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Monogalactosyldiacylglycerols, potent nitric oxide inhibitors from the marine microalga *Tetraselmis chui*

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Methanolic extracts of some marine and freshwater microalgae were tested for their nitric oxide (NO) inhibitory activity on lipopolysaccharide-induced NO production in RAW264.7 macrophage cells. Among the tested extracts, *Tetraselmis chui* extract showed the strongest NO inhibitory activity, thus selected for further study. NO inhibitory activity guided isolation led to identification of two monogalactosyldiacylglycerols (MGDGs) (2*S*)-1-*O*-(6*Z*,9*Z*,12*Z*,15*Z*-octadecatetranoyl)-2-*O*-(4*Z*,7*Z*,10*Z*,13*Z*-hexadecatetranoyl)-3-*O*-*β*-D-galactopyranosylglycerol (**1**) and (2*S*)-1-*O*-(9*Z*,12*Z*,15*Z*-octadecatrinoyl)-2-*O*-(4*Z*,7*Z*,10*Z*,13*Z*-hexadecatetranoyl)-3-*O*-*β*-D-galactopyranosylglycerol (**2**) from the MeOH extract of *T. chui*. The stereo-chemistry of **1** was elucidated by classical degradation method. MGDGs **1** and **2** showed strong NO inhibitory activity compared to N^G-methyl-L-arginine acetate salt, a well known NO inhibitor used as a positive control. Isolated MGDGs suppressed NO production through down-regulation of inducible NO synthase protein. A structure activity relationship study suggested that the polyunsaturated fatty acids of the MGDGs are responsible for NO inhibition. Moreover, increasing unsaturation on the fatty acid side chains enhanced the NO inhibitory potency of the MGDGs.

Keywords: monogalactosyldiacylglycerol; nitric oxide; *Tetraselmis chui*; iNOS; microalgae; galactolipid; glycolipid

1. Introduction

Natural products are leading sources of novel molecules that have been used in the pharmaceutical and nutraceutical industries since their inception. The majority of the natural products currently on the market as therapeutic agents or as health supplements are derived from terrestrial organisms including plants, animals and micro-organisms, even though the oceans cover more than 70% of the Earth's surface. The oceans contain the majority of the biodiversity of the planet and are an enormous resource for new biologically active compounds. Omega-3 fatty acids derived from fish oil provide an example of valuable natural products from the ocean with several known health benefits (Simopoulos, 1991) and are widely used in the functional food and nutraceutical industries. Microalgae, which are at the bottom of the marine food chain, are considered a primary source of diverse bioactive natural products (Skulberg, 2000).

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Considerable interest has been focused on these unicellular photosynthetic organisms due to their potential capacity to produce biofuels and as a source of a wide range of commercially interesting byproducts including proteins, fats, sugars, carotenoids and many others (Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). Currently, a small number of microalgal species are intensively cultivated to support commercial shellfish aquaculture. These include members of the genus *Tetraselmis* isolated for their favourable nutritional qualities, such as high levels of the essential fatty acid 20:5 $n-3$ (Wikfors et al., 1996).

Nitric oxide (NO) is an important signalling molecule that acts in many tissues to regulate a diverse range of physiological processes. It is a weak radical produced from L-arginine via the enzyme NO synthase (NOS). NO acts as a host defence mechanism by damaging pathogenic DNA and as a regulatory molecule in homoeostatic activities (Kuo & Schroeder, 1995). However, excessive production of NO catalysed by inducible NOS (iNOS) is pathogenic to host tissues (Aktan, 2004). Thus, effective inhibition of NO accumulation represents a beneficial therapeutic strategy for the treatment of NO-mediated disorders.

National Research Council (NRC) Canada recently launched a national programme to develop novel microalgae cultivation technologies with the long-term aim of converting CO_2 emissions from large point source emitters into renewable fuels and other value-added products through the scaled production of microalgal biomass. Through this programme, a diverse collection of algal strains have been cultivated and tested for potential biological applications. In this report, we describe the isolation and identification of two monogalactosyldiacylglycerols (MGDGs) from a marine microalga *Tetraselmis chui*, and their NO inhibitory activity in lipopolysaccharide (LPS)-induced NO production in RAW264.7 cells.

2. Results and discussion

Marine and freshwater microalgae, namely *Botryococcus braunii, Chlorella sorokiniana, Chlorella vulgaris, Isochrysis galbana, Nannochloropsis granulata, Neochloris oleoabundans, Pavlova lutheri, Pavlova pinguis, Phaeodactylum tricornutum, Porphyridium aerugineum, Scenedesmus dimorphus* and *T. chui*, acquired from various culture collections were cultivated independently in f/2 medium under tightly controlled conditions in a 500 L internally illuminated photobioreactor (Craigie, Armstrong, Staples, & Bauder, 2003). Harvested algal biomass was freeze dried and extracted with MeOH. The MeOH extracts were subsequently tested for NO inhibitory effects. MeOH extract of *T. chui* showed strong and dose-dependent NO inhibition, reducing 47.7% of NO production in LPS-induced RAW264.7 macrophage cells at 50 $\mu g\,mL^{-1}$ concentration (Figure 1 and Table S1), and was selected for further NO inhibitory activity guided isolation. The extract was then partitioned into hexane, chloroform and aqueous fractions by liquid–liquid extraction. The hexane and chloroform fractions showed significant NO inhibitory activity while the aqueous fraction had very weak NO inhibitory activity (Figure 1a). The ^{1}H-NMR spectra of both hexane and chloroform fractions had identical signals corresponding to polyunsaturated fatty acids (PUFAs) and sugar in addition to chlorophyll signals, indicating that both fractions contained similar compounds responsible for NO inhibition. The hexane fraction was sub-fractionated into three sub-fractions by preparative TLC. The sub-fraction 2 containing MGDGs showed relatively stronger NO inhibition among the three sub-fractions (Figure 1b), which led to isolation of two MGDGs i.e. **1** and **2** by semi-preparative HPLC (Figure 2).

(2*S*)-1-*O*-(6*Z*,9*Z*,12*Z*,15*Z*-octadecatetranoyl)-2-*O*-(4*Z*,7*Z*,10*Z*,13*Z*-hexadecatetranoyl)-3-*O*-β-D-galactopyranosylglycerol (**1**) was isolated as a colourless film with the molecular

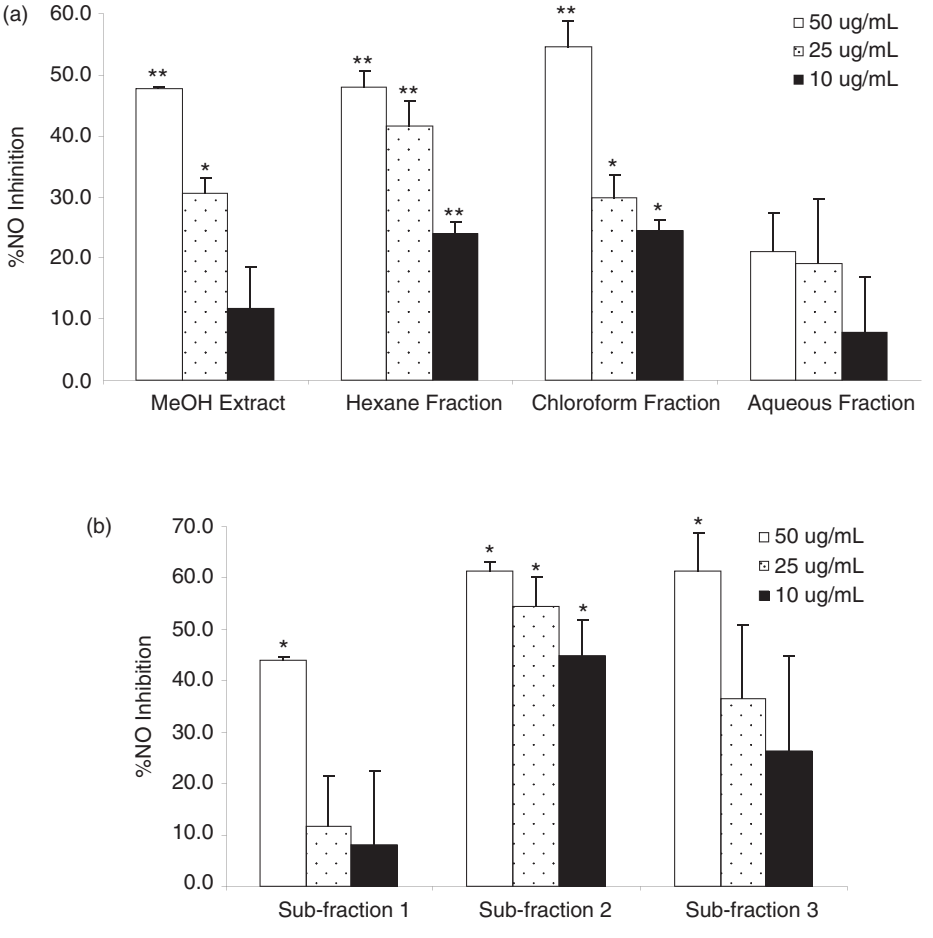

Figure 1. NO inhibitory effects of *T. chui* extract, fractions and sub-fractions: (a) MeOH extract and the hexane, chloroform and aqueous fractions and (b) sub-fractions 1, 2 and 3 obtained after preparative TLC of hexane fraction. Significantly different from the LPS-treated control: $*p < 0.05$ and $**p < 0.01$.

formula $C_{43}H_{66}O_{10}$. The ^{1}H- and ^{13}C-NMR spectra of **1** (Table S2) displayed signals corresponding to 8 unsaturated double bonds, 6 oxygenated methine groups, 17 methylene groups including 3 oxygenated methylene, 2 primary methyl groups and two 2 carbonyl groups suggesting that **1** was composed of a sugar, a glycerol and two PUFAs. In depth, spectral analyses including 2D-NMR led to the identification of a terminal *β*-D-galactopyranose and a glycerol moiety. This was further confirmed by alkaline hydrolysis, which gave 2 *R*-1-*O*-*β*-galactopyranosylglycerol (Oshima, Yamada, Matsunaga, Moriya, & Ohizumi, 1994), and two fatty acid methyl esters. The fatty acids were identified as 6,9,12,15-octadecatetranoic acid and 4,7,10,13-hexadecatetranoic acid attach to glycerol at C-1 and C-2, respectively, via ester bond by spectral analysis (Figures S1 and S2) and further confirmed by GC/mass spectrometry (MS) analysis of the methyl esters obtained after alkaline hydrolysis. Since, allylic carbon signals of *Z*- and *E*-isomers are observed at δ_C 27–28 and 32–33, respectively (Andrianasolo et al., 2008; Jung, Lee, & Kang, 1996), the 26.5 and 26.4 ppm shifts of all *bis*-allylic methylene carbons in **1** suggested that all

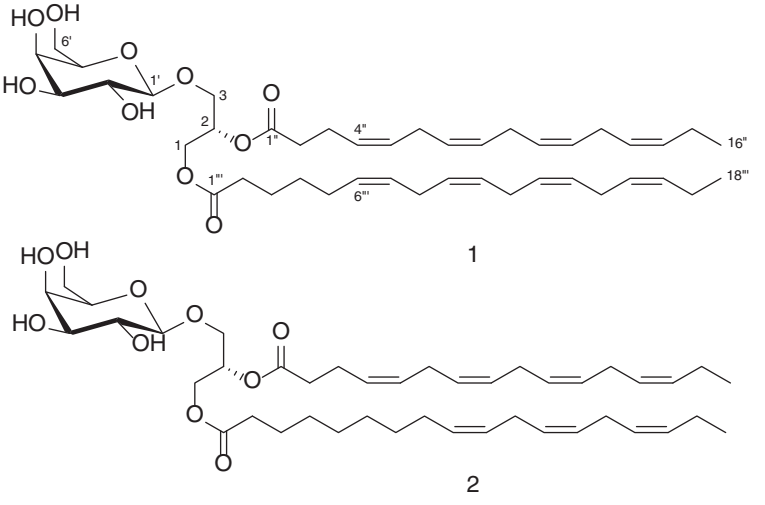

Figure 2. Structure of MGDGs (**1** and **2**) isolated from *T. chui*.

double bonds have a *cis*-geometry (*Z*). Accordingly, stereo-structure of **1** was elucidated as (2*S*)-1-*O*-(6*Z*,9*Z*,12*Z*,15*Z*-octadecatetranoyl)-2-*O*-(4*Z*,7*Z*,10*Z*,13*Z*-hexadecatetranoyl)-3-*O*-*β*-D-galactopyranosylglycerol. Most recently, Xu, Chen, Yan, Chen, and Zhou (2010) identified a similar MGDG from a marine diatom *Stephanodiscus sp*. using UPLC coupled with electrospray ionisation-quadrupole-time of flight MS, though that report neither described the position of the double bonds on the fatty acid chains nor their geometry. The stereo-chemistry of the glycerol moiety at C-2 chiral centre was also unsolved. It is worthwhile to mention here that this study describes the first time **1** was isolated and stereo-structure was well established by spectral analyses and classical chemical degradation method.

(2*S*)-1-*O*-(9*Z*,12*Z*,15*Z*-octadecatrinoyl)-2-*O*-(4*Z*,7*Z*,10*Z*,13*Z*-hexadecatetranoyl)-3-*O*-*β*-D-galactopyranosylglycerol (**2**) was also isolated as a colourless solid with the molecular formula $C_{43}H_{68}O_{10}$. The ^{1}H- and ^{13}C-NMR signals of **2** were identical to that of **1** except the absence of one double bond. The fatty acid at C-1 position of **2** was determined as 9,12,15-octadecatrinoyl acid instead of 6,9,12,15-octadecatetranoic acid as in **1**. Furthermore, spectral data of **2** were identical to the previously reported data (Greca, Monaco, Previtera, & Parrilli, 1989). To the best of our knowledge this is the first report of **2** from *T. chui*.

Both monogalactosylglycerols **1** and **2** isolated from MeOH extract of *T. chui* were tested for their NO inhibitory activity and demonstrated a potent NO inhibitory effect as compared to N^{G}-methyl-L-arginine acetate salt (shortly L-NMMA), a well-known NO inhibitor used as a positive control in this study (Figure 3). These results strongly suggested that MGDGs are primarily responsible for the NO inhibitory activity of *T. chui* MeOH extract. This is also supported by the fact that **1** and **2** are the major components of the chloroform fraction as determined by HPLC analysis (Figure S4). Galactolipids and pigments including carotenoids present in sub-fractions 1 and 3 and their possible synergistic effect may also contribute to the NO inhibition of MeOH extract as these fractions also possessed NO inhibitory activity (Murakami et al., 2000). MGDG **1,** having an additional double bond on the PUFA side chain attached at C-1 of the glycerol moiety

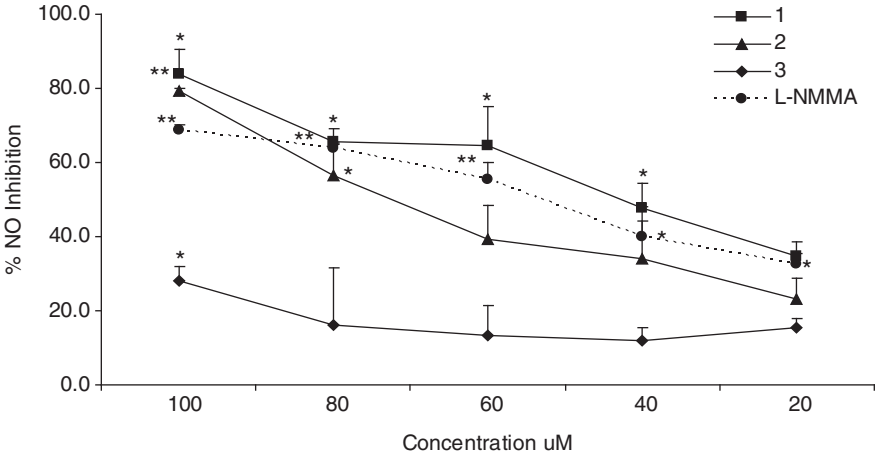

Figure 3. NO inhibitory effects of **1–3** and L-NMMA. Significantly different from the LPS-treated control: $*p < 0.05$ and $**p < 0.01$.

possessed better NO inhibitory activity than **2**, suggesting that increasing unsaturation in the PUFA side chain may enhance the NO inhibitory activity of the glycolipid. To examine the structure activity relationship, we further tested 2 *R*-1-*O*-*β*-D-galactopyranosylglycerol obtained after NaOMe mediated hydrolysis. 2 *R*-1-*O*-*β*-Galactopyranosylglycerol showed no NO inhibition even at 100 μM concentration suggesting that the PUFAs are responsible for NO inhibition. This was further supported by the fact that the glycolipid with saturated fatty acids (**3**) (Figure S3) possessed very weak NO inhibitory activity as compared to **1** and **2**.

O'Donnell et al. (1999) described that unsaturated fatty acids react with the reactive nitrogen species derived from NO to yield nitrated oxidation products. In order to determine whether or not the isolated MGDGs simply inhibit NO production by reacting with NO radicals, we further tested the effect of both MGDGs on iNOSs enzyme regulation in RAW264.7 cells. Stimulation of macrophages by LPS increases iNOS protein levels resulting in a high output of NO, one of the most important elements of inflammatory reaction. Both MGDGs down regulated the iNOS protein levels (Figure 4) in LPS-stimulated RAW264.7 macrophage cells suggesting MGDGs **1** and **2** inhibited the NO production through the down-regulation of iNOS accumulation.

In a recent study, Jo et al. (2010) reported the anti-inflammatory effects of an 80% MeOH extract of *Tetraselmis suecica*. They concluded that metabolites present in the hexane and ethyl acetate fractions of the 80% MeOH extract are responsible for the inhibition of NO production in LPS-stimulated RAW 264.7 macrophage cells. Based on our finding in *T. chui* and almost identical extraction and fractionation techniques employed in both studies, it is possible that MGDGs are responsible for the anti-inflammatory activity of *T. suecica* as well. Similarly, Bruno et al. (2005) and Murakami, Nakamura, Koshimizu, and Ohigashi (1995) reported that MGDGs isolated from a blue–green alga and *Citrus hystrix*, respectively, showed grater anti-inflammatory activity than indomethacin in *in vivo* experimental models. These finding further suggest that MGDGs **1** and **2** may have the potential to be new anti-inflammatory agents and further study is warranted.

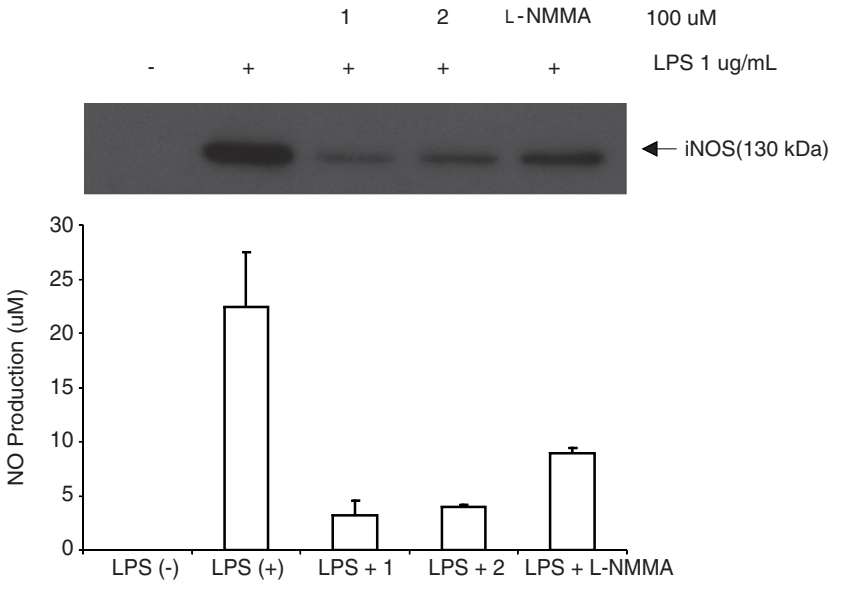

Figure 4. Inhibitory effect of MGDGs (**1** and **2**) and L-NMMA on iNOS expression and the production of NO. Raw 264.7 cells were treated with LPS (1 μg mL^{-1}) for 24 h with or without tested compounds. Protein levels of iNOS were determined by western blot analysis while NO production was determined by absorbance spectrometry in the presence of the Griess reagent.

3. Conclusions

This study demonstrated that the MGDGs isolated from *T. chui* inhibit NO production by reducing iNOS accumulation in LPS-stimulated RAW 264.7 macrophage cells. The structure activity relationship study suggested that the PUFA side chains are essential for NO inhibition of the MGDGs.

Supplementary material

Experimental details are available online, alongside Tables S1–S3 and Figures S1–S4.

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