BrtB is an O-alkylating enzyme that generates fatty acid-bartoloside esters

João P.A. Reis¹, Sandra A.C. Figueiredo¹, Maria Lígia Sousa¹ & Pedro N. Leão¹

Esterification reactions are central to many aspects of industrial and biological chemistry. The formation of carboxyesters typically occurs through nucleophilic attack of an alcohol onto the carboxylate carbon. Under certain conditions employed in organic synthesis, the carboxylate nucleophile can be alkylated to generate esters from alkyl halides, but this reaction has only been observed transiently in enzymatic chemistry. Here, we report a carboxylate alkylating enzyme – BrtB – that catalyzes O-C bond formation between free fatty acids of varying chain length and the secondary alkyl halide moieties found in the bartolosides. Guided by this reactivity, we uncovered a variety of natural fatty acid-bartoloside esters, previously unrecognized products of the bartoloside biosynthetic gene cluster.

¹Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Avenida General Norton de Matos, s/n, 4450-208, Matosinhos, Portugal. Email: pleao@ciimar.up.pt
Esters, in particular carboxyesters, are fundamental to both industrial and biological chemistry. A number of synthetic methods have been developed to generate carboxyesters, with most exploiting nucleophilic attack of an alcohol onto the carboxylic carbonyl, as typified by the century-old Fischer-Speier esterification. Esterification reactions that are independent of alcohols and in which the carboxylate anion acts as nucleophile are also well-known in organic synthesis, in particular with primary alkyl halides as the electrophilic partner. Likewise, biological carboxyester formation most often takes advantage of the electrophilicity of a carbonyl group, via acylation of a suitable alcohol (e.g., lipases and esterases, thioesterases, condensation of α-hydroxy acids in non-ribosomal peptide synthetases), or through direct oxidation by Baeyer–Villiger monoxygenases. Carboxylate nucleophiles can also give rise to carboxyesters through SAM-dependent methylation of carboxylates, e.g., ref. 8 or intramolecular esterifications as those catalyzed by the 3-carboxy-cis,cis-muconate lactonizing enzyme (CML) or the more recently described radical S-adenosylmethionine (rSAM) NosN (Fig. 1a–c). However, to our knowledge, enzymatic C—C bond formation involving a carboxylate nucleophile and an alkyl halide electrophile has only been reported to occur transiently during catalysis by haloalkane dehalogenases and certain haloacid dehalogenases. In their catalytic cycles, an ester bond is formed through attack of a side-chain carboxylate onto a halogenated position and is subsequently hydrolyzed to generate an alcohol product (Fig. 1d).

Recently, Balskus and co-workers unveiled a biological C-alkylation involving the biosynthesis of cyclophane natural products in cyanobacteria. This reaction requires the previous stereoselective chlorination of an unactivated carbon center by the CylC halogenase. To create the final cyclophane dimeric scaffold, an alkylating enzyme (CylK) catalyzes C—C bond formation between C-2 of each alkylresorcinol monomer and the halogenated carbon of the other monomer (Fig. 1e). A number of cyanobacterial biosynthetic gene clusters (BGCs) were found to feature both CylC and CylK homologs, suggesting that analogous C—C bond formation could be a common feature of secondary metabolite biosynthesis in cyanobacteria. One such BGC—brt—encodes the biosynthesis of the bartolosides, a group of chlorinated alkylresorcinols. In brt clusters, the homolog of the CylC halogenase (BrtJ) is likely responsible for the mid-chain chlorinations that are present in all bartolosides. However, the role played by the encoded CylK homolog (BrtB) has remained unclear. The reasons for this are twofold: first, the action of a CylK homolog is not necessary to explain the biosynthesis of currently known bartolosides, second, CylK requires a free C-2 resorcinol as a nucleophile, a position that is unavailable (it is alkylated) in the bartolosides. Here, we show that BrtB is an O-alkylating enzyme that catalyzes the esterification of free (non-activated) fatty acids with the chlorinated positions found in the bartolosides, generating a diversity of fatty acid-bartoloside esters (Fig. 1g).

**Results**

**Bartoloside esters are formed upon fatty acid supplementation.** The biosynthesis of the dialkylresorcinol skeleton in the bartolosides involves recruitment of fatty acid derivatives from primary metabolism. We envisioned that this could be exploited to incorporate terminal alkylene moieties into the bartolosides and generate click chemistry-accessible versions of these natural products for probing their biological role, e.g., ref. 16. To this end, we supplemented cultures of the cyanobacterium *Synechocystis salina* LEGE 06099 with 50 mg L⁻¹ of 5-hexynoic or 6-heptynoic (2) acids. LC-HRESIMS analysis of the resulting cell extracts revealed a massive depletion of the major metabolite 1 and several of its analogues (Fig. 2a, Supplementary Fig. 1) in supplemented cultures, yet, surprisingly, we did not detect any ions compatible with bartolosides containing terminal alkynes in their dialkylresorcinol skeleton. Instead, we observed a series of m/z values consistent with the incorporation of one or two units of the supplemented fatty acids into the depleted bartolosides and the concomitant loss of one or both Cl atoms, respectively (Fig. 2a, Supplementary Fig. 2). This was supported by LC-HRESIMS/MS analysis of these species, which showed fragments corresponding to the intact alkyl precursor or to their neutral losses (Supplementary Fig. 2). To unequivocally establish the identity of the newly observed compounds, we isolated two major metabolites (3 and 4) resulting from the supplementation with 2. Subsequent structure elucidation using 1D and 2D NMR as well as HRESIMS/MS analyses, clarified that 3 and 4 were esters of bartolosides A (1) and G (S), respectively, in which 2 was now esterified to the previously chlorinated positions (Supplementary Note 1, Supplementary Fig. 3). Supplementation of *S. salina* LEGE 06099 with butyric, caprylic, lauric and palmitic acids, as well as with 7-bromohexanoic acid led to the formation of the corresponding monoesters and diesters (Supplementary Fig. 4). Overall, our results show that exogenously provided fatty acids are converted in vivo into fatty acid-bartoloside esters by *S. salina* LEGE 06099.

**BrtB esterifies free fatty acids with bartoloside A.** We set out to investigate whether BrtB, the only enzyme in the *brt* gene cluster with no ascribed function, could be responsible for ester formation. Following the protocol reported by Schultz et al., we expressed and purified a Strep-Tag®-recombinant version of BrtB (NStrep-BrtB) in *Escherichia coli* BL21 DE3 Rosetta cells and tested its ability to convert 1 and 2 into 3 in vitro. We found that adding NStrep-BrtB to a reaction mixture composed of 1, 2 and Ca²⁺ and Mg²⁺-containing buffer was necessary and sufficient to generate diester 3 as well as monoester(s) 6a and/or 6b (Fig. 2b, Supplementary Fig. 5). In line with the in vivo data, we found that BrtB is also able to esterify palmitic acid with 1 to generate bartoloside A palmitate(s) 7a and/or 7b as well as bartoloside A dipalmitate (8) (Fig. 2c, Supplementary Fig. 6). Thus, we show that BrtB catalyzes C—O bond formation through esterification of a free fatty acid with a secondary alkyl halide.

**BrtB is a promiscuous O-alkylating enzyme.** Because our in vivo supplementation experiments showed that *S. salina* LEGE 06099 cells generated bartoloside esters of fatty acids ranging from C₄ to C₁₆ (Supplementary Fig. 4), we next tested the substrate scope of BrtB in enzymatic assays. BrtB could generate the predicted mono- and diester products for all the tested fatty acids, ranging in length from C₂ to C₁₆ (Fig. 3a, Supplementary Fig. 7) but not for other carboxylic acids (Supplementary Fig. 7). Potential nucleophiles with amide, alcohol or phosphonic acid functionalities did not lead to any observable product (Supplementary Fig. 7) when incubated with BrtB and 1. Primary alkyl halides and an aliphatic secondary alkyl halide were not esterified with 2 either (Supplementary Fig. 7). We additionally tested BrtB activity at 15, 25, 37, and 55 °C and observed formation of substrate in all tested temperatures, with maximum activity at 37 °C (Supplementary Fig. 8). Having found that BrtB could use acetate as a substrate, we performed in vitro assays with [¹⁸O]acetate to gain some mechanistic insight into the reaction. As expected, LC-HRESIMS/MS analysis revealed that two or four ¹⁸O atoms were incorporated into the corresponding bartoloside mono- and diesters, respectively (Fig. 3b). The fragmentation pattern
indicated that these atoms were part of the esterified acetate moiety (Supplementary Fig. 9), proving that the fatty carboxylate acts as a nucleophile in this reaction. Carrying out the esterification in D2O-resuspended reaction buffer did not lead to deuterium incorporation into the reaction products (Supplementary Fig. 10), dismissing the possibility of a two-step elimination and nucleophilic addition mechanism. Overall, we show that BrtB is highly promiscuous as to fatty acid length and catalyzes O–C bond formation between a fatty carboxylate nucleophile and the chlorinated carbons in the bartolosides.

**The brt cluster generates diverse bartoloside esters.** Having established that the observed esterification was catalyzed by a brt-encoded enzyme, we hypothesized that fatty acid-bartoloside esters are products of the brt cluster, in which case their production by cyanobacterial cells should occur without exogenous fatty acid supply. In fact, the LC-HRESIMS data for non-supplemented controls in our in vivo fatty acid supplementation experiments showed an abundant compound with m/z values and retention time consistent with a bartoloside A monopalmitate (7a/7b, Supplementary Fig. 4). We thoroughly explored LC-HRESIMS data
from a CH2Cl2/MeOH (2:1) cellular extract of a batch culture of S. salina LEGE 06099 and detected species with m/z values or retention times to those of more abundant bartoloside esters. Neither 7a, nor the alkyne-containing 3 and 4 showed appreciable cytotoxicity (up to 10 μM), against immortalized cell lines or antibacterial activity at 0.5 mg mL−1 in agar disk diffusion assays (Supplementary Figs. 14 and 15). Our findings revealed that the brt gene cluster generates a large diversity of natural products, not only through the incorporation of fatty acid derivatives of different chain length during dialkylresorcinol formation and different halogenation patterns13,14, but also through the relaxed specificity of BrtB, which is able to alkylate a variety of endogenous fatty acids.

Regioselectivity and kinetics of BrtB. Because palmitic acid and 1 were found to be abundant natural substrates of BrtB, we performed 10 h kinetic assays to determine how esterification proceeds, in particular whether BrtB had selectivity for a particular alkyl chain in the first esterification or for a particular monoester in the second esterification. LC-HRESIMS analysis of assays quenched at different time points indicated that BrtB is slightly regioselective (two-fold factor) for the C-2 alkyl chain over the C-5 alkyl chain, but performed the second esterification at similar rates with either 7a or 7b as substrates (Fig. 4d, e, f).

Fig. 2 Bartoloside-fatty acid esters are formed in vivo and in vitro by BrtB. In cultures of S. salina LEGE 06099 supplemented with 50 mg L−1 of 6-heptynoic acid (2), formation of the bartoloside esters 3, 6a/6b and 4 with concomitant depletion of the corresponding bartolosides (A, 1 or G, 5) was observed by LC-HRESIMS (a). Analysis (LC-HRESIMS) of the NStrep-BrtB-mediated O-alkylation of bartoloside A (1) with 2 to generate diester 3 and monoesters 6a/6b (b) or with palmitic acid to generate monoesters 7a/7b and diester 8, the latter in much lower amount (c). Full reactions contained 1 μM recombinant NStrep-BrtB, 100 μM of 1 and 200 μM of either 2 (b) or palmitic acid (c).
Vmax values; however, it showed higher substrate conversions in substrates for 10 h. Conversion values were obtained from areas of reaction NATURE COMMUNICATIONS | (2020) 11:1458 | https://doi.org/10.1038/s41467-020-15302-z | www.nature.com/naturecommunications 24 h assays than BrtB under similar conditions15. p presents a considerably higher Vmax18,19. Regarding CylK, the fatty acid in reaction buffer.

observed apparent Km and Vmax values of 234 ± 41 μM and 0.13 ± 0.01 μM min−1 (standard error, n = 3) for the formation of 4 (Supplementary Fig. 17). Compared to haloalkane dehalogenases, BrtB has a similar apparent Km but presents a considerably higher Vmax18,19. Regarding CylK, the single BrtB homolog characterized, there are no reported Km or Vmax values; however, it showed higher substrate conversions in 24 h assays than BrtB under similar conditions15.

Supplementary Fig. 16). To obtain further insight into the activity of BrtB, we carried out 20-min kinetic assays with 5 (with a single chlorinated position) and varying concentrations of 2, and observed apparent Km and Vmax values of 234 ± 41 μM (standard error, n = 3) and 0.13 ± 0.01 μM min−1 (standard error, n = 3) for the formation of 4 (Supplementary Fig. 17). Compared to haloalkane dehalogenases, BrtB has a similar apparent Km but presents a considerably higher Vmax18,19. Regarding CylK, the single BrtB homolog characterized, there are no reported Km or Vmax values; however, it showed higher substrate conversions in 24 h assays than BrtB under similar conditions15.

Fig. 4 Fatty acid-bartoloside esters are natural products. a LC-HRESIMS analysis of an organic extract of S. salina LEGE 06099 suggests the presence of bartoloside A (1)-palmitic acid esters. b Key HMBC and COSY correlations obtained from 1D and 2D NMR analysis of bartoloside A palmitates. Shown are LC-HRESIMS analysis-derived conversions (from EIC peak areas and assuming similar ionization behavior) in assays quenched at different time points for the first esterification (d n = 2 independent assays) showing that 7a is the preferred product and for the second esterification (e n = 2 independent assays) indicating that both 7a and 7b are converted into 8 at comparable rates.
Discussion
Our work brings to light an example, in biochemical chemistry, of halogenation as an intermediate step towards a non-halogenated product. Cyanobacterial secondary metabolism seems to be particularly rich in such functionalization strategies (e.g., cyclophanes, curacin)\(^{12,21}\). This strategy for O–C bond formation is catalyzed by BrtB, a member of a poorly studied group of beta-propeller enzymes from which only the C–C bond forming CylK had been characterized\(^{12,15}\). These enzymes are often annotated in the GenBank as hemolysins, calcium-binding proteins, or beta-propeller proteins and contain a number of NHL repeats\(^{14}\). Several alginate C5 epimerases e.g., \(^{22}\), virginiamycin B lyase e.g., \(^{23}\) and eukaryotic CMLEs\(^{9}\) have some structural homology to CylK\(^{24}\). Remarkably, both the reverse reaction of virginiamycin B lyase and the forward reaction of CML involve a carboxylycule nophile\(^{25,26}\). A phylogenetic analysis of BrtB/CylK homologs (Supplementary Fig. 18) shows a large number of cyanobacterial homologs within BGCs, which most prominently encode type I polyketide synthases, fatty acid activating enzymes, non-ribosomal peptide synthetases, along with varied tailoring functionalities. Such BGCs represent clear opportunities for the discovery of natural products\(^{12}\) and, in particular, the colocalization of halogenases and BrtB/CylK homologs in a variety of cyanobacterial BGCs\(^{12}\) (Supplementary Fig. 18) points towards additional cryptic halogenation events. The reactivity and substrate flexibility of BrtB is also relevant for biocatalysis, owing to the ubiquity of esters in industrial and pharmaceutical chemicals, the large number of alkyl halide building blocks available and the existing precedent of using biocatalysts to generate esters at the industrial scale\(^{25}\). In particular, we envision that the ability of BrtB to esterify fatty acids of different lengths can be of use for reactions employing bio-based feedstocks such as hydrolysates from oils and fats\(^{26}\). As in the case of CylK, whether BrtB-catalyzed esterification occurs via a Sn1 or Sn2 mechanism is yet to be found\(^{11,12}\); we will focus future efforts on its structural and mechanistic characterization. Our discovery of a O–C bond forming reaction paved the way for identifying a series of natural products, the bartoloside esters, encoded by the bartoloside BGC (Supplementary Fig. 18) points towards additional cryptic halogenation events. The reactivity and substrate flexibility of BrtB is also relevant for biocatalysis, owing to the ubiquity of esters in industrial and pharmaceutical chemicals, the large number of alkyl halide building blocks available and the existing precedent of using biocatalysts to generate esters at the industrial scale. In particular, we envision that the ability of BrtB to esterify fatty acids of different lengths can be of use for reactions employing bio-based feedstocks such as hydrolysates from oils and fats. As in the case of CylK, whether BrtB-catalyzed esterification occurs via a Sn1 or Sn2 mechanism is yet to be found; we will focus future efforts on its structural and mechanistic characterization. Our discovery of a O–C bond forming reaction paved the way for identifying a series of natural products, the bartoloside esters, encoded by the bartoloside BGC (Supplementary Fig. 18) points towards additional cryptic halogenation events.
subfractions that were analyzed by LC-HRESIMS/MS were separated (10 µL at 0.5 mg mL⁻¹ injected) using a gradient from 9:1 to 7:13 eluent A/eluent B in 5 min, increasing to 82% eluent B over 15 min and held for 5 min before returning to the initial conditions.

Analysis of enzymatic reaction samples was carried out by injecting 10 µL of supernatant from the methanol/acetonitrile-quenched reaction mixture (see Enzymatic assays). Separation involved an isocratic step of 9.1 eluent A/eluent B over 2 min, followed by a linear gradient to 7:13 eluent A/eluent B over 3 min and held for 10 min, followed by a linear gradient to 3:7 eluent A/eluent B over 3 min and held for 8 min before returning to the initial conditions. For the activity dependence on temperature, esterification in D₂O assay and assays for the determinants of selectivity, parameters were employed an isocratic step of 9:1 eluent A/eluent B over 2 min, followed by a linear gradient to 7:13 eluent A/eluent B and held for 7 min before returning to the initial conditions. A longer program that allowed for the separation of monomers 7a/7b was used to analyze the assays designed to study the selectivity of the esterification of 1 and palmitic acid. It consisted of a flow rate of 0.6 mL min⁻¹ and an isocratic step of 9:1 eluent A/eluent B over 2 min, followed by a linear gradient to 9:11 eluent A/eluent B over 25 min, held for 38 min, then by a linear gradient to 3:7 eluent A/eluent B over 5 min and was held for 8 min before returning to the initial conditions.

MS/MS analysis. MS/MS parameters for the LC-HRESIMS/MS analysis of crude extracts, HPLC fractions and enzymatic assays were: resolution of 35000, with a 1 m/z isolation window, a loop count of 3, AGC target of 5 × 10⁴ and collision energy 35 eV.

HRESIMS/MS analysis of purified compounds 3 and 4 was performed by direct injection (0.1 mg mL⁻¹ solutions) into the spectrometer, using a resolution of 35000, a 1 m/z isolation window, a loop count of 3 and an AGC target of 5 × 10⁴. Stepwise collision energies of 35, 40, and 45 eV were applied. To obtain structural information regarding the dialysed-sonicated moiety, in-source-formations were selected for ddMS² events. For purified compounds 3 and 4, the in-source collision induced dissociation (CID) energy was set to 90 and 65 eV, respectively (to isolate species corresponding to loss of xylosyl, C₅H₉O and C₆H₈O for 3 as well as loss of xylosyl and C₅H₉O for 4), resolution of 35,000, with 1 m/z window 15 mass units, a CID collision energy was set to 55 eV. For purified compounds 7a and 7b the in-source collision induced dissociation energy was set to 90 eV (to isolate a species corresponding to the loss of xylosyl and HCl).

Isolation of bartoloside esters 3 and 4. The freeze-dried biomass (5.6 g, d.w.) from a 20 L culture of S. salina LEG0699 in 28 medium supplemented with 6-heptynoic acid (details in Feeding Experiments section) was extracted by repeated percolation using a mixture of CH₂Cl₂/MeOH (2:1, v/v) at room temperature. The resulting crude extract (754.6 mg) was fractionated by normal phase flash chromatography (Si gel 60, 0.040–0.063 mm, Macherey-Nagel) using a gradient of increasing polarity from hexane to EtOAc to MeOH, yielding eight fractions (1–8). Fraction 5 (102.2 mg), eluting with 2:3 and 1:1 EtOAc/hexane, contained MeOH (aq) isocratically over 65 min and with a

Isolation of bartoloside esters 3 and 4. The freeze-dried biomass (5.6 g, d.w.) from a 20 L culture of S. salina LEG0699 in 28 medium supplemented with 6-heptynoic acid (details in Feeding Experiments section) was extracted by repeated percolation using a mixture of CH₂Cl₂/MeOH (2:1, v/v) at room temperature. The resulting crude extract (754.6 mg) was fractionated by normal phase flash chromatography (Si gel 60, 0.040–0.063 mm, Macherey-Nagel) using a gradient of increasing polarity from hexane to EtOAc to MeOH, yielding eight fractions (1–8). Fraction 5 (102.2 mg), eluting with 2:3 and 1:1 EtOAc/hexane, contained MeOH (aq) isocratically over 65 min and with a

Isolation of bartoloside esters 3 and 4. The freeze-dried biomass (5.6 g, d.w.) from a 20 L culture of S. salina LEG0699 in 28 medium supplemented with 6-heptynoic acid (details in Feeding Experiments section) was extracted by repeated percolation using a mixture of CH₂Cl₂/MeOH (2:1, v/v) at room temperature. The resulting crude extract (754.6 mg) was fractionated by normal phase flash chromatography (Si gel 60, 0.040–0.063 mm, Macherey-Nagel) using a gradient of increasing polarity from hexane to EtOAc to MeOH, yielding eight fractions (1–8). Fraction 5 (102.2 mg), eluting with 2:3 and 1:1 EtOAc/hexane, contained MeOH (aq) isocratically over 65 min and with a

Isolation of bartoloside esters 3 and 4. The freeze-dried biomass (5.6 g, d.w.) from a 20 L culture of S. salina LEG0699 in 28 medium supplemented with 6-heptynoic acid (details in Feeding Experiments section) was extracted by repeated percolation using a mixture of CH₂Cl₂/MeOH (2:1, v/v) at room temperature. The resulting crude extract (754.6 mg) was fractionated by normal phase flash chromatography (Si gel 60, 0.040–0.063 mm, Macherey-Nagel) using a gradient of increasing polarity from hexane to EtOAc to MeOH, yielding eight fractions (1–8). Fraction 5 (102.2 mg), eluting with 2:3 and 1:1 EtOAc/hexane, contained MeOH (aq) isocratically over 65 min and with a

Isolation of bartoloside esters 3 and 4. The freeze-dried biomass (5.6 g, d.w.) from a 20 L culture of S. salina LEG0699 in 28 medium supplemented with 6-heptynoic acid (details in Feeding Experiments section) was extracted by repeated percolation using a mixture of CH₂Cl₂/MeOH (2:1, v/v) at room temperature. The resulting crude extract (754.6 mg) was fractionated by normal phase flash chromatography (Si gel 60, 0.040–0.063 mm, Macherey-Nagel) using a gradient of increasing polarity from hexane to EtOAc to MeOH, yielding eight fractions (1–8). Fraction 5 (102.2 mg), eluting with 2:3 and 1:1 EtOAc/hexane, contained MeOH (aq) isocratically over 65 min and with a

Isolation of bartoloside esters 3 and 4. The freeze-dried biomass (5.6 g, d.w.) from a 20 L culture of S. salina LEG0699 in 28 medium supplemented with 6-heptynoic acid (details in Feeding Experiments section) was extracted by repeated percolation using a mixture of CH₂Cl₂/MeOH (2:1, v/v) at room temperature. The resulting crude extract (754.6 mg) was fractionated by normal phase flash chromatography (Si gel 60, 0.040–0.063 mm, Macherey-Nagel) using a gradient of increasing polarity from hexane to EtOAc to MeOH, yielding eight fractions (1–8). Fraction 5 (102.2 mg), eluting with 2:3 and 1:1 EtOAc/hexane, contained MeOH (aq) isocratically over 65 min and with a

Isolation of bartoloside esters 3 and 4. The freeze-dried biomass (5.6 g, d.w.) from a 20 L culture of S. salina LEG0699 in 28 medium supplemented with 6-heptynoic acid (details in Feeding Experiments section) was extracted by repeated percolation using a mixture of CH₂Cl₂/MeOH (2:1, v/v) at room temperature. The resulting crude extract (754.6 mg) was fractionated by normal phase flash chromatography (Si gel 60, 0.040–0.063 mm, Macherey-Nagel) using a gradient of increasing polarity from hexane to EtOAc to MeOH, yielding eight fractions (1–8). Fraction 5 (102.2 mg), eluting with 2:3 and 1:1 EtOAc/hexane, contained MeOH (aq) isocratically over 65 min and with a

Isolation of bartoloside esters 3 and 4. The freeze-dried biomass (5.6 g, d.w.) from a 20 L culture of S. salina LEG0699 in 28 medium supplemented with 6-heptynoic acid (details in Feeding Experiments section) was extracted by repeated percolation using a mixture of CH₂Cl₂/MeOH (2:1, v/v) at room temperature. The resulting crude extract (754.6 mg) was fractionated by normal phase flash chromatography (Si gel 60, 0.040–0.063 mm, Macherey-Nagel) using a gradient of increasing polarity from hexane to EtOAc to MeOH, yielding eight fractions (1–8). Fraction 5 (102.2 mg), eluting with 2:3 and 1:1 EtOAc/hexane, contained MeOH (aq) isocratically over 65 min and with a
This procedure yielded 0.3 mg of NStrep-BrtB per L of culture of the above-mentioned strain which was then used to inoculate (1:100 (Supplementary Fig. 19).

Scientific establishment in (D2O) were carried in similar conditions but with a buffer (20 mM HEPES, 500 mM NaCl, 20 mM CaCl2, 10 mM MgCl2, pH 8.0) to stoichiometric ratio. Reactions were incubated at 37 °C with 180 rpm shaking. The equimolar mixture of MgCl2, 5 mM CaCl2, pH 8.0). Bartoloside G (7a) and palmitic acids and the reactions were incubated at 37 °C. The yeast was grown in Sabouraud Dextrose Agar (BioKar diagnostics).

Enzymatic assays. In vitro enzymatic assays to validate BrtB function were carried out in Eppendorf tubes to a final volume of 100 μL in reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl2, 5 mM CaCl2, pH 8.0). Final concentrations of each substrate were 1 μM for NStrep-BrtB, 100 μM for bartosilide A (1) and 200 μM for both palmitic acids and the reaction was initiated at 37 °C with 180 rpm shaking. Aliquots (30 μL) were collected at 6, 24 and 24 h and quenched with 60 μL of a cold mixture of MeOH/MeCN (1:1, v/v), vortexed immediately and incubated in ice for 10 min. Quenched reactions were then centrifuged at 17 000 × g, 4 °C, 10 min) and analyzed by LC-HRESIMS/MS.

In vitro enzymatic assays for testing the influence of temperature on activity and for substrate scope studies were carried out in Eppendorf tubes to a final volume of 100 μL in reaction buffer (20 mM HEPES, 50 mM NaCl, 10 mM MgCl2, 5 mM CaCl2, pH 8.0), over 24 h (temperature) and 1 h (substrate scope). Experiments using 15O2-labeled and unlabeled acetic acid and deuterium oxide (D2O) were performed under the same conditions. Reaction conditions were then centrifuged at 17 000 × g, 4 °C, 10 min) and analyzed by LC-HRESIMS/MS.

Enzymes were added from an affinity chromatography used 6 mL of a 50% Strept-Tactin Superflow resin and the column equilibrated with 20 mL isocratic buffer. The clarified lysate was transferred to the column, discarding the flow-through. The column was washed with 20 mL isocratic buffer and protein was eluted in multiple 1.5 mL fractions using elution buffer (lysis buffer with 2.5 mM desthiobiotin, pH 8.0). The sample was concentrated to 28.6 μM using Pierce® Protein Concentrator tubes, 10 K MWCO (Thermo Scientific) through two consecutive 15 min centrifugations (8000 × g, 4 °C). The concentrated protein was then incubated at 37 °C and aliquots (20 μL) were collected at different time points (5, 10, 20, 30, and 60 min) and quenched with 60 μL of a cold mixture of MeOH/MeCN (1:1, v/v), vortexed immediately and incubated in ice for 10 min. Quenched reactions were then centrifuged at 17 000 × g, 4 °C, 10 min) and analyzed by LC-HRESIMS/MS.

A calibration curve of purified compound 4 was generated (EIC peak area = 1.70 ± 0.04 × 103 [μM], 3.36 ± 0.02 × 103) using dilutions from a 10 mM standard solution of compound 4 (0.005, 0.01, 0.1, 0.5, 1, and 10 μM) and considering the integration of the EIC peak for m/z 761.522 [M+H + HCOOH]−, 4). The initial reaction rates (d[4]/dt) were calculated from each compound and quenched for the formation of 1A to 1B (considering only time points 5, 10, and 20 min) and were then used to calculate apparent Km and Vmax values using R2.

Bioactivity assays. Cytotoxicity assays against human immortalized cells: Compounds 3 and 4 were tested for cytotoxicity against the HCT116 colon colorectal carcinoma, HT-29 colon colorectal adenocarcinoma and hMECUD3 blood-brain barrier cell lines and compound 7a was tested in the HT-29 cell line. All cell lines were subcultivated and grown in supplemented medium as recommended by the providers. All HCT116 cells were maintained in McCoy’s 5 A modified medium while HT-29 and hMECUD3 cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM). All media were also supplemented with 10% fetal bovine serum (Biowhittaker), 1% of penicillin/streptomycin (Biochrom), and 0.1% of Amphotericin B (GE Healthcare), and all cell lines incubated at 37 °C in 5% CO2.

Antimicrobial agar disk diffusion assays: Solutions of 0.5 mg mL−1 of compounds 3, 4, and 7a, prepared in DMSO, were tested against a Gram positive bacterial strain (Staphylococcus aureus ATCC 29213), Gram-negative bacterial strains (Escherichia coli ATCC 25922 and Salmonella typhimurium ATCC 25241) and a yeast strain (Candida albicans ATCC 10231). The bacteria were grown in Mueller-Hinton agar (MH—BioKar diagnostics) from stock cultures and incubated at 37 °C. The yeast was grown in Sabouraud Dextrose Agar (BioKar diagnostics). For the antimicrobial screening, a disk diffusion test was carried out. Bacterial colonies were picked from overnight cultures in MH and suspended in LB liquid medium, the turbidity adjusted to OD600 = 0.090–0.110 (0.5 McFarland standard) and the MH plates seeded with the resulting inoculum. Blank disks (6 mm in diameter, Oxoid) were placed in the inoculated plates and impregnated with 15 μL stock solution of each compound. Plates were left overnight at room temperature and then incubated overnight at 37 °C. After 24 h, the plates were checked for inhibition halos, indicative of antimicrobial activity. A disk with 15 μL DMSO was used as negative control.

Phylogenetic analysis. Amino acid sequences of the BrtB homologs with lowest e-value (Blastp) were retrieved from the NCBI database (232 homologs retrieved). Their amino acid sequences were aligned, together with those of a set of the distantly related virginiamycin lyases and alginate C5 epimerases and with the sequence of BrtB, using MUSCLE, from within Geneious R11 (Biomatters). The resulting alignment (with a total of 239 sequences) was trimmed to its core region and contained 1558 positions. FastTree 2.1.13 (from within Geneious R11) was used to compute an approximately-maximum-likelihood phylogenetic tree, using pseudocounts and 1000 rate categories of sites.

Availability of materials. Plasmsids and strains are available from the corresponding author upon reasonable request.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request. The source data underlying Supplementary Figs. 17 and 19 are provided as a Source Data file.

Received: 19 August 2019; Accepted: 2 March 2020; Published online: 19 March 2020
References

1. Riemschneider, W. & Bolt, H. M. In Ullmann's Encyclopedia of Industrial Chemistry (American Cancer Society, 2005).
2. Lortie, R. In Encyclopedia of Catalysis (American Cancer Society, 2010).
3. Otera, J. & Nishikido, J. Esterification: Methods, Reactions, and Applications (Wiley, 2009).
4. Fischer, E. & Speier, A. Darstellung der Ester. Ber. der Dtsch. chemischen Ges. 28, 3252–3258 (1895).
5. Fischbach, M. A. & Walsh, C. T. Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: Logic, machinery, and mechanisms. Chem. Rev. 106, 3468–3496 (2006).
6. Magarey, N. A., Ehling Schulz, M. & Walsh, C. T. Characterization of the cerulenid NRPS α-hydroxoy acid specifying modules: activation of α-keto acids and chiral reduction at the assembly line. J. Am. Chem. Soc. 128, 10696–10699 (2006).
7. Leisch, H., Morley, K. & Lau, P. C. K. Baeyer (American Cancer Society, 2005).
8. Fischer, E. & Speier, A. Darstellung der Ester. Ber. der Dtsch. chemischen Ges. 28, 37010–37020 (2012).
9. Kajander, T. et al. The Structure Of Neurospora Crassa 3-carboxy-cis,cis-muconate Lactonizing Enzyme, A β Propeller Cycloisomerase. Structure 10, 483–492 (2002).
10. LaMattina, J. W. et al. NosN, a radical S-adenosylmethionine methylase, catalyzes the biosynthesis of nosiheptide. J. Am. Chem. Soc. 139, 17438–17445 (2017).
11. O’Hagan, D. & Schmidtberger, J. W. Enzymes that catalyse SN2 reaction mechanisms. Nat. Prod. Rep. 27, 900–918 (2010).
12. Nakamura, H., Schulz, E. E. & Balskus, E. P. A new strategy for aromatic ring alkylation in cylindrocyclophane biosynthesis. Nat. Chem. Biol. 13, 916–921 (2017).
13. Leão, P. N. et al. Biosynthesis-assisted structural elucidation of the barbolidoses, chlorinated aromatic glycolipids from cyanobacteria. Angew. Chem. Int. Ed. 54, 11063–11067 (2015).
14. Afonso, T. B. et al. Barbolidoses E–K from a Marine Coccid Cyanobacterium. J. Nat. Prod. 79, 2504–2513 (2016).
15. Schultz, E. E., Braffman, N. R., Luescher, M. U., Hager, H. H. & Balskus, E. P. Biocatalytic Friedel–Crafts alkylation using a promiscuous biosynthetic enzyme. Angew. Chem. Int. Ed. 58, 3151–3155 (2019).
16. Luo, X. et al. Complete biosynthesis of cannabinoids and their unnatural analogues in yeast. Nature 567, 123 (2019).
17. Los, D. A. & Mironov, K. S. Modes of fatty acid desaturation in cyanobacteria: an update. Life 5, 554–567 (2015).
18. Schanstra, J. P., Kingma, J. & Janssen, D. B. Specificity and kinetics of haloalkane dehalogenases. J. Biol. Chem. 271, 14747–14753 (1996).
19. Nagata, Y. et al. Purification and characterization of a haloalkane dehalogenase of a new substrate class from a gamma-hexachlorocyclohexane-degrading bacterium, Sphingomonas paucimobilis UT26. Appl. Environ. Microbiol. 63, 3707–3710 (1997).
20. Aggarwal, V. et al. Enzymatic halogenation and dehalogenation reactions: pervasive and mechanistically diverse. Chem. Rev. 117, 5619–5674 (2017).
21. Gu, L. et al. Metamorphic enzyme assembly in polyketide diversification. Nature 459, 731–733 (2009).
22. Bachinger, E. et al. Structural and functional characterization of the r-modules in alginate C-5 epimerases AlgE4 and AlgE6 from Azotobacter vinelandii. J. Biol. Chem. 289, 31382–31396 (2014).
23. Lipka, M., Filipek, R. & Bochtler, M. Crystal structure and mechanism of the Staphylococcus cohnii Viniiangimycin B lyase (Vgb). Biochemistry 47, 4257–4265 (2008).
24. Nakamura, H. Discovery and Characterization of the Cylindrocyclophane Biosynthetic Pathway (Harvard University, 2016).
25. Ansorge-Schumacher, M. B. & Thum, O. Immobilised lipases in the cosmetics industry. Chem. Soc. Rev. 42, 6475–6490 (2013).
26. Biermann, U., Bornscheuer, U., Meier, M. A. R., Metzger, J. O. & Schäfer, H. J. Oils and fats as renewable raw materials in chemistry. Angew. Chem. Int. Ed. 50, 3854–3871 (2011).
27. Marrakhhi, H., Lanéelle, M.-A. & Daffé, M. Mycotic acid: structures, biosynthesis, and beyond. Chem. Biol. 21, 67–85 (2014).
28. Bausch, T. et al. Distribution of heterocyst glycolipids in cyanobacteria. Phycologia 79, 2034–2039 (2009).
29. Taw, J. & Rock, C. O. Exogenous fatty acid metabolism in bacteria. Biochimie 111, 30–39 (2017).
30. Ramos, V. et al. Cyanobacterial diversity held in microbial biological resource centers as a biotechnological asset: the case study of the newly established LEGE culture collection. J. Appl. Phycol. 30, 1437–1451 (2018).
31. Singh, S. P., Rastogi, R. P., Hader, D. P. & Sinha, R. P. An improved method for genomic DNA extraction from cyanobacteria. World J. Microbiol. Biotechnol. 27, 1225–1230 (2010).
32. Huitema, C. & Horsman, G. Analyzing enzyme kinetic data using the powerful statistical capabilities of R. Preprint at: bioRxiv 316588, https://doi.org/10.1101/316588 (2018).
33. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2—approximately maximum-likelihood trees for large alignments. PLoS ONE 5, e4940 (2010).

Acknowledgements

We acknowledge funding by the European Research Council, through a Starting Grant (Grant Agreement 759840) to P.N.L., and by Fundação para a Ciência e Tecnologia (FCT) through project PTDC/BIA-BQM/29710/2017 and grant IF/01584/2014 to P.N.L. The work was also partially supported by Strategic Funding UIDB/04423/2020 and UIDP/04423/2020 by FCT and the European Regional Development Fund, as part of the program PT2020. We thank Emily Balskus (Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA, USA) for helpful discussions and Ralph Urbatrzka (CIIMAR, University of Porto, Porto, Portugal) for help with cytotoxicity assays.

Author contributions

J.P.A.R. and P.N.L conceived the project, J.P.A.R., S.A.C.F., and M.L.S. performed experimental work, J.P.A.R. and P.N.L. wrote the manuscript with input from S.A.C.F. and M.L.S.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-15302-z.

Correspondence and requests for materials should be addressed to P.N.L.

Peer review information

Nature Communications thanks Qi Zhang and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020