SHORT REPORT

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Rec. Nat. Prod. 10:6 (2016) 812-817

records of natural products

Antioxidant and Hepatoprotective Activities of Flavonoids from *Bauhinia hookeri*

Eman Al-Sayed [1,*†], Mai F. Tolba[2,3†] and Maarit Karonen[4]

[1] *Department of Pharmacognosy, Faculty of Pharmacy, Ain-Shams University, Cairo, 11566, Egypt*
[2] *Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, Cairo, 11566, Egypt*
[3] *Department of Biology, School of Science and Engineering, the American University in Cairo, Egypt*
[4] *Laboratory of Organic Chemistry and Chemical Biology, Department of Chemistry, University of Turku, FI-20014 Turku, Finland*

(Received October 26, 2015; Revised March 01, 2016; Accepted March 05, 2016)

Abstract: In a previous study, the total ethanol extract of *Bauhinia hookeri* showed a significant hepatoprotective effect in CCl_4-induced toxicity model in mice. However, the active components responsible for the activity were not identified. Therefore, this study was undertaken to determine if the activity of *B. hookeri* extract is due to its flavonoid content. The hepatoprotective activity of *B. hookeri* flavonoids was determined by measuring the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the culture medium of HepG2 cells challenged with CCl_4. The lipid peroxidation and antioxidant parameters, superoxide dismutase (SOD) and glutathione (GSH) were estimated in the cell lysates. The isolated flavonoids were identified by mass, UV and NMR spectral data. This study revealed that *B. hookeri* flavonoid fraction and its pure compounds (kaempferol 3-*O*-*β*-D-glucoside, quercetin 3-*O*-*β*-D-glucoside and catechin 3-*O*-*α*-L-rhamnoside) possess a promising hepatoprotective activity as evidenced from the normalized levels of ALT and AST. This was attributed partly to their potent antioxidant activity as demonstrated by the increased GSH levels, SOD activity and reduced lipid peroxidation. The whole flavonoid fraction showed the highest cytoprotective activity and was more effective than silymarin. This study highlights a promising natural hepatoprotective remedy derived from *B. hookeri*.

Keywords: Antioxidant; *Bauhinia hookeri;* cytoprotection; flavonoids; lipid peroxidation; oxidative stress.

1. Plant Source

The leaves of *B. hookeri* F. Mull. (Fabaceae) were collected in July 2011 from the botanical garden of the Faculty of Agriculture, Cairo University, Cairo, Egypt. The plant was botanically identified by Eng. Therese Labib, the taxonomy specialist at the herbarium of El-Orman Botanical Garden, Giza, Egypt. A voucher specimen of *B. hookeri* was deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt (ASU BHF2011).The first objective of this study was to determine if the previously reported activity of the *B. hookeri* extract is due to its flavonoid content. The second objective was to investigate the *in vitro* hepatoprotective activity of *B. hookeri* flavonoids in HepG2 cells challenged with CCl_4. Hepatoprotection was determined by assaying the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the culture medium of HepG2 cells treated with CCl_4. The lipid peroxidation and antioxidant parameters superoxide dismutase (SOD) and glutathione (GSH) were

* Corresponding author: E-Mail: em_alsayed@pharma.asu.edu.eg ; Tel.: +202 01001050293, Fax: +20 2 405 1106. † These authors have contributed equally to this article

The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 05/15/2016 EISSN:1307-6167

estimated in the cell lysates to determine the possible mechanisms of the hepatoprotective activity. The isolated flavonoids were identified by spectral data.

2. Previous Studies

There is now general agreement among hepatologists that the number of available drugs for the treatment of liver diseases is far from sufficient [1]. Silymarin is a popular herbal extract used as a hepatoprotective agent; however, some clinical trials showed that silymarin is ineffective in many patients with chronic liver disease. In addition, serious adverse effects including gastroenteritis have been reported in patients using silymarin [1,2]. In view of the limited drugs available for the treatment of liver diseases, there is an urgent need for the development of safe and effective new candidates, especially from natural products, for the treatment of liver disorders.

The genus *Bauhinia* comprises 300 species, and are known as "cow's paw tree", because of the shape of their leaves [3]. Plants that belong to this genus are widely distributed in Africa, Asia and South America. Their leaves and stem-bark have been used in folk medicine for the treatment of different ailments [3]. Several pharmacological activities have been reported for many *Bauhinia* species, including antioxidant, anti-hyperlipediemic and anti-inflammatory effects [4,5]. In a previous study, we demonstrated that the total ethanol extract of *B. hookeri* possessed a significant hepatoprotective effects and was more potent than silymarin in a chronic CCl_4-induced toxicity model in mice [6]. However, the most active components responsible for the activity of the extract were not identified. To the best of our knowledge, no study has so far been conducted on the hepatoprotective activity of flavonoids obtained from *B. hookeri.*

3. Present Study

3.1. Structure Elucidation of the Isolated Compounds

Fractionation of the flavonoid-rich fraction obtained from the 80% ethanol extract of *B. hookeri* yielded compounds **1-6** (Figure 1). Notably, this is the first report of the isolation of these compounds from *B. hookeri*. All the compounds were identified based on their UV, HRESI-MS/MS, 1D and 2D NMR data (Supporting data), as well as by comparing these data with those previously reported in the literature. The compounds were identified as (+)-catechin 3-*O*-*α*-L-rhamnoside (**1**), kaempferol 3-*O*-*β*-D-glucoside (**2**), quercetin 3-*O*-*β*-D-glucoside (**3**), kaempferol 3-*O*-(6″-*O*-galloyl)-*β*-D-galactoside (**4**), kaempferol 3-*O*-(6″-*O*-galloyl)-*β*-D-glucoside (**5**) and quercetin 3-*O*-(6″-*O*-galloyl)-*β*-D-glucoside (**6**).

3.2. Hepatoprotective activity

The hepatoprotective effect of *B. hookeri* flavonoids was evaluated in HepG2 cells for the first time. The human hepatocellular carcinoma cell line is suggested as a suitable model to screen the hepatoprotective activity of drugs because it retains most of the functional characteristics of normal human hepatocytes [7,8]. The hepatocellular integrity was evaluated by assessing the leakage of ALT and AST into the culture medium. Exposure of HepG2 cells to CCl_4 significantly increased the leakage of ALT into the culture medium by about 4.3 folds compared to the normal control. Pretreatment with silymarin at the tested concentrations 50, 100 and 200 μg/mL significantly ameliorated the CCl_4-induced ALT leakage by 54, 56 and 64%, respectively compared to the CCl_4-treated group. Incubation of the cells with 50 μM of compounds **1**, **2** and **3** significantly protected against CCl_4 damage as evidenced from the reduction of ALT levels by 40, 27 and 38%, respectively relative to the CCl_4 group. Pretreatment with 100 μM significantly reduced the ALT leakage by 43, 40 and 44%, while the highest concentration 200 μM produced a more prominent reduction in ALT levels by 54, 62 and 46%, respectively. Pretreatment with compounds **4**, **5** and **6** at 50 μM concentration reduced ALT levels by 43, 30 and 30%, respectively compared to the CCl_4 group. The median concentration of the same compounds produced reductions by about 46, 45 and 40%, while the highest concentration resulted in 50, 45 and 40% reductions in ALT levels. Pretreatment of the cells with the whole fraction at the concentrations 50, 100, and 200 μg/mL significantly ameliorated the CCl_4-induced damage as indicated from the reduced leakage of ALT by 38, 44 and 66%, respectively compared to the CCl_4 group (Figure 2).

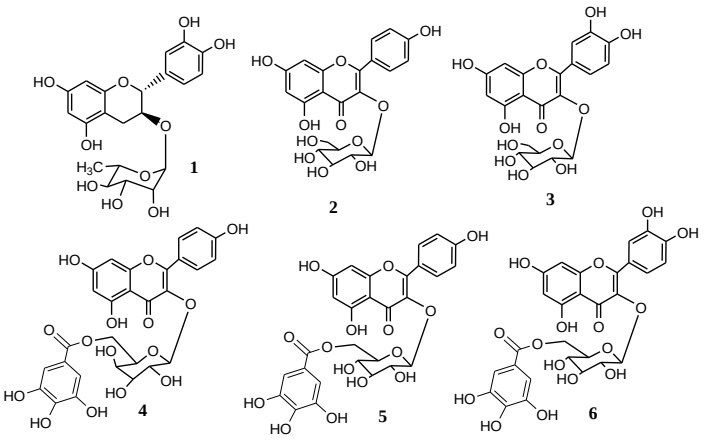

Figure 1. Structures of the isolated flavonoids from *B. hookeri*

Similarly, challenging HepG2 cells with CCl_4 significantly increased the leakage of AST by about 9.3 folds compared to the normal group. Pretreatment with silymarin at 50, 100 and 200 µg/mL significantly reduced CCl_4-induced AST leakage by 51, 51 and 65%, respectively compared to the CCl_4 group. Incubation of the cells with 50 µM of compounds **1**, **2** and **3** significantly ameliorated the AST leakage by 63, 62 and 61%, respectively. Pretreatment with 100 µM reduced AST levels by 63, 65 and 65%. The highest concentration of compounds **1**, **2** and **3** resulted in 67, 70 and 72% reductions in AST levels compared to CCl_4 group. Pretreatment with 50 µM of compounds **4**, **5** and **6** reduced the AST levels by 55, 46 and 46%, respectively. The median concentration of **4**, **5** and **6** produced 60, 50 and 45% reductions in AST levels. The highest concentration of the same compounds produced 61, 72 and 51% decrease in AST leakage. Pretreatment with the whole fraction at the concentrations 50, 100, and 200 µg/mL significantly protected against CCl_4-induced damage as indicated from the reduced leakage of AST by 59, 59 and 77% compared to the CCl_4 group (Figure 2).

Incubation of the cells with CCl_4 significantly reduced the GSH levels in the cell lysates by 46% compared to the normal control group. Silymarin pretreatment at 50, 100 and 200 µg/mL significantly preserved the GSH levels by about 1.2, 1.7 and 2.1 folds compared to the CCl_4 group. Pretreatment with 50 µM of compounds **1**, **2** and **3** significantly increased the GSH levels by 1.6, 1.3 and 1.2 folds, respectively compared to the CCl_4 group. Treatment with compounds **1**, **2** and **3** at 100 µM concentration produced a significant increase in GSH by 2.0, 1.6 and 1.4 folds. Treatment with 200 µM of the same compounds produced the highest elevations in GSH levels by 2.6, 2.2, 2.2 folds. Moreover, pretreatment with 50 µM of compounds **4**, **5** and **6** significantly guarded against CCl_4-induced oxidative stress by boosting the GSH levels by 1.3, 1.3 and 1.5 folds, respectively compared to the CCl_4 group. Pretreatment with 100 µM of compounds **4**, **5** and **6** resulted in a greater increase in the levels of GSH by 1.6, 1.6 and 1.7 folds. While pretreatment with 200 µM of the same compounds resulted in a more significant increase in GSH levels by 2.1, 2.2 and 2.3 folds. Pretreatment of HepG2 cells with the whole fraction at the concentrations 50, 100, and 200 µg/mL significantly enhanced the GSH levels by about 1.2, 1.5 and 2.1 folds, respectively compared to the CCl_4 group (Figure 2).

Similarly, challenging HepG2 with CCl_4 significantly reduced the SOD activity by 60% compared to the normal group. Silymarin pretreatment at 50, 100 and 200 µg/mL significantly improved the SOD activity by about 2.4, 2.9 and 3.0 folds compared to the CCl_4 group. Incubation of the cells with 50 µM of compounds **1**, **2** and **3** before the exposure to CCl_4 significantly enhanced the SOD activity by 2.4, 2.3 and 1.4 folds, respectively, when compared to the CCl_4 group. Additionally, pretreatment with the median concentration of these compounds enhanced the SOD activity by 2.5, 2.7 and 1.7 folds, respectively.

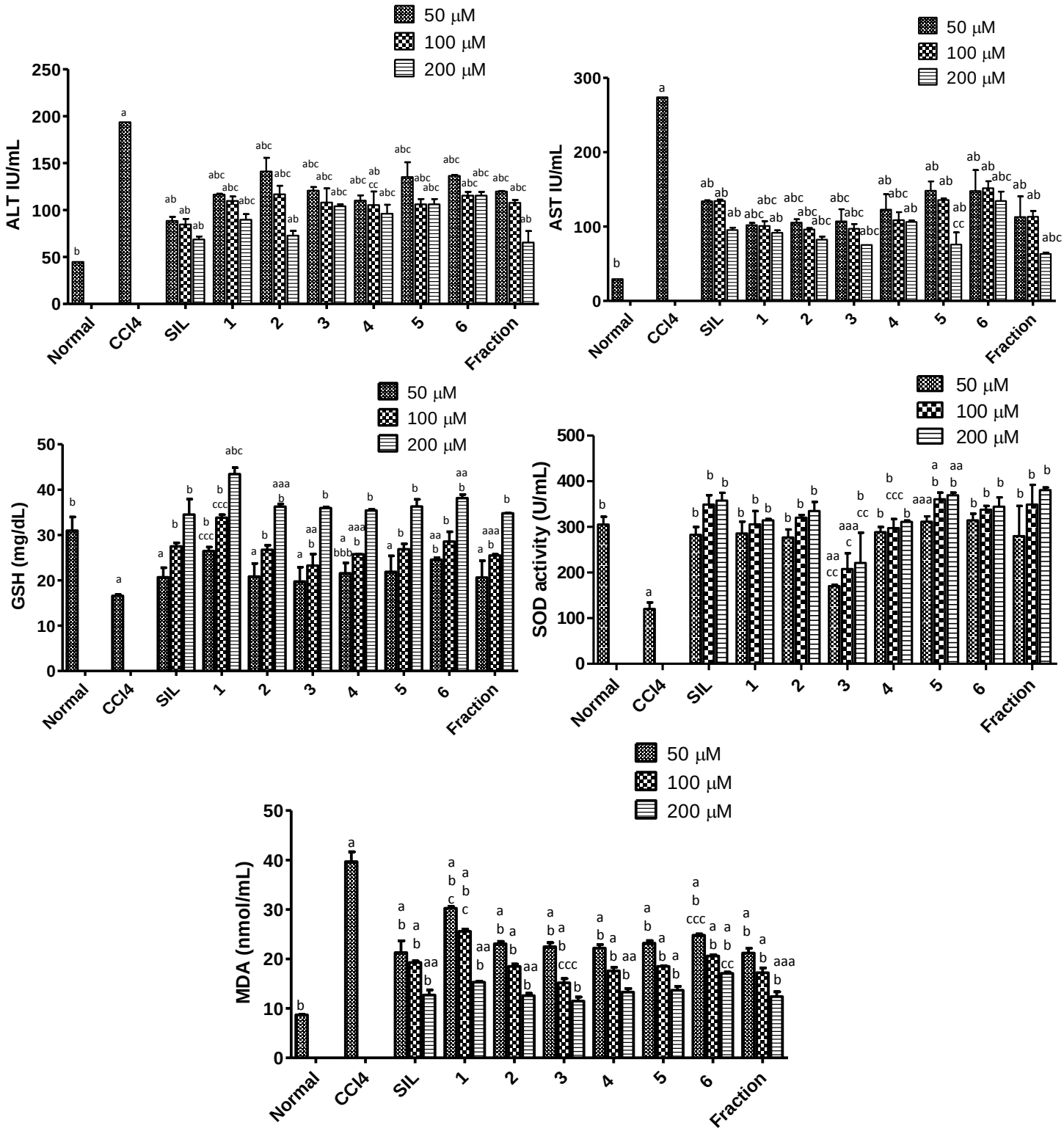

Figure 2. Effect of pretreatment with *B. hookeri* flavonoid fraction, isolated compounds (**1**-**6**) and silymarin (Sil) on the ALT and AST leakage, cellular GSH levels, SOD activity and MDA levels in HepG2 cells challenged with CCl_4. Data are expressed as the means ± SD, (n = 3). The experiment was done in triplicate. The fraction and silymarin were tested in µg/ mL concentration units. a significantly different from the normal group at $P < 0.001$, aa at $P < 0.01$, aaa at $P < 0.05$. b significantly different from the CCl_4 group at $P < 0.001$, bbb at $P < 0.05$. c significantly different from the silymarin groups at $P < 0.001$, cc at $P < 0.01$, ccc at $P < 0.05$.

The higher concentration of the same compounds induced a significant increase in the SOD activity by 2.6, 2.8 and 1.8 folds, respectively. Furthermore, pretreatment with compounds **4**, **5** and **6** at 50 μM enhanced the SOD activity by 2.4, 2.6 and 2.6 folds, respectively. While treatment with compounds **4**, **5** and **6** at 100 μM concentration produced an enhancement in SOD activity by 2.5, 3.0, and 2.8 folds. The highest concentration of compounds **4**, **5** and **6** produced a more significant increase in the activity of SOD by 2.9, 3.1 and 2.6 folds. Pretreatment of the cells with the whole fraction at the concentrations of 50, 100, and 200 μg/ mL significantly enhanced the activity by 2.4, 2.9 and 3.2 folds, respectively compared to the CCl_4-treated group (Figure 2).

Exposure of HepG2 cells to CCl_4 significantly increased lipid peroxidation as evidenced by the increased MDA levels by 4.6 folds compared to the normal control group. Pretreatment with silymarin at 50, 100 and 200 μg/mL significantly reduced the CCl_4-induced increase in MDA levels by about 46, 51and 68%, respectively compared to the CCl_4 group. Pretreatment with 50 μM of compounds **1**, **2** and **3** significantly alleviated the CCl_4-induced elevation in MDA levels by 24, 42 and 43%, respectively. Pretreatment with compounds **1**, **2** and **3** at 100 μM reduced the MDA levels by 36, 53 and 62%, while treatment with the highest concentration of these compounds reduced the levels by 61, 68 and 71%, respectively. Similarly, pretreatment with 50 μM of compounds **4**, **5** and **6** reduced the MDA levels by 44, 42, and 38%, respectively compared to the CCl_4 group. Pretreatment with 100 μM of the same compounds significantly reduced the MDA levels by 56, 53 and 48%, while 200 μM of **4**, **5** and **6** significantly reduced the MDA levels by 67, 66 and 57%, respectively. Pretreatment of HepG2 cells with the whole fraction at the concentrations of 50, 100, and 200 μg/ mL significantly decreased the MDA levels by 47, 57 and 69% compared to CCl_4-treated group (Figure 2).

In conclusion, the current study revealed that the whole flavonoid fraction and compounds **1**, **2** and **3** showed the highest reductions in ALT and AST leakage. The flavonoid fraction showed more cytoprotective activity over silymarin and significantly alleviated the CCl_4-induced leakage of AST and ALT. Kaempferol 3-*O*-*β*-D-glucoside (**2**) showed the highest reduction in ALT level after the flavonoid fraction, followed by catechin 3-*O*-α-L-rhamnoside (**1**). Quercetin 3-*O*-*β*-D-glucoside (**3**) and compound **2** showed the highest reductions in AST levels after the flavonoid fraction, followed by compound **1**. Similarly, the flavonoid fraction and compounds **2** and **3** significantly alleviated the CCl_4-induced increase in MDA levels and were more effective than silymarin. Treatment with compound **1** enhanced the GSH levels over those in the normal and silymarin groups. The other compounds and the flavonoid fraction produced similar enhancement of GSH levels as silymarin. Moreover, treatment with the flavonoid fraction and kaempferol 3-*O*-(6″-*O*-galloyl)-*β*-D-glucoside (**5**) induced a more significant increase in SOD activity over silymarin and the other compounds, which indicated that the antioxidant effect is not the only mechanism which contributes to the cytoprotective activity. Based on this study, the presence of a galloyl group in the glucose moiety (in compounds **4**-**6**) did not enhance the cytoprotective activity. Experimental evidence indicates that whole plant fractions usually possess much better pharmacological activities than single isolated ingredients due to synergistic interactions between the individual components [9,10]. It is also known that mixtures of antioxidant compounds are more active than the individual components of these mixtures [11]. This supports the notion that the flavonoid fraction of *B. hookeri* showed higher cytoprotective activity and antioxidant effects when compared to its single compounds.

Flavonoids, especially flavonols (kaempferol and quercetin) and flavanols (catechin and epicatechin) possess various biological effects that contribute to health benefits including antioxidant activity [11,12]. Flavonols prevent the oxidative stress by direct scavenging of free radicals, metal chelation and induction of antioxidant enzymes. Flavonoids also have a membrane-stabilizing effect [11,12]. The results of the present study are in accordance with previous results which indicated that catechin 3-*O*-α-L-rhamnoside, kaempferol 3-*O*-*β*-D-glucoside and quercetin 3-*O*-*β*-D-glucoside are among the major flavonoids of lotus extract and might be responsible for the hepatoprotective effect of the lotus extract in a CCl_4-induced hepatotoxicity model in rats [13]. However, this study did not determine the hepatoprotective activity of the individual components of lotus extract. It is worth mentioning that the present study represents the first report that demonstrated the hepatoprotective activity of *B. hookeri* flavonoids (**1**-**6**).

Acknowledgments

Professor Jari Sinkkonen, department of chemistry, university of Turku, Finland is acknowledged for measuring the NMR spectra.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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