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Core Structure in Roselipins Essential for Eliciting Inhibitory Activity against Diacylglycerol Acyltransferase

HIROSHI TOMODA*, NORIKO TABATA, YUKAKO OHYAMA and SATOSHI ŌMURA*

Kitasato Institute for Life Sciences & Graduate School of Infection Control Sciences,
Kitasato University, and The Kitasato Institute
Shirokane, Minato-ku, Tokyo 108-8641, Japan

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Fungal roselipins, discovered as inhibitors of diacylglycerol acyltransferase (DGAT), consist of three parts; highly methylated C20 fatty acid, mannose and arabinitol. Demannosyl and/or dearabinitoyl roselipins were prepared chemically or enzymatically. Demannnosyl roselipins conserved the DGAT inhibitory activity, but the others lost the activity, indicating that the arabinitoyl fatty acid core is essential for eliciting the activity.

Roselipins 1A, 1B, 2A and 2B were isolated as inhibitors of diacylglycerol acyltransferase (DGAT) from the culture broth of *Gliocladium roseum* KF-1040[1,2]. They consist of three common parts; 2,4,6,8,10,12,14,16,18-nonamethyl-5,9,13-trihydroxy-2*E*,6*E*,10*E*-icosatrienoic acid, D-mannose glycosylated at 13-OH and D-arabinitol esterified at 1-COOH. The roselipin A and B groups are stereoisomers at the arabinitol moiety, which esterifies the fatty acid from the different hydroxy terminals, and the roselipin 2 group is the derivative acetylated at 6-OH of mannose in the roselipin 1 group[3]. All roselipins showed DGAT inhibitory activity to similar extents and roselipins 1A and 1B showed anti-*Aspergillus niger* activity[2]. It is intriguing to test which part(s) in the structures is involved in the biological activities.

In this paper, demannosyl and/or dearabinitoyl roselipins are prepared chemically or enzymatically, and we show that demannosyl roselipin is the core structure for DGAT inhibition.

Experimental

General Experimental Procedures

UV spectra were recorded on a Shimadzu UV-200S spectrophotometer. IR spectra were recorded on a Horiba FT-210 infrared spectrometer. Optical rotations were obtained with a JASCO DIP-370 digital polarimeter. Melting points were measured with a Yanaco micro melting point apparatus. EI-MS spectra were recorded on a JEOL JMS-D 100 mass spectrometer at 20 eV. FAB-MS spectra were recorded on a JMS-DX300 mass spectrometer. The various NMR spectra were obtained on a Varian XL-400 spectrometer. HPLC was carried out using the JASCO (TRI ROTAR V) system with a YMC-pack column (D-ODS, 20×250 mm) (Yamamura Chem. Co.).

Roselipins

Roselipins 1A, 1B, 2A and 2B were purified from the culture broth of *Gliocladium roseum* KF-1040 as reported previously[1,2].

Preparation of Roselipin Derivatives R-3A and R-3B

Roselipin 1A (8.2 mg) was dissolved in 0.05 M citrate buffer (pH 4.5, 10 ml) and 10 mg/ml sodium taurodeoxycholate (100 μl). Then, β-mannosidase (snail origin, Sigma) was added to the solution on day 0 (20 units) and day 8 (10 units), and the mixture was incubated at 37°C for 30 days. The degraded product was extracted with ethyl acetate and the derivative R-3A was purified by HPLC (solvent, 80% CH_3CN; flow rate, 6 ml/minute; detection, UV at 220 nm). R-3A was eluted as a peak with a retention time of 35 minutes. The peak was collected and the fraction was concentrated to give pure R-3A (3.2 mg) as a colorless

* Corresponding author: tomoda@lisci.kitasato-u.ac.jp or omura-s@kitasato.or.jp

oil.

Roselipin 1B (3.0 mg) was dissolved in 0.05 M citrate buffer (pH 4.5, 10 ml) and 10 mg/ml sodium taurodeoxycholate (100 μl). To the solution was added β-mannosidase on day 0 (20 units) and day 10 (5 units), and the mixture was incubated at 37°C for 30 days. The degraded product R-3B was purified in a similar way. Under the same HPLC conditions, R-3B was eluted as a peak with a retention time of 35 minutes. The peak was collected and the fraction was concentrated to give pure R-3B (1.0 mg) as a colorless oil.

Preparation of Roselipin Derivative R-4

A mixture of roselipins 1A and 1B (50 mg) was dissolved in CH_3OH (1 ml), and was permitted to stand at room temperature for 2 days. After evaporation, derivative R-4 was purified by HPLC (solvent, 80% CH_3CN; flow rate, 6 ml/minute; detection, UV at 220 nm). R-4 was eluted as a peak with a retention time of 64 minutes. The peak was collected and the fraction was concentrated to give pure R-4 (26 mg) as a colorless oil.

Preparation of Roselipin Derivative R-5

A mixture of roselipins (20 mg) was dissolved in 2 N HCl-CH_3OH (1 ml), which was heated at 80°C for 12 hours. After evaporation, derivative R-5 was purified by HPLC (solvent, 100% CH_3CN; flow rate, 6 ml/minute; detection, UV at 220 nm). R-5 was eluted as a peak with a retention time of 58 minutes. The peak was collected and the fraction was concentrated to give pure R-5 (1.4 mg) as a colorless oil.

DGAT Activity

DGAT activity was assayed in an enzyme assay using rat liver microsomes[2,4] and in a cell assay using Raji cells as reported previously[5].

Antimicrobial Activity

Antimicrobial activity was tested using paper disks (6 mm, ADVANTEC). Bacteria were grown on Müller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after a 24-hour incubation at 37°C for

Table 1. Physico-chemical properties of roselipin derivatives R-3A, R-3B, R-4 and R-5.

	Derivative			
	R-3A	R-3B	R-4	R-5
Appearance	Colorless oil	Colorless oil	Colorless oil	Colorless oil
Molecular formula	$C_{34}H_{62}O_9$	$C_{34}H_{62}O_9$	$C_{36}H_{64}O_{10}$	$C_{31}H_{56}O_5$
Molecular weight	614	614	656	508
FAB-MS (*m/z*)				
Positive	615 $[M+H]^+$ 637 $[M+Na]^+$	615 $[M+H]^+$ 637 $[M+Na]^+$	679 $[M+Na]^+$	531 $[M+Na]^+$
Negative	613 $[M-H]^-$	613 $[M-H]^-$	655 $[M-H]^-$	507 $[M-H]^-$
HRFAB-MS (*m/z*)				
	$C_{34}H_{62}O_9Na$ $[M+Na]^+$	$C_{34}H_{62}O_9Na$ $[M+Na]^+$	$C_{36}H_{64}O_{10}Na$ $[M+Na]^+$	$C_{31}H_{56}O_5Na$ $[M+Na]^+$
Calcd:	637.4291	637.4291	679.4397	531.4025
Found:	637.4283	637.4273	679.4391	531.4030
$[\alpha]_D^{24}$	+ 18 °	+ 10 °	+ 12 °	+ 14 °
	(*c* 0.1, MeOH)	(*c* 0.054, MeOH)	(*c* 0.1, MeOH)	(*c* 0.028, MeOH)
UV $\lambda_{max}^{CH_3OH}$ nm (ε)	202 (19,000)	202 (30,100)	202 (17,700)	206 (62,900)
	222 (14,100)	222 (22,700)	221 (13,300)	216 (59,900)
IR υ_{max}^{KBr} (cm^{-1})	3421, 2960, 2926, 2873, 2854, 1714, 1647, 1583, 1456, 1377, 1271, 1227, 1078,1018	3430, 2960, 2929, 2873, 2854, 1701, 1632, 1583, 1458, 1385, 1273, 1228, 1042, 1018	3430, 2962, 2926, 2875, 1714, 1641, 1458, 1379, 1269, 1227, 1124, 1072, 1026	3434, 2962, 2927, 2873, 1718, 1655, 1458, 1377, 1269, 1234, 1128, 1099, 1028
Solubility				
Soluble:	CH_3OH, $CHCl_3$, CH_3CN, acetone, C_2H_5OH, ethyl acetate	CH_3OH, $CHCl_3$, CH_3CN, acetone, C_2H_5OH, ethyl acetate	CH_3OH, $CHCl_3$, CH_3CN, acetone, C_2H_5OH, ethyl acetate	CH_3OH, $CHCl_3$, CH_3CN, acetone, C_2H_5OH, ethyl acetate
Insoluble:	H_2O, *n*-hexane	H_2O, *n*-hexane	H_2O, *n*-hexane	H_2O, *n*-hexane
Color reaction				
Positive:	50% H_2SO_4	50% H_2SO_4	50% H_2SO_4	50% H_2SO_4
Negative:	Ninhydrin reagent	Ninhydrin reagent	Ninhydrin reagent	Ninhydrin reagent

bacteria and after a 48-hour incubation at 27°C for fungi and yeasts.

Results

Structures of Roselipin Derivatives

Physico-chemical properties of roselipin derivatives are summarized in Table 1. In comparison with those of parent roselipins, all the signals of the derivatives observed by NMR experiments were completely assigned as shown in Tables 2 and 3. Eventually, derivative R-3A is demannnosyl roselipin A, R-3B is demannnosyl roselipin B, R-4 is dearabinytoyl roselipin 1 methyl ester, and R-5 is demannnosyl-dearabinitoyl-5-*O*-methylroselipin methyl ester (Fig. 1). The elucidated structures were definitely supported by the physico-chemical properties.

DGAT Inhibitory Activity

Parent roselipins showed DGAT inhibition in the *in vitro* enzymatic assay with IC_{50} values of 15~22 μM. As listed in Table 4, derivatives R-3A and R-3B maintained the inhibitory activity (IC_{50} values; 60 and 33 μM, respectively), although

Table 2. ^{1}H and ^{13}C NMR chemical shifts of roselipin derivatives R-3A and R-3B.

Carbon No.	Derivative R-3A		Derivative R-3B	
	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]
C-1	170.05		169.82	
C-2	128.81		128.73	
C-3	147.57	6.80 (1H, dd, *J*=10.0, 1.5 Hz)	147.71	6.78 (1H, dd, *J*=10.0, 1.5 Hz)
C-4	38.02	2.73 (1H, m)	38.07	2.75 (1H, m)
C-5	83.71	3.82 (1H, d, *J*=8.5 Hz)	83.72	3.82 (1H, d, *J*=9.0 Hz)
C-6	137.14		137.20	
C-7	134.30	5.33 (1H, dd, *J*=9.0, 1.0 Hz)	134.30	5.33 (1H, dd, *J*=9.0, 1.0 Hz)
C-8	37.08	2.62 (1H, m)	37.12	2.63 (1H, m)
C-9	84.33	3.72 (1H, d, *J*=9.5 Hz)	84.31	3.72 (1H, d, *J*=9.5 Hz)
C-10	134.79		134.84	
C-11	134.69	5.56 (1H, dd, *J*=9.5, 1.0 Hz)	134.67	5.57 (1H, dd, *J*=9.5, 1.5 Hz)
C-12	36.20	2.75 (1H, m)	36.24	2.76 (1H, m)
C-13	87.31	3.49 (1H, dd, *J*=7.0, 3.5 Hz)	87.33	3.49 (1H, dd, *J*=7.0, 3.5 Hz)
C-14	34.27	1.87 (1H, m)	34.29	1.87 (1H, m)
C-15	43.93	0.95 (1H, m)	43.97	1.02 (1H, m)
		1.37 (1H, m)		1.42 (1H, m)
C-16	28.85	1.62 (1H, m)	28.90	1.62 (1H, m)
C-17	46.04	0.88 (1H, m)	46.07	0.94 (1H, m)
		1.24 (1H, m)		1.27 (1H, m)
C-18	32.91	1.45 (1H, m)	32.94	1.45 (1H, m)
C-19	29.86	1.08 (1H, m)	29.89	1.22 (1H, m)
		1.42 (1H, m)		1.45 (1H, m)
C-20	11.53	0.89 (3H, t, *J*=7.0 Hz)	11.52	0.89 (3H, t, *J*=6.5 Hz)
C-21	12.88	1.90 (3H, d, *J*=1.5 Hz)	12.87	1.89 (3H, d, *J*=1.5 Hz)
C-22	16.81	0.86 (3H, d, *J*=7.0 Hz)	16.82	0.85 (3H, d, *J*=7.0 Hz)
C-23	11.36	1.68 (3H, d, *J*=1.0 Hz)	11.36[d]	1.67 (3H, d, *J*=1.0 Hz)
C-24	17.82	0.78 (3H, d, *J*=7.0 Hz)	17.82	0.78 (3H, d, *J*=7.0 Hz)
C-25	11.32	1.64 (3H, d, *J*=1.0 Hz)	11.36[d]	1.64 (3H, d, *J*=1.5 Hz)
C-26	18.63	0.99 (3H, d, *J*=7.0 Hz)	18.63	0.99 (3H, d, *J*=7.0 Hz)
C-27	15.56	0.94 (3H, d, *J*=7.0 Hz)	15.58	0.95 (3H, d, *J*=7.0 Hz)
C-28	21.34	0.90 (3H, d, *J*=6.5 Hz)	21.35	0.90 (3H, d, *J*=7.0 Hz)
C-29	20.73	0.90 (3H, d, *J*=6.5 Hz)	20.73	0.90 (3H, d, *J*=7.0 Hz)
C-1'	67.88	4.25 (1H, dd, *J*=12.0, 6.5 Hz)	67.16	4.20 (1H, dd, *J*=11.0, 5.5 Hz)
		4.41 (1H, dd, *J*=12.0, 3.0 Hz)		4.27 (1H, dd, *J*=11.0, 7.0 Hz)
C-2'	70.63	3.94 (1H, ddd, *J*=9.0, 6.5, 3.0 Hz)	69.38	4.14 (1H, ddd, *J*=7.0, 5.5, 2.0 Hz)
C-3'	71.93	3.58 (1H, dd, *J*=9.0, 2.0 Hz)	72.30	3.53 (1H, dd, *J*=8.0, 2.0 Hz)
C-4'	71.64	3.92 (1H, ddd, *J*=6.5, 6.5, 2.0 Hz)	72.71	3.73 (1H, ddd, *J*=8.0, 6.0, 3.5 Hz)
C-5'	64.80	3.65 (2H, ddd, *J*=7.0, 6.5, 6.5 Hz)	65.03	3.64 (1H, dd, *J*=11.0, 6.0 Hz)
				3.81 (1H, dd, *J*=11.0, 3.5 Hz)

[a] Chemical shifts are shown with reference to CD_3OD as 49.8 ppm. [b] Chemical shifts are shown with reference to CD_3OD as 3.30 ppm. [c] Signal was observed as a double doublet with $^{2}J_{CH}$=6.8 Hz. [d] The signals were observed as the same chemical shifts.

Table 3. ^{1}H and ^{13}C NMR chemical shifts of roselipin derivatives R-4 and R-5.

Carbon No.	Derivative R-4		Derivative R-5	
	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]
C-1	170.35		170.35	
C-2	128.55		128.31	
C-3	147.65	6.73 (1H, dd, J=10.0, 1.5 Hz)	147.63	6.67 (1H, dd, J=10.0, 1.5 Hz)
C-4	38.03	2.73 (1H, m)	37.49	2.72 (1H, m)
C-5	83.77	3.80 (1H, d, J=8.5 Hz)	93.51	3.32 (1H, d, J=8.5 Hz)
C-6	137.2		132.48	
C-7	134.35	5.32 (1H, dd, J=9.5, 1.0 Hz)	136.4	5.63 (1H, dd, J=9.5, 1.5 Hz)
C-8	37.12	2.62 (1H, m)	36.44	2.87 (1H, m)
C-9	84.35	3.71 (1H, d, J=9.5 Hz)	78.73	3.87 (1H, brd, J=5.0 Hz)
C-10	134.84		134.5	
C-11	134.67	5.56 (1H, dd, J=9.5, 1.0 Hz)	128.86	5.47 (1H, dd, J=4.0, 1.0 Hz)
C-12	36.23	2.75 (1H, m)	32.13	2.18 (1H, m)
C-13	87.28	3.49 (1H, dd, J=6.5, 3.5 Hz)	80	3.20 (1H, dd, J=6.5, 5.0 Hz)
C-14	34.26	1.87 (1H, m)	32.35	1.80 (1H, m)
C-15	43.95	1.00 (1H, m)	44.12	1.02 (1H, m)
		1.39 (1H, m)		1.46 (1H, m)
C-16	28.88	1.62 (1H, m)	29.19	1.62 (1H, m)
C-17	46.04	0.91 (1H, m)	46.17	0.91 (1H, m)
		1.26 (1H, m)		1.26 (1H, m)
C-18	32.93	1.45 (1H, m)	32.95	1.46 (1H, m)
C-19	29.88	1.08 (1H, m)	29.76	1.08 (1H, m)
		1.42 (1H, m)		1.42 (1H, m)
C-20	11.53	0.89 (3H, t, J=7.0 Hz)	11.56	0.89 (3H, t, J=7.0 Hz)
C-21	12.84	1.87 (3H, d, J=1.5 Hz)	12.8	1.84 (3H, d, J=1.5 Hz)
C-22	16.82	0.84 (3H, d, J=7.0 Hz)	16.93	0.85 (3H, d, J=7.0 Hz)
C-23	11.34	1.67 (3H, d, J=1.0 Hz)	10.91	1.59 (3H, d, J=1.5 Hz)
C-24	17.81	0.78 (3H, d, J=7.0 Hz)	16.63	0.99 (3H, d, J=7.0 Hz)
C-25	11.31	1.64 (3H, d, J=1.0 Hz)	20.49	1.69 (3H, d, J=1.0 Hz)
C-26	18.61	0.99 (3H, d, J=7.0 Hz)	19.39	0.97 (3H, d, J=7.0 Hz)
C-27	15.59	0.95 (3H, d, J=7.0 Hz)	15.54	0.87 (3H, d, J=7.0 Hz)
C-28	21.36	0.90 (3H, d, J=6.5 Hz)	20.79	0.89 (3H, d, J=7.0 Hz)
C-29	20.74	0.90 (3H, d, J=6.5 Hz)	21.03	0.87 (3H, d, J=7.0 Hz)
C-1'	52.2	3.73 (3H, s)	52.22	3.73 (3H, s)
C-1"	102.64	4.49 (1H, bs)		
C-2"	72.69	3.90 (1H, d, J=3.0 Hz)		
C-3"	75.67	3.38 (1H, dd, J=9.5, 3.0 Hz)		
C-4"	68.55	3.57 (1H, dd, J=9.5, 9.5 Hz)		
C-5"	78.25	3.16 (1H, ddd, J=9.5, 5.0, 2.5 Hz)		
C-6"	62.95	3.76 (1H, dd, J=11.5, 5.0 Hz)		
		3.89 (1H, dd, J=11.5, 2.5 Hz)		
C-5-O-Me			56.72	3.15 (3H, s)

[a] Chemical shifts are shown with reference to CD_3OD as 49.8 ppm. [b] Chemical shifts are shown with reference to CD_3OD as 3.30 ppm. [c] Signal was observed as a double doublet with $^{2}J_{CH}$=6.8 H
[d] The signals were observed as the same chemical shifts.

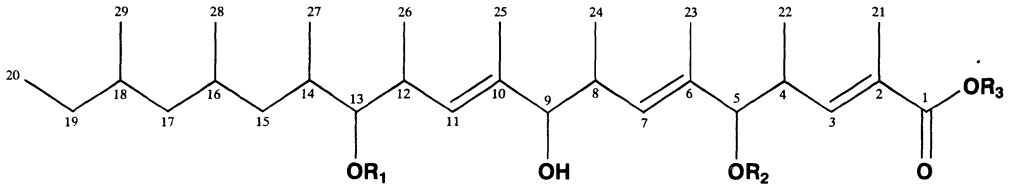
the potency slightly reduced. On the other hand, derivatives R-4 and R-5 completely lost the inhibitory activity.

In the cell assay using Raji cells (Table 4), derivatives R-3A and R-3B inhibited triacylglycerol (TG) synthesis with IC_{50} values of 11 and 10 μM, respectively, showing more potent than parent roselipins in the cell assay. Derivatives R-4 and R-5 showed very weak inhibition as expected from the results of the *in vitro* assay.

Antifungal Activities

Almost no antibacterial activity of the four derivatives was observed at a concentration of 1.0 mg/ml (10 μg/6 mm disk) against the following microorganism; *Bacillus subtilis*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus* and *Staphylococcus aureus*. Regarding their antifungal and

Fig. 1. Structures of roselipin derivatives.



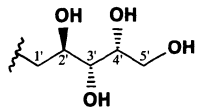
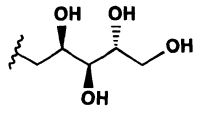
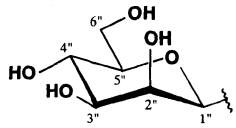
Roselipin derivatives	R_1	R_2	R_3
R-3A	-H	-H	
R-3B	-H	-H	
R-4		-H	-CH_3
R-5	-H	-CH_3	-CH_3

Table 4. DGAT inhibitory activity of roselipin derivatives R-3A, R-3B, R-4 and R-5.

Compound	IC_{50} (μM)	
	Enzyme assay	Cell assay
Roselipin 1A	17	39
1B	15	32
2A	22	24
2B	18	18
Derivative R-3A	60	11
R-3B	33	10
R-4	>760	200
R-5	>960	250

antiyeast activities against *Candida albicans*, *Saccharomyces cerevisiae*, *Pyricularia oryzae*, *Mucor racemosus* and *Aspergillus niger*, derivatives R-3A and R-4 were active against *Mucor racemosus* (diameter of inhibition zone; 14 and 12 mm, respectively) and *Pyricularia oryzae* (0 and 16 mm, respectively) at 1.0 mg/ml (10 μg/6 mm disk). They lost anti-*A. niger* activity, although their parent roselipins 1A and 1B showed the activity. Derivatives R-3B and R-5 showed no antifungal and antiyeast activities at the concentration. Difference in the antifungal activity between derivatives R-3A and R-3B may be that the binding form of the arabinitol to the fatty acid core is significant for antifungal activity. These results are not coincident with those in DGAT inhibition, probably due to its different mode of action.

Discussion

Although they are not good substrates of the enzyme, roselipins 1A and 1B were hydrolyzed gradually by snail β-mannnosidase to produce demannosylated derivatives R-3A and R-3B after a 30 day-incubation. The derivatives maintained the DGAT inhibitory activity, while derivatives R-4 and R-5 lost the activity completely (Table 4), indicating that the arabinitoyl fatty acid core is essential for eliciting DGAT inhibitory activity. It might be that the core structure is mimic to diacylglycerol, the substrate of DGAT enzyme. The results in the cell assay are almost comparable to those in the enzyme assay. Derivatives R-3A and R-3B are more potent in the cell assay than roselipins (Table 4), suggesting that the derivatives are more membrane-permeable than roselipins.

Farese and coworkers identified a gene encoding a mammalian DGAT (DGAT-1)[6]. Its mRNA expression was detected in every mammalian tissue, and the highest expression levels were found in small intestine. These findings are consistent with a proposed role for DGAT in intestinal fat absorption. However, mRNA expression was relatively low in the livers of humans regardless of high DGAT activity, suggesting the existence of a second DGAT in livers. Recently, a second mammalian DGAT (DGAT-2) was cloned and characterized[7]. DGAT-1 and -2 showed no homology, but similar characteristics. Therefore, selectivity of our DGAT inhibitors[1,8~10] in DGAT-1 and -2 inhibition remains to be studied, and they will become the center of researchers' attention[11]. Further understanding DGAT at a molecular level will help us search for DGAT inhibitors, leading to potential approaches for treating hypertriglyceridemia or obesity in humans.

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