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Exploring mutasynthesis to increase structural diversity in the synthesis of highly oxygenated polyketides lactones

Four new highly oxygenated 11-membered lactones (11-14) were synthesized by a combination of metabolic engineering techniques with complex chemical substrate synthesis.
Exploring mutasynthesis to increase structural diversity in the synthesis of highly oxygenated polyketides lactones


The enantioselective synthesis of (2R,3R,4E,8E)-3-hydroxy-2,4,8-trimethyldec-4,8-dienolide (5) by ring-closing metathesis is described. This compound is an analogue of 3,4-dihydroxy-2,4,6,8-tetramethyldec-8-enolide (4) which is a rare 11-membered lactone produced by the fungus, Botrytis cinerea. Mutasynthetic studies with compound 5 using two mutants of B. cinerea led to the isolation of four new highly oxygenated 11-membered lactones (11-14) in which compound 5 has been stereoselectively epoxidized and hydroxylated at sites that were not easily accessible by classical synthetic chemistry.

Introduction

Botrytis cinerea is a grey powdery phytopathogenic mould that affects a large number of commercial crops. Its major phytotoxic metabolites are a family of sesquiterpenes with the botryane carbon skeleton and two groups of polyketides exemplified on the one hand, by the botcinic and botcinoric acids (1) and their cyclic derivatives, the botcinins (2) and on the other hand, by botrylactone (3). A related lactone, 3,4-dihydroxy-2,4,6,8-tetramethyldec-8-enolide (4) has been isolated from a mutant strain of B. cinerea. This lactone possesses the same carbon chain as botrylactone with identical functional groups and stereochemistry in the C1-C4 fragment as both the botcinins and botrylactone. This structural homology and the presence of compound 4 in cultures of the strains of B. cinerea which also produce large amounts of the botcinins, suggests that they have a common biosynthetic origin. Compound 4 may be a ‘shunt’ metabolite in this pathway.

Figure 1. Some polyketides produced by B. cinerea.

The sequencing of the B. cinerea genome led to the development of genetically modified strains lacking the genes which code for the enzymes that are involved in the biosynthesis of the secondary metabolites produced by this fungus. Studies of the metabolites produced by these mutants permitted the identification of the genes involved in the production of the polyketides. The genes responsible for the formation of the per-methylated tetraketide core of both botcinins (2) and botrylactones (3) are all included in the same cluster comprising genes BeBOA1 to BeBOA6. The latter encodes the polyketide synthase (PKS). Naturally-occurring medium-sized lactones (8 to 11-membered lactones) constitute a relatively small number of metabolites which have nevertheless attracted considerable attention because of the range of biological activities which they possess. In particular there are only a few examples of 11-membered lactones that are found in nature. Apart from compound 4 and some complex pyrroolidine and daphniphyllum alkaloids, there are only reports of two insect pheromones, ferrulactone I and II and suspensolide, and four fungal metabolites including the aspercyclines A-C. The latter have anti-inflammatory activity and have the potential to be used in the treatment of allergic disorders such as asthma. The relative thermodynamic instability of 11-membered lactones has made their synthesis and that of their derivatives difficult even using Mitsunobu lactonization or an intramolecular Reformatsky reaction. Recently ring-closing metathesis (RCM) has been developed to provide an efficient method for the preparation of some 11-membered lactones from acyclic precursors. However, RCM reactions in the macrocyclic series tend to give mixtures of the (E) and (Z) isomers of cyclic olefins. A reliable general method for controlling the geometry of the new double bond has yet to be found. Consequently there are few examples of synthetic analogues whose biological activity has been thoroughly evaluated. Although botrylactone was described by Welmar et al. as a powerful antibiotic whilst the botcinins have been reported to show antifungal activity against Magnaporthe grisea, the causal agent of rice blast, nothing is known of the biological activity of compound 4 or its close relatives because of the small amount of material that has been isolated.

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Mutasynthesis is a very interesting strategy that combines chemical synthesis with biosynthetically-patterned biotransformations using genetically engineered microorganisms. The availability of mutants of B. cinerea blocked in the early stages of the pathway but retaining some of the later stages leading to these 11-membered lactones, has allowed us to explore the use of mutasynthesis to generate modified 11-membered lactones that are analogues of compound 4 in order to evaluate their biological activity.

Results and Discussion

We chose compound 5 as a simplified analogue of compound 4 although the stereochemistry of the alcohol at C-3 is different. However it could be easily synthesized in sufficient quantity for the mutasynthesis experiments. The retrosynthetic analysis of the lactone is shown in scheme 1. An RCM cyclization of the ester 6 and a syn aldol reaction of the aldehyde 7 play key roles in the stereoselective construction of the 11-membered lactone ring.

Scheme 1. Retrosynthetic analysis of lactone 5.

The synthetic sequence leading to the lactone 5 is shown in scheme 2. 2-Methyl-2-vinylxirane was treated with commercially available 2-methylallyl magnesium chloride in the presence of Cul to produce the allylic alcohol 8 in a yield of 95%. Oxidation of 8 with TEMPO-BAIB (2,2,6,6-tetramethylpiperidinyloxy-bis(acetoxy)iodobenzene) afforded the aldehyde 7. This was then treated with the boron Z-enolate of the N-propionyl oxazolidinone (-)-(R)-9 to give the syn-aldol product 10 in 70% yield and 88% d.e. Exocyclic cleavage of the oxazolidinone 10 with allyl alcohol and 4 eq. of allylmagnesium bromide at -20°C generated the ester 6 with a yield of 67%. Finally an RCM reaction of 6 under high dilution conditions catalysed by the second generation ruthenium complex A in dry, degassed, refluxing dichloromethane produced the 11-membered lactone 5 with a good regioselectivity and yield (9:1 E/Z ratio, 68% yield). This is in agreement with molecular mechanics calculations (MM2) which predict that the (E)-alkene is the most stable regioisomer.

Scheme 2. Stereoselective synthesis of 5.

The lactone 5 was then incubated with two mutant strains of B. cinerea, bcbot2Δ and bcboαΔbcbot2Δ (bcΔAdI). The first mutant which was obtained by deactivating the botryane biosynthesis gene BcBOT2 that encodes a sesquiterpene cyclase, does not produce the botryanes but does produce a significant amount of botcinic acid (1a) and its relatives. The second mutant, bcΔAdI, is a double mutant which was obtained by deactivating the genes BcBOT2 and BcBOA6 and is consequently unable to produce the three characteristic families of metabolite: the botryanes, the botcinins and botrylactone. Incubation of compound 5 with both mutant strains using liquid surface-culture conditions gave four new 11-membered lactones 11-14 (figure 2). Their distribution and % yields are shown in table 1. Strain bcbot2Δ gave a higher conversion of compound 5. The metabolites 11, 12 and 13 were produced in both cases but there was no apparent incorporation of compound 5 into the botcinin/botrylactone pathway. The structures of the metabolites were established from their 1H and 13C NMR spectra using a combination of 1D and 2D NMR experiments (see ESI).

Figure 2. Metabolites isolated in mutasynthesis.
Compound 11 was obtained as a colourless oil whose HRMS possessed a molecular ion at m/z 240.1353 corresponding to the molecular formula, C₁₉H₂₂O₆. The ¹³C NMR spectrum contained resonances at δC 81.6, 62.9, 59.8 and 58.6 ppm (CH, CH₂, C, and CH carbons respectively) and at δC 174.4 ppm (C carbon) consistent with the presence of four C=O and one lactone C=O carbon atoms in the molecule. The main difference between the ¹H NMR spectra of compounds 5 and 11 was the absence of the olefinic proton H-9 and the appearance of a double-doublet (J 10.0 and 4.2 Hz) at δH 3.01 ppm in compound 11. The presence of an epoxide at this position was confirmed by an H2QC correlation of H-9 with the signal at δC 58.6 ppm (C-9) and HMBC correlations between H-9 and signals at δC 59.8 (C-8) and δC 62.9 ppm (C-10). The stereochemistry of the oxirane ring was established by nOe experiments which were rationalized on the basis of the lowest energy optimized conformer derived from MM2 calculations (figure 3). In particular there were interactions between H-9 and δH 2.16-2.08 (H-6 β), δH 1.08 (H-7 β) and δH 1.75 (4-Me) and δH 1.31 (8-Me). There were also nOe effects involving the signals at δH 3.99 (H-3 β) and δH 1.28 (2-Me β). These were consistent with the epoxidation of compound 5 on the β-face of the ring. Based on the known absolute configuration of compound 5 and the MM2 calculations, compound 11 has the absolute configuration (2R,3R,4E,8S,9S).

The HRMS (m/z 239.1282, M-H⁻) of compound 12 was consistent with a molecular formula, C₁₉H₂₂O₆. The ¹³C NMR spectrum contained 13 signals. Comparison with compound 5 showed that the resonance for δC 38.2 (t) had changed to δC 77.2 (d) in compound 12. There were COSY correlations between δH 4.18 (H-7) and the signals at δH 2.46 and 2.30 which were assigned to H-6 α and H-6 β respectively. There were nOe effects which were observed (figure 3) between the signals at δH 4.18 (H-7), 5.62 (H-9), 5.02 (H-5) and H-6 β, together with δH 4.70 (H-10 β) and H-9. These were consistent with the R configuration at C-7 and hence compound 12 was (2R,3R,4E,7R,8E)-3,7-dihydroxy-2,4,8-trimethyldeca-4,8-dienolide.

The HRMS data for compounds 13 and 14 showed that they both had a molecular formula, C₁₉H₂₂O₆. Their ¹³C NMR spectra contained five C=O signals at δC 81.4 (CH, C-3), 76.9 (CH, C-7), 62.6 (C, C-8) 62.2 (CH₂, C-10) and 56.6 (CH, C-9) ppm for compound 13 and δC 80.9 (C-3), 65.9 (CH, C-6), 62.8 (CH, C-10) 58.6 (CH, C-9) and 58.0 (C, C-8) ppm for 14. These compounds possessed similar ¹H NMR spectra in which the olefinic C-H signals of compound 5 had been replaced by epoxy C-H signals at δH 3.09 (dd, J 10.0, 4.0 Hz) and δH 3.05 (dd, J 10.2, 4.2 Hz) respectively. However compound 13 possessed a new CH(OH) signal at δH 3.22 (dd, J 11.3, 5.2 Hz) (H-7) which showed COSY correlations with signals at δH 2.50 (H-6 α) and δH 2.32 (H-6 β). Compound 14 possessed a new CH(OH) signal at δH 4.64 (triplet J 11.2 of doublets J 4.8 Hz) (H-6) which showed COSY correlations with signals at δH 2.48 (H-7 β), δH 1.12 (H-7 α) and 5.20 (H-5). The nOe effects, shown in figure 3, were in accord with the optimized structures obtained by MM2 calculations and, bearing in mind the origin of the metabolites, led to the absolute stereochemistry for compounds 13 and 14 as 2R,3R,4E,7R,8S,9S and 2R,3R,4E,6R,8S,9S respectively.

Figure 3. MM2 minimized models with decisive nOe correlations for compounds 11-14.

These mutasynthetic transformations have produced derivatives that were hydroxylated at C-6 and C-7 and regioselectively epoxidized at C-8/C9. However there was no hydration of epoxidation of the C4/C5 double bond. Consequently additional synthetic work is necessary in order to develop an efficient route for the synthesis of closer analogues of compound 4. Compounds 11 and 13 did not show any significant antibacterial activity at a concentration of 200 ppm against Escherichia coli, Bacillus subtilis or Streptococcus faecalis. Compounds 11 and 13 did not show any significant phytotoxic activity when tested in vivo on sterilized leaves of Phaseolus vulgaris at a concentration of 500 ppm.

Conclusions

The regioselective ring-closing metathesis of the triene 6 has provided an efficient method for the asymmetric synthesis of (2R,3R,4E,8S)-3-hydroxy-2,4,8-trimethyldeca-4,8-dienolide (E)-5 in 5 steps with 26% overall yield from simple starting materials. This compound is an advanced analogue of 3,4-dihydroxy-2,4,8-tetramethyldec-8-enolide (4) which is a scarce metabolite of B. cinerea. Compound 5 was biotransformed by two mutant strains of B. cinerea, bcbot2A and bcbAdl into four new highly oxygenated 11-membered lactones. These mutasynthetic steps involved regioselective epoxidation of the C8/C9 double bond and hydroxylation at either the C-6 (minor) or C-7 (major) positions. This has proved to be a valuable and versatile approach to increasing the structural diversity of 11-membered lactones. Further
experiments are in progress to develop a synthesis of compound 4.

Experimental

General procedures

Unless otherwise noted, materials and reagents were obtained from commercial suppliers and were used without further purification. Dichloromethane was freshly distilled from CaH2 and tetrahydrofuran was dried over sodium and benzophenone and freshly distilled before use. Air- and moisture-sensitive reactions were performed under argon atmosphere. Purification by semi-preparative and analytical HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with a differential refractometer detector (RI-7490). A LiChrospher® Si 60 (5µm) LiChroCart® (250 mm × 4 mm) column and a LiChrospher® Si 60 (10µm) LiChroCart® (250 mm × 10 mm) were used in isolation experiments. Silica gel (Merck) was used for column chromatography. TLC was performed on a Merck Kieselgel 60 F434, 0.25 mm thick Melting points were measured with a Reichert-Jung Kofler block and are uncorrected. Optical rotations were determined with a digital polarimeter. Infrared spectra were recorded on a FT-IR spectrophotometer and reported as wave number (cm⁻¹).

Microorganisms

B. cinerea mutants, bcbot2.1 and bcaddl1, were previously obtained by deactivating the genes encoding the sesquiterpene synthase BcBOT2.27 and the synthase BcBOT2 and the polyketide synthase BcBOA5, respectively, and are maintained in the BIOGER strain collection INRA (Grignon, France). Conidial stock suspensions of these strains were maintained in glycerol (80%) at -40ºC.

Synthesis of the substrates

(4R,2R,3'S,R,4'E)-4-benzyl-3-[3-hydroxy-2,4,8-trimethylona-4,8-dienoyl]-oxazolidin-2-one (10). n-Dibutylboron triflate (5.2 mL of a 1.0 M solution in CH2Cl2, 5.2 mmol) was added dropwise at 0°C to a stirred solution of (-)-(4R)-4-benzyl-3-propionyloxazolidin-2-one ((-)-(R)-9) (1020 m, 4.36 mmol) in dry CH2Cl2 (6.5 mL) under argon atmosphere conditions. The mixture was stirred for 5 min and then N,N'-diisopropylthylamine was added (0.96 mL, 5.7 mmol). After complete addition, the mixture was stirred at 0°C for 15 min. The yellow solution was re-cooled to -78°C and a saturated ammonium chloride (80 mL) was added (2756 mg, 94 %) as a yellow oil. IR (film) νemax/cm⁻¹: 2925, 2852, 2769 (CHO), 1702 (CO), 1420, 1102, 1090; 1H NMR (400 MHz, CDCl3) δ 1.71 (6H, s, -Me); 2.49 (2H, dq, J 7.2, 0.6, 4-H); 4.70 (1H, br s, 7a-H); 4.75 (1H, br, 7b-H); 4.67 (1H, tq, J 7.2, 1.0, 3-H); 9.38 (1H, s, 1-H); 13C NMR (100 MHz, CDCl3) δc 9.1 (q, 2-Me), 22.2 (q, 6-Me), 26.8 (t, C4), 36.0 (t, C5); 110.8 (t, C7); 139.3 (s, C2); 143.9 (s, C6); 153.9 (d, C3); 195.0 (s, C1); HRMS (CI⁺) calc'd for C12H10O[M⁺]: 138.1044, found 138.1028.

(E)-2,6-dimethylhepta-2,6-dien-1-ol (8). 2-Methylylamine chloride (58.4 mL of a 0.5 M solution in THF, 29.2 mmol) at -30°C was added to a stirred solution of 2-methyl-2-vinylxirane (2 mL, 20.9 mmol) and Cul (199 mg, 1.04 mmol) in dry THF (22 mL) at -30°C. The mixture was stirred at -30°C and after 3h it was treated with a saturated ammonium chloride solution (60 mL) and then allowed to warm to room temperature. The aqueous layer was extracted three times with diethyl ether (90 mL). Combined extracts were washed with HCl 1N (200 mL), saturated sodium bicarbonate (200 mL), water (200 mL), brine (200 mL) and then dried over anhydrous sodium sulphate. Finally, the solvent was concentrated under reduced pressure and the crude was purified by silica gel column chromatography eluted with pentane : Et2O (80:20) to yield compound 8 (2756 mg, 94 %) as a colourless oil. Spectroscopic data of compound 8 were identical to those described in the literature.20

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(2R,3R,4E)- Allyl 3-hydroxy-2,4,8-trimethylnona-4,8-dienoate (6). Allylmagnesium bromide (4.9 mL of a 1.0 M solution in EtO, 4.9 mmol) was added at 0°C to alcohol (15 mL) under argon conditions in a Schlenk flask. The allylic mixture was stirred for 10 min and re-cooled at −20°C. Then, a solution of 10 (886 mg, 2.40 mmol) in allylic alcohol (3 mL) was slowly added. When TLC monitoring indicated the completion of the reaction (3h), a saturated ammonium chloride solution (20 mL) was added and then allowed to warm to room temperature. The aqueous layer was extracted three times with diethyl ether (50 mL). Combined extracts were washed with brine (100 mL), dried over anhydrous sodium sulphate, filtered and the solvent was concentrated under reduced pressure. The crude product was purified by silicon gel column chromatography eluted with petroleum ether:EtOAc (90:10) to give a crude that was purified by semipreparative chromatography, followed by analytical HPLC purification, to yield the ester (90:10). Final purification was carried out by semi-preparative chromatography eluted with petroleum ether:EtOAc (400 mL). The solvent was removed under reduced pressure to give a solid. The crude was filtered over a pad of silica gel and washed with ethyl acetate (77:23), flow = 0.8 mL/min; [M]+ 224.1412, found 224.1397.

Mutatisynthesis experiments

General methods. B. cinerea was grown on a surface culture in Roux bottles on a Czapek-Dox medium (150 mL per flask) comprising (per L of distilled water) glucose (50.0 g), yeast extract (1.0 g), KH2PO4 (5.0 g), NaH2PO4 (2.0 g), MgSO4·7H2O (0.5 g), and FeSO4·7H2O. The pH of the medium was adjusted to pH 7.0 with aqueous NaOH (4 M). Each Roux bottle was inoculated with 2x10^7 fresh conidia or six uniform discs of 0.9 cm diameter mycelial of four-day old culture on malt agar. A filter-sterilised aqueous solution of the labeled precursor or a solution of E-5 in ethanol was fed at a carefully determined optimum time. Roux bottles were incubated at 25±2°C in daylight under static conditions for the optimum period of time. The culture medium and mycelia were then separated by filtration. The broth was separated with NaCl and extracted with ethyl acetate (3x) and dried over anhydrous Na2SO4. The organic extract obtained was evaporated at reduced pressure to dryness.

Feeding of (2R,3R,4E,8E)-3-hydroxy-2,4,8-trimethyldeca-4,8-dienoic acid (E-5) to B. cinerea bcbot2A and bcAdd1. Compound (E)-5 (120 mg), dissolved in EtOH (960 µL), was distributed among 6 Roux bottles containing a 4-day old culture of B. cinerea bcbot2A or bcAdd1 and grown for a further 6 days. Filtration, ethyl acetate extraction and column chromatography, followed by analytical HPLC purification, gave 11, 12, 13 and 14 in the yields shown in table 1.

(2R,3R,4E,7R,8E)-3,7-dihydroxy-2,4,8-trimethyldeca-4,8-dienoic acid (12): Colourless oil; [α]_D^20 +219º (c 0.34 in CHCl3); IR (film) ν_{max} (cm\(^{-1}\)) 3395 (OH), 2923, 1707 (CO), 2759 (C=O); 1^1C NMR (100 MHz, CDCl₃) δ 12.5 (q, 2-Me), 13.1 (q, 6-Me), 19.7 (q, 8-Me), 25.3 (t, 2-Me), 34.0 (s, 8-Me), 38.4 (t, 6-Me), 43.2 (d, C5), 58.5 (d, C9), 59.8 (s, C8), 62.9 (t, C10), 81.6 (t, C11), 126.9 (c, C12), 136.2 (s, C2), 174.4 (s, C4), 174.4 (s, C1); HRMS (CI⁺) dcalcd for C₂₅H₂₄O₄ [M]+ 420.1362, found 240.1353.

(2R,3R,4E,7R,8E,9E)-8,9-epoxy-3-hydroxy-2,4,8-trimethyldeca-4,8-dienoic acid (11): Colourless oil; [α]_D^20 +27 min, petroleum ether: ethyl acetate (77:23), flow = 0.8 mL/min; [α]_D^20 +184º (c 0.34 in CHCl₃); IR (film) ν_{max} (cm\(^{-1}\)) 3448 (OH), 1734 (CO), 1458, 1165, 1022, 887, 764; 1^1C NMR (400 MHz, CDCl₃) δ 7.1 (3H, d, J 13.4, 4.8, 7b-H), 1.28 (3H, d, J 6.6, 2-Me), 3.31 (3H, s, 8-Me), 1.65 (3H, t, J 1.6, 4-Me), 2.08-2.16 (2H, m, 6b-H, 7a-H), 3.70-3.72 (1H, d, J 10.0, 6b-H), 3.72 (1H, d, J 10.0, 6a-H); 1^1H NMR (400 MHz, CDCl₃) δ 1.10 (3H, d, J 6.9, 2-Me), 1.29 (3H, d, J 6.9, 2-Me), 1.39 (3H, s, 8-Me), 1.64 (3H, t, J 1.6, 4-Me), 2.05-2.14 (2H, m, 6b-H, 7a-H), 2.37-2.39 (1H, m, 6a-H), 2.72 (1H, dq, J 10.0, 6.6, 2-H), 3.01 (1H, dd, J 10.0, 4.2, 9-H), 3.53 (1H, dd, J 10.8, 10.0, 10b-H), 3.99 (1H, dd, J 10.0, 1.8, 3-H), 4.86 (1H, dd, J 10.8, 4.2, 10a-H), 5.17 (1H, dd, J 12.0, 3.2, 1.6, 5-H); 1^3C NMR (100 MHz, CDCl₃) δc 10.3 (q, 4-Me), 13.6 (q, 2-Me), 15.9 (q, 8-Me), 24.3 (t, C6), 37.0 (t, C7), 43.6 (d, C2), 58.6 (d, C9), 59.8 (s, C8), 62.9 (t, C10), 81.6 (d, C11), 126.9 (d, C12), 136.2 (s, C2), 174.4 (s, C4); HRMS (CI⁺) dcalcd for C₂₃H₂₂O₄ [M]+ 400.1346, found 240.1353.

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[\alpha]_D^{20} +94.1^\circ$ (c 0.47 in CHCl₃); IR (film) νmax/cm⁻¹ 3431 (OH), 2922, 2855 (CH), 1714 (CO), 1438, 1275, 1254, 1166, 1019, 848, 741; ¹H NMR (400 MHz, CDCl₃) δ(H, d) 1.28 (3H, d, J 6.8, 2-Me), 1.34 (3H, s, 8-Me), 1.77 (3H, d, J 1.6, 4-Me), 2.28-2.35 (1H, m, 6b-H), 2.50 (1H, dt, J 13.6, 11.8, 6a-H), 2.69 (1H, dq, J 9.8, 6.2-H), 3.09 (1H, dd, J 10.0, 4.0, 9-H), 3.22 (1H, dd, J 11.8, 5.2, 7-H), 3.56 (1H, dd, J 10.8, 10.0, 10b-H), 3.97 (1H, d, J 9.8, 3.8-H), 4.89 (1H, dd, J 10.8, 4.0, 10a-H), 5.12 (1H, dd, J 11.8, 3.2, 5-H); ¹³C NMR (100 MHz, CDCl₃) δ(C) 10.2 (q, 8-Me), 10.5 (q, 4-Me), 13.6 (q, 2-Me), 32.5 (t, C6), 43.4 (d, C2), 56.2 (d, C9), 62.2 (t, C10), 62.6 (s, C8), 76.9 (d, C7), 81.4 (d, C3), 122.5 (d, C5), 137.6 (s, C4), 174.4 (s, C1); HRMS (CI⁺) calculated for C₁₁H₁₅O₃ [M+H⁺] 256.1311, found 256.1310.

**Notes and references**


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