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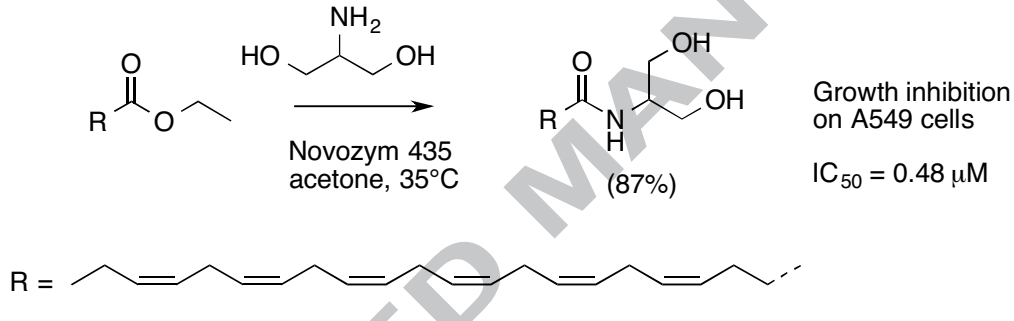

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One-pot Synthesis of Polyunsaturated Fatty Acid Amides with Anti-proliferative Properties

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ABSTRACT

A one-pot environmentally friendly transamidation of ω-3 fatty acid ethyl esters to amides and mono- or diacylglycerols was investigated via the use of a polymer-supported lipase. The method was used to synthesize a library of fatty acid monoglyceryl esters and amides. These new derivatives were found to have potent growth inhibition effects against A549 lung cancer cells.

Keywords: polyunsaturated fatty acid amides, transamidation, environmentally friendly synthesis, growth inhibition

Monoacylglycerols (MAG) of ω-3 polyunsaturated fatty acids (PUFA) have recently been associated with a number of beneficial health effects, such as cardiovascular and cerebral health,[1] and shown some potential against asthma and[2] lipid malabsorption.[3] They also possess anti-inflammatory,[4] anti-proliferative and antioxidant[5] properties which may be beneficial against cancer.[6, 7] Their metabolism is well documented with minimal side effects,[8] and their presence in fish oil extracts has been shown to be beneficial as dietary intake. We have recently demonstrated the anti-proliferative properties of MAG-DHA and MAG-EPA against colon and lung cancer cell lines.[6, 7] In order to better understand the structure-activity relationship (SAR) of this compound class and improve its chemical and metabolic stability compared to native monoacylglycerol esters, a reliable synthetic method allowing rapid analog generation was required. Our first goal was to generate fatty acid amide analogs, with the expectation that they may be more stable compared to their ester counterparts (Fig. 1). Fatty acid amides may additionally possess beneficial anti-inflammatory properties.[9]

We thus embarked on the synthesis of a small library of fatty acid amides of three important polyunsaturated fatty acids: DHA, EPA and DPA. From the onset, we opted for mild, environmentally friendly conditions to effect these transformations. This includes mild reaction conditions, the use of enzymatic or organic catalysis, as well as the preference for class 3 organic solvents[10, 11] and minimal waste generation. Moreover, it should be noted that polyunsaturated fatty acids are prone to double bond migration or degradation when subjected to harsh conditions.[12] Reported PUFA amide derivatives have been prepared by standard peptide coupling reactions from the corresponding acids and amines.[9] In order to avoid additional reagents such as base and peptide coupling reagents, we resorted to direct transamidation from monoethyl esters, which are directly available in large quantities and cheaper.

After careful review of existing methods corresponding to the above requirements[13-19], we retained two approaches: organocatalysis using triazabicyclodecene (TBD), and enzyme-catalyzed transamidation with lipases.

<Insert Figure 1>

Most reported examples of transesterification explored the transformation of triacylglycerol esters to simpler esters such as methyl esters as the final product, using the simpler alcohol as the reaction solvent.[1] This approach turned out to be impractical for the transesterification from ethyl to glyceryl esters or amides, because (1) glycerol and the ω-3 fatty acid ethyl ester are usually non miscible, (2) the high viscosity of glycerol or its derivatives precluded efficient stirring of the reaction mixture, and (3) glyceryl amines being more expensive than glycerol, their use as a solvent would be prohibitive. Furthermore, the method had to

be mild enough to preserve the unconjugated 1,4-*cis*-diene units of the ω-3-PUFA.

Synthesis of glyceryl esters and amides

First attempts of transesterification with triazabicyclodecene, which may have been later transferred onto solid support, were not satisfactory.[20] Reaction optimization along various parameters, including solvent (neat, THF, DMSO, pyridine, water, acetonitrile or mixtures), temperature (0 – 100°C), reagent and catalyst (5-20 mol%) did not produce clean mixtures. The reaction was generally very slow even under the best conditions (4 days for 50% conversion in DMSO at 100°C). Traces of water were detrimental, giving large amounts of the corresponding ω-3-fatty acid as a hydrolysis product. Decomposition and untractable mixtures were frequently observed.

We then turned our attention to lipase-mediated transesterification of DHA ethyl ester to monoacylglycerol as a test reaction. After initial screen we selected two lipases: Lipozyme® and Novozyme 435®, both immobilized on polystyrene beads. After 24 h reaction in glycerol, the reaction with Lipozyme® did not produce any desired MAG ester, while the reaction with Novozyme 435® showed almost complete consumption of the starting ethyl ester by TLC, albeit with several products (Scheme 1). Further analysis by LC-MS and ^{1}H-NMR showed the presence of appreciable amounts of diacylglycerol, some acid, and a small quantity of the desired compound (see below).

<Insert Scheme 1>

For example, reaction of DHA ethyl ester in glycerol at 35°C for 24 h yielded a 16:40:30:14 mixture of monoacylglycerol: diacylglycerol:fatty acid:starting ethyl ester. The predominance of the diester can be explained by the poor solubility of the starting ethyl ester in glycerol compared to the MAG ester, which would favour subsequent reaction of the MAG ester over the ethyl ester. No triacylglycerol was detected in any of these experiments. Formation of the acid was readily explained by the reaction of the ethyl ester with a water molecule; however the occurrence of free fatty acid was much lower in the enzyme-catalyzed compared to the TBD-mediated reaction.

Reaction conditions were subsequently optimized to promote the formation of the MAG ester. Toward this end, several co-solvents able to better mix the starting materials and decrease reaction time were screened. THF, 1,4-dioxane, DMF, DMSO, acetonitrile and acetone were tested at 35°C to optimize enzyme activity,[21] increase fluidity and minimize decomposition. From those experiments, acetone gave the best results (over 50% of the ω-3 fatty acid MAG ester after 5 h of reaction). Acetone is an environmentally benign, low toxicity class 3 solvent[10] so it was chosen as the solvent for further optimization with the amides. Typically, a minimal amount of acetone was required (2 mL per gram of ester).

The first reaction between DHA ethyl ester and 2-Amino-1,3-propanediol gave 87% of the desired amide **1** in 4 h, the main by-product being the acid as a result of hydrolysis. Similar yields were obtained using EPA, DHA and DPA with various amines (Table 1).

<Insert Table 1>

As can be seen in Table 1, the reaction is broadly applicable, both in terms of PUFA ethyl ester and amine. In general, amides were formed preferentially to esters.[22, 23] In the case of more hindered amides (i.e., position 2), an initial reaction of the terminal alcohol followed by intramolecular transamidation may occur to yield the terminal ester as an intermediate, however in those instances no trace of terminally substituted monoester was observed by proton NMR and no other isobaric product was present as measured by LC-MS. Reaction with 1,3-diaminopropan-2-ol provided a mixture of mono amide **2** (48%) and diamide **3** (38%), which were easily separated by flash chromatography. Most derivatives reacted in the same way with similar yields and ease of purification. Exceptions to this observation were the amino acids producing amides **11**, **14** and **20**, which gave lower yields and were more difficult to isolate. In the case of N,N-dimethylaminopropanol, only the alcohol reacted to give ester **27** in low yield. Finally, in **28** the methylamide was obtained preferentially but in low yield, owing to difficult purification.

Growth inhibition results of new derivatives

New compounds were tested for their growth inhibitory effects on lung A549 lung cancer cells (Table 2). An initial screen was performed at a single concentration of 3 μM. In this assay, MAG-DPA gave superior results to MAG-EPA and MAG-DHA, with cell growth inhibition of 74.5%, 51.4% and 59.1%, respectively. In the DHA series, replacement of the glyceryl ester (59.1% growth inhibition) for either a primary (**4**, 88.7%) or secondary amide (**1**, 77.4%) improved growth inhibition. The same held true for diamide **3** (83.8%), whereas monoamide **2** (38.3%) was less efficient than the parent MAG-DHA. Similarly to secondary amide **1**, the analogue with a carboxylate substituent (**20**) displayed reasonable growth inhibition (68.7%). The best compounds in this series were analogs **7** and **15** (98.4 and 98.3%, respectively), whereas truncated mono- and diamide derivatives **16** and **17** (29.3 and 13.0% growth inhibition, respectively) were less efficacious compared to MAG-DHA (59.1% growth inhibition).

<Insert Table 2>

Interestingly, the DHA and DPA series gave remarkably different SARs. Indeed, whereas DHA ethanolamide **16** had modest inhibitory effect on growth of A549 cells, its DPA analog **21** demonstrated a robust effect (29.3 vs 96.9%). On the other hand, both DHA and DPA diamides **3** and **22** displayed robust growth inhibition, the latter being again stronger (83.8 vs 97.7%). Likewise, the same trend was observed for 2-substituted amides **1** and **25** (77.4 vs 97.4%), in favour of DPA.

<Insert Figure 2>

The IC_{50} of DHA analogs **1** and **15** was further determined as indicated in Figure 2, with values of 0.48 and 0.25 μM, respectively, superior to the parent ester MAG-DHA (IC_{50} = 1.25 μM).

Conclusion

A one-pot environmentally friendly method for the synthesis of ω-3 fatty amides derived from various amines and ethyl esters of three important ω-3 polyunsaturated fatty acids has been optimized. The method uses minimal quantities of acetone and mild temperature, combined with enzyme-catalyzed transamidation to afford the desired molecule in high yields, without decomposition or conjugation of the unsaturations. It allowed the synthesis of several amides and supported the rapid determination of the SAR of this new class, particularly with respect to the growth inhibitory properties of the compounds on the A549 lung cancer cell line. These are very promising results, and subsequent biological results will be further reported shortly.

Experimental

Representative procedure: synthesis of amide 25

To 500 mg (1.5 mmol) of DPA ethyl ester as an oil were added 100 mg of Novozym 435© lipase on resin and 5 eq (7.5 mmol) of serinol. A minimum amount of acetone was then added to decrease viscosity (1 mL). The mixture was magnetically stirred for 4-18 h at 35°C until TLC indicated complete disappearance of the ethyl ester (to be noted, LC-MS methods with UV detection are not useful to follow these reactions owing to the very low absorbance of the chromophores). The reaction was then filtered, acetone was evaporated then water added. The mixture was extracted with a minimal amount of diethyl ether (1 mL) thrice. The organic phase was dried with magnesium sulphate, filtered then evaporated under reduced pressure. The crude product was purified by flash chromatography with hexane/ethyl acetate to give the desired amide **25** as a colorless oil in 60% yield (> 90% purity by ^{1}H NMR).

^{1}H NMR (300MHz, $CDCl_3$) δ (ppm): 6.52 (d, 1H, 7.2 Hz), 5.43-5.29 (m, 10H), 4.98 (s, 2H), 3.94-3.91 (m, 1H), 3.81 (dd, 2H, 3.9 Hz, 4.2 Hz), 3.70 (dd, 2H, 4.8 Hz, and 5.1 Hz), 2.89-2.79 (m, 10H), 2.32 (t, 2H, 8.3 Hz), 2.22 (t, 2H, 7.5 Hz), 2.07 (quint, 2H, 7.1 Hz), 1.63 (quint, 4H, 7.2Hz), 1.42-1.30 (m, 6H), 1.26-1.24 (m, 2H), 0.97 (t, 3H, 7.7 Hz).
^{13}C NMR (75.5 MHz, $CDCl_3$) δ (ppm): 178.57, 174.71, 132.02, 130.01, 128.56, 128.41, 128.23, 128.13, 128.01, 127.90, 127.03, 61.93, 52.45, 36.55, 34.19, 29.31, 28.91, 28.80, 27.07, 25.64, 25.53, 24.78, 20.56, 14.29.
IR ($CHCl_3$) ν (cm-1): 3330, 3011, 2931, 2858, 1709, 1650, 1547, 1461, 1266, 1051, 975, 720.
HRMS (M-H$^+$) $C_{25}H_{42}NO_3$; calc: 404.3159; measured: 404.3164

Cell culture

Human A549 lung adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC). A549 cells were maintained in RPMI 1640 (Wisent, St-Bruno, QC, Canada) containing 10 % FBS and 10 units/mL penicillin, 100 µg/mL streptomycin. Cells were grown in a 5% CO_2 incubator at 37°C and used between passage 3 to 6 for all conditions and assays tested in this study.

Growth inhibition assay

A predefined number of A549 cells were allowed to grow in 24 wells plates (1×10^4 cells/well) for 3 days until cells reached 60% confluence. They were then starved in RPMI medium without FBS for 8 h, then the culture medium was replaced with RPMI + 0.2 % FBS and 3 µM of test compound were added to each well. Culture media were changed every 24 h and cells were treated for 48 h. After 48 h, the medium was removed from the culture plates and 0.05% trypsin–EDTA was used to detach the cells from the surface of the culture plates. The harvested cells were counted, both the viable and dead ones, using a Countess Automated Cell Counter (Invitrogen Inc.). Briefly, for each condition an appropriated cell dilution was prepared from the harvested cells and an aliquot was mixed with an equal volume of 0.4% trypan blue, and 10 µL was transferred into each side of a Countess™ chamber slide. All concentrations tested were performed in triplicata and were representative of 5 independent experiments.

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Figures

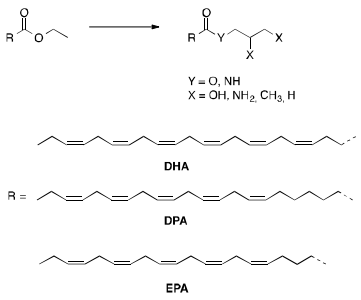
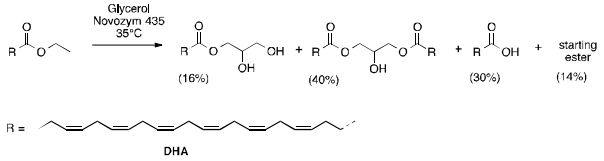


Figure 1. Analogs of DHA, DPA and EPA explored in this study



Scheme 1. First results of transesterification between DHA ethyl ester and glycerol

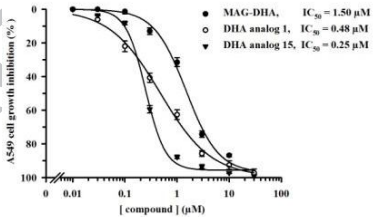


Figure 2. Concentration-response curve to MAG-DHA, analogs **1** and **15** on A549 cell growth

Tables

Table 1 Results of transamidation

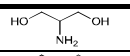
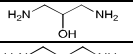
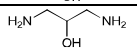
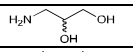
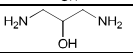
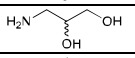
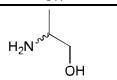
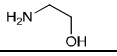
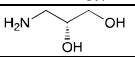
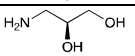
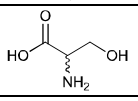
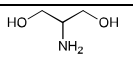
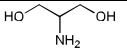
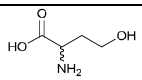
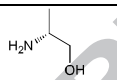
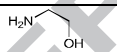
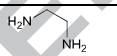
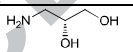
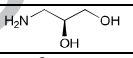
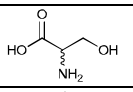
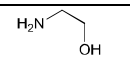
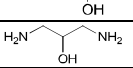
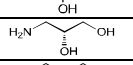
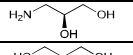
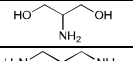
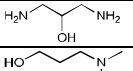
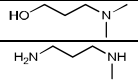
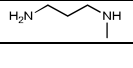
Product (Nr)	Ester[a]	Amine	Product (yield)[b]	Time (h)
1	DHA		Amide (87%)	4
2	DHA		Amide (48%)	8
3	DHA		Diamide 38%)	8
4	DHA		Amide (80%)	8
5	EPA		Amide (40%)	8
6	EPA		Amide (75%)	8
7	EPA		Amide (78%)	8
8	EPA		Amide (80%)	8
9	EPA		Amide (70%)	8
10	EPA		Amide (70%)	8
11	EPA		Amide (60%)	8
12	EPA		Amide (80%)	8
13[c]	EPA		Ester (25%)	18
14	EPA		Amide (55%)	8
15	DHA		Amide (70%)	8
16	DHA		Amide (75%)	8
17	DHA		Diamide (60%)	8
18	DHA		Amide (70%)	8
19	DHA		Amide (65%)	8
20	DHA		Amide (55%)	8
21	DPA		Amide (60%)	8
22	DPA		Diamide (45%)	8
23	DPA		Amide (80%)	8
24	DPA		Amide (70%)	8
25	DPA		Amide (60%)	8
26	EPA		Diamide (40%)	8
27	EPA		Ester (28%)	18
28	EPA		NMe-Amide	16

Table 2 Inhibition of cell growth

Product (Nr)	growth inhibition (%) at 3 μM		Product (Nr)	growth inhibition (%) at 3 μM	
	mean	SEM		Mean	SEM
MAG-DHA	59.1	3.1	**15**	98.3	1.3
MAG-EPA	51.4	2.5	**16**	29.3	3.5
MAG-DPA	74.5	1.7	**17**	13.0	2.0
1	77.4	1.5	**18**	48.1	2.1
2	38.3	5.0	**19**	42.3	2.6
3	83.8	1.4	**20**	68.7	2.5
4	88.7	2.1	**21**	96.9	0.5
7	98.4	0	**22**	97.7	0.5
8	70.9	1.9	**23**	47.4	3.4
9	nd		**24**	31.6	3.2
10	nd		**25**	97.4	0.7
11	nd		**26**	nd	
12	84.2	1.3	**27**	95.0	1.2
13	nd		**28**	67.8	2.3
14	nd		nd: not determined		

			(48%)	

a: DHA= (4Z,7Z,10Z,13Z,16Z,19Z)--4,7,10,13,16,19-docosahexaenoic acid EPA= (5Z,8Z,11Z,14Z,17Z)-5,8,11,14,17-icosapentaenoic acid DPA= (7Z,10Z,13Z,16Z,19Z)-7,10,13,16,19-docosapentaenoic acid b: Isolated yield. c: Excess EPA ethyl ester was used to force the formation of the ester.