

Synthesis and biological evaluation of optically active conjugated γ - and δ -lactone derivatives

Melis Şardan^a, Serdar Sezer^{a,b}, Aslıhan Günel^{a,c}, Mahinur Akkaya^a, Cihangir Tanyeli^{a,*}

^a Department of Chemistry, Middle East Technical University, Ankara 06800, Turkey

^b Research and Development Department, Altaylar Medical Co. Ltd., Ankara 06370, Turkey

^c Department of Chemistry, Ahi Evran University, Kırşehir 40100, Turkey

ARTICLE INFO

Article history:

Received 12 June 2012

Revised 22 July 2012

Accepted 25 July 2012

Available online 2 August 2012

Keywords:

Asymmetric synthesis

Enzymatic resolution

Optically active lactones

ABSTRACT

An efficient synthesis of racemic and both enantiomeric forms of heteroaryl substituted γ - and δ -lactone derivatives derived from allyl and homoallyl alcohol backbones has been accomplished via ring closing metathesis reaction. 2-Heteroaryl substituted allyl and homoallyl alcohols have been efficiently resolved through enzymatic method with high ee (97–99%) and known stereochemistry. Antimicrobial and anti-oxidant activities of target lactones were evaluated.

© 2012 Elsevier Ltd. All rights reserved.

Lactones possess a wide variety of biological activities often depending on the substituents which are mainly on the pyran skeleton. Five- or six-membered rings are the most common naturally occurring lactones because of their stabilities.¹ In particular, α,β -unsaturated γ - and δ -lactone moieties bearing natural products including vitamin C, (*R*)-goniothalamine, yonganin, (*R*)-kavain, jeranolide D, and rubrolide A exhibit valuable biological activities (Fig. 1).^{2–6} This class of lactones has great abundance in both natural and synthetic products which show antimicrobial and cytotoxic activities towards related targets. Additionally, some of the α,β -unsaturated γ -lactones were considered as potent anti-tumor agents, cyclooxygenase or phospholipase A2 inhibitors, or antibiotics.⁷ Moreover, the correlation between chirality and biological activity has become important for the pharmaceuticals which was proved by the growth of chiral drug design and synthesis within the last two decades.⁸ Beside the α,β -unsaturated γ - and δ -lactone derivatives, a couple alternative method for the preparation of chiral saturated γ - and δ -lactones has also been reported.^{9,10}

We have recently been interested in development of new methods for chemoenzymatic synthesis of enantiomerically enriched heteroaryl substituted secondary allylic and homoallylic alcohols using various lipases.¹¹ These alcohols are key intermediates for further transformations depending on oxygen anchored side.¹²

There is a strong demand for a practical route providing an easy access to α,β -unsaturated lactone derivatives, and asymmetric syn-

thesis constitutes an area of considerable current interest. The ring closing metathesis (RCM) reaction has become a very useful process for producing lactone units.^{12–15} Although there are a number of reports on the synthesis of various racemic lactone units via RCM, to the best of our knowledge, only a few work have been reported for the synthesis of chiral lactone derivatives and evaluation of their biological activities.^{16–18}

The aim of this study was to synthesize highly enantiomerically enriched 2-heteroaryl substituted α,β -unsaturated γ - and δ -lactones and test their antibacterial and antioxidant activities. For this purpose, racemic heteroaryl substituted secondary homoallylic and allylic alcohols *rac*-**2a–b** and *rac*-**3a–b** were chosen as proper backbones. We thought to apply well-known Grignard type coupling reaction between 2-heterocarbaldehyde (1 equiv) **1a–b** and allyl and vinylmagnesium bromide (1.2 equiv), respectively (Scheme 1) for the construction of these carbon skeletons.^{19,20} The key step of synthetic strategy is the enantiomeric enrichment of the racemic alcohols by enzymatic resolution in which various lipases were used with 1:1 substrate/enzyme ratio in the presence of THF and vinyl acetate as acyl donor according to the procedures in our previous work.¹¹

Homoallylic and allylic alcohols (*S* or *R* or *rac*)-**2a–b** and (*S* or *rac*)-**3a–b** were converted to acrylate esters **6a–b**, **7a–b**, **10a–b** and **11a–b** by O-acylation reaction (Scheme 2). The compounds (*R*)-**5a–b** could not be isolated due to the decomposition during the chromatographic separation. To a solution of alcohols (1 mmol) in anhydrous DCM (15 mL) was added Et₃N (2.96 mmol). After 10 min. stirring, acryloyl chloride or methacryloyl chloride (1.48 mmol) was added at room temperature to synthesize the

* Corresponding author.

E-mail address: tanyeli@metu.edu.tr (C. Tanyeli).

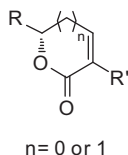
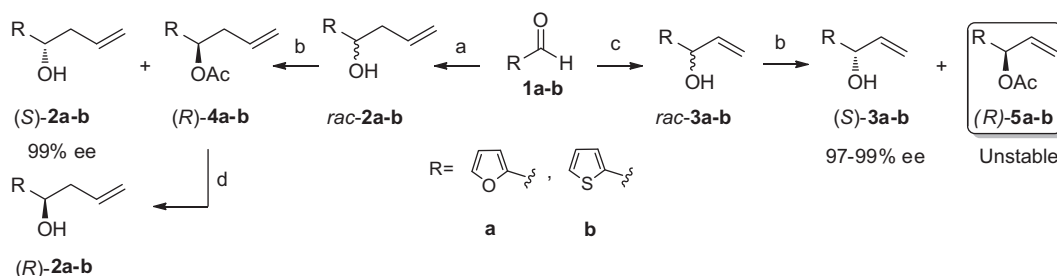
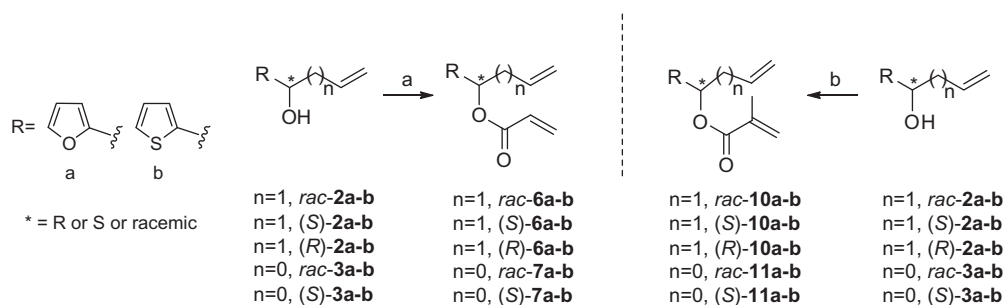


Figure 1. Target Lactone Skeletons.

Scheme 1. Reagent and conditions: (a) Allylbromide, Mg, I₂, dry ether; (b) Lipase, vinyl acetate, THF; (c) vinylmagnesiumbromide (1 M in THF), dry ether; (d) K₂CO₃, MeOH.Scheme 2. Reagent and conditions: (a) Acryloyl chloride, Et₃N, DCM; (b) Methacryloyl chloride, Et₃N, DCM.

desired acrylate esters. For allylic acrylate esters (*S* or *rac*)-**7a-b** and (*S* or *rac*)-**11a-b**, the mixtures were separated by flash column chromatography packed with alumina oxide using EtOAc/hexane (1:20) as eluent system and for the homoallylic acrylate esters **6a-b** and **10a-b**, the crude mixtures were separated by flash column chromatography packed with silica gel using EtOAc/hexane (1:6) as eluent system. After chiral induction and O-acylation reaction, resultant diene systems were converted to target γ - and δ -lactones²¹ (*S* or *R* or *rac*)-**8a-b**, (*S* or *rac*)-**9b** and (*S* or *R* or *rac*)-**12b**, (*S* or *rac*)-**13b** via RCM reaction in the presence of Grubbs' catalysts (Fig. 2). 2-Thiophene substituted α,β -unsaturated γ -lactones **9b** and **13b** were synthesized from O-acyl anchored allylic substrates by adding 0.01 M DCM solution of 10–15% of Grubbs' 1st generation catalyst to 0.01 M refluxing solution of acrylate ester (1 mmol) in DCM at 40 °C. The reaction was stirred 24 h at reflux and controlled by TLC. The solvent was evaporated in vacuum and the purification of crude product was done by column chromatography (silica gel EtOAc/hexane 1:3 for both products). We also tried to synthesize 2-furan substituted α,β -unsaturated γ -lactones **9a** and **13a** via RCM reaction. Furan substituted acrylate esters **7a** and **11a** could not be purified due to their vinylic components which are decomposed on chromatographic separation. Therefore, RCM reaction was carried out immediately. Several conditions were tried for lactonization via RCM reaction such as addition of 10% Grubbs' 1st generation at 40 °C, 5% and 10% Grubbs' 2nd generation at 80 °C. Similarly, the same reaction was carried out with 15% Grubbs' 1st generation at 40 °C in the presence of Ti(OiPr)₄ used as a Lewis acid catalyst.

We only observed some decomposition products. Additionally, optically active α,β -unsaturated δ -lactones **8a-b** and **12b** were synthesized from heteroaryl substituted chiral O-acyl anchored homoallylic substrates via RCM. Since δ -lactones were synthesized at 80 °C, 2nd generation Grubbs' catalyst was used instead of 1st generation Grubbs' catalyst due to its thermal stability. To a solution of acrylate ester (1 mmol) in DCM (8 mL), 5% 2nd generation Grubbs catalyst was added and refluxed for 80 min. Again, furan substituted compound **12a** could not be synthesized. To overcome this problem, the reaction was carried out with different catalyst amounts (6–7.5%) in the presence of different solvents (toluene, DCM) at different temperatures (40, 80, and 110 °C), however, these attempts did not work.

The structures of all compounds were characterized by IR, ¹H NMR, ¹³C NMR, COSY, HMBC, HMQC, and HRMS data. In the IR spectra of all compounds, characteristic carbonyl absorption signal was observed in the range of 1706–1754 cm^{−1}. Moreover, ¹³C NMR spectra of the compounds showed that carbonyl signals characteristically appeared between 161.9 and 172.1 ppm.

Antioxidant activity of compounds was measured with three different methods. These are radical scavenging assay with diphenylpicrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA) assay and metal chelating activity assay for Fe²⁺ ion. These methods were summarized below.

The free radical scavenging activity was determined with the DPPH assay described by Blois.²² In its radical form, DPPH absorbs at 517 nm, but upon reduction with an antioxidant or a radical species its absorption decreases. Concentrations of tested

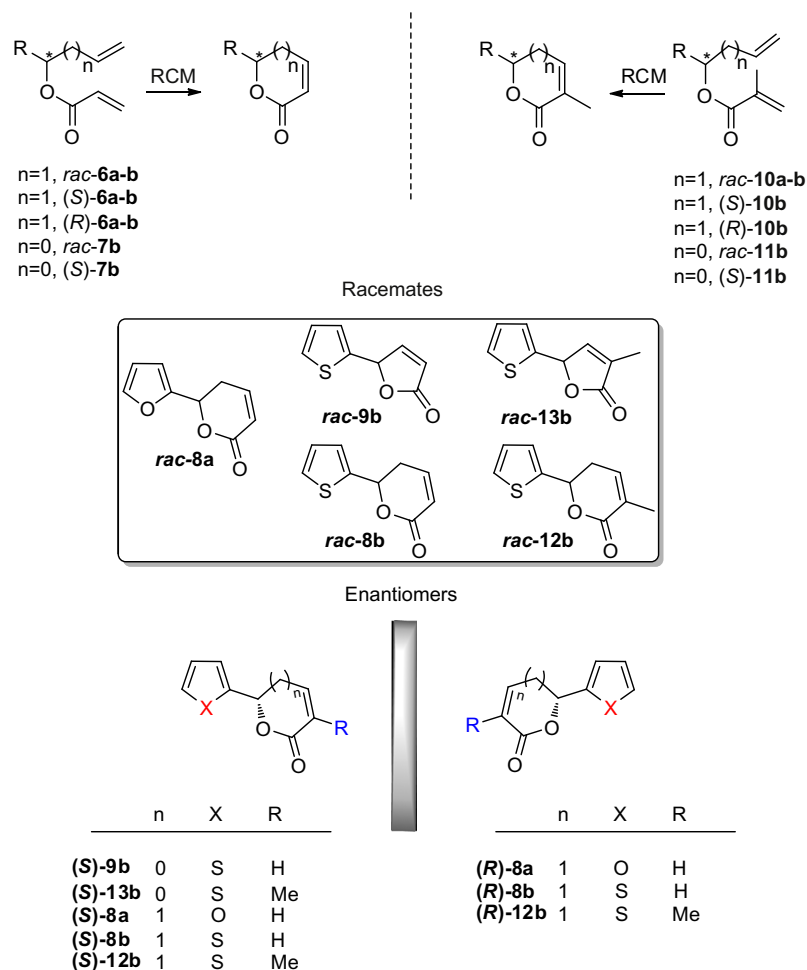


Figure 2. Representation of target furan and pyran skeletons.

compounds are between 10 and 80 μM in methanol. As a control, alpha tocopherol was used. The capability to scavenge the DPPH radical was calculated using the following equation²³ DPPH Scavenging Effect (%) = $[(A_{517\text{nm}}$ of control – $A_{517\text{nm}}$ of sample) / $A_{517\text{nm}}$ of control] $\times$ 100.

For TBA assay, rat liver microsomes were isolated from three Wistar Rats from Animal Care and Use Committee of Marmara University (protocol number:43.2004.Mar) to assess the lipid peroxidation level according to Raj et al.²⁴ The reduced lipid peroxidase activity is considered the indication of antioxidant activity of the sample in use.

Fe^{3+} reducing activity of lactone derivatives synthesized in this study was measured by modified ferrozine method.²⁵ In this assay, $[\text{Fe}^{2+}]$ was calculated using the extinction coefficient value of $27,900 \text{ M}^{-1}\text{cm}^{-1}$ for the $\text{Fe}(\text{ferrozine})^{2+}$ complex.

The antimicrobial activities of the compounds were measured by a modified version of the disc diffusion method (DDM) and by the determination of minimal inhibitory concentration²⁶ (MIC). In tests, as gram-positive bacteria *Staphylococcus aureus* (ATCC 6538) and *Bacillus subtilis* (ATCC 6633); as gram-negative bacteria *Escherichia coli* (ATCC 3509) and *Pseudomonas aeruginosa* (ATCC 9027); and as a fungal sample, *Candida albicans*, (ATCC 10231) were used. The LB agar plates were inoculated with each of the microorganisms at a concentration of 10^6 cells/mL. The filter discs of 6 mm in diameter were saturated in 50 μL of the varying concentrations (10–100 mg/mL in either DMSO or methanol) of each

Table 1
Antioxidant activity assay

Compd	DPPH Assay	TBA Assay (%)	Metal reducing activity ($\mu\text{M Fe}^{2+}/\mu\text{M Compound}$)
(<i>S</i>)- 8a	38.05 ± 1.54^a	7.45	0
(<i>S</i>)- 8b	14.0 ± 3.49	—	0
<i>rac</i> - 8a	38.1 ± 0.39^a	24.48	0
(<i>R</i>)- 8a	—	9.00	0.0092 ± 0.0046
(<i>R</i>)- 8b	—	—	0.0400000 ± 0.0000007
<i>rac</i> - 9b	—	58.8	0.44 ± 0.024
(<i>S</i>)- 9b	—	54.7	0.015 ± 0.0014
(<i>R</i>)- 12b	—	—	ND ^d
(<i>S</i>)- 12b	—	—	ND ^d
<i>rac</i> - 12b	7.11 ± 1.59^b	ND ^d	ND ^d
(<i>S</i>)- 13b	50.65 ± 5.88^c	ND ^d	ND ^d
<i>rac</i> - 13b	93.30 ± 1.39^c	ND ^d	ND ^d
Alpha Tocopherol	66.21 ± 1.21	ND ^d	ND ^d

No inhibition activity.

Values expressed are means $\pm$ SD of three parallel measurements ($p < 0.05$).

^a DPPH assay was performed between 10–200 μM concentration, but above 80 μM concentration results did not change so much. These values represent DPPH activity for 80 μM concentration.

^b This value is measured for 1000–11 μM compound.

^c This values are measured for 11 μM compounds.

^d Not determined.

Table 2
MIC values of compounds

Compd	Antimicrobial Activity MIC Values in µg/mL and zone diameter in mm									
	<i>S. aerus</i>		<i>B. subtilis</i>		<i>P. aeruginosa</i>		<i>E. coli</i>		<i>C. albicans</i>	
	MIC	Zone	MIC	Zone	MIC	Zone	MIC	Zone	MIC	Zone
<i>rac-8b</i>	30	8.5	10	8.5	—	—	20	8.5	25	9
(<i>S</i>)- 8b	50	7	20	8.5	—	—	30	8.5	30	7
(<i>R</i>)- 8b	40	8.5	50	7	—	—	50	7	40	7
(<i>R</i>)- 12b	25	10	25	7	—	—	25	9	25	9
(<i>S</i>)- 12b	25	7	25	11	—	—	25	8	25	9
<i>rac-12b</i>	25	7.5	—	—	—	—	12	8	25	12
(<i>S</i>)- 13b	—	—	—	—	—	—	25	9.5	—	—
<i>rac-13b</i>	—	—	—	—	—	—	—	—	25	7.5
(<i>S</i>)- 8a	—	—	—	—	—	—	—	—	—	—
<i>rac-8a</i>	—	—	—	—	—	—	—	—	—	—
(<i>R</i>)- 8a	—	—	—	—	—	—	—	—	—	—
<i>rac-9b</i>	—	—	—	—	—	—	—	—	100	9
(<i>S</i>)- 9b	—	—	—	—	—	—	—	—	—	—
Streptomycine (50 µg/mL)	—	15	—	—	—	—	—	18	—	—
Gentamicine (50 µg/mL)	—	—	—	17	—	22	—	—	—	—
Nystatin	—	—	—	—	—	—	—	—	—	20

No inhibition activity.

compound. The treated filter discs were placed on the surface of the plates each having different microorganisms and incubated at 37 °C for 24 h to observe the inhibition zone. The inhibition zones which are equal or greater than 7 mm were considered as susceptible to compounds. The solvents, DMSO and methanol, were also tested. As positive controls, antibiotic discs that were treated with gentamicine (10 mg/mL) for *Bacillus subtilis* and *Pseudomonas aeruginosa*, streptomycine (10 mg/mL) for *Escherichia coli* and *Staphylococcus aureus*, and nystatin (10 mg/mL) for *Candida albicans* were used. In the MIC tests, the lowest concentrations of the each compound resulting the biggest zones is considered having the highest antimicrobial activity.

When the analyses of antioxidant activities were examined (Table 1), especially *rac-13b* and (*S*)-**13b** exhibited invaluable radical scavenging activity compared to control compound, alpha tocopherol. (*S*)-**8a** has the highest radical scavenging activity among isomers of these compounds. The compounds, (*S*)-**9b** and *rac-9b* exhibited the highest activities in TBA assay reducing lipid peroxidation activity. The other compounds showed poor or moderate activities. In the case of Fe³⁺ reducing activity, (*R*)-**8a**, (*S*)-**9b** and (*R*)-**8b** showed poor values, where the activity of (*R*)-**8a** was the poorest and *rac-9b* was the highest. Compound *rac-9b* showed higher activity than that of (*S*)-**9b**. The compounds having iron reducing activity can be very important one for a potential treatment of diseases such as in humans. Hemochromatosis can develop because of genetic mutations resulting accumulation of Fe³⁺ or overload of ferritin factors and secondary factors²⁷ such as deficiency of pyruvate kinase^{28,29} and glucose 6-phosphate dehydrogenase.³⁰ Also, iron accumulation increases in cancer progression³¹ via enhancing angiogenesis. As treatment of these cases, iron chelating agents are widely used. Thus, further tests should be conducted with *rac-9b* as an anticancer agent.

In the antimicrobial activity tests, the compounds *rac-8b*, (*S*)-**8b**, (*R*)-**8b** exhibited very low levels of antimicrobial activities against to all microorganisms with respect to others. Interestingly, *P. aeruginosa* was not affected by any of the compounds tested (Table 2). The compounds (*S* or *R* or *rac*)-**8b**, *rac-9b*, (*S* or *R* or *rac*)-**12b** and (*S* or *rac*)-**13b** showed the best antimicrobial activities in very low concentrations (µg/mL). The compounds (*S* or *R* or *rac*)-**12b** showed antimicrobial activity against to all microorganisms except *P. aeruginosa*, which may be due to its anaerobic feature. In compound **12b** derivatives, *rac-12b* did not show any antimicrobial activity against *B. subtilis*, additionally it

showed biggest zone which was against to *C. albicans*. The racemic compounds, *rac-13b* and *rac-9b*, exhibited only antifungal activities. Antifungal activity of *rac-13b* is higher than *rac-9b*. These two compounds showed no antibacterial activity against to tested bacteria.

In conclusion, we have synthesized various heteroaryl substituted γ- and δ-lactone derivatives by RCM and evaluated their antimicrobial and antioxidant activities comparing with their both enantiomers. Among these, compounds *rac-13b* and (*S*)-**13b** showed valuable radical scavenging activity compared to alpha tocopherol. The compounds, (*S*)-**9b** and *rac-9b* exhibited the highest activities in TBA. Moreover, *rac-9b* showed the highest activity regarding Fe³⁺ reducing activity and should be tested as a drug candidate. In the case of antimicrobial activity, all leads compounds except (*S* or *R* or *rac*)-**8a** and (*S*)-**9b** displayed activity towards all microorganisms except *P. aeruginosa*. The compounds, *rac-13b* and *rac-9b* exhibited only antifungal activities. Further biological evaluation studies of new conjugated γ- and δ-lactone derivatives will be reported in due courses.

Acknowledgments

We are grateful to the Turkish Scientific and Technical Research Council for a Grant (No. TBAG-108T072). We thank Neşe Ozgazi and Umran Aydemir Sezer for their help in antimicrobial tests.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2012.07.090>.

References and notes

- Pospisil, J.; Makro, I. E. *Tetrahedron Lett.* **2008**, 49, 1523.
- Boucard, V.; Broustal, G.; Campagne, J. M. *Eur. J. Org. Chem.* **2007**, 225.
- Fatima, A.; Kohn, L. K.; Antonio, M. A.; Carvalhob, J. E.; Pilli, R. A. *Bioorg. Med. Chem. Lett.* **2005**, 13, 2927.
- Amaral, P. A.; Gouault, N.; Roch, M. L.; Eifler-Lima, V. L.; David, M. *Tetrahedron Lett.* **2008**, 49, 6607.
- Sofikiti, N.; Tofi, M.; Montagnon, T.; Vassilikogiannakis, G.; Stratakis, M. *Org. Lett.* **2005**, 7, 2357.
- Zhou, F. S.; Tang, W. D.; Mu, Q.; Yang, G. X.; Wang, Y.; Liang, G. L.; Lou, L. G. *Chem. Pharm.* **2005**, 53, 1387.
- Xavier, N. M.; Silva, S.; Madeira, P. J. A.; Florêncio, M. H.; Silva, F. M. V.; Justino, J.; Thiem, J.; Rauter, A. P. *Eur. J. Org. Chem.* **2008**, 6134.

8. Singh, J.; Hagen, T. J. Chirality and Biological Activity. Burger's Medicinal Chemistry, Drug Discovery and Development, John Wiley & Sons Inc., 2010, pp. 127–166.
9. Davies, S. G.; Nicholson, R. L.; Smith, A. D. *Org. Biomol. Chem.* **2004**, 2, 3385.
10. Watanabe, Y.; Yamazaki, T.; Kubota, T. *Org. Lett.* **2010**, 12, 268.
11. Sezer, S.; Şahin, E.; Tanyeli, C. *Tetrahedron: Asymmetry* **2010**, 21, 476.
12. Ghosh, A. K.; Cappiello, J.; Shin, D. *Tetrahedron Lett.* **1998**, 39, 4651.
13. Bassetti, M.; D'Annibale, A.; Fanfoni, A.; Minissi, F. *Org. Lett.* **2005**, 7, 1805.
14. Marco, J. A.; Carda, M.; Rodriguez, S.; Castillo, E.; Kneeteman, M. N. *Tetrahedron* **2003**, 59, 4085.
15. Fürstner, A. *Angew. Chem. Int. Ed.* **2000**, 39, 3012.
16. Sundby, E.; Perk, L.; Anthonsen, T.; Aasenb, A. J.; Hansen, T. V. *Tetrahedron* **2004**, 60, 521.
17. Fiorelli, C.; Savoia, D. J. *Org. Chem.* **2007**, 72, 6022.
18. Lee, S. M.; Lee, W. G.; Kim, Y. C.; Ko, H. *Bioorg. Med. Chem. Lett.* **2011**, 21, 5726.
19. Tanyeli, C.; Akhmedov, I. M.; Ozdemirhan, D. *Enantiomer* **2001**, 259.
20. Sezer, S.; Ozdemirhan, D.; Sahin, E.; Tanyeli, C. *Tetrahedron: Asymmetry* **2006**, 17, 2981.
21. General Procedure for the Synthesis of γ - and δ -Lactones via RCM: 0.01 M CH_2Cl_2 solution of first generation Grubbs catalyst [varied between 5 and 15 mol %] was added to 0.01 M refluxing solution of acrylate ester (1 mmol) in CH_2Cl_2 by slow addition under argon atmosphere. The reaction was stirred 80 min–24 h at reflux and controlled by TLC. The solvent was evaporated in vacuum and the purification of the crude product was done by column chromatography (silica gel, EtOAc/hexane 1:3 for γ - and 1:2–1:4 for δ -lactones). Representative examples to γ - and δ -lactones are given below, 'respectively'. (S)-(-)-5-(Thiophen-2-yl)furan-2(5H)-one, (S)-(-)-9b yield 60%; orange–brown thick liquid; $[\alpha]_D^{24.6} = -20.1$ (c 1.00, CH_2Cl_2); ν_{max} (cm^{-1}) 1754 (C=O), 1019, 823; ^1H NMR (400 MHz, CDCl_3) δ (ppm) 7.49 (dd, $J = 5.6$ and $J = 1.7$ Hz, 1H), 7.30 (dd, $J = 5.1$ and $J = 1.1$ Hz, 1H), 7.03 (d, $J = 3.1$ Hz, 1H), 6.96 (dd, $J = 5.1$ and $J = 3.6$ Hz, 1H), 6.19 (dd, $J = 5.6$ and $J = 2.1$ Hz, 1H), 6.16 (t, $J = 1.7$, 1H); ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 172.1 (C=O), 154.4, 137.0, 127.7, 127.6, 127.5, 122.3, 79.7; HRMS, Calculated $[\text{M}]^+$ 167.0176, Measured $[\text{M}]^+$ 167.0167.
- (S)-(-)-6-(Furan-2-yl)-5,6-dihydro-2H-pyran-2-one, (S)-(-)-8a yield 65%; light brown liquid; $[\alpha]_D^{23.5} = -101.1$ (c 1.00, CH_2Cl_2); ν_{max} (cm^{-1}) 3122, 2911, 1727 (C=O); ^1H NMR (400 MHz, CDCl_3) δ (ppm) 7.35 (dd, $J = 1.8$ and $J = 0.8$ Hz, 1H), 6.88 (ddd, $J = 9.8$, $J = 5.7$ and $J = 2.8$ Hz, 1H), 6.35 (d, $J = 3.3$ Hz, 1H), 6.30 (dd, $J = 3.3$ and $J = 1.8$ Hz, 1H), 6.01 (ddd, $J = 9.8$, $J = 2.5$ and $J = 1.1$ Hz, 1H), 5.41 (dd, $J = 11.1$ and $J = 4.2$ Hz, 1H), 2.90–2.52 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 161.9 (C=O), 149.5, 143.3, 141.9, 120.5, 109.5, 107.9, 71.2, 26.6; HRMS, Calculated $[\text{M}]^+$ 165.0473, Measured $[\text{M}]^+$ 165.0558.
22. Blois, M. S. *Nature* **1958**, 181, 1199.
23. Gülçin, İ.; Büyükkokuroğlu, M. E.; Oktay, M.; Küfrevioğlu, O. I. *J. Ethnopharmacol.* **2003**, 86, 51.
24. Raj, H. G.; Parmar, V. S.; Jain, S. C.; Goel, S.; Himanshu, P.; Sanjay Malhotra; Singh, A.; Olsen, C. E.; Wengel, J. *Bioorg. Med. Chem. Lett.* **1998**, 6, 833.
25. Koppenol, W. H. *Free Radical Res.* **1993**, 15, 645.
26. Rasoanaivo, P.; Ratsinamamanga-Urverg, S. Biological Evaluation of Plants with Reference to the Malagasy Flora, Napreca, Madagascar 1993, pp. 9–43, 72–83.
27. Beutler, L.; Beutler, E. *Blood Cells Mol. Dis.* **2004**, 33, 40.
28. De Braekeleer, M.; St-Pierre, C.; Vigneault, A.; Simard, H.; De Medicis, E. *Ann. Hematol.* **1991**, 62, 188.
29. Zanella, A.; Berzuini, A.; Colombo, M. B.; Guffanti, A.; Lecchi, L.; Poli, F.; Cappellini, M. D.; Barosi, G. *Brit. J. Haematol.* **1993**, 83, 485.
30. Chiba, Y.; Takizawa, S.; Kishi, K.; Hattori, A.; Shibata, A.; Matsumoto, N.; Fujii, H.; Miwa, S. *J. Jpn. Soc. Int. Med.* **1989**, 78, 41.
31. Whitnall, M.; Howard, J.; Ponka, P.; Richardson, D. *PNAS* **2006**, 103, 14901.