

## Effect of Novel Synthesised Policosanyl Phenolates on Lipid Oxidation

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### Abstract

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Lipophilic derivatisation of phenolic acids could greatly improve their antioxidant activities and solubility in hydrophobic environments, broadening their applications in food, pharmaceutical, and cosmetic industries. In this study, we conducted enzymatic lipophilisation of eight phenolates with policosanols. Vinyl phenolates were used as intermediates to improve the efficiency of enzymatic lipophilisation; and the yields of policosanyl phenolates were in the range of 1.32–20.58%. The antioxidant activities of the resulting phenolipids were compared using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay and linoleic acid peroxidation ferric thiocyanate assay. The synthesised policosanyl phenolates showed lower ABTS radical scavenging capacities ( $IC_{50}$ s > 15 mM); whereas they showed high lipid peroxidation inhibitory activities ( $IC_{50}$ s of peroxidation value < 0.25 mM). The lipid oxidation inhibitory activities of policosanol phenolates were further evaluated using the total oxidation value in a linoleic acid model system and the thiobarbituric acid reactive substances value in a cooked pork model system. Finally, policosanyl 4-hydroxybenzoate, policosanyl syringate, and policosanyl 4-hydroxyphenylacetate showed the highest inhibition effects on lipid oxidation and a potential for use as lipid antioxidants.

**Keywords:** Novozyme 435; lipophilisation; policosanols; phenolic acids; antioxidant

Unsaturated lipids are major oxidation targets in foods (SUN *et al.* 2011). The inhibition of lipid oxidation in foods is important for consumers and industry because the oxidation will decrease food quality and influence wellness. The oxidation of lipid-based products has become a major concern for food industries more now than ever. One of the best ways of inhibiting lipid oxidation is to utilise antioxidants (DECKER *et al.* 2010). However, the amount of antioxidants for food manufacturers to moderate oxidative rancidity is limited and the approval of new antioxidants is challenging (CHAIYASIT *et al.* 2007). Moreover, the concern of using synthetic antioxidants, such as butylated hydroxytoluene (BHT),

is increasing because of their perhaps carcinogenic effects (ITO *et al.* 1986). Therefore, new efficient antioxidants should be developed from natural sources by food scientists.

Phenolic acids are secondary plant metabolites that are ubiquitous in nature. They and their derivatives have wide-ranging biological functions in our diet and are also used as antioxidants in food products (WANG *et al.* 2002). As we have known, the efficiency of antioxidants is affected by their solubility in the phases in which oxidation takes place (DECKER 1998). Therefore, antioxidants in food must be able to partition between several phases and interact with emulsions at the interfaces, and hydrophobicity is

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one of the most important parameters for antioxidant activity. Thus, phenolic acids, as hydrophilic antioxidants, are normally very much restricted in their applications in lipid-based food products.

In recent years, lipophilisation of hydrophilic antioxidant molecules to synthesise antioxidants with a broad range of hydrophobicity has attracted broad interest because they could be accumulated at oil-water or oil-air interfaces to protect against the lipid oxidation (FIGUEROA-ESPINOZA & VILLENEUVE 2005). Various phenolic esters have been synthesised using fatty alcohols or acids (WHITAKER *et al.* 2001; SØRENSEN *et al.* 2012). Recently, various phytosteryl phenolates have also been synthesised and have shown excellent antioxidant capacities in lipids (TAN & SHAHIDI 2011, 2012, 2013; FU *et al.* 2014; WANG *et al.* 2015a).

Policosanols (PLs) are mixtures of long-chain ( $C_{24}$  to  $C_{34}$ ) aliphatic primary alcohols and are natural supplements. They can be obtained from sugarcane, wheat, and beeswax and are used to treat diabetes and hypercholesterolaemia diseases (BERTTNER *et al.* 2006; IRMAK *et al.* 2006; BERTHOLD *et al.* 2014). In a previous study, several esters of policosanols and phenolic acids were synthesised; the resulting policosanyl *p*-coumarate and policosanyl 5-phenylvalerate showed a potential for use as lipid antioxidants (WANG *et al.* 2015b). Hence, in this study, the antioxidant capacities of another eight synthesised policosanyl phenolates were assessed and compared in different systems in order to develop novel more effective food antioxidants.

## MATERIAL AND METHODS

**Chemicals.** Lipase acrylic resin from *Candida antarctica* (Novozyme 435, 5 U/mg) was purchased from Sigma-Aldrich Korea (Seoul, Korea). One unit represents the microequivalents of fatty acid hydrolysed from a triglyceride in 1 h at pH 7.2 at 37°C.

The PLs (average molecular weight is 412), which contained octacosanol (61.87%), triacontanol (20.04%), hexacosanol (12.13%), and other long-chain alcohols (5.99%), were obtained from Riotto Biological Technology Co., Ltd. (Xi'an, China). Ferrous chloride ( $FeCl_2$ ) and ferric chloride ( $FeCl_3$ ), glacial acetic acid, hydrogen chloride, and sulphuric acid were purchased from Shiny Pure Chemicals Co., Ltd. (Minoo Ooka, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), Junsei Chemical Co., Ltd. (Tokyo,

Japan), and Daejung Chemicals & Metals Co., Ltd. (Siheung, Korea), respectively. Ground pork was purchased from a local supermarket in Chuncheon, Korea. Vinyl salicylate, vinyl 4-hydroxybenzoate, vinyl gentisate, vinyl vanillate, vinyl syringate, vinyl veratrate, vinyl 4-chlorophenylacetate, and vinyl 4-hydroxyphenylacetate were produced in our laboratory (WANG *et al.* 2015a). All reagents used in this study were analytical grade and were purchased from Sigma-Aldrich Korea (Korea).

**Preparative synthesis of policosanyl phenolates.** The vinyl phenolates (0.1 mmol) were placed into 10 ml vials to react with the PLs (0.1 mmol) under Novozyme 435 (300 U) catalysis in a binary solvent mixture (3 ml, hexane/2-butanone 8 : 2, v/v) for 4 days at 60°C. The products were separated from the reaction mixture by silica-gel column chromatography with solvent gradient elution (hexane/ethyl acetate from 32 : 1 to 4 : 1, v/v). For instance of policosanyl 4-hydroxybenzoate (2b), a mixture of hexane (2.4 ml), 2-butanone (0.6 ml), vinyl 4-hydroxybenzoate (16.4 mg, 0.1 mmol), policosanols (41.2 mg, 0.1 mmol), and Novozyme 435 (300 U, 60 mg) was stirred using the magnetic stirring bar under nitrogen for 4 days at 60°C. The mixture was filtered, and the solution was evaporated. The residue (57.6 mg) was purified by column chromatography (packed with silica gel Si 60, 63–200  $\mu$ m, Watchers, 6 g) with solvent gradient elution by a mixture of hexane and ethyl acetate from 32 : 1 to 4 : 1 (v/v). Policosanyl 4-hydroxybenzoate (2b) was obtained as a colourless solid (1.1 mg, 1.78%). The reaction and column chromatography were monitored by thin layer chromatography (TLC, silica-gel precoated, flexible TLC sheets, 4 × 10 cm; Merck KGaA, Darmstadt, Germany) using hexane and ethyl acetate (4 : 1, v/v) as an elution solvent. The spots were visualised under ultraviolet at 254 nm using TLC Visualizer (CAMAG Scientific Inc., Wilmington, USA). The percentage yield of the reaction was calculated as the ratio of the obtained amount of product to the maximum theoretical amount, multiplied by 100. The isolated policosanyl phenolates were identified by  $^1H$  NMR, recorded at 400 MHz in  $CDCl_3$  using a Fourier transform NMR spectrometer (Bruker Korea, Seongnam, Korea) with tetramethylsilane as an internal standard. Signal processing and interpretation were performed using the Bruker DPX 400 MHz (9.4T) software package.

**ABTS assay.** The ABTS assay was performed as described by RE *et al.* with slight modifications (RE

*et al.* 1999). Briefly, 2 mM of ABTS diammonium salt was mixed with 3.5 mM of potassium persulphate in distilled water and held in the dark for 24 h before use. The ABTS<sup>+</sup> solution (290 µl) was reacted with the sample (10 µl) in a 90 well plate for 10 minutes. Absorbance at 750 nm was measured using an EL-800 Universal Microplate Reader (Bio-Tek Instruments Inc., Winooski, USA). Percent inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{[(A_{\text{control}} - A_{\text{blank}}) - (A_{\text{sample}} - A_{\text{blank}})]}{(A_{\text{control}} - A_{\text{blank}})} \times 100$$

where:  $A_{\text{control}}$  – absorbance of the ABTS solution (290 µl) with ethyl acetate (10 µl);  $A_{\text{blank}}$  – absorbance of distilled water (290 µl) with ethyl acetate (10 µl);  $A_{\text{sample}}$  – absorbance of the ABTS solution (290 µl) with the test compound (10 µl)

The results were expressed as an  $IC_{50}$ . BHT was used as a positive control.

**Ferric thiocyanate (FTC) assay.** The peroxide value (PV) was determined by FTC assay in a linoleic acid system (SAKANAKA *et al.* 2004). Briefly, 9 ml of 2.5% linoleic acid ethanol solution and 1 ml of the sample were mixed in sealed test tubes and held in the dark at 60°C for 7 days. The control group was prepared without adding the sample. BHT was used as a positive control. The resulting oxidative fatty acid sample (0.5 ml) was reacted with 5 µl of 0.02 M  $FeCl_2$  10% HCl solution and 5 µl of 30% (w/v) ammonium thiocyanate for 3 minutes. Absorbance at 500 nm was measured using a spectrophotometer (Secomam, Alès, France). The same reaction without addition of the fatty acid sample was used as a blank. The PV was calculated from a  $Fe^{3+}$  standard calibration curve using the following equation:

$$PV = [(A_{\text{sample}} - A_{\text{blank}}) \times m] / (55.8 \times 2 \times w)$$

where:  $A_{\text{sample}}$  – absorbance of the sample including the control group at 500 nm;  $A_{\text{blank}}$  – absorbance of the blank at 500 nm;  $w$  – weight of the lipid sample;  $m$  – slope of the calibration curve

The inhibition oxidation (IO) index was calculated from the PVs as follows:

$$\% \text{ IO} = 100 - (PV_{\text{sample}} / PV_{\text{control}}) \times 100$$

where:  $PV_{\text{sample}}$ ,  $PV_{\text{control}}$  – PVs of the sample and control group

The results were expressed as an  $IC_{50}$  for PV inhibition.

**Measurement of the total oxidation value in a linoleic acid model system.** The total oxidation (TOTOX) value represents the total oxidation that lipids have undergone, including primary and secondary oxidation (SHERWIN *et al.* 1978). Nine millilitres of 2.5% linoleic acid ethanol solution and 1 ml of 1 mM samples were mixed in sealed tubes and held in the dark at 60°C. A blank group without the sample was prepared. After the allotted time, the PV and *p*-anisidine values (*p*-AnV) of the samples were determined using the FTC method described above and IUPAC method 2.504 (PAQUOT 1979), respectively. The TOTOX value was obtained by the formula: TOTOX value =  $2 \times PV + p\text{-AnV}$ .

**Measurement of the thiobarbituric acid reactive substances (TBARS) value in a cooked ground pork system.** Fresh ground pork in water suspension (1 g/ml) was homogenised with 100 µM of the sample and cooked at 80°C in a water bath for 15 minutes. The cooked samples were held at 4°C in the dark. BHT was used as a positive control and a blank group was prepared without the sample. After the allotted time, 5 ml of the cooked sample was reacted with 2.5 ml of 10% trichloroacetic acid and 2.5 ml of 0.02 M thiobarbituric acid at 80°C for 15 minutes. After centrifuging at 10 000 rpm for 15 min, the upper absorbance was measured at 532 nm using a spectrophotometer. The TBARS value was calculated based on a malonaldehyde (MDA) standard calibration curve. The results were expressed as µmol MDA/kg meat.

**Statistical analysis.** All experiments were repeated at least three times and the results are given as the means ± SD. All data were analysed using ANOVA. Significant differences were assessed by Duncan's test ( $P < 0.05$ ). The statistical analyses were carried out using IBM SPSS version 19.0 software (IBM, New York, USA).

## RESULTS AND DISCUSSION

**Synthesis of policosanyl phenolates.** For lipophilisation of phenolic acids with PLs, the lipase-catalysed synthesis strategy is the first choice due to the environmental friendly conditions. However, no product was obtained in our preliminary experiment through the direct esterification route. On the one hand, enzymatic reactions often have long reaction times and low yields; and on the other hand, the reactive groups of the materials are perhaps difficult to approach the lipase active site. To improve the

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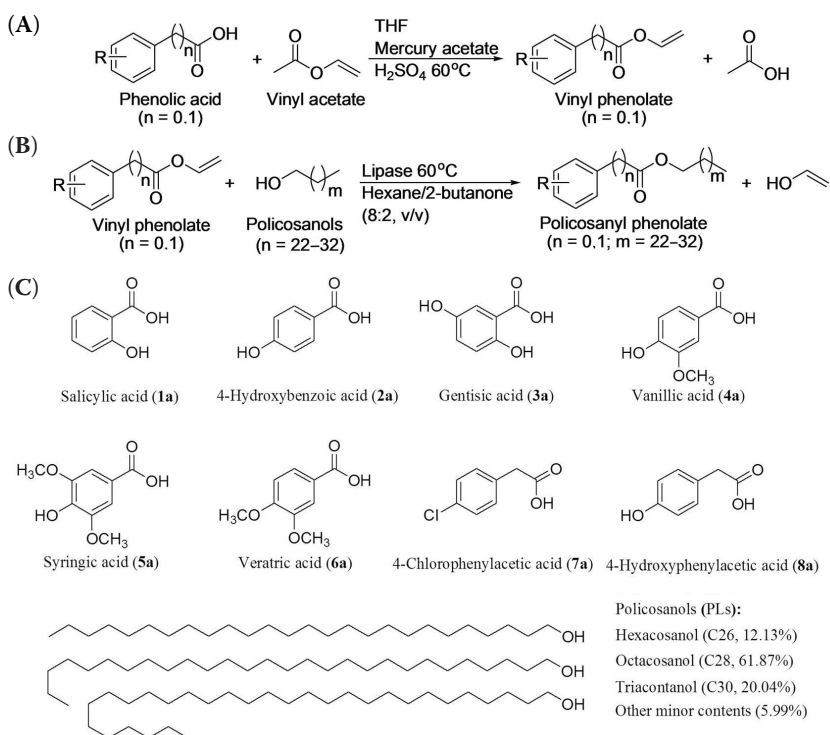


Figure 1. Scheme for policosanyl phenolate synthesis: (A) chemical vinylation; (B) lipase-catalysed esterification; (C) structures of policosanols and phenolic acids.

reaction efficiency, as shown in Figures 1A and B, the vinyl phenolates that were produced in our laboratory were used as activated acyl donors in the present study. The structures of corresponding phenolic acids and PLs are shown in Figure 1C. The purities of synthesised products were confirmed by  $^1\text{H}$  NMR signals, which were over 95%. The structures of the resulting products were confirmed by  $^1\text{H}$  NMR spectrometry (Table 1 and Figure 2), in which the characteristic alkyl peaks of the policosanols were detected in the range of  $\delta$  0.09–1.70 ppm. The change

in the chemical shift of the  $\alpha$ -H in the policosanol from  $\delta$  3.70 ppm to  $\delta$  4.30 ppm indicated the binding of phenolate and PLs. The TLC  $R_f$  values of the policosanyl phenolates are higher than those of their corresponding phenolic acids, indicating that they possess higher hydrophobicity.

In this lipase-catalysed reaction, the vinyl esters are able to easily approach the lipase active site to form the acyl-enzyme intermediate (KWON *et al.* 2007; CHIGORIMBO-MUREFU *et al.* 2009). Despite this, the yields of the policosanyl phenolates remained low at

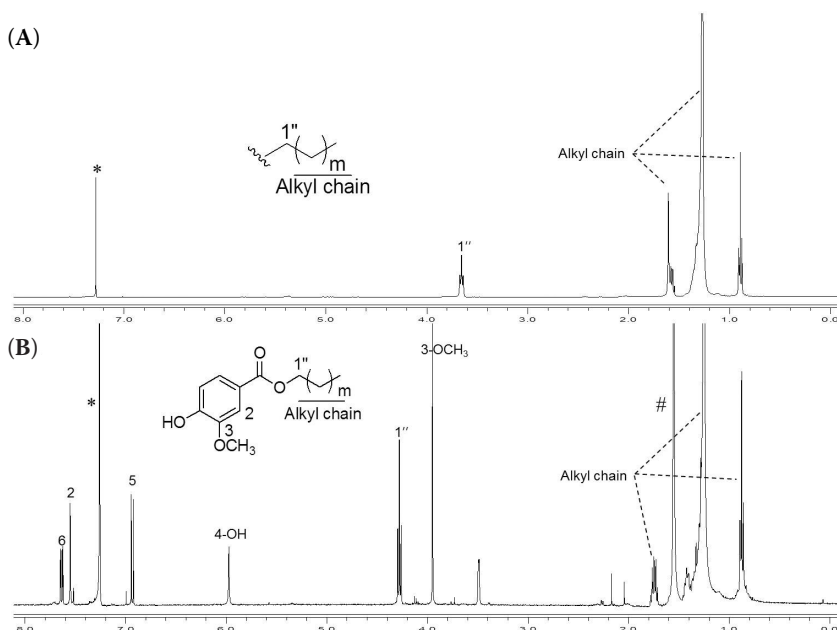
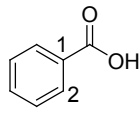
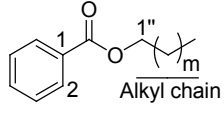


Figure 2.  $^1\text{H}$  NMR spectra of PLs (A) and policosanyl vanillate (B) \*solvent peak ( $\text{CDCl}_3$ ); #water peak

Table 1. Structures, yields, and  $R_f$  values for the synthetic compounds. The TLC elution solvents were hexane and ethyl acetate (4:1, v/v). The yield is the ratio of the amount of product actually obtained to the maximum amount of product possible

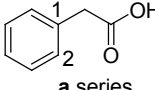


**a series**

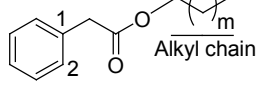


**b series**

No.	Name	$^1\text{H NMR}$ (400 MHz, $\text{CDCl}_3$ , $\delta$ )							Yield (%)	$R_f$ (TLC)
		phenolic group					policosanyl groups			
		2	3	4	5	6	1''	alkyl chains		
–	policosanols	–	–	–	–	–	3.64	0.90–1.70	–	–
1a	salicylic acid	–	6.94	7.43	7.03	7.96	–	–	–	0.23
1b	policosanyl salicylate	–	6.90	7.47	7.00	7.87	4.37	1.08–1.80	1.32	0.87
2a	4-hydroxybenzoic acid	7.96	6.94	–	6.94	7.96	–	–	–	0.08
2b	policosanyl 4-hydroxybenzoate	7.89	6.77	–	6.77	7.89	4.20	0.90–1.67	1.78	0.41
3a	gentisic acid	–	6.77	6.91	–	7.43	–	–	–	0.09
3b	policosanyl gentisate	–	6.81	6.93	–	7.21	4.26	0.81–1.50	4.51	0.53
4a	vanillic acid	7.47	3.73 (-OCH <sub>3</sub> )	–	6.83	7.52	–	–	–	0.06
4b	policosanyl vanillate	7.55	3.95 (-OCH <sub>3</sub> )	5.97 (-OH)	6.94	7.64	4.28	0.88–1.75	8.59	0.48
5a	syringic acid	7.03	3.74 (-OCH <sub>3</sub> )	–	3.74 (-OCH <sub>3</sub> )	7.03	–	–	–	0.06
5b	policosanyl syringate	7.32	3.95 (-OCH <sub>3</sub> )	5.88 (-OH)	3.95 (-OCH <sub>3</sub> )	7.32	4.29	0.88–1.76	3.56	0.31
6a	veratric acid	7.53	3.70 (-OCH <sub>3</sub> )	3.70 (-OCH <sub>3</sub> )	6.87	7.58	–	–	–	0.11
6b	policosanyl veratrate	7.55	3.93 (-OCH <sub>3</sub> )	3.93 (-OCH <sub>3</sub> )	6.89	7.69	4.29	0.88–1.76	20.58	0.58



**a series**



**b series**

No.	Name	$^1\text{H NMR}$ (400 MHz, $\text{CDCl}_3$ , $\delta$ )							Yield (%)	$R_f$ (TLC)	
		phenolic group					policosanyl groups				
		2	3	4	5	6	7	1''			alkyl chains
7a	4-chlorophenylacetic acid	7.04	7.19	–	7.19	7.04	3.51	–	–	–	0.12
7b	policosanyl 4-chlorophenyl acetate	7.22	7.30	–	7.30	7.22	3.58	4.08	0.88–1.58	3.54	0.78
8a	4-hydroxyphenylacetic acid	6.91	6.63	–	6.63	6.91	3.47	–	–	–	0.06
8b	policosanyl 4-hydroxyphenyl acetate	7.15	6.78	4.74 (-OH)	6.78	7.15	3.53	4.07	0.88–1.60	2.69	0.42

1.32–20.58% after a 4-day reaction period. Comparing the yield information of synthesised products in Table 1, we found that the lipase activity can be affected by the functional groups of acyl donors. Specifically, 2b, policosanyl gentisate (3b), policosanyl vanillate (4b), policosanyl syringate (5b), policosanyl

veratrate (6b) in Table 1, the hydroxyl groups look like more strongly inhibited lipase activities than the methoxyl groups; in addition, the effect of a hydroxyl group in the *ortho* position was greater than that of a *para* hydroxyl group. Comparing 2b, 4b, and 5b, the inhibition due to *para* hydroxyl groups of lipase



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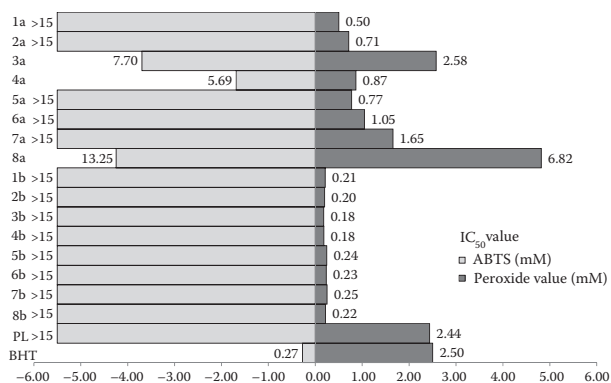


Figure 3. Concentration of antioxidant necessary to reduce oxidation by 50%, determined using ABTS and FTC assays

activity was reduced by *meta* methoxyl groups and a single *meta* methoxyl group inhibited activity more than a double group.

**Antioxidant activities of policosanil phenolates.** Detection of free radical scavenging activity and lipid oxidation inhibition by the resulting phenolipids was carried out using ABTS and FTC assays (Figure 3). The  $IC_{50}$ s for the policosanil phenolates for reducing the ABTS radical were much higher than those for reducing PV, suggesting that the inhibition of lipid oxidation was a predominant mechanism of the antioxidant activity of synthesised samples.

In the ABTS assay, none of the phenolic acids or synthesised policosanil phenolates other than gentisic acid (3a), vanillic acid (4a), and 4-hydroxyphenylacetic acid (8a) showed free radical scavenging activity. The effectiveness of an antioxidant in free radical scavenging is influenced by its chemical properties, such as hydrogen bond energies and resonance delocalisation (SOBRATTEE *et al.* 2005). Therefore, the hydroxyl substituents were necessary for phenolate to act as a hydrogen donor, enabling these compounds to act as efficient free radical scavengers. The reactivity of hydrogen donors can also be improved with the assistance of other function groups (BUETTNER 1993). In contrast, the long aliphatic chain of the policosanil phenolates appears to have limited the reactivity of the hydroxyl donors, or the intramolecular bond interactions were reduced by their complex conformations. Nevertheless, large increases in PV inhibition activity were obtained after lipophilisation of the phenolic acids with PLs. The long alkyl chains of the policosanil phenolates may have stabilised the unsaturated fatty acids. Due to the strong observed potential of these policosanil phenolates for lipid oxidation inhibition, additional confirmatory experiments were conducted.

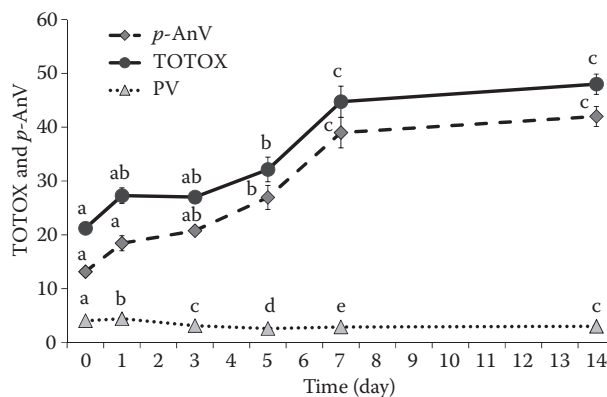


Figure 4. Oxidation trend of linoleic acid. Different letters in one line show significant differences ( $P < 0.05$ )

**Lipid oxidation inhibitory activity of policosanil phenolates in a linoleic acid model system.** The lipid oxidation inhibitory activities of the synthesised policosanil phenolates were measured using the TOTOX value in a linoleic acid model system. The TOTOX value is a combination of PV and *p*-AnV and therefore provides a comprehensive assessment of lipid oxidation. During the storage of linoleic acid without antioxidant treatment, the TOTOX value showed a slow-fast-slow increasing trend and reached a maximum on the 14<sup>th</sup> day (Figure 4). In this process, hydrogen was first abstracted from linoleic acid and hydroperoxide and free radicals were then formed, accompanied by an increase in PV. Further combination of radicals resulted in increasing *p*-AnV; correspondingly, PV substantially decreased. As the oxidation process neared completion, increases in both PV and *p*-AnV slowed and the TOTOX value reached a maximum. However, the TOTOX value on the 14<sup>th</sup> day varied in different samples. Compounds 2b, 5b, and policosanil 4-hydroxyphenyl acetate (8b) effectively inhibited the lipid oxidation after their

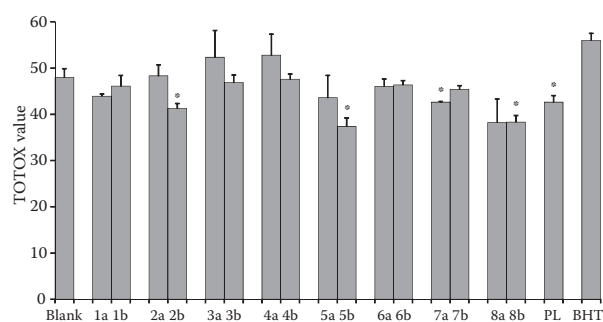


Figure 5. Inhibitory effect on the TOTOX value at 14 days \*means significantly lower than the blank,  $P < 0.05$ ; sample concentrations are 100  $\mu$ M

corresponding phenolic acids were lipophilised with PLs (Figure 5). Inhibition of hydroperoxide and terminal oxidation product formation after 1 week may have been the inhibitory mechanism (Figure 6); in terms of TOTOX, inhibition began to occur after 1 week, likely due to the long alkyl chains of the PLs reacting with other molecules.

**Lipid oxidation inhibitory activity of policosanil phenolates in a cooked ground pork model system.** The lipid oxidation inhibitory activities of the syn-

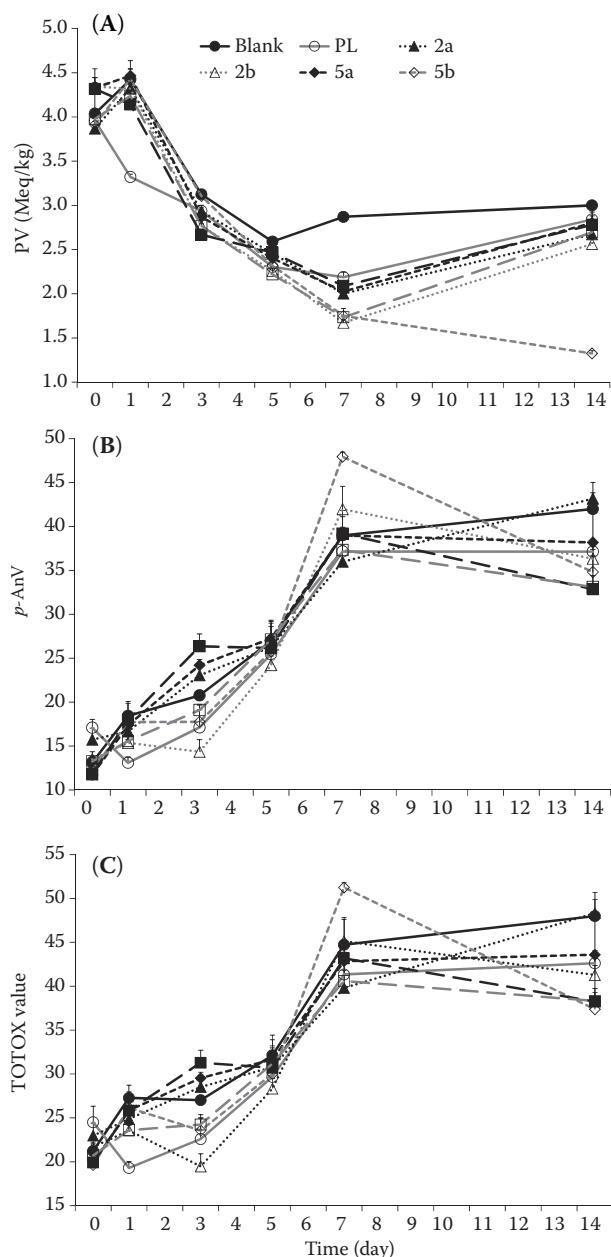


Figure 6. Inhibitory effect on PV (A), *p*-AnV (B), and TOTOX (C) in a linoleic acid model system (sample concentrations are 100  $\mu$ M)

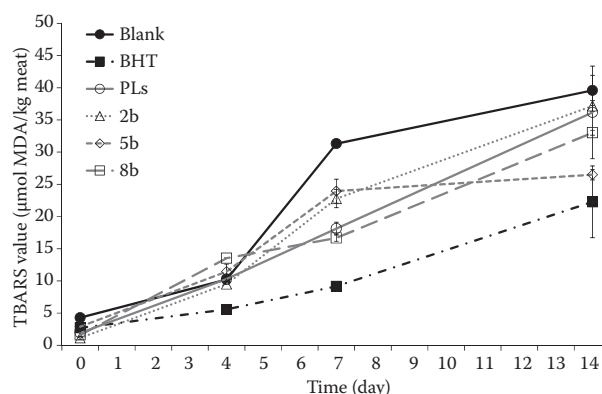


Figure 7. Thiobarbituric acid reactive substance (TBARS) values in cooked pork (sample concentrations are 100  $\mu$ M)

thesised products were confirmed by their TBARS values in the cooked ground pork system (Figure 7). The TBARS values for all meat samples increased up to the 7<sup>th</sup> day due to oxidation. However, the TBARS values of the meat samples treated with policosanil phenolates reflected significant inhibitory effects on lipid oxidation. Compared with BHT, 2b, 5b, and 8b were found to be moderate, long-lasting antioxidants and have the potential to be used as alternative antioxidants in food products.

## CONCLUSIONS

In this study, eight novel policosanil phenolates were synthesised. The lipophilisation of phenolic acids with PLs improved their hydrophobicities and inhibitory activities toward lipid oxidation. Policosanil 4-hydroxybenzoate (2b), policosanil syringate (5b), and policosanil 4-hydroxyphenylacetate (8b) showed a potential as food antioxidants. The availability of a synthetic approach to these phenolipids will greatly facilitate further investigations of their biological properties.

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