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Alichondrichlorin, a Novel Chlorohydrin-Containing Natural Product With Tumoral Cytotoxic Activity Isolated From the *Planctomycetota* Bacterium *Alienimonas chondri* LzC2^T

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Keywords: human breast adenocarcinoma | natural products | *Planctomycetales* | *Planctomycetia* | structural elucidation

ABSTRACT

To address the on-going need for chemical novelty and the limited information on *Planctomycetota* secondary metabolism, we focused on exploring the recently isolated marine planctomycetal strain *Alienimonas chondri* LzC2^T to uncover its potential production of novel compounds. This work contemplates the description of a large-scale cultivation study of strain LzC2^T, followed by metabolite extraction and compound isolation using chromatographic approaches, which resulted in the isolation of a novel molecule designated as alichondrichlorin. Structural elucidation of this new molecule was accomplished by a combination of high-resolution mass spectrometry and nuclear magnetic resonance. The molecule was additionally screened for antiproliferative bioactivity against human tumoral and non-tumoral cell lines. These cytotoxicity assays revealed a targeted effect of alichondrichlorin in the growth of tumoral cell lines, especially human breast adenocarcinoma MCF-7 cell line ($EC_{50} = 4.06\,\mu M$) without effect on the human non-tumoral THLE-2 cell line ($EC_{50} > 50\,\mu M$).

1 | Introduction

Humanity continually seeks the discovery of new molecules that could offer beneficial therapeutical or other biotechnological applications. New classes of compounds are especially sought after, as they can potentially generate new effects and have unique properties. Nature has always been a source for chemical inspiration, due to the inherent complexity and biological functions of natural products. However, some habitats and specific taxonomic groups of organisms have been already extensively researched for this purpose, which often leads to rediscovery of already-known products or simple variations within the same structural classes. An exciting strategy to find novel chemical diversity would be to explore yet uncharted biomes, such as marine microbial communities (Haefner 2003; Molinski et al. 2009; Fenical 2020; Santos et al. 2020).

Planctomycetota form a group of Gram-negative bacteria with diverse uncommon and curious features (Santarella-Mellwig et al. 2013; Acehan, Santarella-Mellwig, and Devos 2014; Devos 2014; Rivas-Marin, Canosa, and Devos 2016; Boedeker et al. 2017; Rivas-Marín and Devos 2018; Wiegand, Jogler, and Jogler 2018; Lage et al. 2019; Devos, Lage, and Sutcliffe 2020; Rivas-Marin et al. 2020; Wiegand et al. 2020). Ecologically, these bacteria have been found to inhabit most environments, particularly in marine microbiomes (Lage and Bondoso 2014; Wiegand, Jogler, and Jogler 2018; Kohn et al. 2020; Wiegand et al. 2020, 2021; Vitorino and Lage 2022), but were also found to be abundant in terrestrial ones, like soils (Ivanova et al. 2016; Brüssow, Bruessow, and Brüssow 2024). *Planctomycetota* characteristically have large genomes, high G + C content and extensive unknown coding regions, which can be correlated with flexible metabolite production (Kallscheuer and Jogler 2021).

Varied studies have highlighted the diversity and complexity of the biosynthetic gene clusters (BGCs) present in planctomycetal strains (Jeske et al. 2013; Graça, Calisto, and Lage 2016; Wiegand et al. 2020; Kallscheuer and Jogler 2021). In vitro bioactivity screenings have also demonstrated that *Planctomycetota* are indeed capable of producing bioactive metabolites, however, the compounds responsible for these effects remain unidentified (Graça, Calisto, and Lage 2016; Jeske et al. 2016; Yadav et al. 2018; Calisto et al. 2019; Belova, Saltykova, and Dedysh 2020; Vitorino, Klimek, et al. 2022; Vitorino, Lobo-da-Cunha, et al. 2022; Vitorino et al. 2024; Kumar et al. 2024).

Based on published studies on *Planctomycetota* chemistry, the existing collection of natural products derived from these bacteria is still rather limited. It encompasses only four chemically distinct structural types of molecules (Figure 1), amounting to a total of 11 individual compounds, all recently reported. These consist of: (1) three carotenoids, including a rare one, of the saproxanthin family identified in two different *Planctomycetia* strains (Kallscheuer et al. 2019), (2) 3,5-dibromo-*p*-anisic acid, a small halogenated molecule that was isolated from a novel yet undescribed planctomycete, likely belonging to the order *Pirellulales* (Panter et al. 2019), which was considered to have herbicidal activity, (3) stieleriacines A-E, novel *N*-acylated tyrosines with mild antimicrobial effects on Gram positive bacteria (Kallscheuer et al. 2020; Sandargo et al. 2020) and (4) two fatty acids obtained from a marine *Rhodopirellula baltica* strain, including a novel chlorinated one and the known malyngic acid (Lee et al. 2011). Out of these, only stieleriacines and the chlorinated fatty acid were classified as novel compounds, while the other molecules were already previously reported as naturally occurring products in other organisms (Venkateswarlu and Chavakula 1995; Shindo et al. 2007; Shindo and Misawa 2014; Campos et al. 2017).

Motivated by the lack of data on *Planctomycetota* sourced natural products, this study focused on investigating the production of novel secondary metabolites by the recently isolated marine planctomycetal strain *Alienimonas chondri* LzC2^T (Vitorino et al. 2020; Vitorino, Albuquerque, et al. 2021), which previously showed bioactivity potential. In the present study, we describe the isolation and chemical characterisation of a novel chlorinated molecule, which was designated as alichondrichlorin, as well as its in vitro anti-proliferative effects on diverse human tumoral and non-tumoral cell lines.

2 | Experimental Procedures

2.1 | Cultivation of *Alienimonas chondri* Strain LzC2^T and Organic Extraction of Metabolites

Alienimonas chondri strain LzC2^T (=CECT 30038^T=LMG 31701^T) is a pink pigmented strain that was previously isolated from the biofilm of the macroalgae *Chondrus crispus*, collected in a rocky beach near Porto, in Portugal (Vitorino et al. 2020; Vitorino, Santos, et al. 2021). The 16S rRNA gene phylogenetic analysis of

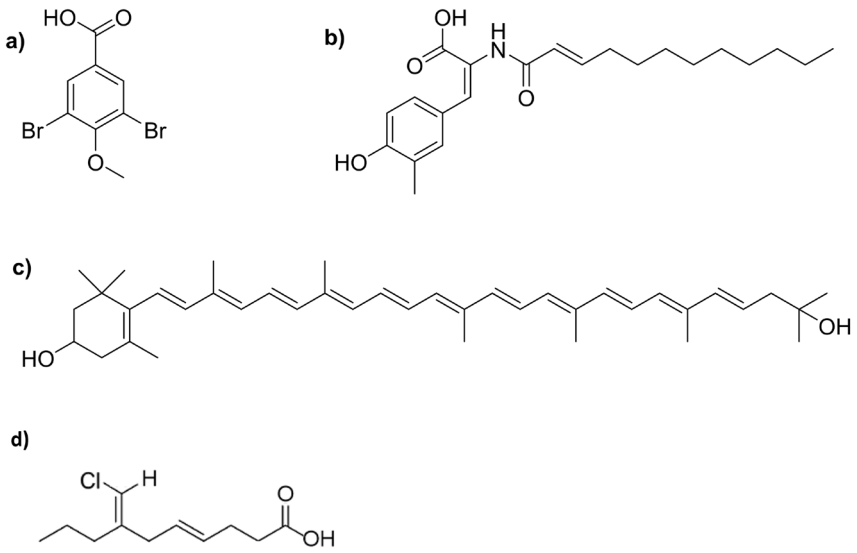

FIGURE 1 | Current collection of planctomycetal chemotypes: (a) 3,5-dibromo-p-anisic acid ($C_8H_6Br_2O_3$) (Panter et al. 2019), (b) stieleriacine A ($C_{22}H_{31}NO_4$) (Kallscheuer et al. 2020), (c) saproxanthin ($C_{40}H_{56}O_2$) (Kallscheuer et al. 2019) and (d) a novel chlorinated fatty acid: (4*E*,7*E*)-7-(Chloromethylene)dec-4-enoic acid (Lee et al. 2011).

this strain classified it as a novel species in the bacterial phylum *Planctomycetota* (class *Planctomycetia*, order *Planctomycetales* and family *Planctomycetaceae*) (Vitorino et al. 2020; Vitorino, Albuquerque, et al. 2021; Vitorino, Santos, et al. 2021).

For the purpose of chemical analysis and molecule isolation, strain LzC2^T was cultivated on a large scale (57 L) in glass flasks of 1 L each containing 750 mL of the culture medium M600 (Figure S1), prepared as previously described (Lage and Bondoso 2011; Vitorino, Santos et al. 2021). Cultures were incubated for 7 days at 25°C under constant shaking (120 rotations per minute-rpm). The biomass was separated from the broth through centrifugation [3600 rpm for 10 min in a 5810R Centrifuge (Eppendorf, Hamburg, Germany)] and the collected cell pellet was freeze-dried to yield 103 g of cellular material. Cells were extracted with an acetone/methanol mixture (1:1) for several consecutive periods of 1 h while under agitation (120 rpm). Organic solvents were then collected by filtration through cheese cloth and Whatman No 1 filter paper in a Büchner funnel and dried in a rotatory vacuum evaporator (Rotavapor R-100, BUCHI, Flawil, Switzerland) to obtain a crude extract (1.5 g).

2.2 | Isolation of Alichondrichlorin

The organic extract previously obtained was mixed with generic silica gel 80 g in a Silia*Sep*80 g cartridge (FLH-R10030B-ISO80, SILICYCLE) and fractioned by flash chromatography in a Pure C-850 FlashPrep equipment (BUTCHI). The mobile phase (80-min run, 12 mL/min flow rate) was constituted by mixture of hexane (A), ethyl acetate (B) and methanol (C), following diverse linear gradients: 30 min ascend from 90% A + 10% B to 0% A + 100% B, 10 min 100% B, 30 min ascend to 0% B + 100% C and 10 min 100% C.

The composition of each fraction obtained was then evaluated by high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) conducted as described previously (Martin et al. 2014), and the obtained analytical data searched against MEDINA's proprietary database (Perez-Victoria, Martin, and Reyes 2016) and the Dictionary of Natural Products (DNP) to dereplicate possible known compounds (Taylor and Francis 2023).

Purification of the fraction containing the compound of interest (**1**) was achieved through high performance liquid chromatography in a Gilson GX-281 HPLC (Gilson Technologies, Middleton, WI, USA) with a reversed-phase C8 column (Zorbax RX-C8, 9.4 × 2.5 nm, 5 μm particle diameter). The mobile phase was composed of HPLC grade water (A) and acetonitrile (B), both supplemented with 0.1% trifluoracetic acid (TFA), eluted in a linear gradient of 75%–87% B in 31 min (3.6 mL/min), with UV detection set at 210 nm and 254 nm. Fractions containing the molecule of interest (**1**) were combined and repurified using the same stationary phase with a linear gradient of 80%–85% B (+0.1% TFA) in 38 min (3.6 mL/min) to yield 0.5 mg of alichondrichlorin with a retention time of 23.7 min.

Alichondrichlorin (**1**). $[\alpha]_D^{25}$-13.0 (*c* 0.25, MeOH); UV (DAD) λ_{max} 210, 254 nm; for ^{1}H and ^{13}C NMR data see Table 1; (+)-ESI-TOFMS *m/z* 583.3762 $[M+H]^+$ (calcd for $C_{33}H_{56}{}^{35}ClO_6{}^+$, 583.3760, Δ +0.3 ppm); 600.4025 $[M+NH_4]^+$ (calcd for $C_{33}H_{59}{}^{35}ClNO_6{}^+$, 600.4025, Δ 0 ppm).

TABLE 1 | NMR data (CD_3OD, 500 MHz) for alichondrichlorin (**1**).

Position	δ_C, type	δ_H, mult. (*J* in Hz)	COSY	HMBC (H to C)
1	172.4[a], C			
2	64.9, CH	4.34, d (4.43)	H-3	C-1, C-3, C-4
3	73.3, CH	4.03, m	H-2, H-4	C-1, C-4, C-5
4	35.1, CH_2	1.57, m	H-3, H-5	C-2, C-3, C-5
5	26.9, CH_2	1.28, m		
6–22	30.6–30.9, CH_2	1.28, m		
23	26.6, CH_2	1.28, m		
24	37.2, CH_2	1.71, m; 1.61, m	H-23, H-25	C-23, C-25, C-26
25	72.6, CH	5.07, hex (6.2)	H-23, H-26	C-1′
26	20.6, CH_3	1.31, d (6.2)	H-25	C-24, C-25
1′	168.1[a], C			
2′	122.9[a], C			
3′, 7′	132.8, CH	7.85, d (8.7)	H-4′, H-6′	C-1′, C-5′
4′, 6′	116.3, CH	6.82, d (8.7)	H-3′, H-7′	C-2′, C-5′
5′	163.6[a], C			

[a]Determined from the indirect dimension of the HMBC spectrum.

2.3 | General Experimental Procedures

Optical rotations were measured in a Jasco P-2000 polarimeter (JASCO Corporation, Tokyo, Japan) in methanol. 1D- and 2D-NMR spectra were recorded on a Bruker Avance III spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) equipped with a 1.7 mm TCI MicroCryoProbe (Bruker Biospin, Fällanden, Switzerland). Chemical shifts were reported in ppm using the signals of the residual solvents as internal reference (δ_H 3.31 and δ_C 49.1 for CD_3OD). LC-UV-ESI-TOF analysis was performed using a Bruker maXis QTOF (Bruker Daltonik GmbH, Bremen, Germany) mass spectrometer coupled to an Agilent 1200 LC (Agilent Technologies, Waldbronn, Germany) under conditions already described (Martin et al. 2014).

2.4 | In Vitro Evaluation of Tumoral Anti-Proliferative Activity

Biological effects of the isolated molecule (**1**) were evaluated using the MTT assay (Mosmann 1983), performed in a high-throughput 96-well-plate format according to MEDINA's workflow (Subko et al. 2021). Pure compound (**1**) was tested in triplicate starting at a concentration of 50 μM and following serial ½ dilutions, for 72 h, against a panel of 5 tumour cell lines including: human skin melanoma A2058 (ATCC CRL-11147), human lung carcinoma A549 (ATCC CCL-185), breast adenocarcinoma MCF-7 (ATCC HTB-22), pancreas carcinoma MIA PaCa-2 (ATCC CRL-1420) and hepatocyte carcinoma Hep G2 (ATCC HB-8065). As a cytotoxicity control, the compound was additionally tested against the non-tumoural human cell line THLE-2 (ATCC, CRL-2706). Data resulting from the assays was interpreted and analysed using the Genedata Screener Software and the EC_{50} values (half maximal effective concentration) determined accordingly (Cautain et al. 2015).

3 | Results

3.1 | Isolation and Structural Elucidation of Alichondrichlorin

A scaled-up cultivation of the recently isolated and characterised marine planctomycetal strain *Alienimonas chondri* LzC2^T (Vitorino et al. 2020; Vitorino, Albuquerque, et al. 2021) was subjected to chemical analyses to search for possible novel bioactive secondary metabolites.

The HPLC-HRMS analysis of one of the fractions obtained after flash chromatography detected the presence of a putative novel molecule that was purified through semi-preparative reversed phase HPLC (Figure S2).

The new compound, alichondrichlorin (**1**) (Figure 2a), was obtained as a white amorphous solid. (+) ESI-TOF analysis identified a protonated adduct $[M+H]^+$ at *m/z* 583.3762 (Figure S3), consistent with a molecular formula of $C_{33}H_{55}ClO_6$ and an isotopic distribution confirming the presence of one chlorine atom in the molecule (Figure S4). Inspection of its ^{1}H, ^{13}C and 2D (COSY, HSQC, TOCSY, NOESY and HMBC) NMR spectra (Figures S5–S12) revealed the presence in the molecule of a 1,4-disubstituted benzene ring (δ_C 132.8 and 116.3; δ_H 7.85 and 6.82 ppm, respectively), three signals accounting for oxygenated/chlorinated methines (δ_C 73.3, 72.6 and 64.9; δ_H 4.03, 5.07 and 4.34, respectively) one doublet methyl group (δ_C 20.6; δ_H 1.31) and signals for a long aliphatic chain (δ_C 30.6–30.9; δ_H 1.28). Key COSY and HMBC correlations (Table 1, Figure 2b,c) allowed to establish the substructures A and B depicted in Figure 2. Particularly, the attachment of the *p*-hydroxybenzoic moiety to C-25 was secured by a weak HMBC correlation between H-25 and C-1′ and the low field chemical shift of H-25 in substructure A. Substructures A and B were connected by a long aliphatic chain whose length was established based on the molecular formula determined for the compound.

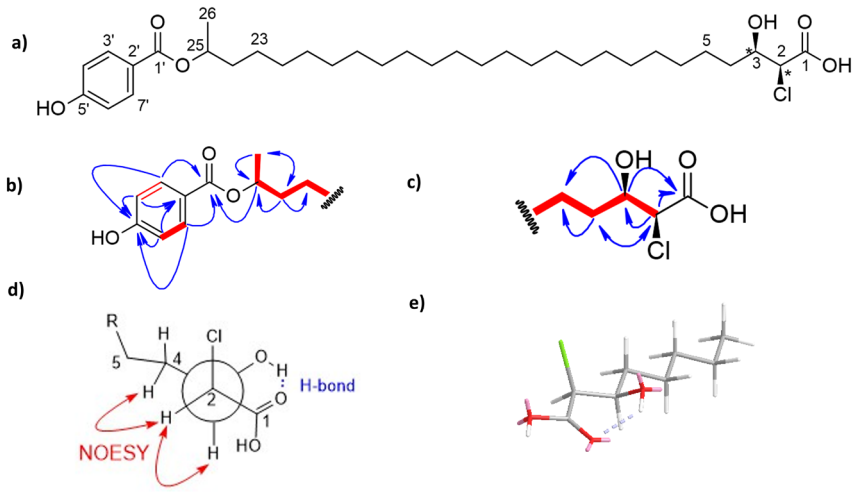

FIGURE 2 | Planar structure of alichondrichlorin (a), key COSY and HMBC correlations of substructures A and B (b and c, respectively), key NOESY correlations showing the relative stereochemistry of the chlorohydrin moiety (d) and proposed main conformation of the chlorohydrin moiety (e).

Once the planar structure of **1** was established, we investigated the relative configuration of the chlorohydrin moiety. It was proposed to be *syn* (*threo*) (Figure 2a,d,e) based on the comparison of the obtained experimental NMR data with published ones for similar structural moieties (Masuda et al. 1994; Kaluzna et al. 2005; Hirose et al. 2008). For **1**, coupling constant $J_{H2\text{-}H3}$ was found to be small (4.3 Hz in CD_3OD and 2.8 Hz in $CDCl_3$). A ^1H-^1H coupling constant of 8 Hz was measured in structurally similar bromohydrins (carrying a carbonyl next to the halogenated position) with *anti* (erythro) configurations (Masuda et al. 1994). These bromohydrin-containing molecules meet the Stiles-House rule due to intramolecular hydrogen bond between the carbonyl and the hydroxy group of the bromohydrin moiety, a behaviour that can also be extended to chlorohydrins next to carbonyl groups (Hirose et al. 2008). Coupling constants reported for α-chloro, β-hydroxy-ethyl esters with both *syn* and *anti* relative configurations for the chlorohydrin moiety can likewise be safely employed for comparison purposes (Kaluzna et al. 2005). Based on these studies, an *anti* (*erythro*) relative configuration in the chlorohydrin moiety of **1** is not compatible with the small observed $J_{H2\text{-}H3}$ and therefore, the relative configuration of the chlorohydrin

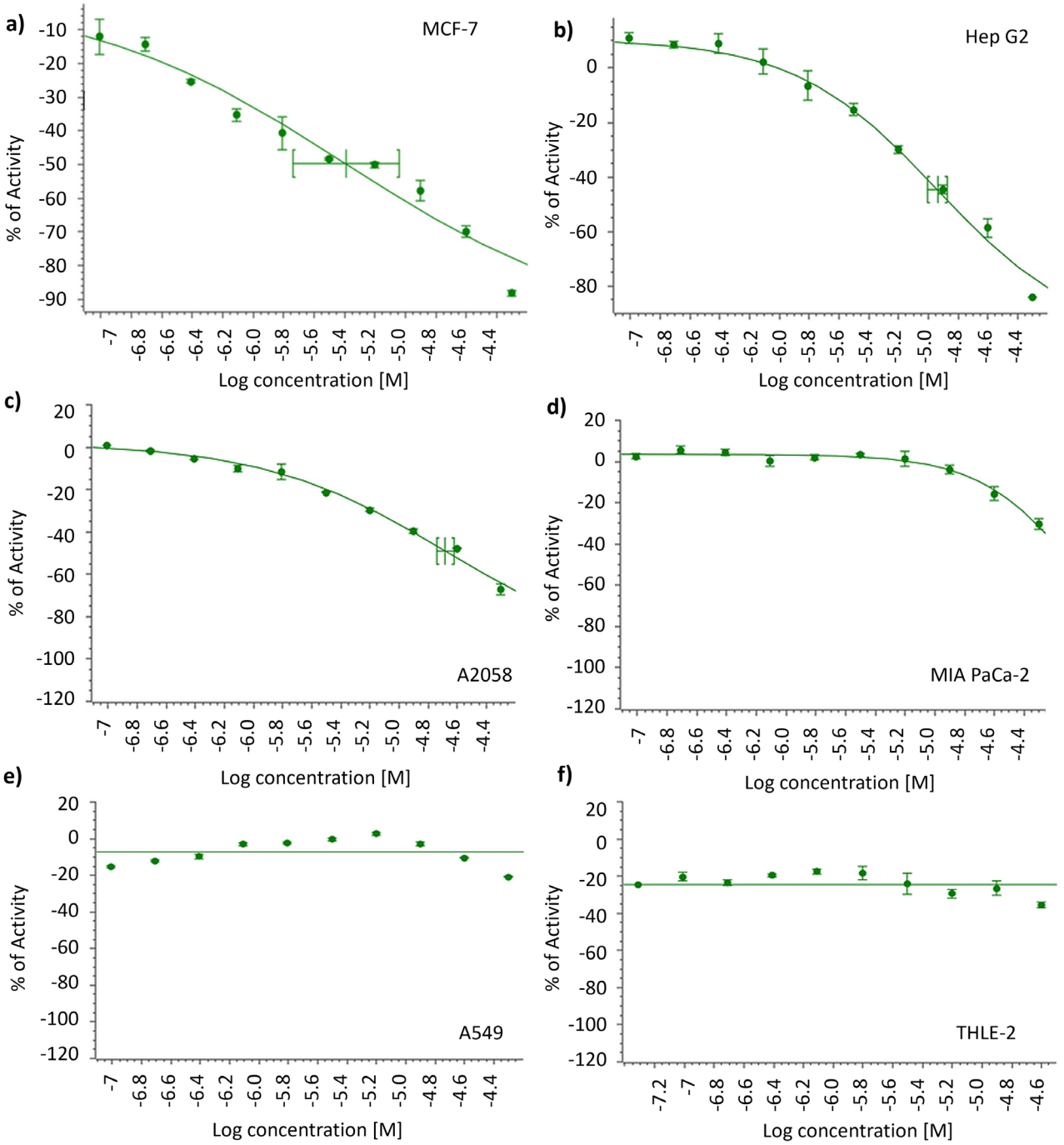

FIGURE 3 | Dose–response curve of alichondrichlorin tested against MCF-7 (a), Hep G2 (b), A2058 (c), MIA PaCa-2 (d) and A549 (e) human tumoral cell lines plus the non-tumoral THLE-2 cell line (f). Highest concentration tested was 50 μM. Activity of −100% corresponds to total inhibition of cellular growth.

moiety was established as *syn* (*threo*). Key NOESY correlations observed in $CDCl_3$ further support this proposal (Figure 2d). The configuration of C-25 remained undetermined. Paucity of sample impeded to perform hydrolysis and Mosher analysis to confirm the absolute configuration of the molecule.

3.2 | Tumoral Cytotoxic Activity of Alichondrichlorin

In vitro biological evaluation of alichondrichlorin was performed against a panel of diverse human tumoral cell lines and one non-tumoral cell line, to evaluate if the effect is specific on tumour cells. Respective dose–response curves obtained are represented in Figure 3. This molecule demonstrated a strong growth inhibitory effect in human breast adenocarcinoma MCF-7, with a calculated EC_{50} value of 4.06 μM (CI 95% 1.82–9.06 μM) (Figure 3a). EC_{50} values of 11.5 μM (CI 95% 9.8–13.5 μM) and 20.8 μM (CI 95% 18.1–23.7 μM) were observed in hepatocyte carcinoma Hep G2 and human skin melanoma A2058 (Figure 3b,c), respectively. At the highest concentration tested (50 μM), no substantial effects were observed against human lung carcinoma A549 or pancreas carcinoma MIA PaCa-2, as well as against the non-tumoral cell line THLE-2 from a human liver (Figure 3d–f), highlighting the targeted cytotoxicity possessed by this molecule.

4 | Discussion

The new molecule obtained in this study was designated as alichondrichlorin after the taxonomic name of its producer species (*Alienimonas chondri*). This report expands the currently limited collection of described bioactive planctomycetal metabolites.

The most striking structural feature in alichondrichlorin is its substructure containing a chlorohydrin moiety (Figure 2, substructure B). Chlorinated natural products are relatively common and of significant pharmacological interest, often exhibiting potent biological effects against various life-threatening health conditions, as well as a wide range of industrial applications (Zeng and Zhan 2019). Chlorine-based drugs (either of natural or synthetic origin) are currently responsible for more than 250 FDA approved medicines, as well as still being relevant candidates in earlier testing phases (Fang et al. 2019). Although chlorine-containing metabolites are commonly found, naturally occurring chlorohydrins, in particular, are rare. Specifically, 2-chloro-3-hydroxy-carboxylate substructures like the one present in alichondrichlorin were only found in other two natural products, utililactone and epiutililactone (Xu et al. 2007). These two molecules are small hemiterpenes isolated from the leaves of the plant *Prinsepia utilis* and were described as having immunosuppressant properties, specifically inhibiting lymphocyte transformation, although no data is available regarding their cytotoxic properties (Xu et al. 2007). Despite their shared moiety, utililactone and epiutililactone are structurally dissimilar from alichondrichlorin, confirming the novelty and interest of our new molecule.

Regarding the other substructure in alichondrichlorin (Figure 2, substructure A), we found for comparison a study that analysed the cytotoxic effects of several compounds with similar (but not equally substituted) hydroxybenzoic moieties (Sandoval-Acuña et al. 2016). In this study, several triphenylphosphonium (TPP+) linked decyl polyhydroxybenzoates were evaluated against several human tumoral breast cell lines (Sandoval-Acuña et al. 2016). The authors found that differently substituted decyl-polyhydroxybenzoates-TPP+ derivatives were potent anti-tumour agents by targeting the mitochondria. However, two control molecules lacking the TPP+ but containing the hydroxybenzoic moiety were inactive towards the same tumour cell lines, while the contrary (control molecule lacking the hydroxybenzoic moiety but containing the TPP+) maintained high activity levels, including towards the MCF-7 tumour cell line, one of those used in our present study. This supports even more our hypothesis that the chlorohydrin moiety in alichondrichlorin is the one most likely conferring the reported biological proprieties to the molecule.

5 | Conclusions

The isolation and characterisation of the novel bioactive secondary metabolite alichondrichlorin is herein described.

The structure of alichondrichlorin contains a chlorohydrin moiety with demonstrated targeted biological activity against a human breast adenocarcinoma cell line, highlighting its possible pharmacological interest. Examples of natural chlorohydrins with these kind of substructure are rare, confirming the novelty and interest of this moiety as a new scaffold conferring interesting biological properties.

Our study has contributed to increase the still very small collection of planctomycetal natural products while it confirms that *Planctomycetota* are indeed promising, but still untapped, sources of chemical novelty likely possessing biotechnological applications.

Outside of the scope of this present study, future work related to alichondrichlorin is essential to help decipher the biosynthetic route responsible for the production of such metabolites. Due to the small amount of alichondrichlorin isolated from a substantial culture volume (not easily obtained in slower-growing organisms), optimisation experiments for metabolite production are crucial to increase the final compound yield. Furthermore, information of the biological effects of this compound can be completed by screening its inhibitory activity against other tumour cell lines, namely drug-resistant ones, as well as performing druggability and mode of action tests.

Author Contributions

Inês R. Vitorino: conceptualization, methodology, investigation, formal analysis, writing – original draft, writing – review and editing. **José D. N. Santos:** methodology, investigation, writing – review and editing. **Gloria Crespo:** methodology, writing – review and editing. **Ignacio Pérez-Victoria:** methodology, investigation, formal analysis, writing – review and editing. **Jesús Martín:** methodology, investigation, formal analysis, writing – review and editing. **Lorena Rodriguez:** methodology, investigation, formal analysis, writing – review and editing. **Maria C. Ramos:** methodology, formal analysis, writing – review and editing. **Teresa P. Martins:** methodology, writing – review and editing. **Pedro N. Leão:** supervision, writing – review and editing. **Francisca Vicente:** supervision, writing – review and editing. **Vítor Vasconcelos:** supervision, writing – review and editing. **Olga M. Lage:** conceptualization, supervision, writing – review and editing. **Fernando Reyes:**

conceptualization, formal analysis, supervision, writing – original draft, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the supporting information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.