

Supplementary Materials

Takeda G protein-coupled receptor 5 and peroxisome proliferator-activated receptor-gamma activation by pinocembrin and pinostrobin isolated from *Lindera sericea*

Ryo Miyata,¹ Masanobu Suzuki,² Yuka Okazaki,² Kento Iwai,^{3,4} Nagatoshi Nishiwaki,³ and Yoshihiro Nakajima¹

¹*Health and Medical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2217-14 Hayashi-cho, Takamatsu, Kagawa 761-0395, Japan*

²*Resources and Environment Division, Kochi Prefectural Industrial Technology Center, 3992-3 Nunoshida, Kochi, Kochi 781-5101, Japan*

³*School of Engineering Science, Kochi University of Technology, Tosayamada, Kami, Kochi 782-8502, Japan*

⁴*Department of Chemistry, Faculty of Science, Nara Women's University, Kita-uoyanishimachi, Nara 630-8506, Japan*

Correspondence

Yoshihiro Nakajima, Health and Medical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2217-14 Hayashi-cho, Takamatsu, Kagawa 761-0395, Japan

E-mail: y-nakajima@aist.go.jp

Contents

S1 Generation of reporter cell line for monitoring TGR5 activation

Figure S1 Effects of INT-777 on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines.	1
Figure S2. Effects of ursolic acid on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines.	2
Figure S3. Effects of forskolin on CREB pathway activation in TGR5(-) negative HepG2 reporter cell line.	3

S2 Evaluation of TGR5 and PPAR γ activation by *L. sericea* methanolic extract

Figure S4 Effects of <i>L. sericea</i> methanol extracts on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines.	4
Figure S5 Reporter cell line for monitoring PPAR γ activation.	5
Figure S6 Effects of <i>L. sericea</i> methanol extracts on PPAR γ activation in A9 reporter cell line.	6

S3 Spectroscopic analysis of *rac*-pinocembrin and *rac*-pinostrobin

Figure S7 HRESIMS spectrum of <i>rac</i> -1.	7
Figure S8 ^1H NMR spectrum (400 MHz, CDCl_3) of <i>rac</i> -1.	8
Figure S9 ^{13}C NMR spectrum (100 MHz, CDCl_3) of <i>rac</i> -1.	9
Figure S10 HRESIMS spectrum of <i>rac</i> -2.	10
Figure S11 ^1H NMR spectrum (400 MHz, CDCl_3) of <i>rac</i> -2.	11
Figure S12 ^{13}C NMR spectrum (100 MHz, CDCl_3) of <i>rac</i> -2.	12

S4 Optical resolutions of *rac*-pinocembrin and *rac*-pinostrobin

Figure S13 Chiral HPLC chromatograms of <i>rac</i> -1, (<i>R</i>)-1, and (<i>S</i>)-1.	13
Figure S14 Chiral HPLC chromatograms of <i>rac</i> -2, (<i>R</i>)-2, and (<i>S</i>)-2.	14
Figure S15 ^1H NMR spectrum (400 MHz, CDCl_3) of (<i>R</i>)-1.	15
Figure S16 ^{13}C NMR spectrum (100 MHz, CDCl_3) of (<i>R</i>)-1.	16
Figure S17 ^1H NMR spectrum (400 MHz, CDCl_3) of (<i>S</i>)-1.	17
Figure S18 ^{13}C NMR spectrum (100 MHz, CDCl_3) of (<i>S</i>)-1.	18
Figure S19 ^1H NMR spectrum (400 MHz, CDCl_3) of (<i>R</i>)-2.	19
Figure S20 ^{13}C NMR spectrum (100 MHz, CDCl_3) of (<i>R</i>)-2.	20
Figure S21 ^1H NMR spectrum (400 MHz, CDCl_3) of (<i>S</i>)-2.	21

Figure S22 ^{13}C NMR spectrum (100 MHz, CDCl_3) of (<i>S</i>)- 2	22
Figure S23 Chiral HPLC chromatogram of <i>L. sericea</i> methanol extracts.	23

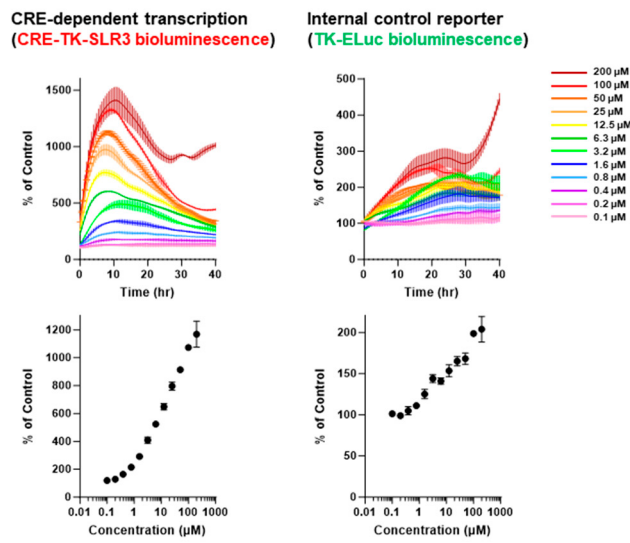
S5 Evaluation of TGR5 and PPAR γ activation by *rac*-pinocembrin and *rac*-pinostrobin

Figure S24 Effects of <i>rac</i> -pinocembrin (<i>rac</i> - 1) on TGR5 activation in TGR5-expressing HepG2 reporter cell line.	24
Figure S25 Effects of <i>rac</i> -pinostrobin (<i>rac</i> - 2) on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines.	25
Figure S26 Effects of <i>rac</i> -pinocembrin (<i>rac</i> - 1) and <i>rac</i> -pinostrobin (<i>rac</i> - 2) on PPAR γ activation in A9 reporter cell line.	26

S6 Evaluation of TGR5 and PPAR γ activation by enantiomer of pinocembrin and pinostrobin

Figure S27 Effects of (<i>R</i>)-pinostrobin ((<i>R</i>)- 2) on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines.	27
Figure S28 Effects of (<i>S</i>)-pinostrobin ((<i>S</i>)- 2) on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines.	28
Figure S29 Effects of (<i>R</i>)-pinocembrin ((<i>R</i>)- 1) and (<i>S</i>)-pinocembrin ((<i>S</i>)- 1) on PPAR γ activation in A9 reporter cell line.	29
Figure S30 Effects of (<i>R</i>)-pinostrobin ((<i>R</i>)- 2) and (<i>S</i>)-pinostrobin ((<i>S</i>)- 2) on PPAR γ activation in A9 reporter cell line.	30

(a) INT-777, TGR5(+)



(b) INT-777, TGR5(-)

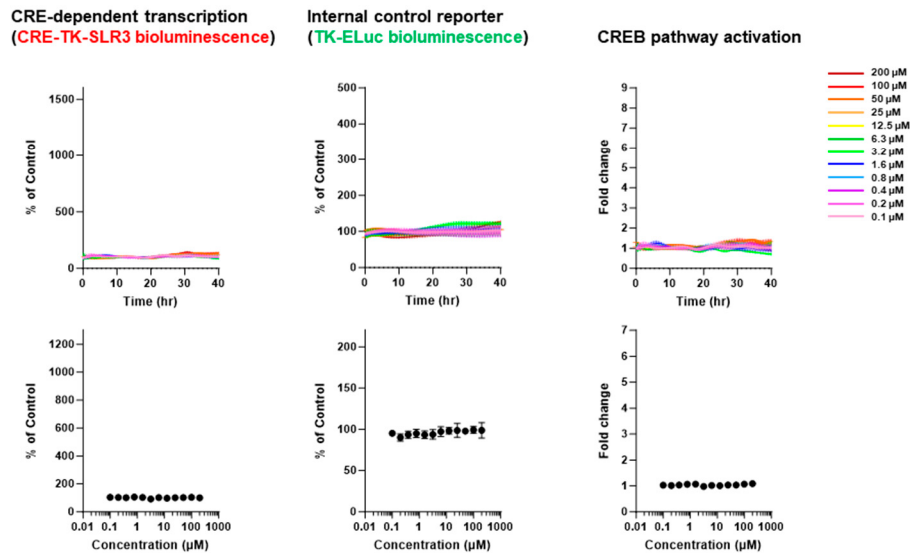
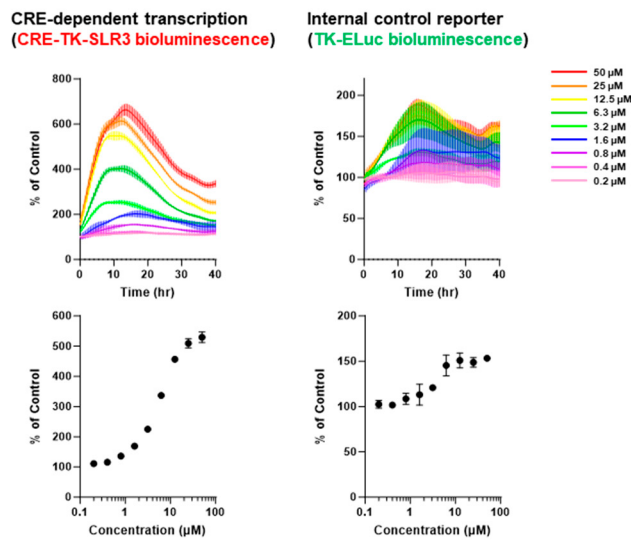


Figure S1 Effects of INT-777 on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines. Kinetics of CRE-dependent transcription monitored by SLR3 and internal control reporter ELuc, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and ELuc bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Fold-change values were normalized to 1 at each time point relative to vehicle control. Values are shown as means $\pm$ SD (n = 3).

(a) Ursolic acid, TGR5(+)



(b) Ursolic acid, TGR5(-)

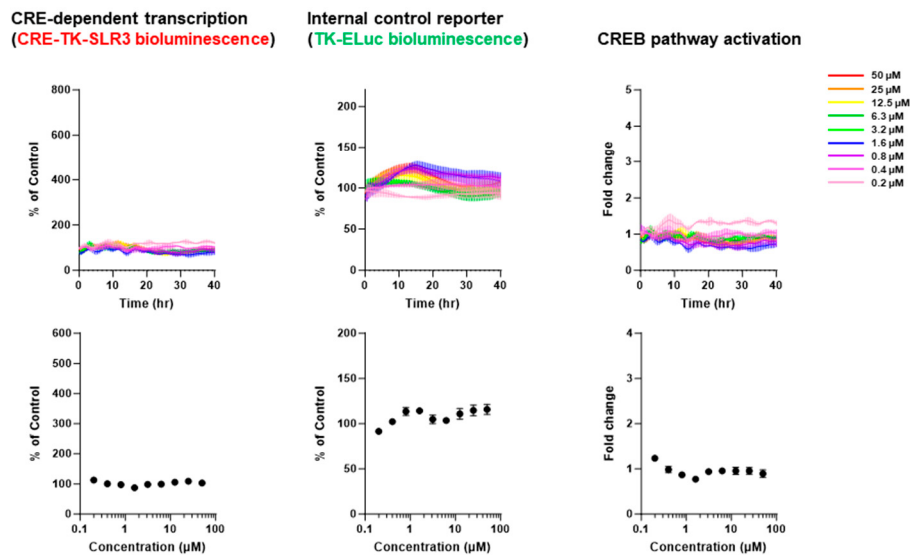


Figure S2 Effects of ursolic acid on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines. Kinetics of CRE-dependent transcription monitored by SLR3 and internal control reporter ELuc, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and ELuc bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Fold-change values were normalized to 1 at each time point relative to vehicle control. Values are shown as means $\pm$ SD (n = 3).

Forskolin TGR5(–)

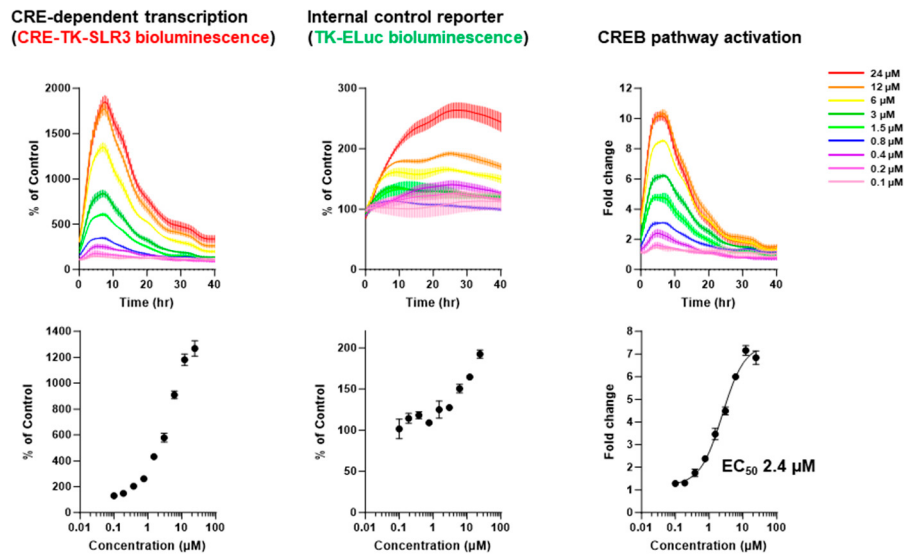
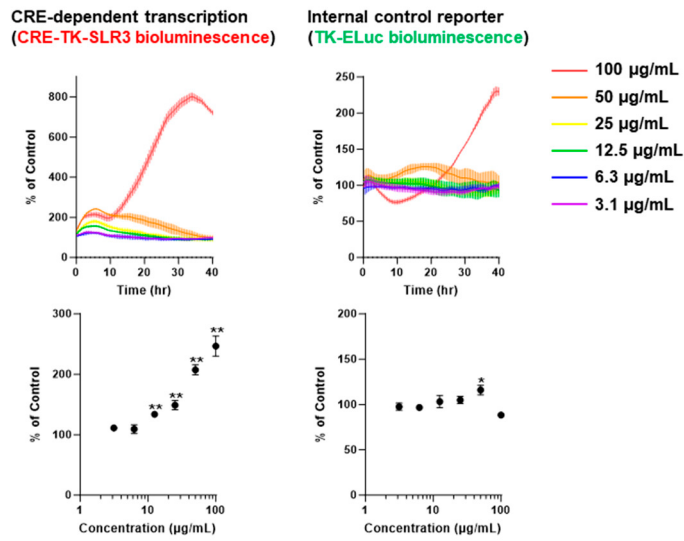


Figure S3 Effects of forskolin on CREB pathway activation in TGR5(–) negative HepG2 reporter cell line. Kinetics of CRE-dependent transcription monitored by SLR3 and internal control reporter ELuc, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and ELuc bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Fold-change values were normalized to 1 at each time point relative to vehicle control. Values are shown as means $\pm$ SD (n = 3).

(a) Methanol extracts, TGR5(+)



(b) Methanol extracts, TGR5(-)

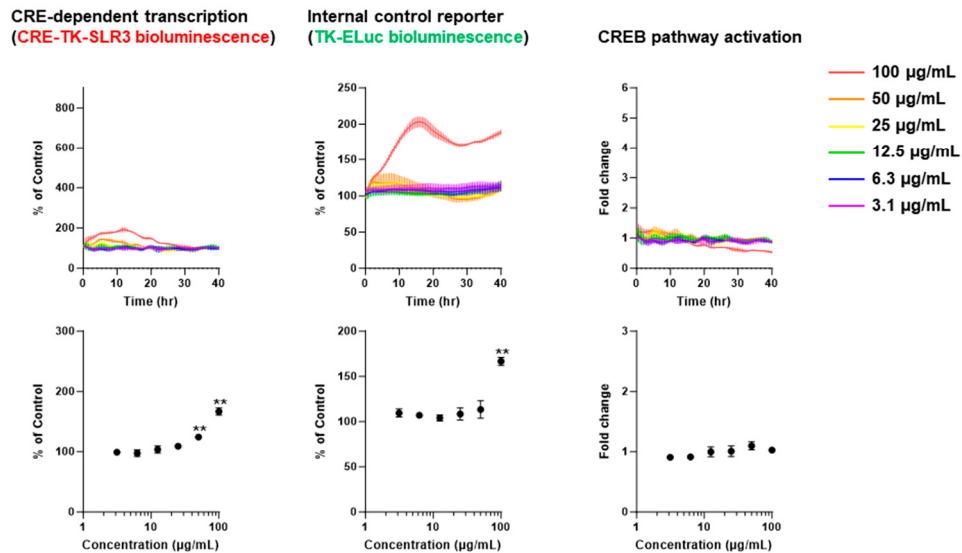


Figure S4 Effects of *L. sericea* methanol extracts on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines. Kinetics of CRE-dependent transcription monitored by SLR3 and internal control reporter ELuc, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and ELuc bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Fold-change values were normalized to 1 at each time point relative to vehicle control. Values are shown as means $\pm$ SD (n = 3).

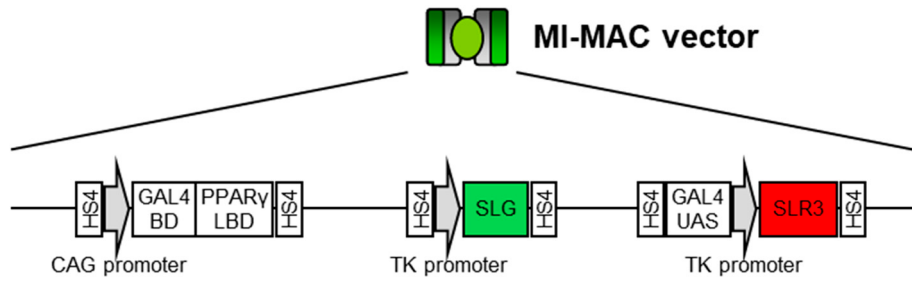


Figure S5 Reporter cell line for monitoring PPAR γ activation. A schematic diagram of constructs established by the site-specific insertion of plasmids carrying the PPAR γ ligand binding domain and luciferase genes into the MI-MAC vector. The plasmids were each inserted into the ϕ C31, R4, and Bxb1 attP sites of the MI-MAC vector by recombinase-mediated homologous recombination in mouse fibroblast A9 cells. Key: HS4, tandem repeat of HS4 insulator; GAL4BD, GAL4 binding domain; PPAR γ LBD, PPAR γ ligand binding domain; SLG, stable luciferase green; GAL4 UAS, GAL4 upstream activation sequence; SLR3, stable luciferase red3.

Methanol extracts, PPAR γ

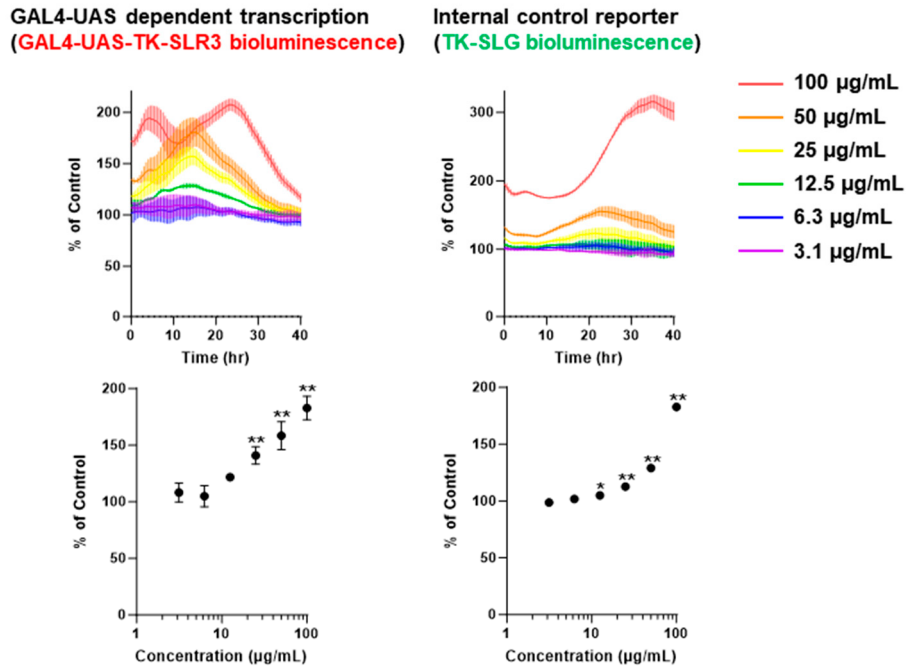


Figure S6 Effects of *L. sericea* methanol extracts on PPAR γ activation in A9 reporter cell line. Kinetics of GAL4-UAS dependent transcription monitored by SLR3 and internal control reporter SLG, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and SLG bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Values are shown as means $\pm$ SD (n = 3).

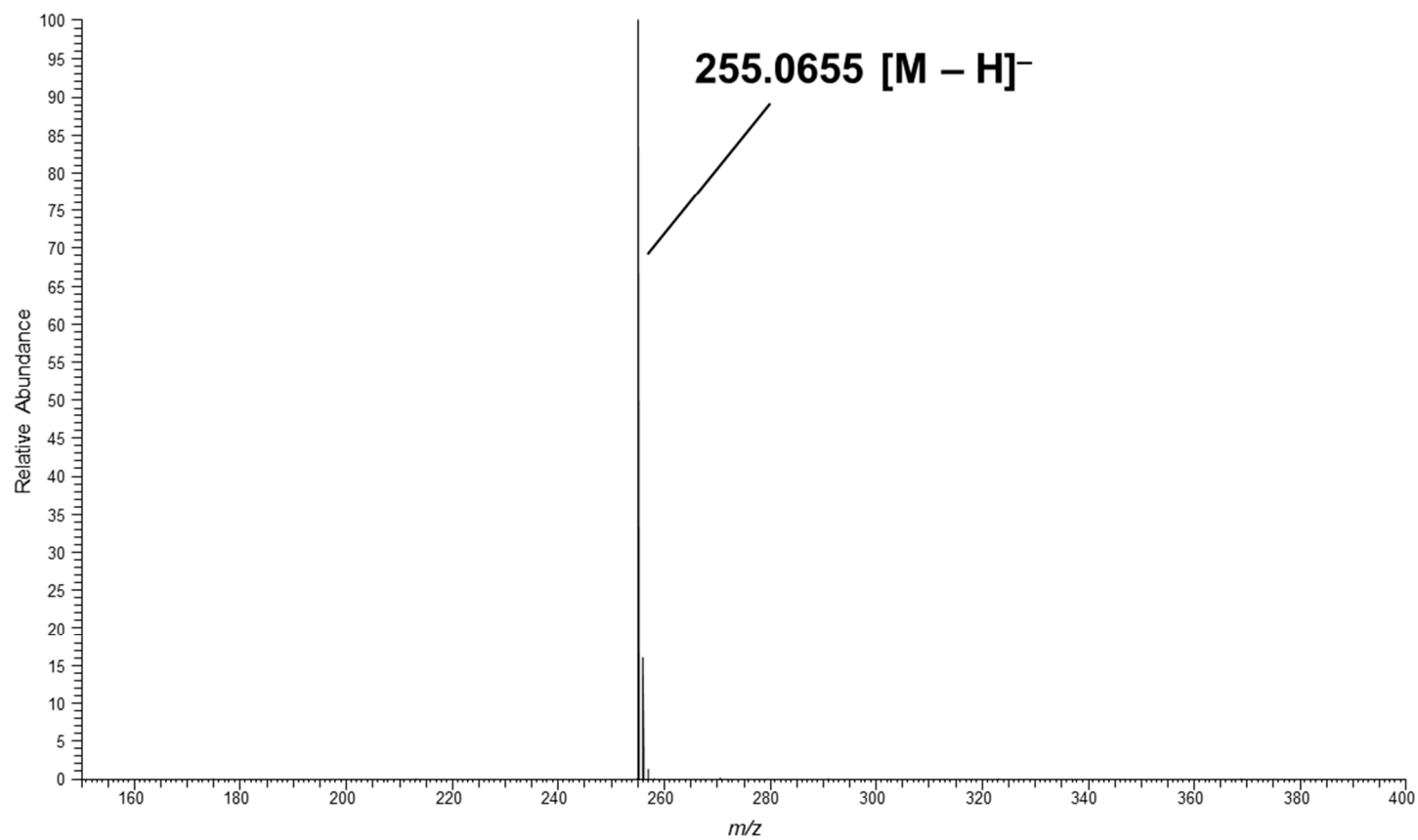


Figure S7 HRESIMS spectrum of *rac*-1.

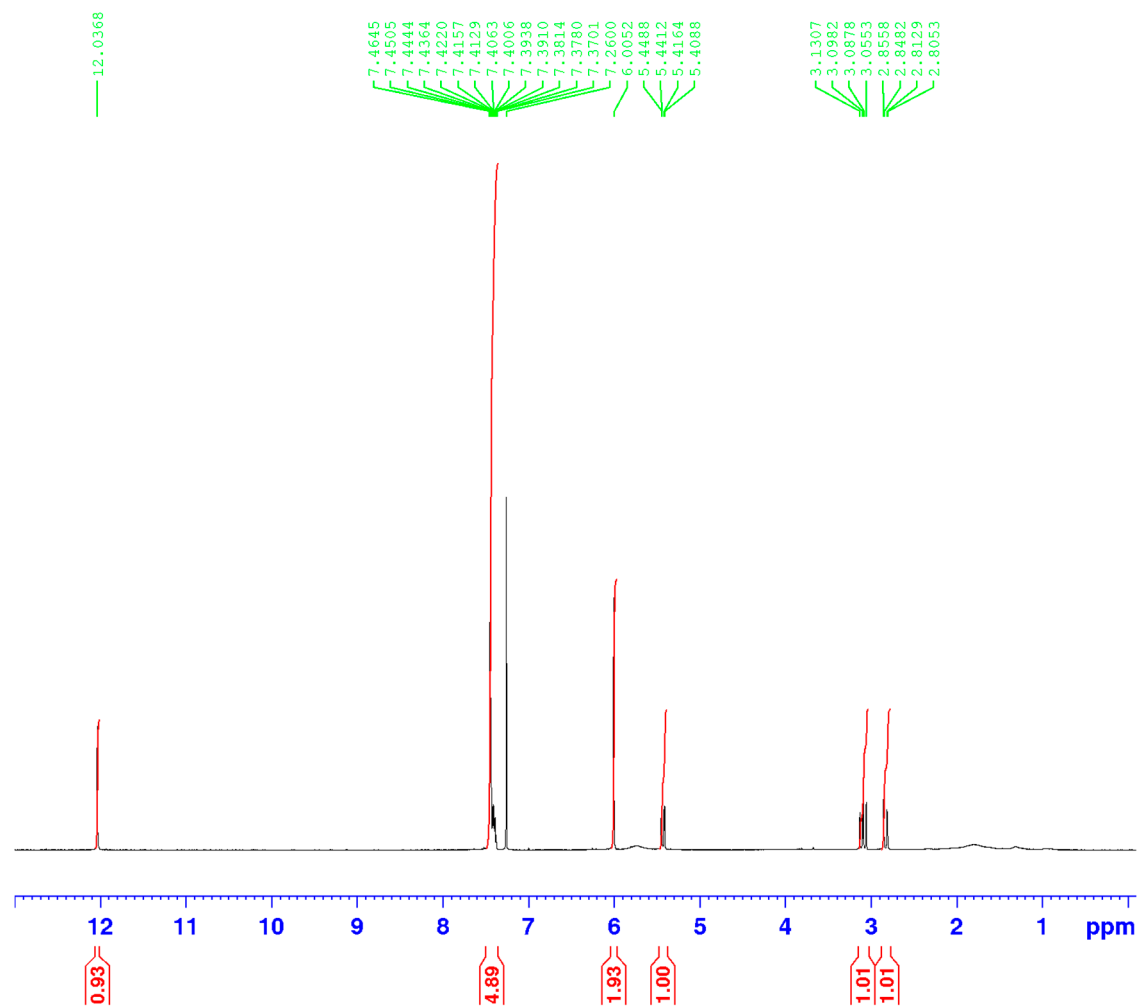


Figure S8 ¹H NMR spectrum (400 MHz, CDCl₃) of *rac*-1.

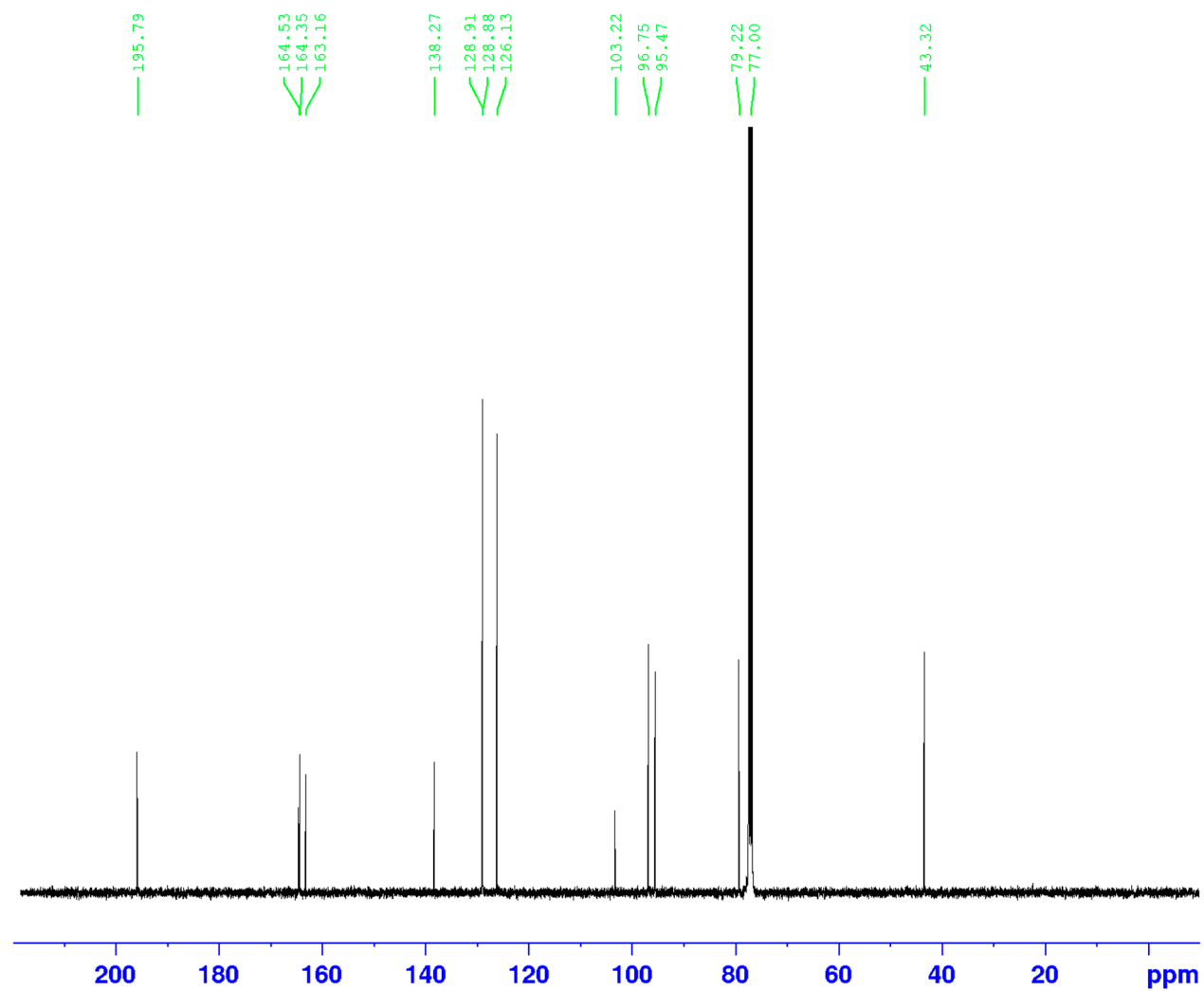


Figure S9 ¹³C NMR spectrum (100 MHz, CDCl₃) of *rac*-1.

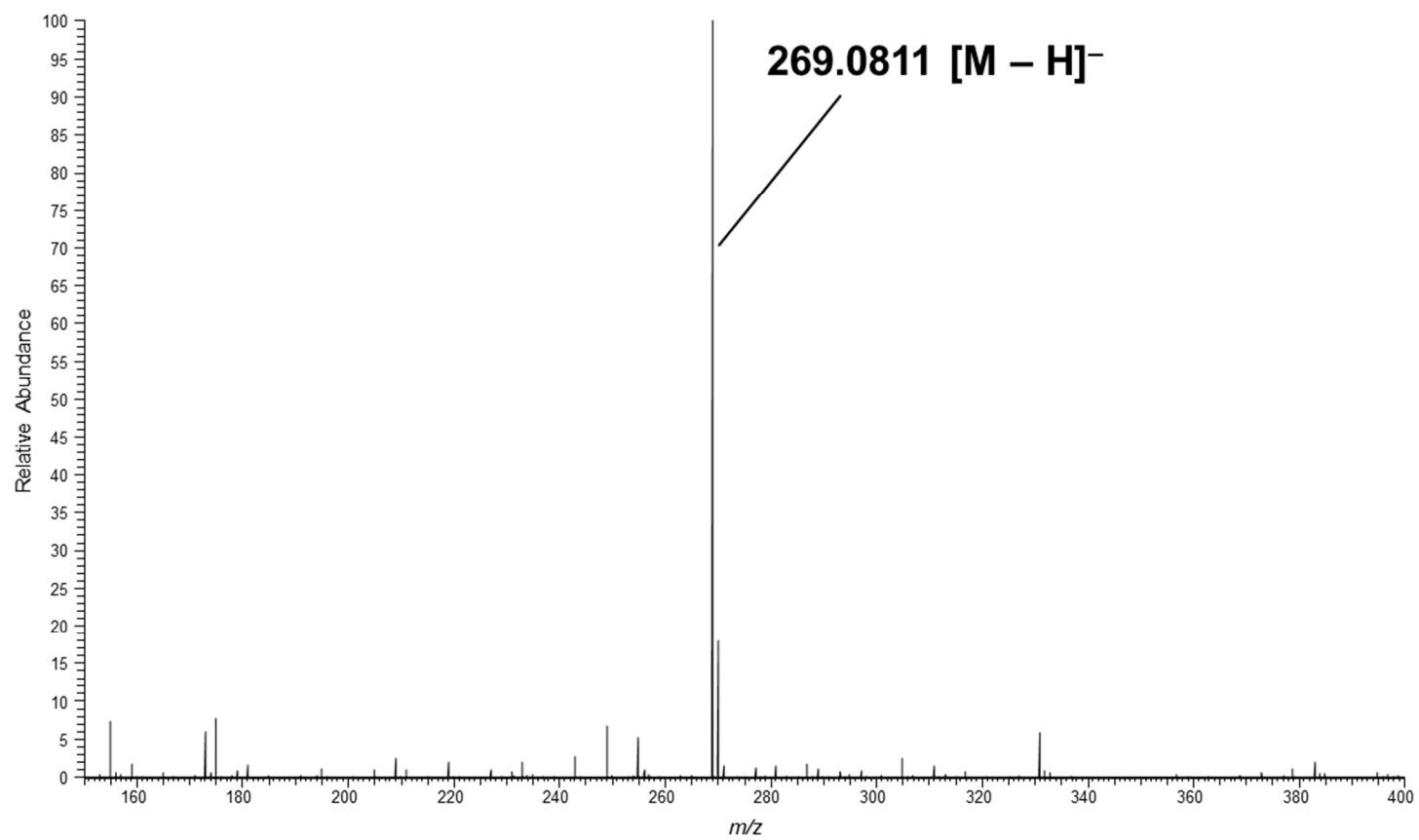


Figure S10 HRESIMS spectrum of *rac*-2.

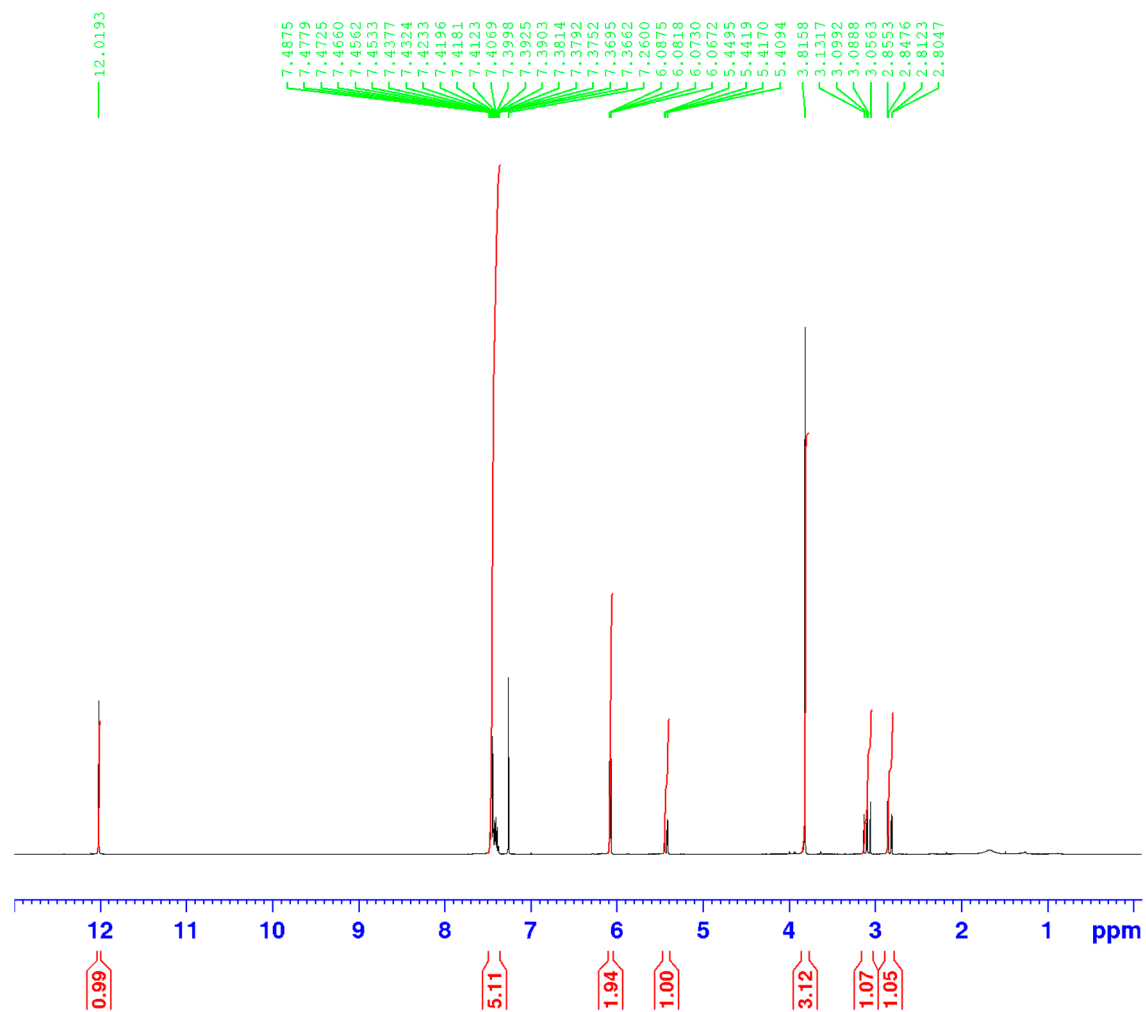


Figure S11 ¹H NMR spectrum (400 MHz, CDCl₃) of *rac*-2.

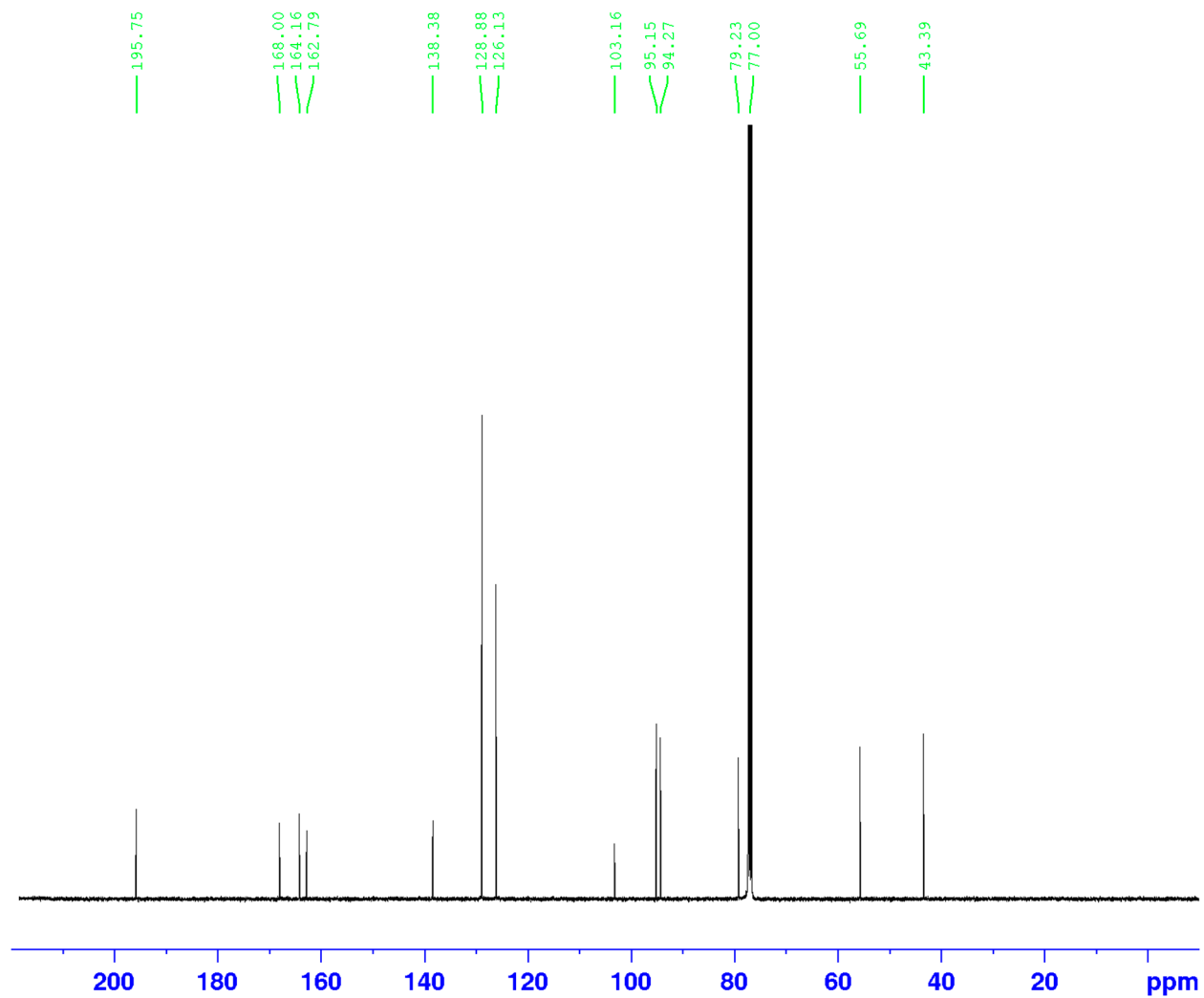


Figure S12 ¹³C NMR spectrum (100 MHz, CDCl₃) of *rac*-2.

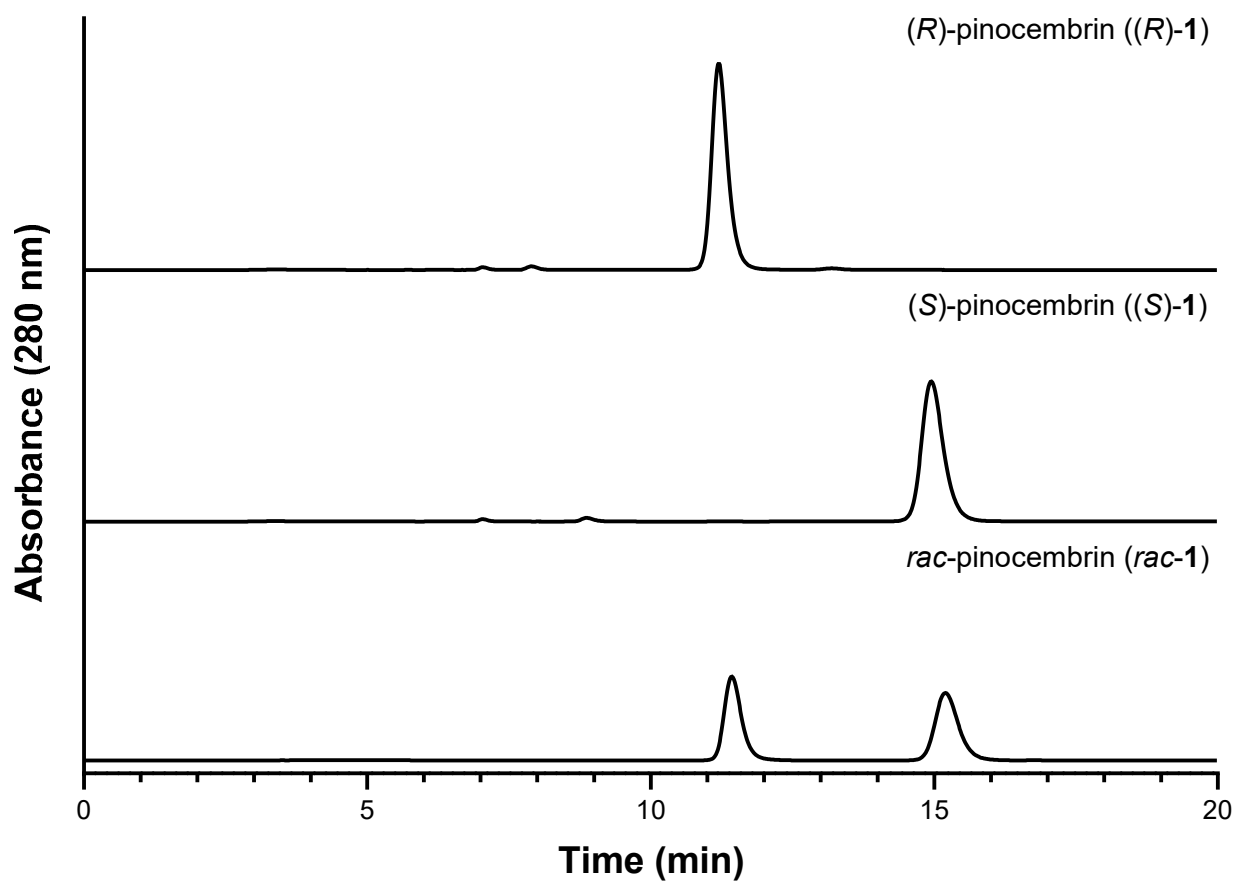


Figure S13 Chiral HPLC chromatograms of *rac*-1, (*R*)-1, and (*S*)-1. HPLC conditions as follows: column, CHIRALPAK IG chiral column (5 μ m, 4.6 $\times$ 250 mm, Daicel); flow rate, 1.0 mL/min; mobile phase, H₂O/acetonitrile (45:55, 0.1% TFA); and detection wavelength, 280 nm.

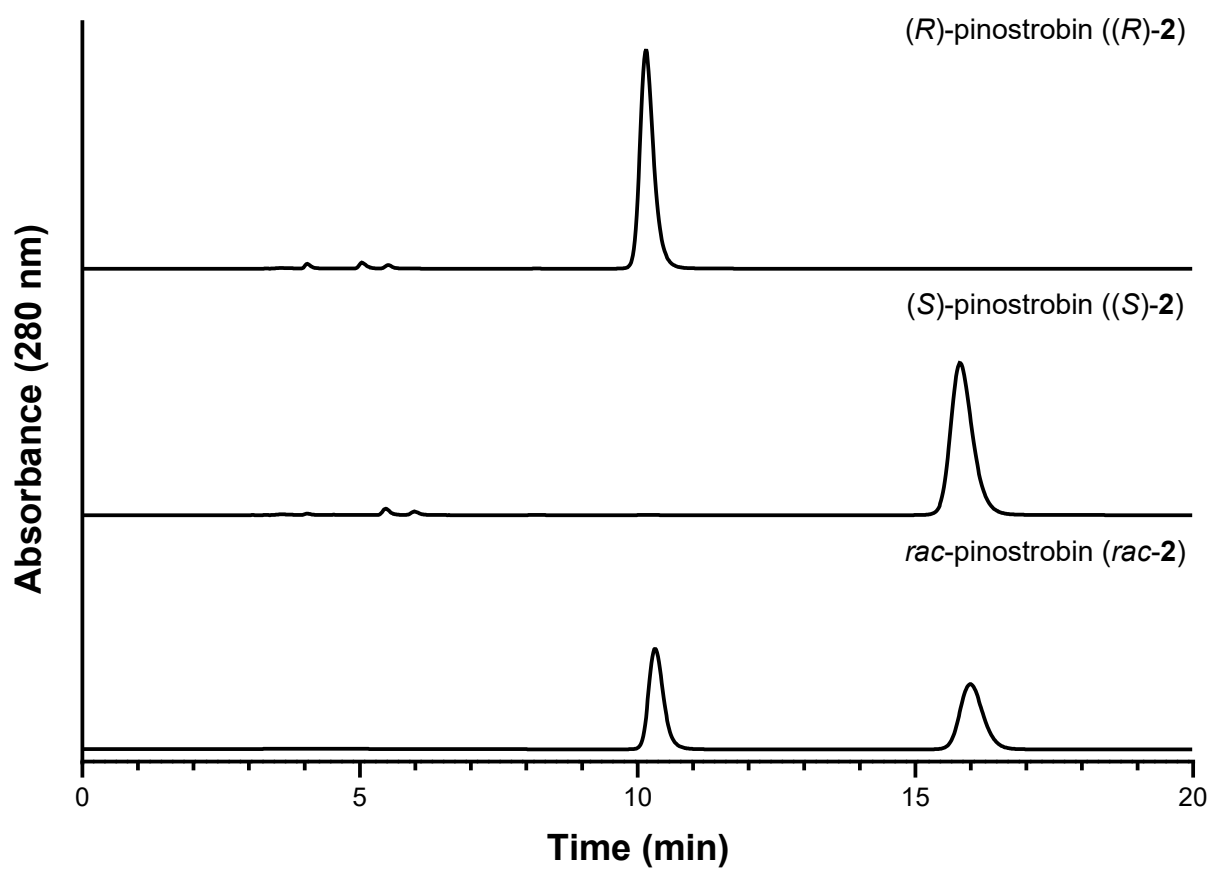


Figure S14 Chiral HPLC chromatograms of *rac*-2, *(R)*-2, and *(S)*-2. HPLC conditions as follows: column, CHIRALPAK IG chiral column (5 μ m, 4.6 $\times$ 250 mm, Daicel); flow rate, 1.0 mL/min; mobile phase, H₂O/acetonitrile (15:85, 0.1% TFA); and detection wavelength, 280 nm.

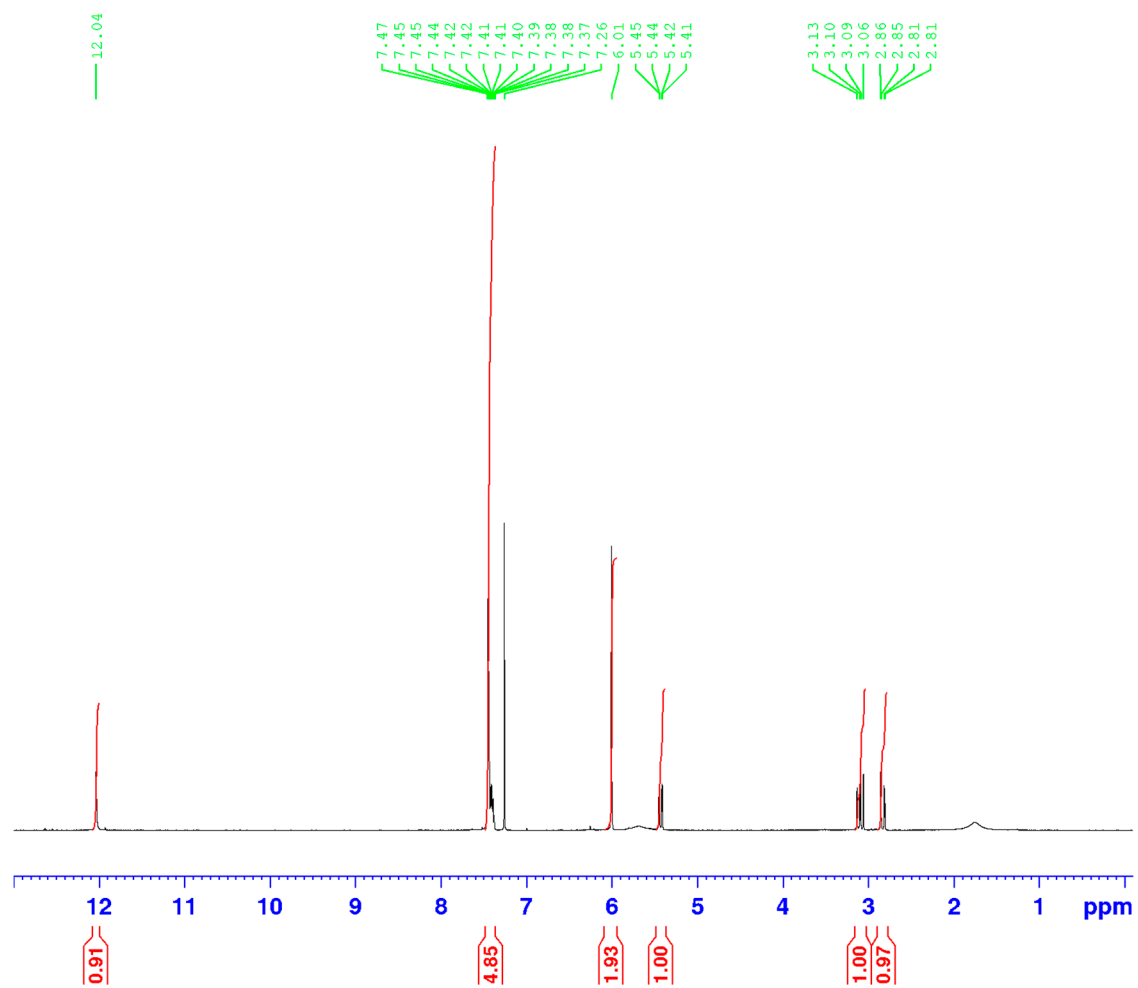


Figure S15 ¹H NMR spectrum (400 MHz, CDCl₃) of (R)-1.

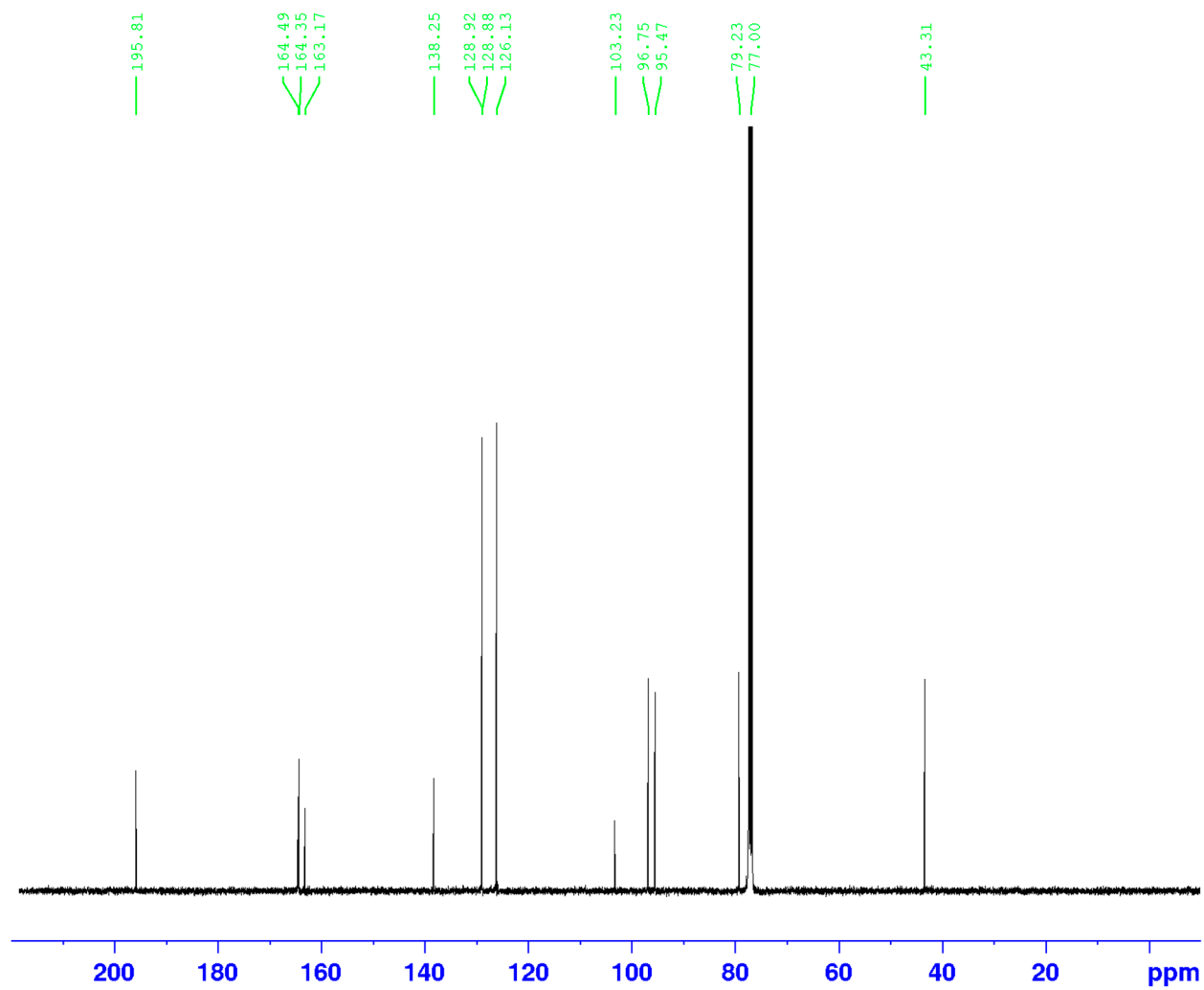


Figure S16 ¹³C NMR spectrum (100 MHz, CDCl₃) of (*R*)-1.

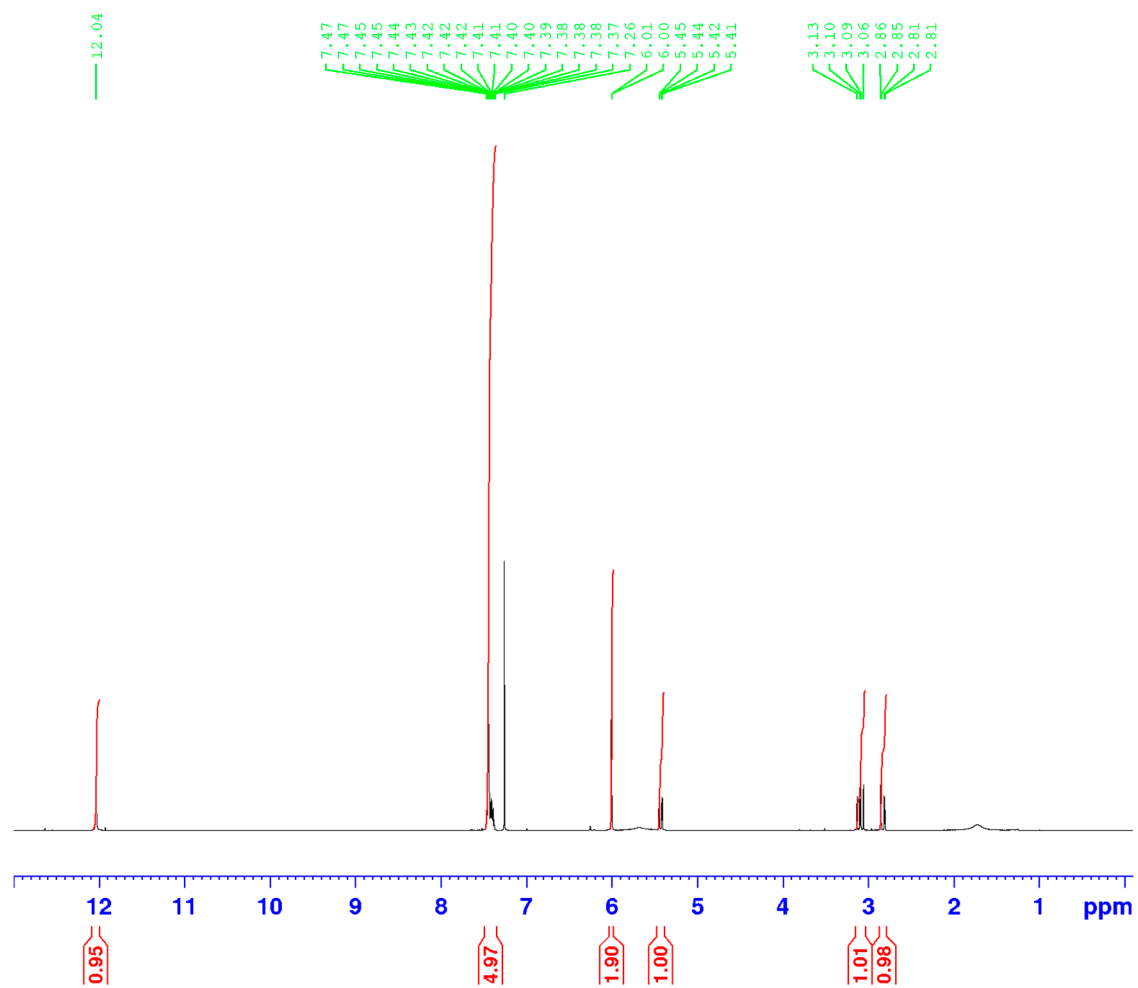


Figure S17 ¹H NMR spectrum (400 MHz, CDCl₃) of (S)-1.

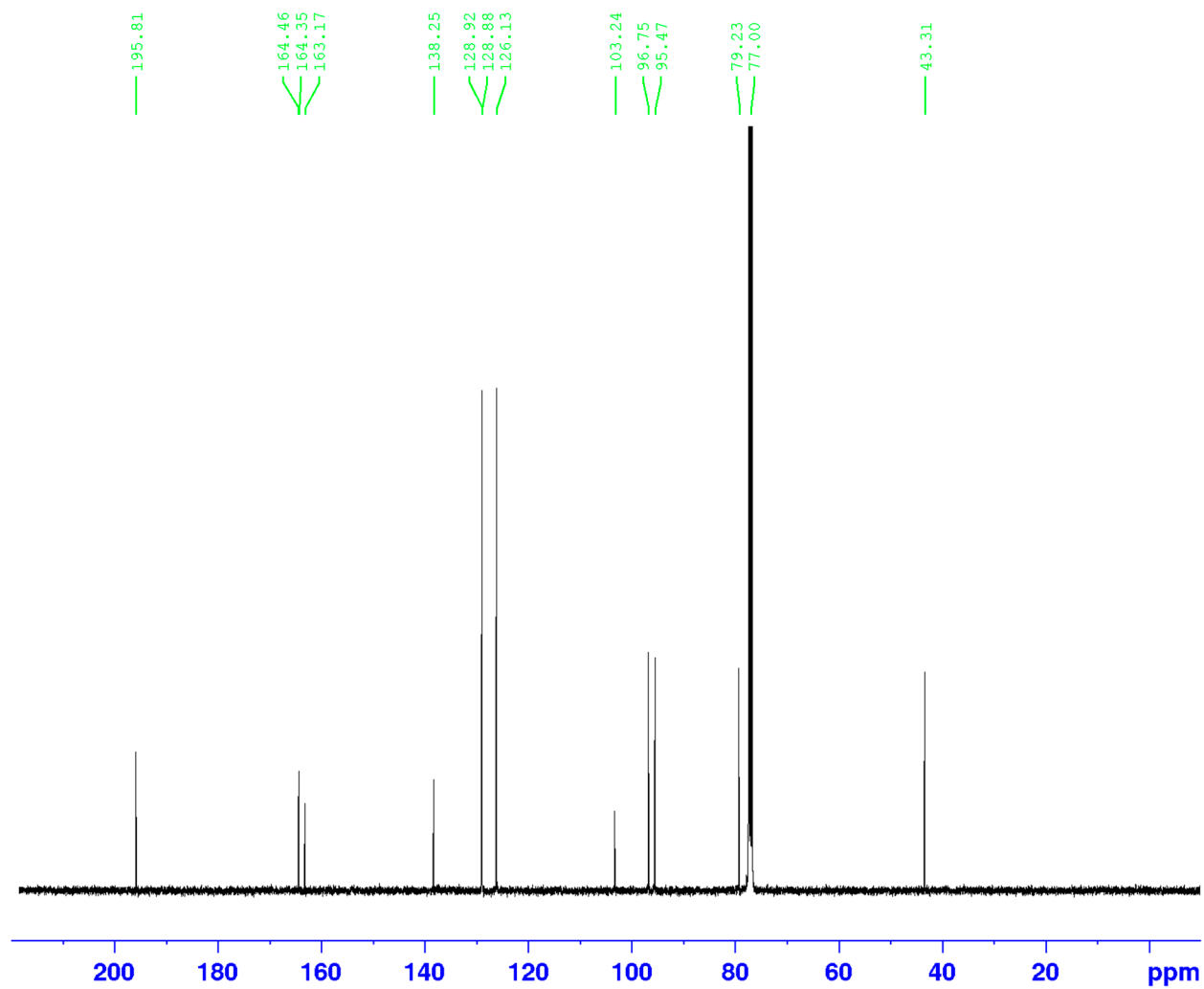


Figure S18 ¹³C NMR spectrum (100 MHz, CDCl₃) of (S)-1.

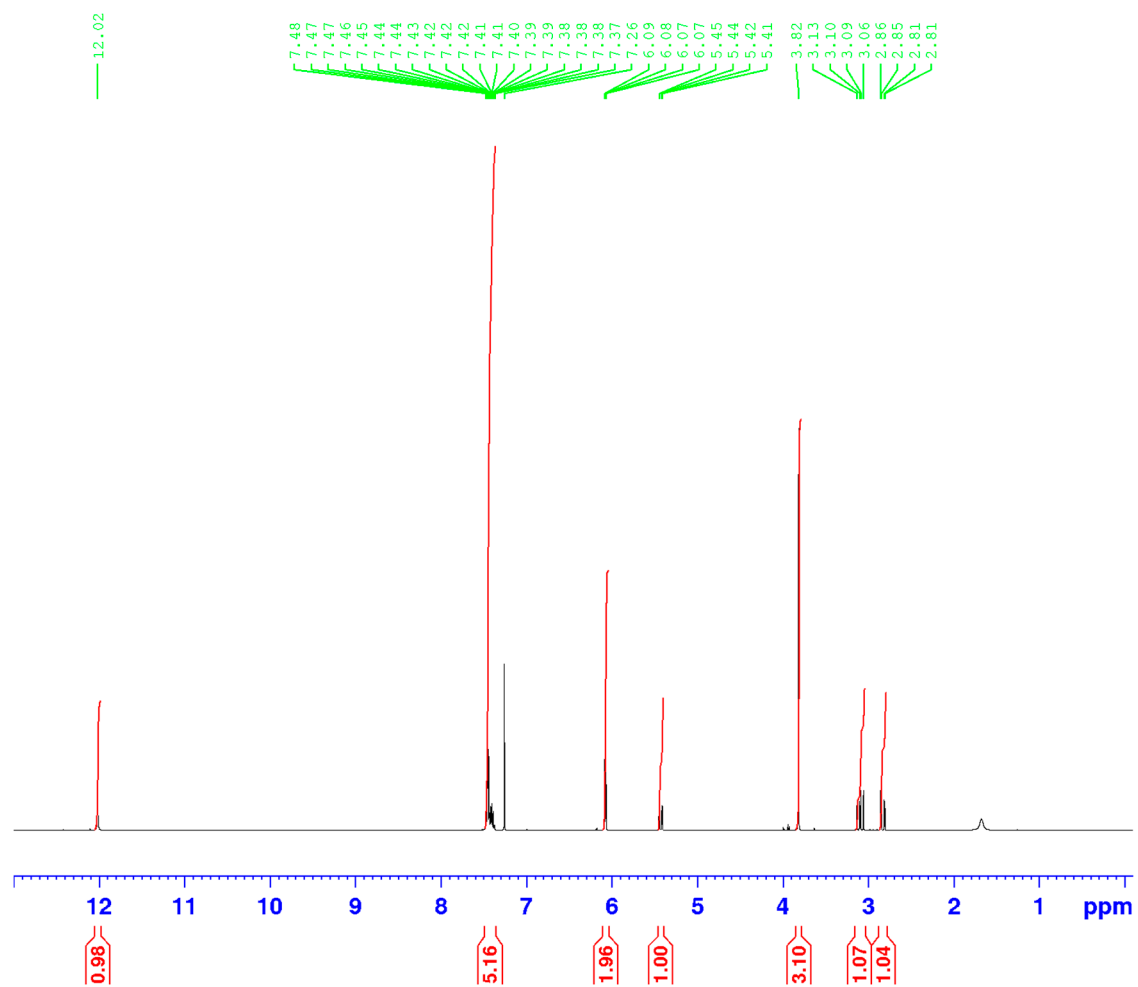


Figure S19 ^1H NMR spectrum (400 MHz, CDCl_3) of (*R*)-2.

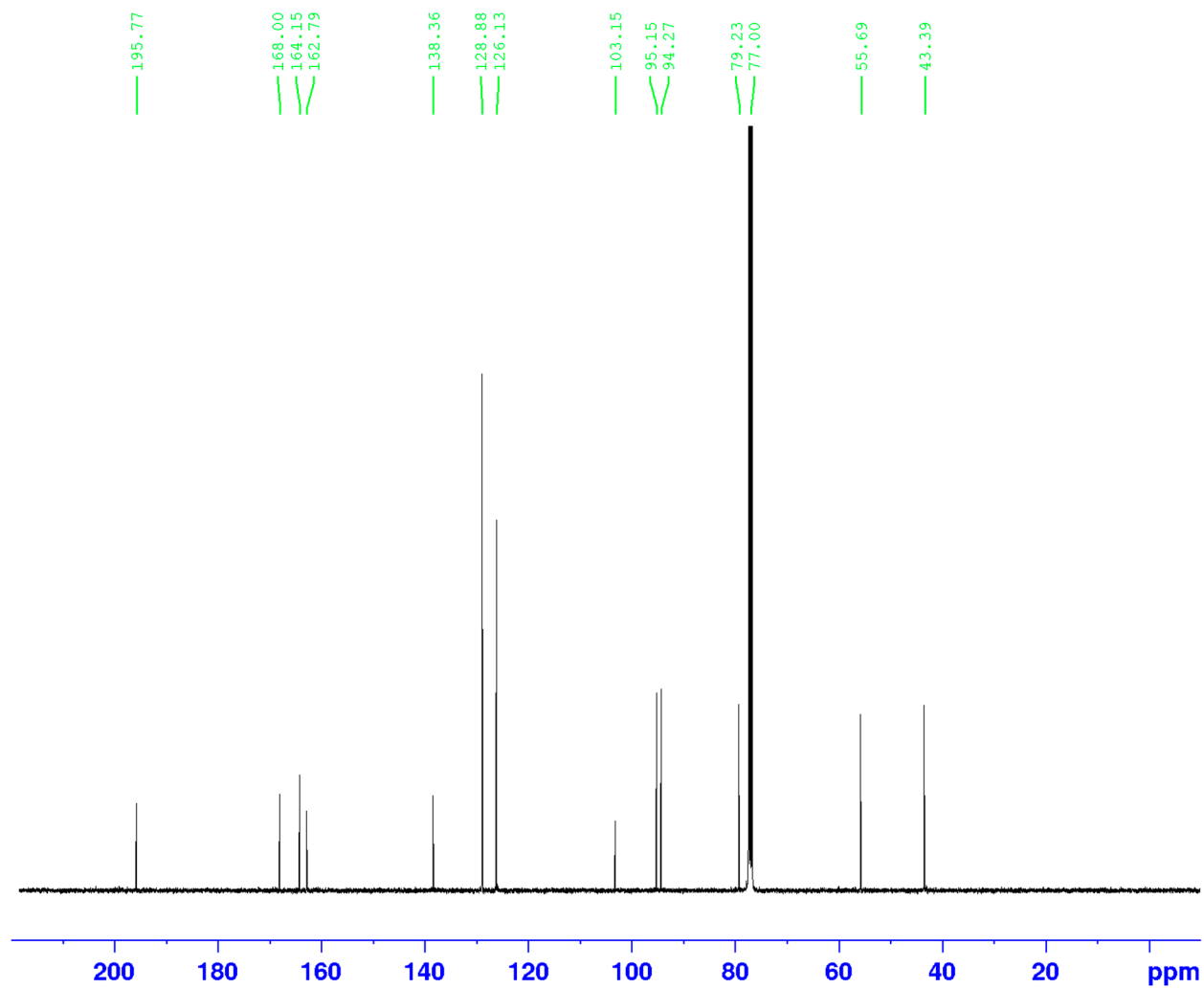


Figure S20 ¹³C NMR spectrum (100 MHz, CDCl₃) of (*R*)-2.

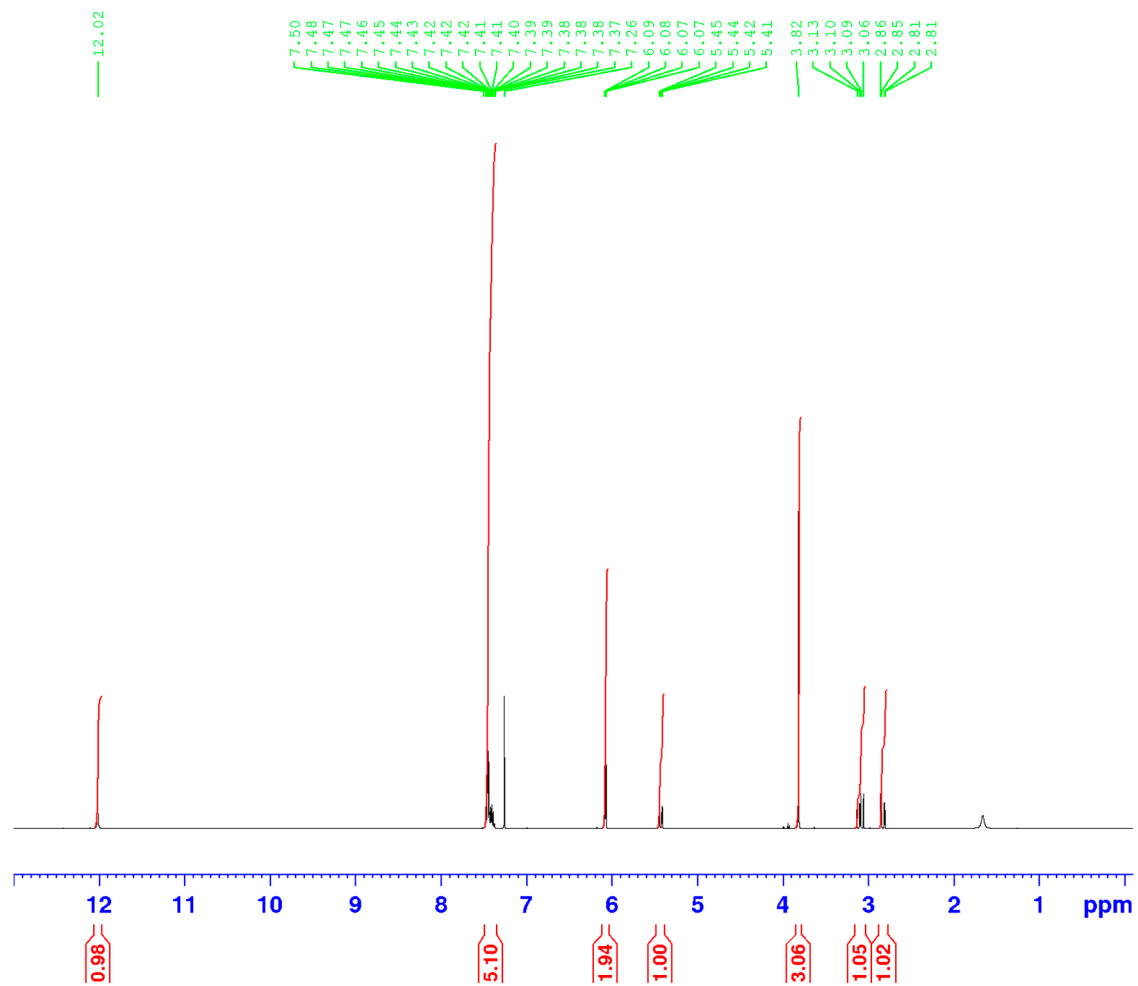


Figure S21 ¹H NMR spectrum (400 MHz, CDCl₃) of (S)-2.

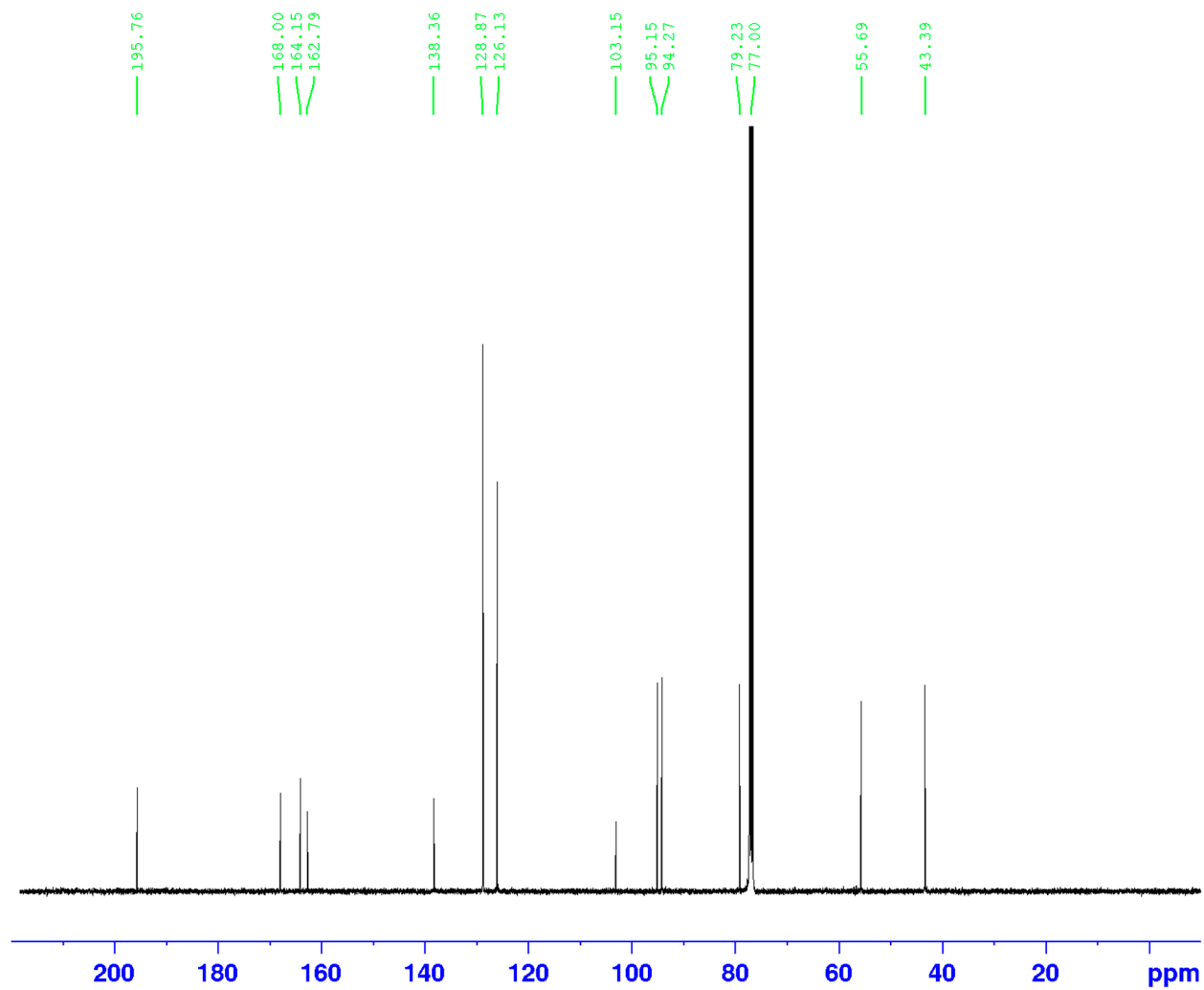


Figure S22 ¹³C NMR spectrum (100 MHz, CDCl₃) of (*S*)-2.

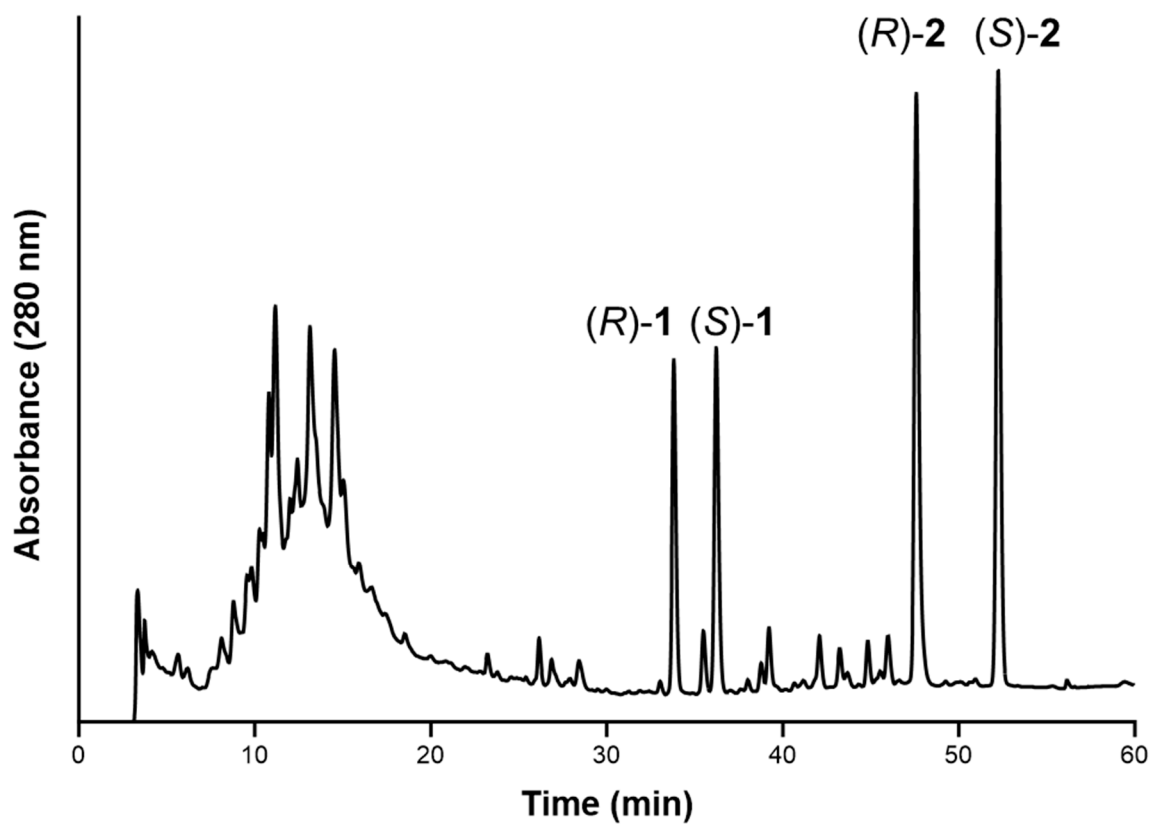


Figure S23 Chiral HPLC chromatogram of *L. sericea* methanol extracts. HPLC conditions as follows: column, CHIRALPAK IG chiral column (5 μ m, 4.6 $\times$ 250 mm, Daicel); flow rate, 1.0 mL/min; mobile phase, mobile phase, H₂O/acetonitrile with 0.1% TFA: gradient, 90:10 (0 min) – 0:100 (50 min) – 0:100 (60 min); and detection wavelength, 280 nm.

rac-1, TGR5(+)

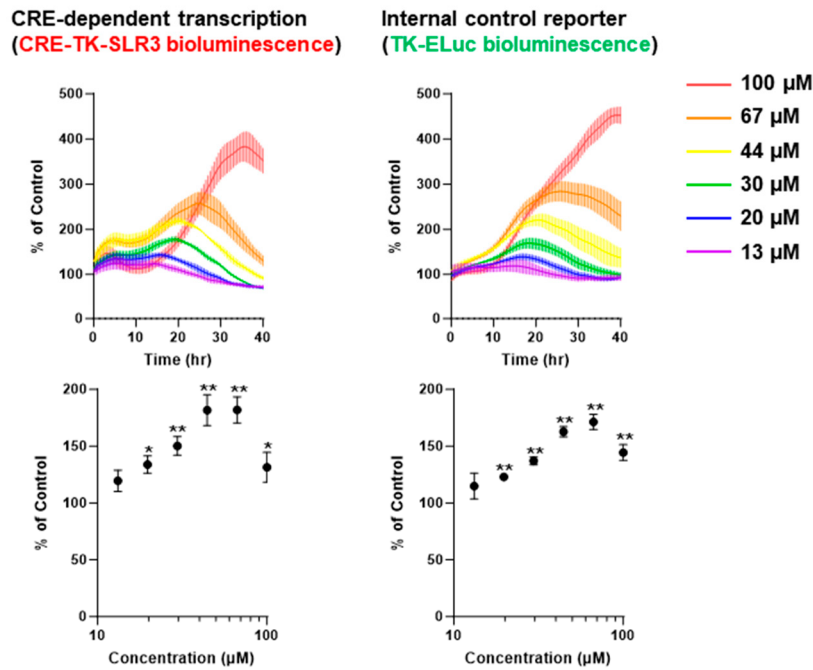
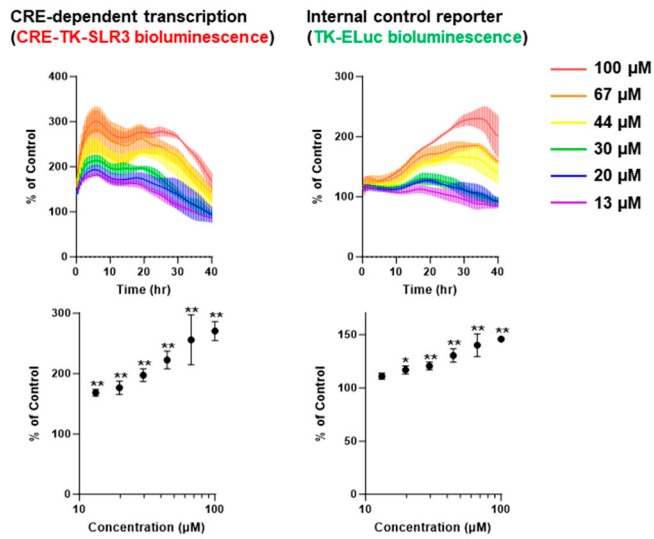


Figure S24 Effects of *rac*-pinocembrin (*rac*-1) on TGR5 activation in TGR5-expressing HepG2 reporter cell line. Kinetics of CRE-dependent transcription monitored by SLR3 and internal control reporter ELuc, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and ELuc bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Values are shown as means $\pm$ SD ($n = 3$).

(a) *rac-2*, TGR5(+)



(b) *rac-2*, TGR5(-)

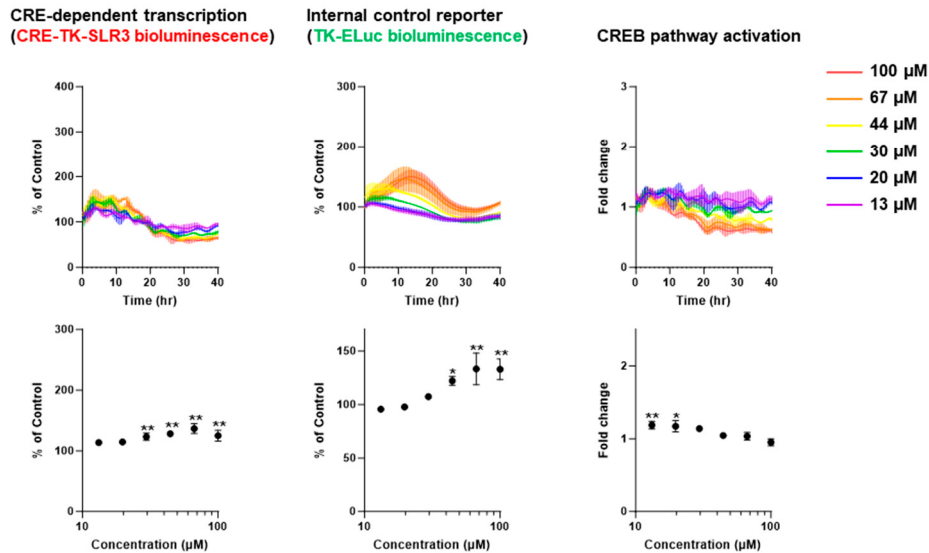
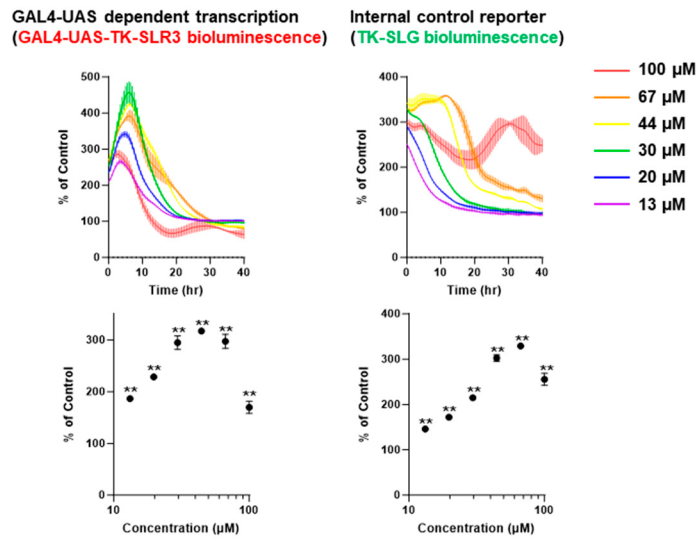


Figure S25 Effects of *rac*-pinostrobin (*rac-2*) on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines. Kinetics of CRE-dependent transcription monitored by SLR3 and internal control reporter ELuc, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and ELuc bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Fold-change values were normalized to 1 at each time point relative to vehicle control. Values are shown as means $\pm$ SD ($n = 3$).

(a) *rac-1*, PPAR γ



(b) *rac-2*, PPAR γ

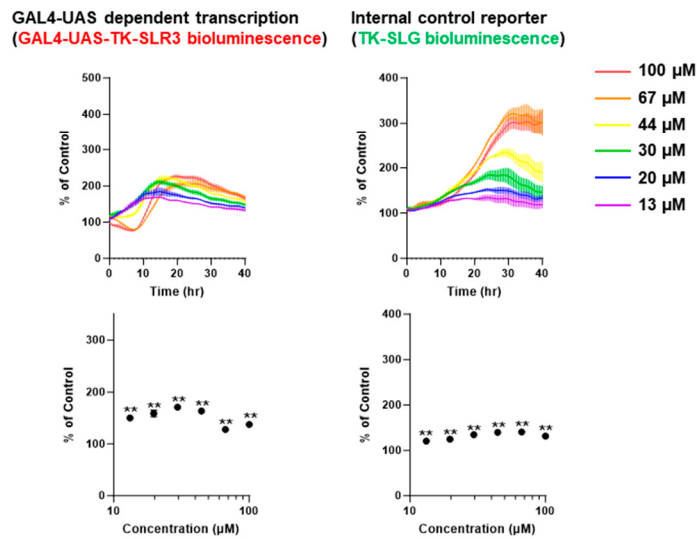
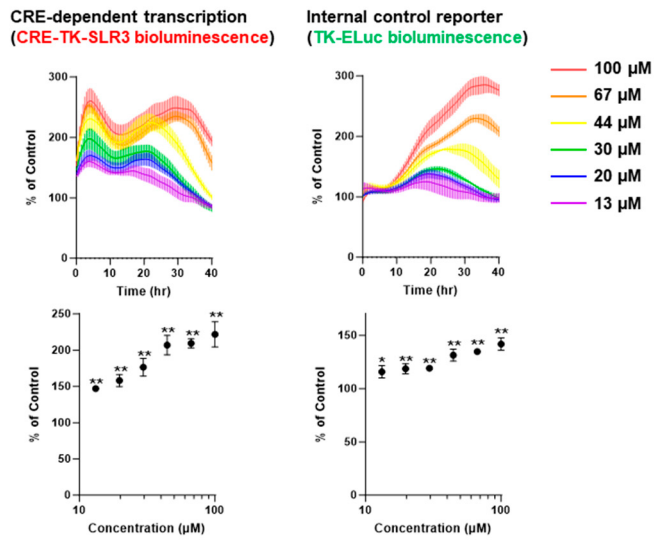


Figure S26 Effects of *rac*-pinocembrin (*rac-1*) and *rac*-pinostrobin (*rac-2*) on PPAR γ activation in A9 reporter cell line. Kinetics of GAL4-UAS dependent transcription monitored by SLR3 and internal control reporter SLG, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and SLG bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Values are shown as means $\pm$ SD (n = 3).

(a) (*R*)-2, TGR5(+)



(b) (*R*)-2, TGR5(-)

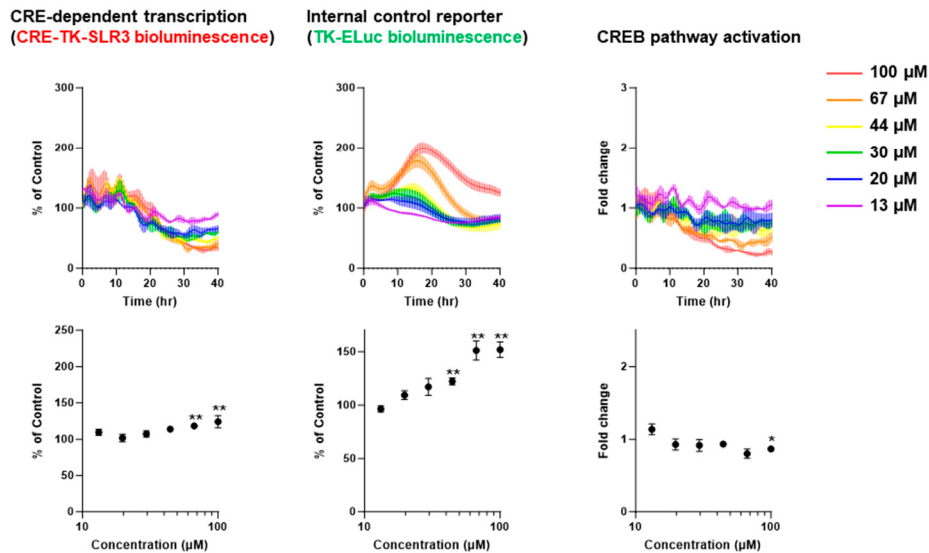
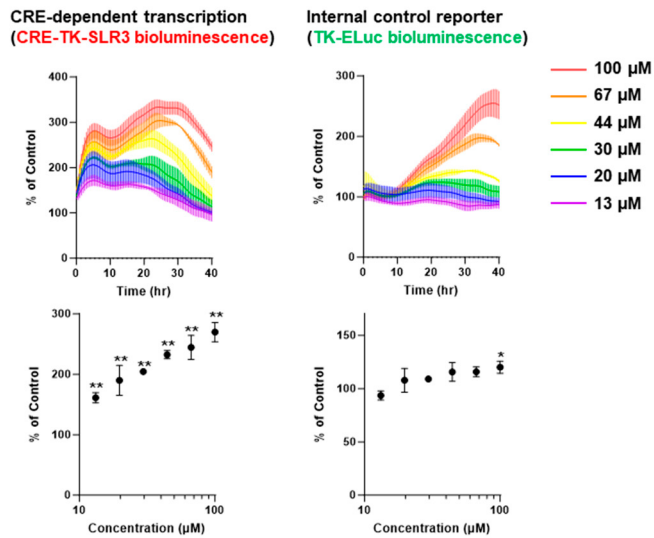


Figure S27 Effects of (*R*)-pinostrobin ((*R*)-2) on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines. Kinetics of CRE-dependent transcription monitored by SLR3 and internal control reporter ELuc, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and ELuc bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Fold-change values were normalized to 1 at each time point relative to vehicle control. Values are shown as means $\pm$ SD ($n = 3$).

(a) (S)-2, TGR5(+)



(b) (S)-2, TGR5(-)

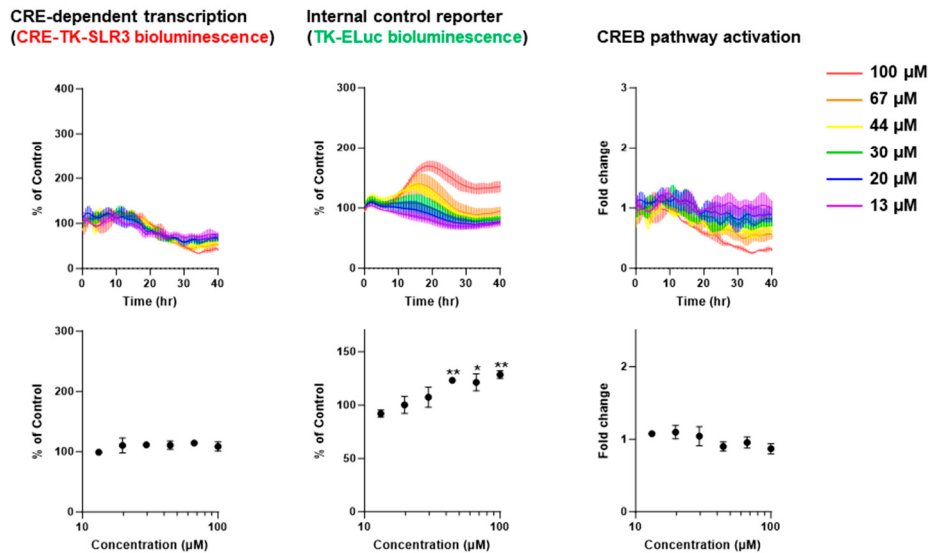
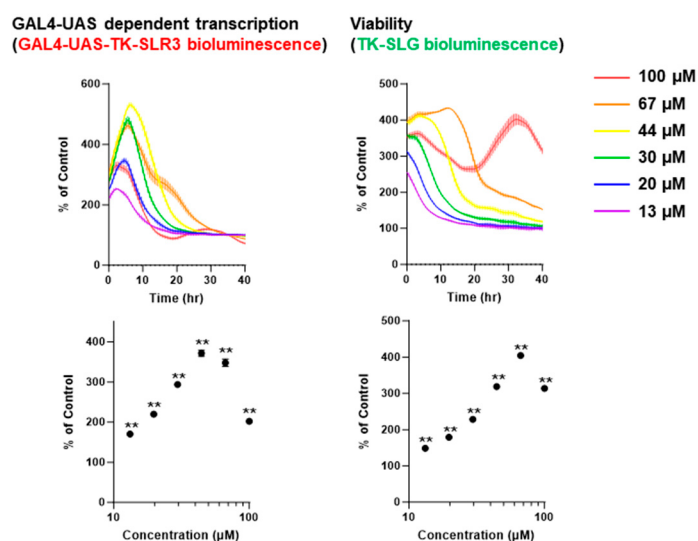


Figure S28 Effects of (S)-pinostrobin ((S)-2) on TGR5 activation in TGR5-expressing (a) and TGR5(-) negative (b) HepG2 reporter cell lines. Kinetics of CRE-dependent transcription monitored by SLR3 and internal control reporter ELuc, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and ELuc bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Fold-change values were normalized to 1 at each time point relative to vehicle control. Values are shown as means $\pm$ SD (n = 3).

(a) (*R*)-1, PPAR γ



(b) (*S*)-1, PPAR γ

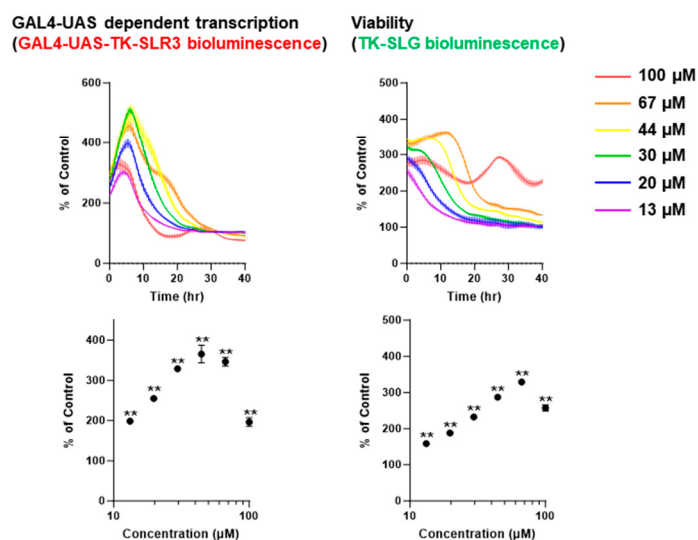
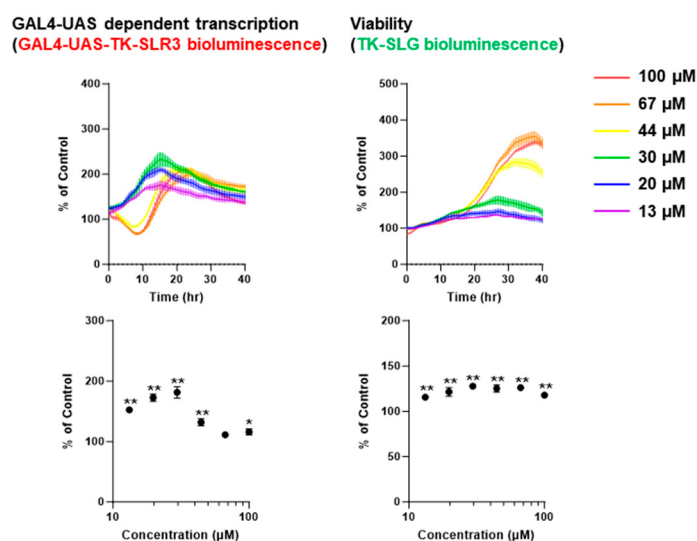


Figure S29 Effects of (*R*)-pinocembrin ((*R*)-1) and (*S*)-pinocembrin ((*S*)-1) on PPAR γ activation in A9 reporter cell line. Kinetics of GAL4-UAS dependent transcription monitored by SLR3 and internal control reporter SLG, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and SLG bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Values are shown as means $\pm$ SD (n = 3).

(a) (R)-2, PPAR γ



(b) (S)-2, PPAR γ

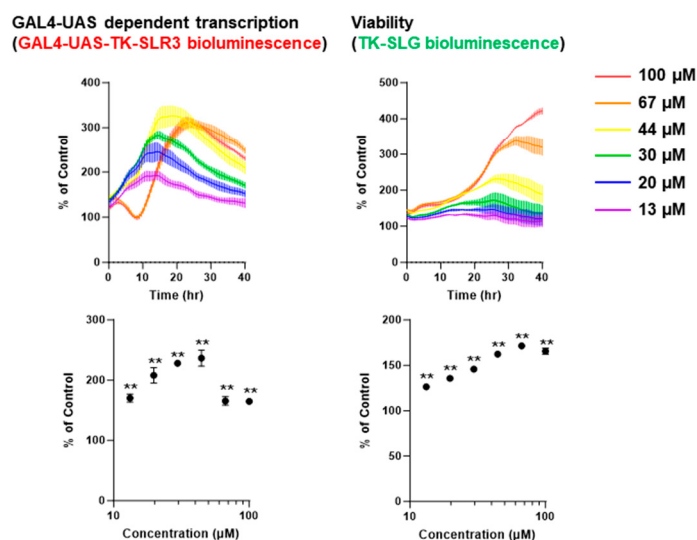


Figure S30 Effects of (R)-pinostrobin ((R)-2) and (S)-pinostrobin ((S)-2) on PPAR γ activation in A9 reporter cell line. Kinetics of GAL4-UAS dependent transcription monitored by SLR3 and internal control reporter SLG, and calculated fold change are shown in the upper panels. Concentration–response curves, summarized as AUC, are shown in the lower panels. SLR3 and SLG bioluminescence intensities are expressed as percentage of vehicle control (set to 100%). Values are shown as means $\pm$ SD (n = 3).