Genomic scanning enabling discovery of a new antibacterial bicyclic carbamate-containing alkaloid

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ABSTRACT

Non-ribosomal peptides are a group of structurally diverse natural products with various important therapeutic and agrochemical applications. Bacterial pyrrolizidine alkaloids (PAs), containing a scaffold of two fused five-membered ring system with a nitrogen atom at the bridgehead, have been found to originate from a multidomain non-ribosomal peptide synthetase to generate indolizidine intermediates, followed by multistep oxidation, catalysed by single Bayer-Villiger (BV) enzymes, to yield PA scaffolds. Although bacterial PAs are rare in natural product inventory, bioinformatics analysis suggested that the biosynthetic gene clusters (BGCs) that are likely to be responsible for the production of PA-like metabolites are widely distributed in bacterial genomes. However, most of the strains containing PA-like BGCs are not deposited in the public domain, therefore preventing further assessment of the chemical spaces of this group of bioactive metabolites. Here, we report a genomic scanning strategy to assess the potential of PA metabolites production in our culture collection without prior knowledge of genome information. Among the strains tested, we found fifteen contain the key BV enzymes that are likely to be involved in the last step of PA ring formation. Subsequently one-strain-many-compound (OSMAC) method, supported by a combination of HR-MS, NMR, SMART 2.0 technology, and GNPS analysis, allowed identification and characterization of a new (5 + 7) heterobicyclic carbamate, legoncarbamate, together with five known PAs, bohemamine derivatives, from Streptomyces sp. CT37, a Ghanaian soil isolate. The absolute stereochemistry of legoncarbamate was determined by comparison of measured and calculated ECD spectra. Legoncarbamate displays antibacterial activity against E. coli ATCC 25922 with an MIC value of 3.1 μg/mL. Finally, a biosynthetic model of legoncarbamate and other bohemamines was proposed based on the knowledge we have gained so far.

1. Introduction

Pyrrolizidines are a group of organic compounds that possess a fused bicyclic five-membered rings with a nitrogen atom at the bridgehead position. While pyrrolizidine alkaloids (PAs) have been mainly found as plant metabolites, some of which are part of plant defence mechanism against insect herbivores, less than 30 PAs have been discovered from bacterial origins [1]. The representatives include clazamycins 1 [2], bohemamine 2 [3], jenamides A1/A2 4 [4], and more recently legonmycins 3 [5], pyrrolizixenamide 5 [6], bohemamine dimer C 6 [7], and azetidomonamide (azabicyclene) 7 [8,9] (Fig. 1A). Bacterial PAs exhibit a broad range of bioactivities and as such they have attracted considerable interest from both academic research groups and the pharmaceutical industry [11].

Recently we disclosed the biosynthetic origin of two bacterial PA specialised metabolites (SMs), denoted as legonmycins A and B [4], isolated from a talented Ghanaian isolate, Streptomyces sp. MA37 [4, 10–16]. We have demonstrated that legonmycins are biosynthesized from an NRPS assembly line to generate the key intermediates, lego

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monooxygenase LgnC acts on 8 and catalyses a multistep Baeyer-Villiger process of ring expansion to afford carbamates, 9 (\(5 + 7\) heterobicyclic ring system). Subsequent ring-opening by hydrolysis and decarboxylation-driven ring contraction finally provides the \([5 + 5]\) heterobicyclic pyrrolizidine ring (Fig. 1B) [4].

The biosynthetic gene clusters (BGCs) that are likely to be responsible for PA productions have been shown to be widely spread in bacterial genomes, suggesting that PA SMs may have important biological functions [6]. One of the key features among these BGCs is that they all encode LgnC-like monooxygenase open reading frames (ORFs) that are likely to be responsible for the biotransformation from \([5 + 6]\) indolizidine intermediates to \([5 + 5]\) PA scaffolds. Bioinformatics analysis of these putative BGCs indicated that the biosynthetic genes encoding tailoring catalytic functions appear to be diverse [6]. In contrast to the biodiversity of BGCs, the chemical space of this group of bacterial PA SMs has not been fully assessed. Many of these bacterial strains are not deposited in the public domain. Thus, it is difficult to assess whether these strains are able to produce PA-like metabolites with chemical diversity under laboratory conditions. Considering that LgnC-type monooxygenases are the key enzymes to biosynthesize PA metabolites, we reasoned that it was possible to apply the PCR-based genomic scanning strategy of using the sequences that are conserved among LgnC-type monooxygenases as probes to identify strains in either public or our own culture collections that may contain PA BGCs, followed by facilitating one strain many compounds (OSMAC) method [17] to isolate new PA metabolites for biological assessments.

Here we report the application of PCR-based genomic scanning of 58 actinomycete strains from NCIMB, the largest bacterial culture collection in the UK and our own culture collection. This resulted in the identification of 15 potential PA producing strains. Among these strains, one of our own cultures, the Ghanaian isolate Streptomyces sp. CT37 (CT37) was found to produce PA-like metabolites. Subsequent chemical workup and structural elucidation allowed the identification of an unusual \([5 + 7]\) heterobicyclic carbamate alkaloid, legoncarbamate 10, and a known PA metabolite, NP25302 11. NOE analysis together with electronic circular dichroism (ECD) measurement and computational calculation confirmed that 10 has (5S, 8S) configuration. Further analysis of the semi-purified extracts from the culture of CT37 using LC-MS-based Global Natural Products Social molecular networking (GNPS) [18], in conjunction with newly developed NMR-based machine learning tool “Small Molecule Accurate Recognition Technology” (SMART 2.0) [19] allowed identification of the presence of four known bohemamine SMs 15–18. In silico analysis of the annotated genome of CT37 together with current knowledge of PA biosynthesis allowed identification of the BGC of the carbamate alkaloid 10 and other PA-related SMs 15–18.

2. Results and discussion

With recently discovered PA metabolites and their corresponding BGCs, we compared the biosynthetic enzymes encoded in these BGCs with key legonmycin biosynthetic enzymes. It appears that this group of
SMs are assembled by multidomain non-ribosomal peptide synthetases (NRPSs) to generate indolizidine intermediates, followed by essential LgnC-like monoxygenases to provide PA bicyclic frameworks. LgnC-like monoxygenases catalyse unusual multistep chemical transformation from indolizidine via carbamate to pyrrolizidine [4] which is clearly different from other Bayer-Villiger (BV) enzymes [20]. Therefore, it is possible to apply degenerate primers, according to the conserved amino acid sequences of these monoxygenases, to scan genomic DNAs of a large amount of available bacterial collections in order to identify potential PA producing strains. To this end, we adopted a bioinformatics-based approach to break the trend in these BV monoxygenases (Fig. S1A). Having compiled data from reported putative BV monoxygenase enzymes together with other types of BV monoxygenases, protein motif elicitor (MEME) [21] analysis enabled us to identify three highly conserved motifs: GxGxxG, YWxxTKN, and GDAAH (where ‘x’ is any amino acid and each independently represents the number of x between each conserved residue) (Fig. S1A). GxGxxG motif has hitherto been used to indicate the presence of FAD which could as readily indicate flavin monoxygenase-related enzymes [22]. However, YWxxTKN, and GDAAH motifs, covering approximate 80 amino acid residues, are unique among LgnC-like monoxygenases and as such these motifs may be used as PCR probe to screen LgnC-like monoxygenase from uncurated genomic DNAs of available bacterial strains (Table S1).

To examine the feasibility of PCR-based genomic scanning approach, we first amplified the internal fragment of LgnC with our designed degenerate primers using the genomic DNA of Strep. albus (Streptomyces albus) as a negative control. The expected PCR product with approximately 240 bp, were observed in DNA electrophoresis analysis (Fig. S1B) in the positive control but not in the negative control. The DNA sequence of the PCR product was further confirmed to be the targeted internal DNA fragment of LgnC by DNA sequencing.

In the collaborative natural product discovery programme with NCIMB Ltd, we obtained 56 Strep. strains (Table S2). We extract the genomic DNAs from these strains together with soil isolates in our laboratory including the new indole alkaloid SM producer, Strep. sp. CT37 (CT37) [23], and performed PCR scanning using these DNAs as templates. Among these strains, we were able to identify fifteen positive PCR results with the expected DNA length. These DNAs were then cloned into a commercially available plasmid via TA cloning method (Method and experiments). The constructs were extracted from the positive clones which was subjected for DNA sequencing. Finally, the resulting DNA sequences were annotated using FramePlot to predict protein-coding regions. The amino acid alignment with the one in LgnC as a control and phylogenetic analysis indicated that these amplified DNAs are indeed encoded the fragments (~80 amino acid (AA) residues) of LgnC-like monoxygenases with high AA similarity (80%-95%), suggesting that these strains potentially possess these BV monoxygenases (Fig. S2).

In order to examine whether these strains can produce PA-like metabolites under laboratory conditions, we applied the OSMAC approach to activate the cryptic PA-like BGCs among these strains. Eight standard Strep. media (ISP2, Modified Bennett’s, ISP3, ISP4, ISP5, ISP6, ISP7, Starch Casein) available in our laboratory were used (Table S3). After small scale fermentation (50 mL, 7 days, 28 °C), one hundred twenty of these culture broths were extracted using ethyl acetate to generate crude metabolites (Fig. S3). The UV spectrum showed similar absorption to legonmycins at 250, 282 and 320 nm (Fig. 4). A comparison of its NMR spectrum to that of known bohemamine analogues indicated that the 1H, 13C and 2D NMR data of compound 11 were identical to NP25302 [3] (Figs. S12–15, Table S5).

Compound 11 was isolated as a yellow powder. Its molecular formula was determined to be C14H18N2O4 based on the HRESIMS data. The UV spectrum showed similar absorption to legonmycins at 250, 282 and 320 nm (Fig. 4). A comparison of its NMR spectrum to that of known bohemamine analogues indicated that the 1H, 13C and 2D NMR data of compound 11 were identical to NP25302 [3] (Figs. S12–15, Table S5).

Taken together, compound 10 is a new carbamate-containing (5 + 7) heterocyclic alkaloid metabolite, which we named legoncarbamate, after its association with Legon, Ghana, the location of the University of Ghana. Compound 10 showed antibacterial activity against E. coli ATCC 25922 with a minimum inhibitory concentration (MIC) value of 3.1 μg/mL (Fig. S21).
Although various LC-MS-based dereplication tools, such as Global Natural Product Social molecular networking (GNPS) [18], have facilitated the targeted isolation of new SMs as well as rapidly dereplicating known ones, unambiguous identification of SMs still requires isolation and structure characterization. Recently, a new NMR-based machine learning tool, Small Molecule Accurate Recognition Technology (SMART 2.0) [26], has been developed for mixture analysis and subsequent discovery and characterization of SMs from environmental isolates. In our case, the minute amount of two semi-purified fractions from the culture of CT37 prevented further purification to obtain the pure substances for structural elucidation. Therefore, we applied this newly developed cheminformatic tool to assign the structures of the SMs present in these two fractions. Subsequently, the HSQC spectra of these two fractions were submitted to SMART NMR. SMART annotation combined with GNPS analysis and NMR interpretation suggested that the majority of the compounds in one fraction are two known bohemamine derivatives, bohemamine B [24] and 5-chlorobohemamine C [24] while bohemamine [30] and bohemamine F [25] in the other fraction (Figs. S16–S20, Tables S6–S7).

The [5 + 7] heterobicyclic SMs containing a carbamate moiety are rare in the natural product inventory. Cyclocarbamide [28] and SB315021 are the only two examples containing such a scaffold.

Fig. 2. Structures of legoncarbamate 10 and NP253021 together with two known metabolites that contain [5 + 7] carbamate ring systems, cyclocarbamide 12, SB315021 13 and the metabolite brabantamide A 14. Compound 10 with key correlation spectroscopy (COSY) (→), key heteronuclear multiple bond correlation (HMBC) (→) and NOE (→) correlations. The structures of compounds 15–18 [3,24,25] in the bracket were deduced from the analyses of NMR-based artificial intelligence SMART 2.0 technology [26] in conjunction with LC-MS-based GNPS [18] and genomic context.

Fig. 3. A. Measured and calculated ECD spectra for 10. B. Two possible structures of 10 that contain cis-configuration between the methyl group at C8 and the hydrogen at C5.

Although various LC-MS-based dereplication tools, such as Global Natural Product Social molecular networking (GNPS) [18], have facilitated the targeted isolation of new SMs as well as rapidly dereplicating known ones, unambiguous identification of SMs still requires isolation and structure characterization. Recently, a new NMR-based machine learning tool, Small Molecule Accurate Recognition Technology (SMART 2.0) [26], has been developed for mixture analysis and subsequent discovery and characterization of SMs from environmental isolates. In our case, the minute amount of two semi-purified fractions from the culture of CT37 prevented further purification to obtain the pure substances for structural elucidation. Therefore, we applied this newly developed cheminformatic tool to assign the structures of the SMs present in these two fractions. Subsequently, the HSQC spectra of these two fractions were submitted to SMART NMR. SMART annotation combined with GNPS analysis and NMR interpretation suggested that the majority of the compounds in one fraction are two known bohemamine derivatives, bohemamine B 15 [24] and 5-chlorobohemamine C 16 [24] while bohemamine [30] and bohemamine F 18 [25] in the other fraction (Figs. S16–S20, Tables S6–S7).

The [5 + 7] heterobicyclic SMs containing a carbamate moiety are rare in the natural product inventory. Cyclocarbamide 12 [28] and SB315021 13 [29] are the only two examples containing such a scaffold.
discovered thus far. It is worth to note that 13 was proposed to be the biosynthetic intermediate of brabantamide A 14, an antibacterial SM isolated from plant-associated Pseudomonas strains [31,32]. Although brabantamide A 14 is a bicyclic pyrroloxazole SM, which is structurally different to all of PA SMs, its biosynthetic gene cluster is remarkably similar to the ones of PAs. One of the key differences between brabantamide A and PA SMs lies on the putative monoxygenase BraC which was proposed to catalyse a BV oxidative ring expansion to yield 13 as the biosynthetic intermediate, the co-occurring metabolite in the producing strains, followed by the ring re-arrangement via allylic 1, 3-transposition [31]. It has yet remained to be determined whether 12 is originated from PA-like pathways or the 14-like pathway. The proposed biotransformation catalysed by LgnC-like monoxygenases and the structural relationship among 10, 12 and other co-occurring bohemamines suggested that they are likely to originate from the same PA biosynthetic pathway. Inspection of the structures of 10 and 12 led to the speculation that the precursors of the core of both compounds are (S)-5-methyl-proline and dehydroalanine, a dehydrated form of serine, a similar model to the one proposed in the recent report of bohemamines [33], respectively. The gene product, LgaE, sharing high AA sequence identity (93%) with LgnC-like monoxygenases, is likely to catalyse the key biotransformation from indolizidine intermediate 18 to pyrrolizidine 20 via bicyclic carbamate intermediate 19 (Fig. 4B). Unlike the legonmycin pathway, the lga BGC contains a series of tailoring enzymes. For example, the gene product LgaA is likely to catalyse the methylation at C-7 position in bohemamine SMs. It is likely that a promiscuous methylation enzyme methylates the key intermediate, carbamate 19, at C8 position during the catalytic cycle of LgaE, to generate legoncarbamate 10. It remains to be elusive whether LgaA is responsible for the methylation at C8 position of 10. The genes, lgaF and lgaG, encode branched-chain α-ketoacid dehydrogenase and isovaleryl CoA dehydrogenase, respectively, which are likely involved in catalysing the oxidative decarboxylation of 4-methyl-2-oxo-pentanoic acid to yield isovaleryl CoA and transfer isovaleryl CoA to 3-methyl pentenyl CoA (Fig. 4B, Table S8).

3. Material and method

3.1. General experimental procedures

The optical rotation was recorded using ADP 410 polarimeter (Bellington + Stanley Ltd.2007, Kent, UK) equipped with a light emitting diode and interference filter. UV spectra were recorded on an Accela PDA detector (Waldbromn, Germany). IR spectra were obtained on a Fourier transform infrared (FTIR) spectrometer (2013, PerkinElmer, UK) equipped with an Attenuated Total Reflection (ATR, PerkinElmer, Buckinghamshire, UK) diamond cell for sample loading was used for infrared spectroscopy experiments. 1D and 2D NMR spectra were

Fig. 4. A, the organisation of the putative lgc BGC in Streptomyces sp. CT37. B, A proposed model of legoncarbamate 10 and bohemamine derivatives, 15–18, identified in CT37.
acquired on a Bruker AVANCE III HD400MHz (AscendTM14.1 T, UK) with Prodigy TCITM cryoprobe at 298 K in CD3OD and DMSO-d6 (Goss Scientific, Massachusetts, MA, USA). Trimethylsilylamine (TMS) was used as an internal standard. HRESIMS data were obtained in positive ESI mode with a mass range of 100–2000 m/z (maximum resolution 30,000) on a Thermo Scientific MS system (LTQ XL/LTQ Orbitrap Discovery, Waldbronn, Germany). Reserpine (m/z 609.2807) was used as a lock mass for internal calibration during data acquisition. The following instrument parameters were used: capillary voltage 45 V, spray voltage 4.5 kV, capillary temperature 200 °C, auxiliary gas flow rate 10–20 arbitrary units, and sheath gas flow rate 5 arbitrary units; furthermore, an automated full dependent MS-MS scan was applied. The injected samples were chromatographically separated in Thermo Instrument HPLC system (Accela PDA detector, Waldbronn, Germany). Accela PDA autosampler and Accela Pump (Agilent Technologies, Waldbronn, Germany) using a C18 (Sunfire 150 × 46 mm) column. The gradient elution for separation was CH3CN/H2O with 0.1% trifluoroacetic acid (TFA) (from 0% to 100% for 30 min, flow rate 1.0 mL/min, UV detection max 340 nm).

3.2. Strain collection

Fifty-eight strains were used for genome scanning which was listed in Table S2. Among which 56 were provided by NCIMB while the remaining two were isolated from soil samples collected in Ghana as described in previous papers [23,36].

3.3. Cultivation and genomic DNA extraction

Strains were cultured in 50 mL ISP2 media and harvested after 7 days. Genomic DNA was extracted using the following protocol to generate a library of genomic DNA. The genomic DNA in this study was extracted from 10 mL cell culture. Cell pellet was harvested by centrifugation and resuspended in 500 µL SET buffer. The cell suspension was mixed with lysozyme (4 mg/mL, final concentration) and incubated at 37 °C for 30 min. SDS (60 µL, 10% (w/v)) and NaCl (200 µL, 5 M) were then added to the mixture, followed by another incubation at 60 °C for 30 min. The protein was precipitated with the mixture of phenol, chloroform and isooamylol (500 µL, ratio of 25:24:1), and the resultant solution was mixed by vortex. The supernatant was separated by centrifuge and transferred to a new Eppendorf tube with isopropanol for DNA precipitation (0.8 vol). The precipitated DNA was dissolved with 75% (v/v) ethanol, followed by the second wash of 100% ethanol. DNA pellet was dried at room temperature and dissolved in sterile Milli-Q water (200 µL).

3.4. Genome scanning of strain library

MEME (Multiple EM for Motif Elicitation) was used for protein motif and degenerate primers design (Table S1) [37]. The DNA fragments were amplified from Streptomyces genomic DNA in the library using Taq DNA polymerase (Invitrogen, UK) with the degenerated primers. The homologues genes of ORF amplified from Streptomyces genomic DNA templates were inserted into pCR™ plasmid using TA Cloning™ Kit (Invitrogen, UK). The constructed plasmid was sequenced by the Dundee sequencing service.

3.5. Cultivation conditions

OSMAC strategy was applied [17] using 8 different fermentation broths (ISP2-ISP7, Modified Bennett’s, Starch Casein, Table S1). These were selected based on the recommended medium for Streptomyces species, which differ with respect to carbon source and salt concentration [38].

The small scale culture (50 mL) of CT37 strain was prepared by inoculating a single colony of the bacteria in a solid medium of choice and incubated at 28 °C, 180 rpm for 7 days (Incu-shake FL16-2, SciQuip, Shrewsbury, UK). Subsequently, Diaion®HP-20 (3 g/50 mL, Mitsubishi Chemical Co., Bisasco, Italy) was added to the fermentation cultures under sterile conditions. The flasks were left at the same shaking temperature and conditions for 18 h.

The culture broths were filtered under vacuum (Buchi pump V100, Buchi, Manchester, UK), and the HP-20 resin was rinsed with Milli-Q water and extracted exhaustively with methanol (MeOH, Fisher Chemical HPLC grade). All the methanol extracts were combined, and concentrated under reduced pressure (Buchi Rotavapor R200, Buchi, Manchester), and subjected to high-resolution electrospray ionization Liquid chromatography mass spectrometry analysis.

3.6. Large scale fermentation

For scale-up fermentation, a seed culture (50 mL) of CT37 was prepared following the same inoculation procedure as small scale cultivation. On the 3rd day, the seed culture was inoculated (1:100) into a 2 L Corning™ polycarbonate baffled flask (contains 250 mL broth). Each of the flasks (8 L in total) were plugged with Fisherbrand™ polyurethane foam stoppers (Fisher Scientific, UK). The cultures were fermented for 7 days under the same condition as described for the small scale fermentation.

3.7. HPLC-HRMS/MS analysis and metabolites annotation using GNPS and SMART 2.0

The MS/MS data were converted from RAW to .mzXML files using the ProteoWizard MScnvert software [39]. A molecular network was generated using Feature-Based Molecular Networking (FBMN) workflow [40] on Global Natural Product Social networking (GNPS) [18] (https://gnps.ucsd.edu). The mass spectrometry data were pre-processed with MZmine v2.38 [41] and exported to GNPS for FBMN analysis. The data were filtered to remove all MS/MS fragment ions within ±17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the ±50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.02 Da with an MS/MS fragment ion tolerance of 0.02 Da to create consensus spectra. The consensus spectra that contained fewer than four spectra were discarded. The edges were filtered to ensure a cosine score above 0.65 and more than four matched peaks. The edges between two nodes were kept in the network if each of the nodes appeared in each other’s respective top 15 most similar nodes. The spectra in the network were then searched against GNPS spectral libraries [18] and annotated by the DEREPLICATOR [42]. The library spectra were filtered in the same manner as the input data, where a score above 0.65 and at least 4 matched peaks are required. The created molecular network was visualized using Cytoscape software v3.4.0 (Seattle, WA, US) [43].

Smart 2.0 was used for rapid structure prediction of major constituents from crude extracts and fractions (https://smart.ucsd.edu/classic). The experimental HSQC data was annotated to generate a digitized HSQC spectrum for library alignment and analysis. Top 6 structures were listed based on cosine similarity score [19].

3.8. HPLC isolation

The compounds of interest were identified in S2 fraction, hence further fractionation was carried out in this fraction using High Pressure Liquid Chromatography (HPLC, Agilent Technologies 1260 infinity, Waldbronn, Germany). The purification was performed using a linear gradient from 10% H2O:MeOH (95:5) to 100% MeOH for 30 min with a solvent flow rate of 1.5 mL/min (C-18 ACE 10 µM 10 × 250 mm column). As a result, 10 (1.0 mg, 11 (1.5 mg) were isolated.

Legoncarbamate (10): white powder, [α]25D +11.3 (c 0.50, MeOH); UV (MeOH) λmax (log ε) 275 nm; ECD (c = 1.0 mg/mL, MeOH) λmax (Δε) 338
In conclusion, we applied genomic scanning strategy to probe our culture collection to identify the potential producers of pyrrolizidine alkaloids (PAs). One of these thirteen potential producers was found to produce known bohemamine derivatives and a new [5 + 7] heterobicyclic alkaloid, legoncarbamate 10, using a combination of chemical workup, NMR-based structural elucidation, SMART 2.0 technology in conjunction with LC-MS-based GNPS analysis. The absolute stereochemistry of legoncarbamate 10 was determined by a comparison of measured and calculated ECD spectra. Legoncarbamate 10 displays antibacterial activity against Gram-negative E. coli ATCC 25922 with an MIC value of 3.1 μg/mL. Finally, a biosynthetic model of legoncarba- mide and other known bohemamine derivatives was proposed based on bioinformation analysis and current knowledge of PA biosynthesis.

Author contributon

Declaration of competing interest
All of the authors declare no conflict of interest.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2021.01.002.

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