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Anti-tumour activity of fatty acid maltotriose esters obtained by enzymatic synthesis

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ABBREVIATIONS: DMSO, dimethylsulfoxide; 2M2B, 2-methyl-2-butanol; MTT, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide.

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ABSTRACT

The anti-tumour properties of two fatty acid esters of maltotriose, 6''-*O*-lauroylmaltotriose and 6''-*O*-palmitoylmaltotriose, in which the 6-OH at the non-reducing end of the maltotriose has been regioselectively acylated, were studied. Both compounds were synthesised by transesterification of vinyl laurate or vinyl palmitate with maltotriose, in presence of immobilized lipase from *Thermomyces lanuginosus*. A mixture formed by 2-methyl-2-butanol and dimethylsulfoxide (80:20 v/v) was the reaction medium. Their cytotoxic activities against two human cancer cell lines, Hep-G2 and HeLa, were studied. 6''-*O*-palmitoylmaltotriose showed 50% inhibition values (IC_{50}) of 2.3 μM (1.7 μg/ml) for Hep-G2 and 3.6 μM (2.7 μg/ml) for HeLa cells, whereas 6''-*O*-lauroylmaltotriose displayed a lower inhibitory effect. 6''-*O*-palmitoylmaltotriose showed a marginal cytotoxicity to rat hepatocytes, confirming its potential as a new anti-tumour agent.

INTRODUCTION

Fatty acid sugar esters, especially those based on sucrose or glucose, are widely used in food and cosmetics as a consequence of their surfactant and antimicrobial properties [1]. Due to their low toxicity, non-antigenicity and biodegradability, carbohydrate esters have been investigated as potential anti-cancer agents.

Maltose tetra- to hexa- fatty acid esters showed cytotoxicity against several tumor cell lines [2,3]. Maltose tetrapalmitate was also employed in syngenic Fischer rats as an immunoadjuvant against a weakly immunogenic transplantable mammary adenocarcinoma [4]. Nishikawa *et al.* studied the effect of fatty acid esters of sucrose [5] and maltose [6] towards Ehrlich ascites carcinoma in mice. The stearic, palmitic and myristic derivatives proved to be more effective than the shorter lauric and caprylic esters [6]. Nishikawa's group also demonstrated that monoesters were more effective than the highly substituted analogs. In fact, water-solubility of sugar esters containing two or more long-acyl-chains is very low, which may detrimentally affect their biological activity. More recently, trehalose diesters of C8 to C12 fatty acids were found to inhibit tumor necrosis factor-α [7]. A similar effect was obtained with *n*-dodecyl-α-D-maltoside, a mimic of a disaccharide monoester [7]. Although different tumour-host systems have been employed, it can be concluded from the different investigations that (i) sucrose esters inhibit in less extension than maltose or trehalose esters, and (ii) fatty acid esters of monosaccharides do not display significant activity.

All the above facts confirm that sugar esters are promising candidates as anti-tumour agents. However, most of the derivatives assayed up to now are complex mixtures of regioisomers, and variations in the position of the acyl group may produce a great change in their molecular cytotoxic effect. Regioselective acylation of carbohydrates is an arduous task due to their multifunctionality [1,8]. The industrial synthesis of sugar esters is usually base-catalysed at high temperatures, has a poor selectivity, and gives rise to coloured side-products [9]. However, the enzyme-catalysed processes are notably more selective [10]. Lipases and proteases are the most useful enzymes for this purpose.

Methodologies for enzymatic sucrose acylation need to find a medium where a polar reagent (the sugar) and a non-polar fatty acid donor are soluble and able to react in presence of the biocatalyst. We developed an enzymatic strategy for the synthesis of sugar

esters, which provides regioselective products [11]. The method was based on the use of a reaction medium formed by a tertiary alcohol (2-methyl-2-butanol) and a polar solvent (dimethylsulfoxide), which represents a compromise between enzyme stability and sugar solubility. We have successfully applied this strategy to the synthesis of esters of a monosaccharide (glucose) [12], several disaccharides (sucrose, maltose, leucrose, α-D-dodecylmaltoside) [11,13,14], and even a trisaccharide (maltotriose) [13]. We have recently reported the antimicrobial effect of some of these derivatives against microorganisms involved in food spoilage [15] or in the development of dental caries [16]. In this work, the synthesis and anti-cancer properties of two maltotriose esters is described.

MATERIALS AND METHODS

Lipase from *Thermomyces lanuginosus* (Lipolase 100L) was kindly donated by Novozymes A/S. Celite (diatomaceous earth, 0.13-0.20 mm, 80-120 mesh) was from BDH. Maltotriose, 2-methyl-2-butanol and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) were from Sigma. Dimethylsulfoxide (DMSO) was supplied by Merck. Vinyl laurate was from Fluka. Vinyl palmitate was from TCI. All other reagents were of the highest available purity. Solvent and liquid reagents were dried over 3 Å molecular sieves (Sigma), at least for 24 h before use.

Enzymatic synthesis of maltotriose esters

Synthesis of 6''-*O*-lauroylmaltotriose and 6''-*O*-palmitoylmaltotriose was carried out according to the methodology developed in our laboratory [13]. The lipase from *Thermomyces lanuginosus* was immobilised on Celite as described [11]. Maltotriose (0.6 mmol) was dissolved in 2 ml of DMSO, and slowly added to 8 ml of 2M2B. The immobilized lipase (0.5 g) and molecular sieves (3 Å, 0.5 g) were then added and the suspension maintained 30 min at 40 ºC with magnetic stirring. Then, fatty acid vinyl ester (3 mmol) was incorporated. After 24 h at 40ºC, the mixture was cooled, filtered and washed with 3 volumes of 2M2B. The maltotriose monoesters were precipitated by addition of 2.5-10 volumes of *n*-heptane, the solid recrystallised in acetone, and dried *in vacuo*. The products were fully characterised by chromatography and spectroscopic techniques (HPLC, NMR, IR, HRMS).

In vitro cytotoxicity assays.

Cell survival was measured by means of the colorimetric MTT test [17]. Dose response curves for each cell line were measured at six different concentrations. All the cytotoxicity and cell viability assays were performed in triplicate. The duration of cell treatment was chosen on the basis of data available in the literature and our data from preliminary experiments. Results are expressed as a percentage of cell growth inhibition compared with a control in absence of ester.

RESULTS AND DISCUSSION

In contrast with the corresponding mono- and disaccharide derivatives, the anti-tumour activity of fatty acid esters of trisaccharides such as maltotriose has not been reported before. As part of our investigation on novel applications of sugar esters, the *in vitro* cytotoxic activity of the enzymatically-synthesised 6''-*O*-lauroylmaltotriose and 6''-*O*-palmitoylmaltotriose against two human hepatocarcinoma cancer cell lines, Hep-G2 and HeLa, was evaluated. These cell lines were selected as a model system because they are commonly employed in studies of anti-tumour effects of potentially active chemicals.

Analytical-grade 6''-*O*-lauroylmaltotriose and 6''-*O*-palmitoylmaltotriose (purity $>99\%$) were regioselectively obtained using the lipase from *Thermomyces lanuginosus* as biocatalyst [13]. Fig. 1 represents the protocol followed to synthesize and purify both compounds. The synthesis was performed by transesterification of vinyl laurate or vinyl palmitate with the sugar in a mixture 2M2B:DMSO 80:20 (v/v). The carbohydrate was dissolved in 2 ml DMSO, and this was slowly added to 8 ml of a 2M2B. Maltotriose solubility in such medium is notably higher than in pure 2M2B, which favours reaction kinetics. In addition, the inactivation of the enzyme is greatly reduced compared with pure DMSO. The above process contrasts with the great difficulties encountered by other researchers to acylate this trisaccharide [18]. Sugar esterification is more difficult when increasing the polymerisation degree of the carbohydrate, because its hydrophilicity makes higher and its solubility in solvents of intermediate polarity such as 2M2B diminishes.

The structure of the purified esters was determined by ^{1}H-NMR and ^{13}C-NMR. Fig. 2 shows the ^{1}H-NMR spectrum of 6''-*O*-lauroylmaltotriose. As the *O*-acylated carbon and the corresponding H-6 protons were shifted downfield, we could unequivocally assign that the 6-OH of glucose at the non-reducing end was regioselectively acylated (Fig. 1). Deng *et al.* also succeeded in the acylation of maltotriose and maltotetraose [19]; they esterified the 6-OH of the second glucose unit from the reducing end, but using a coupled process involving a lipase and a cyclodextrin glucanotransferase.

The novel maltotriose esters here synthesised are excellent non-ionic surfactants [20] and can be used to enhance the bioavailability of polychlorinated biphenyls (PCBs) for further biodegradation [21]. To determine whether these compounds could exert a potential pharmacological effect, studies on differential toxicity were undertaken. The study was

conducted with several cellular models. HepG2 is a cell line derived from a human hepatoma and is representative of a differentiated tumour cell. HepG2 shows a limited, but measurable, hepatic functionality [22-24]. HeLa, on the other hand, is a rather undifferentiated cell line with notable cell growth capability [23].

Cell survival was measured by means of the colorimetric MTT test [17]. Results are shown in Fig. 3, expressed as the percentage of cell growth inhibition compared with a control in absence of ester. A first insight into the experimental data shows that 6''-*O*-palmitoylmaltotriose was more toxic to both cell lines than 6''-O-lauroylmaltotriose. 6''-*O*-palmitoylmaltotriose treatment resulted in a dose-dependent decrease of cell viability of both Hep-G2 and HeLa cells (Fig. 3B), and it became noticeable that the hepatoma cell line showed a somewhat higher susceptibility than the less differentiated HeLa cell. In contrast, 6''-*O*-lauroylmaltotriose showed no significant effect on Hep-G2 and a low inhibitory effect on HeLa (Fig. 3A). Curve analysis allowed graphic estimation of the concentration causing 50% cell growth inhibition (IC_{50}). The IC_{50} values for 6''-*O*-palmitoylmaltotriose against the growth of Hep-G2 and HeLa cancer cell lines were 2.3 μM and 3.6 μM, respectively. Both concentrations correspond to values below 3 μg/ml. These concentrations are lower than those reported for trehalose diesters (7.4-23 μM) [7], dodecyl-α-D-maltoside (23 μM) [7], and highly esterified analogues of maltose (10-100 μM) [2-5].

To investigate whether a real benefit of these compounds could be derived, the toxicity of both compounds on rat hepatocytes was evaluated (Fig. 4). It can be observed that differentiated hepatocytes were clearly less sensitive to palmitoyl maltotriose than transformed cell lines Hep-G2 and HeLa. Such differential toxicity of the palmitoyl ester towards cancer cells –at least 2 orders of magnitude higher than the observed towards differentiated hepatocytes– is a first positive criterion in favour of a potential use of this kind of compounds.

CONCLUSION

To our knowledge this is the first report on the anti-tumour activity of an acylated derivative of a trisaccharide. On the basis of the results presented it can be concluded that novel 6''-*O*-palmitoylmaltotriose is a promising anti-tumour agent with a strong ability to inhibit cancer cell proliferation. Although the IC_{50} values towards HeLa cells are not as low as those reported for other chemicals (e.g. 0.1 μM for several triterpenoids [25]), the values obtained in this work are lower than 4 μg/ml, a limit put forward by NCI for classification of compounds as potential anti-cancer drugs [26]. In addition, the low toxicity and notable hydrophilicity of carbohydrate esters offer practical advantages. Moreover, taking the aforementioned data, we consider that trisaccharide esters are more promising inhibitors than the corresponding mono- and disaccharide counterparts. The possibility of obtaining these compounds in high yield and purity by using our new enzymatic methodology makes pure maltotriose esters easily available. In addition, novel maltotriose esters have been isolated from plants, e.g. from the roots of *Polygala arillata* [27]. Further studies will be necessary to elucidate the inhibition mechanism of tumour cells proliferation by the new maltotriose fatty acid esters hereby described.

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REFERENCES

1. Polat, T. and Linhardt, R. J. (2001) J. Surf. Deterg. **4**, 415-421.
2. Benrezzak, O., Madarnas, P., Pageau, R., Nigam, V. N. and Elhilali, M. M. (1989) Anticancer Res. **9**, 1883-1888.
3. Benrezzak, O. and Nigam, V. N. (1992) Cancer Biochem. Biophys. **13**, 13-22.
4. Bonaventure, J., Nigam, V. N. and Brailovsky, C. A. (1985) J. Biol. Resp. Modif. **4**, 258-263.
5. Nishikawa, Y., Yoshimoto, K., Okada, M., Ikekawa, T., Abiko, N. and Fukuoka, F. (1977) Chem. Pharm. Bull. **25**, 1717-1724.
6. Nishikawa, Y., Yoshimoto, K., Nishijima, M., Fukuoka, F. and Ikekawa, T. (1981) Chem. Pharm. Bull. **29**, 505-513.
7. Okabe, S., Suganuma, M., Tada, Y., Ochiai, Y., Sueoka, E., Kohya, H., Shibata, A., Takahashi, M., Mizutani, M., Matsuzaki, T. and Fujiki, H. (1999) Jpn. J. Cancer Res. **90**, 669-676.
8. Plou, F. J, Cruces, M. A., Pastor, E., Ferrer, M., Bernabé, M. and Ballesteros, A. (1999) Biotechnol. Lett. **21**, 635-639.
9. Watanabe, T. (1999) Foods Food Ingr. J. Japan **180**, 18-25.
10. Plou F. J., Cruces M. A., Ferrer, M., Fuentes, G., Pastor, E., Bernabé, M., Christensen, M., Comelles, F., Parra, J. L. and Ballesteros, A. (2002) J. Biotechnol. **96**, 55-66**.**
11. Ferrer, M., Cruces, M. A., Bernabé, M., Ballesteros, A. and Plou, F. J. (1999) Biotechnol. Bioeng. **65**, 10-15.
12. Tsavas, P., Polydorou, S., Faflia, I., Voutsas, E. C., Tassios, D., Flores, M. V., Naraghi, K., Halling, P. J., Chamouleau, F., Ghoul, M., Engasser, J. M., Ferrer, M. and Plou, F. J. (2002) J. Chem. Eng. Data **47**, 807-810.
13. Ferrer, M., Cruces, M. A., Plou, F. J., Bernabé, M. and Ballesteros, A. (2000) Tetrahedron **56**, 4053-4061.
14. Ferrer**,** M., Plou, F. J., Pastor, E., Fuentes, G., Cruces, M. A., Andersen, L., Kirk, O., Christensen, M. and Ballesteros, A. (2002) Biocatal. Biotransform. **20**, 63-71.

15. Ferrer, M., Soliveri, J., Plou, F. J., López-Cortés, N., Reyes-Duarte, D., Christensen, M., Copa-Patiño, J. L. and Ballesteros, A. (2004) Enzyme Microb. Technol., in the press.
16. Devulapalle, K. S., Gómez de Segura, A., Ferrer, M., Alcalde, M., Mooser, G. and Plou, F. J. (2004) Carbohydr. Res. **339**, 1029-1034.
17. Mosmann, T. (1983) J. Immunol. Meth. **65**, 55-63.
18. Degn, P. and Zimmermann, W. (2001) Biotechnol. Bioeng. **74**, 483-491.
19. Degn, P., Larsen, K. L., Duus, J. O., Petersen, B. O. and Zimmermann, W. (2000) Carbohydr. Res. **329**, 57-63.
20. Ferrer, M., Comelles, F., Plou, F. J., Cruces, M. A., Fuentes, G., Parra, J. L. and Ballesteros, A. (2002) Langmuir **18**, 667-673.
21. Ferrer, M., Golyshin, P. and Timmis, K. N. (2003) World J. Microbiol. Biotechnol. **19**, 637-643
22. Ponsoda, X., Núñez, C., Castell, J. V. and Gómez-Lechón, M. J. (1997) ATLA-Altern. Lab. Anim. **25**, 423-436.
23. Jover, R., Bort, R., Gómez-Lechón, M. J. and Castell, J. V. (2000) in Progress in the Reduction, Refinement and Replacement of Animal Experimentation (Balls, M., van Zeller, A. M. and Halder, M. E., eds), pp 127-137, Elsevier, Amsterdam.
24. Rodríguez-Antona, C., Donato, M. T., Boobis, A., Edwards, R. J., Watts, P. S., Castell, J. V. and Gómez-Lechón, M. J. (2002) Xenobiotica **32**, 505-520.
25. Chen, Y.G., Wu, Z.C., Lu, Y.P., Gui, S.H., Wen, J., Liao, X.R., Yuan, L.M., Halaweish, F. (2003) Arch. Pharmacal. Res. **26**, 912-916.
26. Kettmann, V., Kosfalova, D., Jantova, S., Cernakova, M., Drimal, J. (2004) Pharmazie **59**, 548-551.
27. Kobayashi, W., Miyase, T., Suzuki, S., Noguchi, H. and Chen, X. M. (2000) J. Nat. Prod. **63**, 1066-1069.

FIGURE LEGENDS

FIG. 1. Reaction scheme for the synthesis of 6''-*O*-acylmaltotrioses.

FIG. 2. ^{1}H-NMR spectrum of the synthesized 6''-*O*-lauroylmaltotriose. The spectrum was recorded at 30 °C on a Varian INOVA (300 MHz) spectrometer dissolving the ester in CD_3OD.

FIG. 3. Cell viability of several cellular models after exposure to maltotriose esters. (**A**) 6''-*O*-lauroylmaltotriose and (**B**) 6''-*O*-palmitoylmaltotriose. Hep-G2 (●) and HeLa (○) cell lines were exposed to monoester 24 h, and cell viability was measured by the MTT test after a further 24 h treatment. Standard deviations, calculated from three independent experiments, were in all cases between 5 and 10%. The data were fitted to a four parameter logistic curve using the Sigma Plot 2001 software.

FIG. 4. Toxicity of maltotriose esters towards rat hepatocyte cells. Cell lines were exposed to 6''-*O*-lauroylmaltotriose (●) and 6''-*O*-palmitoylmaltotriose (○) for 24 h, and cell viability was measured by the MTT test after a further 24 h treatment. Standard deviations, calculated from three independent experiments, were in all cases between 5 and 10%.

Fig. 1

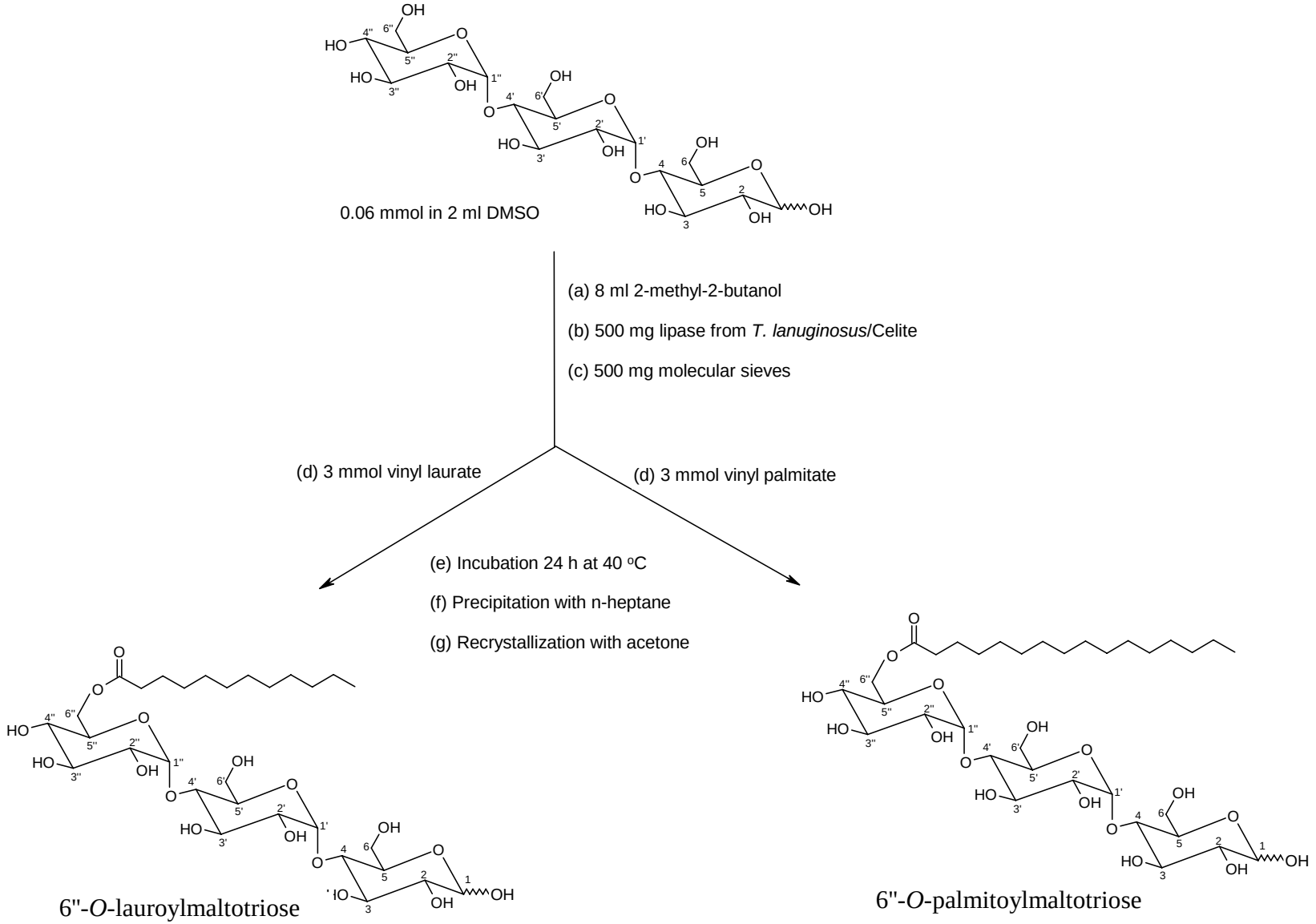
0.06 mmol in 2 ml DMSO
(a) 8 ml 2-methyl-2-butanol
(b) 500 mg lipase from *T. lanuginosus*/Celite
(c) 500 mg molecular sieves
(d) 3 mmol vinyl laurate
(d) 3 mmol vinyl palmitate
(e) Incubation 24 h at 40 °C
(f) Precipitation with n-heptane
(g) Recrystallization with acetone
6"-*O*-lauroylmaltotriose
6"-*O*-palmitoylmaltotriose

Fig. 2

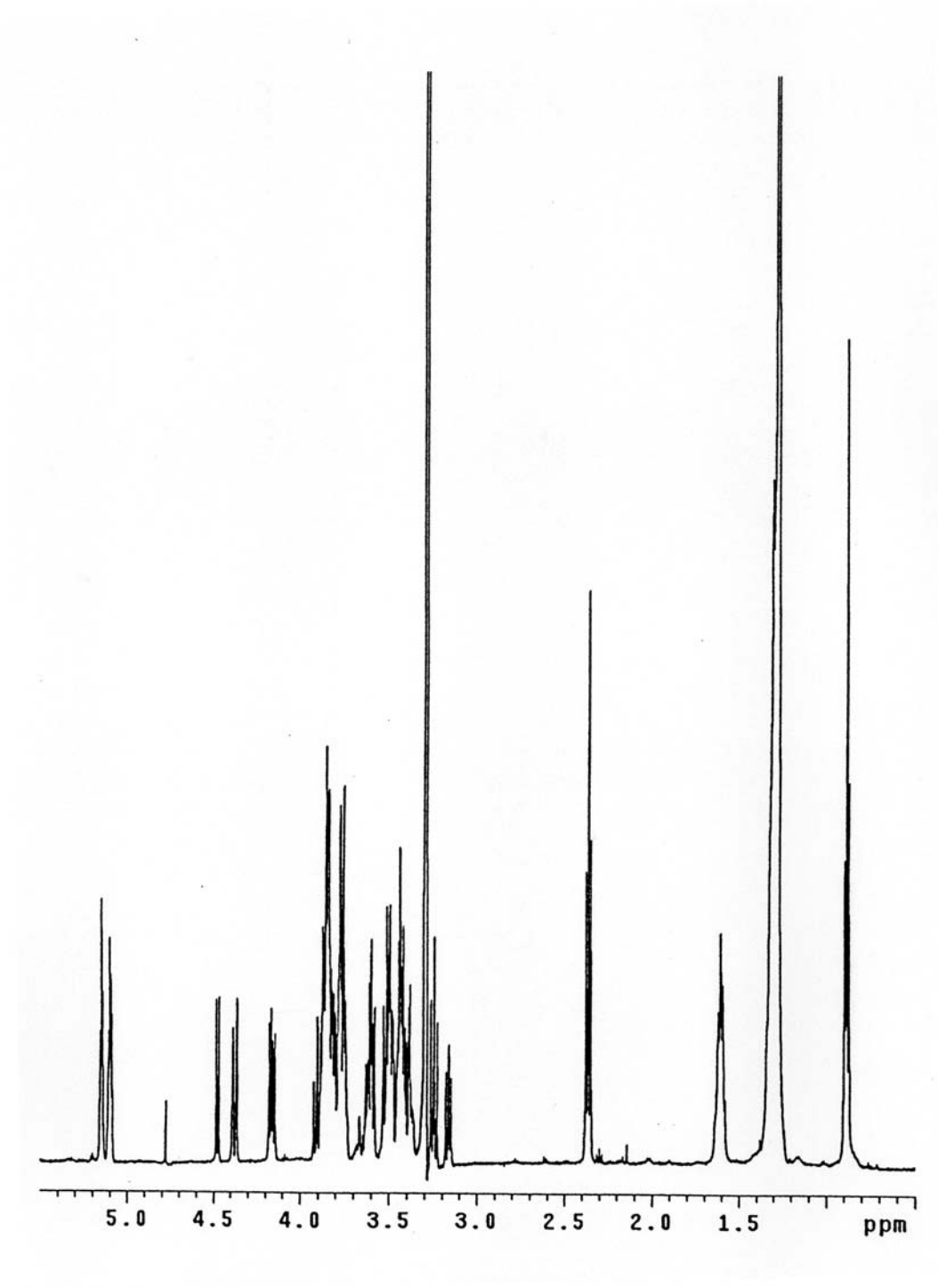

Fig. 3

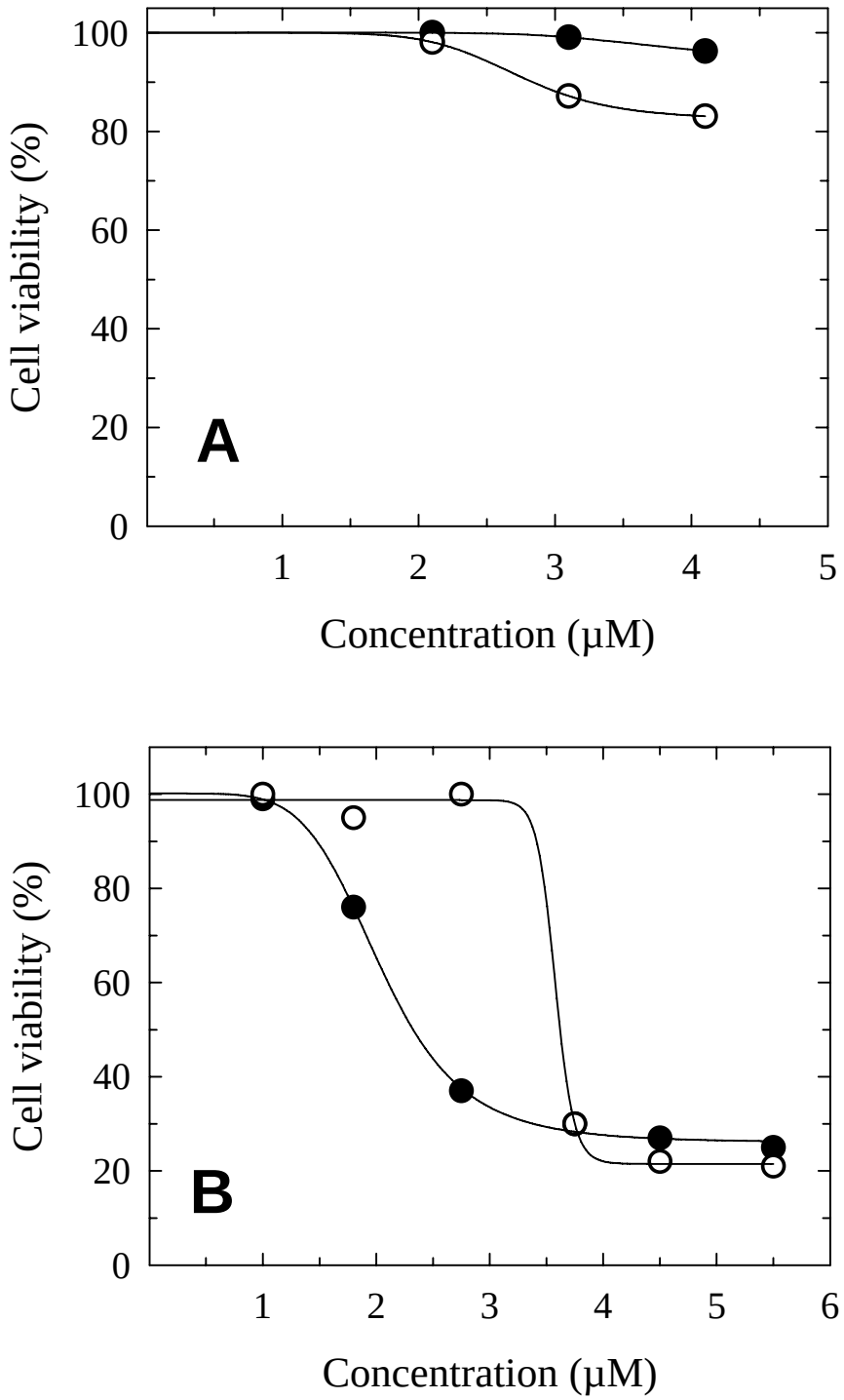
Cell viability (%)
100
80
60
40
20
0
A
1
2
3
4
5
Concentration (μM)
Cell viability (%)
100
80
60
40
20
0
B
1
2
3
4
5
6
Concentration (μM)

Fig. 4