H, H-8'), 5.52 (d, 1H, J = 1.8 Hz, H-3), 1.92 (s, 3H, 3'-C$\underline{H}_3$), 1.81 (d, 3H, J = 7.1 Hz, H-9'); ^{13}C NMR for 70% ^{13}C-labeled sample (125 MHz, CD_3OD) δ 173.0 (br t, J_{CC} ~ 65 Hz, C-4), 167.5 (d, J_{CC} = 80 Hz, C-2), 161.5 (m, C-6), 141.0 (m,

J_{CH} = 151 Hz, C-2'), 138.8 (m, C-6'), 138.3 (m, C-4'), 135.0 (m, C-3'), 133.5 (m, C-7'), 132.0 (m, C-8'), 127.5 (m, J_{CH} ~ 125 Hz, C-5'), 119.0 (m, C-1'), 102.0 (m, J_{CH} = 165 Hz, C-5), 90.0 (m, J_{CH} = 168 Hz, C-3), 18.5 (d, J_{CC} ~ 45 Hz, J_{CH} = 175 Hz, C-9'), 12.0 (weak d, J_{CC} ~ 45 Hz, J_{CH} ~ 170 Hz, 3'-C$\underline{H}_3$); MS (ES) calcd for $C_{15}H_{17}O_3$ (MH^+) 245.11777, found 245.1130.

Ethyl (1*R*,2*S*,4a*R*,6*R*,8a*R*)-1,2,4a,5,6,7,8,8a-Octahydro-2,6-dimethylnaphthalen-1-carboxylate (10). The cyclised NAC ester **7** (2 mg, 0.0065 mmol) was dissolved in dichloromethane (3 mL) and cooled to 0 °C under an argon atmosphere. Sodium metal (1 mg) was dissolved in ethanol (1 mL) and added to the dichloromethane solution. After 1 hour the reaction mixture was warmed to room temperature and left stirring for a further 5 hours. The reaction was quenched by adding water (5 mL). The reaction mixture was then extracted with dichloromethane (2 x 10 mL). The combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo*. Purification by preparative TLC (20% ethyl acetate in hexanes) gave the ethyl ester **10** (1.3 mg, 86%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.54 (ddd, 1H, *J* = 10.0, 4.2, 2.4 Hz, H-3), 5.42 (ddd, 1H, *J* = 10.0, 1.6, 1.5 Hz, H-4), 4.17 (q, 1H, *J* = 7.0 Hz, 1 x H-3'), 4.16 (q, 1H, *J* = 7.0 Hz, 1 x H-3'), 2.52 (dqdd, 1H, *J* = 8.7, 6.1, 4.2, 1.6 Hz, H-2), 2.37 (dd, 1H, *J* = 10.0, 8.7 Hz, H-1), 2.29 (m, 1H, H-4a), 2.05 (m, 1H, H-8a), 1.94-1.83 (m, 1H, H-6), 1.74-1.64 (m, 2H, H-7eq and H-8eq), 1.48-1.35 (m, 2H, H-5eq and H-5ax), 1.38-1.29 (m, 1H, H-8ax), 1.28 (t, 3H, *J* = 7.1 Hz, H-4'), 1.18 (ddd, 1H, *J* = 9.7, 6.0, 5.0 Hz, H-7ax), 0.99 (d, 3H, *J* = 6.1 Hz, 2-C$\underline{H}_3$), 0.97 (d, 3H, *J* = 6.0 Hz, 6-C$\underline{H}_3$). This compound showed identical chromatographic and spectroscopic behaviour as a sample prepared by independent chemical synthesis.[4]

Ethyl (1*R*,2*R*,4a*S*,6*R*,8a*R*)-1,2,4a,5,6,7,8,8a-octahydro-2,6-dimethylnaphthalene-1-carboxylate (11). To a solution of the NAC thioester **8** (5 mg, 0.016 mmol) in dichloromethane (0.52 mL) was added a solution of sodium ethoxide (1.65 mg, 0.024 mmol) in ethanol (0.28 mL) dropwise. The reaction mixture was heated at 60 °C for one hour and 50 °C overnight. The reaction was quenched by adding water (10 mL) at 0 °C, and extracted with chloroform (6 × 5 mL). The combined organic layers were dried (Na_2SO_4) and evaporated. Purification by silica gel column chromatography (3% diethyl ether in hexanes, R_f 0.25) gave the ethyl ester **11** (2.1 mg, 55%). ^{1}H NMR (360 MHz, CD_2Cl_2) δ 5.53 (ddd, 1H, J = 9.7, 4.2, 2.4 Hz, H-3), 5.38 (m, 1H, H-4), 4.13 (q, 2H, J = 7.1 Hz, H-1'), 2.55 (m, 1H, H-1), 2.53 (m, 1H, H-2), 1.95 (m, 1H, H-8eq), 1.77-1.68 (m, 3H, H-4a, H-7eq, H-5eq), 1.52-1.43 (m, 1H, H-6), 1.35 (m, 1H, H-8a), 1.27 (t, 3H, J = 7.2 Hz, H-2'), 1.04 (dddd, 1H, J = 12.0, 12.0, 12.0, 3.0 Hz, H-8ax), 1.00-0.95 (m, 1H, H-7ax), 0.93 (d, 3H, J = 6.5 Hz, 2-Me), 0.90 (d, 3H, J = 6.9 Hz, 6-Me), 0.80 (m, 1H, H-5ax); This compound showed identical chromatographic and spectroscopic behaviour as a sample prepared by independent chemical synthesis.[4]

Ethyl (1*S*,2*S*,4a*R*,6*R*,8a*S*)-1,2,4a,5,6,7,8,8a-Octahydro-2,6-dimethylnaphthalene-1-carboxylate (12). A solution of the triene NAC ester **5** (7 mg, 0.023 mmol) in methanol (150 μL) was converted with a solution of the LNKS enzyme (1 mL, 0.40 mg/ml, activity of 1.05 mU/ml in pellet buffer). The resulting solution was allowed to react on an orbital shaker at room temperature for three days. The reaction mixture was diluted with water (5 mL) and extracted with ethyl acetate (2 x 5 mL) followed by chloroform (2 x 5 mL).

The combined organic layers were then dried ($MgSO_4$) and evaporated *in vacuo*. The resulting residue (8 mg) was dissolved in dichloromethane (3 mL) and cooled to 0 °C under an argon atmosphere. Sodium metal (2 mg) was dissolved in ethanol (1 mL) and added to the dichloromethane solution. After 1 hour the reaction mixture was warmed to room temperature and left stirring for a further 5 hours. The reaction was quenched by adding water (5 mL). The reaction mixture was then extracted with dichloromethane (2 x 10 mL). The combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo*. Purification by preparative TLC (20% ethyl acetate in hexanes) gave a mixture of ethyl esters **10** and **11** (3.2 mg, 60% over two steps) as well as the title compound **12** (0.1 mg, 2% over two steps). Spectroscopic data for **12**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.56 (ddd, 1H, J = 9.9, 4.0, 3.0 Hz, H-3), 5.33 (br d, 1H, J = 9.9 Hz, H-4), 4.15 (q, 2H, J = 7.2 Hz, H-3'), 2.62-2.49 (m, 2H, H-1 and H-2), 2.10-2.00 (m, 1H, H-6), 1.93 (m, 1H, H-4a), 1.72 (dddd, 1H, J = 12.5, 3.9, 3.9, 3.7 Hz, H-8eq), 1.63 (dddd, 1H, J = 13.2, 12.9, 3.9, 3.9 Hz, H-7ax), 1.56-1.53 (m, 1H, H-7eq), 1.52-1.48 (m, 1H, H-5eq), 1.45-1.35 (m, 1H, H-8a), 1.33 (ddd, 1H, J = 13.0, 12.8, 4.8 Hz, H-5ax), 1.26 (t, 3H, J = 7.0 Hz, H-4'), 1.10 (dddd, 1H, J = 12.9, 12.9, 11.9, 3.7 Hz, H-8ax), 0.99 (d, 3H, J = 7.2 Hz, 6-C$\underline{H}_3$), 0.91, (d, 3H, J = 7.0 Hz, 2-C$\underline{H}_3$); ES MS *m/z* 275 (MK^+, 86%), 259 (MNa^+, 32%). This compound showed identical chromatographic and spectroscopic behaviour as a sample prepared by independent chemical synthesis.[4]

Incubation of triene NAC ester 5 with denatured LNKS. A solution of the LNKS enzyme (3 mL, in pellet buffer) was boiled at 100 °C for 1 hour. Analysis using the UV assay described above showed no production of pigments **3** and **4**. To this enzyme

solution (1 mL) was added the triene NAC ester **5** (4 mg, 0.013 mmol) in methanol (150 μL). The resulting solution was shaken on an orbital shaker at room temperature for three days. The reaction mixture was diluted with water (5 mL) and extracted with ethyl acetate (2 x 5 mL) followed by chloroform (2 x 5 mL). The combined organic layers were then dried ($MgSO_4$) and evaporated *in vacuo*. The resulting residue (5 mg) was dissolved in dichloromethane (3 mL) and cooled to 0 °C under an argon atmosphere. Sodium metal (2 mg) was dissolved in ethanol (1 mL) and added to the dichloromethane solution. After 1 hour the reaction mixture was warmed to room temperature and left stirring for a further 5 hours. The reaction was quenched by adding water (5 mL). The reaction mixture was then extracted with dichloromethane (2 x 10 mL). The combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo*. Purification by preparative TLC (20% ethyl acetate in hexanes) gave a mixture of ethyl esters **10** and **11** (1.9 mg, 62% over two steps). Spectral data for **10** and **11** as described above.

References

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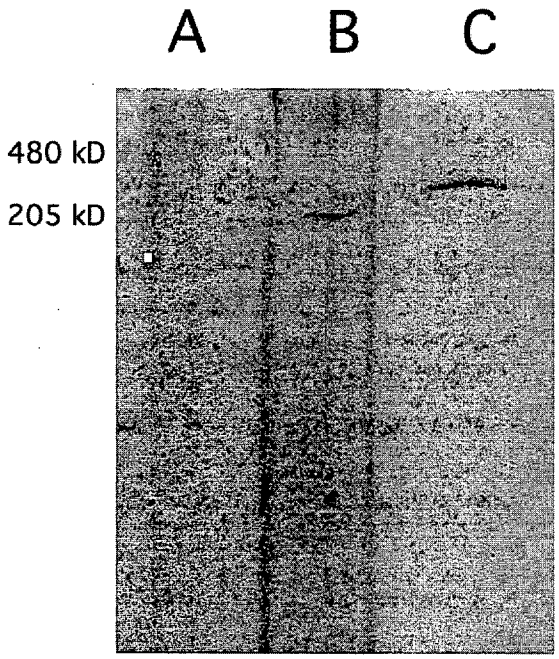
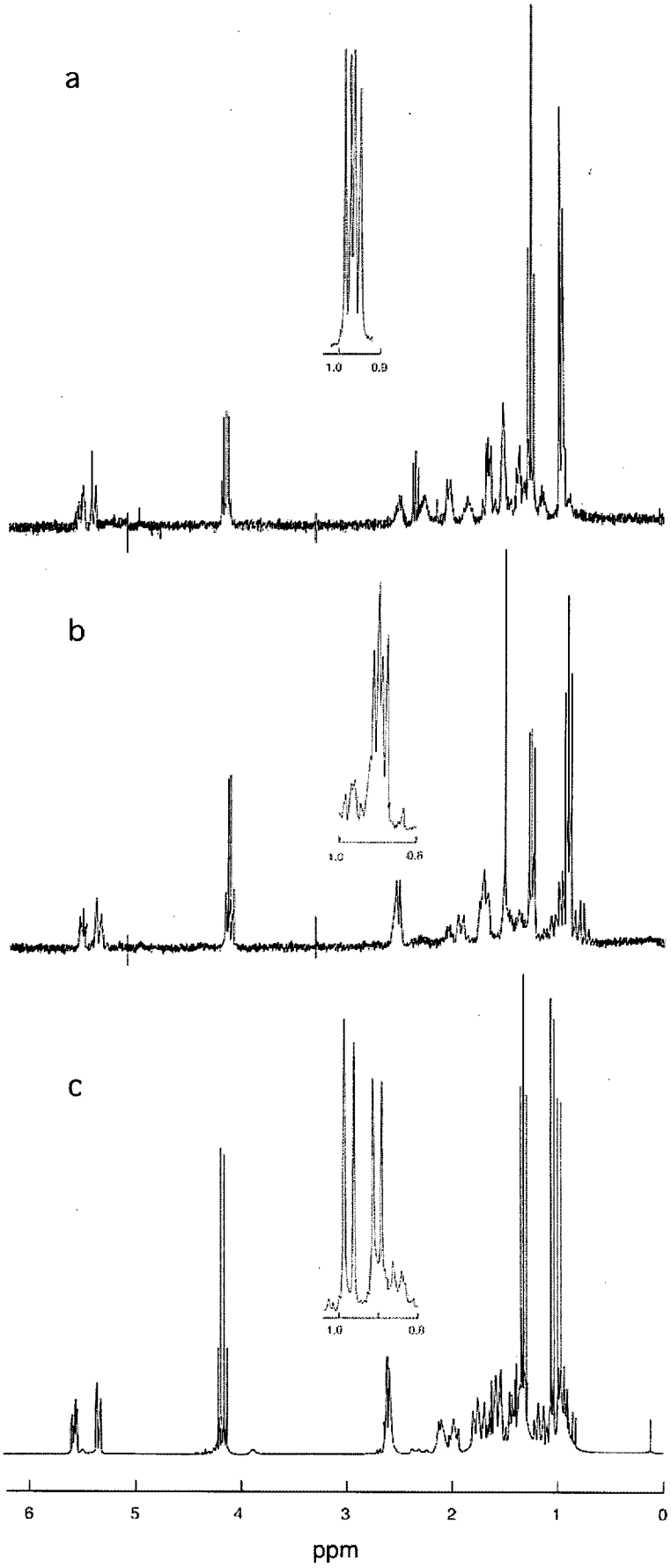
Supporting Information Figure Legends

Figure 1. SDS PAGE analysis of purified LNKS protein. Lane A: apo-ferritin (480 kD); lane B: myosin (205 kD); lane C: purified LNKS.

Figure 2. ^{1}H NMR spectra of the ethyl esters of: a. non-enzymatic *exo* product **10**, b. non-enzymatic *endo* product **11**, and c. enzymatic product **12**. Each spectrum also shows an expansion of the 2- and 6-CH_3 signals.


A
B
C
480 kD
205 kD


a
1.0
0.9
b
1.0
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c
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